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Enzymatic Synthesis of Sulfated Disaccharides using β-D-Galactosidase-catalyzed Transglycosylation

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We have established a unique enzymatic approach for obtaining sulfated disaccharides using *Bacillus circulans* β-D-galactosidase-catalyzed 6-sulfo galactosylation. When 4-methyl umbelliferyl 6-sulfo β-D-galactopyranoside (S6Galβ-4MU) was used as a donor, the enzyme induced transfer of 6-sulfo galactosyl residue to GlcNAc acceptor. As a result, the desired compound 6′-sulfo *N*-acetyllactosamine (S6Galβ1–4GlcNAc) and its positional isomer 6′-sulfo *N*-acetylisolactosamine (S6Galβ1–6GlcNAc) were observed by HPAEC-PAD, in 49% total yield based on the donor added, and in a molar ratio of 1:3.5. With a glucose acceptor, the regioselectivity was substantially changed and S6Galβ1–2Glc was mainly produced along with β-(1–1)α, β-(1–3), β-(1–6) isomers in 74% total yield. When methyl α-D-glucopyranoside (Glcα-OMe) was an acceptor, the enzyme also formed mainly S6Galβ1–2Glcα-OMe with its β-(1–6)-linked isomer in 41% total yield based on the donor added. In both cases, it led to the predominant formation of β-(1–2)-linked disaccharides. In contrast, with the corresponding methyl β-D-glucopyranoside (Glcβ-OMe) acceptor, S6Galβ1–3Glcβ-OMe and S6Galβ1–6Glcβ-OMe were formed in a low total yield of 12%. These results indicate that the regioselectivity and efficiency on the β-D-galactosidase-mediated transfer reaction significantly depend on the anomeric configuration in the glucosyl acceptors.

Key words: *Bacillus circulans*; β-D-galactosidase; sulfated disaccharide; transglycosylation; regioselectivity

Sulfated oligosaccharides are presented in human, rat, and dog milk.[1–5] It has been suggested that these sulfated sugar esters have important roles in simultaneous delivery of calcium and sulfate, two essential nutrients in early life.[1] Sulfation of oligosaccharide units also occurs in glycoconjugates such as glycoproteins, glycolipids, and proteoglycans. These have been known to play numerous roles in biological event such as selectin-binding,[6–8] laminin-binding, neural cell migration,[9] bacteria binding,[10–12] and activation of macrophages.[13] Therefore, the efficient synthetic routes of their oligosaccharide units have become more interesting. Chemical methods for obtaining sulfated oligosaccharides have been developed,[14–17] but it involves various elaborate procedures. From a practical viewpoint, the enzymatic method is an attractive alternative for synthesis of such oligosaccharides.[18,19] Some sulfotransferases involved in biosynthesis of sulfated oligosaccharides have been characterized,[20–22] but the enzymes are not always available, very expensive, and required expensive nucleotides. From such an aspect, we searched for a β-galactosidase showing hydrolytic activity so that it hydrolyzes 4-methyl umbelliferyl 6-sulfo β-D-galactopyranoside into S6Gal and 4-metyl umbelliferone. If such an enzyme was found, it might make it possible to catalyze the direct transfer of a 6-sulfo β-galactose unit to some acceptor substrates.

In this paper, we describe a preparation of 6′-sulfo *N*-acetyllactosamine (S6Galβ1–4GlcNAc), which constitutes L-selectin ligand 6′-sulfo sialyl Le^x,[6] sulfated *O*-[23,24] and *N*-glycan,[25] and its related sulfated disaccharides using β-D-galactosidase-catalyzed 6-sulfo galactosylation.

Materials and Methods

Materials. β-D-Galactosidase II (EC 3.2.1.23) from *Bacillus circulans* was kindly supplied by Daiwa Kasei K. K., Osaka and commercially available β-D-galactosidase from *Escherichia coli* was purchased from Toyobo Co. Ltd., Osaka. 4-Methyl umbel-

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Abbreviations: HPAEC-PAD, high performance anion exchange chromatography-pulsed amperometric detection; S6Galβ-4MU, 6-sulfo β-D-galactopyranoside; Glcα-OMe, methyl α-D-glucopyranoside; Glcβ-OMe, methyl β-D-glucopyranoside; S6Gal, D-galactose-6-sulfate; S6Galβ-*p*NP, *p*-Nitrophenyl 6-sulfo β-D-galactopyranoside; Galβ-*o*NP, *o*-nitrophenyl β-D-galactopyranoside

liferyl 6-sulfo β-D-galactopyranoside (S6Galβ-4MU) was from Toronto Research Chemicals Inc., Ontario. *p*-Nitrophenyl 6-sulfo β-D-galactopyranoside (S6Galβ-*p*NP) was a kind gift from Dr. Hirotaka Uzawa, National Institute of Materials and Chemical Research, Tsukuba, Japan. All other chemicals were obtained from commercial sources.

