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The system of galactans from *Cryptonemia crenulata* (Halymeniaceae, Halymeniales) and the structure of two major fractions. Kinetic studies on the alkaline cyclization of the unusual diad G2S→D(L)6S

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Abstract—*Cryptonemia crenulata* biosynthesizes a family of DL-hybrid galactans that are based on the classical 3-linked β-D-galactopyranosyl→4-linked α-galactopyranosyl alternating sequence (A-units→B-units). The dispersion of structures in these galactans is based on four factors, namely: (a) the amount and position of substituent groups as sulfate (major), pyruvic acid ketals, methoxyl and side substituents of β-D-xylose and/or β-D-galactose; (b) the ratio galactose/3,6-anhydrogalactose in the B-units; (c) the ratio D-/L-galactoses and 3,6-anhydrogalactoses also in the B-units and (d) the sequence of the diads in the linear backbone. Alkali treatment carried out on the major fraction produced a nearly quantitative formation of 3,6-anhydrogalactose units from precursor units (α-galactose 6-sulfate (major) and α-galactose 2,6-sulfate, minor). Kinetic studies show a rate constant, for the diad G2S-D(L) 6-S, of 1.7×10^4 s^{-1} indicating a reaction faster than in λ-carrageenans but slower than in porphyrans.

Keywords: Sulfated galactans; Chemical structure; Methylation analysis; NMR spectroscopy; Kinetic studies; Seaweed; *Cryptonemia crenulata*; Halymeniales

1. Introduction

Most of the polysaccharides in red seaweeds are sulfated galactans. Their backbone is composed of alternating 3-linked β-D-galactopyranosyl residues (A-units) and 4-linked α-galactopyranosyl (or 3,6-anhydrogalactopyranosyl) residues (B-units). This backbone is further modified by different substitutions. 'Ideal' carrageenans are substituted only with sulfate groups, while agarans carry sulfate, pyruvic acid acetals and methyl and glycosyl groups. The B residues are from the D-series in carrageenans or belong to the L-series in agarans.[1] Intermediate structures due to the interchange of substituent groups or to the presence of D- and L-galactose residues in the B-units [DL-hybrids][2] or both have been found in members of different genera of the order Halymeniales as *Grateloupia*[3–11] *Halymenia*[8,12,13] *Pachymenia*,[14–19] *Phyllymenia*[14,15,20] and *Aeodes*.[21–23] Algae from the Halymeniales biosynthesize complex sulfated galactans. Most of them carry sulfate at C-2 of the β-D-galactose unit and then would formally belong to the carrageenan λ-family. They differ from the λ-carrageenans (repeating disaccharide constituted by 3-linked β-D-galactopyranosyl 2-sulfate and 4-linked α-D-galactopyranosyl 2,6-sulfate) in having variable amounts of 3,6-anhydrogalactose and from the carrageenan group in having significant amounts of L-galactose, 3,6-anhydro-2-*O*-methylgalactose and glycosyl lateral substituents. The presence of carrageenan and agaran components is consistent with its classification as DL-hybrid galactans.

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Cryptonemia crenulata (Halymeniaceae, Halymeniales) has been referred to as producing ι-carrageenans.[24] Nevertheless, preliminary results of later research suggest highly complex polysaccharides.[25] The compositions and the in vitro and in vivo antiherpetic properties of crude extracts and main fractions obtained from them by ion-exchange chromatography were determined.[26] We report now an analytical study of the system of galactans biosynthesized by the seaweed, the structural elucidation and DL-hybrid character of two major fractions, and the kinetic analysis of the alkaline modification of one of them.

2. Results

The milled seaweed was extracted with water at room temperature giving C1 (4.0% w/w of the dry and milled seaweed). Sequentially the algal residue was extracted twice with phosphate buffer (pH 6.5) at 80 °C yielding C2 (6.8% w/w) and C3 (1.1% w/w) as previously reported.[26] The three fractions contained galactose as the major sugar (65.0–70.7 mol %), together with small amounts of 3,6-anhydrogalactose (7.3–10.8 mol %) and its 2-*O*-methyl derivative (5.9–8.3 mol %), 2-*O*-methylgalactose (5.9–8.9 mol %) and pyruvic acid (2.9–7.2% w/w). Small-to-trace quantities of 4-*O*- and 6-*O*-methyl galactose were also found. Glucose was detected in similar amounts (5.8–7.5 mol %) in the cold and hot extracts (Table 1). The major counter ion in C1 and C3 was sodium, but in C2 calcium and magnesium predominate (Table 2).

Fractions C1 and C2 were purified by precipitation with 2 M KCl, in which 94% of C1 and 97% of C2 remained soluble (C1S and C2S, respectively),[26] (fraction C3 was discarded due to the low yield). Yields, analysis and rotations as well as the monosaccharide and enantiomeric compositions are reported in Table 1. The compositional schemes of C1S and C2S are similar to those of the raw extracts with substantial amounts of pyruvic acetal groups and xylose, and the enantiomeric determinations showed both D-(major) and L-(minor) forms for the galactose, 3,6-anhydrogalactose and 2-*O*-methylgalactose.

C1S and C2S were fractionated on a DEAE-Sephacel column to give subfractions C1S-1–C1S-3 and C2S-1–

Table 1. Yield, analyses, optical rotation and monosaccharide composition of the galactans obtained from *Cryptonemia crenulata*

Fraction	Yield (%)	SO_3Na (%)	Pyruvic acid (%)	$[\alpha]_D$	Monosaccharaide units[a] (molar ratio)								
					G+D:L[b,c]	DA:LA[c]	LA2M	D2M:L2M[c]	G4M[c,d]	G6M[c]	Xyl[c]	Glc[c]	SO_3Na
C1[e]	4.0	22.0	6.3	+49.3	1	0.12	0.09	0.12	0.02	—	—	0.08	1.10
C2[e]	6.8	28.0	2.9	+40.5	1	0.16	0.12	0.08	0.02	0.02	—	0.11	1.12
C3[e]	1.1	42.0	1.4	+32.0	1	0.19	0.13	0.07:0.02	—	0.04	—	0.11	2.07
C1S[f]	94.0	26.0	7.9	+25.5	1:0.17	0.18:0.04	0.14	0.10:0.02	0.03	0.02	0.17	0.05	1.19
C1S-1[g]	9.6	22.3	n.d.	+14.0	1	0.21	0.04	0.08	—	0.02	0.16	—	0.89
C1S-2[g]	18.0	26.5	2.2	+12.0	1	0.02	—	0.07	0.04	—	0.05	—	0.71
C1S-3[g]	56.0	16.0	0.7	+12.0	1	0.15	0.09	0.11	—	0.12	0.18	—	0.64
C1S-3D[h]	48.0	2.1	n.d.	n.d.	1	0.05	0.03	0.13	—	0.03	0.02	—	0.06
C2S[f]	97.5	27.7	3.7	+27.0	1:0.17	0.04:0.14	0.11	0.07:0.02	0.02	—	0.06	—	1.12
C2S-1[i]	3.2	20.1	n.d.	+11.0	1	0.12:0.01	0.10	0.10	—	0.01	0.05	—	1.16
C2S-2[i]	24.0	25.1	1.9	+6.5	1	0.06:0.04	0.10	0.08	—	—	0.14	0.02	0.80
C2S-3[i]	54.2	28.3	0.5	+23.5	1:0.09	0.09:0.20	0.14	0.05: 0.02	—	—	0.05	—	1.17
C2S-4[i]	3.3	17.5	n.d.	+4.0	1	0.09:0.06	0.13	0.03	—	0.06	—	—	0.54
C2S-3D[h]	96.0	7.1	n.d.	n.d.	1	0.19	0.09	0.05	—	—	0.03	—	0.21
C2S-3T[j]	60.0	n.d.	n.d.	n.d.	1:0.02	0.29:0.25	0.17	0.05	—	—	0.06	—	n.d.
CC[k]	5.0	25.3	n.d.	n.d.	1	0.27	0.28	0.06	—	—	—	—	0.93
C2S-2b[l]	13.5	17.3	n.d.	n.d.	1	0.03	—	0.07	—	—	—	0.28	0.62
C2S-2c[l]	25.0	14.0	4.5	n.d.	1	0.14	0.15	0.09	—	—	—	0.03	0.60
C2S-2d[l]	55.0	20.4	0.4	n.d.	1:0.11	0.01	0.09	0.05:0.03	—	—	—	0.03	0.84

(—) = not detected, n.d. = not determined.

