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Carrageenans from the tetrasporic stages of *Gigartina clavifera* and *Gigartina alveata* (Gigartinaceae, Rhodophyta)

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Abstract

Modern chemical and spectroscopic techniques have been used to characterise the polysaccharides extracted from the tetrasporic life stages of *Gigartina clavifera* and *Gigartina alveata*. Both are predominantly ξ-carrageenans. About one in six of the 3-linked residues in both polysaccharides also have a pyruvate acetal group at the 4- and 6-positions. In addition, a similar proportion of the 4-linked units of each polysaccharide are devoid of sulfate groups, whilst more have sulfate esters on both O-2 and O-6. Some of the 3-linked units contain a sulfate at the 6-position in addition to that at O-2. The polysaccharide from tetrasporophytic *G. alveata* also contains a small but significant number of 3,6-anhydrogalactosyl units, most of which are naturally sulfated at the 2-position.

Keywords: Carrageenan; Galactan, sulfated; *Gigartina clavifera*; *Gigartina alveata*

1. Introduction

Red seaweeds in the family Gigartinaceae are a rich source of the sulfated galactans known as carrageenans. These polysaccharides have applications as gelling, thickening and suspending agents in food processing. A number of *Gigartina* species are endemic to New Zealand, and both *G. clavifera* and *G. alveata* are relatively abundant. *G. clavifera* grows in the lower intertidal range of various habitats. Its range extends from the southern North Island to Stewart Island and the Chatham Islands. *G. alveata* grows intertidally on open coast of the northern North Island of New Zealand and exists

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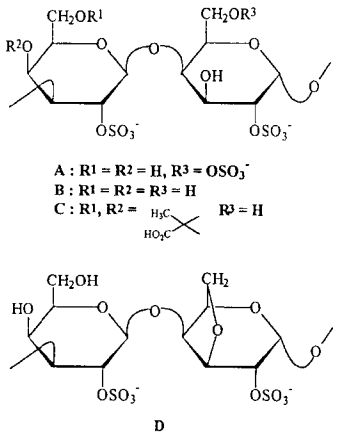

Fig. 1. The structure of λ-type carrageenans. A = λ; B = ξ; C = π; D = θ.

predominantly in the sexual life-stages. In order to describe carrageenan structures, a system of nomenclature has been developed in which idealised disaccharide repeating units are designated by Greek letters. Native carrageenans are often hybrids of more than one of these repeating units. Variations in carrageenan structure occur, not only between different Gigartinacean algae, but also between the life stages of the same algae. Gametophytes contain κ-type carrageenans, while tetrasporophytes contain λ-type carrageenans [1].

The idealised λ-carrageenan structure is that shown in Fig. 1A, in which a 3-linked β-D-galactopyranosyl 2-sulfate residue alternates with a 4-linked α-D-galactopyranosyl 2,6-disulfate residue. The λ-type carrageenan from *Chondrus crispus* tetrasporophytes, for example, differs from this idealised structure in that about 30% of its 3-linked β-D-galactopyranosyl residues do not have the sulfate at the 2-position [2]. In contrast, the tetrasporophytes of certain previously examined *Gigartina* species, such as *G. atropurpurea*, *G. canaliculata* and *G. chamissoi*, also contain ξ-(xi-)carrageenan, in which the 6-sulfate ester of the idealised λ-carrageenan is absent (Fig. 1B), in addition to λ-carrageenan [3]. A further λ-type carrageenan, known as π-carrageenan, has also been characterised [4]. It has the same sulfation pattern as ξ-carrageenan, but the 3-linked units have pyruvate acetal substituents on the 4- and 6-positions (Fig. 1C).

Recent advances in chemical methods for the analysis of sulfated galactans have facilitated the analysis of the λ-carrageenan from *Gigartina decipiens*, which was found to be close to the idealised structure shown in Fig. 1A [5]. Accordingly, we have employed these methods to characterise the polysaccharides from *G. clavifera* and *G. alveata* tetrasporophytes.

2. Experimental

Materials.—Specimens of the material studied have been deposited in the Herbarium of the Museum of New Zealand. Tetrasporophytic specimens of *G. clavifera* were

collected at Island Bay, Wellington, New Zealand, in October, 1992 (WELT A20795), and *G. alveata* at Doubtless Bay, Northland, New Zealand, in November, 1992 (WELT A20794), and air-dried.

