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Synthesis of Sulfated Alkyl Laminara-Oligosaccharides Having Potent Anti-HIV Activity and the Relationship between Structure and Biological Activities

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ABSTRACT: The synthesis of potently anti-HIV-active sulfated alkyl laminara-oligosaccharides composed of glucosidic residues of 5–9 was investigated. The anti-HIV activity and the anticoagulant activity of these sulfated alkyl laminara-oligosaccharides were assessed. The synthesis and separation of respective laminara-oligosaccharides were accomplished in a route starting from acetolysis and hydrolysis of curdlan followed by HPLC. Alkyl oligosaccharides were synthesized using stannic tetrachloride as a Lewis acid catalyst, and then sulfation was carried out with the sulfur trioxide–pyridine complex after deacetylation. Sulfated dodecyl laminarapentaoside through laminaranonaoside showed almost the same anti-HIV activity. Although no cytotoxicity was detected on a series of dodecyl compounds, low-level cytotoxicity appeared with a series of octadecyl compounds. On the other hand, the anticoagulant activity increased as the number of sugar units increased from 5 to 9.

Introduction

Although numerous attempts to develop AIDS drugs have been carried out for almost a decade, only three deoxynucleoside derivatives, i.e., azidothymidine (AZT), dideoxyinosine (DDI), and dideoxycytidine (DDC), are used as effective clinical drugs. However, it has been revealed that the most commonly-used drug AZT, produces an AZT-resistant HIV virus with long-term administration.[1,2]

Soon after it was discovered that a sulfated polysaccharide, dextran sulfate, has an inhibitory effect on HIV infection, we also started to investigate sulfated polysaccharides with potent inhibitory effects on infection by making use of our experience on the synthesis of biologically active sulfated polysaccharides.[3] Lentinan (branched (1→3)-β-glucan) sulfate[4] and curdlan ((1→3)-β-glucan) sulfate with high anti-HIV activities but with low anticoagulant activities were synthesized.[5] The phase I/II tests with the curdlan sulfate have been performed since the end of 1992 in the United States.

Like the interaction of heparin with antithrombin III in anticoagulant activity,[6] the action mechanism of sulfated polysaccharides is supposed to originate from the interaction between a negatively-charged oligosaccharide and a positively-charged protein portion of the virus envelope protein.[7]

Making use of sulfated oligosaccharides which have by themselves very low anti-HIV activities, we have synthesized sulfated alkyl oligosaccharides with potent anti-HIV activities.[8,9] Since the compound is composed both of a hydrophilic sulfated oligosaccharide portion and a hydrophobic long alkyl portion bonded at the reducing terminal, it has the characteristic of a surface-active agent which coagulates together. Several sulfated alkyl laminara-oligosaccharides and malto-oligosaccharides were synthesized to assess their biological activities. It has been

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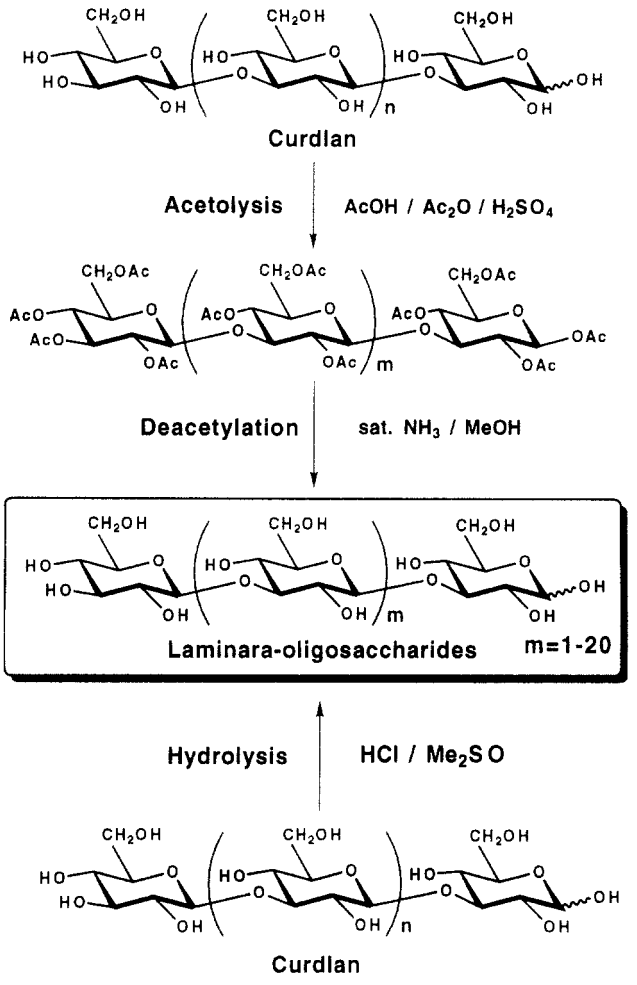
Scheme 1. Synthesis of Laminara-Oligosaccharides by Degradation of Curdlan



