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Galactans from cystocarpic plants of the red seaweed *Callophyllis variegata* (Kallymeniaceae, Gigartinales)

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Abstract—The polysaccharide extracted from cystocarpic *Callophyllis variegata* was fractionated with potassium chloride yielding three small fractions that precipitated in the ranges of 0–0.05 M KCl, 1.20–1.25 M KCl, and 1.80–2.00 M KCl, and a main product soluble in 2.00 M KCl. These fractions were analyzed, and as the first one contained very high amounts of protein, it was not studied further. Structural analysis of the rest of the fractions (F1–F3) was carried out by methylation, desulfation–methylation, IR, and ^{13}C NMR spectroscopy. The results are consistent for F1 with a carrageenan-type backbone mainly constituted by β-D-galactose 2-sulfate linked to α-D-galactose 2,3,6-trisulfate and β-D-galactose 2,4-disulfate linked to 3,6-anhydro-D-galactose 2-sulfate as dominant diads. In F2 these diads are present together with low amounts of β-D-galactose 2-sulfate linked to 3,6-anhydro-D-galactose 2-sulfate, whose contribution becomes higher in F3. In addition, minor but significant amounts of L-galactose were detected. F1–F3 showed potent antiviral activity against herpes simplex types 1 and 2 and dengue type 2.

Keywords: Red algal polysaccharides; Sulfated galactans; Carrageenans; Antiviral activity; *Callophyllis variegata*; Kallymeniaceae

1. Introduction

The genus *Callophyllis* is included within the family Kallymeniaceae. This family formerly placed within the order Cryptonemiales[1] has been transferred to the order Gigartinales *s.s.*[2,3] Recent phylogenetic analysis based on sequences of large-subunit, nuclear ribosomal DNA revealed that the genus *Callophyllis* is polyphyletic, and the type species *C. variegata* was placed in the *Callophyllis s.s.* clade.[4]

Polysaccharide structure can also provide an additional criterion for chemotaxonomy of red algae.[5,6] In general, members of the Kallymeniaceae have been reported to produce complex, highly sulfated, non-gelling galactans, containing low-to-intermediate levels of 3,6-anhydrogalactose, 4-linked residues occurring as either or both D- and L-isomers, and highly heterogeneous patterns of substitution with sulfate ester, methyl ether, and in some cases pyruvate acetal.[7–14] The few previous reports for different *Callophyllis* species inform of carrageenan-type polysaccharides in *C. rhynchocarpa*,[8,12] DL-galactan hybrids in *C. cristata*,[8] a mixture of λ- and θ-carrageenans together with a novel carrageenan structure consisting of 3-linked β-D-galactopyranosyl 2-sulfate units alternating with

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3,6-anhydro-α-D-galactopyranosyl units for *C. hombroniana*,[11,13] and a preliminary report on a carrageenan fraction soluble in 2 M KCl containing L-galactose in *C. variegata*.[14]

C. variegata is one of the six species of the genus described for the Argentinian coasts, with a cold water distribution. The plants consist of flattened, fan-shaped fronds, and grow attached to rocks in the subtidal zone associated with prairies of *Macrocystis pyrifera*. The fact that *C. variegata* is at present widely exploited as an edible seaweed in Asia and Chile[15] provides an additional interest for elucidation of the structures of its phycocolloids.

This paper describes the characterization of the crude polysaccharide extracted from cystocarpic plants of *C. variegata*, its fractionation with potassium chloride, and the structural analysis of the main fractions. Additionally, the antiviral activity of these fractions was assayed against herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), and dengue virus type 2 (DENV-2); F3 was also evaluated against two acyclovir-resistant variants of HSV-1 (TK^- B2006 and TK^- Field) and human cytomegalovirus (HCMV).

2. Results

2.1. Analysis of the native polysaccharide

Cystocarpic *C. variegata* was extracted with water at room temperature giving three extracts (1C–3C) in a total yield of 16.3%. The compositions of 1C–3C are shown in Table 1. Due to their similar carbohydrate composition, these extracts were combined to afford the native polysaccharide.

As only traces of L-galactose were detected in 1C–3C, suggesting the presence of carrageenan-like polysaccharides, a preliminary precipitation curve with KCl was determined by means of a turbidimetric method.[16] This curve indicated that most of the polysaccharides were soluble in 2.00 M KCl, but continuous precipitation was observed from 1.20 M KCl. Preparative fractionation yielded three fractions that precipitated in the ranges of 0–0.05 M KCl, 1.20–1.25 M KCl (F1), and 1.80–2.00 M KCl (F2), and a main product soluble in 2.00 M KCl (F3). The fraction precipitated in the range of 0–0.05 M KCl (yield, 6.0% of the recovered) contained very high amounts of protein (79%), and it was not studied further. The yields and analyses of fractions (F1–F3) are shown in Table 2. The total recovery after fractionation was 65.1%.

Positive optical rotations for F1–F3 were consistent with the carrageenan-like polysaccharides, though these values were lower than expected.[17] Accordingly, low but significant contents of L-galactose were detected in the three fractions, but 3,6-anhydrogalactose in F3 was in the D-configuration. Pyruvic acid values always fell within the error of the method.[18,19]

2.2. Linkage analysis

F1–F3 were converted into the triethylammonium salts[20] and methylated by the Hakomori procedure.[21,22] Table 3 depicts the carbohydrate composition of the permethylated products. Taking into account that significant amounts of 6-*O*-methylgalactose and galactose were detected even after two successive methylation steps, F1 and methylated F2 were subjected to further methylation using lithium chloride–dimethyl sulfoxide to improve the alkylation procedure.[23,24] However, no important quantitative changes in the methylation pattern were observed.

