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Effective one-pot multi-enzyme (OPME) synthesis of monotreme milk oligosaccharides and other sialosides containing a 4-O-acetyl sialic acid†

Hai Yu,† Jie Zeng,† Yanhong Li,‡ Vireak Thon,§ Baojun Shi,‖ and Xi Chen∗‡

Abstract: A facile one-pot two-enzyme chemoenzymatic approach has been established for gram (Neu4,5Ac5Gc) and preparative-scale (Neu4,5Ac5Gc3LnnT) synthesis of monotreme milk oligosaccharides. Other O-acetyl-S-acetyleneuraminic acid (Neu4,5Ac5C) or 4-O-acetyl-5-N-glycolyneuraminic acid (Neu4Ac5Gc)-containing α2–3-sialosides have also been synthesized in preparative scale. Used as an effective probe, Neu4,5Ac5Gc3GalβpNP was found to be a suitable substrate by human influenza A viruses but not bacterial sialidases.

Introduction

Sialic acids commonly presented as the terminal residues on mammalian oligosaccharides or glycoconjugates are key recognition components of bacterial adhesins, viral surface proteins, selectins, Siglecs, and other sialic acid-binding proteins involved in infection, inflammation, cancer metastasis, and immunoregulation.1–4 Sialic acids present tremendous structural diversity in nature and more than 50 members have been identified so far. These include N-acetyleneuraminic acid (Neu5Ac), the non-human sialic acid N-glycolyneuraminic acid (Neu5Gc), 2-keto-3-deoxy-nonulosonic acid (Kdn), their derivatives with single or multiple O-acetylation and less frequent 8-O-methylation, 8-O-sulfation, 9-O-lactylation, or 9-O-phosphorylation.1,4

O-Acetylation is the most frequently occurring modification of sialic acids. Single or multiple O-acetylation at positions 4, 7, 8, and/or 9 of sialic acid has been identified and the presentation varies among different types of species, organs, and cells.1,4 Comparing to 9-O-acetylation which is the most common sialic acid O-acetylation, 4-O-acetylated sialic acids (Fig. 1) including 4-O-acetyl-N-acetyleneuraminic acid (Neu4,5Ac5C, 1) and 4-O-acetyl-N-glycolyneuraminic acid (Neu4Ac5Gc, 2) are less common.5 Neu4,5Ac5C (1) has been found in horse,6 donkey,7 Australian monotreme Echidna,8 Japanese dace,9 South American pit viper,10 rabbit,11 guinea-pig.12 Neu4,5Ac5C-containing oligosaccharides have been found to be the dominant components of the acidic milk oligosaccharides (MOSs) of monotremes echidnas and platypus.12–14 Both Neu4,5Ac5C and Neu4Ac5Gc have been found in horse glycoproteins.15 The presence of Neu4Ac5Gc-GM3 ganglioside in human colon cancer tissues was detected using purified antibodies.15 Neu4Ac5Gc (2) has also been found in α2–8-linked polysialic acids of glycoproteins from unfertilized kokanee salmon egg16 and in the serum of guinea pig in trace amounts.17

4-O-Acetylation of sialic acids provides resistance or decreased sensitivity of sialosides to various sialidases.5,18,19 This property is believed to contribute to the inhibitory activity of Neu4,5Ac5C-rich horse and guinea pig α2-macroglobulins against hemagglutination and infectivity of several human influenza viruses.6,18,19 On the other hand, Neu4,5Ac5C is selectively recognized by the hemagglutinin-esterase (HE)20,21 of mouse hepatitis virus strain S (MHSV), a type of murine coronavirus, and infectious salmon anemia virus (ISAV).22 Nevertheless, the significance of naturally existing 4-O-acetylated sialic acids is not well understood partly due to the lack of access to sufficient amounts of the corresponding sialosides.

To our knowledge, the synthesis of sialosides containing 4-O-acetylated sialic acids by either chemical or enzymatic methods has not been reported. Here we present a facile chemoenzymatic approach for preparative and gram-scale
synthesis of monomeric milk oligosaccharides including Neu4,5Ac2-containing α2–3-linked sialyl lactose (Neu4,5Ac2α3Lac) and sialyl lacto-N-neotetraose (Neu4,5Ac2α3Lnt). Other Neu4,5Ac2- or Neu4Ac5Gc-containing α2–3-linked sialosides representing common terminal sialosides in mammals are also synthesized.

Results and discussion

We envisioned that the desired 4-O-acetyl-sialic acid-containing sialosides could be chemoenzymatically synthesized using an effective one-pot two-enzyme sialylation system containing a cytidine 5’-monophosphate-sialic acid (CMP-Sia) synthetase (CSS) and a sialyltransferase (ST).23 To test the feasibility, the corresponding 4-O-acetyl-sialic acids were chemically synthesized. As shown in Scheme 1, Neu4,5Ac2 (1) was synthesized from N-acetylneuraminic acid (Neu5Ac, 3) by esterification of the C1-carboxyl group with benzyl bromide to provide benzyl ester 4 with a 90% yield. Protection of 4 at C8 and C9 by adding 2,2-dimethoxypropane in the presence of a catalytic amount of p-toluenesulfonic acid produced partially protected 5 in an excellent 92% yield. Regioselectively acetylation of 5 at O-4 using acetic anhydride in pyridine at room temperature provided the key intermediate 6 in a 81% final yield. Final deprotection of the O-isopropylidene group in 6 by treating with 80% acetic acid and removal of the benzyl ester group by catalytic hydrogenolysis with H2 in the presence of Pd/C in methanol resulted in Neu4,5Ac2 (1) in a 82% yield. The obtained product has a proton nuclear magnetic resonance (1H NMR) spectrum consistent with that reported.24 The 4-O-acetyl modification caused a downfield shift of Neu5Ac H-4 signal.

Scheme 1 Chemical synthesis of Neu4,5Ac2 (1).

