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Structural analysis of carrageenans from the red alga, *Callophyllis hombroniana* Mont. Kütz (Kallymeniaceae, Rhodophyta)

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Abstract—The use of a range of modern analytical techniques has facilitated the structural characterisation of the polysaccharide from the New Zealand endemic red alga, *Callophyllis hombroniana*. The native polysaccharide contains a number of structural units with the largest proportion consisting of 3-linked β-D-galactopyranosyl 2-sulfate units, alternating with 4-linked 3,6-anhydro-α-D-galactopyranosyl 2-sulfate units, that is, θ-carrageenan (36 mol %). *C. hombroniana* is the first red seaweed reported to naturally contain such a large proportion of θ-carrageenan.

Keywords: Red seaweed; Carrageenan; Sulfated galactan; *Callophyllis hombroniana*

1. Introduction

The predominant types of polysaccharide from red algae are galactans with a backbone of alternating 3-linked β-D-galactopyranosyl (G) and 4-linked α-galactopyranosyl units. The polysaccharide is an agar-type polysaccharide if the 4-linked α-galactopyranosyl units are in the L-configuration and a carrageenan-type polysaccharide if the 4-linked α-galactopyranosyl units are in the D-configuration. Some red algae contain polysaccharides with both 4-linked α-D-galactopyranosyl (D) and α-L-galactopyranosyl (L) units, sometimes known as 'carragars'. Various hydroxyls may be substituted with sulfate ester (S), pyruvate acetal (P) or methyl ether (M) groups. An additional common feature is the existence of 4-linked 3,6-anhydro-α-galactopyranosyl (A) units.

Very little research has been conducted on the structures and properties of polysaccharides from the *Callophyllis* genus of red seaweeds (family Kallymeniaceae). Preliminary work in the early 1980s on *Callophyllis rhynchocarpa* from the Sea of Japan resulted in a gelling and a non-gelling polysaccharide fraction, both predominantly composed of galactosyl units.[1] More recently, direct constituent sugar analysis of algal biomass from *C. rhynchocarpa* confirmed that the predominant sugar present was galactose.[2] In addition, only carrabiitol peracetate was observed from direct partial reductive hydrolysis of *C. rhynchocarpa* thalli. This was in contrast to the results from *Callophyllis cristata, Callophyllis* sp. 1 and *Callophyllis* sp. 2 where both carrabiitol peracetate and agarobiitol peracetate were observed.[2] Direct constituent sugar analysis of algal biomass from these three *Callophyllis* species also confirmed that the predominant sugar present was galactose.[2] A related South American species, *Callophyllis variegata*, is exploited commercially in Chile as an edible seaweed.[3] Algal samples of *C. variegata* and *C. rangiferina* (both of unknown origin) reportedly contained a λ/θ/α-carrageenan structure or carragar, respectively, by infrared spectroscopic analysis, but no data was given.[4] Life history information was not provided for any of these samples, so it is not known whether different life phases would produce significantly different polysaccharides. Preliminary data on a polysaccharide extract from cystocarpic *C. variegata* has recently been published, which suggested that the total extract contained a carrageenan, but that the predominant fraction (soluble in 2 M KCl) contained approximately 15% L-galactose.[5]

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Eight species in the *Callophyllis* genus occur in New Zealand of which *Callophyllis hombroniana* is a distinctive, endemic species with a cold-water distribution. *C. hombroniana* plants are robust with narrow and flattened fronds often over 30 cm long. It can grow not only in low intertidal pools but also subtidally, frequently on the stalks of sea tulips. Initial results from structural analysis of the native polysaccharide from *C. hombroniana* indicated that it contained predominantly θ-carrageenan (Fig. 1).[6] This was the first report of a red seaweed naturally containing a significant amount of θ-carrageenan. This has recently been supported by ^{13}C NMR spectroscopy of the native polysaccharide from *C. hombroniana*.[7] θ-Carrageenan can be prepared chemically from λ-carrageenan (Fig. 1) using hot aqueous alkali to affect intramolecular ring closure with elimination of sulfate. λ-Carrageenan is commonly found in red seaweeds, sometimes as the dominant structural component, particularly in tetrasporic plants of member of the family Gigartinaceae.[8,9] Certain 4-linked galactopyranosyl 6-sulfate units are known to be the biological precursors of the related 3,6-anhydrogalactopyranosyl units. For example, L6S units are the biosynthetic precursors of LA units in agar. By analogy, G2S,6S units in λ-carrageenan should be the biosynthetic precursors of DA2S units in θ-carrageenan (Fig. 1). Wong and Craigie[10] isolated a sulfohydrolase from the λ-carrageenan-containing tetrasporic plants of the red seaweed *Chondrus crispus* (family Gigartinaceae). This enzyme was capable of converting 4-linked D-galactopyranosyl-6-sulfate (D6S) units (in μ-carrageenan) to 4-linked 3,6-anhydro-D-galactopyranosyl (DA) units (in κ-carrageenan), Figure 1. However, the same enzyme was not only ineffective against, but was actively inhibited by, the λ-carrageenan obtained from the same tetrasporic *C. crispus* plants. As this enzyme did not convert 4-linked D-galactopyranosyl-2,6-disulfate (D2S,6S) units in λ-carrageenan to 4-linked 3,6-anhydro-D-galactopyranosyl 2-sulfate (DA2S) units in θ-carrageenan, it was concluded that λ-carrageenan could not be converted biosynthetically into θ-carrageenan. Although recent investigations of the tetrasporic life-stages of three New Zealand species in the genus *Gigartina* (also family Gigartinaceae) (i.e., *G. alveata*,[11] *Gigartina chapmanii*[9] and *Gigartina atropurpurea*[12]) have identified small amounts of derivatives resulting from DA2S units in polysaccharides that are comprised predominantly of λ-carrageenan, the existence of alternating G2S–DA2S units, that is, θ-carrageenan in these polysaccharides has not been unequivocally established. This report details the structural analysis of a polysaccharide extract from female *C. hombroniana*, in which θ-carrageenan is a significant natural component.

