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# The system of galactans of the red seaweed, *Kappaphycus alvarezii*, with emphasis on its minor constituents

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**Abstract**—The galactans extracted with hot water from *Kappaphycus alvarezii*, after previous extraction at room temperature, are mainly composed of κ-carrageenans (~74%) and μ-carrageenans (~3%). However, a significant percentage of these galactans (at least 14%) is composed of sulfated agarans and, possibly, agaran-type sulfated DL-hybrid galactans. These agarans are partially substituted on C-2 or C-4 or disubstituted on both positions of the β-D-galactose units and on C-3 or C-2 and C-3 of the α-L-galactose residues with sulfate groups or single stubs of β-D-xylopyranose, D-glucopyranose, and galactose or with D-glucopyranosyl-(1→4)-D-glucopyranose side chains. Significant quantities of 2-*O*-methyl- and 3-*O*-methyl-L-galactose units are also present. A great tendency to retain $Ca^{2+}$ and $Mg^{2+}$, in spite of massive treatments with $Na^{+}$ and $K^{+}$ salts, was observed. The complexation between agarans and agarans–κ-carrageenans through divalent cations and the possible zipper-type carbohydrate–carbohydrate interactions would be two complementary mechanisms of interactions.
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## 1. Introduction

Red seaweed galactan sulfates are linear polysaccharides with alternating 3-linked β-D-galactopyranose units and 4-linked 3,6-anhydro-α-galactopyranose or α-galactopyranose units, having different positions and degrees of sulfation. Other substituents, as methyl ethers, pyruvic acid ketals, and single stubs of β-D-xylopyranose and/or other monosaccharides are sometimes present. They have been divided in carrageenans, when the 4-linked residues (B-units) are on the D-configuration, and in agarans, when these residues belong to the L-series. Thus, two diastereomeric polysaccharide groups are defined, and the seaweeds that biosynthesize these polysaccharides are called carrageenophytes and agarophytes, respectively.

Nevertheless, during the last few years it has been shown that seaweeds belonging to the Gigartinaceae and Phyllophoraceae, which are typical carrageenophytes, produce small quantities of sulfated agarans and/or sulfated DL-galactan-hybrids[‡].[1–4] On the other hand, investigation of the polysaccharides from the agarophytes *Digenea simplex* (Ceramiales),[5] and *Rhodomela larix* (Ceramiales)[6] and *Porphyra columbina* (Bangiales)[7] showed the presence of minor amounts of B-units belonging to the D-series. Small quantities of galactan sulfates containing 4-linked α-D-galactose

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‡ DL-Hybrid galactans are galactans with alternating 3-linked β-D-galactopyranose units and 4-linked 3,6-anhydro-α-galactopyranose and/or α-galactopyranose units in which the α-residues have both D- and L-configurations. When the molecule is built up with major amounts of carrageenan diads, having α-galactose units in the D-configuration, the products have been named carrageenan–DL-hybrid galactans; but when the major quantities of disaccharidic units in the molecule are of agaran type, with α-galactose units in L-configuration, they were named agaran–DL-hybrid galactans.

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residues have been isolated also from the agarophyte *Pterocladiella capillacea* (Gelidiales).[8] These types of DL-hybrid structures have been found in many other red seaweeds.[5,9]

*Kappaphycus alvarezii* (Solieriaceae) is a red seaweed of great commercial value that is cultivated mainly in the Far East as raw material for the industrial production of κ-carrageenan.[10,11] Usually, the polysaccharide is extracted with hot, neutral, or alkaline, water from the native or alkali-treated seaweed. Previous work on raw extracts obtained with hot water from the native seaweed showed that they were composed mainly by κ-carrageenan (actually κ/ι-hybrid carrageenan, with small amounts of ι-structure), together with minor quantities of the μ-precursor and small-to-trace amounts of a 'highly methylated galactan' ('methylated carrageenan').[12–15] These results were obtained by the analysis of the composition and $^{13}C$ NMR spectroscopy of raw extracts and $^{13}C$ NMR spectroscopy of μ-enriched extracts. Spectroscopic analysis of the carrageenan of *Kappaphycus striatum* (sacol variety) extracted with hot 0.05 M $NaHCO_3$ indicated similar structural details.[16] No fractionation of the hot-water extracts or attempts of isolation of its different components were carried out in either case.

A recent study of the products extracted with water at room temperature from *K. alvarezii* showed that they consist of low-molecular-weight carrageenans with structural dispersion around a basic κ-pattern. However, significant quantities of 4-linked α-L-galactose residues suggested the presence of agaran structures.[17]

The aims of the present study are to show that this seaweed biosynthesizes, not only an important amount of κ-carrageenan as it is well known, but also significant amounts of sulfated agarans and/or sulfated agaran–DL-hybrid galactans with structures that are not detected by the usual procedures or that are lost during the industrial hydrocolloid workup. Besides, the counterion composition is studied, focusing on the changes that take place with the different chemical treatments carried out. The complexation between agarans and agarans/κ-carrageenans is discussed.

## 2. Results

The milled seaweed, free from any epiphytic and/or epizoic contaminants, was extracted exhaustively with water at room temperature. The extracts were studied and have been reported elsewhere.[17] The residue was further extracted with hot water giving a solution that did not precipitate by addition to 2-propanol; hence, the raw extract (C) was obtained by dialysis and freeze-drying. This procedure was repeated three times more in the same way; the total yield of the extraction procedure, including the room-temperature extracts, was 48% of the milled seaweed, from which C, 74% of the total polysaccharides obtained, was used for further studies. Yield, analysis, and monosaccharide composition of C are given in Table 1. C is composed by galactose and 3,6-anhydrogalactose and small amounts of xylose and glucose and has a number-average molecular weight ($M_n$) of 30 kDa. Enantiomeric analysis (Table 1) of both the major sugars showed small but significant amounts of L-galactose and 3,6-anhydro-L-galactose, suggesting that about 14% of the crude extract has an agaran-type structure. Enantiomeric analysis of a commercial, potassium chloride purified, κ-carrageenan extracted from the same seaweed (K) (Table 1) showed smaller amounts of L-galactose and 3,6-anhydro-L-galactose and a molecular weight of 140 kDa.

The differences between the commercial sample (K) and the raw extract (C) were attributed to the alkaline treatment usually carried out on the seaweed,[10] previous to the extraction of the commercial κ-carrageenan. On that basis, C was submitted to a short alkaline treatment using conditions suitable to avoid degradation.[18] The alkali-modified product (C′) was isolated by 2-propanol precipitation. This product was further dialyzed (MW cutoff 12 kDa) to eliminate small fragments, and by precipitation with 0.125 M KCl, an insoluble product was isolated (C″). The solubility behavior and number-average molecular weight, as well as the composition and enantiomeric analyses of C′ (Table 1) are very similar to those of K.

Cation analyses of C, C′, and C″ (Table 2) show that the divalent counterions $Ca^{2+}$ and $Mg^{2+}$ initially present in C are still preponderant in C′ and C″, in spite of the treatment with hot 1 M NaOH and further precipitation of the modified product with potassium chloride. In the commercial sample of κ-carrageenan (K) the major cation was $K^+$, possibly due to the purification procedure through precipitation with potassium chloride. However, most of it came from absorbed salts that were eliminated by dialysis (K′), leaving $Ca^{2+}$ as the major counterion and $Ca^{2+}$ and $Mg^{2+}$ with higher percentages (64.7%) than those found in C″ (50.4%) and F1 (55.9%) (Table 2). Fraction F3i (Fig. 1), which became insoluble during dialysis (see later), retained 70.8% of $Ca^{2+}$ plus $Mg^{2+}$. On the other hand, T1 (Fig. 1), which also precipitated during dialysis, but after alkaline treatment of F3s with sodium hydroxide (see later), showed sodium as the major cation, but it still retained considerable amounts of divalent cations (46.1%) (Table 2). The methylated derivative of K (Km, Table 2) showed as much $Ca^{2+}$ and $Mg^{2+}$ as monovalent cations, in spite of the anion-exchange chromatography carried out to replace the inorganic cations by triethylammonium and the alkaline methylation. This tendency to retain divalent cations suggests that they are not simple counterions, but that they are tightly bound to the polysaccharides.

**Table 1.** Yields and analyses of a commercial κ-carrageenan, the raw extract (C) and the fractions obtained from it by fractionation with potassium chloride

| Fraction | Range of precipitation (M, KCl) | Yield[a] (%) | Carbohydrates (%) | Sulfate as $SO_3K$ (%) | Monosaccharide composition (mol%) | | | | | | | | $[\alpha]_D$ | Molecular weight (kDa) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | D-Gal | L-Gal | 2-Me-L-Gal | 6-Me-D-Gal | D-AnGal | L-AnGal | D-Xyl | D-Glc | | |
| K | — | — | n.d.[b] | 28.0 | 49.5 | 1.0 | — | — | 48.4 | 1.1 | — | — | +51.8 | 140 |
| K′ | | 75.0 | 54.0 | 28.4 | 47.7 | | — | 2.4 | 48.5 | | tr | 1.4 | n.d. | n.d. |
| C | — | 65.6 | n.d. | 23.9 | 50.2 | 3.1 | tr[c] | tr | 40.0 | 3.8 | 1.1 | 1.8 | +48.6 | 30 |
| C′ | — | 82.0 | 45.0 | 22.0 | 47.8 | tr | tr | 1.2 | 44.6 | 3.1 | 1.3 | 2.0 | n.d. | 125 |
| F1 | 0.1–0.2 | 67.0 | 47.1 | 25.4 | 49.9 | 1.3 | tr | tr | 45.0 | 3.8 | tr | — | +51.2 | 42 |
| F2 | 0.2–0.5 | 7.0 | 49.1 | 26.9 | 48.8 | 1.6 | tr | tr | 46.6 | 3.0 | — | tr | +56.7 | 42 |
| F3[d] | 2.0[e] | 15.0 | n.d. | 22.8 | 41.6 | 18.4 | 5.4 | 3.3 | 14.7 | tr | 10.5 | 6.1 | n.d. | n.d. |
| F3s. | — | 10.6 | 55.0 | 25.1 | 35.9 | 19.2 | 4.3 | 1.3 | 5.1 | | 26.9 | 7.3 | n.d. | 24 |
| F3i.[f] | — | 4.4 | 27.1 | 11.6 | 33.3 | 24.1 | 5.5 | 2.3 | 2.5 | 3.8 | 14.7 | 13.8 | n.d. | n.d. |
| F3i.[g] | — | — | n.d. | — | 46.7 | 33.6 | 7.7 | 3.2 | 3.5 | 5.3 | — | — | n.d. | n.d. |

[a] Yield of K′ is given for 100 g of K. Yield of C is given for 100 g of the residue obtained after exhaustive extraction at room temperature. Yields of C′ and, F1–F3i are given for 100 g of C. Yield of C″ is given for 100 g of C′.
[b] n.d. = not determined.
[c] Percentages lower than 1% are given as trace (tr).
[d] By dissolving F3 in $H_2O$, an insoluble product (F3i) and a soluble product (F3s) were obtained.
[e] Soluble in 2.0 M KCl.
[f] Small percentage of 3-*O*-MeGal was also detected (1.6%).
[g] Composition of the backbone, considering xylose and glucose as side chains or contaminant polysaccharides.