Isolation and analyses of polysaccharides.—Samples were prepared and analysed according to the methods of Falshaw and Furneaux [5]. Briefly, the polysaccharides were extracted using 0.05 M $NaHCO_3$ (60 mL/g weed) at 90°C for 4 h. The cooled extract was treated with amyloglucosidase to digest any floridean starch present before reheating. The extract was then filtered, dialysed and lyophilised.

Infrared spectroscopy was performed on polysaccharide films using a Perkin–Elmer 580 spectrophotometer. ^{13}C NMR spectra were recorded on 3% w/v solutions in 50:50 D_2O–H_2O at 90°C on a Bruker AC 300 spectrometer (75 MHz, 0.885 s acquisition time, 0.5 s delay time and 80° pulse width). Chemical shifts are quoted relative to internal Me_2SO as standard at 39.4 ppm.

Constituent sugar and glycosyl linkage analyses were performed using reductive hydrolysis to prepare (partially methylated) alditol acetate derivatives according to the methods of Falshaw and Furneaux [5]. Samples undergoing glycosyl linkage analysis were methylated according to the method of Furneaux and Stevenson [6], purified by dialysis and recovered by lyophilisation after the first methylation. If required, a second methylation was performed, and the sample was recovered in the same way.

3. Results and discussion

Analysis of native polysaccharides.—The amyloglucosidase-treated, freeze-dried extracts of *G. clavifera* tetrasporophyte (GclT) and *G. alveata* tetrasporophyte (GaT) were white fluffy solids obtained in 52% and 62% yields, respectively, from air-dried seaweed. The infrared spectra of both samples showed an intense band at 1250 cm^{-1} characteristic of sulfate esters generally and a broad band at 820–840 cm^{-1} due to equatorial 2- and 6-sulfate ester groups that are characteristic of λ-type carrageenans [7] (Fig. 2). The spectrum of GaT also showed a weak absorbance at 935 cm^{-1}. This is characteristic of 3,6-anhydrogalactosyl units that are not normally associated with λ-type carrageenans. However, such absorbances have been observed in tetrasporophytic samples from certain Mexican *Gigartina* species [8].

Constituent sugar analyses of GclT and GaT are shown in Table 1. GclT consists of galactose only, as expected for a λ-type carrageenan. GaT contains a small but significant amount of 3,6-anhydrogalactose, consistent with the absorbance at 935 cm^{-1} observed in the infrared spectrum. A portion of each polysaccharide was converted to the triethylammonium salt form, permethylated (KH–Me_2SO then MeI) and subjected to glycosyl linkage analysis [5,6]. Both polysaccharides yielded a significant amount of 2,3,4,6-Gal (i.e., 1,2,3,4,5,6-hexa-*O*-acetyl-galactitol) after a single methylation. This species contains no methyl groups and can be an indication of incomplete methylation of the polymer. Both polysaccharides were subjected to a second methylation. The analysis of GaT was unchanged, but the amount of 2,3,4,6-Gal produced from GclT was reduced. The results shown in Table 2 are, thus, for singly methylated GaT and for doubly methylated GclT.

