A bovine glucuronidase for assembly of β-D-glucuronyl-(1–3)-6-*O*-sulfo-β-D-*gluco*- and *galacto*-pyranosyl linkages†

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Glucuronidase-catalyzed transglycosylation was examined by using 4-nitrophenyl β-D-glucuronide (D-GlcA-*O*-*p*NP) as the glycosyl donor; when *p*NP 6-*O*-sulfo-β-D-*gluco*- and D-*galacto*-pyranosides were used as the acceptors, a bovine enzyme was found to construct β-D-GlcA-(1–3)-linkages with the 6-*O*-sulfo-sugars in both a site- and β-selective way.

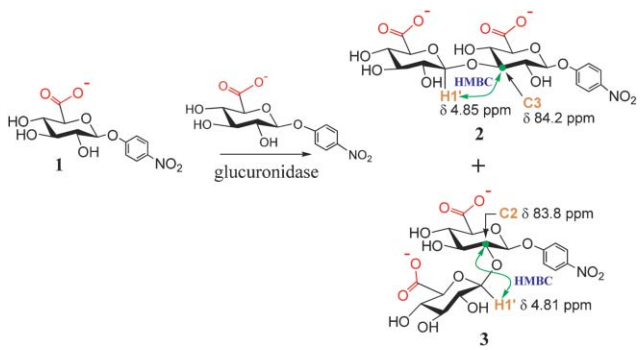
Glycosaminoglycans (GAGs) as represented by heparan, chondroitin, and dermatan sulfates, are *O*- and *N*-sulfated linear glycans found in proteoglycans. These glycans are made up of repeating disaccharides carrying alternating D-hexopyranosyluronic acids and D-hexosamine,[1] and are thought to play major roles in diverse biological events such as blood coagulation, immune systems, cell growth, viral and bacterial infections, and the inflammatory response. Recent studies have suggested that they may play a major role in *in vivo* development of bovine spongiform encephalopathy (BSE) and fatal Creutzfeldt-Jakob disease.[2] Obviously, they and their key fragments have significant potential applications in "carbohydrate-based" biomaterials and medicinal reagents.

The GAGs and their fragments used for biological studies are often heterogeneous, allowing variation in the degree and location of the *O*- and *N*-sulfate groups. To obtain a clearer insight into biological activity and structure relations, the use of such GAG homologues or mimics having a defined chemical structure is essential. Hence, much synthetic effort has focused on these GAG homologues and analogues. Since Sinaÿ *et al.*[3a] described the first synthesis of heparin pentasaccharides, many chemical approaches have been reported.[3b–3d] In recent studies, chemo-enzymatic approaches using sulfotransferases in combination with glycosyl transferase[4] and hydrolytic enzymes such as *endo*-β-D-glycosidases[5] have been explored. Kobayashi and coworkers[6] utilized *exo*-β-D-glycosidases for the oligomerization of GlcA-β(1–3)-GlcNAc-1-oxazolines to obtain a series of GAG homologues.

In our ongoing series of studies on the chemo-enzymatic synthesis and biological applications of sulfo-sugars,[7] our attention has also been directed to GAG mimics with defined *O*-sulfate structures. In preceding papers, we described the synthetic utility of sulfatases[7] and *N*-acetylhexosaminidase.[8] In this communication, we show that a bovine glucuronidase (EC 3.2.1.31) can be used for assembling β-D-glucuronyl-(1–3)-6-*O*-sulfo-β-D-glycopyranosyl linkages. More specifically, the enzyme catalyzes transglycosylation from 4-nitrophenyl (*p*NP) β-D-glucuronate (GlcA-*O*-*p*NP **1**) to the 3-OH position of 6-*O*-sulfo-D-*gluco*- and D-*galacto*-pyranosides.

