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# The structure of the agaran sulfate from *Acanthophora spicifera* (Rhodomelaceae, Ceramiales) and its antiviral activity. Relation between structure and antiviral activity in agarans

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**Abstract**—The sulfated agaran isolated by water extraction from the red seaweed, *Acanthophora spicifera* (Rhodomelaceae, Ceramiales), is made up of A-units highly substituted with sulfate groups on C-2 (28–30%), sulfates on C-2 and 4,6-*O*-(1′-carboxyethylidene) groups (9–15%), and only the C-2 sulfate groups (5–8%) with small amounts of C-6 sulfate, 6-*O*-methyl, and nonsubstituted residues. B-units are formed mainly by 3,6-anhydro-α-L-galactose (15–16%) and its precursor, α-L-galactose 6-sulfate (10–17%), together with lesser amounts of 3,6-anhydro-α-L-galactose 2-sulfate, α-L-galactose 2,6-disulfate, α-L-galactose 2,3,6-tri-sulfate, α-L-galactose 2,6-disulfate 3-xylose, 2-*O*-methyl-α-L-galactose, and unsubstituted α-L-galactose. Small, but significant quantities of β-D-xylose were found in all the fractions, together with small amounts to traces of D-glucose. Some of the fractions have high antiviral activity. Attempts to correlate structure and antiviral activity in agarans are presented.

*Keywords:* Sulfated agarans; Chemical structure; Methylation analysis; NMR spectroscopy; Herpes simplex virus; Antiviral activity; Seaweed; *Acanthophora spicifera*; Rhodomelaceae

## 1. Introduction

Galactans from red seaweeds consist essentially of linear chains of repeating units of 3-linked β-D-galactose (A-unit) and 4-linked α-galactose (B-unit). The sugars in the former always belong to the D-series, while the sugars in B-units are both in the D- and L-configuration in carrageenans and agarans, respectively. In the last years, a new member of the galactan family, the DL-hybrids whose B-units are in both the D- and L-configurations, is under discussion.[1,2] The galactan backbones are usually modified to varying degrees by substituents such as *O*-sulfo, *O*-methyl, 4,6-*O*-pyruvate, and *O*-glycosyl groups.

*Acanthophora spicifera* is a red seaweed of the family Rhodomelaceae (order Ceramiales). Previous reports have indicated that the seaweed produced λ-carrageenans,[3,4] in spite of the fact that the galactans of several species of the order Ceramiales are agarans.

A fraction of a water extract was previously studied suggesting the presence of an agaran backbone with C-2 sulfate groups in the A-unit and, to a lesser degree, a 4,6-*O*-pyruvate acetal in the same residue.[5] This was confirmed by partial reductive hydrolysis of the alkali-treated derivative and isolation of the corresponding disaccharides.[6] We now report the complete structural determination of the polysaccharides of the cold- and

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hot-water extracts, as well as those of their desulfated and alkali-treated derivatives. A correlation was attempted between antiviral activity and structure in the agarans.

## 2. Results

The seaweed was exhaustively extracted three times with water at 25 °C. The extracts were combined as their analyses (not shown) were identical and their yields very low (total yield, 3.6%). The residues were also re-extracted exhaustively three times with hot water, but the last extract was discarded due to a very low yield. The two first hot-water extracts were worked up separately.

Addition of KCl to their aqueous solutions until a 2 M concentration was attained produced a precipitate (20%, 30%, and 25% yields, respectively). These precipitates were composed of protein (12%, 11%, and 5%, respectively), carbohydrate (27%, 51%, and 41%, respectively), and sulfate (8%, 19%, and 20%, respectively) and were kept for further work.

The supernatant yielded C (25 °C), fractions H (first extraction with hot water), and W (second extraction with hot water). Yields, analyses, and monosaccharide compositions are given in Table 1. Galactose and 3,6-anhydrogalactose are the major sugars (80.6%, 88.1%, and 84.9%, respectively, for C, H, and W), with small amounts of 2-*O*-methyl- and 6-*O*-methyl-galactoses, xylose, and pyruvic acid (Table 1). Small amounts to only traces of glucose were found in these fractions (Table 1). The negative rotations in the raw extracts as well as in the fractions (Table 1) suggested a major agaran structure.

The three extracts (C, H, and W) were arbitrarily fractionated by anion-exchange chromatography on DEAE-Sephadex by step elution with water, defined amounts of NaCl (Tables 1 and 2) and NaOH solutions. The alkaline solutions only eluted traces and were discarded. Yields and analyses of the subfractions are given in Table 1. C and H fractions were eluted according to their sulfate content. Low total yields (~65%), in spite of using concentrations of NaCl as high as 4 M or even 0.5 M NaOH suggested the tendency of these products to interact with the column. The compositions of the subfractions (Table 2) were similar to that of the raw extracts with some dispersion in the percentages of 3,6-anhydrogalactose and/or xylose. Some of the subfractions contained major amounts of xylose and glucose. Those with higher yields, in each case (C-3, H-3, and W-4) were further studied. They showed, by HPSEC-MALLS analysis, unimodal symmetrical peaks with MW of 144.1, 149.1, and 249.9 kDa, respectively.

**Table 1.** Yield, analysis, and optical rotation of the agarans C, H, W and their corresponding fractions obtained by anion-exchange chromatography

| Fraction | Eluent (M NaCl) | Yield (%) | Carbohydrate (%) | Sulfate (NaSO$_3$) (%) | Protein (%) | Pyruvic acid (%) | $[\alpha]_D^{25}$ (°) |
|---|---|---|---|---|---|---|---|
| C | — | 3.6 (80)[a] | 57.0 | 12.8 | 1.2 | 3.1 | −55.0 |
| C-1 | 0.25 | 5.4[b] | 68.0 | 6.9 | 3.4 | 0.8 | −32.5 |
| C-2 | 0.5 | 9.0 | 57.0 | 13.6 | 2.4 | 1.0 | −25.0 |
| C-3 | 0.75 | 52.0 | 65.0 | 25.1 | — | 9.4 | −51.0 |
| C-4 | 1.0 | 25.8 | 68.0 | 27.8 | — | 1.7 | −45.0 |
| C-5 | 1.25 | 7.3 | 51.0 | 18.7 | — | 1.5 | −34.8 |
| C-6 | 1.5 | 2.6 | 50.0 | 11.0 | — | 0.6 | Nd |
| H | — | 6.2 (70)[a] | 45.0 | 15.1 | 5.0 | 2.6 | −34.0 |
| H-1 | 0.5 | 8.5[b] | 30.0 | 4.7 | 6.3 | 1.0 | −23.0 |
| H-2 | 0.75 | 24.7 | 44.5 | 18.2 | — | 2.6 | −33.0 |
| H-3 | 1.0 | 53.1 | 56.0 | 24.9 | — | 2.4 | −49.8 |
| H-4 | 1.25 | 9.2 | 56.0 | 26.4 | — | 0.6 | −42.0 |
| H-5 | 1.5 | 1.6 | 38.3 | 19.2 | 1.1 | Nd | Nd |
| H-6 | 1.75 | 1.3 | 30.0 | 17.2 | 1.6 | 1.0 | Nd |
| H-7 | 4.0 | 1.6 | 37.0 | 4.7 | — | Nd | Nd |
| W | — | 6.6 (75)[a] | 50.0 | 26.6 | 0.6 | 3.1 | −49.5 |
| W-1 | 0.25 | 3.7[b] | 30.0 | Nd | Nd | 0.4 | −32.5 |
| W-2 | 0.5 | 3.2 | 31.2 | 19.2 | 5.1 | Nd | −8.0 |
| W-3 | 0.75 | 11.6 | 32.5 | 18.0 | 6.3 | 0.7 | −15.5 |
| W-4 | 1.0 | 54.0 | 55.0 | 22.1 | 1.3 | 5.1 | −79.6 |
| W-5 | 1.25 | 7.4 | 46.0 | 24.2 | — | 1.3 | −61.5 |
| W-6 | 1.5 | 1.0 | 47.0 | 21.1 | 4.7 | Nd | Nd |
| W-7 | 1.75 | 5.3 | 42.4 | 24.6 | — | 1.0 | −24.0 |
| W-8 | 4.0 | 13.8 | 48.5 | 16.6 | 6.8 | Nd | Nd |

Nd = Not determined.