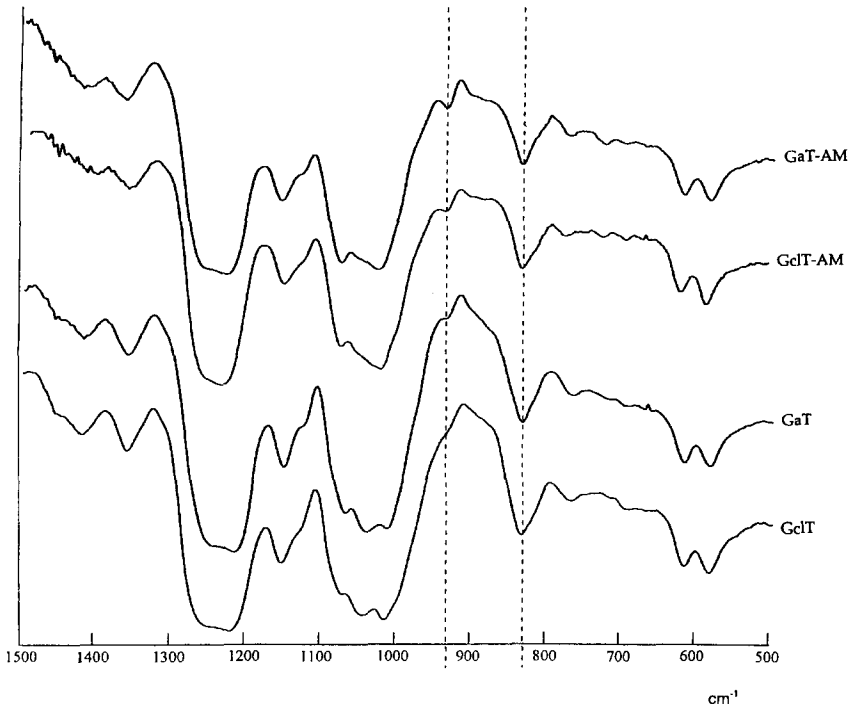

Fig. 2. Infrared spectra of native and alkali-modified (AM) polysaccharide samples from tetrasporophytic *Gigartina clavifera* (GclT) and *Gigartina alveata* (GaT). Dotted lines indicate 830 cm^{-1} and 935 cm^{-1}.

The glycosyl linkage analyses also revealed a number of other residues. In both polysaccharides the predominant units were 2,4-Gal (i.e., 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-galactitol) corresponding to 2-sulfated, 4-linked-galactopyranosyl residues and 2,3-Gal (i.e., 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl-galactitol) corresponding to 2-sulfated, 3-linked-galactopyranosyl residues. These residues represent ξ-carrageenan, Fig. 1B. GclT contained less 2,3-Gal than GaT but more 2,3,6-Gal (corresponding to 2,6-disulfated 3-linked-galactopyranosyl residues). While the latter residue has been observed previously in carrageenans [5,6,9], its occurrence has not been widely associ-

Table 1
Constituent sugar analysis of the polysaccharides from tetrasporophytic *Gigartina clavifera*, and *Gigartina alveata*, and various derivatives (normalised mol%)

Constituent sugar [a]	GclT	GaT	GclT-AM	GaT-AM
AnGal	0	4	12	16
Gal	100	95	88	83
Xyl	0	1	0	1

[a] AnGal was determined as 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol, and Gal as hexa-*O*-acetylgalactitol, etc.

Table 2
Glycosyl linkage analysis of the polysaccharides from tetrasporophytic *Gigartina clavifera, and Gigartina alveata*, and some derivatives thereof (normalised mol%)

Constituent sugar and deduced substitution [a]	GclT [c]	GaT [d]	GclT-AM [c]	GaT-AM [d]	GclT-DS [c]	GaT-DS [d]
3-linked residues						
3-Gal	0	0	0	0	40	41
2,3-Gal	27	40	26	39	0	0
2,3,6-Gal [b]	12	3	12	4	0	0
3,4,6-Gal	0	0	0	0	8	6
2,3,4,6-Gal	8	7	7	7	0	0
4-linked residues						
4-AnGal	0	1	4	2	0	6
2,4-AnGal	0	5	10	13	0	0
4-Gal	7	8	6	8	49	41
2,4-Gal	23	22	23	22	2	3
4,6-Gal	3	0	0	0	0	0
2,4,6-Gal [b]	15	12	7	3	0	0
Terminal/ambiguous residues						
2,3,4-Gal	2	2	3	2	0	0
3,4-Gal	0	0	0	0	1	3
2,6-Gal	1	0	0	0	0	0
2-Gal	2	0	1	0	0	0
2-AnGal	0	0	1	0	0	0

[a] 2,4-Gal means a 2,4-disubstituted and/or linked galactopyranosyl residue, analysed as 1,2,4,5-tetra-*O*-acetyl-3,6-di-O-methylgalactitol etc.
[b] Enantiomeric partially methylated alditol acetates differentiated and determined by deuterium labelling [5].
[c] Methylation × 2.
[d] Methylation × 1.

