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Synthesis of Sulfated Deoxy-Ribofuranans Having Selective Anti-AIDS Virus Activity by Ring-Opening Copolymerization of 1,4-Anhydro Ribose Derivatives

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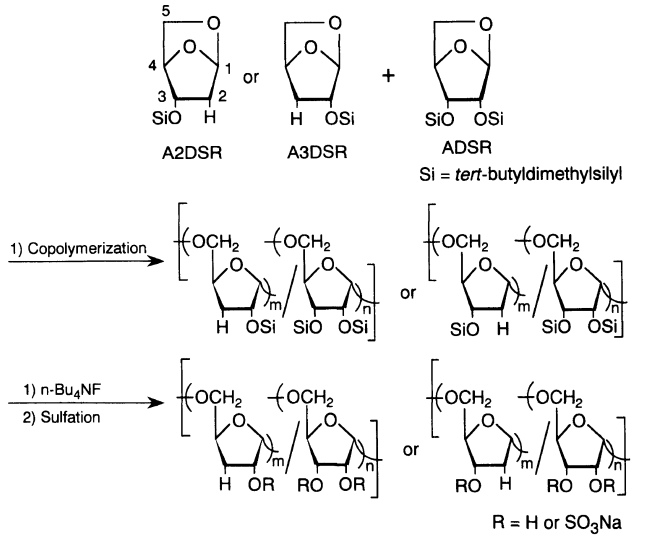
ABSTRACT: Ring-opening copolymerization in various ratios of 1,4-anhydro deoxy-ribose monomers, 1,4-anhydro-2(or 3)-*O*-*tert*-butyldimethylsilyl-3(or 2)-deoxy-α-D-ribopyranose (A3DSR or A2DSR), with 1,4-anhydro-2,3-di-*O*-*tert*-butyldimethylsilyl-α-D-ribopyranose (ADSR) was investigated with boron trifluoride etherate catalyst to give the corresponding copolymers having number average molecular weights of 90.1×10^3—270.0×10^3 for copoly(A3DSR-ADSR)s and 39.1×10^3—93.4×10^3 for copoly(A2DSR-ADSR)s. The molecular weights of copoly(A3DSR-ADSR)s were higher than those of copoly(A2DSR-ADSR)s, suggesting that the substituent at C2 position works more effectively for regulating the ring-opening copolymerization than that at C3. After deprotection of hydroxyl groups followed by sulfation, sulfated heteroribofuranans consisting of various ratios of 2- or 3-deoxy-ribofuranosidic and ribofuranosidic units were obtained. It was found that both sulfated 2- and 3-deoxy-ribofuranans had no anti-AIDS virus activity. The anti-AIDS virus activity increased with decreasing ratio of the deoxy unit to give as high as $EC_{50} = 0.6\,\mu g\,ml^{-1}$, indicating that the number of sulfate groups in the polysaccharide chain was important for the high anti-AIDS virus activity. The sulfated deoxy-ribofuranans had relatively low blood anticoagulant activity of 2—18 unit mg^{-1} compared with sulfated ribofuranan of 56 unit mg^{-1}.

KEY WORDS Copolymerization / Deoxyribofuranan / Sulfation / Anti-AIDS virus activity / Nuclear Magnetic Resonance /

Sulfated polysaccharides such as natural heparin and dextran sulfate have been used as blood anticoagulant agents.[1] Since dextran sulfate was found to inhibit the infection of acquired immunodeficiency syndrome (AIDS) virus (HIV) *in vitro*,[2-4] many sulfated polysaccharides were synthesized and examined on their anti-AIDS virus activity. Among them, a 1,3-β-glucan, curdlan was sulfated with piperidine-*N*-sulfonic acid to give curdlan sulfate, which had a potent anti-AIDS virus activity *in vitro* but a considerably low anticoagulant activity.[5,6,7] The anticoagulant activity is assumed to be a side effect for the AIDS drugs. Thus, curdlan sulfate seems to be a promising candidate of the anti-AIDS drug.[8] Taking into account a well-known action mechanism of a natural sulfated polysaccharide heparin with antithrombin III,[9] it is assumed that the sulfated polysaccharide might inhibit AIDS virus infection by prohibiting a virus surface protein from attaching to a receptor protein of target T-lymphocytes.[10,11] Thus, it is important to develop sulfated polysaccharides with selective anti-AIDS virus activity in order to know the information of the structure–activity relationships.[12]