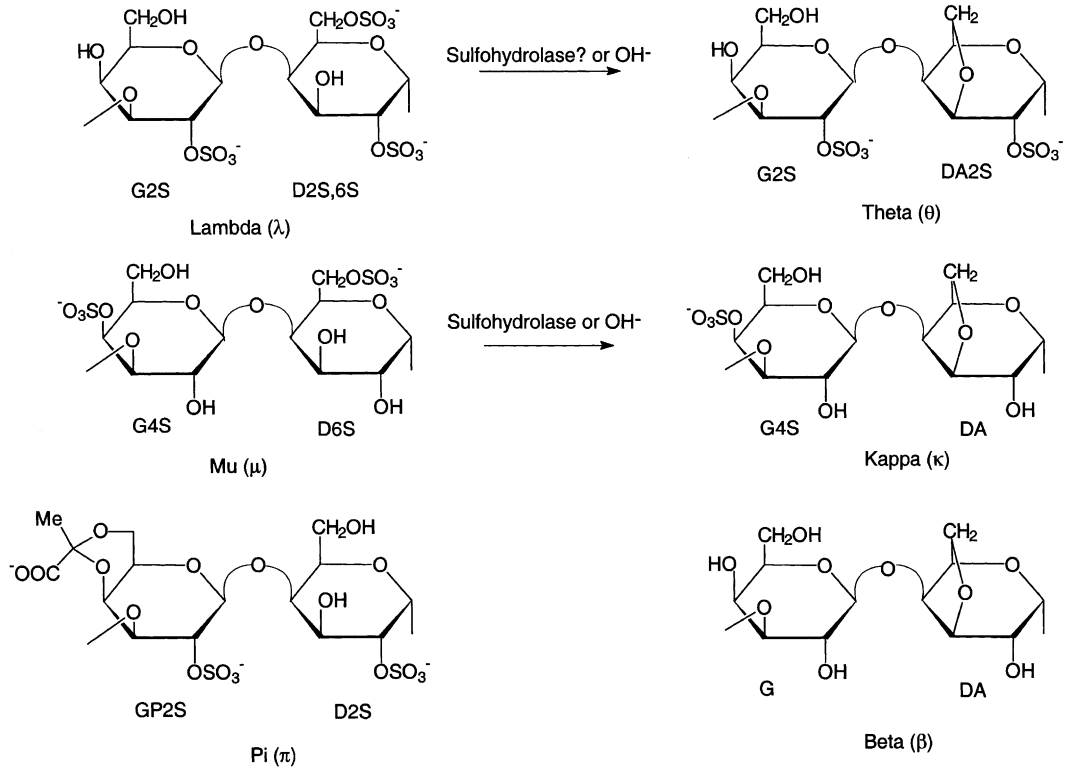


Figure 1. Idealised structures of various carrageenans.

2. Experimental

2.1. Materials

Female *C. hombroniana* was collected as drift at Papatowai, Southeast Otago in February, 1996 and air-dried. A specimen of the material studied has been deposited in the Herbarium of the Museum of New Zealand, Te Papa Tongarewa, WELT A21535.

2.2. Extraction and treatment of the polysaccharide

The polysaccharide was extracted from air-dried seaweed (5 g) using 0.05 M $NaHCO_3$ (60 mL/g of weed) at 95 °C for 3 h.[8] The cooled extract was treated with amyloglucosidase to digest any floridean starch present, reheated, filtered then dialysed and lyophilised to give Ch1-N (1.1 g). The weed residue was re-extracted and purified using the same conditions to give Ch2-N (0.8 g). Alkali treatment of a portion of Ch1-N was performed according to the method of Craigie and Leigh.[13] Briefly, Ch1-N (0.40 g) was dissolved in distilled water (80 mL), sodium borohydride (0.08 g) was added and the solution was stirred at room temperature overnight. Sodium hydroxide (3 M, 40 mL) and sodium borohydride (0.24 g) were then added, and the solution was stirred at 80 °C for 7 h. After cooling, the solution was neutralised then dialysed before lyophilisation to give Ch1-AM (0.26 g). Preparation of the pyridinium salt form and subsequent solvolytic desulfation of Ch1-N (0.40 g) and Ch1-AM (0.13 g) was undertaken by the method described by Falshaw and Furneaux[8] to yield Ch1-N,DS (0.19 g) and Ch1-AM,DS (0.07 g), respectively.

2.3. Chemical methods

Permethylations were performed according to Stevenson and Furneaux.[14] Two methylation steps were performed to maximise methylation. Constituent sugar and glycosyl linkage analyses were undertaken by the methods described by Stevenson and Furneaux[14] and quantified according to Falshaw and Furneaux.[8]

The configuration of the galactosyl units was determined by GLC analysis of their pertrimethylsilylated (*S*)-(+)-2-butyl glycoside derivatives, using the method of Gerwig et al.[15]

Pyruvate acetal substitution was detected as the 2,4-dinitrophenylhydrazone derivative of pyruvic acid following the procedure of Nelson et al.[16]

Reductive partial hydrolysis was conducted as described previously.[17]

The configuration of the 3,6-anhydro-galactosyl units was determined by GLC–MS analysis of their 3,6-anhydro-1-deoxy-1-(1′-phenylethylamino) galactitol peracetate derivatives, using the method of Navarro and Stortz.[18]

2.4. Spectroscopic methods

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 spectra were recorded on 3% (w/v) solutions in 50:50 (v/v) D_2O–H_2O at 90 °C. The spectra of Ch1-AM, Ch1-N,DS and Ch1-AM,DS were acquired on a Bruker AC 300 spectrometer (75 MHz, 0.885 s acquisition time, 0.5 s delay time and 80° pulse width). The spectrum of Ch1-N was acquired on a Varian Unity-500 spectrometer at a carbon frequency of 125 MHz, using a 10-mm broad-band probe, acquisition time of 1.17 s, delay time of 0.8 s and 80° pulse. All chemical shifts are quoted relative to internal Me_2SO as standard at 39.47 ppm.

