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Carbohydrate Research 306 (1998) 427–433

CARBOHYDRATE RESEARCH

Regioselective syntheses of sulfated polysaccharides: specific anti-HIV-1 activity of novel chitin sulfates

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Received 18 July 1997; accepted in revised form 1 November 1997

Abstract

A novel and convenient method for the regioselective syntheses of sulfated analogs of chitin and chitosan is described in relation to studies on structure–biological activity. Fully protected, soluble derivatives of chitosan were found to be useful intermediates for the syntheses of a novel class of sulfated polysaccharides, 2-acetamido-2-deoxy-3-*O*-sulfo-(1→4)-β-D-glucopyranan (3-sulfate, 3S, **4**) and (1→4)-2-deoxy-2-sulfoamido-3-*O*-sulfo-(1→4)-β-D-glucopyranan (2,3-disulfate, 23-S, **3**). These compounds were tested for their activities in (*i*) inhibiting HIV-1 replication in vitro and (*ii*) inhibiting blood coagulation. The results reveal that the selective sulfation at O-2 and/or O-3 affords potent antiretroviral agents showing a much higher inhibitory effect on the infection of AIDS virus in vitro than that by the known 6-*O*-sulfated derivative (6-sulfate, 6S). Moreover, the 23-S product completely inhibited the infection of AIDS virus to T lymphocytes at concentrations as low as 0.28 μg/mL without significant cytotoxicity. The regioselective introduction of sulfate group(s) at O-2 and/or O-3 had little effect on generating anticoagulant activity, whereas 6-*O*-sulfated chitin strongly inhibits blood coagulation. These results suggest that the specific interaction of these new types of chitin sulfates with gp 120 of the AIDS virus depends significantly on the sites of sulfation rather than on the total degree of substitution on sugar residues.

Keywords: Chitin; Chitosan; Regioselective modifications; Chitin sulfates; Anti-HIV

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is the causative retrovirus of acquired immune deficiency syndrome (AIDS) and related disorders [1,2]. Although some nucleoside analogs, such as 3′-azido-3′-deoxythymidine (AZT), dideoxyinosine (ddI), and dideoxycytidine (ddC), have been shown to prolong survival and improve the quality of life of individuals with advanced HIV infection, the

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0008-6215/98/$19.00
PII S0008-6215(97)10081-7

serious toxicity and side effects of these dideoxynucleoside families and the advent of drug-resistant strains of the HIV-1 creates an urgent need to develop new types of anti-HIV-1 reagents having different inhibitory mechanisms from those of nucleoside analogs [3].

Much recent interest has been directed towards the specific modulating effects of a variety of glycosaminoglycans (GAGs) on those extracellular biological processes dependent on protein–carbohydrate interactions [4]. GAGs and chemically designed sulfated polysaccharides have been regarded as a new class of potential inhibitors or effector molecules in biochemical and medicinal fields concerned with cellular surfaces and extracellular matrices (ECM) [5]. The interaction of GAGs or related polyanionic compounds with the major envelope glycoprotein (gp 120) of HIV-1 also is one of the most important factors in connection with regulation of the initial stage of HIV-1 infection in human T lymphocytes [6,7]. In addition to basic studies on the interaction of glycosaminoglycans with envelope glycoproteins, a variety of sulfated polysaccharides have been reported to show potent inhibitory effects on the replication of AIDS virus in vitro. Uryu and his coworkers found that curdlan sulfate, with a linear β-(1→3)-linked glucopyranose backbone, strongly inhibits AIDS virus infection in vitro in drug concentrations as low as 3.3 μg/mL and it was not cytotoxic in vitro [8]. At present, this sulfated polysaccharide is regarded as one of the most potent and practical antiretroviral polysaccharide reagents. *N*-Carboxymethylchitosan *N,O*-sulfate, a heparin-like polysaccharide derived from *N*-carboxymethylchitosan [9] by a random sulfation reaction, was also shown to inhibit HIV-1 replication and viral binding with CD4 [10]. However, it should be noted that much attention must be paid in the design of novel sulfated polysaccharides having specific anti-AIDS activity while lacking unfavorable anticoagulant activity or other side effects. Thus, our interest is focused on the regioselective synthesis of sulfated polysaccharides and structure–activity studies seeking potent antiretrovirus activity with little anticoagulant activity and low cytotoxicity.

