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Additionally sulfated xylomannan sulfates from *Scinaia hatei* and their antiviral activities

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ABSTRACT

Herpes simplex viruses (HSVs) display affinity for cell-surface heparan sulfate proteoglycans with biological relevance in virus entry. This study demonstrates the potential of chemically engineered sulfated xylomannans from *Scinaia hatei* as antiHSV drug candidate. Particularly, a dimethylformamide $-SO_3$/pyridine based procedure has been employed for the generation of anionic polysaccharides. This one-step procedure has the power of providing a spectrum of xylomannans with varying molecular masses (<12–74 kDa), sulfate content (1–50%) and glycosyl composition. Especially, the sulfated xylomannans S1F1 and S2F1 possessed altered activity against HSV-1 and HSV-2 compared to the parental compound (F1) and that too in the absence of drug-induced cytotoxicity. Regarding methodological facet, the directive decoration of hydroxyl functionality with sulfate group plus changes in the molecular mass and sugar composition during isolation by the used reagent opens a door for the production of new molecular entity with altered biological activity from other natural sources.

1. Introduction

The use of mimetics of cellular receptors for viruses for the treatment and/or prevention of viral infections is a promising approach that had already been resulted in the development of novel drugs against influenza virus (Von Itzstein, 2007). Other well-known examples of this approach are mimetics of cell surface heparan sulfate (HS) (for reviews, see Balzarini & Van Damme, 2007; Ghosh et al., 2009; Rusnati et al., 2009; Vaheri, 1964; Witvrouw & De Clercq, 1997), a molecule that serves as an initial receptor for many different viruses including herpes simplex virus (HSV) (WuDunn & Spear, 1989). The inimitably distributed sulfation pattern of HS polysaccharide is believed to regulate its functional specificity (Gama et al., 2006; Liu & Pedersen, 2007). HS mimetics such as sulfated polysaccharides target the virus attachment/entry components, thus preventing adherence of viral particles to cells (Ghosh et al., 2009). Even if sulfated polysaccharides are known to be potent inhibitors of infectivity of HSV, human immunodeficiency virus (HIV), and other HS-binding viruses in cultured cells (Ghosh et al., 2009; Vaheri, 1964; Witvrouw & De Clercq, 1997), intravaginal application of cellulose sulfate provided women no protection against HIV (Cohen, 2008). However, it has been anticipated that next-generation concepts will offer improved prospects for efficacy (Ekblad et al., 2010; Klasse, Shattock, & Moore, 2008). The most plausible approach involves a combination of several drugs, preferentially targeting different steps in the viral infection process. Given that sulfated polysaccharides are safe and acceptable (Bollen et al., 2008; Ghosh et al., 2009; Kilmarx et al., 2008), development of several second-generation combination-formulation based on first generation lead candidates may be more effective (Brache et al., 2007; Liu, Lu, Neurath, & Jiang, 2005; Said et al., 2010). So far, semi synthetic xylomannan sulfates have been studied to a less extent, although structural patterns like, for example, the presence of sulfate groups can also be found in xylomannan sulfates. In addition, comparing study on the biological activity of xylomannan sulfates with respect to the sulfate content, molecular mass and sugar composition can scarcely be found in literature, even though the structural variation may lead to products with altered biological properties. Incidentally, sulfated polysaccharides have typically been generated from plant sources using a two-step process, i.e., an initial extraction of the polysaccharide material followed by a sulfation reaction using various reagents. So, a one-step route for the generation of these macromolecules from natural sources will be economic in that it provides advantages

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in process duration and, consequently, expenditure. Furthermore, the high molecular mass and viscous nature of naturally occurring sulfated polysaccharides impede their application as therapeutic agent. Medium sized compounds having low viscosity may help to overcome this limitation. So, preparation of additionally sulfated and structurally different polysaccharides and screening of their activity against HSV-1 and HSV-2 will be of great consequence.

