Synthesis of Regioselective Substituted Curdlan Sulfates with Medium Molecular Weights and Their Specific Anti-HIV-1 Activities

Ying Gao,[†] Akiko Fukuda,[†] Kaname Katsuraya,[†] Yutaro Kaneko,[‡] Toru Mimura,[‡] Hideki Nakashima,[§] and Toshiyuki Uryu*,[†]

Institute of Industrial Science, University of Tokyo, Roppongi, Minato-ku, Tokyo 106, Japan, Ajinomoto Co., Kyobashi, Chuo-ku, Tokyo 104, Japan, and Yamanashi Medical University, Shimo-kato, Tamaho-cho, Yamanashi 409-38, Japan

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ABSTRACT: To investigate the relationship between the structure of curdlan sulfate with β-[1→3]-D-glucan as main chain and the anti-HIV activity, three regioselective substituted curdlan sulfates (CS's), i.e., CS having sulfate groups at all C6 and some C2 positions (62S), CS having sulfate groups at all C4 and some C2 positions (42S), and CS having sulfate groups at some C6, C4, and C2 positions (642S), were synthesized. Their structures were characterized by ^{13}C NMR spectroscopy. These curdlan sulfates had number average molecular weights in the range 6.2×10^3 to 10.8×10^3. For the various curdlan sulfates, anti-HIV activities and anticoagulant activities were assayed in vitro. It was revealed that these curdlan sulfates showed high anti-HIV activities in the EC_{50} range 0.04−0.4 μg/mL when the degree of sulfation was more than 1.3, and low cytotoxicities as well as low blood anticoagulant activities. Furthermore, it was found that the anti-HIV activity of curdlan sulfates depended on the degree of sulfation but not on the position of sulfate groups.

Introduction

Intensive efforts are underway worldwide to develop chemtherapeutic agents effective against human immunodeficiency virus (HIV). Antiviral nucleoside analogues such as azidothymidine (AZT) (zidovudine), 2′,3′-dideoxyinosine (ddI) (didanosine), 2′,3′-dideoxycytidine (ddC) (zalcitabine), 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T) (stabudine), and 2′-deoxy-3′-thiacytidine (3TC) (lamivudine) have been clinically used to treat the patients suffering from acquired immunodeficiency syndrome (AIDS) and AIDS-related complexes.[1−3] However, since they induce serious side effects and the appearance of drug-resistant viruses,[4] it is necessary to search for a new anti-AIDS agent. Recently, several protease inhibitors have also been used as AIDS drugs.[5]

Until now, remarkable attention has been focused on sulfated polysaccharides such as dextran sulfate, heparin, carrageenan, pentasan polysulfate, and sulfated dextran derivatives which have been proven to be potent inhibitors of HIV-1 replication in vitro.[6−10] Of these, dextran sulfate was reported to have an anti-HIV activity early,[11,12] but it cannot be clinically utilized as an AIDS drug because of its high anticoagulant activity, which is regarded as a side effect for an anti-HIV agent.[13] We have synthesized various sulfated poly- and oligosaccharide derivatives showing potent anti-HIV activity.[14−17] Among them, curdlan sulfate exhibited a high anti-HIV activity of $EC_{50} = 0.17$ μg/mL to completely inhibit HIV infection at a concentration as low as 3.3 μg/mL in vitro but possessed low anticoagulant activity. The phase I/II test of curdlan sulfate was performed in the United States starting in December 1992. It was reported that intravenous administration of curdlan sulfate led to a dosage-related increase in the number of CD4 lymphocytes, which was believed to indicate an effective treatment on AIDS patients.[18] Investigations on the anti-HIV mechanism of curdlan sulfate and its bioactivity have been carried out. The results revealed that curdlan sulfate interferes with the membrane fusion process during HIV-1 infection by the interaction of curdlan sulfate with both the continuous epitopes on the V3 loop and the discontinuous CD4 binding site of gp120, and prevents the virus from penetrating into target cells.[19] In addition, the anticoagulant activity intrinsic to sulfated polysaccharides, which was regarded as a side effect, was found to depend on the method of sulfation.[20]

In the present paper, to study the relation between the anti-HIV activity and the position of sulfate groups in the curdlan sulfate, regioselective substituted curdlan sulfates (CS's), such as CS having sulfate groups at all C6 and some C2 positions (62S), CS having sulfate groups at all C4 and some C2 positions (42S), and CS having sulfate groups at some C6, C4, and C2 positions (642S), were prepared. Their anti-HIV activities and anticoagulant activities were estimated in vitro. Structure analysis of curdlan sulfate was carried out by NMR spectroscopy.

