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Carrageenans with complex substitution patterns from red algae of the genus *Erythroclonium* [1]

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Abstract

Extracellular polysaccharides from three *Erythroclonium* spp. were shown, by a combination of compositional, linkage analyses, and Fourier transform infrared and ^{13}C-nuclear magnetic resonance spectroscopy, to be highly substituted carrageenans with at least five types of repeating disaccharide units. These are the carrabiose 2,4′-disulfate of ι-carrageenan, carrabiose 2-sulfate of α-carrageenan, the 6′-*O*-methylated counterparts of each of these repeating units, and 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate. The polysaccharides also contain significant amounts of unsubstituted, 4-linked galactopyranose and small amounts of 4-linked 3-*O*-methylgalactopyranose and terminal glycosyl residues. The carrageenan preparations of the three species are similar, differing only in the proportions of some components. © 1998 Elsevier Science Ltd.

Keywords: Carrageenan; *Erythroclonium*; Gigartinales; Polysaccharides; Algal; Rhodophyta; Solieriaceae; Structure determination

1. Introduction

Carrageenans are high-molecular-weight, sulfated galactans extractable with hot water from red algae mainly of the order Gigartinales [2]. They are essentially linear polymers composed of repeating disaccharide units of β-(1 → 3)-linked and α-(1 → 4)-linked D-galactopyranose (Gal*p*) residues, although the 4-linked residues often occur as 3,6-anhydrogalactopyranose (AnGal*p*). Carrageenans are also variously substituted with pyruvate acetals, methyl ethers, and single glycosyl residue branches, although carrageenan repeating units are conventionally defined by their AnGal content and pattern of sulfate ester substitution. Knutsen et al. [3] have proposed an adaptable nomenclatural scheme to replace the existing one which uses Greek letters to name carrageenan

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[1] Cell-wall polysaccharides from Australian red algae of the family Solieriaceae (Gigartinales, Rhodophyta). For previous instalment, see ref. [1].

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repeating units, although the new scheme will probably only be approved by IUPAC–IUBMB after some amendments (Prof. D. Horton, pers. comm.). In this article, we use the term 'carrabiose' to refer to the repeating disaccharide 3′-linked *O*-β-D-Gal*p*-(1 → 4)-α-D-AnGal*p*. Carrageenans from some sources have become commercially exploited because of their rheological properties [4,5]. The chemotaxonomic implications of carrageenan chemistry have also proved to be of interest in red algal taxonomy [6–9].

We are screening polysaccharides from Australian red algae with a view towards identifying potential commercial sources of polysaccharides with useful functional properties and applying polysaccharide chemistry to studies of red algal systematics. Among the algae we have studied are several from the family Solieriaceae that produce carrageenans with novel substitution patterns [1,10]. These include algae of the genus *Rhabdonia*, which produce highly methylated carrageenans composed mainly of carrabiose 2,4′-disulfate (the repeating unit of ι-carrageenan) and 6′-*O*-methylated carrabiose 2,4′-disulfate [10]. The carrageenan from *R. coccinea* also contained a significant proportion of carrabiose 2-sulfate units (of α-carrageenan), whereas the carrageenan from *R. verticillata* contained some 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate and some 4-linked Gal*p* residues substituted predominantly with methyl ether at O-3 [10].

The genus *Erythroclonium* is regarded by many phycologists [11–14] as closely related to *Rhabdonia* and thus is a worthy point of departure for further assessing the diversity of galactan structures occurring in the Solieriaceae. *Erythroclonium* comprises five species, four of which are endemic to Australia. These include three species which are the focus of this study: *E. muelleri* Sonder (the lectotype for the genus), which occurs along the southern coasts of Australia from Western Australia to Victoria and Tasmania; *E. sedoides* (Harvey) Kylin, known only from the west of the continent; and *E. sonderi* Harvey, which is distributed along the coasts of Western Australia and South Australia [14].

2. Experimental

Algal samples.—The algae used for this study were: A drift-cast, non-reproductive specimen of *E. muelleri* (MELU-A 042222) collected at Geraldton, Western Australia on 13 November 1995 by Kraft, Saunders, Strachan, and Chiovitti; a drift-cast, cystocarpic specimen of *E. sedoides* (MELU-A 042223) collected at Safety Bay, Western Australia on 7 November 1995 by Kraft, Saunders, and Chiovitti; and a non-reproductive sample of *E. sonderi* (MELU-A 042226) collected using SCUBA at 15–26 m depths from Mostyn's Lump, Houtman-Abrolhos Islands, Western Australia on 11 November 1995 by Kraft and Saunders. Seaweeds collected from the field were sun-dried and stored in plastic bags with silica gel until transported to the laboratory for processing.

Extraction and treatment of the polysaccharides.—Polysaccharides were extracted with hot water, clarified, and precipitated with 2-propanol as described previously [8]. Starch was removed from the preparations by amyloglucosidase digestion [8]. The sulfated galactans were alkali-modified as described by Craigie and Leigh [15].

