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## Note

# $^{13}$C-N.m.r.-spectral analysis of sulfated and desulfated polysaccharides of the agar type

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Agar is a mixture, extracted from certain red marine algae (Rhodophyceae), of hot- and cold-water-soluble polysaccharides having agarose at one extreme, with alternating 4-*O*-linked 3,6-anhydro-α-L-galactopyranose and 3-*O*-linked β-D-galactopyranose as the fundamental backbone structure. This repeating unit is often found substituted to various extents[1–3] by methyl ethers, sulfuric esters, and pyruvic acetals. The alternating, 4-*O*-linked α-L-galactopyranose 6-sulfate (L′) and 3-*O*-linked β-D-galactopyranose (D′) is believed to be the biological precursor to agarose[4] (see Fig. 1A). Chemical analysis of agarose polymers also revealed the existence of 4-*O*-methyl-α-L-galactopyranose, probably linked as a branch to C-6 of 3-*O*-linked β-D-galactopyranose[5,6], as well as a structural irregularity where both the 3,6-anhydro-α-L-galactopyranose and the α-L-galactopyranose 6-sulfate residues are missing, the L sugar being present as L-galactopyranose[7].

We now report the $^{13}$C-n.m.r. chemical-shift assignments for a disaccharide repeating unit, 4-*O*-linked α-L-galactopyranose (L) alternating with 3-*O*-linked β-D-galactopyranose (D) (see Fig. 1B), obtained by solvolytic desulfation of the 4-*O*-linked α-L-galactopyranose 6-sulfate. These data should be useful in evaluating the structure of agars by $^{13}$C-n.m.r. spectroscopy, a technique which has proved to be fast and reliable for the structural determination of the cell-wall polysaccharides[8] of marine red algae. We have also re-evaluated some assignments of the agarose-precursor repeating unit (see Fig. 1A).

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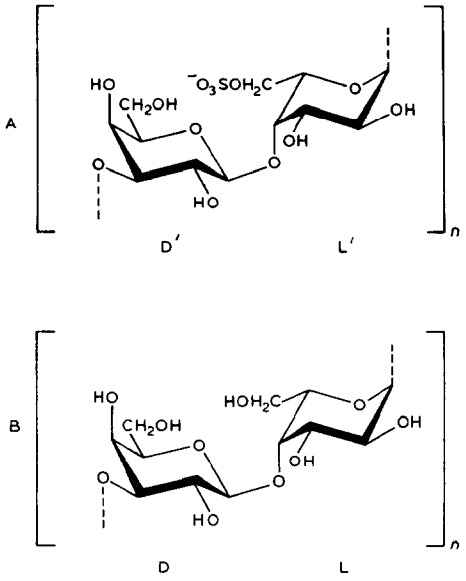


Fig. 1. Chemical structure of: (A), the alternating 3-*O*-linked β-D-galactopyranose (D′) and 4-*O*-linked α-L-galactopyranose 6-sulfate (L′); and (B), the alternating 3-*O*-linked β-D-galactopyranose (D) and 4-*O*-linked α-L-galactopyranose (L) repeating units.

## EXPERIMENTAL

Carrageenans enriched with repeating units containing D-galactopyranose 6-sulfate (the precursor to 3,6-anhydro-galactopyranose) are extracted into water at room temperature[9–11], and we used this method to obtain agarose molecules enriched with L-galactopyranose 6-sulfate. Preliminary experiments showed that the agar from *Porphyra haitenensis* has a high proportion of L-galactopyranose 6-sulfate and a low proportion of 6-*O*-methyl-β-D-galactopyranose. Air-dried *P. haitenensis* was obtained from the People's Republic of China. Hand-sorted and cleaned algae (10 g) were frozen in liquid nitrogen, and ground in a mortar. Algal pieces were dispersed in distilled water (5 g · $L^{-1}$ in 2-L Erlenmeyer flasks), shaken for 12 h at 22°, and filtered through glass-fiber pads (GF/A, Whatman), using a pressure filter (Millipore Corp. No. XX4004740). The algal residues were re-extracted for 12 h and filtered. The filtrates were combined, concentrated *in vacuo*, dialyzed extensively against distilled water, filtered through 1.2- and 0.45-μm membrane filters (Millipore), and freeze-dried. The material recovered, 9.6% of the starting dry weight of the algae, is designated the cold extract. The $^{13}C$-n.m.r. spectrum showed the presence of agarose precursor and agarose repeating units in an estimated ratio of 2:1 (obtained by comparing the signal intensities of the anomeric carbon atom of the 3-*O*-linked D-galactopyranose in the two disaccharides).

