

Synthesis of CMP-NeuAc from *N*-Acetylglucosamine: Generation of CTP from CMP Using Adenylate Kinase^{1,2}

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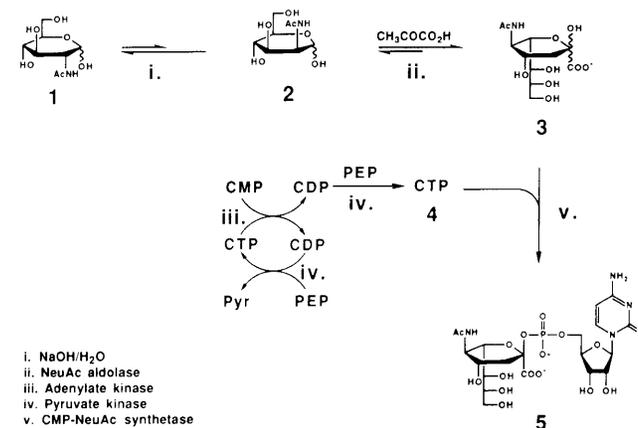
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Abstract: This paper describes a multigram-scale synthesis of cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc) from *N*-acetylmannosamine, pyruvate, cytidine 5'-monophosphate (CMP), and phosphoenolpyruvate (PEP) using three enzymes. *N*-Acetylmannosamine (produced in mole quantities by base-catalyzed epimerization of *N*-acetylglucosamine) and sodium pyruvate react to form *N*-acetylneuraminic acid (NeuAc) in a reaction catalyzed by NeuAc aldolase enclosed in a dialysis membrane. CTP is generated in situ from CMP by using adenylate kinase, pyruvate kinase, and PEP. The only purification step in the synthesis is the final separation of CMP-NeuAc from the reaction mixture by ion-exchange chromatography. CMP-NeuAc generated by this procedure was used to form sialic acid terminated glycoconjugates in reactions catalyzed by sialyltransferases.

Sialic acids (derivatives of neuraminic acid) are components of glycoproteins and glycolipids.⁵⁻⁹ These compounds play an important role in biological recognition. Cell-surface glycoconjugates can serve as binding sites for antibodies, enzymes, hormones, toxins, lectins, bacteria, drugs, and viruses.¹⁰⁻¹⁶ They also can function as cellular labels, sorting newly synthesized proteins between cellular compartments and influencing growth through intercellular contact.¹⁷ Transformed and malignant cells often have modified glycolipids.¹⁸⁻²⁰

Many of the glycoconjugates found in mammalian biochemistry share a similar structural motif: Their terminal carbohydrate residue is a derivative of *N*-acetylneuraminic acid (NeuAc, *N*-acetyl-5-amino-3,5-dideoxy-*D*-glycero-*D*-galacto-2-nonulo-

Scheme I. Generation of CMP-NeuAc from *N*-Acetylglucosamine



pyranosonic acid). NeuAc²¹⁻²⁶ and its glycosides²⁷ have been synthesized chemically and NeuAc has been isolated from natural sources;²⁸⁻³¹ analogues of NeuAc have been synthesized chemically.³² Glycosides of NeuAc pose several synthetic problems in addition to the usual need for procedures for protection and

- (1) Supported by NIH Grants GM 30367 and GM 39589.
- (2) Portions of this work were presented at the 30th National Organic Chemistry Symposium, Vancouver, British Columbia, June 1987.
- (3) 1986-1987 Du Pont Fellow.
- (4) American Cancer Society postdoctoral fellow, Grant PF-2762; 1987.
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deprotection. The glycosidic linkage of NeuAc glycosides is labile to acid, and the correct configuration (α linkage) must be established for biological activity. Only the α -glycosides of sialic acids, for example, bind to the influenza virus haemagglutinin.³³ Further, many glycosidic links between sialic acids and biologically important sugars occur at sterically hindered positions, such as the 3-hydroxyl group of galactose. Current chemical methods have not provided efficient solutions to these problems.²⁷

Biological syntheses of sialylated glycoproteins and glycolipids follow the Leloir pathway, in which enzymes transfer carbohydrates activated as nucleotide phosphates to the growing carbohydrate.³⁴ The sialyl transferases require the activated form of NeuAc, cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc, **5**), to attach NeuAc to a nascent glycoconjugate. We wished to have gram quantities of CMP-NeuAc for use in syntheses of sialylated materials. CMP-NeuAc is commercially available, but is too expensive (\sim \$10⁶/mol) to be used on this scale.

