Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 338 (2003) 1403–1414
CARBOHYDRATE RESEARCH
www.elsevier.com/locate/carres

Analysis of pyruvylated β-carrageenan by 2D NMR spectroscopy and reductive partial hydrolysis

Ruth Falshaw,* Richard H. Furneaux, Herbert Wong

Industrial Research Ltd, P.O. Box 31-310, Lower Hutt, New Zealand

Received 25 November 2002; accepted 24 March 2003

Abstract

A polysaccharide rich in 4′,6′-*O*-(1-carboxyethylidene)-substituted (i.e., pyruvylated) β-carrageenan has been prepared by solvolytic desulfation of a polysaccharide containing predominantly pyruvylated α-carrageenan, which was extracted from the red seaweed, *Callophycus tridentifer*. The ^{13}C and ^{1}H NMR chemical shifts of pyruvylated β-carrageenan have been fully assigned using 2D NMR spectroscopic techniques. The 4′,6′-*O*-(1-methoxycarbonylethylidene) group, generated during chemical methylation of the polysaccharide, has been shown to survive under the conditions of acidic hydrolysis that cleave the 3,6-anhydro-α-D-galactosidic bonds in permethylated samples of both pyruvylated β- and pyruvylated α-carrageenans. As a result, two novel pyruvylated carrabiitol derivatives have been prepared.

Keywords: Carrageenan; Pyruvate acetal; NMR spectroscopy; Reductive partial hydrolysis

1. Introduction

When analysing the structure of a polysaccharide, information on its constituent sugars, their glycosyl linkages and the positions/types of any substituents is required. Information on which sugar unit is connected to which is also fundamentally important. ^{13}C NMR spectroscopy has been particularly valuable in this regard for red algal galactans with a significant degree of structural regularity. The utility of ^{13}C NMR spectroscopy for the structural analysis of carrageenans (red algal galactans with a basic repeating structure of alternating 3-linked β-D-galactopyranosyl and 4-linked α-D-galactopyranosyl units) has recently been reviewed.[1] Many red seaweeds, however, contain sulfated polysaccharides that produce poorly resolved and/or complex ^{13}C NMR spectra, but chemical and spectroscopic analysis of the corresponding desulfated polysaccharides can produce useful information on the polysaccharide backbone, as in the case of those from the red seaweeds *Grateloupia divaricata*,[2] *Plocamium costatum*[3] and *Corrallina pilulifera*.[4] Certain original assignments have been subsequently amended, such as those for C-2, -3 and -4 of 4-linked units and C-2 of 3-linked units in desulfated κ- (i.e., β-)carrageenan.[5,6] Attempts have been made to improve the process of assignment of particular ^{13}C NMR chemical shifts by using prediction from theoretical calculation.[7,8] Assignment of chemical shifts by practical experimentation, especially using 2D NMR spectroscopy as reported herein, is still the preferred method, however, as actual chemical shifts have sometimes been significantly different to the calculated values.[9,10]

In 1997, Chiovitti and co-workers reported the characterisation of the highly pyruvylated carrageenans obtained from red seaweeds in the genus *Callophycus*, using a range of modern analytical techniques.[11] The most highly pyruvylated carrageenan was that from *C. tridentifer*, which contained predominantly 3-linked 4′,6′-*O*-(1-carboxyethylidine)-β-D-galactopyranosyl (GP) units alternating with 4-linked 3,6-anhydro-α-D-galactopyranosyl 2-sulfate (DA2S) units (i.e., pyruvylated α-carrageenan) with a small amount of 3-linked β-D-galactopyranosyl (G) units alternating with 4-linked 3,6-anhydro-α-D-galactopyranosyl 2-sulfate (DA2S)

* Corresponding author. Tel.: +64-4-5690668; fax: +64-4-5690055.
E-mail address: r.falshaw@irl.cri.nz (R. Falshaw).

0008-6215/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0008-6215(03)00171-X

units (i.e., α-carrageenan) and minor amounts of other units. Pyruvylated α-carrageenan provides an ideal substrate for preparing and characterising pyruvylated β-carrageenan i.e., 3-linked 4′,6′-*O*-(1-carboxyethylidine)-β-D-galactopyranosyl (GP) units alternating with 4-linked 3,6-anhydro-α-D-galactopyranosyl (DA) units. This paper details the preparation of pyruvylated β-carrageenan (by desulfation of a sample of pyruvylated α-carrageenan) and the assignment of its ^{13}C and ^{1}H NMR chemical shifts using 2D NMR spectroscopy. The availability of these chemical shift assignments should significantly assist in the interpretation of more complex NMR spectra. In addition, the structures of the partially methylated carrabiitol peracetates obtained by methylation and reductive partial hydrolysis of pyruvylated β-carrageenan and pyruvylated α-carrageenan have been determined, and these provide novel reference compounds.

2. Experimental

2.1. Samples

C. tridentifer Kraft was collected from the northern side of Muttonbird Island, Coff's Harbour, NSW, Australia, 16 September, 1997. A voucher specimen (WELTA 21959) has been deposited at the Museum of New Zealand, Te Papa Tongarewa, Wellington.

2.2. Extraction and treatment of polysaccharides

Air-dried *C. tridentifer* (5 g) was treated with 0.05 M $NaHCO_3$ (60 mL/g weed) at 90 °C for 3 h, and the extract recovered by filtration through a GF-D glass fibre filter. The remaining seaweed residue was then re-extracted twice in fresh 0.05 M $NaHCO_3$ (60 mL/g weed) for a further 2 and 1.5 h at 120 °C. The cooled extracts were combined, filtered then dialysed and lyophilised. A portion of this material was subjected to alkali treatment according to the method of Craigie and Leigh.[12] Preparation of the pyridinium salt form of the alkali-modified polysaccharide and the solvolytic desulfation of this material was undertaken by the method described by Falshaw and Furneaux.[9] Permethylations were performed according to Stevenson and Furneaux.[13] Constituent sugar and glycosyl linkage analyses were undertaken by the reductive hydrolysis method of Stevenson and Furneaux[13] and quantified according to Falshaw and Furneaux.[9]

Reductive partial hydrolysis was conducted as described previously,[14] except that the acetylated products were purified by extraction with dichloromethane (1 mL), and the organic layer was washed with water (1 × 4 mL), 0.5 M sodium carbonate (1 × 4 mL) and water (1 × 4 mL). The resulting carrabiitol derivatives were dissolved in acetone (0.05 mL) and analysed by GLC and GLC–CIMS. GLC was conducted on a Hewlett–Packard 5890 Series II chromatograph fitted with an Alltech RSL-300 column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The sample was introduced by split injection using hydrogen as the carrier gas with a split ratio of 65:1 (column flow: 1.5 mL/min). Injector and FID detector temperatures were 320 °C. The column temperature was held at 250 °C for 1 min rising to 290 °C at 5 °C/min, held for 4 min then rising to 310 °C at 20 °C/min and held for 4 min. GLC–CIMS was conducted as for GLC but using an SGE fused-silica BP-1 column (25 m × 0.22 mm i.d., 0.25 μm film thickness) and a Fisons Trio 1000 quadrapole mass spectrometer with ammonia as the reagent gas, an ion source temperature of 260 °C and helium as carrier gas in splitless mode.

