SULPHATED POLYSACCHARIDES OF THE *Solieriaceae* FAMILY
PART I. A POLYSACCHARIDE FROM *Anatheca dentata*

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ABSTRACT

Hot-water extraction of *Anatheca dentata*, a red seaweed belonging to the family *Solieriaceae*, yielded a mixture of polysaccharides. Fractionation of this mixture with Cetavlon gave a glucomannan as minor component and a highly sulphated, major component, which yielded D- and L-galactose, D-xylose, and traces of 3-*O*-methylgalactose and uronic acid on hydrolysis. Partial hydrolysis of the major component with acid gave 4-*O*-β-D-galactopyranosyl-L-galactose (**1**), 3-*O*-α-L-galactopyranosyl-D-galactose (**2**), 4-*O*-β-D-galactopyranosyl-D-galactose (**3**), *O*-β-D-galactopyranosyl-(1→4)-*O*-α-L-galactopyranosyl-(1→3)-D-galactose (**4**), and *O*-β-D-galactopyranosyl-(1→4)-*O*-α-L-galactopyranosyl-(1→3)-*O*-D-galactopyranosyl-(1→4)-L-galactose (**5**). The results indicate that a substantial part of the molecule has alternating D- and L-galactose residues and α-(1→3)- and β-(1→4)-glycosidic links. However, no role for the excess of D- over L-galactose, or of the xylose, has yet been found.

INTRODUCTION

Anatheca dentata, a red seaweed of the family *Solieriaceae*, is fairly prominent along the coast of Southern Africa, occurring at the lowest level of the intertidal range. This investigation forms part of our study of the sulphated polysaccharides of the red seaweeds.

RESULTS AND DISCUSSION

Anatheca dentata was collected at Kowie Point (*ca.* 120 miles east of Port Elizabeth) in March, 1967. Hot-water extraction of the fresh weed, followed by centrifugation, and precipitation of the mucilage into ethanol, afforded a highly sulphated polysaccharide mixture. Complete hydrolysis of this polysaccharide mixture with acid, and separation of the monosaccharides, gave galactose [as a mixture of D and L isomers (1.57:1)] and D-xylose. Paper-chromatographic evidence was

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obtained for the presence of traces of glucose, mannose, arabinose, and a sugar having a chromatographic mobility of R_{Gal} 2.91 (solvent *B*). In addition, there was present a sugar having R_{Gal} 2.1 (solvent *B*), which was subsequently isolated from a partial, acid hydrolysate of the galactan sulphate and characterised as 3-*O*-methylgalactose.

Fractionation of the polysaccharide mixture with Cetavlon yielded two fractions, the minor one being composed of glucose and mannose residues. Chromatography of the major polysaccharide, on the anion-exchange gel DEAE-Sephadex A-50, gave three fractions. Each fraction yielded galactose and xylose, in approximately the same proportions, on hydrolysis. The properties and molar ratios of the component sugars of the major fraction (*2*) were found to be very similar to those of the Cetavlon-precipitated polymer (Table I). Consequently, all subsequent experiments were performed on the Cetavlon-precipitated polymer. The last fractionation probably effected a separation based chiefly on molecular weight, rather than into structurally different polysaccharides. Although the Cetavlon-precipitated polysaccharide was not entirely homogeneous, there was no evidence, from these experiments, of a separate xylan. The infrared spectrum of the polymer exhibited the general absorption band for ester sulphate at 1240 cm^{-1} but did not show any well-defined bands[1] for axial, equatorial, or primary ester sulphate (800–860 cm^{-1}).

