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Note

A new sulfated β-galactan from clams with anti-HIV activity

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Abstract

A new polysaccharide composed of galactan sulfate with a β-(1→3)-glycosidic linkage has been isolated from the marine clam species *Meretrix petechialis*. The polysaccharide was homogeneous in its composition containing D-galactose. The glycosidic linkage was examined by 2D DQF-COSY and 2D NOESY spectroscopy. The coupling constant of anomeric proton was 7.8 Hz, suggesting a β-galacto configuration. The downfield shift of H-2 of galactose residue demonstrated the presence of 2-*O*-sulfonate group. TQF-COSY confirmed that the C-6 position was substituted with a sulfonate group. The anti-HIV activity of the polysaccharides has been evaluated by the inhibition of syncytia formation. The fusion index and percentage fusion inhibition of sulfated galactan were 0.34 and 56% at 200 μg/mL.

Keywords: Clam polysaccharide; β-(1→3)-Linked galactan sulfate; Anti-HIV activity

Sulfated polysaccharides have been found in a variety of marine animals, plants and microorganisms [1]. Carragenans and fucoidan are sulfated polysaccharides extracted from red seaweed (Rhodphyaceae) and brown algae [1]. Tecogalan sodium, a sulfated polysaccharide isolated from the bacterium *Arthrobacter*, acts as an angiogenesis inhibitor [2] by inhibiting the binding of basic fibroblast growth factor to its cellular receptors [3]. Sulfated polysaccharides, like dextran sulfates and heparin, have also shown a potent inhibitory activity against several different viruses [4,5]. The inhibitory effects of sulfated galactan on the replication of *Herpes simplex* virus (HSV) have been reported [6]. Sulfated polysaccha-

Abbreviations: COSY, correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; DQF, double quantum filtered; TQF, triple quantum filtered; HIV, human immunodeficiency virus.

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rides from the sea alga *Spirulina platensis* inhibited the activity of human immunodeficiency virus (HIV) [7]. This activity presumably results from a direct interaction of the polysaccharide with the HIV binding site at the membrane protein receptor CD4 of the target T-cells [8]. In the present study a new type of D-galactan sulfate was isolated from *Meretrix petechialis* (clam), its structure was characterized and its antiviral activity was examined.

The composition of purified polysaccharide was determined by a post-column HPLC method and galactose was the only major component detected. Methylation analysis of the intact galactan showed 35.5% of 1,3,5-tri-*O*-acetyl 2,4,6-tri-*O*-methyl galactitol, 13.5% 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl galactitol, 49.2% of 1,3,5,6 tetra-*O*-acetyl 2,4-di-*O*-methyl galactitol, and 1.8% of 1,2,3,5,6-penta-*O*-acetyl 4-*O*-methyl galactitol. The chemically desulfated galactan showed 100% of 1,3,5-tri-*O*-acetyl 2,4,6-tri-*O*-methyl galactitol. These results strongly suggest that the polysaccharide has the consecutive structure [→3-Gal(1→3)-Gal-1→], and is partially O-sulfonated at the C-2 and C-6 positions. No amino sugar was detected by Elson–Morgan assay [9]. Inorganic sulfate was also determined by ion chromatography [10]. FTIR spectra showed a broad band at 820 cm^{-1} due to equatorial *O*-sulfonate groups (data not shown). The sample obtained by solvolysis was treated with D-galactose oxidase and β-D-galactosidase. Both enzymes acted on the desulfated polysaccharide, confirming the D-galactose residue was β-D-galactopyranoside. The desulfated galactan was completely degraded into D-galactose monomer by β-D-galactosidase treatment (data not shown), confirming the intact galactan sulfate only contains β-linked D-galactose.

