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^{13}C-N.M.R. SPECTROSCOPIC INVESTIGATION OF METHYLATED AND CHARGED AGAROSE OLIGOSACCHARIDES AND POLYSACCHARIDES*

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ABSTRACT

The ^{13}C-n.m.r. signals of agarose oligomers with various substituted repeating units have been assigned. Enzymic hydrolysis of agaroses gave 2^1-*O*-methylagarobiose, 6^2-*O*-methylagarobiose, the agarobiose biological precursor, agarobiose 4^2-sulfate, 2^1-*O*-methylagarobiose 4^2-sulfate, and pyruvylated agarobiose. The chemical shift data of the oligomers and the parent polymers were compared, and indicated the distribution of the substituents in hybrid polymers.

INTRODUCTION

Agar comprises a family of cell-wall polysaccharides extracted from marine red algae and consists of alternating 3-linked β-D-galactopyranose and 4-linked 3,6-anhydro-α-L-galactopyranose (agarobiose) residues[1] (**1**). Substituents such as sulfate esters, pyruvate acetal, and methyl ethers[1–3] are present and some of the 3,6-anhydro-α-L-galactose residues are replaced by its proposed biological precursor, α-L-galactose 6-sulfate[4]. The physical and rheological properties of agar depend on the extent and type of substitution[5,6], and correlation of the structure with these properties requires characterization of the repeating units. One approach to such an analysis involves the use of an agar-degrading enzyme, such as a β-agarase, and analysis of the products[7–11] using ^{13}C-n.m.r. spectroscopy. This approach has been used to help in the assignment of ^{13}C resonances of algal galactans[9,12–17] and to

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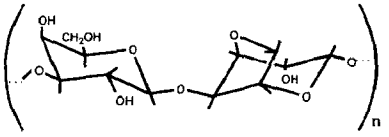
characterize the fine structures of agars and carrageenans[10,11,19–21]. Recently, the assignments of the ^{13}C resonances of agarose were verified from the chemical shift data for the series of neoagaro-oligosaccharides (**2**) generated by the action of β-agarase on agarose[17]. The oligosaccharides contain all the glycosidic linkages present in the polymer and constitute better model compounds than synthetic sugars for the assignment of ^{13}C resonances. We have applied a similar approach to known and novel agarobiose repeating units.



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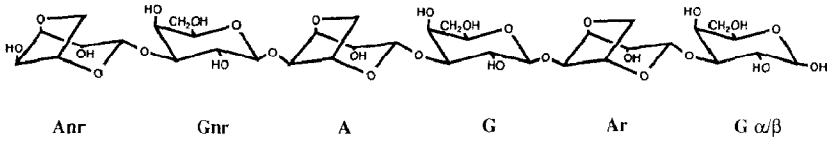


Anr Gnr A G Ar G α/β

2

EXPERIMENTAL

Agar samples. — Solutions (0.5%) of agar fractions, extracted by the sequential solvent method[3], in distilled water were treated with an excess of β-agarase. These fractions were the aqueous 40% ethanol extracts from *Gracilaria crassissima*[22] and *G. compressa*, the aqueous 80% ethanol extract from *G. cervicornis (ferox)*[23], the aqueous 40% ethanol extract from winter-collected *G. pseudoverrucosa*[24], the alkali-modified aqueous 60% ethanol extract from *G. eucheumoides*[3], and the alkali-modified gel matrix left after freezing and thawing *G. eucheumoides* agar gel[25].

Preparation of β-agarase. — β-Agarase (β-agarase-I) was purified[26] from the cell-free culture supernatant solution of the marine bacterium *Pseudomonas atlantica*. Crude β-agarase consisted of the ammonium sulfate-precipitated material from the above cell-free supernatant solution, redissolved in 20mM Tris-HCl buffer (pH 7.5) containing 100mM NaCl. All fractions were treated with an excess of purified β-agarase unless stated otherwise.

Chromatography of agaro-oligosaccharides. — Each enzymic hydrolysate of agar was boiled and applied to a column (26 × 1.1, or 20 × 2.5 cm) of DEAE-Sephadex A 25 (Cl^- form) equilibrated with distilled water at room temperature and eluted first with 3 bed vol. of distilled water, then with a NaCl gradient (0→M, 5 bed vol.). Fractions (4 mL) were analyzed for total carbohydrate by the phenol–sulfuric acid method[27].

Fractions containing neutral and charged oligosaccharides were concentrated *in vacuo* to 10–15 mL and eluted from columns (100 × 4.2 cm) of Bio-Gel P2 and P6 (Bio-Rad Laboratories), respectively, using distilled water and 50mM NaCl, respectively, at 30–50 mL/h. The elution was monitored with a refractive index detector (Pharmacia). The appropriate fractions were combined, concentrated *in vacuo*, desalted on a column (100 × 2.6 cm) of Sephadex G25, and freeze-dried.

Analytical chromatography was performed[28] using a column (2 m × 1.5 cm) of Bio-Gel P2 (200–400 mesh) and elution with H_2O at 65°.

^{13}C-N.m.r. spectroscopy. — Spectra of 4–5% solutions of oligosaccharides in D_2O were recorded at room temperature with a Bruker WH 400 spectrometer operating at 100.6 MHz. Spectral widths of 10–18 kHz and relaxation delays of 0.5–0.7 s were used. Chemical shifts were measured in p.p.m. from the signal for internal or external Me_2SO and converted into values related to Me_4Si (conversion factor, 39.6).

RESULTS AND DISCUSSION

The nomenclature used to designate sugars in the oligosaccharides was as described by Rochas *et al.*[17]. The disaccharide at the non-reducing end is composed of residues of 3,6-anhydro-α-L-galactopyranose (or substituted α-L-galactopyranose) and β-D-galactopyranose (or substituted β-D-galactopyranose) designated as Anr and Gnr, respectively. Internal 3,6-anhydro-α-L-galactopyranose and β-D-galactopyranose residues are designated as A and G, respectively, and 3,6-anhydro-α-L-galactopyranose and α- and β-D-galactopyranose residues at the reducing end are designated as Ar, Gα, and Gβ, respectively (see **2**).

Assignments of the ^{13}C resonances took into account the variations in the intensities of the signals associated with the various repeating units in the oligosaccharides[17]. The number (d.p.) of repeating units was determined from the ratio of the integrated intensities of the signals for C-1: G1α + G1β + G1nr + G1/(G1α + G1β). The ratio G1α + G1β + G1nr + G1/(A1 + A1r + A1nr) was close to 1 when A and G units were present in equal proportion and close to 0.5 or 2 when one of these residues was missing. The d.p. was corrected according to the latter value.

Methylated oligomers formed from Gracilaria eucheumoides *agar containing 2^1-O-methylagarobiose residues.* — Agar extracted from *G. eucheumoides* with boiling aqueous 60% ethanol was alkali-treated[3] and hydrolysed with crude β-agarase. The neutral oligosaccharides, eluted from DEAE-Sephadex A 25 with distilled water, were chromatographed on Bio-Gel P2 to give peaks (I–III) which were identified as tetra-, tri-, and di-saccharides.

