Carbohydrate Research, 43 (1975) 133–143

THE GALACTAN SULPHATE OF THE RED ALGA *Polysiphonia lanosa*

JOHN F. BATEY AND JAMES R. TURVEY

School of Physical and Molecular Sciences, University College of North Wales, Bangor, N. Wales LL57 2UW (Great Britain)

(Received December 10th, 1974; accepted for publication, January 20th, 1975)

ABSTRACT

The structure of the galactan sulphate of *P. lanosa* has been established by a combination of methylation, treatment with alkali, and partial methanolysis of the alkali-treated polysaccharide to give derivatives of agarobiose. The polysaccharide belongs to the agar class, in which 3-linked derivatives of β-D-galactose alternate with 4-linked derivatives of α-L-galactose in a repeating sequence. In addition to D-galactose itself, the 3-linked units include 6-*O*-methyl-D-galactose, D-galactose 6-sulphate, and a hitherto unreported unit, 6-*O*-methyl-D-galactose 4-sulphate. The 4-linked units include L-galactose 6-sulphate, 2-*O*-methyl-L-galactose 6-sulphate, and 3,6-anhydro-L-galactose.

INTRODUCTION

It is generally agreed that a common structural feature of the galactan sulphates of many red algae is an alternating chain of galactose (or galactose derivatives) of the type –A–B–A–B–[1]. The A-units are linked through C-3 and C-1 with the β configuration and are either D-galactose, or methyl ethers or monosulphates of this sugar. The B-units are linked through C-4 and C-1 with the α configuration, but the nature of the unit depends on whether the polysaccharide belongs to the carrageenan or the agar family. In carrageenans[2], B is always a derivative of D-galactose, with the 6-sulphate, the 3,6-anhydro derivative, and, in some cases, additional sulphate at C-2, as common variants. In the agars, B is always a derivative of L-galactose, and typical variants are the 6-sulphate, the 3,6-anhydro sugar, or 2-*O*-methyl derivatives of these two units.

Some exceptions to this general pattern have been reported, and one of these is the galactan sulphate of *Polysiphonia lanosa* (formerly *P. fastigiata*). This polysaccharide contains[3] both D and L enantiomers of galactose, of 3,6-anhydrogalactose, of 6-*O*-methylgalactose, and of galactose 6-sulphate. These findings suggest either a hybrid structure between the agar and carrageenan types, or the presence of two distinct polysaccharides in this alga. The botanical classification of *P. lanosa* places it in the same order and family as *Laurencia pinnatifida*, the galactan of which belongs

to the agar type[4]. We have now re-examined the structure of the galactan sulphate from *P. lanosa*.

RESULTS AND DISCUSSION

Extraction of the seaweed with hot water gave a viscous solution from which a polysaccharide was obtained in 30% yield. Quantitative analysis of the constituent units gave the following molar ratios: 6-*O*-methylgalactose (1.0), galactose (4.5), 3,6-anhydrogalactose (2.5), 2-*O*-methylgalactose (0.07), sulphate ester (4.42), xylose (0.45), and glucose (0.25). The last two monosaccharides were assumed not to be constituents of the major polysaccharide (galactan sulphate), since subsequent studies failed to furnish any evidence for their linkage to the galactan sulphate. It is also known that separate glucans (floridean starch) and xylans are usually found in the water-soluble polysaccharides of many red algae[5]. The monosaccharides obtained by complete acid hydrolysis of the polysaccharide were used to characterise all the galactose derivatives present, with the exception of the 3,6-anhydro sugar, which was obtained as the aldonic acid after oxidative hydrolysis[6] of the polysaccharide. 6-*O*-Methylgalactose was present solely as the D enantiomer, galactose as a mixture of D and L forms in the ratio 3.4:1.1, and 2-*O*-methylgalactose and 3,6-anhydrogalactose as the L forms only. These results conflict with those previously reported[3], which suggested DL mixtures for both 6-*O*-methylgalactose and the anhydro sugar. From our results, the galactan of *P. lanosa* appears to belong to the agar class, as exemplified by porphyran[7] and the galactan of *L. pinnatifida*[4], the former of which contains 6-*O*-methyl-D-galactose and the latter 6-*O*-methyl-D- and 2-*O*-methyl-L-galactose, while both contain 3,6-anhydro-L-galactose.

