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THE GALACTAN SULFATE FROM THE EDIBLE, RED ALGA *Porphyra columbina*

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ABSTRACT

A galactan sulfate has been isolated from the seaweed *Porphyra columbina*, and its structure established by a combination of methylation, methanolysis, treatment with alkali followed by methylation, and ^{13}C-n.m.r. spectroscopy. The polysaccharide belongs to the porphyran class, and consists of 3-linked β-D-galactosyl residues and 4-linked α-L-galactosyl residues. 3,6-Anhydro-L-galactose and L-galactose 6-sulfate residues total approximately half of the sugar units, the other half being made up of D-galactose and 6-*O*-methyl-D-galactose residues. Some evidence is presented that suggests that the galactan sulfate does not have a completely alternating structure.

INTRODUCTION

There is at present considerable interest in New Zealand in the wider use of seaweed polysaccharides for the manufacture of hydrogels for food and non-food industries. It is considered that a knowledge of the structure of the polysaccharides contained in the main New Zealand seaweeds will be valuable for improving their end-use. The aim of our program is, therefore, to investigate the structure and physical properties of polysaccharides obtained from common, New Zealand seaweeds.

One such polysaccharide, a porphyran, has been isolated from *Porphyra columbina*, a red seaweed traditionally eaten by the Maoris of New Zealand, and called by them[1] "karengo". The polysaccharide as extracted does not gel, but very strong gels are readily produced from the alkali-treated porphyran.

RESULTS AND DISCUSSION

A water-soluble polysaccharide from *Porphyra columbina* was obtained by hot-water extraction of air-dried seaweed. Four successive extractions gave a total yield of 21.2%. The products from each extraction were kept separate, and are designated PC1, PC2, PC3, and PC4.

Acid hydrolysis of each polysaccharide extract, and paper chromatography of the hydrolyzates, revealed the presence of compounds having the same R_F values as

TABLE I

COMPOSITION OF GALACTAN SULFATE[a]

Extract	$[\alpha]_D^{20}$ *(degrees)* (c *1*, H_2O)	*6-O-Methyl-* D-*galactose*	*Galactose*[b] (D + L)	L-*Galactose 6-sulfate*[c]	*3,6-Anhydro-* L-*galactose*	*Nitrogen (mass %)*	*Ash (mass %)*
PC1	−55.4	15.5	29.9	29.4	25.2	1.2	12.0
PC2	−61.1	17.9	30.8	31.4	19.8	trace	18.9
PC3	−69.0	16.3	34.2	30.8	18.7	0.6	17.0
PC4	−65.3	17.8	36.0	30.0	16.3	0.4	9.7

[a]All monosaccharide residues expressed as molar percentages. [b]Calculated by subtracting the L-galactose 6-sulfate value in the adjacent column from the total galactose present in the complete hydrolyzate. [c]Based on the sulfur analyses, by assuming that 100% of the total sulfate present occurs as L-galactose 6-sulfate residues (see text).

galactose, 6-*O*-methylgalactose, and 5-(hydroxymethyl)-2-furaldehyde. The last component was almost certainly derived from 3,6-anhydrogalactose residues (in the polysaccharides) which would have decomposed during the acid hydrolysis. Quantitative determination of the constituent sugar-units was performed by g.l.c. of their alditol acetates. The acid-labile 3,6-anhydrogalactose residue was analyzed for by the method of Yaphe and Arsenault[2-4].

The presence of sulfate groups in all four of the polysaccharide extracts was confirmed by infrared (i.r.) spectroscopy, the spectra showing the characteristic[5-7] S=O stretching vibration at 1240 cm^{-1}. In addition, a moderate band at 820 cm^{-1}, characteristic of sulfated primary hydroxyl groups, indicated that virtually all of the sulfate groups are at C-6 of a galactose residue, and this was supported by the absence of other distinct bands for sulfated hydroxyl groups (*e.g.*, at 850 or 830 cm^{-1}). Because of the loss of ester sulfate groups during acid hydrolysis, all sulfated sugar residues would appear as galactitol acetate in g.l.c. analysis. No pyruvate groups were found present in any extract.