3. Results and discussion

3.1. Thallus sample

Galactose was the predominant sugar recovered by direct constituent sugar analysis of a portion of *C. hombroniana* thallus (see Table 1) and is consistent with the results from other *Callophyllis* species (see Introduction). A significant amount of 3,6-anhydrogalactose

Table 1. Constituent sugar analysis of the polysaccharides from *Callophyllis hombroniana*, and various derivatives thereof[a]

Constituent sugar[b]	Ch-thallus	Ch1-N	Ch1-N,DS	Ch1-AM	Ch1-AM,DS	Ch2-N
AnGal	16	21	27	36	40	22
Total Gal	56	76	70	60	57	70
(D-Gal)[c]				(52)		
(L-Gal)[c]				(8)		
Xyl	1	—	1	1	1	1
Glc	27	3	2	3	2	7

[a] Normalised mole %.
[b] AnGal determined as 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol, Gal as galactitol hexaacetate, etc.
[c] D-Gal and L-Gal determined as (*S*)-(+)-2-butyl glycoside derivatives.

was also detected. Although this technique does not distinguish between mono- and polysaccharides, the presence of these sugars indicated that this species of seaweed contained predominantly a galactan.

A portion of *C. hombroniana* thallus was subjected to direct partial reductive hydrolysis. In this technique, mild acid hydrolysis conditions [0.5 M trifluoroacetic acid (TFA), 65 °C, 7.5 h] cleave virtually all the 3,6-anhydrogalactosidic bonds present in red algal galactans, while most of the galactosidic bonds remain intact. In the presence of the relatively acid-stable reducing agent, 4-methylmorpholine-borane (MMB), carrabiitols are formed from carrageenan molecules with contiguous regions of alternating β-D-galactopyranosyl and 4-linked 3,6-anhydro-α-D-galactopyranosyl units while agarobiitols are formed from agars with contiguous regions of alternating β-D-galactopyranosyl and 4-linked 3,6-anhydro-α-L-galactopyranosyl units.[17,19] Sulfate ester substituents present on the agar or carrageenan may survive the mild acid hydrolysis step but are cleaved in the subsequent perchloric acid-catalysed acetylation/acetolysis step.[17] Only carrabiitol peracetate was observed, which indicated that those 3,6-anhydrogalactosyl units present in a polysaccharide structure alternating with galactosyl units were all in the D-configuration.

A large amount of glucose was also detected by direct constituent sugar analysis of a portion of *Callophyllis hombroniana* thallus (see Table 1). This indicated that the seaweed contained floridean starch. To simplify further analyses, two polysaccharide extracts were prepared from *C. hombroniana* thalli and were treated with amyloglucosidase to remove any floridean starch present. Low-molecular weight components, including sugars, were removed by dialysis.

3.2. Polysaccharide samples

The first amyloglucosidase-treated, lyophilised polysaccharide extract of *C. hombroniana* (Ch1-N) was obtained in 22% yield from air-dried seaweed. A second extract, Ch2-N, was obtained in 16% yield. The sulfate groups were removed solvolytically from Ch1-N by heating its pyridinium salt form in a Me_2SO–MeOH–pyridine mixture to give Ch1-N,DS in 48% yield. Ch1-N was treated with hot aqueous alkali to give Ch1-AM in 65% yield. The sulfate groups were removed solvolytically from Ch1-AM to give Ch1-AM,DS in 54% yield.

As expected following purification, the amount of glucose detected in the polysaccharide extracts was considerably lower than that found in the thalli.

3.3. Preliminary analysis of polysaccharide samples

Constituent sugar analysis of the four polysaccharide samples (Ch1-N, -DS, -AM, -AM,DS) indicated that the predominant sugars in all samples were galactose and 3,6-anhydrogalactose, indicating a galactan (see Table 1). Small amounts of xylose were also detected (see Table 1). Also, Ch1-N was found to contain 0.8 wt % pyruvate acetal using colorimetric analysis.

Portions of both Ch1-N and Ch1-AM were subjected to reductive partial hydrolysis,[17] and only carrabiitol peracetate was obtained. This indicates that there are contiguous regions of alternating β-D-galactopyranosyl and 4-linked 3,6-anhydro-α-galactopyranosyl units in these polysaccharides, and that the latter are in the D-configuration. However, this techniques does not identify the configuration of alternating 3- and 4-linked galactosyl units or isolated 4-linked 3,6-anhydro-α-galactopyranosyl units.

The configuration of both 4-linked and 3-linked galactosyl units can be determined by the preparation and GLC analysis of (*S*)-(+)-2-butyl glycoside derivatives. Unfortunately, the butanolysis conditions used in the procedure destroy 4-linked 3,6-anhydro-α-galactopyranosyl units, however, so the configuration of these units cannot be determined by this method. As most of the 4-linked units in Ch1-AM are 3,6-anhydro-α-galactopyranosyl units (Table 1) this technique provides information about the configuration of the 3-linked units and the remaining 4-linked units. Of the (*S*)-(+)-2-butyl glycoside derivatives obtained, 86% were derived from galactosyl units of the D-configuration, and 14% were in the L-configuration. If all the 3-linked units are assumed to be in the D-configuration (as expected from either agars or carrageenans), then most of the 4-linked galactosyl units detected are in the L-configuration (Table 1). The presence of L-galactosyl units in *C. hombroniana* is consistent with the findings of Merino et al. for *C. variegata*.[5]

Recently, a method for determining the configuration of 3,6-anhydro-α-galactopyranosyl units by the preparation and GLC–MS analysis of 3,6-anhydro-1-deoxy-1-(1′-phenylethylamino)galactitol peracetate derivatives was developed by Navarro and Stortz.[18] They quantified a small (<5 mol %) amount of 3,6-anhydro-1-deoxy-1-(1′-phenylethylamino)-D-galactitol peracetate in an alkali-treated polysaccharide extract from *Porphyra columbina* that contained 45–48 mol % of 3,6-anhydro-1-deoxy-1-(1′-phenylethylamino)-L-galactitol peracetate, despite incomplete baseline separation of these two derivatives by GC–MS. A small peak corresponding to 3,6-anhydro-1-deoxy-1-(1′-phenylethylamino)-L-galactitol peracetate was detected in the analysis of Ch1-AM using this method, but it was not sufficiently resolved from the predominant 3,6-anhydro-1-deoxy-1-(1′-phenylethylamino)-D-galactitol peracetate peak to permit quantitation. No clear peak corresponding to 3,6-anhydro-1-deoxy-1-(1′-phenylethylamino)-L-galactitol peracetate was detected in the analysis of Ch1-N.