2.3. NMR spectroscopy

The 1D ^{13}C NMR spectrum was recorded on a 3% w/v solution in 50:50 v/v D_2O–H_2O at 90 °C using a Bruker Avance 300 spectrometer with a 10-mm broadband probe (75 MHz, 5120 scans, 0.885 s acquisition time for the free induction decay, 0.5 s relaxation delay time and 80° pulse width). Chemical shifts are quoted relative to internal Me_2SO at 39.47 ppm for ^{13}C and 2.70 ppm for ^{1}H. As selective excitation[15] is not commonly reported in the polysaccharide literature, some experimental detail is given here. All of the selective excitation ^{1}H NMR spectra were recorded non-spinning on a 3% w/v solution in D_2O at 60 °C with a Bruker 5-mm broadband inverse probe incorporating *z*-axis field gradients. A low-powered 90° gaussian-shaped pulse consisting of 1000 steps truncated at the 1% level was calibrated by selectively exciting the anomeric DA-1 proton signal. The pulse power was found to be 69 dB attenuation (from maximum power) and the pulse length 80 ms (t_p) giving a Gaussian excitation frequency response function where the amplitude of the excitation function ($\pm\pi/t_p$) at the 1% level was expected to be ±39 Hz or ±0.13 ppm from the transmitter. The standard Bruker-supplied selective COSY pulse sequence *selco* was used. The evolution time after the centre of the shaped 90° gaussian until the hard high-powered 90° pulse (11 μs) was set to 75 ms ($= t_p/2 + 35$ ms). A total of 64 scans were taken for the exploratory spectra, and 256 scans were taken for the final selective COSY spectrum with excitation of proton DA-6_{endo}, requiring 40 min of acquisition time.

The double quantum-filtered, heteronuclear multiple quantum coherence (HMQC) COSY 2D spectrum was recorded non-spinning on the same sample as used for the selective experiments, on a Varian Unity 500 spectrometer with a 5-mm broadband inverse probe at 60 °C, as described previously.[10] While general acquisi-

tion parameters and data processing were as previously reported, specific parameters are given in the individual figure legends.

3. Results and discussion

3.1. Sample preparation

A sample of air-dried *C. tridentifer* was extracted, and the resulting native polysaccharide (Ct-N) was obtained in 34 wt.% yield. A portion of the native polysaccharide was alkali-modified using hot $NaOH/NaBH_4$ and purified in 79 wt.% yield to give Ct-AM, which was then subjected to solvolytic desulfation by treatment of the pyridinium salt form with hot Me_2SO/MeOH/pyridine to give Ct-AM,DS in 81 wt.% yield.

3.2. Constituent sugar and glycosyl linkage analysis

Constituent sugar analysis of Ct-AM showed the presence of both 3,6-anhydrogalactosyl (AnGal, 23 mol.%) and galactosyl (Gal, 68 mol.%) units with small amounts of xylosyl (Xyl, 4 mol.%) and glucosyl (Glc, 5 mol.%) units, as expected.[11] Constituent sugar analysis results for Ct-AM,DS were AnGal (39 mol.%); Gal (55 mol.%); Xyl (3 mol.%) and Glc (3 mol.%).

The partially methylated alditol acetate derivatives expected from pyruvylated α-carrageenan are 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methylgalactitol (3,4,6-Gal) and 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol (2,4-AnGal), which in this case correspond to 3-linked 4,6-pyruvylated galactopyranosyl (GP) units and 4-linked 3,6-anhydrogalactopyranosyl 2-sulfate (DA2S) units, respectively. These two partially methylated alditol acetate derivatives and a number of others were previously observed by glycosyl linkage analysis of alkali-modified polysaccharide from *C. tridentifer*.[11] The same species were produced from Ct-AM (Table 1). The presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol (3-Gal) in the glycosyl linkage analysis of Ct-AM is consistent with unsubstituted 3-linked β-D-galactopyranosyl (G) units (Table 1); G units alternating with DA2S units would constitute α-carrageenan, for example. The level of 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylgalactitol (3,4-Gal) and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylgalactitol (3,6-Gal) was higher than obtained previously.[11] 3,4-Gal and 3,6-Gal may correspond to 3-linked β-D-galactopyranosyl (G) units that are substituted at the 4- or 6-position, respectively, with either a sulfate ester (S) or β-D-xylopyranosyl group (X). For example, G4S units alternating with DA2S units would constitute ι-carrageenan. Branching with a β-D-xylopyranosyl group is a possibility, since 4 mol.% of 1,2,3,4,5-penta-*O*-acetyl-xylitol (Xyl) was observed in the constituent sugar analysis of Ct-AM. Although a smaller amount of 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol (T-Xyl) was observed in the corresponding glycosyl linkage analysis, this could be due to oxidative degradation and/or selective losses during processing.[11] Other positions for xylosyl substitution are also possible, based on the current data. Xylosyl substitution at the 6-position of 3-linked β-D-galactopyranosyl units (i.e., G6X) has been found in the xylogalactan sulfate from *Corallina pilulifera*.[4] Alternatively, β-D-xylopyranosyl substitution at the 3-position of 4-linked α-L-galactopyranosyl units (L3X) has been found in the xylogalactan sulfate from *Chondria macrocarpa*,[17] and Chiovitti and co-workers speculated that β-D-xylopyranosyl substitution occurred similarly at the 3-position of 4-linked α-D-galactopyranosyl (i.e., D3X) units in the polysaccharide from *Callophycus laxus*, a species closely related to *C. tridentifer*.[11]

Table 1
Glycosyl linkage analysis (mol.%) of alkali-modified (Ct-AM) and alkali-modified, desulfated (Ct-AM,DS) polysaccharides from *C. tridentifer*

Constituent sugar [a]	Deduced unit [b]	Ct-AM	Ct-AM,DS
3-Gal	G	12	9
3,4-Gal	G4S/G4X?	8	3
3,6-Gal	G6S/G6X?	9	5
3,4,6-Gal	GP	45	30
4-Gal	D	2	4
4-AnGal	DA	-	46
2,4-AnGal	DA2S	23	2
T-Xyl	X	1	1

[a] 3,4-Gal means a 3,4-disubstituted and/or linked galactopyranosyl unit analysed as 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylgalactitol etc.
[b] Nomenclature of Knutsen and co-workers.[27]

Glycosyl linkage analysis of Ct-AM,DS revealed two major derivatives, Table 1. These were 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methylgalactitol (3,4,6-Gal) and 1,4,5-tri-*O*-acetyl-2-*O*-methyl-3,6-anhydrogalactitol (4-AnGal). In this case, the derivative, 3,4,6-Gal is consistent with 3-linked 4′,6′-pyruvylated β-D-galactopyranosyl (GP) units, and 4-AnGal corresponds to 4-linked 3,6-anhydro-α-D-galactopyranosyl (DA) units which, together, are expected from pyruvylated β-carrageenan. Alternating G (3-Gal, Table 1) and DA units correspond to β-carrageenan, consistent with desulfation of α-carrageenan. Small amounts of 3,4-Gal and 3,6-Gal were observed in Ct-AM,DS, as well as Ct-AM. As 52% of the derivatives obtained in the glycosyl linkage analysis of Ct-AM,DS were unequivocally 4-linked (cf. 50% expected for an idealised repeat unit of alternating 3- and 4-linked galactosyl units), it is unlikely that the 3,4-Gal present in either Ct-AM or Ct-AM,DS corresponds to D3X units but, more likely, to G4X or residual G4S

units, Table 1. Similarly, the 3,6-Gal present in the glycosyl linkage analysis of Ct-AM,DS corresponds, most likely, to G6X or residual G6S units.

