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Synthesis of Biologically Active *N*- and *O*-Linked Glycans with Multisialylated Poly-*N*-acetyllactosamine Extensions Using *P. damsela* α2-6 Sialyltransferase

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Supporting Information

ABSTRACT: Sialosides on *N*- and *O*-linked glycoproteins play a fundamental role in many biological processes, and synthetic glycan probes have proven to be valuable tools for elucidating these functions. Though sialic acids are typically found α2-3- or α2-6-linked to a terminal nonreducing end galactose, poly-LacNAc extended core-3 *O*-linked glycans isolated from rat salivary glands and human colonic mucins have been reported to contain multiple internal Neu5Acα2-6Gal epitopes. Here, we have developed an efficient approach for the synthesis of a library of *N*- and *O*-linked glycans with multisialylated poly-LacNAc extensions, including naturally occurring multisialylated core-3 *O*-linked glycans. We have found that a recombinant α2-6 sialyltransferase from *Photobacterium damsela* (Pd2,6ST) exhibits unique regioselectivity and is able to sialylate internal galactose residues in poly-LacNAc extended glycans which was confirmed by MS/MS analysis. Using a glycan microarray displaying this library, we found that Neu5Acα2-6Gal specific influenza virus hemagglutinins, siglecs, and plant lectins are largely unaffected by adjacent internal sialylation, and in several cases the internal sialic acids are recognized as ligands. Polyclonal IgY antibodies specific for internal sialoside epitopes were elicited in inoculated chickens.

Sialosides on *N*- and *O*-linked glycoproteins play many important roles in biological recognition.[1] These glycans present sialic acids as terminal substituents that are ideally positioned to interact with glycan binding proteins,[2] including the siglecs (sialic acid-binding immunoglobulin-type lectins),[3,4] and viral hemagglutinins.[5] The sialic acids on *N*-acetyllactosamine (LacNAc, Galβ1-4GlcNAc) chains of glycoproteins are typically linked α2-3 or α2-6 to a terminal, nonreducing end galactose.[6] However, poly-LacNAc extended core-3 *O*-linked glycans isolated from rat salivary glands have been reported to contain multiple internal Neu5Acα2-6Gal groups (Figure 1A).[7] Similar multi α2-6-sialylated core-3 *O*-linked glycans have been reported on human colonic mucins.[8,9] Although internally sialylated glycans are found in certain gangliosides[10] and milk oligosaccharides,[11] these remain the only reports describing the Neu5Acα2-6Gal epitope on internal Gal residues of poly-LacNAc chains. The extent of expression and biological function of this novel glycan sequence has not been explored due to the lack of specific chemical and biological probes. Here we report the use of a recombinant α2-6 sialyltransferase from *Photobacterium damsela* (Pd2,6ST) to synthesize a library of structures including *N*- and *O*-linked glycans having poly-LacNAc chains with multiple Neu5Acα-2,6-Gal epitopes (**1**−**26**, Figure 1B). The binding specificity of a panel of glycan binding proteins (lectins) and polyclonal IgY antibodies for these multisialylated glycans has been evaluated using a custom glycan microarray.

Pd2,6ST[12,13] has been used previously to synthesize sialosides on glycans with terminal Gal residues and was shown to react with fucosylated and α2-3-sialylated substrates.[14−16] In recent studies, we used recombinant Pd2,6ST to sialylate poly-LacNAc extended glycans, and surprisingly, both ^{1}H NMR and MS analyses suggested that multiple sialic acids were being transferred instead of one onto the terminal Gal as expected. Subsequent MS/MS fragmentation showed that multisialylated glycans were formed by sialylation of the terminal and internal galactose positions along the poly-LacNAc backbone, suggesting a simple enzymatic strategy for synthesis of multisialylated poly-LacNAc chains with sialic acid on both terminal and internal Gal units.

