Supplementary Material for Chemical Communications

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

Hirotaka Uzawa,[a] Takehiro Nagatsuka,[a] Hideo Hiramatsu[b] and Yoshihiro Nishida[c]

[a] *Research Center of Advanced Bionics, National Institute of Advanced Industrial Science and Technology (AIST), Central 5, 1-1-1 Higashi, Tsukuba 305-8565, Japan*

[b] *College of Industrial Technology, Nihon University, 1-2-2 Izumi-cho, Narashino, Chiba 275, Japan*

[c] *Department of Molecular Design and Engineering, Graduate School of Engineering, Nagoya University, Chikusa, Nagoya 464-8603, Japan*

Supporting information

Enzymatic synthesis of dimeric products, β-D-GlcA-(1→3)-β-D-GlcA-*O*-*p*NP (**2**) and β-D-GlcA-(1→2)-β-D-GlcA-*O*-*p*NP (**3**)

Commericially available *p*-nitrophenyl glucuronic acid (*p*NP GlcA) from Sigma was treated with $NaHCO_3$ to give *p*NP glucuronate **1** quantatively. A mixture of **1** (50 mg, 0.15 mmol) and bovine liver glucuronidase (Sigma, 5000U) dissolved in 0.1M AcONa-AcOH buffer (pH 6.0, 100 μL) were incubated at 35°C for 24h. The reaction mixture was boiled for 5 min to stop the enzyme reaction, then the mixture was purified with an ODS HPLC column (Synergi Fusion, Phenomenex. H_2O-MeOH = 7:3 containing 0.05% TFA) to give **2** (10 mg) and **3** (7 mg), respectively.

Compound **2**: $[\alpha]_D^{25} = -45°$ (c 0.87, H_2O); ^{1}H NMR (600 MHz, D_2O, *t*-BuOH 1.23): δ 8.26 (d, *J* = 9.5 Hz, *p*NP), 7.24 (d, *J* = 9.5 Hz, *p*NP), 5.29 (d, *J* = 8.0 Hz, H-1), 4.85 (d, *J* = 8.0 Hz, H-1'), 3.97 (d, *J* = 9.9 Hz, H-5), 3.93 (t, *J* = 9.0 Hz, H-3), 3.87 (t, *J* =6.8 Hz, H-2), 3.75-3.70 (overlap, H-4, H-4'), 3.54-3.54 (m, H-3', 5'), 3.41 (t, 8.6 Hz, H-2'); ^{13}C NMR (150 MHz, D_2O, *t*-BuOH 31.2), δ 163.4 (Ar), 144.3 (Ar), 127.7 (Ar), 118.2 (Ar), 103.9 (C-1), 100.8 (C-1'), 84.2 (C-3), 78.1 (C-4), 77.4 (C-5), 77.0 (C-5'), 74.9 (C-2'), 74.3 (C-2), 73.4 (C-3'), 71.7 (C-4). ESI-MS; 490.2 $[M+H]^-$.

Compound **3**: $[\alpha]_D^{25} = -35°$ (c 0.88, H_2O); ^{1}H NMR (600 MHz, D_2O, *t*-BuOH 1.23): δ 8.22 (d, *J* = 9.0 Hz, *p*NP), 7.19 (d, *J* = 9.0 Hz, *p*NP), 5.77 (d, *J* = 6.6 Hz, H-1), 4.81 (d, *J* = 7.8 Hz, H-1'), 4.24 (d, *J* = 9.6 Hz, H-5), 3.89 (t, *J* = 6.8 Hz, H-2), 3.86-3.84 (m, H-3, 5'), 3.76 (t, J = 9.3 Hz, H-4), 3.55 (t, *J* = 9.3 Hz, H-3'), 3.44 (t, *J* = 9.6 Hz, H-4'), 3.37 (d, *J* = 7.8 Hz, H-2'); ^{13}C NMR (150 MHz, D_2O, *t*-BuOH 31.2), δ 162.7 (Ar), 144.2 (Ar), 127.9 (Ar), 117.3 (Ar), 104.4 (C-1), 99.4 (C-1'), 83.8 (C-2), 76.5 (C-3', 5'), 76.0 (C-5), 74.8 (C-2'), 72.9 (C-4'), 72.1 (C-3), 71.4 (C-4). ESI-MS; 490.2 $[M+H]^-$.

