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

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Abstract: A facile one-pot two-enzyme chemoenzymatic approach has been established for gram (Neu4,5Ac₂α3Lac) and preparative-scale (Neu4,5Ac₂α3LNnT) synthesis of monotreme milk oligosaccharides. Other *O*-acetyl-5-*N*-acetylneuraminic acid (Neu4,5Ac₂-) or 4-*O*-acetyl-5-*N*-glycolylneuraminic acid (Neu4Ac5Gc)-containing α2–3-sialosides have also been synthesized in preparative scale. Used as an effective probe, Neu4,5Ac₂α3GalβpNP 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*-acetylneuraminic acid (Neu5Ac), the non-human sialic acid *N*-glycolylneuraminic 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*-acetylneuraminic acid (Neu4,5Ac₂, **1**) and 4-*O*-acetyl-*N*-glycolylneuraminic acid (Neu4Ac5Gc, **2**) are less common.⁵ Neu4,5Ac₂ (**1**) has been found in horse,⁶ donkey,⁷ Australian monotreme Echidna,^{5, 8}

Japanese dace,⁹ South American pit viper,¹⁰ rabbit,¹¹ guinea-pig.⁶ Neu4,5Ac₂-containing oligosaccharides have been found to be the dominant components of the acidic milk oligosaccharides (MOSs) of monotremes echidnas and platypus.^{12–14} Both Neu4,5Ac₂ and Neu4Ac5Gc have been found in horse glycoproteins.⁵ 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

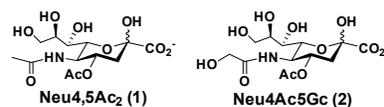


Fig. 1 Structures of naturally occurring 4-*O*-acetyl-*N*-acetylneuraminic acid (Neu4,5Ac₂, **1**) and 4-*O*-acetyl-*N*-glycolylneuraminic acid (Neu4Ac5Gc, **2**).

4-*O*-Acetylation of sialic acids provides resistance or decreased sensitivity of sialosides to various sialidases.^{6, 18, 19} This property is believed to contribute to the inhibitory activity of Neu4,5Ac₂-rich horse and guinea pig α₂-macroglobulins against hemagglutination and infectivity of several human influenza viruses.^{6, 18, 19} On the other hand, Neu4,5Ac₂ is selectively recognized by the hemagglutinin-esterase (HE)^{20, 21} of mouse hepatitis virus strain S (MHV-S), 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

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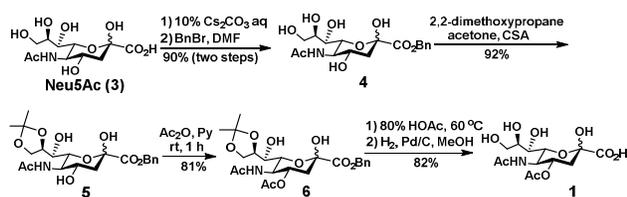
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synthesis of monotreme milk oligosaccharides including Neu4,5Ac₂-containing α 2–3-linked sialyl lactose (Neu4,5Ac₂ α 3Lac) and sialyl lacto-*N*-neotetraose (Neu4,5Ac₂ α 3LNnT). Other Neu4,5Ac₂- or Neu4Ac5Gc-containing α 2–3-linked sialosides representing common terminal sialosides in mammals are also synthesized.

Results and discussion

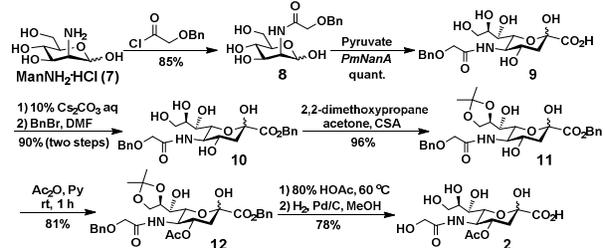
We envisioned that the desired 4-*O*-acetyl-sialic acid-containing sialosides could be chemoenzymatically synthesized using an effective one-pot two-enzyme sialylation system containing a cytidine 5'-monophosphate-sialic acid (CMP-Sia) synthetase (CSS) and a sialyltransferase (ST).²³ To test the feasibility, 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 catalytical amount of *p*-toluenesulfonic acid produced partially protected **5** in an excellent 92% yield. Regioselective acetylation of **5** at *O*-4 using acetic anhydride in pyridine at room temperature provided the key intermediated **6** in a 81% 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**).

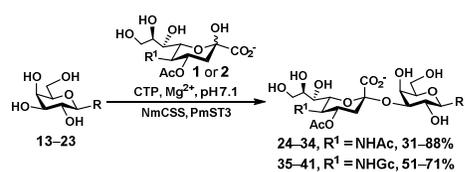
Neu4Ac5Gc (**2**) was chemoenzymatically synthesized from D-mannosamine hydrochloride (ManNH₂·HCl, **7**). As shown in Scheme 2, *N*-(2-Benzyloxyacetyl)-D-mannosamine **8** was obtained by treating ManNH₂·HCl (**7**) with 2-benzyloxyacetyl chloride in the presence of NaHCO₃ in CH₃CN and water. *P. multocida* 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 8,9-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 debenylation 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),²⁶ *Pasteurella multocida* (PmCSS), *Haemophilus ducreyi* (HdCSS), as well as two NmCSS mutants (NmCSS_S81R and NmCSS_Q163A).²⁷ 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, †ESI). 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.²⁸ NmCSS was chosen for further studies and for preparative-scale and gram-scale syntheses.



R = μ NP, Glc, or Glc/GlcNAc-containing glycans, see Table 1 for details.

Scheme 3 One-pot two-enzyme (OP2E) synthesis of sialosides containing Neu4,5Ac₂ (**1**) or Neu4Ac5Gc (**2**). Enzymes: NmCSS, *Neisseria meningitidis* CMP-sialic acid synthetase; PmST3, *Pasteurella multocida* α 2–3-sialyltransferase 3. The pH of the reaction was controlled at 7.1 to minimize de-*O*-acetylation during the enzymatic reaction. Yields were calculated for purified products based on the amounts of acceptors (limiting reagents) used.

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 α 2–3-sialyltransferases from *Pasteurella multocida* (PmST1,²⁹ PmST2,³⁰ PmST3³¹) and PmST1_M144D mutant,³² α 2–6-sialyltransferases Pd26ST³³ and Psp26ST,³⁴ as well as α 2–3/8-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 α 2–3-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.^{31, 37} PmST3 could use oligosaccharides containing a terminal β 1–4- or β 1–3-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^x, 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 α 2–3-linked 4-*O*-acetylated sialosides. Yields were calculated for purified products based on the amounts of acceptors (limiting reagents) used.

