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Chemical structure and antiviral activity of the sulfated heterorhamnan isolated from the green seaweed *Gayralia oxysperma*

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ABSTRACT

A homogeneous sulfated heterorhamnan was obtained by aqueous extraction, then by ultrafiltration from the green seaweed *Gayralia oxysperma*. Besides α-L-rhamnose it contains glucuronic and galacturonic acids, xylose and glucose. The structure was established by methylation analyses of the carboxyl-reduced, carboxyl-reduced/desulfated, carboxyl-reduced/Smith-degraded, and carboxyl-reduced/Smith-degraded/desulfated products and 1D, 2D NMR spectroscopy analyses. The heterorhamnan backbone is constituted by 3- and 2-linked rhamnosyl units (1.00:0.80), the latter being ~50% substituted at C-3 by side chains containing 2-sulfated glucuronic and galacturonic acids and xylosyl units. The 3- and 2-linked rhamnosyl units are unsulfated (20%), disulfated (16%), and mostly monosulfated at C-2 (27%) and C-4 (37%). The branched and sulfated heterorhamnan had high and specific activity against herpes simplex virus.

1. Introduction

Marine green algae (Chlorophyta) synthesize a great variety of water-soluble polysaccharides.[1] Most of them are sulfated and contain predominant amounts of rhamnose[1–12] or galactose[13–15] as well as pyruvic acid ketal.[13–15]

Algae from the genera *Ulva* and *Enteromorpha* (order Ulvales) produce sulfated glucuronoxylorhamnans,[4–8,12] named ulvans.[16] These contain a major repeating disaccharide unit defined as ulvanobiuronic acid 3-sulfate which is composed of 4-linked rhamnose 3-sulfate and 4-linked glucuronic or iduronic acid residues.[3–7,17] Additionally, several other repeating sequences have been identified for ulvans.[4,6–8] Recently, an excellent review describing the structure and properties of ulvans has been published.[18] In contrast, other species of genus *Monostroma* (order Ulvales) biosynthesize 2- and 3-linked rhamnans sulfated at C-2, C-3, or C-4.[9,10]

Sulfated polysaccharides isolated from the genera *Monostroma*, *Ulva*, and *Enteromorpha* genera are known to have a broad range of biological activities, comprising antiviral,[19–22] immunomodulating,[23] anticoagulant,[10,24–26] antioxidant[27,28] besides antihyperlipidemic properties.[29,30]

We now report the structure of the major water-soluble polysaccharide obtained from the green seaweed *Gayralia oxysperma* (Ulvales) and its antiviral activity against herpes simplex virus type 1 (HSV-1) and 2 (HSV-2).

2. Results and discussion

2.1. Extraction and purification of the sulfated polysaccharides from *G. oxysperma*

The milled green alga *G. oxysperma* was successively extracted twice with water at room temperature yielding fractions Go1 and Go2, respectively. The residue was then sequentially extracted four times with the same solvent at 80 °C giving rise to Go3–Go6, respectively. Yields, analyses, and monosaccharide composition of these crude extracts are given in Table 1. The six fractions contained rhamnose as the major neutral sugar (56–88 mol %), together with glucose (3–21 mol %), xylose (4–10 mol %), and arabinose (3–10 mol %). Small amounts of mannose (1–2 mol %) were also detected. GC–MS analysis of acetylated aminoalditol derivatives showed that rhamnose has the L-configuration. All the fractions present high sulfate content (21.2–30.9%) besides uronic acids (12.2–17.0%). On the basis of their monosaccharide composition, the term heterorhamnan is now adopted to define this kind of polysaccharide.

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Table 1
Yield, chemical analyses, specific rotation, and monosaccharide composition of polysaccharidic fractions obtained from *G. oxysperma*

	Go1[a]	Go2	Go3	Go4	Go5	Go6
Yield[b] (%)	1.4	0.6	13.8	12.7	4.5	5.8
Carbohydrate (%)	38.4	44.0	49.6	46.7	45.1	51.7
Sulfate[c] (%)	21.2	22.2	26.1	30.9	23.5	30.9
Uronic acid (%)	12.2	12.9	17.0	16.2	13.7	13.8
Protein (%)	7.3	8.8	3.9	2.0	6.0	4.6
$[\alpha]_D$ (°)	−43.0	−24.5	−38.0	−37.5	−29.0	−33.0
Monosaccharide (mol %)						
Rha	65	56	67	75	88	71
Xyl	10	9	10	9	4	9
Glc	17	21	10	11	3	12
Gal	2	3	6	5	–	3
Ara	4	10	5	–	3	3
Man	2	1	2	–	2	2

– = Not detected.
[a] Fractions are defined in the text.
[b] Percentage based on milled seaweed.
[c] Determined as SO_3Na.

Considering that Go3 had the highest yield (13.8%) among all the crude extracts, it was selected for the structural studies. It was fractionated by ultrafiltration through a 300 kDa cut-off membrane, yielding a retained Go3r (70% yield) and an eluted fraction, Go3e (30% yield). The former is rich in uronic acids (19.0%) and sulfate groups (25.8%), having rhamnose (77 mol %) and xylose (17 mol %) as the major monosaccharides, besides small amounts of glucose (5 mol %) and galactose (1 mol %). The monosaccharide composition of Go3e was different from that of Go3r, since it contains a significant proportion of glucose (20 mol %) and a lower uronic acid content (9.7%), Table 2.

HPSEC-MALLS-RI analysis of Go3r gave a homogeneous profile (Fig. 1) with MW of 1519 kDa (d*n*/d*c* of 0.118), whereas Go3e showed an asymmetric peak when analyzed by the same technique and was kept for further investigation. A great variation in molecular weights, in the range of 91–820 kDa, is reported for ulvans, depending on the species and approach utilized for extraction and analysis.[31–33]

2.2. Carboxyl-reduction of the heterorhamnan

Identification and quantification of the uronic acids present in fraction Go3r were carried out by esterification with 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate followed by reduction of the corresponding products with $NaBD_4$.[34,35] GC–MS analysis of the carboxyl-reduced polysaccharides (Go3r-R, 70.0% yield) showed a net increase of glucitol and galactitol (Table 2), and that 65% and 73% of these alditols corresponded to 1,6,6-trideutero glucitol and galactitol hexaacetate, respectively. These results indicate the presence of 12.3 and 2.9 mol % of glucuronic and galacturonic acid residues, respectively, in the original polymer. In order to determine if the purification procedure carried out with the crude extract Go3 affected its uronic acid composition, it was submitted to carboxyl-reduction (Go3-R, Table 2) followed by GC–MS analysis. The presence of the same uronic acids identified in the purified fraction was confirmed in the crude extract (11 and 3 mol % of glucuronic and galacturonic acids, respectively). Glucuronic acid together with iduronic acid have been reported as components of ulvans isolated from different species of *Ulva* genus.[4–8,17,18] The lack of 2,3,4-tri-*O*-acetyl-1,6-anhydrohexose in the GC–MS analysis of Go3r-R and Go3-R excludes the possibility of the presence of iduronic acid residues in heterorhamnans of *G. oxysperma*.

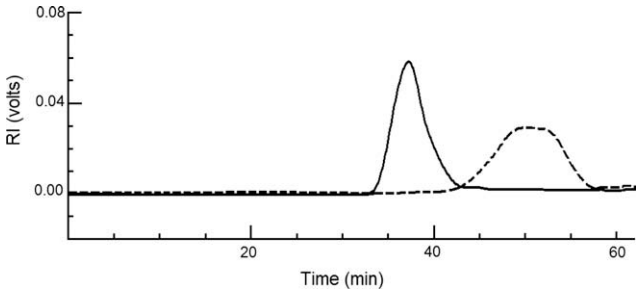


Figure 1. Elution profiles of Go3r (—) and Go3r-S (---) using HPSEC, obtained with a refractive index detector.

