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DL-Galactan hybrids and agarans from gametophytes of the red seaweed *Gymnogongrus torulosus*

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Abstract

Seaweeds from the genus *Gymnogongrus* are known to be carrageenophytes; nevertheless, fractionation techniques used previously for the separation of gel-forming and 'soluble' carrageenans, applied to the galactans of *Gymnogongrus torulosus* together with enantiomeric analysis of the sugar components and (when possible) of the structural units, suggested that the system of galactans biosynthesized by the seaweed was formed by DL-galactan hybrids having major amounts of carrageenan-type or agaran-type chains, with minor quantities of agarans with unusual structural details. © Published by 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Sulfated polysaccharides; Methylation analysis; ^{13}C NMR spectroscopy; DL-Galactan hybrids; Agarans; Carrageenans; *Gymnogongrus torulosus*; Red seaweed; Phyllophoraceae (Rhodophyta)

1. Introduction

Red seaweeds of the family Phyllophoraceae are known to be carrageenophytes;[1–4] gametophytes of seaweeds belonging to this family produce kappa/iota-carrageenans, while tetrasporophytes (when present) biosynthesize lambda-carrageenans.[2] Studies carried out on gametophytes of the genus *Gymnogongrus* and those of the new genus *Anhfeltiopsis*, derived from it[5,6] showed that they produce iota-carrageenans[2,3,7] or kappa/iota-carrageenans.[1–4]

Furneaux and Miller[3] studied the hot-water extract of *Gymnogongrus torulosus* from New Zealand (formerly called *Anhfeltia torulosa*[8]) by ^{13}C NMR spectroscopy, finding that it was a kappa/iota-carrageenan (ratio kappa:iota:(mu + nu) 32:46:22).

In the past few years, it has been found that in seaweeds belonging to the Gigartinaceae (carrageenophytes), there are small but significant amounts of polysaccharides containing α-D- and α-L-galactose and 3,6-anhydro-α-D- and L-galactose units.[9–12] Moreover, recently these units have been detected in the extract obtained with water at room-temperature from the commercially valuable red seaweed *Kappaphycus alvarezii* (Solieriaceae).[13]

We now report the presence of a complex system of possible DL-galactan hybrids and agarans in the red seaweed, *G. torulosus*.

2. Results

The milled seaweed was extracted with water at room temperature and the supernatant

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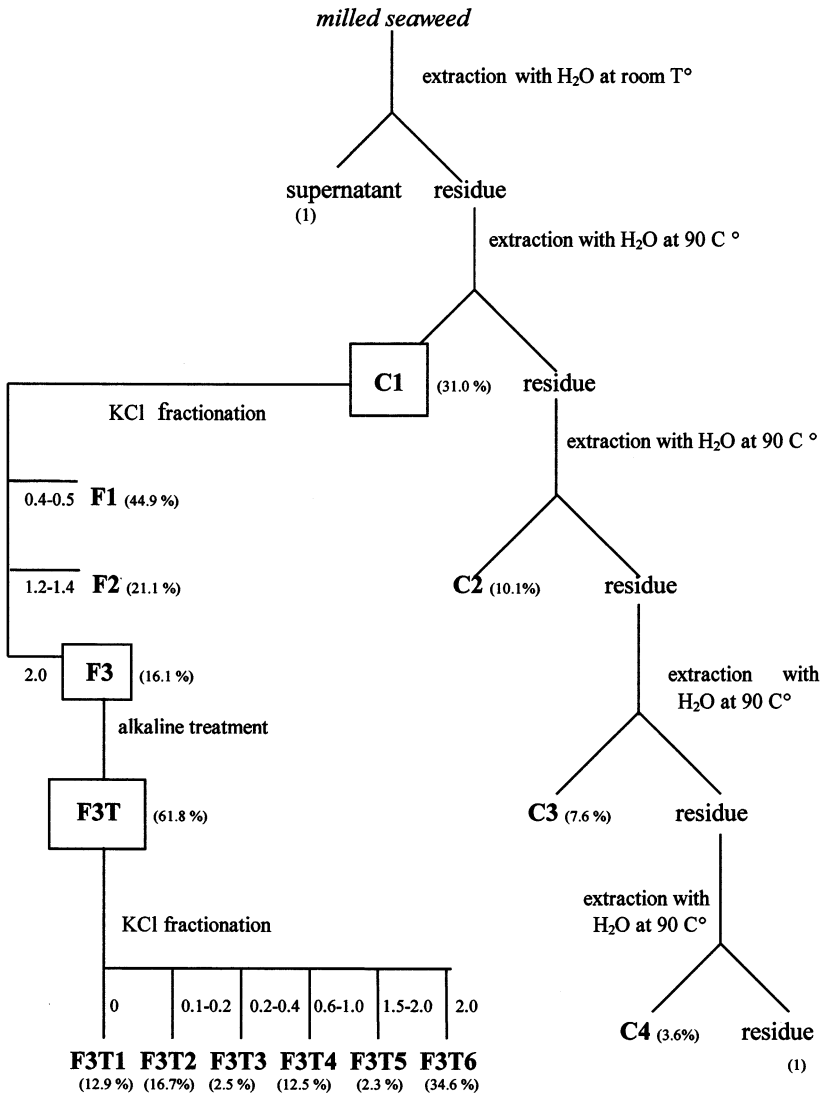

Scheme 1. Extraction, treatment and fractionations of galactans obtained from the red seaweed, *G. torulosus*. (1) Kept for further work. Analyses of C fractions are shown in Table 1, those of F and T fractions are shown in Tables 2 and 3.

was reserved for future work. The residue was sequentially extracted with hot water (90 °C) four times (Scheme 1). Each extract was precipitated with 2-propanol giving samples C1–C4. Yields and analyses of the hot-water extracted 2-propanol-insoluble products are given in Table 1. Most of the material ($\sim$ 59%) was obtained in the first extraction (C1) and contains galactose and 3,6-anhydrogalactose, and small amounts of xylose as sugar constituents. Fractions C2–C4 showed the same major sugars, together with small or trace amounts of xylose, 6-*O*-methylgalactose and glucose. All the extracts have similar galactose:3,6-anhydrogalactose:sulfate molar ratio and contain galactose in a DL molar ratio 1:0.10–0.20, and 3,6-anhydro-galactose in a DL molar ratio 1:0.14–1.11. Optical rotations are in the range of average between those of carrageenans and agarans. When these rotations were plotted against L-galactose plus 3,6-anhydro-L-galactose, they fall into a straight line with a small deviation of C4.

