Synthetic Potential of Molluscan Sulfatases for the Library Synthesis of Regioselectively *O*-Sulfonated D-*Galacto*-Sugars

Hirotaka Uzawa,*[a] Yoshihiro Nishida,[b] Kenji Sasaki,[b] Norihiko Minoura,[a] and Kazukiyo Kobayashi[b]

The substrate specificities of three molluscan sulfatases (E.C. 3.1.6.1; snail, abalone, and limpet origins) were investigated with assorted p*-nitrophenyl (*pNP*) di-*O*-sulfonated β-D-galactopyranosides and β-lactosides [3,6-SO_3 Gal (**1**), 3′,6′-SO_3 Lac (**2**), 4, 6-SO_3 Gal (**3**), 2,6-SO_3 Gal (**4**), 3,4-SO_3 Gal (**5**), and 3,6-SO_3 GalNAc (**6**); Ac, acetyl; Gal, galactose; Lac, lactose] together with mono-*O*-sulfonated β-D-galactopyranoside [pNP 3-SO_3-Gal (**7**)] and tri-*O*-sulfonated α-D-galactopyranoside [2,3,6-SO_3-α-Gal (**11**)]. Some notable differences between the substrate specificity of the three sulfatases were disclosed; snail sulfatase hydrolyzed the 3-*O*- and 2-*O*-sulfo groups of **1** and **4**, respectively, to afford 6-SO_3 Gal (**9**) in high yields, while the abalone enzyme did not act on **4**. Only the limpet enzyme could cleave the 3-*O*-sulfo groups of **7** to give* pNP *β-galactoside. In contrast, every enzyme could utilize **11** as a good substrate to afford a mixture of 6-SO_3-α-Gal (**13**) and 2,6-SO_3 α-Gal (**12**). None of the enzymes could cleave the* O*-sulfo groups of **5** and **6**, which indicates that a primary 6-*O*-sulfo group tends to promote the enzymatic hydrolysis of* O*-sulfo groups at the secondary positions.*

KEYWORDS:

carbohydrates · enzymes · library synthesis · regioselectivity · sulfonation

Introduction

Besides their presence as heparin and keratan sulfates in proteoglycans, *O*-sulfonated sugars are widely distributed in cell-surface glycolipids and glycoproteins. These compounds are believed to play major roles in intercellular recognition processes such as cell adhesion, signal transduction, regulation, and the infection of host cells with viruses.[1] For example, 6-sulfo sialyl Lewis^X (6-sulfo-sLe^X) hexasaccharides are known to serve as the actual L-selectin ligands[2] responsible for the trafficking of leukocytes and cancer cells, which lead to inflammation and metastasis, respectively. Moreover, in mimic syntheses of sLe^X oligosaccharides, 3-sulfo galactosides have been widely used as simple substitutes for natural *N*-acetylneuraminyl α(2–3)galactose linkages.[3] Artificial glycoconjugate polymers with 3- and/or 6-*O*-sulfonated galactosides in the side chain were reported to show high affinity to L- and P-selectins, even though these polymers have no α-L-fucoside or α-sialyl linkages.[4] These reports have shown that *O*-sulfonated D-*galacto*-sugars have great synthetic potential as prominent agents for the treatment of inflammation, metastasis, and autoimmune diseases.[3a, 5]

In connection with our ongoing project on the design of artificial glycopolymers with cell-surface oligosaccharides and their mimics,[6] we have investigated how practical access to *O*-sulfonated mono- and disaccharides can be obtained. These compounds serve as key carbohydrate modules that polyvalent ligands can recognize, and can be used to block the receptor proteins.[7] Previously, we found that regioselective de-*O*-sulfonation of these saccharides by molluscan sulfatases affords 6-*O*-sulfonated hexoses.[8] This finding suggested that molluscan sulfatases have synthetic potential, despite the fact that they have rarely been applied for synthetic purposes. In the study described herein, we investigated the substrate specificity of three sulfatases, from snail, abalone, and limpet, with a series of mono-, di-, and tri-*O*-sulfonated D-*galacto*-sugars (β-Gal, α-Gal, β-GalNAc, and β-Lac; Ac, acetyl; Gal, galactose; Lac, lactose).

Results and Discussion

Several synthetic strategies have been reported for regioselective preparation of *O*-sulfonated *galacto*-sugars.[3, 9] The use of organotin reagents such as Bu_2SnO or $(Bu_3Sn)_2O$ allows the 3-*O*-sulfonation of β-D-galactosides by reaction with either SO_3Me_3N or SO_3pyridine.[3a,b,d–f, 4, 9a, 10] Purely enzymatic and chemoenzymatic approaches have also been proposed in recent studies. *Rhizobium* Nod factor 6-sulfotransferase has been applied in the 6-*O*-sulfonation of GlcNAc residues in chitobiose, chitotriose, and chitotetraose.[11] GlcNAc-6-sulfotransferases were recently used for the study of the biosynthetic pathways of sulfo sLe^x.[12]

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Galactosyltransferase has been used to prepare a 6-sulfo LacNAc residue with 6-sulfo-GlcNAc at the nonreducing end.[13] More recently, a direct enzyme-assisted transglycosylation reaction has been reported that supplies regioselectively sulfonated disaccharides that have the 6-SO_3 GlcNAc unit attached at the nonreducing moiety.[14] In this paper, we have undertaken a clearly distinct alternative approach, which is based on the sequential use of chemical sulfonations and enzymatic desulfonations. Our approach seems effective for the assembly of a library of numerous *O*-sulfonated D-*galacto*-sugars, as described below.

Chemical sulfonation of *p*-nitrophenyl glycosides

In this study, *p*-nitrophenyl (*p*NP) glycosides were employed as the key starting compounds (Scheme 1) because most *p*NP mono- and disaccharides are commercially available and can be easily converted into artificial glycoconjugates and other cluster models.[6a,b, 7, 9, 15] *p*NP 3,6-SO_3-Gal (**1**), 3′,6′-SO_3-Lac (**2**), 3-SO_3-Gal (**7**), and 3′-SO_3-Lac (**8**) were prepared by a stannylene acetal method, as described previously.[8] Other *p*NP sulfoglycosides, that is, disulfo-Gal (**3**–**5**), 3,6-di-SO_3 GalNAc (**6**), and 2,3,6-tri-SO_3 α-Gal (**11**; Scheme 1), were prepared in the following way: *p*NP 2,3-Di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-Gal[16] was derived from *p*NP β-D-galactoside and converted into 4,6-SO_3 Gal (**3**) in three steps: 1) de-*O*-benzylidenation by treatment with an excess amount of camphorsulfonic acid (CSA) in MeOH-CH_2Cl_2-DMF (10:2:1; DMF, dimethyl formamide), 2) sulfonation at *O*-4 and *O*-6 by treatment with 12 mol equiv SO_3Me_3N in DMF, and 3) de-*O*-benzoylation by treatment with NaOMe in MeOH (53% for 3 steps).

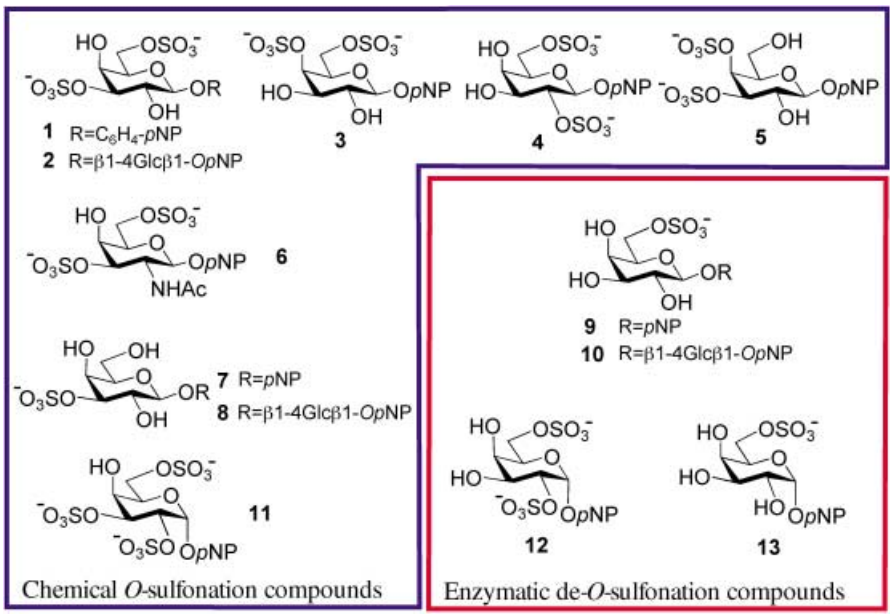
The regio-controlled synthesis of **4** could not be accomplished by the stannylene method because of the *trans* configuration of O-2 and the *O*-*p*NP group. Thus, random *O*-sulfonation of *p*NP Gal was performed by treatment with 2.4 mol equiv SO_3Me_3N, to afford a mixture of **1**, **3**, and **4** (60:16:24, total yield 35%). 2,3,6-SO_3 α-D-Gal (**11**) was prepared in 78% yield by direct *O*-sulfonation of *p*NP α-D-galactoside by reaction with 6 mol equiv SO_3Me_3N. In a manner similar to that of the synthesis of **3**, 3,4-SO_3 Gal (**5**) was derived from *p*NP β-D-galactoside in 5 steps.[17] 3,6-SO_3 GalNAc (**6**) was obtained as the single product of $(Bu_3Sn)_2O$ activated *O*-sulfonation of *p*NP β-D-GalNAc, though the conversion yield was low (19%).

