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A General Strategy for the Chemoenzymatic Synthesis of Asymmetrically Branched *N*-Glycans

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Abstract

A systematic, efficient means of producing diverse libraries of asymmetrically branched *N*-glycans is needed to investigate the specificities and biology of glycan binding proteins. To that end, we describe a core pentasaccharide that at potential branching positions is modified by orthogonal protecting groups to allow selective attachment of unique saccharide moieties by chemical glycosylation. The appendages were selected in such a way that the antenna of the resulting deprotected compounds could be selectively extended by glycosyltransferases to give libraries of asymmetrical multi-antennary glycans. The power of the methodology was demonstrated by the preparation of a series of complex oligosaccharides that were printed as microarrays and screened for binding to lectins and influenza-virus hemagglutinins, which showed that recognition is modulated by presentation of minimal epitopes in the context of complex *N*-glycans.

Most cell surface and secreted proteins are modified by covalently-linked glycans which are essential mediators of biological processes such as protein folding, cell signaling, fertilization, embryogenesis, and the proliferation of cells and their organization into specific tissues (1). Overwhelming data support the relevance of glycosylation in pathogen recognition, inflammation, innate immune responses, and the development of autoimmune diseases and cancer (2, 3). Although the functional importance of glycoprotein glycosylation is well-established, molecular mechanisms by which these compounds exert their functions have been difficult to define. The latter is due to a lack of comprehensive libraries of well-defined complex oligosaccharides that are needed as standards to determine exact structures of glycans in complex mixtures (4, 5) and to examine specificities and biology of glycan binding proteins that occur in nature (6–8).

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Supplementary Materials

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Materials and Methods

Figs. S1 to S24

Tables S1 to S14

References (24, 25, 29, 40–47)

NMR spectra

Naturally occurring glycans are typically isolated in small quantities as mixtures of closely related structures that are difficult to separate, and therefore do not provide a reliable source of well-defined oligosaccharides. Thus, it is widely accepted that chemical- or enzymatic approaches must be employed for the preparation of diverse glycan libraries needed for biological and structural studies (7–11). Despite ongoing progress, the chemical synthesis of complex oligosaccharides remains very time consuming, especially when highly complex structures are targeted (7). The need for more efficient approaches has stimulated the development of chemo-enzymatic methods in which a synthetic oligosaccharide precursor is modified by a range of glycosyltransferases to give more complex derivatives (10, 11). Such an approach can, however, only provide symmetrically branched oligosaccharides.

Naturally occurring branched oligosaccharides often bear unique appendages at each branching point (12). In this respect, the biosynthesis of *N*-linked oligosaccharides is initiated in the endoplasmic reticulum where a dolichol-linked Glc₃Man₉GlcNAc₂ oligosaccharide precursor is transferred *en bloc* to an Asn-X-Ser/Thr sequon on newly synthesized polypeptides. Subsequent trimming and processing of the transferred oligosaccharide results in a GlcNAcMan₃GlcNAc₂ core structure, which is transported to the Golgi where additional *N*-acetyl glucosamine moieties (*O*-GlcNAc) can be added. Subsequent conversion of the *O*-GlcNAc stubs into *N*-acetyllactosamine (Gal(1,4)GlcNAc, LacNAc) provide precursors that can be elaborated by various glycosyltransferases to give rise to enormous structural diversity.

The biosynthesis of complex branched oligosaccharides generally leads to positional isomers, which are structurally difficult to assign by mass spectrometry (4, 5). Furthermore, glycan microarray technology has shown that terminal oligosaccharide motifs of complex glycans mediate biological recognition (13). However, a number of recent studies indicate a more complex picture in which the core structure can influence terminal glycan recognition (14). A synthetic technology that can give libraries of asymmetrically substituted glycans will make it possible to fabricate the next generation of glycan microarray to examine in detail glycan-protein recognition, to develop algorithms for the assignment of MS spectra and to design probes for elucidating pathways of glycoconjugate biosynthesis. Despite the urgent need for libraries of asymmetrically branched *N*-glycans (15), none of the currently available methods can produce collections of such compounds, and previous synthetic efforts have almost exclusively focused on the preparation of symmetrically branched compounds (16–22).

