Enzymes in Carbohydrate Synthesis: *N*-Acetylneuraminic Acid Aldolase Catalyzed Reactions and Preparation of *N*-Acetyl-2-deoxy-D-neuraminic Acid Derivatives

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Abstract: This paper describes the structural characteristics of substrates accepted by *N*-acetylneuraminic acid (Neu5Ac) aldolase (E.C. 4.1.3.3), the results from its stability studies, its use in the synthesis of Neu5Ac and 9-*O*-acetyl-Neu5Ac (Neu5,9Ac$_2$), and the chemical conversion of Neu5Ac to the 2-deoxy derivatives. Values of kinetic parameters (K_m and V_{max}) for 14 aldoses including *N*-acetyl-D-mannosamine (ManNAc) and pyruvate were determined at pH 7.5 and 25 °C in the direction of condensation. The 30–50-mmol-scale synthesis using ManNAc, excess pyruvate, and PAN-immobilized Neu5Ac aldolase provided multigram quantities of Neu5Ac (yield, 87–91% in solution and 67% in isolated products) without a significant loss of enzyme activity. The synthesis using two separate enzyme reactions, acetylation of ManNAc to 6-*O*-acetylManNAc catalyzed by protease N and condensation of 6-*O*-acetyl-ManNAc with pyruvate catalyzed by Neu5Ac aldolase, provided Neu5,9Ac$_2$ in 59% overall yield. To illustrate the utility of Neu5Ac as a synthetic starting material, a potential inhibitor of Neu5Ac-associated enzymes was prepared. Three chemical steps from Neu5Ac provided methyl 4,7,8,9-tetra-*O*-acetyl-*N*-acetyl-2-deoxy-α-neuraminic acid (2-deoxy-α-Neu4,5,7,8,9Ac$_5$OMe) in 50% overall yield. Its structure was analyzed by ^{1}H and ^{13}C NMR spectroscopy and X-ray crystallography.

Sialic acids, nonulosaminic acids, are a family of amino sugars that are derivatives of *N*-acetylneuraminic acid (Neu5Ac). They are widely distributed throughout the animal kingdom and play many important roles in molecular recognition.[1,2] For example, 9-*O*-acetyl-*N*-acetylneuraminic acid (Neu5,9Ac$_2$) is an essential determinant of the cell surface receptors of influenza viruses.[3,4] It is also present in the antigenic epitope of a ganglioside found in the developing rat embryonic cells.[5] Certain types of cancer cells have been shown to contain higher levels of sialic acids than the corresponding normal cells.[6]

N-Acetylneuraminic acid and derivatives are currently prepared from natural sources.[1] Although several chemical methods[7-9] are available for syntheses of these compounds, most of them require complex protection and deprotection methods. The small-scale procedure based on enzymatic catalysis[10-13] indicated that it may provide a better solution with a high degree of selectivity in reaction and simplicity in operation. In this paper, we describe a 30–50-mmol-scale synthesis of Neu5Ac from pyruvate and *N*-acetyl-D-mannosamine (ManNAc) catalyzed by Neu5Ac aldolase (see Table IA) and a small-scale (0.32-mmol) synthesis of Neu5,9Ac$_2$ from ManNAc by two separate enzyme reactions, catalyzed by protease N and Neu5Ac aldolase, respectively (Scheme I). We also describe the structural characteristics of sugar substrates accepted by Neu5Ac aldolase, the results from its stability studies, and the chemical conversion of Neu5Ac to the 2-deoxy derivative (methyl 4,7,8,9-tetra-*O*-acetyl-*N*-acetyl-2-deoxy-α-neuraminate, 2-deoxy-α-Neu4,5,7,8,9,Ac$_5$OMe).

Table I. Kinetic Parameters (K_m, M; V_{max}, U mg^{-1}) of Neu5Ac Aldolase for Several Substrates in the Aldol Condensation in 0.1 M Phosphate (pH 7.5 and 25 °C)

no.	substrate	K_m	V_{max}
	(A) Acceptor + Pyruvate		
1	*N*-acetyl-D-mannosamine (ManNAc)	0.7	25
2	*N*-acetyl-D-glucosamine		0
3	6-*O*-acetyl-ManNAc	0.5	3
4	D-mannose	2.8	50
5	D-glucose	2.3	1.8
6	6-*O*-acetyl-D-mannose	0.8	2.5
7	L-glucose		0
8	D-allose		0.1
9	2-deoxy-D-glucose	1.8	31
10	2-deoxy-D-galactose	1.3	4.5
11	L-fucose		0.9
12	D-lyxose	1.7	3.3
13	D-arabinose		0.8
14	L-xylose		0.3
15	2-deoxy-D-ribose		0.6
16	D-glyceraldehyde		0
17	L-glyceraldehyde		0
18	glycolaldehyde		0
	(B) Donor + ManNAc		
1	pyruvate	0.01	25
2	acetylphosphonate		0
3	3-fluoropyruvate[a]		0
4	3-bromopyruvate[a]		0
5	3-hydroxypyruvate[a]		0
6	acetopyruvate[a]		0
7	acetoacetate[a]		0
8	2-oxobutyrate[a]		0
9	phosphoenolpyruvate[a]		0

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We chose to explore Neu5Ac aldolase (E.C. 4.1.3.3) for two reasons. First, aldol condensations based on enzymatic catalysis[14-18] as a whole can provide an efficient alternative for the

