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Sulfated galactans from Australian specimens of the red alga *Phacelocarpus peperocarpos* (Gigartinales, Rhodophyta)

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Abstract

Polysaccharides from the red alga *Phacelocarpus peperocarpos* were extracted with hot water, clarified, and precipitated with 2-propanol. The native preparation was highly sulfated (36.2% w/w). Alkali modification decreased the sulfate content by 2.0% w/w. The alkali-modified polysaccharide is composed mostly of galactose (Gal, 51 mol%) and 3,6-anhydrogalactose (AnGal, 41 mol%), with minor amounts of a mono-*O*-methylgalactose (MeGal, 1 mol%), xylose (Xyl, 6 mol%), and glucose (Glc, 1 mol%). The FTIR spectrum of the alkali-modified polysaccharide resembled κ-carrageenan with absorption at 930 cm^{-1} (indicative of AnGal) and 850 cm^{-1} (Gal 4-sulfate). However, an additional, major band of absorption occurred at 820 cm^{-1}, indicating the presence of equatorial sulfate ester substitution at O-6 of Gal residues. A combination of linkage and ^{13}C NMR spectroscopic analyses showed that the polysaccharide was composed predominantly of a novel repeating-unit, *O*-β-D-galactopyranosyl 4,6-disulfate)-(1 → 4)-3,6-anhydro-α-D-galactopyranose. Minor structural variations also occurred, including alternative patterns of sulfation and the presence of terminal Xyl*p*. The location of the terminal Xyl*p* residues was not certain but evidence supported their attachment at O-3 of some 4-linked Gal*p* residues. The cell-wall galactans remain unchanged during the life cycle of the alga. © 1996 Elsevier Science Ltd.

Keywords: Carrageenan, 4′,6′-disulfated, κ-, and ω-; Galactans, sulfated; Structure determination; Polysaccharides, algal; Rhodophyta; Gigartinales

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1. Introduction

The major, matrix-phase polysaccharides of marine red macroalgae are hot water-extractable, sulfated galactans (see [1] for a review). These galactans are typically composed of repeating disaccharides of β-(1 → 3)-linked galactopyranose (Gal*p*) and α-(1 → 4)-linked Gal*p*, which are classified either as agars if the 4-linked residue is in the L configuration or carrageenans if the 4-linked residue is in the D configuration. In both classes, the 3-linked residue always has the D configuration. In both agars and carrageenans, 4-linked 3,6-anhydrogalactopyranose (AnGal*p*) residues are formed from precursor Gal*p* residues bearing sulfate ester substitution at O-6 through the action of a sulfohydrolase enzyme in vivo [2–4] or by treatment with alkali in vitro [5]. Red algal galactans may also bear methyl ether or pyruvate acetal substitutions as well as glycosyl substitutions, most commonly as single xylopyranosyl (Xyl*p*) or mono-*O*-methylgalactopyranosyl (MeGal*p*) residues branching from the main galactan chain. To accommodate the growing number of unusual structures being discovered among red algal polysaccharides, Knutsen et al. [6] proposed a versatile nomenclatural system [1].

We are currently surveying cell-wall polysaccharides from Australian red algae of the order Gigartinales both for their potential as commercial sources of phycocolloids [7–9] and to assist with studies of red algal classification [10,11]. One group of algae within this survey is the genus *Phacelocarpus*, currently the sole member of the Phacelocarpaceae and consisting of nine mostly southern-hemisphere species [12,13]. Five *Phacelocarpus* species are found in the waters along southern Australia, three of which also occur in New Zealand [13]. The species *P. peperocarpos* (previously identified as *P. labillardieri* [14]) is a common member of the marine flora of the Australian southern coast and New Zealand. Specimens of *P. peperocarpos* can grow up to 50 cm in length and, typical of the genus, are recognised by their highly branched, flattened fronds, from which irregularly distichously arranged rows of short, determinate branchlets arise from the margins [12,13]. The fruits of female specimens form prominent swellings borne on stalks that protrude from the branch margins between the branchlets [12,13]. Given the abundance of this seaweed in southern Australia, it was selected as a logical starting point for investigating the polysaccharides of the genus *Phacelocarpus*.

