A kit for the investigation of live *Escherichia coli* cell adhesion to glycosylated surfaces

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A) General Synthetic and Analytical Methods

Thin layer chromatography was performed on silica gel plates (GF 254, Merck). Detection was effected by charring with 10% sulfuric acid in EtOH and ninhydrine solution (300 mg ninhydrine, dissolved in 100 mL butanol and 3 mL conc. acetic acid), both followed by heat treatment. Flash chromatography was performed on silica gel 60 (230–400 mesh, particle size 0.040–0.063 mm, Merck) using distilled solvents. Optical rotations were measured with a Perkin–Elmer 241 polarimeter (sodium D-line: 589 nm, length of cell: 1 dm). ^{1}H and ^{13}C NMR spectra were recorded with Bruker DRX-500 and AV-600 spectrometers. Chemical shifts are reported relative to internal MeOD (δ = 3.31) or D_2O (δ = 4.76 ppm). HRESI mass spectra were recorded on an Applied Biosystems (Applera) Mariner ESI-TOF system. Commercial reagents were used without purification unless otherwise noted.

B) Experimental Procedures

B-1 Chemical Synthesis

Synthesis of allyl α -D-mannopyranoside: Allyl alcohol (125 mL, 1.84 mol) was cooled to 0 °C, acetyl chloride (10 mL, 14.0 mmol) was added dropwise, and the mixture was stirred at 0 °C for 1 h. After heating to 70 °C, mannose (10.0 g, 55.6 mmol) was added and the reaction was stirred under reflux for 5 h. It was neutralized with sodium hydrogen carbonate and filtrated over celite. After three co-distillations with toluene, the solvent was removed and the crude product was crystallized from acetone/pentane or purified by flash column chromatography on silica (EtOAc/MeOH, 8:2).

Synthesis of *p*-aminophenyl α-D-mannopyranoside (1): *p*-Nitrophenyl α-D-mannoside (500 mg, 1.84 mmol) and a catalytic amount of palladium on charcoal were dissolved in dry methanol (5.00 mL) and acetic acid (2.50 mL) was added. The mixture was stirred in a hydrogen atmosphere at ambient temperature for 15 h. The catalyst was removed by filtration over a syringe filter (0.45 µm), solvents were removed *in vacuo*, and the crude product was purified by filtration over silica gel. The slightly acidic product was neutralized by addition of Dowex 1 anion exchanger and subsequent filtration over a syringe filter (0.45 µm).

6-Amino-4-thiahexyl α-D-mannopyranoside (3): Allylmannoside (300 mg, 1.36 mmol), cysteamine hydrochloride (774 mg, 6.81 mmol), and a catalytic amount of 2,2'-azobis(isobutyronitrile) (AIBN) were dissolved in dry dioxane (2.5 mL), the reaction mixture was heated up to 65 °C and stirred at this temperature for 7 h. After removal of the solvent *in vacuo*, the crude product was dissolved in water (5 mL) and washed with ethyl acetate (3 x 10 mL). The aqueous phase was concentrated *in vacuo*. Purification by size exclusion chromatography on Sephadex LH20 gel (H_2O) gave the title compound as a light yellow lyophilisate (385 mg, 95 %). ^{1}H NMR (500 MHz, D_2O): δ= 4.88 (d, 1H, $^{3}J_{1,2}$ = 1.7 Hz, H-1), 3.96 (dd, 1H, $^{3}J_{1,2}$ = 1.8 Hz, $^{3}J_{2,3}$ = 3.4 Hz, H-2), 3.92-3.76 (m, 4H, H-3, H-6, H-6', manOC**H**HCH$_2$), 3.67-3.61 (m, 3H, H-4, H-5, manOCH**H**CH$_2$), 3.25 (t, 2H, ^{3}J = 6.7 Hz, CH$_2$C**H$_2$**NH$_2$), 2.89 (t, 2H, ^{3}J = 6.7 Hz, C**H$_2$**CH$_2$NH$_2$), 2.76-2.67 (m, 2H, manOCH$_2$CH$_2$C**H$_2$**S), 2.01-1.88 (m, 2H, manOCH$_2$C**H$_2$**CH$_2$S); ^{13}C NMR (150 MHz, D_2O): δ= 101.5 (C-1), 74.6 (C-4), 72.5 (C-3), 71.9 (C-2), 68.6 (C-5), 67.9 (manO**C**H$_2$CH$_2$), 62.8 (C-6), 40.3 (SCH$_2$**C**H$_2$NH$_2$), 30.2 (manOCH$_2$**C**H$_2$CH$_2$S), 30.1 (S**C**H$_2$CH$_2$NH$_2$), 29.4 (manOCH$_2$CH$_2$**C**H$_2$S) ppm;

