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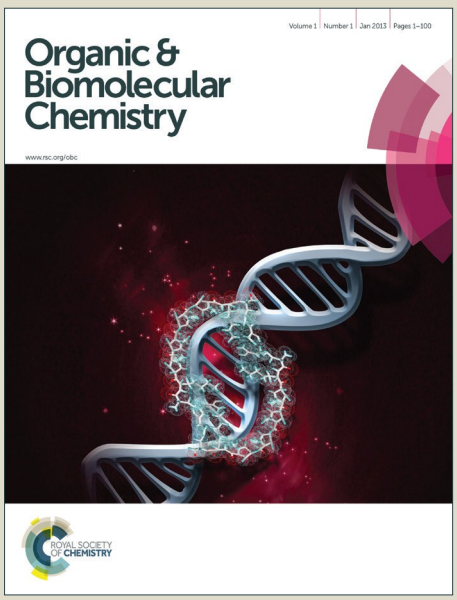

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Development and optimization of a competitive binding assay for the galactophilic low affinity lectin LecA from *Pseudomonas aeruginosa*

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Abstract

Infections with the Gram-negative bacterium *Pseudomonas aeruginosa* result in a high mortality among immunocompromised patients and those with cystic fibrosis. The pathogen can switch from planktonic life to biofilms, and thereby shields itself against antibiotic treatment and host immune defense to establish chronic infections. The bacterial protein LecA, a C-type lectin, is a virulence factor and an integral component for biofilm formation. Inhibition of LecA with its carbohydrate ligands results in reduced biofilm mass, a potential Achilles heel for treatment. Here, we report the development and optimization of a fluorescence polarization-based competitive binding assay with LecA for application in screening of potential inhibitors. As a consequence of the low affinity of D-galactose for LecA, the fluorescent ligand was optimized to reduce protein consumption in the assay. The assay was validated using a set of known inhibitors of LecA and IC_{50} values in good agreement with the known K_d values were obtained. Finally, we employed the optimized assay to screen sets of synthetic thio-galactosides and natural blood group antigens and report their structure-activity relationship. In addition, we evaluated a multivalent fluorescent assay probe for LecA and report its applicability in an inhibition assay.

Introduction

Lectins are carbohydrate-binding proteins and often play prominent roles in bacterial, viral or parasitic infections.[1-5] Microbial lectins enable infection of the host by the pathogen, whereas numerous human lectins of the innate immune system are defense molecules against pathogens. The Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* is a major threat for patients with an impaired immune system or suffering from cystic fibrosis. With 8% of all hospital-acquired infections, it is one of the most common bacteria in the health care setting.[6-8] The bacterium is highly resistant towards antibiotic treatment which is partially caused by its ability to form biofilms.[9] Bacteria embedded in such biofilms are surrounded by a self-produced matrix that shields the pathogen from host immune defense and also from antibiotic treatment, rendering bacteria 10- to 1000-fold more resistant towards antibiotics compared to the free floating planktonic state.[10-12] New therapeutic approaches therefore aim to dismantle the biofilm and thereby restore susceptibility towards antibiotics.[13,14]

LecA and LecB (formerly called PA-IL and PA-IIL) are two soluble lectins produced by *P. aeruginosa* which are important for biofilm formation.[15-17] The sequence of LecA is highly conserved among strains, whereas LecB varies in clinical isolates and two major types prevail, *i.e.*, the PAO1-type and the PA14-type.[18] In environmental isolates, a third variant is widely observed (PA7-type).[19] In addition, both lectins are virulence factors that elicit toxic effects to cells and animals and interfere with human airway ciliary beating.[20-24] Recently, LecA was shown to induce glycolipid-dependent membrane invaginations in artificial vesicles, which was proposed as an uptake mechanism for *P. aeruginosa* into human cells.[25] LecA and LecB have a binding specificity for D-galactose- and L-fucose- or D-mannose-containing carbohydrates or their glycoconjugates, respectively.[16] The crystal structures of both lectins show homotetrameric assemblies and calcium ions mediating the recognition of their carbohydrate ligands.[18,26-29]

In 2001 and 2008, patients with pulmonary *P. aeruginosa* infections were treated with an aerosol containing D-galactose and L-fucose and an efficient reduction of bacterial counts in patient sputum was reported.[30,31] Consequently, the development of inhibitors of both lectins with the aim to provide efficient anti-infectives against this pathogen is a highly active field which is summarized in recent reviews.[13,32-34] Carbohydrate-based ligands of LecA and LecB were shown to inhibit biofilm formation or dissolve established biofilms.[17,35-38] We have previously reported small drug-like glycomimetic inhibitors of the fucophilic LecB with favorable binding kinetics and thermodynamics as well as activity in a bacterial adhesion model.[18,39-43]

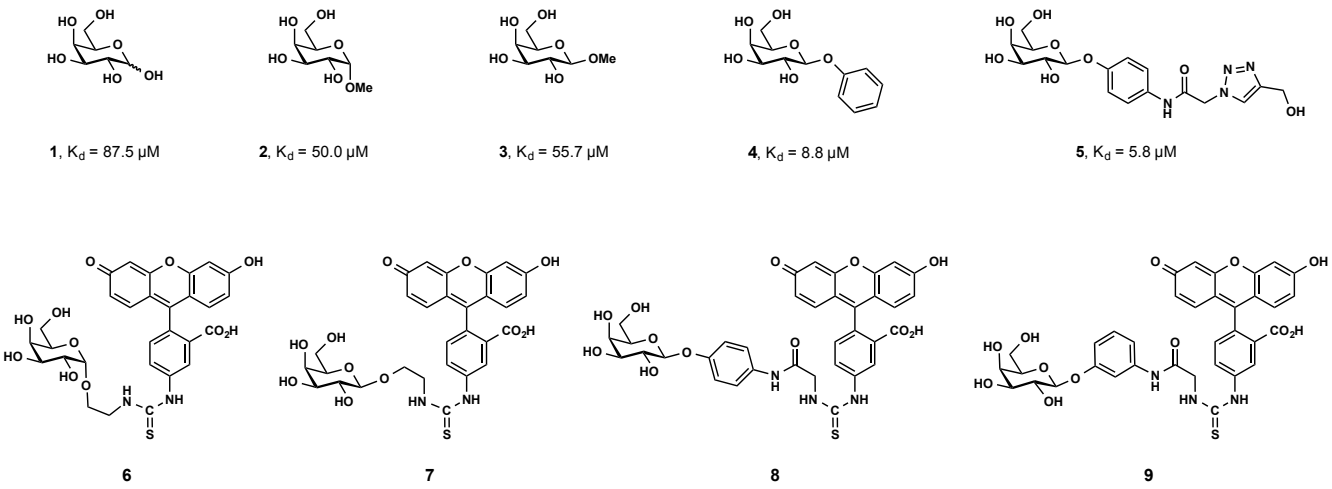


Figure 1: Structures of known LecA ligands **1-5** based on D-galactose, dissociation constants are published in the literature.[44-46] As fluorescent tracers for the competitive binding assay, we designed structures based on aliphatic galactosides **6** and **7**, and phenyl glycoside **8** derived from potent ligand **5**, as well as its *meta*-analog **9**.

The only moderate binding affinity of unmodified D-galactose (**1**, K_d = 87.5 μM, Figure 1) for LecA can be enhanced by the introduction of hydrophobic moieties at the anomeric center.[47] Introducing a methyl-group as aglycon results in modestly enhanced binding affinities of the derivatives **2** and **3**.[44,47] Adding an aromatic ring in β-position to the anomeric center (**4**, K_d = 8.8 μM) results in a tenfold

increased affinity compared to the unmodified monosaccharide **1**.[45,47] Recently, Vidal and co-workers published the monovalent triazol derivative **5** as a potent monovalent inhibitor of LecA with a binding affinity of 5.8 μM.[46] In addition, Roy and co-workers reported naphtyl and umbilliferyl thiogalactosides[44] and Reymond and co-workers described tripeptidylphenyl galactosides[36] with affinities in the same range. In general, the increased affinity of aromatic β-D-galactosides to LecA was assigned to a CH-Π interaction of a histidine residue in LecA (His50) with the aromatic glycoside ligand.[36,44,45] In addition, numerous compounds presenting galactose on various multivalent scaffolds have been reported (for a comprehensive overview, the reader is referred to two recent reviews[32,34]). Very high avidities due to their multivalency have been achieved. An optimal positioning of two galactose residues to simultaneously bind to the two adjacent carbohydrate binding sites in the LecA tetramer was achieved by Pieters and co-workers.[48,49] The proposed binding mode of such highly potent divalent ligands (K_d = 28 nM) was recently experimentally confirmed by X-ray crystallography.[50]

The potency of published LecA inhibitors is usually quantified in hemagglutination inhibition assays (HIA), in enzyme-linked lectin assays (ELLA), by surface plasmon resonance (SPR) or by isothermal microcalorimetry (ITC) and IC_{50}s or K_ds are obtained, respectively (cf. all references of the previous paragraph). SPR and ITC are low throughput methods for obtaining additional data on the kinetics and thermodynamics of binding. Although HIA and ELLA are performed in microtitre plates and can be rapidly performed in parallel, both assays have certain drawbacks. In HI assays, IC_{50} values are usually much higher than the thermodynamic K_d values and ELLAs require numerous incubation and washing steps which results in poor reproducibility.

Fluorescence polarization has emerged as a technique which allows miniaturization for high throughput and *in situ* detection of the potency of a given competitive inhibitor. Despite being established in many fields, its application in the evaluation of carbohydrate - lectin interactions has lagged behind. This is

probably due to the generally low affinity (μM - mM) of such interactions, that require high protein concentrations close to the K_d of the fluorescent tracer. Initially, the method was developed for plant lectins by Khan *et al*. in 1981[51] requiring approx. 200 μM protein. In 1995, a fluorescence polarization-based assay by Jacob *et al*.[52] required 166 μM of the human E-selectin and two decades later it was established for galectins by Leffler *et al*. in 2003 (10 μM protein concentration).[53,54] The method was then successfully developed for high affinity interactions of the *E. coli* lectin FimH at 200 nM concentration by Hultgren and co-workers[55] and by our group for the *P. aeruginosa* lectin LecB[18,39] using 150 nM LecB in the assay. This technology is now used as a routine screening method for these two high-affinity lectins only.[40-43,56]

Here, we report the development and optimization of a fluorescence polarization-based assay for the bacterial lectin LecA and its application in the evaluation of potential inhibitors.

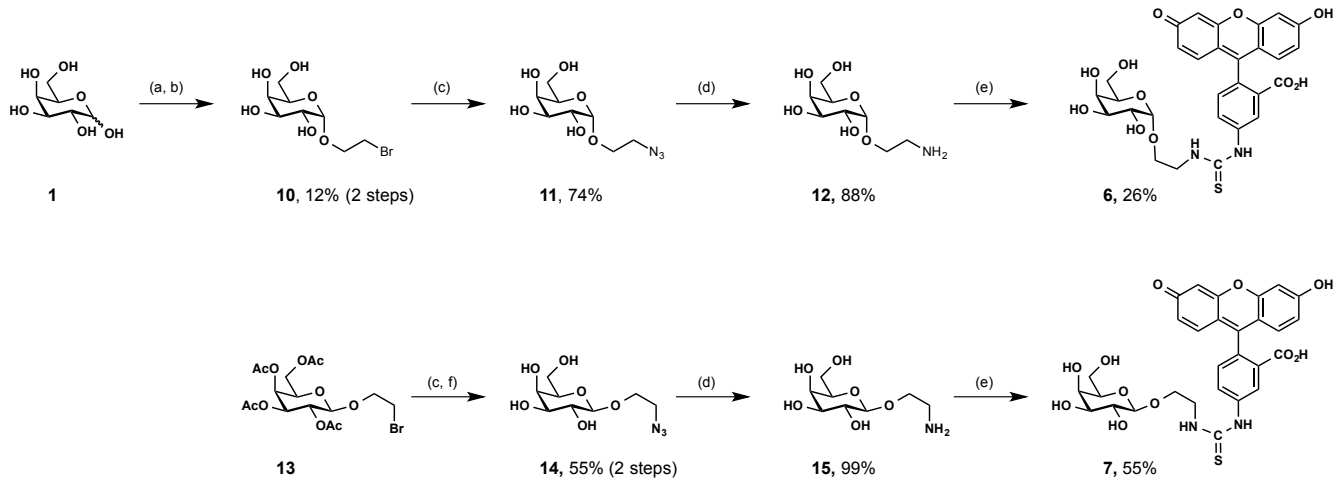
Results and Discussion

Synthesis of fluorescent reporter ligands

To establish a fluorescence polarization assay with LecA, suitable fluorescently labelled ligands were designed and synthesized. D-galactose-based probes specifically binding to the carbohydrate-binding site of LecA were conjugated to fluorescein as fluorophore. To examine the binding properties of potential reporter ligands, four different linkers were selected based on literature data of different galactosides, *i.e.*, **1**-**5** (Figure 1). In the α-galactoside **6**, the fluorescein moiety is oriented towards the solvent based on the crystal structure of LecA with **1**,[26] whereas in its β-anomer **7**, an attractive or repulsive interaction of the aglycone with the protein surface was anticipated. Since phenyl β-D-galactosides are superior inhibitors of LecA, reporter ligand **8** based on the potent LecA inhibitor **5** was designed and its *meta*-analog **9** was included in the study. The latter isomer was deduced from the

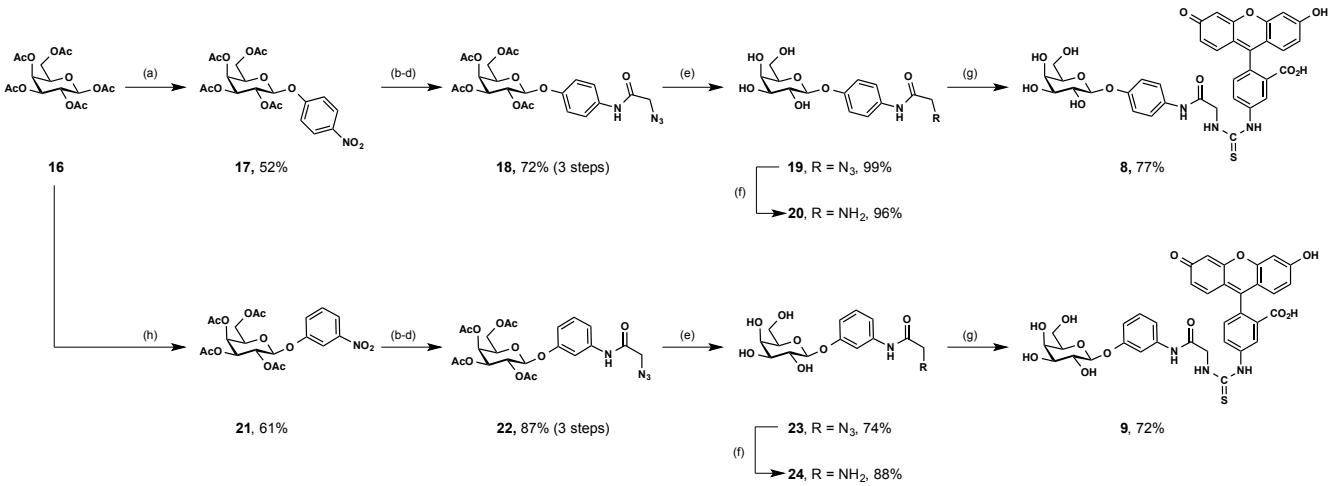
crystal structure of LecA in complex with *para*-nitrophenyl galactoside (pdb code 3ZYF[36]), indicating a solvent exposure for *para*-substituents and the possibility of contacts with the protein of substituents in *meta*-position.

