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Structural analysis of carrageenans from the tetrasporic stages of the red algae, *Gigartina lanceata* and *Gigartina chapmanii* (Gigartinaceae, Rhodophyta)

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Abstract

The use of modern analytical techniques has facilitated the identification of the polysaccharides from tetrasporophytic *Gigartina lanceata* and *Gigartina chapmanii*. The *G. lanceata* polysaccharide corresponds, very closely, to the idealised structure of λ-carrageenan ([G2S-D2S,6S]$_n$). That from *G. chapmanii* contains predominantly ξ-carrageenan ([G2S-D2S]$_n$), but with about one in seven of the 3-linked units also has a pyruvate acetal group at the 4- and 6-positions, one in four of the 4-linked units is also sulfated at the 6-position, and a small but significant number of 4-linked 3,6-anhydrogalactosyl 2-sulfate units are present. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: Carrageenan; Sulfated galactan; *Gigartina lanceata*; *Gigartina chapmanii*

1. Introduction

Industrially important sulfated galactans, known as carrageenans, are found in numerous red seaweeds including those in the family Gigartinaceae. These polysaccharides have a linear structure of alternating 3-linked β-D-galactopyranosyl (G) and 4-linked α-D-galactopyranosyl (D) units. Various hydroxyls may be sulfated (S), pyruvated (P) or, occasionally methylated (M). An additional, common feature is the existence of the 4-linked units in the form of the 3,6-anhydride (A). Traditionally, Greek letters have been assigned to carrageenans comprised of certain idealised carrageenan disaccharide repeating units. However, native carrageenans often contain combinations of these idealised units, with variations in structure occurring not only between different Gigartinacean algae but also within the different life-stages.

Gigartinacean gametophytes contain κ-type carrageenans while tetrasporophytes contain λ-type carrageenans. A new shorthand notation system has recently been developed in which the possible structural features of each unit are defined (see above), and this will be used here in conjunction with the traditional Greek letter system [1].

The idealised structure of λ-carrageenan consists of 3-linked β-D-galactopyranosyl 2-sulfate units alternating with 4-linked α-D-galactopyranosyl

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2,6-disulfate units [$(G2S\text{-}D2S,6S)_n$] (Fig. 1A). In ξ-carrageenan, the 4-linked unit lacks the 6-sulfate ester, and it is thus [$(G2S\text{-}D2S)_n$] (Fig. 1B). π-Carrageenan has the same sulfation pattern as ξ-carrageenan, but the 3-linked units have pyruvate acetal substituents on the 4- and 6-positions, i.e., [$(GP,2S\text{-}D2S)_n$] (Fig. 1C).

The polysaccharide from the tetrasporic life-stage of the endemic New Zealand species *Gigartina decipiens* is close to the ideal structure of λ-carrageenan, but approximately 15% of the 3-linked units contain an additional 6-sulfate, i.e., (G2S,6S) units [2]. Recent investigation of the tetrasporic life-stages of two other New Zealand species, *Gigartina clavifera* and *G. alveata* showed, in each case, the polysaccharides to contain G2S and D2S units as in ξ-carrageenan, but various other minor substitution patterns were also observed [3]. The modern chemical and spectroscopic methods used previously to characterise the above polymers have now been employed to characterise the polysaccharides from the tetrasporic life-stage of two further New Zealand *Gigartina* species, *G. lanceata* and *G. chapmanii*.

Gigartina lanceata is a broad-bladed species that grows on lower intertidal rocks at exposed sites in the lower South Island and southern and eastern offshore islands of New Zealand. *G. chapmanii* is a small bushy species that grows on rocks or shell-fragments in sheltered intertidal pools and muddy sand off the North, South and Stewart Islands of New Zealand. It is of particular interest as it occurs almost exclusively in the tetrasporic form. As such it would constitute a convenient industrial source of λ-type carrageenan. Most other *Gigartina* species occur as mixed populations of gametophytic and tetrasporic life stages, and the harvested material consequently gives an extract containing both κ-type and λ-type carrageenans. Determination of polysaccharide structure is a critical first step in potential commercialisation.

