Synthesis of Sialic Acids, Their Derivatives, and Analogs by Using a Whole-Cell Catalyst

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Abstract: Sialic acids (Sias) are important constituents of cell surface glycans. Ready access to Sias in large quantities would facilitate the development of carbohydrate-based vaccines and small-molecule drugs. We now present a facile method for synthesizing various natural forms and non-natural derivatives or analogs of Sias by using a whole-cell catalyst, which is constructed by adding a plasmid containing necessary enzyme genes into a metabolically engineered strain of *Escherichia coli.* The flexible substrate tolerance of incorporated enzymes (*N*-acetylglucosamine 2-epimerase and *N*-acetylneuraminic acid aldolase) allows the cellular catalyst to convert a wide range of simple and inexpensive sugars into various Sia-related compounds through an easily scalable fermentation process. Further, syntheses using this whole-cell biotransformation in combination with three conventional enzymatic reactions provide a series of complex Sia-containing glycans (sialyloligosaccharides) and their derivatives bearing different substituents. The processes described herein should permit the large-scale and economical production of both Sias and sialyloligosaccharides, and may complement existing chemical and enzymatic strategies.

Introduction

Sialic acids (Sias) represent a class of acidic monosaccharides with a nine-carbon skeleton that are typically found at the terminus of cell surface glycans. The structural and functional diversity of both Sias and Sia-containing glycans (sialyloligosaccharides) makes these carbohydrates important synthetic targets for biological research and drug development.[1,2] Chemical constructions of Sias require complex protection and coupling strategies for the control of both the stereochemistry and regiochemistry of bond formation.[3–6] Syntheses using enzymes involved in natural Sia metabolism are straightforward[7–16] and have been well developed to meet the needs of mass production, as pioneered by the works of Marukin Shoyu Co., Ltd. and Kyowa Hakko Bio Co., Ltd.[17,18] However, the preparation of enzymes and necessary cofactors requires tedious processes, which can make the enzymatic approach expensive and limit its application. Another option is the use of whole-cell catalysis that encapsulates exogenous enzymatic reactions into genetically engineered microorganisms.[19–23] This method allows the in situ generated enzymes, cofactors, and intermediates to be directly employed without purification and, therefore, is particularly attractive for large-scale syntheses.

We previously developed a recombinant bacterium (*Escherichia coli* Δ*nanTEK*/pNA) to convert *N*-acetylglucosamine (GlcNAc) into *N*-acetylneuraminic acid (Neu5Ac), the most abundant Sia found in nature.[24] The desired synthetic pathway in this living-cell catalyst (Scheme 1) was constructed by incorporating a plasmid containing GlcNAc 2-epimerase (AGE) and Neu5Ac aldolase (NanA) genes into a mutant form of *E. coli* K-12 (BW25113), in which the endogenous *nanTEK* gene cluster that is associated with Neu5Ac uptake and the competing *N*-acetylmannosamine (ManNAc) catabolic pathway was deleted. Cultivation of the engineered bacteria in the presence

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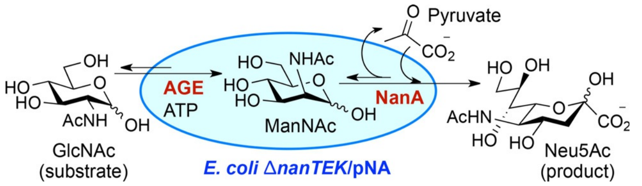


Scheme 1. Synthesis of Neu5Ac by using *E. coli* Δ*nanTEK*/pNA whole-cell catalyst. AGE: GlcNAc 2-epimerase (EC 5.1.3.8), gene from *Anabaena* PCC 7120; ATP: adenosine 5′-triphosphate; NanA: Neu5Ac aldolase (EC 4.1.3.3), gene from *E. coli* K-12; *nanTEK*: a gene cluster encoding NanT (Sia transporter), NanK (ManNAc kinase), and NanE (ManNAc-6-phosphate epimerase); pNA: a plasmid carrying NanA and AGE genes.

of substrates (GlcNAc and pyruvate) at high concentrations drove the reversible reactions toward the product, and multigram quantities of Neu5Ac were obtained in the extracellular fraction with high efficiency (74.2 g L^{-1}, 40 % yield from GlcNAc).

