Published in final edited form as:

Nat Protoc. 2006 ; 1(5): 2485–2492. doi:10.1038/nprot.2006.401.

One-pot three-enzyme chemoenzymatic approach to the synthesis of sialosides containing natural and non-natural functionalities

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Abstract

Chemoenzymatic synthesis, which combines the flexibility of chemical synthesis and the highly selectivity of enzymatic synthesis, is a powerful approach to obtain complex carbohydrates. It is a preferred method for synthesizing sialic acid-containing structures, including those with diverse naturally occurring and non-natural sialic acid forms, different sialyl linkages, and different glycans that link to the sialic acid. Starting from *N*-acetylmannosamine, mannose, or their chemically or enzymatically modified derivatives, sialic acid aldolase-catalyzed condensation reaction leads to the formation of sialic acids and their derivatives. These compounds are subsequently activated by a CMP-sialic acid synthetase and transferred to a wide range of suitable acceptors by a suitable sialyltransferase for the formation of sialosides containing natural and non-natural functionalities. The three-enzyme coupled synthesis of sialosides can be carried out in one pot without the isolation of intermediates. The time for synthesis is 4–18 h. Purification and characterization of the product can be completed in 2–3 d.

INTRODUCTION

Sialic acids are a family of negatively charged α -keto acids with a nine-carbon backbone. In vertebrates, they are mainly found as terminal carbohydrate units on glycoproteins and glycolipids. As the outermost residues, sialic acid residues are directly involved in many biologically important molecular recognition and interaction events and play pivotal roles in a variety of physiological and pathological processes¹.

Glycoconjugates in nature contain heterogeneous forms of glycans. This is especially true for those containing sialic acids. Currently, more than 50 structurally different sialic acid forms have been found in nature. These include three basic sialic acid forms: *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), deaminoneuraminic acid (KDN) (Figure 1), and their derivatives with 8-*O*-methylation, 8-*O*-sulfation, 9-*O*-lactylation, 9-*O*-phosphorylation, and single or multiple *O*-acetylation on the hydroxyl groups at C4, C5, C7, C8, and/or C9^{1,2}. Among them, more than 15 have been found in humans³. In mammalian systems, the naturally occurring structural modifications of three basic sialic acid forms are believed to take place after the formation of sialoglycoconjugates^{4,5}. Some of the sialic acid forms are not abundant in nature. Some of them, such as those containing *O*-acetyl group(s),

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

found in nearly all higher animals and certain bacteria, are labile to commonly used purification procedures^{6–8}. Therefore, it is extremely difficult to isolate sialic acid-containing glycans in homogenous form from natural sources⁹. The chemical synthesis of sialosides containing diverse modifications is also challenging^{10,11}.

Sialyltransferase-catalyzed glycosylation is believed to be the most efficient approach to obtain a variety of sialic acid-containing structures^{12–19}. The sugar nucleotide donor for sialyltransferases, CMP-sialic acid, can be obtained from sialic acid and CTP by CMP-sialic acid synthetase catalyzed reaction. Sialic acid itself can be synthesized from its six carbon precursor, such as *N*-acetylmannosamine (ManNAc) or mannose by sialic acid aldolase catalyzed aldol condensation reaction. Many bacterial enzymes that can be used in the enzymatic synthesis of sialic acid containing structures, including sialic acid aldolases, CMP-sialic acid synthetases, and sialyltransferases, can tolerate a variety of substrate modifications. Herein, we describe a protocol that utilizes a one-pot three-enzyme system for the synthesis of structurally defined naturally and non-naturally occurring sialosides using ‘flexible’ bacterial enzymes that can accommodate a variety of substrates^{3,20} (Figure 2). In this approach, structural modifications at C5 and C7-9 positions on the sialic acid residue of sialoside products can be introduced upstream, chemically or enzymatically, by modifying the C2 and C4-6 positions of their six carbon sugar precursors such as ManNAc or mannose.

