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A nearly idealized 6′-*O*-methylated ι-carrageenan from the Australian red alga *Claviclonium ovatum* (Acrotylaceae, Gigartinales)

Anthony Chiovitti,[a] Antony Bacic,[b] David J. Craik,[c] Gerald T. Kraft[a] and Ming-Long Liao[b,*]

[a]*School of Botany, University of Melbourne, Victoria 3010, Australia*
[b]*CRC for Bioproducts, Plant Cell Biology Research Centre, School of Botany, University of Melbourne, Victoria 3010, Australia*
[c]*Institute for Molecular Bioscience, University of Queensland, St Lucia, Queensland 4072, Australia*

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Abstract—The polysaccharides extracted from *Claviclonium ovatum* were studied by a combination of compositional assays, reductive partial hydrolysis, linkage analysis, Fourier Transform infrared (FTIR) spectroscopy, and ^{13}C, ^{1}H, and $^{13}C/^{1}H$ heteronuclear multiple quantum correlation (HMQC) two-dimensional nuclear magnetic resonance (NMR) spectroscopy. The chemical and spectroscopic data showed that the alkali-modified *C. ovatum* polysaccharides are composed of a nearly idealized repeating unit of 6′-*O*-methylcarrabiose 2,4′-disulfate (the repeating unit of 6′-*O*-methylated ι-carrageenan), although some minor components were also present. The *C. ovatum* galactans are the most highly methylated carrageenans reported.

Keywords: Acrotylaceae; Australia; Carbohydrate analysis; Gigartinales; ι-Carrageenan; *O*-Methyl ether; Polysaccharide structure; Rhodophyta

1. Introduction

The commercially valuable extracellular polysaccharides extracted from red algae are primarily sulfated galactans comprised of repeating disaccharide units.[1,2] The fundamental repeating disaccharide consists of 3-linked β-D-galactopyranose (Gal*p*) and 4-linked α-Gal*p* residues. The configuration of the 4-linked residues determines whether the galactan is classified as an agar (L-) or a carrageenan (D-). The 4-linked residues often occur in the form of 3,6-anhydrogalactopyranose (AnGal*p*), and the repeating units containing L- and D-AnGal*p* as the nominal reducing end sugar are referred to as agarobiose and carrabiose, respectively.[2] The repeating disaccharide units may be substituted to varying degrees by sulfate ester, methyl ether, pyruvate acetal, and/or glycosyl residues. During the last decade, increasingly sophisticated techniques have been applied to the analysis of red algal galactans and have enabled the discovery and characterization of unusual or novel substitution patterns.[3–7] These techniques include chemical methods, such as reductive hydrolysis,[8,9] which permits the quantitative recovery and analysis of the acid-labile AnGal; reductive partial hydrolysis[9,10] and reductive amination[11,12] to derive configurational information about constituent sugars in combination with their linkage patterns; and two-dimensional nuclear magnetic resonance (NMR) spectroscopy.[3–5] A recent report[4] used a combination of essentially all these techniques to characterize pyruvated β-carrageenan and highlighted the benefit of such studies by providing empirically derived reference data for specific substitution patterns.

One notable substitution pattern occurring in red algal galactans is the 6-*O*-methylation of 3-linked D-Gal*p* residues (6-MeGal). The gel setting temperatures of agars have been correlated with their 6-MeGal content,[13,14] and the effect of this substitution on gel setting temperatures has implications for the commercial end use of agars with high 6-MeGal content, which are

* Corresponding author. E-mail: ming@unimelb.edu.au

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regarded as suitable for applications in processed foods but not for microbiological or most biotechnological applications.[15,16] Although 6-MeGal is relatively common and abundant in agars, it is relatively rare in carrageenans. The most conspicuous *O*-methylated repeating disaccharide documented in carrageenans is probably 6′-*O*-methylcarrabiose 2,4′-disulfate (the repeating unit for 6′-*O*-methylated ι-carrageenan). First identified 20 years ago through extensive fractionation as a minor and somewhat anomalous component of the commercially important κ-carrageenan from *Kappaphycus alvarezii* (Doty) Doty (as *Eucheuma cottonii*),[17] this repeating disaccharide unit has more recently been found to be a relatively significant component of carrageenans with comparatively complex substitution patterns from species of *Rhabdonia*, *Erythroclonium*, *Austroclonium*, and *Areschougia*,[6,18–20] as well as a relatively minor component of carrageenans from *Meristotheca* and other *Kappaphycus* species,[21–23] and it has attracted interest for its potential chemotaxonomic and commercial implications.[19,23,24] We have analyzed the sulfated galactans from an Australian endemic red alga, *Claviclonium ovatum* (Lamouroux) Kraft et Min-Thein. The structure of these galactans approaches that of an idealized 6′-*O*-methylated ι-carrageenan, providing the opportunity to characterize in detail the 6′-*O*-methylcarrabiose 2,4′-disulfate disaccharide unit.

