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Streptococcus pneumoniae Sialidase SpNanB-Catalyzed One-Pot Multienzyme (OPME) Synthesis of 2,7-Anhydro-Sialic acids as Selective Sialidase Inhibitors

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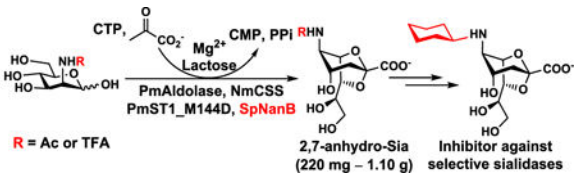
Abstract

Streptococcus pneumoniae sialidase SpNanB is an intramolecular *trans*-sialidase (IT-sialidase) and a virulence factor that is essential for streptococcal infection of upper and lower respiratory tract. SpNanB catalyzes the formation of 2,7-anhydro-*N*-acetylneuraminic acid (2,7-anhydro-Neu5Ac), a potential prebiotic that can be used as the sole carbon source of a common human gut commensal anaerobic bacterium. We report here the development of an efficient one-pot multienzyme (OPME) system for synthesizing 2,7-anhydro-Neu5Ac and its derivatives. Based on crystal structure analysis, an *N*-cyclohexyl derivative of 2,7-anhydro-neuraminic acid was designed, synthesized, and shown to be a selective inhibitor against SpNanB and another *Streptococcus pneumoniae* sialidase SpNanC. This study demonstrates a new strategy of synthesizing 2,7-anhydro-sialic acids in gram scale and the potential application of their derivatives as selective sialidase inhibitors.

TOC Graph



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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Tables S1 and S2, Figure S1 and S2, and NMR spectra of products (PDF).

Notes

The authors declare no competing financial interest.

Keywords

chemoenzymatic synthesis; 2,7-anhydro-sialic acids; sialidase; inhibitor; *Streptococcus pneumoniae*

1. INTRODUCTION

Streptococcus pneumoniae is a common human pathogen that causes pneumonia, otitis media, septicemia, bacteremia, meningitis, and other serious diseases.[1,2] It expresses up to three sialidases including SpNanA,[3,4] SpNanB,[5,6] and/or SpNanC.[7,8] SpNanA is a hydrolytic sialidase catalyzing the hydrolysis of terminal α2–3-, α2–6-, and α2–8-linked sialic acid.[4] SpNanB is an intramolecular *trans*-sialidase (IT-sialidase) which uses α2–3-linked sialosides as substrates to produce 2,7-anhydro-*N*-acetylneuraminic acid (2,7-anhydro-Neu5Ac, **1**) (Figure 1).[9,10] SpNanC catalyzes the selective cleavage of terminal α2–3-linked sialic acid to form 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en, DANA), a transition state analog of most hydrolytic sialidases.[8]

SpNanB, along with SpNanA, provides sialic acid source and helps *Streptococcus pneumoniae* in biofilm formation, nutrition, colonization, and infection of the host.[11–13] SpNanC plays a role of regulating the activity of hydrolytic sialidases including SpNanA as it catalyzes the production and the hydrolysis of hydrolytic sialidase inhibitor Neu5Ac2en.[8] SpNanC has been identified as a *Streptococcus pneumoniae* marker for pneumococcal haemolytic uraemic syndrome in children.[14]

Sialidase inhibitors are proven successful anti-virus drugs,[15] and are potential anti-bacteria reagents.[16] Various types of inhibitors against SpNanA have been identified including Neu5Ac2en and derivatives,[17,18] katsumadain A, artocarpin,[19] and diazenylaryl sulfonic acids.[20] However, effective inhibitors against SpNanB and/or SpNanC are not readily available. Neu5Ac2en inhibited SpNanB and SpNanC only weakly with an IC_{50} value falling in a submillimolar range.[18] Oseltamivir[21] did not show inhibitory activity against SpNanB nor SpNanC with a concentration up to 7.5 mM[22] even though it inhibited *Ruminococcus gnavus* IT-sialidase RgNanH[23] which shares a similar catalytic mechanism as SpNanB and produces the same 2,7-anhydro-Neu5Ac product. 2-*N*-Cyclohexylaminoethanesulfonic acid (CHES) was unexpectedly found to have weak inhibition against SpNanB.[9] A family of β-amino-sulfonic acids was subsequently screened and the most potent inhibitor candidate identified had an IC_{50} value of 38.9 μM.[24] Siastatin B[25] also had a similar inhibitory activity against SpNanB and natural products katsumadain A and artocarpin were reported to be inhibitors against SpNanB.[26] Recently, a natural product malabaricone C was reported to inhibit all three *S. pneumoniae* sialidases, with IC_{50} values in a submicromolar range.[27] However, the control Neu5Ac2en was shown to have an IC_{50} value of 45.1 μM for SpNanB, which disagreed with other reports where Neu5Ac2en was a millimolar inhibitor against SpNanB.[9,18,22,28]

