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Structural analysis of the polysaccharide from *Pachymenia lusoria* (Cryptonemiaceae, Rhodophyta)

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Abstract

The highly complex polysaccharide extracted from the New Zealand red alga *Pachymenia lusoria* (Grev.) J. Ag. has been characterised and certain structural features defined. A reductive hydrolysis procedure was used for constituent sugar and linkage analyses, with trideuteriomethylation being employed to enable the location of natural methyl ether groups to be determined. A reductive partial-hydrolysis procedure allowed agarobiosyl constituent residues to be identified. The analytical results are consistent with the polymer having a linear backbone of 3-linked D-galactopyranosyl alternating with 4-linked D- or L-galactopyranosyl residues. The 3-linked residues are nearly all 2-sulfated, with 1 in 3 also being 6-*O*-methylated and 1 in 5 also bearing a 4,6-pyruvic acetal residue. About one-third of the polymer is comprised of blocks of agarobiosyl repeat units that are 2-sulfated on the β-D-galactopyranosyl and one-third 2-*O*-methylated on the 3,6-anhydro-L-galactosyl constituents. Of the remaining 4-linked residues, half are 2-*O*-methyl-D-galactopyranosyl residues and half are galactopyranosyl residues, of which approximately half are in the L configuration.

Keywords: Pachymenia lusoria; Galactan; Red seaweed; Polysaccharide; Chemical analysis

1. Introduction

In the last 30 years there has been a considerable advance in our knowledge of the constitution of seaweed polysaccharides. Most structural studies of polysaccharides from red seaweeds have concentrated on agars and carrageenans, partly because they are industrially important, and partly because their structures are relatively straightforward.

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Thus agar polysaccharides contain a backbone of alternating 3-linked β-D-galactopyranosyl and 4-linked α-L-galactopyranosyl residues, with the latter predominantly in the form of a 3,6-anhydride. Carrageenans have a similar backbone, except that the 4-linked units are in the D form. Traditionally, agar substitution is thought to consist of low levels of sulfate ester, pyruvic acetal and single branching sugar residues, with the possibility of quite high levels of methyl ether substituents, while carrageenans are considered to have low levels of methyl ether and pyruvic acetal substituents and high levels of sulfate ester groups. Even though the more complex agaroids and carrageenanoids display a wide variation in levels and types of substitution, the constancy of the simple backbone repeating unit greatly assists the structural analysis.

From the mid 1960's, it has been known that the chemical structures of polysaccharides extracted from members of the Cryptonemiaceae are very complex and indeed one view would be that there is no structure in the sense that agar or carrageenan has a structure. Methods of examining the linkage patterns in such polysaccharides have included the analysis of monosaccharides produced by complete hydrolysis and oligosaccharides produced by partial hydrolysis or acetolysis. Methylation of native and desulfated polysaccharides followed by complete hydrolysis prior to analysis has also been used. Thus the extract from *Grateloupia elliptica* [1] consisted of D-galactose, L-galactose, 3,6-anhydro-D-galactose, and sulfate in 9:1:2:8 molar ratios. Additionally, there were small amounts of D-xylose, 2-*O*-methyl-L-galactose, 4-*O*-methyl-D-galactose, and 3,6-anhydro-2-*O*-methyl-L-galactose. Nunn and Parolis [2,3] showed that the polysaccharide from *Aeodes orbitosa* was highly sulfated, and contained predominantly D-galactose and 2-*O*-methyl-D-galactose but also 4-*O*-methyl-L-galactose, 6-*O*-methyl-galactose, and xylose. Periodate oxidation and methylation analysis of the native and desulfated polysaccharide identified the presence of $1 \rightarrow 3$ and $1 \rightarrow 4$ linkages. This showed that elements, at least, of the alternating structure found in carrageenans and agars could be present.

Analysis of the polysaccharide from *Pachymenia carnosa* by partial hydrolysis and acetolysis revealed a complex set of products [4,5]. All indicated that the polysaccharide consisted of 3-linked D-galactopyranosyl residues, some of which were 6-*O*-methylated, and 4-linked D-galactopyranosyl residues, some of which were 2-*O*-methylated. Methylation of the native and desulfated polysaccharide revealed that only the 3-linked units had sulfate ester substituents, which were largely at the 2-position [6]. No overall structure was proposed but ca. 60% of the polymer was considered to consist of an alternation of 3- and 4-linked units, the rest being composed of 3-linked units. Because of this complexity, it could be reasonably assumed that the polysaccharide had no "structure" in the sense that agar does. However, it was noted that some sugars were always linked to other specific sugars [5], so although there may not be a simple structure, the polymer was not a random assembly of its saccharide units.