The sites occupied by the ester sulphate are of interest, in that porphyran contains mainly L-galactose 6-sulphate with suggested traces of a galactose 4-sulphate[7,8], and the galactan of *L. pinnatifida* has both L-galactose 6-sulphate and D-galactose 2-sulphate residues[4]. Autohydrolysis of the galactan in the acid form, with continuous dialysis of the hydrolysate[9], gave a mixture of neutral and sulphated sugars from which two monosaccharide sulphates were isolated. One component was characterised as galactose 6-sulphate by its chromatographic and electrophoretic properties, by methylation and g.l.c. of the derived methyl galactosides, and by periodate oxidation. The specific optical rotation of the barium salt (+16.4°) was, however, too small for the D enantiomer ($[\alpha]_D$ +33.9°) and suggested the presence of a DL mixture. This was confirmed by hydrolysis to galactose, when both the optical rotation and assay for D-galactose using the enzyme D-galactose oxidase[10] showed the presence of the D and L forms in the ratio 2.6:1.0. The second monosaccharide sulphate was identified as 6-*O*-methyl-D-galactose 4-sulphate on the following evidence. Hydrolysis gave 6-*O*-methylgalactose (paper chromatography) and sulphate in the molar ratio 1:0.97. Comparison with authentic 6-*O*-methyl-D-galactose 4-sulphate[11] showed that the two sulphates had identical chromatographic and electrophoretic mobilities, gave the same infrared spectra, and, after methylation and

subsequent methanolysis, gave the same g.l.c. traces of methyl galactosides. The algal compound had $[\alpha]_D^{20}$ +51° (Ba salt in water) compared with +50° for the synthetic compound.

TABLE I

COMPOSITION OF GALACTAN SULPHATE BEFORE AND AFTER ALKALI TREATMENT

Component	*Molar ratio*	
	Original	*Alkali-treated*
6-*O*-Methyl-D-galactose	1.0	1.0
D-Galactose	3.4	3.6
L-Galactose	1.1	
3,6-Anhydro-L-galactose	2.5	3.5
2-*O*-Methyl-L-galactose	0.07	0
Sulphate	4.4	3.4

It is well known that galactose 6-sulphate residues are the biological precursors of 3,6-anhydrogalactose residues in both agar and carrageenan-type polysaccharides. However, it is only the 4-linked B-units (see Introduction) which can form the anhydro sugar. Treatment of such units with alkali in the presence of sodium borohydride can also cause desulphation and formation of the anhydro sugar. Since both D- and L-galactose 6-sulphate are present in the polysaccharide, treatment with alkaline borohydride should distinguish 2- or 4-linked units (B-units) from 3-linked units (A-units). The results of treating the polysaccharide in this way are summarised in Table I; for purposes of comparison, it is assumed that 6-*O*-methyl-D-galactose is unaffected by this treatment. From Table I, it is seen that there is an exact correspondence between the sulphate lost and the increase in 3,6-anhydro sugar. However, the amount of galactose lost was less than the amount of sulphate lost. The discrepancy can be accounted for by the loss of 2-*O*-methyl-L-galactose residues during desulphation. It is known that the analytical method used to measure 3,6-anhydrogalactose also measures the 2-methyl ether of the anhydro sugar[12]. In order to clarify this issue, the alkali-treated polysaccharide was hydrolysed, and the galactose and mono-*O*-methylgalactose fractions were isolated and examined. The galactose isolated was the crystalline D form, and the mono-*O*-methylgalactose fraction contained no trace of 2-*O*-methyl-L-galactose. A further portion of the alkali-treated polysaccharide was completely methanolysed under conditions known to give a mixture of methyl glycosides and acetals of 3,6-anhydrogalactose. Examination of the mixture by t.l.c. revealed 3 components, in addition to those expected for 3,6-anhydrogalactose derivatives. These three components had mobilities and colour responses identical with those of the products obtained by methanolysis of authentic 3,6-anhydro-2-*O*-methyl-L-galactose. It can be assumed, therefore, that both the L-galactose and 2-*O*-methyl-L-galactose are present in the polysaccharide as their respective 4-linked 6-sulphated units, *i.e.*, as B-units, being converted into

anhydro sugars on treatment with alkali. Further evidence in support of this came from the isolation of the monosaccharide sulphates from the alkali-treated polysaccharide, as described above for the native polysaccharide. Two monosaccharide sulphates were isolated, and characterised as D-galactose 6-sulphate and 6-*O*-methyl-D-galactose 4-sulphate.

Confirmation of the linkage positions came from methylation analysis. The native polysaccharide was methylated until the methoxyl content reached a maximum (21.8%), which is close to the theoretical value calculated for complete methylation (21.0%). Oxidative hydrolysis[6] was used to stabilise the anhydro sugar derivatives as aldonic acids, before the hydrolysis of the methylated polysaccharide was completed in the usual way. Separation of the aldonic acids from the neutral methylated sugars led to the characterisation of the sole oxidised anhydro-sugar derivative as 3,6-anhydro-2-*O*-methyl-L-galactonic acid. The 3,6-anhydro-L-galactose residues in the polysaccharide must, therefore, have been linked through C-4. The neutral methylated sugars were separated, and the molar ratios calculated from the amounts actually isolated (Table II); these ratios must be considered as approximations only to the actual proportions present in the methylated polysaccharide.

