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O-Succinyl derivative of ι-carrageenan fragments: Synthesis and characterization

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Abstract

ι-Carrageenan was depolymerized in mild hydrochloric acid condition. Tetrabutylammonium (TBA) salt of ι-carrageenan fragments was acylated with succinic anhydride, 4-dimethylaminopyridine and tributylamine under homogeneous conditions in *N*,*N*-dimethylformamide at 80 °C. Investigation of FT-IR spectrum of the succinylated product showed that a monoester derivative with succinyl group was formed when ι-carrageenan reacted with succinic anhydride. The ^{1}H and ^{13}C NMR spectroscopy has been used to characterize the fine structure of *O*-succinyl derivative of the ι-carrageenan. The ^{13}C and ^{1}H NMR chemical shifts of disaccharide unit of *O*-succinyl ι-carrageenan have been fully assigned using 2D NMR spectroscopic techniques.

Keywords: ι-Carrageenan; Depolymerization; Acylation; Succinic anhydride

1. Introduction

Carrageenans are water soluble linear polysaccharides composed of alternating α(1 → 3) and β(1 → 4) linked D-galactose residues. (Janaswamy & Chandrasekaran, 2002; Myslabodski, Stancioff, & Heckert, 1996). Three primary forms (κ-, ι-, λ-) of carrageenan are identified based on the modification of the disaccharide repeating unit resulting from the occurrence of ester sulfate, or anhydride formation in the 4-linked residue (Karlsson & Singh, 1999), and the corresponding IUPAC inspired names and letter codes are carrageenose 4′-sulfate (G4S–DA), carrageenose 2,4′-disulfate (G4S–DA2S), and carrageenan 2,6,2′-trisulfate (G2S–D2S, 6S).

The carrageenans are the first natural sulfated polysaccharides found to have anti-human immunodeficiency virus (HIV) activity (Nakashima et al., 1987). Interest in the biological activity and pharmaceutical value of sulfated polysaccharides has increased in the last decade. The sulfated polysaccharides, including heparin, dextran sulfate, pentosan polysulfate and others, have been shown to inhibit the replication of HIV (Witvrouw, Desmyter, & De Clercq, 1994). In general, the anti-HIV activity of these compounds is correlated to their anionic character. Increasing in density of negative charge of sulfated polysaccharides favors their anti-HIV activity (Herold et al., 1995). On the other hand, the flexible of backbone of sulfated polysaccharides affect the anti-HIV activity of these compounds, too. The less flexible the backbone of sulfated polysaccharides is, the higher anti-HIV activity of these compounds. However, the flexible of carrageenan molecular, for instance irregular helical form, was found to increase with increasing 3,6-anhydro-α-D-galactopyranosyl units, so the anti-HIV activity of λ-carrageenan is higher than that of κ- and ι-carrageenan (Carlucci et al., 1997). Previously, we have reported the chemical methods to prepare succinylated and maleoylated κ-carrageenan fragments and the structure of the products (Jiang & Guo, 2005; Jiang, Guo, & Tian, 2005).

We undertook the present work on *O*-succinyl derivative of ι-carrageenan fragments for the following purpose:

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introduction of free carboxyl into ι-carrageenan was executed by reaction of monocarboxyl of succinic anhydride with hydroxyl groups of ι-carrageenan to increase the density of the negative charge; these additional charges and the steric hindrance of succinyl carboxylate group at the 2-position of G4S unit will prevent helical formation in iota carrageenan and decrease its chain flexibility. We describe herein the preparation of succinylated ι-carrageenan. The structure of *O*-succinyl derivative of ι-carrageenan is characterized by using FT-IR and NMR spectroscopy.

2. Experimental

2.1. Depolymerization of ι-carrageenan

ι-Carrageenan (lot no. 117H-0151) was purchased from Sigma (St. Louis, MO, USA). Partial hydrolysis was conducted as follow: a solution of ι-carrageenan (2.5 g) in 0.1 M hydrochloric acid (250 ml) was heated at 60 °C and kept for 100 min. The degradation was terminated by neutralization with 0.1 M sodium hydroxide, and then the solution was dialyzed against distilled water for 6 days. The dialyzed solution was filtered through a Millipore membrane (GS, 0.45 μm), and the filtered solution was concentrated in vacuum to a small volume, and then percolated through a column (100 ml) of 732 type (H^+) cation-exchange resin at 4 °C. The pH of the solution was adjusted to 8 by addition of tetrabutylammonium and the TBA salt was obtained after lyophilization and drying for 24 h at 40 °C under vacuum.