ManNAc, mannose, and their derivatives are converted to sialic acids and their derivatives by a flexible sialic acid aldolase. The sialic acids and their derivatives are then activated by a CMP-sialic acid synthetase and transferred to galactose or *N*-acetylgalactosamine-terminated glycosides by sialyltransferases to form structurally defined sialosides. All of these enzyme-catalyzed conversions can be performed in one pot without the isolation of intermediates, thus simplifying the product purification process and avoiding the product loss that occurs during the multiple purification steps required by other approaches. This one-pot multiple-enzyme synthetic approach also uses inexpensive diverse starting materials to avoid the use of high cost commercially available Neu5Ac and CMP-Neu5Ac. Due to the flexible substrate specificity of the bacterial enzymes used in the process, many modifications on the substrates can be tolerated, making this one-pot three-enzyme chemoenzymatic system a powerful approach to obtaining diverse sialosides containing natural and non-natural functionalities. By choosing an appropriate sialyltransferase, either α 2,3- or α 2,6-linked sialosides can be obtained. Together with an *E. coli* K-12 sialic acid aldolase and an *N. meningitidis* CMP-sialic acid synthetase (NmCSS)²¹, a multifunctional *Pasteurella multocida* sialyltransferase²⁰ and a *Photobacterium damsela* α 2,6-sialyltransferase³ have been used in the aforementioned one-pot chemoenzymatic synthesis of α 2,3-linked and α 2,6-linked sialosides, respectively.

As shown in Figure 3, the one-pot three-enzyme approach discussed in this protocol is also suitable for transferring a sialic acid residue, either in its naturally occurring form or non-natural chemically or enzymatically modified form, to glycoconjugates containing a terminal galactose or *N*-acetylgalactosamine residue. Given the mild conditions in which the enzyme-catalyzed process is carried out, the present approach is extremely useful in attaining modifications of the carbohydrate structures of glycoproteins which would otherwise be unachievable by chemical methods. Our approach can also be used to modify directly the glycan structures on cell surfaces. In particular, this methodology allows the labeling of glycoconjugates on cells with isotopes, chromophores, or fluorophores^{22,23}, which are used in the study of the biological function of certain glycans or glycoconjugates.

In principle, any sialic acid aldolases, CMP-sialic acid synthetases, and sialyltransferases can be used in this approach. It is important to note that different enzymes may have different substrate specificities. Some are more flexible than others. It is necessary to carry out small-scale enzymatic assays before setting up preparative-scale reactions. It is also important to set

up a control reaction using natural substrates, such as ManNAc as Neu5Ac precursor and a suitable acceptor for the chosen sialyltransferase (a common one would be lactose).

In this protocol, the aforementioned *E. coli* K-12 sialic acid aldolase, *N. meningitidis* CMP-sialic acid synthetase (NmCSS)²¹, multifunctional *Pasteurella multocida* sialyltransferase²⁰, and *Photobacterium damsela* α 2,6-sialyltransferase are used as sialosides biosynthetic enzymes. Since no significant product inhibition effect²⁴ has been observed during the course of our chemoenzymatic synthesis of sialosides even when the acceptor concentration reaches to 20 mM, phosphatases for degrading CMP and pyrophosphate (PPi) are not included in the reaction mixture. The detailed procedures are described here for the preparation of α 2,3-linked sialoside (Neu5Ac α 2,3Lac β ProN₃) and α 2,6-linked sialoside (Neu5Ac α 2,6Lac β ProN₃) (Figure 4) using ManNAc as Neu5Ac (a predominant sialic acid form) precursor and 3-azidopropyl lactoside (Lac β ProN₃) as an acceptor. As long as the modified substrates can be tolerated by all the enzymes employed in the process, a similar approach can be applied to the synthesis of sialosides with other structural requirements utilizing other sialic acid precursors and acceptors that contain terminal Gal(GalNAc) residues.

MATERIALS

REAGENTS

- Acetic acid (Fisher, cat. no. MAX007375)
- *p*-Anisaldehyde (Sigma-Aldrich, cat. no. A0519-1L)
- Bio-Gel P-2 Gel, fine (Bio-Rad, cat. no. 150-4115)
- Cytidine 5'-triphosphate disodium salt (CTP; Sigma-Aldrich, C1506-1G) ▲
CRITICAL This reagent is not stable when store at room temperature for long duration. Always store at -20 °C immediately after use.
- 95% Ethanol (Fisher, cat. no. S93231)
- Ethyl acetate (Fisher, cat. no. E145-20)
- H₂SO₄ (EM science, cat. no. SX1244-75)
- Hydrochloric acid (Fisher, cat. no. 23749902)
- Methanol (Fisher, cat. no. A412-20)
- MgCl₂·6H₂O (EMD, cat. no. MX0045-2)
- NH₄OH (Fisher, cat. no. AC42330-5000)
- NH₄OAc (Fisher, cat. no. S93116)
- 1-Propanol (n-PrOH; Fisher, cat. no. AC23207-0010)
- Silica gel (Sorbent technologies, cat. no. 20930-15)
- Recombinant sialic acid aldolase from *E. coli* K-12 (enzyme specific activity: 1.1 U/mg protein)
- Recombinant CMP-sialic acid synthetase from *N. meningitidis* (NmCSS) (enzyme specific activity: 290 U/mg protein)
- Recombinant α 2,6-sialyltransferase from *Photobacterium damsela* (Pd2,6ST) (enzyme specific activity: 35 U/mg protein)
- Recombinant multifunctional sialyltransferase from *Pasteurella multocida* (PmST1) (enzyme specific activity: 60 U/mg protein)

