Carbohydrate Research, 214 (1991) 147–154
Elsevier Science Publishers B.V., Amsterdam

A strong inhibition of HIV-induced cytopathic effects by synthetic (1→6)-α-D-mannopyranan sulfate*

Kenichi Hatanaka[†], Yasumi Kurihara, Toshiyuki Uryu[‡]
Institute of Industrial Science, University of Tokyo, Roppongi, Minato-ku, Tokyo 106 (Japan)

Osamu Yoshida, Naoki Yamamoto
Department of Virology and Parasitology, Yamaguchi University School of Medicine, Kogushi, Nishi-ku, Ube-shi, Yamaguchi 755 (Japan)

Toru Mimura, and Yutaro Kaneko
Ajinomoto Co., Inc., Chuo-ku, Tokyo 108 (Japan)

(Received August 13th, 1990; accepted for publication in revised form January 5th, 1991)

ABSTRACT

The anti-HIV effects of mannopyranan sulfate (**1**) were investigated by using MT-4 cells, namely, an HTLV-I-carrying CD-4 positive cell-line, *in vitro*. Stereoregular (1→6)-α-D-mannopyranan, which had been synthesized by ring-opening polymerization of a 1,6-anhydromannose derivative, was sulfated with piperidine-*N*-sulfonic acid to provide **1**. N.m.r. analysis of **1** indicated that the reactivity of hydroxyl groups was in the order, 3-OH > 2-OH >> 4-OH. Mannopyranan sulfate having degree of substitution (d.s.) of 1.19–1.83 effectively inhibited HIV-induced cytopathic effects at a concentration of >3.3 μg/mL. The anticoagulant activity and the adsorption on concanavalin A of **1** indicated the possibility of selective binding of **1** having d.s. of 1.19–1.83 to HIV-protein.

INTRODUCTION

Such natural sulfated polysaccharides as heparin and heparan sulfate, and such synthetic heparinoids as dextran sulfate show anticoagulant activity. We reported that sulfation of linear glycans, been synthesized by ring-opening polymerization of anhydro sugar derivatives, gave new heparinoids having high anticoagulant activity[1]. It was relatively easy to synthesize heparinoids with various proportions of sulfate groups[2,3].

Recently, it was reported that sulfated polysaccharides showed anti-HIV (human immunodeficiency virus = AIDS virus) effects. Lentinan sulfate[2,4] and curdlan sulfate[5,6], prepared by sulfation of the natural polysaccharides, strongly inhibited HIV-induced cytopathic effects *in vitro*. Such sulfated synthetic linear pentofuranans as ribofuranan sulfate[3,7] and xylofuranan sulfate[7], also had anti-HIV activity. These sulfated polysaccharides showed a very low toxicity against MT-4 cell growth.

---

* Presented at the 15th International Carbohydrate Symposium, Yokohama, Japan, August 12–17, 1990.

[†] Present address: Department of Biomolecular Engineering, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Midori-ku, Yokohama 227, Japan.

[‡] To whom correspondence should be addressed.

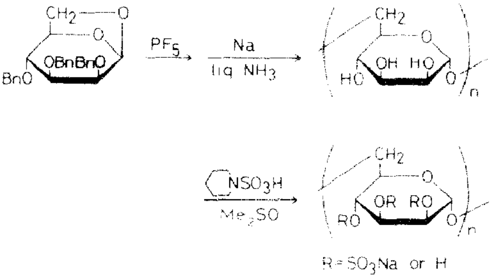
0008-6215/91/$03.50

In this paper, we report the sulfation of stereoregular linear (1→6)-α-D-mannopyranan[8] prepared by ring-opening polymerization of 1,6-anhydro-2,3,4-tri-*O*-benzyl-β-D-mannopyranose followed by deprotection of the product. The effect of the degree of sulfation on anti-HIV activity of mannopyranan sulfate was investigated *in vitro*. The *in vitro* anticoagulant activity of mannopyranan sulfate, and the binding of mannopyranan sulfate to concanavalin A–Sepharose are also reported.

RESULTS AND DISCUSSION

A stereoregular (1→6)-α-D-mannopyranan was prepared by ring-opening polymerization of 1,6-anhydro-2,3,4-tri-*O*-benzyl-β-D-mannopyranose with phosphorus pentafluoride as a catalyst at −60° followed by debenzylation[8]. The mannopyranan, having $[\alpha]_D$ +102° and number-average molecular weight of 7.5×10^4 was sulfated with piperidine-*N*-sulfonic acid (**2**) in dimethyl sulfoxide[9] (Scheme I). The results are summarized in Table I.

