Published in final edited form as:
Carbohydr Res. 2017 November 08; 451: 51–58. doi:10.1016/j.carres.2017.09.003.

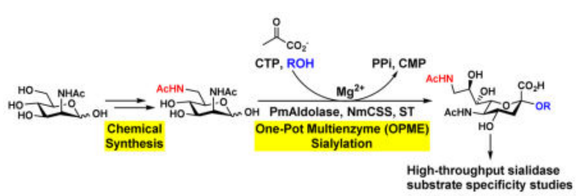
Chemoenzymatic synthesis of Neu5Ac9NAc-containing α2–3- and α2–6-linked sialosides and their use for sialidase substrate specificity studies

Wanqing Li, **An Xiao**, **Yanhong Li**, **Hai Yu**, and **Xi Chen**[*]
Department of Chemistry, University of California-Davis, One Shields Avenue, Davis, California 95616, USA

Abstract

O-Acetylation of sialic acid (Sia) modulates its recognition by sialic acid-binding proteins and plays an important role in biological and pathological processes. 9-*O*-Acetylation is the most common modification of sialic acid in human. However, study of *O*-acetylated sialoglycans is hampered due to the instability of *O*-acetyl group towards pH changes and sensitivity to esterases. Our previous studies demonstrated a chemical biology method to this problem by replacing the oxygen atom in the C9 ester group of sialic acid by a nitrogen to form an amide. Here, we synthesized a library of sixteen new 9-acetamido-9-deoxy-*N*-acetylneuraminic acid (Neu5Ac9NAc)-containing α2–3- and α2–6-linked sialosides with various underlying glycans using efficient one-pot three-enzyme (OP3E) sialylation systems. Neu5Ac9NAc-containing compounds with a *para*-nitrophenol aglycon have been used together with their 9-*O*-acetyl analogs in microtiter plate-based high-throughput substrate specificity studies of nine different sialidases including those from humans and bacteria. In general, similar to 9-*O*-acetylation, 9-*N*-acetyl modification of sialic acid in the substrates lowers sialic acid-cleavage activity of most sialidases. In most cases, Neu5Ac9NAc is a good analog of 9-*O*-acetyl sialic acid. However, exceptions do exist. For example, 9-*N*- and 9-*O*-acetyl modifications have different effects on the sialosides cleave efficiencies of a commercially available *C. perfringens* sialidase as well as recombinant *Streptococcus pneumoniae* sialidase SpNanC and *Bifidobacterium infantis* sialidase BiNanH2. The mechanism for the difference awaits further investigation.

Graphical Abstract



[*]Corresponding author. Department of Chemistry, University of California-Davis, One Shields Avenue, Davis, CA 95616, USA. Tel.: +1 530 7546037; fax: +1 530 7528995. xiichen@ucdavis.edu (X. Chen).

Keywords

9-*N*-acetyl sialic acid; 9-*O*-acetyl sialic acid; chemoenzymatic synthesis; one-pot multienzyme; sialic acid

Sialic acids (Sia) are 9-carbon monosaccharides commonly presented at the glycan termini of glycoconjugates. They are directly involved in many molecular recognition events, including immune regulation, cell-cell interaction, inflammation, cancer metastasis, as well as bacterial and viral infection [1]. More than 50 structurally distinct sialic acid forms have been found in nature including three basic forms *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid (Kdn), and their derivatives with modifications at various locations [2–4]. Among numerous modifications, *O*-acetylation is the most common which can occur at C-4, 7, 8, and 9 positions [5]. In nature, *O*-acetyl group spontaneously migrates from C7 and C8 to C9, which makes *O*-acetylation at C-4 and C-9 the most common naturally occurring sialic acid modifications [6, 7]. It has been shown that 9-*O*-acetylation is an effective biomarker in monitoring Acute Lymophoblastic Leukemia and Visceral Leishmaiasis [5]. It also regulates tissue morphogenesis during development, protects sialosides against sialidase cleavage, and inhibits binding by some proteins [8]. The 9-*O*-acetyl Neu5Ac (Neu5,9Ac$_2$) is recognized by influenza C virus hemagglutinin [9] and by Factor H [10]. Nevertheless, study of the detailed biological significances of 9-*O*-acetyl sialic acids is hampered by the instability of *O*-acetyl ester group towards pH change and its sensitivity to esterases. As shown previously [11], a chemical biology strategy to partially solve this problem is to replace the oxygen atom in the ester group in Neu5,9Ac$_2$ by an “NH” functionality in 9-acetamido-9-deoxy-*N*-acetylneuraminic acid (Neu5Ac9NAc) (Figure 1). The conformational resemblance of Neu5Ac9NAc and Neu5,9Ac$_2$ in a sialyl trisaccharide has been confirmed by computational molecular dynamics, similar mammalian cell incorporation and surface expression, and similar interaction with Neu5,9Ac$_2$-specific proteins by microarray studies. On the other hand, much improved stability of Neu5Ac9NAc compared to Neu5,9Ac$_2$ has been demonstrated in binding and cell feeding studies [11]. A similar strategy of replacing sialic acid *O*-acetyl modification by an *N*-acetyl has also been successfully applied by others to investigate the roles of sialic acid *O*-acetylation [12, 13].

Herein, we show that one-pot three-enzyme (OP3E) sialylation systems [14] are efficient for high-yield production of a library of Neu5Ac9NAc-containing α2–3- and α2–6-linked sialosides with diverse underlying glycans from chemically synthesized 6-acetamido-6-deoxy-*N*-acetylmannosamine (ManNAc6NAc). Among these, *para*-nitrophenylated α2–3- and α2–6-linked sialyl galactosides have been used in microtiter plate-based high-throughput substrate specificity studies of nine different sialidases including those from humans and bacteria [15–18].

To synthesize a library of Neu5Ac9NAc-containing α2–3- and α2–6-linked sialosides, chemically synthesized ManNAc6NAc [11] was used directly in an efficient one-pot multienzyme (OPME) sialylation system [14] containing *Pasteurella multocida* sialic acid aldolase (PmAldolase) [19], *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS)

[20, 21], and a sialyltransferase (Scheme 1). In this system, PmAldolase was used to catalyze the aldol addition reaction of ManNAc6NAc and sodium pyruvate (used in an excess to drive the reaction towards sialic acid formation) to obtain Neu5Ac9NAc. NmCSS was used for catalyzing the formation of the activated sugar nucleotide donor, cytidine 5′-monophosphate-Neu5Ac9NAc (CMP-Neu5Ac9NAc), for the subsequent sialyltransferase-catalyzed glycosylation reaction. *Pasteurella multocida* sialyltransferase 1 [22] M144D mutant (PmST1 M144D) [23] with decreased sialidase and donor hydrolysis activities was used for synthesizing α2–3-linked sialosides. *Photobacterium damselae* α2–6-sialyltransferase (Pd2,6ST) [24] or *Photobacterium* sp. α2–6-sialyltransferase [25] A366G mutant (Psp2,6ST A366G) with an improved expression level [26] was used for synthesizing α2–6-linked sialosides. The reactions were carried out in Tris-HCl buffer (100 mM) with a pH of 8.5 to optimize the activity of NmCSS while retain high activity of PmAldolase and sialyltransferases used. The products were obtained in good to excellent (61–98%) yields after purification using a gel filtration column and a C18 reverse phase column. The structures and the purities of the products were confirmed by high resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) spectroscopy.

As shown in Table 1, using PmST1 M144D as the sialyltransferase, α2–3-linked sialosides (**1**–**7**) were obtained in 61–86% yields. These are comparable to previously reported synthesis of α2–3-sialyllactoside, Neu5Ac9NAcα2–3Galβ1–4GlcβProN_3, which was obtained in 84% yield [11]. Two α2–6-sialyltransferases were used for synthesizing α2–6-linked sialosides (**8**–**16**). Psp2,6ST A366G mutant with an increased expression level and improved activities in sialylating Tn antigens [25, 26] was used for synthesizing Neu5Ac9NAcα2–6GalNAcαProN_3 (**16**) with 71% yield. For other α2–6-linked Neu5Ac9NAc-containing sialosides (**8**–**15**) synthesized, both Pd2,6ST and Psp2,6ST A366G could provide similar yields in small scale reactions. Pd2,6ST was used for the synthesis of Neu5Ac9NAcα2–6GalβProN_3 (**9**) and Neu5Ac9NAcα2–6Galβ1–3GalNAcαProN_3 (**12)** with excellent 92% and 98% yields, respectively. Due to the higher expression level of Psp2,6ST A366G, it was used for the synthesis of the rest of the α2–6-linked sialoside targets (**8**, **10**–**11**, **13**–**15**). Good to excellent yields (64–96%) were obtained.

