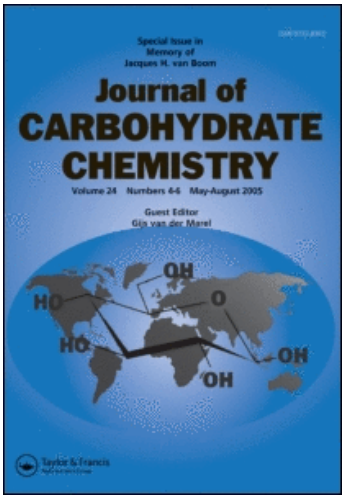

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Glucuronidase-assisted Transglycosylation for the Synthesis of Highly Functional Disaccharides: β-D-Glucuronyl 6-*O*-Sulfo-β-D-Gluco- and -β-D-Galactopyranosides

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Glucuronidase-assisted Transglycosylation for the Synthesis of Highly Functional Disaccharides: β-D-Glucuronyl 6-*O*-Sulfo-β-D-Gluco- and -β-D-Galactopyranosides

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The substrate specificity of snail (*Helix pomatia* and *Helix aspersa*), limpet (*Patella vulgata*), and bovine glucuronidases was examined by using *p*-nitrophenyl glucuronide (GlcA-*O*-*p*NP) and *p*-nitrophenyl 6-*O*-sulfo-β-D-glycopyranosides as the glycosyl donor and acceptors, respectively. When the donor was treated with these enzymes in the absence of the acceptors, β(1→3) glucuronyl disaccharides were obtained as the major products together with β(1→2) isomers as the result of an enzymatic "self-transglycosylation" reaction. When *p*-nitrophenyl 6-*O*-sulfo-β-D-glucopyranosides (6-*O*-sulfo-Glc-*O*-*p*NP and 6-*O*-sulfo-Glc-*S*-*p*NP) were applied as acceptor substrates, every glucuronidase transferred the GlcA residue to either the *O*-3 or *O*-2 position in 6-*O*-sulfo-Glc to yield a mixture of GlcAβ(1→3)- and GlcAβ(1→2)-linked disaccharides in a ratio of 12:1 ∼ 1:1. On the other hand, when *p*-nitrophenyl 6-*O*-sulfo-β-D-galactopyranosides (6-*O*-sulfo-Gal-*O*-*p*NP and 6-*O*-sulfo-Gal-*S*-*p*NP) were applied, limpet and bovine glucuronidases gave a GlcAβ(1→3)-linked disaccharide regioselectively, while the snail enzymes showed no reactivity.

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Keywords Transglycosylation, Glucuronidases, Glucuronic acids, Sulfo sugars

INTRODUCTION

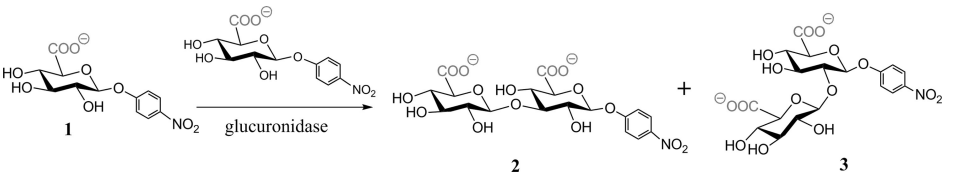
Glucuronylated *O*-sulfo-sugars are widely distributed in nature as repeating disaccharide units in glycosaminoglycans (GAGs), such as those in heparin, and chondroitin sulfate. Glucuronic acid is involved in HNK-1, known as a carbohydrate epitope in neurocells.[1] Therefore, such highly functional disaccharides as those bearing both carboxyl and sulfate groups in a structure have attracted attention in view of their synthetic and medicinal applications. Heparin-based GAG mimics have been synthesized by chemical methods.[2] A simpler and more eco-friendly approach may be desirable from a practical viewpoint.

In our continuous effort on the library assembly of regioselectively *O*-sulfated mono- and disaccharides,[3–7] we have recently found that a bovine glucuronidase (EC 3.2.1.31) transfers the GlcA moiety in GlcA-*O*-*p*NP to the *O*-3 or *O*-2 position of 6-*O*-SO_3-Glc-*O*-*p*NP and 6-*O*-SO_3-Gal-*O*-*p*NP, giving the corresponding glucuronyl disaccharides.[8] The derived disaccharides, possessing both carboxyl and *O*-sulfate groups in one molecule, are close to the repeating disaccharide in GAGs. Thus, it seems probable that the enzymatic reactions may be extended to mimic assembly of GAG fragments such as those in heparin, heparan sulfate, and chondroitin sulfate. Moreover, in the absence of the 6-*O*-sulfo-sugars, the enzyme utilizes the GlcA-*O*-*p*NP also as the acceptor substrate to produce glucuronyl $\beta(1\rightarrow3)$- and $\beta(1\rightarrow2)$-glucuronosides. The self-transglycosylated glucuronyl disaccharides are considered to be mimics of the disaccharide component in glycyrrhizinic acid from the roots of *Glycyrrhiza* sp.[9,10] Although the substrate specificity of glucuronidases has been investigated in the literature,[11,12] the transglycosylation to sulfo-sugars was unknown. These results prompted us to study in further detail the substrate specificity of glucuronidases. In the present study, we employed snail (*Helix pomatia* and *Helix aspersa*), limpet (*Patella vulgata*), and bovine glucuronidases. As the acceptors, GlcA-*O*-*p*NP (for self-transglycosylation), 6-*O*-sulfo-β-D-glucopyranosides (6-*O*-sulfo-Glc-*O*-*p*NP and 6-*O*-sulfo-Glc-*S*-*p*NP), and 6-*O*-sulfo-β-D-galactopyranosides (6-*O*-sulfo-Gal-*O*-*p*NP and 6-*O*-sulfo-Gal-*S*-*p*NP) were employed. As the donor substrate, GlcA-*O*-*p*NP was employed.

