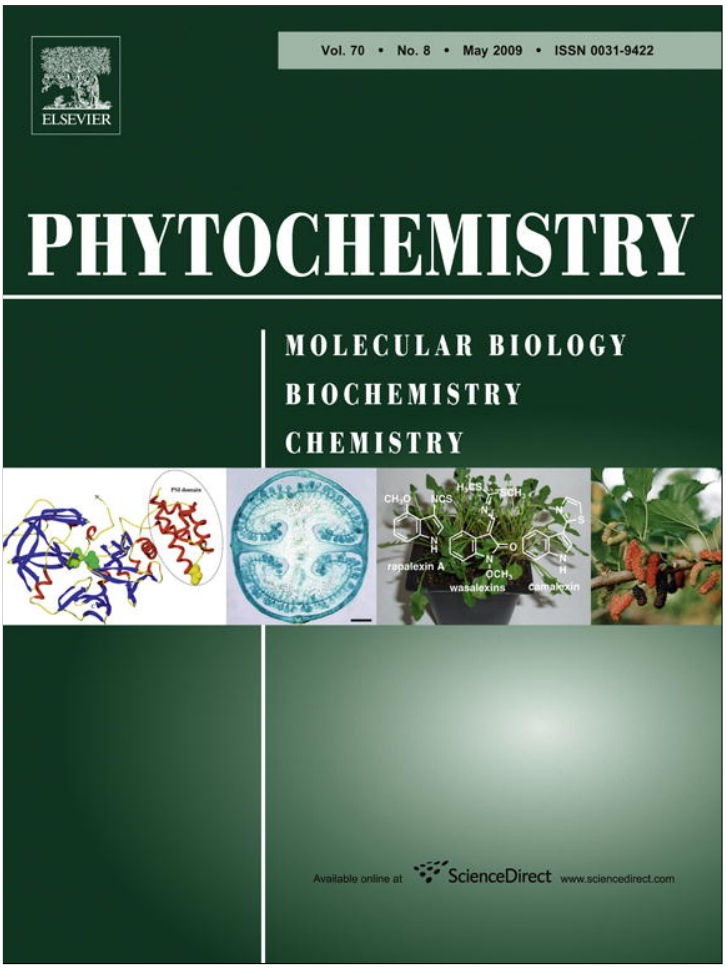

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Sulfated mannans from the red seaweed *Nemalion helminthoides* of the South Atlantic

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ABSTRACT

Nemalion helminthoides, collected in the Argentine South Atlantic coast, was extracted with hot water and the crude product fractionated using cetrimide. The complexed material was subjected to fractional solubilization in solutions of increasing sodium chloride concentration and seven fractions were separated and analyzed. Structural analysis of the main fractions, those soluble in 3.0 and 4.0 M NaCl (yields 21.0% and 13.8%, respectively) and those insoluble in 4.0 M NaCl (yield 20.0%), indicated that this seaweed biosynthesizes (1 → 3)-linked α-D-mannans that are sulfated at positions 4 and 6. Three mannan fractions comprising considerable amounts of xylose were also isolated in very low total yield (2.0%). The fractions that were soluble in 3.0 and 4.0 M NaCl showed low antiherpetic activity whereas this activity was considerable for the fraction solubilized in 2.0 M NaCl (yield 0.5%) which contained single stubs of β-D-xylose. A xylan, soluble in cetrimide solution, containing (1 → 3, 1 → 4)-linked β-D-xylose residues, was also isolated in minor amount.

1. Introduction

The major water-soluble polysaccharides extracted from marine red macroalgae are sulfated galactans (Usov, 1992). However, other types of polysaccharides are synthesized by red seaweeds. The presence of xylans containing both (1 → 3, 1 → 4)-linked β-D-xylose residues and sulfated xylomannans has been reported for the Nemaliales *Nothogenia erinaceum* (formerly classified as *Chaetangium erinaceum*; Nunn et al., 1973), *Nemalion vermiculare* (Usov et al., 1973, 1974, 1975a,b; Usov and Yarotskii, 1975), *Nothogenia fastigiata* (Cerezo et al., 1971; Cerezo, 1972; Matulewicz and Cerezo, 1987; Kolender et al., 1995, 1997) and *Liagora valida* (Usov and Dobkina, 1991). In *N. fastigiata* a sulfated xylogalactan of the agaran-type was also found (Haines et al., 1990; Matulewicz et al., 1994).

The xylomannans from the Nemaliales have a linear backbone of (1 → 3)-linked α-D-mannopyranose units with sulfation at the 4- and 6-position for *N. vermiculare* and *L. valida* and at the 2- and 6-position for *N. fastigiata*. In addition, the polysaccharides from *N. vermiculare* and *N. fastigiata* have single stubs of (1 → 2) β-D-xylopyranose, while in the polysaccharide from *L. valida*, β-D-xylopyranose or 3-*O*-methyl-D-xylopyranose, or (1 → 4)-linked β-D-xylopyranose short chains attached to the 2-position were found. A recent publication describes that the mannan from the Nemalial *Scinaia hatei* (Mandal et al., 2008) was only sulfated at the 4-position and that single stubs of β-D-xylose were located at the 2-, 4- or 6-position.

