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Cell-wall polysaccharides from Australian red algae of the family Solieriaceae (Gigartinales, Rhodophyta): novel, highly pyruvated carrageenans from the genus *Callophycus*

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Abstract

Cell-wall polysaccharides from six species of red algae of the genus *Callophycus* were mainly galactans comprised predominantly of galactose (Gal) and 3,6-anhydrogalactose (AnGal), and were rich in pyruvate and sulfate. The Fourier Transform Infrared (FTIR) spectra of the polysaccharides superficially resembled that of α-carrageenan (composed of the repeating disaccharide carrabiose 2-sulfate), with major bands of absorption indicative of 4-linked AnGal, axial 2-sulfate on 4-linked AnGal, and unsulfated, 3-linked Gal. The FTIR spectra of solutions of *Callophycus* polysaccharides in D_2O–phosphate buffer displayed absorption, corresponding to the carboxylate anion of the pyruvate acetal substituent. Methylation analysis showed that 3,4,6-linked Gal*p* (interpreted as 4,6-pyruvated, 3-linked Gal*p*) and 2,4-linked AnGal*p* (interpreted as 4-linked AnGal*p* 2-sulfate) were the dominant linkages, together with significant quantities of 3-linked Gal*p*. Proton-decoupled ^{13}C nuclear magnetic resonance (NMR) spectroscopy showed the polysaccharides to be composed predominantly of pyruvated carrageenans. The ^{13}C NMR spectra were completely assigned by a *J*-modulated spin-echo pulse sequence and 2D experiments employing gradient Heteronuclear Multiple Bond Correlation (HMBC), $^{13}C/^{1}H$ Heteronuclear Multiple Quantum Coherence (HMQC), and HMQC Total Correlation Spectroscopy (HMQC-TOCSY). The *Callophycus* galactans thus consist predominantly of the novel repeating disaccharide 4′,6′-*O*-(1-

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carboxyethylidene)carrabiose 2-sulfate and minor amounts of the α-carrageenan repeating unit (carrabiose 2-sulfate), and other structural variations. © 1997 Elsevier Science Ltd.

Keywords: *Callophycus*; Gigartinales; Carrageenan, pyruvated; Polysaccharides, red algal; Solieriaceae; Structure determination; Galactans, sulfated

1. Introduction

Most marine red algae elaborate high-molecular-weight, sulfated galactans as the major, matrix-phase components of their cell walls [1,2]. The sulfated galactans from some species are used extensively in the food industry because of their versatile solution properties and protein-reactivity [3,4]. Red algal galactans are fundamentally linear polymers in which the backbones are composed of repeating disaccharides of β-(1 → 3)-linked and α-(1 → 4)-linked galactopyranosyl (Gal*p*) residues. The 3-linked residue of the repeating disaccharide has the D configuration, whereas the configuration of the 4-linked residue, which commonly occurs as 3,6-anhydrogalactopyranose (AnGal*p*), defines the class of the galactan as either an agar (L) or a carrageenan (D). Red algal galactans are a spectrum of polysaccharides bearing a variety of non-glycosyl substitutions. Generalisations of the substitution patterns of agars and carrageenans have been discussed [1,2]. Agars are typically low in sulfate ester substitution, but those from numerous sources are rich in methyl ether or pyruvate acetal substitution. Conversely, carrageenans are comparatively rich in sulfate ester substitution but poor in methyl ether substitution, and, except for those extracted from the diploid phase of some gigartinacean species, they rarely contain significant levels of pyruvate acetal substitution. Carrageenan repeating units are thus traditionally defined according to their pattern of sulfation as well as their AnGal content. However, recent reports of highly methylated carrageenans [5,6] demonstrate that a greater diversity of substitution patterns exists among carrageenans. More-complex red algal galactans are also known in which the substitution patterns are polydisperse or the backbone structures are irregular [7–11], but these have received less attention than the structurally regular agars and carrageenans because they are not commercially exploited and they pose problems for analysis [11]. A nomenclatural scheme was proposed by Knutsen et al. [12] to accommodate the growing collection of combinations of substitutions becoming recognised in red algal galactan structure but it is yet to be approved by IUPAC-IUBMB.

We are currently screening cell-wall galactans from Australian red algae of the family Solieriaceae. The Solieriaceae is a significant component of the Australian marine flora, represented by over 25 species in at least 10 genera [13,14]. This family is also of interest because it contains important sources of commercial phycocolloids, such as the genera *Eucheuma* and *Kappaphycus* [15], and because it is a morphologically and anatomically heterogeneous assemblage [16] for which data on cell-wall carbohydrates offer chemotaxonomic assessment of its constituent taxa [17]. Furthermore, the Solieriaceae is a fruitful source of unusual cell-wall polysaccharides, such as the highly methylated carrageenans extracted from *Rhabdonia* spp. [6]. In the course of our survey, we have screened the cell-wall galactans from Australian representatives of the genus *Callophycus*. This genus contains nine species, most of which are endemic to mainland Australia, including *C. dorsiferus* (the type species) and *C. costatus*, which are both restricted to relatively short stretches of coastline along Western Australia; *C. oppositifolius* and *C. harveyanus*, which both occur along the western and southern coasts of the continent; *C. laxus*, which occurs in South Australia and Victoria; and the essentially tropical species *C. tridentifer* from New South Wales and Queensland [14,18,19]. In this paper we report the results of this survey which demonstrate that Australian *Callophycus* spp. produce novel, highly pyruvated carrageenans.

2. Experimental

Algal samples.—The specimens used for this study are summarised in Table 1.

Extraction and alkali modification of the polysaccharides.—Methods used for the preparation of algal samples and for the hot water-extraction, clarification, and 2-propanol-precipitation of the polysaccharide preparations have been described [20]. All dry weights were determined after drying overnight at 60 °C in vacuo over P_2O_5. Extraction of the polysaccharide from *C. laxus* by the described method resulted in very low yield (4.1% w/w of the dry

Table 1
Collection information for the algae used in this study

Species	Voucher No.	Collection			Collectors
		Locality	Date	Notes	
Callophycus dorsiferus (C. Agardh) Silva	MELU-A, 042225	Geraldton, Western Australia	13 Nov 1995	Drift-cast	G. Kraft, G. Saunders, I. Strachan, and A. Chiovitti
Callophycus costatus (Harvey) Silva	MELU-A, 042219	Garden Island, Western Australia	23 Jul 1992	3–5 m depth, from rocky reef	J. Huisman and I. McKirnan
Callophycus oppositifolius (C. Agardh) Silva	MELU-A, 042221	Garden Island, Western Australia	23 Jul 1992	3–5 m depth, from rocky reef	J. Huisman and I. McKirnan
Callophycus harveyanus (J. Agardh) Silva	MELU-A, 042224	Safety Bay, Western Australia	7 Nov 1995	Drift-cast	G. Kraft, G. Saunders, and A. Chiovitti
Callophycus laxus (Sonder) Silva	MELU-A, K8741	Port MacDonnell, South Australia	1 Dec 1991	Drift-cast	G. Kraft
Callophycus tridentifer Kraft	MELU-A, 042228	North side of Muttonbird Island, Coffs Harbour, New South Wales	29 Nov 1992	7 m depth, on rocks	A. Millar

algal meal). An improved yield of the *C. laxus* polysaccharide preparation was obtained by heating a 1% (w/v) mixture of algal meal in water, adjusted to pH 9 with NaOH, at 121 °C for 1 h and filtration, clarification, and precipitation as described before [20]. The polysaccharides from all six *Callophycus* spp. were alkali-modified by the method described by Craigie and Leigh [21].

Chemical analyses.—Pyruvate content was determined by the lactate dehydrogenase method of Duckworth and Yaphe [22]. Sulfate content was determined by the $BaCl_2$ turbidimetric method of Tabatabai [23] as modified by Craigie et al. [24]. The constituent monosaccharides were derivatised for analysis as described by Stevenson and Furneaux [25] and were separated, identified, and quantified using gas chromatography (GC) and GC–mass spectrometry (MS) as described previously [26].

