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Synthesis of Sulfated Alkyl Malto-oligosaccharides with Potent Inhibitory Effects on AIDS Virus Infection

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ABSTRACT: Sulfated alkyl malto-oligosaccharides with potent anti-human immunodeficiency virus (HIV) activities were synthesized. Malto-oligosaccharides composed of glucose residues of 4−7 were used as the starting materials. The synthesis of alkyl malto-oligosaccharides was carried out by reacting the peracetylated oligosaccharides with aliphatic alcohols by hetero-polyacids or Lewis acid as catalyst. After deacetylation, OH-free alkyl malto-oligosaccharides were sulfated with a sulfur trioxide−pyridine complex. Sulfated alkyl oligosaccharides in which the number of glucose residues ranged from 5 to 7 had potent anti-HIV activities comparable to the very strong activity of curdlan sulfate having the strongest activity of so-far synthesized sulfated polysaccharides. However, the tetraose derivatives had considerably low activities. The cytotoxicity was considerably high in the case of sulfated alkyl oligosaccharides with long alkyl groups such as an octadecyl group. The anticoagulant activity of almost all sulfated alkyl malto-oligosaccharides was almost zero.

Introduction

A number of anti-human immunodeficiency virus (HIV) agents have been examined to find effective acquired immunodeficiency syndrome (AIDS) drugs. At present, only three drugs functioning as the reverse transcriptase inhibitor, i.e., azidothymidine (AZT),[1] dideoxyinocine (DDI),[2] and dideoxycytidine (DDC),[2] have been clinically used. It was revealed that the long-term administration of AZT produces AZT-resistant viruses, causing a remarkable decrease in the anti-HIV activity of the agent.[3,4]

De Clercq anticipated that polyanionic compounds such as dextran sulfate and poly(methacrylic acid) will be possible targets for AIDS drugs.[5] In fact, dextran sulfate showed an inhibitory effect on HIV infection.[6,7] Since we had been preparing sulfated synthetic polysaccharides with high anticoagulant activity,[8] these polysaccharides showed high anti-HIV activities.[9] Then, we successfully synthesized lentinan (branched (1→3)-β-glucan) sulfate with high anti-HIV activity but medium anticoagulant activity.[10,11]

Curdlan ((1→3)-β-glucan) sulfate obtained by sulfating the bacterial polysaccharide curdlan showed high anti-HIV activity but low anticoagulant activity as well as low toxicities in animal tests.[12] Since December 1992, the Phase I/II test of the curdlan sulfate has been performed. Intravenous administration of curdlan sulfate produced marked dose-related increases in CD4 lymphocytes in HIV-infected patients.[13]

As a next target, we aimed at synthesizing medium-molecular-weight carbohydrate derivatives having high anti-HIV activities. Sulfated alkyl oligosaccharides were synthesized in which the long alkyl group bound at the reducing end had high anti-HIV activities.[14]

Recently, it was revealed that sulfated dodecyl laminari-oligosaccharides consisting of pentaose to nonaose expressed anti-HIV activities comparable to the very high activity of the curdlan sulfate.[15,16] However, since these oligosaccharides are scarce in nature, synthesis must be started from the degradation of polysaccharides. Malto-oligosaccharides are potentially available

Table 1. Acetylation of Malto-oligosaccharides[a]

	malto-oligosaccharide		yield[b]		
no.	length	wt (g)	wt (g)	%	α:β[c]
1	4	3.00	4.97	88	22:78
2	5	3.05	5.07	89	17:83
3	6	2.99	5.00	91	20:80
4	7	3.00	5.04	92	23:77

[a] Acetic anhydride, 300 mL; sodium acetate, 3.0 g; 140 °C; 2 h. [b] α and β mixture. [c] Determined by ^{1}H NMR.

