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Sulfated glycofuranans as inhibitors of melanoma lung metastasis

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Abstract Chemically synthesized (1 → 5)-β-D-glucofuranan, (1 → 5)-β-D-galactofuranan, (1 → 5)-β-D-xylofuranan, (1 → 5)-α-L-arabinofuranan, natural xylan, and curdlan were sulfated to investigate their inhibitory activities on B16-BL6 lung metastasis and anticoagulant activities. (1 → 5)-β-D-Glucofuranan sulfate, (1 → 5)-β-D-galactofuranan sulfate, xylan sulfate, and curdlan sulfate had binding abilities with B16-BL6 melanoma lysate. The inhibitory activities of sulfated polysaccharides on B16-BL6 lung metastasis selected by heparin binding assay were in the order (1 → 5)-β-D-galactofuranan sulfate > (1 → 5)-β-D-glucofuranan sulfate > xylan sulfate >> curdlan sulfate. Furthermore, (1 → 5)-β-D-galactofuranan sulfate, (1 → 5)-β-D-glucofuranan sulfate, and xylan sulfate had not only high inhibitory activity on B16-BL6 lung metastasis but also low anticoagulant activity. The correlation between chemical structure and biological activity is discussed.

Key words Glycofuranan · Sulfated polysaccharide · Melanoma lung metastasis · Anticoagulant activity · Heparin

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Introduction

The plant polysaccharides cellulose and hemicellulose are important renewable resources. It is important to use polysaccharides not only as raw materials but also as advanced materials. Plant polysaccharides consist of D-glucose, D-xylose, D-galactose, L-arabinose, and others. After decomposition of natural polysaccharides into monosaccharides, monosaccharides can be converted into artificial polysaccharides as pharmaceuticals by cationic ring-opening polymerization of anhydro saccharides and saccharide orthoesters. Reconstruction of a polysaccharide can make biomass valuable.

Not only natural polysaccharides including several types of wood hemicellulose but also substituted natural polysaccharides and artificial polysaccharides have biological activity.[1–4] Chemically modified polysaccharides (i.e., sulfated polysaccharides) have inhibitory activity on melanoma lung metastasis. Irimura et al. reported that heparanase activity correlates with the lung-colonization ability of murine B16 melanoma cells and is inhibited by heparin.[5] Recently, four independent groups reported gene cloning of mammalian heparanase. Those reports confirmed that heparanase plays an important role in cancer metastasis.[6–9] However, the molecular mechanisms of antimetastasis by heparin have not been clarified.[10] To metastasize, cancer cells must be able to cross basal laminae, which consist of extracellular matrix (ECM). Heparan sulfate proteoglycans (HSPGs) are composed of a protein core covalently linked to heparan sulfate (HS) chains that interact closely with other ECM components. Heparanase secreted by cancer cells is believed to degrade the HS side chains of HSPGs, and as a result cancer cells are able to metastasize. If true, it is necessary to survey heparanase inhibitors to develop antimetastatic agents. Parish et al. have recently designed sulfated oligosaccharide-based inhibitors of tumor growth and metastasis.[11]

Heparin also functions as an anticoagulant by accelerating the binding of antithrombin III with thrombin and a number of other serine proteases of the coagulation

cascade.[12] It is important to design sulfated compounds that have antimetastatic properties without anticoagulant activity. It appears to be a paradox that a heparin-like compound with low anticoagulant activity has high antimetastatic activity. That is why there has been no investigation to develop sulfated polysaccharides with low anticoagulant activity.

Here we report that novel sulfated polysaccharides with low anticoagulant activity that have inhibitory activity on melanoma lung metastasis. It was confirmed that the lysate of B16-BL6 melanoma cells with high potent metastatic ability binds to heparin. The lysate contains heparanase.[13] To explore heparin-like compounds, we adopted a competitive binding assay that polysaccharides with the ability to bind to melanoma lysate are selected using [^{3}H]heparin. Polysaccharides screened by the competitive binding assay are expected to inhibit heparanase, resulting in inhibition of melanoma lung metastasis. Thus, we tested the inhibitory activity of selected polysaccharides on B16-BL6 lung metastasis. The anticoagulant activity was calculated by means of an activated partial thromboplastin time (APTT) test.[14] Correlations between the chemical structure of sulfated polysaccharides and biological activities are discussed.

