Lipochitin Oligosaccharides Immobilized through Oximes in Glycan Microarrays Bind LysM Proteins

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Glycan microarrays have emerged as novel tools to study carbohydrate–protein interactions. Here we describe the preparation of a covalent microarray with lipochitin oligosaccharides and its use in studying proteins containing LysM domains. The glycan microarray was assembled from glycoconjugates that were synthesized by using recently developed bifunctional chemoselective aminooxy reagents without the need for transient carbohydrate protecting groups. We describe for the first time the preparation of a covalent microarray with lipochitin oligosaccharides and its use for studying proteins containing LysM domains. Lipochitin oligosaccharides (also referred to as Nod factors) were isolated from bacterial strains or chemoenzymatically synthesized. The glycan microarray also included

peptidoglycan-related compounds, as well as chitin oligosaccharides of different lengths. In total, 30 ligands were treated with the aminooxy linker molecule. The identity of the glycoconjugates was verified by mass spectrometry, and they were then immobilized on the array. The presence of the glycoconjugates on the array surface was confirmed by use of lectins and human sera (IgG binding). The functionality of our array was tested with a bacterial LysM domain-containing protein, autolysin p60, which is known to act on the bacterial cell wall peptidoglycan. P60 showed specific binding to Nod factors and to chitin oligosaccharides. Increasing affinity was observed with increasing chitin oligomer length.

Introduction

Glycobiology, in particular the study of carbohydrate–protein interactions and the events that follow, has become an important research focus in recent decades. Preparation of homogeneous carbohydrate ligands in suitable amounts for studying such interactions is one of the technological challenges of glycobiology.^[1] As carbohydrates are secondary gene products, they are difficult to express in microorganisms by conventional molecular biological methods. Chemical synthesis is complicated due to the complexity of carbohydrate structures, including linkage positions and anomeric configurations, as well as branching in many cases.^[2] The interest in glycobiology reflects

the various important biological roles in which carbohydrates are involved. Central elements include cell–cell recognition events that mediate immune responses,^[3] post-translational glycosylations that alter protein properties,^[4] and other signaling processes that occur between cells and even between different organisms. One example of a key biological system that is initiated through a complex molecular signaling network is legume–*rhizobium* symbiosis.^[5] The initial communication between plant host and bacteria is facilitated by lipochitin oligosaccharide signaling molecules known as Nod factors.^[5] The putative Nod factor receptors located in the host plant root hairs have extracellular LysM domains.^[6] These domains are also found in certain chitinases^[7] and peptidoglycan binding proteins.^[8] The glycan microarray described below was developed to investigate interactions involving LysM domain-containing proteins.

LysM domain-containing proteins play important biological roles; however, only a few studies of these carbohydrate-binding proteins have been reported. Although LysM domains have been reported for all kingdoms except Archaea,^[9] only one bacterial LysM domain structure has been solved by NMR,^[9] and the crystal structure of another, YkuD, which contains a LysM domain, has been solved at high resolution (2 Å).^[10] These structures have laid the basis for modeling studies.^[11] Recently, *Arabidopsis thaliana* CERK1 (plant immune receptor) has been cocrystallized with chitin, revealing the first LysM–ligand binding site.^[12] Most recently, the structure of the fungal Ecp6 protein has been determined, and an additional

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high affinity binding site has been identified.^[13] LysM binding constants have very recently been determined^[14] by use of surface plasmon resonance techniques. The remainder of the reported LysM domains have been predicted based on amino acid sequences,^[15] and several of these proteins are of unknown function. A better understanding of this group of proteins could therefore lead to enhanced insight into biological processes in a wide range of organisms.

Glycan microarrays have recently emerged as a technology for unraveling glycan–protein interactions.^[16] Starting in 2004, the Consortium for Functional Glycomics (CFG) developed an elaborate glycan array containing more than 600 ligands, including many complex carbohydrate antenna structures as first reported by Blixt et al.^[17] This relied on multi-step syntheses to obtain the glycoconjugates. More recent studies have paved the way for on-surface enzymatic syntheses of the carbohydrate ligands,^[18] analysis of immobilized ligands by MALDI-TOF-MS on the surface,^[19] and even evaluation of binding constants.^[20]

The total synthesis of complex glycoconjugates generally requires many chemical steps with a significant number of protecting group conversions. Glycan microarrays (glycochips), in theory, should be able to present glycans, including glycans isolated from naturally occurring specimens, in a biologically meaningful fashion. Several approaches to covalent glycochips have been described in recent years. Most protocols require the carbohydrate probes to be constructed through a multi-step synthesis in order to fashion them with the spacer moiety able to react with the solid support of choice. Alternatively, glycan–linker conjugates can be constructed directly by using highly chemoselective reactions between the aldehyde of native, underivatized glycans and a bifunctional linker. They can then be characterized and subsequently coupled to microarray surfaces. A less common approach is the use of reactive functionalized surfaces for direct immobilization of glycans.^[21] Strong α -nucleophiles such as hydrazines, hydrazones, sulfonylhydrazides, and hydroxylamines have been used.^[22] Some glycochips have already been designed to take advantage of these chemoselective couplings.^[23]