Neu4Ac5Gc (2) was chemoenzymatically synthesized from D-mannosamine hydrochloride (ManNH2·HCl, 7). As shown in Scheme 2, N-(2-Benzoyloxyacetyl)-D-mannosamine 8 was obtained by treating ManNH2·HCl (7) with 2-benzyloxyacetyl chloride in the presence of NaHCO3 in CH2CN and water. P. multocida sialic acid aldolase (PmNanA)25-catalyzed aldol reaction of 8 with sodium pyruvate at 37 °C for 48 h produced the corresponding sialic acid derivative 9 in an excellent quantitative yield. The introduction of 4-O-acetyl group was carried out similarly to that described above for the synthesis of Neu4,5Ac2 (1). Briefly, selective benzylation of 9 with benzyl bromide formed benzyl ester 10 in a 90% yield. Treating 10 with 2,2-dimethoxypropane produced 9,9-isopropylidene-protected 11 in a 96% yield. Selective acetylation of benzoate 11 with Ac2O and pyridine produced the corresponding 4-O-acetyl derivative 12 in a good yield (81%). Subsequent removal of the isopropylidene group using HOAc/H2O followed by debenzylation using catalytic hydrogenation produced the target Neu4Ac5Gc (2) in a 78% yield.

Scheme 2 Chemoenzymatic synthesis of Neu4Ac5Gc (2).

Sialyltransferase-catalyzed reactions use CMP-sialic acids which can be potentially accessed by a suitable CMP-Sia synthetase (CSS, EC 2.7.7.43). The tolerance of using Neu4,5Ac2 (1) as a potential substrate was tested for several bacterial CSSs including those from Neisseria meningitidis (NmCSS),36 Pasteurella multocida (PmCSS), Haemophilus ducreyi (HdCSS), as well as two NmCSS mutants (NmCSS_S81R and NmCSS_Q163A).37 Small-scale reactions carried out in Tris-HCl buffer (100 mM, pH 7.1) at 37 °C for 15 h followed by thin-layer chromatography (TLC) and mass spectrometry (MS) analyses indicated that all CSSs tested could use Neu4,5Ac2 (1) efficiently (Fig. S1, ES1). These results were quite exciting as a previous report showed that CSSs purified from calf brain, bovine, and equine submaxillary glands could not use Neu4,5Ac2 (1) as a substrate for the synthesis of the corresponding CMP-sialic acid.38 NmCSS was chosen for further studies and for preparative-scale and gram-scale syntheses.

A coupled enzymatic assay was used to evaluate several bacterial sialyltransferases in using CMP-Neu4,5Ac2 and CMP-Neu4Ac5Gc synthesized in situ by NmCSS from Neu4,5Ac2 (1) and Neu4Ac5Gc (2), respectively, as potential donor substrates. Sialyltransferases tested include α2–3-sialyltransferases from Pasteurella multocida (PmST1,29 PmST2,30 PmST3)31 and PmST1_M144D mutant;32 α2–6-sialyltransferases Pd26ST33 and Psp26ST,34 as well as α2–3/8-sialyltransferase CstII.35, 36 Among these sialyltransferases, only PmST3 showed good activity as shown by TLC and MS studies. PmST3 was previously characterized as a monofunctional α2–3-sialyltransferase encoded by Pm1174 gene in Pasteurella...
multocida strain Pm70. It had broad acceptor substrate specificity and could use oligosaccharides, glycolipids, and glycopeptides as acceptors.\textsuperscript{31, 37} PmST3 could use oligosaccharides containing a terminal β1–4- or β1–3-linked galactose as acceptors, but had poor activity on using galactosides containing a monosaccharide unit as acceptors. It could not use fucosylated oligosaccharides, such as Le\(^\text{a}\), as acceptor substrates. It was delightful to identify the unique efficiency of PmST3, among various bacterial sialyltransferases, in catalyzing the synthesis of sialosides containing 4-O-acetyl sialic acid.

Table 1 One-pot two-enzyme (OP2E) preparative-scale synthesis of α2–3-linked 4-O-acetylated sialosides. Yields were calculated for purified products based on the amounts of acceptors (limiting reagents) used.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Acceptor</th>
<th>Donor</th>
<th>Sialosides (Neu4,5Ac(\beta)Neu4Ac(\alpha)Glc(\alpha)Glc(\alpha)OR)</th>
<th>yield</th>
</tr>
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<tbody>
<tr>
<td>a</td>
<td>Ga IPorLac(13)</td>
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<td>Neu4Ac(\beta)Neu4Ac(\alpha)Glc(\alpha)Glc(\alpha)OR</td>
<td>82%</td>
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<td>78%</td>
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<tr>
<td>d</td>
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<td>31%</td>
</tr>
<tr>
<td>e</td>
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</tr>
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<td>k</td>
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<tr>
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<tr>
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<td>62%</td>
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Bifidobacterium longum (OP4E) GlcNAc activation and transfer systems containing NahK_ATCC55813), (NmLgtA).

Monoterm milk oligosaccharides Neu4,5Ac2Gal3Lac (24) and Neu4,5Ac2α3LnnT (25) were readily produced from lactose (Gal)4Glc or Lac, 13 and previously synthesized lacto-Neotetraose (Gal)4GlcNacβ3Galβ4Glc or LnnT, 14 in 82% and 78% yields, respectively (Table 1). Neu4,5Ac2Gal3LnnT (26) was also synthesized from commercially available lacto-N-tetraose (Galβ3GlcNacβ3Galβ4Glc or LNT, 15) in a 65% yield. GalβPnP (16) with a monosaccharide unit was a less efficient acceptor and Neu4,5Ac2α3GalβPnP (27) was obtained in a 31% yield (the yield could not be improved further by adding more enzymes). Other α2,3-linked sialylated disaccharides containing a propyl azide glycolyne (28–33) were also successfully synthesized from the corresponding disaccharides 29, 40–41 17–22 in 74–88% yields. To demonstrate the efficiency of the OP2E system, monoterm milk trisaccharide Neu4,5Ac2α3Lac (24) was further synthesized in a gram scale (1.33 g) with a 71% yield.