2. Experimental

2.1. Algal material

The sample of *C. ovatum* used for this study (MELU #A40751) was a sterile specimen collected by Dr. J. Huisman from the drift at Mangles Bay, Point Peron, Western Australia on 10 June 1993.

2.2. Extraction and treatment of the polysaccharides

The dried algal fronds were cleaned of surface debris and epiphytes and milled to a powder. The polysaccharides were extracted from the milled algal material in distilled water at 95 °C, clarified, and precipitated with 2-propanol as described previously.[25] A portion of this polysaccharide preparation was subsequently alkali-treated according to the method of Craigie and Leigh.[26]

2.3. Compositional analysis

The sulfate content was determined by the turbidimetric method of Tabatabai[27] as modified by Craigie et al.[28] Constituent monosaccharides were analyzed as their alditol acetates generated from the polysaccharides by reductive hydrolysis.[8] The alditol acetates were separated by gas chromatography (GC) on a BPX70 (SGE, Australia) capillary column, detected by electron impact ionization mass spectrometry (MS), and identified by their retention times relative to *myo*-inositol and their mass spectra. The response factors used for quantitation were determined for each sugar as described previously.[29]

2.4. Reductive partial hydrolysis

The configuration of the AnGal residues was determined by analysis of acetylated derivatives of disaccharide alditols (i.e., 3,6-anhydro-4-*O*-β-D-galactosylgalactitols, or 'biitols').[10] Naturally 6′-*O*-methylated biitol hexaacetates were generated from the alkali-modified *C. ovatum* polysaccharide preparation by the reductive partial-hydrolysis procedure[10] and separated from minor reaction products by reverse-phase liquid chromatography (LC) on a 300 μm i.d. × 5 m C_{18} column (3 μm particle size, LC-18, LC Packings, Sunnyvale, CA) using a linear gradient of 30–80% (v/v) acetonitrile in 0.5% (v/v) acetic acid over 40 min. A constant stream of the mobile phase was pumped into the ion source (Rheodyne, Cotati, CA) of the mass spectrometer at 3 μL/min using a syringe pump (Harvard Apparatus, South Natick, MA). The mono-*O*-methylated biitol acetates were detected by electrospray-ionization-MS on a Finnigan MAT 95 reverse-geometry sector mass spectrometer equipped with a Finnigan electrospray and a DEC 3100 data system. Spectra were acquired in the positive-ion mode using a 5 kV accelerating potential. The mass-to-charge (m/z) range scanned was m/z 150–800. For comparison, a native agar preparation from *Curdiea angustata* (Sonder) Millar with almost complete 6′-*O*-methylation[24] was subjected to the same reductive partial-hydrolysis and analysis procedure.

2.5. Linkage analysis

The polysaccharides were converted into their Me_2SO-soluble triethylammonium form and methylated with CD_3I essentially as described by Stevenson and Furneaux,[8] except that a $NaOH/Me_2SO$ suspension was used to generate the alkoxide.[30] The permethylated alditol acetates were derived by reductive hydrolysis and acetylation,[8] and separated by GC as described for the constituent monosaccharide analysis. The eluted derivatives were detected by MS, identified by their unique mass spectra and retention times[31] and quantified from reconstructed ion chromatogram.[18]

2.6. Spectroscopic methods

Polysaccharide films for Fourier transform infrared (FTIR) spectroscopy were prepared from 15 mg samples as described by Liao et al. [32] and the FTIR spectra recorded on a Perkin–Elmer Series 2000 FTIR spectrometer, collecting 32 scans at a resolution of 4 cm^{-1}.

For NMR spectroscopy, a sample of the alkali-modified polysaccharide was dissolved in D_2O (30 mg/mL). All spectra were recorded at 80 °C. A ^1H NMR spectrum was acquired on a Bruker ARX500 spectrometer (operating at 500.13 MHz) with a spectral width of 2.0 kHz, 4 K data points, a 90° pulse, an acquisition time of 1.02 s, a relaxation delay of 1.0 s, and eight scans. The water proton signal was suppressed by low power irradiation during the relaxation delay. The ^1H chemical shifts were reported relative to Me_2SO at 2.71 ppm. The proton decoupled ^{13}C NMR spectrum was acquired on the same spectrometer (operating at 125.8 MHz) with a spectral width of 27.8 kHz, a 45° pulse, an acquisition time of 0.29 s, a relaxation delay of 1.0 s, and ~92,000 scans. The ^{13}C chemical shifts were reported relative to Me_2SO at 39.6 ppm. The methylene carbons were identified with an attached proton test by recording a spectrum with the *J*-modulated spin-echo pulse sequence[33] on a Bruker AMX300 WB spectrometer with a spectral width of 18.5 kHz, an acquisition time of 0.44 s, a relaxation delay of 1.0 s, a modulation delay (τ) of 7.1 ms, and ~45,000 scans. A $^{13}C/^1H$ heteronuclear multiple quantum coherence (HMQC) 2D spectrum[34] was recorded on the Bruker ARX500 spectrometer with a ^1H spectral width of 2.0 kHz, an acquisition time of 512 ms, and a relaxation delay of 1.5 s, during which the residual HOD signal was presaturated. The spectral width in the ^{13}C dimension was 12.0 kHz (95 ppm) and was recorded using 292 free induction decays.