RESULTS AND DISCUSSION

Self-Transglycosylation of the Donor Substrate, GlcA-*O*-*p*NP 1

At the beginning of this study, we examined the self-transglycosylation of GlcA-*O*-*p*NP **1** (Sch. 1). Each of the transglycosylation reactions was tested



Scheme 1:

using ca. 1 M of **1** in 50 mM acetate buffer (pH 6.0) and glucuronidase (ca. 1000U). The enzymatic reactions were traced with HPLC using reference compounds with established structures.[8]

Figure 1 indicates that the *Helix aspersa* glucuronidase (entry 1, Table 1) is able to produce self-transglycosylated disaccharide **2** as the major product together with its isomer **3**, consuming donor substrate **1**. The disaccharide products (**2** + **3**) reached equilibrium after 48 h. Neither the $\beta(1\rightarrow4)$ transfer product nor glucuronyl trisaccharides were detected in the products. Each product was purified with ODS (C-18) column and assigned by NMR and MS analyses. The HMBC NMR spectra of the major product **2** showed the presence of β1-3 linkage; a long-range ^{13}C-^{1}H correlation was observed between H-1′ (δ 4.853 ppm, $J_{1',2'}$ 8.1 Hz) and C-3 (δ 84.2 ppm).[8] In the ^{13}C NMR spectrum of compound **2**, the C-3 signal had clearly shifted downfield (δ 84.2 ppm) in comparison with other ring carbons (71.7–78.1 ppm). ESI-MS spectrum also supported the structure of glucuronyl disaccharide product (490.2 $[M-H]^-$). In a manner similar to **2**, the minor product **3** having β1-2 linkage was assigned by a long-range correlation between H-1′ (δ 4.814 ppm, $J_{1',2'}$ 8.1 Hz) and C-2(δ 83.8 ppm).

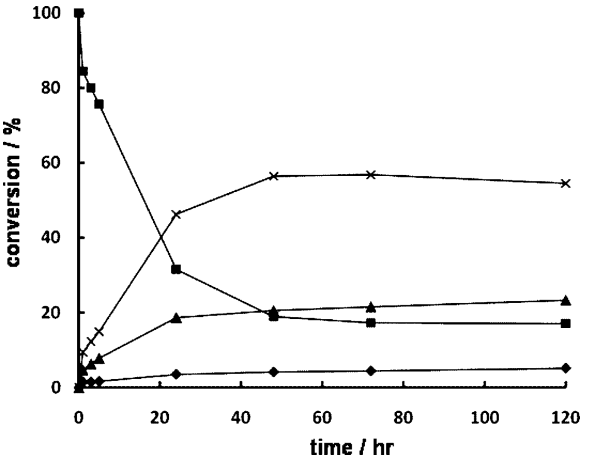


Figure 1: Time course of glucuronidase-catalyzed self-transglycosylation with an enzyme from *Helix aspersa* in acetate buffer (50 mM, pH 6.0). ■, **1**; ▲, **2**; ♦, **3**; ×, *p*NP.

Table 1: Self-transglycosylation of **1** by Glucuronidases

Entry	Enzyme	Time (h)	Disaccharide products[a,b] 2 (%)	3 (%)
1	*H. aspersa*	24	19	4
2	*H. pomatia*	24	16	5
3	*P. vulgata*	24	7	3
4	Bovine	24	42[c]	11[c]
5	*E. coli*	12	—[c,d]	—[c,d]

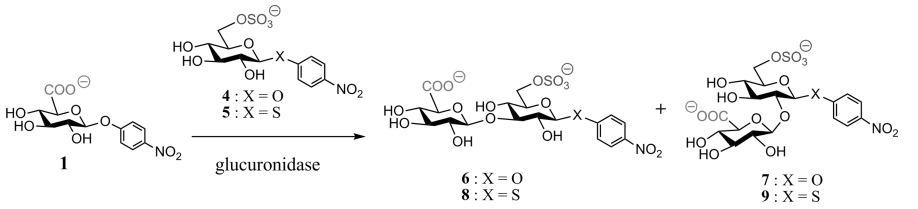
[a]Determined by HPLC analysis.
[b]In 50 mM AcONa-AcOH buffer (pH 6.0).
[c]In 100 mM AcONa-AcOH buffer (pH 6.0).
[d]<1.5%.

Another snail glucuronidase and the limpet one gave similar results (entries 2 and 3, Table 1), and the bovine enzyme showed higher conversion than the molluscan enzymes (entry 4). In the case of the *E. coli* glucuronidase, the enzyme consumed donor **1**, while conversion to **2** and **3** was less than 1.5% (entry 5, Table 1). This enzyme may hydrolyze these disaccharide products soon after conversion, or the acceptor substrates may not be involved in the reaction. Obviously, every enzyme except for *E. coli* catalyzes $\beta(1\rightarrow3)$ and $\beta(1\rightarrow2)$ self-transglycosylation reactions. Among those enzymes, the bovine glucuronidase gave the best results in terms of selectivity and transfer yield in the self-transglycosylation reaction.

Substrate Specificity of Hetero-Transglycosylation for the Synthesis of Glucuronyl 6-*O*-Sulfo-β-D-gluco- and -β-D-galactopyranosides

p-Nitrophenyl 6-*O*-sulfo-β-D-glucopyranoside (6-*O*-sulfo-Glc-*O*-*p*NP, **4**), *p*-nitrophenyl 6-*O*-sulfo-1-thio-β-D-glucopyranoside (6-*O*-sulfo-Glc-*S*-*p*NP, **5**), *p*-nitrophenyl 6-*O*-sulfo-β-D-galactopyranoside (6-*O*-sulfo-Gal-*O*-*p*NP, **10**), and *p*-nitrophenyl 6-*O*-sulfo-1-thio-β-D-galactopyranoside (6-*O*-sulfo-Gal-*S*-*p*NP, **11**) were synthesized by conventional chemical sulfation and applied as the acceptor substrate.