The only report on polysaccharides of *N. helminthoides* was found in an abstract of a PhD thesis (Haslin, 2000) which also mentioned the chemical characterization of the Nemaliales *L. viscida*, *S. furcellata and Cumagloia andersonii*. In this abstract, only a general structure was proposed for the polysaccharides of these seaweeds: (1 → 3)-linked α-D-mannans having sulfate groups and D-xylose side-chains at the 2-, 4- or 6-position.

In addition, the sulfated polysaccharides from *N. fastigiata* and *S. hatei* exhibited potent antiviral activity against several strains of herpes simplex type 1 (HSV-1) and type 2 (HSV-2) replication *in vitro* (Kolender et al., 1997; Mandal et al., 2008).

Herein we report the extraction, fractionation and characterization of the water-soluble polysaccharides from *N. helminthoides* from the South Atlantic and their activity *in vitro* against HSV-1.

2. Results and discussion

2.1. Extraction and fractionation of the polysaccharides

The seaweed was treated with water at 90 °C, the precipitate was removed by centrifugation and the crude polysaccharides were isolated from the supernatant according to two different procedures: (a) precipitation with isopropanol (PP1–2) and (b) dialysis followed by concentration and freeze-drying (PD1–3). Table 1 shows the yields and analyses of these products. The total yield

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Table 1
Analyses of products obtained by extraction with water at 90 °C and further precipitation with *iso*-propanol (PP1–2) or dialysis (PD1–3).

Product	Yield[a] (%)	Molecular Weight (Da)	Sulfate (% $NaSO_3$)	Protein (%)	Monosaccharide composition (mol%)[b]			
					Man	Xyl	Gal	Glc
PP1	28.3	n.d.[c]	28.2	7.6	88	11	1	–
PP2	5.1	11,300	18.9	10.2	68	29	3	–
PD1	20.0	17,500	22.4	5.9	88	9	2	1
PD2	2.6	10,100	17.1	13.0	50	34	16	–
PD3	0.5	6000	11.1	23.6	54	20	22	4

[a] Yields are given per 100 g of dry seaweed.
[b] PP1 contains traces of rhamnose and arabinose. PD1 contains traces of rhamnose, fucose and arabinose. PD2 contains 2% of rhamnose.
[c] n.d. = Not determined.

of the first procedure was higher than that of the second and it was clearly observed that PP1 and PD1 had similar protein contents and monosaccharide compositions but the sulfate percentage of the former was higher. Compared to PP1 and PD1, PP2 and PD2 had major amounts of xylose. Mannose and xylose of the crude polysaccharides were ascribed as belonging to the D-series, considering the absolute configuration reported for these monosaccharides in the sulfated xylomannans and neutral xylans from *N. vermiculare* (Usov, 1992) and *N. fastigiata* (Cerezo et al., 1971; Matulewicz and Cerezo, 1987).

PP1 was dissolved in water and the sulfated polysaccharides were precipitated with cetrimide and the insoluble complexes were subjected to fractional solubilization in solutions of increasing sodium chloride concentration. Table 2 shows the yield and analyses of fractions N1-7 which were separated in this way: NS, the fraction soluble in cetrimide solution, was also analyzed (Table 2) but was left for further studies.

The main fractions obtained were N5 and N6, soluble in 3.0 and 4.0 M NaCl, respectively, and N7, insoluble in 4.0 M NaCl; these fractions constituted 75.2% of the recovered. It can be observed that N1 contained major amounts of galactose, mannose and xylose, while in the rest of the fractions the main monosaccharide was mannose and only minor quantities of galactose were present. In addition, in N2–6 the xylose content decreased with sodium chloride molarity.

The data in this table suggest that fractionation in the 0.5–2.0 M NaCl range (fractions N1–4) was based on the sulfate content which increased with the sodium chloride molarity. Separation of N4 and N5, with similar sulfate percentages, was probably achieved according to the molecular weight but the influence of D-xylose cannot be discarded. For N5 and N6, with similar sulfate contents and molecular weights, fractionation could be attributed to a different distribution of sulfate ester groups in the polysaccharide chain. N5 and N6 had different optical rotations: $[\alpha]_D$ = +80.5 (*c* 0.21, 0.1 M NaCl) and $[\alpha]_D$ = +58.4 (*c* 0.08, 0.1 M NaCl), respectively, consistent with the aforementioned hypothesis of different sulfate distributions and probably different conformations in solution. The optical rotation of N6 was identical to that reported by Usov et al. (1975a) for a sulfated mannan of *N. vermiculare* ($[\alpha]_D$ = +58.8 (*c* 0.05, water). However, the optical rotations of N5 and N6 were higher than those previously reported for the mannans of *N. fastigiata*. HPSEC–RID analyses of N5 and N6 gave homogeneous but considerably polydisperse elution profiles.

Fraction N7 had a similar carbohydrate composition to those of N5 and N6, but lower sulfate content and higher protein percentage. The insolubility in 4 M NaCl could be due to the formation of packed aggregates precluding solvation of the polysaccharide chains (Matulewicz and Cerezo, 1987) and thus the elimination of the hexadecyltrimethylammonium counterion, which was clearly observed in the ^{13}C NMR spectrum (see below).