Herein, we report the use of *E. coli* Δ*nanTEK*/pNA to synthesize various natural Sias and non-natural Sia derivatives or analogs from simple starting materials (Figure 1). We also describe an efficient approach for the synthesis of sialyloligosaccharides by using this engineered bacterium in combination with three enzymatic catalysts, a CMP-Sia synthetase and two sialyltransferases. The present study is based on previous findings that many carbohydrate-processing enzymes, particularly those from microbial sources, have broad substrate scope and have been extensively used for the construction of diverse carbohydrate-related molecules.[25–27]

Results and Discussion

The substrate specificity of purified AGE and NanA was first investigated to ensure that the *E. coli* Δ*nanTEK*/pNA can convert structurally diverse aldoses. In nature, AGE catalyzes the 2-epimerization of GlcNAc to form ManNAc by using adenosine 5′-triphosphate (ATP) as a cofactor and NanA catalyzes the aldol addition reaction between ManNAc and pyruvate to generate Neu5Ac. Both reactions are reversible. The characteristics of substrates acceptable to both AGE[28] and NanA[7–9,15] have been well studied previously. Sixteen aldoses were examined here and the results are summarized in Table 1. In addition to its natural substrates GlcNAc (**1**) and ManNAc (**10**), AGE accepted a range of GlcNAc derivatives (**2**–**8**) with modifications at different positions of the sugar backbone, affording their corresponding epimerization products (**18**–**24**) in 9–35 % equilibrium yields. No product formation was confirmed for the AGE-catalyzed reactions of all the non-nitrogenous sugars tested (**11**–**16**), in agreement with the catalytic mechanism of AGE reported previously.[28,29] In contrast to AGE, NanA accepted ManNAc and the non-nitrogenous sugars, but not GlcNAc and its derivatives as substrates, consistent with previous studies.[7–9,15] The aldol addition reactions of **10**–**13** with pyruvate catalyzed by NanA gave isomerically pure Neu5Ac (**25**), deaminoneuraminic acid (KDN, **34**), and KDN analogs **35** and **36** in