$[\alpha]_D^{21}$ = + 34°; (c= 1.0, MeOH)

HRESI-MS: Calcd for $[C_{11}H_{23}NO_6S + H]^+$: 298.1324. Found: 298.1395.

6-*N*-(2-Methoxy-3,4-dioxocyclobuten-1-yl)amin-3-thiahexyl α-D-mannopyranoside (5): The amino-functionalized mannoside **3** (275 mg, 925 µmol) and squaric acid dimethylester (250 mg, 1.76 mmol) were dissolved in dry methanol (3 mL), triethylamine (150 µL, 1.08 mmol) was added and the mixture was stirred at ambient temperature under nitrogen for 20 h. The crude product was filtrated over a syringe filter (0.45 µm) and purified by MPLC on an RP 8-column (EtOAc/MeOH, 30:70 → 40:60, 120 min), followed by size exclusion chromatography on Sephadex LH20 gel (MeOH) to give the white title compound after lyophilization from

water (253 mg, 67 %). ^{1}H NMR (600 MHz, CD_3OD): δ= 4.75 (bs, 1H, H-1), 4.40, 4.37 (each bs, Σ3H, OCH_3), 3.83 (dd, 1H, $^3J_{5,6}$ = 2.4, $^3J_{6,6'}$ = 11.7 Hz, H-6), 3.85-3.81 (m, 1H, manOCH**H**CH_2), 3.81-3.79 (m, 1H, H-2), 3.77 (t, 1H, ^3J = 6.7 Hz, SCH_2C**H**HNHsquaric acid), 3.71 (dd, 1H, $^3J_{5,6'}$ = 5.8 Hz, $^3J_{6,6'}$ = 11.7 Hz, H-6'), 3.70-3.67 (m, 1H, H-3), 3.61 (dd~t, 1H, $^3J_{4,5}$ = 9.6 Hz, H-5), 3.58 (t, 1H, ^3J = 6.7 Hz, SCH_2CH**H**NHsquaric acid), 3.54-3.49 (m, 2H, H-4, manOC**H**HCH_2), 2.73 (t, 2H, ^3J = 6.8 Hz, SC**H$_2$**CH_2NHsquaric acid), 2.70-2.64 (m, 2H, manOCH_2CH_2C**H$_2$**S), 1.90-1.83 (m, 2H, OCH_2C**H$_2$**CH_2S); ^{13}C NMR (126 MHz, CD_3OD): δ= 190.1, 189.7, 185.0, 184.8, 178.4, 178.2 (squaric acid-C), 101.6 (C-1), 74.6 (C-4), 72.6 (C-3), 72.1 (C-2), 68.6 (C-5), 66.9 (manO**C**H_2CH_2), 62.9 (C-6), 61.2, 61.1 (O**C**H_3), 45.2, 44.6 (SCH_2**C**H_2NHsquaric acid), 33.7, 33.3 (S**C**H_2CH_2NHsquaric acid), 30.6, 30.5 (manOCH_2**C**H_2CH_2S), 29.5, 29.3 (manOCH_2CH_2**C**H_2S) ppm;

$[\alpha]_D^{21}$ = + 46° (c= 0.8, MeOH)

HRESI-MS: Calcd for $[C_{16}H_{25}NO_9S + Na]^+$: 430.1148. Found: 430.1169.