TABLE II

MOLAR RATIOS OF PRODUCTS FROM METHYLATED POLYSACCHARIDE

Galactose derivative isolated	*Molar ratio*
3,6-Anhydro-2-*O*-methyl-L-[a]	4.6
3-*O*-Methyl-L-	0.20
2-*O*-Methyl-D-	0.20
2-*O*-Methyl-L-	0.18
2,3-Di-*O*-methyl-L-	1.0
2,4-Di-*O*-methyl-D-	5.8
2,6-Di-*O*-methyl-D-	0.88
2,4,6-Tri-*O*-methyl-D-	0.90
2,3,4,6-Tetra-*O*-methyl-D-	0.15

[a]Isolated as the derived aldonic acid.

From the results in Table II, it is concluded that the polysaccharide contains D-galactose at the non-reducing end, and as 3-linked units which give the 2,4,6-tri-*O*-methyl derivative. The D-galactose 6-sulphate is also 3-linked, giving the 2,4-di-*O*-methyl derivative, while the 6-*O*-methyl-D-galactose 4-sulphate, also 3-linked, gives the 2,6-dimethyl ether. In the L series, the 3,6-anhydro sugar is 4-linked (see above), and the L-galactose 6-sulphate and 2-*O*-methyl-L-galactose 6-sulphate are similarly linked, since the 2,3-dimethyl ether was the other major product in the L series. The presence of small quantities of mono-*O*-methylgalactoses cannot be interpreted, since they could arise from branching points, from under- or de-methylation, or from disulphated units. It is significant, however, that in no case was there obtained a D-galactose derivative methylated at C-3 (except for end groups), or an L-galactose

derivative methylated at C-4, in keeping with the classification of this polysaccharide as an agar-type.

Final proof of the classification of this polysaccharide requires that the 3-linked D-galactose residues alternate with the 4-linked units having the L configuration. The native polysaccharide contains so many variants on the basic units that some simplification of the structure seemed desirable before methods of partial fragmentation were attempted. Alkali-treated polysaccharide was chosen since, as shown above, in this modified polysaccharide, the L-galactose and 2-*O*-methyl-L-galactose 6-sulphates have been converted into the corresponding 3,6-anhydro sugars. Clingman *et al.*[13] have shown that, under controlled conditions, methanolysis of an agar can lead to the isolation of agarobiose (3,6-anhydro-4-*O*-β-D-galactopyranosyl-L-galactose) as its dimethyl acetal. When subjected to these conditions, the alkali-treated polysaccharide gave a mixture of products, which were separated by a combination of chromatography on charcoal and t.l.c. In addition to methyl glycosides and acetals of galactose, 3,6-anhydrogalactose, 3,6-anhydro-2-*O*-methyl-galactose, and xylose (all identified chromatographically), 42% of the total material isolated was agarobiose dimethyl acetal. A further 16% consisted of acetals of mono-*O*-methyl- and di-*O*-methyl-agarobiose. A minimum of 58% of the alkali-treated polysaccharide must, therefore, have the alternating sequence of D and L units expected of an agar type, and the figure is probably much higher.

CONCLUSIONS

The idealised structure for agar is an alternating sequence of D-galactose and 3,6-anhydro-L-galactose residues, but this sequence is frequently masked by variants on these two sugars[1]. The galactan sulphate of *P. lanosa* belongs to this general family of agar types with a masked repeating structure. It resembles porphyran[7] in having 6-*O*-methyl-D-galactose and L-galactose 6-sulphate residues as masking units, but, in addition, contains residues of 2-*O*-methyl-L-galactose 6-sulphate, D-galactose 6-sulphate, and 6-*O*-methyl-D-galactose 4-sulphate. Although the first three residues are also constituents of the galactan sulphate from the botanically related species *L. pinnatifida*, the last two differ in that *L. pinnatifida* galactan contains D-galactose 2-sulphate as the only sulphated derivative of D-galactose[4].

The presence of monomethyl ethers of D- and L-galactose in algal polysaccharides has been reported many times[14], but this is the first reported occurrence of a sulphated 6-*O*-methyl-D-galactose. It is obvious that, within the limits defined by the A–B–A–B sequence, other variations in the form of methyl ethers, additional sulphate groups, and pyruvic acid (as the 4,6-acetal) will be frequently encountered in these polysaccharides.

