Botanica Marina Vol. 45, 2002, pp. 1–8 © 2002 by Walter de Gruyter · Berlin · New York

Evaluation of the Structure of the Polysaccharides from *Chondria macrocarpa* and *Ceramium rubrum* as Determined by ^{13}C NMR Spectroscopy

I. J. Miller[a]* and J. W. Blunt[b]

[a] Carina Chemical Laboratories Ltd, P. O. Box 30366, Lower Hutt, New Zealand
[b] Department of Chemistry, University of Canterbury, Private Bag 4800, Christchurch, New Zealand

* Corresponding author: ian.miller@xtra.co.nz

The polysaccharides from *Chondria macrocarpa* and *Ceramium rubrum* were examined by ^{13}C NMR spectroscopy and their spectra were analysed by means of set theory. The spectra of the polysaccharides from *Chondria macrocarpa* collected in autumn were in accord with previous chemical analysis but polysaccharides from seaweed collected in spring comprise a higher level of agar precursors, much lower levels of galactose 2-sulfate, and the polysaccharide is separable into two components, the predominant one of which is essentially free of anhydrogalactose. The signal subset due to the xylosyl unit substituted at the 3-position of 4-linked L-galactose could be assigned from the spectrum of desulfated polysaccharide from *Chondria macrocarpa*. The extract from *Ceramium rubrum* consisted of a backbone of 3-linked D-galactosyl units alternating with the 4-linked units 3,6-anhydro-L-galactosyl and L-galactosyl in a ratio of 2:1 respectively, to which substitution by xylose occurred at approximately 8 % of the total sugar units. The natural polymer had moderate levels of sulfate ester, with galactose-6-sulfate being the predominant unit, some of which survives alkaline treatment and was consistent with being 3-linked. There is also evidence of low levels of 3-linked galactose-2-sulfate.

Introduction

Red algal galactans invariably consist of linear backbones comprising alternating 3-linked β-D-galactosyl and 4-linked α-linked galactosyl or 3,6-anhydrogalactosyl units which may be in the D or L configuration. Their hydroxyl groups may be substituted with hydrolysable linkages such as sulfate ester (S) or with the non-hydrolysable methyl ether (M), while substitution to a galactosyl unit (G) with xylose (X) or pyruvate acetal (P) also occurs. Accordingly, a wide range of structures is possible. Since some galactans are of commercial interest, and since the structures may be of chemotaxonomic interest, we have undertaken a program for determining structural features through ^{13}C nuclear magnetic resonance (NMR) spectroscopy.

In order to determine structural features from complex spectra, we have proposed a procedure to rationalize regularities in changes of chemicals shifts due to changes of substitution of the underlying polysaccharide. These regularities can be expressed in terms of set theory, matrices, and mathematical matrix operators corresponding to physical operations. We now extend this work, employing the previous nomenclature and conventions (Miller and Blunt 2000a, b) to investigate the polysaccharides from two algae for which structural data have been reported.

Chemical linkage analyses have been reported for the polysaccharide from *Chondria macrocarpa* Hook. f. *et* Harv. (Furneaux and Stevenson 1990). The 3-linked units were sulfated with approximately 50 % frequency at O-2, and at frequency of about 20–30 % at O-6. Approximately 40 % of the 4-linked units were present as 3,6-anhydro-L-galactose and a further 25 % as its precursor L-galactose-6-sulfate. The remaining 4-linked units largely consisted of L-galactose-6-sulfate which was also substituted at O-3 by a D-xylosyl group. This structure would be expressed in our set nomenclature as: Structure (CM)

=[{3DG, 3DG2S, 3DG6S, 3DG2S6S}-4L{AG, G6S, G3X6S}] (1)

The frequency or probability of substitution, if constant, can be expressed as subscripts. Reasons for further investigation of material already examined include examining the constancy of the structure, and also to assess whether our techniques for analysing NMR spectra can be applied to a spectrum originally considered to be too complex to be interpretable.

The polysaccharide from *Ceramium rubrum* (Hudson) C. Agardh has been examined previously by Turvey and Williams (1976), who found the polysaccharide had the alternating agar diad structure, with the 3-linked units consisting of D-galactose, 6-*O*-methyl-D-galactose, D-galactose-6-sulfate, together with some D-galactose-2-sulfate and possibly 4-sulfate while the 4-linked residues consisted of L-galactose, L-galactose-6-sulfate, 3,6-anhydro-L-galactose and small amounts of 2-*O*-methyl-L-galactose.

Agaroids consist of two basic structural units, which give the ^{13}C NMR spectral sets (Lahaye *et al.* 1989):

S [3DG-4LG] = {103.7, 100.9, 81.2, 79.3, 75.9, 72.2, 71.0, 69.8, 69.4, 68.8, 61.4, 61.2} (2)

$$\mathbf{S}\ [3DG\text{-}4LAG] = \{102.3, 98.2, 82.2, 80.0, 77.3, 75.6, 75.3, 70.2, 69.9, 69.3, 68.7, 61.4\} \quad (3)$$

If these entities are present in a polysaccharide, all signals should be present in its spectrum. The absence of any signal (provided the spectrum is of sufficient quality) proves the absence of the entity. A structure is therefore obtained when the intersection of all data sets is consistent with only one structure.

Materials and Methods

Chondria macrocarpa was collected at South Muritai (Wellington Harbour) in November 1998, and a sample was deposited in the Museum of New Zealand, Te Papa, Wellington, with the herbarium number WELT AO 25411. A further sample was collected from the same location in March 2000, this was not registered.

Ceramium rubrum was collected at Princes Bay (Wellington Harbour) in December 1997, and a sample was deposited in the Museum of New Zealand, Te Papa, Wellington, with the herbarium number WELT A 22156.

