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Xylans from *Scinaia hatei*: Structural features, sulfation and anti-HSV activity

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ABSTRACT

Natural compounds offer interesting pharmacological perspectives for antiviral drug development with regard to broad-spectrum antiviral properties and novel modes of action. In this study, we have analyzed alkali-extracted xylan of *Scinaia hatei*. Alkali extraction of this red alga yielded a xylan shown to have a molecular mass of 120 kDa and a linear structure of (1 → 4)-linked β-D-xylopyranosyl residues. Derivatives (S1, S2, S3 and S4) generated by chemical sulfation from this macromolecule had degree of sulfation between 0.93 and 1.95, and contained strong anti-HSV activity with inhibitory concentration 50% (IC_{50}) from 0.22 to 1.37 μg/ml. Furthermore, they had no direct inactivating effect on virions in a virucidal assay. Sulfate groups account for their *in vitro* antiviral activity. Interestingly, sulfated xylans already exerted anti-HSV activity when only pre-incubated with the cultured cells prior to infection, thus pointing to a main inhibitory effect on viral entry.

1. Introduction

Herpes simplex viruses (HSV) cause various forms of disease such as lesions on the lips, eyes, or genitalia to encephalitis and even disseminated disease in immunocompromised individuals [1]. The prevalence of herpes simplex virus type 1 (HSV-1) infection increases progressively from childhood, the seroprevalence being inversely related to socioeconomic background. Primary HSV-1 infections in children can give rise to mucocutaneous vesicular eruptions which might be reactivated frequently in adults [2]. Genital herpes, generally caused by herpes simplex virus type 2 (HSV-2) is characterized by a high seroprevalence worldwide (8–40%), with an increasing trend in the last 20 years in nearly all countries [3]. Among the high-risk population, HSV-2 infection is a major public health problem in young adults. Herpes viruses are circulated and amplified by transmission through direct contact with a lesion or the body fluid of an infected individual. Transmission may also occur through skin-to-skin contact during periods of asymptomatic shedding. The adsorption of viruses to the host cell surface is the initial and critical step for viral infection. HSV interaction with target cells involves cell surface heparan sulfate (HS) interacting with several viral envelope proteins, especially glycoproteins gB, gC, and gD [4–7]. gD interacts with herpes virus entry mediator (HVEM), nectin-1, and specific sites on heparan sulfate generated by certain 3-*O*-sulfotransferases [7–9]. The uniquely distributed sulfation pattern of HS polysaccharide is believed to regulate its functional specificity [10,11]. The antiviral properties of sulfated polysaccharides have been known for almost 50 years [12]. Accordingly, heparin (heparan sulfate analogue) and several sulfated carbohydrate polymers including dextran, fucoidan, galactan and xylomannan have been found to inhibit HSV because of their structural similarity to HS and therefore are potential anti-HSV drug candidates [13,14]. To date, the performance of these macromolecules in efficacy trials has been disappointing [15], but next-generation concepts now in or approaching clinical trials offer improved prospects for efficacy [16]. The most plausible approach involves a combination of several drugs, preferentially targeting different steps in the viral infection process. Because sulfated polysaccharides are safe and acceptable [17], development of several second-generation combination formulation based on first generation lead candidates may be more effective [14,18]. The identification of active polysaccharides from marine algae may identify macromolecules with superior efficacy. But, naturally occurring sulfated polysaccharides are mostly complex and heterogeneous. Hence, these macromolecules could bind to a variety of physiologically important proteins, and thus, the risk of toxicity to the host is increased. This risk could, however, be lowered by using structurally defined polysaccharides and sulfation using SO_3-pyridine is an important route for the synthesis of these macromolecules.

In a preceding paper [19] it was shown that a sulfated xylomannan containing fraction of the red seaweed *Scinaia hatei* possesses *in vitro* anti-HSV activity. The present study reports isolation and

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chemical characterization of an alkali-extracted xylan. Using chemical and chromatographic methods, and various forms of NMR spectroscopy including COSY, NOESY and HSQC we have been able to deduce structural features of this linear xylan. The possibility to generate xylan derivatives by chemical sulfation in the *O*-positions along the polysaccharide has led to the synthesis of various sulfated derivatives with different degrees of sulfation and charge distribution. With these tailored modifications a range of compounds have been generated that have potential anti-HSV activity and low cytotoxicity.

