Carbohydrates

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Protecting-Group-Controlled Enzymatic Glycosylation of Oligo-*N*-Acetyllactosamine Derivatives

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Abstract: *We describe a chemoenzymatic strategy that can give a library of differentially fucosylated and sialylated oligosaccharides starting from a single chemically synthesized tri-N-acetyllactosamine derivative. The common precursor could easily be converted into 6 different hexasaccharides in which the glucosamine moieties are either acetylated (GlcNAc) or modified as a free amine ($GlcNH_2$) or Boc (GlcNHBoc). Fucosylation of the resulting compounds by a recombinant fucosyl transferase resulted in only modification of the natural GlcNAc moieties, providing access to 6 selectively mono- and bis-fucosylated oligosaccharides. Conversion of the $GlcNH_2$ or GlcNHBoc moieties into the natural GlcNAc, followed by sialylation by sialyl transferases gave 12 differently fucosylated and sialylated compounds. The oligosaccharides were printed as a microarray that was probed by several glycan-binding proteins, demonstrating that complex patterns of fucosylation can modulate glycan recognition.*

N- and *O*-glycans, which decorate the surface of all eukaryotic cells, are involved in a multitude of biological and disease processes.[1] These compounds usually have highly complex architectures, in which branched core structures are extended by various numbers of *N*-acetyllactosamine repeating units.[2] The termini of these chains can be further elaborated by several forms of fucosylation and sialylation to create Lewis antigens and ABO blood groups. The internal LacNAc moieties of the poly-LacNAc chains can also be fucosylated to create more complex epitopes. Furthermore, sulfation of the C-6 position of GlcNAc and Gal and branching at Gal can occur to create I-antigens.[3]

Although the chemical and enzymatic synthesis of terminal fucosylated and sialylated epitopes has received considerable attention,[4] there are no general solutions for the selective incorporation of fucosides and other functionalities into poly-*N*-acetyllactosamine backbones.[5] The lack of such compounds makes it difficult to examine how complex fucosylation patterns may influence binding of glycan-binding proteins and mediate downstream biological processes.

Here, we report a chemoenzymatic strategy that can give easy access to a library of differentially fucosylated and sialylated oligo-LacNAc derivatives starting from advanced precursor. It is based on the finding that lactosamine derivatives with a free amine or the amino function modified by a *tert*-butyloxycarbonyl (Boc) protecting group, are resistant to fucosylation by recombinant FUT5 and Hp39-FT (Figure 1a).[6] To exploit this finding, we prepared precursor **1** (Figure 1b), which through simple modifications could be converted into six different hexasaccharides (**2**–**7,** Figure 1c) in which the glucosamine moieties are either acetylated (GlcNAc) or modified with a free amine ($GlcNH_2$) or Boc (GlcNHBoc). As anticipated, fucosylation of the resulting compounds by a recombinant fucosyl transferase resulted only in modification of the natural GlcNAc moieties. After conversion of the $GlcNH_2$ or GlcNHBoc moieties into the natural GlcNAc counterparts followed by sialylation, a panel of differently fucosylated and sialylated compounds was obtained (**8a**,**b**–**13a**,**b**, Figure 1d). These compounds and a number of controls (**14a**,**b**, **15**, and **16a**,**b**, Figure 1e) were printed as a glycan microarray, which was examined for binding of a number of lectins, glycan-binding proteins, and influenza A virus (IAV) hemagglutinins (HAs). The data showed that the pattern of fucosylation can modulate the interaction with glycan-binding proteins.

To implement a strategy in which protecting groups can control the regioselectivity of enzymatic modifications of oligo-LacNAc chains, we prepared a range of lactosamine derivatives in which the amine was modified to form NHAc (**17**), NHTFA (**18**), NHCbz (**19**), NHBsmoc (**20**),[7] or NHBoc (**21**, Figure 2). These substrates were exposed to the microbial fucosyl transferase Hp39-FT in the presence of GDP-fucose, and the conversion to the corresponding Lewis X (Le^x)-containing oligosaccharides **23**–**27** was monitored by capillary electrophoresis–mass spectrometry (CE-MS).[8] It was found that LacNAc **17** and LacNHTFA **18** were readily converted into the Le^x-containing products **23** and **24**. On the other hand, the presence of the Cbz- and Bsmoc-protecting groups of **19** and **20,** respectively, considerably slowed down the fucosylation and only partial product formation was observed. Strikingly, no conversion to Le^x was observed for LacNHBoc **21** and $LacNH_2$ **22**. Similar results were obtained when FUT5 was employed.