The analysis of enantiomeric monomethylated units in permethylated F3 indicated that 6-*O*-methylgalactose belonged to the D-series. In addition, it confirmed that the 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methylgalactitol detected in the GC analysis of the partially methylated alditol acetates (Table 3) derived mainly from 3- and 4-*O*-methyl-D-galactose (1:2 molar ratio) and that only trace amounts of 3-*O*-methyl-L-galactose were present. Galactose was in the D-configuration.

2.3. Infrared spectroscopy

FTIR analysis of the three fractions were similar, showing a peak at 935 cm^{-1} and a broad band (range, 880–800 cm^{-1}) centered at 844–835 cm^{-1} with a small shoulder at 805–803 cm^{-1}. In the spectrum of the carrageenan

Table 1. Yields and analyses of the products obtained from *C. variegata* by extraction with water at room temperature

Product	Yield[a] (%)	Sulfate[b] (as KSO_3)	Gal:AnGal:Xyl:sulfate (molar ratio)	Protein (%)	Monosaccharide composition[c] (mol %)					
					D-Gal	L-Gal	3-Me Gal	D-AnGal[d]	Xyl	Glc
1C	10.1	23.6	1.00:0.29:0.03:0.83	8.9	72	tr[e]	1	21	2	4
2C	3.0	17.4	1.00:0.26:0.09:0.86	13.8	71	tr	—	18	6	5
3C	3.2	28.5	1.00:0.24:0.04:1.52	14.2	76	tr	—	18	3	3

[a] Yields are given for 100 g of seaweed.
[b] The sulfate contents are possibly underestimated due to the presence of high amounts of calcium and magnesium as counterions.
[c] Traces of fucose and mannose were detected in 1C–3C.
[d] Only 3,6-anhydro-D-galactose was detected in the ^{13}C NMR spectra.
[e] Percentages lower than 1% are considered as traces (tr).

Table 2. Yields and analyses of the fractions F1–F3 isolated from the native carrageenan by precipitation with potassium chloride, their desulfated derivatives (F1DS–F3DS), alkali-treated F3 (F3T), and F3T1–F3T3 obtained after further fractionation with potassium chloride

Fraction	Range of precipitation (M KCl)	Yield[a,b] (%)	Sulfate (% KSO_3)	$[\alpha]_D$	Gal:AnGal:Xyl:sulfate (molar ratio)	Protein (%)	Molecular weight	Monosaccharide composition (mol %)					
								D-Gal	L-Gal	3-Me Gal[c]	D-AnGal[d]	Xyl	Glc	Man
F1	1.20–1.25	9.7	33.6	+10.8	1.00:0.21:0.11:1.43	10.5	185,000	64	7	3	15	8	3	—
F2	1.80–2.00	11.0	40.9	+13.5	1.00:0.23:0.04:1.94	2.0	356,000	60	10	2	16	2	4	6
F3[e]	2.00[f]	73.3	31.6	+20.0	1.00:0.29:0.09:1.49	3.1	95,000	57	10	2	20	6	4	1
F1DS	—	70.9	10.0	+19.8	1.00:0.27:0.02:0.21	n.d.[g]	9260	68	9	—	21	2	—	—
F2DS	—	54.5	8.8	+19.2	1.00:0.27:0.04:0.21	n.d.	14,283	65	11	—	21	3	—	—
F3DS	—	47.3	8.8	+6.4	1.00:0.33:0.05:0.23	n.d.	9657	61	12	—	24	3	—	—
F3T	—	97.3	40.3	+7.0	1.00:0.23:0.06:1.43	n.d.	n.d.	61	16	—	18	5	—	—
F3T1	0.00–0.05	4.0	25.2	n.d.	1.00:0.29:0.10:1.00	n.d.	n.d.		72	—	21	7	—	—
F3T2	1.80–2.00	2.0	20.2	n.d.	1.00:0.27:0.10:0.73	n.d.	n.d.		73	—	20	7	—	—
F3T3	2.00[f]	94.0	29.4	+5.7	1.00:0.32:0.10:1.02	n.d.	n.d.	59	11	—	23	7	—	—

[a] Yields are given as percentages of the recovered for F1–F3 (65.1%) and F3T1–F3T3 (81.3%).
[b] A 6.0% yield (as percentage of the recovered) fraction with 79% protein and 20% carbohydrate precipitated at 0.00–0.05 M KCl and was discarded.
[c] Determined after hydrolysis of the sample and further derivatization of monosaccharides to the alditol acetates.
[d] Only 3,6-anhydro-D-galactose was detected in the ^{13}C NMR spectra. For F3 and F3T3 this was also confirmed using an analytical procedure (see Experimental).
[e] F3 contained the following cations (expressed as wt %): Na^+, 0.38; K^+, 0.09; Ca^{2+}, 1.8; Mg^{2+}, 0.81.
[f] Fraction soluble in 2.00 M KCl.
[g] n.d. = not determined.