Although CMP-NeuAc has been produced enzymatically from NeuAc and CTP, previously reported syntheses³⁵⁻⁴⁴ have yielded quantities less than 0.5 mmol (\sim 250 mg). We found that the reported purification methods are not useful on a gram scale. In addition, the high cost of commercially available CTP (\sim \$10⁴/mol) limits the practical scale of syntheses of CMP-sialic acids using this compound as a starting material.

Our synthesis of CMP-NeuAc addresses the problem of the availability of CTP by introducing a new method of generating this compound from the readily available CMP using adenylate kinase (EC 2.7.4.3, rabbit muscle), pyruvate kinase (EC 2.7.1.40), and phosphoenolpyruvate (PEP) (Scheme I).⁴⁵ We also present two practical methods of purification, one based on ion-exchange chromatography and one based on differential precipitation, that make the isolation of CMP-NeuAc straightforward on a gram scale. We synthesize NeuAc itself using *N*-acetylneuraminic acid aldolase (NeuAc aldolase, EC 4.1.3.3) enclosed in a dialysis membrane (membrane-enclosed enzymatic catalysis, MEEC)⁴⁶ to allow the reuse of the protein without subjecting it to immobilization.

CMP-NeuAc synthesized according to this method was satisfactory for use in enzyme-catalyzed synthetic reactions; we used CMP-NeuAc in reactions catalyzed by 2,6-sialyltransferase to produce 2,6-sialyl-*N*-acetylglucosamine and 2,6-sialyllactose.¹¹

Results and Discussion

Our synthesis of **5** proceeds in four stages (Scheme I): chemical/enzymatic synthesis of NeuAc from GlcNAc; conversion of CMP to CTP; enzyme-catalyzed coupling of CTP and NeuAc to form CMP-NeuAc; purification of CMP-NeuAc. The last step may not be necessary for certain applications, but because phosphate inhibits sialyltransferases we have devised methods that

eliminate phosphate in addition to other impurities.

GlcNAc \rightarrow NeuAc. Isomerization of *N*-acetylglucosamine (**1**) to *N*-acetylmannosamine (**2**) was accomplished on a multigram scale with sodium hydroxide.⁴⁷⁻⁴⁹ The ratio of ManNAc to GlcNAc at equilibrium was approximately 1:3, but ManNAc (88% pure) could be extracted into hot ethanol; the recovered GlcNAc was recycled and the crude ManNAc was used directly in the next stage. Aldol condensation^{50,51} between **2** and excess sodium pyruvate catalyzed by NeuAc aldolase using the MEEC technique,⁴⁶ followed by purification by ion-exchange chromatography, produced **3** on a gram scale. Purification of NeuAc by ion-exchange chromatography was not necessary for the synthesis of CMP-NeuAc: The crude solution containing NeuAc could be used immediately in the synthesis of CMP-NeuAc or stored at -10°C for at least 2 months prior to use. The enclosure of NeuAc aldolase in a dialysis bag with bovine serum albumin (to minimize destruction of the enzyme by proteases) allowed reuse of the enzyme. We reused the enzyme-containing bag in similar reactions over the course of several months. In this application, we feel that the MEEC technique is superior to other methods of immobilization.

CMP \rightarrow CTP. Adenylate kinase catalyzes the equilibration of AMP, ATP, and ADP; the equilibrium constant for this reaction is approximately unity. Pyruvate kinase, in turn, catalyzes the formation of ATP and pyruvate from ADP and PEP with an equilibrium constant of $\sim 10^3$. The practical enzymatic synthesis of CTP from CMP was based upon the discovery that CMP is a substrate for adenylate kinase.⁵² The literature reports that adenylate kinase is highly specific for adenosine nucleotides,⁵³ but we have found that other nucleotides are accepted in synthetic reactions.^{45,54} By coupling these two reactions together, we were able to form CTP from CMP and a catalytic amount of CTP (to initiate the adenylate kinase reaction). Although large quantities of adenylate kinase were required to achieve useful rates, the enzyme has a high specific activity and a low cost.⁵⁵ Immobilization^{54,56} or use of the MEEC technique⁴⁶ allowed reuse of the enzymes. We also were able to generate CTP from CMP using the nucleoside kinases,⁴⁴ but in our hands the system involving adenylate kinase and PEP was more practical and efficient, based on a comparison of the cost and stability of the enzymes.