We hypothesized that the derivatives of SpNanB product, 2,7-anhydro-Neu5Ac (**1**), could be suitable selective inhibitors against SpNanB. 2,7-Anhydro-Neu5Ac (**1**) was initially characterized in 1982 as a sialic acid methanolysis byproduct,[29] and later found in rat urine

and human wet cerumen.[30] It was shown to be a selective carbon source to support the growth of *Ruminoccocus gnavus*, a common human gut commensal anaerobic bacterium.[31] Nevertheless, the roles and potential applications of 2,7-anhydro-Neu5Ac remain largely underexplored, partially due to the limited access to the 2,7-anhydro-sialic acids. There are only a few reports describing the chemical synthesis of 2,7-anhydro-sialic acids.[32–34] Although the overall yield was improved and the synthetic route was shortened,[34] the chemical synthetic methods required multistep protection and deprotection steps. Due to their high efficiency, excellent regio- and stereoselectivity, environmental friendly feature, as well as increasing accessibility, enzymes have been increasingly applied in organic synthesis. Especially, the use of enzymes in the synthesis of carbohydrates is growing rapidly.[35,36] Recently, an enzymatic method was reported to produce 2,7-anhydro-Neu5Ac in milligram scales in a 33% overall yield by treating sialylglycoprotein fetuin with *Ruminoccocus gnavus* IT-sialidase RgNanH.[37] An efficient method for large-scale synthesis of 2,7-anhydro-Neu5Ac and its derivatives is needed.

Herein, we report an efficient one-pot multienzyme (OPME) system for synthesizing 2,7-anhydro-Neu5Ac and its derivatives in gram-scale and preparative-scale with good overall yields. Moreover, we have demonstrated that it is possible to develop 2,7-anhydro-sialic acid derivatives as potential selective inhibitors against certain sialidases.

2. RESULTS AND DISCUSSION

Gram-Scale Enzymatic Synthesis of 2,7-Anhydro-Neu5Ac (1).

Similar to the function of leech IT-sialidase NanL,[38] SpNanB was reported to be able to catalyze the formation of 2,7-anhydro-Neu5Ac directly from Neu5Ac.[10] Nevertheless, our attempts to synthesize 2,7-anhydro-Neu5Ac (**1**) directly from *N*-acetylneuraminic acid (Neu5Ac) using SpNanB resulted in low yields (<20% by thin-layer chromatography analysis) despite the efforts in varying buffer, pH, SpNanB amount, and the concentration of Neu5Ac.

In order to obtain 2,7-anhydro-Neu5Ac (**1**) in gram-scale, a one-pot multienzyme (OPME) system containing four enzymes was developed. As shown in Scheme 1, *Pasteurella multocida* sialic acid aldolase (PmAldolase),[39] *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS),[40] and *Pasteurella multocida* sialyltransferase 1 M144D mutant (PmST1_M144D)[41,42] were used for the in situ formation of α2–3-linked sialyllactose (3'-sialyllactose), which was the substrate of SpNanB for the production of 2,7-anhydro-Neu5Ac (**1**). PmAldolase was responsible for the formation of *N*-acetylneuraminic acid (Neu5Ac), the most common sialic acid form,[43] from *N*-acetylmannosamine (ManNAc) and pyruvate. The Neu5Ac formed was activated by NmCSS to form cytidine 5'-monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac) in the presence of cytidine 5'-triphosphate (CTP). PmST1_M144D was responsible for catalyzing the transfer of the Neu5Ac in CMP-Neu5Ac donor to lactose as the acceptor to form 3'-sialyllactose, which was used as the substrate of SpNanB for the formation of 2,7-anhydro-Neu5Ac as the target product.

Similar to the OPME synthesis of 2,3-dehydro-2-deoxy-sialic acids (Sia2ens),[18] the optimal conditions for SpNanB-dependent OPME synthesis of 2,7-anhydro-Neu5Ac (**1**) were explored. Tris-HCl buffer at pH 7.5 was found to be a well suited condition to balance the activities of all four enzymes in the system. Lactose was chosen as the sialyltransferase acceptor due to its commercial availability and low cost. As lactose produced by the sialidase SpNanB-catalyzed reaction can be reused as the acceptor for sialyltransferase PmST1_M144D-catalyzed reaction (Scheme 1), it was used at 0.5 equivalent of the molar amount of the sialic acid precursor ManNAc in the system. The desired 2,7-anhydro-Neu5Ac (**1**) was obtained in 1.10 grams with a yield of 78% using this OPME method.

