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Bacterial Adhesion

Rationally Designed Chemically Modified Glycodendrimer Inhibits *Streptococcus suis* Adhesin SadP at Picomolar Concentrations

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Abstract: Host cell surface carbohydrate receptors of bacterial adhesins are attractive targets in anti-adhesion therapy. The affinity of carbohydrate ligands with adhesins is usually found in the low μм range, which poses a problem for the design of effective inhibitors useful in therapy. In an attempt to increase the inhibitory power of carbohydrate ligands, we have combined the approach of chemical modification of ligands with their presentation as multivalent dendrimers in the design of an inhibitor of streptococcal adhesin SadP binding to its galactosyl-α1-4-galactose (galabiose) receptor. By using a phenylurea-modified galabiose-containing trisaccharide in a tetravalent dendrimeric scaffold, inhibition of adhesin at a low picomolar level was achieved. This study has resulted in one of the most potent inhibitors observed for bacterial adhesins and demonstrates a promising approach to develop anti-adhesives with the potential of practical applicability.

Introduction

The superfluous and inappropriate use of antibiotics in the treatment of human infections and in the management of livestock animals has contributed to the emergence and spread of multiresistant bacterial pathogens, which constitute a serious problem for general health.[1,2] Bacterial adhesion to host cell surfaces, mediated by the binding of bacterial adhesins to cell surface carbohydrate receptors, is a key step in the establishment of colonization and biofilm formation.[3,4] Anti-adhesion compounds have thus been proposed as alternatives to antibiotics to block the initial step in the infectious process.[5–8] Treatment of pathogens with anti-adhesion drugs targeting host-specific colonization factors could prevent the selection of resistant clones, because the acquired adhesin mutants become unable to bind to the receptor and they would be eliminated by the host's innate immunity mechanisms.[9] In addition, a therapy that specifically targets only a subset of the bacteria significantly reduces the burden to the normal microbiome, in contrast to broad-spectrum antibiotics.[10,11]

Streptococcus suis, an emerging pig and human pathogen, causes meningitis and sepsis. *S. suis* binds to galactosyl-α1-4-galactose, galabiose, in the surface carbohydrates of host cells.[12] Galabiose-containing carbohydrates are present in blood group P glycolipids of mammals and in some non-mammalian glycoproteins.[13–17] Recently, an LPXTG-anchored *S. suis* surface protein was identified as the streptococcal adhesin P (SadP) responsible for binding to the galabiose-containing globotriaosylceramide (Gb3) receptor.[18]

A major obstacle to successful anti-adhesion therapy has been the low avidity of carbohydrates towards adhesins.[6] Galabiose-containing di- and trisaccharides inhibit SadP binding at micromolar concentrations[12] and would therefore most likely not be useful for the prevention of adhesion in vivo. A chemical library of galabiose derivatives[19,20] as well as galabiose dendrimers in di- to octavalent form have both displayed inhibitory powers at the nanomolar level.[21–23] We wanted to investigate whether it is possible to combine these two approaches to increase the inhibitory power of galabiose derivatives. We thus synthesized a tetravalent glycodendrimer of a C3′′-phenylurea derivative of Gb3 trisaccharide (**10**, **14**, Schemes 1 and 2) as the prototype of a novel class of inhibitors. The C3′′-phenylurea derivative **10** was selected because a C3′-phenylurea derivative of galabiose (IC_{50} = 0.04 μм) was reported to be almost eight-fold more potent than the corre-

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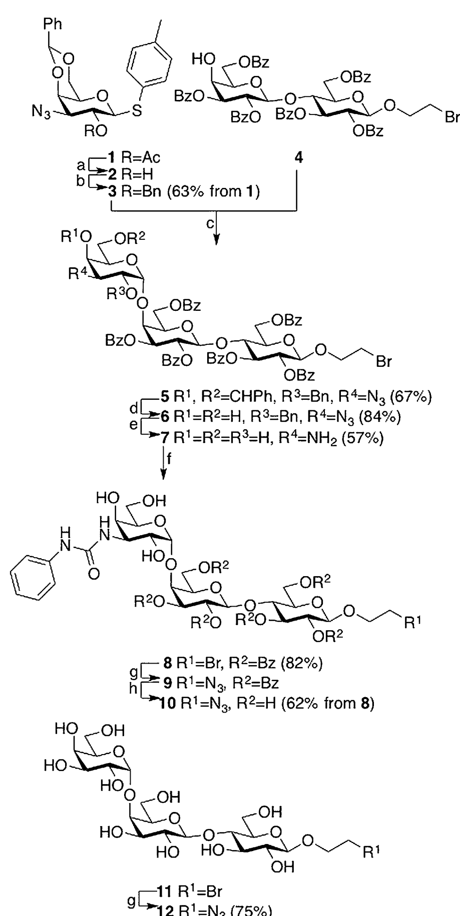


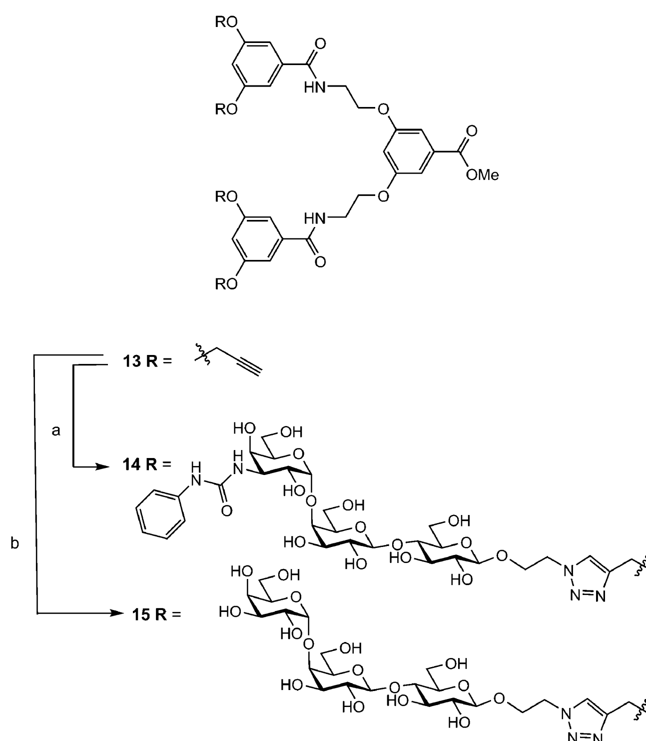
Scheme 1. Synthesis of 2-azidoethyl Gb3 glycosides **10** and **12**. a) NaOMe, MeOH, rt, overnight; b) BnBr, DMF, NaH, 0 °C, 2 h; c) NIS, TMSOTf, CH_2Cl_2, MS 4 Å, 0 °C, 3 h; d) 90 % AcOH, 80 °C, 2 h; e) Pd/C, AcOH, H_2 atm., 2 days; f) phenylisocyanate, Na_2CO_3, THF, rt, 30 min; g) NaN_3, DMF, 60 °C, overnight; h) NaOMe, MeOH, rt, overnight, DOWEX H^+ resin.