Compositional analyses.—The sulfate content of the polysaccharide preparations was determined by the method of Tabatabai [16] as modified by Craigie et al. [17]. The pyruvate content of the polysaccharides was determined by the method of Duckworth and Yaphe [18]. For the quantitative determination of constituent sugars, alditols from the polysaccharide preparations were derived by reductive hydrolysis and acetylation as described by Stevenson and Furneaux [19]. The alditol acetates were separated by gas chromatography (GC) and identified by their retention times relative to *myo*-inositol hexaacetate as described previously [20]. For quantification, molar response factors (RFs) were determined relative to *myo*-inositol for galactose (Gal), 6-*O*-methylgalactose (6-MeGal), glucose (Glc), and xylose (Xyl) obtained from commercial sources. The RF for 3-*O*-methylgalactose (3-MeGal) and 4-*O*-methylgalactose (4-MeGal) was assumed to be the same as that for 6-MeGal. The RF of 3,6-anhydrogalactose (AnGal) was derived by analysis of commercially available κ-carrageenan from '*Eucheuma cottonii*' [presumably *Kappaphycus alvarezii* (Doty) Doty] known to contain Gal and AnGal in approximately equimolar proportions [19]. To assist with the identification of constituent sugars using GC–mass spectrometry (MS), deuterium-reduced alditol acetates were generated by hydrolysis in aqueous trifluoroacetic acid followed by reduction with $NaBD_4$ and acetylation essentially as described by Harris et al. [21].

Linkage analysis.—To determine their linkage and substitution patterns, polysaccharide preparations were converted to their Me_2SO-soluble triethylammonium salts and methylated with CD_3I essentially by the protocol described by Stevenson and Furneaux

[19], except that a NaOH–Me_2SO suspension was used to generate the alkoxide [22]. The two procedures used for hydrolysis, reduction, and acetylation were the same as those used for constituent sugar analysis.

The pertrideuteriomethylated alditol acetates were separated by GC on a BPX70 (SGE, Australia) capillary column, detected by electron impact ionisation–MS, and identified by their mass spectra and their retention times relative to *myo*-inositol hexaacetate as described by Lau and Bacic [23]. The pertrideuteriomethylated species were quantified by a combination of reconstructed ion chromatogram and selected ion monitoring (SIM) techniques [10].

Samples of the alkali-modified polysaccharide preparations were also treated with periodate prior to linkage analysis. Vials containing polysaccharide preparations (6.5 mg) dissolved in 2 mL of 0.1 M $NaIO_4$ solution were wrapped in aluminium foil and shaken gently at room temperature for 2 d. The periodate was quenched with 12 mL 1,2-ethanediol and the oxidised polysaccharides were reduced with 40 mg $NaBH_4$ at room temperature for 24 h. Following neutralisation with acetic acid, the solutions were dialysed exhaustively against Milli-Q water prior to converting the periodate-treated polysaccharides to their triethylammonium salts and analysing their linkages as above.

Spectroscopic analyses.—Polysaccharide films for Fourier transform infrared (FTIR) spectroscopy were prepared [7] and the FTIR spectra were recorded on a Perkin–Elmer series 2000 FTIR spectrometer in transmittance mode (8 scans, collected at a resolution of 4 cm^{-1}).

For ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectroscopy, the polysaccharide samples were dissolved in D_2O (3% w/v). The proton-decoupled ^{13}C-NMR spectra were recorded at 80 °C on a Bruker ARX500 spectrometer (operating at 125.8 MHz). The parameters for the acquisitions were a spectral width of 27.8 kHz, 45° pulse (8.0 μs), an acquisition time of 0.29 s, and a relaxation delay of 1.0 s for 44,000–63,000 scans. ^{13}C signals were referenced to internal Me_2SO at 39.6 ppm. The methylene and quaternary carbons were assigned using the *J*-modulated spin-echo experiment [24]. The *J*-mod spectrum of the alkali-modified preparation from *E. sedoides* was recorded on the same spectrometer at 80 °C with a spectral width of 27.8 kHz, a 90° pulse (16.0 μs), an acquisition time of 0.29 s, a relaxation delay of 1.0 s, and a modulation delay of 6.8 ms, for approximately 60,000 scans.

3. Results and discussion

Polysaccharide composition.—The yield of the hot water-extractable polysaccharides from the three *Erythroclonium* spp. was between 15% and 26% (w/w). The crude preparations were de-starched with amyloglucosidase to generate the 'native' polysaccharides, which were subsequently modified with hot alkali. Alkali-modification leads to an elimination reaction in which the sulfate ester at O-6 of 'precursor' 4-linked Gal*p* residues (and their 2-sulfates) is removed with concomitant formation of AnGal*p* residues (and their 2-sulfates) [15]. The alkali-modified preparations from the three species were highly

Table 1
Composition of polysaccharide preparations from *Erythroclonium* species

Source	Preparation	Sulfate content[a,b] (% w/w)	Pyruvate content[a] (% w/w)	Monosaccharides (mol%)						
				AnGal	Gal	6-MeGal	4-MeGal[c]	3-MeGal[c]	Xyl	Glc
E. muelleri	native	nd	nd	24	52	16	1.5	2	1.5	3
	alk.-mod.	20.9	1.0	28	45.5	18	2	3.5	1	2
E. sedoides	native	nd	nd	25	41	24	1.5	4.5	1	3
	alk.-mod.	19.3	0.8	26.5	39	24	1.5	4.5	2	2.5
E. sonderi	native	nd	nd	21	52	23	tr	2	1	1
	alk.-mod.	19.9	0.3	27	44	25	tr	2	1	1

AnGal = 3,6-anhydrogalactose; Gal = galactose; 6-MeGal = 6-*O*-methylgalactose; 4-MeGal = 4-*O*-methylgalactose; 3-MeGal = 3-*O*-methylgalactose; Xyl = xylose; Glc = glucose; tr = trace (< 0.8%); nd = not determined; alk.-mod. = alkali-modified.

