Overproduction of CMP-Sialic Acid Synthetase for Organic Synthesis

Jennifer Lin-Chun Liu,[†] Gwo-Jenn Shen,[†] Yoshitaka Ichikawa,[†] James F. Rutan,[†] Gerardo Zapata,[‡] Willie F. Vann,[‡] and Chi-Huey Wong*,[†]

Contribution from the Department of Chemistry, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037, and Laboratory of Bacterial Polysaccharides, Center for Biologics Research and Review, 8800 Rockville Pike, Bethesda, Maryland 20892.
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Abstract: The gene coding for *Escherichia coli* CMP-sialic acid synthetase (E.C. 2.7.7.43) was cloned and overexpressed in *E. coli* through a primer-directed polymerase chain reaction. Two plasmids were constructed to produce the native enzyme and a modified enzyme fused with a decapeptide at the C-terminus. The decapeptide tag was used for detection of the enzyme production. Both enzymes produced from *E. coli* were isolated and purified with an Orange A dye resin and FPLC. Their properties were compared with respect to their kinetic parameters, stability, pH profiles, and substrate specificities. Both enzymes have similar k_{cat} and K_m for NeuAc and CTP but different pH profiles. Contrary to the native enzyme, the modified enzyme is more active at higher pH. Studies on specificity indicate that both enzymes have a high specific activity for C-9 modified NeuAc derivatives at neutral pH. Some C-5 modified (hydroxy, deoxy, and deoxyfluoro) NeuAc derivatives are not acceptable as substrates. The modified enzyme has been used in the synthesis of CMP-NeuAc from ManNAc and CMP and sialyl *N*-acetyllactosamine (Neuα2,6Galβ1,4GlcNAc) with in situ generation of NeuAc and regeneration of CMP-NeuAc. The 6-*O*-acyl derivatives of ManNAc were prepared via transesterification in anhydrous dimethylformamide by using an engineered stable subtilisin variant as a catalyst, and the products were used as substrates in sialic acid aldolase-catalyzed synthesis of 9-*O*-acyl-NeuAc derivatives.

Introduction

Sialic acids, derivatives of neuraminic acid, occur as important components of glycoproteins, gangliosides, and oligosaccharides and are usually found in their terminal positions. Homopolymers of *N*-acetylneuraminic acid (NeuAc) in α (2,8) linkages were found in mammalian brain tissues[1,2] and capsular polysaccharides in *Escherichia coli*.[3] Among the many biological and pathological processes, sialic acids and sialic acid-containing oligosaccharides play crucial roles in the regulation of molecular recognition and cell adhension.[4,5] Cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc), which is formed from CTP and NeuAc catalyzed by CMP-sialic acid synthetase (E.C. 2.7.7.43), is the activated form of NeuAc used in the biosynthesis of sialyl oligosaccharides. The enzyme, CMP-sialic acid synthetase, isolated from several mammalian tissues[6-10] has been used in the synthesis of CMP-NeuAc.[11] The application of microbial CMP-NeuAc synthetase[12,13] to organic synthesis, however, is not well studied.

The gene coding for CMP-sialic acid synthetase from *E. coli* has been cloned and sequenced by Vann et al.[13-15] and recently overexpressed for use in gram-scale synthesis of CMP-NeuAc from CTP and NeuAc by Shames et al.[16] Since the original plasmid pW1, which contained 2.7 kb of p6 and CMP-sialic acid synthetase genes, did not express the enzyme efficiently,[15] a plasmid pWG123 modified from pW1 was further constructed (in this report) and used for the production of the native CMP-sialic acid synthetase. This expression system produces CMP-NeuAc synthetase at a level approximately 40-fold higher than that of the wild type. In order to further improve the expression level and to facilitate screening, we have developed a new cloning system based on a lambda phage vector.[17] A level of approximately 1000-fold overproduction of the enzyme was achieved by using this system. The gene coding for the enzyme was fused with a decapeptide tag sequence at the 3'-terminus and cloned into lambda ZAP vector at the *Eco*RI and *Xba*I sites and overexpressed in *E. coli* Sure strain (Stratagene, San Diego). This overproduction strain is now available from ATCC (ATCC 68531). The decapeptide tag is served as a marker to facilitate the selection of positive clones; it can be removed by another PCR (polymerase chain reaction) using the primers that contained no decapeptide tag sequence. In this paper, the properties of both the native and modified enzymes with respect to their stabilities in buffer at room temperature, pH profiles, and substrate specificities were investigated. *N*-Acetylneuraminic acid and analogues with modification at C-5 or C-9 position were prepared via sialic acid aldolase reactions and used for the substrate specificity study. The 6-*O*-acyl-ManNAc derivatives used in the aldolase reactions were prepared via transesterification catalyzed by an engineered subtilisn[18,26] stable in dimethylformamide (DMF). A gram-scale synthesis of CMP-NeuAc from CMP and ManNAc using the modified enzyme coupled with in situ generation of NeuAc from ManNAc via a sialic acid aldolase reaction was also demonstrated.

Results and Discussion

Overexpression and Purification of the Native and the Modified CMP-Sialic Acid Synthetase. The construction of plasmid pWG123 from plasmid pWA1 was performed by removing the p6 gene and by modifying the ribosome binding site. The final construction is shown in Figure 1A. This plasmid was used for

[†] The Scripps Research Institute.
[‡] Center for Biologics Research and Review.

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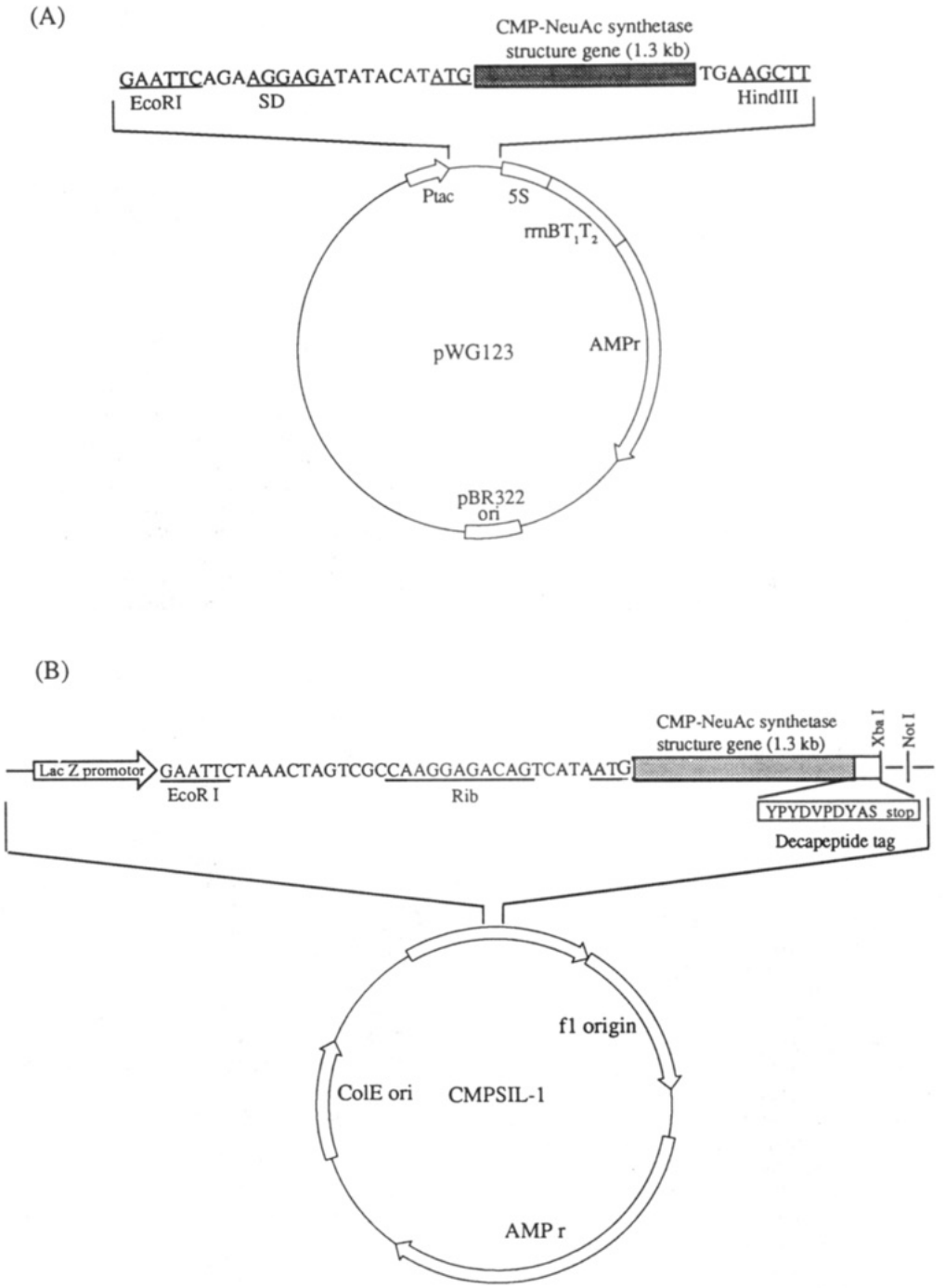

Figure 1. Plasmids pWG123 and CMPSIL-1 for overexpression of the native and tagged CMP-NeuAc synthetase.

expression of the native enzyme, and about 4 units of enzyme were obtained from 1 L of cell culture. The low productivity of pWG123 may be due to the difference of ribosome binding site connected to the enzyme structure gene.

Subcloning the gene fused with a decapeptide sequence into the lambda phage system (plasmid CMPSIL-1) composed of a Lac Z promotor and ribosome binding site allowed overexpression of the tagged enzyme (Figure 1B).[17] The decapeptide tag (shown below) served as a marker for selection of the modified enzyme.

Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Ser

Positive clones were picked with the use of the antidecapeptide monoclonal antibody conjugated with alkaline phosphatase based on the ELISA method.[19] A deep blue color resulting from the reaction of alkaline phosphatase with 5-bromo-4-chloro-3-indolyl phosphate (nitro blue tetrazolium)[20] indicated the successful insertion of the target gene. Approximately 100 units/L of the modified CMP-sialic acid synthetase were obtained from this expression system. By subcloning the CMP-sialic acid synthetase gene obtained from another PCR using the primers that contained no decapeptide tag sequence into the CMPSIL-1 plasmid, the native CMP-sialic acid synthetase was expressed at the level of about 35 units/L of the cell culture.

