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Article

# Enzyme-Sialylation-Controlled Chemical Sulfation of Glycan Epitopes for Decoding the Binding of Siglec Ligands

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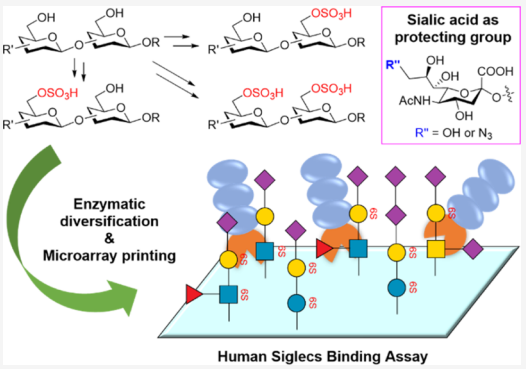
**ABSTRACT:** Widely distributed in nature, sulfated glycan epitopes play important roles in diverse pathophysiological processes. However, due to their structural complexity, the preparation of glycan epitopes with structurally defined sulfation patterns is challenging, which significantly hampers the detailed elucidation of their biological functions at the molecular level. Here, we introduce a strategy for site-specific chemical sulfation of glycan epitopes, leveraging enzymatic sialylation and desialylation processes to precisely control the regio-specificity of sulfation of disaccharide or trisaccharide glycan backbones. Using this method, a sulfated glycan library covering the most common sialylated glycan epitopes was prepared in high yield and efficiency. By screening a microarray prepared with this glycan library, we systematically probed their binding specificity with human Siglecs (sialic acid-binding immunoglobulin-type lectins), many of which function as glyco-immune checkpoints to suppress immune system activation. Our investigation revealed that sulfation and sialylation patterns serve as important determinants of Siglec binding affinity and specificity. Thus, these findings offer new insights for the development of research tools and potential therapeutic agents targeting glyco-immune checkpoints by modulating the Siglec signaling pathway.




## INTRODUCTION

Glycan sulfation is a ubiquitous and crucial post-translational modification that widely occurs in glycoproteins, glycolipids, and proteoglycans.[1,2] Sulfation imparts negative charges to the modified glycans, giving them specificity for interaction with numerous human and microbial glycan binding proteins to regulate diverse biological processes.[3−7] On the other hand, abnormal glycan sulfation has been linked to human diseases, such as cancers and osteoarthritis.[8−12] Sialic acid-binding immunoglobulin-type lectins (Siglecs) are a family of transmembrane receptors with restricted expression on immune cells that regulate the immune cell activities through the engagement of sialoglycans that are often sulfated.[13−15] In humans, 15 Siglec receptors have been identified.[16,17] According to their intracellular signaling domains, Siglec receptors can be classified into three subgroups, including nonsignaling receptors (Siglec-1 and -4), activating receptors (Siglec-14, -15, and -16), and inhibitory receptors (all other Siglecs).[17,18] In the immunological synapse, inhibitory Siglecs engage with sialylated glycan epitopes, initiating inhibitory signaling that suppresses immune cell activation in a manner similar to the interaction observed between PD-1 and PD-L1.[19,20] Thus, such Siglecs are designated as glyco-immune checkpoints. A growing body of evidence has shown that sulfation occurring on the primary hydroxyls of terminal monosaccharide residue, for example, Gal, GlcNAc, and GalNAc, of sialoglycans is involved in modulating the binding affinity of many Siglecs.[21−23] Sulfated glycan epitopes, which are defined as structures containing disaccharide or trisaccharide backbone, can be explored as ligands to deliver diagnostic or therapeutic agents to diverse immune cells that express specific Siglecs.[21,24−30] For these applications, there is an urgent need to comprehend the biological mechanisms underlying subtle differences in sulfation patterns governing the Siglec binding specificities. However, sulfated glycans are not readily accessible due to their structural heterogeneity and complexity.[1] Therefore, the detailed roles that sulfated glycans played in living cells are still largely unexplored.

Given the significance of sulfated glycan epitopes in both fundamental research and pharmaceutical chemistry, many chemical approaches have been explored for their preparation.[31−34] Total chemical synthesis of sulfated glycans needs complex protection−deprotection manipulations for the site-specific introduction of the sulfate group. Moreover, the poor solubility of sulfated glycans in organic solvents and the

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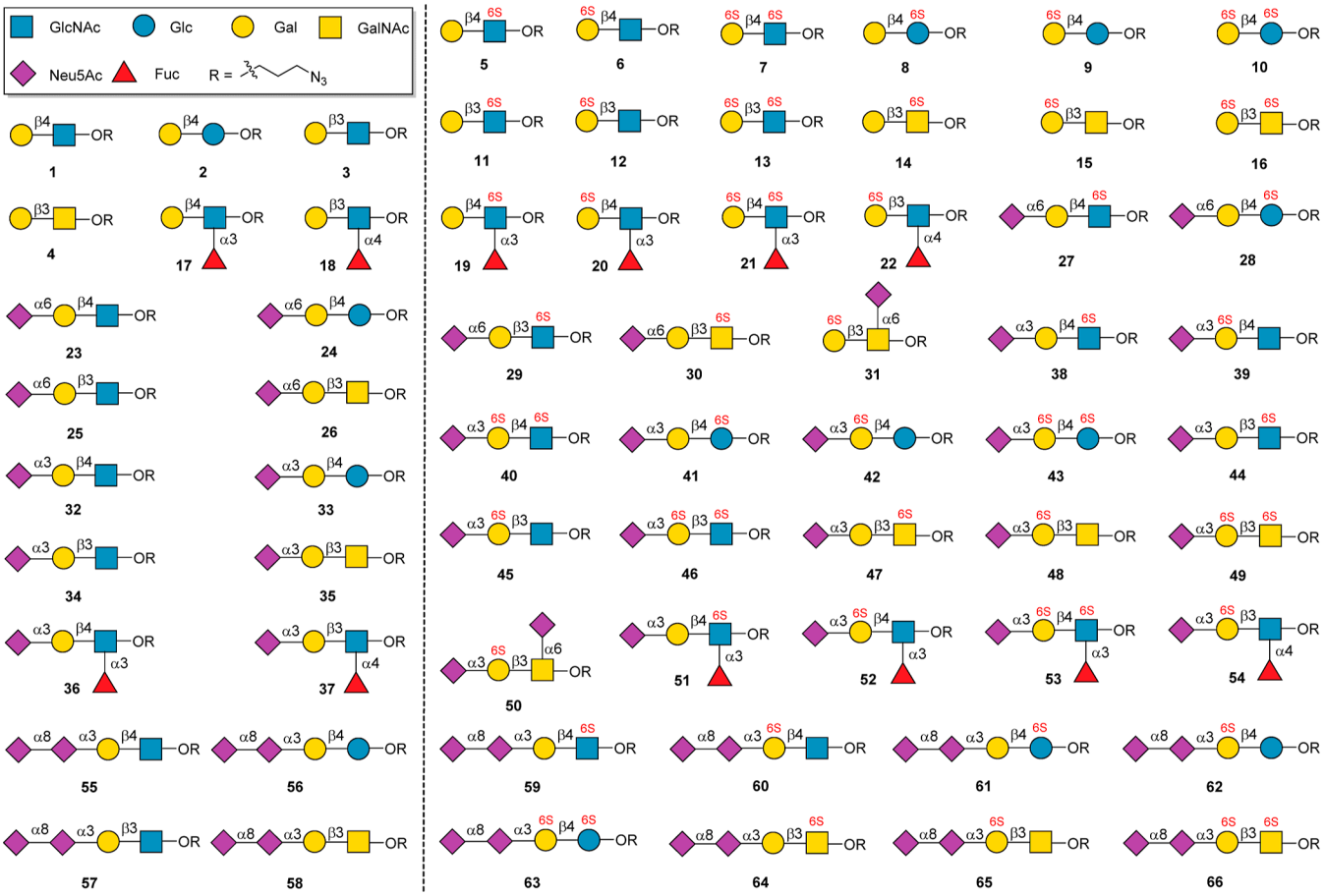