Each fraction was submitted to GPC on Sephadex G-100, using 1 M sodium chloride as eluant. In all the cases, unimodal single peaks were obtained (Fig. 1). Number-average and weight-average molecular weights of the fractions were in the range of 20,000 (Table 1). Noteworthy is the very low dispersion of the molecular weights. In contrast, molecular

Table 1
Yields and analyses of extracts C1–C4

Fraction	Yield [a] (%)	Sulfate as SO_3K (%)	Gal:AnGal:sulfate [b] (molar ratio)	Proteins (%)	Monosaccharide composition (mol%)							$[\alpha]_D$ (°)	Molecular weight		
					Gal		6-*O*-Me-Gal	AnGal		Xyl	Glc		M_n [c]	M_n [d]	M_w [d]
					D-	L-		D-	L-						
C1	31.1	29.9	1:0.7:1.4	tr [e]	54.2	6.1	tr	28.7	9.6	1.4	tr	+25.0	44,000	19,500	19,700
C2	10.1	22.9	1:0.5:1.1	4.4	54.5	5.9	2.5	22.4	11.0	1.4	2.4	+18.4	45,000	20,400	20,600
C3 [f]	7.6	20.2	1:0.5:1.6	9.5	54.7	6.8	3.3	14.2	15.7	1.0	4.3	+13.8	23,000	19,600	19,600
C4	3.6	17.1	1:0.7:1.1	16.8	42.6	8.4	2.7	26.9	3.7	6.4	9.3	−3.5	18,000	20,000	20,100

[a] Yield of C1–C4 are given for 100 g of seaweed.
[b] From colorimetric measurements.
[c] Determined by the method of Park and Johnson.[14]
[d] Determined by g.p.c.
[e] Percentages lower than 1% are given as trace (tr).
[f] Traces of 3-*O*-methyl galactose were found in C3.

weights calculated from determination of the reducing ends[14] are ~45,000 for C1 and C2, but similar to that obtained from GPC for C3 and C4 (Table 1).

FTIR spectra of the extracts are identical, with sharp peaks at 847.5–849.3 cm^{-1} and 804.4–806.4 cm^{-1}, indicating the presence of β-galactose 4-sulfate and 3,6-anhydro-α-galactose 2-sulfate, respectively. The 3,6-anhydro structure was confirmed by the peak at 930.9–933.7 cm^{-1}.

Data from Table 1, GPC, and FTIR spectra indicate that extracts C1–C4 are not different products, but members of the same family of galactans, differing by the usual molecular weight and compositional dispersion. In order to avoid possible contamination with glucose and further structural dispersion (monomethyl galactoses), the major extract C1 was chosen for study. Other fractions, especially C4 whose optical rotation is not in agreement with its enantiomeric composition, were kept for further work.

C1 was shown by methylation analysis (Table 3) to be a kappa/iota-carrageenan (ratio kappa:iota 45:55). Small amounts of non-sulfated β- and α-galactose units were also present. The 100 MHz ^{13}C NMR spectrum of C1 showed the signals corresponding to kappa-, iota-, and nu-structures[15–17] in agreement with results published before.[3]

Nevertheless, there are some data indicating the presence of different structures, namely, the low optical rotation, the presence of L-galactose (6.1%) and 3,6-anhydro-L-galactose (9.6%) residues (Table 1), and the presence of 2,6-di-*O*-methyl-L-galactose (2.0%) and 3,6-anhydro-L-galactose substituted on C-2 (6.5%) between the partially methylated monosaccharides obtained in the methylation analysis (Table 3).

To determine whether the 2-*O*-methyl 3,6-anhydrogalactose units belong to the D- or L-series, they were derivatized to the corresponding diastereomeric *sec*-butyl 2-*O*-methyl-3,6-anhydrogalactonates or the *sec*-butyl and methylbenzyl amides, but efforts carried out to separate them were unsuccessful.

C1 was submitted to potassium chloride fractionation (Scheme 1), yielding two fractions that precipitated at 0.4–0.5 M (F1,

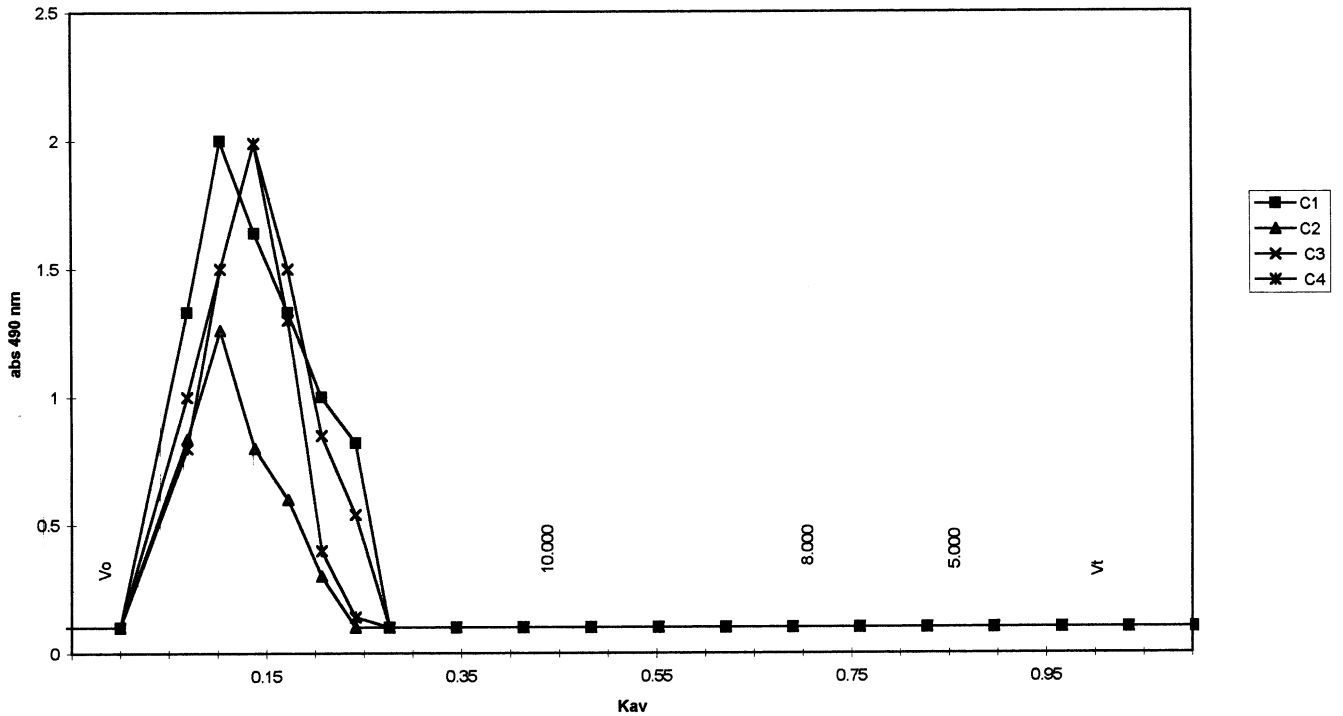

Fig. 1. Gel-permeation chromatography of the four extracts obtained with hot water from gametophytes of the red seaweed *G. torulosus*.