[a] In parenthesis, percentages of the soluble material recovered after 2 M KCl treatment.

[b] Percentage of the recovered material after anion-exchange chromatography. Yields of the fractionations were ~65%.

**Table 2.** Monosaccharide composition (mol%) of the agarans C, H, W and of its anion-exchange chromatography fractions

| Fractions[a] | Gal[b] | AnGal | 2AnGal | 6Gal | 2Gal | Xyl | Glc |
|---|---|---|---|---|---|---|---|
| C | 55.4 | 22.0 | 3.5 | 2.5 | 6.6 | 5.9 | 4.1 |
| C-1 | 68.3 | 2.1 | — | 2.8 | 1.4 | 13.0 | 12.4 |
| C-2 | 45.0 | 7.2 | — | 3.4 | 1.9 | 17.8 | 24.7 |
| C-3 | 69.0 | 18.6 | — | 3.2 | 3.4 | 5.8 | — |
| C-4 | 77.5 | 11.4 | — | 2.6 | 2.0 | 6.5 | — |
| C-5 | 77.7 | 7.6 | — | 5.3 | 2.4 | 4.8 | 2.2 |
| C-6 | 40.0 | 40.0 | 2.8 | 4.5 | — | 9.2 | 3.5 |
| H | 55.5 | 28.9 | 3.7 | 2.2 | 3.5 | 4.6 | 1.6 |
| H-1 | 65.1 | 9.3 | — | 2.8 | 1.7 | 12.4 | 8.7 |
| H-2 | 64.8 | 21.5 | Tr[c] | 3.6 | 3.8 | 4.7 | 1.6 |
| H-3 | 75.2 | 17.2 | Tr | 1.4 | 1.4 | 4.8 | — |
| H-4 | 80.5 | 10.8 | Tr | 1.7 | 1.8 | 5.2 | — |
| H-5 | 64.5 | 9.5 | 3.2 | 4.4 | 5.1 | 5.9 | 7.4 |
| H-6 | 73.3 | 11.9 | 1.6 | 2.6 | 2.1 | 5.8 | 2.7 |
| H-7 | 50.4 | 11.6 | — | 7.7 | 5.6 | 10.2 | 14.5 |
| W | 63.2 | 21.0 | 0.7 | 4.8 | 2.7 | 6.5 | 1.1 |
| W-1 | 61.6 | 10.7 | — | 3.3 | 1.0 | 13.0 | 10.4 |
| W-2 | 55.1 | 14.9 | — | 2.6 | — | 15.6 | 11.7 |
| W-3 | 71.2 | 9.5 | — | 6.5 | 3.3 | 5.9 | 3.6 |
| W-4 | 70.6 | 20.9 | — | 1.8 | 2.0 | 4.7 | — |
| W-5 | 64.4 | 22.9 | — | 2.6 | 1.0 | 5.7 | 3.4 |
| W-6 | 74.3 | 12.0 | — | 4.1 | 3.8 | 4.5 | 1.3 |
| W-7 | 55.7 | 32.6 | 3.2 | 4.0 | 2.4 | — | 2.1 |
| W-8 | 34.0 | 14.1 | — | 2.3 | 1.2 | 44.1 | 4.3 |

[a] Fractions as defined in text.

[b] AnGal means a 3,6-anhydrogalactosyl residue, 2AnGal a 3,6-anhydro-2-*O*-methyl-L-galactosyl residue.

[c] Tr = trace (<1.0 %).

C-3, H-3, and W-4 were permethylated, and the derivatives showed major amounts of 4,6-di-*O*-methylgalactose, galactose, and 2-*O*-methyl-3,6-anhydrogalactose (Table 3). This galactose from the permethylated agarans could be produced by (1 → 3)-linked units (3-linked galactose 2-sulfate 4,6-ketal) or by (1 → 4)-linked residues (4-linked galactose 2,3,6-trisulfate and 4-linked galactose 2,6-disulfate 3-xylose). The differentiation

**Table 3.** Linkages analysis of the constituent sugars of the native, partially desulfated (D) and alkali treated (T) galactans from *A. spicifera*

| Monosaccharide | Fractions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | C-3 | C-3D | C-3T | H-3 | H-3D | H-3T | W-4 | W-4T |
| *3-Linked units* | | | | | | | | | |
| 2,4,6-Gal | G | 5.0 | 31.6 | 5.6 | 4.0 | 34.0 | 3.0 | 8.0 | 7.0 |
| 2,4-Gal | G6S | 1.6 | 1.4 | 1.1 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 4,6-Gal | G2S | 33.3 | 8.0 | 28.7 | 30.0 | 2.4 | 34.0 | 31.1 | 26.7 |
| 2-Gal | G4,6P | 6.7 | 13.5 | 6.4 | 7.0 | 7.8 | 5.0 | 8.0 | 5.3 |
| Gal[a] | G2S,4,6P | 6.9 | — | 5.7 | — | — | — | 9.0 | 8.5 |
| *4-Linked units* | | | | | | | | | |
| Gal[a] | L2,3,6S | 5.1 | 1.3 | 3.8 | 5.1 | — | — | — | — |
| | L2,6S3X | 3.0 | — | 3.1 | 3.0 | 3.9 | 3.3 | — | — |
| 2,6-Gal | L3S | — | 2.1 | — | — | — | — | — | — |
| 2,3,6-Gal | L | Tr | 6.6 | Tr | 4.2 | 27.0 | 3.2 | Tr | Tr |
| 2,3-Gal | L6S | 5.6 | 7.3 | — | 16.0 | 2.4 | — | 17.0 | — |
| 3-Gal | L2,6S | 3.1 | 1.1 | 1.1 | 4.0 | — | 1.0 | 3.6 | — |
| 2-AnGal | LA | 18.0 | 18.5 | 35.2 | 15.0 | 15.1 | 36.0 | 15.7 | 45.3 |
| AnGal | LA2R | 6.0 | 6.0 | 3.5 | 6.0 | 4.4 | 7.5 | 3.8 | 3.5 |
| *Terminal units* | | | | | | | | | |
| 2,3,4-Xyl | TX | 3.0 | 2.0 | 3.1 | 2.8 | 3.9 | 3.3 | 2.8 | 2.7 |

[a] The galactose from the permethylated agarans could be produced by 3-linked units (galactose 2-sulfate 4,6-ketal) or by 4-linked units (galactose 2,3,6-trisulfate and 2,6-disulfate 3-xylose). The differentiation between these units was carried out according to: (a) the composition of the desulfated derivatives (fractions D, Table 4), (b) the percentages of pyruvic acid in the native fractions (Table 1) and (c) the percentages of xylose (Table 2) and terminal xylose (Table 3). Tr $\leqslant$ 1%.

**Table 4.** Yields, sulfate, and monosaccharide composition of the desulfated (D) and alkali-treated (T) fractions

| Fractions[a] | Yield (%) | Sulfate ($NaSO_3$) (%) | Monosaccharide (mol%) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Gal[b] | AnGal | 2AnGal | 6Gal | 2Gal | Xyl |
| C-3D | 85 | 11.4 | 66.2 | 24.0 | — | 2.0 | 3.1 | 4.7 |
| C-3T | 65 | Nd | 42.3 | 42.8 | 3.8 | 5.6 | — | 5.5 |
| H-3D | 85 | 7.4 | 71.0 | 22.2 | Tr | 1.4 | 1.4 | 4.0 |
| H-3T | 68 | Nd | 51.9 | 39.4 | 1.9 | 1.7 | — | 5.1 |
| W-4T | 66 | Nd | 53.8 | 37.8 | 2.9 | 2.1 | — | 3.4 |

[a] Fractions as defined in text.