[a] Based on dry weight of the polysaccharide preparation.

[b] Expressed as SO_3Na.

[c] Confirmed by mass spectral analysis of $NaBD_4$-reduced alditol acetates.

sulfated (19.3–20.9% w/w as SO_3Na, Table 1) and contained small quantities of pyruvate (0.3–1.0% w/w, Table 1).

Constituent sugar analysis (Table 1) of the native and alkali-modified polysaccharide preparations from the three *Erythroclonium* spp. demonstrated they were substituted galactans, with Gal, AnGal, and mono-*O*-methylgalactose (MeGal) comprising over 95 mol% of the sugars of each sample. The 6-MeGal content of the polysaccharide preparations was particularly high, in the range 16–25 mol% for the three species. Small quantities (2–6 mol%) of 3-MeGal and 4-MeGal were also present. Alkali-modification of the native polysaccharides resulted in a slight increase in the proportion of AnGal (1.5–6 mol%) with a concomitant decrease in the proportion of Gal. The effect was most evident for the preparations from *E. sonderi* and least for those from *E. sedoides*. These data indicated the presence of small amounts of 4-linked precursor residues in the native polysaccharides. Xyl and Glc were also detected, generally in small quantities (Table 1). The monosaccharide compositions of the crude, untreated preparations from *E. muelleri* and *E. sedoides* were especially rich in Glc (18.5 and 15 mol%, respectively), which was shown to be derived from contaminating floridean starch by ^{13}C-NMR spectroscopy and susceptibility to amyloglucosidase digestion. The spectrum (not shown) of the crude preparation from *E. muelleri* contained characteristic signals at 100.2, 72.2, 73.9, 78.0, 71.9, and 61.3 ppm corresponding to C-1 to C-6 of the α-(1 → 4)-linked D-glucopyranose of floridean starch [25,26].

FTIR spectroscopic analysis.—The FTIR spectra of the native and alkali-modified preparations from the three *Erythroclonium* spp. were recorded (Fig. 1). The FTIR spectra displayed an intense band of absorption at 1240 cm^{-1}, indicative of sulfate ester [27]. The diagnostic region (940–800 cm^{-1}) of the spectra was comparable to that of the FTIR spectra of hybrid α-/ι-carrageenans from Burmese and Thai specimens of *Catenella nipae* [28,29]. Bands at 935 and 805 cm^{-1} demonstrated the presence of AnGal*p* residues (consistent with constituent sugar analysis, Table 1) and axial sulfate esters located at O-2 of AnGal*p* residues, respectively [27]. The band at 900 cm^{-1} suggested the polysaccharides contained unsulfated, 3-linked Gal*p* and/or 6-MeGal*p* residues [7,26,28,30,31]. This band has also been shown to be associated with unsulfated, 3-linked Gal*p* residues bearing pyruvate acetal substitution [1]. In addition, a weaker absorption band observed at 850 cm^{-1} in the FTIR spectra demonstrated the presence of axial sulfate ester substitution at O-4 of some 3-linked Gal*p* and/or 6-MeGal*p* residues [27].

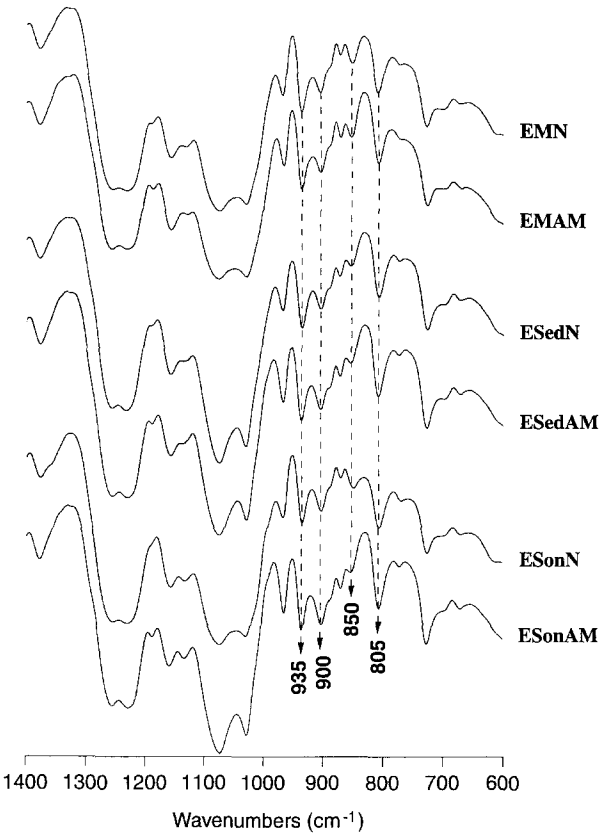

Fig. 1. Fourier transform infrared spectra of polysaccharide preparations from *Erythroclonium*. EMN = *E. muelleri* native preparation; EMAM = *E. muelleri* alkali-modified preparation; ESedN = *E. sedoides* native preparation; ESedAM = *E. sedoides* alkali-modified preparation; ESonN = *E. sonderi* native preparation; ESonAM = *E. sonderi* alkali-modified preparation.