The analytical data obtained for the four polysaccharide extracts are given in Table I. The values for the various monosaccharide residues in this Table are expressed as molar percentages of total carbohydrate present, and the D and L assignment of monosaccharide residues follows directly from the D-galactose oxidase experiments and ^{13}C-n.m.r. analyses described. Nitrogen and ash contents are given as mass percentages. From Table I, it may be seen that, for the four fractions, the molar percentages of the 3,6-anhydro-L-galactose and the L-galactose 6-sulfate are in the range of 16 to 25% and 29 to 31%, respectively, and the sum of the two ranges between 46 and 57%. The sum of 6-*O*-methyl-D-galactose residues and galactose residues is 45–54 mol %. These values, which are in the range found by other workers[8] for galactan sulfate polysaccharides, show that the molar ratio of D to L residues is ~1:1.

As the four extracts described in Table I appear similar, most of the following work was restricted to only one of these, namely, PC3. This fraction was examined for homogeneity by using the precipitation procedure with cetyltrimethylammonium bromide, and also ion-exchange chromatography on DEAE-Sephadex A-25. Both techniques yielded only one major fraction, which had a specific rotation, monosaccharide analysis, and sulfate content similar to those of the original PC3, from which it was concluded that the direct extraction of *Porphyra columbina* with hot water had given an essentially homogeneous galactan sulfate.

To study the relationship between the 3,6-anhydro-L-galactose and L-galactose 6-sulfate residues in the polysaccharide, the alkali elimination of sulfate from galactose 6-sulfate, with the concomitant formation of 3,6-anhydrogalactose units[9], was examined. The conversion involves inversion from the $^1C_4$ to the $^4C_1$ conformation. The four extracts from *Porphyra columbina* were treated with alkali. The products isolated from the treatment exhibited a tendency to gel that was not shown by the original polysaccharides. After freeze-drying, the white, fibrous polysaccharides were analyzed for 3,6-anhydrogalactose and sulfate content. In all cases, the alkali treatment caused a marked increase in the 3,6-anhydrogalactose content, and the sulfate groups were completely removed. For example, the molar percentages of sugar residues in alkali-treated PC3 were galactose, 37.5; 6-*O*-methylgalactose, 16.2; 3,6-anhydrogalactose, 46.3; and sulfate, 0.00. Little or no loss of galactose or 6-*O*-methylgalactose occurred during the alkali treatment, and, within experimental error, the molar ratio of D to L residues remained $\sim 1:1$.

The strong gelling tendency of the alkali-treated polysaccharides, and their high 3,6-anhydrogalactose content, show that these modified galactans closely resemble agarose. The ^{13}C-n.m.r. work described confirmed this, as did the methylation results.

The values for "galactose, D + L" given in Table I include all D and L residues that are unsubstituted in the original, polysaccharide fractions. Measurement of the galactose content of the hydrolyzate of fraction PC 3 before and after treatment with D-galactose oxidase showed that 51.5% of the total galactose present was the L enantiomer. This relatively high value is a consequence of the hydrolysis of sulfate groups from the L-galactose 6-sulfate residues in the galactan sulfate, and, after accounting for these, the remaining 2.7 mol % of L-galactose represents the unsubstituted L residues in the original polysaccharide. Small, variable proportions of unsubstituted L-galactose residues had been detected in polysaccharides of the galactan sulfate type, and, in one instance, 9% of the total L-galactose residues were, in fact, unsubstituted[8].

Although the Hakomori method of methylation[10] has many advantages, it has not been used extensively on sulfated polysaccharides of red algae, apparently because of the difficulty in completely dissolving these polysaccharides in dimethyl sulfoxide. Incomplete solubility in this solvent invariably leads to incomplete methylation[11], and consequently, to overcome this problem, the polysaccharide was acetylated prior to methylation. The methylated polysaccharide obtained from a single Hakomori methylation of extract PC3 had a methoxyl content of 25.51% and a sulfur content

of 3.57%. Examination by i.r. spectroscopy showed a small hydroxyl absorption at 3600–3400 cm^{-1}. Further methylation of an aliquot by the same procedure did not raise the methoxyl content.

A portion of the methylated galactan sulfate was remethylated by using the modified, Haworth method described by Dolan and Rees[12]. The methoxyl content was raised to 27.79%, and the sulfur content was 3.33%.