[b] AnGal means a 3,6-anhydrogalactosyl residue, 2AnGal a 3,6-anhydro-2-*O*-methyl-L-galactosyl residue.

between these units was carried out according to (a) the composition of the desulfated derivatives (fractions D, Table 4), (b) the percentages of pyruvic acid in the native fractions (Table 1), and (c) the percentages of xylose (Table 2) and terminal xylose (Table 3). The FTIR spectra showed a well-defined peak at 819.7 cm$^{-1}$ corresponding to anhydrogalactosyl residues, together with a broad peak at 820–830 cm$^{-1}$ in agreement with the primary sulfate groups and C-2 sulfate (equatorial) groups in the A-units.[7,8] Fractions C-3 and H-3 were desulfated giving C-3D and H-3D. Yields, analyses, and monosaccharide compositions are given in Table 4. Even if the quantitative composition of the desulfated derivatives is slightly different from that of the parent compounds, they do not suggest degradation, which is in agreement with the high yields obtained. Methylation analysis of the desulfated derivatives compared to that of the parent compounds showed decreased amounts of 4,6-di-*O*-methyl-galactose and galactose and increased percentages of 2,4,6-tri-*O*-methyl- and 2-*O*-methyl-galactose, corresponding to the loss of C-2 sulfate (Table 3).

Alkali treatment of C-3, H-3, and W-4 gave the corresponding cyclized derivatives (C-3T, H-3T, and W-4T, respectively) (Table 4). In C-3T the molar ratio Gal/AnGal is ~1, showing that all the (1 → 4)-linked α-galactose in C-3 was (1 → 4)-linked α-galactose 6-sulfate. In H-3T and W-4T that ratio is ~1.3 and ~1.4, respectively, indicating that some (1 → 4)-linked α-galactose did not produce the 3,6-anhydro derivative in the alkaline treatment, and it was not α-galactose 6-sulfate or that its C-3 OH was substituted. Methylation analyses showed that, in the three cases, all the 2,3-di-*O*-methyl and 3-*O*-methyl (1 → 4)-linked galactoses in the parent compounds did not show up in the cyclic derivatives, indicating the cyclization of (1 → 4)-linked α-galactose 6-sulfate. Only small changes were detected in the percentages of 3,6-anhydro-α-galactose 2-sulfate consistent with the decrease of (1 → 4)-linked 3-*O*-methyl-α-galactose, suggesting the presence of small amounts to only traces of (1 → 4)-linked α-galactose 2,6-disulfate (Table 3).

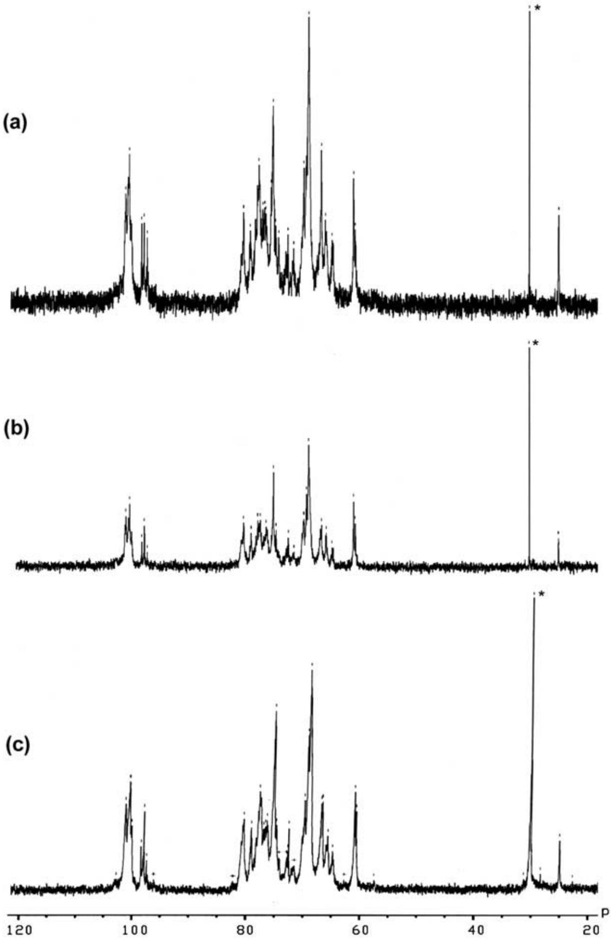

**Figure 1.** $^{13}$C NMR spectra of the agarans: (a) C-3, (b) H-3, (c) W-4. *Acetone signal.

The anomeric region of the $^{13}$C NMR spectra of C-3, H-3, and W-4 showed a complex signal at 101.4–101.1 ppm corresponding to the C-1 of β-D-galactose 2-sulfate with a 1′-*O*-carboxyethylidene group at C-4 and C-6, or without this substituent (101.4–100.9 ppm) linked to α-L-galactose (6-sulfate) or to 3,6-anhydro-α-L-galactose (98.5–97.5 ppm) (Fig. 1). In the alkali-treated derivatives C-3T and H-3T, only two peaks are shown at 101.1–100.7 and 98.1 ppm corresponding to the anomeric carbons of the diad β-D-galactose 2-sulfate → 3,6-anhydro-α-L-galactose. Finally, the $^{13}$C NMR spectra of C-3D and H-3D are rather different: four well-defined absorptions appeared in the anomeric zone of H-3D corresponding to β-D-galactose

(103.2 ppm), β-D-galactose 2-sulfate (101.8 ppm), α-L-galactose (6-sulfate) (100.4 ppm), and 3,6-anhydro-α-L-galactose (98.0–97.9 ppm). Those peaks at 103.0, 100.6, and 97.8 ppm are clearly seen in the spectrum of C-3D, with a complex absorption at 101.2–100.8 ppm.

The spectra showed weak signals at 175.6 and 25 ppm corresponding to the carboxyl and methyl group in the R form of the acetal.[9] The signal at 101.4 ppm corresponding to the acetal carbon[10–12] is possibly hidden by the resonance of the anomeric carbon of the β-D-galactose 2-sulfate. The DEPT spectrum of C-3 showed inverted signals at 66.8 ppm (C-6 sulfate), 64.8 ppm (C-6 of an β-D-galactose 4,6-ketal), 61.1 and 60.1 ppm (non-substituted C-6), and 65.0 ppm (C-5 from β-D-xylose).[13] No resonance at ~104.0 ppm appears in any of the spectra of the fractions or in those of the desulfated and alkali-treated derivatives, suggesting that the β-D-xylose is not linked to the C-6 of the β-D-galactose units.[14] A β-D-xylose linked to C-3 of the B-units would resonate at 101.0 ppm,[15,16] and the small peak would be lost in the complex signals in that zone. No resonances were found at fields higher than 97.5 ppm (see latter), suggesting the absence of α-D-galactose (6-sulfate) and 3,6-anhydro-α-D-galactose. A small peak at 96.2 ppm was found in H-3, which disappears in the desulfated and alkali-treated derivatives, indicating a small amount of α-D-galactose (6-sulfate) in this fraction.

