An Antivirally Active Sulfated Polysaccharide from *Sargassum horneri* (TURNER) C. AGARDH

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A sulfated polysaccharide was isolated from the hot water extract of a brown alga, *Sargassum horneri* (TURNER) C. AGARDH. Fucose was detected as the main component sugar of this polysaccharide. This compound showed potent antiviral activity against herpes simplex virus type 1, human cytomegalovirus and human immunodeficiency virus type 1. Time-of-addition experiments suggested that it inhibited not only the initial stages of viral infection, such as attachment to and penetration into host cells, but also later replication stages after virus penetration.

Key words *Sargassum horneri*; brown alga; fucan sulfate; sulfated polysaccharide; anti-HSV-1 activity

Herpes simplex virus type 1 (HSV-1) is an enveloped virus which causes a variety of infections, remains latent in the host for a lifetime, and is reactivated by the proper provocative stimulus to cause recurrent disease. Patients compromised by either immunosuppressive therapy, cancer, or malnutrition are in danger of severe HSV infections.^{1,2)} Organ transplant recipients are at particular risk for increased severity of HSV infection.³⁾ Recently, the severity of HSV disease in severely immunocompromised patients of acquired immunodeficiency syndrome has been noted.⁴⁾

Some nucleoside analogues such as acyclovir (ACV) have been used clinically as effective antiviral agents against HSV-1 infections.⁵⁾ However, severe side effects and the emergence of drug-resistant mutants during long-term medication with these drugs has often limited their administration to patients. Therefore, the development of new antiviral agents with different mechanism is desired.

To find novel anti-HSV-1 agents, screening assays of the antiviral activity of extracts from algae have been performed.⁶⁾ In continuation of this study, an aqueous extract from *Sargassum horneri* was found to show potent antiviral activity. *S. horneri* is a Sargassaceae (brown algae) and is distributed over the shallow sea of Japan, except east of Hokkaido. The boiled alga has been used as a savory food in several places along the Japan sea (e.g., Noto Peninsula of Ishikawa and Toyama Prefectures, and Akita Prefecture). Bioactivity-guided fractionation of the hot water extract from *S. horneri* has led to the isolation of a novel fucan sulfate as an antiviral component. The present paper describes the structural features of this fucan sulfate and its characteristic antiviral activity.

MATERIALS AND METHODS

Materials *S. horneri* (TURNER) C. AGARDH was collected at Notojima, Ishikawa Prefecture, Japan, in February 1995. A standard molecular weight marker of pullulans (Shodex Standard P-82) was purchased from Showa Denko Co., Ltd. (Japan). Dextran sulfate sodium salt (MW 50000) was purchased from Sigma-Aldrich Japan K. K.

General Experimental Procedures Optical rotation was determined in H₂O using a 1 cm light path length cell

with a JASCO DIP-1000 digital polarimeter. UV absorptions were measured with a JASCO V-530 UV/VIS spectrophotometer. IR spectra were recorded in a KBr disk or liquid film using a Hitachi 260-10 infrared spectrophotometer. ¹H-NMR spectra were recorded on a Variant Unity 500 spectrometer at 499.65 MHz. A solution of polysaccharide in D₂O was measured with 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an external reference. Metal elements were analyzed by a Hitachi scanning electron microanalyzer X-650. GC was carried out on a Shimadzu GC-9A or GL-Science GC-353 gas chromatograph equipped with a hydrogen flame-ionization detector (FID). GC-MS analysis was carried out on a Hewlett Packard HP-7980 series II gas chromatograph equipped with a JASCO JMS-AX505HAD Mass Spectrometer. Carbohydrates were determined by the phenol-H₂SO₄ method.⁷⁾

Extraction and Fractionation of Polysaccharides Fresh material (2.0 kg) was extracted twice with 2 l of boiling water for 1 h. The hot water extract was freeze-dried. After treatment of the extract with 10% trichloroacetic acid (TCA), the TCA-soluble fraction obtained by centrifugation (3000 rpm×10 min) was extensively dialyzed for 3 d against distilled water. The nondialyzed portion was lyophilized to give a brownish residue (H). Yield: 37 g.

Gel Filtration of H H (2.75 g) was dissolved in 0.01 M citrate buffer, pH 7.0, containing 0.1 M NaCl, and the soluble portion was applied to a column of Sepharose 6B (Pharmacia Biotech, Uppsala, Sweden, 4.0×60 cm). The column was eluted with the same buffer, and fractions of 10 ml were collected. The eluted fractions were separated into four fractions (H-1—H-4) according to the elution profile prepared on the basis of the phenol-H₂SO₄ method at 480 nm and the UV absorbance at 260 nm. Yield: H-1, 490 mg; H-2, 1.13 g; H-3, 23 mg; H-4, 40 mg.

