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

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Supporting information

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

Commericially available p-nitrophenyl glucuronic acid (pNP GlcA) from Sigma was treated with NaHCO₃ to give pNP glucuronate 1 quantitatively. 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. H₂O-MeOH = 7:3 containing 0.05% TFA) to give 2 (10 mg) and 3 (7 mg), respectively.

Compound 2; [α]D²⁵ = −45° (c 0.87, H₂O); ¹H NMR (600 MHz, D₂O, t-BuOH 1.23): δ 8.26 (d, J = 9.5 Hz, pNP), 7.24 (d, J = 9.5 Hz, pNP), 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'); ¹³C NMR (150 MHz, D₂O, t-BuOH 31.2), δ 163.4 (Ar), 144.3 (Ar), 127.7 (Ar), 118.2 (Ar), 104.4 (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; [α]D²⁵ = −35° (c 0.88, H₂O); ¹H NMR (600 MHz, D₂O, t-BuOH 1.23): δ 8.22 (d, J = 9.0 Hz, pNP), 7.19 (d, J = 9.0 Hz, pNP), 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'); ¹³C NMR (150 MHz, D₂O, t-BuOH 31.2), δ 162.7 (Ar), 144.3 (Ar), 127.7 (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-pNP (4)
A mixture of pNP 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$_2$O); $^1$H NMR (400 MHz, D$_2$O): 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), 3.66-3.55 (m, 3H, H-2, 3, 4), 2.88 (s, 9H, N(Me)$_3$); $^{13}$C NMR (150 MHz, D$_2$O, tBuOH 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-pNP (5)
A mixture of pNP 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$_2$O); $^1$H NMR (400 MHz, D$_2$O): 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$_2$O, tBuOH 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-pNP (10)
A mixture of pNP 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$_2$O); $^1$H NMR (400 MHz, D$_2$O): 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$_2$O, tBuOH 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), 70.6 (C-4), 68.7 (C-6), 46.3 (NMe$_3$).
Chemical synthesis of a glycosyl acceptor, 6-O-sulfo-Gal-S-pNP (11)
A mixture of pNP 1-thio-β-D-galactopyranoside from Sigma (300 mg, 0.95 mmol) and SO3-NMe3 (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 %).

[α]D25 = −86.7 (c 1.95, H2O); 1H NMR (600 MHz, D2O): 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); 13C NMR (150 MHz, D2O, tBuOH 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 (NMe3). ESI-MS; 396.1 [M]-.

Enzymatic synthesis of β-D-GlcA-(1→3)-β-D-6-O-sulfo-Glc-O-pNP (6) and β-D-GlcA-(1→2)-β-D-6-O-sulfo-Glc-O-pNP (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. H2O-MeOH = 7:3 containing 0.05% TFA ) to give 6 (32mg) and 8 (10mg) based on the consumed donor, respectively.

Compound 6: [α]D25 = −58° (c 0.48, H2O); 1H NMR (600 MHz, D2O, 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'); 13C NMR (150 MHz, D2O, 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: [α]D25 = −34° (c 0.65, H2O); 1H NMR (600 MHz, D2O, 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,
Enzymatic synthesis of $\beta$-D-GlcA-(1→3)-$\beta$-D-6-O-sulfo-Glc-S-pNP (7) and $\beta$-D-GlcA-(1→2)-$\beta$-D-6-O-sulfo-Glc-S-pNP (9)

A mixture of 1 (162 mg, 0.48 mmol), 5 (335 mg, 0.73 mmol) and bovine liver glucuronidase (Sigma, 5000 U) dissolved in 0.1 M AcONa-AcOH buffer (pH 6.0, 2.0 mL) were incubated at 35°C for 24 h. 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$_2$O-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$_2$O); $^1$H NMR (600 MHz, D$_2$O, t-BuOH = 1.23) $\delta$ 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), 3.870 (dd, 1H, $J$ = 1.8, 5.1 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$_2$O, t-BuOH = 31.2) $\delta$ 146.4 (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'), 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]; 594.1 [M+Na].

Compound 9: $[\alpha]_{D}^{25}$ = -9 (c 0.65, H$_2$O); $^1$H NMR (600 MHz, D$_2$O, t-BuOH = 1.23) $\delta$ 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, 11.4 Hz, 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$_2$O, t-BuOH = 31.2) $\delta$ 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'), 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 $\beta$-D-GlcA-(1→3)-$\beta$-D-6-O-sulfo-Gal-O-pNP (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: [α]_25^oD = −61.8° (c 1.60, H2O); \(^1\)H NMR (600 MHz, D_2O, t-BuOH 1.23): \(\delta \) 8.253 (d, \(J = 9.1 \text{ Hz, pNP}\)), 7.260 (d, \(J = 9.2 \text{ Hz, pNP}\)), 5.251 (d, \(J = 7.7 \text{ Hz, H-1}\)), 4.725 (d, \(J = 7.7 \text{ Hz, H-1'}\)), 4.331 (d, \(J = 3.3 \text{ Hz, H-4}\)), 4.275 (dd, \(J = 9.5 \text{ Hz, H-6a}\)), 4.223 - 4.178 (m, H-6b, H-5), 4.025 (dd, \(J = 7.7, 9.9 \text{ Hz, H-2}\)), 3.965 (dd, \(J = 3.3, 9.9 \text{ Hz, H-3}\)), 3.744 (d, \(J = 9.5 \text{ Hz, H-5'}\)), 3.553-3.506 (m, H-3', H-4'), 3.444 (t, \(J = 8.6 \text{ Hz, H-2'}\)); \(^{13}\)C NMR (100 MHz, t-BuOH 31.2), \(\delta \) 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 \(\beta\)-D-GlcA-(1→3)-\(\beta\)-D-6-O-sulfo-Gal-S-pNP (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: [α]_25^oD = −78° (c 0.78, H2O); \(^1\)H NMR (600 MHz, CD_3OD, t-BuOH 1.40): \(\delta \) 8.360 (d, \(J = 9.2 \text{ Hz, pNP}\)), 7.912 (d, \(J = 9.2 \text{ Hz, pNP}\)), 5.082 (d, \(J = 9.9 \text{ Hz, H-1}\)), 4.778 (d, \(J = 7.7 \text{ Hz, H-1'}\)), 4.438 (brd, \(J = 2.6 \text{ Hz, H-4}\)), 4.408 (d, \(J = 5.5 \text{ Hz, H-6a, 6b}\)), 4.239 (t, \(J = 5.7 \text{ Hz, H-5}\)), 4.058 (t, \(J = 9.5 \text{ Hz, H-2}\)), 3.942 (dd, \(J = 2.8, 12.1 \text{ Hz, H-3}\)), 3.813 (d, \(J = 7.3 \text{ Hz, H-5'}\)), 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), \(\delta \) 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].