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Synthesis, Structure and Antiviral Activity of Sulfates of Cellulose and its Branched Derivatives

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ABSTRACT

Cellulose and branched cellulose having D*-glucopyranose or* D*-galactopyranose as side chains were sulfated with piperidine N-sulfonic acid or SO_3–dimethylformamide complex. The structures of the sulfated polysaccharides were analysed by elemental analysis and ^{13}[C]-NMR spectroscopy. The ^{13}[C]-NMR spectra of cellulose sulfate were assigned and degree of substitution values of each hydroxyl group were obtained. The reactivity was in the order C-6 ≫ C-2 > C-3. Sulfated polysaccharides with high degree of substitution values showed high anti-human-immunodeficiency-virus activity.*

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INTRODUCTION

We have tried to synthesize antitumor branched polysaccharides having cellulose ((1→4)-β-D-glucan) or curdlan ((1→3)-β-D-glucan) as the main chain and various kinds of sugars as the side chains (Matsuzaki *et al.*, 1985, 1986*a–c*, 1988; Yamamoto *et al.*, 1988) to simulate naturally occurring antitumor polysaccharides such as lentinan and krestin. Some of them, especially branched polysaccharides derived from curdlan having D-glucopyranose, D-galactopyranose, D- or L-arabinofuranose or their oligomers as side chains showed high antitumor activity against Sarcoma 180 implanted in mice. Antitumor activity of cellulose derivatives was generally low.

It has been reported that a sulfated polysaccharide in sea algae has anti-human-immunodeficiency-virus (anti-HIV) activity *in vitro* (Nakashima *et al.*, 1987). Furthermore, sulfated polysaccharides derived from lentinan and sulfates of branched polysaccharides derived from (1→3)-β-D-glucan as the main chain, having D-arabinofuranose oligomer or D-galactopyranose as side chains, showed high anti-HIV activity (Yoshida *et al.*, 1988).

In this paper, sulfation of cellulose and its branched derivatives, and structure and antiviral activity of the sulfated polysaccharides are reported. We found that the sulfated polysaccharides derived from cellulose showed high antiviral activity as well as sulfated lentinan and sulfates of branched polysaccharides which have structures similar to lentinan.

EXPERIMENTAL

Materials

Cellulose: cellulose acetate (degree of substitution (DS), 1·74; degree of polymerization 110, Daicel Co.) was deacetylated with 0·5 N NaOH solution at room temperature for 17 h. Regenerated cellulose was washed with water and acetone, and dried *in vacuo.*

Branched polysaccharides derived from cellulose

Branched polysaccharides (CEGL and CEGA) were synthesized by the reaction of 3,4,6-tri-*O*-acetyl-(1,2-*O*-ethylorthoacetyl)-α-D-glucopyranose or 3,4,6-tri-*O*-acetyl-(1,2-*O*-ethylorthoacetyl)-α-D-galactopyranose with cellulose acetate in chlorobenzene with 2,6-dimethyl-pyridinium

perchlorate as catalyst. The degree of branching (number of branches per 100 glucosidic residues in the main chain) was 97% (CEGL) and 66% (CEGA), respectively.

Sulfation of branched polysaccharides with piperidine N-sulfonic acid (CEGLS-1 and CEGAS-1)

Branched polysaccharide (0·5 g) was reacted with piperidine *N*-sulfonic acid (3 g) (Nagasawa & Yoshidome, 1969) in DMSO (150 ml) at 90°C for 1 h. The sulfated polymer solution was neutralized with saturated sodium hydrogen carbonate solution and dialysed against saturated sodium hydrogen carbonate solution for 12 h, and then against deionized water for 1 week. The solution was concentrated under reduced pressure and freeze-dried. The yield was 0·76 g for CEGLS-1 and 0·52 g for CEGAS-1.

When the elemental analysis of sulfated polysaccharides indicated a nitrogen content of more than 0·5%, the samples were purified by passing through a column of ion exchange resin (Diaion SK-1B, sodium sulfonate form) to remove piperidine.

