

# Chemoselective modification of proteins for the synthesis of structurally defined multivalent scaffolds

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## General information

NMR spectra were recorded on a Bruker ECX 400 (400 MHz for <sup>1</sup>H and 101 MHz for <sup>13</sup>C) as well as Delta JEOL Eclipse 500 (500 MHz for <sup>1</sup>H). Protein masses were analysed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) using an Ultraflex-II TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a 200 Hz solid-state Smart beam™ laser. The mass spectrometer was operated in the positive linear mode. MS spectra were acquired over an m/z range of 4,000–15,000 and data was analysed using FlexAnalysis® software provided with the instrument. Sinapinic acid was used as the matrix and samples were spotted using the dried droplet technique.

## Materials

Reactions sensitive to moist or air were performed under argon. Dry solvents were obtained by Acros Organics or Sigma-Aldrich. All solvents and reagents were purchased from commercial suppliers as reagent grade and used without further purification. Thin-layer chromatography (TLC) was carried out on aluminium-backed silica gel plates (60 F<sub>254</sub>, 0.2 mm, Merck, Darmstadt, Germany) and detected by UV or charring with 10% sulphuric acid in ethanol and subsequent heating. For column chromatography silica gel 60 Å (230-400 mesh) was used. Dialysis of the proteins was performed with Spectra/POR® biotech-grade cellulose-ester tubings (MWCO = 1kDa) from Spectrum Laboratories, Rancho Dominguez, California, USA, followed by concentration with Amicon Ultra-0.5 mL Centrifugal Filters (Millipore, Billerica, Massachusetts, USA). Water for protein applications was purified with a MilliQ system (Millipore).

## Methods

The herein described sugar azides were known to the literature and synthesized as indicated. Generally, deacetylation reactions were performed under modified Zemplen<sup>1</sup> conditions.

The protein scaffold –  $\psi$ -b\*4M[Hpg] – was expressed and purified as before.<sup>2</sup>

## Synthesis of 2,3,4,6-tetra-O-acetyl- $\beta$ -D-galactopyranose azide (Ac1)<sup>3</sup>

The title compound was obtained as described before. Briefly, D-(+)-galactose (1.8 g, 10.0 mmol) was dissolved in acetic anhydride (5 mL) and cooled with an ice bath. HBr/AcOH (30%, 2.8 mL, 10.4 mmol)

was added and the reaction mixture was allowed to stir at room temperature for 45 minutes. Subsequently it was cooled again to 0 °C and HBr/AcOH (30%, 5.5 mL, 20.4 mmol) was added. The reaction was monitored by TLC (EtOAc/cyclo-hexane 1:1) and reagents were removed under reduced pressure after 2.5 hours. The brown syrup was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (55 mL) and sodium azide (1.3 g, 20.0 mmol) and tetrabutylammonium hydrogen sulfate (TBAHS) (515 mg, 1.5 mmol) and aqueous K<sub>2</sub>CO<sub>3</sub> (1 M, 70 mL) were added consecutively. The 2-phase reaction mixture was stirred vigorously at room temperature over night. After diluting with CH<sub>2</sub>Cl<sub>2</sub> the organic layer was separated, washed with water, aq. sat. NaHCO<sub>3</sub> and brine. Subsequently it was dried (MgSO<sub>4</sub>), filtered and evaporated to dryness. The crude product was purified by reduced pressure column chromatography (EtOAc/cyclo-hexane 3:1). The obtained product was then further purified by recrystallization (cyclo-hexane/diethylether 1:1), yielding 1.49 g (4.0 mmol, 40%) of the title compound. The obtained spectra were in accordance with the literature. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 5.42 (dd, *J* = 3.3, 1.1 Hz, 1H), 5.16 (dd, *J* = 10.3, 8.7 Hz, 1H), 5.03 (dd, *J* = 10.4, 3.4 Hz, 1H), 4.59 (d, *J* = 8.7 Hz, 1H), 4.19 (dd, *J* = 10.0, 5.5 Hz, 1H), 4.14 (dd, *J* = 10.1, 4.9 Hz, 1H), 4.01 (td, *J* = 6.5, 1.2 Hz, 1H), 2.17 (s, 3H), 2.09 (s, 3H), 2.06 (s, 3H), 1.99 (s, 3H).

#### Synthesis of β-D-galactopyranose azide (**1**)<sup>4</sup>

The peracetylated sugar azide **Ac1** (2 mmol, 753 mg) was dissolved in MeOH (6 mL) and stirred at room temperature for 10 minutes. Subsequently a solution of sodium methoxide in MeOH (5.55 M, 0.3 mL) was added and the reaction was further stirred at room temperature until completion. The reaction mixture was then diluted with MeOH and neutralized with an ion-exchange resin. Filtration followed by evaporation of the solvent gave 371 mg (1.81 mmol, 90%) of the title compound. The obtained spectra were in accordance with the literature. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): δ 4.63 (d, *J* = 8.7 Hz, 1H), 3.93 (d, *J* = 3.4 Hz, 1H), 3.80 – 3.67 (m, 3H), 3.65 (dd, *J* = 9.9, 3.4 Hz, 1H), 3.47 (dd, *J* = 9.5, 9.0 Hz, 1H). <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O): δ 90.62, 77.27, 72.70, 70.38, 68.57, 61.01. HRMS (ESI) calcd. for C<sub>6</sub>H<sub>11</sub>N<sub>3</sub>O<sub>5</sub>Na<sup>+</sup> [M+Na<sup>+</sup>]: 228.0591, found: 228.0605.

