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Accessibility of *N*-acyl-D-mannosamines to *N*-acetyl-D-neuraminic acid aldolase

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Abstract—*N*-Acetyl-D-neuraminic acid (NeuNAc) aldolase is an important enzyme for the metabolic engineering of cell-surface NeuNAc using chemically modified D-mannosamines. To explore the optimal substrates for this application, eight *N*-acyl derivatives of D-mannosamine were prepared, and their accessibility to NeuNAc aldolase was quantitatively investigated. The *N*-propionyl-, *N*-butanoyl-, *N*-*iso*-butanoyl-, *N*-pivaloyl-, and *N*-phenylacetyl-D-mannosamines proved to be as good substrates as, or even better than, the natural *N*-acetyl-D-mannosamine, while the *N*-trifluoropropionyl and benzoyl derivatives were poor. It was proposed that the electronic effects might have a significant influence on the enzymatic aldol condensation reaction of D-mannosamine derivatives, with electron-deficient acyl groups having a negative impact. The results suggest that *N*-propionyl-, *N*-butanoyl-, *N*-*iso*-butanoyl-, and *N*-phenylacetyl-D-mannosamines may be employed to bioengineer NeuNAc on cells.

Keywords: *N*-Acyl-D-mannosamine; *N*-Acetyl-D-mannosamine; *N*-Acetyl-D-neuraminic acid; Sialic acid; Aldolase

1. Introduction

As the terminal and thereby the most exposed sugar unit of a majority of oligosaccharides of natural glycoproteins and glycolipids on cell surfaces, *N*-acetyl-D-neuraminic acid (NeuNAc, **1**, also known as *N*-acetylsialic acid) plays a critical role in various biological events.[1–3] For example, the overexpression of NeuNAc is closely correlated with many phenotypes of tumors, so sialylated carbohydrate antigens on tumor cells are important molecular templates and targets for cancer vaccine design and other biological investigations.[4–10]

The biosynthesis of NeuNAc, and therefore sialoglycoconjugates, utilizes *N*-acetyl-D-mannosamine (ManNAc, **2**) as a key substrate.[11] It is well recognized that this biosynthetic pathway can tolerate unnatural substrates, especially ManNAc derivatives with modified *N*-acyl groups, and the phenomenon was extensively exploited by Reutter[12–14] and Bertozzi[15–17] and their co-workers in the bioengineering of NeuNAc on cell surfaces. Based upon this pioneering work, we recently developed a new strategy to overcome the problem of immunotolerance to tumor-associated carbohydrate antigens (TACAs) for cancer vaccine development.[18] The underlying principle is that after a cancer patient or an animal is immunized with a synthetic, artificial TACA bearing modified sialic acid(s) to establish a specific immune response, the patient or animal is treated with a correspondingly modified biosynthetic precursor of sialic acid to initiate the expression of the artificial TACA on tumor cells. The pre-stimulated immune system will then wipe out the marked tumors.

Our preliminary studies utilizing *N*-propionyl-D-mannosamine (ManNPr **3**) as the glycoengineering precursor and polysialic acid on cancer cells as the target antigen[18] gave encouraging results with regard to the glycoengineering and selective immunotargeting of cancer. However, the initial design was not necessarily the optimal one, because more distinctive modifications of NeuNAc may produce more effective vaccines and

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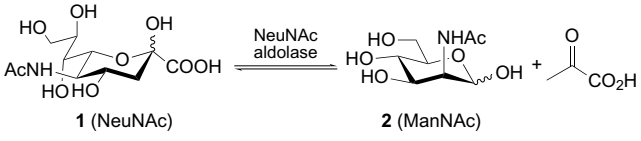
make the glycoengineered tumors more distinguishable. In this context, the caveat is that the modified D-mannosamine as precursor must be available to the biosynthetic pathways of sialic acid and sialoglycoconjugates.

Recently Bertozzi and her co-workers[19] identified that the bottleneck for the biosynthetic machinery of sialic acid to use *N*-acyl-D-mannosamines was its 6-*O*-phosphorylation, the first biosynthetic step, while the enzymes involved in the remaining steps were more permissive. The efficient incorporation of various *N*-acyl-D-mannosamines into cell-surface sialoglycoconjugates[12,19] suggests that the modified D-mannosamine precursors can somehow bypass the substrate-specific step. It was proposed that the conversion of unnatural D-mannosamines to sialic acids took advantage of Neu NAc aldolase,[19] an enzyme that usually catalyzes the degeneration of sialic acid (Scheme 1). However, since this reaction is completely reversible, NeuNAc aldolase is also involved in the biosynthesis of NeuNAc.[20] Moreover, it has been observed that NeuNAc aldolase can tolerate a variety of modifications of D-mannosamine. Thus, NeuNAc aldolase has been used to synthesize a variety of NeuNAc derivatives or analogs by an enzymatic method.[19–27]

To find out the optical substrates for the bioengineering of NeuNAc on tumor cells, and other cells as well, by utilizing the permissibility of NeuNAc aldolase, it is necessary to understand the reactions of various *N*-acyl-D-mannosamines with the enzyme. For this purpose, the accessibility of a number of *N*-acyl derivatives of D-mannosamine to NeuNAc aldolase was investigated.

2. Results and discussion

Eight *N*-acyl-D-mannosamines, including *N*-acetyl (ManNAc, **2**), *N*-propionyl (ManNPr, **3**), *N*-butanoyl (ManNBu, **4**), *N*-*iso*-butanoyl (ManN*i*-Bu, **5**), *N*-pivaloyl (ManNPiv, **6**), *N*-phenylacetyl (ManNPhAc, **7**), *N*-benzoyl (ManNBz, **8**), and *N*-trifluoropropionyl (ManNTFP, **9**) derivatives, were prepared from commercial D-mannosamine (**10**) by reaction with the corresponding acyl anhydrides or, for ManNTFP, by reaction with 1-hydroxybenzotriazole (HOBt) ester of trifloropropionic acid in methanol (Scheme 2). The expected products were obtained in 68–75% yield after silica gel column chromatography and subsequent recrystallization from mixtures of ethanol and ethyl acetate.

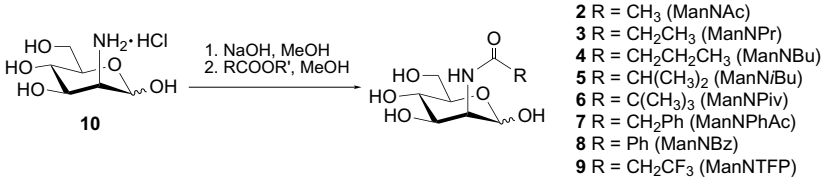


Scheme 1. NeuNAc aldolase-catalyzed reaction.