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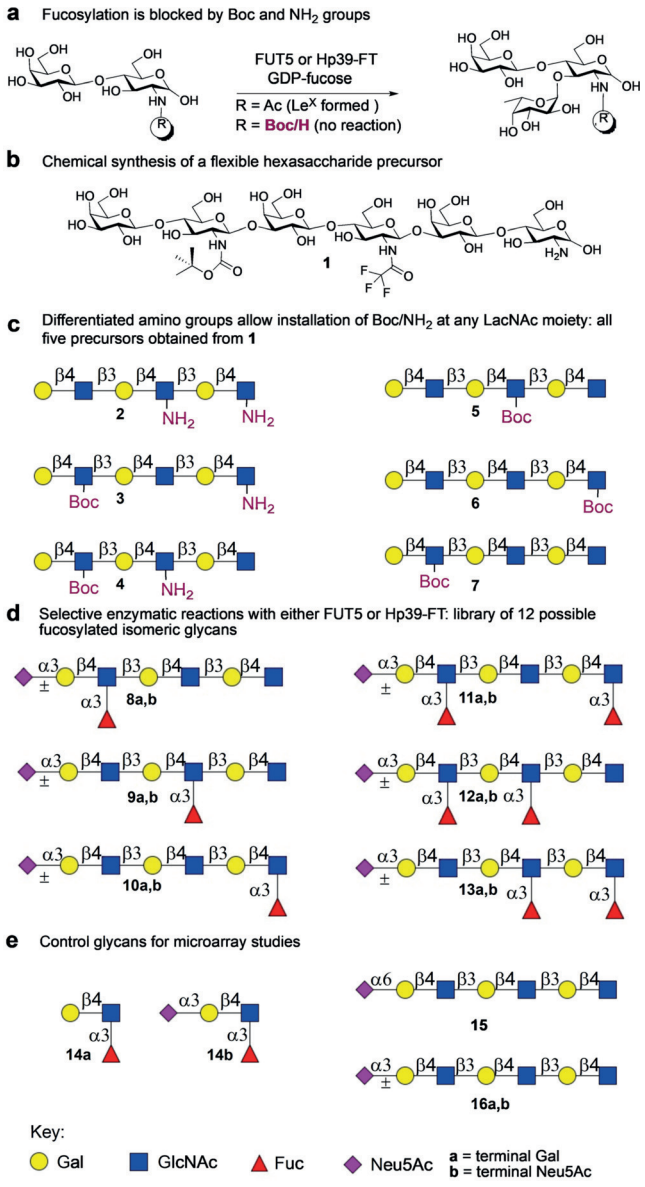


Figure 1. Synthetic hexasaccharide **1** was used to create a library of 12 differentially fucosylated and sialylated derivatives.

