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Structural analysis and antiviral activity of a sulfated galactan from the red seaweed *Schizymenia binderi* (Gigartinales, Rhodophyta)

Betty Matsuhiro,[a,*] Ana F. Conte,[b] Elsa B. Damonte,[b] Adriana A. Kolender,[c] María C. Matulewicz,[c] Enrique G. Mejías,[a] Carlos A. Pujol[b] and Elisa A. Zúñiga[a]

[a]*Departamento de Ciencias Químicas, Facultad de Química y Biología, Universidad de Santiago de Chile, Casilla 40, Santiago 33, Chile*
[b]*Laboratorio de Virología, Departamento de Química Biológica, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón 2, 1428 Buenos Aires, Argentina*
[c]*Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón 2, 1428 Buenos Aires, Argentina*

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Abstract—Aqueous extraction of gametophytic *Schizymenia binderi* afforded a polysaccharide composed of galactose and sulfate groups in a molar ratio of 1.0:0.89 together with uronic acids (6.8 wt %) and minor amounts of other neutral sugars. Alkali-treatment of the polysaccharide afforded a polysaccharide devoid of 3,6-anhydrogalactose. ^{13}C NMR spectroscopy of the desulfated alkali-treated polysaccharide showed a backbone structure of alternating 3-linked β-D-galactopyranosyl and 4-linked α-galactopyranosyl units that are predominantly of the D-configuration and partly of the L-configuration. Methylation, ethylation and NMR spectroscopic studies of the alkali-treated polysaccharide indicated that the sulfate groups are located mainly at positions O-2 of 3-linked β-D-galactopyranosyl residue and at position O-3 of 4-linked-α-galactopyranosyl residues, the latter is partially glycosylated at position O-2. The sulfated galactan from *S. binderi* exhibited highly selective antiviral activity against *Herpes simplex* virus types 1 and 2, with selectivity indices (ratio cytotoxicity/antiviral activity) >1000 for all assayed virus strains. This compound was shown to interfere with the initial adsorption of viruses to cells.

Keywords: *Schizymenia binderi*; Rhodophyta; Sulfated galactan; Uronic acids; Natural antiviral

1. Introduction

Soluble polysaccharides from a few species of the genus *Schizymenia* (Gigartinales, Rhodophyta) have been studied. Whyte et al.[1] in a study of red algae from British Columbia, reported that the polysaccharide from *Schizymenia pacifica* was a sulfated D-galactan with a low amount of 3,6-anhydrogalactose. Bourgougnon and co-workers,[2,3] found that the polysaccharide from gametophytic *Schizymenia dubyi* is a sulfated glucuronogalactan. No 3,6-anhydrogalactose was detected and 45% of total galactose was in the L-form. On the other hand, Deslandes et al.[4] reported that the water-soluble polysaccharide from *S. dubyi* was a λ-carrageenan type polysaccharide.

Sulfated polysaccharides from red seaweeds have shown antiviral activities.[5–9] For example, the aqueous extract from *S. pacifica* inhibited in vitro avian retrovirus and mammalian retrovirus reverse transcriptase.[10] In other studies, the sulfated polysaccharide isolated from this algae showed antihuman immunodeficiency virus (HIV) activity in vitro,[11] while the sulfated glucuronogalactan from *S. dubyi* inhibited the in vitro replication of HIV.[12,13] Cáceres et al.[14] reported that the polysaccharides from cystocarpic and tetrasporic *Stenogramme interrupta* showed antiviral effects against different

* Corresponding author. Tel.: +56 2 6812575; fax: +56 2 6812108; e-mail: bmatsuhi@lauca.usach.cl

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strains of herpes simplex virus (HSV). The antiherpetic activity of these polysaccharides was similar to that previously found for the κ/ι- and λ-carrageenans isolated from *Gigartina skottsbergii*.[15] This work presents the results of the structural analysis of the soluble polysaccharide from *Schizymenia binderi* and its antiviral activity.

2. Results and discussion

2.1. Native polysaccharide

Aqueous extraction of gametophytic *S. binderi* J. Ag. afforded 17.6 dry weight percent of a polysaccharide composed mainly of galactose and hemi-ester sulfate in a molar ratio of 1.0:0.89, together with 6.8 wt % of uronic acids. Gel permeation chromatography analysis on Sephadex G-200 suggested that it was homogeneous, and the average molecular weight was estimated at 380,000. This value was in good agreement with that determined spectrophotometrically by the reducing-end method (310,800). Reductive hydrolysis of the polysaccharide, followed by acetylation and subsequent GC–MS analysis of the resulting acetylated alditols, indicated the presence of minor amounts of other neutral sugars (Table 1). The FT-IR spectrum showed a shoulder around 1750 cm^{-1} and strong absorbance at 1261.3 cm^{-1} due to the S=O asymmetric stretching vibration of sulfate groups, and a medium intensity band at 844.5 cm^{-1} assigned to S–O stretching vibration of sulfate groups on C-4 of galactose residues.[16,17] The second-derivative spectrum showed new signals, one at 1741.4 cm^{-1} (assigned to the C=O stretching vibration of a carboxyl acid group) and another, a small signal at 817.8 cm^{-1}, which was at lower wave number than the expected value for the S–O stretching vibration of primary sulfate group (820 cm^{-1}).[18] One more band at 583.8 cm^{-1}, due to O–S–O asymmetric deformation of sulfate groups, was present.[19] Furthermore, the second-derivative FT-IR spectrum showed signals at 1643.8, 1549.5 and 1384.1 cm^{-1} assigned to bands I, II and III of the amide function of proteins.[16] No band due to the presence of 3,6-anhydrogalactosyl residues appeared at 930 cm^{-1}, which is in agreement with the results obtained by reductive hydrolysis analysis of the polysaccharide. Moreover, 3,6-anhydrogalactose was not detected by the colorimetric assay of Yaphe and Arsenault.[20] HPLC analysis of the acidic fraction of the product obtained by total acid hydrolysis of the polysaccharide indicated the presence of glucuronic acid and an unknown uronic acid in a 3:1 ratio. No galacturonic, mannuronic or guluronic acid was detected. The nature of the uronic acids was determined by carboxyl reduction of the native polysaccharide followed by total hydrolysis and GC analysis of the alditol acetates. Co-chromatography with authentic samples of peracetates of D-glucitol and L-iditol confirmed the presence of glucuronic acid and identified iduronic acid as the minor uronic acid in the native polysaccharide.