We examined two glucuronidases from *Escherichia coli* and bovine liver (Sigma) as potential glycosyl enzymes, which have rarely been evaluated for synthetic purpose.[9] In the present study, as the acceptors, we used *p*NP 6-sulfo-β-D-g*luco*- and D-*galacto*-pyranosides having either an *O*- or *S*-glycosidic linkage, *viz.* 6-*O*-sulfo-Glc-*O*-*p*NP **4**, 6-*O*-sulfo-Glc-*S*-*p*NP **5**, 6-*O*-sulfo-Gal-*O*-*p*NP **10**, and 6-*O*-sulfo-Gal-*S*-*p*NP **11**. The *S*-linkage is resistant to enzymatic hydrolysis, and thus, may be useful for extensional enzymatic reactions and biological applications. These substrates (**4**, **5**, **10** and **11**) were prepared according to a previously reported method *via* chemical 6-*O*-sulfation reactions.[7] The donor **1** at *ca.* 150–260 mM was applied to the acceptor at *ca.* 300–430 mM in aqueous buffers [100 mM phosphate buffer (pH 7.4) and 100 mM acetate buffer (pH 6.0)].

As an initial approach, we examined a self-condensed transglycosylation of **1**, where this sugar was applied at the high concentration of 1500 mM (Scheme 1). The results summarized in Table 1 show that the *E. coli* glucuronidase produced only a trace amount (less than 1.5%) of disaccharide products (**2** + **3**), while giving a mixture of *p*NP and GlcA as major products as the result of hydrolyzing the *p*NP *O*-glycosyl linkage (Entries 1 and 2). The use of the two different buffers had no substantial effect in this case. In contrast, the bovine liver glucuronidase was found to transfer the β-GlcA residue in **1** to produce a mixture of disaccharides **2** and **3** in *ca.* a 2 : 1 to 4 : 1 ratio (LC-MS analysis) (Entries 3 and 4). Further, the transglycosylation reaction conducted in acetate buffer (Entry 4) tended to exhibit a higher



Scheme 1

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Table 1 Regioselectivity of glucuronidase-catalyzed transglycosylation[a]

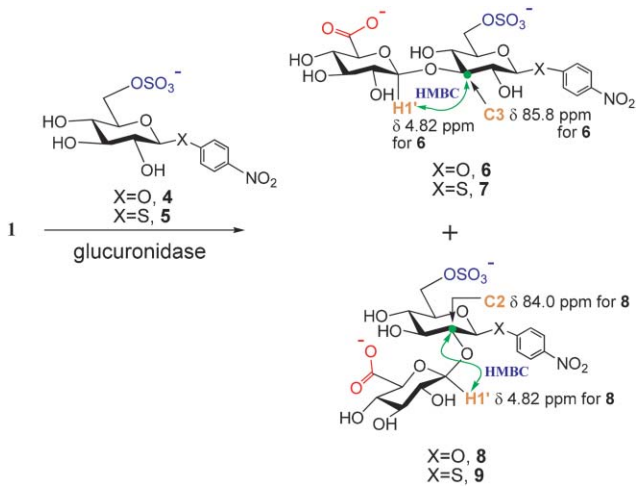
Entry	Substrate	Enzyme	Time (h)	Products (conversion, %)[d]
1	**1**	*E. coli*[b]	15	*trace*[e]
2	**1**	*E. coli*[c]	24	*trace*[e]
3	**1**	bovine[b]	24	**2** (17) + **3** (9)
4	**1**	bovine[c]	24	**2** (42) + **3** (11)
5	**4**	bovine[c]	24	**6** (35) + **8** (3)
6	**5**	bovine[c]	24	**7** (28) + **9** (6)
7	**10**	bovine[c]	24	**12** (43)
8	**11**	bovine[c]	24	**13** (40)

[a] Commercially available glucuronidases from *E. coli* and bovine liver were used. Typical procedure is shown in footnote. [b] Reactions in 0.1 M phosphate buffer (pH 7.4). [c] Reactions in 0.1 M acetate buffer (pH 6.0). [d] LC-MS analysis based on an amount of consumed donor. [e] The dimeric products (**2** + **3**) were detectable in a trace amount by LC-MS.

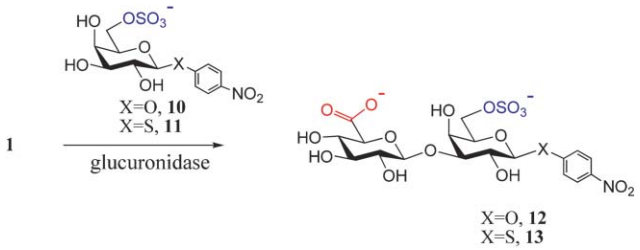
site-selectivity and reaction yield than that in phosphate buffer (Entry 3).