For the extraction of *Chondria macrocarpa*, because the seaweed decomposes quickly when removed from seawater (Furneaux and Stevenson 1990), freshly collected wet seaweed (120 g) was transferred to the laboratory in salt water where it was rinsed and placed in water (1.5 L) containing sodium hydrogen phosphate (4 g). The pH was adjusted to 7, and extraction was commenced within an hour of the completion of collection. The mixture was extracted (2 h) at 120 °C and the resultant solution filtered, cooled, treated with amyloglucosidase and left to stand overnight. The solution was then boiled, the volume was reduced to approximately 150 mL by evaporation, then the solution was left to stand overnight. An insoluble gel/precipitate formed for the November 1998 extract and this was separated from the mother liquor by straining. The solid fraction was then washed with water (2 x 20 mL) containing salt (4 %) and the liquid washings were combined with the mother-liquors. Each fraction was separately dialysed against distilled water, then evaporated to give two solid fractions: CM1 (5.88 g) from the non-gelling fraction; CM2 (2.55 g) from the gelling fraction. The extract from the March 2000 sample did not form two phases, and it was dialysed and evaporated, to give 7.5 g of polysaccharide.

A sample of CM1 (1 g) was further purified by dissolving it in water (100 mL) then boiling (5 min) in the presence of activated charcoal (0.5 g), filtering, then chlorine was bubbled through the solution until no further colour change occurred. Excess chlorine was neutralized with sodium bisulfite. After adjusting the pH to 9 by adding triethylamine, the solution was dialysed against distilled water (x 5) before the water was removed by evaporation. The yield was 0.65 g. A sample (0.3 g) was dissolved in distilled water (50 mL) and was treated in a pulse sequence (3 s on, 1 s off) for 40 min with ultrasound ($\approx$ 40 mW, 20 kHz) from a Sonics and Materials Inc sonicator model CV26.

A sample of the fraction actually analysed by Furneaux and Stevenson (1990) was designated CM3.

A similar procedure was followed for *Ceramium rubrum*, except that air dried alga (30 g) was used and no intermediate separation was achieved. A fraction CR (8.58 g) was obtained.

Alkali treatment of the *Ceramium rubrum* polysaccharide was carried out as follows. Natural polysaccharide (1 g) was dissolved in water (50 mL) at 90 °C, then NaOH (8 g) and $NaBH_4$ (0.3 g) were added and the solution was stirred at 90 °C for 2 h. The solution was then cooled to 50 °C and neutralized by adding acetic acid (glacial) dropwise until the pH was approximately 8. A small amount of precipitate formed, which was removed, and the precipitate and liquors were separately dialysed to give, after evaporation/drying: from the precipitate CR $|OH^-|$ (a) (0.13g); from the mother liquors CR $|OH^-|$ (b) (0.49 g).

Desulfation followed the method of Miller and Blunt (1998), methylations were carried out as described previously (Miller 1998), while the detection of pyruvate acetal followed the procedure of Miller and Furneaux (1982). Reagents and solvents were reagent grade materials and were used without further purification.

Films for infrared spectra were made by slowly drying at 60 °C a 0.75 % solution cast on a polyethylene surface, and the spectra were recorded on a Shimadzu IR 408 spectrometer. The proton decoupled ^{13}C NMR spectra were recorded for saturated solutions in D_2O at 80 °C on a Varian XL300 spectrometer using an acquisition time of 0.5 s and a flip angle of 64°. Spectral sets **S** are expressed as chemical shifts in ppm, referenced to dimethyl sulfoxide at 39.6 ppm. Elements with a star (*) are of higher intensity, and are likely to represent signal coincidences. For the purposes of comparing signals in different spectra, a signal is considered to have an equivalent chemical shift if the difference between the two is <0.5 ppm. (This permits but does not require the signals to have an equivalent origin.) For semi-quantitative estimations, the area under a signal was compared with that of a corresponding signal, i. e. a C-5 signal would be compared with another C-5 signal.

Results and Discussion

Chondria macrocarpa

The infrared spectrum for the natural polymer CM1 showed the following signals (cm^{-1}): {1270–1220 (strong), 1145 (medium), 1020–1070 (strong) 990 (shoulder) 845–800 (medium, broad)}.

As expected from the chemical analysis, the infrared spectrum showed significant levels of sulfate ester (1220–1270 cm^{-1}), together with a broad signal at