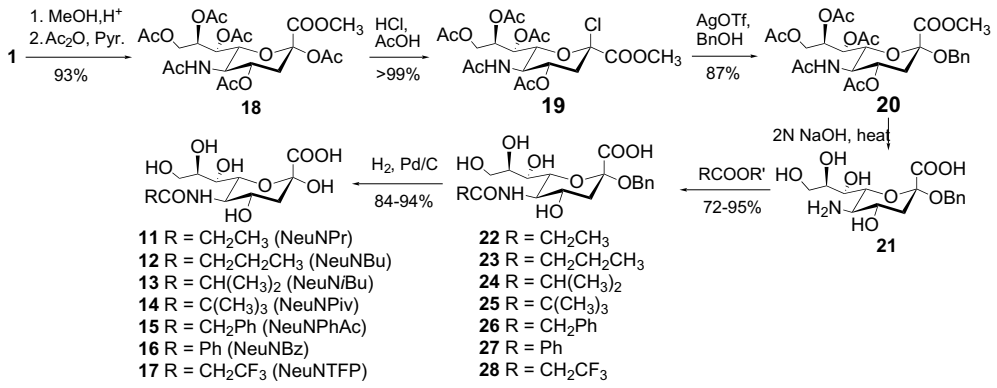
Standard samples of *N*-acylsialic acids (**11**–**17**) were prepared according to Scheme 3. Direct hydrolysis of NeuNAc under either acidic or basic conditions caused its decomposition, probably due to the involvement of its keto α-hydrogens in the open-chain form. Therefore, NeuNAc (**1**) was first converted into the benzyl glycoside **20**[28–30] that could be hydrolyzed under basic conditions smoothly to afford **21**. Selective acylation of the free amino group of **21** using various acyl anhydrides or the active ester of trifluoropropionic acid was followed by catalytic debenzylation to give *N*-acylsialic acids **11**–**18**. All synthetic intermediates and final products in Schemes 2 and 3 were positively identified by NMR spectroscopy and by mass spectrometry.

Commercial NeuNAc aldolase purchased from CALBIOCHEM® was utilized to investigate the enzymatic accessibility of *N*-acyl-D-mannosamines. The reactions were carried out at 37 °C in a buffer (pH 7.4, I = 0.1) containing 0.05 M potassium phosphate and 7.5% (v/v) dithiothreitol.[23,24] Pyruvic acid was used in large excess (10 equiv), and its concentration could be considered as a constant during the treatment of reaction kinetics. The sialic acid products of the reactions were assayed with the modified Svennerholm (resorcinol) method.[31]

The UV–vis absorption spectra of the Svennerholm reaction products of *N*-acylsialic acids **1** and **11**–**17** were analyzed first. As shown in Figure 1, although they had nearly identical λ_{max} (587 nm), their molar extinction coefficients (ε) were different. Since the structure of Svennerholm product is not well defined,[31] how the acyl groups affected the ε was not clear. Nevertheless, it seemed that bulky (Piv) and electron deficient (Bz, PhAc, and TFP) acyl groups resulted in a decrease in the ε values of the Svennerholm reaction products. Moreover, the product of NeuNBz showed a much stronger absorption band at ca. 425 nm than all other derivatives. Due to the differences of the UV–vis spectra, the indi-



Scheme 2. Preparation of *N*-acyl-D-mannosamines (**2**–**9**).



Scheme 3. Preparation of *N*-acyl-D-neuraminic acids (**11**–**17**).

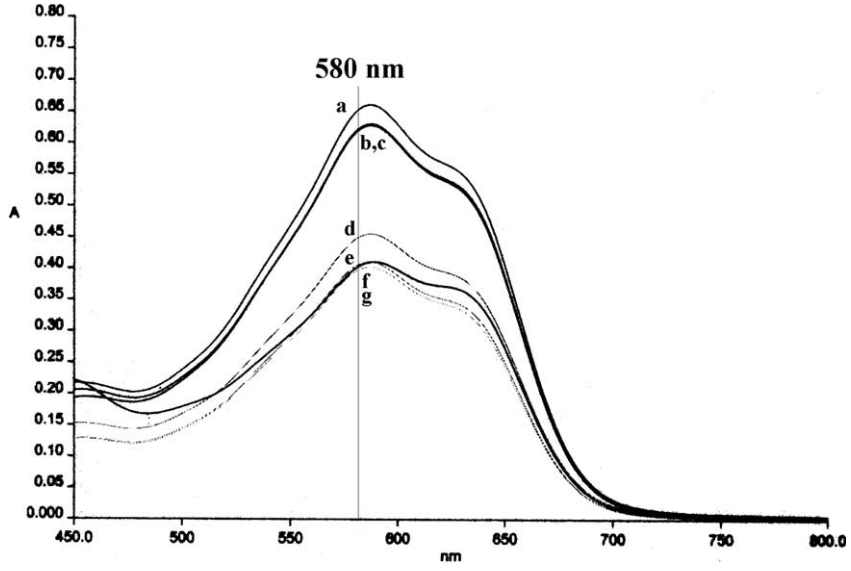


Figure 1. UV–vis spectra of the Svennerholm reaction products of: (a) NeuNAc, (b) NeuNPr, (c) NeuNBu and NeuN*i*-Bu, (d) NeuNPhAc, (e) NeuNTFP, (f) NeuNPiv, and (g) NeuNBz.

vidual calibration curves of **1** and **11**–**17** with regard to their absorbance at 580 nm were obtained using sialic acid concentrations ranging from 0 to 0.2 mM, all of which gave excellent linear correlations. It was also proved that *N*-acyl-D-mannosamines, pyruvic acid or their mixture had no influence on the sialic acid analysis under the Svennerholm conditions. Therefore, the method could be safely used to assay the enzymatic reactions.

Next, we established the suitable concentrations of *N*-acyl-D-mannosamine (5–50 mM) and aldolase (0.9 unit/mL) for the convenient and accurate analysis of sialic acid products by the Svennerholm method. The transformation of *N*-acyl-D-mannosamines to *N*-acylsialic acids was thus studied with 18.2 mM of *N*-acyl-D-mannosamine, 0.9 unit/mL of aldolase and 182 mM of pyruvic acid.

For the reaction analysis, exact aliquots of the reaction mixtures were withdrawn at various time points and examined by Svennerholm method.[31] The results are summarized in Figure 2a and b. The reactions had two phases (Fig. 2a). During the first 5 h, they exhibited the pseudo zero-order kinetics (Fig. 2b). Thereafter, most reactions leveled off and eventually reached a plateau within 24 h. The reaction of ManNTFP was the slowest of all eight derivatives tested, but it reached a similar plateau ca. 50 h later.

It is well established that aldolase-catalyzed reactions are reversible (Eq. 1),[21,22] and the reaction rate can then be expressed by Eq. 2.[32] At the initial stage, the concentration of NeuNR was rather low, so the reverse reaction (k_{-1}[NeuNR][Enz]) could be neglected, and Eq. 2 can be simplified to Eq. 3. Meanwhile, pyruvic acid was used in large excess and the enzyme remained unchanged. Thus, the concentrations of enzyme [Enz] and pyruvic acid [PA] could also be taken as constants. Although the substrate concentration [ManNR]