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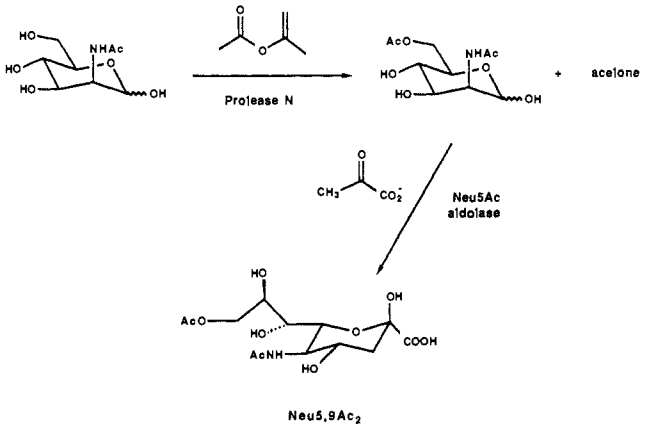
Scheme I. Two-Step Enzymatic Synthesis of Neu5,9Ac$_2$ from ManNAc



asymmetric C–C coupling, which is one of the most interesting and challenging problems in synthetic organic chemistry.[19-26] Most of the current methods used in the aldol reaction depend on the formation of metal–enolate complexes, which are unstable in aqueous media. Second, aldol condensation catalyzed by a specific enzyme, Neu5Ac aldolase, can provide a practical route to natural and unnatural Neu5Ac derivatives that are of great use in research biochemistry and medicinal chemistry.[1,2] The Neu5Ac derivatives can be used as a glycosyl donor via CMP-Neu5Ac for the enzymatic synthesis of oligosaccharides present in glycoproteins and glycolipids[27-29] or as a chiral template for the synthesis of some enzyme inhibitors including 2-deoxy-Neu5Ac (a potential inhibitor of CMP-Neu5Ac synthetase, a key enzyme in the biosynthesis of glyconjugates[30,31]).

Neu5Ac aldolase from microbial sources is commercially available and fairly inexpensive.[32] Industrial-scale hyperproduction of this enzyme by the gene-cloned strain of *Escherichia coli* (pNAL1) has recently been reported.[33] The *E. coli* enzyme is a trimer with a molecular weight of about 98 000.[34] The equilibrium constant[34] for the Neu5Ac aldolase catalyzed reaction in the direction of formation of Neu5Ac is 12.7 M^{-1}; this requires a 7–10-fold excess of pyruvate for the high conversion of the more expensive ManNAc to Neu5Ac.

Results and Discussion

Substrate Specificity. Seventeen carbohydrates and acetylphosphonate, in addition to the natural substrates ManNAc and pyruvate, have been tested as substrates for Neu5Ac aldolase. To obtain values for kinetic parameters (K_m and V_{max}), catalytic activities of the enzyme were measured in 0.1 M phosphate at pH 7.5 and 25 °C in the direction of condensation. Figure 1 shows representative kinetic data for the condensation of D-mannose with pyruvate catalyzed by Neu5Ac aldolase. Our results, together

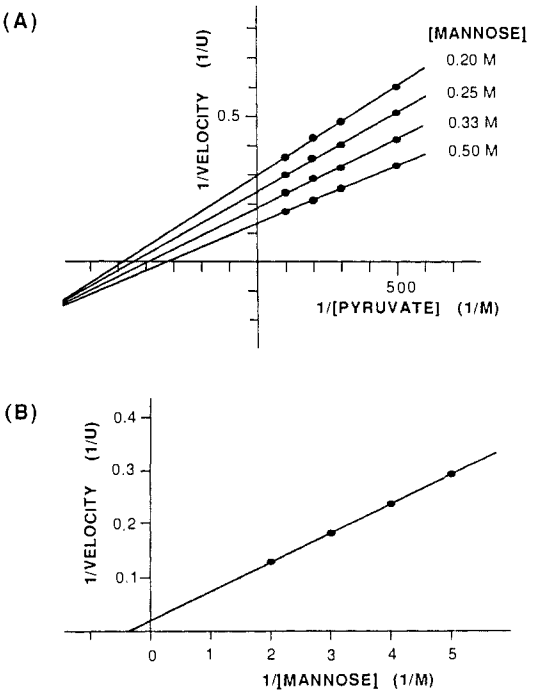


Figure 1. Lineweaver–Burk plots of the aldol condensation of D-mannose with pyruvate catalyzed by Neu5Ac aldolase in 0.1 M phosphate (pH 7.5 and 25 °C): (A) $1/V$ versus 1/[pyruvate] in the presence of different fixed concentration of D-mannose; (B) $1/V$ axis intercept replot against 1/[D-mannose].

with those obtained by others,[34] are summarized in Table I.

The most important observation from Table I is that this enzyme accepted 12 of the 15 tested aldoses as the electrophilic component but only pyruvate as the nucleophilic component. The second observation concerns the relationship between the reactivity and stereochemistry of the carbohydrates tested. The highly reactive substrates—ManNAc, D-mannose, 6-*O*-acetyl-D-mannose, and 2-deoxy-D-glucose—are stereochemically relevant; D-mannose and 2-deoxy-D-glucose express higher V_{max} values than the natural substrate. The moderately reactive ones with the exception of 6-*O*-acetyl-ManNAc are stereochemically close to those highly reactive substrates. Examples are D-glucose, 2-deoxy-D-galactose, and D-lyxose. The poorly reactive ones are stereochemically quite different from ManNAc though they retain the same stereochemistry at C-2. Example are L-fucose, D-arabinose, L-xylose, and 2-deoxy-D-ribose. *N*-Acetyl-D-glucosamine, L-glucose, and two- and three-carbon carbohydrates are unreactive. The third observation concerns the high values for the K_m of the reactive carbohydrates. They range from 0.5 to 2.8 M. The unexpectedly high values may indicate that the open forms of the sugars are the substrates. Since only a small portion of the aldose sugars exists in the aldehyde form in aqueous solution (for example, the composition of aldehyde form of D-mannose is 0.005% in D_2O[35]), high concentrations of the sugars are required to effect appreciable rates (Figure 1). We conclude from these substrate specificity studies that Neu5Ac aldolase accepts a fairly broad range of aldose sugars as the substrates. Table I summarizes the structural requirements of the substrates that are accepted by Neu5Ac aldolase at a rate >10% that of ManNAc.