ated with λ-type carrageenans. In the classical λ-carrageenan from the tetrasporophytic polysaccharide from *Chondrus crispus*, 30% of the 3-linked galactosyl units are not 2-sulfated [2]. None of the partially methylated alditol acetate corresponding to 3-Gal (indicative of 3-linked galactopyranosyl residues) was observed in the methylation analyses of either GclT or GaT, however, indicating that the polysaccharides are fully 2-sulfated on their 3-linked galactosyl residues. As discussed previously, the analyses of both samples after two methylation steps also revealed the alditol acetate corresponding to 2,3,4,6-Gal. Whilst the presence of this species is often an indication of incomplete methylation (see above), it can also correspond to 2-sulfated, 3-linked galactosyl units with a pyruvate acetal substituted on the 4- and 6-positions as in π-carrageenan, Fig. 1C as appears to be the case for GclT and GaT. 2,4-AnGal (i.e., 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol, 5%) and 4-AnGal (i.e., 1,4,5-tri-*O*-acetyl-2-*O*-methyl-3,6-anhydrogalactitol, 1%) were observed for GaT, indicating that most of the 3,6-anhydrogalactosyl units present in the polysaccharide are 2-sulfated. 3,6-Anhydrogalactosyl residues have seldom been identified in native λ-type carrageenans. As the extraction conditions used here were slightly basic, it is conceivable that some anhydrogalactose formation had

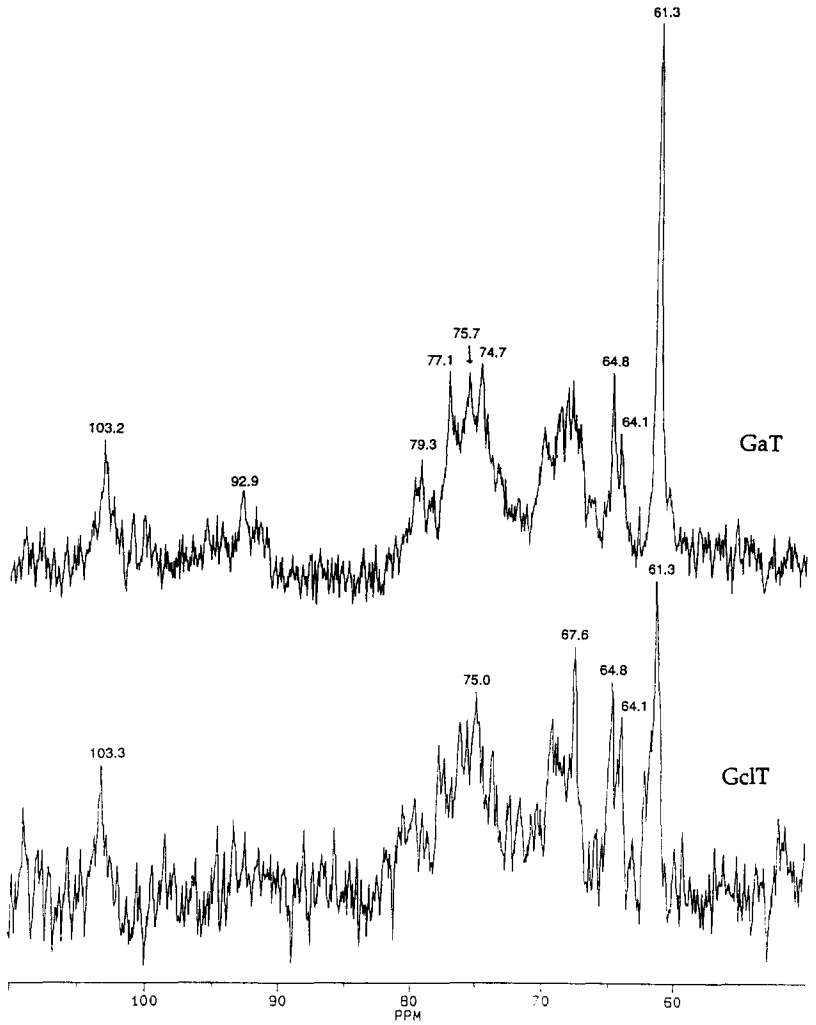

Fig. 3. ^{13}C NMR spectra of λ-type carrageenans from tetrasporophytic *Gigartina clavifera* (GclT, 5383 scans) and tetrasporophytic *Gigartina alveata* (GaT, 7607 scans).

occurred at this stage, although similar materials, such as, GclT do not contain any. However, AnGal was identified in the constituent sugar analysis of native *G. alveata* seaweed. Small amounts of 2-Gal and 2,6-Gal were observed in the analysis of GclT. These could be from terminal 2-sulfated and 2,6-disulfated galactopyranosyl residues, respectively.