We envisaged that oligosaccharide **1** would be an attractive starting material for the preparation of libraries of asymmetrically branched *N*-glycans (Fig. 1). This pentasaccharide resembles the core structure common to all eukaryotic *N*-linked glycans (12) and is modified at positions where branching points can occur with the protecting groups levulinoyl (Lev), fluorenylmethyloxycarbonate (Fmoc), allyloxycarbonate (Alloc), and 2-naphthylmethyl (Nap). It is shown here that these protecting groups are orthogonal and therefore it was expected that libraries of complex branched bi-, tri-, and tetra-antennary structures could be generated by sequential removal of the protecting groups followed by chemical glycosylations using a diverse set of glycosyl donors. Furthermore, it was anticipated that the use of LacNAc and GlcNAc donors **2–5** followed by removal of all protecting groups except the acetyl esters, would give precursor glycans that at each antenna could be selectively extended by a panel of glycosyltransferases to rapidly give large numbers of highly complex asymmetrically substituted *N*-glycans. Selective extension was expected to be feasible because many relevant glycosyltransferases recognize LacNAc but not GlcNAc as a substrate (17). The latter moiety can, however, be converted into LacNAc by enzymatic galactosylation and the resulting derivative can then be elaborated by other glycosyltransferases. Furthermore, acetylation should render LacNAc and GlcNAc moieties

inactive for enzymatic modification; however, the removal of these esters would give an appropriate substrate for extension by glycosyltransferases.

Some applications, such as the use of synthetic glycans as standards for mass spectrometry, require compounds having an unmodified reducing end. Other uses, such as the development of glycan microarrays, need compounds modified with a reactive anomeric linker. To ensure the glycans prepared by the chemo-enzymatic approach can be employed for multiple purposes, the anomeric center of compound **1** was protected as a benzyl glycoside. This protecting group will be removed during the deprotection stage to give glycans having an unmodified reducing end. The latter type of compound can, however, easily be derivatized by a reactive anomeric linker by reaction with an appropriate reagent such as 2-((methylamino)oxy)ethanamine (**23**).

Pentasaccharide **1** was readily assembled from appropriately protected monosaccharide building blocks (Fig. S2). The Fmoc group of **1** could be selectively removed by the non-nucleophilic base triethylamine to give **6** whereas treatment with the nucleophilic base hydrazine acetate led to cleavage of the Lev ester to provide **7** without affecting the other base sensitive protecting groups (Fig. 2A). Treatment of **1** with Pd(PPh₃)₄ affected only the Alloc protecting group providing the corresponding hydroxyl **8** and oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) resulted in the removal of the Nap ether to give **9** in high yield.

Having demonstrated the orthogonality of the temporary protecting groups, attention was focused on the preparation of tri-antennary oligosaccharide **15**, which was expected to be an appropriate precursor for branch-specific enzymatic modification (Fig. 2). Glycosyl acceptor **6** was coupled with **2** using trifluoromethanesulfonic acid (TfOH) (**24**, **25**) as the promoter to give heptasaccharide **10**. The Nap ether of **10** was removed by oxidation with DDQ and the resulting acceptor **11** was glycosylated with **3** to provide nonasaccharide **12**. Next, the Lev ester of **12** was cleaved with hydrazine acetate to give **13**, which was coupled with **4** to give fully protected deca-saccharide **14**. Partial deprotection of **14** to give target compound **15** was accomplished by cleavage of the Alloc carbonate with Pd(PPh₃)₄ followed by removal of the 2,2,2-trichloroethoxycarbamate (Troc) groups with Zn in acetic acid, acetylation of the resulting free amines with acetic anhydride, and catalytic hydrogenolysis of the benzyl ethers. Detailed NMR analysis of **15** showed that the acetyl esters were still intact, and thus a compound was obtained that has unique saccharide appendages at each antenna allowing selective modification by a panel of glycosyltransferases.

In addition to compound **15**, pentasaccharide **1** is an appropriate starting material for the chemical synthesis of other bi-, tri-, and tetra-antennary precursor oligosaccharides by changing the number and sites of attachment of the appendages (**2-5**). For example, a positional isomer of **15** was readily prepared by the sequential removal of the Fmoc, Alloc, Lev groups of **1** and glycosylations with glycosyl donors **2**, **3**, and **4**, respectively (Fig. S3).

The precursor oligosaccharide **15** was further extended by glycosyltransferases to demonstrate the possibility of selective modification of each antenna to form highly complex asymmetrically branched *N*-glycans (Fig. 3). Many human *N*-glycans contain terminal sialic acids either exclusively β (2,3)- or β (2,6)-linked to *N*-acetylglucosamine or a combination of these two linkages (26). Furthermore, Lewis antigens such as Lewis^y (Le^y), Le^x, and sialyl Lewis^x (SLe^x) are found on many biologically important glycans. Therefore, attention was focused on the preparation of heptadecasaccharide **22** which has SLe^x and Le^x appendages at the C-2 and C-4 arm, respectively and a di-LacNAc moiety extended by β (2,6)-linked sialoside at the C-6 arm. A key aspect of this strategy is that relatively few glycosyltransferases are needed to elaborate these terminal glycan sequences, and enzyme

expression systems that produce these and many other mammalian and bacterial glycosyltransferases useful in chemo-enzymatic synthesis have already been described (27, 28).

