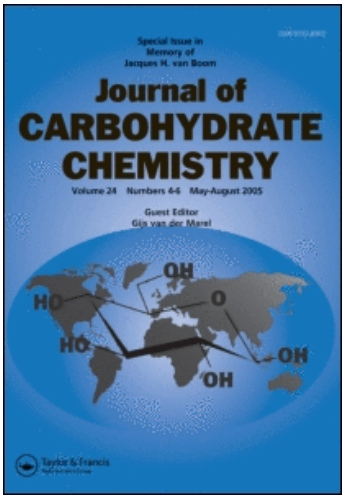

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EFFECT OF DEGREE OF SULFATION ON ANTI-HIV ACTIVITY OF SYNTHETIC (1→5)-α-D-RIBOFURANAN SULFATE[1]

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ABSTRACT

Stereoregular (1→5)-α-D-ribofuranan prepared by selective ring-opening polymerization of anhydro-ribose derivatives was sulfated with piperidine *N*-sulfonic acid to give ribofuranan sulfates (RFS) with various degrees of sulfation (DS). NMR analysis of RFS indicated a similar reactivity of two hydroxyl groups (2-OH and 3-OH). Anti-HIV effects of RFS were investigated by using MT-4 cells, i.e., an HTLV-I-carrying CD-4 positive cell line *in vitro*. RFS with higher DS showed remarkably higher anti-HIV activity. Anticoagulant activity of RFS was also investigated. RFS with a high DS interacted more selectively with virus protein rather than other proteins in the blood than RFS with a low DS.

INTRODUCTION

It is well-known that polysaccharides and oligosaccharides play an important role in biochemical reactions. Relationships between the biological activity and the chemical structure of poly(oligo)saccharides have been investigated. One of the general methods is to analyze the

molecular structure of the active compound, which is prepared from natural polysaccharides. On the other hand, by using 100%-pure synthetic polysaccharide, effects of the chemical structure of the polysaccharide on physiological activities can be made clearer.[3,4]

Heparin and Dextran sulfate, i.e., the polysaccharides having anionic groups, show such biological activities as anticoagulant and lipemia clearing activities. These activities were known to depend on the sulfur content and the molecular weight of the polysaccharides. We reported the high anticoagulant activity of sulfated (1→5)-α-D-ribofuranan and -xylofuranan,[5] which had been synthesized by selective ring-opening polymerization of 1,4-anhydro-D-ribose[6,7] and -xylose derivatives,[8] respectively. Recently, it was reported that sulfated polysaccharides inhibited HIV (human immunodeficiency virus) -induced cytopathic effects *in vitro*.[9,10] Among sulfated polysaccharides, lentinan sulfate,[11,12] curdlan sulfate,[13] and synthetic ribofuranan and xylofuranan sulfates[14] showed a high anti-HIV activity and a very low toxicity against MT-4 cell growth.

In this study, we report the synthesis and characterization of (1→5)-α-D-ribofuranan sulfates with various amounts of sulfate groups. The relationship between the degree of sulfation of ribofuranan sulfate and the anti-HIV effect was investigated *in vitro*. The *in vitro* anticoagulant activity of ribofuranan sulfate is also reported.

RESULTS AND DISCUSSION

Stereoregular (1→5)-α-D-ribofuranan, which was prepared by ring-opening polymerization of 1,4-anhydro-2,3-di-*O*-benzyl-α-D-ribopyranose with boron trifluoride etherate as a catalyst at -40 °C and followed by debenzylation of the obtained polymer,[6] was used as a starting polysaccharide. Ribofuranan with number-average molecular weight of 7.7×10^5 was sulfated with piperidine *N*-sulfonic acid in dimethyl sulfoxide[15] under various conditions. Results are summarized in TABLE 1.

When 1.5 times as much piperidine *N*-sulfonic acid (PSA) as hydroxyl groups of the ribofuranan by mole was used, ribofuranan sulfates (RFS) with a sulfur content of 4.9 to 13.9% were obtained (no. 1 - 3). When more PSA was used (twice, no. 4 - 8; 3 times, no. 9 - 11), the sulfur content of RFS increased. Higher reaction temperatures gave RFS with a higher degree of substitution. The number of sulfate groups per