Stability. The stabilities of *N*-acetylneuraminic acid aldolase in its soluble and insoluble PAN-immobilized forms were evaluated. The enzyme preparations were stirred in 50 mM phosphate (pH 7.5 and 23 °C) without protection from atmospheric oxygen. Aliquots of the mixtures were removed periodically and assayed for remaining enzyme activity. The results are described in Figure 2.

We conclude from this study that Neu5Ac aldolase is insensitive to oxygen and need not be used in degassed solutions or the

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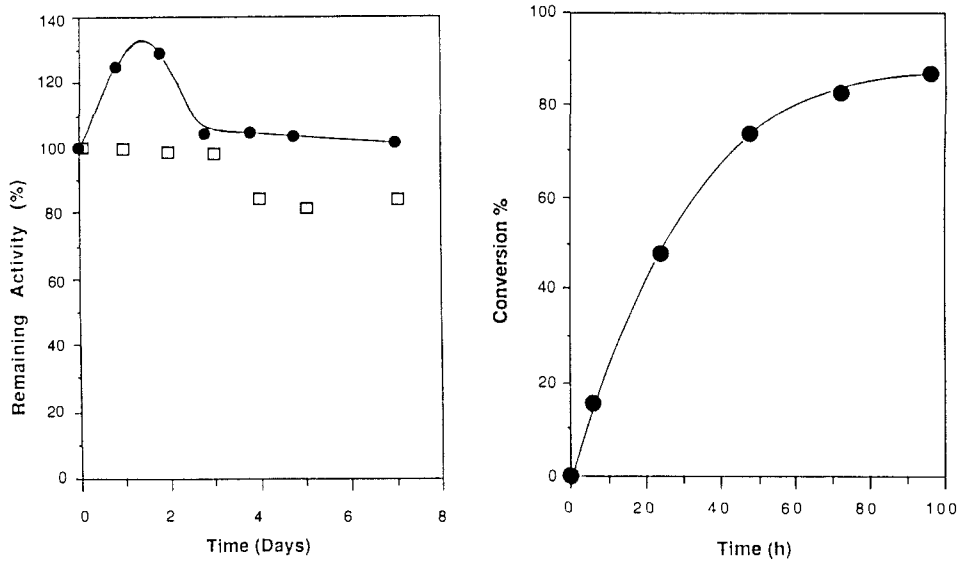

Figure 2. (Left) Stability of Neu5Ac aldolase in two different forms: soluble (□) and insoluble PAN-immobilized (●). The enzymes in 50 mM phosphate (pH 7.5) were stirred without protection against atmospheric dioxygen at 23 °C. The activities of enzymes with time were measured in the direction of the cleavage of Neu5Ac. The initial increase in activity for the immobilized enzyme may be due to the dispersion of the enzyme-containing gel particles into finer particles by stirring. (Right) Progression of Neu5Ac aldolase-catalyzed condensation of ManNAc with pyruvate to Neu5Ac (pH 7.5 and 25 °C). The initial solution (220 mL) contained 30 mmol of ManNAc, 210 mmol of pyruvate, and 30 U of enzyme.

presence of reducing agents such as dithiothreitol or β-mercaptoethanol. This study also established that Neu5Ac aldolase, in its insoluble PAN-immobilized form, has excellent stability under synthetic reaction conditions.

Synthesis of Neu5Ac. Two reactions for the synthesis of Neu5Ac were carried out with ManNAc and pyruvate as the substrates and insoluble PAN-immobilized Neu5Ac aldolase as the catalyst on 30–50-mmol scales. The first reaction was run under the following conditions (pH 7.5 and room temperature): 30 mmol of ManNAc, 210 mmol of pyruvate, 1 mmol of phosphate, and 30 U of enzyme in 220 mL of solution. The excess of pyruvate was required to drive the reaction toward product formation and maximize the consumption of the more expensive ManNAc substrate. The reaction was monitored by ^{1}H NMR spectroscopy, and the progression of the reaction is described in Figure 2. The reaction was stopped when about 90% of the initial ManNAc had reacted (4 days), the enzyme-containing gels were removed, and the products were isolated by ion-exchange column chromatography in 67% yield (based on ManNAc) and 97% purity. The recovery of enzyme was 97% as determined by enzymatic assay.

The second reaction was run with the recovered enzyme under the same conditions with exceptions in the amounts of substrates (40 mmol of ManNAc and 400 mmol of pyruvate) and the volume of solution (260 mL). The 10-day operation gave 91% conversion of ManNAc to Neu5Ac. The recovered enzyme retained 50% of its initial activity, and the products in solution were stored at −10 °C.

This synthesis deserves a few comments. First, the high conversion of the more expensive ManNAc to Neu5Ac was achieved by using a 7–10-fold excess of pyruvate. The excess of pyruvate did not make the isolation of products complicated. Second, it uses highly stable and fairly inexpensive microbial enzymes in the PAN-immobilized form. Thus, we believe that it is possible to prepare large quantities (>50 g) of products by repeating the use of the enzyme several times. Third, though we used the expensive commercial ManNAc as a starting material, it can be prepared readily by the base-catalyzed isomerization of *N*-acetyl-D-glucosamine (GluNAc).[9] On the other hand, the GluNAc epimerase-catalyzed isomerization[36] (when the enzyme is readily available) coupled with NeuNAc aldolase reaction might provide a better solution for the direct synthesis of NeuNAc from inexpensive GluNAc. Overall, this synthesis illustrates a practical use of Neu5Ac aldolase in the synthesis of carbohydrates.