Enzyme assay. β-D-Galactosidase activity was assayed as follows: Mixture containing 2 mM *o*-nitrophenyl β-D-galactopyranoside (Galβ-*o*NP) in 0.9 ml of 50 mM sodium phosphate buffer (pH 6.0) and an appropriate amount of enzyme in a total volume of 0.1 ml were incubated for 10 min at 40°C. The reaction was stopped by adding 0.5 ml of 1.0 M Na_2CO_3, and then the liberated *o*-nitrophenol was measured spectrophotometrically at 420 nm. One unit of enzyme was defined as the amount hydrolyzing 1 μmole of Galβ-*o*NP per min.

Analytical method. HPAEC-PAD analysis was done on a DX-300 Bio-LC system equipped with a pulsed amperometric detector (Dionex, Osaka, Japan). Oligosaccharides were separated on a CarboPac P-1 column (Dionex, φ4×250 mm) at a flow rate of 1 ml/min at room temperature. The elution for S6Galβ1-4GlcNAc and S6Galβ1-6GlcNAc was done with 0.2 M CH_3COONa in 100 mM NaOH for 20 min, and then with 0.2 to 0.4 M CH_3COONa in 100 mM NaOH from 20 to 60 min. The elution for other sulfated disaccharides was done with 0.2 M CH_3COONa in 100 mM NaOH for 25 min. The sulfated disaccharides, which were obtained by β-D-galactosidase-mediated 6-sulfo galactosylation, were measured by HPAEC-PAD analysis coupled with the absolute standard curve method. ^1H- and ^{13}C-NMR spectra were recorded on a JEOL JNM-LA 500 spectrometer at 25°C. Chemical shifts are expressed in δ relative to sodium 3-(trimethylsilyl) propionate as an external standard.

Hydrolytic reaction of β-D-galactosidases on sulfated β-D-galactopyranosides. The hydrolytic activity of β-D-galactosidase on S6Galβ-4MU, S6Galβ-*p*NP, and S3Galβ-*p*NP was investigated by incubating a mixture (50 μl) containing 5 mM of substrates in 50 mM sodium phosphate buffer (pH 6.0) at 40°C. The hydrolyzates were analyzed by TLC developed with 4:1 acetonitrile-water (v/v) and stained with orcinol-sulfric acid method.

6-Sulfo β-D-galactopyranoside hydrolyzing activity of β-D-galactosidase. A mixture containing 2 mM S6Galβ-4MU in 300 μl of 50 mM sodium phosphate buffer (pH 7.0) and an appropriate amount of enzyme were incubated at 40°C. The samples (50 μl) were taken at intervals (0, 24, 48, and 72 h) during the incubation, and inactivated by adding 50 μl of 1.0 M Na_2CO_3. The liberated 4-methylumbelliferon was measured by a microplate reader (Biolumin 960, Amersham Pharmacia, Sweden) at Ex. 330 nm and Em. 460 nm. One unit of enzyme was defined as the amount hydrolyzing 1 μmole of S6Galβ-4MU per min.

The initial rates of enzymatic reaction were evaluated from kinetic curves of the product accumulation as described above. The parameters of Michaelis-Menten type kinetics were evaluated by a 1/v-1/[S] plot and the least squares method.

Preparation of 6′-sulfo N-acetyllactosamine (1) and 6′-sulfo N-acetylisolactosamine (2) with B. circulans β-D-galactosidase. GlcNAc (132 mg, 597 μmol) and S6Galβ-4MU (50 mg, 120 μmol) were dissolved in 750 μl of 100 mM sodium acetate buffer (pH 5.0), followed by 200 U of *B. circulans* β-D-galactosidase. The mixture was incubated for 100 h at 40°C and the reaction was stopped by boiling for 5 min. The precipitate was removed by centrifugation (12,000 rpm, 15 min), and the supernatant was put onto a Sep-pak accel QMA column (φ2×4 cm) equilibrated with H_2O. The column was eluted with 40 ml of H_2O and 0 (60 ml)–0.5 (60 ml) M pyridine-acetate buffer (pH 5.4) of a linear gradient, and the effluent solution was monitored by measuring the absorbance at 210 nm (characteristic absorption of the *N*-acetyl group). The chromatogram showed two peaks (F-1, tubes 3–13; F-2, tubes 38–60). The second peak was presumed to contain the transfer product. F-1 contained GlcNAc used as an acceptor substrate. The F-2 fraction was put onto a Dowex 50W×8 column (Na^+ form, φ1.5×15 cm) equilibrated with H_2O. The unadsorbed fraction was put onto a TSKgel Amide 80 column (φ2.15×30 cm) equilibrated with 80% CH_3CN containing 15 mM potassium phosphate buffer (pH 5.4). The column was eluted with a linear gradient of 80 (200 ml) to 50% (200 ml) CH_3CN containing 15 mM potassium phosphate buffer (pH 5.4) in 80 min at a flow rate of 5.0 ml/min. The chromatogram showed three peaks (F-1, tubes 36–43; F-2, tubes 44–48; F-3, tubes 49–55). F-1 was presumed to contain 6-sulfo galactose released during the incubation. F-2 and F-3 were combined, concentrated, and desalted with Dowex 50 ×8. The resulting disaccharide fractions were lyophilized to afford compounds **1** (4.7 mg) and **2** (5.7 mg), respectively, in a 18% total yield based on the donor added.