Pure 2,7-anhydro-Neu5Ac (**1**) was used to confirm a previous observation that similar to leech IT-sialidase,[38] SpNanB can hydrolyze 2,7-anhydro-Neu5Ac to form Neu5Ac.[9] For synthetic purpose, the 2,7-anhydro-Neu5Ac (**1**) hydrolysis activity of SpNanB was able to be minimized by controlling the reaction time and the amount of SpNanB.

Substrate Specificity Studies of SpNanB.

Substrate specificity studies of SpNanB confirmed that the enzyme was specific to α2–3-linked sialosides. A library of *para*-nitrophenol (*p*NP)-tagged α2–3-linked sialosides[44] with derivatization at various positions of Neu5Ac showed that C5- and C9-modifications of Neu5Ac, including *N*-glycolylneuraminic acid (Neu5Gc), were well tolerated. However, sialosides containing 2-keto-3-deoxynonulsonic acid (Kdn) and its derivatives were not suitable substrates for SpNanB (Table S1 and Figure S1). The substrate promiscuities of SpNanB and the other three enzymes in the OPME system make it possible to synthesize 2,7-anhydro-sialic acids and derivatives with modifications at various positions.

It was interesting to notice that C7-modified sialic acids were also good substrates for SpNanB. These were unexpected as the hydroxyl group on C-7 was the nucleophile that attacked the anomeric carbon of sialic acid during the intramolecular trans-sialidase reaction.[8] To confirm the observation from the colorimetric plate assay and identify the product, thin layer chromatography (TLC), mass spectrometry, and nuclear magnetic resonance (NMR) studies were carried out using Neu5Ac7N_3α2–3Galβ*p*NP, a sialoside with a C7-modified Neu5Ac, as the substrate for SpNanB. TLC analysis showed only two product spots throughout the reaction process, one of which representing Galβ*p*NP. Mass spectrometry results showed a m/z value of 333.1051 (Figure S2), corresponding to 7-azido-Neu5Ac (7N_3Neu5Ac) which was confirmed by NMR studies. These observations indicated that SpNanB can catalyze the hydrolysis of sialosides containing 7-modified sialic acids directly when the 7-hydroxyl group required for intramolecular trans-sialidase reaction is missing.

Design of a 2,7-Anhydro-Neu5Ac Derivative-Based SpNanB Inhibitor.

One of our research goals is to develop selective inhibitors against bacterial sialidases as chemical biological probes. We hypothesize that SpNanB product with substitutions that better fit the SpNanB substrate binding pocket would be selective inhibitors against SpNanB. Analysis of crystal structures of SpNanB complexed with various inhibitors indicated the presence of a hydrophobic pocket near the active site of SpNanB.[9] In the

structure (PDB ID: 4XHB) of SpNanB complexed with 2-*N*-cyclohexylaminoethansulfonic acid (CHES), the cyclohexyl group of CHES occupied the hydrophobic pocket, replacing the acetamido methyl group in 2,7-anhydro-Neu5Ac (**1**) bond to SpNanB in the structure complex.[9,10] Further studies demonstrated that many functional groups could fit in this pocket with varying inhibitory activities against SpNanB.[24] Superimposition of the bound 2,7-anhydro-Neu5Ac, CHES, and 2-[(3-chlorobenzyl)ammonio)ethanesulfonate revealed that the acetyl group in 2,7-anhydro-Neu5Ac occupies the same hydrophobic pocket in SpNanB as the cyclohexyl group and the 3-chlorobenzyl group of inhibitors (Figure 2). Therefore, we hypothesized that replacing the acetyl group in 2,7-anhydro-Neu5Ac (**1**) by a hydrophobic group such as cyclohexyl could result in a selective inhibitor against SpNanB.

Chemoenzymatic Synthesis and Derivatization of 2,7-Anhydro-Sialic Acids.

An efficient strategy to obtain *N*-substituted analogs of 2,7-anhydro-Neu5Ac (**1**) is to synthesize 2,7-anhydro-*N*-trifluoroacetylneuraminic acid (2,7-anhydro-Neu5TFA, **2**) as a precursor for further chemical modification, since the *N*-trifluoroacetyl (*N*-TFA) group was shown previously to be a good mimic of *N*-acetyl group in enzyme-catalyzed reactions and can be easily removed and replace by other groups.[45,46] Indeed, 2,7-anhydro-Neu5TFA (**2**) was successfully obtained from *N*-trifluoroacetylmannosamine (ManNTFA) as the starting material for the OPME system (Scheme 1). A lower yield (55%) obtained was most likely due to the lability of the *N*-trifluoroacetyl (*N*-TFA) group which could fall off during the reaction.[46] The trifluoroacetyl group of the produced 2,7-anhydro-Neu5TFA (**2**) was readily removed under mild basic condition to form 2,7-anhydro-neuraminic acid (2,7-anhydro-Neu, **3**) which was coupled with cyclohexanone by reductive amination to produce the desired 2,7-anhydro-*N*-cyclohexyl neuraminic acid (2,7-anhydro-Neu5Cyclohexyl, **4**) in almost quantitative yield (Scheme 2).