EXPERIMENTAL

General methods. — The methods of paper chromatography, t.l.c., and paper electrophoresis in neutral and in borate buffer have been described[4]. Paper electro-

phoresis in germanate buffer[15] employed 0.05M germanate at pH 10.7. Spray reagents used, in addition to those described before[4], were (*a*) the modified Seliwanoff reagent of Yaphe[16] for 3,6-anhydrogalactose derivatives, and (*b*) the aniline–xylose reagent[17] for glyconic acids. T.l.c. of 3,6-anhydrogalactose derivatives was performed either on cellulose plates with ethyl acetate–acetic acid–formic acid–butanone–water (34:6:2:30:5), or on silica gel G with benzene–methanol (7:3).

Quantitative analysis of sugars by g.l.c. of their glycitol acetates has been described[4]. Sulphate was determined by the method of Lloyd *et al.*[18], and 3,6-anhydrogalactose by the method of Yaphe and Arsenault[19]. D-Galactose was determined in the presence of L-galactose by the specific assay method using D-galactose oxidase[10]. Total galactose, or other aldoses, was determined by the phenol–sulphuric acid method[20].

Extraction of polysaccharide. — The alga was collected in February in the Menai Straits, hand-sorted, and dried at 30° for 2 days before being milled to a fine powder. The powder (330 g) was extracted with water (5 l) at 100° for 24 h, and the residue removed by centrifugation. The residue was extracted as before with water (2 l), and the insoluble material was rejected. The combined extracts were concentrated at 30° to 1.5 l, and polysaccharide was precipitated with ethanol (2 vol.). A solution of the precipitate in water (2 l) was dialysed against tap water for 4 days, and freeze-dried to give a white, fibrous powder (100 g). Before analysis, the powder was dried *in vacuo* over P_2O_5. The analytical figures are given in the Discussion.

Characterisation of the sugars. — Polysaccharide (10 g) was hydrolysed in 0.75M sulphuric acid for 3 h at 100°, the solution was neutralised with barium carbonate, and insoluble salts were removed by centrifugation. The hydrolysate was concentrated, and the residue (4.9 g) was separated into bands by preparative paper chromatography. Chromatographically pure galactose and xylose were obtained, but a further band contained a mixture of mono-*O*-methylgalactoses. This mixture was resolved by preparative paper electrophoresis in borate buffer to give 6-*O*-methyl- and 2-*O*-methyl-galactose.

Galactose. The syrup (490 mg) had $[\alpha]_D^{20}$ +42° (water), which indicated a DL-mixture having a D to L ratio of 3.4:1.0. Assay for total galactose and then for D-galactose using D-galactose oxidase gave a D to L ratio of 3.2:1.0. A portion of the syrup (50 mg) was converted into the phenylosazone (12 mg), m.p. 185–189°, $[\alpha]_D^{20}$ 0° (methanol), indicating that a racemic mixture had crystallised; lit.[21] m.p. 185–187°.

6-O-Methyl-D-galactose. The syrup (260 mg) crystallised on standing and, after recrystallisation from ethanol, the product had m.p. 119–120°, $[\alpha]_D^{20}$ +120→71° (24 h, water); lit.[22] m.p. 122–123°, $[\alpha]_D$ +117→+77.3°. The derived phenylosazone had m.p. 195–196°, $[\alpha]_D^{20}$ +135→+92° (24 h, pyridine); lit.[23] m.p. 195–196°, $[\alpha]_D$ +130→+92°.

2-O-Methyl-L-galactose. The syrup (18 mg) migrated with authentic 2-*O*-methyl-D-galactose on electrophoresis in borate buffer, gave a red–orange colour with the *p*-anisidine hydrochloride spray reagent, but was not detected with the triphenyltetrazolium hydroxide spray, indicating a substituent at C-2. It had $[\alpha]_D^{20}$ −79.3°

(water); lit.[23] $[\alpha]_D$ −78°. The derived aldonolactone had $[\alpha]_D^{20}$ +20.6° (water), lit.[23] $[\alpha]_D$ +20.2°.

3,6-Anhydro-L-galactose. — The polysaccharide (10 g) in 0.25M sulphuric acid (800 ml) containing bromine (2 ml) was heated at 60° for 23 h, with periodic additions of bromine to replace that lost by evaporation. The products of oxidative hydrolysis were isolated as described previously[6]. 3,6-Anhydro-L-galactonic acid was separated from other products by t.l.c. on cellulose and obtained as a syrup (340 mg), $[\alpha]_D^{20}$ −35° (water); lit.[6] for D form, $[\alpha]_D$ +33°. The derived 2,4,5-tri-*O*-*p*-nitrobenzoyl derivative had m.p. 189–193°, $[\alpha]_D^{20}$ +12.5° (acetone); lit.[6] for D form, m.p. 187–191°, $[\alpha]_D$ −14°.