44.9%) and 1.2–1.4 M (F2, 21.1%), while a third one (F3, 16.1% of the starting material), was soluble in 2.0 M KCl. Analyses and monosaccharide composition of these fractions are given in Table 2.

The pattern of precipitation is formally similar to that obtained for carrageenans of cystocarpic samples of *Gigartina skottsbergii*[18] and *Iridaea undulosa*[19] (Gigartinaceae), but in this case, F1 and F3 contain significant amounts of L-galactose (1.4 and 25.4%) and 3,6-anhydro-L-galactose (11.4 and 5.8%, respectively). F2, which is the fraction closer in composition to an ideal carrageenan, contains only a small quantity of 3,6-anhydro-L-galactose (6.0%). All these fractions contain xylose, but its amount is significant only in F3. The FTIR spectra are identical to that of the parent compound (C1). Optical rotations are also an average between those of carrageenans and agarans (Table 2) and proportional to the L-galactose plus 3,6-anhydro-L-galactose content.

Methylation analysis of fractions F1–F3 (Table 3) shows for F1 and F2 the typical pattern of kappa/iota-carrageenans, while F3 is noteworthy for the diversity of structural units. It contains β-D-galactose 4-sulfate (34.9%) and 3,6-anhydrogalactose (14.0%) and 3,6-anhydrogalactose 2-sulfate (12.9%) units as major sugar constituents, but also non-substituted β-D-galactose (8.2%) and β-D-galactose substituted with single stubs of xylose on C-6 (8.4%) and non-substituted α-D-galactose (2.0%), 3-*O*-methyl-α-D-galactose (2.6%), α-L-galactose 3-sulfate (3.2%), and α-galactose 2,6-disulfate (4.9%) units.

4,6-Di-*O*-methylgalactose (3.5%) was also detected. This sugar indicates the presence of β-D-galactose 2-sulfate units and the presence of a small amount of lambda-structure in F3, as has previously been found in the soluble fraction obtained from the carrageenans extracted from cystocarpic *G. skottsbergii*.[17]

Estimation of 4-linked α-galactose 6- and 2,6-disulfate residues[20] in F3 showed that all the cyclizable units were 2,6-disulfated ($\sim$ 2.0%) in agreement with the methylation analysis.

^{13}C NMR spectra of F1–F3 are given in Fig. 2. Spectra of F1 and F2 are very similar among each other, and also similar to

Table 2
Yields and analyses of extract C1 and of the fractions obtained from it by potassium chloride fractionation

Fraction	Range of precipitation (M, KCl)	Yield [a] (%)	Sulfate as SO_3K (%)	Gal:AnGal:sulfate [b] (molar ratio)	Proteins (%)	Monosaccharide composition (mol%)						$[\alpha]_D$ (°)	Molecular weight [c]
						Gal		3-*O*-Me-Gal	AnGal		Xyl		
						D-	L-	D-	D-	L-			
C1		40.4	23.9	1:0.71:1.44	tr [d]	54.2	6.1	tr	28.7	9.6	1.4	+26.3	44,000
F1	0.4–0.5	44.9 (54.7)	29.2	1:0.80:1.53	2.6	47.4	1.4		38.1	11.4	1.7	+40.0	56,000
F2	1.2–1.4	21.1 (25.7)	26.3	1:0.79:1.45	1.1	54.8	tr		36.7	6.0	2.5	+47.9	77,000
F3 [e]	2.0 [f]	16.1 (19.6)	15.8	1:0.36:1.08	5.9	36.5	25.4	2.6	16.8	5.8	8.6	−15.5	18,000

[a] Yield of C1 is given for 100 g of the residue obtained after exhaustive extraction at room temperature. Yields of F1–F3 are given for 100 g of C1. In parentheses, percentage of the total recovered.
[b] From colorimetric measurements.
[c] Determined by the method of Park and Johnson.[14]
[d] Percentages lower than 1% are given as trace (tr).
[e] Small amounts (4.3%) of glucose were found in F3.
[f] Soluble in 2.0 M KCl.

that of the parent product (C1), even though the proportion of nu-structure is higher in F2.[15–17] No other structures were detected in these spectra.

In the spectrum of F3, the same diads are also present (ratio kappa:iota 1:1). The signals corresponding to a nu-structure are small, indicating that the high solubility of this fraction in potassium chloride is not due to the presence of non-cyclized structures of the kappa-family.

The signals at 103.9 and 101.6–101.0 ppm were assigned to β-D-galactose units linked to α-L-galactose residues, substituted in part with sulfate on C-3.[21,22] The signal at 67.8 ppm corresponds to C-2 of the latter unit.[22] Signals corresponding to single stubs of β-D-xylose linked to C-6 of β-D-galactose units are important,[23–25] in agreement with the higher xylose content detected in this fraction and higher mobility of this residue. The possible signal at 57.2 ppm would correspond to a methoxyl linked to C-3 of an α-D-galactose unit,[26] in agreement with the monosaccharide composition of F3 (Table 2) and the spectrum of F3T6 (Fig. 3).

Table 3
Composition of partially methylated monosaccharides produced by permethylation and hydrolysis of C1, F1, F2 and F3

Monosaccharide [a]	C1	F1	F2	F3
2,3,6-Gal	1.6	tr [b]	tr	4.6
2,4,6-Gal	3.0	tr	tr	8.2
2,6-D-Gal	53.7	46.8	60.7	38.4 [c]
2,6-L-Gal	2.0	tr	tr	3.3
2,4-Gal	tr	tr		8.4
6-Gal	tr	tr	tr	2.3
3-Gal	tr	1.4		4.9
2-Gal	tr			1.6
2-AnGal	17.9	21.8	15.6	14.0
D-AnGal	15.3	8.2	14.9	tr
L-AnGal	6.5	21.8	8.8	12.9
2,3,4-Xyl	tr	tr	tr	1.4

[a] Mol% of monosaccharide having methyl groups at the positions indicated.

[b] Percentages lower than 1% are given as trace (tr).