Potassium chloride fractionation of C (Table 1; Fig. 1) gave one insoluble fraction (74.0%), arbitrarily divided in F1 and F2, and one soluble in 2.0 M KCl, F3. Part of F3 became insoluble during the dialysis carried out to eliminate the potassium salts, giving F3s and F3i (Table 1). All the fractions contained L-sugars that appeared in small amounts in F1 and F2, in agreement with their optical rotation and molecular weight, but in important quantities in F3s and in F3i (Table 1). The last two fractions also contained xylose and glucose in significant amounts, together with small quantities of 2-*O*-methyl- and 6-*O*-methyl-galactose.

F1 and F2 gave well-resolved FTIR spectra with strong absorptions at 930 and 849 $cm^{-1}$ as expected for κ-carrageenans,[19] The spectrum of F3s was ill defined, but it still showed the same peaks.

Methylation analyses of K, C, F1, F2, and F3i are shown in Table 3. The same methylation pattern was produced by K, C, F1, and F2 corresponding, as expected, to a nearly ideal κ-carrageenan.

The $^{13}C$ NMR spectra of F1 and F2 (Fig. 2) showed the 12 signals of a κ-carrageenan.[20] In the spectrum of F1 no other signals are present, while in that of F2, very small signals at 105.3 and 71.0 ppm (C-1 and C-2 of the β-D-galactose units) and at 98.5, 68.2, and 68.0 ppm (C-1, C-5, and C-6 of the α-D-galactose residues), corresponding to precursor structures (μ-carrageenan), were detected.[21]

Permethylation of F3i was not achieved under the usual conditions; so the sample, suspended in water, was sonicated to give a cloudy solution, which was passed through a cation-exchange column to give the triethylammonium salt in 70% yield. During the alkylation step, the sample was dissolved in a $Me_2SO$ solution containing LiCl and heated at 80 °C for 2 h before the addition of the reagents. In spite of the procedure described, three methylation steps were needed to achieve permethylation of the galactan backbone and constancy of the methylated derivatives of xylose and glucose. The methylation pattern of the galactan backbone of F3i (Table 3) is different from those of F1 and F2. Part of the β-D-galactose units are nonsubstituted, and substitution occurs either on C-2 or on C-4 of the other β-D-galactose units of the galactan backbone. There is a great variety of 4-linked α-galactose units: α-L-galactose, possibly methylated on C-2, α-L-galactose units substituted on C-3 or C-2 and C-3 and small amounts of 6- and 2,6-disubstituted α-galactose units, 3,6-anhydrogalactose and 3,6-anhydrogalactose substituted on C-2. The high percentage of 6-*O*-methyl-D-galactose between the partially methylated monosaccharides could correspond to β-D-galactose substituted on C-2 and C-4 or α-D-galactose substituted on C-2 and C-3. Xylose and glucose side chains show an ‘all or none’ methylation scheme (Table 3) (see later).

**Table 2.** Cation composition[a] of commercial κ-carrageenan, derivatives, and of fractions isolated from *K. alvarezii*

| Galactans[b] | $Na^+$ | $K^+$ | $Ca^{2+}$ | $Mg^{2+}$ | $Ca^{2+}+Mg^{2+}$ (%) | Total amount | Sulfate as $SO_3^-$ |
|---|---|---|---|---|---|---|---|
| C | 47.8 (20.2) | 4.1 (1.7) | 140.0 (59.2) | 44.2 (18.7) | 77.9 | 236.1 | 200.8 |
| C′ | 73.9 (34.2) | 2.8 (1.3) | 90.0 (41.5) | 50.0 (23.1) | 64.6 | 216.7 | 184.9 |
| C″ | 117.9 (34.6) | 51.2 (15.0) | 150 (44.1) | 21.4 (6.3) | 50.4 | 340.5 | n.d.[c] |
| F1 | 74.2 (25.5) | 53.8 (18.5) | 137 (47.1) | 25.6 (8.8) | 55.9 | 290.6 | 213.4 |
| F3i | 56.5 (28.2) | 2.1 (1.0) | 100.0 (50.0) | 41.6 (20.8) | 70.8 | 200.2 | 97.5 |
| T1 | 139.1 (51.3) | 7.2 (2.6) | 70.0 (25.8) | 55.0 (20.3) | 46.1 | 271.3 | 84.0 |
| K | 41.3 (11.2) | 197.4 (53.5) | 125.0 (33.9) | 5.0 (1.4) | 35.3 | 368.7 | 235.3 |
| K′ | 59.1 (21.3) | 33.3 (12.0) | 140.0 (50.4) | 45.2 (16.3) | 66.7 | 277.6 | n.d. |
| Km | 179.0 (50.5) | 10.0 (2.8) | 120.0 (33.9) | 45.2 (12.8) | 46.7 | 354.2 | n.d. |

[a] In mequiv/100 g. In parenthesis, percentages.
[b] For nomenclature, see text.
[c] n.d. = not determined.

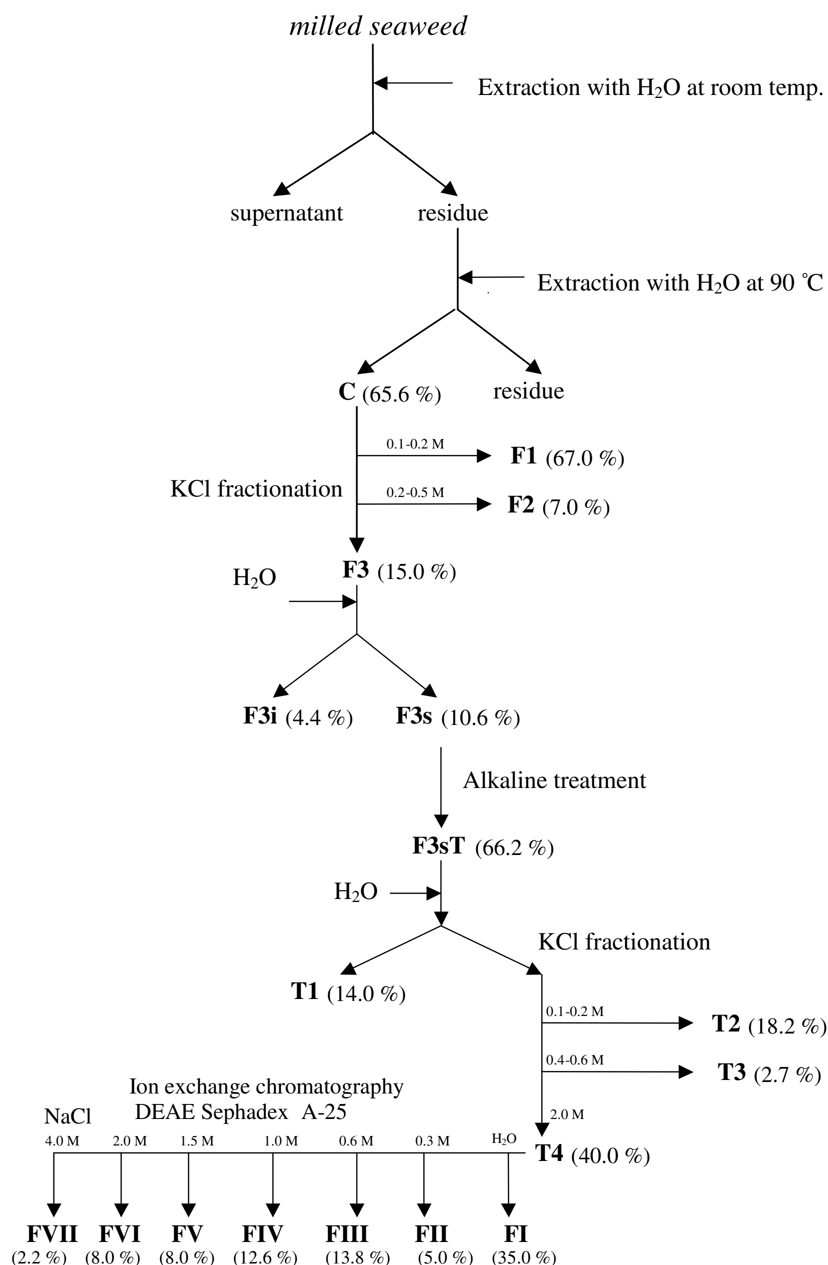


**Figure 1.** Extraction, treatment, and fractionations of the polysaccharide system of the red seaweed *K. alvarezii.*

**Table 3.** Composition of partially methylated monosaccharides produced by permethylation and hydrolysis of product K, C, F1, F2, and F3i