3.4. Polysaccharide backbone

From above, galactose and 3,6-anhydro-galactose are the predominant sugars of the polysaccharide from *C. hombroniana*. Almost all of the 4-linked 3,6-anhydrogalactosyl units are in the D-configuration. Some of the 4-linked galactosyl units are in the D-configuration, and some are in the L-configuration. However, no information concerning the substitution/linkage patterns of the various sugar units is obtained from constituent sugar analysis so the structure of the glycosidic backbone of the polysaccharide from *C. hombroniana* was first established. This was undertaken by simplifying the native polysaccharide structure by the removal of sulfate ester substituents using solvolytic desulfation. Further information on the polysaccharide backbone structure was obtained by the intramolecular conversion of any 4-linked α-galactopyranosyl 6-sulfate units in the native polysaccharide to the corresponding 3,6-anhydro-α-galactopyranosyl units using hot aqueous alkali, followed by the solvolytic removal of any other sulfate ester substituents present.

3.4.1. Major units. Glycosyl linkage analysis of both Ch1-N,DS and Ch1-AM,DS revealed approximately equimolar amounts of 3-linked and 4-linked galactosyl units (Table 2), consistent with the polysaccharide having a backbone composed of these units alternating in a disaccharide repeat structure. The predominant derivatives obtained in both cases were 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol (3-Gal) and 1,4,5-tri-*O*-acetyl-3,6-anhydro-2-*O*-methyl-galactitol (4-AnGal). The total amount of 3,6-anhydrogalactose detected by glycosyl linkage analysis for Ch1-N,DS and Ch1-AM,DS (28 and 41 mol %, respectively, Table 1) was also consistent with that obtained by constituent sugar analysis (27 and 40 mol %, respectively, Table 1). While constituent sugar and glycosyl linkage analyses do not provide any information about the configuration of these units, such information can be obtained by ^{13}C NMR spectroscopy. The ^{13}C NMR spectra of Ch1-DS and Ch1-AM,DS were both well resolved (Fig. 2) and contained major signals having chemical shifts expected for β-carrageenan, that is, alternating 3-linked β-D-galactopyranosyl (G) and 4-linked 3,6-anhydro-α-D-galactopyranosyl (DA) units (Fig. 1).[7,21] This is consistent with the presence of carrabiitol peracetate from the partial reductive hydrolysis of Ch1-N and Ch1-AM.

3.4.2. Minor units. Small amounts of other derivatives were also observed from the glycosyl linkage analysis of both Ch1-N,DS and Ch1-AM,DS (Table 2). Ch1-N,DS contained 16 mol % 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-galactitol (4-Gal) and the ^{13}C NMR spectrum

Table 2. Glycosyl linkage analysis of the native and alkali-modified polysaccharides from *Callophyllis hombroniana*, and the corresponding desulfated polysaccharides[a]

Constituent sugar and deduced substitution[b]	Deduced unit type	Ch1-N	Ch1-AM	Ch1-N,DS	Ch1-AM,DS	Ch2-N
3-Linked units						
3-Gal	G	4	4	40	40	2
2,3-Gal	G2S	38	40	2	2	40
3,6-Gal	G6S	1	2	—	—	—
2,3,6-Gal[c]	G2S,6S	2	1	2	1	1
3,4,6-Gal	GP	5	4	4	4	5
Total 3-linked units		*50*	*51*	*48*	*47*	*48*
4-Linked units						
4-AnGal	DA	7	10	26	40	11
2,4-AnGal	DA2S	18	24	2	1	19
4-Gal	D/L	4	2	16	6	3
2,4-Gal	D2S/L2S	1	2	5	4	1
2,4,6-Gal[c]	D2S,6S	8	1	—	—	7
Total 4-linked units		*38*	*39*	*49*	*51*	*41*
Terminal/ambiguous units						
3,4-Gal		4	2	1	2	2
2,3,4-Gal		3	3	2	—	7
2,3,4,6-Gal		5	5		—	2
T-Gal		Tr	Tr	Tr	Tr	Tr
Total terminal/ambiguous-linked units		*12*	*10*	*3*	*2*	*11*

[a] Normalised mole %.
[b] 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.
[c] Enantiomeric partially methylated alditol acetates (2,3,6-Gal and 2,4,6-Gal) differentiated by deuterium labelling and determined by GC–MS analysis.[8]