Signals of the saccharides in peaks I and III were assigned by comparison with reported[17] chemical shift data (Table I). The signals at 59.5, 59.4, 59.3 p.p.m. were assigned to methyl ether groups, but those for A2r, A2nr, A3r, A3nr, A4r, and A4nr, observed or neoagaro-oligosaccharides, were absent. Instead, reso-

TABLE I

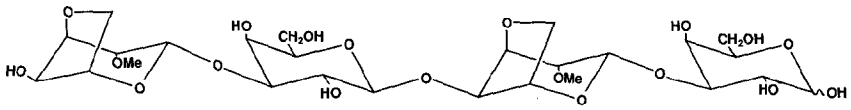
^{13}C-N.M.R. ASSIGNMENTS AT ROOM TEMPERATURE OF NEOAGAROTETRAOSE (AGAROSE) AND NEUTRAL SUBSTITUTED NEOAGARO-OLIGOSACCHARIDES OBTAINED AFTER HYDROLYSIS OF AGARS FROM *Gracilaria eucheumoides* AND *G. cervicornis* WITH β-AGARASE BY ELUTION FROM BIO-GEL P2

Sample	*G. eucheumoides*			*Agarose*[a]	*G. cervicornis*		*Agarose*
Elution peak[b]	III	II	I		I	II	
D.p.	1	1.5	2	2	2	3	3
Structure	**4**	**5**	**3**		**7**	**6**	
Carbon atom							
G1β	97.1	97.1	97.1	97.1	97.1	97.1	97.1
G1α	93.1	93.1	93.1	93.1	93.1	93.1	93.1
G1nr		103.0	102.9	102.7	102.7	102.7	102.7
G1						102.7	102.7
G2β	71.7	71.7	71.7	71.9	71.8	71.8	71.9
G2α	68.2	68.2	68.2	68.4	68.3	68.3	68.4
G2nr		71.2	70.4	70.6	70.5	70.5	70.5
G2						70.5	70.5
G3β	83.3	83.3	83.3	82.9	82.4	82.4	82.9
G3α	80.1	80.1	80.1	79.8	79.7	79.7	79.8
G3nr		73.3	83.0	82.4	82.4	82.4	82.4
G3						82.5	82.5
G4β	69.3	69.3	69.3	69.2	69.5	69.5	69.2
G4α	69.9	69.9	69.9	69.8	70.1	70.1	69.8
G4nr		69.2	69.1	69.1	69.4	69.4	69.1
G4						69.4	69.1
G5β	75.5	75.5	75.5	75.6	73.7	73.7	75.6
G5α	70.9	70.9	70.9	71.0	69.1	69.1	71.0
G5nr		76.0	75.6	75.7	74.0	74.0	75.7
G5						74.0	75.7
G6β	61.7	61.7	61.7	61.8	72.5	72.5	61.8
G6α	61.9	61.9	61.9	61.9	72.6	72.6	61.9
G6nr		61.6	61.6	61.8	72.3	72.3	61.8
G6						72.3	61.8
A1r		99.1	99.1	98.7	98.9	98.9	98.7
A1						98.9	98.7
A1nr	98.9		99.0	98.5	98.7	98.7	98.5
A2r		78.8	78.9	70.1	70.1	70.1	70.1
A2						70.1	70.1
A2nr	78.8		78.9	70.0	70.0	70.0	70.0
A3r		78.7	78.7	80.4	80.5	80.5	80.4
A3						80.5	80.4
A3nr	79.5		79.6	81.3	81.4	81.4	81.3
A4r		77.9	77.9	77.7	77.8	77.8	77.7
A4						77.8	77.7
A4nr	70.5		70.5	70.4	70.5	70.5	70.4
A5r		75.9	75.9	75.9	75.9	75.9	75.9
A5						75.9	75.9
A5nr	77.7		77.8	77.8	77.8	77.8	77.8
A6r		69.7	69.7	69.7	69.8	69.8	69.7
A6						69.8	69.7
A6nr	69.5		69.5	69.5	69.5	69.5	69.5
Methyl[c]							
rα	59.3	59.3	59.3		59.2	59.2	
rβ	59.4	59.4	59.4		59.4	59.4	
nr			59.5		59.5	59.5	
internal						59.5	

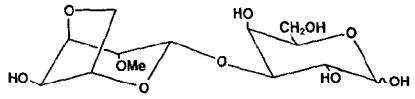
[a]From ref. 17. [b]Refers to Bio-Gel P2. [c]Refers to the MeO associated with sugars at the reducing end (rα, rβ), non-reducing end (nr), and internal repeating unit.

nances at 78.9, 78.7, 79.6, 77.9, and 70.5 p.p.m. were observed and assigned to A2r, A2nr, A3r, A3nr, A4r, and A4nr, respectively, by comparison with data reported for methyl 3,6-anhydro-2-*O*-methyl-α-D-galactopyranoside[29]. Based on the intensities of the peaks, the methyl signals were assigned to the anhydrogalactose residues as follows: A2nr, 59.5 p.p.m.; A2rβ (Ar unit linked to β-D-galactopyranose), 59.4 p.p.m.; and A2rα (Ar unit linked to α-D-galactopyranose), 59.3 p.p.m. A signal at 60.2 p.p.m. was assigned to the Tris buffer originating from the enzymic preparation[30].

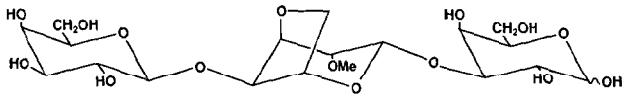
The saccharides in peaks I and III were identified as $2^2,2^4$-di-*O*-methylneoagarotetraose (**3**) and 2^2-*O*-methylneoagarobiose (**4**), respectively.



3 $2^2,2^4$-di-*O*-methylneoagarotetraose



4 2^2-*O*-methylneoagarobiose

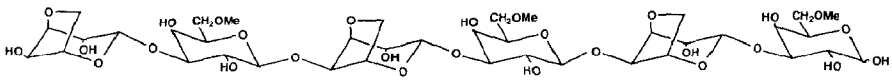


5 2^2-*O*-methylagarotriose

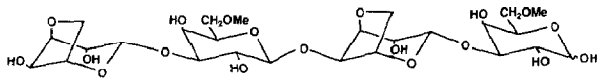
Resonances for the Gα, Gβ, and Ar units in **3** were observed in the ^{13}C-n.m.r. spectrum of the saccharides in peak II (Table I), but signals for the Anr unit in the methylated tetrasaccharide were absent. Instead, signals at 103.0, 71.2, 73.3, 69.2, 76.0, and 61.6 p.p.m. were observed and assigned to C-1,2,3,4,5, and 6, respectively, of a β-D-galactopyranosyl unit at the non-reducing end (Table I) by comparison with data reported by Messer *et al.*[31]. The saccharide in this fraction was identified as *O*-β-D-galactopyranosyl-(1→4)-*O*-(3,6-anhydro-2-*O*-methyl-α-L-galactopyranosyl)-(1→3)-D-galactopyranose (**5**, 2^2-*O*-methylagarotriose). The prefix "agaro" connotes the presence of β-D-galactopyranose at the non-reducing end[32]. The formation of a trisaccharide by the hydrolysis of agar with *P. atlantica* β-agarase is unusual since the major end-products are neoagarotetraose and, to a lesser extent, neoagarobiose[33–35]. The agarotriose (**5**) may correspond to the non-reducing and/or reducing ends of the polysaccharides and may indicate their relatively low molecular weight. Similar results were obtained using a purified β-agarase.

