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Glycopeptides Hot Paper

Fucose Binding Motifs on Mucin Core Glycopeptides Impact Bacterial Lectin Recognition**

*Sandra Behren[+], Jin Yu[+], Christian Pett, Manuel Schorlemer, Viktoria Heine, Thomas Fischöder, Lothar Elling, and Ulrika Westerlind**

Abstract: Mucin glycoproteins are essential components of the mucosal barrier, which protects the host from pathogens. Throughout evolution, bacteria have developed strategies to modulate and penetrate this barrier, and cause virulence by interacting with mucin *O*-glycans at the epithelial cell-surface. *O*-fucosylated glycan epitopes on mucins are key ligands of many bacterial lectins. Here, a chemoenzymatic synthesis strategy is described to prepare a library of fucosylated mucin core glycopeptides to enable studies of mucin-interacting and fucose-binding bacterial lectins. Glycan cores with biologically important Lewis and H-antigens were prepared decorating the peptide backbone at different sites and densities. The fucosylated mucin glycopeptides were applied in microarray binding studies to explore the importance of glycan core and peptide backbone presentation of these antigens in binding interactions with the *P. aeruginosa* lectin LecB and the *C. difficile* toxin A.

Introduction

Mucin glycoproteins are central players in the host-defense machinery directed against invading pathogens.[1] In addition to the dense glycan shield formed by membrane-bound mucin glycoproteins on epithelial cell surfaces, secreted mucins cover the epithelial tissues as the major constituents of the mucus. Thus, the majority of potential infections is prevented by mucus clearance, but bacteria and viruses have co-evolved with the human host and developed strategies to promote immune escape and virulence.[2] Pathogenic bacteria interact with carbohydrate ligands of membrane-bound mucins to promote bacteria cell-adhesion, biofilm formation, or cause an inflammatory environment. Additionally, bacteria may manipulate the glycan structures of the host, for instance by using specific glycosidases, to degrade mucus or to build-up their own glycan shield for immune escape.[1] Mucin glycans on the host epithelial cells are also targets of bacterial protein toxins that promote cell adhesion to allow intracellular protein toxin delivery.[2a]

Fucose residues decorate terminal positions of mucin carbohydrate ligands and other glycoconjugates, which are typically presented in the blood group A-, B- and H-antigens or on Lewis epitopes. These fucosylated structures are critical players in diverse bacteria and virus interactions. For example, blood group O-individuals are presenting the H-antigen structure, which increases the susceptibility for severe cholera infection caused by *Vibrio cholerae*, and gastroenteritis caused by the Norwalk virus.[3] Patients suffering from cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD) have, in addition to high mucin secretion, an increased presentation of fucosylated glycans on mucins in the lung.[4] These fucosylated glycans are targets for lectins of many bacterial pathogens. [5] *Pseudomonas aeruginosa* is a Gram-negative opportunistic bacterium that often infects patients suffering from COPD and CF. The soluble lectin LecB is secreted from *P. aeruginosa* and causes critical virulence due to its involvement in bacterial biofilm formation.[6] LecB is a tetrameric adhesin that specifically recognizes and binds to L-fucosides, which are included in Lewis[a] (Le[a]) antigen structures and have been reported to be preferred LecB ligands. [5, 7] The progressing multi-drug resistance of *P. aeruginosa* highlights the need to develop new strategies to fight this bacterial

[*] Dr. S. Behren,[+] Dr. C. Pett, Dr. M. Schorlemer, Prof. Dr. U. Westerlind
Department of Chemistry, Umeå University
90187 Umeå (Sweden)
E-mail: ulrika.westerlind@umu.se

Dr. S. Behren,[+] Dr. J. Yu,[+] Dr. C. Pett, Dr. M. Schorlemer, Prof. Dr. U. Westerlind
Leibniz-Institut für Analytische Wissenschaften – ISAS – e.V.
44227 Dortmund (Germany)

Dr. J. Yu[+]
Glycosciences Laboratory, Imperial College London
London W12 0NN (UK)

Dr. V. Heine, Dr. T. Fischöder, Prof. Dr. L. Elling
Laboratory for Biomaterials, Institute of Biotechnology and Helmholtz-Institute for Biomedical Engineering, RWTH Aachen University
Pauwelsstraße 20, 52074 Aachen (Germany)

[+] These authors contributed equally to this work.

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infection. Glycoconjugates and glycomimetic structures interfering with LecB binding are interesting candidates in the development of new antimicrobial therapies. So far, multivalent presentation of short glycan epitopes on peptides, dendrimers or polymers has been found to efficiently mimic natural glycan presentation and increase lectin avidity.[8] For example, multivalent presented α-L-fucose monosaccharides were applied as potential LecB inhibitors.[9] Also, the abilities of glycodendrimers presenting the terminal Le^a epitope to inhibit LecB were explored.[9b] Titz and co-workers developed glycomimetic *C*-glycosides of amides and sulfonamides to further elucidate LecB structure–activity relationships.[10] Another opportunistic bacterium that often causes recurrent mild to severe gastrointestinal infections in immune compromised patients is the Gram-positive *Clostridium difficile*.[11] Toxin A (TcdA) and toxin B (TcdB) are secreted from *C. difficile* and are two multi-domain toxins that inactivate critical host GTPases, including Rac, Cdc42 and the Rho A protein family, by monoglucosylation, thus inducing cell death.[12] Therapeutic treatments to combat *C. difficile* infections are limited and often rely on strong antibiotics or therapeutic antibody administration. Consequently, better knowledge of TcdA binding interactions would facilitate the development of novel glycomimetic anti-adhesives. For example, inhibition of TcdA-mediated cell toxicity was recently demonstrated with multivalent bovine serum albumin (BSA) neo-glycoproteins bearing the Lewis antigens $Lewis^y$ and $Lewis^x$.[11b] Furthermore, a mucin-type fusion protein carrying the Galα-1,3 Galili epitope was also found to interact with *C. difficile* TcdA and showed inhibition in a rabbit erythrocyte hemagglutination assays.[13]

Besides their importance in lectin binding interactions, the presentation of the unique terminal mucin glycan epitopes by the underlying core structures and the peptide backbone is potentially essential for the fine binding specificities and biological functions of these lectins. However, this knowledge is often neglected in studies of lectin interactions, even if these structures may define the glycan orientation, structural rigidity or possible limitations for ligand recognition.

In this work, we explored the molecular fine-specificities of TcdA and LecB interactions with fucosylated mucin tandem repeat glycopeptides. We employed a library of fucosylated structures displayed on glycan cores of mucin 1 (MUC1) and mucin 5B (MUC5B) tandem repeat peptides to elucidate the roles of different terminal fucose motifs, and of glycan presentation on different glycosylation sites of the peptide backbone in bacterial lectin recognition events. Therefore, 63 synthetic α-1,2-, α-1,3- and α-1,4-fucosylated mono- and bivalent MUC1 and MUC5B glycopeptides were prepared presenting different Lewis- and H-antigen structures. The glycopeptides carried differently fucosylated LacNAc core-1 to core-4 structures on distinct mucin peptide tandem repeat glycosylation sites. The obtained fucose glycopeptides were immobilized on NHS-activated microarray slides and applied to elucidate binding interactions of the *P. aeruginosa* lectin LecB and the *C. difficile* toxin A.