revealed that the laminara-oligosaccharide was more suitable for an AIDS drug candidate than the malto-oligosaccharide.[10]

In this study, we examined the anti-HIV activity and anticoagulant activity of several sulfated alkyl laminara-oligosaccharides which were individually prepared by the use of five pure oligosaccharides from laminarapentaose to laminaranonaose. In addition, influences of the length of the alkyl chain and the number of glucose residues on the biological activities were examined to optimize the chemical structure for an AIDS drug.

Results and Discussion

Synthesis of Sulfated Alkyl Laminara-Oligosaccharides. Previously, it was reported that laminara-oligosaccharides composed of less than five glucose residues were prepared by hydrolysis of a polysaccharide laminaran with hydrochloric acid.[11] However, a large-scale synthesis of laminara-oligosaccharides which have more than five glucose residues has not been published except for that of laminarapentaose by an enzymatic degradation method of curdlan.[12] Thus, an acetolysis of the 1,3-β-linked glucose polymer, curdlan, was first examined. This was accomplished by finding an optimum condition to give peracetylated laminara-oligosaccharides having the molecular weight distribution maximum between the pentaose and the nonaose.

Deacetylation of the peracetylated laminara-oligosaccharides was performed with ammonia-saturated methanol. High-performance liquid chromatography could thoroughly separate the individual laminara-oligosaccharides from pentaose to nonaose to produce approximately 100 mg each.

Table 1. Optical Rotation of Laminara-Oligosaccharides

laminara-oligosaccharide	$[\alpha]_D^{20}$,[a] deg	$[\alpha]_D^{20}$,[b] deg
pentaose	−8.8	−0.7
hexaose	−13.6	−8.7
heptaose	−7.5	−4.3
octaose	−6.9	−4.7
nonaose	c	−6.9
decaose	c	c

[a] Measured in H_2O (c = 1.0); the sample solution stood for over 24 h before measurement. [b] Measured in dimethyl sulfoxide (c = 1.0); the sample solution stood for over 24 h before measurement. [c] The solubility was poor.

Table 2. Acetylation of Laminara-Oligosaccharides[a]

	sugar					acetylated saccharide			
						yield[d]			
no.	length	wt, mg	amt of KOAc, mg	temp, °C	time, min	mg	%	β/α[c]	$[\alpha]_D^{20}$,[d] deg
1	5	200	200	140	60	360	98	3.8	−48.6
2	6	210	210	140	65	137	35	3.6	−45.3
3	7	70	100	140	65	61	47	3.9	−49.2
4	8	120	150	140	140	73	33	3.4	−50.9
5	9	120	150	140	90	85	39	3.6	−50.9

[a] Acetic anhydride (10 mL/100 mg of sugar). [b] α- and β-mixture. [c] Determined by ^{1}H NMR. [d] Measured in $CHCl_3$ (c = 1.0).

Hydrolysis of curdlan with hydrochloric acid was used as an alternative preparation method. Both acetolysis and hydrolysis methods produced pure laminara-oligosaccharides from hexaose to nonaose. However, the former method was suitable for preparing decaose. The specific rotation of the laminara-oligosaccharides is shown in Table 1.

For the oligosaccharides larger than octaose, the solubility in water was very low or zero, like their parent polysaccharide curdlan which has no solubility in water because of a helical structure in the solid state.[13]

Individual free OH group oligosaccharides were acetylated with a potassium acetate and acetic anhydride mixture in order to obtain as many proportions as possible of the peracetylated oligosaccharides having the β-configuration at the reducing terminal.[9] Results of the acetylation are summarized in Table 2.

Although laminarapentaose was acetylated to the peracetylated laminarapentaose in high yield (98%), the oligosaccharides larger than hexaose gave the peracetylated oligosaccharides in considerably low yields under the same conditions. The low yield might be ascribed to a decrease in the reactivity of the hydroxyl group by formation of the intramolecular hydrogen bond.