B-2 Biological Assays

Media and buffer solutions:

LB-medium (+AMP) (HB101pPKL4): tryptone (10.0 g), sodium chloride (10.0 g), and yeast extract (5.00 g) were dissolved in bidest. water (1 L); after sterilization, ampicillin (100 mg) was added; LB-medium (+AMP, +CAM) (PKL1162): tryptone (10.0 g), sodium chloride (10.0 g), and yeast extract (5.00 g) were dissolved in bidest. water (1.00 L); after sterilization, ampicillin (100 mg) and chloramphenicol (50.0 mg) were added; carbonate buffer solution (pH 8.2 and pH 9.5): sodium carbonate (1.59 g) and sodium hydrogen carbonate (2.52 g) were dissolved in bidest. water (1.00 L) with subsequent pH adjustment; carbonate buffer solution (pH 9.6): sodium carbonate (10.6 g) and sodium hydrogen carbonate (8.40 g) were dissolved in bidest. water; PBS buffer solution (pH 7.2): sodium chloride (8.00 g), potassium chloride (200 mg), sodium hydrogen phosphate-dihydrate (1.44 g), and potassium dihydrogenphosphate (200 mg) were dissolved in bidest. water (1.00 L); PBST buffer solution (pH 7.2): PBS buffer + 0.05 % v/v Tween® 20; substrate buffer solution (pH 4.5): sodium citrate-dihydrate solution (0.1 M in bidest. water) was adjusted to pH 4.5 with 2 M citric acid solution; ABTS solution: ABTS (10.0 mg) was dissolved in substrate buffer (10.0 mL); hydrogen peroxide solution (250 µL, 0.1 % v/v in bidest. water) was added directly before use. pH-Values were adjusted with 0.1 M HCl or 0.1 M NaOH, respectively, unless otherwise noted.

Microtiter plate functionalization

Mannan-coating: Black (nunc Maxisorp) or transparent (Sarstedt) 96-well plates were filled with a solution of mannan from *Saccharomyces cerevisiae* (1.2 mg/mL in carbonate buffer, pH 9.5; 100 µL solution per well) and allowed to dry in at 37 °C overnight. The plates were washed with PBST (3 x 150 µL/well) and stored at 4 °C. Before use the wells were blocked with BSA (5 % in PBS, 120 µL/well) for 2 h at 37 °C and then washed with PBST (3 x 150 µL/well). Blocked plates can be stored at 4°C for max. two days.

Covalent functionalizations: Serially diluted derivatives **3** and **5** were immobilized on Black Immobilizer Amino™ F96 MicroWell™ plates and CovaLink™ NH modules (nunc™), respectively under different pH conditions to deduce the mannoside concentration and pH of the immobilization buffer needed for maximum readout. This procedure factors the eventually different coupling efficiency in the different immobilization protocols.

Covalent functionalization using amino derivatives: Black Immobilizer Amino™ F96 MicroWell™ plates (nunc) were incubated with a solution of the amino-functionalized mannoside (**1** or **3** 10 mM in carbonate buffer, pH 9.6; 100 µL solution per well) overnight at ambient temperature and under gentle agitation. After washing with PBST (3 x 150 µL/well), unreacted functional groups on the microtiter plate surface were blocked with ethanolamine (10 mM solution, 120 µL/well, 2 h) at ambient temperature under slight agitation. Then, the wells were washed with PBST (3 x 150 µL/well).

Covalent functionalization using squaric acid monoesters: Microtiter plate functionalization: Adapting the literature-known procedure,[30] twelve 8-well transparent CovaLink™ NH modules (nunc™) were incubated

with a solution of the squaric acid-functionalized mannoside **5** (100 µL/well, 10 µM in carbonate buffer, pH 8.2) overnight at ambient temperature and under gentle agitation. The wells were washed with PBST (3 x 150 µL/well).

Biotin-streptavidin-based bacterial adhesion assay

E. coli bacteria (HB101pPKL4) were washed with carbonate buffer (pH 8.2) and suspended in the same buffer (10 mg bacteria/mL). NHS-biotin solution (10 mg/mL in DMSO, 2.5 µL) was added to *E. coli* suspension (1 mL) and this mixture was incubated for 2.5 h at rt. The biotin-labelled bacteria were washed with PBS (3 x 10 mL) and suspended in PBS (2 mg labelled bacteria/mL). A serial dilution of the examined inhibitor was prepared in transparent mannoside-coated, BSA-blocked 96-well plates. The bacteria suspension (50 µL/well) was added and the plates were incubated (45 min., 37 °C) under slight agitation (80 rpm). After washing with PBS (3 x 150 µL/well), the plate was incubated with a solution of streptavidin-HRP (100 µL/well, 1:2000 in PBS) for 1 h at 37 °C under slight agitation (80 rpm). The wells were washed with PBS (2 x 150 µL/well) and substrate buffer (1 x 150 µL). ABTS solution was added (100 µL/well) and it was incubated (1 h, rt). The enzyme reaction was stopped by addition of oxalic acid (100 µL/well) and the optical density was read out at 435 nm.