3.3. ^{13}C NMR spectroscopy

The ^{13}C NMR spectrum of Ct-AM (not shown) was well resolved and was very similar to that published previously with the major signals corresponding to both α- and pyruvylated α-carrageenans.[11] An additional, small signal was present at 72.1 ppm in the ^{13}C NMR spectrum of Ct-AM. This signal was not observed in the previously reported spectrum from alkali-modified polysaccharide from *C. tridentifer*. This signal corresponds to C-4 of 3-linked β-D-galactopyranosyl 4-sulfate (G4S) units in ι-carrageenan,[5] and this indicates that at least some of the 3,4-Gal observed in the glycosyl linkage analysis of Ct-AM corresponds to G4S units, Table 1.

The ^{13}C NMR spectrum of Ct-AM,DS was well resolved (Fig. 1). Twelve major signals were visible as expected for the major disaccharide repeat unit of 3-linked 4′,6′-*O*-(1-carboxyethylidine)-β-D-galactopyranosyl (GP) units alternating with 4-linked 3,6-anhydro-α-D-galactopyranosyl (DA) units. In addition, there were signals at 101.3 ppm (shown) and at 25.5 and 175.1 ppm (not shown), corresponding to the acetal, methyl and carboxyl carbons, respectively, of the pyruvate acetal group (Table 2). Small signals were observed in the ^{13}C NMR spectrum of Ct-AM,DS at 102.5, 94.6, 80.4, 75.2 and 61.3 ppm corresponding to the G-1, DA-1, G-3, G-5 and G-6 carbons of β-carrageenan (Fig. 1, Table 3),[6] as expected, since Ct-AM contained a small amount of α-carrageenan (G-DA2S), which on desulfation, would produce β-carrageenan (G-DA).

3.4. 2D NMR spectroscopy

For the 3-linked (GP) unit of Ct-AM,DS, if the resonance at 102.1 ppm is assigned to the anomeric carbon, then the chemical shifts of H-1, H-2, H-3 and H-4 and of C-2, C-3 and C-4 can be assigned unambiguously from the ^{1}H/^{1}H and ^{13}C/^{1}H COSY spectra (Figs. 2 and 3, and Table 2). The chemical shifts for C-4 and C-2 are very similar, but the ^{13}C/^{1}H COSY clearly shows C-2 being 0.2 ppm downfield of C-4. This is the reverse of an assignment made by Miller.[8] The H-4–H-5 coupling in β-D-galactopyranosides is very small, so that no corresponding cross-peak was observed.[10] However, the methylene resonance at 65.3 ppm was assigned to C-6 from a DEPT experiment (not shown), and from this, H-6 and -6′ were individually located at 3.97 and 4.05

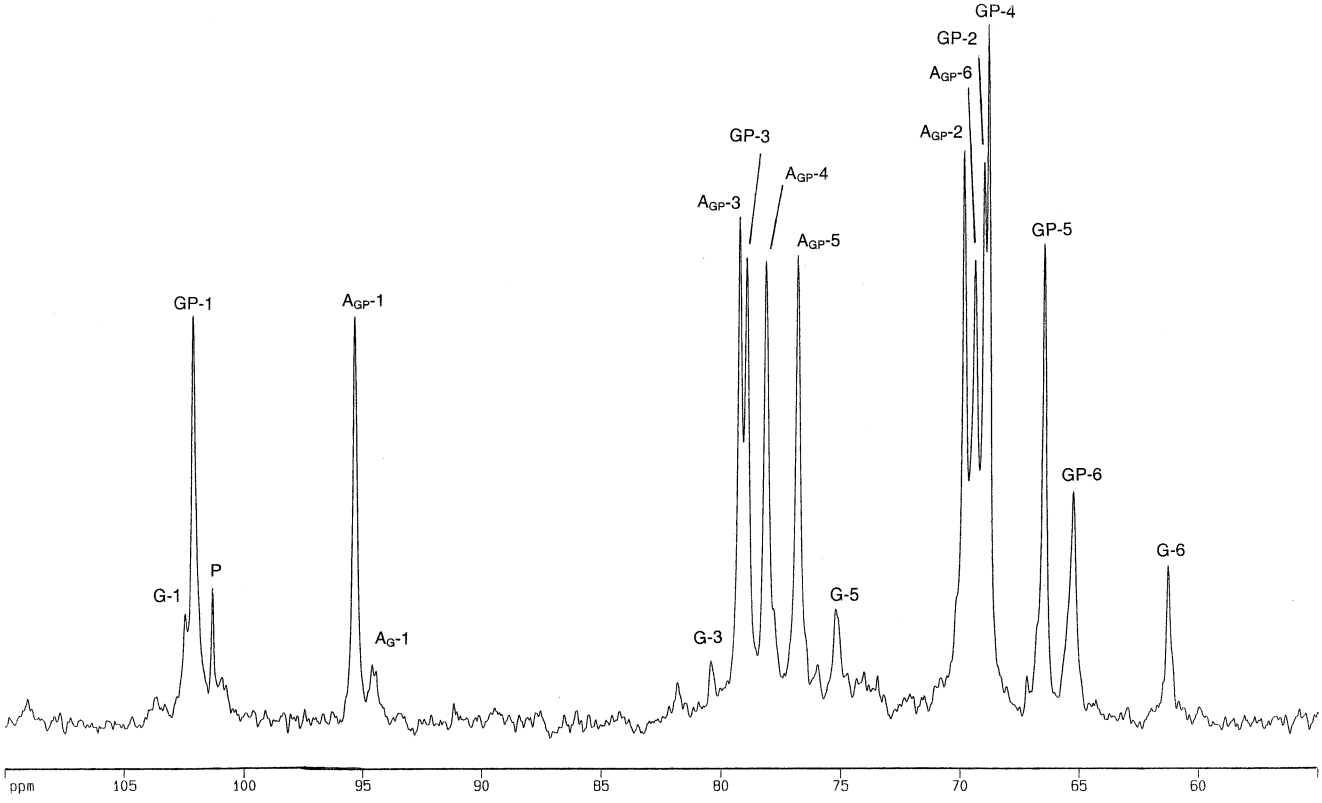


Fig. 1. ^{13}C NMR spectrum (50–110 ppm) of alkali-modified, desulfated *C. tridentifer* polysaccharide. (DA abbreviated to A for clarity; adjacent unit shown as subscript).

Table 2
Assignment of pyruvylated β-carrageenan chemical shifts (ppm) in the ^{13}C and ^{1}H NMR spectra of alkali-modified, desulfated *C. tridentifer* polysaccharide

	GP (3-linked unit)						DA (4-linked unit)						Pyuvate acetal		
	1	2	3	4	5	6	1	2	3	4	5	6	Carboxyl	Acetal	Methyl
Proton	4.62	3.68	3.88	4.36	3.60	3.97 [a]/4.05 [b]	5.17	4.16	4.54	4.59	4.68	4.26 [c]/4.12 [d]			1.44
Carbon	102.1	69.0	78.9	68.8	66.5	65.3	95.3	69.8	79.2	78.1	76.8	69.4	175.1	101.3	25.5

[a] G-6 proton.
[b] G-6′ proton.
[c] DA-6_{exo} proton.
[d] DA-6_{endo} proton.

ppm, respectively, from the $^{13}C/^{1}H$ COSY. Their ^{1}H chemical shift values were sufficiently different that distinct cross-peaks were discernible from the diagonal of the $^{1}H/^{1}H$ COSY spectrum. H-5 was then assigned from the weak H-6′,5 cross-peak. Having located H-5, C-5 was assigned unambiguously.