The FTIR spectra of the alkali-modified preparations differed from those of the native preparations in the diminished absorption at 820–830 cm^{-1}, the region associated with sulfate esters at equatorial O-2 and O-6 positions of 4-linked precursor residues [27,32]. Consistent with changes in constituent sugars (Table 1), the effect was most pronounced for the preparations from *E. sonderi* and least for those from *E. sedoides*. Alkali-modification also resulted in other changes in the FTIR spectra including increased absorption at 870 and 970 cm^{-1}.

Linkage analyses.—The linkage patterns of the native (N) and alkali-modified (AM) polysaccharide preparations from the three *Erythroclonium* spp. are summarised in Table 2, with glycosidic linkage through O-1 assumed for all residues. All constituent sugars were interpreted as pyranosyl residues. Sulfate

Table 2
Linkage analysis of constituent sugars (mol%) of polysaccharide preparations from *Erythroclonium* species

Constituent monosaccharide	*E. muelleri*			*E. sedoides*			*E. sonderi*		
Deduced linkage[a]	N	AM	PO	N	AM	PO	N	AM	PO
AnGal*p*									
2,4-	28.5	32	39.5	33	32.5	37	27.5	32	36
Gal*p*									
Terminal[b]	–	tr	–	–	–	–	1	tr	–
3-[c]	6.5	8	10.5	7.5	8	8	7	8	9
4-[d]	12	10	–	6	6	–	8.5	8	–
3,4-[e]	24.5	23.5	22	21	22	21	20	21	23
2,4,6-[f]	3	–	–	1	–	–	5.5	–	–
3,4,6-	6.5	5	4	6	6	6	2.5	2.5	2
2,3,4,6-	–	–	–	–	–	tr	1	–	–
6-MeGal*p*									
3-[c]	4	4	7	11	11.5	12	10	11.5	11.5
3,4-[e]	12.5	13.5	15	10.5	11	12.5	13	13.5	15
4-MeGal*p*									
Terminal[b]	–	tr	–	1	tr	–	tr	tr	–
3-MeGal*p*									
Terminal[b]	1	2	1	1.5	1.5	2.5	1	1	2
4-[d]	1.5	1	1	1.5	1.5	1	2	1.5	1.5
Xyl*p*									
Terminal	tr	1	tr	–	tr	–	1	1	–

N = native polysaccharide; AM = alkali-modified polysaccharide; PO = alkali-modified polysaccharide subjected to periodic oxidation; AnGal*p* = 3,6-anhydrogalactopyranose; Gal*p* = galactopyranose; 6-MeGal*p* = 6-*O*-methylgalactopyranose; 4-MeGal*p* = 4-*O*-methylgalactopyranose; 3-MeGal*p* = 3-*O*-methylgalactopyranose; Xyl*p* = xylopyranose; tr = trace (< 0.8%); – = not detected.

[a]2,4-Linked AnGal*p* deduced from 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol, terminal Gal*p* deduced from 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-trideuteriomethylgalactitol, 3-linked 6-MeGal*p* deduced from 1,3,5-tri-*O*-acetyl-2,4-di-*O*-trideuteriomethyl-6-*O*-methylgalactitol, etc.

[b,c,d,e]Co-eluting sets of pertrideuteriomethylated alditol acetates. The proportion of Gal*p* and MeGal*p* with the same linkage was determined by SIM–MS of their diagnostic, primary fragment ions. See ref. [10] for details.

[f]Confirmed by mass spectral analysis of $NaBD_4$-reduced, pertrideuteriomethylated alditol acetates.

esters and pyruvate acetals were stable during trideuteriomethylation but were subsequently released during the acid hydrolysis. The location of these substitutions therefore manifested as 'linkage' positions in the results.

The most dominant features of the native and alkali-modified *Erythroclonium* polysaccharides were 2,4-linked AnGal*p*, 3,4-linked Gal*p*, and 3,4-linked 6-MeGal*p*. Consistent with the FTIR data, the 2,4-linked AnGal*p* was interpreted as 4-linked AnGal*p* 2-sulfate. The two 3,4-linked sugars were interpreted as mainly 3-linked Gal*p* 4-sulfate and 3-linked 6-MeGal*p* 4-sulfate. The FTIR spectra of the polysaccharides demonstrated that sulfate esters were indeed present at the axial O-4 position of some 3-linked Gal*p* and 6-MeGal*p* residues. However, the quantity of putative 3-linked, 4-sulfated residues estimated from linkage analysis was greater than expected from the intensity of the 850 cm^{-1} band in the FTIR spectra. This situation is not unique to the *Erythroclonium* polysaccharides. Polysaccharide preparations from *Catenella nipae* exhibited similar FTIR absorption patterns to those of the *Erythroclonium* polysaccharides, but were shown by linkage analyses to contain 20–30 mol% 3,4-linked Gal*p*, which was proved to be derived from 3-linked Gal*p* 4-sulfate by 1D- and 2D-NMR spectroscopy [29]. In addition, a proportion of the 3,4-linked residues in the *Erythroclonium* polysaccharides could represent other types of linkage patterns, such as glycosyl branch points in the galactan backbone or 4-linked Gal*p* and/or 6-MeGal*p* bearing sulfate ester at O-3.