Results and Discussion

Generation of an O-Fucosyl MUC1 and MUC5B glycopeptide library

To study the interactions of the fucose binding lectins LecB and TcdA, LacNAc (type-1 (Galβ1,3GlcNAc) and type-2 (Galβ1,4GlcNAc)) elongated mucin core 1–4 glycosylated amino acids were prepared and incorporated into to the human mucin MUC1 and MUC5B peptide tandem repeat sequences, PAHGVTSAPD**T***RPAPGS**T***A and A**T***PSS**T***PG**T***THTP (**T*** = modified glycosylation sites), by Fmoc-solid-phase peptide synthesis (Fmoc-SPPS) (Figure 1A, Supporting Information Figure S2). The different mucin core threonine building blocks, including core 1 type-1 and type-2, core 2 type-1 and type-2 tetrasaccharide and hexasaccharide, core 3 type-1 and type-2, and core 4 type-2, were reported previously.[14] The core 4 type-1 glycosylated amino acid **Compound 6** was here synthesized analogously to the reported core 2 type-1 synthesis.[14a] **Compound 6** was assembled in 59 % yield by a [3+2] glycosylation using the LacNAc type-1 disaccharide donor **Compound 1** and the type-1 core 3 glycosylated amino acid acceptor **Compound 2** (Figure 1A, Supporting Information Figure S1).[15] The *tert*-butyldimethylsilyl protecting group (TBS) was then removed under acidic conditions using 80 % acetic acid (AcOH), followed by acetylation with acetic anhydride in pyridine to obtain **Compound 4**. The *N*-Troc groups were removed by reductive elimination using zinc dust in AcOH,[16] followed by acetylation to obtain the corresponding acetamide **Compound 5**. Finally, the amino acid *tert*-butyl ester was cleaved using trifluoroacetic acid (TFA)[17] and anisole[18] leading to the formation of the desired type-1 core 4 amino acid **Compound 6** in 38 % yield over four steps. Next, the glycosylated threonine building blocks were introduced into the above-mentioned peptide sequences by applying our reported automated Fmoc-SPPS protocol for glycopeptide synthesis (Figure 1A, Supporting Information Figure S2).[14,19] In brief, preloaded Fmoc-AA-Trt-resins were used. Fmoc-groups were removed with 20 % piperidine and standard Fmoc-amino acids (8 equiv) were coupled using HBTU, HOBt and DIPEA. The glycosylated amino acids (1.5 equiv) were coupled at a higher concentration and with longer reaction times using the stronger activating reagent HATU together with HOAt and DIPEA. At the *N*-terminus a triethylenglycol spacer (3 equiv) was incorporated using HBTU activation. The peptides were released from the resin using TFA : TIPS : H_2O in a ratio of 95 : 5 : 5 and desalted on a C18 cartridge. Glycan deacetylation was performed using NaOMe in MeOH at pH 9.5, or NaOH in MeOH : H_2O 1 : 1 at pH 11.5, followed by final C18 preparative HPLC purification (Supporting Information Section 2). The synthesis of the type-1 and type-2 core 4 MUC1 peptides and type-2 core 1 and type-2 core 2 hexasaccharide MUC5B peptides (**P1**–**P7**, yields and analytical data are described in the Supporting Information Section 2.3), were here described. Meanwhile the MUC1 and MUC5B peptids **P8**–**P31** were previously reported.[14,19] The obtained glycopeptides were enzymatically modified with LacNAc and/or $Lewis^a$

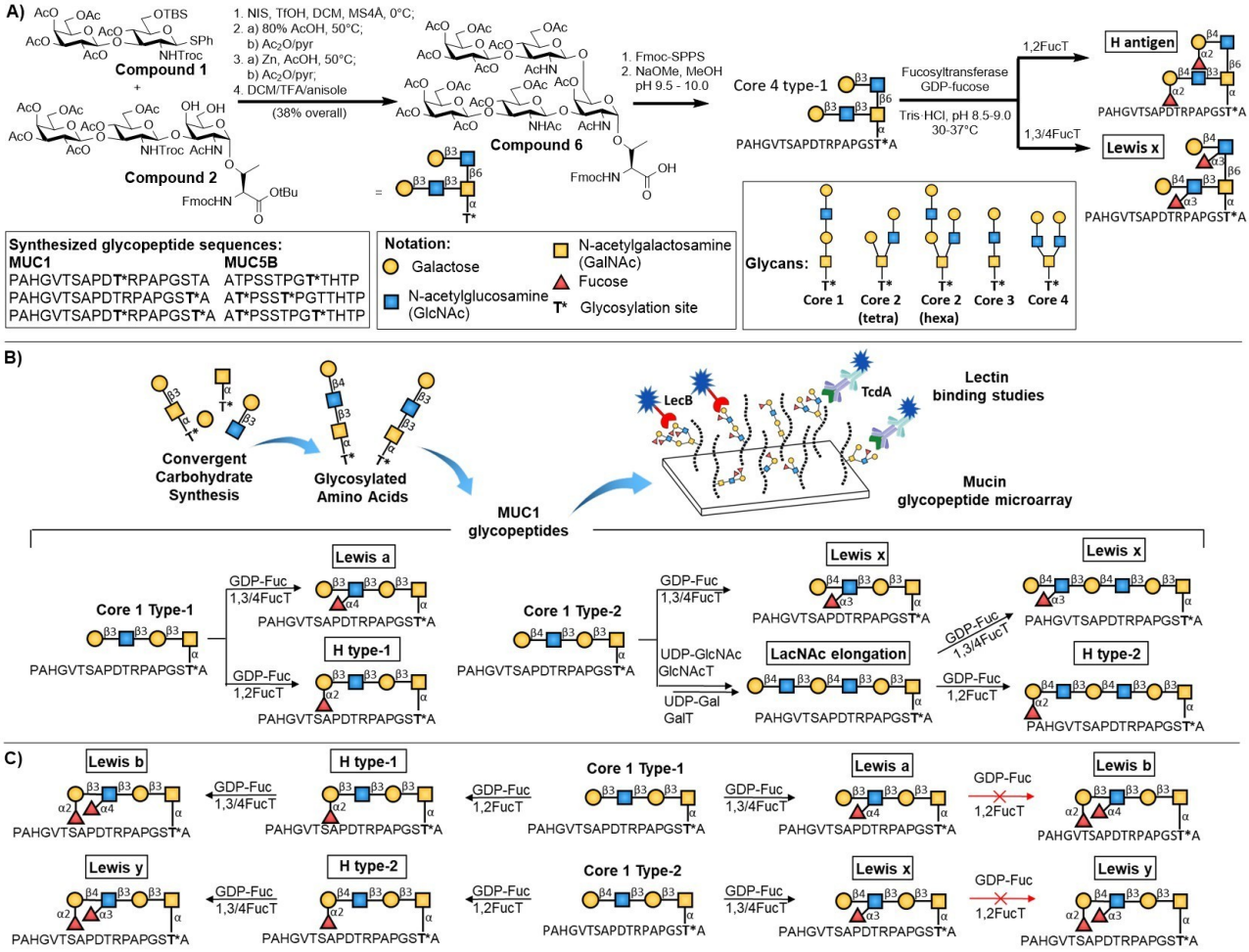

Figure 1. A) Synthesis overview of prepared fucosylated MUC1 and MUC5B *O*-glycopeptides. An example is given for synthesis of the mucin core 4 type-1 glycosylated amino acid building block followed by Fmoc-SPPS glycopeptide synthesis and enzymatic modification; B) Example of enzymatic transformations (fucosylations and additional LacNAc elongation) made for the generation of a fucosyl glycopeptide microarray library; C) Synthesis of Lewis^b and Lewis^y modified glycopeptides.