TABLE 1. Sulfation of (1→5)-α-D-Ribofuranan with Piperidine *N*-sulfonic Acid

No.	Polymer (g)	PSA[a] (g)	DMSO (ml)	Temp (°C)	Time (min)	Yield (g)	S-content (%)	DS[b]	$\bar{M}_n$ (x10^3)
1	0.1	0.38	30	77	60	0.10	4.85	0.26	7.0
2	0.1	0.38	30	81	40	0.12	12.03	0.92	7.9
3	0.1	0.38	30	84	60	0.07	13.91	1.19	6.0
4	0.1	0.50	30	77	60	0.12	13.79	1.28	7.9
5	0.1	0.50	30	77	80	0.12	13.95	1.36	6.6
6	0.2	1.00	60	78	50	0.33	15.07	1.41	7.5
7	0.1	0.50	30	85	60	0.16	15.01	1.48	8.1
8	0.1	0.50	30	90	60	0.18	15.66	1.71	7.1
9	0.1	0.75	30	74	60	0.12	6.35	0.36	6.9
10	0.1	0.75	30	80	60	0.17	16.53	1.80	7.8
11	0.1	0.75	30	90	60	0.18	16.10	1.74	7.3

a. Piperidine *N*-sulfonic acid.

b. Number of sulfate groups per sugar unit.

sugar unit (= DS) calculated with elemental analysis data was 0.26 to 1.80 as shown in Table 1. Number-average molecular weights of RFS were estimated as 6.0 x 10^3 to 8.1 x 10^3, indicating that the main chain scission occurred during sulfation.

^{1}H NMR spectrum of RFS with DS of 1.74 (no. 11) is shown in FIG. 1. Assignments of the protons were carried out by the two-dimensional ^{1}H-^{1}H COSY method. Even in the spectrum of RFS substituted by 87% (Fig. 1), there were resonances assigned to two kinds of mono-substituted ribofuranose units sulfated at C-2 or C-3 in addition to the large peaks which were due to di-substituted ribofuranose units. The intensity of C-2-substituted units was almost the same as that of C-3-substituted unit, regardless of DS, indicating that there is almost no difference in reactivities between 2-OH and 3-OH. This similarity of reactivity was confirmed also by analyzing ^{13}C NMR spectra of RFS (results not shown). From the polymer composition, it was indicated that the mono-substituted unit was less reactive than the non-substituted unit, probably because