Bourgougnon et al.[3] reported that the soluble polysaccharide from gametophytic *S. dubyi* contained 33.7% of glucuronic acid. The presence of uronic acids in polysaccharides isolated from Rhodophyta has been reported in a few cases.[21–25] Agarose-carrageenan hybrid polysaccharides from *Lomentaria catenata*, containing glucuronic acid have been characterized. This uronic acid is present as a single unit branch at O-4 of the →3-β-D-galactopyranosyl residues.[26] The xylogalactan from *Palmaria decipiens* contained 4.8% uronic acids, which were identified as galacturonic and glucuronic acid in the ratio 1.5:1.0.[27] It is interesting to note that some calcareous red algae synthesized alginic acid, which is the major polysaccharide of Phaeophyta.[28,29]

The ^{13}C NMR spectrum at 70 °C of the partially hydrolyzed polysaccharide was not well resolved and was very complex. It showed a signal at 173.5 ppm, assigned to the carbonyl carbon of the uronic acids, and two groups of anomeric carbons that were tentatively assigned with the aid of literature data.[30–32] In the β-anomeric region, a major broad signal at 104.8 ppm was assigned to the anomeric carbon of an unsulfated 3-linked β-D-galactopyranosyl residue and/or monosulfated at position O-4, linked to a →4-α-D-galactopyranosyl residue monosulfated at position O-3. The signal at 103.7 ppm was assigned to the anomeric carbon of 3-linked β-D-galactopyranosyl residues linked to a →4-α-L-galactopyranosyl residue. In the α-anomeric region, a signal at 101.1 ppm was attributed to C-1 of unsulfated 4-linked α-L-galactopyranosyl residue and/or sulfated at position O-3. The signal at

Table 1. Chemical composition (wt %) of the polysaccharides from *Schizymenia binderi*

Components	Native (%)	Alkali treated (%)
Total sugars	55.3	58.7
Neutral sugars		
Galactose	43.4	50.9
D-galactose[a]	35.8	43.2
L-galactose[a]	7.6	7.7
Glucose	1.1	Tr
Xylose	1.8	2.1
3-*O*-Methyl-galactose[b]	1.5	1.6
3,6-Anhydro-galactose	Tr	Tr
Uronic acids	6.8	3.0
Proteins	9.4 (8.9)[c]	4.9 (5.8)[c]
Sulfate (as $NaSO_3$)	22.2	26.4
Pyruvic acid	ND	ND

Percentages lower than 1% are given as trace (Tr).
ND = not detected.
[a] By GC analysis of diastereomeric derivatives produced by reductive amination with (*S*)-1-amino-2-propanol.
[b] By GC–MS analysis.
[c] By Hartree–Lowry method.

96.7 ppm was tentatively assigned to C-1 of 4-linked α-D-galactopyranosyl residue carrying sulfate groups at position O-3, considering the value of 96.9 ppm, referenced to internal Me_2SO, reported by Errea and Matulewicz.[33] At higher field, two signals at 61.5 and 61.1 ppm indicated the presence of unsubstituted primary alcoholic groups in galactopyranosyl residues. The ^1H NMR spectrum was very complex. Four broad singlets in the α-anomeric region could be found, but the rest of the spectrum was poorly resolved and gave no further useful information. Attempts to obtain information using two-dimensional techniques were unsuccessful.

2.2. Alkali-treated polysaccharide

On alkaline-treatment of the polysaccharide, a modified polysaccharide (SB1) was obtained in 76% yield. Its monosaccharide composition was studied by reductive hydrolysis, acetylation and subsequent GC analysis of the alditol acetates produced (Table 1). A very small amount of 3,6-anhydrogalactitol acetate was found. This result indicates that there was not a significant amount of 4-linked galactopyranose residues sulfated at position O-6, which could be cyclized into the corresponding 3,6-anhydro residues. Accordingly, no change in the content of sulfate residue was expected, but an increase of sulfate content was found (Table 1). This might be explained by taking into consideration that the protein linked through a covalent bond to the native polysaccharide was partly hydrolyzed in alkaline medium increasing the relative amount of sulfate group in the polysaccharide.[27] Moreover, the galactose:sulfate molar ratio (1.0:0.91) in SB1 was very similar to that of the native polysaccharide. The FT-IR spectrum of the alkali-modified polysaccharide showed a strong signal at 1256.3 cm^{-1} and a signal of medium intensity at 846.3 cm^{-1}. As in the native polysaccharide, no signal assigned to 3,6-anhydrogalactose was found. Analysis by GC of the 1-deoxy-1-(2-hydroxy-propylamino) alditol acetates derived from the reductive hydrolysis, reductive amination and acetylation product of SB1 indicated that 15.1% of galactose showed the L-configuration.

2.3. Alkali-treated, desulfated polysaccharide

The alkali-treated polysaccharide was solvolytically desulfated to give SB1 DS and analyzed by ^{13}C NMR spectroscopy. The ^{13}C NMR spectrum (Fig. 1) is similar to those reported for the desulfated polysaccharides from *Champia novae-zealandiae* and *Plocamium costatum*.[34,35] Both polysaccharides showed a backbone structure of alternating 3-linked-β-D-galactopyranosyl and 4-linked α-galactopyranosyl residues. The galactose in the latter was present in L- and D-configuration in variable proportions. The ^{13}C resonances of the spectrum in

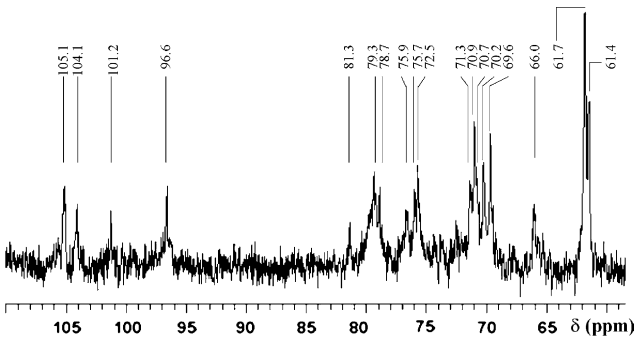

Figure 1. ^{13}C NMR spectrum (100.62 MHz) in D_2O of the desulfated alkali-treated polysaccharide from *Schizymenia binderi*.