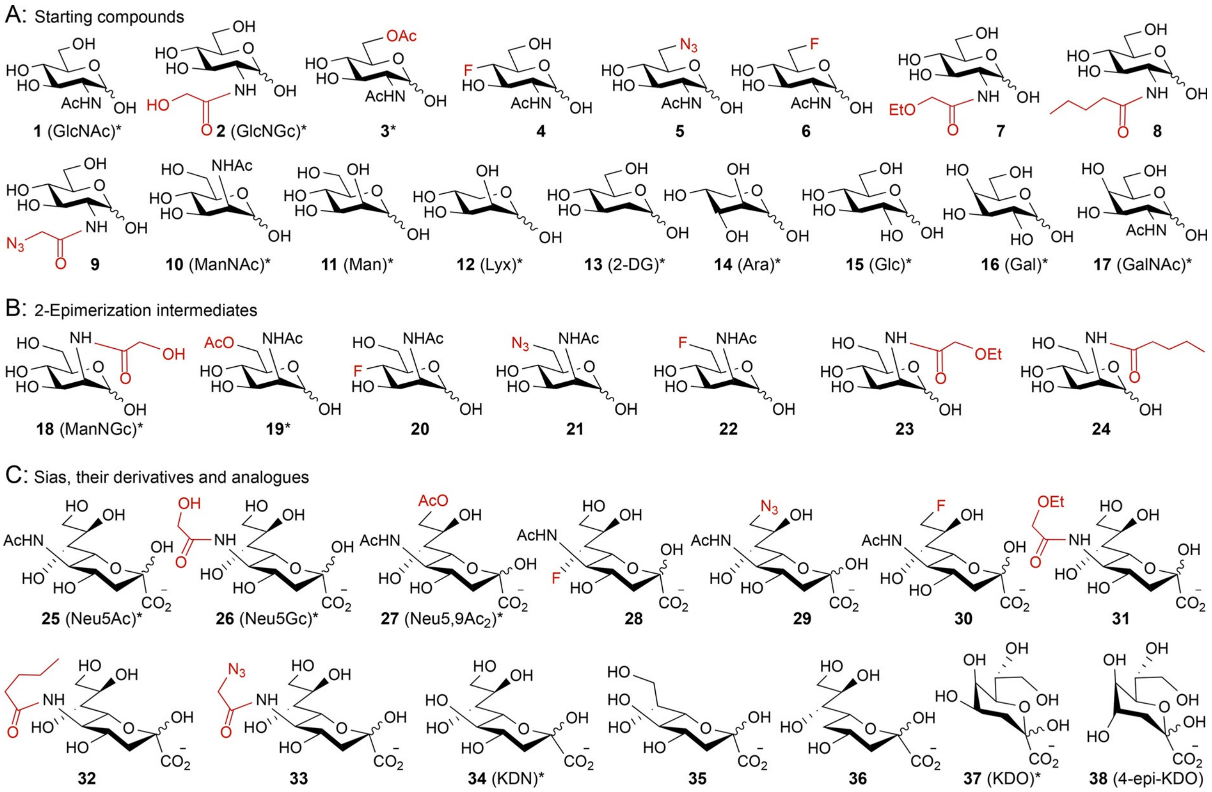


Figure 1. Structures of sugar substrates (A), epimerization (B), and Sia-related products (C). All of these carbohydrates are the D-enantiomers. Compounds marked with an asterisk are naturally occurring.

Table 1. Substrate specificity of AGE and NanA.[a]

Substrate	Product (yield [%])[b]		
	AGE	NanA	AGE + NanA
1	**10** (22)	×	**25** (37)
2	**18** (27)	×	**26** (36)
3	**19** (35)	×	**27** (22)
4	**20** (14)	×	**28** (18)
5	**21** (18)	×	**29** (25)
6	**22** (18)	×	**30** (42)
7	**23** (33)	×	**31** (40)
8	**24** (9)	×	**32** (13)
10	**1** (80)	**25** (83)	–
11	×	**34** (92)	–
12	×	**35** (80)	–
13	–	**36** (80)	–
14	×	**37** + **38** (90)[c]	–
15	×	mixture (63)[c]	–
16	×	mixture (61)[c]	–
17	×	×	–

[a] Reactions were carried out by incubating the substrate in Tris-HCl buffer (pH 8.0) in the presence of AGE (+ATP), NanA (+pyruvate), or both enzymes (+ATP and pyruvate). [b] Equilibrium yields were determined by HPLC. Chromatograms are provided in the Supporting Information. ×: no reaction; –: not tested. [c] An isomeric product mixture was determined by MS and NMR spectroscopy after HPLC isolation. Previous reports have confirmed that the product of the aldol addition reaction with Ara (**14**) is composed of 3-deoxy-D-*manno*-2-octulosonic acid (KDO, **37**) and its 4-epimer (**38**).[8,9]

Table 2. Synthesis of Sias and their derivatives or analogs catalyzed by *E. coli* Δ*nanTEK*/pNA.[a]

Substrate	Product	Yield [%][b]
1	**25**	40
2	**26**	45
3	**27**	36
4	**28**	32
5	**29**	39
6	**30**	42
7	**31**	55
8	**32**	48
9	**33**	44
11	**34**	39
12	**35**	44
13	**36**	51
14	**37** + **38**	36

[a] Reactions were typically carried out by cultivating bacteria ($\mathrm{OD}_{600\,\mathrm{nm}} = 30$) in the presence of sugar substrate (0.2 mmol) and pyruvate (5 equiv) in Tris-HCl buffer (2 mL). [b] Isolated yields. Products were characterized by MS and NMR spectroscopy. Characterization data are provided in the Supporting Information.

high yields, respectively, whereas isomeric mixtures of products were observed in the reactions with starting aldoses **14**–**16**. An inversion of stereoselectivity during the nucleophilic attack of pyruvate at the anomeric center of the aldose may occur in Sia aldolase-catalyzed reactions.[8,9] Although the isomers in these mixtures were not identified in the present study, the above results demonstrate that both AGE and NanA have fairly flexible substrate tolerances. Additionally, neither AGE nor NanA accepted *N*-acetylgalactosamine (GalNAc, **17**) as a substrate.