[a] Knutsen's nomenclature.[29]

[b] G+D = β-D- plus α-D-galactose and L = α-L-galactose.

[c] Only one number when the enantiomeric analyses were not carried out.

[d] The GC–MS analyses showed after acid hydrolysis, reduction with $NaBD_4$ and acetylation *m/z* 129, 158, 189 and 218 corresponding to 4-*O*-methylgalactosyl residues.

[e] Crude extracts.

[f] Soluble fractions in 2 M KCl.

[g] Subfractions of C1S and C2S, respectively.

[h] Partially desulfated fractions from C1S-3 and C2S-3.

[i] Subfractions of C1S and C2S, respectively.

[j] Alkali-treated fraction from C2S-3.

[k] Partially depolymerized fraction from C2S-3.

[l] Subfractions of C2S-2.

Table 2. Cation composition of the raw extracts from *Cryptonemia crenulata*

Fraction	M equiv/g			
	Na^+	K^+	Ca^{2+}	Mg^{2+}
C1	2.96	0.25	0.15	0.04
C2	0.35	0.25	1.25	0.42
C3	2.60	0.10	0.07	0.17

C2S-4, respectively (Table 1). C2S-2 was refractionated on the same column giving four fractions (C2S-2a–C2S-2d) (fraction C2S-2a was discarded due to the low yield) (Table 1). The subfractions C2S-2c–C2S-2d showed the same compositional scheme as above and, when enantiomeric analysis was carried out, the D- and L-forms of galactose, 3,6-anhydrogalactose and 2-*O*-methylgalactose were determined (Table 1). The major subfractions (C1S-3 and C2S-3) were homogeneous by HPSEC–MALLS analysis and showed M_w of 315,000 and 236,200, respectively.

The FTIR spectra of C1S-3 and C2S-3 were qualitatively similar, but the bands showed different intensities. The intensity of the 1250–1260 cm^{-1} peak was higher in C2S-3 than in C1S-3, as well as the total absorption of the diagnostic region (940–800 cm^{-1}) in agreement with their sulfate content. In C2S-3 a major band at 832 cm^{-1}, together with a shoulder at 820–805 cm^{-1}, indicates major amounts of equatorial secondary sulfate groups together with minor quantities of these groups on C-6 of the galactose residues and on C-2 of the 3,6-anhydrogalactose units. In C1S-3 the major peak was found at 820–800 cm^{-1} with a less intense band at 832 cm^{-1} in consistency with the small percentage of β-D-galactose 2-sulfate (Table 4). A medium band at 932 cm^{-1} indicated the presence of the 3,6-anhydro ring in both cases.[27,28]

The ^{13}C NMR spectra of both subfractions (Figs. 1a and 2a) have common absorptions (Table 3) corresponding to the diads [Knutsen's nomenclature],[29] G (102.4–102.8 ppm)→LA2M (98.7 ppm)[30] and G2S (101.1 ppm, C-2 at 78.0 ppm)→D(6S) (96.1 ppm, C-6 sulfated at 67.7 ppm) and/or LA2S (96.1 ppm, C-2 at 75.6 ppm and C-3 at 78.3 or 78.1 ppm).[31] C1S-3 shows G (104.4 ppm)→D2M (93.3 ppm)[32] or D(6S) (96.2 ppm).[7] The spectra also showed absorptions at 25.0 and 175.0 ppm attributed to the methyl and carboxyl groups of pyruvic acid acetal.[33]

Methylation analyses of C1S-3 and C-2S-3 are shown in Table 4, while trideuteromethylation of C2S-3 is shown in Table 5. The pattern of methylation is qualitatively similar but differs from the quantitative point of view. The major 3-linked unit in C1S-3 is the

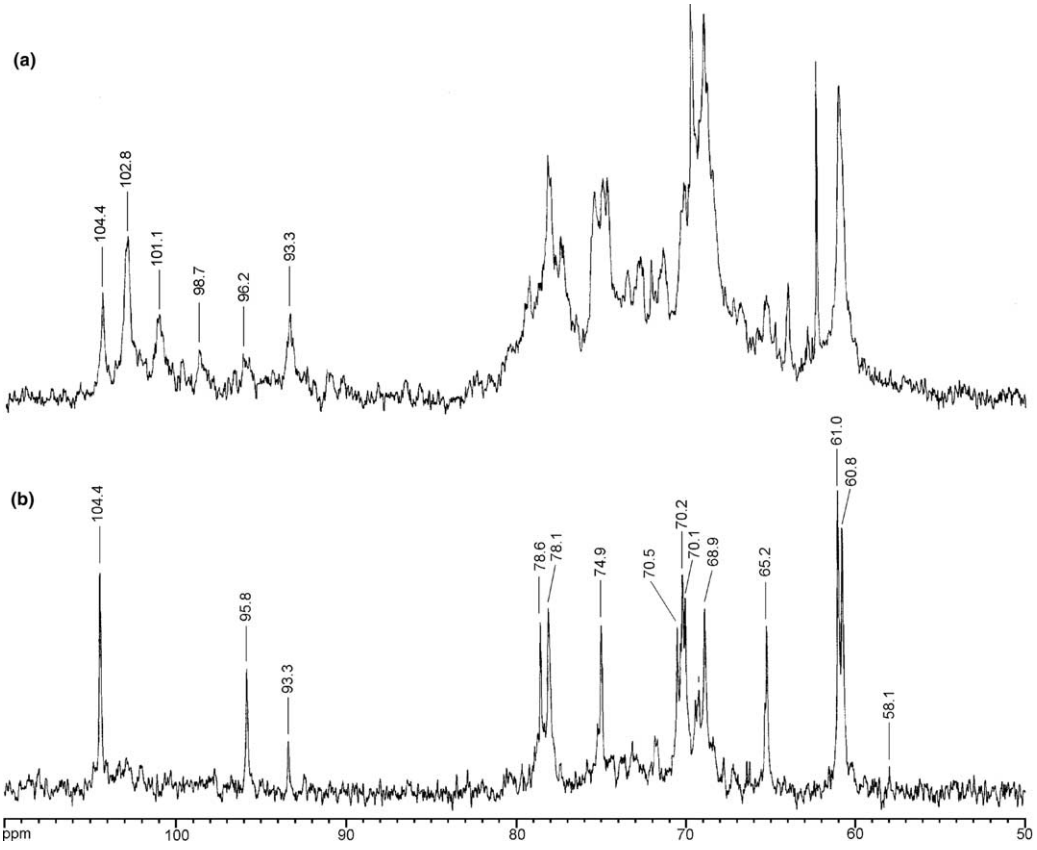

Figure 1. ^{13}C NMR spectra of (a) fraction C1S-3, (b) desulfated C1S-3 (C1S-3D).