Ion-Exchange Column Chromatography of H-1 H-1 (300 mg) dissolved in H₂O was applied to a column of DEAE-cellulose (Wako Pure Chemical Industries, Ltd., Kyoto, Japan, OH[−] form, 2.0×70 cm). The column was eluted with a linear gradient of 0—1.0 M NaCl, and fractions of 5 ml were collected. H-1b was concentrated, dialyzed, and lyophilized to yield 127 mg (H-1b). H-1b was rechromatographed with a Sepharose 6B column (2.0×70 cm) to

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give a colorless polysaccharide (Na-HOR, 63 mg).

Estimation of Molecular Weight The average molecular weight of the polysaccharide was estimated by gel filtration with a Sepharose 6B column using pullulans as standard molecular markers.

Reduction of Carboxyl Group of Uronic Acid The carboxyl group of uronic acid in the polysaccharide was reduced according to the method of Taylor and Conrad.⁸⁾ Briefly, Na-HOR (30 mg) in 10 ml of H₂O was adjusted to pH 4.75 with 0.1 N HCl, followed by adding 192 mg (1 mmol) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), then stirring. The reaction mixture was kept at pH 4.75 with 0.1 N HCl. After the hydrogen ion uptake ceased, 2 M NaBH₄ (25 ml) was added dropwise at room temperature over a period of 1 h, and the pH was maintained at 7.0 by adding 4 N HCl. A drop of 1-octanol was sometimes added as an antifoaming agent. The mixture was dialyzed and lyophilized to obtain the carboxyl-reduced polysaccharide.

Identification of Component Sugars The polysaccharide (5 mg) was dissolved in 2 ml of 0.2 N trifluoroacetic acid (TFA) and sealed. After hydrolysis was carried out for 1 h at 121 °C in a steam autoclave,⁹⁾ TFA was removed by evaporation of the reaction mixture to dryness. The hydrolysates were reduced with NaBH₄ and acetylated. The alditol acetates were analyzed by GC using a Shimadzu GC-9A instrument fitted with a 3% ECNSS-M (Gaschrom Q) glass column (2 m×3 mm i.d.) at an oven temperature of 185 °C. Nitrogen was used as a carrier gas.

The carboxyl-reduced polysaccharide was hydrolyzed with 2 N H₂SO₄ for 2 h at 100 °C. The hydrolysates were converted into TFA alditol derivatives. They were analyzed by GC through an OV-1701 fused silica capillary column at an oven temperature of 170 °C. Nitrogen was used as a carrier gas. The uronic acid content was also measured by the *m*-hydroxydiphenyl method.¹⁰⁾

Microdetermination of Sulfur Sulfur content was determined by the flask combustion method under O₂ gas in the presence of H₂O₂.¹¹⁾ After the sample in the combustion flask was ignited under the O₂ atmosphere with an aliquot of H₂O₂, the inner solution was titrated with a standard solution of 50 mM BaCl₂ using 0.1% dimethylsulfonazo III reagent as an indicator.

Desulfation of Na-HOR¹²⁾ Na-HOR (30 mg) was passed through a Dowex 50W-X8 column (H⁺ form, 1×5 cm). The eluate was neutralized with pyridine, then lyophilized. The pyridinium salt thus obtained was dissolved in 10% MeOH-DMSO (15 ml). The mixture was stirred continuously for 5 h at 80 °C under reflux and dialyzed against distilled water for 4 d. The desulfated polysaccharide was obtained after lyophilization (20 mg).

Methylation Analysis Na-HOR (10 mg) was converted to a triethylamine salt (TEA-HOR) by Furneaux's method.¹³⁾ TEA-HOR and desulfated polysaccharide (DS-HOR) were methylated three times by Hakomori's method.¹⁴⁾ Methylated polysaccharides were suspended in 72% H₂SO₄ on an ice bath for 1 h. After the reaction mixture was diluted with H₂O to 8% H₂SO₄, it was hydrolyzed for 2 h at 100 °C. The hydrolysates were converted to alditols with NaBH₄, then acetylated. The partially methylated alditol acetates were analyzed by GC and GC-MS. GC analysis was carried out using a glass column (2 m×3 mm i.d.) packed with 3%

ECNSS-M on Gaschrom Q at an isothermal column temperature of 170 °C under the N₂ gas used as a carrier. GC-MS analysis was performed using a fused-silica capillary column (25 m×0.32 mm i.d.) of HP-5 with a programmed temperature increase of 2 °C per min from 100 to 240 °C. Helium was used as a carrier gas.