A number of green algae are grown along Indian coastal line, with *Scinaia hatei* as a predominant species (Wealth of India, 1985). Mandal et al. (2008) reported the presence of a160 kDa sulfated xylomannan containing α-(1,3)-linked Man*p* residues substituted at C-2, C-4 and C-6 with single stub of β-Xyl*p* residues in *S. hatei*. Sulfate groups, when present, are located at C-4 of α-(1,3)-linked Man*p* units. Pérez, Carlucci, Noseda, & Matulewicz (2012) studied the structural elements and antiviral activity of a chemically modified polysaccharide from this alga. The current manuscript presents our research on structural elements and antiviral properties of additionally sulfated xylomannans from *S. hatei*. Herein, we have used a single pot strategy based on cost-effective extraction of polysaccharide as well as concurrent additional sulfation of hydroxyls that modify the biological activity of resulting derivatives. This approach relies on the polar nature of DMF $-SO_3$/pyridine reagent and its ability to alter the structural elements of xylomannan present in *S. hatei*. The effects of chemically altered xylomannan sulfates on HSV-1 and HSV-2 infection were studied through an *in vitro* assay using monkey kidney Vero cells. The scope of this contribution should not be understood as mere introduction of antiviral compounds but, also, to encourage scientists to employ this one-pot methodology for the generation of chemically engineered compounds with altered biological activities from other sources. In addition, it will increase the chances of generating library of macromolecules having altered biomolecular properties.

2. Experimental

2.1. General experimental procedures

All chemicals were of reagent grade unless otherwise specified. Evaporations were carried out with an Eyela N-1100 Rotary Evaporator under reduced pressure below 50 °C. Small volumes of sample solutions were freeze-dried by a ScanVac Cool Safe 55-F freeze drier. Dialysis (molecular cut-off 12 kDa; Sigma-Aldrich) against distilled H_2O were performed with continuous stirring. Moisture was determined by drying ground material in an oven at 110 °C for 3 h. Total carbohydrate and uronic acid contents were determined by the PhOH—H_2SO_4 reaction (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and *m*-hydroxydiphenyl-sulfuric acid (Ahmed & Labavitch, 1977) assay, respectively. Neutral sugars were analyzed after hydrolysis with 1 M H_2SO_4 (3 h, 100 °C). The released monosaccharide residues were reduced ($NaBH_4$), acetylated (Ac_2O) and analyzed as their alditol acetate by GC and GC–MS (Blakeney, Harris, Henry, & Bruce, 1983). *Myo*-inositol was used as internal standard. GC was carried out with a Shimadzu GC-17A chromatograph fitted with a flame ionization detector, and a DB-225 column (30 m × 0.53 mm i.d.), using a program that maintained an isocratic temperature of 210 °C for 18 min and helium as gas vector. GC–MS was performed with a Shimadzu QP 5050A GC–MS instrument at 70 eV. Conditions for GC–MS were as described previously (Mazumder, Morvan, Thakur, & Ray, 2004). UV–vis spectra were measured on a Shimadzu UV-2450 spectrophotometer. Fourier transform infrared (FT-IR) spectra of samples were recorded with a Spectrum RX1 spectrometer (PerkinElmer, CITY, ST, Singapore) with 400–4000 cm^{-1} spectral range, 4 cm^{-1} resolution, and 40 number of scans. Sulfated xylomannan were pressed into KBr pellet with a sample: KBr ratio 1:200 (w/w).

2.2. Source of materials

Samples of *S. hatei* (Rhodophyta), collected from the Okha coast of Gujarat, India, were converted into the depigmented algal powder (DAP) as described (Mandal et al., 2008). Briefly, the gathered material was washed, dried immediately by forced air circulation at 35–40 °C and then grounded. The powdered algal biomass (270 g) was treated sequentially with petroleum ether and acetone in a Soxhlet for 2 × 28 h and then air dried to yield 166 g of depigmented algal powder (DAP).

2.3. Generation of sulfated xylomannans from Scinaia hatei

Consecutive isolation and chemical sulfation of polysaccharide from the depigmented algal powder (DAP) of *S. hatei* was carried out using a one-step strategy. Dry algal powder (2 g) was suspended in 30 mL of anhydrous N,N-dimethylformamide (DMF) and the suspension heated at 90 °C for 8 h under vacuum. After cooling to room temperature, the suspension was mixed with 60 mL of DMF containing 7.2 g sulfur trioxide/pyridine complex and the mixture was allowed to react under two time intervals (30 min and 60 min) at 90 °C. Following neutralization with 20% NaOH, the reaction mixtures were filtered and desalted using Sephadex G-25 column (2.6 cm × 90 cm; Amersham Pharmacia Biotech AB, Uppsala, Sweden). Solutions eluted between K_{av} values 0 and 0.8 were collected and lyophilized. Thus, two different chemically engineered fractions, namely S1 and S2 were generated by using two reaction periods, 30 min and 60 min, respectively. In addition, to compare the antiviral activity of the additionally sulfated derivatives with parental xylomannan (F1) of *S. hatei*, we have separately isolated F1 from the depigmented algal powder (DAP) using water (Mandal et al., 2008).