Autohydrolysis of the polysaccharide[9]. — The polysaccharide (2 g) in water (100 ml) was converted into the acid form by stirring with Zeocarb 225(H^+) resin. The mixture was filtered into a dialysis tube, and fresh resin (2 g) was added. The tube was placed in a beaker containing water (700 ml) and barium carbonate (2 g), which was stirred at 80°. The contents of the tube were also stirred. The water and barium carbonate in the beaker were replaced at 1-h intervals during 6 h. The combined dialysate was filtered and concentrated to a syrup (0.90 g). Paper electrophoresis in neutral buffer indicated the presence of neutral sugars, monosaccharide monosulphates, and oligosaccharide sulphates. Preparative paper electrophoresis in neutral buffer was used to separate the monosaccharide sulphates (530 mg). Examination by paper chromatography, t.l.c., and electrophoresis in borate buffer indicated that galactose 6-sulphate was the main constituent, but that a minor amount of a second monosulphate was present. Complete, acid hydrolysis of the mixture showed that galactose was the major constituent sugar with 6-*O*-methylgalactose in smaller amounts. Preparative paper chromatography of a portion (100 mg) of the mixture, using ethyl acetate–acetic acid–formic acid–water (18:8:3:9), separated the two sugar sulphates, which were isolated as barium salts.

(*a*) *Galactose 6-sulphate* (38.6 mg). A portion (20 mg) in water (2 ml) was converted into the sodium form by passage through a column of Zeocarb 225 (sodium form) resin, and the eluate was concentrated to a small volume (5 ml). Galactose and sulphate were determined on a portion of this solution, and the molar ratio of galactose to sulphate was 1.0:1.05. On oxidation of another portion in 15mM sodium metaperiodate[24], 3.0 moles of oxidant per mole of sugar sulphate were consumed in 25 min, rising to 3.5 moles in 24 h. Authentic D-galactose 6-sulphate oxidised under the same conditions consumed 3 moles in 20 min, rising to 3.7 moles in 24 h. A further portion of the solution was evaporated to dryness, the residue was methylated in *N*,*N*-dimethylformamide[25], and the product was methanolysed and examined by g.l.c.[23]. Peaks with retention times of those of an equilibrium mixture of glycosides from 2,3,4-tri-*O*-methyl-D-galactose were obtained. The original barium salt had $[\alpha]_D^{20}$ +16.4°, which is between the values for the D and L forms of galactose 6-sulphate, and indicated a D to L ratio of 2.8:1.0. A portion of the sugar sulphate (10 mg) was hydrolysed in 0.75M sulphuric acid (50 ml) at 100° for 2 h, the acid was neutralised, and the products were desalted on Bio-Deminrolit mixed-bed resin (carbonate form). The product was galactose (paper chromatography) with $[\alpha]_D^{20}$ +37° (water), in-

dicating a D to L ratio of 2.7:1.0. Assay by D-galactose oxidase indicated a D to L ratio of 2.5:1.0.

6-O-Methyl-D-galactose 4-sulphate. The second sugar sulphate (7.5 mg) isolated gave 6-*O*-methylgalactose only (paper chromatography in 3 solvents) on hydrolysis. The molar ratio of 6-*O*-methylgalactose to sulphate was 1.0:0.97. The sulphate had chromatographic and electrophoretic mobilities identical with those of authentic 6-*O*-methyl-D-galactose 4-(barium sulphate)[11], and it had $[\alpha]_D^{20}$ +51° (water); lit.[11] $[\alpha]_D$ +50°. Methylation of a portion (2 mg), and methanolysis and g.l.c. of the derived methyl glycosides[11] gave a pattern of peaks identical with those obtained when authentic D-galactose 4-sulphate was similarly treated.

Alkali-treated polysaccharide. — The polysaccharide (20 g) in water (600 ml) containing sodium borohydride (2 g) was left at 20° for 3 days. The solution was diluted with water to 2.3 l, and sodium borohydride (28 g) and sodium hydroxide (93 g) were added. The mixture was heated at 80° and portions (1 ml) were removed at intervals for analysis for 3,6-anhydrogalactose. A constant value was obtained after 8 h, and the solution was then cooled, dialysed for 4 days, concentrated, and freeze-dried; yield, 16.0 g. The analytical results are given in Table I.