Even though, as aforementioned, the Nemaliales biosynthesize xylans containing both (1 → 3, 1 → 4)-linked β-D-xylose residues, all attempts to isolate these from PD1 and PP1 failed. On the other hand, analysis of the monosaccharide composition of PP2 and PD2 (Table 1) strongly suggested the presence of this type of polysaccharide whose resonances were clearly observed in the corresponding ^{13}C NMR spectra (see below).

Thus, PD2, which was easier to dissolve in water than PP2, was subjected to a cetrimide treatment, with the soluble fraction dialyzed and freeze-dried. Further purification by anion-exchange chromatography and elution with water yielded a pure xylan which was characterized by ^{13}C NMR spectroscopy and methylation analysis (see below).

2.2. Methylation analysis of native and desulfated N5–7, and the neutral xylan

Mannans N5 and N6 were converted into their triethylammonium salts and methylated according to the Ciucanu and Kerek method (1984). A constant monosaccharide composition was

Table 2
Analyses of fractions after treatment of PP1 with cetrimide and further redissolution of the precipitate with increasing sodium chloride concentration.

Fraction	M NaCl	Yield[a] (%)	Molecular Weight (Da)	Sulfate (% $NaSO_3$)	Man:Xyl:Sulfate (molar ratio)	Protein (%)	Monosaccharide composition (mol%)						
							Man	Xyl	Gal	Glc	Rha	Fuc	Ara
NS	–	15.0	n.d.[b]	10.4	n.d.	12.9	82	10	3	2	tr[c]	tr	3
N1	0.5	1.1	3300	6.8	1.00:0.81:0.34	6.3	27	22	33	9	2	4	3
N2	1.0	0.7	8600	17.1	1.00:0.76:0.47	3.5	51	39	2	–	6	–	2
N3	1.5	0.8	13,600	19.4	1.00:0.37:0.45	1.2	64	24	8	2	–	2	tr
N4	2.0	0.5	11,700	22.9	1.00:0.22:0.84	2.0	74	17	4	tr	2	1	2
N5	3.0	21.0	42,800	22.8	1.00:0.00:0.68	1.5	100	tr	–	–	–	–	–
N6	4.0	13.8	43,800	23.4	1.00:0.01:0.64	<4	98	1	tr	tr	–	–	1
N7	Ins. 4.0[d]	20.0	n.d.	13.1	n.d.	14.8	93	4	1	1	–	tr	1

[a] Yields of all fractions are given as percentages of PP1. Redissolution of PP1 in water gave a residue (yield, 2.5%).
[b] n.d. = Not determined.
[c] Percentages lower than 1% were considered as traces (tr).
[d] Insoluble in 4.0 M NaCl.

achieved for N5 when this procedure was repeated once more. However, for N6, considerable undermethylation was observed even after three methylation steps. Thus, N6 was first treated by the Haworth method as described by Cerezo (1973) and then fully methylated twice according to Ciucanu and Kerek. Methylation of N7 was directly achieved by two Ciucanu and Kerek methylation steps, using lithium chloride–dimethylsulfoxide as solvent (Petruš et al., 1995) in the first one and dimethylsulfoxide in the second.

Table 3 depicts the carbohydrate composition of the methylated products. This composition was consistent, for the three fractions, with a (1 → 3)-linked mannan backbone with ~55% of the residues monosulfated on C-4 or C-6.

In order to determine unequivocally the glycosidic linkages and the substitution pattern on the backbone it was necessary to carry out desulfation–methylation analysis on the polysaccharides. The three fractions were desulfated with 0.1 M methanolic hydrogen chloride and this treatment led to elimination of 50% (N7) to 75% (N5 and N6) of the sulfate content. Desulfated N5 was methylated by the Ciucanu and Kerek procedure but the low degree of methylation achieved was not improved by further treatment using lithium chloride–dimethylsulfoxide as solvent. It is interesting to point out that linkage analysis of this methylated derivative showed only the presence of 2,4,6-tri-*O*-methylmannose (mol%, 49%) and mannose (mol%, 41%) with only insignificant amounts of 2,6- and 2,4-di-*O*-methylmannose.

Full methylation of desulfated N5-7 was accomplished when the samples were first treated by the Haworth method and then twice by the Ciucanu and Kerek procedure (Table 3). The increase of 2,4,6-tri-*O*-methylmannose together with the concomitant decrease of 2,6- and 2,4-di-*O*-methylmannose was in agreement with the previously proposed structure. The small percentages of 2,3,4-tri-*O*-methylxylose and 4,6-di-*O*-methylmannose detected in N5DS and N6DS suggested the presence of very low amounts of xylose side-chains at the 2-position of the mannan chain.

When the neutral xylan isolated from PD2 was subjected to methylation analysis, the following mol% composition was obtained: 2,3,4-tri-*O*-methylxylose (3%), 2,4-di-*O*-methylxylose (10%), 2,3-di-*O*-methylxylose (86%) and 2-*O*-methylxylose (1%). Thus, this methylation pattern was ascribed to a (1 → 3, 1 → 4)-linked structure with high prevalence of 4-linked xylopyranosyl residues. The number-average molecular weight calculated from methylation analysis (6600 Da) was similar to the value determined by end-group analysis (6200 Da).