Preparation of 6-sulfo β-D-Gal-(1–1)-α-Glc (3), 6-sulfo β-D-Gal-(1–2)-Glc (4), 6-sulfo β-D-Gal-(1–3)-Glc (5), and 6-sulfo β-D-Gal-(1–6)-Glc (6) with B. circulans β-D-galactosidase. Glucose (108 mg, 600 μmol) and S6Galβ-4MU (50 mg, 120 μmol) were dissolved in 750 μl of 100 mM sodium acetate buffer (pH 5.0), followed by 200 U of *B. circulans* β-D-

galactosidase. The mixture was incubated for 100 h at 40°C and the reaction was stopped by boiling for 5 min. The precipitate was removed by centrifugation (12,000 rpm, 15 min), and the supernatant was put onto a Sep-pak accel QMA column ($\phi 2 \times 4$ cm) equilibrated with H_2O. The column was eluted with 40 ml of H_2O and 0 (60 ml)–0.5 (60 ml) M pyridine-acetate buffer (pH 5.4) of linear gradient, and the effluent solution was monitored by measuring the absorbance at 485 nm (neutral sugar content, phenol-sulfuric acid method). The chromatogram showed two peaks (F-1, tubes 3–13; F-2, tubes 38–60). The second peak was presumed to contain the transfer product. The F-2 fraction was put onto a Dowex 50W × 8 column (Na^+ form, $\phi 1.5 \times 15$ cm) equilibrated with H_2O. The unadsorbed fraction was combined, concentrated. This fraction was further put onto a TSKgel Amide 80 column ($\phi 2.15 \times 30$ cm) equilibrated with 80% CH_3CN containing 15 mM potassium phosphate buffer (pH 5.4). The column was eluted with a linear gradient of 80 (200 ml) to 50% (200 ml) CH_3CN containing 15 mM potassium phosphate buffer (pH 5.4) in 80 min at a flow rate of 5.0 ml/min. As shown in Fig. 1, the chromatogram showed four peaks (F-1, tubes 51–54; F-2, tubes 55–58; F-3, tubes 59–60; F-4, tubes 61–63). Fractions F-1 to 4 were combined, concentrated, and desalted with Dowex 50W × 8. The resulting disaccharide fraction was lyophilized to afford **3** (6.6 mg), **4** (9.6 mg), **5** (8.3 mg) and **6** (1.7 mg), respectively, in a 48% total yield based on the donor added.

Preparation of 6′-sulfo β-D-Gal-(1–2)-α-D-Glc-OMe (7), 6′-sulfo β-D-Gal-(1–6)-α-D-Glc-OMe (8), 6′-sulfo β-D-Gal-(1–3)-β-D-Glc-OMe (9), and 6′-sulfo β-D-Gal-(1–6)-β-D-Glc-OMe (10) with B. circulans β-D-galactosidase. Glcα-OMe (11.6 mg, 60 μmol) and S6Galβ-4MU (5 mg, 12 μmol) were dissolved in 75 μl of 100 mM sodium acetate buffer (pH 5.0), followed by 20 U of *B. circulans* β-D-galactosidase. The mixture was incubated for 100 h at 40°C and was used in successive chromatographies on Sep-pak accel QMA and Dowex 50W × 8 (Na^+ form) columns as described above. A mixture of **7** and **8** was lyophilized to afford 2.3 mg (41% yield based on the donor added).

Compounds **9** and **10** were synthesized from Glcβ-OMe (11.6 mg, 60 μmol) and S6Galβ-4MU (5 mg, 12 μmol) in a similar manner. The reaction mixture was used in successive chromatographies on Sep-pak accel QMA, Dowex 50W × 8 (Na^+ form), and TSKgel Amide 80 columns as described above. Two transfer products were collected, concentrated, and lyophilized to afford **9** (0.07 mg), **10** (0.1 mg), respectively, in a 3% total yield based on the donor added.

Preparation of 2 and 6 by E. coli β-D-galactosidase. GlcNAc (132 mg, 597 μmol) and S6Galβ-4MU (50 mg, 120 μmol) were dissolved in 750 μl of 100 mM sodium phosphate buffer (pH 7.0), followed by 200 U of *E. coli* β-D-galactosidase. The mixture was incubated for 24 h at 40°C. The transfer product was separated by successive chromatographies on Sep-pak accel QMA and Dowex 50W × 8 columns, and then concentrated and lyophilized to yield 2.3 mg (4.6% yield based on the donor added). The ^1H- and ^{13}C-NMR data (D_2O) of the transfer product were almost identical to those of **2**. In a similar manner, the transglycosylation reaction with the Glc acceptor instead of GlcNAc gave 2.4 mg of the transfer product in a 4.8% yield based on the donor added. The ^1H- and ^{13}C-NMR data (D_2O) of the transfer product were almost identical to those of **6**.