Synthesis of 9-*O*-Acetyl-Neu5Ac (Neu5,9Ac$_2$). This synthesis comprises two separate enzyme-catalyzed reactions, the acetylation of ManNAc to 6-*O*-acetyl-ManNAc catalyzed by protease N and the condensation of 6-*O*-acetyl-ManNAc with pyruvate to Neu5,9Ac$_2$ catalyzed by Neu5Ac aldolase (Scheme I). Soluble enzymes were used for both of the reactions. The acetylation of ManNAc in the presence of isopropenyl acetate catalyzed by protease N provided 6-*O*-acetylManNAc in 70% yield with high regioselectivity. The condensation of 6-*O*-acetylManNAc with pyruvate catalyzed by Neu5Ac aldolase subsequently provided Neu5,9Ac$_2$ in 84% yield. This synthesis has the advantage that it requires only two enzymatic steps that are highly regio- and stereoselective. It eliminates the need for the complex, several-step chemical synthesis of 6-*O*-acetyl-ManNAc from ManNAc reported previously.[12] On the basis of our experience in the preparation of Neu5Ac described in the previous section, we believe that this synthesis provides a convenient route to gram quantities of Neu5,9Ac$_2$ from ManNAc.

Chemical Synthesis and Characterization of Methyl 4,7,8,9-Tetra-*O*-acetyl-*N*-acetyl-2-deoxy-α-D-neuraminate (2-Deoxy-α-Neu4,5,7,8,9Ac$_5$OMe). To illustrate the synthetic utility of Neu5Ac, we studied the synthesis of 2-deoxyNeu5Ac derivatives, potential inhibitors of Neu5Ac-associated enzymes, from Neu5Ac. Scheme II outlines the synthesis of 2-deoxy-α-Neu4,5,7,8,9Ac$_5$OMe (**5**). The methyl ester of Neu5Ac (**2**) was prepared by the method of Kuhn et al.[37] The ester was simultaneously acetylated and chlorinated by treatment with acetyl chloride at room temperature to give **3**.[38] Hydrogenolytic cleavage of the carbon–chlorine bond was achieved with hydrogen gas over 10% palladium-on-carbon in the presence of triethylamine. By this method **5** was obtained in 50% overall yield from Neu5Ac. The β anomer of 2-deoxyNeu4,5,7,8,9Ac$_5$ (**6**) was also obtained in approximately 5% yield. Separate experiments confirmed that the β anomer **6** is formed from the catalytic hydrogenation of the dehydro intermediate **4**.

The α anomer **5** was characterized by ^{1}H and ^{13}C NMR spectroscopy and by both low- and high-resolution mass spectroscopy. The proton-coupling relationships in **5** were established by a proton phase-sensitive double-quantum filtered homonuclear correlated spectroscopy (DQCOSY) experiment[39,40] run at 400

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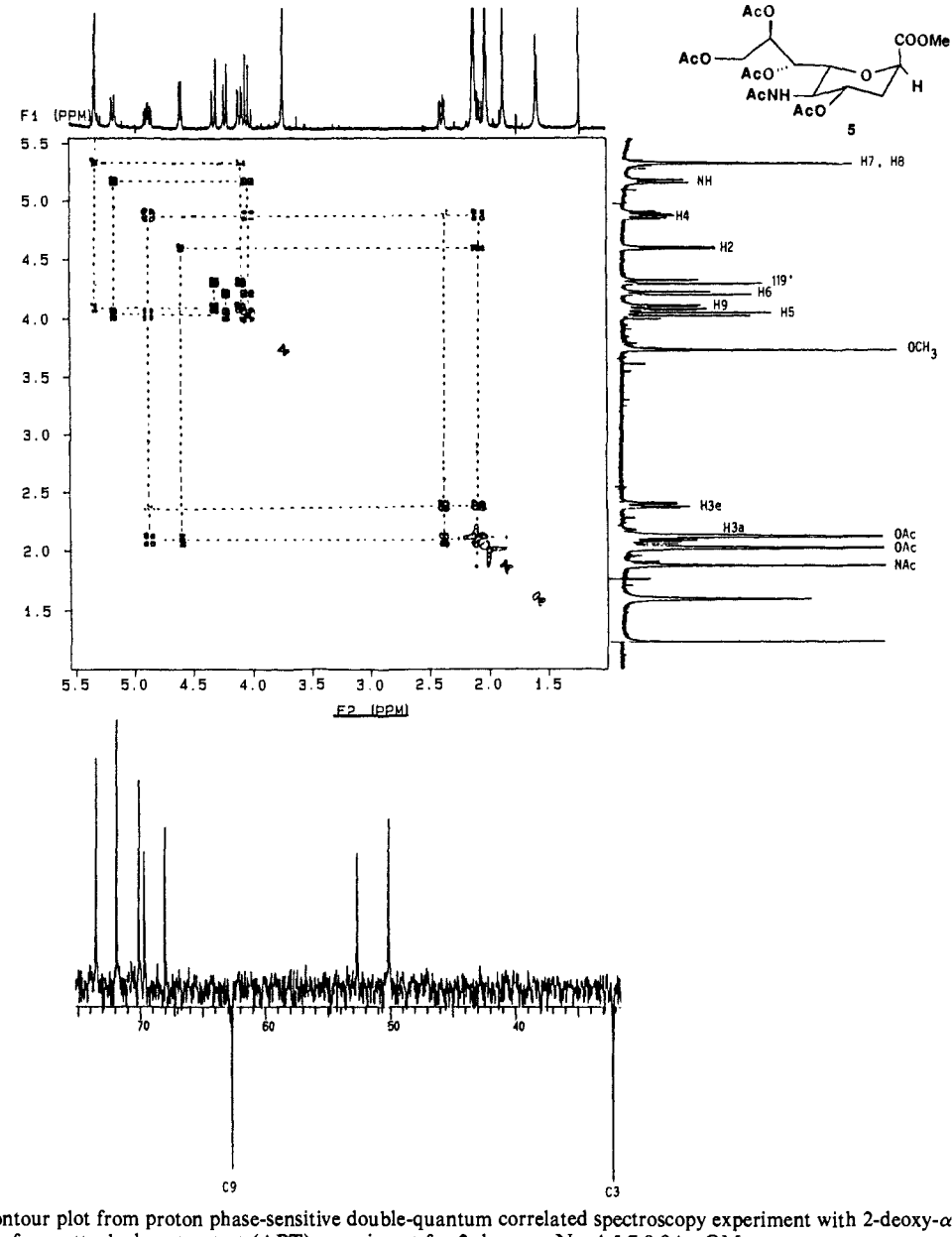