sponding parent galabiose derivative (IC_{50} = 0.31 μM) in hemagglutination inhibition experiments with *S. suis*.[20]

Results and Discussion

Synthesis of oligosaccharides and dendrimers

The synthesis of the Gb3 derivatives and the dendrimer conjugates is described in Schemes 1 and 2. Glycosylation of the 2-bromoethyl lactoside **4**,[24] carrying a primary bromide glycon amenable for later stage derivatizations, with a phenylthio 3-azido-2-*O*-benzyl-galactoside donor **3** and *N*-iodosuccinimide (NIS)/TMSOTf as the promotor system[25] gave the 3′-azido globotrioside derivative **5**. The galactosyl donor **3** was readily obtained from the known corresponding 2-*O*-acetate **1**[26] in two steps involving transesterification of the acetate to give **2**, followed by 2-*O*-benzylation. Hydrolysis of the benzylidene acetal of **5** with 90 % aq. acetic acid at 80 °C gave **6**, which was hydrogenated over Pd/C in AcOH to give the amine **7**. The crude amine **7** was reacted with phenylisocyanate in the presence of sodium carbonate in THF to afford compound **8**. The primary bromide in **8** was substituted with azide to give **9**, followed by



Scheme 2. Synthesis of Gb3 and phenylurea-substituted Gb3 dendrimers **14** and **15**. a) **10**, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, DMF/H_2O 9:1, 47 %; b) **12**, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, DMF/H_2O 9:1, 38 %.

de-*O*-benzoylation with methanolic sodium methoxide to yield the phenylurea-substituted Gb3 derivative **10**. The azidoethyl glycoside of globotriose **12** was synthesized by azide substitution of the corresponding bromide **11**.[27] Both globotriose derivatives **10** and **12** were conjugated to the tetravalent dendrimer **13** by Cu-catalyzed azide–alkyne cycloaddition (CuAAC), yielding the pure conjugates **14** and **15** after preparative HPLC purification (Scheme 2).

Purification and analysis of SadP recombinant proteins

The purified SadP proteins were analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), native PAGE, and gel filtration (Figure 1). The purified proteins run in the SDS-PAGE with a mobility corresponding to a size of 150 kDa for full length SadP and 40 kDa for its N-terminal domain (Figure 1 a). The mobilities in native gel electrophoresis indicated that the apparent molecular size of full-length SadP would be 440 kDa and that of the N-terminal domain 60 kDa (Figure 1 b). Gel filtration chromatography indicated that full-length SadP has a size of 490 kDa suggesting that it could be an oligomer (Figure 1 c). The apparent M_W of the N-terminal domain of SadP was 73 kDa, close to the expected M_W for a dimer.

Modeling of SadP interaction with Gb3 (12) and phenylurea-Gb3 (10) derivatives

Our previous findings suggested that the SadP galabiose combining site can adopt substituents in the terminal galactose 3′′-

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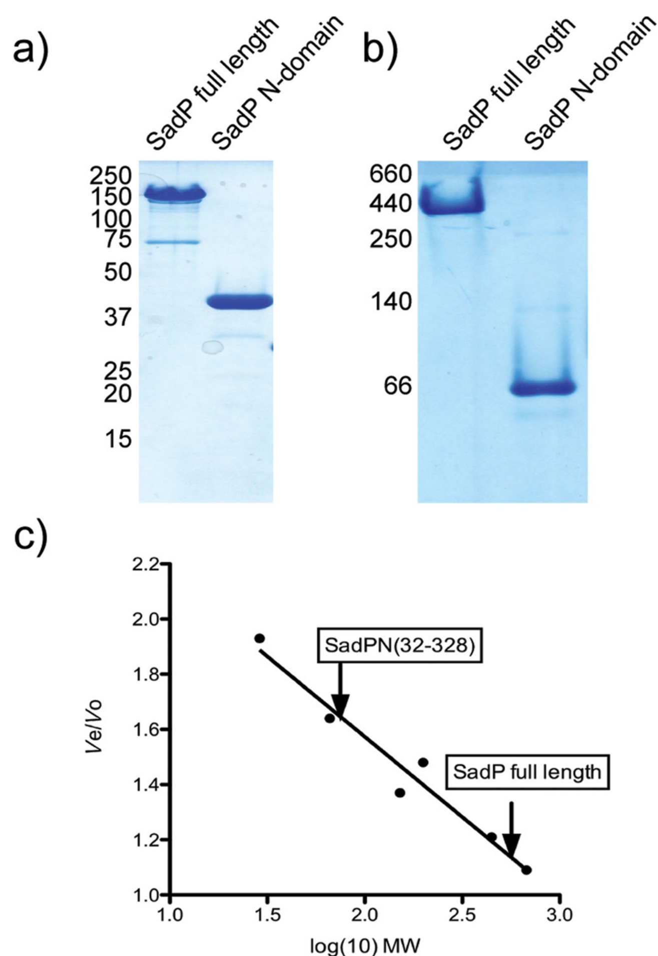

Figure 1. Molecular size of the recombinant adhesins. a) SDS-PAGE and b) native PAGE of C-terminally 6×His-tagged full-length SadP and N-terminally 6×His-tagged galabiose-binding domain of SadPN(31-328); c) gel filtration analysis with HiPrep Sephadex 16/60 column. Gel filtration molecular size markers (Sigma) 29, 66, 150, 200, 443, 669 kDa.

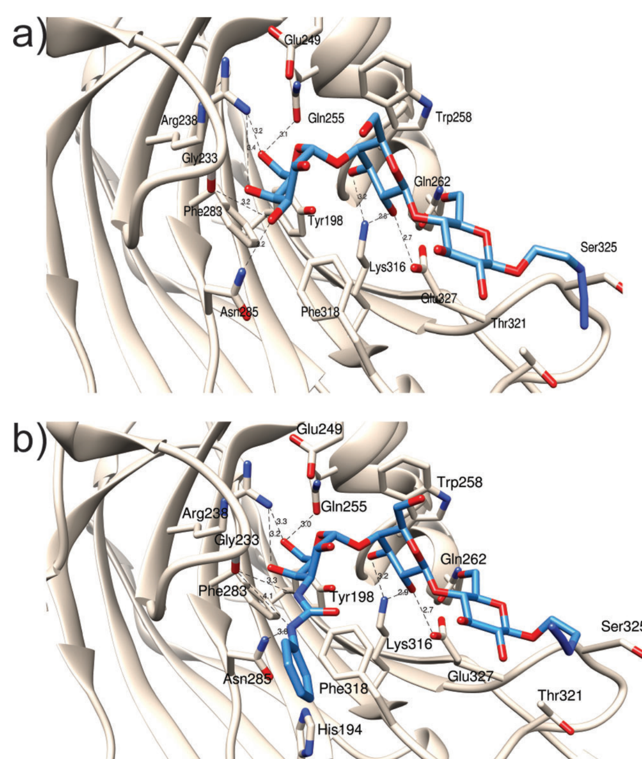

Figure 2. Modeling of SadP binding to a) Gb3 (**12**) and b) phenylurea-Gb3 (**10**) derivatives.

