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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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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 $Glc_3Man_9GlcNAc_2$ 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 $GlcNAcMan_3GlcNAc_2$ 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 (βGal(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 $Pd(PPh_3)_4$ 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 decasaccharide **14**. Partial deprotection of **14** to give target compound **15** was accomplished by cleavage of the Alloc carbonate with $Pd(PPh_3)_4$ followed by removal of the 2,2,2-trichloroethyoxycarbamate (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 α(2,3)- or α(2,6)-linked to *N*-acetyllactosamine 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 heptadecasacharide **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 α(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 α2,3-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-α3 arm which is expected to be available for enzymatic transformations. Indeed, fucosylation of **17** with α1,3-fucosyltransferase (α3FucT) (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 β1,4-galactosyltransferase (GalT-1), uridine 5′-diphosphogalactose (UDP-Gal), and CIAP to give **19**. Treatment of **19** with β1,3-*N*-acetylglucosaminyltransferase (β1,3GlcNAcT) (30), UDP-GlcNAc, and CIAP resulted in a selective addition of a β(1,3)-linked GlcNAc moiety to the LacNAc moiety of the β1-6 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 β1,6-branch was further extended by GalT-1 and α2,6-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 de-acetylation and bis-fucosylation of **15** to give Le^x moieties at the β2 and β4 arm was followed by galactosylation to form a LacNAc moiety at the β6 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-α (2-3) (to give **27**) or bis-α (2-6)-sialylation followed by extension of the β6 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 α(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 crystagalli* 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**). *Sambuccus nigra* agglutinin (SNA) specific for terminal α(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 Neu5Acα(2-6)Gal and Neu5Acα(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 Neu5Acα(2-3)Gal consistent with the consensus receptor specificity of avian viruses (33, 38). Notably, this cloned HA did not recognize the Neu5Acα(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 Neu5Acα(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 Neu5Acα(2–6)Gal moiety on only one branch of a biantennary glycan were bound weakly (**I**, **J**), while those that had the Neu5Acα (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 Neu5Acα(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 Neu5Acα(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 μg/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.

Acknowledgments

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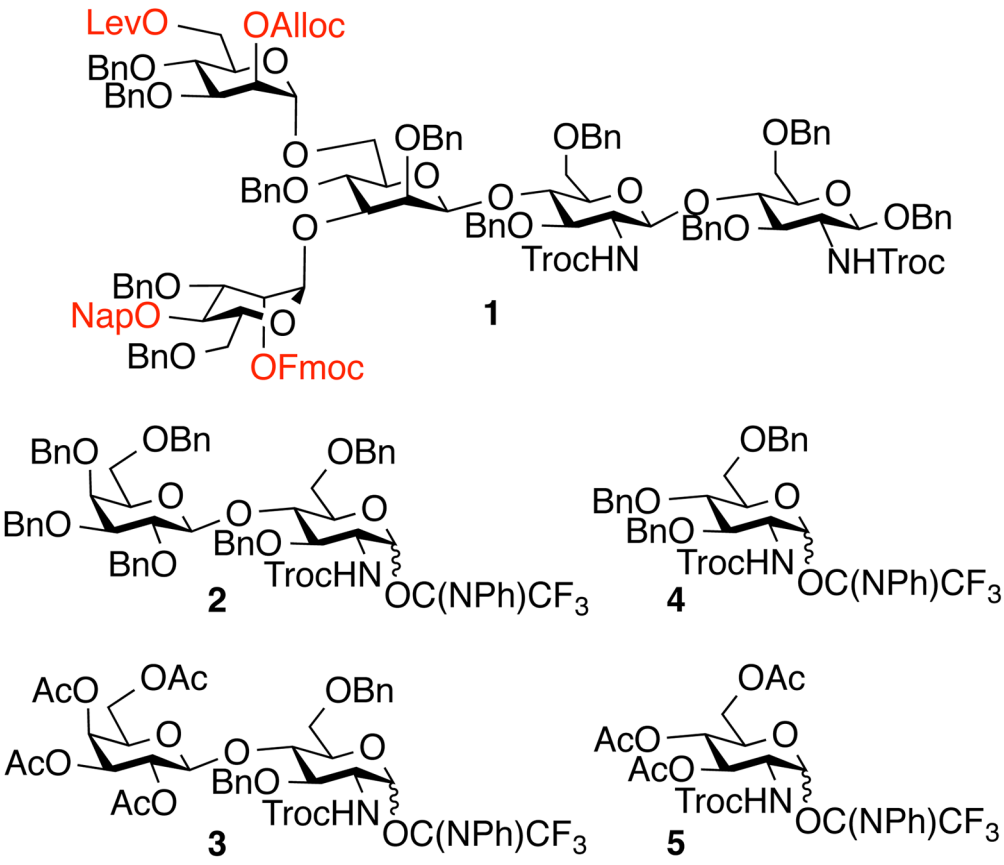

Fig. 1.

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

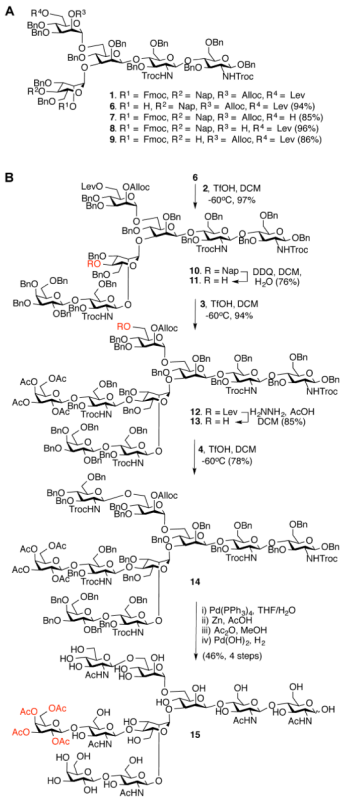

Fig. 2.