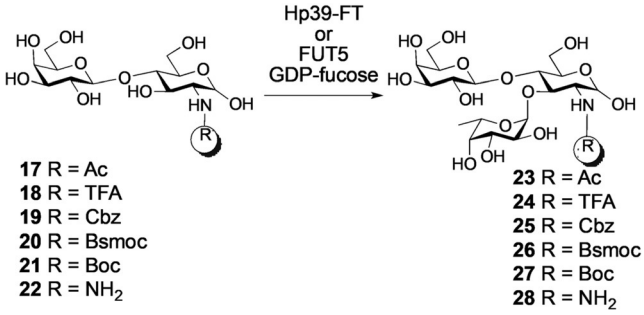
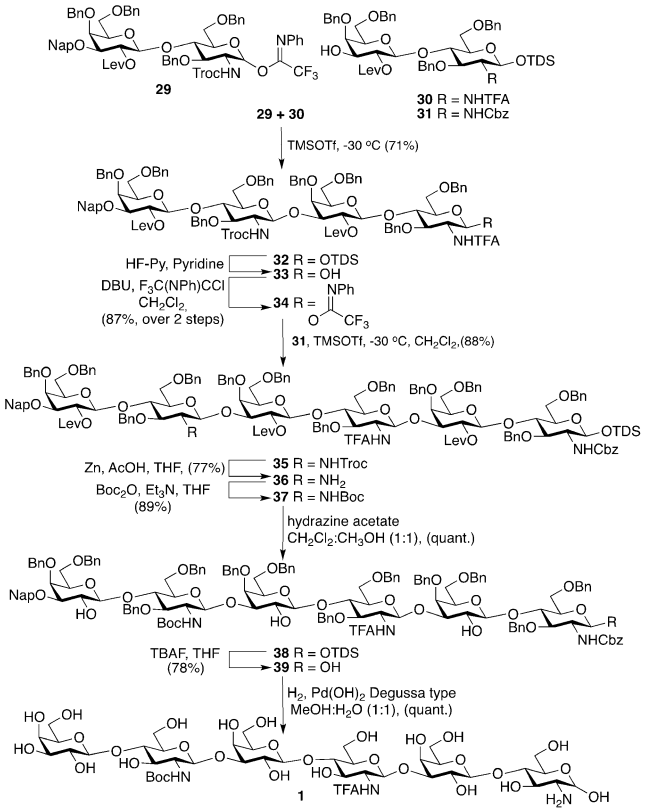


Figure 2. Set of model disaccharides used to identify protecting groups that can temporarily block fucosylation by Hp39-FT or FUT5.

The next challenge was to prepare hexasaccharide **1**, which we anticipated would provide a universal substrate for the synthesis of oligo-LacNAc derivatives having different patterns of NHAc, NHBoc or NH_2 for regioselective fucosylation by Hp39-FT or FUT5. Compound **1** was assembled from building blocks **29–31** to provide hexasaccharide **35** in which the amino functions of the LacNAc moieties are protected with trifluoroacetamido (TFA), 2,2,2-trichloroethoxycarbonyl (Troc), and benzyloxycarbonyl (Cbz) (Scheme 1). The Troc protecting group can be selectively removed by treatment with Zn[9] to give amine **36** that can then be modified as Boc to provide hexasaccharide **37**, which after hydrogenation would yield key intermediate **1**. Installation of Boc at a late stage of the synthesis was important because glycosyl donors with this function at C-2 are prone to oxazolidinone formation.



Scheme 1. Chemical synthesis of required building blocks.

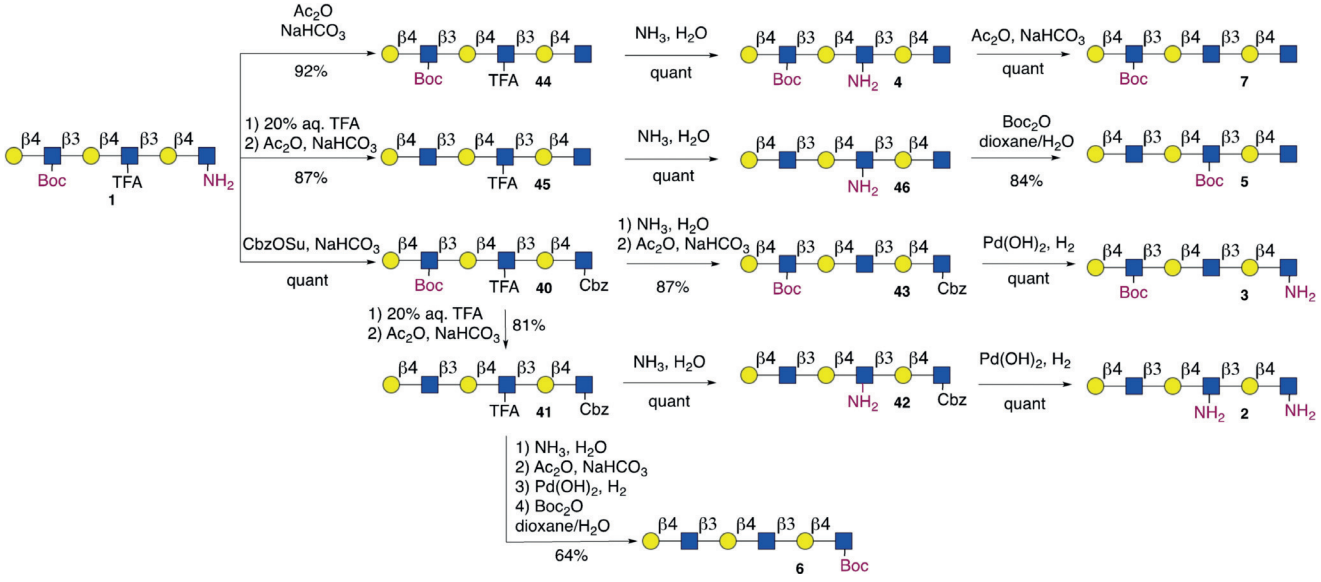
Building blocks **29–31** were prepared from a common disaccharide in which the amine was masked as an azide, while the anomeric center and the C-3′ hydroxyl were temporary protected with TDS[10] and Nap ether, respectively (Supporting Information, Scheme S1). A trimethylsilyl trifluoromethanesulfonate (TMSOTf)-catalyzed glycosylation of **29** with **30** furnished tetrasaccharide **32** in 71% yield. HF/pyridine-mediated cleavage of the anomeric TDS group of **32** gave lactol **33**, which was treated with 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride in the presence of DBU to provide donor **34** (87%, two steps). Glycosylation of **34** with **31** in the presence of TMSOTf provided the hexasaccharide **35** (88%). The latter compound was treated with Zn dust in the presence of acetic acid in THF to afford amine **36**