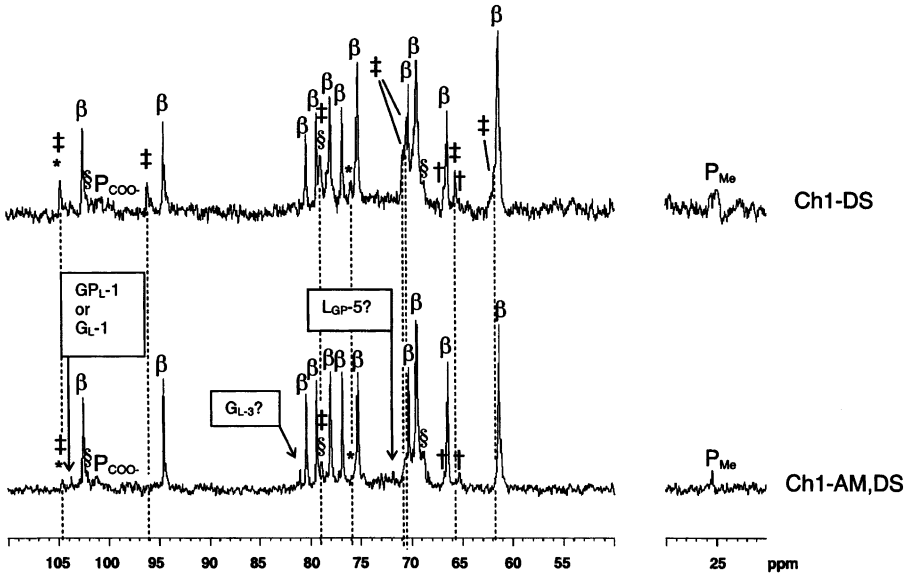


Figure 2. ^{13}C NMR spectra (20–30 ppm and 50–110 ppm) of Ch1-N,DS and Ch1-AM,DS (see text for details). Signals labelled as follows: β,β-carrageenan (G–DA repeat structure);[21] †—signals characteristic of GP units in general;[20] ‡—G–D repeat structure;[27] *—GP–D repeat structure;[20] §—GP–DA repeat structure;[20] P_{Me}—methyl carbon of pyruvate acetal group and P_{coo-}—acetal carbon of pyruvate acetal group; $G(P)_L$—a (pyruvylated) 3-linked β-D-galactosyl unit adjacent to a 4-linked α-L-galactopyranosyl unit; $L_{G(P)}$—a 4-linked α-L-galactopyranosyl unit adjacent to a (pyruvylated) 3-linked β-D-galactosyl unit.

of Ch1-N,DS contained distinct signals/shoulders at 104.8, 96.2, 78.9, 65.8 and 61.6 ppm (Fig. 2), characteristic of G-1, D-1, G-3, G-4 and D-6, respectively, in the repeating structure $[(G–D)_n]$.[7,8] These signals were much smaller or not observed in the ^{13}C NMR spectrum of Ch1-AM,DS, and Ch1-AM,DS also contained less (6 mol %) 4-Gal than Ch1-N,DS. This is consistent with the presence in Ch1-N of a significant number of 6-sulfated 4-linked galactosyl units (which were also 2-sulfated). In Ch1-AM,DS, these units should have been converted to DA2S units during the alkali-modification step prior to desulfation and then converted to DA units by desulfation.

A small amount of 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-galactitol (2,4-Gal) was also observed from the glycosyl linkage analysis of both Ch1-N,DS and Ch1-AM,DS (Table 2). The fact that 6 mol % 4-Gal and 4 mol % 2,4-Gal were present in Ch1-AM,DS could be due either to 4-linked galactopyranosyl units in the native polysaccharide that lacked a 6-sulfate group so were not converted to the corresponding 4-linked 3,6-anhydrogalactopyranosyl units during alkali treatment of Ch1-N prior to desulfation, or due to incomplete alkali modification. The existence of L-galactosyl units in Ch1-AM (see above) suggests that most of the 4-Gal and 2,4-Gal (i.e., 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-galactitol) observed in the glycosyl linkage analysis of Ch1-AM,DS may have originated from 4-linked α-L-galactopyranosyl (2-sulfate) [L(2S)] units. These would not be susceptible to alkali modification.

The derivative 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methyl-galactitol (3,4,6-Gal, 4 mol %), was observed from the glycosyl linkage analysis of both Ch1-N,DS and Ch1-AM,DS (Table 2). In this case, this derivative most likely corresponded to 4,6-pyruvated 3-linked-D-galactopyranosyl (GP) units, which is consistent with the detection of pyruvate acetal substituents from the native polysaccharide from *C. hombroniana* (see above). Also, a distinct signal was observed at 25 ppm in the ^{13}C NMR spectra of both Ch1-DS and Ch1-AM,DS (Fig. 2), which corresponds to the methyl group of a pyruvate acetal substituent group in the *R* configuration.[7,22] A weak and poorly resolved signal was also observed at 101.1 ppm in the ^{13}C NMR spectrum of both Ch1-N,DS and Ch1-AM,DS, which is characteristic of the acetal carbon of a pyruvate acetal group. Signals at 66.5 and 65.3 ppm may be due to carbons-5 and -6 of GP units connected to either D, DA, L or LA units.[20,23] A small signal at 76.1 ppm in the ^{13}C NMR spectrum of Ch1-DS may be due to GP-3 in a GP–D repeating unit.[20] The chemical shift for GP-1 in a GP–D repeat unit is at 104.4 ppm[20] and may be obscured by G-1 in G–D at 104.8 ppm in the ^{13}C NMR spectrum of Ch1-DS. A distinct signal at 104.4 ppm is visible in the ^{13}C NMR spectrum of Ch1-AM,DS [where interference from G–D signals is reduced (see above)], so a GP–D structure may be present in Ch1-AM,DS, but no clear signals for either GP-3 (at 76.1 ppm) or GP-4 (at 67.6 ppm) in a GP–D repeat structure are visible. However, signals at 102.1, 78.9 and 68.8 ppm are visible in both spectra (Fig. 2) and may be due to carbons-1, -3 and -4, respectively, of a GP unit in a GP–DA repeat unit.