Recently, the ring-opening polymerization of 1,4-anhydro-3(or 2)-*O*-*tert*-butyldimethylsilyl-2(or 3)-deoxy-α-D-ribopyranose, *i.e.*, A2DSR or A3DSR, was investigated for their steric factors and electronic effects of the substituent, leading to the synthesis of stereoregular 3-deoxy-(1→5)-α-D-ribofuranan.[13] Deoxy sugars play important roles for biological and functional materials *in vivo* such as 2-deoxy-D-ribose as a component of DNA.

In this study, to clarify relationships between the polymer structure and the anti-AIDS virus activity, we wish to describe firstly the synthesis of sulfated deoxy-ribofuranans having various ratios of the deoxy-sugar



Scheme 1. Synthesis of sulfated deoxy-ribofuranan with a definite structure.

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units by the ring-opening copolymerization of 1,4-anhydro-2(or 3)-*O*-*tert*-butyldimethylsilyl-3(or 2)-deoxy-α-D-*erythro*-pentopyranose (=1,4-anhydro-2(or 3)-*O*-*tert*-butyldimethylsilyl-3 (or 2)-deoxy-α-D-ribopyranose, *i.e.*, A3DSR or A2DSR) with 1,4-anhydro-2,3-di-*O*-*tert*-butyldimethylsilyl-β-D-ribopyranose (ADSR) (Scheme 1). In addition, the anti-AIDS virus activity of the sulfated deoxy-ribofuranans is examined by the MTT method using MT-4 cell line.[14] The relationship between the anti-AIDS virus activity and the structure of the sulfated deoxy-ribofuranans is discussed.

EXPERIMENTAL

Monomers

ADSR, A3DSR, and A2DSR were synthesized by vacuum pyrolysis of D-ribose followed by regioselective *tert*-butyldimethylsilylation according to the previously described methods.[15,16]

Copolymerization

Typical procedure of copolymerization is as followed. In a sealed glass ampoule, a methylene chloride solution (0.5 ml) of both A3DSR (0.156 g, 0.68 mmol) and ADSR (0.244 g, 0.68 mmol) were polymerized with boron trifluoride etherate (3 mol% to the feed) at −40°C for 5 h under high vacuum (10^{-5}—10^{-6} mmHg). After polymerization was terminated by addition of methanol, the reaction mixture was dissolved in chloroform. The chloroform layer was neutralized with sodium bicarbonate, washed with water several times, dried over anhydrous sodium sulfate, and concentrated. Purification was carried out by dissolution-reprecipitation three times through a chloroform–methanol solvent system, and the polymer was isolated by freeze-drying from benzene. Yield, 73%. (No. 3 in Table III). Copolymerization of A2DSR and ADSR was also carried out with the same procedure as above.

Deprotection

To a tetrahydrofuran (THF) solution (20 ml) of copoly(A2DSR-ADSR) (0.2 g) (2-deoxy unit: 87 mol%) was added 1 M *tetra-n*-butylammonium fluoride in THF solution (4 ml), and then the mixture was stirred under reflux for 1.5 h. The reaction was terminated by addition of water, and the mixture was dialyzed with deionized water for 24 h to give 2-deoxyribofuranans (2-deoxy unit: 83 mol%) as a white and powdery precipitates. The yield after freeze-drying from water was 95%. (No. 2 in Table II).

Sulfation

A solution of 3-deoxy-ribofuranan (3-deoxy unit: 46 mol%) (70 mg) in dimethyl sulfoxide (DMSO) (20 ml) was stirred for 1.5 h at 80°C with piperidine-*N*-sulfonic acid (0.4 g). After the reaction mixture was cooled to room temperature, it was neutralized with $NaHCO_3$ solution, and then the mixture was dialyzed with deionized water at room temperature for more than 24 h. The cellulose dialyzer tubing VT-803 (Nacalai Tesque Co., Ltd.) was used for dialysis. Finally, sulfated deoxyribofuranan (0.12 g) was obtained by freeze-drying from water in 82% yield. (No. 6 in Table III).