2.3. NMR spectroscopy of N4–7 and the neutral xylan

The main fractions N5–7, and N5DS, were analyzed by ^{13}C NMR spectroscopy. The spectra of N5 and N6 were determined in deuterium oxide, the spectrum of N7 in dimethyl-d_6 sulfoxide and that of N5DS in 0.5 M NaOH in 1:1 deuterium oxide–water. Table 4 indicates the assignment of the signals and Fig. 1 shows the spectra of N5 and N5DS; the spectrum of N6 (not shown) was similar giving the same resonances found for N5. The assignment was carried out taking into account the previously reported displacements of ^{13}C resonances on sulfation of methyl 3-*O*-methyl-α-D-mannopyranoside (Usov et al., 1975c) and the chemical shifts observed for the xylomannans of *N. fastigiata* at room temperature (Kolender et al., 1995, 1997) and at 80 °C (Kolender, 2003).

Table 3
Composition (mol%) of monosaccharides produced by permethylation and hydrolysis of N5–7 and their desulfated derivatives.

Monosaccharide	N5	N5DS	N6	N6DS	N7	N7DS
2,3,4-Me_3 Xyl	–	2	2	2	1	tr[a]
2,3,4,6-Me_4 Man	2	2	2	3	–	tr
2,4,6-Me_3 Man	36	74	33	81	34	65
4,6-Me_2 Man	–	2	tr	2	tr	4
2,6–Me_2 Man	22	11	19	7	22	16
2,4-Me_2 Man	33	5	35	4	34	7
2-Me Man	3	2	3	–	2	4
4-Me Man	1	–	1	–	–	–
Man	3	2	3	1	7	4

[a] Percentages lower than 1% are considered as traces (tr).

Table 4
^{13}C NMR spectroscopic assignment of N5, N5DS and N4.

Unit	C-1	C-2	C-3	C-4	C-5	C-6
Mannan N5						
α-D-Man	103.1–103.0	70.6	79.4	67.0	74.5	61.9
α-D-Man 6-sulfate	103.3	70.7	79.1	67.2	72.5	68.6
α-D-Man 4-sulfate	102.7–102.5	73.1	76.5	75.2	74.3	62.2
Desulfated mannan N5						
α-D-Man	103.0	70.5	79.8	67.1	74.6	61.9
Xylomannan N4						
α-D-Man	103.1–103.0	70.6	79.4	67.0	74.5	61.9
α-D-Man 6-sulfate	103.3	70.8	79.1	67.3	72.5	68.6
α-D-Man 4-sulfate	102.7–102.5	73.1	76.5	75.2	74.3	62.2
α-D-Man 2-Xyl	100.8	78.9	79.4	67.6	74.5	61.9
α-D-Man 2-Xyl 6-sulfate	101.4	78.9	78.9	67.6	72.5	67.6
β-D-Xyl	104.2	73.8	76.8	70.4	66.0	

In the anomeric region of N5 and N6 signals at 103.3, 103.1 and 103.0, and 102.7 and 102.5 ppm were found. The resonance at 103.3 ppm was assigned to 3-linked α-D-mannose 6-sulfate residues, while the two signals at 103.1 and 103.0 were attributed to 3-linked α-D-mannose units either linked to α-D-mannose 6-sulfate or to α-D-mannose 4-sulfate units, even though it was not possible to establish a unique diad combination. These assignments were made taking into account that in the 75-MHz spectrum of a partially 6-sulfated mannan of *N. fastigiata* (named F5'), determined at 80 °C, resonances at 102.9 and 102.8 ppm were assigned to 3-linked α-D-mannose 6-sulfate and 3-linked α-D-mannose, respectively (Kolender, 2003). As sulfation on the 4-position should shift upfield the C-1 of the mannopyranosyl units, resonances at 102.7 and 102.5 ppm could be due, with the same criterion as above, to α-D-mannose 4-sulfate residues either linked to α-D-mannose/α-D-mannose 6-sulfate or to α-D-mannose 4-sulfate units.

In the 79–81 ppm region, resonances 79.4 and 79.1 ppm were attributed, according to our previous results and the model compounds, to the C-3 of α-D-mannose and α-D-mannose 6-sulfate units, respectively. Even though signals at 80.1 and 79.6 ppm remain unassigned, these could be ascribed to the C-3 of the aforementioned units linked to α-D-mannose 4-sulfate residues. In the same way, the resonance at 71.2 ppm could be assigned to the C-2 of α-D-mannose and α-D-mannose 6-sulfate units linked to 4-sulfated mannose residues.

When the DEPT 135 pulse technique was applied on N5, signals at 68.6 and 61.9–62.2 ppm gave inverted peaks which corresponded to C-6 of mannose 6-sulfate and mannose/mannose 4-sulfate residues, respectively.

The ^{13}C NMR spectrum of N7 (not shown), recorded in dimethyl-d_6 sulfoxide, was similar to those of N5 and N6 but it showed a upfield shift of all the resonances of ~2 ppm and the signals of the alkyl carbons of the hexadecyltrimethylammonium counterion were clearly observed in the 10–30 ppm region.