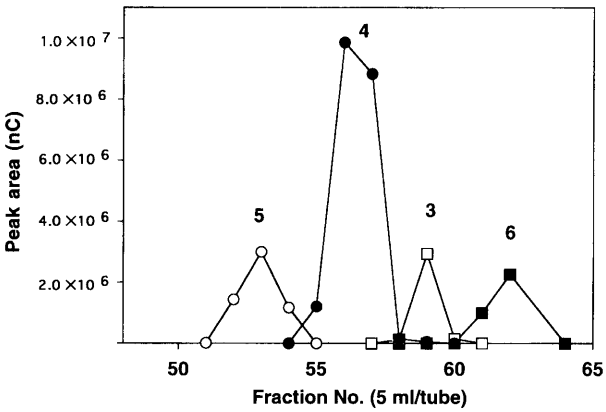


Fig. 1. TSKgel Amido-80 Chromatography of Transglycosylation Products Formed from S6Galβ-4MU and Glucose by *B. circulans* β-D-Galactosidase.

Each fraction was analyzed by HPAEC-PAD analysis and then peak areas of **3** (□), **4** (●), **5** (○), and **6** (■) were plotted.

Results and Discussion

Hydrolytic reaction of β-D-galactosidases on sulfated β-D-galactopyranosides

Hydrolytic activity of *E. coli* β-D-galactosidase on S6Galβ-4MU and S6Galβ-*p*NP was examined by TLC analysis as in Fig. 2. The enzyme hydrolyzed the 6-*O*-sulfated β-D-galactopyranosides into S6Gal and, respective 4MU and *p*NP. On the other hand, it did not act on S3Galβ-*p*NP. These data were also the case for *B. circulans* β-D-galactosidase (data not shown). Kinetic parameters of two β-D-galactosidases on S6Galβ-4MU were measured with Galβ-*o*NP as a control substrate and are listed in Table 1. The introduction of the 6-*O*-substituted sulfonyl group to Galβ-4MU does not appreciably influence the K_m, but did significantly for the V_{max}. As a result, the catalytic efficiency of S6Galβ-4MU was greatly reduced in comparison with that of Galβ-*o*NP and the k_0/K_m of S6Galβ-4MU for *B. circulans* β-D-galactosidase was 0.4% of that of Galβ-*o*NP. However, S6Galβ-4MU still acted as a substrate for

Table 1. Kinetic Parameters of β-D-Galactosidases on 6-*O*-Sulfated and Non-sulfated β-D-Galactosides

Source	Substrate	K (mM)	V_{max} (μmol/mg/min)	k_0 (sec^{-1})	k_0/K_m ($mM^{-1}\cdot sec^{-1}$)
B. circulans	Galβ-*o*NP	0.47	6.7	1.8×10^2	3.9×10^2
	S6Galβ-4MU	0.11	0.04	0.18	1.6
E. coli	Galβ-*o*NP	0.19	53	4.8×10^5	2.5×10^6
	S6Galβ-4MU	0.24	0.47	36	1.5×10^2

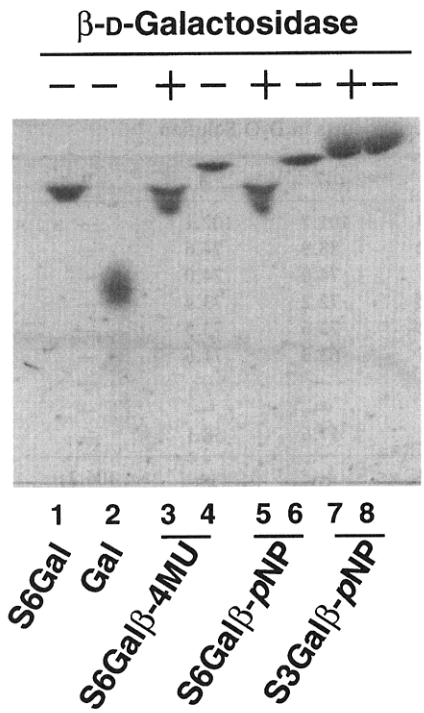


Fig. 2. Hydrolytic Reaction of *E. coli* β-D-Galactosidase on 6- or 3-*O*-Substituted β-D-galactopyranosides.

Reaction mixtures were analyzed by TLC analysis as described as Materials and Methods. Lanes 1 and 2, galactose-6-sulfate and galactose, respectively, as authentic compounds; lanes 3 and 4, S6Galβ-4MU; lanes 5 and 6, S6Galβ-*p*NP; lanes 7 and 8, S3Galβ-*p*NP. +, Presence of the enzyme; −, absence of the enzyme.

the enzyme despite the 6-*O*-sulfation. This is the first report that β-D-galactosidase has a hydrolytic ability acting on 6-*O*-sulfated β-D-galactopyranosides.