- Sodium hydroxide (Fisher, cat. no. S71990)
- Sodium pyruvate (Fisher, cat. no. BP356-100)
- Thin-layer silica gel plates (Sorbent technologies, cat. no. 4115126)
- Tris base (Fisher, cat. no. BP154-1)

EQUIPMENT

- Buchi Rotary evaporator (Fisher, cat. no. 04-987-222)
- C25KC incubator shaker (Fisher, cat. no. 14-278-178)
- Fisher Isotemp economy analog-control water bath Model 105Q (Cat. no. 15-460-5)
- Freeze-dry systems (Fisher, cat. no. 10-271-16)
- Hotplate and Stirrer (Fisher, cat. no 11-497-7A, Corning no. 6795 420)
- Kontes flexcolumn economy column (Fisher, cat. no. K420401-2511)
- Model 2110 fraction collector (Bio-Rad, cat. no. 731-8120)
- Sorvall legend T/RT benchtop centrifuge (Fisher, cat. no. 75-004-377)
- Sterile 50 ml centrifuge tubes (Biologix Research Company, cat. no. 10-9501)

EQUIPMENT SETUP

Packing a gel filtration column with Bio-Gel P-2 Gel

In order to pack a column with about 400 ml hydrated bed volume, slowly add 140 g dry Bio-Gel P-2 gel (typical hydrated bed volume of dry gel is 3 ml/g) to 800 ml deionized water in a 2000 ml beaker and allow it to hydrate for 4 hours at room temperature. In order to remove the gas naturally dissolved in water and that introduced during the process, decant half of supernatant, transfer the gel suspension to a filter flask and attach to a water aspirator. Degas for 10 min with occasional swirling (do not use a stir bar, as it may damage the resin). Add 800 ml degassed water and swirl gel suspension gently. Allow gel to settle for 30 min, decant supernatant to remove fine particles (mainly gel powder and small gel particles). Pour the even slurry into the column (2.5 cm i.d $\times$ 100 cm length) in a single, smooth moment, being careful to avoid producing bubbles. When a 2 cm bed has formed, allow column to flow until the column is packed. Pass 1000 ml degassed water through the column using gravity.

REAGENT SETUP

Enzymes

Enzymes suitable for this protocol, including sialic acid aldolases, CMP-sialic acid synthetases, and sialyltransferases, can be recombinant proteins or purified enzymes obtained in individual laboratories or those from commercially available sources. The examples shown in the protocol use the recombinant enzymes that are cloned in our laboratory. Commercially, sialic acid aldolase (alternative names: *N*-acetylneuraminic acid aldolase, *N*-acetylneuraminate pyruvate lyase, *N*-acetylneuraminic acid lyase, NANA aldolase) from *E. coli* can be obtained from Sigma (Cat. no. A6680) or Fluka (Cat. no. 47153); recombinant α 2,6-(*N*)-sialyltransferase from rat is available from EMD CalBioChem and Fisher (EMD CalBioChem Cat. no. 566222, Fisher Cat. no. NC9364351); recombinant α 2,3-(*N*)-sialyltransferase from rat is available from EMD CalBioChem and Fisher (EMD Calbiochem Cat. no. 566218, Fisher Cat. no. NC9718611), recombinant α 2,3-(*O*)-sialyltransferase from rat is available from EMD CalBioChem (Cat. no. 566227). Several sialyltransferases are available from Japan Tobacco Inc. <http://www.jti.co.jp/biotech/glyco/products/index.html>, including recombinant α 2,6-

sialyltransferase from *Photobacterium damsela* JT0160, α 2,6-sialyltransferase purified from cells of *Photobacterium damsela*, α 2,3-sialyltransferase from *Photobacterium* sp. JT-ISH-224, and α 2,3-sialyltransferase from *Photobacterium phosphoreum* JT-ISH-467. **p-Anisaldehyde sugar stain** Add 25 ml *p*-anisaldehyde to 425 ml ice cold methanol. With vigorous stirring without splashing, cautiously add 50 ml concentrated H₂SO₄ drop wise during a 60-minute period to the methanol solution pre-chilled in an ice/water bath. Store the prepared light yellow staining solution at -20 °C before use.