A similar result was obtained by Allsobrook et al. [7–9] from analysis of the polysaccharide from *Aeodes ulvoidea*. Di-, tri-, and tetra-saccharides were isolated and characterised, and it was concluded that this polymer consisted mainly of alternating 3- and 4-linked D-galactosyl residues. A few of the 3-linked units had a 6-*O*-methyl substituent, while some of the 4-linked units had a 2-*O*-methyl substituent. There was a significant level of 4-*O*-methyl-L-galactose as a branch residue at the 6-position of some

of the D-galactosyl residues. Most of the 6-*O*-methyl-D-galactosyl residues were linked to 2-*O*-methyl-D-galactosyl residues and most of these sugars were concentrated in certain parts of the polymer. In addition, most of the sulfate ester was on the 2-position of 3-linked units, there was some pyruvic acetal present, and some of the 4-linked units were L-galactose. All larger fragments were consistent with an alternating structure.

Usov et al. [10] applied similar techniques to the polysaccharide from *Grateloupia divaricata* Okam, which was concluded to be heavily sulfated, and to contain some 3,6-anhydrogalactose. This polymer also consisted of 3- and 4-linked galactosyl residues [11]. The 3-linked residues in oligosaccharides obtained by acetolysis always had the D configuration, while the 4-linked residues were found in both the D and L configuration [12]. Usov et al. also used ^{13}C NMR spectroscopy as part of their analysis [13,14]. Interpretation of the spectra of the above oligosaccharides aided analysis of the native polysaccharide and its desulfated derivative. The spectrum of the polysaccharide remaining after treatment with methanolic HCl was fully assigned. Whilst such treatment resulted in the loss of xylosyl and 3,6-anhydrogalactosyl residues in addition to sulfate, the structure of the remaining material was greatly simplified. It emerged, interestingly, that 3- and 4-linked galactosyl residues alternated along the backbone of the polymer. Whilst complete assignment of the spectrum of the native polysaccharide was not possible, the L configuration of 3,6-anhydrogalactosyl residues was confirmed and the presence of agarobiosyl units in the polysaccharide was demonstrated.

Rees and co-workers [15,16], and later Parolis [17] using the same sample, examined the structure of the polysaccharide from *Pachymenia himantophora* collected in New Zealand in 1965. From 1939 the name *P. himantophora* was used for plants collected in the North Island of New Zealand, while *P. lusoria* was the name in use for those collected in the South Island, although, in fact, the type specimen for *P. lusoria* originated from the Bay of Islands in the northern North Island [18]. In 1974, however, Chapman and Parkinson [19] considered that *Pachymenia lusoria*, *P. himantophora*, and *P. dichotoma* were conspecific, representing "clinal variation in a single taxon", and applied the oldest name *P. lusoria* to this group. Thus, our sample, collected from the west coast of the South Island, has been identified as *P. lusoria* according to the current understanding of this widespread morphologically variable species. (There are two other species of *Pachymenia* reported from New Zealand that are morphologically distinctive from *P. lusoria*.) If *P. lusoria* is the major widespread *Pachymenia* species in New Zealand, it should exhibit only one polysaccharide structure, allowing that seaweed polysaccharides can show some natural variability. We now report on the determination of the major structural features of the polysaccharide extract from a South Island sample of *P. lusoria*, and show that it is similar to, but different in several important features from, the polysaccharide reported previously from "*P. himantophora*".

2. Experimental

Materials.—Pachymenia lusoria (Grev.) J. Ag. was collected at Constant Bay (Buller, New Zealand) in October 1992. A herbarium sample has been lodged with the Museum of New Zealand, as sample WELT A 20126.

Extraction.—Dried material (10 g) was extracted with water (600 mL), buffered at pH 6.25 with sodium hydrogen/dihydrogen phosphate buffer, at 120°C for 90 min in a pressure vessel. The solution was cooled to 90°C and filtered. The filtrate was then evaporated to reduce the volume to ca. 200 mL, the solution dialysed, and the polysaccharide recovered by evaporation at 60°C.

Alkali modification.—The polysaccharide (1 g) was dissolved in aq 4% NaOH–0.25% $NaBH_4$ (100 mL) and heated for 3 h at 95°C. The solution was then neutralised with AcOH and dialysed, and the polymer recovered by evaporation as above, with a recovery of 40%.

Desulfation.—Desulfation of the polysaccharide followed the method of Furneaux and Stevenson [20].

Spectroscopic analysis.—The ^{13}C NMR spectrum of the polymer was recorded for a 5% w/v solution in D_2O–H_2O at 90°C on a Bruker AC300 spectrometer (acquisition time, 0.8 s; delay, 0.5 s; 90° pulse). Films for infrared spectroscopy were made by slowly drying at 60°C a 0.75% solution cast on a polyethylene surface.

Chemical analysis.—The presence of pyruvic acetal was determined by hydrolysis and TLC analysis of the derived 2,4-dinitrophenylhydrazones following the procedure outlined by Miller and Furneaux [21].

Constituent sugar and methylation analyses for determining substitution patterns followed the reductive hydrolysis procedure of Stevenson and Furneaux [22].