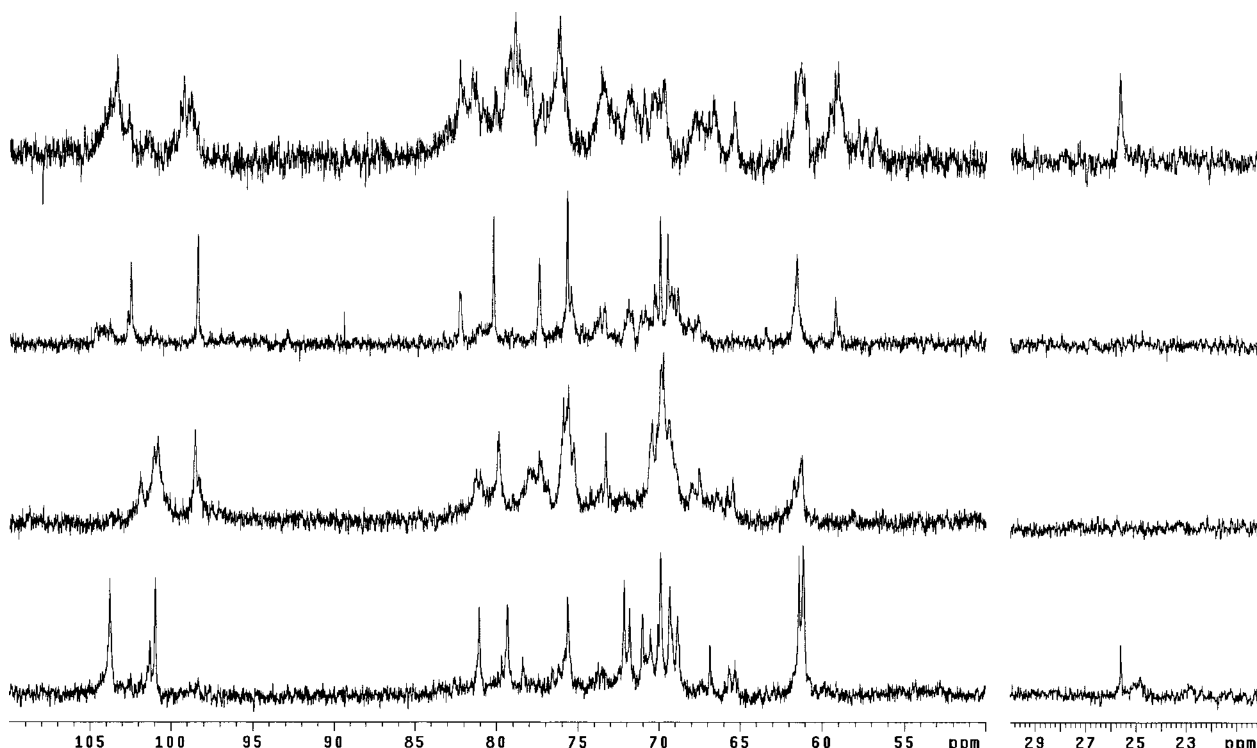

Fig. 1. ^{13}C NMR spectra of polysaccharides from *Chondria macrocarpa*: (top to bottom) (a) **S** CM1 |M|, (b) **S** CM1 |)))|, (c) **S** CM3, (d) **S** CM1 |–S|.

845–800 cm^{-1} without a clear absorption maximum, and consistent with being due to several overlapping signals. Chemical analysis (Furneaux and Stevenson 1990) shows the presence of 3DG2S or G6S, but not 3DG4S, and also anhydrogalactosyl units, which should give a signal at 935 cm^{-1}, are present, but this is absent in CM1. The origin of infrared signals in sulfated galactans between 800–900 cm^{-1} has been reviewed (Matsuhiro 1996), and the only recorded source of a signal due to sulfate ester at 845 cm^{-1} (Rochas *et al.* 1986) is 3DG4S. However, such signals are not solely due to the presence of sulfate ester, as shown by the spectrum of the methylated desulfated polysaccharide listed below.

(i) Desulfated polymer. The spectrum of the desulfated non-gelling polysaccharide (Fig. 1) could be separated into two sets on the basis of signal height:

S CM1 |–S| (major) = {103.8, 100.9, 81.0, 79.3, 75.6, 72.2, 71.0, 69.9, 69.3, 68.8, 61.4, 61.1} (4)

S CM1 |–S| (minor) = {101.3, 79.7, 78.4, 76.6, 76.1, 73.7, 73.5, 71.8, 70.5, 70.0, 66.8, 65.7, 65.3, 59.2, 25.6} (5)

An absence of 4-linked D-galactosyl units is indicated by the absence of signals between 90–97 ppm, as expected for agaroids. To within a 0.5 ppm error range, from (2)

S CM1 |–S| (major) = **S** [3DG-4LG] (6)

but from (3)

S [3DG-4LAG] $\not\subset$ **S** CM1 |–S| (minor) (7)

With no signal between 96–100 ppm, CM1 contains no detectable 3,6-anhydro-L-galactose, despite Furneaux and Stevenson (1990) finding that almost half the 4-linked units consisted of this sugar unit. Furneaux and Stevenson (1990) did not report pyruvate acetal although they did report 2 % of 3,4,6-substituted galactose which survived desulfation. From Lahaye *et al.* (1989) the signals due to pyruvylation in agar are

S [3DG(4,6)P-4LAG] ~**S** [3DG-4LAG] = {79.4, 71.5, 66.8, 65.2, 25.6} (8)

and

S [3DG(4,6)P-4LAG] ~**S** [3DG-4LAG] $\subset$ **S** CM1 |–S| (minor) (9)

The signal at 25.6 ppm is a strong indicator of pyruvate acetal, the presence of which was confirmed through the thin layer chromatographic analysis of 2,4-dinitrophenylhydrazones (Miller and Furneaux 1982). Thus CM1 has a structure quite different from that of the sample analysed by Furneaux and Stevenson (1990).

From chemical analysis (Furneaux and Stevenson 1990) xylose is a substituent, present as 4LG3X. From Usov *et al.* (1997), xylose in G6X gives

S X = {103.8, 76.1, 73.5, 69.8, 65.8} (10)

Hence, for signals sufficiently remote not to be affected by the point of substitution,

S (X~X$\underline{1}$) $\subset$ **S** CM1 |–S| (minor) (11)

There is a close coincidence of two signals at 101.3 ppm, one of intensity approximately equal to the signals assigned to xylose and the other somewhat weaker. Accordingly we assign the weaker of these two the quaternary anomeric carbon of pyruvate acetal, which is usually at approximately 101.4 ppm (Chiovitti *et al.* 1997) and the stronger signal, which has approximately equal intensity to the other xylosyl signals, to X$\underline{1}$. Usov and Elashvili (1991) assigned the corresponding signal from a sulfated galactan with a 3-xylosyl signal as 101.0 ppm, in good agreement with the above analysis. From ^{13}C NMR signal intensities, the degree of substitution is approximately 25 % for both pyruvate acetal and xylose.

One of these two signals remained in the DEPT 135 spectrum, and as the pyruvate signal should disappear, this appears to confirm the assignment, however as the two signals are at best only barely resolved, such a conclusion is unreliable. It appears the signal 3DG(4,6)P-4LG$\underline{1}$ has the same chemical shift as 3DG-4LG$\underline{1}$. Now

S CM1 |–S| (minor) ~ {**S** (X) $\cup$ **S** [3DG(4,6)P-4LAG] $\cup$ **S** [3DG-4LAG]} = {78.4, 73.7, 70.5, 59.2} (12)

From Lahaye *et al.* (1989),

S [3DG6M] ~ **S** [3DG] = {59.2, 71.6, 73.7} (13)

and

{59.2, 71.8, 73.5} $\subset$ **S** CM1 |–S| (minor) (14)

While the signal at 59.2 ppm is broadened and weak, the unit is quite unambiguously identified below.