Table 3. Composition (mol %) of sugars produced by permethylation and hydrolysis of F1–F3, F1DS–F3DS, and F3T3

Monosaccharide	F1	F1DS	F2	F2DS	F3	F3DS	F3T3
2,4,6-Me_3 Gal	5	37	3	38	2	42	7
2,3,6-Me_3 Gal	3	19	3	12	5	18	5
4,6-Me_2 Gal	23	—	26	—	31	—	17
2,6-Me_2 Gal	—	8	—	11	—	11	5
3,6-Me_2 Gal	—	6	—	9	1	12	4
2,3-Me_2 Gal	—	—	—	—	—	—	—
2,4-Me_2 Gal	—	2	—	2	1	4	3
6-Me Gal[a]	22	tr[b]	22	tr	20	—	14
2-Me Gal	2	4	2	3	4	2	5
3-/4-Me Gal[a]	3	—	5	—	3	—	6[c]
Gal[a]	20	3	20	4	10	tr	8
AnGal	18	1	15	tr	12	—	8
2-Me AnGal	2	15	1	17	5	11	13
2,3,4-Me_3 Xyl	tr	tr	—	—	3	tr	—
Xyl	2	—	3	—	2	—	—
Glc	—	5	—	4	1	tr	5

[a] Determination of the absolute configuration indicated these galactose derivatives belonged to the D-series.
[b] Percentages lower than 1% are considered as traces (tr).
[c] Hydrolysis of the samples and further derivatization of the monosaccharides to the aldononitrile acetates showed the presence of 3-Me Gal (2%) and 4-Me Gal (4%).

from tetrasporic *Gigartina lanceata*, a broad band centered at 837 cm^{-1} with a discernible shoulder at 820 cm^{-1} was observed, and this was assigned to the equatorial 2-sulfate and 6-sulfate ester groups, respectively.[25] In the spectra of *C. variegata* fractions, the signal at 935 cm^{-1} is characteristic of the 3,6-anhydrogalactopyranosyl residues, and the shoulder at 805–803 cm^{-1} indicates sulfation at the 2-position.[25] However, according to Anderson et al.[26] the broad signal centered at 844–835 cm^{-1} could also include signals of axial 4-sulfate in 3-linked galactopyranosyl units and equatorial 3-sulfate in 4-linked galactopyranosyl units, as well as the presence of primary 6-sulfate in the galactan backbone.

2.4. Desulfation

In order to determine unequivocally the glycosidic linkages and the substitution pattern in the backbone, desulfation–methylation analysis of the fractions was carried out. In a first approach the pyridinium salt of F3 was heated in 89:10:1 dimethyl sulfoxide–methanol–pyridine, but no desulfation was observed.[27–30] At the same time, treatment with chlorotrimethylsilane (TMSCl) in anhydrous pyridine at 100 °C for 8 h gave 45% desulfation.[31] When heating was applied to the polysaccharide in dimethyl sulfoxide–pyridine in the presence of pyromellitic acid and As_2O_3,[30] the partially desulfated derivative of F3 (F3DS, 72% of desulfation) was obtained in ~47% yield. For desulfated F1 (F1DS, 70% of desulfation) and F2 (F2DS, 78% of desulfation) the yields were ~71% and ~55%, respectively (Table 2). It can be seen that, although this method of

desulfation led to molecular weight reduction, the monosaccharide compositions of the original and desulfated products remained similar.

F1DS–F3DS were subjected to methylation analysis (Table 3). In the three desulfated derivatives, considerable amounts of 2,4,6-tri-*O*-methylgalactose, 2,3,6-tri-*O*-methylgalactose and 3,6-anhydro-2-*O*-methylgalactose were detected, concomitant with the disappearance of 4,6-di-*O*-methylgalactose, 6-*O*-methylgalactose, and 3,6-anhydrogalactose, and an important decrease of galactose. In addition, significant molar percentages of 2,6-di-*O*-methylgalactose and 3,6-di-*O*-methylgalactose were found.

These results were consistent with an α-(1→3)-, β-(1→4)-alternating galactan structure sulfated at the 2-position of the 3-linked galactose and at the same position of the 3,6-anhydrogalactose. However, the presence of 6-*O*-methylgalactose and galactose, in the permethylated native polysaccharides precluded any further assignment by desulfation–methylation analysis. Thus, 2,3,6-tri-*O*-methylgalactose detected in the permethylated desulfated derivative could derive from 4-linked galactopyranosyl 2,3-disulfate or from 4-linked galactopyranosyl 2,3,6-trisulfate units in the native fraction. Similarly, 2,4,6-tri-*O*-methylgalactose could arise from 3-linked galactopyranosyl 2,4-disulfate or from 3-linked galactopyranosyl 2,4,6-trisulfate residues.

2.5. Alkaline treatment of F3

Alkaline treatment, of the modified product[32] followed by fractionation with potassium chloride, provides an alternative method to separate different types of carrageenans for further analysis.

F3 was treated with aqueous sodium hydroxide to afford F3T, which by further fractionation with potassium chloride, gave two minor fractions that precipitated in the ranges 0–0.05 M KCl (F3T1) and 1.80–2.00 M KCl (F3T2), and a main product soluble in 2.00 M KCl (F3T3). Table 2 shows the composition of F3T and F3T1–F3T3. The galactose:3,6-anhydrogalactose:sulfate molar ratio of F3T (1.00:23:1.43) was very similar to that of F3 (1.00:0.29:1.49), exhibiting only a small decrease in the degree of sulfation and in 3,6-anhydrogalactose. This result indicated either the absence of sulfation on the 6-position of the 4-linked galactopyranosyl unit or sulfation on the 6-position and substitution on the 3-position. However, the major product F3T3 showed a lower degree of sulfation (galactose: sulfate molar ratio, 1.00:1.02) than F3T, suggesting loss of sulfated fragments during the dialysis step after fractionation with potassium chloride.

Some differences were observed in the methylation analysis of F3T3 in comparison with F3: an increase in the content of 3,6-anhydro-2-*O*-methylgalactose and the appearance of 2,6-di-*O*-methylgalactose, together with a decrease in 4,6-di-*O*-methylgalactose and 6-*O*-methylgalactose (Table 3).