We used two methods to initiate the reaction catalyzed by adenylate kinase: one used a catalytic amount of CTP, the other ATP. Isolation of CTP in pure form is simplified by using a catalytic amount of CTP. In cases for which the nucleoside triphosphate is extremely expensive or not available, or in cases for which a 1-5% impurity of ATP can be tolerated, it is more economical to use ATP to initiate the reaction. For simplicity, in this work we used CTP.

A dialysis bag containing 700 units (U) of adenylate kinase converted a solution containing 5 g of CMP to CTP ($\sim 90\%$ conversion) in 9 days. We used this solution without purification in the following step to synthesize CMP-NeuAc. If necessary, isolation and purification of CTP was accomplished by first precipitating inorganic phosphate as the magnesium salt⁵⁷ and

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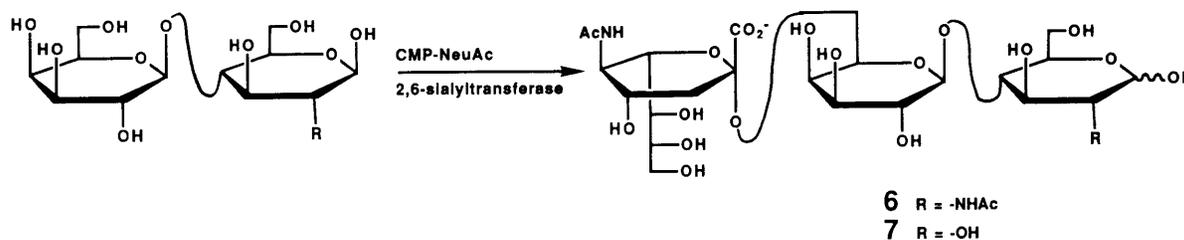
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Scheme II. CMP-NeuAc Generated by the Procedure of Scheme I Was Used To Form α -2,6-Sialyllactosamine (**6**) and α -2,6-Sialyllactose (**7**)

then precipitating the nucleoside triphosphate with a mixture of water and ethanol (1:1, v/v).⁴⁵ This procedure removed all impurities but PEP (<5%), although the recovery of CTP was only ~50% based on CMP. Ion-exchange chromatography, at the expense of slightly more effort, afforded a higher overall yield of CTP (74%).

CTP + NeuAc \rightarrow CMP-NeuAc + PP_i. Addition of a crude solution of **3** and cytidine 5'-monophospho-*N*-acetylneuraminic acid synthetase (CMP-NeuAc synthetase, EC 2.7.7.43) to a solution of crude CTP produced **5**. CMP-NeuAc synthetase was isolated from calf brains according to a procedure described by Paulson³⁵ and van den Eijnden³⁷ and was used in soluble form. We did not use the MEEC technique because large amounts of the ammonium magnesium salt of phosphate precipitate during the reaction and probably hinder transport across the membrane. Others^{36,44} have immobilized the enzyme.

CMP-NeuAc synthetase is most active near pH 9.5 in the presence of 30 mM MgCl₂ or near pH 7.5 in the presence of 5 mM MnCl₂.³⁵ In this report we chose alternative conditions (pH 8.5, 50 mM MgCl₂) to compensate for the loss of magnesium ions (as a precipitate with pyrophosphate) and to maintain the activity of adenylate kinase (which requires Mg²⁺ and is most active near pH 7.5). The reaction may also proceed well under other conditions.

We also converted Man-NAc directly to CMP-NeuAc in the presence of pyruvate, unpurified CTP, NeuAc aldolase, and CMP-NeuAc synthetase on a small scale. In practice, the method using solutions of CTP and NeuAc generated in independent reactions was easier.

Previous reports used an excess of CTP to synthesize CMP-NeuAc. In our hands, the reaction proceeded only to 40–50% completion in the presence of 1 equiv of CTP per 1 equiv of NeuAc. We thought that the apparent equilibrium would be shifted by removing one of the products, pyrophosphate. In situ cleavage of pyrophosphate using pyrophosphatase did not, however, alter the apparent equilibrium, but addition of 3 equiv of CTP resulted in complete conversion of NeuAc to CMP-NeuAc. We also found that prior addition of CMP-NeuAc to the reaction mixture did not inhibit the activity of CMP-NeuAc synthetase. We therefore conclude that the need for excess CTP is simply due to the activity of phosphatases that contaminate the CMP-NeuAc synthetase.

