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THE DESULPHATED POLYSACCHARIDE OF *Anatheca dentata**

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ABSTRACT

The polysaccharide of *Anatheca dentata* has been desulphated and then subjected to periodate oxidation and methylation analysis. The present and previous results indicate that the polysaccharide is composed of alternating 3-linked β-D-galactopyranosyl and 4-linked α-D-galactopyranosyl and 4-linked α-L-galactopyranosyl 2,3,6-trisulphate residues. Some of the 3-linked β-D-galactopyranosyl residues carry 4,6-*O*-(1-carboxyethylidene) groups, and others carry xylopyranosyl branches. D-Glucopyranosyluronic acid residues form part of the polysaccharide chain.

INTRODUCTION

The polysaccharide of *Anatheca dentata* is composed[1,2] of galactose, xylose, and sulphate ($NaSO_3$) in the ratios 6.0:1.0:6.8, together with a small proportion of glucuronic acid and a trace of 3-*O*-methylgalactose. The galactosyl residues are present in both the D and L forms, in the ratio of 1.57:1. Partial hydrolysis with acid revealed that the polysaccharide has an alternating sequence of 3-linked β-D-galactopyranosyl and 4-linked α-L-galactopyranosyl residues as its main structural feature. Some of the D-galactose residues have been shown[1] to possess 4,6-*O*-(1-carboxyethylidene) groups, and some of the L-galactosyl residues have been shown, by the isolation of 4-*O*-(α-D-glucopyranosyluronic acid)-L-galactose, to occur adjacent to D-glucosyluronic acid residues. The isolation of L-galactose 3- and 6-sulphates from the partial hydrolysate, coupled with the alkali-stability of the ester sulphate units, led to the suggestion[1] that those L-galactosyl residues which carry sulphate must either be the site of branch points, or be present as trisulphated residues. The role of xylose and the excess of D- over L-galactose has not been established. We now report on a study of the desulphated polysaccharide.

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DISCUSSION

The polysaccharide was extremely difficult to desulphate, and had to be treated repeatedly with methanolic hydrogen chloride before a desulphated polysaccharide (SO_4^{2-}, 1.2%) was obtained in 59% yield. The decrease in viscosity of a solution of the desulphated polysaccharide is indicative of considerable depolymerisation during desulphation. Paper chromatography of an acid hydrolysate of the methanol-soluble material revealed that both galactose and xylose were cleaved. The infrared spectrum of the desulphated polysaccharide was devoid of bands in the 800–860 and 1240 cm^{-1} regions, but showed a prominent peak at 1725 cm^{-1} which is attributed mainly to D-glucosyluronic acid residues, because few of the carboxyethylidene groups are expected to have survived the desulphation conditions.

Periodate oxidation of the desulphated polysaccharide showed no definite end-point. After 96 h, the rate of oxidation was extremely low and the polysaccharide had consumed 718 mmol of oxidant per "anhydrohexose" unit. The native polysaccharide under similar conditions[3] consumed 243 mmol of periodate per "anhydrohexose" unit. If allowance is made for the sulphate contents of the two polysaccharides, the consumption of periodate becomes 377 and 726 mmol per sulphate-free "anhydrohexose" unit for the native and desulphated polymers, respectively. Paper chromatography of an acid hydrolysate of the periodate-oxidised polysaccharide showed the absence of xylose.

Methylation of the desulphated polysaccharide was effected by repeated treatment of a solution in dimethyl sulphoxide with solid sodium hydroxide and dimethyl sulphate. This yielded a chloroform-soluble gum having a methoxyl content of 36.0%. On hydrolysis, the chloroform-insoluble material gave the same products, and in similar proportions, as did the chloroform-soluble gum, but it could not be rendered chloroform-soluble on remethylation, and was therefore not further investigated. After three treatments with Purdie's reagents[4], the chloroform-soluble gum had a methoxyl content of 40.3% and a very weak i.r. absorption for hydroxyl. Further treatment with Purdie's reagents failed to increase the methoxyl content. The methylated, desulphated polysaccharide was hydrolysed, and the products were fractionated by elution from a charcoal–Celite column with a linear gradient of 0→3% butanone in water. The methylated galactoses obtained were 2,3,4,6-tetra-*O*-methyl-DL-galactose, 2,3,6-tri-*O*-methylgalactose (mainly the L isomer), 2,4,6-tri-*O*-methyl-D-galactose, a di-*O*-methyl fraction, a mono-*O*-methyl fraction, and a small proportion of 2,3,4-tri-*O*-methyl-DL-galactose. The isolation of 2,3,6-tri-*O*-methylgalactose predominantly as the L isomer is consistent with the isolation of 4-*O*-β-D-galactopyranosyl-L-galactose as the major, and 4-*O*-β-D-galactopyranosyl-D-galactose as the minor, 4-linked disaccharide and indicates that all of the L-galactosyl residues in the polymer are 4-linked. The isolation of 2,4,6-tri-*O*-methylgalactose solely as the D isomer was expected from the results of the partial hydrolysis study[1,2] and indicates that all of the 3-linked galactosyl residues have the D configuration. The presence of 2,3,4,6-tetra-*O*-methylgalactose in the hydrolysate as a racemate indicates that the