Unlike 2,7-anhydro-Neu5Ac (**1**) which can be hydrolyzed by SpNanB, 2,7-anhydro-Neu5Cyclohexyl (**4**) was tested and was shown to be resistant to SpNanB hydrolysis.

Sialidase Inhibition Studies of 2,7-Anhydro-Sialic Acids.

The designed inhibitor 2,7-anhydro-Neu5Cyclohexyl (**4**), along with other 2,7-anhydro-sialic acids (**1**−**3**), were tested for their inhibitory activities against several sialidases categorized in the glycoside hydrolase GH33 family in the Carbohydrate Active Enzyme (CAZy) database (www.cazy.org).[47–49] Neu5Acα2–3Galβ*p*NP was used as the substrate in a 384-well plate colorimetric assay.[50,51] Each reaction mixture contained an inhibitor, the substrate, a sialidase of interest, and a β-galactosidase in an amount sufficient to cleave all Galβ*p*NP generated from the sialidase-catalyzed reaction process to release *para*-nitrophenol (*p*NP), whose reading at $A_{405\ nm}$ in a buffer of pH higher than 9.5 is correlated to the activity of the sialidase. The bacterial sialidases tested include SpNanA,[52] SpNanB,[52] and SpNanC,[18] *Pasteurella multocida* sialyltransferase with α2−3-sialidase activity (PmST1),[41] *Bifidobacterium infantis* sialidase BiNanH2,[53] as well as commercially available sialidases from *Arthrobacter ureafaciens* (AuSialidase), *Clostridium perfringens* (CpNanI), and *Vibrio cholerae* (VcSialidase). Recombinant human cytosolic sialidase hNEU2[54] was also tested.

At a concentration of 1 mM, no significant inhibition against any sialidases tested was observed for 2,7-anhydro-Neu5Ac (**1**) or 2,7-anhydro-Neu (**3**) (Table S2). In comparison, noticeable inhibitory activity was observed for 2,7-anhydro-Neu5Cyclohexyl (**4**) against SpNanA, SpNanB, and SpNanC. 2,7-Anhydro-Neu5TFA (**2**), the intermediate designed for the synthesis of 2,7-anhydro-Neu5Cyclohexyl (**4**), also showed some inhibitory activity against SpNanA, SpNanB, AuSialidase, and VcSialidase.

IC_{50} values were obtained for compounds which showed more than 50% inhibitory activity at 1 mM. As shown in Table 1, 2,7-anhydro-Neu5Cyclohexyl (**4**) was a micromolar inhibitor against SpNanB (IC_{50} = 180 ± 23 μM) and SpNanC (IC_{50} = 58.4 ± 2.4 μM). 2,7-Anhydro-Neu5TFA (**2**) was a high micromolar inhibitor against SpNanA (IC_{50} = 145 ± 16 μM) and AuSialidase (IC_{50} = 225 ± 34 μM).

The inhibitor 2,7-anhydro-Neu5Cyclohexyl (**4**) that we designed showed a noticeable improvement in inhibiting SpNanB and SpNanC compared to the non-modified SpNanB product 2,7-anhydro-Neu5Ac (**1**). Another improvement was that 2,7-anhydro-Neu5Cyclohexyl (**4**), but not 2,7-anhydro-Neu5Ac (**1**), was resistant to SpNanB hydrolysis. Although the IC_{50} values were still in a high micromolar range, 2,7-anhydro-Neu5Cyclohexyl (**4**) showed selectivity for the inhibition of all three *Streptococcus pneumoniae* sialidases among all sialidases tested. Therefore, we have demonstrated here that 2,7-anhydro-sialic acids with the potential for further improvement, could be a new type of scaffold for designing potential selective inhibitors against certain sialidases.

3. CONCLUSIONS

In conclusion, a novel one-pot multienzyme (OPME) strategy was developed for gram-scale and preparative synthesis of 2,7-anhydro-Neu5Ac (**1**) and 2,7-anhydro-Neu5TFA (**2**). The latter was further used to synthesize 2,7-anhydro-Neu (**3**) and 2,7-anhydro-Neu5Cyclohexyl (**4**), a designed sialidase inhibitor which showed improved inhibitory activity for SpNanA and more significantly for SpNanB and SpNanC, but not other sialidases tested. Both 2,7-anhydro-Neu5TFA (**2**) and 2,7-anhydro-Neu5Cyclohexyl (**4**) were shown to be high micromolar inhibitors selectively against certain bacterial sialidases. This study demonstrated an effective synthetic strategy for 2,7-anhydro-sialic acids and a new idea of exploring the family of 2,7-anhydro-sialic acids as potential selective sialidase inhibitors.