2.6. ^{13}C NMR spectroscopy

In the spectrum of F1 four anomeric signals were observed at 101.3, 100.0, 94.1, and 92.6 ppm (Fig. 1). From the methylation results and considering D-configuration for galactose, the following nine diads could be tentatively proposed: G2S→DA2S (nomenclature of Knutsen et al., θ-carrageenan), G2S→D2S,3S, G2S→D2S,3S,6S, G2S,4S→D2S,3S, G2S,4S→DA2S, G2S,4S→D2S,3S,6S, G2S,4S,6S→D2S,3S, G2S,4S,6S→DA2S, and G2S,4S,6S→D2S,3S,6S.[33]

An examination of the anomeric signals present in the ^{13}C NMR spectrum of this fraction excluded the presence of G2S→DA2S (absence of a signal at 95.6 ppm due to C-1 of DA2S, relative to internal Me_2SO at 39.4 ppm).[34] The signals at 101.3 and 92.6 ppm were tentatively assigned to the C-1 of G2S,4S and DA2S in the diad G2S,4S→DA2S. In fact, the resonance at 101.3 ppm is in agreement with the chemical shift previously reported for the C-1 of G2S,4S in G2S,4S→DA, and the resonance at 92.6 ppm is coincident with the C-1 of DA2S in τ-carrageenan where the α-unit is also linked to a β-D-galactose sulfated on the 4-position.[35] In addition, the higher intensity of the peak at 101.3 ppm suggested that this signal could include the C-1 of both units in the diad G2S→L.[36]

Hence the galactose, detected in the methylation analysis, should mainly derive from D2S,3S,6S, and the resonances at 100.0 and 94.1 ppm would correspond to the C-1 of G2S and D2S,3S,6S in G2S→D2S,3S,6S. Taking into account linkage analysis, the diad

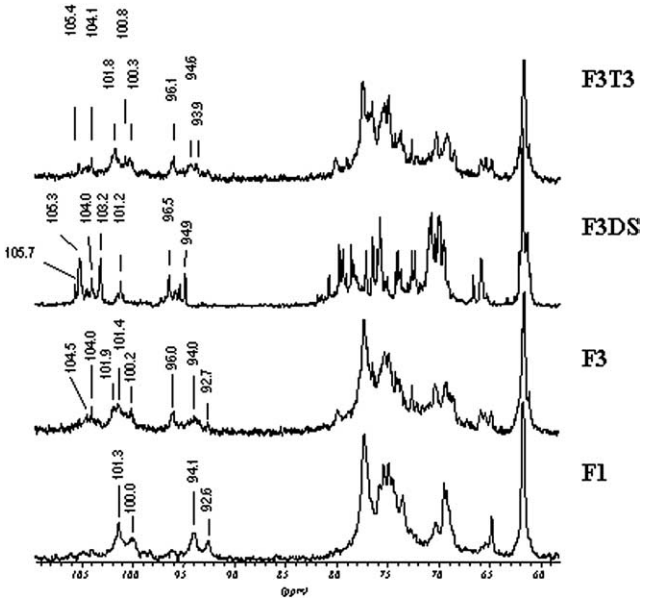

Figure 1. ^{13}C NMR spectra of F1, F3, F3DS, and F3T3.

G2S,4S→D2S,3S,6S should be present in minor amounts. Table 4 shows the chemical shifts of the anomeric signals of the various diads present in the ^{13}C NMR spectrum of F1.

The spectrum of F2 (not shown) exhibited the same anomeric signals as F1, together with a resonance at 96.3 ppm. The signal at 96.3 ppm was assigned to the C-1 of DA2S in G2S→DA2S (θ-carrageenan) (Table 4).[34]

The signals in the anomeric region of F3 (Fig. 1) were similar to those of F2, but differed in the presence of signals at 104.5, 104.0, and 101.9 ppm (Table 4). The small signal at 104.5 ppm (together with the ones at 65.9 and 76.5 ppm) can be ascribed to β-xylopyranosyl sidechains linked to C-6 of the galactan backbone.[37] The peak at 104.0 ppm is consistent with G linked to L (101.4 ppm).[38] An examination of the spectrum indicated a higher contribution of the θ-carrageenan diad and a smaller one of G2S,4S→DA2S than in F2. The resonance at 101.9 ppm remains unassigned.

In the anomeric region of the spectrum of desulfated F1 (not shown), signals at 105.1, 104.5, 104.0, 103.2, 101.2, 96.4, 95.8, 95.4, and 94.9 ppm were observed (Table 4). The main resonances at 105.1 and 96.4 ppm, and 103.2 and 94.9 ppm were assigned to G→D and G→DA, respectively.[31,34] Small peaks at 104.0 and 101.2 ppm are in agreement with the presence of G→L. As stated before, the peak at 104.5 ppm could derive from single stubs of β-(1→6)-linked D-xylose. The minor signals at 95.8 and 95.4 ppm remain unassigned. The signals in the anomeric region of F3DS were similar to those of F1DS, but differed in the presence of a resonance at 105.7 ppm and the relative higher intensity of the signals at 104.0, 101.2, 95.9, and 95.5 ppm. The higher intensity of the signals at 104.0 and 101.2 ppm are consistent with higher content of L-galactose detected in this fraction. The resonance at 105.7 ppm also remains unassigned.