The LacNAc moiety of decasaccharide **15** was sialylated by α ,3-sialyltransferase (ST3Gal-IV), cytidine-5' monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac), and Calf Intestine Alkaline Phosphatase (CIAP), and as expected only one of the three antennae was modified to give exclusively compound **16**. Next, the acetyl esters of **16** were removed by treatment with aqueous ammonia to give compound **17**, which has now an unmasked LacNAc moiety at the C-4 of the Man- β arm which is expected to be available for enzymatic transformations. Indeed, fucosylation of **17** with α ,3-fucosyltransferase (β FucT) (29) resulted in the modification of the LacNAc and sialyl-LacNAc moieties to give bis-fucosylated derivative **18**. The GlcNAc moiety at the C-6 antenna of **18** was converted into a LacNAc moiety by employing α ,4-galactosyltransferase (GalT-1), uridine 5' diphosphogalactose (UDP-Gal), and CIAP to give **19**. Treatment of **19** with α ,3-*N*-acetylglucosaminyltransferase (α ,3GlcNAcT) (30), UDP-GlcNAc, and CIAP resulted in a selective addition of a β (1,3)-linked GlcNAc moiety to the LacNAc moiety of the α -6 branch to give **20**. The Le^x moiety of **19** was unaffected highlighting the feasibility of exploiting inherent substrate specificities of glycosyltransferases for the selective modification of multi-antennary glycans. The α ,6-branch was further extended by GalT-1 and α ,6-sialyltransferase (ST6Gal-1) to provide target compound **22**, which has unique oligosaccharide appendages at each of the three antennae.

After each step, the product was purified by size exclusion chromatography and the resulting compound fully characterized by NMR and mass spectrometry of the permethylated derivative. If any starting material was observed, the compound was resubjected to the enzyme until a homogeneous product was obtained. In addition to target compound **22**, each intermediate of the enzymatic extension (**17-21**) can in principle be employed for biological or biophysical studies. The precursor oligosaccharide **15** is an attractive starting material for the preparation of many other highly complex glycans. To illustrate this feature, compounds **23-27** were prepared (Figs. S14 and S15), which are asymmetrical and have varying numbers of 2,3- or 2,6-linked sialic acids at the various antennae (26). Thus, subsequent deacetylation and bis-fucosylation of **15** to give Le^x moieties at the α and β arm was followed by galactosylation to form a LacNAc moiety at the β arm that was capped with 2,6-Neu5Ac to form **23** or further extended with 2,6-Neu5Ac-LacNAc to provide **24**. Similarly, compounds **25-27** were synthesized by either bis- β (2-3) (to give **27**) or bis- β (2-6)-sialylation followed by extension of the β arm to provide **25** and **26** (Fig S15).

It was anticipated that compounds **22-27** would be useful for examining the activity of the various biologically relevant glycan epitopes in the context of their presence on multiantennary asymmetric structures. Thus, a glycan microarray was constructed composed of the asymmetrical tri-antennary glycans (**22-27**) and previously prepared linear and bi-antennary glycans having a terminal β (1-4)Gal (**A-D**), α (1-3)-Fuc (**E-F**), α (2-6)-Neu5Ac (**G-L**), or β (2-3)-Neu5Ac (**M-Q**) moiety (Table S14). Compounds **22-27** were modified with an amino-containing linker by treatment with 2-((methylamino)oxy)ethanamine (23) and the resulting derivatives were printed on NHS-activated glass slides with the reference compounds (31).

Probing the array with the *Erythrina crista-galli* agglutinin (ECA) specific for terminal LacNAc sequences detected the corresponding reference compounds **A-D** and compounds **I** and **J**; two biantennary compounds that have one branch modified with a LacNAc structure (Fig. 4). Of the synthetic triantennary compounds ECA lectin bound strongly to **25** and weakly to **22-24**. The latter compounds contain LacNAc substituted with a fucoside, which

is known to reduce the affinity of ECA (32). In contrast, the fucose-specific *Aleuria aurantia* lectin (AAL) robustly recognized the fucoside containing glycans **22-24** as well as the three reference compounds containing a Le^x epitope (**E**, **F**, and **M**). *Sambucus nigra* agglutinin (SNA) specific for terminal $\alpha(2-6)$ Neu5Ac recognized all structures containing this epitope (**G-L** and **22-26**).