The results summarized in Scheme 2 and Table 2 show that every enzyme catalyzes GlcA$\beta(1\rightarrow3)$ and $\beta(1\rightarrow2)$ transfer reactions to give the corresponding



Scheme 2:

Table 2: Transglycosylation Using **4** and **5** as the Acceptors[a]

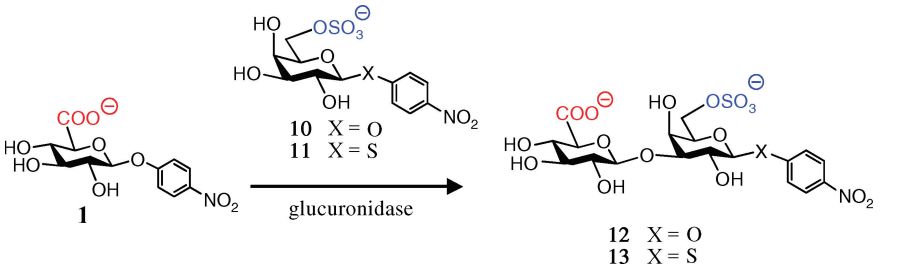
Entry	Enzyme	Acceptor	Buffer	Time (h)	Products[b,c]
1	*H. pomatia*	**4**	Acetate	48	**6** (8%) + **7** (3%)
2	*H. pomatia*	**4**	Phosphate	48	**6** (5%) + **7** (4%)
3	*H. pomatia*	**5**	Acetate	48	**8** (8%) + **9** (3%)
4	*H. pomatia*	**5**	Phosphate	72	**8** (9%) + **9** (2%)
5	*H. aspersa*	**4**	Acetate	48	**6** (9%) + **7** (4%)
6	*H. aspersa*	**4**	Phosphate	48	**6** (9%) + **7** (4%)
7	*H. aspersa*	**5**	Acetate	48	**8** (9%) + **9** (4%)
8	*H. aspersa*	**5**	Phosphate	72	**8** (10%) + **9** (3%)
9	Limpet	**4**	Acetate	48	**6** (7%) + **7** (4%)
10	Limpet	**4**	Phosphate	48	**6** (7%) + **7** (3%)
11	Limpet	**5**	Acetate	48	**8** (7%) + **9** (4%)
12	Limpet	**5**	Phosphate	72	**8** (8%) + **9** (3%)
13	Bovine	**4**	Acetate	24	**6** (35%) +**7** (3%)
14	Bovine	**5**	Acetate	24	**8** (28%) + **9** (6%)

[a]**1** was used as the glycosyl donor.
[b]Determined by LC-MS analysis.
[c]Based on the amount of the donor used for reactions.

glucuronyl 6-*O*-sulfo sugars (**6**–**9**). The reactions proceeded regardless of *O*- or *S*-glycosides in the acceptor substrates and buffer systems employed in this study. The products were assigned by means of NMR and MS analyses in the same manner as described in a preceding section. For example, the NMR spectra of the major product **6** gave a long-range correlation between H-1′ (δ 4.820 ppm, $J_{1',2'}$ 7.7 Hz) and C-3 (δ 85.8 ppm), and also between C-1′ (δ 105.0 ppm) and H-3 (δ 3.916 ppm, $J_{2,3}$ 9.0 and $J_{3,4}$ 9.0 Hz), indicating the presence of a $\beta(1\rightarrow3)$-linkage.

The yields of the hetero-transglycosylation [max. 38% for $\beta(1\rightarrow2)+\beta(1\rightarrow3)$] were lower than those of the self-transglycosylation [max. 53% for $\beta(1\rightarrow2)+\beta(1\rightarrow3)$], and the $\beta(1\rightarrow3)$ selectivity was higher than that of $\beta(1\rightarrow2)$ (the ratios = 12:1 ~ 1:1). Apparently, the bovine enzyme gave the best results in terms of both selectivity and yield (entries 13 and 14, Table 2).

When β-D-galactosides **10** and **11** were employed as the acceptors (Sch. 3), the snail enzymes showed no transglycosylation reaction (entries 1–6,



Scheme 3:

Table 3: Transglycosylation Using **10** and **11** as the acceptors[a]

Entry	Enzyme	Acceptor	Buffer	Time (h)	Products[b,c]
1	*H. pomatia*	**10**	Acetate	48	—[d]
2	*H. pomatia*	**11**	Acetate	48	—[d]
3	*H. pomatia*	**11**	Phosphate	48	—[d]
4	*H. aspersa*	**10**	Acetate	48	—[d]
5	*H. aspersa*	**11**	Acetate	48	—[d]
6	*H. aspersa*	**11**	Phosphate	48	—[d]
7	Limpet	**10**	Acetate	48	—[d]
8	Limpet	**10**	Phosphate	48	—[d]
9	Limpet	**11**	Acetate	96	**13** (7%)
10	Limpet	**11**	Phosphate	48	**13** (3%)
11	Bovine	**10**	Acetate	24	**12** (43%)
12	Bovine	**11**	Acetate	24	**13** (40%)

[a] **1** was used as the glycosyl donor.
[b] Determined by LC-MS analysis.
[c] Based on the amount of the donor added.
[d] <1%.

Table 3). The limpet enzyme produced glucuronyl 6-*O*-sulfo-galactosyl disaccharide **13** (entries 9 and 10, Table 3) from *S*-glycoside **11**, and the transfer reaction was $\beta(1\rightarrow3)$ specific. The same enzyme disfavored *O*-glycoside **10** (entries 7 and 8, Table 3) as the acceptor substrate, although the difference of their structures seems too subtle to be differentiated by the enzyme. The bovine enzyme is able to utilize both **10** and **11** to yield **12** and **13** in acceptable yields (entries 11 and 12, Table 3). In the case of galactosyl acceptors (**10** and **11)**, the reactivity depended strongly on the origins of the enzymes.