Substrate selectivity and regioselectivity in the sulfatase-catalyzed reactions of *p*NP sulfo-Gal compounds 1–7

As we have already reported,[8] all three sulfatases catalyzed regioselective desulfonation at the 3-*O*-sulfo group of 3,6-SO_3 Gal (**1**) to afford 6-SO_3 Gal (**9**; Table 1, Entry 1). When 3′,6′-disulfo-β-lactoside (**2**) was used as a substrate, limpet sulfatase was found to show the highest activity towards cleavage of the 3′-*O*-sulfo group to give *p*NP 6′-sulfo lactoside (**10**; Table 1, Entry 2). The activities of the sulfatases decreased in the order limpet > abalone ≫ snail. In contrast, when 4,6-SO_3 Gal (**3**) was used as the substrate, only the abalone enzyme could hydrolyze the 4-*O*-sulfo group, to give 6-SO_3 Gal (**9**) in 90% yield.

A similar but more critical difference was observed between the three sulfatases when a mixture of 2,6-SO_3 Gal (**4**), 3,6-SO_3 Gal (**1**), and 4,6-SO_3 Gal (**3**) (**4**:**1**:**3** = 24:60:16) was used as substrate (Entry 4). Snail sulfatase hydrolyzed the respective 2-*O*- and 3-*O*-sulfo groups of **4** and **1** almost simultaneously, to give a mixture of 6-SO_3 Gal (**9**; approximately 84%) and 4,6-SO_3 Gal (**3**; approximately 16%). Abalone sulfatase hydrolyzed the 3-*O*-sulfo group of **1** and 4-*O*-sulfo group of **3**, while limpet sulfatase hydrolyzed only the 3-*O*-sulfo group of **1**. These results are consistent with the data obtained from the reactions of each of the compounds **1** and **3** (Table 1, Entries 1 and 3). Thus, it is clear that limpet sulfatase recognizes the 3-*O*-sulfo group of β-galactosides with high specificity, while snail and abalone sulfatases show broader recognition and also act on the 2-*O*- and 4-*O*-sulfo groups, as summarized in Scheme 2.

None of the enzymes accepted 3,4-disulfo Gal (**5**) and 3,6-disulfo GalNAc (**6**; Entries 5 and 6 in Table 1, and Scheme 3) as substrate. These results suggest that vicinal *O*-sulfo groups (3-SO_3/4-SO_3 in **5**) and *O*-sulfo/*N*-Ac groups (as in **6**) may not be accepted by the enzymes. Only limpet sulfatase could use 3-SO_3 Gal (**7**; Entry 7), though the sulfatide (3-sulfo β-D-Gal-ceramide) is considered to be a common substrate of the three sulfatases in nature.[18] The low reactivity for the sulfatide mimic **7** can be ascribed to the lower hydrophobicity and self-assem-



Scheme 1. *Structures of* pNP *sulfo-*D-galacto-*sugars* ***1***–***13*** *derived by the chemoenzymatic approach described herein. Compounds* ***1***–***8*** *and* ***11*** *were sulfonated by using a chemical approach, while compounds* ***9***, ***10***, ***12***, *and* ***13*** *were synthesized by the process of enzymatic de-*O*-sulfonation described in the text.*

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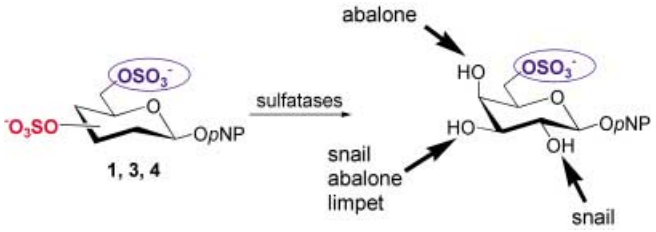
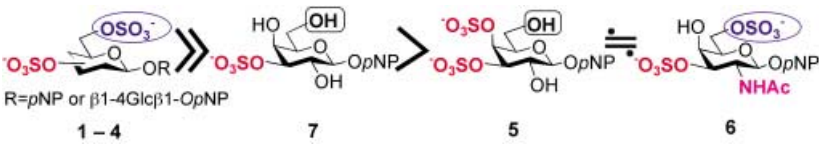
Table 1. *Substrate specificity of molluscan sulfatases[a] for sulfonated D-galacto-sugars.[b]*

Entry	Substrate	Location of de-*O*-sulfonation			Products (yield)[c]		
		Snail	Abalone	Limpet	Snail	Abalone	Limpet
1	3,6-SO_3 Gal (**1**)	*O*-3	*O*-3	*O*-3	**9**(94%[d])	**9**(>95%[d])	**9**(>95%[d])
2	3′,6′-SO_3 Lac (**2**)	—[e]	*O*-3′	*O*-3′	—[d]	**10**(40%[d])	**10**(>95%[d])
3	4,6-SO_3 Gal (**3**)	—	*O*-4	—	—	**9**(90%)	—
4	A mixture of 2,6-SO_3Gal (24%; **4**), 3,6-SO_3Gal (60%; **1**), 4,6-SO_3Gal (16%; **3**)	*O*-2/*O*-3	*O*-3/*O*-4	*O*-3	**9**(84%)	**9**(76%)	**9**(60%)
					3(16%)[f]	**4**(24%)[f]	**4**(24%)[f] + **3**(16%)[f]
5	3,4-SO_3 Gal (**5**)	—	—	—	—	—	—
6	3,6-SO_3 GalNAc (**6**)	—	—	—	—	—	—
7	3-SO_3 Gal (**7**)	—	—	*O*-3	—	—	β-Gal(30%)
8	2,3,6-SO_3α-Gal (**11**)	*O*-3 > *O*-2	*O*-3 > *O*-2	*O*-3 > *O*-2	**12**(42%) + **13**(10%)	**12**(38%) + **13**(16%)	**12**(62%) + **13**(9%)

[a] Sulfatases (E.C. 3.1.6.1) from snail (*Helix pomatia*; 16.1 units mg^{-1}), abalone (not specified; 23 units mg^{-1}), and limpet (*Patella vulgata*; 7.6 units mg^{-1}) were purchased from Sigma and used without further purification. A typical experiment is as follows: A mixture of **1** (80 mg) and sulfatase (from *Helix pomatia*, 5 mg) was dissolved in 0.25 M AcONa – AcOH buffer (pH 6.8, 2 mL) at 37 °C over 2 days to give **9** (60 mg, 94%). See the Experimental Section for details. [b] *p*NP D-glycopyranosides. [c] Isolated yields or yields determined by ^{1}H NMR spectroscopy. [d] See ref. [8]. [e] No reaction. [f] Recovered products and yields.