! CAUTION All reagents are toxic. Handle in a fume hood with care. Wear gloves and safety goggles!

? TROUBLESHOOTING

PROCEDURE

Small scale enzymatic assays

1. Small scale enzymatic assays are commonly performed in a 0.5 ml microcentrifuge tube before preparative scale synthesis. Add *E. coli* K-12 sialic acid aldolase (10 mU), *N. meningitidis* CMP-sialic acid synthetase (NmCSS) (10 mU), and *Photobacterium damsela* α 2,6-sialyltransferase Pd2,6ST (5 mU) (for synthesizing α 2,6-linked sialosides) or *Pasteurella multocida* sialyltransferase PmST1 (5 mU) (for synthesizing α 2,3-linked sialosides) to a Tris-HCl buffer (100 mM, pH = 8.8) containing MgCl₂ (20 mM), 3-azidopropyl lactoside (Lac β ProN₃) as the acceptor (5 mM), ManNAc as a precursor for sialic acid (7.5 mM), sodium pyruvate (40 mM), and CTP (7.5 mM). Add water to bring the total volume of the reaction mixture to 50 μ l. **▲ CRITICAL STEP** a Tris-HCl buffer of pH = 7.5 is used for substrates containing base sensitive functionalities, e.g. acetyl or lactyl groups.
2. Carry out three negative control assays in three different tubes at the same time as described in Step 1. Control I is the assay missing the sialic acid aldolase (this control can be considered as a standard reference mixture containing all starting materials); control II is the assay missing CMP-sialic acid synthetase; control III is the assay missing sialyltransferase.
3. Incubate the microcentrifuge tubes containing the reaction mixture or control reactions at 37°C in a water bath for 1 hour.
4. Monitor the reaction using Thin-layer chromatography (TLC) using two different developing solvents. **! CAUTION** Handle Steps 7–9 in a fume hood with care. Wear gloves and safety goggles! The *R_f* value (retention factor) is calculated as follows: $R_f = (\text{distance from the middle of spot of a compound to the starting line}) / (\text{distance of the solvent front to the starting line})$.
5. Prepare two 10 cm $\times$ 4.2 cm TLC plates. Lightly draw a line that is about 1 cm from the bottom edge using a lead pencil across the short dimension of the plates. Using a centimeter ruler, lightly mark off 0.5 cm intervals on the line starting from 0.6 cm away from the edge.
6. For both plates, on the line drawn on plate, starting from left to right, spot ManNAc, Lac β ProN₃, sample, and controls. Allow the spots to dry (This step can be sped up by using a hair dryer or a hot plate).
7. Develop two TLC plates separately using as developing solvents EtOAc:MeOH:H₂O:HOAc = 4:2:1:0.1 (by volume) and 95% EtOH:1 M NH₄OAc = 14:5 (by volume), respectively, in wide-mouth screw-cap jars. Remove the plates

when the solvent front is about 1 cm from the top of the plate. Mark the position of the solvent front.

8. Allow the plates to dry in a fume hood.
9. Use a pair of forceps to grip one of the top corners of one plate. Carefully but quickly immerse the whole plate to *p*-anisaldehyde sugar staining solution in a wide-mouth screw-cap jar. Immediately take out the plate. Wipe off the extra solvent in the back of the plate. Place the plate on a hotplate with heat on at about 100 °C for 15–60 sec. until the spots appear. Do the same for the other plate. In the first developing solvent, EtOAc:MeOH:H₂O:HOAc = 4:2:1:0.1 (by volume), Neu5Ac, CMP-Neu5Ac, and CTP stay at the original spots having an $R_f \sim 0$. One can observe on the TLC plate developed with this solvent that, in comparison to control I, where no enzymatic reaction occurs, the spot of ManNAc is weakened significantly in control II and control III, and a new greenish black spot of sialoside product can be seen in the sample (For Neu5Ac α 2,3Lac β ProN₃: R_f = 0.35; for Neu5Ac α 2,6Lac β ProN₃, R_f = 0.24). The formation of Neu5Ac (R_f = 0.39) in control II and CMP-Neu5Ac (R_f = 0.18) in control III can be observed in the TLC plate developed using the second developing solvent: 95% EtOH:1 M NH₄OAc = 14:5 (by volume).