Figure 1 shows the spectrum of F3T3 where the same signals as for F3 were observed, together with resonances at 105.4, 100.8, and 94.6 ppm (Table 4). The resonance at 105.4 ppm would correspond to the C-1 of G in G→D, while the signals at 100.8 and 94.6 ppm would account for the C-1 of G2S and DA in the diad G2S→DA. The chemical shift of the anomeric signal of the DA unit is similar to the value reported for DA (94.7 ppm) in β-carrageenan.[31]

Table 4. Anomeric chemical shifts (ppm) of various diads present in the ^{13}C NMR spectra of F1–F3, F1DS, F3DS, and F3T3

Fraction	Diads	3-Linked unit	4-Linked unit
F1	G2S→D2S,3S,6S	100.0	94.1
	G2S,4S→DA2S	101.3	92.6
	G2S→L	101.3	101.3
F2	G2S→D2S,3S,6S	100.3	94.0
	G2S,4S→DA2S	101.3	92.6
	G2S→L	101.3	101.3
	G2S→DA2S	100.3	96.3
F3	G2S→D2S,3S,6S	100.2	94.0
	G2S,4S→DA2S	101.4	92.7
	G2S→L	101.4	101.4
	G2S→DA2S	100.2	96.0
	G→L	104.0	101.4
F1DS	G→D	105.1	96.4
	G→DA	103.2	94.9
	G→L	104.0	101.2
F3DS	G→D	105.3	96.5
	G→DA	103.2	94.9
	G→L	104.1	101.2
F3T3	G2S→D2S,3S,6S	100.3	93.9
	G2S,4S→DA2S	101.8	92.7
	G2S→DA2S	100.3	96.1
	G2S→DA	100.8	94.6
	G→D	105.4	96.1
	G→L	104.1	101.8

2.7. Antiviral activity

Table 5 summarizes the results of the antiviral activity of the fractions in a plaque-reduction assay for HSV-1 (strain F), HSV-2 (strain G), and DENV-2 (strain NGC). F1–F3 were potent inhibitors of HSV-1 and HSV-2, with values of IC_{50} ranging from 0.16 to 2.19 μg mL^{-1}, and DENV-2 with values of IC_{50} from 0.10 to 0.41 μg mL^{-1}. The desulfated derivatives F1DS and F2DS were significantly less active than the fractions F1–F2 (IC_{50} 7.53–43.76 μg mL^{-1}). Moreover, the desulfated derivative F3DS was inactive up to a concentration of 100 μg mL^{-1}. Despite the fact that F3 was treated with alkali, no variations in the IC_{50} values of F3T were observed, and F3T3 retained its antiviral properties although the IC_{50} value was approximately twofold higher. In order to increase the antiviral activity spectrum, F3 was also tested against two TK$^-$ acyclovir-resistant variants of HSV-1 and against HCMV (strain Davis). The values of IC_{50} obtained for HSV-1 TK$^-$

Table 5. Antiviral activity of F1–F3, F1DS–F3DS, F3T, and F3T3 expressed as IC_{50} (μg mL^{-1})

Fraction[a]	HSV-1 (F)	HSV-2 (G)	DENV-2 (NGC)
F1	0.16 ± 0.02	0.24 ± 0.01	0.41 ± 0.12
F1DS	9.99 ± 0.67	7.53 ± 0.48	n.d.[b]
F2	1.55 ± 0.37	2.19 ± 0.70	0.10 ± 0.01
F2DS	43.76 ± 1.13	31.92 ± 3.88	n.d.
F3[c,d]	0.18 ± 0.07	0.21 ± 0.08	0.29 ± 0.06
F3DS	>100	>100	n.d.
F3T[c]	0.18 ± 0.02	0.21 ± 0.01	n.d.
F3T3[c]	0.46 ± 0.02	0.47 ± 0.07	n.d.

[a] Cytotoxicity (CC_{50})>1000 μg mL^{-1} for all the tested fractions.
[b] n.d. = not determined.
[c] Virucidal concentration (VC_{50}) for HSV-1 (F) > 10 μg mL^{-1}.
[d] IC_{50} (μg mL^{-1}) also tested against HSV-1 TK$^-$ B2006 (0.47 ± 0.01), HSV-1 TK$^-$ Field (0.35 ± 0.13), and HCMV (0.79 ± 0.2).

B2006, HSV-1 TK$^-$ Field, and HCMV were 0.47 ± 0.01, 0.35 ± 0.13, and 0.79 ± 0.2 μg mL^{-1}, respectively. The virucidal concentration (VC_{50}) was also estimated for F3 against HSV-1 (strain F) and resulted at least two orders of magnitude higher than the IC_{50} (>10 μg mL^{-1}). None of the products were cytotoxic at concentrations as high as 1000 μg mL^{-1}.

3. Discussion

The polysaccharides, extracted with water at room temperature, from *C. variegata* have a highly sulfated backbone. The results indicate, as previously deduced that the galactan backbone of F1 consists mainly of G2S,4S→DA2S and G2S→D2S,3S,6S. In F2 these diads are present together with low amounts of G2S→DA2S (θ-carrageenan) whose contribution becomes higher in F3. In addition, if no departure from the α-(1→3)-, β-(1→4)-alternating structure is assumed,[39] some of the 6-*O*-methylgalactose units detected in the methylation analysis of F3 should derive from α-D-galactopyranosyl 2,3-disulfate residues.

However, the polysaccharides of *C. variegata* are not true carrageenans because they also contain minor amounts of L-galactose. ^{13}C NMR spectroscopy is consistent with the presence of G2S→L in the three fractions and of G→L in F3. This last diad is clearly observed in the spectra of desulfated F1 and F3.