The peracetylated laminara-oligosaccharide was reacted with n-dodecyl alcohol and n-octadecyl alcohol by use of stannic tetrachloride as glycosylation catalyst to afford n-dodecyl and n-octadecyl laminara-oligosaccharide peracetates, respectively. Results of the glycosylation of peracetylated laminara-oligosaccharides are summarized in Table 3.

Although the reactivity of the peracetylated pentaose to the aliphatic alcohols was considerably high, that of the higher oligosaccharides was low. Moreover, a tendency that dodecyl alcohol gives lower yields than octadecyl alcohol was observed. Individual alkyl laminara-oligosaccharide peracetates were deacetylated to give the corresponding alkyl laminara-oligosaccharides. As is summarized in Table 4, the alkyl laminara-oligosaccharides were sulfated with the sulfur trioxide–pyridine complex to afford the corresponding sulfated alkyl laminara-

Table 3. Synthesis of Peracetylated Alkyl Laminara-Oligosaccharides by using Stannic Chloride

sample	acetylated laminara-oligosaccharide: glucose unit	acetylated laminara-oligosaccharide: amt, mg (mmol)[a]	n-alkyl alcohol: carbon no. of alkyl chain	n-alkyl alcohol: amt, mg (equiv)[b]	amt of $SnCl_4$, mol %	yield,[c] %	$[\alpha]_D^{20}$,[d] deg
L5C12	5	500 (0.32)	12	111 (2.0)	100	53	-52.9
L6C12	6	170 (0.09)	12	39 (2.4)	110	19	n.d.
L7C12	7	160 (0.08)	12	27 (2.1)	170	18	n.d.
L8C12	8	170 (0.07)	12	26 (2.0)	200	38	-52.2
L9C12	9	140 (0.05)	12	27 (3.0)	200	20	n.d.
L5C18	5	150 (0.10)	18	55 (2.0)	200	50	-49.8
L6C18	6	140 (0.08)	18	40 (2.0)	200	23	-47.5
L7C18	7	150 (0.7)	18	40 (2.0)	200	34	-52.0
L8C18	8	120 (0.05)	18	30 (2.0)	200	33	-52.6
L9C18	9	80 (0.03)	18	16 (2.0)	200	37	-53.0

[a] Equivalent to acetylated oligosaccharide. [b] Equivalent to acetylated oligosaccharide. [c] After purification by using column chromatography. [d] Measured in $CHCl_3$ (c = 1.0).

Table 4. Sulfation of Alkyl Laminara-Oligosaccharide with the Sulfur Trioxide–Pyridine Complex[a]

sample	alkyl oligosaccharide: length of sugar unit	alkyl oligosaccharide: length of alkyl chain	alkyl oligosaccharide: amt, mg	yield, mg	DS[b]		sulfated alkyl oligosaccharide: elem anal[c] % C	% H	% N	% S	% Na	$[\alpha]_D^{20}$,[d] deg
L5C12S	5	12	100	190	3.0	calcd	19.2	2.3	0.0	19.5	14.0	
						found	17.8	3.0	0.0	17.0	12.2	-13.2
L6C12S	6	12	20	31	2.3	calcd	18.6	2.2	0.0	19.7	14.0	
						found	13.7	2.9	0.0	14.2	12.9	n.d.
L7C12S	7	12	13	11								
L8C12S	8	12	42	58	2.6	calcd	17.9	2.0	0.0	19.9	14.2	
						found	15.8	2.8	0.0	16.8	13.5	-13.4
L9C12S	9	12	10	15								
L5C18S	5	18	50	70	3.4	calcd	21.2	2.7	0.0	18.9	13.6	
						found	17.9	3.0	0.0	16.7	12.0	-12.3
L6C18S	6	18	28	36	3.2	calcd	20.4	2.5	0.0	19.2	13.7	
						found	15.3	2.5	0.0	16.5	13.2	-12.3
L7C18S	7	18	36	73	2.8	calcd	19.7	2.4	0.0	19.3	13.9	
						found	16.6	2.9	0.0	17.2	14.4	-12.3
L8C18S	8	18	29	60	2.9	calcd	19.3	2.3	0.0	19.5	14.0	
						found	16.2	2.8	0.0	17.5	14.3	-13.6
L9C18S	9	18	25	45	2.4	calcd	18.9	2.2	0.0	19.6	14.0	
						found	17.4	3.0	0.0	15.9	12.7	-9.4

[a] 2.2 equiv of sulfur trioxide pyridine complex to OH group was used. [b] Degree of sulfation (DS) designates the number of sulfate groups per a glucose residue. [c] Calculated as a persulfated sample. [d] Measured in water (c = 1.0).

oligosaccharides. All sulfated compounds have high sulfur contents ranging from 12.0% to 14.4%.