#### Synthesis of 1-azidoethoxy-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranose (**Ac2**)<sup>5</sup>

1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranoside (0.3 mmol, 117 mg) was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (600 μL). After addition of 2-azidoethanol (0.6 mmol, 52 mg) the mixture was cooled to 0 °C. Subsequently BF<sub>3</sub>·OEt<sub>2</sub> (0.39 mmol, 50 μL) was added dropwise to the solution. The reaction was then stirred for 21 hours and was allowed to reach room temperature. The mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> and washed with aq. sat. NaHCO<sub>3</sub>, water and brine. The organic layer was then dried over MgSO<sub>4</sub> and concentrated. The resultant product mixture was purified by column chromatography (EtOAc/cyclo-hexane 45:55), yielding 42 mg (0.1 mmol, 33%) of the title compound. The obtained spectra were in accordance with the literature. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 5.39 (dd, *J* = 3.4, 1.1 Hz, 1H), 5.23 (dd, *J* = 10.4, 8.0 Hz, 1H), 5.01 (dd, *J* = 10.5, 3.4 Hz, 1H), 4.55 (d, *J* = 8.0 Hz, 1H), 4.20-4.08 (m, 3H), 4.04 (ddd, *J* = 10.7, 4.6, 3.6 Hz, 1H), 3.92 (td, *J* = 6.7, 0.9 Hz, 1H), 3.68 (ddd, *J* = 10.7, 8.4, 3.3 Hz, 1H), 3.50 (ddd, *J* = 13.2, 8.6, 3.5 Hz, 1H), 3.29 (ddd, *J* = 13.4, 4.6, 3.4 Hz, 1H), 2.14 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 1.97 (s, 3H).

#### Synthesis of 1-azidoethoxy-β-D-galactopyranose (**2**)<sup>5</sup>

Per-acetylated **Ac2** (0.21 mmol, 87 mg) was treated as described for the synthesis of sugar azide **1**, yielding 44 mg (0.18 mmol, 84%) of compound **2**. The obtained spectra were in accordance with the

literature. <sup>1</sup>H-NMR (400 MHz, CD<sub>3</sub>OD): δ 4.27 (d, *J* = 7.5 Hz, 1H), 4.02 (dt, *J* = 10.8, 5.3 Hz, 1H), 3.84 (dd, *J* = 3.2, 0.9 Hz, 1H), 3.74 (dtd, *J* = 8.9, 6.2, 2.8 Hz, 3H), 3.54-3.50 (m, 2H), 3.49-3.46 (m, 3H). <sup>13</sup>C NMR (176 MHz, D<sub>2</sub>O) δ 102.96, 75.24, 72.79, 70.77, 68.70, 68.43, 61.01, 50.63. HRMS (ESI) calcd. for C<sub>8</sub>H<sub>15</sub>N<sub>3</sub>O<sub>6</sub>Na<sup>+</sup> [M+Na<sup>+</sup>]: 272.0853, found: 272.0866.

### Synthesis of 2-[2-(2-azidoethoxy)ethoxy]ethoxy-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (Ac3)<sup>6</sup>

To 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranoside (12.8 mmol, 5 g) in dry CH<sub>2</sub>Cl<sub>2</sub> (60 mL), 2-[2-(2-chloroethoxy)ethoxy]ethanol (25.6 mmol, 4.3 g) was added and the mixture was stirred for 20 minutes at -4 °C. Subsequently TMSOTf (17.5 mmol, 3.9 g, 3 mL) was added dropwise via syringe over 30 minutes. After stirring for 4 hours at -4 °C, the reaction mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> (40 mL) and consecutively washed with aq. sat. NaHCO<sub>3</sub>, water and brine. The organic layer was then dried (MgSO<sub>4</sub>), filtered and concentrated *in vacuo*. 2.8 g of the purified intermediate (column chromatography: EtOAc/cyclo-hexane gradient 1:3 → 1:1) were dissolved in DMF (15 mL) and NaN<sub>3</sub> (11 mmol, 715 mg) was added. It was then stirred for 20 hours at 90 °C. The reaction mixture was then diluted with EtOAc (85 mL) and washed with water and brine. After drying (MgSO<sub>4</sub>), filtration and concentration, 1.7 g (3.4 mmol, 27%) of the title compound were obtained. The obtained spectra were in accordance with the literature. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 5.38 (dd, *J* = 3.4, 1.0 Hz, 1H), 5.20 (dd, *J* = 10.5, 8.0 Hz, 1H), 5.01 (dd, *J* = 10.5, 3.4 Hz, 1H), 4.57 (d, *J* = 8.0 Hz, 1H), 4.17 (dd, *J* = 11.2, 6.6 Hz, 1H), 4.12 (dd, *J* = 11.2, 6.8 Hz, 1H), 3.96 (dt, *J* = 11.0, 4.2 Hz, 1H), 3.90 (td, *J* = 6.7, 1.0 Hz, 1H), 3.75 (ddd, *J* = 11.0, 6.9, 4.0 Hz, 1H), 3.70 - 3.61 (m, 8H), 3.39 (t, *J* = 5.2, 2H), 2.14 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 1.98 (s, 3H).