2.4. Size exclusion chromatography (SEC)

Solutions (3–5 mL) of chemically engineered sulfated xylomannan containing fractions (S1 and S2) in 500 mM Na acetate buffer, (pH 5.5) were separately loaded onto Superdex^{TM} 75 column (2.6 × 26 cm^2; Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with the same solvent. Then the column was eluted (15 mL/h) with the same buffer at 30–35 °C. The elution of polymer was expressed as a function of the partition coefficient K_{av}. The column was calibrated with standard dextrans (500, 70, 40, 10 and 1 kDa). Fractions were collected using a Pharmacia Biotech RediFrac fraction collector and analyzed for total sugar content using the PhOH–H_2SO_4 reaction (Dubois et al., 1956).

2.5. Desulfation and sulfate estimation

The xylomannan sulfate S1F1 was converted into its pyridinium salt and then desulfated by solvolysis using the method of Falshaw and Furneaux (1998) as described (Mandal et al., 2008). The solution was dialyzed and lyophilized to yield the desulfated compound (S1F1D). Desulfation using CH_3OH—HCl and auto-desulfation (Percival & Wold, 1963) was also carried out to identify the optimal procedure. Estimation of sulfate by the modified barium chloride method (Craigie, Wen, & vanderMeer, 1984) was carried out as described (Mandal et al., 2008).

2.6. Glycosidic linkage analysis

Methylation was carried out by the method of Blakeney and Stone (1985). Prior to methylation, samples were converted into their pyridinium forms. In the methylation procedure, free hydroxyl groups in the carbohydrates were deprotonated (lithium dimethylsulfinyl anion), methylated (CH_3I), and then the glycosidic

linkages were hydrolyzed (2M CF_3CO_2H, 75 min, 120 °C). Afterward, the liberated partially methylated monosaccharides were reduced ($NaBD_4$), acetylated (Ac_2O), and analyzed by GC and GCMS. Molar ratios of PMAAs were calculated from peak areas using response factors as described (Sweet, Shapiro, & Albersheim, 1975).

2.7. Cells and viruses

Vero (African green monkey kidney) cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum. For maintenance medium (MM), the serum concentration was reduced to 1.5%. HSV-1 strain F and HSV-2 strain G were propagated and titrated by plaque formation in Vero cells.

2.8. Cytotoxicity assay

Vero cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoleum bromide; Sigma-Aldrich, St. Louis, MO, USA) method. Confluent cultures in 96-well plates were exposed to different concentrations of the sulfated xylomannans, with three wells for each concentration, with incubation conditions alike to those used in the antiviral assays. Afterward 10 μL of MM containing MTT (final concentration 0.5 mg/mL) was added to each well. Following 2 h of incubation at 37 °C, the supernatant was eliminated and 200 μL of ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance at 595 nm was measured in a microplate reader. The cytotoxic concentration 50% (CC_{50}) was calculated as the compound concentration required to reduce cell viability by 50%.

2.9. Antiviral assay

AntiHSV 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 compounds. Following 1 h of adsorption at 37 °C, residual inoculum was substituted by maintenance medium containing 0.7% methylcellulose and the corresponding dose of each molecule. Plaques were counted after 2 days of incubation at 37 °C. The inhibitory concentration 50% (IC_{50}) was calculated as the compound concentration required for reducing virus plaques by 50%. All analyses were carried out twice and each in duplicate.