A longer propyl azide-containing oligosaccharide Galβ3GlcNacβ3Galβ4GlcβPnPr β (23) was synthesized from LcβPnPr β (17) using a sequential two-step one-pot multienzyme (OPME) 28 process (Scheme 3) similar to that reported previously for LnnT. Briefly, Lc3 trisaccharide GlcNacβ3Glcβ4GlcβPnPr β was synthesized from LacβPnPr β (17) and GlcNac in a 94% yield using a one-pot four-enzyme (OP4E) GlcNac activation and transfer system consisting of Bifidobacterium longum N-acetylhexosamine-1-kinase (BLNahk, Nahk_ATCC55813), 13 Pasteurella multocida N-acetylgalactosamine uridylyltransferase (PmglmU), 6 Pasteurella multocida inorganic pyrophosphatase (PpmPpa), 40 and Neisseria meningitidis β1-3-N-acetylgalactosaminyltransferase (Nmlgta). 45–47 LnnTβPnPr β (23) was then produced from Lc3 and galactose in an excellent (99%) yield using a OP4E galactosylation system 37, 39 containing Escherichia coli galactokinase (Ecgalk), 48 Bifidobacterium longum UDP-sugar pyrophosphorylase (Bulpus), 49 PpmPpa, 40 and Neisseria meningitidis β1-4-galactosyltransferase (Nmlgtb). 40, 47 Synthesis of Neu4,5Ac2α3LnnTβPnPr β (34) from LnnTβPnPr β (23) and Neu4,5Ac2 (1) was successfully achieved in a good 81% yield using NmCSS and PmST3 in one-pot (Scheme 3).

Although less efficient compared to the synthesis of their Neu4,5Ac2-containing matching pairs, Neu4Ac5Gc-containing α2,3-linked sialosides with a propyl azide glycolyne (35–41) were successfully obtained from Neu4Ac5Gc (2) and the corresponding disaccharides (17–22) or tetrasaccharide (23) in 51–71% yields using the OP2E sialylation reaction (Scheme 3). This indicated that CMP-Neu4,5Ac2 generated in situ is a better donor substance than in situ generated CMP-Neu4Ac5Gc for PmST3. Longer reaction time needed for the synthesis of Neu4Ac5Gc-containing compounds also led to de-O-acetylation which also contributed to lower sialylation yields.

All sialoside products synthesized in preparative scale were purified by gel filtration chromatography and semi-prep high-performance liquid chromatography (HPLC) using a reverse-phase C18 column. The structures were characterized by 1H and 13C nuclear magnetic resonance (NMR) spectroscopy as well as high resolution mass spectrometry (HRMS). Neu4,5Ac2α3Lac (24) synthesized in gram scale was purified (1.33 g) using a CombiFlash® RF 200i system with a ODS-SM column (51 g, 50 µM, 120 Å, Yamazen).

The synthesized pNP-tagged sialoside Neu4,5Ac2α3GalβPnP (27) was used in a 384-well plate-based high-throughput colorimetric assay 29 for substrate specificity studies of the α2,3-sialidase activity of Pasteurella multocida sialyltransferase 1 (PmST1), 29 four commercially available bacterial sialidases, and four human influenza A viruses. 51 While all bacterial sialidases tested including PmST1 as well as commercially available sialidases from Arthrobacter ureafaciens (Prozyme), Clostridium perfringens (Prozyme), Vibrio cholerae (Prozyme), and Streptococcus pneumoniae (Prozyme) did not show any sialidase activity towards Neu4,5Ac2α3GalβPnP (27) under the conditions used, human influenza A viruses (presumably the surface neuraminidases) could hydrolyze it (Fig. 2 black columns). As expected, all bacterial sialidases and viruses tested could cleave non-O-acetylated sialoside Neu5Acα3GalβPnP (50) which was used as a positive control (Fig. 2 white columns).
In order to test whether the cleavage of Neu4,5Ac₂ by human influenza A viruses was caused by de-O-acetylation or direct de-sialylation, Neu4,5Ac₂3Gal was incubated with purified human influenza A virus strain A/Puerto Rico/3/8/ H1N1 (A/PR8) followed by high-resolution mass spectrometry analysis. Major product peak for Neu4,5Ac₂ was seen and only a trace amount of Neu5Ac was observed, indicating a direct de-sialylation of Neu4,5Ac₂ mechanism was used by human influenza A viruses.

Conclusions

In conclusions, we report here a convenient and efficient chemoenzymatic sialylation approach for synthesizing α-2–3-linked sialosides containing a 4-O-acetylated sialic acid. PmST3, but not other bacterial sialyltransferases, was identified as a unique sialyltransferase that can catalyze the transfer of 4-O-acetylated sialic acid. Using chemically or chemoenzymatically synthesized Neu4,5Ac₂ (1) or Neu4Ac5Gc₂ (2), α-2–3-linked sialosides were readily obtained by a one-pot two-enzyme (OP2E) system containing NmCSS and PmST3. Using Neu4,5Ac₂3GalpNP as an effective probe, substrate specific studies showed that while Neu4,5Ac₂ was not hydrolyzed by bacterial sialidases tested, it was effectively removed by human influenza A virus neuraminidases.