(77%), which was reacted with Boc_2O to yield the key hexasaccharide **37** (Scheme 1). Next, an orchestrated sequence of deprotection steps was performed. The Lev esters of **37** were hydrolyzed with hydrazine acetate in a mixture of CH_2Cl_2 and CH_3OH to give triol **38**, which was treated with TBAF to cleave the anomeric TDS group to provide **39** (78%). Finally, the benzyl ethers, the Nap ether, and Cbz carbamate were simultaneously removed by catalytic hydrogenation to give the target compound **1**. This efficient one-step global deprotection was achieved using a Degussa-type $Pd(OH)_2$ catalyst, and under the mild reaction conditions the TFA and Boc groups remained intact.

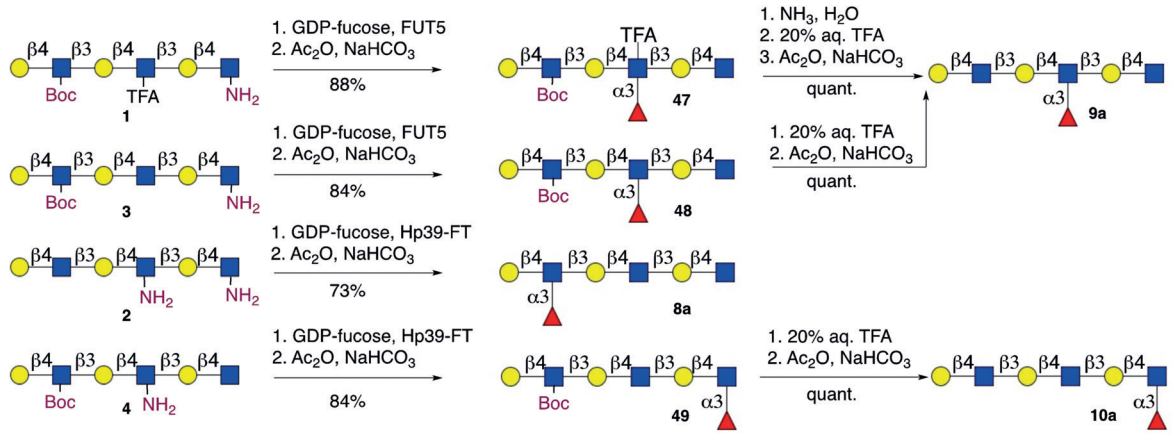
Each of the three amino moieties of **1** can be uniquely manipulated, therefore this compound provides an attractive intermediate for the synthesis of compounds **2**–**7** with different patterns of NAc/NHBoc and NH_2. The TFA group can be cleaved under mild basic conditions (dilute aqueous ammonia), while the Boc group is removable with dilute aqueous trifluoroacetic acid (20%). The Cbz group is stable to both basic and acidic conditions, but can readily be cleaved by catalytic hydrogenation over Pd/C without affecting the TFA and Boc protecting groups.