(Le^a, α-1,4-fucosylation), Lewis^x (Le^x, α-1,3-fucoslyaltion), and/or H-type (H, α-1,2-fucosylation) core glycans (Figure 1B, C, Figure 2 and Supporting Information Figure S3–S5). Therefore, *Helicobacter pylori* β-1,3-*O*-*N*-acetylglucosaminyltransferase (β3GlcNAcT)[20] and a fusion protein of the human β-1,4-*O*-galactosyltransferase (His$_6$-Propeptide-catβ4GalT-1, β4GalT)[21] were first applied to enzymatically extend selected glycopeptides with additional LacNAc units. Then, the glycopeptides were further modified with fucosylation using *Helicobacter mustelae* α-1,3/4-*O*-fucosyltransferase (Hmα1,3/4FucT), resulting in Le^x and Le^a antigens,[22] or *H. mustelae* α-1,2-*O*-fucosyltransferase (Hmα1,2FucT), resulting in H-type-1 or H-type-2 antigens[23] (Figure 1B, Supporting Information Figure S3, S4). By combining the two fucosyltransferases on the terminal type-1 or type-2 LacNAc units, bi-fucosylated Lewis^b (Le^b, α-1,4- and α-1,2-fucosylation) and Lewis^y (Le^y, α-1,3- and α-1,2-fucosylation) motifs were generated in a high yield by first introducing a α-1,2 fucose residue, followed by α-1,3/4-fucosylation. However, the reverse enzymatic route was also explored, but the desired bi-fucosylated products were not obtained in satisfactory yields, which is consistent with a recent report modifying *O*-glycans (Figure 1C, Supporting Information Figure S6).[24] Yields and structures of all *O*-fucosylated glycopeptides are given in Figure 2 and the Supporting Information. The glycopeptide library was then printed on NHS-activated hydrogel slides (Nexterion® slide H, Schott). In conclusion, we were able to generate an extensive *O*-fucosyl MUC1 and MUC5B glycopeptide microarray library with well-defined and closely related glycans that provides a unique platform to explore fine specificities of mucin-recognizing and fucose-binding proteins (Supporting Information Table S1). Besides synthesis of analogs of the PSGL-1 selectin glycopeptide ligand,[25] only a handful of fucosylated *O*-glycopeptides have been previously reported.[26] The three-dimensional presentation of the terminal fucosylated mucin glycan epitopes on the underlying core structures and the natural mucin peptide backbone might impact and limit ligand recognition by these proteins. Consequently, we explored the influence of glycosylation site placement and

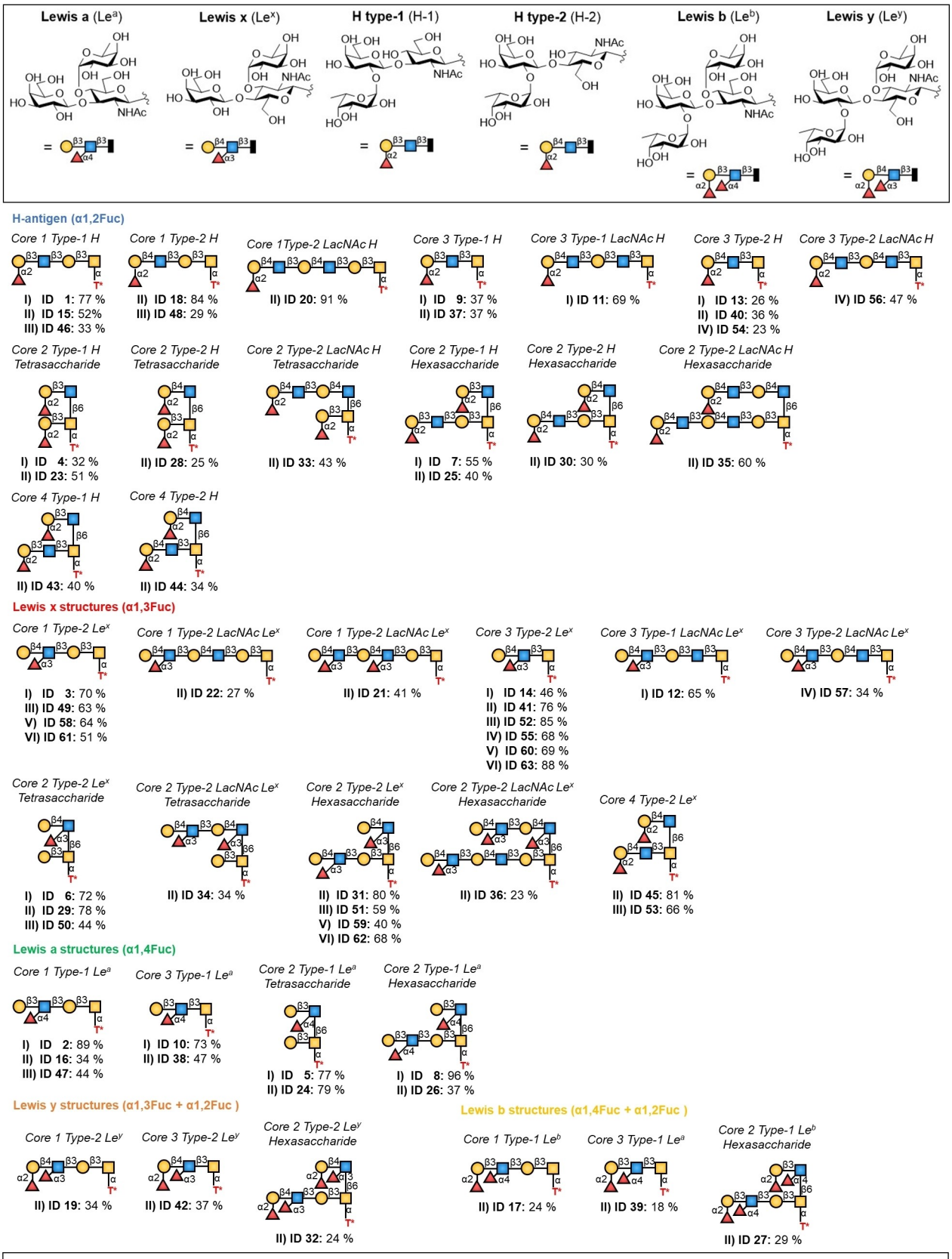

Figure 2. Schematic representation of the here prepared fucosylated MUC1 and MUC5B glycopeptides and their isolated yields from enzymatic *O*-fucosylation. ID = Microarray ID.

glycan density on the peptide backbone, as well as the mucin core structure, LacNAc extension and different fucosyl epitopes on LecB and TcdA binding.