Scheme 2. *Discrimination between secondary O-sulfo groups in the 6-O-SO_3 D-galacto-sugars **1**, **3**, and **4** in sulfatase reactions. Limpet sulfatase recognized only the 3-O-SO_3 group in **1** with high specificity, while snail and abalone sulfatases showed broader recognition for 2-O-SO_3 and 4-O-SO_3 in **4** and **3**, respectively, as well as for 3-O-SO_3 in **1**.*



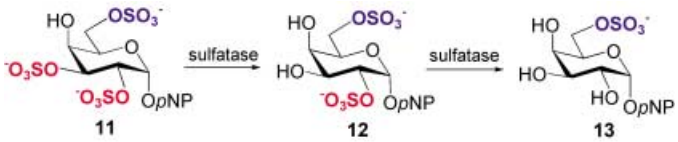
Scheme 3. *Relative activities of O-sulfonated D-galacto-sugars in sulfatase reactions. The presence of a primary 6-O-SO_3 group promotes de-O-sulfonation at the secondary positions, that is, 3-O-SO_3 in **1**, 3′-O-SO_3 in **2**, 4-O-SO_3 in **3**, and 2-O-SO_3 in **4**. The absence of 6-O-SO_3 in **5** and **7**, and the presence of NHAc in **6** did not aid de-O-sulfonation at the secondary sulfonates.*

bling properties of this compound at the aglycon structure compared to those of the sulfatide (*p*NP versus ceramide). It is of more significance, however, that 3,6-disulfo Gal (**1**) is a better substrate than **7** for all three enzymes, as shown in Scheme 3. This result suggests the existence of natural substrates with 3,6-di-*O*-sulfo groups.

Time courses of enzyme reactions with *p*NP 2,3,6-SO_3 α-Gal (**11**)

The reactions of substrates **1** – **4** led us to assume that a primary 6- (or 6′)-*O*-sulfo group may promote de-*O*-sulfonation at the secondary positions (Scheme 3). To substantiate this assumption, we performed enzyme-catalyzed reactions with 2,3,6-tri-SO_3 α-Gal (**11**; see Entry 8 in Table 1 and Scheme 4), which possesses vicinal *O*-sulfo groups as well as a primary *O*-sulfo group at C-6. The reactions revealed that all three enzymes could utilize the substrate **11** to produce 2,6-di-SO_3 α-Gal (**12**) and 6-mono-SO_3 α-Gal (**13**). In this case, the vicinal *O*-sulfo groups could be recognized by these enzymes, which supports the hypothesis that the 6-*O*-sulfo group promotes hydrolysis at the secondary sulfo groups.

To obtain more precise information on the enzyme-catalyzed reactions of **11**, we monitored each of the enzyme reactions by ^{1}H NMR spectroscopy. The time courses of reactions catalyzed by the snail enzyme (Figure 1) show that the sulfatase converted **11** mainly into the 2,6-disulfo compound **12** in the first 24 h of reaction (34% conversion) and then started to produce the 6-sulfo compound **13**. The yield of **13** reached 4% after 45 h and increased steadily thereafter. The amount of **12** present reached saturation after about 70 h, and the amounts of both **11** and **12** decreased steadily thereafter. After 195 h, the yields of the 2,6-disulfo product **12** and the 6-sulfo product **13** were 42% and 10%, respectively. In this experiment, no production of the 2,3- and 3,6-disulfo compounds could be



Scheme 4. *Stepwise de-O-sulfonation of tri-O-sulfonated **11** by sulfatases. All the sulfatases hydrolyzed the 3-O-SO_3 group in **11** to produce the 2,6-di-O-sulfonated compound **12**, and then hydrolyzed the 2-O-SO_3 group in **12** to afford the 6-mono-O-sulfonated compound **13**.*

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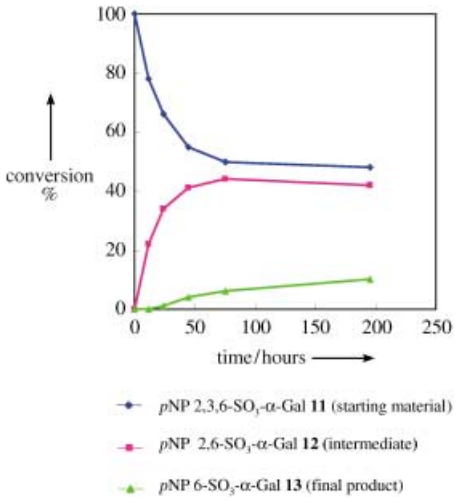
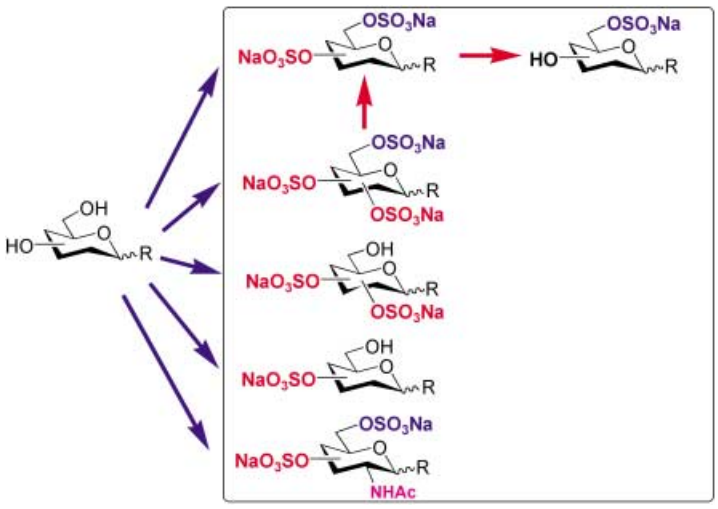


Figure 1. Time course of snail sulfatase reactions with pNP *2,3,6-SO$_3$-α-D-Gal (**11**; starting material, shown in blue) and* pNP *2,6-SO$_3$-α-Gal (**12**; intermediate, shown in pink). The product,* pNP *6-SO$_3$-α-Gal (**13**), is shown in green.*



Scheme 5. Library synthesis of sulfo D-galacto-sugars by the chemoenzymatic method described herein. Blue arrows: Chemical O-sulfonation by stannylene acetal technology, conventional chemical transformation, or random synthesis. Red arrows: Enzymatic de-O-sulfonation by sulfatases.

detected. These results indicate that the snail enzyme recognizes the 3-*O*-sulfo group of **11** selectively and catalyzes reaction at this group to produce **12**, and then acts at the 2-*O*-sulfo group of **12** to yield **13**. Abalone and limpet sulfatases behaved similarly to the snail enzyme, though the abalone enzyme showed the highest reactivity for the conversion of **12** into **13** (16% yield in 195 h). The limpet enzyme also yielded the two products **12** and **13** in 62% and 9%, respectively (141 h). The production of **13** by the limpet enzyme indicated that this enzyme also hydrolyzes the 2-*O*-sulfo group, though little reactivity was detectable for the β-isomer (**4**). This result may be explained as the result of an anomer effect changing the regioselectivity, though further experiments are needed to determine the precise mechanism.

Conclusion

The substrate selectivities and regioselectivities of three molluscan sulfatases were examined with a series of mono-, di-, and tri-*O*-sulfonated *galacto*-sugars. We observed a unique phenomenon: the hydrolysis of *O*-sulfo groups at the secondary positions is promoted by the presence of a 6-*O*-sulfo group in the same substrate. Although these enzymes have rarely been applied for synthetic purposes, it is now clear that they possess great potential for application to the library assembly of *O*-sulfonated sugars. In our approach, which combined enzymatic and chemical reactions, we were able to obtain 13 types of *p*NP sulfo-D-*galacto*-sugar, nine of which were obtained by use of organotin and random sulfonation methods (blue arrows in Scheme 5) and four of which were the products of enzyme reactions (red arrows).

Experimental Section

General: Sulfatases from snail (*Helix pomatia*, 16.1 units mg^{-1}), abalone entrails (not specified, 23 units mg^{-1}) and limpet (*Patella vulgata*, 7.6 units mg^{-1}) were from Sigma and used without further purification. All other reagents were obtained from Aldrich or Sigma and used as received.

Reactions were performed in well-dried glassware under an inert atmosphere of N_2. Unless stated otherwise, all chemical reactions were performed under anhydrous conditions at 40 °C. Reactions were followed by TLC analysis performed on Silica Gel 60 F-254 precoated plates (E. Merck). Compounds were visualized under UV light and by spraying with 20% H_2SO_4 in EtOH, followed by charring at 140 °C. Flash chromatography was carried out on E. Merck Silica Gel (230–400 mesh) or Silica Gel 60 RP-18 (40–63 μm). Gel filtration was performed on Sephadex LH-20 (Amersham Pharmacia Biotech). Optical rotations were measured on a JASCO DIP-1000 polarimeter at ambient temperature.

^{1}H, ^{13}C and ^{1}H-^{1}H COSY spectra were recorded on a Varian G300, Bruker ASX-400, Bruker AVANCE-500, or JEOL LA-600 instrument. All data are assumed to be first order. Mass spectra were obtained in a Hitachi MS-80B or JEOL DX303 spectrometer. Combustion analyses were performed on a Carlo Elba EA-1108 or Perkin-Elmer EA-2400 instrument.

