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Structural analysis of antiviral sulfated α-D-(1 → 3)-linked mannans

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Abstract

The structures of two α-(1 → 3)-α-D-*xylo*-mannans were determined. The different antiviral activity of the *xylo*-mannans from *Nothogenia fastigiata* was explained on the basis of a flexible backbone, molecular size, content and distribution of sulfate groups and of the single stubs of β-(1 → 2)-linked D-xylose.

Keywords: α-(1 → 3)-Linked mannans; α-(1 → 3)-Linked mannans, sulfated; β-(1 → 2)-D-Xylose modified; Antiherpes activity

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1. Introduction

The red seaweed *Nothogenia fastigiata* synthesizes a complex system of xylogalactan [1,2] and xylomannan sulfates [3]. These products show erratic solubility behavior associated with composition-, temperature-, time- and conformation-dependent molecular associations [1–3].

Herein we report the structural analysis of two sulfated (1 → 3)-α-D-mannans, one efficiently inhibiting the replication of several enveloped viruses and the other not antivirally active. Their structures are compared with that of a sulfated (1 → 3)-α-D-xylomannan with lower activity previously studied [3], in an attempt to throw some light on the structural basis of the antiviral behavior of these compounds.

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2. Experimental

Isolation and fractionation of the sulfated polysaccharides.—*Nothogenia fastigiata* was extracted with boiling water, and the water-soluble polysaccharides were fractionated with Cetrimide. The acid-complexed material was subjected to solubilization in solutions of increasing sodium chloride concentration as previously reported [3]. Fractions 5 and 6, soluble in 3.0 M and 4.0 M NaCl, respectively, were redissolved in sodium chloride solutions of the corresponding concentration. These solutions were centrifuged, extracted with 1-pentanol, dialyzed, concentrated, and freeze-dried yielding mannans 5′ and 6′.

General methods.—Hydrolyses of polysaccharides were carried out with 2 M trifluoroacetic acid for 16 h at 95°C, and the sugar mixtures were derivatized to the alditol acetates [4] and aldononitrile acetates [5] for analysis by GLC and GLC–MS. Sulfate (expressed as $NaSO_3$) was analyzed by the turbidimetric method of Dodgson and Price [6]. Nitrogen was determined with a Carlo Erba EA 1108 elemental analyzer with a CHNS-O configuration, and the protein content was calculated by multiplying the nitrogen content by 6.25. The optical rotations (Na D-line) were measured at room temperature in a Perkin–Elmer 141 polarimeter using 0.05–0.22% solutions of polysaccharide in H_2O, DMSO, 1:1 H_2O–DMSO, 0.1 M NaCl and 0.5 M NaCl. Molecular weights were determined by the colorimetric method of Park and Johnson [7].

GLC was carried out on a Hewlett–Packard 5890 instrument, fitted with a flame-ionization detector, using a fused silica capillary column (0.25 mm i.d. × 30 m) WCOT-coated with a 0.25 μm film of SP–2330. Chromatography was performed (*i*) isothermally at 220°C for the alditol acetates and the aldononitrile acetates and (*ii*) isothermally at 210°C for the methylated alditol acetates and methylated aldononitrile acetates. N_2 was used as carrier at a flow rate of 1 mL min^{-1} and the split ratio was 100:1. The injector and detector temperature was 240°C.

GLC–MS of methylated alditol acetates [8,9] and aldononitrile acetates [10–12] was carried out with a Hewlett–Packard 5890 gas–liquid chromatograph coupled to a Trio–2 VG Masslab. Chromatography was performed on the SP–2330 capillary column, programmed from 150°C for 1 min and then at 9°C min^{-1} to 230°C. The He flow rate was 1 mL min^{-1}, the injector temperature 240°C, and the split ratio 100:1. Mass spectra were recorded over a mass range of 40–500 amu using an ionizing potential of 70 eV.

The 50 MHz ^{13}C NMR ^1H-decoupled spectra were recorded on a Bruker AC 200 spectrometer, at room temperature, and with an external reference to Me_4Si; solutions of the samples (ca. 40 mg) in 1:1 H_2O–D_2O (0.4 mL) and a 5-mm tube were used. Specific parameters included a pulse angle of 45°, an acquisition time of 0.400 s, no pulse delay, a spectral width of 11 kHz, and 518, 094–555, 627 scans.

The Fourier-transform infrared spectra were recorded with a 510P Nicolet FTIR spectrophotometer, using a KBr pellet, at 4000–250 cm^{-1}; 32–64 scans were taken with a resolution of 2–4 cm^{-1} [13]. Derivation, including Savitzky–Golay algorithm with 23 smoothing points, was performed using the PCIR software package incorporated into the hardware of the instrument.

Desulfation of mannan 6′.—Mannan 6′ (100 mg) was desulfated as described previously [3]; dialysis was carried out with tubing with a molecular weight cutoff of 1000.