Table 2. ^{13}C NMR chemical shifts (ppm) of desulfated SB1 from *Schizymenia binderi*

Polysaccharide	C-1	C-2	C-3	C-4	C-5	C-6
→3-*O*-β-D-Galp-1→[a]	104.1	70.2	81.3	69.6	75.9	61.7
→4-*O*-α-L-Galp-1→[a]	101.2	69.6	71.30	79.3	72.5	61.4
→3-*O*-β-D-Galp-1→[b]	105.1	70.9	79.3	66.0	75.7	61.4
→4-*O*-α-D-Galp-1→[b]	96.6	69.6	71.3	78.7	70.7	61.7

[a] Assigned according to Ref. 36.
[b] Assigned according to Ref. 30.

Figure 1 were assigned according to the literature,[30,36] and are presented in Table 2. In the results presented herein, signals were referenced to internal CH_3OH and show 0.2–0.4 ppm differences with those reported in the literature, except for C-4 (difference of 0.7 ppm) of the β-D-galactose residue linked to α-L-galactose. The D:L-ratio of galactose (5.4:1.0) determined by the peak area ratio of the anomeric carbons was consistent with the value determined by GC of the 1-deoxy-1-(2-hydroxy-propylamino) alditol acetates method (5.6:1.0). The native polysaccharide was also desulfated and analyzed by ^{13}C NMR spectroscopy. The spectrum is very similar to that of SB1 DS, but some signals, probably due to proteins, appeared at high field. Altogether, these results indicate that the sulfated galactan from *S. binderi* and SB1 comprised a backbone of alternating 3-linked β-D-galactopyranosyl and 4-linked α-galactopyranosyl units that are predominantly of the D-configuration and partly of the L-configuration with predominance of a carrageenan type backbone. The concomitance of 4-linked α-D- and α-L-galactose units in red seaweed galactans is well known.[26,34,35,37–40] Estevez et al.,[41] by fractionation techniques, isolated from gametophytes of *Gymnogongrus torulosus* (Phyllophoraceae) DL-galactan hybrids of the carrageenan- and agaran-types, together with minor quantities of agarans. The authors suggested that the hybrids galactans would correspond to a junction zone in a block copolymer. Recently, Zibetti et al.[42] found that in the complex DL-hybrid galactans from *Cryptonemia crenulata*, when the α-linked residues were galactopyranosyl residues they were in D-configu-

ration while the 3,6-anhydro-galactopyranosyl units were all in the L-configuration.[42]

2.4. Alkylation analysis

The glycosyl linkages and sulfate positions on the galactan were studied by methylation and ethylation analyses of the alkali-treated polysaccharide (SB1). The native polysaccharide was not analyzed because, in polar solvents, it formed precipitates upon standing, probably due to the presence of proteins. SB1 was subjected to carboxyl reduction to give SB1 CR prior to methylation. After two rounds of methylation, desulfation, two more rounds of methylation and GC analysis of the partially methylated galactitol acetates, under methylation was suspected due to the relatively high content of the 2,6-di-*O*-methyl-galactitol acetate. No derivatives of glucose or idose were detected, probably due to the low amount of uronic acid present in the alkali-treated polysaccharide.

To improve methylation, a sample of SB1 was submitted to two rounds of methylation using CH_3I–NaOH and then to a third methylation cycle using the Purdie method.[43] Good results were obtained as presented in Table 3. Better results were obtained by ethylation analysis of SB1, which allowed the identification of 3-*O*-methyl-2,4,6-tri-*O*-ethyl-galactitol acetate by GC–MS and the separation of 4,6-di-*O*-ethyl-galactitol acetate from 2,6-di-*O*-ethyl-galactitol acetate (Table 3). Comparison of methylation and ethylation data of SB1 showed good correlation, but ethylation data are discussed quantitatively. The 3-linked D-galactopyranosyl units in SB1 originated from D-galactopyranosyl (18.2%), D-galactopyranosyl 2-sulfate (14.0%) and D-galactopyranosyl 4-sulfate. The presence of 2,6-di-*O*-ethyl-galactitol acetate could be ascribed either to 4-substituted, 3-linked galactopyranosyl units or 3-substituted, 4-linked galactopyranosyl units. Taking into consideration the total amount of 2,4,6-tri-*O*-ethyl galactitol acetate after desulfation (41.3%) and the contributions of the different sulfated units to its formation, it can be deduced that a minor amount (~5%) of 3-linked galactopyranosyl units sulfated at O-4 is present. Then, the major amount of 2,6-di-*O*-ethyl galactitol acetate could have originated from 4-linked galactopyranosyl units sulfated at position O-3. The presence of 3,6-di-*O*-ethyl-galactitol acetate (10.4%) after desulfation indicates glycosylation at *O*-2 of 4-linked galactopyranosyl units. Because only 2% of 3-*O*-methyl-2,4,6-tri-*O*-ethyl galactitol acetate was detected, branching could originate also from uronic acids and xylose residues. It is known that 2,3,4-tri-*O*-methyl-xylitol acetate is quite volatile and could have been lost during the analysis.[44] Therefore, it can be deduced that ~10% of 6-*O*-ethyl-galactitol acetate comes from 4-linked galactopyranosyl units sulfated at position O-3 and branched at position

Table 3. Linkage analysis of the constituent sugars of the alkali-treated polysaccharide (SB1) from *Schizymenia binderi*