For the 4-linked (DA) unit of Ct-AM,DS, if the resonance at 95.3 ppm is assigned to the anomeric carbon, then the chemical shifts of H-1, H-2 and C-2 can then be assigned. H-3 was identified at 4.54 ppm from the strong H-2,3 cross-peak and, from this, C-3 was located at 79.2 ppm (Table 2). After C-1, C-3 was clearly the next-most-downfield resonance for the DA unit, but this was assigned to C-4 by Miller.[8] 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.[18,19] The resonance at 69.4 ppm was identified by a DEPT experiment (not shown) as due to a methylene carbon, and is assigned to C-6. H-6_{endo} and H-6_{exo} can then be identified from the $^{13}C/^{1}H$ COSY (Fig. 3 and Table 2). An H-6_{endo},6_{exo} cross-peak was also observed in the $^{1}H/^{1}H$ COSY (Fig. 2). Although an H-5,6_{endo} cross-peak was observed in the $^{1}H/^{1}H$ COSY of ι-carrageenan,[10] no H-5,6_{endo} cross-peak was observed here for pyruvylated β-carrageenan in Ct-AM,DS.

A selective COSY experiment was undertaken to resolve the chemical shifts for C-4 and C-5 of the DA unit. The reliability of this experiment was verified by selectively exciting the anomeric proton signal at 5.17 ppm and observing that the only noticeable antiphase correlation peak was indeed proton H-2 (at 4.16 ppm). Selective COSY excitation of H-6_{exo} (at 4.26 ppm) gave only a weak COSY correlation signal at 4.54 ppm (corresponding to H-3), resulting from unavoidable excitation of the near-neighbouring H-2 resonance at 4.16 ppm. Selective COSY excitation of H-6_{endo} at 4.12 ppm gave a relatively strong correlation signal at 4.54 ppm (H-3) and a weak correlation signal at 5.17 ppm (H-1), which again corresponds to unavoidable excitation of the near-neighbouring resonance H-2 at 4.16 ppm. More important, however, was the weak COSY correlation peak noted at 4.68 ppm, which is assigned to proton H-5. This then leaves H-4 at 4.59 ppm. Having determined the proton chemical shifts, the corresponding carbon shifts can thus be determined (i.e., 78.1 ppm for C-4 and 76.8 ppm for C-5) from the $^{13}C/^{1}H$ COSY spectrum, Fig. 3 and Table 2. The shift at 78.1 ppm was previously assigned to C-3 by Miller using set theory.[8] Miller considered chemical shift differences of <0.5 ppm to be within the range of uncertainty for the set theory technique (and hence equivalent to zero difference) but, in this case, the method was unable to correctly assign shifts that are more than 1 ppm apart.

Table 3
The effect of pyruvate acetal (P) or sulfate (S) substitution on the chemical shifts (ppm) of various carbon atoms in 3-linked β-D-galactopyranosyl (G) units adjacent to 4-linked α-D-galactopyranosyl (D), 4-linked 3,6-anhydro-α-D-galactopyranosyl (DA) or 4-linked 3,6-anhydro-α-L-galactopyranosyl (LA) units

Carbon	GP-LA[a]	G-LA [a]	Δ	GP-DA	G-DA [b]	Δ	GP-DA2S [c]	G-DA2S [c]	Δ	GP-D [d]	G-D [e]	Δ	G4S,6S-DA [f]	G-DA [b]	Δ
3-Linked unit															
C-1	102.2	102.4	−0.2	102.1	102.5	−0.4	101.9	102.5	−0.6	104.4	104.8	−0.4	102.8	102.5	0.3
C-2	70.0	70.2	−0.2	69.0	69.5	−0.5	69.1	69.9	−0.8		70.6		69.6	69.5	0.1
C-3	79.5	82.2	−2.7	78.9	80.4	−1.5	76.7	82.1	−5.4	76.1	79.0	−2.9	78.9	80.4	−1.5
C-4	71.6	68.8	2.8	68.8	66.4	2.4	67.4	67.0	0.4	67.6	65.7	1.9	74.0	66.4	7.6
C-5	66.7	75.3	−8.6	66.5	75.3	−8.8	66.7	75.2	−8.5	66.7	75.4	−8.7	72.7	75.3	−2.6
C-6	65.3	61.4	3.9	65.3	61.3	4.0	65.5	61.3	4.2	65.5	61.2	4.3	68.2	61.3	6.9
4-Linked unit															
C-1	98.4	98.3	0.1	95.3	94.7	0.6	91.4	94.8	−3.4	95.2	96.2	−1.0	95.2	94.7	0.5
C-2	69.9	69.9	0.0	69.8	70.2	−0.4	75.2	75.2	0.0		69.2		69.9	70.2	−0.3
C-3	80.1	80.1	0.0	79.2	79.4	−0.2	77.7	78.0	−0.3		70.9		79.5	79.4	0.1
C-4	77.5	77.4	0.1	78.1	78.0	0.1	78.3	78.3	0.0		78.5		78.9	78.0	0.9
C-5	75.7	75.7	0.0	76.8	76.8	0.0	77.1	77.1	0.0		70.6		77.0	76.8	0.2
C-6	69.4	69.4	0.0	69.4	69.5	−0.1	69.9	69.9	0.0		61.5		69.6	69.5	0.1

Δ is the change in chemical shift on 4′,6′-pyruvylation of the G unit.
[a] Lahaye and co-workers.[20]
[b] Usov and Shashkov.[6]
[c] Chiovitti and co-workers.[11]
[d] Assignments of Falshaw and Furneaux,[22] revised by Miller and Blunt.[21]
[e] Usov and co-workers.[5]
[f] Liao and co-workers.[23]