Influenza viruses recognize sialic acids as receptors, and it is well documented that human and avian viruses exhibit differential specificity for glycans with Neu5Ac $\alpha(2-6)$ Gal and Neu5Ac $\alpha(2-3)$ Gal linkages, respectively. This difference in specificity represents a major barrier for transmission of avian viruses into humans (33, 34), and increasing attention is placed on glycan microarray analysis to understand the receptor requirements of avian and human virus hemagglutinins (HA) required for species tropism (35-37). To assess the potential for influenza HA to distinguish between symmetric and asymmetric glycans we evaluated the specificity of an HA from an exemplary H5N1 avian virus (VN/04), a human seasonal H1N1 virus (KY/07) and an H1N1 virus from the 2009 influenza pandemic (CA/05).

The H5 HA from VN/04 recognized compounds **N-Q** and **27**, which contain the Neu5Ac $\alpha(2-3)$ Gal consistent with the consensus receptor specificity of avian viruses (33, 38). Notably, this cloned HA did not recognize the Neu5Ac $\alpha(2-3)$ Gal in the fucosylated sequence SLe^x in compound **22** or the reference compound **M**. In contrast, the HA from the two human influenza viruses exhibited binding only to glycans containing the Neu5Ac $\alpha(2-6)$ Gal epitope (Fig. 4), but otherwise exhibited different fine specificities. The HA from the H1N1 seasonal strain A/Kentucky/07 (KY/07) recognized all the reference compounds (**G-L**) and all the triantennary compounds (**22-26**) that contained this linkage. However, relative to the linear reference compounds (**G**, **H**), the compounds that have a Neu5Ac $\alpha(2-6)$ Gal moiety on only one branch of a biantennary glycan were bound weakly (**I**, **J**), while those that had the Neu5Ac $\alpha(2-6)$ Gal sequence on only one branch of the triantennary glycans (**23**, **24**) were recognized equally well. Thus, this HA distinguishes structures with a single sialic acid in the context of linear or biantennary and triantennary *N*-linked glycan chains. More dramatic differences are seen comparing the seasonal H1 and the pandemic HA H1 from A/California/05/09 (CA/05). The CA/05 HA recognized only reference compounds **H** and **L** and a single triantennary glycan, namely **26**. These compounds have in common the Neu5Ac $\alpha(2-6)$ epitope linked to an extended dimeric-LacNAc moiety. However, this motif is also present in triantennary glycans **22** and **24**, which are not recognized by this HA. Compounds **L** and **26** also have in common at least two Neu5Ac $\alpha(2-6)$ epitopes on different antennae, but so do compounds **K** and **25**, which have a single LacNAc extension and are not recognized. These results reflect differences in the specificity of these HAs, and not simple differences in avidity, since similar array results were obtained when concentration of the HA applied to the array was titrated down in 2-fold dilutions from 100 to 6 μ g/mL (Fig. S24).

These results demonstrate that glycan epitopes presented on asymmetrically branched *N*-linked glycans can be distinguished from the same epitopes on linear or symmetrically branched glycans. Such context-dependent recognition can be due to extended binding sites, unfavorable interactions by neighboring antennae and multivalency by proper spacing of minimal epitopes at two or more antennae. As illustrated by the selected influenza HAs, these differences are relevant to the recognition of receptors by human pathogens. A complete understanding of influenza receptor specificity and its relevance to adaptation of animal viruses to human hosts will require an extensive panel of asymmetric and symmetric glycan structures representative of those found on human and animal airway epithelia (37). Such libraries of glycans, which can be produced by the methodology presented here, will begin to define the human glycome, and provide tools to understand the biology mediated by

both microbial and mammalian glycan binding proteins that mediate host pathogen interactions and innate and adaptive immune responses (13, 39).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References and Notes