Sulfation of mannopyranan was carried out at 75–85° for 1 h using **2** of 1–3 times the molar quantity of hydroxyl groups in the mannopyranan. Mannopyranan sulfates (**1**) having sulfur content of 10–16% were obtained. In the range of 75–85°, the degree of



(**1**)

Scheme I.

TABLE I

Sulfation of mannopyranan with piperidine-*N*-sulfonic acid[a]

No.	*Mannan (g)*	**2**[b] *(g)*	*Me₂SO (mL)*	*Temp. (°C)*	*Yield (g)*	*S-content (%)*	D.s.[c]	$\bar{M}_n$ ($\times 10^4$)
1	0.1	0.31	30	80	0.13	10.13	0.98	9.3
2	0.1	0.46	30	80	0.16	11.50	1.19	12.1
3	0.1	0.46	30	85	0.14	11.79	1.22	10.1
4	0.2	0.92	60	80	0.33	13.81	1.56	16.4
5	0.15	0.69	45	85	0.30	15.07	1.83	10.8
6	0.1	0.61	30	75	0.22	15.20	1.97	9.2
7	0.1	0.61	30	80	0.18	15.65	1.99	10.7
8	0.1	0.92	30	80	0.21	16.06	2.12	4.7

[a] Reaction time, 1 h. [b] Piperidine-*N*-sulfonic acid. [c] Number of sulfate groups per sugar unit.

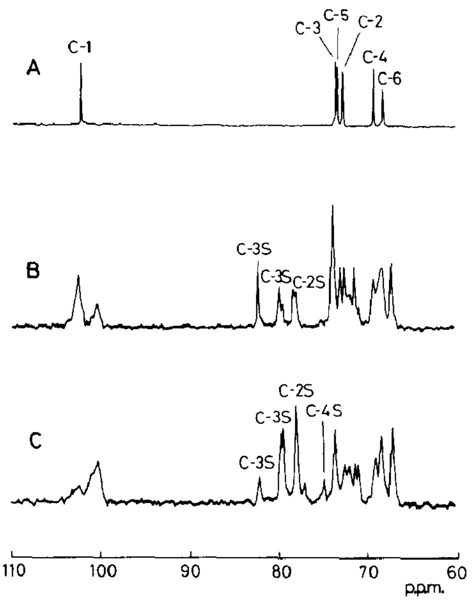


Fig. 1. ^{13}C-N.m.r. spectra of (A): (1→6)-α-D-mannopyranan, (B): **1** (d.s. 1.19), and (C): **1** (d.s. 1.97).

sulfation of the **1** obtained was not much affected by reaction temperature but was governed by the amount of **2**. When **2** of 1–2 times the molar quantity of the hydroxyl groups was used, main-chain scission in the mannopyranan scarcely occurred, whereas the number-average molecular weight was decreased when 3 times as much **2** was used (no. 8 in Table I). The number of sulfate groups per sugar unit (= d.s.) as calculated from elemental analysis data was 0.98–2.12, as shown in Table I, indicating that 30–70% of the hydroxyl groups of the mannopyranan had been sulfated under these conditions.

The ^{13}C-n.m.r. spectra of **1** and OH-free mannopyranan are shown in Fig. 1. Assignments were performed by the ^{1}H–^{13}C COSY method. In the spectrum of **1** of d.s. 1.19 (Fig. 1B), the resonance assignable to substituted C-4 carbon was almost negligible. On the other hand, in a polymer of higher d.s. (1.97), 11% of C-4 was substituted with sulfate groups, as shown in the ^{13}C-n.m.r. spectrum (Fig. 1C). The distribution of sulfate

TABLE II

Distribution of sulfate groups in the mannopyranan sulfate

No.	*D.s.*[a]	*Monosulfated at C-2*	*Monosulfated at C-3*	*Disulfated at C-2 and C-3*	*Sulfated at C-4*
2	1.19	0.04	0.34	0.40	0
6	1.97	0.09	0.15	0.76	0.11

[a] Calculated based on elemental analysis.

groups on C-2, C-3, and C-4 is shown in Table II. A sample of **1** of higher d.s. (no. 6) consisting mainly of disubstituted (C-2 and C-3) product, whereas **1** of lower d.s. was relatively rich in residues monosubstituted at C-3. The reactivity of hydroxyl groups was in the order, 3-OH > 2-OH > > 4-OH.