The ^{13}C NMR spectrum of N5DS gave the six signals expected for an (1 → 3)-linked α-D-mannan; these resonances were coincident with those previously reported by Usov and Dobkina (1991) for a water-soluble desulfated xylomannan fraction from *L. valida*, with the exception of the C-3 which appeared at 79.8 ppm, that is with a downfield displacement of 1.1 ppm (Table 4, Fig. 1). However, it should be kept in mind that our experiment was carried out in alkaline solution due to the insolubility of the sample in water.

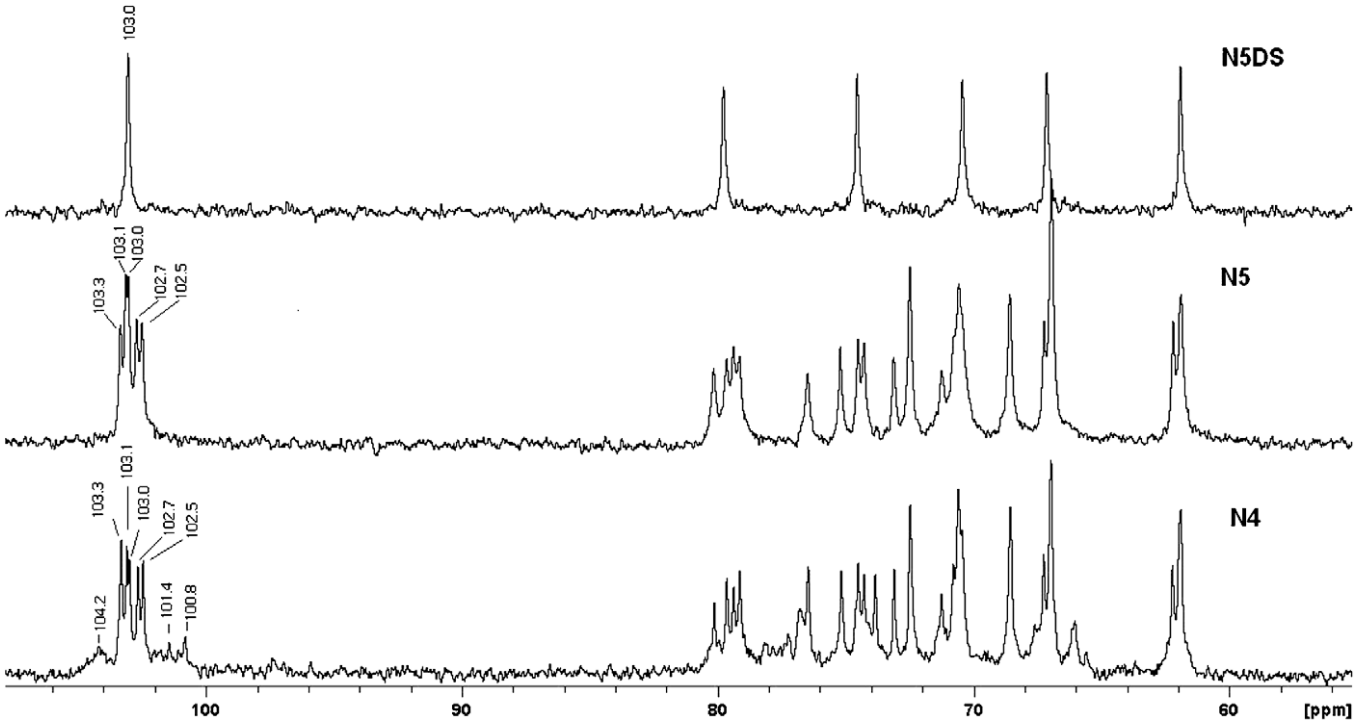

Fig. 1. ^{13}C NMR spectra of N5DS, N5 and N4.

The anomeric signal at 5.14, with a shoulder at 5.17 ppm, observed in the ^{1}H NMR spectra of N5 and N6 (not shown), was assigned to mannose/mannose 6-sulfate and mannose 4-sulfate, respectively.

N4, which was obtained in very low yield (0.5%), showed a considerable antiviral activity (see below) and therefore its ^{13}C NMR spectrum was determined (Table 4, Fig. 1). The general pattern of the spectrum was similar to those of N5 and N6, but it showed small signals consistent with the presence of 3-linked α-D-mannose/α-D-mannose 6-sulfate both with single stubs of (1 → 2)-linked β-D-xylose. However, attachment of xylose to the 6-position could not be discarded due to the broad anomeric resonance of D-xylose at 104.2 ppm found together with small signals at 69.7 ppm (C-6) and 72.7 ppm (C-5) of a 3-linked α-D-mannopyranosyl unit substituted on C-6 (Usov and Dobkina, 1991).

The ^{13}C NMR spectrum of the xylan confirmed a (1 → 3, 1 → 4)-linked β-D-structure (Table 5); the spectrum was assigned taking into account the values reported for model xylo-oligosaccharides (Kovač et al., 1980) and the xylans from *Leptosarca simplex* (Adams et al., 1988) and *N. fastigiata* (Matulewicz et al., 1992). The isolation of this xylan with (1 → 4)- and (1 → 3)-linked β-D-xylopyranosyl residues in a ~9:1 ratio, only after the second extraction step with water, is in agreement with the presence of large blocks of β-(1 → 4) linkages (Matulewicz et al., 1992). In *N. fastigiata* the same type of polysaccharides was obtained in the first extraction step, but they contained (1 → 4)- and (1 → 3)-linked β-D-xylopyranosyl residues in a ~3–5:1 ratio, with the (1 → 3) linkages interspersed throughout the chain rather than grouped contiguously (Cerezo et al., 1971; Matulewicz et al., 1992). A 4:1 ratio was determined for a xylan from *N. vermiculare* isolated only after extraction of the seaweed with 1 M NaOH (Usov et al., 1974).