Isolation of CMP-NeuAc. We found that the best way to separate CMP-NeuAc from CTP, PEP, CMP, cytidine, pyruvate, dipyruvate, Glc-NAc, phosphate, and inorganic salts was by ion-exchange chromatography using the formate form of anion-exchange resin (AG 1-X2). The column was eluted with a gradient of aqueous ammonium bicarbonate. The use of ammonium bicarbonate prevented hydrolysis of acid-labile CMP-NeuAc and provided it as the ammonium salt. Excess ammonium bicarbonate was easily removed by stirring the fractions containing CMP-NeuAc with cation-exchange resin (Dowex 50W-X8, H⁺ form) until the pH of the solution was 7. To make the separation easier, residual nucleotides and excess PEP were hydrolyzed by using alkaline phosphatase before chromatography. The purity of CMP-NeuAc was >95%.

We also developed a simple method based on differential precipitation to isolate CMP-NeuAc. Pure NeuAc was used so

that the initial concentration of pyruvate and dipyruvate (a side product formed from pyruvate during the synthesis of NeuAc and during the generation of CTP) was minimal. Excess CTP was removed by precipitation in a mixture of ethanol and 10 mM NH₄OH (1:1, v/v); some CMP-NeuAc also precipitated in this step. Residual nucleotides and excess PEP were hydrolyzed with alkaline phosphatase. CMP-NeuAc was precipitated from the resulting solution (which contained cytidine, pyruvate, and dipyruvate) by a mixture of ethanol and 10 mM NH₄OH (9:1, v/v). The differential precipitation steps could be repeated to increase the purity of the product, although for our purposes the product obtained at this stage was sufficiently pure (>90%) for use in other enzymatic syntheses following desalting on Biogel P-2.

Use of CMP-NeuAc. The CMP-NeuAc purified by differential precipitation was used in conjunction with sialyltransferases according to the procedure of Sabesan and Paulson¹¹ to produce α -2,6-sialyllactosamine (NeuAc- α (2,6)Gal- β (1,4)GlcNAc, **6**) and α -2,6-sialyllactose (NeuAc- α (2,6)Gal- β (1,4)Glc, **7**) (Scheme II).

Conclusion

This report represents the first reported synthesis of CMP-NeuAc practical on a multigram scale. Generation of CTP from CMP on a preparative scale allows the use of large quantities of CTP; the MEEC technique allows repeated reuse of NeuAc aldolase without any special effort; only one simple purification step is necessary. The synthesis presently works on a multigram scale and is limited primarily by the (relatively modest) effort required to isolate CMP-NeuAc synthetase.

The one-pot synthesis of CMP-NeuAc from Man-NAc demonstrates the ability to carry out several enzymatic reactions simultaneously; in this example, a phosphoryl-transfer reaction (synthesis of CMP-NeuAc) drives the stereoselective formation of a carbon-carbon bond (synthesis of NeuAc). For practical application, however, it is easier to generate and store crude solutions of NeuAc and CTP rather than to generate them simultaneously.

The purification based on differential precipitation illustrates a simple alternative for the isolation of mixtures of charged species in aqueous solutions. Because some CMP-NeuAc precipitates with CTP, the method based on ion-exchange chromatography is preferable for the isolation of CMP-NeuAc. The technique based on differential precipitation could be useful for sensitive compounds that are not stable to ion-exchange chromatography or for separations in which the components exhibit large differences in charge or solubility.

The enzymatic route allows the possibility of preparing labeled compounds and the ability to generate and to use NeuAc in situ, bypassing all purification steps. The scale with which NeuAc itself can be synthesized by the procedures given here is limited by the cost of NeuAc aldolase.

The need for an excess of CTP because of traces of phosphatase activity present in the preparation of CMP-NeuAc synthetase lead us to develop a practical method for the preparation of this compound on a large scale. The results show that adenylate kinase is a useful catalyst for the production of CTP⁴⁵ (a previous report⁵⁴ demonstrates the preparation of ribavirin triphosphate). This enzyme may be useful in the synthesis of other nucleoside triphosphates; further work on this subject is in progress.