In a first sequence of reactions, the free amine of key intermediate **1** was acetylated with Ac_2O in the presence of $NaHCO_3$ to give **44**, which was treated with dilute aqueous ammonia to remove the TFA protecting group, providing target compound **4** with a free amine and Boc group at the central and distal lactosamine moieties, respectively. Acetylation of the free amine of **4** gave the second target compound **7** (Scheme 2). Alternatively, exposure of **1** to aqueous TFA to remove the Boc group, followed by acetylation of the resulting amine gave **45**, which was subsequently treated with aqueous ammonia to give **46**, and Boc anhydride to provide **5**. In another sequence of reactions, the amine of **1** was protected with Cbz by reaction with CbzOSu to give **40**, which was subsequently treated with aqueous ammonia and Ac_2O to convert the trifluoracetamido into an acetamido moiety providing **43**, which was subjected to catalytic hydrogenation over $Pd(OH)_2$ to remove the Cbz protecting group, resulting in the formation of **3**. Derivative **2**, with free amines at the proximal and central LacNAc moieties, was also prepared starting from **40** by treatment with 20% trifluoracetic acid, followed by acetylation of the resulting amine with acetic anhydride to convert Boc into acetamido to give **41**, which was followed by hydrolysis of the TFA protecting group with ammonia and cleavage of the Cbz moiety by catalytic hydrogenation. Finally, hexasaccharide **6**, with a blocking Boc moiety at the proximal LacNAc, was obtained by conversion of TFA into acetamido, while transforming Cbz into Boc by standard manipulations in four steps in 64% overall yield from intermediate **41**.

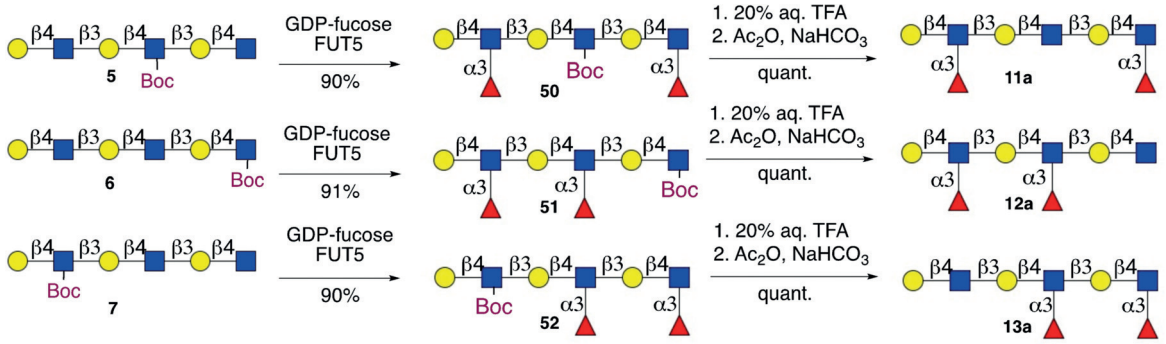
Next, attention was focused on the selective fucosylation of compounds **2**–**7** to give mono-fucosylated **8a**–**10a** (Scheme 3) and di-fucosylated **11a**–**13a** (Scheme 4). We selected the mammalian fucosyl transferase FUT5, which preferentially forms internal Le^x moieties and Hp39-FT that favors fucosylation of terminal LacNAc acceptors.[20] Gratifyingly, treatment of **1** with FUT5 in presence of GDP–fucose followed by *N*-acetylation of the free amine gave the expected mono-fucosylated heptasaccharide **47** in a yield of 88% after purification by HPLC using a semi-preparative HILIC column (Scheme 3). Full assignment of ^1H NMR spectra (750 MHz) combined with ^1H–^1H COSY, ^1H–^1H TOCSY, and ^1H–^{13}C HSQC experiments confirmed the position of the fucoside of **47**. Having both the TFA and the Boc group in **47** assists in identification of otherwise overlapping GlcNAc H-2 signals, which proved to be useful in compound characterization. A downfield shift of H-2 of GlcNHTFA from 3.90 to 4.09 ppm (t, $J = 9.7$ Hz), along with the appearance of one anomeric fucoside at 5.04 ppm (d, $J = 4.1$ Hz), confirmed the presence of the fucoside at the central lactosamine moiety. In this respect, it is known that $\alpha(1,3)$-fucosylation of LacNAc is accompanied by a downfield shift of H-2 of GlcNAc along with an upfield shift of Gal H-4.[5,11] The H-2 signal of GlcNHBoc and GlcNHAc of **47** were unchanged (3.48 ppm, dd, $J = 10.3$, 8.3 Hz) providing further support for the site of



Scheme 2. Diversification of common intermediate **1** into substrates **2**–**7**.