Anti-HIV Activity and Anticoagulant Activity. The anti-HIV activity of sulfated alkyl laminara-oligosaccharides was assayed by use of the MTT method employing the MT-4 cell line and HIV-$1_{HTLV\text{-}III}$.[14] Results of the measurement of anti-HIV activities are shown in Table 5.

All sulfated dodecyl laminara-oligosaccharides had potent anti-HIV activities, exhibiting EC_{50}'s of 0.10–0.18 μg/mL. Since these compounds had low cytotoxicities, i.e., CC_{50}'s larger than 1000 μg/mL, they showed a high ratio of cytotoxicity to activity (SI). The anti-HIV activity of the sulfated dodecyl laminara-oligosaccharides is almost equivalent to that of highly anti-HIV-active curdlan sulfate (EC_{50} of 0.18 μg/mL). Although we reported that a sulfated dodecyl laminara-oligosaccharide having 11 glucose residues exhibited the highest anti-HIV activity,[8] it was revealed that the anti-HIV activity of sulfated dodecyl laminara-oligosaccharides did not depend on the length of carbohydrate moiety from pentaose to nonaose. This might be due to the high purity and the high degree of sulfation of the sulfated dodecyl laminara-oligosaccharides prepared in this experiment.

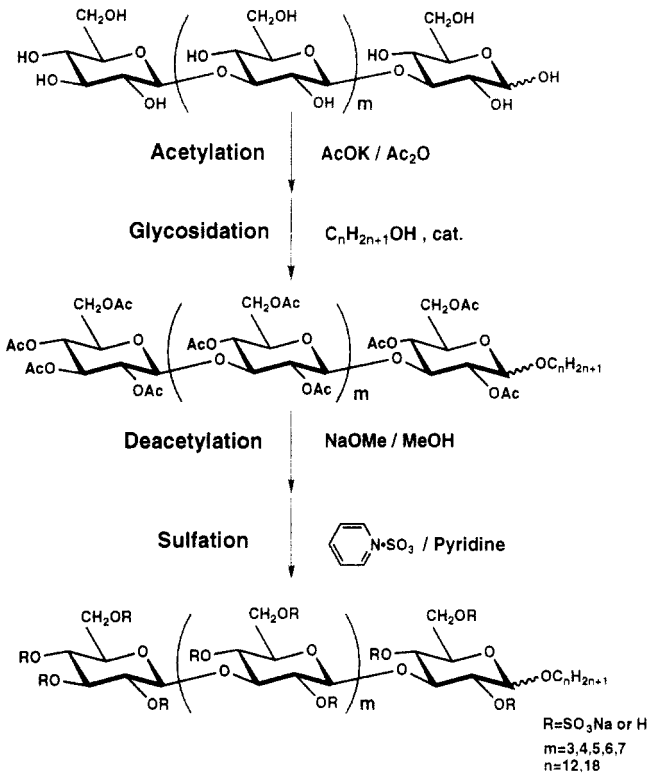
Sulfated octadecyl laminara-oligosaccharides in which the carbohydrate moiety ranged from 5 to 9 exhibited anti-HIV activities almost equivalent to those of the dodecyl analogues, that is, EC_{50}'s of 0.20–0.63 μg/mL. However, their cytotoxicities were considerably higher, CC_{50}'s of 180–240 μg/mL. Thus, the anti-HIV activity does not depend on the alkyl length, while the cytotoxicity increases with increasing alkyl length.

The anticoagulant activity of the sulfated alkyl oligosaccharides was determined according to a modification of the United States Pharmacopoeia using bovine plasma (Table 5).[3] The sulfated dodecyl laminara-oligosaccharides smaller than the heptaoside had no anticoagulant activity as well as the sulfated octadecyl laminara-oligosaccharides smaller than the hexaoside, while both dodecyl and octadecyl oligosaccharides showed small anticoagulant activities of 2–6 units/mg. Although it is known that sulfated polysaccharides with molecular weights more than approximately 10 000 exhibit the anticoagulant activity,[15] that of the oligosaccharide derivatives has not yet been examined.