[c] Hydrolysis of the permethylated polysaccharide and derivatization to the corresponding aldononitrile acetates showed 3.5% of 4,6-di-*O*-methyl- and 34.9% of 2,6-di-*O*-methyl-galactose.

F3 was submitted to an alkaline treatment to give F3T, part of which insolubilized during the dialysis (F3T1, 12.9%). The remaining material was fractionated with potassium chloride (Scheme 1), producing four gelling fractions, which precipitated at 0.1–0.2 M (F3T2, 16.7%), 0.2–0.4 M (F3T3, 2.5%), 0.6–1.0 M (F3T4, 12.5%) and 1.5–2.0 M KCl (F3T5, 2.3%). A fraction soluble in 2.0 M KCl (F3T6, 34.6%) was also obtained. Analyses, monosaccharide and enantiomeric composition of these fractions are given in Table 4.

The monosaccharide and enantiomeric composition, as well as the optical rotation and sulfate content show that F3T1 is an agaran.

Fractions F3T2–F3T5 would be carrageenans according to their gelling properties and sulfate content, but they contain major to significant amounts of L-galactose and 3,6-anhydro-L-galactose. Optical rotations are positive (carrageenans), but their values are lowered by the presence of L-sugars.

F3T6 shows ~73% of agaran- and ~27% of carrageenan character with a low sulfate content and a negative optical rotation (agaran) with an absolute value reflecting the presence of the carrageenan structure.

Methylation analysis indicates for F3T1 (Table 5) an agaran-like structure comprised of β-D-galactose, β-D-galactose 4-sulfate and β-D-galactose with single stubs of xylose linked to C-6, together with α-L-galactose and α-L-galactose 3-sulfate and 3,6-anhydro-α-L-galactose and 3,6-anhydro-α-L-galactose, probably, substituted with sulfate on C-2. The 21.1% of 2,3,6-tri-*O*-methylgalactose should involve the 6.2% of 3-*O*-methyl-α-D-galactose units found in this sample (Table 4).

Fractions F3T2–F3T5 (Table 5) show methylation patterns of kappa/iota-carrageenans. Enantiomeric analysis of the partially methylated sugars allowed us to detect small quantities of 2,6-di-*O*-methyl-α-L-galactose in all these samples and to determine that substantial amounts of the 3,6-anhydro-α-galactose 2-sulfate units in fraction F3T2 and F3T4 are in the L configuration.

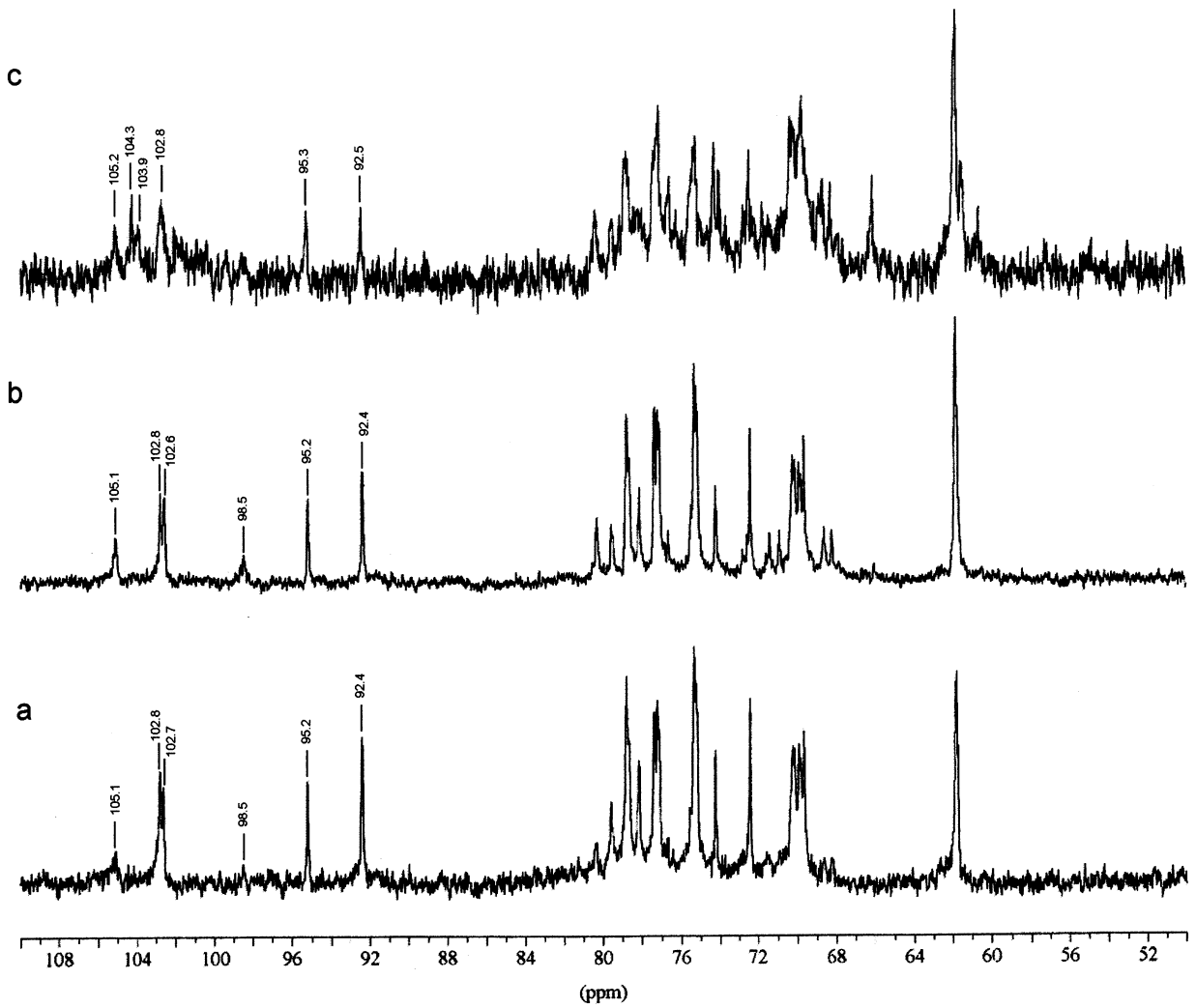

Fig. 2. ^{13}C NMR spectra of (a) F1; (b) F2; and (c) F3.

F3T6 shows a complex pattern of methylation, indicating the presence of non-substituted β-D-galactose, β-D-galactose 4-sulfate and β-D-galactose substituted with single stubs of xylose on C-6, together with major amounts of α-L-galactose 3-sulfate units; small quantities of 3-*O*-methyl-α-D-galactose and α-D-galactose 2,6-disulfate units were also detected. The 3,6-anhydrogalactose residues are in the D and L forms.