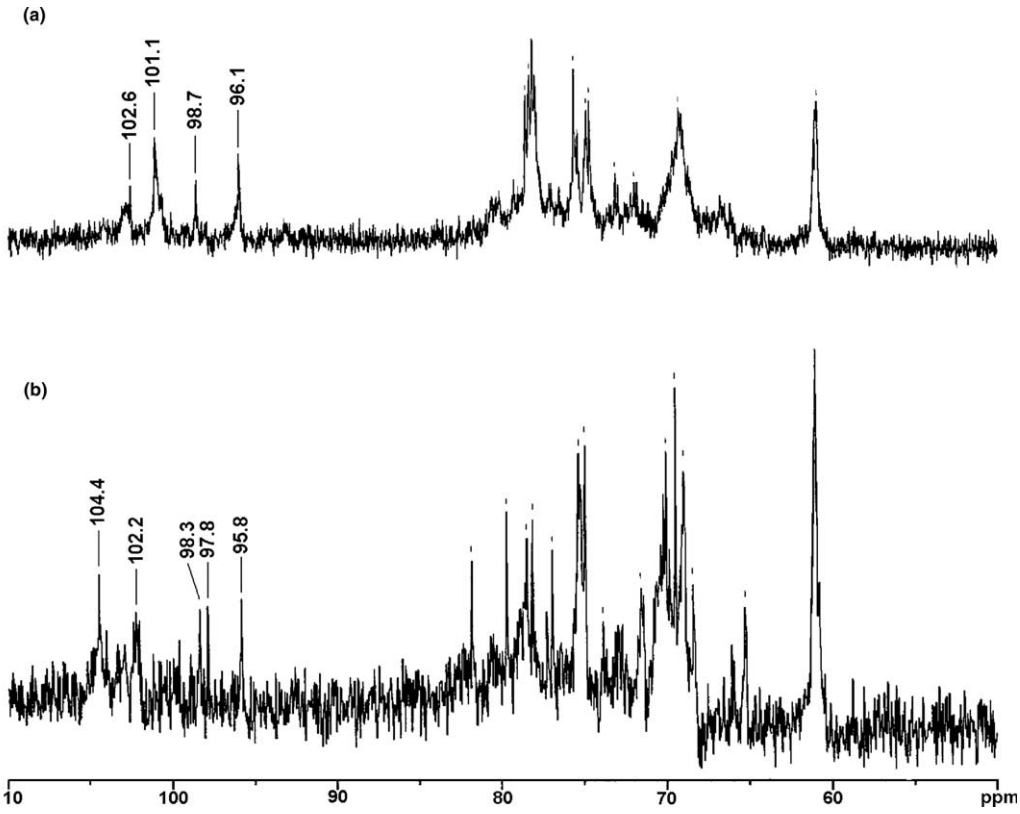

Figure 2. ^{13}C NMR spectra of (a) fraction C2S-3, (b) desulfated C2S-3 (C2S-3D).

Table 3. Chemical shifts (ppm) of various diads present in the ^{13}C NMR spectra of *C. crenulata* galactans

Diads	A-unit	B-unit
G→LA2M[a]	102.4–102.8	98.7
G2S→D(6S) and/or LA2S[a]	101.1	96.1
G→D(6S)[b]	104.4	96.2
G→D2M[b,c,d]	104.4	93.3
G→D(6S)[d]	104.3	96.1
G(P)→L(6S)[d]	103.1	100.7
G(P)→LA2M[d]	102.1–102.5	98.7
G(P)→LA2S[d]	102.1–102.5	96.1–96.7
G→LA[e]	102.2	97.8
G→LA2M[e]	102.2	98.3
G→D[e,c]	104.4	95.8

[a] Common diads in C1S-3 and C2S-3 spectra.
[b] Diads in C1S-3 spectrum.
[c] Diads in C1S-3D spectrum.
[d] Diads in CC spectrum.
[e] Diads in C2S-3D spectrum.

unsubstituted one, while in C2S-3 it is the 2-sulfated residue. The original 2-*O*-methyl D- or L-galactoses in the subfractions are always found as 4-linked units. In C2S-3 the 3,6-anhydro residues are composed by the 2-sulfated, 2-methylated or non-substituted units. The main difference between the 4-linked units in both subfractions is that in the first one there are only small amounts of 'precursor units', while in the latter they amounted to 15.6%. Also that all the 3,6-anhydrogalactose (16.7 mol %) was 2-*O*-methylated or/and non-methylated but not 2-sulfated in C1S-3, while in C2S-3 half of the 3,6-anhydro residues (D: 2 mol % and L: 11.6 mol %) were sulfated and the other half were C-2 methylated (8.6 mol %) or unsubstituted (3.5 mol %). The presence of Gal (Table 4) among the products of methylation for both fractions was attributed to 4,6-*O*-(1-carboxyethylidene)-galactopyranosyl 2-sulfate units. Thus, C1S-3 and C2S-3 show 1 pyruvate acetal group every 30 and 40 units, respectively. The degrees of pyruvylation are in agreement with the pyruvate percentages obtained by the Koepsell and Sharpe[34] method, corrected for the interference of 3,6-anhydrogalactose[35] (Table 1).

Alkali treatment of C2S-3 (60% yield) (C1S-3 contained only small amounts of 'precursor units') at 80 °C for 5 h produced a nearly quantitative cyclization of the precursor units (Table 4) without modification of the carbohydrate backbone (Table 1). Enantiomeric analysis of C2S-3T showed a major increase of the 3,6-anhydro-α-D-galactose (4.8→12.4 mol %) and only a minor increase of the L-form (11.0→14.3 mol %) in agreement with the total amount of (1→4) linked α-galactose 6-sulfate units in the parent compound and showing the proportions of the D- (7.6 mol %) and L- (3.3 mol %) forms in this residue. The small increase in 3,6-anhydrogalactose 2-sulfate agrees with the percentage (2.4 mol %) of the (1→4)-linked α-galactose 2,6-disulfate in the parent compound. Methylation analysis

Table 4. Methylation analysis of the native galactans (C1S-3, C2S-3) and their partially desulfated (C1S-3D, C2S-3D), alkali-treated (C2S-3T) and partially hydrolyzed (CC) subfractions

Derivative[a]	C1S-3	C1S-3D	C2S-3	C2S-3D	C2S-3T	CC
3-Linked residues						
2,4,6-Gal	24.0	40.0	3.0	42.6	4.0	17.8
4,6-Gal	6.5	3.1	31.4	2.0	33.7	9.2
2,4-Gal	7.6	3.0	—	2.0	—	—
4-Gal[b]	2.3	—	9.8	—	9.0	4.7
Gal	3.3	—	2.5	—	2.5	7.5
4-Linked residues						
2,3,6-Gal	11.5	42.6	7.5[c]	23.4[c]	7.7	13.3
2,3-Gal	3.7	—	13.2[c]	—	—	9.2
3-Gal[b]	—	—	2.4	—	—	—
2AG	16.7	2.0	12.1[c]	23.6[c]	24.2	10.8
AG	—	—	13.6	2.0	16.0	19.2
Undefined residues						
2,6-Gal	7.1	—	2.2	—	—	—
2-Gal	8.2	3.4	—	2.4	—	3.2
Terminal residues						
2,3,4-Xyl	3.7	2.4	2.3	2.0	2.9	2.4
2,3,4,6-Gal	5.4	3.5	—	—	—	2.7

[a] Mol % of monosaccharide having methyl or trideuteromethyl groups at the positions indicated.
[b] Determined after acid hydrolysis, reduction with $NaBD_4$ and acetylation. The relative proportion of the 4- and 3-Gal was determined using fragment ions, *m/z* 132 and 133, respectively.
[c] GC–MS analysis showed natural methoxyl on C-2.

Table 5. Trideuteromethylation of the native (N) and partially desulfated (D) galactan C2S-3

Derivative	Composed by	Corresponding units		Mol %		Mass fragments (*m/z*)[a]
		N	D	N	D	
2,4,6-Gal	2,4,6-CD_3Gal	G	G + G2S + G2,6S	3.0	42.6	
4,6-Gal	4,6-CD_3Gal	G2S	G2S[b]	31.4	2.0	
2,4-Gal	2,4-CD_3Gal		G2S6X[c]	—	2.0	
4-Gal[d]	4-CD_3Gal	G2,6S + G2S6X		9.8	—	132, 192
2-Gal	2-CD_3Gal		GP2S	—	2.4	
Gal	Gal	GP2S		2.5	—	
2,3,6-Gal	2,3,6-CD_3Gal	DL	D/L + D6S + L6S + D2,6S/L2,6S	4.5[e]	17.8[e]	107, 120, 167
	2Me-3,6-CD_3Gal	D2M/L2M	D2M/L2M + L2M6S/D2M6S	3.0[e]	5.6[e]	104, 117, 164
2,3-Gal	2,3-CD_3Gal	D6S[f]		7.6[g]	—	107, 120, 167
		L6S[f]		3.3[g]	—	107, 120, 167
	2Me-3-CD_3Gal	L2M6S/D2M6S		2.3[g]	—	104, 117, 164
3-Gal[d]	3-CD_3Gal	D2,6S/L2,6S		2.4	—	133, 193
2AG	2-CD_3 AnGal	DA	DA + LA2S/DA2S	3.5[h]	14.9[h]	120
	2Me-3-CD_3 AnGal	LA2M	LA2M	8.6[h]	8.7[h]	117
AG	AG	LA2S/DA2S	LA2S/DA2S[b]	13.6	2.0	
2,6-Gal	2,6-CD_3Gal	G4S/D3S/L3S		2.2	—	
2,3,4-Xyl	2,3,4-CD_3Xyl	X	X	2.3	2.0	