2. Materials and methods

2.1. Isolation of polysaccharide

Air-dried seaweed, depigmented by sequential treatment with petroleum ether and acetone in a Soxhlet extractor (24 h each), was extracted with water as described [19]. Xylan rich fractions were extracted from the water un-extracted residue by sequential treatment with 1 M (fraction Sh1OH) and 4 M KOH (fraction Sh4OH). The recovery of the polysaccharides from alkaline solution was made by acidification to pH 5.5 with AcOH, dialysis and lyophilisation. The crude xylan was purified further by three fractional precipitations with EtOH from a solution in water.

2.2. Size exclusion chromatography

The xylan sample (Sh4OH) was dissolved in a small amount of NaOAc (0.5 M) at 60–80 °C, centrifuged and further diluted with the same solvent to a xylan concentration of about 0.6% (6 mg/ml). The SEC analysis has been carried out on Sephacryl S-300 column (90 cm × 2.6 cm, Pharmacia) using 0.5 M sodium acetate buffer (pH 5.0) as eluent. The column, injector system and the collector were maintained at 30–35 °C during the analysis. The eluent was pumped at a flow rate of 0.5 ml/min. In a separate experiment the apparent molecular mass of the purified xylan (F2) was determined on the same column. This column was calibrated with dextran standards (gift from Dr. T. Vuorinen) in the range 0.8–500 kDa.

2.3. Estimation of sulfate groups

Sulfate groups were estimated by IR-spectrometric [20] and modified turbidometric barium chloride [21] methods as described [22].

2.4. Sugar analysis

Total sugars and uronic acids were determined by the phenol–sulfuric acid [23] and m-hydroxydiphenyl-sulfuric acid [24] assays using glucose and glucuronic acid, respectively as standard. Neutral sugars were released by hydrolysis in 2 M TFA and analyzed as their alditol acetates [25] by gas liquid chromatography (GLC; Shimadzu GC-17A, Shimadzu, Kyoto, Japan) and gas liquid chromatography–mass spectrometry (GLC–MS; Shimadzu QP 5050 A, Shimadzu). Liberated monosaccharides were also analyzed by thin layer chromatography [26]. Alternatively, TMS derivatives of methyl glycosides were analyzed by GLC [27].

2.5. Sulfation

Polysaccharide containing fraction F2 was sulfated using the method as described [28]. The freeze-dried polysaccharide (100 mg) was soaked in dry DMF (2 ml) and SO_3–pyridine complex dissolved in 2.5 ml DMF mixed with the polysaccharide. For every mol of SO_3–pyridine complex, 1 mol of pyridine was added to the mixture. The reaction was carried out under nitrogen atmosphere at 90 °C. After cooling to room temperature, 25 ml water was added and then the pH of the solution was adjusted to 7 with 1 M NaOH. Finally, sulfated polysaccharides were dialyzed against distilled water and freeze-dried. The sulfated derivatives S1, S2, S3 and S4 were obtained when the reaction times were 0.5, 1, 1.5 and 2 h, respectively.

2.6. Linkage analysis

Four milligrams of each of the xylan (F2) and its sulfated derivative (S4) were subjected to two rounds of methylation [29], with the modifications suggested by Stevenson and Furneaux [30]. The permethylated materials were converted into their partially methylated alditol acetates and analyzed by GLC–MS as described [31].

2.7. NMR analysis

The ^{1}H NMR spectra of samples were acquired at probe temperatures of 25 and 60 °C with Bruker 600 spectrometer (Bruker Biospin AG, Fallanden, Switzerland) operating at 600 MHz for ^{1}H. Samples were dissolved in D_2O (99.96 atom%D), two-dimensional (2D) correlated spectroscopy (COSY), 2D nuclear Overhauser enhancement and exchange spectroscopy (NOESY), and heteronuclear single quantum correlation (HSQC) were recorded using standard pulse sequences. The ^{13}C NMR was recorded on a Bruker 300 spectrometer. The chemical shifts are reported relative to an internal acetone standard at 2.225 and 31.55 ppm for ^{1}H and ^{13}C NMR spectra, respectively.