2. Experimental

Algal samples.—All specimens of *P. peperocarpos* were collected at Warrnambool, Victoria. Detailed carbohydrate analyses were carried out on a drift-cast, tetrasporic specimen (MELU-A 042229) collected on 28 October 1990 by Dr. G.T. Kraft. Another tetrasporic specimen (MELU-A 042230) was collected on 2 December 1991 by Dr. G.T. Kraft and Dr. G.W. Saunders at a depth of 3–5 m. The drift-cast specimen of the female gametophyte (MELU-A 042227) was collected on 25 June 1992 by Dr. G.T. Kraft.

[1] This system has not been submitted for review by the IUPAC–IUBMB Joint Commission for Biochemical Nomenclature. The trivial name "carrabiose" used in this article denotes the disaccharide *O*-β-D-galactopyranosyl-(1 → 4)-3,6-anhydro-α-D-galactopyranose.

Extraction and alkali modification of the polysaccharides.—The procedures used for preparing the algal samples and for hot water-extraction, clarification, and 2-propanol-precipitation of the polysaccharide preparations have been described previously [15]. The polysaccharides were alkali modified by the method described by Craigie and Leigh [5].

Chemical analyses.—Sulfate content was determined by the method of Tabatabai [16] as modified by Craigie et al. [17]. Pyruvate content was assayed by the lactate dehydrogenase method of Duckworth and Yaphe [18]. The optical rotation of an aqueous solution of the alkali-modified polysaccharide (0.3% w/v) was measured at room temperature on a Perkin–Elmer model 241C polarimeter at 589 nm wavelength. Alditol acetates of the constituent monosaccharides were prepared for analysis by the reductive hydrolysis method of Stevenson and Furneaux [19] and were separated, identified, and quantified using gas chromatography (GC) and GC–mass spectrometry (MS) as described previously [20].

Linkage and substitution patterns were investigated by preparing dimethyl sulfoxide (Me_2SO)-soluble triethylammonium salts of the polysaccharides and methylating as described by Stevenson and Furneaux [19], except using a NaOH–Me_2SO suspension to generate the alkoxide [21]. The partially methylated alditol acetates were generated by reductive hydrolysis [19], and were separated by GC on a BPX70 capillary column, detected by electron impact (70 eV) ionisation-MS, and identified by their mass spectra and their retention times relative to *myo*-inositol hexaacetate as described by Lau and Bacic [22]. The partially methylated species were quantified using the total ion current.

Spectroscopic analyses.—Fourier transform infrared (FTIR) spectra of polysaccharide films (prepared as described in [10]) were recorded on a Perkin–Elmer Series 2000 FTIR spectrometer in transmittance mode (8 scans, collected at a resolution of 4 cm^{-1}).

For nuclear magnetic resonance (NMR) spectroscopy, the polysaccharide was dissolved in D_2O (4% w/v) with Me_2SO added as an internal standard referenced at 39.6 ppm. The proton-decoupled ^{13}C NMR spectrum was recorded at 80 °C on a Bruker AMX300 WB spectrometer (operating at 75.5 MHz) with a spectral width of 17.9 kHz, a 45° pulse angle, an acquisition time of 0.46 s, and a relaxation delay of 0.2 s for approximately 70,000 scans. The methylene carbons were assigned using the *J*-modulated spin-echo experiment [23]. This spectrum was recorded at 80 °C with a spectral width of 20.0 kHz, an acquisition time of 0.41 s, a relaxation delay of 1.5 s, and a modulation delay of 7.1 ms for approximately 29,000 scans.