2.3. Solvolytic desulfation of the carboxyl-reduced heterorhamnan

Solvolytic desulfation[36] of Go3r-R removed 92% of the sulfate giving rise to the partially desulfated fraction Go3r-RD (70.0% yield, Table 2). Monosaccharide analysis of Go3r-RD was similar to that obtained for the parent fraction indicating lack of degradation during the solvolytic treatment (Table 2).

2.4. Methylation analyses of fractions Go3r-R and Go3r-RD

Methylation analysis was initially carried out with two polysaccharidic fractions, the carboxyl-reduced one (Go3r-R) and the carboxyl-reduced and desulfated fraction (Go3r-RD). The results of

Table 2
Yield, chemical analyses, specific rotation, and monosaccharide composition of polysaccharidic fractions obtained from Go3

	Go3-R[a]	Go3e	Go3r	Go3r-R	Go3r-RD	Go3r-RS	Go3r-RSD	Go3r-S	Go3r-SD
Yield (%)	68.3	30.0[b]	70.0[b]	70.0	70.0	50.0	80.0	50.0	72.0
Carbohydrate (%)	45.9	39.0	49.8	51.7	78.4	52.7	79.9	54.0	55.0
Sulfate[c] (%)	25.3	29.5	25.8	25.0	2.0	26.1	3.0	26.0	3.0
Uronic acid (%)	–	9.7	19.0	–	–	–	–	4.0	n.d.
Protein (%)	n.d.	3.7	–	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$[\alpha]_D$ (°)	n.d.	−20.0	−45.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Monosaccharide (mol %)									
Rha	59	54	77	64	64	91	92	95	95
Xyl	9	16	17	13	13	3	3	3	3
Glc	21	20	5	19	18	6	5	2	2
Gal	9	4	1	4	5	–	–	–	–
Ara	1	3	–	–	–	–	–	–	–
Man	1	3	–	–	–	–	–	–	–

– = Not detected. n.d. = not determined.
[a] Fractions are defined in the text.
[b] Percentage based on material recovered after ultrafiltration of Go3. Yields of ultrafiltration were 90%.
[c] Determined as SO_3Na.

Table 3
Methylation analysis of the carboxyl-reduced (Go3r-R), carboxyl-reduced, and Smith-degraded (Go3r-RS) fractions and their partially desulfated products (Go3r-RD and Go3r-RSD) obtained from Go3r

Derivative[a]	Deduced linkage	Fractions			
		Go3r-R	Go3r-RD	Go3r-RS	Go3r-RSD
2,3,4-Rha[b]	Rha*p*-(1→	–	–	3	3
2,4-Rha	→3)-Rha*p*-(1→	6	35	15	50
3,4-Rha	→2)-Rha*p*-(1→	4	15	1	33
2-Rha	→3,4)-Rha*p*-(1→	11	1	17	1
3-Rha	→2,4)-Rha*p*-(1→	9	1	16	2
4-Rha	→2,3)-Rha*p*-(1→	19	13	20	5
Rha	→2,3,4)-Rha*p*-(1→	17	1	20	–
2,3,4-Xyl	Xyl*p*-(1→	5	5	–	–
2,4-Xyl	→3)-Xyl*p*-(1→	2	2	3	2
2,3-Xyl	→4)-Xyl*p*-(1→	4	4	–	–
2/3-Xyl	→3,4/2,4)-Xyl*p*-(1→	1	1	–	–
2,3,4,6-Glc	Glc*Ap*-(1→	–	5	–	–
3,4,6-Glc	→2)-Glc*Ap*-(1→	5	–	–	–
2,3,6-Glc	→4)-Glc*Ap*-(1→	3	7	–	4
3,6-Glc	→2,4)-Glc*Ap*-(1→	4	–	5	–
2,3,6-Glc	→4)-Glc*p*-(1→	4	4	–	–
2,3,4,6-Gal	Gal*Ap*-(1→	–	4	–	–
3,4,6-Gal	→2)-Gal*Ap*-(1→	5	1	–	–
2,3,4,6-Gal	Gal*p*-(1→	1	1	–	–

– = not detected. / = and/or.
[a] Mol % of monosaccharide bearing methyl groups at the positions indicated.
[b] 2,3,4-Rha analyzed as 1,5-di-*O*-acetyl-2,3,4-*O*-methylrhamnitol, etc.

these analyses are shown in Table 3. Although the methylation process was sequentially repeated three-times to obtain the per-O-methylated product, the content of nonmethylated rhamnose (Rha) for Go3r-R remained unaltered and was thus attributed to 2,3,4-trisubstituted rhamnosyl units. Besides this, monomethylated rhamnose (4-Rha, 3-Rha, and 2-Rha) and dimethylated rhamnose residues (2,4-Rha and 3,4-Rha) were also found indicating the presence of disubstituted (39 mol %), 3-linked (6 mol %), and 2-linked (4 mol %) rhamnosyl units in Go3r-R. Xylosyl units were mainly detected as nonreducing terminal residues (2,3,4-Xyl, 5 mol %) followed by lower amounts of 3-linked, 4-linked, and 2,4- plus 2,3-disubstituted xylosyl units. Additionally, 3,4,6-Glc, 2,3,6-Glc and 3,6-Glc (reduced glucuronic acid), 3,4,6-Gal (reduced galacturonic acid), and nonreducing terminal galactose residues were also identified.

Methylation analysis of the desulfated product (Go3r-RD), when compared with that of the parent compound (Go3r-R), showed the disappearance of previously present 2-, 2,4-substituted glucuronic acid and a decrease of 2-substituted galacturonic acid with a concomitant appearance of nonreducing glucuronic (5 mol %) and galacturonic acid (4 mol %) residues, and an increase of 4-substituted glucuronic acid (3–7 mol %). These results showed that ~80% of the uronic acids are sulfated on C-2 either as nonreducing or 4-linked residues and that ~20% are nonsulfated 4-linked residues. Nevertheless, the major derivatives among the Go3r-RD methylation products were 2,4-Rha (35 mol %), 3,4-Rha (15 mol %), and 4-Rha (13 mol %) corresponding to 3-linked, 2-linked, and 2,3-disubstituted rhamnosyl units. The later represents branching points in the heterorhamnan in agreement with the amount of nonreducing terminal residues (14 mol %) detected among the partially O-methylated derivatives from Go3r-RD (Table 3). After desulfation, the increase of 3-linked and 2-linked rhamnose residues (2,4-Rha and 3,4-Rha, respectively) along with the decrease of 2-Rha, 3-Rha, Rha, and 4-Rha, showed that the parent polysaccharide contains 3- and 2-linked rhamnosyl units with sulfation at C-4 (10 and 8 mol %, respectively). In addition, it could contain 3-linked units sulfated at C-2 and/or C-2,C-4 (19 mol %), besides 2-linked rhamnosyl units sulfated on C-3 and/or C-3,C-4 positions (3 mol %). It follows that the results of these methylation analyses were not sufficient to determine the position of all the sulfate groups.

Table 4
Analysis of rhamnose derivatives from Go3r-R after successive methylation, desulfation, and trideuteromethylation

Derivative	Composed by[a]	Corresponding units[b]	Mol %	Mass fragments (*m/z*)[c]
4-Rha	4-Me Rha	2L3R/3L2R	6	131, 203, 262, 290
	4-CD_3 Rha	2L3R4S/3L2R4S	7	134, 206, 265, 293
2,4-Rha	2,4-Me Rha	3L	6	118, 131, 187, 234, 262
	2,4-CD_3 Rha	3L2,4S	5	121, 134, 193, 240, 268
	2-CD_3 4-Me Rha	3L2S	13	121, 134, 190, 237, 265
	2-Me 4-CD_3 Rha	3L4S	10	118, 131, 190, 237, 265
3,4-Rha	3,4-Me Rha	2L	4	115, 130, 131, 190, 262
	3,4-CD_3 Rha	2L3, 4S	3	121, 133, 134, 193, 268
	3-Me 4-CD_3 Rha	2L4S	8	118, 130, 134, 190, 265

/ = and/or.
[a] Me = methyl, CD_3 = trideuteromethyl.
[b] L, R, S correspond to glycosidic linkage, side chain and sulfate at the position indicated, respectively.
[c] Fragment ions observed in the partially trideuteromethylated alditol acetates.