TABLE I

COMPARISON BETWEEN CETAVLON-PRECIPITATED POLYSACCHARIDE AND FRACTION *2* OBTAINED BY CHROMATOGRAPHY ON DEAE-SEPHADEX A-50

	Cetavlon-precipitated polysaccharide	*Fraction* 2
$[\alpha]_D^{21}$ (degrees)	−40.5 (*c* 0.99)	−33 (*c* 0.96)
SO_4^{2-} (%)	35.5	35.2
$NaSO_3^-$ [*A*] (%)	38.1	37.8
Galactose [*B*] (%)	59.0	57.0
Xylose [*C*] (%)	8.2	7.7
Molar proportions of *A*:*B*:*C*	6.78:6.01:1.00	6.96:6.00:0.97
3-*O*-Methylgalactose	Trace	Trace

Partial hydrolysis of the polysaccharide with acid, followed by separation of the neutral products on a charcoal–Celite column, yielded a mixture of D- and L-galactose, D-xylose, a minute trace of glucose, a small proportion of 3-*O*-methylgalactose, and several oligosaccharides. The major oligosaccharide was 4-*O*-β-D-galactopyranosyl-L-galactose (**1**), followed, in smaller amounts, by 3-*O*-α-L-galactopyranosyl-D-galactose (**2**), 4-*O*-β-D-galactopyranosyl-D-galactose (**3**), *O*-β-D-galactopyranosyl-(1 → 4)-*O*-α-L-galactopyranosyl-(1 → 3)-D-galactose (**4**), and *O*-β-D-galactopyranosyl-(1 → 4)-*O*-α-L-galactopyranosyl-(1 → 3)-*O*-D-galactopyranosyl-(1 → 4)-*O*-L-galactose (**5**). Oligosaccharides **1**, **3**, and **4** were obtained crystalline. Several other oligosaccharide fractions were obtained but were in insufficient amount and purity for analysis. The structures of these compounds, except **3** which is known[2], were obtained by the following series of experiments. The reducing end-group was esta-

blished by reduction of the oligosaccharide with sodium borohydride, followed by partial hydrolysis of the product with acid and chromatographic examination of the resulting mixture. The positions of the glycosidic linkages were established by methylation of the oligosaccharide followed by either methanolysis and examination of the resulting mixture by g.l.c., or hydrolysis followed by examination of the mixture by t.l.c. or paper chromatography. In those cases (**1** and **2**) where g.l.c. of the methyl glycosides was used, quantitative determination of the latter was effected. The results of these analyses, which are set out in Table II, are consistent with the structures of the oligosaccharides as formulated in the first column.

It is evident from the analysis of the oligosaccharides that a substantial part of the macromolecule must be composed of alternating D- and L-galactose residues linked in an alternating α-(1 → 3) and β-(1 → 4) sequence. Furthermore, because of the preponderance of D- over L-galactose in the hydrolysis mixture of the polysaccharide, and because of the presence of 4-*O*-β-D-galactopyranosyl-D-galactose (**3**) amongst the partial hydrolysis products, D-galactose must replace part of the L-galactose in the repeating unit. The structure is further complicated by the presence of xylose which has so far not appeared in any of the partial hydrolysis products. It is evident that a fragment larger than a tetrasaccharide will have to be isolated and characterised before any more-definite conclusions can be drawn.

Work on this and on the position of the sulphate ester groups is in progress.

EXPERIMENTAL

Paper chromatography was carried out on Whatman No. 1 paper with the following solvent systems: (*A*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4), (*B*) ethyl acetate–pyridine–water (8:2:1), and (*C*) butyl alcohol–ethanol–water (40:11:9). Spray reagents used were (*1*) *p*-anisidine hydrochloride[3], (*2*) periodate–benzidine[4], (*3*) Bromocresol Green[5] (0.1% solution in 95% aqueous ethanol just made alkaline with sodium hydroxide), and (*4*) 20% sulphuric acid in ethanol. R_{Gal} values refer to rates of movement relative to that of galactose. Infrared spectra were recorded on a Beckman IR-8 spectrophotometer, using KBr discs. Concentration of solutions was carried out at 40°/20 mmHg, and specific rotations were determined for aqueous solutions. Sulphate was determined with 4′-chlorobiphenyl-4-ylamine[6].