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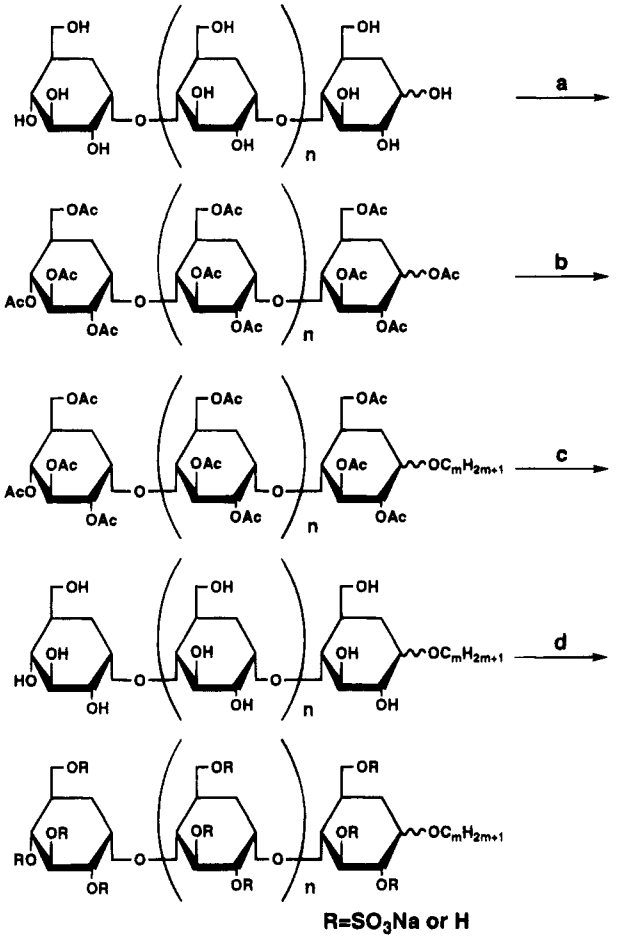
Scheme 1. Synthesis of Sulfated Alkyl Malto-oligosaccharides Starting from Malto-oligosaccharides



oligosaccharides which can be prepared by hydrolysis of cyclodextrins.[17,18]

In this study, we report the synthesis of sulfated alkyl malto-oligosaccharides with potent anti-HIV activities but with low or no anticoagulant activities. The glycosidation of peracetylated malto-oligosaccharides composed of 5−7 glucose residues with long-chain alcohols was successfully performed by a ferric chloride catalyst or hetero-polyacid catalysts such as phosphomolybdic and phosphotungstic acids. To examine effects of the length of the alkyl group bound at the reducing end on the anti-HIV activity, the alkyl groups of dodecyl through octadecyl groups were used as the alkyl group.

Results and Discussion

Synthesis of Sulfated Alkyl Malto-oligosaccharides. Sulfated alkyl malto-oligosaccharides were prepared according to the synthetic route represented in Scheme 1.

Malto-oligosaccharides were acetylated with a mixture of sodium acetate and acetic anhydride in order to obtain as much as possible proportions of the peracetylated oligosaccharides having the β-configuration at the reducing end. Results of the acetylation are summarized in Table 1. Malto-oligosaccharides consisting of maltotetraose through maltoheptaose were individually acetylated to afford respective peracetylated malto-oligosaccharides having the β/α ratio of 3.3 to 4.9 in high yields (88−92%).

In the next glycosidation step, the peracetylated malto-oligosaccharide was reacted with various n-alkyl alcohols by use of a Lewis acid $FeCl_3$ or hetero-polyacids as the catalyst. Results of the glycosidation are summarized in Table 2. The yield of the alkyl malto-oligosaccharide peracetates was not high, 49−59%, except for the *n*-hexyl group (68%). The reason for the intermediate yield was attributable to the very low reactivity of the α-acetyl oligosaccharide.[16] However, when the hetero-polyacids were used as the catalyst, the yield was increased to 50−76%. In no. 8 in Table 2, the α-acetate, i.e., α-acetyl peracetylated maltopentaoside, was reacted with *n*-octadecyl alcohol by phosphotungstic acid catalyst. Octadecyl maltopentaoside peracetate was obtained in a 41% yield. Accordingly, the phosphotungstic acid as catalyst can bind the *n*-alkyl group at the reducing end of malto-oligosaccharides. Previously, the glycosidation of laminari-oligosaccharides with alcohols was carried out by Lewis acid catalysts, giving alkyl laminari-oligosaccharides in about 19−53% yield.[15]

All alkyl malto-oligosaccharide peracetates were richer in the β-anomer than in the α-anomer. In general, both with increasing number of glucose residues and with increasing carbon number of alkyl groups, the α/β ratio of the products decreased, that is, the proportion of the β-anomer increased. For example, octadecyl maltoheptaoside peracetates (no. 13 in Table 2) were composed of a mixture of 10% α-octadecyl and 90% β-octadecyl. Thus, it is assumed that large malto-oligosaccharides