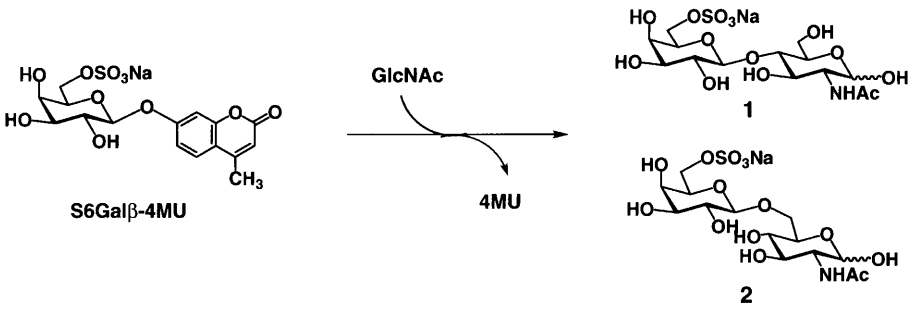
B. circulans β-D-galactosidase-catalyzed 6-sulfo galactosylation

B. circulans β-D-galactosidase has been widely used for synthesis of β-D-galactose-containing oligosaccharides,[26-32] because of its high transglycosylation activity. We have already reported that the enzyme catalyzed predominant β-D-galactosyl transfer from lactose to OH-4 over the OH-6 position of GlcNAc. The enzymatic methodology was expanded to the synthesis of **1** from S6Galβ-4MU as a donor to GlcNAc as an acceptor. The reaction was done in a high substrate concentration of 18% as much as possible and in a large excess of catalytic amount (200 U/mL). The desired **1** and its positional isomer **2** were analytically observed by HPAEC-PAD in 49% total yield, and in a molar ratio of 1:3.5 as shown in Figs. 3(a) and 5(a). They were separated by successive chromatographies on Sep-pak accel QMA, Dowex 50W × 8, and TSK-gel Amide 80 columns. No β-(1-3) or β-(1-1)-linked disaccharides were observed during the reaction. Compounds **1** and **2** were characterized to be the desired compound S6Galβ1-4GlcNAc and its positional isomer S6Galβ1-6GlcNAc, respectively, by NMR analysis (Table 2).

When Glc was an acceptor instead of GlcNAc, four transfer products **3**, **4**, **5**, and **6** were observed in a high total yield of 74% based on the donor added and in a molar ratio of 1:6.7:2.1:1.4, respectively, as in Figs. 3(b) and 5(b). They were easily separated by a TSK-gel Amide-80 column as in Fig. 1. Their structures were also characterized by ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR analyses (Table 2). Compound **4** preponderated over the β-(1-1)α, β-(1-3), and β-(1-6)-linked isomers during the reaction. To understand the highly regioselective formation of **4**, further transglycosylations were done with methyl α- and β-D-glucopyranosides (Glcα-OMe and Glcβ-OMe) as acceptor substrates. With the former acceptor, β-(1-2)-linked disaccharide **7** predominated and there was a small proportion of β-(1-6)-linked isomer **8**. Compounds **7** and **8** were observed in a total yield of 41% based on the donor and in a molar ratio of 3.8:1 (Figs. 3(c) and 5(c)). In contrast, with the corresponding Glcβ-OMe acceptor, β-(1-3)-linked disaccharide **9** and β-(1-6)-linked isomer **10** were observed in a low total yield of 12% and in a molar ratio of 1:3 (Figs. 3(d) and 5(d)). No S6Galβ1-2Glcβ-OMe was detected during the reaction.

Regioselectivity of sulfated disaccharide formation

The positions of 6-sulfo galactosylation mediated by *B. circulans* β-D-galactosidase with GlcNAc, Glc, Glcα-OMe, and Glcβ-OMe acceptors are depicted by arrows in Fig. 5. The 6-sulfo galactosylation profile catalyzed by the β-D-galactosidase with S6Galβ-4MU and GlcNAc was shown in Fig. 4. The β-(1-6)-linked disaccharide was preferentially formed over β-(1-4)-linked disaccharide during the entire course of the reaction. Maximum production of desired **1** was observed after 120 h, although its production was about one fourth that of **2** (Fig. 5(a)). The observed regioselectivity with S6Galβ-4MU was substantially

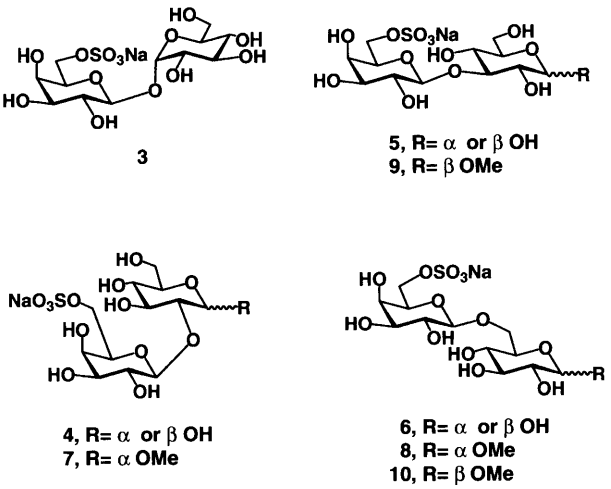


Scheme 1.