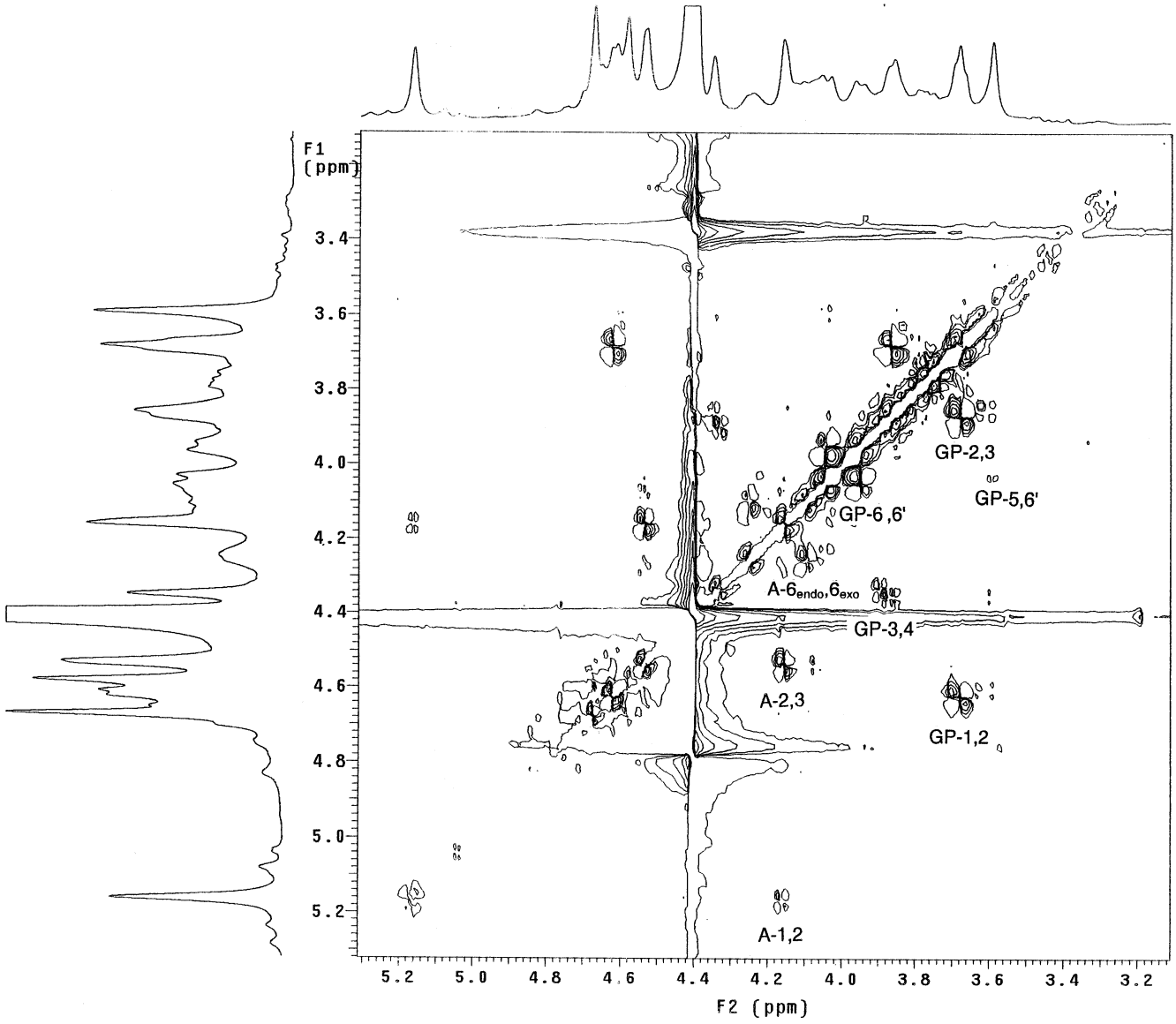


Fig. 2. $^{1}H/^{1}H$ COSY of alkali-modified, desulfated *C. tridentifer* polysaccharide. A total of 400 fid's were collected in 5 h. For each fid, the sweep width was 2600.9 Hz with a data acquisition time of 0.394 s, resulting in 1000 complex data points. A total of 32 scans were collected per fid with a recycle time of 1.39 s. (DA abbreviated to A for clarity).

3.5. Effect of pyruvate acetal substitution on ^{13}C NMR chemical shifts

Pyruvate acetal substitution at the 4- and 6-positions of 3-linked β-D-galactopyranosyl units would be expected to have most impact on the chemical shifts of the substituted carbon atoms (i.e., C-4 and C-6; the α-effect) and those adjacent to them (i.e., C-3 and C-5; the β-effect). The effect on the chemical shifts of these carbons on 4′,6′-pyruvylation of G-DA is shown in Table 3, along with the corresponding chemical shifts of the equivalent carbons on 4′,6′-pyruvylation of G-DA2S,[6,11] agar [i.e., β-D-galactopyranosyl (G) units alternating with 4-linked 3,6-anhydro-α-L-galactopyranosyl (LA) units][20] and a carrageenan with β-D-galactopyranosyl (G) units alternating with 4-linked α-D-galactopyranosyl (D) units.[5,21,22] The effect of 4′,6′-pyruvylation on C-5 and C-6 of the 3-linked units is similar in all four cases (~ -8.7 and $\sim +4.0$ ppm, respectively), but was more variable for both C-4 and C-3. C-4 of the 3-linked units was deshielded about 2 ppm for unsulfated structures, but only 0.4 ppm for G-DA2S, while C-3 was shielded by 1.5 ppm for G-DA to 5.4 ppm for G-DA2S. The effect of 4′,6′-pyruvylation of G-DA2S has been discussed previously in relation to the corresponding 4′,6′-pyruvylation of G-LA.[11] The α-effects on C-4 and C-6 of the 3-linked units upon direct substitution by a pyruvate acetal group are much smaller than that for

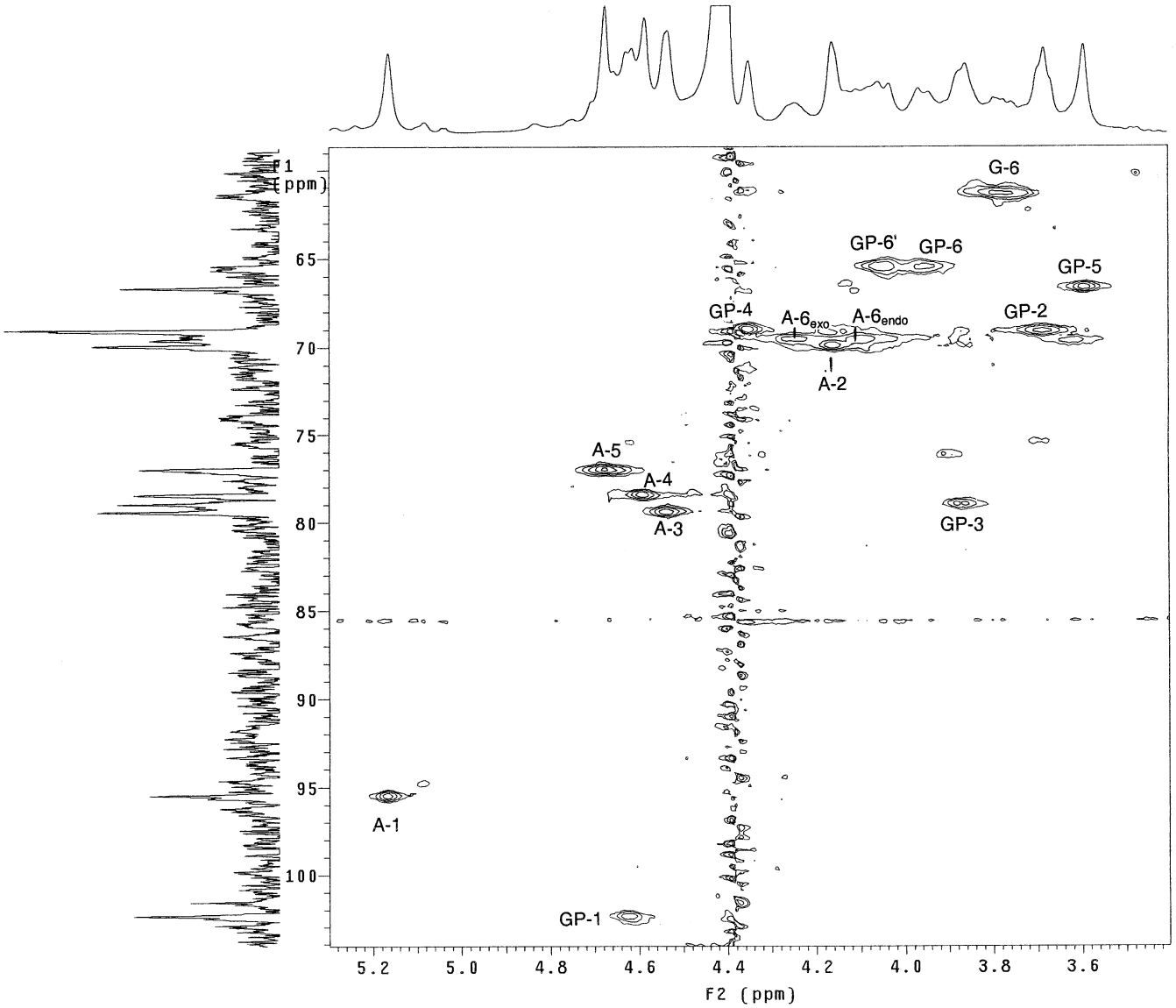


Fig. 3. $^{13}C/^{1}H$ COSY of alkali-modified, desulfated *C. tridentifer* polysaccharide. A total of 400 fid's were collected in 22 h. For each fid, the sweep width was 2600.9 Hz with a data acquisition time of 0.197 s, resulting in 512 complex data points. A total of 128 scans were collected per fid, with a recycle time of 1.50 s. (DA abbreviated to A for clarity.)