Sulfation of branched polysaccharides with chlorosulfonic acid (CEGLS-2 and CEGAS-2)

Chlorosulfonic acid (10 ml) was added dropwise in pyridine (60 ml) at 0°C. The branched polysaccharide (0·5 g) swollen in pyridine was then added into the chlorosulfonic acid solution and the solution was heated at 100°C for 1 h. After the reaction, the solution was cooled, neutralized with 2·5 N sodium hydroxide solution, dialysed against deionized water, and concentrated. The sulfate was obtained by freeze-drying. The yield was 0·91 g for CEGLS-2 and 0·92 g for CEGAS-2.

Sulfation of cellulose with SO_3-DMF complex

Various methods for sulfation of cellulose were discussed by Schweiger (1972). He found that SO_3–DMF complex is the most suitable reagent for sulfation of cellulose, in order to avoid degradation of cellulose and to attain a high degree of substitution. Cellulose (2 g) was stirred in DMF (20 ml) at room temperature for 12 h. The SO_3–DMF complex (DMF 7·3 g, weight of SO_3 shown in Table 1), was then added and the mixture stirred at room temperature for 4 h and then at 70°C for 30 min. After the reaction, the slurry was poured into water (200 ml), neutralized with 1 N sodium hydroxide solution, and precipitated into methanol (1·2 liters). The precipitate was redissolved in water (300 ml) and dialysed against water for 5 days. The insoluble portion was removed by centrifugation. The solution was concentrated and freeze-dried. The yield is

TABLE 1
Synthesis and Elemental Analyses of Cellulose Sulfates

Sample no.	*Synthetic condition*[a]		*Elemental analyses*			*Degree of substitution*
	SO_3 (g)	*Yield (g)*	*C (%)*	*H (%)*	*S (%)*	
CES-4	0·5	0·04	n.d.[b]	n.d.	n.d.	n.d.
CES-5	1·0	0·31	25·14	3·47	8·6	0·77
CES-8	1·5	1·10	20·45	2·78	13·2	1·45
CES-1	2·0	1·36	15·21	2·06	17·0	2·51
CES-2	4·0	1·19	13·61	1·74	18·4	3·05
CES-3	8·0	0·59	12·55	1·98	16·0	2·87

[a]Cellulose, 2·0 g; DMF, 7·3 g.
[b]n.d., not determined.

shown in Table 1. The polymer samples were purified with ion exchange resin columns, when necessary.

Methods

Elemental (C, H and N) analyses of the sulfated polysaccharides were carried out with a Perkin–Elmer 240B elemental analyser. Sulfur content was determined by the Toray Research Center. The sample was burnt in a Pyrex flask with an aqueous solution of hydrogen peroxide. The sulfate ion produced was determined with a DIONEX ion chromatograph apparatus.

^{13}[C]-NMR spectra were recorded on D_2O solutions at 50°C with a Bruker AC 250 spectrometer at 62·90 MHz with sodium 2,3-dimethyl-2-silapentane-5-sulfonate as the reference under the following conditions: data points 65536, pulse width 1·5 ms, acquisition time 1·966 s, number of scans 20 000–30 000.

Gel permeation chromatography was carried out with a Toso HLC-802A HPLC equipped with Toso TSK gel G-4000SW, G-3000SW and G-2000SW columns. The eluent was 0·1 M phosphate buffer. Standard dextrans were used as the reference for molecular weight.