Thin-layer chromatography (t.l.c.) was carried out on glass plates coated with silica gel G (Merck) containing calcium sulphate as binder, employing butanone–water (85:7) as solvent. R_{TMG} values of methylated sugars refer to the rates of travel relative to that of tetra-*O*-methyl-D-galactose. Gas–liquid chromatography (g.l.c.) was carried out on a Beckman GC-4 chromatograph equipped with dual flame-ionisation detectors and nitrogen as carrier gas. For the investigation of the methyl glycosides of the methylated sugars, the stationary phase [15% by weight of poly(butane-1,4-diol succinate)] was supported on acid-washed Celite (80–100 mesh) and maintained at the operating temperature of 175°. Retention times (*T*) are relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.

For the quantitative analysis of sugars as their glycitol acetates by g.l.c., the

TABLE II

STRUCTURAL ANALYSIS OF OLIGOSACCHARIDES

Oligosaccharide	*Products of total hydrolysis*	*Products of partial hydrolysis*	*Products of partial hydrolysis of reduced oligosaccharide*	*Hydrolysis of methanolysis products of methylated oligosaccharide*[a]
O-β-D-Gal*p*-(1→4)-L-Gal (1)	DL-Galactose		D-Galactose	*A*:*B* = 1.0:1.02
O-α-L-Gal*p*-(1→3)-D-Gal (2)	DL-Galactose		L-Galactose	*A*:*C* = 1.0:1.05
O-β-D-Gal*p*-(1→4)-*O*-α-L-Gal*p*-(1→3)-D-Gal (4)	D- and L-Galactose; ratio 2:1	1, 2	1	*A*, *B*, *C*
O-β-D-Gal*p*-(1→4)-*O*-α-L-Gal*p*-(1→3)-*O*-D-Gal*p*-(1→4)-L-Gal (5)	DL-Galactose	1, 2, 4	4	

[a] *A* = 2,3,4,6-Tetra-*O*-methylgalactose; *B* = 2,3,6-tri-*O*-methylgalactose; *C* = 2,4,6-tri-*O*-methylgalactose.

stationary liquid phase (20% Apiezon M) was supported on Chromosorb W (80–100 mesh; acid-washed and treated with chlorodimethylsilane) and maintained at an operating temperature of 200°. The preparation of the glycitol acetates was carried out essentially by the method of Bowker and Turvey[7].

Extraction and purification of polysaccharide. — Wet *Anatheca dentata* (5.6 kg) was macerated and mixed with hot water, and the pH was adjusted to 3 with glacial acetic acid. Steam was passed into the mixture for 0.50 h with constant stirring, after which the solution was strained through muslin and centrifuged hot, yielding a clear, pale-pink mucilage. When the extract was set aside for 24 h at 4°, a colloidal precipitate appeared which was removed by centrifugation. Precipitation into ethanol (5 vol.) afforded a fibrous, white product which was washed with ether, and dried (280 g; 5% on a wet-weight basis). Purification of the polysaccharide was effected by dissolution in water, centrifugation of the solution, and precipitation into ethanol (5 vol.). The recovered polysaccharide was washed with ether and dried *in vacuo* at 45°. [Found (on material dried at 70°/0.5 mmHg): N, 0.53; SO_4^{2-}, 30.5)]. The polysaccharide was not precipitated from solution on the addition of potassium chloride solution, and in this respect resembles the λ-fraction of carrageenan[8]. The polymer was not precipitated from solution when treated with Fehling's solution.

Chromatographic examination (solvents *A*, *B*, and *C*) of a neutralised acid-hydrolysate revealed spots corresponding to galactose (major), xylose, and traces of two other sugars having R_{Gal} 2.1 and 2.91 (solvent *B*) with spray *1*. Spray *3* revealed the presence of a small amount of uronic acid.

