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An Expeditious Route to *N*-Glycolylneuraminic Acid Based on Enzyme-catalyzed Reaction

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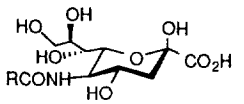
Abstract: A new preparative way of *N*-glycolylneuraminic acid (NeuGc), one of the important family of sialic acids, from *N*-acetylglucosamine (GlcNAc) *via N*-acetylmannosamine (ManNAc) was established based on the combination of chemical and enzymatic reactions. In a kinetic study of the key enzymatic reaction for this process, aldolase-catalyzed synthesis of sialic acid, an inhibitory effect of *gluco*-isomer on the enzymatic reaction was quantitatively clarified, and the importance of isomerically pure substrate with *manno*-configuration for aldolase-catalyzed reaction was suggested. A newly developed method, selective degradation of GlcNAc contaminating in the substrate by use of *Rhodococcus rhodochrous* IFO 15564 provided pure ManNAc to avoid such inhibitory effect of the *gluco*-isomer for aldolase. Starting from pure ManNAc, *via* mannosamine hydrochloride, acetoxyacetyl chloride was applied for introducing a protected form of glycolyl group to give *N*-acetylglycolylmannosamine. For the removal of acetyl protective group, a lipase from *Aspergillus niger* was effectively used under a mild and neutral condition to afford *N*-glycolylmannosamine (ManNGc), the substrate of aldolase. NeuGc was prepared in 25% yield and 7 steps from GlcNAc.

Introduction

N-Glycolylneuraminic acid (NeuGc, **1a**)[2] has been characterized as one of the family of sialic acids.[3] In contrast to *N*-acetylneuraminic acid (NeuAc, **1b**), the distribution of NeuGc is rather limited to glycolipids and glycoproteins in certain species of animals[4] and specific organs.[5] However, the NeuGc-containing glycoconjugates have important biological relevance, for example, as a marker of cancer,[6] and in the infection of influenza virus from animals to human. Thus, increasing interests are currently centered at the synthesis[7] and biology[8] of NeuGc and the corresponding glycoconjugates.

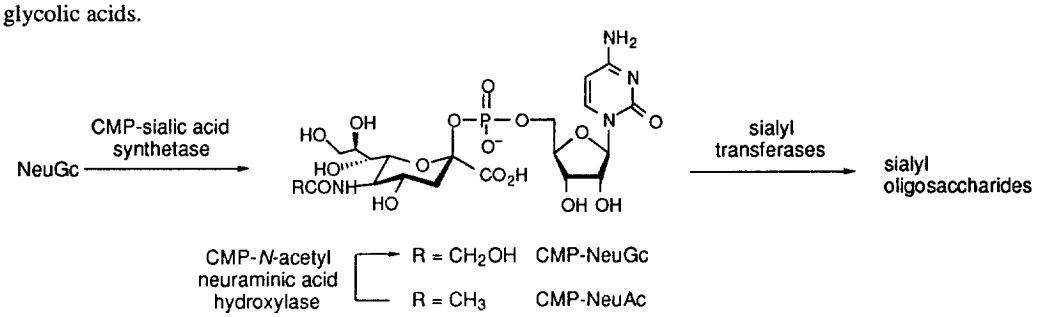


1a : R = CH_2OH *N*-glycolylneuraminic acid (NeuGc)
1b : R = CH_3 *N*-acetylneuraminic acid (NeuAc)

Figure 1

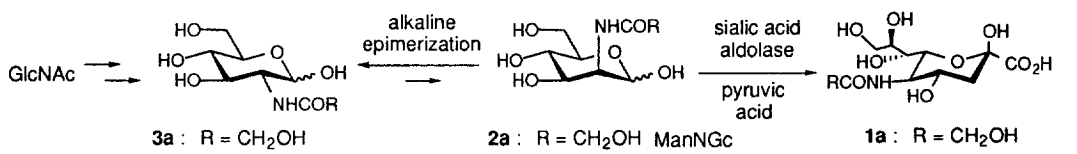
Enzyme-catalyzed syntheses have emerged as one of the viable approaches to the oligosaccharide synthesis, whose potential has been particularly highlighted in the synthesis of NeuAc-containing oligosaccharides.[9] Such enzyme-based synthesis follows the biosynthetic pathway: 1) the formation of activated form of sialic acid by cytidine monophosphoneuraminic acid (CMP-sialic acid) synthetase followed by 2) regiospecific glycosylation of sugar acceptor by sialyl transferases. In the present context, it is important to point out that NeuGc is well accepted by this enzyme,[10] although it is not the natural substrate of CMP-sialic acid synthetase. The resulting activated form (CMP-NeuGc) works as the substrate for sialyl transferases.[10,11] This fact is the possible basis of enzymatic syntheses of NeuGc-related oligosaccharides. Indeed, an effort aiming a preparative-scale synthesis of CMP-NeuGc in a chemical manner[12] has also been reported.

In the biosynthesis[13] of NeuGc-containing oligosaccharides, an extra hydroxyl group of NeuGc is introduced on the *N*-acetyl group at the stage of CMP-NeuAc (Scheme 1). As readily judged from this situation, there is no abundant source and/or precursors of NeuGc in nature.[14] At present, NeuGc and its glycosides are mostly supplied from NeuAc by chemical synthesis, which requires multi-steps and tedious procedures: after glycosidic bond formation, the *N*-acetyl group is removed and replaced with protected forms of glycolic acids.



Scheme 1

Sialic acid aldolase-catalyzed synthesis of NeuGc starting from *N*-glycolylmannosamine (ManNGc, **2a**) which merits simple procedure was reported by Augé and co-workers (Scheme 2).[10,15] However, two drawbacks have been left unsolved. The first limiter is the low availability of the substrate, ManNGc, and the second is the low efficiency of the aldolase-catalyzed reaction. Herein we report our investigation on a preparative-scale synthesis of NeuGc based on the sialic acid-aldolase catalyzed reaction that is able to solve these two problems.

