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A Chemoenzymatic Synthron Strategy for Synthesizing *N*-Acetyl Analogs of *O*-Acetylated *N. meningitidis* W Capsular Polysaccharide Oligosaccharides

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Abstract

O-Acetylated sialic acid has been found in the *Neisseria meningitidis* serogroup W (NmW) capsular polysaccharide (CPS) and is a required structural component of clinically used NmW CPS-based polysaccharide and polysaccharide-conjugate vaccines. The role of sialic acid *O*-acetylation in NmW CPS, however, is not clearly understood. This is partially due to the lack of a precise control of the percentage and the location of *O*-acetylation which is labile and susceptible to migration. We explore chemoenzymatic synthetic strategies of preparing *N*-acetylated analogs of *O*-acetylated NmW CPS oligosaccharides which can serve as structurally stable probe mimics. Substrate specificity studies of NmW CPS polymerase (NmSiaD_W) identified 4-azido-4-deoxy-*N*-acetylmannosamine (ManNAc4N₃) and 6-azido-6-deoxy-*N*-acetylmannosamine (ManNAc6N₃) as suitable chemoenzymatic synthons for synthesizing *N*-acetyl analogs of NmW CPS oligosaccharides containing 7-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,7Ac₂) and/or 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9Ac₂). The synthesis was achieved by NmSiaD_W-dependent sequential one-pot multienzyme (OPME) strategy with *in situ* generation of the corresponding sugar nucleotides from simple monosaccharides or derivatives to form N₃-oligosaccharides which were converted to the desired NAc-oligosaccharides by an efficient one-step chemical transformation.

Graphical Abstract

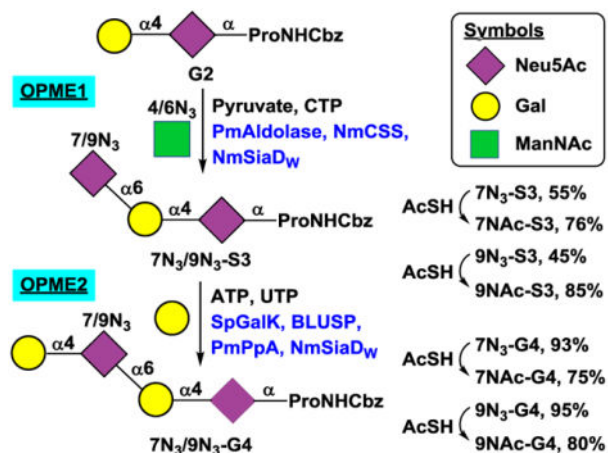
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Supporting Information

Structures of CMP-sialic acids and precursors; HSQC spectra comparison of S3 and 7N₃-S3 or 9N₃-S3; ¹H, ¹³C, HSQC and HSQC-TOCSY NMR spectra of the products. The Supporting Information is available free of charge on the ACS Publications website.

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Keywords

capsular polysaccharide; chemoenzymatic synthesis; glycosyltransferase; *N*-acetyl sialic acid; *O*-acetyl sialic acid

INTRODUCTION

O-Acetylation is a common modification of carbohydrates^{1–2} including sialic acids which are a family of monosaccharides belonging to nine-carbon α -keto acids (so called nonulosonic acids).^{3–5} In addition to be an important structural component of animal glycomes, sialic acids have been found in numerous bacteria. Among more than 50 different sialic acid forms found in nature, *N*-acetylneuraminic acid (Neu5Ac) is the most common form. Neu5Ac in the capsular polysaccharides (CPSs) or the lipopolysaccharides (LPSs) of bacteria is often *O*-acetylated.² For example, among six *Neisseria meningitidis* (Nm) serogroups (A, B, C, W, X, and Y) that cause life threatening invasive meningococcal diseases (IMDs) including meningitis and septicemia,^{6–8} four (except for serogroups A and X) have Neu5Ac in their CPSs and Neu5Ac *O*-acetylation has been observed for CPSs from three Nm serogroups (C, W, and Y).⁹ NmW strains have various levels of CPS *O*-acetylation.^{9–11} It was shown that *O*-acetylation of Neu5Ac in NmW CPS (a heteropolymer with a repeating unit of $-6\text{Gal}\alpha 1-4\text{Neu5Ac}\alpha 2-$) (Figure 1)^{12–14} may not be immunologically essential^{15–16} but can influence the efficacy of CPS periodate-treatment for conjugation with protein carriers in the development of conjugate vaccines.¹⁵ The World Health Organization (WHO) also specified the requirement of having minimal 0.3 mmol *O*-acetyl content per gram of polysaccharide in the NmW CPS polysaccharide vaccine.¹⁷

Nevertheless, the role of *O*-acetylation in NmW CPS remains unclear and the percentage of *O*-acetylation is suggested to be a parameter for vaccine quality control.² The lack of the understanding is partially due to the low abundance of *O*-acetylation in the CPSs of some NmW strains,² variation of *O*-acetylation levels due to manufacturing, instability of *O*-acetylation under basic conditions,¹⁰ and *O*-acetyl migration leading to the variation of site distribution of *O*-acetyl group.⁹

The detailed biosynthetic process for the formation of *O*-acetylated NmW CPS is also not clear despite the identification of the corresponding *O*-acetyltransferase encoded by *oatWY*¹⁸ and the report of its crystal structures in the presence or the absence of coenzyme A (CoA), acetyl-CoA and its non-hydrolyzable analog.¹⁹ For example, *O*-acetylation was observed at either C7 or C9 of Neu5Ac in NmW CPS while only one *O*-acetyltransferase gene *oatWY* was identified from NmW and NmY strains.¹⁸ The absence of a suitable acceptor in the crystal structures of NmOatWY¹⁹ and *O*-acetyl migration from C7 to C9 in Neu5Ac of NmW CPS observed by nuclear magnetic resonance (NMR) spectroscopy studies⁹ did not allow the confirmation of the specific *O*-acetylation site of the *O*-acetyltransferase NmOatWY. It was shown that NmOatWY was able to catalyze the transfer of acetyl from acetyl-CoA to NmY CPS polysaccharides and disaccharide (at a lower efficiency) but was unable to acetylate monosaccharide Neu5Ac or cytidine 5'-monophosphate-Neu5Ac (CMP-Neu5Ac).¹⁹ However, the detailed relationship of its reaction efficiency and the structures of its carbohydrate acceptor substrates is not clear. Therefore, it is impractical to obtain structurally defined NmW CPS with *O*-acetyl groups at designated locations by biosynthesis. Furthermore, the high-cost of acetyl-CoA, the required activated acetyl donor for acetyltransferases, prohibits large-scale enzymatic synthesis of *O*-acetylated NmW CPS or oligosaccharides by NmOatWY. On the other hand, chemical synthesis of *O*-acetylated NmW CPS oligosaccharides is possible but will be challenging. The *O*-acetyl migration, the sensitivity of *O*-acetyl group to pH variation and esterase-catalyzed cleavage²⁰ will cause obstacles in accurately interpreting results even if pure products are used in biological studies including immunological evaluations of the vaccine candidates.

We showed previously that replacing the labile *O*-acetyl group in 9-*O*-acetylated sialosides by the more stable *N*-acetyl group^{21–22} was a promising strategy to overcome the challenges of *O*-acetyl cleavage and/or migration in investigating their functional roles. For example, 9-acetamido-9-deoxy-Neu5Ac (Neu5Ac9NAc) was designed as a stable mimic of 9-*O*-acetyl-Neu5Ac (Neu5,9Ac₂).²¹ As determined by systematic NMR spectroscopic and molecular dynamics simulation studies, Neu5,9Ac₂-containing GM3 ganglioside glycan and its Neu5Ac9NAc analog are similar on their overall secondary structures and conformations without substantial differences on the dihedral angles of their glycosidic bonds.²² Neu5Ac9NAc-containing sialosides were applied in protein-binding, cell feeding, and sialidase substrate specificity studies as good mimics of the corresponding Neu5,9Ac₂-counterparts but with a higher stability.^{21, 23–24} Neu5Ac9NAc and 4-acetamido-4-deoxy-Neu5Ac (Neu5Ac4NAc), a stable mimic of 4-*O*-acetyl-Neu5Ac (Neu4,5Ac₂), were also used in a protein crystallography study of viral hemagglutinin-esterases.²⁵

Herein, we explore chemoenzymatic strategies for synthesizing structurally defined *N*-acetyl analogs of *O*-acetylated NmW CPS oligosaccharides using two one-pot multienzyme (OPME) glycosylation systems containing NmW CPS polysaccharide synthase NmSiaD_W with *in situ* generation of uridine 5'-diphosphate-galactose (UDP-Gal) and CMP-Sia from the corresponding monosaccharides Gal and sialic acid or sialic acid precursor, respectively.