The α2–3/6-sialosides obtained include Neu5Ac9NAcα2–3/6Galβ*p*NP (**1** and **8**) and propyl azide (ProN_3)-containing ones (**2**–**7** and **9**–**16**) such as disaccharides Neu5Ac9NAcα2–3/6GalβProN_3 (**2** and **9**), sialyl type I glycans Neu5Ac9NAcα2–3/6Galβ1–3GlcNAcβProN_3 (**3** and **10**), sialyl type II glycans Neu5Ac9NAcα2–3/6Galβ1–4GlcNAcβProN_3 (**4** and **11**), sialyl type III glycans Neu5Ac9NAcα2–3/6Galβ1–3GalNAcαProN_3 (**5** and **12**), sialyl type IV glycans Neu5Ac9NAcα2–3/6Galβ1–3GalNAcβProN_3 (**6** and **13**), Neu5Ac9NAcα2–3/6Galβ1–3GlcNAcαProN_3 (**7** and **14**), α2–6-sialyl type VI glycan [27] Neu5Ac9NAcα2–6Galβ1–4GlcβProN_3 (**15**), and sialyl Tn antigen Neu5Ac9NAcα2–6GalNAcαProN_3 (**16**). These represent common terminal sialyl glycan structures in vertebrates.

Among the compounds synthesized, two *p*NP-tagged Neu5Ac9NAc-containing sialosides Neu5Ac9NAcα2–3Galβ*p*NP (**1**) and Neu5Ac9NAcα2–6Galβ*p*NP (**8**) were used conveniently in a 384-well plate-based high-throughput colorimetric assay for substrate specificity studies [15] of nine sialidases. Four additional *p*NP-tagged sialosides including Neu5Ac-containing ones such as Neu5Acα2–3Galβ*p*NP (**17**) and Neu5Acα2–6Galβ*p*NP

(**18**) as well as Neu5,9Ac$_2$-cotaining ones such as Neu5,9Ac$_2$α2–3Galβ*p*NP (**19**) and Neu5,9Ac$_2$α2–6Galβ*p*NP (**20**) [15] (Figure 2) were also used as sialidase substrates for comparison purpose. Sialidases used include six recombinant sialidases and three commercially available sialidases. The recombinant sialidases used were human cytosolic sialidase hNEU2 [28], bacterial sialidases PmST1 (a multifunctional sialyltransferase which also has sialidase activity) [22], *Bifidobacterium infantis* sialidase NanH2 [29], and three *Streptococcus pneumoniae* sialidases SpNanA [30], SpNanB [30], and SpNanC [31, 32]. Three commercial bacterial sialidases used were those from *Arthrobacter ureafaciens, Vibrio cholerae*, and *Clostridium perfringens*. In this assay method, individual sialosides were incubated in duplicates at 37 °C for 30 min with an appropriate amount of a sialidase as well as an excess amount of β-galactosidase. The reactions were stopped by adding *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer (0.5 M, pH 10.5) to adjust the pH value of the solution to higher than 9.5 to convert the *para*-nitrophenol formed in the enzymatic reactions to *para*-nitrophenolate which was quantified by a microplate reader at A_{405nm} [15].

As shown in Figure 3, substituting the 9-hydroxy group of the Neu5Ac in sialosides by an acetamido group led to significant reduction of the activities of hNEU2 (Fig. 3A) and *V. cholerae* sialidase (Fig. 3B), in which the 9-*N*-acetyl modification protected sialosides against sialidase cleavage. The effect was similar to that observed for Neu5Ac 9-*O*-acetyl modification (Fig. 3A and 3B). In comparison, both 9-*N*- and 9-*O*-acetyl modifications decreased the sialoside cleavage efficiencies of *A. ureafaciens* sialidase (Fig. 3C), SpNanA (Fig. 3D), PmST1 (Fig. 3E), and SpNanB (Fig. 3F) only moderately or slightly. Overall, these examples (Fig. 3A–3G) showed that Neu5Ac9NAc-sialosides are good mimics of Neu5,9Ac$_2$-sialosides in probing the activities of these sialidases. Nevertheless, there were exceptions. Different effects were observed for 9-*N*- and 9-*O*-acetylation of Neu5Ac in affecting sialoside cleavage by SpNanC (Fig. 3G), *C. perfringens* sialidase (Fig. 3H), and BiNanH2 (Fig. 3I). While 9-*N*-acetylation of Neu5Ac did not alter the sialidase activity of SpNanC significantly, 9-*O*-acetylation of Neu5Ac improved the efficiency of sialoside cleavage by SpNanC (Fig. 3G). In comparison, 9-*N*-acetylation of Neu5Ac decreased the efficiency of sialoside cleavage by *C. perfringens* sialidase while 9-*O*-acetylation of Neu5Ac did not alter its activity significantly (Fig. 3H). On the other hand, Neu5Ac 9-*N*-acetylation did not have significant effect on the sialic acid cleavage efficiency of BiNanH2, but Neu5Ac 9-*O*-acetylation completely blocked its activity (Fig. 3I). A possible factor to consider for the differences is that “NH” in the amide in Neu5Ac9NAc can serve as both a hydrogen bond donor and a hydrogen bond acceptor while the oxygen atom in the ester in Neu5,9Ac$_2$ can only serve as a hydrogen bond acceptor. The mechanism for these differences, however, needs further investigation.

The results obtained by the microtiter plate-based assays for PmST1 (Fig. 3E), SpNanC (Fig. 3G), *C. perfringens* sialidase (Fig. 3H), and BiNanH2 (Fig. 3I) were further confirmed by high-performance liquid chromatography (HPLC)-based assays (Figures S1–S4, ESI). In addition, time course studies for BiNanH2 (Figure S5, ESI) and more detailed kinetics studies for SpNanC and BiNanH2 (Table 2) were carried out. As shown in Table 2, SpNanC catalyzes the cleavage of Neu5Ac9NAcα2–3Galβ*p*NP (**1**) and Neu5Acα2–3Galβ*p*NP (**17**) with similar efficiencies (k_{cat}/K_M = 137 s^{-1} mM^{-1} and 130 s^{-1} mM^{-1}, respectively). In contract, The catalytic efficiency of SpNanC for Neu5,9Ac$_2$α2–3Galβ*p*NP (**19**) (k_{cat}/K_M = 302 s^{-1} mM^{-1}) is much (2.2–2.3 fold) higher. For BiNanH2, it has similar catalytic efficiencies towards Neu5Ac9NAcα2–3Galβ*p*NP (**1**) and Neu5Acα2–3Galβ*p*NP (**17**) (k_{cat}/K_M = 59.3 s^{-1} mM^{-1} and 66.7 s^{-1} mM^{-1}, respectively). Its catalytic efficient towards Neu5Ac9NAcα2–6Galβ*p*NP (**8**) (k_{cat}/K_M = 179 s^{-1} mM^{-1}) is slightly lower than that of Neu5Acα2–6Galβ*p*NP (**18**) (k_{cat}/K_M = 242 s^{-1} mM^{-1}). In comparison, its activity towards Neu5,9Ac$_2$α2–3Galβ*p*NP (**19**) and Neu5,9Ac$_2$α2–6Galβ*p*NP (**20**) are not high enough for obtaining apparent kinetics parameters. These results validated those shown in Figure 3.

In summary, a library of sixteen new α2–3/6-linked Neu5Ac9NAc-containing sialosides have been successfully synthesized in good to excellent (61–98%) yields using highly efficient one-pot three-enzyme sialylation systems. Among the sialosides synthesized, *para*-nitrophenylated α2–3- and α2–6-linked Neu5Ac9NAc-containing sialyl galactosides have been used together with their Neu5Ac-, and Neu5,9Ac$_2$-sialoside analogs in microtiter plate-based high-throughput substrate specificity studies of various sialidases. In general, Neu5Ac9NAc-sialosides are good mimics of Neu5,9Ac$_2$-sialosides in probing the activities of most sialidases. Nevertheless, exceptions do exist and different effects were observed for Neu5Ac 9-*N*- and 9-*O*-acetylation in affecting sialoside cleavage by SpNanC, *C. perfringens* sialidase, and BiNanH2. Further investigation will be needed to elucidate the mechanism for these differences.