Table 5
^{13}C NMR spectroscopic assignment (ppm) of the neutral xylan.

β-D-Xylp	C-1		C-2		C-3	C-4	C-5
	(1 → 3)	(1 → 4)	(1 → 3)	(1 → 4)			
3-Linked	103.9	102.4	73.3	73.3	84.1	68.4	65.6
4-Linked	103.9	102.4	74.1	73.5	74.4	77.0	63.7

2.4. Antiviral activity

Table 6 indicates that N3 and N4 were the more active fractions against HSV-1(F), with IC_{50} of 9.68 and 5.43 μg/ml, respectively. N5 and N6 showed very low activity even in concentrations of 40 μg/ml. In comparison, the active fractions had higher xylose contents and lower molecular weights than N5 and N6. No cytotoxicity, even in concentrations of 1000 μg/ml, was observed for N3 and N4.

It has been reported that the (1 → 3)-linked α-D-mannans of *N. fastigiata* sulfated on C-2 and C-6 showed a high antiherpetic activity (Kolender et al., 1997) and this activity was attributed to the fact that these structures mimic the minimal binding domain of cellular heparan sulfate (Damonte et al., 2004; Pujol et al., 2007). In addition, the lower activity of the fractions with D-xylose side chains suggested that branching could preclude binding to the virus. However, a similarly high antiherpetic activity was found in a (1 → 3)-linked α-D-mannan from *S. hatei*, sulfated on C-4 and

Table 6
Antiviral activity of fractions from *Nemalion helminthoides*.

Fraction	IC_{50}[a](μg/ml)
N1	>40
N2	>40
N3	9.68 ± 1.72
N4	5.43 ± 1.45
N5	>40
N6	>40
DS8000[b]	2.1 ± 0.1
Heparin[b]	1.3 ± 0.1

[a] Inhibitory concentration 50%: Compound concentration required to reduce virus plaques by 50%. Mean of two determinations ± SD.
[b] Dextran sulfate with molecular weight 8000 (DS8000) and heparin were used as control compounds.

substituted with single stubs of D-xylose on C-2, C-4 and C-6 (Mandal et al., 2008).

The mannans of *N. helminthoides*, sulfated on C-4 and C-6 (N5 and N6), showed a low antiherpetic activity, indicating the importance of sulfation on C-2 present in *N. fastigiata*. On the other hand, N4 which contained D-xylose side chains was the most active fraction suggesting, as observed for *S. hatei*, that branched structures do not inhibit the antiherpetic activity.

3. Conclusions

N. helminthoides, collected in Mar del Plata, in the Argentine South Atlantic coast, biosynthesizes mainly a system of (1 → 3)-linked α-D-mannans 4- and 6-sulfated and having low amounts of single stubs of β-D-xylose. A (1 → 3, 1 → 4)-linked β-D-xylan was also isolated in minor yield. Usov et al. (1973, 1974, 1975a,b) reported the presence of a neutral xylan and a sulfated mannan with similar structures in *N. vermiculare*. The same mannan backbone but with different substitution patterns has been found in other seaweeds belonging to the same order Nemaliales.

The mannans N5 and N6 of *N. helminthoides* showed low antiviral activity against HSV-1(F) but the activity increased for N3 and N4 which contained D-xylose side chains. The most active fraction (named F6') from *N. fastigiata* was a (1 → 3)-linked α-mannan sulfated on C-2 and C-6 but devoid of xylose and with a similar molecular weight to that of N5 and N6 (Kolender et al., 1997). Thus, the results obtained for *N. helminthoides* suggest again the importance of sulfation on the 2-position for antiherpetic activity.

4. Experimental

4.1. Materials

Gametophytic *N. helminthoides* (Valley Betters, 1902) was collected from the rocks of Cabo Corrientes (38° 01′S, 57° 31′W) (Mar del Plata, Provincia de Buenos Aires, Argentina) in April 2006 and in December 2006. Each collection was washed with sea water, carefully hand sorted and freeze-dried. A voucher specimen (BA 46.775) of collection in April 2006 was deposited in the herbarium of the Museo de Ciencias Naturales Bernardino Rivadavia (Buenos Aires, Argentina).

4.2. Extractions

Materials of both collections were pooled before the extractions and isolation of the polysaccharides was carried out according to two different procedures:

4.2.1. Extraction followed by precipitation

The dry seaweed (145 g), previously milled, was extracted with H_2O (2.7 l) for 4 h at 90 °C. The residue was removed by centrifugation and the supernatant was poured into four volumes of *iso*-PrOH. The resulting ppt. was separated by centrifugation, washed with Me_2CO (×4) and dried at room temp. to obtain PP1 (41 g). This procedure was repeated twice to give PP2 (7.3 g) and PP3 (0.012 g). PP1 was used for further fractionation.