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RESULTS AND DISCUSSION

Donor substrate specificity studies of the α 2–6-sialyltransferase activity of NmSiaD_W.

NmSiaD_W is a bifunctional polysaccharide synthase that has both α 1–4-galactosyltransferase and α 2–6-sialyltransferase activities for the formation of NmW CPS containing a disaccharide repeating unit of –6Gal α 1–4Neu5Ac α 2-. It was used successfully in a sequential OPME system for the synthesis of structurally defined NmW CPS oligosaccharides ranging from disaccharide to deca-saccharide from a benzyloxycarbonyl (Cbz)-tagged sialylmonosaccharide 2-*O*-(*N*-benzyloxycarbonyl) aminopropyl α -*N*-acetylneuraminide (Neu5Ac α ProNHCbz).²⁶ The hydrophobic UV-detectable Cbz-tag was shown to facilitate reaction progress monitoring, enzyme biochemical characterization, and product purification processes.²⁶

Using previously synthesized galactosyldisaccharide Gal α 1–4Neu5Ac α ProNHCbz (**G2**)²⁶ as the acceptor substrate, the donor substrate specificity study for the α 2–6-sialyltransferase activity of NmSiaD_W was investigated using a one-pot two-step reaction (Scheme 1) similar to that described previously.²⁷ In step 1, CMP-sialic acid or its analog was generated *in situ* from a sialic acid, its analog, or its precursor in the presence of *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS)^{28–29} with or without *Pasteurella multocida* sialic acid aldolase (PmAldolase)³⁰ and sodium pyruvate. The yields were determined by ultra-high performance liquid chromatography (UHPLC) and mass spectrometry (MS) analyses. In step 2, **G2** and NmSiaD_W were added and the reactions were carried out in a short time (10 min) with a lower concentration (10 μ g/mL) of NmSiaD_W (to compare the efficiency of different substrates) or a longer time (10 h) with a higher concentration (3.3 mg/mL) of NmSiaD_W (to test the suitability of the compounds as potential substrates for synthesis).

As shown in Table 1, all donor precursors tested (entries **1–11**, Table 1) (see Figure S1 for structures) could be catalyzed by NmCSS without (entries **1–6**, Table 1) or with (entries **7–11**, Table 1) PmAldolase and sodium pyruvate to produce CMP-sialic acid analogs. The α 2–6-sialyltransferase activity of NmSiaD_W was shown to be promiscuous towards donor substrate and derivatives. In addition to the *N*-acetyl group (e.g. in CMP-Neu5Ac, entry **1**), *N*-glycolyl (e.g. in CMP-Neu5Gc, entry **2**), *N*-azidoacetyl (e.g. in CMP-Neu5Az, entry **7**), and OH (e.g. in CMP-Kdn, entry **6**) at the C5 of sialic acids in CMP-sialic acids were all tolerated by NmSiaD_W. A C5-N₃ group substitution (e.g. in CMP-Neu5N₃, entry **11**) was also tolerated but to a lower extent. In addition, while an 4-*O*-acetyl (e.g. in CMP-Neu4,5Ac₂, entry **4**) or 9-*O*-acetyl (e.g. in CMP-Neu5,9Ac₂, entry **5**) was not tolerated by NmSiaD_W, a 7-N₃ (e.g. in CMP-Neu5Ac7N₃, entry **8**), 8-OMe (e.g. in CMP-Neu5Ac8OMe, entry **3**), or 9-N₃ (e.g. in CMP-Neu5Ac9N₃, entry **9**) substitution of Neu5Ac in CMP-Neu5Ac was tolerated. In comparison, 9-*N*-acetyl (e.g. in CMP-Neu5Ac9NAc, entry **10**) was only weakly tolerated.

The tolerance of CMP-Neu5Gc (entry **2**), CMP-Neu5Ac8OMe (entry **3**), CMP-Kdn (entry **6**), CMP-Neu5Az (entry **7**), CMP-Neu5Ac7N₃ (entry **8**), and CMP-Neu5Ac9N₃ (entry **9**) as donor substrates for the α 2–6-sialyltransferase activity of NmSiaD_W provides an opportunity for chemoenzymatic synthesis of derivatives of NmW CPS oligosaccharides. The corresponding azido-containing donor precursor analogs (ManNAz, entry **7**),

ManNAc4N₃ (entry **8**), and ManNAc6N₃ (entry **9**) can be further explored as potential probes for metabolic engineering of NmW CPS. The latter two (ManNAc4N₃³¹ and ManNAc6N₃³²) can be used as chemoenzymatic synthons for synthesizing structurally defined *N*-acetyl analogs of 7-*O*- and/or 9-*O*-acetylated NmW CPS oligosaccharides by OPME reactions followed by conversion of the N₃-groups in the oligosaccharide products to *N*-Ac groups. A strategy using a monosaccharide diazido derivative as a chemoenzymatic synthon has been successfully developed and applied previously by us for synthesizing glycans containing a terminal 5,7-di-*N*-acetyllegionaminic acid (Leg5,7diNAc, a bacterial nonulosonic acid).³³ It has not, however, been tested in chemoenzymatic synthesis of bacterial polysaccharides containing internal sialic acid residues.

It is interesting to notice the lower degree of donor substrate promiscuity of the α 2–6-sialyltransferase activity of NmSiaD_W compared to the α 2–3-sialyltransferase activity of *Pasteurella multocida* sialyltransferase 1 (PmST1)³⁴ and α 2–6-sialyltransferase activity of *Photobacterium damsela* α 2–6-sialyltransferase (Pd2,6ST).³⁵ Both PmST1 and Pd2,6ST were able to tolerate all^{24, 34–36} except one (CMP-Neu4,5Ac₂, entry **4**)³⁷ *in situ*-generated CMP-Sia and analogs shown in Table 1. For example, *in situ*-generated CMP-Neu5,9Ac₂ (entry **5**)^{35–36} not tolerated by NmSiaD_W and CMP-Neu5Ac9NAc (entry **10**)²⁴ that was only weakly accepted by NmSiaD_W were both suitable donor substrates for PmST1 and Pd2,6ST.

Preparative-scale synthesis of sialyltrisaccharides containing different sialic acid forms.

With a good understanding of the donor substrate specificity of the α 2–6-sialyltransferase activity of NmSiaD_W, preparative-scale chemoenzymatic synthesis of structurally defined *N*-acetyl analogs of *O*-acetyl NmW CPS oligosaccharides was carried out using previously synthesized Cbz-tagged NmW CPS galactosyldisaccharide Gal α 1–4Neu5Ac α ProNHCBz (**G2**)²⁶ as the acceptor substrate and chemoenzymatic synthons ManNAc4N₃ and ManNAc6N₃ as donor precursors. As shown in Scheme 2, NmSiaD_W was used together with PmAldolase³⁰ and NmCSS²⁸ in an OPME α 2–6-sialylation system (**OPME1**) to sialylate **G2** for the formation of sialyl trisaccharides containing Neu5Ac, Neu5Ac7N₃, or Neu5Ac9N₃. In this system, sodium pyruvate (excess amount, 5–10 equiv.) and ManNAc, ManNAc4N₃, or ManNAc6N₃ were used by PmAldolase to produce Neu5Ac or its monoazido-analog Neu5Ac7N₃ or Neu5Ac9N₃. NmCSS then used CTP to activate Neu5Ac or its derivative to form CMP-Neu5Ac or its analog which was used by the sialyltransferase activity of NmSiaD_W to form α 2–6-linked sialosides (Scheme 2). From 150 mg **G2**, trisaccharide **S3** (220 mg, 97%), **7N₃-S3** Neu5Ac7N₃ α 2–6Gal α 1–4Neu5Ac α ProNHCBz (120 mg, 55%), or **9N₃-S3** Neu5Ac9N₃ α 2–6Gal α 1–4Neu5Ac α ProNHCBz (98 mg, 45%) was obtained in an excellent to a moderate yield.

The structure and the purity of the products were confirmed by nuclear magnetic resonance (NMR), high-resolution mass spectrometry (HRMS), and ultra-high performance liquid chromatography (UHPLC). For example, replacing the OH at C7 of the terminal Neu5Ac in **S3** by an N₃ in **7N₃-S3** led to an upfield shift of the C7 ¹³C signal from 68.24 ppm to 61.19 ppm. The influence on the C7 ¹H chemical shift was weaker, with 0.01 ppm difference from 3.57 ppm in **S3** to 3.56 ppm in **7N₃-S3** (Figure S2). For **9N₃-S3**, the ¹³C chemical shift of substituted Neu5Ac C9 moved upfield from 62.60 ppm in **S3** to 52.97 ppm. Two

diastereotopic protons on substituted C9 in **9N₃-S3** appeared at 3.68 ppm and 3.50 ppm, respectively, both being upfield of the corresponding protons (3.86 ppm and 3.63 ppm) in **S3** (Figure S3).