We used CMP-NeuAc produced according to this method to transfer sialic acid to lactose and *N*-acetylglucosamine, using 2,6-sialyltransferase. The availability of large quantities of

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CMP-NeuAc should facilitate studies on the use of the sialyltransferases in organic synthesis.

Experimental Section

Materials and Methods. CMP-NeuAc synthetase was isolated from 10 fresh calf brains (obtained from a local slaughter house) according to the method of Higa and Paulson;³⁵ the protein pellet obtained after the second ammonium sulfate precipitation was used without further purification. CMP-NeuAc:Gal- β -(1,4)GlcNAc- α -(2,6)sialyltransferase (EC 2.4.99.1) was from Genzyme. NeuAc aldolase⁵⁸ and alkaline phosphatase were obtained from Sigma as lyophilized powders; all other enzymes were purchased as suspensions in ammonium sulfate. *N*-Acetylglucosamine and CMP were obtained from United States Biochemical Corp. All other chemicals and biochemicals were reagent grade and were used without further purification.

Dialysis membranes (Spectrapor 2, M_r 12000–14000 cutoff, 16 mm) were obtained from Spectrum Medical Industries, were boiled in water for 15 min, and were then rinsed with water before use. To prepare an enzyme-containing bag for use in enzyme-catalyzed transformations, the tubing was tied shut at one end, a solution of enzymes in the reaction mixture was added, and the other end of the tubing was tied shut, taking care to exclude as much air as possible. Ion-exchange resin was washed with methanol or ethanol and water before use.

Water was distilled from glass in a Corning Ag 1b still. Welding-grade nitrogen was used without further purification. Evaporation of water was done at 0.1 Torr with a rotary evaporator equipped with a cold finger containing acetonitrile and dry ice. Centrifugation was carried out at 4 °C in a Sorvall RC-5B refrigerated centrifuge or, if noted, in an IEC Model 428 desktop centrifuge. An LKB 2120 peristaltic pump connected to a Horizon pH controller afforded pH control of certain reactions.

¹H NMR and ¹³C spectra were obtained with a Bruker AM 500 or AM 300 instrument and were referenced to DSS and dioxane respectively; ³¹P NMR spectra were recorded on a Bruker WM 300 machine and were referenced internally to inorganic phosphate.

***N*-Acetylmannosamine (2).**^{47,48} A 6-L Erlenmeyer flask equipped with a magnetic stirring bar was charged with 1 kg (4.5 mol) of *N*-acetylglucosamine (**1**) in 4.5 L of water. The solution was adjusted to pH 11 with 10 N NaOH and stirred at room temperature for 3 days. The resulting pale yellow solution was neutralized with concentrated HCl and concentrated by rotary evaporation at 0.1 Torr. Heating the resulting solid for 10 min on a steam bath in 4 L of boiling 95% ethanol dissolved most of the **2** present while most of **1** did not dissolve. The solution was left at 4 °C for 3 h, passed through a Büchner funnel, and rinsed with cold 95% ethanol. Concentration of the filtrate by rotary evaporation at reduced pressure yielded 143 g of a solid containing 88% of **2**, according to analysis by ¹H NMR spectroscopy; **1** and ethanol were also present. The procedure was repeated twice using the recovered **1** to provide 462 g of a crude solid containing ~88% of **2** (1.8 mol) according to analysis by ¹H NMR spectroscopy. Removal of all traces of ethanol prior to use of the material in enzymatic synthesis was accomplished by twice dissolving crude **2** in water followed by concentration in vacuo. Unreacted **1** was recovered.

***N*-Acetylneuraminic Acid (3).** A solution containing 1.5 g (6.0 mmol) of *N*-acetylmannosamine (**2**), 6 g of sodium pyruvate (96%, 52 mmol), and 25 mg of sodium azide⁵⁹ in 25 mL of distilled water was placed in a 100-mL graduated cylinder containing a magnetic stirring bar. After the pH of the solution had been adjusted to pH 7.5 with dilute (typically 0.25 M) NaOH, a dialysis bag containing NeuAc aldolase (5 U) and 10 mg of bovine serum albumin dissolved in 4 mL of the reaction solution was added to the reaction vessel. The bag was allowed to stir freely in the solution at room temperature, and daily the solution was adjusted to pH 7.5 with dilute NaOH. After 5 days, ~90% of **2** had been converted to **3**. The enzyme-containing bag was removed, stored at 4 °C, and reused in similar reactions for 2 months. The reaction solution was stored at -10 °C for 2 months prior to its use without further purification in the synthesis of CMP-NeuAc. In other experiments, purification of **2** using ion-exchange chromatography (AG 1X-2 resin, formate form) with a 0–2 N gradient of formic acid provided pure material whose spectral data agreed with that of a commercial sample (Sigma): ¹H NMR (500 MHz, D₂O) δ 1.80 (apparent t, $J = 12$ –13 Hz, 1 H), 1.98 (s, 3 H), 2.22 (dd, $J = 13.0$, 4.8 Hz, 1 H), 3.47 (d, $J = 11.2$ Hz, 1 H), 3.54 (dd, $J = 11.9$, 6.4 Hz, 1 H), 3.56–3.52 (m, 1 H), 3.77 (dd, $J = 11.8$, 2.5 Hz, 1