Table 2. Glycosidation of the Malto-oligosaccharides with Alcohols[a]

no.	acetylated malto-oligosaccharide[b] glucose unit	acetylated malto-oligosaccharide[b] g (mmol)	carbon no. of *n*-alkyl alcohol	catalyst kind	catalyst wt % to sugar	temp (°C)	time (min)	yield[c] (%)	α:β[d]
1	4	1.00 (0.80)	12	PW[e]	50	80	20	62	26:74
2	4	1.00 (0.80)	18	PW	50	80	20	69	28:72
3	5	1.00 (0.65)	12	$FeCl_3$	50	60	20	58	24:76
4	5	0.50 (0.33)	18	$FeCl_3$	59	60	60	59	19:81
5[f]	5	0.10 (0.07)	18	$FeCl_3$	47	60	60	trace	n.d.
6	5	0.50 (0.32)	12	PW	50	80	30	59	18:82
7	5	0.50 (0.32)	18	PW	50	80	20	76	24:76
8[f]	5	0.20 (0.13)	18	PW	100	80	60	41	31:69
9	5	0.50 (0.32)	18	PMo[g]	59	80	20	70	18:82
10	6	1.00 (0.55)	12	PW	50	80	30	48	18:82
11	6	0.50 (0.27	18	PW	50	80	20	52	21:79
12	7	1.00 (0.47)	12	PW	50	80	120	45	12:88
13	7	1.00 (0.47)	18	PW	50	80	30	57	10:90

[a] 1.5 equiv of *n*-alkyl alcohols to acetylated sugars was used. [b] A mixture of α and β anomers (α:β ≅ 20:80) of peracetylated sugars. [c] After purification by using column chromatography. [d] Determined by HPLC. [e] Phosphotungustic acid. [f] Only α-acetyl peracetyl malto-oligosaccharide was used. [g] Phosphomolybdic acid.

Table 3. Sulfation of Alkyl Malto-oligosaccharides with the Sulfur Trioxide–Pyridine Complex[a]

	alkyl oligosaccharide			sulfated alkyl oligosaccharide				
					elem anal.[b] (found)			
sample	no. of glucose units $n+2$	carbon no. of alkyl chain m	wt (g)	yield (g)	% C	% H	% S	DS[c]
M4C12S	4	12	0.10	0.18	15.1	2.5	18.0	4.0
M4C18S	4	18	0.10	0.21	17.7	3.0	17.9	4.0
M5C12S	5	12	0.10	0.18	17.1	2.9	18.3	3.4
M5C18S	5	18	0.10	0.18	15.1	2.4	16.8	4.0
M6C12S	6	12	0.10	0.13	15.0	2.5	16.3	3.3
M6C18S	6	18	0.10	0.22	14.7	2.7	18.2	4.2
M7C12S	7	12	0.10	0.09	17.5	2.9	18.8	3.1
M7C18S	7	18	0.10	0.23	17.1	3.1	17.7	3.3

[a] Sulfur trioxide–pyridine complex (3 equiv) at 80 °C for 90 min in dry pyridine. [b] Nitrogen was not found in all samples. [c] Degree of sulfation (DS) designates the number of sulfate groups per glucose residue.

have a regulating power to produce higher proportions of β-glycosides by controlling the approaching direction of alcohols.

Sulfation of alkyl malto-oligosaccharides was carried out with a sulfur trioxide–pyridine complex which usually causes a high degree of sulfation without degrading the oligosaccharide chain.[16] Results of sulfation are summarized in Table 3. Sulfated alkyl malto-oligosaccharides with high sulfur contents of 16.3–19.2% were produced in high yields.

The structure of the sulfated alkyl malto-oligosaccharides was confirmed by ^{13}C NMR measurements. Although the maximum theoretical degree of sulfation is 3.1–3.3, several samples represented higher values, probably due to sodium sulfate included in the sulfated alkyl oligosaccharides even after dialysis. This phenomenon was also observed in the previous report.[14]

Anti-HIV Activity and Anticoagulant Activity. The anti-HIV activity of sulfated alkyl malto-oligosaccharides was assayed by use of the MTT method employing an MT-4 cell line and HIV-$1_{HTLV-III}$.[19] Results of the measurement of anti-HIV activities are shown in Table 4.