***p*NP 3,6-di-*O*-sulfo-β-D-galactopyranoside, sodium salt (1)**: A mixture of *p*NP galactopyranoside (600 mg, 1.99 mmol) and $(Bu_3Sn)_2O$ (0.764 mL, 1.5 mmol) was refluxed in tetrahydrofuran (THF)/benzene (1:1, total volume 50 mL) for 3 h with continuous azeotropic removal of water. The reaction mixture was concentrated in vacuo, and the residual stannylene acetal was treated with SO_3NMe_3 (1.4 g, 0.01 mol) in DMF at 60 °C for 3 h. The reaction mixture was diluted with benzyl alcohol (8 mL) and concentrated in vacuo. The residue was then successively purified by size exclusion chromatography on a Sephadex LH-20 column, reverse-phase chromatography on a column of octadecylsilyl (ODS-C18; E. Merck) and ion-exchange chromatography on a Dowex Na^+ column to afford **1** (980 mg, 97%): $[\alpha]_D = -35°$ ($c = 0.7$ in H_2O); ^{1}H NMR (D_2O, *t*BuOH = 1.23 ppm): $\delta =$ 8.271 and 7.278 (d, $J = 9.3$ Hz, *p*NP), 5.330 (d, $J = 7.8$ Hz, H-1), 4.484 (dd, $J = 3.3$ and 9.6 Hz, H-3), 4.429 (d, $J = 3.3$ Hz, H-4), 4.34–3.93 (m, H-6 and H-6′), 4.013 (dd, $J = 7.8$ and 9.6 Hz, H-2) ppm; ^{13}C NMR (*t*BuOH = 31.3 ppm): $\delta = 163.6$, 144.5, 128.0, 118.4, 101.3, 81.3, 74.7, 70.2, 68.8, 68.3 ppm; FAB-MS: 527 $[M+Na-1]^+$.

***p*NP 3′,6′-di-*O*-sulfo-β-D-lactoside, sodium salt (2)**: A mixture of *p*NP lactoside (79 mg, 0.17 mmol) and Bu_2SnO (128 mg, 0.51 mmol) was

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refluxed in MeOH (total volume 200 mL) for 5 h with continuous azeotropic removal of water. The reaction mixture was concentrated in vacuo and the stannylene acetal intermediate treated with SO_3NMe_3 (71 mg, 0.51 mmol) in DMF at 50 °C for 16 h. The reaction mixture was diluted with benzyl alcohol (2 mL) and concentrated in vacuo. The residue was then purified by sequential column chromatography with Sephadex LH-20, ODS C-18, and ion exchange resin (Dowex Na^+) to afford **2** (53 mg, 40%). $[\alpha]_D = -39°$ ($c = 1.1$ in H_2O). ^1H NMR (600 MHz, D_2O, *t*BuOH = 1.23 ppm): $\delta = 8.270$ and 7.251 (d, $J = 9.2$ Hz, *p*NP), 5.313 (d, $J = 8.1$ Hz, H-1), 4.629 (d, $J = 7.7$ Hz, H-1′), 4.38–4.32 (dd, $J = 3.3$ and 9.5 Hz, H-3′, and br d, $J = 3.3$ Hz, H-4′), 4.27–4.20 (m, H-6 and H-6′ of galactose residue), 4.07–4.02 (br t, $J = 6.2$ Hz, H-5′), 4.02–3.97 (br d, $J = 12.0$ Hz, H-6 of glucose residue), 3.805 (br t, $J = 9.9$ Hz, H-3), 3.90–3.77 (m, H-5) ppm. ^{13}C NMR (*t*BuOH = 31.3 ppm): $\delta = 163.7$, 144.6, 128.1, 118.4, 104.5, 101.1, 81.6, 80.7, 76.8, 75.8, 74.4, 74.3, 70.8, 69.1, 68.5, 61.8 ppm. FAB-MS (pos.): 668 $[M+1]^+$, 690 $[M+Na]^+$.

***p*NP 4,6-di-*O*-sulfo-β-D-galactopyranoside, sodium salt (3)**:

A) Synthesis of *p*NP 4,6-*O*-benzylidene-β-D-galactopyranoside: A mixture of *p*NP galactopyranoside (2 g, 6.6 mmol), benzaldehyde dimethyl acetal (1.3 mL, 8.7 mmol), and camphorsulfonic acid (154 mg, 0.1 mmol) was dissolved in CH_3CN and DMF (1:1, 100 mL) at RT over a 20-h period. Triethylamine (4 mL) was added. Ethyl acetate (200 mL) was added and the mixture was washed with $NaHCO_3$ and NaCl. After evaporation, the residue was crystallized from diethyl ether and hexane to give *p*NP 4,6-*O*-benzylidene-β-D-galactopyranoside (2.4 g, 94%). $[\alpha]_D = -120°$ ($c = 0.6$ in DMF). ^1H NMR (500 MHz, CD_3OD (3.35 ppm)): $\delta = 8.264$ and 7.315 (d, $J = 9.2$ Hz, *p*NP), 7.57 and 7.39 (m, C_6H_5CH), 5.677 (s, C_6H_5CH), 5.186 (d, $J = 7.7$ Hz, H-1), 4.338 (br d, $J = 3.4$ Hz, H-4), 4.30–4.18 (m, H-6, H-6′), 3.979 (dd, $J = 7.8$ and 9.8 Hz, H-2), 3.861 (br s, H5), 3.811 (dd, $J = 3.4$ and 9.8 Hz, H-3) ppm. ^{13}C NMR (CD_3OD (49.0 ppm)): $\delta = 163.8$, 143.9, 139.6, 129.9, 129.0, 127.6, 126.6, 117.8, 102.5, 101.9, 77.3, 73.4, 71.5, 70.0, 68.4 ppm. Elemental analysis: calcd. for $C_{19}H_{19}NO_8$: C, 58.61; H, 4.92; N, 3.60; found: C, 58.62; H, 4.84; N, 3.56. FAB-MS (pos.) 390 $[M+1]^+$, 412 $[M+Na]^+$.

B) Synthesis of *p*NP 2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-galactopyranoside: A mixture of the compound produced by Method A (2 g, 5.1 mmol), *N*,*N*-dimethylaminopyridine (13.3 mg, 0.109 mmol), benzoyl chloride (3.1 mL, 26.6 mmol), triethylamine (0.15 mL, 1.08 mmol), and pyridine (50 mL) was stirred for 48 h at RT. After cooling, crushed ice and sat. $NaHCO_{3(aq)}$ were added, then the mixture was stirred for 0.5 h at RT, diluted with ethyl acetate, washed with sat. $NaHCO_{3(aq)}$ and water, dried ($MgSO_4$) and evaporated. The residue was eluted from a column of silica gel (200 g) with toluene/ethyl acetate (4:1) to give the desired product (2.7 g, 90%). $[\alpha]_D = +79°$ ($c = 0.42$ in $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$ (TMS)): $\delta = 8.152$ and 7.118 (d, $J = 9.3$ Hz, *p*NP), 8.001 (d, $J = 8.5$ Hz, benzoyl), 7.948 (d, $J = 8.5$ Hz, benzoyl), 7.57–7.35 (m, C_6H_5CH and benzoyl), 6.173 (dd, $J = 8.0$ and 10.4 Hz, H-2), 5.606 (s, C_6H_5CH), 5.483 (dd, $J = 3.4$ and 10.4 Hz, H-3), 5.470 (d, $J = 8.0$ Hz, H-1), 4.701 (br d, $J = 3.4$ Hz, H-4), 4.452 (br d, $J = 11.9$ Hz, H-6), 4.214 (br d, $J = 11.9$ Hz, H-6′), 3.910 (br s, H-5) ppm. ^{13}C NMR ($CDCl_3$ (77.7 ppm)): $\delta = 166.8$, 165.8, 162.2, 143.8, 137.8, 134.3, 134.1, 130.7, 130.4, 129.8, 129.6, 129.2, 128.9, 126.9, 126.4, 117.7, 101.7, 99.8, 73.9, 73.0, 69.4, 69.2, 67.8 ppm. FAB-MS (pos.): 596 $[M-2]^+$, 619 $[M+Na-2]^+$. Elemental analysis: calcd. for $C_{33}H_{27}NO_{10}$: C, 66.3; H, 4.55; N, 2.34; found: C, 66.5; H, 4.56; N, 2.22.

