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A Chemoenzymatic Synthon 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 ($ManNAc4N_3$) and 6-azido-6-deoxy-*N*-acetylmannosamine ($ManNAc6N_3$) as suitable chemoenzymatic synthons for synthesizing *N*-acetyl analogs of NmW CPS oligosaccharides containing 7-*O*-acetyl-*N*-acetylneuraminic acid ($Neu5,7Ac_2$) and/or 9-*O*-acetyl-*N*-acetylneuraminic acid ($Neu5,9Ac_2$). 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 N_3-oligosaccharides which were converted to the desired NAc-oligosaccharides by an efficient one-step chemical transformation.

Graphical Abstract

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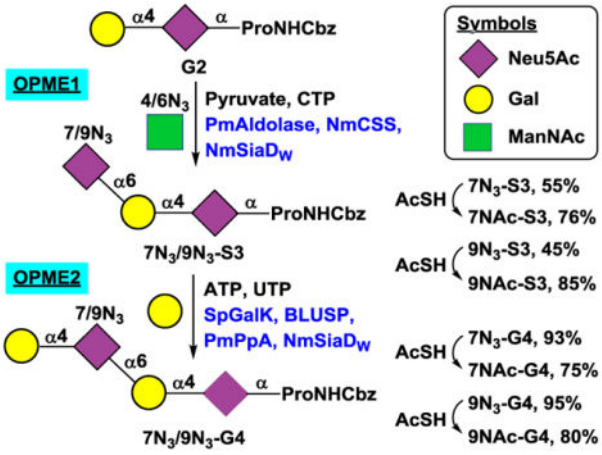
Supporting Information

Structures of CMP-sialic acids and precursors; HSQC spectra comparison of **S3** and **$7N_3$-S3** or **$9N_3$-S3**; ^1H, ^{13}C, HSQC and HSQC-TOCSY NMR spectra of the products. The Supporting Information is available free of charge on the ACS Publications website.

The authors declare no competing financial interest.



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 −6Galα1–4Neu5Acα2-) (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 NmOatWY[19] and *O*-acetyl migration from C7 to C9 in Neu5Ac of NmW CPS observed by nuclear magnetic resonance (NMR) spectroscopy studies[9] 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 cleavage[20] 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 (Neu5,9Ac_2).[21] As determined by systematic NMR spectroscopic and molecular dynamics simulation studies, Neu5,9Ac_2-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 Neu5,9Ac_2-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 (Neu4,5Ac_2), 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.[26]

RESULTS AND DISCUSSION

Donor substrate specificity studies of the α2–6-sialyltransferase activity of $NmSiaD_W$.

$NmSiaD_W$ is a bifunctional polysaccharide synthase that has both α1–4-galactosyltransferase and α2–6-sialyltransferase activities for the formation of NmW CPS containing a disaccharide repeating unit of −6Galα1–4Neu5Acα2-. It was used successfully in a sequential OPME system for the synthesis of structurally defined NmW CPS oligosaccharides ranging from disaccharide to decasaccharide from a benzyloxycarbonyl (Cbz)-tagged sialylmonosaccharide 2-*O*-(*N*-benzyloxycarbonyl) aminopropyl α-*N*-acetylneuraminide (Neu5AcαProNHCbz).[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 Galα1–4Neu5AcαProNHCbz (**G2**)[26] as the acceptor substrate, the donor substrate specificity study for the α2–6-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 μg/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 α2–6-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-N_3 group substitution (e.g. in CMP-Neu5N_3, entry **11**) was also tolerated but to a lower extend. In addition, while an 4-*O*-acetyl (e.g. in CMP-Neu4,5Ac_2, entry **4**) or 9-*O*-acetyl (e.g. in CMP-Neu5,9Ac_2, entry **5**) was not tolerated by $NmSiaD_W$, a 7-N_3 (e.g. in CMP-Neu5Ac7N_3, entry **8**), 8-OMe (e.g. in CMP-Neu5Ac8OMe, entry **3**), or 9-N_3 (e.g. in CMP-Neu5Ac9N_3, 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-Neu5Ac7N_3 (entry **8**), and CMP-Neu5Ac9N_3 (entry **9**) as donor substrates for the α2–6-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**),