3- and 4-linked galactosyl residues in the native polysaccharide are equally susceptible to cleavage by methanolic hydrogen chloride.

The mono-*O*-methyl fraction consisted of 2-*O*-methyl-, 3- and/or 4-*O*-methyl-, and 6-*O*-methyl-galactose, with 2-*O*-methylgalactose constituting 80% of the fraction. Only the 2-*O*-methylgalactose is considered to have structural significance and arises from those 3-linked D-galactosyl residues that carry pyruvate. The other monomethylgalactoses were almost certainly undermethylation products.

The variety of di-*O*-methylgalactoses detected in the di-*O*-methyl fraction suggests that some of these were undermethylation products, whereas others represent the sites of branch points. The branch points are therefore mainly located on D-galactosyl residues. The 2,3,4-tri-*O*-methyl-DL-galactose is considered to have arisen by demethylation of some of the 2,3,4,6-tetra-*O*-methyl-DL-galactose during hydrolysis.

The major, methylated xylose was the 2,3,4-tri-*O*-methyl derivative; minor amounts of all three possible di-*O*-methylxyloses were also obtained. The latter are considered to be undermethylation products, a result supported by the observation[1] that all of the xylose residues in the polymer were cleaved by periodate. These results, together with the fact that xylose was not encountered in any of the oligosaccharides obtained on partial hydrolysis[2] with acid, indicate that the xylose occurs as a non-reducing end-group. A small proportion of uronic acid was detected in acid hydrolysates of the polysaccharide, and its presence was confirmed by the isolation of 2,3,4-tri-*O*-methyl-D-glucuronic acid from the hydrolysate of the methylated, desulphated polymer. In addition, a minute amount of a di-*O*-methyluronic acid was detected in the hydrolysate. From the quantity of 2,3,4-tri-*O*-methyl-D-glucuronic acid obtained, it is estimated that the sulphated polysaccharide contains ~3% by weight of D-glucuronic acid.

Methylation of the native polysaccharide was unsuccessful (a methoxyl content of only 10% was achieved)[3], presumably due to the high content of sulphate and branching in the macromolecule, which prevented the acquisition of further information on the location of the sulphate ester groups.

The present and previous[1,2] results show that ~80% of the main polysaccharide chain in the native polysaccharide can be accounted for in terms of alternating 4-linked α-L-galactopyranosyl and 3-linked β-D-galactopyranosyl residues. The remaining 20% of the main chain has the L-galactopyranosyl residues replaced by D-galactopyranosyl residues. The failure to isolate oligosaccharides bearing 4-linked α-D-galactopyranosyl residues and with a d.p. higher than 2 strongly suggests that the 4-linked D-galactopyranosyl residues are most likely to be found interspersed throughout the macromolecule, rather than in a separate region of alternating 4- and 3-linked D-galactopyranosyl residues. A further novel feature of *Anatheca dentata* polysaccharide is the replacement of some (~10%) of the 3-linked D-galactopyranosyl residues by D-glucopyranosyluronic acid residues, and the presence of 4,6-*O*-(1-carboxyethylidene) groups on some of the 3-linked D-galactopyranosyl residues.

The location of the ester sulphate groups in the polysaccharide also appears to be novel. These groups have been shown[1], by the isolation of L-galactose 3- and

6-sulphates, to reside on the L-galactosyl residues only. Since these residues are all 4-linked, the sulphate groups are expected to be alkali-labile. Treatment of the polysaccharide with alkali[1], however, has established that the ester sulphate units are alkali-stable. It therefore follows that the L-galactosyl residues carrying sulphate must either be the site of branch points or trisulphated. The latter is the most likely explanation, since branching probably occurs on the D-galactopyranosyl residues. The trisulphated units would be expected to be stable to base, since base hydrolysis (so far unrecorded in carbohydrate sulphates) of one of the sulphate groups would be a prerequisite for the elimination of sulphate. In order to account for the high sulphate content of the polysaccharide, all of the L-galactosyl residues would have to be trisulphated. The analytically determined L-galactose–sulphate ratio is 1:3.