We first examined the reactivity of Pd2,6ST on LacNAc oligomers of varying length and Gal composition (Scheme 1). Substrates **27**−**30** were prepared as previously described.[17] Reaction of **27**, which contains a single internal Gal residue, with Pd2,6ST readily gave monosialylated **31** in 87% yield. Treatment of **28** and **29** with Pd2,6ST gave glycans **32** and **33**, respectively. NMR and MS analysis confirmed the addition of two Neu5Ac residues in both. Finally, sialylation of tri-LacNAc **30** using Pd2,6ST and excess CMP-Neu5Ac gave **34** which contains three Neu5Ac residues. Sialosides **31**−**34** were subjected to hydrogenation conditions to give **1**, **3**−**5** respectively. In contrast, recombinant human α2-6-sialyltransferase (hST6Gal-I),[18] an enzyme that catalyzes the α2-6-sialylation of terminal Gal residues, gave no reaction on the internal Gal of **27**. However, we found that disialo **32** could also be prepared by galactosylation of internal sialylated **31** using β1-4-galactosyltransferase/UDP-4′-Gal-epimerase fusion protein (β4GalT-GalE),[18] followed by

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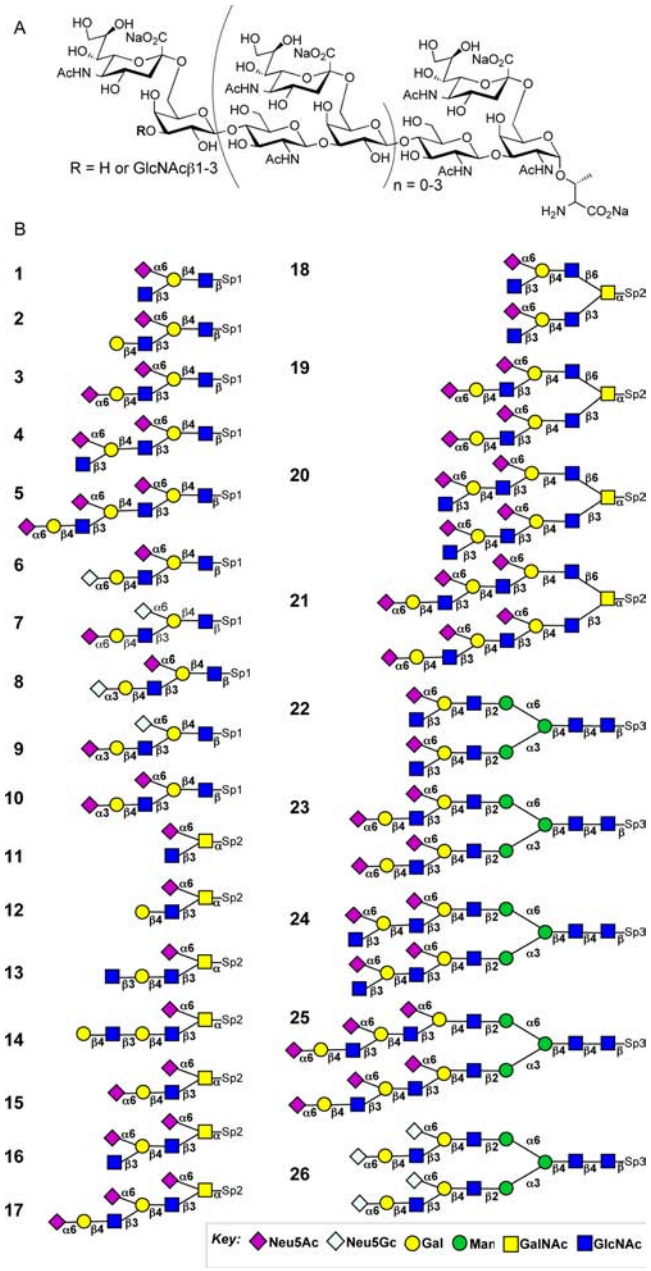

Figure 1. (A) Poly-LacNAc extended Core-3 *O*-linked glycans with multiple Neu5Acα2-6Gal groups isolated from rat salivary[7] and human colonic[8,9] mucins. (B) Synthetic sialoside targets (**1**–**26**), including Core-3 *O*-linked structures (**15**–**17**) isolated from colonic mucins. Sp1 = *O*-ethyl amine, Sp2 = *O*-threonine, and Sp3 = *N*-(KVA)NKT.