1. Experimental

1.1. Materials

All chemicals were obtained from commercial suppliers and used without further purification. ^{1}H NMR (400 or 800 MHz) and ^{13}C NMR (400 or 800 MHz) spectra were recorded on a Bruker Avance-400 Spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C) or a Avance-800 Spectrometer (800 MHz for ^{1}H, 200 MHz for ^{13}C). High resolution electrospray ionization (ESI) mass spectra were obtained using a Thermo Electron LTQ-Orbitrap Hybrid MS at the Mass Spectrometry Facility in the University of California, Davis. Column chromatography was performed using Redi*Sep* Rf silica columns or an ODS-SM column (51 g, 50 μm, 120 Å, Yamazen) on the CombiFlash® Rf 200i system. Analytical thin-layer chromatography was performed on silica gel plates 60 GF_{254} (Sorbent technologies) using anisaldehyde stain for detection. Gel filtration chromatography was performed using a column (100 cm × 2.5 cm) packed with BioGel P-2 Fine resins (Bio-Rad, Hercules, CA, USA). *Arthrobacter ureafaciens* sialidase, *Vibrio cholerae* sialidase, and *Clostridium perfringens* sialidase (CpNanH) were purchased from Prozyme (Hayward, CA, USA). Recombinant PmAldolase [19], NmCSS [20, 21], PmST1 M144D mutant [23], Pd2,6ST [24], Psp2,6ST A366G mutant [26], hNEU2 [28], PmST1 [22], SpNanA [30], SpNanB [30], SpNanC [32], and BiNanH2 [29], were expressed as described previously. Neu5Acα2–3Galβ*p*NP (**17**), Neu5Acα2–6Galβ*p*NP (**18**), Neu5,9Ac$_2$α2–3Galβ*p*NP (**19**), Neu5,9Ac$_2$α2–6Galβ*p*NP (**20**) [15], and ManNAc6NAc [11] were synthesized as reported previously.

1.2. One-pot three-enzyme synthesis of α2–3/6-linked sialosides

An acceptor (30–50 mg, 10 mM) and ManNAc6NAc (1.2–1.5 equiv.) were incubated at 37 °C in Tris-HCl buffer (100 mM) (pH 8.5 for synthesizing α2–3-sialosides or α2–6-sialosides using Psp2,6STA366G, pH 7.5 for synthesizing α2–6-sialosides using Pd2,6ST) containing sodium pyruvate (6.0–7.5 equiv.), CTP (1.5 equiv.), $MgCl_2$ (20 mM), an appropriate amount of PmAldolase (1.5 mg), NmCSS (2.5 mg), and Pd2,6ST (2.5 mg), Psp2,6ST A366G mutant (2.5 mg), or PmST1 M144D mutant (2.5 mg). The reaction was monitored by thin-layer chromatography (TLC) using a developing solvent consisting of EtOAc:MeOH:H_2O = 5:2:1 (by volume) and the TLC plates were stained with a *p*-anisaldehyde sugar stain. After 1–24 h, the reaction was quenched by adding the same volume of pre-chilled ethanol and the reaction mixture was centrifuged to remove precipitates. The supernatant was concentrated and passed through a BioGel P-2 gel filtration column eluting with water followed by a C18 column (H_2O:CH_3CN= 1:0 – 4:1) to obtain the target products.

1.2.1. 4-Nitrophenyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-O-β-D-galactopyranoside (Neu5Ac9NAcα2–3GalβpNP, 1)—Yield 80%; 87 mg, White foam. ^{1}H NMR (400 MHz, D_2O) δ 8.32–8.15 (m, 2H), 7.32–7.17 (m, 2H), 5.29 (d, *J* = 7.8 Hz, 1H), 4.25 (dd, *J* = 3.2 and 9.8 Hz, 1H), 4.06 (d, *J* = 3.2 Hz, 1H), 3.98–3.84 (m, 4H), 3.81–3.64 (m, 4H), 3.61–3.46 (m, 2H), 3.29 (dd, *J* = 7.6 and 14.2 Hz, 1H), 2.80 (dd, *J* = 4.6 and 12.4 Hz, 1H), 2.04 (s, 3H), 1.95 (s, 3H), 1.84 (t, *J* = 12.1 Hz, 1H); ^{13}C NMR (100 MHz, D_2O) δ 174.95, 174.32, 173.75, 161.70, 142.47, 126.07(2C), 116.41(2C), 99.94, 99.70, 75.50, 75.45, 72.76, 70.00, 69.60, 68.77, 68.29, 67.29, 60.67, 51.67, 42.10, 39.67, 22.03, 21.75; HRMS (ESI) Anal. Calcd for $C_{25}H_{34}N_3O_{16}$ $[M-H]^-$: 632.1945, Found: 632.1957.

1.2.2. 3-Azidopropyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyrano-sylonic acid)-(2→3)-O-β-D-galactopyranoside (Neu5Ac9NAcα2–3Galβ ProN$_3$, 2)—Yield 72%; 73 mg, White foam. ^{1}H NMR (400 MHz, D_2O) δ 4.47 (d, *J* = 7.8 Hz, 1H), 4.08 (dd, *J* = 3.2 and 9.8 Hz, 1H), 4.05–3.63 (m, 10H), 3.59–3.45 (m, 5H), 3.32 (dd, *J* = 7.8 and 14.2 Hz, 1H), 2.76 (dd, *J* = 4.6 and 12.4 Hz, 1H), 2.04–2.03 (m, 6H), 1.96–1.89 (m, 2H), 1.81 (t, *J* = 12.2 Hz, 1H); ^{13}C NMR (100 MHz, D_2O) δ 174.93, 174.40, 173.80, 102.54, 99.93, 75.85, 74.88, 72.70, 69.95, 69.54, 69.13, 68.31, 67.52, 67.14, 60.93, 51.66, 47.90, 42.09, 39.61, 28.25, 22.02, 21.82; HRMS (ESI) *m/z* calcd for $C_{22}H_{36}N_5O_{14}$ $[M-H]^-$: 594.2264, found 594.2282.

1.2.3. 3-Azidopropyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-O-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranoside (Neu5Ac9NAcα2–3Galβ1–3GlcNAcβProN$_3$, 3)—Yield 61%; 38 mg, White foam. ^{1}H NMR (400 MHz, D_2O) δ 4.58 (d, *J* = 7.8 Hz, 1H), 4.50 (d, *J* = 7.8 Hz, 1H), 4.07 (dd, *J* = 3.2 and 9.8 Hz, 1H), 4.03–3.47 (m, 19H), 3.39 (t, *J* = 6.4 Hz, 2H), 3.27 (dd, *J* = 7.6 and 14.2 Hz, 1H), 2.76 (dd, *J* = 4.8 and 12.8 Hz, 1H), 2.13–2.03 (m, 9H), 1.89–1.77 (m, 3H); ^{13}C NMR (100 MHz, D_2O) δ 174.79, 174.35, 174.25, 173,79, 103.25, 100.76, 99.70, 82.41, 75.52, 75.25, 74.95, 72.54, 69.82, 69.40, 69.03, 68.64, 68.24, 67.17, 67.02, 60.89, 60.62, 54.33, 51.55, 47.67, 41.93, 39.56, 27.98, 22.14, 21.92, 21.69; HRMS (ESI) *m/z* calcd for $C_{30}H_{49}N_6O_{19}$ [M-H] $^-$: 797.3058, found 797.3064.

1.2.4. 3-Azidopropyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-O-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (Neu5Ac9NAcα2–3Galβ1–4GlcNAcβProN$_3$, 4)—Yield 86%; 48 mg, White foam. ^{1}H NMR (400 MHz, D_2O) δ 4.56–4.52 (m, 2H), 4.11 (dd, *J* = 3.2 and 10.0 Hz, 1H), 4.04–3.82 (m, 6H), 3.80–3.54 (m, 12H), 3.50 (dd, *J* = 1.8 and 9.0 Hz, 1H), 3.39 (td, *J* = 1.8 and 6.6 Hz, 2H), 3.28 (dd, *J* = 7.8 and 14.0 Hz, 1H), 2.76 (dd, *J* = 4.6 and 12.4 Hz, 1H), 2.12–1.98 (m, 9H), 1.92–1.74 (m, 3H); ^{13}C NMR (100 MHz, D_2O) δ 174.93, 174.46, 174.39, 173.79, 102.54, 101.13, 99.87, 78.23, 75.53, 75.14, 74.76, 72.74, 72.33, 69.95, 69.58, 69.37, 68.30, 67.46, 67.11, 61.01, 59.99, 55.06, 51.65, 47.76, 42.12, 39.61, 28.08, 22.14, 22.02, 21.81; HRMS (ESI) *m/z* calcd for $C_{30}H_{49}N_6O_{19}$ [M-H] $^-$: 797.3058, found 797.3079.