Following Usov and Elashvili (1991), for 4LG3X, the signals due to LG{$\underline{2}$, $\underline{3}$, $\underline{4}$} occur at {67.3, 73.5, 76.1}. While these signals are present, they are also already assigned, and unless the additional signals {70.5, 78.4} are assigned for this purpose, then they represent a further unidentified component. We provisionally consider 70.5 ppm may be better assigned to 4LG$\underline{2}$3X, and the signal at 78.4 ppm to either $\underline{3}$ or $\underline{4}$ of this species.

Four signals: {65.7, 65.3, 61.4, 61.1} inverted in a DEPT 135 experiment, which inverts methylene signals. This confirmed the above assignment, with one of {65.7, 65.3} being the xylosyl methylene group, and the other the methylene carbon to which the pyruvate is attached.

(ii) Natural polymer. The samples CM1 and CM2 gave poor spectra, with numerous broad signals that could not be listed as discrete signals. However, in both samples, the largest anomeric signal was at about 103.8 ppm, showing from (2) that the major component involved the 3DG-4LG backbone.

A purified form of CM1 gave an improved (but still poor) spectrum with signals including

S CM1 = {104.3, 103.9, 102.5, 101.8, 101.0, 99.7, 98.5, (79.6–82), 77.8, (76–77), 75.9, (72.8–74), 72.4, (70.8–72), (69.5–70.3), 69.3, 68.9, 67.4–67.8, 66.8, 65.6, 65.3, 62.0, 25.7} (15)

where () indicates a number of signals, many of which may indicate splitting due to long-range environmental differences. Given that CM1 |–S| contained no detectable anhydrogalactose, and the signals at 102.5 and 98.5 are almost certainly the anomeric signals of the 3DG-4LAG diad, either selective concentration or the non-alkaline purification involving chlorine gas, bisulfite, and activated carbon seems to have initiated some anhydrogalactosyl formation. Attempts to simplify the spectrum by partially depolymerizing this sample with ultrasonication (represented by |)))|) led to a spectrum (Fig. 1) with three sets of signals, the two strongest of which were

S CM1 |)))|(major) = {102.5, 98.3, 82.2, 80.1, 77.3, 75.6, 75.4, 73.3, 71.8, 70.9, 70.3, 69.9, 69.4, 69.2, 68.9, 68.1, 67.8, 61.5, 59.1} (16)

From (5)

S 3DG-4LAG $\subset$ **S** CM1 |)))| (17)

Thus from (13), (16) and (17) CM1 |)))|(major) is a mixture of agar and agar methylated at the 6 position of the 3-linked unit, the degree of methylation being approximately 30 %. The minor signals present in **S** CM1 |)))| are consistent with the major signals of (15), except that pyruvate acetal was missing. Thus it seems that the ultrasonication must have converted the 4-linked galactose-6-sulfate to 3,6-anhydrogalactose, and presumably removed the pyruvate. Accordingly, a major difficulty in analysing this polymer was obtaining a good spectrum without modifying the polymer.

The presence of 6-*O*-methylgalactose is not surprising from taxonomic considerations, as 6-*O*-methylgalactose was found in *Chondria decipiens* Kylin (Usov *et al.* 1983). Furneaux and Stevenson (1990) did not report the presence of this sugar, although they did report minor amounts of methylated hexosyl residues.

The samples CM1 and CM2 clearly had low levels of 2-sulfation, however this was not the case for the sample CM3 from Furneaux and Stevenson (1990). For this sample the spectrum was relatively well-resolved (Fig. 1) and had many features different from the spectra of CM1 and CM2:

S CM3 = {102.2, 101.9, 101.0, 100.8, 98.5, 81.0, 79.9, 78.1, 77.4, 75.9, 75.6, 75.3, 74.3, 73.3, 72.2, 70.4, 69.7, 69.4, 67.5, 66.5, 65.4, 61.2} (18)

Since Furneaux and Stevenson collected their sample in March, we also collected a sample and repeated the extraction following our method above. No separation into gelling/non-gelling components was achieved, and the spectrum we obtained from the bulk polymer was almost identical to **S** CM3. Accordingly, we conclude that the polysaccharide of *Chondria macrocarpa*

varies from what is predominantly an agar precursor in late spring to a more sulfated agar in autumn, both of which also contain the xylogalactan.

The most striking feature about **S** CM3 is the lack of signals downfield from 102.2 ppm, and hence from (5)

S [3DG-4LG6{H,S}] $\not\subset$ **S** CM3 (19)

However, by chemical analysis (Furneaux and Stevenson 1990) the 4LG unit is present, and since 3DG$\underline{1}$-4LG occurs at approximately 103.7 ppm, virtually all 4LG units must be in an environment where sulfate ester alters the 3DG$\underline{1}$ chemical shift. From (1), this implies that most 4LG units occur in [3DG2S-4LG3{H,X}].

Two signals of 3DG4LAG, namely {82.2, 68.7}, do not occur in **S** CM3, hence this structure appears to be excluded. However, the signal at 69.4 ppm has a very broad upfield 'tail' and there appears to be a broad signal of low intensity at approximately 82 ppm, hence [3DG6{H,S}-4LAG] may be present at low levels.

From Miller and Blunt (2000b)

S [3DG2S-4LAG] = {101.1, 98.3, 80.9, 79.6, 78.2, 77.4, 75.7, 75.1, 69.9, 69.4, 69.0, 61.4} (20)

Hence **S** [3DG2S-4LAG] $\subset$ **S** CM3 (21)

There is no recorded spectrum **S** [3DG2S-4LG], and while the species is most likely to be present in **S** CM1, there was insufficient resolution to reasonably obtain a signal set.