Linkage and substitution patterns were investigated by preparation of Me_2SO-soluble triethylammonium salts of the polysaccharides and methylation as described by Stevenson and Furneaux [25], except that a NaOH–Me_2SO suspension was used to generate the alkoxide [27]. The partially methylated alditol acetates were generated by reductive hydrolysis and acetylation [25]. The partially methylated alditol acetates were separated by GC on a BPX70 capillary column (SGE, Australia), 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 [28]. The partially methylated species were quantified by calculating their molar ratios from the total ion current. For quantification of permethylated AnGal*p* derivatives, a response factor was calculated by analysis of commercially available κ-carrageenan (Sigma, no. C-1263) from '*Eucheuma cottonii*' [presumably *Kappaphycus alvarezii* (Doty) Doty] known to contain Gal and AnGal in approximately equimolar proportions [25]. To distinguish between co-eluting derivatives by GC–MS, partially methylated alditol acetates were also generated by hydrolysis in aqueous trifluoroacetic acid followed by $NaBD_4$ reduction and acetylation [29].

Spectroscopic methods.—Fourier transform infrared (FTIR) spectra of dried films of the polysaccharides [30] were recorded. To detect the presence of carboxyl groups, additional FTIR spectra were recorded of 0.75–1.00% (w/v) solutions of selected *Callophycus* polysaccharides in D_2O–phosphate buffer (pD 7.2) prepared as described by Bociek and Welti [31]. Solutions were injected into a cell composed of calcium fluoride windows separated by a teflon spacer of 0.2 mm pathlength. All FTIR spectra were recorded on a Perkin–Elmer Series 2000 FTIR spectrophotometer (8 scans, at a resolution of 4 cm^{-1}), scanning between 4000 and 400 cm^{-1} for the dried films and between 1900 and 1500 cm^{-1} for the D_2O solutions.

For nuclear magnetic resonance (NMR) spectroscopy, the polysaccharide samples were dissolved in D_2O (3% w/v). A ^1H NMR spectrum of the polysaccharide from *C. laxus* was recorded at 80 °C on a Bruker DRX500 spectrometer (operating at 500.13 MHz) using a spectral width of 4.0 kHz, 16K data points, 90° pulse (7.1 μs), a relaxation delay of

2 s and 32 scans. The water proton signal was suppressed by low-power irradiation during the relaxation delay. The ^{1}H chemical shifts were reported relative to internal Me_2SO at 2.71 ppm [32]. The proton-decoupled ^{13}C NMR spectrum of the *C. costatus* polysaccharide was recorded at 80 °C on a Bruker AMX600 spectrometer (operating at 150 MHz), using a spectral width of 31.3 kHz, 45° pulse (8.0 μs), an acquisition time of 0.26 s, and a relaxation delay of 1 s for ~ 52,000 scans. ^{13}C NMR spectra of polysaccharide preparations from the other *Callophycus* spp. were recorded at 80 °C on a Bruker AMX300 WB spectrometer (operating at 75.5 MHz). The spectrum of the preparation from *C. tridentifer* was recorded with a spectral width of 19.2 kHz, 45° pulse, an acquisition time of 0.43 s, and a relaxation delay of 1 s for ~ 72,000 scans. The parameters for the other acquisitions were a spectral width of 12.2–20.0 kHz, 45° pulse (7.0 μs), an acquisition time of 0.41–0.67 s, and a relaxation delay of 0.4–1.0 s for 35,000–51,000 scans. ^{13}C signals were referenced to internal Me_2SO at 39.6 ppm. The methylene carbons of the Gal*p* and AnGal*p* residues and the acetal and carboxyl carbons of the pyruvate substituent were identified by recording proton-decoupled ^{13}C NMR spectra using a *J*-modulated spin-echo pulse sequence [33] at 80 °C on the Bruker AMX300 WB spectrometer. The *J*-mod spectrum of the preparation from *C. tridentifer* was recorded with a spectral width of 15.2 kHz, an acquisition time of 0.54 s, a relaxation delay of 1.5 s, and a modulation delay (τ) of 7.1 ms for ~ 100,000 scans.

Heteronuclear Multiple Quantum Coherence (HMQC) [34], HMQC Total Correlation Spectroscopy (HMQC-TOCSY), and Heteronuclear Multiple Bond Correlation (HMBC) [35] 2D spectra were recorded on a Bruker ARX500 spectrometer at 80 °C. Pulsed field gradients were used for coherence selection in the HMBC experiment [36]. The 2D spectra were recorded with a ^{1}H spectral width of 4.0 kHz, an acquisition time of 256–512 ms, and relaxation delay of 1.5 s, during which the residual HOD signal was presaturated. The spectral width in the ^{13}C dimension was 12.0–18.0 kHz (95–150 ppm) which was recorded using 256–512 FIDs (Free induction decays). The mixing delays in HMQC-TOCSY and HMBC spectra were 80 and 55 ms, respectively.

3. Results and discussion

Compositional analyses.—The yields of the crude polysaccharides obtained from the six *Callophycus* spp. were in the range 11–26% w/w (Table 2). The compositions of the alkali-modified *Callophycus* polysaccharide preparations were similar (Table 2). The preparations had a high pyruvate content (in the range 8.0–10.5% w/w, Table 2) and a high sulfate content (in the range 15.9–16.8% w/w as SO_3Na, Table 2). Constituent sugar analyses revealed that Gal and AnGal were the dominant sugars in the preparations, accounting for 87–93 mol% of the total monosaccharides. Small quantities of mono-*O*-methylgalactose (MeGal), xylose (Xyl), and glucose (Glc) were also present.

FTIR spectroscopy.—The FTIR spectra of the dried films of the alkali-modified *Callophycus* polysaccharides (Fig. 1a) were similar. They exhibited a

Table 2
Composition of alkali-modified polysaccharide preparations from *Callophycus* species

Polysaccharide source	Yield [a] (% w/w)	Sulfate content [b,c] (% w/w)	Pyruvate content [b] (% w/w)	Monosaccharides [d] (mol%)					
				Gal	AnGal	3-/4-MeGal	6-MeGal	Xyl	Glc
C. dorsiferus	17	16.2	9.9	59.5	33	tr [e]	tr	2	5.5
C. costatus	20	16.3	10.1	57	32	1	1	3	6
C. oppositifolius	12	16.2	8.7	63.5	28.5	1	– [e]	3	4
C. harveyanus	11	16.2	8.6	61	26	1	tr	4	8
C. laxus	26	15.9	8.0	58	30	1	–	6	5
C. tridentifer	21	16.8	10.5	65	28	–	–	3	4

[a] Yield of crude polysaccharide, based on dry weight of the seaweed. Alternative extraction conditions used for *C. laxus* (see Experimental for details).
[b] Based on dry weight of the alkali-modified polysaccharide preparation.
[c] Expressed as SO_3Na.
[d] Gal = galactose; AnGal = 3,6-anhydrogalactose; 3-/4-MeGal = 3-*O*-methylgalactose and/or 4-*O*-methylgalactose; 6-MeGal = 6-*O*-methylgalactose; Xyl = xylose; Glc = glucose.
[e] tr = trace (< 0.8 mol%); – = not detected.

strong band of absorption at 1240 cm^{-1}, indicative of sulfate ester substitution [37]. The diagnostic region between 945 and 800 cm^{-1} of the FTIR spectra superficially resembled that of α-carrageenan [composed of carrabiose 2-sulfate units, where the term 'carrabiose' refers to the repeating disaccharide 3′-linked *O*-β-D-Gal*p*-(1 → 4)-α-D-AnGal*p*], reported from a Burmese specimen of *Catenella nipae* [38]. A band of absorption at 935 cm^{-1} confirmed the presence of AnGal residues and a band at 805 cm^{-1} indicated that the AnGal residues carried axial sulfate esters at O-2 [37]. Another major band occurred at 900 cm^{-1}. This signal has been observed in the IR spectra of α-carrageenan, agarose, and hybrid β/κ-carrageenan (composed of carrabiose and carrabiose 4′-sulfate units) and was assigned to unsulfated, β-(1 → 3)-linked Gal residues [30,38–41]. A weak peak was also observed at 825 cm^{-1} in the FTIR spectra, the region associated with sulfate ester at primary and secondary equatorial positions of Gal*p* residues [37].