3. Results and discussion

Preparation and properties of the polysaccharides.—Hot water-extraction of the three *Phacelopcarpus peperocarpos* specimens, filtration of the extracts, and alcohol-precipitation gave crude polysaccharide preparations in 24–45% yield (w/w of the dried seaweed meal). The crude preparation from the specimen MELU-A 042229 was treated with amyloglucosidase to give the preparation of the native cell-wall polysaccharides, which was subsequently treated with hot alkali. The alkali-modified preparation was dextrorotatory ($[\alpha]_D = +23°$, *c* 0.3 in H_2O). In the presence of either KCl or $CaCl_2$

Table 1
Constituent sugars (mol%) of polysaccharide preparations from *Phacelocarpus peperocarpos*

Constituent monosaccharide	Tetrasporophyte (MELU-A 042229)		Tetrasporophyte (MELU-A 042230), crude	Gametophyte (MELU-A 042227), crude
	native	alkali-modified		
Gal	64	51	61	57
AnGal	29	41	29	29
3/4-MeGal	1	1	1	tr
Xyl	5	6	5	6
Glc	1	1	4	8

Gal = galactose, AnGal = 3,6-anhydrogalactose, 3/4-MeGal = 3- and/or 4-*O*-methylgalactose, Xyl = xylose, Glc = glucose.

(0.2% w/v), solutions of both the native and the alkali-modified preparations from MELU-A 042229 were non-gelling at a concentration of 2% (w/v) at room temperature, indicating differences between the *P. peperocarpos* polysaccharide and commercial red algal galactans such as agarose, κ-, and ι-carrageenans.

Chemical analyses.—Constituent sugar analysis showed (Table 1) that the native and alkali-modified preparations from MELU-A 042229 were predominantly galactans, with Gal and AnGal the dominant sugars. The remainder comprised 3-MeGal and/or 4-MeGal, Xyl, and glucose (Glc). The amyloglucosidase-treated, native preparation had a sulfate content of 36.2% (w/w of the dried polysaccharide preparation, calculated as SO_3Na), which decreased to 34.2% (w/w) after alkali modification. The sulfate content of the alkali-modified preparation was comparable to that of typical ι-carrageenan [24], which has two sulfate esters for every repeating disaccharide unit. Alkali modification also resulted in a decrease in the proportion of Gal residues (by 13 mol%) and an approximately equimolar increase in AnGal residues (Table 1). The changes in sulfate content and constituent sugars associated with alkali modification demonstrated the presence of precursor residues in the native preparation. The ratio of Gal:AnGal in the alkali-modified preparation (~ 5:4) was above the 1:1 ratio expected for a galactan composed of an idealised repeating disaccharide and suggested the polysaccharide probably contained some irregularity in structure. The alkali-modified preparation contained no pyruvate.

The constituent sugars of crude preparations from another diploid tetrasporophyte (MELU-A 042230) and a haploid, female gametophyte (MELU-A 042227) of *P. peperocarpos* were essentially the same as those of the native preparation from MELU-A 042229 (Table 1).

FTIR spectroscopic analysis.—The FTIR spectra (Fig. 1) of the native and alkali-modified preparations from sample MELU-A 042229 contained strong absorption bands at 1240 cm^{-1}, indicative of high-sulfate-containing galactans, in agreement with the sulfate content of the preparations [25]. The diagnostic region of the FTIR spectra (between 940 and 800 cm^{-1}) of both the native and the alkali-modified preparations contained three major absorption bands. Two of these were characteristic of κ-carrageenan (polymer of carrabiose 4′-sulfate), (see [6] and [1]). These occurred at 930 cm^{-1},

