Carbohydrate Research, 62 (1978) 313–320

THE STRUCTURE OF THE POLYSACCHARIDE OF *Pachymenia carnosa**

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(Received May 9th, 1977; accepted for publication, June 24th, 1977)

ABSTRACT

The sulphated polysaccharide of *Pachymenia carnosa* and its desulphated derivative have been studied by methylation analysis. Depolymerization during the desulphation process has been shown to occur mainly through the cleavage of (1 → 3) linkages. The methylation results indicate that the ratio of (1 → 4) to (1 → 3) linkages in the native polysaccharide is 1:2.26. The sulphate groups occur on positions 2, 4, and 2,6 of (1 → 3)-linked galactose residues. Methylations carried out in methyl sulphoxide with the Purdie reagents lead to extensive desulphation; 2-sulphate units appear to be more susceptible to desulphation than 4- or 6-sulphate units. Desulphation does not occur during methylation by the Hakomori method.

INTRODUCTION

Pachymenia carnosa, a red seaweed of the Grateloupiaceae, yields a methylated, sulphated galactan[2] which, on acid hydrolysis, affords D-galactose, 2-*O*-methyl-D-galactose, 6-*O*-methyl-D-galactose, and 4-*O*-methyl-D,L-galactose in the molar ratios 9.22:1.5:0.78:0.31, together with sulphate (SO_3Na, 32.5%). Partial, acid hydrolysis and acetolysis studies[2,3] have established that the polysaccharide consists mainly of (1 → 3)-α-D-galactopyranosyl (or its 6-methyl ether) and (1 → 4)-β-D-galactopyranosyl (or its 2-methyl ether) residues arranged in an alternating sequence. In addition, the majority of the sulphate groups in the polysaccharide[2] were shown to be alkali-stable. In this respect, the polysaccharide of *P. carnosa* and other Grateloupiaceae polysaccharides differ from λ, μ, and ν carrageenans[4]. We now report the results of a methylation analysis of the native and desulphated polysaccharides of *P. carnosa*, which was undertaken in order to determine the precise location of the sulphate groups and whether the polysaccharide is composed entirely of alternating (1 → 3) and (1 → 4) linkages.

RESULTS AND DISCUSSION

The native polysaccharide was substantially desulphated by treatment with methanolic hydrogen chloride, to give a product that contained 1.7% of sulphate

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*Sulphated Polysaccharides of the *Grateloupiaceae* Family: Part X. For Part IX, see Ref. 1.

and had $[\alpha]_D$ +70°. Hydrolysis of the desulphated polysaccharide gave galactose, 2-*O*-methylgalactose, 6-*O*-methylgalactose, and 4-*O*-methylgalactose (p.c.), in the molar ratios (alditol acetates) 9.22:1.50:0.92:0.20. These ratios are practically the same as the molar ratios (9.22:1.50:0.78:0.31) of the sugars present in the native polysaccharide[2]. It is therefore reasonable to assume that the glycosidic linkages present in the native polysaccharide are also present in the desulphated polysaccharide. However, because glycosidic cleavage occurs during desulphation with methanolic hydrogen chloride and because all glycosidic bonds do not show the same susceptibility to attack by acidic reagents[5], the various linkage types are probably not present in the same proportions in the native and desulphated polysaccharides. Methylation analysis of the desulphated polysaccharide should therefore only furnish information concerning the various linkage-types present in the native polysaccharide.

Methylation of the desulphated polysaccharide was performed in methyl sulphoxide with sodium methylsulphinylmethanide and methyl iodide[6]. The polysaccharide was completely methylated after two treatments. P.c. and t.l.c. examination of an acid hydrolysate of the product revealed the presence of 2,3,6- and 2,4,6-tri-*O*-methylgalactose in approximately equal proportions (by comparison with standard solutions), together with small proportions of 2,3,4,6-tetra-*O*-methylgalactose and of at least two di-*O*-methylgalactoses. The results of the g.l.c. analysis of the alditol acetates derived from the hydrolysis products of the methylated polysaccharide are given in Table I. The tri-*O*-methylgalactoses were separated by p.c. and characterized. The presence of large proportions of 2,3,6-tri- and 2,4,6-tri-*O*-methyl-D-galactose complements the results of partial hydrolysis[2] and acetolysis[3], and shows that the native polysaccharide is composed entirely of (1 → 3)- and (1 → 4)-linked units, although not necessarily in equal proportions. The 2,3,4,6-tetra-*O*-methylgalactose is considered to have arisen partly from the non-reducing end-groups produced by depolymerization during desulphation of the native polysaccharide and (in the absence of 2,3,4-tri-*O*-methylgalactose from amongst the hydrolysis products) partly from 4-*O*-methylgalactose end-groups in the polysaccharide. This interpretation is supported by the observation[7] that all of the 4-*O*-methylgalactose residues present in the native and desulphated polysaccharides are cleaved by periodate. The structural significance of the small proportion of di-*O*-methylgalactoses present amongst the hydrolysis products of the methylated, desulphated polysaccharide will be discussed later.