Binding Preferences of the Pseudomonas aeruginosa lectin LecB

To determine the binding preferences of LecB, the glycopeptide microarray library was incubated with a dilution series of LecB-biotin (31 nM–16 μM), followed by incubation with Cy5-labeled streptavidin for fluorescent detection. Surface dissociation constants Surf. K_D (see Supporting Information Equation 1) for LecB binding to the fucosylated MUC1 and MUC5B peptides were determined and are reported in Table 1 and Supporting Information Table S2. The microarray IDs of the printed glycopeptides are used henceforth in the discussion. Microarray analysis showed that the fine specificities of LecB strongly depended on the different fucose motifs, presenting peptide backbone, underlying core structures, LacNAc-extension as well as placement of the glycosylation sites (Figure 3 and 4, Supporting Information Figure S7–S10). The lectin exhibited a broad selectivity towards all fucosylated glycopeptides and bound to all fucosylated MUC1 and MUC5B glycopeptides in a high nanomolar to low micromolar range ($K_{D,Muc1}$ Surf = 0.16–2.97 μM and $K_{D,Muc5B}$ Surf = 0.39–2.91 μM).

Table 1: K_D surf values for mono- and bivalent[a] H-type, Lewis^x and Lewis^a MUC1 glycopeptides determined by incubation of LecB at 8 different concentrations at 31 nM–16 μM; Hex = hexasaccharide; Tet = tetrasaccharide.

H-type			Lewis x			Lewis a		
ID	Core	K_D [μM]	ID	Core	K_D [μM]	ID	Core	K_D [μM]
PDT*R			**PDT*R**			**PDT*R**		
4	Core 2_{Tet} type-1	0.55	3	Core 1 type-2	1.19	8	Core 2_{Hex} type-1	0.24
7	Core 2_{Hex} type-1	0.63	6	Core 2_{Tet} type-2	1.72	5	Core 2_{Tet} type-1	0.31
9	Core 3 type-1	1.02	14	Core 3 type-2	2.97	10	Core 1 type-1	0.36
1	Core 1 type-1	1.10				2	Core 3 type-1	0.42
GST*A			**GST*A**			**GST*A**		
25	Core 2_{Hex} type-1	0.53	31	Core 2_{Hex} type-2	0.91	26	Core 2_{Hex} type-1	0.19
23	Core 2_{Tet} type-1	0.54	45	Core 4 type-2	1.17	24	Core 2_{Tet} type-1	0.25
43	Core 4 type-1	0.55	29	Core 2_{Tet} type-2	1.72	16	Core 1 type-1	0.26
15	Core 1 type-1	0.64	41	Core 3 type-2	1.76	38	Core 3 type-1	0.31
37	Core 3 type-1	0.82	**Bivalent[b]**			**Bivalent[b]**		
30	Core 2_{Hex} type-2	0.28	51	Core 2_{Hex} type-2	0.54	47	Core 1 type-1	0.16
28	Core 2_{Tet} type-2	0.37	53	Core 4 type-2	0.75			
44	Core 4 type-2	0.37	49	Core 1 type-2	0.79			
18	Core 1 type-2	0.48	50	Core 2_{Tet} type-2	1.12			
40	Core 3 type-2	0.60	52	Core 3 type-2	1.26			
Bivalent[b]								
48	Core 1 type-2	0.35						
46	Core 1 type-1	0.50						

[a] Bivalent means that two glycosylation sites of the peptide backbone are modified.

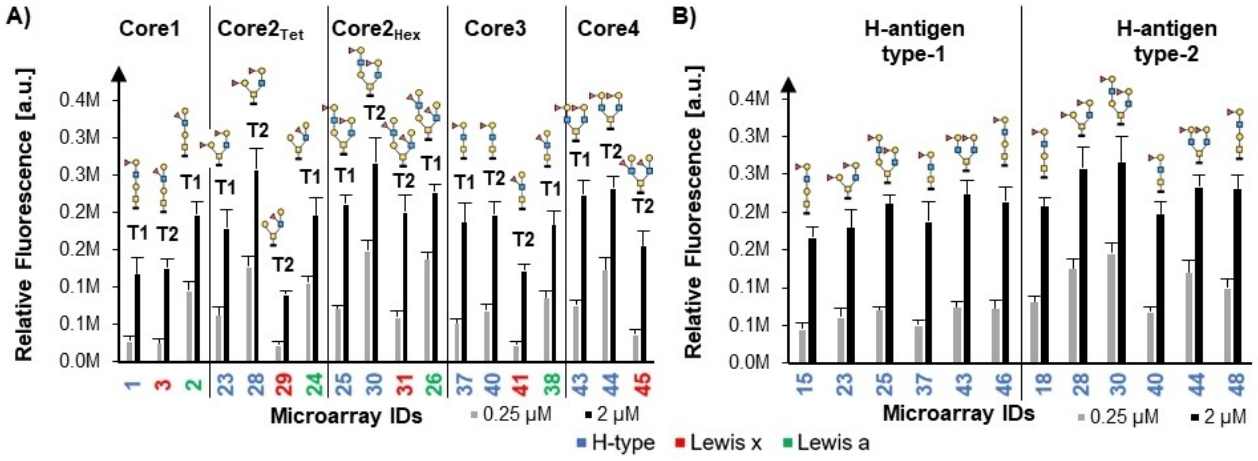

Figure 3. Overview of the LecB recognized fucosylated motifs presented on different *O*-glycan core structures of MUC1 glycopeptides. A) Binding of LecB here shown at two selected concentrations at 0.25 μM and 2 μM to different H-type (blue), Le^x (red) and Le^a (green) modified MUC1 core structures; B) Comparison of LecB-binding affinity towards H-type-1 and type-2 MUC1 glycopeptides (blue); Notation for carbohydrates see Figure 1; Hex = hexasaccharide; Tet = tetrasaccharide; T1 = type-1 (Galβ1,3GlcNAc); T2 = type-2 (Galβ1,4GlcNAc); Microarray IDs see Sup. Info Table S1.