substitution by a sulfate ester group at the corresponding positions e.g., G4S,6S-DA[23], Table 3. In contrast, the β-effect of 4′,6′-pyruvylation on C-5 of the 3-linked units is much larger than that for sulfation at C-4 and C-6, Table 3. We attribute this large β-effect to donation of electron density from lone-pair orbitals on carboxylic oxygen to an antibonding σ* orbital on C-5. The overlap between these orbitals is similar to that between a lone-pair orbital on O-5 and an antibonding σ* orbital on C-1 in α-D-glycopyranosyl residues, which is the source of the anomeric effect that displaces the C-1 signal of the α-anomer approx 4 ppm upfield from that of the β-anomer.[24]

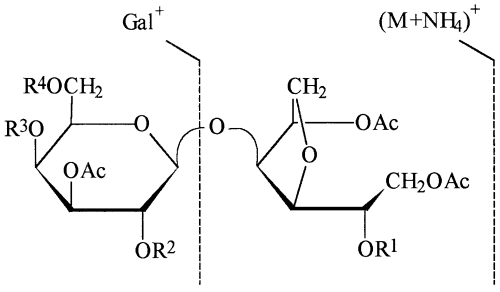
Either sulfate ester or pyruvate acetal substitution at the 4- and 6-positions of the 3-linked unit has a much smaller effect on other carbon atoms (generally 1 ppm or less). The exception is C-1 of the 4-linked unit in GP-DA2S, which is shifted 3.4 ppm upfield compared with C-1 of the 4-linked unit in G-DA2S (Table 3), which has been postulated as due to conformational changes.[11]

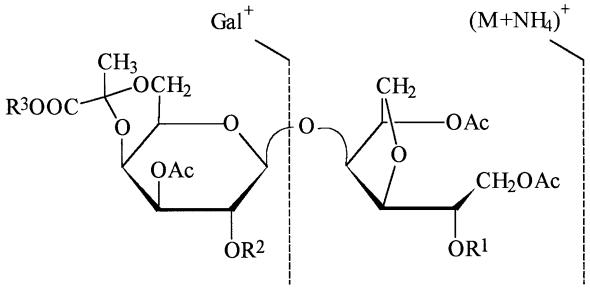
3.6. Reductive partial-hydrolysis of methylated polysaccharides

A portion of methylated Ct-AM,DS (prepared for glycosyl linkage analysis above) was subjected to reductive partial hydrolysis. In this technique, mild acidic 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 and

their permethylated derivatives, while most of the galactosidic bonds remain intact. In the presence of the relatively acid-stable borane reducing agent, 4-methylmorpholineborane (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.[14,25] Sulfate ester substituents present on the carrageenan may survive the mild acid hydrolysis step but are cleaved in the subsequent perchloric acid-catalysed acetylation/acetolysis step.[14] Recently, Gonçalves and co-workers have shown that pyruvate acetal groups also survive the mild acid hydrolysis step.[16] However, the fate of pyruvate acetal groups in a methylated polysaccharide under mild acidic hydrolysis conditions was not known.

Permethylated Ct-AM,DS was subjected to the reductive partial hydrolysis procedure. The major species observed by GC had a retention time of 7.75 min. This did not correspond to any of the partially methylated carrabiitol derivatives prepared previously[14] when run under identical GC conditions. CIMS of the major species produced ions at *m/z* 582 and 303, corresponding to the $(M+NH_4)^+$ ion and a major fragment $(Gal)^+$ ion, respectively. This could be interpreted as arising from a partially methylated carrabiitol acetate with a total of two methyl groups, one on the β-D-galactosyl moiety and one on the 3,6-anhydrogalactitol moiety,[14] such as 2,2′-di-*O*-methylcarrabiitol pentaacetate (**1a**), the product expected if the pyruvate acetal had been removed during the reductive partial hydrolysis procedure. However, 4′,6′-*O*-[1-methoxycarbonylethylidene]-2,2′-di-*O*-methyl carrabiitol triacetate (**2a**) is isomeric with **1a** and is the product expected if the pyruvate acetal were methylated then survived the reductive partial hydrolysis procedure, Schemes 1 and 2. To confirm the identity of the major species, a sample of Ct-AM,DS was trideuteromethylated prior to reductive partial hydrolysis. The resulting carrabiitol derivative had a characteristic mass ion at *m/z* 591 $(M+NH_4)^+$ indicative of a carrabiitol peracetate with a total of three trideuteromethyl groups. In addition, the Gal^+ ion at *m/z* 309 indicated that two trideuteromethyl groups existed on the β-D-galactosyl moiety, Scheme 2. By difference, the remaining trideuteromethyl group must occur on the 3,6-anhydrogalactitol moiety of the carrabiitol. A structure consistent with these results would be 4′,6′-*O*-[1-trideuteromethoxycarbonylethylidene]-2,2′-di-*O*-trideuteromethyl carrabiitol triacetate (**2b**) rather than 2′,2-di-*O*-trideuteromethylcarrabiitol pentaacetate (**1b**), Scheme 1. Thus, the carboxylic acid group of the pyruvate acetal is (trideutero)methyl esterified under the polysaccharide (trideutero)methylation conditions, and this (trideutero)methyl esterified pyruvate acetal substituent survives both the mild acid hydrolysis and the perchloric acid-catalysed acetylation conditions of the reductive partial hydrolysis procedure, Scheme 2. Additional small peaks were also identified as 2,2′,6′-tri-*O*-methylcarrabiitol tetraacetate (**1c**) and 2,2′,4′,6′-tetra-*O*-methylcarrabiitol triacetate (**1d**) corresponding to κ- and β-carrageenans, respectively (Scheme 1, Table 4).[14] This indicates that some G4S units are linked to DA units and also that some G units are linked to DA units in Ct-AM,DS, Table 4.