2.2. O-Succinyl derivative of ι-carrageenan fragments

A solution of ι-carrageenan TBA salt (986 mg, corresponding to 2.08 mmol of OH group) in dry *N*,*N*-dimethylformamide (DMF) (20 ml) under N_2 was added to 4-dimethylaminopyridine (58.9 mg, 0.59 mmol), succinic anhydride (612 mg, 6.24 mmol), and tributylamine (1.50 ml, 6.24 mmol). The solution was heated at 80 °C for 6 h. After the reaction, the mixture was cooled to 4 °C in ice water, and then a cold saturated ethanolic solution of sodium acetate (150 ml) was added. The mixture was allowed to stand for 1 h at 4 °C. After centrifugation, the precipitate was dissolved in distilled water, and dialyzed against 5% aq sodium hydrogen carbonate for 2 days, followed by distilled water for 4 days. The solution was lyophilized to give the *O*-succinyl ι-carrageenan fragments.

2.3. Characterization

The molecular weight of ι-carrageenan derivative was determined from gel permeation chromatography (GPC) on a Shimadzu LC-6A apparatus (column, Asahipak GS-220H, GS-310H, and GS-510H) with dextran sulfate as standard. The column set was kept at 30 °C. The mobile phase was 0.1 M $NaNO_3$ (pH 7.0) at a flow rate of 1.0 ml min^{-1}.

FT-IR spectra were obtained by using a Nicolet Magna 750 spectrometer. Sample was ground together KBr in an agate mortar before pressing to form a optically clear pellet. The spectral resolution was 4 cm^{-1}.

^1H spectrum of D_2O solution was recorded at 35 °C on a Bruker AVANCE DRX-500 spectrometer with a 5-mm ^1H probe. The parameters were as follows: pulse angle: 90°; acquisition time: 3 s; relaxation delay: 2 s; scans: 32; spectral width, 5251 Hz. Chemical shifts (δ) were given in ppm relative to the water peak ($\delta = 4.68$ ppm). ^{13}C NMR of MeOD solution was run at 35 °C using a Bruker AVANCE DRX-500 spectrometer with a 5-mm ^1H probe (125 MHz, 9036 scans, 1 s acquisition time, 1.8 s relaxation delay time, 30,303 Hz spectral width).

$^1H/^1H$ COSY measurement was performed with a Bruker AVANCE DRX-500 apparatus with a 1024×512 data matrix. A 4194 Hz spectral width was used in both dimensions. The spectrum was processed by using a sine-bell filtering function in both dimensions after zero-filling to a 1024×512 matrix. $^1H/^{13}C$ HMQC spectrum was acquired using a 1024×256 data matrix on a Bruker AVANCE DRX-500 spectrometer, and an acquisition time was 0.157 s.

3. Results and discussion

3.1. Depolymerization of ι-carrageenan

Common methods for depolymerization of carrageenan include acid hydrolysis (Hjerde, Smidsrød, & Christensen, 1996; Karlsson & Singh, 1999; Yu et al., 2002), reductive hydrolysis (Falshaw, Furneaux, & Wong, 2003; Stevenson & Furneaux, 1991), methanolysis (Knutsen & Grasdalen, 1992), and active oxygen species fragmentation (Yamada, Ogamo, Saito, Uchiyama, & Nakagawa, 2000; Yamada et al., 1997). ι-Carrageenan is stable to desulfation during mild acid hydrolysis (Karlsson & Singh, 1999; Yu et al.,

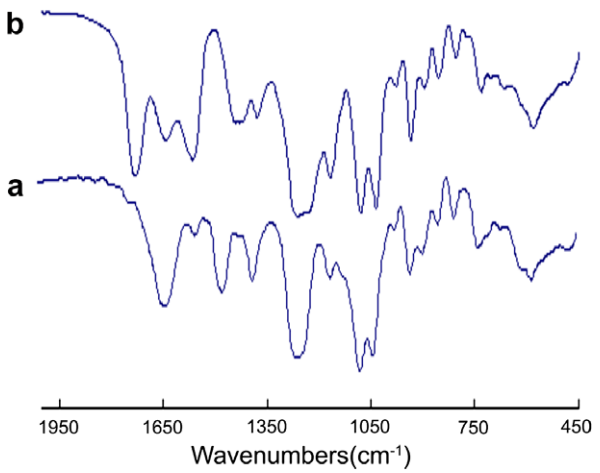

Fig. 1. FT-IR spectra of ι-carrageenan fragments and its *O*-succinyl derivative. (a) Starting ι-carrageenan fragments; (b) *O*-succinyl ι-carrageenan fragments.