### Synthesis of 2-[2-(2-azidoethoxy)ethoxy]ethoxy-β-D-galactopyranoside (3)<sup>7</sup>

Per-acetylated Ac3 (25 mg, 50 μmol) was treated as described for the synthesis of sugar azide **1**, yielding 14 mg (0.04 mmol, 80%) of compound **3**. The obtained spectra were in accordance with the literature. <sup>1</sup>H-NMR (400 MHz, MeOH-*d*<sub>4</sub>): δ 4.26 (d, *J* = 7.5 Hz, 1H), 4.05 - 3.99 (m, 1H), 3.82 (dd, *J* = 3.3, 1.0 Hz, 1H), 3.76 - 3.64 (m, 11H), 3.56 - 3.49 (m, 2H), 3.47 (dd, *J* = 9.7, 3.3 Hz, 1H), 3.39 (t, *J* = 5.0, 2H). <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O) δ 102.90, 75.21, 72.76, 70.82, 69.80, 69.65, 69.54, 69.26, 68.69, 61.03, 50.18. HRMS (ESI) calcd. for C<sub>12</sub>H<sub>23</sub>N<sub>3</sub>O<sub>8</sub>Na<sup>+</sup> [M+Na<sup>+</sup>]: 360.1377, found: 360.1391.

### Synthesis of 2-azido-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (Ac4)<sup>3</sup>

D-lactose (6.5 mmol, 2.22 g) was suspended in acetic anhydride (53 mmol, 5 mL) and the mixture was cooled with an ice-water bath. HBr/AcOH (33%, 6.5 mmol, 1.6 mL) was added in one portion and the reaction was further stirred for 30 minutes at room temperature. Subsequently another portion of HBr/AcOH (33%, 13 mmol, 3.2 mL) was added over 30 minutes while the reaction was cooled with an ice bath. The reaction was then stirred at room temperature for 1 hour, and left at -14 °C for 60 hours. Removal of solvents and excess of reagents by co-evaporation with toluene at reduced pressure furnished a slightly yellow residue, which was taken up in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (10 mL). Consecutively NaN<sub>3</sub> (13 mmol, 845 mg) and tetrabutylammonium bisulfate (975 μmol, 331 mg) were added and overlaid with K<sub>2</sub>CO<sub>3</sub> (1 M in H<sub>2</sub>O, 10 mL). Vigorous stirring started the two-phase reaction, which was diluted with CH<sub>2</sub>Cl<sub>2</sub> after 4 hours. The layers were separated and the organic phase was washed with aq. sat. NaHCO<sub>3</sub> (1x), water

(1x) and brine (2x). The organic layer was then dried over  $\text{MgSO}_4$ , filtered and evaporated to dryness, yielding thick, yellow syrup, which was pure according to  $^1\text{H-NMR}$  (4.23 mmol, 2.8 g, 65%).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  5.43 (d,  $J = 3.4$  Hz, 1H), 5.29 (t,  $J = 9.2$  Hz, 1H), 5.18 (dd,  $J = 10.2, 8.0$  Hz, 1H), 5.04 (dd,  $J = 10.3, 3.3$  Hz, 1H), 4.94 (t,  $J = 9.1$  Hz, 1H), 4.71 (d,  $J = 8.5$  Hz, 1H), 4.61-4.56 (m, 2H), 4.22-4.16 (m, 3H), 3.96 (t,  $J = 6.7$  Hz, 1H), 3.90 (t,  $J = 9.5$  Hz, 1H), 3.78 (ddd,  $J = 10.0, 5.0, 1.8$  Hz, 1H), 2.23 (s, 3H), 2.21 (s, 3H), 2.15 (2x s, 6H), 2.13 (s, 6H), 2.04 (s, 3H).

#### Synthesis of 2-azido- $\beta$ -D-galactopyranosyl)-(1 $\rightarrow$ 4)-2,3,6-tri-O-acetyl- $\beta$ -D-glucopyranoside (4) <sup>4</sup>

Previously obtained **Ac4** (3.93 mmol, 2.6 g) were dissolved in MeOH (40 mL). To this a solution of NaOMe in MeOH (0.1 M, 800  $\mu\text{L}$ ) was added and the reaction was stirred at room temperature for 3 hours. Subsequently another portion of NaOMe sol. (0.1 M, 1.2 mL) was added and the reaction was further stirred over night at room temperature. White crystals formed and were filtered off.  $^1\text{H-NMR}$  (400 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  4.76 (d,  $J = 8.8$  Hz, 2H), 4.44 (d,  $J = 7.8$  Hz, 1H), 3.97 (d,  $J = 12.0$  Hz, 1H), 3.91 (d,  $J = 3.4$  Hz, 1H), 3.84-3.80 (m, 1H), 3.79-3.74 (m, 2H), 3.72-3.69 (m, 1H), 3.65 (dd,  $J = 2.5, 0.9$  Hz, 1H), 3.64 (d,  $J = 3.3$  Hz, 1H), 3.55-3.51 (m, 1H), 3.33 (s, 2H), 3.32-3.30 (m, 1H).  $^{13}\text{C-NMR}$  (101 MHz,  $\text{D}_2\text{O}$ )  $\delta$  102.92, 89.98, 77.75, 76.74, 75.40, 74.39, 72.57, 72.55, 70.98, 68.59, 61.09, 59.89. HRMS (ESI) calcd. for  $\text{C}_{12}\text{H}_{21}\text{N}_3\text{O}_{10}\text{Na}^+$  [ $\text{M}+\text{Na}^+$ ]: 390.1119, found: 390.1135.

