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Structural analysis of carrageenans from Burmese and Thai samples of *Catenella nipae* Zanardini

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Abstract

The carrageenans extracted from samples of the red seaweed *Catenella nipae* Zanardini from Burma and Thailand have been characterised by recently developed chemical derivatisation procedures (utilising reductive hydrolysis and reductive partial-hydrolysis techniques) combined with GLC–MS analysis and by IR and NMR spectroscopy. Both polysaccharides are linear polymers composed primarily of 4-linked 3,6-anhydro-α-D-galactopyranosyl-2-sulfate residues alternating with 3-linked β-D-galactopyranosyl residues that are either unsubstituted (α-carrageenan) or 4-sulfated (ι-carrageenan). The Burmese sample has a somewhat higher proportion of α-carrageenan residues. The Thai *C. nipae* carrageenan was shown to have minor proportions of β- and κ-carrageenan residues. $^{1}H-^{1}H$ COSY, $^{13}C-^{1}H$ COSY and TOCSY NMR spectroscopic examinations of polymer segments produced from this polysaccharide as well as ι-carrageenan itself, have permitted the ^{1}H and ^{13}C NMR assignments for ι-carrageenan to be independently verified and for characteristic ^{1}H and ^{13}C chemical shifts for α-carrageenan to be identified and assigned for the first time. © 1996 Elsevier Science Ltd.

Keywords: Carrageenan, α-, β-, ι-, and κ-; Galactans, sulfated; Structure determination; Reductive hydrolysis; Reductive partial-hydrolysis; Polysaccharides

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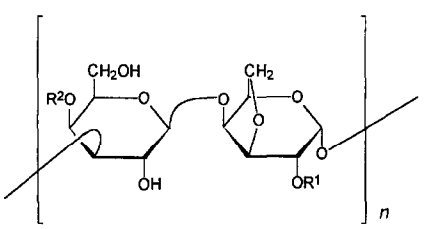
1. Introduction

Red seaweeds from a number of families of the Rhodophyta contain sulfated galactans known as carrageenans. These have an essentially linear backbone of alternating 3-linked β-D-galactopyranosyl and 4-linked α-D-galactopyranosyl residues that can carry various substituents in different quantities. In addition, some of the 4-linked galactosyl residues may exist in the form of the 3,6-anhydride. In order to describe this range of structures, Greek letters have been assigned to various idealised disaccharide repeating units. Thus in 1986, the letter α was assigned to the carrageenan with alternating 3-linked β-D-galactopyranosyl and 4-linked 3,6-anhydro-α-D-galactopyranosyl-2-sulfate units (Fig. 1, A) that had been isolated from a Burmese sample of *Catenella nipae* [1]. α-Carrageenan differs from the more commonly occurring ι-carrageenan (Fig. 1, B) in that there is no sulfate ester group on the 4-position of the 3-linked units. More recently, samples of *C. nipae* from Australia and the Philippines and *C. caespitosa* from France and South America have been examined [2,3], but all contain ι-carrageenan as the dominant component. The original analysis of the *C. nipae* (Burma) polysaccharide was a preliminary one based on infrared spectroscopy and milk reactivity [1]. We have now re-examined this sample using modern chemical and spectroscopic techniques and have compared it with material from a sample of *C. nipae* from Thailand.

2. Experimental

The origin and extraction of *C. nipae* Zanardini (from Burma, MSD #32428) have been described previously [1]. The extraction, alkali-modification, infrared and ^{13}C NMR spectroscopy, and constituent sugar analysis were performed on this sample according to the methods described by Liao et al. [2]. Pyruvate content was determined by the method of Duckworth and Yaphe [4]. Methylation analysis of the alkali-modified polysaccharide preparation was performed as described by Stevenson and Furneaux [5].



A : $R^1 = SO_3^-$, $R^2 = H$ α-carrageenan
B : $R^1 = R^2 = SO_3^-$ ι-carrageenan
C : $R^1 = H$, $R^2 = SO_3^-$ κ-carrageenan
D : $R^1 = R^2 = H$ β-carrageenan

Fig. 1. The structures of some carrageenans.

C. nipae Zanardini (Thailand) was collected from Lam Sok, Trat Province. The material consisted mostly of diploid thalli, and a voucher specimen has been deposited at the Museum of New Zealand WELT A21020. The seaweed was extracted with 0.2% sodium hydroxide. Infrared and standard ^{13}C NMR spectroscopy and constituent sugar and methylation analyses of the sample from the Thai *C. nipae* sample were performed as described by Falshaw and Furneaux [6]. Alkali treatment was performed according to the method of Craigie and Leigh [7]. Reductive partial-hydrolyses of the polysaccharide (with and without prior methylation) were performed according to the method of Falshaw and Furneaux [8].

^{1}H NMR, double quantum filtered COSY, Heteronuclear Multiple Quantum Coherence (HMQC) COSY and HMQC TOCSY spectra were collected on a Varian Unity-500 spectrometer with an inverse probe at 30 to 50 °C. Samples (~ 40 mg) in D_2O (1 mL) contained in 5 mm o.d. NMR tubes were referenced to internal Me_2SO at 39.47 ppm for carbon and 2.70 ppm for proton. Since specific acquisition parameters varied widely depending on the spectrum, these are described in the individual figure legends and only general details are given here. The ^{1}H NMR projection shown in the 2D plot of Fig. 7 (see Results and discussion) is an actual 1D spectrum which was aquired as follows: sixty-four scans were collected with 4.096 s data acquisition time for the free induction decay (fid) after a 6 s relaxation delay and 90° pulse with a free 8000 Hz sweep width resulting in 32K complex data points. No digital filtering or zero filling of the fid was used before a complex fourier transformation. All 2D NMR spectra were acquired in the phase-sensitive States hypercomplex mode in which the number of t_2-fids is twice the number of t_1 increments [9]. Gaussian weighting functions were used in both dimensions to ensure that the weighted t_2-fids or t_1-interferograms had decayed to zero to avoid truncation wiggles. Except for one of the HMQC spectra, no zero filling of the t_2-fids was used, but t_1-interferograms were zero filled to give 512 complex data points. High-power 90° pulse widths varied depending on the sample but were generally between 11 and 12 μs for ^{1}H and 12 and 14 μs for ^{13}C. Except for one of the HMQC spectra, sweep widths in the ^{1}H dimension varied from 2.5 to 3.5 ppm. The double quantum filtered $^{1}H-^{1}H$ COSY spectra had the highest digital resolution so as to better resolve out the detail in the contours. Negative signals are drawn as a single contour trace, whereas positive signals are drawn as multi-level contours. All pulse sequences were used as supplied by Varian. HMQC incorporates a special BIRD pulse with a relaxation delay time (null) to assist in cancelling unwanted ^{1}H signals [10]. During data acquisition time, WALTZ composite pulse decoupling was applied at low power corresponding to a ^{13}C 90° pulse time of 37 μs.

HMQC–TOCSY spectra were acquired with parameters similar to those used for HMQC, but with the addition of a windowed MLEV-17 ^{1}H spin locking period during which the ^{1}H pulse power was lowered, corresponding to a ^{1}H 90° pulse width of 24 μs [11]. The addition of windows (set to the equivalent of the time required for a 180° ^{1}H pulse) aids the suppression of interfering artifacts. The windows also assist in lowering the average ^{1}H pulse power.

Samples for COSY experiments were depolymerised by the periodate oxidation, borohydride reduction, mild acid hydrolysis sequence developed by Dea et al. [12], under the conditions reported by Furneaux and Stevenson [13].