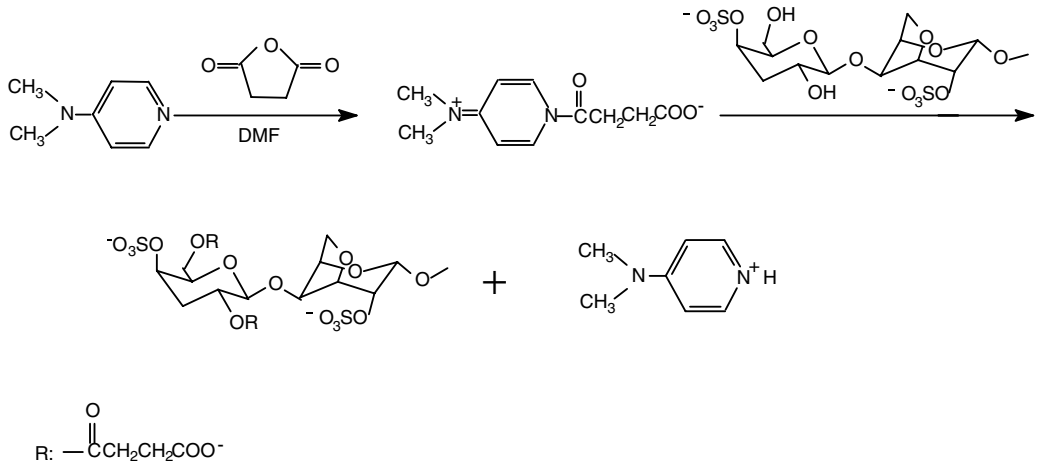

Fig. 2. Synthesis of *O*-succinyl ι-carrageenan fragments.

2002). Therefore, depolymerization of ι-carrageenan was performed in 0.1 M hydrochloric acid at 60 °C. FT-IR spectrum of ι-carrageenan fragments is shown in Fig. 1a. Absorption peaks appearing at 1250 cm^{-1}, 850 cm^{-1} and 805 cm^{-1} are contributed to sulfate group, galactose-4-sulfate and 3, 6-anhydrogalactose-2-sulfate, respectively. Meanwhile, the band at 930 cm^{-1} is characteristic of C–O of 3, 6-anhydrogalactose. These characteristic absorption peaks found in the FT-IR spectra are indicative of the structural integrity of ι-carrageenan in the degradation process.

3.2. *O*-Succinyl derivative of ι-carrageenan

Yamada et al. (2000) synthesized *O*-acylated derivatives of κ-carrageenans by introducing straight chain carboxylic acids of different length (C4, C6, C12). Three free hydroxyl groups of κ-carrageenan were all acylated by using *N*,*N*-dimethylformamide as the solvent in the presence of 4-dimethylaminopyridine and tributylamine as catalysts at room temperature. We found that *O*-succinyl derivative of ι-carrageenan was not obtained under these conditions. Petitou et al. (1992) acylated the tributylammonium salt of dermatan sulfate using succinic anhydride as acylating agent and DMF as solvent in the presence of a catalytic amount of 4-dimethylaminopyridine at 60 °C. Under similar reaction conditions, succinylation of the tetrabutylammonium salt of ι-carrageenan fragments proceeded successfully at 80 °C. The reaction formula for ι-carrageenan reacted with succinic anhydride is shown in Fig. 2. The weight average molecular weight of ι-carrageenan derivative is 38,500. Fig. 1b shows the FT-IR spectrum of product after the reaction for 6 h. Observation of the region around 1740 cm^{-1} indicates the carbonyl stretching vibration of ester linkage. The absorption band at 1590 cm^{-1} and 1430 cm^{-1} are attributed to asymmetrical stretching vibration and symmetrical stretching vibration of $–COO^-$ of carboxylate, respectively (Ke & Dong, 1998). This

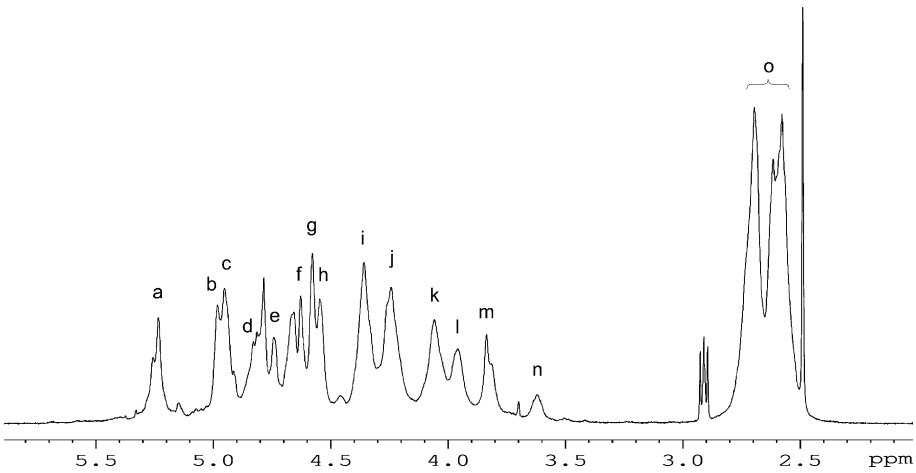

Fig. 3. ^1H NMR spectrum of *O*-succinyl ι-carrageenan fragments. a, DA2S-1; b, G4S-4; c, G4S-2; d, G4S-1; e, DA2S-3; f, DA2S-4; g, DA2S-5; h, DA2S-2; i, G4S-6; j, G4S-3 and DA2S-6b; k, G4S-5; l, DA2S-6a; m, G4S-5 and G4S-6 unsubstituted; n, G4S-2 unsubstituted; o, methylene of acyl chain.