Monosaccharide	Deduced unit and substitution pattern	Polysaccharide[a]			
		Methylation		Ethylation	
		SB1	SB1DS[b]	SB1	SB1DS
2,3,4,6-Gal	T[c]	4.3	5.7	1.3	3.9
2,4,6-Gal	→3-Gal	21.9	39.7	18.2	41.3
2,3,6-Gal	→4-Gal →3-Gal-4S[d]	2.9	32.8	3.1	29.7
2,6-Gal	+ →4-Gal-3S →3-Gal-6S	—	—	34.3	8.7
2,4-Gal	+ →3-Gal-6R[e] →4-Gal-2S	2.0	1.8	2.9	1.8
3,6-Gal	+ →4-Gal-2R	ND[f]	8.3	1.3	10.4
6-Gal	→3-Gal-2,4S + →4-Gal-2,3S + →4-Gal-2R,3S →3-Gal-4,6S +	12.1	ND	16.8	ND
2-Gal	→4-Gal 3,6S	6.1	3.0	4.1	2.0
3-Gal	→4-Gal-2,6S	2.1	ND	1.9	ND
4,6-Gal	→3-Gal-2S	—	—	14.0	ND
2,6 + 4,6-Gal[g]		48.6	8.8	—	—
3-*O*-Me-2,4,6-tri-*O*-ethyl-Gal	T	—	—	2.0	2.1

[a] Normalized mol % of monosaccharide having methyl or ethyl groups at the positions indicated.
[b] SB1DS = desulfated SB1.
[c] T = terminal.
[d] S = $-SO_3^-$.
[e] R = glycosyl unit.
[f] ND = not detected.
[g] According to GC–MS analysis.

O-2. The rest of the 6-*O*-ethyl-galactitol acetate could arise from 3-linked galactopyranosyl and/or 4-linked galactopyranosyl disulfate units. The presence of 2,6-di-*O*-alkyl derivatives in the final alkylation products may be due to incomplete desulfation (5–6% of remaining sulfate) and/or under alkylation, although glycosylation at position O-4 in 3-linked D-galactopyranosyl units as in the hybrid galactan from *Lomentaria catenata*,[26] and/or at position O-3 of 4 linked galactopyranosyl units could not be excluded. A small amount of 2-*O*-methyl-galactitol acetate was observed, and because pyruvic acid was not detected by the lactate hydrogenase method, the presence of a pyruvic acid ketal at positions O-4 and O-6 of 3-linked galactopyranosyl units can be excluded. Thus, this species could originate from 4-linked and 3-linked galactopyranosyl units disubstituted by sulfate or glycosyl groups at positions O-3 and O-6, and at positions O-4 and O-6, respectively. Minor amounts (~2%) of 2,4-di-*O*-galactitol acetate indicate glycosylation at position O-6 of 3-linked galactopyranosyl units.

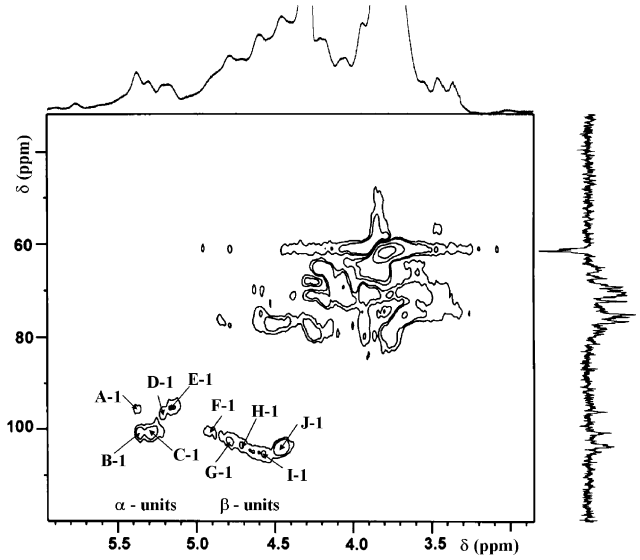

Figure 3. $^{13}C/^{1}H$ 2D HSQC spectrum in D_2O of the partially hydrolyzed alkali-treated polysaccharide from *Schizymenia binderi*. A-1 means C-1 and H-1 of unit A.

2.5. NMR studies of partially acid-hydrolyzed SB1

To confirm the results of alkylation analysis, NMR studies on a sample of partially acid-hydrolyzed SB1 were performed. In the ^{13}C NMR spectrum (Fig. 2), the signal at 104.8 ppm was assigned to C-1 of 3-linked β-D-galactopyranosyl residue linked to →4-α-D-galactopyranosyl unit unsulfated or sulfated at position O-3 according to the chemicals shifts reported for the basic carrageenan structure.[30] The remaining β-anomeric carbon signals could belong to unsulfated and 2-sulfated β-D-galactopyranosyl residues.[30–32,34] The DEPT 135 spectrum showed only three inverted signals between 60 and 62 ppm indicating that most of the primary alcoholic groups in the galactopyranosyl residues were not substituted by glycosyl groups or by sulfate groups, which was in agreement with the results obtained in the alkaline treatment of the native polysaccharide. A two-dimensional HSQC spectrum (Fig. 3) allowed the identification of the anomeric ^{13}C and ^{1}H spin systems of the galactopyranosyl residues (Table 4). The ^{1}H–^{1}H COSY spectrum (Fig. 4) allowed, starting from H-1, the assignments of H-2 and some H-3 signals of the galactopyranosyl units (Table 4). In the α-anomeric region of the HSQC spectrum, connectivities among five anomeric ^{13}C–^{1}H systems were observed. From the value of the ^{13}C chemical shift of C–1,[30] unit A corresponds to →4-α-D-galactopyranosyl units unsulfated or sulfated at position O-3. According to Mulloy et al.[45] the chemical shifts of protons at positions of sulfation in sulfated fucans are typically shifted by 0.6–0.7 ppm to low field. Therefore, for unit A, the sulfation at position O-3 was deduced from the downfield shifts of H-3 compared with the chemical shifts of H-3 in

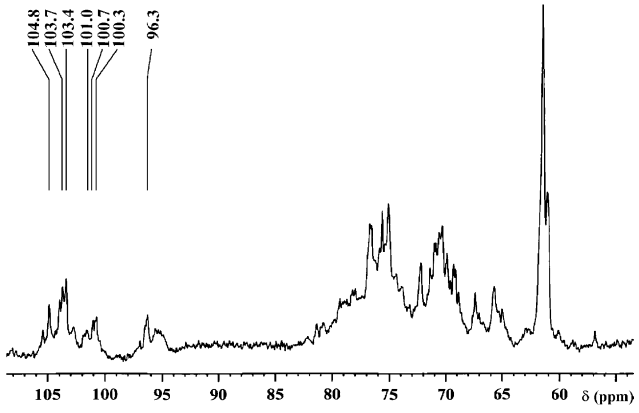

Figure 2. ^{13}CNMR spectrum (100.62 MHz) in D_2O of the partially hydrolyzed alkali-treated polysaccharide from *Schizymenia binderi*.