Furneaux and Stevenson (1990) found that almost half the 3-linked residues did not have 2-sulfate esters and stated that the sulfate ester was stable throughout the procedure. If so, given the absence of a signal due to 3DG$\underline{1}$ at 103.8 ppm, then the 3DG$\underline{1}$2H signal must be shifted upfield. Apart from 3DG-4LAG at the low levels constrained by the very low intensity signal at approximately 82.3 ppm (Fig. 1), the only available substituent is xylosyl. Thus either the 3-xylosyl substituent shifts the 103.7 ppm signal due to adjacent 3DG$\underline{1}$ upfield by almost 2 ppm (which requires verification) or it would appear that some desulfation must have occurred in the analysis.

If 3DG-4LG3X6S is a unit of the natural polymer, it follows

S [3DG-4LG3X6S~{$\underline{4}$,$\underline{5}$,$\underline{6}$}] $\subset$ **S** CM1 $\cap$ **S** CM3 (22)

Now **S** CM1 $\cap$ **S** CM3 = {101.1, 100.8, 81.0, 79.7, 78.2, 76.1, 75.6, 73.9, 73.4, 72.2, 70.4, 69.7, 69.3, 66.5, 65.5, 61.3} (23)

If X$\underline{1}$ is at 101.1 or 100.8 ppm,

X $\subset$ **S** CM1 $\cap$ **S** CM3 (24)

as required.

From our assignments above

S [3DG~$\underline{1}$-4LG3X~$\underline{5}$~$\underline{6}$] = {100.8, 81.0, 78.7, 75.5, 73.5, 70.5, 69.9, 68.8, 61.3} (25)

By comparing (23) and (25) with **S** CM3 and allowing for the broad signals in **S** CM3 we would suspect but could not prove the presence of signals due to 3DG-4LG3X in **S** CM3. A definitive identification of the presence of xylosyl substitution by NMR techniques is likely to require the spectrum of the desulfated species.

The spectra of the methylated species

S CM1 |M| = {103.4, 102.7, 101.6, 99.3, 98.8, 82.3, 81.5, 80.1, 79.6, 79.2, 78.9, 78.6, 77.9, 77.6, 77.2, 76.2, 75.8, 73.6, 71.8, 71.0, 70.5, 69.7, 67.8, 66.7, 65.4, 62.1, 61.6, 61.3, 59.1, 57.8, 56.6, 25.6} (26)

S CM1 |M, –S| = 103.4, 102.5, 101.5, 100.7, 99.5, 99.3, 98.6, 97.9, 83.4, 82.1, 81.4, 80.6, 80.1, 79.2, 78.9, 77.9, 77.4, 75.8, 73.6, 72.5, 71.4, 70.5, 69.7, 68.6, 66.7, 65.3, 62.2, 61.3, 61.0, 60.2, 59.3, 59.1, 57.9, 56.7, 25.6} (27)

were largely in accord with the above analysis, allowing for the fact that alkali will convert 4LG6S to 4LAG.

From Miller (2001)

S [3DG-4LG] |M| = {103.2, 98.4, 81.8, 79.3, 79.0, 77.8, 77.1, 73.0, 72.0, 71.2, 70.3, 61.4, 61.2, 59.1, 59.0, 58.7, 56.8} (28)

From Miller and Blunt (2000a, b)

S [3DG-4LAG] |M| = {102.7, 99.5, 82.5, 81.0, 79.1, 78.1, 77.6, 76.0, 74.0, 71.7, 69.5, 61.7, 59.7, 59.3} (29)

S [3DG2S-4LAG] |M| = {101.4, 99.3, 80.5, 79.6, 79.3, 78.7, 77.4, 75.8, 75.2, 73.8, 71.7, 69.5, 61.3, 59.2, 59.0} (30)

As can be seen, if 4LAG2$\underline{M}$ is assigned to 59.1 ppm

S [3DG-4LAG] |M| $\subset$ **S** CM1 |M| (31)

The spectrum (28) was run in cold water because of solubility problems, and differences in chemical shift are expected. Apart from the signal at 73.0 ppm, which has to be accommodated in the broad upfield tail of the signal at 73.6 ppm,

S [3DG-4LG] |M| $\subset$ **S** CM1 |M| (32)

while provided the signal at 75.2 ppm is included in the broad tail to the signal at 75.8 ppm

S [3DG2S-4LAG] |M| $\subset$ **S** CM1 |M| (33)

However, the signal at 101.4 ppm is a very low intensity signal showing that although the species indicated by (33) is present, it is present only at low levels, a result consistent with the above conclusion that the spring sample has low levels of galactose-2-sulfate. That there was no anhydrogalactose in CM1 by desulfation indicates that all anhydrogalactose in the methylated species arose through base catalysed cyclization. The poorer quality of the spectrum for **S** CM1 |M| (Fig. 1) reduces the reliability of conclusions drawn from (31)–(33), however they were supported by

S [3DG4LAG] |M| ⊂ **S** CM1 |M, –S| (34)

S [3DG-4LG] |M| ⊂ **S** CM1 |M, –S| (35)

Ceramium rubrum

The infrared spectrum for the natural polymer CR showed the following signals (cm^{-1}): {1270–1220 (medium), 1150 (medium), 1030–1090 (strong), 990 (shoulder), 932 (medium), 890 (weak), 815 (shoulder), 770 (medium, broad)}. The polymer was clearly substituted by sulfate ester, probably at either a 2-position or a 6-position, but the degree of sulfation, judging from the lack of signal strength at 1200–1300 cm^{-1}, would be less than that of porphyran, which has approximately one sulfate ester per four saccharide units. The spectrum of CR1 |M, –S| (methylated, then desulfated) gave the following weak to medium signals in the region 700–1000 cm^{-1}: {975, 935, 900, 870, 820, 790–760}. There were no signals between 1200–1300 cm^{-1}, thus showing that desulfation was completed.