The ester sulphate groups present in the native polysaccharide are stable towards alkali[2]; such groups may only be located on the following sugar units: *1*, (1 → 3)-galactose; *2*, (1 → 3)-6-*O*-methylgalactose; *3*, (1 → 3)-2-*O*-methylgalactose; *4*, (1 → 4,6)-2-*O*-methylgalactose; and *5*, (1 → 2,4,6)-galactose. The results of the methylation analysis of the desulphated polysaccharide rule out the possible presence of units *4* and *5*. It therefore follows that only (1 → 3)-linked units in the polymer carry sulphate. Of these, unit *3* may be ruled out, because all of the 2-*O*-methylgalactose-containing oligosaccharides isolated from partial degradations[2,3] of the polysaccharide have this sugar linked through position 4.

TABLE I

G.L.C. ANALYSIS OF THE METHYLATED ALDITOL ACETATES FROM THE DESULPHATED POLYSACCHARIDE

	Column 1		*Column* 2	
	Retention time	*Molar ratio*	*Retention time*	*Molar ratio*
2,3,4,6-Tetra-*O*-methylgalactose	1.00	1.07	1.00	0.72
2,4,6-Tri-*O*-methylgalactose	1.71	18.90	1.34	18.90
2,3,6-Tri-*O*-methylglaactose	1.80	19.79	1.16	18.85
2,6-Di-*O*-methylgalactose	2.67	0.90	1.44	0.35
4,6-Di-*O*-methylgalactose	2.67		1.55	0.58
2,3-Di-*O*-methylgalactose	4.08	1.00	1.83	0.88
2,4-Di-*O*-methylgalactose	4.46	0.61	2.20	0.65

TABLE II

G.L.C. ANALYSIS OF THE METHYLATED ALDITOL ACETATES FROM THE METHYLATED, NATIVE POLYSACCHARIDE *B*

	Retention time		*Molar ratio*
	Column 1	*Column* 2	*Column* 2
2,3,4,6-Tetra-*O*-methylgalactose	1.00	1.00	0.23
2,4,6-Tri-*O*-methylgalactose	1.73	1.36	0.46
2,3,6-Tri-*O*-methylgalactose	1.82	1.18	7.50
2,6-Di-*O*-methylgalactose	2.64	1.46	4.00
4,6-Di-*O*-methylgalactose	2.64	1.57	9.45
2,3-Di-*O*-methylgalactose	4.07	1.85	0.25
4-*O*-Methylgalactose	7.46	2.56	3.49

TABLE III

STRUCTURAL UNITS PRESENT IN THE NATIVE POLYSACCHARIDE

Methylated sugar	*Units in polysaccharide*
2,3,4,6-Tetra-*O*-methylgalactose	4-*O*-Methylgalactosyl
2,3,6-Tri-*O*-methylgalactose	(1,4)-Galactose (2 methyl ether)
2,4,6-Tri-*O*-methylgalactose	(1,3)-Galactose (6 methyl ether)
4,6-Di-*O*-methylgalactose	(1,3)-Galactose (6 methyl ether) 2-sulphate
2,6-Di-*O*-methylgalactose	(1,3)-Galactose (6 methyl ether) 4-sulphate
2,3-Di-*O*-methylgalactose	(1,4,6)-Galactose (2 methyl ether)
4-*O*-Methylgalactose	(1,3)-Galactose 2,6-disulphate

The precise location of the sulphate groups on (1 → 3)-linked units in the polysaccharide was established by methylation analysis. The native polysaccharide was first methylated by the Haworth procedure[8], to afford a partially methylated polysaccharide (*A*) which was soluble in methyl sulphoxide. A portion of *A* was methylated twice by the Hakomori method[6], to afford a fully methylated, native polysaccharide (*B*). Table II shows the g.l.c. analysis (alditol acetates) of the products of hydrolysis of *B*, and Table III indicates the units in the polysaccharide from which the methylated products are derived. That the monomethylalditol acetate was derived from 4- and not 3-*O*-methylgalactose was established when 4-*O*-methylgalactose was isolated from the hydrolysis products of the methylated, native polysaccharide and characterized.