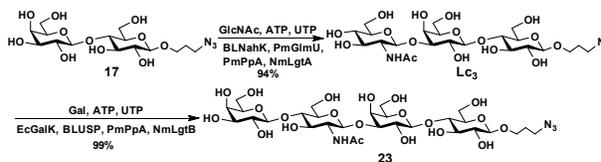
Entry	Acceptor	Donor	Sialosides (Neu4,5Ac ₂ /Neu4Ac5Gc α 3OR)	yield
a	Galβ4Glc or Lac (13)	Neu4,5Ac ₂ (1)	24	82%
b	Galβ4GlcNAcβ3Galβ4Glc or LNT (14)	1	25	78%
c	Galβ3GlcNAcβ3Galβ4Glc or LNT (15)	1	26	65%
d	GalβpNP (16)	1	27	31%
e	Galβ4GlcβProN ₃ or LacβProN ₃ (17)	1	28	88%
f	Galβ4GlcNAcβProN ₃ or LacNAcβProN ₃ (18)	1	29	85%
g	Galβ3GlcNAcβProN ₃ (19)	1	30	86%
h	Galβ3GlcNAc α ProN ₃ (20)	1	31	80%
i	Galβ3GalNAcβProN ₃ (21)	1	32	81%
j	Galβ3GalNAc α ProN ₃ (22)	1	33	74%
k	Galβ4GlcNAcβ3Galβ4GlcβProN ₃ or LNTβProN ₃ (23)	1	34	81%
l	17	Neu4Ac ₅ Gc (2)	35	67%
m	18	2	36	71%
n	19	2	37	65%
o	20	2	38	56%
p	21	2	39	51%
q	22	2	40	52%
r	23	2	41	62%

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NmCSS and PmST3 were used in an efficient one-pot two-enzyme (OP2E) sialylation system^{23, 38} (Scheme 3) for preparative-scale synthesis of target sialosides. To minimize loss of the 4-*O*-acetyl group, enzymatic reactions were carried out at 37 °C in a Tris-HCl buffer (100 mM, pH 7.1) containing NmCSS, PmST3, Mg²⁺, cytidine 5'-triphosphate (CTP), Neu4,5Ac₂ or Neu4Ac5Gc, and a galactoside acceptor. Monotreme milk oligosaccharides Neu4,5Ac₂α3Lac (**24**) and Neu4,5Ac₂α3LNnT (**25**) were readily produced from lactose (Galβ4Glc or Lac, **13**) and previously synthesized lacto-*N*-neotetraose (Galβ4GlcNAcβ3Galβ4Glc or LNnT, **14**)³⁹ in 82% and 78% yields, respectively (Table 1). Neu4,5Ac₂α3LNT (**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 aglycone (**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, monotreme 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 Galβ4GlcNAcβ3Galβ4GlcβProN₃ or LNnTβProN₃ (**23**) was synthesized from LacβProN₃ (**17**) using a sequential two-step one-pot multienzyme (OPME)³⁸ process (Scheme 4) similar to that reported previously for LNnT.³⁹ Briefly, Lc₃ trisaccharide GlcNAcβ3Galβ4GlcβProN₃⁴² was synthesized from LacβProN₃ (**17**) and GlcNAc in a 94% yield using a one-pot four-enzyme (OP4E) GlcNAc activation and transfer system containing *Bifidobacterium longum* *N*-acetylhexosamine-1-kinase (BLNahK, NahK_ATCC55813),⁴³ *Pasteurella multocida* *N*-acetylglucosamine uridylyltransferase (PmGlmU),⁴⁴ *Pasteurella multocida* inorganic pyrophosphatase (PmPpA),⁴⁰ and *Neisseria meningitidis* β1–3-*N*-acetylglucosaminyltransferase (NmLgtA).^{45–47} LNnTβProN₃ (**23**)⁴² 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 (BLUSP),⁴⁹ PmPpA,⁴⁰ and *Neisseria meningitidis* β1–4-galactosyltransferase (NmLgtB).^{40, 47} Synthesis of Neu4,5Ac₂α3LNnTβProN₃ (**34**) from LNnTβProN₃ (**23**) and Neu4,5Ac₂ (**1**) was successfully achieved in a good 81% yield using NmCSS and PmST3 in one-pot (Scheme 3).



Scheme 4 Sequential one-pot multienzyme (OPME) synthesis of LNnTβProN₃ (**23**). Enzymes: BLNahK or NahK_ATCC55813, *Bifidobacterium longum* *N*-acetylhexosamine-1-kinase; PmGlmU, *Pasteurella multocida* *N*-acetylglucosamine uridylyltransferase; PmPpA, *Pasteurella multocida* inorganic pyrophosphatase; NmLgtA, *Neisseria meningitidis* β1–3-*N*-acetylglucosaminyltransferase; EcGalK, *Escherichia coli* galactokinase; BLUSP, *Bifidobacterium longum* UDP-sugar pyrophosphorylase; NmLgtB, *Neisseria meningitidis* β1–4-galactosyltransferase.

Although less efficient compared to the synthesis of their Neu4,5Ac₂-containing matching pairs, Neu4Ac5Gc-containing α2–3-linked sialosides with a propyl azide aglycone (**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 substrate 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 assay⁵⁰ 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 Neu5Acα3GalβpNP⁵⁰ which was used as a positive control (Fig. 2 white columns).

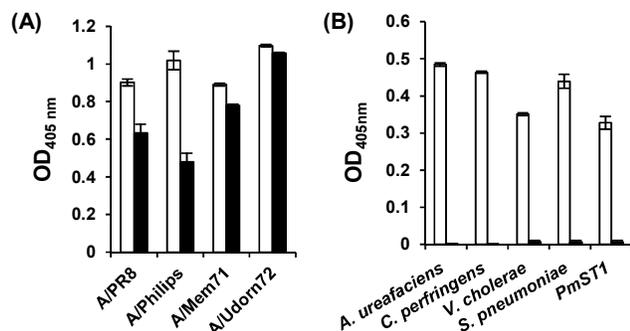


Fig. 2 Human influenza A virus (A) and bacterial sialidase (B) substrate specificity studies using Neu4,5Ac α 3Gal β pNP (27) as the substrate (black columns). Non-*O*-acetylated sialoside Neu5Ac α 3Gal β pNP⁵⁰ (white columns) was used as a positive control. Human influenza A viruses (purified) used: A/PR8, A/Puerto Rico/34/8 H1N1; A/Philips, A/Philippines/2/82/X-79 H3N2; A/Mem71, A/Memphis/71 H3N1; A/Udorn72, A/Udorn/307/72 H3N2.