Scheme 3. Selective enzymatic mono-fucosylation.



Scheme 4. Selective enzymatic di-fucosylation followed by removal of Boc groups.

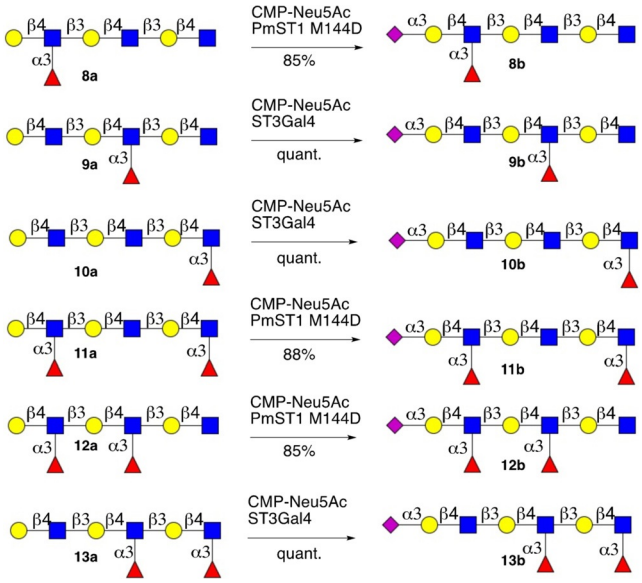
fucosylation. A small amount (10%) of a regioisomer was formed in which the reducing end was fucosylated (see the Supporting Information for full compound characterization). After *N*-acetylation, this by-product could readily be removed by HPLC. The required compound **9a** was obtained by cleavage of the TFA and Boc blocking groups of **47** using standard procedures followed by acetylation of the resulting free amines. A similar regioselectivity was observed when **3** was subjected to FUT5 to give, after *N*-acetylation, compound **48** which by a simple two-step procedure could also be transformed into **9a**.

Treatment of hexasaccharide **2** with Hp39-FT and GDP–fucose followed by acetylation of the two free amines resulted in the exclusive formation of **8a**, which was isolated in yield of 73% after purification by HPLC using a HILIC column. NMR spectroscopic analysis established that the fucoside was located at the distal LacNAc moiety (see the Supporting Information). Similarly, fucosylation of **4** with Hp39-FT followed by *N*-acetylation resulted in the clean formation of mono-fucosylated glycan **49**. The latter derivative was converted into **10a** by removal of Boc with 20% aqueous trifluoracetic acid followed by acetylation of the amines with acetic anhydride in the presence of $NaHCO_3$. These conditions did not affect the sensitive fucosides.

Exposure of **5** to FUT5 and GDP–fucose resulted in the formation of di-Le^x-containing derivative **50** as the only product, which was isolated in a yield of 90% (Scheme 4). The di-fucosylated positional isomers **51** and **52** were obtained by similar procedures starting from glycans **6** and **7**, respectively (see the Supporting Information for compound characterization). Compounds **50**, **51**, and **52** were converted into **11a**, **12a**, and **13a** by removal of the Boc group, followed by acetylation of the amines using standard procedures.