#### Synthesis of 2-azidoethoxy-(2,3,4,6-tetra-O-acetyl- $\beta$ -D-galactopyranosyl)-(1 $\rightarrow$ 4)-2,3,6-tri-O-acetyl- $\beta$ -D-glucopyranoside (Ac5) <sup>8</sup>

$\alpha$ -D-lactosyl bromide hepta-O-acetate (**7**, 1.12 mmol, 784 mg) was dissolved in dry acetonitrile (3 mL) and added to a mixture of  $\text{HgBr}_2$  (0.56 mmol, 202 mg),  $\text{Hg}(\text{CN})_2$  (0.56 mmol, 141 mg) and 1-azidoethanol (1.23 mmol, 107 mg) in dry acetonitrile (5 mL). The reaction was stirred over night (16 hours) at room temperature ( $\approx 22^\circ\text{C}$ ). The reaction was then cooled to  $4^\circ\text{C}$ , diluted with  $\text{CH}_2\text{Cl}_2$  (50 mL) and consecutively washed with aq. sat.  $\text{NaHCO}_3$  (15 mL), water (15 mL) and brine (15 mL). The organic phase was dried over  $\text{MgSO}_4$ , filtered and evaporated to dryness, yielding a white solid. The crude product was subjected to column chromatography (EtOAc/cyclo-hexane gradient from 25:75  $\rightarrow$  75:25), resulting in 480 mg (0.68 mmol, 60 %) of the title compound. The obtained spectra were in accordance with the literature.  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  5.34 (dd,  $J = 3.4, 1.0$  Hz, 1H), 5.20 (t,  $J = 9.2$  Hz, 1H), 5.11 (dd,  $J = 10.4, 7.9$  Hz, 1H), 4.94 (ddd,  $J = 17.3, 9.9, 5.6$  Hz, 2H), 4.56 (d,  $J = 7.9$  Hz, 1H), 4.53 (dd,  $J = 12.2, 2.3$  Hz, 1H), 4.49 (d,  $J = 7.9$  Hz, 1H), 4.16 - 4.05 (m, 3H), 3.99 (ddd,  $J = 10.7, 5.0, 3.5$  Hz, 1H), 3.90 - 3.85 (m, 1H), 3.85 - 3.79 (m, 1H), 3.71 - 3.65 (m, 1H), 3.62 (ddd,  $J = 9.9, 4.9, 2.1$  Hz, 1H), 3.51 - 3.44 (m, 1H), 3.27 (ddd,  $J = 13.5, 5.0, 3.4$  Hz, 1H), 2.15 (s, 3H), 2.12 (s, 3H), 2.06 (s, 3H), 2.04 (s, 9H), 1.96 (s, 3H).

#### Synthesis of 2-azidoethoxy-( $\beta$ -D-galactopyranosyl)-(1 $\rightarrow$ 4)- $\beta$ -D-glucopyranoside (5) <sup>8,9</sup>

Compound **Ac5** (0.25 mmol, 176 mg) was dissolved in methanol (2.5 mL). To this a solution of NaOMe in MeOH (0.1 M, 0.13 mL) was added and the reaction was stirred at room temperature ( $\approx 22^\circ\text{C}$ ) for 5 hours. It was then neutralized with DOWEX<sup>®</sup>  $\text{H}^+$ -ion exchange resin (HCR-W2) and evaporated to dryness. 83 mg (0.20 mmol,  $\approx 80\%$ ) of the title compound were obtained as a white solid. The obtained spectra were in accordance with the literature.  $^1\text{H-NMR}$  (400 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  4.36 (dd,  $J = 7.7, 4.4$  Hz, 2H), 4.04 - 3.98 (m, 1H), 3.92 (dd,  $J = 12.1, 2.5$  Hz, 1H), 3.86 - 3.81 (m, 2H), 3.81 - 3.75 (m, 2H), 3.75 - 3.68 (m, 2H), 3.61 - 3.45 (m, 8H), 3.44 - 3.40 (m, 1H), 3.27 (dd,  $J = 8.7, 8.1$  Hz, 1H).  $^{13}\text{C-NMR}$  (101 MHz,  $\text{D}_2\text{O}$ )  $\delta$  102.98, 102.22,

78.36, 75.40, 74.86, 74.38, 72.81, 72.56, 71.00, 68.68, 68.59, 61.07, 60.09, 50.57. HRMS (ESI) calcd. for  $C_{14}H_{25}N_3O_{11}Na^+$  [ $M+Na^+$ ]: 434.1381, found: 434.1397.