Preparative scale synthesis of sialosides

10. Take out the following compounds from a –20 °C freezer: 3-azidopropyl lactoside (Lac β ProN₃) as the sialyltransferase acceptor (other substrates containing terminal Gal or GalNAc residues can also be used in this protocol as acceptors), ManNAc (other compounds, including mannose and ManNAc/mannose derivatives can also be used in this protocol) as the sialic acid precursor, sodium pyruvate, and CTP. Allow them to warm to room temperature for 30 min. **CRITICAL STEP** Avoid keeping these compounds at room temperature for longer than 2 hours. Store at –20 °C after use.
11. Weigh out 50 mg (0.117 mmol) Lac β ProN₃, 39 mg (0.176 mmol, 1.5 equiv.) ManNAc, 65 mg (0.588 mmol, 5 equiv.) sodium pyruvate, 99 mg (0.176 mmol, 1.5 equiv.) CTP, and 41 mg (0.20 mmol, 20 mM in final concentration) MgCl₂·6H₂O into a 50 ml centrifuge tube.
12. Add 5 ml deionized H₂O to the tube. Swirl the tube to allow compounds to dissolve completely.
13. Add 1 ml Tris-HCl buffer stock solution (1 M, pH 8.8) to the tube. Swirl the tube to mix. **CRITICAL STEP** For synthesizing sialosides containing acetyl or lactyl group, the pH of the reaction solution should be adjusted to 7.5 using Tris-HCl buffer stock solution (1 M, pH 7.5) to avoid the cleavage of the ester group.
15. Add *E. coli* K-12 sialic acid aldolase (6.0 U), *N. meningitidis* CMP-sialic acid synthetase (NmCSS) (6.0 U), and Pd2,6ST (3.0 U) (for synthesizing α 2,6-linked sialoside Neu5Ac α 2,6Lac β ProN₃) or PmST1 (3.0 U) (for synthesizing α 2,3-linked sialoside Neu5Ac α 2,3Lac β ProN₃).

▲ **CRITICAL STEP** Any sialic acid aldolases, CMP-sialic acid synthetases, and sialyltransferases can be used in this approach. An α 2,6-sialyltransferase (e.g. Pd2,6ST) is used for the synthesis of α 2,6-linked sialosides. An α 2,3-sialyltransferase (e.g. PmST1) is used for the synthesis of α 2,3-linked sialosides. The amount of an enzyme that is suitable for a particular reaction is calculated based on the specific activity of individual enzymes and the amount of the acceptor. One unit is the amount of enzyme that is required to produce 1.0 μ mole of product per min at 37 °C. Usually an excess amount of enzyme (1.5–3 fold of the amount that is calculated for converting

the limiting substrate to the product in 1 hour) is used to make sure that the reaction goes smoothly and to accommodate structural modification on the substrates. For example, in order to convert 50 mg (0.117 mmol) Lac β ProN₃ to product in 1 hour, the theoretical amount of sialyltransferase that is required is 0.117 mmol/60 min = 1.95 μ mol/min = 1.95 U. An excess amount (3 U, about 1.5 fold of the theoretical amount) is used here to make sure that high yield production can be achieved in 2 hours.

15. Add deionized H₂O to bring the total volume of the reaction mixture to 10 ml.
16. Incubate the tube containing reaction mixture in a C25KC incubator shaker at 37 °C with agitation at 140 rpm. The reaction can be carried out at room temperature overnight (e.g. 15 h) if incubator shaker is not available.
17. Using the sample from small-scale enzymatic assay as a standard solution and EtOAc:MeOH:H₂O:HOAc = 4:2:1:0.1 (by volume) as developing solvent, monitor the reaction using thin-layer chromatographic analysis after 1 hour and every 30 min. after that. Reactions usually can be completed in 2 hours.

▲ **W CRITICAL STEP** In some cases the acceptor and the corresponding sialoside product cannot be separated well in the developing solvent mentioned above. If this occurs, adjust the ratio of individual solvents in the developing solvent. Another commonly used developing solvent for separating products from sugar starting materials (acceptors and donor precursors) is a mixture of *n*-PrOH:H₂O:NH₄OH = 7:2:1 (by volume).