OH group[12,20] and thus this site could be used in the rational design of high-affinity inhibitors. The interaction mechanisms at the atomic level between the SadP galabiose-binding pocket and the unmodified Gb3 trisaccharide derivative **12** on one hand and its phenylurea derivative **10** on the other hand (Figure 2) were therefore modeled with Chimera by using the published crystal structure of factor H-binding protein (Fhb; 100% identity to SadP; PDB id: 5BOA).[28] The predicted binding of **12** is presented in Figure 2a and shows that SadP interacts with galabiose's hydroxyls HO-4′′, HO-6′′, HO-2′, and HO-3′ facing the SadP ligand binding groove, whereas glucose hydroxyls are not critically involved in the binding to **12**, which is in line with the previous studies with Gb3 deoxy analogs.[29] The docking of phenylurea-Gb3 derivative **10** suggests (Figure 2b) that the phenylurea group protrudes out of the binding pocket to avoid clashes with residues at the binding site. The urea's NH groups are at appropriate distances to interact with the SadP Gly233 carbonyl oxygen, contributing to the binding of **10**. In addition, Phe318 and His194 may stabilize the phenyl ring of phenylurea through aromatic stacking interactions, thus improving the affinity of the compound. The NH groups are situated further away from Asn285 owing to repulsive forces. It is possible, however, that water molecules could mediate interactions between Asn285 and urea's NH groups to optimize the binding of derivative **10**. The model is in agreement with previous findings on the binding inhibition with natural oligosaccharides and derivatives[12,20] and justifies the selection of the phenylurea derivative **10** for the design of a multivalent inhibitory construct **14**.

Picomolar inhibition activity of the dendrimers 14 and 15 to SadP

In attempts to determine the inhibitory power of the dendrimers **14** and **15**, it became evident that conventional methods, such as solid-phase binding assays used before,[12,18] were not sensitive enough to characterize compounds with extraordinarily low IC_{50} values. To increase the sensitivity to detect Galα1-4Gal binding activity when using picomolar concentrations of the adhesin, an amplified luminescent proximity homogeneous assay (AlphaScreen, Perkin Elmer) was set up by using recombinant 6×His-tagged SadP and a pigeon ovomucoid, which contains terminal galabiose in its N-glycans. The principle of the method is outlined in Figure 3a. The detection of the 6×His-tagged adhesin–biotinylated glycoprotein complex is based on their attachment to donor beads that are labeled with streptavidin and acceptor beads labeled with an anti-6× His antibody. The assay can detect interaction based on energy transfer if the beads are drawn to a distance of ≤200 nm. The assay conditions of AlphaScreen were optimized and adjusted

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to be able to use picomolar concentrations of SadP (Figure S1 a,b in the Supporting Information), which allows the determination of the low dendrimer IC_{50} values. Biotinylated pigeon ovomucoid (1 nM) and 6×His-tagged N-terminal domain of SadP (250 pM) were used in the inhibition assay. The carbohydrate inhibition assay was validated with less potent inhibitors active in mM concentrations and negative controls (Figure S1 c in the Supporting Information) and showed that 80 mM galactose almost completely inhibited SadP interaction, whereas lactose and mannose only partially inhibited and glucose was a negative inhibitor, which correlates well with earlier findings.[12] The assay identified IC_{50} values for the monovalent oligosaccharides in the micromolar range and for the dendrimers in the nano- to low picomolar range (Figure 3 b).

The inhibitory powers of the dendrimers compared with the monovalent unmodified trisaccharide **12** were 276-fold for the Gb3-dendrimer **15** and 26 500-fold for the modified phenylurea-C3′′-Gb3 dendrimer **14** (Table 1). The corresponding inhibitory power of **14** compared with phenylurea-modified trisaccharide **10** was 2790-fold (Table 1). Thus, as a result of the C3′′-substitution, the phenylurea-modified dendrimer **14** was 100-fold more potent as an inhibitor than the Gb3 dendrimer **15** and exhibited an IC_{50} of 26 pM.

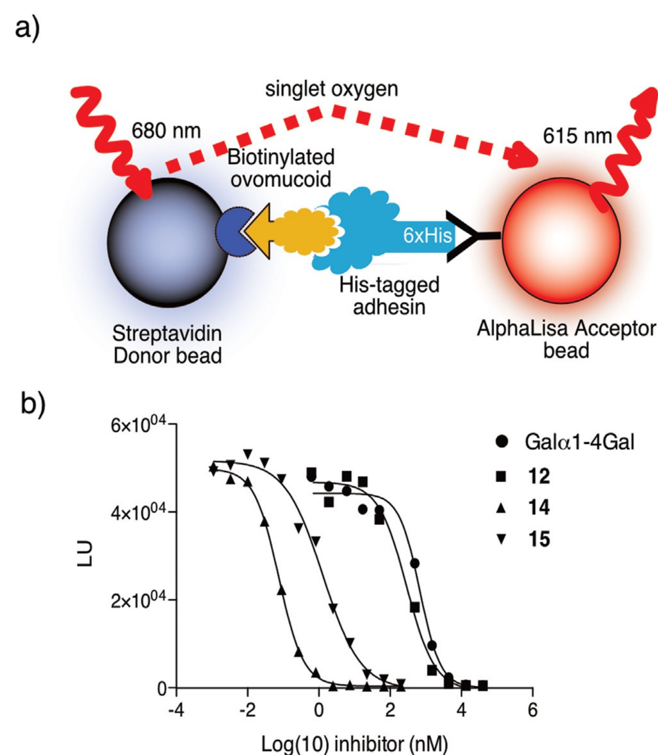

Figure 3. a) Scheme of the experimental setup for homogenous SadP–ovomucoid binding interaction analyzed with AlphaScreen. Streptavidin donor beads are illuminated with 680 nm, the photosensitizer phthalocyanine converts oxygen to reactive singlet oxygen $^{1}O_2$, and the energy is transferred to a thioxene contained in the acceptor beads, which emit luminescence at 615 nm. b) Competitive inhibition by using SadPN(31-328). Inhibitors were added in three-fold dilutions, 40 000 to 0.06 nM for monovalent oligosaccharides Galα1-4Gal and **12** and 200 to 0.001 nM for dendrimers **14** and **15**. The *x*-axis is log(10) of inhibitor concentration and the *y*-axis is the luminescent signal. Oligosaccharide derivatives phenylurea-C3′′-Gb3 dendrimer **14** (black up-pointing triangle), Gb3 dendrimer **15** (black down-pointing triangle), monovalent trisaccharide **12** (black square), and monovalent reference galabiose (Galα1-4Gal) disaccharide (black circle).