A one-pot reaction combining the catalysts AGE and NanA was next examined for its ability to synthesize Sia-related products from GlcNAc (**1**) and its derivatives (**2**–**8**). All compounds **1**–**8** could be converted into the expected Neu5Ac (**25**) and its derivatives (**26**–**32**) through this one-pot approach in 13–42% equilibrium yields (Table 1, column AGE + NanA). As NanA alone did not accept **1**–**8** as substrates, the one-pot reaction must proceed via the intermediates **10** and **18**–**24**, which were generated in the AGE-catalyzed epimerization reactions and then coupled in situ to pyruvate in the NanA-catalyzed aldol addition reactions. Taken together with the result that NanA directly converted non-nitrogenous sugars **11**–**16**, this study of combined AGE-NanA catalysis provided a basis to investigate the *E. coli* Δ*nanTEK*/pNA for the synthesis of various Sias and their derivatives or analogs.

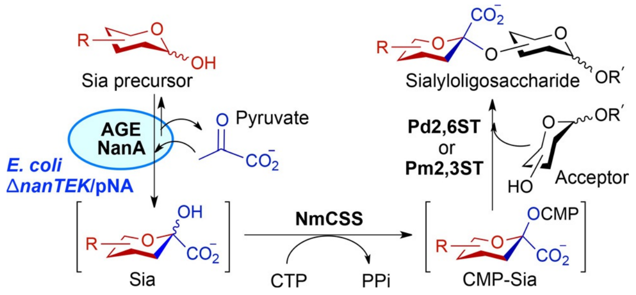
The scope of *E. coli* Δ*nanTEK*/pNA-catalyzed reactions was explored by using the exogenous addition of substrates (sugars and pyruvate) with the endogenous supply of ATP. A high concentration of substrates was used to drive the reversible reactions toward product formation. This engineered bacterium transformed **1**–**9** and **11**–**14** into different natural Sia forms (**25**–**27**, **34**), their derivatives (**28**–**33**), and analogs (**35**–**38**; Table 2). In the conversion of GlcNAc (**1**) and its derivatives (**2**–**8**), the yields from whole-cell catalysis (32–55%) were generally higher than those of the corresponding in vitro enzymatic reactions (13–42%, Table 1, column AGE + NanA). This increased catalytic efficiency could primarily be attributed to the knockout of the *nanT* gene in the bacteria,[24] which resulted in the abrogation of uptake of extracellularly transported products and thus ensured the progress of intracellular reactions towards the desired direction. The removal of the *nanE* and *nanK* genes in the bacteria could prevent the requisite epimerization intermediates from entering the bacteria-intrinsic ManNAc metabolic pathways. This also contributed to improvement in the efficiency of cell-based conversions of **1**–**8**. However, the whole-cell catalysis of non-nitrogenous aldoses **11**–**14** showed much lower yields (36–51%) than the corresponding enzymatic approaches (80–92%, Table 1, column NanA). In addition to the exogenously incorporated Sia-synthetic pathway, the endogenous metabolic machinery of *E. coli* also employs Man (**11**) or arabinose (Ara, **14**) as sources of carbon and energy.[30,31] It is very likely that their stereochemically relevant analogs lyxose (Lyx, **12**) and 2-deoxy-glucose (2-DG, **13**) were similarly consumed during normal cell metabolism, leading to decreased efficiencies of cell-based transformations of these compounds relative to isolated enzymatic reactions. Nevertheless, the conversion yields of all sugar substrates tested for the whole-cell catalysis were around 40% (for the space-time yields, see Table S1 in the Supporting Information). For example, $74.2\ \mathrm{g\,L^{-1}}$ of Neu5Ac (**25**) was afforded from an initial input of $132.7\ \mathrm{g\,L^{-1}}$ GlcNAc (**1**). These values represent the highest level reported to date for cell-based generation of Neu5Ac.[19–23] We therefore conclude that the *E. coli* Δ*nanTEK*/pNA is an efficient biocatalyst for the synthesis of various Sia-related compounds.