Experimental Section

Materials and methods. Chemicals were purchased and used as received. NMR spectra were recorded in the NMR facility of University of California, Davis on a Varian Inova 400 (400 MHz for 1H, 100 MHz for 13C) and a Bruker Avance-800 NMR spectrometer (800 MHz for 1H, 200 MHz for 13C). Chemical shifts are reported in parts per million (ppm) on the δ scale. High resolution (HR) 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. Silica gel 60 Å (230–400 mesh, Sorbent Technologies) was used for flash column chromatography. Thin layer chromatography was performed on silica gel plates (Sorbent Technologies) using anisaldehyde sugar stain for detection. Gel filtration chromatography was performed with a column (100 cm × 2.5 cm) packed with Bio-Gel P-2 Fine resins (Bio-Rad). D-Galactose (Gal) and N-acetyl-D-glucosamine (GlcNAc) were from Fisher Scientific. GalβpNP was from Sigma. N-Acetyl-D-mannosamine (ManNAC) and N-acetyl-neuraminic acid (Neu5Ac) was from Inalco (Italy). Adenosine 5′-triphosphate (ATP), uridine 5′-triphosphate (UTP), and cytosine 5′-triphosphate (CTP) were purchased from Sangon Biotech Co. Ltd. Lacto-N-tetraose (LNT) was from Elicynt (Crolles, France). *Aspergillus oryzae* β-glucosidase was from Sigma (St. Louis, MO). Recombinant enzymes *Bifidobacterium longum* strain ATCC55113 N-acetyhexosamine-1-kinase (BLNahK or NahK_ATCC55113), *Pasteurella multocida* N-acetylglucosamine uridylyltransferase (PmGuml), *Pasteurella multocida* inorganic pyrophosphatase (PmPpAP), *Neisseria meningitidis* β-1-3-N-acetylgalactosaminyltransferase (Nmlgt), *Escherichia coli* galactokinase (EcGalK), *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP), *Neisseria meningitidis* β-1-4-galactotransferase (NmlgtB), *Pasteurella multocida* sialic acid aldolase (PmPnA), *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS), *Pasteurella multocida* CSS (PmCSS), *Haemophilus ducreyi* CSS (HdCSS), and two NmCSS mutants (NmCSS_S186R and NmCSS_Q163A), and *Pasteurella multocida* multifunctional α-2–3-sialyltransferase 3 (PmST3) were expressed and purified as described previously. Purified human influenza A viruses A viruses A/Puerto Rico/3/8/ H1N1 (A/PR8), A/Philippines/2/82/X-79 H3N2 (A/Phil), A/Memphis/71 H3N1; A/Udorn72, A/Udorn307/72 H3N2 were described previously. 

**Chemical synthesis of 5-N-acetyl-4-O-acetyleneuraminic acid (Neu4,5Ac₂, 1)***

**Benzy l 5-acetamido-3,5-dideoxy-D-glycerol-β-D-galacto-2-nonolyporanosonate (4).** To a stirring solution of Neu5Ac (3, 5 g, 16.2 mmol) in water (20 mL), 10% Cs₂CO₃ was added dropwise to adjust the pH value of solution to neutral. The reaction mixture was then evaporated and dried for overnight using a vacuum pump. The obtained solid was dissolved in N,N-dimethylformamide (20 mL) and benzyl bromide (3 mL) was added dropwise. The mixture was stirred under argon for overnight at room temperature and then filtered. The filtrate was evaporated to produce a syrup. Crystallization using 2-propanol produced 4 (5.8 g, 90%). 

**Benzy l 5-acetamido-3,5-dideoxy-8,9-O-isopropyldiene-D-glycerol-β-D-galacto-2-nonolyporanosonate (5).** Compound 4 (3.1 g, 7.76 mmol) was dissolved in 2,2-dimethoxypropane (50 mL) and camphorsulfonic acid (50 mg) was added. The mixture was stirred for overnight at room temperature. Then trimethylamine (100 μL) was added to the mixture until the pH reached 7. Then the mixture was evaporated to dryness and the residue was purified by silica gel chromatography.
was added and the mixture was stirred for 15 min before it was concentrated. The residue was purified by a silica gel column (EtOAc:MeOH = 15:1, by volume) to produce compound 5 (3.7 g, 92%). 1H NMR (800 MHz, CDCl3) δ 7.59–7.41 (m, 5H), 5.29 (d, J = 12.8 Hz, 1H), 5.22 (d, J = 12.0 Hz, 1H), 4.27–3.84 (m, 7H), 2.23 (dd, J = 12.8 and 4.8 Hz, 1H), 2.08 (s, 3H), 2.01 (t, J = 12.8 Hz, 1H), 1.44 (s, 3H), 1.38 (s, 3H); 13C NMR (200 MHz, CDCl3) δ 173.97, 169.61, 134.97, 128.48 (2C), 128.38, 128.00 (2C), 109.30, 95.43, 74.15, 71.19, 69.82, 69.72, 67.36, 67.05, 53.09, 39.06, 26.70, 25.07, 22.52.

**Benzyl 5-acetamido-4-O-acetyl-3,5-dideoxy-8,9-O-isopropylidene-D-glycero-β-D-galacto-2-nonulopyranosonate (1).** To a solution of compound 6 (2.0 g, 4.55 mmol) in pyridine (4 mL), acetic anhydride (2 mL) was added. The mixture was stirred for 1 h at room temperature and then ethanol (10 mL) was added. The solution was evaporated to dryness and the residue was purified by a silica gel column (EtOAc:Hexane = 2:1, by volume) to produce compound 6 (1.7 g, 81%). 1H NMR (400 MHz, CDCl3) δ 7.36–7.29 (m, 5H), 6.22 (d, J = 8.0 Hz, 1H, NH), 5.36 (td, J = 10.8 and 5.6 Hz, 1H, H-4), 5.26 (d, J = 12.4 Hz, 1H), 5.12 (d, J = 12.4 Hz, 1H), 4.54–3.82 (m, 7H), 2.23 (dd, J = 12.8 and 4.8 Hz, 1H), 2.04 (t, J = 12.8 Hz, 1H), 2.03 (s, 3H), 1.97 (s, 3H), 1.34 (s, 3H), 1.28 (s, 3H); 13C NMR (100 MHz, CDCl3) δ 172.78, 172.23, 168.76, 134.85, 128.68 (2C), 128.62, 128.16 (2C), 109.19, 94.92, 74.17, 71.80, 69.81, 68.93, 67.89, 67.44, 51.91, 51.35, 51.54, 25.22, 23.04, 21.09.