Anti-HIV Assay

The anti-AIDS virus activity of sulfated deoxy-ribofuranans was determined by the MTT method by using MT-4 cells and $HIV_{HTLV\text{-}IIIB}$ virus.[15] The MTT method is a convenient assay method of AIDS drugs based on the change of coloures of a yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to a blue formazan product by metabolically active MT-4 cells (viable cells). MT-4 cells were infected with HIV at the multiplicity of 0.01. Then, the HIV- or mock-infected MT-4 cells (1.5×10^5 cells ml^{-1}, respectively) were incubated for 5 days at 37°C in the presence of various concentrations of the sulfated 2- and 3-deoxy-ribofuranans. The uninfected cells were measured spectrophotometrically by changing of the coloures of test solutions. The activity was represented as EC_{50} which denotes the concentration of a test compound for protecting 50% of the HIV infection. The cytotoxicity was designated as CC_{50} which indicates the 50% cytotoxic concentration of the sulfated 2- and 3-deoxy-ribofuranans against MT-4 cells.

Anticoagulant Activity

Anticoagulant activity was determined by the modified method of the United States Pharmacopoeia using bovine plasma.[17] Dextran sulfate (Meito Sangyo Co., Ltd.) was used as a reference with an anticoagulant activity of 22.7 unit mg^{-1}.

Characterization

400 MHz ^1H and 100 MHz ^{13}C NMR spectra were recorded on a JEOL EX-400 in D_2O or $CDCl_3$ solution. TMS or DSS was used as an internal standard. Number-average molecular weight of water-soluble or THF-soluble polymers was estimated by gel permeation chromatography (column: TOSOH TSK-gel, G2500PW, G3000PW, 7.5 mm × 600 mm × 2; eluent, 66.7 mmol of phosphate buffer, pH = 6.86 or TOSOH TSK-gel, $G3000H_{XL}$, $G4000H_{XL}$, $G5000H_{XL}$, 7.5 mm × 600 mm × 3) with standard pullulan (Shodex Standard P-82) or standard polystyrene (Shodex Standard SM-105) as a reference. Specific rotations were measured in chloroform or water at 25°C by a JASCO DIP-140 polarimeter in a 1-dm cell.

RESULTS AND DISCUSSION

Copolymerization of A2DSR or A3DSR with ADSR

When we investigated the ring-opening polymerization of 1,4-anhydro-deoxy-ribose derivatives such as A3DSR and A2DSR with Lewis acid catalysts, it was revealed that the substituent at the C2 position play an important role for a stereoregularity of the resulting polymers.[13] Therefore, to examine the copolymerizability of the deoxy-ribose monomers, the copolymerization of A3DSR or A2DSR with 2,3-di-*O*-*tert*-butyldimethylsilylated monomer ADSR was carried out. The results are summarized in Table I.

The copolymerization was performed with $BF_3 \cdot OEt_2$ catalyst under high vacuum at −40°C to give the corresponding copolymers having various ratios of the deoxy-sugar units. The number-average molecular weight and specific rotation of the copolymers increased with

Table I. Ring-opening copolymerization of A3DSR or A2DSR with ADSR

No.[a]	Deoxymonomer		ADSR feed	Time	Yield	$\bar{M}_n$[b] ($\times 10^3$)	$[\alpha]_D^{25}$[c]	A3DSR unit in polymer[d]	α-Content[e]
	g	mol%	g	h	%		deg	%	%
A3DSR									
1	0.25	100	0	1	82	38.2	+98	100	95
2	0.20	84	0.05	1	72	90.1	+107	79	98
3	0.15	67	0.10	0.5	70	100.0	+113	75	99
4	0.125	58	0.125	0.5	84	160.4	+119	67	100
5	0.10	47	0.15	0.5	84	200.1	+129	47	100
6	0.05	25	0.20	0.5	85	270.0	+140	22	100
A2DSR									
7	0.25	100	0	1	63	24.4	+113	100	78
8	0.20	84	0.05	1	42	39.1	+114	71	81
9	0.15	67	0.10	0.5	50	48.3	+118	64	80
10	0.125	58	0.125	1.0	62	52.5	+121	51	83
11	0.10	47	0.15	0.5	57	53.3	+126	44	86
12	0.05	25	0.20	0.5	86	93.4	+135	25	93
13	0	0	0.25	0.5	86	380.0	+149	0	100

[a] Solvent, CH_2Cl_2 0.5 ml; catalyst, $BF_3 \cdot OEt_2$ 3.0 mol%, temperature, −40°C. [b] Determined by GPC (polystyrene standard). [c] Measured in $CHCl_3$ (*c*, 1%). [d] Calculated from ^1H NMR spectrum. [e] Calculated from ^{13}C NMR spectrum.