However, when the carboxyl-reduced polysaccharide Go3r-R was sequentially per-O-methylated, desulfated, and trideuteromethylated, the sulfation pattern was clarified. The results of this analysis (Table 4) showed that 4-Rha, 2,4-Rha, and 3,4-Rha derivatives were partially methylated and/or trideuteromethylated, and the CD_3 location was interpreted as the sulfation position in the original polysaccharide. The 4-Rha derivative gave rise to fragments at *m/z* 131 and 134, indicating the presence of CH_3 and CD_3 at C-4 in the proportion of 0.88:1.00, respectively. Therefore, from the 13 mol % of 4-Rha, detected among the Go3r-RD products of methylation (Table 3), corresponding to branched rhamnosyl units, 6 mol % was unsulfated and 7 mol % C-4 sulfated.

The 2,4-Rha derivative showed characteristic fragments ions at *m/z* 234 and 240 indicating the presence of CH_3 and CD_3 groups, respectively, at both C-2 and C-4 positions, besides a fragment ion at *m/z* 237 corresponding to a CD_3 group at C-2 (CH_3 at C-4) and/or at C-4 (CH_3 at C-2). Therefore, from the ratios of these fragments (0.26:0.21:1.00, respectively), the 35 mol % of 2,4-Rha observed in the methylation analysis of Go3r-RD (Table 3) was derived from 3-linked nonsubstituted rhamnopyranosyl units (6 mol %), sulfated on both C-2 and C-4 (5 mol %), sulfated on C-2 (13 mol %) and sulfated on C-4 (10 mol %).

Similarly, the 3,4-Rha derivative (Table 4) gave fragment ions at *m/z* 130 and 133 indicating the presence of CH_3 and CD_3 groups, respectively, at C-3 position together with fragment ions at *m/z* 131 and 134 indicative of CH_3 and CD_3 groups, respectively, at the C-4 position. The ratio of the above mentioned fragments (0.95:0.37:0.31:1.00, respectively) confirmed that the 2-linked rhamnosyl units are unsulfated (27%) and C-4 sulfated (53%), as previously determined from the methylation analysis of the Go3r-RD polysaccharide (Table 3). Taking into account that the 4-Rha derivative detected among the products of methylation analysis of Go3r-R (19 mol %, Table 3) corresponds to unsulfated branching points (6 mol %) and to 3-linked 2-sulfated rhamnosyl units (13 mol %), it is possible to conclude that 2-linked 3-sulfated rhamnosyl units are not present in this polysaccharide. Therefore, these data indicate that the 3,4-disulfated rhamnosyl units gave rise to 20% of the 2-linked residues after desulfation.

Thus, the polysaccharide fraction Go3r, isolated from *G. oxysperma*, is a branched and sulfated heterorhamnan with 2,3-substituted, and 3- and 2-linked rhamnose residues in a molar ratio of 0.37:1.00:0.43, respectively, corresponding to one branching point every five rhamnose residues. The 3- and 2-linked rhamnose

residues are mostly 4-sulfated (37%) and to a lesser extent 2-sulfated and disulfated (27% and 16%, respectively). Notwithstanding of the fact that the branching position was not completely established (at C-2 or C-3 of the 2,3-disubstituted rhamnosyl units), approximately 50% of these are sulfated on C-4. The complexity of the sulfation pattern is increased by the presence of glucuronic and galacturonic acids, each 2-sulfated, as nonreducing end-units and 2-sulfated 4-linked glucuronic acid residues.

Ulvans isolated from genus *Ulva* contain ulvanobiuronic acid ([→4)-β-D-Glc*p*A-(1→4)-α-L-Rha*p* 3-sulfate-(1→]) as the major disaccharide repeating structure.[4–8,17,18] This is also substituted on C-2 of its rhamnose 3-sulfate residue by single stubs of glucuronic acid together with other disaccharide repeating structures containing xylose and xylose 2-sulfate, which were also identified in ulvans.[18] A heteroglycan produced by *Enteromorpha compressa* (Ulvales) also contains rhamnose 3-sulfate besides 4-linked glucuronic acid and xylose 2-sulfate residues.[11] In contrast, *Monostroma* spp. produced branched sulfated rhamnans with different proportions of 2- and 3-linked rhamnosyl units[9,10,25] with sulfate substitution mainly at C-2,[9,10] C-3, or C-4.[9] Hence, the glycosidic linkage and sulfation patterns of the heterorhamnan of *G. oxysperma* are distinctly different from those observed for other green algae belonging to genera *Ulva* and *Enteromorpha*.

2.5. Controlled Smith degradation and methylation analysis of the Smith-degraded product

In order to complement the structural information discussed above, the carboxyl-reduced heterorhamnan Go3r-R was submitted to a controlled Smith degradation[37,38] giving rise to the carboxyl-reduced and Smith-degraded product (Go3r-RS, 50% yield, 26.1% SO_3Na) that was sequentially submitted to solvolytic treatment (Go3r-RSD, ~88% of desulfation) (Table 2). Monosaccharide composition of both products was similar and showed mainly rhamnose (91–92 mol %) besides minor amounts of xylose (3 mol %) and glucose (6–5 mol %) (Table 2). Methylation analysis of resulting Go3r-RS (Table 3) showed that the nonreducing end-units, as well as other units susceptible to periodate oxidation (2-linked rhamnose, 4-linked xylose, and 4-linked glucose) were removed. Methylation analysis of Go3r-RSD (Table 3) showed the presence of 4-Rha derivative (5 mol %) indicating that the Smith-degraded and desulfated product still retained a low level of branching (one branching point every eighteen rhamnosyl units). Although the composition and length of the branches were partially determined, in addition to uronic acids and xylose units they must also contain rhamnosyl units, in accord with the detection of 2,3,4-Rha amongst the methylation derivatives of both sulfated (Go3r-RS) and desulfated (Go3r-RSD) products (3 mol %). Go3r-RSD methylation data also showed that the 2,3-substituted 3-linked, 2-linked rhamnosyl units are present in a molar ratio of 0.10:1.00:0.66, respectively. When compared with Go3r-RD, the Smith-degraded product Go3r-RSD showed an increase of 2-linked rhamnosyl (from 0.43 to 0.66) with a concomitant decrease of 2,3-disubstituted rhamnosyl units from 0.37 to 0.10. This result could indicate that, at least in part, the branches are located at C-3 of the 2-linked rhamnosyl units in the heterorhamnan of *G. oxysperma*.

The native polysaccharide Go3r was also submitted to a controlled Smith degradation to afford Go3r-S (Table 2). Its HPSEC-MALLS-RI analysis gave a homogeneous profile with MW of 109 kDa (d*n*/d*c* of 0.251). The HPSEC-RI elution profile of Go3r-S is shown in Figure 1. The ~14-fold decrease of the molecular weight, when compared with that of Go3r, suggests that the heterorhamnan backbone contains units susceptible to periodate oxidation, and that these are randomly distributed along the polysaccharide.

2.6. FTIR analyses

FTIR spectra of Go3r-R and Go3-R were similar and showed absorptions of high intensity at 1250 cm^{-1} related to sulfate groups (stretching vibration of S–O of sulfate) together with two other bands at 850 and 829 cm^{-1}. Absorptions at 850 cm^{-1} were observed in the FTIR spectra of ulvans from *Ulva lactuca* and *E. compressa* and attributed to axial sulfate on C-2 of rhamnose.[39,40] However, the FTIR spectrum of the ulvan from *U. 'rigida'* bearing sulfate groups at equatorial position[3] (on C-3 of rhamnose) also showed absorption at 850 cm^{-1}. From these results, the authors[3] suggested that this absorption did not correspond to sulfate groups at an axial position. Taking into account these results, the absorptions at 850 and 829 cm^{-1} present in the FTIR spectra of Go3r-R and Go3-R cannot be employed as diagnostic stretching vibrations of C–O–S in equatorial and/or axial positions.