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

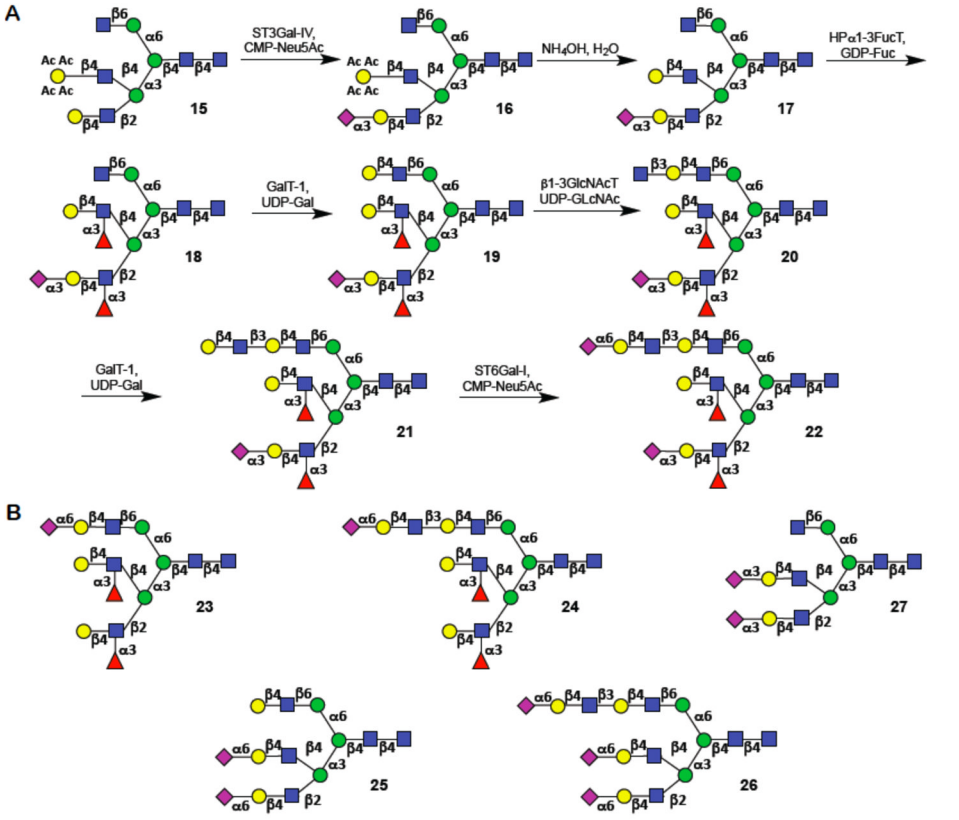

Fig. 3.

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, ◆); D-galactose (Gal, ●); *N*-acetyl-D-glucosamine (GlcNAc, ■); D-mannose (Man, ●); L-fucose (Fuc, ▲).

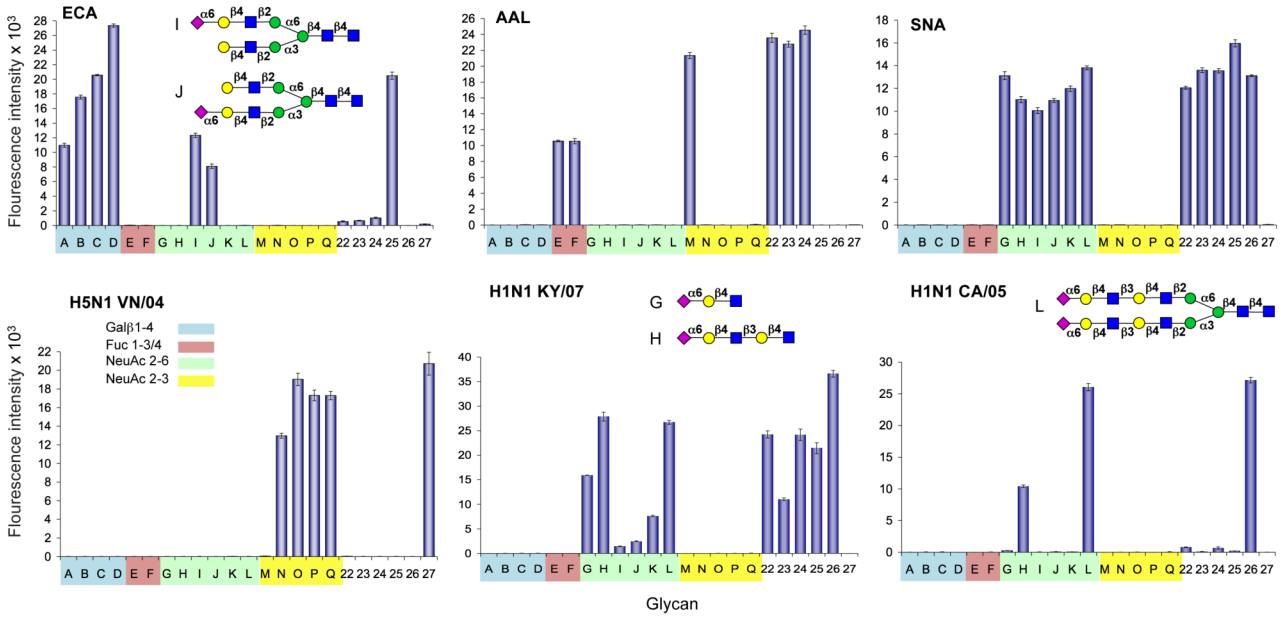

Fig. 4.

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.