The *Erythroclonium* preparations contained significant levels of 3-linked 6-MeGal*p* and unsubstituted, 3-linked Gal*p* residues. There were also small but significant proportions of 3,4,6-linked Gal*p* residues,

which were assigned on the basis of the results of the pyruvate assay (Table 1) to pyruvated, 3-linked residues occurring as 4,6-*O*-(1-carboxyethylidene)-Gal*p* [33]. Both unsulfated and pyruvated 3-linked Gal*p* residues probably contributed to the intense band of absorption at 900 cm^{-1} in the FTIR spectra of the polysaccharides [1,29].

Linkage analyses also provided evidence for 4-linked residues other than 4-linked AnGal*p* 2-sulfate. These included significant quantities of unsubstituted, 4-linked Gal*p* and a small amount of 4-linked 3-MeGal*p*. To confirm the identity of the 4-linked residues, the alkali-modified preparations were treated with periodate. In this procedure, sugar residues with vicinal free hydroxyls were destroyed. Linkage analyses of the periodate-treated preparations (PO, Table 2) revealed that the unsubstituted, 4-linked Gal*p* residues were totally destroyed, whereas the 4-linked 3-MeGal*p* residues survived the treatment.

Other structural variations in the polysaccharides included small amounts of 2,4,6-linked Gal*p* in the native preparations which were absent from the alkali-modified preparations and therefore interpreted as 4-linked Gal*p* 2,6-disulfate, the precursor residue to 4-linked AnGal*p* 2-sulfate. Small amounts of terminal 3-MeGal*p*, 4-MeGal *p*, and Xyl *p* residues were also present.

Linkage analyses revealed the complex structure of the polysaccharides, indicating heterogeneous patterns of substitution including methylation, sulfation, pyruvation, and incomplete replacement of the 4-linked residues with AnGal*p*. The preparations from the three *Erythroclonium* spp. were nonetheless relatively similar, except for differences in the proportions of some of the residues. The constituent sugar analyses (Table 1) showed that the *E. muelleri* polysaccharide contained ca. 17 mol% 6-MeGal, whereas the polysaccharides from *E. sedoides* and *E. sonderi* contained significantly more (ca. 24 mol%). Linkage analyses of the polysaccharides indicated that the 'extra' 6-MeGal in the polysaccharides of the latter two species occurred mainly as unsulfated, 3-linked residues (Table 2). Furthermore, the preparations from *E. sonderi* contained less 4,6-pyruvated, 3-linked Gal*p* residues than the preparations from *E. muelleri* and *E. sedoides*, consistent with their pyruvate contents (Table 1). The relative amount of the precursor residue, 4-linked Gal*p* 2,6-disulfate, was highest in the native preparation from *E. sonderi* and least in the native preparation from *E. sedoides*, consistent with observations from constituent sugar (Table 1) and FTIR (Fig. 1) analyses.

^{13}C-NMR spectroscopic analysis.—Proton-decoupled ^{13}C-NMR spectra of the three alkali-modified preparations from *Erythroclonium* were similar, except for differences in the intensities of some resonances. The ^{13}C-NMR spectrum of one representative, *E. sedoides*, is shown in Fig. 2 and the resonance assignments for the five dominant repeating disaccharide units inferred from the linkage data are summarised in Table 3.

Evidence from ^{13}C-NMR spectroscopy demonstrated that the *Erythroclonium* polysaccharides were highly substituted carrageenans. The ^{13}C-NMR spec-

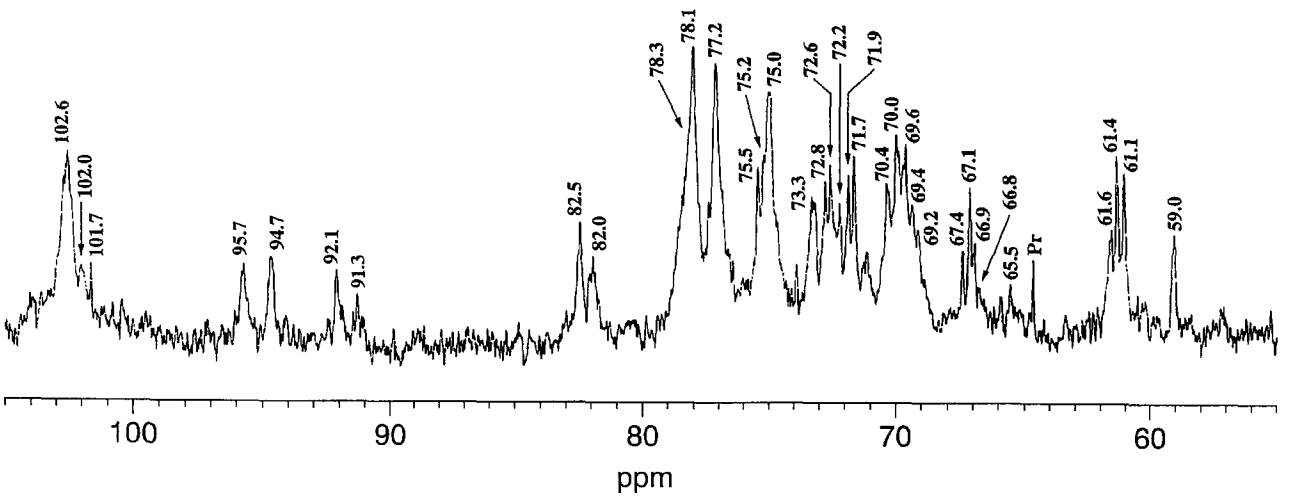

Fig. 2. Proton-decoupled ^{13}C-nuclear magnetic resonance spectrum of the alkali-modified polysaccharide preparation from *Erythroclonium sedoides*. The signal labelled 'Pr' (at 64.7 ppm) arises from the residual 2-propanol in the sample.