ManNAc4N_3 (entry **8**), and ManNAc6N_3 (entry **9**) can be further explored as potential probes for metabolic engineering of NmW CPS. The latter two (ManNAc4N_3[31] and ManNAc6N_3[32]) 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 N_3-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 α2–6-sialyltransferase activity of $NmSiaD_W$ compared to the α2–3-sialyltransferase activity of *Pasteurella multocida* sialyltransferase 1 (PmST1)[34] and α2–6-sialyltransferase activity of *Photobacterium damselae* α2–6-sialyltransferase (Pd2,6ST).[35] Both PmST1 and Pd2,6ST were able to tolerate all[24, 34–36] except one (CMP-Neu4,5Ac_2, entry **4**)[37] *in situ*-generated CMP-Sia and analogs shown in Table 1. For example, *in situ*-generated CMP-Neu5,9Ac_2 (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 α2–6-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 Galα1–4Neu5AcαProNHCbz **(G2)**[26] as the acceptor substrate and chemoenzymatic synthons ManNAc4N_3 and ManNAc6N_3 as donor precursors. As shown in Scheme 2, $NmSiaD_W$ was used together with PmAldolase[30] and NmCSS[28] in an OPME α2–6-sialylation system (**OPME1**) to sialylate **G2** for the formation of sialyl trisaccharides containing Neu5Ac, Neu5Ac7N_3, or Neu5Ac9N_3. In this system, sodium pyruvate (excess amount, 5–10 equiv.) and ManNAc, ManNAc4N_3, or ManNAc6N_3 were used by PmAldolase to produce Neu5Ac or its monoazido-analog Neu5Ac7N_3 or Neu5Ac9N_3. 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 α2–6-linked sialosides (Scheme 2). From 150 mg **G2**, trisaccharide **S3** (220 mg, 97%), **7N_3-S3** Neu5Ac7N_3α2–6Galα1–4Neu5AcαProNHCbz (120 mg, 55%), or **9N_3-S3** Neu5Ac9N_3α2–6Galα1–4Neu5AcαProNHCbz (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 N_3 in **7N_3-S3** led to an upfield shift of the C7 ^{13}C signal from 68.24 ppm to 61.19 ppm. The influence on the C7 ^{1}H chemical shift was weaker, with 0.01 ppm difference from 3.57 ppm in **S3** to 3.56 ppm in **7N_3-S3** (Figure S2). For **9N_3-S3**, the ^{13}C 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 **$9N_3$-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 **$7N_3$-S3** and **$9N_3$-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** Neu5Ac7NAcα2–6Galα1–4Neu5AcαProNHCbz (13 mg, 76%) and **9NAc-S3** Neu5Ac9NAcα2–6Galα1–4Neu5AcαProNHCbz (12 mg, 85%) in good yields.

Acceptor substrate specificity of the α1–4-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 α1–4-galactosyltransferase activity of $NmSiaD_W$. Therefore, sialyltrisaccharides **S3**, **$7N_3$-S3**, **$9N_3$-S3**, **7NAc-S3**, and **9NAc-S3** obtained above were tested as acceptor substrates for the α1–4-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±3%) was lower than others (quantitative yields). These results suggest that **$7N_3$-S3** and **$9N_3$-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 **$7N_3$-S3** and **$9N_3$-S3**, in addition to **S3**, were suitable acceptor substrates for the α1–4-galactosyltransferase activity of $NmSiaD_W$, preparative-scale synthesis of galactosyltetrasaccharides were carried out using an OPME α1–4-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 α1–4-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 **$7N_3$-S3** or **$9N_3$-S3**, tetrasaccharide **G4** (56 mg, 97%), **$7N_3$-G4** Galα1–4Neu5Ac$7N_3$α2–6Galα1–4Neu5AcαProNHCbz (54 mg, 93%), and **$9N_3$-G4** Galα1–4Neu5Ac$9N_3$α2–6Galα1–4Neu5AcαProNHCbz (55 mg, 95%) were formed in excellent yields.

Similar to that described above, the azido group in **$7N_3$-G4** and **$9N_3$-G4** was converted to *N*-acetyl group using thioacetic acid in a saturated sodium bicarbonate solution[33, 38] to obtain **7NAc-G4** Galα1–4Neu5Ac7NAcα2–6Galα1–4Neu5AcαProNHCbz (20 mg, 75%) and **9NAc-G4** Galα1–4Neu5Ac9NAcα2–6Galα1–4Neu5AcαProNHCbz (21 mg, 80%), respectively, in good yields.

Donor and acceptor substrate specificity studies of the α2–6-sialyltransferase activity of $NmSiaD_W$ with galactosyltetrassacharide acceptors.