The thermodynamic stability of addition products between aldehydes and α -nucleophiles is known to be highest for products formed from aminooxy nucleophiles.^[24] Also, N-alkyl substituted aminooxy moieties can direct the stereochemistry at the reducing unit of the saccharide towards the retention of the ring-closed pyranose structure (either exclusively, as with glucose, or predominantly, as with mannose and galactose).^[25] However, this comes at the cost of slower formation and lower stability of the conjugate. When interrogating glycans, especially shorter oligosaccharides, it is of crucial importance for functional recognition by proteins that the reducing end is present in the ring-closed form.^[26] However, it is important to realize that Nod factors naturally have a free, that is, nonglycosidic, reducing end, which is likely in equilibrium between the ring-closed and ring-open forms. Reaction of the Nod factor with the AMB aminooxy linker will generate a carbohydrate oxime, which is also in equilibrium between the closed and open forms. Several examples of glycoconjugates with carbo-

hydrate oximes have previously been reported for neoglycolipid probes absorbed onto nitrocellulose microarrays^[27] and by Thygesen, Sauer, and Jensen^[28] for gold nanoparticles.

We have previously developed a series of chemoselective bifunctional linkers allowing solution-phase glycan–linker formation and subsequent covalent attachment onto a slide with activated esters.^[23,29] Both the aminooxy and the N-alkyl aminooxy versions of these linkers formed a sufficient proportion of ring-closed glycan–linker conjugates for recognition in a microarray. Importantly, we also observed that the dynamic equilibrium between open and closed forms of unsubstituted carbohydrate oximes can be shifted towards the closed form by binding to proteins.^[28] Here, two of these linkers were evaluated for the first time in the reaction with *rhizobial* Nod factors. However, in the reaction with chitin oligosaccharides, only the aminooxy compound provided satisfactory conversion of the glycan–linker conjugate.

Glycochips represent a promising tool for investigating Nod factor receptor specificity and thereby advancing knowledge of the specificity of signaling in legume–*rhizobium* symbiosis. We present here the first application of these bifunctional linker reagents for the construction of glycan microarrays with complex carbohydrates. The carbohydrate ligands obtained for immobilization on the glycochip described here were chosen based on predictions derived from the known or expected binding capacities of LysM domains. The ligands fall into three categories: chitin oligosaccharides, peptidoglycan (PGN)-related compounds, and Nod factor variants. Thirty ligands were obtained and immobilized as glycoconjugates to form an array. Their presence on the array surface was verified by binding with lectins and human IgG. For initial demonstration of the efficacy of the array, the LysM protein p60, known to process bacterial cell wall PGN, was chosen. This represents the first analysis of a LysM domain protein on a glycan microarray.

Results and Discussion

The carbohydrate ligands obtained for immobilization on the present microarray fall into three categories: the chitin oligosaccharides, the PGN-related compounds, and a range of Nod factor variants. The first step was to obtain a range of lipochitin oligosaccharides, as well as other carbohydrates and PGN-related compounds. These were then chemoselectively converted into glycoconjugates by using our *N*-(2-aminoethyl)-4-(aminooxymethyl)benzamide^[29] (AMB) reagent, which provided a free amine ready for printing on an activated array surface, as well as the partially ring-closed structure, which mimics the natural compounds. The chitin oligomers, Nod factors, and PGN-related structures were synthesized or extracted from bacterial cultures when commercial products were unavailable.

The glycoconjugates were purified on a hydrophilic interaction chromatography (HILIC) column. The bifunctionality of the AMB linker allowed the hydroxylamine to react with the reducing end (aldehyde) of any unprotected reducing carbohydrate in aqueous solution. A key advantage of the linker is its chromogenic properties, which enable easy visualization and quantification. The resulting carbohydrate linker conjugate exists in

equilibrium between the open and closed forms (Scheme 2). The ratio of open versus closed form depends on the glycan to which the aminoxy linker is attached. On the array presented in this paper, the reducing end carbohydrate forming the oxime is GlcNAc in most cases. The ratio of open versus closed forms in the cases of LacNAc,^[30] GlcNAc,^[31] and (Glc)_{1–3}^[28] has been reported to be 8:1, 7:3, and 4:1, respectively. This correlates well with the AMB linker conjugates obtained (data not shown) and is a feature to be considered when interpreting binding data. The glycoconjugate, for example, GlcNAc-AMB, was designated **1A** to indicate the linker attachment to GlcNAc, **1**.

Nod factor isolation

Nod factors are lipochitin oligosaccharides (LCOs). As the name implies, the backbone is composed of GlcNAc assembled by $\beta(1\rightarrow4)$ linkages in lengths of three to six units.^[5] The lipid chain is situated at the nonreducing end. Additional chemical groups are located mainly at the terminal residues and vary between *rhizobial* strains (Scheme 1). Nod factors used in this study are listed in Table 1.

Scheme 1. Generic Nod factor structure. The systematic name for a Nod factor is Nod-*n*-II(R',R'',lipid,R'''), as suggested by Heidstra and Bisseling.^[32] Nod indicates that it is a Nod factor molecule, and the Roman numeral corresponds to the number of GlcNAc units in the chitin backbone. The chemical ornamentations and lipid chain are indicated in brackets. Those to the left of the lipid chain indication are situated at the nonreducing end (R', methyl, and R'', carbamoyl in the *M. loti* Nod factor) of the chitin oligomer, whereas those to the right (R''', acetyl-fucosyl in the *M. loti* Nod factor) are located at the reducing end terminus.

