Synthesis and in Vitro Inhibitory Effect of L-Glycosyl-Branched Curdlan Sulfates on AIDS Virus Infection

Takashi Yoshida

Division of Biological Sciences, Graduate School of Science, Hokkaido University, Kita-ku, Sapporo 060, Japan

Yuichi Yasuda and Toshiyuki Uryu*

Institute of Industrial Science, University of Tokyo, Roppongi, Minato-ku, Tokyo 106, Japan

Hideki Nakashima and Naoki Yamamoto

Tokyo Medical and Dental University School of Medicine, Yushima, Bunkyo-ku, Tokyo 113, Japan

Toru Mimura and Yutaro Kaneko

Ajinomoto Company, Inc., Chuo-ku, Tokyo 108, Japan

Received May 16, 1994; Revised Manuscript Received August 3, 1994®

ABSTRACT: Such natural and nonnatural sugars as D- and L-glucoses and D- and L-mannoses were reacted with curdlan to form branched curdlans, respectively. These structures were analyzed by means of ^{13}C NMR spectroscopy and methylation analysis. The branched curdlans were sulfated with piperidine-*N*-sulfonic acid in DMSO to give branched curdlan sulfates. It was revealed that these branched sulfates had high anti-AIDS virus activities in the EC_{50} range of 0.3–1.2 μg/mL in vitro using MT-4 cells and exhibited low cytotoxicities as well as low anticoagulant activities. Furthermore, for L-glycosyl-branched curdlan sulfates, retention times in rat in vivo calculating from their anticoagulant activities were a few hours.

Introduction

Since the discovery of acquired immunodeficiency syndrome (AIDS) in 1981[1] and the isolation of the causative virus called human immunodeficiency virus (HIV) in 1983,[2] several antiviral drugs have been developed and then clinically tried to cure AIDS. Azidothymidine (AZT) and its related compounds such as dideoxyinosine (ddI) and dideoxycytidine (ddC) showed clinical improvements for the AIDS patients. These modified nucleosides have an action mechanism as a potent reverse transcription inhibitor to terminate virus DNA replication. However, these drugs in clinical trials caused serious side effects and an AZT-resistant virus appeared by a long-term treatment.[3] In addition, development of an effective AIDS vaccine is a difficult task because of a multiplicity of HIV strains.[4]

A branched 1,3-β-glucan lentinan has a strong antitumor activity[5] and has been clinically used as an antitumor drug in Japan. Potent antitumor activity of arabinosyl-branched curdlan which was prepared by the branching reaction of a linear polysaccharide curdlan with arabinose–orthoacetates was reported.[6] In general, some natural branched polysaccharides have unique antitumor[7] and antiinflammatory[8] activities. We have successfully synthesized lentian sulfates and curdlan sulfates by sulfation of a naturally occurring branched 1,3-β-glucan, lentian, and a linear 1,3-β-glucan curdlan with piperidine-*N*-sulfonic acid, respectively.[9–12] Lentian sulfates having a sulfur content of 16.2% completely inhibited the infection of the AIDS virus (HIV-1) at concentrations as low as 3.3 μg/mL and showed a moderate anticoagulant activity of 21 units/mg. On the other hand, curdlan sulfate with a sulfur content of 14.4% also exhibited potent anti-AIDS virus activity at 3.3 μg/mL and had a low anticoagulant activity of less than 10 units/mg by using bovine plasma according to a modified procedure of the United States Pharmacopeia.[13] Since the anticoagulant activity of sulfated polysaccharides is regarded as a side effect for the anti-AIDS virus activity, it is worth examining the cause for the difference between a very high activity of the curdlan sulfate and a medium anticoagulant activity of the lentinan sulfate. It has been revealed that the half-life of curdlan sulfates in vivo using rats depended on molecular weights of curdlan sulfates, i.e., 60 min for a curdlan sulfate of 7×10^4 and 180 min for 17×10^4.[11]

It was also found that sulfates of synthetic polysaccharides ribofuranan, ribopyranan, xylofuranan, and dextran completely protected against the infection of the AIDS virus at 3.3–10 μg/mL and were not cytotoxic in vitro.[14–16]

Chemical synthesis of branched polysaccharides is important to elucidate the relation between the biological activity and the polysaccharide structure. In this study, we report the synthesis of branched curdlan sulfates by the branching reaction of curdlan with D- and L-glucosyl and D- and L-mannosyl orthoacetates followed by sulfation of the obtained branched curdlans. The structure of branched curdlans was determined by NMR spectroscopy and methylation analysis. In addition, the dependence of retention time in the blood in vivo on the concentration of L-glycosyl- and L-mannosyl-branched curdlan sulfates is calculated from their anticoagulant activities.