The anti-HIV effects of **1** were based on the inhibition of HIV-induced cytopathic effects (CPE) and the expression of HIV-specific antigen in MT-4 cells, which are[4,5] a human CD4-positive cell-line carrying HTLV-I. The decrease in 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 day after HIV infection.

At any concentrations < 5000 μg/mL, **1** did not inhibit cell growth (Fig. 2, open bars), revealing that the toxicity of **1** to cell growth is very low. Compound **1** of d.s. 0.98 (no. 1 in Table I), did not show anti-HIV effect (Fig. 2A), whereas samples of **1** having d.s. of 1.19–1.83 (no. 2–5), effectively protected MT-4 cells from destruction by HIV infection at a concentration of 3.3 μg/mL (Fig. 2B). However, more-substituted **1** (no. 6–8, d.s. = 1.19–2.12) protected MT-4 cells at higher concentrations (Fig. 2C). The effect of the degree of sulfation on anti-HIV activity of **1** is shown in Fig. 3, and compared with the activity of ribofuranan sulfate (**3**)[2]. The moderately substituted **1** showed the highest anti-HIV activity, even though it was reported[5] that more-substituted **3** had higher activity than less-substituted **3**[2].

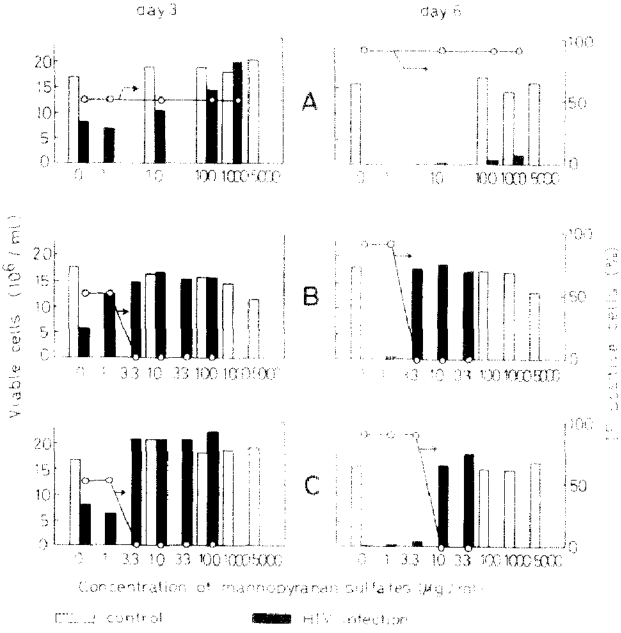


Fig. 2. Effects of **1** on cell growth, HIV-induced cytopathic effects, and percentage of immunofluorescence-positive cells. A: d.s. 0.98; B: d.s. 1.22; C: d.s. 1.99.

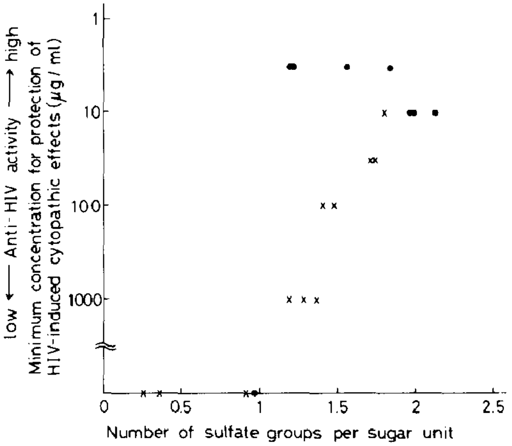


Fig. 3. Effect of degree of sulfation of **1** (●) and ribofuranan sulfate (**3**)[3] (×) on the anti-HIV activity.

It is well-known that such sulfated polysaccharides as heparin binds to blood proteins such as antithrombin III to inhibit coagulation of the blood. The effect of degree of sulfation on the anticoagulant activity of **1** is shown in Fig. 4. By analogy[3] with **3**[3], **1** of higher d.s. showed higher anticoagulant activity. As may be seen from Table III, **1** having degrees of sulfation of 1.19–1.83 showed the highest anti-HIV activity and relatively low anticoagulant activity, suggesting that **1** having certain levels of sulfation might slightly favor binding to AIDS virus protein over reacting with other plasma proteins.