On the other hand, the polysaccharides extracted from *C. hombroniana* with hot water[11,13] were less sulfated and contained a large proportion of θ-carrageenan, together with minor amounts of λ-carrageenan and G2S→DA, and L-galactose residues; in addition the content of pyruvate was estimated as up to 0.8%.[11] The infrared spectrum of this polysaccharide showed an intense band at 1250 cm^{-1} and a broad band at 830 cm^{-1} with a broad, but discernible, shoulder at 816 cm^{-1}; a peak due to 3,6-anhydrogalactosyl at 935 cm^{-1} was also observed. The ^{13}C NMR spectrum of the desulfated polysaccharide mainly exhibited the signals of β-carrageenan.

It is known that seaweeds concentrate divalent cations. In the case of *C. variegata*, it is noteworthy that the polysaccharides of F3 retained calcium and magnesium as the major counterions, in spite of being treated with massive amounts of potassium chloride (Table 2). It is possible that the high percentage of calcium and magnesium could be related with the rather low sulfate values determined (Tables 1 and 2).

Recently, Miller[40] reported that the polysaccharides from *Trematocarpus acicularis* and *Sarcodia montagneana* contained high amounts of 2,4,6- and/or 2,3,6-trisulfated galactopyranosyl units. In the ^{13}C NMR spectrum of the polysaccharide from *Sarcodia*, a signal at 94.9 ppm was assigned to the C-1 of L2S,3S,6S in GP2S→L2S,3S,6S, but only a small resonance, corresponding to a sulfated C-6, was detected at ~68 ppm. In our case, no signal was observed around 68 ppm, although considerable amounts (10–20%) of unmethylated galactose were identified even after exhaustive methylation analysis. However, a comparison of the ^{13}C NMR spectra of F1 and F2 with those of F3 and F3T3 (Fig. 1) indicates that a peak at 69.5 ppm could be tentatively assigned to that resonance.

Potent antiviral activity of the polysaccharides from *C. variegata* is in agreement with the structures proposed, since they fulfill features such as adequate molecular weight and sulfate on the 2-position of the 3-linked unit and on the 2-position of the 4-linked unit in the diads G2S→DA2S and G2S,4S→DA2S.[41] In the diad G2S→D2S,3S,6S sulfation in the 2-position of the 3-linked unit and on the 6-position of the 4-linked unit is consistent with the sulfation of the minimal binding domain to the virus in λ-carrageenan;[41] however, the influence of sulfation on the 3-position remains uncertain. Since virucidal concentration of F3 highly exceeded the antiviral IC_{50} for HSV-1, the inhibitory effect of this sulfated polysaccharide appears to be based mainly on its ability to interfere with the replication cycle of HSV-1, in particular with the initial attachment of the virus to the target cell. The potent antiviral activity exerted by the compounds and the low cytotoxicity revealed high selectivity indices, indicating that they can be considered very promising antiviral agents.

The study of representatives from families, which have received little attention up to the moment, is revealing unusual polysaccharide structures or, at least, departing from the traditional ones,[39] as in the present case. Within the order Gigartinales *s.s.*,[3] the families Kallymenicaceae and Dumontiaceae include most of the species known to produce galactans containing substantial amounts of agarans together with carrageenans. Previous reports on different *Callophyllis* species have coincided in classifying the galactans from these algae as highly sulfated and complex, that is, agarans and carrageenans in *C. rangiferina*[6] and *C. cristata*,[8] carrageenans in *C. rhynchocarpa*,[8,12] λ/θ/α-carrageenans in *C. variegata* (only by infrared spectroscopy),[6] and chiefly θ-carrageenan in *C. hombroniana*.[11,13] On the other hand, molecular biology data showed that *C. cristata* falls in a different clade than *Callophyllis s.s.*[4] Unfortunately, no molecular biology data are available for *C. hombroniana*, *C. rhynchocarpa* and *C. rangiferina*, and no polysaccharide structures have been reported for the totality of *Callophyllis* species, but the ratio of agaran/carrageenan, as well as the major type of carrageenan, might prove to be a significant chemotaxonomic marker for the genus.[5,6]

Sulfohydrolase isolated by Wong and Craigie[42] from tetrasporic plants of *Chondrus crispus* catalyzes the conversion of μ- to κ-carrageenan, yet cannot convert λ- to θ-carrageenan. According to Falshaw et al.,[11]

θ-carrageenan would be a significant natural component in *C. hombroniana*, and the same seems to be true for *C. variegata*. The presence of the unusual structure G2S,4S→DA2S could represent a precursor for θ-carrageenan. Note that fractions enriched in θ-carrageenan are poorer in G2S,4S→DA2S. Sulfation in the 2- and 4-position of the 3-linked unit has been also suggested for the polysaccharide of *Kallymenia berggrenii*.[10]

4. Experimental

4.1. Materials

C. variegata was collected from Cueva de los Leones, Puerto Deseado (Santa Cruz, Argentina) in April of 1993, dried in the open under strong winds and carefully hand sorted. A voucher specimen (HRP 6139 *C. variegata*) was deposited in the herbarium of A. Boraso (Comodoro Rivadavia, Chubut, Argentina).

4.2. Extractions

Cystocarpic plants (45 g), previously milled, were extracted with water (1.5 L) with mechanical stirring for 24 h at rt. The residue was removed by centrifugation, and the supernatant was dialyzed (see General Methods), concentrated, and freeze-dried. The residue was extracted (×2) in the same way; the crude products corresponding to the three extractions were pooled to afford the native polysaccharide (yield, 7.5 g).