CONCLUSION

We have examined enzyme-assisted transglycosylation by using glucuronidases with different origins. We confirmed that all of the snail, limpet, and bovine enzymes are able to transfer GlcA from GlcA-*O*-*p*NP to the *O*-2 and *O*-3 positions of 6-*O*-sulfo-β-D-glucopyranoside. The limpet and bovine enzymes can also utilize the *O*-3 position of 6-*O*-sulfo-β-D-galactopyranosides for transglucuronylation. The enzymes also catalyze self-transglycosylation to afford *O*-2- or *O*-3-linked disaccharide. Obviously, the bovine enzyme shows the highest reactivity for providing a practical enzymatic approach to highly functional saccharides like compounds **12** and **13** bearing both carboxyl and sulfate groups in one molecule. The disaccharide products obtained here are good candidates for testing biomedical activities such as antiblood coagulation and anti-inflammation,[2] and may become leading compounds for carbohydrate-based drug design.[13–15] Moreover, the *p*NP group introduced at the aglycon can be chemically reduced to a *p*-aminophenyl group and led to carbohydrate-based materials such as biosensors and glyco-chips.[16–19]

EXPERIMENTAL

General Methods

Glucuronidases (EC 3.2.1.31) from *Helix pomatia* (99 units/mL), *Helix aspersa* (393 units/mg), *Patella vulgata* (1900 units/mg), bovine (10,400 units/mg), and *Escherichi coli* (13,400 units/mg) were purchased from Sigma (St. Louis, MO, USA) and used without further purification. One unit of enzymatic activity was defined as the amount of enzyme that releases 1 μg of phenolphthalein from phenolphthalein glucuronide per hour at 37°C (pH 5.0). All other reagents were obtained from Aldrich or Sigma and used as received. Enzymatic reactions were monitored by HPLC-MS (SHIMADZU LC-MS 2010A) with a column of Synergi Fusion-RP 80 (2.0 × 250 mm) eluted with H_2O/MeOH (7/3, v/v) containing 0.1% HCOOH at a flow rate of 0.2 mL/min and with a UV detector monitored at 300 nm, or HPLC (TOSOH CCP&8020) with an ODS column of Synergi Fusion-RP 80 (4.6 × 250 mm) eluted with H_2O/MeOH (7/3, v/v) containing 0.1% HCOOH at a flow rate of 1.0 mL/min and with a UV detector monitored at 300 nm. The products were purified with a preparative HPLC (SHIMADZU) using an ODS column of Luna C18 (21.2 × 250 mm) eluted with H_2O/MeOH (7/3, v/v) containing 0.05% TFA, or flash column chromatography on silica gel 60 RP-18 (ODS-C18, Yamazen, Japan, 40–63 μm). Optical rotations were measured with a JASCO DIP-1000 digital polarimeter at ambient temperature, using a 10-cm micro cell. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL LA-600 or a Varian INOVA 400 spectrometer for solutions in D_2O. Chemical shifts are given in ppm and referenced to *tert*-butyl alcohol (*t*-BuOH) (δ_H 1.23 in D_2O, 1.40 in CD_3OD,[20] δ_C 31.2 in D_2O, or 31.1 in CD_3OD) as an internal reference. All data are assumed to be first order with an apparent doublet and triplet reported as d and t, respectively. Resonances that appear broad are designated br. ESI mass spectra (ESI-MS) were directly recorded using a SHIMADZU LC-MS 2010A mass spectrometer.

*HPLC Analysis of the Starting Materials, Glucuronyl Dimeric Products [p-Nitrophenyl β-D-Glucuronyl-(1→3)-β-D-glucuronic Acid (**2**), and p-Nitrophenyl β-D-Glucuronyl-(1→2)-β-D-glucuronic Acid (**3**)] and Liberated p-Nitrophenol*

p-Nitrophenyl glucuronic acid (GlcA-*O*-*p*NP) was treated with $NaHCO_3$ to give *p*NP glucuronate, sodium salt **1** quantitatively. To a solution of 4.6 mg (0.014 mmol) of **1** in 10 μL of 50 mM AcONa-AcOH buffer (pH 6.0), snail glucuronidase (2.7 mg, 1061U) from *Helix aspersa* dissolved in the same buffer (5 μL) was added and incubated at 36°C. The reaction mixture (1 μL) was taken out at appropriate time intervals (0, 1, 3, 5, 24, 48, 72, and 120 h) during the incubation and was heated over a water bath at 95°C for 2 min to stop each enzyme reaction. Every sample was then analyzed by HPLC with an

ODS column of Synergi Fusion (4.6 × 250 mm) eluted with H_2O/MeOH (7/3, v/v) containing 0.1% HCOOH at a flow rate of 1.0 mL/min and with a UV detector monitored at 300 nm. Each peak area was plotted versus reaction time to give a time course (Fig. 1). In a similar way to the snail enzyme, *Helix pomatia* (10.1 μL, 1000 U), limpet (0.4 mg, 760 U), bovine (0.096 mg, 1000 U), or *E. coli* (0.075 mg, 1000 U) glucuronidase was used.

*LC-MS Analysis of the Starting Materials, Glucuronyl 6-O-Sulfo-Glc Disaccharide Products [p-Nitrophenyl β-D-Glucuronyl-(1→3)-6-O-sulfo-β-D-glucopyranoside (**6**) and p-Nitrophenyl Glucuronyl-(1→2)-6-O-sulfo-β-D-glucopyranoside (**7**)], and Liberated p-Nitrophenol*

To a solution of 3.5 mg (0.010 mmol) of **1** and 10.0 mg (0.025 mmol) of **4** in 20 μL of 50 mM AcONa-AcOH buffer (pH 6.0), snail glucuronidase (2.5 mg, 1000 U) from *Helix aspersa* dissolved in the same buffer (10 μL) was added and incubated at 35°C. The reaction mixture (1 μL) was taken out at appropriate time intervals (0, 1, 3, 5, 24, and 48 h) during the incubation and was heated over a water bath at 95°C for 2 min to stop each enzyme reaction. Every sample was then analyzed by HPLC-MS with an ODS column of Synergi Fusion (2.0 × 250 mm) eluted with H_2O/MeOH (7/3, v/v) containing 0.1% HCOOH at a flow rate of 0.2 mL/min and with a UV detector monitored at 300 nm. In a similar way to the snail enzyme, *Helix pomatia* (10.1 μL, 1000 U), limpet (0.53 mg, 1000 U), bovine (0.096 mg, 1000 U), or *E. coli* (0.075 mg, 1000 U) glucuronidase was used in this study. Similarly, other glycosyl acceptors such as 10.0 mg of 6-*O*-sulfo-Glc-*S*-*p*NP (**5**), 10.0 mg of 6-*O*-sulfo-Gal-*O*-*p*NP (**10**), or 10.0 mg of 6-*O*-sulfo-Gal-*S*-*p*NP (**11**) were used, respectively, to monitor the starting materials, products, and liberated *p*NP at various intervals (0, 1, 3, 5, 24, and 48 h).