Alkali-treated polysaccharide (5.0 g) was completely hydrolysed, as described previously, to give a sugar syrup (2.0 g). Galactose and mono-*O*-methylgalactose fractions were separated from the syrup by preparative paper chromatography. The galactose fraction, which crystallised on standing, had $[\alpha]_D^{20}$ +77° (24 h, water), in close agreement with the figure (+80°) for the D form. The mono-*O*-methylgalactose fraction was examined by electrophoresis in borate buffer. Only 6-*O*-methyl-D-galactose was detected, and this was confirmed by the rotation, $[\alpha]_D^{20}$ +76°, of the syrup; lit.[22] $[\alpha]_D$ +77.3°. A further portion (100 mg) of alkali-treated polysaccharide was methanolysed in 3% methanolic hydrogen chloride for 6 h under reflux, and the solution was cooled, neutralised (silver carbonate), filtered, and concentrated. The resulting mixture of glycosides and acetals was examined by t.l.c. on silica gel, with controls prepared by methanolysing authentic 3,6-anhydro-D-galactose and its 2-methyl ether. The latter component gave a pattern of three spots with characteristic red colours when sprayed with the Seliwanoff reagent. Spots with identical mobilities, and giving the same colour, were present in the methanolysate of the polysaccharide, in addition to those expected for 3,6-anhydrogalactose derivatives.

Alkali-treated polysaccharide (2 g) was autohydrolysed, as described above, and galactose 6-sulphate isolated as the barium salt (24 mg). The barium salt, precipitated with ethanol, had $[\alpha]_D^{20}$ +34.7° (water), identical with the value found for authentic D-galactose 6-(barium sulphate). Confirmation that only the D enantiomer was present was obtained by hydrolysis, which gave D-galactose, $[\alpha]_D^{20}$ +79°.

Methylation of the polysaccharide. — The native polysaccharide (20 g) was methylated as described by Dolan and Rees[26] until the methoxyl content was constant at 21.8%. The yield was 8.1 g. Methylated polysaccharide (5.0 g) was subjected to oxidative hydrolysis[6], and the products were stirred with Amberlite IR-120(H^+) resin before being slowly passed down a column (30 × 3.5 cm) of

Sephadex A-25 (formate form). Elution with water (1 litre) gave syrupy, neutral products (3.0 g). Acidic products were then eluted from the column with 2% aqueous formic acid (2 l), and the eluate was concentrated (20°) by vacuum distillation, water being repeatedly added during concentration until all formic acid had been removed. Examination of the residual syrup (1.05 g) by t.l.c. on cellulose indicated that 3,6-anhydro-2-*O*-methylgalactonic acid was the sole, migrating product; traces of methylated aldonic acids were present at the origin. Preparative t.l.c. gave chromatographically pure 3,6-anhydro-2-*O*-methyl-L-galactonic acid which crystallised (819 mg). After recrystallisation from ethyl acetate, it had m.p. 139–141°, $[\alpha]_D^{20}$ −77.5° (methanol); lit.[27] m.p. 141–142°, $[\alpha]_D$ −70.3°. The derived methyl ester had m.p. 90–91°, $[\alpha]_D^{20}$ −71.0° (methanol); lit.[27] m.p. 90–91°, $[\alpha]_D$ −70.8°.

The neutral products from the column were fractionated by preparative paper chromatography in several solvent systems until chromatographically pure fractions were obtained, as follows (all physical constants are taken from ref. 28, unless shown otherwise). 2,4-Di-*O*-methyl-D-galactose (0.90 g), m.p. 106–108° (from acetone), $[\alpha]_D^{20}$ +108 → +84.5° (24 h, water); lit. m.p. 105–108°, $[\alpha]_D$ +113° → +85°. The derived anilide had m.p. 215–216°; lit. m.p. 216°. 2,4,6-Tri-*O*-methyl-D-galactose (149 mg) crystallised on standing for several weeks and, after recrystallisation from ether, had m.p. 101–102°, $[\alpha]_D^{20}$ +113 → +86° (24 h, water); lit. m.p. 102–105°, $[\alpha]_D^{20}$ +124 → +90°. Its identity was confirmed by g.l.c. of the derived methyl glycosides, and co-chromatography with an authentic sample of 2,4,6-tri-*O*-methyl-D-galactose. The anilide had m.p. 166°; lit. m.p. 166°. 2,3,4,6-Tetra-*O*-methyl-D-galactose (26 mg) crystallised from ethanol with m.p. 69°, $[\alpha]_D^{20}$ +137 → +114° (24 h, water); lit. m.p. 72°, $[\alpha]_D$ +142 → +117°. A second di-*O*-methylgalactose fraction (288 mg) was homogeneous by paper chromatography, but was separated by preparative paper electrophoresis in germanate buffer[15] into two components with M_G 0.1 and 0.5. The zones were eluted with water, and the eluates neutralised with Bio-Deminrolit resin (carbonate form) and evaporated to dryness. Extraction of the residues with boiling ethyl acetate, and evaporation, gave the products in pure form. 2,3-Di-*O*-methyl-L-galactose (M_G 0.1 in germanate) was a syrup (144 mg) with $[\alpha]_D^{20}$ −84°; lit.[23] $[\alpha]_D$ −80°. The anilide had m.p. 124–127°, mixture m.p. 125–128° with an authentic sample[23]. 2,6-Di-*O*-methyl-D-galactose (M_G 0.5 in germanate) crystallised (125 mg) from ethyl acetate with m.p. 126–128°, mixture m.p. with an authentic sample 128°, $[\alpha]_D^{20}$ +83°; lit. $[\alpha]_D$ +87.5°. The anilide had m.p. 119–120°; lit. m.p. 121–122°. 2-*O*-Methylgalactose (57 mg) was isolated as a syrup, $[\alpha]_D^{20}$ +2.9° (water), indicating a mixture of D and L forms. Its identity was established by its electrophoretic mobility (M_G 0.46 in borate buffer), by its R_F value in three solvent systems, and by its colour reactions with spray reagents (orange–red with *p*-anisidine hydrochloride, no colour with triphenyltetrazolium hydroxide). The anilide had m.p. 163°; lit. for D form, m.p. 165°. 3-*O*-Methyl-L-galactose (30 mg) was identified by its M_G value (0.69) in borate buffer, by its R_F value in three solvent systems, and by $[\alpha]_D^{20}$ −100° (water); lit. for D form, $[\alpha]_D$ +108°.