These modifications are thought to determine species-specific recognition most likely mediated by the Nfr1 and Nfr5 receptor kinases described for *Lotus japonicus* roots and homologous receptors found in other legumes.^[33,34] Nod factors were

Scheme 2. Chemoenzymatic synthesis of a Nod factor and a biotinylated chitotetraose.

extracted and purified from the *Mesorhizobium loti* strain R7A^[35] and mutants derived from this strain. To obtain the Nod factors, *M. loti* growth media was extracted with butanol after removal of the cells, then evaporated and further purified by reversed-phase chromatography. All Nod factor products were verified by mass spectrometry. The characteristic fragmentation pattern arose from the cleavage of glycosidic bonds, which

Table 1. Ligands immobilized on the array.^[a]

Chitin	(GlcNAc) ₈ (7)	Nod factors ^[b]	Nod factors ^[b]	PGN compounds
GlcNAc (1)	(GlcNAc) ₄ -biotin (8)	NodMI.R7Awt-V(Me,Cb,C18:1,Fuc,Ac) (13)	NodMI.R7AΔ <i>nod</i> O-V(Me,C18:0) (19)	MurNAc (25)
(GlcNAc) ₂ (2)	controls	NodMI.R7Awt-V(Me,Cb,C18:0,Fuc,Ac) (14)	NodMI.R7AΔ <i>nod</i> O-V(Me,C16:0) (20)	GlcNAc-MurNAc (26)
(GlcNAc) ₃ (3)	cellotetrose (9)	NodMI.R7Awt-V(Me,Cb,C18:1,Fuc) (15)	NodMI.R7AΔ <i>nod</i> O-V(Me,C18:1,Fuc,Ac) (21)	GlcNAc-MurNAc-A-iso-q (27)
(GlcNAc) ₄ (4)	maltotetrose (10)	NodMI.R7Awt-V(Me,Cb,C16:0,Fuc,Ac) (16)	NodMI.R7AΔ <i>nod</i> O-V(Me,C18:0,Fuc,Ac) (22)	A-e-K-a (C-terminal; 28)
(GlcNAc) ₅ (5)	N-stearyl-GlcN (11)	Nod-V(C18:1) ^[c] (17)	NodMI.R7AΔ <i>nod</i> S _{no} O-V(C18:1,Fuc,Ac) (23)	A-e-K-a (N-terminal; 29)
(GlcNAc) ₆ (6)	AMB linker (12)	NodMI.R7AΔOZ-V(Me,C18:1) (18)	NodMI.R7AΔ <i>nod</i> L _{no} O-V(Me,C18:1,Fuc) (24)	GlcNAc-MurNAc-A-e-K-a (30)

[a] The compounds conjugated with the AMB linker are designated by an A next to the compound number, so GlcNAc-AMB becomes **1A**. [b] Nod factor nomenclature as suggested by Heidstra and Bisseling.^[32] MI indicates that it originated from *M. loti*; R7A has been included in this paper to indicate the strain and which *nod* genes have been knocked out. [c] Chemoenzymatically prepared Nod factor. [d] Cb: carbamoyl; Fuc: fucose; Me: methyl; wt: wild type.

Figure 1. Mass spectrum of the major Nod factor product, NodMI.R7Awt-V(Me,Cb,18:1,Fuc,Ac), obtained from the unmodified *M. loti* R7A. The fragmentation patterns are easily recognized by the stepwise loss of a GlcNAc residue.

revealed the expected chemical traits at the reducing and non-reducing termini. The major Nod factor produced by *M. loti* R7A was the NodMI-V(Cb,Me,18:1,Fuc,Ac) (Figure 1), although minor Nod factor products with other lipid chains were also observed. Nod factors containing C_{18:0} and C_{16:0} chains were present, and these were isolated for immobilization on the array as well. The remainder of the Nod factors reported here were obtained from mutant strains of *M. loti* R7A defective in one or more genes required for complete Nod factor biosynthesis.^[35] The mutant strains produced different Nod factor yields, which might reflect an altered transmembrane transport efficiency^[36] or the need for a certain structural trait to be present for proper recognition by the other modifying enzymes. The identity of Nod factors was established by electrospray mass spectrometry (ESI-MS), and purity was assessed by HPLC (Figure 1 and Figure S3 in the Supporting Information). Table 1 provides an overview of the Nod factors obtained; the genotypes of the mutant strains involved are included.

Lipochitin oligosaccharides and peptidoglycan synthesis

Although some Nod factors were purified from extracts produced by using *M. loti* nod gene mutants, a chemoenzymatic synthesis approach was used to produce LCOs **8** and **17** (Table 1). The *M. loti* nodB gene was expressed in *E. coli* to enable purification of the resulting deacetylase. The enzyme product, NodB, catalyzes the deacetylation reaction, thereby making an amino group available at the nonreducing end of the chitin oligosaccharide.^[37] Having deacetylated the product, a biotin group (**8**) or a lipid chain (**17**) could be attached by using an *N*-hydroxysuccinimide (NHS) ester as depicted in Scheme 2.

A similar strategy for synthesizing a Nod factor, V(C18:0), was utilized by Huang and Mei^[38] by using an acid chloride for

acylation. However, in our hands, the NHS approach was preferred because of its stability and water compatibility. The control compound, **11**, was likewise synthesized by treating an NHS-activated stearic acid with glucosamine. Compound **8** was chosen for the array in order to view chitin-degrading activity if it was encountered. Isolated and synthesized Nod factors were reacted with an excess of AMB to provide glycoconjugates for array printing (Scheme 2).