Cells and Viruses Vero and HEL cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS). HSV-1 (HF strain) was propagated on Vero cells. Human cytomegalovirus (HCMV, Towne strain) was grown on HEL cells. MT-4, Molt-4 and Molt-4/HTLV-IIIB, a human immunodeficiency virus type 1 (HIV-1)-producing cell line, were cultured in RPMI-1640 medium containing 10% FCS.

Cytotoxicity For cell growth inhibition studies, Vero and MT-4 cells were cultured for 72 h, and HEL cells were cultured for 120 h, at 37 °C, in the presence of increasing amounts of compounds. Viable cell yield was determined by the trypan blue exclusion test. The inhibition data were plotted as dose-response curves, from which the 50% cell growth inhibitory concentration (CC₅₀) was obtained.

Anti-HSV-1 and Anti-HCMV Activities Cell monolayers in 48-well plates were infected with a virus at 0.2 plaque-forming unit (PFU) per cell at room temperature. After 1 h of viral infection, the monolayers were washed three times with phosphate-buffered saline and incubated in a maintenance medium (MEM plus 2% FBS) at 37 °C. Drugs were added during infection and throughout the incubation thereafter, or immediately after the virus infection (0 h). Virus yields were determined by a plaque assay at the 1-d incubation point for HSV-1, and at the 5-d incubation point for HCMV. The antiviral activity was expressed as the 50% or 90% replication inhibitory concentration, IC₅₀ or IC₉₀, which was the lowest drug concentration that reduced plaque numbers by 50% or 90%, respectively, in the treated cultures compared with untreated ones. In time-of-addition experiments, cells were infected with a virus at 10 PFU per cell, and drugs were added at the indicated time.

Anti-HIV Activity The 50% tissue culture infection dose (TCID₅₀) of the supernatant from Molt-4/HTLV-IIIB cells was determined as previously described.⁶⁾ MT-4 cells (2×10⁴ cells/well) were infected with HIV at 20 TCID₅₀/well and incubated at 37 °C. After 5 d, viable cells were counted by the trypan blue exclusion test. Drugs were added during infection and throughout the incubation thereafter, or immediately after the virus infection.

RESULTS AND DISCUSSION

The hot water extract of the alga was fractionated as shown in Chart 1. The non-dialyzable portion (H) of the 10% TCA soluble fraction was antivirally active and contained polysaccharide because the phenol-H₂SO₄ reaction was positive. As shown in Fig. 1, H was separated by gel filtration on Sepharose 6B to give four fractions. The most potent fraction (H-1) was then separated by ion-exchange column chromatography on DEAE cellulose (Fig. 2). The second eluate (H-1b) was further purified by gel filtration on Sepharose 6B to yield a colorless polysaccharide (Na-HOR) as an antiviral component. Na-HOR was eluted as a single peak, and its apparent molecular weight was estimated to be 2.29×10⁵ by

gel filtration on Sepharose 6B. This polysaccharide consisted of fucose (96%) and glucuronic acid (4%), as determined by GC analysis and the *m*-hydroxydiphenyl method. Consequently, we determined that the main part of this polysaccha-

ride was fucan on the basis of the ratio of fucose and glucuronic acid. The polysaccharide had $[\alpha]_D -71.0^\circ$ (H_2O , $c=0.5$). In the 1H -NMR spectrum, methyl proton signals were observed at δ 1.2–1.4 ppm, which were assigned to the methyl groups of fucose residues. Anomeric signals spreading at δ 5.0–5.5 ppm were detected as singlets. These results suggest that fucose residues have an α -L-configuration. In addition, X-ray microanalysis revealed the presence of sodium and sulfur in the molecule. The sulfur was suggested to be present as a sulfate ester because of the observation of an S=O stretching absorption band at 1250 cm^{-1} in the IR spectrum. The degree of substitution of sulfate ester in this polysaccharide was calculated to be 0.26 on the basis of sulfur content. No detection of nitrogen indicated the absence of protein, aminosugars and nucleic acid. These results suggest that Na-HOR is a fucan sulfate (sodium salt).