3. Results and discussion

3.1. Compositional analyses

The native polysaccharide preparation was obtained in 32% yield (w/w of the dried algal material, Table 1) and contained a relatively high level of sulfate (35.8% w/w of the polysaccharide preparation, Table 1). Constituent monosaccharide analysis of the native preparation (Table 1) confirmed that the polysaccharides were mainly galactans, with 93 mol% of the total monosaccharides comprising 6-MeGal, AnGal, and Gal. The remainder consisted of Glc (6 mol%) and Xyl (1 mol%).

Following alkali modification, the sulfate content decreased to 34.2% (w/w) (Table 1). The monosaccharide composition of the alkali-modified preparation was similar to that of the native preparation, except that the proportion of Gal was 8 mol% lower and the proportion of AnGal was 7 mol% higher (Table 1). These observations indicated that alkali-modification resulted in the conversion of precursor 4-linked Gal*p* 6-sulfate residues (and/or their 2-sulfated counterparts) into AnGal*p* residues (and/or their 2-sulfated counterparts) with concomitant loss of sulfate ester from O-6.[35] As a consequence, 6-MeGal (45 mol%) and AnGal (42 mol%) were the dominant sugars in the alkali-modified preparation and occurred in nearly equimolar proportions.

3.2. Reductive partial hydrolysis

The compositional data indicated that the 6-MeGal and the AnGal sugars formed the dominant repeating unit of the galactans from *C. ovatum*. To determine whether the galactans were composed mainly of 6′-*O*-methylated agarobiose or carrabiose units, the alkali-modified preparation was subjected to reductive partial hydrolysis and acetylation[10] to generate biitol derivatives, which were analyzed by LC–MS. For comparison, a sample of the polysaccharide from a species of *Curdiea*, *Cu. angustata*, was used to generate the standard for 6′-*O*-methylagarobiitol hexaacetate.[24]

Reverse-phase LC revealed that reductive partial hydrolysis of the alkali-modified *C. ovatum* polysaccharide yielded one dominant biitol acetate with a retention time of 27.9 min. Under the same chromatographic conditions, this component eluted 1 min later than the dominant biitol acetate (6′-*O*-methylagarobiitol hexaacetate) generated from the *Cu. angustata* agar (26.9 min). The ESI mass spectra of the dominant derivatives from the two polysaccharides (Fig. 1) were, however, comparable and both contained diagnostic pseudomolecular ions with m/z 615 and 631 for the sodium and potassium adducts, respectively, of the 6′-*O*-methylated biitol hexaacetates ($C_{25}H_{36}O_{16}$). Fragmentation and the loss of acetic acid (60 amu) from the sodium adducts gave pseudomolecular ions with m/z 556. Fragmentation at the glycosidic bond gave tri-*O*-acetylated 6-MeGal$^+$ ions with m/z 303 (cf. m/z 331 for tetra-*O*-acetylated Gal$^+$ ions).[10] Further fragmentation of the 6-MeGal$^+$ ion and the loss of acetic acid produced ions with m/z 243 and 183. These data showed that the

Table 1. Yield and composition of the polysaccharide preparations from *C. ovatum*

	Yield[a] (% w/w)	Sulfate[b] (% w/w)	Constituent monosaccharides[c] (mol%)				
			Gal	AnGal	6-MeGal	Xyl	Glc
Native	32	35.8	14	35	44	1	6
Alkali modified	ND[d]	34.2	6	42	45	1	6

[a] Based on the dry weight of the seaweed.
[b] Based on the dry weight of the polysaccharide preparation and expressed as SO_3Na.
[c] Monosaccharides: Gal = galactose, AnGal = 3,6-anhydrogalactose, 6-MeGal = 6-*O*-methylgalactose, Xyl = xylose, Glc = glucose.
[d] ND = not determined.