Table 2. ^{1}H- and ^{13}C-NMR Chemical Shifts of Transfer Products in D_2O Solution

	1	2	3	4	5	6	7	8	9	10
C-1α	93.0	93.6	102.3	94.6	93.2	94.9	101.7	102.1	—	—
C-2α	56.3	56.8	73.4	83.9	72.1	74.2	83.9	74.6	—	—
C-3α	71.7	73.3	71.0	73.9	87.6	75.5	74.0	74.0	—	—
C-4α	82.3	72.7	74.1	72.2	68.8	72.2	72.2	73.4	—	—
C-5α	72.7	73.3	74.4	73.6	72.4	73.2	73.6	73.5	—	—
C-6α	62.6	71.5	61.9	63.3	62.1	71.5	63.3	71.6	—	—
COCH3α	24.5	24.7	—	—	—	—	—	—	—	—
C=Oα	177.5	177.5	—	—	—	—	—	—	—	—
Meα	—	—	—	—	—	—	57.6	58.1	—	—
C-1β	97.4	97.7	—	97.6	97.0	98.7	—	—	106.2	106.3
C-2β	58.8	59.4	—	84.8	74.4	76.8	—	—	75.1	75.3
C-3β	75.4	76.6	—	75.6	87.6	77.6	—	—	88.1	77.6
C-4β	81.7	72.7	—	72.2	68.9	72.4	—	—	71.2	72.4
C-5β	77.3	77.5	—	78.3	76.7	78.4	—	—	78.3	78.5
C-6β	72.3	72.3	—	62.9	62.2	72.0	—	—	63.5	71.9
COCH3β	24.8	24.9	—	—	—	—	—	—	—	—
C=Oβ	177.3	177.3	—	—	—	—	—	—	—	—
Meβ	—	—	—	—	—	—	—	—	60.0	59.9
C-1′α	105.6	106.3	105.0	107.2	104.7	106.1	107.2	106.3	—	—
C-2′α	73.3	73.4	72.6	74.4	73.7	73.4	74.6	75.3	—	—
C-3′α	74.9	75.4	74.8	75.2	74.3	75.3	75.4	75.5	—	—
C-4′α	70.9	71.1	70.3	71.2	69.8	71.1	71.2	71.2	—	—
C-5′α	74.9	75.2	74.9	75.3	76.7	75.4	75.5	75.8	—	—
C-6′α	69.8	69.8	69.4	70.2	69.7	69.8	70.3	69.9	—	—
C-1′β	105.5	106.1	—	105.9	105.0	106.2	—	—	105.7	106.1
C-2′β	73.3	73.4	—	74.4	73.7	73.4	—	—	73.9	73.4
C-3′β	74.9	75.5	—	75.2	74.4	75.3	—	—	75.4	75.5
C-4′β	70.9	71.1	—	71.2	69.8	71.1	—	—	71.1	71.2
C-5′β	74.9	75.2	—	75.3	76.7	75.4	—	—	75.7	75.9
C-6′β	69.9	70.0	—	70.1	69.8	69.9	—	—	70.2	70.0
H-1α	5.07	5.13	5.14	5.45	5.18	5.18	5.10	5.10	—	—
	3.4 Hz	3.7 Hz	2.5 Hz	3.4 Hz	3.7 Hz	3.7 Hz	3.4 Hz	3.4 Hz		
H-1β	4.61	4.67	—	4.68	4.70	4.62	—	—	4.43	4.40
	7.3 Hz	8.3 Hz		7.8, 7.8 Hz	8.0 Hz	7.9 Hz			8.2 Hz	8.0 Hz
H-1′α	4.38	4.39	4.51	4.52	4.65	4.42	4.53	4.45	—	—
	8.0 Hz	7.7 Hz	8.0 Hz	7.6 Hz	7.9 Hz	7.7 Hz	7.7 Hz	6.7 Hz		
H-1′β	4.38	4.40	—	4.52	4.42	4.41	—	—	4.65	4.47
	8.1 Hz	7.7 Hz		7.6 Hz	7.9 Hz	7.0 Hz			8.0 Hz	8.0 Hz

different from that with lactose, because the transgalactosylation from lactose to GlcNAc using the enzyme causes the preferential formation of LacNAc over IsoLacNAc.[26] In a similar way, replacement of Glc acceptor by GlcNAc increased the efficiency of 6-sulfo galactosylation, but diminished the regioselectivity (Fig. 5(b)). Yanahira *et al.* have reported that the enzyme catalyzed the formation of Galβ1-2Glc, Galβ1-3Glc, Galβ1-4Glc, and Galβ1-6Glc in a molar ratio of 1:7.6:8.2:3 through the galactosylation from *p*-nitrophenyl β-D-galactopyranoside donor to glucose acceptor.[28] The β-(1-4) linked disaccharide



Scheme 2.