Anti-HIV activity was assayed as follows: MT-4 cells were infected with HIV at a multiplicity of infection of 0·002 at 37°C for 60 min. After washing a mixture of uninfected and infected MT-4 cells, the number of

TABLE 2
Elemental analyses and Degree of Substitution of Sulfates of Branched Polysaccharides Derived from Cellulose

Sample[a] *no.*	*C (%)*	*H (%)*	*S (%)*	*Degree of substitution*
CEGLS-1	18·80	3·90	14·76	1·77
CEGLS-2	13·16	3·19	17·04	2·90
CEGAS-1	19·93	3·81	15·06	1·70
CEGAS-2	13·21	3·31	15·97	2·72

[a]CEGLS and CEGAS indicate sulfates of branched polysaccharides derived from cellulose with glucosyl and galactosyl side chains, respectively. 1 and 2 indicate sulfates synthesized with piperidine *N*-sulfonic acid and chlorosulfonic acid, respectively.

cells was adjusted to 3×10^5 cells/ml and cultured in the absence or presence of varying concentrations of sulfated polysaccharides. On the third and sixth day after infection, the number of viable cells was counted by the trypan blue dye exclusion method.

RESULTS AND DISCUSSION

Sulfation of branched polysaccharides is carried out easily with piperidine *N*-sulfonic acid in DMSO or with chlorosulfonic acid in pyridine. Table 2 shows the results of elemental analyses and DS values calculated from elemental analyses. The table indicates that DS values of CEGLS-1 and CEGAS-1 obtained with piperidine *N*-sulfonic acid are *c.* 1·7, whereas those of CEGLS-2 and CEGAS-2 obtained with chlorosulfonic acid are 2·7–2·9, indicating that higher substitution takes place on sulfation with chlorosulfonic acid.

Therefore, sulfation of cellulose was carried out using the SO_3-DMF complex. Table 1 shows that there is an optimum amount of SO_3 required to get the highest yield of cellullose sulfate. The elemental analyses indicate that DS values are 0·77–3·05.

Since ^{13}[C]-NMR spectra of sulfates of branched polysaccharides are complicated, we first tried to elucidate the structure of the linear cellulose sulfate. The NMR spectra of cellulose sulfates were reported by Kamide and Okajima (1981) and by Philippe *et al.* (1987). Kamide and Okajima determined proton and ^{13}[C]-NMR spectra of a cellulose sulfate obtained by reaction with the SO_3–DMF complex. Although the resolution of the NMR spectra was poor, they concluded that the

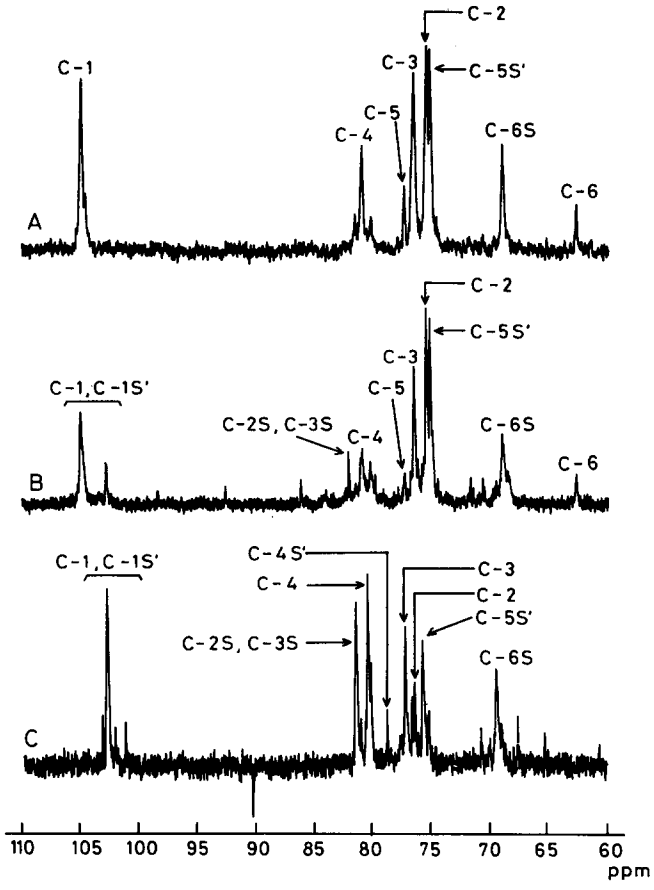

Fig. 1. ^{13}[C]-NMR spectra of cellulose sulfates. A: CES-5 (DS, 0·76); B: CES-8 (DS 1·12); C: CES-2 (DS 1·60).