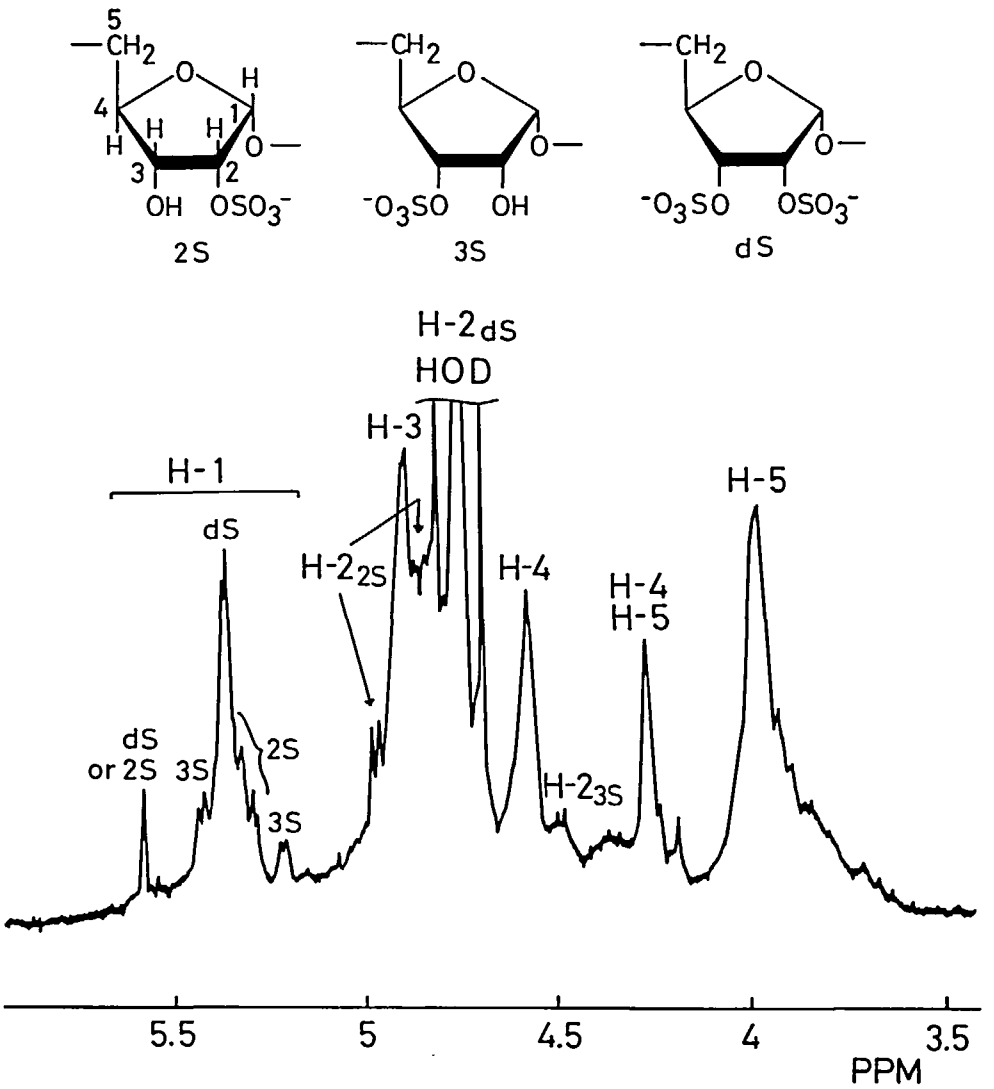

FIG. 1. 270 MHz ^{1}H NMR spectrum of RFS (no. 11 in TABLE 1).

the 2-OH and 3-OH are in *cis*-configuration, which may cause steric hindrance and/or electric repulsion during the sulfation reaction.

Anti-HIV effect of RFS was assayed in terms of the inhibition of HIV-induced cytopathic effects and the expression of viral antigen in MT-4 cells, which are human CD4-positive cell line carrying HILV-