3. Results and discussion

Constituent sugar analysis.—Constituent sugar analysis of the polysaccharide from Thai *C. nipae* showed the presence of both 3,6-anhydrogalactosyl (AnGal) and galactosyl (Gal) residues. Very similar results were obtained for the Burmese sample (Table 1). As both α- and ι-carrageenans contain a disaccharide repeating unit, a 1:1 ratio of Gal to AnGal would be expected, but in both samples the amount of AnGal detected was lower than expected. This may be due to the presence of 4-linked 2,6-disulfated galactosyl "precursor" residues. Such residues can be converted to the corresponding 3,6-anhydride by treatment with hot alkali. However, alkali-treatment of an ι-carrageenan-dominant sample from South Australian *C. nipae* had not resulted in a significant increase in AnGal content [2]. This was also the case here (Table 1). Alternatively, it is known that under standard reductive hydrolysis conditions the presence of a 2-sulfate ester group on 3,6-anhydrogalactosyl units inhibits the hydrolysis of its glycosidic bond, making it difficult to release all the AnGal residues before the reducing agent has been consumed [5]. In such cases, addition of extra reducing agent (4-methylmorpholine-borane, 0.05 mL) to the sample before and after the 80 °C prehydrolysis step has been shown to improve the recovery of AnGal residues [6]. Application of these amended conditions to the polysaccharide from Thai *C. nipae* led to a higher recovery of AnGal (44% cf. 40%, Table 1), although the Gal to AnGal ratio was still not 1:1.

Reductive partial-hydrolysis.—The use of alditol acetate derivatives in the structural analysis of polysaccharides provides no information on the configuration of the sugar residues present. From the information provided so far, it is still possible that these polysaccharides (or part of them) have an agaroid nature, i.e., the 4-linked 3,6-anhydrogalactosyl units could be of the L-configuration as opposed to D- in the case of carrageenans. The polysaccharide from *Dasyclonium incisum*, for example, is an agar equivalent of κ-carrageenan, being sulfated at O-4 of the 3-linked units [14]. It is, therefore, necessary to obtain independent confirmation of the absolute configuration of the constituent sugar residues. The traditional method of determining the configuration of aldoses involves the production and analysis of (+)-(*S*)-2-butyl glycosides [15], but

Table 1
Constituent sugar analysis (normalised mol-%) of the original (X) and alkali-modified (AM) *Catenella nipae* polysaccharides from Burma and Thailand

Constituent sugar [a]	*C. nipae*			
	X		AM	
	Burma	Thailand [b]	Burma	Thailand
AnGal	40	40 (44)	43	40
Gal	59	60 (56)	56	60
Xyl	1	0 (0)	1	0
Glc	Tr	Tr (Tr)	Tr	Tr

[a] AnGal determined as 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol, Gal as galactitol hexa-acetate, etc.
[b] Numbers in parentheses refer to the results obtained with extra reducing agent.

Table 2
Glycosyl-linkage analysis (normalised mol-%) of the polysaccharides from *Catenella nipae* (Burma, alkali-modified) and *C. nipae* (Thailand, original)

Constituent sugar and deduced substitution [a]	*C. nipae*	
	Burma	Thailand
3-Gal	32	24
3,4-Gal	20	31
3,4,6-Gal	4	5
4-AnGal	2	6
2,4-AnGal	42	34

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

this method is unsuitable for 3,6-anhydrogalactosyl units as they are degraded under the conditions used. The configuration of the 3,6-anhydrogalactopyranosyl units was determined using the partial-hydrolysis technique of Falshaw and Furneaux [8]. This technique utilises the susceptibility of 3,6-anhydrogalactosidic linkages to selective reductive hydrolysis. The disaccharide alditols (''biitols'') produced from agars and carrageenans (agarobiitol and carrabiitol, respectively) are diastereomers, and their corresponding peracetates are separable by GLC. The polysaccharide from Thai *C. nipae* was subjected to this procedure, and one peak was observed by GLC corresponding to carrabiitol peracetate. Thus the 3,6-anhydrogalactopyranosyl units in this material are of the D-configuration.

Methylation analysis.—Methylation analysis of the polysaccharides from Thai and Burmese *C. nipae* in the triethylammonium salt form was complete in one step, based on the absence of unmethylated Gal residues, and the results are shown in Table 2. This analysis revealed three major residues in both samples: 2,4-AnGal (i.e., 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol) corresponding to 4-linked 2-sulfated 3,6-anhydrogalactopyranosyl residues; 3,4-Gal (i.e., 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-galactitol) corresponding to 3-linked 4-sulfated galactopyranosyl residues; and 3-Gal (i.e., 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol) corresponding to 3-linked galactopyranosyl residues. 3,4-Gal and 2,4-AnGal are the expected products from ι-carrageenan, whilst 3-Gal and 2,4-AnGal are expected from α-carrageenan. These results indicate an α-/ι-carrageenan hybrid in both polysaccharide preparations, with the Burmese sample containing a higher proportion of α-carrageenan residues. In addition, small amounts of 4-AnGal and 3,4,6-Gal were also observed. The presence of 4-AnGal indicates that some of the 3,6-anhydrogalactosyl units lack a 2-sulfate ester as in κ- or β-carrageenan (Fig. 1, C and D, respectively). The presence of 3,4,6-Gal suggests 3-linked galactopyranosyl units substituted at both the 4- and 6-position, as when a pyruvate ketal is present. Assay of the Burmese sample using the lactate dehydrogenase method gave a pyruvate content of 0.6%, thus confirming the presence of ketal substitution. The ratios of 3-linked galactosyl to 4-linked 3,6-anhydrogalactosyl moieties identified in the methylation analysis were 60:40 and 56:44 for the Thai (as extracted) and Burmese (alkali-modified) samples, respectively (Table 2), almost the same as obtained by constituent sugar

analysis. The addition of extra reducing agent during derivatisation of the methylated Thai sample did not alter this ratio. It has been noted previously that the effect of extra reducing agent during the analysis of methylated polysaccharides is less than that for constituent sugars [6]. No 2,4,6-Gal corresponding to 4-linked 2,6-disulfated galactosyl "precursor" residues was observed, although such residues may have been converted to the corresponding 3,6-anhydride under the alkaline methylation (and extraction for the Thai sample) conditions employed.

Permethylation and reductive partial-hydrolysis.—The use of reductive partial-hydrolysis has been extended to permethylated polysaccharides. The GLC–CIMS analysis of the partially methylated partially acetylated biitols produced allows information on the substitution patterns of adjacent residues to be determined [8]. Thus a permethylated sample of a commercial ι-carrageenan (Sigma Chemical Co.) yields 2′,6′-di-*O*-methyl-carrabiitol penta-acetate (i.e., 3,4-di-*O*-acetyl-2,6-di-*O*-methyl-β-D-galactopyranosyl-(1 → 4)-1,2,5-tri-*O*-acetyl-3,6-anhydro-D-galactitol). The permethylated polysaccharide from Thai *C. nipae* produced not only 2′,6′-di-*O*-methyl-carrabiitol penta-acetate but also 2′,4′,6′-tri-*O*-methyl-carrabiitol tetra-acetate, thus confirming the partial absence of a substituent on O-4 of the 3-linked galactosyl residues, and hence the presence of α-carrageenan disaccharide units in the sample.