Experimental Section

General Information. HPLC purification was carried out on a Toso Series 8000 computer-controlled liquid chromatograph system equipped with packed columns of TSKGel Amide-80 (21.5 mm i.d. × 30 cm × 2 pieces). The charcoal powder for open column chromatography was a Wako activated charcoal (particle sizes coarser than 297 μm, 40%; 297–63 μm, 50% finer than 63 μm, 10%). The optical rotation was measured on a solution in $CHCl_3$, H_2O, or dimethyl sulfoxide by means of a Perkin-Elmer 241 polarimeter using a 1-mL cell. NMR spectra were measured on a JEOL GX-270 spectrometer in $CDCl_3$ using Me_4Si or D_2O using 3-(trimethylsilyl)propanesulfonic acid sodium salt as the internal standard. For column chromatography, silica gel (Mercks Kiesel-gel 60, 70–230 mesh ASTM) was used. The Sulfur

Scheme 2. Synthetic Route for Sulfated Alkyl Laminara-Oligosaccharides



trioxide–pyridine complex (Tokyo Kasei Kogyo Co., Ltd.) was used without further purification.

Synthesis of Laminara-Oligosaccharides. Method A. To a suspension containing of 10 g of curdlan in 40 mL of acetic anhydride and 40 mL of acetic acid was slowly added 10 mL of concentrated H_2SO_4 while the solution was maintained below 30 °C with an ice-cooled bath. The mixture was stirred for 30 h at 35 °C, poured into ice water, and filtered off. The residue was stirred vigorously in aqueous $NaHCO_3$ and filtered off. After it was dissolved in acetone, the acetone solution was reprecipitated with water. The peracetylated product was stirred in methanol (1000 mL) saturated with NH_3 for about 30 h, and then the solvent was removed under diminished pressure to provide free OH group oligosaccharides. The residue was dissolved in a small amount of water, and the product was precipitated by adding acetone, washed with acetone, and freeze-dried from water to afford white laminara-oligosaccharides in 80% yield. Free laminara-oligosaccharide mixtures (5.9 g) were roughly separated by HPLC at 70 °C with an acetonitrile and water mixture (50:50) as the eluent, to afford 2.6 g of the crude product containing from pentaose to undecaose. The crude product was further purified by using the same HPLC system at 70 °C, with an acetonitrile and water mixture (60:40) as the eluent. Finally, 104 mg of hexaose, 116 mg of heptaose, 83 mg of octaose, 80 mg of nonaose, and 61 mg of decaose were collected.

Method B. After 25 g of curdlan was suspended in 300 mL of dimethyl sulfoxide and the mixture was heated to 105 °C, 30 mL of 0.5 N HCl was added. The clear solution was stirred for 90 min, and then the solution was cooled to room temperature. Then it was neutralized with $NaHCO_3$, followed by precipitating with the addition of acetone. After freeze-drying from water, an off-white laminara-oligosaccharide mixture was obtained in 85% yield. Laminara-oligosaccharide mixtures (11.8 g) were charged on an open column of charcoal (2.0 kg, 7.5 cm i.d. × 90 cm) and were eluted with aqueous ethanol gradient (19–52%). Each fraction was analyzed by HPLC equipped with the analytical TSKGel Amide-80 column. After workup, 0.38 g of hexaose, 0.50 g of heptaose, 0.72 g of octaose, and 0.80 g of nonaose were obtained.

Acetylation of Laminara-Oligosaccharides. To boiling acetic anhydride (20 mL) in a three necked flask was added 200 mg of potassium acetate. Then 200 mg of laminarapentaose was gradually added under vigorous stirring. The solution was kept for 1 h at 140 °C and then cooled to room temperature. After the workup procedure, 360 mg (98%) of peracetylated laminarapentaose was obtained. Other laminara-oligosaccharides were acetylated by the same procedure. All peracetates were purified by silica gel column chromatography.

Glycosylation of Peracetylated Laminara-Oligosaccharide with *n*-alkyl Alcohol. Peracetylated laminarapentaose (500 mg) and 111 mg of *n*-dodecyl alcohol were added to 30 mL of dry 1,2-dichloroethane at 45 °C, followed by adding 83 mg of stannic tetrachloride. The mixture was stirred for 5 h. After the workup procedure, 0.30 g of *n*-dodecyl laminarapentaoside peracetate was obtained in 53% yield. Other glycosides were also synthesized by the same manner.