For the synthesis of ethyl-linked ligand α-**6**, bromoethanol was first glycosylated with D-galactose (**1**) in a Fischer-type reaction (Scheme 1). The resulting inseparable mixture of anomers was treated with β-galactosidase to remove the β-anomer and pure α-**10** was obtained. Nucleophilic displacement of the bromide with sodium azide (**10→11**), hydrogenolysis of the azide (**11→12**) and conjugation with fluoresceine isothiocyanate (FITC) gave α-**6** in good overall yield in this protecting group-free reaction sequence (Scheme 1). The β-anomer **7** was synthesized starting from protected bromoethyl β-D-galactoside **13**,[57] treatment with sodium azide and subsequent Zemplén deprotection to the unprotected azide (**13→14**, 2 steps). After hydrogenolysis of the azide **14**, the β-ethyl linked reporter ligand (**7**) was obtained by reaction of amine **15** with fluoresceine isothiocyanate.



Scheme 1: Synthesis of α-ethyl linked reporter ligand **6** and β-ethyl linked ligand **7**. Reagents and conditions: (a) $La(OTf)_3$, 2-bromoethanol, 70 °C, 24 h, 35%; (b) *Aspergillus oryzae* β-galactosidase, aq. phosphate buffer, 37 °C, 22 h, 33%; (c)

NaN_3, DMF, 75 °C, 5-16 h; (d) H_2, Pd/C, MeOH, r.t., o.n.; (e) fluoresceine isothiocyanate, $NaHCO_3$, DMF, r.t., o.n.; (f) NaOMe, MeOH, r.t., 3 h.



Scheme 2: Synthesis of phenyl-linked reporter ligand *para*-**8** and *meta*-**9**. Reagents and conditions: (a) *p*-nitrophenol, BF_3•OEt_2, CH_2Cl_2, 0 °C- r.t., 24 h; (b) H_2, Pd/C, CH_2Cl_2, 12 h; (c) $BrCH_2COBr$, NEt_3, CH_2Cl_2, 0°C – r.t. 2 h; (d) NaN_3, nBu_4NI, DMF, 50 °C, 12 h; (e) NaOMe, MeOH, r.t., 2 h; (f) H_2, Pd/C, MeOH, r.t., 2 h; (g) fluoresceine isothiocyanate, $NaHCO_3$, DMF, r.t., 14 h; (h) *m*-nitrophenol, BF_3•OEt_2, CH_2Cl_2, 0 °C- r.t., 24 h.

The fluorescent reporter ligands with phenyl spacers **8** and **9** were synthesized starting from pentaacetyl galactose **16** (Scheme 2). For the *para*-substituted ligand **8**, *para*-nitrophenol was galactosylated to give the glycoside **17** in 52% yield. Then, a three-step one-pot reaction sequence was performed with acetylated **17** according to Cecioni *et al*.:[46] 1) reduction of the nitro group to an aniline, 2) acetylation of the aniline with bromoacetyl bromide and 3) nucleophilic substitution of the bromide with sodium azide. The resulting acetyl protected azide **18** was obtained in 72% yield over three steps after one purification. Deacetylation (**18→19**), hydrogenolysis of the azide (**19→20**), and coupling of **20** with fluoresceine isothiocyanate yielded *para*-**8** in 73% yield over 3 steps.

Following the same protocol, *meta*-**9** was obtained after glycosylation of *meta*-nitrophenol with **16** and subsequent manipulation of the arene moiety (Scheme 2). In an analogous three-step one-pot sequence, azide **22** was obtained from nitrophenyl glycoside **21** in 87% yield over three steps. The azide was deacetylated (**22**→**23**), reduced to the amine **24** and coupled with fluoresceine isothiocyanate to give *meta*-**9** in 47% over three steps.

Binding of fluorescent ligands to LecA observed by fluorescence polarization

To evaluate the binding of the synthesized galactose-derived ligands **6**-**9** to LecA, a direct titration with increasing amounts of LecA was performed (Figure 2). All four ligands showed a dose-dependent increase in fluorescence polarization with increasing concentrations of LecA. All systems showed sigmoidal curves with varying maximal fluorescence polarization. The phenyl-β-galactoside derived ligands had K_d values in the low μM-range (K_d 8.1 μM for **8**, K_d 7.4 μM for **9**). The two alkyl-linked ligands **6** and **7** possessed K_d values of 19.2 μM and 27.4 μM, respectively. This data was consistent with the literature describing increased affinities of LecA for galactoside ligands with β-linked aromatic aglycones (see Figure 1). Interestingly, the absolute signal intensities vary significantly between the different ligands from approx. 85 mP for **8** to 200 mP for **6**. It should be noted that the intensity of fluorescence polarization in the bound state, *i.e.* at high protein concentrations depends on the residual mobility of the fluorophore in the bound state (propeller effect). The higher polarization values of **6** indicated a less flexible fluorophore as observed for compound **8**. However, the amount of protein required for application in a competitive binding assay largely depends on the affinity of the fluorescent compound to the protein. Therefore, compounds **8** and **9** were superior to the alkyl linked tracers **6** and **7**.

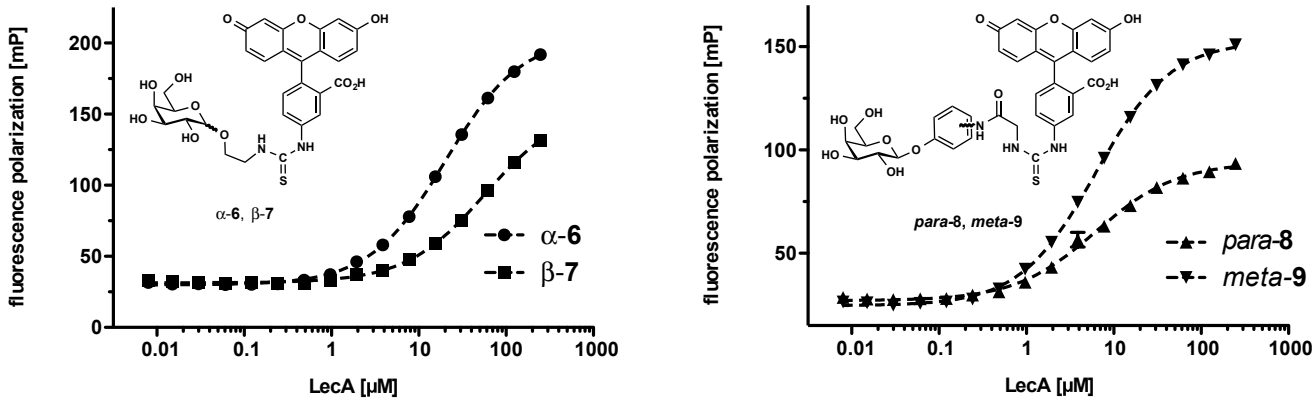


Figure 2: Titration of ethyl-linked reporter ligands **6**, **7** and phenyl-linked ligands **8**, **9** with LecA. Dissociation constants were obtained from a four-parameter fitting procedure to the dose-dependent increase in fluorescence polarization. Ethyl-linked ligands **6** (K_d 19.2 ± 4.8 μM) and **7** (K_d 27.4 ± 20.3 μM), showed weaker binding affinity than the phenyl-linked ligands **8** (K_d 8.1 ± 2.1 μM) and **9** (K_d 7.4 ± 2.8 μM). One representative titration experiment of triplicates on one plate is shown. Dissociation constants and standard deviations given were from at least three independent replicates of triplicates on three plates each.

Validation of the competitive binding assay: Determination of IC_{50} values of para- and meta-substituted phenyl β-D-galactosides

In order to determine IC_{50} values of inhibitors in competition experiments, the protein concentration should lie within a range of 30-90% of the maximal signal intensity in fluorescence polarization assays.[58,59] Due to the high binding affinity for the rigid-linked reporter ligands **8** and **9** (Figure 2), ligand **8** was chosen as assay probe with the aim to reduce the protein consumption needed to reach this signal range. In practice, both **8** and **9** are suitable tracers with comparable properties. To validate the competitive binding assay, we tested known LecA ligands methyl α-D-galactoside (**2**),[44] p-nitrophenyl β-D-galactoside (**25**),[47] compound **5**[46]-derived para-substituted phenyl β-D-galactoside **19** and their meta-isomers **26** and **23**, respectively (Figure 3). All tested compounds inhibited the binding of LecA to

the fluorescent tracer **8** in a dose-dependent manner with IC_{50}s in the one- to two-digit μM-range. Methyl galactoside **2** inhibited LecA with an IC_{50} of 38.6 μM which is in good agreement to the corresponding literature known dissociation constant (K_d 50 μM, see Figure 1). All phenyl β-D-galactoside derivatives were more potent inhibitors of LecA (IC_{50}s 8.5-19.0 μM), with comparable potencies of the *meta*- and their *para*-regioisomers: highest inhibition was observed for the amido-phenyl derivatives **19** (IC_{50} 9.8 μM) and **23** (IC_{50} 8.5 μM) and nitrophenyl galactosides **25** and **26** were two-fold weaker (IC_{50}s 19.0 μM and 13.4 μM, respectively).

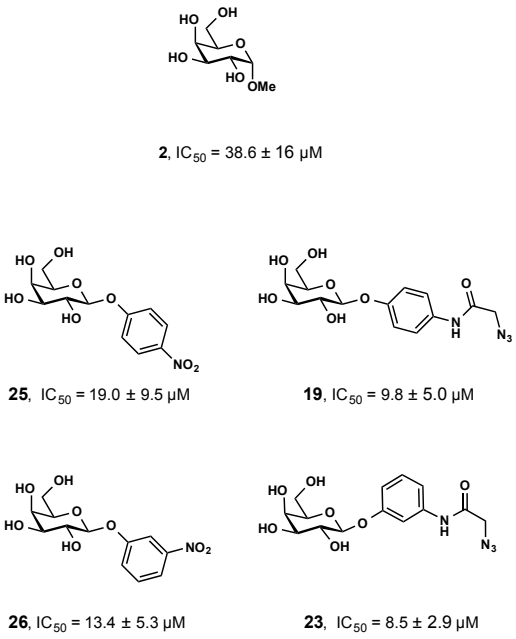


Figure 3: Validation of the competitive binding assay yielded IC_{50} values for known LecA inhibitors. Averages and standard deviations were obtained from three independent experiments.

Structure-activity relationship of thiogalactoside LecA inhibitors

After validation of the competitive binding assay using fluorescent probe **8**, a panel of thiogalactosides was screened in a 384-well microtitre plate format (Table 1). In addition, all thiogalactosides were also

tested with ethyl-linked **7** as a probe that reached increased fluorescence polarization values when bound to LecA in the direct titration experiment (Figure 2) to assess its suitability for this assay format. The *O*-glycosides methyl α-D-galactoside (**2**) and phenyl β-D-galactoside (**4**) were included as reference compounds. In general, all tested galactosides showed inhibition of LecA with IC_{50}s in the range of 10 to 75 μM using probe **8**. When tested with assay probe **7**, a similar trend was observed with generally slightly higher IC_{50} values (13-146 μM). However, relative potencies of inhibitors are comparable between both systems. A common trend observed was the increased potencies of phenyl thio-β-D-galactoside derivatives (IC_{50}s 9.3-23.7 μM using fluorescent ligand **8**) over alkyl- or benzyl-derivatives (26.2-74.9 μM). Positioning of the aromatic moiety one methylene group further away from the anomeric center as in phenethyl thio-β-D-galactoside (**38**), resulted in gain of potency (13.8 μM) compared to the shorter benzyl derivatives (IC_{50}s 31-64 μM). The latter fact has been observed for **38** (K_d = 15.6 μM) and was explained by a beneficial coordination of the arene group by molecular modeling.[45] In general, *para*-tolyl **31** was the most potent LecA inhibitor in this series with fourfold higher potency in both assay variants (using probe **7** or **8**) than reference compound **2**.