| Monosaccharide[a] | Assignment | K | C | F1 | F2 | F3i | |
|---|---|---|---|---|---|---|---|
| | | | | | | a[b] | b[c] |
| 2,3,6-Gal | D; L | tr[d] | 1.0 | tr | tr | 7.7 | 9.0 |
| 2,4,6-Gal | G | tr | 1.7 | — | — | 18.8 | 21.9 |
| 2,6-D-Gal | G4S; D3S | 49.8 | 51.5 | 49.3 | 51.6 | 13.0 | 15.2 |
| 2,6-L-Gal | L3S | tr | tr | tr | tr | 7.9 | 9.2 |
| 4,6-Gal[e] | G2S | — | — | — | — | 12.2 | 14.2 |
| 2,3-Gal | D6S; L6S | 2.2 | tr | tr | tr | 2.9 | 3.4 |
| 6-Gal | G2,4S; D2,3S | tr | 1.4 | 1.3 | 1.6 | 13.4 | 15.6 |
| 3-Gal[e] | G2,6S; L2,6S | tr | 1.1 | tr | tr | 1.9 | 2.2 |
| 2-Gal | G4,6S; D3,6S; L3,6S | tr | tr | 3.6 | tr | 1.9 | 2.2 |
| 2-AnGal | DA; LA | 48.0 | 43.3 | 45.8 | 46.8 | 3.1 | 3.6 |
| AnGal | DA2S; LA2S | — | — | — | — | 3.0 | 3.5 |
| 2,3,4,6-Gal | Galtnr | — | — | — | — | 1.2 | |
| 2,3,4-Xyl | Xyltnr | — | tr | — | — | 6.2 | |
| Xyl | | | | | | 3.1 | |
| 2,3,4,6-Glc | Glctnr | — | — | — | — | 1.1 | |
| 2,3,6-Glc | →1)-Glc-(4→ | — | — | — | — | 1.0 | |
| Glc | | — | — | — | — | 1.6 | |

[a] Mol% of monosaccharide having methyl groups at the positions indicated.
[b] Mol% of monosaccharides in the whole product having methyl groups at the position indicated.
[c] Mol% of monosaccharides in the backbone having methyl groups at the position indicated.
[d] Percentages lower than 1% are given as trace (tr).
[e] Confirmed from the analysis of the corresponding aldononitrile acetates.

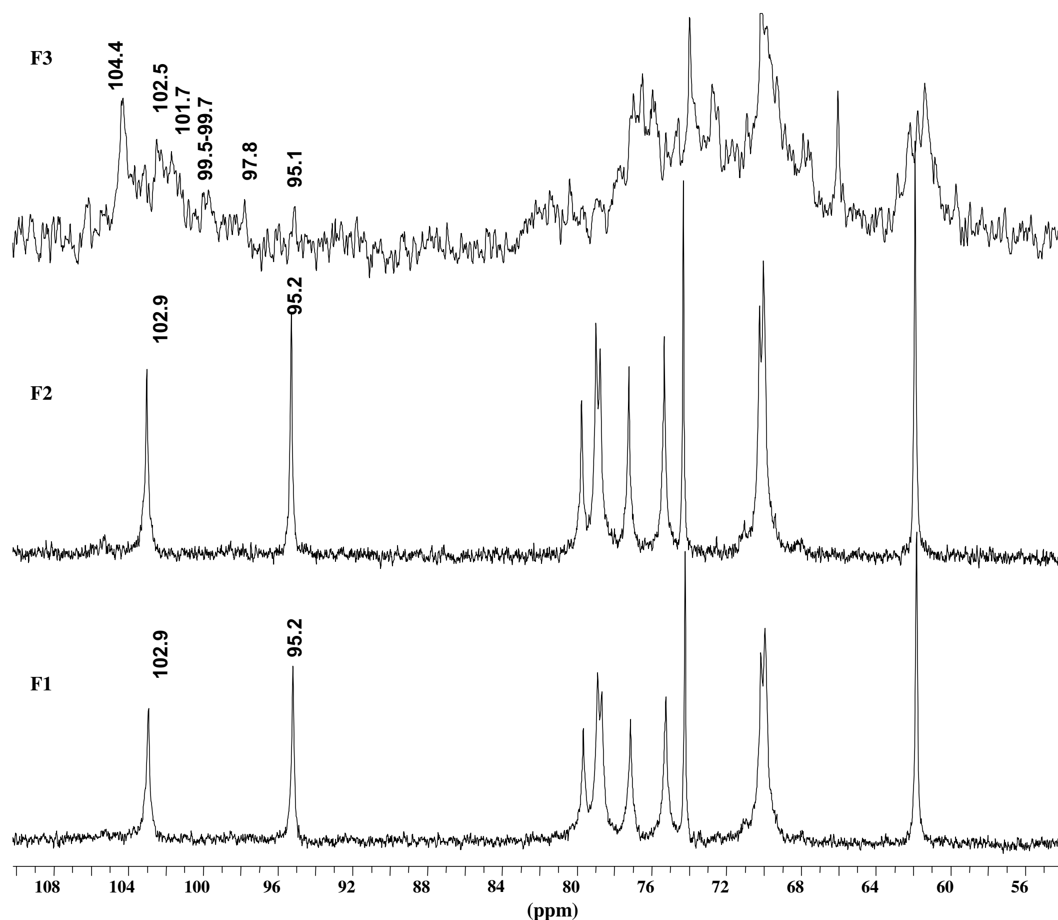


**Figure 2.** $^{13}$C NMR spectra of F1, F2, and F3.

The $^{13}$C NMR spectrum of F3i (Fig. 2) is very complex and has a low signal-to-noise ratio. Only those clearly observable absorptions were interpreted with the aid of the previous chemical analysis. The peak at 103.9 ppm corresponds to β-D-galactose units linked either to (2-*O*-methyl)-α-L-galactose (C-1 shoulder at 99.5 ppm) or 3-substituted α-L-galactose (C-1 at 101.3 ppm).[22] The signals at 102.3 and 97.8 ppm could be attributed to C-1 of a 2-substituted β-D-galactose linked to 2,3-disubstituted α-D-galactose residue.[23] while the peaks at 104.5 (shoulder) and 99.7 ppm would derive from C-1 of a 4-substituted β-D-galactose linked to 2,3-disubstituted α-L-galactose units.[24] The important signal at 104.4 ppm was attributed to β-D-xylose units linked to certain positions of the backbone that could be either C-2 or C-4 of the β-D-galactose units. C-6 of these units was discarded as the linkage position based on the methylation analysis (Table 3), in spite of the data published before.[25,26] On the other hand, C-1 of a β-D-xylose linked to C-3 of an α-L-galactose unit was reported at 101.7 ppm.[27] A signal at 101.7 ppm was also attributed to C-1 of a β-D-xylose unit linked to C-4 of a β-D-galactose residue of the agarans extracted from *Georgiella confluens*.[22] Hence, it is more likely that for the galactans of *K. alvarezii*, the position of substitution is C-2.

F3s was submitted to an analytical alkaline treatment in two different conditions: those for cyclization of a porphyran[28] and those for a μ/ν-carrageenan.[18] In both cases, the monosaccharide composition of the treated product (not shown) was the same, suggesting that most of the β-D-galactose units linked to α-galactose 6-sulfate are either not sulfated or are 4-sulfated.[18,28] The latter conditions were used for the preparative alkaline treatment, obtaining F3sT (70.2% yield). The alkaline solution was neutralized and then dialyzed; a precipitate was obtained (T1). Fractionation of the remaining product with potassium chloride gave two gelling fractions, T2 and T3 and one soluble in 2.0 M KCl, T4. Composition and enantiomeric analysis of T2 and T3 showed that their major sugars were those corresponding to a κ/ι-carrageenan, but that they also contained significant quantities of L-sugars (Table 4). These results are in agreement with the low optical rotation of T2. Methylation analysis of this fraction (Table 5) showed the defining units of κ-carrageenan, together with a small amount of 2-substituted 3,6-anhydro-D-galactose.

The $^{13}$C NMR spectrum of T2 (not shown) showed important signals corresponding to a κ-carrageenan. Small signals at 98.4 and 97.8 could be attributed to C-1 of 3,6-anhydro-L-galactose units,[29] while a small peak at 92.4 ppm corresponds to C-1 of 3,6-anhydro-D-galactose 2-sulfate units of an ι-carrageenan.[20]

T1 was constituted by major amounts of galactose (ratio D-/L- 1:0.8), and xylose, together with small quantities of 2-*O*-methyl-L-galactose, 6-*O*-methyl-D-galactose and glucose (Table 4). Permethylation of T1 was only

**Table 4.** Yields and analyses of the fractions obtained by alkaline treatment of F3s and further fractionation with potassium chloride

| Fraction | Range of precipitation (M, KCl) | Yield[a] (%) | Carbohydrates (%) | Sulfate as $SO_3K$ (%) | Monosaccharide composition (mol%) | | | | | | | | | $[\alpha]_D$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | D-Gal | L-Gal | 2-Me-L-Gal | 3-Me-L-Gal | 6-Me-D-Gal | D-AnGal | L-AnGal | D-Xyl | D-Glc | |
| T1 | Insol.[b] | 14.8 | 24.0 | 10.0 | 39.4 | 31.2 | 2.3 | — | 3.5 | 3.6 | 3.0 | 12.9 | 4.1 | n.d.[c] |
| | | | | | 47.5[d] | 37.6 | 2.8 | — | 4.2 | 4.3 | 3.6 | | | |
| T2 | 0.1–0.2 | 18.2 | 51.6 | 31.8 | 43.9 | 4.5 | — | — | 1.0 | 45.7 | 1.7 | 1.4 | 1.8 | +20.0 |
| T3 | 0.4–0.6 | 2.7 | n.d. | 31.1 | 47.0 | tr[e] | — | — | 3.1 | 34.0 | 12.5 | 1.6 | 1.8 | n.d. |
| T4 | 2.0[f] | 40.0 | 48.0 | 19.3 | 29.0 | 14.2 | 6.1 | 2.9 | 2.8 | 5.3 | 4.6 | 18.9 | 16.2 | −40.0 |
| | | | | | 44.6[d] | 21.9 | 9.4 | 4.5 | 4.3 | 8.2 | 7.1 | | | |

[a] Yields are given for 100 g of treated F3s.
[b] Insoluble in water.
[c] n.d. = not determined.
[d] Composition of the agaran backbone, supposing that xylose and glucose are lateral chains or contaminating polysaccharides.
[e] Percentages lower than 1% are given as trace (tr).
[f] Soluble in 2 M KCl.