- Hart GW, Copeland RJ. Glycomics hits the big time. *Cell*. 2010; 143:672. [PubMed: 21111227]
- Freeze HH. Genetic defects in the human glycome. *Nat Rev Genet*. 2006; 7:537. [PubMed: 16755287]
- Ohtsubo K, Marth JD. Glycosylation in cellular mechanisms of health and disease. *Cell*. 2006; 126:855. [PubMed: 16959566]
- North SJ, Hitchen PG, Haslam SM, Dell A. Mass spectrometry in the analysis of *N*-linked and *O*-linked glycans. *Curr Opin Struct Biol*. 2009; 19:498. [PubMed: 19577919]
- Marino K, Bones J, Kattla JJ, Rudd PM. A systematic approach to protein glycosylation analysis: a path through the maze. *Nat Chem Biol*. 2010; 6:713. [PubMed: 20852609]
- Laurent N, Voglmeir J, Flitsch SL. Glycoarrays--tools for determining protein-carbohydrate interactions and glycoenzyme specificity. *Chem Commun*. 2008:4400.
- Boltje TJ, Buskas T, Boons GJ. Opportunities and challenges in synthetic oligosaccharide and glycoconjugate research. *Nat Chem*. 2009; 1:611. [PubMed: 20161474]
- Lepeniev B, Yin J, Seeberger PH. Applications of synthetic carbohydrates to chemical biology. *Curr Opin Chem Biol*. 2010; 14:404. [PubMed: 20227905]
- Zhu XM, Schmidt RR. New principles for glycoside-bond formation. *Angew Chem, Int Ed*. 2009; 48:1900.
- Palcic MM. Glycosyltransferases as biocatalysts. *Curr Opin Chem Biol*. 2011; 15:226. [PubMed: 21334964]
- Schmaltz RM, Hanson SR, Wong CH. Enzymes in the synthesis of glycoconjugates. *Chem Rev*. 2011; 111:4259. [PubMed: 21749134]
- Moremen KW, Tiemeyer M, Nairn AV. Vertebrate protein glycosylation: diversity, synthesis and function. *Nat Rev Mol Cell Biol*. 2012; 13:448. [PubMed: 22722607]
- Rillahan CD, Paulson JC. Glycan microarrays for decoding the glycome. *Annu Rev Biochem*. 2011; 80:797. [PubMed: 21469953]
- Gabius HJ, Andre S, Jimenez-Barbero J, Romero A, Solis D. From lectin structure to functional glycomics: principles of the sugar code. *Trends Biochem Sci*. 2011; 36:298. [PubMed: 21458998]
- Ranzinger R, Frank M, von der Lieth CW, Herget S. Glycome-DB.org: a portal for querying across the digital world of carbohydrate sequences. *Glycobiology*. 2009; 19:1563. Analysis of the glycomeDB database shows that 85% of known glycan structures are asymmetrical. Almost all of

the known N-linked glycans have fewer than 25 monosaccharides and such compounds should be accessible by the strategy presented here. [PubMed: 19759275]

16. Unverzagt C. Chemoenzymatic synthesis of a sialylated undecasaccharide-asparagine conjugate. *Angew Chem, Int Ed.* 1996; 35:2350.
17. Hanashima S, Manabe S, Ito Y. Divergent synthesis of sialylated glycan chains: combined use of polymer support, resin capture-release, and chemoenzymatic strategies. *Angew Chem, Int Ed.* 2005; 44:4218.
18. Jonke S, Liu KG, Schmidt RR. Solid-phase oligosaccharide synthesis of a small library of *N*-glycans. *Chem-Eur J.* 2006; 12:1274. [PubMed: 16273561]
19. Sun B, Srinivasan B, Huang XF. Pre-activation-based one-pot synthesis of an alpha-(2,3)-sialylated core-fucosylated complex type Bi-antennary *N*-glycan dodecasaccharide. *Chem-Eur J.* 2008; 14:7072. [PubMed: 18601234]
20. Unverzagt C, et al. Synthesis of multiantennary complex type *N*-glycans by use of modular building blocks. *Chem-Eur J.* 2009; 15:12292. [PubMed: 19806620]
21. Serna S, Etxebarria J, Ruiz N, Martin-Lomas M, Reichardt NC. Construction of *N*-glycan microarrays by using modular synthesis and on-chip nanoscale enzymatic glycosylation. *Chem-Eur J.* 2010; 16:13163. [PubMed: 20878803]
22. Walczak MA, Danishefsky SJ. Solving the convergence problem in the synthesis of triantennary *N*-glycan relevant to prostate-specific membrane antigen (PSMA). *J Am Chem Soc.* 2012; 134:16430. [PubMed: 22954207]
23. Bohorov O, Andersson-Sand H, Hoffmann J, Blixt O. Arraying glycomics: a novel bi-functional spacer for one-step microscale derivatization of free reducing glycans. *Glycobiology.* 2006; 16:21C.
24. Schmidt RR, Kinzy W. Anomeric-oxygen activation for glycoside synthesis - the trichloroacetimidate method. *Adv Carbohydr Chem Biochem.* 1994; 50:21. [PubMed: 7942254]
25. Yu B, Tao HC. Glycosyl trifluoroacetimidates. Part 1: Preparation and application as new glycosyl donors. *Tetrahedron Lett.* 2001; 42:2405.
26. Spik G, Debruyne V, Montreuil J, van Halbeek H, Vliegthart JF. Primary structure of two sialylated triantennary glycans from human serotransferrin. *FEBS Lett.* 1985; 183:65. [PubMed: 3979568]
27. More than 800 expression constructs of human glycosylation enzymes in multiple expression vectors and protocols to produce the enzymes for biochemical, enzymatic synthesis, and structural studies are available at <http://glycoenzymes.ccr.c.uga.edu>
28. Blixt O, Razi N. Chemoenzymatic synthesis of glycan libraries. *Methods Enzymol.* 2006; 415:137. [PubMed: 17116472]
29. Wang W, et al. Chemoenzymatic synthesis of GDP-L-fucose and the Lewis X glycan derivatives. *Proc Natl Acad Sci U S A.* 2009; 106:16096. [PubMed: 19805264]
30. Sauerzapfe B, et al. Chemo-enzymatic synthesis of poly-*N*-acetylactosamine (poly-LacNAc) structures and their characterization for CGL2-galectin-mediated binding of ECM glycoproteins to biomaterial surfaces. *Glycoconjugate J.* 2009; 26:141.
31. Blixt O, et al. Printed covalent glycan array for ligand profiling of diverse glycan binding proteins. *Proc Natl Acad Sci U S A.* 2004; 101:17033. [PubMed: 15563589]
32. Itakura Y, et al. Systematic comparison of oligosaccharide specificity of *Ricinus communis* agglutinin I and *Erythrina* lectins: a search by frontal affinity chromatography. *J Biochem.* 2007; 142:459. [PubMed: 17652328]
33. Chandrasekaran A, et al. Glycan topology determines human adaptation of avian H5N1 virus hemagglutinin. *Nat Biotechnol.* 2008; 26:107. [PubMed: 18176555]
34. Imai M, Kawaoka Y. The role of receptor binding specificity in interspecies transmission of influenza viruses. *Curr Opin Virol.* 2012; 2:160. [PubMed: 22445963]
35. Chen LM, et al. *In vitro* evolution of H5N1 avian influenza virus toward human-type receptor specificity. *Virology.* 2012; 422:105. [PubMed: 22056389]
36. Pearce MB, et al. Pathogenesis and transmission of swine origin A(H3N2)v influenza viruses in ferrets. *Proc Natl Acad Sci U S A.* 2012; 109:3944. [PubMed: 22355116]