The high viscosity of aqueous λ-carrageenan solutions, even when hot, makes it difficult to obtain well-resolved ^{13}C NMR spectra. The ^{13}C NMR spectra of both native GclT and GaT were complex and poorly resolved (Fig. 3). In both spectra a characteristic signal was observed at 64.1 ppm, close to that corresponding to the C-4 of 3-linked

units in λ-carrageenan (64.2 ppm) [5], but in each case another more intense signal at 64.8 ppm was visible. We assign this to C-4 of 3-linked units in ξ-carrageenan. Both spectra show a signal just above 103 ppm. The C-1 of 2-sulfated 3-linked units in λ-carrageenan occurs at 103.4 ppm [5] and suggests the presence of this species here. However, structural differences on the adjacent ring such as the absence of a 6-sulfate (i.e., λ- vs ξ-) may not affect this chemical shift. For example, structural differences on the 4-linked units between κ- and ι-carrageenans do not cause a difference in shift for C-1 of the 3-linked units. Thus a signal at about 103 ppm may also be indicative of ξ-carrageenan. The spectrum of GclT contains an unassigned signal at 109 ppm, but it was too poorly resolved to observe a signal in the 90–96 ppm region corresponding to C-1 of 4-linked units. For GaT, a signal was observed at 92.9 ppm, 1.2 ppm higher than that for λ-carrageenan. We assign this to C-1 of 4-linked galactosyl 2-sulfate units in ξ-carrageenan. The signal at 61.3 ppm is unusually intense in the spectrum of GaT. The presence of pyruvate acetal groups was confirmed in GaT by a signal in the ^{13}C NMR spectrum at 25.4 ppm (not shown) corresponding to the methyl carbon of the pyruvate group. Such a signal was not discernible in the noisy spectrum of GclT.

Alkali-modified polysaccharide.—Treatment of polysaccharides containing 4-linked galactosyl 6-sulfate residues with hot alkali results in the formation of 3,6-anhydrogalactosyl residues through intramolecular displacement of the 6-sulfate group [10]. Alkali-modification of idealised λ-carrageenan would yield a polysaccharide composed of alternating 3-linked β-D-galactosyl 2-sulfate and 4-linked 3,6-anhydro-α-D-galactosyl 2-sulfate residues, known as θ-carrageenan (Fig. 1D). The native polysaccharides from tetrasporophytic *G. clavifera* and *G. alveata* were treated with alkali using the method of Craigie and Leigh [7] to give GclT-AM and GaT-AM, respectively. Infrared analysis of both samples showed a peak at 935 cm^{-1} that is characteristic of 3,6-anhydrogalactosyl residues. In the case of GaT-AM, this absorbance was more intense than in the native sample. For GclT-AM, this was a new peak and was less intense than for GaT-AM. A strong peak remained at 820–830 cm^{-1} in both alkali-modified samples (Fig. 2). This is the region associated with equatorial 2-sulfate. Alkali modification of λ-carrageenan changes the conformation of 4-linked units so that the 2-sulfate is axial but the 2-sulfate on the 3-linked unit is still equatorial. However, previously published spectra of alkali-modified λ-carrageenans [5,7,11] show peaks of only weak intensity in this region suggesting that the 2-sulfate on 3-linked galactosyl units does not produce an intense signal. Therefore, the peak observed in GclT-AM and GaT-AM would appear not to be associated with an equatorial 2-sulfate from 3-linked residues. However, as most of the 4-linked units in GclT and GaT lack 6-sulfate, alkali-treatment has little effect, and most of the 4-linked units in the products still contain equatorial 2-sulfate.