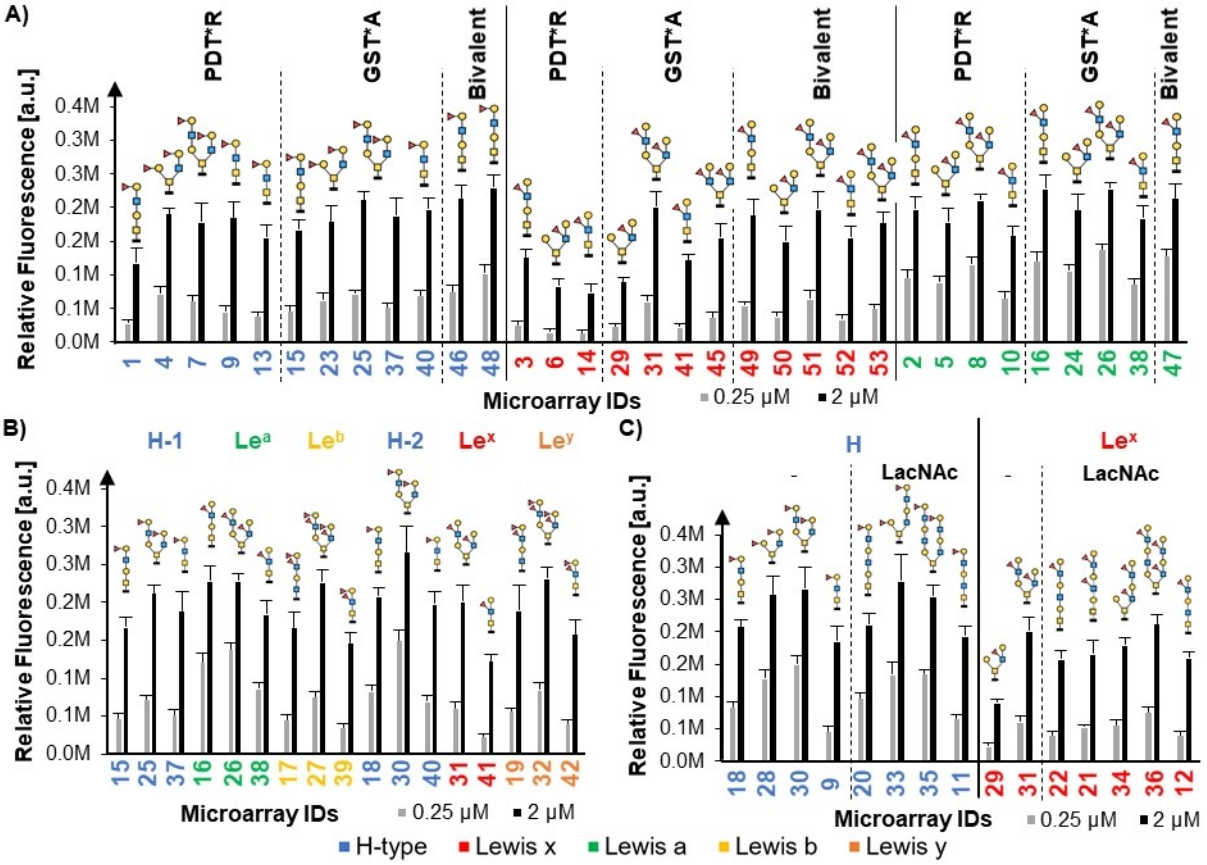

Figure 4. A) Elucidating the impact of glycosylation sites and multivalency: Comparison of LecB-binding towards monovalent H-type (blue), Le^x (red) and Le^a (green) epitopes presented on core 1–4 MUC1 peptides glycosylated in two different glycosylation sites; PDT*R or GST*A, and the corresponding bivalent glycopeptides; B) Influence of LacNAc elongation on LecB-binding towards H-type and Le^x MUC1 glycopeptides; C) Comparison of LecB-binding between Le^b (yellow) and Le^y (orange); and the respective mono-fucosylated Lewis/H-antigen glycopeptides; LacNAc = extension with a *N*-acetyllactosamine unit; Microarray IDs see Sup. Info Table S1.

Influence of different fucose containing motifs on MUC1 glycopeptides on LecB binding

First, we explored the impact of different *O*-fucosyl motifs on LecB binding, including Le^a, Le^x, Le^y, Le^b and blood H epitopes. Our findings showed that Le^a and H-type-2 MUC1 glycopeptides were better binders than the respective H-type-1 and Le^x glycopeptides with the Le^x glycans being the weakest binders. This glycopeptide binding pattern was consistent with findings from previous glycan recognition studies of LecB.[8a–c] Here, we observed that LecB bound Le^a glycans 1.2–3.1-fold and 3.3–7.0-fold better than the respective H-antigen type-1 and Le^x glycopeptides. For instance, compare Le^a peptide **ID 26** with H-antigen peptide **ID 25** and Le^x peptide **ID 31** (Figure 3A, Table 1).

This observation could be explained by the ability of the Le^a antigen to create an additional hydrogen bond with the protein backbone due to the favorable steric location of the GlcNAc *O*-6 position. However, the GlcNAc *N*-acetyl group of the Le^x glycan located in the same position would lead to sterical hindrance and the Le^x glycan must adapt into a less favorable conformation upon binding to LecB.[8d,e] In agreement with previous studies, H-antigen type-2 structures were better LecB-binders than the type-1 derivatives.[8a,c] Here, we observed a 1.3–1.9-fold increase in affinity for the H-type-2 glycans with the GST*A core 3 peptides (compare **ID 37** and **ID 40**) being the only exception (Figure 3B, Table 1).

Then, differences in LecB-binding between bi-fucosylated Le^b and Le^y core structures and their respective mono-fucosylated Le^a, Le^x and H-type analogs were determined. Le^b and Le^y modified glycan structures possess two fucose moieties that could potentially enhance binding strength through multivalent interactions. However, we could not observe an increase in LecB binding affinity for these structures (Figure 4B).[7a,c] Instead, the Le^b and Le^y peptides were weaker binders than both the H-antigen and Le^a modified glycopeptides, indicating that the presence of a second fucose residue may sterically hinder LecB binding (Table 2B). On the contrary, Le^y core structures were better binders than the respective Le^x glycans, but weaker than the H-type glycopeptides.

In conclusion, these findings suggest that the linkage between the fucosyl moiety and the underlying glycan core is important for LecB recognition.

Influence of different glycan core MUC1 glycopeptides on LecB binding

Next, we tested the importance of the underlying mucin core structures on LecB recognition. The mucin core 1 to core 4 glycans are structures commonly found in glycoproteins. Microarray analysis showed that LecB exhibited varying affinities for the different mucin core structures (Figure 4A). Generally, LecB preferably bound to branched (core 2 and core 4) over linear (core 1 and core 3) H-type, Le^x and Le^a glycans (Table 1, Figure 3A, Figure 4A).

We found that on the respective glycosylation site, the linear H-antigen glycans carrying only one fucosyl residue showed comparable binding affinities to LecB. However, the branched structures that exhibit two fucose residues, one on each arm, showed higher binding affinities. These terminal epitopes situated on the branched core structures are oriented in opposite directions based on predicted energy minimized structures (www.glycam.org, for example search on: DGalpb1-4DGlcpNAcb1-6[DGalpb1-3]DGalpNAca1-OME), and can potentially facilitate intra- or intermolecular multivalent lectin interactions through their spatial arrangement.