Infrared spectroscopy.—The IR spectrum of the polysaccharide from Burmese *C. nipae* was essentially the same as that reported previously [1]. It contained strong absorptions at 805 and 930 cm^{-1} that are characteristic of a 2-sulfate ester on a 3,6-anhydrogalactosyl residue and a 3,6-anhydrogalactosyl moiety itself, respectively [7], as found in both α- and ι-carrageenan. Absorption at 850 cm^{-1} is characteristic of

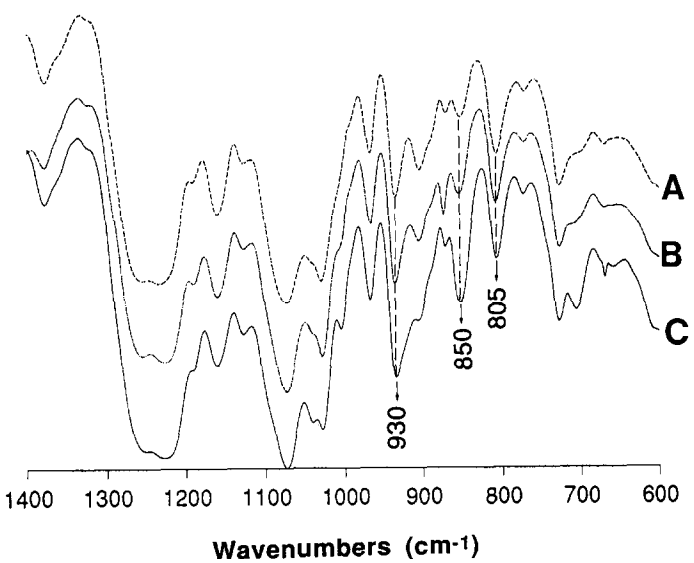


Fig. 2. Infrared spectra of polysaccharides from A: *Catenella nipae* (Burma), B: *C. nipae* (Thailand), C: ι-carrageenan ("*Eucheuma spinosa*" ex. Sigma Chemical Co.).

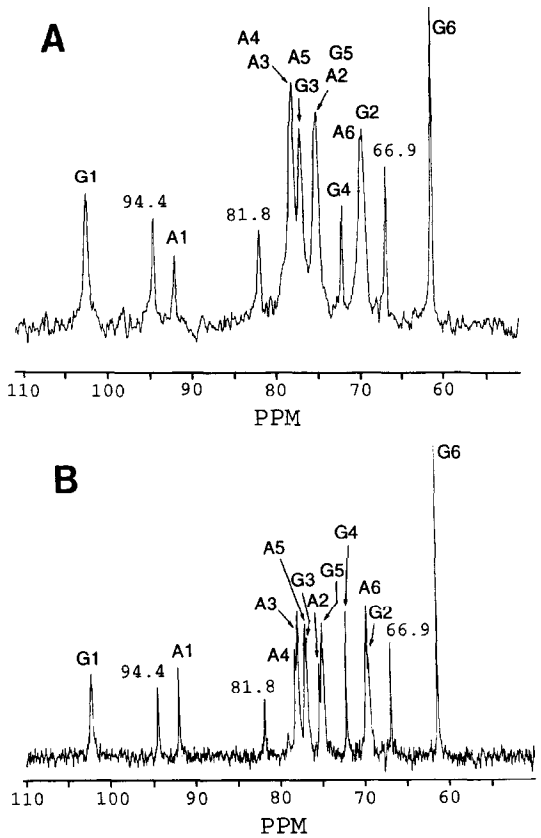


Fig. 3. ^{13}C NMR spectra of polysaccharides. A: *Catenella nipae* (Burma), B: *C. nipae* (Thailand), 56524 scans and 4157 scans respectively. G1–G6 and A1–A6 are twelve signals corresponding to the disaccharide repeating unit of ι-carrageenan. G represents the 3-linked galactosyl unit and A the 4-linked 3,6-anhydrogalactosyl unit. The signals at 66.9, 81.8, and 94.4 ppm (referenced to Me_2SO at 39.47 ppm) are proposed as the characteristic resonances of α-carrageenan.

axial 4-sulfate as found in ι- but not α-carrageenan. The presence of this band, although only of weak intensity, was revealed without ambiguity by Fourier-transform infrared spectroscopy (Fig. 2, A). The IR spectrum of Thai *C. nipae* polysaccharide was very similar to that of the Burmese sample except that the intensity of the band at 850 cm^{-1} was greater (Fig. 2, B). This suggests the presence of more ι-carrageenan in the Thai sample, being consistent with the methylation data. However, the intensity of this band is not as strong as that seen in ι-carrageenan (ex. Sigma Chemical Co., Fig. 2, C).

^{13}C NMR spectroscopy.—The ^{13}C NMR spectra of the two polysaccharides after extraction differed only in the relative intensities of the peaks at 66.9, 72.1, 91.9, and 94.4 ppm (Fig. 3). We conclude that these differences reflect the greater level of ι-carrageenan disaccharide units in the Thai sample. Twelve signals corresponding to the disaccharide repeating unit of ι-carrageenan are visible and have been labelled in each case [16]. G represents the 3-linked galactosyl unit and A the 4-linked 3,6-anhydroga-

lactosyl unit. This leaves three obvious signals at 66.9, 81.8, and 94.4 ppm which we propose are characteristic of α-carrageenan.

The ^{13}C NMR spectra of various carrageenans, including β-, κ-, and ι-carrageenan, have been fully assigned [16,17], but that for α-carrageenan has not been reported. The effects of removing a 4-sulfate ester group in going from κ- to β-carrageenan are chemical shift movements for the G4 carbon of −7.7 ppm, the G3 carbon of +1.6 ppm and the A1 carbon of −0.6 ppm [17]. If the same shifts were applied in going from ι- to α-carrageenan, one would expect α-carrageenan to give signals at 64.4, 78.3, and 91.3 ppm for G4, G3, and A1, respectively, but these are not observed. In fact, the three unknown signals are similar though not identical to those observed for β-carrageenan: β-G4 at 66.3 ppm, β-G3 at 80.3 ppm, and β-A1 at 94.5 ppm.

Minor κ- and β-carrageenan components shown to be present in the Thai *C. nipae* sample by ^{1}H NMR spectroscopy (see later) were not detected by ^{13}C NMR spectroscopy, partly because of the relatively poor sensitivity of the technique and partly because the chemical shifts for the A1 and G1 resonances for these units are so similar to those of the major α- and ι-constituents.

Our inability to utilise existing ^{13}C NMR data on β-, κ-, and ι-carrageenan to predict those of α-carrageenan can be explained if, in solution, ι-carrageenan adopts a significantly different average conformation about its glycosidic linkages than the other three carrageenans. Only ι-carrageenan has sulfate groups on adjacent sugar residues. Electrostatic interactions between these groups are likely to be repulsive (but could be attractive if mediated by a suitable cation) and are the most likely cause of such a change in conformation [18].