GFP-based bacterial adhesion assay

A serial dilution of the examined inhibitor was prepared in black mannoside-coated, BSA-blocked 96-well plates. The bacteria suspension (2 mg bacteria/mL, 50 µL solution per well) was added and the plates were agitated (80 rpm) and incubated for 45 min. at 37 °C. After washing with PBS (3 x 150 µL), the wells were filled with PBS (100 µL/well) and the fluorescence intensity (485nm/535nm) was determined.

C) Detailed Adhesion-Inhibition Assay Protocols

E. coli bacteria were grown in LB-media at 37 °C under slight agitation. Strain HB101 pPKL4 was grown in LB-media + AMP (100mg ampicillin/L), strain PKL1162 was grown in LB-media + AMP + CAM (100mg ampicillin, 50 mg chloramphenicol/L).

All assays are performed, using at least duplicate samples of each well.

C-1 ELISA

96-well microtiter plate			HB101pPKL4		
time	**temp**	**procedure**	**time**	**temp**	**procedure**
10 min		coating with mannan solution 1.5 mg/ml in carbonate buffer, pH 9.5, 120 µl/well	over night	37 °C	grow bacteria in LB medium + ampicillin (100 µg/ml)
over night	37 °C	dry open microtiter plate in incubator			
10 min	rt	wash three times with PBS, 150 µl/well			
2 h	37 °C	block with 5% skimmed milk powder in PBS, 120 µl/well			
10 min	rt	wash three times with PBS, 150 µl/well			
all previous steps can be performed one day in prior					
5 min	rt	fill with PBS, 50 µl/well	12 min	rt	harvest cells centrifuge 10 min at 2000 rpm
10 min	rt	inhibitor solution, highest conc. in well A-C 2, 50 µl/well, serial dilution	13 min	rt	wash cells with PBS
			5 min	rt	suspend to, 2 mg/ml in PBS
5 min	rt	add *E. coli* suspension 50 µl/well			
45 min	37 °C	incubate in closed container			
10 min	rt	wash three times with PBS, 150 µl/well			
5 min	rt	fill with AB 1 solution, 1:2000 in 2 % skimmed milk in PBS			
30 min	37 °C	incubate in closed container			
10 min	rt	wash three times with PBST, 150 µl/well			
5 min	rt	fill with AB 2 solution, 1:4000 in 2 % skimmed milk in PBS			
30 min	37 °C	incubate in closed container			
10 min	rt	wash twice with PBST and once with citrate puffer, 150 µl/well			
30 min	rt	add substrate solution*, 100 µl/well, stain in a dark place * (10 mg ABTS in 10 ml citrate buffer + 250 µl 0.1 % H_2O_2)			
5 min	rt	add 2 % oxalic acid, 50 µl/well to stop the enzymatic staining			
5 min	rt	fill with PBS, 100 µl/well			
measure fluorescence intensity at 485nm/535nm					
altogether 3h, 30 min					