### Synthesis of 2-[2-(2-azidoethoxy)ethoxy]ethoxy-(2,3,4,6-tetra-*O*-acetyl- $\beta$ -D-galactopyranosyl)-(1 $\rightarrow$ 4)-2,3,6-tri-*O*-acetyl- $\beta$ -D-glucopyranoside (Ac6) <sup>9</sup>

$\beta$ -D-lactosyl octa-*O*-acetate (2.21 mmol, 1.5 g) was transferred into a round-bottom flask containing dry  $CH_2Cl_2$  (15 mL) and molecular sieves (4Å, 1 g). 2-[2-(2-chloroethoxy)ethoxy]ethanol (6.63 mmol, 963  $\mu$ L) was added and the solution was stirred for 1 hour at room temperature ( $\approx 22^\circ C$ ). It was then cooled to  $0^\circ C$  with an ice-salt bath and TMSOTf (4.41 mmol, 975  $\mu$ L) was added over 5 minutes. The mixture was further stirred for 15 minutes at  $0^\circ C$  and then for 30 minutes at room temperature. Subsequently the reaction was left at  $0^\circ C$  for 5 hours. It was then diluted with  $CH_2Cl_2$  (90 mL) and consecutively washed with aqu. sat.  $NaHCO_3$  (2x 45 mL) and brine (45 mL). The organic layer was dried over  $MgSO_4$ , filtered and concentrated *in vacuo*, yielding slightly yellow syrup. Excess of 2-[2-(2-chloroethoxy)ethoxy]ethanol was removed by decanting with water and column chromatography (EtOAc/cyclo-hexane gradient from 50:50  $\rightarrow$  66:33). The intermediate product was dissolved in DMF (10 mL) and reacted with  $NaN_3$  (3.05 mmol, 198 mg) at  $80^\circ C$  for 18 hours. After cooling to room temperature the reaction mixture was diluted with EtOAc (200 mL) and washed with water (2x 30 mL) and brine (2x 30 mL). The organic phase was then dried over  $MgSO_4$ , filtered and evaporated to dryness, yielding clear syrup (0.38 mmol, 300 mg, 17%). The obtained spectra were in accordance with the literature. <sup>1</sup>H-NMR (400 MHz,  $CDCl_3$ )  $\delta$  5.34 (dd,  $J = 3.5, 1.1$  Hz, 1H), 5.23 – 5.15 (m, 1H), 5.10 (dd,  $J = 10.4, 7.9$  Hz, 1H), 4.95 (dd,  $J = 10.4, 3.5$  Hz, 1H), 4.89 (dd,  $J = 9.6, 8.0$  Hz, 1H), 4.56 (d,  $J = 7.9$  Hz, 1H), 4.49 – 4.46 (m, 1H), 4.09 (dt,  $J = 11.1, 7.1$  Hz, 3H), 3.94 – 3.84 (m, 2H), 3.82 – 3.75 (m, 1H), 3.75 – 3.57 (m, 11H), 3.41 – 3.36 (m, 2H), 2.14 (s, 3H), 2.11 (s, 3H), 2.05 (s, 3H), 2.05(3x s, 9H), 1.96 (s, 3H).

### Synthesis of 2-[2-(2-azidoethoxy)ethoxy]ethoxy-( $\beta$ -D-galactopyranosyl)-(1 $\rightarrow$ 4)- $\beta$ -D-glucopyranoside (6) <sup>10</sup>

To a solution of Ac6 (0.25 mmol, 198 mg) in methanol (2.5 mL) NaOMe in MeOH (0.1 M, 0.13 mL) was added. The reaction mixture was then stirred at ambient temperature for 5 hours. Subsequently it was diluted with methanol (5 mL) and neutralized with DOWEX® H<sup>+</sup>-ion exchange resin (HCR-W2). After filtration and removal of the solvent, the title compound was obtained as syrup (97 mg, 0.19 mmol,  $\approx 79\%$ ). The obtained spectra were in accordance with the literature. <sup>1</sup>H-NMR (500 MHz,  $CD_3OD$ )  $\delta$  4.35 (t,  $J = 8.1$  Hz, 2H), 4.05 – 3.98 (m, 1H), 3.90 (dd,  $J = 12.1, 2.4$  Hz, 1H), 3.83 (dt,  $J = 11.3, 6.1$  Hz, 2H), 3.79 – 3.73 (m, 2H), 3.71 (dd,  $J = 5.6, 4.0$  Hz, 3H), 3.70 – 3.66 (m, 7H), 3.61 – 3.55 (m, 3H), 3.52 (d,  $J = 8.6$  Hz, 1H), 3.49 (dd,  $J = 9.7, 3.2$  Hz, 1H), 3.44 – 3.40 (m, 1H), 3.40 – 3.37 (m, 2H), 3.27 (t,  $J = 8.4$  Hz, 1H). <sup>13</sup>C NMR (176 MHz,  $D_2O$ )  $\delta$  103.00, 102.14, 78.48, 75.40, 74.82, 74.37, 72.88, 72.59, 71.01, 69.73, 69.63, 69.54, 69.23, 68.79, 68.60, 61.05, 60.16, 50.20. HRMS (ESI) calcd. for  $C_{18}H_{33}N_3O_{13}Na^+$  [ $M+Na^+$ ]: 522.1906, found: 522.1936.

### Synthesis of THPTA <sup>11</sup>

3-Azidopropyl acetate (4 mmol, 573 mg), tripropargylamine (1 mmol, 132 g) and  $Cu(MeCN)_4PF_6$  were dissolved in dry tetrahydrofuran (10 mL), put under an argon atmosphere and refluxed over night. The solvent was removed, and the reaction crude was purified with column chromatography (chloroform/methanol, gradient 100:0  $\rightarrow$  94:6), yielding 370 mg (0.66 mmol, 66%) of the desired