Table 3
Assignments of resonances[a] observed in the ^{13}C-NMR spectrum of the alkali-modified polysaccharide preparation from *E. sedoides*[b]

Repeating unit	Sugar	Carbon atom									
		C-1	C-2	C-3	C-4	C-5	C-6	O-Me	Pyruvic methyl	Pyruvic acetal	Pyruvic carboxyl
G4S-DA,2S	3-linked	102.6	69.4	77.2[c]	72.2	75.0[d]	61.4				
	4-linked	92.1	75.0[d]	78.1	78.3	77.2[c]	70.0				
G4S,6M-DA,2S	3-linked	102.6	69.4	77.2[c]	72.6	73.3	71.9	59.0			
	4-linked	92.1	75.0[d]	78.1	78.3	77.2[c]	70.0				
G-DA,2S	3-linked	102.6	69.6	82.0	66.9	75.2[e]	61.4				
	4-linked	94.7	75.5	78.1	78.3	77.2[c]	70.0				
G6M-DA,2S	3-linked	102.6	69.6	82.0	67.1	73.3	71.7	59.0			
	4-linked	94.7	75.5	78.1	78.3	77.2[c]	70.0				
GP-DA,2S	3-linked	102.0	69.2	77.2[c]	67.4	66.8	65.5		25.5	101.7	175.7
	4-linked	91.3	75.2[e]	78.1	78.3	77.2[c]	70.0				

Repeating units: G4S-DA,2S = carrabiose 2,4′-disulfate of ι-carrageenan; G4S,6M-DA,2S = 6′-*O*-methylated carrabiose 2,4′-disulfate; G-DA,2S = carrabiose 2-sulfate of α-carrageenan; G6M-DA,2S = 6′-*O*-methylated carrabiose 2-sulfate; GP-DA,2S = 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate.
[a]Chemical shifts in ppm referenced to Me_2SO at 39.6 ppm.
[b]A 3% w/v solution in D_2O recorded at 80 °C.
[c,d,e]Coincident resonances.

tra of the *Erythroclonium* preparations (Fig. 2) contained sets of signals attributable to the repeating disaccharide of ι-carrageenan (carrabiose 2,4′-disulfate), including diagnostic resonances for C-1 of the 4-linked residue at 92.1 ppm and C-4 of the 3-linked residue at 72.2 ppm (Table 3). The signals were assigned by comparison with reported data [29,34–36]. The signals for the 6′-*O*-methylated counterpart of carrabiose 2,4′-disulfate were assigned in accordance with the ^{13}C-NMR data reported for the 6′-*O*-methylated carrageenans from *Rhabdonia* [10]. These included a set of diagnostic signals at 72.6, 73.3, 71.9, and 59.0 ppm (Fig. 2, Table 3) attributed to C-4, C-5, C-6, and 6-*O*-methyl carbon, respectively, of the 3-linked 6-MeGal*p* 4-sulfate residue.

A set of signals was assigned to the repeating disaccharide of α-carrageenan (carrabiose 2-sulfate) by comparison with data reported by Falshaw et al. [29]. Diagnostic resonances for α-carrageenan included resonances for C-1 of the 4-linked residue at 94.7 ppm, and C-3 and C-4 of the unsubstituted, 3-linked Gal*p* residue at 82.0 and 66.9 ppm, respectively (Table 3).

Linkage analysis data revealed that the *Erythroclonium* polysaccharides were rich in 3-linked 6-MeGal*p*. These residues were presumed to alternate mainly with 4-linked D-AnGal*p* 2-sulfate residues in the form of 6′-*O*-methylated carrabiose 2-sulfate. Assignment of signals for the repeating unit of 6′-*O*-methylated α-carrageenan were based on those of α-carrageenan [29] but with consideration given to the shifts expected with 6′-*O*-methylation, drawn by analogy from the spectral changes observed with 6′-*O*-methylation of agarose and ι-carrageenan [10,34,37,38]. The signal for C-6 of the 3-linked residue of α-carrageenan coincides with that of ι-carrageenan at 61.4 ppm (Fig. 2, Table 3) [29,34–36]. In addition to the signal at 71.9 ppm assigned to C-6 of the 3-linked residue of 6′-*O*-methylated carrabiose 2,4′-disulfate, another signal was observed at 71.7 ppm, and both signals were inverted in a *J*-mod experiment (spectrum not shown). The latter signal was assigned to the *O*-methylated C-6 of the 3-linked residue of 6′-*O*-methylated carrabiose 2-sulfate. The ca. 10.3 ppm downfield shift of this signal is comparable to the α-shift of 8–11 ppm observed for the C-6 signal with *O*-methylation for agarose and ι-carrageenan [10,34,37,38]. This signal was also present in the spectra of other polysaccharides rich in putative 6′-*O*-methylated carrabiose 2-sulfate (Liao et al., manuscript in preparation). A diagnostic signal for the unsubstituted C-4 of the 3-linked Gal*p* residue of carrabiose 2-sulfate was observed in the spectra of the *Erythroclonium* polysaccharides (66.9 ppm, Fig. 2). An additional signal was, however, observed at 67.1 ppm in the spectra of the *Erythroclonium* polysaccharides (Fig. 2). This signal was assigned (Table 3) to C-4 of the 3-linked 6-MeGal*p* residue of 6′-*O*-methylated carrabiose 2-sulfate. The ca. 0.2 ppm downfield shift of this signal is comparable to the