The configuration of galactosyl residues was determined by heating a sample of alkali-modified polysaccharide (1 mg) for 1 h at 120°C with aq CF_3CO_2H (2 M, 0.25 mL). The hydrolysate was then cooled and evaporated at 40°C using a stream of dry air. Toluene (0.5 mL × 2) was then added and evaporated. (*S*)-(+)-2-Butyl glycosides were prepared from the resulting sample and analysed by GLC as their pertrimethylsilylated derivatives following the methods of Gerwig et al. [23]. The retention times were compared with those of standards prepared from D-galactose, L-galactose, 6-*O*-methyl-D-galactose, and 2-*O*-methyl-L-galactose, the latter obtained from a hydrolysed sample of the agar from *Curdiea coriacea* [24]. (*R*)-(−)-2-Butyl glycoside derivatives of 2-*O*-methyl-L-galactose and 6-*O*-methyl-D-galactose, which are enantiomeric with and hence have the same retention times as the (*S*)-(+)-2-butyl glycoside derivatives of 2-*O*-methyl-D-galactose and 6-*O*-methyl-L-galactose, respectively, were also prepared.

A reductive partial-hydrolysis procedure [25], developed from the method of Usov and Klochova [26], was used to identify the constituent agarobiosyl residues in both native and permethylated polysaccharide samples.

3. Results and discussion

Constituents of the polysaccharide.—The general expectation was that the extract would be a complex polysaccharide based on galactose, with a range of substituents including sulfate esters, methyl ether groups, pyruvic acetal, and branching sugar units. These could occur in a variety of positions, possibly with more than one substituent per saccharide unit. This was found to be the case.

Table 1
Constituent sugars (normalised mol%) of the native (N), alkali-modified (A), and partially desulfated (D) polysaccharides of *Pachymenia lusoria*

Constituent sugar [a]	Polymer N	Polymer A	Polymer D
AnGal	10	10	7
2-Me-AnGal	5	5	4
Gal	43	47	45
6-Me-Gal	21	19	22
2-Me-Gal	18	17	20
Xyl	3	2	2

[a] AnGal refers to 3,6-anhydrogalactosyl residues determined as 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol; 2-Me-AnGal to 3,6-anhydro-2-*O*-methylgalactosyl residues, etc.

The presence of pyruvic acetal was indicated in the ^{13}C NMR spectrum, which showed a strong signal for a *C*-methyl group at 25.3 ppm, and this was confirmed by a qualitative chemical method [21]. The presence of sulfate ester substituents was confirmed by infrared spectroscopy, and methyl ethers and xylosyl residues by constituent sugar analysis.

Infrared spectroscopy showed the absorptions expected of a polysaccharide, with a strong OH stretch at 3200–3500 cm^{-1}, a strong OH bend at 1620–1640 cm^{-1}, and a strong CO stretch at 1010–1070 cm^{-1}. Additionally, there was a strong absorption at 1220–1260 cm^{-1} and a medium strong absorption at 1360–1370 cm^{-1} indicative of sulfate ester generally, a weak signal at 930 cm^{-1} indicative of a low level of 3,6-anhydrogalactose, and a signal of weak to moderate intensity at 830 cm^{-1} indicative of an equatorial sulfate ester substituent [27].

Constituent sugar analysis of the native polysaccharide from *Pachymenia lusoria* (Polymer N, Table 1) showed it to contain 3,6-anhydrogalactosyl, 3,6-anhydro-2-*O*-methylgalactosyl, galactosyl, 6-*O*-methylgalactosyl, 2-*O*-methylgalactosyl, and xylosyl residues. Substituent and linkage (''methylation'') analysis (Table 2) indicated that the polysaccharide was composed predominantly of 4-linked 3,6-anhydrogalactosyl (4-AnGal), 4-linked galactosyl (4-Gal), and 2,3-linked/substituted galactosyl (2,3-Gal) residues. These may be naturally *O*-methylated since this information is masked on permethylation. In addition, the presence of 2,3,4,6-substituted galactosyl residues (2,3,4,6-Gal) suggests 2-sulfated-3-linked galactosyl residues with a pyruvic acetal on the 4- and 6-positions. This is supported by evidence of the presence of pyruvic acetal by ^{13}C NMR spectroscopy and by chemical analysis. If this interpretation is accepted, then from Table 2 we see that 45% of the sugars are 4-linked, and 45% 3-linked, with 10% ambiguous or terminal sugars. These figures are quite suggestive that the polymer does follow the alternating 3-linked and 4-linked galactosyl backbone noted by Usov et al. [14] for *Grateloupia divaricata*, and which is found in most other red algal galactans. The constituent sugar analysis of the alkali-treated polymer (Polymer A, Table 1) is identical to that of the native polymer. The results from the methylation analysis of the alkali-treated polymer are essentially the same as those of the native sample (Table 2) except that there are fewer ambiguous residues and a correspondingly higher level of 4-Gal. This suggests that methylation of this sample was more complete although the reaction conditions were the same.