Experimental Section

Characterization. ^{13}C NMR spectra were recorded by a JEOL JMN GX-270 spectrometer working at 67.8 MHz. Samples were measured on a D_2O solution at 37 °C or a DMSO-d_6 solution at 80 °C. Sodium 4,4-dimethyl-4-sila-1-pentanesulfonate (DSS) was used as an internal reference. Specific rotations were taken on a Perkin-Elmer 241 polarimeter in a 1-dm cell (10-cm length). Molecular weights were estimated by aqueous-phase gel permeation chromatography (column: Toyo Soda TSK-gel, G2000SW, G3000SW, G4000SW, 7.6 mm × 600 mm × 3; eluent,

® Abstract published in *Advance ACS Abstracts*, September 15, 1994.

0024-9297/94/2227-6272$04.50/0

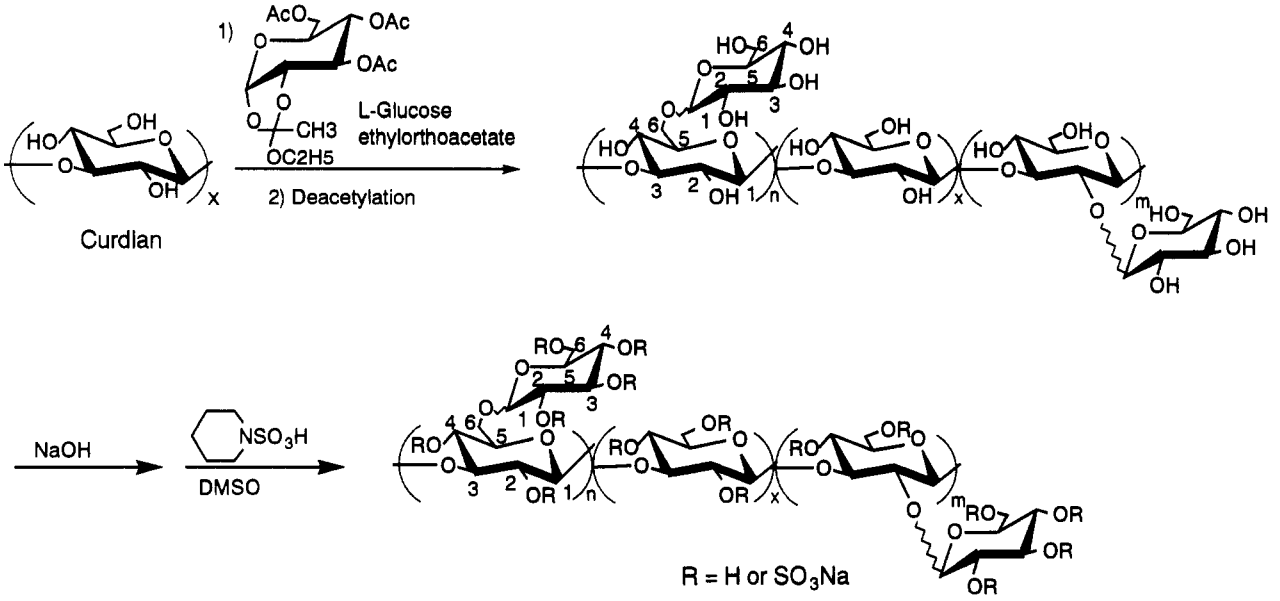

Figure 1. Synthesis of L-glucosyl-branched curdlan sulfate.

66.7 mmol of phosphate buffer, pH = 6.86) using pullulan (Shodex Standard P-82) as a reference. GC-MS spectra were measured at 70 eV with a JEOL JMS DX-300 spectrometer (column: HP OV-1, 0.31 mm × 25 m).

Materials. Curdlan ($\bar{M}_n$ = 8.9 × 10^4) was purchased from Wako Pure Chemical Industries. Piperidine-*N*-sulfonic acid was prepared by piperidine and chlorosulfonic acid according to the method of Nagasawa and Yoshidome.[17]

Repeated Glycosydations of Curdlan with Orthoacetates. To a suspension of curdlan (0.5 g) in chlorobenzene (30 mL) was added 3,4,6-tri-*O*-acetyl α-D-glucose-(1,2-ethyl orthoacetate) (2.0 g). The mixture was heated at a reflux temperature, and then a small amount of water was distilled away as the chlorobenzene azeotrope at atmospheric pressure. 2,6-Lutidinium perchlorate (20 mg) was added to the mixture, which was stirred under reflux for 6 h. The insoluble product was filtered through a 4-Å glass filter and washed with acetone several times to give a branched curdlan after dryness. This procedure was repeated three times to increase the degree of substitution.