Experimental Section

Materials and General Methods. Commercial curdlan ($\bar{M}_n = 8.9 \times 10^4$, Wako Pure Chemical Ind., Ltd.), the sulfur trioxide−pyridine complex, and pivaloyl chloride (Tokyo Kaisei Kogyo Co., Ltd.) were used without further purification. Dimethyl sulfoxide (DMSO) and pyridine were distilled before use. ^{13}C NMR spectra were recorded with a JEOL LA400 spectrometer working at 100 MHz. Samples were measured on D_2O or DMSO-d_6 solutions at 37 °C and room temperature using sodium 4,4-dimethyl-4-sila-1-pentanesulfonate (DSS) as an internal standard. Molecular weights of curdlan sulfates were measured by aqueous-phase gel permeation chromatography (Columns: TS-gel G2000SW, G3000SW, and G4000SW; 7.6 mm × 600 mm × 3. Eluent: phosphate buffer solution (pH = 7.02)) using standard pullulans as reference. Molecular weights of curdlans were obtained by measuring the molecular weight of acetylated curdlan by means of GPC in chloroform solutions.

* Corresponding author.
† University of Tokyo.
‡ Ajinomoto Co.
§ Kamanashi Medical University.
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Hydrolysis. To a suspension of curdlan ($\bar{M}_n = 8.9 \times 10^4$) (1.0 g) in 15 mL of DMSO was added 0.2 M H_2SO_4 (2.5 mL). The mixture was heated to 100 °C, and then stirred vigorously at 100 °C for 1 h in nitrogen atmosphere. After the reaction mixture was cooled to room temperature, acetone (200 mL) was added until precipitates appeared. The precipitate was collected by centrifugation and then washed with acetone three times. The product was dried in vacuum to give curdlan with a $\bar{M}_n$ of 9.2×10^3.

Pivaloylation. Pivaloyl chloride (1.5 mL) was added gradually to the suspension of curdlan (1.0 g) in pyridine (40 mL). The suspension was stirred vigorously for 1 h at 80 °C. Then, pyridine was evaporated at a reduced pressure. The residue was washed with H_2O (300 mL) three times, and the precipitate was collected by centrifugation. An off-white powdery 6-pivaloylcurdlan (6-piva-curdlan) was obtained by freeze-drying from water. Yield: 83%.

Sulfation. Method A. A SO_3−pyridine complex (4.0 g) was added to a 6-piva-curdlan (1.0 g) solution in DMSO (20 mL). The mixture was kept at room temperature for 75 min. Then, it was neutralized with saturated Na_2CO_3 solution and dialyzed against deionized water overnight. The dialyzate was concentrated at reduced pressure and then freeze-dried from water to give 6-piva-curdlan sulfate.

Method B. Low molecular weight curdlan was sulfated according to the above procedure instead of 6-piva-curdlan.

Depivaloylation. 6-Piva-curdlan sulfate (1.0 g) was dissolved in a 3% NaOH aqueous solution (100 mL). The solution was stirred for 4 h at room temperature, followed by dialyzing against deionized H_2O for 2 days. After the dialyzate was evaporated at lower than 50 °C, the concentrated solution was neutralized to pH = 6.8−7.2 by 0.1 N NaOH solution. 6-OH-free curdlan sulfate was obtained by freeze-drying from water.