Methylation analysis.—Mannan 6′ (23.1 mg) was converted into the triethylammonium salt [14] before the methylation; this sample, desulfated mannan 6′ (29.9 mg) and mannan 5′ (14.2 mg) were methylated according to the Hakomori procedure [15]. The methylated derivatives were recovered by dialysis (molecular weight cutoff 3500) and freeze-drying. Yields: mannan 5′, 14.7 mg; mannan 6′, 12.1 mg; desulfated mannan 6′, 27.9 mg.

Antiviral activity.—Antiviral essays were carried out as previously reported [16]. Vero cells as monolayers grown in 24-well plates (6×10^5 cells/well) were infected with 100 PFU/well of herpes simplex type 1 (F-strain), in the absence or presence of varying concentrations of the test compound, and then overlaid with MEM (Eagle's minimum essential medium) containing 0.5% methylcellulose with or without compound. After 3 days of incubation, plaques were counted.

3. Results

The sulfated polysaccharides of the red seaweed *Nothogenia fastigiata* were precipitated with Cetrimide and the insoluble complexes were subjected to fractional solubilization in solutions of increasing sodium chloride concentration; in this way, seven fractions were separated and analyzed [3]. More homogeneos samples (mannans 5′ and 6′), which contained mainly mannose and only small amounts of xylose, were isolated from fractions 5 and 6 (soluble in 3.0 M and 4.0 M NaCl, respectively). Table 1 shows their monosaccharide composition, sulfate and protein content, molecular weight, molar ratio xylose:mannose 2-sulfate:mannose 6-sulfate:mannose and antiviral activity.

Mannans 5′ and 6′ were methylated, and Table 2 gives the composition of the permethylated derivatives. These data indicate for both mannans a linear backbone of (1 → 3)-linked D-mannoses with minor D-xylose substitution. Mannan 5′ is mainly 6-sulfated with sulfation restricted to ~ 34% of the units. On the contrary, mannan 6′ is

Table 1
Composition, molecular weight and antiviral activity of the sulfated *xylo*-mannans

xylo-Mannan	Sugar composition		Sulfate % SO_3Na	Protein %	Xyl:2S:6S:Man [a] molar ratio	Molecular weight	Antiviral activity [b] IC_{50} [c]
	Mannose mol%	Xylose mol%					
3	78.3	21.7	26.1 (~ 29) [d]	10.6	31:45:44:100	39,100	14.4 [e]
5′	97.5	2.5	15.3 (~ 16)	5.9	5:3:29:100	6,400	> 100 [e]
6′	97.9	2.1	31.7 (~ 33)	6.6	3:52:35:100	30,000	0.6 [f]

[a] Xylose:mannose 2-sulfate:mannose 6-sulfate:total mannose; mannose 6-sulfate includes the units with single stubs of β-(1 → 2)-linked D-xylose.

[b] Evaluated against Herpes simplex type 1 (F-strain).

[c] IC_{50}, inhibitory concentration 50%.

[d] Calculated from methylation analysis.

[e] E. Damonte and C.E. Coto, personal communication.

[f] Ref. [16].

Table 2
Carbohydrate composition (mol per 100 moles of mannose) of the permethylated *xylo*-mannans

Sugar	*xylo*-Mannan		
	3 [a]	5′	6′
2,3,4-Me_3 Xyl	31.1	5.2	3.0
2,4,6-Me_3 Man	7.1	60.8	9.4
2,3,6-Me_3 Man	—	tr [b]	tr
4,6-Me_2 Man	45.0	3.0	52.2
2,4-Me_2 Man	15.2	29.1	30.9
6-Me Man	3.5	—	1.4
4-Me Man	29.2	tr	4.6
2-Me Man	tr	2.1	tr
Man	—	5.0	1.5

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

heavily sulfated at the 2-position (52.2%) and at the 6-position (30.9%), with the possibility of minor quantities of disulfated units.

The FTIR spectra of mannan 6′ and the previously reported *xylo*-mannan (fraction 3) [3] exhibited broad peaks with a maximum at 843 cm^{-1} and a shoulder at 815–820 cm^{-1}. The second derivative mode provided in both cases two sharper signals at 843 and 825 cm^{-1}. The spectrum of mannan 5′ showed a broad band centered at 802 cm^{-1}; in the second derivative mode, a strong absorption at 823 cm^{-1} was observed.

The ^{13}C NMR spectra of mannans 5′ and 6′ (Table 3) gave, in the anomeric region, absorptions at 103.1–103.4 ppm [3-linked α-D-mannose (6-sulfate) units] and 100.4 ppm (3-linked α-D-mannose 2-sulfate units) [17,18]. The first signal predominated strongly in the former sample, while in the latter both peaks were of similar size. In the