Table 2
Linkage points on saccharide units as obtained from methylation analysis on polymers N, A, and D (see Table 1)

Linkages [a]	(Normalised mol%)		
	Polymer N	Polymer A	Polymer D
3-Linked units			
3-Gal	1	1	21
2,3-Gal	28	28	12
3,6-Gal		1	4
2,3,6-Gal	6	6	
3,4,6-Gal	2	1	7
2,3,4,6-Gal	8	7	5
4-Linked units			
4-AnGal	18	16	10
4-Gal	25	32	34
2,4-Gal	2	1	
Ambiguous and terminal			
3,4-Gal	5	3	3
2,3,4-Gal	4	1	2
T-Gal	1	1	1
T-Xyl		2	1

[a] 3-Gal determined as 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol; T(erminal)-Xyl as 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol, etc. See text for further explanation.

Position of natural O-*methyl substituents.*—The combination of the sugar analysis and methylation analysis leads to some further conclusions. Thus the 3,6-anhydrogalactosyl residues are not 2-sulfated, but one in three is naturally 2-*O*-methylated. There is, however, a problem in that the naturally occurring methylation in the galactosyl residues is masked during methylation analysis. This problem can be overcome by the use of trideuteriomethyl iodide as the reagent in the procedure. The natural methylation and the introduced trideuteriomethylation can be differentiated in the mass spectra of the derivatives. Hence it is possible to determine the linkage pattern of the naturally *O*-methylated galactosyl residues.

As an example of the method, for 2-substituted, 3-linked galactosyl residues (2,3-Gal), 4,6-bis-*O*-(trideuteriomethylation) results in fragments at m/z 167, 107, and 48, while 4-*O*-trideuteriomethylation of naturally 6-*O*-methylated residues results in fragments at m/z 164, 104, and 45. The amounts of these three sets of fragment ions present in the 2,3-Gal peak by GLC–MS analysis were m/z 167 (37%), 164 (63%); 107 (40%), 104 (60%); and 48 (49%), 45 (51%). Therefore, of the 28 mol% of 2,3-Gal residues in the base-treated polymer (Table 2), 18–14 mol% would be 6-*O*-methylated. Since the total 6-*O*-methylated galactose as determined by constituent sugar analysis was 19 mol% (Table 1), we are confident that all 6-*O*-methylation occurs on these 2,3-Gal residues. No significant levels of 6-*O*-methylation were detected in any of the other species present. In turn this implies that all of the 6-*O*-methylgalactosyl residues are 3-linked and have a sulfate ester (or other hydrolysable substituent) on O-2, and no other substitution. Similarly the trideuteriomethylation–GC–MS method was used to demon-

strate that approximately half the 4-linked galactosyl residues (4-Gal), i.e.. 16 mol%, are naturally 2-*O*-methylated. This is close to the total amount of 2-MeGal (17 mol%) found by constituent sugar analysis. A third of the 3,6-anhydrogalactosyl residues are naturally 2-*O*-methylated (Table 1, data for Polymers N and A). This was also confirmed by GC–MS analysis of the trideuteriomethylated sample.

We conclude that essentially all 2-*O*-methylation occurs on 4-linked galactosyl or 3,6-anhydrogalactosyl residues. These results are in agreement with those of Parolis [6] for *Pachymenia carnosa*, and of Allsobrook et al. [9] for *Aeodes ulvoidea*, which strongly suggests that this type of substitution is common for polysaccharides from species within the Cryptonemiaceae, and is possibly typical of them.

Location of sulfate ester substituents.—A sample of the native polymer was also desulfated by solvolysis of the pyridinium salt form in Me_2SO–methanol–pyridine according to the procedure of Furneaux and Stevenson [20]. From the linkage analysis of this material (Polymer D, Table 2) it is clear that desulfation was incomplete but nevertheless some conclusions can be drawn. Of the 3-linked galactosyl residues, the amount of 3-linked units (3-Gal) rose dramatically while the level of 2,3-linked units (2,3-Gal) and 2,3,6-linked units (2,3,6-Gal) fell significantly, and 3,6-linked units (3,6-Gal) increased. From this, it seems reasonable to conclude that much of the sulfate ester is at the 2-position of 3-linked galactosyl residues. 2-Sulfate ester groups were removed to a significant extent during desulfation. The appearance of 3,6-linked galactosyl residues is consistent with the terminal sugars (xylose, and perhaps some galactose) being linked to the 6-position of a 2-sulfated 3-linked galactosyl residue. The increase of 3,4,6-linked galactosyl residues following desulfation is consistent with the pyruvic acetal being on a 2-sulfated 3-linked galactosyl residue in the native polymer, although the residual level of the 2,3,4,6-substitution in the desulfated polymer is rather high.

Of the 4-linked units, the level of 4-linked galactosyl residues (4-Gal) increases at the expense of 4-linked anhydrogalactosyl residues (4-AnGal) in the native polymer. This may be due to some degradation occurring during desulfation. The removal of the 2,4-linked galactosyl residue (2,4-Gal) on desulfation is consistent with a small amount of the 4-linked units also being sulfated at the 2-position.