Separation and characterisation of the components of the polysaccharide. — Polysaccharide (6.0 g) was hydrolysed with 0.5M sulphuric acid (50 ml) for 16 h on a boiling-water bath. After neutralisation with barium carbonate, the solution was evaporated to a partially crystalline syrup (4.45 g) and applied to a cellulose column (50 × 5.4 cm) which was eluted with butyl alcohol–water (95:5). Fractions (*ca.* 25 ml) were collected and, on the basis of paper chromatography, combined into three fractions which were evaporated to dryness.

Fraction I. The syrup (0.59 g) was shown by paper chromatography to contain xylose, and traces of glucose, mannose, arabinose, and two other sugars having R_{Gal} 2.1 and 2.91 (solvent *B*). An aqueous solution of the syrup was decolourised with charcoal in water, filtered, and evaporated to dryness. Recrystallisation of the residue from ethanol yielded D-xylose (120 mg), m.p. and mixed m.p. 143–144°, $[\alpha]_D^{23}$ +45 (6 min) → +18.9° (*c* 0.5). The dibenzylidene dimethyl acetal derivative[9] had m.p. and mixed m.p. 212–213° (Kofler hot stage) with an authentic sample.

Fraction II. The syrup (0.58 g) was shown by paper chromatography (solvents *A* and *B*) to contain galactose (major sugar), xylose, glucose (trace), mannose (trace), and a sugar (trace) having R_{Gal} 2.1 (solvent *B*).

Fraction III. A portion of this fraction (3.05 g) was recrystallised from ethanol to give galactose, m.p. 161–162°, $[\alpha]_D^{22}$ +17.7° (*c* 0.50); the value obtained for the specific rotation indicated a D:L ratio of 1.57:1. Oxidation with nitric acid–water (1:1) yielded galactaric acid, m.p. and mixed m.p. 212–213°.

Fractionation of the polysaccharide with Cetavlon. — A solution of the polysaccharide (1.0 g) in water (50 ml) to which a 10% aqueous solution of cetyltrimethylammonium bromide (Cetavlon; 15 ml) had been added was allowed to stand overnight. The precipitated complex was centrifuged off and washed four times with water. The supernatant liquid was retained for further investigation (see later). The complex was redissolved by stirring overnight in 4M potassium chloride (50 ml). The insoluble residue was removed by centrifugation and discarded, and the clear solution was poured into ethanol (5 vol.). The precipitated polysaccharide was washed with ethanol and then redissolved in water (50 ml), and the solution was dialysed against running water, concentrated, and poured into ethanol (5 vol.). The resulting polysaccharide was washed with ethanol and finally ether, and dried at 50° in a vacuum (yield, 0.73 g). Chromatographic examination of an acid hydrolysate (solvents *A* and *B*) showed the presence of galactose (major), xylose, traces of two other sugars R_{Gal} 2.1 and 2.91 (solvent *B*), and a minute trace of glucose. To the solution remaining after removal of the precipitated complex, was added sufficient potassium iodide to precipitate the excess of Cetavlon. After removal of the Cetavlon–iodide complex by centrifugation, the supernatant was dialysed against running tap-water, concentrated, and freeze-dried to an off-white foam (31 mg). Chromatographic examination of an acid hydrolysate (solvent *B*) showed the presence of glucose and mannose.

All subsequent experiments were performed on the Cetavlon-precipitated polysaccharide. Further purification of the polysaccharide for analysis was effected by repeated (twice) dissolution in water, centrifugation of the solution, and pouring the supernatant into ethanol (5 vol.). After collection by centrifugation, the polysaccharide was washed with ether, and dried in a vacuum at 45°; it had $[\alpha]_D^{25}$ −40.5° (*c* 0.99) [Found (on material dried at 60°/0.5 mmHg): 3,6-anhydrogalactose[10], 0.41; OMe, 0.0; N, 0.0; SO_4^{2-}, 35.5%; equiv. wt. (from SO_4^{2-} detn.), 271].