CD_3 = Trideuteromethyl.
/ = and/or, — = not detected.
[a] Fragment ions used to determine the relative proportions of the various partially methylated, partially trideuteromethylated alditol acetates.
[b] Alternatively could represent 2-*O*-glycosylated units.
[c] Single stubs of xylose (X) at the indicated position.
[d] Determined after acid hydrolysis, reduction with $NaBD_4$, acetylation and GC–MS analyses of the native polysaccharide.
[e] Values calculated using the relative abundance of mass fragments [*m/z* (%)] of the derivative 2,3,6-Gal.
[f] The enantiomeric indications were based in enantiomeric analyses of the content of 3,6-anhydrogalactose in the native and alkali-treated galactans.
[g] Values calculated using the relative abundance of mass fragments [*m/z* (%)] of the derivative 2,3-Gal.
[h] Values calculated using the relative abundance of mass fragments [*m/z* (%)] of the derivative 2AG.

of the derivative (C2S-3T) confirms these results (Table 4). Although the percentage of 3,6-anhydro-2-*O*-methyl-α-galactose remained practically constant through the treatment (Table 1), the trideuteromethylation of the native galactans (Table 5) showed the presence of small amounts of (1→4) linked 2-*O*-methylgalactose 6-sulfate

in C2S-3. In the ^{13}C spectrum of the polysaccharide C2S-3T (not shown), there was one new signal, of low intensity, at 95.3 ppm. This signal was attributed to the C-1 of 3,6-anhydro-α-D-galactosyl residues (formed in the alkali treatment) linked to G(P)2S.

The cyclization reaction of C2S-3 follows a pseudo first-order kinetics (similar determination on C1S-3 was not possible due to the small amounts of 'precursor units'). At 80 °C the cyclization showed a rate constant, in M sodium hydroxide, of $1.7 \times 10^4\ s^{-1}$ and a half-life of 68.6 min. These values are intermediate between those of the disaccharide α-D-galactopyranosyl 2,6-disulfate-(1→3)-D-galactose[36] and λ-carrageenan[37] (Table 6).

Solvolytic desulfation of both fractions C1S-3 and C2S-3 removed most of the sulfate (87% in C1S-3 and 75% in C2S-3), giving C1S-3D and C2S-3D in yields of 48% and 96%, respectively. Compositional analysis of the desulfated derivatives indicates that the solvolytic treatment had not significantly modified the composition of C2S-3, with only a small decrease of the anhydro sugars (Table 1). On the other hand, the desulfated derivative of C1S-3 showed degradation with significant decrease of anhydrogalactosyl units (Table 1). The ^{13}C NMR spectrum of C1S-3D (Fig. 1b) shows, in agreement with the above results, two diads [Knutsen's nomenclature],[29] namely:

G (C-1–C-6: 104.4, 70.2, 78.6, 65.2, 74.9, 60.8 ppm) linked to D (C-1–C-6: 95.8, 68.9, 70.5, 78.1, 70.1, 61.0 ppm)[7] or its 2-*O*-methyl derivative (C-1 at 93.3 ppm and methyl group at 58.1 ppm).[32] The signal at 104.4 ppm would also correspond to single stubs of β-D-xylopyranose (C-5 at 65.4 ppm) and β-D-galactopyranose linked to C-6 of β-D-galactopyranose units[38] (methylation analysis). In contrast with the spectrum of the native polysaccharide (Fig. 1a and Table 3), the signal attributed to the L-sugar, LA2M (98.7 ppm), as well as the signal corresponding to methyl (25.1 ppm, not shown) of the pyruvate acetal, are not observed. Therefore, the remaining block resembles a partially desulfated 'λ-carrageenan' with β-D-xylose and β-D-galactose single stubs on C-6 of some A-units and C-2 methoxyls on part of the B-units.

Methylation analysis (Table 4) of both desulfated fractions shows the removal of most of the sulfate groups. Trideuteromethylation of C2S-3D (Table 5) confirmed the results obtained with the respective native polysaccharide. Therefore the A-units are 93% 2-sulfated with some of these bearing sulfate groups or xylosyl units at O-6 or acetal of pyruvic acid at O-4 and O-6. B-units are also heavily substituted on C-2 by sulfate groups (33%, anhydrogalactosyl 2-sulfate plus galactosyl 2,6-sulfate) and by methoxyl groups (28%) on the anhydrogalactosyl and galactosyl units. The last units are also, in part, 6-sulfated.

The ^{13}C NMR spectrum of C2S-3D (Fig. 2b) shows, in agreement with the above results, three diads [Knutsen's nomenclature],[29] namely:

G (C-1–C-6: 102.2, 70.0, 81.8, 68.4, 74.9, 60.9 ppm)→LA (C-1–C-6: 97.8, 69.4, 79.7, 77.3, 75.3, 69.0 ppm);[30]
G (C-1–C-6: 102.2, 70.0, 82.3, 68.4, 74.9, 60.9 ppm)→LA2M (C-1–C-6: 98.3, 78.5, 78.1, 77.3, 75.3, 69.0 ppm, methyl group at 58.8 ppm)[30] and
G (C-1–C-6: 104.4, 70.0, 78.5, 65.2, 74.9, 60.9 ppm)→D (C-1–C-2 and C-4–C-6: 95.8, 69.0 ppm and 78.1, 70.0, 60.9 ppm).[7] Signals at 25.0, 65.2 and 66.4 ppm were attributed to the methyl carbon of the pyruvate acetal and the C-6 and C-5 of the pyruvated β-D-galactopyranose units.[30,39,40]

Fraction C2S-3 was partially depolymerized with 0.375 M TFA at 65 °C for 6 h in a reducing medium to preserve the anhydro sugars.[41] The mixture obtained was submitted to anion-exchange chromatography (Sephadex A-50). The subfractions CC and CD were further submitted to gel filtration on Bio-Gel P-2. The main subfraction (CC) proved to be homogeneous on HPSEC–MALLS and Bio-Gel P-30 with a molecular weight of 25,000 kDa. Monosaccharide composition is given in Table 1. The oligosaccharide contains major amounts of galactose, 3,6-anhydro-α-galactose and

Table 6. Rate constants and half-lives of the alkaline cyclization reactions in carrageenan and agaran diads[a,b]

Temp (°C)	Diads					
	G4S→D2,6S G4S→D6S[c]	G→L6S[d]	G→D2,6S[e]	G→D2,6S[f]	G2S→D(L)6S[g]	G2S→D2,6S[h]
70	13.0 (9.0)	1.5 (77.0)	—	—	—	0.35 (320)
80	26.0 (4.5)	4.9 (23.0)	4.8 (24.7)	3.2 (38.3)	1.7 (68.6)	0.67 (170)

[a] For experimental details, see Experimental.
[b] Rate constants are given in $\times 10^4\ s^{-1}$ and half-lives (in parenthesis) in minutes.
[c] The partially cyclized μ/ν-carrageenan sample ($1C_3$)[37] contained about 80% of G4S→D2,6S and 20% of G4S→D6S of cyclizable diads.
[d] From *Porphyra columbina*.[64]
[e] Average values from degraded λ-carrageenan $T_{2(26)}$ and $T_{2(35)}$ containing this diad.[36]
[f] Average values from a di- and a trisaccharide containing this diad from the partial acid hydrolysis of a λ-carrageenan.[42]
[g] Fraction C2S-3 from galactan of *C. crenulata* containing about 70% of G2S→D6S and 30% of G2S→L6S.
[h] Lambda-carrageenan ($1T_2$)[37] with 100% of G2S→D2,6S cyclizable diad.

3,6-anhydro-2-*O*-methyl-α-galactose, with a small amount of 2-*O*-methylgalactose and 25.3% of sulfate (as SO_3Na).

Methylation analysis (Table 4) showed a pattern of partially methylated galactoses as complex as that of the parent compound, the main difference being in the increase of 2,4,6-tri-*O*-methylgalactose concomitant with the decrease of the 4,6-di-*O*-methylgalactose. The loss of the sulfate group at C-2 of the A-units is in agreement with the desulfation and with previous results in the autohydrolysis of λ-carrageenans.[42] Methylation shows major quantities of 2,3,6-tri-*O*-methylgalactose together with 3,6-anhydrogalactose and 3,6-anhydro-2-*O*-methylgalactose. Lesser, but significant, amounts of 2- and 4-*O*-methylgalactoses, 2,3- and 4,6-di-*O*-methylgalactoses and galactose were also found. The high percentages of single stubs of galactose (Table 4) are noteworthy.