The obtained galactosyltetrasaccharides **G4**, **$7N_3$-G4**, **$9N_3$-G4**, **7NAc-G4**, and **9NAc-G4** as well as donor precursors $ManNAc4N_3$ and $ManNAc6N_3$ 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-$Neu5Ac7N_3$, and CMP-$Neu5Ac9N_3$ were obtained readily *in situ* from the corresponding precursors Neu5Ac, $ManNAc4N_3$, and $ManNAc6N_3$, respectively, in a reaction catalyzed by NmCSS with (for $ManNAc4N_3$ and $ManNAc6N_3$) or without (for Neu5Ac) PmAldolase in step 1. The analysis of the $NmSiaD_W$-catalyzed α2–6-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 N_3-substitution at the C7 or C9 of the Neu5Ac in the acceptor was better tolerated than the corresponding *N*-acetyl-substitution.

When $ManNAc4N_3$ (entries **6–10** in Table 3) or $ManNAc6N_3$ (entries **11–15** in Table 3) was used as the donor precursor for the α2–6-sialyltransferase activity of $NmSiaD_W$, **G4**, **$7N_3$-G4**, and **$9N_3$-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 **$9N_3$-G4** was used as the acceptor substrate and $ManNAc4N_3$ (entry **8** in Table 3) or $ManNAc6N_3$ (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 substates 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. $ManNAc4N_3$ and $ManNAc6N_3$, the six-carbon precursors for $Neu5Ac7N_3$ and $Neu5Ac9N_3$, 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 α2–6-

sialyltransferase activity of $NmSiaD_W$ and the tolerance of the resulting oligosaccharides as acceptors for the α1–4-galactosyltransferase activity of $NmSiaD_W$ showed the potential of using $ManNAc4N_3$ and $ManNAc6N_3$ 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, ^{13}C 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 μm, 2.1×50 mm, 959757–902), an AdvanceBio Glycan Map (1.8 μm, 2.1×150 mm, 859700–913) column from Agilent Technologies or an Dionex CarboPac PA100 (8.5 μm, 4×250 mm, 043055) column from Thermo Scientific. Reverse phase chromatography purification of products was performed with a C18 column (ODS-SM, 50 mm, 120 Å, 3.0×20 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. $ManNAc4N_3$,[31] $ManNAc6N_3$,[32] and Galα1–4Neu5AcαProNHCbz (**G2**)[26] were synthesized as described previously.

Two-step donor substrate specificity study for the α2–6-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 μL in a buffer (Tris-HCl, 100 mM, pH 8.5; pH 7.5 for reactions with $Neu4,5Ac_2$ or $Neu5,9Ac_2$) containing $MgCl_2$ (10 mM), sialic acid or its analog (6 mM), CTP (5 mM), and NmCSS (15 μg). If a sialic acid precursor (6 mM) was used as a starting material, sodium pyruvate (25 mM) and PmAldolase (40 μg) 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 $Neu4,5Ac_2$ or $Neu5,9Ac_2$) containing $MgCl_2$ (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 EcilpsePlusC18 column or an AdvancBio Glycan Map column (Agilent), as well as by Bruker UltraFlextreme MALDI-TOF in a negative mode. Sialic acids and derivatives tested were Neu5Ac, Neu5Gc, Neu5Ac8OMe,[43] $Neu4,5Ac_2$,[37] $Neu5,9Ac_2$,[44] and Kdn. Sialic acid precursors and derivatives tested were ManNAz,[34] $ManNAc4N_3$,[31] $ManNAc6N_3$,[32] ManNAc6NAc,[21] and $Man2N_3$.[35]

Acceptor substrate specificity study for the α1–4-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 $MgCl_2$ (10 mM), UDP-Gal (2 mM), a sialyltrisaccharide **S3** or analog (**$7N_3$-S3**, **$9N_3$-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 EcilpsePlusC18 column (Agilent) and by Bruker UltraFlextreme MALDI-TOF in a negative mode.

Donor and acceptor substrate specificity studies of the α2–6-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, $ManNAc4N_3$, or $ManNAc6N_3$) (6 mM), CTP (5 mM), sodium pyruvate (25 mM), $MgCl_2$ (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 (**$7N_3$-G4**, **$9N_3$-G4**, **7NAc-G4**, or **9NAc-G4**) (1 mM), $MgCl_2$ (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 UltraFlextreme MALDI-TOF in a negative mode.