Deacetylation. Branched curdlan (0.1 g) was suspended in 5% NaOH solution (50 mL), and the mixture was stirred at room temperature for 2 h. The solution was dialyzed against deionized water for a day. The dialyzate was freeze-dried to give a free-OH branched curdlan.

Methylation Analysis. Dry D-glucose-branched curdlan (50 mg) was dissolved in 10 mL of DMSO. To the mixture was added 2 M methyl sulfinylmethylsodium in DMSO (2 mL). After the mixture was stirred at room temperature for 2 h under a nitrogen atmosphere, the mixture was cooled in an ice–salt bath for 45 min. After methyl iodide (3 mL) was added, the frozen solution was warmed slowly to reach room temperature and then stirred for 4 h. The reaction mixture was poured into water, and the fully methylated branched curdlan was isolated by dialysis against deionized water for 2 days after extraction with CH_2Cl_2. The yield was 45 mg. A mixture of methylated curdlan (5 mg) and 2 N trifluoroacetic acid (TFA; 0.5 mL) was heated at 110 °C for 1 h. The mixture was cooled and evaporated under a stream of nitrogen to dryness. Water (0.5 mL) was added to the residue, and it was evaporated again to dryness. Sodium borohydrate solution, which was prepared by mixing 1 mL of 1 M NH_4OH and 20 mg of $NaBH_4$, was added to the dry hydrolyzate, and the mixture was stirred for 1 h at 50 °C. Acetic acid (3 drops) was added to the mixture, and then it was evaporated to dryness under a nitrogen stream at less than 35 °C. After adding 0.2 mL of a MeOH–AcOH (9:1) solution, the mixture was evaporated again, followed by repeated evaporations from MeOH three times. A mixture of alditol derivatives was obtained. Acetic anhydride (0.3 mL) was added to the alditol, and the mixture was stirred at 100 °C for 1 h. To the cooled mixture was added water (0.5 mL), and the solution was stirred at room temperature overnight. The products were extracted with methylene chloride. The methylene chloride layer was washed with water several times and dried over anhydrous sodium sulfate to give a mixture of alditol acetate after evaporation. The reaction of hydrolysis, reduction, and acetylation was continuously carried out in the same 1-mL flask.

Sulfation. Branched curdlan was sulfated by piperidine-*N*-sulfonic acid in dry DMSO at 85 °C for 1 h to give branched curdlan sulfates. The procedure was described in the previous paper.[12]

Anti-AIDS Virus Assay. The anti-AIDS virus activity of linear and branched curdlan sulfates was determined by the MTT method,[18] in which the viability of both HIV- and mock-infected cells was assayed spectrophotometrically via the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenases of metabolically active cells to a blue formazan product. MT-4 cell (a human T4-positive cell line carrying human T-lymphotropic virus type I) was infected with HIV-1 at the multiplicity of 0.01, and HIV- and mock-infected MT-4 cells were incubated in the presence of various concentrations of curdlan sulfates for 5 days at 37 °C in a CO_2 incubator. The concentration of curdlan sulfates achieving the 50% inhibition of the MT-4 cells from HIV infection was defined as the 50% effective dose (EC_{50}). The cytotoxicity (CC_{50}) was determined by the 50% cytotoxic concentration of curdlan sulfate on the MT-4 cells.

Anticoagulant Activity. Anticoagulant activity was determined by use of normal rat plasma APTT doubling dosages.[19]

Results and Discussion

Synthesis of Branched Curdlans and Their Structural Analysis. A bacterial polysaccharide curdlan is composed of linear 1,3-β-D-glucosidic linkages.[20] L-Glycosyl-branched curdlan sulfates were synthesized by the route as illustrated in Figure 1. To increase the degree of branching, curdlan was subjected to the repeated branching reaction with glucosyl and mannosyl orthoacetates in the presence of 2,6-lutidinium perchlorate as a catalyst. The results of branching reaction are summarized in Table 1. Branching of curdlan occurred to a very low extent by a single reaction (no. 1). This difficulty might be due to the rigid structure of curdlan. However, by the repeated condensation, 20 mol % of D-glucosyl branches were introduced into curdlan (no. 2). When the condensation was repeated three times, the ratio of branches reached to 39 mol % (no. 3). By the reaction repeated three times, branched curdlans consisting of L-glucosyl, D-mannosyl,