The peptides included on the array were chosen based on the enzymatic activity of the protein involved in PGN synthesis and remodeling.^[39] The four amino acid sequences, which included two D-amino acids, constitute the linking residues connecting the GlcNAc-MurNAc chains in the bacterial cell wall PGN.^[40] The compounds were obtained through solid-phase peptide synthesis and were either designed with an aldehyde at the C-terminus (**28**) or N-terminus (**29**) to allow oxime formation with the UV-active AMB linker prior to immobilization on the array surface, or linked to GlcNAc-MurNAc (compound **30**). These are depicted in Scheme 3.

Array printing

Every compound was printed in triplicate at four spot densities to avoid false positive readouts and to increase the likelihood of optimal conditions for proper binding (e.g., distance to ligand in cases of multiple binding sites). Lectin binding did indeed lead to more intense fluorescence readouts in the areas with high ligand density; this reflects the binding of more lectin molecules (Figure S6).

Carbohydrate linker conjugates were printed on NHS-activated glass slides^[17] in four different concentrations (60, 20, 6.7, and 2.2 μM) in triplicate, thus having different ligand densities which decrease in steps of 33% on the array surface. The identity of the glycoconjugates was verified by mass spectrometry

Scheme 3. The PGN-related structures **28**, **29**, and **30** were designed to include an aldehyde to facilitate AMB linker conjugation as with the reducing carbohydrates. L-amino acids are designated by capital letters; D-amino acids are written in lower case.

prior to array printing. The quality of the printing was evaluated and then accepted after a “red scan” in which the geometrical placement and morphology was probed by a red laser immediately after printing to confirm spot appearance (Scheme 4).

Verification of immobilized ligands

Solanum tuberosum lectin (STL) was used to verify the presence of GlcNAc-containing carbohydrates on the array, whereas sera from humans were acquired to visualize GlcNAc-containing peptidoglycans, as demonstrated previously.^[17]

To detect protein binding to the array ligands, STL was biotinylated, and streptavidin was used as secondary binder containing either a Cyanine3 (Cy3) or an Alexa label. STL is a dimeric protein with four carbohydrate binding sites.^[41] The K_d values of STL binding to the unconjugated chitin oligomers and NodMI-V(Cb,Me,18:1,FucAc; **10**) in solution were obtained by thermophoresis for comparison (Table 2).

The data obtained from the solution binding studies corresponded well qualitatively with the on-chip results from the glycan microarray. STL binds β -pGlcNAc glycosides; hence in chitin oligomers, the reducing end GlcNAc would not be rec-

Table 2. STL binding constants determined by thermophoresis.

Ligands	K_d [nM]	Standard deviation
GlcNAc	no binding	–
chitobiose	115 488	9148
chitotriose	5565	1150
chitotetraose	976	67
chitopentaose	731	218
chitohexaose	504	73
chitooctaose	685	64
NodMI-V(Cb,Me,18:1,FucAc)	1422	554

ognized. In solution, the chitin tetramer is required for full recognition. Interestingly, this correlates well with the results from on-chip binding, where the glycans are covalently linked through an oxime linkage. The oxime linkage is in a dynamic equilibrium between the open-chain form and the ring-closed form, existing primarily as the β -glycoside. STL specificity towards GlcNAc oligosaccharides was further demonstrated by a competition experiment with *N*-pentaacetyl chitopentaose as outlined in Figure S6.

Studies have indeed shown that the GlcNAc monomer cannot inhibit the agglutinating activity of STL, whereas chitobiose has only a weak ability to do so.^[42] As previously mentioned, the linked carbohydrate exists in a dynamic equilibrium between the open and closed forms, whereas only the closed form is expected to be recognized by proteins. However, previous work performed using a surface plasmon resonance (SPR) technique revealed that, despite the availability of the closed (pyranose) form of GlcNAc, wheat germ agglutinin (WGA) did not recognize the LacNAc at all.^[36] This result was contrary to ECA binding, which requires the entire disaccharide for recognition. In this case, the dissociation rate was higher and the association rate lower compared to the entirely ring-closed form, reflecting unstable binding due to the dynamic nature of the oxime coupling.^[36] WGA was tested on the array (Figure 2C) and yielded an identical pattern to STL. Therefore, for these particular lectins, the first GlcNAc residue should not be considered as a part of the recognized structure, and two or more ring-closed GlcNAc residues must be available for proper binding. However, this notion does not account for all GlcNAc-recognizing lectins, as WGA did bind chitotriose (**3**) on the array (Figure 2A).

It is likely that the chitin backbone is the primary part of the Nod factors that was recognized by the lectins we examined. Like STL, WGA (Figure 2C) recognized all Nod factors. However, looking at compound **22** (NodMI.R7A Δ noI/O-V(Me,18:0,FucAc)) and **18** (NodMI.R7A Δ noI/O-V(Me,18:1)), there seems to be a difference in lectin binding affinities. This difference is even more apparent when looking at WGA and DSL. These two lectins only recognized about half of the Nod factors on the array, and WGA did not bind to compound **21** (NodMI.R7A Δ noI/O-V(Me,18:1,FucAc)), unlike the rest of the lectins. One possibility is that the accessibility of the backbone determines the observed binding or lack thereof. However, if this was the only criterion, one would expect the binding patterns to be more

Scheme 4. Carbohydrate AMB linker conjugation. Nod factors were obtained through several steps of bacterial growth and compound extraction (see structure in Scheme 1). The reaction with AMB linker leads to the corresponding oxime, which exists in a dynamic equilibrium between open and closed forms. The linker conjugates were then printed on slides to generate the microarray glycochips.

similar. Thus, the ornamentation of Nod factors could play an important role in recognition.