Deacetylation of Alkyl Oligosaccharide Peracetate. Alkyl oligosaccharide peracetate (100 mg) was stirred in methanol containing 0.3 equiv of sodium methoxide to the acetyl group at room temperature for 5 h, followed by neutralizing with H^+ type ion exchange resin (Daia Ion SK-1B) to pH = 6.0–6.5. A colorless alkyl oligosaccharide was obtained in quantitative yield after solvent evaporation.

Sulfation of *n*-Dodecyl Laminarapentaoside. A solution containing 100 mg of *n*-dodecyl laminarapentaoside in dry pyridine (5 mL) was heated to 85 °C, and then 340 mg (2.2 equiv to the hydroxyl groups) of the sulfur trioxide–pyridine complex was added, followed by stirring for 90 min. After cooling, the solution was neutralized with a saturated barium hydroxide solution to pH = 7.5–8.0. The precipitated $BaSO_4$ was separated by centrifugation, and the supernatant was passed through a Na^+ type ion exchange resin column. The raw product was purified by dissolving in a very small amount of water followed by precipitating from acetone. The aqueous solution of the compound was neutralized to pH = 6.9–7.2 by 0.2 N HCl. Finally, the product was freeze-dried from water to give 190 mg of off-white powdery sulfated *n*-dodecyl laminarapentaoside.

Table 5. Anti-HIV Activity and the Anticoagulant Activity of Sulfated Alkyl Laminara-Oligosaccharides

sample	no. of glucose unit	carbon no. of alkyl chain	anti-HIV activity[a] EC_{50}, μg/mL	cytotoxic effect[b] CC_{50}, μg/mL	SI[c] (CC_{50}/EC_{50})	anticoagulant activity, units/mg
L5C12S	5	12	0.10	>1000	>10000	0[d]
L6C12S	6	12	0.18	>1000	>5600	0[d]
L7C12S	7	12	0.14	>1000	>7100	0[d]
L8C12S	8	12	0.14	>1000	>7100	6
L9C12S	9	12	0.18	>1000	>5600	3
L5C18S	5	18	0.63	220	350	0[d]
L6C18S	6	18	0.62	220	360	0[d]
L7C18S	7	18	0.20	180	900	2
L8C18S	8	18	0.59	210	360	4
L9C18S	9	18	0.59	240	410	6
curdlan sulfate		0	0.18	>1000	>5600	10
dextran sulfate		0	0.65	>1000	>1500	21
curdlan		0	>1000	>1000	<>1	n.d.
dextran		0	>1000	>1000	<>1	n.d.

[a] Concentration of the drug inhibiting 50% virus infection. [b] Drug concentration for 50% cytotoxicity. [c] Selectivity index. [d] Under the limit of determination by this method.

Anti-HIV Assay. The anti-HIV activity of a series of sulfated alkyl oligosaccharides against HIV infection was determined by the protection from HIV-induced cytopathic effects (CPE).[16] HIV-$1_{HTLV\text{-}IIIB}$ was prepared from the culture supernatant of MOLT-4/HTLV-IIIB cells which were persistently infected with HTLV-IIIB. Human T-Lymphotropic virus type I (HTLV-I), ppsitive human T-cell line, MT-4, which was established by cocultivation with cord blood lymphocytes and the peripheral blood lymphocytes of an ATL patient,[17] was subcultured twice a week at a concentration of 3×10^5 cells/mL in an RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin. MT-4 cells were infected with HTLV-IIIB at the multiplicity of infection (MOI) of 0.01. HIV- or mock-infected MT-4 cells (1.5×10^5 cells/mL, 200 mL) were incubated in the presence of various concentrations of the test compounds. The cell viability was quantified by a colorimetric assay which monitors the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue-colored formazan product according to Pauwels *et al.*[14] The absorbances were determined in a microcomputer-controlled photometer (Titertek Multiskan, Labsystem Oy, Helsinki, Finland). The activity is represented as EC_{50} which denotes 50% inhibition of HIV infection, and cytotoxicity is represented as CC_{50}.

Anticoagulant Activity. Anticoagulant activity was measured according to a modification of the United States Pharmacopoeia using bovine plasma.[3] Dextran sulfate was used as a reference compound with an anticoagulant activity of 21.0 units/mg.

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