*p-Nitrophenyl β-D-Glucuronyl-(1→3)-β-D-glucuronic Acid (**2**) and p-Nitrophenyl β-D-Glucuronyl-(1→2)-β-D-glucuronic Acid (**3**)*

A mixture of **1** (50 mg, 0.15 mmol) and bovine liver glucuronidase (0.48 mg, 5000 U) dissolved in 100 mM AcONa-AcOH buffer (pH 6.0, 100 μL) was incubated at 35°C for 24 h. The reaction mixture was boiled for 5 min to stop the enzyme reaction, and then the mixture was purified with an ODS HPLC column (Luna C18, H_2O-MeOH = 7:3 containing 0.05% TFA) to give **2** (10 mg) and **3** (7 mg). **2**: $[\alpha]_D^{25} = -45^\circ$ (*c* 0.87, H_2O);^{1}H NMR (600 MHz, D_2O): δ 8.258 (d, J 9.5 Hz, aromatic), 7.241 (d, J = 9.5 Hz, aromatic), 5.286 (d, $J_{1,2}$ = 8.0 Hz, H-1), 4.853 (d, $J_{1',2'}$ = 8.1 Hz, H-1′), 3.974 (br d, $J_{4,5}$ = 9.9 Hz, H-5), 3.928 (dd, $J_{3,4}$ = 8.8, $J_{2,3}$ = 9.1 Hz, H-3), 3.868 (dd, $J_{1,2}$ = 8.0, $J_{2,3}$ = 9.1 Hz, H-2), 3.768–3.687 (m, H-4, H-4′), 3.560–3.503 (m, H-3′, H-5′), 3.407 (dd, $J_{1',2'}$ = 8.1, $J_{2',3'}$ = 8.8 Hz, H-2′); ^{13}C NMR (150 MHz, D_2O), δ 163.4, 144.3, 127.7, 118.2, 103.9 (C-1′), 100.8 (C-1), 84.2 (C-3), 78.1, 77.4, 77.0, 74.9, 74.3, 73.4, 71.7.

ESI-MS (negative); 490.2 [M-H]$^-$. **3**: $[\alpha]_D^{25} = -35^\circ$ (*c* 0.88, H_2O); ^{1}H NMR (600 MHz, D_2O): δ 8.224 (d, $J = 9.0$ Hz, aromatic), 7.193 (d, $J = 9.0$ Hz, aromatic), 5.556 (d, $J_{1,2} = 7.0$ Hz, H-1), 4.814 (d, $J_{1',2'} = 8.1$ Hz, H-1′), 4.241 (br d, $J_{4,5} = 9.5$ Hz, H-5), 3.901 (dd, $J_{1,2} = 7.0$, $J_{2,3} = 9.1$ Hz, H-2), 3.862 (dd, $J_{2,3} = 9.1$, $J_{3,4} = 9.1$ Hz, H-3), 3.848 (d, $J_{4',5'} = 9.5$ Hz, H-5′), 3.756 (dd, $J_{3,4} = 9.1$, $J_{4,5} = 9.5$ Hz, H-4), 3.554 (dd, $J_{2',3'} = 9.2$, $J_{3',4'} = 9.5$ Hz, H-3′), 3.455 (dd, $J_{3',4'} = 9.5$, $J_{4',5'} = 9.5$ Hz, H-4′), 3.359 (dd, $J_{1',2'} = 8.1$, $J_{2',3'} = 9.2$ Hz, H-2′); ^{13}C NMR (150 MHz, D_2O), δ 162.7, 144.2, 127.9, 117.3, 104.4 (C-1′), 99.4 (C-1), 83.8 (C-2), 76.5, 76.3, 76.0, 74.8, 72.9, 72.1. ESI-MS (negative); 490.2 [M-H]$^-$.

*p-Nitrophenyl 6-O-sulfo-β-D-glucopyranoside, trimethylamine salt (**4**)*

A mixture of *p*NP β-D-glucopyranoside (1 g, 3.32 mmol) and SO_3-NMe_3 (1.38 g, 9.9 mmol) was dissolved in DMF (35 mL) at 40°C. After 90 min, the reaction mixture was diluted with MeOH (10 mL) and concentrated in vacuo. The residue was then purified by a column of ODS C-18 (Yamazen) to afford **4** (994 mg, 68%). $[\alpha]_D^{25} = -88.9$ (*c* 1.79, H_2O); ^{1}H NMR (600 MHz, D_2O): δ 8.265 (d, $J = 9.2$ Hz, aromatic), 7.253 (d, $J = 9.2$ Hz, aromatic), 5.270 (d, $J_{1,2} = 7.7$ Hz, H-1), 4.377 (dd, $J_{5,6a} = 2.2$, $J_{6a,6b} = 11.4$ Hz, H-6a), 4.224 (dd, $J_{5,6b} = 5.7$, $J_{6a,6b} = 11.4$ Hz, H-6b), 3.96–3.90 (m, H-5), 3.68–3.61 (m, H-2, H-3), 3.61–3.55 (m, H-4), 2.879 (s, NMe_3); ^{13}C NMR (150 MHz, D_2O) δ 163.2, 144.1, 127.7, 118.1, 101.0 (C-1), 76.8, 75.7, 74.2, 70.6, 68.4, 46.3 (NMe_3). ESI-MS (negative); 380.1 [M-Me_3NH]$^-$.