The small proportion of 2,6-, 2,4-, and 4,6-di-*O*-methylgalactose present in the hydrolysis products of the desulphated polysaccharide arises from those units in the desulphated polysaccharide (SO_3Na, 1.7%) which carry sulphate. The 2,4-di-*O*-methylgalactose is derived from (1 → 3)-linked 6-sulphate units in the desulphated polysaccharide. These units must have been produced by the removal (during desulphation) of the 2-sulphate groups from the 2,6-disulphate units in the native polysaccharide. The 2,3-di-*O*-methylgalactose residues present in both the native and desulphated polysaccharides must, in the absence of alkali-labile sulphate, represent a genuine branch-point and hence the site of attachment of the 4-*O*-methylgalactosyl end-groups.

Calculations based on the methylation results of polysaccharide *B* indicate a polymer containing 33.2% of sulphate (SO_3Na), which would be expected to consume 0.129 mol of periodate per hexosyl residue. These data are in agreement with the experimental results[2,7]: *viz*, 32.5% of sulphate and 0.129 mol of periodate per hexosyl residue. In addition, the presence of 2-, 4-, and 6-sulphate groups in the polysaccharide is supported by its infrared spectrum[2], which shows a broad band between 800 and 860 cm^{-1}, with a peak at 825 cm^{-1}, and by the isolation of D-galactose 6-sulphate from a partial hydrolysate[7] of the polysaccharide.

A comparison of the methylation results of the desulphated and native polysaccharides (Tables I and II) shows that the ratio of (1 → 4) to (1 → 3) linkages alters from 1:2.26 in the native polymer to 1:1.04 in the desulphated polysaccharide, clearly showing that (1 → 3)-linked units are more susceptible to cleavage by methanolic hydrogen chloride than (1 → 4)-linked units. A similar susceptibility has been

TABLE IV

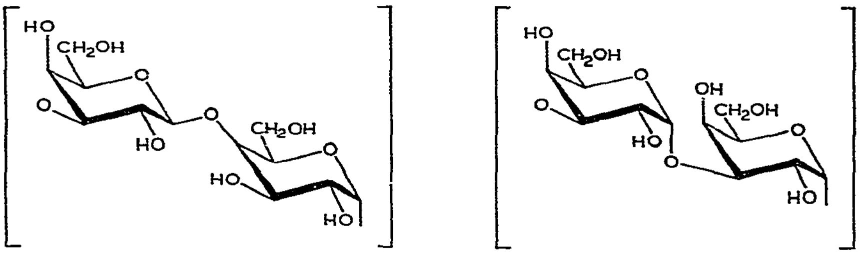
G.L.C. ANALYSIS OF THE METHYLATED ALDITOL ACETATES FROM THE METHYLATED, NATIVE POLYSACCHARIDE *C*

	Column 2	
	Retention time	*Molar ratio*
2,3,4,6-Tetra-*O*-methylgalactose	1.00	0.19
2,4,6-Tri-*O*-methylgalactose	1.35	7.14
2,3,6-Tri-*O*-methylgalactose	1.16	8.85
2,6-Di-*O*-methylgalactose	1.44	2.13
4,6-Di-*O*-methylgalactose	1.55	2.37
2,4-Di-*O*-methylgalactose	2.19	3.35
2,3-Di-*O*-methylgalactose	1.82	0.25
4-*O*-Methylgalactose	2.52	1.08

demonstrated with aqueous sulphuric acid[3,5]. These results show that the interpretation of methylation results of polysaccharides that have been desulphated[9,10] with methanolic hydrogen chloride, especially where desulphation is difficult[11] and forcing conditions are required, should be treated with caution; production of 2,3,6- and 2,4,6-tri-*O*-methylgalactose in almost equimolecular proportions can be taken as indicative of the presence of an equal number of (1 → 3) and (1 → 4) linkages in the original polysaccharide sulphate only when evidence is available to show that the different linkages present in the native polymer are equally susceptible to cleavage with methanolic hydrogen chloride. In addition, the practice[3] of partially desulphating a highly sulphated galactan with methanolic hydrogen chloride prior to an acetolysis study (because of the possible effect of the sulphate groups on the cleavage pattern) is not recommended, as acetolysis studies are carried out in order to produce oligosaccharides rich in (1 → 3) linkages; these linkages are cleaved to a lesser extent than (1 → 4) linkages under acetolysis conditions. It is noteworthy that the only tetrasaccharide isolated from the acetolysate of the partially desulphated polysaccharide of *P. carnosa*[3] was *O*-β-D-galactopyranosyl-(1 → 4)-*O*-α-D-galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-D-galactose, whereas the tetrasaccharide *O*-α-D-galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-*O*-α-D-galactopyranosyl-(1 → 3)-D-galactose was isolated in large yield from the acetolysates of native *A. ulvoidea*[1] polysaccharide and λ-carrageenan[5].