4. EXPERIMENTAL SECTION

Materials.

Recombinant sialidases were expressed and purified as reported previously for human cytosolic sialidase hNEU2,[54] as well as bacterial sialidases from *Streptococcus pneumoniae* (SpNanA,[52] SpNanB,[52] and SpNanC[18]), *Pasteurella multocida* sialyltransferase 1 with α2–3-sialidase activity (PmST1),[41] and *Bifidobacterium infantis* sialidase BiNanH2.[53] Commercially available bacterial sialidases including those from *Arthrobacter ureafaciens* (Prozyme), *Clostridium perfringens* CpNanI (Sigma-Aldrich), and *Vibrio cholerae* were from Sigma-Aldrich. *Aspergillus oryzae* β-galactosidase was purchased from Sigma-Aldrich. *Pasteurella multocida* sialic acid aldolase (PmNanA),[39] *Neisseria meningitidis*

CMP-sialic acid synthetase (NmCSS),[40] and *Pasteurella multocida* sialyltransferase 1 M144D mutant (PmST1_M144D)[41,42] were expressed and purified as described previously. Siaα2–3Galβ*p*NP[44] used for substrate specificity studies were synthesized as described previously.

General methods.

Nuclear Magnetic Resonance (NMR) spectra were recorded in the NMR facility of the University of California, Davis on a Bruker Avance-400 NMR spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C). Chemical shifts are reported in parts per million (ppm) on the δ scale. High resolution electrospray ionization (ESI) mass spectra were obtained using a Thermo Electron LTQ-Orbitrap Hybrid MS at the Mass Spectrometry Facility at the University of California, Davis. Specific rotation was recorded on a Rudolph Research Analytical Autopol IV automatic polarimeter. Column chromatography was performed using Redi*Sep* Rf silica columns or an ODS-SM (C18) column (51 g, 50 μm, 120 Å, Yamazen) on the CombiFlash® Rf 200i system. Thin layer chromatography (TLC) 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). All reagents were at least of reagent grade and were used as supplied without further purification unless indicated.

One-pot multienzyme (OPME) synthesis of 2,7-anhydro-sialic acids.

ManNAc (1.0 g) or ManNTFA (300 mg), lactose (0.5 equiv.), sodium pyruvate (5 equiv.), CTP (1.5 equiv.) were dissolved in Tris-HCl buffer (100 mM, pH 7.5, 100 or 20 mL) containing 20 mM of $MgCl_2$. The pH of the solution was further adjusted to 7.5 with 4 M of NaOH. PmAldolase (8.0 or 4.0 mg), NmCSS (4.0 or 2.0 mg), PmST1_M144D (8.0 or 4.0 mg), SpNanB (4.0 or 2.0 mg) were added and the reaction was incubated in an isotherm incubator for 16 h at 37 °C with agitation at 100 rpm. The reaction was quenched by adding the same volume of ice-cold ethanol and incubating at 4 °C for 1 h. The formed precipitates were removed by centrifugation and the supernatant was concentrated. The residue was purified gradually by passing it through a BioGel P-2 gel filtration column, a silica column (EtOAc:MeOH:H_2O = 4:2:0.2, by volume), followed by a C18 column (100% H_2O) to produce the pure compound.

5-Acetamido-2,7-anhydro-3,5-dideoxy-α-D-glycero-D-galacto-non-2-ulopyranosonic acid (2,7-anhydro-Neu5Ac, 1).

1.10 g, 78%, white solid. $[\alpha]_D^{23} + 46.3^{o}$(*c* 0.5, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 4.51 (bs, 1H, H-6), 4.40 (dd, *J* = 7.8, 0.8 Hz, 1H, H-7), 3.95–3.90 (m, 1H, H-4), 3.89 (bs, 1H, H-5), 3.72 (dd, *J* = 11.8, 2.8 Hz, 1H, H-9), 3.56 (dd, *J* = 11.8, 6.0 Hz, 1H, H-9'), 3.50 (ddd, *J* = 7.8, 6.0, 2.8 Hz, 1H, H-8), 2.14 (dd, *J* = 15.2, 5.6 Hz, 1H, H-3ax), 2.00 (s, 3H, COCH_3), 2.00–1.92 (m, 1H, H-3eq); ^{13}C NMR (100 MHz, D_2O) δ 174.0, 173.5, 105.5, 77.1, 76.6, 71.9, 66.8, 62.2, 51.9, 35.2, 21.8; HRMS (ESI) Anal. Calcd for $C_{11}H_{16}NO_8$ $[M-H]^-$: 290.0881, Found: 290.0886.