C) Synthesis of *p*NP 2,3-di-*O*-benzoyl-β-D-galactopyranoside: *p*NP 2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-galactopyranoside (2.6 g, 4.5 mmol) and camphorsulfonic acid (310 mg, 1.3 mmol) were dissolved in MeOH (50 mL), CH_2Cl_2 (10 mL), and DMF (5 mL). After 43 h, Et_3N (1 mL) was added and the reaction mixture was diluted with AcOEt and washed with $NaHCO_3$ and NaCl. After evaporation, the residue was crystallized from diethyl ether/hexane to give the desired compound (2 g, 89%). $[\alpha]_D = +103°$ ($c = 0.39$ in DMF). ^1H NMR (500 MHz, CD_3OD (3.35 ppm)): $\delta = 8.230$ and 7.259 (d, $J = 9.5$ Hz, *p*NP), 8.021 (d, $J = 8.5$ Hz, benzoyl), 7.975 (d, $J = 8.5$ Hz, benzoyl), 7.65–7.40 (m, benzoyl), 6.043 (dd, $J = 8.0$ and 10.4 Hz, H-2), 5.717 (d, $J = 8.0$ Hz, H-1), 5.517 (dd, $J = 3.1$ and 10.4 Hz, H-3), 4.432 (br d, $J = 2.8$ Hz, H-4), 4.130 (m, H-5), 3.929 (dd, $J = 6.7$ and 11.6 Hz, H-6), 3.881 (dd, $J = 5.5$ and 11.6 Hz, H-6′) ppm. ^{13}C NMR (CD_3OD (49.0 ppm)): $\delta = 167.2$, 167.0, 163.2, 144.2, 134.6, 134.5, 130.8, 130.6, 129.6, 129.5, 126.7, 117.9, 100.0, 77.4, 75.9, 71.1, 67.9, 62.4 ppm. FAB-MS (pos.): 510 $[M]^+$, 532 $[M+Na-1]^+$. Elemental analysis: calcd. for $C_{26}H_{23}NO_{10}$: C, 61.3; H, 4.55; N, 2.75; found: C, 61.3; H, 4.44; N, 2.69.

D) Synthesis of *p*NP 2,3-di-*O*-benzoyl-4,6-di-*O*-sulfo-β-D-galactopyranoside: The above-described diol galactose derivative (593 mg, 1.2 mmol) was sulfonated by treatment with SO_3Me_3N (2 g, 14.4 mmol) in DMF (10 mL) at 60 °C for 23 h. The reaction mixture was evaporated. Caution: The disulfo derivative immediately decomposes in MeOH solution, while in MeOH/H_2O (1:1) the compound is stable. The residue was purified on a ODS C-18 (40 g) column eluted with $H_2O \rightarrow H_2O$/MeOH (1:1) to give the disulfo derivative (831 mg, quant.). $[\alpha]_D = +55°$ ($c = 0.6$ in H_2O). ^1H NMR (500 MHz, D_2O (*t*BuOH = 1.23 ppm, Me_3N^+ salt)): $\delta = 8.117$ and 7.135 (d, $J = 9.2$ Hz, *p*NP), 7.913 (br d, $J = 7.4$ Hz, benzoyl), 7.817 (br d, $J = 7.7$ Hz, benzoyl), 7.560 (br t, $J = 7.5$ Hz, benzoyl), 7.486 (br t, $J = 7.5$ Hz, benzoyl), 7.392 (br t, $J = 7.8$ Hz, benzoyl), 7.298 (br t, $J = 7.8$ Hz, benzoyl), 5.851 (dd, $J = 8.0$ and 10.1 Hz, H-2), 5.789 (d, $J = 8.0$ Hz, H-1), 5.675 (dd, $J = 3.4$ and 10.1 Hz, H-3), 5.190 (br d, $J = 3.4$ Hz, H-4), 4.69–4.64 (m, H-5), 4.456 (dd, $J = 3.1$ and 11.8 Hz, H-6), 4.310 (dd, $J = 8.9$ and 11.8 Hz, H-6′), 2.873 (s, Me_3N^+ salt) ppm. FAB-MS (pos.): 735 $[M+Na-2]^+$. Elemental analysis: calcd. for $C_{26}H_{21}NNa_2O_{16}S_2 \cdot 2H_2O$: C, 41.66; H, 3.36; N, 1.87; S, 8.55; found: C, 41.50; H, 3.25; N, 2.21; S, 8.22.

E) Synthesis of **3**: The disulfonated derivative (57 mg, 0.08 mmol) obtained as described in (D) was immediately dissolved in 0.1 M NaOMe/MeOH. After 10 min, the reaction mixture was neutralized with Dowex H^+ to give **3** (24.2 mg, 60%). The deprotected disulfo compound **3** was stable in MeOH, H_2O, or a mixture of both. $[\alpha]_D = -63°$ ($c = 0.3$ in H_2O). ^1H NMR (500 MHz, D_2O (*t*BuOH = 1.23 ppm, Na^+ salt)): $\delta = 8.280$ and 7.283 (d, $J = 9.0$ Hz, *p*NP), 5.248 (d, $J = 7.7$ Hz, H-1), 4.77–4.68 (m, H-4), 4.40–4.33 (m, H-6 and H-6′), 4.196 (m, H-5), 3.955 (dd, $J = 3.0$ and 9.8 Hz, H-3), 3.893 (br dd, $J = 7.6$ and 9.8 Hz, H-2) ppm. ^{13}C NMR (D_2O (*t*BuOH = 31.3 ppm)): $\delta = 168.9$, 144.4, 127.9, 118.3, 101.5, 77.7, 74.4, 73.1, 72.0, 69.3 ppm. Elemental analysis: calcd. for $C_{12}H_{13}NNa_2O_{14}S_2 \cdot 2H_2O$: C, 26.62; H, 3.16; N, 2.59; S, 11.84; found: C, 26.93; H, 2.99; N, 2.51; S, 11.67.

Mixture of *p*NP 2,6-di-*O*-sulfo-β-D-galactopyranoside, sodium salt (4) with compounds 1, and 3: A mixture of *p*NP galactopyranoside (1 g, 3.3 mmol) and SO_3NMe_3 (1.1 g, 8.0 mmol) was dissolved in DMF at 80–90 °C over a 14-h period. The reaction mixture was then diluted with methanol (10 mL) and concentrated in vacuo. The residue was purified by sequential column chromatography with Sephadex LH-20, ODS C-18, and ion-exchange resin (Dowex Na^+) to afford a mixture of **4**, **1**, and **3** (580 mg, 35%, **4**/**1**/**3** = 24:60:16). ^1H NMR (500 MHz, D_2O, *t*BuOH = 1.23 ppm) for **4**: $\delta = 8.280$ and 7.283 (br d, $J = 9.2$ Hz, *p*NP), 5.377 (d, $J = 7.7$ Hz, H-1), 4.583 (dd, $J = 7.7$ and 9.5 Hz, H-2), 4.127 (br d, $J = 3.4$ Hz, H-4), 3.973 (dd, $J = 3.4$ and 9.5 Hz, H-3) ppm. The ratio **4**/**1**/**3** was determined by integration of the respective H-1 signals at 5.377 (**4**), 5.330 (**1**), and 5.248 (**3**) ppm. FAB-MS (pos.): 506 $[M+1]^+$, 528 $[M+Na]^+$.

***p*NP 3,4-di-*O*-sulfo-β-D-galactopyranoside, sodium salt (5)**:

A) Synthesis of *p*NP 3,4-isopropylidene-β-D-galactopyranoside: A mixture of *p*NP galactopyranoside (3 g, 10 mmol) and Dowex H^+

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(1.2 g) was dissolved in acetone (500 mL) at RT over a period of 3 h. Triethylamine (8 mL) was added and the reaction mixture was filtered. After evaporation, the residue was purified by silica gel (400 mL) chromatography with CH_2Cl_2/MeOH (10:1) containing 1% triethylamine to give *p*NP 3,4-*O*-isopropylidene-β-D-galactopyranoside (2.2 g, 65%). $[\alpha]_D = -59°$ ($c = 1.7$ in MeOH). M.p.: 154–157 °C. ^{1}H NMR (300 MHz, CD_3OD (*t*BuOH = 1.23 ppm)): $\delta = 8.231$ and 7.236 (d, $J = 9.3$ Hz, *p*NP), 5.044 (d, $J = 7.8$ Hz, H-1), 4.313 (dd, $J = 2.1$ and 5.7 Hz, H-4), 4.37–4.10 (m, H-3, H-5), 3.87–3.72 (m, H-2, H-6, H-6′), 1.528 and 1.371 (2 s, CH_3 of isopropylidene) ppm. ^{13}C NMR (75 MHz, CD_3OD (*t*BuOH = 31.3 ppm)): $\delta = 164.2$, 144.3, 127.0, 118.1, 111.6, 101.5, 80.9, 75.7, 75.2, 74.0, 62.6, 28.6, 26.7 ppm.