**Table 5.** Composition of partially methylated monosaccharides produced by permethylation and hydrolysis of T1, T2, and T4

| Monosaccharide[a] | Assignment | T1 | | T2 | | T4 | |
|---|---|---|---|---|---|---|---|
| | | a[b] | b[c] | a[b] | b[c] | a[b] | b[c] |
| 2,3,6-Gal | D; L | 7.4 | 9.4 | — | — | 13.3 | 17.1 |
| 2,4,6-Gal | G | 21.1 | 26.9 | — | — | 8.0 | 11.1 |
| 2,6-Gal | G4S; D3S; L3S | 12.9[d] | 15.5[e] | 46.7 | 46.7 | 19.1[f] | 25.3[g] |
| 4,6-Gal | G2S | 8.3 | 10.5 | — | — | 7.8 | 10.5 |
| 6-D-Gal | G2,4S; D2,3S; L2,3S | 6.9 | 8.9 | — | — | 14.0[h] | 19.2[i] |
| 4-Gal | G2,6S | 2.3 | 3.2 | — | — | 2.3 | 3.2 |
| 2-AnGal | DA; LA | 17.1 | 21.9 | 46.3 | 46.3 | 4.9 | 6.8 |
| AnGal | A2S | 2.9 | 3.7 | 7.0[j] | 7.0 | 4.9 | 6.8 |
| 2,3,4,6-Gal | Galtnr | tr[k] | | — | | 3.6 | |
| Gal | | 1.4 | | — | | 2.5 | |
| 2,3,4-Xyl | Xyltnr | 3.9 | | tr | | 4.0 | |
| Xyl | | 3.6 | | — | | 3.0 | |
| 2,3,4,6-Glc | Glctnr | 4.1 | | — | | 3.6 | |
| 2,3,6-Glc | →1)-Glc-(4→ | 6.8 | | — | | 4.0 | |
| Glc | | — | | — | | 5.0 | |

[a] Small percentages of 2,3-Gal and 2-D-Gal were also detected in all reactions.
[b] Mol% of monosaccharides in the whole product having methyl groups at the position indicated.
[c] Mol% of monosaccharides in the backbone having methyl groups at the position indicted.
[d] 5.2% and 7.7% in the D- and L-series, respectively.
[e] 6.2% and 9.3% in the D- and L-series, respectively.
[f] 8.1% and 11.0% in the D- and L-series, respectively.
[g] 10.8% and 14.7% in the D- and L-series, respectively.
[h] 12.2% and 1.8% in the D- and L-series, respectively.
[i] 16.8% and 2.4% in the D- and L-series, respectively.
[j] In D-configuration.
[k] Percentages lower than 1% are given as trace (tr).

achieved by a procedure similar to that applied to F3i. The structure of T1 (Table 5) is similar to that of F3i. The most significant difference between these fractions is the important percentage of 3,6-anhydro-2-*O*-methylgalactose detected between the partially methylated monosaccharides that should arise from α-galactose 6-sulfate residues present in F3s that were not cyclized during the alkaline treatment, but reacted during the methylation procedure. Accordingly, these residues would be in the L-configuration.[18,28] Comparable amounts of 2,3,4,6-tetra-*O*-methyl- and 2,3,6-tri-*O*-methyl-glucose suggest short chains of 4-linked glucose units linked to the galactan backbone, while the presence of wholly methylated and non-methylated xylose showed the previously mentioned 'all or none' pattern.

The $^{13}$C NMR spectrum of T1 showed a very low signal-to-noise ratio, even though the sample was previously sonicated, and it was useless for structural analysis.

T4 contains all the products that, after alkaline treatment, did not insolubilize by interaction with potassium chloride (Fig. 1). It contains similar amounts of D- and L-galactose, suggesting that it is a mixture of agaran-type polysaccharides, in agreement with its negative optical rotation. Data from methylation analysis of T4 (Table 5) were analyzed, as produced from an agaran backbone with branches of xylose, glucose, and galactose residues. Accordingly, it comprises a ratio of backbone monosaccharidic units:substituents of 1:0.8, from which approximately 60% are sulfate groups and the other substituents are sugar side chains (as estimated from the total carbohydrate content and data from Tables 4 and 5). The main constituent units of the backbone are: nonsubstituted β-D-galactose units, β-D-galactose units substituted on C-4 and substituted on C-2 in similar quantities. The 4-linked α-galactose units are partially substituted on C-3; 3,6-anhydro-α-galactose and 3,6-anhydro-α-galactose substituted on C-2 also contribute in minor quantities to the structure of T4. The 6-*O*-methylgalactose detected between the methylated monosaccharides, mostly in the D-configuration (Table 5), could arise from 2,4-disubstituted β-D-galactose units or 2,3-disubstituted α-D-galactose residues, or from both. Besides, some of the α-L-galactose residues are methylated on C-2, and some on C-3; on the other hand, some of the β-D-galactose units are methylated on C-6. There are significant quantities of galactose and xylose side chains as well the presence of similar quantities of terminal and 4-linked glucose units suggests disaccharidic side chains.

The $^{13}$C NMR spectrum of T4 is complex (Fig. 3); however, with the aid of the analysis outlined above, it was partially interpreted. The most important signals of the spectrum are those of the side chains, possibly

due to the higher mobility of these units. The signal at 104.3 ppm is small relative to that of 66.0 ppm, suggesting that the single stubs of β-D-xylose are linked at least to two different positions; the signals corresponding to C-2–C-5 are clear in the spectrum.[25] The signals arising from nonsubstituted and 4-substituted β-D-glucopyranose units[25] are in agreement with the presence of β-D-glucopyranosyl(1→4)-β-D-glucopyranose side chains.

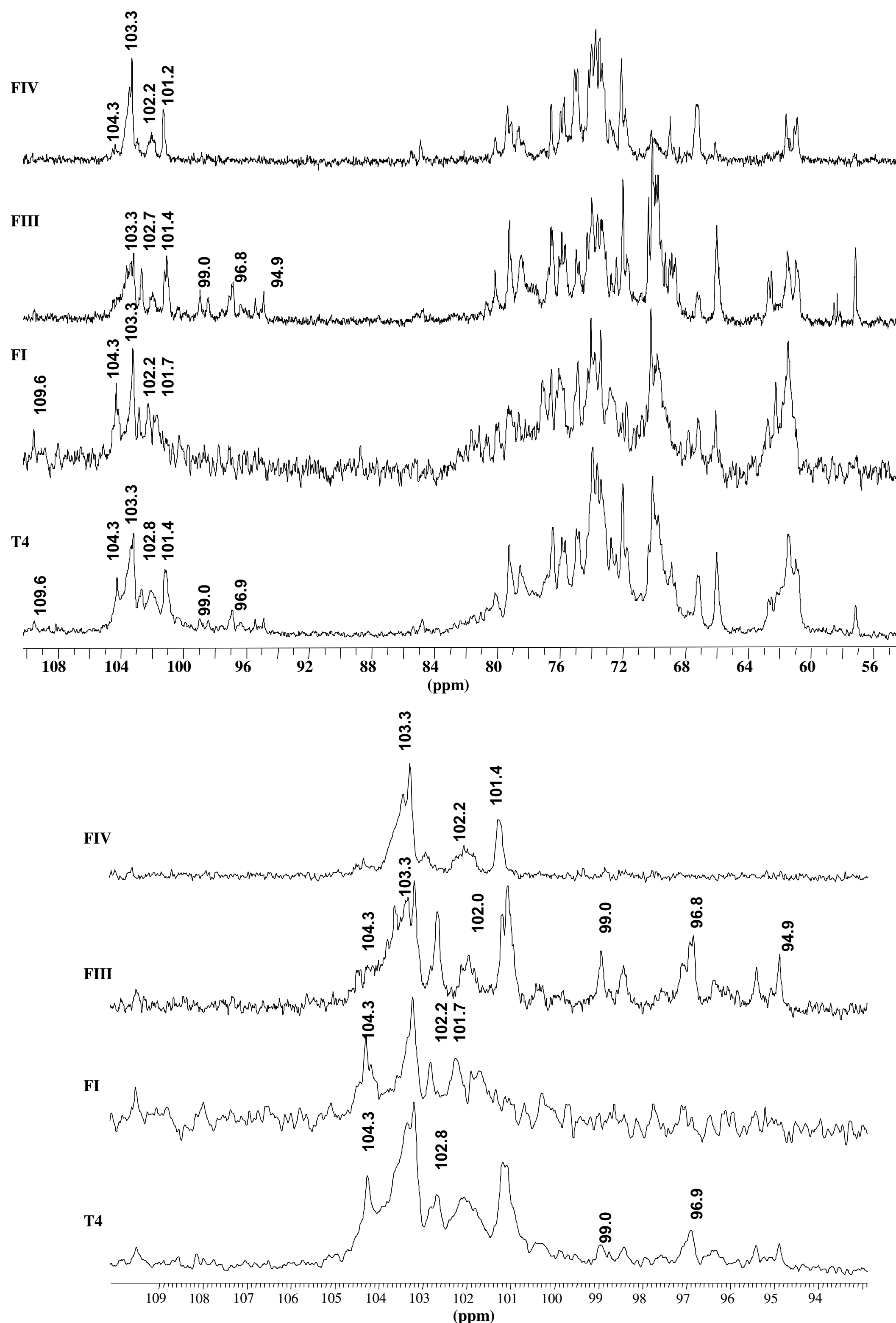


**Figure 3.** $^{13}$C NMR spectra of FIV, FIII, FI, and T4.

The signals at 109.6, 81.1, 78.6, 84.9, 71.8, 62.8 ppm that could correspond to C-1–C-6 of single stubs of β-D-galactofuranose[25] are also clear. Nevertheless, specific ions for these units were not found in the mass spectra of methylated galactoses obtained from permethylated T4. The signal of C-6 of these units should appear, according to the literature,[25] at 63.6 ppm; however, there is no signal in this clean region of the spectrum. Hence, the signal at 62.8 ppm was tentatively assigned to this carbon atom. The signals assigned to the backbone structures are indicated in Table 6.