2D NMR spectroscopy of ι-carrageenan segments.—Because of the "anomalous" ^{13}C NMR chemical shifts identified for α-carrageenan, we considered it important to first re-evaluate the spectral assignments for ι-carrageenan and then to determine the assignments for α-carrageenan. The ^{1}H NMR spectrum of ι-carrageenan has been fully assigned previously by Welti [19] and the ^{13}C NMR spectrum by Usov and Shashkov [16], and Greer et al. [20]. Welti subsequently made corrections to his original data and reversed the assignments for H-2 and H-3 in the anhydrogalactosyl residue, the data being published in a review by Usov [21]. These authors relied partly upon analogy to chemical shifts observed for other galactan sulfates and for model compounds, although Welti also utilised data from homonuclear decoupling and spectral simulation experiments. Greer et al. used DEPT spectroscopy to identify the two methylene carbon resonances. To avoid the use of arguments involving chemical shift analogies as far as possible, we undertook $^{1}H-^{1}H$ and $^{13}C-^{1}H$ COSY 2D NMR experiments. This required the use of samples of reduced viscosity. It is thought that carrageenans contain occasional "kinking" residues which are susceptible to periodate oxidation. Polysaccharide segments of lower molecular weight and hence lower viscosity can thus be produced by sequential periodate oxidation, borohydride reduction, and very mild acid hydrolysis, as demonstrated previously for ι-carrageenan [12,19]. Samples of ι-carrageenan (ex. Sigma Chemical Co., "*Eucheuma spinosa*" presumably *E. denticulatum*) and the Thai *C. nipae* polysaccharide were, thus, subjected to this depolymerisation sequence prior to COSY analysis.

An independent assignment of the ^{1}H and ^{13}C NMR spectra of ι-carrageenan could

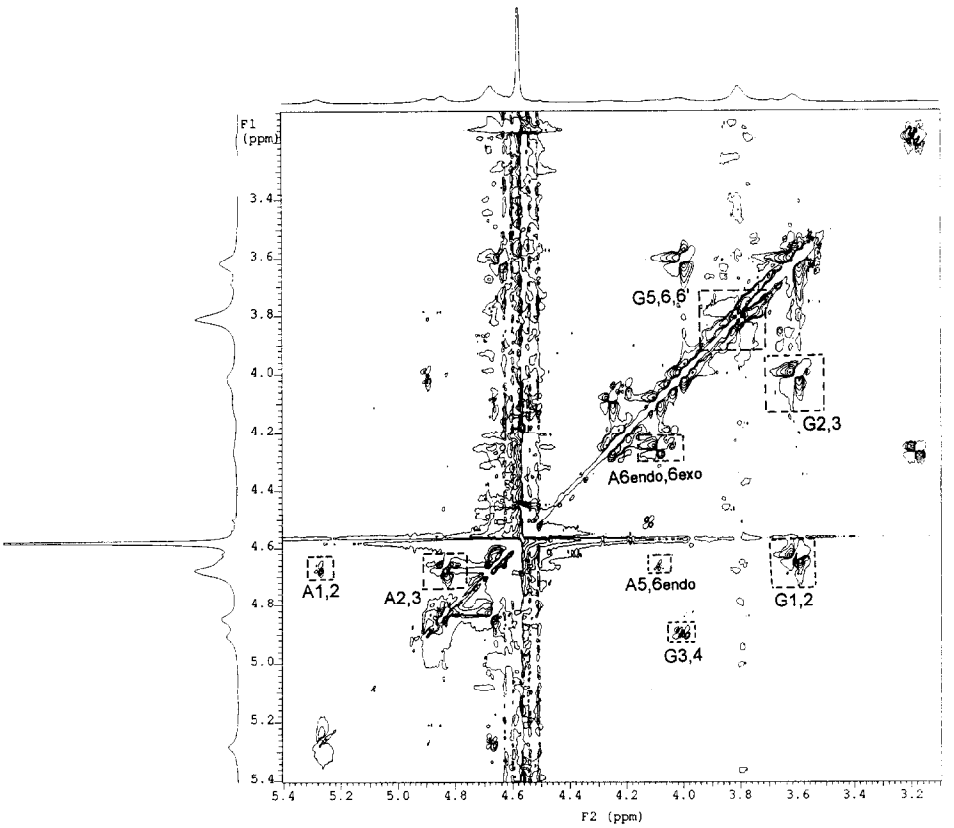


Fig. 4. $^1H-^1H$ COSY of segmented ι-carrageenan (''*Eucheuma spinosa*'' ex. Sigma Chemical Co.). A total of 470 fids were collected in 11 h at 40 °C. For each fid the sweep width was 1497.7 Hz with a data acquisition time of 0.684 s, resulting in 1K complex data points. A total of 64 scans were collected per fid with a recycle time of 1.49 s.

be made from the 2D NMR spectra of the ι-carrageenan segments in Figs. 4–6, using only: (a) knowledge of the typical values for $^1H-^1H$ coupling constants in β-D-galactopyranoside and 3,6-anhydro-α-D-galactopyranoside moieties (as detailed in ref. [19]) to predict absent $^1H-^1H$ cross-peaks; (b) data from the DEPT spectrum to identify the two methylene carbons resonances [20]; and (c) chemical shift analogies to differentiate the anomeric and methylene carbon resonances as belonging to the ι-G or ι-A residue to provide entry points for analysis of the 2D spectra.

A simplified notation is used to identify cross-peaks in the 2D spectra (Figs. 4–9). In the 2D NMR experiments reported here, the G and A residues are independent spin systems and can be discussed separately. The symbols α- or ι- indicate that the cross-peak is associated with an α- or ι-carrageenan disaccharide repeat unit, respectively, with the sulfate substitution patterns shown in Fig. 1. In the $^1H-^1H$ COSY spectrum of the ι-carrageenan segments (Fig. 4), for example, the cross-peak connecting H-1 and H-2 in the 4-linked 3,6-anhydrogalactosyl-2-sulfate residue is identified as

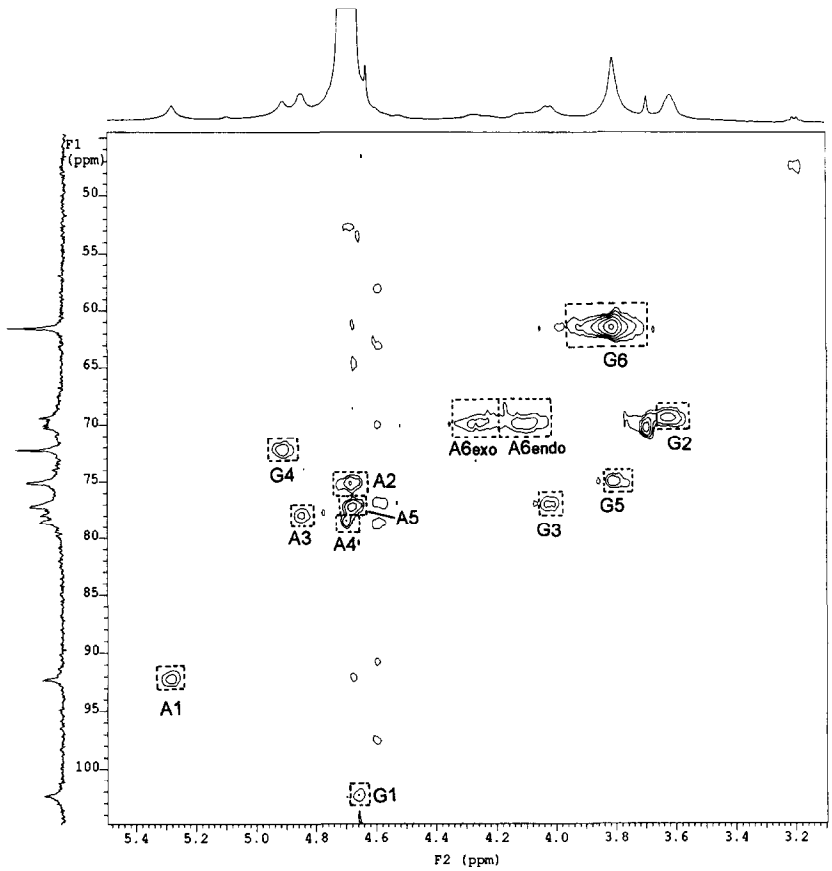


Fig. 5. ^{13}C–^{1}H COSY of segmented ι-carrageenan ("*Eucheuma spinosa*" ex. Sigma Chemical Co.). A total of 236 fids were collected in 4 h at 30 °C. For each fid the sweep width was 2488.8 Hz with a BIRD null time of 0.3 s and a data acquisition time of 0.206 s, resulting in 512 complex data points. A total of 64 scans were collected per fid with a recycle time of 1.10 s.