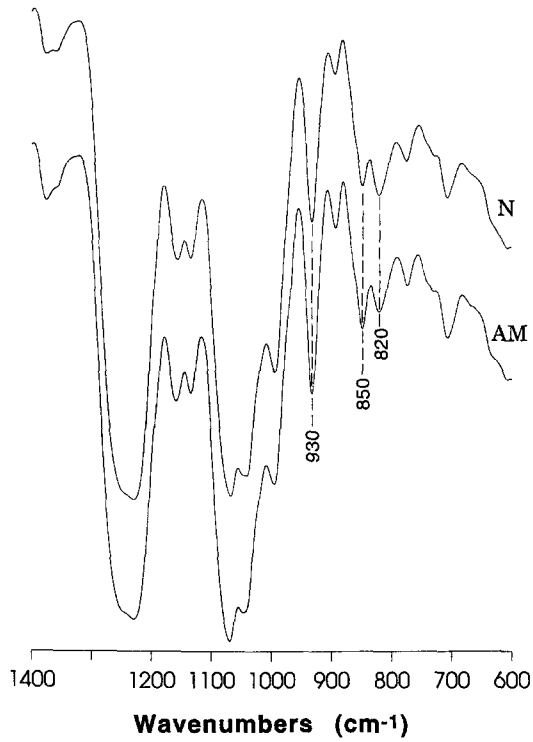

Fig. 1. FTIR spectrum of the native (N) and alkali-modified (AM) polysaccharide preparations from *Phacelocarpus peperocarpos* (sample MELU-A 042229).

indicating the presence of AnGal residues, and at 850 cm^{-1}, indicating the presence of axial sulfate ester substitution at O-4 of 3-linked Gal residues [25].

Another major band occurred at 820 cm^{-1} in the FTIR spectra of both the native and alkali-modified preparations. This band is diagnostic for equatorial sulfate ester substitution at O-6 of 4-linked and/or 3-linked Gal residues. It is ordinarily observed in the IR spectra of λ-type carrageenans or red algal galactans rich in precursor residues, so alkali modification usually decreases its intensity [25,26]. For the *P. peperocarpos* polysaccharide, the intensity of the band at 820 cm^{-1}, relative to the 850 cm^{-1} band, diminished after alkali modification, whereas the band at 930 cm^{-1} (AnGal) increased. These data were consistent with the constituent sugar analysis and indicated that alkali modification removed sulfate esters at O-6 of the 4-linked Gal residues with concomitant formation of AnGal residues. Nevertheless, the band at 820 cm^{-1} remained intense in the spectrum of the alkali-modified preparation, suggesting that a significant proportion of 6-sulfate esters were not alkali-labile. This band could arise from 6-sulfate esters either on 3-linked Gal residues or on 4-linked precursor residues bearing additional substitution at O-3 which prevented the alkali elimination of sulfate from O-6. These possibilities can be distinguished by a combination of linkage and NMR spectroscopic analyses (see below).

Table 2
Linkage analysis of constituent sugars of the alkali-modified polysaccharide preparation from *Phacelocarpus peperocarpos* (sample MELU-A 042229)

Constituent monosaccharide	Deduced linkage [a]	Mol%
AnGal*p*	4-	39.5
	2,4-	2.5
Gal*p*	3,4-	4
	3,6-	9
	3,4,6-	35.5
	2,3,4,6-	5.5
Xyl*p*	terminal	3
Glc*p*	4-	1

AnGal*p* = 3,6-anhydrogalactopyranose, Gal*p* = galactopyranose, Xyl*p* = xylopyranose, Glc*p* = glucopyranose.
[a] 4-Linked AnGal*p* deduced from 1,4,5-tri-*O*-acetyl-2-*O*-methyl-3,6-anhydrogalactitol, etc.

The FTIR spectra (not shown) of crude preparations from the other tetrasporophyte (MELU-A 042230) and the female gametophyte (MELU-A 042227) were essentially the same as that of the native preparation from MELU-A 042229. Combined with constituent sugar analysis, these data indicated that the chemistry of the galactan did not alter appreciably during the life cycle of the alga.