B) Synthesis of *p*NP 3,4-*O*-isopropylidene-2,6-di-*O*-benzoyl-β-D-galactopyranoside: A mixture of the diol compound described above (1.75 g, 5.1 mmol), *N*,*N*-dimethylaminopyridine (50 mg, 0.41 mmol), and benzoyl chloride (1.77 mL, 15 mmol) was dissolved in pyridine (50 mL) and stirred for 17.5 h at RT. The reaction mixture was cooled to 0 °C, then crushed ice and $NaHCO_3$ were added. After 1 h, the mixture was diluted with CH_2Cl_2, washed with $NaHCO_3$ and NaCl, and evaporated to afford dibenzoyl compound (2.9 g, quant.). $[\alpha]_D = +4.6°$ ($c = 1.6$ in $CHCl_3$). M.p.: 177–178 °C. ^{1}H NMR (300 MHz, $CDCl_3$ (TMS)): $\delta = 8.08$–8.03 (m, benzoyl), 7.976 and 7.005 (d, $J = 9.3$ Hz, *p*NP), 7.69–7.40 (m, benzoyl), 5.564 (dd, $J = 6.3$ and 7.5 Hz, H-2), 5.253 (d, $J = 7.5$ Hz, H-1), 4.792 (dd, $J = 3.6$ and 11.7 Hz, H-6), 4.641 (dd, $J = 8.4$ and 11.7 Hz, H-6′), 4.534 (br t, $J = 6.3$ Hz, H-3), 4.48–4.38 (m, H-4 and H-5), 1.682 and 1.413 (2 s, CH_3 of isopropylidene) ppm. ^{13}C NMR (100 MHz, $CDCl_3$ (77.0 ppm)): $\delta = 166.2$, 165.2, 161.3, 149.9, 142.9, 135.9, 133.6, 133.5, 129.8, 129. 6, 129.3, 128.6, 128.5, 125.5, 123.7, 116.7, 111.5, 98.0, 73.0, 72.5, 71.7, 63.5, 27.4, 26.1 ppm. Elemental analysis: calcd. for $C_{29}H_{27}NO_{10}$: C, 63.38; H, 4.95; N, 2.55; found: C, 63.38; H, 4.95; N, 2.49.

C) Synthesis of *p*NP 2,6-di-*O*-benzoyl-β-D-galactoryranoside: The fully protected galactoside (1 g, 1.8 mmol) described in (B) was dissolved in CH_2Cl_2/MeOH (1:2, v/v) in the presence of CSA (0.17 g, 0.73 mmol) over a period of 2 days. Et_3N (1 mL) was added and the reaction mixture was evaporated. The residue was dissolved in AcOEt, and the organic solvent was washed with sat. $NaHCO_{3(aq)}$ and H_2O. After evaporation, a white solid was obtained (0.78 g, 84%). $[\alpha]_D = +14°$ ($c = 0.68$ in MeOH). M.p.: 192–194 °C. ^{1}H NMR (400 MHz, $CDCl_3$, (TMS)): $\delta = 8.061$ and 7.042 (d, $J = 8.7$ Hz, *p*NP), 8.04–8.00 (m, benzoyl), 7.70–7.40 (m, benzoyl), 5.513 (dd, $J = 7.9$ and 9.2 Hz, H-2), 5.280 (d, $J = 7.9$ Hz, H-1), 4.791 (dd, $J = 4.9$ and 11.8 Hz, H-6), 4.645 (dd, $J = 7.7$ and 11.7 Hz, H-6′), 4.2–3.9 (m, H-3, H-4, H-5) ppm. ^{13}C NMR (75 MHz, DMSO-d_6, 39.6 ppm): $\delta = 165.8$, 165.4, 161.8, 142.2, 133.9, 129.6, 129.1, 125.8, 116.8, 97.7, 73.4, 72.3, 71.0, 68.7, 64.1 ppm. Elemental analysis: calcd. for $C_{26}H_{23}NO_{10}$: C, 61.30; H, 4.55; N, 2.75; found: C, 60.94; H, 4.42; N, 2.62.

D) Synthesis of **5**: The diol galactose derivative (80 mg, 0.16 mmol) obtained as described in (C) and SO_3Me_3N (157 mg, 1.1 mmol) were dissolved in DMF at 50 °C for 24 h. MeOH (2 mL) was added and the reaction mixture was evaporated. The residue was purified with silica gel (10 g, $CHCl_3$/MeOH (10:3)) to give *p*NP 2,6-di-*O*-benzoyl-3,4-di-*O*-sulfo-β-D-galactopyranoside (37.4 mg, 35%). $[\alpha]_D = +37°$ ($c = 0.06$ in MeOH/CH_2Cl_2 (10:1, v/v)). The above-mentioned disulfogalactopyranoside (34 mg, 0.048 mmol) was then dissolved in MeOH/THF (5:1, 12 mL), and 28% NaOMe/MeOH solution (100 μL) was added. After 30 min, the reaction solution was neutralized with Dowex 50 (H^+), filtered, and evaporated. The residue was purified with ODS (C-18, H_2O as the eluent) to give **5** (21.5 mg, 89%). $[\alpha]_D = -25°$ ($c = 0.27$ in H_2O). ^{1}H NMR (300 MHz, D_2O (*t*BuOH = 1.23 ppm)): $\delta = 8.275$ and 7.274 (d, $J = 9.3$ Hz, *p*NP), 5.384 (d, $J = 7.8$ Hz, H-1), 5.047 (br d, $J = 3.0$ Hz, H-4), 4.536 (dd, $J = 3.0$ and 10.2 Hz, H-3), 4.16–4.09 (m, H-5), 4.033 (dd, $J = 7.8$ and 10.2 Hz, H-2), 3.90–3.75 (m, H-6, H-6′) ppm. ^{13}C NMR (100 MHz, D_2O (*t*BuOH = 31.2 ppm)): $\delta = 163.4$, 144.5, 127.9, 118.3, 101.2, 79.1, 76.6, 76.2, 70.2, 62.3 ppm.

***p*NP 3,6-di-*O*-sulfo-2-deoxy-*N*-acetyl-β-D-galactosaminide, sodium salt (6)**: A mixture of *p*NP 2-deoxy-*N*-acetyl-β-D-galactosaminide (70 mg, 0.23 mmol) and $(Bu_3Sn)_2O$ (179 μL, 0.35 mmol) was refluxed in MeOH/THF/benzene (1:1:1, total volume 300 mL) → MeOH/benzene (1:1, total volume 300 mL) → benzene (total volume 100 mL) for 12 h with continuous azeotropic removal of water. The reaction mixture was concentrated in vacuo and the stannylene acetal intermediate treated with SO_3NMe_3 (130 mg, 0.93 mmol) in DMF at 60 °C for 34 h. The reaction mixture was diluted with methanol (10 mL) and concentrated in vacuo. The residue was then purified by sequential column chromatography with Sephadex LH-20, ODS C-18, and ion-exchange resin (Dowex Na^+) to afford **6** (24 mg, 19%). $[\alpha]_D = +7.8°$ ($c = 0.47$ in H_2O). ^{1}H NMR (500 MHz, D_2O, *t*BuOH = 1.23 ppm): $\delta = 8.252$ and 7.227 (d, $J = 9.2$ Hz, *p*NP), 5.401 (d, $J = 8.6$ Hz, H-1), 4.573 (dd, $J = 3.1$ and 10.7 Hz, H-3), 4.388 (br d, $J = 3.1$ Hz, H-4), 4.371 (dd, $J = 8.6$ and 10.7 Hz, H-2), 4.32–4.20 (m, H-5, H-6, H-6′), 2.001 (s, NH*Ac*) ppm. Elemental analysis: calcd. for $C_{14}H_{16}N_2Na_2O_{14}S_2 \cdot 1\,H_2O$: C, 29.79; H, 3.21; N, 4.96; found: C, 30.19; H, 3.68; N, 5.15. FAB-MS (pos.): 568 $[M+Na-1]^+$.