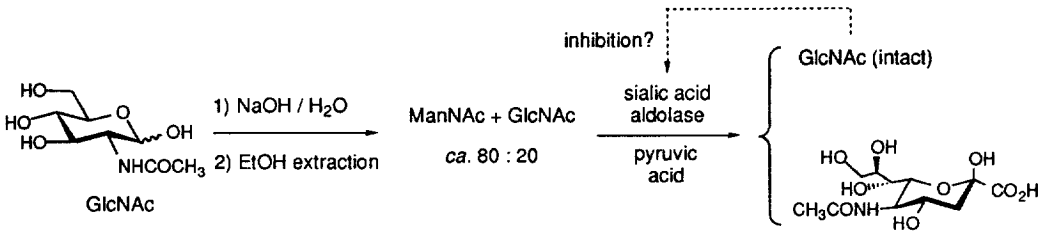


Scheme 2

Problem in sialic acid aldolase-catalyzed synthesis: Inhibitory effect of *gluco*-isomer

Sialic acid aldolase-catalyzed reaction between sugars and pyruvic acid has recently been widely applied for synthesizing sialic acids and related compounds,[10,15-22] because of the increasing availability of the enzyme.[23] A distinctive advantage of this enzymatic reaction comes from the substrate specificity of this enzyme: only *manno*- isomer provides sialic acid while *gluco*- isomer remains intact, and accordingly, an epimeric mixture of *manno*- and *gluco*- isomers can be employed as the substrate (Scheme 2). This specificity allowed the development of a combination of aldolase-catalyzed reaction and the *in situ* epimerization process of *gluco*- to *manno*-isomer by enzymatic[20] or chemical[21] means.

ManNAc, the direct precursor for NeuAc, is conventionally prepared from inexpensive GlcNAc and its derivatives by way of an epimerization at 2-position.[19-22,24-28] The alkaline epimerization of GlcNAc itself, which affords a thermodynamically equilibrated mixture of *manno*- and *gluco*-isomers (*ca.* 20 : 80), has been developed by Kuhn,[26] Roseman,[27] and Whitesides'[19] groups. After extraction with a proper solvent system, the ratio of *manno*- and *gluco*-isomers can be raised to *ca.* 80 : 20, which serves as the substrate for aldolase-catalyzed reaction[19,22,27] (Scheme 3).



Scheme 3

While it has been generally recognized that the coexisting *gluco*-isomer is intact in the aldolase-catalyzed reaction, which justified the use of a mixture of isomers as the substrate, we observed that the enzyme reaction became considerably slow. This experience raised the following question: Does *gluco*-isomer really have an inhibitory effect? Does *gluco*-isomer bind with sialic acid aldolase or not?

If this enzyme reaction is applied to the substrates whose reaction rate is slow, such inhibitory effect would become very serious, and we should start from isomerically pure *manno*-isomer in order to maximize the efficiency of aldolase-catalyzed reaction. Many reports described that the *gluco*-isomer had little, if any, interaction to the aldolase. There has been a comment by Kragl and Wandrey : GlcNAc has a weak inhibitory effect (19%) on the aldolase at a concentration of 200 mM.[20]

Thus, we decided to confirm the inhibitory effect of GlcNAc on sialic acid aldolase. Toward this end, the kinetic studies of sialic acid aldolase-catalyzed reaction were performed in the presence of various concentration of GlcNAc. Mannose (**4**) was used as the substrate, instead of ManNAc, because of a high cost of ManNAc in pure state. It has been reported that the reaction rate of mannose was comparable to ManNAc.[17,18]

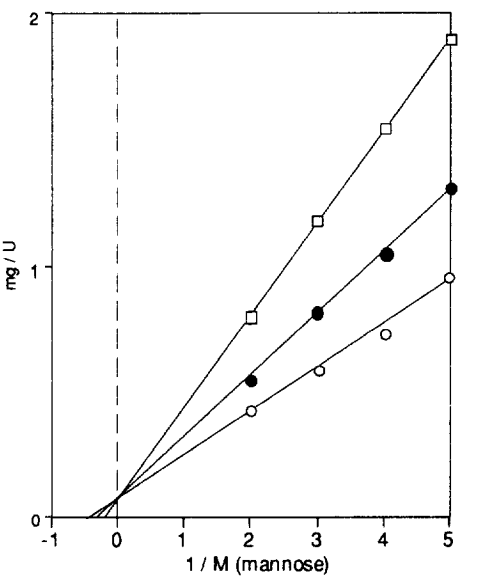


Figure 2. Lineweaver–Burk plot of the measurement of sialic acid aldolase-catalyzed reaction at the varied concentration of GlcNAc.
□ : GlcNAc 0.8 M; ● : GlcNAc 0.4 M; ○ : GlcNAc 0 M.

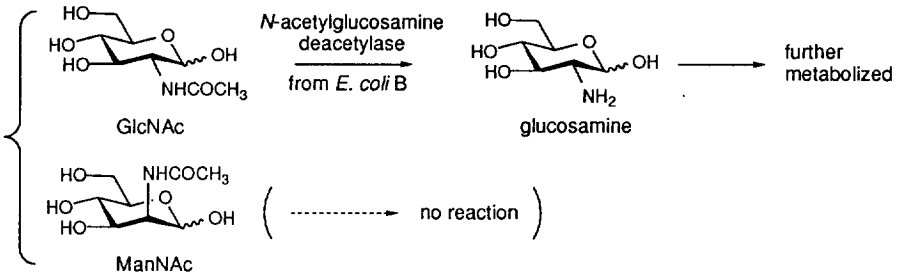
The results were shown in a Lineweaver–Burk plot (Figure 2). Km and Vmax values determined were 3.3 M and 41.7 units per mg, and were in good accordance with those reported previously by Wong and co-workers.[18] GlcNAc works obviously as a competitive inhibitor to the aldolase, Ki value being 0.85 M. Comparing with the reported Km value of ManNAc (0.7 M),[18] it is concluded that GlcNAc and ManNAc interact with sialic acid aldolase in a similar extent.