Adsorption of **1** on concanavalin A (conA)–Sepharose was also investigated. More than 70% of **1** applied onto a conA–Sepharose column was not adsorbed and was

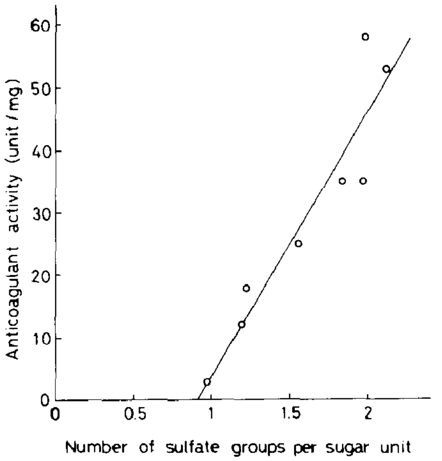


Fig. 4. Relationship between deree of sulfation on **1** and the anticoagulant activity.

TABLE III

Biological activities of mannopyranan sulfate

No.	*S-content* (%)	*D.s.*[a]	$\bar{M}_n$ ($\times 10^4$)	*Anti-HIV*[b] ($\mu g/mL$)	A. A.[c] (U/mg)
1	10.13	0.98	9.3	> 1000	3
2	11.50	1.19	12.1	3.3	12
3	11.79	1.22	10.1	3.3	18
4	13.81	1.56	16.4	3.3	25
5	15.07	1.83	10.8	3.3	35
6	15.20	1.97	9.2	10	35
7	15.65	1.99	10.7	10	58
8	16.06	2.12	4.7	10	53

[a] Number of sulfate groups per mannose unit. [b] Minimum concentration for protection of HIV-induced cytopathic effects. [c] Anticoagulant activity.

eluted by water. The adsorbed **1** could not be eluted off with 0.3M methyl α-D-mannopyranoside but was eluted with 0.5M NaCl, indicating that the interaction between **1** and conA–Sepharose was of ionic character.

Anti-HIV activity, anticoagulant activity, and the adsorption on conA-Sepharose of **1** were compared with those of **3**[3] and lentinan sulfate (**4**)[2] in Table IV. These data also indicate the possibility of a slightly selective reaction of **1** having d.s. of 1.22 with HIV.

TABLE IV

Biological activities and adsorption on conA Sepharose of sulfated polysaccharides

Kind	*D.s.*	*Anti-HIV activity* ($\mu g/mL$)	*Anticoagulant activity* (unit/mg)	*Adsorption on* conA *Sepharose* (%)	*Ref.*
1	1.22	3.3 (+ + + +)[a]	18 (+)	7.1 (+)	
1	1.99	10 (+ + +)	58 (+ + +)	22.9 (+ + +)	
3[b]	1.41	100 (+)	14 (+)	13.9 (+ +)	3
3	1.71	33 (+ +)	24 (+ +)	22.6 (+ + +)	3
3	1.80	10 (+ + +)	30 (+ +)	20.9 (+ + +)	3
4[c]	1.69	3.3 (+ + + +)	54 (+ + +)	21.1 (+ + +)	2
4	1.95	3.3 (+ + + +)	21 (+ +)	24.3 (+ + +)	2

[a] The number of plus marks shows relative strength of the activity or the adsorption. [b] Ribofuranan sulfate. [c] Lentinan sulfate.

Further investigations on conformational analysis between **1** and viral protein, and the effect of homogeneity and molecular weight of **1** should be done to clarify the interaction between sulfated polysaccharides and HIV-protein.

EXPERIMENTAL

*Mannopyranan sulfate (***1***).* — 1,6-Anhydro-2,3,4-tri-*O*-benzyl-β-D-mannopyranose was polymerized with PF_5 as catalyst to give a stereoregular benzylated (1→6)-α-D-mannopyranan according to the precedure of Fréchet and Schuerch[8]. The linear mannopyranan derivative was debenzylated with Na in liquid NH_3 and the OH-free mannopyranan obtained was sulfated[9] with piperidine-*N*-sulfonic acid (**2**) in Me_2SO under various conditions, as shown in Table I. The mixture was made neutral by saturated $NaHCO_3$ solution in an ice bath, and poured into acetone. Then crude mannopyranan sulfate was separated by centrifugation, washed with acetone, and dialyzed overnight with running distilled water. The product **1** was freeze-dried from water, it was moisture sensitive and was stored at -20°.