***p*NP 3-*O*-sulfo-β-D-galactopyranoside, sodium salt (7)**: A mixture of *p*NP β-D-galactopyranoside (640 mg, 2.13 mmol) and Bu_2SnO (540 mg, 2.15 mmol) was refluxed in THF/toluene (1:1, total volume 100 mL) for 4.5 h with continuous azeotropic removal of water. The reaction mixture was concentrated in vacuo, and the residue was treated with SO_3NMe_3 (450 mg, 3.23 mol) in DMF (20 mL) at 60 °C for 12.5 h. The reaction mixture was diluted with methanol (8 mL) and concentrated in vacuo. The residue was purified on a silica gel column ($CHCl_3$:MeOH = 5:1) and treated with ion-exchange resin (Dowex Na^+) to afford **7** (814 mg, 95%): $[\alpha]_D = -41.8°$ ($c = 1.3$ in MeOH); m.p.: 186–194 °C; ^{1}H NMR (D_2O, RT): $\delta = 8.22$ and 7.23 (dd, $J = 2.1$ and 7.5 Hz, *p*NP), 5.30 (d, $J = 7.5$ Hz, H-1), 4.46 (dd, $J = 3.3$ and 9.6 Hz, H-3), 4.39 (d, $J = 3.3$ Hz, H-4), 4.04–3.97 (m, H-2 + H-6S), 3.80 (d, H-5 + H-6R) ppm. FAB-MS 426 $[M+Na]^+$. Elemental analysis: calcd. for $C_{12}H_{14}O_{11}NSNa$: C, 35.375; H, 3.577; N, 3.438; S, 7.870; found: C, 35.185; H, 3.577; N, 3.348; S, 7.336.

***p*NP 3′-*O*-sulfo-β-D-lactoside, sodium salt (8)**: A mixture of *p*NP lactoside (79 mg, 0.17 mmol) and Bu_2SnO (43 mg, 0.17 mmol) was refluxed in MeOH (total 200 mL) for 3 h with continuous azeotropic removal of water. The reaction mixture was concentrated in vacuo and the stannylene acetal intermediate treated with SO_3NMe_3 (24 mg, 0.17 mmol) in DMF at 50 °C for 16 h. The reaction mixture was diluted with methanol (10 mL) and concentrated in vacuo. The residue was then purified by sequential column chromatography with Sephadex LH-20, ODS C-18, and ion-exchange resin (Dowex Na^+) to afford **8** (56 mg, 57%). $[\alpha]_D = -39°$ ($c = 1.3$ in H_2O). ^{1}H NMR (600 MHz, D_2O, *t*BuOH = 1.23 ppm): $\delta = 8.235$ and 7.225 (d, $J = 9.2$ Hz, *p*NP), 5.283 (d, $J = 7.8$ Hz, H-1), 4.596 (d, $J = 8.1$ Hz, H-1′), 4.342 (dd, $J = 3.0$ and 9.8 Hz, H-3′), 4.297 (br d, $J = 3.0$ Hz, H-4′), 4.03–3.97 (br d, $J = 11.0$ Hz, H-6 of glucose residue), 3.89–3.74 (m, H-6, H-6′ and H-6″ of glucose and galactose residues), 3.796 (br t, $J = 9.5$ Hz, H-3) ppm. ^{13}C NMR (*t*BuOH = 31.3 ppm): $\delta = 163.6$, 144.5, 128.0, 118.3, 104.4, 101.1, 81.8, 79.6, 76.8, 76.7, 75.8, 74.2, 70.8, 68.6, 62.7, 61.5 ppm.

***p*NP 6-*O*-sulfo-β-D-galactopyranoside, sodium salt (9)**: Sulfatases that are commercially available were used without purification. The typical procedure is as follows: A mixture of **1** (80 mg) and sulfatase (from *Helix pomatia*, 5 mg) was dissolved in sodium acetate buffer (pH 6.8, 0.25 M; 2 mL) at 37 °C over a 2-day period. The reaction mixture was purified by sequential column chromatography with Sephadex LH-20, ODS, and ion-exchange resin (Dowex Na^+) to give **9** (60 mg, 94%): $[\alpha]_D = -64.4°$ ($c = 3.1$ in H_2O); ^{1}H NMR (D_2O, *t*BuOH = 1.23 ppm): $\delta = 8.195$ and 7.213 (d, $J = 9.2$ Hz, *p*NP), 5.174 (d, $J = 7.3$ Hz,

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H-1), 4.29 – 4.17 (m, H-6 and H-6′), 4.075 (br d, $J = 3.0$ Hz, H-4), 3.875 (dd, $J = 7.3$ and 9.9 Hz, H-2), 3.823 (dd, $J = 3.0$ and 9.9 Hz, H-3) ppm; ^{13}C NMR (*t*BuOH = 31.3 ppm): $\delta = 163.5$, 144.1, 127.8, 118.2, 101.6, 74.9, 73.9, 71.9, 69.9, 68.7 ppm. FAB-MS 426 $[M+Na]^+$. Elemental analysis: calcd. for $C_{12}H_{14}NNaO_{11}S \cdot 2H_2O$: C, 32.81; H, 4.13; N, 3.19; S, 7.30; found: C, 32.42; H, 3.44; N, 2.95; S, 6.88.

***p*NP 6′-*O*-sulfo-β-D-lactoside, sodium salt (10)**: A mixture of **2** (20 mg) and sulfatase (from abalone entrails, 1.8 mg) was dissolved in acetic acid/sodium acetate buffer (pH 6.8, 0.25 M; 0.5 mL) at 37 °C over a period of 3 days. The reaction mixture was purified by sequential column chromatography with Sephadex LH-20, ODS C-18, and ion-exchange resin (Dowex Na^+) to give **10** (6.8 mg, 40 %). $[\alpha]_D = -44°$ ($c = 0.37$ in H_2O). ^{1}H NMR (600 MHz, D_2O, *t*BuOH = 1.23 ppm): $\delta = 8.264$ and 7.244 (d, $J = 9.2$ Hz, *p*NP), 5.304 (d, $J = 8.1$ Hz, H-1), 4.506 (d, $J = 7.8$ Hz, H-1′), 4.25 – 4.20 (m, H-6 and H-6′ of galactose residue), 4.03 – 3.96 (H-4′ ($J = 3.3$ Hz), H-5′ and H-6 of glucose residue ($J = 12.0$ Hz)), 3.90 – 3.74 (m, H-5), 3.756 (br t, $J = 9.0$ Hz, H-3), 3.73 – 3.66 (m, H-3′ and H-2) ppm. ^{13}C NMR (*t*BuOH = 31.3 ppm): $\delta = 163.6$, 144.6, 128.0, 118.3, 104.8, 101.0, 80.6, 76.8, 75.8, 74.7, 74.2, 72.6, 70.8, 70.1, 69.1, 61.7 ppm. FAB-MS (pos.): 566 $[M+1]^+$, 588 $[M+Na]^+$.

***p*NP 2,3,6-tri-*O*-sulfo-α-D-galactopyranoside, sodium salt (11)**: A mixture of *p*NP α-D-galactopyranoside (79.8 mg, 0.26 mmol) and SO_3NMe_3 (222 mg, 1.6 mmol) was dissolved in DMF at 50 °C. After 17 h, the reaction mixture was diluted with MeOH (5 mL) and concentrated in vacuo. The residue was then purified by sequential column chromatography with Sephadex LH-20, ODS C-18, and ion-exchange resin (Dowex Na^+) to afford **11** (128 mg, 78 %). $[\alpha]_D = +129°$ ($c = 0.6$ in H_2O). ^{1}H NMR (500 MHz, D_2O, *t*BuOH = 1.23 ppm): $\delta = 8.273$ and 7.350 (d, $J = 9.2$ Hz, *p*NP), 6.154 (d, $J = 3.7$ Hz, H-1), 4.877 (dd, $J = 3.1$ and 10.4 Hz, H-3), 4.82 – 4.74 (m, H-2), 4.513 (br d, $J = 2.8$ Hz, H-4), 4.17 – 4.13 (m, H-5), 4.191 (dd, $J = 4.3$ and 11.0 Hz, one of H-6), 4.137 (dd, $J = 8.1$ and 11.0 Hz, one of H-6) ppm. ^{13}C NMR (*t*BuOH = 31.3 ppm): $\delta = 162.9$, 144.4, 127.7, 119.1, 97.0 (C-1), 76.2, 73.6, 71.3, 69.1, 68.9 ppm. Elemental analysis: calcd. for $C_{12}H_{12}NNa_3O_{17}S_3 \cdot 4H_2O$: C, 21.21; H, 2.97; N, 2.06; S, 14.16; found: C, 21.13; H, 2.78; N, 2.06; S, 14.35.