Table 4. Assignments of chemical shifts (ppm) presents in the HSQC and COSY NMR spectra of SB1

Unit		δ (ppm)			
		C_1	H_1	H_2	H_3
A	→4-α-D-Gal-3S[a]	96.3	5.38	4.08	4.63
B	→4-α-L-Gal-3S	100.7	5.35	3.80	4.47
C	→4-α-L-Gal	101.0	5.30	3.90	3.67
D	→4-α-D-Gal-2,3S or 2R[b],3S	96.7	5.22	4.22	4.76
E	→4-α-D-Gal	95.3	5.17	3.96	
F	→3-β-D-Gal-2S	100.3	4.90	4.29	
G	→3-β-D-Gal-4S	102.4	4.80	3.72	
H	→3-β-D-Gal-2S	103.4	4.72	4.20	
I	→3-β-D-Gal	104.8	4.58	3.62	
J	→3-β-D-Gal	103.7	4.46	3.83	

[a] S = $-SO_3^-$.
[b] R = glycosyl unit.

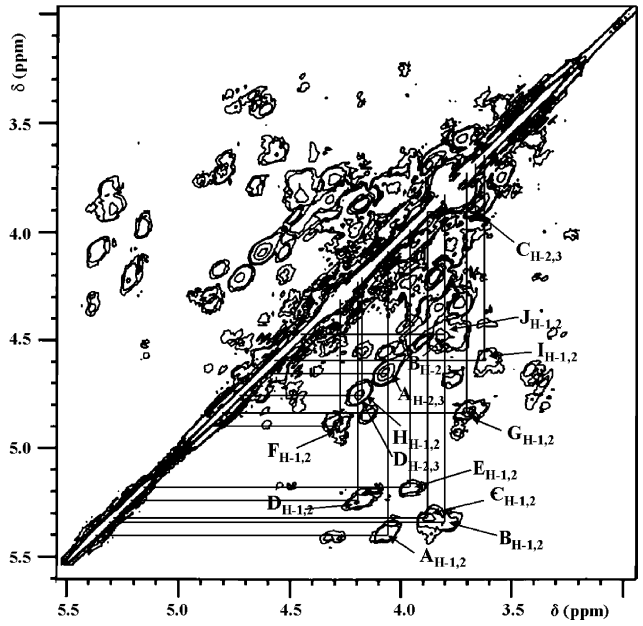

Figure 4. $^{1}H/^{1}H$ 2D COSY spectrum in D_2O of the partially hydrolyzed alkali-treated polysaccharide from *Schizymenia binderi*.

4-linked α-D-galactopyranosyl unit (3.95 ppm).[31] Similarly, sulfation at position O-3 in unit B was deduced from the chemical shift of H-3. In unit D, the signals of H-2 and H-3 are at lower field than in the unsubstituted 4-linked α-D-galactopyranosyl residue, which is indicative of the presence of disulfated derivative or most probably, in accordance to ethylation analysis, of 4-linked α-D-galactopyranosyl units glycosylated at position O-2 and sulfated at position O-3. The signal at 104.8 ppm was assigned to C-1 of unit I. This unit (C-2–C-6: 70.9, 79.3, 65.7, 75.6 and 61.5 ppm)[30] is linked to unit A. The ^{13}C chemical shifts for unit A (C-2–C-6: 67.3, 79.3, 76.7, 71.4 and 61.5 ppm) were assigned according to the ^{13}C chemical shifts reported for methyl α-D-galactopyranoside 3-SO_3^-,[31] considering a 8 ppm glycosylation shift on C-4.[46] The signal at 103.7 ppm was assigned to C-1 of unit J (C-2–C-6: 70.4, 81.4, 68.9, 75.6 and 61.5 ppm),[36,41] linked to unit B (C-2–C-6: 68.1, 76.6, 77.8, 72.2 and 60.0 ppm),[31] and probably to unit C (C-2–C-6: 69.6, 71.0, 79.3, 72.2 and 61.0 ppm).[36] The signal at 103.4 ppm was assigned to C-1 of β-D-galactopyranosyl-2-SO_3^- (unit H) as in λ-carrageenan.[32] A downfield shift of ~0.5 ppm of the signal of H-2 in this unit compared with H-2 of unit J is indicative of sulfation at position O-2. Unit H is probably linked to unit D. The minor signal at 102.4 ppm (not labelled in Fig. 2) is assigned to the anomeric carbon of 3-linked β-D-galactopyranosyl unit sulfated at position O-4.[31] The signal at 100.3 ppm (not labelled in Fig. 2) was assigned to C-1 of β-D-galactopyranosyl unit sulfated at position O-2, probably also substituted at position O-4.

The results obtained by NMR spectroscopy analysis of SB1 corroborate those obtained by methylation and ethylation analyses and indicate that the alkali-treated polysaccharide from *S. binderi* is mainly composed of 3-linked β-D-galactopyranosyl residues unsulfated, sulfated at position O-2 and in minor proportion at O-4 and 4-linked α-D-galactopyranosyl residues predominantly sulfated at position O-3 and glycosylated at O-2. The 4-linked α-L-galactopyranosyl residues are partially sulfated at position O-3. Moreover, the ^{13}C NMR data confirm most of the assignments of the chemical shifts in the spectrum of the partially hydrolyzed native polysaccharide and strongly suggest that its basic structure was maintained on alkaline treatment. Similar sulfation patterns were observed by Bourgougnon et al.[3] in the study of substitution by sulfate groups in the polysaccharide from *S. dubyi*. Also, they identified the presence of L-galactose, although they found a seasonal variation in L-galactose content from traces in spring to 42% in autumn.