The azido group in trisaccharides **7N₃-S3** and **9N₃-S3** was readily converted to an *N*-acetyl group using a simple one-step reduction and simultaneous acetylation process by adding thioacetic acid in a saturated sodium bicarbonate solution^{33, 38} to obtain **7NAc-S3** Neu5Ac7NAc α 2-6Gal α 1-4Neu5Ac.ProNHCBz (13 mg, 76%) and **9NAc-S3** Neu5Ac9NAc α 2-6Gal α 1-4Neu5Ac.ProNHCBz (12 mg, 85%) in good yields.

Acceptor substrate specificity of the α 1-4-galactosyltransferase activity of NmSiaD_W.

Successful synthesis of longer *N*-acetyl analogs of *O*-acetylated NmW CPS oligosaccharides also depends on the acceptor substrate promiscuity of the α 1-4-galactosyltransferase activity of NmSiaD_W. Therefore, sialyltrisaccharides **S3**, **7N₃-S3**, **9N₃-S3**, **7NAc-S3**, and **9NAc-S3** obtained above were tested as acceptor substrates for the α 1-4-galactosyltransferase activity of NmSiaD_W and uridine-5'-diphosphate galactose (UDP-Gal) was used as the donor substrate. As shown in Table 2, all sialyltrisaccharides except for **7NAc-S3** were suitable acceptors although the efficiency with **9NAc-S3** (68 \pm 3%) was lower than others (quantitative yields). These results suggest that **7N₃-S3** and **9N₃-S3**, instead of **7NAc-S3** or **9NAc-S3**, will be the preferred substrates for NmSiaD_W-catalyzed enzymatic extension for the synthesis of longer *N*-acetyl NmW CPS oligosaccharide derivatives.

Preparative-scale synthesis of galactosyltetrasaccharides containing different sialic acid forms.

With the confirmation that both **7N₃-S3** and **9N₃-S3**, in addition to **S3**, were suitable acceptor substrates for the α 1-4-galactosyltransferase activity of NmSiaD_W, preparative-scale synthesis of galactosyltetrasaccharides were carried out using an OPME α 1-4-galactosylation system (**OPME2**) (Scheme 2) containing *Streptococcus pneumoniae* TIGR4 galactokinase (SpGalK),³⁹ *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP),⁴⁰ *Pasteurella multocida* inorganic pyrophosphatase (PmPpA),⁴¹ and NmSiaD_W. In this system, SpGalK was responsible for the formation of galactose-1-phosphate (Gal-1-P). It was then used by BLUSP for *in situ* formation of activated sugar nucleotide UDP-Gal, which was used by NmSiaD_W to produce α 1-4-linked galactosides. PmPpA was included to hydrolyze the inorganic pyrophosphate (PPi) formed in the BLUSP-catalyzed reaction to drive the reaction towards the formation of UDP-Gal.⁴⁰ From 50 mg of **S3** or its derivative **7N₃-S3** or **9N₃-S3**, tetrasaccharide **G4** (56 mg, 97%), **7N₃-G4** Gal α 1-4Neu5Ac7N₃ α 2-6Gal α 1-4Neu5Ac.ProNHCBz (54 mg, 93%), and **9N₃-G4** Gal α 1-4Neu5Ac9N₃ α 2-6Gal α 1-4Neu5Ac.ProNHCBz (55 mg, 95%) were formed in excellent yields.

Similar to that described above, the azido group in **7N₃-G4** and **9N₃-G4** was converted to *N*-acetyl group using thioacetic acid in a saturated sodium bicarbonate solution^{33, 38} to obtain **7NAc-G4** Gal α 1-4Neu5Ac7NAc α 2-6Gal α 1-4Neu5Ac.ProNHCBz (20 mg, 75%) and **9NAc-G4** Gal α 1-4Neu5Ac9NAc α 2-6Gal α 1-4Neu5Ac.ProNHCBz (21 mg, 80%), respectively, in good yields.

Donor and acceptor substrate specificity studies of the α 2–6-sialyltransferase activity of NmSiaD_W with galactosyltetrasaccharide acceptors.

The obtained galactosyltetrasaccharides **G4**, **7N₃-G4**, **9N₃-G4**, **7NAc-G4**, and **9NAc-G4** as well as donor precursors ManNAc4N₃ and ManNAc6N₃ allowed the exploration of the potential of NmSiaD_W in synthesizing longer oligosaccharides with desired *N*-acetylation patterns. A one-pot two-step reaction process similar to that shown in Scheme 1 above for the donor substrate specificity studies of the sialyltransferase activity of NmSiaD_W with **G2** as the acceptor was used. As shown in Table 3, CMP-Neu5Ac, CMP-Neu5Ac7N₃, and CMP-Neu5Ac9N₃ were obtained readily *in situ* from the corresponding precursors Neu5Ac, ManNAc4N₃, and ManNAc6N₃, respectively, in a reaction catalyzed by NmCSS with (for ManNAc4N₃ and ManNAc6N₃) or without (for Neu5Ac) PmAldolase in step 1. The analysis of the NmSiaD_W-catalyzed α 2–6-sialyltransfer process in step 2 showed that when Neu5Ac was used as the donor precursor, all galactosyltetrasaccharides tested were well tolerated by NmSiaD_W to form sialylpentasaccharide products (see Scheme 2 for structures) in high yields (83–97%, entries **1–5** in Table 3). Nevertheless, differentiation of the efficiency of the acceptors was observed when the reactions were carried out with a lower concentration of NmSiaD_W in a shorter period of time. The N₃-substitution at the C7 or C9 of the Neu5Ac in the acceptor was better tolerated than the corresponding *N*-acetyl-substitution.

When ManNAc4N₃ (entries **6–10** in Table 3) or ManNAc6N₃ (entries **11–15** in Table 3) was used as the donor precursor for the α 2–6-sialyltransferase activity of NmSiaD_W, **G4**, **7N₃-G4**, and **9N₃-G4** were suitable acceptor substrates but **7NAc-G4** and **9NAc-G4** were not. Therefore, the data support the potential of chemoenzymatic synthesis of *N*-acetyl analogs of longer NmW CPS oligosaccharides with defined *N*-acetylation pattern although optimization is needed to further improve the yields of the reactions when **9N₃-G4** was used as the acceptor substrate and ManNAc4N₃ (entry **8** in Table 3) or ManNAc6N₃ (entry **13** in Table 3) was used as the donor precursor.

The observation that 7NAc or 9NAc-analogs of NmW CPS oligosaccharides are poor or unsuitable substrates for either the galactosyltransferase or the sialyltransferase activity of NmSiaD_W indicates that NmW CPS polysaccharide is most likely formed before NmOatWY-catalyzed *O*-acetylation. This is consistent with the previous results from biochemical characterization of NmOatWY.¹⁹

CONCLUSIONS

In conclusion, the donor and acceptor substrate promiscuity of NmSiaD_W was demonstrated. ManNAc4N₃ and ManNAc6N₃, the six-carbon precursors for Neu5Ac7N₃ and Neu5Ac9N₃, were confirmed to be suitable chemoenzymatic synthons in NmSiaD_W-dependent sequential one-pot multienzyme (OPME) chemoenzymatic synthesis of structurally defined stable *N*-acetyl analogs of *O*-acetylated NmW CPS oligosaccharides. The obtained oligosaccharide products are important probes for binding studies of antibodies against NmW CPS and for investigating the functions of its *O*-acetylation. They can also be used to investigate host-microbe interactions. The tolerance of an azido group substitution at the C7 or C9 of Neu5Ac in the *in situ*-generated CMP-Neu5Ac donor derivatives by the α 2–6-

sialyltransferase activity of NmSiaD_W and the tolerance of the resulting oligosaccharides as acceptors for the α 1–4-galactosyltransferase activity of NmSiaD_W showed the potential of using ManNAc4N₃ and ManNAc6N₃ or their per-acetylated analogs for metabolic engineering studies.⁴² The chemoenzymatic synthon strategy can be extended for the synthesis of the *N*-acetyl analogs of other oligosaccharides containing *O*-acetyl sialic acids.

EXPERIMENTAL SECTION

Materials and General Methods.