Table 3
^{13}C NMR spectral assignment of the *xylo*-mannans

Unit	C-1	C-2	C-3	C-4	C-5	C-6
Mannan 3						
α-D-Man 2-sulfate	100.7	77.5	77.5	67.1	74.6	61.8
α-D-Man 6-sulfate	103.4	70.8	79.3	67.1	72.3	68.2
α-D-Man 6-sulfate [a]	101.8	79.3	79.3	68.2	72.3	68.2
α-D-Man	103.4	70.8	79.3	67.1	74.6	61.8
β-D-Xyl	104.6	73.7	76.4	70.2	67.1	
Mannan 5′						
α-D-Man	103.1	70.6	79.1	67.0	74.4	62.0
α-D-Man 6-sulfate	103.1	70.6	79.1	67.0	72.4	68.1
α-D-Man 2-sulfate	100.4	77.2	77.2	67.0	74.4	62.0
Mannan 6′						
α-D-Man 2-sulfate	100.4	77.5	77.5	66.7	74.3	61.7
α-D-Man 6-sulfate	103.4	70.6	78.9	67.1	72.3	68.4
α-D-Man	103.4	70.6	78.9	67.1	74.3	61.7

[a] With single stubs of β-(1 → 2)-linked D-xylose.

spectrum of mannan 5′, the ratio of the areas of the C-5 resonances at 74.4 and 72.4 ppm was in agreement with the methylation analysis (~ 2:1), indicating that most of the mannose units were nonsubstituted. In this spectrum, weak signals due to C-1 (α) (95.0 ppm) and C-1 (β) (94.3 ppm) of the reducing end-chain mannose units were observed [17].

The ^{13}C NMR spectrum of *xylo*-mannan 3 (Table 3) showed, in the anomeric region, three major signals at 104.6, 101.8 and 100.7 ppm. The resonance at 104.6 ppm was assigned to the single stubs of β-(1 → 2)-linked D-xylose [19]. The peak at 100.7 ppm (broad) was mainly due to 3-linked α-D-mannose 2-sulfate units, even though small amounts of 3-linked α-D-mannose units with single β-(1 → 2)-linked D-xylopyranosyl side-chains could also contribute to this signal [17,20]. The resonance at 101.8 ppm corresponds to 3-linked α-D-mannose 6-sulfate units with single stubs of β-(1 → 2)-linked D-xylose [17,18,20]. The spectrum also showed a minor signal at 103.4 ppm due to 3-linked mannose (6-sulfate) units.

Mannan 6′ was desulfated with 0.1 M methanolic hydrogen chloride with elimination of 87.4% of the sulfate groups and very small degradation. The desulfated derivative was only sparingly soluble in water and contained mannose and xylose in a molar ratio 42.5:1.0 and sulfate ($NaSO_3$, 4.0%); its molecular weight was 11,600. Methylation analysis showed major amounts of 2,4,6-tri-*O*-methylmannose (75.5%); small amounts of 2,3,4-tri-*O*-methylxylose (4.6%); dimethylated mannoses, namely: 4,6-di-*O*-methyl- (6.9%), 2,6-di-*O*-methyl- (6.8%) and 2,4-di-*O*-methyl-mannose (6.1%); 6-*O*-methylmannose (1.4%) and mannose (3.3%).

Each of the *xylo*-mannans gave similar optical rotations in different solvents (see experimental) as follows: *xylo*-mannan 3, +13.1° to +18.6°; mannan 5′, +74.3° to +79.5°; mannan 6′, +55.2° to +64.2°. For mannan 5′ the optical rotation calculated for the nonsulfated derivative (+93.2°, water) was similar to that reported for the methyl 3-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (+94.8°, water) [21]. For the non-sulfated mannan 6′, the rotation was slightly lower (+80.3°, water), but at higher ionic strength the calculated rotation (+92.6°, 0.1 M NaCl) nearly equalled that of the disaccharide. These data suggest that these *xylo*-mannans are in solution in a random coil-like conformation and that the sulfate groups are not determinant for a more ordered shape.

4. Discussion

The red seaweed *Nothogenia fastigiata* synthesizes a system of α-(1 → 3)-linked D-mannans 2-and 6-sulfated and having single stubs of β-(1 → 2)-linked D-xylose [3]. Most of the fractions isolated were active against herpes simplex type 1, but only mannan 6′ elicited marked activity and proved also active against several other viruses [16].

The mannans studied follow the same structural pattern but also show significant differences, namely: (*i*) fraction 3′ is a *xylo*-mannan highly substituted with single stubs of β-(1 → 2)-linked D-xylose, while mannans 5′ and 6′ are "true mannans" containing only small amounts of xylose side-chains; (*ii*) the molecular weight of 5′ is much smaller than those of the other mannans; and (*iii*) its sulfate content and sulfation on C-2 are lower.

The antiviral activity of sulfated polysaccharides depends in most cases on their ability to block the virus receptors necessary for adsorption to the target cells [22–25]. This activity does not depend directly on the primary structure as the backbone acts as carrier of the sulfate groups [25–28]. The sulfated polymer–virus "complex" could be stabilized if a cooperative mechanism [29] of interaction between the sulfate groups and the positive virus receptors is involved, explaining the variation of activity with the sulfate content [25,26,28,30] and the molecular weight (manans 5′ and 6′) [23,25,26]. High substitution of the polysaccharide backbone (*xylo*-mannan 3) would make difficult the formation of the "complex" resulting in lower antiviral activity. The differential activity of an antiviral sulfated polysaccharide against several viruses might be related to the topographycal distribution of the receptors on these viruses [25].

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