**Figure 1.** Structures of 66-membered glycan library.

instability of the sulfate group in acid conditions add extra difficulty to the already challenging synthesis of the sulfated glycan backbones.[35] Alternatively, Chen and co-workers reported a chemoenzymatic approach to prepare sulfated sialyl Lewis x (SLeX) glycan epitope.[36] By this method, the sulfated glycan backbones, which need to be prepared by total chemical synthesis, were modified by sialyltransferases to produce SLeX antigens. Similarly, Cao and co-workers prepared sulfated O-Mannose glycan epitopes.[37] Recently, Wang and co-workers synthesized sulfated antibody glycoforms (sulfated Gal$\beta$1,4GlcNAc backbone) using human GlcNAc-6-*O*-sulfotransferase and human $\beta$1,4 galactosyltransferase.[38] Later, Boons and co-workers synthesized complex sulfated keratan glycans and *N*-glycans (sulfated Gal$\beta$1,4GlcNAc backbone) by using the same enzymes.[39,40] Now, there is still a lack of a general method for the rapid construction of a number of structure-defined sulfated glycans containing other glycan backbones.

Here, we describe an enzyme-sialylation-controlled strategy for the site-specific chemical sulfation of glycan epitopes bearing disaccharide or trisaccharide backbones. Taking advantage of enzyme-catalyzed sialylation as a protecting group, which could protect the highly reactive C-6 or C-3 hydroxyl group (OH) of Gal residue, the sulfate group could be introduced into disaccharide or trisaccharide backbones specifically in mild chemical reaction conditions. The protective sialic acid residue can be removed later by neuraminidase, resulting in general glycan backbones containing mono- or disulfate groups. The further extension of these sulfated glycan backbones by several glycosyltransferases produced a 66-membered glycan library covering the most common sialylated glycan epitopes (Figure 1). The constructed well-defined glycan microarray was subsequently used to probe the binding specificities of human Siglecs, revealing many new findings about the specificity of Siglecs for sulfated glycans.

## RESULTS AND DISCUSSION

Although human glycan structures are complex, the backbones are much simple. Gal$\beta$1,4GlcNAc (Type II LacNAc), Gal$\beta$1,4Glc (Lactose), Gal$\beta$1,3GlcNAc (Type I LacNAc), and Gal$\beta$1,3GalNAc are the four basic backbones of *N*-glycans, *O*-glycans, and glycolipids. Glycan sulfation is mediated by sulfotransferases using 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as the sulfate donor in living cells. However, it is challenging to scale up the synthesis of PAPS in laboratories since PAPS is unstable in solution and commercial PAPS is prohibitively expensive for large-scale glycan sulfation. Moreover, human sulfotransferases need to be expressed by using an eukaryotic expression system, and many sulfotransferases suffer from low expression.[41] In addition, sulfotransferases are often associated with low activities, and a mass of enzymes need to be added in the reaction system.[39,40] For these reasons, we sought to develop a chemical approach that would enable the incorporation of sulfate and Neu5Ac site, specifically onto the most common glycan epitopes found in *N*- and *O*-linked glycans, i.e., Gal$\beta$1,4GlcNAc, Gal$\beta$1,4Glc, Gal$\beta$1,3GlcNAc, and Gal$\beta$1,3GalNAc.[42]



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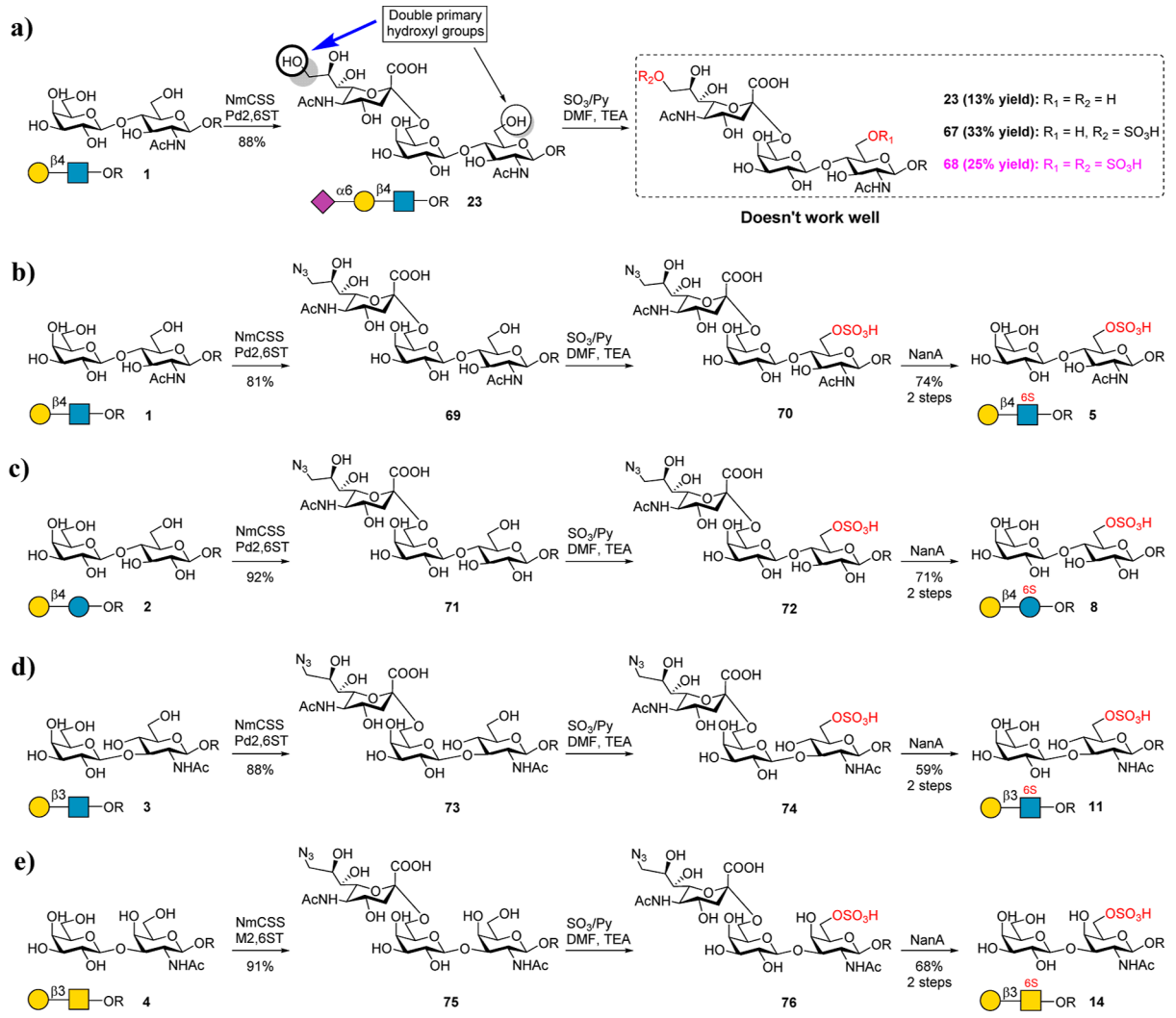


**Figure 2.** Chemoenzymatic preparation of disaccharide backbones **5**, **8**, **11**, and **14**. (a) Use of Neu5Ac as a protecting group to prepare **5**. The condition of chemical sulfation is $SO_3$/Py (12 equiv), DMF/TEA (V/V = 7:3), 0 °C−r.t., and 2 h. (b−e) Use of 9-$N_3$−Neu5Ac as the protecting group to prepare **5**, **8**, **11**, and **14**. The condition of chemical sulfation is $SO_3$/Py (8 equiv), DMF/TEA (V/V = 9:1), 0 °C−r.t., and 1−2 h. The condition of 9-$N_3$−Neu5Ac hydrolysis is NaOAc buffer (pH 6.5), NanA, and 4 h.