FTIR spectra recorded of *Callophycus* polysaccharides in D_2O–phosphate buffer (pD 7.2), exemplified by that of *C. costatus* (Fig. 1b), provided further evidence for the unusually high level of pyruvate acetal substitution. The spectra exhibited an intense band of absorption at 1605 cm^{-1} which was associated with the carboxylate anion [31] of the pyruvate moiety. Interference from the broad absorption band of H_2O at ~1640 cm^{-1} was eliminated by the experimental conditions employed. Application of this technique to red algal galactans lacking pyruvate substitution (e.g. κ- and ι-carrageenans from Sigma Chemical Co.) gave spectra (not shown) devoid of absorption bands in the entire range 1900–1500 cm^{-1}.

Linkage analysis.—The linkage patterns of the alkali-modified polysaccharides were studied by methylation and subsequent reductive hydrolysis [25] (Table 3). All sugars were interpreted as pyranosyl residues. Pyruvate acetals and sulfate esters are stable during methylation, but are subsequently hydrolysed in acid, and therefore manifest as 'linkage' positions in the partially methylated alditol acetates.

The dominant linkage in the polysaccharides was 3,4,6-linked Gal*p*, and, consistent with the results of the pyruvate assay and FTIR analysis (Fig. 1b), provided further evidence for 3-linked Gal*p* residues bearing the pyruvate acetal, occurring as 4,6-*O*-(1-carboxyethylidene)-Gal*p* [42]. The polysaccharides also contained a significant proportion of unsubstituted, 3-linked Gal*p* residues and it is likely that both the 4,6-pyruvated and the unsubstituted, 3-linked residues contributed to the intense absorption at 900 cm^{-1} in the FTIR spectra of the polysaccharide films (Fig. 1a). The AnGal*p* was present only as 2,4-linked AnGal*p*, and was interpreted from FTIR data (Fig. 1a) as 4-linked AnGal*p* 2-sulfate.

In addition to the dominant linkages, the polysaccharide from *C. laxus* also contained significant

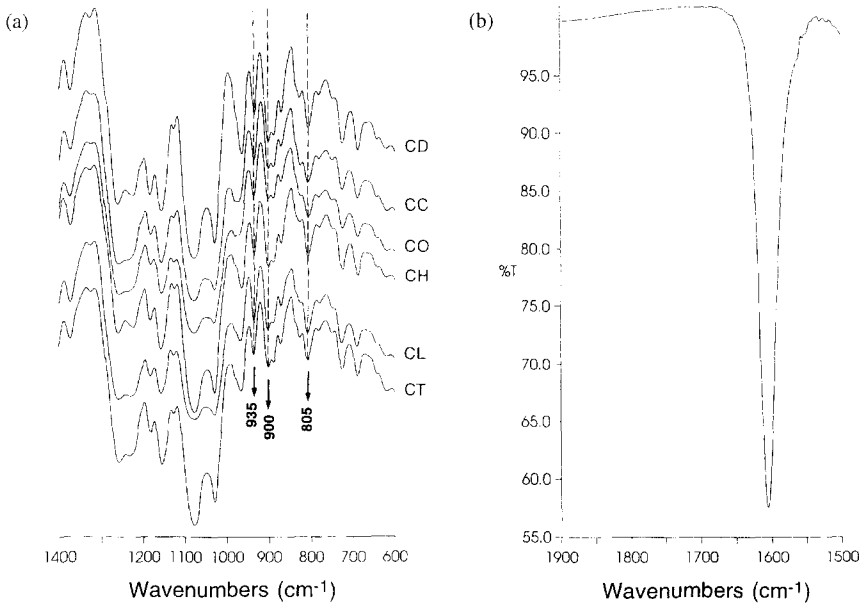

Fig. 1. (a) FTIR spectra of dried films of alkali-modified polysaccharide preparations from *Callophycus* species. CD = *C. dorsiferus*; CC = *C. costatus*; CO = *C. oppositifolius*; CH = *C. harveyanus*; CL = *C. laxus*; CT = *C. tridentifer*. (b) FTIR spectrum of a solution of the alkali-modified polysaccharide preparation from *C. costatus* in D_2O–phosphate buffer (pD 7.2).

Table 3
Linkage analysis (mol%) of the alkali-modified polysaccharide preparations from *Callophycus* species

Polysaccharide source	Constituent sugar [a]:	Gal*p*						AnGal*p*	Xyl *p*
	Deduced linkage [b]:	3-	4-	2,4-	3,4-	3,6-	3,4,6-	2,4-	Terminal
C. dorsiferus		12	2	1.5	2	tr [c]	47.5	34	1
C. costatus		12.5	tr	– [c]	3	–	49.5	34	1
C. oppositifolius		16.5	6	–	7	–	44.5	26	–
C. harveyanus		14.5	6	2	6	2	43	25.5	1
C. laxus		17	3	–	17.5	–	32.5	30	tr
C. tridentifer		10	2	–	4	1	54	29	–

[a] Gal*p* = galactopyranose; AnGal*p* = 3,6-anhydrogalactopyranose; Xyl *p* = xylopyranose.
[b] 3-Gal*p* deduced from 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol, etc.
[c] tr = trace (< 0.8%); – = not detected.

amounts of 3,4-linked Gal *p* (17.5 mol%), but the source of this component was uncertain (see below). Smaller quantities of 3,4-linked Gal*p* were also present in the other *Callophycus* polysaccharides. All the *Callophycus* preparations contained small quantities of other linkages, including 4-linked Gal*p*, indicating either minor structural variations in the cell-wall galactans or the presence of minor amounts of other polysaccharides.

Linkage analysis of the native, unmodified preparations from *C. dorsiferus*, *C. costatus*, *C. oppositifolius*, *C. harveyanus*, and *C. tridentifer* gave essentially the same results (data not shown) as those of the alkali-treated preparations (Table 3), except for the presence (< 5 mol%) of precursor residues (4-linked Gal*p* 2,6-disulfate) which were detected as 2,4,6-linked Gal*p*.

NMR spectroscopy.—The *Callophycus* polysaccharides were investigated by both proton-decoupled ^{13}C NMR spectroscopy and ^{13}C NMR spectroscopy employing the *J*-modulated spin-echo pulse sequence. The spectra of the polysaccharides from the six species were similar. The NMR spectra of the polysaccharide from one representative, *C. tridentifer*, are shown in Fig. 2 and the assignments of the signals are summarised in Table 4.