The ^{13}C NMR spectrum of the desulfated polysaccharide **S** CR|–S| (Fig. 2) was divided into subsets, based on relative intensity. Signals marked † were inverted in a DEPT 135 experiment, showing them to be methylene carbon atoms. The major subset was

S CR|–S| (a) = {102.4, 98.3, 82.3, 80.2, 77.4, 75.7, 75.4, 70.3, 69.9, 69.4†, 68.8, 61.4†} (36)

From (3) **S** CR|–S| (a) = **S** [3DG-4LAG] (37)

that is, the major component is the agar skeleton. The next strongest subset was

S CR|–S| (b) = {103.8, 101.2, 101.0, 81.0, 79.3, 78.8, 73.3, 72.2, 71.1, 70.8, 70.5, 67.6†, 61.7†, 61.2†} (38)

From (2),

S [3DG-4LG] ⊂ **S** CR |–S| (39)

Minor signals included

S CR|–S| (c) = {101.6, 74.7, 74.2, 73.6, 65.5†} (40)

From (10) **S** X ⊂ **S** CR|–S| (41)

if the downfield broadened signal at 75.7 includes the signal at 76.1. Thus xylose is likely to be a low-frequency component. If so, there remain the unaccounted signals

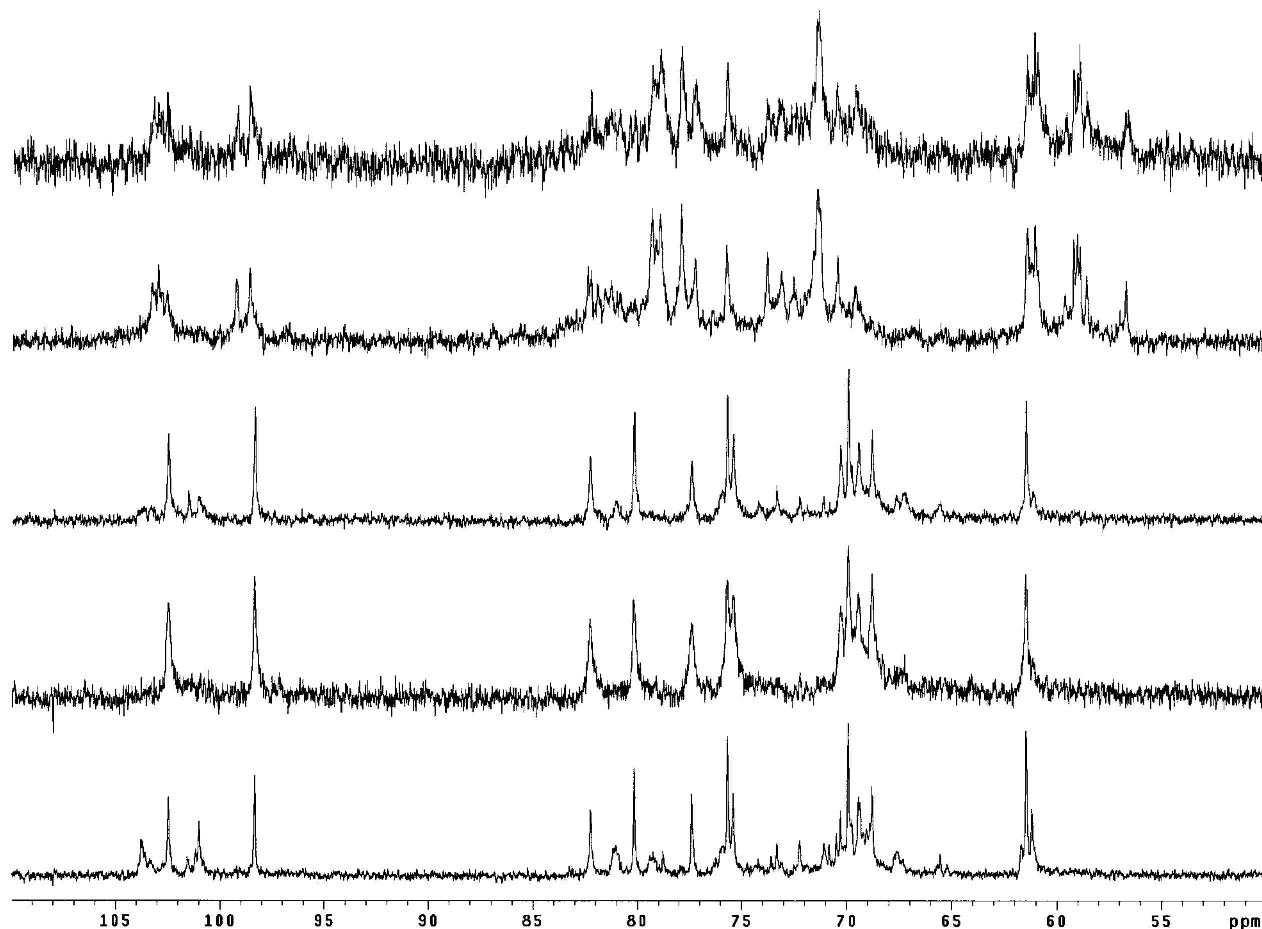

Fig. 2. ^{13}C NMR spectra of polysaccharides from *Ceramium rubrum*: (top to bottom) (a) **S** CR |M, –S|, (b) **S** CR |M|, (c) **S** CR |OH$^-$|, non-gelling fraction, (d) **S** CR |OH$^-$|, gelling fraction, (e) **S** CR |–S|.

S CR|–S| U = {101.5, 101.2, 78.8, 74.7, 74.2, 73.3, 71.1, 70.5, 67.6} (42)

The position of substitution of xylose is unclear. Firstly, from Usov *et al.* (1997) the substitution is not at 3DG6, as this signal would be inverted at approximately 69 ppm in a DEPT 135 experiment. If our assignment following (12) of the signals {78.7, 70.5} is correct, since {78.7, 70.5} $\subset$ **S** CR|–S|, then the result is indicative of 4LG3X, however the low signal intensities of these signals attributed to xylose do not permit us to be more definitive.