H), 3.85 (apparent t, $J = 10.4$ Hz, 1 H), 4.1–3.9 (m, includes a d, $J = 10.7$ Hz, 2 H).

Generation of CTP (4). The potassium salt of PEP⁵⁶ (4.75 g, 95% by enzymatic assay,⁶⁰ 22 mmol) was dissolved in 30 mL of water and placed in a 50-mL polypropylene centrifuge tube, and the pH was adjusted to pH 8.5 with 5 N NaOH. CMP (4.65 g, 10 mmol) was added, followed by 1 mL of a basic solution containing MgCl₂·6H₂O (1.3 M) and NH₄Cl (3.8 M) to precipitate any inorganic phosphate present.⁵⁷ The reaction mixture was centrifuged (IEC centrifuge, 1200g, 5 min), and the supernatant was decanted into a clean tube containing a magnetic stirring bar. MgCl₂·6H₂O (3.0 g, 15 mmol) was added, followed by a catalytic amount of CTP (100 mg, 0.2 mmol). The solution was degassed with nitrogen for 15 min, and 5 μ L of 2-mercaptoethanol was added. A dialysis bag containing 1 mL of a suspension of adenylate kinase in ammonium sulfate (~700 U) and 0.5 mL of a suspension of pyruvate kinase (~1400 U) in ammonium sulfate dissolved in 2 mL of the reaction mixture was then added to the reactor. During the course of the reaction, performed at room temperature, nitrogen was slowly bubbled through the solution and addition of 1 N HCl via a peristaltic pump maintained the pH of the solution near 8. A white precipitate, presumably the ammonium magnesium salt of P_i, formed during the course of the reaction. After 9 days, ¹H NMR and ³¹P NMR spectroscopy indicated that the ratio of CTP to CMP was 85:15; CDP was not detected. The pale yellow solution (pH 8.5) was transferred to a 250-mL polypropylene bottle containing a magnetic stirring bar for use in the next step. The enzyme-containing bag was left in the reaction mixture.

Synthesis of CMP-NeuAc (5). Cytidine 5'-monophosphoneuraminic acid synthetase was dissolved in a solution of **3** and was added to a solution of CTP; both preparations were crude mixtures generated as described above. The mixture was adjusted to pH 8.5, and MgCl₂·6H₂O (1.42 g, 50 mM) was added. The reaction was stirred at room temperature, and addition of 1 N NaOH via a peristaltic pump maintained the pH of the reaction near pH 8.5. Copious amounts of a white precipitate formed during the reaction, and after 2 days, an additional 1.0 g of MgCl₂·6H₂O was added. After 3 days, analysis by ¹H NMR spectroscopy indicated that 90–95% of **4** had been converted to **5**. The enzyme-containing bag was removed and dialyzed twice against 50 mL of H₂O for periods of 1 h. After the bag was stored in H₂O at 4 °C for 5 days, 70 U of adenylate kinase and 60 U of pyruvate kinase activity remained.