For visualization of the PGN-related structures that did not bind to any lectins on the slide (**25–30**), we used anti human-IgG serum antibodies, as they contain a broad repertoire of anti-glycan reactivities, including against peptidoglycans.^[17] Antibodies binding to the ligands were then visualized by adding fluorescently labeled goat anti-human IgG as the secondary binder. IgG is predominantly involved in the adaptive immune system but also recognizes various pathogenic bacteria and fungi. Sera from seven individuals were examined, and each sera exhibited slightly different readouts (Figure S9). These small differences were likely due to differences in pathogen exposure of the individuals but, taken together, they covered the PGN-related structures on the array, thereby confirming the presence of the PGN structures. An interesting observation was the recognition of compounds **27** and **30** in several of the tested sera samples. Compound **27**, referred to in the literature as GlcNAc-MurNAc dipeptide (GMDP),^[43] has been reported to exhibit immunomodulatory properties.

This observation suggests that recognition might require the chitin moiety as well as certain chemical features provided by the Nod factors. Antibody binding to other carbohydrates on

the array (**2, 3, 5, 6, 26**, and **27**) has been previously confirmed in similar array setups.^[44]

P60 autolysin on chip

Binding of the lectins and the serum antibodies verified the presence of ligands on the array surface and proved that they were accessible. Only the mono- to trisaccharides **1–3** could not be visualized when using these proteins. The functionality of the array was subsequently tested with a LysM domain protein. P60 is an autolysin essential for the virulence of the human pathogen *Listeria monocytogenes*.^[45] The protein consists of two LysM domains, an SH3 domain, and a catalytic domain, which cleaves the peptide sequence connecting the GlcNAc-MurNAc chains in PGN.^[45] Cleavage is predicted to occur between D-iGlu, that is, at the side-chain amide bond, and mDAP in the sequence MurNAc-L-Ala-D-iGlu-mDAP-D-Ala-D-Ala. For this study, p60 was expressed in *E. coli* and biotinylated upon purification. The array readout (Figure 3) showed binding of p60 to the longer chitin oligosaccharides and to some of the Nod factors but not to the peptidoglycans (**25–30**) present on the array. Recent studies have indeed proven the ability of a plant receptor, CERK1, to bind a tetrameric chitin molecule through direct hydrogen bond interactions

Figure 2. Lectin binding on array. A) WGA; B) DSL; C) LEL; D) STL. All four lectins are known to recognize GlcNAc. The chitin oligomers (1–8) and Nod factor variants (13–24) are indicated on the LEL readout in the lower left corner, and the compound numbers are given in the lower right array readout. Compounds 11 and 12 were reference molecules. Each compound was spotted in three replicates at four concentrations to give a density range.

and van der Waals interactions with at least three of the GlcNAc units to a shallow groove on its LysM domain surface.^[12] Assuming that the presently observed LysM–chitin binding events take place in a similar fashion, it would be expected that longer chitin oligosaccharides would lead to higher affinity, as this would allow further contributions to the interaction.

Based on the predicted cleavage site of p60, binding to peptidoglycans (25–30) would be expected. However, such an interaction between peptide and the p60 catalytic domain is likely to be transient and thus not visible in the present setup. Instead, it is possible that p60 was associated with the PGN GlcNAc–MurNAc chains through its LysM domains, thus bringing it into the vicinity of the peptide ligands to which its activity is to be exerted. However, binding was not observed for compounds 26, 27, and 30, which contain the GlcNAc–MurNAc moiety, but the chain length could be of critical importance to binding. Thermophoresis and isothermal calorimetry experiments also indicated an absence of binding to the peptidoglycans and GlcNAc–MurNAc moiety-containing compounds (data not shown). Longer repeats of GlcNAc–MurNAc would be highly relevant to include on the array. This would allow comparison to the chitin oligosaccharide fragments, which are highly similar with regard to structure. However, tighter binding to GlcNAc–MurNAc repeats may be expected, as these carbohydrates, in this case, must be considered the natural ligand. Unfortunately, GlcNAc–MurNAc oligosaccharides are very difficult to obtain. Purification of such well-defined PGN fragments and chemical synthesis have proven difficult and tedious, although great efforts have been made and impressively resulted in highly complex PGN-related structures.^[46]

Interestingly, selective and differential binding to Nod factors was observed for p60. This suggests that this enzyme exhibits a binding specificity that is dependent upon the structure of the lipid chain and the additional ornamentation. The possibility of a combined effect of these to determine the binding specificity cannot be excluded. Such a combination might influence the accessibility of the chitin backbone, which might be the primary cause of the interaction.

Figure 3. P60 binding to chitin oligosaccharides and Nod factors. Ligand compounds are positioned in numerical order starting with compounds 1–6 on the top row. One compound is represented by 12 spots, with four concentrations in three replicates. Compound details are listed in Table 1. The bright spot at the bottom, second from the left, represents the biotinylated chitotetraose designed to detect chitin degrading activity by the disappearance of the signal.