Anti-HIV Assay. The anti-HIV activity of regioselective substituted curdlan sulfates was assayed by the MTT method.[21] MT-4 cells (a human T4-positive cell line carrying human T-lymphotropic virus type I) were infected with HIV-$1_{HTLV-IIIB}$ at the multiplicity of 0.01, and HIV-1- and mock-infected MT-4 cells were incubated in the presence of various concentrations of the test material for 5 days at 37 °C in a CO_2 incubator. The viability of both HIV-1- and mock-infected cells was assayed spectrophotometrically via the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT). The anti-HIV activity is represented as the EC_{50}, which denotes the concentration of the test material inhibiting 50% infection of MT-4 cells from HIV. The cytotoxitity CC_{50} was determined by the 50% cytotoxic concentration of the test material on the MT-4 cell.

Anticoagulant Activity. Anticoagulant activity was evaluated by a modified United States Pharmacopoeia method using bovine plasma.[22] Dextran sulfate with an anticoagulant activity of 21.0 unit/mg was used as a reference sample.

Results and Discussion

Synthesis and Structure Analysis. Formerly, curdlan sulfates were prepared by sulfation of high molecular weight curdlan which was subjected to degradation during sulfation. In this study, regioselective substituted curdlan sulfates with medium molecular weights were synthesized by the two routes.

Curdlan sulfated predominately at the C6 position was obtained according to the synthetic route represented in Scheme 1. First, in order to obtain curdlan sulfate having medium molecular weight, medium molecular weight curdlan was sulfated. For this purpose, a commercial high molecular weight curdlan was hydrolyzed with sulfuric acid to give medium molecular weight curdlan (small curdlan) having a $\bar{M}_n$ of 9200 in 90% yield.

The small curdlan was sulfated with a sulfur trioxide−pyridine complex. The results of the sulfations are summarized in Table 1. The degree of sulfation estimated by elemental analysis was in the range 0.66−1.55. Their number average molecular weights were 8.8

Scheme 1. Synthetic Route of Curdlan Sulfated at All C6 and Some C2 Positions

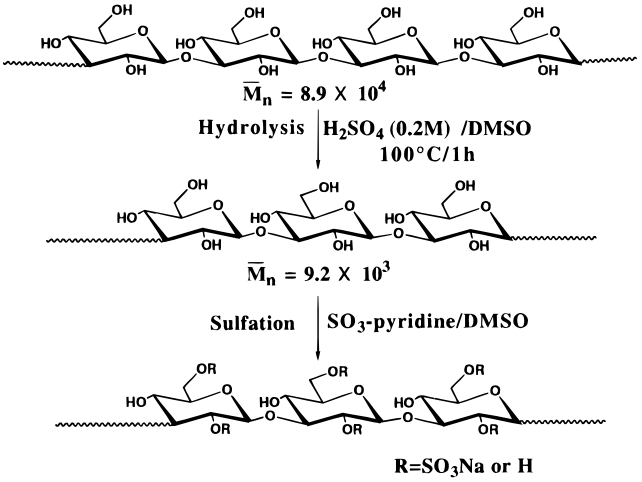


Table 1. Sulfation of Curdlan[a]

no.	curdlan (g)	SO_3−py[b] (equiv)	time (min)	yield (g)	$\bar{M}_n$[c] ($\times 10^3$)	DS[d]	elem anal. C	H	S
1	1.0	4.0	60	1.0	8.8	1.55	20.8	3.8	14.3
2	1.0	3.0	60	1.1	10.8	1.52	20.7	3.7	14.0
3	1.0	2.0	90	0.4	*e*	0.66	31.5	3.7	6.6

[a] Small curdlan ($\bar{M}_n = 9200$) stirred in DMSO at room temperature. [b] The sulfur trioxide−pyridine complex was equivalent to a glucose unit. [c] Determined by GPC. [d] Calculated by elemental analysis. [e] Failed to measure for low solubility caused by low degree of sulfation.

$\times 10^3$ and 10.8×10^3, respectively, when curdlan ($\bar{M}_n = 9200$) reacted with 4.0 and 3.0 equiv of SO_3−pyridine (nos. 1 and 2). The molecular weight of curdlan sulfate decreased with increasing amount of SO_3−pyridine because of the degradation of curdlan during sulfation. In the case of 2.0 equiv of the sulfating agent, the molecular weight of sample no. 3 could not be measured because of the insolubility in water due to the low degree of sulfation.