2.7. NMR analyses

2.7.1. NMR analyses of the sulfated polysaccharidic fractions

The ^{13}C NMR spectra of the crude extracts Go1–Go6 showed the same signal pattern (data not shown), so that it was decided to perform the NMR analyses with Go3, as a representative of the water-soluble polysaccharide system produced by *G. oxysperma*.

The ^{1}H and ^{13}C NMR spectra of the native polysaccharide and its modified products were partially assigned using COSY, TOCSY, and HMQC experiments and by comparison with the chemical shift data of similarly substituted sugar residues.[41–45] As these references correspond to unsulfated polysaccharides (bacterial rhamnans), we complemented their chemical shifts considering the α- and β-effect of sulfation.[4,6,7]

The ^{13}C NMR analyses of the sulfated polysaccharides (Go3, Go3r) show complex spectra with broadened signals in agreement with the heterogeneous pattern of glycosylation and sulfation of the rhamnosyl units together with the presence of other monosaccharides such as xylose and uronic acids. In addition, the presence of sulfated and nonsulfated branching points in the polysaccharidic fractions results in spectra with a high degree of overlapping. In accord with this structural complexity the ^{13}C NMR spectra of Go3, Go3r (Figs. 2a and b), and Go3r-R show several anomeric resonances, at ~105 ppm attributed to β-glycosidic configurations[46] with those between 103 and 98 ppm being mainly assigned to α-rhamnosyl units.

The very intense high-field signals at 18.2–17.9 ppm (Figs. 2a–c) arise from C-6 of rhamnosyl units in agreement with major amounts of this 6-deoxysugar (Tables 1 and 2) and its complex pattern of substitution (Table 3). After carboxyl-reduction, the disappearance of the lower-field signal at 175.5 ppm (not shown) from carboxyl groups of uronic acids, along with the increase of the signal corresponding to unsubstituted C-6 (61.7 ppm), is in agreement with the conversion of glycopyranosiduronic acids to the corresponding neutral monosaccharides.

The α-anomeric configuration of rhamnosyl units was deduced from H-5 signals (data not shown) at 3.77–3.88 ppm (compared with the β-anomer at 3.39 ppm)[47] and C-5 signal at 70.6 ppm (compared with that of the β-anomer at 72.3–73.4 ppm),[48,49] present in all the ^{13}C NMR spectra (Figs. 2a–c and 3a, b).

Due to the relatively low glucuronic acid and xylose contents (Tables 1 and 2), their heterogeneous substitution patterns (Table 3), and overlapping of signals, it was very difficult to identify their chemical shifts. However, the low intensity signal at 105.2 ppm present in the spectrum of Go3r (Fig. 2b) could arise from nonreducing terminal β-D-xylosyl units based on the corresponding methyl glycoside resonance[46] as well as from 4-linked β-D-xylose and β-D-glucuronic acid residues.[6] In addition, the C-1 signal at 102.6 ppm showed HMQC correlation with its linked proton at

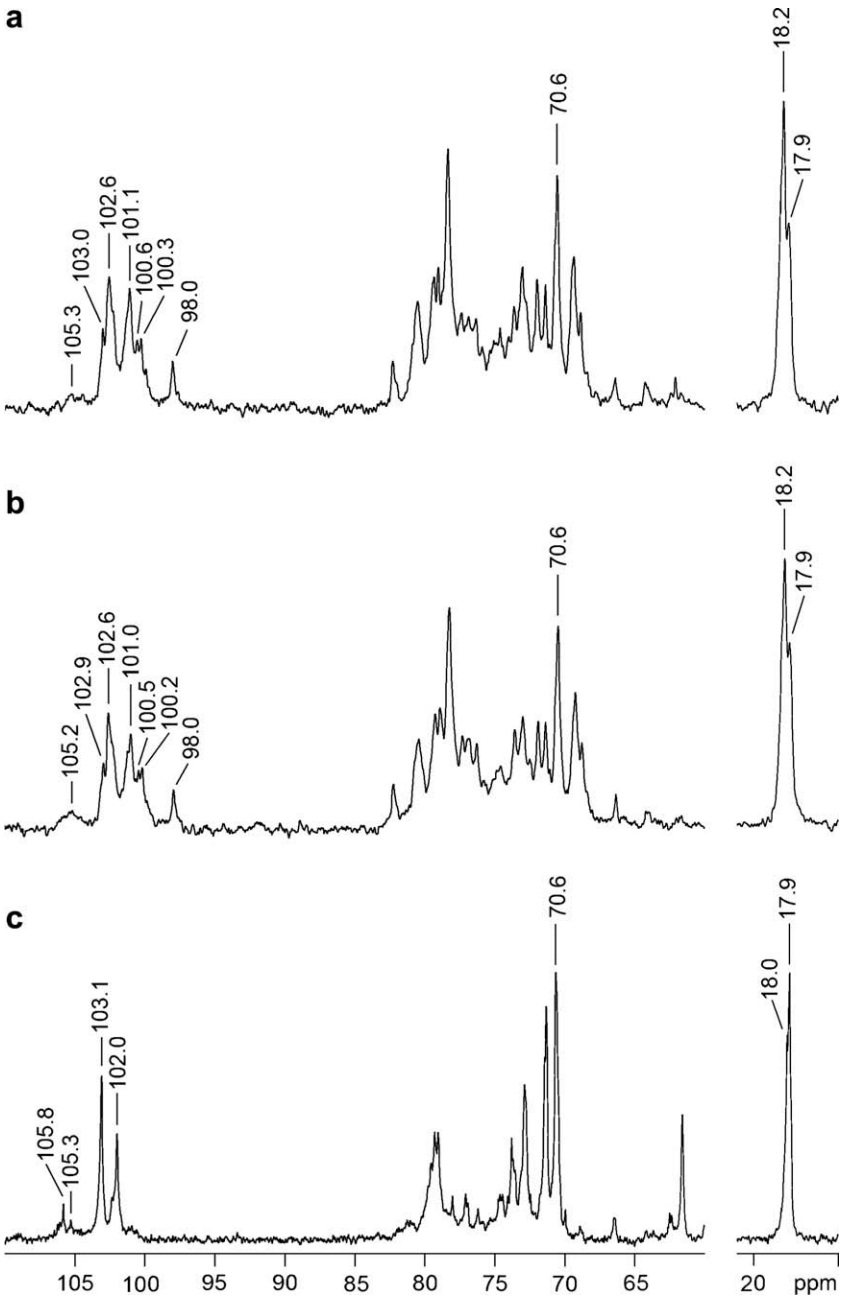


Figure 2. ^{13}C NMR spectra of the crude extract Go3 (a), purified polysaccharide Go3r (b), and the carboxyl-reduced and desulfated derivative Go3r-RD (c).

4.79 ppm, and could arise from β-D-glucuronic acid 2-sulfate residues. These resonances are in close agreement with those reported for 4-linked 2-substituted glucuronosyl residues present in an exopolysaccharide produced by a deep-sea bacterium[50] taking into account the additional effect of sulfation as well as those reported for 4-linked 2-sulfated glucuronosyl units.[51,52] These assignments were corroborated by the disappearance of the correlation in the HMQC spectrum of the desulfated polysaccharide.