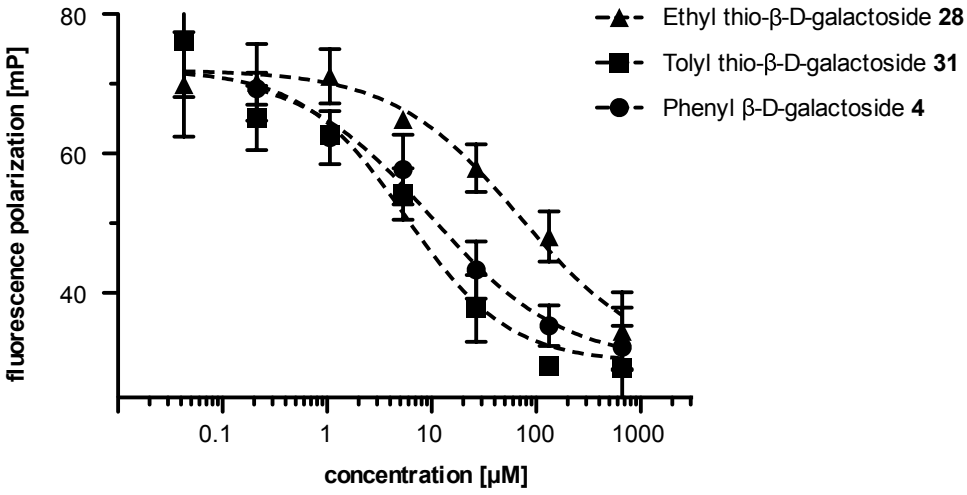
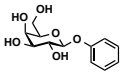
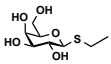
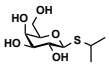
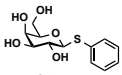
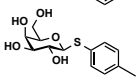
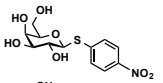
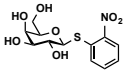
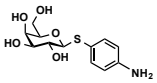
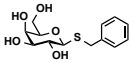
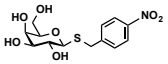
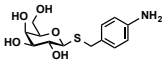
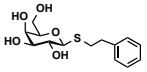


Figure 4: Competitive inhibition of LecA and fluorescent probe **8** with selected *O*- and *S*-galactosides. One representative titration of triplicates on one plate is shown. IC_{50}s given in Table 1 are calculated from three independent experiments on three plates.

Table 1: Evaluation of synthetic galactoside inhibitors of LecA using two different fluorescent probes with flexible linker compound **7** or phenyl-linked **8**. Averages and standard deviations were obtained from three independent experiments. [a]relative potencies compared to **2**.

	compound	8 IC_{50} [μM]	rel. potency[a]	7 IC_{50} [μM]	rel. potency[a]
2		38.6 ± 16.4	1	54.4 ± 32.4	1
4		13.2 ± 7.7	2.2	24.5 ± 6.4	2.2
27		74.9 ± 46.1	0.5	146.0 ± 69.3	0.4
28		32.4 ± 13.1	1.2	50.1 ± 24.5	1.1
29		26.2 ± 8.2	1.5	38.5 ± 21.9	1.4
30		9.9 ± 4.7	3.8	24.0 ± 14.1	2.3
31		9.3 ± 4.2	4.2	12.5 ± 4.9	4.4
32		10.8 ± 2.7	3.6	26.5 ± 14.8	2.1
33		12.1 ± 3.8	3.2	12.5 ± 0.7	4.4
34		23.7 ± 7.2	1.6	25.0 ± 9.9	2.2
35		63.6 ± 16.4	0.6	49.0 ± 2.8	1.1
36		31.7 ± 22.7	1.2	39.0 ± 0.0	1.4
37		30.9 ± 10.9	1.2	71.0 ± 21.2	0.8
38		13.8 ± 8.2	2.8	28.5 ± 10.6	1.9

Structure-activity relationship of human blood group antigens

Gilboa-Garber and co-workers tested 90 patient blood samples for agglutination with LecA and a preference for the blood group B-type was observed.[60] The two LecB variants from strains PAO1 and

PA14 were previously shown to bind strongly to the Lewis[a] blood group antigen.[18,61] We therefore tested a set of human blood group antigens from the A-, B-, H- and Lewis-type blood groups in the established assay (Table 2). In accordance to the agglutination experiments, a weak competitive inhibition of LecA was observed for blood group B-antigens **43** and **48** with IC_{50}s in the low mM-range and even weaker for blood group A antigens **42** and **47**. All other antigens tested did not show inhibition of LecA binding to the fluorescent probe **8** at concentrations up to 1 mM of the oligosaccharides. Recently, a significant preference for *P. aeruginosa* induced sepsis with blood group B infants compared to A-, AB- and O-type was reported indicating a further potential link to the role of LecA in the infection process.[62]

	compound	inhibition
39	Lewis[a]	n.i.
40	Lewis[b]	n.i.
41	H type I	n.i.
42	A type I	26% at 1 mM
43	B type I	44% at 1 mM

	compound	inhibition
44	Lewis[x]	n.i.
45	Lewis[y]	n.i.
46	H type II	n.i.
47	A type II	32% at 1 mM
48	B type II	45% at 1 mM

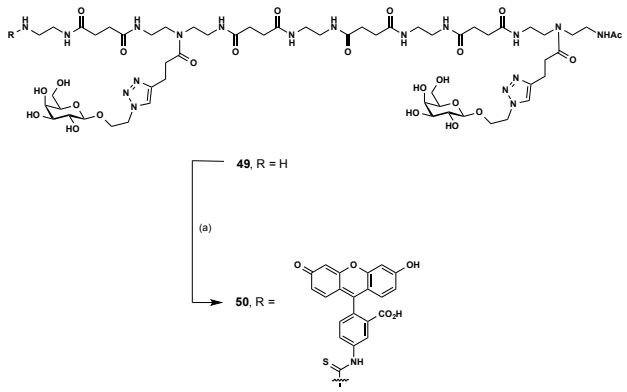
Table 2: Evaluation of various human blood group antigens as ligands of LecA in the competitive binding assay with phenyl-linked **8** as fluorescent probe. n.i. = no inhibition. Averages and standard deviations were obtained from triplicates on one plate.

The observed selectivity of LecA for the B-type antigens can be explained with the presence of one terminal unsubstituted galactose residue. Despite the fact that in Lewis^a and Lewis^b structures also one terminal galactoside is present, no inhibition was observed by these Lewis-type antigens. This could result from two reasons: (i) that the galactose moiety is conformationally stacked via its β-face on the α-face of the adjacent fucose residue in these two Lewis-type structures[63,64] and thus coordination of this moiety to LecA as observed in the crystal structures for simple galactosides[26,44,45,50,65] is probably hindered; or (ii) from a reduced affinity for β-galactosides in such LacNAc containing structures as previously detected by glycan array experiments[65] and inhibition studies of LecA with structurally related lactose.[47] The estimated IC_{50} value for the B-type antigens was approximately thirty-fold higher than the one for methyl galactoside **2**, which may also result from steric hindrance induced by the neighboring glycoside residues in these epitopes.

Can multivalent fluorescent probes improve the assay and reduce protein consumption?

In high affinity lectin-carbohydrate interactions, protein consumption in fluorescence polarization-based competitive binding assays can be drastically reduced into the nanomolar range.[18,39,43,55] Such high affinity systems are suitable assay conditions for large screenings where protein consumption is an important factor. The binding of LecA to phenyl galactosides reaches only low 5-10 μM affinities (Figure 1) and thus, 10-15 μM protein was needed in the assay described above. The 12.9 kDa polypeptide chain of LecA forms a homotetrameric assembly with four carbohydrate binding sites.[26] Polyvalent display of galactosides was shown to significantly increase binding affinity of ligands towards this protein.[32,34,49] The use of oligo- or multivalent fluorescently-labelled assay probes could thus further decrease the amount of LecA needed in the competitive binding assay. For this purpose, we synthesized the divalent LecA inhibitor **49** in a solid phase assembly according to a previously

published procedure[66] and attached fluoresceine to the free amino group to give the bivalent assay probe **50** (Scheme 3). **49** was identified from a series of sequence-defined glycopolymer segments presenting galactose in analogy to Ponader *et al*. for mannose and the multivalent lectin ConA[66] and using a short ethyl spacer building block (SDS) as potential divalent ligand of LecA.



Scheme 3: Synthesis of bivalent fluorescent assay probe **50** from its glycopeptide precursor **49**. Reagents and conditions (a) 6-FITC, PBS, pH 9, DMF, o.n., 36%.

Binding to the target protein was then examined by titration of **50** with increasing amounts of LecA (Figure 5A). A K_d of 1.54 μM was determined for the divalent ligand and the calculated Z' factor for this ligand **50** was 0.93. The applicability of **50** in a competitive binding assay at optimal protein concentration (2-5 μM) showed a good agreement of the determined IC_{50} values of selected LecA inhibitors (Figure 5B) with those obtained from the assay using the monovalent ligands **7** and **8**.

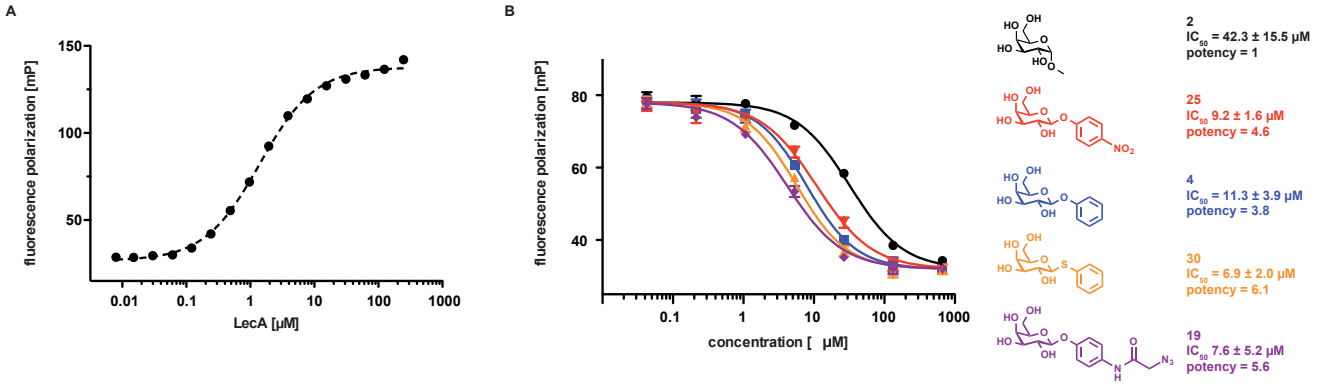


Figure 5: A. Direct titration of the bivalent ligand **50** with increasing amounts of LecA; one site binding fit gave a determined K_d of 1.54 ± 0.13 μM. One representative titration of triplicates on one plate is shown. The K_d and standard deviation were calculated from three independent experiments on three plates. B. Competitive binding assay to LecA (2 μM) of five different galactosides with the bivalent ligand **50**. All potencies were calculated with respect to **2**. One representative titration of triplicates on one plate is shown. IC_{50}s and standard deviations given were calculated from at least four independent experiments on four plates for compound **2** and **30**; or from two independent experiments for **4, 19** and **25**.

Conclusion

Competitive fluorescence polarization-based assays are valuable tools for rapid screening of potential inhibitors. Due to the generally low affinity nature of lectin-carbohydrate interactions, the technique requires high amounts of protein in the range of the K_d to achieve sufficient fluorescence polarization of the probe. Here, we developed a competitive binding assay for the bacterial lectin LecA. Four different fluorescein-linked monovalent galactosides were evaluated and the dissociation constants determined were in the 10-25 μM range. Due to their increased potency, phenyl-linked **8** and **9** required less protein in the competitive binding assay compared to ethyl-linked **6** or **7**. Therefore, the competitive binding assay was established for assay probe **8** (Z' = 0.99) with a set of known LecA inhibitors and good

agreement of the obtained IC_{50} values with published K_d values was achieved. We then screened a set of thiogalactosides with various aglycones for inhibition and established a structure-activity relationship that compares to previously published *O*-linked analogs. Aryl thioglycosides were potent inhibitors whereas alkyl or benzyl derivatives showed a reduced inhibition. We furthermore tested human blood group antigen epitopes for inhibition of LecA but only a weak inhibition by blood group B was observed. To further reduce the required amount of LecA in the assay system, the fluorescently-labeled divalent LecA inhibitor **50** was designed and synthesized. The successful use of this bivalent assay probe in inhibitor screening is a promising possibility for the low affinity system galactose-LecA to further reduce the amount of protein (2-5 μM) needed in high throughput assays. Here, comparable IC_{50} values were obtained with two monovalent and the bivalent assay probe. Due to the presence of an additional linking moiety in **50** between its two galactose residues, inhibitors binding to LecA at positions occupied by this linker could be detected as hits despite a binding pose outside the carbohydrate binding site. This could theoretically yield inhibitors of the association of the linker with LecA rather than functional inhibitors of the carbohydrate-binding functionality of LecA.

In summary, a robust competitive binding assay for LecA (Z' up to 0.99) was developed and optimized with a set of assay probes. All synthesized galactose-based fluorescent tracers showed good performance in binding to LecA and three were tested in the competitive binding assay. Aryl-linked ligands, such as **8** and **9** (K_ds of 8.1 and 7.4 μM, respectively), showed highest potency in the monovalent series, further reduction of protein consumption from 18 μM to 2-5 μM could be achieved by employing the potent divalent assay probe **50** (K_d = 1.5 μM). The assay can be used for these potent monovalent and bivalent probes in a 384-well format for miniaturization and protein-economic use in inhibitor screening.