Sialylation of **9a**, **10a**, and **13a**, which have a terminal LacNAc moiety, was accomplished by the mammalian sialyl transferase ST3Gal4 in the presence of CMP–Neu5Ac to give **9b**, **10b**, and **13b**, respectively (Scheme 5). On the other hand, sialylation of glycans **8a**, **11a**, and **12a** bearing a terminal Le^x moiety was achieved by using the mutant bacterial sialyl transferase PmST1 M144D.[12]

Compounds **8a**,**b**–**13a**,**b** (Figure 1d) and control derivatives **14a**,**b**, **15**, and **16a**,**b** (Figure 1e) were modified with a 2-[(methylamino)oxy]ethanamine linker,[13] and then printed on *N*-hydroxysuccinimide (NHS)-activated glass slides. Subarrays were exposed to a range of plant lectins including *Aleuria aurantia* (AAL), *Erythrina cristagalli* (ECL), *Maackia amurensis* II (MAL-II), wheat germ agglutinin (WGA), and *Sambucus nigra* agglutinin (SNA, Supporting Information, Figure S2), the mammalian glycan-binding proteins E-selectin and DC-SIGN and several recombinant HAs of IAVs (Figure 3). Detection of binding was accomplished by using AlexaFluor635 (lectins, E-selectin, DC-SIGN) or AlexaFluor647 (HAs).



Scheme 5. Sialylation of compounds **8a**–**13a** by either ST3Gal4 or PmST1 M144D to give the sialosides **9b**–**13b**, respectively.

The array results uncovered that the pattern of fucosylation of poly-*N*-acetyllactosamine chains can influence lectin binding. For example, a number of plant lectins, such as AAL, and ECL recognize terminal epitopes, and fucosylation of the central or reducing LacNAc moiety did not greatly impact binding (for detailed discussion on the binding of plant lectins see the Supporting Information). On the other hand, MAL-II binds to sialyl LacNAc and does not tolerate a fucoside at this position. DC-SIGN, which is a protein expressed by dendritic cells that plays an important role in pathogen detection and innate immunity,[14] shows yet another binding pattern (Figure 3a) and recognizes compounds with terminal (**8a, 11a, 12a**) and central (**9a** and **13a**) Le^x moieties but does not tolerate Le^x at the reducing end (**10a** and **10b**).

Next, we examined binding properties of E-selectin, which is an inflammatory protein that recognizes fucosylated glycans on leukocytes, thereby initiating rolling and tethering of these cells to sites of inflammation. There is conflicting data regarding the ligand requirements of E-selectin.[15] On the one hand, sialyl-Le^x (SLe^x) is a well-recognized ligand for E-selectin. On the other hand, it has been proposed that VIM-2, which is a sialylated glycosphingolipid expressed on human neutrophils containing an internal fucoside, is the functional E-selectin receptor.[15,16] Studies with isolated compounds have indicated that positional isomers of VIM-2 are poorer receptors, including SLe^x containing glycans. In our microarray screen, only compounds containing a SLe^x moiety (**8b, 11b**, **12b**) were recognized by E-selectin (Figure 3b). Internally fucosylated motifs, such as **9b**, **10b**, and **13b**, showed no detectable binding. Thus, the E-selectin appears to bind only to SLe^x-containing glycans, yet it cannot be excluded that additional functionalities and molecular environment could enhance binding affinities of compounds such as VIM-2, and for example the presence of a ceramide may be an important receptor determinant.