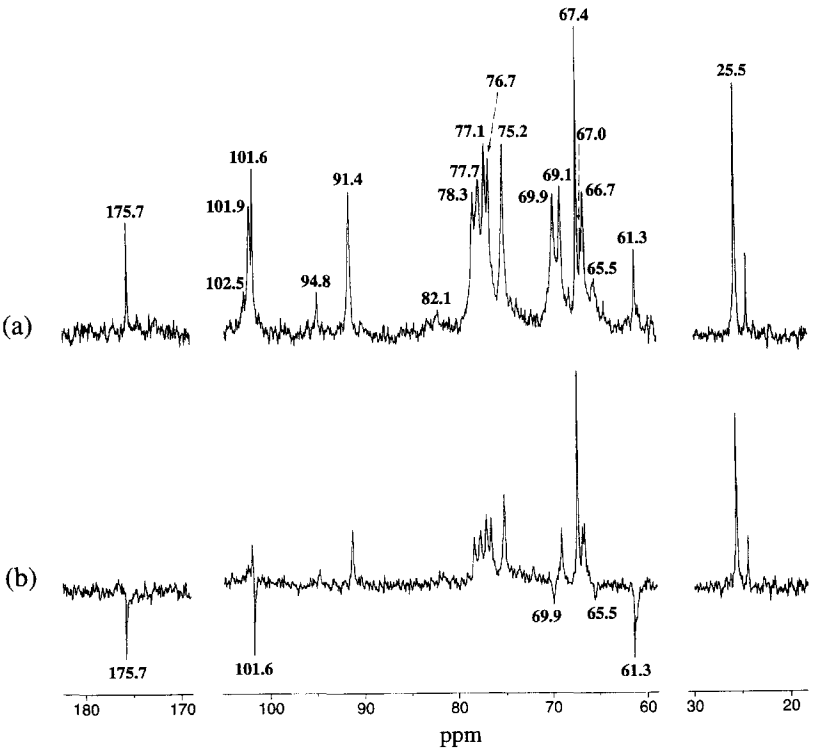

Fig. 2. ^{13}C NMR spectra of the alkali-modified polysaccharide preparation from *Callophycus tridentifer*. (a) Proton-decoupled ^{13}C NMR spectrum. (b) Spectrum recorded with the *J*-modulated spin-echo pulse sequence. The most upfield resonance (at 24.4 ppm) arises from the residual 2-propanol in the polysaccharide preparation.

Table 4
Assignments of resonances [a] observed in the ^{13}C NMR spectrum of the alkali-modified polysaccharide preparation from *Callophycus tridentifer* [b]. The major repeating unit GP-DA,2S = 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate; the minor repeating unit G-DA,2S = carrabiose 2-sulfate of α-carrageenan

Repeating unit	Sugar	Carbon atom								
		C-1	C-2	C-3	C-4	C-5	C-6	Pyruvate acetal		
								Methyl	Acetal	Carboxyl
GP-DA,2S	3-linked	101.9	69.1	76.7	67.4	66.7	65.5	25.5	101.6	175.7
	4-linked	91.4	75.2 [c]	77.7	78.3	77.1	69.9 [d]			
G-DA,2S	3-linked	102.5	69.9 [d]	82.1	67.0	75.2 [c]	61.3			
	4-linked	94.8	75.2 [c]	78.0	78.3	77.1	69.9 [d]			

[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] Coincident resonances.

The signals for the three carbons comprising the pyruvate acetal substituent were assigned by comparison with those for model compounds and agarose oligosaccharides containing pyruvate acetal substitution [43,44]. The signal at 25.5 ppm corresponded to that of the methyl carbon. The chemical shift of this signal indicated that the methyl group was equatorial to the dioxane ring and that the acetal carbon of the pyruvate substituent must therefore have the *R* configuration [45]. The most downfield signal at 175.7 ppm, which was inverted in the *J*-mod experiment (Fig. 2b), was assigned to the carboxyl carbon of the pyruvate acetal substituent. The signal at 101.6 ppm was assigned to the acetal carbon. For agarose oligosaccharides containing the pyruvate acetal, a comparable signal for the acetal carbon was observed at 101.7 ppm [44]. Proof for the assignment of the non-protonated acetal carbon was obtained by its appearance as a negative inflection in the *J*-mod experiment (Fig. 2b) and by HMBC spectroscopy. In

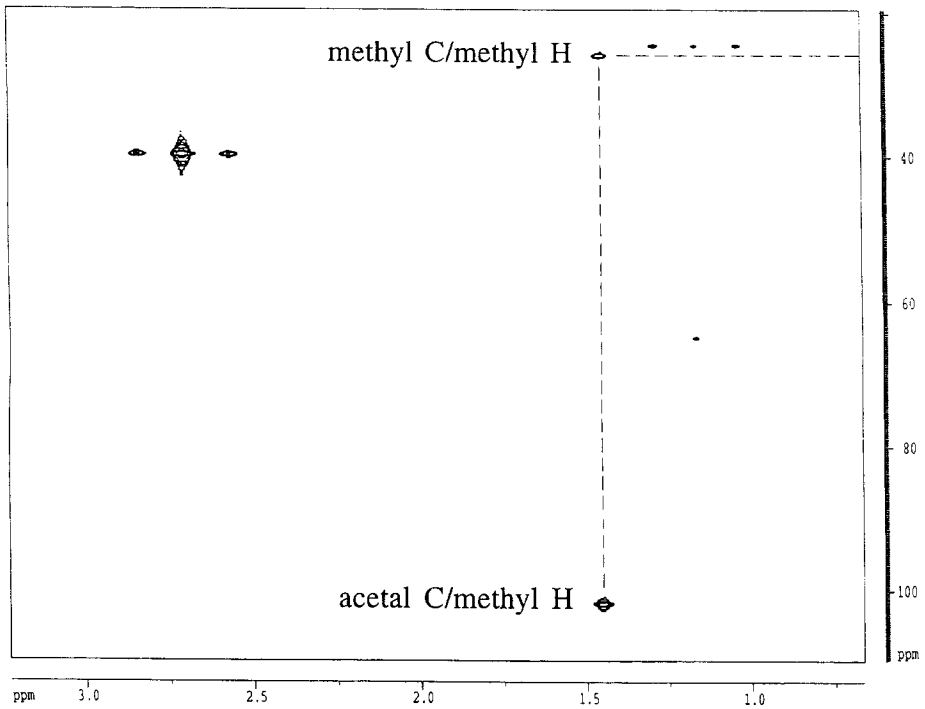

Fig. 3. Gradient Heteronuclear Multiple Bond Correlation (HMBC) spectrum of the alkali-modified polysaccharide preparation from *Callophycus laxus*. The cross-peak at 39.6/2.71 ppm corresponds to the geminal carbon nucleus and methyl protons of the Me_2SO standard added to the sample. Minor cross-peaks at 24.4/1.17 ppm and 64.5/1.17 ppm arise from the geminal and vicinal carbon/methyl protons, respectively, of residual 2-propanol in the polysaccharide preparation.

the HMBC spectrum recorded of the polysaccharide from *C. laxus* (Fig. 3), methyl protons were correlated with geminal and vicinal carbon nuclei. From the compositional data (Table 2), the only methyl group in significant proportions in the *Callophycus* polysaccharides was that belonging to the pyruvate acetal substituent. The corresponding carbon nuclei must therefore be those of the methyl carbon (geminal) and the acetal carbon (vicinal). In the HMBC spectrum, these assignments were confirmed by the $^{13}C/^{1}H$ cross-peaks at 25.5/1.45 ppm and 101.6/1.45 ppm, respectively.

The high content of the pyruvate acetal substituent in the *Callophycus* polysaccharides enabled the detection and assignment of the acetal carbon of the substituent in the proton-decoupled ^{13}C NMR spectra of the unfractionated preparations. Ordinarily, the resonance for the acetal carbon of the pyruvic group is not observed in the ^{13}C NMR spectra of unfractionated samples of red algal galactans [46] because, if present, the pyruvate substituent is usually at concentrations too low to be detected and because the high viscosity of such samples leads to line-broadening and therefore loss of resolution in the spectrum. The relatively long T_1 relaxation time of the acetal carbon further impedes its detection by ^{13}C NMR spectroscopy, even when the substituent is present at a significant level [43]. For red algal galactans, the acetal carbon signal has only been reported in ^{13}C NMR spectra of oligosaccharides generated from the agar extracted from *Gracilaria compressa* [44].