4.3. Fractionation with potassium chloride

The native polysaccharide (6.8 g) was dissolved in water (2.7 L). Solid, finely divided KCl was added in small portions with constant and violent mechanical agitation so that the concentration was increased by 0.05 M each time. After each addition, stirring continued for 5–16 h to ensure equilibration of the system; the upper limit of potassium chloride concentration was 2.00 M. The precipitates, separated by centrifugation, as well as the residual solution, were dialyzed, concentrated, and freeze-dried.

Alkali-treated F3 (F3T, 560 mg) was dissolved in water (225 mL) and fractionated with potassium chloride as described for the native polysaccharide.

4.4. Alkaline treatment

For analytical treatment, sample F3 (103 mg) was dissolved in water (50 mL), and $NaBH_4$ (5 mg) was added. After 24 h at rt, 3 M NaOH was added (25 mL) with a further quantity of $NaBH_4$ (3 mg). The solution was heated at 80 °C for 15 h, and the content of 3,6-anhydrogalactose was determined on the samples removed at intervals (2, 4, 6, 8, and 15 h). A constant value for 3,6-anhydrogalactose was obtained after 2 h.

For preparative treatment, F3 (755 mg) was dissolved in water (375 mL), and $NaBH_4$ (39 mg) added. After 24 h at rt, 3 M NaOH was added (188 ml) with a further quantity of $NaBH_4$ (30 mg). The solution was heated at 80 °C for 2 h. The solution was cooled at room temperature, dialyzed, concentrated, and freeze-dried. Yield of F3T: 680 mg.

4.5. General methods

Carbohydrate content was analyzed by the phenol–H_2SO_4 method without previous hydrolysis of the polysaccharide.[43] The presence of 3,6-anhydrogalactose was determined independently by the resorcinol–HCl method.[44] Sulfate was measured using the turbidimetric method of Dodgson and Price after hydrolysis of the samples with 1 M HCl for 4–5 h at 105–110 °C.[45] For 1C–3C, nitrogen was analyzed by the method of Dumas and Pregl, and protein was calculated by multiplying the nitrogen content by 6.25.[46] For fractions F1–F3 protein was determined by the method in Ref. 47. Molecular weight was estimated by the method of Park and Johnson.[48] Pyruvic acid was determined by the method of Ref. 18. Optical rotations (Na D-line) were measured in a Perkin–Elmer 343 polarimeter using 0.2–0.4% solutions of the polysaccharides in 0.1 M NaCl. Unless otherwise stated, dialyses were carried out with tubing with a molecular weight cutoff of 3500 Da.

Na^+, K^+, Ca^{2+}, and Mg^{2+} determinations were carried out by flame atomic absorption spectrometry using a Shimadzu AA 6800 atomic absorption spectrometer.

The Fourier-transform infrared spectra were recorded with a 510P Nicolet FTIR spectrophotometer, using a film of the sample, at 4000–250 cm^{-1}, 32–64 scans were taken with a resolution of 2–4 cm^{-1}. Derivation was performed using the Omnic software package incorporated into the hardware of the instrument.

Reductive hydrolysis of the samples and acetylation of the sugar mixtures were performed as described in Ref. 22. The permethylated samples were also hydrolyzed with 2 M TFA at 121 °C for 2 h, and the monosaccharides were further derivatized to the aldononitrile acetates.[49]

GLC of alditol acetates and aldononitrile acetates were carried out on a Hewlett–Packard 5890A gas chromatograph equipped with a flame-ionization detector and fitted with a fused-silica column (0.25 mm i.d × 30 m) WCOT-coated with 0.20-μm film of SP-2330. Chromatography was carried out at (a) 220 °C isothermally for alditol acetates; (b) from 180 °C (2-min hold) to 230 °C at 1 °C min^{-1}, followed by a 30-min hold, for partially methylated alditol acetates; (c) from 180 °C (2-min hold) to 210 °C at 1 °C min^{-1} then at 2 °C min^{-1} to 230 °C, followed by a 30-min hold, for

partially methylated aldononitrile acetates. Nitrogen was used as carrier at a flow rate of 1 mL min^{-1}. The split ratio was 80:1. The injector and detector temperature was 240 °C.

Conversion of GLC areas to molar basis was calculated for the partially methylated alditol acetates according to the effective carbon response theory.[50] For 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrolgalactitol and 1,4,5-tri-*O*-acetyl-3,6-anhydro-2-*O*-methylgalactitol, values of 0.72 and 0.64, respectively, were used.[22]

GLC–MS of the methylated alditol acetates was carried out on a GCMS-QP 5050A gas chromatograph/mass spectrometer (Shimadzu Corporation). Chromatography was performed on the SP-2330 capillary column using the programme temperature (b). The He total flow rate was 4.4 mL min^{-1}, the head pressure 12 psi; the injector temperature 250 °C, and the split ratio 10:1. Mass spectra were recorded over a mass range of 30–600 amu, using an ionization potential of 70 eV.

The ratio of D-:L-galactose in the native and alkali-treated samples and the absolute configuration of galactose and the monomethylated units in the permethylated polysaccharides was estimated by the method of Cases et al.[51] The absolute configuration of 3,6-anhydrogalactose was determined by the method of Errea et al.[52]

For ^{13}C NMR spectroscopy, samples (10–30 mg) were dissolved in 1:1 H_2O–D_2O (0.5 mL) and 5-mm tubes were used. The 125-MHz ^{13}C NMR ^{1}H-decoupled spectra were recorded at rt on a Bruker AM 500 spectrometer using a spectral width of 26.0/29.0 kHz, a 58°/64° pulse, an acquisition time 0.6 s, and a relaxation delay 0.2 s; for 22,600–54,040 scans. In all cases, signals were referenced to internal Me_2SO at 39.6 ppm.