*p-Nitrophenyl 6-O-sulfo-1-thio-β-D-glucopyranoside, trimethylamine salt (**5**)*

A mixture of *p*NP 1-thio-β-D-glucopyranoside (400 mg, 1.26 mmol) and SO_3-NMe_3 (277 mg, 1.99 mmol) was dissolved in DMF (11 mL) at 40°C for 90 min. The reaction mixture was then processed in the same way described for compound **4** to give **5** (337 mg, 59%). $[\alpha]_D^{25} = -83.9$ (*c* 3.88, H_2O); ^{1}H NMR (600 MHz, D_2O): δ 8.142 (d, $J = 9.1$ Hz, aromatic), 7.614 (d, $J = 9.1$ Hz, aromatic), 5.034 (d, $J_{1,2} = 9.9$ Hz, H-1), 4.375 (dd, $J_{5,6a} = 2.2$, $J_{6a,6b} = 11.3$ Hz, H-6a), 4.193 (dd, $J_{5,6b} = 5.8$, $J_{6a,6b} = 11.3$ Hz, H-6b), 3.88–3.81 (m, H-5), 3.589 (dd, $J_{2,3} = 9.2$, $J_{3,4} = 9.2$ Hz, H-3), 3.523 (dd, $J_{3,4} = 9.2$, $J_{4,5} = 9.2$ Hz, H-4), 3.485 (dd, $J_{1,2} = 9.9$, $J_{2,3} = 9.2$ Hz, H-2), 2.880 (s, NMe_3); ^{13}C NMR (150 MHz, D_2O) δ 147.6, 144.9, 130.6, 125.7, 87.2 (C-1), 79.2, 78.6, 73.2, 70.6, 68.7, 46.3 (NMe_3). ESI-MS (negative); 396.1 [M-Me_3NH]$^-$.

*p-Nitrophenyl 6-O-sulfo-β-D-galactopyranoside, trimethylamine salt (**10**)*

A mixture of *p*NP β-D-galactopyranoside (200 mg, 0.66 mmol) and SO_3-NMe_3 (554 mg, 3.98 mmol) was dissolved in DMF (18 mL) at 40°C for 90 min. The reaction mixture was then processed in the same way described for compound **4** to give **10** (198 mg, 68%). $[\alpha]_D^{25} = -63.4$ (*c* 2.59, H_2O); ^{1}H NMR (600 MHz, D_2O): δ 8.262 (d, $J = 9.2$ Hz, aromatic), 7.263 (d, $J = 9.2$ Hz, aromatic),

5.204 (d, $J_{1,2}$ = 7.7 Hz, H-1), 4.28–4.17 (m, H-6a, H-6b, H-5), 4.068 (br d, $J_{3,4}$ = 3.3 Hz, H-4), 3.867 (dd, $J_{1,2}$ = 7.7, $J_{2,3}$ = 9.9 Hz, H-2), 3.813 (dd, $J_{2,3}$ = 9.9, $J_{3,4}$ = 3.3 Hz, H-3), 2.881 (s, NMe_3); ^{13}C NMR (150 MHz, D_2O) δ 163.4, 144.2, 127.7, 118.1, 101.5 (C-1), 74.8, 73.8, 71.9, 69.8, 68.6, 46.3 (NMe_3). ESI-MS (negative); 380.1 [M-Me_3NH]$^-$.

*p-Nitrophenyl 6-O-sulfo-1-thio-β-D-galactopyranoside, trimethylamine salt (**11**)*

A mixture of *p*NP 1-thio-β-D-galactopyranoside (300 mg, 0.95 mmol) and SO_3-NMe_3 (277 mg, 1.99 mmol) was dissolved in DMF (11 mL) at 40°C for 90 min. The reaction mixture was then processed in the same way described for compound **4** to give **11** (277 mg, 64%). $[\alpha]_D^{25} = -86.7$ (*c* 1.95, H_2O); ^{1}H NMR (600 MHz, D_2O): δ 8.158 (d, J = 9.2 Hz, aromatic), 7.632 (d, J = 9.2 Hz, aromatic), 5.016 (d, $J_{1,2}$ = 9.3 Hz, H-1), 4.221 (dd, $J_{5,6a}$ = 4.4, $J_{6a,6b}$ = 11.0 Hz, H-6a), 4.182 (dd, $J_{5,6b}$ = 7.7, $J_{6a,6b}$ = 11.0 Hz, H-6b), 4.14–4.10 (m, H-5), 4.083 (br d, $J_{3,4}$ = 3.2 Hz, H-4), 3.763 (dd, $J_{3,4}$ = 3.2, $J_{2,3}$ = 9.3 Hz, H-3), 3.731 (dd, $J_{1,2}$ = 9.3, $J_{2,3}$ = 9.3 Hz, H-2), 2.880 (s, NMe_3); ^{13}C NMR (150 MHz, D_2O) δ 147.4, 145.6, 130.1, 125.8, 87.6 (C-1), 78.2, 75.3, 70.6, 70.0, 69.0, 46.3 (NMe_3). ESI-MS (negative); 396.1 [M-Me_3NH]$^-$.

*p-Nitrophenylβ-D-Glucuronyl-(1→3)-6-O-sulfo-β-D-glucopyranoside, sodium salt (**6**) and p-Nitrophenylβ-D-Glucuronyl -(1→2)-6-O-sulfo-β-D-glucopyranoside, sodium salt (**7**)*