2,7-Anhydro-3,5-dideoxy-5-trifluoroacetamido-α-D-glycero-D-galacto-non-2-ulopyranosonic acid (2,7-anhydro-Neu5TFA, 2).

219.8 mg, 55%, white solid. $[\alpha]_D^{23} + 44.7^o$(*c* 0.6, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 4.65 (bs, 1H, H-6), 4.48 (dd, *J* = 7.8, 0.8 Hz, 1H, H-7), 4.07–4.04 (m, 2H, H-4, H-5), 3.78 (dd, *J* = 11.6, 2.8 Hz, 1H, H-9), 3.62 (dd, *J* = 11.6, 6.2 Hz, 1H, H-9'), 3.57 (ddd, *J* = 7.8, 6.2, 2.8 Hz, 1H, H-8), 2.24 (dd, *J* = 15.2, 5.4 Hz, 1H, H-3ax), 2.05 (d, *J* = 15.2 Hz, 1H, H-3eq); ^{13}C NMR (100 MHz, D_2O) δ 173.9, 158.5 (q, *J* = 38.0 Hz), 115.7 (q, *J* = 284.0 Hz), 105.6, 76.8, 76.5, 71.9, 66.1, 62.2, 53.0, 35.4; HRMS (ESI) Anal. Calcd for $C_{11}H_{13}NO_8F_3$ $[M-H]^-$: 344.0599, Found: 344.0591.

Chemical derivatization at C-5 of 2,7-anhydro-sialic acids.

5-Amino-2,7-anhydro-3,5-dideoxy-α-D-glycero-D-galacto-non-2-ulopyranosonic acid (2,7-anhydro-Neu, 3).—2,7-Anhydro-Neu5TFA (30.0 mg, 0.0817 mmol) was dissolved in Na_2CO_3 aqueous solution (2 mL, pH = 9) and the reaction was stirred for 16 h. Without neutralization, the solution was directly passed through a BioGel P-2 gel filtration column to produce 2,7-anhydro-Neu (20.1 mg, 99%) as a white solid. $[\alpha]_D^{23} + 43.6^o$(*c* 0.25, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 4.60 (bs, 1H, H-6), 4.42 (dd, 1H, *J* = 7.8, 0.6 Hz, H-7), 4.09–4.02 (m, 1H, H-4), 3.77 (dd, *J* = 11.6, 2.8 Hz, 1H, H-9), 3.61 (dd, *J* = 11.6, 6.0 Hz, 1H, H-9'), 3.56 (ddd, *J* = 7.8, 6.0, 2.8 Hz, 1H, H-8), 3.16 (bs, 1H, H-5), 2.26 (dd, *J* = 15.4, 5.6 Hz, 1H, H-3ax), 2.04 (d, *J* = 15.4 Hz, 1H, H-3eq); ^{13}C NMR (100 MHz, D_2O) δ 173.9, 105.7, 77.6, 76.6, 71.9, 67.3, 62.3, 52.8, 34.8; HRMS (ESI) Anal. Calcd for $C_9H_{14}NO_7$ $[M-H]^-$: 248.0776, Found: 248.0771.

2,7-Anhydro-5-cyclohexylamido-3,5-dideoxy-α-D-glycero-D-galacto-non-2-ulopyranosonic acid (2,7-anhydro-Neu5Cyclohexyl, 4).

To a stirred solution of 2,7-anhydro-Neu (15.0 mg, 0.0602 mmol) in a mixed solvent of DMF/H_2O/AcOH (7:2:1, 2 mL), cyclohexanone (62 μL, 0.60 mmol) and $NaBH_3CN$ (38 mg, 0.60 mmol) were added and the reaction was stirred for 30 min. The solvent was concentrated in *vacuo*. The crude product was re-dissolved in water (1 mL) and was passed through a BioGel P-2 gel filtration column to produce 2,7-anhydro-Neu5Cyclohexyl (20.8 mg, 98%) as a white solid. $[\alpha]_D^{23} + 48.2^o$(*c* 1.1, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 4.77 (bs, 1H, H-6), 4.45 (d, *J* = 7.6 Hz, 1H, H-7), 4.23–4.16 (m, 1H, H-4), 3.77 (dd, *J* = 11.8, 2.8 Hz, 1H, H-9), 3.62 (dd, *J* = 11.8, 5.8 Hz, 1H, H-9'), 3.57 (ddd, *J* = 7.6, 5.8, 2.8 Hz, 1H, H-8), 3.36 (bs, 1H, H-5), 3.21 (bs, 1H, H-1' hexyl), 2.29 (dd, *J* = 15.4, 5.8 Hz, 1H, H-3ax), 2.17–1.98 (m, 3H, H-3eq, H hexyl), 1.93–1.77 (m, 2H, H hexyl), 1.75–1.61 (m, 1H, H hexyl), 1.45–1.24 (m, 4H, H hexyl), 1.25–1.09 (m, 1H, H hexyl); ^{13}C NMR (100 MHz, D_2O) δ 173.5, 105.6, 76.7, 75.3, 71.8, 64.4, 62.2, 56.0, 55.5, 35.3, 30.0, 29.7, 24.7, 24.19, 24.15; HRMS (ESI) Anal. Calcd for $C_{15}H_{24}NO_7$ $[M-H]^-$: 330.1558, Found: 330.1559.