2.6. Antiviral activity

The native galactan from *S. binderi* was evaluated for in vitro antiviral activity against diverse strains of human herpes viruses by a plaque reduction assay in Vero cells. The polysaccharide was active against all viruses assayed, inhibiting reference strains of both serotypes HSV-1 and HSV-2 to approximately the same extent (Table 5). In addition, the compound was also highly effective against two TK^- strains of HSV-1 resistant to acyclovir, the drug presently in clinical use for

Table 5. Antiviral activity of sulfated galactan from *Schizymenia binderi* against herpes viruses

Virus	*Schizymenia binderi*		Dextran sulfate 8000	
	EC_{50} (μg/mL)[a]	SI[b]	EC_{50} (μg/mL)[a]	SI[b]
HSV-1 (F)	0.76 ± 0.01	>1316	2.1 ± 0.1	>472
TK^- HSV-1 (B2006)	0.18 ± 0.05	>5556	1.7 ± 0.2	>591
TK^- HSV-1 (Field)	0.21 ± 0.08	>4762	2.6 ± 0.2	>378
HSV-2 (G)	0.63 ± 0.02	>1574	1.1 ± 0.2	>990

[a] EC_{50} (effective concentration 50%): concentration required to reduce plaque number in Vero cells by 50%. Each value is the mean of two determinations ± standard deviation. The CC_{50} (cytotoxic concentration 50%: concentration required to reduce 50% the number of viable cells) was >1000 μg/mL.

[b] SI (selectivity index) = ratio CC_{50}/EC_{50}.

treatment of human herpetic infections.[47] No cytotoxic effects were observed when cell viability was evaluated in monolayers of Vero cells at a concentration up to 1000 μg/mL. From these data, selectivity indices (ratio CC50/EC50) were estimated to be in the range 1316–5556 for the different strains of HSV-1 and HSV-2 (Table 5). Dextran sulfate 8000 was tested as a reference compound of known antiherpetic activity.[5] The polysaccharide from *S. binderi* exhibited a more effective antiviral action and selectivity indices 1.5- to 12.5-fold higher than dextran sulfate 8000 (Table 5). These results indicate that the content of the sulfate groups (66.3% for dextran sulfate and 22.2% for sulfated galactan) and sulfation at position O-6 in dextran sulfate are not determinant factors on antiviral activity. Based on these results, this sulfated galactan can be considered a highly selective antiherpetic compound, with antiviral effectiveness, particularly against the TK^- strains of HSV-1, slightly superior not only to the standard polysaccharide dextran sulfate but also in comparison with other previously reported sulfated galactans of algal origin, such as those compounds derived from *S. dubyi*, *Pterocladia capillacea*, *Cryptopleura ramosa*, *Nothogenia fastigiata* and *Bostrychia montagnei*.[2,7,9,47–49] To analyze the possibility that this polysaccharide may act directly on the virus particle leading to infectivity inactivation, a virucidal assay against HSV-1, strain F and HSV-2, strain G, was carried out. The compound was unable to inactivate both virion serotypes after 60 min of incubation at 37 °C, even at a concentration of 100 μg/mL, more than 100-fold higher than the antiviral EC_{50} values. Consequently, these results allow us to conclude that the polysaccharide exerts a true antiviral action against HSV, interfering with its multiplication in infected cells.

Next, the inhibitory effect of the polysaccharide on virus multiplication was studied by performing a virus plaque reduction assay to determine the EC_{50} under different treatment conditions. When the antiviral assay was performed omitting the presence of the galactan in the adsorption period and including the compound only in the plaquing medium after adsorption, no significant reduction in the number of virus plaques was detected, even at a concentration of 2.5 μg/mL (Fig. 5). By contrast, the presence of the galactan only at virus adsorption was as effective as the treatment throughout the whole incubation period, at and after adsorption. The EC_{50} values obtained from data shown in Figure 5 were 0.95 ± 0.15 μg/mL (compound only present at adsorption) and 0.78 ± 0.06 μg/mL (compound present during and after adsorption), indicating that HSV adsorption is the main target for the antiviral effect of this polysaccharide. Similar results were obtained when dextran sulfate 8000 was used, indicating the same mode of action for both sulfated polysaccharides, although the EC_{50} values for this compound were 2.1 ± 0.1 when only present at adsorption and 2.0 ± 0.2 when present throughout the

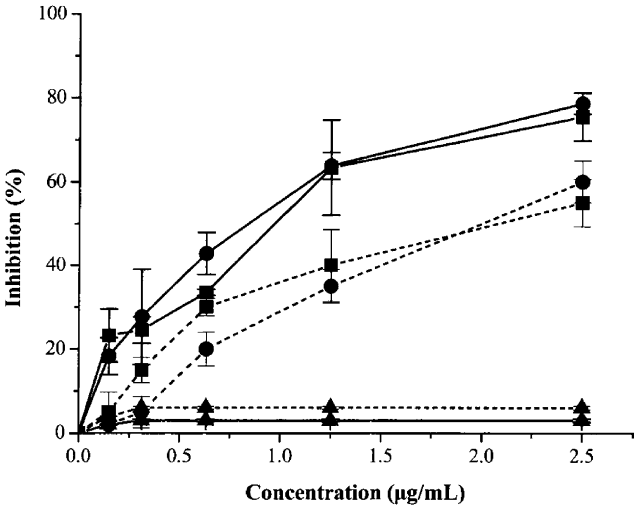

Figure 5. Influence of different treatment conditions on the anti-HSV-1 activity of the sulfated galactan from *Schizymenia binderi*. Vero cells were infected with 50 PFU of HSV-1 and a plaque reduction assay was performed under different treatment conditions: compound present only during adsorption (■); compound present only after adsorption (▲); compound present during and after adsorption (●). Solid lines correspond to polysaccharide from *S. binderi* and dotted lines to dextran sulfate 8000. EC_{50} values are indicated as dashed lines parallel to the axes that intersect at the concentration corresponding to 50% inhibition. Each value is the mean of duplicate determinations ± standard deviation.

experiment. Both serotypes of HSV use the heparan sulfate residues of cellular proteoglycans as the primary receptor for virion binding and adsorption,[50] thus the sulfated galactan of *S. binderi* would exert its inhibitory effect by interference with the interaction of HSV-heparan sulfate.