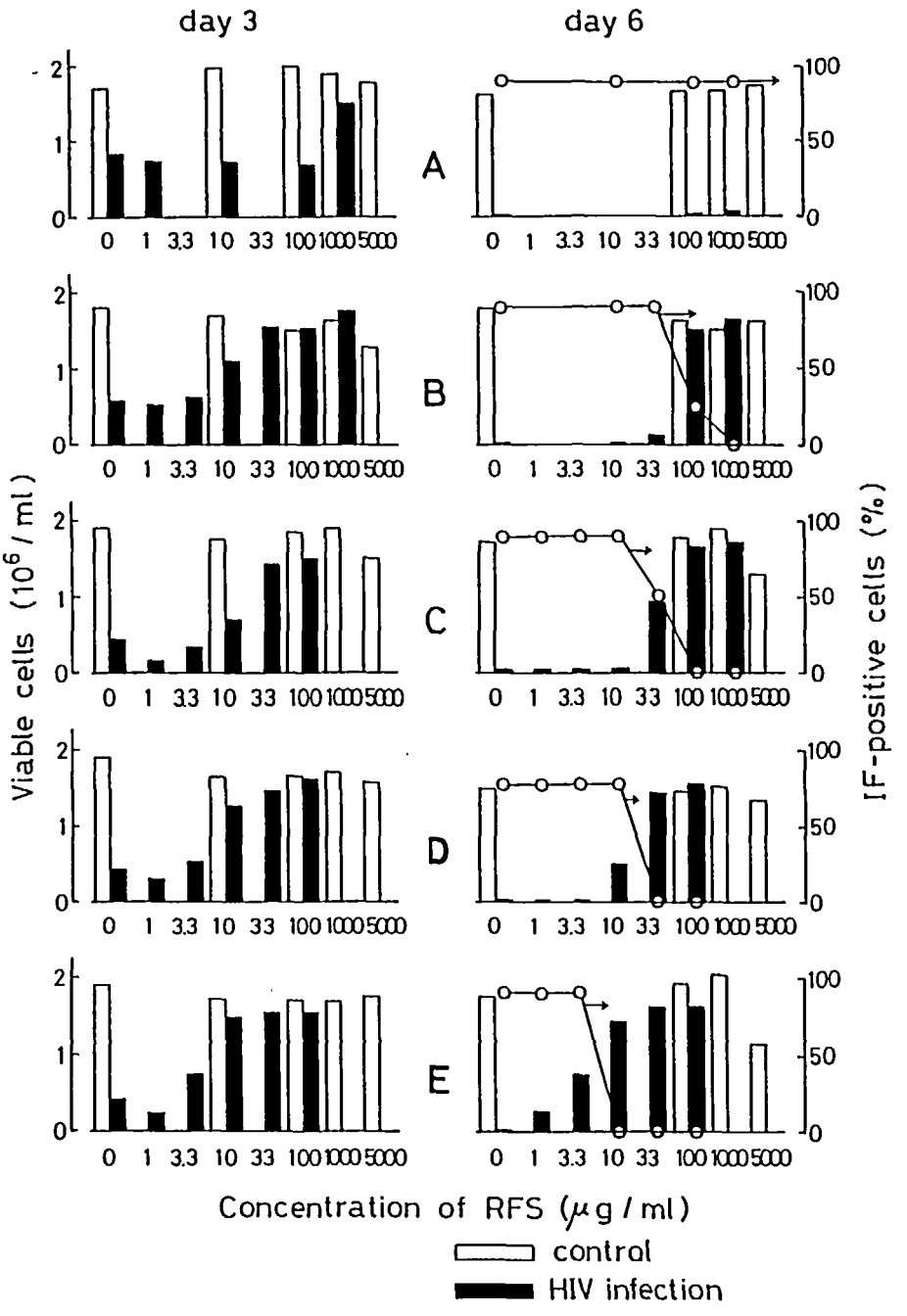

FIG. 2. Effects of RFS on cell growth, HIV-induced cytopathic effects, and percentage of immunofluorescence-positive cells. A: DS=0.92; B: DS=1.28; C: DS=1.48; D: DS=1.74; E: DS=1.80.

I.[11,14] The number of viable cells and the percentage of viral antigen-positive cells were determined by the trypan blue dye exclusion method and indirect immunofluorescence method, respectively, on the 3rd and 6th days after HIV infection.

As shown in FIG. 2 (open bars), all RFSs did not suppress the cell growth at less than 1000 μg/mL. At a concentration as low as 10 μg/mL, RFS no. 10, which has the highest DS of 1.80, effectively protected MT-4 cells from destruction by HIV infection (FIG. 2E). So far, lentinan sulfate and curdlan sulfate exihibited the highest anti-HIV activity, that is, those sulfated polysaccharides concentration of 3.3 μg/mL.[12,16] On the other hand, RFSs, which have a smaller number of sulfate groups per ribose unit, did not protect MT-4 cells from HIV-induced cytopathic effects at any concentration (FIG. 2A) or protected MT-4 cells at higher concentrations (FIG. 2B - D). Effect of the degree of sulfation on anti-HIV activity is depicted in FIG. 3. Where the logarithm of the reciprocal of minimum concentration for effective protection is plotted against DS, a straight line is obtained, indicating that higher substitution of RFS causes much higher anti-HIV activity. For example, RFS with DS of 1.80 inhibited the HIV-induced cytopathic effects at a concentration 100-fold lower than that of RFSs with DS of 1.19 to 1.36.

Sulfated polysaccharides such as lentinan sulfate, dextran sulfate, and RFS are known to bind not only to the virus glycoprotein (gp 120) but also to other proteins in the blood. In this study, the attention was focused on an interaction between RFS and blood coagulation factors. RFS with DS of more than 1.2 had anticoagulant activity which increased linearly with increasing DS, as shown in FIG. 4 and TABLE 2. Since RFS with higher DS showed higher anticoagulant activity like dextran sulfate, it can be suggested that the interaction between RFS and blood coagulation factors is of ionic character based on electrostatic attraction between negatively charged sulfate groups of the polysaccharide and positively charged portions in the proteins.

Compared with the anticoagulant activity, the anti-HIV activity depended remarkably on the degree of substitution of the sulfated polysaccharide. Therefore, it is assumed that ribofuranan sulfates having more sulfate groups might react more strongly and selectively with HIV protein (gp 120) than with blood coagulation proteins.

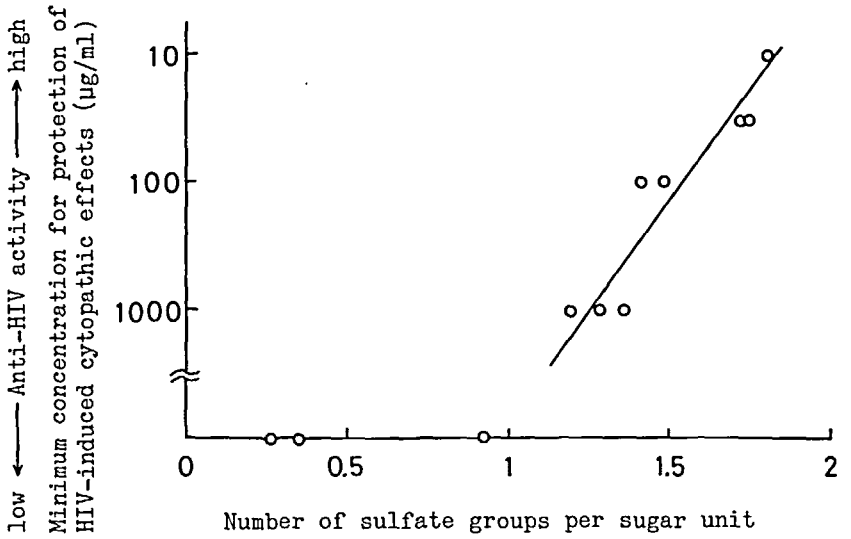

FIG. 3. Relationship between degree of sulfation and the anti-HIV activity.