C-2 Biotin-Streptavidin-based Assay

96-well microtiter plate			HB101 pPKL4		
time	**temp**	**procedure**	**time**	**temp**	**procedure**
10 min	rt	coating with mannan solution 1.5 mg/ml in carbonate buffer, pH 9.5, 120 µl/well	over night	37 °C	grow bacteria in LB-medium + ampicillin (100 µg/ml)
over night	37 °C	dry open microtiter plate in incubator	12 min	rt	harvest cells centrifuge 10 min at 2000 rpm
10 min	rt	wash three times with PBST, 150 µl/well	13 min	rt	wash cells with carbonate buffer, pH 8.2
2 h	37 °C	block with 5 % BSA in PBS, 120 µl/well	5 min	rt	suspend to 10 mg/ml (1 M carbonate buffer, pH 8.2)
10 min	rt	wash three times with PBST, 150 µl/well	5 min		add NHS-biotin*, 25 µg per mg cell pellet *(10 mg/ml solution in DMSO)
5 min	rt	fill with PBS, 50 µl/well	2.5 h	rt	biotinylation
10 min	rt	inhibitor solution, highest conc. in well A-C 2, 50 µl/well, serial dilution	45 min	rt	wash three times with PBS
			5 min	rt	suspend to, 2 mg/ml in PBS
altogether 2 h, 45 min			altogether 3h, 45 min		
5 min	rt	add *E. coli* suspension 50 µl/well			
45 min	37 °C	incubate in closed container			
10 min	rt	wash three times with PBST, 150 µl/well			
1 h	37 °C	add streptavidin-HRP, 1:2000 in PBS, 100 µl/well, incubate in closed container			
10 min	rt	wash twice with PBS and once with citrate puffer, 150 µl/well			
30 min	rt	add substrate solution*, 100 µl/well, stain in a dark place * (10 mg ABTS in 10 ml citrate buffer + 250 µl 0.1 % H_2O_2)			
5 min	rt	add 2 % oxalic acid, 50 µl/well to stop the enzymatic staining			
measure optical density at 405 nm, (ref 490 nm)					
altogether 2h, 45 min					