OPME synthesis of Neu5Ac$7N_3$α2–6Galα1–4Neu5AcαProNHCbz ($7N_3$-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), $ManNAc4N_3$ (75 mg, 0.30 mmol), sodium pyruvate (253 mg, 2.3 mmol), CTP disodium salt (218 mg, 0.41 mmol), $MgCl_2$ (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 **7N$_3$-S3** as a sodium salt (120 mg, 55%).

^{1}H NMR (800 MHz, D_2O) δ 7.47–7.38 (m, 5H, Ar-H), 5.11 (s, 2H, O-$\underline{CH_2}$-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-$\underline{CH_2}$-CH_2), 3.24–3.16 (m, 2H, CH_2-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’-CH_3-CO), 2.04 (s, 3H, H”’-CH_3-CO), 1.78–1.69 (m, 3H, O-CH_2-$\underline{CH_2}$-CH_2-NH; H”’−3_{ax}), 1.61 (t, *J* = 12.0 Hz, 1H, H’−3_{ax}). ^{13}C{^{1}H} NMR (200 MHz, D_2O) δ 174.53, 174.37, 173.34, 173.16, 158.37 (NH-COO), 136.56 (O-CH_2-$\underline{Ar}$), 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-$\underline{CH_2}$-Ar), 62.58, 62.54, 62.40, 62.06 (O-$\underline{CH_2}$-CH_2), 61.23 (C”’−7), 52.51 (C”’−5), 49.54 (C’−5), 39.88 (C”’−3), 37.47 (CH_2-NH), 36.78 (C’−3), 28.89 (O-CH_2-$\underline{CH_2}$-CH_2-NH), 22.43 (C’-$\underline{CH_3}$-CO), 22.11 (C”’-$\underline{CH_3}$-CO). HRMS (ESI-Orbitrap) m/z: [M - H]$^-$ Calcd for $C_{39}H_{57}N_6O_{23}$ 977.3475; found 977.3476.

Chemical synthesis of Neu5Ac7NAcα2–6Galα1–4Neu5AcαProNHCbz (7NAc-S3).

7N$_3$-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 CH_3CN in H_2O gradient as the elution solvent. **7NAc-S3** was obtained as a white solid (13 mg, 76% yield). ^{1}H NMR (800 MHz, D_2O) 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). ^{13}C{^{1}H} NMR (200 MHz, D_2O) 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 $C_{41}H_{61}N_4O_{24}$ 993.3681; found 993.3696.

OPME synthesis of Neu5Ac9N$_3$α2–6Galα1–4Neu5AcαProNHCbz (9N$_3$-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), ManNAc6N_3 (75 mg, 0.30 mmol), sodium pyruvate (203 mg, 1.85 mmol), CTP disodium salt (194 mg, 0.37 mmol), $MgCl_2$ (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 **$7N_3$-S3**. **$9N_3$-S3** was obtained as a sodium salt (98 mg, 45%). ^{1}H NMR (800 MHz, D_2O) δ 7.46–7.37 (m, 5H, Ar-H), 5.11 (s, 2H, O-$\underline{CH_2}$-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-$\underline{CH_2}$-CH_2; H”’−9), 3.24–3.16 (m, 2H, CH_2-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’-CH_3-CO), 2.03 (s, 3H, H”’-CH_3-CO), 1.75 (p, *J* = 6.6 Hz, 2H, O-CH_2-$\underline{CH_2}$-CH_2-NH), 1.70 (t, *J* = 12.2 Hz, 1H, H”’−3_{ax}), 1.61 (t, *J* = 12.0 Hz, 1H, H’−3_{ax}). ^{13}C{^{1}H} NMR (200 MHz, D_2O) δ 174.93, 174.50, 173.44, 173.30, 158.37 (NH-COO), 136.56 (O-CH_2-$\underline{Ar}$), 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-$\underline{CH_2}$-Ar), 62.87, 62.51, 62.05 (O-$\underline{CH_2}$-CH_2), 53.03 (C”’−9), 51.81 (C”’−5), 49.54 (C’−5), 40.04 (C”’−3), 37.47 (CH_2-NH), 36.71 (C’−3), 28.89 (O-CH_2-$\underline{CH_2}$-CH_2-NH), 22.39 (C’-$\underline{CH_3}$-CO), 22.01 (C”’-$\underline{CH_3}$-CO). HRMS (ESI-Orbitrap) m/z: [M - H]$^-$ Calcd for $C_{39}H_{57}N_6O_{23}$ 977.3475; found 977.3479.