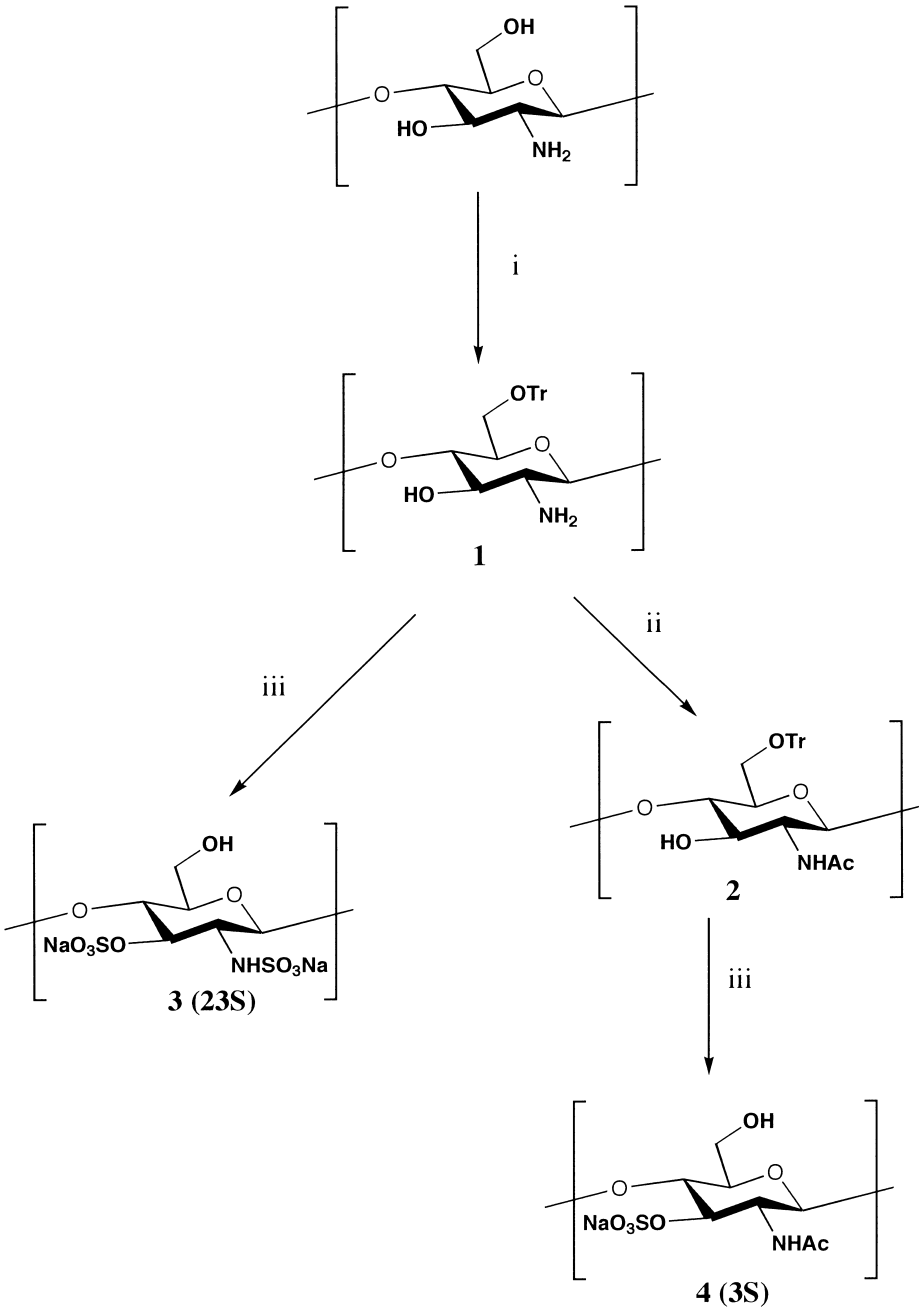
Chitin and chitosan are naturally abundant and simple β-(1→4) glycans composed of 2-acetamido-2-deoxy-D-glucopyranose or 2-amino-2-deoxy-D-glucopyranose residues, and they have served as key starting materials for the efficient preparation of bioactive polysaccharides having anticoagulant activity [11–13], immunomodulating effects [14], and inhibitory effects on lung metastasis of melanoma cells [15,16]. Since these bioactive macromolecules are usually prepared from chitin by direct chemical modifications under heterogeneous reaction systems, standard acylations [17] and alkylations [18] are known to occur predominantly at O-6 (primary hydroxyl groups) owing to the low reactivity of the O-3 secondary hydroxyl groups because of rigid hydrogen bonds from secondary hydroxyl groups at C-3 and acetamido groups at C-2 [19]. Therefore, selective modifications at C-2 and/or C-3 have long been an acknowledged feature for innovation in chitin chemistry and technology.

