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# THE LINKAGE OF 4-*O*-METHYL-L-GALACTOSE IN THE SULPHATED POLYSACCHARIDE OF *Aeodes ulvoidea**

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ABSTRACT

Six disaccharides and one trisaccharide, namely, 6-*O*-β-D-galactopyranosyl-D-galactose, 4-*O*-β-D-galactopyranosyl-D-galactose, 6-*O*-(4-*O*-methyl-α-L-galactopyranosyl)-D-galactose, 3-*O*-(2-*O*-methyl-α-D-galactopyranosyl)-D-galactose, 4-*O*-β-D-galactopyranosyl-2-*O*-methyl-D-galactose, *O*-(β-D-galactopyranosyl)-(1→4)-*O*-[4-*O*-methyl-α-L-galactopyranosyl-(1→6)]-D-galactose, 2-*O*-methyl-4-*O*-(6-*O*-methyl-β-D-galactopyranosyl)-D-galactose were isolated and characterized from a partial, acid hydrolysate of the sulphated polysaccharide from *Aeodes ulvoidea*. These results demonstrate, for the first time, the mode of linkage of 4-*O*-methyl-L-galactose in a red-seaweed polysaccharide.

## INTRODUCTION

The presence of 4-*O*-methylgalactose in Nature was first demonstrated by Araki *et al.*[2], who isolated a small amount of the L isomer from the agar of *Gelidium amansii*. Since then, the sugar has been obtained (mainly as the L isomer) from several polysaccharides isolated from seaweeds belonging to the *Grateloupiaceae* family[3–7]. However, only in the case of the polysaccharide of *Aeodes ulvoidea*[6] does the sugar occur in more than trace quantity. Acid hydrolysis of the polysaccharide of *A. ulvoidea*[6] afforded D-galactose, 4-*O*-methyl-L-galactose, and 2-*O*-methyl-D-galactose, in the molar ratios 10.5:1.1:1, and ester sulphate (19.9%). In addition, the presence in the polymer of trace amounts of xylose, mannose, and 6-*O*-methylgalactose has been demonstrated[6]. Periodate oxidation of the polymer resulted in the cleavage of all of the 4-*O*-methyl-L-galactose residues, while methylation analysis of the partially desulphated polymer suggested that the 4-*O*-methyl-L-galactose residues are present as non-reducing end-groups and that the polymer contained mainly (1→3) and (1→4) linkages[6]. We now report the results of a partial-hydrolysis study performed on the native polymer in order to obtain more information concerning the mode of linkage of the 4-*O*-methyl-L-galactose residues.

*Suiphated polysaccharides of the *Grateloupiaceae* Family: Part VIII. For Part VII, see Ref. 1.

## RESULTS AND DISCUSSION

Partial hydrolysis of the polysaccharide of *Aeodes ulvoidea* followed by fractionation of the neutral products, using a combination of gradient elution from a charcoal–Celite column and paper chromatography, resulted in the isolation and characterization of six disaccharides and one trisaccharide. The saccharides are listed in Table I, together with data essential for the determination of their structures.

The isolation of disaccharide **3** demonstrates, for the first time, the mode of linkage of 4-*O*-methyl-L-galactose in a red-seaweed polysaccharide. This sugar has previously been shown to be present as a non-reducing end-group, by periodate-oxidation studies on the native polymer and by methylation studies on the desulphated polymer[6]. The isolation of the branched trisaccharide **6** demonstrates that the 4-*O*-methyl-L-galactose is linked as a single branch-unit to the (1→4),(1→3)-linked polysaccharide chain.

The isolation of disaccharide **1** from the hydrolysate of a sulphated, red-algal polysaccharide is also novel. This disaccharide has previously been reported as a component of the sulphated polysaccharide isolated from the green alga *Cladophora rupestris*[8]. It is possible that **1** may have arisen from a contaminating, green-seaweed polysaccharide, since *Aeodes ulvoidea* is often associated with a green, endophytic alga, which manifests itself as red patches on the greenish-red thallus. Although great care was taken to remove all visible traces of the endophyte, some of it may have remained. This suggestion tends to be supported by the result of the ultracentrifuge examination of the polysaccharide[6].