The 1D ^{1}H NMR spectra of the intact polysaccharide (a) and the product obtained from solvolysis (b) are shown in Fig. 1. The chemical shifts and coupling constants of the intact polysaccharide are presented in Table 1. The spectrum of galactan sulfate shows the heterogeneity of the core structure compared with that of the sample obtained by solvolysis. The presence of anomeric proton was observed as a doublet at 4.83 ppm and its coupling constant was 7.8 Hz in the solvolyzed sample, suggesting that all of the anomers of each galactose residue contain the β configuration. Based on the chemical shift and integration of each signal, some of the H-2 and H-6 protons are O-sulfonated. The integrations of signals of 2-O- and 6-O-sulfonated galactose residues at 4.45 and 4.21 ppm showed that 20% of C-2 and 80% of C-6 hydroxy groups were O-sulfonated. These values were estimated by the integration of all signals corresponding to galactose ring protons. The positions of the *O*-sulfonate groups were confirmed by methylation analysis, but quantification by this method was not possible as the permethylation procedure can result in partial removal of O-sulfonated groups. A 1D differential NOE experiment of O-desulfonated galactan showed 7.4% of nuclear Overhauser enhancement between the anomeric proton and the H-3 proton of the galactose residue. This observation confirms the (1→3)-linkage of the galactan polysaccharide. Interestingly, there was no branched structure in the polysaccharide based on the permethylation analysis of O-desulfonated galactan (data not shown). Two-dimensional TOCSY and NOESY experiments suggested the structure shown in Fig. 1. Comparison of the NMR data of intact and O-desulfonated galactan confirms the proposed structure.

Fig. 2 shows 2D DQF COSY spectrum of the intact polysaccharide. Several cross-peaks

Table 1
^{1}H NMR data of sulfated galactan from *M. petechialis*

	Chemical shift (ppm)		Coupling constant (Hz)
H-1	4.65	$J_{1,2}$	7.6–7.8
H-1 [a]	4.83		
H-2	3.83	$J_{2,3}$	7.8–8.2
H-2 [a]	4.45		
H-3	3.84	$J_{3,4}$	<1.5
H-3 [a]	3.97		
H-4	4.26	$J_{4,5}$	n.d.
H-4 [b]	4.26		
H-5	3.75	$J_{5,6}$	n.d.
H-5 [b]	3.96		
H-6	3.77		
H-6 [b]	4.21		

[a] 2-O-Sulfonated Gal residue.
[b] 6-O-Sulfonated Gal residue.

between H-1 and H-2 suggest that the polysaccharide contain both sulfated and unsulfated galactose residues. The chemical shift of H-2 in the neutral galactose residue in the solvolyzed sample resonates at around 3.83 ppm. A TQF COSY experiment confirmed that the additional *O*-sulfonate groups were attached to C-6 of galactose residues (data not shown).

