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Recognition of sialylated poly-LacNAc on *N*- and *O*-linked glycans by human and avian influenza A virus hemagglutinins**

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The initial stages of influenza A virus infection are mediated by the binding of the viral hemagglutinin (HA) to sialylated glycan receptors on host epithelial cells.^[1] The specificity of the HA is believed a key determinant of viral host range.^[2] While all 16 influenza HA subtypes are found in avian viruses, only three are found in viruses adapted to humans (H1, H2 and H3), each resulting in a major pandemic. HAs from avian and human viruses are characterized by their preference for α 2–3 and α 2–6 linked sialic acids, respectively. Studies now suggest that other elements of sialoglycan sequence are also important factors of HA specificity that contribute to the species barrier.^[3] Recently, human and swine respiratory epithelial cells were shown to express sialylated *N*-glycans with extended poly-*N*-acetylglucosamine (poly-LacNAc) chains.^[4] Poly-LacNAc chains are Gal β 1–4GlcNAc β 1–3 tandem repeats that extend *N*- and *O*-linked glycans of glycoproteins and contribute to the biology mediated by glycan binding proteins.^[5] Sasisekharan and coworkers have suggested that human HAs bind preferentially to extended α 2–6 sialosides and may be critically important for viral adaptation to humans.^[4a, 6]

Studies on the preference of influenza HAs for extended glycans have employed synthetic sialosides that are linear terminal fragments of natural *N*- and *O*-linked glycans, which differ in their core structure and are often branched.^[4a, 7] To more fully address the influence of poly-LacNAc chains on HA specificity in the context of natural glycans, we have synthesized a series of sialylated poly-LacNAc structures on intact *O*- (**4–9**, **16–21**) and *N*-linked glycan (**10–12**, **22–24**) cores (Figure 1). These sialosides were incorporated into a custom glycan microarray alongside the linear terminal fragments (**1–3**, **13–15**) for analysis of specificities of human and avian influenza HAs.

Several groups have reported chemical and chemo-enzymatic syntheses of poly-LacNAc structures.^[8] For synthesis of extended natural *N*- and *O*-linked glycans, our strategy relied on enzymatic elaboration of advanced core structures. The α 2–3 and α 2–6 sialoside targets comprised *O*-linked (Cores 2–4) and *N*-linked cores with up to two and three LacNAc repeats, respectively. Representative syntheses for *N*- (**11** and **23**) and Core-2 *O*-linked glycans (**5** and **17**) are described in Scheme 1. Key LacNAc extensions were attained by alternating reactions using recombinant *Helicobacter pylori* β 1–3-*N*-acetylglucosaminyltransferase (β 1–3GlcNAcT)^[8b] and the bacterial β 1–4-galactosyltransferase/UDP-4'-Gal-epimerase fusion protein (GalT-GalE).^[9] Reaction of *N*-glycan **25** with UDP-GlcNAc (4 eq.) using β 1–3GlcNAcT followed by treatment with UDP-Glc (4 eq.) and GalT-GalE allowed efficient construction of LacNAc on both antennae affording **27** (Scheme 1a). Divergent sialylation of **27** using rat α 2–3-sialyltransferase (rST3Gal-III) or human α 2–6-sialyltransferase (hST6Gal-I), with CMP-Neu5Ac gave the desired α 2–3 **11** and α 2–6 **23** products, respectively. The synthesis of *O*-linked cores 3–4 and the tri-LacNAc *N*-linked glycans were conducted following similar conditions (Schemes S1–S6 in the Supporting information).