G.l.c. of the methanolysates of methylated **1** and **3** revealed, in addition to peaks due to 2,3,4-tri- and 2,3,4,6-tetra-*O*-methylgalactose, a peak at *T* 3.71. This peak is considered to be due to an alkaline-degradation product formed during the methylation of the disaccharides. A similar degradation has been shown to occur during the methylation of (1→3)-linked galactose disaccharides[1]. This is not surprising, since it has been shown[9] that 3-*O*-α-D-galactopyranosyl-D-galactose and galactose trisaccharides possessing a terminal (1→3)-linkage are degraded by saturated, aqueous calcium hydroxide, whereas 4-*O*-β-D-galactopyranosyl-D-galactose and trisaccharides having a terminal (1→4)-linkage survive this treatment. Significantly, g.l.c. of methanolysed, methylated disaccharides **2**, **5**, and **7** gave only those peaks expected of (1→4)-linked galactose disaccharides. The branched trisaccharide **6**, which was shown, by partial hydrolysis, to possess both (1→4) and (1→6) terminal linkages, appeared to be even more susceptible to alkaline degradation than disaccharides **1** and **3**. G.l.c. of the methanolysate of the methylated trisaccharide afforded a large peak due to 2,3,4,6-tetra-*O*-methylgalactose and several minor, anomalous peaks.

Significantly, the major disaccharides (**2** and **5**) isolated from the partial, acid hydrolysate possess β-D-(1→4) linkages. Disaccharides **2**, **4**, **5**, and **7** were previously isolated from the partial hydrolysate of the polysaccharide from *Pachymenia carnosa* (*Grateloupiaceae*).

TABLE I

SACCHARIDES OBTAINED BY PARTIAL HYDROLYSIS

| | *Saccharide*[a] | *Total hydrolysis products* | *Partial hydrolysis products* | *Partial hydrolysis products after reduction* | *Hydrolysis or methanolysis products of methylated oligosaccharide*[d] |
|---|---|---|---|---|---|
| **1** | β-D-Gal-(1→6)-D-Gal (syrup) | Gal | Gal | | *A, C* |
| **2** | β-D-Gal-(1→4)-D-Gal (cryst.)[b] | Gal | Gal | | *A, B* |
| **3** | α-L-4MeGal-(1→6)-D-Gal (syrup) | Gal, 4MeGal | Gal, 4MeGal | 4MeGal | *A, C* |
| **4** | α-D-2MeGal-(1→3)-D-Gal (syrup)[c] | Gal, 2MeGal | | 2MeGal | |
| **5** | β-D-Gal-(1→4)-D-2MeGal (cryst.)[b] | Gal, 2MeGal | Gal, 2MeGal | Gal | |
| **6** | β-D-Gal-(1→4)-[α-L-4MeGal-(1→6)]-D-Gal (syrup) | Gal, 4MeGal | Gal, 4MeGal, 2,3 | Gal,4MeGal | *A* (see Discussion) |
| **7** | β-D-6MeGal-(1→4)-D-2MeGal (syrup) | 6MeGal, 2MeGal | 6MeGal, 2MeGal | 6MeGal | *A, B* |

[a]2MeGal, 2-*O*-methylgalactose; 6MeGal, 6-*O*-methylgalactose; 4MeGal, 4-*O*-methylgalactose. [b]Melting points compared with authentic samples. [c]Chromatographically identical with 3-*O*-(2-*O*-methyl-α-D-galactopyranosyl)-D-galactose. [d]*A*, 2,3,4,6-tetra-*O*-methylgalactose; *B*, 2,3,6-tri-*O*-methylgalactose; *C*, 2,3,4-tri-*O*-methylgalactose.

In addition to the saccharides listed in Table I, chromatographic evidence was obtained for the presence of 4-*O*-β-D-galactopyranosyl-L-galactose in the partial hydrolysate. This disaccharide is a major structural unit of the polysaccharide of *Anatheca dentata* (Solieriaceae)[10], and has also been isolated in trace amount from the sulphated polysaccharide of *Phyllymenia cornea* (*Grateloupiaceae*)[11].

At present, no unique structure can be proposed for the polysaccharide of *Aeodes ulvoideae*. However, the accumulated evidence indicates that the polymer, like other polymers isolated from seaweeds of the *Grateloupiaceae* family, is composed of a polysaccharide chain containing α-D-(1→3) and β-D-(1→4) linkages. In addition, 4-*O*-methyl-L-galactose was shown to occur as single branch-units attached to position 6 of 4-linked galactose residues in the main polysaccharide chain. No evidence is available at present to suggest that the α-D-(1→3)- and β-D-(1→4)-linked units occur in an alternating sequence, as in other *Grateloupiaceae* polysaccharides.