4.2.2. Extraction followed by dialysis

The dry seaweed, previously milled (25 g) was extracted with H_2O (100 ml) for 4 h at 90 °C. The residue was removed by centrifugation and the supernatant was dialyzed with tubing of molecular weight cutoff of 6000–8000 Da, first against running-tap H_2O and then distilled H_2O, concentrated and freeze-dried (PD1, 5.0 g). This procedure was repeated once more (PD2, 0.65 g).

4.3. Fractionation of the sulfated polysaccharides

PP1 (20 g) was dissolved in H_2O (2.2 l) and the soln. was centrifuged at 10 °C during 20 min at 13131 *g* (9000 rpm) to give a residue which was freeze-dried (0.5 g). To the soln., a 10% (w/v) aq. soln. of cetrimide (270 ml) was added slowly with stirring, until no further complex formation occurred; stirring was continued for 16 h. The complexes were removed by centrifugation and suspended in 0.5 M NaCl (1.46 l) with stirring continued overnight. The ppt. was centrifuged off and washed with 0.5 M NaCl ((3 × 100 ml); the supernatant of the first centrifugation and the washings were pooled. The resulting soln. was extracted with 1-pentanol (3 × 300 ml), dialyzed with tubing of molecular weight cutoff of 3500 Da, concentrated and freeze-dried. The ppt. was subjected to successive similar procedures so that the concentration of NaCl was increased by 0.5–1.0 M each time; the upper limit of the NaCl concentration was 4.0 M (N1–N6). The residual precipitate was suspended in water and the suspension was dialyzed and freeze-dried to give N7.

In order to complete the removal of the cetrimide counterion, fractions N1 and N5 were redissolved in 0.5 and 3.0 M NaCl, respectively, and the polysaccharides were isolated as described before for the original fractions. Thus, Table 2 shows the yields and analyses of the purified fractions.

The supernatant from the cetrimide precipitation was retreated with cetrimide but no further complex formation was observed. The soln. was dialyzed, concentrated and freeze-dried giving NS.

4.4. Isolation of the neutral xylan from PD2

A soln. of PD2 (610 mg) in H_2O (60 ml) was centrifuged at 10 °C during 20 min at 4904 *g* (5500 rpm) and the insoluble material (30 mg) was separated. To the supernatant, a 10% aq. soln. of cetrimide (7 ml) was added with the stirring continuing overnight. After centrifugation at 10 °C during 20 min at 5836 *g* (6000 rpm), the supernatant was dialyzed with tubing of molecular weight cutoff of 3500 Da, concentrated and freeze-dried to give the crude xylan (78 mg). An analytical column (9.5 × 1.0 cm) was filled with DEAE Sephadex A-25 (Cl^-) previously swollen in water for 2 h in a boiling water bath. An aq. soln. (5 ml) containing the crude xylan (23 mg) was applied to the column which was eluted with H_2O, with fractions (1 ml) collected and analyzed for carbohydrate content by the PhOH–H_2SO_4 reaction. Fractions corresponding to the same peak were pooled, concentrated and freeze-dried to give the neutral xylan (20 mg).

4.5. General experimental procedures

Carbohydrate contents were analyzed by the PhOH–H_2SO_4 method (Dubois et al., 1956) without previous hydrolysis of the polysaccharide using mannose as standard. Sulfate was measured using the turbidimetric method of Dodgson and Price (1962) after hydrolysis of the samples with either 1 M HCl for 4–5 h at 105–110 °C, or by ion chromatography with conductimetric detection. For the latter, samples were hydrolyzed in 2 M TFA at 121 °C for 2 h, evaporated to dryness under N_2 and redissolved in high purity H_2O from a Milli-Q system. A DIONEX DX-100 ion chromatography system was used with an AS4A column (250 × 4 mm), an AMMS-II micromembrane suppressor and a conductivity detector (eluent: 1.8 mM Na_2CO_3/1.7 mM $NaHCO_3$, flow rate: 2 ml min^{-1}). For the soluble samples, protein was estimated by the method of Lowry et al. (1951); for the insoluble samples, nitrogen content was determined with a CE-440 Elemental Analyzer (Exeter Analytical Inc.) and the protein content was calculated by multiplying the nitrogen content by 6.25. Number-average molecular weight was determined using the colorimetric method of Park and Johnson

(1949) which measures the content of reducing end-chain residues based upon reduction of ferricyanide ions in alkaline soln. followed by formation of Prussian blue (ferric ferrocyanide). Optical rotations were measured at room temperature in a Perkin–Elmer 343 polarimeter (sodium D-line) using 0.08–0.21% soln. of polysaccharide in 0.1 or 0.02 M NaCl. Centrifugation was carried out using a Sigma 4K15 Centrifuge (rotor 12256).

Hydrolysis of polysaccharides was carried out with 2 M TFA for 2 h at 121 °C, and the sugar mixtures were derivatized to the alditol acetates for analysis by GC and GC–MS.