? TROUBLESHOOTING

18. When no further formation of the production is observed, stop the reaction by adding 10 ml pre-chilled (at 4 °C) 95% ethanol. Mix gently by inverting the tube. Incubate the mixture at 4 °C for 30 min.
19. Centrifuge for 30 min. at 5,000 $\times$ *g* in a Sorvall legend T/RT benchtop centrifuge. Transfer the clear supernatant to a 100 ml round bottom flask.
20. Wash the protein precipitates with 5 ml deionized water. Centrifuge for 30 min. at 5,000 $\times$ *g* in a Sorvall legend T/RT benchtop centrifuge. Transfer the clear supernatant in this wash step to the 100 ml round bottom flask containing the clear supernatant of the reaction mixture.
21. Concentrate the combined supernatant using a rotary evaporator at 30 °C under vacuum.

▪ **PAUSE POINT** The round bottom flask can be sealed by Parafilm and left at 4 °C overnight.

Purification and characterization of products

22. Open the outlet of a Bio-Gel P-2 gel filtration column (2.5 $\times$ 80 cm) to allow the water flow down to the top level of the gel bed.
23. Dissolve the residue from step 21 in 0.5 ml deionized water. Carefully load this solution to the gel filtration column and allow the sample to flow down to the level of the gel bed.
24. Wash the 100 ml round bottom flask with 0.5 ml water and carefully load the resulting solution to the column. Repeat this washing process for one more time.
25. Add 5 ml water to the column. Cap the column. Allow deionized water (about 600 mL) flow via gravity from a 4 L flask into the column. Collect flow through in 6 ml fractions using a fraction collector. **PAUSE POINT** This procedure can be set up at

the end of the day so that it is performed overnight. **CRITICAL POINT** Make sure that the fraction collector works properly.

26. Quick TLC screening to identify fractions that contain carbohydrates: Use a lead pencil to draw light parallel lines at 1 cm intervals across one dimension of an 8 × 5 cm TLC plate. Draw light parallel lines at 1 cm intervals across the other dimension (perpendicular to the first dimension). In the end, 40 squares will be obtained.
27. Starting from the 20th tube collected, spot sequentially the samples from every two tubes on the areas drawn on the TLC plate.
28. Allow spots to dry (this step can be sped up by using a hair dryer or a hot plate).
29. Use a pair of forceps to grip one corner of the plate. Carefully but quickly immerse the whole plate to *p*-anisaldehyde sugar staining solution in a wide-mouth screw-cap jar. Immediately take out the plate. Wipe off the extra solvent in the back of the plate. Place the plate on a hotplate with heat on at about 100 °C for 15–60 sec. until the spots appear.
30. Identify the fractions that contain carbohydrates (carbohydrates will appear as colored spots on the TLC plate).
31. Using EtOAc:MeOH:H₂O:HOAc = 4:2:1:0.1 (by volume) as developing solvent, perform TLC analysis as in Step 17 for all the fractions that contain carbohydrates. Assume that chromatography fractions preceded *and* followed by carbohydrate-containing fractions contain carbohydrates themselves.
32. Combine the fractions containing the sialoside product into a round bottom flask of an appropriate size (the volume of the solution should be less than half of the flask maximum volume). Concentrate the combined fractions in a rotary evaporator at 30 °C under vacuum.

? TROUBLESHOOTING

33. Dissolve the residue in 2 ml deionized water. Transfer the solution to a 20 ml vial.
34. Wash the round bottom flask with 1 ml deionized water and transfer the solution to the 20 ml vial containing the product. Repeat the washing step one more time.
35. Freeze dry the sample using a freeze dryer.
 - **PAUSE POINT** The sample thus dried can be stored at –20 °C safely for one year.
36. (This step and the ones that follow it are optional) Purify the sialoside further by flash chromatography. Carry this step only when necessary. The necessity for additional silica gel chromatography purification is determined by examining the mass spectrum and the ¹H NMR and ¹³C NMR spectra of the purified product. In most cases, sialoside products can be purified by simply passing Bio-Gel P-2 gel filtration column. Occasionally, the NMR spectra of the purified product show peaks from the same unknown impurities. In these cases, the products require additional silica gel chromatography purification.
37. Dissolve the impure sialoside (50–100 mg) in 2 ml deionized water in a 100 ml flask.
38. Add 2 g silica gel to the solution and evaporate the water using a rotary evaporator at 30 °C under high vacuum. Compounds including sialoside and impurities are absorbed by the silica gel.
39. Pack a chromatography column (3 cm i.d × 20 cm length with a 250 ml capacity) with 35 g silica gel.