This result indicates that a complete conversion of the substrate would not be achieved if *gluco*- and *manno*-mixture is used as the substrate. Our observation also accounts for a low yield (59%) of NeuGc in case of using a mixture of ManNGc and GlcNGc (3 : 2) as the substrate.[10] Thus, we came to the conclusion that

the use of a pure *manno*-isomer would enhance the efficiency of aldolase-catalyzed synthesis of sialic acid and its analogs.

Pure ManNAc: the use of *N*-acetylglucosamine deacetylase from a new source of enzyme

We have already established an expeditious preparation of a mixture of ManNAc and GlcNAc in a ratio of 90 : 10 to 80 : 20 based on the Ca^{2+}-catalyzed epimerization of GlcNAc.[28] Prior to the aldolase-catalyzed reaction, we need, however, to remove the undesired *gluco*-isomer from this mixture,[cf. 29] as discussed above.



Scheme 4

The pioneering work of Roseman gave us an important clue to solve this problem. In the late 1950's, through extensive studies on the deoxy amino sugars, Roseman reported a biocatalytic metabolic degradation of GlcNAc (Scheme 4). For example, *E. coli* B possesses an *N*-acetylglucosamine deacetylase as the key enzyme. Due to the substrate specificity of this enzyme, GlcNAc could be selectively degraded from a mixture with ManNAc and thus, pure ManNAc is available.[30,31]

We became interested in this enzyme[32] and decided to secure a new source of enzyme which shows a higher and selective degrading activity to GlcNAc. Through a screening of microorganisms, *Rhodococcus rhodochrous* IFO15564[33] was found as a potent candidate. With this desired microorganism in hand, we then further optimized the incubation conditions as is indexed by the degrading activity of GlcNAc itself.

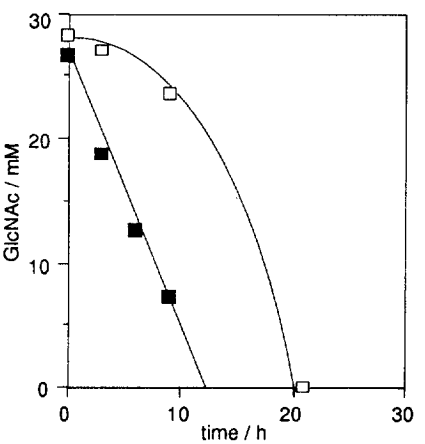


Figure 3. Incubation of GlcNAc with the cells of *R. rhodochrous*. □: incubation with the cells grown on glucose; ■: incubation with the cells grown on GlcNAc at the stage of pre-incubation.

Effect of inducer. Through an experiment with cells pre-incubated in a glucose medium, we became aware of that the consumption of GlcNAc during the incubation with this microorganism was slow at the initial stage, and then accelerated after *ca.* 10 h from the beginning of the incubation (Figure 3). This observation suggested that GlcNAc works not only as the substrate, but also as the inducer of the metabolic degradation system. As expected, it was revealed that the cells grown on GlcNAc itself showed a much higher degrading activity of GlcNAc from the beginning of incubation (Figure 3).

The stage of the growth for the highest degrading activity. In a glucose medium, the growth (30 °C) of this microorganism reached the stationary phase with an OD_{660} of *ca.* 15, after 40 h from the beginning. The cells at the end of growth phase were more active for the degradation than those at the stationary phase, and the ones which had been once frozen and thawed. The growth of this microorganism in GlcNAc medium was similar with that in glucose medium and the activity was quite high (Figure 3).

Optimum pH for the metabolic degradation of GlcNAc. When the reaction was carried out under slightly acidic condition (pH 6), the degrading activity was 1.8 times as high as that in alkaline medium (pH 8).

Effect of cell concentration during the metabolic degradation. Next, the cell concentration for the efficient degradation of GlcNAc was optimized. We performed experiments with the various cell concentration with the substrate concentration being fixed. The results are listed in Table 1. The optimum cell concentration for the high degradation activity turned out to be 125 g per liter, which means that harvested grown cells should be re-suspended in a buffer solution with a volume of 1/4 of the pre-cultivation broth.

Table 1. Relative rate for degrading activity of GlcNAc with resting cells of *R. rhodochrous*[a)]

concentration of cell (g / L)	relative rate
31.3	1.0
62.5	2.0
125	5.1
250	2.4
325	0.47

a) For the reaction conditions, see experimental.

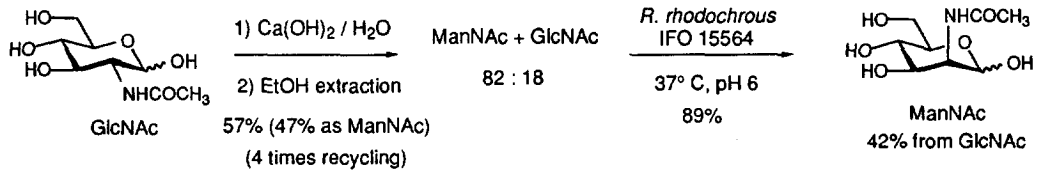
Table 2. Comparison between *E. coli* B and *R. rhodochrous* IFO 15564 in regard to degrading activity of GlcNAc[a)]

strain	units[b)]	recovery (%) of GlcNAc[c)]
E. coli B	31.4	22.4
R. rhodochrous IFO 15564	89.8	0

a) All the experiments were carried out by using cells from 200 mL of the culture broth. For the detail of reaction conditions, see experimental.
b) Unit = mg of degraded GlcNAc per 1 h.
c) The amount of remaining GlcNAc was determined after 21 h from the beginning of the reaction.