Chemicals were obtained from commercial suppliers and used without further purification. ¹H NMR, ¹³C NMR, HSQC, and HSQC-TOCSY (90 ms and 10 ms) spectra were recorded on an 800 MHz Bruker Avance III spectrometer in the NMR facility of the University of California, Davis. Structural assignments were made with additional information from HSQC and HSQC-TOCSY experiments. High-resolution electrospray ionization (ESI) mass spectra were obtained using a Thermo Electron LTQ-Orbitrap Hybrid mass spectrometer at the mass spectrometry facility in the University of California, Davis or an LTQ-Orbitrap Eilte mass spectrometer at the Georgia State University. Matrix-assisted laser desorption/ionization (MALDI) mass spectra were obtained using Bruker UltraFLEXtreme MALDI-TOF at the mass spectrometry facility in the University of California, Davis. UHPLC assays were performed using Agilent 1290 Infinity LC with an EclipsePlus C18 (Rapid Resolution HD, 1.8 μ m, 2.1 \times 50 mm, 959757–902), an AdvanceBio Glycan Map (1.8 μ m, 2.1 \times 150 mm, 859700–913) column from Agilent Technologies or an Dionex CarboPac PA100 (8.5 μ m, 4 \times 250 mm, 043055) column from Thermo Scientific. Reverse phase chromatography purification of products was performed with a C18 column (ODS-SM, 50 mm, 120 Å , 3.0 \times 20 cm) from Yamazen Corporation on a CombiFlash Rf 200i system. Galactose was from Fisher Scientific, Inc. *N*-Acetylneuraminic acid (Neu5Ac) was from Inalco (Italy). Adenosine 5'-triphosphate (ATP), cytosine 5'-triphosphate (CTP), and uridine 5'-triphosphate (UTP) were purchased from Hangzhou Meiya Pharmaceutical Co. Ltd. Recombinant enzymes *Pasteurella multocida* sialic acid aldolase (PmAldolase),³⁰ *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS),²⁸ *Streptococcus pneumoniae* TIGR4 galactokinase (SpGalK),³⁹ *Bifidobacterium longum* UDP sugar pyrophosphorylase (BLUSP),⁴⁰ *Pasteurella multocida* inorganic pyrophosphatase (PmPpA),⁴¹ *Neisseria meningitidis* serogroup W capsular polysaccharide polymerase (NmSiaD_W)²⁶ were expressed and purified as reported previously. ManNAc4N₃,³¹ ManNAc6N₃,³² and Gal α 1–4Neu5Ac₂ProNHCBz (**G2**)²⁶ were synthesized as described previously.

Two-step donor substrate specificity study for the α 2–6-sialyltransferase activity of NmSiaD_W using disaccharide **G2** as an acceptor.

In the first step of the assay, CMP-sialic acid or its analog was synthesized from sialic acid, sialic acid analog, or its precursor. If Neu5Ac or its analog was used as a starting material, the step 1 reactions were performed in duplicate at 30 °C for 4 h in a total volume of 20 μ L in a buffer (Tris-HCl, 100 mM, pH 8.5; pH 7.5 for reactions with Neu4,5Ac₂ or Neu5,9Ac₂) containing MgCl₂ (10 mM), sialic acid or its analog (6 mM), CTP (5 mM), and NmCSS (15 μ g). If a sialic acid precursor (6 mM) was used as a starting material, sodium pyruvate (25 mM) and PmAldolase (40 μ g) were included in the reaction mixture. Step 2 sialylation

reactions were performed in duplicate at 30 °C for 10 min (with 0.10 µg of NmSiaD_W) or 10 h (with 33 µg of NmSiaD_W) in a total volume of 10 µL in a buffer (Tris-HCl, 100 mM, pH 8.5; pH 7.5 for reactions with Neu4,5Ac₂ or Neu5,9Ac₂) containing MgCl₂ (10 mM), a reaction mixture from step 1 (2.5 µL), acceptor **G2** (1 mM), and NmSiaD_W. Reactions were quenched by adding 10 µL of pre-chilled ethanol followed by incubation of the mixture at -20 °C for 30 min. Samples were analyzed using UHPLC with an EclipsePlusC18 column or an AdvancBio Glycan Map column (Agilent), as well as by Bruker UltraFlex extreme MALDI-TOF in a negative mode. Sialic acids and derivatives tested were Neu5Ac, Neu5Gc, Neu5Ac8OMe,⁴³ Neu4,5Ac₂,³⁷ Neu5,9Ac₂,⁴⁴ and Kdn. Sialic acid precursors and derivatives tested were ManNAz,³⁴ ManNAc4N₃,³¹ ManNAc6N₃,³² ManNAc6NAc,²¹ and Man2N₃.³⁵

Acceptor substrate specificity study for the α 1–4-galactosyltransferase activity of NmSiaD_W using sialyltrisaccharide **S3** and analogs obtained.

Assays were performed in duplicate at 30 °C for 10 min (with 0.20 µg of NmSiaD_W) or 10 h (with 12 µg of NmSiaD_W) in a total volume of 10 µL in a buffer (MES, 100 mM, pH 6.5) containing MgCl₂ (10 mM), UDP-Gal (2 mM), a sialyltrisaccharide **S3** or analog (**7N₃-S3**, **9N₃-S3**, **7NAc-S3**, or **9NAc-S3**) (1 mM), and NmSiaD_W. Reactions were quenched by adding 10 µL of pre-chilled ethanol followed by incubation of the mixture at -20 °C for 30 min. Samples were analyzed using UHPLC with an EclipsePlusC18 column (Agilent) and by Bruker UltraFlex extreme MALDI-TOF in a negative mode.

Donor and acceptor substrate specificity studies of the α 2–6-sialyltransferase activity of NmSiaD_W using **G4** or its analogs.

In the first step of the assay, CMP-sialic acid or its analog was synthesized in duplicate at 30 °C for 4 h in a total volume of 10 µL in a buffer (Tris-HCl, 100 mM, pH 8.5) containing a sialic acid or precursor (Neu5Ac, ManNAc4N₃, or ManNAc6N₃) (6 mM), CTP (5 mM), sodium pyruvate (25 mM), MgCl₂ (10 mM), NmCSS (15 µg), and PmAldolase (40 µg). Step 2 sialylation reactions were performed in duplicate at 30 °C for 10 min (with 0.10 µg of NmSiaD_W) or 10 h (with 33 µg of NmSiaD_W) in a total volume of 10 µL in a buffer (Tris-HCl, 100 mM, pH 8.5) containing a reaction mixture from step 1 (2.5 µL), a galactosyltetrasaccharide **G4** or analog (**7N₃-G4**, **9N₃-G4**, **7NAc-G4**, or **9NAc-G4**) (1 mM), MgCl₂ (10 mM), and NmSiaD_W. Reactions were quenched by adding 10 µL of pre-chilled ethanol followed by incubation of the mixture at -20 °C for 30 min. Samples were analyzed using UHPLC with a Dionex CarboPac PA100 column (Thermo Scientific) and by Bruker UltraFlex extreme MALDI-TOF in a negative mode.

OPME synthesis of Neu5Ac7N₃ α 2–6Gal α 1–4Neu5Ac α ProNHCBz (**7N₃-S3**).

A reaction mixture in a total volume of 10 mL containing Tris-HCl buffer (100 mM, pH 8.5), galactosyldisaccharide **G2** (150 mg, 0.22 mmol), ManNAc4N₃ (75 mg, 0.30 mmol), sodium pyruvate (253 mg, 2.3 mmol), CTP disodium salt (218 mg, 0.41 mmol), MgCl₂ (20 mM), PmAldolase (20 mg), NmCSS (7 mg) and NmSiaD_W (4 mg) was incubated in a 50 mL centrifuge tube in a shaker (100 rpm) at 30 °C for 2 days. The reaction progress was monitored by UHPLC (EclipsePlus C18, Agilent, 5–12% Acetonitrile + 0.1% TFA in water

over 7 min, monitored at 215 nm). When an optimal yield was achieved, pre-chilled ethanol (10 mL) was added and the resulting mixture was incubated at 4 °C for 30 min. The precipitates were removed by centrifugation (4300 g) at 4 °C for 30 min. The supernatant was concentrated and purified by a C18 column using a CombiFlash Rf 200i system with a gradient (0–100% acetonitrile) of water with 0.1% TFA (v/v) and acetonitrile for elution. Fractions containing the product were collected, neutralized, concentrated, and further purified by a C18 column to produce **7N₃-S3** as a sodium salt (120 mg, 55%).