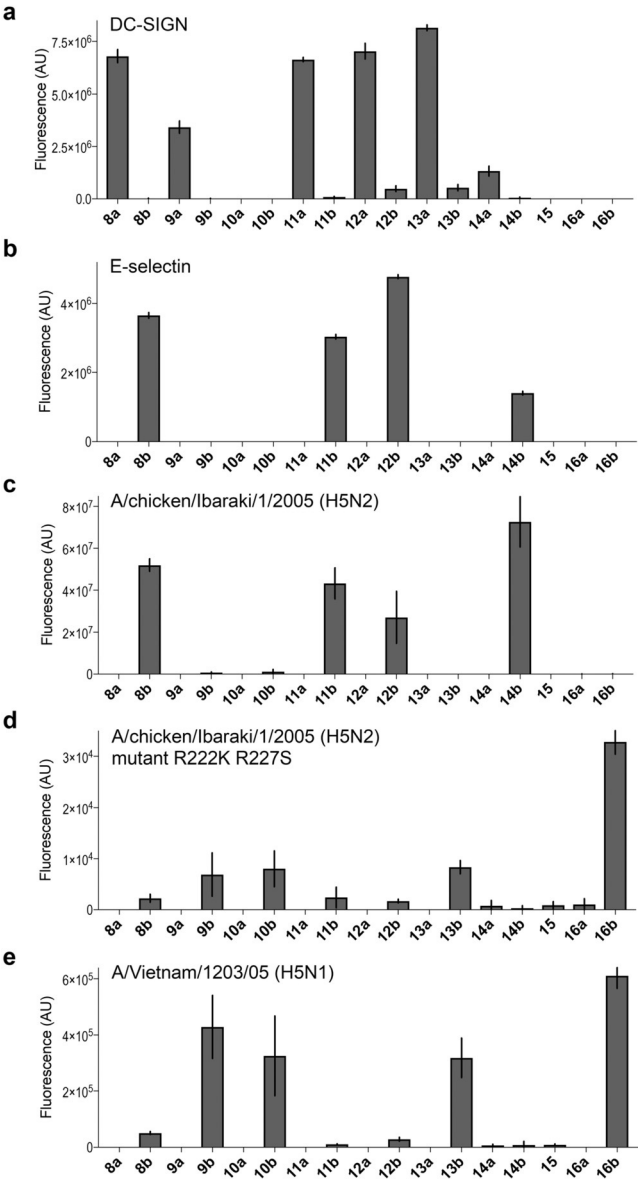


Figure 3. Microarray results of synthetic glycan library at 100 μM with a) DC-SIGN (10 μg mL^{-1}); b) E-selectin (2 μg mL^{-1}); c) A/chicken/Ibaraki/1/2005 (H5N2) (50 μg mL^{-1}); d) A/chicken/Ibaraki/1/2005 (H5N2) mutant R222K R227S (50 μg mL^{-1}; and e) A/Vietnam/1203/05 (H5N1) (50 μg mL^{-1}). The lowest concentration required for good responsiveness in the optimum dynamic range was selected for all proteins examined. Bars represent the mean ± SD.

Finally, we examined receptor usage of HAs of several IAVs. It is generally accepted that avian and human IAV bind α(2,3)- and α(2,6)-sialosides, respectively. Evidence is emerging that this binary differentiation is an oversimplification and other features, such as branching and the presence of extended LacNAc moieties, can modulate HA binding.[17] Furthermore, the presence of a fucosyl residue at a terminal LacNAc moiety to create SLe^x may be a species barrier between chicken and duck.[18] To gain further insight in IAV transmission between the latter two species, the glycan array was used to examine the receptor requirements of IAVs isolated from a chicken and a zoonotic human infection

(Figure 3c–e). A chicken influenza virus, A/chicken/Ibaraki/1/2005 (H5N2), showed specificity for SLe^x-containing glycans (**8b**, **11b**, **12b**, **14b**). Computational analysis has indicated that this glycan-binding specificity is determined by amino acid residues at positions 222 and 227. Therefore, we also examined the R222K R227S mutant, and as expected this HA recognized non-fucosylated α(2,3)-sialoside **16b**. Interestingly, a fucosyl moiety at the terminal LacNAc moiety (**8b**, **11b**, **12b**) almost abolished binding, whereas such a residue at the central and non-reducing LacNAc moiety (**9b**, **10b**, **13b**) reduced responsivenss, indicating this HA preferentially binds to extended and unmodified sialyl LacNAc epitopes. As a control, we analyzed A/Vietnam/1203/05 (H5N1) that contains K222 and S227. This HA recognized α(2,3)-sialyl-LacNAc-containing compounds (**9b**, **10b**, **13b**, **16b**) and fucosylation of this residue greatly reduced binding (**8b**, **11b**, **12b**).[19] Fucosylation of the central and reducing LacNAc moiety had only a minor influence on binding. Thus, terminal SLe^x is indeed a species barrier in H5Nx viruses.

In conclusion, a methodology is described to control the enzymatic fucosylation of oligo-LacNAc derivatives using temporary blocking groups. It is to be expected that other types of terminal modifications can be installed and for example exposure of glycans **8a**–**13a** to FUT2 should create an H-type antigen or Le^y epitope. We also anticipate that the activity of other enzymes that modify poly-*N*-acetyllactosamine chains, such as specific sulfotransferases and I-branching GlcNAc transferases, will be impacted by chemical modifications on LacNAc, thereby further expanding the scope of the methodology.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: glycan microarray · glycosyl transferases · glycosylation · lectin · regioselectivity

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