A1,2. In the corresponding ^{13}C–^{1}H COSY spectrum (Fig. 5), the cross-peak connecting H-1 and C-1 in the same residue is identified as A1; a single number is sufficient as only geminal connectivity is revealed.

For the ι-G residue, if the resonance at 102.4 ppm is assigned to the anomeric carbon, then the chemical shifts of H-1, -2, -3, and -4 and of C-2, -3, and -4 can be assigned unambiguously. H-4–H-5 coupling is very small in β-D-galactopyranosides, so that no ι-G4,5 cross-peak was observed; this situation was encountered in the COSY spectrum of a κ-neocarratetraose oligosaccharide [22] and for β-galactosides generally [23,24]. If the methylene resonance at 61.4 ppm is assigned to C-6, then H-6, -6′, and -5 can be located together in a multiplet centred at 3.84 ppm; their chemical shift values are sufficiently similar that no cross-peaks are discernable from the diagonal. Having located H-5, C-5 can be assigned unambiguously.

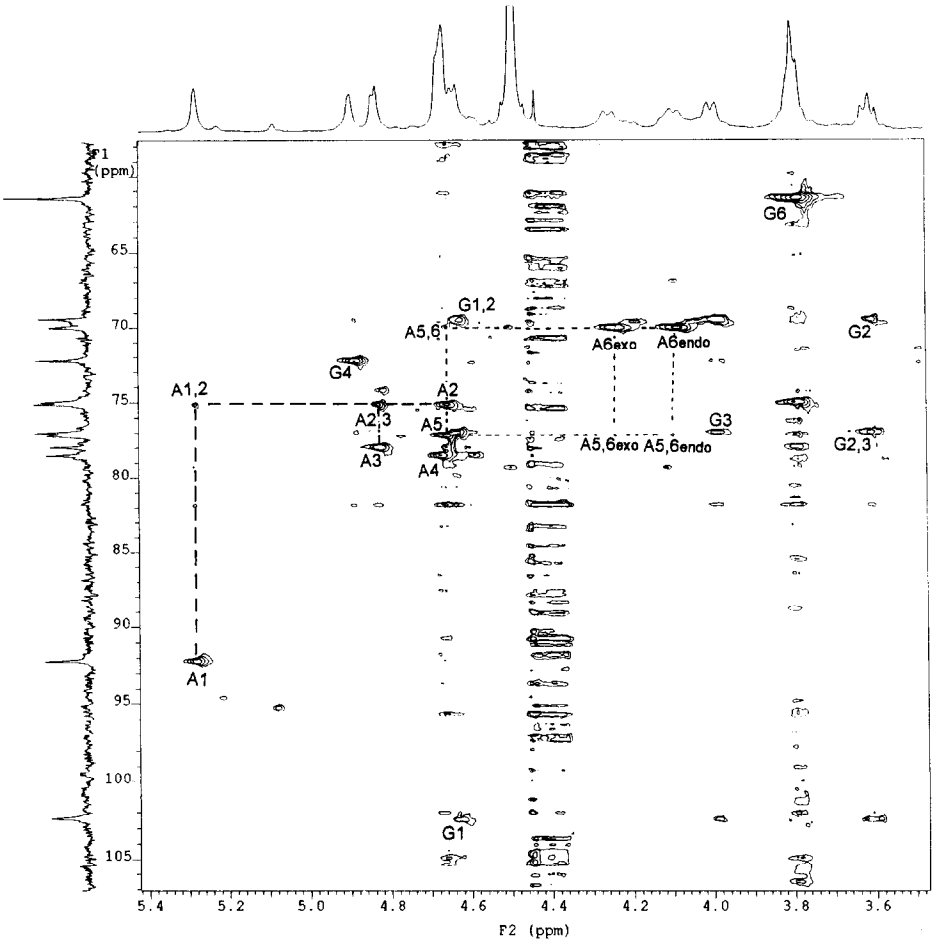
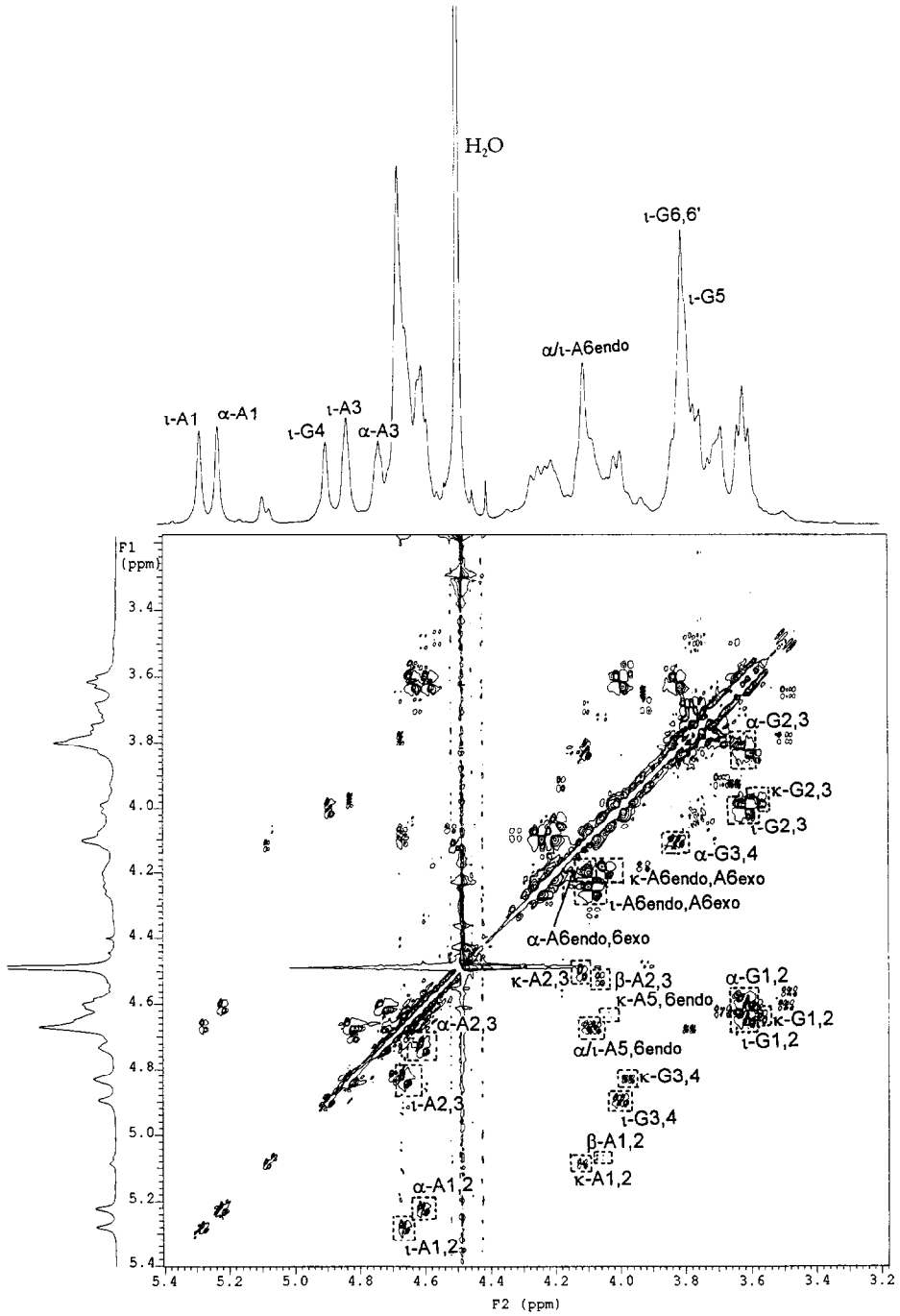