SpNanB-catalyzed hydrolysis assays of 2,7-anhydro-Neu5Ac (1) and 2,7-anhydro-Neu5Cyclohexyl (4).

2,7-Anhydro-Neu5Ac (**1**) or 2,7-anhydro-Neu5Cyclohexyl (**4**) (10 mM final concentration) was treated with SpNanB (1 mg/mL) in NaOAc buffer (100 mM, pH = 6.0) or Tris-HCl

buffer (100 mM, pH = 7.0) for 24 h. Thin layer chromatography (TLC) and mass spectrometry showed that most 2,7-anhydro-Neu5Ac (**1**) was converted to Neu5Ac, whereas 2,7-anhydro-Neu5Cyclohexyl (**4**) remained intact.

Substrate specificity studies of SpNanB.

Substrate specificity assays were carried out in duplicates in 384-well plates in a final volume of 20 μL in NaOAc buffer (200 mM, pH 5.5) containing a sialoside selected from Siaα2–3Galβ*p*NP or Siaα2–6Galβ*p*NP (0.3 mM), β-galactosidase (12 μg), and SpNanB (0.4 μg). The reactions were incubated for 30 min at 37 °C, and were stopped by adding 40 μL of 0.5 M CAPS buffer (*N*-cyclohexyl-3-aminopropane sulfonic acid, pH 10.5) to each well. The amount of the *para*-nitrophenolate formed was determined by measuring the $A_{405\ nm}$ of the reaction mixtures using a microplate reader.

Inhibition assays.

Inhibition assays were carried out in duplicates in 384-well plates in a final volume of 20 μL containing Neu5Acα2–3Galβ*p*NP (0.3 mM) and β-galactosidase (12 μg) with or without inhibitors. The assay conditions varied for different sialidases as described below: SpNanA (0.0015 μg), NaOAc buffer (100 mM, pH 6.0); SpNanB (0.003 μg), NaOAc buffer (100 mM, pH 6.0); SpNanC (0.01 μg), MES buffer (100 mM, pH 6.5); AuSialidase (1.0 mU), NaOAc buffer (100 mM, pH 5.5); CpNanI (1.3 mU), MES buffer (100 mM, pH 5.0); VcSialidase (0.57 mU), NaCl (150 mM), $CaCl_2$ (10 mM), NaOAc buffer (100 mM, pH 5.5); PmST1 (0.4 μg), CMP (0.4 mM), NaOAc buffer (100 mM, pH 5.5); BiNanH2 (0.029 μg), NaOAc buffer (100 mM, pH 5.0); hNEU2 (1.2 μg), MES buffer (100 mM, pH 5.0). The reactions were incubated for 30 min at 37 °C, and were stopped by adding 40 μL of 0.5 M CAPS buffer (*N*-cyclohexyl-3-aminopropane sulfonic acid, pH 10.5) to each well. The amount of the *para*-nitrophenolate formed was determined by measuring the $A_{405\ nm}$ of the reaction mixtures using a microplate reader.

The percentage inhibition was determined using a concentration of 1 mM of each inhibitor. The reaction without any inhibitors was used as a control. IC_{50} values were obtained by varying the concentrations of inhibitors from 0 to 1000 μM to obtain concentration-response plots of the inhibitors. The values of IC_{50} were calculated by the software Grafit 5.0.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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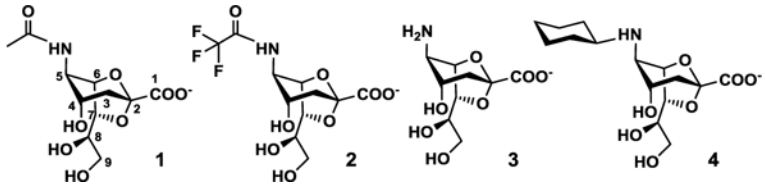


Fig. 1.

Structures of enzymatically synthesized 2,7-anhydro-Neu5Ac (**1**) and its chemoenzymatically synthesized derivatives **2**–**4**.