Conclusions

A glycan microarray containing 30 ligands was assembled through solid-phase peptide synthesis, chemoenzymatic synthesis, and compound isolation from *M. loti* R7A mutant strains, as well as highly chemoselective reactions with a bi-functional linker. The successful assembly was confirmed by lectin binding, as well as antibody recognition from human sera. The first study with these carbohydrate linker conjugates in an array setup involved binding of LysM domain-containing proteins. Application of the array was demonstrated by using the bacterial p60 autolysin, which surprisingly binds Nod factor variants, most likely through GlcNAc recognition. Our results demonstrate the application of novel bifunctional chemoselective aminoxy reagents without the need for transient carbohydrate protecting groups in an array setup to assess binding of LysM domain-containing protein.

Experimental Section

HPLC and mass spectrometry: Preparative HPLC and analytical HPLC-MS was performed on a Dionex Ultimate 3000 HPLC with Chromeleon 6.80SP3 software by using columns from FeF Chemicals A/S (C-18, 250×21.2 mm, 5 μ m, 300 Å) and Phenomenex (Gemini C-18, 50×4.60 mm, 3 μ m, 110 Å), respectively. Purification of glycoconjugates was carried out by using a Waters HPLC system with a Dionex Acclaim Mixed-Mode HILIC-1 150×5 mm, 5 μ m, 120 Å. Elution conditions were as follows: preparative HPLC for Nod factor purification; 10 mL min⁻¹ linear gradient flow of B (CH₃CN) in A (H₂O) over 35 min. Analytical HPLC-MS for structure confirmation of Nod factors and glycoconjugates; 1.0 mL min⁻¹ linear gradient flow of B (CH₃CN, 0.1% formic acid) in A (H₂O, 0.1% formic acid) over 10 min. HPLC on analytical HILIC column for glycoconjugate purification; 1.0 mL min⁻¹ linear gradient flow of D (CH₃CN/NH₄OAc 9:1, v/v, 100 mM, pH 5.8) in C (NH₄OAc 10 mM, pH 5.8) over 15 min. The Dionex MSQ Plus mass spectrometer was set to a cone voltage of 70 V in electrospray ionization positive mode.

Commercially available carbohydrates: GlcNAcMurNAc was purchased from Toronto Research Chemical, Inc., GlcNAcMurNAc-L-Ala-D-iso-Gln from CalBioChem, MurNAc from Carbosynth, maltotetraose from Dextra Laboratories, Ltd., and chitin oligosaccharides and cellotetraose from Seikagaku Biobusiness Corporation.

NodB expression and purification: Primers were designed to amplify the *M. loti* R7A *nodB* gene. Both primers contained ligation-independent cloning (LIC) overhang sequences, and the forward primer also contained a ribosome binding site and tobacco etch virus (TEV) protease cleavage site. The *nodB* PCR product was inserted into the pET-30 Ek/LIC vector and transformed into *E. coli* BL21 (DE3) to enable His tag purification of the expressed NodB protein.

Nod factor isolation: Plasmid pMP2112^[47] containing *nodD* from *Rhizobium leguminosarum* was transferred to *M. loti* strains to allow induction of *nod* gene expression by using naringenin.^[48] The Nod factors were then extracted from the growth medium by using 1/4 volume butanol, followed by reversed-phase preparative HPLC as previously reported.^[49] Products were lyophilized prior to conjugation with the AMB linker as described by Cló, Blixt, and Jensen.^[29]

Ligand synthesis: Peptides were synthesized by solid-phase peptide synthesis (SPPS). Coupling of amino acids was carried out with microwave-assisted SPPS on an automated peptide synthesizer (SyrWave, Biotage) with 9-fluorenylmethoxycarbonyl (Fmoc) for protection of *N*^α-amino groups. TentaGel S Amide resin (0.27 mmol g⁻¹, Rapp Polymere GmbH) was used to assemble peptide **28**, whereas TentaGel S Rink Amide resin (0.24 mmol g⁻¹, Rapp Polymere GmbH) was used for peptides **29** and **30**. Side-chain-protecting groups were *tert*-butyl on Glu and *tert*-butoxycarbonyl on Lys. *N*^α-Fmoc amino acids (5.0 equiv) were coupled by using *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium-hexafluorophosphate-*N*-oxide (HBTU; 4.75 equiv), 1-hydroxy-7-azabenzotriazole (HOAt; 5 equiv) and *N,N*-diisopropylethylamine (DIEA; 9.75 equiv) as coupling reagents in DMF for 2×10 min at 75 °C. *N*^α-Fmoc deprotections were performed with piperidine/DMF (2:5) for 3 min, followed by 2×piperidine/DMF (1:4) for 15 min. Between couplings and deprotections, the resin was washed with 3×NMP, DCM, and then 3×NMP. The peptides were released from the solid support by treatment with trifluoroacetic acid (TFA)/H₂O (95:5) for 2 h. The TFA solutions were concentrated by nitrogen flow, and the compounds were precipitated with diethyl ether to yield the crude material. Amino acids, coupling reagents, DIEA, piperidine, DMF, and NMP were all obtained from Iris Biotech GmbH, Germany and Sigma-Aldrich, Denmark. The crude peptides were purified by RP-HPLC (on a Dionex Ultimate 3000 system) on a preparative C18 column (Phenomenex 110 Å, 10 μ m, C18 particles, 2.1×200 mm). The purity of the peptide was established by analytical HPLC, and identification was carried out by ESI-MS (MSQ Plus Mass Spectrometer, Thermo).