In contrast to the α-1,2-fucosylated (H-antigen) glycopeptides, the Le^a and Le^x core 2 tetrasaccharides contain only one fucose residue on the 6-arm and therefore show a similar binding affinity as the linear structures. Since the branched Le^a and Le^x core 2 hexasaccharide and core 4 contain an additional fucosylated LacNAc unit on the 3-arm, which can participate in multivalent interactions leading to an overall higher avidity, they showed better binding than the respective tetrasaccharide derivative.

We can conclude that the mucin core structure presenting the different fucosylated epitopes strongly influences lectin binding. Here, branched core structures can facilitate multivalent binding and thus show enhanced affinities for LecB compared to the linear mucin core structures.

Influence of glycosylation site placement and bivalent ligand presentation on MUC1 glycopeptides on LecB binding

The placement of the glycan epitope on the peptide backbone and the amino acid sequence might influence lectin binding. Additionally, an increased glycan density caused by multivalent ligand presentation on the same peptide backbone can enhance lectin binding due to avidity binding effects. Consequently, we compared LecB binding between the monovalent MUC1 glycopeptides (glycosylation either in the PDT*R or GST*A region) and also to the respective bivalent analogs (glycosylation in the PDT*R and GST*A regions). We could determine a slight preference of LecB for glycans presented in the GST*A region over the PDT*R region (Table 1). As expected, avidity effects with stronger binding of LecB toward bivalent over monovalent MUC1 glycopeptides were observed (Figure 4A): The bivalent H-antigen glycopeptides **ID 46** ($K_D = 0.50$ μM) and **ID 48** ($K_D = 0.35$ μM) were better binders than their corresponding monovalent PDT*R and GST*A peptides **ID 1**; and **ID 15** and **ID 18**, respectively (Table 1). Again, LecB showed preferred binding to the H-type-2 glycan over the corresponding type-1 structure. Consistently, the bivalent Le^a peptide **ID 47** ($K_D = 0.16$ μM) was a better binder than the corresponding monovalent glycopeptides **ID 10** and **ID 16**. Also, the bivalent Le^x glycopeptides **ID 49** ($K_D = 0.79$ μM), **ID 50** ($K_D = 1.12$ μM), **ID 51** ($K_D = 0.54$ μM), **ID 52** ($K_D = 1.26$ μM) and **ID 53** ($K_D = 0.75$ μM) showed enhanced binding compared to the respective monovalent glycans (Figure 4A, Table 1).

In conclusion, an increased glycan density enhanced LecB binding, and the placement of the glycan epitope on the peptide backbone was found to influence ligand recognition.

Influence of LacNAc elongation on MUC1 glycopeptides on LecB binding

The recognition of LecB towards a selection of LacNAc elongated Le^x and H-type modified MUC1 core structures was evaluated (Figure 4C). It was previously reported that the increased length of oligosaccharides carrying Le^a and Le^x structures can enhance the affinity of LecB since each added LacNAc unit carries an additional fucose moiety that could potentially participate in multivalent binding with LecB.[7b] In agreement with these findings, we found that LacNAc elongation on different mucin core structures increased LecB-binding up to 3.3-fold (Table 2A). In contrast, only a minor increase in the binding affinity was

Table 2: A) Surf. K_D values of LecB-binding towards LacNAc elongated and un-elongated MUC 1 glycopeptides; B) K_D surface values of LecB-binding towards of Le^b, Le^y modified glycans, and their respective mono-fucosylated glycopeptides; LacNAc = extension with an *N*-acetyl-lactosamine unit; Hex = hexasaccharide; Tet = tetrasaccharide.

A)			B)		
ID	**Core Structure**	**K_D [μM]**	**ID**	**Core Structure**	**K_D [μM]**
	H-type			**Lewis y**	
18	Core 1 type-2	0.48	18	Core 1 type-2+H	0.48
20	Core 1 type-2_{LacNAc}	0.32	19	Core 1 type-2+Le^y	0.72
28	Core 2_{Tet} type-2	0.37	30	Core 2_{Hex} type-2+H	0.28
33	Core 2_{Tet} type-2_{LacNAc}	0.34	31	Core 2_{Hex} type-2+Le^x	0.91
30	Core 2_{Hex} type-2	0.28	32	Core 2_{Hex} type-2+Le^y	0.51
35	Core 2_{Hex} type-2_{LacNAc}	0.29	40	Core 3 type-2+H	0.60
9	Core 3 type-1	1.02	41	Core 3 type-2+Le^x	1.76
11	Core 3 type-1_{LacNAc}	0.54	42	Core 3 type-2+Le^y	1.18
	Lewis x			**Lewis b**	
22	Core 1 type-2_{LacNAc}	0.90	15	Core 1 type-1+H	0.64
21	Core 1 type-2_{LacNAc}	0.79	16	Core 1 type-1+Le^a	0.26
29	Core 2_{Tet} type-2	1.72	17	Core 1 type-1+Le^b	0.82
34	Core 2_{Tet} type-2_{LacNAc}	0.76	25	Core 2_{Hex} type-1+H	0.53
31	Core 2_{Hex} type-2	0.91	26	Core 2_{Hex} type-1+Le^a	0.19
36	Core 2_{Hex} type-2_{LacNAc}	0.49	27	Core 2_{Hex} type-1+Le^b	0.54
14	Core 3 type-2	2.97	37	Core 3 type-1+H	0.82
12	Core 3 type-1_{LacNAc}	0.91	33	Core 3 type-1+Le^a	0.38

observed for LacNAc extended linear core 1 and core 3 H-type peptides, and no increase for the elongated branched LacNAc core 2 structures **ID 35** and **ID 33** compared with **ID 28** and **ID 30**. This observation might be explained by the fact that, in contrast to the Le^x glycans, only the terminal galactose residues are α-1,2-fucosylated. Consequently, LacNAc extension of these structures only elongates the glycan branches without incorporating additional fucosyl residues, and LecB binding cannot be enhanced through an increased fucose presentation on the mucin core structures. The increase in binding affinity for LacNAc extended H-type core 1 and core 3 glycans indicates that the elongation of the GalNAc 3-arm leads to a more optimal ligand presentation for the LecB binding sites, potentially facilitating intermolecular multivalent binding interactions.

However, our data stand in contrast to a previous study on *N*-glycans where a di-LacNAc bi-antennary H-type-2 glycan structure showed an enhanced affinity compared to the mono-LacNAc analog due to a favored sterical fit towards the LecB binding pockets.[27] This might be explained by the structural difference of mucin type *O*-glycans compared to mannose containing *N*-glycans. It can be assumed that the spatial orientations of the mucin core branches in relation to the LecB binding pockets promote different binding modes compared with *N*-glycans. Interestingly, the extended H-antigen peptides were still better binders than the corresponding Le^x peptides despite the increased presentation of fucose residues in the LacNAc-modified Le^x peptides.