This whole-cell catalysis has particular advantages for large-scale preparation. The *E. coli* Δ*nanTEK*/pNA can be constructed quickly and economically, as its precursor *E. coli* K-12 is a routinely used biological engineering tool with many genetically modified variants readily available.[32] Further, a large number of *E. coli* variants have been used as platforms for industrial production of diverse molecules and their fermentation processes have been well developed.[33,34] Most of the whole-cell reactions in this study were carried out on milligram scales. However, for the synthesis of several natural Sias, Neu5Ac (**25**), *N*-glycolylneuraminic acid (Neu5Gc, **26**), 9-*O*-acetyl-Neu5Ac (Neu5,9Ac$_2$, **27**), and KDN (**34**), the reactions were scaled up to gram levels without apparent decreases in catalytic efficiencies. We also confirmed that the whole-cell catalyst could be reused at least five times without a significant loss of activity.

The Sia-related products listed in Table 2 may be very useful standards or probes for relevant biological research and drug development. Neu5Ac (**25**) is a common distal end moiety of mammalian glycoproteins and glycolipids that are involved in many physiological and pathological processes such as cellular recognition and tumorigenesis.[1] Neu5Gc (**26**) is distributed in non-human mammals but can be metabolically incorporated by human cells from dietary sources, which results in human susceptibility to bacterial toxins.[35,36] Neu5,9Ac$_2$ (**27**) is found in higher animals and serves as the cell-surface receptor of influenza C and D viruses.[37,38] KDN (**34**) occurs in bacteria or lower vertebrates and is associated with the binding of trout sperm to eggs.[39,40] The eight-carbon Sia analog KDO (**37**) is a component of lipopolysaccharides in Gram-negative bacteria and is essential to bacterial viability.[41] The non-natural Neu5Ac derivatives (**28**–**33**) with substitutions at different positions of the sugar skeleton can be used as probes in the study of sialidases, sialyltransferases, and Sia-binding proteins.[25] Complementary to existing chemical and enzymatic methods,[3–16] the whole-cell catalysis illustrated above provides an attractive route to readily access these valuable compounds.

The usefulness of *E. coli* Δ*nanTEK*/pNA was further explored in this study with the construction of sialyloligosaccharides (Scheme 2). Synthesis was initiated with the cell-catalyzed transformation of a Sia precursor into a Sia, which was then converted into its nucleotide-activated form by using CMP-Sia synthetase NmCSS,[42] followed by the transfer of Sia to a glycosyl acceptor by using sialyltransferase Pd2,6ST[43] or Pm2,3ST.[44] The whole-cell and enzymatic reactions were carried out consecutively in Tris-HCl buffer without isolating the intermediates. Combinations of eight Sia precursors with six glycosyl acceptors (**39**–**44**, Figure S1 in the Supporting Information) afforded 15 oligosaccharides (**45**–**59**, Table 3) with a sialyl α2,3- or α2,6-linkage in 66–98% isolated yield. Among these products, the Neu5Ac-, Neu5Gc-, Neu5,9Ac$_2$-, and KDN-containing structures (**45**–**53** and **59**) represent sequences found in natural glycoconjugates, and the oligosaccharides derivatives carrying an azide (**55**) or an alkynyl group (**47**–**50** and **52**–**59**) at a specific position on the sugar carbon backbone can be selectively manipulated downstream in a chemical synthesis through “click” ligation.[45]

At present, the large-scale synthesis of such complex sialyloligosaccharides primarily relies on enzymatic processes, which typically use ManNAc or its derivatives as starting materials, and Neu5Ac aldolase, CMP-Sia synthetase, and sialyltransferase as a catalyst combination.[43,44,46] ManNAc is commercially available, but its present price is even higher than that of Neu5Ac (Carbosynth Ltd., Berkshire, UK). This can make these enzymatic approaches expensive and limit their application. A GlcNAc 2-epimerase such as AGE could be included into the catalyst combination to confer the ability to synthesize sialyloligosaccharides from the inexpensive GlcNAc. Unfortunately, these enzymes catalyze the reversible conversion between ManNAc and GlcNAc, with a preference for GlcNAc formation.[47] The inefficiency of GlcNAc 2-epimerases to generate ManNAc, as also observed for AGE in this study (Table 1, column AGE), precludes their direct application to the enzymatic synthesis of sialyloligosaccharides. However, our approach that uses a combined catalysis of whole cell and conventional enzymes enabled various sialyloligosaccharides to be prepared in large amounts directly from GlcNAc and the readily available derivatives of GlcNAc.