***p*NP 2,6-di-*O*-sulfo-α-D-galactopyranoside, sodium salt (12) and *p*NP 6-*O*-sulfo-α-D-galactopyranoside, sodium salt (13)**: The typical procedure of enzymatic desulfonation for the preparation of **12** and **13** is as follows: A mixture of **11** (29.5 mg) and limpet sulfatase (8.77 mg) was dissolved in acetic acid/sodium acetate buffer (pH 6.8, 0.25 M; 0.8 mL) at 37 °C over a 141.5-h period. The reaction mixture was purified by sequential column chromatography with Sephadex LH-20, ODS C-18, and ion-exchange resin (Dowex Na^+) to give **12** (15.1 mg, 62 %), **13** (1.8 mg, 9 %), and recovered **11** (3.8 mg, 13 %). **12**: $[\alpha]_D = +147°$ ($c = 0.2$ in H_2O). ^{1}H NMR (500 MHz, D_2O, *t*BuOH = 1.23 ppm): $\delta = 8.262$ and 7.322 (d, $J = 9.3$ Hz, *p*NP), 6.114 (d, $J = 3.7$ Hz, H-1), 4.640 (dd, $J = 3.7$ and 10.3 Hz, H-2), 4.295 (m, H-4), 4.246 (dd, $J = 3.3$ and 10.3 Hz, H-3), 4.20 – 4.15 (m, H-5 and H-6), 4.119 (dd, $J = 8.0$ and 10.7 Hz, H-6) ppm. ^{13}C NMR (*t*BuOH = 31.3 ppm): $\delta = 163.1$, 144.4, 127.7, 119.1, 97.1(C-1), 76.4, 71.5, 70.6, 68.9, 68.8 ppm. FAB-MS (pos.): 506 $[M+1]^+$, 528 $[M+Na]^+$. Elemental analysis: calcd. for $C_{12}H_{13}NNa_2O_{14}S_2 \cdot 2H_2O$: C, 26.62; H, 3.16; N, 2.59; found: C, 26.91; H, 2.77; N, 2.46. **13**: $[\alpha]_D = +188°$ ($c = 0.53$ in H_2O). ^{1}H NMR (500 MHz, D_2O, *t*BuOH = 1.23 ppm): $\delta = 8.258$ and 7.301 (d, $J = 9.2$ Hz, *p*NP), 5.824 (d, $J = 3.7$ Hz, H-1), 4.24 – 4.20 (m, H-5), 4.175 (dd, $J = 4.0$ and 11.0 Hz, H-6), 4.029 (dd, $J = 3.7$ and 9.7 Hz, H-2), 4.14 – 4.06 (m, H-3, H-4, H-6) ppm. ^{13}C NMR (*t*BuOH = 31.3 ppm): $\delta = 163.1$, 144.0, 127.6, 118.7, 98.6(C-1), 71.4, 70.8, 70.4, 69.4, 68.8 ppm. FAB-MS (pos.): 426 $[M+Na]^+$. Elemental analyis: calcd. for $C_{12}H_{14}NNaO_{11}S \cdot 1H_2O$: C, 34.21; H, 3.83; N, 3.33; S, 7.61; found: C, 33.94; H, 3.85; N, 3.37; S, 7.44.

Analysis of the changes in the amounts of 11, 12, and 13 over the time course of the reaction by ^{1}H NMR spectroscopy: The reaction mixture was monitored by ^{1}H NMR spectroscopy of samples taken after approximately 12, 24, 45, 75, and 195 h. Abalone (0.55 mg), snail (0.81 mg), and limpet (0.71 mg) sulfatases were present in the mixture together with **11** (10 mg), under the conditions described above. Each solution sample (about 10 μL) was exchanged with D_2O (0.5 mL) and evaporated. This procedure was repeated at least three times. The residue was dissolved in D_2O (0.4 mL) with the internal standard (*t*BuOH) and measured by 500 MHz ^{1}H NMR (512 times). The ratio of the H-1 signals of **11**, **12**, and **13** was calculated from the integral curves.

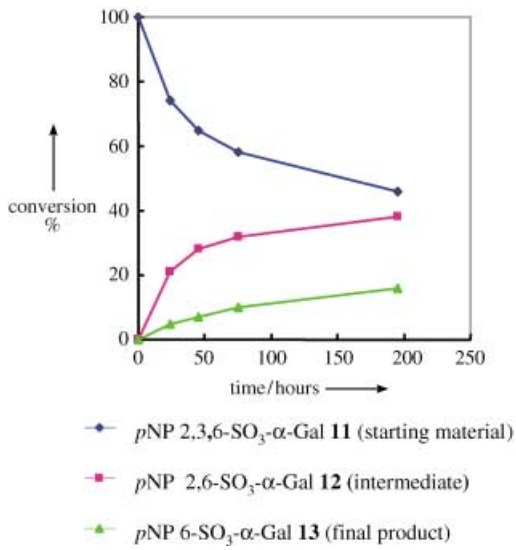

Figure 2. Time course of abalone sulfatase reactions with pNP 2,3,6-SO_3-α-D-Gal (**11**; *starting material, shown in blue) and* pNP 2,6-SO_3-α-Gal (**12**; *intermediate, shown in pink). The product,* pNP 6-SO_3-α-Gal (**13**), *is shown in green.*

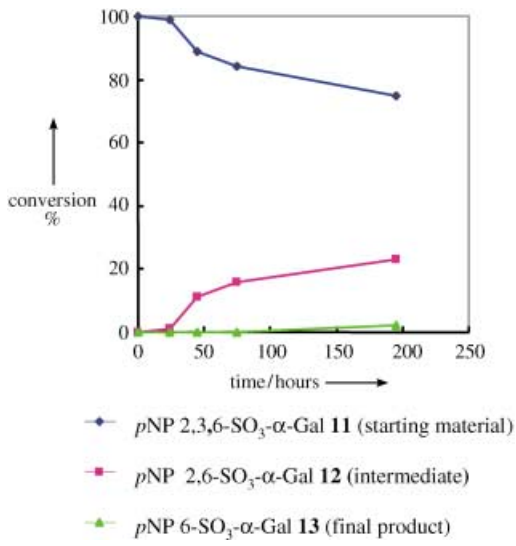

Figure 3. Time course of limpet sulfatase reactions with pNP 2,3,6-SO_3-α-D-Gal (**11**; *starting material, shown in blue) and* pNP 2,6-SO_3-α-Gal (**12**; *intermediate, shown in pink). The product,* pNP 6-SO_3-α-Gal (**13**), *is shown in green.*

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Snail sulfatase: Reaction time (hours) and yields of **11**(%), **12**(%), **13**(%): (0, 100, 0, 0), (12, 78, 22, 0), (24, 66, 34, 1), (45, 55, 41, 4), (75, 50, 44, 6), (195, 48, 42, 10). The time course is shown in Figure 1.

Abalone sulfatase: Reaction time (hours) and yields of **11**(%), **12**(%), **13**(%): (0, 100, 0, 0), (24, 74, 21, 5), (45, 65, 28, 7), (75, 58, 32, 10), (195, 46, 38, 16). A plot of the conversion of **11**, **12**, and **13** versus time is shown in Figure 2.

Limpet sulfatase: Reaction time (hours) and yields of **11**(%), **12**(%), **13**(%): (0, 100, 0, 0), (24, 99, 1, 0), (45, 89, 11, 0), (75, 84, 16, 0), (195, 75, 23, 2). The conversion of **11**, **12**, and **13** versus time is shown in Figure 3.

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[16] The starting material, *p*NP 2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-Gal, was synthesized by 2,3-di-*O*-benzoylation by treatment with BzCl and pyridine of *p*NP 4,6-*O*-benzylidene-β-D-Gal, which was readily available by selective 4,6-*O*-benzylidenation of *p*NP β-D-Gal.

[17] *p*NP 3,4-SO_3 Gal (**5**) was synthesized from *p*NP β-D-Gal as follows: 1) 3,4-*O*-isopropylidenation in acetone in the presence of ion-exchange resin (Dowex H^+), 2) 2,6-di-*O*-benzoylation with BzCl and pyridine, 3) de-*O*-isopropylidenation with CSA, 4) 3,4-di-*O*-sulfonation with 6.9 mol equiv SO_3NMe_3 in DMF, and 5) de-*O*-benzoylation with NaOMe in MeOH (17% after 5 steps).

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