Experimental details

Chemical Synthesis

General Experimental Details

Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker Avance III 400, 500 or 600 UltraShield spectrometer at 400/500/600 MHz (^{1}H) or 101/126/151 MHz (^{13}C). Chemical shifts are given in ppm and were calibrated on residual solvent peaks[67]. Multiplicities were specified as s (singlet), m (multiplet) or interpreted according to 1st order and higher order where possible. The signals were assigned with the help of ^{1}H,^{1}H-COSY, DEPT-135-edited ^{1}H,^{13}C-HSQC and ^{1}H,^{13}C-HMBC experiments. High resolution mass spectra were obtained on a ESI Bruker micrOTOF II spectrometer. Analytical HPLC-MS was performed on a Thermo Dionex Ultimate 3000 HPLC coupled to a Bruker Amazon SL ESI-MS system. Data were analyzed using DataAnalysis from Bruker. Thin layer chromatography (TLC) was performed using silica gel 60 coated aluminum sheets containing fluorescence indicator (Merck KGaA, Darmstadt, Germany) using UV light (254 nm) and by charring either in aqueous $KMnO_4$ solution or in a molybdate solution (a 0.02 M solution of ammonium cerium sulfate dihydrate and ammonium molybdate tetrahydrate in aqueous 10% H_2SO_4) with heating. Medium pressure liquid chromatography (MPLC) was performed on a Teledyne Isco Combiflash Rf200 system using pre-packed silica gel 60 columns from Teledyne Isco, SiliCycle or Macherey-Nagel. Commercial chemicals and solvents were used without further purification. D-galactose was purchased from Dextra Laboratories (Reading, UK) and fluorescein isothiocyanate isomer I (FITC) from Serva Biochemicals (Heidelberg, Germany). Deuterated solvents were from Eurisotop (Saarbrücken, Germany). Methyl galactoside **2** and thioglycosides **27**-**33**, **36, 37** were purchased from Carbosynth (Compton, UK) and thioglycosides **34** and **38** from Sigma-Aldrich (Steinheim, Germany). Blood group oligosaccharides **39**-**48** were purchased from Elicityl Oligotech (Crolles, France).

2-Bromoethyl α-D-galactopyranoside (10). D-galactose (**1**, 360 mg, 2 mmol) and $La(OTf)_3$ (38 mg, 65 μmol) were suspended in 2-bromoethanol (2.1 mL, 30 mmol), heated to 70 °C under nitrogen atmosphere and subsequently stirred for 24 h in analogy to Dasgupta *et al*..[68] The resulting solution was cooled to r.t., directly loaded onto a silica gel column and purified by MPLC (SiO_2, CH_2Cl_2/EtOH, 5 - 20%) to give an anomeric mixture of the glycoside as inseparable syrup (202 mg, 35%, α/β ratio = 7/3). The mixture (150 mg) was dissolved in 10 mL aqueous phosphate buffer (20 mM NaH_2PO_4, pH = 6.0) and *Aspergillus oryzae* β-galactosidase (110 mg Lactrase, 18% enzyme, Pro Natura, Germany) was added. The reaction was incubated at 37 °C and shaking at 180 rpm for 22 h. The mixture was filtered over celite, the solvents were removed *in vacuo*, the residue was purified by MPLC (SiO_2, CH_2Cl_2/EtOH, 5 - 15%) and pure α-galactoside **10** (48.5 mg, 33%) was obtained. ^{1}H NMR (500 MHz, MeOH-d4) δ 4.93 (d, *J* = 3.4 Hz, 1H, H-1), 4.02 (dt, *J* = 11.3, 6.5 Hz, 1H, 1H of OC*H*$_2$CH$_2$Br), 3.98-3.95 (m, 1H, H-5), 3.94 (dd, *J* = 3.0, 1.3 Hz, 1H, H-4), 3.89 (dt, *J* = 11.5, 5.9 Hz, 1H, 1H of OC*H*$_2$CH$_2$Br), 3.82 (dd, J = 10.1, 3.4 Hz, 1H, H-2), 3.79 (dd, J = 10.3, 3.0 Hz, 1H, H-3), 3.75 (dd, J = 11.5, 6.7 Hz, 1H, H-6a), 3.73 (dd, *J* = 11.4, 5.4 Hz, 1H, H-6b), 3.64 (t, *J* = 6.2 Hz, 2H, OCH$_2$C*H*$_2$Br); ^{13}C NMR (126 MHz, MeOH-d4) δ 100.73 (C-1), 72.71 (C-5), 71.40 (C-3), 71.04 (C-4), 70.12 (C-2), 69.62 (O*C*H$_2$CH$_2$Br), 62.70 (C6), 31.33 (OCH$_2$*C*H$_2$Br). ESI-MS $[C_8H_{15}BrO_6+Na]^+$ calcd. 308.99, found 309.0. The synthesis of **10** was first reported by Grandjean *et al*. without disclosure of analytical data.[69]

2-Azidoethyl α-D-galactopyranoside (11). Bromide **10** (48.5 mg, 0.17 mmol) was dissolved in DMF (1.7 mL) and NaN_3 (54 mg, 0.84 mmol) was added. The reaction was stirred at 75 °C for 16 h, cooled to r.t., filtered over celite and the volatiles were removed *in vacuo*. The product was purified by MPLC (SiO_2, CH_2Cl_2/EtOH, 5 - 15%) and the pure product was obtained as solid (30.9 mg, 74%). ^{1}H NMR (500 MHz, MeOH-d4) δ 4.86 (d, *J* = 2.9 Hz, 1H, H-1), 3.94 – 3.89 (m, 2H, H-4, 1H of OC*H*$_2$CH$_2$N$_3$),

3.87 (ddd, *J* = 6.8, 5.3, 1.3 Hz, 1H, H-5), 3.80 (dd, *J* = 9.8, 2.9 Hz, 1H, H-2), 3.77 (dd, *J* = 9.8, 2.5 Hz, 1H, H-3), 3.74 (dd, *J* = 11.5, 6.8 Hz, 1H, H-6a), 3.70 (dd, *J* = 11.5, 5.6 Hz, 1H, H-6b), 3.64 (ddd, *J* = 10.6, 5.6, 3.5 Hz, 1H, 1H of OC*H*$_2$CH$_2$N$_3$), 3.57 (ddd, *J* = 13.2, 7.6, 3.5 Hz, 1H, 1H of OCH$_2$C*H*$_2$N$_3$), 3.39 (ddd, *J* = 13.2, 5.7, 3.4 Hz, 1H, 1H of OCH$_2$C*H*$_2$N$_3$); ^{13}C NMR (126 MHz, MeOH-d4) δ 100.70 (C-1), 72.64 (C-5), 71.31 (C-3), 71.09 (C-4), 70.06 (C-2), 68.11 (O*C*H$_2$CH$_2$N$_3$), 62.76 (C-6), 51.82 (OCH$_2$*C*H$_2$N$_3$); ESI-MS [C$_8$H$_{15}$N$_3$O$_6$+Na]$^+$ calcd. 272.09, found: 272.1; **11** was first reported by Wang, [70] the NMR data correspond to those reported by Park *et al*. in D$_2$O.[71]

2-Aminoethyl α-D-galactopyranoside (12). Azide **11** (30.9 mg, 124 μmol) was dissolved in MeOH (1.0 mL) and Pd/C (3.1 mg, 10 wt-%) was added. The reaction vessel was flushed several times with hydrogen and subsequently stirred under hydrogen atmosphere (1 bar) over night. The reaction was filtered over celite, the volatiles were removed *in vacuo* and the pure product was obtained as an oil (24.4 mg, 88%) which was used without further purification in the next step. ^{1}H NMR (500 MHz, MeOH-d4) δ 4.85 (1H, H-1, overlapped by HDO peak, assigned by HSQC and COSY), 3.89 (dd, *J* = 2.8, 1.2 Hz, 1H, H-4), 3.87 – 3.81 (m, 2H, H-5, 1H of OC*H*$_2$CH$_2$NH$_2$), 3.81 – 3.78 (m, 1H, H-2), 3.76 (dd, *J* = 9.8, 2.7 Hz, 1H, H-3), 3.73 (dd, *J* = 11.0, 6.5 Hz, 1H, H-6a), 3.69 (dd, *J* = 11.4, 5.2 Hz, 1H, H-6b), 3.50 (ddd, *J* = 10.5, 7.4, 3.7 Hz, 1H, 1H of OC*H*$_2$CH$_2$NH$_2$), 2.96 (ddd, *J* = 13.2, 5.5, 3.6 Hz, 1H, 1H of OCH$_2$C*H*$_2$NH$_2$), 2.91 (ddd, *J* = 13.5, 7.3, 3.7 Hz, 1H, 1H of OCH$_2$C*H*$_2$NH$_2$); ^{13}C NMR (126 MHz, MeOH-d4) δ 100.54 (C-1), 72.62 (C-5), 71.47 (C-3), 71.09 (C-4), 70.30 (C-2), 69.03 (O*C*H$_2$CH$_2$NH$_2$), 62.84 (C-6), 41.74 (OCH$_2$*C*H$_2$NH$_2$).

***N*-(fluorescein-5-yl)-*N*'-(α-D-galactopyranosyl-*O*-ethyl-)-thiocarbamide (6)**. Amine **12** (23 mg, 103 μmol) was dissolved in DMF (4.0 mL) and NaHCO$_3$ (0.093 mmol, 7.8 mg) was added. After addition of FITC (103 μmol, 40 mg), the reaction was protected from light and stirred at r.t. over night. The solvent was removed under reduced pressure and the residue was purified by MPLC (SiO$_2$, CH$_2$Cl$_2$/ EtOH/5% AcOH, gradient of 5%-40% EtOH) to give **6** as an orange solid (16.2 mg, 26%). ^{1}H NMR

(500 MHz, MeOH-d4) δ 8.12 (d, *J* = 2.1 Hz, 1H, ArH), 7.77 (d, *J* = 8.9 Hz, 1H, ArH), 7.17 (d, *J* = 8.2 Hz, 1H, ArH), 6.81 (d, *J* = 8.8 Hz, 2H, ArH), 6.68 (d, *J* = 2.3 Hz, 2H, ArH), 6.58 (dd, *J* = 8.8, 2.4 Hz, 2H, ArH), 4.89 (d, *J* = 3.5 Hz, 1H, H-1), 3.99 - 3.92 (m, 2H, 1H of OC*H*$_2$CH$_2$NHR, 1H of OCH$_2$C*H*$_2$NHR), 3.92 - 3.85 (m, 2H, H-4, -5), 3.84 - 3.78 (m, 3H, 1H of OCH$_2$C*H*$_2$NHR, H-2, -3), 3.78 - 3.73 (m, 2H, 1H of OC*H*$_2$CH$_2$NHR, H-6a), 3.70 (dd, *J* = 11.4, 4.9 Hz, 1H, H-6b). ^{13}C NMR (126 MHz, MeOH-d4) δ 182.89 (C=S), 130.92 (2C, ArCH), 130.0 (ArCH, from HSQC), 126.8 (ArCH, from HSQC), 120.8 (ArCH, from HSQC), 112.61 (2C, ArCH), 103.61 (2C, ArCH), 100.79 (C-1), 72.69 (C-5), 71.47 (C-3), 71.12 (C-4), 70.21 (C-2), 67.98 (O*C*H$_2$CH$_2$NHR), 62.93 (C-6), 45.59 (OCH$_2$*C*H$_2$NHR). ESI-MS [C$_{29}$H$_{28}$N$_2$O$_{11}$S+Na]$^+$ calcd. 635.13, found 613.1; R$_f$ 0,45 (CH$_2$Cl$_2$/ EtOH 2:1+ 2% AcOH).

2-Bromoethyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (13), first reported by Coles *et al*.,[72] was synthesized according to Dahmen *et al*..[57] ^{1}H NMR (400 MHz, CDCl$_3$) δ 5.38 (d, *J* = 3.3 Hz, 1H, H-4), 5.22 (dd, *J* = 10.4, 8.0 Hz, 1H, H-2), 5.02 (dd, *J* = 10.5, 3.4 Hz, 1H, H-3), 4.53 (d, *J* = 7.9 Hz, 1H, H-1), 4.20 - 4.08 (m, 3H, H-6a, -6b, 1H of OC*H*$_2$CH$_2$Br), 3.88 - 3.92 (m, 1H, H-5), 3.85 – 3.76 (m, 1H, 1H of OC*H*$_2$CH$_2$Br), 3.50 - 3.43 (m, *2*H, OCH$_2$C*H*$_2$Br), 2.14 (s, 3H), 2.07 (s, 3H), 2.04 (s, 3H), 1.98 (s, 3H). ^{13}C NMR (101 MHz, CDCl$_3$) δ 170.49, 170.32, 170.24, 169.64 (4C, CH$_3$*C*O), 101.66 (C-1), 70.96 (C-5), 70.87 (C-3), 69.87 (O*C*H$_2$CH$_2$Br), 68.68 (C-2), 67.09 (C-4), 61.38 (C-6), 30.04 (OCH$_2$*C*H$_2$Br), 20.97, 20.79, 20.77, 20.69 (4C, *C*H$_3$CO).

2-Azidoethyl β-D-galactopyranoside (**14**). **14** was synthesized from **13** by nucleophilic displacement with sodium azide to give 2-azidoethyl-2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside according to D'Agata *et al*..[73] The azido galactoside was deprotected under Zemplén conditions as previously reported by Susaki *et al*.[74] with the following modifications: the reaction was stopped by neutralization with Amberlite IR-120H$^+$, filtered and the volatiles were removed *in vacuo*. Deprotected Azide **14** was obtained in 55% over 2 steps. ^{1}H NMR data corresponds to the assignment reported by Susaki *et al*..

^{13}C NMR (101 MHz, MeOH) δ 105.10 (C-1), 76.75 (C-5), 74.95 (C-3), 72.45 (C-2), 70.29 (C-4), 69.20 (O<u>C</u>H$_2$CH$_2$N$_3$), 62.49 (C-6), 52.11 (OCH$_2$<u>C</u>H$_2$N$_3$).