Table 1. Glycosylation of Curdlan with Orthoacetates[a]

no.	curdlan or branched curdlan	repeated times of condensation	orthoacetate	yield (g)	degree of branching[b] (%)
1	0.50	1	D-glucose	0.65	<5
2	0.55	2	D-glucose	0.63	20
3	0.55	3	D-glucose	0.60	39
4	0.60	3	L-glucose	0.75	35
5	0.38	3	D-mannose	0.37	19
6	0.48	3	L-mannose	0.47	17

[a] Solvent, chlorobenzene; orthoacetate, 2.0 g; catalyst, 2,6-lutidinium perchlorate, 15–20 mg; temp, reflux; time, 6 h. [b] Determined from the ^{13}C NMR spectrum.

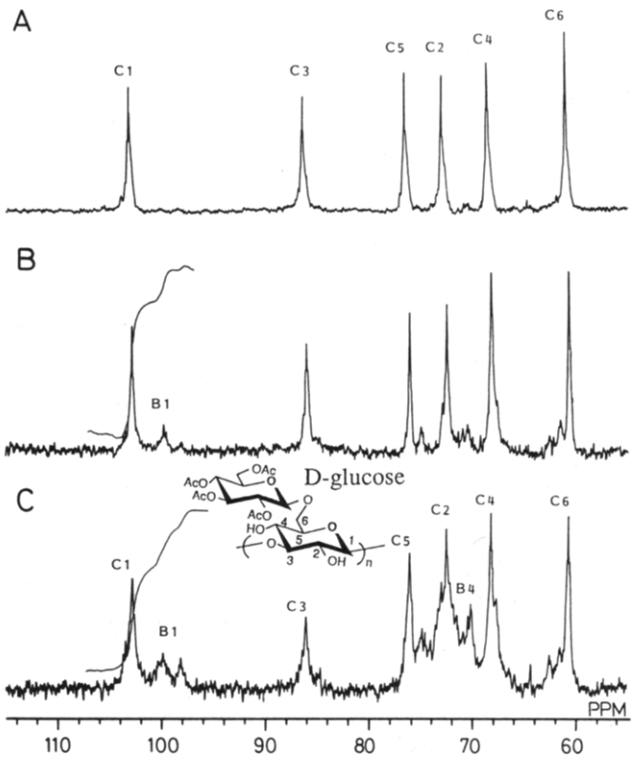

Figure 2. 67.8-MHz ^{13}C NMR spectra of D-glucosyl-branched curdlan: (A) after one condensation and (B) after two-times-repeated and (C) after three-times-repeated condensations with D-glucosyl orthoacetate (DMSO-d_6 as solvent at 80 °C).

and L-mannosyl branches were also prepared in the branching ratio of 17–35 mol %, respectively.

The structural analysis of branched curdlans was attempted by a combination of ^{13}C NMR spectroscopy and methylation analysis according to the method of Hakomori.[21] ^{13}C NMR spectra of D-glucosyl-branched curdlans are shown in Figure 2. Absorption of the C1 carbon in the curdlan main chain appeared at 101.5 ppm. As the ratio of D-glucosyl branches in the curdlan main chain increases, C1 carbon absorption of the branches appears as two peaks at 99 and 100 ppm, suggesting that the branches were introduced into two different positions of curdlan. In spectrum C, the estimation of the C1 peaks gave the degree of branching of 39 mol %. The line broadening of absorptions is observed as the branches increase. In the region of 60–80 ppm in spectrum C, many peaks overlap and it was difficult to analyze the detailed structure of the branched curdlan.

Methylation analysis of free-OH branched curdlans was performed by Hakomori's procedure to determine the ratio and position of the branches.[21] The fully methylated branched curdlans were converted into partially methylated alditols by hydrolysis with a 2 N TFA solution. Reduction with $NaBH_4$ followed by acetylation with acetic

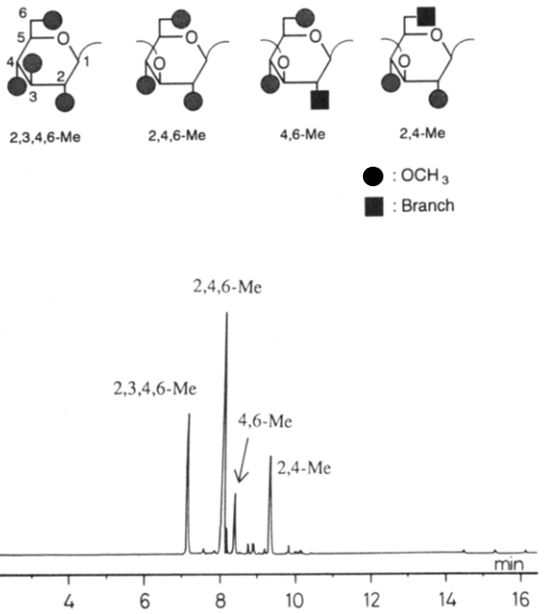

Figure 3. Gas–liquid chromatography of alditol acetates from L-glucosyl-branched curdlan. Condition: column, methyl silicone, 0.25 mm × 30 m; temp, 150 °C (1 min) → 250 ° (7 min), 10 °C/min.