Polysaccharide *A* was also methylated in methyl sulphoxide with silver oxide and methyl iodide, to afford polysaccharide *C*. G.l.c. analysis of the derived alditol acetates is shown in Table IV. A comparison of these results with those (Table II) obtained for polysaccharide *B* clearly shows that extensive desulphation (40% loss of sulphate) occurred during Purdie methylation, as evidenced by the large increase in the proportion of 2,4,6-tri-*O*-methylgalactose and the drastic decrease in the proportions of 2,6- and 4,6-di-*O*-methylgalactose and 4-*O*-methylgalactose. The production of 2,4-di-*O*-methylgalactose, not observed amongst the hydrolysis products of polysaccharide *B*, is indicative of the removal of the 2-sulphate groups from 2,6-disulphate units. Furthermore, the results indicate that the ease of desulphation is in the order 2 > 4 > 6 sulphate. The loss of sulphate with the concomitant production of 2,4,6-tri-*O*-methylgalactose and 2,4-di-*O*-methylgalactose is further proof that the sulphate groups reside on (1 → 3)-linked units in the native polysaccharide. The methylation results obtained by the Hakomori procedure[6] show that desulphation does not occur with this method. This conclusion was verified when polysaccharide *B* gave the same results on g.l.c. analysis after being subjected to a further two Hakomori methylations.

The present results show that the main polysaccharide chain of *P. carnosa* is composed entirely of (1 → 3) and (1 → 4) linkages; the (1 → 3) linkages account for 70%, and the (1 → 4) linkages 30%, of the polysaccharide structure. This conclusion implies that only 60% of the polysaccharide can be accounted for in terms of an alternating structure of (1 → 3) and (1 → 4) linkages. The polysaccharide structure is best considered in terms of the units X and Y (Fig. 1); X must account



X Y

Fig. 1. Structural units (X and Y) for the polysaccharide.

for 60%, and Y for 40%, of the polysaccharide structure. A polysaccharide composed solely of X units would have a classic, alternating structure of α-1,3- and β-1,4-linked units. To account for the methylation results, the *P. carnosa* polymer must be composed of three X units for every two Y units. The possible arrangements of these five units are shown in formulae **1–6**.

(1) $[XXXYY]_n$, (2) $[YXXXY]_n$, (3) $[XXYXY]_n$, (4) $[XXYYX]_n$,
(5) $[XYXYX]_n$, (6) $[(....XXX...)_n(....YY....)_n]$

Formula **6** shows units X and Y in two structurally dissimilar regions in the polysaccharide, whereas formulae **1–5** have these units mixed and repeated in a regular fashion throughout the polysaccharide chain. It is not possible to say whether any, or a combination of some or all, of formulae **1–6** represents the actual polysaccharide chain. Indeed, it is not possible to say whether all of the structural features are found in a single polysaccharide or whether the whole extract consists of several polysaccharide types. Further comment must await an investigation of the polysaccharide's homogeneity.

The polysaccharide of *P. carnosa* and other polysaccharides extracted from seaweeds belonging to the Grateloupiaceae family[1–4,9,10,12–15] differ significantly from the "KCl soluble fraction" or λ-fraction polysaccharides (λ, μ, ν, and ε)[4,14] of the carrageenan family; all of the β-1,4-linked units in the *P. carnosa* polymer are devoid of sulphate, and hence all the sulphate resides on α-1,3-linked units and is stable to alkali. In contrast, the λ-fraction polysaccharides have both 1,3- and 1,4-linked units sulphated, and hence a feature of these polysaccharides is their ability to release sulphate, with the concomitant formation of 3,6-anhydro-D-galactosyl residues, when treated with alkali. It is therefore suggested that the term *aeodan* be introduced to distinguish between these two polysaccharide types; *aeodan* would then imply a polysaccharide composed entirely of α-1,3- and β-1,4-linked D-galactose (or methyl ethers, but not 3,6-anhydride) residues with only the 1,3-linked units carrying sulphate.