The well-known chemical approach to install the sulfate group relies on the use of $SO_3$ complexes, such as $SO_3$/Py, which primarily installs sulfate onto the C-6 OH of sugar residues[43,44] but cannot differentiate C-6 OH located on different sugar residues. Sometimes, $SO_3$/Py also introduces sulfate onto C-3 OH.[44] To achieve site-specific sulfation, we took advantage of $\alpha$2,3 sialyltransferase- or $\alpha$2,6 sialyltransferase-mediated sialylation to introduce a sialic acid as a temporary "protecting group", which specifically blocks the C-6 OH or C-3 OH of the terminal Gal residue, respectively. Chemical sulfation can then be conducted to introduce a sulfate group to the C-6 OH of a desired monosaccharide residue. Finally, removal of the protecting group by neuraminidase produced the sulfated glycan backbones (Figures 2 and 4−6), which can be further elaborated by glycosyltransferases to give structure-defined sulfated glycans. Notably, in 2019, Cao and co-workers pioneered the use of sialic acid as a temporary "protecting group" for site-specific enzymatic introduction of fucose residue in the LacNAc backbone.[7]

To test the proposed strategy, we first synthesized **5** from **1**. A linker containing an azido group (azido propane, Pro$N_3$) was designed for microarray printing. Initially, we tried to use Neu5Ac as a protecting group to block the C-6 OH of Gal for site-selective chemical sulfation of the C-6 OH of GlcNAc (Figure 2a). To this end, **23** was prepared from **1** by a one-pot reaction using CMP-Sialic acid synthetase from *Neisseria meningitidis* (NmCSS)[45] and $\alpha$2,6 sialyltransferase from *Photobacterium damselae* (Pd2,6ST).[46] The enzymes were prepared as reported previously.[47,48] Subsequently, **23** was treated with $SO_3$/Py in a mixed solvent of DMF and TEA at room temperature for 2 h (Figure 2a). However, the reaction produced a mixture, and three components were isolated, including starting material **23** (13% yield), **67** (9*S*-Neu5Ac$\alpha$2,6Gal$\beta$1,4GlcNAcPro$N_3$; 33% yield), and **68** (9*S*-Neu5Ac$\alpha$2,6Gal$\beta$1,4(6*S*)GlcNAcPro$N_3$; 25% yield) (Figure S1). This result indicated that the C-9 OH of Neu5Ac possesses a higher reactivity than the C-6 OH of GlcNAc in **23**. Although **68** can also be hydrolyzed by neuraminidase from *Streptococcus pneumoniae* (NanA, $\alpha$2,3/6/8 sialidase)[49] to give the target product **5**, the total yield was very low.

To address this issue, we chose 9-$N_3$−Neu5Ac as a temporary "protecting group" due to its lack of a primary OH at the C-9 position (Figure 2b). 9-$N_3$−Neu5Ac was


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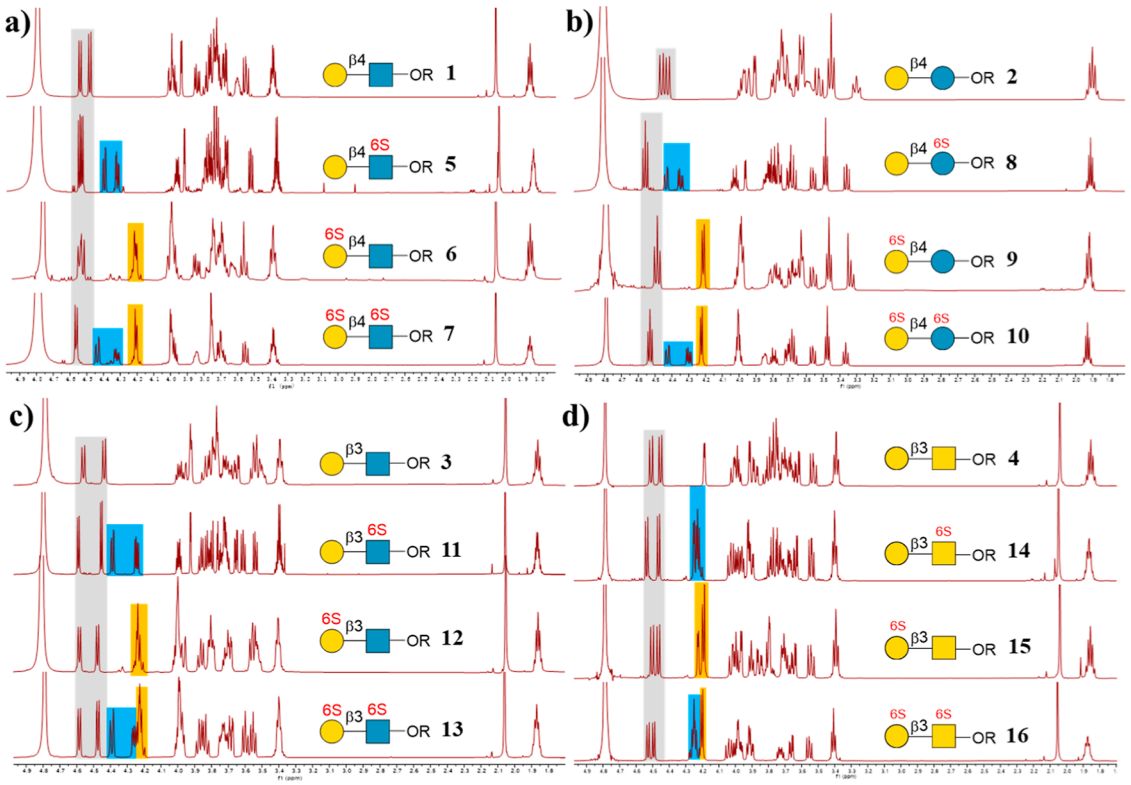


**Figure 3.** Presentation of the $^1$H NMR spectrum of nonsulfated and sulfated glycan backbones. The gray squares indicate the anomeric protons. The blue squares indicated the C-6 position protons of GlcNAc, Glc, or GalNAc. The yellow squares indicate the C-6 position protons of Gal.