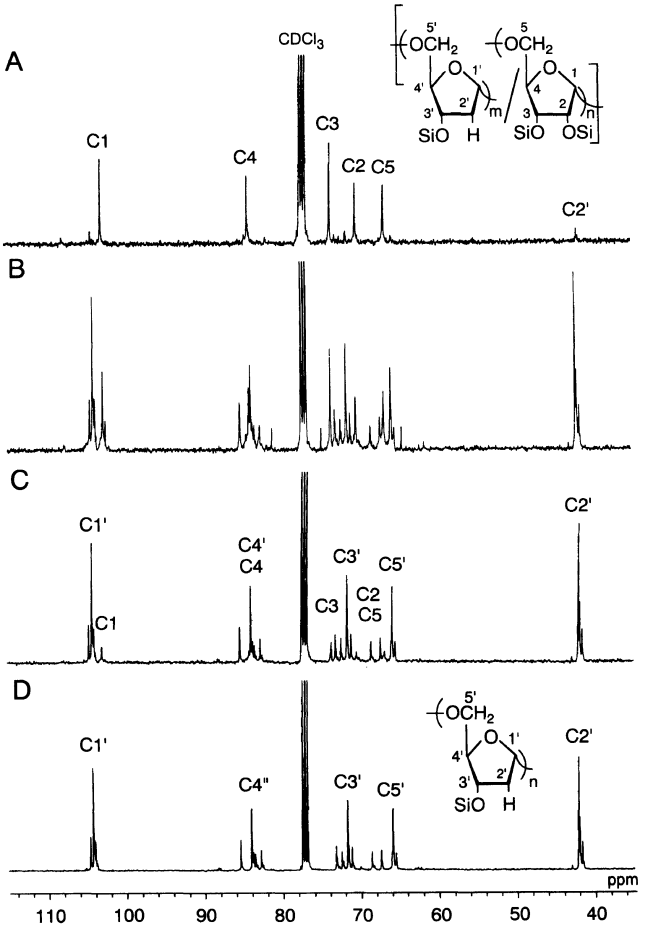

Figure 1. 100 MHz ^{13}C NMR spectra of copoly(A2DSR-ADSR)s (A)—(C) and poly(A2DSR) (D) ($CDCl_3$ as solvent). Mole fraction of A2DSR unit in copolymer: (A), 18; (B), 64; (C), 87 mol%.