1.2.5. 3-Azidopropyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-O-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside (Neu5Ac9NAcα2–3Galβ1–3GalNAcαProN$_3$, 5)—Yield 64%; 57 mg, White foam. ^{1}H NMR (400 MHz, D_2O) δ 4.92 (d, *J* = 3.8 Hz, 1H), 4.55 (d, *J* = 7.8 Hz, 1H), 4.32 (dd, *J* = 3.8 and 11.2 Hz, 1H), 4.28–4.23 (m, 1H), 4.16–3.39 (m, 20H), 3.28(dd, *J* = 7.6 and 14.2 Hz, 1H), 2.75 (dd, *J* = 4.6 and 12.4 Hz, 1H), 2.13–2.03 (m, 9H), 1.95–1.88 (m, 2H), 1.80 (t, *J* = 12.2 Hz, 1H); ^{13}C NMR (100 MHz, D_2O) δ 174.92, 174.51, 174.37, 173.90, 104.35, 99.88, 97.15, 77.44, 75.64, 74.72, 72.65, 70.60, 69.93, 69.49, 69.14, 68.51, 68.34, 67.43, 64.92, 61.20, 60.97, 51.66, 48.84, 48.66, 48.17, 42.03, 39.62, 27.94, 22.01, 21.82; HRMS (ESI) *m/z* calcd for $C_{30}H_{49}N_6O_{19}$ [M-H] $^-$: 797.3058, found 797.3072.

1.2.6. 3-Azidopropyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-O-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-galactopyranoside (Neu5Ac9NAcα2–3Galβ1–3GalNAcβProN$_3$, 6)—Yield 84%; 14.8 mg, White foam. ^{1}H NMR (600 MHz, D_2O) δ 4.51–4.46 (m, 2H), 4.16 (d, *J* = 3.2 Hz, 1H), 4.03–3.93 (m, 3H), 3.97–3.50 (m, 15H), 3.47–3.45 (m, 1H), 3.36 (td, *J* = 1.5 and 6.8 Hz, 2H), 3.23 (dd, *J* = 7.6 and 14.1 Hz, 1H), 2.71 (dd, *J* = 4.6 and 12.3 Hz, 1H) 2.03–1.96 (m, 9H), 1.87–1.80 (m, 2H), 1.75 (t, *J* = 12.1 Hz, 1H); ^{13}C NMR (151 MHz, D_2O) δ 174.80, 174.56, 174.27, 173.85, 104.39, 101.28, 99.72, 79.95, 75.45, 74.63(2C), 72.53, 69.82, 69.42, 68.95, 68.26, 67.71, 67.29, 66.88, 60.88, 60.83, 51.55, 51.04, 48.74, 41.94, 39.53, 28.00, 22.16, 21.92, 21.71. HRMS (ESI) *m/z* calcd for $C_{30}H_{49}N_6O_{19}$ [M-H] $^-$: 797.3058, found 797.3064

1.2.7. 3-Azidopropyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-O-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-α-D-glucopyranoside (Neu5Ac9NAcα2–3Galβ1–3GlcNAcαProN$_3$, 7)—Yield 63%; 85 mg, White foam. ^{1}H NMR (400 MHz, D_2O) δ 4.88 (d, *J* = 3.6 Hz, 1H), 4.53 (d, *J* = 7.8 Hz, 1H), 4.09 (ddd, *J* = 3.4, 7.4 and 10.0 Hz, 2H), 4.01–3.42 (m, 20H), 3.28 (dd, *J* = 7.6 and 14.2 Hz, 1H), 2.76 (dd, *J* = 4.6 and 12.4 Hz, 1H), 2.04–

2.03 (m, 9H), 1.95–1.89 (m, 2H), 1.80 (t, *J* = 12.2 Hz, 1H); ^{13}C NMR (100 MHz, D_2O) δ 174.91, 174.35(2C), 173.88, 103.26, 99.87, 97.04, 80.47, 75.67, 75.01, 72.66, 71.57, 69.94, 69.47, 69.22, 68.71, 68.34, 67.32, 64.96, 60.97, 60.51, 52.43, 51.66, 48.17, 42.01, 39.64, 27.93, 22.02, 21.99, 21.81; HRMS (ESI) *m/z* calcd for $C_{30}H_{49}N_6O_{19}$ [M-H] $^-$: 797.3058, found 797.3071.

1.2.8. 4-Nitrophenyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-β-D-galactopyranoside (Neu5Ac9NAcα2–6GalβpNP, 8)—Yield 64%; 69 mg, White foam. ^{1}H NMR (400 MHz, D_2O) δ 8.26–8.15 (m, 2H), 7.26–7.12 (m, 2H), 5.09 (d, *J* = 7.6 Hz, 1H), 3.95 (dd, *J* = 4.0 and 7.6 Hz, 2H), 3.91–3.56 (m, 8H), 3.47 (dd, *J* = 2.8 and 14.2 Hz, 1H), 3.36 (dd, *J* = 1.6 and 8.8 Hz, 1H), 3.14 (dd, *J* = 8.0 and 14.2 Hz, 1H), 2.67 (dd, *J* = 4.4 and 12.4 Hz, 1H), 1.94 (s, 3H), 1.86 (s, 3H), 1.67–1.49 (m, 1H); ^{13}C NMR (100 MHz, D_2O) δ 174.94, 174.30, 173.48, 161.84, 142.51, 126.12(2C), 116.39(2C), 100.42, 99.88, 74.04, 72.47, 72.33, 70.25, 69.96, 69.69, 68.36, 68.15, 63.02, 51.80, 42.15, 40.14, 21.98, 21.72; HRMS (ESI) Anal. Calcd for $C_{25}H_{34}N_3O_{16}$ [M-H] $^-$: 632.1945, Found: 632.1955.

1.2.9. 3-Azidopropyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-β-D-galactopyranoside (Neu5Ac9NAcα2–6GalβProN$_3$, 9)—Yield 92%; 107 mg, White foam. ^{1}H NMR (400 MHz, D_2O) δ 4.39 (d, *J* = 7.8 Hz, 1H), 4.05–3.88 (m, 4H), 3.87–3.57 (m, 8H), 3.54–3.44 (m, 4H), 3.28 (dd, *J* = 7.8 and 14.0 Hz, 1H), 2.73 (dd, *J* = 4.8 and 12.4 Hz, 1H), 2.04–2.02 (m, 6H), 1.95–1.89 (m, 2H), 1.70 (t, *J* = 12.2 Hz, 1H); ^{13}C NMR (100 MHz, D_2O) δ 174.98, 174.37, 173.41, 102.90, 100.47, 73.39, 72.57, 72.48, 70.65, 69.94, 69.69, 68.54, 68.17, 67.45, 63.29, 51.83, 47.87, 42.13, 40.17, 28.30, 22.01, 21.82; HRMS (ESI) *m/z* calcd for $C_{22}H_{36}N_5O_{14}$ [M-H] $^-$: 594.2264, found 594.2285.

1.2.10. 3-Azidopropyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranoside (Neu5Ac9NAcα2–6Galβ1–3GlcNAcβProN$_3$, 10)—Yield 68%; 34 mg, White foam. ^{1}H NMR (400 MHz, D_2O) δ 4.58 (d, *J* = 8.4 Hz, 1H), 4.39 (d, *J* = 7.8 Hz, 1H), 4.05–3.45 (m, 20H), 3.39 (td, *J* = 1.2 and 6.6 Hz, 2H), 3.30 (dd, *J* = 7.6 and 14.2 Hz, 1H), 2.70 (dd, *J* = 4.8 and 12.4 Hz, 1H), 2.10–1.89 (m, 9H), 1.89–1.83 (m, 2H), 1.70 (t, *J* = 12.2 Hz, 1H); ^{13}C NMR (100 MHz, D_2O) δ 174.84, 174.59, 174.38, 173.46, 103.89, 100.88, 100.11, 84.01, 75.46, 73.61, 72.37, 72.28, 70.48, 69.93, 68.91(2C), 68.48, 68.25, 67.14, 63.56, 60.87, 54.30, 51.78, 47.78, 42.15, 40.12, 28.08, 22.19, 22.03, 21.83; HRMS (ESI) *m/z* calcd for $C_{30}H_{49}N_6O_{19}$ [M-H] $^-$: 797.3058, found 797.3065.