Purification of CMP-NeuAc: Ion-Exchange Chromatography. The reaction mixture was centrifuged (25000g, 20 min), the pellet was resuspended in 100 mL of 10 mM NH₄OH, and the solution was centrifuged again; this wash procedure was repeated. Combination of the washings and dialysates (see above) yielded a solution whose pH was adjusted to pH 9.5 with 1 N NaOH. Alkaline phosphatase (1 mg, ~1000 U) and MgCl₂·6H₂O (1.2 g) were added to the solution. A white precipitate formed during the reaction, and addition of 0.5 N NaOH maintained the solution near pH 9. After 30 min, analysis by ¹H NMR spectroscopy indicated that CTP, CMP, and PEP had been converted to cytidine, inorganic phosphate, and pyruvate. The solution was centrifuged (10000g, 10 min), and the pellet was resuspended in 25 mL of 10 mM NH₄OH and centrifuged again; this wash step was repeated. The combined supernatants were concentrated to 30 mL and purified by ion-exchange chromatography (AG 1-X2, formate form; 5 \times 20 cm; eluant, 50 mM ammonium bicarbonate followed by a 50 mM–1 M gradient of ammonium bicarbonate). The fractions containing CMP-NeuAc, identified by thin-layer chromatography (silica gel; eluant, *n*-BuOH/AcOH/H₂O, 5:3:2), were pooled and concentrated to give a white powder. Excess ammonium bicarbonate was removed by dissolving the powder in 25 mL of H₂O and adding cation-exchange resin (Dowex 50W-X8, H⁺ form) to the stirred solution until the pH was 7. The solution was filtered and concentrated to yield the ammonium salt of CMP-NeuAc (2.7 g, 3.7 mmol, 62% overall yield from **2**) in >95% purity; dipyruvate was also present. Spectral data agreed with the literature⁶¹ and with that of a sample from Sigma: ¹H NMR (500 MHz, D₂O) δ 1.55 (ddd, $J = 13.2$, 11.6, 5.7 Hz, 1 H), 1.95 (s, 3 H), 2.39 (dd, $J = 13.2$, 4.7 Hz, 1 H), 3.35 (d, $J = 9.7$ Hz, 1 H), 3.50 (dd, $J = 6.6$, 11.8 Hz, 1 H), 3.79 (dd, $J = 11.8$, 2.4 Hz, 1 H), 3.84–3.82 (m, 1 H), 3.86 (d, $J = 10.5$ Hz, 1 H), 4.18–4.11 (m, 3 H), 4.20 (apparent t, $J = 4.7$ –5.0 Hz, 1 H), 4.25 (apparent t, $J = 4.7$ –4.8 Hz, 1 H), 5.88 (d, $J = 4.7$ –4.8 Hz, 1 H), 5.88 (d, $J = 4.5$ Hz, 1 H), 6.02 (d, $J = 7.6$ Hz, 1 H), 7.87 (d, $J = 7.6$ Hz, 1 H); ¹³C NMR (82 MHz, D₂O) δ 174.7, 174.2, 166.2, 157.7, 141.6, 100.1 (d, $J = 7.8$ Hz), 96.5, 89.1, 82.9 (d, $J = 7.8$

(58) NeuAc aldolase is also available in 1000-U quantities at lower price from Toyobo (distributed by Shinko American Inc., 1450 Broadway, New York, NY). The enzyme has been cloned: Ohta, Y.; Shimosaka, M.; Murata, K.; Tsudaka, Y.; Kimura, A. *Appl. Microbiol. Biotechnol.* **1986**, *24*, 386. For a study of NeuAc aldolase, see: Uchida, Y.; Tsudaka, Y.; Sugimori, T. *J. Biochem.* **1984**, *96*, 507.

(59) Sodium azide (0.1%) prevents bacterial growth in the sugar solution.

(60) *Methods of Enzymatic Analysis*, 3rd ed.; Bergmeyer, H. U., Bergmeyer, J., Grassl, M., Eds.; Verlag-Chemie: Weinheim, Germany, 1983; Vol. VI, pp 555–561.

(61) Haverkamp, J.; Beau, J.-M.; Schauer, R. *Hoppe-Seyler's Z. Physiol. Chem.* **1979**, *360*, 159.

Hz), 74.2, 71.7, 69.6 (d, $J = 9.9$ Hz), 69.3, 68.8, 66.8, 64.8 (d, $J = 5.3$ Hz), 63.0, 51.8, 41.1 (d, $J = 9.7$ Hz), 22.1.