Thus the results of this desulfation experiment indicate that most of the 2-sulfate ester was cleaved. While solvolytic desulfation was not fully effective, the alternative method, which uses methanolic hydrogen chloride, results in the loss of information on the anhydrogalactosyl residues. This was also found to be the case for *Grateloupia divaricata* [11]. We felt that the less efficient solvolytic method was preferable since sufficient desulfation occurred to give a reasonably firm indication of the location of the sulfate esters, and the anhydrogalactosyl residues were retained.

Enantiomeric configuration of sugar residues.—Further structural information can be obtained by determining the configuration of the sugar species present using chemical methods. By converting enantiomeric sugar residues into diastereomeric pertrimethylsilylated (+)-2-butyl glycosides and separating them by GLC [23], it was found that the polymer from *Pachymenia lusoria* contained both D- and L-galactose in an approximate ratio of 4:1. In addition, the 6-*O*-methyl- and 2-*O*-methyl-galactosyl residues were found to be exclusively in the D configuration, which was also found to be the case for the

polysaccharide from *Pachymenia carnosa* by Farrant et al. [4,5]. Unfortunately the 3,6-anhydrogalactosyl residues were unstable under the conditions used in this method, but information on their configuration and the way they are linked can be partly determined by partial reductive hydrolysis, as described in the next section. The ^{13}C NMR spectrum of the desulfated polymer was more complex than that reported by Usov et al. [14] but a clear signal was present, corresponding to 4-linked L-galactopyranosyl residues. If a little under 50% of the residues are galactosyl, then we infer that approximately half of the 4-linked galactosyl residues are in the L configuration.

Constituent agarobiitol residues.—Mild acid hydrolysis preferentially cleaves 3,6-anhydrogalactosidic bonds, so a disaccharide is formed where 3,6-anhydrogalactosyl and galactosyl residues alternate in the polymer. Reduction of such a disaccharide results in an agarobiitol if the anhydrogalactose is in the L configuration, or a carrabiitol if the anhydrogalactose is in the D configuration.

Following the work of Usov et al. [26], a "*reductive partial-hydrolysis procedure*" that incorporates an acid-catalysed acetylation step [25] has been developed. This step effects cleavage of sulfate ester substituents and per-*O*-acetylation. The resulting per-acetylated biitols can be separated and identified by GC and GC–MS.

Standards for the possible species present in *Pachymenia lusoria* polysaccharide, were prepared, i.e., agarobiitol, 6′-*O*-methylagarobiitol, 2-*O*-methylagarobiitol, and 2,6′-di-*O*-dimethylagarobiitol from agars from *Pterocladia lucida, Curdiea codiodes, Dasyclonium incisum*, and *Curdiea coriacea*, respectively, carrabiitol from Sigma, and 6′-*O*-methylcarrabiitol from κ-carrageenan a partially methylated iota-carrageenan [25]. The prime indicates a substituent on the non-reducing terminal galactosyl residue. Each acetylated biitol had a unique retention time and a mass spectrum characteristic of the number of methyl ether substituents on each sugar residue.

The *Pachymenia* sample gave two disaccharide-alditol derivatives with retention times corresponding to agarobiitol and 2-*O*-methylagarobiitol. Had other biitols been formed, they would have been detected and accordingly this indicates a high level of selectivity in forming these agarobiitols. This confirms that the 3,6-anhydrogalactosyl residues in this polysaccharide are of the L configuration, and they are substituted at O-4 by D-galactosyl residues which are not 6-*O*-methylated.

Hydrolysis of the linkage at C-1 of the anhydrogalactosyl residue is known to be faster than for other linkages, but to obtain agarobiose, and hence biitols, it is also necessary to cleave the linkage at O-3 of the D-galactosyl residue in the agar. If the polysaccharide exists in agarobiose blocks, this result would be expected and accordingly the simplest interpretation of these results is that we have evidence for the existence of the agarobiose as blocks within the polymer.

A second experiment was conducted to obtain a quantitative measure of the recovery of agarobiitol peracetate. A known weight of base-treated *Pachymenia lusoria* polysaccharide was derivatised. A known weight of inositol hexaacetate was added to the product and its response was related to the response of a carrabiitol acetate standard. Of the sample, 10 mol% was found to have been converted into agarobiitol peracetate. Since base-treated *Pachymenia lusoria* contained 10 mol% 3,6-anhydrogalactosyl residues by constituent sugar analysis, this confirms that, within the limits of experimen-