In order to test whether the cleavage of Neu4,5Ac α 2 by human influenza A viruses was caused by de-*O*-acetylation or direct de-sialylation, Neu4,5Ac α 2 α 3Lac was incubated with purified human influenza A virus strain A/Puerto Rico/34/8 H1N1 (A/PR8) followed by high-resolution mass spectrometry analysis. Major product peak for Neu4,5Ac α 2 was seen and only a trace amount of Neu5Ac was observed, indicating a direct de-sialylation of Neu4,5Ac α 2 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 Neu4,5Ac α 2 (1) or Neu4Ac5Gc (2), α 2–3-linked sialosides were readily obtained by a one-pot two-enzyme (OP2E) system containing NmCSS and PmST3. Using Neu4,5Ac α 2 α 3Gal β pNP as an effective probe, substrate specific studies showed that while Neu4,5Ac α 2 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 ^{13}C) and a Bruker Avance-800 NMR spectrometer (800 MHz for ^1H , 200 MHz for ^{13}C). 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 \times 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. Gal β pNP was from Sigma. *N*-Acetyl-D-mannosamine (ManNAc) and *N*-acetylneuraminic acid (Neu5Ac) was from Inalco (Italy). Adenosine 5'-triphosphate (ATP), uridine 5'-triphosphate (UTP), and cytosine 5'-triphosphate (CTP) were purchased from Hangzhou Meiya Pharmaceutical Co. Ltd. Lacto-*N*-tetraose (LNT) was from Elicityl (Crolles, France). *Aspergillus oryzae* β -galactosidase was from Sigma (St. Louis, MO). Recombinant enzymes *Bifidobacterium longum* strain ATCC55813 *N*-acetylhexosamine-1-kinase (BLNahK or NahK_ATCC55813),⁴³ *Pasteurella multocida* *N*-acetylglucosamine uridylyltransferase (PmGlmU),⁴⁴ *Pasteurella multocida* inorganic pyrophosphatase (PmPpA),⁴⁰ *Neisseria meningitidis* β 1–3-*N*-acetylglucosaminyltransferase (NmLgtA),^{45, 46} *Escherichia coli* galactokinase (EcGalK),⁴⁸ *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP),⁴⁹ *Neisseria meningitidis* β 1–4-galactosyltransferase (NmLgtB),⁴⁰ *Pasteurella multocida* sialic acid aldolase (PmNanA),²⁵ *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS),²⁶ *Pasteurella multocida* CSS (PmCSS), *Haemophilus ducreyi* CSS (HdCSS), and two NmCSS mutants (NmCSS_S81R and NmCSS_Q163A),²⁷ and *Pasteurella multocida* multifunctional α 2–3-sialyltransferase 3 (PmST3)³¹ were expressed and purified as described previously. Purified human influenza A viruses 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) were described previously.⁵¹

Lac β ProN $_3$ (17), LacNAc β ProN $_3$ (18), disaccharides 19–22, tetrasaccharide LNnT (14) were prepared as described previously.^{29, 40, 41} Tetrasaccharide LNnT β ProN $_3$ (23) was chemoenzymatically synthesized via sequential one-pot multienzyme (OPME) glycosylation approach from Lac β ProN $_3$ (17) as described below:

Chemical synthesis of 5-*N*-acetyl-4-*O*-acetylneuraminic acid (Neu4,5Ac α , 1)

Benzyl 5-acetamido-3,5-dideoxy-D-glycero- β -D-galacto-2-nonulopyranosonate (4). To a stirring solution of Neu5Ac (3, 5 g, 16.2 mmol) in water (20 mL), 10% Cs $_2$ CO $_3$ was added dropwisely 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 dropwisely. 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%). ^1H NMR (800 MHz, D $_2$ O) δ 7.59–7.41 (m, 5H), 5.27 (dd, J = 27.2 and 10.8 Hz, 2H), 4.06–4.03 (m, 2H), 3.89 (t, J = 10.4 Hz, 1H), 3.80 (dd, J = 12.8 and 3.2 Hz, 1H), 3.67 (m, 1H), 3.58 (dd, J = 12.0 and 6.4 Hz, 1H), 3.53 (d, J = 8.8 Hz, 1H), 2.29 (dd, J = 12.8 and 5.6 Hz, 1H), 2.03 (s, 3H), 1.88 (t, J = 12.8 Hz, 1H); ^{13}C NMR (200 MHz, D $_2$ O) δ 174.71, 170.47, 134.80, 132.95, 130.65, 129.03, 128.77, 128.21, 95.20, 70.30, 69.98, 68.19, 68.02, 66.53, 63.00, 51.92, 39.90, 21.94.

Benzyl 5-acetamido-3,5-dideoxy-8,9-*O*-isopropylidene-D-glycero- β -D-galacto-2-nonulopyranosonate (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 μ L)

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, CDCl_3) 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), 2.33 (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); ^{13}C NMR (200 MHz, CDCl_3) 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 (6). To a solution of compound **5** (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, CDCl_3) 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); ^{13}C NMR (100 MHz, CDCl_3) 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, 35.90, 27.00, 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 produce a syrup which was dissolved in methanol (20 mL) and treated with H_2 in the presence of 10% Pd/C for 2 h at room temperature. The solution was then filtered through Celite and concentrated. The residue was purified by a silica gel column (EtOAc:MeOH:H₂O = 5:2:1, by volume) to produce a white powder **1** (658 mg, 82%). ^1H NMR (800 MHz, D_2O) δ 5.27 (td, J = 11.2 and 4.8 Hz, 1H, H-4), 4.19 (d, J = 10.4 Hz, 1H, H-8), 4.15 (t, J = 10.4 Hz, 1H, H-5), 3.82 (dd, J = 12.0 and 2.4 Hz, 1H, H-9a), 3.74 (m, 1H, H-6), 3.60 (dd, J = 12.0 and 6.4 Hz, 1H, H-9b), 3.56 (d, 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), 1.97 (s, 3H); ^{13}C NMR (200 MHz, D_2O) δ 174.39, 173.31, 173.29, 95.16, 70.00, 69.98, 69.91, 67.95, 63.02, 49.31, 35.95, 21.76, 20.22; HRMS (ESI) calculated for $\text{C}_{13}\text{H}_{20}\text{NO}_{10}$ (M-H) 350.1093, found 350.1093.