The cold extract (200 mg) was dissolved in water, and agarose repeating units

were removed by treatment with an excess of β-agarase I, purified according to Morrice *et al.*[12]. The hydrolyzate was fractionated on a column (100 × 4.2 cm diam.) of Bio-Gel P6 (Bio-Rad Laboratories, Richmond, CA) with 50mM NaCl in distilled water as the eluant at a flow rate of 30 mL/h, and a resistant fraction, recovered in the void volume, was desalted on Sephadex G-25 (Pharmacia Fine Chemicals), and freeze-dried (107 mg). The enzyme-resistant fraction was shown by $^{13}$C-n.m.r. spectroscopy to be enriched in agarose-precursor repeating unit, with an estimated ratio of precursor to agarose repeating units of 6:1 (as determined before). A slight increase in 6-*O*-methyl-β-D-galactopyranose-containing repeating unit was also noted.

Several solvolytic desulfations have been described in the literature[13], but the procedure we used was as follows. The cold extract (645 mg) was placed in a 250-mL, round-bottomed flask containing 100 mL of methanol (Fisher Scientific Co.) and a magnetic stirring bar. The flask was closed with a plug of glass wool and $P_2O_5$ (Fisher). Acetyl chloride (3 mL, Fisher) was slowly added with stirring, the mixture stirred for 6 h at room temperature, and the acid neutralized by the addition of sodium carbonate (Fisher, 6 g) dissolved in a small amount of distilled water and by an additional 3 g of sodium carbonate powder. The suspension was concentrated *in vacuo*, dialyzed against distilled water, and freeze-dried. The incompletely anhydrous state of our reagents may have created acidic conditions in the reaction mixture, resulting in degradation of the cold extract, as only a 30% yield was obtained.

Solutions (4–5%, w/v) in $D_2O$ of the cold-extract, enzyme-resistant fraction and of desulfated cold extract, filtered through glass-fiber pads (GF/A, Whatman), were analyzed on a Bruker WH 400 spectrometer at 100.62 MHz. Proton-decoupled $^{13}$C-n.m.r. spectra were recorded at room temperature and at 80°. The chemical shifts (p.p.m.) were measured relative to internal dimethyl sulfoxide (39.6 p.p.m.) and converted into values relative to external $Me_4Si$. Distortionless enhancement by the polarization-transfer (DEPT) pulse technique was used, as described[14], at room temperature.

The $^{13}$C-n.m.r. chemical shifts for the cold-extract, enzyme-resistant fraction are consistent with structure A (see Fig. 1) and agree with those published[15,16], with the exception of the C-5 (L′) signal originally assigned[15] at 73.5 and later reassigned[16] at 67.7 p.p.m. (see Table I). The DEPT sequence permitted definitive assignment of the C-6 (L′) signal at 68.10 p.p.m. (room temperature) by the characteristic, $CH_2$ negative signal (see Fig. 2). This assignment is shifted to 67.67 p.p.m. in the spectrum obtained at 80°. The C-5 (L′) atom was assigned, by deduction, to the signal at 70.16 p.p.m. at 80° or at 70.38 p.p.m. at room temperature. It appears that the assignment of C-5 (L′) reported by Shashkov *et al.*[15] at 73.5 p.p.m. could correspond to C-6 of the D-galactopyranose residue when this carbon atom bears a methoxyl group[17], as they analyzed a methylated agar derived from a different alga, *Bangia fuscopurpurea*, having 6-*O*-methyl-β-D-galactopyranose in the repeating unit[15]. A signal at 67.5 p.p.m., assigned by Usov *et al.*[16] to C-6 (L′)