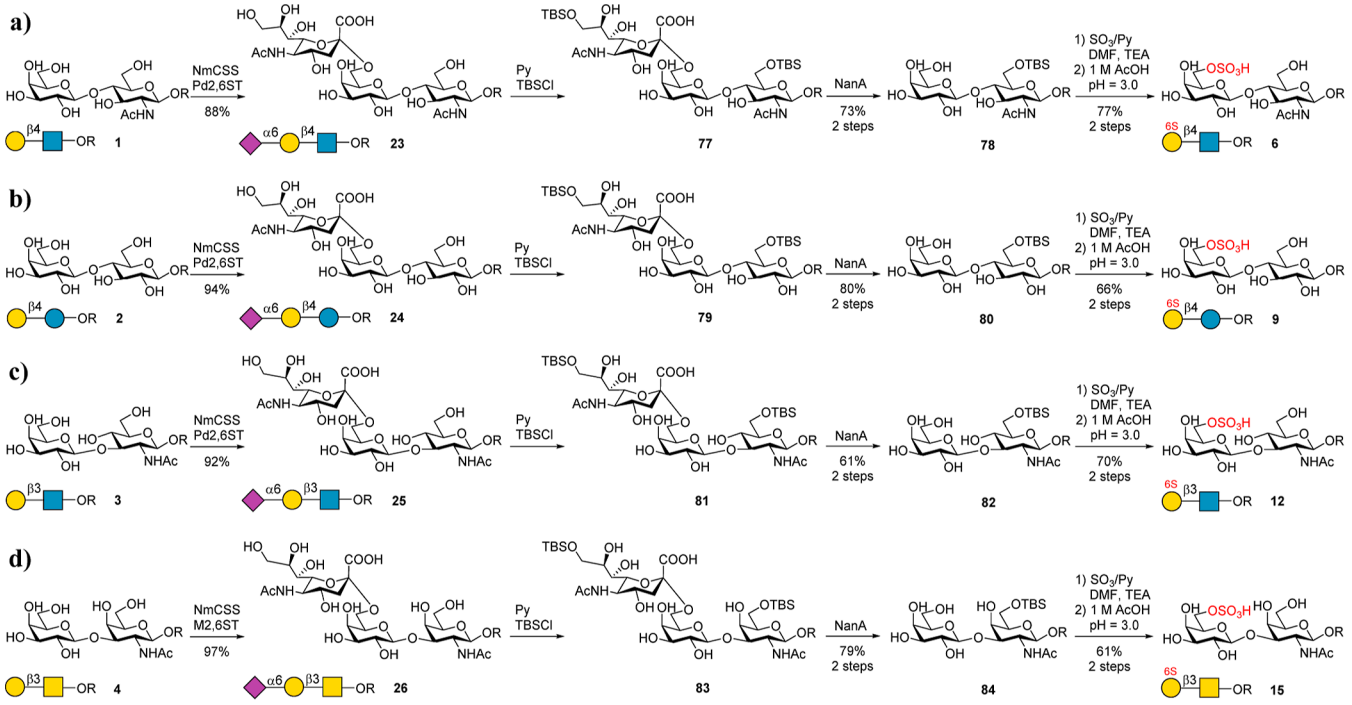


**Figure 4.** Chemoenzymatic preparation of disaccharide backbones **6**, **9**, **12**, and **15**. The condition of chemical sulfation is $SO_3$/Py (4 equiv), DMF/TEA (V/V = 1:1), 0 °C−40 °C, and 3−4 h. Deprotection of TBS is performed by adjusting pH to 3.0 using 1 M AcOH(aq).

prepared from Neu5Ac through three facile chemical steps.[50,51] It was then used to prepare **69** by using Pd2,6ST and NmCSS in a one-pot reaction. Next, **69** was treated with $SO_3$/Py in a mixed solvent of DMF and TEA to produce target intermediate **70** as a primary product (Figure 2b). Further treatment of **70** with NanA successfully gave the target backbone **5** with a high yield (74%, two steps from **69**). Notably, although three chemical steps are needed to prepare 9-$N_3$−Neu5Ac, 9-$N_3$−Neu5Ac can be recycled after hydrolysis and purification by a size-exclusion column for further



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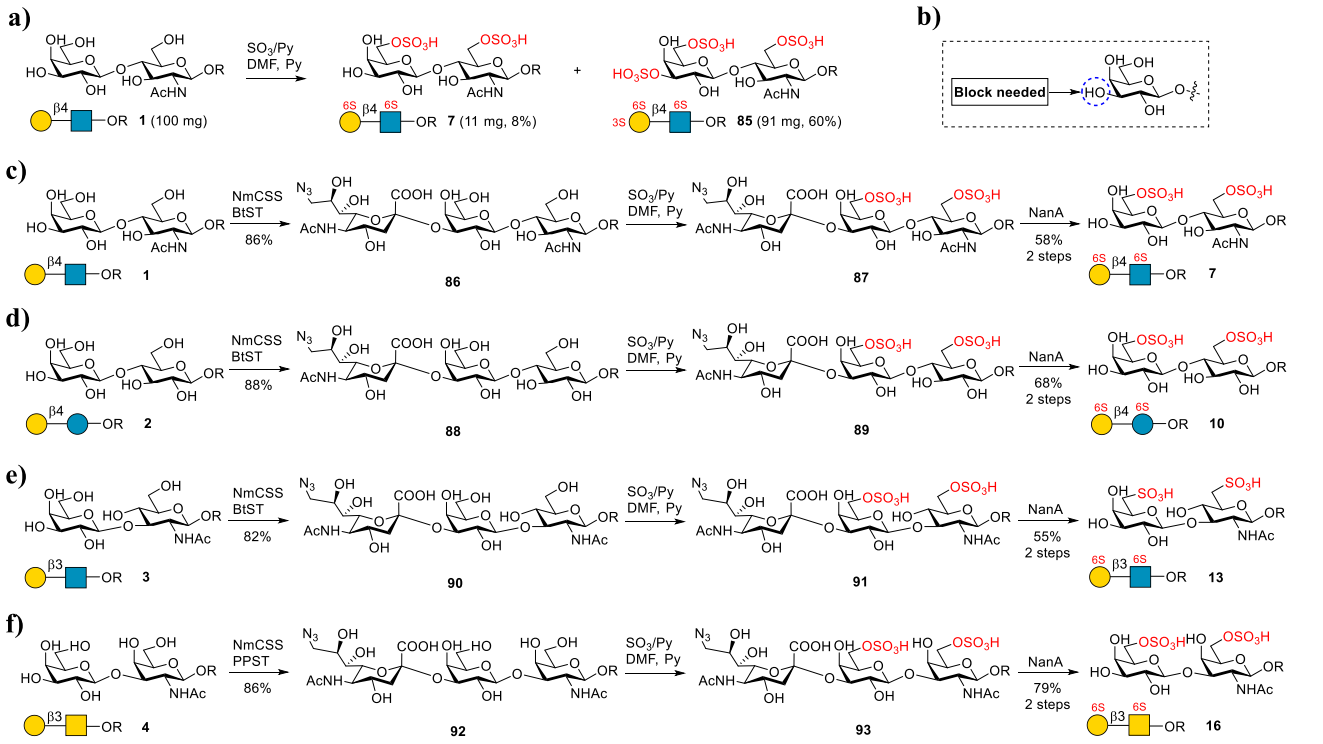


**Figure 5.** Chemoenzymatic preparation of backbones **7**, **10**, **13**, and **16**. The condition of chemical sulfation is $SO_3$/Py (15 equiv), DMF/Py (V/V = 1:1), 0 °C, and 1−2 h.

enzymatic reaction. Similarly, sulfated disaccharides **8**, **11**, and **14** were also synthesized successfully in high yields (Figure 2c−e). All products were confirmed by 1D and 2D NMR and HRMS analyses (see the Supporting Information). The sulfate group position of the products can be clearly distinguished by comparison with the $^1$H NMR spectrum of nonsulfated structures (Figure 3). Indeed, it was reported that several $\beta$1,4 galactosyltransferases can recognize 6*S*-GlcNAc and convert 6*S*-GlcNAc to Gal$\beta$1,4(6*S*)GlcNAc using UDP-Gal as a donor.[38,43] However, other backbones Gal$\beta$1,4Glc, Gal$\beta$1,3GlcNAc, and Gal$\beta$1,3GalNAc cannot be synthesized enzymatically due to the lack of suitable galactosyltransferases. Although chemical synthesis of **5**, **8**, **11**, and **14** can also be achieved by the strategy design,[44,52−54] the processes are tedious and suffer from low yields. For example, the chemical synthesis of **5** needs more than 12 steps with a total yield of less than 8.5%.[52] Moreover, each backbone needs a different synthetic route. Thus, we present here a general strategy for the efficient preparation of **5**, **8**, **11**, and **14**.