¹H NMR (800 MHz, D₂O) δ 7.47–7.38 (m, 5H, Ar-H), 5.11 (s, 2H, O-CH₂-Ar), 5.04 (d, *J* = 3.9 Hz, 1H, H^{''}-1), 4.03 (t, *J* = 10.2 Hz, 1H, H[']-5), 4.00–3.74 (m, 12H), 3.74–3.59 (m, 7H), 3.56 (dd, *J* = 9.2, 2.0 Hz, 1H, H^{'''}-7), 3.50 (dt, *J* = 9.7, 6.2 Hz, 1H, O-CH₂-CH₂), 3.24–3.16 (m, 2H, CH₂-NH), 2.87 (dd, *J* = 12.5, 4.7 Hz, 1H, H[']-3_{eq}), 2.72 (dd, *J* = 12.5, 4.7 Hz, 1H, H^{'''}-3_{eq}), 2.07 (s, 3H, H[']-CH₃-CO), 2.04 (s, 3H, H^{'''}-CH₃-CO), 1.78–1.69 (m, 3H, O-CH₂-CH₂-CH₂-NH; H^{'''}-3_{ax}), 1.61 (t, *J* = 12.0 Hz, 1H, H[']-3_{ax}). ¹³C{¹H} NMR (200 MHz, D₂O) δ 174.53, 174.37, 173.34, 173.16, 158.37 (NH-COO), 136.56 (O-CH₂-Ar), 128.75 (Ar), 128.27 (Ar), 127.56 (Ar), 100.58 (C[']-2), 100.51 (C^{'''}-2), 94.87 (C^{''}-1), 72.89, 72.15, 71.85, 71.61, 70.97, 69.33, 69.17, 68.76, 68.37, 68.07, 67.83, 66.75 (O-CH₂-Ar), 62.58, 62.54, 62.40, 62.06 (O-CH₂-CH₂), 61.23 (C^{'''}-7), 52.51 (C^{'''}-5), 49.54 (C[']-5), 39.88 (C^{'''}-3), 37.47 (CH₂-NH), 36.78 (C[']-3), 28.89 (O-CH₂-CH₂-CH₂-NH), 22.43 (C[']-CH₃-CO), 22.11 (C^{'''}-CH₃-CO). HRMS (ESI-Orbitrap) *m/z*: [M - H]⁻ Calcd for C₃₉H₅₇N₆O₂₃ 977.3475; found 977.3476.

Chemical synthesis of Neu5Ac7NAcα2–6Galα1–4Neu5AcαProNHCBz (7NAc-S3).

7N₃-S3 (17 mg) was added to a round bottom flask (50 mL) containing saturated sodium bicarbonate aqueous solution (2 mL), thioacetic acid (200 μL) was then added drop-wisely. The reaction was heated in an oil bath under argon at 65 °C for 30 h. After completion of the reaction, the solvent was removed, and the compound was purified by silica gel chromatography using a mixed solvent of ethyl acetate:methanol (6:1 by volume) as an eluent and then by a C18 column in a CombiFlash Rf 200i system using CH₃CN in H₂O gradient as the elution solvent. **7NAc-S3** was obtained as a white solid (13 mg, 76% yield). ¹H NMR (800 MHz, D₂O) δ 7.46–7.39 (m, 5H), 5.11 (s, 2H), 5.09 (d, *J* = 3.9 Hz, 1H), 4.05 (t, *J* = 10.2 Hz, 1H), 3.98–3.92 (m, 3H), 3.89–3.84 (m, 5H), 3.82–3.76 (m, 3H), 3.74–3.68 (m, 3H), 3.66–3.60 (m, 3H), 3.56 (ddd, *J* = 9.9, 8.4, 4.5 Hz, 2H), 3.52–3.46 (m, 2H), 3.20 (h, *J* = 7.3 Hz, 2H), 2.91 (dd, *J* = 12.6, 4.7 Hz, 1H), 2.74 (dd, *J* = 12.4, 4.6 Hz, 1H), 2.05 (s, 3H), 1.99 (s, 3H), 1.94 (s, 3H), 1.75 (dd, *J* = 14.8, 9.0 Hz, 3H), 1.62 (t, *J* = 12.0 Hz, 1H). ¹³C{¹H} NMR (200 MHz, D₂O) δ 174.4, 173.9, 173.8, 173.3, 158.4, 136.6, 128.8, 128.3, 127.6, 100.6, 100.2, 94.2, 72.4, 72.1, 71.8, 71.6, 71.6, 69.6, 69.2, 69.2, 68.6, 68.0, 67.9, 66.8, 63.8, 62.5, 62.4, 62.1, 52.0, 49.5, 49.2, 40.2, 37.5, 36.4, 28.9, 22.3, 22.1, 21.9. HRMS (ESI-Orbitrap) *m/z*: [M - H]⁻ Calcd for C₄₁H₆₁N₄O₂₄ 993.3681; found 993.3696.

OPME synthesis of Neu5Ac9N₃α2–6Galα1–4Neu5AcαProNHCBz (9N₃-S3).

A reaction mixture in a total volume of 10 mL containing Tris-HCl buffer (100 mM, pH 8.5) galactosyldisaccharide **G2** (150 mg, 0.22 mmol), ManNAc6N₃ (75 mg, 0.30 mmol), sodium pyruvate (203 mg, 1.85 mmol), CTP disodium salt (194 mg, 0.37 mmol), MgCl₂ (20 mM), PmAldolase (15 mg), NmCSS (5 mg), and NmSiaD_W (4 mg) was incubated in a 50 mL

centrifuge tube in a shaker (100 rpm) at 30 °C for 2 days. Procedures for reaction progress monitoring, centrifugation, concentration, purification, collection, and neutralization were similar to that described above for **7N₃-S3**. **9N₃-S3** was obtained as a sodium salt (98 mg, 45%). ¹H NMR (800 MHz, D₂O) δ 7.46–7.37 (m, 5H, Ar-H), 5.11 (s, 2H, O-CH₂-Ar), 5.05 (d, *J* = 3.9 Hz, 1H, H''-1), 4.06–3.99 (m, 2H), 3.95 (d, *J* = 3.8 Hz, 1H, H''-3), 3.89–3.74 (m, 8H), 3.73–3.60 (m, 8H), 3.58 (dd, *J* = 9.1, 1.8 Hz, 1H, H'''-7), 3.53–3.48 (m, 2H, O-CH₂-CH₂; H'''-9), 3.24–3.16 (m, 2H, CH₂-NH), 2.88 (dd, *J* = 12.5, 4.8 Hz, 1H, H'-3_{eq}), 2.72 (dd, *J* = 12.5, 4.7 Hz, 1H, H'''-3_{eq}), 2.07 (s, 3H, H'-CH₃-CO), 2.03 (s, 3H, H'''-CH₃-CO), 1.75 (p, *J* = 6.6 Hz, 2H, O-CH₂-CH₂-CH₂-NH), 1.70 (t, *J* = 12.2 Hz, 1H, H'''-3_{ax}), 1.61 (t, *J* = 12.0 Hz, 1H, H'-3_{ax}). ¹³C{¹H} NMR (200 MHz, D₂O) δ 174.93, 174.50, 173.44, 173.30, 158.37 (NH-COO), 136.56 (O-CH₂-Ar), 128.75 (Ar), 128.27 (Ar), 127.56 (Ar), 100.58 (C'-2), 100.23 (C'''-2), 94.68 (C''-1), 72.80, 72.28, 72.11, 71.84, 70.23, 69.51, 69.17, 68.96, 68.87, 68.34, 68.03, 67.83, 66.75 (O-CH₂-Ar), 62.87, 62.51, 62.05 (O-CH₂-CH₂), 53.03 (C'''-9), 51.81 (C'''-5), 49.54 (C'-5), 40.04 (C'''-3), 37.47 (CH₂-NH), 36.71 (C'-3), 28.89 (O-CH₂-CH₂-CH₂-NH), 22.39 (C'-CH₃-CO), 22.01 (C'''-CH₃-CO). HRMS (ESI-Orbitrap) *m/z*: [M - H]⁻ Calcd for C₃₉H₅₇N₆O₂₃ 977.3475; found 977.3479.

Chemical synthesis of Neu5Ac9NAcα2-6Galα1-4Neu5AcαProNHCBz (9NAc-S3).