To summarise, the underlying polymer framework as deduced from the desulfated natural polymer consists mainly of 3-linked D-galactosyl units alternating with the 4-linked units 3,6-anhydro-L-galactosyl and L-galactosyl, represented by the approximate formula 3DG-4L{$AG_{0.67}$, $G_{0.33}$} to which approximately 8 % of the total galactosyl units are substituted by xylose.

The signals (not shown) for the natural polymer similarly fell into three subsets, based on declining relative signal intensity

S CR(a) = {102.5, 98.3, 82.3, 80.2, 77.4, 75.7, 75.4, 70.3, 69.9, 69.8, 69.4, 69.2, 68.8, 61.7, 61.5} (43)

S CR(b) = {103.6, 101.5, 101.2, 81.0, 78.8, 76.1, 75.9, 73.3, 72.2, 71.1, 70.8, 70.1, 67.6, 61.1} (44)

S CR(c) = {103.3, 79.5, 78.8, 74.2, 65.5} (45)

Thus **S** [3DG-4LAG] $\subset$ **S** CR(a) (46)

hence the unsubstituted agar diad is the major component of the natural polysaccharide. Also,

S [3DG-4LG] $\subset$ **S** CR(a) $\cup$ **S** CR(b) (47)

From information published by Lahaye *et al.* (1989)

S [3DG-4LG6S]~**S** [3DG-4LG] = {72.1, 67.9} (48)

Accordingly **S** [3DG-4LG6S] $\subset$ **S** CR(b) (49)

Following the same reasoning as above, xylose signals can also be seen at a low level.

As noted above the signals characteristic of [3DG2S-4LAG] are {101.1, 78.2, 80.9} (Miller and Blunt 2000b). There is no clear signal at 78.2 ppm, although there is a long downfield 'tail' to the major 77.4 ppm signal and there are signs of a separate low intensity signal at about 77.9 ppm.

An alkali treatment was carried out to cyclise L-galactose-6-sulfate to 3,6-anhydrogalactose. After neutralisation 13 % by weight of the initial material was recovered as insoluble material, which after dialysis was dissolved in hot water. The ^{13}C NMR spectrum of this material (Fig. 2) contained twelve signals of (3). A non-gelling material was also obtained in 49 % by weight of the original material. Greater than 80 % of this material was also non-substituted agar, but there was also a set of minor signals (Fig. 2).

S CR|OH| ~ **S** [3DG-4LAG] = {103.8, 103.5, 101.5, 101.0, 81.0, 75.9, 73.3, 72.2, 71.0, 67.6, 67.3, 65.7, 61.1} (50)

From (10) **S** (X~X$\underline{1}$) $\subset$ **S** CR|OH| (51)

gives

S (CR)|OH| ~**S** [3DG-4LAG] ~ **S** (X~X$\underline{1}$) = {103.8, 103.5, 101.5, 101.0, 81.0, 72.2, 71.0, 67.6, 67.3, 61.1} (52)

The substitution 3DG6S requires (Usov *et al.* 1983)

S 3DG{$\underline{4}$, $\underline{5}$, $\underline{6}$} = {68.4, 73.0, 67.5} (53)

and as {68.8, 73.3, 67.6} $\subset$ **S**(CR) (54)

3DG6S is likely to be present.

The signal at 81.0 ppm in (52) would normally be attributed to 3DG$\underline{3}$-4LG, however this was eliminated since 79.3 $\notin$ **S** CR|$\overline{\text{OH}}$| ~ **S** [3DG-4LAG] ~ **S** (X~X$\underline{1}$). It could also be 3DG2S$\underline{3}$-4LAG, which also requires signals at 78.2 and 101.1 ppm (Miller and Blunt 2000b). There is no clear signal at 78.2 ppm, but there is a broad downfield 'tail' from the signal at 77.4 ppm.

From (2) **S** [3DG-4LG] $\not\subset$ **S** CR|OH| (55)

because the only signal present required for 4LG$\underline{4}$ at $\approx$ 79.1 is the very weak signal at about 79.4 ppm. On the other hand, the signals at {103.8, 103.5, 101.5, 101.0} are strongly suggestive that at least one 3DG4LG unit is present with some degree of substitution.

Upon methylation the spectrum **S** CR |M| was obtained (Fig. 2)

S CR |M| = {103.3, 103.0, 102.6, 99.3, 98.6, 82.4, 82.0, 81.3, 79.3, 79.0, 77.9, 77.3, 75.7, 73.8, 73.1, 72.5, 71.4, 70.5, 69.6, 61.4, 61.1, 59.6, 59.2, 59.1, 58.9, 58.6, 56.7} (56)

Also present were a number of minor signals of very low intensity which are not recorded here. From (29)

S [3DG-4LAG] |M| $\subset$ **S** CR |M| (57)

and from (28)

S [3DG-4LG] |M| $\subset$ **S** CR |M| (58)

This is confirmed by the signals at {58.6, 56.7} which are due to 4LG2$\underline{\text{M}}$, 4LG3$\underline{\text{M}}$, respectively (Miller and Blunt 2000a). The signal at 67.6 ppm, stable to alkali shown by (52), is removed by methylation. This suggests that the methylation procedure itself has not retained sulfate ester fully. This issue requires further investigation, as methylation is a cornerstone of polysaccharide structural analysis.

Methylation also allows us to assess the levels of 2-sulfation. From the previous spectral assignment for 3DG2S-4LAG|M| we can confirm that the necessary signals are present as minor components in **S** CR |M| (signals at {101.4, 75.2, 79.6}) but they are very much minor signals. Similarly,

S CR |M, –S| = {103.3, 102.6, 99.2, 98.6, 82.3, 81.8–81.3 (b), 80.2, 78.9 (b), 77.9, 77.2, 75.7, 73.8, 73.4, 73.2, 72.5 (b), 71.4, 70.5, 69.6, 61.4, 61.1, 59.6, 59.2, 58.9 (b), 58.6, 56.7} (59)

where (b) refers to a broadened signal.