It is difficult, if not impossible, to calculate a theoretically expected methoxyl content for a porphyran type of polysaccharide. If it is assumed that (*i*) the polymer has a disaccharide repeating-unit consisting of a (1→3)-linked galactopyranose residue and a (1→4)-linked 3,6-anhydrogalactopyranose residue, and (*ii*) complete desulfation occurs during methylation, the methoxyl content of the fully methylated polysaccharide would be 34.2%. On the other hand, if it is assumed that the repeating unit is a tetrasaccharide consisting of two (1→3)-linked galactopyranose residues, a (1→4)-linked 3,6-anhydrogalactopyranose residue, and a (1→4)-linked galactopyranose 6-sulfate residue, the methoxyl content of the fully methylated polysaccharide would be 33.4%. This work shows that such a tetrasaccharide repeating-unit is the closer to the structure of the present galactan sulfate. Desulfation during methylation should not affect the theoretical methoxyl content very much, although it is unlikely that much desulfation had actually occurred. For example, Parolis[13] claimed that ester sulfate groups are generally stable towards the alkaline medium used in methylations by the Hakomori method.

TABLE II

G.L.C. ANALYSIS OF THE METHYLATED ALDITOL ACETATES FROM THE PARTIAL AND COMPLETE HYDROLYSIS OF METHYLATED GALACTAN SULFATE

Component	*90% Formic acid (1 h)*[a]		*90% Formic acid (1 h) + 16 h in 0.13M sulfuric acid*		*0.5M Sulfuric acid (16 h)*	
	Mole %	*Corrected mole %*	*Mole %*	*Corrected mole %*	*Mole %*	*Corrected mole %*
2,3,4,6-Tetra-*O*-methyl-D-galactose	0.7	0.7	0.4	0.3	0.9	0.7
3,6-Anhydro-2-*O*-methyl-L-galactose	21.7	18.7	4.2	18.7	0.0	18.7
2,4,6-Tri-*O*-methyl-D-galactose	44.7	46.5	53.9	45.7	58.9	47.8
2,3,6-Tri-*O*-methyl-L-galactose	1.6	1.7	2.4	2.0	1.7	1.4
2,6-Di-*O*-methyl-D-galactose	1.3	1.4	2.6	2.2	2.8	2.3
2,3-Di-*O*-methyl-L-galactose	29.9	31.1	36.6	31.0	35.8	29.1

[a]This procedure accounts for ~62% of the total hydrolysis of the polysaccharide.

The absence of excessive desulfation during methylation of the present galactan sulfate is further supported by the recovery of a large proportion of 2,3-di-*O*-methyl-L-galactose in the hydrolyzate (see Table II), suggesting that nearly all of the sulfate groups are attached to C-6 and are retained during the methylation. Also, the fact that the methylated polysaccharide contains 10% of sulfate is further evidence in support of incomplete desulfation, as it is assumed that, on complete methylation of such a polysaccharide as PC3, having a sulfate content of 12.4%, the sulfate content of the methylated product should be 9.3%. This indicates that 0.7% of the so-called sulfate is probably due, in fact, to tenaciously held dimethyl sulfoxide, or side reactions[14] involving the retention of sulfur therefrom.

The low methoxyl content of the fully methylated galactan sulfate is, therefore, unexplained. It is noteworthy, however, that other workers have also found that, when methylated fully, sulfated polysaccharides give apparently low methoxyl values. For example, Anderson and Rees[15] reported low values for the porphyran from *Porphyra umbilicalis*, and Dolan and Rees[12] gave a range of values, some as low as 20.5%, for a methylated carrageenan, and attributed this to "contamination". Nunn and Parolis[16,17] also found it impossible to achieve satisfactory methoxyl contents for their methylated, sulfated polysaccharides. In view of these reports, it was concluded that, for the present methylated polysaccharide, the value of 27.8% is not a satisfactory indication of the degree of methylation, and it has been assumed that complete methylation was achieved, because the hydrolyzate of the permethyl ether was free from galactose and mono-*O*-methylgalactose.

Methylated polysaccharide PC3 was partially hydrolyzed by heating in 90% formic acid for 1 h at 100°, and completely hydrolyzed by heating at 100° with either 90% formic acid followed by 0.13M sulfuric acid, or with 0.5M sulfuric acid alone. The results of g.l.c. analysis of the alditol acetates of the hydrolyzates are given in Table II. The mol % values in Table II were calculated directly from the g.l.c. analyses, and it is noteworthy that hydrolysis with 90% formic acid alone gives a greater proportion of 3,6-anhydro-2-*O*-methyl-L-galactose than hydrolysis with formic acid followed by sulfuric acid, and that sulfuric acid alone results in a hydrolyzate containing no anhydro compound. This suggests that formic acid stabilizes the free anhydro compound.