The enzyme was purified from cell-free extract in two steps: Orange A dye resin column chromatography[16] provided the partially purified enzyme with a specific activity of 0.72 unit/mg. This preparation was directly used for the synthetic purpose. Further purification was carried out by FPLC Superose 12 gel filtration chromatography, to obtain the enzyme with a specific activity of ca. 2 units/mg and about 95% pure as judged by

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Table I. Kinetic Parameters for the Native and Tagged CMP-NeuAc Synthetase

	k_{cat}, s^{-1}	K_m, mM NeuAc	K_m, mM CTP	V_{max}, units/mg
native enzyme	1.8	4	0.31	2.1
tagged enzyme	1.9	4.8	1.99	2.3

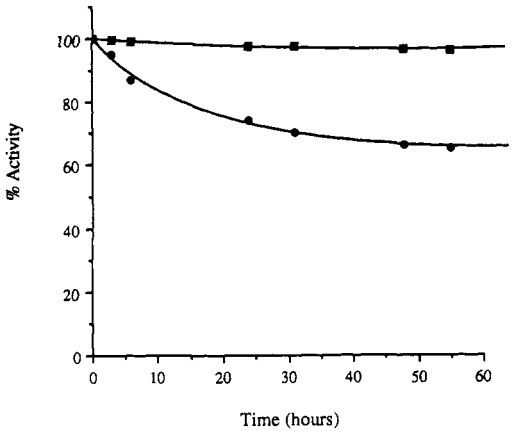


Figure 2. Stability study of both the native (■) and the tagged (●) CMP-sialic acid synthetase.

SDS-polyacrylamide gel electrophoresis.

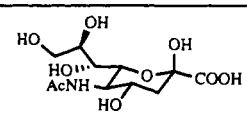
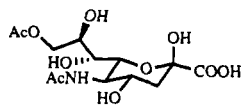
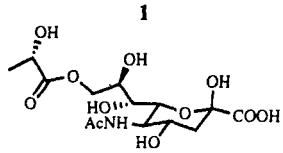
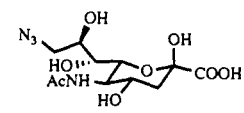
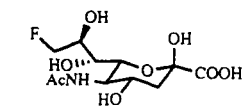
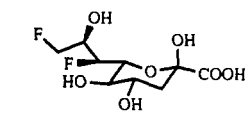
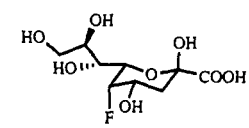
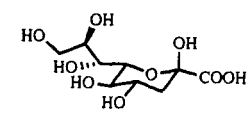
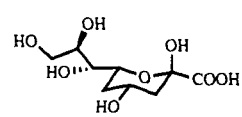
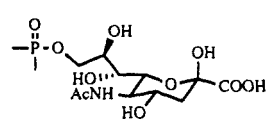
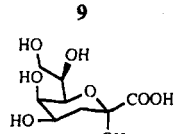
Kinetic Study of the Native and Modified CMP-Sialic Acid Synthetase. The specific activities and kinetic constants of the two enzymes are very similar. The native and the modified enzymes had the specific activities of 2.1 units/mg and 2.3 units/mg, respectively. For the native enzyme, the k_{cat} is 1.8 s^{-1} and the K_m values for the two substrates, NeuAc and CTP, are 4 and 0.31 mM, respectively.[13] The tagged enzyme had the k_{cat} of 1.9 s^{-1} and K_m's of 4.8 and 1.99 mM respectively for NeuAc and CTP (Table I).

Enzyme Stability. The enzyme stabilities were studied at room temperature (Figure 2) for 3 days. The native enzyme has a half-life of 800 h in a phosphate buffer (pH 7.5) at room temperature. On the other hand, the tagged enzyme has a half-life of 80 h, approximately 10 times less stable than the wild type. The difference in their stability is possibly due to the additional decapeptide sequence.

pH Profile. The enzyme activities were studied from pH 4.5 to 10.5 in the presence of two different metal ions, Mg^{2+} and Mn^{2+}, which were known to affect the enzyme activity. Similar to the enzyme isolated from the mammalian tissue,[9] the native microbial CMP-sialic acid synthetase was found to have an optimum pH at pH 7.5 in the presence of Mn^{2+}, and at pH 9.5 in the presence of Mg^{2+} (Figure 3B). The tagged enzyme however, showed an optimum activity at pH 9.5 in the presence of either Mg^{2+} or Mn^{2+} (Figure 3A).

Substrate Specificity. CMP-sialic acid synthetase from a variety of mammalian tissues was found to be specific for CTP and sialic acids.[21] It accepts some C-9 and C-8 modified sialic acid analogues including fluorescent probes attached at the 9-position.[22-25] The enzyme from the mammalian system also accepts C-5 modified substrates such as KDN and 5-*N*-glycolylneuraminic acid as substrates.[16,23] The cloned enzymes from the *E. coli* system have similar substrate specificity to the enzyme from mammalian systems. Several sialic acid analogues were synthesized and tested as substrates for the native and the tagged enzymes at pH 7.5 and 9.0. Both enzymes have high activity for the C-9 modified sialic acid analogues (9-*O*-acetyl-, 9-*O*-lactyl-, 9-deoxy-9-fluoro-, and 9-azido-9-deoxy-NeuAc); however, the C-5 modified analogues (KDN, 5-deoxy-KDN, and 5-deacetamido-5-epi-5-fluoro-NeuAc) were not substrates. This suggests that the 5-acetamido group of the sialic acids is critical for substrate recognition by the microbial enzymes.

Table II. Substrate Specificities of the Native and Tagged CMP-NeuAc Synthetase

	tagged enzyme		native enzyme	
	pH 7.5	pH 9.0	pH 7.5	pH 9.0
	1	1	1	1
 **1**	0.98	2.26	0.92	0.48
 **2**	0.92	1.7	0.95	0.46
 **3**	1.2	1.34	0.99	0.49
 **4**	1.1	1.2	0.98	0.52
 **5**	<0.05	<0.05	<0.05	<0.05
 **6**	<0.05	<0.05	<0.05	<0.05
 **7**	<0.05	<0.05	<0.05	<0.05
 **8**	<0.05	<0.05	<0.05	<0.05
 **9**	<0.05	<0.05	<0.05	<0.05
 **10**	<0.05	<0.05	<0.05	<0.05

(21) Kean, E. L.; Roseman, S. *Methods Enzymol.* **1966**, *8*, 208.
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(25) Gross, H. T.; Brossmer, R. *Eur. J. Biochem.* **1988**, *177*, 583.

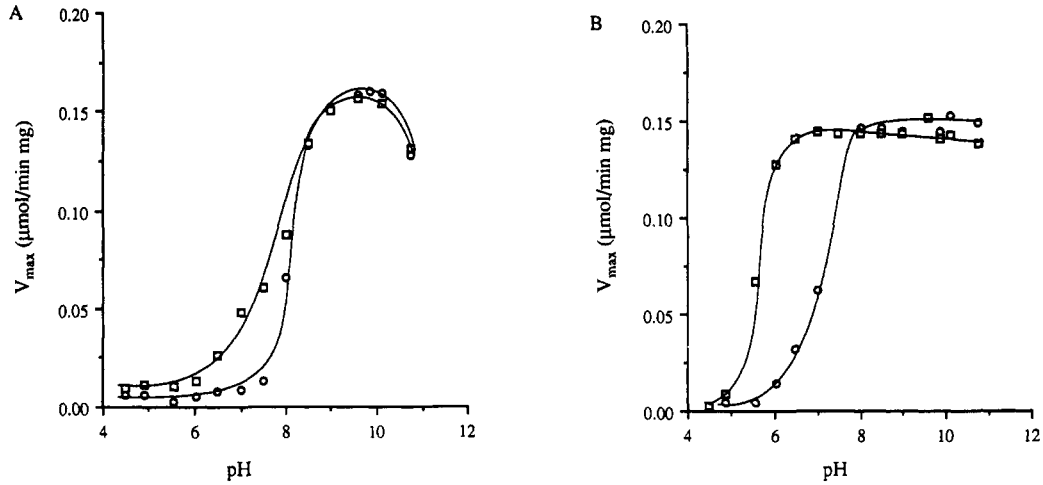
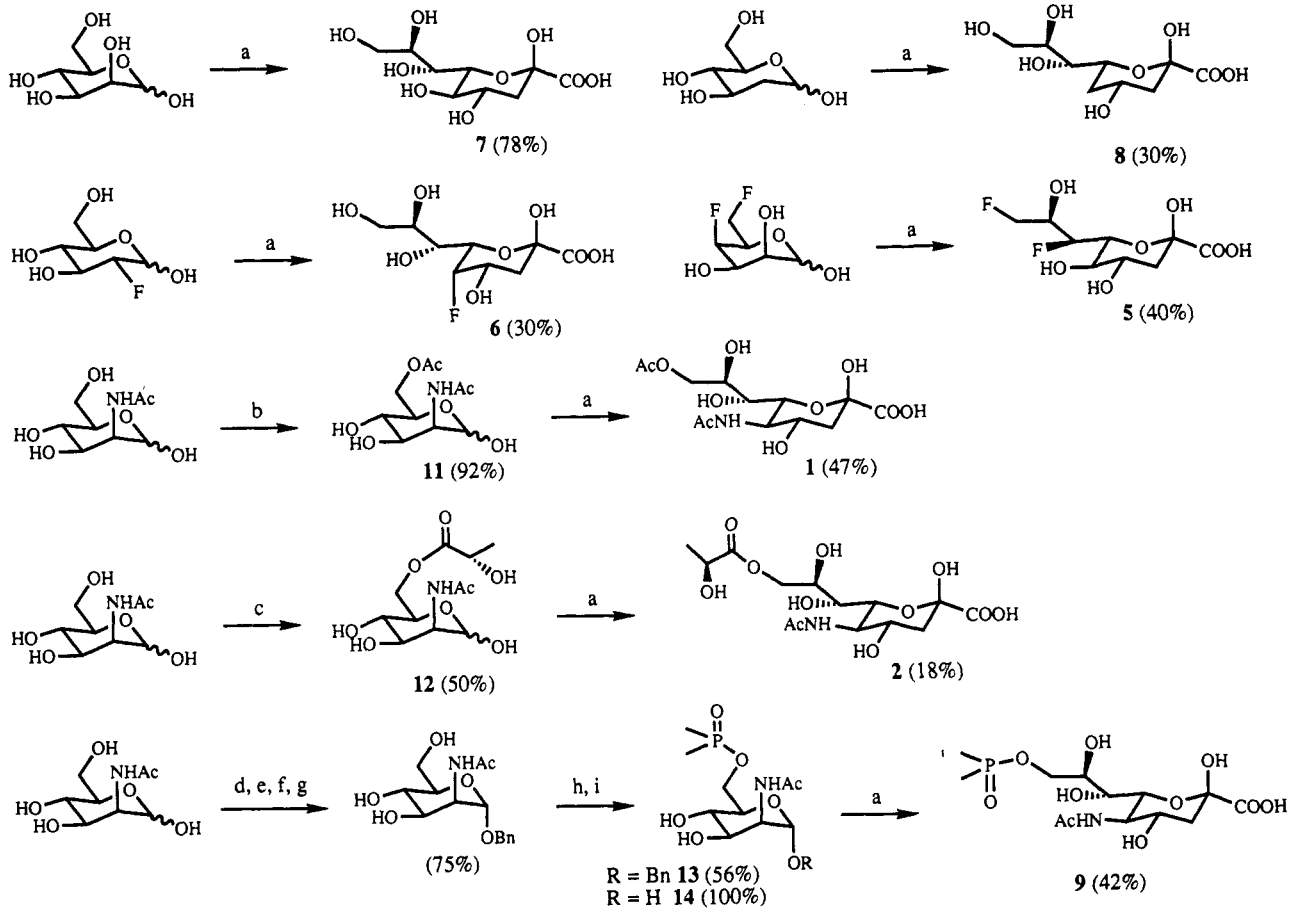


Figure 3. (A) pH profile of the tagged CMP-sialic acid synthetase. (B) pH profile of native CMP-sialic acid synthetase: (O) in the presence of 6 mM $MnCl_2$; (□) in the presence of 20 mM $MgCl_2$. The buffers were prepared in 50 mM Na cacodylate and 0.2 mM DTT, from pH 4.5 to 7.5, and in 0.2 M Tris and 0.2 mM DTT, from pH 8 to 10.8.