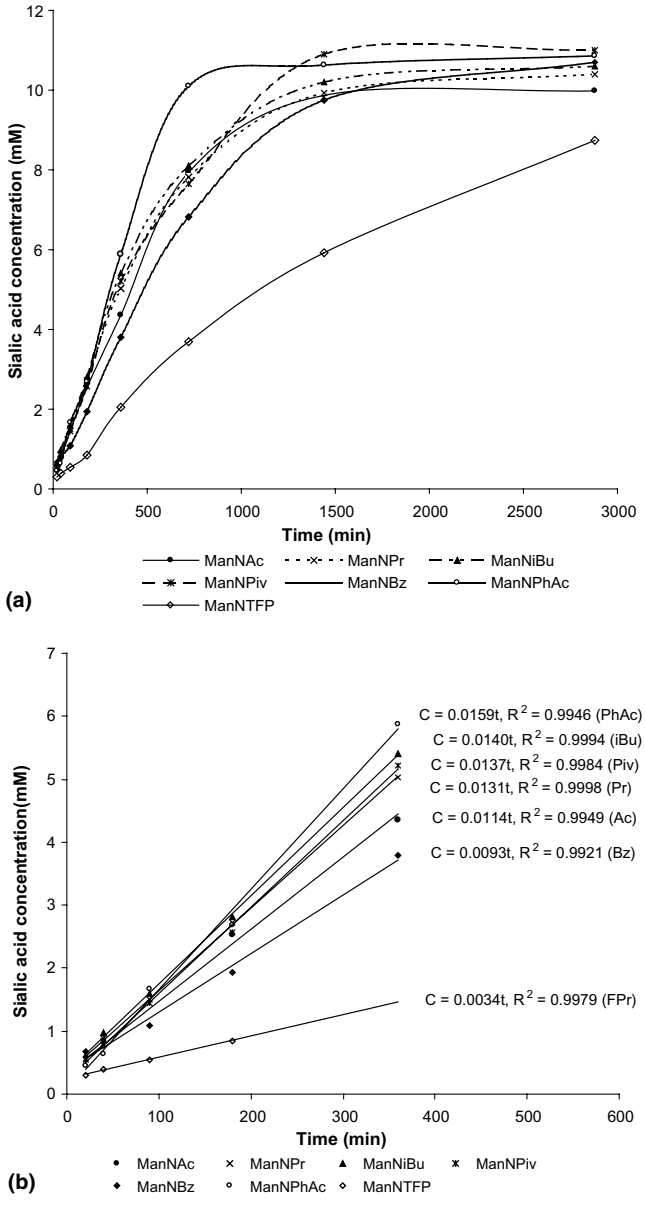


Figure 2. The reaction kinetics of *N*-acyl-D-mannosamines (18.2 mM) with pyruvic acid (182 mM) and NeuNAc aldolase (0.9 unit/mL): 37 °C, 0.05 M potassium phosphate buffer (pH 7.4, $I = 0.1$) containing 7.5% (v/v) dithiothreitol.

changed with the proceeding of reactions, at the initial stage this change was relatively small, and [ManNR] could be considered as a consistent. Eq. 3 can thus be simplified to Eqs. 4 and 5, showing the zero-order kinetics.

$$\text{ManNR} + \text{PA} + \text{Enz} \underset{(k_{-1})}{\overset{\text{K}\ (k_1)}{\rightleftharpoons}} \text{NeuNR+Enz} \qquad (1)$$

$$\begin{aligned}\text{reaction rate}(v) &= \text{d[NeuNR]}/\text{d}t \\ &= k_1[\text{ManNR}][\text{PA}][\text{Enz}] \\ &\quad - k_{-1}[\text{NeuNR}][\text{Enz}] \qquad (2)\end{aligned}$$

$$\text{d[NeuNR]}/\text{d}t = k_1[\text{ManNR}][\text{PA}][\text{Enz}] \qquad (3)$$

$$\text{d[NeuNR]}/\text{d}t = k' \qquad (4)$$

$$[\text{NeuNR}] = k'\text{t} \qquad (5)$$

On the other hand, with the accumulation of NeuNR, the term k_{-1}[NeuNR][Enz] of Eq. 2 would become more and more significant, while the consumption of ManNR reduced k_1[ManNR][PA][Enz]. As a result, the reactions slowed down and gradually reached an equilibrium (the plateau).

The initial reaction rates of various *N*-acyl-D-mannosamine derivatives were different (Fig. 2b), even though their conversion rates at the equilibriums were similar (69% ± 1.8%). Compared to ManNAc ($k' = 0.0114$), the reactions of ManNPr ($k' = 0.0131$), ManN*i*-Bu ($k' = 0.0140$), ManNPiv ($k' = 0.0137$) and ManNPhAc ($k' = 0.0159$) were slightly faster, but the reactions of ManNBz ($k' = 0.0093$) and ManNTFP ($k' = 0.0034$) were obviously slower. Since **5**–**7** that bear rather bulky *N*-acyl groups such as *i*-Bu, Piv, and PhAc were as good substrates as ManNAc, it seems that the steric effect is not critical for determining the enzymatic accessibility of these substrates. On the contrary, ManNTFP and ManNBz with strongly electron-deficient acyl groups were poor substrates, which may indicate that the electronic effect probably has a significant influence on the accessibility of *N*-acyl-D-mannosamines to the aldolase, and the highly electron-deficient acyl groups retard the reaction. However, ManNPhAc with a mildly electron-deficient acyl group is a good substrate, which suggests that, besides electronic effect, other factors such as hydrophobic interactions, may also affect the substrate–aldolase binding.

ManNPhAc was especially interesting to us, as it was the best substrate for NeuNAc aldolase and thus possibly the most efficient precursor for engineering cell-surface sialic acid. Meanwhile, since a phenylacetyl group is distinctly different from an acetyl group, the phenylacetyl derivatives may form effective vaccines. The reaction of ManNPhAc was then studied in detail. As shown in Figure 3a, with pyruvic acid in excess, the reaction rate increased with the concentration of ManNPhAc from 1.82 to 182 mM. At low concentrations, the reaction rate (k') had a linear correlation with [ManNPhAc] (Figure 2b), which can be explained by Eq. 3. However, the reaction rate leveled off at higher [ManNPhAc]. The results fit a Lineweaver–Burk plot (Fig. 2c).[32] Because cellular levels of mannosamine are usually much lower than the tested concentrations, the NeuNAc aldolase-catalyzed reaction rate will probably observe a linear correlation with the concentration of ManNPhAc in the biological system. Therefore, any measures that can improve the cellular concentrations of ManNPhAc would be able to increase the biosynthesis of PhAc-modified sialic acid by cells and thereby im-