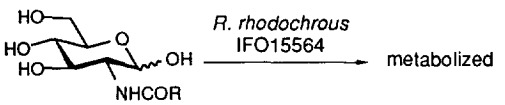
Preparative-scale synthesis: a comparison with *E. coli* B. Table 2 shows the best result using *R. rhodochrous* , together with the one which has been reported by Roseman by using *E. coli* B. *R. rhodochrous* showed obviously a higher activity for degrading GlcNAc. In addition, it was revealed that *ca.* 1/5 of fed GlcNAc still remained under Roseman's conditions, even after a prolonged incubation.

We applied the established method to the preparative-scale synthesis of ManNAc. Starting from an 82 : 18 mixture of ManNAc and GlcNAc, pure ManNAc was obtained in 89% yield (Scheme 5). For preparing 1 g of pure ManNAc, 360 mL of the culture broth of *R. rhodochrous* was necessary. We found that the degrading activity was influenced by the aeration into the incubation broth during the reaction, especially in a large-scale experiment. This procedure, combined with the calcium hydroxide-catalyzed epimerization of GlcNAc,[28] afforded pure ManNAc in 42% overall yield (see experimental). We thus established a preparative-scale synthesis of ManNAc, which had been found as a component of bacterial polysaccharide,[34] and would be useful as the starting material in synthetic organic chemistry,[35] as the probe for substrate-enzyme interaction,[36] as inducer of enzymes,[37] and as an accelerator of regeneration of dentine.[38]



Scheme 5

Substrate specificity. We should add here that this new microorganism also showed the degrading activity toward some related glucosamine derivatives (Scheme 6). When the substituent on amino group was changed from acetyl to glycolyl (**3a**) or acetylglycolyl group (**3c**), both amino sugars worked as the substrates as shown in Table 3, although the rates were lower than that of GlcNAc itself.



3a : R = CH_2OH GlcNGc
3b : R = CH_3 GlcNAc
3c : R = CH_2OAc GlcNGcAc

Scheme 6

Table 3. Relative rate for degrading activity of glucosamine derivatives with resting cells of *R. rhodochrous*[a)]

substrate	relative rate
GlcNGc	29
GlcNAc	100
GlcNGcAc	15

a) For the reaction conditions, see experimental.

Chemo-enzymatic preparation of pure ManNGc and NeuGc, from pure ManNAc

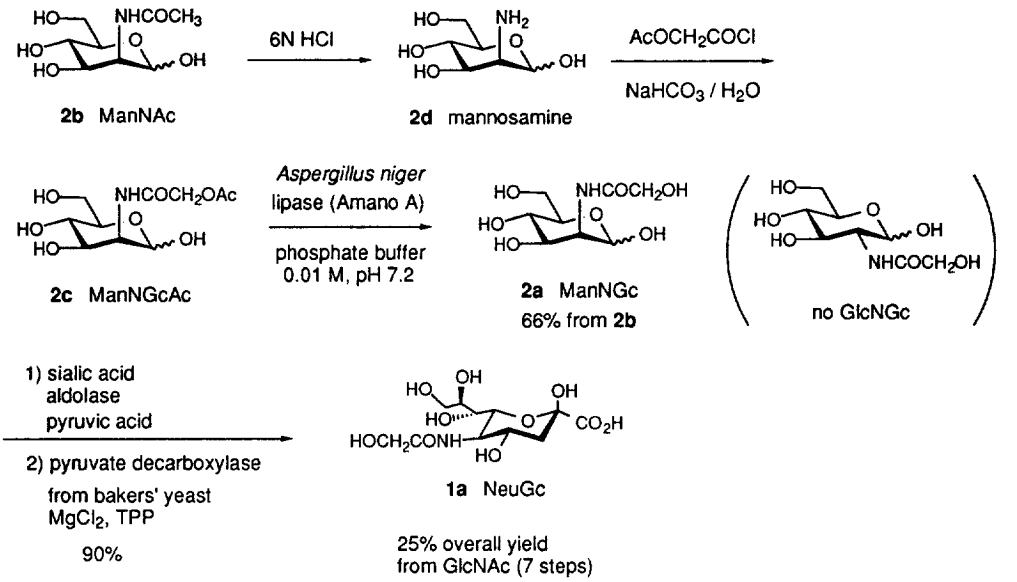
As mentioned before, it is desirable that the substrate for the sialic acid aldolase-catalyzed reaction is pure ManNGc, *i.e.*, free from the corresponding *gluco*-isomer. For this reason, the starting material was decided to be pure ManNAc, which is now available as above. Moreover, the use of inexpensive and commercially available acetoxyacetyl chloride as the glycolylating reagent of mannosamine (**2d**) was proposed, in place of 1,3-dioxolane-2,4-dione[15,39] and *p*-nitrophenyl glycolate[15] which are expensive and unstable.

The hydrolysis of acetamide group in ManNAc (**2b**) was performed with 6 N hydrochloric acid at 80 °C for 50 min to give mannosamine (**2d**) as its hydrochloride. Prolonged reaction brought about a complex mixture of the products. Subsequent treatment with acetoxyacetyl chloride in saturated sodium hydrogen carbonate solution[*cf.*40] provided *N*-acetylglycolylmannosamine (ManNGcAc, **2c**) in 51% total yield from **2b**.