Fig. 1. Structures of synthetic polysaccharides. D-Glucofuranan sulfate: $R_1 = R_2 = SO_3Na$ or H, $R_3 = CH_2OSO_3Na$, CH_2OH. D-Xylofuranan sulfate: $R_1 = R_2 = SO_3Na$ or H, R_3 = H. D-Galactofuranan sulfate: $R_1 = R_2 = SO_3Na$ or H, $R_3 = CH_2OSO_3Na$ or CH_2OH. L-Arabinofuranan sulfate: $R_1 = R_2 = SO_3Na$ or H, R_3 = H

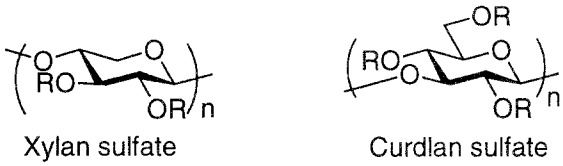

Fig. 2. Structures of natural polysaccharides. Xylan sulfate: R = SO_3Na or H. Curdlan sulfate: R = SO_3Na or H

Materials and methods

Materials

Heparin sodium salt (molecular weight 6–30 kDa) and dextran sulfate (1500 kDa) were purchased from Sigma Chemical and Nacalai Tesque, respectively, as standard compounds for three assays. As shown in Fig. 1, (1 → 5)-β-D-glucofuranan,[15] (1 → 5)-β-D-galactofuranan,[16] (1 → 5)-β-D-xylofuranan,[17] and (1 → 5)-α-L-arabinofuranan[18] were synthesized by ring-opening polymerization and subsequent deprotection. The number-average degrees of polymerization ($\overline{DP_n}$s) of (1 → 5)-β-D-glucofuranan, (1 → 5)-β-D-galactofuranan, (1 → 5)-β-D-xylofuranan, and (1 → 5)-α-L-arabinofuranan before deprotection were 22.8, 38.8, 14.4, and 65.6, respectively, determined by gel permeation chromatography (GPC) using polystyrene standards (columns: Shodex KF 802 + KF802.5 + KF803; eluent: tetrahydrofurane (THF); 1 ml/min).

The chemical structures of the natural polysaccharides used in this study are described in Fig. 2. Xylan from oat-spelt[19] was purchased from Nacalai Tesque (Japan). Curdlan was purchased from Wako Pure chemicals.

Sulfation method

A SO_3–pyridine complex (10 equivalents per OH group) was added to a solution or a suspension of polysaccharide in *N*,*N*-dimethylformamide (DMF). The mixture was stirred at room temperature for 1–7 days and then neutralized with a 2 N NaOH solution. The sulfated polysaccharide was purified by gel filtration chromatography (column: Biorad biogel P-6; eluent: water), or by dialysis against deionized water for some days. The solution was freeze-dried to give a colorless powder.

Measurements

The ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectra were recorded with a Varian INOVA300 FT-NMR (300 MHz) spectrometer in D_2O with sodium 3-(trimethylsilyl)propionate-2,2,3,3,-d_4 as external standard. Molecular weight distributions of the sulfated polymers were analyzed by GPC in 1/15 N phosphate buffer (pH 6.90). Calibration curves were obtained using poly (ethylene oxide) standards (TOSOH). A Shimadzu liquid chromatograph injector (LC-10ATvp), Shimadzu column oven (CTO-10Avp), Shimadzu UV-VIS detector (SPD-10Avp), Shimadzu refractive index detector (RID-10A), Shimadzu LC workstation (CLASS-LC10), and TSK column (TSKgel GMPWXL: 7.8 mm × 30 cm) were used. The flow rate was 1.0 ml/min.

B16-BL6 tumors

The B16-BL6 cells were cultured as monolayers in RPMI 1640 medium (Nikken Biomedical Laboratories, Kyoto, Japan) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY, USA) and penicillin (50 units/ml)–streptomycin (50 μg/ml) (ICN Biomedicals, Aurora, OH, USA). B16-BL6 cells were grown in a 5% CO_2/95% air atmosphere at 37°C.