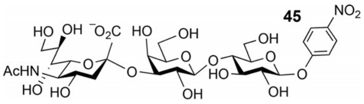
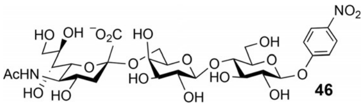
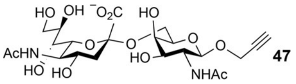
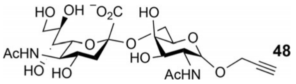
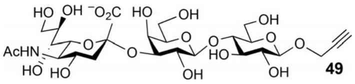
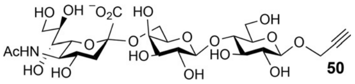
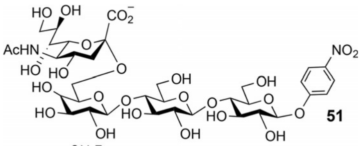
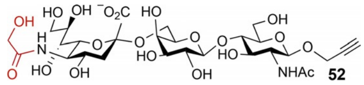
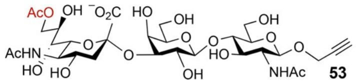
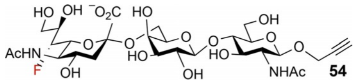
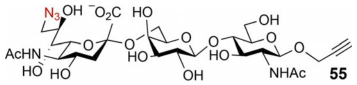
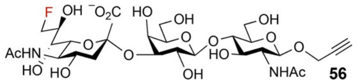
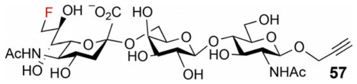
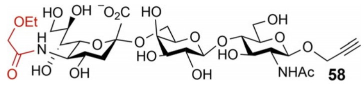
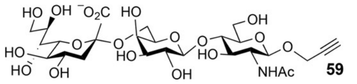


Scheme 2. Synthesis of sialyloligosaccharides by using *E. coli* Δ*nanTEK*/pNA, CMP-Sia synthetase (NmCSS), and sialyltransferase (Pd2,6ST or Pm2,3ST) as catalysts. The cell-catalyzed reactions gave Sia-containing mixtures in Tris-HCl buffer after removing the bacteria by centrifugation. Incubation of these mixtures in the presence of an acceptor, NmCSS, CTP, and Pd2,6ST (or Pm2,3ST) afforded sialyloligosaccharide products. CMP: cytidine 5′-monophosphate; CTP: cytidine 5′-triphosphate; PPi: inorganic pyrophosphate.

Conclusion

The *E. coli* Δ*nanTEK*/pNA represents a new biocatalyst for the facile synthesis of natural Sias as well as their non-natural derivatives or analogs. Coupled with a CMP-Sia synthetase and two sialyltransferases, this whole-cell catalyst also allows a range of sialyloligosaccharides to be prepared efficiently and economically. The catalytic processes described in this study are easy to scale up, and the large-scale syntheses of both Sias and sialyloligosaccharides should benefit their use in biological and pharmaceutical applications. Given the distinct advantages of microbial fermentation in the mass production of biomolecules, engineering of new bacteria-based catalysts that can directly synthesize sialyloligosaccharides and even sialylated glycoconjugates from simple materials represents a challenge for the future. Indeed, research efforts along these lines are ongoing.[48,49]

Table 3. Structure and yield of sialyloligosaccharide products.[a]

Precursor	Product	Yield [%][b]
1	 **45**	98
1	 **46**	98
1	 **47**	95
1	 **48**	95
1	 **49**	92
1	 **50**	95
1	 **51**	92
2	 **52**	89
3	 **53**	72
4	 **54**	92
5	 **55**	66
6	 **56**	90
6	 **57**	88
7	 **58**	82
11	 **59**	92

[a] Reactions were carried out generally on a preparative scale by using 40 μmol acceptors and 5 equivalents of Sia precursors, except for the syntheses of **49** and **50**, which were scaled up by 100-fold and gave these compounds in gram quantities. [b] Isolated yield based on acceptor.