In summary, LacNAc elongation of fucosylated MUC1 glycopeptides can enhance LecB binding.

Influence of structural modifications of MUC5B glycopeptides on LecB binding

To determine if the mucin peptide sequence strongly influences the lectin binding preferences, LecB binding to selected H-type and Le^x MUC5B glycopeptides was evaluated. In accordance with the MUC1 data, the H-type glycopeptides were better binders than the corresponding Le^x glycans (Supporting Information Table S2, Figure S10). Additionally, LecB bound to Le^x MUC5B core structures in a similar manner as to the respective MUC1 analogs (core 3 < core 1 < core 2 hexasaccharide); and LacNAc elongation enhanced LecB binding. Unexpectedly and in contrast to the MUC1 data, bivalent glycan presentation on the MUC5B peptide backbone did not have a major impact on LecB-binding. These data suggest that the presentation of the ligand(s) on the peptide backbone as well as on distinct glycosylation sites might be important for LecB binding, in particular to gain optimal effects from bidentate and multivalent binding interactions. However, additional experiments with more fucosylated MUC5B and other mucin tandem repeat peptides are necessary to further conclude the impact of peptide backbone presentation in LecB recognition.

Our data provide insight into the specific preferences of LecB and the factors that influence its binding affinity towards different glycopeptides. We could show that the structural properties of the fucosylated mucin glycopeptides, including different fucosyl antigens, the underlying core structure, LacNAc extension of the core glycans, the glycosylation site placement and glycan density on the peptide backbone, are important factors that define the fine binding specificities of LecB.

Binding Preferences of the Clostridium difficile toxin A

To determine the binding preferences of *C. difficile toxin A* (TcdA), the glycopeptide microarray library was incubated with a dilution series of TcdA (27 nM–3.5 μM), followed by incubation with a mouse anti-TcdA mAb for fluorescent detection. Surface K_D values for TcdA binding to the fucosylated MUC1 and MUC5B peptides were determined and are reported in Supporting Information Table S3. Microarray analysis (Figure 5, Supporting Information Figure S11–S13) showed that TcdA binding strongly depended on the different fucose motifs, presenting peptide backbone, underlying core structures, LacNAc-extension as well as placement of the glycosylation sites.

Influence of different fucose motifs on MUC1 glycopeptides on TcdA binding

The *C. difficile* toxin A was previously reported to recognize the Galili epitope, but also the fucosylated Le^y, Le^x, sialyl-Le^x and sulfo-Le^x glycans.[11,13] Interestingly, toxins A from two other pathogens (*P. luminescens* TcdA1 (PI-TcdA1) and *Morganella morganii* TcdA4) have recently been identified to also recognize simpler Le^x and Le^y oligosaccharides.[28] Therefore, we were interested to evaluate the binding recognition of *C. difficile* TcdA towards Le^a, Le^x, Le^y, Le^b and blood H-antigens presented on the natural mucin core glycopeptide backbone. Interestingly, we found that TcdA selectively recognized Le^x and Le^y antigens on the glycan core structures of MUC1 and MUC5B peptides, whereas H-type, Le^a and Le^b modified glycopeptides were not recognized at all (Supporting Information Figure S11–S12). TcdA bound to Le^x and Le^y modified glycopeptides in a high nanomolar to low micromolar range (Surf. $K_D = 0.28$–2.46 μM). Next, TcdA recognition of mono-fucosylated Le^x and bi-fucosylated Le^y core structures were explored. TcdA bound to the bi-fucosylated Le^y structures **ID 32** and **ID 42** with a similar strength as to the Le^x glycopeptides **ID 31** and **ID 41** (Figure 5D).

In summary, TcdA selectively bound to Le^x glycopeptides, and recognized Le^y modified structures with similar affinity.

Influence of different glycan cores on MUC1 glycopeptides on TcdA binding

We found that TcdA showed differences in binding strength towards different MUC1 core structures: TcdA preferably

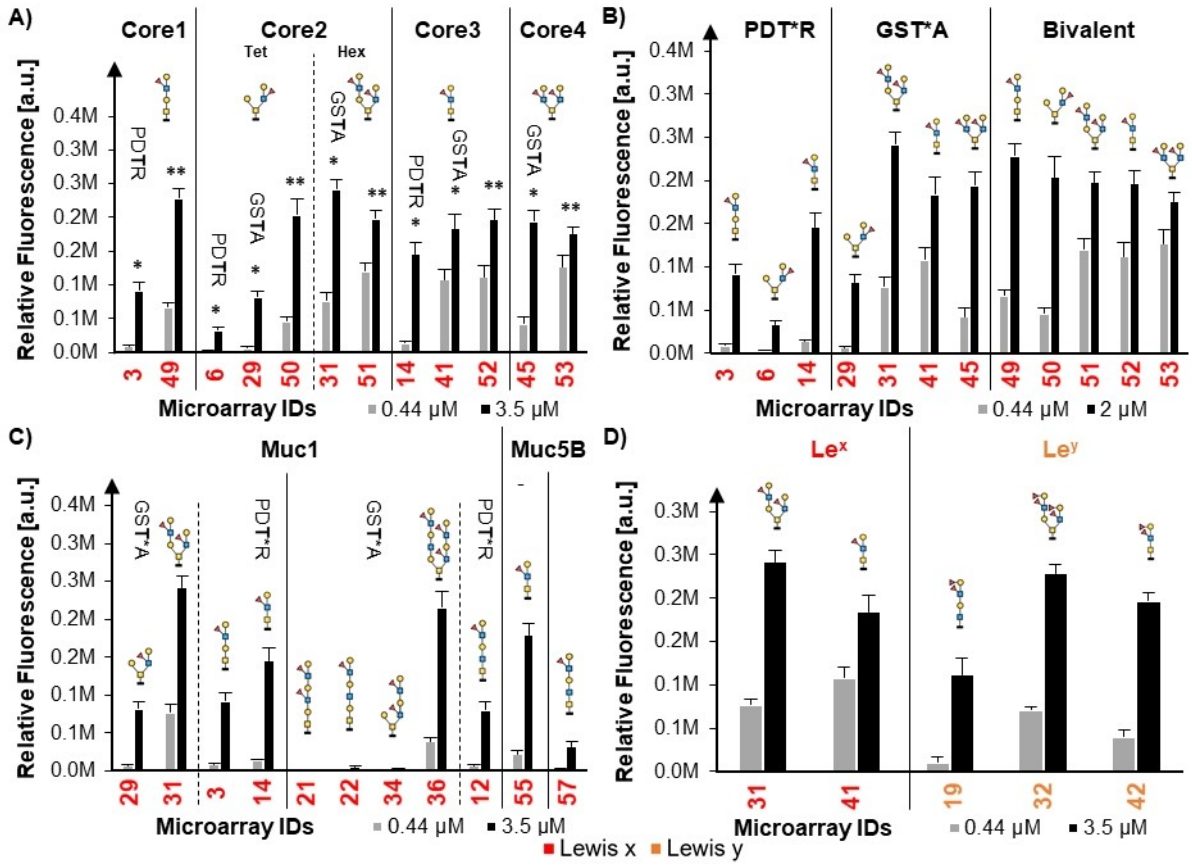

Figure 5. A) TcdA-binding towards different Le^x (red) MUC1 core structures; B) Comparison of TcdA-binding towards monovalent Le^x MUC1 peptides glycosylated in the PDT*R or GST*A region, and the corresponding bivalent glycopeptides; C) Influence of LacNAc elongation on TcdA-binding towards Le^x MUC1 glycopeptides; D) Comparison of TcdA-binding between and Le^y (orange) glycans; and the corresponding Le^x glycopeptides; Hex = hexasaccharide; Tet = tetrasaccharide; * = monovalent glycopeptides; ** = bivalent glycopeptides; Microarray IDs see Sup. Info Table S1.