Desulfation of T4 gave T4D (74.9% yield, 5.4% of sulfate). The monosaccharide composition of T4D was similar to that of T4, with a small increase in the D-galactose content and a decrease in the 3,6-anhydrogalactose content, possibly as the result of a small degradation. Methylation analysis of T4D, which was very difficult to achieve, showed an important increase in the percentage of 2,4,6-tri-*O*-methylgalactose (11.0% → 30.6%) and a concomitant decrease in the percentage of 2,6-di-*O*-methyl- (35.7 → 24.8%) and 6-*O*-methyl-galactose (19.2% → 15.8%) in the agaran backbone. These results indicate that the sulfate groups were linked to C-4 of non-(major) or 2-substituted (minor) or to C-2 of a nonsubstituted 3-linked β-D-galactose units and to C-3 of non-(major) or 2-substituted (minor) 4-linked α-L-or α-D-galactose residues.

The ^{13}C NMR spectrum of T4D (not shown) was ill defined and with a small signal-to-noise ratio. However, as expected, it is similar to that of the parent polysaccharide. Absorptions in the anomeric region were detected in the range 109.5–100.9 ppm, as in the spectrum of T4, but not at higher fields, in agreement with the lesser amounts of 3,6-anhydrogalactose. The signal at 67.2 is very clear, suggesting that the substituent on C-3 of the α-L-galactose units is not sulfate, but mostly a sugar side chain.

Ion-exchange chromatography of T4 (Fig. 4) produced seven fractions (FI–FVII), which were eluted with increasing concentrations of sodium chloride, according to their sulfate content. Yields, analyses, composition of these fractions are given in Table 7. Although all the fractions contained D- and L-galactose, and 3,6-anhydro-D- and-L-galactose, the relative amounts of these sugars are very variable. However, the ratio D/L-galactose is quite constant in FI–FV, but the L-sugar content is much lower in FVI and FVII. Significant amounts of xylose and glucose and small-to-trace quantities of monomethylated galactose units were also present, with the exception of FII, where the percentage of 2-*O*-methyl-L-galactose is important, and of FVI with 10.4% of 6-*O*-methyl-D-galactose.

Fractions FI, FIII, FIV, and FV showed a similar qualitative scheme of methylation, also similar to that

**Table 6.** Signal assignment of the ^{13}C NMR spectrum of T4

| Unit | Ref. | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 | OMe |
|---|---|---|---|---|---|---|---|---|
| *Side chains* | | | | | | | | |
| T β-D-Glucopyranose | | 103.4 | 74.0 | 75.7 | 70.4 | 76.5 | 61.5 | |
| 4-Linked β-D-glucopyranose | 25 | 103.3 | 73.7 | 75.0 | 79.3 | 75.7 | 61.0 | |
| T β-D-Xylopyranose | 25 | 104.3 | 74.0 | 76.5 | 70.2 | 66.1 | | |
| | 26 | 101.7 | | | | | | |
| *Backbone* | | | | | | | | |
| Main units | | | | | | | | |
| 3-Linked β-D-galactose | | 103.9 (sh.)[a] | 70.1 | 81.5 | 69.1 | 75.9 | 61.5 | |
| 3-Linked β-D-galactose 4-sulfate[b] | | 103.9 (sh.) | 70.4 | 79.3 | 77.1 | 75.0-7 | 61.5 | |
| 4-Linked α-L-galactose | 29 | 101.2 | 69.8 | 71.0 | 79.0 (sh.) | 72.5 | 61.4 | |
| 4-Linked α-L-galactose 3-sulfate | 22 | 101.1 | 67.3 | 79.0 (sh.) | 76.9 | 71.8 | 61.4 | |
| 4-Linked 2-*O*-methyl-α-L-galactose | 22 | 99.0 | 79.3 | 69.8 | 79.3 | 72.8 | 61.4 | 58.6 |
| Minor units (tentative) | | | | | | | | |
| 3-Linked β-D-galactose | | 102.8 | | | | | | |
| 4-Linked 3,6-anhydro-α-L-galactose | 29 | 98.5 | | | | | | |
| 3-Linked β-D-galactose 2-sulfate | | 102.1 | | | | | | |
| 4-Linked α-L-galactose 2,3-disulfate | 23 | 96.4 | | | | | | |
| Carregeenan structures | | | | | | | | |
| 3-Linked β-D-galactose | | 102.8 | | | | | | |
| 4-Linked 3,6-anhydro-α-D-galactose | 20 | 95.0 | | | | | | |
| 3-Linked β-D-galactose 4-sulfate | | 102.8 | | | | | | |
| 4-Linked 3,6-anhydro-α-D-galactose | 20 | 95.5 | | | | | | |

[a] Shoulder.

[b] Substitution with sulfate or side chains.

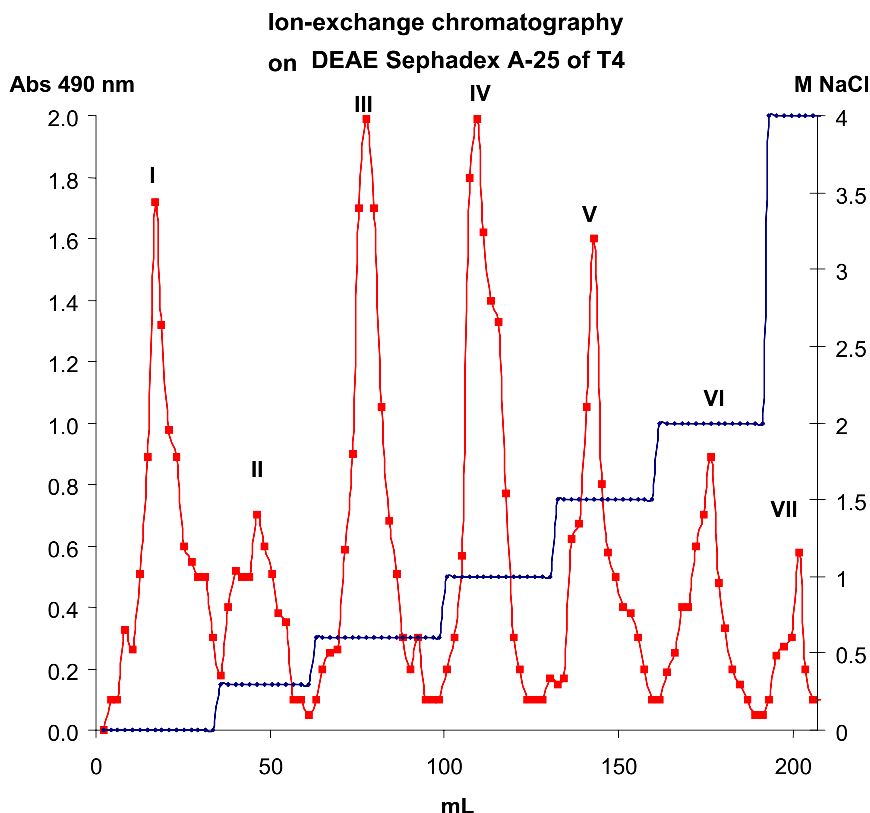


**Figure 4.** Elution pattern of the ion-exchange chromatography of T4.

**Table 7.** Yields and analyses of the fractions obtained from T4 by ion-exchange chromatography on Sephadex DEAE A-25

| Fraction | Eluant solvent M, NaCl | Yield[a] (%) | Sulfate as $SO_3Na$ (%) | Monosaccharide composition (mol%)[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | D-Gal | L-Gal | 2-Me-L-Gal | 3-Me-L-Gal | 6-Me-D-Gal | AnGal | D-Xyl | D-Glc |
| FI | 0.0 | 35.0 | 5.8 | 31.7 | 25.7 | 3.5 | 1.6 | 1.3 | 5.7 | 9.6 | 20.9 |
| FII | 0.3 | 5.0 | 11.9 | 23.9 | 15.1 | 16.0 | 2.8 | tr | 23.4 | 14.4 | 1.8 |
| FIII | 0.6 | 13.8 | 13.4 | 28.7 | 16.9 | 5.1 | 2.3 | — | 36.3 | 7.0 | 2.4 |
| FIV | 1.0 | 12.6 | 15.6 | 32.5 | 18.3 | 1.0 | — | 5.3 | 15.1 | 14.5 | 13.3 |
| FV | 1.5 | 8.0 | 20.6 | 31.9 | 16.3 | 1.0 | — | 4.7 | 16.1 | 12.9 | 17.1 |
| FVI | 2.0 | 8.0 | 21.3 | 60.8 | 4.7 | tr | — | 10.4 | 9.2 | 8.8 | 6.1 |
| FVII | 4.0 | 2.2 | 27.8 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

[a] Yields are given for 100 g of T4.
[b] Man (1.0–3.8%) was also present.

of T4, but with important variations in the amounts of the partially methylated derivatives (Table 8).