Table 1. Inhibitory powers of synthetic dendrimers of the SadP–galabiose interaction.

	IC_{50} [nM] ± SD	Inhibitory power	Power [mol_{Gb3}^{-1}]
Gb3 (**12**)	690.0 ± 316	1	1
Ph-urea-Gb3 (**10**)	72.5 ± 5.9	9.5	9.5
4-valent Gb3 (**15**)	2.5[a] ± 1.1	276	69
4-valent Ph-urea-Gb3 (**14**)	0.026[a] ± 0.020	26500	6630

[a] $P=0.0054$. Compounds **10** and **12** were measured in triplicate and **14** and **15** in quadruplicates by using separate protein purifications for each measurement.

The glycodendrimers **14** and **15** inhibited bacterial adhesion-receptor interaction at low picomolar concentrations, an affinity range seen with toxins,[7] but not to the same degree for bacterial anti-adhesives. Several hypotheses exist for the mechanisms that cause the multivalent effect.[30] A chelating effect model assumes that the four phenylurea moieties of **14** interact simultaneously with a multivalent SadP adhesin, which increases the avidity of the interaction. This can lead to large potency enhancements, already for a divalent system, of 3 to 4 orders of magnitude.[31] Alternatively, statistical rebinding of the phenylurea moieties of **14** is an option owing to the proximity of the receptor analogs in the dendrimer **14** scaffold, which would increase the probability of the adhesin to interact with the ligands, thus enhancing potency, yet usually not to the same magnitudes as chelation. The chelating effect model is supported by the molecular size measurements. In the native-PAGE and gel filtration analysis (Figure 1 b,c), the apparent molecular size of the N-terminal domain is indicated to be a dimer, which supports the chelating effect model. Observations with whole *S. suis* bacteria and divalent inhibitors show increased inhibitory power compared with monovalent galabiose.[19,22] Also, the crystal structure of the adhesin has six adhesin molecules in the asymmetric unit, suggesting that oligomer formation is possible.[28] Furthermore, the oligomerization of full-length SadP (Figure 1 b,c) indicates that multivalent interactions could increase the inhibitory power of the dendrimeric inhibitors. The interaction of SadP with the dendrimers **14** and **15** is most likely due to chelation with a contribution of statistical rebinding, the latter being especially likely owing to mismatch of the valency between the divalent protein and tetravalent ligand. Such a mismatch may favor ligand rebinding as ligand trisaccharide moieties do not need to unbind a binding site prior to rebinding a different site.

Conclusion

The 100-fold inhibitory power between the tetravalent phenylurea-modified Gb3 and the unmodified tetravalent Gb3 was ten times more compared with the 9.5-fold inhibitory power between the corresponding monovalent oligosaccharides, sug-

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gesting a potency enhancement owing to the use of phenylurea modification. The 26 500-fold inhibitory power and the picomolar IC_{50} of the tetravalent phenylurea-C3′′-deoxy-Gb3 dendrimer **14** compared with monovalent unmodified trisaccharide **12** suggests that combining the chemical modification of oligosaccharides with dendrimer approaches can lead to strong synergistic multivalency effects on affinities and thus opens a new pathway for the design of anti-adhesives that could be applied for the prevention and treatment of bacterial infections.

Experimental Section

General synthetic methods

All solvents were dried prior to use according to standard methods. Commercial reagents were used without further purification unless otherwise stated. Analytical TLC was performed on Silica Gel 60-F254 (Merck) with detection by fluorescence and by charring following immersion in a 10% ethanolic solution of sulfuric acid. Flash chromatography was performed with silica gel 60 (40–60 μm). Preparative reverse-phase (RP)-HPLC was performed with a Waters 600 Series HPLC with Waters Symmetry C18 column, 5 μm, 19×100 mm, gradient of MeCN in H_2O (0.1% TFA). Optical rotations for the final compounds were measured at the sodium D-line at ambient temperature, with a Perkin Elmer 141 polarimeter. ^1H and ^{13}C NMR spectroscopy was recorded with a Bruker Avance 400 MHz spectrometer by using residual $CHCl_3$ or CHD_2OD peaks as internal standards with a reference to Me_4Si. HRMS was determined by direct infusion with a Waters XEVO-G2 QTOF mass spectrometer using electrospray ionization (ESI) and with a Shimadzu Axima-CFR (MALDI-TOF).

Synthesis of phenylurea-Gb3 10 and unmodified Gb3 12 2-azidoethyl glycosides

***p*-Tolyl 3-azido-3-deoxy-2-*O*-benzyl-4,6-*O*-benzylidene-1-thio-α-D-galactopyranoside (3)**: A solution of *p*-tolyl 2-acetyl-3-azido-3-deoxy-4,6-*O*-benzylidene-1-thio-α-D-galactopyranoside **1**[26] in MeOH was treated with 0.5 M NaOMe and stirred at room temperature for overnight, neutralized with DOWEX H^+ resin, filtered, evaporated under reduced pressure, and dried under vacuum to give crude **2**. The crude **2** (0.78 g, 1.95 mmol) was dissolved in dry DMF (5 mL) at 0 °C, and NaH (0.18 g, 7.8 mmol) and BnBr (0.34 mL, 2.84 mmol) were added. The reaction mixture was stirred for 2 h, neutralized with MeOH, and solvents were evaporated in vacuo. The residue was dissolved in CH_2Cl_2 (50 mL) and washed with H_2O (100 mL). The organic layer was collected, dried over anhydrous Na_2SO_4, and concentrated. Flash chromatography (heptane/EtOAc 3:2) as an eluent gave **3** (602 mg, 63%). $[\alpha]^{20}_D$: −11.5 (*c* = 1.2 in $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ = 7.51 (d, *J* = 8.4 Hz, 2 H, ArH), 7.42 (m, 10 H, ArH), 7.04 (d, *J* = 8.0 Hz, 2 H, ArH), 5.57 (s, 1 H, C*H*Ph), 4.91 (d, *J* = 10.0 Hz, 1 H, C*H*₂Ph), 4.66 (d, *J* = 10.0 Hz, 1 H, C*H*₂Ph), 4.63 (d, *J* = 9.6 Hz, 1 H, H-1), 4.40 (dd, *J* = 1.6, 12.4 Hz, 1 H, H-6), 4.23 (dd, *J* = 3.2, 0.8 Hz, 1 H, H-4), 4.05 (dd, *J* = 1.6, 12.4 Hz, 1 H, H-6), 3.92 (t, *J* = 9.6 Hz, 1 H, H-2), 3.56 (dd, *J* = 3.6, 10.0 Hz, 1 H, H-3), 3.46 (d, *J* = 0.8 Hz, 1 H, H-5), 2.33 ppm (s, 3 H, SCH_3); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 137.9, 137.6, 137.5, 133.0, 129.8, 129.2, 128.6, 128.5, 128.2, 128.1, 126.5, 101.2 (*C*HPh), 87.1, 75.1, 75.0, 74.7, 70.0, 69.3, 65.1, 21.2 ppm; HRMS (ESI-TOF): *m/z* = 512.1622 (*M* + Na; $C_{21}H_{29}BrNaO_{11}$ requires *m/z* = 512.1620).