Subsequently, we attempted to synthesize sulfated disaccharides **6**, **9**, **12**, and **15**, which contain a sulfate group at the C-6 OH of Gal (Figure 4). The challenge is to selectively protect the C-6 OH of GlcNAc to prevent its sulfation. The *tert*-butyldimethylsilyl (TBS) was chosen as the protecting group. **23** was treated with *tert*-butyldimethylsilyl chloride (TBSCl) in pyridine (Figure 4a), affording absolute selectivity to produce compound **77** as the major product, which, upon hydrolysis by NanA, gave the intermediate **78**. Then, **78** was treated with $SO_3$/Py and TEA in DMF at 40 °C for 2 h to chemically sulfate the C-6 OH of Gal. It is worth mentioning that the C-3 OH of Gal was also sulfated to some extent. This byproduct can be avoided by controlling the reaction time and temperature. The evaporated reaction crude was then dissolved in water, and the pH was adjusted to 3.0 with 1 M aqueous AcOH to deprotect the TBS group, giving the target product **6** in 77% yield with respect to **78**. In contrast, the chemical synthesis of **6** needs 15 steps (13% yield).[36] Similarly, the backbones (**9**, **12**, and **15**) were also prepared successfully in excellent yields (Figure 4b−d). All products were confirmed by 1D and 2D NMR and HRMS analysis (see the Supporting Information). The sulfate group position of the products can be clearly distinguished by comparison with the $^1$H NMR spectrum of nonsulfated structures (Figure 3).

As the next step, we synthesized disulfated backbones **7**, **10**, **13**, and **16**. Initially, we tried to prepare **7** from **1** by treating **1** directly with $SO_3$/Py and TEA in DMF as described above. However, the reaction was rather messy, and NMR analysis indicated that the main product contained only one sulfate group (data not shown). Therefore, **1** was treated in a more intensive sulfation reaction condition (Figure 5a). However, the main product was a trisulfated glycan **85** containing an additional sulfate group at C-3 OH of Gal (Figures 5b and S2). This result again proved that the C-3 OH of the Gal residue has a relatively higher reactivity under intensive sulfation conditions. To address this issue, we designed a strategy as shown in Figure 5c−f to prepare disulfated backbones **7**, **10**, **13**, and **16**. In this approach, the C-3 OH of the terminal Gal was protected by an $\alpha$2,3-linked 9-$N_3$−Neu5Ac by using $\alpha$2,3 sialyltransferase. To synthesize **7** from **1** (Figure 5c), **1** was first converted to **86** by using *Bibersteinia trehalosi* $\alpha$2,3 sialyltransferase (BtST).[55,56] Comparing with the well-known $\alpha$2,3-sialyltransferase from *Pasteurella multocida* (PmST1), BtST processes low sialidase activity and donor hydrolysis activity, while no $\alpha$2,6-sialyltransferase and *trans*-sialidase activity were observed. In addition, the use of 9-$N_3$−Neu5Ac as a "protecting group" could avoid unwanted byproducts. **86** was further treated with $SO_3$/Py at 0 °C for 1 h. As expected, disulfated glycan **87** was produced as the main product. **87** was


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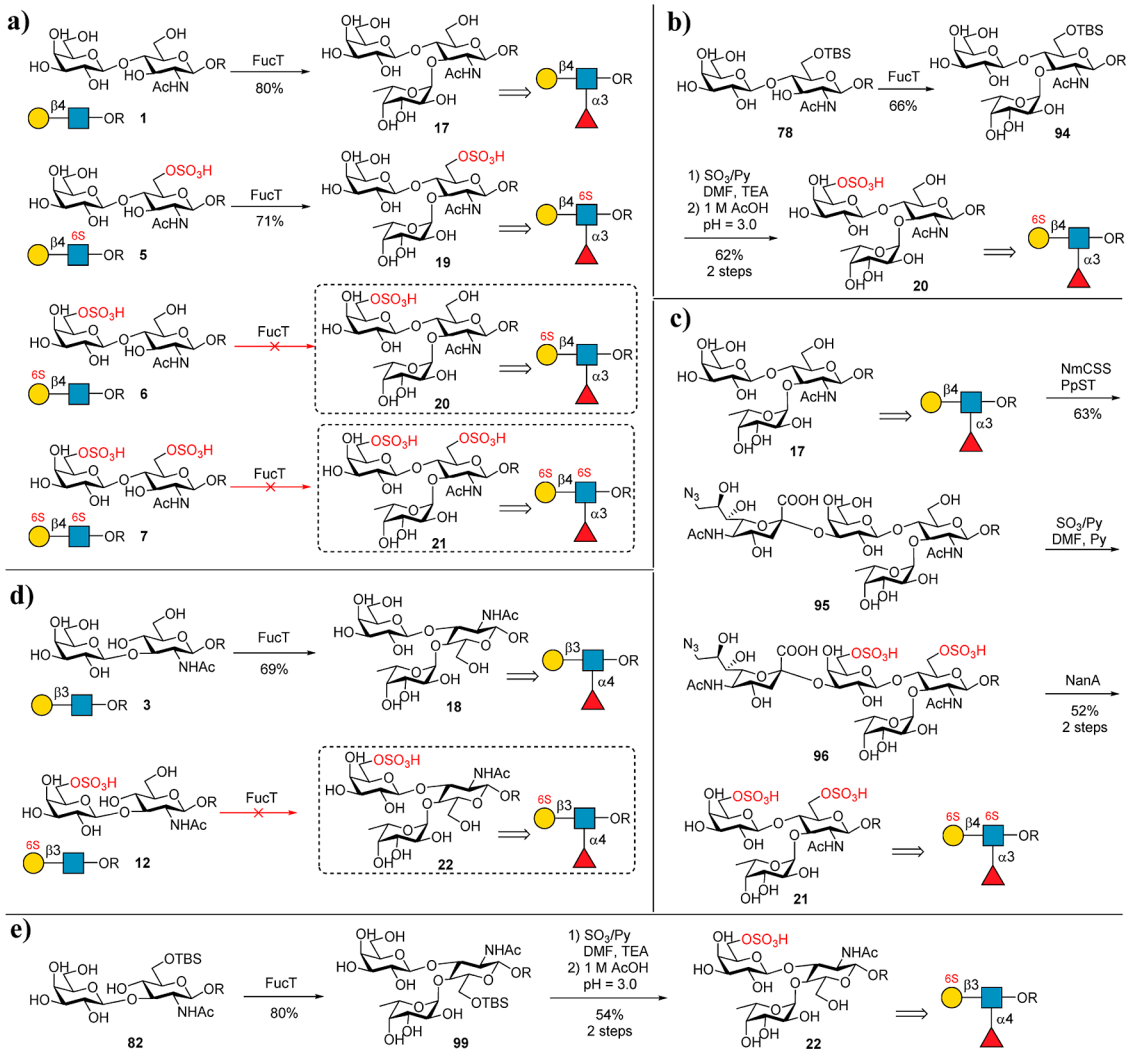


**Figure 6.** Chemoenzymatic preparation of fucosylated backbones **17**, **18**, **19**, **20**, **21** and **22**. The condition of chemical sulfation is $SO_3$/Py (4 equiv), DMF/TEA (V/V = 1:1), 0–40 °C, and 3–4 h for (b,e) and $SO_3$/Py (15 equiv), DMF/Py (V/V = 1:1), 0 °C, and 1–2 h for (c).

then treated with NanA to hydrolyze 9-$N_3$–Neu5Ac to give the target product **7** in a total yield of 50% for 3 steps. Meanwhile, total chemical synthesis of **7** needs over 16 steps.[36] Similarly, backbones **10**, **13**, and **16** were also successfully prepared using the described strategy (Figure 5d–f). All products were confirmed by 1D and 2D NMR and HRMS analysis (see the Supporting Information). As mentioned above, the sulfate group position of products can be clearly distinguished by comparison with the $^1$H NMR spectrum of nonsulfated structures (Figure 3).