sialylation of the terminal Gal using hST6Gal-I (Scheme S1 in Supporting Information [SI]). Thus, the ability of hST6Gal-I to sialylate the glycans having internal sialic acids suggests a plausible biosynthetic mechanism where the multisialylated poly-LacNAc extensions are formed by iterative terminal sialylation by ST6-Gal I followed by LacNAc chain extension. We next explored Pd2,6ST catalyzed sialylations of poly-LacNAc on various *N*- and *O*-linked core structures (Scheme 1). Core-3 *O*-linked glycans **12**–**14** with sialic acid on the GalNAc residue were prepared from **36** using recombinant chicken ST6GalNAc-I (Scheme S2 in SI).[19] Treatment of **12**–**14** with Pd2,6ST using excess CMP-Neu5Ac readily gave the multisialylated glycans **15**–**17**. The addition of the Neu5Ac residues was confirmed by

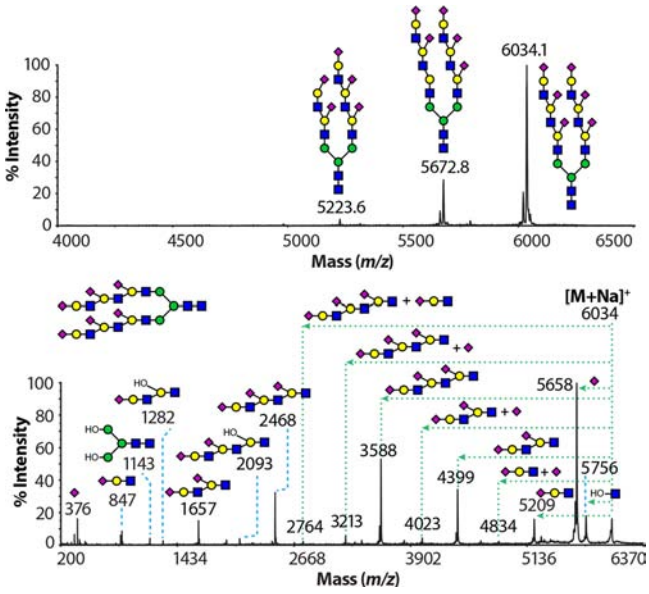

Figure 2. Mass spectrometric analysis of *N*-linked glycan **25**. (*top*) MS analysis of per-methylated product mixture; (*bottom*) MS/MS fragmentation analysis of $[M + Na]^+$ peak *m*/*z* 6034.

integration of the Neu5Ac H-3ax and H-3eq methylene protons in the ^{1}H NMR and was supported by MS analysis. This report represents the first synthesis of the naturally occurring glycans **15**–**17**.

Also, we sialylated a panel of poly-LacNAc extended biantennary core-4 *O*-linked (**37**–**40**) and *N*-linked glycans (**41**–**44**) (Scheme 1). The substrates **37**–**44** were prepared as previously described.[20] Remarkably, reaction using Pd2,6ST and excess CMP-Neu5Ac (or CMP-Neu5Gc for **26**) gave the multisialylated products **18**–**26**. MS analysis of the per-methylated **19**, **20**, and **25** showed the multisialylation products with minor amounts of under-sialylated products (Figures 2 and S3–S5 in SI). MS/MS fragmentation revealed that in addition to the terminal Gal residue of poly-LacNAc, including extended *N*- and *O*-linked glycans, Pd2,6ST can also sialylate internal Gal in α2,6-linkage, and that adjacent LacNAc units are concurrently modified. Typical yields for the Pd2,6ST-catalyzed sialylations ranged between 80 and 95%.