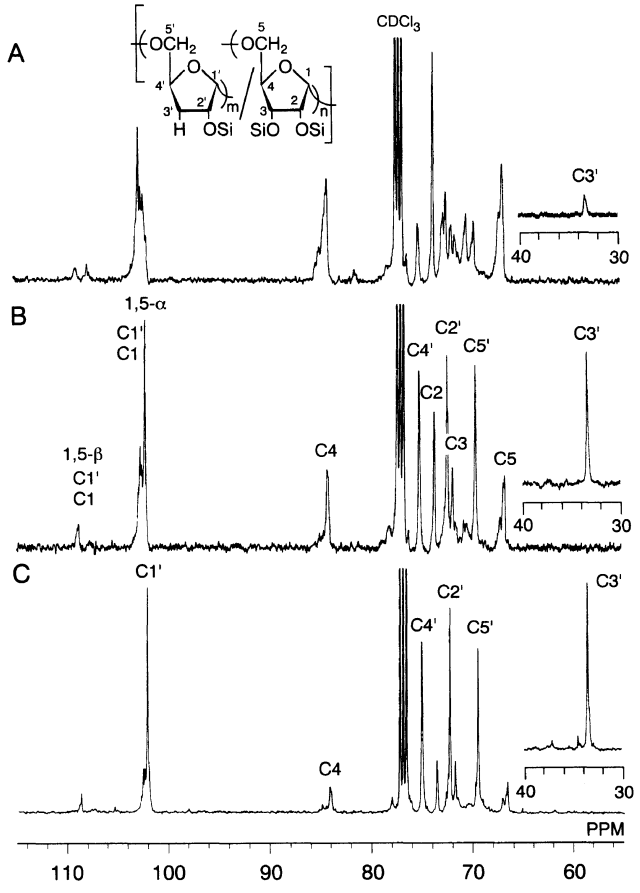

Figure 2. 100 MHz ^{13}C NMR spectra of copoly(A3DSR-ADSR)s ($CDCl_3$ as solvent). Mole fraction of A3DSR unit in copolymer: (A), 23; (B), 46; (C), 75 mol%.

increasing proportion of 2,3-di-*O*-*tert*-butyldimethylsilylated ribofuranose units in the copolymer backbone. Mole fractions of the 2- and 3-deoxy-sugar units in both copolymer chains were almost the same as those in feeds, respectively. The yields (72—85%), the molecular weights ($\bar{M}_n = 90.1 \times 10^3$—$270.0 \times 10^3$), and a 1,5-α stereoregularity of copoly(A3DSR-ADSR)s were higher than those (25—71% yields and $\bar{M}_n = 39.1 \times 10^3$—$93.4 \times 10^3$) of copoly(A2DSR-ADSR)s. These results indicate that the C2 substituent regulated to a considerable extent the copolymerization.

The structural analysis of the resulting copolymers was

Table II. Deprotection of silylated deoxyribofuranans into free deoxyribofuranans[a]

No.	Deoxycopolymer				Free deoxyribofuranan			
	Feed	$\bar{M}_n$[b]	$[\alpha]_D^{25}$[c]	Deoxy unit	Yield	$\bar{M}_n$[b]	$[\alpha]_D^{25}$[d]	Deoxy unit
	g	($\times 10^3$)	deg	mol%	% (mg)	($\times 10^3$)	deg	mol%
Copoly(A2DSR-ADSR)								
1	0.15	8.7	+127.6	100	98 (85)	4.3	+96.6	100
2	0.20	10.5	+117.3	87	95 (93)	4.9	+98.8	83
3	0.30	16.4	+110.2	64	85 (114)	6.5	+97.0	68
4	0.13	30.2	+107.9	18	97 (65)	15.7	+99.2	16
5	0.30	17.3	+106.2	0	99 (119)	9.6	+105.5	0
Copoly(A3DSR-ADSR)								
6	0.20	31.1	+90.4	100	79 (81)	15.7	+92.0	100
7	0.30	13.7	+98.0	46	77 (97)	8.5	+99.2	45

[a] *n*-Bu_4NF, 300 mol%; solvent, THF; temp, 60°C; time, 1.5 h. [b] Determined by GPC. [c] Measured in chloroform at 25°C (*c*, 1%). [d] Measured in water at 25°C (*c*, 1%).

carried out by means of ^{13}C NMR measurements as shown in Figures 1 and 2. In Figure 1D, poly(A2DSR) was found to have the mixed structures, because each carbon absorption due to the 2-deoxy-ribose unit appeared as multiple peaks. The 1,5-α stereoregularity of copoly(A2DSR-ADSR)s consisting of higher ratios of ADSR unit was low (Figures 1B and 1C), although ADSR gave a 1,5-α stereoregular polymer. The C2 absorption of the deoxy unit appeared in higher magnetic field at 42.0 ppm than that of the non-deoxy unit.

In Figure 2, it was revealed that the carbon signals of copoly(A3DSR-ADSR)s also appeared as multiple peaks due to the sequential differences of the monomeric units in the copolymer chain. Their 1,5-α stereoregularity ranged in 90—95 mol% calculated from intensities of C1 peaks around 102 and 108.5 ppm. The small absorption around 108.5 ppm might be assigned to the C1 carbon of 1,5-β 3-deoxy-furanosidic unit. These NMR results also suggest that the existence of C2 substituent in the ribofuranosidic ring influenced the stereoregularity of the resulting copolysaccharides.