The ^{13}C NMR spectra of CC (Fig. 3) and CD (the small amount of sample preclude any other determination) were visually very different, as in CC the peaks were sharp and well resolved, while those of the spectrum of CD were broad and not neat. Analysis of the peaks in the anomeric zone required data from the methylation analysis. Peaks at 24.9 and 175.3 ppm and at 101.1 ppm correspond to methyl and carboxyl[33] and acetal carbon[43] of the pyruvate acetal, while those at 104.2–104.3 ppm would, in part, correspond to β-D-xylose[38] and/or β-D-galactose linked to C-6 of the A-units. The following diads were also determined:

G (104.3 ppm)→D(6S) (96.1 ppm)[7] or D2M (93.3 ppm);[32]
G(P) (103.1 ppm)→L(6S) (100.7 ppm);[44]
G(P) (102.1–102.5 ppm)→LA2M (98.7 ppm);[30] or LA2S (96.1–96.7 ppm).[31]

In addition, the signal at 101.1 ppm was attributed to C-1 of G(P)2S.

There were also evident absorptions at 66.8 ppm (C-6 sulfate), 65.0–64.8 and 63.6 ppm (C-6 and C-5 of a β-D-galactose 4,6-acetal). Signals corresponding to methyl groups of 3,6-anhydro-2-*O*-methylgalactose (59.9 ppm)[30] and 2-*O*-methylgalactose (58.1 ppm)[38] were also observed. The anomeric signal at 93.3 ppm would be attributed to both D2M and/or DA2S.

Both spectra (CC and CD) show absorptions corresponding to 3,6-anhydrogalactitol units (C-1 at 62.8 ppm, C-3 and C-4 at 83.0 and 86.2 ppm, respectively),[41] indicating the cleavage of a part of anhydrogalactosidic bonds. Previous results (not shown) demonstrated that oligosaccharides with a low-degree of polymerization (di- and tetrasaccharides) were obtained only after 14 h of partial hydrolysis using the original conditions.[41] Milder partial reductive hydrolysis conditions, in comparison with those of the original method,[41] used in this work, together with the sulfate group on C-2 of 3,6-anhydrogalactosyl units, could explain the resistance of some of the anhydrogalactosidic linkages giving rise to high-molecular mass oligosaccharides.

3. Discussion

The only structural information on the polysaccharides of *C. crenulata* reported the seaweed as an ι-carrageenan producer.[24] However further studies on these products

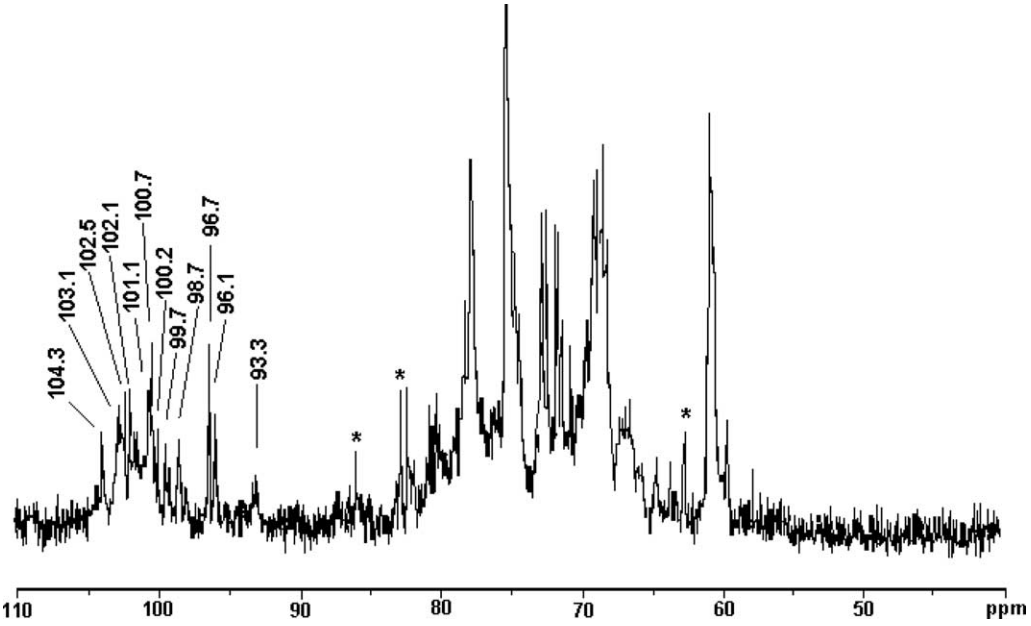

Figure 3. ^{13}C NMR spectra of fraction CC. *Anhydrogalactitol signals (see text).

did not agree with this structure, indicating more complex ones.[25]

3.1. The structure of the major fractions of the matrix polysaccharides from *C. crenulata*

Both major fractions (C1S-3 and C2S-3) contain qualitatively similar A-units constituted by non-sulfated and 2-sulfated residues. These residues can also have minor substitution on C-2 and C-6 (sulfate) and C-4,6 (pyruvic acid acetal). Non-sulfated units predominate in C1S-3, but 2- and 2,6-sulfated residues are the major ones in C2S-3. B-units are rather different in both fractions: C1S-3 is characterized by the small amounts of 'precursor units' and 3,6-anhydro-2-*O*-methyl-α-L-galactose, the lack of 3,6-anhydrogalactose 2-sulfate and substitution in C-6 with single stubs of β-D-galactose and β-D-xylose. In C2S-3 the α-galactose 6-sulfate and the 3,6-anhydrogalactose and 3,6-anhydro-2-*O*-methylgalactose units are predominant, together with a small amount of 2-*O*-methyl α-L- and α-D-galactose, and substitution at C-6 with single stubs of β-D-xylose. The pattern of naturally methylated galactoses is slightly different from those obtained with *Pachymenia carnosa*[14,16] and *Aeodes ulvoidea*[22,23] where all the methylation occurs on 4-linked galactosyl or 3,6-anhydrogalactosyl residues. In *Pachymenia lusoria* all the *O*-methyl galactosyl units are in the D-configuration, even when 4-linked. The 4-linked galactosyl units may be either in the D- or L-configuration, but the 3,6-anhydro units are all in the L-configuration.[18] The glycosyl substituents are β-linked to C-6 of a (1→3) linked β-D-galactopyranose (2-sulfate) as in the DL-hybrid galactan from *P. lusoria*[18] (Halymeniaceae, Halymeniales) and from gamethophytes of *Gymnogongrus torulosus*[45] (Phyllophoraceae, Gigartinales). In an agaran from *Joculatus maximus*,[46] (Corallinales), β-D-xylosylation is at *O*-6 of ca. 50% of the A-units. Similar xylosylation has been demonstrated in a xylogalactan sulfate from *Corallina officinalis*[47] (Corallinales). Side branches of β-D-xylose residues were found at *O*-3 of the B-units in a galactan sulfate from *Chondria macrocarpa*[48] (Ceramiales) and in *Laurencia nipponica*[41] (Ceramiales).

Carrageenans are linear molecules, and the presence of these branches of β-D-xylose and/or β-D-galactose, as well as those of unusual major diads (see later), suggest that the structure of carrageenan blocks in DL-hybrids may be somewhat different to that of classical carrageenans.

3.2. The family of matrix galactans synthesized by *C. crenulata*

The compositional and enantiomeric analyses of all the fractions and subfractions (Table 1), as well as the structural units of CS1-3 and CS2-3, indicate that *C. crenulata* biosynthesizes a family of galactans based on the classical 3-linked β-D-Gal→4-linked α-Gal alternating sequence. The dispersion of structures is based on four factors, namely: (a) the amount and position of substituent groups as sulfate, pyruvic acid acetals, methoxyl and lateral chains of β-D-xylose and/or β-D-galactose; (b) the ratio galactose/3,6-anhydrogalactose in the B-units; (c) the ratio D-/L-galactoses and 3,6-anhydrogalactoses also in the B-units and (d) the sequence of the diads in the linear backbone. Details of the major and minor A- and B-units are given in Tables 3 and 4 and in the preceding section.