Scheme I. Preparation of *N*-Acetylneuraminic Acid Derivatives via Sialic Acid Aldolase-Catalyzed Aldol Condensation[a]



[a] (a) NeuAc aldolase, 0.5 M pyruvate, 10 mM DTT, pH 7.5; (b) vinyl acetate, DMF, subtilisin 8399, 45 °C, 5 days; (c) lactic acid ethyl ester, subtilisin 8399, 50 °C, 3 days; (d) Ac_2O, pyridine; (e) BnOH, BF_3·OEt_2, CH_3NO_2; (f) MeONa, MeOH; (g) Dowex 50W-X8 [H^+]; (h) dimethylphosphinyl chloride, 2,6-lutidine, DMF.

Although both microbial enzymes have very similar substrate specificity at pH 7.5, they have different specificity at pH 9.0. At pH 7.5, the native enzyme was found to be specific for NeuAc; the relative rates for the C-9 modified NeuAc derivatives decreased to ca. 50%. On the contrary, the relative rates of the C-9 modified analogues for the tagged enzyme are higher than that of *N*-acetylneuraminic acid (Table II).

The C-5 and C-9 modified sialic acid derivatives used in this study were prepared via sialic acid aldolase-catalyzed condensation of C-2 and C-6 modified *N*-acetylmannosamine (ManNAc) derivatives with pyruvate (Scheme I). The 6-*O*-acylated ManNAc derivatives were prepared via transesterification in DMF catalyzed by a subtilisin variant (8399 or 8397) engineered to be stable in organic solvent.[26] The wild type enzyme (BPN′) can also be used but requires more enzyme since the variant is about 100 (for 8399) to 1000 times (for 8397) more stable than the wild type. We

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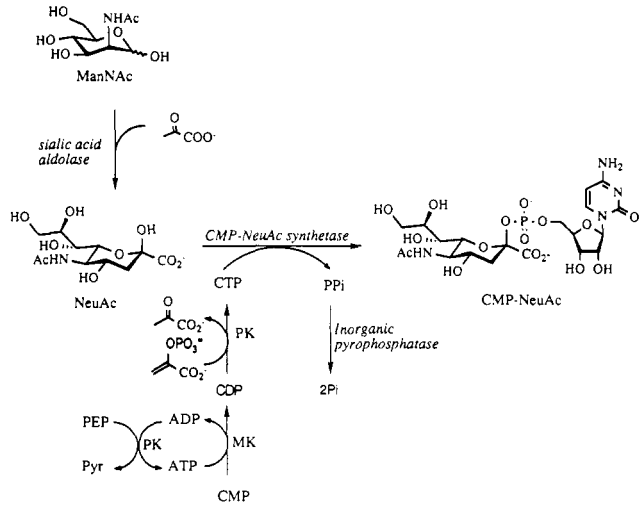
Scheme II. CMP-Sialic Acid Synthetase-Catalyzed Synthesis of CMP-NeuAc with in Situ Generation of NeuAc and CTP[a]



[a] Abbreviations: PEP, phosphoenolpyruvate; Pyr, pyruvate; PPi, inorganic pyrophosphate; PK, pyruvate kinase; MK, myokinase.

believe the enzymatic procedure described here for the preparation of 6-*O*-acyl sugars is much more effective than the reported chemical procedure.[27]

Synthesis of CMP-*N*-Acetylneuraminic Acid Using a Cofactor Regeneration System. CMP-NeuAc was synthesized directly from *N*-acetylmannosamine (ManNAc) and CMP via a cofactor regeneration system as shown in Scheme II. Although the Whitesides group reported the use of calf brain enzyme in multigram-scale synthesis of CMP-NeuAc, they prepared CTP and NeuAc separately.[11a] A small-scale conversion of ManNAc to CMP-NeuAc in the presence of pyruvate, CTP, NeuAc aldolase, and unpurified CMP-NeuAc synthetase from calf brains was also described.[11a] Thiem and Stangier reported the synthesis of CMP-NeuAc using the calf enzyme from NeuAc with in situ generation of CTP from CMP; however, the yield of CMP-NeuAc was only 15% yield.[11b]

Our synthesis started with the less expensive materials, ManNAc and CMP, and catalytic amounts of ATP (1% that of CMP). NeuAc was generated from ManNAc and pyruvic acid in situ with sialic acid aldolase and converted irreversibly to CMP-NeuAc. CMP was converted to CDP with myokinase and ATP. The released ADP was converted to ATP with pyruvate kinase and phosphoenolpyruvate (PEP).[28] CDP was then converted to CTP with pyruvate kinase and PEP. CTP reacted with NeuAc with the aid of the tagged CMP-NeuAc synthetase. In 2 days, 98% yield of CMP-NeuAc was obtained on the basis of the added CMP with a turnover number of 100 for ATP. The overall yield was significantly improved as compared to the reported procedure[11] on the basis of the crude enzyme preparation from calf brain. This procedure provides a straightforward method for the preparation of CMP-NeuAc without using the expensive starting materials such as CTP and NeuAc. Another advantage is that the system eliminates the problem of substrate inhibition caused by CTP (K_i = 1.97 mM, Figure 4).

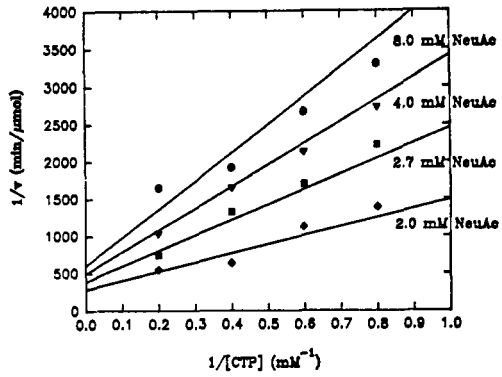
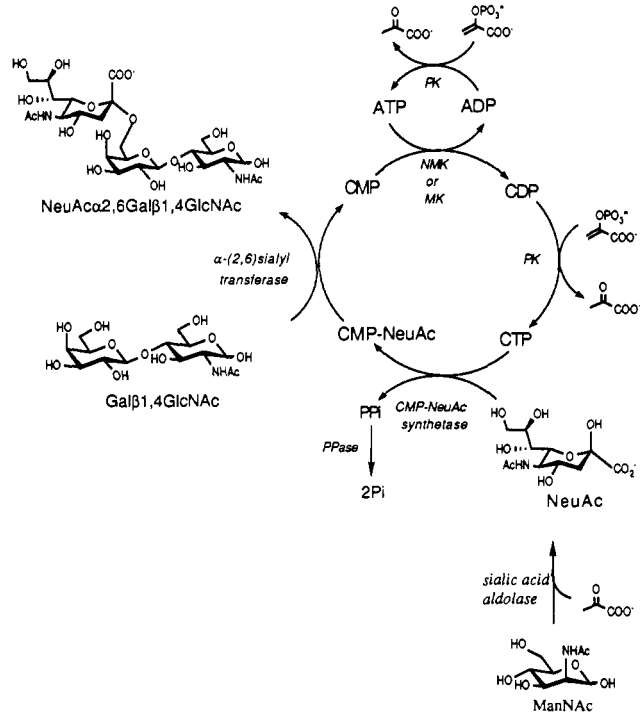


Figure 4. Lineweaver–Burk plot of tagged CMP-sialic acid synthetase for the determination of kinetic parameters. The initial rates at different substrate concentration were accumulated to fit in the equation $v = V[A][B]/([A][B] + K_a[B] + K_b[A] + K_{ia}K_b)$, where K_a and K_b represent the Michaelis constants for CTP and NeuAc and are 1.99 and 4.8 mM, respectively. K_{ia} is the dissociation constant (or inhibition constant) for CTP and is 1.97 mM.

Scheme III. Enzymatic Synthesis of Sialyl *N*-Acetyllactosamine[a]



[a] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; CMP, cytidine 5′-monophosphate; PK, pyruvate kinase; NMK, nucleoside monophosphate kinase; MK, myokinase; PPase, pyrophosphatase.

Synthesis of Sialyl Oligosaccharide with in Situ Regeneration of CMP-Sialic Acid. The above CMP-NeuAc synthesis system was further coupled with α(2,6)sialyl transferase-catalyzed incorporation of NeuAc from CMP-NeuAc into *N*-acetyllactosamine using a procedure described previously.[17] Sialic acid was introduced irreversibly to a disaccharide acceptor starting from ManNAc without a separate preparation of NeuAc and CMP-NeuAc. The pyruvate generated from PEP in cofactor regeneration is used as a substrate for sialic acid aldolase. In a synthesis of NeuAcα(2,6)Galβ(1,4)GlcNAc as shown in Scheme III, CMP was converted to CDP catalyzed by myokinase (MK) in the presence of PEP. CDP was further converted to CTP with PEP catalyzed by PK. CTP was then reacted with NeuAc catalyzed by CMP-NeuAc synthetase. The byproduct inorganic pyrophosphate (PPi) was decomposed by pyrophosphatase (PPase). Sialylation of Galβ(1,4)GlcNAc was accomplished by CMP-NeuAc and α(2,6)sialyl transferase. The released CMP was again

(27) For 9-*O*-acetyl-*N*-acetylneuraminic acid and 9-*O*-lactyl-*N*-acetylneuraminic acid: (a) Auge, C.; David, S.; Gautheron, C.; Veyrieres, A. *Tetrahedron Lett.* **1984**, *25*, 4663 (6-*O*-acetyl-ManNAc was prepared chemically). (b) Kim, M.-J.; Hennen, W. J.; Sweers, M.; Wong, C.-H. *J. Am. Chem. Soc.* **1988**, *110*, 6481 (6-*O*-acetyl-ManNAc was prepared via protease N reaction). (c) Auge, C.; David, S.; Gautheron, C.; Malleron, A.; Cavaye, B. *New J. Chem.* **1988**, *12*, 733 (for 9-*O*-lactyl-NeuAc from chemically synthesized 6-*O*-lactyl-ManNAc). For 9-azido-9-deoxy-*N*-acetylneuraminic acid: (d) Brossmer, R.; Rose, U. *Biochem. Biophys. Res. Commun.* **1980**, *96*, 1282 (no physical data were reported). For 3-deoxy-L-glycero-L-galactononulosonic acid (KDN): (e) Auge, C.; Gautheron, C.; David, S. *Tetrahedron* **1990**, *46*, 201. For 3,5-dideoxy-L-glycerol-L-galactononulosonic acid (5-deoxy-KDN): (f) Auge, C.; Bouxom, B.; Cavaye, B.; Gautheron, C. *Tetrahedron Lett.* **1989**, *30*, 2217.