Partial methanolysis of alkali-treated polysaccharide. — The alkali-treated

polysaccharide (2 g) was dried over P_2O_5, and then suspended in 1% methanolic hydrogen chloride (50 ml) and heated under reflux for 1 h. The solution was cooled, neutralised with silver carbonate, filtered, and concentrated to a syrup (1.9 g). The syrup in water (5 ml) was chromatographed on a column (42×4 cm) of charcoal (Ultrasorb S.C. 120/240, British Carbonorit Union) and Celite (1:1) as described by Whelan *et al.*[29]. Eluting solvents were 2% (5 l), 3% (5 l), and 5% aqueous ethanol (5 l), followed by a linear gradient of 5→26% aqueous ethanol over 45 l. Fractions (50 ml) were collected, tested for carbohydrate with the phenol–sulphuric acid reagent, and combined as suggested by the elution curve. Combined fractions were evaporated to dryness and the sugars were extracted with methanol. The sugars eluted with 2% ethanol were methyl glycosides of galactose (200 mg), identified chromatographically and by hydrolysis to galactose. 3% Ethanol eluted a mixture (57 mg) of glycosides of galactose and xylose. No sugars were eluted with 5% ethanol. The fractions eluted with the ethanol gradient were combined into 5 groups (*A* to *E*). Fraction *A* (50 mg) was identified by paper chromatography and t.l.c. as mixed glycosides and acetals of 6-*O*-methylgalactose and 3,6-anhydrogalactose. Acid hydrolysis gave 6-*O*-methylgalactose and 5-hydroxymethyl-2-furaldehyde. Fraction *B* (174 mg) contained glycosides and acetals of 3,6-anhydrogalactose, oxidative hydrolysis giving 3,6-anhydrogalactonic acid (t.l.c.). Fraction *C* (470 mg) contained some minor constituents, but the major portion was identified as the dimethyl acetal of agarobiose (3,6-anhydro-4-*O*-β-D-galactopyranosyl-L-galactose). This compound was separated from a portion of the mixture by t.l.c. on cellulose in a chromatographically pure form, $[\alpha]_D^{20}$ −26.3° (water); lit.[30] $[\alpha]_D$ −29°. The derived hexa-acetate had m.p. 134°, $[\alpha]_D^{20}$ −13°; lit.[13] m.p. 137–138°, $[\alpha]_D$ −13.5°. Fraction *D* (200 mg) contained a mixture of agarobiose dimethyl acetal with glycosides and acetals of 3,6-anhydro-2-*O*-methylgalactose and the dimethyl acetal of a mono-*O*-methylagarobiose. The components were separated by preparative t.l.c., and the mono-*O*-methylagarobiose derivative (50 mg) was subjected to oxidative hydrolysis, the products being examined by t.l.c. The major products were 6-*O*-methyl-D-galactose and 3,6-anhydro-L-galactonic acid, with smaller amounts of D-galactose and 3,6-anhydro-2-*O*-methyl-L-galactonic acid. Fraction *E* (95 mg) contained the mono-*O*-methylagarobiose acetals as major component, with a di-*O*-methylagarobiose dimethyl acetal as a minor component. The latter was isolated (20 mg) by t.l.c. and subjected to oxidative hydrolysis. The only products (t.l.c.) were 6-*O*-methyl-D-galactose and 3,6-anhydro-2-*O*-methyl-L-galactonic acid, suggesting that the original compound was the dimethyl acetal of 3,6-anhydro-2-*O*-methyl-4-*O*-(6-*O*-methyl-β-D-galactopyranosyl)-L-galactose.