2-Bromoethyl 3-azido-3-deoxy-2-*O*-benzyl-4,6-*O*-benzylidene-α-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (5): Molecular sieves (4 Å, activated, 1.5 g) were added to a solution of lactoside acceptor **4**[24] (0.91 g, 0.85 mmol) and compound **3** (0.5 g, 1.02mmol) in CH_2Cl_2 and the mixture was stirred under N_2 for 1 h. The mixture was then cooled to 0 °C and NIS (0.31 g, 1.36 mmol) and TMSOTf (0.1 equiv) were added. The reaction temperature was increased to room temperature. The progress of the reaction was followed with TLC (12:1 toluene/EtOAc) and mass spectrometry. After 2 h, TLC showed the complete disappearance of the donor **3** and formation of the desired product **5**. The reaction mixture was diluted with dichloromethane and filtered through Celite. The dichloromethane solution was washed successively with 10% aq. $Na_2S_2O_3$ and 1 M aq. $NaHCO_3$, dried (Na_2SO_4), and concentrated. Flash chromatography (SiO_2; toluene/EtOAc, 12:1 to 6:1 gradient) gave **5** (820 mg, 67%). $[\alpha]^{20}_D$: +25 (*c* = 1.0 in $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ = 8.07–8.05 (m, 2 H, ArH), 8.01–7.94 (m, 8 H, ArH), 7.82–7.80 (m, 2 H, ArH), 7.63–7.28 (m, 23 H, ArH), 7.19–7.13 (m, 5 H, ArH), 5.79 (t, *J* = 9.2 Hz, 1 H, H-3), 5.64 (t, *J* = 10.8 Hz, 1 H, H-2′), 5.44 (m, 2 H, C*H*Ph, H-2), 5.00 (dd, *J* = 2.8, 8.4 Hz, 1 H, H-3′), 4.85 (d, *J* = 8.0 Hz, 1 H, H-1′), 4.82 (d, *J* = 3.2 Hz, 1 H, H-1′′), 4.75 (d, *J* = 7.6 Hz, 1 H, H-1), 4.65 (dd, *J* = 12.0, 1.6 Hz, 1 H, H-6′), 4.56–4.48 (m, 3 H, C*H*₂Ph, H-6′), 4.31–4.20 (m, 4 H, H-4′, H-4, H-6′′, H-4′′), 4.11–4.02 (m, 2 H, H-6′′, OC*H*₂CH₂), 3.96–3.92 (m, 2 H, H-2′′, H-3′′), 3.79 (m, 1 H, H-5′), 3.70 (m, 2 H, H-5, H-6), 3.66 (m, 2 H, H-5′′, OC*H*₂CH₂Br), 3.38 ppm (m, 3 H, H-6, OCH₂C*H*₂Br); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 165.26, 165.21, 137.36, 137.08, 133.74, 133.65, 133.42, 133.37, 133.31, 133.27, 133.16, 130.04, 129.79, 129.71, 129.69, 129.60, 129.57, 129.55, 129.44, 129.27, 129.23, 128.99, 128.96, 128.92, 128.85, 128.7, 128.62, 128.57, 128.50, 128.45, 128.41, 128.38, 128.29, 128.19, 128.13, 128.04, 126.06, 101.13 (*C*HPh), 100.86 (C-1′), 100.59 (C-1), 100.26 (C-1′′), 76.17, 75.41, 74.12, 74.01, 73.18, 73.14, 72.89, 72.68, 71.69, 69.67, 69.59, 68.92, 63.34, 62.27, 61.54, 58.79, 29.46, 28.53 ppm; HRMS (ESI-TOF): *m/z* = 1460.3433 (*M* + Na; $C_{76}H_{68}N_3O_{21}NaBr$ requires *m/z* = 1460.3426).

2-Bromoethyl 3-azido-3-deoxy-2-*O*-benzyl-α-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-galactopyranosyl-(1→4) 2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (6): Compound **5** (0.8 g, 0.57 mmol) in 90% aq. AcOH (10 mL) was stirred at 90 °C for 2 h until TLC (*n*-heptane/EtOAc, 1:1) showed complete conversion of the starting material to a slower moving spot. The solvents were co-evaporated in vacuo with toluene and the syrupy residue was purified by flash chromatography (heptane/EtOAc, 3:1) to give **6** (630 mg, 84%). $[\alpha]^{20}_D$: +15.2 (*c* = 1.0 in $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz): δ = 8.07–8.04 (m, 4 H, ArH), 7.97–7.91 (m, 6 H, ArH), 7.86–7.83 (m, 2 H, ArH), 7.62–7.28 (m, 18 H, ArH), 7.10–7.00 (m, 5 H, ArH), 5.76 (t, *J* = 9.6 Hz, 1 H, H-3), 5.55 (dd, *J* = 8.0, 7.6 Hz, 1 H, H-2′), 5.43 (dd, *J* = 7.6, 8.0 Hz, 1 H, H-2), 5.13 (dd, *J* = 10.8, 2.4 Hz, 1 H, H-3′), 4.80 (d, *J* = 8.0 Hz, 1 H, H-1′), 4.72 (d, *J* = 8.0 Hz, 1 H, H-1), 4.62–4.59 (m, 2 H, H-1′′, H-6), 4.50–4.30 (m, 4 H, H-6, H-6′, C*H*₂Ph), 4.24 (t, *J* = 9.6 Hz, 1 H, H-4), 4.04–4.00 (m, 3 H, H-4′, H-4′′, H-6′), 3.94–3.86 (m, 3 H, H-6, H-6′′, C*H*₂CH₂Br), 3.82–3.74 (m, 4 H, H-5, H-5′, H-6′′, OC*H*₂CH₂), 3.68 (t, *J* = 10.4 Hz, 1 H, H-3′′), 3.35–3.31 (m, 2 H, OCH₂C*H*₂Br), 3.19 ppm (dd, *J* = 2.8, 10.4 Hz, 1 H, H-4′′); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 165.79, 165.76, 165.69, 165.65, 165.13, 165.10, 137.3, 133.6, 133.5, 133.41, 133.37, 133.3, 133.2, 129.9, 129.8, 129.72, 129.69, 129.52, 129.51, 129.36, 129.34, 129.2, 128.7, 128.6, 128.53, 128.47, 128.40, 128.30, 128.1, 128.0, 101.3 (C-1′), 100.5 (C-1), 97.9 (C-1′′), 75.9, 75.2, 73.5, 73.4, 73.24, 73.18, 72.6, 71.5, 70.5, 69.9, 69.7, 69.4, 62.8, 62.2, 61.1, 29.4 ppm; HRMS (ESI-TOF): *m/z* = 1372.3127 (*M* + Na; $C_{69}H_{64}N_3O_{21}NaBr$ requires *m/z* = 1372.3113).