Only the hydrolysis of the acetyl protective group of **2c** would provide the desired product, ManNGc (**2a**). This step, however, turned out to be troublesome, because ManNGc is prone to undergo epimerization to the thermodynamically stable *gluco*-isomer. Even under mild basic conditions such as in sodium hydrogen carbonate solution at room temperature, the corresponding *gluco*-isomer was detected before completion of the hydrolysis. On the other hand, under acidic conditions, concomitant cleavage of the amide group occurred and the reaction gave a complex mixture.

At this point, we turned our attention to enzymatic hydrolysis[41] of the acetyl protective group under neutral conditions. An *Aspergillus niger* lipase (Amano A) turned out to be most effective through screening of lipases, proteases and related enzymes as well as microorganisms. The hydrolysis smoothly proceeded

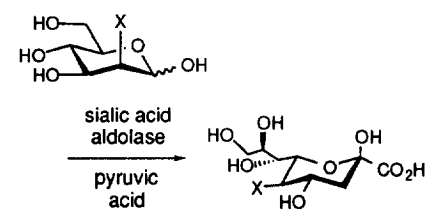
without any epimerization to give ManNGc (**2a**) in 75%. When the crude products at each stage were used without further purification, **2a** was obtained in 66% total yield from **2b** (Scheme 7).



Scheme 7

As pure ManNGc (**2a**) and ManNGcAc (**2c**) became in hand, their relative rate of reaction as the substrate on sialic acid aldolase were compared to that of ManNAc (**2b**) and mannose (**4**, Scheme 8), naturally abundant substrates. The results in Table 4 show that ManNGc is a slightly poor substrate compared with ManNAc.

The aldolase-catalyzed reaction of ManNGc (**2a**) in a preparative-scale was examined. As expected, the use of pure *manno*-isomer as the substrate much improved the efficiency of the reaction (90%) than that where the epimeric mixture was employed as the substrate.[10] In a large-scale preparation, the removal of excess pyruvic acid by means of pyruvate decarboxylase from bakers' yeast facilitated the purification of **1**.[28] In contrast to the previous results where 40% and 22% of the product (NeuAc and KDN, respectively) had been lost in this step,[28] the decarboxylative removal of excess pyruvate worked very well resulting in a quantitative recovery of NeuGc. All the reactions from GlcNAc could be totally combined and the yield of NeuGc (**1**) is 25% in 7 steps from GlcNAc.



2a : X = $NHCOCH_2OH$ ManNGc
2b : X = $NHCOCH_3$ ManNAc
2c : X = $NHCOCH_2OAc$ ManNGcAc
4 : X = OH mannose

Scheme 8

Table 4. Relative rate for aldolase-catalyzed synthesis of sialic acid derivatives[a)]

substrate	relative rate
ManNGc	58
ManNAc	100
ManNGcAc	31
mannose	80

a) For the reaction conditions, see experimental.

Conclusion

A new preparative way of NeuGc has been established, which is characterized by several advantages: 1) Inexpensive GlcNAc and acetoxyacetyl chloride are used as the starting material and the glycolylating reagent, respectively; 2) A new method provides pure ManNAc and ManNGc as intermediates, which are devoid of the *gluco*-isomer with the inhibitory effect in sialic acid aldolase-catalyzed reaction; 3) All the reactions can be carried out in water, because the carbohydrate intermediates carry the least protective groups on their hydroxyl groups.

EXPERIMENTAL

^1H and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz on a JEOL JNM-GX 400 or JNM α-400 spectrometer. Acetonitrile (δ 1.7) was used as an internal standard for the chemical shifts of ^{13}C NMR in D_2O. Column chromatography was carried out with Katayama 60 K070-WH silica gel of 70-230 mesh. Absorbance in UV and visible light were recorded on a JASCO UVIDEC 610A or Shimadzu UV-2100S spectrometer. AC-220-10 on Asahi Chemical Micro Acylyzer S1 was used for desaltation. Amicon Stirred Ultrafiltration Cell 8200 was used for ultrafiltration.

Kinetic measurements. The rates of aldolase-catalyzed reactions were determined by measuring the amount of remaining pyruvic acid.[18] The reactions were carried out in 1 mL of 0.1 M phosphate buffer (pH 7.5) containing: varied concentration of sodium pyruvate (2.0, 3.33, 5.0, and 10.0 mM); varied concentration of mannose (0.20, 0.25, 0.33, and 0.50 M); varied concentration of GlcNAc (0, 0.40, and 0.80 M). Each solution was incubated at 37 °C. A small aliquot (25-100 μL) was withdrawn periodically and mixed with 1.4 mL of 0.1 M phosphate (pH 7.5) buffer containing 0.3 mM NADH, and 20-30 units of L-lactate dehydrogenase. The amount of unreacted pyruvic acid was determined from the observed decrease in absorbance at 340 nm based on 6220 $M^{-1}cm^{-1}$ for the molecular absorbance of NADH. The kinetic parameters were obtained from the Lineweaver–Burk plots as shown in Figure 1.

For the relative rate measurements, the concentration of sodium pyruvate and sugars (**2a**, **2b**, **2c**, **4**) were fixed at 10 mM and 0.25 M, respectively. Other conditions were the same as above.

Incubation of the microorganism. The incubation of *Rhodococcus rhodochrous* IFO 15564 was carried out according to the reported procedure[33] with a slight modification. The ingredients of the sterilized medium were as follows: $MgSO_4 \cdot 7H_2O$ (500 mg), yeast extract (1 g), KH_2PO_4 (4.10 g), and K_2HPO_4 (1.22 g) in distilled water (900 mL), at pH 7.2. To a 90 mL of this medium in a 500-mL Erlenmeyer flask with two internal projections, were added glucose or GlcNAc (1.5 g, in 10 mL of water) through a sterilized cellulose acetate filter (Advantec TOYO, DISMIC-25cs), and a loopful of *R. rhodochrous* IFO 15564 was inoculated. The flask was shaken at 174 r.p.m. on a gyrorotary shaker at 30 °C. At the end of the growth phase (38-40 h), the OD660 reached 13-17. The cells from two incubation flasks were combined and harvested at 1870 x g for 20 min at 4 °C and washed with phosphate buffer (0.1 M, pH 6.0). The collected cells were then resuspended in the same buffer solution and the total volume of the mixture was adjusted to 50 mL.