ACKNOWLEDGMENTS

We thank Dr. D. A. Rees for the gift of specimens, and S.R.C. for financial support.

REFERENCES

1 N. S. ANDERSON, T. C. S. DOLAN, AND D. A. REES, *Nature (London)*, 205 (1965) 1060–1062.
2 A. PENMAN AND D. A. REES, *J. Chem. Soc. Perkin I*, (1973) 2182–2187.
3 M. MCKENZIE, Ph.D. Thesis, University of Edinburgh, 1953.
4 D. M. BOWKER AND J. R. TURVEY, *J. Chem. Soc. C*, (1968) 983–988.
5 J. R. TURVEY AND E. L. WILLIAMS, *Phytochemistry*, 9 (1970) 2383–2388.
6 N. S. ANDERSON, T. C. S. DOLAN, AND D. A. REES, *J. Chem. Soc. C*, (1968) 596–601.
7 S. PEAT, J. R. TURVEY, AND D. A. REES, *J. Chem. Soc.*, (1961) 1590–1595; N. S. ANDERSON AND D. A. REES, *J. Chem. Soc.*, (1965) 5880–5887.
8 T. P. WILLIAMS, Ph.D. Thesis, University of Wales, 1961; D. A. REES, *J. Chem. Soc.*, (1961) 5168–5171.
9 T. J. PAINTER, *Chem. Ind. (London)*, (1959) 1488.
10 H. ROTH, S. SEGAL, AND D. BERTOLI, *Anal. Biochem.*, 10 (1965) 32–52.
11 J. F. BATEY AND J. R. TURVEY, *Carbohyd. Res.*, 38 (1974) 316–319.
12 C. ARAKI, *Mem. Fac. Ind. Arts, Kyoto Tech. Univ., Sci. Technol.*, 2B (1953) 17–52.
13 A. L. CLINGMAN, J. R. NUNN, AND A. R. STEPHEN, *J. Chem. Soc.*, (1957) 197–203.
14 C. ARAKI, in E. G. YOUNG AND J. L. MCLACHLAN (Eds.), *Proc. Intern. Seaweed Symp., 5th, Halifax*, Pergamon Press, London, 1966, pp. 3–17.
15 B. LINDBERG AND B. SWAN, *Acta Chem. Scand.*, 14 (1960) 1043–1050.
16 W. YAPHE, *Can. J. Microbiol.*, 3 (1957) 987–993.
17 J. SAARNIO, E. NISKASAARI, AND C. GUSTAFSSON, *Suomen. Kem.*, 25B (1952) 25–29.
18 P. F. LLOYD, B. EVANS, AND R. J. FIELDER, *Carbohyd. Res.*, 11 (1969) 129–136.
19 W. YAPHE AND G. P. ARSENAULT, *Anal. Biochem.*, 13 (1965) 143–148.
20 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
21 N. K. RICHTMYER, *Methods Carbohyd. Chem.*, 2 (1963) 127–131.
22 J. R. NUNN AND M. M. VON HOLDT, *J. Chem. Soc.*, (1957) 1094–1097.
23 D. M. BOWKER AND J. R. TURVEY, *J. Chem. Soc. C*, (1968) 989–992.
24 G. O. ASPINALL AND R. J. FERRIER, *Chem. Ind. (London)*, (1957) 1216.
25 R. KUHN, H. TRISCHMANN, AND I. LÖW, *Angew. Chem.*, 67 (1955) 32.
26 T. C. S. DOLAN AND D. A. REES, *J. Chem. Soc.*, (1965) 3534–3539.
27 C. ARAKI AND S. HIRASE, *Bull. Chem. Soc. Jap.*, 33 (1960) 291–295.
28 F. SMITH AND R. MONTGOMERY, *The Chemistry of Plant Gums and Mucilages*, Reinhold, New York, 1959; R. L. WHISTLER AND J. N. BEMILLER, *Methods Carbohyd. Chem.*, 5 (1965) 298–357.
29 W. J. WHELAN, J. M. BAILEY, AND P. J. P. ROBERTS, *J. Chem. Soc.*, (1953) 1293–1298.
30 C. ARAKI AND K. ARAI, *Bull. Chem. Soc. Jap.*, 30 (1957) 287–293.