Multigram scale synthesis of Neu4,5Ac₂ (1). A multigram scale synthesis of Neu4,5Ac₂ (**1**) was also achieved using the procedure described above. Briefly, Neu5Ac (10.1 g, 32.65 mmol) was dissolved in water (50 mL) and 10% Cs_2CO_3 was added to adjust the solution to neutral. After removal of the solvent and dried under vacuum, the obtained solid was dissolved in dry DMF (60 mL), and benzyl bromide (6 mL) was added at 0 °C. The mixture was stirred at room temperature under argon for overnight and then filtered. The filtrate was evaporated to a syrup which was crystallized using 2-propanol to produce **4** (11.1 g, 85%). Compound **4** (10.08 g, 25.23 mmol) was then dissolved in acetone (150 mL), and 2,2-dimethoxypropane (20 mL) and camphorsulfonic acid (150 mg) were added. The mixture was stirred at room temperature for 2 hours. Trimethylamine (500 μL) was added and the mixture was stirred for 15 min before it was concentrated. The obtained residue was purified using a 150 g RediSep Rf silica column (EtOAc:MeOH = 5:1, by volume) on a CombiFlash® Rf 200i system to produce **5** (10.5 g, 95%). Compound **5** (9.89 g, 22.50 mmol) was dissolved in pyridine

(40 mL) and Ac_2O (15 mL) was added. The mixture was stirred for 1 h at room temperature and ethanol (30 mL) was added at 0 °C. After being concentrated, the residue was purified using a 150 g RediSep Rf silica column (EtOAc:MeOH = 10:1, by volume) on a CombiFlash® Rf 200i system to produce compound **6** (9.1 g, 84%). A solution of compound **6** (8.58 g, 17.83 mmol) in 90% acetic acid (40 mL) was stirred at 60 °C for 2 h. The reaction mixture was evaporated and the obtained syrup was dissolved in methanol (50 mL) and treated with H_2 in the presence of 10% Pd/C for 2 h at room temperature. The solution was filtered and concentrated. The residue was purified using a ODS-SM column (51 g, 50 μM , 120 Å, Yamazen) on a CombiFlash® Rf 200i system and was eluted with a gradient of 0–100% acetonitrile in water containing 0.05% formic acid over 30 minutes with a 30 mL/min flow rate. The fractions containing the pure product were collected and concentrated to produce the final product **1** (5.2 g, 83%) as a white powder.

Synthesis of Neu4Ac5Gc (2)

N-(2-Benzoyloxyacetyl)-D-neuraminic acid (9). Solid K_2CO_3 (14.4 g, 104.3 mM) was added to a stirred mixture of ManNH_2HCl (**7**, 4.5 g, 20.8 mM) in dry MeOH (100 mL). The mixture was stirred for 30 min and benzyloxyacetyl chloride (5.8 g, 31.3 mM) was added dropwisely. When the reaction was completed as monitored by TLC, the mixture 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 isotherm incubator for 48 h at 37 °C with gentle shaking. The reaction progress was monitored by TLC ($n\text{PrOH}:\text{H}_2\text{O}:\text{HOAc}$ = 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, D_2O) 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 (d, J = 9.6 Hz, 1H), 2.21 (dd, J = 12.8 and 4.8 Hz, 1H), 1.82 (t, J = 12.8 Hz, 1H); ^{13}C NMR (200 MHz, D_2O) δ 176.65, 173.01, 136.45, 128.72(2C), 128.56(2C), 128.48, 96.33, 73.20, 70.30, 69.88, 68.38, 68.33, 66.86, 63.10, 51.88, 39.37. HRMS (ESI) m/z calcd for $\text{C}_{18}\text{H}_{24}\text{NO}_{10}$ (M-H) 414.1400, found 414.1399.

Benzyl 5-glycolylamido-3,5-dideoxy-D-glycero- β -D-galacto-2-nonulopyranosonate (10). To a stirring solution of Neu5GcOBn (**9**, 3.0 g, mmol) in water (25 mL), 10% CsCO_3 was added dropwisely 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, CD_3OD) δ 7.35–7.24 (m, 10H), 5.26 (m, 2H, PhCH_2), 4.65 (m, 2H, PhCH_2), 4.39–3.53 (m, 9H), 2.36 (dd, J = 12.8 and 4.8 Hz, 1H), 2.17 (t, J = 12.8 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 172.27, 170.95, 132.14, 131.50,

127.86 (4C), 127.78, 127.70 (4C), 126.90, 97.80, 73.37, 71.18, 70.23, 69.22, 68.92, 68.61, 67.31, 66.96, 53.05, 39.06.

Benzyl 5-glycolylamido-3,5-dideoxy-8,9-O-isopropylidene-D-glycero-β-D-galacto-2-nonulopyranosonate (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 trimethylamine (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** (1.56 g, 96%) as a white solid. ¹H NMR (800 MHz, D₂O) δ 7.43–7.37 (m, 10H), 7.17 (d, *J* = 8.8 Hz, 1H), 5.27 (d, *J* = 12.0 Hz, 1H), 5.20 (d, *J* = 12.0 Hz, 1H), 4.60 (d, *J* = 12.0 Hz, 1H), 5.27 (d, *J* = 11.2 Hz, 1H), 4.49 (d, *J* = 6.4 Hz, 1H), 4.25–3.99 (m, 9H), 3.59 (t, *J* = 7.2 Hz, 1H), 2.36 (dd, *J* = 12.0 and 4.0 Hz, 1H), 2.15 (t, *J* = 12.0 Hz, 1H), 1.45 (s, 3H), 1.37 (s, 3H); ¹³C NMR (200 MHz, D₂O) δ 172.51, 169.48, 136.54, 134.94, 128.61(2C), 128.58(2C), 128.45, 128.27, 128.01(2C), 127.99(2C), 109.15, 95.39, 74.59, 73.41, 71.23, 69.56, 68.96, 67.61, 66.93, 66.76, 52.44, 38.98, 26.97, 25.28.