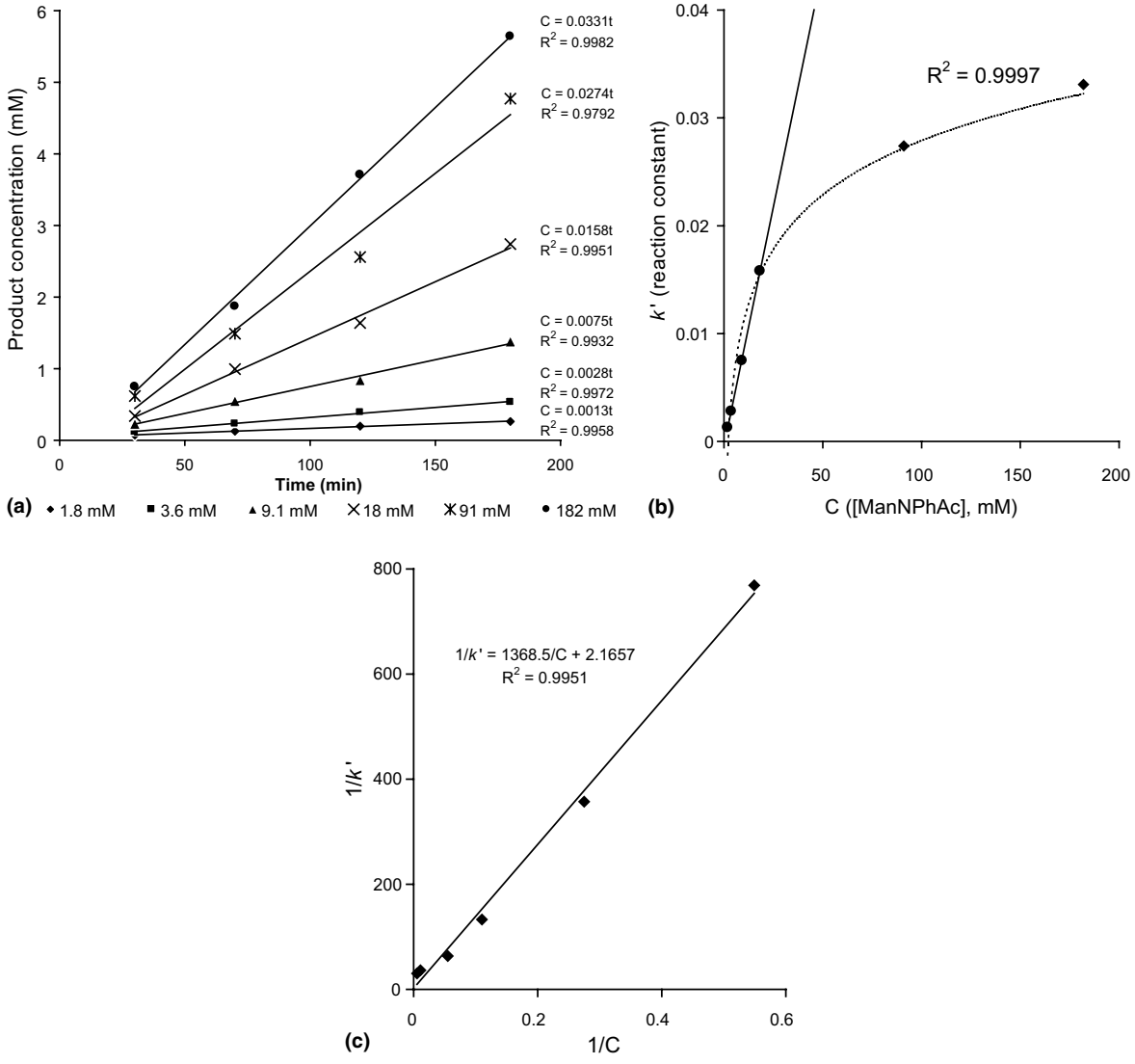


Figure 3. The impact of ManNPhAc concentrations (1.8, 3.6, 9.1, 18, 91, and 182 mM) on its reaction with pyruvic acid (182 mM) and NeuNAc aldolase (0.9 unit/mL): 37 °C, 0.05 M potassium phosphate buffer (pH 7.4, $I = 0.1$) containing 7.5% (v/v) dithiothreitol.

prove the efficiency of cell-surface sialic acid engineering.

In conclusion, a number of *N*-acyl derivatives of D-mannosamine were prepared, and their accessibility to NeuNAc aldolase was studied. It was demonstrated that NeuNAc aldolase could tolerate a range of *N*-modifications of D-mannosamine, and as substrates of NeuNAc aldolase, ManNPr, ManNBu, ManN*i*-Bu, ManNPiv, and ManNPhAc were as good as or even better than the natural ManNAc. These results suggest that ManNPr, ManNBu, ManN*i*-Bu, ManNPiv, and ManNPhAc may be used as effective biosynthetic precursors to engineer cell-surface sialic acid. Coupled with the fact that artificially modified sialooligosaccharides are highly immunogenic,[33–35] these results should be valuable for the development of new cancer immunotherapies. Among the potentially useful biosynthetic precursors identified, ManN*i*-Bu and ManNPhAc are especially interesting to us, because our initial studies also indicated that *N*-*i*-Bu and *N*-PhAc modified sialic acids could induce robust IgG antibodies desirable for cancer immunotherapy.[36] Therefore, we are currently investigating in detail the bioavailability of ManN*i*-Bu and ManNPhAc to cells, as well as their efficiency to glycoengineer tumors in vitro and in vivo.

3. Experimental

3.1. General methods

NMR spectra were recorded on a Gemini-300 FT NMR spectrometer. Proton chemical shifts are reported in ppm (δ) downfield from tetramethylsilane (TMS).

Coupling constants (J) are reported in Hertz (Hz). The fast-atom-bombardment mass spectra (FABMS) were obtained with a Kratos MS-25RFA spectrometer. Optical rotations were determined with a Perkin–Elmer 241 polarimeter, and thin-layer chromatography (TLC) was performed on silica gel GF_{254} plates with detection by charring with phosphomolibdic acid–EtOH or 5% H_2SO_4–EtOH. Anhydrous solvents and other reagents were purchased from vendors and used without further purification.

3.2. *N*-Acyl derivatives (2–8) of D-mannosamine

To a solution of D-mannosamine·HCl (300 mg, 1.39 mmol) in 5 mL MeOH and 0.5 mL of 3 N NaOH was added dropwise 1.0 mL of an acyl anhydride while cooling the mixture in an ice-water bath. After the reaction was finished (within 3 h as indicated by TLC), the mixture was neutralized with 2 N HCl and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography (eluent: MeOH–CH_2Cl_2) to afford *N*-acylmannosamine that was further purified by passing through a Bio-Gel P2 column using distillated water as eluent. The fractions containing the expected product were combined, freeze-dried, and finally recrystallized from EtOH and EtOAc to give a white crystalline product.

3.2.1. *N*-Acetyl-D-mannosamine (2). 210 mg (70%); $[\alpha]_D^{20}$ +11 (c 1.0, H_2O) (lit.[37] +9.7); ^{1}H NMR (D_2O, 300 MHz): (α-isomer) δ 5.10 (d, 1H, $J_{1,2}$ 1.3 Hz, H-1), 4.30 (dd, 1H, $J_{2,3}$ 4.6 Hz, H-2), 4.02 (dd, 1H, $J_{3,4}$ 9.7 Hz, H-3), 3.81–3.83 (m, 2H, H-6,6′), 3.80 (m, 1H, H-5), 3.59 (dd, 1H, $J_{4,5}$ 9.0 Hz, H-4), 2.03 (s, 3H, Ac); (β-isomer) 5.00 (d, 1H, $J_{1,2}$ 1.6 Hz, H-1), 4.42 (dd, 1H, $J_{2,3}$ 4.4 Hz, H-2), 3.81 (m, 1H, H-3), 3.80–3.85 (m, 2H, H-6,6′), 3.50 (dd, 1H, J 9.9, 9.6 Hz, H-4), 3.41–3.43 (m, 1H, H-5), 2.07 (s, 3H, Ac); FABMS: m/z 204.0 [M+H$^+$−H_2O], 221.1 [M$^+$], 222.1 [M+H$^+$], 244.1 [M+Na$^+$]; HRFABMS: Calcd for $C_8H_{15}NO_6$ [M+H$^+$] m/z 222.0972; found 222.1016.