We reported a facile and an efficient method for preparing a key starting material, 2-deoxy-2-phthalimido-(1→4)-β-D-glucopyranan, having excellent solubility in general organic solvents, through the complete *N*-phthaloylation of chitosan [20]. The enhanced solubility of *N*-phthaloylchitosan prompted us to prepare useful "standardized intermediates" [21]. Indeed, the versatility of these soluble intermediates has been demonstrated in the first example of an easy preparation of amphiphilic polysaccharides that show the capacity to form self-assembling systems [22]. We therefore considered that the use of precisely protected intermediates, such as 6-*O*-tritylchitosan, would permit the systematic construction of new chitin sulfates in satisfactory yield with excellent regioselectivity. The present paper describes the feasibility of "standardized intermediates" for regioselective syntheses of new sulfated derivatives of chitin and chitosan having specific anti-AIDS activity.

2. Results and discussion

Synthesis.—Scheme 1 outlines the synthetic procedure for (1→4)-2-deoxy-2-sulfoamido-3-*O*-sulfo-β-D-glucopyranan (**3**, 23S) and (1→4)-2-acetamido-2-deoxy-3-*O*-sulfo-β-D-glucopyranan (**4**, 3S). First, the known intermediate 2-amino-3-hydroxyl derivative **1** [21], readily accessible from *N*-phthaloylchitosan, was selected as a convenient precursor for the preparation of 23S, since this partially protected derivative has been reported to show much better solubility in general organic solvents than chitosan itself. Next, selective *N*-acetylation of **1** was performed, to synthesize a precursor of 3S, by treatment of **1** with acetic

anhydride–dimethylformamide (DMF) in the presence of an excess of methanol. This afforded 6-*O*-trityltchitin (**2**) in 87% yield. Although the hydrogen atom of the 2-NHAc group of derivative **2** could potentially reform a rigid crystalline structure through hydrogen-bonding interactions, fortunately, this precursor **2**, having an unprotected hydroxyl group at C-3, exhibited excellent solubility in such organic solvents as pyridine, DMF, and dimethyl sulfoxide. Possibly, the bulky 6-*O*-trityl functionalities along the polymer chain, causing disruption of molecular of packing by such hydrogen bonds, might contribute, along with hydrophobic factors, to the excellent solubility of compound **2**. This conclusion was further supported by the X-ray diffraction diagrams of these materials. Changes in the X-ray diffraction diagrams of derivatives **1** and **2** in contrast with native crab chitin or chitosan, were evident, as shown in Fig. 1. The original chitin powder showed three



Scheme 1. (i) *(a)* Phthalic anhydride, ethylenglycol, DMF, 130 °C, *(b)* TrCl, pyridine–4-dimethylaminopyridine, 90 °C, *(c)* NH_2–$NH_2{\cdot}H_2O$, 100 °C (93% from chitosan); (ii) Acetic anhydride, DMF-MeOH (87%); (iii) *(a)* SO_3–pyridine, pyridine, 80 °C, *(b)* dichloroacetic acid, 20 °C, *(c)* aq. 1 M NaOH pH 8∼9, dialysis and gel filtration by Sephadex G-25 (62% for **3** and 80% for **4**).