2-Aminoethyl β-D-galactopyranoside (**15**), first reported by Chiang *et al*.,[75] was synthesized from azide **14** (200 mg, 0.8 mmol) by hydrogenation (1 bar) over Pd/C (10-wt%, 24 mg) in MeOH (4.8 mL) at r.t. over night. The solution was filtered through a pad of celite and the volatiles were removed *in vacuo* to give the title compound as white solid (190 mg, 99%). ^{1}H NMR (400 MHz, D$_2$O) δ 4.44 (d, *J* = 7.9 Hz, 1H, H-1), 4.06 – 3.97 (m, 1H, 1H of OC<u>*H*$_2$</u>CH$_2$NH$_2$), 3.95 (dd, *J* = 3.4, 0.7 Hz, 1H, H-4), 3.88 – 3.75 (m, 3H, H-6a, -6b, 1H of OC<u>*H*$_2$</u>CH$_2$NH$_2$), 3.73 (ddd, *J* = 7.8, 4.3, 0.8 Hz, 1H, H-5), 3.68 (dd, *J* = 9.9, 3.5 Hz, 1H, H-3), 3.57 (dd, *J* = 9.9, 7.8 Hz, 1H, H-2), 2.93 (ddd, *J* = 6.7, 4.6, 2.0 Hz, 2H, OCH$_2$C<u>*H*$_2$</u>NH$_2$). NMR data were in agreement with Susaki *et al*..[74]

***N*-(fluorescein-5-yl)-*N*'-(β-D-galactopyranosyl-*O*-ethyl)-thiocarbamide** (**7**). Amine **15** (0.235 mmol, 52 mg) was dissolved in DMF (1.5 mL), FITC (0.235 mmol, 86 mg) and NaHCO$_3$ (2.15 mmol, 180 mg) were added. The reaction was stirred at r.t. over night, filtered and the remaining solids were washed with DMF and filtered. The solvent of the combined filtrates was removed under reduced pressure. The residue was purified by MPLC (SiO$_2$, CH$_2$Cl$_2$/EtOH/2% AcOH, gradient of 5%-40% EtOH) to give the title compound as orange foam after lyophilization from water (76 mg, 55%). ^{1}H NMR (400 MHz, MeOH-d4) δ 8.23 (s, 1H, ArH), 7.84 (dd, J = 8.3, 1.9 Hz, 1H, ArH), 7.15 (d, J = 8.3 Hz, 1H, ArH), 6.69 - 6.64 (m, 4H, ArH), 6.54 (dd, J = 8.7, 2.2 Hz, 2H, ArH), 4.30 (d, J = 7.5 Hz, 1H, H-1), 4.05 - 3.88 (m, 3H, 2H of OC<u>*H*$_2$</u>CH$_2$NHR, 1H of OCH$_2$C<u>*H*$_2$</u>NHR), 3.86 – 3.74 (m, 3H, H-4, -6a,b), 3.66 – 3.53 (m, 2,%-2, -5H), 3.50 (dd, J = 9.7, 3.3 Hz, 1H, H-3). ^{13}C NMR (101 MHz, MeOH-d4) δ 182.72 (C=S), 175.26 (C=O), 171.30 (C=O), 154.14 (ArC), 130.35 (ArC), 113.56 (ArCH), 111.45 (2C, ArCH), 105.53 (ArCH), 103.48 (C-1), 101.39 (ArCH), 76.90 (C-5), 74.88 (C-3), 72.55 (C-2), 70.37 (C-4), 70.15 (O<u>C</u>H$_2$CH$_2$NH) 62.81 (C-6), 46.11 (OCH$_2$<u>C</u>H$_2$NH); R$_f$ = 0.25 (CH$_2$Cl$_2$/EtOH 3:1 + 2% AcOH); HRMS calcd. C$_{29}$H$_{27}$N$_2$O$_{11}$S$^-$: 611.1341 [M-H]$^-$; found: 611.1345.

1,2,3,4,6-Penta-*O*-acetyl-β-D-galactopyranoside (16) was synthesized according to Cohen *et al*..[76]

***p*-Nitrophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (17).** Molecular sieves (5 g, 3Å) were dried under vacuum at 350 °C in a two-necked flask for 30 min. After cooling to r.t., the flask was flushed with argon and dry CH_2Cl_2 (25 mL) was added. Galactosyl donor **16** (5.0 g, 12.8 mmol) and p-nitrophenol (2.2 g, 15.9 mmol) were added, the reaction mixture was cooled (0 °C) and BF_3•OEt_2 (8.2 mL, 65.3 mmol) was added dropwise. Afterwards, the mixture was allowed to warm to r.t. and stirred for 1 d. The reaction was poured onto ice water (200 mL) and the aqueous phase was extracted with aqueous saturated $NaHCO_3$ (5 x 50 mL). The combined organic layers were washed with brine (25 mL) and dried over anhydrous Na_2SO_4. After filtration, the solvent was removed *in vacuo*. The crude product was dissolved in warm EtOH and left at 4 °C overnight. Precipitated pure **17** (3.1 g, 6.6 mmol, 52%) was obtained as light yellow amorphous solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.25-8.17 (m, 2H, ArH), 7.10-7.05 (m, 2H, ArH), 5.51 (dd, J = 10.4, 7.9 Hz, 1H, H-2), 5.47 (d, J = 3.3 Hz, 1H, H-4), 5.17 (d, J= 7.9 Hz, 1H, H-1), 5.13 (dd, J= 10.4 3.4 Hz, 1H, H-3), 4.25-4.10 (m, 3H, H-5,-6a,b), 2.18 (s, 3H, C$\underline{H}_3$) 2.06 (s, 6H, C$\underline{H}_3$) 2.01 (s, 3H, C$\underline{H}_3$). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 170.41 (C=O), 170.23 (C=O), 170.15 (C=O), 169.38 (C=O), 161.30 (ArC), 143.31 (ArC), 125.91 (2C, ArCH), 116.68 (2C, ArCH), 98.69 (C-1), 71.57 (C-5), 70.68 (C-3), 68.37 (C-2), 66.79 (C-4), 61.46 (C-6), 20.82 (C$\underline{H}_3$), 20.79 (C$\underline{H}_3$), 20.76 (C$\underline{H}_3$), 20.68 (C$\underline{H}_3$); R_f = 0.27 (CH_2Cl_2/MeOH 12:1); ESI-MS: $[C_{20}H_{23}NO_{12}+Na]^+$ calcd. 492.1 found 492.1. **17** was first described by Goebel and Avery,[77] NMR data obtained are in agreement with the literature ^{1}H-NMR data by Apparu *et al*..[78]

***p*-(α-Azidoacetamido)-phenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (18)** was synthesized from protected **17** in 3 chemical steps in analogy to the reaction sequence of Vidal and co-workers[46] who started from unprotected nitrophenyl β-D-galactoside and acetylated as last step. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.01 (s, 1H, N$\underline{H}$), 7.51-7.42 (m, 2H, ArCH), 7.04-6.93 (m, 2H, ArCH), 5.46 (dd, *J* = 10.3, 7.9 Hz, 1H, H-2), 5.44 (dd, *J* = 3.4, 1.2 Hz, 1H, H-4), 5.09 (dd, *J* = 10.5, 3.4 Hz, 1H, H-3), 4.99

(d, *J* = 8.0 Hz, 1H, H-1), 4.21 (dd, *J* = 11.3, 7.0 Hz, 1H, H-6a), 4.13 (s, 2H, $C\underline{H_2}N_3$), 4.18-4.10 (m, 1H, H-6b), 4.06-4.01 (m, 1H, H-5), 2.17 (s, 3H, $C\underline{H_3}$), 2.07 (s, 3H, $C\underline{H_3}$), 2.05 (s, 3H, $C\underline{H_3}$), 2.00 (s, 3H, $C\underline{H_3}$); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 170.50 ($CH_3\underline{C}O$), 170.37 ($CH_3\underline{C}O$), 170.25 ($CH_3\underline{C}O$), 169.53 ($CH_3\underline{C}O$), 164.63 ($\underline{C}ONH$), 154.12 (ArC), 132.33 (ArC), 121.81 (2C, ArCH), 117.75 (2C, ArCH), 100.05 (C-1), 71.17 (C-5), 70.90 (C-3), 68.71 (C-2), 66.97 (C-4), 61.48 (C-6), 53.01 ($\underline{C}H_2N_3$), 20.88 ($\underline{C}H_3$), 20.81 ($\underline{C}H_3$), 20.79 ($\underline{C}H_3$), 20.72 ($\underline{C}H_3$); R_f = 0.23 (PE/EtOAc 1:1); ESI-MS $[C_{22}H_{26}N_4O_{11}+H]^+$ calcd. 523.17, found 523.1. Our data match the previously published NMR spectra by Cecioni *et al*.,[46] though the peak assignment by Cecioni *et al*. differs from the one reported here.

***p*-(2-Azidoacetamido)-phenyl β-D-galactopyranoside (19)**. A small portion of sodium was dissolved in dry MeOH (5 mL) and tetraacetate **18** (164 mg, 0.31 mmol) was added. The solution was stirred for 2 h at r.t., neutralized with Amberlite IR-120/H^+ (Merck), the resin was filtered and the solvent was removed *in vacuo* to give **19** (102 mg, 0.31 mmol, 99%) as a white solid. ^{1}H NMR (400 MHz, MeOH-d4) δ 7.49-7.43 (m, 2H, ArH), 7.14-7.04 (m, 2H, ArH), 4.82 (d, J = 7.7 Hz, 1H, H-1), 3.99 (s, 2H, CH_2), 3.90 (d, J = 3.5 Hz, 1H, H-4), 3.82-3.72 (m, 3H, H-2, -6a,b), 3.70-3.64 (m, 1H, H-5), 3.57 (dd, J = 9.7, 3.4 Hz, 1H, H-3); ^{13}C NMR (101 MHz, MeOH-d4) δ 168.25 (C=O), 156.15 (ArC), 133.68 (ArC), 122.82 (2C, ArCH), 118.16 (2C, ArCH), 103.28 (C-1), 76.99 (C-5), 74.85 (C-3), 72.27 (C-2), 70.23 (C-4), 62.43 (C-6), 53.23 (CH_2N_3); HRMS: $[C_{14}H_{18}N_4O_7+Na]^+$ calcd: 377.1068; found: 377.1056; R_f = 0.68 (EtOAc/EtOH 5:3).

***p*-(α-Aminoacetamido)-phenyl β-D-galactopyranoside (20)** was synthesized from the azide **19** (25 mg, 0.07 mmol) by hydrogenation (1 bar) over Pd-C (8-wt%, 2 mg) in MeOH for 2 h. The solution was filtered through a pad of celite and the volatiles were removed *in vacuo*. After MPLC, (SiO_2, EtOAc/ EtOH /5% NH_4OH, gradient 2-100% EtOH) the title compound was obtained as a colorless oil (22 mg, 96%). ^{1}H NMR (500 MHz, D_2O) δ 7.37-7.32 (m, 2H, Ar-H), 7.13-7.08 (m, 2H, Ar-H), 5.00 (d, J = 7.5 Hz, 1H, H-1), 3.96 (d, J = 3.2 Hz, 1H, H-4), 3.82 (t, J = 6.20 Hz, 1H, H-5), 3.79-3.69 (m, 4H, H-2, -3,

-6a,b), 3.46 (s, 2H, C$\underline{H_2}$); ^{13}C NMR (126 MHz, D_2O) δ 173.56 ($\underline{C}$O), 154.25, (ArC), 131.50 (ArC), 123.84 (2C, ArCH), 117.02 (2C, ArCH), 100.92 (C1), 75.42 (C5), 72.53 (C3), 70.51 (C2), 68.45 (C4), 60.72 (C6), 43.84 (C$\underline{H_2}$); MS: $[C_{14}H_{20}N_2O_7+H]^+$ calcd 329.1, found 329.1.

Para-substituted phenyl-linked reporter ligand 8. Amine **20** (22 mg, 0.07 mmol) was dissolved in dry DMF (4 mL). Fluoresceine isothiocyanate (26.1 mg, 0.07 mmol, 1 eq) and $NaHCO_3$ (5 mg, 0.06 mmol, 0.9 eq) were dissolved in dry DMF (1 mL) and this solution was added to **20** and stirring was continued at r.t. for 14 h. The solvent was removed and the crude product was purified by MPLC, (SiO_2, CH_2Cl_2/EtOH containing 1% HOAc, gradient of 5 to 60% EtOH). To remove the residual AcOH the residue was co-evaporated with hexane (3 x 25 mL) and subsequently lyophilized. **8** was obtained as an orange solid (37 mg, 0.05 mmol, 77%). ^{1}H NMR (500 MHz, DMSO-d6) δ 10.44 (br s, 1H, NH/OH), 10.08 (s, 1H, NH/OH), 8.40-8.22 (m, 2H, ArH, NH), 7.79 (d, J = 7.5 Hz, 1H, ArH), 7.51 (d, J = 8.9 Hz, 2H, ArH), 7.15 (d, J = 8.2 Hz, 1H, Ar-H), 7.00 (d, J = 8.9 Hz, 2H, ArH), 6.73-6.62 (m, 3H, ArH), 6.54-6.33 (m, 4H, ArH), 4.75 (d, J = 7.7 Hz, 1H, H-1), 4.37 (s, 2H, C$\underline{H_2}$NHR), 3.69 (d, J = 3.1 Hz, 1H, H4), 3.58-3.46 (m, 5H, H-2, -3, -5, -6a,b6); ^{13}C NMR (126 MHz, DMSO-d6) δ 180.70 (CS), 166.76 (CO), 153.47 (ArC), 133.00 (ArC), 129.68 (2C, ArCH), 120.38 (2C, ArCH), 116.55 (2C, ArCH), 110.35 (ArC), 102.49 (ArCH), 101.41 (C-1), 75.47 (C-2/3/5), 73.32 (C-2/3/5), 70.32 (C-2/3/5), 68.14 (C-4), 60.39 (C-6), 47.47 ($\underline{C}H_2$); HRMS: $[C_{35}H_{31}N_3O_{12}S+H]^+$ calcd. 718.1701, found 718.1661; R_f = 0.14 (CH_2Cl_2/EtOH/AcOH 3:1:0.08).