The alkali-treated fraction H-3T was submitted to partial reductive hydrolysis that utilizes the susceptibility of 3,6-anhydrogalactosidic linkages, yielding oligosaccharides with 3,6-AnGalOH as the terminal unit.[16] As a consequence of alkaline modification, the main products of the partial reductive hydrolysis were two disaccharide alditols that were analyzed by negative-ion mode ESIMS. The first, which eluted with water on anion-exchange chromatography, was characterized as agarobiitol ($M_n = 326$) with a negative ion $m/z$ 325 $[M-H]^-$, and the second (0.1 M NaCl) as agarobiitol 2-sulfate ($M_n = 428$) with $m/z$ 405 $[M-Na]^-$. These oligosaccharides were also obtained from the alkali-treated crude extract of *A. spicifera*.[6] However, in fraction H-3T another oligosaccharide alditol (eluted with 0.2 M NaCl) was detected by the ESIMS technique (Fig. 2). The analysis in the negative-ion mode showed an ion with $m/z$ 813, corresponding to the monosodium salt $[M-Na]^-$ of a disulfated tetrasaccharide alditol ($M_n = 836$). These data suggest that the related oligosaccharide is β-D-Gal*p*-2-sulfate-(1 → 4)-3,6-An-α-L-Gal*p*-(1 → 3)-β-D-Gal*p*-2-sulfate-(1 → 4)-3,6-An-L-GalOH (agarotetraitol $2^2$, $2^4$ disulfate). Its HMQC spectrum contains signals at 63.0/3.68 and 99.1/4.71 ppm corresponding to C-1–H-1 of reduced 3,6-anhydro-α-L-galactose and β-D-galactose 2-sulfate, respectively. These residues, as internal components of the tetrasaccharide, show signals at 100.5/4.73 and 98.1/5.10 ppm, respectively. These results are in agreement with methylation and $^{13}$C NMR analyses of H-3T fraction.

The antiviral activity of the raw extracts (C, H, and W) and of their corresponding fractions, obtained by anion-exchange chromatography, as well as the fraction derived from the alkali-modified agaran H-3T, were evaluated in Vero cells by a virus plaque-reduction

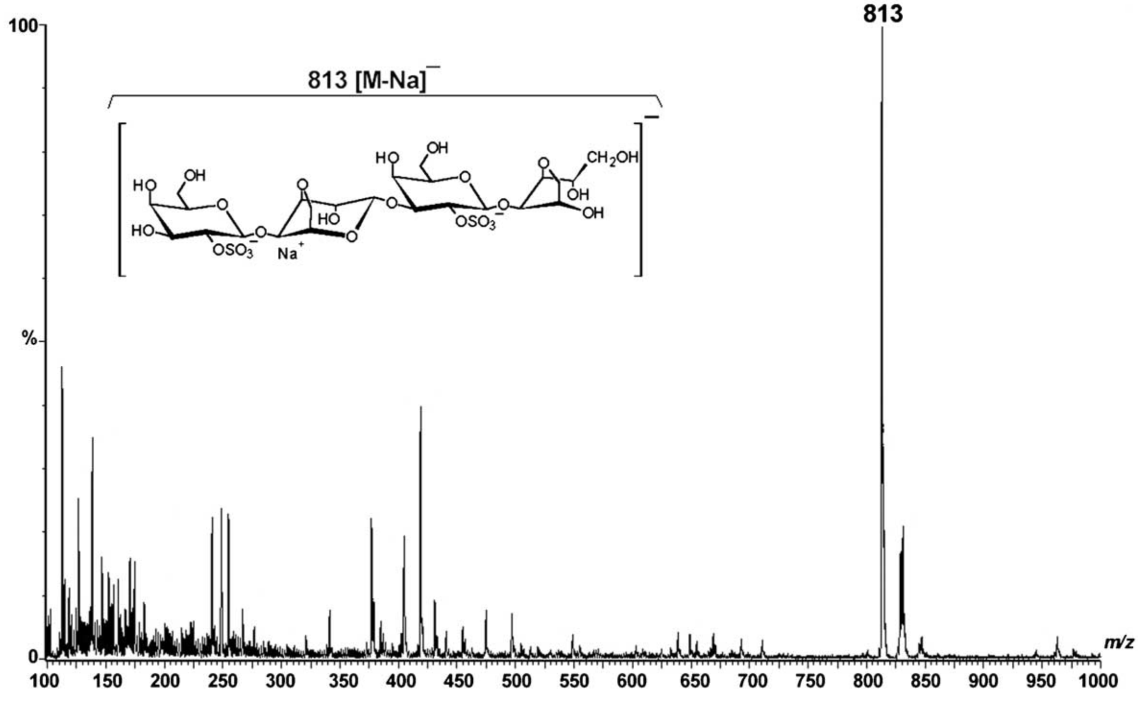

**Figure 2.** ESIMS spectrum of agarotetraitol $2^2$, $2^4$ disulfate, negative-ion mode.

**Table 5.** Antiherpetic activity and selectivity indices of the agarans from *A. spicifera*

| Compound | IC$_{50}$ (μg/mL)[a] | | SI (CC$_{50}$/IC$_{50}$)[b] | |
|---|---|---|---|---|
| | HSV-1 | HSV-2 | HSV-1 | HSV-2 |
| C | 1.8 ± 0.2 | 1.8 ± 0.1 | >555 | >555 |
| C-1 | >50 | >50 | <20 | <20 |
| C-2 | 21.6 ± 0.5 | 43.6 ± 4.1 | >46 | >23 |
| C-3 | 4.2 ± 0.8 | 4.2 ± 0.5 | >238 | >238 |
| C-4 | 1.0 ± 0.1 | 2.4 ± 0.3 | >1000 | >417 |
| C-5 | 4.5 ± 0.4 | 2.8 ± 0.6 | >222 | >357 |
| C-6 | 19.2 ± 1.1 | 18.7 ± 0.8 | >52 | >53 |
| H | 0.8 ± 0.1 | 0.9 ± 0.1 | >1250 | >1111 |
| H-1 | 8.0 ± 2.9 | 4.5 ± 2.9 | >125 | >222 |
| H-2 | 0.8 ± 0.2 | 1.4 ± 0.2 | >1250 | >714 |
| H-3 | 1.4 ± 0.1 | 2.0 ± 0.1 | >714 | >500 |
| H-3T | >5 | >5 | <200 | <200 |
| H-4 | 0.6 ± 0.1 | 1.4 ± 0.3 | >1666 | >714 |
| H-5 | 2.0 ± 1.1 | 2.8 ± 1.0 | >500 | >357 |
| H-6 | 1.2 ± 0.1 | 1.7 ± 0.6 | >833 | >588 |
| H-7 | >50 | >50 | <20 | <20 |
| W | 2.6 ± 0.1 | 3.0 ± 0.1 | >385 | >333 |
| W-1 | >50 | >50 | <20 | <20 |
| W-2 | >50 | 35.6 ± 1.1 | <20 | >28 |
| W-3 | 35.0 ± 0.8 | 38.3 ± 0.6 | >28 | >26 |
| W-4 | 4.4 ± 1.1 | 1.2 ± 0.3 | >227 | >833 |
| W-5 | 13.5 ± 0.4 | 8.0 ± 0.1 | >74 | >125 |
| W-7 | 7.2 ± 0.7 | 8.0 ± 0.1 | >139 | >125 |

[a] IC$_{50}$ (inhibitory concentration 50%): concentration required to reduce plaque number in Vero cells by 50%. Mean of two determinations ±SD.