Table 1
Assignment of *O*-succinyl ι-carrageenan fragments chemical shifts (ppm) in the ^{1}H and ^{13}C NMR spectra

	G4S (3-linked unit)						DA2S (4-linked unit)						Carboxyl of easter linkage	Free carboxyl	Methylene
	1	2	3	4	5	6	1	2	3	4	5	6			
Proton	4.83	4.96(3.62)	4.26	4.98	4.06	4.36(3.32)	5.23	4.55	4.74	4.63	4.58	3.96[a]/4.26[b]			2.58–2.69
Carbon	102.2	72.6	76.5	74.5	74.5	65.9(63.5)	94.5	76.8	79.9	80.6	78.9	72.1	177.1[c] 177.9[d]	182.7	32.6–33.6

Chemical shits of proton and carbon unsubstituted are shown in parentheses.
[a] DA2S H-6a.
[b] DA2S H-6b.
[c] Carbonyl carbon of ester linkage linked to G4S-2.
[d] Carbonyl carbon of ester linkage linked to G4S-6.

revealed that a monoester derivative with succinyl group was formed when ι-carrageenan reacted with succinic anhydride.

3.3. ^{1}H NMR spectroscopy

The ^{1}H NMR spectrum of *O*-succinyl ι-carrageenan was well resolved (Fig. 3). Twelve major signals at 3.9–5.3 ppm region are visible for the major disaccharide repeating unit of *O*-succinyl ι-carrageenan. A multiplet was present at 2.50–2.80 ppm region, corresponding to the methylene proton of succinyl group. The methylene proton number was obtained by integrating the proton signals of the acyl chain. The degree of substitution of the product is given by:

$$\mathrm{DS} = \text{proton number of } \delta\ 2.50 - 2.80/4 = 6.0/4 = 1.5.$$

Small signal at 3.62 ppm was assigned to G4S-2 proton unsubstituted, and the corresponding proton numbers is 0.19. In addition, signal was observed at 3.82 ppm corresponding to G4S-6 protons unsubstituted whose resonance overlaps with that of G4S H-5 (Fig. 3, Table 1). The integration of this signal showed that the corresponding proton numbers is 0.90.

3.4. ^{13}C NMR spectroscopy

^{13}C NMR studies on acetylated derivatives of oligo- and polysaccharides have demonstrated that the chemical shift of carbonyl carbon signals is remarkably sensitive to their position of substitution in the sugar residue, to the type of sugar residue, and to the mode of the linkage between them (Goux & Weber, 1993). A full-range ^{13}C NMR spectrum of *O*-succinyl ι-carrageenan is shown in Fig. 4. There are signals at 177.9 ppm and 177.1 ppm corresponding to the carbonyl carbons of ester linkage linked to G4S-6 and G4S-2, respectively. However, the carbonyl carbon signal of free carboxyl overlaps at 182.7 ppm.

The carbon signal of disaccharide repeating unit of ι-carrageenan substituted group at 60–110 ppm. In addition, there are signals at 32.6–33.6 ppm corresponding to the succinyl methylene carbons. A small signal is observed at 63.5 ppm corresponding to G4S-6 carbon unsubstituted (Fig. 4 and Table 1).

3.5. 2D NMR spectroscopy

For the 3-linked (G4S) unit of *O*-succinyl ι-carrageenan, if the resonances at 102.2 ppm and 65.85 ppm are assigned to C-1 and C-6, then H-1 and H-6 can be located at 4.83 and 4.36 ppm, respectively, from the $^{1}H/^{13}C$ HMQC spectrum (Fig. 5). The H-2 signal at 4.96 ppm shows correlations to H-1 and to H-3 at 4.26 ppm in the $^{1}H/^{1}H$ COSY spectrum (Fig. 6). The H-6 coupling with H-5 leads to the identification of the H-5 at 4.06 ppm. H-3–H-4 and H-4–H-5 coupling is so small that no corresponding cross-peaks would be observed. The $^{1}H/^{13}C$ HMQC spectrum allowed the transfer of the H-2, H-3 and H-5 proton