In conclusion, the water-soluble polysaccharide from *S. binderi* can be considered as an agaran–carrageenan hybrid, with predominance of a carrageenan backbone. The unusual sulfation pattern of this sulfated galactan, which is different from those of known carrageenans,[51] may explain the highly selective antiviral activity.

3. Experimental

3.1. Materials and general procedures

S. binderi J. Ag. was collected in Navidad Bay (33° 55′S, 71° 51′W) in the month of January, and identified by M. E. Ramírez from Museo Nacional de Historia Natural, Santiago, Chile, where a specimen is held (Herbarium No. SGO 151032). FT-IR spectra of polysaccharides were obtained in KBr pellets according to the method described earlier.[17] ^1H and ^{13}C NMR spectra were registered on a Bruker Avance DRTX 400 spectrometer operating at 400.13 MHz (^1H) and 100.62 MHz (^{13}C), at 80 °C after isotopic exchange with D_2O (3×0.75 mL) using D_2O as solvent with CH_3OH as

internal reference (^{13}C: 48.952 ppm). Two-dimensional spectra were registered using standard Bruker software.

3.2. Chemical analyses

Total sugar content was determined by phenol-sulfuric acid method.[52] 3,6-Anhydro-galactose content was determined by the resorcinol method of Yaphe and Arsenault.[20] Uronic acid was determined according to the method of Filisetti–Cozzi and Carpita.[53] Pyruvic acid was determined according to the lactate dehydrogenase method.[54] Molecular weight determination by the reducing end assay was performed as described earlier.[14] Absorbance was registered with a Genesys 5 double beam spectrophotometer. Sulfate and protein ($N \times 6.25$) contents were determined by microanalysis in Facultad de Química, Universidad Católica de Chile. Proteins were also determined by the Hartree–Lowry method.[55,56] High performance liquid chromatography (HPLC) was performed on a Merck Hitachi model 655A-chromatograph with a Whatman Partisil 10-SAX column using D-glucuronolactone (Sigma), D-galacturonic acid (Sigma), D-mannuronic and L-guluronic acids as standards.[57] The ratio of D- to L-galactose was determined through the formation of diastereomeric derivatives with (*S*)-1-amino-2-propanol and GC analysis of the corresponding 1-deoxy-1-1-(2-hydroxy-propylamino) alditol acetates on an Ultra 2 column under the conditions described by Cases et al.[58] Reductive hydrolysis of the polysaccharides and GC of the acetylated alditols was performed according to the method of Stevenson and Furneaux.[44] Ethyl iodide was synthesized according to the literature.[59]

3.3. Extraction

The dried algal material (50 g) was extracted with distilled H_2O (2.5 L) at 90 °C during 3 h with stirring. The residue was removed by centrifugation and the supernatant was dialyzed (molecular weight cut-off of 3500 Da) against distilled H_2O. The resulting solution was concentrated in vacuo and poured into 5 vol of EtOH. The precipitate was dissolved in H_2O and freeze dried (17.6% yield).

3.4. Total hydrolysis

An aliquot of polysaccharide was heated with 2 M TFA during 2 h at 120 °C. The acid was removed in vacuo by repeated co-evaporations with distilled H_2O. The resulting syrup was dissolved in a minimum concentration of H_2O and applied to a column (30 cm × 2.5 cm) of DEAE Sephadex A-25 (Cl^-). The column was eluted with distilled H_2O, until the eluant monitored by phenol–sulfuric acid reagent became free from carbohydrates, and then with 10% formic acid. The fraction eluted with water was concentrated to dryness and the neutral sugars were reduced with $NaBH_4$, acetylated with Ac_2O in anhydrous pyridine and analyzed by GC.[60] The acidic fraction was concentrated in vacuo, the acid removed by repeated additions of water and evaporations and the residue was examined by HPLC for uronic acids as described before.[57]

3.5. Reduction of uronic acid residues in polysaccharides and total hydrolysis

The reduction of the uronic acid residues in both the native and alkali-treated polysaccharides was accomplished according to the method of Taylor et al.[61] The polysaccharide (100 mg) was dissolved in distilled water (10 mL) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (300 mg) was added with stirring. After 2 h, $NaBH_4$ (35 mg) solution was added and the mixture was stirred for 2 h, dialyzed against distilled water and freeze dried. The procedure was repeated once more and the reduced polysaccharides were hydrolyzed as described in Section 3.4. The resulting sugars were reduced with $NaBH_4$, acetylated with Ac_2O in dry pyridine and analyzed by GC.[58]

3.6. Gel permeation chromatography

The native polysaccharide (3 mg) was purified by chromatography on a Sephadex G-200 column (100 × 1.5 cm) using 0.4 M NaCl as eluant. The column was calibrated with 0.4% Blue dextran 2000 and D-glucose solutions. Fractions of 3 mL were collected and elution was monitored spectrophotometrically with the phenol–sulfuric acid reagent.[52] The column was calibrated, furthermore, with polysaccharides of narrow molecular weight distributed dextran sulfates (500 and 8 kDa) (Sigma), dextrans (482, 71.327, 41.272 and 10.200 kDa) (Sigma) and gel filtration standard proteins (670, 158, 44, 17 and 1.35 kDa) (Biorad).

3.7. Alkaline treatment

The native polysaccharide (100 mg) was dissolved in water (30 mL) and $NaBH_4$ (25 mg) was added. After 12 h of stirring, $NaBH_4$ (60 mg) and NaOH (450 mg) were added, and the mixture was heated at 80 °C for 4 h.[62] The residue was removed by centrifugation and the supernatant was concentrated, dialyzed against distilled water and freeze dried to give SB1 (76 mg).

3.8. Solvolytic desulfation

The native polysaccharide and SB1 were converted into the pyridinium salts and the solvolytic desulfation was performed according to the method described by Falshaw and Furneaux.[63] Briefly, the sample (20 mg) was

dissolved in a mixture of anhydrous DMSO–CH_3OH–pyridine (89:10:1 v/v/v, 8 mL) and heated at 100 °C for 4 h. After cooling, distilled water (5 mL) was added and the mixture was dialyzed against distilled water and freeze dried (35–40% yield).