Methylated saccharides containing 6²-O-methylagarobiose residues formed from Gracilaria cervicornis (ferox) *agar.* — The neutral oligosaccharides formed by hydrolysis with β-agarase of agar extracted from *G. cervicornis (ferox)* with aqueous 80% ethanol[23] were chromatographed on Bio-Gel P2, to give peaks II and I identified as hexa- and tetra-saccharides, respectively.

The ^{13}C resonances of the Ar, A, and Anr units, and for C-1,2,3 of the G, Gnr, Gβ, and Gα units were given by both fractions (Table I). Signals for the OMe groups were observed at 59.2, 59.4, and 59.5 p.p.m., and the signal at 60.4 p.p.m. was assigned to the Tris buffer[30]. The resonances at 70.1, 69.1, 72.6, and 59.2 p.p.m. were assigned to C-4,5,6 and to MeO, respectively, of 6-*O*-methyl-α-D-galactopyranose at the reducing end by comparison with data reported by Gorin and Mazurek[36]. Similarly, by comparison with data reported by Brasch *et al.*[37], the signals at 69.5, 73.7, 72.5, and 59.4 p.p.m. were assigned to C-4,5,6, and to MeO, respectively, of the 6-*O*-methyl-β-D-galactopyranose residue at the reducing end. Finally, the resonances at 69.4, 74.0, 72.3, and 59.5 p.p.m. were assigned to C-4,5,6, and to MeO of the G and Gnr units by comparison with the values observed for the 6-*O*-methyl-β-D-galactopyranose at the reducing end (Table I). Thus, the saccharides in peaks II and I were identified as 6¹,6³,6⁵-tri-*O*-methylneoagarohexaose (**6**) and 6¹,6³-di-*O*-methylneoagarotetraose (**7**), respectively. The oligosaccharides **6** and **7** differed from those obtained from *Porphyra umbilicalis* agar[10] in having a methoxyl group at position 6 of the D-galactopyranose residue at the reducing end.



6 6¹, 6³, 6⁵-tri-*O*-methylneoagarohexaose



7 6¹, 6³-di-*O*-methylneoagarotetraose

T.l.c. (1-butanol–ethanol–water, 3:1:1) was used occasionally to follow the enzymic degradation of agars. On detection with the naphthoresorcinol reagent[38], faint yellow spots were obtained under u.v. light from 2-methylated oligomers instead of the blue spots usually observed for the 3,6-anhydrogalactose-containing oligomers. Furthermore, due to the different hydration of the methylated oligomers, different positions of elution were obtained by gel-permeation chromatography on Bio-Gel P2 as compared to the non-substituted neoagarobiose oligomers (Fig. 1).

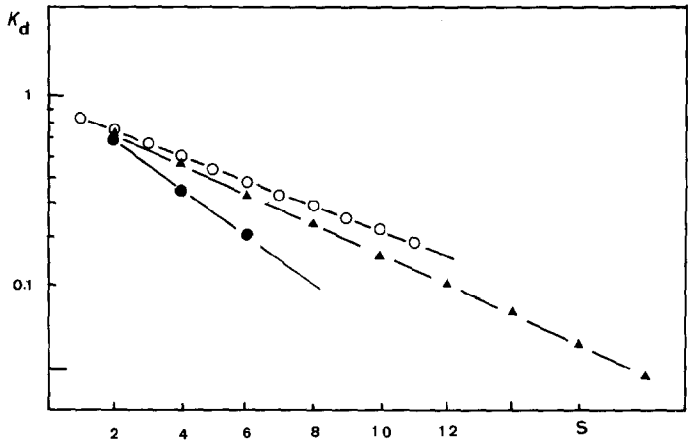
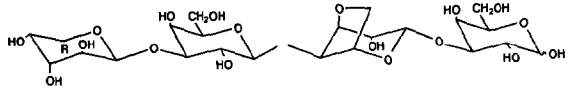


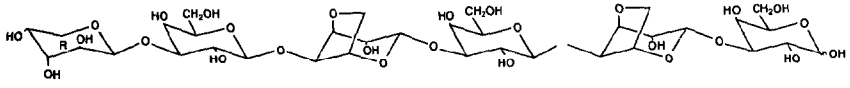
Fig. 1. Partition coefficient (K_d) *versus* the number of sugars (S) for malto-oligosaccharides (○), neoagaro-oligosaccharides (▲), and 2-*O*-methylated neoagaro-oligosaccharides (●). $K_d = V_e - V_o/V_p$, where V_o is the void volume, V_e is the volume of elution of the sugar, and V_p is the bed volume of the column.

Sulfated oligomers containing the biological precursor to the agarobiose repeating unit formed from Gracilaria crassissima *agar.* — The charged oligosaccharides obtained by hydrolysis with β-agarase of agar extracted from *G. crassissima*[22] with aqueous 40% ethanol were recovered from the DEAE-Sephadex A 25 column as one major fraction, and chromatographed on a Bio-Gel P6 column to give peaks II and I identified as a mono-sulfated tetra- (**8**) and a mono-sulfated hexa-saccharide (**9**), respectively.

The ^{13}C-n.m.r. spectrum of the former was similar to that of the sulfated tetrasaccharide obtained from *Porphyra umbilicalis* agar[10]. The ^{13}C assignments are presented in Table II. The chemical shift data for the Gα, Gβ, Ar, A, and G units were identical to those reported for neoagaro-oligosaccharides[17]. The chemical



8 preneoagarotetraose 6^4-sulfate R = $CH_2OSO_3^-$



9 preneoagarohexaose 6^6-sulfate R = $CH_2OSO_3^-$

shifts of the Gnr residue were affected by the change in conformation of the non-reducing end unit from $^4C_1$ in 3,6-anhydro-α-L-galactopyranose to $^1C_4$ in α-L-galactopyranose 6-sulfate[6]. The resonance of G1nr was assigned to the peak at 102.7 p.p.m. by comparison with data for neoagarotetraose, and accords with the attribution by Morrice *et al.*[10] taking into account a shift of 0.7 p.p.m. The resonance of A1nr was observed at 101.7 p.p.m. and corresponded to that of C-1 of α-L-galactopyranose 6-sulfate at the non-reducing end of neoagaro-oligosaccharides[10]. The signal of A2nr was assigned to the peak at 69.4 p.p.m. by comparison with data for C-2 in methyl α-D-galactopyranoside[36] and 4-linked α-L-galactopyranose[9]. The assignments of the other carbons were made by comparison with those reported for α-D-galactose 6-sulfate[39]. The latter data may be imprecise due to the different configuration and, thus, the environment of the model sugar as compared to the Anr residue. Proposed names for oligosaccharides **8** and **9** are preneoagarotetraose 6^4-sulfate and preneoagarohexaose 6^6-sulfate, respectively. The prefix "preneoagaro" indicates the presence of α-L-galactopyranose at the non-reducing end instead of 3,6-anhydro-α-L-galactose.