Preparation of B16-BL6 cell extracts

Subconfluent murine B16-BL6 melanoma cells (B16-BL6) were harvested by treatment with 0.02% EDTA-4Na in

phosphate-buffered saline [PBS(−)] and 0.25% trypsin. After suspension into single cells, they were washed twice with PBS(−) and checked for viability (usually >95%) by trypan blue dye exclusion. Cells were suspended in chilled 50 mM Tris-HCl buffer, pH 7.5, containing 0.2% Triton X-100 at a concentration of 6×10^6 cells/ml. Cell suspensions (1 ml) were sonicated for 20 s at 4°C at constant power using an ultrasonic disrupter UD-201 (Tomy Seiko, Tokyo, Japan). The supernatant was collected after centrifugation at 9800 *g* for 5 min and stored at −80°C.[6]

Heparin binding assay

[^{3}H]Heparin-containing buffer 130 μl (final concentration 0.25 μg/ml [^{3}H]heparin sodium salt specific activity 10.7 MBq/mg) (Du Pont-New England Nuclear Research Products, Boston, MA, USA), 50 mM sodium acetate buffer (pH 5.6), 20 mM D-saccharic acid 1,4-lactone (Nacalai Tesque, Kyoto, Japan), 150 mM NaCl, $MgCl_2$ 10 μM, $CaCl_2$ 10 μM and bovine serum albumin fraction V (BSA) 0.3 mg/ml (Sigma, St. Louis, MO, USA) 20 μl of sample, and 50 μl of B16-BL6 cell extract diluted in 50 mM Tris-HCl buffer, pH 7.5, were mixed in 96-well tissue culture plates and incubated at room temperature for 30 min. Bound [^{3}H]heparin was filtered with a glass filter, washed in 50 mM Tris-HCl buffer, and counted on a Matrix 96 (Packard, Meriden, CT, USA).[20]

B16-BL6 lung metastasis

Female C57BL/6 mice, 7 weeks old, were obtained from Charles River Japan (Yokohama, Japan). The B16-BL6 [5×10^6 cells/ml in PBS(−)] were incubated with heparin (Sigma Chemical), dextran sulfate (Nacalai Tesque), and the sample (0.5 mg/ml) or PBS(−) at 4°C for 1 h. The viability of B16-BL6 cells at the end of the incubation was more than 95%. Treated cells (1×10^6 cells/mouse, 0.1 mg/mouse) were inoculated intravenously in the mice at a volume of 0.2 ml on day 0. On day 17 the mice were killed, and the lung was removed. The wet weight of lung was measured.[5]

Hematology

Blood was obtained under ether anesthesia from the abdominal aorta of male Sprague-Dawley rats (Charles River Japan) and anticoagulated with trisodium citrate. The plasma was separated by centrifugation. The APTT was measured by a light-scattering technique at 37°C using a CA-5000 (Toa Medical Electronics, Kobe, Japan). The citrated plasma was incubated with the sample and Sysmex APTT II (Toa Medical Electronics) solution containing elaidic acid and rabbit brain cephalin for 3 min. Coagulation was then initiated by adding 25 mM calcium chloride.[14]

Anticoagulant activities of sulfated polysaccharides were calculated using a calibration curve for heparin (Sigma) 162 units/mg. Anticoagulant activity (units/mg) = 1000 (μg)/conc. (μg/ml) × (APTT − 14.5)/125.[14]

Results and discussion

Sulfation of polysaccharides

Sulfation with an SO_3–pyridine complex (10 equivalents per OH group) is a general method to obtain a highly sulfated polysaccharide.[21–23] We applied this method to all polysaccharides. In general, a signal of a proton adjacent to the electron-withdrawing group shifts to the lower field in the ^{1}H-NMR spectrum. The sulfate group is an electron-withdrawing group. As shown in Figs. 3 and 4, no signal appears at the same chemical shift between nonsulfated polysaccharides and sulfated ones. All of the ring proton signals of sulfated polysaccharides are shifted to a lower field than those of polysaccharides without sulfate groups. This indicates that sulfation of polysaccharides proceeded. Although the NMR spectra of polysaccharides before sulfation have good resolution, the NMR spectra of sulfated polysaccharides do not have good resolution because these polysaccharides are not completely sulfated. Each proton peak could