The structure analysis of curdlan sulfate was performed by ^{13}C NMR spectroscopy. Figure 1a shows the ^{13}C NMR spectrum of curdlan sulfate having the degree of sulfation 1.55 (no. 1). Assignment of peaks was determined by reference to the previous works.[16,20,23] The C6 absorption of curdlan, which appeared at 63 ppm, shifted completely to around 70 ppm after sulfation. Thus, all the hydroxyl groups of curdlan at the C6 position were sulfated, when it was sulfated with SO_3−pyridine. This phenomenon was consistent with the previous result using piperidinesulfonic acid as sulfating agent.[20] The C2 absorption of curdlan shifted partially from 76 to 82 ppm, indicating that the C2 position was partially sulfated. Since sulfation of the C4 position was much less than that of the C6 and C2 positions, the degree of sulfation was negligibly small.

Curdlans sulfated at all C4 and some C2 positions (42S) and at some C6, C4, and C2 positions (642S) were prepared by the synthetic route shown in Scheme 2. Protection of the 6-hydroxyl group in curdlan was performed with pivaloyl chloride. The results of the pivaloylations are shown in Table 2. Protection of the 6-hydroxyl group of curdlan with a pivaloyl group was accomplished at 80 °C to give 6-pivaloylated curdlan (6-piva-curdlan). The degree of pivaloylation calculated by means of ^{13}C NMR spectroscopy ranged from 13% to 100% by controlling of the quantity of pivaloyl chloride and the reaction time.

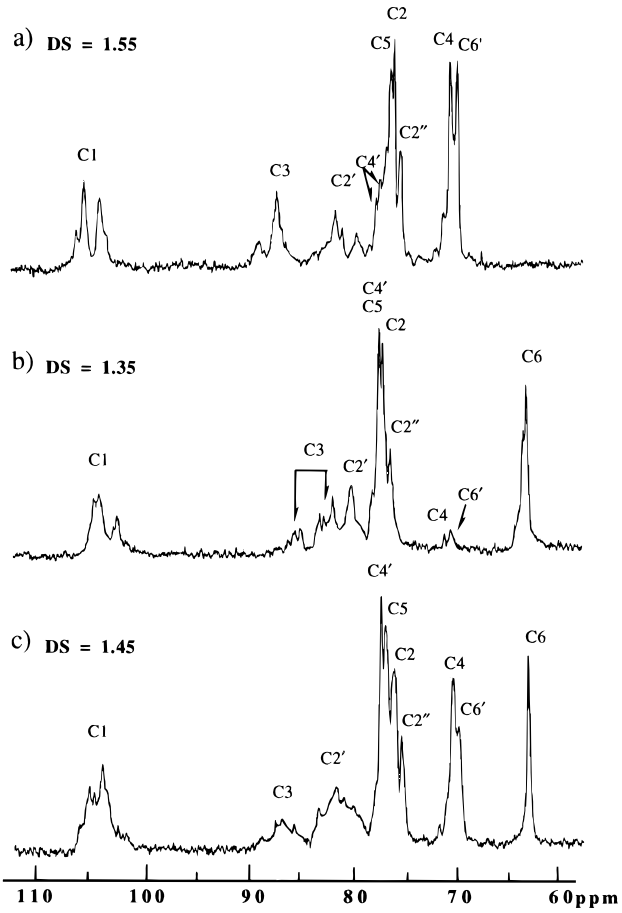
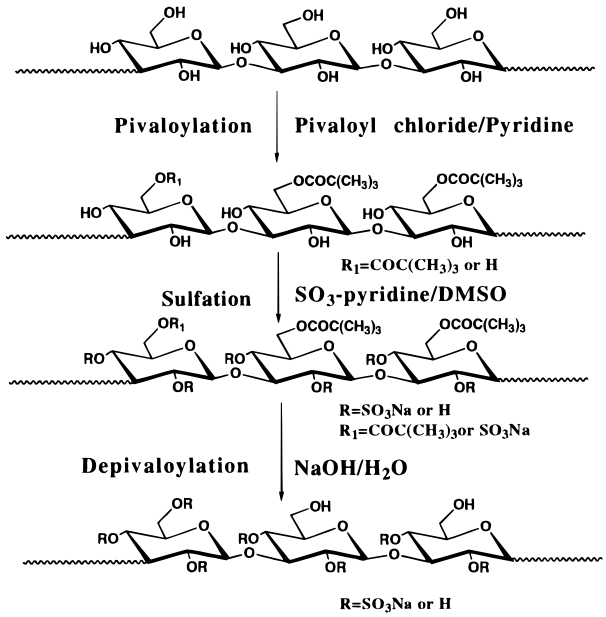