The structural units can be organized as diads according to the ^{13}C NMR spectral data (Table 3). The arrangements of different amounts of these diads in sequences would build up the polysaccharide chains, but the emerging complexities make it impossible, at this stage, to construct a model of the polysaccharide. These possible arrangements would be severely restricted if the hybrid is a block copolymer of carrageenan and agaran structures as previously supposed[2] (or a mixture of carrageenans and agarans). Both hypotheses are consistent with the degradation of C1S-3 to a 'carrageenan' during the desulfation. With this view in mind, the carrageenan blocks (or molecules) could be grossly described by the major G2S→D6S and G→D6S diads and its 3,6-anhydro and substituted derivatives.

These parent diads have not been found as major components in natural carrageenans. The agaran blocks would consist in A-units similar to those of the carrageenan and B-units as non-substituted or 6-sulfated L-galactose and 3,6-anhydro-α-L-galactose. The G2S→L6S diad was found in agarans from *Acanthophora spicifera*.[49] These diads G2S→D(L)6S are structurally analogous to the minimal binding sequence necessary for the interaction of heparan sulfate with the glycoprotein gC of the herpes virus HSV-1[50] and are considered responsible for the high-antiherpectic activity of these type of polysaccharides.[26,49]

It is not actually known whether the above 'block DL-hybrid' hypothesis is correct or whether the polysaccharide extracts constitute a mixture of carrageenan-type and agaran-type molecules. Attempts at fractionation of the raw extracts or of different fractions and subfractions by KCl differential precipitation or ion-exchange chromatography, as well as by several similar fractionations used for other similar products[5,7,11,22] always failed, and to the best of our knowledge, there is no evidence that any seaweed of the Halymeniaceae (Halymeniales) contain more than one type of galactan. Whether this is a proof of the existence of DL-hybrid galactan molecules or only shows the failure of present-day techniques to fractionate mixtures of 'diastereoisomeric' polysaccharides it is not known, but if we apply the classical definition of homogeneity, these products should be considered as DL-hybrid galactan until a

successful fractionation demonstrates the contrary. On the other hand, it must be kept in mind that the seaweeds concentrate calcium and magnesium, and that these divalent cations should contribute to the complexation of the sulfated polysaccharides making their separation extremely difficult.[51] It is worth noting that the concentration of Ca^{2+} and Mg^{2+} increased about 10 times in the extract (C2) obtained under the harshest conditions (Table 2).

Present evidence suggests that the presence of both carrageenan and agaran structures in red seaweeds is not exceptional but a general fact[5,7,11,18,22,23,25,52–63] and that the traditional division of the red seaweeds in carrageenophytes and agarophytes can be maintained only if it refers to the major type (and not the only one) of polysaccharides biosynthesized by the seaweed.

3.3. Kinetic studies on the alkaline 3,6-anhydro cyclization in C2S-3 diads

According to the methylation and ^{13}C NMR spectroscopic data of C2S-3, there are several structural possibilities (diads) for 3,6-anhydrogalactose formation. The rate constant of the cyclization reaction would be determined by the presence of two different A-units and two different B-units (four possible cyclizable diads). Nevertheless, previous kinetics indicated that the enantiomeric character of the B-units,[64] as well as the presence of a C-2 sulfate in them (when the A-unit carries a sulfate at C-4),[37,65] would not influence that rate. Non-sulfated and C-2 sulfated residues are present in percentages of 9% and 91%, respectively, in the A-units (Tables 4 and 5), while 2,6-disulfated and 6-sulfated α-galactoses accounted for 30% and 70% of the cyclizable B-residues (Tables 4 and 5). The lesser units would produce opposite effects, that non-sulfated A-units (porphyrans) would increase the reaction rates[36] (Table 6) while 2-sulfated B-units (λ-carrageenans) could slow them[37] (Table 6). It is expected then, that the system of parallel reactions would be undetected and that the rate constant value would be in between those of porphyrans and λ-carrageenans. Actually, the rate constant ($1.7 \times 10^4\ s^{-1}$) shows a reaction that is about two times faster than in λ-carrageenans,[37] but slower than in porphyrans.[64] The driving force of the cyclization is the ionization of the C-3 hydroxyl group in the B-unit. In diads, which carry a sulfate group on C-2 of the A-unit (*C. crenulata*) or sulfate groups on C-2 of the A- and B-units (λ-carrageenans), an interaction of these groups with the C-3 hydroxyl group (B-unit) is expected, shielding the C-3 hydroxyl from ionization, and, therefore, reducing the cyclization rate.[36] Molecular models suggest an appropriate geometry for this interaction, which has also been reported for heparin sulfates.[66]

The destabilization of the $^4C_1$ chair conformation of the B-unit and its change to a $^1C_4$ chair conformation is due to the repulsion between the negative charges on the C-2 and C-3 hydroxide ions (G2S→D(L)6S diad, *C. crenulata*) or on the C-2 sulfate and C-3 hydroxide ion (λ diad, G2S→D2,6S). The negative charge on the C-2 is preexistent in the λ diad (sulfate group) and is formed firstly on C-2 of the *C. crenulata* diad due to the higher acidity of this hydroxyl group.[67,68] It is expected that the interaction of the closer hydroxide ions of rigid geometry would destabilize the B-unit $^4C_1$ conformation to a $^1C_4$ one, easier than that of the hydroxide and sulfate ions producing, in the first case, a faster cyclization. As in the κ-family (with the sulfate group on C-4 of the A-unit),[37,65] it is suggested that the change of the C-2 sulfate in the B-unit from the equatorial to the axial position during the conversion of the $^4C_1 \rightarrow {}^1C_4$ chair conformation does influence the cyclization rate. This lack of influence was attributed to the low 1,3-axial repulsion of the axial group, which is further relieved by the distortion of the chair caused by the 3,6-anhydro bridge.[69]

4. Experimental

4.1. Extraction and purification

The samples of *C. crenulata* were collected and worked up as previously reported.[26] Briefly, the seaweed was extracted with water at 25 °C for 5 h yielding C1. Sequentially the algal residue was extracted twice with hydrogen/dihydrogen phosphate buffer (pH 6.5) at 80 °C yielding C2 and C3. The polysaccharides (C1, C2 and C3) were obtained by precipitation with EtOH. C1 and C2 were submitted to KCl fractionation[26] giving the soluble (2 M KCl) fractions C1S and C2S, respectively. The insoluble fractions were discarded. C1S and C2S were submitted to anion-exchange chromatography (DEAE-Sephacel) using water and an aqueous solution of NaCl of increasing concentration. From C1S were obtained the following fractions: C1S-1 (0.3 M), C1S-2 (0.4 M) and C1S-3 (0.7 M), whereas from C2S were obtained the fractions C2S-1 (water), C2S-2 (0.5 M), C2S-3 (M) and C2S-4 (1.5 M) (Table 1).

C2S-2 was refractionated using the same column of DEAE-Sephacel. Elution was performed with water, then with a solution of NaCl to give four fractions, C2S-2a (0.3M), C2S-2b (0.35 M), C2S-2c (0.45 M) and C2S-2d (M) (Table 1).

4.2. Analytical methods

Total carbohydrate was determined by the phenol–sulfuric acid method,[70] using D-galactose as the standard. Sulfate content and protein were determined by the

turbidimetric method of Dodgson and Price[71] and Lowry et al.,[72] respectively. Pyruvic acid and protein were determined by the method of Koepsell and Sharpe.[34] The results of this determination were corrected for the presence of 3,6-anhydrogalactose.[35] The cation composition was determined by flame atomic absorption spectrometry. The ratio of D- and L-galactose was estimated after reductive amination with chiral 1-amino-2-propanol, followed by acetylation and GC analysis (Ultra-2 column, Hewlett–Packard), whereas the configuration of 2-*O*-methylgalactose was determined using α-methylbenzylamine, as the amine.[73] Derivatization of 3,6-anhydrogalactonates, following GC analysis (SP2330 column) of the corresponding diastereomeric *sec*-butyl esters, was used to estimate the ratio of D- to L-3,6-anhydrogalactose.[74] The determination of monosaccharide composition and analysis of the methylation products were carried out by reductive hydrolysis[75] using extra reducing agent (4-methylmorpholine·borane) before and after prehydrolysis steps.[76] The reactions were followed by GC and GC–MS of the alditol acetates. To distinguish between co-eluting derivatives by GC–MS, partially methylated alditol acetates were also generated by hydrolysis in aqueous formic acid (45% for 16 h at 100 °C), followed by $NaBD_4$ reduction and acetylation. These GC analyses were carried out on an HP-5890 gas–liquid chromatograph equipped with a flame-ionization detector (FID), using a fused silica capillary column (30 m × 0.25 mm) coated with DB-225 (Durowax) eluted isothermically at 210 °C. Nitrogen was used as carrier gas at flow rate of 1 mL min^{-1} and a split ratio of 100:1. GC–MS analysis was performed using a Varian 3300 chromatograph and a Finnigan Mat ITD spectrometer equipped with two different columns, DB-225 or a DB-23. Helium was used as carrier gas at 1 mL min^{-1}.