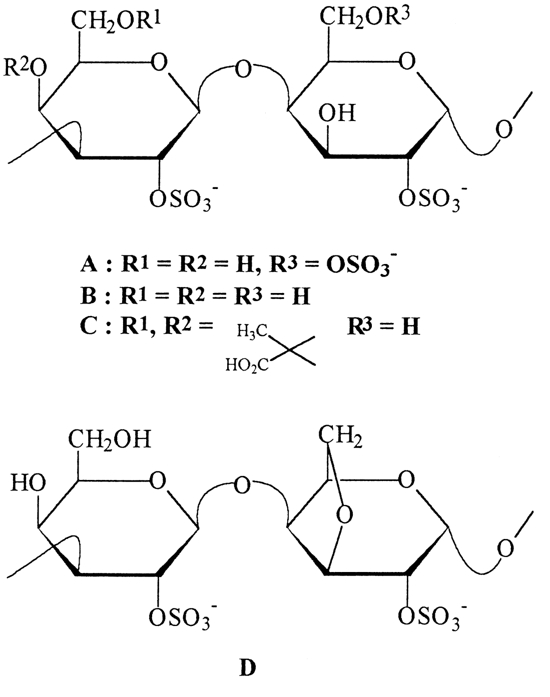

Fig. 1. Idealised disaccharide repeat units for λ-type carrageenans. A = λ; B = ξ; C = π; D = θ.

2. Experimental

Materials.—Specimens of the material studied have been deposited in the Herbarium of the Museum of New Zealand, Te Papa Tongarewa. Tetrasporophytic specimens of *G. lanceata* were collected at Aramoana, Dunedin in March 1994 (WELT A21034), and of *Gigartina chapmanii* at Kau Bay, Wellington in December 1994 (WELT A21036), and air-dried.

Isolation of polysaccharides.—Samples were prepared and analysed according to the methods described in Falshaw and Furneaux [2]. Briefly, the polysaccharides were extracted using 0.05 M $NaHCO_3$ (60 mL/g weed) at 90 °C for 4 h. The cooled extract, in each case, was treated with amyloglucosidase to digest any floridean starch present, reheated, filtered then dialysed and lyophilised.

Infrared spectroscopy.—Infrared spectroscopy was performed using a Perkin–Elmer 1605 FTIR spectrophotometer. Samples were analysed as films, prepared by drying 0.4% solutions on silanised glass dishes.

^{13}C NMR spectroscopy.—Spectra were recorded on 3% w/v solutions in 50:50 v/v 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.

Alkali modification.—Polysaccharides (100 mg) were dissolved in water (20 mL), $NaBH_4$ (20 mg) was added, and the mixtures were stirred overnight. $NaBH_4$ (60 mg) and aqueous NaOH (3 M, 10 mL) were added, and the solutions were heated at 80 °C for 7 h [4]. After being cooled, neutralised with glacial acetic acid, and dialysed against distilled water (×3), the solutions were lyophilised to give GlT-AM and GchT-AM in 82 and 80% yields, respectively.

Solvolytic desulfation.—The native polysaccharides were converted to the pyridinium salt form by dialysis against pyridinium hydrochloride ($\sim$0.1 M, adjusted to pH 6.8) three times, then twice against distilled water, and finally lyophilised. A sample of each of the resulting materials was dissolved in 89:10:1 v/v Me$_2$SO–MeOH–pyridine, 0.4 mL/mg and heated for 4 h at 100 °C [2]. After cooling, the samples were dialysed against tap water, then distilled water ($\times$3) and isolated by lyophilisation to give GlT-DS and GchT-DS in 51 and 60% yields, respectively.

Constituent sugar and glycosyl-linkage analyses.—These were performed using reductive hydrolysis (with *N*-methylmorpholine borane in aqueous trifluoroacetic acid) to prepare (partially methylated) alditol acetate derivatives according to the methods described in Stevenson and Furneaux [5], and Falshaw and Furneaux [2]. Samples undergoing glycosyl-linkage analysis were converted to the triethylammonium salt form and methylated (MeI/Me$_2$SO$^-$K$^+$) according to the method of Stevenson and Furneaux [5], purified by dialysis [H$_2$O$\times$1; Et$_3$NHCl (0.1 M, pH 7)$\times$1; H$_2$O$\times$2] and recovered by lyophilisation after the first methylation. A second methylation was performed, and the sample was recovered in the same way.

3. Results and discussion

Native polysaccharides.—The amyloglucosidase-treated, lyophilised extracts of tetrasporic *G. lanceata* (GlT) and *G. chapmanii* (GchT) were fluffy white solids obtained in 42 and 47% yields, respectively, from air-dried seaweed.