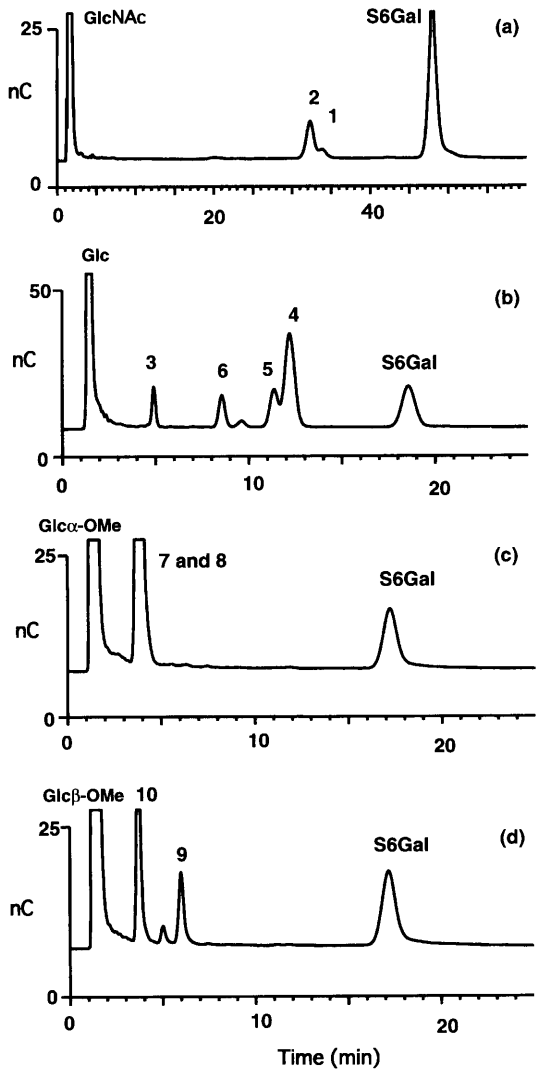


Fig. 3. HPAEC-PAD Analyses of Reaction Mixtures Obtained by *B. circulans* β-D-Galactosidase-catalyzed 6-Sulfo Galactosylation with S6Galβ-4MU as a Donor Substrate.

Acceptor substrates: (a), GlcNAc; (b), Glc; (c), Glcα-OMe; (d), Glcβ-OMe. The numbers show the transfer products.

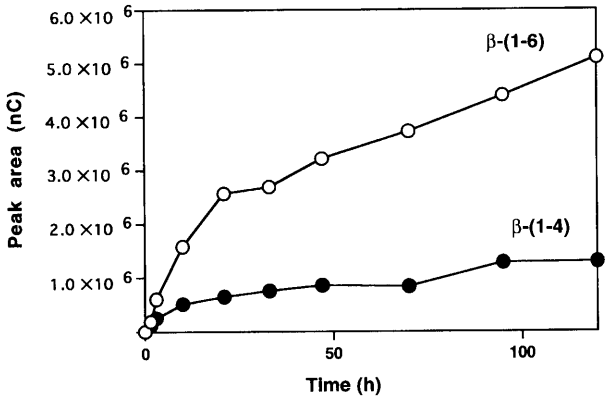


Fig. 4. Course of *B. circulans* β-D-Galactosidase-catalyzed 6-Sulfo Galactosylation with S6Galβ-4MU and GlcNAc.

Reaction mixtures were analyzed by HPAEC-PAD during incubation and peak areas of **1** (●) and **2** (○) were plotted.

was produced much more than the β-(1-2) linked isomer. On the contrary, 6-sulfo galactosylation with Glc produced mainly the β-(1-2)-linked product **4** along with β-(1-1)α, β-(1-3), and β-(1-6) isomers, and no desired β-(1-4)-linked isomer, which occurs in rat mammary gland, was detected during the reaction. The regioselectivity of *B. circulans* β-D-galactosidase was significantly affected by introduction of a 6-*O*-substituted sulfonyl group into Galβ-4MU. The predominant formation of **4** may be related to adopting a suitable orientation along C-1 to C-2 of the Glc acceptor. In order to analyze the regioselective formation, transglycosylation reactions with Glcα-OMe and Glcβ-OMe instead of Glc were done. When Glcα-OMe was an acceptor, the enzyme formed mainly β-(1-2)-linked disaccharide **7** with its β-(1-6)-linked isomer **8** in a high yield (Fig. 5(c)). In contrast, with the corresponding Glcβ-OMe acceptor, mainly β-(1-6)-linked disaccharide **10** with its β-(1-3)-linked isomer **9** was formed in a low yield (Fig. 5(d)). Thus, replacement of Glcβ-OMe by Glcα-OMe did change the direction of S6Gal transfer and diminish the efficiency of 6-sulfo galactosylation. The regioselectivity of the S6Gal transfer to the acceptor by the enzyme was strongly influenced by the 1,2-*cis* configuration of 1-methoxy and 2-hydroxyl groups, which probably adopt an orientation favorable to the enzyme. These results suggest that the preponderant formation of **4** with Glc acceptor is induced by recognizing α-glucose as an acceptor molecule rather than β-glucose.