The degrading reaction of GlcNAc from the mixture of GlcNAc (30 mM) and ManNAc (*ca.* 200 mM) was carried out by using cell suspension as above (50 mL) with stirring at 30 °C. The remaining amounts of GlcNAc (**3b**) and ManNAc (**3a**) were determined by ^1H NMR as follows: The sample solution (1.2 mL) was mixed with a solution of methyl β-D-glucopyranoside (13.3 mM, 250 μL) and centrifuged. From the resulting supernatant solution, a portion (1 mL) was taken and concentrated *in vacuo*. The residue was dissolved in D_2O (0.5 mL) and concentrated *in vacuo*. The substitution of exchangeable protons with deuterium was carried out once again. The determination of GlcNAc (**3b**) and ManNAc (**3a**) was performed by comparing

the integration of signals in ^{1}H NMR with that of methyl β-D-glucopyranoside: GlcNAc (**3b**) δ 4.68 (d, *J*= 8.6 Hz, H-1, β-isomer), 5.17 (d, *J*= 3.7 Hz, H-1, α-isomer); ManNAc (**3a**) δ 5.00 (d, *J*= 1.7 Hz, H-1), 5.10 (d, *J*= 1.5 Hz, H-1); methyl β-D-glucopyranoside δ 4.35 (d, *J*= 8.1 Hz, H-1). For the determination of other sugars (**3b, 3c**), the integration of observed anomeric protons were similarly compared with that of an added external standard. *E coli* B was incubated according to the reported procedure[31] and used for comparing its degrading activity.

***N*-Acetyl-D-mannosamine (ManNAc, 2b).** The incubation of *Rhodococcus rhodochrous* IFO 15564 was carried out as described above. The collected cells from four flasks were then resuspended in the same buffer solution and the total volume of the mixture was adjusted to 100 mL. To this was added a mixture of GlcNAc (550 mg, 2.5 mmol) and ManNAc (2.48 g, 11.2 mmol). This was divided into two Sakaguchi incubation flasks (500 mL volume) and the flasks were shaken on a reciprocal shaker 115 c.p.m at 37 °C for 8 h. The disappearance of GlcNAc was confirmed as described above. The cells were removed by centrifugation at 18780 x g for 20 min at 20 °C. The precipitated cells were further washed twice with water (30 mL). The combined supernatant and washing were concentrated to a small volume, and the residue was diluted with water (40 mL). At this point, the recovery of ManNAc was determined to be 93% by the NMR measurement with internal standard as stated above. Celite (4.5 g) was added to this solution, and the mixture was lyophilized at –20 °C overnight. The residual solid was charged on the column of silica gel (30 g) and eluted with a mixture of ethyl acetate-isopropyl alcohol-water (9 : 4 : 2). The combined eluates containing pure material were concentrated *in vacuo*, and diluted with water (100 mL). The resulting solution was desalted, and lyophilized at –20 °C overnight to afford ManNAc (**2b**, 2.59 g). The purity of this material was estimated to be 85% by the NMR measurement with internal standard and accordingly, the yield was 89%. No significant signals due to organic compounds were observed by ^{1}H NMR measurement.

2b: $[\alpha]^{22}_D$ +11.2° (c 1.85, H_2O, after 1 h equilibration as a solution) [lit.[31] $[\alpha]^{20}_D$ +9.7° (c 10 for monohydrate, H_2O, after equilibration as a solution)], ^{1}H NMR (400 MHz, D_2O) δ 5.09 (d, 0.56H, *J* = 1.2 Hz), 4.99 (d, 0.44H, *J* = 1.5 Hz), 4.41 (dd, 0.44H, *J* =3.2, 1.5 Hz), 4.28 (dd, 0.56H, *J* = 4.6, 1.2 Hz), 4.01 (dd, 0.56H, *J* = 9.8, 4.6 Hz), 3.87-3.78 (m, 2.56H), 3.77 (dd, 0.44H, *J* = 12.5, 4.9 Hz), 3.59 (dd, 0.56H, *J* = 9.8, 9.3 Hz), 3.49 (dd, 0.44H, *J* = 10.0, 9.8 Hz), 3.38 (ddd, 0.44H, *J* = 10.0, 4.9, 2.2 Hz), 2.06 and 2.02 (each s, total 6H); ^{13}C NMR (100 MHz, D_2O) δ 176.5, 175.6, 93.9, 93.8, 77.1, 72.9, 72.8, 69.7, 67.6, 67.3, 61.2, 54.9, 54.0, 22.9, 22.7. The ^{1}H NMR spectrum was in good accordance with that of an authentic sample (Sigma A8176).

On a large-scale incubation of *R. rhodochrous* IFO 15564, the seed culture (100 mL, after 24 h incubation with 6.8 of OD660) as obtained above was added to the 1000 mL of the same incubation medium containing GlcNAc (1.5%) in a 5 L Erlenmeyer flask with two internal projections. The flask was shaken at 250 r.p.m. at 30 °C for 24 h on a gyrorotary shaker. During the incubation, the flask was purged with air (6 L / min). At the end of the growth phase (22-24 h), the OD660 reached 13-17. The cells were harvested as above and re-suspended in a phosphate buffer solution (0.1 M, pH 6.0) and the total volume was adjusted to 500 mL. Then a mixture of GlcNAc (1.38 g, 6.2 mmol) and ManNAc (6.21 g, 28.0 mmol) was added and the mixture was poured into Erlenmeyer incubation flask with two internal projection (5 L volume). The flask was shaken on a gyrorotary shaker 250 r.p.m at 30 °C for 7 h with a continuous purge of air (6 L / min) in the flask. The recovery of ManNAc was determinated to be 85%. The subsequent workup was in the same manner as described above; ManNAc (**2b**, 5.74 g, 86% purity, 22.3 mmol, 80% yield).