5-Acetamido-4-O-acetyl-3,5-dideoxy-D-glycero-β-D-galacto-2-nonulopyranosonate (1). A solution of compound 6 (1.1 g, 2.28 mmol) in 90% acetic acid was stirred for 2 h at 60 °C. The reaction mixture was evaporated to dryness to produce a syrup, which was dissolved in water (50 mL) and treated with HCl in the presence of 10% Pd/C for 2 h at room temperature. The solution was filtered and concentrated. The residue was purified by a silica gel column (EtOAc:MeOH:H2O = 5:2:1, by volume) to produce compound 1 (1.1 g, 85%). Compound 1 δ 7.36–7.29 (m, 5H), 6.22 (d, J = 11.2 and 4.8 Hz, 1H), 5.36 (td, J = 10.4 Hz, 1H, H-4), 4.15 (t, J = 10.4 Hz, 1H, H-5), 3.82 (dd, J = 12.0 and 2.4 Hz, 1H, H-6), 3.60 (dd, J = 12.0 and 6.4 Hz, 1H, H-9b), 3.56 (dd, J = 9.6 Hz, 1H, H-7), 2.32 (dd, J = 12.8 and 5.6 Hz, 1H, H-3eq), 2.04 (s, 3H), 1.99 (t, J = 11.2 Hz, 1H, H-3ax); 13C NMR (200 MHz, CDCl3) δ 174.39, 173.13, 173.29, 95.16, 70.00, 69.98, 69.91, 67.95, 65.02, 49.31, 35.95, 21.76, 20.22; HRMS (ESI) calculated for C31H32NO10 (M+H) 530.1973, found 530.1970.

**Synthesis of Neu4GcSG (2).** 1-(N-[2-Benzoyl oxyacetyl]-N-deamino acid (9). Solid K2CO3 (14.4 g, 104.3 mmol) was added to a stirred mixture of ManNH2·HCl (7.45 g, 20.8 mM) in dry MeOH (100 mL). The mixture was stirred for 30 min and filtered. The solution was concentrated and purified by flash chromatography (DCM:MeOH = 10:1, by volume) to produce ManNGc analog 8 (5.8 g, 85%). Compound 8 (3.5 g, 10.7 mM) and pyruvate (4.11 g, 37.4 mM) were dissolved in water (35 mL) in a 50 mL centrifuge tube. After the addition of an appropriate amount of PmNanA (52 mg), the reaction mixture was incubated in an isothermic incubator for 48 h at 37 °C with gentle shaking. The reaction progress was monitored by TLC (nPrOH:H2O:HAc = 5:2:1, by volume) and mass spectrometer (MS). When the reaction was completed, ice-cold ethanol (35 mL) was added and the reaction mixture was incubated at 4 °C for 30 min. The mixture was centrifuged and the supernatant was concentrated. The residue was purified by silica gel column flash chromatography (EtOAc:MeOH:H2O = 6:2:1 then 4:2:1, by volume) to produce the desired Neu5GcObn 9 (4.4 g, quant.) as a white solid. 1H NMR (800 MHz, D2O) δ 7.45–7.41 (m, 5H), 4.64 (s, 2H), 4.12 (s, 2H), 4.09 (m, 1H), 4.05 (d, J = 10.4 Hz, 1H), 3.96 (t, J = 10.4 Hz, 1H), 3.81 (dd, J = 12.0 and 3.2 Hz, 1H), 3.75 (m, 1H), 3.58 (dd, J = 12.0 and 6.4 Hz, 1H), 3.50 (t, J = 9.6 Hz, 1H), 2.21 (dd, J = 12.8 and 4.8 Hz, 1H), 1.82 (t, J = 12.8 Hz, 1H); 13C NMR (200 MHz, D2O) δ 176.65, 173.01, 136.45, 128.72(2C), 128.56(2C), 128.48, 96.33, 73.70, 70.39, 69.88, 68.38, 68.33, 66.86, 63.10, 51.88, 39.37. HRMS (ESI) m/z 1238 (C64H41NO16) calculated for C64H41NO16: 1238.0083, found 1238.0080.

**Benzyl 5-glycolylamido-3,5-dideoxy-D-glycero-β-D-galacto-2-nonulopyranosonate (15).** To a stirred solution of Neu5GcObn 9 (3.0 g, 7.25 mmol) in water (25 mL), 10% CeO2 was added dropwise to adjust pH to neutral. The solution was then evaporated and the residue was dried for overnight by a vacuum pump to produce the cesium salt of Neu5GcObn. The salt was dissolved in N,N-dimethylformamide (40 mL), benzyl bromide (1.5 mL) was then added to the solution. The mixture was stirred under argon for 2 h at room temperature. After evaporation, the residue was purified by silica gel column chromatography (EtOAc:MeOH:H2O = 6:2:1, by volume) to produce compound 10. 1H NMR (400 MHz, CD3OD) δ 7.35–7.24 (m, 10H), 5.26 (m, 2H, PhCH2), 4.65 (s, 2H, PhCH2), 3.43–3.53 (m, 9H), 2.36 (dd, J = 12.8 and 4.8 Hz, 1H), 2.17 (t, J = 12.8 Hz, 1H); 13C NMR (100 MHz, CD3OD) δ 172.27, 170.95, 132.14, 131.50,
**Benzyl 5-glycolylamido-3,5-dideoxy-8,9-O-isopropylidene-D-glycero-D-galacto-2-nonulopyranosyl (11).** To a solution of compound 10 (1.5 g, 2.97 mmol) in acetone (40 mL), 2,2-dimethoxypropane (4 mL) and camphorsulfonic acid (15 mg) were added. The mixture was stirred for 3 h at room temperature. Then trimethylene (200 μL) was added and the solution was stirred for 15 min. The reaction mixture was concentrated and the residue was purified by a silica gel column (EtOAc:Hexane = 5:1, by volume) to produce compound 11 (0.31 g, 78%).