Constituent sugar analysis of GclT-AM now revealed both galactosyl (Gal) and 3,6-anhydrogalactosyl (AnGal) residues, and for GaT the amount of AnGal had increased (Table 1). In both cases, the amount of AnGal present was smaller than would have been expected if all of the 4-linked 2,6-disulfated residues in the corresponding native sample had been converted. Methylation analysis showed that 2,4-AnGal had indeed been formed, but also that 2,4,6-Gal residues remained (Table 2). For GclT, the small amounts of 4,6-Gal and 2,6-Gal present in the native sample had been converted to the corresponding 4-AnGal and 2-AnGal. Alkali modification of both samples had

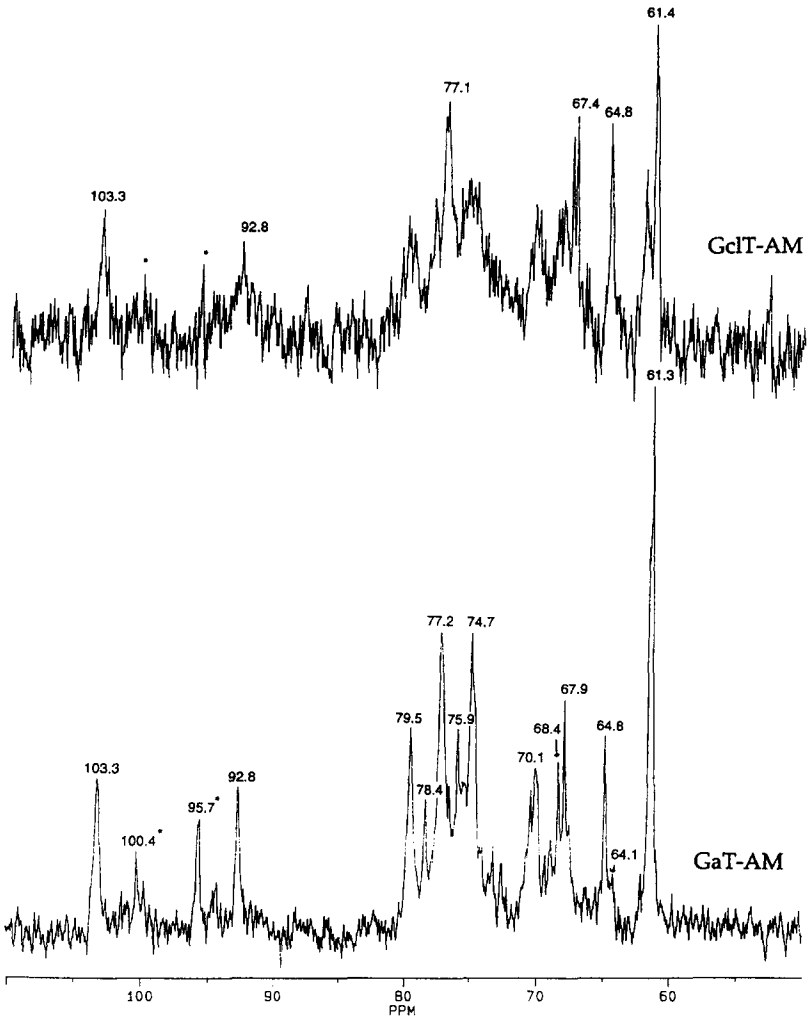

Fig. 4. ^{13}C NMR spectra of alkali-modified λ-type carrageenans from tetrasporophytic *Gigartina alveata* (GaT-AM, 6301 scans) and from tetrasporophytic *Gigartina clavifera* (GclT-AM, 5633 scans).

been incomplete under these "standard" conditions. This has been previously observed with *G. decipiens* [5]. The ^{13}C NMR spectrum of GaT-AM was reasonably well resolved (Fig. 4). The intensity of the signal at 64.1 ppm was much lower than that for the native material. Signals at 100.4 and 95.7 ppm, corresponding to the anomeric carbons of alkali-modified λ-carrageenan (i.e., θ-carrageenan) are clearly visible and are marked (*). Other signals are present with shifts attributable to θ-carrageenan, but the poor resolution of the spectrum from the native polymer in the 65–85 ppm range makes it impossible to distingiush between original and new signals. However, certain signals visible in the native sample were obviously still present, particularly those at 64.8, 92.8

and 103.3 ppm. We have already assigned the first two signals, and we assign the third to C-1 of 3-linked galactosyl units in ξ-carrageenan. Although this is coincident with the corresponding signal for λ-carrageenan (see above), it is too intense here to be accounted for by unreacted λ-carrageenan. The ^{13}C NMR spectrum of GclT-AM is less