anhydride gave the alditol acetates. Figure 3 shows the GC chromatography of the partially methylated alditol acetates obtained from D-glucosyl-branched curdlan. The peaks having retention times of 7.22, 8.17, 8.48, and 9.42 min were assigned to 2,3,4,6-tetra-*O*-methyl-, 2,4,6-tri-*O*-methyl-, 4,6-di-*O*-methyl-, and 2,4-di-*O*-methylgluditol acetates, respectively, by comparison with the authentic samples and by measuring GC-MS spectra. These four alditol acetates originated from a D-glycosyl branches, an unsubstituted main chain, a main chain glucose residue with a branch at C2 position, and a main chain glucose residue with a branch at C6 position, respectively, as also illustrated in Figure 2. No 4-*O*-methyl- and 2,6-di-*O*-methylgluditol acetates were detected in these experiments, suggesting that bisubstitution at C2 and C6 positions and a single substitution at the C4 position did not occur, probably because of steric hindrance of the curdlan main chain. The relative molar ratio of branches is summarized in Table 2. L-Glucosyl- and D-mannosyl-branched curdlans were also analyzed, and the ratios of branches were 28–33 mol % calculated from their peak areas of GC chromatography. These values were roughly comparable to the results of NMR spectroscopy. The relative ratios of branches in the curdlan main chain were 12–16 mol % at the C2 position and 21–28 mol % at the C6 position, respectively, indicating that the primary alcohol of the C6 position was advantageous for the substitution of branching.

Sulfation of Branched Curdlans. The removal of acetyl groups of branches by a 5% sodium hydroxide solution provided free-OH branched curdlans. The free-OH branched curdlans were sulfated with piperidine-*N*-sulfonic acid (PSA) in DMSO to give branched curdlan sulfates as shown in Table 3. The degree of sulfation (DS) estimated by elemental analysis was in the range of 1.3–1.8. D-Mannosyl- and L-mannosyl-branched curdlan sulfates (nos. 4 and 5) had a higher value, 1.8. Although the DS of branched sulfates increased with increasing amount of PSA, the number-average molecular weights decreased. The branched curdlan exhibited specific rotations around +10°, except for L-glucosyl-branched curdlan sulfate (no. 2), which had a specific rotation of

Table 2. Relative Molar Ratio and Retention Time of Partially Methylated Alditol Acetates from Branched Curdlan on an S2149 (Methyl Silicone) Glass Capillary Column[a]

	relative molar ratio (mol %) and retention time (min)				ratio of branches (mol %)		relative ratio of branches (mol %)[b]		
branch	2,3,4,6-Me	2,4,6-Me	4,6-Me	2,4-Me	GC	NMR	position 2	position 4	position 6
D-glucose	19.5 (7.22)	35.9 (8.17)	9.5 (8.48)	13.4 (9.42)	33	39	16	~0	22
L-glucose	20.0 (7.21)	41.7 (8.15)	7.7 (8.46)	13.1 (9.40)	32	35	12	~0	21
D-mannose	16.0 (7.21)	32.2 (8.16)	9.1 (8.48)	16.0 (9.42)	28	26	16	~0	28

[a] GC conditions: column, methyl silicone, 30 m; temp, 150 °C (1 min) → 250 °C (7 min), 10 °C/min. [b] Calculated from GC data.

Table 3. Sulfation of Branched Curdlan[a]

	branched curdlan						elemental anal. (%)			
no.	branch (mol %)	g	PSA,[b] g (mol %)	yield (g)	$\bar{M}_n$[c] × 10^{-4}	$[\alpha]^{25}_D$[d] (deg)	C	H	S	DS[e]
1	D-glucose (39%)	0.30	0.73 (216)	0.35	4.2	+10.0	22.46	3.75	13.2	1.3
2	L-glucose (35%)	0.39	0.90 (206)	0.40	4.7	+0.9	22.00	3.60	13.2	1.3
3	D-glucose (39%)	0.30	1.00 (294)	0.41	3.6	+10.5	20.35	3.26	14.4	1.6
4	D-mannose (19%)	0.26	1.20 (400)	0.24	1.3	+12.5	19.70	3.31	15.1	1.8
5	L-mannose (17%)	0.33	1.50 (400)	0.27	1.2	+10.9	19.07	3.19	15.2	1.8

[a] Solvent, DMSO, 20–30 mL; temp, 85 °C; time, 1 h. [b] Piperidine-*N*-sulfonic acid. [c] Determined by GPC. [d] Measured in H_2O (*c* 1%). [e] The number of sulfate groups per sugar unit in curdlan sulfate.