Attempted fractionation of Cetavlon-precipitated polysaccharide by chromatography on DEAE-Sephadex A-50. — The anion-exchange gel DEAE-Sephadex A-50 was swollen and equilibrated with 0.1M potassium chloride before being packed in a column (30 × 4.5 cm). Polysaccharide (200 mg) in water (14 ml) was applied to the column, which was then sequentially eluted with the following solutions: (*a*) 0.75M potassium chloride (2.8 l), (*b*) 1.0M potassium chloride (2.8 l), and (*c*) 1.5M potassium chloride (1.0 l). Fractions (20 ml) were collected and analysed for carbohydrate with the phenol–sulphuric acid reagent[11]. This yielded three fractions, the solutions of which, after dialysis, evaporation, and freeze-drying, gave fractions *1* (46 mg), *2* (112 mg), and *3* (14.5 mg). These fractions were eluted with potassium chloride solutions *a*, *b*, and *c*, respectively. No further material was recovered from the column by increasing the concentration of potassium chloride. The properties of fraction *2* (major) and those of the Cetavlon-precipitated polysaccharide are compared in Table I. The properties of fractions *1* and *3* were very similar to those of fraction *2* and of Cetavlon-precipitated material, so there does not appear to have been a separation into different polysaccharides.

Partial hydrolysis of the Cetavlon-precipitated polysaccharide. — Polysaccharide

(1.0 g) in 0.5M sulphuric acid (20 ml) was heated by immersion of the flask in a boiling-water bath. Samples (1 ml) were removed at regular intervals, neutralised ($BaCO_3$), concentrated, and examined on paper chromatograms (solvent *A*). The chromatograms were characterised by streaking, but it appeared that treatment for 2–2.5 h under the above conditions gave the maximal concentration of oligosaccharides. A large quantity of polysaccharide (18.0 g) was then hydrolysed similarly for 2 h, and the hydrolysate, after neutralisation ($BaCO_3$) and centrifugation, was deionised [Amberlite IR-120(H^+) and IRA-400(acetate) resins]. The aqueous eluate containing the neutral fragments was concentrated to syrup (3.82 g). The acid components were eluted from the IRA-400 (acetate) resin with 0.5M sulphuric acid (4.0 l). The eluate was neutralised with barium carbonate, concentrated to about 200 ml, and passed through a column of Amberlite IR-120 (H^+) resin. After removal of the acetic acid from the latter eluate by freeze-drying, the product was dissolved in water (50 ml) and neutralised with ammonia. The ammonium salts (13.0 g) were isolated by freeze-drying.

Separation and characterisation of the neutral components of the partial hydrolysate. — A solution of the neutral syrup (3.82 g) in the minimal quantity of water was applied to a charcoal–Celite column (1:1; 5.4 × 60 cm). Monosaccharides were eluted with water and oligosaccharides with aqueous alcohol of increasing strength, as indicated below. Fractions (*ca.* 30 ml) were subsequently combined on the evidence of paper-chromatographic analysis.

Fraction I. The syrup (2.69 g), eluted with water (10 l), contained (paper chromatography, solvents *A* and *B*) galactose (major), xylose, and a minute trace of glucose.

Fraction II. The syrup (25 mg), eluted with 2% aqueous ethanol (1.5 l), showed the presence of galactose and a substance with R_{Gal} 2.1 (solvent *B*). The syrup was fractionated on Whatman No. 1 paper (solvent *A*). Extraction of the appropriate portions of the paper with 50% aqueous methanol, followed by concentration, afforded a chromatographically pure product (9 mg) having R_{Gal} 2.1 (solvent *B*), $[\alpha]_D^{21}$ +28° (*c* 0.5). The sugar moved with the mobility of authentic 3-*O*-methylgalactose in solvent systems *A*, *B*, and *C*, and gave spots of the same colour as given by this sugar with spray *I*. Demethylation[3] with hydrobromic acid gave (paper chromatography) galactose and unchanged material. G.l.c. examination of the glycitol acetate, prepared from the sugar (3 mg), gave a peak having the same retention time as that of the glycitol acetate of authentic 3-*O*-methylgalactose. The low value obtained for the optical rotation (*cf.* 3-*O*-methyl-D-galactose[12] $[\alpha]_D$ +109°) is possibly due to the "sugar" being a mixture of 3-*O*-methyl-D- and 3-*O*-methyl-L-galactose.