GC of alditol acetates was carried out on a Hewlett–Packard 5890A gas chromatograph equipped with a flame-ionization detector and fitted with a fused-silica column (30 m × 0.25 mm) WCOT-coated with 0.20 m film of SP-2330. Chromatography was carried out at (a) 220 °C isothermally for alditol acetates; (b) from 160 to 210 °C at 2 °C/min and from 210 to 240 °C at 5 °C/min followed by a 30-min hold, for partially methylated alditol acetates. N_2 was used as carrier at a flow rate of 1 ml/min. The split ratio was 80:1. The injector and detector temperature was 240 °C.

Conversion of GC areas to molar basis was calculated for the partially methylated alditol acetates according to the effective carbon response theory (Sweet et al., 1975).

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

N4–6 (20–30 mg), previously exchanged with deuterium by repeated evaporations in D_2O, were dissolved in D_2O (0.5 ml); N7 (15 mg) was dissolved in DMSO-d_6. Spectra were recorded using 5-mm tubes, at 70 °C, on a Bruker Avance DRX400 spectrometer. For 400-MHz ^1H NMR experiments, the parameters were a spectral width of 6.1 kHz, a 90° pulse, an acquisition time of 5.2 s for 16–32 scans. For 100.63-MHz ^{13}C NMR ^1H-decoupled experiments the parameters were a 90° pulse, a relaxation delay of 0.11 s, an acquisition time of 0.59 s, a spectral width of 20.1 kHz, for 33,000–90,000 scans. ^{13}C NMR DEPT spectra were obtained at $\theta z = 135°$ where *C*H and *C*H_3 signals appear in a positive phase with *C*H_2 in a negative phase. Chemical shifts (ppm) were measured relative to internal Me_2CO at 2.21 ppm for ^1H NMR spectra of N5 and N6 and at 31.1 ppm for ^{13}C NMR spectra of N4–6; the ^{13}C NMR spectrum of N7 was referenced to internal DMSO-d_6 at 39.8 ppm.

The ^{13}C NMR spectrum of N5DS was determined in 0.5 M NaOH in 1:1 D_2O–H_2O at 40 °C using the same parameters mentioned for N5 and fixing at 61.9 ppm the signal corresponding to C-6.

For ^{13}C NMR spectroscopy of the xylan, the sample (15 mg) was dissolved in 1:1 D_2O–H_2O (0.5 ml) and a 5-mm tube was used. The 125-MHz ^{13}C NMR ^1H-decoupled spectrum was recorded at room temp. on a Bruker AM 500 spectrometer using a spectral width of 29.4 kHz, a 51.4 pulse, an acquisition time of 0.56 s and a relaxation delay of 0.6 s, for 30,000 scans.

4.6. HPSEC of N5 and N6

The determination of the homogeneity was performed on a Waters High-Performance Size Exclusion Chromatograph (Waters, USA) consisting of a Waters pump (Model 515) and a differential refractometer (Model 2410 RID). Samples were analyzed using four Waters Ultrahydrogel columns (2000, 500, 250 and 120) connected in series and 0.1 M $NaNO_2$ soln., containing NaN_3 (200 ppm) as preservative, was used as eluent at a flow rate of 0.6 ml/min. The samples (1 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. HPSEC data were collected and analyzed by the Wyatt Technology ASTRA program. These experiments were carried out at 25 °C.

4.7. Desulfation of fractions

Four portions of N5 (40 mg) were suspended in 0.1 M methanolic hydrogen chloride (30 ml) and the mixtures were stirred at room temp. for 24, 48, 72 and 96 h, respectively. H_2O (10 ml) was added to each mixture which were then individually neutralized with aq. $NaHCO_3$, dialyzed (molecular cutoff 3500) and freeze-dried. After 24 h, a constant sulfate content (expressed as $NaSO_3$, ~5%) was obtained. Thus, N6 and N7 (100 mg) were desulfated during 48 h using the same procedure described above.

4.8. Methylation analysis

N5 (25.5 mg) was converted into the corresponding triethylammonium salt (19.6 mg) and was methylated by the method of Ciucanu and Kerek (NaOH–CH_3I in DMSO; Ciucanu and Kerek, 1984) with the sample left overnight during the first treatment with NaOH. A constant monosaccharide composition was achieved when this procedure was repeated once more; yield, 10 mg.

N6 (20 mg), N5DS (22 mg), N6DS (21 mg) and N7DS (24 mg) were first treated by the Haworth method (aq. NaOH–$(CH_3O)_2SO_2$) as described by Cerezo (1973), and then methylated twice according to Ciucanu and Kerek as described above. Yields: N6, 3 mg; N5DS, 12.4 mg; N6DS, 4 mg; N7DS, 11.5 mg.

N7 (20 mg) was methylated by two Ciucanu and Kerek methylation steps, using LiCl–DMSO as solvent (Petruš et al., 1995) in the first one and DMSO in the second. Permethylation was achieved in the first step; yield, 5.5 mg.

In all cases, the methylated derivatives were recovered by dialysis (molecular weight cutoff 3500 Da) and freeze-dried.

4.9. Antiviral assays

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

4.10. Cytotoxicity test

Vero cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method. Confluent cultures in 96-well plates were exposed to four different concentrations of the polysaccharide, with three wells for each concentration, using incubations 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 50% cytotoxic concentration (CC50) was calculated as the compound concentration required reducing cell viability by 50%.

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