3. Results and discussion

3.1. Additional sulfation of free hydroxyls

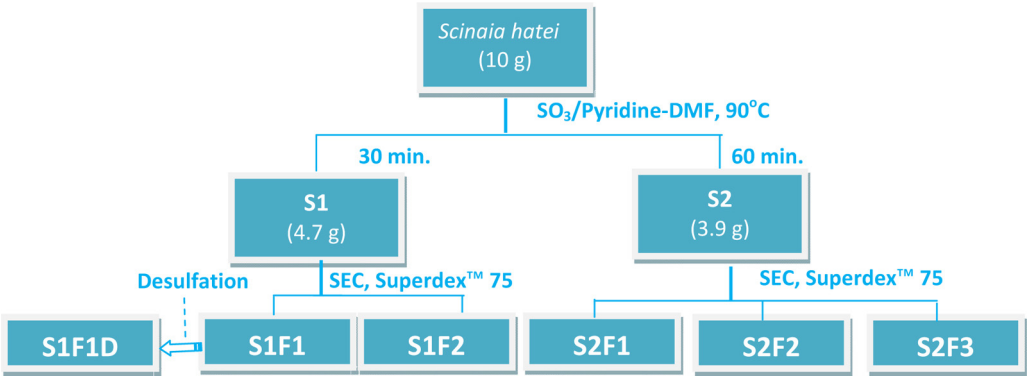
The seaweed *S. hatei* was used to obtain chemically engineered xylomannan sulfates with varying sulfate contents, glycosyl composition and molecular masses. The detailed structural characterization of the parental xylomannan has previously been published (Mandal et al., 2008). Briefly, the presence of a xylomannan sulfate (F1) with an apparent molecular mass of 160 kDa and containing 23% xylose and 77% mannose has been reported. This polymer, which contained 8% sulfate (w/w), had a backbone of α-(1,3)-linked Man*p* residues substituted with single stubs of β-linked terminal Xyl*p* residues. Herein, a one-step strategy was employed for the generation of chemically altered sulfated polysaccharides from depigmented algal (*S. hatei)* powder (DAP). In particular, SO_3/pyridine-DMF reagent treatment of DAP for 30 min produced a transparent solution. Upon neutralization with 20% NaOH followed by desaltation and lyophilization, a sulfated xylomannan containing fraction named as S1 was obtained (47%). Notably, DMF-SO_3/pyridine reagent, which is polar in nature, was found to be efficient for the isolation of sulfated xylomannan. Nonetheless, this class of biomacromolecule contains hydroxyl and other polar groups. Additionally, the studied reagent may assist in the dissolution process by breaking covalent cross-links of the matrix polymers and other linkages such as hydrogen bonds and ionic bonds present in the starting algal material. Subsequently, S1 was further purified by size exclusion chromatography (SEC) using a Superdex™ 75 column yielding S1F1 and S1F2 (Scheme 1). Aiming at the diversification of the sulfate contents and molecular mass of the chemically engineered xylomannan sulfates, the algal powder was treated with the same reagent for other interval of time. Thereby, S2 (39%) was generated from DAP on 60 min treatment followed by desaltation on a Sephadex G-25 column. The S2 fraction upon subjection to SEC on Superdex™ 75 produced S2F1, S2F2 and S2F3. Consequently, a spectrum of macromolecules with different structural elements has been generated. The utility of this method lies in the use of DMF-SO_3/pyridine reagent that efficiently extracts the xylomannan plus decorates it with sulfate groups in one pot.

To compare the antiviral activity of these chemically altered molecules with parental xylomannan (F1), we have separately isolated the later molecule (F1) from DAP (Mandal et al., 2008).

3.2. Glycosyl compositional analysis showed abundance of xylomannans

The sugar compositions of the chemically altered polysaccharides as listed in Table 1 are qualitatively similar to parental xylomannan (F1), but quantitatively there are differences.

Irrespective of reaction time the high molecular mass fraction contained higher amounts of mannose residues, whereas the low molecular mass fractions contained elevated amounts of xylose residues.



Scheme 1. Generation and purification of chemically altered xylomannans (S1F1, S1F2, S2F1, S2F2, S2F3 and S1F1D) from the marine alga *S. hatei*.

Table 1
Sulfate content, molar mass and glycosyl composition of chemically altered xylomannans from *S. hatei*.

	F1	S1F1	S1F2	S2F1	S2F2	S2F3	S1F1D
Sulfate[a]	8	48.1	11.1	50.1	46.9	11.3	<1
Molar mass (kDa)	160	74–12	<12	74–58	58–12	<12	
Xylose[b]	23	24.8	42.8	19.4	31.2	41.1	18.3
Mannose[b]	77	75.2	57.2	80.6	68.8	58.9	81.7
Mannose/Xylose	3.34	3.03	1.33	4.15	2.2	1.43	4.46

[a] Percent weight of fraction dry weight.
[b] Mole percent of anhydro sugar.