Chemical synthesis of a glycosyl acceptor, 6-*O*-sulfo-Glc-*O*-*p*NP (**4**)

A mixture of *p*NP β-D-glucopyranoside from Sigma (1 g, 3.32 mmol) and SO_3-NMe_3 from Sigma (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 sequential column chromatography with Sephadex LH-20, ODS C-18 and ion exchange resin (Dowex Na^+) to afford **4** (994 mg, 68 %).

$[\alpha]_D^{25}$ = –88.9 (c 1.79, H_2O); ^{1}H NMR (400 MHz, D_2O): δ 8.26 (d, 2H, *J* = 9.2 Hz, Ar), 7.25 (d, 2H, *J* = 9.2 Hz, Ar), 5.27 (d, 1H, *J* = 8.0 Hz, H-1), 4.38 (dd, 1H, *J* = 2.0, 11.2 Hz, H-6a), 4.23 (dd, 1H, *J* = 5.6, 11.2 Hz, H-6b), 3.94 (ddd, 1H, *J* = 2.0, 5.6, 9.6 Hz, H-5), 3.66-355 (m, 3H, H-2, 3, 4), 2.88 (s, 9H, $N(Me)_3$); ^{13}C NMR(150 MHz, D_2O, *t*BuOH 31.2) δ 163.2 (Ar), 144.1 (Ar), 127.7 (Ar), 118.1 (Ar), 101.0 (C-1), 76.8 (C-3), 75.7 (C-5), 74.2 (C-2), 70.6 (C-4), 68.4 (C-6), 46.3 (NMe_3). ESI-MS; 380.1 $[M]^-$.

Chemical synthesis of a glycosyl acceptor, 6-*O*-sulfo-Glc-*S*-*p*NP (**5**)

A mixture of *p*NP 1-thio-β-D-glucopyranoside from Sigma (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 (400 MHz, D_2O): δ 8.22 (d, 2H, *J* = 9.2 Hz, Ar), 7.69 (d, 2H, *J* = 9.2 Hz, Ar), 5.08 (d, 1H, *J* = 9.6 Hz, H-1), 4.40 (dd, 1H, *J* = 2.0, 11.6 Hz, H-6a), 4.21 (dd, 1H, *J* = 6.0, 11.6 Hz, H-6b), 3.87 (ddd, 1H, *J* = 2.0, 6.0, 9.6 Hz, H-5), 3.61-3.46 (m. 3H, H-2, 3, 4), 2.88 (s, 9H, $N(Me)_3$); ^{13}C NMR (150 MHz, D_2O, *t*BuOH 31.2) δ 147.6 (Ar), 144.9 (Ar), 130.6 (Ar), 125.7 (Ar), 87.2 (C-1), 79.2 (C-5), 78.6 (C-3), 73.2 (C-2), 70.6 (C-4), 68.7 (C-6), 46.3 (NMe_3). ESI-MS; 396.1 $[M]^-$.

Chemical synthesis of a glycosyl acceptor, 6-*O*-sulfo-Gal-*O*-*p*NP (**10**)

A mixture of *p*NP β-D-galactopyranoside from Sigma (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 (400 MHz, D_2O): δ 8.26 (d, 2H, *J* = 9.2 Hz, Ar), 7.26 (d, 2H, *J* = 9.2 Hz, Ar), 5.21 (d, 1H, *J* = 7.2 Hz, H-1), 4.26-4.19 (m, 3H, H-6a, 6b, 5), 4.07 (brd, 1H, *J* = 2.8 Hz, H-4), 3.87 (dd, 1H, *J* = 7.2, 10.0 Hz, H-2), 3.81 (dd, 1H, *J* = 3.6, 10.0 Hz, H-3), 2.88 (s, 9H, $N(Me)_3$); ^{13}C NMR (150 MHz, D_2O, *t*BuOH 31.2) δ 163.4 (Ar), 144.2 (Ar), 127.7 (Ar), 118.1 (Ar), 101.5 (C-1), 74.8 (C-5), 73.8 (C-3), 71.3 (C-2),

69.8 (C-4), 68.6 (C-6), 46.3 (NMe_3). ESI-MS; 380.1 $[M]^-$.