Exploiting the unique regioselectivity of Pd2,6ST, we were also able to synthesize di-LacNAc structures with varied sialylation patterns between the terminal and internal Gal positions (Scheme S3 in SI). The terminal Gal residue of **28** was first capped with either an α2-6 (**45**, **46**) or α2-3-linked (**47**, **48**) sialic acid by reaction using hST6Gal-I or *Pasteurella multocida* α2-3-sialyltransferase 1 (PmST1)[21] respectively. Next, the internal Gal positions of each were α2-6-sialylated by treatment with Pd2,6ST to give **49**–**53**, following the hydrogenation conditions to give **6**–**10** with ethyl amine linkers. MS analysis of the products confirmed that a second sialic acid was added which matched the integrations in the ^{1}HNMR.

To assess the potential of these novel compounds (**1**–**26**) to serve as ligands for glycan binding proteins known to mediate cell surface biology, a custom glycan microarray was constructed from this sialoside library and screened against several sialoside binding proteins (Figure 3A–E). Glycans with a terminal α2-3- or α2-6-linked sialic acid or no sialic acid were included as controls (**A**–**M**, Figure 3A–E). Controls **L** and **M** were only included in the screen against Siglec-F and polyclonal IgY

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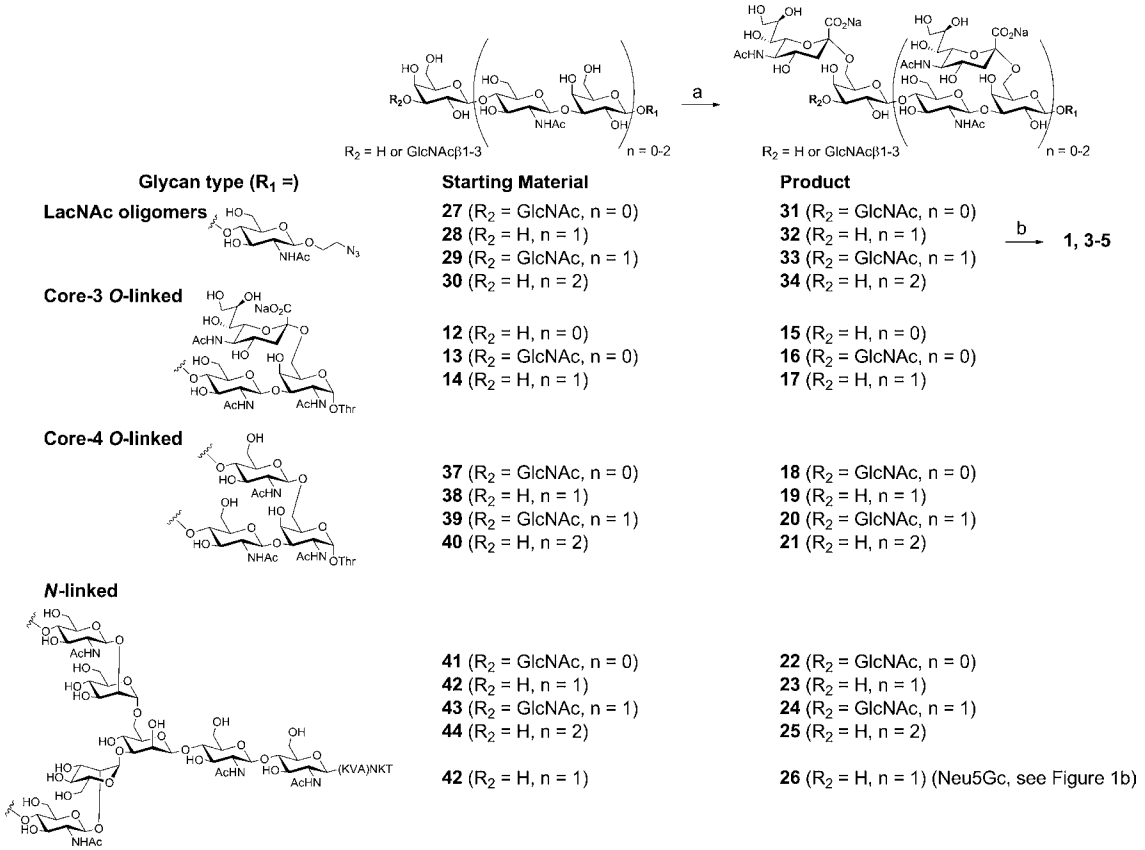
Scheme 1. Enzymatic transformations: Pd2,6ST-Catalyzed Sialylation of Poly-LacNAc Extended Glycans[a]