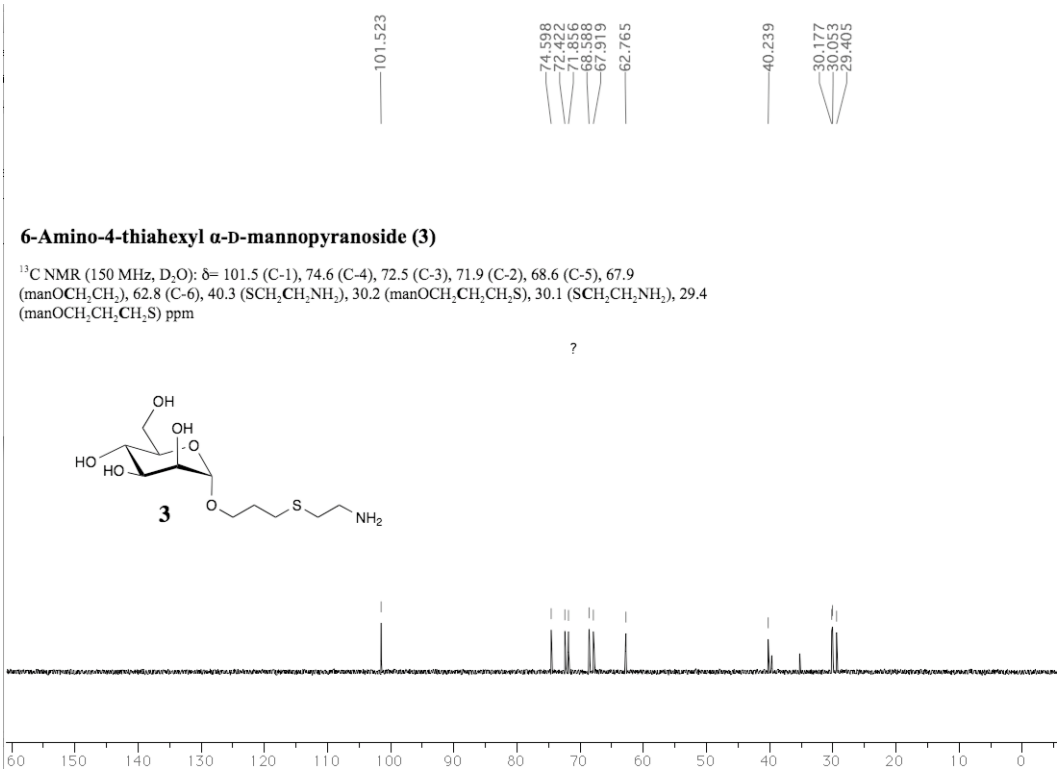
C-3 GFP-based Assay

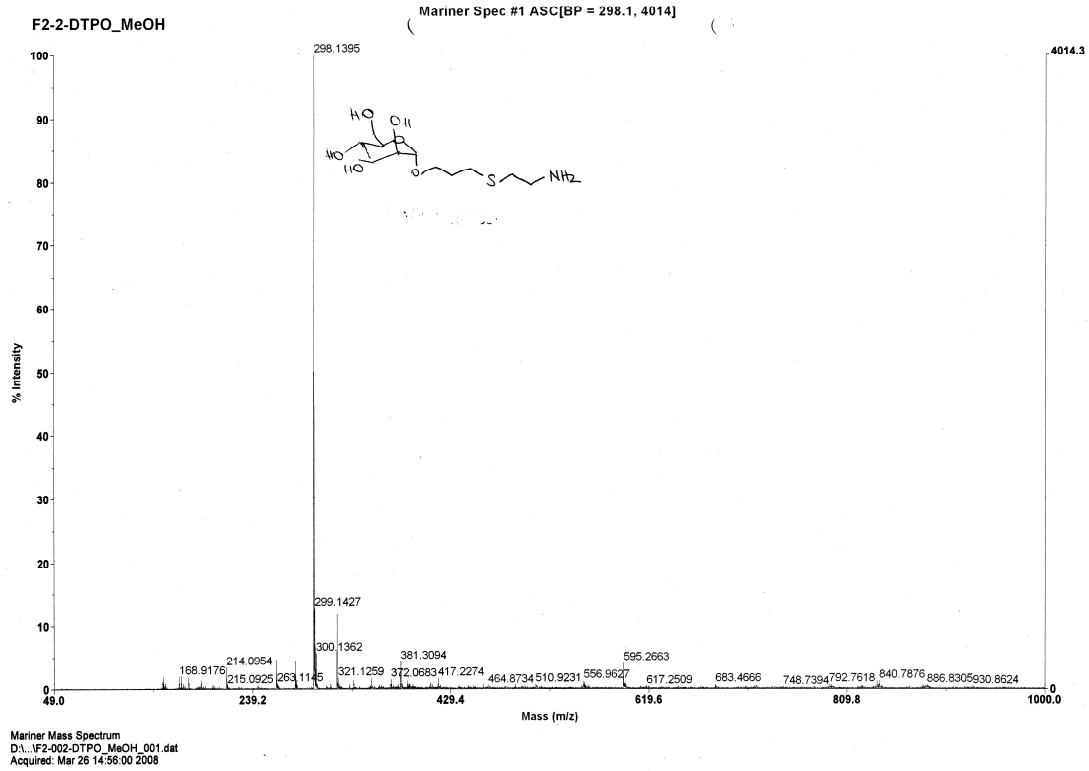
96-well microtiter plate			PKL1162		
time	**temp**	**procedure**	**time**	**temp**	**procedure**
10 min	rt	coating with mannan solution 1.5 mg/ml in carbonate buffer, pH 9.5, 120 µl/well	over night	37 °C	grow bacteria in LB medium + ampicillin (100 µg/ml) + chloramphenicol (50 µg/ml)
over night	37 °C	dry open microtiter plate in incubator			
10 min	rt	wash three times with PBST, 150 µl/well			
2 h	37 °C	block with 5 % BSA in PBS, 120 µl/well			
10 min	rt	wash three times with PBST, 150 µl/well			
all previous steps can be performed one day in prior					
5 min	rt	fill with PBS, 50 µl/well	12 min	rt	harvest cells centrifuge 10 min at 2000 rpm
10 min	rt	inhibitor solution, highest conc. in well A-C 2, 50 µl/well, serial dilution	13 min	rt	wash cells with PBS
			5 min	rt	suspend to, 2 mg/ml in PBS
5 min	rt	add *E. coli* suspension 50 µl/well			
45 min	37 °C	incubate in closed container			
10 min	rt	wash three times with PBS, 150 µl/well			
5 min	rt	fill with PBS, 100 µl/well			
measure fluorescence intensity at 485nm/535nm					
altogether 1h, 25 min					

Abbreviations: BSA: bovine serum albumin, LB: lysogeny broth, NHS: N-hydroxy succinimide, rpm: revolutions per minute, PBS: phosphate buffered saline, PBST: PBS + 0.05 % Tween 20.

D) ^{13}C NMR and Mass Spectra

6-Amino-4-thiahexyl α-D-mannopyranoside (**3**):





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6-*N*-(2-Methoxy-3,4-dioxocyclobuten-1-yl)amin-3-thiahexyl α-D-mannopyranoside (**5**):