Methylated and sulfated oligomers containing 2^2-O-methylagarobiose 4^1-sulfate residues formed from Gracilaria eucheumoides *agar.* — The gel matrix recovered after freeze-and-thaw cycles of *G. eucheumoides* agar was alkali-treated[23] and then hydrolysed with crude β-agarase. The charged oligosaccharides produced were eluted as one major fraction from DEAE-Sephadex A 25 with a NaCl gradient (0→M), and chromatographed on a Bio-Gel P6 column to give series of peaks (Fig. 2).

The oligosaccharide in peak I was identified as a tetrasaccharide. The ^{13}C resonances of the Ar, Gα, and Gβ units and C-1,6 of the Gnr unit were assigned by comparison with data reported for $2^2,2^4$-di-*O*-methylneoagarotetraose (**3**, Table II). The resonance for G4nr in the neutral methylated oligosaccharide was absent, but there was a signal at 77.2 p.p.m. that was assigned to a sulfated G4nr, using the chemical shift difference (7.8 p.p.m. downfield) observed between the resonances of C-4 in methyl 3-*O*-methyl-β-D-galactopyranoside and its 4-sulfate[41] and by comparison with the chemical shift (77.5 p.p.m.) of the C-4 resonance in β-D-galactopyranose 4-sulfate[39]. This substitution also affected the chemical shift of the G3nr, G5nr, and G2nr resonances and those of the Anr unit by 0.1–0.2 p.p.m. (Table II). Similarly, the signal for the methyl carbon on A2nr was shifted downfield to 59.8 p.p.m., whereas the ^{13}C resonances of the Ar unit were identical to those for the neutral oligosaccharide (Tables I and II). Consequently, the oligosaccharide in peak I was identified as $2^2,2^4$-di-*O*-methylneoagarotetraose 4^3-sulfate (**10**).

Peak II contained hexasaccharides with an average of 3 methylated repeating units. All the ^{13}C resonances were assigned by comparison with those observed for $2^2,2^4$-di-*O*-methylneoagarotetraose and $2^2,2^4$-di-*O*-methylneoagarotetraose 4^3-sulfate (Table II). An average of 1 methylated repeating unit was sulfated on C-4 of the β-D-galactopyranosyl residue as determined from the integral of G4 and G4nr of the non-sulfated residue. At least two different types of sulfated oligo-

TABLE II

^{13}C-N.M.R. ASSIGNMENTS AT ROOM TEMPERATURE OF CHARGED NEOAGARO-OLIGOSACCHARIDES OBTAINED AFTER HYDROLYSIS OF AGAR FROM *Gracilaria crassissima*, *G. eucheumodes*, *G. pseudoverrucosa*, AND *G. compressa* WITH β-AGARASE BY ELUTION FROM BIO-GEL P6

Sample	*G. crassissima*		*G. eucheumoides*		*G. pseudoverrucosa*		*G. compressa*
Elution peak[a]	II	I	I	II	I	II	I
D.p.	2	3	2	3	2	3	2
Structure	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Carbon atom							
G1β	97.1	97.1	97.1	97.1	97.1	97.1	97.1
G1α	93.1	93.1	93.1	93.1	93.1	93.1	93.1
G1nr	102.7	102.7	102.8	102.8	102.6	102.6	102.4
G1		102.7		102.8		102.7	
G2β	71.9	71.9	71.7	71.7	71.8	71.8	71.9
G2α	68.4	68.4	68.2	68.2	68.3	68.3	68.4
G2nr	70.0	70.0	70.9	70.9[b]/70.5[c]	71.2	71.1[b]/70.6[c]	70.6
G2		70.6		70.9[b]/70.5[c]		70.8[b]/70.6[c]	
G3β	83.0	83.0	83.3	83.3	83.0	83.0	83.0
G3α	79.8	79.8	80.2	80.1	79.8	79.8	79.8
G3nr	81.2	81.2	79.9	79.9[bd]/83.0[c]	77.5	77.8[b]/82.5[c]	79.7
G3		82.6		80.1[bd]/83.0[c]		78.1[b]/82.5[c]	
G4β	69.3	69.3	69.2	69.2	69.2	69.2	69.3
G4α	69.9	70.0	69.9	69.9	69.8	69.4	70.0
G4nr	69.4	69.4	77.2	77.2[b]/69.0[c]	77.5	77.6[b]/69.1[c]	72.2
G4		69.1		77.4[b]/69.4[c]		77.6[b]/69.1[c]	
G5β	75.6	75.6	75.5	75.5	75.6	75.6	75.6
G5α	71.0	71.0	70.9	70.9	71.0	71.0	71.0
G5nr	76.3	76.3	75.5	75.5[b]/75.6[c]	75.4	75.4[b]/75.8[c]	67.0
G5		75.8		75.5[b]/75.6[c]		75.7[b]/75.8[c]	75.7
G6β	61.8	61.8	61.7	61.7	61.8	61.8	61.8
G6α	62.0	62.0	61.9	61.9	62.0	61.9	62.0
G6nr	62.0	62.1	61.7	61.7[b]/61.5[c]	61.7	61.8	65.7
G6		61.8		61.7[b]/61.5[c]		61.7	
A1r	98.8	98.8	99.1	99.1	98.8	98.8	98.9
A1		98.8		99.1		97.4[b]/98.6[c]	
A1nr	101.7	101.7	98.9	98.9	97.1	97.1[b]/98.6[c]	98.9
A2r	70.2	70.2	78.8	78.8	70.1	70.1	70.1
A2		70.2		78.8		70.0[b]/70.2[c]	
A2nr	69.4	69.4	79.0	79.0	70.2	70.2	70.0
A3r	80.4	80.4	78.6	78.6	80.3	80.4	80.4
A3		80.4		78.6		81.3[b]/80.4[c]	
A3nr	70.3	70.3	79.8	79.8[bd]/79.6[c]	81.2	81.3	81.4
A4r	77.7	77.7	78.0	78.0	77.8	77.8	77.7
A4		77.7		78.0		78.0[b]/77.8[c]	
A4nr	70.8	70.8	70.3	70.3	70.4	70.4	70.4
A5r	75.9	75.9	75.9	75.9	75.9	76.0	75.9
A5		75.9		75.9		75.9	
A5nr	70.0	70.0	77.8	77.8	77.8	77.8	77.9
A6r	69.8	69.8	69.7	69.7	69.7	69.7	69.8
A6		69.8		69.7		69.6[b]/69.7[c]	
A6nr	69.0	69.0	69.5	69.5	69.5	69.5	69.5
Methyl[e]							
rα			59.3	59.3			
rβ			59.4	59.4			
nr			58.5	59.5			
internal							
Pyruvate							
methyl							26.0
carbonyl							176.5
acetal							101.7

[a]Refers to Bio-Gel P6. [b]For the sulfated repeating unit. [c]For the non-sulfated repeating unit. [d]May be reversed. [e]Refers to MeO associated with the residue in the internal repeating unit or at the reducing end (rα, rβ) and non-reducing end.