Chemical synthesis of a glycosyl acceptor, 6-*O*-sulfo-Gal-*S*-*p*NP (**11**)

A mixture of *p*NP 1-thio-β-D-galactopyranoside from Sigma (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 8.20 (d, 2H, *J* = 9.2 Hz, Ar), 7.66 (d, 2H, *J* = 9.2 Hz, Ar), 5.04 (d, 1H, *J* = 9.0 Hz, H-1), 4.22 (dd, 1H, *J* = 4.8, 11.4 Hz, H-6a), 4.18 (t, 1H, *J* = 9.3 Hz, H-5) 4.13 (dd, 1H, *J* = 4.8, 11.4 Hz, H-6b), 4.08 (d, 1H, *J* = 2.2 Hz, H-4), 3.78-3.71 (m, 2H, H-2, 3), 2.88 (s, 9H, $N(Me)_3$); ^{13}C NMR (150 MHz, D_2O, *t*BuOH 31.2) δ 147.4 (Ar), 145.6 (Ar), 130.1 (Ar), 125.8 (Ar), 87.6 (C-1), 78.2 (C-5), 75.3 (C-3), 70.6 (C-2), 70.0 (C-4), 69.0 (C-6), 46.3 (NMe_3). ESI-MS; 396.1 $[M]^-$.

Enzymatic synthesis of β-D-GlcA-(1→3)-β-D-6-*O*-sulfo-Glc-*O*-*p*NP (**6**) and β-D-GlcA-(1→2)-β-D-6-*O*-sulfo-Glc-*O*-*p*NP (**8**)

A mixture of **1** (121mg, 0.36 mmol), **4** (207mg, 0.47 mmol) and bovine liver glucuronidase (Sigma, 5000U) dissolved in 0.1M AcONa-AcOH buffer (pH 6.0, 1.4mL) were incubated at 35°C for 24h. The reaction mixture was boiled for 5 min to stop the enzyme reaction, then the mixture was purified with an ODS HPLC column (Synergi Fusion, Phenomenex. H_2O-MeOH = 7:3 containing 0.05% TFA) to give **6** (32mg) and **8** (10mg) based on the consumed donor, respectively.

Compound **6**: $[\alpha]_D^{25}$ = –58° (*c* 0.48, H_2O); ^{1}H NMR (600 MHz, D_2O, *t*-BuOH 1.23) δ 8.265 (d, 2H, *J* = 9.1 Hz, Ar), 7.255 (d, 2H, *J* = 9.1 Hz, Ar), 5.300 (d, 1H, *J* = 7.7 Hz, H-1), 4.820 (d, 1H, *J* = 7.7 Hz, H-1'), 4.392 (dd, 1H, *J* =2.2, 11.4 Hz, H-6a), 4.227 (dd, 1H, *J* = 2.2, 11.4 Hz, H-6b), 3.970 (ddd, 1H, *J* = 1.8, 5.5, 9.9 Hz, H-5), 3.916 (t, 1H, *J* = 9.0 Hz, H-3), 3.854 (t, 1H, *J* = 9.0 Hz, H-2), 3.743 (d, 1H, *J* = 9.5 Hz, H-5'), 3.673 (dd, 1H, *J* = 8.8, 9.9 Hz, H-4), 3.558-3.512 (m, 2H, H-3', H-4'), 3.425-3.400 (m, 1H, H-2'); ^{13}C NMR (150 MHz, D_2O, *t*-BuOH = 31.2) δ 178.3 (C-6'), 164.2 (Ar), 145.2(Ar), 128.6 (Ar), 119.0 (Ar), 105.0 (C-1'), 101.7 (C-1), 85.8 (C-3), 78.2 (C-5'), 77.9 (C-3'), 76.3 (C-5), 75.8 (C-2'), 75.1 (C-2), 74.3 (C-4'), 70.2 (C-4), 69.4 (C-6). ESI-MS; 556.2 $[M+H]^-$; 578.2 $[M+Na]^-$.

Compound **8**: $[\alpha]_D^{25}$ = –34° (c 0.65, H_2O); ^{1}H NMR (600 MHz, D_2O, *t*-BuOH = 1.23) δ 8.243 (d, 2H, *J* = 9.2 Hz, Ar), 7.204 (d, 2H, *J* = 9.2 Hz, Ar), 5.505 (d, 1H, *J* = 7.3 Hz, H-1), 4.821 (d, 1H, *J* = 8.0 Hz, H-1'), 4.356 (dd, 1H, *J* = 2.2, 11.4 Hz, H-6a), 4.217 (dd, 1H, *J* = 5.5, 11.4 Hz, H-6b), 3.944 (ddd, 1H, *J* =1.8, 5.2, 9.9 Hz, H-5), 3.876-3.804 (m, 2H, H-2,

5'), 3.819 (t, 1H, *J* = 9.2 Hz, H-3), 3.633 (t, 1H, *J* = 9.5 Hz, H-4), 3.555 (t, 1H, *J* = 9.5 Hz, H-3'), 3.436 (t, 1H, *J* = 9.3 Hz, H-4'), 3.360-3.313 (overlap, H-2'); ^{13}C NMR (100 MHz, D_2O, *t*-BuOH = 31.2) δ 162.8 (Ar), 144.1 (Ar), 128.0 (Ar), 117.3 (Ar), 104.4 (C-1'), 99.6 (C-1), 84.0 (C-2), 76.5 (C-4'), 76.3 (C-3), 75.3 (C-5), 74.8 (C-3'), 72.9 (C-5'), 71.4 (C-2'), 70.1 (C-4), 68.3 (C-6). ESI-MS; 556.2 $[M+H]^-$.