[b] CC$_{50}$ (cytotoxic concentration 50%): concentration required to reduce 50% the number of viable Vero cells after 48 h of incubation with the compounds. This concentration was >1000 μg/mL for all the compounds.

assay. As shown in Table 5, some agarans (C-3, H-3, and W-4) showed high, or very high, activity against both HSV-1 and HSV-2, as well as their parent raw extracts (C, H, and W) from which they are major fractions, being H (first extraction with hot water) the most active with IC$_{50}$ values of 0.8 and 0.9 μg/mL for HSV-1 and HSV-2, respectively. In general, those fractions that eluted in the range of 0.75–1.0 M of NaCl (C-3, H-2, H-3, and W-4) show higher antiviral activity and have percentages of sulfate in the range of 18.2–25.1% than those eluted with smaller ionic strength (between 0.25 and 0.5 M) and which have low percentages of sulfate (6.9–19.2%). It is worth noting that H-1 showed high activity in spite of a low content of sulfate groups (4.7%) (Tables 1 and 5). The fractions C-4, C-5, H-4–6 showed good activity with IC$_{50}$ values between 0.6 and 4.5 μg/mL, while C-6, W-5, and W-7 were less inhibitory against both viruses. H-7 was inactive up to a concentration of 50 μg/mL (Table 4). The alkali treatment carried out to cyclize L-galactose 6-sulfate in agaran H-3 produced a decrease in the IC$_{50}$ values for both HSV-1 and HSV-2 strains (at least 3.6 and 2.5 times, respectively, Table 5).

No cytotoxicity was observed with any of the polysaccharides when cell viability was evaluated in Vero cells in the presence of concentrations up to 1000 μg/mL. Thus, selectivity indices were estimated in the range of 20–1666.

In order to evaluate the anticoagulant activity of the agarans, the activated partial thromboplastin times

**Table 6.** Anticoagulant activity of the agarans from *A. spicifera*

| Fractions | APTT (s)[a] | | | TT (s)[b] | | |
|---|---|---|---|---|---|---|
| | Concentration (μg/mL) | | | Concentration (μg/mL) | | |
| | 250 | 500 | 1000 | 250 | 500 | 1000 |
| H-2 | 38 | 48 | 63 | 23 | 25 | 30 |
| H-3 | 65 | 67 | >120 | 26 | 28 | 30 |
| H-4 | 64 | 86 | >120 | 29 | 34 | 35 |
| H-5 | 76 | 103 | >120 | 29 | 36 | 41 |
| H-6 | 52 | 65 | 84 | 27 | 27 | 29 |
| H-7 | 37 | 38 | 45 | 24 | 25 | 25 |

[a] APTT for control sample without agaran 32 s.

[b] TT for control sample without agaran 20 s, APTT for heparin (5 μg/mL) >120 s, TT for heparin (5 μg/mL) >100 s. The data are the mean values of two experiments.

(APTT) and thrombin times (TT) were measured. For this purpose, the most active fractions of the hot water extract H (H-2–6) and H-7 were employed. As shown in Table 6, the value of 32 s, corresponding to APTT for the control sample without agaran, showed a minimal increment or, in some cases, was duplicated, when the polysaccharides were added at a concentration of 250 μg/mL, while the TT (20 s for control sample) only raised slightly. Furthermore, the APTT values increased 2- to 3-fold (with the exceptions of H-3–5 >120 s), and the TT values experimented a little increment, when the agarans were tested at concentrations as high as 1000 μg/mL. By contrast, heparin at concentration of 5 μg/mL showed APTT and TT times higher than 120 and 100 s, respectively.

## 3. Discussion

Many species of the order Ceramiales have been studied, all of them producing agaran structures. Different sulfation, methoxylation, and xylosyl substitution patterns appear in the many polysaccharides studied in the order, from the genera *Laurencia*,[16–20] *Polysiphonia*,[21–24] *Odonthalia*,[25,26] *Chondria*,[27] *Cladhymenia*,[28] *Delesseria*,[29–33] *Ceramium*,[34] and *Rhodomela*,[35,36] among others. However, a reinvestigation of the polysaccharide from *Rhodomela larix*[36] has found minor amounts of B-units belonging to the D-series suggesting the presence of polysaccharides containing both agaran- and carrageenan-type backbones[23] (DL-hybrids) as in other polysaccharides from seaweeds from the Halymeniales and Rhodymeniales orders.

As other seaweeds of the order Ceramiales, *A. spicifera* synthesized agarans. Fractionation of the agaran and compositional and structural studies of the fractions and of its desulfated derivatives indicated that they were parts of a sulfated, pyruvated agaran with a narrow structural dispersion. Narrow structural dispersion was also found in the agaran of another member of the Rhodomelaceae, *Bostrychia montagnei*.[37]

The agarans from *A. spicifera* have A-units highly substituted with sulfate groups on C-2 (28–30%), sulfates at the C-2 position, and 4,6-*O*-(1′-carboxyethylidene) groups (9–15%), and only C-2 sulfated species (5–8%) with small amounts of C-6 sulfates, C-6 methyl or nonsubstituted β-D-galactoses. B-units are formed mainly by 3,6-anhydro-α-L-galactose residues (15–16%) and their precursor, α-L-galactose 6-sulfate (10–17%), together with lesser amounts of 3,6-anhydro α-L-galactose 2-sulfate, α-L-galactose 2,6-disulfate, α-L-galactose 2,3,6-trisulfate, α-L-galactose 2,6-disulfate 3-xylose, 2-*O*-methyl, and unsubstituted α-L-galactose. Agarans of the *Laurencia* genus (Rhodomelaceae) share major parts of that complex substitution pattern.[16–20] 3-Linked β-D-galactose in small amounts (2.2%) was found in *Gelidium purpurascens*[38] and together with 4-linked 2-*O*-methyl-α-L-galactose in *Gelidium lingulatum*.[39]

All the data but the optical rotations in previous works that suggested the presence of $\lambda$-like carrageenan can be understood on the basis of the above structure. No explanation was found for the positive rotation found in Ref. 4.

Some agarans isolated from the red seaweed *A. spicifera* showed a very selective and potent antiviral activity against both HSV-1 and HSV-2 (Table 5), while others did not. Table 7 shows an attempt of correlation between the molecular weights, sulfate contents, percentages of methylated galactoses and xylose single stubs of those fractions, and their antiviral activity. None of them showed any cytotoxic effects, and all were also lacking anticoagulant properties (Table 6).

The inhibitory effects of sulfated polysaccharides appears to be based mainly on their ability to interfere with the initial attachment of the virus to the target cell, leading to the blockade of virus entry. The initial contact between an envelope virus and the host cell occurs by the ionic interaction between positively charged regions of the viral external glycoproteins and the negatively charged substituents of the cell surface. The initial step is the binding of viral glycoprotein C (gC), and in some cases glycoprotein B (gB), to cell-surface heparan sulfate (HS).[40–42]

After attachment, a new glycoprotein D (gD) binds to a set of coreceptors, which include protein members of the tumor necrosis factor family and the immunoglobulin superfamily,[43] as well as 3-sulfated heparan sulfate,[44] all of which are required for entry. The interaction between gD–correceptor triggers the fusion of the virion envelope with the cell membrane to allow further penetration.

A cluster of basic and hydrophobic amino acids in the N-terminal of gC has been identified as the major HS-binding domain.[45] Hosoya et al.[46] reported that all the retroviruses and myxoviruses sensitive to polysulfates shared a tripeptide (Phe-Leu-Gly) in the external glycoprotein, whereas other nonsusceptible viruses of the same family lacked this sequence, indicating that a hydrophobic zone may be involved, together with the positive charges, in the inhibitory action of these compounds. This is consistent with the higher activity of fucans compared to those of carageenans or dextran sulfate, possibly due to the (C-6 methyl group) hydrophobic character of the fucose.[47] This could also be linked to the fact that alkyl glycosides of sulfated oligosaccharides with long hydrophobic alkyl chains have much higher activities than their parent sulfated oligosaccharides.[48]

The minimum binding sequence in the interaction of heparan sulfate with the glycoprotein C of HSV-1 (HS/HSV-1 gC) is the structure