Figure 3. (Top) Contour plot from proton phase-sensitive double-quantum correlated spectroscopy experiment with 2-deoxy-α-Neu4,5,7,8,9Ac$_5$OMe. (Bottom) Spectrum from attached proton test (APT) experiment for 2-deoxy-α-Neu4,5,7,8,9Ac$_5$OMe.

MHz. Figure 3 (top) contains the contour plot from the DQCOSY experiment. The coupling correlations derived from the DQCOSY experiment clearly show that the new absorption at 4.59 ppm is coupled to H3e and H3a and to no other protons in the molecule, thus confirming the assignment of this absorption to the H2 proton. The observed coupling constants for the H2 proton in **5** were 6.8 and 1.2 Hz, respectively. The ^{1}H NMR spectrum of the minor product displayed a new absorption at 4.63 ppm with coupling constants of 12.2 and 3.2 Hz. The smaller coupling constants of the major product compared to the coupling constants of the minor product further confirm the major product to be the α anomer **5** and the minor product to be the β anomer **6**.

Figure 3 (bottom) contains a portion of the spectrum of **5** obtained from an attached proton test (APT)[41,42] run at 50 MHz. The inverted peaks in this spectrum correspond to the methylene and quaternary carbon absorptions; the upright peaks correspond to the methine absorptions. As expected, the absorption corresponding to the quaternary carbon in the starting material is absent, and an additional methine carbon absorption is observed.

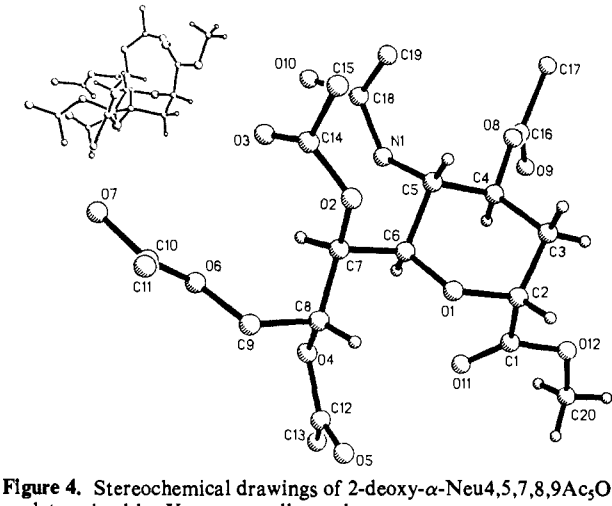

Figure 4. Stereochemical drawings of 2-deoxy-α-Neu4,5,7,8,9Ac$_5$OMe as determined by X-ray crystallography.

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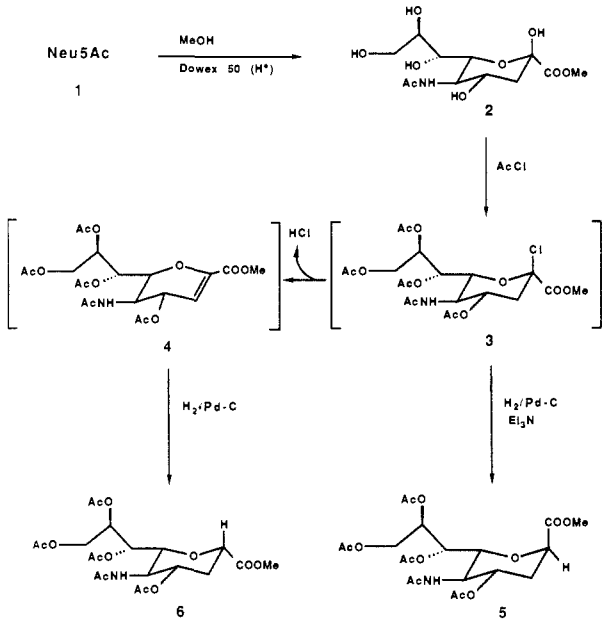
Scheme II. Synthesis of 2-Deoxy-α-Neu4,5,7,8,9Ac$_5$OMe



The structure of the α anomer in a chair conformation was confirmed by single-crystal X-ray crystallography[43] as shown in Figure 4.

Each of the following reactions were attempted[44] but failed to provide either **5** or **6**. The 2-OH group of Neu5Ac methyl ester tetraacetate was converted to the phenylthiocarbonate derivative and the mixed oxalate ester with *N*-hydroxy-2-thiopyridone. Neither of these compounds was successfully reduced in radical chain reactions with AIBN/Bu_3SnH or AIBN/*tert*-butyl mercaptan. The 2-chloride **3** was inert toward AIBN/Bu_3SnH or chromous acetate as was the 2-OH derivative in the presence of triethylsilane/BF_3. The 2-bromide was too labile to manipulate.

In summary, Neu5Ac aldolase has been proven to be another synthetically useful catalyst for C–C bond formation without the need of cofactors, especially in the synthesis of sialic acid-related carbohydrates. The 2-deoxy derivative **5** or **6** prepared from the enzyme-produced Neu5Ac may be an inhibitor of Neu5Ac-associated enzymes. Work is in progress to determine its biological activity.