Fraction III. The syrup (0.531 g), eluted with 5% aqueous ethanol (6.5 l), was shown by paper chromatography to be a mixture of three oligosaccharides having R_{Gal} 0.24 (major), 0.31, and 0.17 (trace) (solvent *B*). An aqueous solution of the syrup was decolourised with charcoal, filtered, and evaporated to a syrup. Crystallisation from 80% aqueous ethanol gave colourless needles (185 mg) which, after recrystallisation from the same solvent, had m.p. 237–238° (dec.), $[\alpha]_D^{19}$ −48° (*c* 0.5) (final), R_{Gal} 0.24 (solvent *B*) and 0.34 (solvent *A*). Paper chromatography (solvent *A*) of a

partial, acid hydrolysate (2 mg) revealed galactose and the original material. A portion (40 mg) was reduced with sodium borohydride (40 mg) in water (5 ml) for 24 h, and then shaken with Amberlite IR-120(H^+) resin. After the resin had been filtered off, the solution was evaporated to dryness, and the borate was removed by repeated distillation with methanol. Acid hydrolysis of the non-reducing syrup, followed by separation of the resultant mixture on Whatman No. 1 paper (solvent *A*), afforded two fractions. The first fraction was recrystallised from methanol (charcoal) to give galactitol, m.p. and mixed m.p. 186–187°. The second fraction was recrystallised from methanol (charcoal) to give D-galactose, $[\alpha]_D^{17}$ +117 (5 min)→ +78° (final) (*c* 0.56).

Another portion (20 mg) of the oligosaccharide was hydrolysed (0.5M sulphuric acid for 3 h at 100°), and the neutralised ($BaCO_3$) hydrolysate was centrifuged and evaporated to dryness. The residue, after two crystallisations from methanol, gave galactose, m.p. 160–161°, $[\alpha]_D^{18}$ 0° (*c* 0.57). A further portion of the oligosaccharide (10 mg) in redistilled *N*,*N*-dimethylformamide (1.0 ml) was cooled to 0°, and redistilled methyl iodide (1.0 ml) and dry silver oxide (1.0 g) were added[13]. The mixture was stirred in the dark for 3 h at 0° and then for 45 h at room temperature. The product was filtered, and the silver salts were thoroughly washed with chloroform. The combined filtrate and washings were concentrated to dryness, and traces of *N*,*N*-dimethylformamide were removed under high vacuum (0.1 mmHg, 40°, 5 min). A solution of the dry residue in chloroform was filtered and concentrated to a syrup (9.2 mg), which was given one treatment with the Purdie reagents[14]. T.l.c. (spray *4*) of the product indicated that methylation was complete. A portion of the methylated product on hydrolysis (0.5M sulphuric acid) and examination by t.l.c. (spray *4*) revealed spots having the mobilities of 2,3,4,6-tetra-*O*-methylgalactose (blue-grey; R_{TMG} 1) and 2,3,6-tri-*O*-methylgalactose (brown-grey; R_{TMG} 0.88). The remainder of the methylated product was refluxed with 3% methanolic hydrogen chloride for 6 h, and the derived methyl glycosides were examined by g.l.c. Peaks corresponding to 2,3,4,6-tetra-*O*-methylgalactose (*T* 1.88) and 2,3,6-tri-*O*-methylgalactose (*T* 3.29, 4.02, 4.26, and 4.58), in the molar ratio 1.0:1.02, were observed. The above evidence suggests that this disaccharide is 4-*O*-β-D-galactopyranosyl-L-galactose (**1**). The β-D configuration is assumed from the specific rotation. The α-D-linked disaccharide would be expected to have a positive, specific rotation.