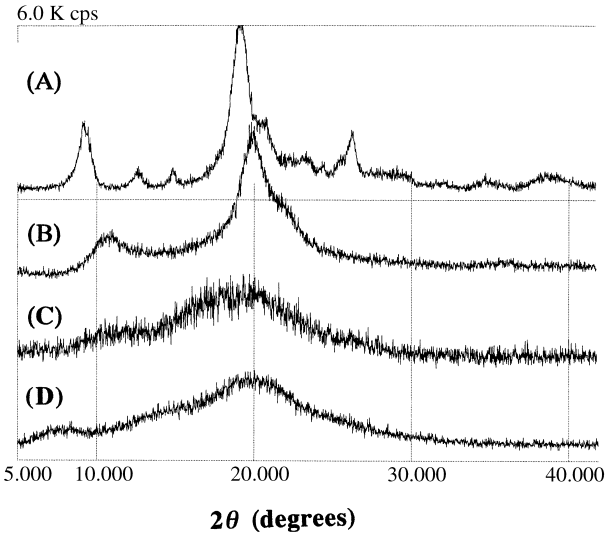


Fig. 1. X-ray powder diffraction patterns of shrimp shell chitin and its derivatives: (A) chitin; (B) chitosan; (C) 6-*O*-Tritylchitosan (**1**); (D) 6-*O*-Tritylchitin (**2**).

major crystalline peaks at $2\theta = 26.2°$, 19.2°, and 9.2° and two major peaks were observed around $2\theta = 20.0°$ and 10.8° in the case of chitosan with a degree of deacetylation of 1.00. On the other hand, the peaks in the X-ray diffraction diagrams of 6-*O*-tritylated derivatives **1** and **2** were apparently broader than those of chitin and chitosan, suggesting lower crystallinity or a less-ordered structure. It is concluded that destruction of the rigid crystalline structure of chitin and chitosan is of great importance for successful solubilization by sterically hindered trityl groups, introduced through systematic protection strategy under homogeneous conditions, using *N*-phthaloyl chitosan as a key starting material.

Reaction of **1** with SO_3–pyridine complex (10 equiv) in pyridine proceeded smoothly in homogeneous solution, and the crude product obtained by subsequent detritylation (by treatment with an excess of dichloroacetic acid) was purified by dialysis and gel filtration with Sephadex G-25 to afford 23S (**3**) in 62% overall yield from **1**. Based on elemental analytical data, the calculated best-fit degree of sulfation (d.s.) is 1.7. Sulfation of precursor **2** and subsequent detritylation were also performed as for the preparation of 23S and yielded 3S (**4**) with a d.s. value of 0.44 in 80% yield from **2**. Compounds **3** and **4** were readily characterized as the desired regioselectively sulfated derivatives by their ^{13}C-NMR spectra, which showed signals for C-2 and C-3 (**3**) or C-3 (**4**) downshifted by the sulfation reaction. The signals for C-6 were observed at δ 63.3 (**3**) and δ 62.6 (**4**), indicating that primary hydroxyl groups at C-6 were not sulfated.

The ^{13}C-NMR chemical shifts of derivatives **3**, **4**, and 6-*O*-sulfated chitin (6S) [23] are summarized in Table 1. Considering the steric effect by other substituent groups at C-2, C-4, and C-6, as well as the low reactivity of 3-OH groups, it seems that 30% of the secondary hydroxyl groups at O-3 of compound **3** were not sulfated. This conclusion is supported by the NMR spectrum of **3**, in which significant signals at δ 72.5 for unsubstituted C-3 and δ 77.5 for sulfated C-3 were observed. The average molecular weights of these compounds as determined by GPC were in the approximate range 10^4 to 2.8×10^4.

Table 1
^{13}C Chemical shifts of chitin sulfates in ppm from DSS[a]

Compd	6S	3(3S)	4(23S)
C-1	102.7	103.2	103.1
C-2	57.7	57.7	—
C-2S	—	—	58.9
C-3	75.9	75.1	72.5
C-3S	—	78.5	77.5
C-4	82.0	82.1	80.4
C-5	78.1	77.0	75.9
C-6	—	62.6	63.3
C-6S	69.6	—	—
CH_3	25.5	25.4	—
C=O	177.3	177.4	—

[a] 4,4-Dimethyl-4-silapentanesulfonic acid sodium salt.

Table 2
Anti-HIV-1 activity and anticoagulant activity of chitin sulfates in vitro

Test drugs	D.s.[a]	S(%)	Mw (KDa)	EC_{50}[b](μg/mL)	CC_{50}[c](μg/mL)	SI[d]	Anticoagulant activity (unit/mg)
6S	0.87	10.2	58	57.0	> 1000	> 18	93
3(3S)	0.44	5.4	28	9.6	> 1000	> 103	< 3
4(23S)	1.69	16.5	16	0.28	> 1000	> 3570	< 3
Curdlan sulfate	1.30	14.1	79	0.10	> 1000	> 10,000	23

[a] Degree of sulfation.
[b] 50% effective concentration.
[c] 50% cytotoxic concentration.
[d] Selective index (CC_{50}/EC_{50}).