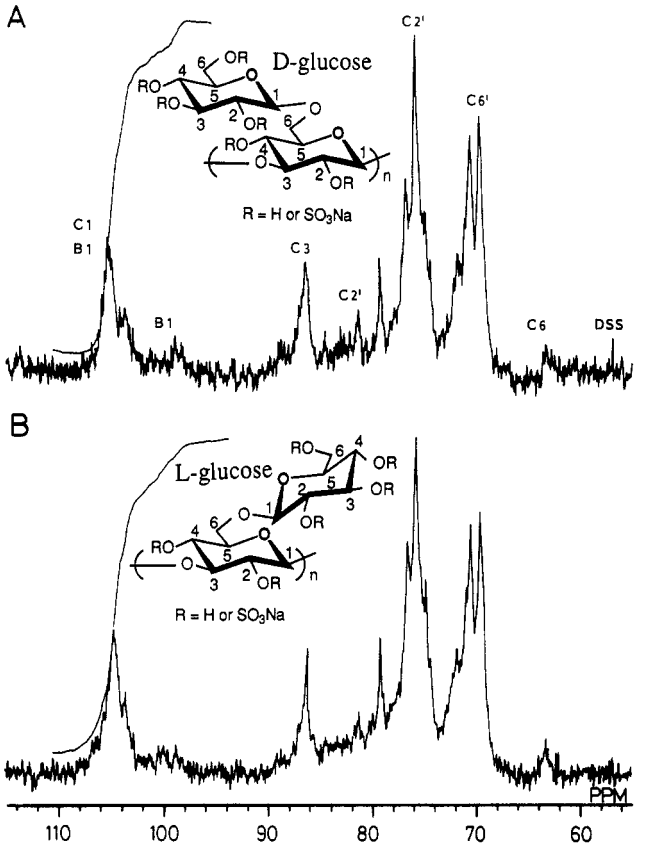

Figure 4. 67.8-MHz ^{13}C NMR spectra of (A) D-glucosyl- and (B) L-glucosyl-branched curdlan sulfates (D_2O as solvent at 37 °C).

+0.9°. These branched curdlan sulfates were soluble in water.

Figure 4 shows ^{13}C NMR spectra of D-glycosyl- and L-glucosyl-branched curdlan sulfates, both of which have a DS of 1.3. The peak assignment was carried out by reference to that of linear curdlan sulfates, which was reported previously.[12] The C6 absorption of the curdlan main chain appeared at 63 ppm, while it was shifted downfield appearing around 70 ppm, indicating that the hydroxyl group at C6 was substituted by the sulfate group. The peaks originating from sulfated glucosyl branches were unclear, because of overlapping and line broadening. However, C1 peaks of both D- and L-glycosyl branches appeared explicitly around 100 ppm. The degree of sulfation calculated from both spectra was in agreement with that (DS = 1.3) from the elemental analysis.

Table 4. Anti-AIDS Virus Activity of Branched Curdlan Sulfates

no.	curdlan sulfate[a] (branch)	S content (%)	$\bar{M}_n$ × 10^{-4}	$[\alpha]^{25}_D$ (deg)	EC_{50}[b] (μg/mL)	CC_{50}[c] (μg/mL)	SI
1	D-GCS1 (39%)	13.2	4.2	+10.0	0.9	>1000	>1030
2	D-GCS2 (39%)	14.4	3.6	+10.5	0.3	>1000	>3430
3	L-GCS1 (35%)	13.2	4.7	+0.9	1.2	>1000	>780
4	D-MCS1 (19%)	15.1	1.7	+12.5	0.6	>1000	>1660
5	L-MCS1 (17%)	15.2	1.2	+10.9	0.5	>1000	>2000
6	CS	14.1	7.9		0.43	>1000	>2320
7	DS	18	0.7		0.91	>1000	>1100
8	AZT (mM)				0.0019	6.43	3380

[a] D-GCS: D-glucose-branched curdlan sulfate. L-GCS: L-glucose-branched curdlan sulfate. D-MCS: D-mannose-branched curdlan sulfate. L-MCS: L-mannose-branched curdlan sulfate. CS: curdlan sulfate. DS: dextran sulfate. [b] 50% effective concentration. [c] 50% cytotoxic concentration. [d] Selectivity index: CC_{50}/EC_{50}.