The mother liquor from Fraction III, after the crystallisation of 4-*O*-β-D-galactopyranosyl-L-galactose, was fractionated on Whatman No. 1 paper (solvent *B*). This afforded a chromatographically homogeneous syrup (25 mg) having R_{Gal} 0.31 (solvent *B*) and 0.34 (solvent *A*), $[\alpha]_D^{19}$ −20° (*c* 0.6). Paper chromatography of a partial, acid hydrolysate revealed the presence of galactose and the original material. Complete hydrolysis of an aliquot (9 mg) gave galactose having $[\alpha]_D^{18}$ 0° (*c* 0.43). Reduction of another aliquot (12 mg), followed by hydrolysis and separation of the products on Whatman No. 1 paper (solvent *A*), gave chromatographically homogeneous galactose, $[\alpha]_D^{21}$ −73° (*c* 0.48). Methylation of a further aliquot (5.7 mg), as above, gave, on g.l.c. examination of the glycosides, peaks with the retention times of

2,3,4,6-tetra-*O*-methylgalactose (*T* 1.80) and 2,4,6-tri-*O*-methylgalactose (*T* 4.05 and 4.50), in the molar ratio 1.0:1.05. The presence of these sugars was confirmed by paper chromatography of an acid hydrolysate of a portion of the fully methylated oligosaccharide. These results indicate that this disaccharide is 3-*O*-α-L-galactopyranosyl-D-galactose (**2**). The α-L configuration is assumed from the negative, specific rotation. The β-L-linked disaccharide would be expected to have a positive, specific rotation.

Fraction IV. The syrup (0.108 g), eluted with 5% aqueous ethanol (11 l), was shown by paper chromatography (solvent *A*) to be a mixture of oligosaccharides, R_{Gal} 0.36 (major), 0.34, 0.26, 0.17, and 0.13 (the last three in minute traces). Fractionation on Whatman No. 1 paper (solvent *A*) yielded a chromatographically homogeneous syrup (41 mg) having R_{Gal} 0.36 (solvent *A*), which readily crystallised from methanol (charcoal) to give 4-*O*-β-D-galactopyranosyl-D-galactose, m.p. and mixed m.p. 203–205°, $[\alpha]_D^{17}$ +84 (3 min) → +70° (final) (*c* 0.5). The infrared spectrum was identical with that of 4-*O*-β-D-galactopyranosyl-D-galactose (**3**). A portion of the disaccharide was methylated and methanolysed, and the derived methyl glycosides were examined by g.l.c. Peaks corresponding to 2,3,4,6-tetra-*O*-methylgalactose (*T* 1.78) and 2,3,6-tri-*O*-methylgalactose (*T* 3.14, 3.78, 4.14, and 4.45), in the molar ratio of 1.0:1.06, were observed.

Fraction V. The syrup (18 mg), eluted with 7.5% aqueous ethanol (8.5 l), was shown by paper chromatography (solvent *A*) to be a mixture of three oligosaccharides having R_{Gal} 0.13, 0.17, and 0.36. The fraction was not further examined.

Fraction VI. The syrup (0.206 g), eluted with 10% aqueous ethanol (15 l), contained four oligosaccharides having R_{Gal} 0.1 (major), 0.32, 0.17 (trace), and 0.24 (trace) (solvent *A*). Separation on Whatman No. 1 paper (solvent *A*), followed by extraction of the appropriate portions of the papers with 50% aqueous methanol, afforded a chromatographically homogeneous syrup (80 mg), R_{Gal} 0.1 (solvent *A*), which, after crystallisation from methanol, had m.p. 229–230° (dec.), $[\alpha]_D^{19}$ −36 (3 min) → −44° (final) (*c* 0.50). Paper chromatography of a neutralised, partial, acid hydrolysate revealed the presence of 4-*O*-β-D-galactopyranosyl-L-galactose, 3-*O*-α-L-galactopyranosyl-D-galactose, galactose, and the original material. Complete hydrolysis with acid gave galactose having $[\alpha]_D^{20}$ +28° (*c* 0.49). The value for the specific rotation indicates a D:L-isomer ratio of 2:1. Reduction of the saccharide, followed by paper chromatography of a partial, acid hydrolysate of the non-reducing syrup, revealed the presence of 4-*O*-β-D-galactopyranosyl-L-galactose and galactose. A portion of the oligosaccharide (5 mg) was methylated and hydrolysed, and the products were examined by paper chromatography (solvent *B*; spray *1*). Spots with the mobilities of 2,3,4,6-tetra-*O*-methylgalactose, 2,4,6-tri-*O*-methylgalactose (R_{TMG} 0.73 in solvent *B*), and 2,3,6-tri-*O*-methylgalactose (R_{TMG} 0.83 in solvent *B*) were observed. The latter two are indistinguishable in solvent *A*. These results indicate that this trisaccharide is probably *O*-β-D-galactopyranosyl-(1 → 4)-*O*-α-L-galactopyranosyl-(1 → 3)-D-galactose (**4**).