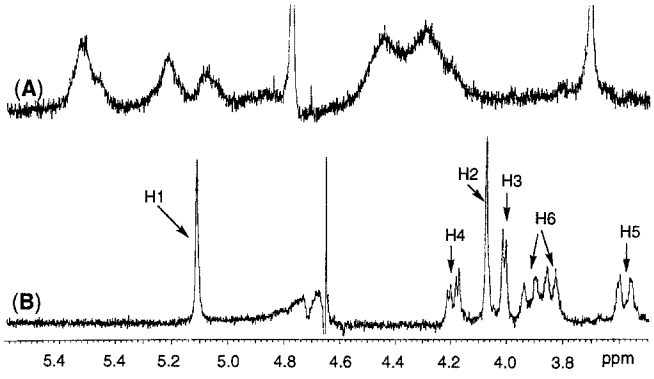

Fig. 3. The 300-MHz ^{1}H-NMR spectra of sulfated (1 → 5)-β-D-glucofuranan (**A**) and (1 → 5)-β-D-glucofuranan (**B**) (D_2O as solvent)

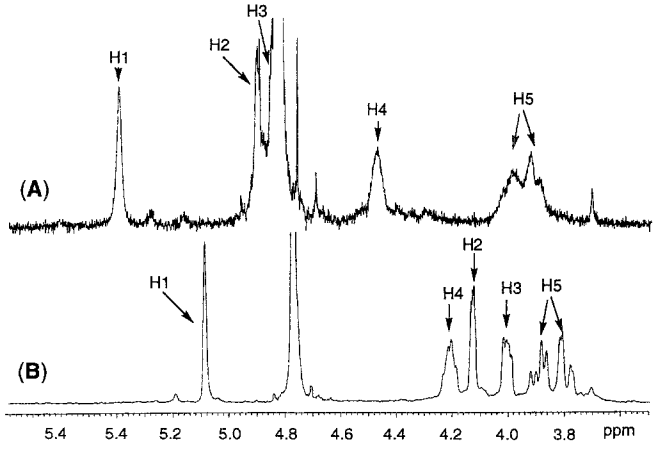

Fig. 4. The 300-MHz ^{1}H-NMR spectra of sulfated (1 → 5)-α-L-arabinofuranan (**A**) and (1 → 5)-α-L-arabinofuranan (**B**) (D_2O as solvent)

Table 1. Molecular weight of polysaccharides

Polysaccharide	Molecular weight (kDa)	Degree of polymerization
Heparin	6–30	
Dextran sulfate	1500	
(1 → 5)-β-D-Glucofuranan sulfate	14	29
(1 → 5)-β-D-Galactofuranan sulfate	21	44
(1 → 5)-β-D-Xylofuranan sulfate	22	66
(1 → 5)-α-L-Arabinofuranan sulfate	27	81
Xylan sulfate	ND	
Curdlan sulfate	98	209

ND, not determined

Table 2. Inhibitory activity of sulfated polysaccharides on the heparin binding assay

Sulfated compound	Inhibition (%) at concentrations of 1–100 μg/ml		
	1	10	100
Heparin	6	47	95
(1 → 5)-β-D-Glucofuranan	34.0	81.5	100
(1 → 5)-β-D-Galactofuranan	32.1	100	100
(1 → 5)-β-D-Xylofuranan	No data	0	91
(1 → 5)-α-L-Arabinofuranan	No data	0	19
Xylan	No data	72.1	100
Curdlan	35.4	100	100

barely be identified because the configurations of all artificial polysaccharides are C-1 and C-2 proton singlets. Detailed structural analyses of these sulfated polysaccharides will be reported elsewhere.

Molecular weight distributions of sulfated polysaccharides were determined by means of size exclusion chromatography (Table 1). The molecular weights of these sulfated polysaccharides, estimated by poly (ethylene oxide) standards, were larger than the calculated molecular weights of the fully sulfated polysaccharides. The degrees of polymerization of sulfated polysaccharides, which were calculated as completely sulfated polysaccharides, did not decrease after sulfation. This indicates that the degradation of polysaccharides did not occur during the sulfation process. The increase in molecular weight after sulfation may depend on the difference in the eluents and standards for organic solvent-soluble and water-soluble polymers. With increasing sulfation Baumann and Faust[23] observed a drastic increase in hydrodynamic volume and thus an increase in the relative molecular weight of the derivatives compared to that of pullulan standards. Our data were in agreement with their results. However, there is a possibility that the sulfated xylofuranan aggregates in 1/15 N phosphate buffer solution. A correlation between molecular weight and biological activity cannot be discussed because the polysaccharides described below do not have the same molecular weight. A study to solve this problem is now in progress. However, it is clear that the molecular weights of four synthetic polysaccharides are lower than those of natural polysaccharides.