Infrared spectra of both samples (Fig. 2) showed an intense band at 1250 cm^{-1}, which is characteristic of sulfate esters in general. The spectrum of GlT also showed a broad band centred at 837 cm^{-1} with a discernible shoulder at 820 cm^{-1}. These are characteristic of equatorial 2- and 6-sulfate ester groups, respectively, as found in λ-carrageenan. The corresponding signal in the IR spectrum of GchT was much narrower with one discernible maximum at 833 cm^{-1} indicating the presence of equatorial 2-sulfate in GchT but less sulfate than in GlT as found in ξ-carrageenan, which has a (G2S-D2S) repeat unit, and π-carrageenan, which has a (GP,2S-D2S) repeat unit. The GchT IR spectrum contained a weak, but discernible, signal at 935 cm^{-1}, whereas the GlT spectrum contained only a slight shoulder at this position. This signal is characteristic of 3,6-anhydrogalactosyl units not normally associated with λ-type carrageenans but observed previously in these materials from certain New Zealand and Mexican *Gigartina* species [3,6].

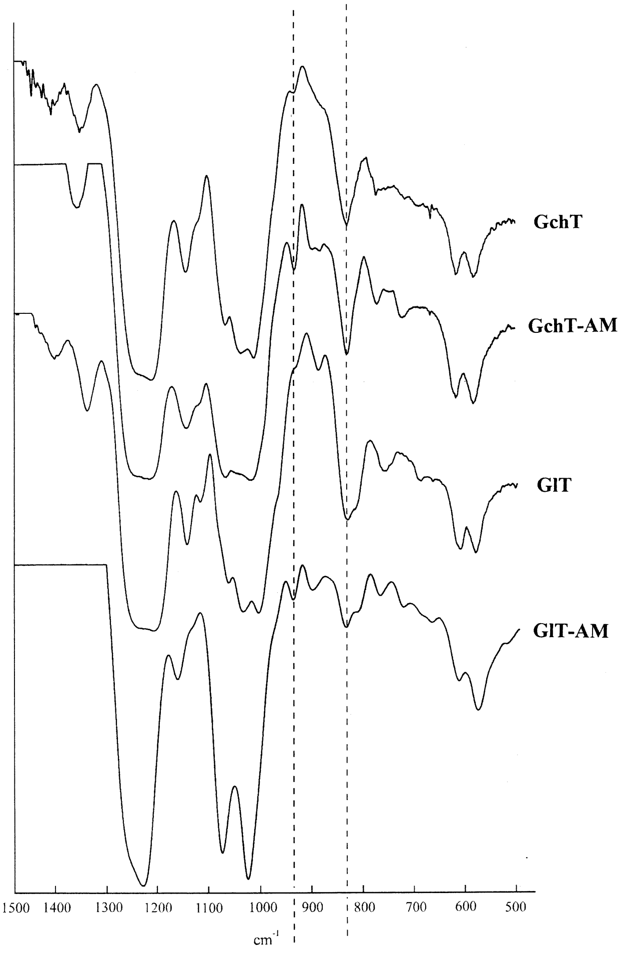

Fig. 2. Infrared spectra of native and alkali modified (AM) polysaccharide samples from tetrasporophytic *G. lanceata* (GlT) and *G. chapmanii* (GchT). Dotted lines are shown at 835 and 935 cm^{-1}.

Constituent sugar analyses of GlT and GchT are shown in Table 1. GlT consisted almost exclusively of galactose as expected for a λ-type carrageenan, while GchT contained a significant level, 5 mol%, of 3,6-anhydrogalactosyl units, determined as 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol, (AnGal), consistent with the 935 cm^{-1} signal in the IR spectrum. The same level of AnGal was observed in the constituent sugar analysis of *G. chapmanii* seaweed (Table 1), indicating that it is a component of the native polymer and not an artefact of

Table 1
Constituent sugar analysis of the polysaccharides from tetrasporophytic *G. lanceata* and *G. chapmanii*, and various derivatives thereof

(Normalised mole %)

Constituent sugar[a]	GlT	GchT	Tetrasporic *G. chapmanii* weed	GlT-AM	GchT-AM	GlT-DS	GchT-DS
AnGal	1	5	5	35	16	1	7
Gal	99	95	87	65	84	99	93
Xyl			1				
Glc			7				

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

extraction conditions. A similar level of AnGal has been found previously in tetrasporic *G. alveata* seaweed and in the λ-type carrageenan extracted from it [3].