In the anomeric region of the ^{13}C NMR spectra (Fig. 2) there were two major signals, other than that of the acetal carbon, at 101.9 and 91.4 ppm. The signal at 101.9 ppm was assigned to C-1 of the pyruvated, 3-linked Gal*p* residue. The signal at 91.4 ppm could only belong to C-1 of 4-linked AnGal*p* 2-sulfate, but its chemical shift did not coincide with the C-1 signal of AnGal*p* reported for any other red algal galactan [7,44,46–49]. The chemical shift of the C-1 signal of AnGal*p* is configuration-dependent, those for D-AnGal*p* residues (carrageenans) occurring up to 5 ppm upfield of those for corresponding L-AnGal*p* residues (agars) [2,7,47]. The C-1 signal of the AnGal*p* 2-sulfate residue of the pyruvated polysaccharides was extremely upfield, clearly in the region characteristic of D-AnGal*p* and not L-AnGal*p* residues. Furthermore, the intensities of the two major C-1 signals were comparable. ^{13}C NMR spectroscopy therefore demonstrated that the dominant component of the *Callophycus* preparations was a repeating unit of pyruvated carrabiose 2-sulfate. Pyruvated carrabiose 2-sulfate and the repeating unit of ι-carrageenan (carrabiose 2,4′-disulfate) are similar in having one anionic group per sugar residue. The signals for C-1 of the 4-linked residues of the pyruvated carrageenans from *Callophycus* (Table 4) and ι-carrageenan (at ~92.2 ppm [47,49]) are also both further upfield than those reported for other AnGal-containing carrageenans [47,49]. However, the unique position of the signal for C-1 of AnGal*p* 2-sulfate of the pyruvated carrageenans suggested that these carrageenans adopt a significantly different conformation about their glycosidic bonds which is affected by both sulfation at O-2 of the D-AnGal*p* residues and the pyruvate acetal substituent on the neighbouring 3-linked Gal*p* residues.

The non-anomeric signals of the D-AnGal*p* 2-sulfate residue were assigned (Table 4) by comparison with data reported for the AnGal*p* 2-sulfate residue of ι-carrageenan [47,48]. These assignments are also consistent with those of the AnGal*p* 2-sulfate residue of α-carrageenan [49].

The chemical and spectroscopic data indicated that the polysaccharides from *Callophycus* spp. were composed predominantly of a repeating structure of 4,6-pyruvated, 3-linked Gal*p* alternating with 4-linked D-AnGal*p* 2-sulfate. This repeating structure is termed 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate, but the polysaccharide could be described trivially as 4′,6′-pyruvated α-carrageenan. The other known pyruvate-containing carrageenan, π-carrageenan (composed of alternating 4,6-pyruvated, 3-linked Gal*p* 2-sulfate and 4-linked Gal*p* 2-sulfate), has sulfate ester substitution at O-2 of both the 3-linked Gal*p* and the 4-linked Gal*p* residues but lacks sulfate ester substitution at O-6 of the 4-linked Gal*p* residues [42,50]. AnGal*p* residues therefore cannot be formed within π-carrageenan either by the action of a sulfohydrolase enzyme ('dekinkase') in vivo [51–53] or by alkali treatment in vitro.

Some of the non-anomeric ^{13}C NMR resonances of the pyruvated, 3-linked Gal*p* residue of the *Callophycus* polysaccharides were assigned (Table 4) as chemical shifts arising from the pyruvation of α-carrageenan by analogy with the effect of 4′,6′-pyruvation on agarose ^{13}C NMR resonances [44,46]. The peak at 65.5 ppm, which was inverted in the *J*-mod experiment (Fig. 2b), was assigned to C-6 of the pyruvated, 3-linked residue. The ~4 ppm-downfield shift of this signal relative to that of the unsubstituted C-6 of α-carrageenan was consistent with the α-effect of the 4′,6′-pyruvation of agarose [44,46]. The low intensity of this CH_2 signal resulted from its

broad line width. This was most likely due to a shortening of its T_2 relaxation time (which is inversely proportional to line width) relative to the CH signals. The signal at 66.7 ppm was assigned to C-5 of the pyruvated, 3-linked Gal*p*. Its position relative to that of the corresponding signal for α-carrageenan (at 75.3 ppm) was in accord with the ~ 8.6 ppm-upfield shift of the C-5 signal associated with the 4′,6′-pyruvation of agarose [44,46]. Among the three remaining major resonances, the most downfield one at 76.7 ppm was assigned to C-3 of the pyruvated, 3-linked Gal*p* residue. The ~ 5.2 ppm-upfield shift of the C-3 signal relative to α-carrageenan was significantly greater, however, than the 2.7 ppm-upfield shift of the corresponding signal observed with the 4′,6′-pyruvation of agarose [44,46]. Notably, a comparable disparity between α-carrageenan and agarose is also observed for the effect on the signal for C-3 of the 3-linked residue with sulfation of O-4. The signal for C-3 of the 3-linked residue of ι-carrageenan is ~ 4.8 ppm upfield of that of α-carrageenan [49], whereas the chemical shift of the corresponding signal for agarose 4′-sulfate is only ~ 2–3 ppm upfield of that of agarose [44,46].

Two major signals remained to be assigned in the ^{13}C NMR spectra of the *Callophycus* polysaccharides at 69.1 and 67.4 ppm which, by a process of elimination, must belong to C-2 and C-4 (or vice versa) of the pyruvated, 3-linked Gal*p* residue. Their assignments were not entirely certain, however. In the ^{13}C

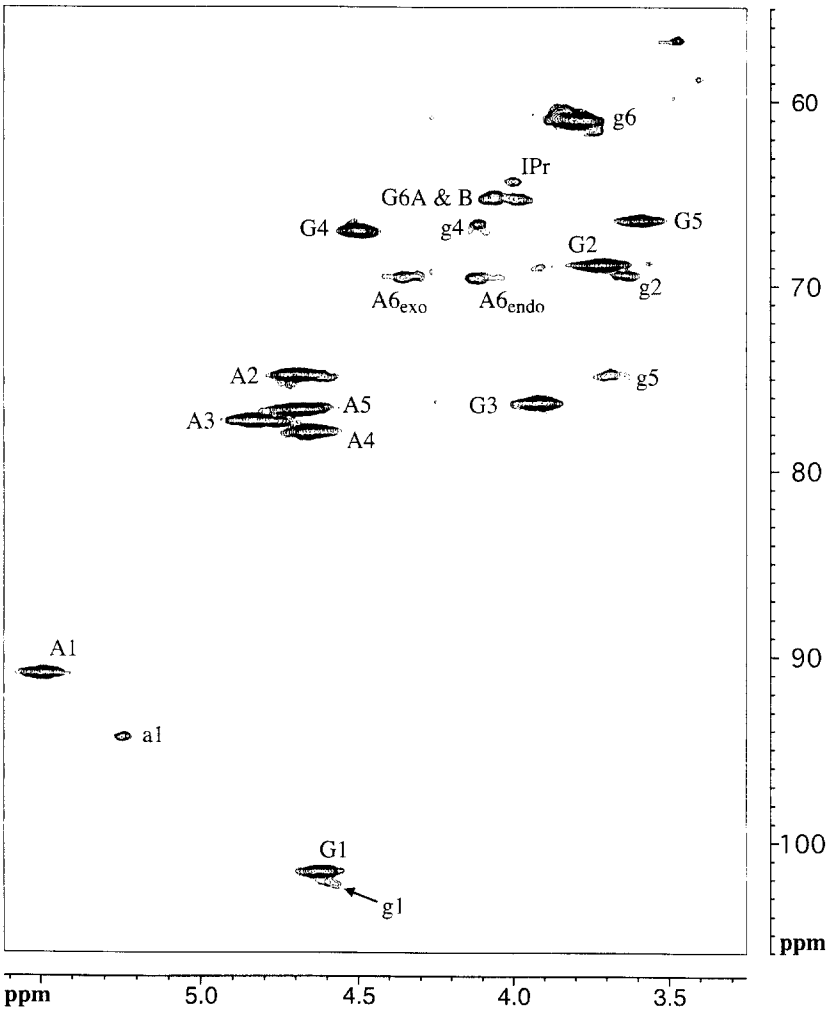

Fig. 4. ^{13}C/^{1}H Heteronuclear Multiple Quantum Coherence (HMQC) spectrum of the alkali-modified polysaccharide preparation from *Callophycus dorsiferus*. The geminal carbon/proton cross-peaks of the dominant repeating disaccharide, 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate, are labelled with capital letters (G = 3-linked residue; A = 4-linked residue). Those of the minor repeating disaccharide, carrabiose 2-sulfate of α-carrageenan, are labelled with lower case letters (g = 3-linked residue; a = 4-linked residue). The cross-peak labelled 'IPr' arises from the residual 2-propanol in the polysaccharide preparation.