Anti-HIV assay. — The anti-HIV activity of **1** was analyzed by measuring the number of viable cells and the percentage of viral antigen-positive cells[10]. The anti-HIV activity of **1** was assayed in terms of the inhibition of HIV-induced cytopathic effects in MT-4 cells, a human CD4-positive cell-line carrying HTLV-I. MT-4 cells and MT-4 cells infected with HIV at a multiplicity of infection of 0.002 were adjusted to 3×10^5 cells/mL and cultured in the presence of various concentrations of the compounds. The effects of the compounds were monitored by counting the viable cells (identified by the Trypan Blue dye exclusion method) 3 and 6 days after infection. The inhibitory effect of **1** was also investigated in an assay of the HIV-specific antigens. MT-4 cells infected with HIV at a multiplicity of infection of 0.002 were cultured in the presence of various concentration of **1**. The inhibitory effect of **1** on the expression of HIV-specific antigen was determined by the indirect immunofluorescence (IF) method, using a seropositive anti-HIV human serum and an anti-human IgG conjugated with a fluorescent substance. More than 500 celles were counted under a fluorescence microscope and the percentage of IF-positive cells was calculated 3 and 6 days after infection.

Anticoagulant activity. — The anticoagulant activity of **1** was measured by use of bovine serum according to a modification of the United States Pharmacopoeia method as described previously[1]. Dextran sulfate (Meito Sangyo NC-1032) was used as a control.

Adsorption on concanavalin A–Sepharose. — An aqueous solution of **1** (0.2 mg/mL, 2 mL) was applied to the concanavalin A–Sepharose (Sigma) column (1 mL). After washing with water (1 mL, twice), **1** was eluted by 0.5M NaCl (1 mL, twice). Washings and eluates were mixted with 1 mL of Toluidine Blue solution and the absorbance was measured at 516 nm.

Measurements. — N.m.r. spectra of **1** were recorded on a JEOL GX-270 spectrometer in D_2O using sodium 4,4-dimethyl-4-silapentane-1-sulfonate as internal standard. The number-average molecular weight of **1** was determined by gel-permeation chromatography (columns, Tosoh TSK gel; eluant, 66.7mM phosphate buffer, pH 6.86) using standard dextran as reference. Absorptions were measured by a Jasco Uvidec-505 digital spectrophotometer. Elemental analyses were performed by Toray Research Center, Inc.

REFERENCES

1 K. Hatanaka, T. Yoshida, S. Miyahara, T. Sato, F. Ono, T. Uryu, and H. Kuzuhara, *J. Med. Chem.*, 30 (1987) 810–814.

2 K. Hatanaka, T. Yoshida, T. Uryu, O. Yoshida, H. Nakashima, N. Yamamoto, T. Mimura, and Y. Kaneko, *Jpn. J. Cancer Res.*, 80 (1989) 95–98.

3 K. Hatanaka, I. Nakajima, T. Yoshida, T. Uryu, O. Yoshida, N. Yamamoto, T. Mimura, and Y. Kaneko, *J. Carbohydr. Chem.*, in press.

4 H. Nakashima, O. Yoshida, T. S. Tochikura, T. Yoshida, T. Mimura, Y. Kido, Y. Motoki, Y. Kaneko, T. Uryu, and N. Yamamoto, *Jpn. J. Cancer Res.*, 78 (1987) 1164–1168.

5 T. Yoshida, K. Hatanaka, T. Uryu, Y. Kaneko, F. Suzuki, H. Miyano, T. Mimura, O. Yoshida, and N. Yamamoto, *Macromolecules*, in press.

6 Y. Kaneko, O. Yoshida, R. Nakagawa, T. Yoshida, M. Date, S. Ogihara, S. Shioya, Y. Matsuzawa, N. Nagashima, Y. Irie, T. Mimura, H. Shinkai, N. Yasuda, K. Matsuzaki, T. Uryu, and N. Yamamoto, *Biochem. Pharm.*, 39 (1990) 793–797.

7 O. Yoshida, H. Nakashima, T. Yoshida, Y. Kaneko, I. Yamamoto, T. Uryu, K. Matsuzaki, and N. Yamamoto, *Biochem. Pharm.*, 37 (1988) 2887–2891.

8 J. Fréchet and C. Schuerch, *J. Am. Chem. Soc.*, 91 (1969) 1161–1164.

9 K. Nagasawa, H. Harada, S. Hayashi, and T. Misawa, *Carbohydr. Res.*, 21 (1972) 420–426.

10 Y. Hamamoto, H. Nakashima, T. Mitsui, A. Matsuda, T. Ueda, and N. Yamamoto, *Antimicrob. Agents Chemother.*, 31 (1987) 907–910.