2.8. 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 MS were propagated and titrated by plaque formation in Vero cells.

2.9. Cytotoxicity assay

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

2.10. Antiviral 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 compounds. After 1 h of adsorption at 37 °C, residual inoculum was replaced by MM containing 0.7% methylcellulose and the corresponding dose of each compound. Plaques were counted after 2 days of incubation at 37 °C. The inhibitory

concentration 50% (IC_{50}) was calculated as the compound concentration required to reduce virus plaques by 50%. All determinations were performed twice and each in duplicate.

2.11. Virucidal activity

A virus suspension of HSV-1 (F) containing 4×10^6 PFU was incubated with an equal volume of MM with or without various concentrations of the polysaccharides for 2 h at 37 °C. The samples were then diluted in cold MM to determine residual infectivity by plaque formation. The sample dilution effectively reduced the drug concentration to be incubated with the cells at least 100-fold to assess that titer reduction was only due to cell-free virion inactivation. The virucidal concentration 50% (VC_{50}), defined as the concentration required to inactivate virions by 50% was then calculated.

3. Results and discussion

3.1. Isolation and purification of a sulfated xylan

The central goal of this study is to develop anti-HSV drug candidates from the hemicellulosic polysaccharides of the marine alga *Scinaia hatei*. To achieve this, we have removed pigments and xylomannans from the algal powder as described [19] and then extracted the water insoluble residue with alkali as shown in Fig. 1. The 1 M (Sh1OH) and 4 M KOH (Sh4OH) extracted fractions amounted to 5% and 6%, respectively of algal dry weight and are made up mostly of xylose residues (Table 1).

The major hemicellulosic fraction (Sh4OH) was subjected to further fractionation. Size exclusion chromatography on Sephacryl S-300 column separated it into three sub-fractions (F1, F2 and F3). These sub-fractions accounted for 27%, 54% and 19%, respectively of the total sugar eluted from the column (Fig. 2) and all of them contained xylose as the major constituent sugar. Uronic acid was not detected during TLC analysis of the acid liberated monosaccharides, GLC analysis of the TMS derivatives of the generated methyl glycosides and IR spectroscopic analysis (no band for carbonyl group was observed in the acid form of the polymers). They also had similar ^{1}H NMR spectra. The only difference between these three samples, as judged by size exclusion chromatography, seems to be the molecular mass. The fractionation range of the Sephacryl S-300 column was 1000–300,000 for globular protein and for dextrans about the same. All these fractions were eluted within the fractionation range of the column. Fraction F2 was subjected to further chemical analysis.

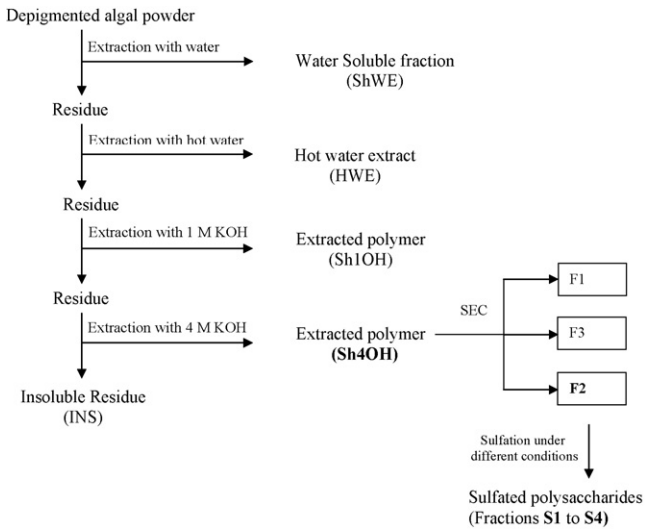

Fig. 1. Scheme for the generation of sulfated xylans from red alga *Scinaia hatei*.

Table 1
Chemical composition of fractions[a] generated from *Scinaia hatei*.