The ^{1}H NMR spectra of Go3r and the Smith-degraded product (Go3r-S) were qualitatively similar, and the anomeric resonances between 5.79 and 5.03 ppm were assigned to α-rhamnosyl units (Fig. 4a and c). The ^{13}C NMR spectra of Go3r (and Go3r-S) showed in the anomeric region several signals between 102.9 and 98.0 (103.6–98.0) ppm mainly attributed to α-L-rhamnosyl units (Figs. 2b and 3a). From the HMQC and COSY experiments of Go3r and (Go3r-S), the anomeric signals at 100.5 (100.4)/5.33, 100.2 (100.1)/5.48 (5.50), and 98.0/5.78 (5.79) ppm were assigned to 3-linked rhamnosyl units sulfated at C-2 and at both C-2 and C-4. Their C-2 signal appeared at 78.2 ppm, in agreement with the α-effect (+6.9 ppm) of sulfation of the 3-linked rhamnose residues (see Table 5). The H-1–H-2 cross-peaks, 5.48(5.50)/4.70 and 5.33/4.70 ppm, are consistent with sulfation at C-2. The influence of sulfation on these resonances was confirmed by the lack of those signals in the spectra of the desulfated products Go3r-RD and Go3r-SD (Figs. 2c, 3b, and 4b, d). The H-1, C-1, and C-2 resonances are in good agreement with those reported for the related α-D-mannose 2-sulfate units in the 3-linked mannans isolated from the red seaweed *Nothogenia fastigiata*.[53]

In the HMQC spectra of Go3r and (Go3r-S), the anomeric signals at 101.3 (101.4) and 101.0 ppm correlated with their geminal hydrogens at 5.22 (5.23) and 5.27 (5.28) ppm that were ascribed to 3,4- and 4-sulfated 2-linked rhamnose residues (C-4 at

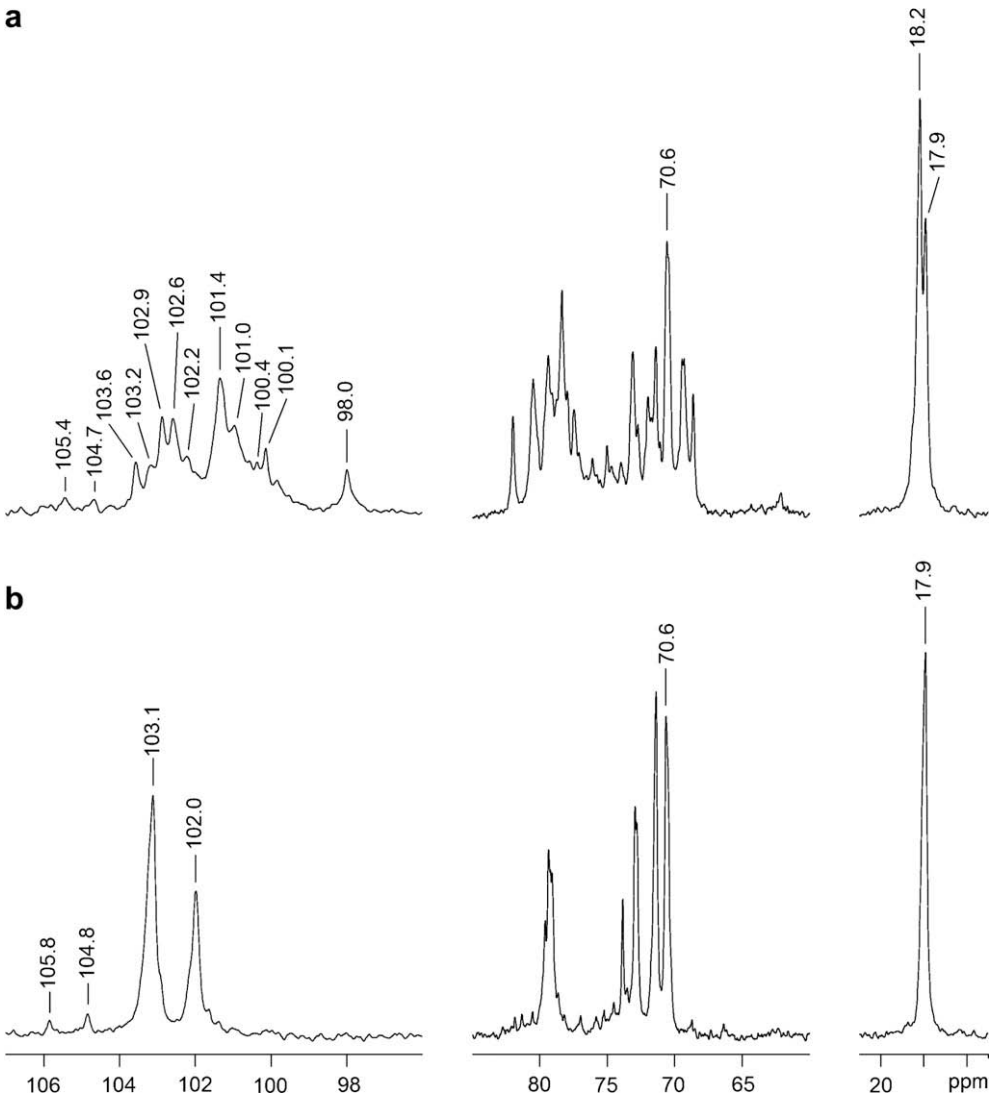


Figure 3. ^{13}C NMR spectra of the Smith-degraded Go3r-S (a) and Smith-degraded and desulfated Go3r-SD (b) products.

82.0 ppm). Additionally these anomeric signals correspond to 2,3-substituted 4-sulfated or unsulfated rhamnose residues,[43] showing that part of the branching points remained after controlled Smith degradation. The anomeric signals at 102.9–102.6 and (103.6–102.6) ppm showed correlations with their geminal protons at 5.03–5.10 and (5.05–5.09) ppm, respectively. These resonances were attributed to 3-linked and 3-linked 4-sulfated rhamnose residues. The values for C-2 (71.4 ppm) and H-2 (4.17 ppm) are consistent with the lack of C-2 substitution, and are in accord with literature data for 3-linked and 3-linked 4-substituted rhamnose residues.[41,42,54] For Go3r-S, the 4-sulfated residue gave C-4/H-4 and C-5/H-5 resonances at 80.5/4.36 and 69.3–69.2/3.94 ppm, respectively. In the spectrum of Go3r, similar resonances at 80.4/4.34 and 69.2/3.94 ppm were assigned to C-4/H-4 and C-5/H-5 of the 3-linked 4-sulfated rhamnose residues. In agreement with C-4 sulfation, these carbon resonances are shifted downfield (~7.6 ppm) and upfield (~1.1 ppm), respectively, when compared with the corresponding signals of the 3-linked rhamnose residues in the spectrum of the desulfated product (Figs. 2c and 3b, Table 5).

NMR analyses of the sulfated heterorhamnan thus confirm the presence of several types of sulfated 3- and 2-linked rhamnose residues as determined by methylation analysis.