bound to short linear over branched Le^x glycopeptides (Figure 5A, Figure 5B). These results indicate that branched structures, although presenting two Le^x units instead of one, are sterically less favored than the linear core 1 and core 3 glycans. Particularly interesting is the significant drop in affinity of the Core 2 type-2 tetrasaccharide modified peptides, which do not have an extension with a Le^x antigen on the GalNAc 3-arm in contrast to the other core structures (compare peptides **ID 29** vs **ID 31** and **ID 50** vs **ID 51** in Figure 5A), implying that fucosylation on the 3-arm might be a better sterical fit for the TcdA binding pocket than fucosylation on the 6-arm.

In conclusion, the underlying mucin core structure impacts TcdA binding significantly, and TcdA shows a preference for linear Le^x structures over the branched analogs.

Influence of glycosylation site placement and bivalent ligand presentation on MUC1 glycopeptides on TcdA binding

The placement of the glycan epitope on the peptide backbone, and an increased ligand density by bivalent glycan presentation might influence lectin binding. Therefore, TcdA binding preferences were elucidated comparing the monovalent MUC1 glycopeptides (glycosylation either in the PDT*R or GST*A region) with the respective bivalent analogs (glycosylation in the PDT*R and GST*A regions). Microarray analysis showed that TcdA preferably bound to MUC1 peptides glycosylated in the GST*A over the PDT*R region (Figure 5B). We also found that bivalent peptides were better TcdA binders than the respective monovalent glycopeptides. These findings can again be related to the multivalent binding effects.

Influence of LacNAc elongation on MUC1 glycopeptides on TcdA binding

Interestingly and in contrast to the LecB recognition, LacNAc elongation decreased binding of TcdA towards Le^x modified MUC1 peptides (Figure 5C). For example, the shorter core glycopeptides **ID 14**, **ID 29** and **ID 31** were better binders than their LacNAc elongated analogues **ID 12**, **ID 34** and **ID 36**. This may be explained by the lower entropic penalty for the less flexible shorter core structures

presenting the Le^x epitope. Previous NMR and molecular dynamics studies of monomeric and dimeric Le^x oligosaccharides have shown that the rigid and well defined "stacked" conformation of the Le^x antigen remains in both structures.[29] However, the dimeric Le^x, which also is a representative structure in our LacNAc elongated glycopeptides, has flexibility in the β-D-GlcNAc-(1→3)-D-Gal glycosidic bond connecting the two Le^x trisaccharides, which results in two different conformations. These two conformations were in the previously reported work suggested to result in the formation of two different antibody binding epitopes,[29] and probably also have an impact on the lectin recognition observed in this study, for instance compare binding to peptides **ID 29** and **ID 34** (Figure 5C).

In conclusion, TcdA showed a preference for shorter, less flexible glycan structures over the LacNAc elongated analogs even though those presented an additional fucose moiety for potential multivalent binding. The shorter glycan structures might have a better sterical fit to the TcdA binding sites and their higher rigidity might lead to a beneficial entropy effect.

Influence of structural modifications of MUC5B glycopeptides on TcdA binding

Finally, we evaluated TcdA regarding its binding specificities towards fucosylated MUC5B glycopeptides. In accordance with the recognition of the MUC1 glycopeptides, TcdA did not recognize H-type MUC5B glycopeptides and only bound to the Le^x glycopeptides (Supporting Information Table S3, Figure S12, Figure S13). In agreement with the binding recognition of MUC1 glycopeptides, LacNAc elongation decreased the TcdA binding affinity compared with the respective shorter core glycopeptides. In contrast to the MUC1 data, the bivalent Le^x peptides **ID 60** and **ID 63** were better binders than the monovalent glycopeptide **ID 55**. The placement of the second glycosylation site of the compared bivalent glycopeptides was not important for the lectin affinity.

In summary, similar TcdA binding patterns were observed for MUC1 and MUC5B glycopeptides. However, multivalent presentation of the glycan ligands on different mucin peptide backbones can create different three-dimensional presentation of the glycans and thus a different binding behavior of TcdA.

Conclusion

A library of fucosylated mucin core 1–4 MUC1 and MUC5B tandem repeat glycopeptides was generated to study the fine binding specificities of the fucose-recognizing bacterial lectins LecB from *Pseudomonas aeruginosa* and toxin A from *Clostridium difficile.* Selected glycopeptides were extended with additional LacNAc units using the *Helicobacter pylori* β-1,3-*O*-*N*-acetylglucosaminyltransferase (β3GlcNAcT) and a fusion protein of human β-1,4-*O*-galactosyltransferase (His_6-Propeptide-catβ4GalT-1, β4GalT). Subsequently, the different fucose motifs, including the Le^a, Le^x and H-type as well as bi-fucosylated Le^b and Le^y antigens, were enzymatically prepared using *Helicobacter mustelae* α-1,3/4-*O*-fucosyltransferase (Hmα1,3/4FucT) and/or *H. mustelae* α-1,2-*O*-fucosyltransferase (Hmα1,2FucT). Thereby, the order of the applied fucosyltransferases was crucial to prepare the Le^b and Le^y determinants. The obtained fucosylated mucin glycopeptide library was printed on microarrays, which were applied to determine the binding preferences of LecB and TcdA. Whereas TcdA exclusively bound to α-1,3-fucosylated MUC1 and MUC5B core structures consisting of Le^x and Le^y epitopes, LecB exhibited a broader selectivity toward all presented fucosylated glycopeptides. Additionally, both lectins exhibited unique fine specificities that strongly depended on the different fucose motifs, presenting peptide backbone, underlying core structures, LacNAc-extension as well as placement of the glycosylation sites on the MUC1 and MUC5B glycopeptides. The preference of the TcdA lectin towards the shorter core structures presenting Le^x epitopes is particular interesting and raise the question if simple Le^x glycomimetics with mono- or multivalent structures could be developed to inhibit TcdA-promoted interactions. Together, this study highlights the importance of the evaluated structural glycopeptide properties in lectin binding interactions, which defines the glycan orientation, structural rigidity or possible limitations for ligand recognition.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

Keywords: Fucose · Glycopeptides · Glycosylation · Lectins · Microarrays

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