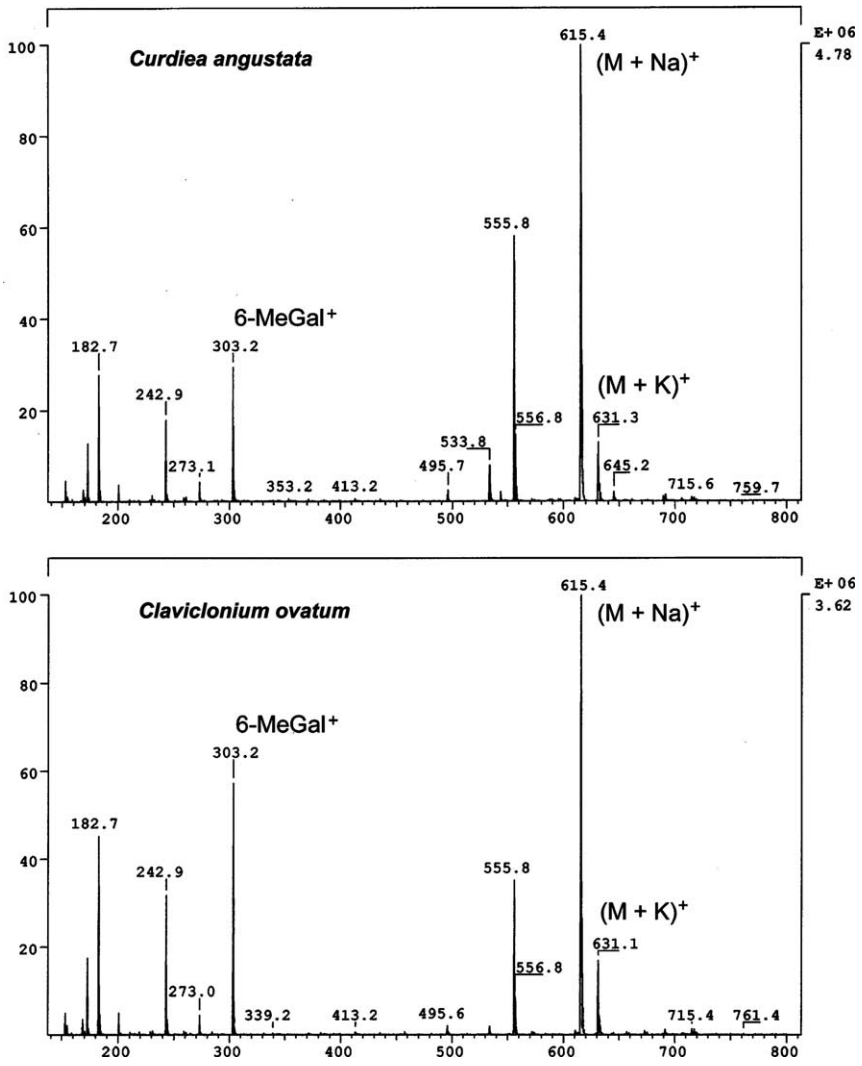

Figure 1. Mass spectra of the 6′-*O*-methylagarobiitol hexaacetate derivative from *Cu. angustata* and the 6′-*O*-methylcarrabiitol hexaacetate derivative from *C. ovatum*. Diagnostic ions are indicated. M = molecular ion of 1,2,5-tri-*O*-acetyl-3,6-anhydro-4-*O*-[2,3,4-tri-*O*-acetyl-6-*O*-methyl-β-D-galactopyranosyl]galactitol. 6-MeGal$^+$ = fragment ion for the 2,3,4-tri-*O*-acetyl-6-*O*-methyl-β-D-galactopyranosyl moiety.

mono-*O*-methylated biitol acetates derived from the galactans of *C. ovatum* and *Cu. angustata* were diastereoisomers, and thus the backbone of the *C. ovatum* galactan was composed essentially of a 6′-*O*-methylated carrabiose repeating unit.

3.3. FTIR spectroscopy

The FTIR spectrum of the native and alkali-modified polysaccharide preparations are presented in Fig. 2. The spectra contained a strong band of absorption at 1230–1240 cm^{-1}, which was indicative of substantial levels of sulfate ester substitution[36] and consistent with the results of the compositional assays (Table 1). The diagnostic region (800–950 cm^{-1}) of the spectrum of the native preparation resembled that of ι-carrageenan[36] with absorption bands at 935 cm^{-1} (attributable to AnGal residues), 855 cm^{-1} (indicative of axial sulfate ester at O-4 of 3-linked Gal), and 810 cm^{-1} (indicative of axial sulfate ester at O-2 of 4-linked AnGal*p*). The spectrum of the alkali-modified preparation displayed essentially the same absorption bands as that of the native preparation, although it showed diminished absorption in the region 820–835 cm^{-1}, which is associated with equatorial sulfate esters at O-6 and O-2 of 4-linked precursor residues,[17,36] and this was consistent with the differences observed in the constituent sugar analysis (Table 1). Alkali modification also enhanced absorption at 970 cm^{-1} of the spectrum, an effect noted previously for the galactans from *Erythroclonium* species[6] and observed for the FTIR spectra of the galactans from *Rhabdonia* species.[18]

The one notable difference between the spectra of the *C. ovatum* galactans and that of classical ι-carrageenan[36] was that the band at 935 cm^{-1} in the spectra of the *C. ovatum* galactans, particularly that of the alkali-