5-Glycolylamido-4-O-acetyl-3,5-dideoxy-D-glycero-β-D-galacto-2-nonulopyranosonate (2). To a solution of 8,9-O-isopropylidene-Neu5GcOBn (**11**, 1.1 g, 2.02 mmol) in pyridine (4 mL), acetic anhydride (2 mL) was added. The mixture was stirred for 1 h at room temperature and ethanol (10 mL) was added. The solution was evaporated to dryness and the residue was purified by a silica gel column (EtOAc:toluene = 1:2, by volume) to produce compound **12** (0.96 g, 81%). A solution of compound **12** (0.8 g, 1.36 mmol) in 90% acetic acid was stirred for 2 h at 60 °C. The reaction mixture was evaporated to give a syrup which was dissolved in methanol (20 mL) and treated with H₂ in the presence of 10% Pd/C for 2 h at room temperature. The solution was filtered through Celite and concentrated. The residue was purified by a silica gel column (EtOAc:MeOH:H₂O = 5:2:1, by volume) to produce a white powder **2** (0.31 g, 78%). ¹H NMR (800 MHz, D₂O) δ 5.36 (td, *J* = 11.2 and 4.8 Hz, 1H, H-4), 4.32 (d, *J* = 10.4 Hz, 1H, H-8), 4.23 (t, *J* = 10.4 Hz, 1H, H-5), 4.05 (d, *J* = 6.4 Hz, 2H, OCH₂-), 3.82 (dd, *J* = 12.0 and 2.4 Hz, 1H, H-9a), 3.75 (m, 1H, H-6), 3.61 (dd, *J* = 12.0 and 6.4 Hz, 1H, H-9b), 3.56 (d, *J* = 8.8 Hz, 1H, H-7), 2.35 (dd, *J* = 12.8 and 5.6 Hz, 1H, H-3eq), 2.04 (s, 3H), 2.01 (t, *J* = 12.8 Hz, 1H, H-3ax); ¹³C NMR (200 MHz, D₂O) δ 75.34, 173.25, 172.97, 95.06, 69.99, 69.75, 69.70, 67.86, 62.98, 60.71, 48.97, 35.98, 20.22; HRMS (ESI) calculated for C₁₃H₂₀NO₁₁ (M-H) 366.1042, found 366.1042.

Chemoenzymatic synthesis of GlcNAcβ3Galβ4GlcβProN₃ (Lc₃βProN₃) and Galβ4GlcNAcβ3Galβ4GlcβProN₃ (LNnTβProN₃, **23**)

One-pot four-enzyme preparative-scale synthesis of trisaccharide GlcNAcβ3Galβ4GlcβProN₃ (Lc₃βProN₃). A reaction mixture in a total volume of 40 mL containing Tris-HCl buffer (100 mM, pH 7.5), LacβProN₃ (**17**, 0.45 g, 1.06 mmol), *N*-acetylglucosamine (GlcNAc, 0.304 g, 1.38 mmol), ATP (0.758 g, 1.38 mmol), UTP (0.756 g, 1.38 mmol), MgCl₂ (20 mM), BLNahK (12.0 mg), PmGlmU (6.0 mg), NmLgtA (6.0 mg), and PmPpA (4.0 mg) was incubated in a shaker with agitation (100 rpm) at 37 °C for 15 h and then 72 h at room temperature. The product formation was monitored by TLC (EtOAc:MeOH:H₂O:HOAc = 4:2:1:0.2 by volume) and detected by *p*-anisaldehyde sugar stain and mass spectrometry (MS). When the reaction was completed, the same volume (40 mL) of 95% ethanol was added to the reaction mixture and the mixture was incubated at 4 °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 Lc₃ trisaccharide GlcNAcβ3Galβ4GlcβProN₃⁴² (0.625 g, 94%). ¹H NMR (800 MHz, D₂O) δ 4.67 (d, *J* = 8.0 Hz, 1H), 4.48 (d, *J* = 8.0 Hz, 1H), 4.43 (d, *J* = 8.0 Hz, 1H), 4.15 (d, *J* = 3.2 Hz, 1H), 4.01–3.97 (m, 2H), 3.90 (d, *J* = 11.2 Hz, 1H), 3.76–3.55 (m, 13H), 3.47–3.44 (m, 4H), 3.30 (d, *J* = 8.0 Hz, 1H), 2.03 (s, 3H), 1.91 (m, 2H); ¹³C NMR (200 MHz, D₂O) δ 174.85, 102.81, 102.74, 101.99, 81.83, 78.20, 75.52, 74.76, 74.65, 74.23, 73.43, 72.66, 69.87, 69.54, 68.21, 67.24, 60.83, 60.34, 59.92, 55.53, 47.73, 28.10, 22.03. HRMS (ESI) *m/z* calculated for C₂₃H₄₀N₄O₁₆Na (M+Na) 651.2337, found 651.2337.

One-pot four-enzyme preparative-scale synthesis of Galβ4GlcNAcβ3Galβ4GlcβProN₃ (LNnTβProN₃, **23).** The reaction mixture in a total volume of 30 mL containing Tris-HCl buffer (100 mM, pH 8.0), Lc₃ 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 (12.0 mg), BLUSP (10.0 mg), NmLgtB (7.0 mg), and PmPpA (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*-PrOH: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 by silica gel chromatography (EtOAc:MeOH:H₂O = 5:3:1.5) and finally Bio-Gel P-2 column (eluted with H₂O) to produce Galβ4GlcNAcβ3Galβ4GlcβProN₃⁴² (**23**, 0.613 g, 99%). ¹H NMR (800 MHz, D₂O) δ 4.69 (d, *J* = 8.0 Hz, 1H), 4.48 (d, *J* = 7.2 Hz, 1H), 4.47 (d, *J* = 8.0 Hz, 1H), 4.43 (d, *J* = 7.2 Hz, 1H), 4.15 (d, *J* = 3.2 Hz, 1H), 4.01–3.92 (m, 4H), 3.85–3.57 (m, 19H), 3.53 (t, *J* = 8.8 Hz, 1H), 3.46 (t, *J* = 7.2 Hz, 2H), 3.30 (d, *J* = 8.0 Hz, 1H), 2.03 (s, 3H), 1.90 (m, 2H); ¹³C NMR (200 MHz, D₂O) δ 174.80, 102.82, 102.73, 102.64, 101.99, 81.92, 78.20, 78.00, 75.23, 74.75, 74.65, 74.42, 74.23, 72.66, 72.37, 72.05, 70.84, 69.83, 68.42, 68.20, 67.24, 60.91, 60.83, 59.92, 59.72, 55.06, 47.73, 28.10, 22.05. HRMS (ESI) *m/z* calculated for C₂₉H₅₀N₄O₂₁Na (M+Na) 813.2865, found 813.2865.

General procedures for one-pot two-enzyme (OP2E) preparative-scale synthesis of α2–3-linked sialosides containing a 4-O-acetyl-sialic acid. An acceptor (10 mM, 1.0 eq, chosen from **13–23**), 4-O-acetyl-sialic 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

containing the pure product were collected and concentrated to provide the final purified product.

Neu4,5Ac₂α3Galβ4Glc (24). 56 mg, yield 82%; white solid. ¹H 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, 75.41, 75.02, 74.68, 74.20, 73.67, 72.74, 71.63, 71.04, 69.23, 67.78, 67.05, 62.41, 60.91, 59.93, 49.68, 36.54, 21.75, 20.20; HRMS (ESI) *m/z* calcd for C₂₅H₄₀N₂O₂₀ (M-H) 674.2144, found 674.2149.