A portion of each extract was converted into the triethylammonium salt form and subjected to methylation, dialysis then lyophilisation before being treated to a second methylation procedure. Two methylation steps were performed as a precaution to avoid incomplete methylation. Previous experience had shown that, while the carrageenans from tetrasporic *G. decipiens* and *G. alveata* were successfully methylated in one step [2,3], the carrageenan from tetrasporic *G. clavifera* had required two methylations [3]. The permethylated polysaccharides were converted to partially methylated alditol acetates by reductive hydrolysis and acetylation and analysed by GC and GC–EIMS. The results are shown in Table 2. The main species obtained for GlT were 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 units and 2,4,6-Gal (i.e., 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methyl-galactitol) corresponding to 2,6-disulfated 4-linked-galactopyranosyl units as expected for an idealised λ-carrageenan [$(\mathrm{G2S\text{-}D2S,6S})_n$]. A small

Table 2
Glycosyl-linkage analysis of the polysaccharides from tetrasporophytic *G. lanceata* and *G. chapmanii*, and some derivatives thereof

(Normalised mole %)

Constituent sugar and deduced substitution[a]	Unit type	GlT	GchT	GlT-AM	GchT-AM	GlT-DS	GchT-DS
3-linked units							
3-Gal	G	2	2	2	3	49	44
2,3-Gal	G2S	48	39	46	40	1	0
2,3,6-Gal[b]	G2S,6S	1	1	1	1	1	0
3,6-Gal	G6S	0	0	0	0	Tr	1
3,4,6-Gal	GP	0	0	0	0	0	6
2,3,4,6-Gal	GP,2S	0	6	0	5	0	0
4-linked units							
4-AnGal	DA	0	0	0	0	0	5
2,4-AnGal	DA2S	2	6	33	15	0	0
4-Gal	D	1	1	0	2	45	38
2,4-Gal	D2S	1	28	0	26	1	1
2,4,6-Gal[b]	D2S,6S	37	12	6	2	0	0
Terminal/ambiguous units							
2,3,4-Gal		1	0	0	0	0	0
3,4-Gal		2	1	2	1	1	1
2,6-Gal		5	2	1	0	0	0
2-Gal		0	2	0	3	0	0
2-AnGal		0	0	9	2	0	0
T-AnGal		0	0	0	0	0	2
T-Gal		0	0	0	0	2	2

[a] 2,4-Gal means a 2,4-disubstituted and/or linked galactopyranosyl unit, analysed as 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-galactitol, etc.

[b] Enantiomeric partially methylated alditol acetates (2,3,6-Gal and 2,4,6-Gal) differentiated by deuterium labelling and determined by GC–MS analysis [2].

amount of 2,6-Gal was observed in the analysis of GlT. This is thought to arise from terminal 2,6-disulfated galactopyranosyl units as discussed below.

For GchT, the predominant units identified by linkage analysis were 2,3-Gal and 2,4-Gal. If they occur alternately, the units these species represent comprise ξ-carrageenan, i.e., [(G2S-D2S)$_n$]. There was also a significant amount of 2,4,6-Gal, which, with 2,3-Gal, represents λ-carrageenan. The analysis of GchT also revealed the alditol acetate corresponding to 2,3,4,6-Gal. Whilst the presence of this latter species is often an indication of incomplete methylation, it can also correspond to 2-sulfated, 3-linked galactosyl units with a pyruvate acetal substituent on the 4- and 6-positions, and this appears to be the case for GchT (see below). These units alternating with 2-sulfated 4-linked-galactopyranosyl units would represent π-carrageenan [(GP,2S-D2S)$_n$] (Fig. 1C). The 3,6-anhydro-galactosyl units present in the polysaccharides are 2-sulfated (i.e., DA2S) since the species 2,4-AnGal was observed in the glycosyl-linkage analyses. Whether the GP and DA2S units occur in hybrid carrageenan structures or in separate molecules is unclear at this stage.

Only a small amount of the partially methylated alditol acetate corresponding to 3-Gal (indicative of 3-linked galactopyranosyl units) was observed in the methylation analyses of either GlT or GchT, however, indicating that these polysaccharides are almost fully 2-sulfated on their 3-linked galactosyl units. Neither GlT or GchT contained significant amounts of 2,3,6-Gal, which has been previously observed in the corresponding λ-type carrageenans from *G. decipiens* and *G. clavifera* [2,3].