***m*-Nitrophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (21).** Molecular sieves (5 g, 3Å) were dried under vacuum at 350 °C in a two-necked flask for 30 min. After cooling to r.t., the flask was flushed with argon and dry CH_2Cl_2 (25 mL) was added. Galactosyl donor **16** (5.0 g, 12.8 mmol) and m-nitrophenol (2.2 g, 15.9 mmol) were added, the reaction mixture was cooled (0 °C) and BF_3•OEt_2 (8.2 mL, 65.3 mmol) was added dropwise. Afterwards, the mixture was allowed to warm to r.t. and stirred for 1 d. The reaction was poured onto ice water (250 mL) and the aqueous phase was extracted with

aqueous saturated $NaHCO_3$ (5 x 50 mL). The combined organic layers were washed with brine (25 mL) and dried over anhydrous Na_2SO_4. After filtration, the solvent was removed *in vacuo*. The crude product was dissolved in warm EtOH and left at 4 °C overnight. Precipitated pure **21** (3.3 g, 7.1 mmol, 55%) was obtained as light yellow amorphous solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.94 (ddd, J = 8.2, 2.1, 0.9 Hz, 1H, ArH), 7.88 (t, J = 2.3 Hz, 1H, ArH), 7.46 (t, J = 8.2 Hz, 1H, ArH), 7.31 (ddd, J = 8.3, 3.5, 0.9 Hz, 1H, ArH), 5.52 (dd, J = 10.5, 7.9 Hz, 1H, H-2), 5.48 (d, J = 3.4 Hz, 1H, H-4), 5.14 (d, J = 7.7 Hz, 1H, H-1), 5.12 (dd, J = 10.5, 3.4 Hz, 1H, H-3), 4.24-4.13 (m, 3H, H-5, -6a,b), 2.18 (s, 3H, $C\underline{H}_3$), 2.10 (s, 3H, $C\underline{H}_3$), 2.08 (s, 3H, $C\underline{H}_3$), 2.01 (s, 3H, $C\underline{H}_3$); ^{13}C-NMR (400 MHz, $CDCl_3$): δ 170.73 (C=O), 170.27 (C=O), 170.16 (C=O), 169.44 (C=O), 157.15 (ArC), 149.20 (ArC), 130.34 (ArCH), 123.88 (ArCH), 118.33 (ArCH), 111.38 (ArCH), 99.32 (C-1), 71.81 (C-5), 70.76 (C-3), 68.44 (C-2), 67.07 (C-4), 61.94 (C-6), 20.87 ($\underline{C}H_3$), 20.79 ($\underline{C}H_3$), 20.75 ($\underline{C}H_3$), 20.70 ($\underline{C}H_3$); ESI-MS: $[C_{20}H_{23}NO_{12}+Na]^+$ calcd. 492.1, found 492.1; R_f = 0.33 (PE/EtOAc 3:2). The title compound was first synthesized by Iversen and Johansson starting from tetraacetylgalactosyl bromide.[79]

***m*-(α-Azidoacetamido)-phenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (22)**. Nitrophenyl galactoside **21** (650 mg, 1.39 mmol) was dissolved in dry CH_2Cl_2 (30 mL) and Pd/C (60 mg) was added. After three vacuum/H_2 cycles the reaction was stirred under H_2 atmosphere (1 atm) for 16 h at r. t.. After cooling to 0 °C, NEt_3 (230 μL, 1.67 mmol) followed by bromoacetylbromide (145 μL, 1.67 mmol) were added dropwise. After stirring for 1 h at 0 °C, it was warmed to r.t. and stirred for an additional hour. The reaction was filtered and the organic phase was washed 1 N HCl (2 x 50 mL), saturated $NaHCO_3$ (3 x 50 mL) and water (2 x 50 mL). After drying of the organic layer over Na_2SO_4 the solvent was removed *in vacuo*. Crude bromoacetylated galactoside was obtained as yellow foam and dissolved in dry DMF (20 mL). NaN_3 (452 mg, 6.95 mmol) and the catalyst Bu_4NI (52 mg, 0.14 mmol) were added and the reaction was stirred for 16 h at 50 °C. After filtration, the organic phase was washed with saturated aqueous $NaHCO_3$ (2 x 50 mL), water (2 x 50 mL), and brine (2 x 50 mL). The

organic layer was dried over Na_2SO_4, filtered and the volatiles removed *in vacuo*. The crude yellow oil was purified by MPLC (SiO_2, PE/EtOAc 0-100%) to give pure azide **22** as a solid (615 mg, 1.19 mmol, 86% over three steps). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.22 (s, 1H, NH), 7.40 (t, 2.2Hz, 1H, ArH), 7.24 (t, J = 8.1 Hz, ArH), 7.16 (ddd, J = 8.2, 2.0, 1.0 Hz, 1H, ArH), 6.78 (ddd, J = 8.2, 2.5, 1.0 Hz, 1H, ArH), 5.46 (dd, J = 10.6, 8.0 Hz, 1H, H-2), 5.44 (d, J = 3.2 Hz, 1H, H-4), 5.09 (dd, J= 10.5, 3.5 Hz, 1H, H-3), 5.06 (d, J = 7.9 Hz, 1H, H-1), 4.24-4.06 (m, 5H, H-5, -6a,b, CH_2N_3), 2.16 (s, 3H, $C\underline{H}_3$), 2.06 (s, 3H, $C\underline{H}_3$), 2.04 (s, 3H, $C\underline{H}_3$), 2.00 (s, 3H, $C\underline{H}_3$); ^{13}C-NMR (101 MHz, $CDCl_3$) δ 170.85 ($CH_3\underline{C}O$), 170.68 ($CH_3\underline{C}O$), 170.54 ($CH_3\underline{C}O$), 169.87 ($CH_3\underline{C}O$), 163.00 ($\underline{C}ONH$), 157.77 (ArC), 138.60 (ArC), 130.35 (ArC), 115.07 (ArCH), 113.74 (ArCH), 109.23 (ArCH), 99.93 (C-1), 71.50 (C-5), 71.23 (C-3), 68.99 (C-2), 67.30 (C-4), 61.75 (C-6), 53.32 ($\underline{C}H_2N_3$), 21.19 ($\underline{C}H_3$), 21.10 ($\underline{C}H_3$), 21.07 ($\underline{C}H_3$), 21.02 ($\underline{C}H_3$); ESI-MS $[C_{22}H_{26}N_4O_{11}+Na]^+$ calcd. 545.15, found 545.1; R_f = 0.21 (PE/EtOAc 1:1).

***m*-(α-Azidoacetamido)-phenyl β-D-galactopyranoside (23).** A small portion of sodium was dissolved in dry MeOH (5 mL) and tetraacetate **22** (100 mg, 0.28 mmol) was added. The solution was stirred for 2 h at r.t., then neutralized with Amberlite IR-120/H^+ (Merck), and the resin was filtered. The solvent was removed *in vacuo* to give **23** (91 mg, 0.31 mmol, 98%) as a white solid. ^{1}H-NMR (400 MHz, MeOH-d4) δ 7.44 (t, J = 2.0 Hz, 2H, ArH), 7.26-7.19 (m, 2H, ArH, NH), 6.91-6.86 (m, 1H, ArH), 4.87 (d, J= 7.8 Hz, 1H, H-1), 4.00 (s, 2H, $C\underline{H}_2$), 3.91 (dd, J = 3.4, 1.0 Hz, 1H, H-4), 3.82-3.73 (m, 3H, H-2, -6a,b), 3.72-3.68 (m, 1H, H-5), 3.58 (dd, J = 9.7, 3.4 Hz, 1H, H-3); ^{13}C-NMR (101 MHz, MeOH-d4) δ 168.40 ($\underline{C}O$), 159.55 (ArC), 140.23 (ArC), 130.58 (ArC), 115.09 (ArCH), 113.87 (ArCH), 109.97 (ArCH), 102.88 (C-1), 76.97 (C-4), 74.88 (C-2), 72.24 (C-3), 70.21 (C-5), 62.41 (C-6), 53.32 (CH_2N_3); HRMS: $[C_{14}H_{18}N_4O_7+Na]^+$ calcd 377.1068, found 377.1053; R_f = 0.47 (EtOAc/EtOH 4:1).

***m*-(α-Aminoacetamido)-phenyl β-D-galactopyranoside (24).** Azide **23** (30 mg, 0.08 mmol) was hydrogenolyzed unter H_2 (1 atm) over Pd-C (10-wt%, 3 mg) in MeOH for 2 h. The solution was filtered through a pad of celite and the volatiles were removed *in vacuo*. After MPLC, (SiO_2, EtOAc/EtOH/5%

NH_4OH, gradient 2-100% EtOH) compound **24** was obtained as a colorless oil (23 mg, 88%). ^1H NMR (500 MHz, D_2O) δ 7.40 (t, *J* = 8.2 Hz, 1H, ArH), 7.33 (t, *J* = 2.2 Hz, 1H, ArH), 7.17 (dd, *J* = 8.1, 1.1 Hz, 1H, ArH), 7.00 (dd, J = 8.4, 2.5 Hz, 1H, ArH), 5.08 (d, *J* = 7.5 Hz, 1H, H-1), 4.03 (dd, *J* = 3.2, 1.0 Hz, 1H, H-4), 3.92-3.87 (m, 1H, H-5), 3.85-3.76 (m, 4H, H-2, -3, -6a,b), 3.56 (s, 2H, C$\underline{H_2}$); ^{13}C NMR (126 MHz, D_2O) δ 157.73 (ArC), 138.590 (ArC), 130.94 (ArCH), 116.67 (ArCH), 113.88 (ArCH), 110.49 (ArCH), 101.33 (C-1), 76.10 (C-5), 73.18 (C-3), 71.14 (C-2), 69.10 (C-4), 61.39 (C-6), 44.44 (C$\underline{H_2}N_3$); HRMS: $[C_{14}H_{20}N_2O_7+H]^+$ calcd. 329.1343, found 329.1327; R_f = 0.2 (EtOH/NH_4OH 6:1).

Meta-substituted phenyl-linked reporter ligand 9. Amine **24** (23 mg, 0.07 mmol) was dissolved in dry DMF (4 mL). Fluoresceine isothiocyanate (26.1 mg, 0.07 mmol) and $NaHCO_3$ (5 mg, 0.06 mmol) were separately dissolved in dry DMF (1 mL) and added to **24** under stirring at r.t.. After 14 h, the solvent was removed and the crude product was purified by MPLC (SiO_2, CH_2Cl_2/EtOH/1% AcOH, gradient of 5 to 60% EtOH). For removal of the remaining AcOH the residue was co-evaporated with hexane (3 x 25 mL) and lyophilized. The title compound **9** (38 mg, 0.05 mmol, 71%) was obtained as orange solid. ^1H NMR (500 MHz, DMSO-d6) δ 10.40 (s, 1H, NH/OH), 10.20 (s, 1H, NH/OH), 10.10 (s, 1H, NH/OH), 8.36 (s, 1H, ArH), 8.25 (br s, 1H, $CH_2N\underline{H}$), 7.79 (dd, J = 8.3, 1.9 Hz, 1H, ArH), 7.39 (s, 1H, ArH), 7.35-7.18 (m, 4H, NH, ArH), 6.78-6.74 (m, 1H, ArH), 6.70-6.53 (m, 7H, ArH), 5.18 (d, J = 5.25 Hz, 1H, OH-2), 4.86 (d, J = 5.35 Hz, 1H, OH-3), 4.78 (d, J = 7.70 Hz, 1H, H-1), 4.68-4.61 (m, 1H, OH-6), 4.51 (d, J = 4.65 Hz, 1H, OH-4), 4.43-4.37 (m, 2H, C$\underline{H_2}$NH), 3.73-3.69 (m, 1H, H4), 3.60-3.51 (m, 3H, H-2, -5, -6a), 3.51-3.45 (m, 1H, H-6b), 3.43-3.39 (m, 1H, H-3); ^{13}C NMR (126 MHz, DMSO-d6) δ 180.69 (CS), 168.50 (CO), 167.12 (CO), 159.55 (ArC), 157.87 (ArC), 151.92 (ArC), 141.22 (ArC), 139.77 (ArC), 129.51 (ArCH), 129.08 (ArCH), 126.70 (ArC), 124.23 (ArCH), 116.57 (ArCH), 112.64, 110.97, 109.73, 107.61, 102.25 (ArCH), 101.15 (C-1), 75.38 (C-2/C-5), 73.29 (C-3), 70.29 (C-2/C-5), 67.95 (C-4), 60.11 (C-6), 47.65 (CH_2NH); ESI-MS $[C_{35}H_{31}N_3O_{12}S+H]^+$ calcd. 718.17, found 718.2; R_f = 0.12 (CH_2Cl_2/EtOH/AcOH 3:1:0.08).

***p*-Nitrophenyl β-D-galactopyranoside (25)** was obtained after deprotection of acetate **17** following Zemplén conditions. **25** was first described by Goebel and Avery.[77]

***m*-Nitrophenyl β-D-galactopyranoside (26)** was obtained after deprotection of acetate **21** following Zemplén conditions. **26** was first described by Csuros *et al*.[80]

Phenyl β-D-galactopyranoside (4) was prepared from galactose pentaacetate **16** according to Curcio *et al*.[81]

Benzyl β-D-thiogalactopyranoside (35). Galactose pentaacetate **16** was treated with thioacetic acid and BF_3•OEt_2 according to Caraballo *et al*.[82] After deprotection of 1-thio-galactose pentaacetate under Zemplén conditions, the resulting 1-thiogalactose was treated with sodium hydride and benzyl bromide to give thiogalactoside **35** after chromatographic purification.[83] ^{1}H NMR (400 MHz, MeOH-d4) δ 7.39-7.34 (m, 2H, ArH), 7.32-7.25 (m, 2H, ArH), 7.24-7.18 (m, 1H, ArH), 4.15 (d, J = 9.7 Hz, 1H, H-1), 4.03 (d, J = 12.9 Hz, 1H, 1H of $PhCH_2$), 3.89-3.83 (m, 2H, H-4, 1H of $PhCH_2$), 3.79 (dd, J = 11.5, 6.9 Hz, 1H, H-6a), 3.71 (dd, J = 11.5, 5.1 Hz, 1H, H-6b), 3.64-3.53 (m, 1H, H-2), 3.45 (t, J = 6.0 Hz, 1H, H-5), 3.38 (dd, J = 9.2, 3.4 Hz, 1H, H-3); ^{13}C NMR (101 MHz, MeOH-d4) δ 139.53 (ArC), 130.28 (2C, ArCH), 129.41 (2C, ArCH), 127.93 (ArC), 85.79 (C-1), 80.66 (C-5), 76.28 (C-3), 71.45 (C-2), 70.59 (C-4), 62.73 (C-6), 34.38 ($PhCH_2$). The title compound was first described by Helferich and Türk.[84]

Experimental procedure for β-D-Gal(1,4)-4-SDS-FITC (50)

The synthesis is following previously established protocols for the solid phase synthesis of glycomacromolecules applying alkyne-functionalized TDS building block and short ethyl spacer building block SDS.[66] Commercially available Tentagel chlorotrityl resin modified with ethylene diamine (EDA) was used as resin for solid phase synthesis. After swelling 0.05 mmol of resin in DCM

for 30 min, the initial coupling was performed by dissolving 0.25 mmol (5 eq.) of building block and 0.25 mmol (5 eq.) of PyBOP in DMF (2 mL), followed by the addition of 1.0 mmol (20 eq.) DIPEA. This mixture was shaken for 30 sec and purged with nitrogen for 1 min. Then the mixture was added to the resin and was shaken for 1.5 h. After that, the resin was washed from unreacted reagent 10 times with DMF. The Fmoc protecting group was then cleaved by adding a solution of 0.4 M LiCl in DBU/ piperidine/DMF (1:1:48, v:v) for 20 min and 25% piperidine in DMF for 20 and then 10 min. After the last deprotection step the resin was washed 10 times with DMF.