The periodate-oxidation studies[1,3] lend further support to the above conclusions. Only the D-xylopyranosyl and the nonsulphated, 4-linked D-galactopyranosyl residues would be expected to be cleaved by periodate. The expected reduction of periodate per sulphate-free "anhydrohexose" unit is 390 mmol, with which the experimentally determined value of 377 mmol is in good agreement.

The polysaccharide of *Anatheca dentata* thus differs significantly from other[5-7] polysaccharides extracted from seaweeds belonging to the *Solieriaceae* family.

EXPERIMENTAL

The analytical methods have been previously described[1,2].

Desulphation of the polysaccharide. — Polysaccharide (13 g; dried *in vacuo* over P_2O_5 for 48 h) was shaken with 0.15M methanolic hydrogen chloride at room temperature for 48 h, after which the undissolved polysaccharide was removed, dialysed against running water, and isolated by freeze-drying. After a further 4 such treatments, the polysaccharide (5 g) had a sulphate content of 1.2%. P.c. (solvents *A* and *B*) of an acid hydrolysate revealed galactose and xylose.

Desulphated polysaccharide. — (*a*) *Periodate oxidation.* To a solution of polysaccharide (19.4 mg) in water (5 ml) was added 30mM sodium metaperiodate (5 ml), and the solution was stored in the dark at room temperature. Aliquots (0.10 ml) were withdrawn at intervals and diluted 250 times, and the reduction of periodate (per "anhydrohexose" unit), measured spectrophotometrically[8], was as follows.

Time (h)	4	12	24	48	72	96
Periodate reduced (mmol)	382	549	597	690	711	718

(*b*) *Methylation analysis.* To a solution of polysaccharide (4.75 g) in dimethyl sulphoxide (100 ml) was added powdered sodium hydroxide (30 g) and dimethyl sulphate (15 ml) with stirring during 5 h. The mixture was stirred for a further 16 h, and sufficient concentrated ammonia was then added, followed by vigorous shaking, to decompose the dimethyl sulphate. After the addition of water (200 ml), the mixture

was dialysed against distilled water for 5 days. The solution was concentrated to a small volume and the polysaccharide (5.0 g) was isolated by freeze-drying. The above methylation procedure was repeated and, after a third methylation, the solution containing the partially methylated polymer was dialysed, concentrated, and extracted with chloroform (5 × 100 ml). Concentration of the combined chloroform solutions yielded a gum (A, 1.0 g). The aqueous solution containing the chloroform-insoluble material, on concentration and freeze-drying, yielded a partially methylated polysaccharide (3.62 g). This fraction was remethylated and extracted with chloroform as before, yielding a gum (B, 0.20 g). The chloroform-insoluble material (2.90 g) was not further investigated.

Fractions A and B were combined (1.20 g) (Found: OMe, 36.0%) and dissolved in methyl iodide (25 ml). Silver oxide (8 g) was added in small portions and the mixture was gently boiled under reflux for 8 h with stirring[4]. After filtration, the silver salts were thoroughly washed with chloroform, and the combined filtrate and washings were concentrated to yield the polysaccharide (0.93 g). Two further treatments with Purdie's reagents[4] afforded a product (0.94 g) (Found: OMe, 40.3%. Calc. for 6:1 galactose–xylose: OMe, 44.5%). The product (in dry $CHCl_3$) showed a very weak i.r. hydroxyl absorption. Further treatments with Purdie's reagents failed to increase the methoxyl content.

A solution of the methylated, desulphated polysaccharide (0.94 g) in 90% aqueous formic acid (30 ml) was kept at 100° for 1 h, cooled, and concentrated under diminished pressure. The syrupy residue was hydrolysed (100° for 16 h) with 0.25M sulphuric acid (30 ml), and the hydrolysate was neutralised ($BaCO_3$), centrifuged, and concentrated. The syrupy residue (0.70 g) was eluted from a column (32 × 4 cm) of charcoal–Celite (1:1) with a linear gradient of 0 → 3% butanone in water (total volume, 8 litres). Fractions (~25 ml) were analysed by p.c. and combined to give the following fractions.