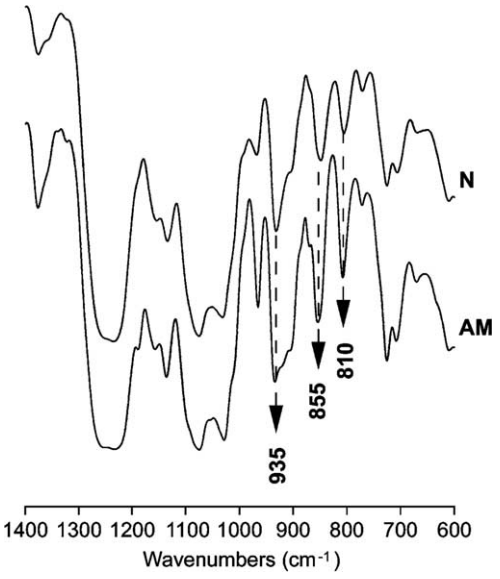

Figure 2. FTIR spectra of the native (N) and alkali-modified (AM) polysaccharide preparations from *C. ovatum*.

modified preparation, had a broad shoulder with enhanced absorption at ~900 cm^{-1}. The FTIR spectra of classical ι-carrageenans often show weak absorption in this region (e.g., see spectra presented in Ref. 19), but absorption in this region for the *C. ovatum* galactans was comparatively intense. Absorption in the region at ~900 cm^{-1} is normally associated with unsulfated[3,37] or pyruvate-substituted 3-linked residues.[5]

3.4. Linkage analysis

The linkage and substitution patterns of the constituent sugars in the alkali-modified preparation (Table 2) were studied by methylation with CD_3I to determine the linkage of the 6-MeGal units. All the sugars were linked through O-1 and O-5 and interpreted as pyranosyl (*p*) residues glycosidically linked through O-1. The two dominant linkage patterns were 2,4-linked AnGal*p* (42 mol%) and 3,4-linked 6-MeGal*p* (50 mol%), which were interpreted on the basis of FTIR data as 4-linked AnGal*p* 2-sulfate and 3-linked 6-MeGal*p* 4-sulfate, respectively. The data from the compositional analyses, reductive partial hydrolysis, FTIR spectroscopy, and linkage analysis together indicated that the alkali-modified *C. ovatum* galactans were composed of a nearly idealized repeating unit of 6′-*O*-methylcarrabiose 2,4′-disulfate (the repeating unit of 6′-*O*-methylated ι-carrageenan).

A heterogeneous mixture of minor linkage and substitution patterns, each of which occurred at low levels (≤2 mol%), was also detected (Table 2). These included 3-, 4-, and 3,4-linked Gal*p* residues. In line with the FTIR data, the 3,4-linked Gal*p* was interpreted as mainly 3-linked Gal*p* 4-sulfate residues. Glc was present

Table 2. Linkage analysis of the constituent monosaccharides in the alkali-modified polysaccharide preparation from *C. ovatum*

Constituent monosaccharide[a]	Deduced linkage[b]	Mol%
AnGal*p*	2,4-	42
Gal*p*	3-	2
	4-	2
	3,4-[c]	2
	2,3,4-	Tr
	2,3,6-/2,4,6-	Tr
6-MeGal*p*	3,4-[c]	50
Glc*p*	4-	2

Tr = trace (0.8 mol%).

[a] Monosaccharides as in Table 1; *p* = pyranose.

[b] 2,4-Linked AnGal*p* deduced from 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol, 3-linked Gal*p* deduced from 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-trideuteriomethylgalactitol, 3,4-linked 6-MeGal*p* deduced from 1,3,4,5-tetra-*O*-acetyl-6-*O*-methyl-2-*O*-trideuteriomethylgalactitol, etc.

[c] Co-eluting derivatives were distinguished by permethylation with CD_3I and quantified by mass spectrometric analysis of their diagnostic primary fragment ions.[18]

only as 4-linked Glc*p*, and indicated the preparation contained small amounts of floridean starch.

In contrast to the indications given by the FTIR data, linkage analysis provided evidence for only a small amount of unsulfated 3-linked Gal*p* or 6-MeGal*p* residues (2 mol% of the total linkages, Table 2) and no evidence for pyruvated 3-linked Gal*p* residues, which would have been detected as 3,4,6-linked Gal*p* residues.[5,38] This observation suggested that absorption in the region at ~900 cm^{-1} of the IR spectra of the *C. ovatum* galactans was affected by 6′-*O*-methylation of the carrabiose 2,4′-disulfate repeating disaccharide. In previous investigations of relatively highly 6′-*O*-methylated carrageenans, such as those from species of *Rhabdonia*, *Erythroclonium*, and *Areschougia*,[6,18,20] moderate to relatively intense absorption in the region at ~900 cm^{-1} of the FTIR spectra was reported. However, these galactans were comparatively more complex than those of *C. ovatum*. They contained relatively less 6-MeGal (in the range 15–35 mol% of constituent sugars) and varying proportions of 3-linked unsulfated Gal*p*, unsulfated 6-MeGal*p*, and pyruvated Gal*p* residues (with sums in the range of 5–25 mol% of the total linkages), which were qualitatively correlated with absorption at ~900 cm^{-1} in the FTIR spectra. Consequently, in the earlier studies, any effect on the FTIR spectra due to 6-*O*-methylation of 3-linked residues could not be distinguished from effects due to other substitution patterns.