Purification of CMP-NeuAc: Differential Precipitation. CMP-NeuAc was generated as described above except that the NeuAc (1 g) used had been purified by ion-exchange chromatography. The following steps were performed at 4 °C. The reaction mixture was centrifuged (25000g, 20 min), and the pellet was resuspended in 100 mL of 10 mM NH₄OH and centrifuged again. This wash procedure was repeated. Combination of the washings and dialysates (see above) yielded a solution whose pH was adjusted to pH 9.5 and was then concentrated; the pH must be checked when the volume is approximately halved and adjusted up to pH 9 during this step to prevent hydrolysis. The concentrate was desalted on Biogel P-2 (100 × 4.5 cm; eluant, 10 mM NH₄OH), and the fractions containing CMP-NeuAc (determined by TLC as above) were pooled and concentrated to 30 mL. Slow addition of ethanol (30 mL) precipitated CTP, and the solution was centrifuged (10000g, 10 min). The supernatant containing CMP-NeuAc was decanted, and the pellet was resuspended in 30 mL of 10 mM NH₄OH. The precipitation and centrifugation steps were repeated. The combined supernatants were concentrated to near dryness and redissolved in 50 mL of 10 mM NH₄OH. The pH of the solution was adjusted to pH 9, and alkaline phosphatase (~200 U) and MgCl₂·6H₂O (1.1 g) was added as above. After 90 min, the pH of the solution was adjusted to 9.5 and the solution was centrifuged (10000g, 10 min). The supernatant was saved, the pellet was resuspended in 25 mL of 10 mM NH₄OH, and the solution was centrifuged again. This wash procedure was repeated. The combined supernatants were concentrated to 30 mL, and the addition of ethanol (270 mL) precipitated **5**. The supernatant was concentrated to 30 mL, and the precipitation was repeated. The collected precipitates were desalted on Biogel P-2 as above to yield 1.2 g of a solid containing ~90% CMP-NeuAc (~50% yield from **2**); pyruvate, dipyruvate, and an unidentified contaminant were also present.

(5-Acetamido-1-carboxy-3,5-dideoxy- α -D-glycero-D-galacto-2-nonu-

lopyranosyl)-(2,6)- β -D-galactopyranosyl-(1,4)-2-acetamido-2-deoxy- α , β -D-glucopyranose (2,6-Sialyllactosamine, **6).** A solution of 43 mg of sodium cacodylate (200 μ mol), 10 mg of CMP-NeuAc (14 μ mol), and 17 mg of *N*-acetylglucosamine (43 μ mol; synthesized from glucose⁶²) in 2 mL of D₂O was placed in a plastic, 15-mL polypropylene tube. Addition of 0.4 N HCl in D₂O adjusted the solution to pH 6.8, 0.1 mL (~17 mU) of 2,6-sialyltransferase was added, and the tube was capped and left at room temperature. ¹H NMR spectroscopy (500 MHz) indicated that the reaction was >90% complete after 2 days. Purification by ion-exchange chromatography¹¹ yielded ~3 mg (4 μ mol) of **6**, identified by comparison of its ¹H NMR spectrum to that reported in the literature.¹¹

(5-Acetamido-1-carboxy-3,5-dideoxy- α -D-glycero-D-galacto-2-nonulopyranosyl)-(2,6)- β -D-galactopyranosyl-(1,4)- α , β -D-glucopyranose (2,6-Sialyllactose, **7).** A solution of 43 mg of sodium cacodylate (200 μ mol), 10 mg of CMP-NeuAc (14 μ mol), and 800 mg of lactose (155 μ mol) in 2 mL of D₂O was placed in a plastic, 15-mL polypropylene tube. Addition of 0.4 N HCl in D₂O adjusted the solution to pH 6.8, 0.1 mL (~17 mU) of 2,6-sialyltransferase was added, and the tube was capped and left at room temperature. ¹H NMR spectroscopy (500 MHz) indicated that the reaction was 50% complete after 1 week. Purification by ion-exchange chromatography¹¹ yielded ~1 mg (2 μ mol) of **7**, identified by comparison of its ¹H NMR spectrum to that reported in the literature.¹¹

Registry No. 1, 7512-17-6; **2**, 3615-17-6; **3**, 131-48-6; **5**, 3063-71-6; **6**, 78969-47-8; **7**, 35890-39-2; CTP, 65-47-4; CMP, 63-37-6; CDP, 63-38-7; *N*-acetylglucosamine, 32181-59-2; lactose, 63-42-3; adenylate kinase, 9013-02-9; pyruvate kinase, 9001-59-6; acetylneuraminic acid aldolase, 9027-60-5; CMP-acetylneuraminic acid synthetase, 9067-82-7; 2,6-sialyltransferase, 9075-81-4.

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