Fig. 6. $^{13}C-^{1}H$ TOCSY of segmented ι-carrageenan ("*Eucheuma spinosa*" ex. Sigma Chemical Co.). A total of 512 fids were collected in 19 h at 50 °C. For each fid the sweep width was 1297.8 Hz with a BIRD null time of 0.5 s, TOCSY mixing time of 35 ms and a data acquisition time of 0.395 s, resulting in 512 complex data points. A total of 80 scans were collected per fid with a recycle time of 1.63 s.

For the ι-A residue, if the resonance at 92.2 ppm is assigned to the anomeric carbon, then the chemical shifts of H-1 and H-2 can be unambiguously assigned. H-2 is found at 4.67 ppm, in a complex 4-proton resonance in the range 4.65–4.70 ppm. Welti had originally assigned H-2 as the separate resonance at 4.85 ppm, though on admittedly inconclusive evidence [19], and later reversed the assignments for H-2 and H-3 [21]. H-3–H-4 and H-5–H-6_{exo} coupling is very small in 3,6-anhydro-α-D-galactopyranosides, so that no intense cross-peaks connecting these protons would be expected [19,21]. H-3 can then be identified from the strong cross-peak that must be ι-A2,3 rather than ι-A3,4, and from this C-3 can be located. If the resonance at 69.9 ppm identified by a DEPT experiment as due to a methylene carbon is assigned to C-6, then H-6_{endo}, -6_{exo},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ι-G5
α/ι-A6endo
ι-A1
α-A1
ι-A3
ι-G4
α-A3
F1 (ppm)
F2 (ppm)
α-G2,3
κ-G2,3
ι-G2,3
α-G3,4
κ-A6endo,A6exo
ι-A6endo,A6exo
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β-A2,3
α-G1,2
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κ-G1,2
ι-G1,2
α/ι-A5,6endo
α-A2,3
κ-G3,4
ι-A2,3
ι-G3,4
β-A1,2
κ-A1,2
α-A1,2
ι-A1,2

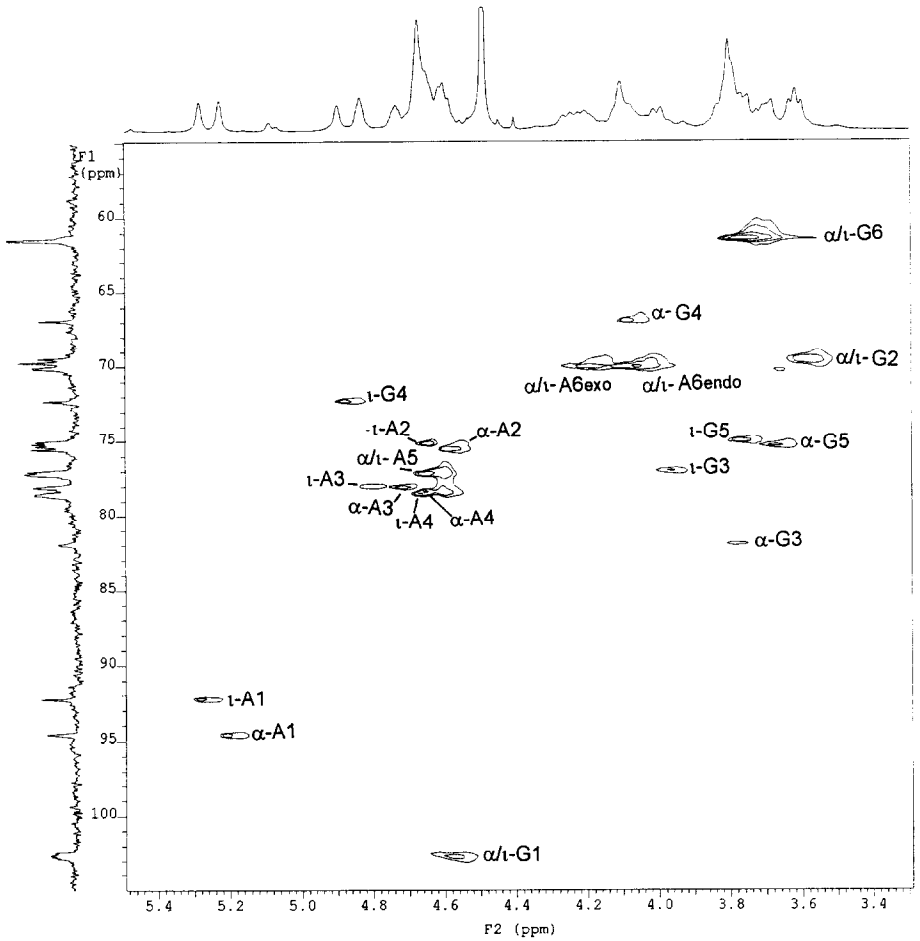

Fig. 8. $^{13}C-^{1}H$ COSY of segmented *Catenella nipae* (Thailand) polysaccharide. A total of 782 fids were collected in 14 h at 50 °C. For each fid the sweep width was 1330 Hz with a BIRD null time of 0.5 s and a data acquisition time of 0.192 s, resulting in 256 complex data points which was then zero filled to 512 complex data points. A total of 64 scans were collected per fid with a recycle time of 0.99 s.

-5, and C-5 can then be identified. Since the chemical shifts of H-2 and H-5 are the same, C-2 and C-5 cannot be unambiguously assigned in the $^{13}C-^{1}H$ COSY. A $^{13}C-^{1}H$ TOCSY was performed which connects adjoining protons and carbons (Fig. 6). In this spectrum, A-1 (92.2/5.28 ppm) can be seen to connect to A-2, and A-2 connects strongly to A-3 (77.9/4.85 ppm). Thus A-2 is assigned to (75.2/4.67 ppm). The

Fig. 7. $^{1}H-^{1}H$ COSY of segmented *Catenella nipae* (Thailand) polysaccharide. A total of 612 fids were collected at 50 °C in 23 h. For each fid the sweep width was 1248.9 Hz with a data acquisition time of 0.820 s, resulting in 1000 complex data points. A total of 64 scans were collected per fid with a recycle time of 1.92 s. A 1D ^{1}H NMR spectrum is shown on the *x*-axis (see text for aquisition details).