Neu4,5Ac₂α3Galβ4GlcNAcβ3Galβ4Glc (25). 34 mg, yield 78%; white solid. ¹H NMR (800 MHz, D₂O) δ 5.21 (d, *J* = 4.0 Hz, 0.4H, α-isomer), 4.97 (m, 1H), 4.69 (d, *J* = 8.8 Hz, 0.4H, α-isomer), 4.68 (d, *J* = 8.0 Hz, 0.6H, β-isomer), 4.65 (d, *J* = 8.0 Hz, 0.6H, β-isomer), 4.55 (d, *J* = 8.0 Hz, 1H), 4.43 (d, *J* = 8.0 Hz, 1H), 4.15 (d, *J* = 4.0 Hz, 1H), 4.13 (dd, *J* = 3.2 and 9.6 Hz, 1H), 4.09 (t, *J* = 9.6 Hz, 1H), 3.96–3.26 (m, 28H), 2.76 (dd, *J* = 4.8 and 12.0 Hz, 1H), 2.06 (s, 3H), 2.02 (s, 3H), 1.96 (s, 3H), 1.94 (t, *J* = 12.0 Hz, 1H). ¹³C NMR (200 MHz, D₂O) α/β isomers: δ 174.77, 174.45, 173.08, 172.92, 102.80, 102.77, 102.67, 102.40, 95.61, 81.92, 81.90, 78.25, 78.14, 77.88, 75.42, 74.99, 74.77, 74.68, 74.42, 74.23, 73.65, 72.35, 72.03, 71.44, 71.28, 71.00, 70.86, 70.00, 69.87, 69.84, 69.25, 68.21, 67.78, 67.39, 62.50, 60.87, 60.85, 59.94, 59.72, 55.06, 49.12, 46.55, 36.41, 22.05, 21.77, 20.20. HRMS (ESI) *m/z* calculated for C₃₉H₆₃N₂O₃₀ (M-H) 1039.3466, found 1039.3442.

Neu4,5Ac₂α3Galβ3GlcNAcβ3Galβ4Glc (26). 29 mg, yield 65%; white solid. ¹H NMR (800 MHz, D₂O) δ 5.20 (d, *J* = 3.2 Hz, 0.4H, α-isomer), 4.93 (m, 1H), 4.71 (d, *J* = 8.0 Hz, 1H), 4.65 (d, *J* = 8.0 Hz, 0.6H, β-isomer), 4.50 (d, *J* = 8.0 Hz, 1H), 4.43 (d, *J* = 8.0 Hz, 1H), 4.13 (d, *J* = 3.2 Hz, 1H), 4.09 (dd, *J* = 3.2 and 9.6 Hz, 1H), 4.07 (t, *J* = 9.6 Hz, 1H), 3.94–3.26 (m, 27H), 2.75 (dd, *J* = 4.0 and 12.0 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) β-isomer: δ 174.81, 174.41, 173.41, 173.09, 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, 59.93, 54.47, 49.14, 36.70, 22.17, 21.76, 20.21. HRMS (ESI) *m/z* calculated for C₃₉H₆₃N₂O₃₀ (M-H) 1039.3466, found 1039.3487.

Neu4,5Ac₂α3GalβpNP (27). 13 mg, yield 31%; white solid. ¹H NMR (800 MHz, D₂O) δ 8.27 (d, *J* = 8.0 Hz, 2H), 7.25 (d, *J* = 8.0 Hz, 2H), 5.30 (d, *J* = 8.0 Hz, 1H), 4.95 (m, 1H), 4.25 (dd, *J* = 9.6 and 4.0 Hz, 1H), 4.09 (t, *J* = 10.4 Hz, 1H), 4.04 (d, *J* = 2.4 Hz, 1H), 3.93–3.57 (m, 10H), 2.79 (dd, *J* = 12.0 and 4.8 Hz, 1H), 2.06 (s, 3H), 1.95 (s, 3H), 1.92 (t, *J* = 12.0 Hz, 1H); ¹³C NMR (200 MHz, D₂O) δ 174.48, 173.37, 173.10, 163.03, 162.87, 161.61, 142.42, 126.03, 116.31, 99.69, 99.51, 75.43, 75.31, 72.28, 71.69, 71.02, 68.66, 67.79, 67.16, 62.43, 60.54, 49.17, 36.63, 21.82, 20.27; HRMS (ESI) calculated for C₂₅H₃₃N₂O₁₇ (M-H) 633.1779, found 633.1807.

Neu4,5Ac₂α3Galβ4GlcβProN₃ (28). 28 mg, yield 88%; white solid. ¹H NMR (800 MHz, D₂O) δ 4.98 (m, 1H), 4.51 (d, *J* = 8.0 Hz, 1H), 4.47 (d, *J* = 8.0 Hz, 1H), 4.11 (dd, *J* = 9.6 and 3.2 Hz, 1H), 4.07 (t, *J* = 10.4 Hz, 1H), 3.98–3.94 (m, 3H), 3.89–3.55 (m, 14H), 3.44 (t, *J* = 6.4 Hz, 2H), 3.29 (t, *J* = 8.8 Hz, 1H), 2.75 (dd, *J* = 12.8 and 4.8 Hz, 1H), 2.10 (s, 3H), 1.95 (s, 3H), 1.91 (t, *J* = 12.8 Hz, 1H), 1.89 (m, 2H); ¹³C NMR (200 MHz, D₂O) δ 174.44, 173.42, 173.09, 102.51, 102.02, 99.63,

78.12, 75.41, 75.02, 74.65, 74.22, 72.68, 72.26, 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 C₂₈H₄₅N₄O₂₀ (M-H) 757.2627, found 757.2633.

Neu4,5Ac₂α3Galβ4GlcNAcβProN₃ (29). 32 mg, yield 85%; white solid. ¹H 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, 172.78, 102.42, 101.02, 99.29, 78.17, 75.39, 74.97, 74.61, 72.36, 72.22, 71.36, 70.79, 69.24, 67.78, 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 C₃₀H₄₈N₅O₂₀ (M-H) 798.2893, found 798.2893.

Neu4,5Ac₂α3Galβ3GlcNAcβProN₃ (30). 24 mg, yield 86%; white solid. ¹H NMR (800 MHz, D₂O) δ 4.98 (m, 1H), 4.54 (d, *J* = 8.0 Hz, 1H), 4.48 (d, *J* = 8.0 Hz, 1H), 4.10–4.07 (m, 2H), 3.96–3.36 (m, 20H), 2.75 (dd, *J* = 12.0 and 4.8 Hz, 1H), 2.06 (s, 3H), 2.02 (s, 3H), 1.95 (s, 3H), 1.94 (t, *J* = 12.0 Hz, 1H), 1.83 (m, 2H); ¹³C NMR (200 MHz, D₂O) δ 174.46 (2C), 173.12, 172.33, 103.23, 100.77, 98.92, 82.44, 75.53, 75.24, 74.85, 72.36, 71.28, 70.61, 68.96, 68.61, 67.77, 67.26, 67.03, 62.53, 60.80, 60.60, 54.32, 49.06, 47.66, 36.29, 27.97, 22.17, 21.78, 20.19; HRMS (ESI) calculated for C₃₀H₄₈N₅O₂₀ (M-H) 798.2893, found 798.2900.