3.3. Estimation of molecular masses

Molecular masses of all chemically engineered sulfated xylomannans were determined by size exclusion chromatography (SEC). Fraction S1 upon SEC on Superdex 75 column yielded two fractions S1F1 and S1F2. The elution profile for S1F1 revealed that this product is homogeneous (Fig. 1). Based on calibration with standard dextran, the apparent molecular mass of S1F1 could be in the range of app. 74–12 kDa. The low molecular mass fraction isolated from S1 has a molecular mass of <12 kDa (Table 1). On similar treatment, S2 yielded three fractions (Fig. 1): a high molecular mass fraction S2F1 (74–58 kDa), a second medium molecular mass fraction S2F2 (58–12 kDa) and a low molecular mass fraction S2F3 (<12 kDa). Remarkably, molecular mass of the parental xylomannan (160 kDa) of *S. hatei* (Mandal et al., 2008) significantly reduced (74–<12 kDa) during generation of chemically sulfated compounds (Table 1). Indeed, following 30 min reaction time two chemically altered sulfated xylomannan fractions were generated, whereas 60 min reaction time yielded three fractions: all with molecular masses considerably lower than parental compound. Furthermore, the amount of lowest molecular mass fraction (S2F3) following 60 min reaction time (Fig. 1) is significantly larger compared to 30 min reaction product (S1F2). Evidently, longer reaction time generates larger amount of low molecular mass chemically altered sulfated xylomannan. Presumably, SO_3/pyridine–DMF reagent that assisted in the dissolution process also cleaved glycosidic bonds of the parental molecule resulting in decrease in the molecular mass of chemically altered product. Taken together, this reagent not only extracts the polysaccharide and decorates it with sulfate groups, but also brings out changes in the molecular mass of the resulting polysaccharide.

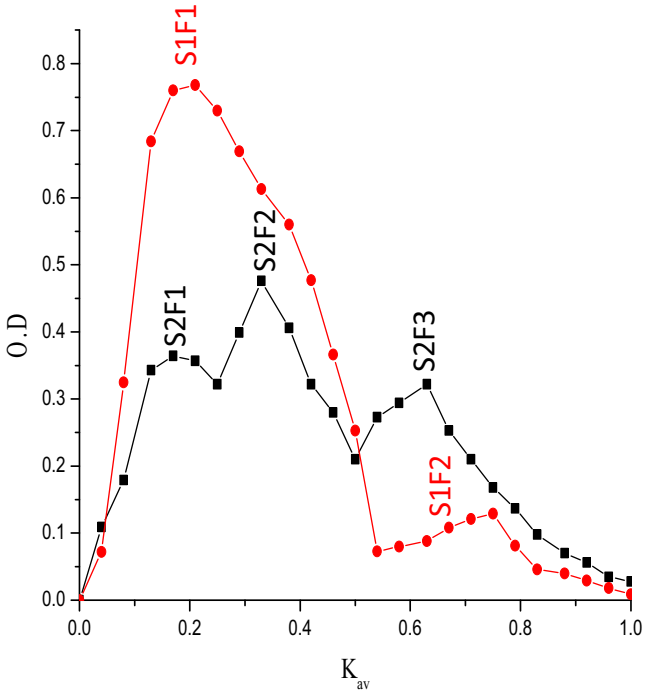


Fig. 1. Elution profiles of (a) S1 and (b) S2 fractions on Superdex 75 column with 500 mM Na. acetate buffer (pH 5.5) at 15 mL/h. Fractions S1 and S2 were generated from *S. hatei* by a one-step procedure using SO_3/pyridine–DMF at 90 °C for 30 min and 60 min, respectively. Elution of polysaccharides was expressed as a function of the partition coefficient K_{av} [$K_{av} = \{V_e - V_0\}/\{V_t - V_0\}$, where V_t and V_0 being were the total and void volume of the column determined using potassium hydrogen phthalate and dextran (500 kDa), respectively and V_e is the elution volume of the sample].