Core-2 *O*-linked glycans are commonly extended with poly-LacNAc off the β 1–6 branch. Initial galactosylation of **28** added Gal β 1–4 to GlcNAc giving **29** (Scheme 1b). As both branches of **29** present terminal Gal, two sites were potentially reactive for GlcNAc addition. Regioselective reaction on the β 1–6 branch was anticipated as β 1–3GlcNAcT demonstrates higher selectivity for Gal β 1–4GlcNAc substrates. Thus, under controlled conditions using UDP-GlcNAc (2 eq.), selective elongation of the β 1–6 branch was achieved to afford **30**.^[10] NMR and MS analysis confirmed addition of a single GlcNAc

unit. The asialo di-LacNAc structure **31** was prepared by reaction of **30** with UDP-Glc catalyzed by GalT-GalE. Finally, selective sialylation of **31** was performed with either rST3Gal-III or hST6Gal-I and CMP-Neu5Ac (2 eq). Both sialyltransferases show preference for Gal β 1-4GlcNAc substrates and gave **5** and **17**, respectively. The mono-sialylated products were confirmed by NMR and MS analysis.

The 24 glycans in the sialoside library (Figure 1) contain either the terminal Neu5Ac α 2-3Gal (**1–12**) or Neu5Ac α 2-6Gal (**13–24**) sequence. A glycan microarray was constructed from this library to study the binding properties of influenza A virus HA.^[7a, 11] The aglycone of each sialoside was equipped with a free amine for direct printing on *N*-hydroxy succinimide-activated slides (Figure S1 in the Supporting information). Recombinant HAs from selected avian and human influenza A viruses were then screened to assess the effects on HA binding of both length and presentation of sialylated poly-LacNAc.

As expected, the avian HAs preferentially recognized α 2-3-linked sialosides (Figure 2 and Figure S2 in the Supporting information). However, while H4 (A/duck/Czech/1/56) bound strongly to nearly all α 2-3 structures, other avian HAs showed more selective binding patterns. For instance, H3 (A/duck/Ukr/1/63), a progenitor of the 1968 Hong Kong pandemic,^[12] only bound the linear glycans (**2, 3**), and the *O*- (**8**) and *N*-linked (**10**) glycans. Remarkably, all avian HAs, including H5 (A/Vietnam/1203/04), a highly pathogenic human isolate of the bird flu,^[13] showed strong preference for short *N*-linked structures, binding strongly to **10**, and reduced or no binding to the longer glycans (**11, 12**).

Although human HAs demonstrated classic preference for α 2-6-sialosides, they exhibited varied fine specificity for the extended *N*- and *O*-linked glycans (Figure 2 and Figure S2 in the Supporting Information). As previously reported, the human HAs bound best to the linear sialosides with di- and tri-LacNAc extensions (**13–15**).^[4] Significantly, however, the same sequences were not uniformly recognized when presented on *N*- and *O*-linked glycan cores. For instance, while the H1 (A/SC/1/18) and the H2 (A/Japan/305/57) HAs bound strongly to the linear sialoside with the di-LacNAc extension (**14**), they bound poorly to the same sequence presented on Core 3 (**19**) and Core 4 (**21**) glycans. Surprisingly, these same two HAs exhibited strong binding to *N*-glycans with the di-LacNAc sequence (**23**), but dramatically reduced binding to the same sequence with the tri-LacNAc repeat (**24**).

In summary, we have synthesized a panel of novel glycans containing sialylated poly-LacNAc on intact *N*- and *O*-linked glycan cores as candidates of the natural glycan receptors of influenza viruses. While all avian and human virus HAs retained their basic α 2-3 and α 2-6 linkage specificity, respectively, the *N*- and *O*-linked glycan cores differentially impacted the ability of individual HAs to recognize the sialic acid as a receptor. The lack of a consistent recognition pattern for human HAs suggests that the fine specificity of the virus for receptor(s) may drift under antigenic selective pressure, while retaining the ability to bind to a subset of α 2-6-sialosides sufficient to mediate infection and transmission. It should also be noted that the branched *N*-linked and Core 4 *O*-linked glycans produced with our synthetic strategy are symmetric di-sialylated glycans. However, glycans extended on a single branch also occur in nature.^[4a, 4b] Thus, it will also be of interest to investigate the role of asymmetric glycans on influenza receptor biology.