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2-Bromoethyl 3-amino-3-deoxy-α-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-galactopyranosyl-(1→4) 2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (7): A solution of **6** (0.6 g, 0.466 mmol) in AcOH (24 mL) and Pd/C (10%, 200 mg) was hydrogenated (H_2, 1 atm) for 2 days at room temperature. The reaction was followed by mass spectrometry. After the completion of the reaction, Pd/C was filtered over Celite and the solution was divided into three parts and dried under reduced pressure and then in vacuo to give compound **7** (312 mg, 57%), which was used for the next step without any further purification.

2-Bromoethyl 3-phenylurea-3-deoxy-α-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (8): The crude compound **7** (0.1 g, 0.08 mmol) was dissolved in THF (5 mL), Na_2CO_3 (58 mg, 0.55 mmol) was added, followed by phenylisocyanate (0.087 mL, 0.82 mmol) and stirred at room temperature. After 30 min, the solution was concentrated and purified by flash chromatography (CH_2Cl_2/MeOH, 9:1) to give compound **8** (89 mg, 82%). $[\alpha]^{20}_{D}$: +10.5 (c=1.0 in $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): δ=8.07–7.87 (m, 13H, ArH, N*H*), 7.61–7.54 (m, 2H, ArH), 7.49–7.31 (m, 17H, ArH), 7.24 (m, 2H, ArH), 7.17 (m, 2H, ArH), 6.97 (m, 1H, ArH), 5.80 (m, 2H, H-3, H-2′), 5.42 (dd, J=8.0, 9.6 Hz, 1H, H-2), 5.28 (dd, J=2.4, 8.4 Hz, 1H, H-3′), 4.98 (d, J=3.2 Hz, 1H, H-1′′), 4.88 (d, J=7.6 Hz, 1H, H-1′), 4.60 (m, 2H, H-1, H-6′), 4.41 (m, 3H, H-6′, H-6, H-4′), 4.19 (br s, 1H, H-4), 4.06–3.93 (m, 5H, H-6, H-5′′, OCH_2CH_2Br, H-2′′), 3.81–3.70 (m, 3H, H-4′′, H-5′, H-6′′), 3.62 (m, 1H, H-5), 3.46 ppm (m, 4H, H-6′′, H-3′′, OCH_2CH_2Br); ^{13}C NMR ($CDCl_3$, 100 MHz): δ=166.3, 165.8, 165.73, 165.71, 165.6, 165.2, 157.3, 139.0, 133.9, 133.6, 133.5, 133.4, 133.2, 129.8, 129.71, 129.66, 129.64, 129.55, 129.4, 129.3, 129.04, 128.96, 128.8, 128.59, 128.58, 128.55, 128.51, 128.45, 128.3, 122.9, 119.8, 101.0 (C-1′), 100.9 (C-1), 99.9 (C-1′′), 76.3, 73.7, 73.5, 73.4, 73.0, 72.8, 71.6, 70.5, 70.2, 70.0, 69.0, 62.5, 62.2, 61.4, 52.4, 29.4 ppm; HRMS (ESI-TOF): m/z=1353.3286 (M+H; $C_{69}H_{66}N_2O_{22}Br$ requires m/z=1353.3291).

2-Azidoethyl 3-phenylurea-3-deoxy-α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (10): Compound **8** (50 mg, 0.037 mmol) was dissolved in DMF (5 mL), NaN_3 (5 mg, 0.074 mmol) was added, and the reaction mixture was stirred overnight at 60 °C. After the completion of the reaction, the solvent was evaporated. The crude compound **9** (34 mg, 0.025mmol) was dissolved in MeOH (5 mL), 0.5 M NaOMe was added, and the mixture was stirred overnight at room temperature. After the TLC and mass spectrum showed complete conversion of the starting material, the reaction was neutralized with DOWEX H^+ resin, filtered, evaporated, and purified by preparative RP-HPLC to afford compound **10** (11.4 mg, 64%) as a white amorphous solid. Purity was 95.2% according to analytical UHPLC (Waters Acquity system, column Waters Acquity CSH-C18 1.7 μm, 2.1×100 mm, 0.5 mL min^{-1} H_2O/MeCN gradient 3–95% over 9.5 min with 0.1% formic acid, column temperature 60 °C, detection at 254 nm). $[\alpha]^{20}_{D}$: +7.3 (c=1.0 in MeOH); ^{1}H NMR (CD_3OD, 400 MHz): δ=7.37 (t, J=8.8 Hz, 2H, ArH), 7.26 (t, J= 8.4 Hz, 2H, ArH), 6.98 (t, J= 7.6 Hz, 1H, ArH), 5.00 (d, J=3.6 Hz, 1H, H-1′′), 4.43–4.40 (m, 2H, H-1, H-3′), 4.40 ppm (d, J=7.6 Hz, 1H, H-1′); ^{13}C NMR (CD_3OD, 100 MHz): δ=156.9, 139.6, 128.4, 128.2, 122.0, 118.7, 104.0, 102.9, 100.5, 79.6, 77.6, 75.3, 75.1, 75.0, 73.4, 73.2, 71.3, 71.2, 68.5, 68.1, 67.7, 61.6, 60.5, 60.0, 51.6, 50.6 ppm; HRMS (ESI-TOF): m/z= 714.2453 (M+Na; $C_{27}H_{41}N_5O_{16}Na$ requires m/z=714.2466).

2-Azidoethyl α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (12): Compound **11**[27] (0.16 g, 0.26 mmol) was dissolved in DMF (15 mL), NaN_3 (34 mg, 0.524 mmol) was added, and the reaction mixture was stirred overnight at 60 °C. After the completion of the reaction, solvent was evaporated and the crude material was purified by preparative RP-HPLC to give compound **12** (114 mg, 75% yield). Purity was 93.3% according to analytical UHPLC (Waters Acquity system, column Waters Acquity CSH-C18 1.7 μm, 2.1×100 mm, 0.5 mL min^{-1} H_2O/MeCN gradient 3–95% over 9.5 min with 0.1% formic acid, column temperature 60 °C, detection at 212 nm). $[\alpha]^{20}_{D}$: +47 (c=1.0 in MeOH); ^{1}H NMR (CD_3OD, 400 MHz): δ=4.96 (d, J=3.6 Hz, 1H, H-1′′), 4.43 (d, J=6.8 Hz, 1H, H-1), 4.37 (d, J=8.0 Hz, 1H, H-1′), 4.28 (t, J=6.0 Hz, 1H, H-5′′), 4.04–3.67 (m, 13H, $CH_2CH_2N_3$, H-6′, H-6′′, H-3, H-4′′, H-2′′, H-3′′), 3.60–3.51 (m, 4H, H-2, H-4, H-3′), 3.49 (m, 3H, H-3, $CH_2CH_2N_3$), 3.35 ppm (m, 1H, H-6′); ^{13}C NMR (CD_3OD, 100 MHz): δ=105.3 (H-1), 104.2 (H-1′), 102.6 (H-1′′), 81.0, 79.7, 76.5, 76.4, 76.3, 74.7, 74.6, 72.8, 72.6, 71.2, 71.0, 70.5, 69.4, 62.6, 61.9, 61.5, 52.0 ppm; HRMS (ESI-TOF): m/z=574.2099 (M+H; $C_{20}H_{36}N_3O_{16}$ requires m/z=574.2096).