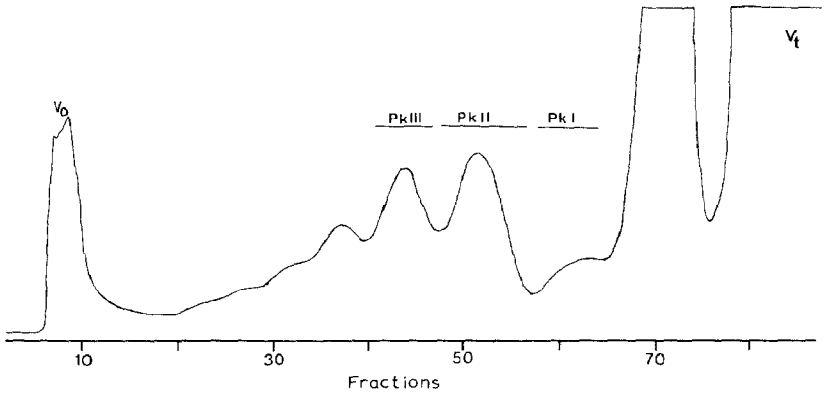
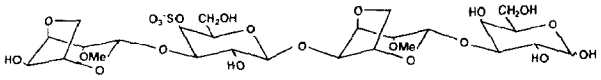


Fig. 2. Bio-Gel P6 chromatogram of the charged oligosaccharides recovered after the hydrolysis of *Gracilaria eucheumoides* agar with β-agarase; V_o and V_t refer to the void and total volumes, respectively.

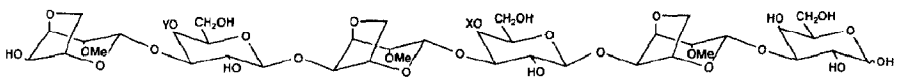
saccharides were expected in this fraction, namely, $2^2,2^4,2^6$-tri-*O*-methylneoagarohexaose 4^3- and 4^5-sulfate (**11**). The location of the sulfated disaccharide in the oligomers affected the chemical shifts of C-4 (77.4 and 77.2 p.p.m.) and C-3 (80.1 and 79.9 p.p.m.) in the β-D-galactopyranosyl 4-sulfate residue as well as those of other carbons in the adjacent sugars (Table II).

The oligosaccharides in peak III (Fig. 2) contained an average of 5.5 methylated repeating units. Of the β-D-galactopyranosyl residues, ~3 were 4-sulfated, as estimated by the integration of the resonances for G4 and G4nr of the non-sulfated residues. Due to the heterogeneity of this fraction, precise identification of these oligosaccharides was not possible.

Sulfated oligomers containing agarobiose 4'-sulfate residues formed from Gracilaria pseudoverrucosa *agar.* — Agar extracted from *Gracilaria pseudoverrucosa*[24] with boiling aqueous 40% ethanol was alkali-treated and hydrolysed with β-agarase. The charged oligosaccharides eluted with the NaCl gradient in one



10 2^2, 2^4-di-*O*-methylneoagarotetraose 4^3-sulfate

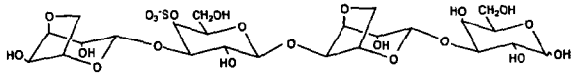


11 $2^2,2^4,2^6$-tri-*O*-methylneoagarohexaose 4^3-sulfate (X = SO_3, Y = H) or 4^5-sulfate (X = H, Y = SO_3)

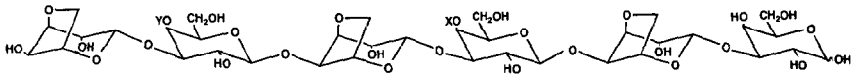
major peak from DEAE-Sephadex A25 were chromatographed on Bio-Gel P6 to give peaks I and II.

The ^{13}C resonances of the saccharide in peak I were assigned to Gα, Gβ, and Ar units and to G1nr, G6nr, A3nr, A4nr, and A6nr, by comparison with data for neoagarotetraose (Table II). The signal for G4nr at 69.1 p.p.m. for neoagarotetraose was not observed, but there was a signal at 77.5 p.p.m. which was assigned to a sulfated G4nr by comparison with results obtained with 2^2,2^4-di-*O*-methyl-neoagarotetraose 4^3-sulfate from *G. eucheumoides*. However, the signal for G3nr, expected from the above results to be at ~80 p.p.m., was absent. Since the resonance at 77.5 p.p.m. had a double intensity compared to those for A3nr (81.2 p.p.m.) or A6nr (69.5 p.p.m.), for example, it was assigned to G3nr. The signal for G5nr was shifted upfield by 0.3 p.p.m., whereas that for G2nr was moved downfield, from 70.6 to 71.2 p.p.m. A small downfield shift (0.2 p.p.m.) was also observed for the signal of A2nr, and a marked effect was observed for the A1nr resonance that was shifted to 97.1 p.p.m. and coincided with the resonance of G1β. Because of this overlap, the mean number of 2 repeating units in this oligosaccharide fraction was determined by comparing the integrated intensities of the signals assigned to G5nr, G5β, and G5α, and A6r and A6nr. The differences in the chemical shifts from those observed with the methylated, sulfated oligomers may be related to the proximity of the methoxyl and sulfate groups in *G. eucheumoides* oligosaccharides, which could affect the conformation of the sugars. The saccharide in peak I was therefore identified as neoagarotetraose 4^3-sulfate (**12**).

The ^{13}C resonances of the saccharides in peak II were identified by comparison with those observed for neoagaro-oligosaccharides and for the oligomers in peak I (Table II). A mean number of three repeating units was determined using the integrated intensities of the signals for G1 and G1nr (102.7 and 102.6 p.p.m., respectively) and G3α and G3β (83.0 and 79.8 p.p.m., respectively). This fraction appeared to contain neoagarohexaose 4^5- and 4^3-sulfate (**13**). The ^{13}C resonances of the internal sulfated repeating unit had slightly different chemical shifts as compared to those in the sulfated repeating unit at the non-reducing end (Table II).