Experimental Section

Materials and Methods. Neu5Ac aldolase (microorganisms, E.C. 4.1.3.3) was obtained from Toyobo. Protease N from *Bacillus subtilis* was obtained from Amano. L-Lactate dehydrogenase (rabbit muscle, E.C. 1.1.1.27) was obtained from Sigma. ManNAc, pyruvic acid sodium salt, and NADH were obtained from Sigma. Neu5Ac aldolase was immobilized as described elsewhere.[45] The purities of Neu5Ac and 9-*O*-acetylNeu5Ac were determined with Neu5Ac aldolase, L-lactate dehydrogenase, and NADH in a coupled reaction. Diethyl acetylphosphonate was prepared as described elsewhere.[46] Tetrahydrofuran and 1,4-dioxane were distilled from sodium–benzophenone ketal. Methanol was distilled from calcium hydride. Triethylamine was distilled from barium oxide. All other solvents and chemicals were of reagent grade and were used as received. UV absorbance changes were measured on a Beckman DU-70 spectrophotometer. ^{1}H NMR spectra were obtained on Varian XL-200 and XL-400 spectrometors at 200 and 400 MHz, respectively. ^{13}C NMR spectra were obtained on a Varian XL-200 spectrometer operating at 50 MHz. Low-resolution mass spectra were obtained on a Hewlett-Packard 5995C quadrupole gas chromatograph–mass spectrometer operating at 70 eV. High-resolution mass spectra were obtained on a VG Analytical 70S high-resolution double-focusing magnetic sector mass spectrometer also operating at 70 eV. Nicolet R3m/V X-ray diffractometer and SHELXTL (version 5) software were used in the single-crystal X-ray structure determination. Melting points were determined on a Fischer-Johns capillary melting point apparatus. Thin-layer chromatography was done on 0.25-mm layers of silica gel (60 Å) on glass-backed plates supplied by J. T. Baker Co., and the compounds were visualized by spraying the plates with a 10% sulfuric acid in ethanol solution and heating. Silica gel column chromatography was done with Baker flash chromatography silica gel (230–400 mesh).

Kinetic Measurements. The rates for aldolase-catalyzed reactions were obtained by enzymatically measuring the amount of reacted pyruvate. The reactions were carried out in 0.1 M phosphate (pH 7.5 and 25 °C) with the following: 0.03 mg of enzyme; 2.0, 2.5, 3.33, 5, and 10 mM pyruvate; 0.2, 0.25, 0.33, and 0.50 M carbohydrate in 3 mL of solution. Each solution was incubated at 25 °C. Periodically, a small aliquot (50–100 μL) was withdrawn and mixed into an assay solution (2.8 mL) containing 0.1 M phosphate (pH 7.5), 0.2 mM NADH, and 20–30 U of L-lactate dehydrogenase. The decrease in absorbance was measured at 340 nm and converted into the amount of the unreacted pyruvate using 6220 for the molecular absorbance of NADH. The kinetic parameters were obtained from the Lineweaver–Burk plots.

Stability. The enzyme-containing solutions (0.05 M phosphate, pH 7.5) were stirred at 23 °C. Periodically, a small aliquot (100 μL) was withdrawn and mixed into an assay solution (1.1 mL) containing 100 mM phosphate, 10 mM Neu5Ac, 0.2 mM NADH, and 20 U of L-lactate dehydrogenase. The decrease in absorbance was followed at 340 nm and 25 °C, and the activity of the enzyme was calculated from the change in absorbance.

Acetylphosphonic Acid. Diethyl acetylphosphonate (0.9 g, 5 mmol) was cooled to 0 °C under nitrogen, and bromotrimethylsilane (4 mL, 30 mmol) was added dropwise. The reaction mixture was allowed to stir at room temperature overnight. The excess of reagent was removed under reduced pressure. Aqueous $NaHCO_3$ (0.5 M, 10 mL) was added at 0 °C and the solution stirred for 1 h. After it was degassed under reduced pressure, the solution was freeze-dried to yield 0.8 g of a hygroscopic white solid: purity 60% (based on acetylphosphonic acid disodium salt as determined by ^{1}H NMR spectroscopy using pyruvate as standard); yield 57%; ^{1}H NMR (D_2O/DSS) δ 2.36 (d, J_{PCC} = 3.7 Hz, 3 H); ^{13}C NMR (D_2O/DSS) δ 33.0 (d, J_{PCC} = 43.5 Hz) and 231.4 (d, J_{PC} = 157 Hz). The product was tested as the substrate for Neu5Ac aldolase without further purification.

***N*-Acetylneuraminic Acid (Neu5Ac).** In a 500-mL, three-necked flask equipped with a magnetic stirring bar were placed 6.64 g (30 mmol) of *N*-acetyl-D-mannosamine, 24.1 g (210 mmol) of sodium pyruvate, 0.1 g of sodium azide, 140 mL of distilled water, and 10 mL of 0.1 M phosphate buffer (pH 7.5). The solution was adjusted to pH 7.5 with 1 N NaOH, and the volume of the solution was increased to 180 mL by the addition of distilled water. The reaction was initiated by the addition of 40 mL of enzyme gel suspension (30 U). The solution was stirred at room temperature, and the reaction was monitored by ^{1}H NMR spectroscopy as follows. Periodically, a small aliquot (200 μL) was withdrawn and the enzyme-containing gels were removed by centrifugation. The mother liquor was freeze-dried, and the solids were dissolved in D_2O-containing DSS. A ^{1}H NMR spectrum of this solution was recorded. The ratio of ManNAc to Neu5Ac was calculated based on the integrals of the peaks from the *N*-acetyl groups of both compounds (2.06 ppm for Neu5Ac, 2.10 ppm for ManNAc) and from C_2-methylene group of Neu5Ac. The reaction was stopped when the conversion of ManNAc to Neu5Ac reached 87% (4 days). The enzyme-containing gels were removed by centrifugation and washed twice with distilled water. The recovery of the enzyme was 97%. The combined mother liquor was subjected to Bio-Rad AG 1-X8 (formate form, 100–200-mesh) column chromatography as described elsewhere[47] to yield 6.21 g (20.1 mmol, 67%). The ^{1}H NMR spectrum of the product in D_2O was identical with that of authentic Neu5Ac and found to be 96% pure by enzymatic assay.