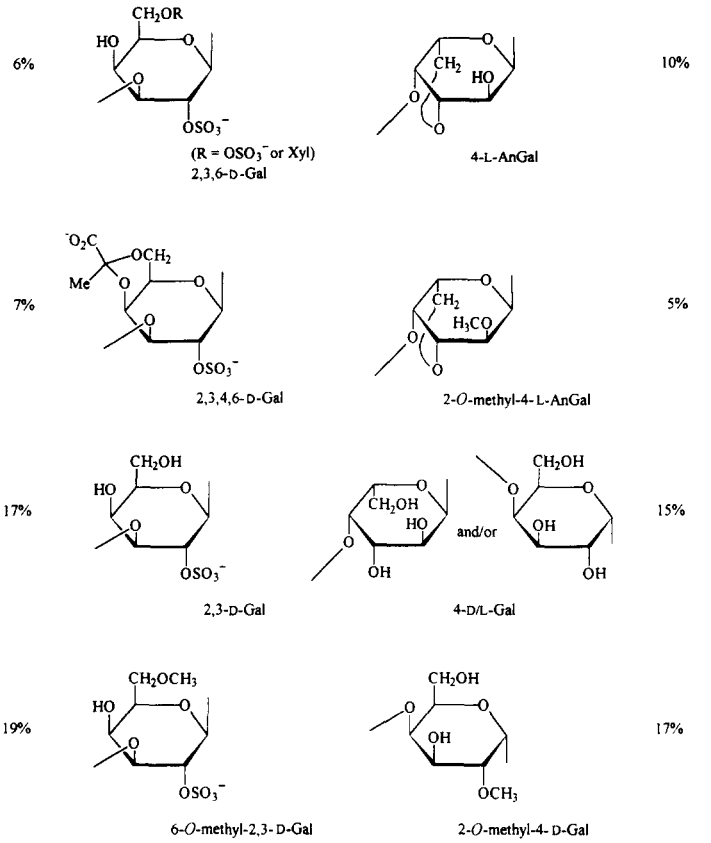

Fig. 1. Major component residues in the polysaccharide from base-treated *Pachymenia lusoria*.

tal accuracy, all of the agarobiose units within the polymer are converted into agarobiitol derivatives.

There are four major 4-linked sugar residues and (at least) four major 3-linked residues in *Pachymenia lusoria* (see Fig. 1). Evidence from the reductive partial-hydrolysis experiments on underivatised base-treated material showed that galactosyl residues substituted naturally with a 6-*O*-methyl group are not linked to 3,6-anhydrogalactosyl residues or their 2-*O*-methyl analogues. However, there still remain three possible major substitution patterns for β-D-galactosyl residues that are linked to 3,6-anhydro-L-galactosyl units, i.e., (*i*) sulfate ester on O-2 (2,3-Gal), (*ii*) sulfate ester on O-2 plus pyruvic acetal on O-4 and O-6 (2,3,4,6-Gal), and (*iii*) sulfate ester on O-2 and a sugar branch at O-6 (2,3,6-Gal).

The alternatives were evaluated by application of the reductive partial-hydrolysis procedure to permethylated samples. The resulting partially methylated, partially acetylated biitols were analysed by GC–CIMS with ammonia as reagent gas. The various species are characterised by their $[M + NH_4]^+$ ions. In addition, the mass of the fragment ion corresponding to the β-D-galactosyl unit of the biitol is indicative of the

number of *O*-methyl substituents it contains. The positions of the *O*-methyl groups are determined by comparing GC retention times of the biitol derivatives with those of various standards (where possible).

The GC–CIMS total ion chromatograph of the sample from methylated, base-treated *Pachymenia lusoria* revealed a major component at 8.1 min and two minor components at 7.7 and 9.4 min. The major peak (8.1 min) gave an $[M + NH_4]^+$ ion of m/z 554, which corresponds to a biitol with three *O*-methyl groups. The major fragment ion of m/z 275 indicates that there are two methyl groups on the β-D-galactosyl residue. As shown in Fig. 2, we propose that this species is 2,4′,6′-tri-*O*-methylagarobiitol tetraacetate (**2**), which would be obtained from disaccharide **1** comprised of a 2-sulfated β-D-galactosyl residue linked to the 4-position of a 3,6-anhydro-L-galactosyl residue; the position of natural methylation is determined below.

The minor component peak at 7.7 min with the same MS ions as **2** was identified as 2,2′,6′-tri-*O*-methylagarobiitol tetraacetate (**6**) by comparison with standards, and arises from disaccharide **5** comprised of a 4-sulfated 3-linked β-D-galactosyl residue connected to the 4-position of a 3,6-anhydro-L-galactosyl residue. Another small peak was observed at 9.4 min. The presence in its mass spectrum of ions at m/z 582 and 303 indicate a di-*O*-methylbiitol with one methyl group per sugar unit. We propose that this species is 2,4′-di-*O*-methylagarobiitol pentaacetate (**8**) arising from disaccharide **7** comprised of a 2,6-disulfated 3-linked β-D-galactosyl residue connected to the 4-position of a 3,6-anhydro-L-galactosyl residue.