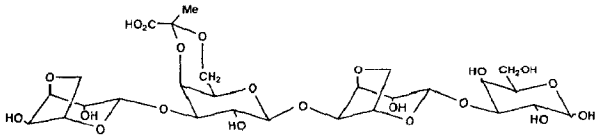


12 **neoagarotetraose 4^3-sulfate**



13 **neoagarohexaose 4^3-sulfate** (X = SO_3, Y = H) **or 4^5-sulfate** (X = H, Y = SO_3)

Charged oligosaccharides containing pyruvylated agarobiose repeating units isolated from the agar of Gracilaria compressa. — Oligosaccharides generated by the action of β-agarase on the agar[23] extracted from *Gracilaria compressa* with aqueous 40% ethanol were fractionated on DEAE-Sephadex A25, and the major fraction, eluted with a NaCl gradient (0→M), was chromatographed on Bio-Gel P6 to give one major peak. The saccharides in this fraction had, on average, two repeating units. The ^{13}C resonances were assigned to the Gα, Gβ, Ar, and Anr units and to C-1,2 of the Gnr unit by comparison with data for neoagarotetraose (Table II). The signals at 176.5, 101.7, and 26.0 p.p.m. were attributed to the carboxyl, quaternary, and methyl carbon in a pyruvate acetal[40]. The signals for G3nr, G4nr, G5nr, and G6nr of neoagaro-oligosaccharides were not observed, but resonances at 79.7, 72.2, 67.0, and 65.7 p.p.m. were present and assigned to G3nr, G4nr, G5nr, and G6nr, respectively, of a 4,6-*O*-carboxyethylidene-β-D-galactopyranosyl moiety in the repeating unit at the non-reducing end of the tetrasaccharide. These assignments were made using the chemical shift differences observed for the respective ^{13}C resonances for methyl 3-*O*-methyl-β-D-galactopyranoside and its 4,6-*O*-benzylidene derivative[41], and by the DEPT technique to assign the methylene carbons. A shift was also observed for the resonances of G1nr (102.7 to 102.4 p.p.m.), A1 and A1nr (+0.2 and +0.4 p.p.m., respectively, Table II). Consequently, the saccharides in this peak were identified as $4^3,6^3$-*O*-carboxyethylideneneoagarotetraose (**14**). The *R* configuration of the pyruvate group and an equatorial orientation of the methyl group were consistent[42] with the chemical shift of the resonance of the methyl carbon at 26.0 p.p.m.



14 $4^3,6^3$-*O*-carboxyethylideneneoagarotetraose

Comparison of the ^{13}C-n.m.r. data for substituted oligosaccharides and related polysaccharides. — Rochas *et al.*[17] demonstrated that the chemical shifts of the ^{13}C resonances for the Ar, A and Gnr, and G units in the neoagaro-oligosaccharides were close to those for the internal A and G units of agarose, respectively. Accordingly, the present results show that the chemical shifts for the ^{13}C resonances of the Ar and Gnr units of $2^2,2^4$-di-*O*-methylneoagarotetraose isolated from *G. eucheumoides* agar and of the A and G units of $6^1,6^3,6^5$-tri-*O*-methylneoagarohexaose isolated from *G. cervicornis (ferox)* agar (Table I) were in good agreement with those reported[37,41,43] for the A and G units, respectively, of the methylated agarose (**15, 16,** Fig. 4, Table III) and obtained by comparison with chemical shift data for synthetic model sugars.

TABLE III

^{13}C-N.M.R. CHEMICAL SHIFT DATA (P.P.M.) AT 80° FOR AGAROBIOSE REPEATING UNITS (SEE FIG. 4)

Structure	*Ref.*	*Unit*[a]	*Carbon*							
			1	*2*	*3*	*4*	*5*	*6*	*CH_3*	*COOH*
1	17	G	102.4	70.2	82.2	68.8	75.3	61.4		
		A	98.3	69.9	80.1	77.4	75.7	69.4		
15	36	G	102.7	70.2	82.7	68.8	75.3	61.4		
		A	98.7	78.9	78.5	77.6	75.7	69.5	59.2	
16	36, 41	G	102.4	70.2	82.2	69.0	73.6	71.8	59.1	
		A	98.3	69.9	80.2	77.4	75.7	69.4		
17	9	G	103.7	69.8	81.2	69.1	75.9	61.6		
		A	101.3	69.2	71.0	79.0	70.2	67.7		
						78.7		67.5		
18		G	102.5	70.5	79.8	76.6	75.2	61.4		
		A	98.2	79.1	78.5	77.7	75.7	69.9	59.1	
19		G	102.4	70.8	80.0	77.0	75.0	61.4		
				71.0						
		A	96.8	69.9	80.1	77.4	75.7	69.4		
20		G	102.2	70.0	79.5	71.6	66.7	65.3	25.7	176.3
		A	98.4	69.9	80.1	77.5	75.7	69.4		

[a]A and G refer to the 4-linked and 3-linked sugars, respectively.

Following the same approach, it was hoped that, by using the chemical shift data for charged oligosaccharides produced from the agar rich in α-L-galactose 6-sulfate residues from *G. crassissima*[22], the assignment of the ^{13}C resonances of the precursor repeating unit in the parent polysaccharide could be confirmed. Unfortunately, the absence of a 4-linkage on the α-L-galactosyl 6-sulfate unit at the non-reducing end of the oligosaccharides obtained did not permit a comparison of the assignment of most of the ^{13}C resonances of this unit with those of the sulfated sugar in the polymers, with the exception of A1 which had chemical shift data close to those of A1nr (Tables II and III). Furthermore, although the chemical shift data of the ^{13}C resonances of the β-D-galactopyranosyl unit at the non-reducing end agreed closely with those for the β-D-galactopyranose residue in the polymer (Table III), the chemical shift data for G1nr in the oligosaccharide and G1 in the polymer could not be compared since, in the latter molecule, this carbon is linked to α-L-galactose 6-sulfate in the $^1C_4$ conformation, whereas, in the present oligosaccharide, it is linked to an anhydrogalactose residue in the $^4C_1$ conformation[6].