3.2.2. *N*-Propionyl-D-mannosamine (3).[38] 215 mg (68%); $[\alpha]_D^{20}$ +7.0 (c 1.0, H_2O); ^{1}H NMR (D_2O, 300 MHz): (α-isomer) δ 5.08 (d, 1H, $J_{1,2}$ 1.6 Hz, H-1), 4.29 (dd, 1H, $J_{2,3}$ 4.8 Hz, H-2), 4.02 (dd, 1H, $J_{3,4}$ 9.7 Hz, H-3), 3.75–3.86 (m, 3H, H-5,6,6′), 3.58 (t, 1H, J 9.5, H-4), 2.29 (q, 2H, J 7.6 Hz, Pr), 1.07 (t, 3H, J 7.6 Hz, Pr); (β-isomer) δ 4.99 (d, 1H, $J_{1,2}$ 1.7 Hz, H-1), 4.42 (dd, 1H, $J_{2,3}$ 4.4 Hz, H-2), 3.75–3.86 (m, 3H, H-3,6,6′), 3.48 (t, 1H, J 9.5, H-4), 3.33–3.43 (m, 1H, H-5), 2.33 (q, 2H, J 7.7 Hz, Pr), 1.09 (t, 3H, J 7.7 Hz, Pr); FABMS: m/z 218.1 [M+H$^+$−H_2O], 236.1 [M+H$^+$], 258.1 [M+Na$^+$]; HRFABMS: Calcd for $C_9H_{17}NO_6$ [M+H$^+$] m/z 236.1129; found 236.1133.

3.2.3. *N*-Butanoyl-D-mannosamine (4).[39] 264 mg (75%); $[\alpha]_D^{20}$ +9.0 (c 1.0, H_2O); ^{1}H NMR (D_2O, 300 MHz): (α-isomer) δ 5.07 (d, 1H, $J_{1,2}$ 1.3 Hz, H-1), 4.28 (dd, 1H, $J_{2,3}$ 4.8 Hz, H-2), 4.02 (dd, 1H, $J_{3,4}$ 9.7 Hz, H-3), 3.75–3.84 (m, 3H, H-5,6,6′), 3.58 (dd, 1H, $J_{4,5}$ 10.8 Hz, H-4), 2.22 (t, 2H, J 7.6 Hz, Bu), 1.47–1.60 (m, 2H, Bu), 0.86 (t, 3H, J 7.5 Hz, Bu); (β-isomer) δ 5.0 (d, 1H, $J_{1,2}$ 1.7 Hz, H-1), 4.41 (d, 1H, $J_{2,3}$ 4.3 Hz, H-2), 3.74–3.84 (m, 3H, H-3,6,6′), 3.48 (dd, 1H, J 9.8, 9.3 Hz, H-4), 3.32–3.45 (m, 1H, H-5), 2.25 (t, 2H, J 7.3 Hz, Bu), 1.47–1.68 (m, 2H, Bu), 0.89 (t, 3H, J 7.4 Hz, Bu); FABMS: m/z 232.2 [M+H$^+$−H_2O], 250.3 [M+H$^+$]; HRFABMS: Calcd for $C_{10}H_{19}NO_6$ [M+H$^+$] m/z 250.1285; found 250.1293.

3.2.4. *N*-iso-Butanoyl-D-mannosamine (5). 252 mg (73%); $[\alpha]_D^{20}$ +10 (c 1.0, H_2O); ^{1}H NMR (D_2O, 300 MHz): (α-isomer) δ 5.11 (d, 1H, $J_{1,2}$ 1.2 Hz, H-1), 4.32 (dd, 1H, $J_{2,3}$ 4.7 Hz, H-2), 4.07 (dd, 1H, $J_{3,4}$ 9.7 Hz, H-3), 3.77–3.92 (m, 3H, H-5,6,6′), 3.63(t, 1H, J 9.6 Hz, H-4), 2.54–2.70 (m, 1H, *i*-Bu), 1.10 (d, 6H, J 6.9 Hz, *i*-Bu); (β-isomer) δ 5.04 (d, 1H, $J_{1,2}$ 1.3 Hz, H-1), 4.45 (dd, 1H, $J_{2,3}$ 3.9 Hz, H-2), 3.77–3.92 (m, 3H, H-3,6,6′), 3.52 (dd, 1H, J 9.9, 9.6 Hz, H-4), 3.38–3.44 (m, 1H, H-5), 2.54–2.70 (m, 1H, *i*-Bu), 1.11 (d, 6H, J 6.9 Hz, *i*-Bu); FABMS: m/z 250.1 [M+H$^+$], 272.1 [M+Na$^+$], 232.1 [M+H$^+$−H_2O]; HRFABMS: Calcd for $C_{10}H_{19}NO_6$ [M+H$^+$] m/z 250.1285; found 250.1273. Anal. Calcd for ($C_{10}H_{18}NO_6$) C, 48.19; H, 7.68; N, 5.62. Found: C, 47.79; H, 7.73; N, 5.44.

3.2.5. *N*-Pivaloyl-D-mannosamine (6). 273 mg (76%); $[\alpha]_D^{20}$ +10 (c 1.0, H_2O); ^{1}H NMR (D_2O, 300 MHz): (α-isomer) δ 5.13 (s, 1H, H-1), 4.33 (dd, 1H, $J_{1,2}$ 1.6 Hz, $J_{2,3}$ 4.6 Hz, H-2), 4.09 (dd, 1H, $J_{3,4}$ 9.6 Hz, H-3), 3.80–3.92 (m, 3H, H-5,6,6′),3.63 (t, 1H, J 9.1 Hz, H-4), 1.21 (s, 9H, Me); (β-isomer) δ 5.08 (d, 1H, $J_{1,2}$ 1.6 Hz, H-1), 4.47 (dd, 1H, $J_{2,3}$ 4.1 Hz, H-2), 3.8–3.9 (m, 3H, H-5,6,6′), 3.47 (t, 1H, J 9.4 Hz, H-4), 3.40–3.46 (m, 1H, H-5),1.23 (s, 9H, Me); FABMS: m/z 246.1 [M+H$^+$−H_2O], 264.1 [M+H$^+$]; HRFABMS: Calcd for $C_{11}H_{21}NO_6$ [M+H$^+$] m/z 264.1442; found 264.1455. Anal. Calcd for ($C_{11}H_{20}NO_6$) C, 50.18; H, 8.04; N, 5.32. Found: C, 50.39; H, 8.12: N, 5.22.

3.2.6. *N*-Phenylacetyl-D-mannosamine (7). 300 mg (73%); $[\alpha]_D^{20}$ −10 (c 1.0, H_2O); ^{1}H NMR (D_2O, 300 MHz): (α-isomer) δ 7.33–7.45 (m, 5H, H–Ar), 5.11 (d, 1H, $J_{1,2}$ 1.5 Hz, H-1), 4.33 (dd, 1H, $J_{2,3}$ 4.7 Hz, H-2), 4.08 (dd, 1H, $J_{3,4}$ 9.9 Hz, H-3), 3.80–3.87 (m, 3H, H-5,6,6′), 3.63 (dd, 1H, $J_{4,5}$ 9.5, Hz, H-4), 3.69, 3.65 (2d, 2H, J 15.0 Hz, Bn); (β-isomer) δ 7.33–7.45 (m, 5H, H–Ar), 5.04 (d, 1H, $J_{1,2}$ 1.5 Hz, H-1), 4.46 (dd, 1H, $J_{2,3}$ 4.4 Hz, H-2), 3.80–3.87 (m, 2H, H-3,6), 3.77 (dd, 1H, J 12.0, 5.4 Hz, H-6′), 3.75 (s, 2H, Bn), 3.51 (dd, 1H, J 9.7, 9.5 Hz, H-4), 3.40 (ddd, 1H, J 9.7, 4.8, 2.3 Hz, H-5); FABMS: m/z 280.1 [M+H$^+$−H_2O], 298.1 [M+H$^+$], 320.1 [M+Na$^+$], 336.1 [M+K$^+$]; HRFABMS: Calcd for $C_{14}H_{19}NO_6$ [M+H$^+$] m/z 298.1285; found 298.1348. Anal. Calcd for

($C_{14}H_{18}NO_6$) C, 56.56; H, 6.44; N, 4.71. Found: C, 56.16; H, 6.39: N, 4.46.