## EXPERIMENTAL

Concentration of solutions was carried out below 45° under diminished pressure, and specific rotations were measured in water on a Perkin–Elmer 141 automatic polarimeter. Paper chromatography (p.c.) was carried out on Whatman No. 1 paper, using (*1*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4), (*2*) butyl alcohol–pyridine–water (9:2:2), and (*3*) ethyl acetate–pyridine–water (8:2:1). Sprays *a* and *b* were, respectively, 2% *p*-anisidine hydrochloride in half-saturated butyl alcohol[12], and 5% triphenyltetrazolium chloride in methanol mixed with an equal part of a mixture of 2 ml of 2.5M sodium hydroxide in 3 ml of methanol, just before spraying[13]. $R_{GAL}$ values refer to the rates of movement of sugars on chromatograms relative to that of D-galactose in solvent *1*. Thin-layer chromatography (t.l.c.) was performed on silica gel G containing calcium sulphate as binder, employing butanone–water (85:7) as solvent, and detection with either aniline–diphenylamine–phosphoric acid[14] followed by heating at 110°, or 10% sulphuric acid in ethanol followed by charring on a hot plate. G.l.c. of the methyl glycosides of methylated sugars was carried out on a Perkin–Elmer 900 gas chromatograph, using a flame-ionization detector and nitrogen as carrier gas. The column packing used was 20% of butane-1,4-diol succinate polyester on Gaschrom P (80–100 mesh) at an operating temperature of 190°. Retention times (*T*) are relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside. Infrared spectra were recorded on a Beckman IR-8 spectrophotometer, using KBr discs.

*Partial hydrolysis of the polysaccharide.* — Polysaccharide (20 g) was hydrolysed with sulphuric acid (250 ml, 0.375M) for 2 h on a boiling water bath, and the hydrolysate was neutralised ($BaCO_3$), centrifuged, and concentrated. The resulting syrup, after dissolution in water (200 ml), was deionized (Amberlite IR-120 and IRA-400 resins) and concentrated to a neutral syrup (7.5 g), which was applied to a charcoal–Celite column (1:1 w/w; 56 × 5.5 cm) and eluted with water and aqueous ethanol (0–16% ethanol in water), using the gradient technique. Fractions (~30 ml) were collected, analysed by paper chromatography, and combined as follows.

*Fraction 1.* The syrup (2.52 g), eluted with water, was shown by p.c. to be mainly galactose.

*Fraction 2.* The crystalline solid (190 mg), eluted with water, was mainly 4-*O*-methylgalactose.

*Fraction 3.* The semi-crystalline syrup (900 mg), eluted with water, contained mostly 4-*O*-methylgalactose and 2-*O*-methylgalactose.

*Fraction 4.* The white solid (136 mg), eluted with 1% aqueous ethanol, contained (p.c., solvents *1* and *3*) 2-*O*-methylgalactose (major component), 4-*O*-methylgalactose, and 6-*O*-methylgalactose. Further fractionation on Whatman No. 1 paper (solvent *3*) resulted in the isolation of a syrup (2 mg), which had $[\alpha]_D$ +30° (*c* 0.2) and was chromatographically identical with 6-*O*-methylgalactose (solvents *1* and *3*, sprays *a* and *b*).

*Fraction 5.* The white solid (140 mg), eluted with 2% aqueous ethanol, contained (p.c., solvent *1*, spray *a*) three oligosaccharides with $R_{GAL}$ 0.21, 0.33, and 0.36. Fractionation of the mixture on Whatman 3MM paper (solvent *1*) afforded a chromatographically homogeneous disaccharide **1** (16 mg), $[\alpha]_D^{25}$ +11° (*c* 0.58), $R_{GAL}$ 0.21, d.p. 2.1, revealed with spray *b*. Hydrolysis of the disaccharide gave galactose only, while partial hydrolysis afforded galactose and starting material. The specific rotation of the acid hydrolysate indicated the presence of D-galactose only. Methylation of **1** by a modified Hakomori procedure, followed by t.l.c. and p.c. of an acid hydrolysate, revealed products having the mobilities of 2,3,4,6-tetra- and 2,3,4-tri-*O*-methylgalactose, and some degradation material. Methanolysis of the hydrolysate followed by g.l.c. of the derived methyl glycosides revealed peaks corresponding to methyl 2,3,4,6-tetra-*O*-methylgalactoside (*T* 1.58), methyl 2,3,4-tri-*O*-methylgalactoside (*T* 5.81), and a degradation product (*T* 3.71). The above facts indicated that **1** was 6-*O*-β-D-galactopyranosyl-D-galactose.