The presence of alkali-stable L-galactose 3-sulfate units, xylosyl side chains and traces of 3-*O*-methylgalactose were previously found in the polysaccharide extracted from *Anatheca dentata* (Solieriaceae, Gigartinales).[27–29]

Traces of 4,6-di-*O*-methylgalactose were detected between the methylated sugars obtained from F3T2, F3T4, and F3T6, indicating the presence of small amounts of lambda-structures.

The ^{13}C NMR spectrum of F3T2 (Fig. 3) is characteristic of a kappa/iota-carrageenan; no other signals were found, even though (Table 3), this fraction contains α-L-galactose and 3,6-anhydro-α-L-galactose units (10.3 and 15.8%, respectively). This fact could be explained taking into account that 13% of this fraction was found to be insoluble during preparation of the sample for NMR analysis.

The spectrum of F3T6 (Fig. 3) is completely different to those of the other fractions. Signals corresponding to a kappa/iota-carrageenan are not present. The signals at 104.3, 74.0, 76.6, 70.2, and 66.1 were assigned to C-1–C-5, respectively, of single stubs of β-D-xylose linked to C-6 of some of the β-D-

Table 4
Yields and analyses of the carrageenans obtained by alkaline treatment of F3 and of the fractions obtained from it by further fractionation with potassium chloride

Fraction	Range of precipitation (M, KCl)	Yield [a] (%)	Sulfate as SO_3K (%)	Gal:AnGal:sulfate [b] (molar ratio)	Proteins (%)	Monosaccharide composition (mol%) [c]							$[\alpha]_d$ (°)	Molecular weight [d]
						Gal		3-*O*-Me-Gal		AnGal		Xyl		
						D-	L-	D-	L-	D-	L-			
F3T		61.8	21.5	1:0.3:0.6	tr [e]	46.0	20.1	3.6	1.0	—21.0—		8.3	nd [f]	nd
F3T1	ins [g]	12.9 (15.8)	8.6	1:0.1:0.2		43.9	32.1	6.2			10.0	7.8	−114.3	39,700
F3T2	0.1–0.2	16.7 (20.5)	27.3	1:1.0:1.0	1.9	41.2	10.3	—tr—		31.2	15.8	1.4	+33.3	35,000
F3T3	0.2–0.4	2.5 (3.5)	23.6	nd	nd	48.9	10.3	—tr—		—40.8—		tr	nd	nd
F3T4	0.6–1.0	12.5 (15.3)	19.0	1:0.7:0.6	tr	55.1	4.6	—tr—		19.2	19.8	1.3	+46.3	45,000
F3T5	1.5–2.0	2.3 (2.5)	18.5	nd	nd	50.9	6.9	—tr—		—42.2—		tr	nd	nd
F3T6	2.0 [h]	34.6 (42.4)	16.1	1:0.1:0.4	3.6	45.9	29.3	4.2		8.3	4.2	8.1	−56.9	22,500

[a] Yield of F3T is given for 100 g of F3. Yields of F3T1–F3T6 are given for 100 g of F3T. In parenthesis, percentage of the total recovered.
[b] From colorimetric determinations.
[c] Small amounts of glucose are present in all the fractions.
[d] Determined by the method of Park and Johnson.[14]
[e] Percentages lower than 1% are given as trace (tr).
[f] nd = not determined.
[g] Insolubilized during dialysis (see Section 4).
[h] Soluble in 2.0 M KCl.

galactose units.[23–25] Nevertheless, other signals are clear in the anomeric region: the major ones are those at 104.1–103.9 and 101.7–101.4 ppm corresponding to chains of alternating 3-linked β-D-galactose and 4-linked α-L-galactose units. Considering the whole spectrum and data from methylation analysis (Table 5), it was inferred that the β-D-galactose units are, in part, substituted with β-D-xylose on C-6 and the α-L-galactose residues are, in part, sulfated on C-3 or C-6. Other important signals on the anomeric region are at 102.1 and 99.0 ppm (with a shoulder at 98.9 ppm). They would correspond to a linear