1.2.11. 3-Azidopropyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (Neu5Ac9NAcα2–6Galβ1–4GlcNAcβProN$_3$, 11)—Yield 91%; 56 mg, White foam. ^{1}H NMR (400 MHz, D_2O) δ 4.62–4.52 (m, 1H), 4.45 (d, *J* = 7.8 Hz, 1H), 4.04–3.89 (m, 5H), 3.88–3.51(m, 14H), 3.45 (dd, *J* = 1.8 and 9.0 Hz, 1H), 3.39 (td, *J* = 1.4 and 6.6 Hz, 2H), 3.30 (dd, *J* = 7.8 and 14.0 Hz, 1H), 2.76 (dd, *J* = 4.6 and 12.4 Hz, 1H), 2.16–1.98 (m, 9H), 1.89–1.83 (m, 2H), 1.71 (t, *J* = 12.2 Hz, 1H); ^{13}C NMR (100 MHz, D_2O) δ 174.83, 174.51, 174.39, 173.49, 103.46, 100.95, 100.18, 80.74, 74.45, 73.68, 72.39(2C), 70.70, 69.87(2C), 68.39, 68.17, 67.09, 63.44, 61.75, 60.34, 54.83, 51.85, 47.77, 42.14, 40.06, 28.09, 22.27, 22.01, 21.83; HRMS (ESI) *m/z* calcd for $C_{30}H_{49}N_6O_{19}$ [M-H] $^-$: 797.3058, found 797.3063.

1.2.12. 3-Azidopropyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside (Neu5Ac9NAcα2–6Galβ1–3GalNAcαProN$_3$, 12)—Yield 98%; 86 mg, White foam. ^{1}H NMR (400 MHz, D_2O) δ 4.91 (d, *J* = 3.6 Hz, 1H), 4.46 (d, *J* = 7.8 Hz, 1H), 4.33 (dd, *J* = 3.8 and 11.2 Hz, 1H), 4.29–4.24 (m, 1H), 4.10–3.41 (m, 20H), 3.31 (dd, *J* = 7.6 and 14.0 Hz, 1H), 2.74 (dd, *J* = 4.8 and 12.4 Hz, 1H), 2.13–1.99 (m, 9H), 1.95–1.89 (m, 2H), 1.66 (t, *J* = 12.2 Hz, 1H); ^{13}C NMR (100 MHz, D_2O) δ 174.98, 174.51, 174.40, 173.40, 104.52, 100.39, 97.16, 77.27, 73.20, 72.43, 72.37, 70.73, 70.48, 69.81, 69.65, 68.67, 68.56, 68.20, 64.90, 63.48, 61.42, 51.86, 48.72, 48.19, 42.08, 40.19, 27.96, 22.00, 21.98, 21.82; HRMS (ESI) *m/z* calcd for $C_{30}H_{49}N_6O_{19}$ [M-H] $^-$: 797.3058, found 797.3079.

1.2.13. 3-Azidopropyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-galactopyranoside (Neu5Ac9NAcα2–6Galβ1–3GalNAcβProN$_3$, 13)—Yield 96%; 16.8 mg, White foam. ^{1}H NMR (600 MHz, D_2O) δ 4.47–4.38 (m, 2H), 4.17 (dd, *J* = 3.2 Hz, 1H), 3.98–3.55 (m, 17H), 3.51–3.42 (m, 2H), 3.35 (td, *J* = 4.5 and 6.5 Hz, 2H), 3.26–3.21 (m, 1H), 2.68 (dd, *J* = 4.6 and 12.4 Hz, 1H), 2.11–1.89 (m, 9H), 1.84–1.77 (m, 2H), 1.64 (t, *J* = 12.2 Hz, 1H); ^{13}C NMR (151 MHz, D_2O) δ 174.85, 174.58, 174.28, 173.31, 104.79, 101.38, 100.35, 79.80, 74.85, 73.00, 72.40, 72.38, 70.43, 69.81, 69.60, 68.48, 68.08, 67.72, 67.11, 63.26, 60.85, 52.24, 51.70, 51.07, 42.07, 40.11, 28.06, 22.11, 21.90, 21.72, HRMS (ESI) *m/z* calcd for $C_{30}H_{49}N_6O_{19}$ [M-H] $^-$: 797.3058, found 797.3067

1.2.14. 3-Azidopropyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-α-D-glucopyranoside (Neu5Ac9NAcα2–6Galβ1–3GlcNAcαProN$_3$, 14)—Yield 76%; 51mg, White foam. ^{1}H NMR (400 MHz, D_2O) δ 4.87 (d, *J* = 3.6 Hz, 1H), 4.39 (d, *J* = 7.8 Hz, 1H), 4.10 (dd, *J* = 3.6 and 10.6 Hz, 1H), 4.03–3.37 (m, 21H), 3.30 (dd, *J* = 7.7 and 14.1 Hz, 1H), 2.70 (dd, *J* = 4.7 and 12.4 Hz, 1H), 2.09–1.94 (m, 9H), 1.94–1.90 (m, 2H), 1.71 (t, *J* = 12.2 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) δ 174.83, 174.42, 174.38, 173.48, 103.71, 100.12, 96.91, 81.81, 73.60, 72.42, 72.29, 71.58, 70.53, 69.93, 69.91, 68.79, 68.50, 68.26, 64.91, 63.51, 60.64, 52.27, 51.78, 48.14, 42.15, 40.14, 27.96, 22.03, 21.93, 21.82; HRMS (ESI) *m/z* calcd for $C_{30}H_{49}N_6O_{19}$ [M-H] $^-$: 797.3058, found 797.3078.

1.2.15. 3-Azidopropyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-O-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (Neu5Ac9NAcα2–6Galβ1–4GlcβProN$_3$, 15)—Yield 94%; 73

mg, White foam. ^{1}H NMR (400 MHz, D_2O) δ 4.50 (d, *J* = 8.0 Hz, 1H), 4.44 (d, *J* = 7.8 Hz, 1H), 4.04–3.24 (m, 23H), 2.71(dd, *J* = 4.8 and 12.4 Hz, 1H), 2.14–2.03 (m, 6H), 1.96–1.89 (m, 2H), 1.74 (t, *J* = 12.2 Hz, 1H); ^{13}C NMR (100 MHz, D_2O) δ 174.82, 174.39, 173.43, 103.18, 101.98, 100.30, 79.62, 74.62, 74.59, 73.68, 72.71, 72.33, 70.75, 69.93, 69.89, 68.52, 68.33, 67.31, 63.62, 60.24, 51.75, 48.85, 47.86, 42.16, 40.06, 28.22, 22.04, 21.84; HRMS (ESI) *m/z* calcd for $C_{28}H_{46}N_5O_{19}$ [M-H] $^-$: 756.2792, found 756.2812.

1.2.16. 3-Azidopropyl O-(5,9-diacetamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→6)-2-acetamido-2-deoxy-α-D-galactopyranoside (Neu5Ac9NAcα2–6GalNAcαProN$_3$, 16)—Yield 71%; 78 mg, White foam. ^{1}H NMR (400 MHz, D_2O) δ 4.89 (d, *J* = 3.6 Hz, 1H), 4.15 (dd, *J* = 3.8 and 11.2 Hz, 1H), 4.06–4.00 (m, 2H), 3.96–3.89 (m, 3H), 3.84–3.78 (m, 2H), 3.74–3.41 (m, 8H), 3.29 (dd, *J* = 7.8 and 14.0 Hz, 1H), 2.73 (dd, *J* = 4.8 and 12.4 Hz, 1H), 2.13–2.03 (m, 9H), 1.94–1.88 (m, 2H), 1.69 (t, *J* = 12.2 Hz, 1H); ^{13}C NMR (100 MHz, D_2O) δ 174.94, 174.51, 174.37, 173.37, 100.37, 97.04, 72.40, 69.96, 69.74, 69.49, 68.49, 68.21, 67.46, 65.15, 63.79, 51.82, 49.92, 48.14, 42.14, 40.23, 27.91, 22.01, 21.92, 21.82; HRMS (ESI) *m/z* calcd for $C_{24}H_{39}N_6O_{14}$ [M-H] $^-$: 635.2530, found 635.2554.