3.4. Sulfate content of the chemically engineered xylomannans

Sulfate content of the xylomannans generated from *S. hatei* was determined by methods as described (Mandal et al., 2008). As listed in Table 1, the high and medium molecular mass derivatives (S1F1, S2F1 and S2F2) contain considerable amount of sulfate (48.1, 50.1, 46.9%, w/w, respectively), but for low molecular mass derivatives (S1F2 and S2F3) these values are small (11.1 and 11.3%, respectively).

3.5. FT-IR Analysis of sulfated xylomannans

Structural changes were also demonstrated by the use of Fourier transformed-IR spectra, comparing high molecular mass xylomannan sulfates such as S1F1 and S2F1, with the medium molecular mass macromolecule S2F2 each compound being engineered chemically from *S. hatei*. As highlighted in Fig. 2 all compounds showed intense absorption band around 1250 cm^{-1} related to the

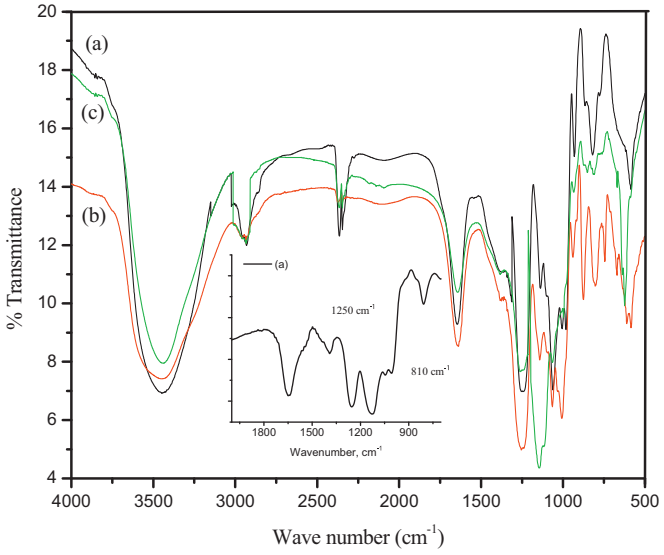


Fig. 2. FT-IR spectra of chemically altered xylomannan sulfates (a) S1F1, (b) S2F1 and (c) S2F2 from *S. hatei*. The band at 1250 cm^{-1} characteristic of sulfate groups is present in all spectra (arrow). The presence of a primary sulfate group (810 cm^{-1}) is also indicated. Inset: The expansion of spectrum 700–2000 cm^{-1} indicates the presence of a primary sulfate group (810 cm^{-1}) in S1F1 (a).

Table 2
Partially methylated alditol acetates derived from a sulfated xylomannan fraction S1F1 and its desulfated derivative S1F1D.

Methylation products[a]	*m/z* values of fragment ions	Peak area[b]	
		S1F1	S1F1D
2,3,4-Me_3-Xyl_p	43, 101, 102, 117, 118, 161 and 162	21	20
2,3,4,6- Me_4-Man_p	43, 45, 118, 161, 162 and 205	Nd[c]	3
2,4,6-Me_3-Man_p	43, 45, 101, 118, 129, 161 and 234	9	47
2,6-Me_2-Man_p	43, 45, 118, 129, 203 and 305	8	11
2,4-Me_2-Man_p	43, 118, 129, 189 and 234	28	8
4-Me-Man_p	43,129,189 and 262	27	11
Man_p		9	Trace

[a] 2,3,4-Xyl denotes 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol, etc.
[b] Percentage of total area of the identified peaks.
[c] Nd, not detected.

Table 3
Anti-HSV activities and selectivity indices of the chemically engineered xylomannans from *S. hatei*.

Compound	IC_{50} (μg/ml)[a]		SI (CC_{50}/IC_{50})[b]	
	HSV-1	HSV-2	HSV-1	HSV-2
F1	0.6–1.53	1.2	>2000	>2000
S1	1.12 ± 0.19	1.29 ± 0.1	>893	>775
S1F1	0.67 ± 0.07	0.22 ± 0.0	>1492	>4545
S1F2	88.62 ± 16	38.55 ± 2.9	>11	>26
S2	0.98 ± 0.30	0.86 ± 0.17	>1020	>1163
S2F1	0.62 ± 0.07	0.75 ± 0.2	>1613	>1333
S2F2	0.88 ± 0.07	1.28 ± 0.03	>1136	>781
S1F1D	>100	>100		
Heparin	4.1–9.1	0.5	>769	>476

[a] IC_{50} (inhibitory concentration 50%), concentration required to reduce HSV plaque number by 50%.
[b] SI (selectivity index), ratio of CC_{50} (cytotoxic concentration 50%)/IC_{50}. CC_{50} > 1000 μg/mL for all samples.