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 GlT and GchT were complex and poorly resolved and are not shown. 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) [2], but for GchT another more intense signal at 64.8 ppm was visible, corresponding to C-4 of 3-linked units in ξ-carrageenan [3]. Both spectra show a signal just above 103 ppm which could correspond to the C-1 of 3-linked units in either λ- or ξ-carrageenan [3]. For GchT, the signal in the region expected for C-1 of the 4-linked units was observed at 92.9 ppm corresponding to C-1 of 4-linked galactosyl 2-sulfate units in ξ-carrageenan, whereas for GlT it was at ~91.7 ppm as expected for λ-carrageenan.

Alkali-modified polysaccharides.—The treatment of polysaccharides containing 4-linked galactosyl 6-sulfate units with hot alkali results in the formation of 3,6-anhydrogalactosyl units through intramolecular displacement of the 6-sulfate group. 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 units, known as θ-carrageenan [(G2S-DA2S)$_n$] (Fig. 1D). The native polysaccharides from tetrasporophytic *G. lanceata* and *G. chapmanii* were treated with alkali using the method of Craigie and Leigh [4] to give GlT-AM and GchT-AM, respectively. Infrared spectroscopic analysis of both samples (Fig. 2) showed a peak in the region characteristic of 3,6-anhydrogalactosyl units around 935 cm^{-1}. In the case of GchT-AM, this signal was more intense than in the native sample. For GlT-AM, the slight shoulder observed in the IR of the native polymer was now a distinct peak. For GchT-AM a strong, sharp peak remained at 833 cm^{-1} due to equatorial 2-sulfate on both the 3- and 4-linked galactosyl units. Such G2S and D2S units are unaffected by alkali treatment. For GlT-AM, the intensity of the band at 837 cm^{-1} was much lower than in the native sample, indicating the significant conversion of equatorial 2-sulfate on 2,6-disulfated 4-linked galactosyl units to axial 2-sulfate on 3,6-anhydrogalactosyl units. The extent of conversion to the 3,6-anhydride, although significant, appears incomplete for two reasons. The remaining signal at 837 cm^{-1} is too intense to come from equatorial 2-sulfate on 3-linked units, only, so some equatorial 2-sulfate must still be present on 4-linked units as in the native polymer. From the examination of samples where the conversion of 4-linked units into 2-sulfated 3,6-anhydro-α-D-galactosyl units is complete (as in θ-carrageenan) [2,4,7] it appears that the equatorial 2-sulfate on 3-linked units in these polymers gives rise to signals of only weak intensity in the region of 835 cm^{-1}. Also a broad peak, centred at 816 cm^{-1}, was visible in the IR spectrum of GlT-AM indicating that equatorial 6-sulfate (peak expected at 820 cm^{-1}) remained as well as axial 2-sulfate on 3,6-anhydrogalactosyl units (peak expected at 805 cm^{-1}) having been formed.

Constituent sugar analysis of both GlT-AM and GchT-AM showed that the amount of AnGal had increased compared with the corresponding native samples (Table 1). Recovery of AnGal from 3,6-anhydrogalactosyl units can be incomplete by the reductive hydrolysis procedure when these units are 2-sulfated, due to the stabilising effect of this 2-sulfate ester group [2]. Nevertheless, the amount of AnGal present, in both cases, was smaller than would have been expected if all of the 4-linked 2,6-disulfated units in the corresponding native sample had been converted to the corresponding 3,6-anhydride. Methylation analysis showed that for both GlT-AM and GchT-AM, 2,4-AnGal had indeed been formed, but also that some 2,4,6-Gal units had survived alkali treatment (Table 2). Alkali modification of both samples was clearly incomplete under these "standard" conditions. This is consistent with a slow rate of 3,6-anhydro-galactosyl ring formation in λ-type carrageenans [8], and has been observed previously with carrageenans from other tetrasporic New Zealand *Gigartina* species [2,3].