**Temperature.** The product formation was monitored by TLC (EtOAc:O:HOAc = 4:2:1:0.2 by volume) at 37 °C for 30 min. After centrifugation, the supernatant was concentrated and passed through a Bio-Gel P-2 gel filtration column (water was used as an eluent). The fractions containing the product were collected, concentrated, further purified by silica gel column (EtOAc:MeOH:H₂O = 5:2:1, by volume) and finally by a Bio-Gel P-2 column (eluted with H₂O) to produce Lc trisaccharide GlcNAc[β3Gal][β4Glc][βProN]₄⁻(23, 0.613 g, 99%).

**One-pot four-enzyme preparative-scale synthesis of Gal[β4GlcNAc[β3Gal][β4Glc][βProN]₄[βN][βProN] (LmN)[βProN] (23).** The reaction mixture in a total volume of 30 mL containing Tris-HCl buffer (100 mM, pH 8.0), Lc trisaccharide (0.55 g, 0.875 mmol), galactose (0.204 g, 1.14 mmol), ATP (0.627 g, 1.14 mmol), UTP (0.626 g, 1.14 mmol), MgCl₂ (20 mM), ECGalK (120 mg), BUsp (10 mg), NmLgtB (70 mg), and PmpP (8 mg) was incubated in a shaker with agitation (100 rpm) at 37 °C for 5 hrs. The product formation was monitored by TLC (n-ProOH:H₂O:NH₄OH = 5:2:1 and detected by p-anisaldehyde sugar stain) and mass spectrometry. When an optimal yield was achieved, ethanol (30 mL) was added and the mixture was incubated at 4 °C for 30 min. The precipitates were removed by centrifugation and the supernatant was concentrated and purified by a Bio-Gel P-2 gel column (water was used as an eluate). Further purification was achieved using silica gel chromatography (EtOAc:MeOH:H₂O = 5:3:1.5) and finally Bio-Gel P-2 column (eluted with H₂O) to produce Gal[β4GlcNAc[β3Gal][β4Glc][βProN]₄ (23, 0.191 g, 99%).

**One-pot two-enzyme OPZE preparative-scale synthesis of α2-3-linked disialoses containing a 4-O-acetyl sialic acid.** An acceptor (10 mM, 1.0 eq, chosen from 13–23), 4-O-acetylsialic acid (1.2 eq, 1 or 2), and CTP (2.0 eq), MgCl₂ (20 mM) were dissolved in Tris-HCl buffer (100 mM, pH 7.1), containing NmCSS (3 mg/mL), and PmST3 (5 mg/mL). The reaction mixture was incubated at 37 °C in an incubator with agitation (100 rpm) for 14 h. The product formation was monitored by MS. The reaction was terminated by adding the same volume (10 mL) of ice-cold EtOH followed by incubation at 4 °C for 30 min. The mixture was centrifuged to remove precipitates. The supernatant was concentrated and passed through a Bio-Gel P-2 gel filtration column with water as the eluate to obtain the desired product. The compound was further purified by a reverse-phase C18 column (Phenomenex, 10 μm, 21.2 × 250 mm) with a flow rate of 10 mL/min using a gradient elution of 0–100% acetonitrile in water containing 0.05% formic acid over 20 minutes. Mobile phase A: 0.05% formic acid in water (v/v); Mobile phase B: acetonitrile (v/v); Gradient: 0% B for 3 minutes, 0% to 100% B over 12 minutes, 100% B for 2 minutes, then 100% to 0% B over 3 minutes. The fractions were collected, concentrated, and purified by silica gel chromatography (EtOAc:MeOH:H₂O = 5:2:1, by volume) and finally by a Bio-Gel P-2 column (eluted with H₂O) to produce 4GlcNAc[β3Gal][β4Glc][βProN]₄⁻(13, 0.625 g, 94%).
containing the pure product were collected and concentrated to provide the final purified product.

**Neu4,5Ac2α2Galβ3GlcNAcβ3Galβ4Glc (24).** 56 mg, yield 82%; white solid. H NMR (800 MHz, D2O) δ 5.20 (d, J = 4.0 Hz, 0.4H, α-isomer), 4.94 (m, 1H), 4.64 (d, J = 8.0 Hz, 0.6H, β-isomer), 4.51 (d, J = 8.0 Hz, 1H), 4.12–3.25 (m, 18H), 2.75 (dd, J = 12.0 and 4.8 Hz, 1H), 2.05 (s, 3H), 1.94 (s, 3H), 1.91 (t, J = 12.0 Hz, 1H); 13C NMR (200 MHz, D2O) δ 174.43, 173.42, 173.09, 102.49, 99.62, 95.65, 78.03, 75.31, 75.02, 74.02, 74.20, 73.67, 72.74, 71.63, 71.04, 69.23, 67.78, 67.35, 67.24, 62.41, 60.90, 59.92, 49.16, 47.74, 36.54, 28.11, 21.76, 20.21; HRMS (ESI) calculated for C35H50N3O20 (M+H) 757.2627, found 757.2633.