intermediate, tris-[(3-hydroxypropyltriazol-4-yl)methyl]amine acetate. This intermediate (280 mg, 0.50 mmol) was then dissolved in 2 M NH<sub>3</sub> in MeOH (7 mL) and stirred at 40 °C for 16 hours. The reaction mixture was then cooled to room temperature, followed by concentration *in vacuo*. The white solid was then dispersed in MeCN, filtered and dried under vacuum, yielding tris-[(3-hydroxypropyltriazol-4-yl)methyl]amine (141 mg, 0.33 mmol, 65%). The obtained spectra were in accordance with the literature. **<sup>1</sup>H-NMR** (400 MHz, DMSO-*d*<sub>6</sub>): δ 8.03 (s, 3H), 4.67 (t, *J* = 5.0 Hz, 3H), 4.41 (t, *J* = 7.1 Hz, 6H), 3.62 (s, 6H), 3.40 (q, *J* = 5.6 Hz, 6H), 1.96 (quintet, *J* = 6.7 Hz, 6H). **<sup>13</sup>C-NMR** (101 MHz, DMSO-*d*<sub>6</sub>): δ 143.40, 124.00, 57.48, 47.07, 46.57, 33.00.

#### General procedure for functionalisation of ψ-b\*4M[Hpg] via CuAAC<sup>10</sup>

Modification of ψ-b\*4M[Hpg] was carried out the same way for each sugar azide. First ψ-b\*4M[Hpg] (0.07-0.2 mg/mL, Dulbecco's PBS incl. 10% glycerol, pH 8, 150 µL) were transferred to a 0.5 mL-reaction vessel (Protein LoBind tube, Eppendorf). Subsequently buffer (Dulbecco's PBS, pH 7.3, 32.5 µL) was added, followed by sugar azide solution (250 mM in MilliQ-water). CuSO<sub>4</sub>·5H<sub>2</sub>O (20 mM in MilliQ-water, 2.5 µL) and THPTA (50 mM in MilliQ-water, 5 µL) were mixed and the whole volume (7.5 µL) was transferred to the reaction vessel. Aminoguanidine·HCl (100 mM in MilliQ-water, 25 µL) was added, and the reaction was started with sodium ascorbate (100 mM in MilliQ-water, 25 µL). The vessel was sealed and stirred at 2-4 °C for 64 hours. The mixture was then diluted to 500 µL with buffer (Dulbecco's PBS incl. 2 mM EDTA, pH 7.4) and dialyzed for a total of 22 hours. The dialysed samples were then concentrated with centrifugal filter tubes for further analysis and SPR measurements.

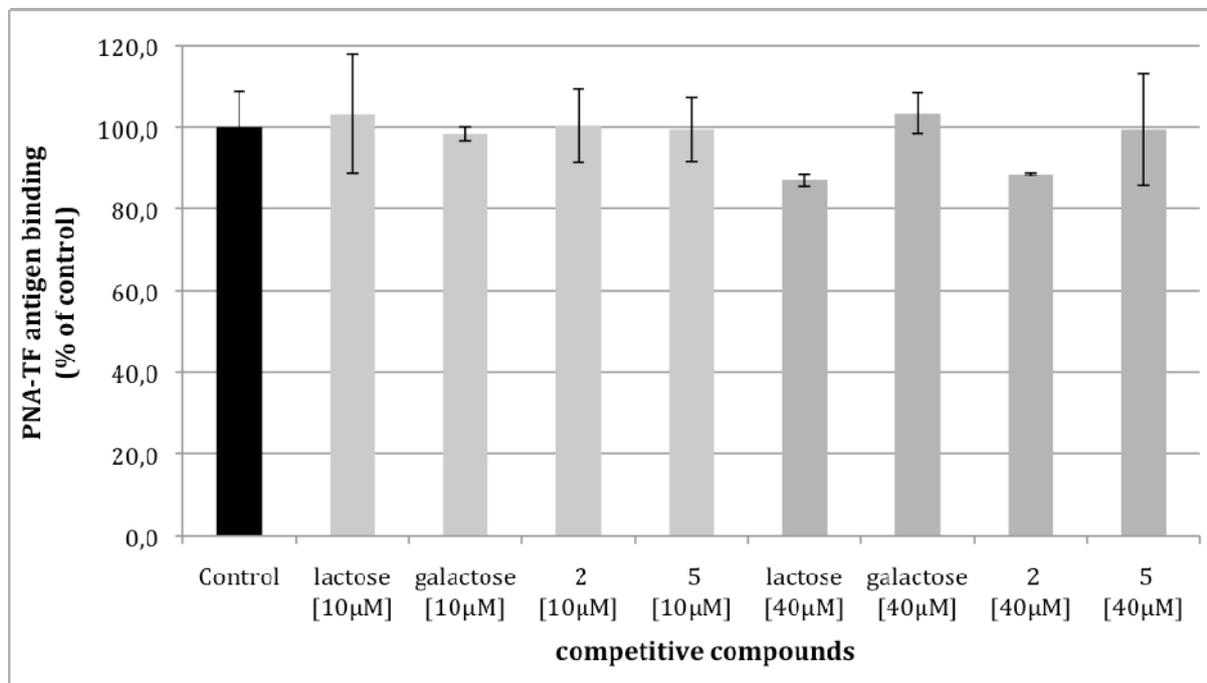
#### Surface Plasmon Resonance (SPR) measurements