The spectra of fractions FI, FIII, and FIV were qualitatively similar to that of their parent compound, but with significant differences in the intensity of the peaks; only these differences are discussed: In FI a major peak at 104.3 ppm was correlative to a major one at 66.1 ppm, while a minor peak at 66.3 ppm is consistent with another minor absorption at 101.7 ppm, showing the existence of two types of xylose side chains linked, possibly, to C-2 and/or C-4 of the β-D-galactose units or C-2 and/or C-3 of the α-L-galactose residues. On the contrary, in FIII a high signal at 101.4 ppm corresponds to a major peak at 66.0 ppm, while a minor one at 104.3 ppm corresponds to a shoulder at 65.9 ppm. The spectrum of FI does not show any absorption corresponding to methyl groups, in agreement with monosaccharide composition (Table 7), while that of FIII shows a peak at 57.1 ppm (3-*O*-methyl-L-galactose) and minor ones at 58.3 and 58.5 ppm corresponding to 2-*O*-methyl-L-galactose residues. In the anomeric zone, at fields higher than 100.0 ppm, FI showed only one small signal at 97.8 ppm, possibly due to C-1 of 2,3-disubstituted α-D-galactose units linked to 2-or 2,4-disubstituted β-D-galactose units.[23] On the contrary, FIII shows in this region, major absorptions at 98.9, 98.4, 97.1 (traces), 96.8 (small), 96.4, 95.4, and 94.9, due to C-1 of 3,6-anhydro-D/L-galactose units[21,29] and to 2,3-disub-

**Table 8.** Composition of partially methylated monosaccharides produced by permethylation and hydrolysis of FI, FIII, FIV, and FV

| Monosaccharide[a] | Assignment[b] | FI | | FIII | | FIV | | FV | |
|---|---|---|---|---|---|---|---|---|---|
| | | a[b] | b[c] | a | b | a | b | a | b |
| 2,3,6-Gal | D; L | 6.1 | 11.1 | tr | tr | 5.0 | 6.5 | — | — |
| 2,4,6-Gal | G | 6.7 | 12.2 | 4.8 | 6.4 | 7.1 | 9.3 | 5.4 | 9.5 |
| 2,6-D-Gal[e] | G4S; D3S | 9.5 | 17.3 | 21.8 | 28.4 | 24.8 | 32.3 | 16.7 | 29.1 |
| 2,6-L-Gal[e] | L3S | 9.5 | 17.3 | 6.0 | 8.0 | 9.9 | 12.9 | 5.0 | 8.8 |
| 6-D-Gal | G2,4S; D2,3S | 6.5 | 11.8 | 1.6 | 2.1 | 7.1 | 9.3 | 2.9 | 5.1 |
| 6-L-Gal | L2,3S | 2.6 | 4.7 | 3.5 | 4.8 | 1.3 | 1.7 | 2.2 | 3.8 |
| 4-Gal | G2,6S | 1.6 | 2.9 | 1.0 | 1.3 | 4.1 | 5.4 | 1.7 | 3.0 |
| 2-D-Gal | G4,6S; D3,6S | tr | tr | 1.6 | 2.1 | 2.1 | 2.7 | 2.4 | 4.2 |
| 2-AnGal | DA; LA | 10.6 | 19.4 | 31.9 | 42.3 | 10.8 | 14.1 | 14.2 | 24.8 |
| AnGal | DA2S | 1.8 | 3.3 | 3.1 | 4.6 | 4.4 | 5.8 | 6.7 | 11.7 |
| 2,3,4,6-Gal | Galtnr | 5.7 | | 3.0 | | 3.5 | | 7.2 | |
| Gal | | 1.0 | | 2.1 | | 1.8 | | 1.9 | |
| 2,3,4-Xyl | Xyltnr | 6.8 | | 5.5 | | 3.5 | | 4.0 | |
| Xyl | | 12.5 | | 6.4 | | 3.3 | | 12.6 | |
| 2,3,4,6-Glc | Glctnr | 7.7 | | 2.2 | | 1.1 | | 6.3 | |
| 2,3,6-Glc | →1)-Glc-(4→ | 1.7 | | tr | | 3.0 | | 1.0 | |
| Glc | | 9.7 | | 5.5 | | 7.2 | | 9.8 | |

[a] Small percentages of 2,3-Gal were also detected in all fractions.
[b] Mol% of monosaccharides in the whole product having methyl groups at the position indicted.
[c] Mol% of monosaccharides in the backbone having methyl groups at the position indicted.
[d] 4,6-Gal was not determined.

stituted D/L-galactose residues[23,24] linked to β-D-galactose residues substituted on different positions. Disaccharidic units, like the latter ones, give absorptions appearing in a broad range, due to different substitution and/or different environment.

## 3. Discussion

### 3.1. The system of galactans of *K. alvarezii*

The system of galactans from *K. alvarezii* (Solieriaceae, Gigartinales) is composed by major quantities of strongly gelling κ-carrageenans, obtained only by hot water extraction, together with a nongelling fraction containing small amounts of a precursor μ-carrageenan and important quantities of agarans and agaran-type DL-hybrid galactans. On the other hand, significant amounts (6% of the milled seaweed) of low-molecular-weight galactans with κ-structure, minor quantities of ι-structure, agarans, and agaran-type DL-hybrids are also biosynthesized by this seaweed.

To study the system of galactans of *K. alvarezii*, it was possible to selectively extract the low-molecular-weight galactans from the milled seaweed with water at room temperature, while the higher molecular weight κ-carrageenans, together with L-galactose-containing galactans were further obtained with hot water. The same type of preliminary fractionation was done with the galactans from *Gymnogongrus torulosus* (Phyllophoraceae, Gigartinales), but not with the galactans of seaweeds belonging to the Gigartinaceae, as they were mostly extracted at room temperature. This different behavior was attributed to a different cellular arrangement between these seaweeds: on one hand, in the two former families there is a compact arrangement of the cells along the multiaxial tallus with a pseudoparenchymatous medulla,[30] while in the latter one there is a filamentous medulla.[31,32]

It is worth noting some differences between the sulfated galactans obtained from these red seaweeds (Gigartinales). In the Solieriaceae, the higher specificity in the site of sulfation, as well as the higher degree of conversion of the precursor units, produce a 'nearly pure' structure and consequently, high yields of strongly gelling carrageenans (κ-carrageenan from the genus *Kappaphycus*[33] ι- from *Eucheuma*[33,34] and β- from *Betaphycus*[34]), precipitating at low concentrations of potassium chloride. Galactans from cystocarpic plants of the Gigartinaceae are wholly extracted at room temperature and they are made up of similar amounts of low-gelling (κ/ι-) and nongelling (partially cyclized μ/ν-) carrageenans, together with small amounts of L-galactose and 3,6-anhydro-L-galactose-rich galactans.[1,2,35] On the other hand, gametophytes of *Gymnogongrus torulosus* (Phyllophoraceae) biosynthesize major quantities of DL-hybrid galactans, together with carrageenans of the κ-family with predominance of ι-structure.[4]

### 3.2. The raw extract and the commercial κ-carrageenan

The raw extract (C) obtained with hot water from *K. alvarezii*, after eliminating the fraction obtained at

room temperature, showed clear differences with the commercial sample of κ-carrageenan (K) obtained from the same seaweed, namely: (a) it was soluble in 75% 2-propanol; (b) it had much lower number-average molecular weight; (c) it contained larger amounts of L-galactose units; and (d) it also contained higher quantities of xylose and glucose (Table 1). The most significant preparative differences between both samples were that the commercial sample (K) was extracted directly with hot water from a seaweed previously treated with alkali.[10] On the basis that the alkaline medium could not only cyclize the precursor units but could also release and/or degrade small agaran-type fragments, eventually separated and lost during the 2-propanol precipitation or in the further dialysis, C was submitted to a short alkaline treatment producing C′ (Table 1). This product, which was insoluble in 75% 2-propanol, had higher number-average molecular weight and a lower percentage of L-galactose. These characteristics are similar to those of the commercial sample K and are consistent with the above hypothesis of complex formation.

Different types of carrageenans and agarans form complexes that precipitate at narrow ranges of potassium chloride concentrations[4,35–39] and cannot be separated, that is, by anion-exchange chromatography[2] but are readily fractionated after alkaline treatment.[35,37–39] Complexes of the raw κ-carrageenan with small fragments of agarans and/or agaran DL-hybrids (see later) in C would protect κ-carrageenan from coalescence and precipitation in 75% 2-propanol, lower its number-average molecular weight, and increase its content of L-galactose, xylose, and glucose.

### 3.3. Counterions

The raw extract C contains major amounts (77.9%) of divalent ($Ca^{2+}$ and $Mg^{2+}$) cations (Table 2) in agreement with the known capacity of the seaweeds to concentrate these salts.[40] These counterions were maintained in high percentages in fractions (Table 2) that were obtained through fractionation procedures involving contact with massive amounts of potassium chloride or hot and concentrated sodium hydroxide solutions or sequences of both. It is worth noting that permethylation of the polysaccharides involves as the first step the preparation of the triethylammonium salts by ion-exchange chromatography; nevertheless, the methylated derivative contains as much inorganic cations as the parent polysaccharide (Table 2). Similar difficulties to exchange the divalent counterions were found in a commercial sample of κ-carrageenan from *Eucheuma cottonii* (usual commercial name for *K. alvarezii*), which originally contained $K^+$ (0.17 equiv/%) and $Ca^{2+}$ (0.12 equiv/%, ~40% of $Ca^{2+}$). After cation-exchange chromatography at 80 °C on a $K^+$ pre-regenerated resin, it showed $K^+$ (0.22 equiv/%), but it still contained ~15% of $Ca^{2+}$ (0.04 equiv/%).[41] To obtain the nearly pure sodium form of another commercial κ-carrageenan (Deltagel 379, Quest International), it was necessary to treat the polysaccharide with hot 500 mM NaCl, 50 mM NaOH, and 50 mM EDTA solution, further twice redissolve in NaCl solution and precipitate with 2-propanol.[42] On the other hand, it was shown that the mixed ionic presence of both $K^+$ and $Ca^{2+}$ was crucial for the gel formation of κ-carrageenans.[41]

Consequently, $Ca^{2+}$ binding in these polymers is not a simple function of availability of anion binding sites, but of a more complex $Ca^{2+}$–polysaccharide interaction, as was shown in some other cases,[43,44] leading to the formation of interchain complexes (see later).