Anti-AIDS Virus Activity. The anti-AIDS virus activity in vitro was determined by the MTT method using MT-4 cells,[18] which have proven to be the most sensitive cell line of AIDs virus infection. The complete cell death occurred within 4–5 days after the virus infection. Table 4 shows the results of anti-AIDS virus activity of branched curdlan sulfates. The protective effects of branched curdlan sulfates on the AIDS virus (HIV-1) induced cytopathic effects and were assayed on the fifth day after inflection. A curdlan sulfate as a reference compound has a EC_{50} of 0.43 μg/mL, which corresponds to the complete inhibition concentration of 3.3 μg/mL. These branched curdlan sulfates inhibited the cytopathic effect caused by AIDS virus infection at low concentrations. The EC_{50} ranged from 0.3 to 1.2 μg/mL, suggesting that the branched curdlan sulfates had potent anti-AIDS virus activities. It was revealed that the cytotoxicities, CC_{50}, were extremely low, because these sulfates did not inhibit the cell growth at concentrations of more than 1000 μg/mL.

The anticoagulant activity of D- and L-glycosyl-branched curdlan sulfates was calculated from an activated partially thromboplastin time (APTT) by using a curdlan sulfate and heparin as references. The results are summarized in Table 5. It is noteworthy that the branched sulfate had anticoagulant activities of 18.5 and 17.2 units/mg which are almost equivalent to that of the curdlan sulfate of 19.0 units/mg. The activities were roughly one-tenth of that of heparin (150 units/mg), suggesting that the rigid rodlike structure of curdlan sulfates was related to the weak interaction with the coagulating enzymes in plasma.

Table 5. Anticoagulant Activity (APTT) of Branched Curdlan Sulfates[a]

curdlan sulfate (branch)	S content (%)	$\bar{M}_n \times 10^{-4}$	AA[b] (units/mg)
D-GCS1 (D-glucose 39%)	13.2	4.2	18.5
L-GCS1 (L-glucose 35%)	13.2	4.7	17.2
CS[c]	14.1	4.6	19.0

[a] APTT: activated partially thromboplastin time. [b] Anticoagulant activity: standard; heparin, 150.0 units/mg. [c] Standard curdlan sulfate.

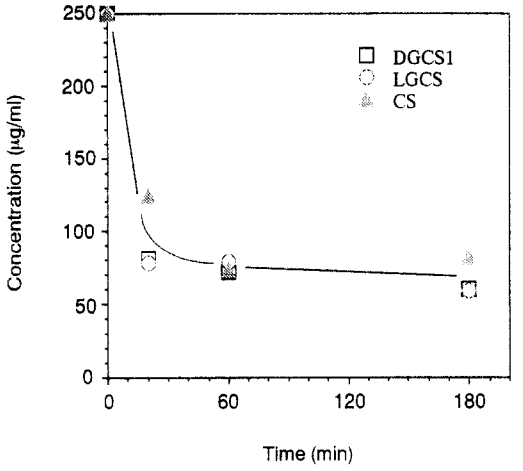

Figure 5. Retention time of branched curdlan sulfates in plasma in vivo calculated from their anticoagulant activities.

Time dependences on the concentration of D- and L-glycosyl-branched curdlan sulfates in rat in vivo are shown in Figure 5. The branched sulfates were intravenously injected into rats at a dose of 10 mg/kg; that is, the initial concentration in blood is calculated to be 250 μg/mL. The blood samples were withdrawn in 20, 60, and 180 min after injection, and then the concentrations of the sulfates in plasma were evaluated from the APTT. The concentration of the branched sulfate 3 h after injection was a third of the initial concentration. However, there was almost no significant difference in the behaviors in the blood between D- and L-glycosyl-branched curdlans and also between the linear and the branched curdlans. It was revealed recently that the disappearance of curdlan sulfates from blood was due to absorption mainly in such tissues as liver, bone marrow, kidney, and lymph node without degradation for 10 days.[22] This fact might be preferred to protect from infection of the AIDS virus because AIDS viruses are found often in the first step of infection in the lymph node.[23]

Acknowledgment. The authors thank Professor Tetsuo Miyakoshi of Meiji University of measuring the GC and GC-MS spectra and many useful suggestions.