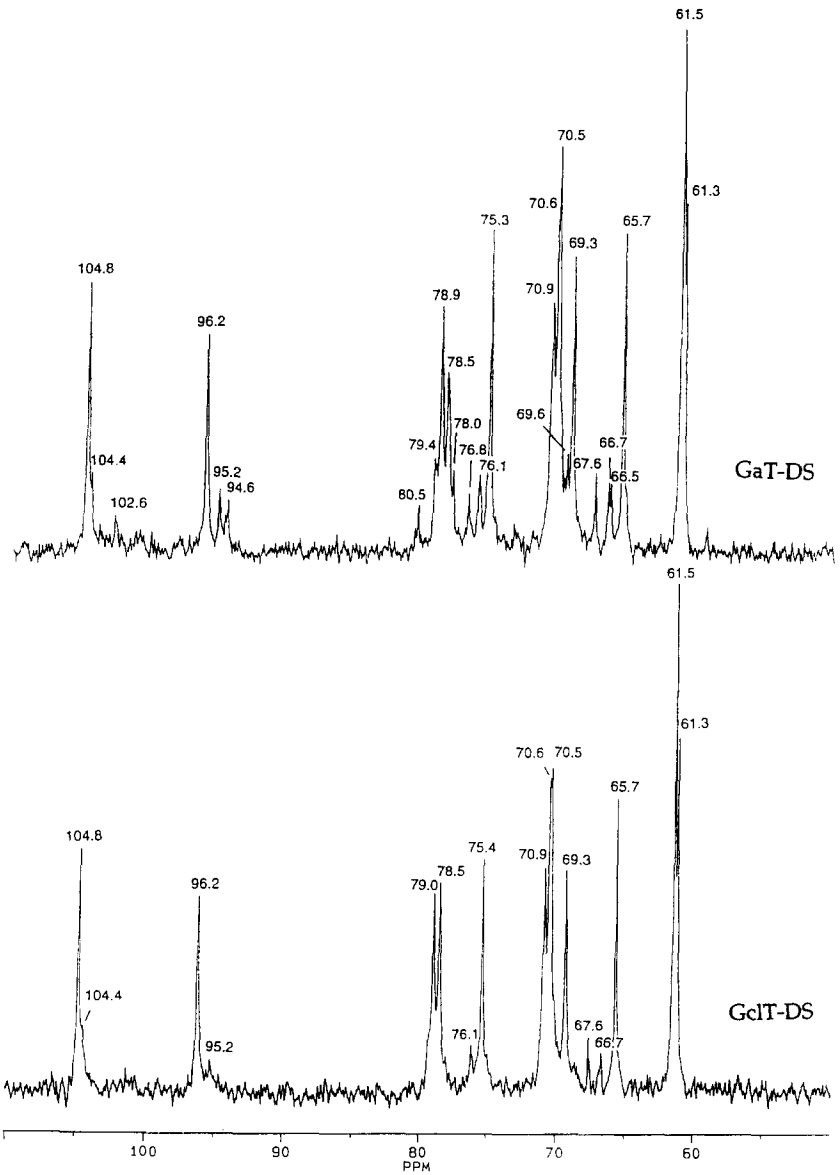

Fig. 5. ^{13}C NMR spectra of desulfated λ-type carrageenans from tetrasporophytic *Gigartina clavifera* (GclT-DS, 1355 scans) and tetrasporophytic *Gigartina alveata* (GaT-DS, 2369 scans).