TABLE I

$^{13}$C-N M R CHEMICAL SHIFTS (p.p.m.) AT 80° AND AT ROOM TEMPERATURE (r.t.) OF *Porphyra haitenensis* COLD-EXTRACT, ENZYME-RESISTANT FRACTION, AND DESULFATED COLD EXTRACT

| *Carbon atom* | *Literature values) (Usov* et al.[16]) | | *Sample* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Cold-extract, enzyme-resistant fraction* | | | | *Desulfated cold extract* | | | |
| | *80°* | | *80°* | | *r.t.* | | *80°* | | *r.t.* | |
| | *D'*[a] | *L'*[a] | *D'* | *L'* | *D'* | *L'* | *D*[a] | *L*[a] | *D* | *L* |
| C-1 | 103.7 | 101.2 | 103.67 | 101.26 | 103.90 | 101.72 | 103.73 | 100.94 | 103.82 | 101.17 |
| C-2 | 70.5 (69.7)[b] | 69.8 | 69.79 | 69.23 | 69.87 | 69.30 | 69.95 | 69.37 | 69.99 | 69.41 |
| C-3 | 81.1 | 71.0 | 81.20 | 71.00 | 81.32 | 71.23 | 81.04 | 71.04 | 81.04 | 71.20 |
| C-4 | 69.1 | 79.0 | 69.05 | 79.00 | 69.30 | 79.26 | 68.90 | 79.32 | 69.02 | 79.38 |
| C-5 | 75.9 | 67.7 (73.5)[b] | 75.85 | 70.16 | 76.20 | 70.38 | 75.65 | 72.20 | 75.80 | 72.39 |
| C-6 | 61.8 (61.4)[b] | 67.5 | 61.63 | 67.67 | 61.96 | 68.10 | 61.39 | 61.18 | 61.56 | 61.31 |

[a]D', L', D, and L refer to sugars in the repeating units depicted in Figs. 1A and 1B, respectively. [b]Values reported by Shashkov *et al.*[15].

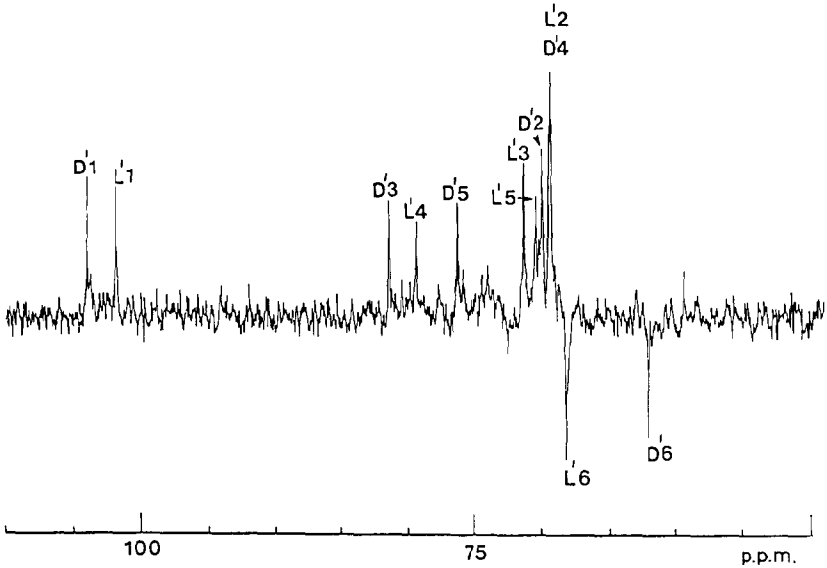


Fig. 2. DEPT sequence of the cold-extract, enzyme-resistant fraction recorded at room temperature with 800 scans, a spectral width of 10 kHz, an acquisition time of 0.819 s, and a relaxation delay of 1.0 s.