(28) Hirschblein, B. L.; Mazenod, F. P.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 3765.

converted to CDP, to CTP, and to CMP-NeuAc. Starting from LacNAc, ManNAc, PEP, and catalytic amounts of CTP and ATP, sialyl LacNAc was synthesized in 89% isolated yield. We have also demonstrated that *N*-acetyllactosamine can be generated in situ from a galactosyl transferase-catalyzed reaction coupled with regeneration of UDP-Glc and UDP-Gal.[29]

Conclusion

In summary, we have developed an efficient method for the overproduction of a modified CMP-NeuAc synthetase. The system allows for a rapid selection of a positive clone for expression of the desired enzyme. This modified enzyme had similar specificity and kinetic properties to the native enzyme, though small differences in stability and pH optimum were observed. If necessary, however, the tag can be removed via a second cloning. We have also developed new preparative syntheses of NeuAc derivatives and CMP-NeuAc, and coupling of the synthesis of CMP-NeuAc with a sialyl transferase is now available for the preparation of various sialosides on large scales.

Experimental Section

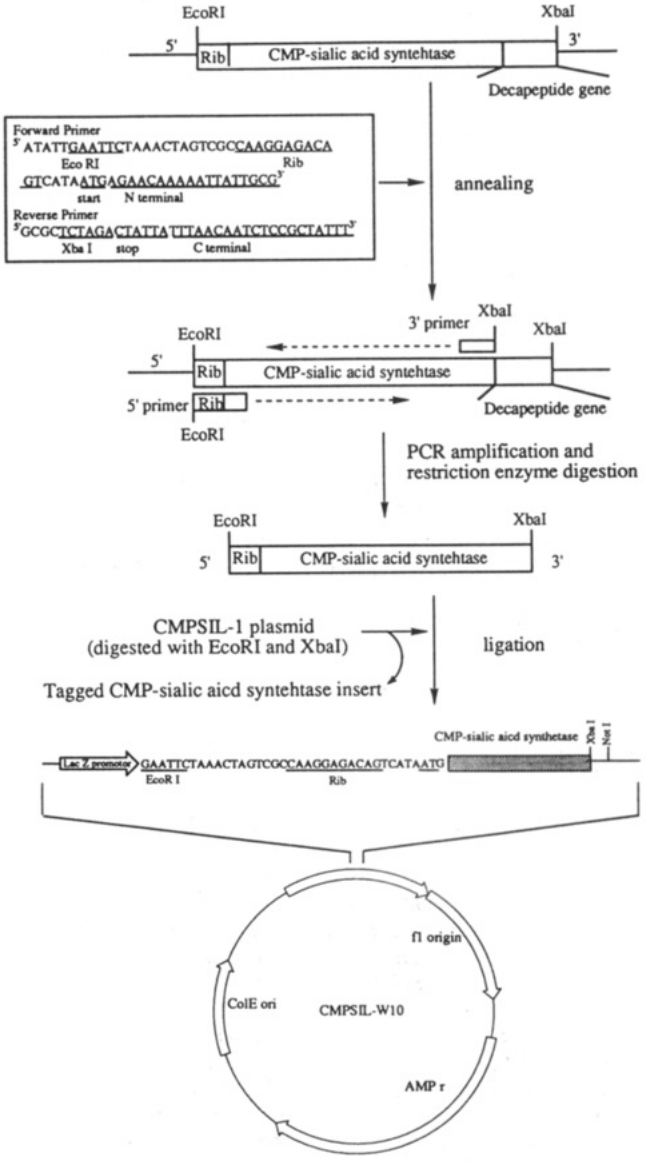
General. All chemicals were purchased from commercial sources as reagent grade. Dye affinity chromatography was performed on Amicon Orange A matrix gels. Gel filtration chromatography was conducted on a Bio-Gel P-2, 200–400 mesh (Bio-Rad). Fast protein liquid chromatography (FPLC) was performed on a Pharmacia system composed of two P-500 pumps, a GP-250 gradient programmer, and a single-path UV-1 monitor with a Sepharose 12 GPC column. UV–visible spectrum was recorded on a Beckman DU-70 spectrometer. SDS–PAGE was carried out on Pharmacia Phast System. ^1H and ^{13}C NMR spectra were recorded at 250, 300, and 500 MHz on Bruker AC-250, AM-300, and AMX-500, respectively. High-resolution mass spectra (HRMS) were recorded on a VG ZAB-ZSE mass spectrometer under electron impact (EI) or fast atom bombardment (FAB) conditions. Thin-layer chromatography was conducted on Baker Si250F silica gel TLC plates with fluorescent indicator. Column chromatography was conducted with silica gel, grade 62, 60–200 mesh, and 150 Å.

Plasmids for the Native and Tagged CMP-*N*-Acetylneuraminic Acid Synthetase. The 1.3-kb NeuAc gene coding for the native CMP-sialic acid synthetase was amplified by PCR[30] by using the primers shown in Figure 1 with pWA1 plasmid DNA as a template. The PCR product was purified by phenol extraction followed by gel filtration on a Bio-Gel P-10 spin column in TE buffer. The purified oligonucleotide was digested with *Eco*RI and *Hin*dIII and purified by an agarose gel electrophoresis in low melting point agarose. This fragment was then ligated into the pKK 223-3 under the control of the tac promotor.[15,31] This plasmid was assigned as pWG123. pWG123 was then transformed into *E. coli* Sure strain obtained from Stratagene Co.

The construction of plasmid CMPSIL-1, which contains the modified CMP-NeuAc synthetase gene, was described previously.[17] The CMP-NeuAc synthetase gene was fused with a decapeptide tag sequence, Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Ser, at the C-terminus and was cloned into the lambda ZAP vector at *Eco*RI and *Xba*I sites and overexpressed in the *E. coli* Sure strain. The method for removing the decapeptide tag from the modified enzyme is described in Scheme IV. To subclone the CMP-sialic acid synthetase gene insert containing no decapeptide tag sequence into CMPSIL-1, the primers in Scheme IV were used for PCR as described previously.[17b] The amplified PCR inserts and CMPSIL-1 plasmid were then digested with *Eco*RI and *Xba*I (40 units/mg of DNA) for 1 h at 37 °C and the digested DNA was purified on 1% agarose gel. The purified native CMP-sialic acid synthetase gene insert and the same restriction enzyme digested CMPSIL-1 plasmid were ligated with T4 DNA ligase (4 units from Stratagene Co.) at 4 °C overnight. The ligated DNA was then transformed into *E. coli* Sure strain and plated on LB agar plates containing 250 μg/mL ampicillin. The positive clones were selected by the assay of CMP-NeuAc synthetase activity after growth on LB medium and induced with IPTG as described previously.[17b]

Preparation of the Native and Modified CMP-NeuAc Synthetases. The bacteria that harbor plasmids were grown on LB-rich media (Bacto Trypton, 25 g; yeast extract, 10 g; NaCl, 3 g; pH 7.0 in 1 L) containing 100 μg/mL ampicillin to midlogarithmic phase (OD_{660} about 0.6–0.7)

Scheme IV. Removal of the Decapeptide Sequence



at 37 °C and then induced with 0.5 mM IPTG (isopropyl β-D-thiogalactopyranoside) for 10 h at 30 °C with shaking. The cells were harvested by centrifugation (10000*g*, 20 min, 4 °C) and disrupted by a French pressure cell at 16000 lb/in^2. The cell debri was removed by centrifugation at 23000*g* for 60 min and the supernatant (cell-free extract) was used for enzyme purification. The cell-free extract (30 mL) from a 1 L culture was passed through the Orange A dye column (1.5 mg/mL of gel, 3 cm × 30 cm) and washed with 200 mL of 0.2 M Tris-HCl buffer containing 0.02 M $MgCl_2$ and 0.2 mM dithiothreitol, pH 7.5. The enzyme was eluted with a linear gradient from 0 to 1 M KCl in the same buffer (total 200 mL). The active fractions were pooled and dialyzed in 2 L of 0.2 M Tris-HCl buffer (pH 7.5) containing 0.02 M $MgCl_2$ and 0.2 mM dithiothreitol. This enzyme preparation was used for synthesis directly. For kinetic studies, the enzyme was further purified to about 95% purity by FPLC on a Superose 12 HR 10/30 column from Pharmacia.

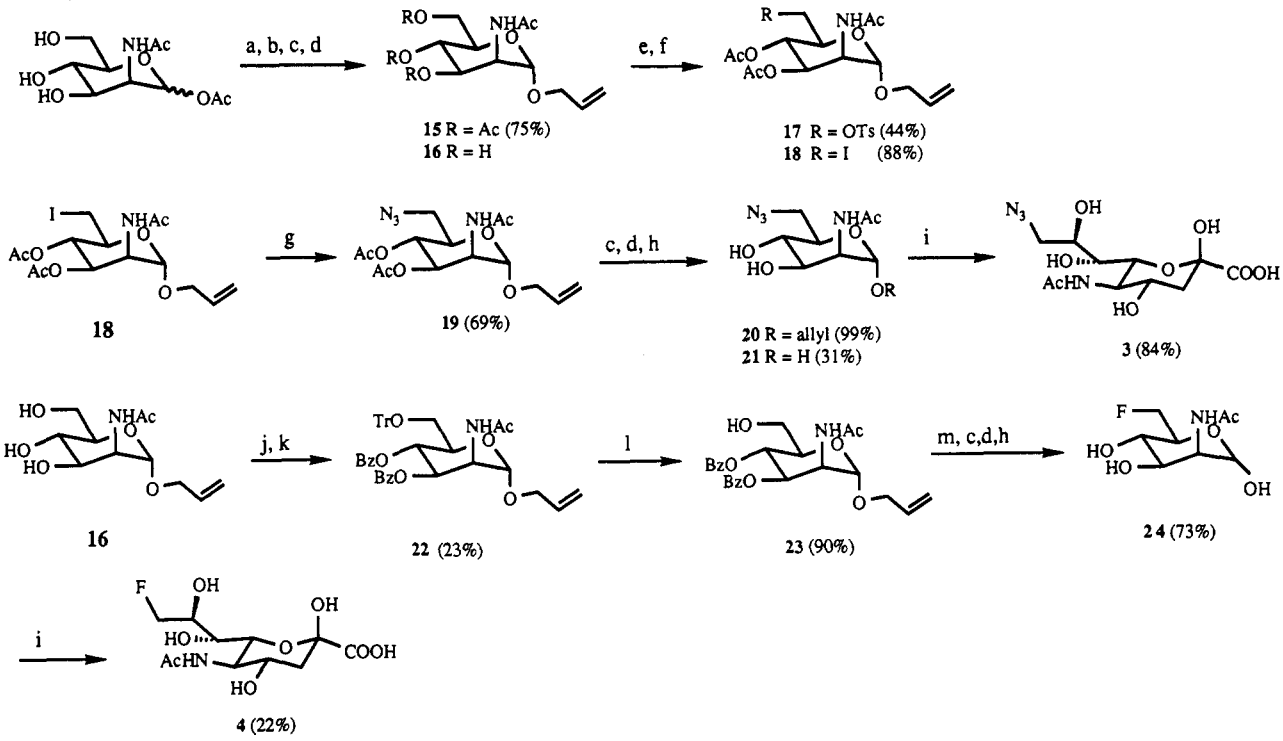
Enzyme Assay and Kinetics. The activity of the native or the modified CMP-NeuAc synthetase was assayed on the basis of the thiobarbituric acid method described by Vann et al.[13] The enzyme was incubated in 250 μL of buffer containing 5.5 mM CTP, 2.8 mM *N*-acetylneuraminic acid, 0.2 M Tris, 20 mM $MgCl_2$, and 0.2 mM DTT, pH 9.0. After the mixture was incubated at 37 °C for 30 min, 50 μL of 1.6 M $NaBH_4$ was added to destroy excess NeuAc at room temperature for 15 min. The mixture was then put in the ice bath and 50 μL of H_3PO_4 was added to decompose $NaBH_4$. The mixture was kept at 0 °C for 5 min then incubated at 37 °C for 10 min to cleave the phosphoester bond of the formed CMP-NeuAc. The free NeuAc was oxidized with 50 μL of 0.2 M $NaIO_4$ at room temperature for 10 min, and 400 μL of 4% $NaAsO_2$ in 0.5 N HCl was added. The solution mixture was then transferred to

(29) Ichikawa, Y.; Liu, J. L.-C.; Shen, G.-J.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 6300.