40. Load the silica gel adsorbed with compounds from step 38 to the top of the gel bed in the silica gel column using a long-stem funnel. Cover the top with a layer of sand (1 cm thickness).
41. Elute the column in sequence with 250 ml ethyl acetate and 250 ml EtOAc:MeOH:H₂O = 4:2:0.5 (by volume).
42. Collect the flow through in 6 ml fractions (fractions of sialoside may contain tiny amount of silica gel because of the high polarity of the eluent used. Additional gel permeation can be used to remove these impurities). Analyze each fraction using TLC.
43. Combine the fractions containing the sialoside products into a round bottom flask of appropriate size (the volume of the solution should be less than half of the flask maximum volume). Concentrate the mixture in a rotary evaporator at 30 °C under vacuum.
44. Dissolve the substance in 2 ml deionized water. Transfer the solution to a 20 ml vial.
45. Wash the round bottom flask with 1 ml deionized water and transfer the solution to the 20 ml vial containing the product. Repeat the washing step one more time.
46. Free dry the sample using a freeze dryer.
47. Characterize the sialoside product by ¹H NMR, ¹³C NMR, and high resolution mass spectrometry (HRMS).

• TIMING

Timeline

Steps 1–3: 2 hrs

Steps 4: 30 min

Steps 5–9: 30 min

Steps 10–11: 2–15 hrs

Steps 12–15: 2 hrs

Steps 16–25: 8 hrs

Steps 26: 8 hrs

Steps 27: 3 hrs

? TROUBLESHOOTING

See Table 1 for troubleshooting advice.

ANTICIPATED RESULTS

The described protocol allows preparative-scale syntheses of diverse sialosides with yields ranging from 61% to 99%.

For example, the results for one-pot three-enzyme preparative synthesis of an α2,3-linked sialoside and an α2,6-linked sialosides using ManNAc as a sialic acid precursor and LacβProN₃ as an acceptor are shown in the following (Figure 5). The TLC plate is developed using developing solvent EtOAc:MeOH:H₂O:HOAc = 4:2:1:0.1 (by volume). The sialoside

products show greenish black after stained with *p*-anisaldehyde sugar stain. The R_f values of Neu5Ac α 2,3Lac β ProN₃ and Neu5Ac α 2,6Lac β ProN₃ are 0.35 and 0.24, respectively.

Analytical data

3-Azidopropyl O-(5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosylonic acid)-(2 $\rightarrow$ 6)-O- β -D-galactopyranosyl-(1 $\rightarrow$ 4)- β -D-glucopyranoside (Neu5Ac α 2,6Lac β ProN₃)—Yield, 98%; white foam. ¹H NMR (400 MHz, D₂O) δ 4.34 (d, 1H, J = 8.0 Hz), 4.28 (d, 1H, J = 8.0 Hz), 3.88–3.78 (m, 4H), 3.75–3.58 (m, 7H), 3.73–3.36 (m, 9H), 3.33 (t, 2H, J = 6.4 Hz), 3.19 (t, 1H, J = 8.8 Hz), 2.56 (dd, 1H, J = 12.4 and 4.8 Hz, H-3_{eq}"), 1.88 (s, 3H), 1.77 (m, 2H), 1.58 (t, 1H, J = 12.4 Hz, H-3_{ax}"); ¹³C NMR (100 MHz, D₂O) δ 175.08, 173.64, 103.36, 102.16, 100.45, 79.76, 74.79, 74.70, 73.84, 72.89, 72.68, 72.52, 71.95, 70.95, 68.67, 68.51, 68.53, 67.49, 63.71, 62.80, 60.41, 51.95, 48.04, 40.26, 28.40, 22.24. HRMS (ESI) m/z calcd for C₂₆H₄₃N₄O₁₉Na₂ (M+Na) 761.2317, found 761.2339.

3-Azidopropyl O-(5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-2-nonulopyranosylonic acid)-(2 $\rightarrow$ 3)-O- β -D-galactopyranosyl-(1 $\rightarrow$ 4)- β -D-glucopyranoside (Neu5Ac α 2,3Lac β ProN₃)—Yield, 84%; white foam. ¹H NMR (400 MHz, D₂O) δ 4.35 (d, 1H, J = 8.0 Hz), 4.30 (d, 1H, J = 8.4 Hz), 3.93 (dd, 1H, J = 3.2 and 10.0 Hz), 3.85–3.77 (m, 3H), 3.73–3.37 (m, 16H), 3.28 (t, 2H, J = 6.8 Hz), 3.13 (t, 1H, J = 8.8 Hz), 2.57 (dd, 1H, J = 12.4 and 4.4 Hz, H-3_{eq}"), 1.85 (s, 3H), 1.73 (m, 2H), 1.62 (t, 1H, J = 12.4 Hz, H-3_{ax}"); ¹³C NMR (100 MHz, D₂O) δ 175.13, 174.02, 102.76, 102.26, 99.91, 78.33, 75.60, 75.29, 74.89, 74.47, 72.99, 72.92, 71.89, 69.49, 68.48, 68.20, 67.58, 67.48, 62.68, 61.15, 60.16, 51.80, 47.98, 39.75, 28.36, 22.15; HRMS (ESI) m/z calcd for C₂₆H₄₃N₄O₁₉Na₂ (M+Na) 761.2317, found 761.2317.