The disaccharide with $R_{GAL}$ 0.33 was chromatographically identical with 4-*O*-β-D-galactopyranosyl-L-galactose[10], while the component with $R_{GAL}$ 0.36 was chromatographically identical with disaccharide **2**.

*Fraction 6.* The semi-crystalline syrup (780 mg), eluted with 3% aqueous ethanol, crystallised readily from aqueous methanol to afford disaccharide **2** which, after recrystallisation, had m.p. 203–204°, $[\alpha]_D^{20}$ +87 (5 min) → +64° (*c* 0.66), $R_{GAL}$ 0.36. A mixture m.p. with an authentic sample of 4-*O*-β-D-galactopyranosyl-D-galactose (m.p. 196°) was 199–201°. Partial and complete hydrolysis of **2** afforded galactose only, while t.l.c. and p.c. of an acid hydrolysate of methylated **2** showed the presence of 2,3,4,6-tetra-*O*-methylgalactose and 2,3,6-tri-*O*-methylgalactose. G.l.c. of a methanolysate of hydrolysed, methylated **2** showed peaks corresponding to methyl 2,3,4,6-tetra-*O*-methylgalactosides (*T* 1.59) and methyl 2,3,6-tri-*O*-methylgalactosides (*T* 2.74, 3.27, 3.58, and 3.86).

*Fraction 7.* The syrup (250 mg), eluted with 5% ethanol, contained (p.c.) mainly a mixture of disaccharide **2** and a disaccharide with $R_{GAL}$ 0.47. Fractionation of the syrup on Whatman 3MM paper (solvent *1*) afforded disaccharide **3** (50 mg) as a chromatographically pure (solvents *1* and *2*) syrup, $[\alpha]_D^{25}$ −69° (*c* 1.18), $R_{GAL}$ 0.47,

revealed with spray *b*. It gave galatose and 4-*O*-methylgalactose on acid hydrolysis, and galactose, 4-*O*-methylgalactose, and starting material on partial hydrolysis. Reduction of **3** with sodium borohydride followed by hydrolysis yielded 4-*O*-methylgalactose as the only reducing sugar. Methylation of **3** by the Hakomori procedure[15], followed by t.l.c. and p.c. of an acid hydrolysate, revealed the presence of 2,3,4,6-tetra- and 2,3,4-tri-*O*-methylgalactose, and some degradation material. Methanolysis of the hydrolysate followed by g.l.c. of the derived methyl glycosides revealed peaks corresponding to methyl 2,3,4,6-tetra-*O*-methylgalactoside (*T* 1.60), methyl 2,3,4-tri-*O*-methylgalactoside (*T* 5.85), and an unidentified peak at *T* 3.76 (*cf.* disaccharide **1**). These results indicate that **3** was 6-*O*-(4-*O*-methyl-α-L-galactopyranosyl)-D-galactose. The α-L configuration was assumed from the optical rotation.

*Fraction 8*. The syrup (246 mg), eluted with 5–10.5% aqueous ethanol, was further fractionated by p.c. (Whatman No. 1, solvent *1*) to afford disaccharide **4** (12 mg) as a syrup, $[\alpha]_D^{25}$ +100° (*c* 0.40), $R_{GAL}$ 0.61. Hydrolysis of **4** gave galactose and 2-*O*-methylgalactose. Reduction of **4** with sodium borohydride followed by acid hydrolysis gave 2-*O*-methylgalactose only. The disaccharide was chromatographically identical with 3-*O*-(2-*O*-methyl-α-D-galactopyranosyl)-D-galactose.