(30) Innis, M. A.; Gelfand, D. H.; Sinsky, J. J.; White, T. J. *PCR Protocols, A guide to methods and applications*; Academic Press: San Diego, CA, 1990.

(31) Tabor, S.; Richardson, C. C. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 1074.

Scheme V. Synthesis of 9-Azido-9-deoxy-*N*-acetylneuraminic Acid and 9-Deoxy-9-fluoro-*N*-acetylneuraminic Acid[a]



[a] (a) Ac_2O, pyridine; (b) $BF_3{\cdot}OEt_2$, CH_3NO_2, allyl alcohol; (c) MeONa, MeOH; (d) Dowex 50W-X8 [H^+]; (e) tosyl chloride, pyridine–CH_2Cl_2; (f) NaI, 2-butanone; (g) NaN_3, DMF, 100 °C; (h) $PdCl_2$, NaOAc, 95% AcOH, 60 °C; (i) NeuAc aldolase, 0.5 M pyruvate, 10 mM DTT, pH 7.5; (j) trityl chloride, pyridine, 72 °C; (k) BzCl, 0 °C; (l) 80% AcOH; (m) (diethylamino)sulfur trifluoride, dry diglyme, 3 h, 40 °C.

a test tube containing 1 mL of 0.6% thiobarbituric acid in 0.5 M Na_2SO_4 and heated in boiled water for 15 min. After the solution was cooled, 1 mL of the solution was taken out and mixed with 1 mL of cyclohexanone. The mixture was shaken and centrifuged, and the upper layer was taken for absorbance measurement at 549 nm ($\epsilon = 4.11\ mM^{-1}\ cm^{-1}$).

Initial velocities were measured at various concentration of CTP (1.25–5 mM) and NeuAc (2–8 mM) for kinetic studies. The accumulated data were fitted into the sequential bi-bi substrate rate equation, eq 1 to derive the Michaelis constants and maximum velocity (V) by

$$v = \frac{VAB}{AB + K_aB + K_bA + K_{ia}K_b} \quad (1)$$

using a Sigma plot program from Sigma Co. In this equation, A stands for [CTP], B stands for [NeuAc], K_a and K_b are the Michaelis constants for CTP and NeuAc, respectively, and K_{ia} is the dissociation constant (or inhibition constant) for CTP. A typical Lineweaver–Burk plot for the determination of kinetic parameters is shown in Figure 4.

Enzyme Stability Study. The native and modified enzymes were incubated at room temperature in a 0.2 M Tris buffer, pH 7.5, containing 0.02 M $MgCl_2$ and 0.2 mM dithiothreitol. At defined time intervals, 30-μL aliquots were removed and assayed for remaining activity as described above.

pH Profile. Both enzymes were assayed for activity in 0.2 M Tris buffers, from pH 8 to 10.8, and in 50 mM sodium cacodylate buffers, from pH 4.5 to 7.5. These buffers were prepared in the presence of 20 mM $MgCl_2$ and 6 mM $MnCl_2$ separately, each containing 0.2 mM dithiothreitol. The assay solution containing 5.5 mM CTP and 2.8 mM NeuAc was incubated at 37 °C for 30 min, and the amounts of CMP-NeuAc formed were determined on the basis of the thiobarbituric acid assay.

Substrate Specificity. The substrate specificity was determined in a 250-μL assay solution containing 2.8 mM of each substrate, 5.5 mM CTP, CMP-NeuAc synthetase, and 0.2 M Tris buffer with 20 mM $MgCl_2$ and 0.2 mM DTT at pH 7.5 and 9.0. The incubation time varied from 15 min to 5 h, depending on the activity of the enzymes toward the substrate analogues. The formation of CMP-NeuAc derivatives was determined by the thiobarbituric acid assay.

CMP-*N*-Acetylneuraminic Acid. To 100 mL of HEPES buffer (200 mM, pH 7.5) was added ManNAc (1.44 g, 6 mmol), PEP sodium salt (1.88 g, 8 mmol), pyruvic acid sodium salt (1.32 g, 12 mmol), CMP (0.64 g, 2 mmol), ATP (0.011 mg, 0.02 mmol), pyruvate kinase (300 units), myokinase (750 units), inorganic pyrophosphatase (3 units), *N*-acetylneuraminic acid aldolase (100 units), and CMP-sialic acid synthetase (1.6 units), and the reaction mixture was stirred at room temperature for 2 days under argon until CMP disappeared. The reaction mixture was concentrated by lyophilization and directly applied to a Bio-Gel P-2 (200–400 mesh, 3 × 90 cm) column and eluted with water at a flow rate of 6 mL/40 min at 4 °C. The CMP-NeuAc fractions were pooled, applied to Dowex-1 (formate form), and eluted with a 0.1–0.5 M ammonium bicarbonate gradient. The CMP-NeuAc fractions free of the nucleotides were pooled and lyophilized. Excess ammonium bicarbonate was removed by addition of Dowex 50W-X8 (H^+ form) to the stirred solution of the residual powder until pH 7.5. The resin was filtered off and the filtrate was lyophilized to yield the ammonium salt of the CMP-NeuAc (1.28 g, 87.8% yield). ^1H NMR (500 Hz, D_2O) δ 1.5 (1 H, ddd, J = 13, 11.6 Hz, H-3ax), 1.9 (3 H, s, NAc), 2.33 (1 H, dd, J = 13, 4.7 Hz, H-3eq), 3.29 (1 H, d, J = 9.7 Hz, H-7), 3.46 (1 H, dd, J = 6.6, 11.8 Hz, H-9a), 3.73 (1 H, dd, J = 11.8, 2.4 Hz, H-9b), 3.78 (1 H, dd, J = 9.7, 2.4 Hz, H-8), 3.8 (1 H, dd, J = 9.7, 9.7 Hz, H-5), 3.92 (1 H, ddd, J = 9.7, 4.7, 11.6 Hz, H-4), 3.98 (1 H, dd, J = 9.7, 1.4 Hz, H-6), 4.09 (3 H, m, H-4′, H-5′), 4.15 (1 H, dd, J = 5, 5 Hz, H-3′), 4.18 (1 H, dd, J = 5, 5 Hz, H-2′), 5.83 (1 H, d, J = 5 Hz, H-1′), 5.97 (1 H, d, J = 7.6 Hz, H-5″), 7.32 (1 H, d, J = 7.6 Hz, H-6″).

Enzyme Immobilization on Eupergit C. A solution of the native CMP-NeuAc synthetase enzyme was dialyzed against a 0.2 M Tris buffer (pH 7.5) containing 0.02 M $MgCl_2$ for 2 h. To the enzyme solution, free of dithiothreitol, with enzyme activity of 2 units was added 1 g of Eupergit C gel (Accurate Chemical and Scientific Corp.), and the mixture was shaken with a roter at 80 rpm for 3 days. Mercaptoethanol, 200 μL, was added to the suspension to destroy the excess oxirane group, and the resulting suspension was shaken for another 3 days. The enzyme solution was then filtered through a glass funnel and the Eupergit C gel was washed with 0.2 M Tris buffer (pH 7.5) containing 0.02 M $MgCl_2$ and 0.2 mM dithiothreitol (4 × 4 mL). The enzyme-immobilized Eupergit C gel, 300 mg, was suspended in 2 mL of Tris buffer, pH 9.0, containing 0.02 M $MgCl_2$ and 0.2 mM dithiothreitol, and 50 μL of 137.5 mM CTP and 50 μL of 70 mM *N*-acetylneuraminic acid were then added to reach the final concentration of 3.3 mM for CTP and 1.7 mM for *N*-acetylneuraminic acid. The reaction mixture was incubated at 37 °C for 30 min. The supernatant, 250 μL, was taken and quenched with 50 μL of 1.6 M $NaBH_4$. The formation of CMP-*N*-acetylneuraminic acid was determined by the thiobarbituric acid assay. The enzyme activity of 0.03 unit was found in the individual assay and the total enzyme activity in 1 g of Eupergit C gel was 0.83 unit (41.5% yield).

CMP-NeuAc Synthesis by Immobilized CMP-NeuAc Synthetase. In 5 mL of pH 9.0 buffer containing 200 mg of NeuAc, 352 mg of CTP,

0.2 M Tris base, 0.02 M $MgCl_2$, and 0.2 mM DTT was suspended 0.4 U of Eupergit C immobilized with CMP-NeuAc synthetase (500 mg). The reaction was incubated at 37 °C with a rotary shaker for 48 h. The Eupergit C gel was then filtered and the filtrate was directly applied to Dowex-1 (formate form) and eluted with 0.1–0.5 M ammonium bicarbonate gradient (total volume 600 mL). The CMP-NeuAc containing fractions were pooled and lyophilized. Excess ammonium bicarbonate was removed by addition of Dowex 50W-X8 [H^+] to a stirred solution of the residual powder in water (15 mL) until pH 7.5. The resin was filtered and the filtrate was lyophilized to yield the ammonium salt of CMP-NeuAc in 82% yield (325 mg).