Tetravalent phenylurea-modified galabioside (14): A solution of compound **13** (2.0 mg, 3.0 μmol), compound **10** (9.8 mg, 0.021 mmol), NaAsc (5.6 mg, 0.028 mmol), and $CuSO_4 \cdot 5H_2O$ (3.5 mg, 0.014 mmol) in DMF/H_2O (2 mL, 9:1) was prepared. The mixture was heated in a microwave to 80 °C for 30 min. After cooling to room temperature, the copper salts were removed by the Cuprisorb resin and filtered off. The solvents were removed under reduced pressure and the residue was purified by preparative HPLC to afford compound **12** (4.8 mg, 1.40 μmol, 47%) as a white powder. ^{1}H NMR (500 MHz, D_2O with 30% CD_3CN): δ=8.14 (s, 4H, 4×$CH_{triazole}$), 7.40–7.28 (m, 16H, 16×CH, PhNH), 7.15 (s, 2H, 2×CH, aryl), 7.06 (dd, J=7 Hz, 4H, 4×CH, PhNH), 7.01 (s, 4H, 4×CH, aryl), 6.82 (s, 3H, 3×CH, aryl), 5.18 (s, 8H, 4×$OCH_2C_{triazole}$), 4.96 (s, 4H, 4×H-1′′), 4.63 (s, 8H, 4×$N_{triazole}CH_2$), 4.44 (d, J=8 Hz, 4H, 4×H-1), 4.41–4.33 (m, 8H, 4×H-1′, 4×H-3′), 4.27–4.15 (m, 10H, 2×OCH_2), 4.12–3.95 (m, 20H, 4×$OCH_2CH_2N_{triazole}$), 3.93–3.77 (m, 25H, OCH_3), 3.76–3.71 (m, 36H, 2×CH_2NH), 3.70–3.59 (m, 22H), 3.59–3.43 (m, 16H), 3.24 ppm (t, J=8 Hz, 4H, 4×H-2′); ^{13}C NMR (125 MHz, D_2O with 30% CD_3CN): δ=161.7, 161.0, 159.1 (C, aryl), 144.8 ($C_{triazole}$) 140.7 (C, PhNH), 138.1 (C, aryl), 131.1 (CH, PhNH), 127.7, 126.9 (CH, triazole), 125.2 (CH, PhNH), 120.9 (CH, PhNH), 110.4, 108.7, 107.4 (CH, aryl), 105.3, 104.2 (C-1, C-1′), 101.9 (C-1′′), 81.1, 79.3, 77.3, 76.7, 76.3, 74.5 (C-2′), 73.1, 72.9 (C-3′), 69.7, 69.6 (OCH_2, $OCH_2CH_2N_{triazole}$), 69.2, 63.0 ($OCH_2C_{triazole}$), 62.2, 62.0, 54.2 (OCH_3), 52.3, 52.0 ($N_{triazole}CH_2$), 41.1 ppm (CH_2NH); HRMS (MALDI-TOF): m/z=3467.943 (M+Na; $C_{146}H_{198}N_{22}O_{74}Na$ requires m/z=3467.244).

Tetravalent galabioside (15): A solution of compound **13** (3 mg, 4.42 μmol), compound **12** (12 mg, 0.021 mmol), NaAsc (8.2 mg, 0.041 mmol), and $CuSO_4 \cdot 5H_2O$ (5.31 mg, 0.021 mmol) in DMF/H_2O (2 mL, 9:1) was prepared. The mixture was heated in a microwave to 80 °C for 30 min. After cooling to room temperature, the copper salts were removed by the Cuprisorb resin and filtered off. The solvents were removed under reduced pressure and the residue was purified by preparative HPLC to afford **10** (5 mg, 1.68 μmol, 38%) as a white powder. ^{1}H NMR (500 MHz, D_2O): δ=8.06 (s, 4H, 4×$CH_{triazole}$), 6.87 (s, 2H, 2×CH, aryl), 6.79 (s, 4H, 4×CH, aryl), 6.54 (s, 2H, 2×CH, aryl), 6.51 (s, 1H, CH, aryl), 5.00–4.90 (m, 12H, 4×H-1′′, 4×$OCH_2C_{triazole}$), 4.60 (br s, 8H, 4×$N_{triazole}CH_2$), 4.41 (d, J=8 Hz, 8H, 4×H-1, 4×H-1′), 4.35 (t, J=6.5 Hz, 4H, 4×H-3′), 4.26–4.19 (m, 4H, 2×OCH_2), 4.08–3.97 (m, 16H, 4×$OCH_2CH_2N_{triazole}$), 3.95–3.80 (m, 26H), 3.79–3.76 (m, 30H), 3.76–3.66 (m, 25H, OCH_3), 3.66–3.53 (m, 14H, 2×CH_2NH), 3.53–3.45 (m, 10H), 3.25 ppm (t, J=9 Hz, 4H, 4×H-2′); ^{13}C NMR (125 MHz, D_2O): δ=160.3, 159.7 (C, aryl), 143.6 ($C_{triazole}$), 134.1 (C, aryl), 126.94, 126.88, 125.9 (CH, triazole), 109.3, 107.62, 107.60, 106.1 (CH, aryl), 104.4, 103.3 (C-1, C-1′), 101.4 (C-1′′), 79.9, 78.3, 76.3, 75.7, 75.2, 73.6 (C-2′), 73.1, 71.8, 71.7 (C-3′), 69.8, 68.9 (OCH_2, $OCH_2CH_2N_{triazole}$), 67.4, 61.9 ($OCH_2C_{triazole}$), 61.3, 61.1, 53.5 (OCH_3), 52.1, 51.1 ($N_{triazole}CH_2$), 40.3 ppm (CH_2NH); HRMS (MALDI-

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TOF): $m/z = 2994.722$ ($M+Na$; $C_{118}H_{174}N_{14}O_{74}Na$ requires $m/z = 2994.028$).

Bacterial strains

Escherichia coli NovaBlue (Novagen) was used for the cloning of SadP and strain BL21(DE3) for expression of the adhesin of serotype 2 *S. suis* with subtype P_N adhesin.[12,18] The *E. coli* strains were supplemented with 100 μg mL^{-1} of ampicillin (vector SadPN(32-328)-pET46EkLIC) or with 30 μg mL^{-1} of kanamycin (vector SadP-pET28b).