The GC trace of the sample from reductive partial-hydrolysis of the trideuteriomethylated, base-treated *Pachymenia lusoria* polysaccharide also comprised one major peak but on mass spectrometry this peak resulted in two $[M + NH_4]^+$ ions. The ion at m/z 563 corresponding to tris-*O*-(trideuteriomethyl)agarobiitol tetraacetate was more intense than that at m/z 560 which corresponds to a mono-*O*-methyl-bis-*O*-(trideuteriomethyl)agarobiitol tetraacetate. The presence of a major fragment ion at m/z 281 and the absence of a fragment ion at m/z 275 indicated that both methyl groups on the β-D-galactopyranosyl residue were trideuterated. The species present are considered to be 2,4′,6′-tris-*O*-(trideuteriomethyl)agarobiitol tetraacetate (**3**) and 2-*O*-methyl-4′,6′-bis-*O*-(trideuteriomethyl)agarobiitol tetraacetate (**4**). Such species would arise from 2-sulfated 3-linked β-D-galactopyranosyl residues alternating with 4-linked 3,6-anhydro-α-L-galactopyranosyl residues some of which are naturally 2-*O*-methylated (i.e., disaccharides **1**, R = H or Me). Two small peaks corresponding to the minor components detected in the preceding experiment were also observed by GC–MS, but in this case molecular ions were not visible, so unambiguous identification was not possible.

These analyses do not reveal if 6-*O*-xylosylated or 4,6-*O*-pyruvated 3-linked β-D-galactosyl residues are connected to 3,6-anhydrogalactosyl residues. We do not yet have standards for the biitol derivatives concerned, but suspect that they would not be sufficiently volatile for GC analysis.

^{13}C NMR spectroscopy.—We have determined that there are at least twelve sugar units present in *Pachymenia lusoria*, which can be differentiated by substitution. The ^{13}C NMR spectra of the polymers are complex, the signals are broad, and unfortunately only limited information can be deduced from these spectra. Apart from confirming the

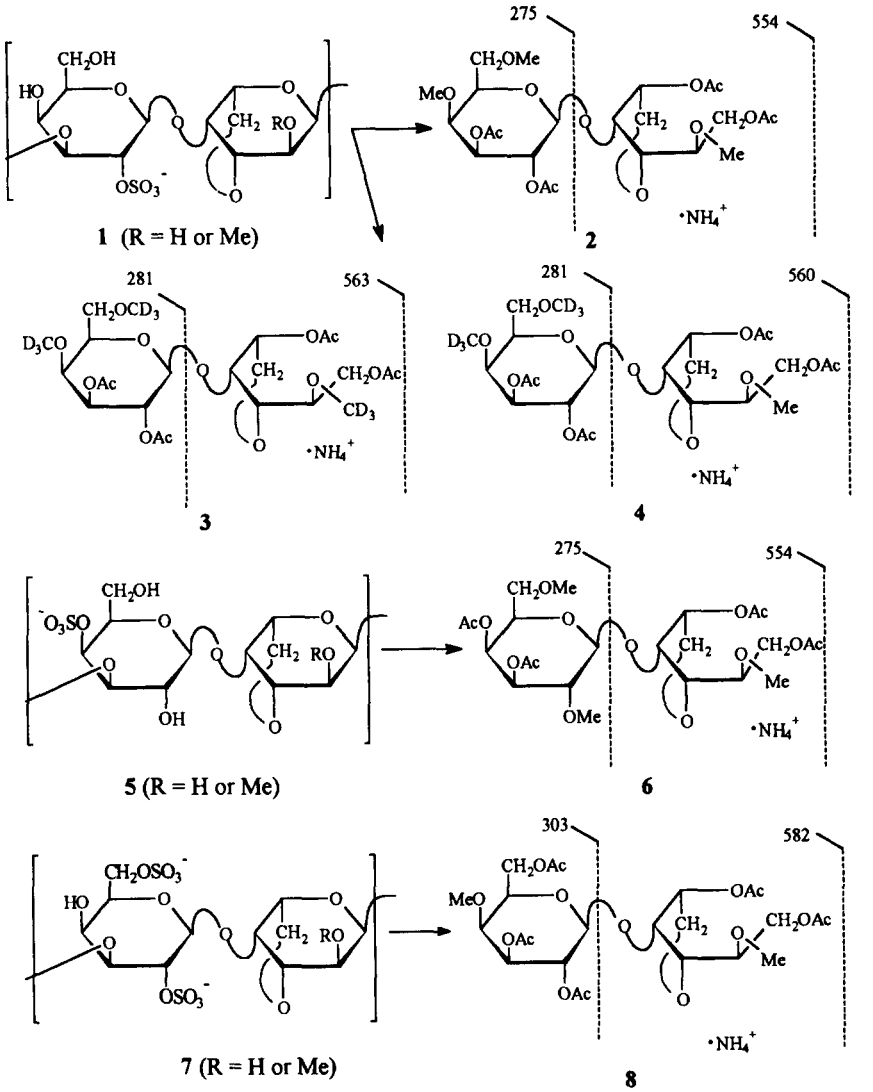

Fig. 2. Origins of partially methylated, partially acetylated agarobiitol derivatives and their major mass-spectral ions.

presence of pyruvic acetal, 4-linked-L-galactosyl residues, 3-linked 2-sulfated galactosyl residues, 3,6-anhydro-L-galactosyl residues, and the 2-*O*-methyl derivative of the latter (by comparison with known spectra), the main feature is the presence of distinct signals in the C-1 region. These indicate that the polymer is not a random assembly of different units.