The inhibitory effects on growth of HIV-1 in vitro, and the anticoagulant activity of chitin sulfates, were determined as reported previously [24,25]. Table 2 summarizes the physical properties and the biological activities of chitin sulfates in comparison with that of curdlan sulfate [8] as a positive control. The concentration of reagent that protects 50% of the HIV-1-induced cytopathic effects was defined as the 50% effective concentration (EC_{50}). As shown in Table 2, 23S (**3**) showed the highest inhibitory effect among the chitin sulfates used in this experiment. This compound inhibited virus infection with an EC_{50} of 0.28 μg/mL. Interestingly, sulfation at O-6 of the GlcNAc residue seems to decrease the anti-HIV-1 activity of chitin sulfates, because 3S (**4**) with a lower degree of sulfation (d.s. 0.44) inhibited HIV-1 infection more effectively than 6S (d.s. 0.87). No significant cytotoxicity of chitin sulfates was noted over a wide concentration range. Among chitin sulfates employed here [23], only 6S showed strong anticoagulant activity (93 units/mg) while 23S and 3S exhibited little activity. It was assumed that sulfate groups at O-3 and/or O-2 of chitin sulfates specifically interact with gp120 of HIV-1 and the O-6 site might contribute efficiently to the anticoagulant activity through non-specific ionic interaction with thrombin or other blood-coagulation factors. Further studies using synthetic peptides that correspond to basic regions are of gp120 are under way.

In summary, novel sulfated analogs of chitin, 23S and 3S, have been regioselectively prepared via standardized intermediates under homogeneous conditions, and are shown to have potent and specific inhibitory effects on HIV-1 infection. These new inhibitors of retrovirus infection showed both low cytotoxicity and low anticoagulant activity.

3. Experimental section

Materials and general methods.—Chitosan was prepared by repeated *N*-deacetylation of chitin from shrimp shells [26]. The degree of deacetylation of this starting material was 1.00 (100%), as determined by elemental analysis and conductometric titration. 2-Amino-2-deoxy-6-*O*-(triphenylmethyl)-(1→4)-β-D-glucopyranan (**1**) was prepared from 2-deoxy-2-phthalimido-(1→4)-β-D-glucopyranan according to the method reported previously [21].[1]

2-Acetamido-2-deoxy-6-*O*-sulfo-(1→4)-β-D-glucopyranan (6S) was prepared from chitin by direct partial *O*-sulfation according to the method reported previously [23] and characterized as shown in Table 1. The degree of 6-*O*-sulfation as calculated from the analytical data was 0.87. Dextran sulfate as a standard sample for anticoagulant activity was a gift from Meito Sangyo Co. Ltd.; its sulfur content is 18.4%. Unless otherwise stated, all commercially available solvents and reagents were used without further purification. Pyridine was stored over molecular sieves (4Å) for several days before use. Optical rotations were determined with a Perkin–Elmer 343 digital polarimeter at 23 °C. ^{1}H and proton-decoupled carbon NMR spectra were recorded at 300 and 75.47 MHz, respectively, by use of a Bruker AMX-300 spectrometer in chloroform-*d*, dimethyl sulfoxide-d_6, or deuterium oxide, using tetramethylsilane (Me_4Si), methanol, or 3 4,4-dimethyl-4-silapentanesulfonic acid sodium salt

[1]Although our previous method for solubilization of chitosan based on simple *N*-phthaloylation facilitated further derivatization and functionalization of this aminopolysaccharide [20–22], it was reported by other researchers that differences in properties of the starting chitosan material, such as molecular weight, crystallinity, and degree of deacetylation, might promote gel formation, owing to a significant degree of phthaloylation at O-6 and/or O-3 as a side-reaction [27]. Here we have improved the selective and quantitative *N*-phthaloylation by using a small excess of ethylenglycol to prevent *O*-phthaloylation. A typical example of the improved method for quantitative *N*-phthaloylation is as follows: A mixture of chitosan (500 mg, 3.10 mmol), phthalic anhydride (13.85 g, 6.21 mmol), and ethylene glycol (0.346 mL, 6.41 mmol) in DMF (13 mL) was heated with stirring at 130 °C under nitrogen. The reaction system became a viscous yellow solution within 1–2 h and the mixture was filtered through cotton to remove a small amount of unreacted chitosan. The precipitate obtained by pouring the solution into ice–water was collected by filtration, successively washed with EtOH ether, and dried over P_2O_5, to give 2-deoxy-2-phthalimido-(1→4)-β-D-glucopyranan (746 mg, 83.3%).