The results of methylation analysis, as summarized in Table 1, showed that Na-HOR was composed of terminal, 1,3-linked, 1,4-linked and 1,3,4-linked fucose residues. When the result of methylation analysis of Na-HOR was compared with that of its desulfated compound (DS-HOR), 3,4-di-*O*-methyl fucose was revealed to be present in Na-HOR, suggesting the presence of 2-*O*-substituted sulfate

Chart 1. Fractionation of Hot H_2O Extract from *Sargassum horneri*

Fig. 1. Elution Profile of H by Column Chromatography on Sepharose 6B

The sample was dissolved in 0.01 M citrate buffer (pH 7.0) containing 0.1 M NaCl, and the column was eluted using the same buffer. ○, absorbance at 260 nm; ●, absorbance at 480 nm (phenol- H_2SO_4 method).

Fig. 2. Elution Profile of H-1 by Column Chromatography on DEAE Cellulose

The sample was dissolved in H_2O , and the column was eluted with a linear gradient of NaCl (0→1.0 M). ○, absorbance at 260 nm; ●, absorbance at 480 nm (phenol- H_2SO_4 method).

Table 1. Methylation Analysis of Na-HOR and Its Desulfated Compound (DS-HOR)

Methylated sugar	Primary mass fragments (<i>m/z</i>)	Mol %		Substitution sites
		Na-HOR	DS-HOR	
2,3,4- <i>O</i> -Me ₃ -Fuc ^{a)}	117, 131, 161, 175	12.9	20.8	none
2,3- <i>O</i> -Me ₂ -Fuc	117, 161, 203, 247	13.6	27.1	4
2,4- <i>O</i> -Me ₂ -Fuc	117, 131, 233, 247	9.2	37.5	3
3,4- <i>O</i> -Me ₂ -Fuc	131, 175, 189, 233	10.0		2
2- <i>O</i> -Me-Fuc	117, 231, 275	27.5	14.6	3,4
4- <i>O</i> -Me-Fuc	131, 261	15.0		2,3
Unmethylated Fuc	145, 159, 217, 231, 289	11.8		2,3,4

a) Identified as 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl fucitol.

Table 2. Anti-HSV-1 Activity of the Na-HOR from *S. horneri* and Dextran Sulfate (DS)

Sample	Cytotoxicity CC ₅₀ ^{a)}	Antiviral activity				Selectivity index	
		IC ₅₀ ^{b)}		IC ₉₀ ^{c)}		CC ₅₀ /IC ₅₀	
		A ^{d)}	B ^{e)}	A	B	A	B
Na-HOR	>10000	1.0	1.4	3.0	16	>10000	>7140
DS	6200	1.9	51	6.9	120	3300	120

a) Concentration (μg/ml) required to reduce the growth of Vero cells by 50%. b) Concentration (μg/ml) required to reduce HSV-1 replication by 50% when measured by the plaque yield reduction method. c) Concentration (μg/ml) required to reduce HSV-1 replication by 90%. d) Each compound was added to the medium at the same time as the viral infection for 1 h and throughout the incubation thereafter. e) Each compound was added to the medium immediately after viral infection.

Table 3. Anti-HIV-1 and Anti-HCMV Activity of Na-HOR and DS

Sample	Anti-HIV activity							Anti-HCMV activity						
	Cytotoxicity CC ₅₀ ^{a)}	Antiviral activity				Selectivity index		Cytotoxicity CC ₅₀ ^{d)}	Antiviral activity				Selectivity index	
		IC ₅₀ ^{b)}		IC ₉₀ ^{c)}		CC ₅₀ /IC ₅₀			IC ₅₀		IC ₉₀		CC ₅₀ /IC ₅₀	
		A ^{e)}	B ^{f)}	A	B	A	B		A	B	A	B	A	B
Na-HOR	2350	1.2	5.4	4.0	12	1958	435	3500	3.3	8.5	5.0	14	1060	412
DS	2440	2.8	8.4	4.9	40	871	290	2500	2.9	8.3	6.2	12	862	301

a) Concentration (μg/ml) required to reduce the growth of MT-4 cells by 50%. b) Concentration (μg/ml) required to reduce viral replication by 50%. c) Concentration (μg/ml) required to reduce viral replication by 50%. d) Concentration (μg/ml) required to reduce the growth of HEL cells by 50%. e) Each compound was added to the medium at the same time as the viral infection for 1 h and throughout the incubation thereafter. f) Each compound was added to the medium immediately after viral infection.