Because 3,6-anhydro-2-*O*-methyl-galactose is degraded during the acid hydrolyses, the mol % values in Table II were corrected by taking the mol % of the 3,6-anhydrogalactose in the original, unmethylated polysaccharide as 18.7 (see Table I) and calculating the mol % of 3,6-anhydro-2-*O*-methyl-galactose from this. All of the mol % values were then corrected accordingly, and the corrected values are also given in Table II.

The large proportion of 2,4,6-tri-*O*-methyl-D-galactose in the hydrolyzate of methylated PC3 proves that part of the D-galactose or 6-*O*-methyl-D-galactose residues, or of both, in the original polysaccharide is linked through O-3. It also suggests that there is little or no sulfate group present at C-2 of a galactose residue, as has been found in some of the galactans from red algae[16,18].

The 2,3,6-tri-*O*-methyl-L-galactose is a minor component of the hydrolyzate, confirming that a small proportion of unsubstituted, (1→4)-linked L-galactose residues is present. The values obtained from the methylation analyses (see Table II) are in reasonable agreement with those found by using the D-galactose oxidase method.

Another minor component was identified as 2,6-di-*O*-methylgalactose. A possible source could be a (1→3)-linked D-galactose having a sulfate group on C-4. The 2,6-di-*O*-methylgalactose also occurs in the hydrolyzate of the methylated, alkali-treated porphyran, and, as alkali removes all sulfate groups, these cannot be the cause of the 2,6-di-*O*-methylgalactose in the alkali-treated case. It is more likely, therefore, that the small proportion of 2,6-di-*O*-methylgalactose results from undermethylation. If this is so, it probably originates from undermethylated, 3-linked D-galactose. Another possibility is that the 2,6-di-*O*-methylgalactose may arise from a position of branching, both O-3 and O-4 being linked to other sugar units. However, so far, no conclusive proof of any branching in these types of polysaccharides has been found. Anderson and Rees[15] also detected traces of 2,6-di-*O*-methylgalactose in the hydrolyzates of a methylated galactan sulfate, and were unsure as to the degree to which it could be due to undermethylation, as the proportion was so small.

The diacetate of 2,3,4,6-tetra-*O*-methylgalactitol was identified in all of the g.l.c. analyses, and it is due to (terminal) nonreducing galactosyl groups. The mol % from the three hydrolyses varied from 0.3 to 0.7, indicating that, provided that no branching occurs, the number-average molecular weight of the galactan may be in the vicinity of 30,000 to 60,000 if it is assumed that all of the nonreducing (terminal) groups in the methylated polysaccharide are 2,3,4,6-tetra-*O*-methyl-D-galactosyl.

The presence of a large proportion of 2,3-di-*O*-methyl-L-galactose in the hydrolyzate suggests two possibilities. Firstly, the galactose may be linked to other sugar residues at O-4 and O-6. Secondly, the galactose could be sulfated at C-6 and linked at O-4, or *vice versa*. The link through O-4 of an L-galactose 6-sulfate was found to be the case, as it satisfies the following conditions. Alkali treatment of the sulfated galactan gave an approximately equivalent amount of anhydro residues. Thus, as a (1→4)-linked galactose residue can only form an anhydro ring between C-3 and C-6, the major portion of the sulfate groups in the original polysaccharides must be on either C-3 or C-6, or both. The fact that the hydrolyzate of the methylated PC3 extract gives such a large proportion of 2,3-di-*O*-methyl-L-galactose indicates that the bulk of the sulfate groups must be on C-6 of galactose residues which are linked glycosidically through O-1 and O-4. Furthermore, methylation and hydrolysis of the alkali-treated galactan PC3 gave no 2,3-di-*O*-methyl-L-galactose at all, thus confirming the fact that the 2,3-di-*O*-methyl-L-galactose isolated from the methylated, original polysaccharide (without alkali treatment) arises mainly from the (1→4)-linked L-galactose 6-sulfate units.

The methylated galactan sulfate PC3 was also methanolyzed. Methyl glycosides of 2,4,6-tri-*O*-methyl-D-galactose and 3,6-anhydro-2-*O*-methyl-L-galactose were the major products. Methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-

O-methyl-L-galactose, and 2,3-di-*O*-methyl-L-galactose were minor products. Traces of the glycosides of 2,6-di-*O*-methyl-D-galactose were also present. These methanolysis results are consistent with the methylation results already described.