Chemical synthesis of Neu5Ac9NAcα2–6Galα1–4Neu5AcαProNHCbz (9NAc-S3).

$9N_3$-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%). ^{1}H NMR (800 MHz, D_2O) 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). ^{13}C{^{1}H} NMR (200 MHz, D_2O) 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 $C_{41}H_{61}N_4O_{24}$ 993.3681; found 993.3691.

OPME synthesis of Galα1–4Neu5Ac7N_3α2–6Galα1–4Neu5AcαProNHCbz ($7N_3$-G4).

A reaction mixture in a total volume of 10 mL containing Tris-HCl buffer (100 mM, pH 8.5), sialyltrisacchairde **$7N_3$-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), $MgCl_2$ (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 **$7N_3$-S3**. **$7N_3$-G4** was obtained as a sodium salt (54 mg, 93%). ^{1}H NMR (800 MHz, D_2O) δ 7.47–7.38 (m, 5H, Ar-H), 5.11 (s, 2H, O-$\underline{CH_2}$-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-$\underline{CH_2}$-CH_2), 3.25–3.15 (m, 2H, CH_2-NH), 2.90–2.85

(dd, *J* = 12.8, 4.8 Hz, 2H, H'−3_{eq}; H"'−3_{eq}), 2.08 (s, 3H, H'-CH_3-CO), 2.05 (s, 3H, H"'-CH_3-CO), 1.75 (p, *J* = 6.7 Hz, 2H, O-CH_2-CH_2-CH_2-NH), 1.68 (t, *J* = 12.1 Hz, 1H, H"' −3_{ax}), 1.61 (t, *J* = 12.0 Hz, 1H, H'−3_{ax}). $^{13}C\{^1H\}$ NMR (200 MHz, D_2O) δ 174.51, 174.07, 173.37, 172.81, 158.37 (NH-COO), 136.57 (O-CH_2-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-CH_2-Ar), 62.77, 62.48, 62.40, 62.06 (O-CH_2-CH_2), 61.24 (C"'−7), 60.70, 50.34 (C"'−5), 49.55 (C'−5), 37.48 (CH_2-NH), 36.78 (C"'−3), 36.66 (C'−3), 28.90 (O-CH_2-CH_2-CH_2-NH), 22.43 (C'-CH_3-CO), 22.15 (C"'-CH_3-CO). HRMS (ESI-Orbitrap) m/z: $[M - H]^-$ Calcd for $C_{45}H_{67}N_6O_{28}$ 1139.4003; found 1139.4007.

Chemical synthesis of Galα1–4Neu5Ac7NAcα2–6Galα1–4Neu5AcαProNHCbz (7NAc-G4).

To a round bottom flask containing **$7N_3$-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, D_2O) 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). $^{13}C\{^1H\}$ NMR (200 MHz, D_2O) 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 $C_{47}H_{71}N_4O_{29}$ 1155.4209; found 1155.4214.

OPME synthesis of Galα1–4Neu5Ac9N_3α2–6Galα1–4Neu5AcαProNHCbz ($9N_3$-G4).

A reaction mixture in a total volume of 10 mL containing Tris-HCl buffer (100 mM, pH 8.5), sialyltrisacchairde **$9N_3$-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), $MgCl_2$ (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 **$7N_3$-S3**. **$9N_3$-G4** was obtained as a sodium salt (55 mg, 95%). ^1H NMR (800 MHz, D_2O) δ 7.46–7.37 (m, 5H, Ar-H), 5.10 (d, J = 4.1 Hz, 3H, O-CH_2-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, CH_2-NH), 2.88–2.82 (m, 2H,, H'−3_{eq}; H"'−3_{eq}), 2.06 (s, 3H, H'-CH_3-CO), 2.03 (s, 3H, H"'-CH_3-CO), 1.78–1.69 (m, 4H, O-CH_2-CH_2-CH_2-NH; H"' −3_{ax}; H'−3_{ax}). $^{13}C\{^1H\}$ NMR (200 MHz, D_2O) δ 174.47, 174.33, 171.77, 171.37, 158.35 (NH-COO), 136.54 (O-CH_2-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-CH_2-Ar), 63.29, 62.80, 62.06 (O-CH_2-CH_2), 60.72, 53.43 (C"'−9), 49.36 (C"'−5), 49.31 (C'−5),, 37.37 (CH_2-NH), 36.01 (C"'−3) 35.56 (C'−3), 28.77 (O-CH_2-CH_2-CH_2-NH), 22.30 (C'-CH_3-CO),

22.11 (C"'-$\underline{CH_3}$-CO). HRMS (ESI-Orbitrap) m/z: $[M - H]^-$ Calcd for $C_{45}H_{67}N_6O_{28}$ 1139.4003; found 1139.4021.