### 3.4. Carrageenans

The gelling (κ-carrageenan) fractions (F1 and F2) or those (partially cyclized μ-carrageenan) gelling after alkaline treatment (T2 and T3) of *K. alvarezii* contain significant amounts of agaran-like structures and little glucose and/or xylose (Tables 1 and 4), which are lost during the methylation procedure. Although important quantities of potassium salts were used in the isolation of F1, it still maintained a high percentage of $Ca^{2+}$ (Table 2). Two hypotheses are consistent with all these facts: (a) these fractions are not 'pure' carrageenans, but carrageenan-type DL-hybrids containing small amounts of agaran structures, which are degraded and lost in alkaline medium or (b) they are 'pure' carrageenans complexed with low-molecular-weight agaran-type fragments, similar to those extracted from the same seaweed at room temperature.[17] These complexes would be partially broken by alkaline treatment or formed again during the freeze-drying of the samples, as part of the agaran fragments could remain in the dialysis bag, due to the small pore size used (MW cutoff 3.5 kD). The complex would be again broken, and the agaran fragments would be degraded and finally lost during the methylation procedure.

The second hypothesis looks, at the moment, more reliable, considering that in fractions isolated by potassium chloride fractionations, carrageenans are usually complexed with small amounts of KCl-soluble galactans[1–4,35] and that the complexation decreases after the alkaline treatment.[45,35]

### 3.5. Agarans

Some of the agarans spontaneously retrograded during fractionation of the raw extract (F3i) or after alkaline treatment of the soluble fraction F3s (T1). F3i and T1 are agarans with a great structural dispersion, as the

3-linked β-D-galactose units are substituted in part on C-4 or on C-2, or disubstituted on C-2 and C-4. The major B-units are 4-linked α-L-galactose substituted on C-3, nonsubstituted L-galactose D/L-galactose substituted on C-2 and C-3 and 3,6-anhydro-L- and -D-galactose and their derivative substituted on C-2, together with small amounts of other less important units (Tables 3 and 5). These insoluble products are almost 'pure' agarans (90–93%, of agaran structure, Tables 1 and 4), and have characteristics similar to those of the insoluble fraction, obtained in comparable situation as T1, from the alkali-treated, KCl-soluble fraction of *G. torulosus* (F3T1),[4] suggesting that these insoluble agarans are usual minor components of the polysaccharide system of the carrageenophytes.

The alkali-treated product gave, after precipitation of T1, two gelling fractions (T2 and T3, Table 4) and a soluble one (T4). T4 is also mostly an agaran with a substitution pattern similar to that of T1 and F3i, in spite of its different solubility behaviors. T4 contains major amounts of agaran constituent sugars, and the linear backbone is heavily substituted with β-D-xylose and D-glucose-disaccharidic side chains, and in lesser amounts, with galactose (Table 4). All the sulfate groups are linked either to C-2 or to C-4 of the β-D-galactose units (major) and to C-3 of the 4-linked α-L-galactose residues (minor) (Table 5). Ion-exchange chromatography of T4 produced seven fractions (Table 7) separated as a function of their sulfate content. All of them contained both D- and L-galactose residues, although their molar ratio was similar in the first three fractions (FI–FIII) and very different, with high predominance of D-galactose, in the latter ones (FVI, FVII). In these fractions there is also a heavy substitution with xylose, glucose, and galactose (Tables 7 and 8). Monomethylated galactose units are present in all the fractions, but 2-*O*-methyl-L-galactose is predominant in FII, while an important percentage of 6-*O*-methyl-D-galactose is present in FVI, which has a carrageenan-like structure. These units are usually found in agarans,[46] generally with methyl groups at positions 2 or 6, while the presence of 3-*O*-methyl-D-galactose was only mentioned for a few agarans.[4,7,21,47–49] The major fractions (FI, FII, FIV, and FV) showed the same structural units as T4, but in different proportions, in agreement with the structural dispersion of T4.

### 3.6. Side chains

Several fractions (F3s, F3i, T1, T4, and FI–FVII) contain small to significant amounts of xylose, glucose, and galactose linked to C-2 and/or C-4 of the 3-linked β-D-galactose units and C-3 and/or C-2 and C-3 of the 4-linked α-D- and/or α-L-galactose residues, as short chains or single stubs. Those units, which appear methylated in the permethylated agarans (Tables 3 and 5) do not arise from contaminating xylans, glucans, or galactans, as the ratio terminal-to-internal units is always very high. These units have been found in several agarans[22,50–52] or agaran-type DL-hybrids.[1,4,5,17] In the agarans from *Bostrychia montagnei* (Ceramiales), terminal β-D-xylose units are linked to C-6 of the β-D-galactose units and C-3 of the α-L-galactose residues.[50] They have also been found linked to C-6 of the β-D-galactose units in the 'corallinans'.[51,52] In the agarans from *G. confluens* (Ceramiaceae, Ceramiales),[22] they are linked to C-4 of the β-D-galactose units.

The anomeric configurations of D-glucose single stubs and side chain oligosaccharide units are not certain. Absorption at 100.8 ppm was found in the $^{13}$C NMR spectrum of a fraction soluble in 2 M KCl of the L-galactose containing galactans from *G. skottsbergii*, and it was attributed to floridean starch.[1] The presence of glucose in the main chain of the galactan was postulated in the soluble fraction obtained from the tetrasporic stage of *Sarcothalia crispata* (formerly *I. undulosa*).[35] However, only in the cases of some fractions of the room temperature-extracted products of *K. alvarezii*[17] and *S. crispata*[35] where glucose was the major monosaccharide (54.8–84.7%), the presence of a linear glucan with α-(1→6) linkages and trace amounts of α-(1→4) linkages was evident from methylation analysis and $^{13}$C NMR spectroscopy; but, in the case of *K. alvarezii* it was attributed to a contaminant polysaccharide.

A disaccharide, obtained by acid hydrolysis of the sulfated polysaccharides isolated from *Lomentaria catenata* (Rhodymeniales),[53] was attributed to α-D-glucopyranosyl-(1→3)-D-galactose, based on methylation analysis and an $[\alpha]_D$ +40 ($c$ 0.55, $H_2O$). However, for this disaccharide obtained by synthesis, an $[\alpha]_D$ +105 ($c$ 9, $H_2O$) was observed.[54] These polysaccharides are not carrageenans or even carrageenan DL-hybrids, but agaran DL-hybrids, and the lateral chains of glucose would be inserted in the minor carrageenan block of an agaran DL-hybrid. If this is true, the structure of the carrageenan block in the agaran DL-hybrid would be somewhat different from that of the usual carrageenans.

On the other hand, the water-insoluble fibrillar cell wall of *K. alvarezii* contains not only cellulose, but also a (1→3) (1→4) β-D-glucan sulfated on C-6, among other polysaccharides.[55]

In the spectrum of T4, only signals in the range 104.4–101.1 ppm that could be due to C-1 of β-D-glucose units are important in the anomeric region, and the signals at 103.4 and 103.3 (Table 6) were tentatively attributed to C-1 of terminal and 4-linked β-D-glucose units, respectively.

Absorptions at 106.0–109.6 ppm in some of the $^{13}$C NMR spectra of the fractions suggest that some of the galactose residues could be β-D-galactofuranose;

however, the specific fragments of the mass spectrum of the 2,3,5,6-tetra-*O*-methylgalactose could not be found by GC–MS analysis of the mixture of partially methylated alditol acetates obtained from the methylated fractions. However, they could have been lost during the methylation procedure. Terminal galactose units were found in the galactan sulfate from *B. montagnei*[50] linked to the same positions as xylose, and in the soluble fractions of the tetrasporic[3] and cystocarpic[1,2,35] *Gigartina skottsbergii* and *Sarcothalia crispata*.

In the 'all or none' methylation scheme of these sugars (Tables 3, 5, and 8), significant amounts of free xylose, glucose, and galactose appear. They could be ascribed to zones of the side chains blocked by the carbohydrate–carbohydrate zipper-type interactions[56] (see later). Alternatively, they could be components of low-molecular-weight fibrillar xylans, glucans, and galactans, but in that case, they would be expected to produce submethylation schemes.

### 3.7. Carrageenan–agaran and agaran–agaran interactions

Carrageenan molecules have few intermolecular interactions,[36] and this fact has been the basis of the potassium chloride fractionation procedure. To the best of our knowledge, the only clear carrageenan–carrageenan interaction is the cosolubilization of λ-carrageenans with μ/ν-carrageenans in 2 M KCl.[37–39,57] On the other hand, the retention of small, but significant amounts of soluble L-galactose-containing galactans by KCl-precipitated κ/ι-carrageenans from *G. skottsbergii*[2] and *K. alvarezii*[17] and *G. torulosus*,[4] which were reduced or disappear after methylation, is consistent with the aggregation of these carrageenans with low-molecular-weight fragments of agarans, similar to those extracted with water at room temperature from *K. alvarezii*[17] and *G. torulosus* (unpublished results). This complexation could be reduced or eliminated by alkaline treatment. On the contrary, agarans are readily prone to complexation.[58]

It is known that seaweeds concentrate $Ca^{2+}$ and $Mg^{2+}$ salts,[40] and these cations remain linked to the polysaccharides after the massive treatment with potassium salts (potassium chloride fractionation) and/or hot sodium hydroxide (alkaline treatment) or both (Table 2). $Ca^{2+}$ showed high affinity for sulfated L-galactans from different ascidian species,[59] which increases with increasing amounts of nonsulfated sugar branches in the molecule. $Ca^{2+}$ affinity in the ascidian L-galactans increases as the mean distance between charged groups decreases, suggesting that the $Ca^{2+}$ binding requires more than one sulfate group per each calcium atom. Similar results were reported for glycosaminoglycans.[60] In this case, it has been shown that sulfate groups are capable of binding $Ca^{2+}$ with a stronger affinity than that expected from simple salt formation. All these results indicate a complex $Ca^{2+}$–polysaccharide interaction.