Methylation of alkali-treated polysaccharide PC3 was very much easier than that of the original, untreated galactan sulfate. One Hakomori treatment gave a fully methylated product (as shown by i.r. analysis) which was hydrolyzed with 90% formic acid for 1 h at 100°, followed by 0.13M sulfuric acid for 16 h at 100°. As expected, the major component of the hydrolyzate was 2,4,6-tri-*O*-methyl-D-galactose. Only small quantities of 3,6-anhydro-2-*O*-methyl-L-galactose were detected, and no 2,3-di-*O*-methyl-L-galactose was found. Very small amounts of 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-L-galactose, and 2,6-di-*O*-methyl-D-galactose were also present. The similarity of these results to those obtained for the hydrolyzate of a sample of methylated agarose suggests that, in the original PC3 fraction, the two types of L residue and the two types of D residue are structurally equivalent, and that chain branching is very unlikely.

Good agreement was obtained between the data given in Table I and the methylation data for original and alkali-treated PC3, as may be seen from the summary of results presented in Table III. In this Table, the methylation data on alkali-treated PC3 have been corrected, as described previously, to allow for the degradation of the 3,6-anhydro-L-galactose derivative during hydrolysis. A comparison of the mole percentages of L-galactose 6-sulfate (30.8) with the methylation data for the 2,3-di-*O*-methyl-L-galactose residue (31.0) confirmed that virtually all of the sulfate is attached to C-6 of an L-galactose residue and, hence, the suggestion

TABLE III

COMPARISON OF THE ANALYTICAL AND METHYLATION DATA ON THE GALACTAN SULFATE (EXTRACT PC3)

Component	*Original PC3 (mole%)*	*Alkali-treated PC3 (mole%)*	*Methylation data on PC3 (corrected moles%)*[a]	*Methylation data on alkali-treated PC3 (corrected moles%)*[b]
L-Galactose 6-sulfate	30.8	0.0		
2,3-Di-*O*-methyl-L-galactose			31.0	0.0
3,6-Anhydro-L-galactose	18.7	46.3		
3,6-Anhydro-2-*O*-methyl-L-galactose			18.7	48.4
6-*O*-Methyl-D-galactose	16.3	16.2		
D-Galactose	31.5	34.6		
2,4,6-Tri-*O*-methyl-D-galactose[b]			47.9	49.7
L-Galactose	2.7	2.7		
2,3,6-Tri-*O*-methyl-L-galactose			2.0	1.3
2,3,4,6-Tetra-*O*-methyl-L-galactose			0.3	0.6

[a]The data in this column were taken from the combined formic–sulfuric acid hydrolysis results in Table II. [b]This value includes the small amount of 2,6-di-*O*-methyl-D-galactose.

that the ester sulfate is mostly stable towards the alkali medium used in the methylation procedure, as already discussed, is justified.

The present structural studies on the galactan sulfate from *Porphyra columbina* are most readily explained by assuming that the polysaccharide contains a linear chain of galactose residues linked alternately by (1→3) and (1→4) glycosidic bonds. Anderson and Rees[15] interpreted the results of their investigation of the galactan sulfate from *Porphyra umbilicalis* as clearly supporting such an alternating, linear structure. If the arrangements of the (1→3)- and (1→4)-linked residues in the fully methylated, alkali-treated galactan sulfate are considered, the following are possible sequences.

-D-D-L-L-D-D-L-L-D-D-L-L-D-D-L-L-D-D-L-L-D-D-L-L- (1)
-D-D-D-D-D-D-D-D-D-D-D-L-L-L-L-L-L-L-L-L-L- (2)
-D-D-D-L-L-D-L-L-L-D-D-L-D-D-D-L-L-D-L-L-L- (3)
-D-L-D-L-D-L-D-L-D-L-D-L-D-L-D-L-D-L-D-L-D-L (4)

where D represents a 2,4,6-tri-*O*-methyl-D-galactose residue, and L, a 3,6-anhydro-2-*O*-methyl-L-galactose residue in the methylated polymer.