	Sh1OH	Sh4OH	F2	S1	S2	S3	S4
Total sugar[b]	36	43	60	46	37	34	31
DS[c]	Nd[d]	Nd[d]	Nd[d]	0.93	1.42	1.64	1.95
Xylose[e]	98	98	98	98	98	98	98
Mannose[e]	1	1	1	1	1	1	1
Galactose[e]	1	Nd[d]	Nd[d]	Nd[d]	Nd[d]	Nd[d]	Nd[d]
Glucose[e]	Nd[d]	1	1	1	1	1	1

[a] See text for the identification of fractions.
[b] Percent weight of fraction dry weight.
[c] DS, degree of sulfation i.e., numbers of sulfate group per monosaccharide residue.
[d] Nd, not detected.
[e] Mole percent of neutral sugars.

3.2. Molecular mass

The elution profile of this macromolecule on Sephacryl S-300 suggests that this glycan is homogeneous (Fig. 2). Based on calibration with standard dextrans, the apparent molecular mass of this purified xylan would be 120 kDa. It should, however, be noted that a linear polysaccharide may have a different hydrodynamic

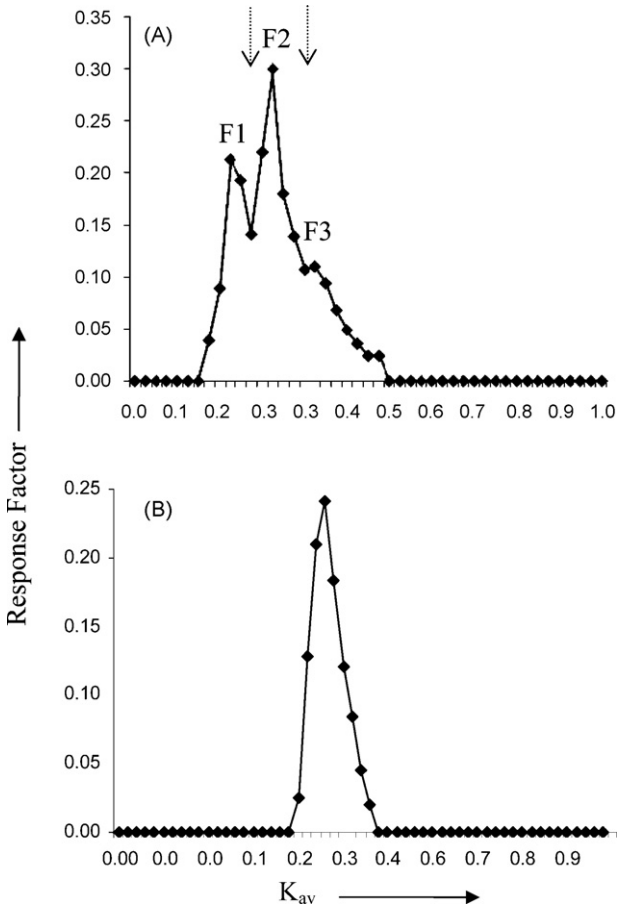

Fig. 2. Size exclusion chromatographic elution profile of (A) the hemicellulosic polysaccharides (Sh4OH) of the marine red alga *Scinaia hatei* on Sephacryl S-300 column and (B) re-chromatography of sub-fraction F2. Elution of polysaccharide was expressed as a function of the partition coefficient K_{av} [$K_{av} = (V_e - V_0)/(V_t - V_0)$ with V_t and V_0 being the total and void volume of the column determined as the elution volume of glucose and standard dextran (500 kDa), respectively, and V_e is the elution volume of the sample].

volume than dextrans and, therefore, elute at a different rate than expected on the basis of their molecular mass. This water soluble polymer had a negative specific rotation $[\alpha]_D^{32}$ −61° (c 0.2, H_2O).

3.3. Sulfation and glycosidic linkage analysis

Degree of sulfation (DS) affects the antiviral activity of polysaccharides [32]. In general, for a particular class of polysaccharide, the higher the charge density, the higher is its activity [14]. In addition to the well-documented DS dependence, the specific position of the sulfate group also appears to be important for the antiviral activity of sulfated polysaccharide [14,33,34]. To study the effect of sulfate groups, we have sulfated the xylan (F2) under various conditions as given in Section 2 to yield sulfated derivatives S1, S2, S3 and S4. Their sugar contents and degrees of sulfation are given in Table 1. The FT-IR spectra of these sulfated xylans showed a band around 1250 cm^{-1} assigned to the >S=O stretching vibration of the sulfate group (Supplementary Fig. 1). In the spectrum of the native polymer Sh4OH this band characteristic of sulfate groups was not present.