3.2.7. *N*-Benzoyl-D-mannosamine (8). 300 mg (74%); $[\alpha]_D^{20}$ −17 (*c* 1.0, H_2O); ^1H NMR (D_2O, 300 MHz): (α-isomer) δ 7.5–7.8 (m, 5H, H–Ar), 5.26 (s, 1H, $J_{1,2}$ 1.1 Hz, H-1), 4.58 (dd, 1H, $J_{2,3}$ 4.7 Hz, H-2), 4.17 (dd, 1H, $J_{3,4}$ 9.8 Hz, H-3), 3.8–3.9 (m, 3H, H-5,6,6′), 3.65–3.78 (t, 1H, $J_{2,3}$ 9.7 Hz, H-4); (β-isomer) δ 7.5–7.8 (m, 5H, H–Ar), 5.14 (s, 1H, H-1), 4.72–4.73 (m, 1H, H-2), 3.8–3.9 (m, 3H, H-3,6,6′), 3.64 (t, 1H, *J* 9.8 Hz, H-4), 3.44–3.50 (m, 1H, H-5); FABMS: *m*/*z* 266.3 [M+H$^+$−H_2O], 284.3 [M+H$^+$]; HRFABMS: Calcd for $C_{13}H_{17}NO_6$ [M+H$^+$] *m*/*z* 284.1129; found 284.1131.

3.3. *N*-Trifluoropropionyl-D-mannosamine (9)

To the solution of 1-hydroxybenzotriazole (297 mg) and *N*,*N*′-dicyclohexylcarbodiimide (454 mg) in 5 mL of DMF was added 194 μL of 3,3,3-trifluoropropionic acid (2.2 mmol). After the reaction mixture was stirred at 0 °C for 1 h and at rt for additional 3 h, D-mannosamine·HCl (300 mg, 1.39 mmol) was added. The reaction mixture was stirred overnight, and the precipitate was then filtered off. The filtrate was condensed under reduced pressure, and the residue was purified by silica gel column chromatography (eluent: MeOH–CH_2Cl_2) to afford **9** that was further purified by a Bio-Gel P2 column and finally recrystallized from EtOH and EtOAc to give white crystalline **9** (329 mg, 82%). $[\alpha]_D^{20}$ +3.0 (*c* 0.5, H_2O); ^1H NMR (D_2O, 300 MHz): (α-isomer) δ 5.11 (d, 1H, $J_{1,2}$ 1.6 Hz, H-1), 4.35 (dd, 1H, $J_{2,3}$ 4.6 Hz, H-2), 4.05 (dd, 1H, $J_{3,4}$ 9.7 Hz, H-3), 3.78–3.86 (m, 3H, H-5,6,6′), 3.58 (dd, 1H, *J* 9.9, 9.7 Hz, H-4), 3.32 (q, 2H, J_{HF} 10.7, CH_2CF_3); (β-isomer) δ 5.02 (d, 1H, $J_{1,2}$ 1.6 Hz, H-1), 4.48 (dd, 1H, $J_{2,3}$ 4.4 Hz, H-2), 3.78–3.86 (m, 3H, H-3,6,6′), 3.48 (t, 1H, *J* 9.9 Hz, H-4), 3.38–3.42 (m, 1H, H-5), 3.36 (q, 2H, J_{HF} 10.7, CH_2CF_3); FABMS: *m*/*z* 272.3 [M+H$^+$−H_2O], 290.1 [M+H$^+$], 312.1 [M+Na$^+$]; HRFABMS: Calcd for $C_9H_{14}NO_6F_3$ [M+H$^+$] *m*/*z* 290.0846; found 290.0856. Anal. Calcd for ($C_9H_{13}NO_6F_3$): C, 37.38; H, 4.88: N, 4.84. Found: C, 37.01; H, 4.70: N, 5.09.

3.4. Benzyl glycoside of D-neuraminic acid (21)

A solution of compound **20**[30] (2.4 g, 4.1 mmol) refluxed in 2 N NaOH (50 mL) for 6 h was cooled to rt, and concd HCl was added to neutralize it to pH 6. The mixture was then concentrated under reduced pressure, and the residue was extracted with MeOH to remove NaCl. The extracts were combined, concentrated, and purified by column chromatography (1:6 MeOH–EtOAc) to give **21** (1.25 g, 85%). $[\alpha]_D^{20}$ −40 (*c* 0.5, H_2O); R_f 0.59 (eluent: 1:1:1:1 BuOH–HOAc–EtOAc–H_2O); ^1H NMR (D_2O, 300 MHz): δ 7.45 (m, 5H, H–Ar), 4.70, 4.53 (2d, 2H, *J* 10.9 Hz, CH_2Ph), 4.15 (dd, 1H, *J* 10.2, 1.9 Hz, H-6), 3.81–3.94 (m, 4H), 3.76 (dd, 1H, *J* 12.6, 6.1 Hz, H-9), 3.31 (t, 1H, *J* 10.3 Hz, H-5), 2.85 (dd, 1H, *J*, 12.4, 4.6 Hz, H-3e), 1.79 (t, 1H, *J* 12.4 Hz, H-3a).

3.5. Benzyl glycosides of *N*-acyl-D-neuraminic acid (22–27)

To a solution of **21** (150 mg, 0.42 mmol) in MeOH (4 mL) was added an anhydride (1.26 mmol) at 0 °C slowly. The mixture was stirred at 0 °C for 1 h and then at rt until TLC showed that the reaction had gone to completion. Workup as shown in Section 3.2 and column chromatography (1:4 to 1:1 MeOH–EtOAc) of the product afforded **22**–**27**.

3.5.1. Benzyl glycoside of *N*-propionyl-D-neuraminic acid (22). 165 mg (95%); R_f 0.59 (eluent: 1:1:1:1 BuOH–HOAc–EtOAc–H_2O); ^1H NMR (D_2O, 300 MHz): δ 7.40 (m, 5H, H–Ar), 4.80, 4.52 (2d, 2H, *J* 11.3 Hz, Bn), 3.96–3.60 (m, 6H), 3.58 (d, 1H, *J* 9.0 Hz, H-7), 2.88 (dd, 1H, *J* 4.4 Hz, 12.2 Hz, H-3e), 2.34 (m, 2H, $COCH_2$), 1.71 (t, 1H, *J* 11.8 Hz, H-3a), 1.10 (t, 3H, *J* 7.5 Hz, CH_3); HRFABMS: Calcd for $C_{19}H_{27}NO_9$ [M+Na$^+$] *m*/*z* 436.1584; found 436.1584.