37. Walther T, et al. Glycomic analysis of human respiratory tract tissues and correlation with influenza virus infection. *PLoS Pathog.* 2013; 9:e1003223. [PubMed: 23516363]
38. Stevens J, et al. Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. *Science.* 2006; 312:404. [PubMed: 16543414]
39. van Kooyk Y, Rabinovich GA. Protein-glycan interactions in the control of innate and adaptive immune responses. *Nat Immunol.* 2008; 9:593. [PubMed: 18490910]
40. Crich D, Smith M. 1-Benzenesulfinyl piperidine/trifluoromethanesulfonic anhydride: a potent combination of shelf-stable reagents for the low-temperature conversion of thioglycosides to glycosyl triflates and for the formation of diverse glycosidic linkages. *J Am Chem Soc.* 2001; 123:9015. [PubMed: 11552809]
41. Gaunt MJ, Yu JQ, Spencer JB. Rational design of benzyl-type protecting groups allows sequential deprotection of hydroxyl groups by catalytic hydrogenolysis. *J Org Chem.* 1998; 63:4172.
42. Maiti KK, et al. Chemical synthesis and proinflammatory responses of monophosphoryl lipid A adjuvant candidates. *Eur J Org Chem.* 2010:80.
43. Boltje TJ, Li C, Boons GJ. Versatile set of orthogonal protecting groups for the preparation of highly branched oligosaccharides. *Org Lett.* 2010; 12:4636. [PubMed: 20845911]
44. Schmidt RR, Stumpp M. Glycosylimidates. 8. Synthesis of 1-thioglycosides. *Liebigs Ann Chem.* 1983:1249.
45. Logan SM, et al. Novel biosynthetic functions of lipopolysaccharide rfaJ homologs from *Helicobacter pylori*. *Glycobiology.* 2005; 15:721. [PubMed: 15814825]
46. de Vries RP, et al. The influenza A virus hemagglutinin glycosylation state affects receptor-binding specificity. *Virology.* 2010; 403:17. [PubMed: 20441997]
47. de Vries RP, et al. Glycan-dependent immunogenicity of recombinant soluble trimeric hemagglutinin. *J Virol.* 2012; 86:11735. [PubMed: 22915811]