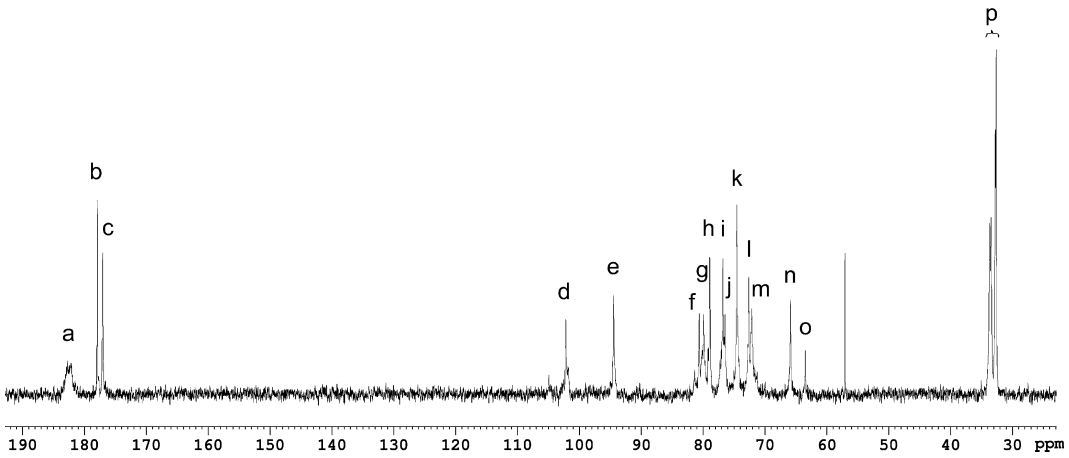

Fig. 4. Full-range ^{13}C NMR spectrum of *O*-succinyl LMW κ-carrageenan. a, Free carboxyl; b, carbonyl carbon of ester linkage linked to G4S-6; c, carbonyl carbon of ester linkage linked to G4S-2; d, G4S-1; e, DA2S-1; f, DA2S-4; g, DA2S-3; h, DA2S-5; i, DA2S-2; j, G4s-3; k, G4S-4, 5; l, G4S-2; m, DA2S-6; n, G4S-6; o, G4S-6 unsubstituted; p, methylene of acyl chain.

assignments to the corresponding carbon atoms (Fig. 5 and Table 1).

For the 4-linked (DA2S) unit of *O*-succinyl ι-carrageenan, if the resonance at 94.5 ppm is assigned to the anomeric carbon, then the signal at 5.23 ppm can be assigned to H-1 from the $^{1}H/^{13}C$ HMQC spectrum (Fig. 5). The correlation between H-1 and H-2 allows the identification of the H-2 at 4.55 ppm which correlates to the H-3 at 4.74 ppm in the