Experimental Section

General information

All chemical regents and solvents, including compounds **1**, **2**, **8**, and **10–17**, were obtained from commercial source and used as purchased, unless otherwise noted. The GlcNAc derivatives **3**,[50] **4**,[51] **5**,[52] **6**,[53] and **9**[54] were synthesized as described previously, and **7** was synthesized as described in the Supporting Information. The glycosyl acceptors (Figure S1 in the Supporting Information), **39**,[55] **40**,[56] **41**,[57] **42**,[58] **43**,[55] and **44**,[59] were synthesized as described previously. All aqueous solutions were prepared by using ultrapure water (≥18 MΩ, Milli-Q, Millipore). TLC analysis was performed with Merck silica gel glass plates 60F$_{254}$. Sephadex G-10 used for gel-filtration chromatography was purchased from GE Healthcare (China). Silica gel (200–300 mesh) used for column chromatography was obtained from Qingdao Haiyang Chemical Co., Ltd. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were measured with a Bruker Advance spectrometer with the signal of deuterated solvents as the internal standard. ESIMS and HR-ESIMS spectra were recorded with a Bruker Apex IV FTMS or an Agilent 6520 QTOFMS spectrometer. HPLC was performed with an Agilent 1200 system coupled with a UV detector (detection at 210 nm). Optical rotations were measured with a PerkinElmer 241 polarimeter.

Bacteria and enzymes

The method used for the construction of *E. coli* Δ*nanTEK*/pNA has been detailed previously[24] and is briefly summarized in the Supporting Information. The procedures used for the preparation of recombinant enzymes (AGE,[24] NanA,[24] NmCSS,[42] Pd2,6ST,[43] Pm2,3ST[44]) have been detailed previously and are also briefly described in the Supporting Information.

AGE-catalyzed epimerization reaction

AGE (0.1 U) was added to a solution of a sugar substrate (**1**–**8**, **10**–**12**, or **14**–**17**, 0.4–0.6 mg, 2 μmol), ATP (0.1 mg, 0.2 μmol), and MgCl$_2$ (0.4 mM) in Tris-HCl buffer (50 mM, pH 8.0, 100 μL). The mixture was incubated at 37 °C for 6 h. The reaction was then quenched by heating at 100 °C for 5 min. After removal of precipitates from the mixture by centrifugation (6000 rpm, 30 min, 4 °C), the supernatant was analyzed by HPLC to determine the equilibrium yield of product (**1**, **10**, and **18**–**24**), which ranged from 9% to 80% as summarized in Table 1. Chromatographic conditions: Bio-Rad Aminex HPX-87H column (300×7.8 mm), aqueous sulfuric acid solution (6 mM) as eluent, flow rate at 0.3 or 0.35 mL min^{-1}, UV detection at 210 nm.

NanA-catalyzed aldol addition reaction

A solution of a sugar substrate (**1**–**8** or **10**–**17**, 0.4–0.6 mg, 2 μmol), sodium pyruvate (1.1 mg, 10 μmol, 5.0 equiv), and NanA (0.1 U) in Tris-HCl buffer (50 mM, pH 8.0, 100 μL) was incubated overnight at 37 °C. The reaction was quenched by adding an aqueous ethanol solution (95%, 100 μL) followed by incubation at 0 °C for 30 min. The precipitates were then removed from the mixture by centrifugation (6000 rpm, 30 min, 4 °C) and the supernatant was analyzed by HPLC to determine the equilibrium yield of product (**25**, **34**–**36**, and the isomeric product mixtures), which ranged from 61% to 92% as summarized in Table 1. The isomeric product mixtures were further isolated by HPLC for the NMR and MS analyses. The HPLC was performed by using the same conditions as described for the AGE-catalyzed epimerization reaction.