1.3. Microtiter plate-based sialidase substrate specificity assays

Assays were carried out in duplicates. For each reaction in a final volume of 20 μL, a sialoside was incubated with an appropriate amount of a sialidase and an excess amount of β-galactosidase (12 μg) in a buffer solution in a 384-well plate at 37 °C for 30 min. The sialidase amounts and buffers used were: *A. ureafaciens* sialidase (0.5 mU), NaOAc buffer (100 mM, pH 5.5); *C. perfringens* sialidase (0.75 mU), MES buffer (100 mM, pH 5.0); *V. cholerae* sialidase (1.5 mU), NaCl (150 mM), $CaCl_2$ (10 mM), NaOAc buffer (100 mM, pH 5.5); SpNanA (1.5 ng), NaOAc buffer (100 mM, pH 6.0); SpNanB (3 ng), NaOAc buffer (100 mM, pH 6.0); SpNanC (20 ng), MES buffer (100 mM, pH 6.5); PmST1 (0.4 μg), NaOAc buffer (100 mM, pH 5.5), CMP (0.4 mM); hNEU2 (1.3 μg), MES buffer (100 mM, pH 5.0); BiNanH2 (4 ng), NaOAc buffer (100 mM, pH 5.0). The reactions were stopped by adding 40 μL of *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer (0.5 M, pH 10.5) to adjust pH to higher than 9.5 and $A_{405\ nm}$ values of samples were read by a microplate reader [15].

1.4. HPLC-based assays for PmST1, SpNanC, C. perfringens sialidase, and BiNanH2

Assays were carried out as described above for microtiter plate-based assays. The reactions were stopped by adding 40 μL of pre-chilled ethanol. The mixtures were then centrifuged and the supernatants were analyzed by Agilent 1290 Infinity HPLC system at 315 nm. A C14 reverse phase Rapid Resolution High Definition column (BONUS RP RRHD 1.8 μm, 2.1 × 150 mm, Agilent) was used for analyzing samples with Neu5Acα2–3Galβ*p*NP (**17**), Neu5,9Ac$_2$α2–3Galβ*p*NP (**19**), Neu5Ac9NAcα2–6Galβ*p*NP (**8**), or Neu5Acα2–6Galβ*p*NP (**18**). A C18 reverse phase Rapid Resolution High Definition column (EclipsePlusC18 RRHD 1.8 μm, 2.1 × 50 mm, Agilent) was used for analyzing samples with Neu5Ac9NAcα2–3Galβ*p*NP (**1**) or Neu5,9Ac$_2$α2–6Galβ*p*NP (**20**). The mobile phases used were acetonitrile (ACN) in H_2O mixed solvent with varied percentages of acetonitrile: 4.5% for Neu5Acα2–6Galβ*p*NP (**18**); 6.5% for Neu5Ac9NAcα2–3Galβ*p*NP (**1**) or

Neu5,9Ac$_2$α2–6GalβpNP (**20**); 9% for Neu5Ac9NAcα2–6GalβpNP (**8**); and12% for Neu5Acα2–3GalβpNP (**17**) or Neu5,9Ac$_2$α2–3GalβpNP (**19**).

1.5. Time course studies for BiNanH2

Time course studies for BiNanH2 were carried out in duplicate at 37 °C in reaction mixtures (200 μL each) containing NaOAc buffer (100 mM, pH 5.0), BiNanH2 (220 ng/mL), and a sialidase substrate (0.3 mM) selected from Neu5Acα2–3GalβpNP (**17**), Neu5Acα2–6GalβpNP (**18**), Neu5Ac9NAcα2–3GalβpNP (**1**), and Neu5Ac9NAcα2–6GalβpNP (**8**). Aliquots (20 μL each) were taken at 5, 10, 15, 20, 30, 45, or 60 min intervals and added to microcentrifuge tubes (500 μL) containing 40 μL of pre-chilled ethanol. The mixtures were centrifuged on a bench-top centrifuge (13,000 rpm × 3 min). The supernatants (45 μL) were analyzed by Agilent 1290 Infinity HPLC system at 315 nm as described above.

1.6. Kinetic studies for BiNanH2

The kinetic studies for BiNanH2 were performed in duplicates at 37 °C for 10 min in a total volume of 20 μL each containing NaOAc buffer (100 mM, pH 5.0), a sialidase substrate [selected from Neu5Ac9NAcα2–3GalβpNP (**1**), Neu5Ac9NAcα2–6GalβpNP (**8**), Neu5Acα2–3GalβpNP (**17**), and Neu5Acα2–6GalβpNP (**18**)], and BiNanH2 (5.8 ng when compound **1** or **17** was used as the substrate and 1.5 ng when compound **8** or **18** was used as the substrate). The reactions were stopped by adding 40 μL of pre-chilled ethanol. The mixtures were then centrifuged and the supernatants were analyzed by the HPLC system described above for HPLC-based assays. Apparent kinetic parameters were obtained by varying substrate concentrations from 0.1–40 mM (0.1, 0.2, 0.4, 1, 2, 4, 10, 20, and 40 mM) and fitting the data (the average values of duplicate assay results) into the Michaelis–Menten equation using Grafit 5.0.

1.7. Kinetic studies for SpNanC

The kinetic studies for SpNanC were performed in duplicates at 37 °C for 10 min in a total volume of 20 μL each containing MES buffer (100 mM, pH 6.5), a sialidase substrate [selected from Neu5Ac9NAcα2–3GalβpNP (**1**), Neu5Acα2–3GalβpNP (**17**), and Neu5,9Ac$_2$α2–3GalβpNP (**19**)], and SpNanC (2.5 ng when compound **1** or **17** was used as the substrate and 1.5 ng when compound **19** was used as the substrate). After stopping the reactions by by adding 40 μL of pre-chilled ethanol, the mixtures were centrifuged and the supernatants were analyzed by the HPLC system as described above. Apparent kinetic parameters were obtained by varying substrate concentrations from 0.1–40 mM (0.1, 0.2, 0.4, 1, 2, 4, 10, 20, and 40 mM) and fitting the data (the average values of duplicate assay results) into the Michaelis–Menten equation using Grafit 5.0.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was partially supported by National Institutes of Health (NIH) Common Fund grant U01GM120419. Bruker Avance-800 NMR spectrometer was funded by National Science Foundation grant DBIO-722538. Y.L.,

H.Y., and X.C. are co-founders of Glycohub, Inc., a company focusing on the development of carbohydrate-based reagents, diagnostics, and therapeutics. Glycohub, Inc. played no role in the design, execution, interpretation, or publication of this study.