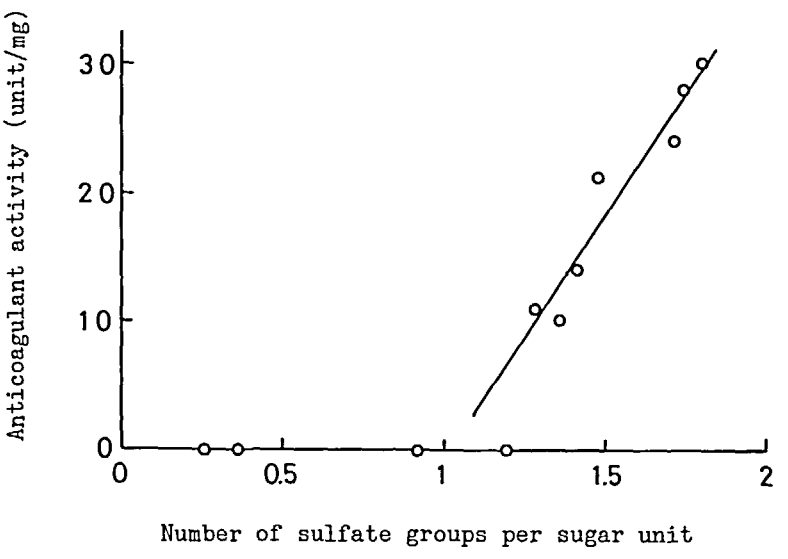

FIG. 4. Effect of degree of sulfation on the anticoagulant activity.

TABLE 2. Biological Activities of Ribofuranan Sulfate

No.	S-content (%)	DS[a]	$\bar{M}_n$ ($\times 10^3$)	Anti-HIV[b] (mg/ml)	A. A.[c] (U/mg)
1	4.85	0.26	7.0	>1000	0
9	6.35	0.36	6.9	>1000	0
2	12.03	0.92	7.9	>1000	0
3	13.91	1.19	6.0	1000	0
4	13.79	1.28	7.9	1000	11
5	13.95	1.36	6.6	1000	10
6	15.07	1.41	7.5	100	14
7	15.01	1.48	8.1	100	21
8	15.66	1.71	7.1	33	24
11	16.10	1.74	7.3	33	28
10	16.53	1.80	7.8	10	30

a. Number of sulfate groups per sugar unit.

b. Minimum concentration for protection of HIV-induced cytopathic effects.

c. Anticoagulant activity.

EXPERIMENTAL

Ribofuranan Sulfate (RFS). 1,4-Anhydro-2,3-di-*O*-benzyl-α-D-ribopyranose was polymerized with boron trifluoride etherate as catalyst to give a stereoregular 2,3-di-*O*-benzyl-(1→5)-α-D-ribofuranan.[6] After debenzylation with sodium in liquid ammonia, the obtained OH-free (1→5)-α-D-ribofuranan was sulfated with piperidine *N*-sulfonic acid in DMSO[15] under various conditions as summarized in TABLE 1. The reaction mixture was neutralized by saturated $NaHCO_3$ solution in an ice bath, and poured into acetone. Then crude ribofuranan sulfate was separated with centrifugation, washed with acetone, and dialyzed overnight with running distilled water. RFS was freeze-dried from water. RFS was moisture sensitive and was stored at -20 °C.

Anti-HIV Assay. Anti-HIV effect of RFS was assayed by measuring the decrease in the number of viable cells and indirect immunofluorescence method using MT-4 cells.[17]

Anticoagulant Activity. Anticoagulant activity of RFS was determined by use of bovine serum according to a modification of the United States Pharmacopoeia method.[5] Dextran sulfate (Meito Sangyo NC-1032) was used as a control.

Measurements. NMR spectra of RFS were recorded on a JEOL GX-270 spectrometer in deuterium oxide using sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) as internal standard. Number-average molecular weights of RFSs were estimated by gel permeation chromatography (columns, Tosoh TSK gel; eluent, 66.7 mM phosphate buffer, pH 6.86) using a series of standard dextrans as reference. Elemental analysis was performed by Toray Research Center, INC.

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