3.5.2. Benzyl glycoside of *N*-butanoyl-D-neuraminic acid (23). 167 mg (93%); R_f 0.65 (eluent: 1:1:1:1 BuOH–HOAc–EtOAc–H_2O); ^1H NMR (D_2O, 300 MHz): δ 7.40 (m, 5H, H–Ar), 4.77, 4.53 (2d, 2H, *J* 11.3 Hz, Bn), 3.96–3.60 (m, 7H), 2.80 (dd, 1H, *J* 4.4 Hz, 13.2 Hz, H-3e), 2.30 (t, 2H, *J* 7.2 Hz, $COCH_2$), 1.72 (t, 1H, *J* 11.8 Hz, H-3a), 1.64 (q, 2H, *J* 7.2 Hz, CH_3CH_2), 0.94 (t, 3H, *J* 7.2 Hz, CH_3); HRFABMS: Calcd for $C_{20}H_{29}NO_9$ [M+Na$^+$] *m*/*z* 450.1740; found 450.1740.

3.5.3. Benzyl glycoside of *N*-*iso*-butanoyl-D-neuraminic acid (24). 51 mg (84%); R_f 0.80 (eluent: 1:1:1:1 BuOH–HOAc–EtOAc–H_2O); ^1H NMR (D_2O, 300 MHz): δ 7.40 (s, 5H, H–Ar), 4.75, 4.52 (2d, 2H, *J* 10.8, CH_2Ph), 3.89–3.55 (m, 6H), 3.56 (d, 1H, *J* 8.6 Hz, H-7), 2.81 (dd, 1H, *J* 4.4, 13.2 Hz, H-3e), 2.55 (m, 1H, COCH), 1.71 (t, 1H, *J* 12.8, H-3a), 1.15 (2 d, 6H,*J* 7.0 Hz, $2CH_3$); HRFABMS: Calcd for $C_{20}H_{29}NO_9$ [M+Na$^+$] *m*/*z* 450.1740; found 450.1747.

3.5.4. Benzyl glycoside of *N*-trimethyl-D-neuraminic acid (25). 176 mg (95%); R_f 0.64 (eluent: 1:1:1:1 BuOH–HOAc–EtOAc–H_2O); ^1H NMR (D_2O, 200 MHz): δ 7.37 (m, 5H, H–Ar), 4.70, 4.48 (2d, 2H, *J* 10.8, CH_2Ph), 4.05 (d, 1H, *J* 10.3, H-6), 3.85–3.73 (m, 4 H), 3.64–3.53 (m, 1H), 3.47 (m, 1H), 2.82 (dd, 1H, *J* 3.6, 12.4 Hz, H-3e), 1.64 (t, 1H, *J* 12.1 Hz, H-3a), 1.14 (s, 9H, $3CH_3$); HRFABMS: Calcd for $C_{19}H_{27}NO_9$ [M+Na$^+$] *m*/*z* 436.1584; found 436.1584.

3.5.5. Benzyl glycoside of *N*-phenylacetyl-D-neuraminic acid (26). 181 mg (91%); R_f 0.78 (eluent: 1:1:1:1 BuOH–HOAc–EtOAc–H_2O); ^{1}H NMR (D_2O, 200 MHz): δ 7.3–7.5 (m, 10H, H–Ar), 4.67, 4.52 (2d, 2H, J 9.5 Hz, CH_2Ph), 3.75–3.68 (m, 6H), 3.60 (s, 2H, CH_2Ph), 3.49 (dd, 1H, J 12.3, 7.1 Hz, H-9), 2.74 (dd, 1H, J 4.0, 12.4 Hz, H-3e), 1.64 (t, 1H, J 12.2 Hz, H-3a); HRFABMS: Calcd for $C_{24}H_{29}NO_9$ [M+Na$^+$] m/z 498.1740; found 498.1740.

3.5.6. Benzyl glycoside of *N*-benzoyl-D-neuraminic acid (27). 174 mg (90%); R_f 0.59 (eluent: 1:1:1:1 BuOH–HOAc–EtOAc–H_2O); ^{1}H NMR (D_2O, 300 MHz): δ 8.01–7.43 (m, 10H, H–Ar), 4.70, 4.54 (2d, 2H, J 10.0 Hz, CH_2Ph), 4.05 (d, 1H, J 10.3, H-6), 4.2–3.6 (m, 6H), 2.83 (dd, 1H, J 4.8, 12.5 Hz, H-3e), 1.77 (t, 1H, J 12.3 Hz, H-3a); HRFABMS: Calcd for $C_{23}H_{27}NO_9$ [M+Na$^+$] m/z 484.1584; found 484.1570.

3.6. Benzyl glycoside of *N*-trifluoropropionyl-D-neuraminic acid (28)

To a solution of 1-hydroxybenzotriazole (149 mg) and *N*,*N*′-dicyclohexylcarbodiimide (227 mg) in 5 mL of DMF was added 97 μL of 3,3,3-triflouropropionic acid (1.1 mmol). The reaction mixture was stirred at 0 °C for 1 h, then at rt for 3 h and **21** (150 mg, 0.42 mmol) was added to it. The reaction mixture was then stirred overnight, and the resulting precipitate was filtered off. The filtrate was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography to afford **28** (140 mg, 72%). R_f 0.79 (eluent: 1:1:1:1 BuOH–HOAc–EtOAc–H_2O); ^{1}H NMR (D_2O, 300 MHz): δ 7.40 (br, 5H, H–Ar), 4.74, 4.54 (2d, 2H, J 10.2 Hz, CH_2Ph), 3.30 (q, 2H, J 10.9 Hz, CH_2CF$_3$), 2.79 (dd, 1H, J 12.5, 4.6 Hz, H-3e), 1.86 (t, 1H, J 12.5 Hz, H-3a); HRFABMS: Calcd for $C_{19}H_{24})F_3NO_9$ m/z [M+Na$^+$] 490.1301; found 490.1354.

3.7. *N*-Acyl-D-neuraminic acids 11–17

A mixture of **22**–**28** (0.1 mmol) and 10% palladium-on-carbon (0.01 mol) in MeOH (5 mL) was stirred vigorously at rt under a hydrogen atmosphere until TLC showed the disappearance of the starting material. The catalyst was filtered off, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (eluent: 20:80:1 to 50:50:1 MeOH–EtOAc–AcOH) to give **11**–**17** that were further purified by a Bio-Gel P-2 column using distilled water as eluent. Recrystallization from 1:2 EtOH–EtOAc afforded pure products.

3.7.1. *N*-Propionyl-D-neuraminic acid (11). 30 mg (94%); $[\alpha]_D^{20}$ −21 (c 1.0, H_2O); R_f 0.28 (eluent: 1:1:1:1 BuOH–HOAc–EtOAc–H_2O); ^{1}H NMR (D_2O, 300 MHz): (β-isomer) δ 4.07–3.98 (m, 3H), 3.85–3.83 (m, 2H), 3.66 (dd, 1H, J 12.5, 7.2 Hz, H-9), 3.59 (d, 1H, J 8.1 Hz, H-7), 2.34 (q, 2H, J 7.9 Hz, COCH$_2$), 2.26 (dd, 1H, J 4.2, 13.0 Hz, H-3e), 1.86 (t, 1H, J 12.4 Hz, H-3a), 1.15 (t, 3H, J 7.6 Hz,CH$_3$). HRFABMS: Calcd for $C_{12}H_{21}NO_9$ m/z [M+Na$^+$] 346.1114; found 346.1117.