Enzymatic synthesis of β-D-GlcA-(1→3)-β-D-6-*O*-sulfo-Glc-*S*-*p*NP (**7**) and β-D-GlcA-(1→2)-β-D-6-*O*-sulfo-Glc-*S*-*p*NP (**9**)

A mixture of **1** (162mg, 0.48 mmol), **5** (335mg, 0.73 mmol) and bovine liver glucuronidase (Sigma, 5000U) dissolved in 0.1M AcONa-AcOH buffer (pH 6.0, 2.0 mL) were incubated at 35°C for 24h. The reaction mixture was boiled for 5 min to stop the enzyme reaction, then the mixture was purified with an ODS HPLC column (Synergi Fusion, Phenomenex. H_2O-MeOH = 7:3 containing 0.05% TFA) to give **7** (15 mg) and **9** (10 mg), respectively.

Compound **7**: $[\alpha]_D^{25} = -51$ (c 0.27, H_2O) ; ^{1}H NMR (600 MHz, D_2O, *t*-BuOH = 1.23) δ 8.148 (d, 2H, *J* = 9.1 Hz, Ar), 7.633 (d, 2H, *J* = 9.1 Hz, Ar), 5.034 (d, 1H, *J* = 9.9 Hz, H-1), 4.800 (d, 1H, *J* = 7.7 Hz, H-1'), 4.391 (dd, 1H, *J* = 1.9, 11.4 Hz, H-6a), 4.198 (dd, 1H, *J* = 6.2, 11.4 Hz, H-6b), 3.873-3.843 (m, 1H, H-5), 3.848 (t, 1H, *J* = 9.0 Hz, H-3), 3.742 (d, 1H, *J* = 7.3 Hz, H-5'), 3.684 (dd, 1H, *J* = 9.2, 9.9 Hz, H-4), 3.566-3.514 (m, 2H, H4', 3'), 3.400 (t, 1H, *J* = 8.6 Hz, H-2'); ^{13}C NMR (150 MHz, D_2O, *t*BuOH = 31.2) δ 146.4 (Ar), 142.7 (Ar), 131.3 (Ar), 125.7 (Ar), 104.0 (C-1'), 87.1 (C-1), 86.9 (C-3), 79.0 (C-5), 76.7 (C-3'), 76.0 (C-5'), 74.6 (C-2'), 72.7 (C-2, C-4'), 69.2 (C-6). ESI-MS; 572.2 $[M+H]^-$; 594.1 $[M+Na]^-$.

Compound **9**: $[\alpha]_D^{25} = -9$ (c 0.65, H_2O); ^{1}H NMR (600 MHz, D_2O, *t*-BuOH = 1.23) δ 8.242 (d, 2H, *J* = 9.1 Hz, Ar), 7.214 (d, 2H, *J* = 9.2 Hz, Ar), 5.500 (d, 1H, *J* = 7.3 Hz, H-1), 4.798 (d, 1H, *J* = 7.7 Hz, H-1'), 4.361 (dd, 1H, *J* = 1.8, 11.4 Hz, H-6a), 4.220 (dd, 1H, *J* = 5.5, 11.4Hz, H-6b), 3.946 (ddd, 1H, *J* = 1.8, 5.1, 9.9 Hz, H-5), 3.870 (dd, 1H, *J* = 7.7, 9.2 Hz, H-2), 3.826 (t, 1H, *J* = 9.0 Hz, H-3), 3.713 (d, 1H, *J* = 9.5 Hz, H-5'), 3.628 (t, 1H, *J* = 9.5 Hz, H-4), 3.534 (t, 1H, *J* = 9.3 Hz, H-3'), 3.419 (t, 1H, *J* = 9.3 Hz, H-4'), 3.362-3.325 (m, 1H, H-2'); ^{13}C NMR (150 MHz, D_2O, *t*-BuOH = 31.2) δ 164.2 (Ar), 143.1(Ar), 131.0 (Ar), 125.6 (Ar), 104.5 (C-1'), 84.9 (C-1), 79.2 (C-2), 78.8 (C-5), 76.7 (C-3'), 76.3 (C-5'), 74.7 (C-2'), 72.8 (C-4'), 71.2, 70.5 (C-3, 4), 68.7 (C-6). ESI-MS; 572.2 $[M+H]^-$, 594.2 $[M+Na]^-$.