*Fraction 9*. The syrup (270 mg), eluted with 10.5% aqueous ethanol, crystallized on trituration with methanol to afford disaccharide **5** which, after recrystallization, had m.p. 211–214° alone and in admixture with authentic 4-*O*-β-D-galactopyranosyl-2-*O*-methyl-D-galactose. Disaccharide **5** failed to react with spray *b*, gave galactose and 2-*O*-methylgalactose on hydrolysis, and only galactose on reduction followed by hydrolysis. The infrared spectrum of **5** was identical with that of authentic 4-*O*-β-D-galactopyranosyl-2-*O*-methyl-D-galactose.

*Fraction 10*. The syrup (95 mg), eluted with 15% aqueous ethanol, consisted (p.c., solvent *1*) mainly of two sugars having $R_{GAL}$ 0.1 and 0.14. Further fractionation on Whatman No. 1 paper (solvent *1*) afforded two sub-fractions, each of which contained both the major saccharides. Hydrolysis of each sub-fraction followed by p.c. revealed the presence of galactose only.

*Fraction 11*. The syrup (180 mg), eluted with 16% aqueous ethanol, contained (p.c.) one major oligosaccharide ($R_{GAL}$ 0.15) together with traces of a number of oligosaccharides. Fractionation of the mixture on Whatman No. 1 paper, using solvent *1*, afforded trisaccharide **6** as a syrup (39 mg), $[\alpha]_D^{21}$ −32° (*c* 0.41), $R_{GAL}$ 0.15; **6** gave galactose and 4-*O*-methylgalactose (p.c., solvents *1* and *2*) on acid hydrolysis, and gave galactose, 4-*O*-methylgalactose, disaccharides **2** and **3**, and starting material on partial, acid hydrolysis. Reduction of **6** followed by partial hydrolysis afforded galactose and 4-*O*-methylgalactose as the only reducing sugars. Neither **2** nor **3** (both of which are relatively stable to acid hydrolysis) was detected in the partial hydrolysate. Examination of the methyl glycosides derived from the methylated (Hakomori procedure[15]), hydrolysed trisaccharide showed a single, large peak corresponding to methyl 2,3,4,6-tetra-*O*-methylgalactosides (*T* 1.60). In addition, very much smaller peaks with *T* values of 2.00, 2.80, 3.22, 3.67, and 3.95 were observed. No peak corresponding to methyl 2,3-di-*O*-methylgalactosides (*T* 8.38) were observed.

The trisaccharide was assigned the structure *O*-(β-D-galactopyranosyl)-(1→4)-*O*-[4-*O*-methyl-α-L-galactopyranosyl-(1→6)]-D-galactose.

*Fraction 12.* The syrup (100 mg), eluted with 16% aqueous ethanol, contained several slow-moving oligosaccharides as well as one fast-moving disaccharide ($R_{GAL}$ 1.85). Fractionation of the mixture on Whatman No. 1 paper (solvent *1*) afforded disaccharide **7** (35 mg) as a syrup that was chromatographically pure in solvents *1* and *3*, and had $[\alpha]_D^{22}$ +15° (*c* 0.83), $R_{GAL}$ 1.85. It gave only 6-*O*-methylgalactose and 2-*O*-methylgalactose on acid hydrolysis, and these two sugars and the starting material on partial, acid hydrolysis. Hydrolysis of reduced **7** afforded 6-*O*-methylgalactose (solvent *2*) as the only reducing sugar. The disaccharide was methylated by the method of Haq and Percival[16], and hydrolysis of the methylated disaccharide followed by paper chromatography (solvent *3*, spray *a*) revealed spots corresponding to 2,3,4,6-tetra-*O*-methylgalactose and 2,3,6-tri-*O*-methylgalactose. G.l.c. of the methanolysed hydrolysate revealed peaks corresponding to methyl 2,3,4,6-tetra-*O*-methylgalactosides (*T* 1.63) and methyl 2,3,6-tri-*O*-methylgalactosides (*T* 2.78, 3.34, 3.64, and 3.91). The disaccharide was assigned the structure 2-*O*-methyl-4-*O*-(6-*O*-methyl-β-D-galactopyranosyl)-D-galactose. The β-D configuration was assumed from the low, positive specific rotation.

*Fraction 13.* The rest of the material (500 mg), eluted with up to 50% aqueous ethanol, was a mixture of oligosaccharides with very low chromatographic mobility, and was not further investigated.

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