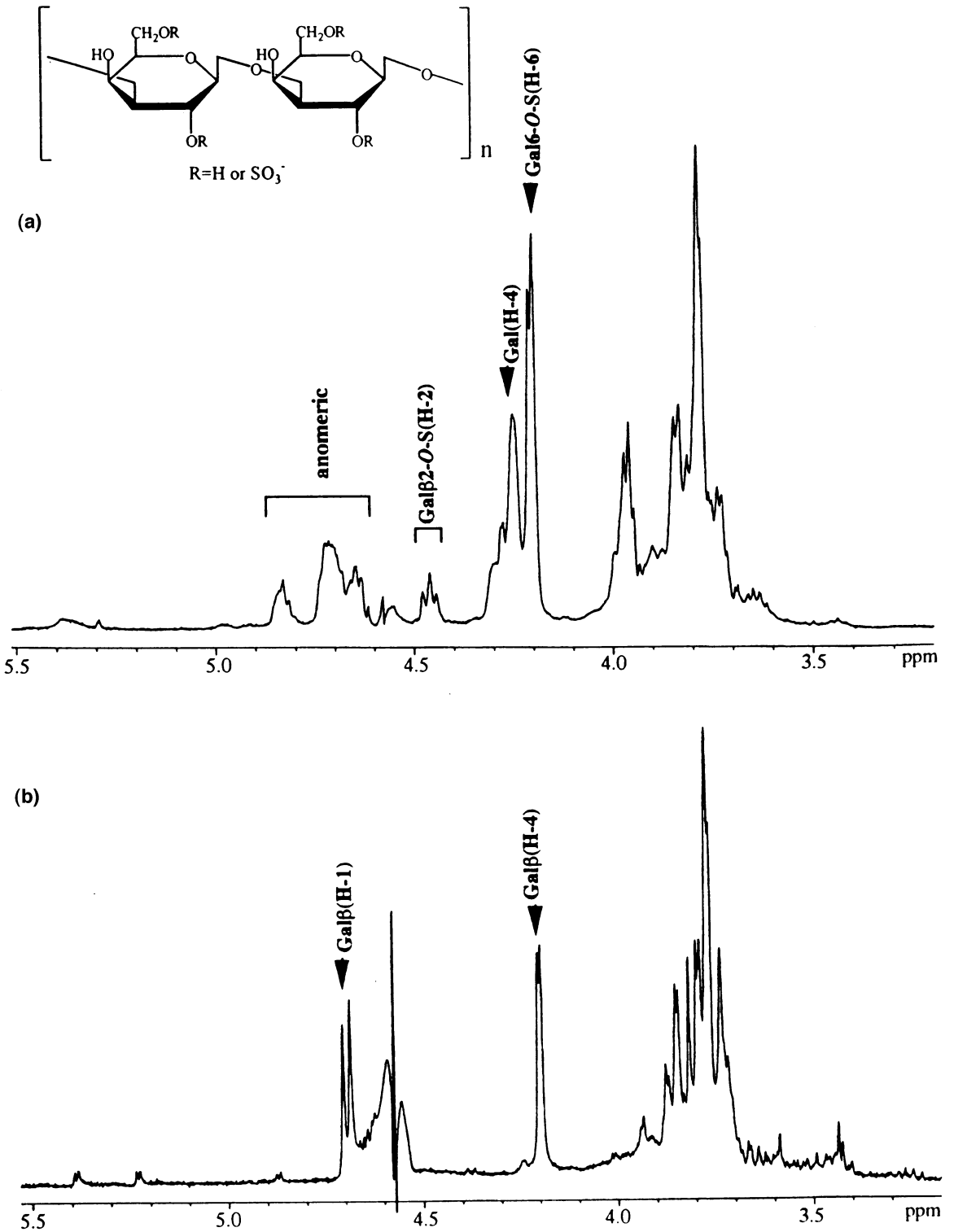

Fig. 1. ^{1}H NMR spectra of galactan sulfate and desulfated galactan. The inset in (a) indicates a proposed structure of galactan sulfate polysaccharide.