Cloning of recombinant SadP constructs

The galabiose binding N-terminal domain of SadP was cloned into pET46EkLIC vector (Novagen) as follows. The primer pairs SadPDER48 (gacgacgacaagatagaatcgctagaaccagatgtt) and SadPDER49 (gaggagaagcccggtttattcttctcaagggtaatctc) were designed to clone the 882 bp fragment of the adhesin N-terminal galabiose binding domain. The fragments were amplified with Phusion HotStart II DNA polymerase and were cloned into LIC-vector (LIC, ligation independent cloning) pET46EkLIC. The ligation products were transformed into NovaBlue competent cells. Construct yielded a 33.4 kDa 6×His-tagged fusion protein, SadPN(32-328). The sequence of the N-terminal domain of SadP was verified by sequencing with T7 promoter and T7 terminator primers. The vector SadPN(32-328)-pET46EkLIC was transformed into expression strain BL21(DE3). The C-terminally tagged full-length SadP of strain D282 was cloned into vector pET28b (Novagen) NcoI and XhoI restriction sites. The gene encoding full-length adhesin was amplified with Phusion HotStart II DNA polymerase with primers SAPcS (tataccatggaatcgctagaaccag) and SAPcR (tatctcgagacttgttcgcctgtat) to produce a 2127 bp fragment for cloning into the vector pET28b to produce a vector D282-pET28b. The construct was transformed into strain BL21(DE3) to produce a C-terminally His-tagged recombinant protein.

Expression and purification of SadP

The bacteria were grown at 30 °C, 250 rpm, to an OD600 of 0.5, and the protein expression was induced with 0.2 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) for 3.5 h. Bacteria were harvested by centrifugation at 3000×g, at 4 °C, and stored at −84 °C. The recombinant protein was purified with Ni-NTA affinity chromatography (NTA = nitrilotriacetic acid). Briefly, bacteria were lysed with 0.4 mg mL^{-1} hen egg lysozyme (Sigma) in 50 mM sodium phosphate buffer pH 8.0, containing 0.5 M NaCl, EDTA-free protease inhibitor cocktail (Pierce), 20 mM imidazole, 20 μg mL^{-1} deoxyribonuclease, and 1 mM $MgCl_2$ on ice for 30 min. The lysate was sonicated to further homogenize the cell debris and was centrifuged at 20 000×g, 4 °C, for 30 min. The filtered lysate was purified with Ni-NTA affinity chromatography at 25 °C by using a HiPrep FF column connected to Äktaprime plus (GE Healthcare). Further purification was done with a gel filtration column HiLoad 16/60 Superdex 200 (GE Healthcare) by using Tris-Cl, pH 7.5, and 0.15 M NaCl (TBS) running buffer. The molecular weights were analyzed by using gel filtration marker kits (Sigma–Aldrich, 29 000 to 700 000 Da). The calibration curve was fitted and the molecular weight of the SadP proteins was estimated by using Prism software. The purity of the recombinant proteins was analyzed with SDS-PAGE chromatography. For native gel electrophoresis, the recombinant proteins were diluted with sample buffer without SDS and were separated with 8 % polyacrylamide gel.

Amplified luminescent proximity homogeneous assay

Pigeon ovomucoid was isolated as described before[18] and was biotinylated by using Sulfo-NHS-LC-Biotin (Pierce). The ovomucoid concentration was measured with the Bradford method (Bio-Rad). The molar ratio of biotinylation was analyzed with immunopure HABA (4′-hydroxyazobenzene-2-carboxylic acid, Pierce) according to the manufacturer's protocol using hen egg avidin. The biotin/ovomucoid ratio was calculated to be 3.9 biotin mole^{-1} of ovomucoid. The labeled ovomucoid was aliquoted and stored at −20 °C. The AlphaScreen was set up by using AlphaScreen Streptavidin Donor beads and Ni-NTA Acceptor or AlphaLisa anti-6×His acceptor beads (Perkin Elmer). The assay conditions were first optimized by finding the hook point for the interaction by using the N-terminally His-tagged N-terminal domain of SadP, and biotinylated ovomucoid. The cross-titration of adhesin and glycoprotein was analyzed by setting up a matrix of proteins in 96-well AlphaPlates (Perkin Elmer) with the final concentrations of biotinylated ovomucoid of 0, 0.3, 1, 3, 10, and 30 nM and His-tagged adhesin of 0, 10, 50, 100, 300, and 500 nM in TBS (Tris-buffered saline), 0.2 % BSA (bovine serum albumin), 0.05 % TWEEN 20 (TBST–0.2 % BSA). The His-tagged adhesin (5 μL) and biotinylated ovomucoid (5 μL) at concentrations indicated above were pipetted into the wells and the plates were centrifuged for 1 min at 1000×g. The plates were incubated at 25 °C for 1 h. This was followed by addition of Ni-NTA- or anti-6×His-acceptor beads in TBST–0.2 % BSA to a final concentration of 20 μg mL^{-1} and incubation for 1.5 h. Finally, streptavidin donor beads diluted in TBST–0.2 % BSA were added to a final concentration of 20 μg mL^{-1} and the mixtures were incubated for 30 min and measured with an Ensight multimode reader (Perkin Elmer) using an excitation wavelength of 680 nm with donor beads and measurement of the emission wavelength of 615 nm from acceptor beads (Figure S2 a in the Supporting Information). To test SadP at picomolar concentrations, the hook point for SadP was tested by using constant biotinylated 1 nM pigeon ovomucoid, dilutions of His-tagged N-terminal domain of SadP, and anti-6×His acceptor beads (Figure S2 b in the Supporting Information). For inhibition assays, the compounds **10**, **14**, and **15** were diluted in TBST–0.2 % BSA buffer and were mixed with the adhesin and biotinylated ovomucoid. The binding was measured as above and the experiments were performed as duplicates. The binding inhibition data was fitted and the IC_{50} values were calculated by using Prism with settings of log(inhibitor) versus response slope (four parameters). An average of the IC_{50} values from three separate experiments was calculated.

Molecular modeling of SadP–galabiose interaction

Modeling of the SadP interaction with unmodified Gb3 trisaccharide **12** and phenylurea-Gb3 **10** were performed with Chimera[32] by using the available crystal structure of SadP (SadP designated as Fhbp[28]). Docking of Gb3 trisaccharide **12** and phenylurea-Gb3 trisaccharide **10** was performed by using Autodock Vina as implemented in Chimera.[33] Dock Prep was used through Chimera to prepare the model receptor and ligands for docking. Hydrogens were added and partial charges were assigned. The search space was defined by a grid that encompassed the site of interest as potential binding site for the compounds. The grid was adjusted manually and visualized on screen. After a docking run, AutoDock Vina generated nine different binding poses along with their respective scores. The docking results were saved, visualized in Chimera, and the SadP–ligand interactions were investigated. The best docked pose was selected based on energy considerations, plausible bond formations with the active site, the experimentally

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determined structure of Fhb with Galα1-4Gal-oligosaccharide, and the proposed binding mode of the glucose moiety.

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Conflict of Interest

The authors declare no conflict of interest.

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