Neu4,5Ac₂α3Galβ3GlcNAcαProN₃ (31). 21 mg, yield 80%; white solid. ¹H 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), 1.91–1.88 (m, 3H); ¹³C NMR (200 MHz, D₂O) δ 174.42, 174.33, 173.40, 173.10, 103.20, 99.49, 96.97, 80.21, 75.61, 74.90, 72.19, 71.73, 71.48, 71.04, 69.01, 68.55, 67.73, 67.16, 64.82, 62.30, 60.87, 60.39, 52.36, 49.13, 48.04, 36.68, 27.84, 21.92, 21.76, 20.21; HRMS (ESI) calculated for C₃₀H₄₈N₅O₂₀ (M-H) 798.2893, found 798.2895.

Neu4,5Ac₂α3Galβ3GalNAcβProN₃ (32). 22 mg, yield 81%; white solid. ¹H 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 (d, *J* = 3.2 Hz, 1H), 4.09–4.06 (m, 2H), 4.16–3.59 (m, 16H), 3.52 (dd, *J* = 9.6 and 8.0 Hz, 1H), 3.36 (m, 2H), 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), 1.83 (m, 2H); 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 C₃₀H₄₈N₅O₂₀ (M-H) 798.2893, found 798.2903.

Neu4,5Ac₂α3Galβ3GalNAcαProN₃ (33). 18 mg, yield 74%; white solid. ¹H 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 174.47, 174.43, 173.43, 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, 49.15, 48.57, 48.06, 36.65, 27.85, 21.93, 21.76, 20.21; HRMS (ESI) calculated for C₃₀H₄₈N₅O₂₀ (M-H) 798.2893, found 798.2902.

Neu4,5Ac₂α3Galβ4GlcNAcβ3Galβ4GlcβProN₃ (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), 1.94 (s, 3H), 1.91 (t, *J* = 12.0 Hz, 1H), 1.89 (m, 2H); ¹³C NMR (200 MHz, D₂O) δ 174.78, 174.44, 173.40, 173.08, 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, 74.23, 72.65, 72.27, 72.02, 71.62, 71.03, 69.83, 69.25, 68.20, 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 C₄₂H₆₈N₅O₃₀ (M-H) 1122.3949, found 1122.3965.

Neu4Ac5Gcaβ3Galβ4GlcβProN₃ (35). 15 mg, yield 67%; white solid. ¹H NMR (800 MHz, D₂O) δ 4.98 (dt, *J* = 11.2 and 4.8 Hz, 1H, H-4''), 4.52 (d, *J* = 8.0 Hz, 1H), 4.47 (d, *J* = 8.0 Hz, 1H), 4.16–3.55 (m, 21H), 3.44 (t, *J* = 6.4 Hz, 2H), 3.30 (t, *J* = 8.8 Hz, 1H), 2.78 (dd, *J* = 12.0 and 4.8 Hz, 1H), 2.05 (s, 3H), 1.92 (t, *J* = 12.0 Hz, 1H), 1.89 (m, 2H); ¹³C NMR (200 MHz, D₂O) δ 175.38, 173.45, 173.05, 102.51, 102.01, 99.61, 78.11, 75.39, 75.02, 74.64, 74.21, 72.68, 72.04, 71.67, 70.77, 69.24, 67.72, 67.35, 67.24, 62.39, 60.90, 60.71, 59.92, 48.88, 47.74, 36.65, 28.11, 20.23; HRMS (ESI) calculated for C₂₈H₄₅N₄O₂₁ (M-H) 773.2576, found 773.2500.

Neu4Ac5Gcaβ3Galβ4GlcNAcβProN₃ (36). 17 mg, yield 71%; white solid. ¹H NMR (800 MHz, D₂O) δ 5.02 (dt, *J* = 11.2 and 4.8 Hz, 1H, H-4''), 4.54 (d, *J* = 8.0 Hz, 1H), 4.51 (d, *J* = 8.0 Hz, 1H), 4.15 (t, *J* = 11.2 Hz, 1H), 4.13 (dd, *J* = 10.4 and 3.2 Hz, 1H), 4.07–3.55 (m, 20H), 3.37 (m, 2H), 2.80 (dd, *J* = 12.0 and 4.8 Hz, 1H), 2.05 (s, 3H), 2.03 (s, 3H), 1.95 (s, 3H), 1.93 (t, *J* = 12.0 Hz, 1H), 1.82 (m, 2H); ¹³C NMR (200 MHz, D₂O) δ 175.38, 174.38, 173.45, 173.05, 102.45, 101.03, 99.63, 78.17, 75.39, 75.02, 74.62, 72.22, 72.04, 71.67, 70.77, 69.26, 67.73, 67.38, 67.04, 62.39, 60.91, 60.71, 59.90, 54.96, 48.88, 47.65, 36.64, 27.98, 22.05, 20.24; HRMS (ESI) calculated for C₃₀H₄₈N₅O₂₁ (M-H) 814.2842, found 814.2870.

Neu4Ac5Gcaβ3Galβ3GlcNAcβProN₃ (37). 14 mg, yield 65%; white solid. ¹H NMR (800 MHz, D₂O) δ 5.02 (dt, *J* = 11.2 and 4.8 Hz, 1H, H-4''), 4.54 (d, *J* = 8.0 Hz, 1H), 4.48 (d, *J* = 8.0 Hz, 1H), 4.15–3.34 (m, 24H), 2.78 (dd, *J* = 12.0 and 4.8 Hz, 1H), 2.05 (s, 3H), 2.02 (s, 3H), 1.90 (t, *J* = 12.0 Hz, 1H), 1.83 (m, 2H); ¹³C NMR (200 MHz, D₂O) δ 175.34, 174.45, 173.43, 173.05, 103.31, 100.78, 99.43, 82.35, 75.54, 75.25, 74.95, 71.96, 71.75, 70.75, 68.94, 68.59, 67.67, 67.14, 67.04, 62.25, 60.89, 60.71, 60.59, 54.33, 48.85, 47.66, 36.79, 27.97, 22.18, 20.22; HRMS (ESI) calculated for C₃₀H₄₈N₅O₂₁ (M-H) 814.2842, found 814.2869.