Figure 1. 100-MHz ^{13}C NMR spectra of sulfated curdlans (a) 62S, (b) 42S, and (c) 642S (D_2O as solvent at 37 °C).

Scheme 2. Synthetic Route of Curdlan Sulfated at All C4 and Some C2 Positions (42S) and Some C6, C4, and C2 Positions (642S)



The structure of 6-piva-curdlan was confirmed by ^{13}C NMR spectroscopy. As shown in Figure 2, the C6 absorption of 6-piva-curdlan was observed at 65 ppm, while that of the original curdlan appeared at 63 ppm. Since the C6 peak at 63 ppm disappeared and shifted completely to 65 ppm, the degree of pivaloylation was estimated to be 100% by ^1H NMR measurement after acetylation of the remaining hydroxyl groups. The C5 peak shifted upfield because of the substitution at the 6-hydroxyl group. Pivaloylation of hydroxyl groups at the C2 and C4 positions was not observed.

Table 2. Protection of C6 Hydroxyl Group of Curdlan with Pivaloyl Chloride[a]

no.	curdlan (g)	pivaloyl chloride mL	pivaloyl chloride equiv[c]	temp (°C)	time (min)	yield (g)	D_{piv}[b]
1	1.0	3.7	5.0	rt	>1000	*d*	0
2	1.0	0.5	0.7	80	60	0.9	0.13
3	1.0	1.1	1.5	80	60	0.7	0.37
4	1.0	1.5	2.0	80	60	1.2	0.83
5	1.0	2.2	3.0	80	30	1.1	0.95
6	1.0	3.5	4.0	80	45	1.0	1.00

[a] Reacted in pyridine. [b] Degree of pivaloylation was determined by ^{13}C NMR spectroscopy. [c] Equivalent to a glucose unit. [d] Failed to react.

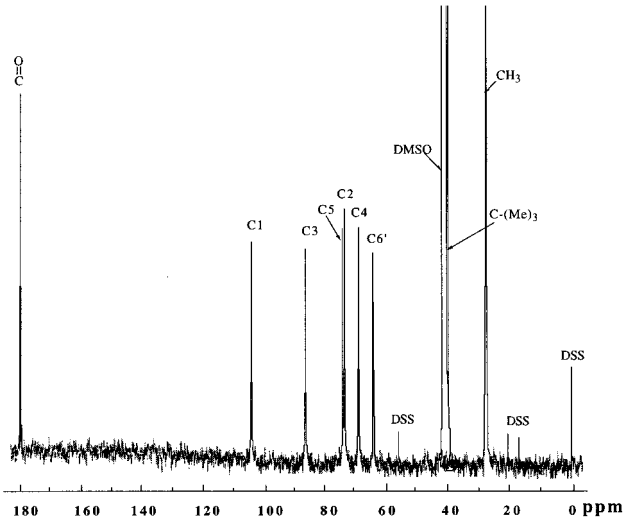


Figure 2. 100-MHz ^{13}C NMR spectrum of 6-piva-curdlan (DMSO-d_6 as solvent at 25 °C).