Fraction VII. The syrup (0.144 g), eluted with 10–15% aqueous ethanol (11.5 l), was shown (paper chromatography) to be a mixture of Fractions VI and VIII.

Fraction VIII. The syrup (53 mg), eluted with 20% aqueous ethanol (4 l), contained an oligosaccharide having R_{Gal} 0.04, and traces of three other oligosaccharides having R_{Gal} 0.01, 0.1, and 0.14 (solvent *A*). Fractionation on Whatman No. 1 paper (solvent *A*) gave a chromatographically pure syrup (24 mg), R_{Gal} 0.04, $[\alpha]_D^{18}$ −66° (*c* 0.58). Partial, acid hydrolysis gave (paper chromatography; solvents *A* and *B*) galactose, 4-*O*-β-D-galactopyranosyl-L-galactose, 3-*O*-α-L-galactopyranosyl-D-galactose, *O*-β-D-galactopyranosyl-(1→4)-*O*-α-L-galactopyranosyl-(1→3)-D-galactose, an oligosaccharide having R_{Gal} 0.13 (solvent *A*), and the starting material. Complete, acid hydrolysis gave galactose having $[\alpha]_D^{22}$ 0 ±3° (*c* 0.35). Reduction of the oligosaccharide as above, followed by partial hydrolysis of the non-reducing syrup with acid, gave (paper chromatography) *O*-β-D-galactopyranosyl-(1→4)-*O*-α-L-galactopyranosyl-(1→3)-D-galactose, 4-*O*-β-D-galactopyranosyl-L-galactose, 3-*O*-α-L-galactopyranosyl-D-galactose, and galactose. A portion of the oligosaccharide was methylated and hydrolysed, and paper-chromatographic examination (solvents *A* and *B*; spray *I*) of the products revealed the presence of 2,3,4,6-tetra-*O*-methylgalactose, 2,3,6-tri-*O*-methylgalactose, and 2,4,6-tri-*O*-methylgalactose. These results indicate that this is a tetrasaccharide with a probable composition of *O*-β-D-galactopyranosyl-(1→4)-*O*-α-L-galactopyranosyl-(1→3)-*O*-D-galactopyranosyl-(1→4)-L-galactose (**5**). We prefer not to speculate on the anomeric configuration of the remaining (1→4)-glycosidic link in **5**, because we have been unable to characterise a trisaccharide with the composition *O*-L-Gal*p*-(1→3)-*O*-D-Gal*p*-(1→4)-L-Gal among the products of partial hydrolysis.

Fraction IX. The syrup (28 mg), eluted with 20–25% aqueous ethanol (4.5 l), was a mixture of two oligosaccharides, R_{Gal} 0.04 and 0.06 (trace) (solvent *A*).

Fraction X. The syrup (22 mg), eluted with 25–40% aqueous ethanol (2.5 l), consisted mainly of an oligosaccharide having R_{Gal} 0.02, together with traces of two other oligosaccharides, R_{Gal} 0.04 and 0.06 (solvent *A*).

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