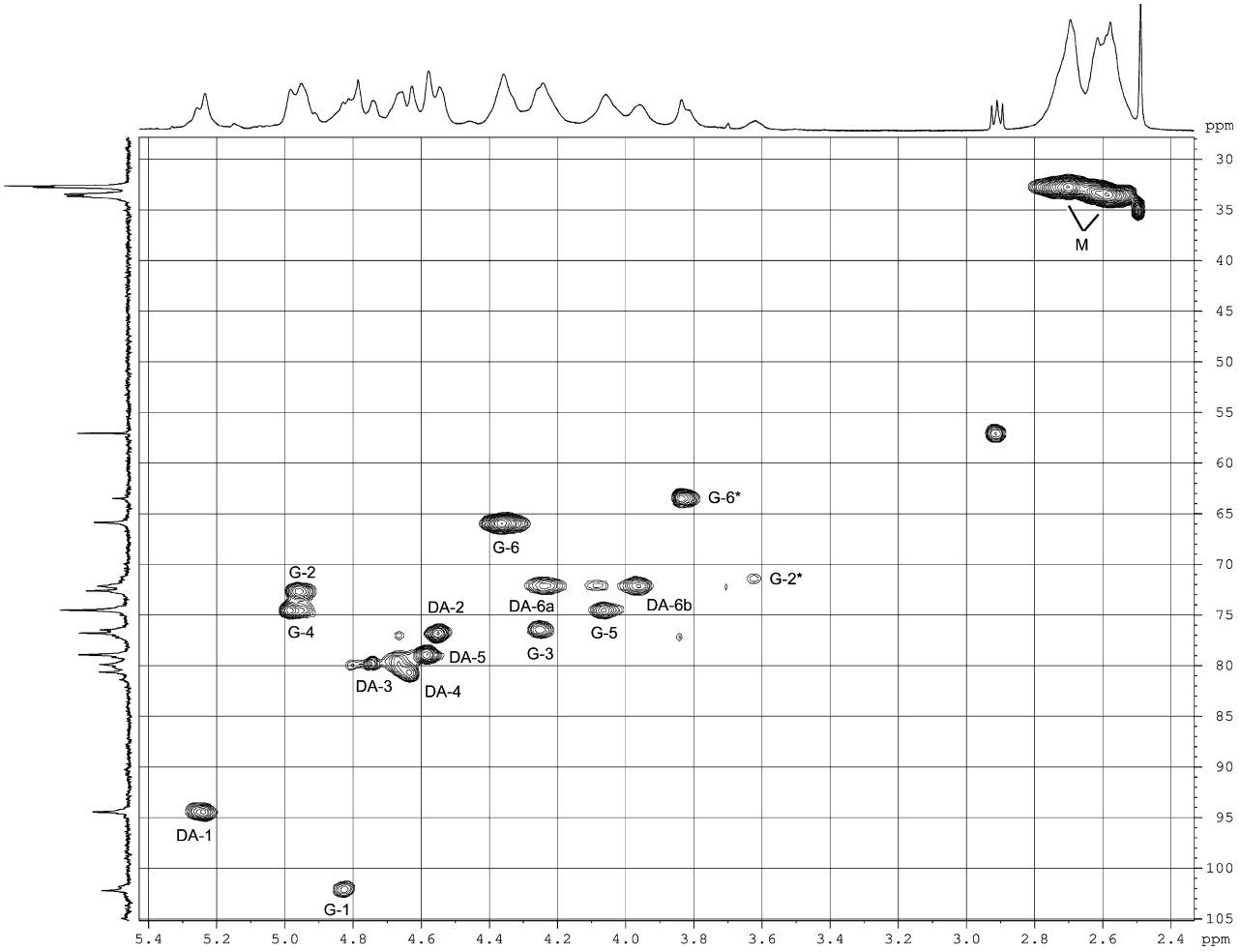

Fig. 5. $^{1}H/^{13}C$ HMQC spectrum of *O*-succinyl ι-carrageenan fragments. G-6*, G4S-6 unsubstituted; G-2*, G4S-2 unsubstituted; M, methylene of acyl chain (G4S abbreviated to G and DA2S abbreviated to DA for clarity).

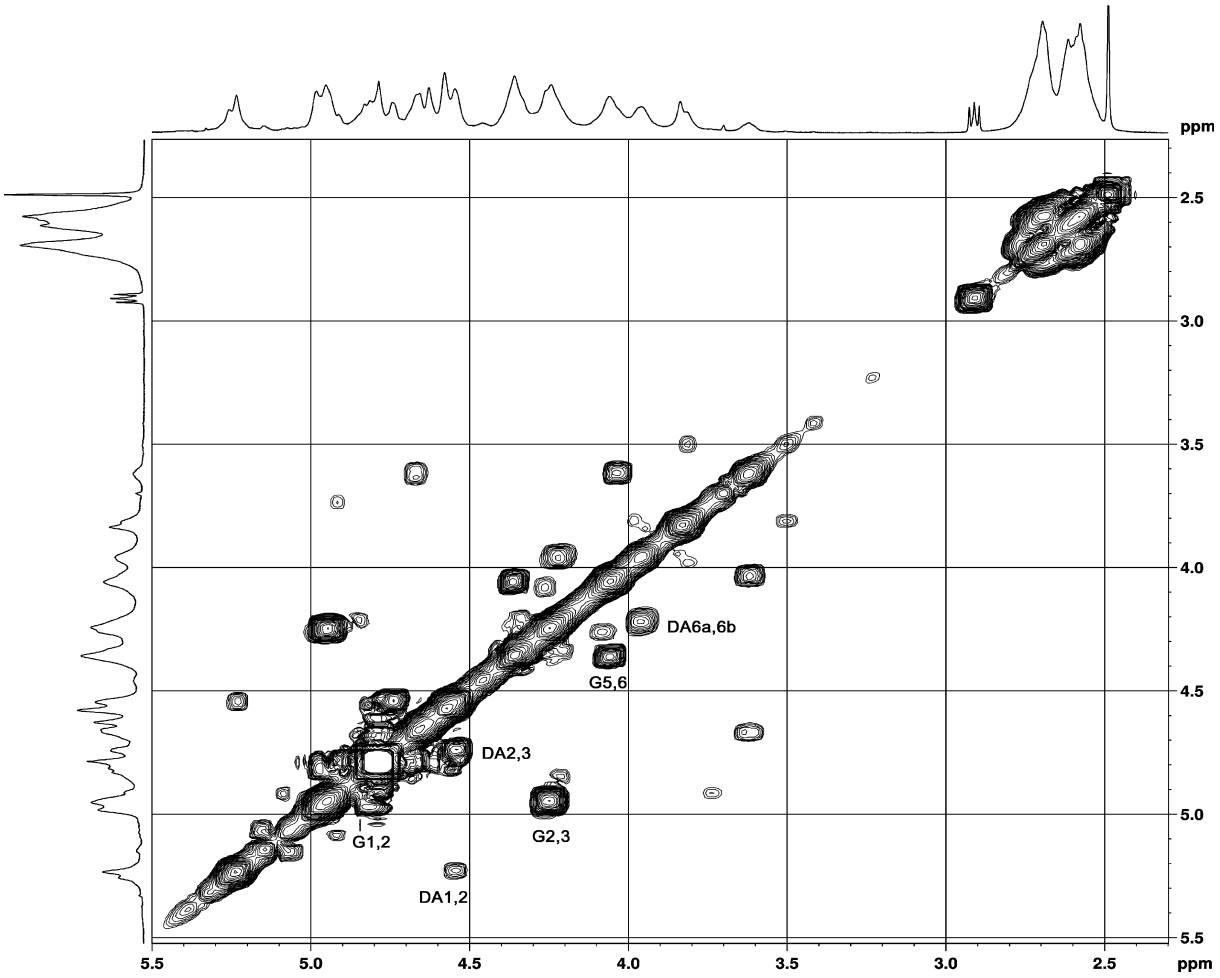

Fig. 6. $^{1}H/^{1}H$ COSY spectrum of *O*-succinyl ι-carrageenan fragments (G4S abbreviated to G and DA2S abbreviated to DA for clarity).