Chemical synthesis of Galα1–4Neu5Ac9NAcα2–6Galα1–4Neu5AcαProNHCbz (9NAc-G4).

To a round bottom flask containing **$9N_3$-G4** (26 mg), saturated sodium bicarbonate solution (3 mL) was added, and then 300 μL 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%). ^{1}H NMR (800 MHz, D_2O) 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). ^{13}C{^{1}H} NMR (200 MHz, D_2O) 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 $C_{47}H_{71}N_4O_{29}$ 1155.4209; found 1155.4223.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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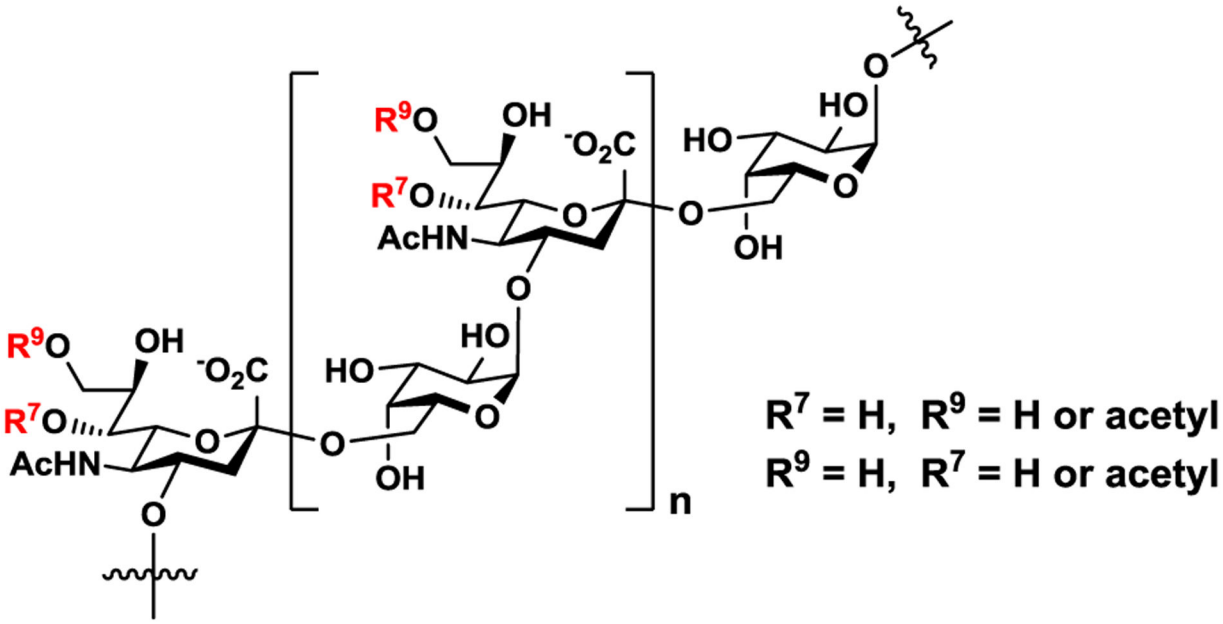
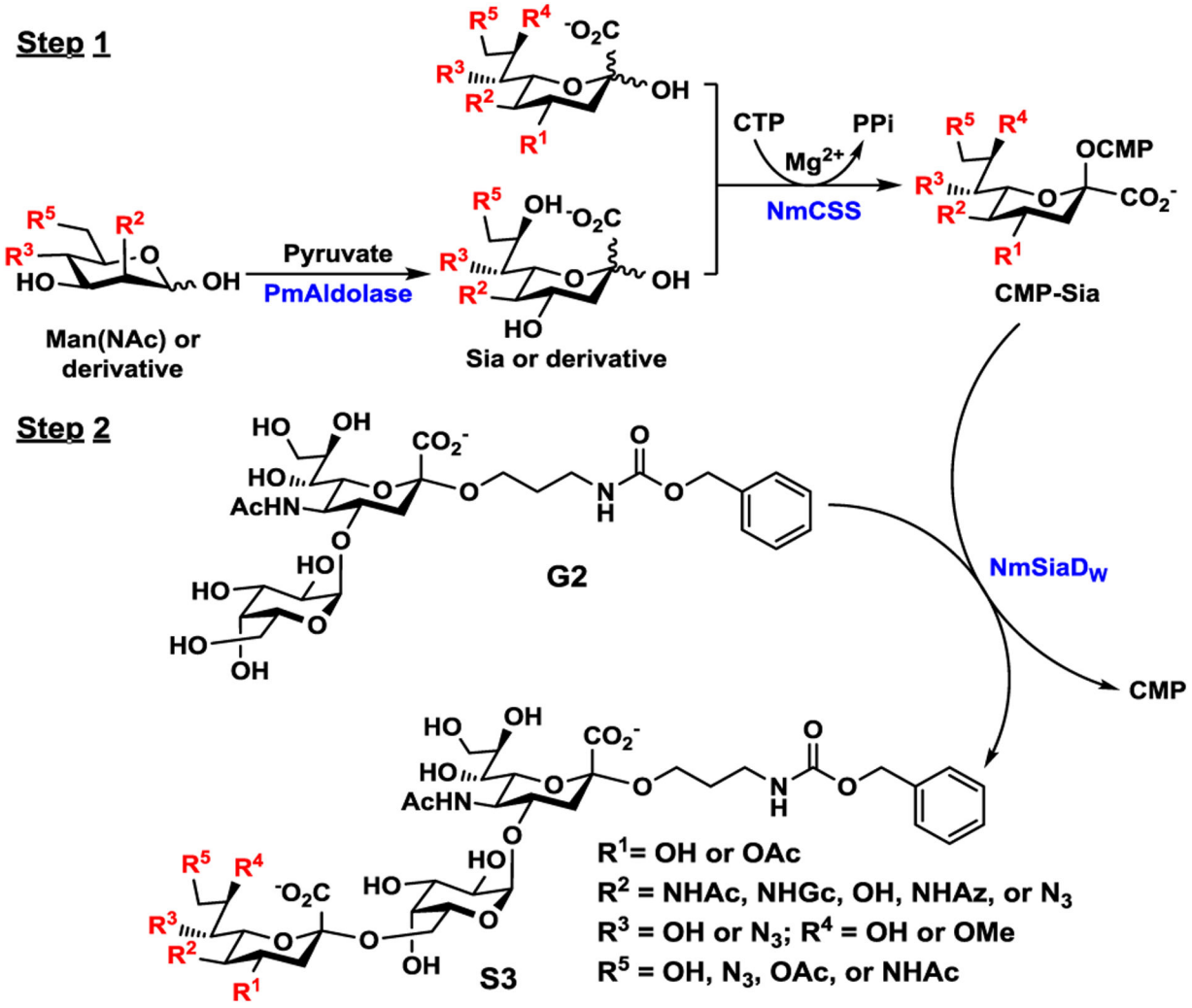