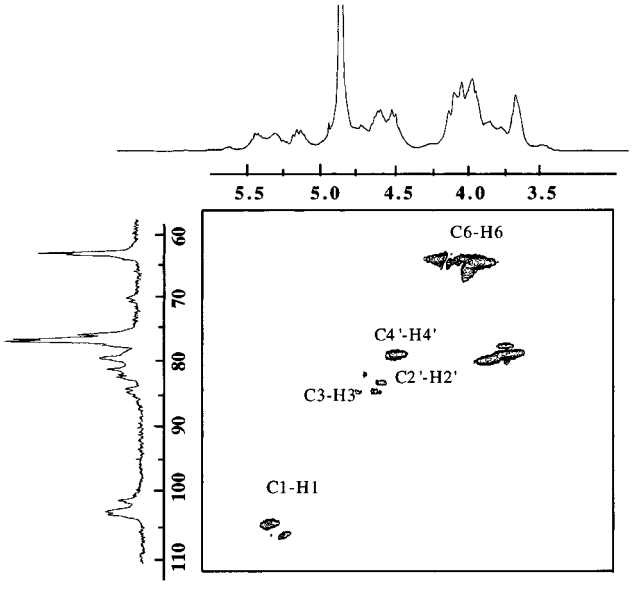


Figure 3. 2D $^{13}C-^1H$ cosy-fg NMR spectra of curdlan sulfate (42S) (D_2O as solvent at 37 °C).

6-Piva-curdlan was sulfated with a sulfur trioxide–pyridine complex in DMSO at room temperature. By depivaloylation of 6-piva-curdlan sulfate in a 3% sodium hydroxide solution, curdlans sulfated at all C4 and some C2 positions (42S) and some C6, C4, and C2 positions (642S) were prepared. Results of the sulfations and depivaloylations are summarized in Table 3. These curd-

Table 3. Sulfation of 6-Pivaloylcurdlan with a SO_3−Pyridine Complex and Depivaloylation in Aqueous NaOH Solution[a]

no.	6-PCD[b] *D*piv[g] (%)	SO_3−py[c] (equiv)	time (min)	yield (g)	DS[d]	assignment of sulfate[e] at C6	at C4	at C2	$\bar{M}_n$[f] ($\times 10^3$)	$\bar{M}_w/\bar{M}_n$	elem anal. C	H	S
42S													
1	100	6.2	75	0.63	1.35	0	1.0	0.35	8.0	2.95	21.8	4.07	13.1
2	100	5.5	75	0.42	1.35	0	1.0	0.35	9.1	3.07	21.6	4.15	13.0
3	100	4.7	90	0.90	1.39	0	1.0	0.39	8.6	3.30	21.3	4.30	13.2
642S													
4	95	4.6	70	0.65	1.51	0.05	*h*	*h*	9.3	3.15	20.6	3.90	13.9
5	83	4.4	70	0.45	1.36	0.17	*h*	*h*	10.2	4.23	23.8	4.00	14.4
6	37	3.6	70	0.60	1.45	0.63	*h*	*h*	7.7	2.70	20.3	4.07	13.1
7	13	3.6	90	0.74	1.79	0.87	*h*	*h*	7.1	2.52	19.3	2.90	15.4

[a] 6-Piva-curdlan (1.0 g) was sulfated in DMSO at room temperature. [b] 6-Piva-curdlan. [c] The SO_3−pyridine complex was equivalent to a glucose unit. [d] The number of sulfate groups per sugar unit in curdlan sulfate was determined by elemental analysis. [e] Assigned by combination to ^{13}C NMR and elemental analysis. [f] Determined by GPC. [g] Degree of pivaloylation. [h] Existing but difficult to calculate.

Table 4. Anti-HIV Activity of Curdlan Sulfates with Medium Molecular Weights

no.	proportion of sulfate group[a] at C6	at C4	at C2	$\bar{M}_n$[b] ($\times 10^3$)	S (%)	DS[c]	anti-HIV activity EC_{50}[d] (μg/mL)	CC_{50}[e] (μg/mL)	SI[f] (CC_{50}/EC_{50})
62S									
1	1.0	*g*	0.55	8.8	14.3	1.55	0.04	>1000	>26920
2	1.0	*g*	0.52	10.8	14.0	1.52	0.16	>1000	>6250
42S									
3	0	1.0	0.35	8.0	13.1	1.35	0.25	>1000	>3990
4	0	1.0	0.35	9.1	13.0	1.35	0.05	936	>17240
5	0	1.0	0.39	8.6	13.2	1.39	0.16	>1000	>6250
6	0	0.9	0.24	7.8	11.9	1.14	1.04	>1000	>960
7	0	0.8	0.24	6.2	11.4	1.04	3.08	>1000	>320
642S									
8	0.87	*h*	*h*	7.1	15.4	1.79	0.19	>1000	>5260
9	0.63	*h*	*h*	7.7	13.1	1.45	0.22	>1000	>2550
10	0.17	*h*	*h*	10.2	14.4	1.36	0.19	>1000	>5260
11	0.05	*h*	*h*	9.3	13.9	1.51	0.32	>1000	>3120
CS[i]					14.1		0.17	>1000	>6070