Deprotection

Deprotection of the silylated deoxy-ribofuranans was carried out with tetra-*n*-butylammonium fluoride in THF to give deoxy-ribofuranans having free hydroxyl groups in good yields as summarized in Table II. The molecular weights ranged from 4.3×10^3 to 15.7×10^3 by means of GPC with phosphate buffer as eluent. Figure 3 shows the ^{13}C NMR spectra of the copoly-riboses consisting of 2-deoxy-ribofuranosidic and ribofuranosidic units in the ratio of 16, 68, and 83 mol% for the 2-deoxy unit. All signals were assigned by two dimensional NMR spectroscopies such as H–H COSY and HMQC. The signals due to the 2-deoxy unit appeared as multiple peaks, while absorptions due to the ribofuranosidic unit appeared as single peaks. The peaks at 107.5 and 105.5 ppm were assigned to the C1 carbons of the 2-deoxy- and ribofuranosidic units, respectively, and at 43.5 ppm the C2 carbon of the 2-deoxy unit. The ratio of the 2-deoxy units after deprotection was almost the same as that before deprotection. The structure of copoly-riboses consisting of ribofuranosidic and 3-deoxy-ribofuranosidic units were determined by the same experimental and analytical procedures as above.

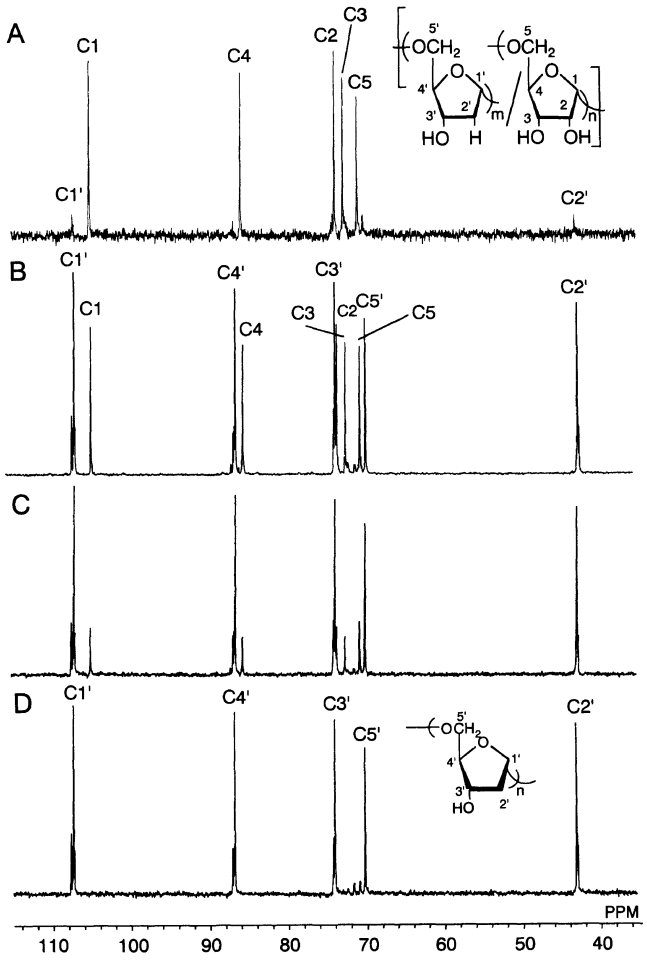

Figure 3. 100 MHz ^{13}C NMR spectra of ribofuranans having deoxy ribose unit in the ratio of (A) 16, (B) 68, and (C) 83 mol%, and (D) poly(2-deoxy-ribofuranose) (D_2O as solvent).

Sulfation

The deoxy-ribofuranans were sulfated with piperidine-*N*-sulfonic acid in DMSO to give sulfated deoxy-ribofuranans, of which ^{13}C NMR spectra are exhibited in Figure 4. The number-average molecular weights of the sulfated deoxy-ribofuranans were 5.8×10^3—20.1×10^3. Each absorption was broadened by the effect of sulfate groups. No peaks due to the unsulfated deoxy-ribofuranosidic and ribofuranosidic units were observed in

the spectra, suggesting that almost all hydroxyl groups were sulfated. The number of sulfate groups per deoxy-ribose unit (degree of sulfation) calculated from elemental analysis data ($S = 10.64$—16.53%) was 0.8—1.4.