Linkage analysis.—The linkage and substitution patterns of the alkali-modified preparation from sample MELU-A 042229 were determined by methylation and subsequent reductive hydrolysis [19] (Table 2). All sugars were in the pyranose form. Sulfate esters were stable during the methylation but were subsequently released during hydrolysis and therefore manifested as 'linkages' in the results. Almost all the AnGal*p* (39.5 mol%) was linked through O-4, as expected for red algal galactans, although a small proportion (2.5 mol%) also occurred as 2,4-linked AnGal *p*, interpreted as 4-linked AnGal*p* 2-sulfate. The dominant linkage for Gal*p* was 3,4,6-Gal*p* (35.5 mol%) and, in combination with the FTIR data and the lack of pyruvate acetal substitution revealed by enzymic assay, provided evidence for 3-linked Gal*p* 4,6-disulfate as major residues. Smaller proportions of 3,6-linked (9 mol%) and 3,4-linked Gal*p* (4 mol%) suggested the presence of some monosulfated residues, occurring as 3-linked Gal*p* 6-sulfate and 3-linked Gal *p* 4-sulfate, respectively. The 2,3,4,6-linked Gal*p* residues possibly represented variously substituted Gal*p* residues and/or undermethylation, but their precise nature is uncertain. The Xyl*p* occurred as terminal residues, which probably occurred as single residues branching from the galactan chain. Detection of a minor amount of 4-linked Glc*p* was indicative of residual floridean starch, which survived digestion by amyloglucosidase.

NMR spectroscopic analysis.—A proton-decoupled ^{13}C NMR spectrum was recorded of the alkali-modified preparation (Fig. 2A). To assist with the assignment of the methylene (C-6) carbons of the Gal*p* and AnGal*p* residues, a spectrum employing the *J*-modulated spin-echo pulse sequence was also recorded (Fig. 2B). All of the major signals in the ^{13}C NMR spectrum, except the two at 72.7 and 68.2 ppm, were assigned

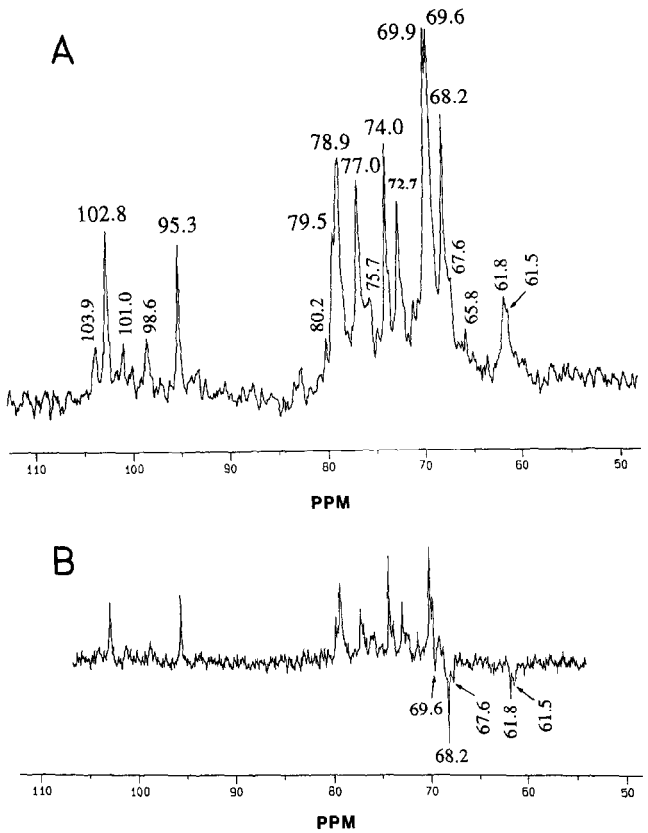

Fig. 2. Proton-decoupled ^{13}C NMR spectrum (A) and ^{13}C NMR spectrum recorded with the *J*-modulated spin-echo pulse sequence (B) of the alkali-modified polysaccharide preparation from *Phacelocarpus peperocarpos* (sample MELU-A 042229).

(Table 3) by comparison with data reported for κ-carrageenan [27,28], with anomeric resonances at 102.8 and 95.3 ppm for the 3-linked D-Gal*p* and 4-linked D-AnGal*p* residues, respectively.