A small signal at 103.8 ppm in the ^{13}C NMR spectra of both Ch1-DS and Ch1-AM,DS may be due to car-

bon-1 of a G or GP unit in the repeating structure [(G–L)$_n$ or (GP–L)$_n$].[7,23,24] A signal at 81.0 ppm in the ^{13}C NMR spectrum of Ch1-AM,DS may be due to carbon-3 of a G unit in the repeating structure [(G–L)$_n$] and the signal at 71.6 ppm may be due to carbon-5 of an L unit in the repeating structure [(GP–L)$_n$].[23] Some other minor signals have chemical shifts that cannot be uniquely assigned to one structure (Fig. 2). Whether these minor components exist as part of a hybrid molecule or as separate polymers is not known. Preliminary results do indicate, however, that L-galactosyl units also occur in the polysaccharide extract from *C. variegata* and that they occur in a distinct fraction.[5]

3.5. Polysaccharide sulfation pattern

A number of substitution patterns are possible for a polysaccharide backbone of G–DA units, and qualitative information on substitution patterns can be obtained from ^{13}C NMR spectroscopy. The ^{13}C NMR spectrum of Ch1-N was reasonably well resolved (Fig. 3) and was dominated by a series of signals uniquely characteristic of 3-linked β-D-galactopyranosyl 2-sulfate (G2S) alternating with 4-linked 3,6-anhydro-α-D-galactopyranosyl 2-sulfate (DA2S) units, that is, θ-carrageenan.[7,8] The major partially methylated alditol acetate derivatives detected in the glycosyl linkage analysis of Ch1-N were 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl-galactitol (2,3-Gal) and 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol (2,4-AnGal), respectively (Table 2). These two species correspond to G2S and DA2S units, respectively, and support the ^{13}C NMR data that the major repeat structure in Ch1-N is the G2S–DA2S repeat structure of θ-carrageenan. This is the first time that θ-carrageenan has been found as the predominant structure in a native polysaccharide from a red seaweed and was not simply generated by alkali modification of its chemical precursor, λ-carrageenan [i.e., 3-linked β-D-galactopyranosyl 2-sulfate (G2S) alternating with 4-linked α-D-galactopyranosyl 2,6-disulfate (D2S,6S) units] (Fig. 1).

Some λ-carrageenan also existed in the native polysaccharide extract as shown by characteristic signals for carbons-1 and -4 of a G2S unit linked to a D2S,6S unit at 103.6 and 64.3 ppm, respectively, in the ^{13}C NMR spectrum of Ch1-N. Also, the signal at 75.9 ppm, while not unique to λ-carrageenan, can be assigned to carbon-3 of a G2S unit linked to a D2S,6S unit in this case. The presence of λ-carrageenan in Ch1-N was confirmed by the detection of the derivative 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methyl-galactitol (2,4,6-Gal) in addition to 2,3-Gal in the glycosyl linkage analysis of Ch1-N (Table 2). These two species correspond to D2S,6S and G2S units, respectively, consistent with λ-carrageenan.

The presence of a significant amount of 4-AnGal in the native polysaccharide is also noteworthy. As the predominant 3-linked unit is 2-sulfated, the most likely diad is G2S-DA. A G2S-DA repeating structure has not been reported previously as a major component of a carrageenan, and no distinct signals could be unequivocally assigned to a G2S-DA repeating structure in the ^{13}C NMR spectrum of Ch1-N. A signal was observed at 59.9 ppm, which was not observed in the ^{13}C NMR spectra of Ch1-AM, Ch1-DS or Ch1-AM,DS. This signal remains unassigned.

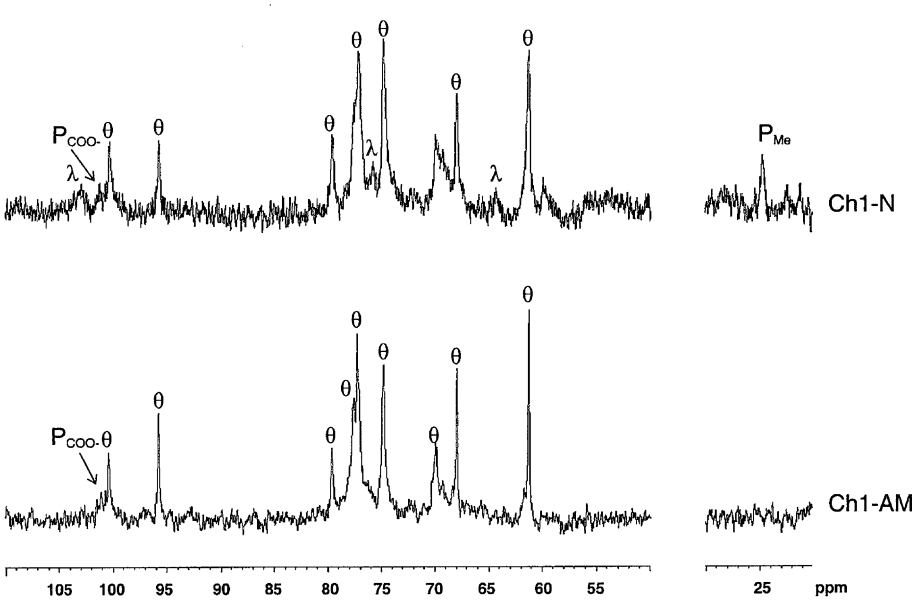


Figure 3. ^{13}C NMR spectra (20–30 ppm and 50–110 ppm) of Ch1-N, and Ch1-AM (see text for details). Signals labelled as follows: θ,θ-carrageenan (G2S–DA2S repeat structure);[8] λ,λ-carrageenan (G2S-D2S,6S repeat structure);[8] P$_{Me}$—methyl carbon of pyruvate acetal group and P$_{coo-}$—acetal carbon of pyruvate acetal group.