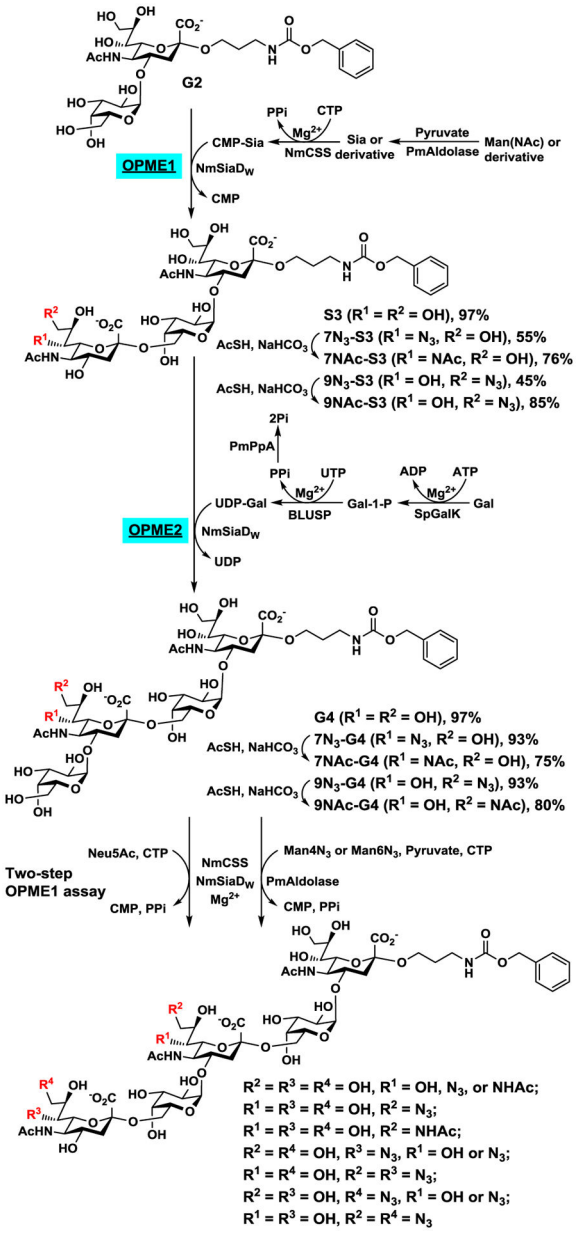
Figure 1.