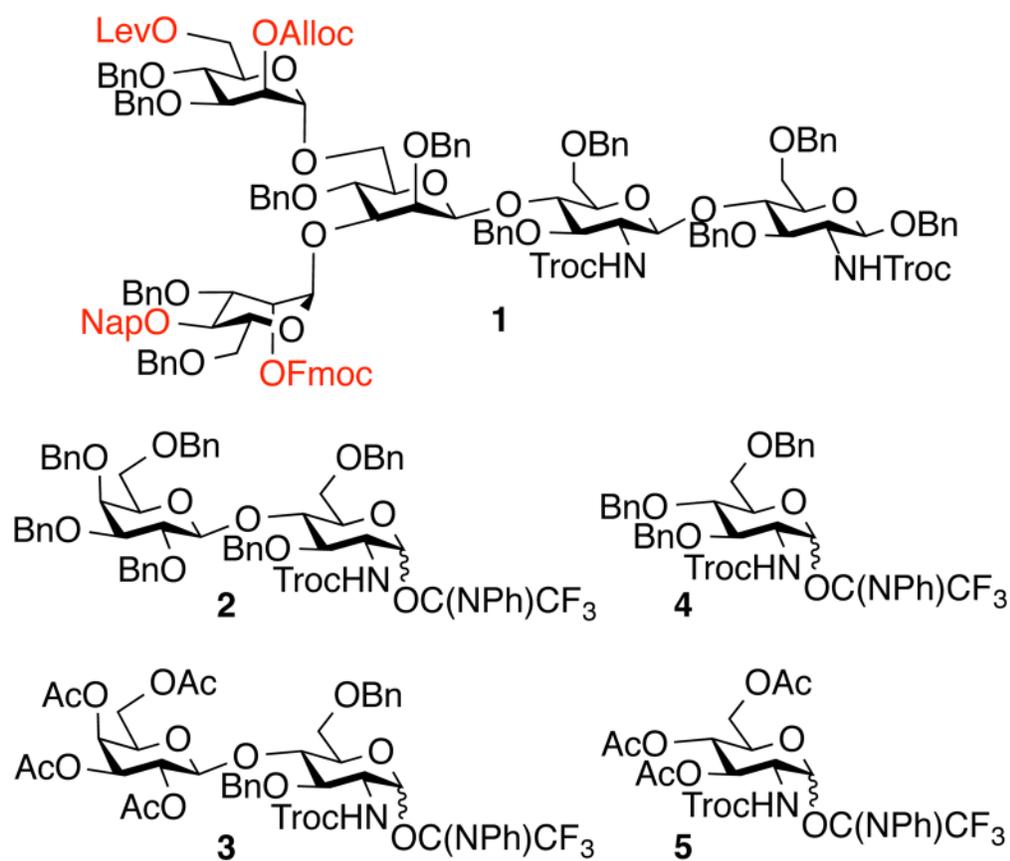


Fig. 1. Orthogonally protected core pentasaccharide **1** and glycosyl donors **2-5** for extensions in a parallel combinatorial manner to give oligosaccharide precursors to enzyme substrates.

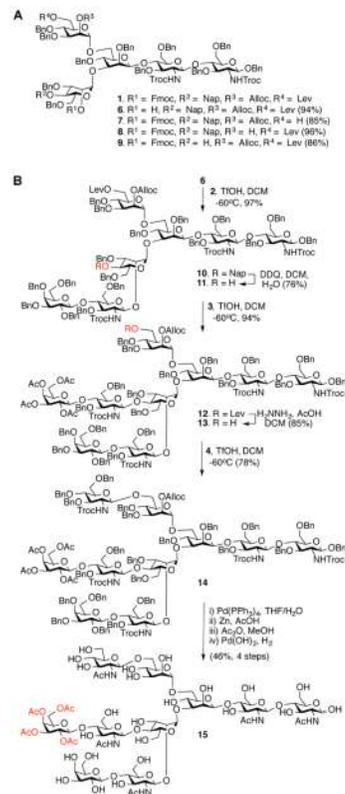


Fig. 2. Chemical synthesis of deca-saccharide **15** for branch-specific enzymatic extensions. **(A)** Selective removal of temporary protecting groups. **(B)** Preparation of glycan precursor for enzymatic extension.