Fraction I was a syrup (149 mg) that contained (p.c., solvents *A* and *B*) mono- and di-*O*-methylgalactoses. The syrup was fractionated on Whatman No. 1 paper (solvent *B*, 6 h), to give the mono-*O*-methyl derivatives as a syrup (35 mg). Further fractionation (p.c., solvent *B*) gave (*1*) a syrup (7 mg), $[\alpha]_D^{20}$ −3° (*c* 0.7), which was chromatographically (solvents *A* and *B*) identical with 3- and/or 4-*O*-methylgalactose; (*2*) a syrup (5 mg) which, on reduction (sodium borohydride) and acetylation, gave products having g.l.c. retention times identical with those of the glycitol acetates of 6-, 2-, and 3- and/or 4-*O*-methylgalactose in the molar ratios ~2:2:1; and (*3*) a chromatographically pure syrup (21 mg), which, after crystallisation from methanol–ethyl acetate, had m.p. 152–153° alone and in admixture with 2-*O*-methyl-D-galactose, $[\alpha]_D^{20}$ +14 (4 min) → +62° (*c* 0.42); lit.[9] m.p. 146–149°, $[\alpha]_D^{16}$ +84.9° (final) (*c* 0.53).

The di-*O*-methyl fraction was a syrup (111 mg) that contained (p.c., solvent *B*, spray *1*) 2,6-di-*O*-methylgalactose (R_{Gal} 6.0, red, major component), 2,4-di-*O*-methylgalactose (R_{Gal} 4.8, yellow, goes red on storage), and 2,3-di-*O*-methylgalactose (R_{Gal} 5.6, yellow, goes red on storage). A portion (13 mg) of the syrup was reduced

with borohydride and acetylated. G.l.c. revealed glycitol acetates having retention times corresponding to those of 2,6-di-*O*-methylgalactose (*T* 3.94, major component), 2,4-di-*O*-methylgalactose (*T* 6.26), 4,6-di-*O*-methylgalactose (*T* 4.27, trace), and 2,3-di-*O*-methylgalactose (*T* 5.12). Preparative p.c. (solvent *B*, 12 h) of the syrup (98 mg) yielded 2,6-di-*O*-methylgalactose (10 mg), $[\alpha]_D^{20}$ +56° (*c* 0.5) {lit.[10] $[\alpha]_D$ +84° (*c* 0.4)}, and 2,4-di-*O*-methylgalactose (16 mg), $[\alpha]_D^{20}$ +30° (*c* 0.53) {lit.[10] $[\alpha]_D$ +85° (*c* 0.3)}.

Fraction II was a syrup (63 mg) that contained (p.c., solvent *B*, spray *1*) 2,3-di-*O*-methylxylose (R_{TMG} 0.93, pink), 2,4-di-*O*-methylxylose (R_{TMG} 0.86, pink), 2,3,4-tri-*O*-methylgalactose (R_{TMG} 0.68, brown), and a methylated uronic acid (trace; R_{TMG} 0.43, solvent *A*, pink). Fractionation of the syrup on Whatman No. 1 paper (solvent *B*, 6 h) and extraction with 50% aqueous methanol yielded the three major components. 2,3,4-Tri-*O*-methylgalactose (11 mg), $[\alpha]_D^{20}$ +11° (*c* 0.55), gave a peak (*T* 7.41) in g.l.c. identical with that obtained from methyl 2,3,4-tri-*O*-methylgalactosides. 2,4-Di-*O*-methyl-D-xylose (8 mg), $[\alpha]_D^{20}$ +21° (*c* 0.57), gave peaks (*T* 1.46, 1.92) identical with those obtained from methyl 2,4-di-*O*-methylxylosides. 2,3-Di-*O*-methyl-D-xylose (8 mg), $[\alpha]_D^{20}$ +20° (*c* 0.6), gave peaks (*T* 1.47, 1.74) identical with those given by methyl 2,3-di-*O*-methylxylosides, as well as a peak (*T* 1.30) probably due to a small proportion of methyl 3,4-di-*O*-methylxylosides. The 2,3-di-*O*-methylxylose was detected with triphenyltetrazolium hydroxide, and demethylation[11] yielded xylose as the only monosaccharide (p.c.).

Fraction III was a syrup (49 mg) that contained (p.c., solvent *B*, spray *1*) 2,4,6-tri-*O*-methylgalactose (R_{TMG} 0.73, red-brown) together with a trace of the 2,3,4-isomer. Crystallisation from ether–light petroleum gave a product having $[\alpha]_D^{16}$ +120 (4 min) → +89° (*c* 0.63), m.p. 102–103° alone and in admixture with 2,4,6-tri-*O*-methyl-D-galactose[12] {m.p. 104–106°, $[\alpha]_D^{20}$ +96° (*c* 1.4)}. The anilide had m.p. 173–174° alone and in admixture with 2,4,6-tri-*O*-methyl-*N*-phenyl-D-galactosylamine[13] (m.p. 170.5–171.5°).