***N*-Acetylglycolyl-D-mannosamine (ManNGcAc, 2c).** To a solution of **2b** (500 mg, 2.26 mmol) in water (1 mL) was added 6 N hydrochloric acid (1.5 mL) and the resulting mixture was stirred at 80 °C for 50 min. After cooling, hydrochloric acid was removed by an azeotropic concentration *in vacuo* with water and butanol

five times. The residue was dissolved in cooled water (20 mL) and $NaHCO_3$ (3.5 g) was added. After confirming its pH to be *ca.* 9, the mixture was stirred vigorously with ice-cooling and acetoxyacetyl chloride (Aldrich 30236-8, 1.3 mL, 12.1 mmol) was added in several portions. The mixture was further stirred for 20 min with ice-cooling. After the precipitate was filtered off, the mixture was neutralized by adding 2 N hydrochloric acid. The mixture was concentrated *in vacuo* to a small volume and diluted with water (30 mL). The resulting solution was desalted and concentrated *in vacuo*, and diluted with water (5 mL). To the solution, Celite (2.5 g) was added and the mixture was lyophilized at –20 °C overnight. The residual solid was charged on the column of silica gel (30 g) and eluted with ethyl acetate-isopropyl alcohol-water (27 : 8 : 4). The combined eluates containing pure material were concentrated *in vacuo*, diluted with a small volume of water, and lyophilized at –20 °C overnight to afford ManNGcAc (**2c**, 401 mg). The purity of this material was estimated to be 80% by the NMR measurement with internal standard and accordingly, the yield was 51%. No significant signals from organic compound were observed by ^1H NMR measurement, therefore the contaminant was concluded to be water.

$[\alpha]^{23}_D$ +7.7° (*c* 1.55, H_2O, after 1 h equilibration as a solution); ^1H NMR (400 MHz, D_2O) δ 5.11 (d, 0.59H, *J* = 1.5 Hz), 5.02 (d, 0.41H, *J* = 1.5 Hz), 4.71 (s, 0.82H,), 4.66 (d, 1.18H, *J* = 3.4 Hz), 4.33 (dd, 0.59H, *J* = 4.6, 1.5 Hz), 4.03 (dd, 0.59H, *J* = 9.8, 4.6 Hz), 3.87–3.79 (m, 2.59H), 3.76 (d, 0.41H, *J* = 12.2, 4.9 Hz), 3.58 (dd, 0.59H, *J* = 9.8 Hz), 3.48 (dd, 0.41H, *J* = 9.8 Hz), 3.39 (ddd, 0.41H, *J* = 9.8, 4.9, 2.4 Hz), 2.15 and 2.14 (each s, total 6H), ^{13}C NMR (100 MHz, D_2O) δ 174.4, 174.3, 172.4, 171.6, 93.9, 93.9, 77.4, 73.0, 69.8, 67.7, 67.5, 63.6, 63.6, 61.4, 55.2, 54.2, 21.0, 21.0. The ^1H NMR spectrum was in good accordance with that reported previously.[15]

***N*-Glycolyl-D-mannosamine (ManNGc, 2a).** Commercially available Lipase A (Amano Pharmaceutical, from *Aspergillus niger*) was treated as follows: Lipase (500 mg) was dissolved in a phosphate buffer (pH 7.2, 10 mM, 4 mL) was placed in a dialysis bag. This was dialyzed twice with the same buffer solution (400 mL) at 4 °C overnight.

To a solution of **2c** (198 mg, 0.71 mmol) in a phosphate buffer solution (pH 7.0, 1 M, 1.3 mL) was added the dialyzed lipase A solution as described above (3.6 mL). The mixture was diluted with water (4.6 mL) and was stirred at 37 °C for 36 h. Lipase was removed from the reaction mixture by dialyzing twice against water (240 mL). The mixture was concentrated *in vacuo* to a small volume and diluted with water (30 mL). The resulting solution was deionized concentrated *in vacuo* to a small volume (5 mL). To the solution, Celite (1.5 g) was added and the mixture was lyophilized at –20 °C overnight. The residual solid was charged on the column of silica gel (30 g) and eluted with ethyl acetate-isopropyl alcohol-water (9 : 4 : 2). The combined eluates containing pure material were concentrated *in vacuo*, diluted with water and lyophilized at –20 °C overnight to afford ManNGc (**2c**, 148 mg). The purity of this material was determinated to be 85% by the NMR measurement with internal standard and accordingly, the yield was 75%. No significant signals from organic compound were observed by ^1H NMR measurement.