SPR measurements were carried out at 25 °C on a Biacore X instrument (GE Healthcare, Freiburg, Germany). Binding probe was the commercial available Thomsen-Friedenreich (TF) antigen linked to a biotinylated polyacrylamide (PAA) carrier: Galβ1-3GalNAcα-(CH<sub>2</sub>)<sub>3</sub>-PAA-(CH<sub>2</sub>)<sub>6</sub>-biotin (Lectinity Holdings Inc., Moscow, Russia). The probe had a molecular weight of ~ 30 kDa and contained 20 mol% TF antigen and 5 mol% biotin. Via the strong biotin-streptavidin interaction the probe was coupled to 671 resonance units (RU) on a streptavidin functionalized sensor chip SA (GE Healthcare, Freiburg, Germany). Before immobilization, the sensor chip was conditioned with three consecutive 1 min injections of 1 M NaCl and 50 mM NaOH. The TF antigen probe was diluted to 4.2 µg/ml in HBS-EP buffer (GE Healthcare) and passed over one lane of the chip surface, the second lane remained untreated and served as a reference. After the immobilization procedure, the chip surface was equilibrated with three consecutive 1 min injections of running buffer, containing 20 mM HEPES, pH 7.4; 150 mM NaCl and 1 mM CaCl<sub>2</sub>. A 35 µL sample volume was injected over both lanes, whereas the final binding signals were obtained by subtraction of data from the free reference lane. The association phase was set to 105 s followed by a 180 s dissociation phase. The response values were calculated by subtraction of the report point at the beginning of the sample injections (0 s) from the report point at the end of the dissociation phase (285 s). Regeneration of the chip was performed after each run, with a 60 s flow of 4 M MgCl<sub>2</sub>.

Binding analyses were performed with running buffer at a flow rate of 20 µl/min. To measure peanut agglutinin (PNA) interaction to immobilized TF antigen, a 800 nM solution of PNA (Axxora GmbH, Loerrach, Germany) was used and the resulting RU value was set to 100% binding (positive control). For

all competitive measurements PNA was preincubated for 18 min at room temperature with barstar-conjugates at a final concentration of 10  $\mu\text{M}$  protein before injection. The resulting RU values were calculated as X% binding of the control and converted to % inhibition.

#### Competition of PNA-TF binding by the free sugars lactose, galactose and their linker derivatives

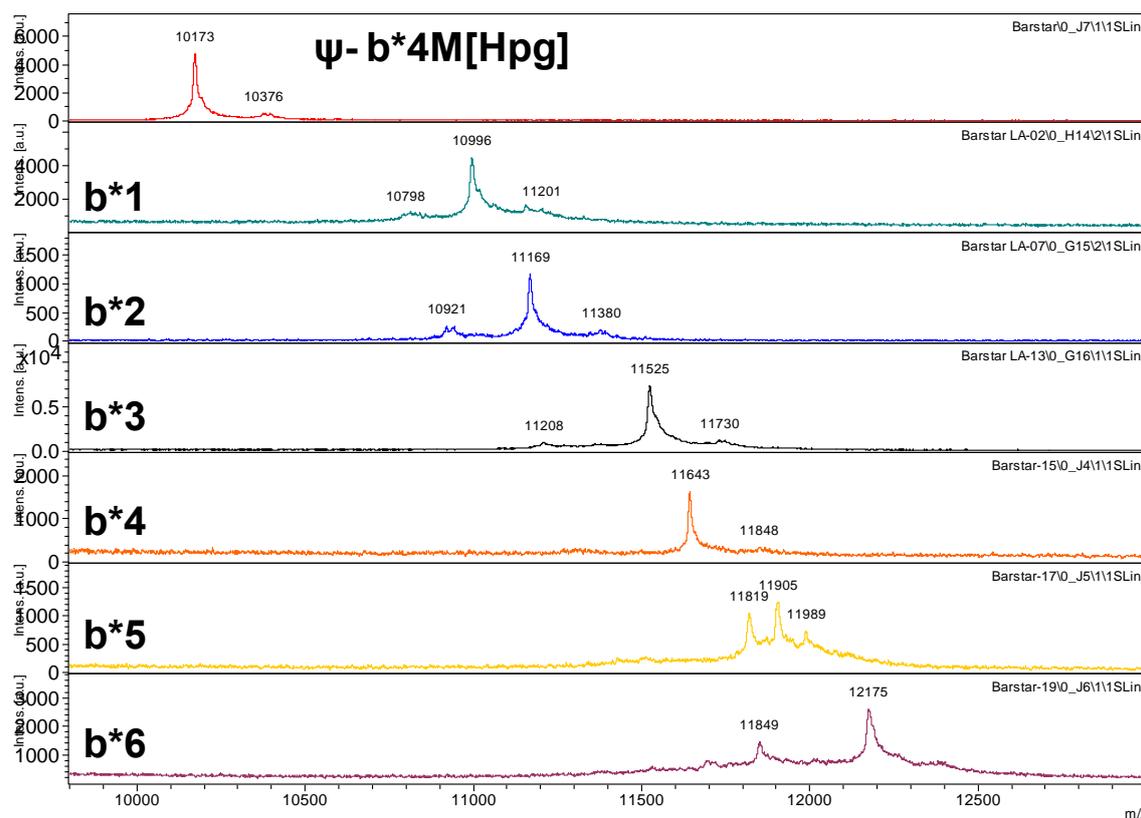


By the competitive SPR binding assay inhibition of PNA binding to immobilized TF-antigen was analyzed for 10 and 40 mM concentrations of free sugars lactose, galactose and their respective ethyl azide linker containing derivatives 2 and 5. PNA preincubation with 10 mM of all compounds did not lead to any inhibitory effect and is comparable to the untreated control. At 40 mM concentrations only lactose and the lactose-linker compound 5 showed a slight inhibition of ~12%. In contrast to the soluble compound, 5 fourfold linked to barstar protein (b\*5) inhibited PNA-TF antigen interaction to 44% at 10 mM protein concentration (manuscript, Figure 1A).