2.7.2. NMR analyses of the desulfated polysaccharidic fractions

The desulfated polysaccharide (Go3r-RD) and the Smith-degraded and desulfated product (Go3r-SD, Table 2) were analyzed using 1D and 2D NMR spectroscopy. ^{13}C NMR spectra of Go3r-RD (Fig. 2c) and Go3r-SD (Fig. 3b) showed two main anomeric signals attributed to 2,3-linked, 2-linked (102.0 ppm), and 3-linked (103.1 ppm) rhamnose residues in agreement with methylation results. The HMQC spectra of Go3r-RD and Go3r-SD (Fig. 5) contain well-defined cross-peaks for these anomeric nuclei at 102.0/5.20 for the 2-linked rhamnose residues[41,42] (designed as residue **A** in Chart 1) and at 103.1/5.07 and 103.1/4.99 assigned to 3-linked rhamnose-(1→3)- and 3-linked rhamnose-(1→2)-rhamnose residues, respectively (residues **C** and **D**, respectively, Chart 1). The assignments for **C** and **D** residues (Table 5) are in accordance with those reported for the rhamnosyl repeating units present in the O-polysaccharide chains of the lipopolysaccharide from *Pseudomonas syringae pv. garcae* NCPPB 2708.[41] In addition, the signals at 5.26 and 5.27 ppm present in the spectra of Go3r-RD and Go3r-SD (Fig. 4b and d, respectively) were attributed to H-1 of 2,3-disubstituted rhamnose residues (residue **B**, Chart 1). For Go3r-RD, the HMQC spectrum showed that the H-1 signal at 5.26 ppm correlated with a carbon at 102.0 ppm. These assignments are in good agreement with the values reported for 2-linked 3-substituted

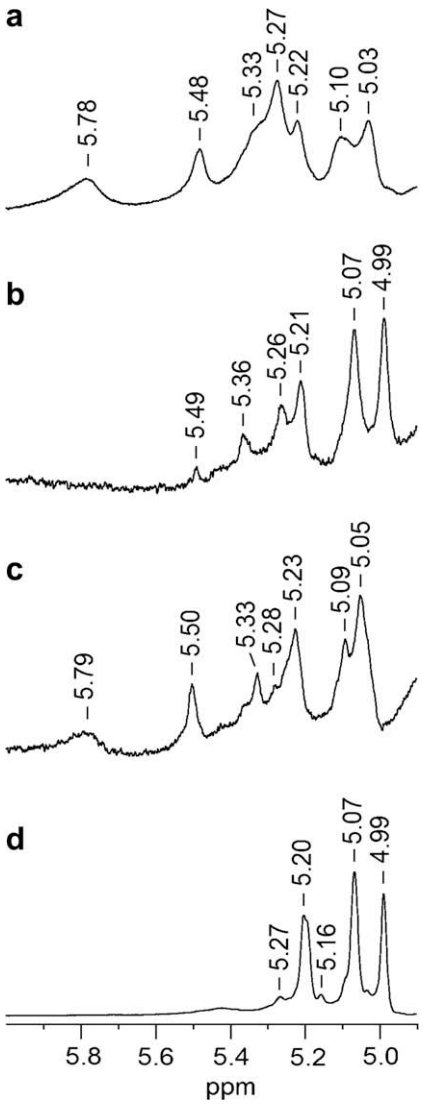


Figure 4. ^{1}H NMR spectra of the purified polysaccharide Go3r (a) carboxyl-reduced and desulfated derivative Go3r-RD (b), Smith-degraded Go3r-S (c), and Smith-degraded and desulfated Go3r-SD (d) products.

rhamnose residues from the O-polysaccharide of *P. syringae pv.* tomato GSPB 483.[43]

The H-1 resonances of Go3r-RD and Go3r-SD at 5.26–5.27, 5.20, 5.07, and 4.99 ppm (Fig. 4b,d, Table 5) had relative integrals of 0.6:0.7:1.1:1.0 and 0.2:1.4:1.7:1.0, respectively. The H-1 signal intensity for 2,3-linked rhamnose residues (5.27 ppm) in Go3r-SD spectrum decreased in comparison with the corresponding signal (5.26 ppm) in the Go3r-RD spectrum. This result is in agreement with methylation analysis, which showed a decrease of the branching points after controlled Smith degradation. Furthermore, after this modification the intensity of the signal at 5.20 ppm increased with a concomitant decrease of that at 5.27 ppm, indicating the presence of branching points at C-3 of the 2-linked rhamnose residues. Therefore, NMR analyses complement and confirm methylation data and allow us to propose the major structural features of the heterorhamnan Go3r that are shown in Chart 2.

2.8. Cytotoxicity test of the sulfated heterorhamnans from G. oxysperma

The extracts with the highest yields Go3 and Go4 and the purified fraction Go3r, obtained on ultrafiltration, were initially evaluated for cytotoxicity by assessing their effects on Vero cell viability. For comparative purposes, heparin was simultaneously assayed as a known reference polysaccharide. No effect on cell viability was observed with any of these compounds at concentrations up to 1000 μg mL^{-1}.

2.9. Antiviral activity of the sulfated heterorhamnans against herpes simplex virus

The three polysaccharidic fractions were then screened for antiviral activity against strain F of HSV-1 and strain MS of HSV-2, by a virus plaque reduction assay on Vero cells. As shown in Table 6, Go3, Go3r, and Go4 exhibited potent in vitro antiherpetic activity with IC_{50} values ranging from 0.27 to 0.3 μg mL^{-1} for HSV-1 and values 10-fold lower from 0.036 to 0.054 μg mL^{-1} for HSV-2. Heparin was simultaneously assayed as a reference substance, and it was found less effective than the fractions obtained from *G. oxysperma* to inhibit the multiplication of HSV-1 and HSV-2.

Good anti HSV activity has also been reported with structurally different sulfated polysaccharides produced by green algae from genera *Enteromorpha, Monostroma, Caulerpa, Chaetomorpha*, and *Codium*.[22,55] A rhamnan sulfate isolated from *Monostroma latissimum* showed potent antiviral effect against human cytomegalovirus (HCMV) and HIV-1. The antiviral action of the rhamnan sulfate was not only due to the inhibition of virus adsorption, but also might involve the later replication of virus in host cells.[20]

Given the lack of cytotoxicity exhibited by the sulfated heterorhamnans analyzed in this work, and the very good antiviral activity against the two serotypes of HSV tested, they present very high selectivity indices (SI: ratio CC_{50}/IC_{50}), indicating the specificity of the inhibitory effect against herpesviruses.

Table 5
Chemical shift assignments of NMR spectra of Go3r-RD and Go3r-SD

Rhamnosyl residues	Chemical shift (ppm)						Chemical shift (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6	H-1	H-2	H-3	H-4	H-5	H-6
C[a]	103.1 (103.1)	71.3 (71.4)	79.3 (79.3)	72.9 (72.8/72.9)	70.6 (70.6)	17.9/18.0 (17.9)	5.07 (5.07)	4.18 (4.15)	3.92 (3.91)	3.60 (3.60)	3.76–3.88 (3.78–3.88)	1.30–1.33 (1.32)
D[a]	103.1 (103.1)	71.3 (71.4)	79.0 (79.1)	72.9 (72.8/72.9)	70.6 (70.6)	17.9/18.0 (17.9)	4.99 (4.99)	4.18 (4.15)	3.87 (3.86)	3.62 (3.62)	3.76–3.88 (3.78–3.88)	1.30–1.33 (1.29)
A[a]	102.0 (102.0)	79.3 (79.3)	71.3 (71.4)	73.8 (73.8)	70.6 (70.6)	17.9/18.0 (17.9)	5.21 (5.20)	4.09 (4.08)	3.96 (3.95)	3.53 (3.51)	3.76–3.88 (3.78–3.88)	1.30–1.33 (1.32)
B[b]	102.0 (102.0)					17.9/18.0 (17.9)	5.26 (5.27)	4.09				1.30–1.33 (1.29–1.32)

In parentheses chemical shifts in Go3r-SD ^{1}H and ^{13}C NMR spectra.
[a] In accordance with Refs. 41 and 42.
[b] In accordance with Ref. 43.