The chemical shifts of the ^{13}C resonances of the sulfated biological-precursor repeating units in agar extracted from different species of algae varied slightly. Split signals for C-4 and C-6 of the α-L-galactose 6-sulfate residue have been observed in a number of spectra recorded for different agar preparations (Fig. 3). These variations have been observed for the sulfated disaccharide repeating units distributed randomly or in blocks in fragments of agar from *Porphyra umbilicalis*[10]. Assignments of the ^{13}C resonances for the biological precursor of the agarose re-

peating unit have been reported from the high-molecular-weight segments enriched in blocks of these sulfated repeating units[9]. These segments resulted from hydrolysis with β-agarase of the cold-water-soluble polysaccharide extracted from *Porphyra hatainensis*. The chemical shift data of this sulfated repeating unit in *G. crassissima* agar vary slightly. This agar was mainly composed of randomly distributed sulfated units, since the charged fraction of the hydrolysate comprised monosulfated tetra- and hexa-saccharides. The slight variation in chemical shifts is therefore ascribed to the different distribution. For example, C-4 and C-6 of *Porphyra* agar resonated at 79.0 and 67.7 p.p.m., respectively, and at 78.7 and 67.5 p.p.m., respectively, for *G. crassissima* agar[22]. Variations also occurred for C-1 of the β-D-galactopyranose residue and C-1,3 of the α-L-galactopyranose 6-sulfate residue, and, possibly, for the overlapping resonances between 69.0 and 71.0 p.p.m. belonging to these residues in the sulfate disaccharide (Fig. 3). Thus, ^{13}C-n.m.r. spectra of agar showing signals for C-4,6 of the α-L-galactose 6-sulfate

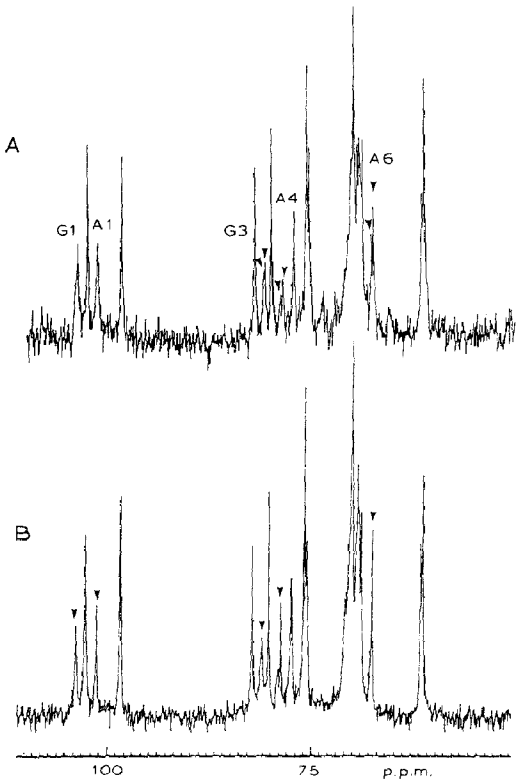


Fig. 3. ^{13}C-N.m.r. spectrum at 80° of the 22°-water-soluble agar from **A**, *Gracilaria verrucosa* (1636 transients); and **B**, *Gracilaria crassissima* (3581 transients); A and G refer to the α-L-galatose 6-sulfate and β-D-galactose residues, respectively, of the biological precursor of the agarobiose repeating unit. The arrows indicate the split signals.

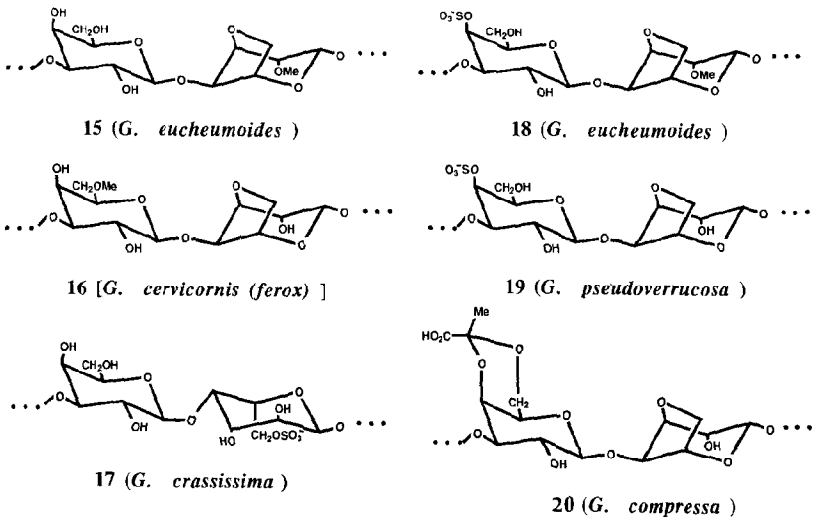


Fig. 4. Structures of the agarobiose repeating units, and the associated algae: **15**, 2^1-*O*-methylagarobiose; **16**, 6^2-*O*-methylagarobiose; **17**, the agarobiose biological precursor; **18**, 2^1-*O*-methylagarobiose 4^2-sulfate; **19**, agarobiose 4^2-sulfate; and **20** pyruvylated agarobiose.

residue indicate the presence of both random and block sequences of the sulfated disaccharide and may be used to estimate their proportions.

Based on the ^{13}C resonances for carbons of the Ar, Anr, and Gnr units of 2^2,2^4-di-*O*-methylneoagarotetraose 4^3-sulfate obtained from *G. eucheumoides* (Table II), the ^{13}C resonances at 98.2, 79.8, 79.1, 76.6, 75.2, 70.5, and 69.9 p.p.m. for the alkali-treated parent polysaccharide[3] were assigned to A1, G3, A2, G4, G5, G2, and A6, respectively, of the 2^1-*O*-methylagarobiose 4^2-sulfate repeating unit in the polysaccharides (Table III, Fig. 4, **18**).

The signals at 96.8, 77.0, 75.0, and 71.0/70.8 p.p.m. in the spectrum of fractions[24] of the agar from *G. pseudoverrucosa* (Table III) were assigned to A1, G4, G5, and G2, respectively, of the agarobiose 4^2-sulfate repeating unit (Fig. 4, **19**), by comparison with data for neoagarotetraose 4^3-sulfate (Table II). The signal at 80.0 p.p.m. for the 4-sulfated agar was tentatively assigned to G3, although, for the sulfated oligosaccharides, the chemical shifts for the resonances of G3nr and G4nr coincided. The split signal for G2 (71.0/70.8 p.p.m.) is probably due to a different distribution of the sulfated disaccharide in the polymer, as already discussed above for the biological precursor to the agarobiose repeating unit.

The location of the sulfate group on C-4 of the galactose residue in the agar from *G. eucheumoides* and *G. pseudoverrucosa* was confirmed by the characteristic[24,25] i.r. band at 845 cm^{-1} and by ^1H-n.m.r. spectroscopy[25].

^{13}C-N.m.r. data for agarobiose repeating units having a sulfate group on C-4 of the 6-*O*-methyl-β-D-galactose residues have been reported for the agar from *Ondothalia corymbifera*[41]. However, due to the methoxyl group on C-6 of the β-D-

galactopyranose residue, these assignments could not be compared with those observed here.