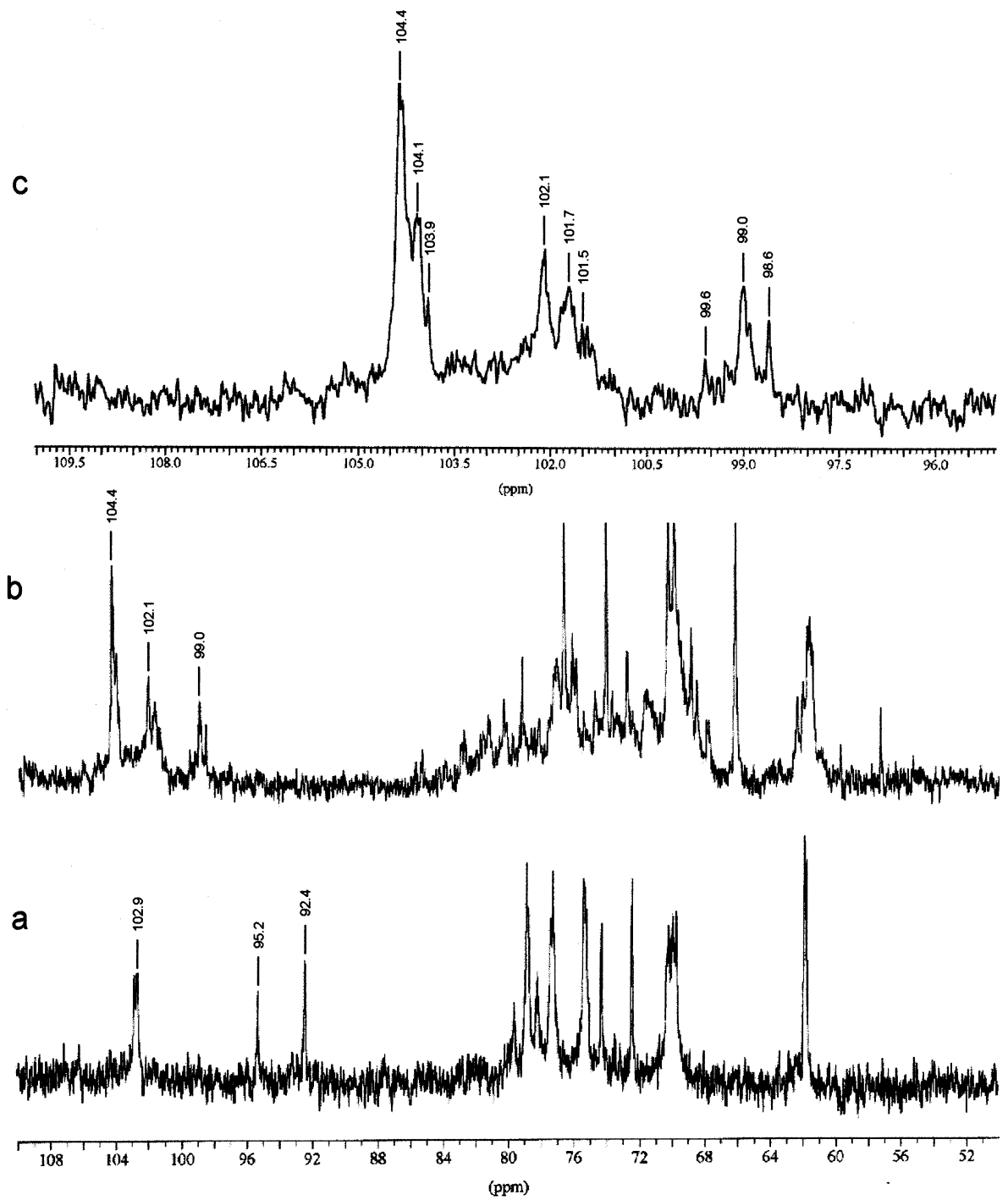

Fig. 3. ^{13}C NMR spectra of (a) F3T2; (b) F3T6; and (c) F3T6 (anomeric region).

Table 5
Methylation analyses of F3T and of the fractions obtained from it by potassium chloride fractionation

Monosaccharide [a]	F3T	F3T1	F3T2	F3T3	F3T4	F3T5	F3T6
2,3,6-Gal	6.8	21.1	tr [b]	tr	1.6	tr	3.0
2,4,6-Gal	7.2	14.5	tr	tr	1.2	1.5	10.4
2,6-D-Gal	25.9	7.8	47.2	43.1 [c]	53.2	47.0 [d]	6.2
2,6-L-Gal	9.1	19.4	2.1	1.6	1.6	1.7	27.4
2,4-Gal	6.5	7.9	tr				6.2
6-Gal	tr	3.3				tr	4.0
3-Gal	3.0	tr		tr	tr	1.9	7.0
2-Gal	tr	tr				tr	2.0
Gal	1.0	1.2					tr
Glc	3.4	3.6			tr		
2-AnGal	14.6	11.0	29.4	29.2	14.6	11.6	25.1
AnGal	20.0	4.2 [e]	17.7 [f]	19.4	26.2 [g]	32.3	4.8
2,3,4-Xyl	2.5	6.0	3.6	6.7	1.6	4.0	3.9

[a] Mol% of monosaccharide having methyl groups at the positions indicated.
[b] Percentages lower than 1% are given as trace (tr).
[c] Hydrolysis of the permethylated polysaccharide and derivatization to the corresponding aldononitrile acetates showed 1.3% of 4,6-di-*O*-methyl- and 41.8% of 2,6-di-*O*-methyl-galactose.
[d] Hydrolysis of the permethylated polysaccharide and derivatization to the corresponding aldononitrile acetates showed 1.0% of 4,6-di-*O*-methyl- and 46.0% of 2,6-di-*O*-methyl-galactose.
[e] All in D-series.
[f] 7.2 and 10.5% in the D- and L- series, respectively.
[g] 1.0 and 25.2% in the D- and L- series, respectively.

chain of alternating 3-linked β-D-galactose and 3,6-anhydro-α-L-galactose units. The fact that the signal at 102.1 ppm appears 0.6–0.7 ppm displaced to higher fields in comparison with data reported previously[21,30–32] would be due to substitution or conformational factors that are not clear. Small signals at 105.2 and 98.6 ppm correspond the anomeric carbon atoms of a nu-diad.[16,17]

Analysis of this spectrum showed an important proportion of porphyran structure and some nu-diads, and the difference between the percentage of 3,6-anhydrogalactose informed in the monosaccharide composition and that obtained after the methylation procedure (12.5 and 29.9%, respectively, Tables 4 and 5), indicated that the alkaline treatment carried out on F3 had not been complete.

F3T6 was submitted to further alkaline treatment, taking into account the conditions for cyclization of a porphyran,[33] obtaining complete cyclization.

3. Discussion

Galactans from red seaweeds have structures based on a linear chain of alternating 3-linked β-D-galactopyranosyl residues (A units) and 4-linked α-D- or α-L-galactopyranosyl units (B units). The simplest classification is made according to whether the 4-linked residue belongs to the D-series (carrageenans) or to the L-series (agarans) (Fig. 4). However, a third group in which the B-units could have D or L configuration in the same molecule is under study. They are known as hybrid or intermediate galactans. The denomination 'hybrid' has been used in the field of carrageenans to describe products whose structures are built up by a mixture of idealized repeating units (i.e., kappa/iota-carrageenans). To avoid uncertainties we use the term 'carrageenan hybrids' and adopt the term 'DL-galactan hybrids' for the third group.[34]

The existence of DL-galactan hybrids has not been proved beyond a doubt because in no case has an oligosaccharide containing carrageenan and agaran structures been isolated. Actually, partial hydrolysis of suspected 'DL-galactan hybrids' has produced only carrageenan and/or agaran fragments.[35] It is possible that the carrageenan–agaran domain would correspond to a junction zone in a

block copolymer.[35] In this case the yield of DL-hybrid oligosaccharides in a random hydrolysis should be low. Moreover, if we consider that carrageenans and agarans may self-complex in solution, the irregular zones may be slightly more susceptible to hydrolysis, and the yield in DL-oligosaccharides would be even lower than that expected in a random hydrolytic process.

The criterion of fractionation of a possible 'DL-galactan hybrid' into carrageenans and agarans fails in its practical application as no methodology for the separation of 'diasteromeric' polymers is known. The only partially successful methodology known at the time is based on the gel-forming properties of kappa/iota-like regular chains, depending on their specific interactions with potassium ion.

When this technique is applied to DL-galactan hybrid systems, the hybrids containing gel-forming kappa/iota-carrageenan chains (kappa/iota DL-hybrids) will precipitate, while those with mu/nu- or partially cyclized mu/nu-carrageenan chains (mu/nu DL-hybrids) will remain soluble, together with agarans and DL-hybrids with predominant agaran chains (agaran DL-hybrids), in the 2 M KCl-soluble fraction. Further alkaline treatment results in longer and/or more numerous helical regions in the carrageenan domain, and a new precipitation with potassium chloride separates the original mu/nu DL-hybrids.