Heparin binding assay (screening for B16-BL6 lung metastasis)

To focus on possibly useful polysaccharides, we tested a competitive binding assay wherein polysaccharides with the ability to bind to melanoma lysate are selected using [^{3}H]heparin. As shown in Table 2, two synthetic glycofuranans, (1 → 5)-β-D-glucofuranan sulfate and (1 → 5)-β-D-galactofuranan sulfate, inhibited heparin binding to B16-BL6 cell lysate. Natural polysaccharide derivatives (i.e., a xylan sulfate and a curdlan sulfate) also inhibited heparin binding to B16-BL6 cell lysate. Compared with the inhibitory activity of heparin binding by (1 → 5)-β-D-glucofuranan sulfate and (1 → 5)-β-D-galactofuranan sulfate, that by (1 → 5)-β-D-xylofuranan sulfate and (1 → 5)-α-L-arabinofuranan sulfate was significantly low. In general, sulfated polysaccharides with higher molecular weight have higher biological activity. As shown in Table 1, the number-average molecular weights of (1 → 5)-β-D-glucofuranan sulfate and (1 → 5)-β-D-galactofuranan sulfate are lower than those of (1 → 5)-β-D-xylofuranan sulfate and (1 → 5)-α-L-arabinofuranan sulfate, so molecular weight does not affect heparin binding activity much. On the other hand, (1 → 5)-β-D-glucofuranan and (1 → 5)-β-D-galactofuranan consist of hexoses, and (1 → 5)-β-D-xylofuranan and (1 → 5)-α-L-arabinofuranan consist of pentoses. This indicates that the sulfated hydroxymethyl group at C-5, that is, the 6-*O* sulfate group, in a (1 → 5)-β-D-hexofuranan skeleton, plays an important role in the inhibitory activity of sulfated polysaccharides on heparin binding to the B16-BL6 cell lysate.

Inhibition of B16-BL6 lung metastasis

Figure 5 shows lungs with metastatic B16-BL6 melanoma cells and normal lung. The black colonies in the lungs are B16-BL6 melanoma cells. There is no melanoma lung colony on normal lung (Fig. 5, upper right), but after experimental lung colonization the weight of the lung increases. There are many visible melanoma lung colonies on control lung (Fig. 5, upper middle). A few colonies on the lung indicate that sulfated polymer is effective for lung colonization of B16-BL6 melanoma cells in mice. We did not test the inhibitory activities of B16-BL6 lung metastasis of (1 → 5)-β-D-xylofuranan sulfate and (1 → 5)-α-L-arabinofuranan

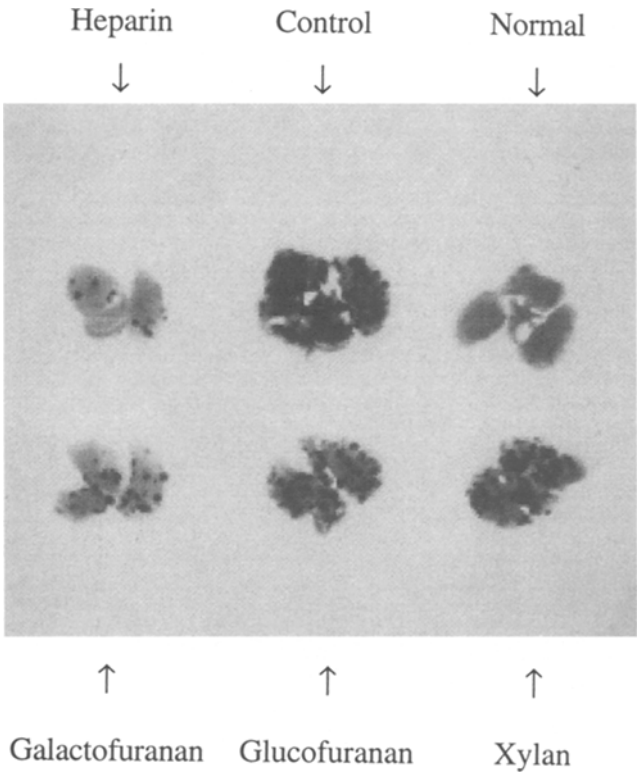

Fig. 5. Effect of sulfated polysaccharides on lung colonization of B16-BL6 melanoma cells in mice

sulfate because these polymers did not have inhibitory activity in the heparin binding assay. As shown in Table 3, all tested polysaccharides inhibited lung metastasis except for curdlan sulfate. The fact that curdlan sulfate activated lung metastasis indicates that there is another system recognizing the sulfated polysaccharide.