Methylation analysis of the purified xylan (F2) revealed a linear structure. The presence of 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylxylitol as the only product suggest that the xylan is (1 → 4)-linked (Supplementary Fig. 2). Moreover, terminal residues were not detected confirming the results of size exclusion chromatography that the molecular mass of this macromolecule is high. In order to determine the positions of sulfate groups' fraction S4 was also subjected to methylation analysis. GLC–MS analysis of the partially methylated alditol acetates revealed the presence of xylitol penta-acetate as the only sugar indicating that all of its hydroxyl groups were sulfated.

3.4. NMR analysis

To specify the anomeric configuration and to confirm glycosidic linkage pattern and ring form (pyranose or furanose) of the xylosyl residues, the sulfated xylan fraction S4 was analyzed by ^1H and ^{13}C NMR (Supplementary Fig. 3) spectrometry. The ^1H NMR spectrum of S4 is given in Fig. 3. Complete assignments of all the signals were made by COSY (Supplementary Fig. 4) and HSQC (Fig. 4) experiments. The NOESY spectrum of S4 shows a correlation between H-1 and H-4 of xylopyranosyl residues (Supplementary Fig. 5) indicative of (1 → 4)-linkage. The carbon resonances were assigned using heteronuclear (HSQC) and homonuclear (COSY) correlation NMR experiments. The presence of only one anomeric signal confirms that fraction S4 is hyper-sulfated as was evident from the result of methylation analysis. In addition, the presence of a doublet centered at δ 102.2 with $J_{C\text{-}1,H\text{-}1}$ value of 161 Hz in the ^{13}C, ^1H-coupled ^{13}C NMR spectrum of S4 indicates that the anomeric configuration of the xylose residue is β. Taken together with the optical rotation value $[\alpha]_D$ −61° of the purified xylan, this result confirms the presence of β-glycosidic configurations.

3.5. Antiviral activity

Cytotoxicity for Vero cells and antiviral activity against both serotypes HSV-1 and HSV-2 were tested by MTT and plaque reduction assays, respectively, for the extracted original fraction Sh4OH and the four F2 sulfated derivatives S1, S2, S3 and S4. Dextran sulfate 8000 (DS8000) was tested in parallel as reference substance. All samples exhibited a full lack of toxicity for Vero cells since no effect on cell viability was observed with any of these fractions at concentrations up to 1000 μg/ml (Table 2). As seen in Table 2, the extracted and purified algal fraction Sh4OH may be considered a moderate anti-herpetic inhibitor with IC_{50} values of 7.6 and 11.7 μg/ml against HSV-1 (strain F) and HSV-2 (strain MS), respectively. On the other hand, the four sulfated derivatives S1 to S4 exhibited a more potent antiviral activity in comparison to the native xylan Sh4OH, with values of IC_{50} ranging from 0.41 to 1.37 μg/ml against HSV-1, and 0.22 to 0.39 μg/ml against HSV-2. These data confirm the importance of the anionic features of the molecules, given mainly by the presence of sulfate groups, in their antiviral effectiveness. Furthermore, the four sulfated fractions exhibited high selectivity indices against both HSV serotypes (>730 to >4545) due to their lack of toxicity for Vero cells and low inhibitory concentration 50% value. In all cases, the sulfated derivatives showed a higher inhibitory effect and selectivity for HSV when compared with other well known sulfated polysaccharide such as DS8000, assayed as reference substance.