reactivity of hydroxyl groups is in the order C-2 > C-3 > C-6. Philippe and co-workers determined NMR spectra of cellulose sulfates obtained by reacting SO_2 or SO_3 with cellulose dissolved in NO_2–DMF. They observed that the preferential substitution occurs at C-6, but did not give detailed assignments for the NMR spectra.

Figure 1 shows ^{13}[C]-NMR spectra of cellulose sulfates with various DS values. The assignment was carried out assuming that a large low field shift of the carbon occurs when bonded to the substituent and a small high field shift of the neighboring carbon occurs by substitution. The change in chemical shifts by glucosylation (Matsuzaki *et al.*, 1986b) was also considered. The assignment is shown in Table 3. S indicates substituted carbon and S′ indicates neighboring carbon to the substituted

TABLE 3
^{13}[C]-NMR Chemical Shifts of Cellulose Sulfates (ppm)

Sample degree of substitution[a]	*CES-5 0·76*	*CES-8 1·12*	*CES-2 1·60*
C-1	104·9	104·8	
C-1S′	104·6	102·8	101·3–103·3
C-2	75·5	75·4	76·5
C-2S		82·2	81·5
C-3	76·6	76·5	77·2
C-4	81·0	81·0	80·5
C-4S′			78·9
C-5	77·4	77·4	
C5S′	75·2	75·1	75·8
C-6	62·7	62·7	
C-6S	69·0	68·9	69·4

[a]Determined by ^{13}[C]- NMR spectra. S: Substituted carbon; S′: neighboring carbon to the substituted carbon.

TABLE 4
Degree of Substitution of Hydroxyl Groups in Cellulose Sulfates

	CES-5	*CES-8*	*CES-2*
Degree of substitution:[a]	0·76 (0·77)	1·12 (1·45)	1·60 (3·05)
C-6	0·76	0·87	1·00
C-3	0	0	0·11
C-2	0	0·25	0·49

[a]Values in parentheses are calculated from elemental analyses.

carbon. The chemical shifts for samples CES-5 and CES-8 agree well, but those for CES-2 shifted a little. The shifting may be due to change of chain conformation of molecules induced by dissociating groups of high density.

The DS values at C-6 were obtained by comparison of the unsubstituted C-6 signal with the substituted C-6S signal (or comparison of the C-5 signal with the C-5S′ signal). The DS values at C-3 were obtained by comparing the C-4 signal with the C-4S′ signal. The DS values at C-2 were obtained by comparing the area of the C-2S + C-3S signal with C-2 and C-3 signals, and substracting the C-3 substitution. The sum of the C-6, C-3 and C-2 substitution shown in Table 4 generally agrees with the

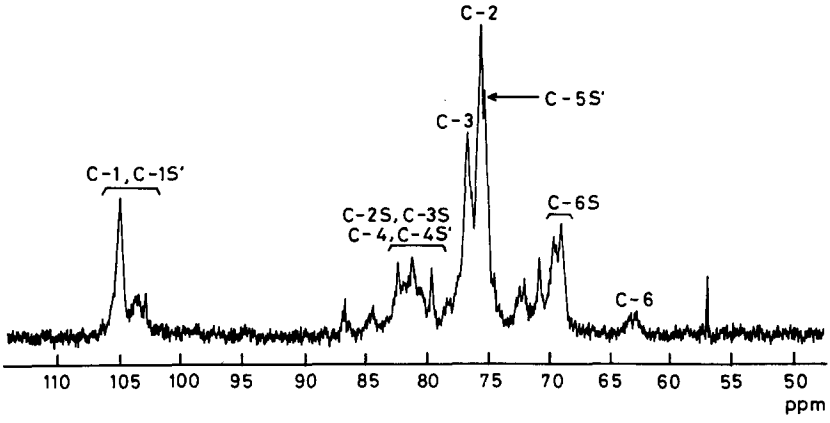

Fig. 2. ^{13}[C]-NMR spectrum of sulfate of a branched cellulose having glucosyl branches (degree of branching 15%).