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 α2–6-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 α2–6-sialyltransferase activity of $NmSiaD_W$ using *in situ* generated CMP-sialic acids and analogs.

	Donor precursor	Percentage conversion (%)		
		CMP-Sialic acid	Sialyltransfer	
			10 μg/mL, 10 min	3.3 mg/mL, 10 h
1	Neu5Ac	[a]CMP-Neu5Ac (Quant.)	30±0.3	Quant.
2	Neu5Gc	[a]CMP-Neu5Gc (Quant.)	32±2	Quant.
3	Neu5Ac8OMe	[a]CMP-Neu5Ac8OMe (Quant.)	0	83±7
4	$Neu4,5Ac_2$	[a,c]$CMP\text{-}Neu4,5Ac_2$ (Quant.)	0	0
5	$Neu5,9Ac_2$	[a,c]$CMP\text{-}Neu5,9Ac_2$ (Quant.)	0	0
6	Kdn	[a]CMP-Kdn (Quant.)	0	98±2
7	ManNAz	[b]CMP-Neu5NAz (Quant.)	28±2	Quant.
8	$ManNAc4N_3$	[b]$CMP\text{-}Neu5Ac7N_3$ (Quant.)	0	78±3
9	$ManNAc6N_3$	[b]$CMP\text{-}Neu5Ac9N_3$ (Quant.)	0	90±2
10	ManNAc6NAc	[b]CMP-Neu5Ac9NAc (Quant.)	0	18±1
11	$Man2N_3$	[b]$CMP\text{-}Neu5N_3$ (Quant.)	0	64±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 [a]without (for **1–6**) or [b]with (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 [c]Tris-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 α1–4-galactosyltransferase activity of $NmSiaD_W$ using sialyltrisaccharides as potential acceptors and UDP-Gal as the donor.

	Substrates	Percentage conversion (%)	
		20 μg/mL, 10 min	1.2 mg/mL, 10 h
1	**S3**	22±1	Quant.
2	**$7N_3$-S3**	11±1	Quant.
3	**$9N_3$-S3**	0	Quant.
4	**7NAc-S3**	0	0
5	**9NAc-S3**	0	68±3

Table 3.

Substrate specificity study for the α2–6-sialyltransferase activity of $NmSiaD_W$ using galactosyltetrasaccharides as well as Neu5Ac, $ManNAc4N_3$, and $ManNAc6N_3$.

	Acceptor	Percentage conversion (%)		
		CMP-Sialic acid	Transferase reaction	
			10 μg/mL, 10 min	3.3 mg/mL, 10 h
1	G4	[a]CMP-Neu5Ac (Quant.)	35±0.1	97±1
2	$7N_3$-G4		13±1	83±1
3	$9N_3$-G4		14±0.2	89±3
4	7NAc-G4		0	85±2
5	9NAc-G4		0	97±1
6	G4	[b]CMP-Neu5Ac$7N_3$ (Quant.)	0	90±1
7	$7N_3$-G4		0	82±2
8	$9N_3$-G4		0	36±1
9	7NAc-G4		0	0
10	9NAc-G4		0	0
11	G4	[b]CMP-Neu5Ac$9N_3$ (Quant.)	0	97±1
12	$7N_3$-G4		0	83±2
13	$9N_3$-G4		0	31±1
14	7NAc-G4		0	0
15	9NAc-G4		0	0

Step 1 of the reaction was carried out with [a]Neu5Ac, [b]$ManNAc4N_3$, or [b]$ManNAc6N_3$ (1.2 equiv.) in the presence of NmCSS (1.5 mg/mL), CTP (1 equiv.) and Tris-HCl (pH 8.5) [a]without or [b]with PmAldolase (4 mg/mL) and sodium pyruvate (5 equiv.).