A mixture of **1** (121 mg, 0.36 mmol), **4** (207 mg, 0.47 mmol), and bovine liver glucuronidase (0.48 mg, 5000 U) dissolved in 100 mM AcONa-AcOH buffer (pH 6.0, 1.4 mL) was incubated at 35°C for 24 h. The reaction mixture was boiled for 5 min to stop the enzyme reaction. The mixture was purified with an ODS HPLC column (Luna C18, H_2O-MeOH = 7:3 containing 0.05% TFA) and then treated with ion exchange resin (Dowex Na^+) to give **6** (32 mg) and **7** (10 mg). **6**: $[\alpha]_D^{25} = -58°$ (*c* 0.48, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 8.268 (d, J = 9.4 Hz, aromatic), 7.257 (d, J = 9.4 Hz, aromatic), 5.300 (d, $J_{1,2}$ = 7.7 Hz, H-1), 4.820 (d, $J_{1',2'}$ = 7.7 Hz, H-1′), 4.392 (dd, $J_{5,6a}$ = 2.2, $J_{6a,6b}$ 11.3 Hz, H-6a), 4.227 (dd, $J_{5,6b}$ = 5.9, $J_{6a,6b}$ = 11.3 Hz, H-6b), 4.00–3.95 (m, H-5), 3.916 (dd, $J_{2,3}$ = 9.0, $J_{3,4}$ = 9.0 Hz, H-3), 3.854 (dd, $J_{1,2}$ = 7.7, $J_{2,3}$ = 9.0 Hz, H-2), 3.743 (d, $J_{4',5'}$ = 9.5 Hz, H-5′), 3.673 (dd, $J_{3,4}$ = 9.0, $J_{4,5}$ = 9.9 Hz, H-4), 3.58–3.50 (m, H-3′, H-4′), 3.411 (dd, $J_{1',2'}$ = 7.7, $J_{2',3'}$ = 9.1 Hz, H-2′); ^{13}C NMR (150 MHz, D_2O) δ 178.3 (C-6′), 164.2, 145.2, 128.6, 119.0, 105.0 (C-1′), 101.7 (C-1), 85.8 (C-3), 78.2, 77.9, 76.3, 75.8, 75.1, 74.3, 70.2, 69.4. ESI-MS (negative); 556.2 [M-Na]$^-$; 578.2 [M-H]$^-$. **7**: $[\alpha]_D^{25} = -34°$ (*c* 0.65, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 8.243 (d, J = 9.2 Hz, aromatic), 7.204 (d, J = 9.2 Hz, aromatic), 5.505 (d, $J_{1,2}$ = 7.3 Hz, H-1), 4.821 (d, $J_{1',2'}$ = 8.0 Hz, H-1′), 4.356 (dd, $J_{5,6a}$ = 2.2, $J_{6a,6b}$ = 11.4 Hz, H-6a), 4.217 (dd, $J_{5,6b}$ = 5.5, $J_{6a,6b}$ = 11.4 Hz, H-6b), 3.944 (m, H-5), 3.869 (br

d, $J_{5',4'}$ = 9.2 Hz, H-5′), 3.862 (dd, $J_{1,2}$ = 7.3, $J_{2,3}$ = 9.2 Hz, H-2), 3.819 (dd, $J_{2,3}$ = 9.2, $J_{3,4}$ = 9.2 Hz, H-3), 3.633 (dd, $J_{3,4}$ = 9.2, $J_{4,5}$ = 9.2 Hz, H-4), 3.555 (dd, $J_{2',3'}$ = 9.2, $J_{3',4'}$ = 9.5 Hz, H-3′), 3.436 (dd, $J_{3',4'}$ = 9.5, $J_{4',5'}$ = 9.2 Hz, H-4′), 3.345 (dd, $J_{1',2'}$ = 8.0, $J_{2',3'}$ = 9.2 Hz, H-2′); ^{13}C NMR (100 MHz, D_2O) δ 162.8, 144.1, 127.9, 117.3, 104.4 (C-1′), 99.6 (C-1), 84.0 (C-2), 76.5, 76.3, 75.3, 74.8, 72.9, 71.4, 70.1, 68.3. ESI-MS (negative); 556.2 $[M\text{-}Na]^-$.

*p-Nitrophenyl β-D-Glucuronyl-(1→3)-6-O-sulfo-1-thio-β-D-glucopyranoside, sodium salt (**8**) and p-Nitrophenyl β-D-Glucuronyl-(1→2)-6-O-sulfo-1-thio-β-D-glucopyranoside, sodium salt (**9**)*

A mixture of **1** (162 mg, 0.48 mmol), **5** (335 mg, 0.73 mmol), and bovine liver glucuronidase (0.48 mg, 5000 U) dissolved in 100 mM AcONa-AcOH buffer (pH 6.0, 2.0 mL) was incubated at 35°C for 24 h. The reaction mixture was boiled for 5 min to stop the enzyme reaction. The mixture was purified with an ODS HPLC column (Luna C18, H_2O-MeOH = 7:3 containing 0.05% TFA) and then treated with ion exchange resin (Dowex Na^+) to give **8** (15 mg) and **9** (10 mg). **8**: $[\alpha]_D^{25} = -51$ (*c* 0.27, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 8.149 (d, J = 8.8 Hz, aromatic), 7.634 (d, J = 8.8 Hz, aromatic), 5.034 (d, $J_{1,2}$ = 9.9 Hz, H-1), 4.800 (d, $J_{1',2'}$ = 7.7 Hz, H-1′), 4.391 (dd, $J_{5,6a}$ = 1.9, $J_{6a,6b}$ = 11.3 Hz, H-6a), 4.198 (dd, $J_{5,6b}$ = 6.2, $J_{6a,6b}$ 11.3 Hz, H-6b), 3.873–3.843 (m, H-5), 3.848 (dd, $J_{2,3}$ = 9.2, $J_{3,4}$ 9.2 Hz, H-3), 3.742 (d, $J_{4',5'}$ = 9.5 Hz, H-5′), 3.684 (dd, $J_{1,2}$ = 9.9, $J_{2,3}$ = 9.2 Hz, H-2), 3.607 (dd, $J_{3,4}$ = 9.2, $J_{4,5}$ = 9.9 Hz, H-4), 3.571–3.507 (m, H-4′, H-3′), 3.400 (dd, $J_{1',2'}$ = 7.7, $J_{2',3'}$ = 8.8 Hz, H-2′); ^{13}C NMR (150 MHz, D_2O) δ 147.9, 144.2, 131.3, 125.7, 104.0 (C-1′), 87.1 (C-1), 86.9 (C-3), 79.0, 76.7, 76.0, 74.6, 72.7, 69.2, 68.8. ESI-MS (negative); 572.2 $[M\text{-}Na]^-$; 594.1 $[M\text{-}H]^-$ **9**: $[\alpha]_D^{25} = -9$ (*c* 0.65, H_2O); ^{1}H NMR (600 MHz, D_2O) δ 8.242 (d, J = 9.2 Hz, aromatic), 7.214 (d, J = 9.2 Hz, aromatic), 5.501 (d, $J_{1,2}$ = 7.3 Hz, H-1), 4.799 (d, $J_{1',2'}$ = 8.0 Hz, H-1′), 4.362 (dd, $J_{5,6a}$ = 2.2, $J_{6a,6b}$ = 11.8 Hz, H-6a), 4.220 (dd, $J_{5,6b}$ = 5.5, $J_{6a,6b}$ = 11.8 Hz, H-6b), 3.978–3.922 (m, H-5), 3.870 (dd, $J_{1,2}$ = 7.3, $J_{2,3}$ = 8.8 Hz, H-2), 3.826 (dd, $J_{2,3}$ = 8.8, $J_{3,4}$ = 9.3 Hz, H-3), 3.710 (br d, $J_{4',5'}$ = 9.5 Hz, H-5′), 3.628 (dd, $J_{3,4}$ = 9.3, $J_{4,5}$ = 9.3 Hz, H-4), 3.534 (dd, $J_{2',3'}$ = 9.5, $J_{3',4'}$ = 9.2 Hz, H-3′), 3.419 (dd, $J_{3',4'}$ = 9.2, $J_{4',5'}$ = 9.5 Hz, H-4′), 3.353 (dd, $J_{1',2'}$ = 8.0, $J_{2',3'}$ = 9.5 Hz, H-2′); ^{13}C NMR (150 MHz, D_2O) δ 173.5 (C-6′), 147.7, 144.5, 131.0, 125.6, 104.5 (C-1′), 84.9 (C-1), 80.2 (C-2), 79.2, 78.8, 76.7, 76.3, 74.7, 72.8, 70.5, 68.7. ESI-MS (negative); 572.2 $[M\text{-}Na]^-$, 594.2 $[M\text{-}H]^-$.