As can be seen in Figure 2 the spectra **S** CR |M, –S| and **S** CR |M| are very similar, the major components of both being permethylated agar, with the major differences being a loss of intensity at 79.3 ppm coupled with an increase at about 82 ppm for the desulfated entity, which would be expected following desulfation if 3DG2S4M6M was a minor component.

Conclusions

The structure of the polysaccharides extracted from *Chondria macrocarpa* varied depending on season. Our March extract was equivalent to that obtained by Furneaux and Stevenson (1990) however an extract from samples obtained in November could be separated into two fractions, one of which contained no anhydrogalactosyl units and little 2-sulfate ester. The November fraction also contained significant levels of pyruvate acetal, not found in the original chemical analysis, significant levels of 6-*O*-methylation on the 3-linked unit, and only low levels of 2-sulfation on the 3-linked unit. The March sample appears to have all 3G attached to 4LG in the form 3DG2S.

Ceramium rubrum was also found to consist of two similar polysaccharides. Both contained agarobiose and the precursor diads, but following alkali treatment, a gelling fraction which was indistinguishable from agar by ^{13}C NMR spectroscopy was obtained, together with another fraction which consisted mainly of agarobiose diads, but had low levels of additional substitution. The natural polysaccharide from *Ceramium rubrum* consists of [3DG-4L{$AG_{0.67}$, $G6\{S,H\}_{0.33}$}] with low levels of xylosyl substitution, possibly at more than one location. There are only very low levels of additional sulfation, and we can confirm that 3DG2S is present at the limits of detection, but we can find no positive indication for 3DG4S, and can definitely rule out 3DG4S-4LAG at our levels of detection. From the methyl signals, the intensities of the signals 4LG2$\underline{M}$ and 4LG3$\underline{M}$ are approximately equal, which suggests that the 4LG units that do not form anhydrogalactosyl units are essentially unsubstituted at these positions, and hence, since they do not cyclise, are also non-sulfated at the 6-position. This would explain why, despite the low level of sulfation, the bulk of the polysaccharide does not form a gel.

Acknowledgements

We would like to thank Dr W. Nelson for assistance with seaweed identification, Mr Rewi Thompson for running the NMR spectra, Dr R. H. Furneaux for a sample of the *Chondria macrocarpa* extract he analysed, and the Foundation for Research, Science and Technology for finance under contract CRL 801.

Accepted 10 July 2001.

References

Chiovitti, A., A. Bacic, D. J. Craik, S. L. A. Munro, G. T. Kraft and M.-L. Liao. 1997. Cell-wall polysaccharides from Australian red algae of the family Solieriaceae (Gigartinales, Rhodophyta): novel, highly pyruvated carrageenans from the genus *Callophycus*. *Carbohydr. Res. 299*: 229–243.

Furneaux, R. H. and T. T. Stevenson. 1990. The xylogalactan from *Chondria macrocarpa* (Ceramiales, Rhodophyta). *Hydrobiologia 204/205*: 615–620.

Lahaye, M., W. Yaphe, M. T. P. Viet and C. Rochas. 1989. ^{13}C NMR spectroscopic investigation of methylated and charged agarose oligosaccharides and polysaccharides. *Carbohydr. Res. 190*: 249–265.

Matsuhiro, B. 1996. Vibrational spectroscopy of seaweed galactans. *Hydrobiologia 326/327*: 481–489.

Miller, I. J. 1998. The structure of a pyruvylated carrageenan extracted from *Stenogramme interrupta* as determined by ^{13}C NMR spectroscopy. *Bot. Mar. 41*: 305–315.

Miller, I. J. 2001. Evaluation of the structure of the polysaccharide from *Myriogramme denticulata* as determined by ^{13}C NMR spectroscopy. *Bot. Mar. 44*: 583–587.

Miller, I. J. and J. W. Blunt. 1998. Desulfation of algal galactans. *Carbohydr. Res. 309*: 39–43.

Miller, I. J. and J. W. Blunt. 2000a. New ^{13}C NMR methods for determining the structure of algal polysaccharides. Part 1. The effect of substitution on the chemical shifts of simple diad galactans. *Bot. Mar. 43*: 239–250.

Miller, I. J. and J. W. Blunt. 2000b. New ^{13}C NMR methods for determining the structure of algal polysaccharides. Part 2. Galactans consisting of mixed diads. *Bot. Mar. 43*: 251–261.

Miller, I. J. and R. H. Furneaux. 1982. Agars from New Zealand red algae in the family Gelidiceae: a structural study. *N. Z. J. Sci. 25*: 15–18.

Rochas, C., M. Lahaye and W. Yaphe. 1986. Sulfate content of carrageenan and agar determined by infrared spectroscopy. *Bot. Mar. 24*: 335–340.

Turvey, J. R. and E. L. Williams. 1976. The agar-type polysaccharide from the red alga *Ceramium rubrum*. *Carbohydr. Res. 49*: 419–425.

Usov, A. I. and M. Ya. Elashvili. 1991. Polysaccharides of the algae 44. Investigation of the sulfated galactan from *Laurencia nipponica* Yamada (Rhodophyta, Rhodomelaceae) using partial reductive hydrolysis. *Bot. Mar. 34*: 553–560.

Usov, A. I., M. I. Bilan and A. S. Shashkov. 1997. Structure of a sulfated xylogalactan from the calcerous red alga *Corallina pilulifera* P. *et* R. (Rhodophyta, Corallinaceae). *Carbohydr. Res. 303*: 93–102.

Usov, A. I., E. G. Ivanova and A. S. Shashkov. 1983. Polysaccharides of the algae 33: Isolation and ^{13}C NMR spectral study of some new gel-forming polysaccharides from Japan Sea red seaweeds. *Bot. Mar. 26*: 285–294.