The identification by methylation analysis of 5 mol% of 2,6-Gal in GlT, and 9 mol% of 2-AnGal in the GlT-AM, respectively, requires some comment. In principle, the 2,6-Gal from GlT could be derived from main-chain, branching, or nonreducing terminal sugar units. The first option of main-chain 2- or 6-linked units can be ruled out because the desulfated sample GlT-DS gave no 2-Gal, 6-Gal or 2,6-Gal, and so the species attached to both the 2- and 6-positions must, therefore, be sulfate esters. The second option of galactosyl 2,6-sulfated branch units requires that (a) the alkali-modified sample GlT-AM should contain 5 mol% of [2-AnGal + 2,6-Gal] (but there is 10%!) and (b) the desulfated sample should contain 5 mol% of T-Gal (there is only 2 mol%) and also 5 mol% of derivatives from disubstituted or more highly substituted main-chain unit to which the branch is attached. There is 1 mol% of each of four such units, but three of these probably result from incomplete desulfation of the major units in the native polymer. We consider the third option, of nonreducing terminal units, to be the most plausible. The native GlT carrageenan is a viscous, high molecular weight polymer likely to have only an insignificant proportion of such units. To generate the level of 2,6-Gal and 2-AnGal seen, significant chain cleavage by breaking (1→4)-glycosyl linkages must therefore have taken place, probably under alkaline conditions during methylation analysis. We have previously invoked this phenomenon to account for smaller amounts of equivalent units generated during multiple methylation of certain carrageenan samples [5], e.g. the 2 mol% of 2-Gal from a predominantly ξ-carrageenan from *G. clavifera* [3]. Why somewhat more severe chain cleavage occurred here in the case of the *G. lanceata* samples and not noticeably for the closely related λ-carrageenan from *G. decipiens* [2] is not clear.

The ^{13}C NMR spectrum of GlT-AM (not shown) was well resolved with strong signals corresponding to those published previously for θ-carrageenan [9]. Some weaker signals were also visible for unreacted λ-carrageenan. The ^{13}C NMR spectrum of GchT-AM (not shown) was also well resolved and was very similar to that of alkali-modified tetrasporic *G. alveata* [3] with strong signals present at 64.8, 92.8 and 103.3 ppm indicating a predominantly ξ-carrageenan structure. Anomeric carbon signals at 100.3 and 95.7 ppm indicated that some θ-carrageenan had been created.

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 GlT-DS and GchT-DS. Desulfation of idealised λ- or ξ-carrageenan would yield the same polysaccharide, composed of alternating 3-linked β-D-galactopyranosyl and 4-linked α-D-galactopyranosyl units [$(G\text{-}D)_n$]. The ^{13}C NMR spectrum of GlT-DS (not shown) was composed predominantly of 12 well-resolved peaks having chemical shifts corresponding to those assigned by Usov et al. [9] for a methanolic HCl-desulfated commercial λ-carrageenan. Such has also been seen for solvolytically desulfated λ-carrageenan from *G. decipiens* [2]. Linkage analysis of GlT-DS revealed approximately equimolar amounts of 3-linked and 4-linked galactosyl units (Table 2), consistent with a structure composed of these as a repeating disaccharide unit.

The ^{13}C NMR spectrum of GchT-DS (not shown) corresponded to that of the desulfated carrageenan from tetrasporic *G. alveata* [3]. It contained 12 major signals corresponding to alternating 3-linked β-D- and 4-linked α-D-galactopyranosyl units. Smaller signals were also visible at 104.4, 95.2, 76.1, 67.6 and 66.7 ppm corresponding to 4,6-pyruvated 3-linked β-D-galactopyranosyl units. This is to be expected as the methylation analysis of GchT-DS (Table 2) indicates a number of pyruvated 3-linked galactosyl units (as 3,4,6-Gal) that were sulfated at the 2-position in native and alkali-modified GchT (seen as 2,3,4,6-Gal). In addition, 12 minor signals were visible with the same chemical shifts as in the corresponding *G. alveata* sample, which correspond to those of β-carrageenan (i.e., 3-linked β-D-galactopyranosyl units alternating with 4-linked 3,6-anhydro-α-D-galactopyranosyl units, $[(\text{G-DA})_n]$ [10]. This is consistent with the presence of 4-AnGal in the methylation analysis of GchT-DS resulting from the removal of sulfate ester from the 2-position of 3,6-anhydrogalactosyl units in the native polymer (seen as 2,4-AnGal).

4. Conclusions

The use of modern analytical techniques has facilitated the identification of the polysaccharides from tetrasporophytic *G. lanceata* and *G. chapmanii*. *G. lanceata* corresponds, almost completely, to the idealised structure of λ-carrageenan whilst *G. chapmanii* is predominantly a ξ-carrageenan. About one in seven of the 3-linked units also has a pyruvate acetal group at the 4- and 6-positions. About one in four of the 4-linked units is additionally sulfated at the 6-position, and there is also a small but significant number of 3,6-anhydrogalactosyl units that are sulfated at the 2-position.

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