NMR spectrum of α-carrageenan, the signal for unsubstituted C-4 of the 3-linked Gal*p* residue occurs at 66.9 ppm [49]. For 4′,6′-pyruvated agarose, the α-shift reported for C-4 of the 3-linked residue was 2.8 ppm [44,46]. If a similar α-shift occurred in the ^{13}C NMR spectra of the pyruvated carrageenans from *Callophycus*, the signal for C-4 of the 3-linked residue would be expected at 69.7 ppm. However, the signal for unsubstituted C-2 of the 3-linked residue of α-carrageenan was reported at 69.7 ppm (when referenced to Me_2SO at 39.6 ppm) [49]. The unassigned signal at 69.1 ppm observed in the spectra of the *Callophycus* polysaccharides could thus equally be assigned to either C-2 or C-4 of the pyruvated, 3-linked residue. By contrast, assignment of the 67.4 ppm signal in the spectra of the *Callophycus* polysaccharides to either carbon is not predicted. The assignments of these two signals were resolved by 2D HMQC and HMQC-TOCSY spectra.

In $^{13}C/^{1}H$ HMQC experiments, exemplified by the spectrum of the *C. dorsiferus* polysaccharide (Fig. 4), cross-peaks corresponding to all the geminally connected $^{13}C/^{1}H$ nuclei of the pyruvated repeating disaccharide were observed, including those for the anomeric nuclei of the pyruvated, 3-linked residue at 102.0/4.61 ppm and its neighbouring 4-linked AnGal*p* 2-sulfate residue at 91.4/5.48 ppm. Notably, among the $^{13}C/^{1}H$ cross-peaks for the non-anomeric nuclei, two were observed corresponding to C-6/H-6A and C-6/H-6B of the 3-linked residue locked in the ring formed through the pyruvate acetal (at 65.5/4.06 and 65.5/3.97 ppm, respectively). As

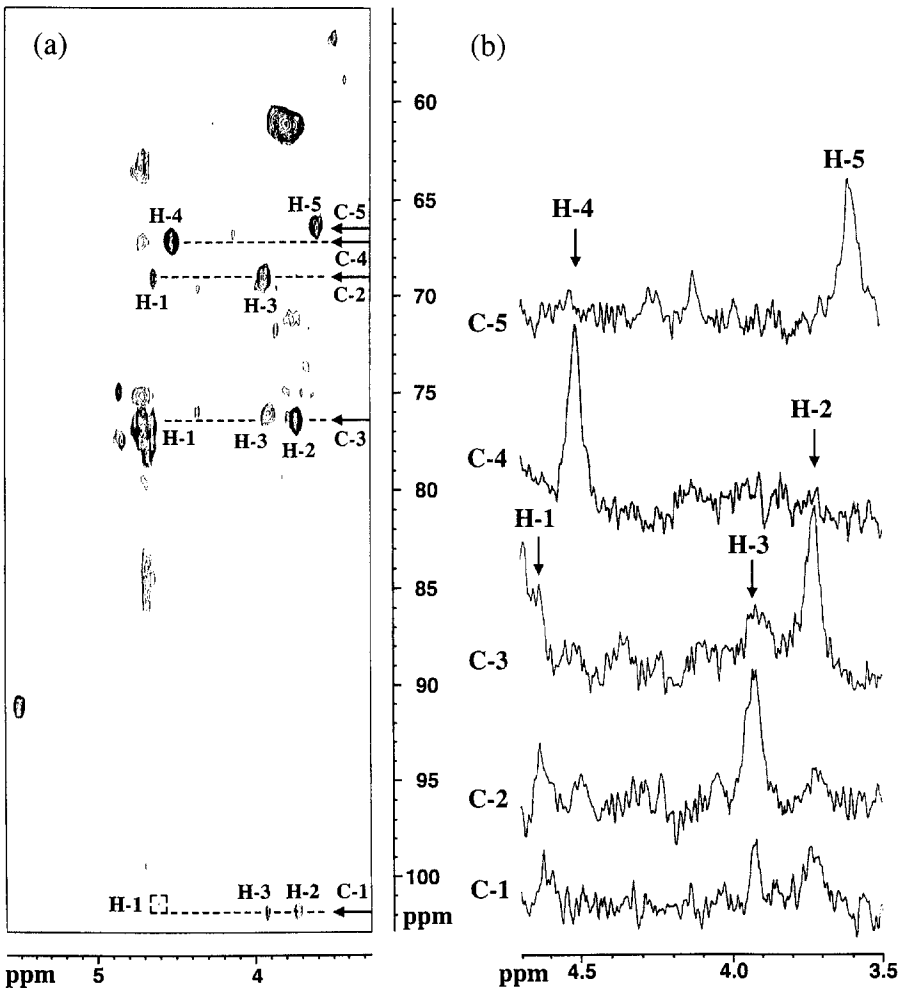

Fig. 5. $^{13}C/^{1}H$ Heteronuclear Multiple Quantum Coherence Total Correlation Spectroscopy (HMQC-TOCSY) spectrum of the alkali-modified polysaccharide preparation from *Callophycus dorsiferus*. (a) Conventional contour plot. The boxed cross-peak, C-1/H-1, is evident at a lower contour level. (b) Traces through the spectrum at ^{13}C shifts corresponding to the ring carbon atoms (C-1 to C-5) of the 4,6-pyruvated, 3-linked β-D-galactopyranosyl residue.

expected, a cross-peak corresponding to the acetal carbon of the pyruvate substituent was absent from the HMQC spectrum.

The two major signals that remained unassigned in the ^{13}C NMR spectra (Fig. 2) gave $^{13}C/^{1}H$ cross-peaks in the HMQC spectrum (Fig. 4) at 67.4/4.49 ppm and 69.2/3.71 ppm. These must be assigned to C-2/H-2 and C-4/H-4 (or vice versa) of the pyruvated, 3-linked Gal*p* residue. From ^{1}H NMR data compiled for α-, ι-, and κ-carrageenan and various oligosaccharides of the neo-carrabiose series, the resonances for H-2 of the 3-linked residue unsubstituted at O-2 were observed at ~3.6 ppm and were much further upfield (by ~0.50–1.30 ppm) than those for H-4 [47,49,54,55], irrespective of whether O-4 was substituted. For α- and ι-carrageenan, these observations were further confirmed by $^{1}H/^{1}H$ COSY [49]. By analogy with the ^{1}H NMR data of these polysaccharides, we assigned the proton resonance at 3.71 ppm to H-2 and that at 4.49 ppm to H-4 of the pyruvated, 3-linked Gal*p* residue. Their corresponding $^{13}C/^{1}H$ cross-peaks in the HMQC spectrum led us to assign the signals at 69.1 and 67.4 ppm in the ^{13}C NMR spectrum (Fig. 2) to C-2 and C-4, respectively, of the pyruvated, 3-linked residue (Table 4).