9N₃-S3 (14 mg) was added to a round bottom flask (50 mL) containing saturated sodium bicarbonate aqueous solution (2 mL), thioacetic acid (200 μL) was then added drop-wisely. Reaction conditions and procedures for concentration and purification were similar to that described above for **7NAc-S3**. **9NAc-S3** was obtained as a white solid (12 mg, 85%). ¹H NMR (800 MHz, D₂O) δ 7.46–7.38 (m, 5H), 5.11 (s, 2H), 5.06 (d, *J* = 3.9 Hz, 1H), 4.03 (t, *J* = 10.2 Hz, 1H), 3.95 (d, *J* = 3.4 Hz, 1H), 3.90 (ddd, *J* = 9.0, 7.9, 2.9 Hz, 1H), 3.84 (dddd, *J* = 26.4, 12.7, 7.7, 3.6 Hz, 6H), 3.80–3.75 (m, 2H), 3.71–3.60 (m, 7H), 3.55 (dd, *J* = 14.0, 2.9 Hz, 1H), 3.52–3.49 (m, 1H), 3.47 (dd, *J* = 9.0, 1.8 Hz, 1H), 3.31 (dd, *J* = 14.1, 7.9 Hz, 1H), 3.24–3.16 (m, 2H), 2.88 (dd, *J* = 12.5, 4.7 Hz, 1H), 2.72 (dd, *J* = 12.4, 4.7 Hz, 1H), 2.07 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.75 (p, *J* = 6.6 Hz, 2H), 1.70 (t, *J* = 12.2 Hz, 1H), 1.62 (t, *J* = 12.0 Hz, 1H). ¹³C{¹H} NMR (200 MHz, D₂O) δ 174.9, 174.5, 174.4, 173.5, 158.4, 136.1, 128.8, 128.3, 127.6, 100.3, 94.7, 72.8, 72.3, 72.1, 71.8, 70.0, 69.8, 69.5, 69.2, 68.9, 68.3, 68.0, 67.8, 66.4, 62.8, 62.5, 62.1, 51.8, 49.1, 42.1, 40.0, 37.5, 36.7, 28.9, 22.4, 22.0, 21.8. HRMS (ESI-Orbitrap) *m/z*: [M - H]⁻ Calcd for C₄₁H₆₁N₄O₂₄ 993.3681; found 993.3691.

OPME synthesis of Galα1-4Neu5Ac7N₃α2-6Galα1-4Neu5AcαProNHCBz (7N₃-G4).

A reaction mixture in a total volume of 10 mL containing Tris-HCl buffer (100 mM, pH 8.5), sialyltrisaccharide **7N₃-S3** (50 mg, 0.05 mmol), galactose (12 mg, 0.07 mmol), ATP disodium salt (38 mg, 0.07 mmol), UTP trisodium salt (38 mg, 0.07 mmol), MgCl₂ (20 mM), SpGalK (1 mg), BLUSP (1 mg), PmPpA (1 mg), and NmSiaD_W (0.5 mg) was incubated in a 50 mL centrifuge tube in a shaker (100 rpm) at 30 °C for 16 h. Procedures for reaction progress monitoring, centrifugation, concentration, purification, collection and neutralization were similar to that described above for **7N₃-S3**. **7N₃-G4** was obtained as a sodium salt (54 mg, 93%). ¹H NMR (800 MHz, D₂O) δ 7.47–7.38 (m, 5H, Ar-H), 5.11 (s, 2H, O-CH₂-Ar), 5.08 (d, *J* = 4.0 Hz, 1H, H'''-1), 5.05 (d, *J* = 3.9 Hz, 1H, H''-1), 4.07 (t, *J* = 10.2 Hz, 1H, H'''-5), 4.03 (t, *J* = 10.3 Hz, 1H, H'-5), 4.00–3.90 (m, 5H), 3.89–3.59 (m, 20H), 3.50 (dt, *J* = 9.7, 6.1 Hz, 1H, O-CH₂-CH₂), 3.25–3.15 (m, 2H, CH₂-NH), 2.90–2.85

(dd, $J = 12.8, 4.8$ Hz, 2H, H[']-3_{eq}; H^{'''}-3_{eq}), 2.08 (s, 3H, H[']-CH₃-CO), 2.05 (s, 3H, H^{'''}-CH₃-CO), 1.75 (p, $J = 6.7$ Hz, 2H, O-CH₂-CH₂-CH₂-NH), 1.68 (t, $J = 12.1$ Hz, 1H, H^{'''}-3_{ax}), 1.61 (t, $J = 12.0$ Hz, 1H, H[']-3_{ax}). ¹³C{¹H} NMR (200 MHz, D₂O) δ 174.51, 174.07, 173.37, 172.81, 158.37 (NH-COO), 136.57 (O-CH₂-Ar), 128.76 (Ar), 128.27 (Ar), 127.56 (Ar), 100.58 (C[']-2), 100.54 (C^{'''}-2), 95.19 (C^{'''}-1), 94.62 (C[']-1), 73.06, 72.56, 72.16, 71.81, 71.43, 71.06, 70.79, 69.36, 69.22, 69.17, 69.03, 68.80, 67.99, 67.85, 67.75, 66.75 (O-CH₂-Ar), 62.77, 62.48, 62.40, 62.06 (O-CH₂-CH₂), 61.24 (C^{'''}-7), 60.70, 50.34 (C^{'''}-5), 49.55 (C[']-5), 37.48 (CH₂-NH), 36.78 (C^{'''}-3), 36.66 (C[']-3), 28.90 (O-CH₂-CH₂-CH₂-NH), 22.43 (C[']-CH₃-CO), 22.15 (C^{'''}-CH₃-CO). HRMS (ESI-Orbitrap) m/z: [M - H]⁻ Calcd for C₄₅H₆₇N₆O₂₈ 1139.4003; found 1139.4007.

Chemical synthesis of Galα1-4Neu5Ac7NAcα2-6Galα1-4Neu5AcaProNHCBz (7NAc-G4).

To a round bottom flask containing 7N₃-G4 (26 mg), saturated sodium bicarbonate solution (3 mL) was added, 300 μL of thioacetic acid was then added drop-wisely. Reaction conditions and procedures for concentration and purification were similar to that described above for 7NAc-S3. 7NAc-G4 was obtained as a white solid (20 mg, 75%). ¹H NMR (800 MHz, D₂O) δ 7.51-7.34 (m, 5H), 5.11 (s, 2H), 5.08 (d, $J = 3.9$ Hz, 1H), 5.07 (d, $J = 4.0$ Hz, 1H), 4.07-3.99 (m, 2H), 3.96-3.85 (m, 7H), 3.85-3.76 (m, 6H), 3.76-3.60 (m, 11H), 3.55-3.46 (m, 2H), 3.28-3.16 (m, 2H), 2.90 (ddd, $J = 12.0, 6.9, 4.7$ Hz, 2H), 2.06 (s, 3H), 1.99 (s, 3H), 1.95 (s, 3H), 1.75 (p, $J = 6.8$ Hz, 2H), 1.68 (t, $J = 12.0$ Hz, 1H), 1.62 (t, $J = 12.0$ Hz, 1H). ¹³C{¹H} NMR (200 MHz, D₂O) δ 174.4, 173.8, 173.7, 173.3, 172.9, 158.4, 136.6, 128.8, 128.3, 127.6, 100.4, 94.9, 94.3, 72.8, 72.5, 72.1, 71.8, 71.4, 71.4, 71.0, 69.6, 69.3, 69.2, 69.2, 69.0, 68.0, 67.9, 67.9, 66.8, 63.9, 62.5, 62.4, 62.1, 60.7, 49.8, 49.6, 49.3, 37.5, 37.0, 36.5, 28.9, 22.3, 22.1, 21.8. HRMS (ESI-Orbitrap) m/z: [M - H]⁻ Calcd for C₄₇H₇₁N₄O₂₉ 1155.4209; found 1155.4214.

OPME synthesis of Galα1-4Neu5Ac9N₃α2-6Galα1-4Neu5AcaProNHCBz (9N₃-G4).

A reaction mixture in a total volume of 10 mL containing Tris-HCl buffer (100 mM, pH 8.5), sialyltrisaccharide 9N₃-S3 (50 mg, 0.05 mmol), galactose (12 mg, 0.07 mmol), ATP disodium salt (38 mg, 0.07 mmol), UTP trisodium salt (38 mg, 0.07 mmol), MgCl₂ (20 mM), SpGalK (1 mg), BLUSP (1 mg), PmPpA (1 mg), and NmSiaD_W (2 mg) was incubated in a 50 mL centrifuge tube in a shaker (100 rpm) at 30 °C for 2 days. Procedures for reaction progress monitoring, centrifugation, concentration, purification, collection and neutralization were similar to that described above for 7N₃-S3. 9N₃-G4 was obtained as a sodium salt (55 mg, 95%). ¹H NMR (800 MHz, D₂O) δ 7.46-7.37 (m, 5H, Ar-H), 5.10 (d, $J = 4.1$ Hz, 3H, O-CH₂-Ar; H^{'''}-1), 5.06 (d, $J = 3.9$ Hz, 1H, H[']-1), 4.08 (td, $J = 10.3, 5.5$ Hz, 2H, H[']-5; H^{'''}-5), 4.01 (ddd, $J = 8.9, 6.0, 2.7$ Hz, 1H), 3.98-3.53 (m, 24H), 3.49 (dd, $J = 13.2, 6.0$ Hz, 1H, H^{'''}-9), 3.22-3.18 (m, 2H, CH₂-NH), 2.88-2.82 (m, 2H, H[']-3_{eq}; H^{'''}-3_{eq}), 2.06 (s, 3H, H[']-CH₃-CO), 2.03 (s, 3H, H^{'''}-CH₃-CO), 1.78-1.69 (m, 4H, O-CH₂-CH₂-CH₂-NH; H^{'''}-3_{ax}; H[']-3_{ax}). ¹³C{¹H} NMR (200 MHz, D₂O) δ 174.47, 174.33, 171.77, 171.37, 158.35 (NH-COO), 136.54 (O-CH₂-Ar), 128.76 (Ar), 128.29 (Ar), 127.57 (Ar), 99.42 (C[']-2), 99.23 (C^{'''}-2), 94.72 (C^{'''}-1), 94.27 (C[']-1), 72.38, 72.26, 72.15, 71.61, 71.05, 70.96, 69.77, 69.54, 69.32, 69.25, 69.06, 69.02, 68.67, 68.01, 67.88, 67.78, 66.77 (O-CH₂-Ar), 63.29, 62.80, 62.06 (O-CH₂-CH₂), 60.72, 53.43 (C^{'''}-9), 49.36 (C^{'''}-5), 49.31 (C[']-5), 37.37 (CH₂-NH), 36.01 (C^{'''}-3) 35.56 (C[']-3), 28.77 (O-CH₂-CH₂-CH₂-NH), 22.30 (C[']-CH₃-CO),