**Table 7.** Correlation between sulfate content, molecular weight, percentages of methoxylated galactoses and xylose single stubs with antiviral activity in sulfated agarans

| Agaran from | $M_w$ (kDa) | Sulfate ($NaSO_3$) (%) | Methoxylated galactoses[a] (%) | Xylose single stubs (%) | $IC_{50}$ (μg/mL) | |
|---|---|---|---|---|---|---|
| | | | | | HSV-1 | HSV-2 |
| *A. spicifera* | | | | | | |
| C-1 | Nd | 6.9 | 4.2 | 13.0 | >50 | >50 |
| C-2 | Nd | 13.6 | 5.3 | 17.8 | 21.6 | 43.6 |
| C-3 | 144.1 | 25.1 | 6.6 | 6.0 | 4.2 | 4.2 |
| C-4 | Nd | 27.8 | 4.6 | 6.5 | 1.0 | 2.4 |
| C-5 | Nd | 18.7 | 7.7 | 4.8 | 4.5 | 2.8 |
| C-6 | Nd | 11.0 | 7.3 | 9.2 | 19.2 | 18.7 |
| H-1 | Nd | 4.7 | 4.5 | 12.4 | 8.0 | 4.5 |
| H-2 | Nd | 18.2 | 7.4 | 4.7 | 0.8 | 1.4 |
| H-3 | 149.9 | 24.9 | 2.8 | 4.8 | 1.4 | 2.0 |
| H-4 | Nd | 26.4 | 3.5 | 5.2 | 0.6 | 1.4 |
| H-5 | Nd | 19.2 | 12.8 | 5.9 | 2.0 | 2.8 |
| H-6 | Nd | 17.2 | 6.3 | 5.8 | 1.2 | 1.7 |
| H-7 | Nd | 4.7 | 13.3 | 10.7 | >50 | >50 |
| W-1 | Nd | Nd | 4.3 | 13.0 | >50 | >50 |
| W-2 | Nd | 19.2 | 2.6 | 15.6 | >50 | >50 |
| W-3 | Nd | 18.0 | 9.8 | 5.9 | 35.0 | 38.3 |
| W-4 | 249.9 | 22.1 | 3.8 | 4.7 | 4.4 | 1.2 |
| W-5 | Nd | 24.2 | 3.6 | 5.7 | 13.5 | 8.0 |
| W-7 | Nd | 24.6 | 9.6 | — | 7.2 | 8.0 |
| *C. ramosa*[51] | 2.8 | 35.2 | 7.9 | 4.6 | 1.6 | 4.2 |
| *P. capillaceae*[52,53] | Nd | 27.0 | 1.7 | 6.1 | 3.2–6.1 | 7.5 |
| *B. montagnei*[37,54] | | | | | | |
| B1 | 31.3 | 15.0 | 19.7 | 4.7 | >50 | >50 |
| B2 | 5.6 | 11.2 | 6.8 | 19.8 | >50 | >50 |
| B3 | 11.9 | 16.2 | 16.8 | 8.9 | >50 | >50 |
| B4 | 43.7 | 22.0 | 18.1 | 3.1 | 15.4 | 12.4 |
| B5 | 42.5 | 24.0 | 12.8 | 3.2 | 25.7 | 46.2 |
| B6 | 34.0 | 22.0 | 12.8 | 4.9 | 13.1 | 16.4 |
| *N. fastigiata*[55] | | | | | | |
| F1 | Nd | 10.8 | 3.2 | 13.5 | 20.0 | 24.4 |
| F7 | Nd | 18.1 | — | 11.9 | 15.0 | 32.6 |
| *C. officinales*[58,59] | | | | | | |
| 2I | 2.7 | 6.4 | 47.0 | 17.0 | >50 | >50 |
| 4II | 21.0 | 12.5 | 33.0 | 12.5 | >50 | >50 |
| *G. confluens*[56,57] | | | | | | |
| CP | 13.5 | 12.6 | 38.0 | 13.0 | >50 | >50 |
| F2 | 19.6 | 12.6 | 38.0 | — | >50 | >50 |

Nd = not determined.

[a] Corresponds to 2-*O*-, 6-*O*-methylgalactosyl, and 3,6-anhydro-2-*O*-methyl-L-galactosyl.

$$\rightarrow 4)\text{-}\beta\text{-}\textsc{l}\text{-}({}^{1}C_{4})\text{-IdoA2-OSO}_3\text{-}(1 \rightarrow 4)\text{-}\alpha\text{-}\textsc{d}\text{-}({}^{4}C_{1})\text{-GlcNR6}'\text{-OSO}_3\text{-}(1 \rightarrow$$

which, for maximum binding, must be expressed in relation to other structural components as *N*-sulfo, additional *O*-sulfo groups, and carboxyl groups, and situated in a chain with, at least, 10–12 repeating units.[49] No structural suggestions have been made for the hydrophobic counterpart. The average pattern of sulfation of the agarans from *A. spicifera* permits the possibility of disaccharide units formally similar to that minimal sequence of the HS

$$\rightarrow 3)\text{-}\beta\text{-}\textsc{d}\text{-}({}^{4}C_{1})\text{-Gal}p\text{2-OSO}_3\text{-}(1 \rightarrow 4)\text{-}\alpha\text{-}\textsc{l}\text{-}({}^{1}C_{4})\text{-Gal}p\text{6}'\text{-OSO}_3\text{-}(1 \rightarrow$$

that would be responsible for the activity (Fig. 3).

When molecular models of the ionic minimal binding zones are examined with the 2- and 6′-sulfate groups on the same side of the molecule, they show, through axial C–H bonds and unshared electron pair orbitals, upper and lower surfaces with hydrophobic characteristics[50] (Fig. 3). The accommodation of the negative charges and hydrophobic surfaces in the polysaccharide to their counterparts in the binding zone of the glycoprotein C

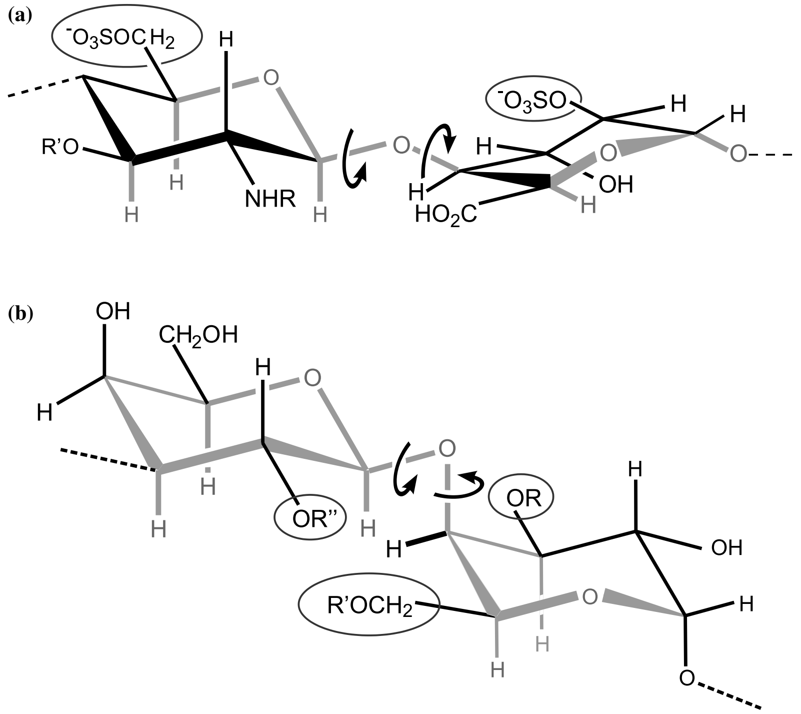

**Figure 3.** (a) Minimal binding structures in HS/heparin. Different binding possibilities can be generated by the flexibility of the Ido ring having its sulfate on C-2 in axial or equatorial position. Besides, rotation around the glycosidic bonds produces a set of structures that offer possibilities for optimal ionic and hydrophobic interactions. Hydrophobic zones are shown in gray, while ionic ones are circles. $R' = H$, minimal binding structure for the interaction with glycoprotein C (gC). $R' = SO_3^-$, minimal binding structure for the interaction with glycoprotein D (gD). (b) Minimal binding structures in agarans. Rotation around the glycosidic bonds produces a set of structures, which offer possibilities for optimal ionic and hydrophobic interactions. Hydrophobic zones are shown in gray while ionic are circled. $R = H$, $R' = SO_3^-$, and $R'' = SO_3^-$; minimal binding structures for interaction with glycoprotein C (gC). $R = SO_3^-$, $R' = SO_3^-$, and $R'' = SO_3^-$; minimal binding structures for interaction with glycoprotein D (gD).