3.7.2. *N*-Butanoyl-D-neuraminic acid (12). 30 mg (90%); $[\alpha]_D^{20}$ −27 (c 1.0, H_2O); R_f 0.31 (eluent: 1:1:1:1 BuOH–HOAc–EtOAc–H_2O); ^{1}H NMR (D_2O, 300 MHz): (β-isomer) δ 4.02–3.98 (m, 2H), 3.95 (d, 1H, J 9.6 Hz, H-9), 3.85–3.74 (m, 2H), 3.61 (dd, 1H, J 12.0, 6.4 Hz, H-9′), 3.55 (d, 1H, J 9.0 Hz, H-7), 2.29 (t, 2H, J 7.2 Hz, COCH$_2$), 2.26 (dd, 1H, J 4.2, 13.0 Hz, H-3e), 1.86 (t, 1H, J 12.4 Hz, H-3a), 1.63 (q, 2H, J 7.2 Hz, CH$_3$CH_2), 0.95 (t, 3H, J 7.2 Hz, CH$_3$). HRFABMS: Calcd for $C_{13}H_{23}NO_9$ [M+Na$^+$] m/z 360.1271; found 360.1247.

3.7.3. *N-iso*-Butanoyl-D-neuraminic acid (13). 31 mg (93%); $[\alpha]_D^{20}$ −28 (c 1.0, H_2O); R_f 0.38 (eluent: 1:1:1:1 BuOH–HOAc–EtOAc–H_2O); ^{1}H NMR (D_2O, 300 MHz): (β-isomer) δ 4.08–3.78 (m, 4H), 3.62 (dd, 1H, J 12.9, 6.6 Hz, H-9), 3.54 (d, 1H, J 8.8 Hz, H-7), 2.58 (m, 1H, COCH), 2.26 (dd, 1H, J 5.2, 12.8 Hz, H-3e), 1.85 (t, 1H, J 12.5 Hz, H-3a), 1.15 (2d, 2×3H, J 7.0 Hz, 2 CH$_3$). HRFABMS: Calcd for $C_{13}H_{23}NO_9$ [M-H+2Na]$^+$ m/z 382.1089; found 382.1084.

3.7.4. *N*-Trimethylacetyl-D-neuraminic acid (14). 32 mg (93%); $[\alpha]_D^{20}$ −31 (c 1.0, H_2O); R_f 0.40 (eluent: 1:1:1:1 BuOH–HOAc–EtOAc–H_2O); ^{1}H NMR (D_2O, 600 MHz): (β-isomer) δ 4.08–3.78 (m, 4H), 3.60 (dd, 1H, J 12.0, 6.0 Hz, H-9), 3.45 (d, 1H, J 9.0 Hz, H-7), 2.25 (dd, 1H, J 4.8, 12.6 Hz, H-3e), 1.84 (t, 1H, J 12.6 Hz, H-3a), 1.06 (s, 9H, 3CH$_3$). HRFABMS: Calcd for $C_{14}H_{25}NO_9$ [M+Na$^+$] m/z 374.1427; found 374.1482.

3.7.5. *N*-Phenylacetyl-D-neuraminic acid (15). 35 mg (91%); $[\alpha]_D^{20}$ −14 (c 1.0, H_2O); R_f = 0.40 (eluent: 1:1:1:1 BuOH–HOAc–EtOAc–H_2O); ^{1}H NMR (D_2O, 300 MHz): (β-isomer) δ 7.42 (m, 5H, H–Ar), 4.08–3.96 (m, 3H), 3.77 (m, 1H), 3.71–3.66 (m, 1H), 3.68 (s, 2H, CH_2Ph), 3.54 (dd, 1H, J 11.7, 6.4 Hz, H-9), 3.49 (d, 1H, J 7.8 Hz, H-7), 2.32 (dd, 1H, J 4.9, 13.1 Hz, H-3e), 1.86 (t, 1H, J 13.0 Hz, H-3a). HRFABMS: Calcd for $C_{17}H_{23}NO_9$ [M+Na$^+$] m/z 408.1270; found 408.1271.

3.7.6. *N*-Benzoyl-D-neuraminic acid (16). 31 mg (84%); $[\alpha]_D^{20}$ −15 (c 1.0, H_2O); R_f 0.39 (eluent: 1:1:1:1 BuOH–HOAc–EtOAc–H_2O); ^{1}H NMR (D_2O, 300 MHz): (β-isomer) δ 7.64–7.43 (m, 5H, H–Ar), 4.15 (s, 3H), 3.83–3.73 (m, 2H), 3.65–3.50 (m, 2H), 2.26 (dd, 1H, J 4.2, 12.5 Hz, H-3e), 1.86 (t, 1H, J 11.6 Hz, H-3a). HRFABMS: Calcd for $C_{16}H_{21}NO_9$ [M+Na$^+$] m/z 394.1114; found 394.1140.

3.7.7. *N*-Trifluoropropionyl-D-neuraminic acid (17). 34 mg (90%); $[\alpha]_D^{20}$ −16 (*c* 1.0, H_2O); R_f 0.43 (eluent: 1:1:1:1 BuOH–HOAc–EtOAc–H_2O); ^1H NMR (D_2O, 300 MHz): (β-isomer) δ 4.13–4.03 (m, 3H), 3.86–3.78 (m, 2H), 3.60 (dd, 1H, *J* 11.4, 6.6 Hz, H-9), 3.55 (d, 1H, *J* 9.0 Hz, H-7), 3.35 (q, 2H, *J* 10.5, CF_3CH_2), 2.32 (dd, 1H, *J* 4.6, 13.2 Hz, H-3e), 1.86 (t, 1H, *J* 13.2 Hz, H-3a). HRFABMS: Calcd for $C_{12}H_{18}F_3NO_9$ $[M+H^+]$ m/z 378.1012; found 378.1013.

3.8. General procedure for the enzymatic reactions

To a 1.5 mL vial was added ManNR (7.27 μmol) in 130 μL of 0.05 M potassium phosphate buffer (pH 7.4, $I = 0.1$), 72.73 μmol pyruvic acid in 100 μL buffer, 2.0 μmol dithiothreitol in 30 μL buffer, and 140 μL diluted NeuNAc aldolase solution (0.35 U). The reaction was kept at 37 °C with occasional shaking. At time points of 20, 40, 90, 180, 360, 720, and 1440 min, 40-μL aliquots were taken out and put on dry ice for 30 min. After that, the samples were directly added into resorcinol reagent and analyzed by the modified Svennerholm method described below.

3.9. Modified Svennerholm method[31] for the analysis of sialic acid products

An aliquot (40 μL) of the enzymatic reaction mixture was added to a 1.5 mL solution of the resorcinol reagent (0.2% w.t. resorcinol, 80% v/v concd HCl and 0.25% v/v 0.1 M $CuSO_4$, in H_2O). After the mixture was heated in a boiling water bath for 30 min and cooled to rt, to it was added 3 mL of the extraction solvent (85:15 BuOAc–BuOH). The mixture was vigorously shaken on a vortex mixer, and after the mixture settled down, the two layers were carefully separated. The absorbance of the organic layer was measured at 580 nm with a UV–vis spectrophotometer using a 1.0-cm quartz cuvette with the extraction solvent as blank. The calibration curves of all sialic acid derivatives were obtained by the same measurement.

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