One-pot reaction catalyzed by the combination of AGE and NanA

AGE (0.1 U) was added to a solution of a sugar substrate (**1**–**8**, 0.4–0.6 mg, 2 μmol), ATP (0.1 mg, 0.2 μmol), and MgCl$_2$ (0.4 mM) in Tris-HCl buffer (50 mM, pH 8.0, 100 μL). The mixture was incubated at 37 °C for 6 h followed by addition of sodium pyruvate (1.1 mg, 10 μmol, 5.0 equiv) and NanA (0.1 U), and further incubation at 37 °C overnight, when TLC analysis (*i*PrOH/H$_2$O/HOAc, 14:6:3, v/v) indicated that the reaction was complete. The reaction was then quenched by addition of an aqueous ethanol solution (95%, 100 μL) and incubation at 0 °C for 30 min. The precipitates were removed from the mixture by centrifugation (6000 rpm, 30 min, 4 °C) and the supernatant was analyzed by HPLC to determine the equilibrium yield of product (**25**–**32**), which ranged from 13% to 42% as summarized in Table 1. The HPLC was performed by using the same conditions as described for the AGE-catalyzed epimerization reaction.

Synthesis of Sia-related compounds (25–38) catalyzed by *E. coli* Δ*nanTEK*/pNA

E. coli Δ*nanTEK*/pNA was grown in Luria-Bertani medium (pH 7.0) containing ampicillin (50 μg mL^{-1}) with shaking (250 rpm) at 37 °C overnight. The bacteria were then inoculated (1% inoculum) into an auto-induction ZYM medium[60] followed by incubation on a shaker (250 rpm) at 37 °C for 16 h to induce the expression of AGE and NanA. After collection by centrifugation (8000 rpm, 5 min, 4 °C) and washing with aqueous 0.85% NaCl solution, the induced bacteria were mixed with a sugar substrate (**1**–**9** or **11**–**14**), sodium pyruvate, and MgCl$_2$ (10 mM) in Tris-HCl buffer (100 mM, pH 8.0, bacterial concentration: $\mathrm{OD}_{600}\,n_{\mathrm{m}}=30$). The reaction mixture was incubated on a shaker (200 rpm) at 30 °C for 16 h. The bacteria were then removed from the mixture by centrifugation (6000 rpm, 30 min, 4 °C) and the supernatant was passed through a Sephadex G-10 column eluted with deionized water to afford compounds **25**–**38**, which were characterized by NMR and MS analyses. The details of reaction scale and product yield are provided in Table S1 (in the Supporting Information).

Synthesis of sialyloligosaccharides (45–59) catalyzed by *E. coli* Δ*nanTEK*/pNA, NmCSS, and Pd2,6ST (or Pm2,3ST)

A reaction mixture containing the induced *E. coli* Δ*nanTEK*/pNA ($\mathrm{OD}_{600}\,n_{\mathrm{m}}=30$), a Sia donor precursors (**1**–**7** or **11**, 30–60 mg, 0.2 mmol), sodium pyruvate (110 mg, 1.0 mmol, 5.0 equiv), and MgCl$_2$ (10 mM) in Tris-HCl buffer (100 mM, pH 8.0, 2 mL) was incubated on a shaker (200 rpm) at 30 °C for 16 h. The bacteria were then removed from the mixture by centrifugation (6000 rpm, 30 min, 4 °C) to give a supernatant, to which were added a glycosyl acceptor (**39**–**44**, 10–25 mg, 0.04 mmol), CTP (32 mg, 0.06 mmol), NmCSS (0.2 U), and Pd2,6ST (or Pm2,3ST, 0.2 U). The mixture was further incubated with shaking (140 rpm) at 25 °C for 2 h, when TLC analysis (EtOAc/MeOH/H$_2$O/HOAc, 8:4:2:1, v/v) indicated that the reaction was complete. The reaction was then quenched by addition of an aqueous ethanol solution (95%, 2 mL) and incubation at 4 °C for 30 min. The precipitates were removed from the mixture by centrifugation (6000 rpm, 30 min, 4 °C) and the supernatant was passed through a Sephadex G-10 column eluted with deionized water to yield compounds **45**–**59** (19–34 mg, 66–98%), which were characterized by NMR and MS analyses. For the synthesis of **46** and **47**, the reactions were also carried out on the gram scale. The details of reaction scale and product yield are provided in Table S2 (in the Supporting Information).

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Conflict of interest

The authors declare no conflict of interest.

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