For the preparation of compound **28**, the TentaGel S Amide resin with a free amino group and no linker was placed in a reaction syringe. A solution containing *o*-PALdehyde^[50] (34.64 mg, 0.2 mmol), HBTU (73.96 mg, 0.195 mmol), HOAt (27.02 mg, 0.2 mmol), and DIEA (46.53 mg, 0.36 mmol) was prepared in 2 mL DMF. This solution was added to the resin and left on a shaker overnight at room temperature. The resin was washed with 3×NMP, 3×DCM, and 3×NMP and placed in a microwave vial. 2,2-Dimethoxyethylamine (105.14 mg, 1 mmol) and sodium cyanoborohydride (62.8 mg, 1 mmol) were added to this, along with MeOH (3 mL) containing 1% acetic acid. The reaction vial was then heated in a microwave instrument for 10 min at 60 °C. The resin was washed with MeOH, and the reaction was repeated to ensure full incorporation. The resin was washed thoroughly with 3×NMP, 3×DCM, 3×NMP, and 3×DCM and placed in a microwave vial to which a solution of Fmoc-Gly (297.4 mg, 1 mmol) and DIC (63.1 mg, 0.5 mmol) in DCM/DMF (10:1) was added. The reaction was heated in the microwave at 60 °C for 10 min. The resin was washed in DCM/DMF (10:1), and the reaction was repeated with fresh reagents. After washing with 3×NMP, 3×DCM, and 3×NMP, the remaining amino acids were attached under the conditions described above. The peptide was cleaved by TFA and then purified by HPLC according to the following procedure: solvent A, water containing 0.1% TFA; solvent B, CH₃CN containing 0.1% TFA. Gradient elution (0–20 min: 5–50%) was applied at a flow rate of 15 mL min⁻¹. Calculated mass for the target compound: C₂₃H₃₉N₇O₉, 557.3 Da. Masses found by ESI-MS: *m/z* 558.2 [*M*+*H*]⁺.

The tetrapeptide in **29** was synthesized according to the general procedure described above. It was then purified by preparative HPLC according to the following procedure: solvent A, water containing 0.1% TFA; solvent B, CH₃CN containing 0.1% TFA. Gradient elution (0–20 min: 5–60%) was applied at a flow rate of 10 mL min⁻¹. The N-terminal modification was done directly on

resin by adding a solution containing 4-(dimethoxymethyl)benzoic acid (78.4 mg, 0.4 mmol), HBTU (147.9 mg, 0.39 mmol), HOAT (54.04 mg, 0.4 mmol), and DIEA (93.06 mg, 0.72 mmol) in DMF (4 mL). This solution was added to the resin-bound peptide and left on a shaker for 2 h at room temperature. The resin was then washed as follows: 3×NMP, 3×DCM, 3×NMP, and 6×DCM, prior to TFA cleavage and subsequent HPLC purification according to the following procedure: solvent A, water containing 0.1% TFA; solvent B, CH₃CN containing 0.1% TFA. Gradient elution (0–25 min: 5–60%) was applied at a flow rate of 10 mL min^{−1}. Calculated mass for the target compound: C₂₅H₃₆N₆O₈ 548.3 Da. Masses found by ESI-MS: *m/z* 549.3 [*M*+H]⁺.

The tetrapeptide in **30** was identical to that in **29** and was thus synthesized by the same approach. The peptide was cleaved from the resin, purified (10 mg, 0.024 mmol), and placed in a microwave vial. To this was added HBTU (3.6 mg, 0.0096 mmol), HOAt (1.4 mg, 0.0104 mmol), and GlcNAc-MurNAc (5.16 mg, 0.0104 mmol), dissolved in 1.5 mL DMF. The solution was basified by adding DIEA (3.2 μL, 0.0192 mmol). The reaction mixture was heated in the microwave at 75 °C for 10 min with stirring. DMF was removed under vacuum, and the crude material was purified by HPLC with solvent A, water containing 0.1% TFA; solvent B, MeOH containing 0.1% TFA. Gradient elution (0–20 min: 5–20%) was applied at a flow rate of 10 mL min^{−1}. Calculated mass for the target compound: C₃₆H₆₂N₈O₁₈ 894.4 Da. Masses found by ESI-MS: *m/z* 895.1 [*M*+H]⁺ and 448.2 [*M*+H]²⁺.

A synthetic Nod factor, **17**, was generated chemoenzymatically. Enzymatic deacetylation at the nonreducing terminal GlcNAc unit of *N*-pentaacetyl chitopentaose was catalyzed by NodB in 20 mM MOPS buffer, pH 7.2. Acylation of the resulting mono-deacetylated chitopentaose was carried out in 80% CH₃CN with an NHS-activated *cis*-vaccenic acid (6 equiv) under basic conditions (3.5 equiv Et₃N) at 35 °C for 16 h. Purification of the target molecule, **17**, was performed on a preparative HPLC, providing a 15% yield. The biotinylated *N*-triacyetyl chitotetraose, **8**, was prepared under similar conditions except for the acylation step, which was carried out in 50% CH₃CN with NHS-biotin from CalBioChem. Stearinylated glucosamine **11** was prepared by treating the NHS-activated stearic acid (1.5 equiv) with glucosamine (1.5 equiv) in DMF and was purified by flash chromatography with an EtOAc/heptane gradient and MeOH to give a 31% yield.