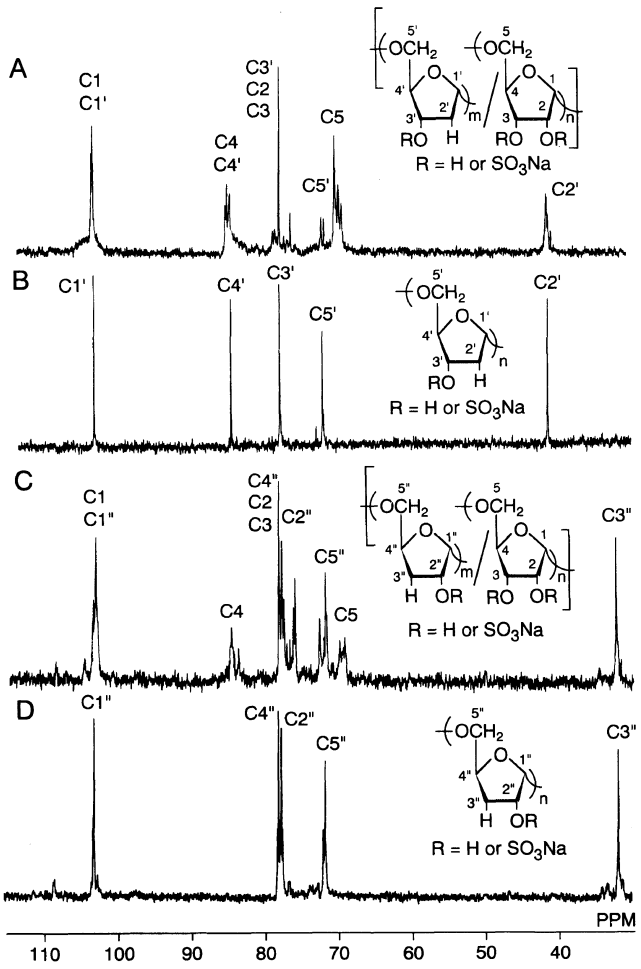

Figure 4. 100 MHz ^{13}C NMR spectra of sulfated polyriboses (D_2O as solvent). (A) Sulfated copoly(2-deoxy-ribofuranose-ribofuranose) (S = 14.74%, 2-deoxy unit: 68 mol%, (B) sulfated poly(2-deoxy-ribofuranose) (S = 10.64%), (C) sulfated copoly(3-deoxy-ribofuranose-ribofuranose) (S = 15.40%, 3-deoxy unit: 46 mol%), and (D) poly(3-deoxy-ribofuranose) (S = 14.15%).

By comparison with the spectra of sulfated 2-deoxy-ribofuranan (Figure 4B) and 2-deoxy-ribofuranan (Figure 3D), all carbon peaks were shifted lower and upper magnetic fields after sulfation, suggesting that the degree of sulfation was approximately 1. Similar NMR results were obtained by the sulfation of 3-deoxy-ribofuranans.

Anti-AIDS Virus and Blood Anticoagulant Activities of Sulfated Deoxy-Ribofuranans

In general, sulfated polysaccharides with higher degree of sulfation exhibited higher anti-AIDS virus activity.[18] However, since there are no reports on the synthesis of sulfated polysaccharides having partial deoxy-sugar units, the relationship between the structure and anti-AIDS virus activity was investigated by the MTT method using MT-4 cell.[14] The MTT method is a convenient procedure for the measurement of anti-AIDS virus activity in which the viability of HIV-infected cells was compared spectrophotometrically with that of mock-infected cells. The MT-4 cell is a human T4-positive cell line carrying HTLV-1 and is sensitive to AIDS viruses. For sulfated deoxy-ribofuranans, the anti-AIDS virus activity and HIV-induced cytopathic effect were measured in 5-day culture, as shown in Table III. Since both EC_{50} values of sulfated 2-deoxy- and 3-deoxy-ribofuranans (No. 1, 4, and 5) were more than 1000 $\mu g\,ml^{-1}$, these deoxy-ribofuranans had no anti-AIDS virus activity.