Preparation of Sialic Acid Analogues. 3-Deoxy-L-*glycero*-L-*galacto*-2-nonulosonic Acid (KDN) (7). To 10 mL of potassium phosphate buffer (0.1 M, pH 7.5) was added 10 mM DTT, 0.5 M pyruvate, 0.1 M D-mannose and 1.5 mg of NeuAc aldolase. The reaction mixture was shaken at 37 °C for 3 days. The reaction mixture was chromatographed with a Dowex-1 (HCO_3^-) resin, eluted with a 0–1 M ammonium bicarbonate gradient. The fractions containing KDN were pooled and lyophilized 3 times repeatedly to remove the volatile salt to give a 78% yield of KDN. The physical data of the product were consistent with the reported.[27]

3,5-Dideoxy-L-*glycero*-L-*galacto*-2-nonulosonic Acid (5-deoxy-KDN) (8). 2-Deoxyglucose (0.1 M) was added to the solution instead of D-mannose, and the preparation procedure is the same as above. The reaction mixture was shaken for 5 days and compound **8** was obtained in 30% yield. The physical data of the product were consistent with the reported data.[27]

***6-O*-Acetyl *N*-Acetylmannosamine (11).** In 2 mL of DMF was suspended 500 mg (2.2 mmol) of *N*-acetylmannosamine. Vinyl acetate (1 mL, 5 equiv) and 160 mg of subtilisin mutant 8399[26] were then added and the suspension was stirred vigorously at room temperature. The reaction progress was monitored by TLC with ethyl acetate/methanol (2/1). After the formation of the diacetylated derivatives began in 5 h, the reaction was stopped by evaporating the vinyl acetate and DMF. Methanol was then added to dissolve the sugar. The enzyme and insoluble salt were filtered off, and the filtrate was concentrated. The residual syrup was chromatographed on a silica gel, with ethyl acetate/methanol (10/1) to give **11** (92%) as an α/β mixture (3:1). α-Anomer: ^{1}H NMR (D_2O) δ 1.98 (3 H, s, NAc), 2.06 (3 H, s, OAc), 3.58 (1 H, dd, J = 9, 9 Hz, H-4), 3.96 (1 H, dd, J = 3.5, 9 Hz, H-3), 4.15–4.4 (4 H, m, H-2,5,6,6'), 5.04 (d, J = 0.9 Hz, H-1). α/β mixture: ^{13}C NMR (D_2O) δ 23.1, 24.7, 56.1, 56.7, 66.5, 66.6, 69.7, 69.8, 71.3, 72.4, 74.6, 76.6, 95.8, 95.9, 176.7, 176.8, 177.6, 178.4. The product was identical with that prepared with protease N reaction.[27]

9-*O*-Acetyl-*N*-acetylneuraminic Acid (1). To a 10 mL of 0.1 M potassium phosphate buffer (pH 7.5) containing 10 mM DTT and 0.5 M pyruvate were added 100 mg of 6-*O*-acetylmannosamine and 1.5 mg of NeuAc aldolase (36 units). The reaction mixture was incubated at 37 °C for 8 days followed by lyophilization. The lyophilized powder was dissolved in a small amount of water and directly applied to a Bio-Gel P-2 column (3 × 90 cm) and eluted with water at a flow rate of 6 mL/40 min at 4 °C. To remove the trace amount of contaminated NeuAc due to the hydrolysis of the product, another gel filtration was required. The 9-*O*-acetyl-NeuAc was obtained in 47% yield. Its physical data were consistent with the reported data.[27b]

6-*O*-Lactyl *N*-Acetylmannosamine (12). ManNAc (200 mg) and 50 mg of subtilisin mutant 8399[25] were added to a mixture of lactic acid ethyl ester (4 mL) and 400 μL of 0.5 N phosphate buffer (pH 7.5). The reaction mixture was shaken under 50 °C for 3 days. The solvent was then evaporated and methanol was added to the residue. After the insoluble materials were filtered off, the filtrate was concentrated. The residue was chromatographed on silica gel with ethyl acetate/methanol (5/1) to give **12** in 50% yield. The physical data were consistent with the reported data.[27c] ^{1}H NMR (D_2O) δ 1.29 (3 H, d, J = 7 Hz, lactyl-CH_3), 1.91 (3 H, s, OAc), 3.51 (1 H, dd, J = 9.5, 9.5 Hz, H-4), 3.92 (1 H, dd, J = 9.5, 4.2 Hz, H-3), 4.16 (1 H, dd, J = 1.8, 4.3 Hz, H-2), 4.2–4.4 (3 H, m, H-5,6), 4.88 (d, J = 0.9 Hz, H-1α), 4.97 (d, J = 1.3 Hz, H-1β); ^{13}C NMR (D_2O) δ 20.10, 22.76, 22.93, 54.17, 54.81, 65.1, 67.6, 67.91, 69.43, 70.61, 93.87. HRMS calcd for $C_{11}H_{19}NO_2$ (M^+): 293.1111. Found: 293.1091.

9-*O*-Lactyl-*N*-acetylneuraminic Acid (2). The procedure was the same as that for the preparation of **1**, except **12** instead of **11** was used. Another gel filtration purification was required to separate the product from NeuAc to give **2** in 18% yield. The physical data of the product were consistent with the reported values.[27c]

Allyl 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-mannopyranoside (15). A solution of ManNAc (5.0 g, 2.6 mmol) and Ac_2O (10 mL) in pyridine (20 mL) was stirred for 10 h at room temperature, and the mixture was concentrated, followed by coevaporation with toluene. A solution of the residue, allyl alcohol (2.63 g, 45.2 mmol; 3.1 mL), and BF_3·OEt_2 (1.60 g, 11.3 mmol; 1.39 mL) in CH_3NO_2 (150 mL) was gently refluxed for 2.5 h. After it cooled, the mixture was concentrated. The residue was chromatographed on silica gel, with toluene–EtOAc (1:2) to give **15** in 6.47 g (74%). ^{1}H NMR ($CDCl_3$) δ 1.99, 2.05, 2.06, 2.12 (3 H, s, 3 × OAc, NHAc), 4.00–4.03 (1 H, m, H-5), 4.07 (1 H, dd, J = 2.45, 12.24 Hz, H-6a), 4.29 (1 H, dd, J = 5.34, 12.23 Hz, H-6b), 4.63 (1 H, ddd, J = 1.43, 4.60, 9.11 Hz, H-2), 4.81 (1 H, d, J = 1.43 Hz, H-1), 5.11 (1 H, t, J = 10.18 Hz, H-4), 5.36 (1 H, dd, J = 4.59, 10.19 Hz, H-3), 5.82 (1 H, d, J = 9.11 Hz, NHAc); ^{13}C NMR ($CDCl_3$) δ 20.67, 20.74, 23.33, 50.29, 62.42, 66.05, 68.02, 68.64, 69.11, 98.02, 118.42, 132.83, 169.89, 169.96, 170.08, 180.55.

Allyl 2-Acetamido-3,4-di-*O*-acetyl-2-deoxy-6-*O*-tolylsulfonyl-α-D-mannopyranoside (17). A solution of **15** (6.46 g, 16.7 mmol) and methanolic NaOMe (2 mL; 1 M solution) in MeOH (100 mL) was stirred for 2 h at room temperature and neutralized by addition of Dowex 50W-X8 [H^+]. After the resin was filtered off, the filtrate was concentrated by coevaporation with pyridine. A solution of tolylsulfonyl chloride (3.50 g, 18.3 mmol) in pyridine (20 mL) and CH_2Cl_2 (30 mL) was added dropwise to a cooled solution of the residue in pyridine (30 mL) and CH_2Cl_2 (50 mL) at 0–5 °C over 30 min, and the mixture was stirred for 10 h at room temperature then cooled. Acetic anhydride (30 mL) was added to the mixture, and the mixture was stirred for 5 h at room temperature. The mixture was concentrated, and the residue was chromatographed on silica gel, with toluene–EtAOc (1:2), to give **17** (3.68 g, 44%). ^{1}H NMR ($CDCl_3$) δ 1.98 (6 H, s, 2 × Ac), 2.04 (3 H, s, Ac), 2.46 (3 H, s, CH_3 of tosyl), 3.96–4.00 (1 H, m, H-4), 4.10 (1 H, dd, J = 4.50, 11.50 Hz, H-6a), 4.30 (1 H, dd, J = 2.00, 11.50 Hz, H-6b), 4.62 (1 H, ddd, J = 1.50, 4.50, 9.00 Hz, H-2), 4.78 (1 H, d, J = 1.50, 4.50, 9.00 Hz, H-2), 4.78 (1 H, d, J = 1.50 Hz, H-1), 5.16 (1 H, t, J = 10.0 Hz, H-4), 5.33 (1 H, dd, J = 4.50, 10.0 Hz, H-3), 6.03 (1 H, d, J = 9.50 Hz, NHAc); ^{13}C NMR ($CDCl_3$) δ 20.59, 20.78, 21.64, 23.26, 50.00, 65.75, 67.99, 68.10, 68.69, 69.20, 98.04, 118.37, 129.89, 132.79, 170.28. HRMS calcd for $C_{22}H_{29}NO_{10}S$ (M^+): 500.1590. Found: 500.1590.

Allyl 2-Acetamido-3,4-di-*O*-acetyl-2,6-dideoxy-6-iodo-α-D-mannopyranoside (18). A solution of **17** (3.68 g, 7.37 mmol) and NaI (2.21 g, 14.7 mmol) in 2-butanone (100 mL) was gently refluxed for 10 h and cooled. The mixture was concentrated, and the residue was chromatographed on silica gel, with toluene–EtOAc (1:2), to give **18** (2.96 g, 88%). ^{1}H NMR ($CDCl_3$) δ 1.99, 2.05, 2.08 (3 H, s, 2 × OAc, NHAc), 3.19 (1 H, dd, J = 7.50, 11.0 Hz, H-6a), 3.35 (1 H, dd, J = 3.0, 11.0 Hz, H-6b), 3.71 (1 H, ddd, J = 3.0, 7.5, 10.0 Hz, H-5), 4.63 (1 H, ddd, J = 1.5, 4.5, 9.5 Hz, H-2), 4.82 (1 H, d, J = 1.5 Hz, H-1), 4.98 (1 H, t, J = 10.0 Hz, H-4), 5.36 (1 H, d, J = 4.5, 10.0 Hz, H-3), 5.79 (1 H, d, J = 9.5, NHAc); ^{13}C NMR ($CDCl_3$) δ 5.49, 20.74, 23.34, 50.17, 68.51, 69.11, 70.07, 97.76, 118.54, 132.75, 169.93, 169.99. HRMS calcd for $C_{15}H_{22}NO_7I$ (M^+): 456.0519. Found: 456.0520.

Allyl 2-Acetamido-3,4-di-*O*-acetyl-6-azido-2,6-dideoxy-α-D-mannopyranoside (19). In 10 mL of DMF was dissolved 0.9 g of **18**, 3 equiv of NaN_3 was then added and the reaction mixture was heated at 100 °C for 10 h. The product had the same R_f value (0.7) as the iodo derivative (**18**) on TLC (ethyl acetate). However, on the TLC, the azido derivative was UV invisible but the iodo derivative was UV visible. After evaporation, the residue was purified by silica gel chromatography (hexane/ethyl acetate, 3/2) to give **19** (0.5 g, 69%). ^{1}H NMR ($CDCl_3$) δ 1.92 (3 H, s, NHAc), 1.98 (3 H, s, OAc), 1.99 (3 H, s, OAc), 3.2 (1 H, dd, J = 3, 13 Hz, H-6a), 3.27 (1 H, dd, J = 6.5, 13 Hz, H-6b), 3.87 (1 H, ddd, J = 3, 6.5, 11.7 Hz, H-5), 3.95 (1 H, dd, J = 6, 18 Hz, allyl-H-1a), 4.11 (1 H, dd, J = 6, 18 Hz, allyl-H-1b), 4.54 (1 H, ddd, J = 2, 5.5, 9.8 Hz, H-2), 4.74 (1 H, d, J = 2 Hz, H-1), 5.02 (1 H, dd, J = 9.8, 11.7 Hz, H-4), 5.14 (1 H, dd, J = 2, 11.3 Hz, allyl-H-3a), 5.23 (1 H, dddd, J = 6, 6, 11.3, 16 Hz, allyl-H-2), 6.17 (1 H, d, J = 9.8 Hz, NH); ^{13}C NMR ($CDCl_3$) δ 20.8, 23.50, 67.68, 68.4, 69.2, 97.5, 117.8, 132.4, 169.8, 170.1.