Backbones Gal$\beta$1,4GlcNAc and Gal$\beta$1,3GlcNAc are frequently fucosylated in natural glycans.[57] Therefore, we next sought to prepare a sulfated version of fucosylated glycan backbones **17** to **22**. Initially, we tried to synthesize **17**, **19**, **20**, and **21** from **1**, **5**, **6**, and **7** (Figure 6a) using *Helicobacter pylori* $\alpha$1,3/4 fucosyltransferase (FucT).[58] However, only **1** and **5** can be accepted by FucT to give glycans **17** (also known as the sulfated Lewis X ($Le^X$) antigen) and **19** in excellent yields, while **6** and **7** cannot be recognized by FucT (Figure 6a). It is possible that the sulfate group at the C-6 OH of Gal inhibits the fucosylation by FucT, whereas FucT accepts the modification at C-6 OH of GlcNAc. Inspired by this assumption, we investigated the activity of FucT toward **78** (Figure 6b). We found that FucT indeed accepts **78** as a substrate to produce **94**, which was then selectively sulfated, as described above. After the removal of the TBS group, the target glycan backbone **20** was formed in 41% total yield with respect to **78**. Compared to the total chemical synthesis of **20**,[36] which requires complicated protection/deprotection manipulations, the strategy described here is more efficient. As FucTs did not accept substrates containing modification at the C-6 OH of the Gal residue, we designed a new synthetic route to prepare backbone **21** from **17** (Figure 6c). However, we found that BtST could not accept **17** as a substrate. The use of PmST1M144D results in a high ratio of byproducts containing $\alpha$2,6-linked Neu5Ac (data not shown). This is probably because PmST1 has weak $\alpha$2,3-sialidase activity, which leads to the cleavage of $\alpha$2,3-sialyl linkages slowly but leaves $\alpha$2,6-sialyl linkages intact. Thus, we cloned and screened many other $\alpha$2,3 sialyltransferases. Finally, we found an $\alpha$2,3 sialyltransferases from *Photobacterium phosphoreum* (PPST),[56,59] could recognize **17** efficiently, while no byproduct containing $\alpha$2,6-linked Neu5Ac was observed. More importantly, it also accepted CMP-9-$N_3$–Neu5Ac as a donor. Using PPST, intermediate **95** was successfully obtained from **17** in a high yield (67%). **95** was then treated with a chemical sulfation


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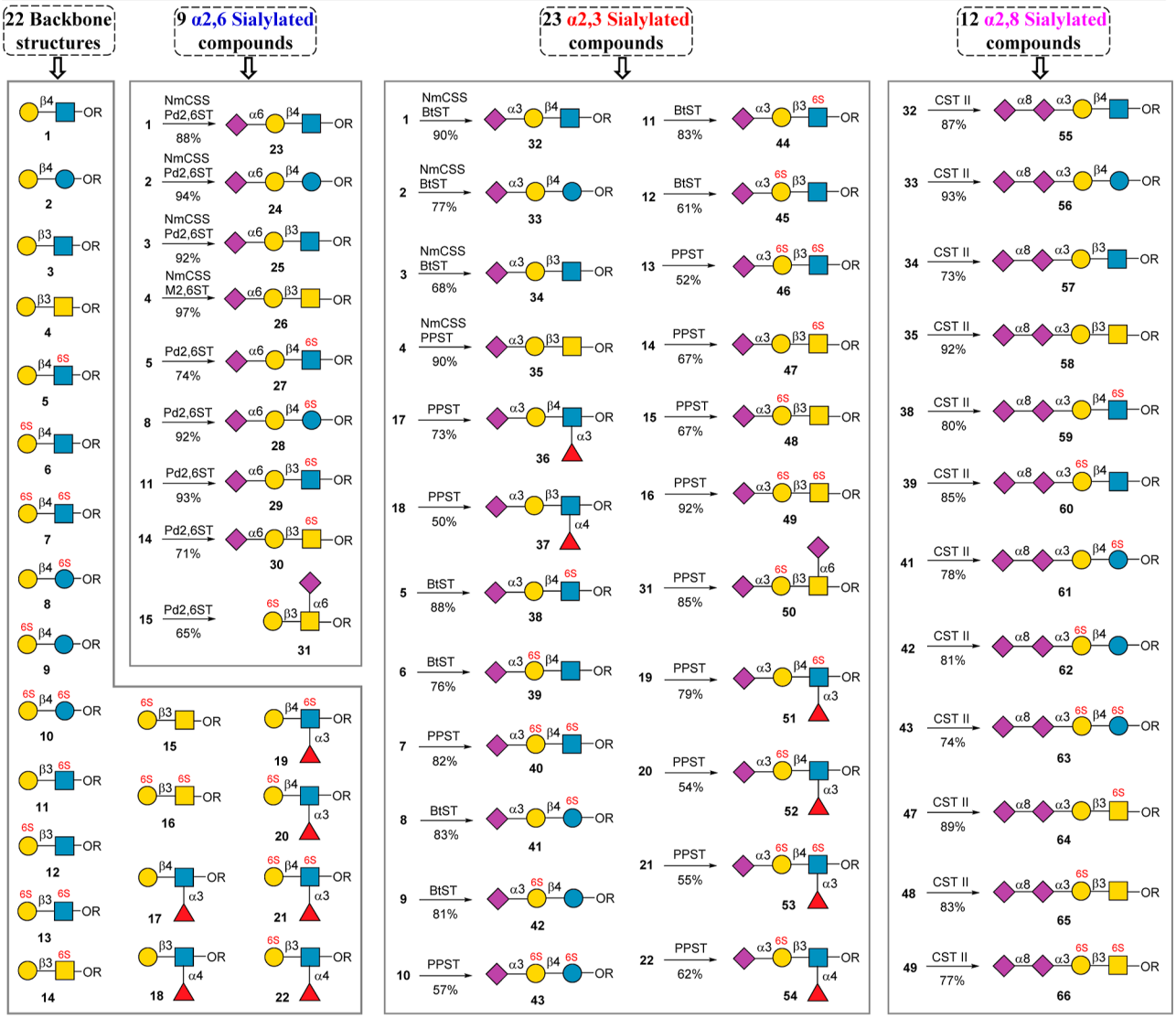


**Figure 7.** Preparation of sialoglycans from 22 backbones.

reaction to introduce two sulfate groups to produce intermediate **96**. The hydrolysis of 9-$N_3$−Neu5Ac by NanA produced the target product **21** in 52% yield with respect to **17**. Similarly, **18** was prepared successfully from **3** in a 69% yield. **22** was prepared from **82** in 54% yield (Figure 6e) as the strategy for the preparation of **20**. Meanwhile, the preparation of **97** and **98** from **11** and **13** is unsuccessful due to the tested α1,3/4 fucosyltransferases from *H. pylori* (NCTC 11369 and UA948) that could not recognize both substrates (Figure S3).