0.2–0.4 ppm downfield shift of the corresponding signal observed with the 6′-*O*-methylation of agarose and ι-carrageenan [10,34,37,38]. It is possible that the broad envelope at 73.3 ppm, assigned to C-5 of the 3-linked residue of 6′-*O*-methylated carrabiose 2,4′-disulfate, included the signal for C-5 of the 3-linked residue of 6′-*O*-methylated carrabiose 2-sulfate. This signal was ca. 1.9 ppm upfield of the corresponding signal for the unsubstituted, 3-linked Gal*p* residue of carrabiose 2-sulfate. A β-shift of 1.7 ppm is observed for the C-5 signal of the 3-linked Gal*p* residue with 6′-*O*-methylation for agarose [34,37,38]. The reduced intensities of the signals at 94.7, 67.2, and 71.7 ppm in the spectrum (not shown) of the *E. muelleri* polysaccharide relative to those in the spectra of the other two *Erythroclonium* polysaccharides were consistent with the relatively lower content of 3-linked 6-MeGal*p* in the *E. muelleri* preparation (Table 2).

Signals for the pyruvated repeating disaccharide, 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate were assigned by comparison with data reported for the carrageenans from *Callophycus* containing unusually high levels of pyruvate acetal substitution [1]. Diagnostic resonances included those for the carboxyl, acetal, and methyl carbons of the pyruvate acetal substituent at 175.7 (not shown in Fig. 2), 101.7, and 25.5 ppm (not shown), respectively, C-1 of the 4-linked residue at 91.3 ppm, and C-1, C-2, C-4, C-5, and C-6 of the pyruvated, 3-linked residue at 102.0, 69.2, 67.4, 66.8, and 65.5 ppm, respectively. These signals were observed in the spectra of the polysaccharides from *E. sedoides* and *E. muelleri*, but were indistinguishable from the noise in the spectrum of the polysaccharide from *E. sonderi*, reflecting the lower pyruvate content of the *E. sonderi* polysaccharide (Tables 1 and 2).

Signals at ca. 95.7 and 82.5 ppm matched those observed in the ^{13}C-NMR spectrum of the polysaccharide from *Rhabdonia verticillata* [10]. In the spectrum of the *R. verticillata* polysaccharide, the former signal was assigned to C-1 of 4-linked 3-MeGal*p* and Gal*p* residues. Similarly, the signal at 95.7 ppm in the ^{13}C-NMR spectra of the *Erythroclonium* polysaccharides was assigned to C-1 of 4-linked 3-MeGal*p* and Gal*p* residues, although, in contrast to the polysaccharide from *R. verticillata* [10], the content of unsubstituted, 4-linked Gal*p* was relatively high but that of 4-linked 3-MeGal*p* was relatively low (Table 2). For the polysaccharide from *R. verticillata*, the signal at 82.5 ppm was suggested to arise either from the C-3 of a 3-linked Gal*p* residue with a neighbouring 4-linked 3-MeGal*p* or Gal*p* residue or from the *O*-methylated C-3 of 4-linked 3-MeGal*p* [10]. The low content of 4-linked 3-MeGal*p* in the *Erythroclonium* polysaccharides (Table 2) versus the relatively high intensity of the signal in their ^{13}C-NMR spectra (Fig. 2) supports the former assignment and precludes the latter one.

In the unsubstituted C-6 region of the ^{13}C-NMR spectra of the *Erythroclonium* polysaccharides, two signals at 61.1 and 61.6 ppm were observed in addition to the one at 61.4 ppm assigned to C-6 of the 3-linked residues of carrabiose 2-sulfate and carrabiose 2,4′-disulfate units. As expected, all three signals were inverted in the *J*-mod experiment (not shown). Based on the presence of 4-linked residues not in the AnGal*p* 2-sulfate form (Gal*p* and 3-MeGal*p*, Table 2) the signals at 61.1 and 61.6 ppm were assigned to unsubstituted C-6 of 3-linked residues and their neighbouring 4-linked residues not in the AnGal*p* 2-sulfate form, respectively, in line with the assignments for desulfated λ-carrageenan [34,39]. Signals were also observed at 70.4 and 72.8 ppm in the spectra of all three *Erythroclonium* polysaccharides (Fig. 2). The resonance at 70.4 ppm, which was not inverted in the *J*-mod spectrum, was tentatively assigned to C-5 of the 4-linked residues not in the AnGal*p* 2-sulfate form (mainly Gal*p*), also in line with assignments for desulfated λ-carrageenan [34,39]. As the signal at 72.6 ppm was assigned to C-4 of 3-linked 6-MeGal*p* 4-sulfate residues having neighbouring 4-linked AnGal*p* 2-sulfate, we speculate that the signal at 72.8 ppm is attributable to C-4 of the same residues having neighbouring 4-linked residues other than AnGal*p* 2-sulfate.