22.11 (C¹³-CH₃-CO). HRMS (ESI-Orbitrap) m/z: [M - H]⁻ Calcd for C₄₅H₆₇N₆O₂₈ 1139.4003; found 1139.4021.

Chemical synthesis of Gal α 1–4Neu5Ac9NAc α 2–6Gal α 1–4Neu5Ac α ProNHCBz (9NAc-G4).

To a round bottom flask containing 9N₃-G4 (26 mg), saturated sodium bicarbonate solution (3 mL) was added, and then 300 μ L of thioacetic acid was added drop-wisely. Reaction conditions and procedures for concentration and purification were similar to that described above for 7NAc-S3. 9NAc-G4 was obtained as a white solid (21 mg, 80%). ¹H NMR (800 MHz, D₂O) δ 7.47–7.37 (m, 5H), 5.11 (s, 2H), 5.08 (d, *J* = 4.0 Hz, 1H), 5.06 (d, *J* = 3.9 Hz, 1H), 4.03 (td, *J* = 10.2, 4.5 Hz, 2H), 3.96 (ddd, *J* = 13.9, 3.4, 1.2 Hz, 2H), 3.92 (ddd, *J* = 9.0, 8.0, 2.9 Hz, 1H), 3.87–3.75 (m, 10H), 3.74–3.67 (m, 7H), 3.67–3.61 (m, 2H), 3.57 (dd, *J* = 14.1, 2.9 Hz, 1H), 3.50 (dt, *J* = 12.5, 4.4 Hz, 2H), 3.30 (dd, *J* = 14.1, 8.0 Hz, 1H), 3.20 (q, *J* = 6.8 Hz, 2H), 2.88 (ddd, *J* = 17.3, 12.5, 4.6 Hz, 2H), 2.07 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.75 (p, *J* = 6.6 Hz, 2H), 1.66 (t, *J* = 12.0 Hz, 1H), 1.61 (t, *J* = 12.0 Hz, 1H). ¹³C{¹H} NMR (200 MHz, D₂O) δ 174.5, 174.5, 174.3, 173.4, 173.1, 158.4, 136.6, 128.8, 128.3, 127.6, 100.6, 100.3, 95.0, 94.5, 73.2, 72.6, 72.1, 72.1, 71.8, 71.0, 70.1, 69.7, 69.5, 69.2, 69.0, 69.0, 68.0, 67.9, 67.8, 66.8, 63.0, 62.5, 62.1, 60.7, 49.6, 49.5, 42.1, 37.5, 36.7, 36.6, 28.9, 22.4, 22.1, 21.8. HRMS (ESI-Orbitrap) m/z: [M - H]⁻ Calcd for C₄₇H₇₁N₄O₂₉ 1155.4209; found 1155.4223.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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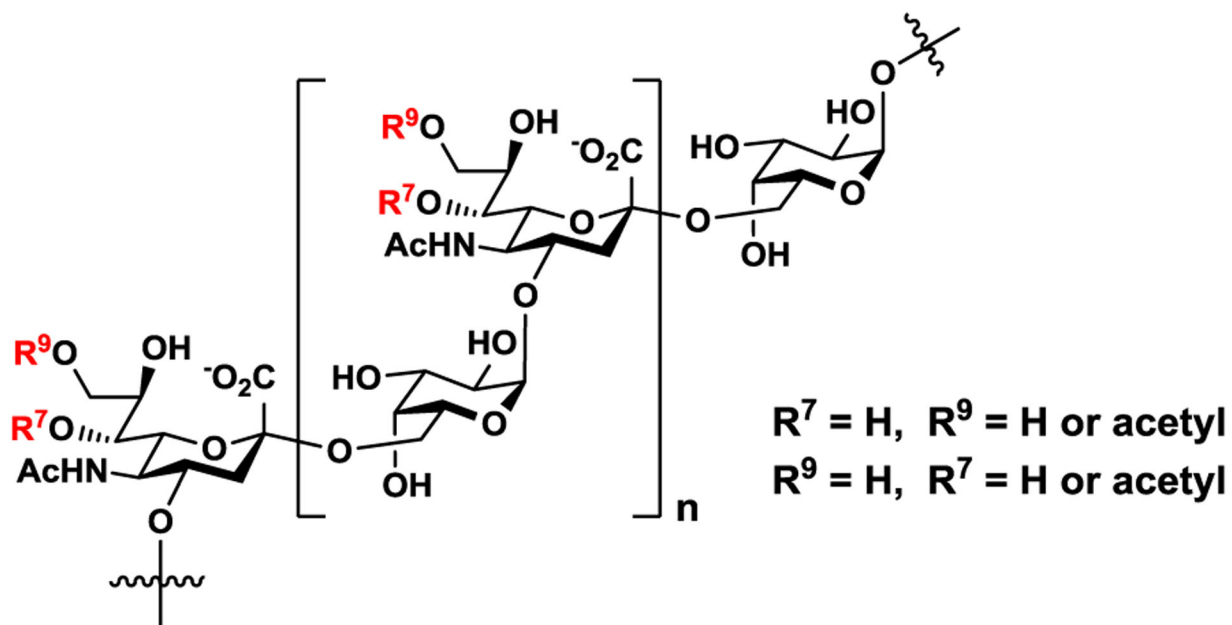
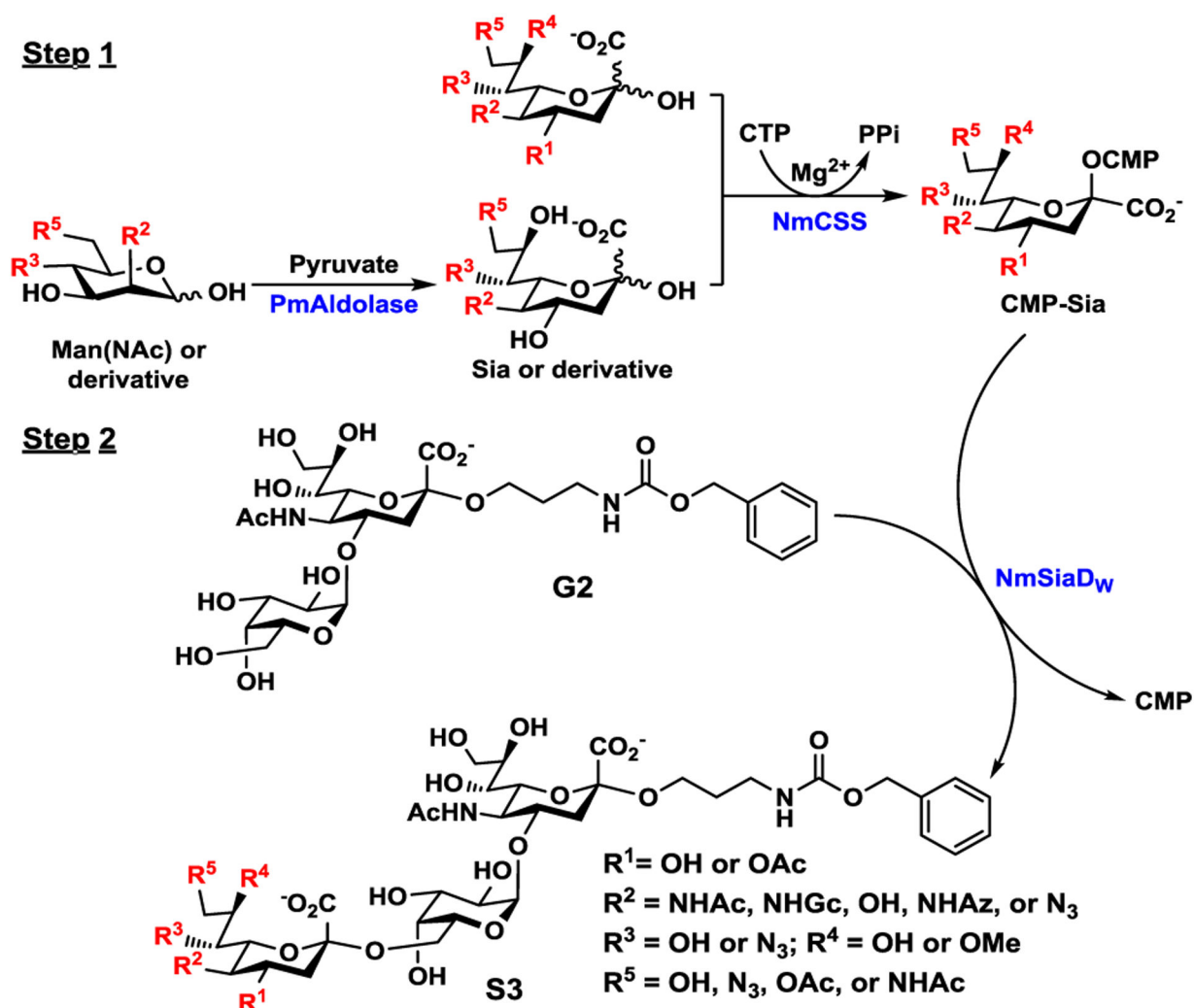