3.5. NMR spectroscopy

^{13}C NMR data have been reported previously for 6′-*O*-methylcarrabiose 2,4′-disulfate occurring as one component of carrageenans with multiple, and often relatively complex, substitution patterns.[6,17,18,20] However, the

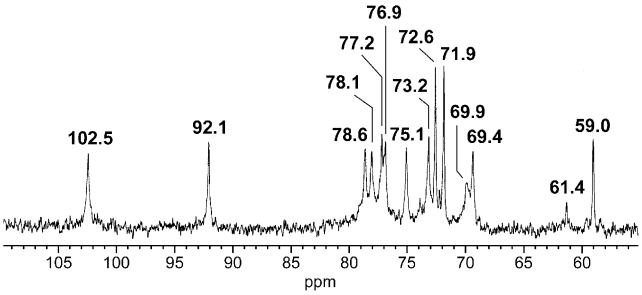

Figure 3. Proton-decoupled ^{13}C NMR spectrum of the alkali-modified polysaccharide preparation from *C. ovatum*.

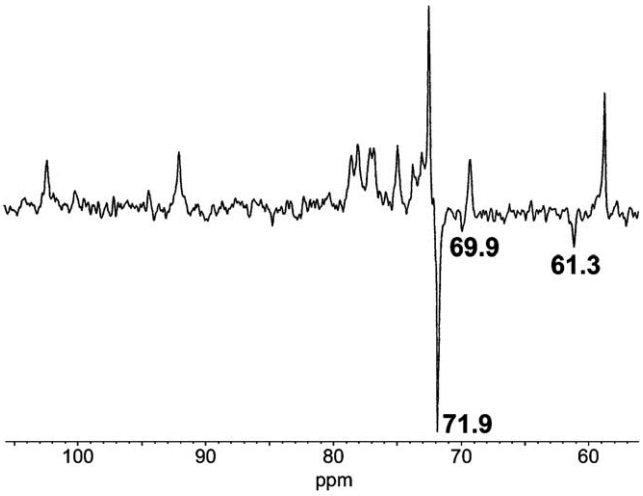

Figure 4. ^{13}C NMR spectrum of the alkali-modified polysaccharide preparation from *C. ovatum* recorded with the *J*-modulated spin echo pulse sequence.

nearly idealized repeating structure of the alkali-modified *C. ovatum* galactans enabled detailed NMR analysis of authentic 6′-*O*-methylcarrabiose 2,4′-disulfate. A proton decoupled ^{13}C NMR spectrum of the alkali-modified *C. ovatum* preparation was recorded (Fig. 3) and the major signals in the spectrum corroborated the earlier data for 6′-*O*-methylcarrabiose 2,4′-disulfate. The signals were assigned according to Ref. 18 and the assignments are summarized in Table 3. The diagnostic signals for the repeating unit included those for A-1, G-4, G-5, G-6, and the *O*-methyl carbon at 92.1, 72.6, 73.2, 71.9, and 59.0 ppm, respectively. The assignments of the G-4, G-5, and G-6 carbons were confirmed with an attached proton test and a $^{13}C/^{1}H$ HMQC 2D spectrum (see below). Compared with the C-6, C-5, and C-4 signals for the 3-linked Gal*p* 4-sulfate residue of classical ι-carrageenan,[3,39,40] the corresponding G-6, G-5, and G-4 signals in the spectrum of the *C. ovatum* galactans showed α-, β-, and γ-shifts, respectively, consistent with the effects of methyl ether substitution at G-6.[6,18,39]

The assignment of the G-6 and A-6 carbon signals was verified by an attached proton test for methylene carbons.[33] To achieve this, a spectrum of the polysaccharide was recorded using the *J*-modulated spin-echo pulse sequence. The carbon signals assigned to G-6 and A-6 at 71.9 and 69.9 ppm, respectively, occurred as negative inflections (Fig. 4).