$[\alpha]^{22}_D$ +10.8° (*c* 1.61, H_2O, after 1 h equilibration as a solution) [lit.[39] $[\alpha]^{25}_D$ +7.6° (H_2O, after equilibration as a solution)], $[\alpha]^{22}_D$ +13.9° (*c* 2.5, MeOH, after overnight equilibration as a solution) [lit.[12] $[\alpha]^{20}_D$ +5.4° (*c* 2.5, MeOH)], ^1H NMR (400 MHz, D_2O) δ 5.12 (d, 0.54H, *J* = 1.5 Hz), 5.04 (d, 0.46H, *J* = 1.5 Hz), 4.45 (dd, 0.46H, *J* = 4.4, 1.5 Hz), 4.33 (dd, 0.54H, *J* = 4.6, 1.5 Hz), 4.15 (s, 1.08H), 4.12 (dd, 0.46H, *J* = 9.5., 4.4 Hz), 4.11 (s, 0.92H), 4.05 (dd, 0.54H, *J* = 9.8., 4.6 Hz), 3.89-3.79 (m, 2.08H), 3.75 (dd, 0.46H, *J* = 12.2, 5.4 Hz), 3.58 (dd, 0.54H, *J* = 9.8 Hz), 3.48 (dd, 0.46H, *J* = 9.8, 9.5 Hz), 3.40 (ddd, 0.46H, *J* = 9.8, 5.4, 2.2 Hz). ^{13}C NMR (100 MHz, D_2O) δ 177.0, 176.1, 93.9, 93.9, 77.5, 73.1, 73.0, 69.8, 67.8, 67.6, 62.0, 61.9, 61.5, 61.4, 54.8, 53.8. The ^1H NMR spectrum was in good accordance with that reported previously.[10]

In a large-scale experiment, the purification at the stage of **2c** was omitted. Starting from **2b** (2.96 g, 13.4 mmol), **2a** (2.47 g, 85% purity, 8.8 mmol, 66% yield) was obtained. In this procedure, the removal of lipase A could be effectively performed by using ultra- filtration (Amicon YM10 Membrane, 4.5 kg/m^2).

The attempted hydrolysis of **2c** by using other enzymes (lipase from *Candida antarctica*, Novo Nordisk, PS from *Pseudomonas cepacia*, Amano Pharmaceutical, OF from *Candida cylindracea*, Meito, from pig pancreas, Sigma, protease N from *Bacillus subtilis*, Amano Pharmaceutical) was carried out in the similar manner as described above. Among them, *Candida antarctica* lipase and protease N were slightly active, but the activities were weaker than that of lipase A.

***N*-Glycolylneuraminic acid (NeuGc, 1a).** According to the reported procedure,[10] ManNGc (**2a**, 120 mg, 0.51 mmol) was treated with sodium pyruvate (419 mg, 3.80 mmol), sodium azide (2.6 mg, 0.03 mmol), phosphate buffer (0.1 M, 170 μL, pH 7.5), and sialic acid aldolase (TOYOBO NAL-301, 3 units) in water (2.5 mL) at pH 7.5 and stirred at 30 °C for 3 days. The pH was occasionally adjusted to 7.5 by 10 M sodium hydroxide. The conversion of NeuGc (**1a**) from **2a** reached to 100%, judging from ^{1}H NMR spectrum of the reaction mixture. The integration of the signals for **1a** (δ 1.82, H-3$_{ax}$ of β-isomer; δ 1.67, H-3$_{ax}$ of α-isomer), and methyl β-D-glucopyranoside (δ 4.36, internal standard) were compared.

Sialic acid aldolase was removed from the reaction mixture by ultra-filtration (Amicon YM10 Membrane, 4.5 kg/m^2). The resulting solution was purified by column chromatography on Dowex-1 (HCO_3^- form, 20-50 mesh; 70 ml, 2 cm x 20 cm) with ammonium hydrogen carbonate (0–0.2 M). The collected eluates were concentrated *in vacuo* to a small volume and diluted with water (10 mL). The resulting solution was desalted and lyophilized to give **1a** (ammonium salt, 157 mg, 99% purity, 0.46 mmol, 90% yield) as colorless amorphous solid. Its purity was determined by ^{1}H NMR with internal standard stated above. No significant signals from organic compound were observed by ^{1}H NMR measurement.

The aldolase-catalyzed reaction was scaled up by using **2a** (1.46 g, 6.17 mmol). In this experiment, the conversion of **1a** from **2a** reached to 93% after 48 h incubation at 30 °C. Then the excess pyruvate was decomposed by pyruvate decarboxylase-catalyzed reaction in a similar manner as already reported.[28]

Further purification of **1a** was achieved by column chromatography on Dowex-1 (HCO_3^- form, 20-50 mesh; 210 mL, 3 cm x 30 cm) with ammonium hydrogen carbonate (0–0.5 M). The collected eluates were concentrated *in vacuo* to a small volume and diluted with water (50 mL). The resulting solution was desalted and lyophilized to give **1a** (ammonium salt, 2.11 g, 90% purity, 5.55 mmol, 90% yield) as colorless amorphous solid. Its spectral properties were in good accordance with those already obtained.

$[\alpha]^{22}_D$ –26.9° (*c* 1.34, H_2O, after 1h equilibration as a solution) [lit.[10] $[\alpha]^{28}_D$ –25° (*c* 0.85, H_2O, after equilibration as a solution)], ^{1}H NMR (400 MHz, D_2O) δ 4.13 (ddd, 1H, *J* = 12.7, 11.5, 4.9 Hz), 4.12 (s, 2H,), 4.08 (dd, 1H, *J* = 10.3, 0.7 Hz), 3.98 (dd, 1H, *J* = 10.3, 10.0 Hz), 3.82 (dd, 1H, *J* = 11.7, 2.7 Hz), 3.74 (ddd, 1H, *J* = 9.0, 6.4, 2.7 Hz), 3.59 (dd, 1H, *J* = 11.7, 6.4 Hz), 3.50 (dd, 1H, *J* = 9.0, 0.7 Hz), 2.22 (dd, 1H, *J* = 12.7, 4.9 Hz), 1.82 (dd, 1H, *J* = 12.7, 11.5 Hz). ^{13}C NMR (100 MHz, D_2O) δ 176.4, 176.0, 96.8, 71.1, 70.9, 69.1, 67.6, 64.0, 61.8, 52.7, 40.0. The ^{1}H NMR and ^{13}C NMR spectra were in good accordance with those reported previously.[10]

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