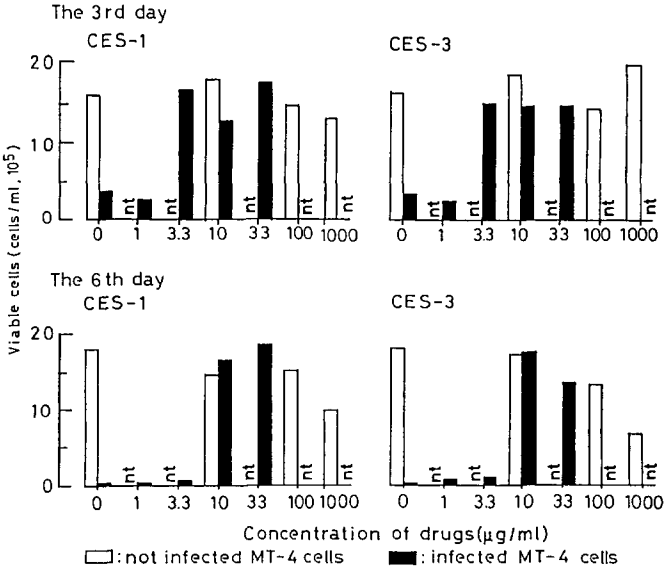

Fig. 3. Anti-HIV activity of cellulose sulfates (CES-1 and CES-3) expressed as number of viable MT-4 cells after 3 and 6 days of incubation.

total substitution calculated from elemental analyses for samples with low DS values, but not for samples with high DS values. The reason for the discrepancy may be due to contamination of samples with unidentified sulfur compounds derived from SO_3. From the table it is seen that

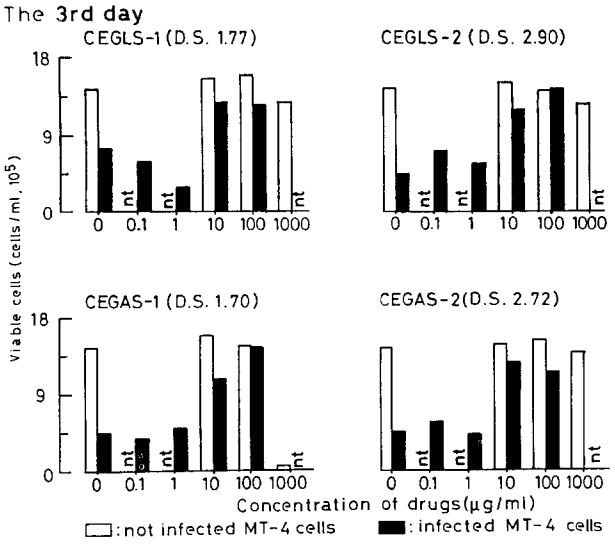

Fig. 4. Anti-HIV activity of sulfates of branched celluloses having glucosyl and galactosyl branches (CEGLS-1 and CEGLS-2, CEGAS-1 and CEGAS-2) expressed as number of viable MT-4 cells after 3 days of incubation.

the reactivity is in the order C-6 ≫ C-2 > C-3, which is the same as for glucosylation with orthoesters (Matsuzaki *et al.*, 1985) and esterification (Takahashi *et al.*, 1986).

Figure 2 shows the ^{13}[C]-NMR spectrum of a sulfate of a branched polysaccharide derived from cellulose having glucosidic side chains (degree of branching 15%). The assignment is shown in the figure. Quantitative estimation of DS value at each hydroxyl group could not be made due to the complexity of the spectrum.