A sulfated malto-heptaoside without an alkyl group (M7C0S) exhibited a low activity of $EC_{50} = 10$ μg/mL. Sulfated alkyl maltopentaoside, -hexaoside, and -heptaoside having long alkyl groups at the terminal of oligosaccharides showed high anti-HIV activities. These high anti-HIV activities were represented by low EC_{50}'s from 0.15 to 0.59 μg/mL. These compounds had low cytotoxicities represented by large CC_{50} values. However, when sulfated alkyl oligosaccharides had too long alkyl groups compared to those of the carbohydrate portion, they showed considerable cytotoxicities ranging from CC_{50} of 380 to 810 μg/mL.

On the other hand, all sulfated alkyl maltotetraosides had low activities, exhibiting EC_{50} of 6.0–13.5 μg/mL, although they possessed long alkyl groups. In the case of laminari-oligosaccharide homologues, it has been revealed that the anti-HIV activity of sulfated dodecyl laminari-oligosaccharides does not depend on the length of carbohydrate moiety from the pentaose to the nonaose and that the anti-HIV activity does not depend on the alkyl length, while the cytotoxicity increases with increasing alkyl length.[16] However, in the malto-oligosaccharide series, it is necessary to balance the length of alkyl portion and carbohydrate moiety and to use the longer carbohydrate moiety than the pentaose in order to produce the drug having high anti-HIV activity and low cytotoxicity.

Sulfated polysaccharides and oligosaccharides usually have anticoagulant activities. Since the anti-HIV agent must be used in human blood where the HIV inhabits, high anticoagulant activities are not desirable for this agent. Thus, the anticoagulant activity of the sulfated dodecyl and octadecyl malto-oligosaccharides was determined according to a modification of the United States Pharmacopoeia procedure using bovine plasma (Table 4).[8] The sulfated malto-oligosaccharides smaller than the hexaoside possessed no anticoagulant activity, while both hexaosides and heptaosides showed small anticoagulant activities of 1–3 unit/mg. The dependence of the anticoagulant activity on the length of

Table 4. Anti-HIV Activity of Sulfated Alkyl Malto-oligosaccharides and Sulfated Oligosaccharides

	sulfated alkyl oligosaccharide					
sample	no. of glucose units	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[d] (units/mg)
M4C12S	4	12	13	>1000	>77	0[e]
M4C14S	4	14	10	810	81	n.d.
M4C16S	4	16	6.0	560	93	n.d.
M4C18S	4	18	9.3	510	55	0[e]
M5C0S	5	0	2.3	>1000	>430	n.d.
M5C6S	5	6	1.1	>1000	>910	n.d.
M5C12S	5	12	0.53	>1000	>1900	0[e]
M5C14S	5	14	0.56	>1000	>1800	n.d.
M5C16S	5	16	0.15	480	3200	n.d.
M5C18S	5	18	0.43	410	950	0[e]
M6C12S	6	12	0.20	>1000	>5000	1
M6C14S	6	14	0.48	>1000	>2100	n.d.
M6C16S	6	16	0.48	>1000	>2100	n.d.
M6C18S	6	18	0.43	410	140	1
M7C0S	7	0	10	>1000	>100	n.d.
M7C12S	7	12	0.19	>1000	>5300	3
M7C14S	7	14	0.59	>1000	>1700	n.d.
M7C16S	7	16	0.48	>1000	>2100	n.d.
M7C18S	7	18	0.37	380	1000	2
curdlan sulfate[f]		0	0.4	>1000	>2300	10

[a] Drug concentration effective for 50% inhibition of virus infection in a 5 day HIV-infected MT-4 cell culture. [b] Drug concentration for 50% cytotoxicity in a 5 day-old MT-4 cell culture. [c] Selectivity index. [d] Dextran sulfate was used for measurement of anticoagulant activity as reference (21.0 units/mg). [e] Under the limit of determination by this method. [f] Curdlan sulfate with a molecular weight of 79×10^3 used for measurement of anti-HIV activity as reference.

sulfated malto-oligosaccharide portions was similar to that for sulfated alkyl laminari-oligosaccharides.[16]

Experimental Section

General Procedures. HPLC analysis was carried out on a Tosol Series 8000 liquid chromatograph system equipped with a packed column of TSKGel Silica-60. 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 (Merck's Kieselgel 60, 70–230 mesh ASTM) was used. Malto-oligosaccharides were kindly supplied by Nihon Shokuhin Kako Co., Ltd., and their purities were over 98% by HPLC. Phosphotungstic acid (Wako Pure Chemical Industries, Ltd.), 12 tungsto(VI)silicic acid 26-water (Wako), phosphomolybdic acid (Wako), a sulfur trioxide–pyridine complex (Tokyo Kasei Kogyo Co., Ltd.) were used without further purification.