Enzymatic synthesis of β-D-GlcA-(1→3)-β-D-6-*O*-sulfo-Gal-*O*-*p*NP (**12**)

A mixture of **1** (110 mg, 0.33 mmol), **10** (287 mg, 0.65 mmol) and bovine liver

glucuronidase (Sigma, 4000U) dissolved in 0.1M AcONa-AcOH buffer (pH 6.0, 1.5 mL) were incubated at 35°C for 24h. The reaction mixture was then processed in the same way described for compounds **6** and **8** to give **12** (59 mg).
Compound **12**: $[\alpha]_D^{25} = -61.8°$ (c 1.60, H_2O); ^{1}H NMR (600 MHz, D_2O, *t*-BuOH 1.23): δ 8.253(d, *J* = 9.1 Hz, *p*NP), 7.260 (d, *J* = 9.2 Hz, *p*NP), 5.251 (d, *J* = 7.7 Hz, H-1), 4.725 (d, *J* = 7.7 Hz, H-1'), 4.331(d, *J* = 3.3 Hz, H-4), 4.275 (dd, *J* = 9.5 Hz, H-6a), 4.223 - 4.178 (m, H-6b, H-5), 4.025 (dd, *J* = 7.7, 9.9 Hz, H-2), 3.965 (dd, *J* = 3.3, 9.9 Hz, H-3), 3.744 (d, *J* = 9.5 Hz, H-5'), 3.553-3.506 (m, H-3', H-4'), 3.444 (t, *J* = 8.6 Hz, H-2'); ^{13}C NMR (100 MHz, *t*-BuOH 31.2), δ 177.5 (C-6'), 163.3 (Ar), 144.2 (Ar), 127.7(Ar), 118.1 (Ar), 105.3 (C-1'), 101.2 (C-1), 83.5 (C-3), 77.8 (C-5'), 76.9 (C-4'), 74.8 (C-5), 74.7 (C-2'), 73.4 (C-3'), 71.0 (C-2), 69.4 (C-4), 69.1 (C-6). ESI-MS; 556.2 $[M+H]^-$.

Enzymatic synthesis of β-D-GlcA-(1→3)-β-D-6-*O*-sulfo-Gal-*S*-*p*NP (**13**)
A mixture of **1** (100 mg, 0.30 mmol), **11** (276 mg, 0.61 mmol) and bovine liver glucuronidase (Sigma, 5000U) dissolved in 0.1M AcONa-AcOH buffer (pH 6.0, 2.0 mL) were incubated at 35°C for 24h. The reaction mixture was then processed in the same way described for compounds **6** and **8** to give **13** (25 mg).
Compound **13**: $[\alpha]_D^{25} = -78°$ (c 0.78, H_2O); ^{1}H NMR (600 MHz, CD_3OD, *t*-BuOH 1.40): δ 8.360 (d, *J* = 9.2 Hz, *p*NP), 7.912 (d, *J* = 9.2 Hz, *p*NP), 5.082 (d, *J* = 9.9 Hz, H-1), 4.778 (d, *J* = 7.7 Hz, H-1'), 4.438 (brd, *J* = 2.6 Hz, H-4), 4.408 (d, *J* = 5.5Hz, H-6a, 6b), 4.239 (t, *J* = 5.7 Hz, H-5), 4.058 (t, *J* = 9.5 Hz, H-2), 3.942 (dd, *J* = 2.8, 12.1 Hz, H-3), 3.813 (d, *J* = 7.3 Hz, H-5'), 3.473-3.409 (m, H-4', H-3'), 3.528-3.484 (m, H-2'); ^{13}C NMR (100 MHz, CD_3OH 49.0), δ 147.2 (Ar), 146.8 (Ar), 129.9 (Ar), 124.9 (Ar), 105.5 (C-1'), 87.5 (C-1), 86.1 (C-3), 78.3 (C-5), 77.5 (C-4'), 75.2 (C-3'), 73.6 (C-2'), 69.8 (C-3'), 69.6 (C-2), 69.4 (C-4) 68.9 (C-6). ESI-MS; 572.2 $[M+H]^-$, 594.2 $[M+Na]^-$.