Fraction IV was a syrup (145 mg) that contained (p.c., solvent *B*) a mixture of 2,3,6- and 2,4,6-tri-*O*-methylgalactose in the ratio ~2:1.

Fraction V was a syrup (80 mg), $[\alpha]_D^{16}$ −56° (*c* 0.5), which was chromatographically identical with 2,3,6-tri-*O*-methylgalactose (R_{TMG} 0.83, solvent *B*; red-brown, spray *1*); lit.[9] $[\alpha]_D^{18}$ +90° (*c* 0.37) for 2,3,6-tri-*O*-methyl-D-galactose. The derived 2,3,6-tri-*O*-methylgalactonolactone had m.p. 97–99° (from ether). The m.p. was lowered on admixture with authentic 2,3,6-tri-*O*-methyl-D-galactono-1,4-lactone[9], m.p. 97–99°; the i.r. spectrum was identical with that of the authentic D-compound.

Fraction VI was a syrup (64 mg) that contained (p.c.) a mixture of 2,3,6-tri-*O*-methylgalactose and a methylated uronic acid (R_{TMG} 0.53, solvent *A*; pink, spray *1*). Fractionation on Whatman No. 1 paper (solvent *B*, 6 h) afforded a chromatographically pure syrup (23 mg), R_{TMG} 0.53 (solvent *A*). The syrup was shaken with Amberlite IR-120 (H^+) resin to yield the free acid, $[\alpha]_D^{17}$ +45° (*c* 0.4); lit.[14] $[\alpha]_D$ +58° for 2,3,4-tri-*O*-methyl-D-glucuronic acid. Treatment of the acid with boiling, 4% methanolic hydrogen chloride for 7 h gave a syrup, g.l.c. of which

revealed components having retention times (*T* 2.50, 3.24) identical with those of the methyl ester methyl glycoside of 2,3,4-tri-*O*-methylglucuronic acid. Carboxyl-reduction of this product (sodium borohydride) gave products having retention times in g.l.c. identical with those (*T* 2.57, 3.69) of methyl 2,3,4-tri-*O*-methylglucosides. Acid hydrolysis of the methyl glycosides (0.5M sulphuric acid, 100°, 3 h) yielded a syrup which was identical (p.c., solvent *B*, spray *1*) with 2,3,4-tri-*O*-methylglucose and which gave glucose on demethylation. Thus, the methylated uronic acid was 2,3,4-tri-*O*-methyl-D-glucuronic acid.

Fraction VII was a syrup (42 mg) that contained (p.c.) 2,3,4,6-tetra-*O*-methylgalactose (R_{TMG} 1.0, solvent *B*; red-brown, spray *1*) and the methylated uronic acid present in fraction VI. Fractionation on Whatman No. 1 paper (solvent *B*, 6 h) gave 2,3,4,6-tetra-*O*-methylgalactose (30 mg), $[\alpha]_D^{17}$ 0° (*c* 0.5). The anilide, after several recrystallisations from ethanol, had m.p. 178–180°, $[\alpha]_D^{20}$ 0° (*c* 0.49, acetone). In admixture with 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-galactosylamine[15] (m.p. 192–194°), the m.p. was 178–190°. The i.r. spectrum was identical with that of the anilide of 2,3,4,6-tetra-*O*-methyl-D-galactose; lit.[16] m.p. 179–180°, $[\alpha]_D$ 0° (*c* 2.5, acetone), for the anilide of 2,3,4,6-tetra-*O*-methyl-DL-galactose.

Fraction VIII was a syrup (36 mg) that contained (p.c., solvents *A* and *B*) a mixture of 2,3,4-tri-*O*-methylxylose and 2,3,4,6-tetra-*O*-methylgalactose.

Fraction IX was a syrup (50 mg), R_{TMG} 1.11, which was identical with 2,3,4-tri-*O*-methylxylose (p.c., solvent *B*; pink, spray *1*). Crystallisation from ether–light petroleum gave material having m.p. and mixture m.p. 89–90°, $[\alpha]_D^{19}$ +48 (4 min) → +19° (*c* 0.50); lit.[17] m.p. 89–90°, $[\alpha]_D^{15}$ +20.3° (*c* 1.1). The derived *p*-nitrobenzoate had m.p. 133–134° (from ethanol); lit.[18] m.p. 135°.

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