[a] Determined by ^{13}C NMR and elemental analysis. [b] Measured by GPC. [c] Degree of sulfation. [d] Anti-HIV activity: drug concentration effective for 50% inhibition of virus infection in 5-day HIV-infected MT-4 cell culture. [e] Cytotoxic effect: drug concentration for 50% cytotoxicity in 5-day MT-4 cell culture. [f] Selectivity index. [g] The degree of sulfation at C4 was negligibly small. [h] Existing but difficult to state. [i] Curdlan sulfate with a molecular weight of 7.9×10^4 was used for measure of anti-HIV as reference.

lan sulfates had number average molecular weights of 7.1×10^3 to 10.2×10^3. Assignment of the sulfate groups at each hydroxyl position was carried out by the combination of ^{13}C NMR spectroscopy and elemental analysis.

Figure 1b shows the ^{13}C NMR spectrum of curdlan sulfate (no. 1: DS = 1.35). The peaks were identified by both two-dimensional NMR measurement (Figure 3) and reference to the previous works.[16,20,23] The C6 absorption of curdlan appeared at 63 ppm. Since the absorption around 70 ppm due to sulfated C6 carbons was very small, it was revealed that the 6-hydroxyl group was almost free ($DS_6 = 0$). The C4 absorption of curdlan shifted from 71 to 77 ppm, indicating that the 4-hydroxyl group was sulfated completely ($DS_4 = 1.0$). It was found that the C3 absorption showed a large upfield shift, since the vicinal C4 hydroxyl group was sulfated. The degree of substitution at the C2 position was calculated by the combination of the peak intensity and the elemental analysis to be $DS_2 = 0.35$. It may be concluded that when a curdlan 100% pivaloylated at the C6 position was sulfated and then depivaloylated, the resulting sulfated curdlan had sulfate groups at all C4 positions and some C2 positions as well as free hydroxyl groups at the C6 position (nos. 1−3). The synthesis of this kind of regioselective substituted curdlan sulfate would be impossible, if the primary 6-hydroxyl group of curdlan had not been protected. Because the 6-hydroxyl group is a primary alcoholic OH, the sulfation occurs more readily at the 6-OH than at the secondary 2- and 4-OH's.

In addition, assignments of the sulfate groups at the C2, C4, and C6 positions were controlled by the degree of pivaloylation at the 6-hydroxyl group of curdlan (nos. 4−7). As shown in Figure 1c (no. 6: DS = 1.45), hydroxyl groups at each position in curdlan were sulfated partially. The degree of sulfation at C6 ($DS_6 = 0.63$) was calculated from the degree of pivaloylation. But, it was difficult to exactly estimate the degree of sulfation at the C2 and C4 positions because of overlapping of the carbon signals.

Anti-HIV Activity and Anticoagulant Activity. The anti-HIV activity of various curdlan sulfates was assayed by the MTT method using the MT-4 cell line and the $HIV_{HTLV-IIIB}$ strain to give the EC_{50} value, which is defined as a drug concentration effective for 50% inhibition of the virus infection to MT-4 cells. Anti-HIV in vitro activities of the curdlan sulfates are shown in Table 4. In spite of the medium molecular weights, all curdlan sulfates exhibited high anti-HIV activities represented by low EC_{50} values ranging from 0.04 to 0.4 μg/ml when the degree of sulfation was more than 1.3. In addition, the cytotoxicities of the curdlan sulfates were low, as shown by CC_{50} values larger than 1000 μg/mL.