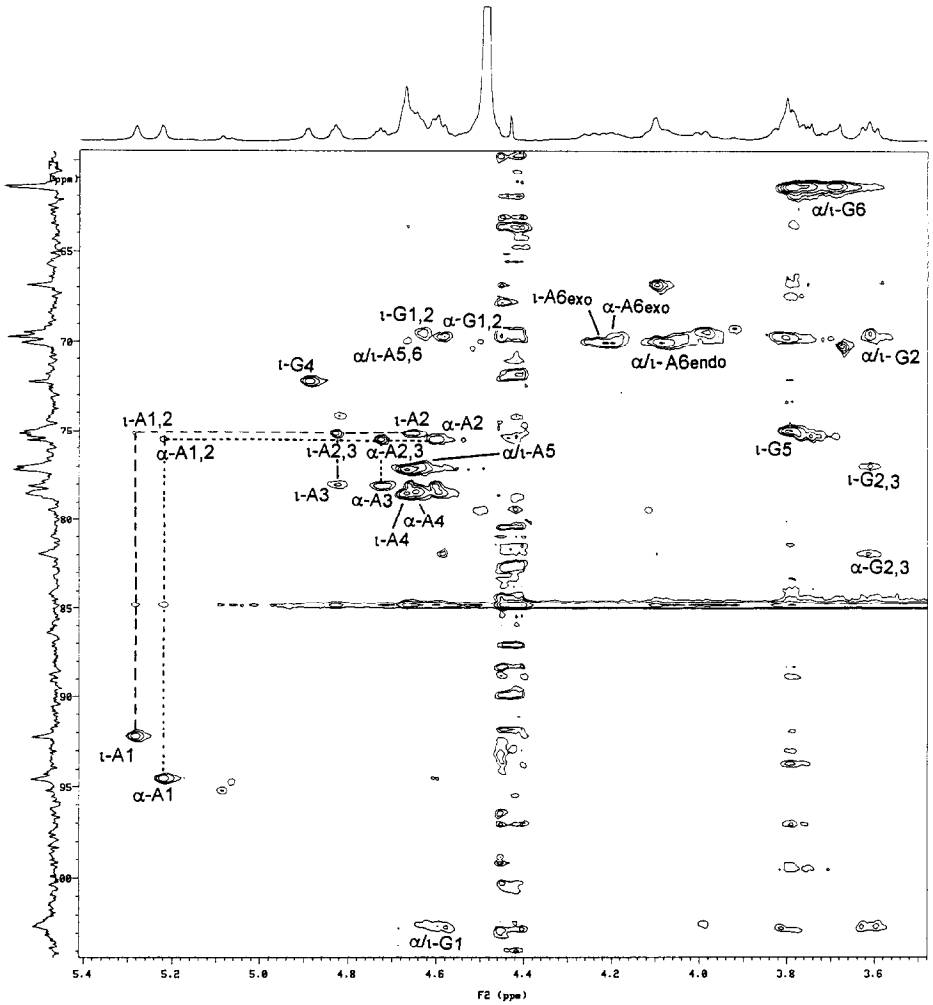

Fig. 9. $^{13}C-^{1}H$ TOCSY of segmented *Catenella nipae* (Thailand) polysaccharide. A total of 484 fids were collected in 18 h at 50 °C. For each fid the sweep width was 1750 Hz with a BIRD null time of 0.5 s, TOCSY mixing time of 70 ms and a data acquisition time of 0.293 s, resulting in 512 complex data points. A total of 80 scans were collected per fid with a recycle time of 1.66 s.

position of A-5 is confirmed by cross-peaks to A-6_{endo} and A-6_{exo}, and from it the carbon and proton shifts are obtained (77.3 and 4.67 ppm, respectively). The remaining peak is thus assigned to A-4 (78.5/4.69 ppm). The chemical shift of H-4 is slightly higher than that of H-5 and H-2, but they are not resolved in the complex four-proton resonance at 4.65–4.70 ppm in the $^{1}H-^{1}H$ COSY. Because the chemical shifts of H-4 and H-5 are so similar, no ι-A4,5 cross-peak is discernable from the diagonal.

The resulting assignments for ι-carrageenan are set out in Tables 3 and 4. Gratifyingly, our assignments are the same as those deduced previously [16,19,21], given Welti's corrected assignments for H-2 and H-3 in the ι-A residue [21]. The chemical

Table 3
Assignment of chemical shifts (ppm) in the ^{1}H NMR spectra of viscosity-reduced ι-carrageenan ("*Eucheuma spinosa*" ex. Sigma Chemical Co.) and *Catenella nipae* (Thailand)

Sample	G (3-linked residue)						A (4-linked residue)						
	H-1	H-2	H-3	H-4	H-5	H-6, -6′	H-1	H-2	H-3	H-4	H-5	H-6_{exo}	H-6_{endo}
Sigma													
ι-Carrageenan [a]	4.65	3.61	4.02	4.91	3.81	3.82	5.28	4.67	4.85	4.69	4.67	4.26	4.09
Catenella													
α-Carrageenan [b]	4.61	3.62	3.82	4.10	3.65	? [c]	5.22	4.61	4.73	4.65	4.67	4.21	4.09
ι-Carrageenan [b]	4.65	3.62	4.00	4.89	3.78	3.80	5.28	4.67	4.83	4.67	4.66	4.27	4.09
κ-Carrageenan [b]	4.64	3.56	3.97	4.84	?	?	5.09	4.12	4.50	?	4.63	4.20	4.03
β-Carrageenan [b]	?	?	?	?	?	?	5.04	4.06	4.52	?	?	?	?

[a] Spectrum acquired at 40 °C.
[b] Spectrum acquired at 50 °C.
[c] ca. 3.8 ppm.

shift values differ slightly from those previously reported, probably because our spectra were recorded at a lower temperature (30–40 °C vs. 80 °C). There was still some interference from the water peak in the ^{1}H–^{1}H COSY spectrum, even though the sample had been lyophilised from D_2O several times.

The 2D spectra from which Figs. 4 and 5 were taken revealed several resonances not associated with ι-carrageenan. These were at 47.6 and 3.20 ppm, 69.6 and 4.28 ppm, and 70.4 and 3.70 ppm for carbons and attached protons, respectively, with the proton resonances at 3.20 and 4.28 ppm being coupled. These resonances were not evident in the ^{13}C–^{1}H TOCSY spectrum from which Fig. 6 was taken, the sample having been further purified by dialysis. Knutsen et al. [25,26] have noted similar impurities arising from the dialysis tubing used in purification procedures, so care was taken to more extensively dialyse the depolymerised *Catenella* polysaccharide sample, and such peaks were of greatly reduced intensity in the resulting spectra.

2D NMR spectroscopy of α-/ι-carrageenan segments.—The methodology used for ι-carrageenan was applied to the Thai *C. nipae* polysaccharide. The ^{1}H–^{1}H and ^{13}C–^{1}H COSY spectra (Figs. 7 and 8) were recorded at 50 °C in order to shift the residual water

Table 4
Assignment of chemical shifts in the ^{13}C NMR spectra of viscosity-reduced ι-carrageenan ("*Eucheuma spinosa*" ex. Sigma Chemical Co.) and *Catenella nipae* (Thailand)

Sample	G (3-linked residue)						A (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
Sigma												
ι-Carrageenan [a]	102.4	69.4	77.0	72.2	75.0	61.5	92.2	75.2	77.9	78.5	77.3	69.9
Catenella												
ι-Carrageenan [b]	102.7	69.6	77.0	72.3	74.9	61.6	92.2	75.2	78.0	78.5	77.1	70.0
α-Carrageenan [b]	102.7	69.6	81.9	66.9	75.3	61.6	94.7	75.4	78.1	78.3	77.1	70.0

[a] Spectrum acquired at 30 °C.
[b] Spectrum acquired at 50 °C.

resonance to an area away from other signals of interest. As a result, some of the signals were shifted slightly from those in Figs. 4 and 5.