*p-Nitrophenyl β-D-Glucuronyl-(1→3)-6-O-sulfo-β-D-galactopyranoside, sodium salt (**12**)*

A mixture of **1** (110 mg, 0.33 mmol), **10** (287 mg, 0.65 mmol), and bovine liver glucuronidase (0.38 mg, 4000 U) dissolved in 100 mM AcONa-AcOH buffer (pH 6.0, 1.5 mL) was incubated at 35°C for 24 h. The reaction mixture was then processed in the same way described for compounds **6** and **7** to give

12 (59 mg). $[\alpha]_D^{25} = -61.8^\circ$ (*c* 1.60, H_2O); ^1H NMR (600 MHz, D_2O): δ 8.258 (d, $J = 9.3$ Hz, aromatic), 7.263 (d, $J = 9.3$ Hz, aromatic), 5.253 (d, $J_{1,2} = 7.7$ Hz, H-1), 4.724 (d, $J_{1',2'} = 8.1$ Hz, H-1′), 4.330 (br d, $J_{3,4} = 3.3$ Hz, H-4), 4.303–4.169 (m, H-6a, H-6b, H-5), 4.025 (dd, $J_{1,2} = 7.7$, $J_{2,3} = 9.9$ Hz, H-2), 3.965 (dd, $J_{3,4} = 3.3$, $J_{2,3} = 9.9$ Hz, H-3), 3.743 (d, $J_{4',5'} = 9.5$ Hz, H-5′), 3.56–3.50 (m, H-3′, H-4′), 3.443 (dd, $J_{1',2'} = 8.1$, $J_{2',3'}$ 9.2 Hz, H-2′); ^{13}C NMR (100 MHz), δ 177.5 (C-6′), 163.3, 144.2, 127.7, 118.1, 105.2 (C-1′), 101.2 (C-1), 83.5 (C-3), 77.8, 76.9, 74.8, 74.7, 73.4, 71.0, 69.4, 69.0. ESI-MS (negative); 556.2 $[M\text{-}Na]^-$.

*p-Nitrophenyl β-D-Glucuronyl-(1→3)-6-O-sulfo-1-thio-β-D-galactopyranoside, sodium salt (**13**)*

A mixture of **1** (100 mg, 0.30 mmol), **11** (276 mg, 0.61 mmol), and bovine liver glucuronidase (0.48 mg, 5000 U) dissolved in 100 mM AcONa-AcOH buffer (pH 6.0, 2.0 mL) was incubated at 35°C for 24 h. The reaction mixture was then processed in the same way described for compounds **6** and **7** to give **13** (25 mg). $[\alpha]_D^{25} = -78^\circ$ (*c* 0.78, H_2O); ^1H NMR (600 MHz, CD_3OD): δ 8.361 (d, $J = 9.0$ Hz, aromatic), 7.913 (d, $J = 9.0$ Hz, aromatic), 5.079 (d, $J_{1,2} = 9.9$ Hz, H-1), 4.778 (d, $J_{1',2'} = 7.3$ Hz, H-1′), 4.438 (br d, $J_{3,4} = 2.9$ Hz, H-4), 4.409 (m, H-6a, H-6b), 4.239 (m, H-5), 4.057 (dd, $J_{1,2} = 9.9$, $J_{2,3} = 9.1$ Hz, H-2), 3.941 (dd, $J_{3,4} = 2.9$, $J_{2,3} = 9.1$ Hz, H-3), 3.818 (br, H-5′), 3.65–3.55 (m, H-4′, H-3′), 3.512 (dd, $J_{1',2'} = 7.3$ Hz, H-2′); ^{13}C NMR (100 MHz, CD_3OD), δ 147.2, 146.8, 129.9, 124.9, 105.4 (C-1′), 87.5 (C-1), 86.1 (C-3), 78.3, 77.5, 75.9, 75.2, 73.6, 69.8, 69.6, 68.9. ESI-MS (negative); 572.2 $[M\text{-}Na]^-$, 594.2 $[M\text{-}H]^-$.

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