$^{1}H/^{1}H$ COSY spectrum (Fig. 6). However, the two protons, H-6a at 3.96 ppm and H-6b at 4.26 ppm whose resonance overlaps with that of G4S H-3 located in the same region, were easily identified due to their correlation to C-6 at 72.1 ppm from the $^{1}H/^{13}C$ HMQC spectrum. Furthermore, an H-6a, 6b cross-peak was also observed in the $^{1}H/^{1}H$ COSY spectrum. The connectivities between H-3 and H-4, H-4 and H-5 and H-5 and H-6 are missing due to small spin–spin coupling. Having located H-2 and H-3, the corresponding carbon atoms were assigned unambiguously (Fig. 5 and Table 1). Obviously, all connectivities of DA unit, except those between H-3 and H-4, H-4 and H-5 and H-5 and H-6, are clearly displayed in the $^{1}H/^{1}H$ COSY spectrum.

The $^{1}H/^{13}C$ HMBC spectrum (Fig. 7) confirmed all the assignments made using the $^{1}H/^{1}H$ COSY and $^{1}H/^{13}C$ HMQC spectra. Meanwhile, the chemical shifts of protons and carbon atoms at 4- and 5-positions of DA2S unit would be assigned from the $^{1}H/^{13}C$ HMBC spectrum. H-4 of DA2S unit was identified at 4.63 ppm from the long-range correlation between DA2S H-4 and G4S C-1. DA2S C-6 signal shows long-range correlation to DA2S H-5 at 4.58 ppm. Having determined the proton chemical shifts, the corresponding carbon shifts can thus be determined (i.e., 80.6 ppm for C-4 and 78.9 ppm for C-5) from the $^{1}H/^{13}C$ COSY spectrum (Fig. 5). The remainder of the long-range correlations are shown in Fig. 7. This then leaves G4S H-4 at 4.98 ppm which correlates to the G4S C-4 at 74.5 ppm whose signal overlaps with G4S C-5 in the $^{1}H/^{13}C$ HMQC spectrum (Fig. 7). In addition, signals of carboxyl carbon of ester linkage at 177.1 ppm and 177.9 ppm show long-range correlations to G4S H-2 and G4S H-6, respectively.

The effect of succinyl substitution at the 2- and 6-positions of G4S unit of ι-carrageenan on the chemical shifts of these carbons of its disaccharide repeating unit is shown in Table 2. In general, this substitution has most impact on the chemical shifts of the substituted carbon atoms (i.e., G4S C-2 and G4S C-6, the α-effect) and those adjacent to them (i.e., G4S C-1, G4S C-3 and G4S C-5, the β-effect). The signals for C-2 and C-6 of the G4S units were all shifted downfield, while that of C-1, C-3, C-5 were shifted upfield due to shielding effect of carbonyl of the succinyl groups at the 2- and 6-positions of G4S unit. Succinyl substitution at the 2- and 6-positions of G4S unit has a much smaller effect on carbon atoms of DA2S unit.