Anderson and Rees[15] selected structure **4** for their porphyran, mainly on the basis of their isolation of relatively large amounts (62% yield) of agarobiose derivatives from the methanolyzate of the fully methylated, alkali-treated polysaccharide. It is known that 3,6-anhydrogalactopyranosyl linkages are very much more labile to acid-catalyzed cleavage than is usual for hexopyranosyl linkages[19], and, even though a completely selective hydrolysis is unlikely to be achieved, structure **4** should never, on either partial or complete hydrolysis, yield greater amounts of 3,6-anhydro-2-*O*-methyl-L-galactose than of 2,4,6-tri-*O*-methyl-D-galactose. The other structures, **1**–**3**, could all give a greater proportion of 3,6-anhydro-2-*O*-methyl-L-galactose. Because, in the work of Anderson and Rees[15], in no case did the molar amount of 3,6-anhydro-2-*O*-methyl-L-galactose exceed that of 2,4,6-tri-*O*-methyl-D-galactose, these workers concluded that their polysaccharide had structure **4**. No evidence was found for oligosaccharides of the types to be expected from structures **1**–**3**.

However, examination of Table II shows that, in the case of the hydrolysis with 90% formic acid, more 3,6-anhydro-2-*O*-methylgalactose is liberated than structure **4** would permit. The molar ratio of anhydrogalactose to (galactose + 6-*O*-methylgalactose) in the original PC3 was ~19:51. When 90% formic acid is used, the ratio of 3,6-anhydro-2-*O*-methylgalactose to 2,4,6-tri-*O*-methyl-galactose is 22:45 (see Table II). It therefore appears likely that, in the *Porphyra columbina* polysaccharide, structure **4** does not give a complete picture, and some adjacent 3,6-anhydrogalactose residues may occur. This point is worthy of further, detailed investigation, as the results in Table II alone are insufficient to prove or disprove the issue unequivocally.

Further information concerning the galactan sulfate structure came from ^{13}C-n.m.r. spectroscopy. Agarose and related types of polysaccharides afford well resolved ^{13}C-n.m.r. spectra[20,21] having characteristic signals in the anomeric region. Four anomeric signals having similar intensity were observed in the ^{13}C-n.m.r. spectrum

of the native polysaccharide. The signals at 104.1 and 101.6 p.p.m. correspond to the anomeric carbon atoms in the part of the polysaccharide in which the 3-linked β-D-galactosyl units alternate with the 4-linked α-L-galactose 6-sulfate units. The other two signals, at 102.7 and 98.7 p.p.m., correspond to the anomeric carbon atoms of the dissacharide repeating-structure in which the 3-linked β-D-galactosyl units alternate with 4-linked 3,6-anhydro-α-L-galactose units. The two latter signals were the only anomeric signals obtained for the agaroses of *Gracilaria secundata* and *Pterocladia* species studied in our laboratories, thus implying that at least part of the galactan sulfate of *Porphyra columbina* contains the same repeating-unit as in agarose (*i.e.*, β-D-galactosyl units alternating with 4-linked 3,6-anhydro-L-galactose units) and this precludes structure **2**. The lower fields of the anomeric regions (102.9–104.1 p.p.m.) are consistent with the β-D configuration of the galactopyranose residues[22], and the signals at 98.7–101.6 p.p.m. are an indication of the presence of α-L-galactopyranose residues. The presence of 6-*O*-methyl-D-galactopyranose residues in the polysaccharide is confirmed by the signal at 59.4 p.p.m., which agrees with information in the literature[23].

The polysaccharide isolated following alkaline treatment of the galactan sulfate PC3 gave only two signals in the anomeric region, at 102.8 and 98.8 p.p.m., and, in this and all other respects, its ^{13}C-n.m.r. spectrum was identical to that of agarose. As the galactose obtained from the hydrolysis of alkali-treated PC3 was virtually all the D enantiomer, it follows that the anhydro residues of this polysaccharide must be derived from L-galactose. Had 3,6-anhydro-D-galactopyranose been present, a signal at 93–96 p.p.m. would have been found in the ^{13}C-n.m.r. spectrum, as has been shown by Yaphe *et al.*[21]. Furthermore, many of the 3,6-anhydro-L-galactose residues of the alkali-treated galactan sulfate were derived from 6-*C*-sulfate residues of the original polysaccharide. It follows that most, if not all, of the sulfate groups in the original, galactan sulfate PC3 are attached to C-6 of L-galactose residues.

The ^{13}C-n.m.r. spectra of the original and the alkali-treated galactan sulfate, together with the enzymic oxidation results, thus confirm the D and L assignments of the various monosaccharide residues listed in Table I, and also give the anomeric configurations of these residues in the polymer.