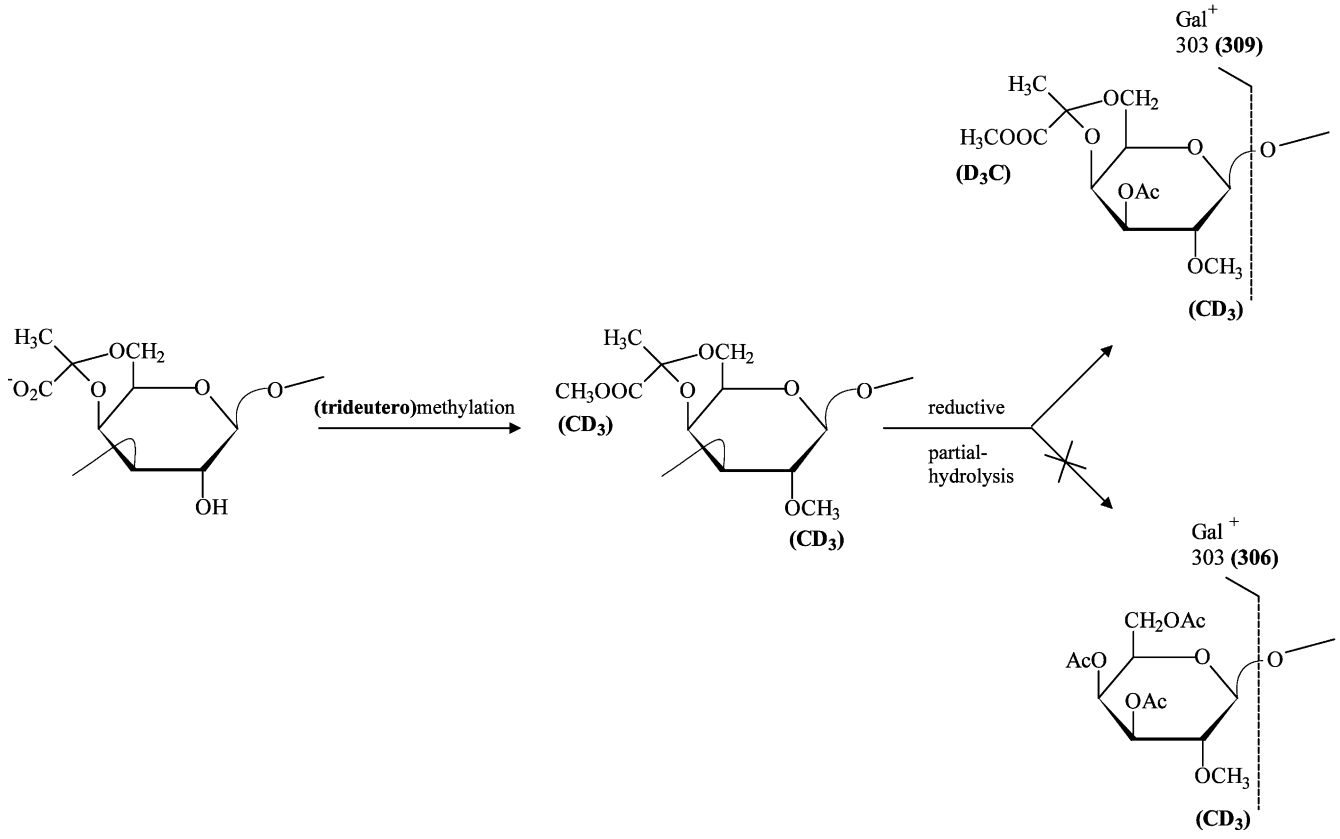




	R^1	R^2	R^3	R^4
1a	CH_3	CH_3	Ac	Ac
1b	CD_3	CD_3	Ac	Ac
1c	CH_3	CH_3	Ac	CH_3
1d	CH_3	CH_3	CH_3	CH_3
1e	Ac	CH_3	Ac	Ac
1f	Ac	Ac	Ac	Ac
1g	Ac	CH_3	Ac	CH_3
1h	Ac	CH_3	CH_3	CH_3

	R^1	R^2	R^3
2a	CH_3	CH_3	CH_3
2b	CD_3	CD_3	CD_3
2c	Ac	CH_3	CH_3

Scheme 1.



Scheme 2.

Since a (trideutero)methyl esterified pyruvate acetal substituent survives reductive partial hydrolysis conditions, then reductive partial hydrolysis of the permethylated, alkali-modified polysaccharide, Ct-AM, would be expected to produce the derivative, 4′,6′-*O*-[1-methoxycarbonylethylidene]-2′-*O*-methylcarrabiitol tetraacetate (**2c**), Scheme 1. The major species produced had the expected CIMS ions of *m*/*z* 610 [corresponding to the $(M + NH_4^+)$ ion] and *m*/*z* 303 (corresponding to the β-D-galactosyl unit), Table 4. Compound **2c** eluted later (8.64 min) than its isomer, 2′-*O*-methylcarrabiitol hexaacetate (**1e**, 7.46 min) or even carrabiitol heptaacetate (**1f**, 8.18 min), (Scheme 1, Table 4).[14] Additional small peaks were also identified as 2′,6′-di-*O*-methylcarrabiitol pentaacetate (**1g**) and 2′,4′,6′-tri-*O*-methylcarrabiitol tetraacetate (**1h**) corresponding to ι- and α-carrageenans, respectively (Scheme 1, Table 4).[14] This is consistent with the ^{13}C NMR data for Ct-AM. The formation of a methyl ester of the 4′,6′-*O*-1-carboxyethylidene group also occurs during methanolysis of

Table 4
CIMS pseudomolecular and major fragment ions and GLC retention times obtained from various partially methylated, partially acetylated carrabiitols (Figures in brackets refer to trideuteromethylated species)

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
4.88	**1d**	G-DA	3	1	247	526
5.44	**1c**	G4S-DA	2	1	275	554
5.56	**1h**	G-DA2S	3	0	247	554
6.25	**1g**	G4S-DA2S	2	0	275	582
7.46	**1e**		1	0	303	610
7.75	**2a** (**2b**)	GP-DA	2	1	303 (309)	582 (591)
8.18	**1f**		0	0	331	638
8.64	**2c**	GP-DA2S	2	0	303	610

[a] Maltose peracetate eluted at 9.36 min.

pyruvylated algal galactans, resulting in a methyl 4′,6′-*O*-(1-methoxycarbonylethylidene)galactoside, which gave a characteristic fragmentation pattern when analysed by GC–EIMS (as its trifluoroacetate derivative).[26] This methanolysis method does not provide information about the structure of the adjacent reducing sugar, however.

3.7. Effect of pyruvate substitution on the loss of 3,6-anhydro-galactose during constituent sugar and glycosyl linkage analysis

The amount of AnGal detected by constituent sugar analysis was lower for Ct-AM (23 mol.%) than the approx 50% expected for a polysaccharide containing predominantly pyruvylated α-carrageenan. However, it is known that under standard reductive hydrolysis conditions, the presence of a 2-sulfate ester group on a 3,6-anhydrogalactosyl unit inhibits the hydrolysis of its glycosidic bond making it difficult to release all the AnGal residues before the reducing agent has been consumed, leading to some degradative loss of AnGal.[13] The amount of AnGal obtained for Ct-AM,DS was also lower (39 mol.%) than the approx 50% expected for an idealised pyruvylated β-carrageenan, even though the 2-sulfate ester groups on 3,6-anhydrogalactosyl units have been removed during the desulfation process. This suggests that the pyruvate acetal group is also affecting recovery of AnGal in some way. Chiovitti and co-workers[11] suggested two possible reasons. Firstly, a pyruvate acetal group might stabilise the glycosidic linkage of an adjacent 3,6-anhydrogalactosyl unit. Secondly, any pyruvic acid released during the initial hydrolysis (2.4 M TFA, 80 °C, 5 min) would compete with 3,6-anhydrogalactose for the reductant, MMB. Pyruvate acetal substituents are definitely cleaved at some stage during the reductive hydrolysis procedure since 1,2,3,4,5,6-hexa-*O*-acetyl-galactitol (Gal) is obtained rather than any pyruvylated species. However, the point at which pyruvate acetal hydrolysis occurs, and the effect that pyruvate acetal substitution has on the rate of glycosidic bond hydrolysis, under either the initial conditions or the subsequent, harsher conditions (2 M TFA, 120 °C, 1 h) of the reductive hydrolysis procedure is still unknown. We note that Gonçalves and co-workers[16] recently showed that a pyruvate acetal substituent on an agar polysaccharide survived milder, but longer, reductive partial hydrolysis conditions (i.e., 0.5 M TFA, 65 °C, 8 h).