The two products were separable with a preparative HPLC column (Synergi Fusion, Phenomenex) and determined by MS to have a molecular weight identical to that of dimeric GlcA-GlcA-*O*-*p*NP. Using HMBC and HMQC 2D-NMR techniques, the major product **2**† was assigned as GlcA-β-(1–3)-GlcA-*O*-*p*NP having a correlation between $\delta_{H1'}$ 4.85 ppm (J = 8.0 Hz) and δ_{C3} 84.2 ppm. The minor product **3**† was GlcA-β-(1–2)-GlcA-*O*-*p*NP with a correlation between $\delta_{H1'}$ 4.81 ppm (J = 7.8 Hz) and δ_{C2} 83.8 ppm.

Next, we used *p*NP 6-*O*-sulfo-sugars (Entries 5 to 8) as the acceptor sugars. The result in Entry 5 shows that the bovine liver glucuronidase transferred the β-GlcA to **4**, yielding a mixture of **6** and **8** in the ratio of 11 : 1 (Scheme 2)‡. These products, with identical molecular weights [*m*/*z* 556.2 $[M + H]^-$], were assigned as the disaccharides possessing GlcA-β(1–3)- and β(1–2)-linkages to the 6-sulfo-Glc-*O*-*p*NP, respectively. A long range correlation was observed between $\delta_{H1'}$ 4.82 ppm (J = 7.7 Hz) and δ_{C3} 85.8 ppm, and also between $\delta_{C1'}$ 105.0 ppm and δ_{H3} 3.92 ppm (J = 9.0 and 9.0 Hz) in the major product **6**,† indicating the presence of a β(1–3)-linkage. In a similar way, the minor product **8**† was determined to have a β(1–2)-linkage. Entry 6 shows that the enzyme also utilized the *p*NP *S*-linked glycoside **5** to afford a mixture of β-D-glucuronyl-(1–3)- and β(1–2)-linked disaccharides (**7** and **9**),†



Scheme 2



Scheme 3

which were separated and identified in the same way as described above.

When the 6-sulfo-D-galactosides (**10** and **11**) were used as the acceptors (Entries 7 and 8), only β(1–3)-linked disaccharides (**12** and **13**)† (Scheme 3) were derived in a site- and β-selective way. The *O*- and *S*-glycosidic linkages were not substantially different with respect to their regioselectivities. Judging from the results in Entries 3 to 6, it is concluded that this enzyme can most likely discriminate differences in the stereochemistries of D-*galacto*- and D-*gluco*-sugars.

In summary, we have described that bovine glucuronidase catalyzes *β-GlcA-transfer* reactions between *p*NP β-D-glucuronate, which is used as the donor, and *p*NP 6-sulfo-β-D-glycopyranosides, which are used as the acceptor sugars. The enzyme produces unnatural disaccharides made up of β-D-GlcA-(1–3)-6-sulfo-β-D-Glc or β-D-GlcA-(1–3)-6-sulfo-β-D-Gal linkages as the major products. The derived disaccharides are hetero-anionic sugars carrying both carboxylate and sulfate groups on one molecule. (All the data for **2** ∼ **13** are given in the supporting electronic information.†)

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Notes and references

‡ Typical procedure for enzymatic transglycosylation. *Synthesis of **6** and **8***. A mixture of **1** (sodium salt, 121 mg), **4** (207 mg) and bovine liver glucuronidase (Sigma, 5000U) dissolved in 0.1 M 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, and then the mixture was purified with an ODS HPLC column (Synergi Fusion, Phenomenex. H_2O–MeOH = 7 : 3 containing 0.05% TFA). After work-up and purification by HPLC, each of the two products was obtained [**6** (32 mg, 14% as a sodium salt) and **8** (10 mg, 5% as a Na salt)]. The ratio of the isolated yields after purification gives no actual information on the ratio of reaction products. The glucuronidase from bovine liver (Sigma) might have been contaminated by a trace amount of glycosidases. However, neither of glycosyl acceptor **4** nor **10** was hydrolyzed during the enzymatic transglycosylation reactions.

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