To examine the dependence of the anti-HIV activity on the chemical structure of curdlan sulfate, the EC_{50} values were plotted against the degree of sulfation as well as the different positions of the sulfate groups (Figure 4A). It was revealed that it is necessary to have a high degree of sulfation to obtain highly anti-HIV active curdlan sulfate, as was the case for dextran sul-

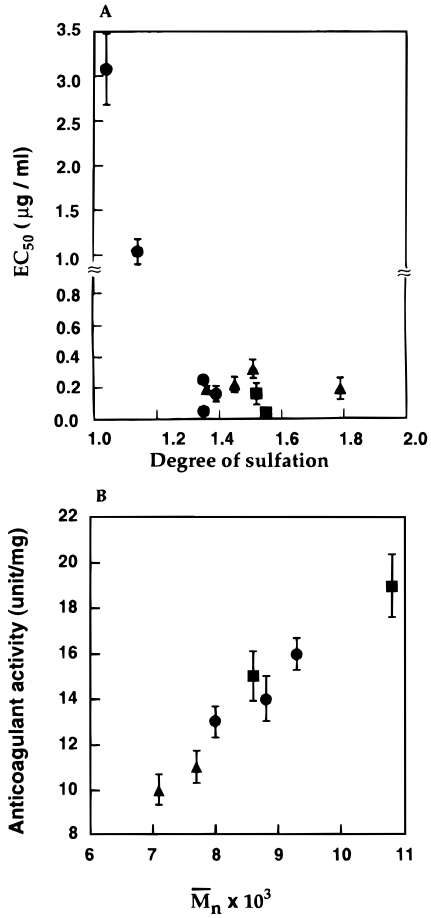


Figure 4. (A) Dependence of the anti-HIV activity EC_{50} of curdlan sulfate on both degree of sulfation and position of sulfate groups (●, 42S; ■, 62S; and ▲, 642S) and (B) dependence of the anticoagulant activity of curdlan sulfate on both molecular weight and position of sulfate groups (●, 42S; ■, 62S; and ▲, 642S).

Table 5. Anticoagulant Activity of Curdlan Sulfate

	proportion of sulfate group[a]						
no.	at C6	at C4	at C2	$\bar{M}_n$[b] ($\times 10^3$)	S (%)	DS[c]	AA[d] (unit/mg)
1	0.85	*e*	*e*	7.1	15.4	1.79	10
2	0.67	*e*	*e*	7.7	13.1	1.45	11
3	0	1.0	0.35	8.0	13.1	1.35	13
4	1.0	*f*	0.55	8.8	14.3	1.55	14
5	0	1.0	0.39	8.6	13.2	1.39	15
6	0	1.0	0.51	9.3	13.9	1.51	16
7	1.0	*f*	0.52	10.8	14.0	1.52	19
CRDS[g]				31.0		2.60	35

[a] Determined by ^{13}C NMR and elemental analysis. [b] Determined by GPC. [c] Degree of substitution was calculated by elemental analysis. [d] Anticoagulant activity; commercial dextran sulfate having 20.6 unit/mg as reference. [e] Existing but difficult to state. [f] Degree of sulfation at C4, much less than that of the C2 and C6 positions, was ignored. [g] Reference 20.

fate.[24] In addition, the anti-HIV activity of the curdlan sulfate did not depend on the positions of the sulfate groups.

We have reported that the anticoagulant activity of curdlan sulfate, calculated from an activated partial thromboplastin time (APTT) using heparin as reference,[15,25] is almost equivalent to that determined by a modified United States Pharmacopoeia method using dextran sulfate as reference.[20] Therefore, we evaluated the anticoagulant activity of the regioselective substituted curdlan sulfates by the modified United States Pharmacopoeia method. The results are shown in Table 5. It has been reported that curdlan sulfates obtained by the SO_3−pyridine method had higher anticoagulant activities than those obtained by the piperidine sulfonic acid method.[20] In the present study, however, curdlan sulfates prepared by SO_3−pyridine exhibited low anticoagulant activities, probably because the curdlan sulfates had medium molecular weights. Furthermore, as shown in Figure 4B, it was revealed that the anticoagulant activity of curdlan sulfates had a tendency to decrease with decreasing molecular weight in the narrow molecular weight range of 7×10^3 to 11×10^3, although there was no dependence of the anticoagulant activity on the position of the sulfate group.

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