Optical rotations of aqueous solution of the polysaccharide samples (0.2%) were measured at 20 °C using a 10-cm cell and the sodium D line (589.3 nm) on a Rudolph Autopol III automatic polarimeter.

4.3. Alkali modification

The sample of polysaccharide C2S-3 (200 mg) was dissolved in water (100 mL) and $NaBH_4$ (10 mg) was added. After 12 h at 4 °C the NaOH concentration was adjusted to M, using 3 M NaOH (50 mL). The cyclization reaction was carried out at 80 °C for 5 h to give C2S-3T. The increase of 3,6-anhydrogalactose was determined by the resorcinol method.[77] To determine the reaction rate and half-life, C2S-3 was submitted to alkaline cyclization at 80 °C as previously described.[37]

4.4. Desulfation

Partial solvolytic desulfation followed the method of Nagasawa et al.[78] The native galactans (C1S-3 and C2S-3) in the pyridinium salt form, were treated with 89:10:1 dimethyl sulfoxide–methanol–pyridine at 100 °C for 4 h to afford C1S-3D and C2S-3D, respectively.

4.5. Methylation

Methylation (C1S-3, C1S-3D and C2S-3T) or trideuteromethylation (C2S-3 and C2S-3D) analysis was carried out by the method of Ciucanu and Kerek[79] with the polysaccharides in the corresponding triethylammonium salt form.[80] Briefly, the samples (15 mg) were dissolved in dimethyl sulfoxide (1 mL), and powdered NaOH (30 mg) was added as base. After 30 min at 25 °C with vigorously stirring, iodomethane or trideuteroiodomethane (CH_3I or CDI_3) (0.1 mL) was added, and the reaction was allowed to proceed as described above. The addition of NaOH and CH_3I or CD_3I was repeated twice. The reaction was interrupted by addition of water, neutralized with HOAc, dialyzed against distilled water and freeze-dried. The polysaccharides were submitted to more three steps of methylation or trideuteromethylation in the same way as described above.

4.6. HPSEC–MALLS analysis

The determination of the homogeneity was performed on a waters high-performance size-exclusion chromatography (HPSEC) apparatus coupled to a differential refractometer (RI) and a Wyatt Technology Dawn-F Multi-Angle Laser Light Scattering detector (MALLS). Waters Ultrahydrogel columns (2000, 500, 250 and 120) were connected in series and coupled with multidetection equipment, using a $NaNO_2$ solution (0.1 M) as eluent, containing NaN_3 (200 ppm) as preservative. The samples (2 mg mL^{-1}) were dissolved in the same solvent under magnetic stirring for 2 h and filtered through a 0.45 and 0.22 μm nitrocellulose membrane (GSWP, Millipore). HPSEC data were collected and analyzed by the Wyatt Technology ASTRA program. The light scattering signal was detected simultaneously at 11 scattering angles, θ, ranging from 35° to 132°. All experiments were carried out at 25 °C.

4.7. Partial depolymerization

The galactan C2S-3 (700 mg) was dissolved in water (52.5 mL), the solution was heated to 65 °C and 4.8 g an acid-stable reducing agent (borane·4-methylmorpholine complex)[41] and TFA 1.5 M (17.5 mL) were then added. The solution was maintained at 65 °C for 6 h. Acid was removed (co-distillation with water), and the residue was freeze-dried and then submitted to anion-exchange chromatography on DEAE-Sephadex A-25 (column 5 × 22 cm) using a water and linear gradient of NaCl (0–0.5 M), 0.6 M NaCl and 0.7 M NaCl as eluent.

The fractions (CC and CD) were mixed with Me_2SO (100 mL) and submitted to mechanical stirring. After centrifugation, the supernatant was treated with $CHCl_3$ (3 vol.), and the precipitate was dried at room temperature. The oligosaccharide fractions were sequentially desalinized using a Bio-Gel P-2 column (2×60 cm). The homogeneity of CC was available on HPSEC–MALLS and Bio-Gel P-30 column (0.5×5.5 cm). The molecular weight was determined on Bio-Gel P-30 using standard dextrans (MW 9400 and 40,200 kDa).

4.8. Spectroscopic analysis

For NMR analysis the lyophilized samples were dissolved in D_2O (20–30 mg). The NMR spectra of the solutions were recorded at 70 °C using a Bruker Avance DRX400 NMR spectrometer. The spectra were obtained using a 5 mm multinuclear inverse detection probe. Fourier-transform infrared (FTIR) spectra of polysaccharide pellets were recorded on a Perkin–Elmer series 2000 FTIR spectrometer in transmittance mode (eight scans, collected at a resolution of 4 cm^{-1}).