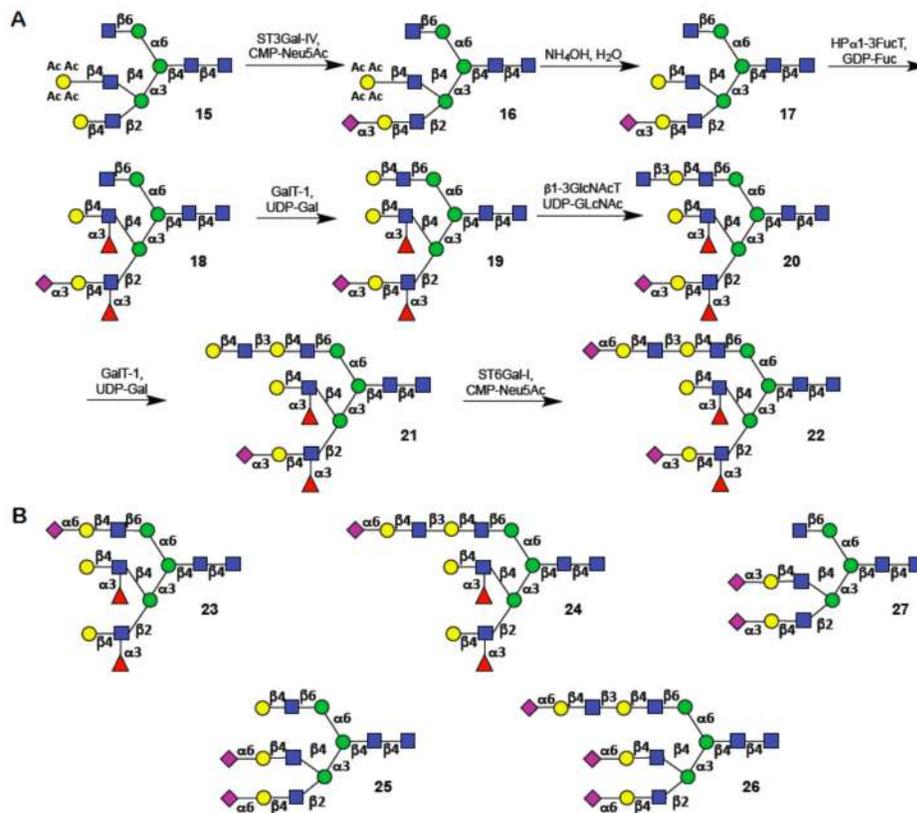


Fig. 3.
 Chemoenzymatic synthesis of **(A)** asymmetrically substituted multi-antennary glycan **22**.
(B) Structures of compounds (**23-26**) prepared by the chemoenzymatic approach. *N*-acetyl neuraminic acid (Neu5Ac, \blacklozenge); D-galactose (Gal, \bullet); *N*-acetyl-D-glucosamine (GlcNAc, \blacksquare); D-mannose (Man, \bullet); L-fucose (Fuc, \blacktriangle).

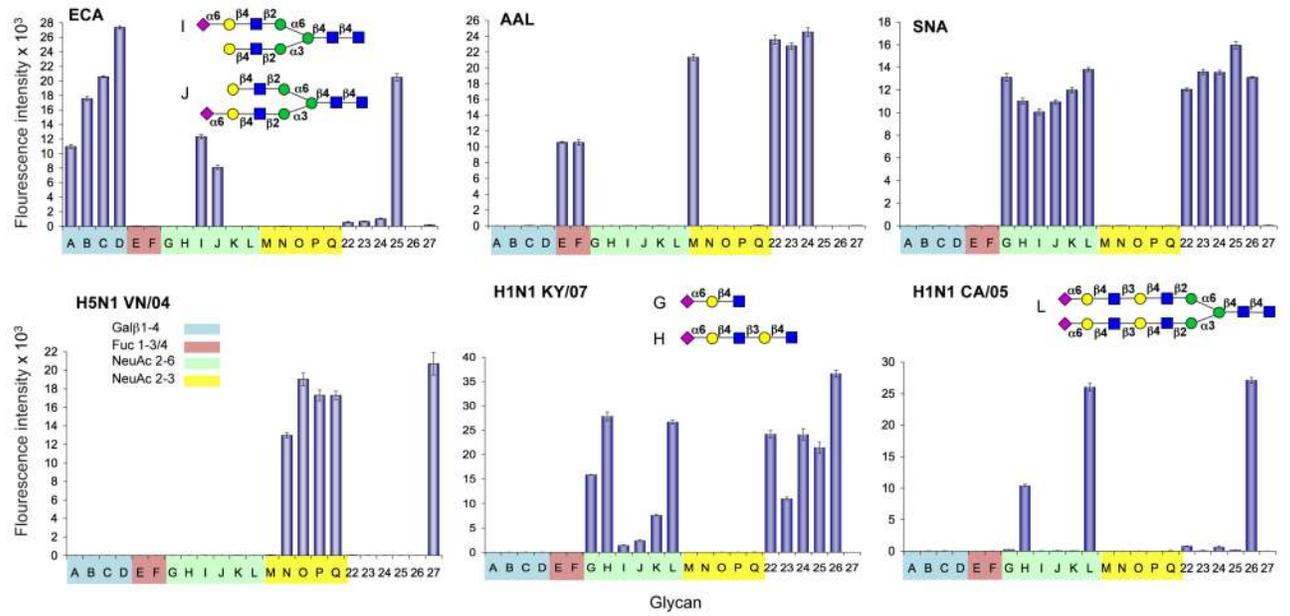


Fig. 4. Glycan microarray binding analyses. Fluorescently labeled lectins (ECA, AAL, and SNA), and recombinant avian (VN/04) and human influenza A (KY07 and CA/05) HA were assessed for binding to the array. Shown is the mean signal and standard error calculated for six independent replicates on the array. Structures of each of the lettered glycans are found in Table S14.