Methylation schemes of the agarans from *K. alvarezii* (Tables 3 and 5) showed a methylated agaran backbone with branches of xylose, glucose, and galactose, which are either totally methylated or free from methoxy groups. This 'all or none' methylation pattern of the branches is consistent with a 'zipper' complexation of side chains.[56]

Thus, the galactans from *K. alvarezii* could form complexes through two different mechanisms, namely: (a) a divalent calcium cation bridging two sulfate groups from different molecules, being the arrangement stabilized by further complexation of the cation and (b) interaction of the 'zipper' type between the side chains of different agaran molecules.[43] Both mechanisms would be complementary, as the $Ca^{2+}$ bridging of sulfate groups from different molecules takes them close enough, to allow the low-range forces of the 'zipper' model to operate.

## 4. Conclusions

The red seaweed *K. alvarezii* biosynthesizes a complex system of sulfated galactans as matrix products, mainly composed by κ-carrageenans of high- and low-molecular-weight and small amounts of μ-structures. A significant part of this polysaccharides system is composed by sulfated agarans, and possibly, sulfated agaran–DL-hybrid galactans. The complexity of the system is enhanced by formation of carrageenan–agaran and agaran–agaran complexes, possibly through two different but complementary mechanisms.

## 5. Experimental

### 5.1. Material

Samples of *K. alvarezii* were kindly provided by Soriano S.A. (Chubut, Argentina). A voucher specimen (B.A. 35,708) has been deposited in the herbarium of the Museo de Ciencias Naturales Bernardino Rivadavia (Buenos Aires, Argentina). Thalli of *K. alvarezii* were washed with filtered seawater and analyzed for epiphytic and epizoic contaminants in a Nikon AFX-II macroscope (Nikon, Japan). For checking the existence of endophytes, cross-sections were obtained manually with a single-edge razor blade and observed on a Zeiss Axioplan microscope (Oberkochen, Germany). Commercial κ-carrageenan (c-1263) was obtained from Sigma Chemical Co (K), purified by reprecipitation at 0.125 M KCl and further by dialysis (K′).

### 5.2. General

For GC, alditol acetates were obtained by reductive hydrolysis and acetylation of the samples.[61] Sulfate

was determined turbidimetrically.[62] The number-average molecular weight ($M_n$) was estimated by the method of Park and Johnson[63] based on the determination of end-chain reducing units. Optical rotations (Na D-line) were measured in a Perkin–Elmer 343 polarimeter, using 0.2–0.4% solutions of the polysaccharides in $H_2O$. The total carbohydrate content was estimated by the phenol–sulfuric acid method[64] without previous hydrolysis of the polysaccharide.

The ratio D:L-galactose and the configuration of monomethylated galactoses, except 2-*O*-methylgalactose, were estimated by the method of Cases et al.[65] through their diastereomeric acetylated 1-deoxy-1-(2-hydroxypropilamino)alditols. The ratio of 2-*O*-methyl-D:L-galactose was determined by derivatization to the diastereomeric acetylated 1-deoxy-1-(1-phenylethylamino)alditols.[65] The ratio of 3,6-anhydro-D:L-galactose was estimated by the method of Errea et al.[66] involving an oxidative hydrolysis to obtain the aldonic acids, with further conversion to the acetylated diastereomeric *sec*-butyl esters. The ratio of 2,6-di-*O*-methyl-D:L-galactose were 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.[67]

### 5.3. Extraction

The residue obtained after exhaustive extraction with $H_2O$ at room temperature (33.0 g),[17] was extracted with $H_2O$ (1.65 L) at 90 °C with mechanical stirring for 5 h. The residue was removed by centrifugation, and the supernatant was poured into three vols of 2-PrOH, whereby the polysaccharide did not precipitate. The supernatant was concentrated, dialyzed (molecular weight cutoff 6.0–8.0 kDa), and freeze-dried, yielding the soluble fraction C (21.6 g).

### 5.4. Alkaline treatment of C and precipitation of C′ with potassium chloride

The sample (196 mg) was dissolved in $H_2O$ (98 mL), and $NaBH_4$ (9.8 mg) was added. After 24 h at rt, 3 M NaOH was added (49 mL) with a further quantity of $NaBH_4$ (9.8 mg). The solution was heated at 80 °C for 20 min, and then cooled to rt, dialyzed (molecular weight cutoff 12.0–14.0 kDa), concentrated, and poured into three vols of 2-PrOH, whereby the polysaccharide precipitated. The product was pressed between filter paper and dried by solvent exchange (EtOH and $Et_2O$) and finally in vacuo, obtaining C′ (160 mg).

C′ (95 mg) was dissolved in $H_2O$, and finely divided KCl was added to give a 0.125 M solution. The precipitate was separated by centrifugation and dialyzed and lyophilized to give C″ (70 mg).

### 5.5. Fractionation of C and F3sT with potassium chloride

The polysaccharide (0.33–7.0 g) was dissolved in $H_2O$ (0.13–2.8 L, 0.25%). 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 cutoff for F1–F3, 6.0–8.0 kDa; for T2–T4, 3.5 kDa), concentrated, and freeze-dried.

### 5.6. Desulfation of T4

Desulfation of fraction T4 (40 mg) was carried out as reported previously[68] by conversion to its pyridinium salt (yield 90%), which was then treated with chlorotrimethylsilane in anhydrous pyridine at 100 °C for 8 h. $H_2O$ (2 mL) was added, and the mixture was dialyzed against tap $H_2O$, 1 M NaOH, distilled $H_2O$, and then lyophylized, obtaining T4D (yield 74.9%).

### 5.7. Insolubilization of F3i and alkaline treatment of F3s

F3 (665 mg) was dissolved in $H_2O$ (332 mL), and two products were obtained: an insoluble product, F3i (195 mg), which was centrifuged off and freeze-dried, and a soluble part, F3s (470 mg). F3s was treated with $NaBH_4$ (20 mg). After 24 h at rt, 3 M NaOH was added (166 mL) with a further quantity of $NaBH_4$ (20 mg). The solution was heated at 80 °C for 3 h and then cooled to rt, dialyzed (molecular weight cutoff 1.0 kDa), concentrated, and freeze-dried to give F3sT.

### 5.8. Alkaline treatment of F3s (porphyran conditions)

F3s (10 mg) was treated as described above, but the solution was heated at 80 °C for 24 h. The percentage of 3,6-anhydrogalactose was determined by GLC of the alditol acetates[61] before and after the treatment and neutralization.

### 5.9. Ion-exchange chromatography of T4

For the analytical fractionation a column ($0.6 \times 2$ cm) was filled with 27 mg DEAE-Sephadex A-25, previously stabilized in $H_2O$. T4 (4 mg) was dissolved in $H_2O$, which was used as first eluant. Then increasing concentrations of NaCl were stepwise applied. The upper concentration was 4 M. Fractions of 0.6 mL were collected, and aliquots were assayed by the phenol–sulfuric acid method.[64] After obtaining blank readings, the eluant was replaced by another with higher concentration of NaCl. For preparative fractionation, a column ($0.9 \times 9$ cm) was filled as indicated above. Fractionation

of T4 (50 mg) was carried out as described before, but in this case, fractions of 1.3 mL were isolated.

### 5.10. GLC

GLC of the alditol acetates, as well as those of the partially methylated alditol and aldononitrile acetates were carried out on a Hewlett–Packard 5890A gas–liquid chromatograph (GLC) 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 μm film of SP-2330. Chromatographic analyses were carried out as described before.[4]

### 5.11. GLC–MS

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

### 5.12. Methylation analysis

The sample (C1, F1–F3s, T2–T4, FI, FIII–FV), was converted into the corresponding triethylammonium salt by ion-exchange chromatography.[61] The dry polysaccharide (5–20 mg) was dissolved in $Me_2SO$ (1–4 mL) and then methylated according to the method of Ciucanu and Kerek.[69] Powdered NaOH (20–80 mg) was added, and the mixture was stirred for 1–2 h at rt. $CH_3I$ (0.2–0.5 mL) was added, and the reaction was allowed to proceed at 25 °C for 1 h. The addition was repeated twice. Water (2 mL) was added to stop the reaction, and the mixture was dialyzed (molecular weight cutoff 1.0–3.5 kD) and lyophilized (yields 80–90%). An aliquot of each methylated sample was derivatized to the acetylated alditols as described for the polysacharides,[61] and another portion was hydrolyzed with TFA for 2 h at 120 °C. The partially methylated sugars were converted into the corresponding aldononitrile acetates.[70]

### 5.13. Solubilization of F3i, T1, and T4D

The sample (~35 mg) was suspended in $H_2O$ (1.5 mL) and sonicated several times (3 × 20 min) with a High Intensity Ultrasonic Processor VC50 (Vibra Cell), working at 20 kHz with a 3-mm probe.

### 5.14. Methylation analysis of F3i, T1, and T4D

The sample (20–15 mg), was solubilized as described before and converted into the corresponding triethylammonium salt (15–11 mg),[61] which was methylated by a modification of the method of Ciucanu and Kerek[69] in order to achieve solubilization: the sample was dissolved and sonicated in a solution of LiCl (15 mg) in $Me_2SO$ (3–4 mL), improving the solubility of the samples, and stirred at 80 °C for 2 h. Finely powdered NaOH (60–20 mg) was added, and the mixture was stirred at 80 °C for 1 h before addition of the methylating reagent. This methylation procedure was repeated (three times) until a constant pattern of methylation was achieved (yields 60–70%).

### 5.15. Preparation of the samples for $^{13}C$ NMR spectroscopy

The sample 20–40 mg was dissolved in 1:1 $H_2O$–$D_2O$ solutions (1 mL) and agitated 24 h at rt. The partially insoluble samples were dissolved as described before. However in F3i, T1, T4, and T4D a precipitate was obtained that represented 5–15% of the original sample.

### 5.16. $^{13}C$ NMR spectroscopy

$^{13}C$ NMR 125 MHz 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 26,000–48,000. Chemical shifts were referenced to internal acetone ($\delta$ 216.2 and 31.1). $\delta$ Values from the literature were corrected considering this standard to allow comparisons.

### 5.17. Cation analysis

Cation analysis was carried out by atomic absorption spectroscopy of the samples in water solutions on a Shimadzu 6800 instrument equipped with an autosampler. Commercial standards (Merk) were used to calibrate.

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