References

1. Varki A, Gagneux P. Ann N Y Acad Sci. 2012; 1253:16–36. [PubMed: 22524423]
2. Chen X, Varki A. ACS Chem Biol. 2010; 5:163–176. [PubMed: 20020717]
3. Angata T, Varki A. Chem Rev. 2002; 102:439–470. [PubMed: 11841250]
4. Schauer R. Glycoconj J. 2000; 17:485–499. [PubMed: 11421344]
5. Klein A, Roussel P. Biochimie. 1998; 80:49–57. [PubMed: 9587662]
6. Varki A, Diaz S. Anal Biochem. 1984; 137:236–247. [PubMed: 6731802]
7. Kamerling JP, Schauer R, Shukla AK, Stoll S, Halbeek H, Vliegenthart J. Eur J Biochem. 1987; 162:601–607. [PubMed: 3830159]
8. Klotz FW, Orlandi PA, Reuter G, Cohen SJ, Haynes JD, Schauer R, Howard RJ, Palese P, Miller LH. Mol Biochem Parasitol. 1992; 51:49–54. [PubMed: 1565137]
9. Rogers GN, Herrler G, Paulson J, Klenk H. J Biol Chem. 1986; 261:5947–5951. [PubMed: 3700379]
10. Varki A, Kornfeld S. J Exp Med. 1980; 152:532–544. [PubMed: 7411019]
11. Khedri Z, Xiao A, Yu H, Landig CS, Li W, Diaz S, Wasik BR, Parrish CR, Wang LP, Varki A, Chen X. ACS Chem Biol. 2017; 12:214–224. [PubMed: 27936566]
12. Herrler G, Gross HJ, Imhof A, Brossmer R, Milks G, Paulson JC. J Biol Chem. 1992; 267:12501–12505. [PubMed: 1618756]
13. Bakkers MJ, Zeng Q, Feitsma LJ, Hulswit RJ, Li Z, Westerbeke A, van Kuppeveld FJ, Boons GJ, Langereis MA, Huizinga EG, de Groot RJ. Proc Natl Acad Sci U S A. 2016; 113:E3111–3119. [PubMed: 27185912]
14. Yu H, Chokhawala HA, Huang S, Chen X. Nat Protoc. 2006; 1:2485–2492. [PubMed: 17406495]
15. Chokhawala HA, Yu H, Chen X. Chembiochem. 2007; 8:194–201. [PubMed: 17195254]
16. Khedri Z, Muthana MM, Li Y, Muthana SM, Yu H, Cao H, Chen X. Chem Commun. 2012; 48:3357–3359.
17. Yu H, Cao H, Tiwari VK, Li Y, Chen X. Bioorg Med Chem Lett. 2011; 21:5037–5040. [PubMed: 21592790]
18. Li Y, Cao H, Dao N, Luo Z, Yu H, Chen Y, Xing Z, Baumgarth N, Cardona C, Chen X. Virology. 2011; 415:12–19. [PubMed: 21501853]
19. Li Y, Yu H, Cao H, Lau K, Muthana S, Tiwari VK, Son B, Chen X. Appl Microbiol Biotechnol. 2008; 79:963–970. [PubMed: 18521592]
20. Yu H, Yu H, Karpel R, Chen X. Bioorg Med Chem. 2004; 12:6427–6435. [PubMed: 15556760]
21. Sun M, Li Y, Chokhawala HA, Henning R, Chen X. Biotechnol Lett. 2008; 30:671–676. [PubMed: 17989925]
22. Yu H, Chokhawala H, Karpel R, Yu H, Wu B, Zhang J, Zhang Y, Jia Q, Chen X. J Am Chem Soc. 2005; 127:17618–17619. [PubMed: 16351087]
23. Sugiarto G, Lau K, Qu J, Li Y, Lim S, Mu S, Ames JB, Fisher AJ, Chen X. ACS Chem Biol. 2012; 7:1232–1240. [PubMed: 22583967]
24. Yu H, Huang S, Chokhawala H, Sun M, Zheng H, Chen X. Angew Chem Int Ed Engl. 2006; 45:3938–3944. [PubMed: 16721893]
25. Ding L, Yu H, Lau K, Li Y, Muthana S, Wang J, Chen X. Chem Commun. 2011; 47:8691–8693.
26. Ding L, Zhao C, Qu J, Li Y, Sugiarto G, Yu H, Wang J, Chen X. Carbohydr Res. 2015; 408:127–133. [PubMed: 25593075]
27. Ye J, Liu X, Peng P, Yi W, Chen X, Wang F, Cao H. ACS Catal. 2016; 6:8140–8844.
28. Li Y, Cao H, Yu H, Chen Y, Lau K, Qu J, Thon V, Sugiarto G, Chen X. Mol BioSyst. 2011; 7:1060–1072. [PubMed: 21206954]

29. Sela DA, Li Y, Lerno L, Wu S, Marcobal AM, German JB, Chen X, Lebrilla CB, Mills DA. J Biol Chem. 2011; 286:11909–11918. [PubMed: 21288901]
30. Tasnima N, Yu H, Li Y, Santra A, Chen X. Org Biomol Chem. 2016; 15:160–167. [PubMed: 27924345]
31. Owen CD, Lukacik P, Potter JA, Sleator O, Taylor GL, Walsh MA. J Biol Chem. 2015; 290:27736–27748. [PubMed: 26370075]
32. Xu G, Kiefel MJ, Wilson JC, Andrew PW, Oggioni MR, Taylor GL. J Am Chem Soc. 2011; 133:1718–1721. [PubMed: 21244006]

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/...

Author Manuscript

Author Manuscript

Author Manuscript

Highlights

- Sixteen new Neu5Ac9NAc-containing sialosides are successfully synthesized
- One-pot multienzyme (OPME) sialylation systems are highly efficient for the synthesis
- High-throughput microtiter plate assay results were confirmed by HPLC-based methods
- 9-*N*-Acetyl Neu5Ac is a good mimic of 9-*O*-acetyl Neu5Ac to probe most sialidases
- Exceptions exist and mechanism needs further investigation

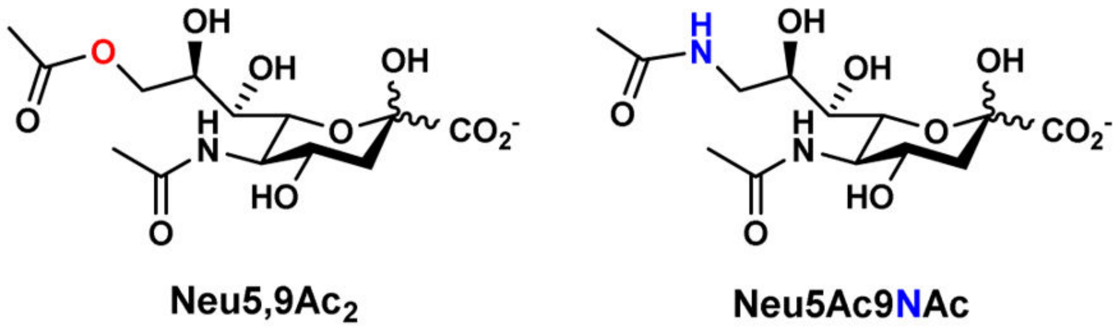


Figure 1.

Structures of 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9Ac$_2$) and its 9-*N*-acetyl analog, 9-acetamido-9-deoxy-*N*-acetylneuraminic acid (Neu5Ac9NAc).

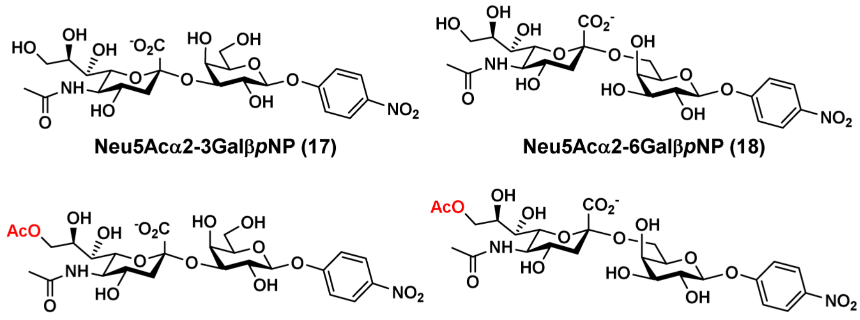


Figure 2.

Structures of Neu5Ac-sialosdies Neu5Acα2–3Galβ*p*NP (**17**) and Neu5Acα2–6Galβ*p*NP (**18**) as well as Neu5,9Ac$_2$-sialosides Neu5,9Ac$_2$α2–3Galβ*p*NP (**19**) and Neu5,9Ac$_2$α2–6Galβ*p*NP (**20**) used as substrates for microtiter plate-based high-throughput sialidase assays.

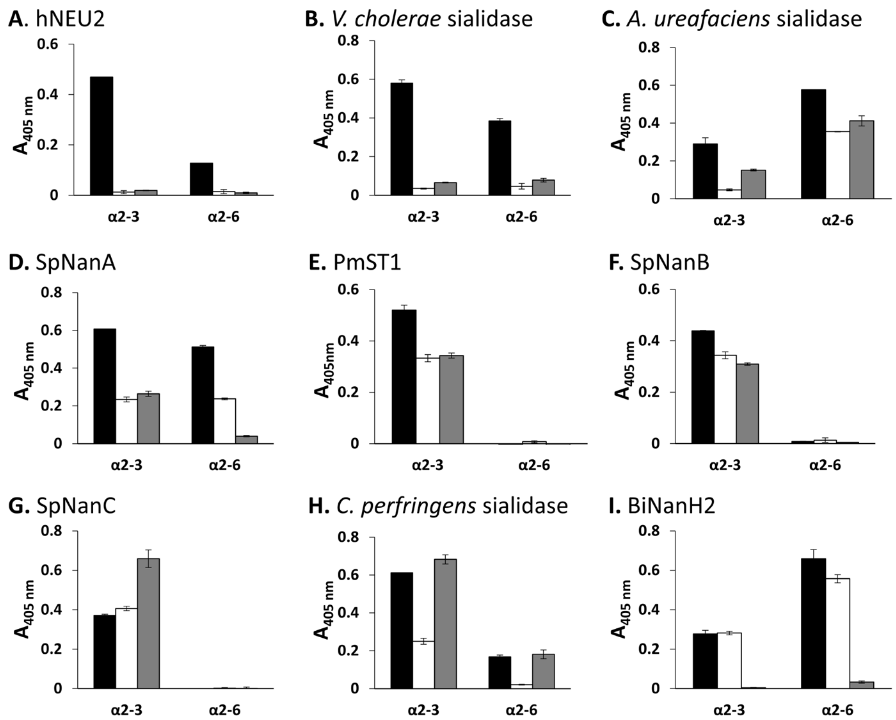
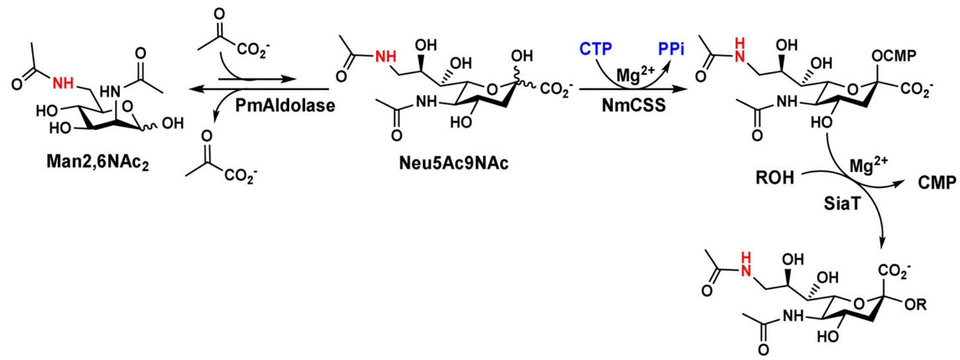