A $^{13}C/^{1}H$ HMQC spectrum was recorded for the alkali-modified *C. ovatum* preparation (Fig. 5). Cross-peaks corresponding to all the geminally connected $^{13}C/^{1}H$ nuclei in the 6′-*O*-methylcarrabiose 2,4′-disulfate repeating unit were observed and they were assigned by using the ^{13}C NMR data (Table 3) as a reference (although see the discussion for assignments of G-4 and G-5 below). The spectrum contained a diagnostic cross-peak for the *O*-methyl substitution at 59.1/3.42 ppm. As expected, the C-6 nuclei each showed two cross-peaks with their geminal H-6 nuclei. These included 71.9/3.77 and 71.9/3.70 ppm for the C-6/H-6a and C-6/H-6b, respectively, of the 3-linked 6-MeGal*p* 4-sulfate residue and 69.9/4.25 and 69.9/4.10 ppm for the C-6/H-6_{exo} and C-6/H-6_{endo}, respectively, of the 4-linked AnGal*p* 2-sulfate residue.

The assignments of the G-4 and G-5 carbon signals for the 3-linked 6-MeGal*p* 4-sulfate residues reported by Chiovitti and co-workers[6,18,20] were the reverse of the corresponding assignments reported earlier by Bellion et al.[17] The cross-peaks corresponding to G4 and G5 in the HMQC spectrum (Fig. 5) showed that, although the ^{13}C signals were relatively close (0.6 ppm apart), the corresponding ^{1}H signals were well separated (0.97 ppm apart). The chemical shifts of the H-4 and H-5 signals for the 3-linked residues in carrageenans and oligosaccharides of the neocarrabiose series vary according to the sites and types of substitution but the H-4 signals consistently occur at $\geqslant$4.10 ppm and are 0.40–1.10 ppm downfield of the H-5 signals.[3–5,39,41,42] The chemical shifts of H-4 of the 3-linked Gal*p* 4-sulfate residues in ι- and κ-carrageenan occur in the range 4.80–4.90 ppm, whereas those for H-5 occur at ~3.77 ppm.[39,41] In line with these observations, the cross-peaks at 72.6/4.87 and 73.2/3.90 ppm in the HMQC spectrum (Fig. 5) were assigned to C-4/H-4 and C-5/H-5, respectively, of the 3-linked 6-MeGal*p* 4-sulfate residues and corroborated the carbon signal assignments of Chiovitti and co-workers.[6,18,20]

Assignment of the ^{13}C and HMQC spectra enabled the assignment of the signals in a ^{1}H spectrum of the alkali-modified *C. ovatum* galactans (Fig. 6, Table 3). In the ^{1}H NMR spectrum, the G-1, A-2, A-4, and A-5 proton signals were incompletely resolved as an intense signal at 4.66 ppm. However, these were separated by dispersion in the carbon dimension of the HMQC spectrum (Fig. 5), identified as cross-peaks at 102.5/4.65, 75.1/4.67, 78.6/4.67, and 77.2/4.66 ppm, respectively, and the protons were assigned accordingly (Table 3).

An intense signal for the *O*-methyl protons was observed at 3.42 ppm in the ^{1}H NMR spectrum (Fig. 6).

Table 3. Assignments of resonances observed in the ^{1}H and proton-decoupled ^{13}C NMR spectra of the alkali-modified polysaccharide preparation from *C. ovatum*

	G4S6M[a] (3-linked unit)							DA2S[a] (4-linked unit)					
	G-1	G-2	G-3	G-4	G-5	G-6	OMe	A-1	A-2	A-3	A-4	A-5	A-6
Carbon	102.5	69.4	76.9	72.6	73.2	71.9	59.0	92.1	75.1	78.1	78.6	77.2	69.9
Proton	4.65	3.63	3.99	4.87	3.90	3.77/3.70[b]	3.42	5.30	4.67	4.84	4.67	4.66	4.25/4.10[c]

[a] Repeating unit: G4S6M–DA2S = 6′-*O*-methylcarrabiose 2,4′-disulfate.
[b] G-6a/G-6b protons.
[c] A-6_{exo}/A-6_{endo} protons.

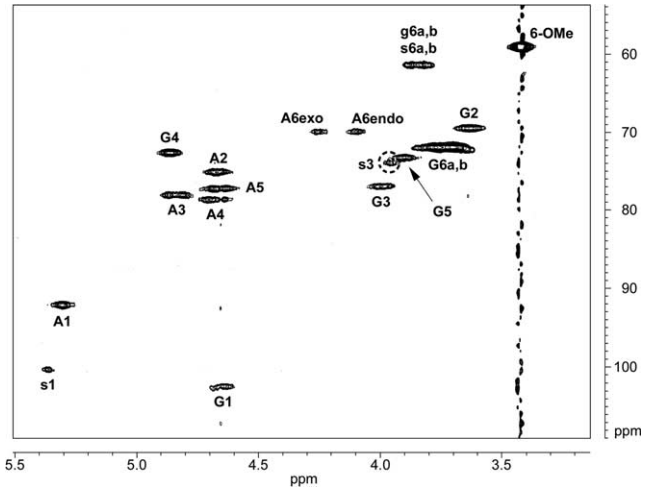

Figure 5. $^{13}C/^{1}H$ 2D heteronuclear multiple quantum coherence (HMQC) spectrum of the alkali-modified polysaccharide preparation from *C. ovatum*. The geminal carbon/proton cross-peaks are labeled with letters corresponding to sugar residues (G = 3-linked 6-*O*-methyl-β-D-galactopyranose 4-sulfate; A = 4-linked 3,6-anhydro-α-D-galactopyranose 2-sulfate; g = 3-linked β-D-galactopyranose, 3-linked β-D-galactopyranose 4-sulfate, and 4-linked α-D-galactopyranose; s = 4-linked α-D-glucopyranose of floridean starch).