Structure analysis was performed by complete acetylation of this compound, and selected spectral and analytical data are as follows.

2-Deoxy-2-phthalimido-(1→4)-β-D-*glucopyranan* (**1**).—IR (KBr) ν_{max}: 1773, 1709, 1465, and 667 cm^{-1}; ^{1}H-NMR chemical shifts (Me_2SO-d_6) δ 7.8–7.3 (m, 4 H, Ph), 5.1 (br d, 1 H, H-1), 4.8 (m, 1 H, H-3), 4.1 (br s, 1 H, H-5), and 3.7–3.1 (m, 4 H, H-2, 4, 6a, and 6b); IR (KBr) ν_{max}: 1773, 1709, 1465, and 667 cm^{-1}; Anal. Calcd for $C_{14}H_{13}O_6N_1 \cdot 0.4\ H_2O$: C, 56.34; H, 4.66; N, 4.69. Found: C, 56.01; H, 4.45; N, 4.32.

3,6-Di-O-*acetyl*-2-*deoxy*-2-*phthalimido*-*(1→4)*-β-D-*glucopyranan*.— IR (KBr) ν_{max}: 1775 and 1223 cm^{-1}; ^{13}C-NMR chemical shifts ($CDCl_3$) δ 169.6 and 169.4 (C=O), 134.1, 131.3, and 123.5 (Ph), 96.9 (C-1), 75.2 (C-3), 72.5 (C-4), 70.2 (C-5), 62.0 (C-6), 55.1 (C-2), and 20.3 (CH_3); Anal. Calcd for $C_{18}H_{17}O_8N_1 \cdot 0.3\ H_2O$: C, 56.78; H, 4.62; N, 3.69. Found: C, 56.78; H, 4.55; N, 3.85.

(TSP) as internal standards. X-Ray powder patterns of chitin and its derivatives were measured at room humidity by a Rigaku Geigerflex X-ray diffractometer RAD-2R using Ni-filtered CuK_α radiation generated at 35 kV and 30 mA. Average molecular weights were estimated by gel-permeation chromatography (GPC) with an Asahipak GS-510 column, and pullulans (5.8, 12.2, 23.7, 48.0, 100, 186, and 380 K, Shodex Standard P-82) as standards. Size-exclusion chromatography was performed on Sephadex G-25 using deionized water as eluant. All extractions were concentrated below 40 °C under diminished pressure.

*2-Acetamido-2-deoxy-6-*O*-(triphenylmethyl)-(1→4)-*β*-*D*-glucopyranan* (**2**).—To a cooled solution of (1→4)-2-amino-2-deoxy-6-*O*-(triphenylmethyl)-*β*-D-glucopyranan (**1**, 5.00 g, 12.4 mmol) in DMF (200 mL) was gradually added MeOH (100 mL), and then Ac_2O (3.51 mL, 37.2 mmol) and the mixture was stirred for 24 h at room temperature under nitrogen. The precipitate obtained by pouring the viscous solution into ice–water was collected by filtration, washed with EtOH and ether, and dried for 6 h at 50 °C over P_2O_5 to give powdery **2** (4.80 g, 87%); IR (KBr) ν_{max}: 3500, 1666, 1518, 750, 710, and 690 cm^{-1}; Anal. Calcd for $C_{27}H_{27}O_5N_1 \cdot 0.6\ H_2O$: C, 71.07; H, 6.23; N, 3.07. Found: C, 70.87; H, 6.24; N, 3.25.