2-Acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose (6-*O*-Acetyl-ManNAc). Protease N (2 g) was dissolved in 0.1 M NaH_2PO_4 (35 mL), and the resulting solution was stirred for 15 min. The pH was then adjusted to 7.8 with 8 N NaOH and the solution freeze-dried. This freeze-dried preparation was used in the synthetic procedure. *N*-Acetyl-β-D-mannosamine monohydrate (Sigma) (478 mg, 2 mmol) was

(43) Details of crystal structure will be published separately.

(44) Although these reagents have been commonly used in deoxygenation of sugars, they did not work in this case: Robins, M. J.; Wilson, J. S.; Hansske, F. *J. Am. Chem. Soc.* **1983**, *105*, 4059. Ogilvie, K. K.; Hakimelahi, G. H.; Proba, Z. A.; Usman, N. *Tetrahedron Lett.* **1983**, *24*, 865. Lewis, M. D.; Cha, J. K.; Kishi, Y. *J. Am. Chem. Soc.* **1982**, 104, 4976. Motherwell, W. B. *Pure Appl. Chem.* **1981**, *53*, 15. Barton, D. H. R.; Basu, N. K.; Hesse, R. H.; Morehouse, F. S.; Pechet, M. M. *J. Am. Chem. Soc.* **1966**, *88*, 3016. The successful procedure we used for the deoxygenation proved to be very efficient. A similar procedure was used in the synthesis of 2-deoxy-KDO: Luthman, K.; Orbe, M.; Waglund, T.; Claesson, A. *J. Org. Chem.* **1987**, *52*, 3777.

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suspended in anhydrous *N*,*N*-dimethylformamide (2 mL). Isopropenyl acetate (600 mg, 6 mmol) was added followed by the enzyme preparation (600 mg). The suspension was shaken at 45 °C and monitored by TLC (silica gel; EtOAc/MeOH/H_2O = 100/10/1). After 44 h the suspension was filtered and the enzyme washed with methanol (2 × 3 mL). The solvents were evaporated under vacuum at 42 °C to give a yellow syrup. This syrup was fractionated on a silica gel column (45 g) eluted with EtOAc/MeOH/H_2O = 100/10/1. Two products were obtained: The first with a higher R_f corresponds to a triacetate compound (30 mg, 10%). The second (major) product was obtained as an amorphous white solid (384 mg, 73%): ^{1}H NMR (D_2O/*p*-dioxane = 3.57 ppm) δ 4.93 (s, H1α), 4.84 (s, H1β), 4.29–4.02 (m, 5 H), 3.87 (dd, H3α), 3.70–3.27 (m, 3 H), 1.95, 1.91, 1.88, and 1.87 (4 s, 6 H, acetyl); ^{13}C NMR (D_2O/*p*-dioxane = 67.46 ppm) δ 176.60, 175.67, 174.96, and 174.92 (all carbonyls), 94.05 (c1β), 93.97 (C1α), 74.78 (Cβ), 72.72 (Cβ), 70.57 (C5α), 69.43 (C^a), 67.94 (Cα), 67.78 (Cβ), 64.61 (C6α), 64.36 (C6β), 54.84 (C2β), 54.18 (C2α); α/β = 76/24; mp 47–51 °C; $[\alpha]^{24}_D$ +15.9° (*c* 1.13, H_2O). Anal. Calcd for $C_{10}H_{16}NO_7$: C, 45.80; H, 6.15; N, 5.34. Found: C, 45.89; H, 6.20; N, 4.95.

6-*O*-Acetyl-D-mannose. The 6-*O*-acetyl sugar was prepared from D-mannose on a 2-mmol scale by the same procedure as described for 2-acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose (vida supra): yield 180 mg (40%); colorless syrup; ^{1}H NMR (D_2O/*t*-BuOH = 1.15 ppm) δ 5.06 (d, H1α, *J* = 1.4 Hz), 4.81, (s, H1β), 4.38–4.07 (m, 3 H), 3.99–3.42 (m, 6 H), 1.97 (s, 3 H, acetyl); ^{13}C NMR (D_2O/*t*-BuOH) δ 175.67 and 175.61 (carbonyl), 95.67 (C1α), 95.30 (C1β), 75.02 (C5β), 74.34 (C3β), 72.58 (C2β), 72.05 (C5α), 71.58 (C2α), 71.49 (C3α), 68.21 (C4α), 67.98 (C4β), 65.22 (C6α,β), 21.93 (acetyl-β), 21.75 (acetyl-α); $[\alpha]^{24}_D$ +13.2° (*c* 1.1, H_2O) [lit.[48] $[\alpha]^{30}_D$ +14.7° (*c* 1.5, H_2O)]; α/β = 64/36.