The assignments for C-2 and C-4 of the pyruvated, 3-linked Gal*p* residue were further confirmed from a HMQC-TOCSY spectrum (Fig. 5), which correlates the chemical shift of a given carbon with not only its attached proton but also with other protons coupled to this proton. In Fig. 5, C-1 showed cross-peaks with H-1, H-2, and H-3; C-2 was correlated with H-1 and H-3; and C-3 was correlated with H-1, H-2, and H-3. Although the C-1/H-1 cross-peak in the 2D spectrum (Fig. 5a) appeared weak at the observed contour level, the trace taken through C-1 confirmed its correlation with H-1 (Fig. 5b). For C-4 and C-5, correlations were observed only with their attached protons. Relayed correlations were not expected, or observed, for C-4 or C-5 with other ring protons due to the small coupling of their attached protons with neighbouring protons in the pyruvated, 3-linked Gal*p* residue. The observations above thus clearly differentiated C-2 from C-4. The established assignment of the resonance for C-4 of the 3-linked residue indicated its apparent downfield shift associated with the 4′,6′-pyruvation of α-carrageenan was only 0.5 ppm, in contrast to 2.8 ppm for agarose. Furthermore, the signal for C-2 of the 3-linked residue was ~0.5 ppm further upfield than the corresponding signal for α-carrageenan.

A combination of the ^{13}C NMR and the HMQC data permitted the assignments of the proton resonances of the pyruvated repeating disaccharide (Table 5) in the ^{1}H NMR spectrum obtained of the *C. laxus* polysaccharide (Fig. 6). The intense, upfield resonance at 1.45 ppm was assigned to the methyl protons of the pyruvate acetal, in agreement with assignments reported for the pyruvate acetal in agars, π-carrageenan, and bacterial polysaccharides [42,56–58].

In the ^{13}C NMR spectra of the *Callophycus* polysaccharides (Fig. 2 and Table 4), minor anomeric signals observed at 102.5 and 94.8 ppm matched those of α-carrageenan [49]. The presence of α-carrageenan was confirmed by a diagnostic signal for C-3 of the 3-linked Gal*p* residue at 82.1 ppm (in the range 81.8–82.2 ppm for the six *Callophycus* polysaccharides) and signals for C-4 and C-6 of the 3-linked Gal*p* residue at 67.0 and 61.3 ppm, respectively [49]. These data were consistent with those from linkage analysis which indicated the presence of 3-linked Gal*p* residues in the *Callophycus* polysaccharides and were thus shown by NMR spectroscopy to alternate with 4-linked D-AnGal*p* 2-sulfate residues. $^{13}C/^{1}H$ cross-peaks diagnostic of α-carrageenan [49] were also observed in the HMQC spectrum (Fig. 4), including one for the anomeric nuclei of the 4-linked AnGal*p* 2-sulfate residue at 94.7/5.23 ppm and four for the 3-linked Gal*p* residue at 102.5/4.59 ppm (C-1/H-1), 69.8/3.63 ppm (C-2/H-2), 67.0/4.12 ppm (C-4/H-4), and 75.2/3.68 ppm (C-5/H-5). The cross-peak at 61.4/3.78 ppm was assigned to C-6/H-6A and C-6/H-6B of the

Table 5
Assignments of proton resonances [a] attributed to the dominant repeating disaccharide 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate in the ^{1}H NMR spectrum of the polysaccharide preparation from *Callophycus laxus* [b]

3-Linked residue								4-Linked residue						
H-1	H-2	H-3	H-4	H-5	H-6A	H-6B	Pyruvate methyl	H-1	H-2	H-3	H-4	H-5	$H\text{-}6_{exo}$	$H\text{-}6_{endo}$
4.61	3.71	3.91	4.49	3.59	4.06	3.97	1.45	5.48	4.70	4.83	4.65	4.67	4.36	4.13

[a] Chemical shifts in ppm referenced to Me_2SO at 2.71 ppm.
[b] A 3% w/v solution in D_2O recorded at 80 °C.

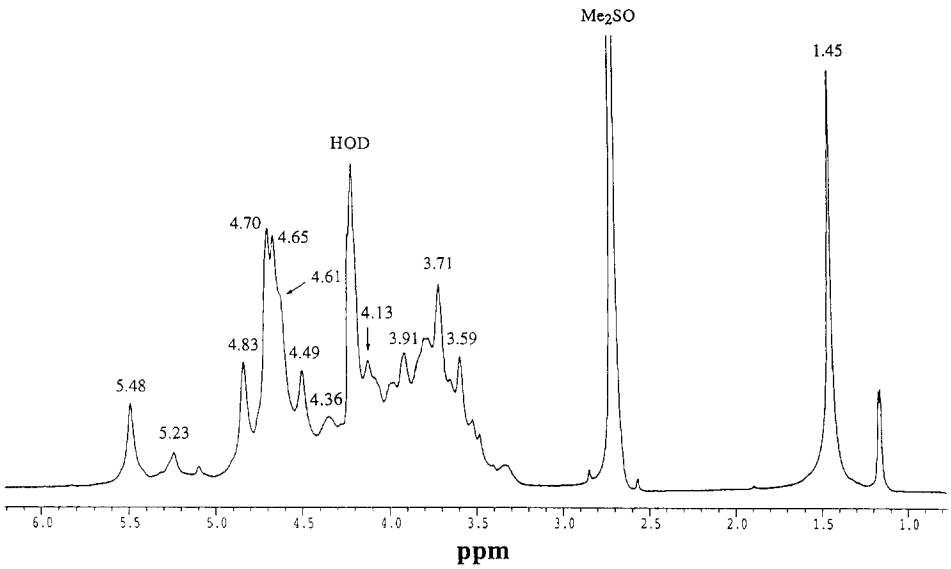

Fig. 6. ^{1}H NMR spectrum of the alkali-modified polysaccharide preparation from *Callophycus laxus*. The most upfield resonance (at 1.17 ppm) arises from the residual 2-propanol in the polysaccharide preparation.

3-linked Gal*p* residue of α-carrageenan and confirmed the tentative proton assignment made previously at ~3.8 ppm [49]. Despite arising from a minor component, this cross-peak had a large intensity probably due to the greater mobility of the $-CH_2OH$ group relative to the rest of the molecule. When the HMQC spectrum was examined at lower intensity levels, another cross-peak was observed at 81.8/3.82 ppm and corresponded to the C-3/H-3 nuclei of the 3-linked Gal*p* residue of α-carrageenan [49]. In the ^{1}H NMR spectrum (Fig. 6), a resonance diagnostic for α-carrageenan was also observed at 5.23 ppm (assigned to H-1 of the 4-linked AnGal*p* 2-sulfate residue) [49].

Minor components of the C. laxus *polysaccharide preparation.*—Linkage analysis of the *C. laxus* polysaccharide preparation indicated approximately equimolar proportions of 3-linked Gal*p* and 3,4-linked Gal*p* residues (Table 3). The 3-linked Gal*p* residues were shown to belong to the minor component, α-carrageenan, since essentially all of the diagnostic signals for α-carrageenan were observed throughout the NMR studies of the *C. laxus* polysaccharide. The source of the 3,4-linked Gal*p*, however, was unclear. Many red algal polysaccharides, such as κ- and ι-carrageenan, contain 3-linked Gal*p* 4-sulfate residues. If the 3,4-linked Gal*p* residues in the *C. laxus* polysaccharide were also 3-linked Gal*p* 4-sulfate residues, these would be expected to alternate mainly with 4-linked D-AnGal*p* 2-sulfate residues in the form of the disaccharide repeat of ι-carrageenan. The IR spectrum of ι-carrageenan shows a strong band of absorption at ~845 cm^{-1} [37]. However, the FTIR spectrum of the *C. laxus* polysaccharide showed no significant absorption at 845 cm^{-1} (Fig. 1a). The ^{13}C NMR spectrum of ι-carrageenan exhibits diagnostic signals, such as those at ~92.2 ppm (C-1 of AnGal*p* 2-sulfate) and ~72.2 ppm (C-4 of 3-linked Gal*p* 4-sulfate) [47,48]. Although certain minor peaks in the ^{13}C NMR spectrum (not shown) of the *C. laxus* polysaccharide could possibly arise from these carbons, they were not clearly distinguishable from the noise. The ^{1}H NMR spectrum of ι-carrageenan exhibits a diagnostic resonance at ~5.28 ppm (H-1 of AnGal*p* 2-sulfate) [49,54] but this resonance was not observed in the ^{1}H NMR spectrum of the *C. laxus* polysaccharide (Fig. 6). When the HMQC spectrum of the *C. laxus* polysaccharide was examined at very low intensity levels, cross-peaks unique to ι-carrageenan (C-1/H-1 of AnGal*p* 2-sulfate and C-4/H-4 of 3-linked Gal*p* 4-sulfate [49]) were visible but their authenticity was also questionable because they were not clearly distinguishable from the noise.