Allyl 2-Acetamido-6-azido-2,6-dideoxy-α-D-mannopyranoside (20). Compound **19** (0.5 g) was dissolved in 10 mL of methanol containing 0.2 M MeONa. After 5 min, Dowex 50 cation exchange resin was added to neutralize the mixture. The resin was filtered and the filtrate was concentrated. The yield was 99%. ^{1}H NMR ($CDCl_3$) δ 2.03 (3 H, s, NAc), 3.4–3.5 (2 H, m, H-6a,6b), 3.59 (1 H, dd, J = 9.8, 9.8 Hz, H-4), 3.75 (1 H, m, H-5), 4.02 (1 H, dd, J = 9.8, 5 Hz, H-3), 4.18, 4.39 (1 H, dd, J = 5, 8.1 Hz, H-2), 4.74 (1 H, br s, OH), 4.8 (1 H, s, H-1), 4.87 (1 H, br s, OH), 5.23 (1 H, dd, J = 1.2, 10 Hz, allyl-H-3a), 5.32 (1 H, dd, J = 17, 1.2 Hz, allyl-H-3b), 5.90 (1 H, dddd, J = 6, 6, 10, 17 Hz, allyl-H-2), 6.69 (1 H, d, J = 8.1 Hz, NH).

2-Acetamido-6-azido-2,6-dideoxy-α-D-mannopyranoside (21). A suspension of **20** (200 mg), 1.2 equiv of $PdCl_2$, and 2.4 equiv of NaOAc was dissolved in 95% acetic acid (5 mL). The reaction mixture was stirred at room temperature overnight and then concentrated. The residue was purified with silica gel chromatography ($CHCl_3$/ethyl acetate/methanol,

5/2/2) to give **15** in 31% yield. ^{1}H NMR (D_2O) δ 1.66 (3 H, s, NAc), 3.46 (1 H, dd, J = 9.8, 9.8 Hz, H-4), 3.45–3.55 (2 H, m, H-6a, 6b), 3.82 (1 H, m, H-5), 3.87 (1 H, dd, J = 4.6, 9.8 Hz, H-3), 4.15 (1 H, d, J = 4.6 Hz, H-2), 4.88 (d, J = 1.2 Hz, H-1β), 4.97 (s, H-1α).

9-Azido-9-deoxy-*N*-acetylneuraminic Acid (3). In a 10 mL of 0.1 M potassium phosphate buffer (pH 7.5) containing 10 mM DTT and 0.5 M pyruvate was dissolved 50 mg of 6-azido-6-deoxy-*N*-acetylmannosamine (**21**) and 1.5 mg of NeuAc aldolase. The starting material was consumed in 14 h. The solution was lyophilized and the mixture was purified with Bio-Gel P-2 gel filtration (3 × 90 cm) chromatography, eluted with water at a flow rate of 6 mL/40 min, at 4 °C. The fractions containing the product were pooled and freezed dry to give **3** in 84% yield. ^{1}H NMR (D_2O) δ 1.66 (1 H, dd, J = 11, 13 Hz, H-3ax), 1.89 (3 H, s, NAc), 2.05 (1 H, dd, J = 4.4, 13 Hz, H-eq), 3.31 (1 H, dd, J = 5.8, 12 Hz, H-9a), 3.37 (1 H, dd, J = 1.2, 10 Hz, H-7), 3.45 (1 H, dd, J = 3.3, 12 Hz, H-9b), 3.7–3.9 (4 H, m, H-4, 5, 6, 8). HRMS calcd for $C_{11}H_{18}N_4D_8$ ($M - H^-$): 333.1046. Found: 333.1046.

4,6-Dideoxy-4,6-difluoro-α-D-talopyranose. Methyl-4,6-dideoxy-4,6-difluoro-α-D-talopyranoside (200 mg) prepared from methyl α-D-mannoside [68% yield; $[\alpha]^{25}_D$ 101.9° (c 1.02, MeOH) (lit. 102.3°, c 1.03 MeOH); ^{13}C NMR (MeOH) δ 103.2 (s, C_1), 90.2 (dd, C6, $J_{C\text{-}6,F\text{-}6}$ = 181.25, $J_{C\text{-}6,F\text{-}4}$ = 6.25 Hz), 83.08 (dd, C_4, $J_{C\text{-}4,F\text{-}4}$ = 167.5, $J_{C\text{-}4,F\text{-}6}$ = 7.5 Hz), 70.3 (s, C_2), 69.31 (dd, C5, $J_{C\text{-}5,F\text{-}5}$ = 22.5, $J_{C\text{-}5,F\text{-}4}$ = 18.75 Hz), 66.5 (d, C_3, $J_{C\text{-}3,F\text{-}4}$ = 16.25 Hz] via reaction with (diethylamino)sulfur trifluoride[32] was mixed with 5 g of Dowex 50 (H^+) resin in 50 mL of water and refluxed with stirring overnight (~18 h). The resin was filtered off and the solution was lyophilized to yield the free pyranose that was used in the aldol reaction without further purification.

5-Deamino-7,9-dideoxy-7-epi-7,9-difluoroneuraminic Acid (5). HRMS calcd: 271.0625. Found: 271.0649. ^{1}H NMR (500 MHz, D_2O) δ 4.7 (m, H-7), 4.5 (dd, H-9, $J_{H,F}$ = 47.5, $J_{H,H}$ = 3 Hz), 4.20 (td, H-8, $J_{H,F}$ = 18.5, $J_{H,H}$ = 3.0 Hz), 3.93 (m, H-6), 3.84 (m, H-4), 3.47 (t, H-5, $J_{H,H}$ = 10.5 Hz), 2.12 (dd, H-3e, $J_{H\text{-}eq,H\text{-}4}$ = 2.6, $J_{H\text{-}eq,H\text{-}3}$ = 12.5 Hz), 1.74 (dd, H-3a, $J_{H,H\text{-}4}$ = 12.5, $J_{H\text{-}3ax,H\text{-}\beta eq}$ = 12 Hz). Peak assignment was accomplished by the use of 2D techniques.

5-Deamino-5-epi-5-fluoroneuraminic Acid (6). Prepared from 2-deoxy-2-fluoroglucose via the aldolase reaction. HRMS calcd: 269.0673. Found: 269.0651. ^{13}C NMR (D_2O, CD_3OD as standard) δ 174.5 (s, C-1), 96.4 (s, C-2), 34.5 (s, C-3), 65.5 (d, C-4, $J_{C\text{-}4,F\text{-}5}$ = 18 Hz), 91.2 (d, C5, $J_{C\text{-}5,F\text{-}5}$ = 191 Hz), 70.7 (d, C-6, $J_{C\text{-}6,F\text{-}5}$ = 18 Hz), 71.9 (s, C-7), 72.8 (s, C-8), 63.5 (s, C-9).

Immobilization of Neu5Ac aldolase. To a suspension of PAN-750[33] (2 g) in 7.0 mL of Hepes buffer (pH 7.5, 0.3 M Hepes, 0.03 M $MgCl_2$, and 20 mM Neu5Ac for active site protection) and 1.275 mL of triethylenetetramine (TET) was added a 1-mL Hepes buffer solution of 15.9 mg Neu5Ac aldolase (6.0 units/mg). After the suspension lyophilized, it was allowed to stand for 1 h at room temperature. The gel was then broken up and rinsed into a 50-mL centrifuge tube with 30 mM Hepes buffer (pH 7.5, containing 10 mM $MgCl_2$ and 50 mM ammonium sulfate). The solution was shaken vigorously and centrifuged at 3000 rpm for 10 min. The solution was decanted and the gel was resuspended in the original 0.3 M Hepes buffer (pH 7.5, containing 30 mM $MgCl_2$). The rinse was repeated, and the gel was obtained by centrifugation. The immobilization yield of Neu5Ac aldolase was 60%.

Large-Scale Neu5Ac Synthesis. A suspension of ManNAc (10.0 g, 41.8 mmol), sodium pyruvate (32.19 g, 292.6 mmol), and the enzyme-containing gels (57 units) in 270 mL of phosphate buffer (pH 7.5, 5 mM) containing 50 mg of sodium azide was stirred at room temperature with an overhead stirrer. The reaction progress was monitored by ^{1}H NMR. After 48 h, the reaction was 90.9% complete. The gel was removed by centrifugation and then placed into a flask for further use. The Neu5Ac was purified by the procedure described below.

A crude preparation of ManNAc (approximately 75% pure) obtained from the base-catalyzed epimerization of *N*-acetylglucosamine (GlcNAc)[11a] was also used. The contamination of GlcNAc is not a problem since it is not accepted by the enzyme.

Purification of Neu5Ac. The crude Neu5Ac obtained from the aldolase reactions was easily purified by a three-step process that eliminated the need for chromatography. The crude solution was first passed through a large column containing excess Dowex 50W-X8 [H^+] resin and eluted with water. The eluent was then lyophilized to yield a white pasty solid, which was then triturated with ethyl acetate to remove pyruvic acid. Only a trace amount of ManNAc can be detected by NMR. The enzymatic synthesis produced at least 85% yield of NeuAc.

Benzyl 2-Acetamido-2-deoxy-6-*O*-(dimethylphosphinyl)-α-D-mannopyranoside (13). A solution of ManNAc (5.0 g, 2.6 mmol), Ac_2O (10 mL), and pyridine (20 mL) was stirred for 10 h at room temperature, and the mixture was concentrated, followed by coevaporation with toluene. A solution of the residue, benzyl alcohol (20 mL), and BF_3·OEt_2 (1.6 g, 11.3 mmol) in CH_3NO_2 (150 mL) was gently refluxed for 2.5 h. After cooling, the mixture was concentrated. The residue was chromatographed on silica gel, with toluene–EtOAc (1:2). The isolated benzyl 2-acetamido-4,5,6-tri-*O*-acetyl-2-deoxy-α-D-mannopyranoside and 0.15 g of NaOMe were dissolved in MeOH (100 mL) and the solution was stirred for 30 min at room temperature and neutralized by addition of Dowex 50W-X8 (H^+). After the resin was filtered off and the filtrate was concentrated, followed by coevaporation with pyridine. A solution of dimethylphosphinic chloride (1 g, 8.8 mmol) in DMF was added to a cold solution of benzyl 2-acetamido-2-deoxy-α-D-mannopyranoside (0.50 g, 1.6 mmol) and 2,6-lutidine (0.34 g, 3.2 mmol) in anhydrous DMF (30 mL) with a dry ice–acetone bath, and the reaction was allowed to slowly warmed to room temperature. The reaction was monitored by TLC with (1 M NH_4OAc/2-propanol/EtOAc, 1/2.4/3.4). After 10 h, the reaction mixture was directly applied to silica gel chromatography, eluted with $CHCl_3$/EtOAc/MeOH (5/2/1) to give **13** (56% yield). ^{1}H NMR (D_2O) δ 1.45 (3 H, d, J = 13.4 Hz, PCH_3), 1.5 (3 H, d, J = 13.4 Hz, PCH_3), 1.89 (3 H, s, NAc), 3.53 (1 H, dd, J = 8, 8 Hz, H-4), 3.76 (1 H, dd, J = 8, 4.3 Hz, H-3), 4.0 (3 H, m H-5, 6), 4.18 (1 H, d, J = 4.3 Hz, H-2), 4.44 (1 H, d, J = 9.2 Hz, BnH-1a), 4.55 (1 H, d, J = 9.2 Hz, BnH-1b), 4.76 (1 H, s, H-1), 7.28 (5 H, s Bn).