Capping of N-terminal Site: After successful assembly of the desired number of building blocks on solid phase, the N-terminal site was capped with an acetyl group. For that, a 0.5 M solution of DIPEA and acetic anhydride in DMF was shaken with the resin for 1 h. Subsequently, it was washed 10 times with DMF.

General CuAAC protocol: To 0.05 mmol of resin loaded with the desired number of building blocks, 0.4 mmol (8 eq.) of 2-azidoethyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside[85] dissolved in 1.5 mL DMF was added. 20 mol% sodium ascorbate and 20 mol% $CuSO_4$ were dissolved in 0.5 mL water and were also added to the resin. The mixture was shaken overnight and was subsequently washed in cycles with a 23 mM solution of sodium diethyldithiocarbamate in DMF, water, DMF and DCM.

The deprotection of the sugar moieties was performed using 5 mL of 0.2 M suspension of NaOMe in MeOH. The resin was shaken with this mixture for 1 h. Subsequently it was washed with water and DCM.

Cleavage from solid support: 30% TFA in DCM was added to the resin and was shaken for 1 h. The filtrate was added to cold Et_2O (40 mL). The resulting precipitate was centrifuged and the ether was decanted. The product was redissolved in MeOH and was again precipitated in cold Et_2O, centrifuged and decanted.

β-D-Gal(1,4)-4-SDS (**49**) was synthesized following the above described general coupling and deprotection protocol with the building block sequence TDS, SDS, SDS, TDS. The primary amine end group was capped by the reaction with acetic anhydride. Two β-D-galactosetetraacetate units were subsequently conjugated to the polymeric scaffold by applying general CuAAC protocol followed by sugar deprotection. Final cleavage from solid support gave the desired glycooligomer **49** with a yield of 58 mg (0.041 mmol, 82%) and 92% purity. MALDI-TOF-MS: m/z $[C_{58}H_{98}N_{18}O_{23}+H]^+$ calcd. 1415.71; found 1415.7. RP-HPLC: (5%/95% MeCN/H_2O to 95%/5% MeCN/H_2O in 30 min): t_r = 4.91 min.

β-D-Gal(1,4)-4-SDS-FITC (50). 19.27 mg (0.014 mmol) of β-D-Gal(1,4)-4-SDS **49** were dissolved in 1.36 mL PBS buffer (pH 9.0) to give a 10 mM solution. 1.36 mL of a 10 mM solution of FITC (5.29 mg, 0.014 mmol) in DMF were added. The reaction mixture was stirred overnight. The solvent was removed under reduced pressure and the obtained crude product was purified by preparative HPLC (5%/95% MeCN/H_2O to 95%/5% MeCN/H_2O in 10 min). The final product **50** was obtained as yellow powder after lyophilization with 96% purity. ^{1}H-NMR (600 MHz, D_2O): δ(ppm) 8.03 (s, 1H, FITC-Ar-H), 7.86 (s, 1H, Ar-H), 7.80 (s, 1H, Ar-H), 7.60 (s, 1H, FITC-ArH), 6.94 (s, 1H, FITC-Ar-H), 6.73 (s, 2H, FITC-Ar-H), 6.67-6.47 (m, 4H, FITC-Ar-H), 4.63–4.50 (m, 4H, N$\underline{CH_2}CH_2O$), 4.36–4.29 (m, 2H, Gal-H), 4.28-3.96 (m, 4H, $N(CH_2)_2O$), 3.92–3.88 (m, 2H, Gal-H), 3.76–3.16 (m, 36H, Gal-H, $NH(CH_2)_2NH$), 2.99-2.81 (m, 4H, NC(O)C$\underline{H_2}CH_2$), 2.78-2.62 (m, 4H, NC(O)$CH_2C\underline{H_2}$), 2.52–2.35 (m, 16H, C(O)C$\underline{H_2}C\underline{H_2}$C(O)), 1.91 (s, 1.5 H, NHC(O)C$\underline{H_3}$), 1.89 (s, 1.5 H, NHC(O)C$\underline{H_3}$). MALDI-TOF-MS: m/z $[C_{79}H_{109}N_{19}O_{28}S+Na]^+$ calcd. 1826.73; found 1826.7. RP-HPLC: (5%/95% MeCN/H_2O to 95%/5% MeCN/H_2O in 30 min): t_r = 12.96 min.

Recombinant expression and purification of LecA

The protein LecA was expressed and purified as described previously.[65] Briefly, *E. coli* BL21(DE3) carrying the plasmid pET25pa1l were grown in 1 L LB supplemented with ampicillin (100 μg/mL) to an OD_{600} = 0.5-0.6 at 37 °C and 180 rpm. Expression was induced with IPTG (0.25 mM final concentration) and bacteria were cultured for 4 h at 30 °C and 180 rpm. The cells were then harvested by centrifugation (3'000 x g, 10 min) and the pellet was washed with PBS. The cells were resuspended in 25 mL TBS/Ca (20 mM Tris, 137 mM NaCl, 2.6 mM KCl at pH 7.4 supplemented with 100 µM $CaCl_2$) with PMSF (1 mM) and lysozyme (0.4 mg/mL) and subsequently disrupted using a homogenizer (5 cycles, Microfluidics). Cell debris was removed by centrifugation (60 min, 10'000 x g) and the supernatant was loaded on galactosylated sepharose CL-6B.[86] The column was washed with TBS/Ca and LecA was eluted by addition of 100 mM D-galactose to the buffer. The eluted fractions were extensively dialyzed against distilled water and then, the protein was lyophilized. Between 20 and 35 mg LecA per liter bacterial culture were obtained. The protein was dissolved in TBS/Ca before use and after centrifugation the concentration was determined by UV spectroscopy at 280 nm using a molar extinction coefficient of 27385 M^{-1} cm^{-1}.[87]

Direct binding of fluorescent ligands to LecA

The fluorescent ligands **6**-**9** were dissolved in DMSO to a final concentration of 3 mg/mL. A 1 µM dilution stock was prepared in TBS/Ca. Approx. 5 mg LecA was dissolved in 1 mL of TBS/Ca and incubated with shaking for 2 h at r.t.. Afterwards, the solution was centrifuged for 30 min at 25'000 x g and 23 °C and the concentration of LecA in the supernatant was determined by UV absorbance at 280 nm (ε = 27385 $M^{-1}cm^{-1}$). A serial dilution of LecA was added in triplicates to one 384-well plate (Greiner Bio-One, Germany, cat no 781900). The solution of one fluorescent ligand was added to a

final concentration of 10 nM, and after incubation for 1 h at r.t. fluorescence polarization was determined using a PheraStar FS microplate reader (BMG Labtech GmbH, Germany). The data were analyzed using a four parameter fit calculated with MARS Data Analysis Software (BMG Labtech GmbH, Germany). A minimum of three independent measurements on three plates was performed for each ligand.

Competitive binding assays

Typically, to 20 µL of a concentrated stock solution of LecA (final assay concentrations of LecA: 20 μM for **7**, or 18 μM for **8**, 2-5 μM for **50**) and fluorescent ligand (final assay concentrations of fluorescent ligands **7**, **8**, or **50**: 5-10 nM) in TBS/Ca were added 10 μL serial dilutions (10 mM to 128 µM) of test compounds in TBS/Ca in triplicates in black 384-well microtiter plates (Greiner Bio-One, Germany, cat no 781900). After addition of the reagents, the microtiter plates were centrifuged at 800 rpm for 1 min at 23 °C and subsequently incubated for 4-6 h at r.t.. Fluorescence was measured on a PheraStar FS plate reader (BMG Labtech GmbH, Germany) or an Infinite F500 (Tecan Deutschland GmbH, Germany) with excitation filters at 485 nm and emission filters at 535 nm. The measured intensities were reduced by the values of only LecA in buffer. The data were analyzed with MARS Data Analysis Software (BMG Labtech GmbH, Germany) or Graphpad Prism and fitted according to the four parameter variable slope model. Bottom and top plateaus were defined by the standard compounds included as controls in each assay (methyl α-D-galactoside (**2**) and phenyl β-D-galactoside (**4**), respectively) and the data was reanalyzed with these values fixed. A minimum of three independent measurements on three plates was performed for each inhibitor.

Associated Content

Supporting Information containing ^{1}H and ^{13}C-NMR spectra of all synthesized compounds; for glycopeptides the ^{1}H-NMR spectrum, MALDI-MS spectra and HPLC traces. HPLC chromatograms for all fluorescent ligands are given.

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References

1. H. Lis, N. Sharon, *Chem. Rev.,* 1998, ***98***(2), 637-674.
2. N. Sharon, *FEBS Lett.,* 1987, ***217***(2), 145-157.
3. Varki A. *Essentials of glycobiology.* : 2nd ed edition. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press; 2009.
4. W. A. Petri, Jr, R. Haque, B. J. Mann, *Annu. Rev. Microbiol.,* 2002, ***56***, 39-64.
5. J. A. Rodrigues, A. Acosta-Serrano, M. Aebi, M. A. J. Ferguson, F. H. Routier, I. Schiller, S. Soares, D. Spencer, A. Titz, I. B. H. Wilson, L. Izquierdo, *PLoS Pathog.,* 2015, ***11***(11), e1005169.

6. A. I. Hidron, J. R. Edwards, J. Patel, T. C. Horan, D. M. Sievert, D. A. Pollock, S. K. Fridkin, National Healthcare Safety Network Team, Participating National Healthcare Safety Network Facilities, *Infect. Control Hosp. Epidemiol.,* 2008, ***29***(11), 996-1011.
7. K. G. Kerr, A. M. Snelling, *J. Hosp. Infect.,* 2009, ***73***(4), 338-344.
8. A. Y. Peleg, D. C. Hooper, *New Engl. J. Med.,* 2010, ***362***(19), 1804–1813.
9. K. Poole, *Front. Microbiol.,* 2011, ***2***, 65.
10. H.-C. Flemming, J. Wingender, *Nat. Rev. Microbiol.,* 2010, ***8***(9), 623-633.
11. D. Davies, *Nat. Rev. Drug Discov.,* 2003, ***2***(2), 114-122.
12. T. Bjarnsholt, O. Ciofu, S. Molin, M. Givskov, N. Høiby, *Nat. Rev. Drug Discov.,* 2013, ***12***(10), 791-808.
13. S. Wagner, R. Sommer, S. Hinsberger, C. Lu, R. W. Hartmann, M. Empting, A. Titz, *J. Med. Chem.,* 2016, ***59***(13), 5929-5969.
14. R. Sommer, I. Joachim, S. Wagner, A. Titz, *CHIMIA,* 2013, ***67***(4), 286-290.
15. D. Tielker, S. Hacker, R. Loris, M. Strathmann, J. Wingender, S. Wilhelm, F. Rosenau, K.-E. Jaeger, *Microbiology,* 2005, ***151***(Pt 5), 1313-1323.
16. N. Gilboa-Garber, *Methods Enzymol.,* 1982, ***83***, 378-385.
17. S. P. Diggle, R. E. Stacey, C. Dodd, M. Cámara, P. Williams, K. Winzer, *Environ. Microbiol.,* 2006, ***8***(6), 1095-1104.
18. R. Sommer, S. Wagner, A. Varrot, C. M. Nycholat, A. Khaledi, S. Haussler, J. C. Paulson, A. Imberty, A. Titz, *Chem. Sci.,* 2016, doi:10.1039/C6SC00696E.
19. A. M. Boukerb, A. Decor, S. Ribun, R. Tabaroni, A. Rousset, L. Commin, S. Buff, A. Doléans-Jordheim, S. Vidal, A. Varrot, A. Imberty, B. Cournoyer, *Front. Microbiol.,* 2016, ***7***, 811.
20. C. Chemani, A. Imberty, S. de Bentzmann, M. Pierre, M. Wimmerová, B. P. Guery, K. Faure, *Infect. Immun.,* 2009, ***77***(5), 2065-2075.
21. O. Bajolet-Laudinat, S. Girod-de Bentzmann, J. M. Tournier, C. Madoulet, M. C. Plotkowski, C. Chippaux, E. Puchelle, *Infect. Immun.,* 1994, ***62***(10), 4481-4487.
22. E. C. Adam, B. S. Mitchell, D. U. Schumacher, G. Grant, U. Schumacher, *Am. J. Respir. Crit. Care Med.,* 1997, ***155***(6), 2102-2104.
23. M. Mewe, D. Tielker, R. Schönberg, M. Schachner, K.-E. Jaeger, U. Schumacher, *J. Laryngol. Otol.,* 2005, ***119***(8), 595-599.
24. R. S. Laughlin, M. W. Musch, C. J. Hollbrook, F. M. Rocha, E. B. Chang, J. C. Alverdy, *Ann. Surg.,* 2000, ***232***(1), 133-142.
25. T. Eierhoff, B. Bastian, R. Thuenauer, J. Madl, A. Audfray, S. Aigal, S. Juillot, G. E. Rydell, S. Müller, S. de Bentzmann, A. Imberty, C. Fleck, W. Römer, *Proc. Natl. Acad. Sci. U. S. A.,* 2014, ***111***(35), 12895-12900.
26. G. Cioci, E. P. Mitchell, C. Gautier, M. Wimmerová, D. Sudakevitz, S. Pérez, N. Gilboa-Garber, A. Imberty, *FEBS Lett.,* 2003, ***555***(2), 297-301.
27. R. Loris, D. Tielker, K.-E. Jaeger, L. Wyns, *J. Mol. Biol.,* 2003, ***331***(4), 861-870.