3.9. Partial acid-hydrolysis of native polysaccharide and SB1

The polysaccharide (100 mg) was stirred in concentrated HCl (1 mL) and concentrated sulfuric acid (0.1 mL) at 20 °C. After 15 min, the solution was poured into acetone (100 mL) and the precipitate was washed thrice with portions acetone (2 mL) and dissolved in water. The resulting solution was dialyzed against 0.1 M sodium acetate followed by distilled water and freeze dried.

3.10. Methylation analysis

3.10.1. With CH_3I–NaOH. The alkali-treated polysaccharide in its carboxyl reduced form was converted into the triethylammonium salt as described by Stevenson and Furneaux,[44] and then methylated with CH_3I–NaOH according to the method of Ciucanu and Kerek.[64] Briefly, the polysaccharide (50 mg) in DMSO (5 mL) was stirred with finally powdered NaOH (400 mg) for 2 h at rt, then CH_3I (5 mL) was added and the mixture was stirred for 1 h. The addition was repeated twice. The reaction was stopped by addition of water (2 mL), dialyzed against distilled water and freeze dried. The solid obtained was submitted to the same methylation procedure. An aliquot of the methylated polysaccharide was hydrolyzed with TFA for 2 h at 120 °C and the partially methylated sugars were reduced with $NaBH_4$ and treated with Ac_2O and anhydrous pyridine 1:1 (v/v). GC of partially methylated alditol acetates was carried out on a Shimadzu GC-17A gas–liquid chromatograph equipped with a SP-2330 column (0.25 mm i.d. × 30 m). GC–MS was performed on a Hewlett–Packard 5890A gas–liquid chromatograph fitted with a SP-2330 column interfaced to a GCMS-QP 5050A mass spectrometer operating at 70 eV and using He as carrier gas as described.[65] Conversion of GC areas to molar basis was calculated for the partially methylated alditol acetates according to the effective carbon response theory of Sweet et al.[66]

3.10.2. Desulfation of the partially methylated SB1. The remainder of the methylated polysaccharide was desulfated as described in Section 3.8 and methylated with CH_3I–NaOH, hydrolyzed and analyzed by GC and GC–MS as alditol acetates as described in Section 3.10.1.

3.10.3. Methylation by Purdie's method. The alkali-treated polysaccharide was submitted to two rounds of methylation as described in Section 3.10.1 and the resulting product was isolated from the reaction mixture and dried in vacuum for 8 h at 50 °C. The partially methylated polysaccharide (20 mg) was heated at reflux with CH_3I (4 mL) and dry Ag_2O (300 mg) for 8 h.[43] The resulting product was desulfated as described in Section 3.8, methylated and analyzed by GC and GC–MS as described in Section 3.10.1.

3.11. Ethylation analysis

Ethylation of the alkali-treated polysaccharide (SB1) was conducted as described in Section 3.10.1 using C_2H_5I instead of CH_3I and a third ethylation cycle was performed using C_2H_5I–Ag_2O as described in Section 3.10.3. A portion of the ethylated polysaccharide was hydrolyzed with 2 M TFA at 120 °C during 2 h and the partially ethylated sugars were reduced with $NaBH_4$ and acetylated with Ac_2O in anhydrous pyridine. The alditol acetates produced were analyzed by GC and GC–MS as for the methylated derivatives (Section 3.10.1) using data reported by Cases et al. for MS interpretation.[67]

The partially ethylated SB1 was desulfated as described in Section 3.8. The desulfated SB1 was submitted to two cycles of ethylation with C_2H_5I–NaOH, hydrolyzed with 2 M TFA and the resulting partially ethylated sugars were analyzed as alditol acetates by GC and GC–MS as described in Section 3.10.1.

3.12. Cells and viruses

Vero (African green monkey kidney) cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 5% calf serum. For maintenance medium (MM), the serum concentration was reduced to 1.5%. HSV-1 strain F and HSV-2 strain G were obtained from the American Type Culture Collection (Rockville, USA) and used as reference strains. B2006 and Field were HSV-1 TK^- strains obtained from Prof. Dr. E. De Clercq (Rega Institute, Belgium). Virus stocks were propagated and titrated by plaque formation in Vero cells.

3.13. Cytotoxicity assay

A stock solution of 2 mg/mL in distilled H_2O was used to evaluate the biological activity of the polysaccharide. 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 polysaccharide, 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) was added to each well. After 2 h of incubation at 37 °C, the superna-

tant was removed and ethanol (200 μL) was added to each well to solubilize the formazan crystals. After vigorous shaking, the 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%.

3.14. Antiviral assays

Antiviral activity was evaluated 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 compound. After 1 h of adsorption at 37 °C, 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. The effective concentration 50% (EC_{50}) was calculated as the compound concentration required to reduce virus plaques by 50%. All determinations were performed twice and each in duplicate.

To test the effect of the incubation time on the antiviral activity of the sulfated galactan, Vero cells grown in 24-well plates were infected with 50 PFU of HSV-1, strain F, in MM with or without various concentrations of the polysaccharide. After 1 h adsorption at 4 °C, medium containing unadsorbed virus was removed and cell cultures were washed twice with PBS. Then, 0.5 mL of MM with or without compound, containing 0.7% methylcellulose, was added to each well. After 2 days of incubation at 37 °C, virus plaques were counted. Dextran sulfate 8000 (Sigma–Aldrich) was used as reference compound. Its sulfate content was determined according to the method of Dodgson and Price.[68]

3.15. Virucidal assay

A virus suspension containing 4×10^5 PFU of HSV-1, strain F, was incubated with an equal volume of MM with or without various concentrations of the compound for 1 h at 37 °C. The samples were then diluted in cold MM to determine residual infectivity by plaque formation. The sample dilution effectively reduced the drug concentration to be incubated with the cells at least 100-fold to assess that titre reduction was only due to cell-free virion inactivation. The virucidal concentration 50% (VC_{50}), defined as the concentration required to inactivate virions by 50%, was then calculated.

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