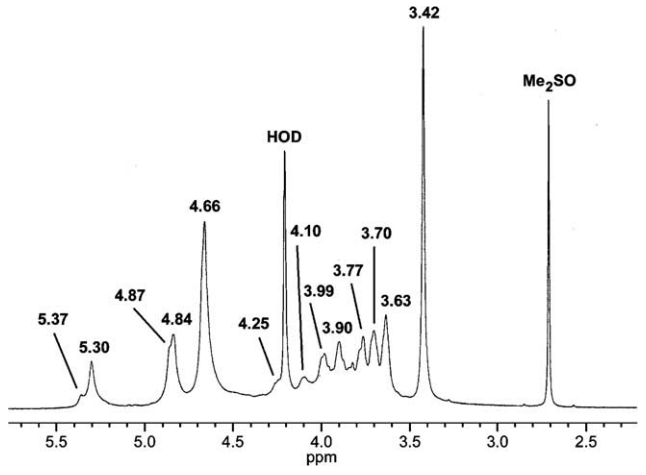

Figure 6. ^{1}H NMR spectrum of the alkali-modified polysaccharide preparation from *C. ovatum*.

The chemical shift of this signal was in the range reported previously for the *O*-methyl protons of red algal galactans (3.40–3.65 ppm)[39] and was essentially consistent with *O*-methyl substitution at C-6 of 3-linked Gal*p* residues reported for agars (3.40–3.41 ppm).[39] The chemical shifts of most of the remaining ^{1}H signals for 6′-*O*-methylcarrabiose 2,4′-disulfate, including the diagnostic anomeric proton signal for A-1 at 5.30 ppm, were within 0.02 ppm of those reported for the corresponding ^{1}H signals for the carrabiose 2,4′-disulfate repeating unit of ι-carrageenan (Ref. 41, but with reversal of the assignments for the A-2 and A-3 protons).[3] The exceptions were the protons at G-4, G-5, and G-6, which were influenced by the *O*-methyl substitution at G-6. The signals for the G-6a, G-6b, and G-4 protons were shifted 0.05, 0.12, and 0.04 ppm upfield, respectively, of their corresponding signals in the ^{1}H spectrum of ι-carrageenan.[3] The G-5 proton signal was shifted 0.09 ppm downfield of the corresponding signal in the ^{1}H spectrum of ι-carrageenan.

NMR spectroscopy indicated that residual floridean starch was present in the alkali-modified *C. ovatum* polysaccharide preparation. The minor signal at 61.3 ppm in the ^{13}C NMR spectrum (Fig. 3) showed a negative inflection in the attached proton test (Fig. 4) and confirmed the presence of unsubstituted C-6. Linkage analysis (Table 2) indicated the signal could have represented the conflated C-6 resonances of unsubstituted 3- and 4-linked Gal*p* and 3-linked Gal*p* 4-sulfate residues of the carrageenans and the 4-linked α-Glc*p* residues of floridean starch. However, two relatively weak cross-peaks at 100.5/5.37 and 73.9/3.96 ppm in the HMQC spectrum (Fig. 5) were assigned by comparison with reported ^{13}C NMR data[43,44] to C-1/H-1 and C-3/H-3, respectively, of the 4-linked α-Glc*p* residues of floridean starch. Consequently, the cross-peaks at 61.4/3.87 and 61.4/3.82 ppm probably arose mainly from the C-6/H-6a and C-6/H-6b, respectively, of the 4-linked α-Glc*p* residues of floridean starch, but they also were possibly partly attributable to unsubstituted C-6 occurring in the *C. ovatum* galactans. From the HMQC spectrum, the weak proton signal at 5.37 ppm in the ^{1}H NMR spectrum (Fig. 6) was assigned to H-1 of the 4-linked α-Glc*p* residues of floridean starch.

4. Conclusion

The chemical and spectroscopic data showed that the alkali-modified *C. ovatum* galactans were composed of a nearly idealized repeating unit of 6′-*O*-methylcarrabiose

2,4′-disulfate, with some minor substitution patterns also present. The *C. ovatum* carrageenans therefore are the most highly methylated carrageenans reported. The ^{13}C, ^{1}H, and HMQC NMR spectra for the repeating unit were fully assigned.

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