4.6. Desulfation

F3 was desulfated using two different methods.

Method 1: F3 (63.3 mg) was converted into the corresponding pyridinium salt. This salt (51.6 mg) was suspended in anhydrous pyridine (6.4 mL) and was treated with TMSCl (2.7 mL) at 100 °C for 8 h, as described in Ref. 31. Yield: F3DSTMSCl, 36.8 mg.

Method 2: F3 (200.6 mg) was suspended in dry Me_2SO (9 mL) and was treated with As_2O_3 (200 mg), pyromellitic acid (150 mg), and anhydrous pyridine (1.5 mL) at 120 °C for 3 h, as described in Ref. 30. Dialysis was carried out with tubing with a molecular weight cutoff of 1000 Da. Yield: F3DS, 82.9 mg.

F1 (137.4 mg) and F2 (170.4 mg) were desulfated using Method 2. Yields: F1DS, 78.0 mg; F2DS, 67.0 mg.

4.7. Methylation analysis

F1 (24.5 mg), F2 (7.9 mg), F3 (33.0 mg), F3T3 (22.7 mg), F1DS (11.3 mg), F2DS (10.8 mg) and F3DS (20.7 mg) were converted into the corresponding triethylammonium salts[20] and were methylated by the Hakomori procedure (sodium methylsulfinylmethanide–iodomethane)[21] as modified by Stevenson and Furneaux.[22] The procedure was repeated to ensure the permethylation. Yields: F1, 23.8 mg; F2, 9.3 mg; F3, 12.0 mg; F3T3, 22.0 mg; F1DS, 12.7 mg; F2DS, 9.5 mg; F3DS, 24.8 mg.

The triethylammonium salt of F1 (18.7 mg) and methylated F2 (8.3 mg) were dissolved in Me_2SO (2 and 1 mL, respectively) containing 7% LiCl.[23] Methylation was carried out by the method of Ciucanu and Kerek (NaOH–iodomethane).[24] Yields: F1, 10.8 mg; methylated F2, 6.7 mg.

4.8. Biological activities

4.8.1. Cells and viruses. Vero (African green monkey kidney) cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum. For maintenance medium (MM), the serum concentration was reduced to 1.5%. The C6/36 HT mosquito cell line from *Aedes albopictus* was cultured at 28 °C in L-15 medium (Leibovitz) supplemented with 0.3% tryptose phosphate broth, 0.02% glutamine, 1% MEM non-essential amino acids solution, and 5% fetal calf serum. The human diploid foreskin fibroblast cell line PH was provided by Dr. G. Carballal (CEMIC, Buenos Aires, Argentina) and propagated in MEM supplemented with 10% fetal calf serum.

HSV-1 (strain F), HSV-2 (strain G), and HCMV (strain Davis) were obtained from the American Type Culture Collection (Rockville, USA). B2006 and Field were HSV-1 TK^- acyclovir-resistant strains obtained from Professor Dr. E. De Clercq (Rega Institute, Belgium). All HSV stocks were propagated and titrated by plaque formation in Vero cells. DENV-2 (strain NGC) was provided by Dr. A. S. Mistchenko (Hospital de Niños Dr. Ricardo Gutiérrez, Buenos Aires, Argentina). DENV-2 stocks were prepared in C6/36 HT cells and titrated by plaque formation in Vero cells. HCMV was propagated and titrated by a cytopathic effect assay in PH cells.

4.8.2. Cytotoxicity assay. Vero cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich) method. Confluent cultures in 96-well plates were exposed to different concentrations of the polysaccharides, with three wells for each concentration, using incubation conditions equivalent to those used in the antiviral assays. Then 10 μL of MM containing MTT (final concentration 0.5 mg mL^{-1}) was added to each well. After 2 h of incubation at 37 °C, the supernatant was removed and 200 μL of ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader at

595 nm. The cytotoxic concentration 50% (CC_{50}) was calculated as the compound concentration required to reduce cell viability by 50%.

4.8.3. Antiviral assays. The antiviral activity against HSV-1, HSV-2, and DENV-2 was determined by a virus plaque-reduction assay. Vero cell monolayers grown in 24-well plates were infected with about 50 PFU/well in the absence or presence of various concentrations of the compounds. After 1 h of adsorption at 37 °C, the residual inoculum was replaced by MM containing 0.7% methylcellulose and the corresponding dose of each compound. Plaques were counted after 2 days of incubation at 37 °C for HSV-1 and HSV-2, or after 7 days for DENV-2. The inhibitory concentration 50% (IC_{50}) was calculated as the compound concentration required to reduce virus plaques by 50%. All determinations were performed twice and each in duplicate.

For HCMV, a cytopathic effect inhibition assay was performed. PH cell monolayers were infected in quadruplicate with HCMV at a multiplicity of infection of 0.1 in the absence or presence of various concentrations of the compounds. Cell controls were included in each experiment. After 7 days of incubation at 37 °C, the cytopathic effect was examined under an inverted microscope. The inhibitory concentration 50% (IC_{50}) was calculated as the concentration required to reduce virus induced cytopathogenicity by 50%.

4.8.4. Virucidal assay. A virus suspension of HSV-1 strain F containing 5×10^4 PFU was incubated with an equal volume of MM with or without compound for 2 h at 37 °C. The samples were then diluted in cold MM to determine residual infectivity in a plaque formation assay using Vero cells.

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