Table 3
Assignments of the major resonances [a] in the ^{13}C NMR spectrum of the alkali-modified polysaccharide preparation from *Phacelocarpus peperocarpos* [b] (sample MELU-A 042229) attributed to the repeating disaccharide carrabiose 4′,6′-disulfate

Sugar residue	Carbon atom					
	C-1	C-2	C-3	C-4	C-5	C-6
3-Linked	102.8	69.6 [c]	78.9 [d]	74.0	72.7	68.2
4-Linked	95.3	69.9	79.5	78.9 [d]	77.0	69.6 [c]

[a] Referenced to Me_2SO at 39.6 ppm.
[b] Spectrum of 4% w/v sample in D_2O recorded at 80 °C.
[c,d] Coincident resonances.

Compared with the ^{13}C NMR spectrum of κ-carrageenan, the intensity of the most upfield signal (the broad envelope centred at 61.7 ppm) for the primary alcohol carbon (C-6 of the 3-linked Gal*p* residues), was significantly decreased, confirming the substitution at this position. The signal at 68.2 ppm, which was inverted in the *J*-mod experiment (Fig. 2B), was thus assigned to C-6 of 3-linked Gal*p* residues bearing sulfate ester substitution at both O-4 and O-6. The 6.8 ppm downfield shift of this C-6 signal relative to that of κ-carrageenan (at 61.4 ppm) was within the range of α-shifts observed for the sulfation of carbon atoms in red algal galactans (~ 5–8 ppm [7,27,28]). Interestingly, the signal for the sulfated C-6 of the 3-linked residue in the spectrum of the *P. peperocarpos* polysaccharide was ~ 0.8–0.9 ppm further downfield than that reported for the C-6 signal of the 3-linked residue of ω-carrageenan (polymer of carrabiose 6′-sulfate) [29,30] and of a sulfated agar (agarose 6′-sulphate) [31,32]. The enhanced downfield shift of the C-6 signal due to 4,6-disulfation was also reported from NMR studies of model compounds [33,34]. The remaining, major signal at 72.7 ppm was assigned to C-5 of the 3-linked residue bearing sulfate esters at both O-4 and O-6. Its position in the spectrum was further upfield than the C-5 signals of the 3-linked residues of galactans composed of monosulfated repeating disaccharides such as κ-carrageenan, ω-carrageenan, agarose 4′-sulfate, and agarose 6′-sulfate [27–32,35]. This observation was consistent with results from NMR studies of monosulfated and disulfated model compounds [33,34]. The broad envelope at 78.9 ppm showed slight splitting (Fig. 2A). We have assigned both the signals for C-3 of the 3-linked residue and C-4 of the 4-linked AnGal*p* residue to this envelope. The ^{13}C NMR spectrum therefore demonstrated that the polysaccharide from *P. peperocarpos* was composed predominantly of the repeating (1 → 3′)-linked disaccharide carrabiose 4′,6′-disulfate (Fig. 3).

Minor structural features.—The detection of minor components in the linkage analysis was reflected in the ^{13}C NMR spectrum by the presence of minor resonances, confirming the existence of structural variations on the major repeating disaccharide carrabiose 4′,6′-disulfate in the *P. peperocarpos* polysaccharide. In particular, terminal Xyl*p* residues were identified (Table 2), which probably arose from single-residue branches. There are at least two possible sites of attachment of the Xyl*p* residues. In the agaroid from *Laurencia nipponica*, they were located at O-3 of 4-linked Gal*p* residues [36], whereas in the agar from *Melanthalia abscissa*, they were found at O-6 of the 3-linked Gal*p* residues [37]. Accordingly, some of the 3,4- and 3,4,6-linked Gal*p* residues in the linkage analysis of the *P. peperocarpos* polysaccharide may be inter-