**Neu4,5Ac2α2Galβ3GlcNAcβ3Galβ4GlcNAcβ4ProN (29).** 32 mg, yield 85%; white solid. H NMR (800 MHz, D2O) δ 4.97 (m, 1H), 4.53 (d, J = 8.0 Hz, 1H), 4.501 (d, J = 8.8 Hz, 1H), 4.12 (dd, J = 10.4 and 3.2 Hz, 1H), 4.08 (t, J = 10.4 Hz, 1H), 3.99–3.54 (m, 18H), 3.36 (m, 2H), 2.75 (dd, J = 12.8 and 4.8 Hz, 1H), 2.05 (s, 3H), 2.02 (s, 3H), 1.95 (s, 3H), 1.94 (t, J = 12.0 Hz, 1H), 1.82 (m, 2H); 13C NMR (200 MHz, D2O) δ 174.44, 174.38, 173.09, 172.89, 102.42, 101.02, 99.29, 78.17, 75.39, 74.97, 74.61, 72.36, 72.22, 71.36, 70.79, 69.24, 67.74, 67.39, 67.00, 62.52, 60.85, 59.90, 54.95, 49.10, 47.63, 36.34, 27.97, 22.02, 21.75, 20.19; HRMS (ESI) calculated for C35H52N3O30 (M+H) 798.2893, found 798.2893.

**Neu4,5Ac2α2Galβ3GlcNAcβ3Galβ4GlcNAcβ4ProN (30).** 24 mg, yield 86%; white solid.

| Neu4,5Ac2α2Galβ3GlcNAcβ3Galβ4GlcNAcβ4ProN (31). | 21 mg, yield 80%; white solid. H NMR (800 MHz, D2O) δ 4.93 (m, 1H), 4.84 (d, J = 4.0 Hz, 1H), 4.50 (d, J = 8.0 Hz, 1H), 4.10–4.05 (m, 3H), 3.93–3.44 (m, 19H), 2.76 (dd, J = 10.8 and 4.8 Hz, 1H), 2.05 (s, 3H), 2.01 (s, 3H), 1.95 (s, 3H), 1.89 (t, J = 12.0 Hz, 1H); 13C NMR (200 MHz, D2O) δ 174.81, 174.41, 174.41, 173.49, 103.25, 102.78, 102.40, 99.45, 95.61, 81.99, 81.80, 78.16, 75.24, 75.08, 74.95, 74.78, 74.68, 74.23, 73.65, 72.19, 71.73, 71.03, 69.89, 68.95, 68.33, 67.74, 67.13, 62.30, 60.91, 60.85, 60.38, 59.53, 54.47, 49.14, 36.70, 22.17, 21.76, 20.21; HRMS (ESI) calculated for C39H56N4O26 (M+H) 1039.3487.

**Neu4,5Ac2α2Galβ3GlcNAcβ3Galβ4GlcNAcβ4ProN (32).** 22 mg, yield 81%; white solid. H NMR (800 MHz, D2O) δ 4.95 (m, 1H), 4.49 (d, J = 8.0 Hz, 1H), 4.48 (d, J = 8.0 Hz, 1H), 4.16 (dd, J = 12.0 and 4.8 Hz, 1H), 2.75 (dd, J = 12.0 and 4.8 Hz, 1H), 2.05 (s, 3H), 2.01 (s, 3H), 1.94 (s, 3H), 1.92 (t, J = 12.0 Hz, 1H); 13C NMR (200 MHz, D2O) δ 174.62, 174.43, 173.10, 172.92, 104.46, 101.28, 99.22, 79.95, 75.49, 74.62, 74.58, 72.26, 71.49, 70.85, 68.86, 67.76, 67.75, 67.30, 66.88, 62.43, 60.82 (2C), 51.03, 49.10, 47.67, 36.46, 27.99, 22.16, 21.76, 20.19; HRMS (ESI) calculated for C39H58N4O28 (M+H) 798.2893, found 798.2893.

**Neu4,5Ac2α2Galβ3GlcNAcβ3Galβ4GlcNAcβ4ProN (33).** 18 mg, yield 74%; white solid. H NMR (800 MHz, D2O) δ 4.94 (m, 1H), 4.89 (d, J = 4.0 Hz, 1H), 4.53 (d, J = 8.0 Hz, 1H), 4.32–3.43 (m, 22H), 2.75 (dd, J = 10.8 and 4.8 Hz, 1H), 2.06 (s, 3H), 2.02 (s, 3H), 1.95 (s, 3H), 1.91–1.88 (m, 3H); 13C NMR (200 MHz, D2O) δ 174.77, 174.43, 173.45, 173.10, 104.35, 99.55, 97.08, 77.27, 75.60, 74.65, 72.20, 71.73, 71.06, 70.52, 68.95, 68.47, 67.75, 67.29, 64.80, 62.35, 61.11, 60.86, 60.15, 48.57, 48.06, 36.65, 27.85, 21.93, 21.76, 20.21; HRMS (ESI) calculated for C39H58N4O28 (M+H) 798.2893, found 798.2902.
**Neu4,5Ac2Galβ3GalNAcβ3Galβ4GlcβProN (34).** 20 mg, yield 81%; white solid. ¹H NMR (800 MHz, D₂O) δ 4.93 (m, 1H), 4.67 (d, J = 8.0 Hz, 1H), 4.54 (d, J = 8.0 Hz, 1H), 4.47 (d, J = 8.0 Hz, 1H), 4.41 (d, J = 8.0 Hz, 1H), 4.14 (d, J = 3.2 Hz, 1H), 4.11 (dd, J = 9.6 and 3.2 Hz, 1H), 4.07 (t, J = 10.4 Hz, 1H), 3.98–3.54 (m, 28H), 3.44 (t, J = 6.4 Hz, 2H), 3.29 (t, J = 8.0 Hz, 1H), 2.75 (dd, J = 12.0 and 4.8 Hz, 1H), 2.05 (s, 3H), 2.01 (s, 3H), 1.91 (t, J = 12.0 Hz, 1H), 1.89 (m, 2H); ¹³C NMR (200 MHz, D₂O) δ 177.48, 174.44, 174.30, 170.08, 102.82, 102.69, 102.41, 101.98, 99.64, 81.93, 78.21, 77.85, 75.41, 75.02, 74.77, 74.65, 74.42, 72.75, 72.65, 72.27, 72.02, 71.62, 71.30, 69.83, 69.25, 68.70, 67.76, 67.36, 67.24, 62.41, 60.91, 60.84, 59.91, 59.70, 55.05, 49.15, 47.73, 36.53, 28.10, 22.04, 21.75, 20.20; HRMS (ESI) calculated for C₁₉⁴H₁₈₁N₄O₇ (M-H) 814.2842, found 814.2874.