(gC) could be optimized by rotation of the sugar units through their glycosidic linkages (Fig. 3). The simultaneous use of both ionic and hydrophobic forces in the formation of the sulfated polysaccharide–virus surface complexes would result in their synergetic stabilization, as the hydrophobic forces would be strengthened by the high ionic strength field produced by the charged groups in their environment. The ionic attractions would also be reinforced due to the lower dielectric constant resulting from the hydrophobic structures.

The antiviral activities of some agarans of known structure have been determined, but in no cases were attempts made to establish structure–activity relationships. The low-molecular-weight (2.8 kDa) sulfated agaran from *Cryptopleura ramosa* has a sulfate content of 35.2% and shows a high antiviral activity (1.6–4.2 μg/mL)[51] A sulfated agaran from *Pterocladia capillacea*[52] (~27% sulfate) was mainly constituted by 4-linked α-L-galactose 3-sulfate (B-units) with lesser amounts of 3-linked β-D-galactose 6-sulfate (A-units) and was active against various herpesviruses at concentrations ranging from 3 to 12 μg/mL.[53]

In the agarans from *B. montagnei*,[37] the 3-linked β-D-galactopyranosyl units carry partial sulfation on C-2 (2.1–7.7%), C-4 (11.6–18.4%), and C-6 (12.4–20.2%). Approximately 50–60% of the 4-linked units have complete sulfation on C-3, with minor amounts of 2- and 6-sulfation (0.8–2.2% and 2.8–5.2%, respectively). Some of the fractions obtained by anion-exchange chromatography of the raw extract (B4–B6) have low activity against HSV-1 (13.1–25.7 μg/mL) and HSV-2 (12.4–46.2 μg/mL), while others (B1–B3) were not active.[54] The activity could be correlated with the sulfate content and the molecular weights (22.0–24.0% and 34–43.7 kDa) in the active ones and (11.2–16.2% and 5.6–31.3 kDa) in the nonactive fractions. The agarans from *Nothogenia fastigiata*[55] (F1 and F7) have sulfate groups on positions 3 and 6 of the B-units, F1 has no sulfates on the A-unit, while F7 has them on position 4. They achieved a dose-dependent inhibition of the replication of HSV-1 in Vero cells, with 50% effective doses in the range of 15.0–32.6%. The low degree of sulfation (10.8–18.1%, F1 and F7, respectively) is compatible with the very low activity. The methylated agaran from *Georgiella confluens*[56] with low 3,6-anhydrogalactose content and bearing xylose single stubs linked at position 4 of the β-D-galactose, and sulfated (12.8% of total sulfate) mainly (~8–9%) at position 3 of the α-L-galactose, with lesser amounts of sulfation at position 4 of the β-D-galactose (~3–4%), did not show any antiviral activity.[57] Two 'corallinans,' sulfated agarans from the calcareous red seaweed *Corallina officinalis*,[58] with low percentages of total sulfate (6.4% and 12.5%, respectively), and partial sulfation on C-6 of the A-units, and C-2 and C-3 of the B-units did not show any antiviral activity.[59]

Table 7 shows an attempt to correlate sulfate content, molecular weights, percentage of methoxylated sugars, and xylose single stubs with antiviral activity.

As in other sulfated polysaccharides the high molecular weights and degree of sulfation are determinants of activity. Nevertheless, the activity of the agaran from *C. ramosa*[51] suggests that when the percentage of sulfate is very high, the molecular weight (if above a limit) becomes less important. Agarans carry two structural details not found in other sulfated polysaccharides: B-units are in the L-configuration and methylated galactoses and 3,6-anhydrogalactoses appear in significant, or high, amounts in most of them. The few data at hand suggest that the B-units in the L-configuration are not of primary importance for the antiviral activity, influencing only the possibility of the polysaccharide to adopt an adequate shape to position the sulfate groups for the formation of a polysaccharide–virus complex. On the other hand, Table 7 suggests that increased amounts of methoxylated galactoses and/or xylose as single stubs at random in the chain, could be deleterious for the activity. The few data produced in the agaran sulfate field suggest that all the sulfate groups, in different positions of the agaran backbone, could contribute to the low inhibitory activity of these galactans. It is known that in the multiple interactions between the cell-surface heparan sulfate and the HSV glycoproteins involved in the early steps of infections, all the sulfate and carboxyl groups, which contribute to the negative charge of the heparan sulfate, are important for viral binding.

Only in the case of the *Acanthophora* sulfated agarans the average sulfation patterns support the above mentioned binding structure. Actually, in most of the cases the major sulfation is on C-3 of the 4-linked α-L-galactopyranosyl units. It is worth noting that specific sites in HS, which include one of the disaccharides,

$\rightarrow$ 4)-β-L-($^{1}C_{4}$)-IdoA2-OSO$_3$-
(1 $\rightarrow$ 4)-α-D-($^{4}C_{1}$)-GlcNH$_2$3′-OSO$_3$6′-OSO$_3$(1 $\rightarrow$

or

$\rightarrow$ 4)-β-L-($^{1}C_{4}$)-IdoA-2-OSO$_3$-
(1 $\rightarrow$ 4)-α-D-($^{4}C_{1}$)-GlcNH$_2$-3′-OSO$_3$(1 $\rightarrow$

can serve as a gD-binding receptor for HSV-1 entry into cells.

It is also worth noting that similar sulfation (3-OSO$_3$) in the central α-D-glucopyranosylamine *N*-(OSO$_3$) 6-(OSO$_3$) unit in the heparin pentasaccharide is a marker component for the ATIII-binding site.[60] It is also known that the 2,3-*O*-disulfation in HS is important for the binding to gB (major) and gC.[61] The agaran from *G. confluens*[56] has no antiviral activity in spite of showing significant sulfation on C-3 of the α-L-galactopyranosyl units. This is possibly due to its low sulfate content (Table 7).

## 4. Experimental

### 4.1. Extraction

Specimens of *A. spicifera* (Vahl) Borgesen were collected in Bombinhas-Santa Catarina, Brazil. A voucher specimen was deposited in the herbarium of Departamento de Botânica da Universidade Federal do Paraná, Brasil with the herbarium number UPCB 46569. The samples were air dried and then milled. The powder was treated with aq MeOH (85%, 4×) to remove pigments, lipids, and low-molecular-weight carbohydrates (yield 75%). The material (65 g) was then extracted with water (2 L, 3×) at 25 °C with mechanical stirring for 18 h. The residue was removed by centrifugation, and the supernatant was added to 3 vol of EtOH, giving the precipitated polysaccharide. Chemical analysis of the three crude extracts showed that they were similar in composition and were thus combined. The combined products were redissolved in water, dialyzed, and freeze-dried (3.6% yield). The residue was re-extracted 3× with hot water (70 °C, 8 h) with mechanical stirring, yielding two crude extracts (6.2% and 6.6% yield, respectively) and a third one that was discarded due to its low yield. The solutions of the three crude extracts (the rt, and the two hot-water ones) (0.25% w/v) were treated with solid, finely divided KCl with constant mechanical agitation, up to 2.0 M. The stirring was continued for 16 h to ensure equilibration of the system. The remaining solutions were dialyzed, concentrated, and freeze-dried to yield the soluble fractions C (rt extracts), H, and W (first two extractions with hot water), respectively. The precipitated fractions were insoluble and after analysis were kept for future work.