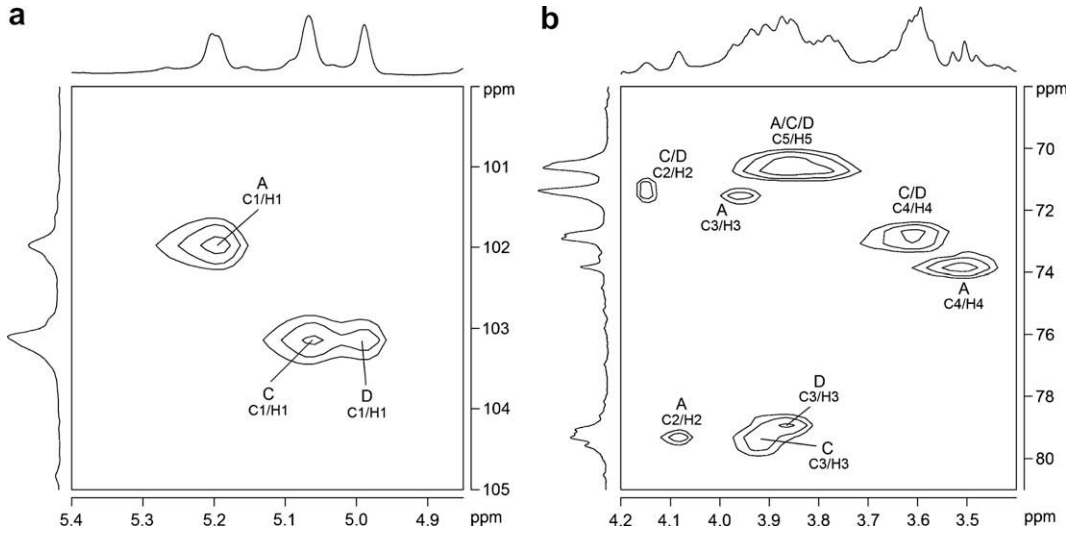


Figure 5. HMQC spectrum of Smith-degraded and desulfated Go3r-SD product, anomeric (a) and C-2–C-5/H-2–H-5 (b) regions. **A**, **C,** and **D** correspond to [→2)-α-L-Rha*p*-(1→], [→3)-α-L-Rha*p*-(1→3)], and [→3)-α-L-Rha*p*-(1→2)] residues, respectively (see Chart 1).

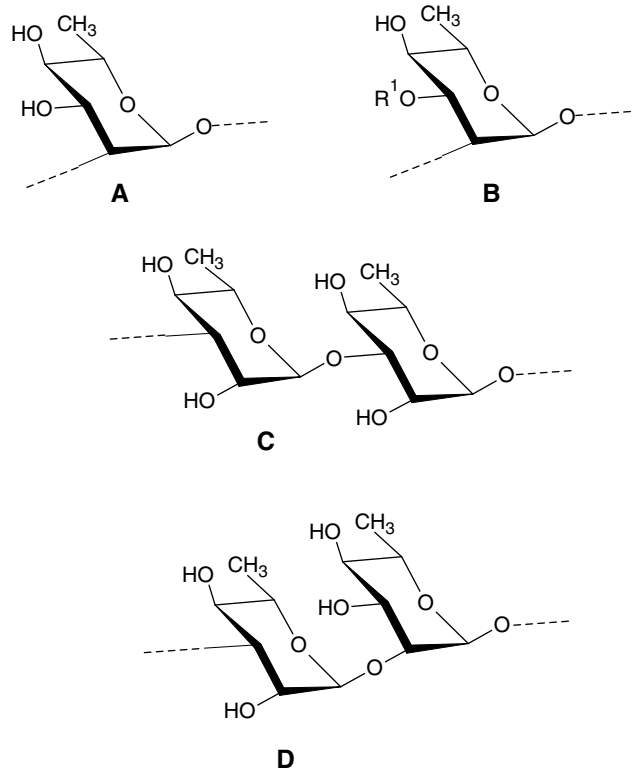


Chart 1. Major rhamnose residues present in the carboxyl-reduced and desulfated polysaccharide Go3r-RD and in the Smith-degraded and desulfated product Go3r-SD. **A**, **B**, **C**, and **D** correspond to [→2)-α-L-Rha*p*-(1→], [→2,3)-α-L-Rha*p*-(1→3)], [→3)-α-L-Rha*p*-(1→3)], and [→3)-α-L-Rha*p*-(1→2)] residues, respectively. Go3r-RD: R^1 = side chains containing Glc, Gal (reduced uronic acids), or Xyl as nonreducing terminal units; Go3r-SD: R^1 = side chains containing Rha as nonreducing terminal units.

The antiviral activity of sulfated polysaccharides is linked to anionic features of the macromolecules as well as to their molecular weight.[56–66] Moreover, as previously reported, the hydrophobic character of the constituent sugars seems to be important for antiviral activity of the polysaccharide.[58,59] Therefore, the sulfated heterorhamnan from *G. oxysperma* presents a combination of structural factors that determine its antiherpetic activity, such as high molecular weight and sulfate content (75% and 80% of the rhamnosyl and uronic acids, respectively are substituted by sulfate groups). Together with these structural characteristics, the hydrophobic character of the methyl group at C-5 of the rhamnosyl units is an additional factor that could contribute to the high and specific activity of the sulfated heterorhamnan isolated from the green seaweed *G. oxysperma*.

3. Experimental

3.1. Collection of specimens

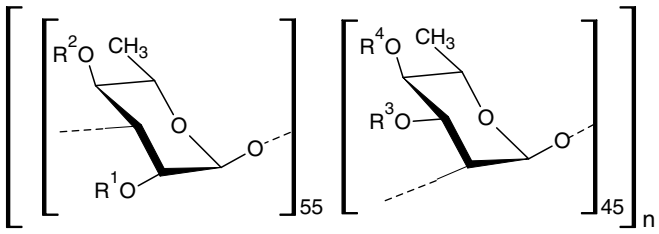
The specimen of the green seaweed *G. oxysperma* (Kützing) K.L. Vinogradova ex Scagel et al. was cultivated at Bahia de Paranaguá, Paraná State (southern coast of Brazil).[67] The voucher specimen was deposited in the herbarium of the Department of Botany, Federal University of Paraná (Curitiba, Brazil) with herbarium number UPCB-58059. The fresh material was cleaned to remove undesirable contaminants, washed with tap water, sun-dried, and milled.

3.2. Extraction and purification of polysaccharides

The milled seaweed *G. oxysperma* was extracted twice with water (5% w/v) at 25 °C with mechanical stirring for 4 h. After centrifugation, the supernatant was precipitated with EtOH (3 vols), and the resulting precipitate was redissolved in water, dialyzed (cut-off 12–14 kDa), concentrated, and freeze-dried to give the crude extract Go1. This was repeated to give fraction Go2. The residue was then submitted to similar four successive aq extractions at 80 °C for 4 h, to afford the crude extracts Go3 to Go6. Fraction Go3 was submitted to ultrafiltration through a 300 kDa cut-off membrane, yielding a retained fraction (Go3r) and an eluted fraction (Go3e).

3.3. Analytical methods

Sulfate and uronic acid contents were determined by the methods of Dodgson and Price[68] and Filisetti-Cozzi and Carpita,[69] respectively. Total carbohydrate content was estimated by the phenol-sulfuric acid method,[70] using rhamnose as standard. Protein content was measured by the method of Lowry using the



$\mathbf{R^1}$ = $\mathbf{R^2}$ = H (10%) $\mathbf{R^3}$ = Side chains (21%) $\mathbf{R^3}$ = $\mathbf{R^4}$ = H (6%)

$\mathbf{R^1}$ = $\mathbf{R^2}$ = SO_3^- (8%) $\mathbf{R^4}$ = H (10%) or SO_3^- (11%) $\mathbf{R^3}$ = $\mathbf{R^4}$ = SO_3^- (5%)

$\mathbf{R^1}$ = H, $\mathbf{R^2}$ = SO_3^- (16%) $\mathbf{R^3}$ = H $\mathbf{R^4}$ = SO_3^- (13%)

$\mathbf{R^1}$ = SO_3^- $\mathbf{R^2}$ = H (21%)

Side chains containing Glc*p*A 2-S (8%) or Gal*p*A 2-S (6%) or Xyl*p* (8%) as NRT

Chart 2. Major rhamnose residues present in the sulfated heterorhamnan Go3r. NRT = nonreducing terminal units.