**Neu4Ac5Gcα2Galβ3GalNAcβ3Galβ4GlcβProN (40).** 11 mg, yield 52%; white solid. ¹H NMR (800 MHz, D₂O) δ 5.02 (dt, J = 10.4 and 4.8 Hz, 1H, H-4'), 4.89 (d, J = 4.0 Hz, 1H), 4.53 (d, J = 8.0 Hz, 1H), 4.30 (dd, J = 11.2 and 4.0 Hz, 1H), 4.23 (d, J = 3.2 Hz, 1H), 4.15 (t, J = 10.4 Hz, 1H), 4.10–3.41 (m, 21H), 2.78 (dd, J = 12.8 and 4.8 Hz, 1H), 2.04 (s, 3H), 2.01 (s, 3H), 1.90 (t, J = 12.8 Hz, 1H), 1.88 (m, 2H); ¹³C NMR (200 MHz, D₂O) δ 175.35, 174.45, 174.44, 173.05, 104.34, 99.51, 97.07, 77.24, 75.58, 74.64, 71.97, 71.75, 70.77, 70.51, 68.95, 68.47, 67.69, 67.27, 64.79, 63.21, 61.10, 60.85, 60.70, 48.85, 48.56, 48.05, 36.74, 27.84, 21.93, 20.22; HRMS (ESI) calculated for C₂₉₀H₁₇₁N₄O₁₂ (M-H) 1138.3989, found 1138.3989.

**Gram-scale OP2E synthesis of monotrope milk trisaccharide Neu4,5Ac β3Gal.** Lactose (1.09 g, 3.05 mmol), Neu4,5Ac (1.07 g, 3.05 mmol), and CTP (1.93 g, 3.66 mmol) were dissolved in water (30 ml) and the pH of the solution was adjusted to 7.1 using NaOH solution (4 M). MgCl₂ (20 mM), Tris-HCl buffer (100 mM, pH 7.1), NmCSS (15 mg), and PmST3 (20 mg) were then added and the total volume of the reaction mixture was brought up to 100 ml by adding H₂O. The reaction mixture was incubated in an incubator shaker with shaking (100 rpm) at 30 °C for overnight. The reaction was monitored by mass spectrometry (MS). Additional CTP (0.96 g) was added to the reaction mixture and the pH was carefully adjusted to pH 7.1 using NaOH solution (4 M). The reaction was allowed to continue for another 20 h at room temperature until an optimal yield was achieved. After adding ethanol (100 ml), the reaction mixture was incubated at 4 °C for 1 h. The precipitates were removed by centrifuge and the supernatant was concentrated. The residue was purified using a QES-SE chromatography (20 cm, 50 μM, 120 Å, Yamazan) on a Combiflash® RF 200i system. Mobile phase A: water; Mobile phase B: acetonitrile; Gradient: 10% B for 6 minutes, 10% to 100% B over 12 minutes, 100% B for 6 minutes, then 100% to 80% B over 1 minute. The fractions containing the pure product were collected and concentrated to produce the final purified product Neu4,5Ac β3Gal (133 g, 71%) as a white powder. The identity and purity of the product were confirmed by NMR and HRMS.

**Sialidase assays.** Sialidase assays were carried out in duplicate at 37 °C for 40 min (for bacterial sialidases) or 30 min (for human influenza A viruses) in a 384-well plate (Fisher Scientific, Chicago, IL) in a final volume of 20 μl containing Neu4,5Ac β3Gal pNP (0.3 mM)
and *Aspergillus oryzae* β-galactosidase (12 μg, 126 mU). The amount of the β-galactosidase required to completely hydrolyze Ga1pβPNP (0.3 mM) within the time frame of the assay was predetermined and confirmed by control assays with Ga1pβPNP (0.3 mM). The reactions were stopped by adding 40 μL of N-cyclohexyl-3-aminopropionic sulfonic acid (CAPS) buffer (0.5 M, pH 11.5). The amount of para-nitrophenolate formed was determined by measuring the A_{405 nm} of the reaction mixtures using a microtiter plate reader. Neu5Acα3GalβPNP\textsuperscript{50} was used as a control.

The assay conditions for bacterial sialidases were: *A. ureafaciens* sialidase (1.5 μU) in sodium acetate buffer (100 mM, pH 6.0); *C. perfringens* sialidase (3 μU) in MES buffer (100 mM, pH 5.0); *V. cholerae* sialidase (1.5 μU) in sodium acetate buffer (100 mM, pH 5.5) containing NaCl (150 mM) and CaCl\textsubscript{2} (10 mM); *S. pneumoniae* sialidase (1.5 μU) in sodium acetate buffer (100 mM, pH 5.0); PmST1 (10 μg) in sodium acetate buffer (100 mM, pH 5.5). Viral sialidase assays were carried out in a MES buffer (100 mM, pH 5.0) containing a purified human influenza A virus strain A/Puerto Rico/34/8 H1N1 (A/PR8), A/Philippines/2/82/X-79 H3N2 (A/Philips), A/Memphis/71 H3N1 (A/Mem71), and A/Udorn/307/72 H3N2 (A/Udorn72).\textsuperscript{51}

To test whether the cleavage of Neu4,5Acβ by human influenza A virus was caused by de-O-acetylation or direct de-sialylation, Neu4,5Acβα3Lac (1 mM) was incubated with purified human influenza A virus strain A/Puerto Rico/34/8 H1N1 (A/PR8), A/Philippines/2/82/X-79 H3N2 (A/Philips), A/Memphis/71 H3N1 (A/Mem71), and A/Udorn/307/72 H3N2 (A/Udorn72).\textsuperscript{51}

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### Notes and references