Figure 3.

Sialidase substrate specificity studies using Neu5Ac-sialosides (black bars) Neu5Acα2–3Galβ*p*NP (**17**) and Neu5Acα2–6Galβ*p*NP (**18**), Neu5Ac9NAc-sialosdies (white bars) Neu5Ac9NAcα2–3Galβ*p*NP (**1**) and Neu5Ac9NAcα2–6Galβ*p*NP (**8**), as well as Neu5,9Ac$_2$-sialosides (grey bars) Neu5,9Ac$_2$α2–3Galβ*p*NP (**19**) and Neu5,9Ac$_2$α2–6Galβ*p*NP (**20**) as substrates.

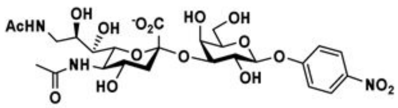
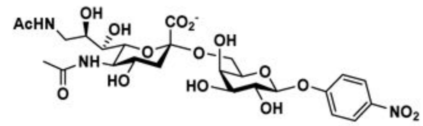
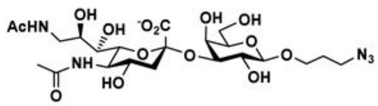
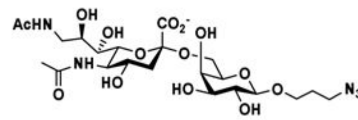
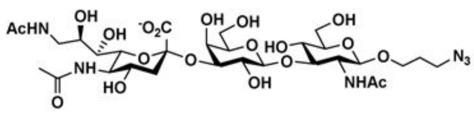
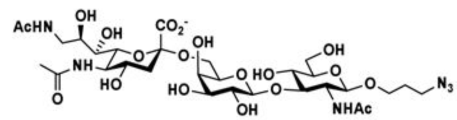
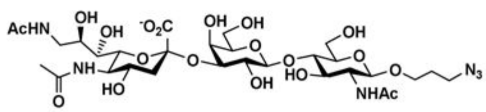
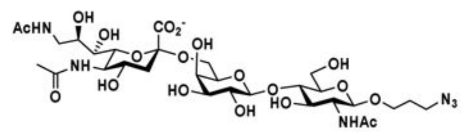
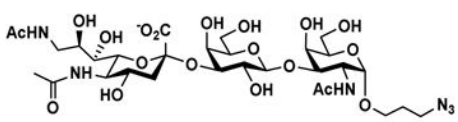
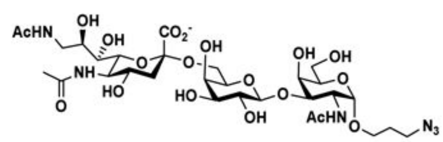
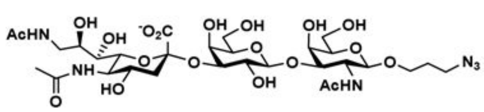
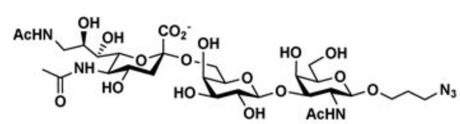
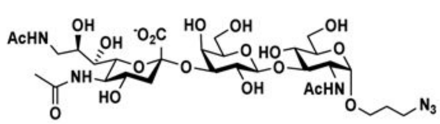
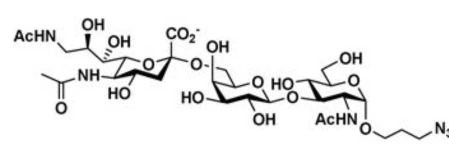


Scheme 1.

One-pot three-enzyme chemoenzymatic synthesis of Neu5Ac9NAc-containing α2–3- and α2–6-linked sialosides.

Table 1

Neu5Ac9NAc-containing sialosides synthesized via the OP3E sialylation system.

α 2–3/6-Sialosides	Yield (Comp#)	α 2–6-Sialosides	Yield (Comp#)
 Neu5Ac9NAcα2–3Galβ*p*NP	80% (**1**)	 Neu5Ac9NAcα2–6Galβ*p*NP	64% (**8**)
 Neu5Ac9NAcα2–3GalβProN_3	72% (**2**)	 Neu5Ac9NAcα2–6GalβProN_3	92% (**9**)
 Neu5Ac9NAcα2–3Galβ1–3GlcNAcβProN_3	61% (**3**)	 Neu5Ac9NAcα2–6Galβ1–3GlcNAcβProN_3	68% (**10**)
 Neu5Ac9NAcα2–3Galβ1–4GlcNAcβProN_3	86% (**4**)	 Neu5Ac9NAcα2–6Galβ1–4GlcNAcβProN_3	91% (**11**)
 Neu5Ac9NAcα2–3Galβ1–3GalNAcαProN_3	64% (**5**)	 Neu5Ac9NAcα2–6Galβ1–3GalNAcαProN_3	98% (**12**)
 Neu5Ac9NAcα2–3Galβ1–3GalNAcβProN_3	84% (**6**)	 Neu5Ac9NAcα2–6Galβ1–3GalNAcβProN_3	96% (**13**)
 Neu5Ac9NAcα2–3Galβ1–3GlcNAcαProN_3	63% (**7**)	 Neu5Ac9NAcα2–6Galβ1–3GlcNAcαProN_3	76% (**14**)

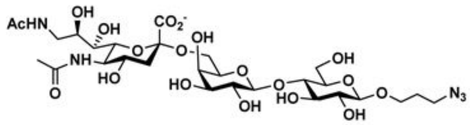
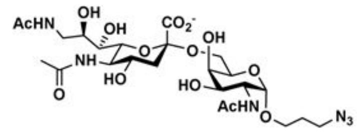
α 2–3/6-Sialosides	**Yield (Comp#)**	**α 2–6-Sialosides**	**Yield (Comp#)**
 Neu5Ac9NAcα2–6Galβ1–4GlcβProN_3	94% (**15**)	 Neu5Ac9NAcα2–6GalNAcαProN_3	71% (**16**)

Table 2

Apparent kinetics parameters for SpNanC and BiNanH2.

Sialidases	Substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})
SpNanC	Neu5Acα2–3Galβ*p*NP (**17**)	(3.65 ± 0.10)×10^2	2.66 ± 0.3	137
	Neu5Ac9NAcα2–3Galβ*p*NP (**1**)	(3.38 ± 0.15)×10^2	2.60 ± 0.4	130
	Neu5,9Ac_2α β2–3Gal *p*NP (**19**)	(3.78 ± 0.12)×10^2	1.25 ± 0.2	302
BiNanH2	Neu5Acα2–3Galβ*p*NP (**17**)	(1.66 ± 0.02)×10^2	2.8 ± 0.1	59.3
	Neu5Ac9NAcα2–3Galβ*p*NP (**1**)	(1.08 ± 0.02)×10^2	1.61 ± 0.2	66.7
	Neu5Acα2–6Galβ*p*NP (**18**)	(3.31 ± 0.06)×10^2	1.37 ± 0.1	242
	Neu5Ac9NAcα2–6Galβ*p*NP (**8**)	(8.07 ± 0.08)×10^2	4.51 ± 0.2	179