Figures 3–5 indicate the anti-HIV activity of sulfated polysaccharides. The anti-HIV activity is expressed as the number of viable cells on the third and sixth days of incubation of partially infected MT-4 cells in the presence of sulfated polysaccharides (shaded bars in the figures). Control experiments on uninfected MT-4 cells in the presence of sulfated polysaccharides were also carried out (open bars), in order to examine the cytotoxicity of the compounds. The toxicity of sodium cellulose sulfates has been discussed by Kamide *et al.* (1983) and Rothschild & Castania (1968). In our control experiments, decrease of viable cells was generally observed at 1000 μg/ml of dose, especially for CEGAS-1 and CEGAS-2. It is deduced that sulfates of galactose units are more toxic than those of glucose units.

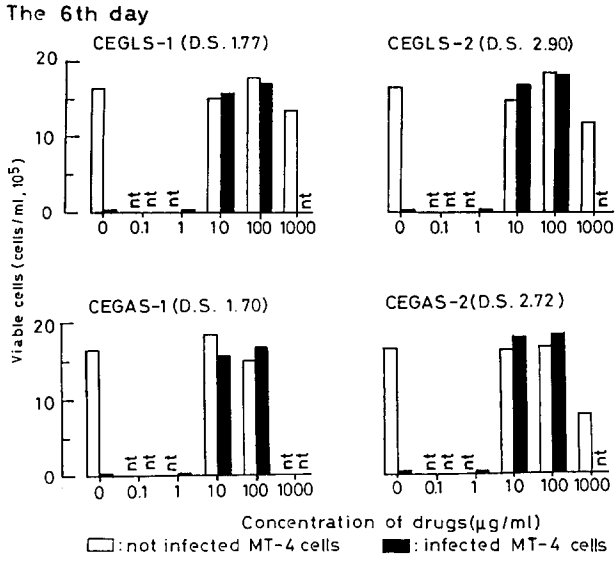

Fig. 5. Anti-HIV activity of sulfates of branched celluloses having glucosyl and galactosyl branches expressed as number of viable MT-4 cells after 6 days of incubation.

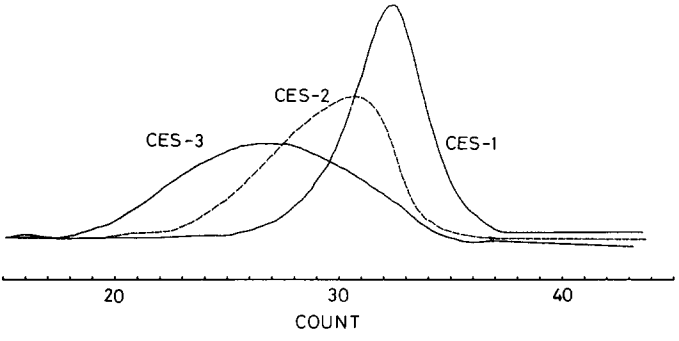

Fig. 6. Gel permeation chromatograms of sulfated celluloses, CES-1, CES-2 and CES-3.

On the sixth day of incubation, MT-4 cells partially infected with HIV were viable in the presence of concentrations of sulfated polysaccharides higher than 10 μg/ml (shaded bars at 10 μg/ml of concentration in Figs 3, 4 and 5), whereas in the absence of the compounds the cells were all dead on the sixth day (shaded bars at 0 μg/ml of concentration in Figs 3, 4 and 5). It is suggested that the anti-HIV activity of these sulfated compounds, is due to the inhibition of viral binding with MT-4 cells (Yarchoan *et al.*, 1988).

Figure 6 shows the gel permeation chromatography elution profiles of some samples. The number-average molecular weights of CES-1, CES-2 and CES-3 calculated from the curves are 4600, 8000 and 13 000, respectively. It is noted that CES-1 of a low molecular weight shows the same anti-HIV activity as that of the polymers of high molecular weights.

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