The 2D NMR spectra were assigned as though each of the constituent disaccharide repeat units occurred in separate polymer molecules or largely in block sequences within the same molecule. In such a case the spectra would just be a superposition of the spectra of the separate carrageenans. The assignments are shown in Figs. 7 and 8. Most of the off-diagonal resonances are accounted for. All the cross-peaks expected for ι-carrageenan were found. Of the remaining cross-peaks, most are due to the α- and smaller amounts of κ- and β-carrageenan components in this material.

At this point it is appropriate to address the question, would the juxtaposition of an α- and an ι-disaccharide repeat unit result in characteristic resonances in the 2D NMR spectra of an α-/ι-carrageenan? Given the relatively broad line widths encountered, only the influence of nearest neighbour residues need be considered. All G-residues in an α-/ι-carrageenan are flanked by 3,6-anhydrogalactosyl-2-sulfate residues. They differ only by the presence or absence of a directly attached 4-sulfate substituent and can be uniquely identified as ι-G or α-G residues, respectively. Their spectra should be independent of the relative distribution of the α- and ι-disaccharide units.

The situation for the A-residues is more complex. A 2-sulfated A-residue ("A2S") could be flanked by either an unsulfated or a 4-sulfated G-residue ("G" or "G4S", respectively), the combinations being:

1. –G4S–A2S–G4S–
2. –G–A2S–G4S–
3. –G4S–A2S–G–
4. –G–A2S–G–

Knutsen and Grasdalen [22], in studies of β-/κ-neocarrabiose oligosaccharides (a situation where line widths are considerably narrower), were able to demonstrate that 4-sulfation of a 3-linked galactosyl residue had a far greater effect on the adjacent anhydrogalactosyl residue towards the nonreducing terminus of the oligosaccharide. In this residue H-1, -2, and -6_{endo}, which are spatially close to the sulfate group, were deshielded by 0.02, 0.06, and 0.05 ppm, respectively, and the rest of the protons were shielded to a small extent. The adjacent anhydrogalactosyl residue towards the reducing terminus, which is more remote from the sulfate substituent, was less affected, with no change in H-1 and H-2, a small deshielding of H-3 (< 0.01 ppm), and only small changes in the shifts of the other protons. Although there is no equivalent ^{13}C NMR data, and the situation could be different in an α-/ι-carrageenan, we propose that the ^{1}H and ^{13}C resonances of the 2-sulfated A-residues in an α-/ι-carrageenan would similarly be affected only by the nature of the adjacent G-residue towards the reducing terminus. In this case, the A-residue in sequences (1) and (2) would give resonances characteristic of an ι-carrageenan, and those in the sequences (3) and (4) would give resonances characteristic of an α-carrageenan.

We conclude, therefore, that it would be difficult to detect differences in the NMR spectra between samples containing adjacent α- and ι-disaccharide repeat units or only contiguous α- or ι-disaccharide sequences. The somewhat misshapen cross-peaks assigned to α-A4 and α-A5 in the $^{13}C-^{1}H$ COSY spectrum (Fig. 8) are perhaps the only candidates.

Thus the $^{1}H-^{1}H$ COSY spectrum (Fig. 7) reveals the A1 proton for ι-carrageenan is at 5.28 ppm, and that for α-carrageenan is at 5.22 ppm. The α-A1,2 and α-A2,3 cross-peaks are visible. No α-A3,4 connection is observed, which is consistent with a negligible value for $J_{3,4}$ in 3,6-anhydrogalactosides, so it is not possible to locate α-A4. Two cross-peaks are visible close to that of ι-$A6_{endo}$,6_{exo}. We have assigned the weaker one to κ-$A6_{endo}$,6_{exo} (which is in agreement with Welti's assignments [19]) and the more intense one to α-$A6_{endo}$,6_{exo}. Thus the α- and ι-$A6_{endo}$ protons are coincident but the α-$A6_{exo}$ proton has a lower ^{1}H chemical shift than ι-$A6_{exo}$. Only one further cross-peak is visible in the α-/ι-$A6_{endo}$ plane which suggests that the α- and ι-A5 protons are also coincident.

There is a strong cross-peak close to that of ι-G1,2. We have assigned this to α-G1,2 and have thus pinpointed the α-G1 and α-G2 proton signals. The α-G2 proton is, in fact, coincident with the ι-G2 proton; however, an intense cross-peak leads to α-G3 at 3.82 ppm, whereas the ι-G3 proton is at 4.00 ppm. The α-G3 proton is connected to a proton at 4.10 ppm, which we assign to α-G4. The $^{1}H-^{1}H$ COSY also shows a complex region around 3.8 ppm, which we consider to be due to the α-G5 and α-G6 protons. The region is too complex to resolve, as it also contains the corresponding proton signals for ι-carrageenan. Cross-peaks corresponding to Welti's assignments of κ-G1, G2, G3, and G4 are observed and marked as such. Some cross-peaks still remain unassigned but may be due to pyruvated galactosyl units (see later). Our assignments for the proton spectrum of the Thai *C. nipae* polysaccharide are shown in Table 3. Having assigned most of the proton spectrum, we can now consider the ^{13}C NMR spectrum of the Thai *C. nipae* through the $^{13}C-^{1}H$ COSY, Fig. 8. Thus the ^{13}C signals at 66.9, 81.9, and 94.7 ppm are assigned to α-G4, α-G3, and α-A1, respectively. These signals are 0.1, 2.9, and 2.6 ppm higher than those predicted by computer modelling for α-carrageenan [27]. The database of computer-generated shifts was considered a starting point for subsequent experimental determinations [28]. Some of the carbon signals for α-carrageenan are coincident with those of ι-carrageenan, but tentative assignments have been made (Table 4). The peak at 75.3/3.65 ppm close to ι-G5 has been assigned to α-G5. Thus the shift for this proton is obtained, which was not possible from the $^{1}H-^{1}H$ COSY. The ι- and α-G6 signals overlap too much in the proton dimension of the $^{13}C-^{1}H$ COSY to pinpoint a proton shift for α-G6. Both the $^{13}C-^{1}H$ COSY and $^{13}C-^{1}H$ TOCSY (Fig. 9) for Thai *C. nipae* show a small peak close to ι-A4, which we assign to α-A4. The $^{13}C-^{1}H$ TOCSY also confirms some of our other assignments for α-carrageenan.

An additional signal was observed in the ^{13}C NMR spectrum of the segmented Thai *C. nipae* sample at 25 ppm, which is indicative of the methyl group of a pyruvate ketal. This is consistent with the presence of 3,4,6-Gal from the methylation analysis. This signal was not observed in the ^{13}C NMR spectrum of the untreated sample, but minor components are often obscured in the spectrum of viscous polysaccharide samples.

4. Conclusions

Results from spectroscopic and chemical analyses have established the presence of the sequence of sugar residues characteristic of α-carrageenan (i.e., 3-linked β-D-galac-

topyranosyl units flanked by 4-linked 3,6-anhydro-α-D-galactopyranosyl-2-sulfate residues) in *C. nipae* from Thailand and confirmed it in *C. nipae* from Burma. The sequence of residues characteristic of ι-carrageenan were also observed.

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