On the other hand, an increase in the proportion of non-deoxy units, *i.e.*, sulfated ribofuranose units, afforded the anti-AIDS virus activity to the sulfated polysaccharides. The EC_{50} increased to 16.6 $\mu g\,ml^{-1}$ and 19.2 $\mu g\,ml^{-1}$ with decreasing ratio of deoxy-ribose units to 68 mol% and 46 mol% for the 2-deoxy- and 3-deoxy-ribose units in the polymer chains (No. 2 and 6), respectively. For a sulfated one with 16 mol% of 2-deoxy-ribose unit, the EC_{50} was 0.6 $\mu g\,ml^{-1}$ (No. 3) which was almost equivalent to that of active curdlan sulfate. These results suggest that the anti-AIDS virus activity depended on the number of sulfate groups in the polymer backbone. As CC_{50} values were more than 561 $\mu g\,ml^{-1}$, that the cytotoxicity of the sulfated deoxy-

Table III. Anti-AIDS virus and anticoagulant activities of sulfated deoxyribofuranans

No.	Sulfated deoxy-ribofuranan					EC_{50}[a]	CC_{50}[b]	Anticoagulant activity[c]
	Deoxy unit / mol%	$\bar{M}_n$ ($\times 10^3$)	S content / %	$[\alpha]_D^{25}$[d] / deg	DS[e]	$\mu g\,ml^{-1}$	$\mu g\,ml^{-1}$	unit mg^{-1}
2-deoxy								
1	100	5.8	10.64	+98.8	0.8	>1000	561	2
2	68	6.4	14.74	+96.6	1.3	16.6	715	2
3	16	20.1	16.53	+100.7	1.4	0.6	>1000	18
3-deoxy								
4	100	8.5	13.92	+89.3	0.9	>1000	>1000	6
5	100	7.2	14.15	+92.0	0.9	>1000	>1000	n.d.
6	46	13.5	15.40	+91.9	1.3	19.2	807	12
RS[f]		17	17.6	+83.0	1.9	3.3[g]	>1000	56
CS[h]		79[i]	14.1			0.43	>1000	<10

[a] 50% Effective concentration. [b] 50% Cytotoxic concentration. [c] Dextran sulfate H-039, 22.7 units mg^{-1}. [d] Measured in water at 25°C (*c*, 1%). [e] Degree of sulfation per sugar unit. [f] Sulfated ribofuranan. [g] Minimum effective concentration for 100% inhibition of AIDS virus infection. [h] Standard curdlan sulfate. [i] Weight average molecular weight.

ribofuranans was low.

It is assumed that the sulfated polysaccharides having negative charges interact with positively charged portions of surface glycoprotein gp120 of AIDS virus to prevent the virus from attaching to human T-lymphocytes.[19] Thus, it was revealed that the interaction between the one sulfate group in a deoxy-ribofuranose unit and the gp120 might be too weak to cause the above effect. Previously, it was reported that sulfated polysaccharides having the molecular weights of higher than 7000 and high degrees of sulfation exhibited the potent anti-AIDS virus activity.[3,20,21] It is assumed that the molecular weights of the sulfated deoxy-ribofuranans of 5.8×10^3 to 8.5×10^3 might be enough high to cause high activities. Accordingly, it was concluded that the low anti-AIDS virus activity of the sulfated deoxy-ribofuranans is attributed to low content of the sulfate group. The position of sulfate groups, at the C2 or C3 position of the ribose ring, did not affect the anti-AIDS virus activity.

Another important biological activity of sulfated polysaccharides, *i.e.*, a blood anticoagulant activity, was examined by use of bovine plasma according to the United States Pharmacopoeia.[17] The anticoagulant activity of the sulfated deoxy-ribofuranans was low in the range of 2 to 18 unit mg^{-1}, which is favorable for an AIDS drug.

In conclusion, it was found that the anti-AIDS virus activity of the sulfated deoxy-ribofuranans was strongly dependent on the number of sulfate groups in the polymer chain, but independent on the position. Further investigations are undergoing on the interactions between the sulfate groups in sulfated polysaccharides and the surface glycoprotein gp 120 of AIDS virus.

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