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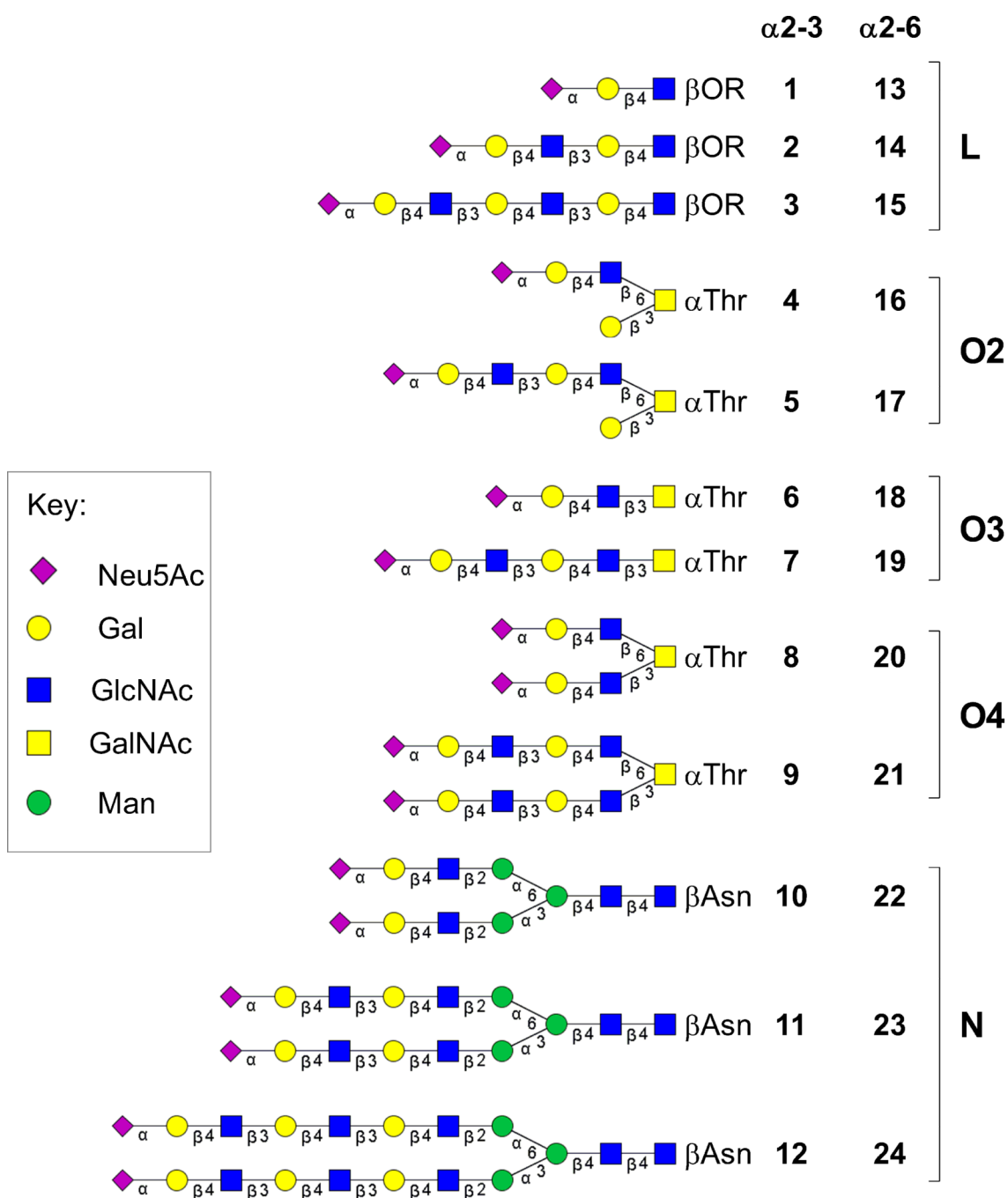


Figure 1. Structures of sialylated poly-LacNAc linear (L) fragments (1–3, 13–15) and the same sequences elaborated on O-linked (O2, O3, O4) (4–9, 16–21) and N-linked (N) glycan cores (10–12, 22–24).