Table 6
Antiviral activities of fractions obtained from *G. oxysperma*

Fractions	IC_{50}[a] (μg mL^{-1})		SI[b] (CC_{50}/IC_{50})	
	HSV-1 (F)	HSV-2 (MS)	HSV-1 (F)	HSV-2 (MS)
Go3	0.30 ± 0.15	0.054 ± 0.006	>3333	>18,518
Go3r	0.27 ± 0.03	0.036 ± 0.001	>3704	>27,778
Go4	0.28 ± 0.12	0.037 ± 0.001	>3571	>27,027
Heparin[c]	1.30 ± 0.1	0.50 ± 0.1	>769	>2000

[a] IC_{50} (inhibitory concentration 50%): concentration required to reduce plaque number in Vero cells by 50%. Each value is the mean of two determinations ± SD.

[b] SI (selectivity index): CC_{50}/IC_{50}. CC_{50} (cytotoxic concentration 50%): concentration required to reduce the number of viable Vero cells by 50% after 48 h of incubation with the fractions. This concentration was >1000 μg mL^{-1} for all the fractions.

[c] Heparin included as a reference substance.

Folin–Ciocalteau reagent with bovine serum albumin as standard.[71] For monosaccharide composition, the polysaccharide samples were hydrolyzed using M TFA at 100 °C for 4 h. Hydrolysis products were reduced with $NaBD_4$, and after acetylation with 1:1 acetic anhydride–pyridine for 12 h at room temperature, the resulting alditol acetates derivatives were analyzed by GC–MS. GC–MS analyses were performed with a Varian 3800 chromatograph, equipped with a fused silica capillary column (30 m x 0.25 mm) coated with DB-225MS (Durabond), and a Varian Saturn 2000R ITD spectrometer. The chromatograph was programmed to run at 50 °C for 1 min, then 50–215 °C at 40 °C min^{-1}, using helium as carrier gas at 1 mL min^{-1}. The enantiomeric configuration of rhamnose was carried out after reductive amination with chiral (*S*)-α-methylbenzylamine as previously described.[72]

3.4. Carboxyl-reduction

The crude extract Go3 was esterified with 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate and the product reduced with $NaBH_4$ to give Go3-R (68% yield).[34] Buffers MES and TES were used to maintain the pH.[35] Fraction Go3r was carboxyl-reduced using the same method described above, but using $NaBD_4$ to give Go3r-R (70% yield).

3.5. Controlled Smith degradation

Go3r-R (130 mg) and Go3r (130 mg) were oxidized with 0.05 M aq $NaIO_4$ (20 mL) for 72 h at 25 °C in the dark.[37] Each sample was dialyzed, and treated with $NaBH_4$ (pH 9–10) for 20 h, neutralized with HOAc, dialyzed, freeze-dried, and submitted to partial acid hydrolysis (M TFA, 20 h, 25 °C).[38] The products of controlled Smith degradation were dialyzed and freeze-dried to afford Go3r-RS and Go3r-S (50% yield).

3.6. Desulfation

Partial solvolytic desulfation was carried out as previously described.[36] The polysaccharides Go3r-R (60 mg), Go3r-RS (40 mg), and Go3r-S (50 mg) in the pyridinium salt form were treated with 89:10:1 Me_2SO–MeOH–pyridine at 100 °C for 4 h. The desulfated polysaccharides were recovered after dialysis and freeze-drying to afford Go3r-RD (70% yield), Go3r-RSD (80% yield), and Go3r-SD (72% yield), respectively.

3.7. Methylation analysis

Per-O-methylation was carried out by the method of Ciucanu and Kerek[73] with the carboxyl-reduced polysaccharides (Go3r-R and Go3r-RD) and carboxyl-reduced and Smith-degraded products (Go3r-RS and Go3r-RSD). The samples (15 mg) in the triethylammonium salt form[74] were dissolved in Me_2SO (1 mL), and powdered NaOH (30 mg) was added. After 30 min at 25 °C with stirring, MeI (0.1 mL) was added, and the reaction was allowed to proceed as described above. The process was repeated twice and the reaction was interrupted by addition of water (2 mL) and neutralized with 50% aqueous AcOH. The products were dialyzed against distilled water, freeze-dried and submitted to more two steps of methylation in the same way as described above. Partially methylated alditol acetates were generated by hydrolysis in aqueous formic acid (45%, for 16 h, at 100 °C), followed by $NaBD_4$ reduction and acetylation. The products were analyzed by GC–MS and identified by their typical electron-impact breakdown profiles and retention times.[75,76] A portion of per-O-methylated Go3r-R was sequentially desulfated,[36] then trideuteromethylated as described above, but using trideuterated iodomethane. The partially methylated and trideuteromethylated alditol acetates were generated and analyzed as described above.

3.8. High-pressure size-exclusion chromatography (HPSEC) analysis

HPSEC-MALLS-RI analysis was carried out with a 1 mg mL^{-1} soln of the polysaccharide, using 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 (MALLS) detector adapted online. Four Waters Ultrahydrogel columns (2000, 500, 250, and 120) were connected in series and coupled with multi-detection equipment. A 0.1 M $NaNO_3$ soln, containing NaN_3 (0.5 g L^{-1}), was used as eluent. The value of d*n*/d*c* (differential refractive index to change in solute concentration) was determined using five concentrations, between 1.0 and 0.2 mg mL^{-1}. HPSEC data were collected and analyzed by the Wyatt Technology ASTRA program. All experiments were carried out at 25 °C.

3.9. Spectroscopic methods

For nuclear magnetic resonance spectroscopy (NMR) analysis, each lyophilized sample was dissolved in D_2O (~30 mg mL^{-1}). The NMR spectrum of the solution was recorded at 70 °C using a Bruker Advance DRX400 NMR spectrometer, equipped with a 5 mm multi-nuclear inverse detection probe, at a base frequency of 100.63 MHz for ^{13}C and 400 MHz for ^{1}H nuclei. Chemical shifts are expressed in ppm using acetone as internal standard at 31.45 and 2.225 ppm for ^{13}C and ^{1}H, respectively. For ^{1}H and 2D NMR experiments, the samples were deuterium exchanged by successive freeze-drying steps in D_2O (99.9%) and then dissolved in D_2O (20–30 mg mL^{-1}). ^{1}H and ^{13}C acquisition parameters were previously reported.[77] 2D ^{1}H, ^{1}H COSY, TOCSY, and ^{1}H, ^{13}C HMQC experiments were carried out using the pulse programs supplied with the Bruker manual.

Fourier-transform infrared (FTIR) spectra were recorded on a Perkin–Elmer series 2000 FTIR spectrometer using the polysaccharides in the KBr pellets form.

3.10. Cells and viruses

Vero (African green monkey kidney) cells were grown in Eagle's minimum essential medium (MEM) (GIBCO, USA) supplemented with 5% calf serum (GIBCO, USA). For maintenance medium (MM), the serum concentration was reduced to 1.5%.

HSV-1 strain F and HSV-2 strain MS were used for the antiviral assays. Virus stocks were propagated and titrated by plaque formation in Vero cells.

3.11. Cytotoxicity test

Vero cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; Sigma–Aldrich, St. Louis, MO, USA) method. Confluent cultures in 96-well plates were exposed to different concentrations of the polysaccharides, with three wells for each concentration, using incubation conditions equivalent to those used in the antiviral assays. Then 10 μL of MM containing MTT (final concentration 0.5 mg mL^{-1}) was added to each well. After 2 h of incubation at 37 °C, the supernatant was removed and 200 μL of 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%.

3.12. Virus plaque reduction assay

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 fractions. After 1 h of adsorption at 37 °C, the residual inoculum was replaced by MM containing 0.7% methylcellulose and the corresponding dose of each fraction. Plaques were counted after 2 days of incubation at 37 °C. The IC_{50} was calculated as the fraction concentration required to reduce virus plaques by 50%. All determinations were performed twice and each in duplicate. Heparin was from Sigma–Aldrich, USA.

Acknowledgments

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