>S═O stretching of sulfate group and an additional absorption band around 810 cm^{-1}. Assignment of band at 1250 cm^{-1} was based on the data originally published by Orr (1954), and Lloyd, Dodgson, Price, and Rose (1961). The band at ~810 cm^{-1} was ascribed to C—O—S stretching of primary sulfate groups of sugar residues on the basis of reports by Lloyd and Dodgson (1961), Lloyd et al. (1961) and Chattopadhyay, Adhikari, Lerouge, and Ray (2007). In the parental xylomannan, sulfate groups are located at C4 of the Man*p* units. Thus, insertion of additional sulfate group in the primary hydroxyl position has been indicated.

3.6. Glycosidic linkage composition and position of sulfate groups

Subsequently, an analysis of glycosidic linkage positions and ring form of constituent sugars was performed by methylation procedure. This method also helped to locate the position of sulfate groups in the chemically engineered xylomannan sulfate S1F1. Initially, we have desulfated S1F1 by solvolysis to yield a derivative (S1F1D) in 25% yield. Glycosidic linkage analysis (Table 2) of the desulfated derivative S1F1D clearly indicates that the xylomannan is branched containing non-reducing end units of Xyl*p* (2,3,4-Me_3-Xyl*p*) and Man*p* (2,3,4,6-Me_4-Man*p*). The Man*p* units were, mainly, (1,3)-, (1,3,4)-, and (1,3,6)-linked, in accord with 2,4,6-Me_3-Man, 2,6-Me_2-Man, and 2,4-Me_2-Man derivatives, respectively. Comparison of these data with the glycosidic linkage composition of chemically altered xylomannan sulfate (S1F1) revealed that sulfate esters were introduced at position C6 of the 1,3-linked Man*p* residues. In the parental xylomannan sulfate ester is placed at C4 of the Man*p* unit. During treatment with SO_3/pyridine-DMF mixture sulfate groups were introduced predominantly at primary hydroxyl position (C6). Nevertheless, this is not surprising as this primary hydroxyl group is more reactive than the secondary one.

In a nutshell, a one-pot procedure for the simultaneous extraction and additional sulfation of xylomannan from *S. hatei* derived algal powder has been employed. This approach not only extracts polysaccharides but, also brings about directive decoration of hydroxyl functionality with sulfate group and modifies the molecular masses of the generated compounds.

3.7. Antiviral activities of the chemically modified polysaccharides

Considering the difference in structures observed between the naturally occurring xylomannan and its chemically modified derivatives, it is expected that the biological activities of the derivatives will also be influenced. So, the preparations from *S. hatei* containing chemically altered sulfated xylomannans were analyzed for antiviral activity. To this end, an established plaque reduction assay for HSV was applied as similarly done in previous investigation (Mandal et al., 2008). Remarkably, compared to *S. hatei* derived xylomannan (F1) described earlier (Mandal et al., 2008), the chemically engineered sulfated xylomannans S1F1 and S2F1 exerted a similar or an even more stringent inhibition of HSV infection of Vero cells (Table 3). The mean values of 50% inhibitory concentration (IC_{50}) ranged over 0.22 ± 0.0 μg/mL and 0.75 ± 0.2 μg/mL against HSV-2, and 0.67 ± 0.07 μg/mL and 0.62 ± 0.07 μg/mL against HSV-1, respectively (Table 3). The strong antiHSV efficacy of S1F1 could be attributed to specific structural elements. Significantly, chemical alteration that occurred during generation of these xylomannan preparations led to substantial variations in activity, ranging from highly active (S2F1), moderately active (S2F2) to less active (S1F2). The differences in potencies arise from the variations of sulfate content, molecular mass and glycosyl makeup of the derived macromolecules. Literature data suggest that molecular mass contributes to antiviral activity, and the higher the average molecular mass of a polysaccharide is, the higher is its antiviral potency (Ghosh et al., 2009). Fraction S2F1 has the highest mass while the mass of S1F2 is the lowest (Table 1). Additionally, the sulfate content has a major impact on the antiviral activity of polysaccharide. The sulfate contents of these fractions are in the order: S2F1 > S2F2 > S1F2. So, it is usual that S2F1 will have the highest whilst the S1F2 fraction has the lowest potency (Table 3). Taken together, the potential of this one step strategy for the production of bioactive polysaccharide depends on the authority of numbers: the higher the amount of altered functionality (i.e., amount of sulfate group in the component and/or the molecular mass of the constituent) in the fraction is, the better is the chance of producing higher potency.