Acetylation of Malto-oligosaccharides. To boiling acetic anhydride (300 mL) in a three-necked flask was added 3.0 g of sodium acetate. Then, 3.0 g of maltopentaose was gradually added under vigorous stirring. The solution was kept for 2 h at 140 °C and then cooled to room temperature. After a workup procedure, 5.1 g (89%) of peracetylated maltopentaose was obtained. Other malto-oligosaccharides were acetylated by the same procedure. All peracetates were purified by recrystallization from ethanol.

Glycosidation of Peracetylated Malto-oligosaccharide with *n*-Alkyl Alcohol. Method A. To a flask containing 10 mL of dry toluene heated at 80 °C were added 0.50 g of peracetylated maltopentaose and 132 mg of *n*-octadecyl alcohol. After the mixture became a clear solution, 253 mg of phosphotungstic acid dried under vacuum at 120 °C was added. The reaction was kept for 20 min, and then it was diluted with 50 mL of ethyl acetate and successively washed with aqueous $NaHCO_3$ twice and with saturated NaCl solution twice. After a workup procedure, the raw product was chromatographed on silica gel using (5/1–1/3) hexane–ethyl acetate as the eluent. An off-white amorphous solid in a 76% yield was recovered. ^{13}C NMR: δ 61.3 (C_6), 62.2 (C_6), 62.3 (C_6), 62.8 (C_6), 63.0 (C_6), 95.7 (C_1, 4C), 100.1 (anomeric C_1).

Method B. Peracetylated maltopentaose (500 mg) and 96 mg of *n*-octadecyl alcohol were added to 10 mL of dry toluene at 60 °C, followed by adding 56 mg of ferric chloride. The mixture was stirred for 1 h. After a workup procedure, 0.34 g of *n*-octadecyl maltopentaoside peracetate was obtained in 59% yield.

Deacetylation of Alkyl Oligosaccharide Peracetate. One gram of alkyl malto-oligosaccharide peracetate was stirred in methanol containing 0.2 equiv of 2 N sodium methoxide to the acetyl group at room temperature for 5 h, followed by neutralizing with an H^+-type ion-exchange resin (Daia Ion SK-1B) to pH = 6.0–6.5. A colorless alkyl oligosaccharide was obtained in a quantitative yield. NMR data of hexyl maltopentaoside follows. ^1H NMR: δ 4.47 (anomeric H_1, J_{12} = 8.0 Hz). ^{13}C NMR: δ 63.0 (C_6, 5C) 102.0 (C_1), 102.2 (C_1, 2C), 102.8 (C_1), 104.4 (anomeric C_1).

Sulfation of *n*-Octadecyl Maltopentaoside. A solution containing 102 mg of *n*-hexadecyl maltotetraoside in 20 mL of dry pyridine was heated to 85 °C, and then 710 mg (3.0 equiv of hydroxyl groups) of a 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. The precipitated $BaSO_4$ was separated by centrifugation, and the supernatant was passed through a Na^+-type ion-exchange resin column. An aqueous solution of the raw product was neutralized to pH = 6.9–7.2 by 0.2 N HCl. Finally, the product was freeze-dried from water to give 211 mg of an off-white powdery sulfated *n*-hexadecyl maltotetraoside. ^{13}C NMR: δ 96.1 (C_1), 96.6 (C_1), 96.9 (C_1, 2C), 103.3 (anomeric C_1).

Anti-HIV Assay. The anti-HIV activity of a series of sulfated alkyl oligosaccharides against HIV infection was determined by protection from HIV-induced cytopathic effects (CPE).[9] MT-4 cells were infected with HTLV-IIIB at the multiplicity of infection (MOI) of 0.01.[20] 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.*[19] 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.[8] Dextran sulfate was used as a reference compound with an anticoagulant activity of 21.0 units/mg. The experimental results were the average of three or four measurements.

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