*2-Deoxy-2-sulfoamido-3-*O*-sulfo-(1→4)-*β*-*D*-glucopyranan* (**3**).—Sulfur trioxide–pyridine complex (989 mg, 6.2 mmol) was added to a stirred solution of aminoalcohol **1** (500 mg, 1.24 mmol) in dry pyridine (20 mL) at room temperature. Stirring was continued for 2 h at 80 °C under nitrogen. After cooling, EtOH (200 mL) was added and the precipitate formed was collected by centrifugation. It was then dissolved in distilled water (50 mL) under cooling with ice and the solution was brought to pH 8∼9 by addition of aqueous 1 M NaOH at 0–5 °C. The resulting pale-yellow solution was dialyzed against deionized water for 2 days and then lyophilized to give 530 mg of crude sulfated material as a yellow, cotton-like product. The crude intermediate (530 mg) was suspended in dichloroacetic acid (10 mL) and stirred at room temperature. After 1 h, the reaction mixture became clear. The white precipitate obtained by pouring the solution into EtOH (200 mL) was collected by centrifugation and dissolved in distilled water (50 mL). The solution was then brought to pH 8∼9 with aqueous 1 M NaOH at 0 °C, dialyzed against deionized water for 2 days, and concentrated to 5 mL. The resulting solution was subsequently chromatographed on column (20×600 mm) of Sephadex G-25 with deionized water as eluant. The pure fractions around the void volume were lyophilized to afford compound **3** (258 mg, 62% from compound **1**); $[\alpha]_D$ −0.7° (*c* 2.0, H_2O); IR (KBr) ν_{max}: 3450, 1230, and 809 cm^{-1}; ^{13}C-NMR chemical shifts (D_2O, 300 MHz) are listed in Table 1. Anal. Calcd for $C_6H_{9.31}O_{9.07}N_1S_{1.69}$: C, 21.60; H, 2.81; N, 4.20; S, 16.16. Found: C, 21.83; H, 2.68; N, 4.63; S, 16.45.

*2-Acetamido-2-deoxy-3-*O*-sulfo-(1→4)-*β*-*D*-glucopyranan* (**4**).—Sulfur trioxide–pyridine complex (893 mg, 5.61 mmol) was added to a stirred solution of 6-*O*-tritylchitin (**2**, 500 mg, 1.122 mmol) in dry pyridine (20 mL) at room temperature. Stirring was continued for 2 h at 80 °C under nitrogen. The procedures for subsequent deprotection and purification followed the method for the preparation of **3**, and gave 3-sulfate **4** (237 mg, 80%); $[\alpha]_D$ −4.3° (*c* 2.0, H_2O); IR (KBr) ν_{max}: 3450, 1650, 1530, 1230, and 809 cm^{-1}; ^{13}C-NMR chemical shifts (D_2O, 300 MHz) are listed in Table 1. Anal. Calcd for $C_8H_{11.36}O_{6.36}N_1S_{0.44}Na_{0.44} \cdot H_2O$): C, 36.46; H, 5.00; N, 5.31; S, 5.35. Found: C, 36.87; H, 5.57; N, 5.31; S, 5.44.

Anti-HIV assay.—HIV-infected and uninfected MT-4 cells (3×10^5 cells/mL), a human T4-positive cell line carrying HTLV-1 (human T-lymphotropic virus type I), were cultured with various concentrations of test compounds and assayed after 6 days of HIV infection by measuring the decrease in the number of viable cells and by the percentage of immunofluorescence (IF)-positive cells as described in a previous report [28]. The HIV-1 strain was prepared from the supernatant of MOLT-4/$HIV_{HTLV\text{-}IIIB}$ cells. The MTT method [24] was also used for the anti-HIV assay, 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 dehydrogeneses of metabolically active cells to a blue formazan product. MT-4 cells were infected with HIV at the multiplicity (MOI) of 0.1, and HIV-infected and mock-infected MT-4 cells were incubated in the presence of various concentrations of chitin sulfates for 5 days at 37 °C in a CO_2 incubator. The dose achieving 50% protection of MYT-4 cells from HIV was defined as the 50% effective dose (EC_{50}), and CC_{50} denotes the 50% cytotoxic dose of drugs.

Anticoagulant activity.—Anticoagulant activity in vitro was performed and evaluated by the use of bovine plasma according to a modification of the United States Pharmacopoeia [27] in comparison with the activity of dextran sulfate.

Acknowledgements

This work was partly supported by The Iwatani Naoji Foundation and also a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture, and Sports, Japan (Grant 06750899).

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