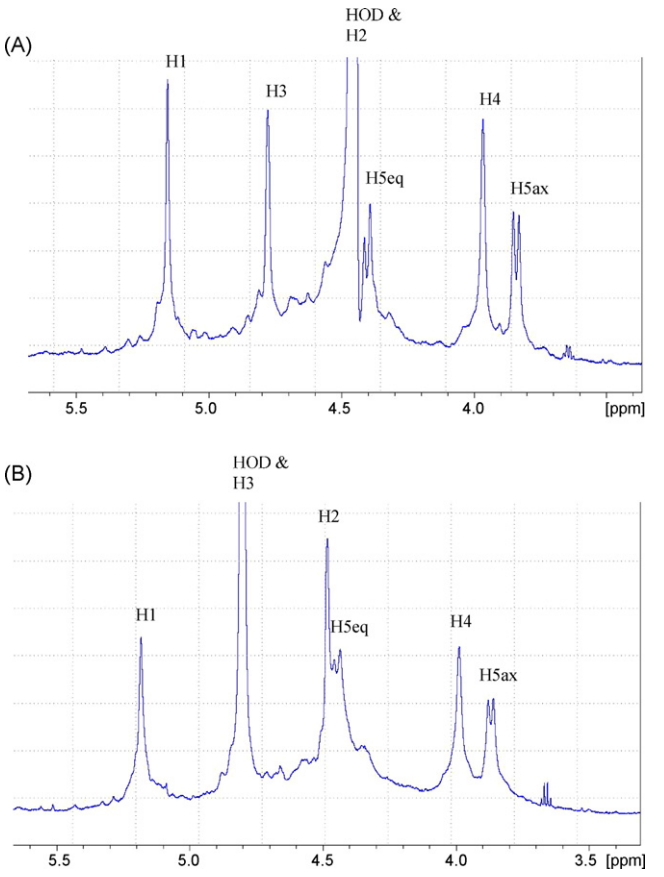

Fig. 3. ^1H NMR spectra at 600 MHz of the sulfated xylan (S4) generated from *Scinaia hatei*. The spectra were recorded at (A) 60 °C and (B) 25 °C in D_2O solution. Chemical shifts are relative to internal acetone at 2.225 ppm. H5eq and H5ax refer to H5 signals of equatorial and axial proton, respectively. The signal for the residual water was designated as HOD.

Table 2
Activity of sulfated xylans generated from *Scinaia hatei* against HSV-1 and HSV-2.

Compound	CC_{50}[a] (μg/ml)	HSV-1 (F)		HSV-2 (MS)	
		IC_{50}[b] (μg/ml)	SI[c]	IC_{50}[b] (μg/ml)	SI[c]
Sh4OH	>1000	7.6 ± 1.4	>132	11.7 ± 1.7	>85
S1	>1000	0.9 ± 0.2	>1111	0.39 ± 0.05	>2564
S2	>1000	1.2 ± 0.2	>833	0.22 ± 0.03	>4545
S3	>1000	0.41 ± 0.01	>2439	0.28 ± 0.01	>3571
S4	>1000	1.37 ± 0.01	>730	0.36 ± 0.03	>2777
DS8000	>1000	2.1 ± 0.1	>476	0.57 ± 0.01	>1754

[a] CC_{50} (cytotoxic concentration 50%): compound concentration required to reduce Vero cell viability by 50%, determined by MTT method.
[b] IC_{50} (inhibitory concentration 50%): concentration required to reduce HSV plaque number by 50%. Mean of two determinations ± standard deviation.
[c] SI (selectivity index): ratio CC_{50}/IC_{50}.