The azido group in the sialosides synthesized can be used conveniently for chemoselective conjugation via Staudinger ligation²⁵ or click chemistry^{26,27}. The azido group can also be reduced to amino group and conjugate to other molecules including proteins through bifunctional crosslinkers^{28,29}.

Acknowledgements

This work was supported by Mizutani Foundation for Glycoscience, NIH R01GM076360, and start-up funds from the Regents of the University of California.

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Figure 1.

Three basic forms of naturally occurring sialic acids: Neu5Ac, Neu5Gc, and KDN.

Figure 2.

Schematic presentation of the one-pot three-enzyme chemoenzymatic approach to the synthesis of sialosides containing natural and non-natural functionalities. In this system, structural modifications at C5, C7-C9 positions on the sialic acid residue of sialoside products can be introduced by chemically or enzymatically modifying the C2, C4-C6 positions in the six carbon sugar precursors (ManNAc or mannose) of sialic acids. These ManNAc or mannose analogs can then be directly converted to the corresponding naturally occurring and unnatural sialosides in one-pot using three enzymes. The first enzyme, sialic acid aldolase is responsible for the formation of sialic acids and their derivatives, these compounds are then activated by the second enzyme, a CMP-sialic acid synthetase, and transferred to galactose or *N*-acetylgalactosamine-terminated glycosides by the third enzyme, a sialyltransferase. Depending on the type of the sialyltransferase used, α 2,3- or α 2,6- linked sialosides can be synthesized conveniently.

Figure 3.

Applications of the one-pot three-enzyme system in modify glycoconjugates or cells containing a terminal galactose or *N*-acetylgalactosamine residue. Sialic acid residues on glycoconjugates or cell surface can be removed by sialidase catalyzed reaction, dialyzed, and then modified with a special form of sialic acid using the one-pot three-enzyme system described in this protocol.

Figure 4.
One-pot three-enzyme synthesis of α2,3- and α2,6-linked sialyltrisaccharides
Neu5Acα2,3LacβProN₃ and Neu5Acα2,6LacβProN₃.

Figure 5.

Thin layer chromatography (TLC) analysis of one-pot three-enzyme synthesis of sialosides using ManNAc as a sialic acid precursor and $\text{Lac}\beta\text{ProN}_3$ as an acceptor substrate. The enzymatic reactions were proceeded for 1 h. The developing solvent used was $\text{EtOAc}:\text{MeOH}:\text{H}_2\text{O}:\text{HOAc} = 4:2:1:0.1$ (by volume). The plate was stained with *p*-anisaldehyde sugar stain. Lanes: 1, ManNAc; 2, $\text{Lac}\beta\text{ProN}_3$; 3, reaction mixture using the one-pot three-enzyme system containing PmST1; 4, reaction mixture using the one-pot three-enzyme system containing Pd2,6ST.

TABLE 1

Troubleshooting table.

PROBLEM	SOLUTION
<p><i>p</i>-Anisaldehyde sugar stain The color of the <i>p</i>-anisaldehyde sugar stain changes from light yellow to dark red.</p> <p>Step 11 The enzymatic reaction does not work or the yield is very low.</p>	<p>Direct light or heat will change the color of the <i>p</i>-anisaldehyde sugar stain and shorten its shelf-life. It is recommended that the stain be stored at -20°C in a wide mouth jar wrapped with aluminum foil. Put the stain back to the freezer after each use.</p> <ol style="list-style-type: none"> 1 Enzymes have no activity or the amount is not enough. Check the enzymes to see if they lost activity during the storage or add more enzymes to the reaction mixtures. 2 Check the pH value of the reaction mixture using pH papers. Adjust the pH value by carefully adding 1 M NaOH solution if necessary. ! CAUTION NaOH is a strong base, handle with care.
<p>Step 22 The sialoside product can not be separated from acceptor by gel filtration chromatography.</p>	<p>Use minimum volume of water (normally 0.5 ml) to dissolve the crude product when load sample to Bio-Gel P-2 column.</p>