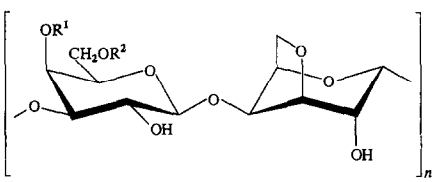

Fig. 3. Summary of proposed repeating structures for the polysaccharide from *Phacelocarpus peperocarpos*. The dominant repeating structure is carrabiose 4′,6′-disulfate ($R^1 = R^2 = SO_3^-$). Possible minor structural features include carrabiose 4′-sulfate ($R^1 = SO_3^-$, $R^2 = H$) of κ-carrageenan and carrabiose 6′-sulfate ($R^1 = H$, $R^2 = SO_3^-$) of ω-carrageenan.

preted as 4-linked Gal*p* and 4-linked Gal*p* 6-sulfate residues bearing terminal Xyl*p* at O-3 or some of the 3,6- and 3,4,6-linked Gal*p* residues as 3-linked Gal *p* and 3-linked Gal*p* 4-sulfate residues bearing terminal Xyl*p* at O-6. We speculate, however, that O-3 of 4-linked Gal*p* is the main site of attachment for terminal Xyl*p* residues since the Gal:AnGal ratio deviated from 1:1 and because the ^{13}C NMR chemical shifts of some minor resonances were in agreement with data reported [36] for terminal Xyl*p* residues in the *Laurencia nipponica* polysaccharide (C-1 at 101.0 ppm, C-3 at 75.7 ppm, and C-5 at 65.8 ppm, Fig. 2A). Xylosylation would consequently obstruct the conversion of some 4-linked precursor residues to AnGal*p* by alkali modification. The two minor anomeric resonances at 98.6 and 103.9 ppm were thus tentatively assigned to the anomeric carbons of 4-linked residues not in the AnGal form and their neighbouring 3-linked residues, respectively. This interpretation was supported by the weak, broad band showing split peaks at 61.5 and 61.8 ppm, which indicated incomplete substitution at O-6 of the 3-linked Gal*p* residues and that some of the 4-linked residues were indeed not in the AnGal form [26,27]. However, another possibility for the downfield positions of the two anomeric resonances at 98.6 and 103.9 ppm was that they reflected the presence of some 4-linked residues in the L configuration, suggesting that the polysaccharide deviated from an authentic carrageenan structure. Complex red algal galactans with 4-linked residues in both the D and the L configuration have been reported from genera such as *Grateloupia* [27] and *Pachymenia* [38]. Other minor, non-anomeric signals in the ^{13}C NMR spectrum at 80.2 and 67.6 ppm, the latter of which was inverted in the *J*-mod experiment, were assigned to C-3 and C-6, respectively, of a monosulfated, 3-linked Gal*p* 6-sulfate residue as in classical ω-carrageenan [29,30].

4. Conclusion

We propose that the polysaccharide from *P. peperocarpos* is a highly sulfated galactan predominantly composed of the repeating $(1 \rightarrow 3')$-linked disaccharide carrabiose 4′,6′-disulfate (Fig. 3). Evidence from linkage analysis and ^{13}C NMR spectroscopy indicated a variety of minor structural variations suggestive of alternative sulfation patterns (Fig. 3) and xylosylation. Comparative studies of cell wall galactans from diploid and haploid generations showed that the galactan chemistry remained consistent during the life cycle of the alga. However, a preliminary investigation of the polysaccharides from New Zealand *P. peperocarpos* var. *novae-zelandiae* (Dr. I.J. Miller, Dr. R. Falshaw, and Dr. R.H. Furneaux, personal communication) indicated a very different structure, suggesting the polysaccharide chemistry of this alga is variable. This possibility is supported by a preliminary survey of other Australian *Phacelocarpus* spp. [A. Chiovitti et al., unpublished], which showed a high degree of structural diversity of the cell wall galactans between some members of this genus as well as between different reproductive states of the same species. These observations have also led us to consider re-examining the taxonomy of both the Australian and New Zealand *Phacelocarpus* spp.

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