With backbones **1** to **22** in hand, we next conducted enzymatic sialylation reactions to construct the glycan library shown in Figure 1. In humans, Neu5Ac is typically linked to the Gal residue by an α2,3 or α2,6 linkage, while Neu5Ac can be linked to another Neu5Ac by an α2,8 linkage. The sialylation reaction toward the nonsulfated sialoglycans was performed in a one-pot reaction manner containing CTP, Neu5Ac, and NmCSS, which could avoid the use of pure CMP-Neu5Ac as a glycosylation donor. Meanwhile, the sialylation reaction toward the sulfated sialoglycans was performed using CMP-Neu5Ac as the glycosylation donor for the convenience of reaction detection by TLC. **23** to **31**, which contain α2,6-linked Neu5Ac, were efficiently synthesized as described above using Pd2,6ST (**26** was prepared using Pd2,6ST mutant(M2,6ST)). α2,3 Sialylation was performed by using BtST or PPST to produce 23 structures (Figure 7). **35** to **37**, **40** to **43**, and **46** to **54** were prepared by using PPST because PPST gives higher yields or many backbones such as **7**, **10**, **13**, **16**, and **17** to **22** cannot be accepted by BtST. Other structures shown in Figure 7 were prepared by using BtST. We next chose 12 important α2,3 sialoglycans for further extension by α2,3/8 sialyltransferase from *Campylobacter jejuni* (CST II).[60] The selected glycans include nonsulfated glycans, monosulfated glycans, and disulfated glycans, and all selected substrates can be well accepted by CST II to produce 12 structures (**55** to **66**) containing both α2,3- and α2,8-linked Neu5Ac. All of the above synthesized compounds were purified by using size-exclusion and ion-exchange columns. The obtained glycans, including 20 nonsulfated glycans and 46 sulfated glycans, were confirmed by 1D and 2D NMR and HRMS analysis (see the Supporting Information).

With the 66 synthetic glycans in hand, we further treated them with $Pd(OH)_2/C$ under $H_2$ pressure to reduce the linker of $ProN_3$ to $ProNH_2$. The reduced glycans were then printed



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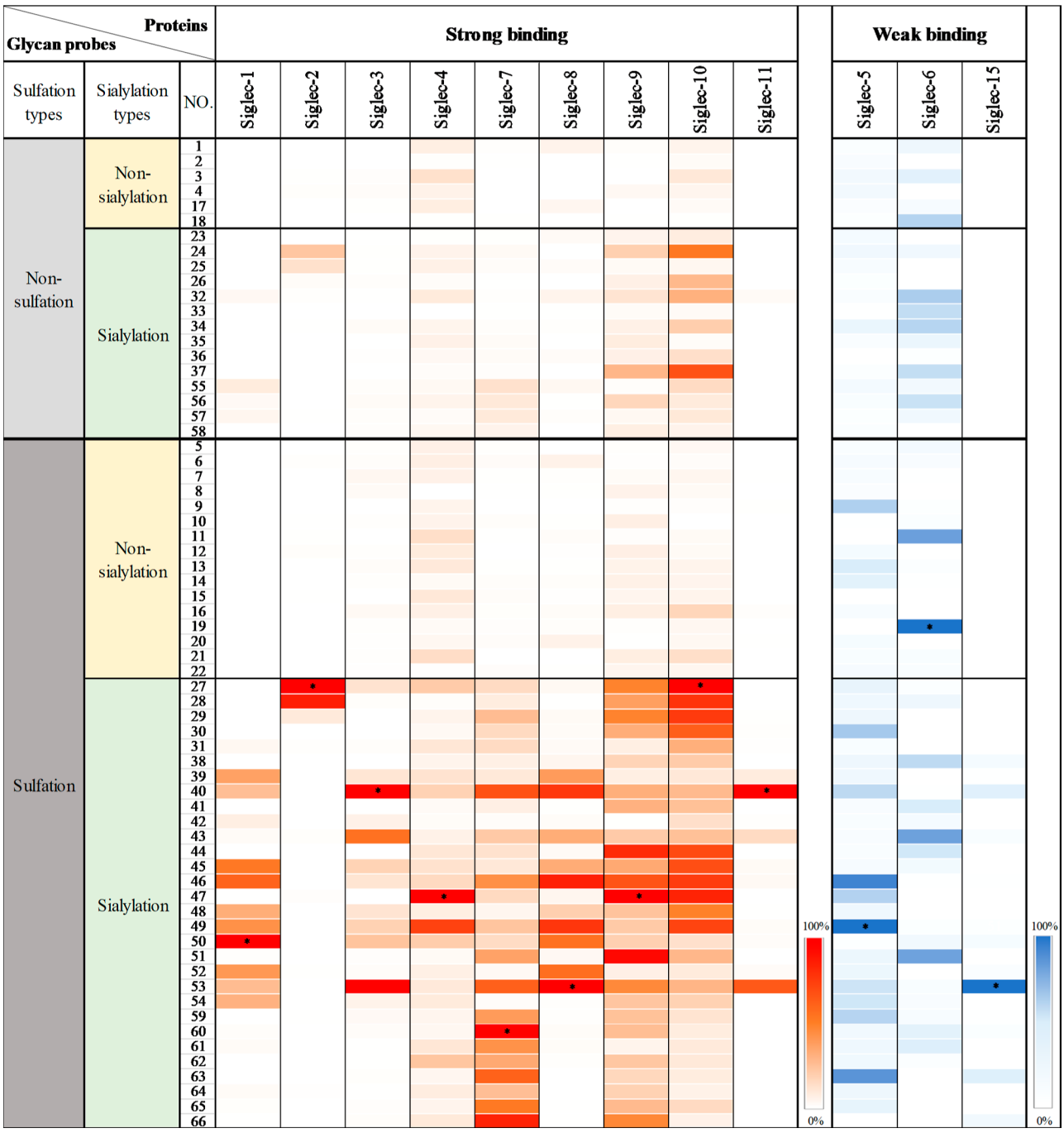


**Figure 8.** Binding profiles of human Siglecs with the 66-membered glycan microarray. *The strongest binding signal of the tested Siglec is defined as 100%. See the histogram charts of binding results in the Supporting Information (Figure S5).

onto amine-reactive *N*-hydroxysuccinimide (NHS)-activated glass slides to construct a glycan microarray. The array was probed with five well-known plant lectins including *Maackia amurensis* Lectin I (MAL I, binds terminal Neu5Ac$\alpha$2,3-Gal$\beta$1,4GlcNAc),[61,62] *Sambucus nigra* Lectin (SNA, binds Neu5Ac$\alpha$2,6Gal$\beta$1,4GlcNAc),[63,64] *Erythrina cristagalli* Lectin (ECL, binds Gal$\beta$1,4GlcNAc),[65] *Arachis hypogaea* Lectin (PNA, binds Gal$\beta$1,3GalNAc),[66,67] and *Aleuria aurantia* Lectin (AAL, binds L-fucose residue).[68,69] Microarray screening provided the expected binding specificities to these plant lectins (Figure S4), confirming the feasibility of the constructed glycan microarray for use in protein-binding studies.