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.[25] Alkali modification of idealised λ-carrageenan results in the formation of θ-carrageenan (Fig. 1). The ^{13}C NMR spectrum of Ch1-AM was also reasonably well resolved (Fig. 3) and contained a series of strong signals characteristic of θ-carrageenan (see above) and a loss of any clear signals characteristic of λ-carrageenan. It has been found previously that the standard alkali modification conditions [7 h @ 80 °C] used by Craigie and Leigh[13] do not result in complete conversion of 4-linked α-D-galactopyranosyl 2,6-disulfate (D2S,6S) units in λ-carrageenan to 4-linked 3,6-anhydro-α-D-galactopyranosyl 2-sulfate (DA2S) units in θ-carrageenan.[8] In this case, however, these conditions did result in the almost complete loss of 2,4,6-Gal and a corresponding increase in 2,4-AnGal, for Ch1-AM (Table 2). The predominant derivatives identified by glycosyl linkage analysis of Ch1-AM were 2,3-Gal and 2,4-AnGal, consistent with 2-sulfated, 3-linked galactopyranosyl units and 2-sulfated, 4-linked 3,6-anhydro-α-D-galactopyranosyl units, respectively, as expected for the repeating disaccharide structure in θ-carrageenan.

As in the ^{13}C NMR spectra of Ch1-N,DS and Ch1-AM,DS, a small signal was observed at 101.1 ppm in the ^{13}C NMR spectra of both Ch1-N and Ch1-AM. This chemical shift is characteristic of the acetal carbon of a pyruvate acetal group ppm, and the corresponding methyl carbon at 25.5 ppm was clearly visible in the ^{13}C NMR spectrum of Ch1-N but, surprisingly, was not visible in the ^{13}C NMR spectrum of Ch1-AM (Fig. 3). The presence of pyruvate acetal groups in Ch1-N was also confirmed colorimetrically (see above). As discussed above, the derivative 3,4,6-Gal observed by glycosyl linkage analysis of both Ch1-N,DS and Ch1-AM,DS (Table 2) most likely corresponds to 4,6-pyruvated 3-linked-D-galactopyranosyl (GP) units. Very similar amounts of 3,4,6-Gal were also observed by glycosyl linkage analysis of both Ch1-N and Ch1-AM (Table 2). This indicates that the GP units are not substituted with a sulfate group at the 2-position in the native or alkali-modified polysaccharide, and so these units are different from those found in π-carrageenan (Fig. 1).

Small amounts of a number of other derivatives were also observed by glycosyl linkage analysis of both Ch1-N and Ch1-AM (Table 2). The total amount of units identified in Table 2 as being unambiguously 4-linked is lower than the 50 mol % expected for a polysaccharide with a structure of repeating 3- and 4-linked units. This suggests that a significant proportion of the derivatives where the linkage position is ambiguous [i.e., 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-galactitol (3,4-Gal), 1,2,3,4,5-penta-*O*-acetyl-6-*O*-methyl-galactitol (2,3,4-Gal) and 1,2,3,4,5,6-hexa-*O*-acetyl-galactitol (2,3,4,6-Gal)] were derived from sulfated 4-linked units. Given the presence of some 4-linked L-galactosyl units in the polysaccharide from *C. hombroniana* (see above), these derivatives are most likely derived from 4-linked L-galactosyl units.

The total amount of 3,6-anhydrogalactose detected by glycosyl linkage analysis of Ch1-N (25 mol %) was consistent with that obtained by constituent sugar analysis (21 mol %), above. This was slightly lower than that obtained from Ch1-N,DS (27–28 mol %, Tables 1 and 2). The total amount of 3,6-anhydrogalactosyl units in Ch1-AM by glycosyl linkage analysis of Ch1-AM was 34 mol % (Table 2), similar to that obtained by constituent sugar analysis (36 mol %, Table 1). This was slightly lower than that obtained from Ch1-AM,DS (40–41 mol %, Tables 1 and 2). However, recovery of the derivative 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol from 2-sulfated 3,6-anhydrogalactosyl units can be incomplete by the reductive hydrolysis procedure, due to the stabilising effect of this 2-sulfate ester group.[8]

The infrared spectrum of Ch1-N (Fig. 4) showed an intense band at 1250 cm^{-1}, characteristic of sulfate esters generally,[13] and a broad band centred at 830 cm^{-1} with a broad, but discernible, shoulder at 816 cm^{-1}. Equatorial 2- and 6-sulfate ester groups give bands at approximately 830 and 820 cm^{-1}, respectively, while the axial 2-sulfate ester group on a 3,6-anhydrogalactosyl unit gives a band at approximately 805 cm^{-1}.[13] The broadness of the 830 cm^{-1} band and its shoulder suggest the possibility of all three types of sulfate ester substitution in Ch1-N. This spectrum also contained a distinct band at 935 cm^{-1}, which is characteristic of 3,6-anhydrogalactosyl units and would be expected for θ-carrageenan. Infrared spectroscopic analysis of Ch1-AM (Fig. 4) showed that the peak at 935 cm^{-1} (characteristic of 3,6-anhydrogalactosyl units) had increased in intensity and still contained a distinct band centred at 830 cm^{-1} that is characteristic of the equatorial 2-sulfate ester group on a galactosyl unit (as found in G2S units). Another broad band was centred at 810 cm^{-1}, which was more defined than in Ch1-N. This, and the shift from 820 cm^{-1} towards 805 cm^{-1}, indicated a reduction in 6-sulfated units and an increase in 3,6-anhydrogalactosyl 2-sulfate units in Ch1-AM.

A portion of Ch2-N was also methylated twice and subjected to glycosyl linkage analysis. The results for Ch2-N were very similar to those of Ch1-N (Table 2).