[a]Reagents and conditions: (a) Pd2,6ST, CMP-Neu5Ac (CMP-Neu5Gc for preparing **26**); (b) H_2, Pd/C, H_2O. CMP = cytidine monophosphate, Neu5Ac = *N*-acetyl neuraminic acid; Neu5Gc = *N*-glycolyl neuraminic acid.

(Figure 3C, E). The array was constructed by direct printing on glass slides activated with *N*-hydroxysuccinimide to react with the free amino group on the aglycone of each glycan to form a covalent amide bond.

Sambucus nigra agglutinin (SNA),[22] which is known to bind terminal α2-6-linked sialosides (e.g., control glycans **B**-**H**), bound all synthetic glycans with terminal α2-6-linked sialic acid (**3**, **5**−**7**, **15**, **17**, **19**, **21**, **23**, **25**, and **26**), and surprisingly also bound two glycans, **16** and **18,** which contained only internal sialic acid (Figure 3A). Several members of the sialic acid binding siglec receptor family were also examined.[3] The B cell siglec CD22 (Siglec-2) is known to be specific for glycans with a terminal Siaα2-6Galβ1-4GlcNAc sequence, exhibiting equal affinity for glycans with the sialic acids Neu5Ac or Neu5Gc.[23] Multisialylated glycans that bound strongly included the linear fragments (**3**, **5**−**7**), biantennary *N*- and *O*-linked glycan structures (**19**, **21**, **23**, **25**, and **26**) and two glycans with internal sialic acid only (**20** and **24**) (Figure 3B). The eosinophil siglec, murine Siglec-F, as expected showed strong preference for the control glycans **L** and **M,** in keeping with its known specificity for these sulfated α2−3-sialylated structures (Figure 3C).[24] However, Siglec-F also bound strongly to many of the novel multisialylated glycans (**4**, **5**, **10**, **18**−**25**), including several with internal sialic acids only (**4**, **18**, **20**, **22**, and **24**). Notably, the switch from Neu5Ac to Neu5Gc largely abrogates binding to Siglec-F. A recombinant hemagglutinin (HA) from a seasonal human influenza virus (H1 A/KY/07)[25] was also examined (Figure 3D). The HA which is known to bind glycans with the terminal sequence Neu5Acα2-6Gal (**C**, **D**, and **F**) bound exclusively to glycans with this terminal sequence (**3**, **5**, **7**, **17**, **19**, **21**, **23**, and **25**) and did not bind to glycans with only internal sialic acids. Lack of binding to glycans with no terminal sialic acids helps document the fidelity of the enzymatic synthesis during repetitive syntheses of LacNAc repeats.

Last, we used the glycan array to screen polyclonal IgY antibodies isolated from eggs of chickens inoculated with glycan **4** displayed on a viral scaffold (see SI, Figure 3E). The chicken polyclonal IgY recognized glycans with internal sialic acid (**1**, **4**, **16**, **18**, **20**, **22**, and **24**) and showed no binding to the α2-6-sialylated control glycans with only terminal α2-6 sialic acids (**B**−**H**). This shows that the chicken immune system can recognize the internal sialic acids distinctly from the terminal sialic acids, suggesting that such antibodies could be used as immune probes to aid in decoding the functions of this class of glycans.