We next investigated Siglecs binding with a constructed glycan microarray. Among 15 Siglecs in humans, Siglec-12 carries a mutation (R122C) and loses the ability to bind sialoglycans.[70] Siglec-5 and -14 and Siglec-11 and -16 are paired receptors that share the same ligand-binding preferences.[71−73] Therefore, we chose to study the binding preference of recombinant Siglec-1-Fc to Siglec-11-Fc and Siglec-15-Fc with the constructed microarray, in which the binding avidity was assessed using fluorescently labeled antihuman Fc followed by scanning with a microarray reader (647 nm).

As summarized in Figure 8, glycan sulfation, in general, enhanced the binding ability of ligands to Siglecs, which is


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consistent with the previous reports that Siglecs have a preference for sulfated glycan epitopes.[13,14,17] For example, in line with what was reported by Crocker et al., **27**, which contains both Neu5Ac $\alpha$2,6 linked to Type II LacNAc (Gal$\beta$1,4GlcNAc) and a sulfate group at the 6-OH position of the inner GlcNAc but not the unsulfated glycan **23**, exhibited strong binding to Siglec-2, a B-cell-associated inhibitory receptor.[15] In accordance with Paulson's observation, a strong binding of **28** to Siglec-2 was also observed. In addition, the glycan-binding patterns for Siglec-3, -7, -8, and -9 revealed by our screening were also consistent with what has been reported previously.[6,22,23] Importantly, we found that the glycan **60**, harboring both $\alpha$2,8 sialylation and Gal-6-*O*-sulfation, presented an additive effect on Siglec-7 binding compared to **39** and **55**.

Significantly, our screening also revealed some novel patterns of Siglec-binding specificity. For example, a previous study showed that Siglec-3 and Siglec-8 share a similar ligand-binding preference.[39] However, we found that the disulfated glycans **46** and **49** with the $\beta$1,3 Gal backbone exhibited a strong binding to Siglec-8 but a weak binding to Siglec-3, suggesting that not only sialylation and sulfation patterns but also the backbone structure of the ligand may influence recognition by Siglecs. Similarly, the ligand backbone structures were also found to influence binding to Siglec-9. Specifically, **44** and **47** displayed binding affinities to Siglec-9 stronger than those of **38** and **41**, suggesting that Siglec-9 prefers sulfated $\alpha$2,3-linked sialoglycans with Type I LacNAc or Gal$\beta$1,3GalNAc backbones. In addition, our screening data showed that disulfated 3′SLN (**40**) and SLe$^x$ (**53**) bound strongly to both Siglec-3 and -8, indicating that the presence of $\alpha$1,3 fucose on Type II LacNAc had no effect on the binding. Similarly, Siglec-11 also showed the highly specific binding to disulfated 3′SLN (**40**) and SLe$^x$ (**53**). On the other hand, the interaction between Siglec-8 and **45** was abrogated when $\alpha$1,4 fucose was conjugated to Type I LacNAc (**54**).

Furthermore, our binding experiments revealed how site-specific sulfation patterns regulate ligand binding to Siglec-1,[74,75] Siglec-4,[22,76] and -10,[77−79] whose ligand specificities were largely unidentified previously. For example, Gal-6-*O*-sulfation of ligands dramatically increases the binding of Siglec-1 (**32** vs **39** and **40**, **34** vs **45** and **46**, and **35** vs **48** and **49**). GalNAc-6-*O* sulfation, rather than Gal-6-*O* sulfation, of Neu5Ac$\alpha$2,3Gal$\beta$1,3GalNAc resulted in an increased binding affinity to Siglec-4 (**47** vs **48**). Interestingly, despite the negatively charged nature of both sulfate and sialic acid, the replacement of GalNAc-6-*O*-sulfation with sialylation (**49** to **50**) resulted in a higher binding affinity to Siglec-1. In contrast to Siglec-1, the replacement of GalNAc-6-*O*-sulfation by sialylation caused a dramatic loss of Siglec-4 binding.

Likewise, sulfation also plays a critical role in fine-tuning the ligand binding affinity to Siglec-10. But unlike the above observations, recognition of the sulfated ligands by Siglec-10 is less affected by the types of a sugar residue at the reducing end and the linkages of either $\beta$1,3 or $\beta$1,4 of LacNAc. 6-*O*-Sulfation of the internal GlcNAc, Glc, or GalNAc residues of $\alpha$2,6-linked sialosides **27**, **28**, **29**, and **30** resulted in a significant increase in Siglec-10 binding ability (**27**, **28**, **29**, and **30** vs **23**, **24**, **25**, and **26**). However, for $\alpha$2,3-linked sialosides (**32**, **33**, **34**, and **35**), the effect of 6-*O*-sulfation on Siglec-10 binding more depends on the $\beta$1,3-linked backbone structure. For example, sulfation of ligand **32** with the Type II LacNAc backbone showed no significant gain in binding (**38**, **39**, and **40**); whereas sulfation of ligand **34** with the Type I LacNAc backbone significantly enhanced binding to Siglec-10 (**44**, **45**, and **46**).

Finally, it is worth pointing out that ligands presented in our array did not show strong binding to other Siglecs, including Siglec-5, -6, and -15 (Figure 8). The strongest signals are close to the background level. This is probably because the epitopes are too small to bind these Siglecs or the absence of specific glycan carriers for these Siglecs. Hence, installation of these epitopes on glycolipidsor glycoproteins may increase the binding signals. Although these bindings are weak, the binding specificity can be summarized. For example, disulfated SLe$^x$ (**53**) is the dominant ligand for Siglec-15.

## CONCLUSIONS

Operating orthogonally to the well-established immune checkpoints, PD-1 and CTLA-4,[80] Siglecs have stimulated enthusiasm for developing strategies to suppress their inhibitory functions for cancer immunotherapy or to harness such function for the treatment of autoimmunity or inflammation. However, the ligand specificity has only been explored for a subfamily of Siglecs. In this study, we successfully developed a strategy using sialylation as a protecting approach for the rapid and efficient preparation of structure-defined glycan epitopes with site-specific sulfation. Using this strategy, 66 sulfated glycans with structures covering a large number of terminal sialylated glycan epitopes were prepared, allowing us to perform a comprehensive characterization of the ligand specificity for the entire Siglec family. Although our strategy is highly efficient for the preparation of sulfated glycan epitopes bearing disaccharide or trisaccharide backbones, it provides low yields when applied to the synthesis of glycans containing four or more monosaccharides, as the regio-specificity of the chemical sulfation reaction decreases.

Our screening revealed that not only sialylation and sulfation patterns but also the backbone structure of the ligands can influence recognition by Siglecs. We confirmed the glycan-binding patterns of Siglec-2, -3, -7, -8, and -9, which are consistent with those reported previously. In addition, our screening also revealed ligand specificity of less-studied Siglec-1, -4, -10, and -11 as well as subtle differences in ligand preference for several other Siglecs. For example, Siglec-4 prefers GalNAc-6-*O* sulfation, rather than Gal-6-*O* sulfation, of Neu5Ac$\alpha$2,3Gal$\beta$1,3GalNAc. It is worth noting that the $\alpha$2,8 linkage of sialylation and sulfation of 6-*O*-Gal has a synergic effect on Siglec-7 binding. Siglec-8, but not Siglec-3, prefers disulfated glycans with the $\beta$1,3Gal backbone. Taken together, our study paves the way for the development of new Siglec-target strategies by providing a clear understanding of how sulfation and sialylation patterns govern the binding specificities of human Siglecs at the molecular level.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.4c08817.

Additional experimental details, materials, and methods; and supporting figures, supporting table, $^1$H NMR spectra, and $^{13}$C NMR spectra for all compounds (PDF)



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### Notes

The authors declare the following competing financial interest(s): The authors have submitted a patent application (China patent/PCT 2024113874483) regarding this work.

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