2-Acetamido-2-deoxy-6-*O*-(dimethylphosphinyl)-α-D-mannopyranoside (14). A solution of **13** (100 mg, 0.26 mmol) in ethanol/water (10 mL; 1/1) was hydrogenated with 50 mg of 10% Pd/C for 10 h. The reaction progress was monitored with TLC (EtOAc/AcOH/H_2O, 8/2/1). The catalyst was filtered and the filtrate was concentrated to give **14** (100% yield). ^{1}H NMR (D_2O) δ 1.51 (6 H, d, J = 13.6 Hz), 1.94 (3 H, s, NAc), 3.45 (2 H, m), 3.9 (1 H, dd, J = 8, 4.3 Hz, H-3), 4.1 (3 H, m, H-5,6), 4.87, 4.98 (1 H, s, H-1).

9-*O*-(Dimethylphosphinyl)-*N*-acetylneuraminic Acid (9). Sialic acid aldolase-catalyzed aldol condensation of compound **14** and pyruvic acid was conducted for 4 days. The product was purified with a Bio-Gel P-2 at 4 °C to give **9** in 42% yield. ^{1}H NMR (D_2O) δ 1.48 (6 H, d, J = 14.2 Hz, P-CH_3), 1.67 (1 H, dd, J = 11,13 Hz, H-3ax), 1.9 (3 H, s, NAc), 2.08 (1 H, dd, J = 5.3, 13 Hz, H-2eq), 3.45 (1 H, d, J = 9.3 Hz, H-7), 3.8–4.0 (6 H, m, H-4,5,6,8,9). HRMS calcd: 385.1137. Found: 385.1309.

Allyl 2-Acetamido-3,4-di-*O*-benzoyl-2-deoxy-6-*O*-trityl-α-D-mannopyranoside (22). A solution of allyl 2-acetamido-2-deoxy-α-D-mannopyranoside (**16**), 2.0 g, and 1.2 equiv of trityl chloride was stirred for 10 h at 72 °C. After the reaction mixture was cooled to 0 °C, 2.5 equiv of benzoyl chloride was added to the mixture. The reaction mixture was allowed to slowly warm to room temperature in 2 h. After the reaction was completed, ice water was added to the reaction mixture and the mixture was extracted with ethyl acetate. The organic extracts were washed with 1 N HCl twice, dried, and concentrated. The residue was applied to silica gel chromatography, eluted with hexane/ethyl acetate (10/1) to give **22** (23% yield). ^{1}H NMR ($CDCl_3$) δ 2.05 (3 H, s, NAc), 3.87 (1 H, dd, J = 9.8, 9.8 Hz, H-4), 4.05 (1 H, dd, J = 6, 12 Hz, allyl), 4.1 (3 H, m, H-5,6,6'), 4.2 (1 H, m, allyl), 4.96 (1 H, d, J = 11.3 Hz, allyl), 5.07 (1 H, d, J = 11.3 Hz, allyl), 5.17 (1 H, m, allyl), 5.97 (1 H, d, J = 7.4 Hz, N*H*Ac).

Allyl 2-Acetamido-3,4-di-*O*-benzoyl-2-deoxy-α-D-mannopyranoside (23). A suspension of 1.5 g of compound **22** in 80% acetic acid (10 mL) was allowed to stir overnight at room temperature. After the reaction mixture was concentrated, the residue was applied to silica gel chromatography, eluted with hexane/ethyl acetate (5/1) to give **23** (90% yield). ^{1}H NMR (D_2O) δ 2.0 (3 H, s, NHAc), 3.78 (2 H, m, H-5,6), 4.04 (1 H, m, H-6), 4.1 (1 H, m, allyl), 4.23 (1 H, ddd, J = 1.26, 5.73, 12.7 Hz, allyl), 4.88 (1 H, dd, J = 4.56, 9.24 Hz, allyl), 4.93 (1 H, d, J = 1.06 Hz, H-1), 5.24 (1 H, dd, J = 1.25, 10.5 Hz, allyl), 5.33 (1 H, dd, J = 1.4, 17.2 Hz, allyl), 5.63 (1 H, dd, J = 10.1, 10.1 Hz, H-4), 5.91 (1 H, dd, J = 4.55, 10.34 Hz, H-3), 5.95 (1 H, m, allyl), 6.83 (1 H, d, J = 9.15 Hz, HNAc).

2-Acetamido-2,6-dideoxy-6-fluoro-α-D-mannopyranoside (24). To a stirred solution of (diethylamino)sulfur trifluoride (0.5 mL) in dry diglyme (2 mL) was added a solution of **23** (100 mg) in dry diglyme (3 mL) at room temperature, and the reaction mixture was stirred for 1 h at room temperature and 3 h at 40 °C. After the starting material was consumed, the reaction mixture was poured onto ice water and extracted with ethyl acetate. The extract was dried and concentrated, and the residue was applied to silica gel chromatography. After the impurity was eluted with hexane, the product was eluted with ether to give the fluorinated product in 89% yield. The produce was then dissolved in 5 mL of 1 N sodium methoxide in methanol to remove the benzoyl group. After 20 min, Dowex 50W X-8 [H^+] was added to neutralize the reaction mixture. The resin was filtered and the filtrate was concentrated to give

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allyl 2-acetamido-2,6-dideoxy-6-fluoro-α-D-mannopyranoside in 99% yield. The product (50 mg), 1.2 equiv of palladium(II) acetate, and 2.5 equiv of sodium acetate in 95% acetic acid (5 mL) were mixed and stirred at 50 °C for 18 h, and the solvent was removed under vacuum. The residue was applied to silica gel chromatography, eluted with ethyl acetate/methanol (2/1) to obtain **24** in 73% yield. ^{1}H NMR (D_2O) δ 1.9 (3 H, s, NAc), 3.5 (1 H, dd, J = 10.3, 10.3 Hz, H-4), 3.72 (1 H, m, H-5), 3.93 (1 H, dd, J = 4.5, 10.3 Hz, H-3), 4.16 (1 H, d, J = 4.5 Hz, H-2), 4.46 (2 H, m, H-6), 4.9, 5.0 (1 H, s, H-1).

9-Deoxy-9-fluoro-*N*-acetylneuraminic Acid (4). A solution of **24** (20 mg) and pyruvic acid sodium salt (255 mg, 30 equiv) in 0.1 M potassium phosphate buffer (pH 7.5, 10 mL) in the presence of *N*-acetylneuraminic acid aldolase (100 units) was incubated at 37 °C for 8 days. The reaction mixture was lyophilized and chromatographed with Bio-Gel P-2 column to give **4** in 22% yield. The physical data were in accordance with reported.[34]

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Total Synthesis of Furanocembranolides. 1. Stereocontrolled Preparation of Key Heterocyclic Building Blocks and Assembly of a Complete *seco*-Pseudopterane Framework

Leo A. Paquette,* Annette M. Doherty,[1a] and Christopher M. Rayner[1b]

Contribution from the Evans Chemical Laboratories, The Ohio State University, Columbus, Ohio 43210. *Received October 15, 1991*

Abstract: A retrosynthetic strategy for the total synthesis of pseudopterolide and allied pseudopteranes is presented. This scheme is dependent upon early elaboration of suitable 2,5-difunctionalized 3-furoate esters. To this end, the pair of useful substrates **21** and **24** was readily synthesized from 2,3-*O*-isopropylidene-D-glyceraldehyde and methyl 4-(phenylthio)acetoacetate. The conversion of both of these intermediates into furanolactone **27** was next studied. The best method for gaining suitable control of stereochemistry involved condensation of **24** with methyl 3-formylpropionate under conditions of boron trifluoride catalysis. Transformation of the (phenylthio)methyl substituent of **27** into the requisite isopentenyl side chain was next accomplished in five steps. Because alkylation α to the lactone carbonyl in **46** could be realized only in modest yield, this final segment of the intended macrocyclic ring was introduced earlier by more convergent means. Indeed, the coupling of **24** to **52** proved to be efficient and highly diastereoselective. Following an unsuccessful attempt to introduce the isopentenyl side chain after elaboration of the butenolide subunit, the chemical sequence was reversed. The dual selenenylation strategy for oxidation of both relevant pendant groups was notably effective for this purpose. The subsequent chemospecific attachment of the isobutenyl fragment onto bromide **62** was achieved by palladium(0)-catalyzed coupling to a vinylstannane in a process that promises considerable versatility. Further chemical manipulation gave rise to the seco-pseudopterane **71**, thereby completing the intermediate stages of the total synthesis of the pseudopterane ring system.

Extensive investigation of marine invertebrates belonging to the genera *Alcyonacea*, *Lophogorgia*, and *Gorgonacea* by several research groups has led to the identification and characterization of a host of cembranoids, many of which possess potent biological activity. The biogenetic considerations underlying formation of these macrocycles have been reviewed,[2] as has synthetic activity in the area.[3] Among the many metabolites produced by these organisms is a small subgroup that possesses furano and butenolide structural segments in combination. Pseudopterolide (**1**), a representative furanocembranolide, is a potent cytotoxic agent that inhibits cell cleavage but not nuclear division,[4] giving an effect similar to that triggered by cytochalasin D. Pukalide (**2a**)[5] and epoxypukalide (**2b**)[6] are also characterized by the presence of one or two fused oxirane rings, an appreciable number of stereogenic centers, as well as carbomethoxy and isopropenyl appendages. However, the central ring is now 14-membered. The structurally related aldehyde known as lophotoxin (**3**) is recognized to be a particularly powerful neuromuscular toxin.[7] More highly oxygenated and nitrogen-containing analogues of these systems continue to be identified at a rapid pace.[8]

A 3-methyl group in the furan ring characterizes other less oxygenated analogues. Of these, kallolide A (**4a**), a potent antiinflammatory agent with efficacy equivalent to indomethacin,[9] shares with kallolide B (**4b**) an irregular pseudopterane carbon skeleton. Their formation presumably stems from the coupling of two geranyl units. Rubifolide (**5**)[10] and coralloidolide A (**6**),[11]

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