The comparable antiHSV potency of derivatives S1F1, S2F1 and S2F2, all possessing high sulfate contents 47 to 50% (w/w), with that of parental polymer F1 (IC_{50} ranged over 0.5 ± 0.1 μg/mL and 0.5 ± 0.2 μg/mL against HSV-1 and HSV-2, respectively) having significantly low (8%) sulfate content (Mandal et al., 2008) seemed to be surprising at the first sight! Nevertheless, the molecular mass of the parental polymer (160 kDa) is much higher than that of chemically engineered xylomannan sulfates (74 to <12 kDa). Additionally, positions of sulfate groups in the studied polymers are also different. In the parental xylomannan (F1) sulfate groups are located mostly at C4, but in the modified polymer they are at C6 and C4 positions. Publications linking antiviral activity and structure of the polysaccharide have emphasized the importance of specific position of the sulfate group, as shown for chondroitin having sulfates at positions C4 and C6 (Bergefall et al., 2005), carrageenan containing 2,6-disulfated Gal*p* residue (Carlucci, Scolaro, Errea, Matulewicz, & Damonte,1997) or the 3-*O*-sulfated octasaccharide generated from heparin (Copeland et al., 2008), on potency. Although position of the

O-sulfate on specific sugar may be important, yet the general pattern as in which position sulfate groups exert the most pronounced antiviral potency has yet to be established. The sugar composition of the studied polymers may be important too. For fractions generated under similar condition it has been observed that the higher the mannose: xylose ratio, the better is the antiHSV potency (Table 3). Then again, derivatives for instance S1F1, S2F1 and S2F2 cause significant reduction in plaque formation while no major effect (IC_{50} >100) was observed for the nonsulfated xylomannan S1F1D (Table 3). Hence, sulfate group is the hallmark of antiHSV activity. Based on the data obtained, we can infer that the xylose and sulfate content, the position of sulfate group and the molecular mass of the derivatives or, even, all could be important factors that control the antiHSV potency of chemically altered xylomannan sulfates. Remarkably, compound S1F1 exerted the highest antiviral activity against HSV-2, while compounds S2F1, S2F2 and F1 showed stronger potency against HSV-1 (Table 3). This finding underlines the high antiherpesviral potency of these sulfated xylomannans and argues against a general, nonspecific block of cell surface accessibility by them. Additionally, the selectivity indices (SI), calculated as the CC_{50} (cytotoxic concentration 50%)/IC_{50} (inhibitory concentration 50%) ratios of the chemically modified derivatives, showed no effect on cell viability for all tested polysaccharides at concentrations up to 200 μg/mL (Table 3). Thus, a very low or no toxicity can be assumed for these compounds. Consequently our results illustrate how the biological activity of naturally occurring polysaccharide can be modified through directed decoration with sulfate functionality, changes in glycosyl make up and variation of molecular masses.

4. Conclusions

This study underlines the great potential of chemically altered xylomannan sulfates from *S. hatei* for antiviral study and drug development. A method for the synchronized extraction and chemical sulfation of xylomannan has been described. The generated new molecular entities demonstrated significant antiviral efficacy over a very limited degree of cytotoxicity. Remarkably, compound with greater sulfate content, less xylose substitution and higher molecular mass possesses higher antiHSV activity. Even if it appears that the position of the *O*-sulfates on specific sugar is important no general pattern in which position sulfate groups exert the most pronounced antiviral activity was established. This is the area in this study that needs clarification. Finally, the key distinctiveness of this study is the promotion of a single step procedure for the generation of a range of chemically engineered xylomannan sulfates with varying structural elements. This commanding strategy, which complements nature's synthetic capability not only by chemically altering hydroxyl function into sulfate group but also by modifying the molecular mass and glycosyl composition of the derived compound, may provide innovative opportunity for the generation of other bioactive compounds.

Conflict of interest

The authors declare no competing financial interest.

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