Table 4. Time-of-Addition Experiments

Treatment No.	Time of addition					Anti-HSV-1 activity (IC ₅₀ ; μg/ml)	
	3 h before infection	During infection	0—1 h p.i. ^{a)}	1—2 h p.i.	2—24 h p.i.	Na-HOR	DS ^{b)}
1	+ ^{c)}	— ^{d)}	—	—	—	>200	>200
2	+	+	—	—	—	1.2	2.5
3	—	+	—	—	—	1.6	0.72
4	—	+	+	—	—	3.4	2.9
5	—	—	+	—	—	>200	>200
6	—	—	+	+	—	>200	>200
7	—	—	—	+	—	>200	>200
8	—	—	—	—	+	3.8	>200
9	—	—	+	+	+	7.6	146
10	—	+	+	+	+	2.4	19
11	+	+	+	+	+	3.0	0.91

a) Post infection. b) Dextran sulfate. c) Cells were incubated in the presence of drug. d) Cells were incubated in the absence of drug.

groups in the terminal fucose residues. An increase in 2,4-di-*O*-methyl fucose, a decrease in 2-*O*-methyl fucose and the disappearance of 4-*O*-methyl fucose in DS-HOR indicated that some 1,3-linked fucose residues carried sulfate groups at C-4 or C-2. Similarly, an increase in 2,3-di-*O*-methyl fucose and a decrease of 2-*O*-methyl fucose indicated that some of the 1,4-linked fucose residues carried sulfate groups at C-3. The disappearance of unmethylated fucose indicated that some 1,3,4-linked fucose residues carried sulfate groups at C-2. The fucoidan (fucan sulfate) from *Fucus vesiculosus* reported previously¹⁵⁾ was composed of 1,3-linked fucose with a branch point at the C-2 or C-4 position. Therefore, Na-HOR was suggested to be a distinct structural feature from the reported fucoidan.

In order to evaluate the antiviral activity of Na-HOR, the cytotoxicity (CC₅₀) and anti-HSV-1 activity (IC₅₀) of Na-

HOR and dextran sulfate (MW=50000, DS), which is a representative synthetic sulfated polysaccharide, were examined. As shown in Table 2, the selectivity indexes (CC₅₀/IC₅₀) of Na-HOR and DS were more than 3000 when they were added to the medium at the same time of viral infection. When added after the viral infection, Na-HOR remained a potent inhibitor, while the potency of DS decreased remarkably. These results suggest that the inhibition mechanism of Na-HOR might be different from that of DS.

So far, sulfated polysaccharides have been known to be as potent inhibitors of enveloped virus replication. Therefore, Na-HOR was evaluated for antiviral activity against enveloped viruses, including HIV-1 and HCMV. As shown in Table 3, Na-HOR also showed potent inhibition against HIV-1 and HCMV.

The mechanism by which sulfated polysaccharides inhibit

virus replication has been generally explained by the inhibition of virus attachment to host cells and by the subsequent virus-cell fusion, or virus penetration.^{16–22)} However, as described above, the inhibition mechanism of Na-HOR was suggested to be different, at least in part, from that of DS. Thus, to delineate the drug-sensitive phase, time-of-addition experiments were carried out with Vero cells infected at a high titer of 10 PFU per cell (Table 4). In the experiments, the anti- HSV-1 activity of Na-HOR was compared with that of DS. Treatment of host cells with the compounds for 3 h before infection showed no inhibitory effect against HSV-1 replication. When they were present during infection (Treatment 2–4, 10, 11), both sulfated polysaccharides suppressed virus replication efficiently, and there was no striking difference in antiviral effect between two compounds. In separate experiments, pretreatment of virus particles with the compounds produced no antiviral effect (data not shown). These results suggest that binding of the polysaccharide to virion or host cells could not be an inhibitory factor of virus replication. There is a possibility that the polysaccharide binds to certain membrane molecules of the host cell different from the receptor, resulting in interference with virus penetration into the host cells, *i.e.*, the virus-cell fusion process. When antiviral activity was evaluated in the cultures treated with the compound immediately after (Treatment 9) or 2 h after infection (Treatment 8) and throughout the incubation thereafter, however, the IC_{50} value for Na-HOR was 20- or 50-fold lower than that for DS, respectively. Thus, unlike dextran sulfate, Na-HOR inhibited HSV-1 replication, even when added at 2 h postinfection when some virus-specific proteins are synthesized.^{23,24)} The interference with the late steps of viral replication by an anionic polymer such as Na-HOR was the first such finding. We are currently attempting to further elucidate the targets at the inhibitory action of Na-HOR in virus-infected cells.

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