On the basis of the chemical shift data for the repeating unit at the non-reducing end of $4^3,6^3$-*O*-carboxyethylideneneoagarotetraose obtained from the agar of *G. compressa* (Table II), the ^{13}C resonances of the parent polysaccharide at 102.2, 79.5, 77.5, 71.6, 66.7, and 65.3 p.p.m. (see ref. 23) were assigned to G1, G3, A4, G4, G5, and G6, respectively, of $4^2,6^2$-*O*-carboxylethylideneagarobiose repeating units (Table III, Fig. 4, **20**). The methyl and carbonyl carbons in the pyruvate acetal resonated at 25.7 and 176.3 p.p.m., respectively. The ^{13}C resonance at 70.0 p.p.m. for pyruvylated agar was assigned to A2 although no shift was observed for the resonance of A2nr of the pyruvylated oligosaccharides. The acetal carbon resonance was not observed in the spectra of the polysaccharides, probably because of its long T_1 value[40].

The combined use of hydrolysis with β-agarase and analysis of the resulting fragments by ^{13}C-n.m.r. spectroscopy permitted the characterization of several agarobiose repeating units and the type of substituent distribution (block or random) in hybrid molecules.

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REFERENCES

1 C. ARAKI, *Proc. Int. Seaweed Symp.*, 5 (1966) 3–17.
2 M. DUCKWORTH AND W. YAPHE, *Carbohydr. Res.*, 16 (1971) 189–197.
3 M. LAHAYE, C. ROCHAS, AND W. YAPHE, *Can. J. Bot.*, 64 (1986) 579–585.
4 D. A. REES, *Biochem. J.*, 81 (1961) 347–352.
5 S. ARNOTT, A. FULMER, W. E. SCOTT, I. C. M. DEA, R. MOORHOUSE, AND D. A. REES, *J. Mol. Biol.*, 90 (1974) 269–284.
6 D. A. REES AND E. J. WELSH, *Angew. Chem. Int. Ed. Engl.*, 16 (1977) 214–224.
7 K. C. HONG, M. E. GOLDSTEIN, AND W. YAPHE, *Proc. Int. Seaweed Symp.*, 6 (1969) 473–482.
8 M. DUCKWORTH AND W. YAPHE, *Carbohydr. Res.*, 16 (1971) 435–445.
9 M. LAHAYE, C. ROCHAS, AND W. YAPHE, *Carbohydr. Res.*, 143 (1985) 240–245.
10 L. M. MORRICE, M. W. MCLEAN, W. F. LONG, AND F. B. WILLIAMSON, *Eur. J. Biochem.*, 133 (1983) 673–684.
11 A. I. USOV AND E. G. IVANOVA, *Bot. Mar.*, 31 (1987) 365–370.
12 G. K. HAMER, S. S. BHATTACHARJEE, AND W. YAPHE, *Carbohydr. Res.*, 54 (1977) c7–c10.
13 S. S. BHATTACHARJEE, W. YAPHE, AND G. K. HAMER, *Carbohydr. Res.*, 60 (1978) c1–c3.
14 S. S. BHATTACHARJEE, W. YAPHE, AND G. K. HAMER, *Proc. Int. Seaweed Symp.*, 9 (1979) 379–385.
15 C. ROCHAS, M. RINAUDO, AND M. VINCENDON, *Int. J. Biol. Macromol.*, 5 (1983) 111–115.
16 C. W. GREER, C. ROCHAS, AND W. YAPHE, *Bot. Mar.*, 28 (1985) 9–14.
17 C. ROCHAS, M. LAHAYE, W. YAPHE, AND M. T. PHAN VIET, *Carbohydr. Res.*, 148 (1986) 199–207.
18 C. BELLION, G. K. HAMER, AND W. YAPHE, *Can. J. Microbiol.*, 28 (1982) 874–880.
19 C. W. GREER AND W. YAPHE, *Bot. Mar.*, 27 (1984) 473–478.
20 C. W. GREER AND W. YAPHE, *Bot. Mar.*, 27 (1984) 479–484.
21 C. W. GREER, I. SHOMER, M. E. GOLDSTEIN, AND W. YAPHE, *Carbohydr. Res.*, 129 (1984) 189–196.
22 M. LAHAYE, J. F. REVOL, C. ROCHAS, J. MCLACHLAN, AND W. YAPHE, *Bot. Mar.*, 31 (1988) 491–501.

23 M. LAHAYE AND W. YAPHE, *Bot. Mar.*, (1988) in press.
24 M. LAHAYE AND W. YAPHE, *Carbohydr. Polym.*, 8 (1988) 285–301.
25 M. LAHAYE, Ph.D. Thesis, McGill University, 1986.
26 L. M. MORRICE, M. W. MCLEAN, F. B. WILLIAMSON, AND W. F. LONG, *Eur. J. Biochem.*, 135 (1983) 553–558.
27 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
28 A. HEYRAUD AND M. RINAUDO, *J. Liq. Chromatogr.*, 4 (1981) 175–293.
29 A. S. SHASHKOV, A. I. USOV, AND S. V. YAROTSKII, *Bioorg. Khim.*, 3 (1977) 46–49.
30 C. BELLION, Ph.D. Thesis, McGill University, 1979.
31 M. MESSER, E. TRIPONOFF, W. STERN, J. G. COLLINS, AND J. H. BRADBURY, *Carbohydr. Res.*, 83 (1980) 327–334.
32 C. ARAKI AND K. ARAI, *Bull. Chem. Soc. Jpn.*, 30 (1957) 287–293.
33 W. YAPHE, *Can. J. Microbiol.*, 3 (1957) 987–993.
34 D. GROLEAU AND W. YAPHE, *Can. J. Microbiol.*, 23 (1977) 672–679.
35 L. M. MORRICE, M. W. MCLEAN, W. F. LONG, AND F. B. WILLIAMSON, *Eur. J. Biochem.*, 137 (1983) 149–154.
36 P. A. J. GORIN AND M. MAZUREK, *Can. J. Chem.*, 53 (1975) 1212–1223.
37 D. J. BRASH, C. T. CHUAH, AND L. D. MELTON, *Aust. J. Chem.*, 34 (1981) 1095–1105.
38 M. DUCKWORTH AND W. YAPHE, *J. Chromatogr.*, 49 (1970) 482–487.
39 P. J. ARCHBALD, M. D. FENN, AND A. B. ROY, *Carbohydr. Res.*, 93 (1981) 177–190.
40 P. A. J. GORIN, M. MAZUREK, H. S. DUARTE, M. IACOMINI, AND J. H. DUARTE, *Carbohydr. Res.*, 100 (1982) 1–15.
41 A. S. SHASHKOV, A. I. USOV, AND S. V. YAROTSKII, *Bioorg. Khim.*, 4 (1978) 74–81.
42 P. J. GAREGG, P.-E. JANSSON, B. LINDBERG, F. LINDH, J. LÖNNGREN, I. KVARNSTROM, AND W. NIMMICH, *Carbohydr. Res.*, 78 (1980) 127–132.
43 F. M. NICOLAISSEN, I. MEYLAND, AND K. SCHAUMBURG, *Acta Chem. Scand., Ser. B*, 34 (1980) 103–107.