9-*O*-Acetyl-*N*-acetylneuraminic Acid (Neu5,9Ac$_2$). To an aqueous solution (3 mL, pH 7.5) containing 0.1 M pyruvate and 0.02 M phosphate were added 6-*O*-acetyl-ManNAc (0.32 mmol) and Neu5Ac aldolase (2 mg). The solution was allowed to stand at room temperature overnight. A small aliquot 0.3 mL) from the solution was freeze-dried and the solid dissolved in D_2O. Analysis of the solution by ^{1}H NMR spectroscopy indicated that the reaction was complete. The D_2O solution combined with the mother liquor was filtered with an ultrafiltration cell to remove the enzymes, and the filtrate was chromatographed at 4 °C at a flow rate of 17 mL/h on a column (1.6 × 60 cm) containing Biogel P-2 equilibrated in water. The fractions containing Neu5,9Ac$_2$ as assayed with the orcinol reagent[47] were pooled and freeze-dried to yield 0.15 g of solid. Its ^{1}H NMR spectrum in D_2O was identical with that reported[49] and indicated that it was almost free from pyruvate. The enzyme assay indicates that it contains 0.27 mmol of product (yield 84%).

Methyl 4,7,8,9-Tetra-*O*-acetyl-*N*-acetyl-2-deoxy-α-D-neuraminate (2-Deoxy-α-Neu4,5,7,8,9Ac$_5$OMe, 5). A sample of methyl 4,7,8,9-tetra-*O*-acetyl-*N*-acetyl-2-chloro-β-neuraminate (3) was prepared from 200 mg of Neu5Ac by esterification of the acid function in methanol containing dried Dowex 50 (H^+) resin[37] followed by simultaneous acetylation and chlorination of the crude ester by acetyl chloride (5.5 mL, 26 °C).[38] After 12 h the volatile components were removed in vacuo, and the residue was azeotroped twice with 8-mL portions of tetrahydrofuran. The residue (320 mg, ca. 100%) was dissolved in 4.5 mL of 1,4-dioxane, and 0.28 mL (3 equiv) of triethylamine was added. The solution was transferred to a Parr hydrogenation bottle with the help of an additional 11.5 mL of 1,4-dioxane. The solution was hydrogenated over 200 mg of 10% palladium-on-carbon under 40 psi of hydrogen gas on a low-pressure Parr hydrogenation apparatus. After 20 h, the excess hydrogen gas was released, the solution filtered, and the catalyst washed with 30 mL of 1,4-dioxane. The combined filtrate and washings were evaporated in vacuo. The residue was taken up in 20 mL of ethyl acetate, washed twice with 10-mL portions of 10% potassium hydrogen sulfate and once with water, and dried over anhydrous sodium sulfate. The solution was filtered and evaporated in vacuo. The residue was dissolved in chloroform and loaded onto a 11 mm × 540 mm silica gel column, which was packed in chloroform. The product was eluted from the column with a 0–2% gradient of methanol in chloroform. The fractions containing the first product were pooled and evaporated to yield 155 mg (50%) of **5**. A sample of **5** was dissolved in dichloromethane and two volumes of hexanes were carefully placed on top of the dichloromethane layer. The loosely covered biphasic mixture was allowed to stand undisturbed at room temperature. As the solutions diffused and evaporated, large prisms were deposited from which suitable crystals were selected for X-ray crystallographic analysis: mp 146–147 °C; ^{1}H NMR ($CDCl_3$) δ 1.87 (s, 3 H, *N*-Ac), 2.01, 2.02, 2.10, 2.11 (4 s, 12 H, OAc), 2.05–2.14 (m, 1 H, H3a), 2.38 (dd, 1 H, H3e, J_1 = 13.2 Hz, J_2 = 1.6 Hz), 3.73 (s, 3 H, OCH_3), 3.99–4.08 (m, 1 H, H5), 4.09 (dd, 1 H, H9, J_1 = 9.3 Hz, J_2 = 4.9 Hz), 4.21 (dd, 1 H, H6, J_1 = 10.4 Hz, J_2 = 1.2 Hz), 4.31 (dd, 1 H, H9′, J_1 = 12.8 Hz, J_2 = 3.5 Hz), 4.59 (dd, 1 H, H2, J_1 = 6.8 Hz, J_2 = 1.2 Hz), 4.87 (ddd, 1 H, H4, J_1 = 11.2 Hz, J_2 = 10.4 Hz, J_3 = 4.9 Hz), 5.18 (d, 1 H, NH, *J* = 10.4 Hz), 5.30–5.36 (m, 2 H, H7 and H8); ^{13}C NMR ($CDCl_3$) δ 20.79 (2 C) 20.92, 21.09, 23.18, 32.00, 49.80, 52.32, 62.31, 67.62, 69.25, 69.69, 71.49, 73.12, 170.02, 170.18 (2 C), 170.73, 170.81, 171.13; MS (low-resolution) *m/e* 476 (M + 1,2.4%), 475 (M, 0.5%), 446 (4.9%), 432 (6.4%), 416 (8.5%), 373 (25.9%), 356 (38.7%), 314 (15.0%), 295 (94.0%), 253 (100%). MS (high resolution; *m/e*) for $C_{20}H_{30}NO_{12}$ (M + 1) calcd 476.176 80, obsd 476.176 25. MS (high resolution; *m/e*) for $C_{20}H_{29}NO_{12}$ (M) calcd 475.168 98, obsd 475.164 96. See Figure 3 for a proton DQCOSY spectrum of **5** and the spectrum of the sugar carbons obtained from an APT experiment. See Figure 4 for a drawing of the X-ray crystallographic structure of **5**. A small portion of a β component **6** was also obtained: ^{1}H NMR ($CDCl_3$) 1.97 (s, 3 H, *N*-Ac), 2.00–2.13 (4 s + m, 13 H, 4-OAc and H3a), 2.15–2.33 (m, 1 H, H3e), 3.91 (s, 3 H, OCH_3), 4.02–4.28 (m, 2 H), 4.30–4.54 (m, 2 H), 4.63 (dd, 1 H, H2, J_1 = 12.2 Hz, J_2 = 3.2 Hz) 5.21–5.44 (m, 2 H), 5.46–5.58 (m, 2 H).

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