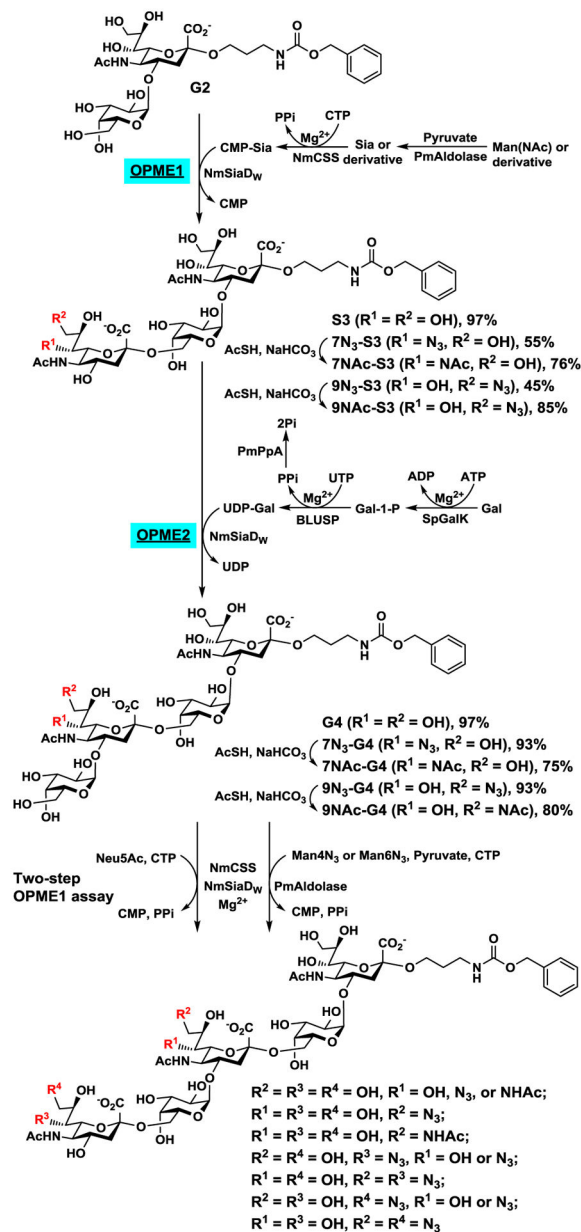
Figure 1.

Structure of *N. meningitidis* serogroup W (NmW) capsular polysaccharide (CPS) containing *O*-acetylation at C7 or C9 of *N*-acetylneuraminic acid (Neu5Ac).



Scheme 1.

Schematic illustration of the reactions for one-pot two-step donor substrate specificity studies of the α 2–6-sialyltransferase activity of NmSiaD_W (in Step 2) using CMP-sialic acids and analogs generated *in situ* in Step 1.

**Scheme 2.**

One-pot multienzyme (OPME) chemoenzymatic systems for the formation of NmW CPS trisaccharides, tetrasaccharides, pentasaccharides, and their azido or *N*-acetyl analogs.

Table 1.

Results of donor substrate specificity studies for the α 2–6-sialyltransferase activity of NmSiaD_W using *in situ* generated CMP-sialic acids and analogs.

	Donor precursor	Percentage conversion (%)		
		CMP-Sialic acid	Sialyltransfer	
			10 μ g/mL, 10 min	3.3 mg/mL, 10 h
1	Neu5Ac	^a CMP-Neu5Ac (Quant.)	30 \pm 0.3	Quant.
2	Neu5Gc	^a CMP-Neu5Gc (Quant.)	32 \pm 2	Quant.
3	Neu5Ac8OMe	^a CMP-Neu5Ac8OMe (Quant.)	0	83 \pm 7
4	Neu4,5Ac ₂	^{a,c} CMP-Neu4,5Ac ₂ (Quant.)	0	0
5	Neu5,9Ac ₂	^{a,c} CMP-Neu5,9Ac ₂ (Quant.)	0	0
6	Kdn	^a CMP-Kdn (Quant.)	0	98 \pm 2
7	ManNAz	^b CMP-Neu5NAz (Quant.)	28 \pm 2	Quant.
8	ManNAc4N ₃	^b CMP-Neu5Ac7N ₃ (Quant.)	0	78 \pm 3
9	ManNAc6N ₃	^b CMP-Neu5Ac9N ₃ (Quant.)	0	90 \pm 2
10	ManNAc6NAc	^b CMP-Neu5Ac9NAc (Quant.)	0	18 \pm 1
11	Man2N ₃	^b CMP-Neu5N ₃ (Quant.)	0	64 \pm 1

Step 1 of the reaction was carried out with a monosaccharide (**1–11**, 1.2 equiv.) in the presence of NmCSS (1.5 mg/mL) and CTP (1 equiv.) for 4 h ^awithout (for **1–6**) or ^bwith (for **7–11**) PmAldolase (4 mg/mL) and sodium pyruvate (5 equiv.). Tris-HCl (pH 8.5) was used for all reactions except for entries **4** and **5** where ^cTris-HCl (pH 7.5) was used to minimize de-*O*-acetylation which would take place at pH 8.5.

Table 2.

Results of the acceptor substrate specificity studies for the α 1–4-galactosyltransferase activity of NmSiaD_W using sialyltrisaccharides as potential acceptors and UDP-Gal as the donor.

	Substrates	Percentage conversion (%)	
		20 μ g/mL, 10 min	1.2 mg/mL, 10 h
1	S3	22 \pm 1	Quant.
2	7N ₃ -S3	11 \pm 1	Quant.
3	9N ₃ -S3	0	Quant.
4	7NAc-S3	0	0
5	9NAc-S3	0	68 \pm 3

Table 3.

Substrate specificity study for the α 2–6-sialyltransferase activity of NmSiaD_W using galactosyltetrasaccharides as well as Neu5Ac, ManNAc4N₃, and ManNAc6N₃.

	Acceptor	Percentage conversion (%)		
		CMP-Sialic acid	Transferase reaction	
			10 μ g/mL, 10 min	3.3 mg/mL, 10 h
1	G4	^a CMP-Neu5Ac (Quant.)	35 \pm 0.1	97 \pm 1
2	7N ₃ -G4		13 \pm 1	83 \pm 1
3	9N ₃ -G4		14 \pm 0.2	89 \pm 3
4	7NAc-G4		0	85 \pm 2
5	9NAc-G4		0	97 \pm 1
6	G4	^b CMP-Neu5Ac7N ₃ (Quant.)	0	90 \pm 1
7	7N ₃ -G4		0	82 \pm 2
8	9N ₃ -G4		0	36 \pm 1
9	7NAc-G4		0	0
10	9NAc-G4		0	0
11	G4	^b CMP-Neu5Ac9N ₃ (Quant.)	0	97 \pm 1
12	7N ₃ -G4		0	83 \pm 2
13	9N ₃ -G4		0	31 \pm 1
14	7NAc-G4		0	0
15	9NAc-G4		0	0

Step 1 of the reaction was carried out with ^aNeu5Ac, ^bManNAc4N₃, or ^bManNAc6N₃ (1.2 equiv.) in the presence of NmCSS (1.5 mg/mL), CTP (1 equiv.) and Tris-HCl (pH 8.5) ^awithout or ^bwith PmAldolase (4 mg/mL) and sodium pyruvate (5 equiv.).