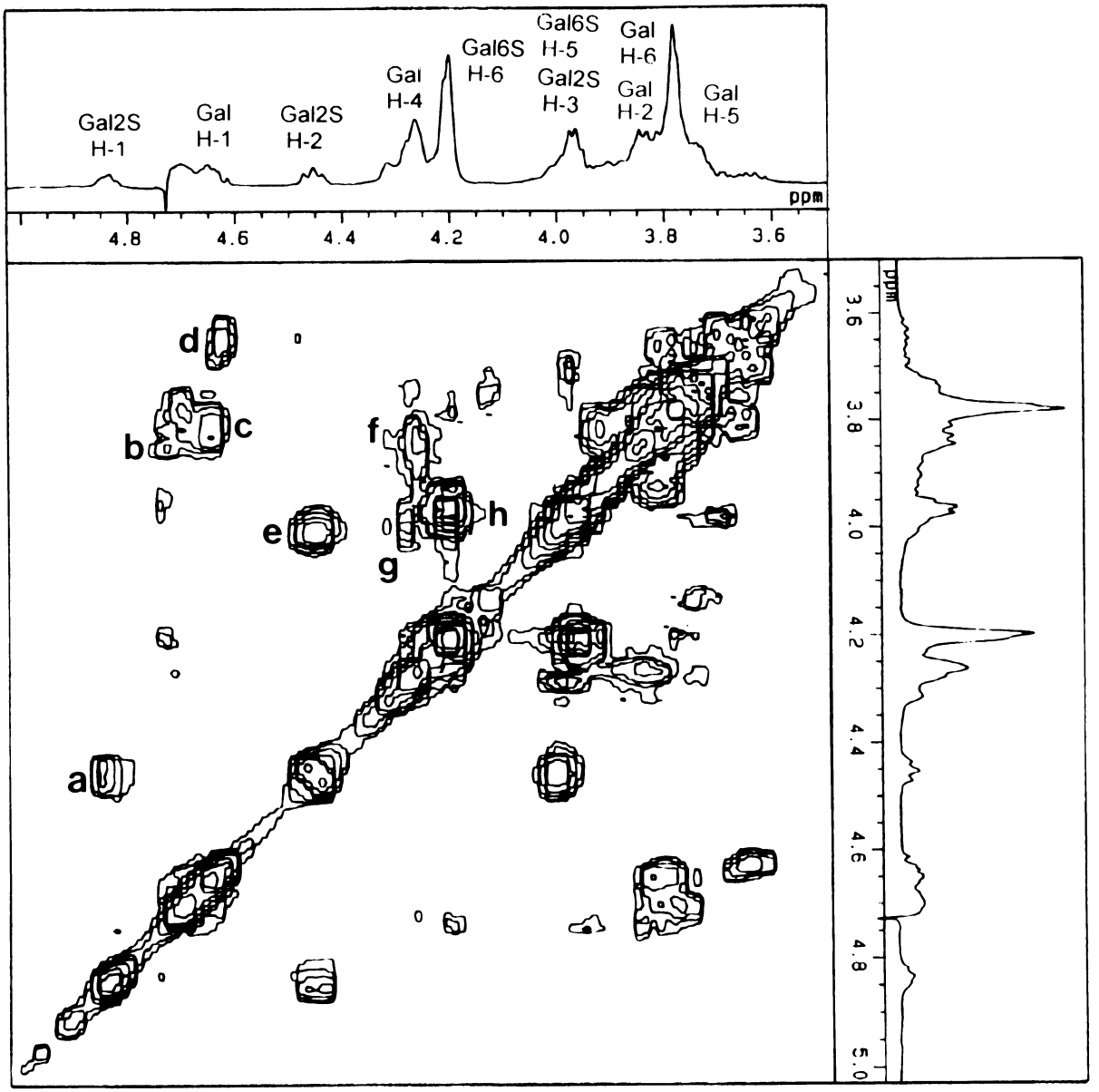

Fig. 2. Two-dimensional ^{1}H-DQF COSY spectrum of galactan sulfate polysaccharide. Cross-peaks: (a) H-1/H-2 of Gal2S; (b, c and d) H-1/H-2 of Gal; (e) H-2/H-3 of Gal2S; (f) H-3/H-4 of Gal; (g) H-3/H-4 of Gal2S; (h) H-5/H-6 of Gal6S (Gal, galactan; Gal2S, galactose 2-*O*-sulfonate; Gal6S, galactose 6-*O*-sulfonate).

The structural features of invertebrate polysaccharides are structurally diverse and are composed of both acidic and neutral polysaccharides. Marine algal species also contain sulfated polysaccharides, which are often present in large quantities and appear to constitute the structural material of the tissues. Polysulfated galactans from sea urchins have been characterized as being comprised of L-galactose, which has a (1→4)-linkage with 3-*O*-sulfonate groups [11]. The present study shows that clam polysaccharide is mainly composed of D-sugars, as demonstrated by the susceptibility of these sugars to both D-galactose oxidase and β-D-galactosidase, with sulfate ester groups at the C-2 or C-6 positions. Solvolytic desulfation indicates that it is a linear β-(1→3)-linked galactopyranose.

The fusion index and percentage inhibition of *v*PE 16-induced syncytia formation by polygalactan sulfate are shown in Table 2. The sulfated galactan inhibited syncytia for-

mation by 33 and 56% at two concentrations, 100 and 200 μg/mL, respectively. In a control experiment, dextran sulfate (MW 5000) inhibited syncytia formation by 65 and 95% at the same two concentrations.