FTIR and NMR data thus appeared to suggest that the 3,4-linked Gal*p* in the linkage analysis of the *C. laxus* polysaccharide was probably derived from two or more sources, so that the quantity of each of the individual components was below the threshold of detection by NMR spectroscopy. Although some of the 3,4-linked Gal*p* residues possibly represented 3-linked Gal*p* 4-sulfate, some were possibly derived

from 4-linked Gal*p* 3-sulfate (the weak peak at 825 cm^{-1} in Fig. 1a could arise from this unusual sulfate ester). By analogy, a small proportion of the 3,4,6-linked Gal*p* residues was possibly derived from disulfated, 4-linked Gal*p* residues, and both the mono- and the di-sulfated, 4-linked Gal*p* residues possibly occurred in all the *Callophycus* preparations (Fig. 1a and Table 3). However, some of the 3,4-linked Gal*p* in the linkage analysis of the *C. laxus* polysaccharide probably represented branch points in the galactan chain. We speculate that such branch points are 4-linked Gal*p* bearing terminal Xyl *p* residues at O-3, a substitution pattern found in some red algal polysaccharides [59]. The *C. laxus* polysaccharide contained a significant amount of Xyl (6 mol%, Table 2) which was detected (< 0.8 mol%) only as terminal residues in the linkage analysis (Table 3). This reduced recovery of Xyl *p* residues during the linkage analysis could arise from oxidative degradation [60] and/or selective losses during processing [61].

Quantification of AnGal.—The NMR spectroscopic data showed that the *Callophycus* polysaccharides were composed of repeating disaccharides of one major type, 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate, and at least one minor type, carrabiose 2-sulfate. Both types of repeating unit have AnGal*p* 2-sulfate as the 4-linked residue. However, compositional and linkage analyses gave ratios of ~ 2:1 for Gal:AnGal (Tables 2 and 3) rather than the expected ratio of ~ 1:1. This discrepancy is rationalised as incomplete recovery of the AnGal sugar by the reductive hydrolysis method.

The reductive hydrolysis procedure involves an initial hydrolysis at 80 °C in the presence of 4-methylmorpholineborane (MMB); harsh hydrolysis at 120 °C, during which the MMB is rapidly destroyed; and finally concentration in the presence of fresh MMB. Recovery of AnGal by this method is essentially quantitative for agarose and κ-carrageenan but is usually incomplete for polysaccharides that contain sulfate ester substitution at O-2 of AnGal*p*, such as ι- or α-carrageenan [25,49]. This situation probably also applied to the carrageenans from *Callophycus* spp. Modification of the reductive hydrolysis procedure (by use of increased amounts of in situ reductant) increased the yield of AnGal residues from α-, ι-, and alkali-modified λ-carrageenan to ~ 45 mol% [25,49,62]. However, when the modified procedure was applied to *Callophycus* polysaccharides, the quantity of AnGal recovered was still considerably less than that from α-, ι-, and alkali-modified λ-carrageenan. It is well known that glycosidic linkages involving acidic sugars (uronic acids) are considerably more resistant to acid hydrolysis than those of neutral sugars [63]. We speculate that the high pyruvate content of the polysaccharide further impeded the recovery of AnGal. Like sulfate ester at O-2 of AnGal *p*, the pyruvate acetal possibly shields the α-(1 → 3) glycosidic linkage, thus further increasing its resistance to hydrolysis. However, it is not certain at which point during the reductive hydrolysis procedure the pyruvate acetal substituents are cleaved from the polysaccharide. If the pyruvate acetals resist hydrolysis and stabilise adjacent 3,6-anhydrogalactosidic linkages during the initial hydrolysis, this would result in an apparent lower AnGal content because the AnGal residues subsequently released during the harsh hydrolysis would be destroyed. Alternatively, if the pyruvate acetal substituents are cleaved during the initial hydrolysis, the released pyruvic acid would compete with the AnGal for the reductant. We have attempted optimising reductive hydrolysis for the recovery of AnGal from the *Callophycus* polysaccharides. Preliminary experiments suggested increasing the concentration of MMB in the hydrolysis medium and conducting the preliminary hydrolysis at higher temperatures (95 °C) increased the recovery of AnGal to ~ 40 mol% of total constituent sugars.

Source of the Glc.—Small quantities of Glc were present in the constituent sugar analyses (Table 2). In the constituent sugar analyses of red algal polysaccharides, Glc is generally regarded as being derived from floridean starch [9]. The Glc was therefore possibly derived from floridean starch that was protected from enzymic digestion, either physically or by the presence of compounds in the *Callophycus* samples which inhibit the amyloglucosidase. However, because Glc was not detected in the linkage or NMR analyses, the source of the Glc was not confirmed.

Structure–property implications.—Pyruvate acetal was first identified as a polysaccharide substituent in commercial agar [64] and has since been identified as a substituent of various red algal and bacterial polysaccharides [10,42,58,65–67]. In compositional analyses of red algal galactans, the pyruvate content rarely exceeds 2% w/w [25,65,66,68], although pyruvate levels of ~ 6.5% w/w, quantified by ^1H NMR, were reported for a polysaccharide rich in π-carrageenan from a member of the Gigartinaceae [42] and ~ 5.5% w/w, quantified colorimetrically as the 2,4-dinitrophenylhydrazone derivative, for the agar from *Gelidium allanii* [68]. The pyruvate contents of the *Callophycus* carrageenans are therefore the highest thus far reported for red algal galactans.

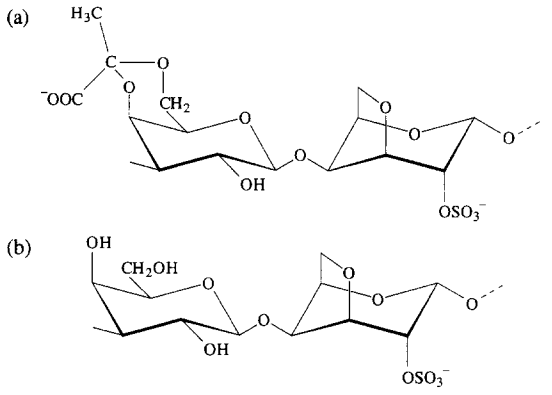

Fig. 7. Summary of proposed structures for the polysaccharides from *Callophycus* species. The dominant repeating disaccharide (a) = 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate. The minor repeating disaccharide (b) = carrabiose 2-sulfate of α-carrageenan.

In the light of the observation that increasing levels of pyruvate acetal substitution dramatically modify the physicochemical properties of polysaccharides such as xanthan gum [69,70], the unusually high level of pyruvate acetal in the *Callophycus* polysaccharides may have important implications for their solution properties.

4. Conclusion

Despite the widespread geographic distribution of the six species of *Callophycus* investigated, chemical and spectroscopic data indicated that the structure of the polysaccharides (summarised in Fig. 7) elaborated by those species are essentially the same. We propose that the major repeating disaccharide is 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate. Minor amounts of the α-carrageenan repeating unit (carrabiose 2-sulfate) and some other structural variations are also present.

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