Methylation analysis coupled to enantiomeric determinations provides a major tool for the study of DL-hybrids. ^{13}C NMR spectroscopy is a useful and rapid complement for structural studies,[36] but due to its inherent low sensitivity, as well as to the low solubility and usual complexity of the spectra of these polysaccharides, small but still significant percentages of minor diastereomeric structures may not be detected.

Seaweeds belonging to the Phyllophoraceae are typical carrageenophytes producing different polysaccharides in the different life stages.[2] Cystocarpic samples of the genus *Gymnogongrus* have been reported to synthesize iota-carrageenans and/or kappa/iota-car-

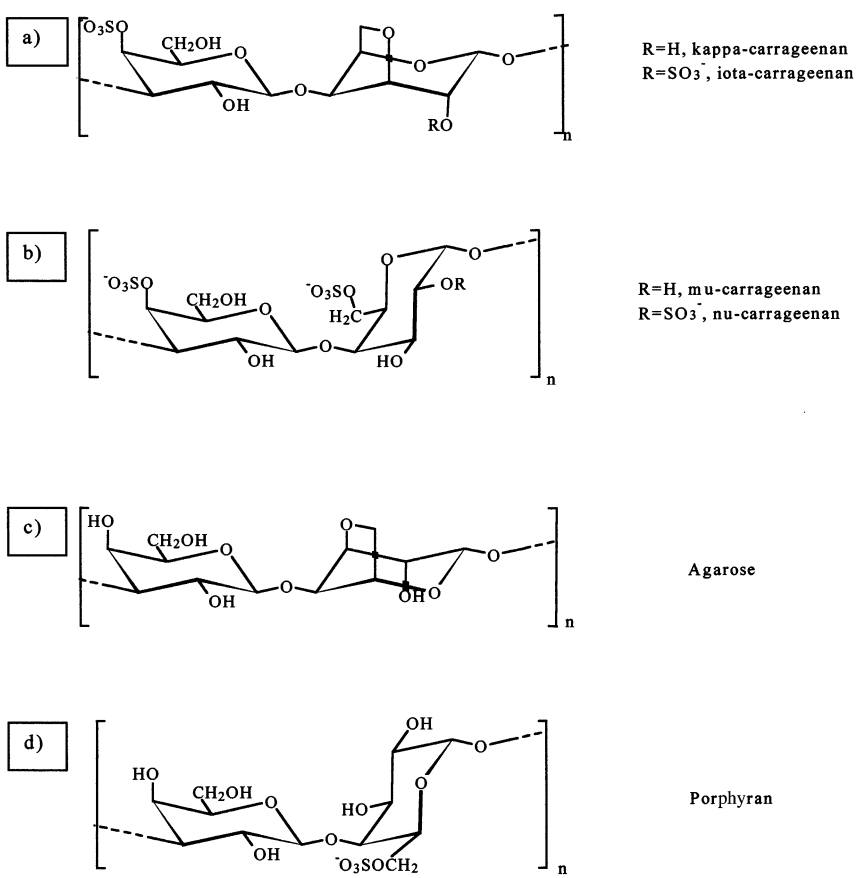

Fig. 4. Idealized repeating structures of (a) gelling and (b) non-gelling carrageenans and (c) gelling and (d) non-gelling agarans.

rageenan hybrids.[1,4–7] To the best of our knowledge, L-galactose was found only in the polysaccharides extracted from *Mastocarpus stellata*,[9] Phyllophoraceae (formerly Petrocelidaceae[6]).

However, in cystocarpic samples of seaweeds belonging to the Gigartinaceae, L-galactose-containing polysaccharides with agaran-like structures have been isolated.[9–12]

The extensive use of the above-described fractionation procedure shows that the red seaweed *G. torulosus* produces a system of galactans formally similar to that biosynthesized by cystocarpic plants of the Gigartinaceae, (i.e., KCl-insoluble carrageenans of the kappa/iota-carrageenan hybrids type together with KCl-soluble mu/nu-carrageenans).[18,19] Nevertheless, enantiomeric analysis of the component sugars (Tables 1, 2 and 4) and structural units (Tables 3 and 5) shows that the gelling components (before and after alkaline treatment) are not carrageenans, in spite of their specific interactions with potassium ions. Thus, in F1 and F2, the 3,6-anhydrogalactose units are in the D- and L-forms, while the 3,6-anhydrogalactose 2-sulfate would be in the D-form giving a carrabiose/agarobiose ratio $\sim 3.5{:}1$ (Table 2). Considering that a chain of 3.5 carrabiose units is too short for gelation,[37] the agarobiose should not be interspersed regularly in the backbone but grouped. This is in agreement with the block-structure theory,[35] and the isolation of carrageenan and/or agaran fragments from the partial hydrolysis of the polysaccharides from *Anatheca dentata* (Gigartinales)[27–29] and *Gratelupia divaricata* (Cryptonemiales).[38]

F3 shows a behavior (not in the case of F3T1) formally similar to other 2 M KCl-soluble fractions from cystocarpic samples of the Gigartinaceae, composed by mu/nu-carrageenans and L-galactose-containing galactans.[10] In these cases, after alkaline treatment, the new 'kappa/iota-carageenans' were separated by precipitation with potassium chloride and the DL-galactans remain in solution. The new 'kappa/iota-carragenans' from F3 (fractions F3T2–F3T5) show the same behavior but contain significant amounts of L-galactose and 3,6-anhydro-L-galactose units (Tables 4 and 5). The carrabiose–agarabiose ratio is 1–2:1 which is lower than in F1 and F2, suggesting that the larger percentages of agaran structures in F3 have contributed to the original solubility in potassium chloride. As in the previous cases of F1 and F2, the gelling behavior of F3T2–F3T5 suggests block copolymers. Fraction F3T6 contains $\sim$ 73% of a structure that shares similarities with agarans and corallinans, together with $\sim 23\%$ of carrageenan backbone (Table 4). The major quantities and diversity of agabiose-like units are in agreement with the observed solubility of F3T6 in potassium chloride solution.

In summary, the use of fractionation techniques based on the precipitation of DL-galactans with major amounts of regular backbones of the kappa/iota-carrageenan-type, before and after alkaline treatment, together with enantiomeric analysis of the sugar components, and when possible of the structural units, strongly suggest that the red seaweed, *G. torulosus* (Phyllophoraceae) synthesizes a complex mixture of galactans comprising DL-galactan hybrids of the carrageenan- and agaran-type, together with minor amounts of agarans with unusual structural details.

4. Experimental

Material.—Samples of cystocarpic and sterile plants of *G. torulosus* were collected during summer of 1998 in Cabo Corrientes, Mar del Plata (Provincia de Buenos Aires), Argentina.