Table 3
Assignment of chemical shifts in the ^{13}C NMR spectra of desulfated polysaccharide samples from tetrasporophytic *Gigartina clavifera* and *Gigartina alveata*

Polysaccharide structure	(3-linked residue)						(4-linked residue)					
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6
Desulfated λ-carrageenan [a,b]	104.8	70.6	78.9	65.7	75.3	61.3	96.2	69.3	70.9	78.5	70.5	61.5
Desulfated π-carrageenan [a,b]	104.4			67.6	76.1	66.7	95.2					
β-carrageenan [b]	102.6	69.6	80.5	66.5			94.6		79.4	78.0	76.8	69.6

[a] GclT-DS. [b] GaT-DS

well resolved (Fig. 4), but signals present at 64.8, 92.8 and 103.3 ppm indicate a predominantly ξ-carrageenan structure, although signals in the region of 100.4 and 95.7 ppm, indicating θ-carrageenan, are just discernible. A signal at 25 ppm (not shown) is visible in GclT-AM, thus providing spectroscopic evidence of pyruvate acetal groups in GclT.

Desulfated polysaccharides.—The sulfate groups were removed solvolytically from the native polysaccharides by heating their pyridinium salt forms in a Me_2SO–methanol–pyridine mixture to give GclT-DS and GaT-DS. Desulfation of idealised λ- or ξ-carrageenan would yield a polysaccharide composed of alternating 3-linked β-D-galactopyranosyl and 4-linked α-D-galactopyranosyl residues. The ^{13}C NMR spectrum of GclT-DS was composed predominantly of twelve, well-resolved peaks (Fig. 5). The chemical shifts of these peaks (Table 3) correspond to those assigned by Usov et al. in 1980 [12] for a methanolic HCl-desulfated commercial λ-carrageenan, and also similar chemical shifts have been seen for solvolytically desulfated *G. decipiens* [5]. A signal at 25.5 ppm (not shown) was clearly visible in the ^{13}C NMR spectrum of GclT-DS (Fig. 5), and minor signals are visible at 104.4, 76.1, 67.6 and 66.7 ppm, which we assign to C-1, C-5, C-4 and C-6 of pyruvated 3-linked galactosyl units, respectively, and 95.2 ppm for the C-1 of the adjoining 4-linked unit (Table 3). GLC–MS analysis of GclT-DS after a single methylation revealed 2,3,4,6-Gal. This could be due to incomplete desulfation or undermethylation. The sample was, therefore, subjected to a second methylation, after which this species was absent. The results indicated the presence of equimolar amounts of 3-linked and 4-linked residues (Table 2), consistent with a structure composed of repeating disaccharide units. The existence of pyruvated units in GclT was confirmed by the presence of 3,4,6-Gal in GclT-DS. The amount observed corresponded to the amount of 2,3,4,6-Gal obtained in the analysis of the doubly methylated native and alkali-modified samples. This indicated that pyruvate acetals occur on 2-sulfated, 3-linked galactosyl units in the native polymer.

The ^{13}C NMR of GaT-DS (Fig. 5) also contained twelve major signals corresponding to alternating 3-linked β-D- and 4-linked α-D-galactopyranosyl residues. Smaller signals corresponding to pyruvated units were also visible as for GclT-DS (Table 3). This is to be expected as the methylation analysis of GaT-DS (Table 2) indicates a similar number of pyruvated 3-linked galactosyl units as in GclT-DS. These, too, were sulfated at the 2-position in native GaT. In the ^{13}C NMR spectrum of GaT-DS, additional minor signals were visible (Fig. 5 and Table 3) that correspond to those of β-carrageenan (3-linked

β-D-galactopyranosyl residues alternating with 4-linked 3,6-anhydro-α-D-galactopyranosyl residues) [13]. This is consistent with the presence of 4-AnGal in the methylation analysis of GaT-DS.

4. Conclusions

The use of modern analytical techniques has facilitated the identification of the polysaccharides from tetrasporophytic *G. clavifera* and *G. alveata*. Both are predominantly ξ-carrageenans. About one in six of the 3-linked residues in both polysaccharides also has a pyruvate acetal group at the 4- and 6-positions. In addition, a similar proportion of the 4-linked units of each polysaccharide are devoid of sulfate groups whilst more have sulfate esters on both O-2 and O-6 (as in λ-carrageenan). Some of the 3-linked units contain a sulfate at the 6-position, in addition to that at O-2. The polysaccharide from tetrasporophytic *G. alveata* also contains a small but significant number of 3,6-anhydrogalactosyl units, most of which are naturally sulfated at the 2-position.

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