Polysulfates exert their anti-HIV activity by either interfering with CD4 binding to gp 120, thus inhibiting syncytica formation, or by binding to the V3 loop, stopping infectivity [7]. Most of the sulfated polysaccharides that have been found to inhibit HIV replication appear to inhibit syncytia formation [12].

1. Experimental

Materials.—Clams (*M. petechialis*) were purchased at a local market in Seoul, South Korea. Dextran sulfate standards, heparin, chondroitin 4-sulfate, Ham's F12, newborn calf serum (NBSC), trypsin (1:250 diluted), and penicillin G–streptomycin solution were purchased from Sigma (St. Louis, MO). All other reagents used were of analytical grade. Spectrapore dialysis membranes with molecular-weight cut-offs of 1000 and 3000 were from Spectrum Medical (Los Angeles, CA). A fused silica capillary column, DB-1, was obtained from J&W Science (Folsom, CA).

General methods.—Neutral sugars were determined by the post-column HPLC method using 2-cyanoacetamide as a fluorogenic reagent [13], and uronic acid was determined by the carbazole method [14]. Hexosamine was also determined by post-column HPLC using a strong cation-exchange column and the Elson–Morgan method [9]. Sulfate groups were determined by ion chromatography [10].

Preparation of galactan sulfate polysaccharide.—The polysaccharide was prepared according to the previously published method [15]. Approximately 0.18 g of the galactan sulfate polysaccharide was obtained as a white powder from 15 g of the acetone de-fatted, dried, whole soft body of the clam (*M. petechialis*).

Methanolysis and methylation analysis of galactan sulfate polysaccharide.—Compositional analysis of monosaccharide was performed by GC after methanolysis and trimethylsilylation [16]. Polysaccharide was thoroughly dried in vacuo over P_2O_5. After methanolysis for 24 h at 80 °C, in 0.5 mL of dry methanolic 1 M HCl under N_2, and neutralizing with pyridine (0.15 mL), amino sugars were N-reacetylated with Ac_2O (0.1 mL) and dried. The methanolyzed sample was trimethylsilylated with 30 μL of a 2:1 (v:v) mixture of *N,O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA)–pyridine at 8 °C for 1 h.

The dried polysaccharide was also permethylated (repeatedly) by Hakomori's method [17]. The product was subjected to acetolysis in 80% AcOH containing 1 M HCl at 80 °C for 24 h and the resulted partially O-methylated monosaccharides were reduced by 5% $NaBH_4$ in 10 mM NaOH. After removal of borate by evaporation as methylborate, partially O-methylated alditols were acetylated [18]. The partially methylated alditol acetates were analyzed by GC and GC–MS. GC on both the permethylated and trimethylsilylated samples was performed with flame ionization detection on a Hitachi G-3000 gas chromatograph equipped with DB-1 fused silica capillary column (375 μm i.d. × 25 m). GC–MS analyses were performed on a Hewlett–Packard model 5890 series II gas chromatograph with a DB-1 fused silica capillary column (375 μm i.d. × 25 m) followed by a mass selective detector model 5971 equipped with a Chemistation data system. All analyses

Table 2
Inhibition of *v*PE 16-induced syncytia formation in CD4 HeLa cell by D-galactan sulfate from *M. petechialis*

Sample	Concentration (μg/mL)	Fusion index (FI)	% Fusion inhibition
Control		0.74 ± 0.07	0
D-Galactan sulfate	100	0.46 ± 0.05	33.18 ± 0.78
	200	0.33 ± 0.03	55.60 ± 4.85
Dextran sulfate (MW 5000)	100	0.27 ± 0.06	63.00 ± 8.54
	200	0.05 ± 0.01	93.27 ± 2.32

were performed in the electron impact ionization mode, and an ionizing voltage of 70 eV was used. The temperature of the injection port and the detector was set at 28 °C. The column temperature was programmed from 120 to 27 °C at the rate of 2 °C/min and then held isothermally at 27 °C.