Neu4Ac5Gcaβ3Galβ3GlcNAcβProN₃ (38). 12 mg, yield 56%; white solid. ¹H NMR (800 MHz, D₂O) δ 5.02 (dt, *J* = 10.4 and 4.8 Hz, 1H, H-4''), 4.85 (d, *J* = 3.2 Hz, 1H), 4.51 (d, *J* = 8.0 Hz, 1H), 4.15 (t, *J* = 10.4 Hz, 1H), 4.12–3.42 (m, 23H), 2.79 (dd, *J* = 12.8 and 4.8 Hz, 1H), 2.05 (s, 3H), 2.02 (s, 3H), 1.91 (t, *J* = 12.8 Hz, 1H), 1.89 (m, 2H); ¹³C NMR (200 MHz, D₂O) δ 175.35, 174.32, 173.43, 173.06, 103.19, 99.48, 96.98, 80.19, 75.60, 74.90, 71.96, 71.76, 71.48, 70.76, 69.02, 68.54, 67.67, 67.14, 64.83, 62.27, 60.87, 60.71, 60.39, 52.36, 48.85, 48.05, 36.78, 27.84, 21.93, 20.23; HRMS (ESI) calculated for C₃₀H₄₈N₅O₂₁ (M-H) 814.2842, found 814.2875.

Neu4Ac5Gcaβ3Galβ3GalNAcβProN₃ (39). 10 mg, yield 51%; white solid. ¹H NMR (800 MHz, D₂O) δ 5.02 (m, 1H), 4.50 (d, *J* = 8.0 Hz, 1H),

4.48 (d, *J* = 8.0 Hz, 1H), 4.16–3.37 (m, 24H), 2.75 (dd, *J* = 12.0 and 4.8 Hz, 1H), 2.06 (s, 3H), 2.05 (s, 3H), 2.02 (s, 3H), 1.90 (t, *J* = 12.0 Hz, 1H), 1.83 (m, 2H); ¹³C NMR (200 MHz, D₂O) δ 175.34, 174.61, 173.48, 173.04, 104.49, 101.28, 99.44, 79.90, 75.49, 74.64, 74.63, 71.94, 71.73, 70.77, 68.85, 67.75, 67.70, 67.20, 66.87, 62.28, 60.86, 60.82, 60.70, 51.04, 48.85, 47.66, 36.78, 27.99, 22.17, 20.21; HRMS (ESI) calculated for C₃₀H₄₈N₅O₂₁ (M-H) 814.2842, found 814.2872.

Neu4Ac5Gcaβ3Galβ3GalNAcβProN₃ (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, 173.44, 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, 62.31, 61.10, 60.85, 60.70, 48.85, 48.56, 48.05, 36.74, 27.84, 21.93, 20.22; HRMS (ESI) calculated for C₃₀H₄₈N₅O₂₁ (M-H) 814.2842, found 814.2874.

Neu4Ac5Gcaβ3Galβ4GlcNAcβ3Galβ4GlcβProN₃ (41). 10 mg, yield 62%; white solid. ¹H NMR (800 MHz, D₂O) δ 5.04 (dt, *J* = 10.4 and 4.8 Hz, 1H, H-4''), 4.70 (d, *J* = 8.8 Hz, 1H), 4.56 (d, *J* = 8.0 Hz, 1H), 4.49 (d, *J* = 8.0 Hz, 1H), 4.44 (d, *J* = 8.0 Hz, 1H), 4.18–3.57 (m, 33H), 3.46 (t, *J* = 6.4 Hz, 2H), 3.12 (t, *J* = 8.0 Hz, 1H), 2.80 (dd, *J* = 12.0 and 4.8 Hz, 1H), 2.07 (s, 3H), 2.03 (s, 3H), 1.91 (t, *J* = 12.0 Hz, 1H), 1.91 (m, 2H); ¹³C NMR (200 MHz, D₂O) δ 174.78, 174.44, 173.40, 173.08, 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, 74.23, 72.65, 72.27, 72.02, 71.62, 71.03, 69.83, 69.25, 68.20, 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 C₄₂H₆₈N₅O₃₁ (M-H) 1138.3898, found 1138.3938.

Gram-scale OP2E synthesis of monotreme milk trisaccharide

Neu4,5Ac₂α3Lac. Lactose (1.09 g, 3.05 mmol), Neu4,5Ac₂ (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 ODS-SM column (51 g, 50 μM, 120 Å, Yamazen) 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 minutes. The fractions containing the pure product were collected and concentrated to produce the final purified product Neu4,5Ac₂α3Galβ4Glc (1.33 g, 71%) as a white powder. The identity and the 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 Neu4,5Ac₂α3GalβpNP (0.3 mM)

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Organic & Biomolecular Chemistry

and *Aspergillus oryzae* β -galactosidase (12 μ g, 126 mU). The amount of the β -galactosidase required to completely hydrolyze Gal β pNP (0.3 mM) within the time frame of the assay was predetermined and confirmed by control assays with Gal β pNP (0.3 mM). The reactions were stopped by adding 40 μ L of *N*-cyclohexyl-3-aminopropane sulfonic acid (CAPS) buffer (0.5 M, pH 11.5). The amount of *para*-nitrophenolate formed was determined by measuring the $A_{405\text{ nm}}$ of the reaction mixtures using a microtiter plate reader. Neu5Ac α 3Gal β pNP⁵⁰ was used as a control.

The assay conditions for bacterial sialidases were: *A. ureafaciens* sialidase (1.5 mU) in sodium acetate buffer (100 mM, pH 5.5); *C. perfringens* sialidase (3 mU) in MES buffer (100 mM, pH 5.0); *V. cholerae* sialidase (1.5 mU) in sodium acetate buffer (100 mM, pH 5.5) containing NaCl (150 mM) and CaCl₂ (10 mM); *S. pneumoniae* sialidase (1.5 mU) in sodium acetate buffer (100 mM, pH 6.0); PmST1 (10 μ g) 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 (1 \times 10⁵ HAU mL⁻¹) selected from 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).⁵¹

To test whether the cleavage of Neu4,5Ac₂ by human influenza A viruses was caused by de-*O*-acetylation or direct de-sialylation, Neu4,5Ac₂ α 3Lac (1 mM) was incubated with purified human influenza A virus strain A/Puerto Rico/34/8 H1N1 (A/PR8) (1 \times 10⁵ HAU mL⁻¹) in a MES buffer (100 mM, pH 5.0) for 30 min at 37 °C. The reaction mixture was then subjected to high-resolution mass spectrometry analysis.

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