28. E. Mitchell, C. Houles, D. Sudakevitz, M. Wimmerova, C. Gautier, S. Pérez, A. M. Wu, N. Gilboa-Garber, A. Imberty, *Nat. Struct. Biol.,* 2002, ***9***(12), 918-921.

29. E. P. Mitchell, C. Sabin, L. Snajdrová, M. Pokorná, S. Perret, C. Gautier, C. Hofr, N. Gilboa-Garber, J. Koca, M. Wimmerová, A. Imberty, *Proteins,* 2005, ***58***(3), 735-746.

30. P. von Bismarck, R. Schneppenheim, U. Schumacher, *Klin. Padiatr.,* 2001, ***213***(5), 285-287.

31. H.-P. Hauber, M. Schulz, A. Pforte, D. Mack, P. Zabel, U. Schumacher, *Int. J. Med. Sci.,* 2008, ***5***(6), 371-376.

32. A. Bernardi, J. Jiménez-Barbero, A. Casnati, C. De Castro, T. Darbre, F. Fieschi, J. Finne, H. Funken, K.-E. Jaeger, M. Lahmann, T. K. Lindhorst, M. Marradi, P. Messner, A. Molinaro, P. V. Murphy, C. Nativi, S. Oscarson, S. Penadés, F. Peri, R. J. Pieters, O. Renaudet, J.-L. Reymond, B. Richichi, J. Rojo, F. Sansone, C. Schäffer, W. B. Turnbull, T. Velasco-Torrijos, S. Vidal, S. Vincent, T. Wennekes, H. Zuilhof, A. Imberty, *Chem. Soc. Rev.,* 2012, ***42***(11), 4709-4727.

33. J.-L. Reymond, M. Bergmann, T. Darbre, *Chem. Soc. Rev.,* 2013, ***42***(11), 4814-4822.

34. S. Cecioni, A. Imberty, S. Vidal, *Chem. Rev.,* 2015, ***115***(1), 525-561.

35. E. M. V. Johansson, S. A. Crusz, E. Kolomiets, L. Buts, R. U. Kadam, M. Cacciarini, K.-M. Bartels, S. P. Diggle, M. Cámara, P. Williams, R. Loris, C. Nativi, F. Rosenau, K.-E. Jaeger, T. Darbre, J.-L. Reymond, *Chem. Biol.,* 2008, ***15***(12), 1249-1257.

36. R. U. Kadam, M. Bergmann, M. Hurley, D. Garg, M. Cacciarini, M. A. Swiderska, C. Nativi, M. Sattler, A. R. Smyth, P. Williams, M. Cámara, A. Stocker, T. Darbre, J.-L. Reymond, *Angew. Chem. Int. Ed. Engl.,* 2011, ***50***(45), 10631-10635.

37. A. M. Boukerb, A. Rousset, N. Galanos, J.-B. Méar, M. Thepaut, T. Grandjean, E. Gillon, S. Cecioni, C. Abderrahmen, K. Faure, D. Redelberger, E. Kipnis, R. Dessein, S. Havet, B. Darblade, S. E. Matthews, S. de Bentzmann, B. Guéry, B. Cournoyer, A. Imberty, S. Vidal, *J. Med. Chem.,* 2014, ***57***(24), 10275-10289.

38. C. Ligeour, O. Vidal, L. Dupin, F. Casoni, E. Gillon, A. Meyer, S. Vidal, G. Vergoten, J.-M. Lacroix, E. Souteyrand, A. Imberty, J.-J. Vasseur, Y. Chevolot, F. Morvan, *Org. Biomol. Chem.,* 2015, ***13***, 8433-8444.

39. D. Hauck, I. Joachim, B. Frommeyer, A. Varrot, B. Philipp, H. M. Möller, A. Imberty, T. E. Exner, A. Titz, *ACS Chem. Biol.,* 2013, ***8***(8), 1775-1784.

40. R. Sommer, T. E. Exner, A. Titz, *PLoS One,* 2014, ***9***(11), e112822.

41. A. Hofmann, R. Sommer, D. Hauck, J. Stifel, I. Göttker-Schnetmann, A. Titz, *Carbohydr. Res.,* 2015, ***412***, 34-42.

42. R. Sommer, D. Hauck, A. Varrot, S. Wagner, A. Audfray, A. Prestel, H. M. Möller, A. Imberty, A. Titz, *ChemistryOpen,* 2015, ***4***(6), 756-767.

43. G. Beshr, R. Sommer, D. Hauck, D. C. B. Siebert, A. Hofmann, A. Imberty, A. Titz, *Med. Chem. Commun.,* 2016, ***7***, 519-530.

44. J. Rodrigue, G. Ganne, B. Blanchard, C. Saucier, D. Giguère, T. C. Shiao, A. Varrot, A. Imberty, R. Roy, *Org. Biomol. Chem.,* 2013, ***11***(40), 6906-6918.

45. R. U. Kadam, D. Garg, J. Schwartz, R. Visini, M. Sattler, A. Stocker, T. Darbre, J.-L. Reymond, *ACS Chem. Biol.,* 2013, ***8***(9), 1925-1930.

46. S. Cecioni, J.-P. Praly, S. E. Matthews, M. Wimmerová, A. Imberty, S. Vidal, *Chem. Eur. J.*, 2012, ***18***(20), 6250-6263.

47. N. Garber, U. Guempel, A. Belz, N. Gilboa-Garber, R. J. Doyle, *Biochim. Biophys. Acta.*, 1992, ***1116***(3), 331-333.

48. F. Pertici, N. J. de Mol, J. Kemmink, R. J. Pieters, *Chem. Eur. J.*, 2013, ***19***(50), 16923-16927.

49. F. Pertici, R. J. Pieters, *Chem. Commun. (Camb)*, 2012, ***48***(33), 4008-4010.

50. R. Visini, X. Jin, M. Bergmann, G. Michaud, F. Pertici, O. Fu, A. Pukin, T. R. Branson, D. M. E. Thies-Weesie, J. Kemmink, E. Gillon, A. Imberty, A. Stocker, T. Darbre, R. J. Pieters, J.-L. Reymond, *ACS Chem. Biol.*, 2015, ***10***(11), 2455-2462.

51. M. I. Khan, N. Surolia, M. K. Mathew, P. Balaram, A. Surolia, *Eur. J. Biochem.*, 1981, ***115***(1), 149-152.

52. G. Jacob, C. Kirmaier, S. Abbas, S. Howard, C. Steininger, J. Welply, P. Scudder, *Biochemistry*, 1995, ***34***(4), 1210–1217.

53. P. Sörme, B. Kahl-Knutsson, M. Huflejt, U. J. Nilsson, H. Leffler, *Anal. Biochem.*, 2004, ***334***(1), 36-47.

54. P. Sörme, B. Kahl-Knutson, U. Wellmar, U. J. Nilsson, H. Leffler, *Methods Enzymol.*, 2003, ***362***, 504-512.

55. Z. Han, J. S. Pinkner, B. Ford, R. Obermann, W. Nolan, S. A. Wildman, D. Hobbs, T. Ellenberger, C. K. Cusumano, S. J. Hultgren, J. W. Janetka, *J. Med. Chem.*, 2010, ***53***(12), 4779-4792.

56. S. Kleeb, L. Pang, K. Mayer, D. Eris, A. Sigl, R. C. Preston, P. Zihlmann, T. Sharpe, R. P. Jakob, D. Abgottspon, A. S. Hutter, M. Scharenberg, X. Jiang, G. Navarra, S. Rabbani, M. Smiesko, N. Lüdin, J. Bezençon, O. Schwardt, T. Maier, B. Ernst, *J. Med. Chem.*, 2015, ***58***(5), 2221-2239.

57. J. Dahmen, T. Frejd, G. Gronberg, T. Lave, G. Magnusson, G. Noori, *Carbohydr. Res.*, 1983, ***116***(2), 303-307.

58. X. Huang, A. Aulabaugh, *Methods Mol. Biol.*, 2009, ***565***, 127-143.

59. G. S. Sittampalam, N. P. Coussens, H. Nelson, M. Arkin, D. Auld, C. Austin, B. Bejcek, M. Glicksman, J. Inglese, P. W. Iversen, Z. Li, J. McGee, O. McManus, L. Minor, A. Napper, J. M. Peltier, T. Riss, O. J. Trask, Jr., J. Weidner. Assay Guidance Manual. 2012. Available at: http://www.ncbi.nlm.nih.gov/books/NBK53196/.

60. N. Gilboa-Garber, D. Sudakevitz, M. Sheffi, R. Sela, C. Levene, *Glycoconj. J.*, 1994, ***11***(5), 414-417.

61. S. Perret, C. Sabin, C. Dumon, M. Pokorná, C. Gautier, O. Galanina, S. Ilia, N. Bovin, M. Nicaise, M. Desmadril, N. Gilboa-Garber, M. Wimmerová, E. P. Mitchell, A. Imberty, *Biochem. J.*, 2005, ***389***(Pt 2), 325-332.

62. K.-C. Kuo, H.-C. Kuo, L.-T. Huang, C.-S. Lin, S.-N. Yang, *J. Microbiol. Immunol. Infect.*, 2013, ***46***(2), 109-114.

63. A. Titz, A. Marra, B. Cutting, M. Smieško, G. Papandreou, A. Dondoni, B. Ernst, *Eur. J. Org. Chem.*, 2012, ***2012***(28), 5534–5539.

64. A. Imberty, C. Breton, R. Oriol, R. Mollicone, S. Pérez, *Adv. Macromol. Carbohydr. Res.*, 2003, ***2***, 67-130.

65. B. Blanchard, A. Nurisso, E. Hollville, C. Tétaud, J. Wiels, M. Pokorná, M. Wimmerová, A. Varrot, A. Imberty, *J. Mol. Biol.,* 2008, ***383***(4), 837-853.

66. D. Ponader, F. Wojcik, F. Beceren-Braun, J. Dernedde, L. Hartmann, *Biomacromolecules,* 2012, ***13***(6), 1845-1852.

67. H. E. Gottlieb, V. Kotlyar, A. Nudelman, *J. Org. Chem.,* 1997, ***62***(21), 7512-7515.

68. S. Dasgupta, V. K. Rajput, B. Roy, B. Mukhopadhyay, *J. Carbohydr. Chem.,* 2007, ***26***(2), 91-106.

69. C. Grandjean, C. Rommens, H. Gras-Masse, O. Melnyk, *Tetrahedron Lett.,* 1999, ***40***(40), 7235-7238.

70. L.-X. Wang, 2005, US2005/244424 A1.

71. S. Park, I. Shin, *Org. Lett.,* 2007, ***9***(9), 1675-1678.

72. H. W. Coles, M. L. Dodds, F. H. Bergeim, *J. Am. Chem. Soc.,* 1938, ***60***(5), 1020-1022.

73. R. D'Agata, G. Grasso, G. Iacono, G. Spoto, G. Vecchio, *Org. Biomol. Chem.,* 2006, ***4***(4), 610-612.

74. H. Susaki, K. Suzuki, M. Ikeda, H. Yamada, H. K. Watanabe, *Chem. Pharm. Bull. (Tokyo),* 1994, ***42***(10), 2090-2096.

75. C. K. Chiang, M. McAndrew, R. Barker, *Carbohydr. Res.,* 1979, ***70***(1), 93-102.

76. R. B. Cohen, K.-C. Tsou, S. H. Rutenburg, A. M. Seligman, *J. Biol. Chem.,* 1952, ***195***(1), 239-249.

77. W. F. Goebel, O. T. Avery, *J. Exp. Med.,* 1929, ***50***(4), 521-531.

78. M. Apparu, M. Blanc-Muesser, J. Defaye, H. Driguez, *Can. J. Chem.,* 1981, ***59***(2), 314-320.

79. T. Iversen, R. Johansson, *Synthesis,* 1979, 823–824.

80. Z. Csuros, G. Deak, M. Haraszthy-Papp, *Acta Chim. Acad. Sci. Hung.,* 1964, ***42***(3), 263-267.

81. P. Curcio, C. Zandanel, A. Wagner, C. Mioskowski, R. Baati, *Macromol. Biosci.,* 2009, ***9***(6), 596-604.

82. R. Caraballo, M. Sakulsombat, O. Ramström, *Chembiochem.* 2010, ***11***(11), 1600-1606.

83. M. Cerný, J. Stanek, J. Pacák, *Monatsh. Chem.,* 1962, ***94***(1), 290-294.

84. B. Helferich, D. Türk, *Chem. Ber.,* 1956, ***89***(10), 2215-2219.

85. L. Wu, N. S. Sampson, *ACS Chem. Biol.,* 2014, ***9***(2), 468-475.

86. N. Fornstedt, J. Porath, *FEBS Lett.,* 1975, ***57***(2), 187-191.

87. M. R. Wilkins, E. Gasteiger, A. Bairoch, J. C. Sanchez, K. L. Williams, R. D. Appel, D. F. Hochstrasser, *Methods Mol. Biol.,* 1999, ***112***, 531-552.