E. coli β-D-galactosidase-catalyzed 6-sulfo galactosylation

E. coli β-D-galactosidase has been also used for oligosaccharide synthesis.[33-36] It has been reported that the enzyme predominantly catalyzed β-D-galactosyl transfer from lactose to the OH-6 position of GalNAc[32] and GlcNAcβ-SEt.[33] The transglycosylation by *E. coli* enzyme was done with S6Galβ-4MU

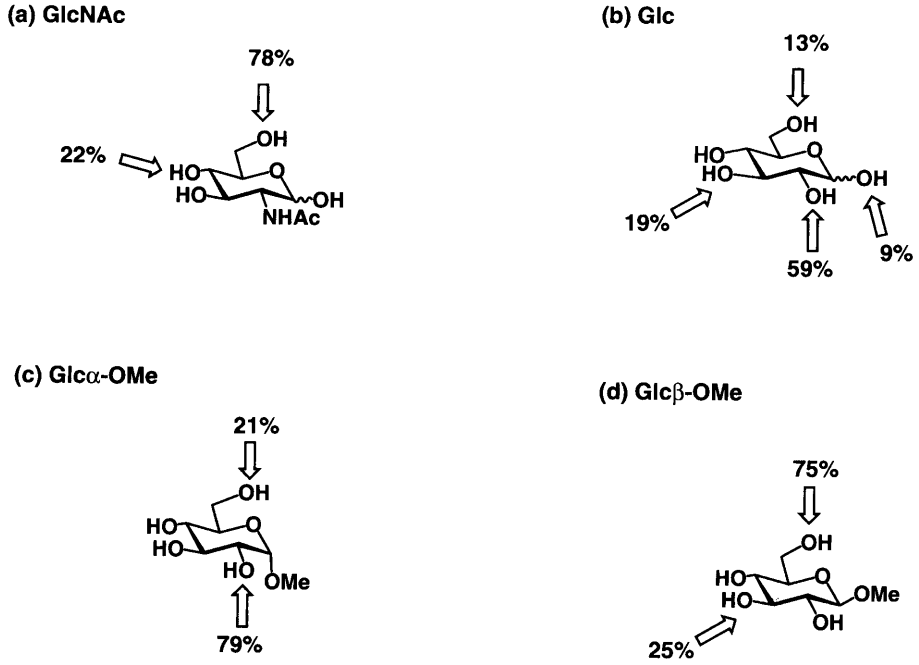


Fig. 5. Regioselectivity of *B. circulans* β-D-Galactosidase-catalyzed 6-Sulfo Galactosylation with S6Galβ-4MU as a Donor.
Acceptor substrates: (a), GlcNAc; (b), Glc; (c), Glcα-OMe; (d), Glcβ-OMe. Arrows indicate the positions of 6-sulfo galactosylation. The numbers show the function of a given 6-sulfo galactosylation compared with the total. The percentages are based on the time at which the total production of 6′-sulfated disaccharides reaches its maximum detected by HPAEC-PAD. When Glcα-OMe was used as an acceptor substrate, **7** and **8** were separated from each other by HPAEC-PAD. Therefore, the ratio of two compounds was estimated by ^{1}H-NMR.

as a donor and GlcNAc or Glc as acceptor. The enzyme formed only β-(1–6)-linked disaccharides **2** and **6** from corresponding acceptors in 4.6 and 4.8%, respectively, based on the donor. The regioselectivity of *E. coli* β-D-galactosidase was not affected by introduction of a 6-*O*-substituted sulfonyl group into Galβ-4MU, but its efficiency on the 6-sulfo galactosylation decreased greatly. These reactivities on the substrates were significantly different from those of *B. circulans* β-D-galactosidase.

Conclusion

An interesting result from this study is the capacity of the β-D-galactosidase to catalyze 6-sulfated galactosyl transfer to GlcNAc and Glc acceptors. The regioselectivity and efficiency greatly depend on the nature of the acceptor substrate and vary with different enzymes. We developed a synthetic method for **1**, which is a component of L-selectin ligand, using β-D-galactosidase from *B. circulans*. This is the first report of the practical synthesis of sulfated galactosyl disaccharides using β-D-galactosidase-catalyzed 6-sulfo galactosylation. Such well-defined 6′-sulfated disaccharides, especially S6Galβ1-4GlcNAc, would be useful as substrates for sialyltransferases and fucosyltaransferases involved in biosynthetic routes for forming glycoconjugates.

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