Potential heterogeneity.—Since the material analysed has such a complex structure, the question arises as to whether more than one polymer is present. We consider that,

within the levels of experimental accuracy, the structural components identified behave as though they originate essentially from one polymer. For instance, although considerable material was lost during base-treatment and desulfation (presumably low molecular weight material removed by dialysis), the structure of the remaining material was consistent with the native sample. Thus, if there were a mixture of polymers present, each would have had to behave in the same way to chemical treatment, purification, and isolation. No evidence has been presented in the literature that any seaweed of the Cryptonemiaceae contains more than one galactan.

4. Conclusion

Fig. 1 summarises what we deduce to be the nature and mol% of the major residues that make up the *Pachymenia lusoria* polysaccharide. On the left side are the four variously substituted 3-linked D-galactopyranosyl residues, and on the right the five types of 4-linked galactopyranosyl residues, three in the L and two in the D configuration. We have not shown the relative proportions of unsubstituted 4-linked D- and L-galactopyranosyl residues, since these numbers could only be derived indirectly, and were judged to be too uncertain. The anomeric configurations shown are not proven, but assumed by analogy with those found in other red algal galactans.

At this stage it is not possible to construct a complete model for this complex polysaccharide. From the fact that agarobiitol derivatives were obtained in good yield from the partial reductive-hydrolysis procedure, it appears that most of the (substituted) agarobiose residues that make up about one-third of the polymer occur in blocks together. These disaccharide residues are composed (at least in part) of 2-sulfated galactosyl residues (but not their 6-*O*-methylated counterparts) linked to the 4-position of 3,6-anhydro-L-galactosyl residues, some of which are 2-*O*-methylated. It is not yet evident, however, how the remaining residues are distributed in the polymer.

Two issues arise from this work. The first is whether the polysaccharides obtained from *Pachymenia lusoria* and *Pachymenia himantophora* are the same. There are similarities, such as the approximately equal amounts of 3- and 4-linked residues, the "consecutive" arrangements of the disaccharide components containing the 3,6-anhydrogalactosyl residues, and the fact that most of the 3-linked residues are sulfated at O-2 while most of the 4-linked residues are not sulfated. However, there are also differences. Some, such as the apparent absence of L-galactose in *P. himantophora*, and the presence of agarobiosyl units in *P. lusoria* but the identification of carrabiosyl units in *P. himantophora*, may be due to the analytical techniques used. For example, interpretation of results of *P. himantophora* may have been affected by poor resolution of acetylated dimethyl acetal derivatives by packed column GLC.

There are aspects of the structural information on these two polysaccharide samples, however, that are inconsistent with their having come from the same red seaweed species. For example, the *P. himantophora* polysaccharide was shown to be devoid of methylated galactose residues, to have little, if any, pyruvate substitution, and (from the methylation analysis conducted by Parolis) to have a high level (27 mol%) of 3-linked D-galactopyranosyl 2,6-disulfate residues. In contrast, the *P. lusoria* polysaccharide

examined here had a high level of natural methylation (5 mol% 2-MeAnGal, 21 mol% 6-MeGal, and 18 mol% 2-MeGal), pyruvate substituents (8 mol%), and a much lower level (6 mol%) of 3-linked D-galactopyranosyl 2,6-disulfate residues.

Whilst we have only examined material from a single collection of *Pachymenia* of undetermined life stage from a single site, we are tempted to conclude that there is more than one species within the current description of *Pachymenia lusoria* in New Zealand and that the polysaccharide structural features identified here may assist in a reevaluation of this species. Interestingly, the northern and southern populations of *Pachymenia lusoria* are separated by a region (Cook Strait, Taranaki, Hawkes Bay, northern coasts of the South Island) where these plants are rare or absent.

The second issue relates to the taxonomic significance of certain structural units. We have identified significant levels of methyl ether, sulfate ester, and pyruvic acetal substituents in our sample of *Pachymenia lusoria*. The data can be reasonably interpreted as if the polymer were composed of a linear backbone of alternating 3- and 4-linked residues. These sugar residues (Fig. 1) are predominantly galactosyl but sometimes 3,6-anhydrogalactosyl units. All *O*-methyl galactosyl units are in the D configuration, even when 4-linked. The 4-linked galactosyl units may be either in the D or L configuration, but the 3,6-anhydrogalactosyl units present are all in the L configuration. Almost all of the 3-linked galactosyl residues are sulfated at O-2. Many of these structural units have been reported in polysaccharides from species of *Grateloupia* and *Aeodes*, as well as other species of *Pachymenia*, but not from unrelated plants. Thus, they may act as a taxonomic marker for a specific botanical grouping. There is insufficient information available as yet to know whether the substituted agarabiose residues identified herein occur in other Cryptonemiaceae.

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