Methyl esterification of pyruvate acetal substituents during permethylation of the polysaccharides Ct-AM and Ct-AM,DS has been demonstrated above. Although these methyl esterified pyruvate acetal substituents survive reductive partial hydrolysis conditions, they are cleaved under reductive hydrolysis conditions to give the partially methylated alditol acetate derivative, 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methylgalactitol (3,4,6-Gal) rather than 1,3,5-tri-*O*-acetyl-4′,6′-*O*-(1-methoxycarboxyethylidine)-2-*O*-methylgalactitol (Table 1). Methylation of Ct-AM,DS prior to reductive hydrolysis led to an increase in total AnGal recovery (48 mol.% cf. 39 mol.%), which suggests that methyl esterification of pyruvate acetal substituents on Ct-AM,DS leads to improved recovery of AnGal from the reductive hydrolysis procedure although the reason for this is not known. Conversely, methylation of Ct-AM prior to reductive hydrolysis had no effect on the recovery of AnGal (23% in both cases). This suggests that, in the case of Ct-AM, the presence of a 2-sulfate ester substituent on the 3,6-anhydrogalactosyl units is still the major factor contributing to the poor recovery of AnGal whether the polysaccharide and its pyruvate acetal substituent are permethylated or not.

4. Conclusions

Pyruvylated β-carrageenan has been prepared by solvolytic desulfation of the pyruvylated α-carrageenan component of a polysaccharide extracted from the red seaweed, *C. tridentifer*. The ^{1}H and ^{13}C NMR chemical shifts for pyruvylated β-carrageenan have been fully assigned experimentally using 2D NMR spectroscopic techniques. Some significant differences were found with previously published, theoretically predicted assignments.

The carboxylic acid group of the pyruvate acetal substituent in pyruvylated α- and β-carrageenans is esterified under the conditions used to methylate the polysaccharides. The resulting methyl esterified pyruvate acetal substituents survive acid hydrolysis conditions that are sufficient to cleave the 3,6-anhydro-α-D-galactosidic bonds. As a result, two new, partially methylated carrabiitol peracetates, 4′,6′-*O*-[1-methoxycarbonylethylidene]-2′,2-di-*O*-methylcarrabiitol triacetate and 4′,6′-*O*-[1-methoxycarbonylethylidene]-2′-*O*-methylcarrabiitol tetraacetate, have been produced and characterised.

Acknowledgements

The authors would like to thank P. Richards (Royal Botanic Gardens, Sydney, NSW, Australia) for the collection of *Callophycus tridentifer*, Dr R.H. Newman (Industrial Research Ltd) for useful comments and the New Zealand Foundation for Research, Science and Technology for funding support.

References

1. Van de Velde, F.; Knutsen, S. H.; Usov, A. I.; Rollema, H. S.; Cerezo, A. S. *Trends Food Sci. Technol.* **2002**, *13*, 73–92.
2. Usov, A. I.; Barbakadze, V. V.; Yarotskii, S. V.; Shashkov, A. S. *Bioorg. Khim.* **1978**, *4*, 1507–1512.
3. Falshaw, R.; Furneaux, R. H.; Miller, I. J. *Bot. Marina* **1999**, *42*, 431–435.
4. Usov, A. I.; Bilan, M. I.; Shashkov, A. S. *Carbohydr. Res.* **1997**, *303*, 93–102.
5. Usov, A. I.; Yarotsky, S. V.; Shashkov, A. S. *Biopolymers* **1980**, *19*, 977–990.
6. Usov, A. I.; Shashkov, A. S. *Bot. Marina.* **1985**, *28*, 367–373.
7. Stortz, C. A.; Cerezo, A. S. *Carbohydr. Polym.* **1992**, *18*, 237–242.
8. Miller, I. J. *Bot. Marina.* **1998**, *41*, 305–315.
9. Falshaw, R.; Furneaux, R. H. *Carbohydr. Res.* **1994**, *252*, 171–182.
10. Falshaw, R.; Furneaux, R. H.; Wong, H.; Liao, M.-L.; Bacic, A.; Chandrkrachang, S. *Carbohydr. Res.* **1996**, *285*, 81–98.
11. Chiovitti, A.; Bacic, A.; Craik, D. J.; Munro, S. L. A.; Kraft, G. T.; Liao, M-L. *Carbohydr. Res.* **1997**, *299*, 229–243.
12. Craigie, J. S.; Leigh, C. In *Handbook of Phycological Methods: Physiological and Biochemical Methods*; Hellebust, J. A.; Craigie, J. S., Eds.; Cambridge University Press: Cambridge, MA, 1978; pp 109–131.
13. Stevenson, T. T.; Furneaux, R. H. *Carbohydr. Res.* **1991**, *210*, 277–298.
14. Falshaw, R.; Furneaux, R. H. *Carbohydr. Res.* **1995**, *269*, 183–189.
15. Kessler, H.; Mronga, S.; Gemmecker, G. *Magn. Reson. Chem.* **1991**, *29*, 527–557.
16. Gonçalves, A. G.; Ducatti, D. R. B.; Duarte, M. E. R.; Noseda, M. D. *Carbohydr. Res.* **2002**, *337*, 2443–2453.
17. Furneaux, R. H.; Stevenson, T. T. *Hydrobiologia* **1990**, *204/205*, 615–620.
18. (a) Welti, D. *J. Chem. Res. Sect. S* **1977**, 312–313; (b) Welti, D. *J. Chem. Res. Sect. M* **1977**, 3566–3587.
19. Usov, A. I. *Bot. Marina.* **1984**, *27*, 189–202.
20. Lahaye, M.; Yaphe, W.; Viet, M. P. T.; Rochas, C. *Carbohydr. Res.* **1989**, *190*, 249–265.
21. Miller, I. J.; Blunt, J. W. *Bot. Marina* **2000**, *43*, 251–261.
22. Falshaw, R.; Furneaux, R. H. *Carbohydr. Res.* **1995**, *276*, 155–165.
23. Liao, M.-L.; Chiovitti, A.; Munro, S. L. A.; Craik, D. J.; Kraft, G. T.; Bacic, A. *Carbohydr. Res.* **1996**, *296*, 237–247.
24. Jarvis, M. C. *Carbohydr. Res.* **1994**, *259*, 311–318.
25. Usov, A.I.; Elashvili, M.Y. F.E.C.S. Fifth Int. Conf. Chem. Biotechnol. Biol. Active Nat. Prod., Varna, Bulgaria, 2, **1989**, 346–350.
26. DiNinno, V. L.; Bell, R. A.; McCandless, E. L. *Biomed. Mass Spectrom.* **1978**, *5*, 671–673.
27. Knutsen, S. H.; Myslabodski, D. E.; Larsen, B.; Usov, A. I. *Bot. Marina* **1994**, *37*, 163–169.