### 4.2. Analytical methods

Sulfate content was determined by the turbidimetric method of Dodgson and Price.[62] Pyruvic acid was determined by the 2,4-dinitrophenylhydrazine colorimetric method of Koepsell and Sharpe[63] after hydrolysis (0.02 M oxalic acid, 100 °C, 1 h). Protein content was determined according to the method of Lowry et al.,[64] using crystalline bovine serum albumin as the standard. Total carbohydrate was determined by the phenol–sulfuric acid method using galactose as the standard.[65]

### 4.3. Anion-exchange chromatography

The fractions C, H, and W were fractionated by anion-exchange chromatography on DEAE-Sephacel. The column was eluted successively with distilled water and then with solutions of increasing NaCl concentrations (0.25–4.0 M) and 0.5 M NaOH. The column eluents were analyzed for carbohydrates by the phenol–sulfuric method.[65] The fractions obtained were concentrated, dialyzed, and freeze-dried (Table 1).

### 4.4. Specific optical rotations

Optical rotations of aqueous solutions of the polysaccharide samples (0.2%), were measured at 20 °C, using a 1 dm cell at the sodium D line (589.3 nm) with a Rudolph Autopol III automatic polarimeter.

### 4.5. HPSEC-MALLS analysis

The determination of the homogeneity of agarans C-3, H-3, and W-4 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 a multi-detection equipment, using a $NaNO_2$ solution (0.1 mol/L) as eluent, containing $NaN_3$ (200 ppm) as preservative. The samples (2 mg/mL) 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, $\theta$, ranging from 35° to 132°. All experiments were carried out at 25 °C.

### 4.6. Aqueous alkaline treatment

The cyclization reaction was carried out as previously described.[66] Briefly, the samples C-3, H-3, and W-4 (50 mg) dissolved in water (25 mL) were reduced with $NaBH_4$ (5 mg) at 4 °C for 16 h. After this period 3 M NaOH were added (12.5 mL), together with a further quantity of $NaBH_4$ (2.5 mg), to give a final concentration of 1 M NaOH. The solutions were heated at 80 °C. Samples were taken at intervals, and the content of 3,6-anhydrogalactose[67] was determined. Heating was continued until the 3,6-anhydrogalactose content remained constant (4 h), yielding the alkali-modified galactans C-3T, H-3T, and W-4T.

### 4.7. Desulfation

Partial solvolitic desulfation of the polysaccharides (C-3 and H-3) was carried out as previously reported[68] at 100 °C for 4 h, using 80:10:1 $Me_2SO$–MeOH–pyridine to yield C-3D and H-3D.

### 4.8. Methylation analysis

The native agarans (C-3, H-3, and W-3) and its corresponding alkali-treated (C-3T, H-3T, and W-3T) and desulfated derivatives (C-3D and H-3D) were converted to their triethylammonium salts by ion-exchange with Dowex-50 resin.[69] Methylation of the polysaccharides as their triethylammonium salts was carried out by the method of Ciucanu and Kerek.[70] The dry polysaccharides (25 mg) were dissolved in $Me_2SO$ (3.0 mL) with stirring. Powdered NaOH (50 mg) was added, and the mixture was stirred for 30 min at room temperature. $CH_3I$ (0.1 mL) was added, and the reaction was allowed to proceed at 25 °C for 30 min. The addition was repeated twice. Water (2 mL) was added to stop the reaction, and the solution was neutralized with HOAc, dialyzed against distilled water, and lyophilized to give the methylated polysaccharides (yield ~87%). A second methylation was carried out following the same procedure (yield 80%).

The partially methylated alditol acetates were generated by reductive hydrolysis and acetylation,[71] analyzed by gas chromatography (GC) and GC–mass spectrometry (GC–MS). Alditol acetates were also generated by hydrolysis in aq $HCO_2H$ (45%), followed by $NaBD_4$ reduction and acetylation, and also analyzed by GC and GC–MS.

### 4.9. Partial reductive hydrolysis

Fraction H-3T was submitted to partial reductive hydrolysis[16] (8 h, 65 °C, TFA 0.5 M) with the acid-stable reducing agent, borane-4-methylmorpholine complex (97%, Sigma–Aldrich). Hydrolysis products were then fractioned and purified by sequential chromatography steps utilizing charcoal–Celite, DEAE-Sephadex A-25 ($Cl^-$), and BioGel P-2 as previously described.[6]

### 4.10. Gas chromatography

GC–MS analyses were carried out with a Varian 3300 chromatograph and a Finnigan Mat ITD spectrometer using a fused silica capillary column (30 m × 0.25 mm) coated with DB-23. Chromatography was run isothermically at 210 °C. Both injector and FID temperatures were 250 °C. Helium was used as carrier gas at a flow rate of 1 mL/min.

### 4.11. Spectroscopic methods

Fourier-transform infrared (FTIR) spectra of KBr pellets of the polysaccharides were recorded in a Perkin–Elmer Series 2000 FTIR spectrophotometer (eight scans, at a resolution of 4 cm$^{-1}$) scanning between 4000 and 400 cm$^{-1}$. For nuclear magnetic resonance (NMR) spectroscopy analysis, the lyophilized polysaccharide was dissolved in $D_2O$ (20–50 mg/mL). NMR spectra of solutions were recorded using a Bruker Avance DRX 400 NMR spectrometer. The samples were analyzed by one-dimensional $^{13}C$ and $^{13}C$ DEPT (DEPT135) at 50 °C. Bi-dimensional $^{13}C$ HMQC (40 °C) was carried out with the oligosaccharide previously exchanged with deuterium by repeated evaporations in $D_2O$ and then dissolved in $D_2O$ (~2 mg/0.4 mL) using a multi-nuclear inverse detection, 5 mm probe. Chemical shifts are expressed in ppm using acetone as the internal standard at 30.2 and 2.225 ppm for $^{13}C$ and $^1H$, respectively.

### 4.12. Electrospray-ionization mass spectrometry

ESIMS analyses were recorded in a Micromass Quattro LC–MS/MS triple quadrupole mass spectrometer. Data acquisition and processing was performed using Maslynx 3.2 software. Samples were diluted in 7:3 acetonitrile–water at 1 mg/mL and introduced into the spectrometer by a syringe pump (KD Scientific Inc.). $N_2$ was used as nebulizer (83 L/h) and desolvation gas (309 L/h). The source was operating at 150 °C and desolvation temperature at 250 °C. The pressure in the analyzer was $1.1\times10^{-5}$ and $2.0\times10^{-5}$ mbar in the gas cell. To provide parent ions, the negative-ion mode parameters were: 4.00 kV, 100, and 1.6 V (for capillary, cone, and ion energy, respectively), giving rise to negative ions for neutral molecules and sulfated oligosaccharide alditols.

### 4.13. Cells and viruses

Vero (African green monkey kidney) cells were grown in minimum essential medium (MEM) supplemented with 5% bovine serum. For maintenance medium (MM), 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, MD, USA).

### 4.14. Cytotoxicity test

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) was added to each well. After 2 h of incubation at 37 °C, the supernatant was removed, and 200 µL of EtOH 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%.

### 4.15. Antiviral assay

Antiviral activity was evaluated by reduction of virus plaque formation. Vero cell monolayers grown in 24-well plates were infected with about 50 plaque-forming units (PFU) of virus/well in the absence or presence of various concentrations of the compounds. After 1 h of adsorption at 37 °C, the residual inoculums were 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 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.

### 4.16. Assay for anticoagulant activity

Anticoagulant activities of the sulfated polysaccharides were determined using the activated partial thromboplastin time (APTT) assay[72] and thrombin time (TT) as described by Carlucci et al.,[73] using heparin (150 units/mg) as a standard and polysaccharides in various concentrations (250–1000 µg/mL).

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