General.—Galactose was analyzed by the phenol–H_2SO_4 method[39] without previous hydrolysis of the polysaccharide. Galactose content was corrected for the presence of 3,6-anhydrogalactose, which was determined independently by the resorcinol method.[40] Sulfate was determined turbidimetrically.[41] The molecular weight was estimated by the method of Park and Johnson[14] based on the determination of end-chain reducing units, while the protein content was determined by the method of Lowry et al.[42] Optical rotations (Na D-line) were measured in a Perkin–Elmer 343 polarimeter, using 0.2–0.4% solutions of

the polysaccharides in water. For GLC, alditol acetates were obtained by reductive hydrolysis and acetylation of the samples.[43] The percentages of α-galactose 6-sulfate and 2,6-disulfate units were estimated by the method of Ref. 21. The ratio of D- to L-galactose and the configuration of monomethylated galactoses (when the percentages present were high enough) were estimated by the method of Cases et al.[44] through its diastereomeric acetylated1-deoxy-1-(2-hydroxypropilamino)alditols. The ratio of D- to L-3,6-anhydrogalactose was estimated by the method of Errea et al.,[45] using an oxidative hydrolysis to obtain the aldonic acids, which are further converted to the acetylated diastereomeric *sec*-butylesters. The ratio of D- to L-2,6-di-*O*-methylgalactose was determined on the permethylated polysaccharide by conversion of the monosaccharides, obtained by hydrolysis of the sample to the diastereomeric acetylated 1-deoxy-1-(1-phenylethylamino)alditols.[46]

Extraction.—Sterile and cistocarpic plants, previously milled (66 g), were extracted with water (3.3 L) at rt with mechanical stirring for 24 h. The residue was removed by centrifugation and the supernatant poured into 3 vol of 2-PrOH, whereby the polysaccharide precipitated as long fibers. The liquors were decanted, and the product was pressed between filter paper and dried by solvent exchange (EtOH and Et_2O) and finally in vacuo. The supernatant was concentrated, dialyzed (molecular weight cut-off 1000) and freeze-dried, which gave the soluble fraction. The residue was extracted twice in the same way. The final residue obtained at rt was freeze-dried (53 g) and resuspended in water (2.7 L) and extracted at 90 °C with mechanical stirring for 4–5 h. The extract was treated as described above. The supernatant of the 2-PrOH precipitation was treated in the same way, but dialyzed with tubing of molecular weight cut off 3500. The residue was extracted four times in the same way.

GPC of extracts C1–C4.—The sample (6–8 mg) was chromatographed on a Sephadex G-100 (column 70 × 1.0 cm i.d.) using 1 M NaCl as eluant. Fractions of 1.3 mL were isolated and the aliquots were assayed by the phenol–H_2SO_4 method.[39] Dextran sulfates of M_r 5000, 8000, and 10,000 (Sigma) were chromatographed in the same conditions.

Fractionation of C1 with potassium chloride.—The polysaccharide (9.0 g) was dissolved in water (3.5 L, 0.25%). A solid, finely divided KCl was added in small portions with constant and violent mechanical agitation so that the concentration was increased by 0.1 M each time. After each addition, stirring was continued for 5–16 h to ensure equilibration of the system. The upper limit of KCl concentration was 2.0 M. The precipitates, as well as the residual solutions were dialyzed (molecular weight cut-off 3500), concentrated and freeze-dried.

Alkaline treatment of F3.—The sample (400 mg) was dissolved in water (200 mL), and $NaBH_4$ (20 mg) was added. After 24 h at rt, 3 M NaOH was added (100 mL) along with a further quantity of $NaBH_4$ (15 mg). The solution was heated at 80 °C for 3 h. The solution was cooled to rt, dialyzed (molecular weight cut-off 1000), concentrated and freeze-dried.

Alkaline treatment of F3T6.—Treatment was carried out as described above, but using 15 mg of F3T6, and the solution was heated at 80 °C for 5 h. The percentage of 3,6-anhydrogalactose was determined colorimetrically[40] before and after the treatment and neutralization, as well as by GLC of the alditol acetates obtained after reductive hydrolysis and acetylation of the sample (previously dialyzed and lyofilized).

GLC.—GLC of the alditol acetates, as well as those of the partially methylated alditol and aldononitrile acetates was carried out on a Hewlett–Packard 5890A gas–liquid chromatograph equipped with a flame ionization detector and fitted with a fused silica column (0.25 mm i.d. × 30 m) WCOT-coated with a 0.20 mμ film of SP-2330. Chromatography were carried out as described before.[13]

GCL–MS.—GLC–MS was performed on a Shimadzu GC-17A gas–liquid chromatograph equipped the SP-2330 (see above) interfaced to a GCMS–QP 5050A mass spectrometer working at 70 eV. Helium was used as the carrier gas.

FTIR.—Fourier-transform infrared spectra were recorded with a 510P Nicolet FTIR spec-

trophotometer, using films prepared by drying aq solutions of the polysaccharides. Scans were carried out over the range of 4000–250 cm^{-1}, with 32–64 scans being taken at a resolution of 2–4 cm^{-1}.

Methylation analysis.—The sample (3–10 mg) was converted into the corresponding triethylammonium salt,[43] and it was methylated according to Ciucanu and Kerek[47] using finely powdered NaOH as base. The methylated samples were derivatized to the acetylated alditols as described for the polysaccharides.[43]. A portion of methylated F3, F3T2, F3T4 and F3T6 were hydrolyzed with TFA for 2 h at 120 °C, and the partially methylated sugars were converted into the corresponding aldononitrile acetates.[48]

Preparation of the samples for ^{13}C NMR spectroscopy.—The sample (20–30 mg) was dissolved in 1:1 water–D_2O solutions (1 mL), agitated 24 h at rt, and centrifuged. In all cases, a precipitate was obtained that represented 5–20% of the original sample.

^{13}C NMR spectroscopy.—100 MHz ^{13}C NMR spectrum of C1 was recorded at 70 °C, in 1:0.25 water–D_2O solutions, with an external reference of Me_4Si. The parameters were as follows: pulse angle 90°, acquisition time 0.6 s, relaxation delay 4.5 s, spectral width 32 kHz and scans 82,924. 125 MHz ^{13}C NMR spectra were recorded at rt, with an external reference of Me_4Si. The parameters were as follows: pulse angle 51.4°, acquisition time 0.56 s, relaxation delay 0.6 s, spectral width 29.4 kHz and scans 33,000–48,000. Chemical shifts were referenced to internal acetone (δ 216.2 and 31.1)

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