EXPERIMENTAL

The analytical methods used were described in Part IV[9]. In addition, g.l.c. analyses were performed on the following columns: *1* (alditol acetates), 3% of

ECNSS-M on Gas Chrom Q (100–120 mesh) at 170°; *2* (alditol acetates), 15% of Apiezon T on Gas Chrom Q (100–120 mesh) at 180°[16]; *3* (methyl glycosides), 15% of poly(butane-1,4-diol succinate) on acid-washed Chromosorb W (80–100 mesh) at 175°.

Desulphation of the polysaccharide. — The polysaccharide (1 g) was shaken with 0.15M methanolic hydrogen chloride (75 ml) for 48 h at room temperature, and the desulphated polysaccharide was isolated in 70% yield (SO_3Na, 1.7%) as previously described[15]. The component sugars of the desulphated polysaccharide were determined by g.l.c. examination of the derived alditol acetates [20% of Apiezon M on Gas Chrom Q (100–120 mesh) at 190°].

Methylation of the desulphated polysaccharide. — The polysaccharide (300 mg) in dry methyl sulphoxide (10 ml) was treated with sodium methylsulphinylmethanide in methyl sulphoxide[6] (3 ml), and the reaction mixture was vigorously stirred for 4 h. Methyl iodide (1 ml) was added with cooling, and stirring was continued for another 1 h. The reaction mixture was then poured into water and dialysed against running distilled water. The polysaccharide was isolated by freeze-drying and shown by its infrared spectrum to be incompletely methylated. A further methylation treatment afforded a fully methylated polysaccharide (250 mg; OMe, 43.6%). A portion of the polysaccharide was converted into the methyl glycosides and examined on column *3*, while another portion was converted into the alditol acetates and examined on columns *1* and *2*.

Hydrolysis of the desulphated, methylated polysaccharide. — Methylated polysaccharide (150 mg) was hydrolysed with 0.5M sulphuric acid for 16 h, and the hydrolysate was neutralised ($BaCO_3$), concentrated, and fractionated by paper chromatography in solvent *1*. 2,4,6-Tri-*O*-methyl-D-galactose, obtained as a syrup (65 mg) that crystallised from ether–light petroleum, had $[\alpha]_D$ +89.0° (*c* 1.0), m.p. and mixture m.p. with an authentic sample[7] 100–102°. 2,3,6-Tri-*O*-methyl-D-galactose was obtained as a syrup (62 mg), $[\alpha]_D$ +97.0° (*c*, 1.0), and was converted into the lactone which had m.p. and mixture m.p. (with authentic 2,3,6-tri-*O*-methyl-D-galactono-1,4-lactone[7]) 99–100°.

Methylation of the native polysaccharide. — The polysaccharide (1.0 g) was exhaustively methylated by the Haworth procedure[8], to afford partially methylated polysaccharide (*A*, 1.06 g; OMe, 15.4%). A portion (400 mg) of the partially methylated polysaccharide was subjected to two Hakomori methylations, as indicated above, to afford the fully methylated, native polysaccharide (*B*). The alditol acetates derived from the hydrolysate of *B* were examined by g.l.c. on columns *1* and *2*. A second portion (500 mg) of *A* was dissolved in methyl sulphoxide (20 ml) and methyl iodide (10 ml), and silver oxide (4 × 1 g at 0.5-h intervals) was added with constant stirring. After 4 h, the addition of silver oxide and methyl iodide was repeated and stirring was continued for a further 16 h, after which the polysaccharide (*C*, 300 mg) was isolated by dialysis, centrifugation, and freeze-drying of the supernatant solution. The alditol acetates derived from polysaccharide *C* were examined by g.l.c. on columns *1* and *2*.

*Isolation of 4-O-methyl-*D*-galactose from polysaccharide* B. — The polysaccharide (150 mg) was hydrolysed with 0.5M sulphuric acid, and the derived syrup was fractionated by paper chromatography (solvent *2*). The component having R_{Gal} 1.80 was eluted with ethanol–water and concentrated to a syrup (12 mg) that crystallised from ethanol–ethyl acetate. The product had $[\alpha]_D$ +74.0° (*c*, 1.0), m.p. and mixture m.p. (with authentic 4-*O*-methyl-D-galactose[9]) 202–204°.

ACKNOWLEDGMENTS

The author thanks the South African Council for Scientific and Industrial Research and Rhodes University for financial support.

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