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References

1. Painter, T. J. In *The Polysaccharides*; Aspinall, G. O., Ed.; Academic: London, 1983; pp 195–285.
2. Stortz, C. A.; Cerezo, A. S. *Curr. Top. Phytochem.* **2000**, *4*, 121–134.
3. Hirase, S.; Araki, C.; Watanabe, K. *Bull. Chem. Soc. Jpn.* **1967**, *40*, 1445–1448.
4. Hirase, S.; Watanabe, K. *Bull. Inst. Chem. Res.* **1972**, *50*, 332–336.
5. Usov, A. I.; Miroshnikova, L. I.; Barbakadze, V. V. *Zh. Obsh. Khim.* **1975**, *45*, 1618–1624.
6. Baeza, P.; Matsuhiro, B. *Bot. Mar.* **1977**, *20*, 355–357.
7. Usov, A. I.; Yarotsky, S. V.; Shashkov, A. S. *Biopolymers* **1980**, *19*, 977–990.
8. Parekh, R. G.; Doshi, Y. A.; Chauhan, V. D. *Indian J. Mar. Sci.* **1989**, *18*, 139–140.
9. Zablackis, E.; Perez, J. *Bot. Mar.* **1990**, *33*, 273–276.
10. Lai, M.-F.; Lii, C. *Bot. Mar.* **1998**, *41*, 223–234.
11. Sen Sr, A. K.; Das, A. K.; Sarkar, K. K.; Siddhanta, A. K.; Takano, R.; Kamei, K.; Hara, S. *Bot. Mar.* **2002**, *45*, 331–338.
12. Parekh, R. G.; Doshi, Y. A.; Chauhan, V. D. *Indian J. Mar. Sci.* **1987**, *16*, 274–276.
13. Lai, M.-F.; Li, C.-F.; Li, C.-Y. *Food Hydrocoll.* **1994**, *8*, 215–232.
14. Farrant, A. J.; Nunn, J. R.; Parolis, H. *Carbohydr. Res.* **1971**, *19*, 161–168.
15. Farrant, A. J.; Nunn, J. R.; Parolis, H. *Carbohydr. Res.* **1972**, *25*, 283–292.
16. Parolis, H. *Carbohydr. Res.* **1978**, *62*, 313–320.
17. Parolis, H. *Carbohydr. Res.* **1981**, *93*, 261–267.
18. Miller, I. J.; Falshaw, R.; Furneaux, R. H. *Carbohydr. Res.* **1995**, *268*, 219–232.
19. Miller, I. J.; Falshaw, R.; Furneaux, R. H.; Hemmingson, J. A. *Bot. Mar.* **1997**, *40*, 119–127.
20. Nunn, J. R.; Parolis, H. *Carbohydr. Res.* **1970**, *14*, 145–150.
21. Nunn, J. R.; Parolis, H. *Carbohydr. Res.* **1968**, *6*, 1–11.
22. Allsobrook, A. J. R.; Nunn, J. R.; Parolis, H. *Carbohydr. Res.* **1971**, *16*, 71–78.
23. Allsobrook, A. J. R.; Nunn, J. R.; Parolis, H. *Carbohydr. Res.* **1974**, *36*, 139–145.
24. Saito, R. M.; Oliveira, E. C. *Hydrobiologia* **1990**, *204/205*, 585–588.
25. Chopin, T.; Kerin, B. F.; Mazerolle, R. *Phycol. Res.* **1999**, *47*, 167–188.
26. Talarico, L. B.; Zibetti, R. G. M.; Faria, P. C. S.; Scolaro, L. A.; Duarte, M. E. R.; Noseda, M. D.; Pujol, C. A.; Damonte, E. B. *Int. J. Biol. Macromol.* **2004**, *34*, 63–71.
27. Stancioff, D. J.; Stanley, N. F. *Proc. Int. Seaweed Symp.* **1969**, *6*, 595–609.
28. Craigie, J. S.; Leigh, C. In *Handbook of Phycological Methods: Physiological and Biochemical Methods*; Hellebust, J. A., Craigie, J. S., Eds.; Cambridge University Press: Cambridge, 1978; pp 109–131.
29. Knutsen, S. H.; Myslabodski, D. E.; Larsen, B.; Usov, A. I. *Bot. Mar.* **1994**, *37*, 163–169.
30. Lahaye, M.; Yaphe, W.; Viet, M. T. P.; Rochas, C. *Carbohydr. Res.* **1989**, *190*, 249–265.
31. Miller, I. J.; Blunt, J. W. *Bot. Mar.* **2000**, *43*, 263–271.
32. Miller, I. J.; Blunt, J. W. *Bot. Mar.* **2000**, *43*, 251–261.
33. Garegg, P. J.; Lindberg, B.; Kvarnström, I. *Carbohydr. Res.* **1979**, *77*, 71–78.
34. Koepsell, H. J.; Sharpe, E. S. *Arch. Biochem. Biophys.* **1952**, *38*, 443–449.
35. Duckworth, M.; Yaphe, W. *Chem. Ind.* **1970**, *23*, 747–748.
36. Noseda, M. D.; Cerezo, A. S. *Carbohydr. Polym.* **1995**, *26*, 1–3.
37. Ciancia, M.; Noseda, M. D.; Matulewicz, M. C.; Cerezo, A. S. *Carbohydr. Polym.* **1993**, *20*, 95–98.
38. Usov, A. I.; Bilan, M. I.; Shashkov, A. S. *Carbohydr. Res.* **1997**, *303*, 93–102.
39. Lahaye, M.; Rochas, C. *Hydrobiologia* **1991**, *221*, 137–148.
40. Chiovitti, A.; Bacic, A.; Craik, D. J.; Munro, S. L. A.; Kraft, G. T.; Liao, M.-L. *Carbohydr. Res.* **1997**, *299*, 229–243.
41. Usov, A. I.; Elashvili, M. *Bot. Mar.* **1991**, *34*, 553–560.
42. Noseda, M. D.; Cerezo, A. S. *Int. J. Biol. Macromol.* **1993**, *15*, 177–181.
43. Gorin, P. A. J.; Mazurek, M.; Duarte, H. S.; Iacomini, M.; Duarte, J. H. *Carbohydr. Res.* **1982**, *100*, 1–15.
44. Lahaye, M.; Yaphe, W.; Rochas, C. *Carbohydr. Res.* **1985**, *143*, 240–245.
45. Estevez, J. M.; Ciancia, M.; Cerezo, A. S. *Carbohydr. Res.* **2001**, *331*, 27–41.
46. Takano, R.; Hayashi, J.; Hayashi, K.; Hara, S.; Hirase, S. *Bot. Mar.* **1996**, *39*, 95–102.
47. Cases, M. R.; Stortz, C. A.; Cerezo, A. S. *Int. J. Biol. Macromol.* **1994**, *16*, 93–97.

48. Furneaux, R. H.; Stevenson, T. T. *Hydrobiologia* **1990**, *204/205*, 615–620.
49. Duarte, M. E. R.; Cauduro, J. P.; Noseda, D. G.; Noseda, M. D.; Gonçalves, A. G.; Pujol, C. A.; Damonte, E. B.; Cerezo, A. S. *Carbohydr. Res.* **2004**, *339*, 335–347.
50. Feyzi, E.; Trybala, E.; Bergström, T.; Lindahl, U.; Spillmann, D. *J. Biol. Chem.* **1997**, *272*, 24850–24857.
51. Kloareg, B.; Quatrano, R. S. *Mar. Biol. Ann. Rev.* **1988**, *26*, 259–315.
52. Craigie, J. S.; Rivero-Carro, H. *Abstr. Papers*, XIVth Int. Seaweed Symp., Brest-St. Malo: France, August 1992; p Abstr. 71.
53. Ciancia, M.; Matulewicz, M. C.; Cerezo, A. S. *Phytochemistry* **1993**, *34*, 1541–1543.
54. Takano, R.; Nose, Y.; Hayashi, K.; Hara, S.; Hirase, S. *Phytochemistry* **1994**, *37*, 1615–1619.
55. Miller, I. J.; Furneaux, R. H. *Bot. Mar.* **1996**, *39*, 141–147.
56. Stortz, C. A.; Cases, M. R.; Cerezo, A. S. *Carbohydr. Polym.* **1997**, *34*, 61–65.
57. Ciancia, M.; Matulewicz, M. C.; Cerezo, A. S. *Phytochemistry* **1997**, *45*, 1009–1013.
58. Takano, R.; Iwane-Sakata, H.; Hayashi, K.; Hara, S.; Hirase, S. *Carbohydr. Polym.* **1998**, *35*, 81–87.
59. Takano, R.; Yokoi, T.; Kamei, K.; Hara, S.; Hirase, S. *Bot. Mar.* **1999**, *42*, 183–188.
60. Estevez, J. M.; Ciancia, M.; Cerezo, A. S. *Carbohydr. Res.* **2000**, *325*, 287–299.
61. Takano, R.; Shiomoto, K.; Kamei, K.; Hara, S.; Hirase, S. *Bot. Mar.* **2003**, *46*, 142–150.
62. Falshaw, R.; Furneaux, R. H.; Miller, I. J. *Bot. Mar.* **1999**, *42*, 431–435.
63. Miller, I. J. *Bot. Mar.* **2001**, *44*, 251–545.
64. Noseda, M. D.; Viana, A. G.; Duarte, M. E. R.; Cerezo, A. S. *Carbohydr. Polym.* **2000**, *42*, 301–305.
65. Viana, A. G.; Noseda, M. D.; Duarte, M. E. R.; Cerezo, A. S. *Carbohydr. Polym.* **2004**, *58*, 455–460.
66. Fransson, L.-A.; Maemström, A.; Söjberg, I.; Huckerby, T. N. *Carbohydr. Res.* **1980**, *80*, 131–145.
67. Sugihara, M. J. *Adv. Carbohydr. Chem.* **1953**, *8*, 1–44.
68. Lenz, R. W. *J. Am. Chem. Soc.* **1960**, *82*, 182.
69. Eliel, E. L.; Allinger, N. L.; Angyal, S. J.; Morrison, G. A. *Conformational Analysis* **1981**.
70. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350–356.
71. Dodgson, K. S.; Price, R. G. *Biochem. J.* **1962**, *84*, 106–110.
72. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
73. Cases, M.; Cerezo, A. S.; Stortz, C. A. *Carbohydr. Res.* **1995**, *269*, 333–341.
74. Errea, M. I.; Ciancia, M.; Matulewicz, M. C.; Cerezo, A. S. *Carbohydr. Res.* **1998**, *311*, 235–238.
75. Stevenson, T. T.; Furneaux, R. H. *Carbohydr. Res.* **1991**, *210*, 277–298.
76. Falshaw, R.; Furneaux, R. *Carbohydr. Res.* **1994**, *252*, 171–182.
77. Yaphe, W.; Arsenault, G. P. *Anal. Biochem.* **1965**, *13*, 143–148.
78. Nagasawa, K.; Inoue, Y.; Kamaia, T. *Carbohydr. Res.* **1977**, *58*, 47–55.
79. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
80. Stortz, C. A.; Cerezo, A. S. *Carbohydr. Res.* **1993**, *242*, 217–227.