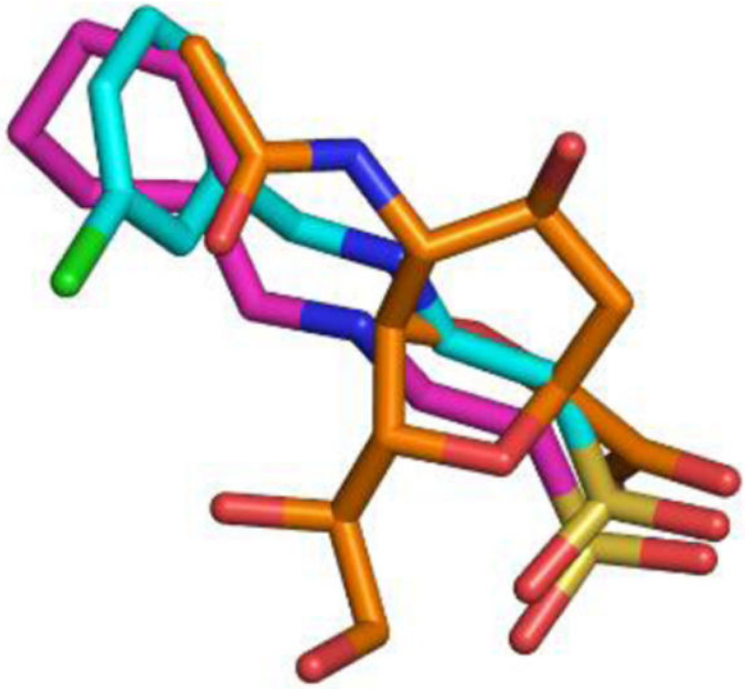
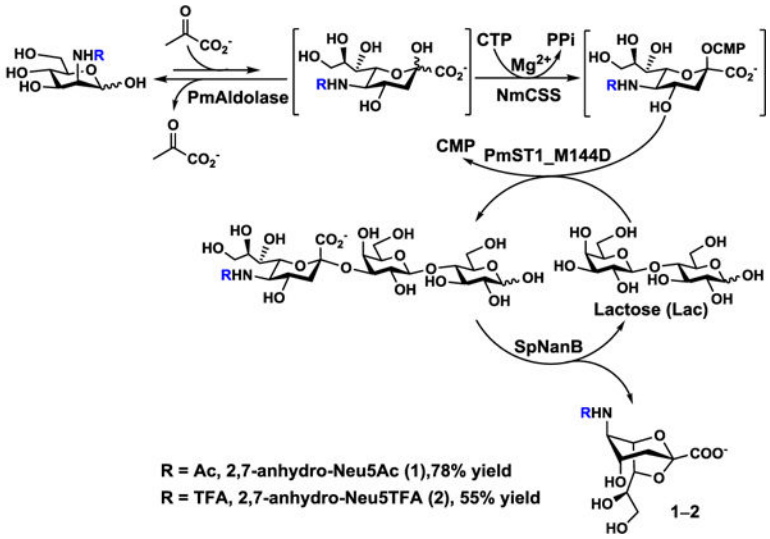


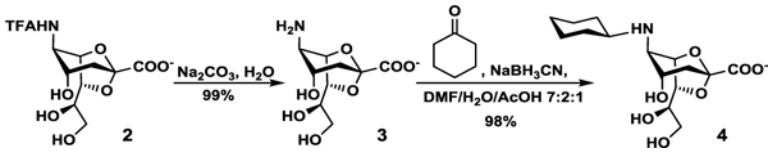
Fig. 2.

Superimposition of 2,7-anhydro-Neu5Ac (in brown sticks), CHES (in pink sticks), and 2-[(3-chlorobenzyl)ammonio)ethanesulfonate (in pale-blue sticks). The coordinates were extracted from Protein Data Bank (PDB IDs: 2WV1, 2VW2, and 4FPF).



Scheme 1.

One-pot multienzyme (OPME) synthesis of 2,7-anhydro-Neu5Ac (1) and 2,7-anhydro-Neu5TFA (2).



Scheme 2.

Synthesis of 2,7-anhydro-Neu5Cyclohexyl (4) from 2,7-anhydro-Neu5TFA (2).

Table 1.

IC_{50} Values of 2,7-anhydro-Neu5TFA (2) and 2,7-anhydro-Neu5Cyclohexyl (4) against bacterial sialidases.

Sialidases	IC_{50} values of different inhibitors (μM)	
	(2)	(4)
SpNanA	145 ± 16	500 − 1000
SpNanB	250 − 500	180 ± 23
SpNanC	> 1000	58.4 ± 2.4
AuSialidase	225 ± 34	> 1000
VcSialidase	500 − 1000	> 1000