NMR spectroscopy.—^{1}H NMR spectroscopy, on 1 mg of dry, exchanged sample in ^2H_2O (0.5 mL, 99.96%), was performed using a Unity-Varian 500 spectrometer with a VXR 5000 computer system (Varian Instruments) or a Jeol 500 MHz instrument equipped with a VAX 32 computer. The operation conditions for one-dimensional spectra were as follows: frequency, 500 MHz; sweep width, 6 kHz; flip angle, 90° (11.1 or 12.8 μs); sampling point, 48 K; accumulation, 256 pulses; temperature, 298 K. The water resonance was suppressed by selective irradiation during the relaxation delay.

Two-dimensional spectra were recorded with 512×2048 data points and a spectral width of 3200 Hz. The water resonance was suppressed by selective irradiation during the relaxation delay. A total of 128–256 scans were accumulated for each t1, with a relaxation delay of 2 s. The digital resolution was 1.6 Hz/point in both dimensions with zero-filling in the t_1 and t_2 dimensions in the case of DQF-COSY, and a Lorentz–Gauss function was applied in all other cases.

Desulfation of galactan sulfate.—Desulfation of the polysaccharide was performed according to the method described by Nagasaki et al. [19]. Briefly, the sodium salt of polysaccharide (10 mg) was dissolved in 100 μL of water, and the solution was passed through a Dowex 50W X8 (H^+, 200–400 mesh) column. The eluate and washings were combined, neutralized with pyridine and lyophilized to obtain the pyridinium salt of polysaccharide as a white powder (11 mg). The pyridinium salt of polysaccharide (10 mg) was dissolved in 200 μL of Me_2SO containing 10% water and heated at 80 °C for 5 h. Then, the content was diluted with water (500 μL) and adjusted to pH 9.0–9.5 by 0.1 M NaOH. The sample solution was dialyzed against distilled water for 24 h and lyophilized.

Determination of configuration.—A sample of 1–2 mg of the polysaccharide was dissolved in 0.1 mL of 2 M HCl. The tube was sealed and heated at 10 °C for 5 h. The hydrolyzate was dried and dissolved in water and was separated by TLC [20]. The chromatogram was sprayed with a solution of 0.1% galactose oxidase and 0.01% peroxidase in 0.1 M acetate buffer of pH 5.2, and then with 0.5% alchoholic dianisidine solution. L-Galactose and D-galactose were used as references. Furthermore, desulfated galactan was treated with β-D-galactosidase from *Aspergillus niger* and the product was confirmed by the HPLC method described above.

Syncytia formation inhibition assay.—This assay followed a previously described procedure [21]. Briefly, the assay used was CD4 HeLa cells and *v*PE 16. Recombinant *v*PE 16 was prepared from a vaccinia virus that expresses HIV-1 envelope protein gp 120 and gp 41 on its surface. CD4 HeLa cells (2×10^5 cells/mL) growing in the log phase were seeded in 12-well culture plates (3 mL cell suspension/well). After 3 days, the plates were washed with fresh medium to remove non-adhering cells and 0.2 mL of culture medium including test compounds or dextran sulfate were added at varying concentrations. The culture plates were incubated for 30 min at 3 °C in a humidified atmosphere containing 5% CO_2. Diluted *v*PE 16 (10 μL of 7.3×10^2 PFU) was added and incubated for an additional 30 min. Incubation was again carried out after 0.8 mL of culture medium was added. After 16–20 h, syncytia formation was observed under a microscope. The fusion index (FI) and the percentage fusion inhibition are calculated as follows:

$$\mathrm{FI} = \frac{\text{Total number of nuclei}}{\text{Total number of cells}} - 1$$

$$\%\ \text{Fusion inhibition} = \left(1 - \frac{\mathrm{FI}_1}{\mathrm{FI}_2}\right) \times 100$$

where FI_1 is the fusion index of the test sample and FI_2 is the fusion index of the control sample.

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