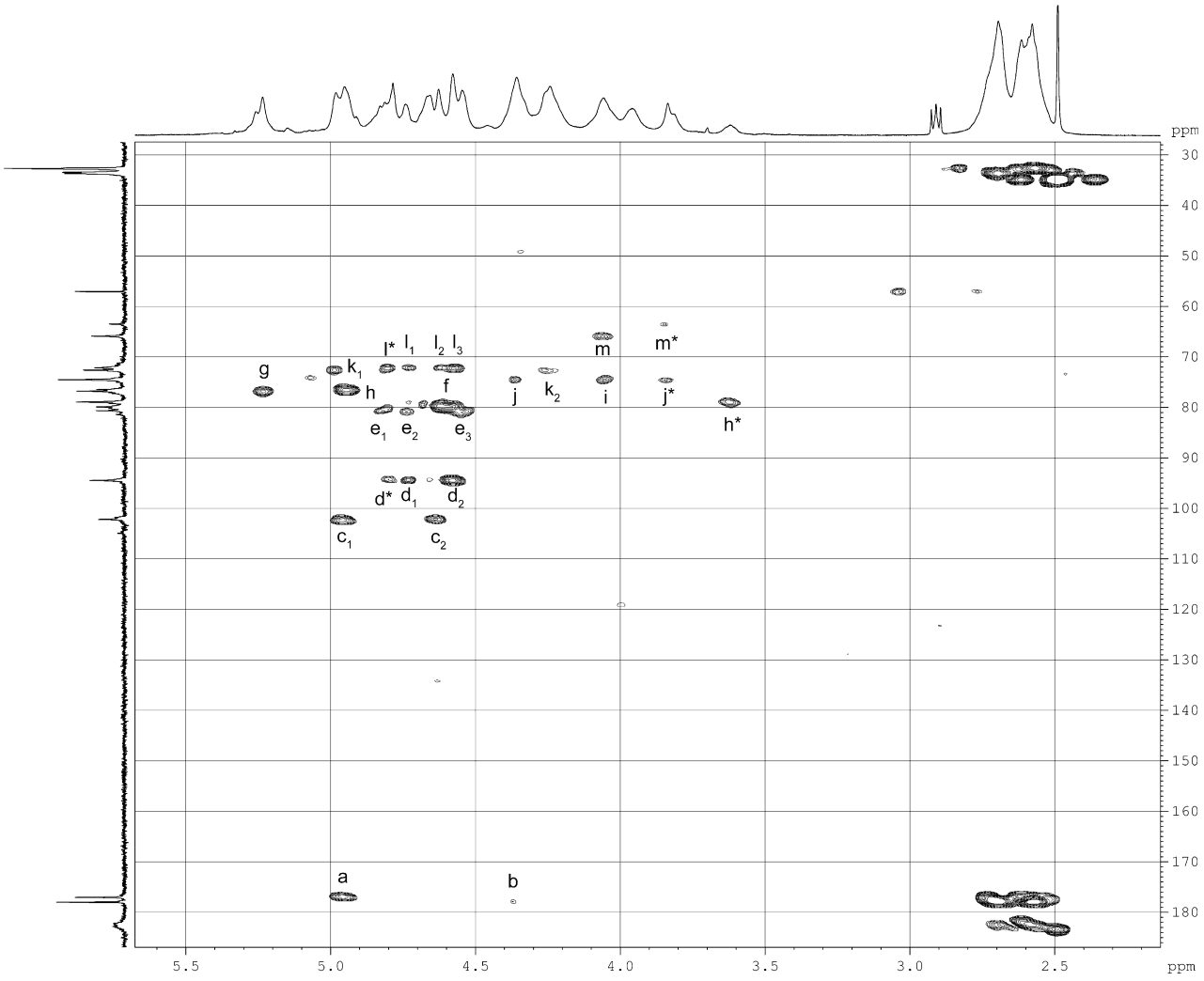

Fig. 7. HMBC spectrum of *O*-succinyl ι-carrageenan fragments. a, C=O/G H-2; b, C=O/G H-6; c_1, c_2: G C-1/ H-2, DA H-4; d_1, d_2: DA C-1/H-3, H-2; e_1, e_2, e_3: DA C-4/G H-1, DA H-3, H-2; f, DA C-3/H-4; g, DA C-2/H-1; h, G C-3/H-2; i, G C-4/H-5; j, G C-5/H-6; k_1, k_2: G C-2/H-4, H-3; l_1, l_2, l_3: DA C-6/H-3, H-4, H-5; m, G C-6/H-5; d*, DA C-1/H-3 unsubstituted; h*, G C-3/H-2 unsubstituted; j*, G C-5/H-6 unsubstituted; i*, G C-4/H-5 unsubstituted; m*, G C-6/H-5 unsubstituted (G4S abbreviated to G and DA2S abbreviated to DA for clarity).

Table 2
The effect of succinyl substitution on the chemical shifts of various carbon atoms in disaccharide repeating unit of ι-carrageenan (ppm)

	G4S (3-linked unit)			DA 2S (4-linked unit)		
	O-Succinyl LMW ι-carrageenan	Starting LMW ι-carrageenan	$\Delta\delta$	*O*-Succinyl LMW ι-carrageenan	Starting LMW ι-carrageenan	$\Delta\delta$
C-1	102.2	104.4	−2.2	94.5	94.3	0.2
C-2	72.6	71.5	1.1	76.8	77.2	−0.4
C-3	76.5	79.1	−2.6	79.9	80.0	−0.1
C-4	74.5	74.3	0.2	80.6	80.6	0.0
C-5	74.5	77.0	−2.5	78.9	79.3	−0.4
C-6	65.9	63.5	2.4	72.1	72.0	0.1

4. Conclusions

O-Succinyl ι-carrageenan has been prepared, and DS of 1.5 could be attained under appropriate conditions. The molecular weight of ι-carrageenan derivative is 38,742. Succinyl substitution at different position of G4S-DA2S is dependent on the position of hydroxyl of sugar residue. The substitution at the 2-position of G4S unit is higher than that at 6-position of G4S unit. The ^{1}H and ^{13}C NMR chemical shifts for disaccharide unit of *O*-succinyl ι-carrageenan fragments have been fully assigned using 2D NMR spectroscopic techniques.

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