References and Notes

(1) Gottlieb, M. S.; Schroff, R.; Schanker, H. M.; Weisman, J. D.; Fan, P. T.; Wolf, R. A.; Saxon, A. *New Engl. J. Med.* **1981**, *305*, 1431.
(2) Barre-Sinoussi, F.; Chermann, J. C.; Chamaret, S.; Gruest, J.; Dauguet, C.; Axler-Blin, C.; Vezinet-Brun, F.; Rouzioux, C.; Rozenbaum, W.; Montagnier, L. *Science* **1983**, *220*, 868.
(3) Larder, B. A.; Darby, G.; Richman, D. D. *Science* **1989**, *243*, 1731.
(4) Haynes, B. F. *Science* **1993**, *260*, 1279.
(5) Chihara, G.; Maeda, Y.; Hamuro, J.; Sasaki, T.; Fukuoka, F. *Nature* **1969**, *222*, 687.
(6) Matsuzaki, K.; Yamamoto, I.; Sato, T.; Oshima, R. *Makromol. Chem.* **1986**, *187*, 317.
(7) Hamuro, J.; Yamashita, Y.; Ohsaka, Y.; Maeda, Y.; Chihara, G. *Nature* **1971**, *233*, 486.
(8) Hara, C.; Kiho, T.; Ukai, S. *Carbohydr. Res.* **1982**, *111*, 143.
(9) Yoshida, O.; Nakashima, H.; Yoshida, T.; Kaneko, Y.; Yamamoto, O.; Matsuzaki, K.; Uryu, T.; Yamamoto, N. *Biochem. Pharmacol.* **1988**, *37*, 2887.
(10) Hatanaka, K.; Yoshida, T.; Uryu, T.; Yoshida, O.; Nakashima, H.; Yamamoto, N.; Mimura, T.; Kaneko, Y. *Jpn. J. Cancer Res.* **1989**, *80*, 95.
(11) Kaneko, Y.; Yoshida, O.; Nakagawa, R.; Yoshida, T.; Date, M.; Ogiwara, S.; Shioya, S.; Matsuzawa, Y.; Nagashima, M.; Irie, Y.; Mimura, T.; Shinkai, H.; Yasuda, N.; Matsuzaki, K.; Uryu, T.; Yamamoto, N. *Biochem. Pharmacol.* **1990**, *39*, 793.
(12) Yoshida, T.; Hatanaka, K.; Uryu, T.; Kaneko, Y.; Suzuki, E.; Miyano, H.; Mimura, T.; Yoshida, O.; Yamamoto, N. *Macromolecules* **1990**, *23*, 3717.
(13) U.S. Pharmacopeia National Formulary, USP XXI, 1985.
(14) Nakashima, H.; Yoshida, O.; Tochikura, T.; Yoshida, T.; Mimura, T.; Kido, Y.; Motoki, Y.; Kaneko, Y.; Uryu, T.; Yamamoto, Y. *Jpn. J. Cancer Res.* **1987**, *78*, 1164.
(15) Yoshida, T.; Katayama, Y.; Inoue, S.; Uryu, T. *Macromolecules* **1992**, *25*, 4051.
(16) Yoshida, T.; Nakashima, H.; Yamamoto, N.; Uryu, T. *Polym. J.* **1993**, *25*, 1069.
(17) Nagasawa, K.; Yoshidome, H. *Chem. Pharm. Bull.* **1969**, *17*, 1316.
(18) Pauwels, R.; Balzarini, J.; Baba, M.; Snoeck, R.; Schols, D.; Herdewijn, P.; Desmyter, J.; Clercq, E. D. *J. Viol. Methods* **1987**, *30*, 810.
(19) Lenahan, J. G.; Frye, S.; Philips, G. *Clin. Chem.* **1966**, *12*, 263.
(20) Harada, T.; Masada, M.; Fugimori, K.; Maeda, I. *Agric. Biol. Chem.* **1966**, *30*, 196.
(21) Hakomori, S. *J. Biochem.* **1964**, *55*, 205.
(22) Uryu, T.; Kaneko, Y.; Yoshida, T.; Mihara, R.; Shoji, T.; Katsuraya, K.; Nakashima, H.; Yamamoto, N. *Carbohydrates and Carbohydrate Polymers*; Yalpani, M., Ed.; ATL Press: Mount Prospect, IL, 1993; pp 101–115.
(23) Pantalao, G.; Graziosi, C.; Demaret, J. F.; Butini, L.; Montroni, M.; Fox, C. H.; Orenstein, J. M.; Kotler, D. P.; Fauci, A. S. *Nature* **1993**, *362*, 355.