Since the completion of this work, a note[24] has appeared that describes the water-soluble polysaccharide obtained from *Porphyra columbina* grown in Chile. That polysaccharide has a much lower content of 3,6-anhydrogalactose and galactose 6-sulfate than the polysaccharide described herein. Furthermore, the Chilean polysaccharide was fractionated by precipitation with cetyltrimethylammonium bromide, whereas our polysaccharide could not be fractionated with this reagent.

EXPERIMENTAL

Isolation of polysaccharide. — The alga *Porphyra columbina* was collected in June from rocks in the intertidal zone of St. Clair beach, Dunedin, New Zealand. It was carefully hand-sorted before drying in air at 30°. The seaweed (259 g, dry

weight) was then extracted with boiling water (2 L) for 2 h. The seaweed residues were removed on a cloth filter, the filtrate concentrated, and the concentrate dialyzed against running tap-water. The dialyzed concentrate was then poured into ethanol (5 L), and 10% sodium hydroxide was added to aid coagulation. The precipitate was removed by filtration, washed successively with ethanol and dry ether, and dried *in vacuo*. The seaweed residues were re-extracted three times with boiling water, after which, no more carbohydrate could be extracted. The four extracts were treated as separate polysaccharides and designated PC1, PC2, PC3, and PC4. They were further purified by dissolving in distilled water, dialyzing against distilled water, and isolating by lyophilization. Yields for each extraction were: 26.2, 13.6, 7.0, and 7.6 g (dry weight).

Materials and methods. — 5-(Hydroxymethyl)-2-furaldehyde was prepared by the method of Haworth and Jones[25]. 6-*O*-Methyl-D-galactose was prepared by methylation of 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose[26], followed by hydrolysis with trifluoroacetic acid[27].

A Perkin–Elmer Model 141 polarimeter was used for measurement of optical rotation. Melting points were determined with a Kofler hot-stage, melting-point apparatus and are uncorrected. A Perkin–Elmer 421 grating spectrophotometer was used for i.r. analyses. All evaporations were conducted at a bath temperature below 40°.

Ash, sulfur, nitrogen, and methoxyl contents were determined by the Microanalytical Laboratory, University of Otago, and moisture contents, by heating an aliquot of the sample at 105° to constant weight. The following solvents were used for paper chromatography by the descending method: (*A*) 10:3:3 (v/v) 1-butanol–pyridine–water, (*B*) 9:2:2 (v/v) ethyl acetate–acetic acid–water, and (*C*) 18:3:1:4 (v/v) ethyl acetate–acetic acid–formic acid–water. Sugars were detected with *p*-anisidine hydrochloride[28] or silver nitrate–sodium hydroxide sprays[29].

Thin-layer chromatography separations were conducted by the ascending method on aluminum foil coated 0.20 mm thick with Kieselgel $60F_{254}$. The following solvent systems were used: (*D*) 1:2 (v/v) toluene–diethyl ether and (*E*) 9:1 (v/v) 2-butanone–water. A 10% sulfuric acid solution was used as a spray for detecting sugars, and 0.3% (2,4-dinitrophenyl)hydrazine in 2M hydrochloric acid for the detection of aldehydes.

Gas–liquid chromatography (g.l.c.) was performed in a Shimadzu model GC-5A gas chromatograph equipped with a flame-ionization detector. The glass column (0.5 × 220 cm) was packed with 3% of ECNSS-M (supplied by Applied Science Laboratories), on Chromosorb W (AW-DMCS), 100–120 mesh. Nitrogen, the carrier gas, flowed at 55 mL/min, and the column temperature was 190°. A sensitivity check* of the chromatograph gave a value of 0.02 $cm^2.s.g^{-1}$. G.l.c.–mass spectrometry (g.l.c.–m.s.) was performed in a Varian Aerograph MAT mass spectro-

*Flame Ionization Detector FID-5, Instruction Manual, Scientific Inst. Div., Shimadzu Seisakusho, Ltd., Kyoto, Japan.

meter CH-7, using a stainless-steel column (0.3 × 220 cm) and the packings and conditions outlined.

The method of Yaphe and co-workers[2–4], with D-fructose as the standard, was used to determine 3,6-anhydrogalactose. Pyruvate was determined by the procedure of Hadjivassiliou and Rieder[30] as modified by Duckworth and Yaphe[31] for polysaccharides. The method of Schlegel and co-workers[32], involving use of D-galactose oxidase, was used to determine the ratio of D- to L-galactose. The D-galactose oxidase (EC 1.1.39; Type V from *Dactylium denroides*) was purchased from Sigma, and used without purification.