Partially methylated, partially acetylated carrabiitols or agarobiitols are formed using mild acidic hydrolysis followed by acetylation/acetolysis from methylated polysaccharides with alternating G and A units.[17] GC and GC–MS analysis of these types of derivatives can provide useful information regarding the substitution patterns of 3-linked D-galactopyranosyl units linked at the 4-position of 3,6-anhydro-D,L-galactopyranosyl units.[17,20,26] As only carrabiitol peracetate was obtained

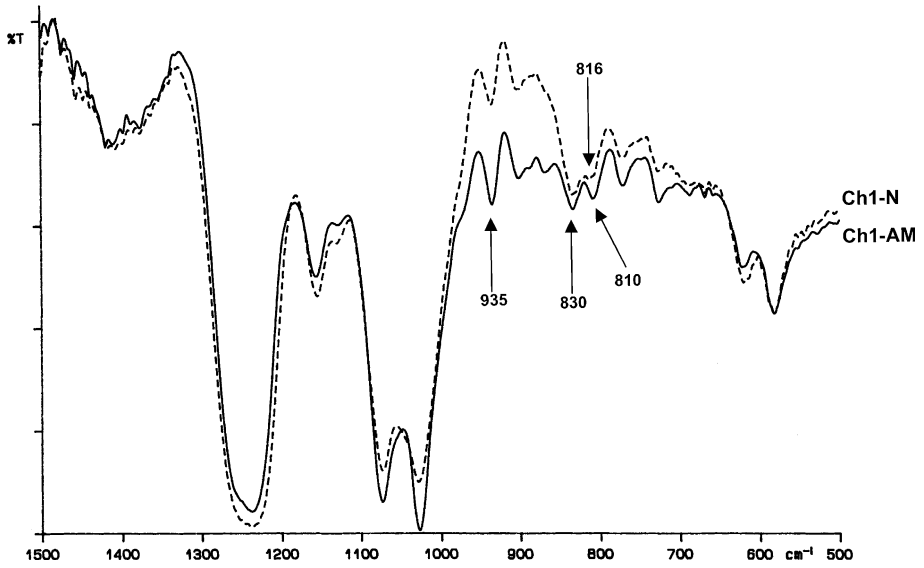
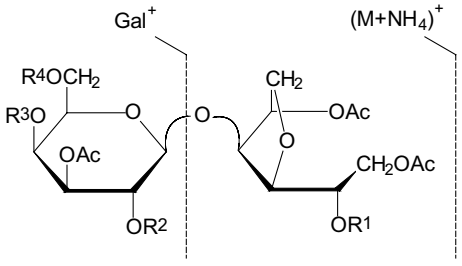


Figure 4. Infrared spectra (1500–500 cm^{-1}) of Ch1-N, and Ch1-AM (see text for details).

from reductive partial hydrolysis of Ch1-AM, only partially methylated carrabiitol derivatives were expected from reductive partial hydrolysis of methylated Ch1-AM. Two peaks were observed corresponding to 86% and 14% of the total integrated area. The larger of the two peaks observed had a retention time corresponding to that of 4′, 6′-di-*O*-methylcarrabiitol pentaacetate (**1a**) (Fig. 5 and Table 3), obtained previously from a fully characterised sample of θ-carrageenan.[17] This peak gave a CI mass spectrum with ions at *m*/*z* 582 and 275, corresponding to the $(M+NH_4)^+$ ion and a major fragment $(Gal)^+$ ion, respectively, corresponding to a partially methylated carrabiitol acetate with a total of two methyl groups, both on the β-D-galactosyl moiety, again as expected for 4′,6′-di-*O*-methylcarrabiitol pentaacetate, arising from regions of alternating G2S and DA2S units as in θ-carrageenan. The smaller, faster eluting peak gave a CI mass spectrum with ions at *m*/*z* 554 and 275, corresponding to the $(M+NH_4)^+$ ion and a major fragment $(Gal)^+$ ion, respectively (Table 3 and Fig. 5). These ions are consistent with a partially methylated carrabiitol peracetate with a total of three methyl groups, two on the β-D-galactosyl moiety and one on the 3,6-anhydrogalactitol moiety.[17] The retention time of this species was longer than that observed for 2,2′,



	R^1	R^2	R^3	R^4
1a	Ac	Ac	CH_3	CH_3
1b	CH_3	CH_3	Ac	CH_3
1c	CH_3	Ac	CH_3	CH_3

Figure 5. CI fragmentation of partially methylated carrabiitol acetate derivatives.

6′-tri-*O*-methylcarrabiitol pentaacetate (**1b**) obtained previously from partial reductive hydrolysis of κ-carrageenan.[17] Since 4-AnGal (corresponding to DA units) and 2,3-Gal (corresponding to G2S units) represented a significant proportion of the derivatives observed by glycosyl linkage analysis of Ch1-AM, the partially

Table 3. CIMS pseudomolecular and major fragment ions, and GLC retention times obtained from various partially methylated, partially acetylated carrabiitols

Retention time (min)[a]	Carrabiitol derivative	Adjacent sugar units with deduced substitution	Number of *O*-methyl groups on β-D-galactosyl moiety	Number of *O*-methyl groups on 3,6-anhydro-D-galactitol moiety	Gal^+ ion	$(M+NH_4)^+$ ion
5.44	**1b**	G4S–DA (κ)	2	1	275	554
5.76	**1c**	G2S–DA	2	1	275	554
6.45	**1a**	G2S–DA2S (θ)	2	0	275	582

[a] Maltose peracetate eluted at 9.36 min.

methylated carrabiitol derivative, 2,4′,6′-tri-*O*-methylcarrabiitol pentaacetate (**1c**), is thus a likely possibility. 2,4′,6′-tri-*O*-methylcarrabiitol pentaacetate is a new carrabiitol derivative not previously reported.

4. Conclusions

The use of various modern analytical techniques has facilitated the structural characterisation of the polysaccharide from *C. hombroniana*. The native polysaccharide contains a number of structural units with the largest proportion consisting of 3-linked β-D-galactopyranosyl 2-sulfate units alternating with 4-linked 3,6-anhydro-α-D-galactopyranosyl 2-sulfate units, that is, θ-carrageenan. *C. hombroniana* is the first red seaweed reported to naturally contain such a large proportion of θ-carrageenan. The native polysaccharide also contains λ-carrageenan and a novel carrageenan structure consisting of 3-linked β-D-galactopyranosyl 2-sulfate units alternating with 4-linked 3,6-anhydro-α-D-galactopyranosyl units. In addition, part of the total polysaccharide extract contains L-galactosyl units. The biosynthesis of θ-carrageenan is beyond the scope of this work, but it is clear that θ-carrageenan is produced naturally by *C. hombroniana*.

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