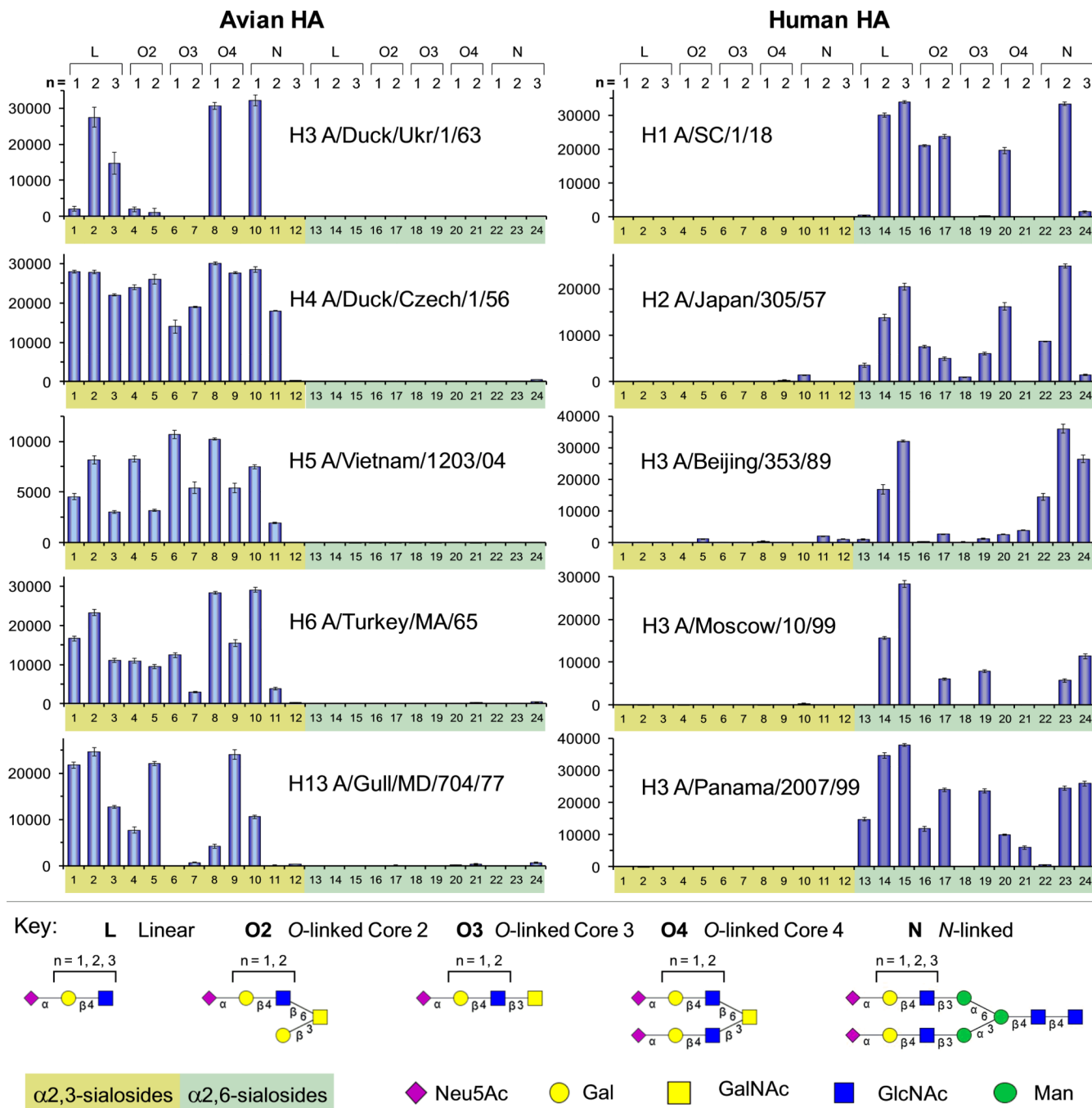
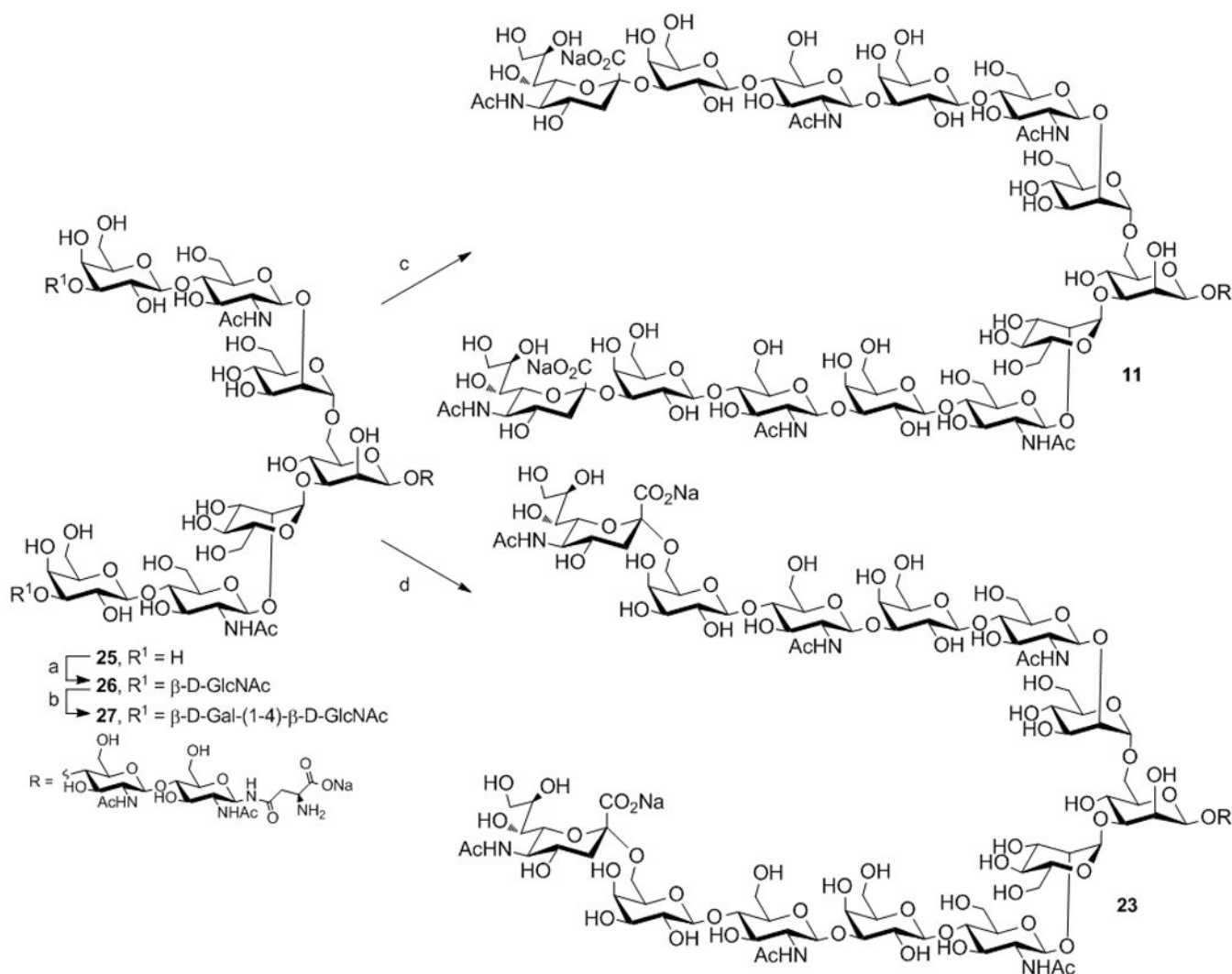
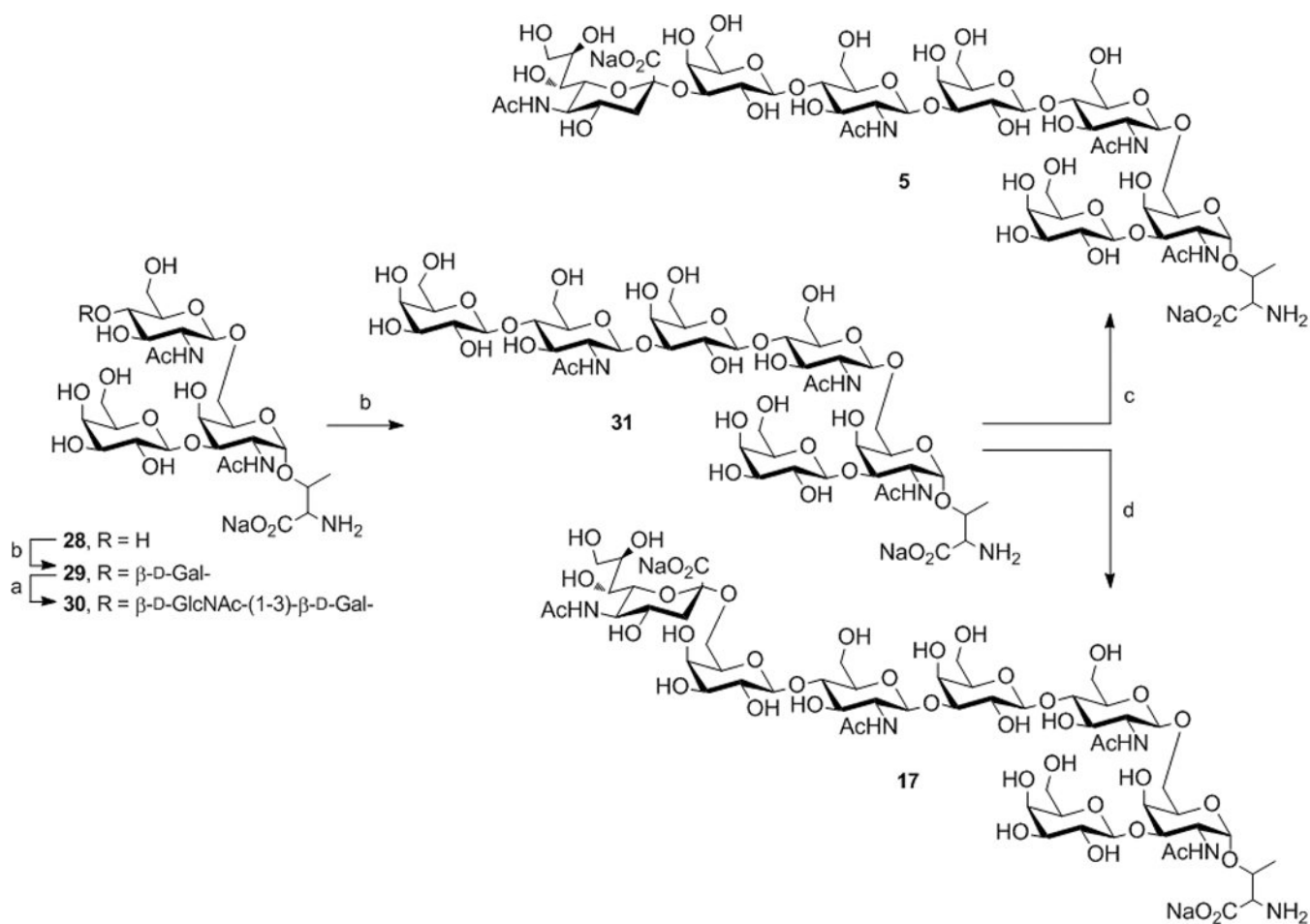


Figure 2. Glycan microarray binding analyses as measured by fluorescence intensity for avian and human influenza A recombinant hemagglutinins. All HAs were evaluated at 15 μ g/ml except for A/SC and A/Beijing, which were evaluated at 150 μ g/ml. See additional details in Supporting information.



**Scheme 1.**

a. Enzymatic transformations of **25** to **11** and **23** a) β 1-3GlcNAcT, UDP-GlcNAc; b) GalT-GalE, UDP-Glc; c) rST3Gal-III, CMP-Neu5Ac; d) hST6Gal-I, CMP-Neu5Ac.

b. Enzymatic transformation of **28** to **5** and **17**. See Scheme 1a for conditions.