^{13}C-N.m.r. spectra were recorded with a JEOL FX60 spectrometer operating at 15.04 MHz in the pulsed, Fourier-transform mode, with broad-band noise-decoupling. Free induction decays were accumulated with a 90° pulse (22 μs) and a repetition time of 0.75 s. Spectra were recorded using 8 k data points and a spectral width of 2.5 kHz. Chemical shifts were measured relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, and are reported relative to external Me_4Si. All measurements were made at 90°, and samples at a concentration of 100 mg/mL of D_2O were contained in a tube (10 mm o.d.).

Attempted fractionation of the polysaccharide. — An aqueous solution of polysaccharide (PC3, 2.0 g) was treated with a solution of cetyltrimethylammonium bromide (10% w/v) by the method described by Scott[33]. The precipitate that formed yielded a polysaccharide {1.5 g; sulfate 12.45%; $[\alpha]_D^{19}$ −59.6° (*c* 0.22, water)} which, on further examination by hydrolysis followed by paper chromatography, was found to contain the same constituent monosaccharides as the original PC3.

A solution of the polysaccharide (PC3, 512 mg) in water (50 mL) was applied to a column (4 × 42 cm) of DEAE-Sephadex A-25, using the method described by Neukom and co-workers[34]. Elution of the column with a linear concentration gradient of sodium chloride (0–4 M) yielded fractions that were hydrolyzed, and the hydrolyzate analyzed by chromatography. The results showed that no fractionation had occurred, and that each fraction contained the same constituent monosaccharides as the original PC3. The major fraction had a sulfate content of 12.9%, and $[\alpha]_D^{19}$ −63.4° (*c* 0.20, water).

Treatment of the polysaccharide with alkali. — The polysaccharide (1 g) in water (50 mL) containing sodium borohydride (0.1 g) was kept for 30 min at 100°; then sodium hydroxide (3 g) and more sodium borohydride (0.05 g) were added. After a further 1 h at 80°, sodium borohydride (0.05 g) was again added, and, after 2 h, the solution was cooled, made neutral with acetic acid, dialyzed, and the product isolated by freeze-drying the dialyzate; yield, 0.75 g. Complete hydrolysis of a portion of the product with acid, followed by paper chromatography (solvents *A* and *C*), showed the presence of galactose, 6-*O*-methylgalactose, and 5-(hydroxymethyl)-2-furaldehyde. The composition of the hydrolyzate was established by g.l.c. of the alditol acetates.

Acetylation of the polysaccharide. — Freeze-dried polysaccharide (PC3, 1.0 g) was dissolved in formamide (100 mL), dry pyridine (170 mL) and acetic anhydride

(150 mL) were added, and the mixture was stirred for 72 h at room temperature. The viscous solution was poured with stirring into ice–water (1.5 L), and the fibrous precipitate recovered by filtration, dissolved in water, the solution dialyzed against distilled water for 3 days, and the product isolated by freeze-drying (1.31 g). The i.r. spectrum showed a trace of hydroxyl absorption at 3600–3460 cm^{-1}, not decreased by reacetylation.

Methylation of the polysaccharide acetate. — The polysaccharide acetate (0.7 g) was methylated by the Hakomori method. The methylated polysaccharide (0.5 g) showed a small hydroxyl peak in its i.r. spectrum. A portion (0.3 g) of the material was therefore further methylated by the Haworth method, to give the methylated polysaccharide (0.25 g); OMe 27.8%; $[\alpha]_D^{20}$ +84.1° (*c* 0.24, chloroform). Further methylation did not change the methoxyl content.

Methylation of alkali-treated polysaccharide. The alkali-treated polysaccharide was fully methylated by using a single Hakomori treatment without prior acetylation.

Hydrolysis of methylated polysaccharides. — Four different methods of hydrolysis were used, namely, (*A*) partial hydrolysis with 90% formic acid for 1 h at 100°, (*B*) 90% formic acid for 1 h at 100°, followed by 0.13M sulfuric acid for 16 h at 100°, (*C*) 0.5M sulfuric acid for 16 h at 100°, and (*D*) methanolysis with 2.3% methanolic hydrogen chloride, to yield the methyl glycosides of the partially methylated sugars and stabilize the methylated 3,6-anhydrogalactose derivatives, using the procedure of Penman and Rees[35]. The identity of the hydrolysis products was determined by comparison with g.l.c. retention-times previously reported[35], and confirmed by g.l.c.–m.s.

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