#### Fluorescence measurements

Fluorescence spectra of proteins were measured using the luminescence spectrometer LS55 (PerkinElmer Life Sciences, Boston, MA, USA) at 20 °C in Dulbecco's PBS. The protein concentrations were adjusted to give emission maxima between 600 and 800 nm. Spectra were recorded from 300 – 500 nm with an excitation wavelength of 280 nm (excitation/emission slits 10/10). Afterwards fluorescence profiles were normalized for purposes of better comparison.

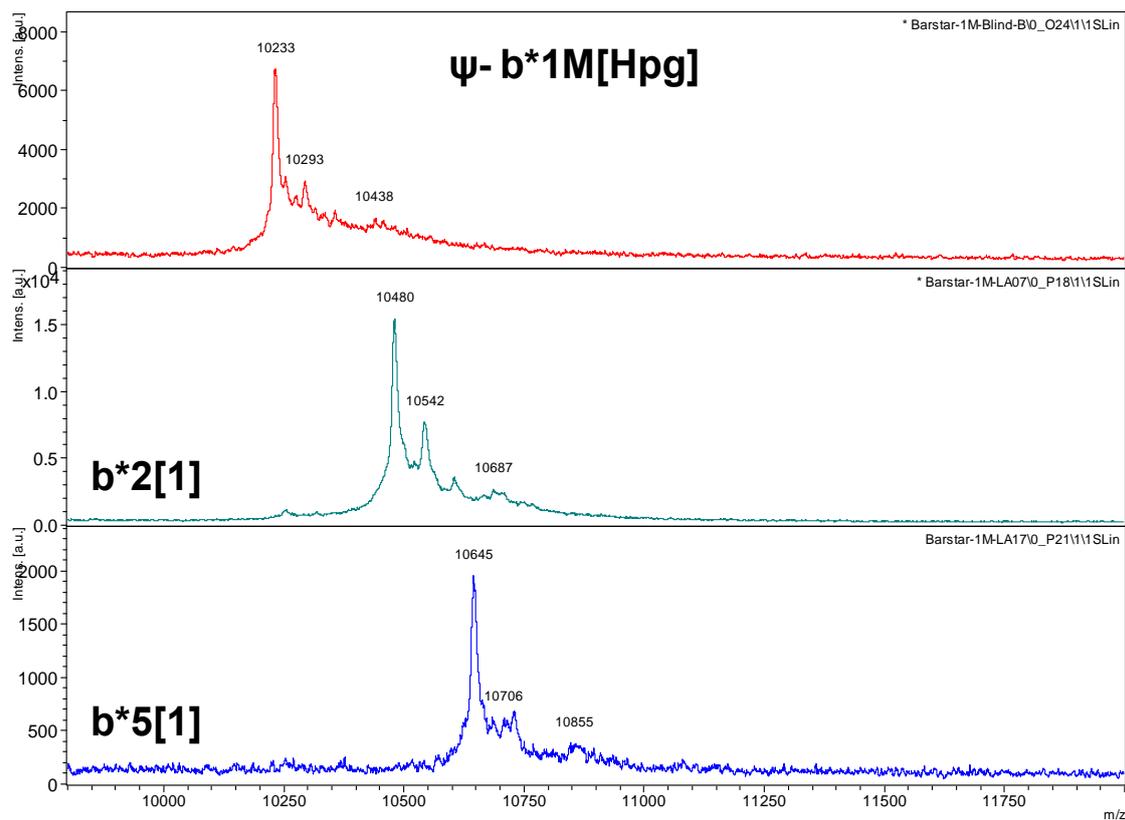
## MALDI-ToF Spectra



MALDI-ToF mass spectra of the unreacted barstar  $\psi$ -b\*4M[Hpg] carrying four alkyne-containing Hpg residues (theoretical masses given in the table below) and its reaction product with various azido sugars; from top to bottom b\*1 – b\*6. In some samples, in addition to the peaks corresponding to the fully reacted products there are smaller side peaks due to incomplete reaction (three sugar residues attached, e.g. b\*1: 10,798, b\*2: 10,921). In most samples, including the unreacted barstar b\*4M, there are small side peaks at a higher mass due to attachment of the matrix molecule sinapinic acid (theoretically +206).

Theoretical and experimental mass (M+H, average)

Name	Theoretical	Experimental
$\psi$ -b*4M[Hpg]	10,174	10,173
b*1	10,994	10,996
b*2	11,170	11,169
b*3	11,523	11,525
b*4	11,643	11,643
b*5	11,819	11,819
b*6	12,171	12,175



MALDI-ToF mass spectra of the unreacted barstar  $\psi$ -b\*1M[HPG] carrying one alkyne-containing Hpg residue (theoretical masses given in the table below) and its reaction product with two different azido sugars. In all samples there are side peaks at +62 (copper adducts) and +206 (attachment of the matrix molecule sinapinic acid).

Theoretical and experimental mass (M+H, average)

Name	Theoretical	Experimental
$\psi$ -b*1M[HPG]	10,240	10,233
b*2[1]	10,489	10,480
b*5[1]	10,651	10,645

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