Array assembly: Ligand–linker conjugates were prepared on a 0.5–5 μmol scale by reacting 3 equiv AMB with the carbohydrates in either CH₃CN/H₂O (1:1) or MeCN/100 mM NH₄OAc buffer pH 4.5 (1:1) for 2 h at 35 °C. Conjugation yields were in the range of approximately 20–50%. Purification of the compounds was carried out on an Acclaim mixed-mode HILIC HPLC column from Dionex. Ligand–linker conjugates were then lyophilized and stored at 4 °C until printing on an array. All linker–conjugate structures were confirmed by MS under the conditions described above. MS data is available in Table S1. Printing was done according to the conditions previously reported by the Blixt lab.^[51] Surface-functionalized glass slides containing NHS-activated carboxyl groups (Nexterion H MPX-16; with 16 subarrays, divided by a teflon layer) was used with the BioRobotics MicroGrid II spotter. Print buffer contained 150 mM phosphate and 0.005% CHAPS, pH 8.5. Linker–conjugate concentrations were determined based on a UV standard curve. All compounds were printed in concentrations of 60, 20, 6.67, and 2.22 μM, respectively, to vary the compound density on the surface. The slides were incubated for 60 min in a humidity chamber immediately after printing. To block excess unreacted NHS-esters, the glycan arrays were submerged in blocking buffer containing

ethanolamine (50 mM) and borate (50 mM), pH 9.2, for 30 min at room temperature.

Array experiments and data representation: Proteins were solubilized in PLI-P (buffer containing 0.5 M NaCl, 3 mM KCl, 1.5 mM KH₂PO₄, 6.5 mM Na₂HPO₄, 1% BSA, 1% Triton-X-100, pH 7.4) and submitted directly onto the array surface at 20 μL per subarray. P60 (0.8 μM), YkuD (10 μM), and STL (0.004 μM; Vector Labs) were incubated on array overnight at 4 °C, followed by addition of the secondary dye, Streptavidin-Cy3 (Invitrogen). The entire slide was washed in PBS containing 0.05% Tween, followed by washing in PBS after each incubation step and drying by centrifugation. Sera (Asterand) were diluted 50 times in PLI-P buffer prior to incubation on the array. Visualization was achieved by adding goat anti-human IgG Alexa 488 (Invitrogen). Images of spots on arrays were retrieved by use of a ProScanArray HT microarray scanner (Perkin Elmer). Data acquisition and handling, such as readout intensity quantification, was performed by using the ProScanArray Express 4.0 software (PerkinElmer). For the data represented in bar diagrams, the bar values represent an average of the three spot readouts at the same concentration. The concentration of 20 μM was chosen to be representative of the data, as the fluorescence intensity did not reach saturation and was thus within the dynamic range. Error bars represent variation based on these three replicate signal readouts.

Expression, purification, and labeling of the p60 protein: The gene encoding the p60 protein from *Listeria monocytogenes* was amplified by PCR from genomic DNA and cloned into the pET30 Ek/Lic vector from Novagen. The 5'-end of the gene encoding the first 26 amino acids (peptide signal) was removed. In order to obtain a catalytically inactive protein, the catalytic residue Cys396 was mutated to Ala by PCR mutagenesis. The resulting construct, pET30:P60mut, allows expression of the protein fused to a His tag and an S tag. A TEV protease site was introduced after the two tags. *E. coli* BL21 cells were used for expression. The protein was purified by three purification steps: first, immobilized metal affinity chromatography (IMAC), followed by TEV protease cleavage and a second IMAC step to get rid of the His and S tags, then size-exclusion chromatography. Prior to labeling, proteins were dialyzed against 50 mM phosphate buffer, pH 7.5, and labeling was done following instructions from the SureLINK Chromophoric Biotin kit.

Microscale thermophoresis: The STL lectin was labeled by using the Monolith NT.115 Protein Labeling Kit BLUE (NanoTemper Technologies) to achieve a 1:1 molar ratio of labeled protein to dye. A titration series was prepared in which the concentration of the labeled proteins was kept constant at 50 nM, and the concentration of the titrant was varied in thermophoresis buffer (50 mM phosphate pH 7.5, 0.4 M NaCl, and 0.1% Tween-20). After incubation for 30 min, MST measurements were performed at room temperature on a Monolith NT.115 instrument (NanoTemper Technologies). For each measurement, the laser was switched on for 30 s and off for 5 s. Each experiment was repeated three times, and average *K_d* values were calculated after fitting the data with GraphPad Prism software.

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FULL PAPERS

Hold still! Complex lipochitin oligosaccharides were immobilized by oxime linkages to form a glycan microarray to evaluate binding of proteins containing LysM domains. Array functionality was evaluated by using LysM domain-containing protein autolysin p60, which targets bacterial peptidoglycan. Specific binding to Nod factors and chitin oligosaccharides was observed, with increasing affinity corresponding to increasing chitin oligomer length.

*N. N. Maolanon, M. Blaise, K. K. Sørensen, M. B. Thygesen, E. Cló, J. T. Sullivan, C. W. Ronson, J. Stougaard, O. Blixt, K. J. Jensen**

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Lipochitin Oligosaccharides Immobilized through Oximes in Glycan Microarrays Bind LysM Proteins