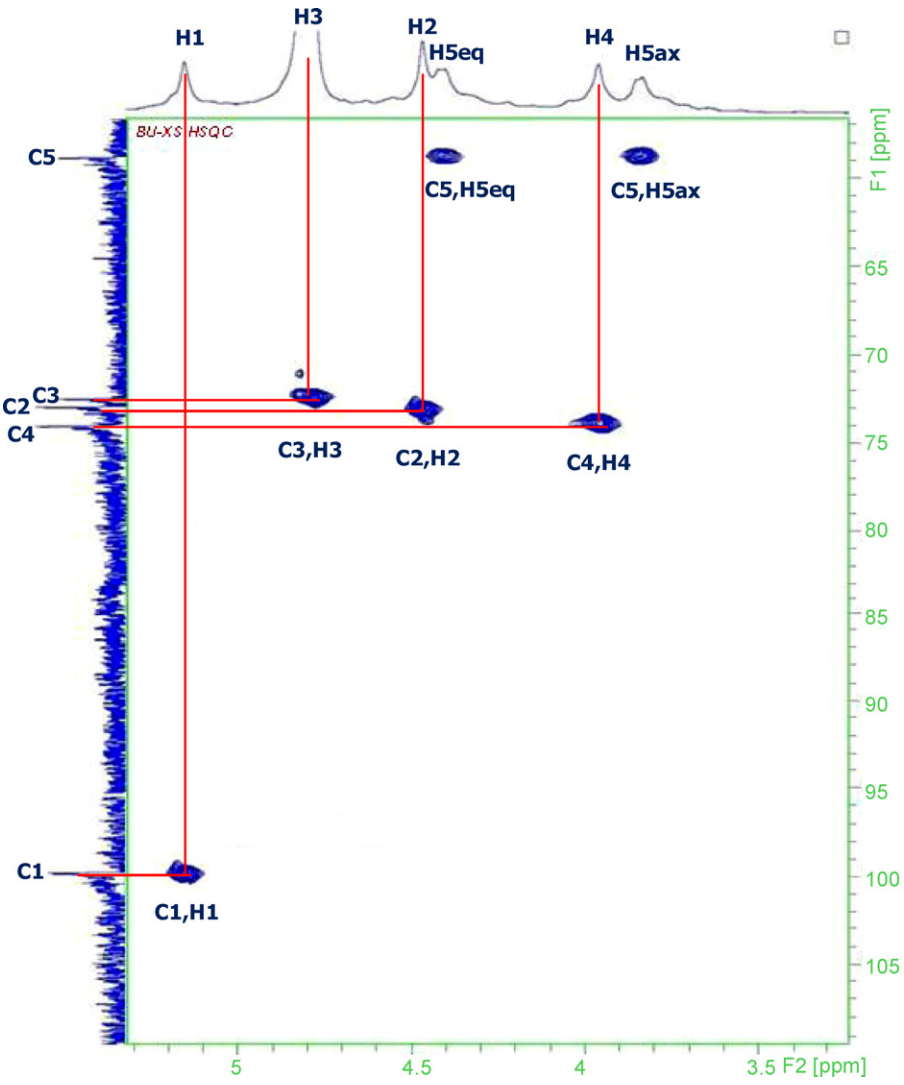

Fig. 4. HSQC spectrum (D_2O, 25 °C) of the sulfated xylan (S4) generated from *Scinaia hatei*. The cross-peaks labeled are the correlations between H's and C's of xylose residues.

Although the extra-sulfation significantly increased the antiviral potential of polysaccharides, the small differences observed in the IC_{50} values of the sulfated derivatives were not directly related to the sulfate content (compare Tables 1 and 2). In the level of DS presented by these derivatives, it appears that there is a correlation between the anti-HSV activity and degree of sulfation up to a DS value of 0.93, but above this apparently limiting value, no direct correlation exists between these parameters.

3.6. Virucidal activity

The virucidal activity was also estimated for the *S. hatei* xylans against HSV-1 in order to elucidate the possibility that these polysaccharides may act directly on the virus particles. Pre-incubation of the virus with the compounds had no significant direct inactivating effect on HSV-1 virions up to 50 μg/ml that was the maximum concentration assayed (data not shown). Thus, the inhibitory effect of these sulfated polysaccharides appears to be based mainly on their ability to interfere with the replication cycle of HSV-1. The lack of virucidal activity for the xylans from *S. hatei* is in accordance with previous studies that found only exceptionally in a lamba-carrageenan form *Gigartina skottsbergii* the presence of potent inactivating properties against HSV-1 [35] whereas most algal sulfated galactans and carrageenans are not able to produce significant virion inactivation [36].

4. Concluding remarks

In summary, the findings of this study highlight several novel and important aspects of *S. hatei* derived polysaccharides with regard to their anti-HSV properties. (i) A number of different soluble polysaccharides could be easily generated from *S. hatei* with high yield, (ii) these substances exerted high biological activity which could be analyzed in cell culture-based assay systems at a low level of cytotoxicity, (iii) strong inhibitory effect was demonstrated against HSV-2 (IC_{50} of S4 was 0.36 ± 0.03 μg/ml), (iv) the DS appeared to be an important hallmark of anti-HSV activity and (v) the main mode of action was directed to viral entry. Further studies will be needed to characterize the mode of antiviral activity in detail and to define the chances of sulfated polysaccharides for their putative use in suitable applications, e.g. topical administration, of antiviral treatment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijbiomac.2009.12.003.

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