Based on these results, it was reconfirmed that inhibition of B16-BL6 lung metastasis is related to inhibitory activities on the heparin binding assay. Consequently, the inhibition of B16-BL6 lung metastasis by four sulfated polysaccharides screened by the heparin binding assay is in the order galactofuranan sulfate > glucofuranan sulfate > xylan sulfate >> curdlan sulfate, judged by inhibitory activity on lung metastasis.

Anticoagulant activity

In general, it is preferable for an antimetastaic agent not to have anticoagulant activity. As shown in Table 4, glycofuranan sulfates have lower anticoagulant activity than heparin and dextran sulfate. It was found that (1 → 5)-β-D-xylofuranan sulfate had low activity (4.3 units/mg). On the other hand, Hatanaka et al. reported that (1 → 5)-α-D-xylofuranan sulfate had anticoagulant activity of 69.1 units/mg.[24] Their activities are therefore very different.

Furthermore, glycofuranans with the R-configuration at the C-4 position [i.e., (1 → 5)-β-D-glucofuranan sulfate and

Table 3. Effect of sulfated polysaccharides on lung colonization of B16-BL6 melanoma cells in mice

Sulfated polysaccharide	B16-BL6 lung metastasis inhibition (%)	Weight of lung[a] (mg)	No. of mice
Control	0	279	15
Heparin	97	144	15
Dextran sulfate	56	221	15
(1 → 5)-β-D-Glucofuranan	66	187	9
(1 → 5)-β-D-Galactofuranan	78	169	5
Xylan	57	200	9
Curdlan	−45	342	9

[a] Normal mouse: 139.5 mg (n = 2)

Table 4. Anticoagulant activity of sulfated polysaccharides

Sulfated polysaccharide	Conc. (μg/ml)	APTT (s)	Anticoagulant activity (units/mg)
Control (saline)	–	16.5	–
Control (5% DMSO)	–	17.0	–
Control (0.5% DMSO)	–	16.2	–
Heparin	50	> 120	162
Dextran sulfate	50	> 120	–
Dextran sulfate	5	39.2	39.5
(1 → 5)-β-D-Glucofuranan	50	49.5	5.6
(1 → 5)-β-D-Galactofuranan	50	23.3	1.4
(1 → 5)-β-D-Xylofuranan	50	30.2	4.3
(1 → 5)-α-L-Arabinofuranan	50	18.9	1.8
Xylan	50	39.0	3.9
Curdlan	50	73.4	9.4

Anticoagulant activity (units/mg) = 1000 (μg)/conc. (μg/ml) × (APTT − 14.5)/125
APTT, activated partial thromboplastin time; DMSO, dimethylsulfoxide

(1 → 5)-β-D-xylofuranan sulfate] tend to have higher anticoagulant activity than those with the S-configuration at the C-4 position [i.e., (1 → 5)-β-D-galactofuranan sulfate and (1 → 5)-α-L-arabinofuranan sulfate]. This indicates that the configuration of the polysaccharide, rather than the molecular weight affects its anticoagulant activity (Table 1). The heparin-binding and anticoagulant activities of polysaccharides do not bear a linear relation to each other. Consequently, the polysaccharide sulfates with inhibitory activity, shown by the heparin binding assay, and low anticoagulant activity that are suitable for medical use are in the order galactofuranan sulfate > xylan sulfate > glucofuranan sulfate > curdlan sulfate (judged by the APTT).

Conclusions

It was found that (1 → 5)-β-D-galactofuranan sulfate, (1 → 5)-β-D-glucofuranan sulfate, and xylan sulfate not only have low anticoagulant activity but also high inhibitory activity on experimental melanoma lung metastasis. It was indicated that the configuration of the polysaccharides is important. Thus, our methodology (i.e., chemical syntheses of nonnatural and natural polysaccharides and subsequent introduction of functional groups) is effective for structure-activity studies. A study of the influence of molecular weight and the position of sulfate groups on the activity is in progress to evaluate sulfated glycofuranans as inhibitors of melanoma lung metastasis.

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