Although they cannot be assigned with certainty, we propose that the signals at 95.7, 82.5, 70.4, 61.6, and 61.1 ppm are associated with the presence of unsubstituted, 4-linked Gal*p* residues and 4-linked Gal*p* residues substituted at O-3 with methyl ether and possibly sulfate ester and/or terminal glycosyl residues. These signals were intense in the ^{13}C-NMR spectra of the *Erythroclonium* polysaccharides, more so than expected from the content of 4-linked Gal*p* and 3-MeGal*p* residues alone in the linkage analysis data (Table 2). The difference may reflect incomplete recovery of 4-linked Gal*p* and 3-MeGal*p* residues and/or inadequate identification of 4-linked Gal*p* residues substituted with sulfate ester and/or glycosyl residues at O-3 (occurring as 3,4-linked Gal*p* residues, Table 2). The chemical shift of the 95.7 signal is more upfield than that of the signal for C-1 of the 4-linked residues of desulfated λ-carrageenan

at 96.2 ppm [34,39] (cf. the signal for C-1 of the 4-linked residues of desulfated porphyran at 100.9 ppm [40]). Furthermore, the chemical shifts for C-1 of the AnGal*p* 2-sulfate residues in the *Erythroclonium* polysaccharides furnish evidence for carrageenan structure. We therefore presume the 4-linked Gal*p* and putative O-3-substituted residues also have the D-configuration.

The diverse collection of structures in the *Erythroclonium* carrageenans raises the question of whether these structures occur in a hybrid polysaccharide or as a mixture of several distinct homopolymers. ^{13}C- and ^{1}H-NMR spectroscopic studies of unfractionated carrageenan preparations [29] and oligosaccharides obtained by fractionation of enzymic digests of carrageenans from a number of sources [32,41–45] have provided evidence that the different disaccharide units of these carrageenans occur as hybrid molecules. By contrast, the occurrence of galactan homopolymers composed of a single type of repeating unit has not yet been demonstrated in red algae [43–45]. Therefore, although it remains to be determined, it is likely that the various structures of the *Erythroclonium* carrageenans form hybrid molecules.

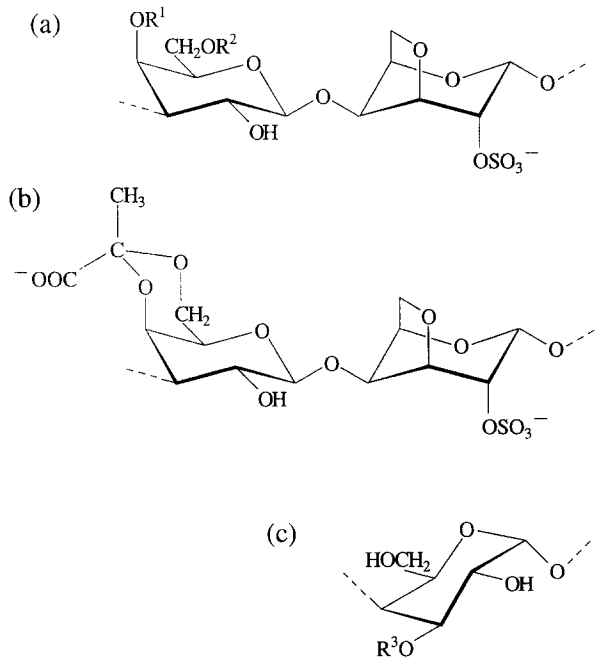

Fig. 3. Proposed structures in the carrageenans from *Erythroclonium*. (a) Carrabiose 2-sulfate of α-carrageenan (R^1 = H, R^2 = H); carrabiose 2,4′-disulfate of ι-carrageenan ($R^1 = SO_3^-$, R^2 = H); 6′-*O*-methylated carrabiose 2-sulfate (R^1 = H, $R^2 = CH_3$); 6′-*O*-methylated carrabiose 2,4′-disulfate ($R^1 = SO_3^-$, $R^2 = CH_3$). (b) 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate. (c) 4-linked D-galactopyranose (R^3 = H); 4-linked 3-*O*-methyl-D-galactopyranose ($R^3 = CH_3$).

4. Conclusion

The *Erythroclonium* polysaccharides are highly substituted carrageenans with a diverse combination of sulfation, methylation, and pyruvation (Fig. 3). The *E. sedoides* polysaccharide typified the carrageenans from *Erythroclonium*. It is rich in both repeating disaccharides of ι- and α-carrageenan and the 6′-*O*-methylated counterparts of both repeating units. It also contains significant quantities of 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate and 4-linked, unsulfated Gal*p*, some of which is methylated at O-3. There is also evidence for terminal MeGal*p* and Xyl*p* residues and the possibility of sulfate ester or glycosyl substitution at O-3 of 4-linked Gal*p* residues. The *E. sonderi* polysaccharide differs by having appreciably less pyruvate acetal substitution and significantly more 4-linked precursor residues in the native polysaccharide, whereas the *E. muelleri* polysaccharide has appreciably less unsulfated, 3-linked 6-MeGal*p*.

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