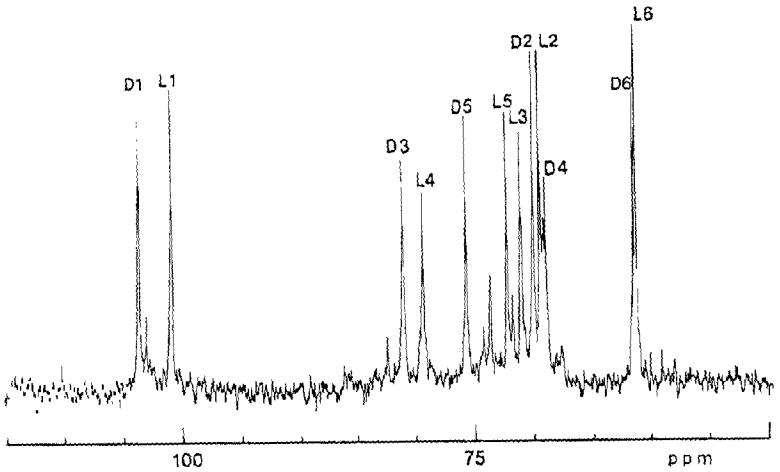


Fig. 3. $^{13}$C-N.m.r. spectrum of the desulfated cold extract, recorded at 80° with 1912 scans, a spectral width of 13.157 kHz, an acquisition time of 0.623 s, and a relaxation delay of 0.5 s.

could correspond to C-2 (L) when C-3 (L) bears a sulfate group, although experimental proof will be required in order to verify this sulfation pattern. Other small signals may be attributed to residual agarose and 6-*O*-methylated agarose repeating units, as well as to the $\alpha$ and $\beta$ anomers of the reducing-end sugar.

Desulfation was not complete, as some residual sulfated repeating unit could be detected in the $^{13}$C-n.m.r. spectra of the desulfated cold extract (see Fig. 3). Other small signals could be attributed to agarose and 6-*O*-methylated agarose repeating units. The spectra of the sulfated and desulfated polymers were compared (see Figs. 1A and 1B, and Table I). A major upfield shift (6.49 p.p.m. at 80°) occurred for C-6 (L), to 61.18 p.p.m. at 80° or to 61.31 p.p.m. at room temperature. A downfield shift (2.04 p.p.m. at 80°) to 72.20 p.p.m. at 80° or 72.39 p.p.m. at room temperature was observed for C-5 (L), and a slight downfield shift was detected for C-4 (L).

This spectrum (see Fig. 3) confirmed the $^{13}$C-n.m.r. chemical shift assignments of the agarose-precursor repeating unit, and provided information for the characterization of an agar having alternating 4-*O*-linked $\alpha$-L-galactopyranose and 3-*O*-linked $\beta$-D-galactopyranose residues. These results may also be helpful in determining the $^{13}$C-n.m.r. chemical-shift assignments of agarose molecules having branched 4-*O*-methyl-$\alpha$-L-galactopyranose. Desulfation of the sulfated agar galactan (see Fig. 1A) brings about a change in the spatial environment of carbon atoms in the D-galactopyranose, and of C-1 and C-2 of the L-galactopyranose, as detected by the small chemical shifts in their assignments. The effects of the enantiomeric form on the $^{13}$C-n.m.r. chemical shifts of the algal polysaccharides having 4-*O*-linked and 3-*O*-linked galactopyranose can be observed by comparing assignments obtained for this D and L polymer with those of the D and D polymer[18] in carrageenan.

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