In summary, we report that recombinant *P. damsela* α2-6-sialyltransferase is able to transfer sialic acid to internal galactose residues in poly-LacNAc backbones, forming multisialylated glycans. This unique specificity has allowed us to synthesize a library of *N*- and *O*-linked glycans with multisialylated poly-LacNAc extensions, including poly-LacNAc extended core-3 *O*-linked glycans previously identified in rat salivary and human colonic mucins. Screening a glycan microarray of this library has demonstrated that sialic acid specific binding proteins, including SNA and members of the siglec family of immune cell receptors can recognize the internal sialic acids as ligands. On the basis of our ability to generate IgY antibodies specific for glycans with internal sialic acids, we anticipate that these novel glycans will

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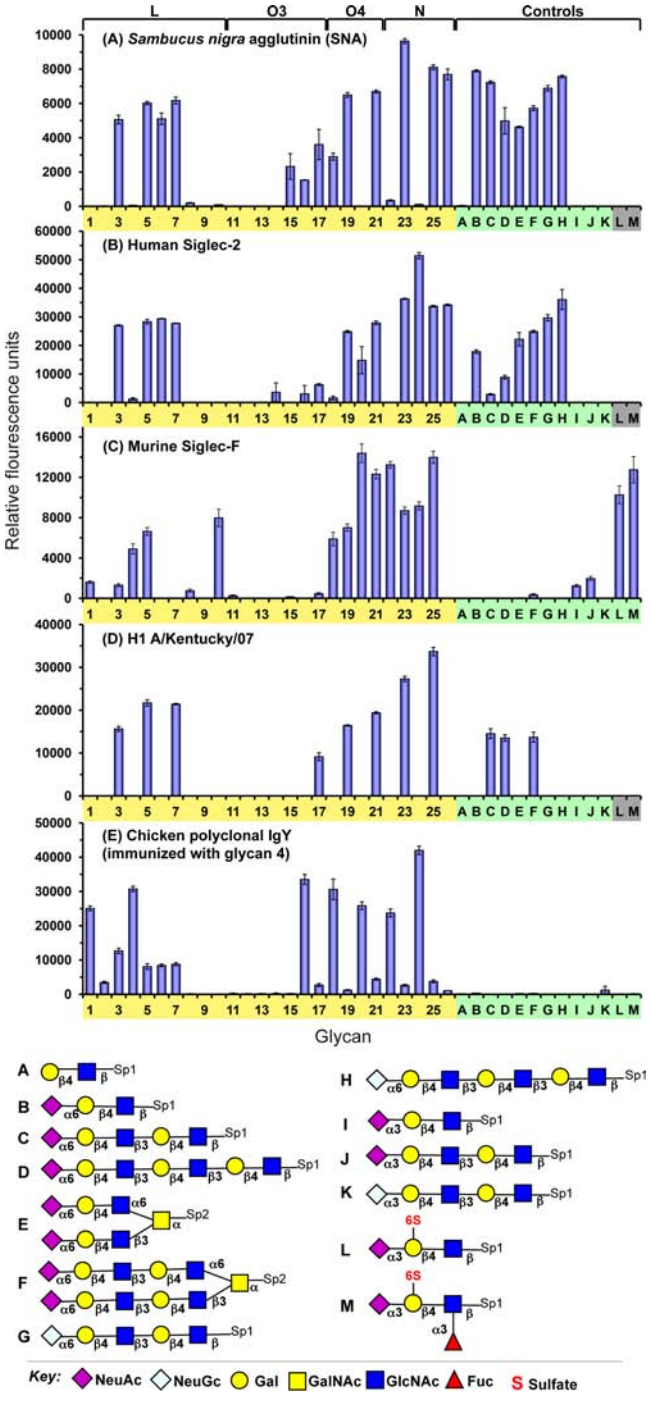

Figure 3. Glycan microarray binding analyses as measured by fluorescence intensity for (A) SNA, (B) recombinant human Siglec-2 Fc chimera, (C) murine Siglec-F Fc chimera, (D) influenza A viral hemagglutinin H1 (A/Kentucky/07), and (E) total chicken polyclonal IgY (elicited with glycan **4**). (*yellow*) Sialosides **1**–**26**; (*green/gray*) controls **A**–**M**. Controls **L** and **M** were only included in the screen against Siglec-F and polyclonal IgY. See SI.

facilitate investigations into the biological roles of these multisialylated poly-*N*-acetyl-lactosamine glycans.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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§C.M.N. and W.P. contributed equally.

Notes

The authors declare no competing financial interest.

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