Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 12 (2004) 6427–6435
Bioorganic & Medicinal Chemistry

Chemoenzymatic synthesis of CMP–sialic acid derivatives by a one-pot two-enzyme system: comparison of substrate flexibility of three microbial CMP–sialic acid synthetases

Hai Yu, Hui Yu, Rebekah Karpel and Xi Chen*

Department of Chemistry, University of California, One Shields Avenue, Davis, CA 95616, USA

Received 2 August 2004; accepted 16 September 2004

Abstract—Three C terminal His_6-tagged recombinant microbial CMP–sialic acid synthetases [EC 2.7.7.43] cloned from *Neisseria meningitidis* group B, *Streptococcus agalactiae* serotype V, and *Escherichia coli* K1, respectively, were evaluated for their ability in the synthesis of CMP–sialic acid derivatives in a one-pot two-enzyme system. In this system, *N*-acetylmannosamine or mannose analogs were condensed with pyruvate, catalyzed by a recombinant sialic acid aldolase [EC 4.1.3.3] cloned from *E. coli* K12 to provide sialic acid analogs as substrates for the CMP–sialic acid synthetases. The substrate flexibility and the reaction efficiency of the three recombinant CMP–sialic acid synthetases were compared, first by qualitative screening using thin layer chromatography, and then by quantitative analysis using high performance liquid chromatography. The *N. meningitidis* synthetase was shown to have the highest expression level, the most flexible substrate specificity, and the highest catalytic efficiency among the three synthetases. Finally, eight sugar nucleotides, including cytidine 5′-monophosphate *N*-acetylneuraminic acid (CMP–Neu5Ac) and its derivatives with substitutions at carbon-5, carbon-8, or carbon-9 of Neu5Ac, were synthesized in a preparative (100–200 mg) scale from their 5- or 6-carbon sugar precursors using the *N. meningitidis* synthetase and the aldolase.

1. Introduction

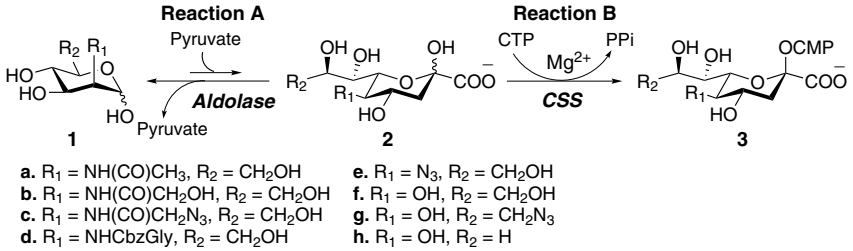
Sialic acids are negatively charged, 9-carbon sugars that have been predominantly found in vertebrates, a few higher invertebrates, and certain types of bacteria.[1] They constitute a remarkable diverse family of more than 50 structurally distinct members. In vertebrates, sialic acids are primarily expressed on the outermost end of carbohydrate structures on cell surface glycoproteins and glycolipids or exist as homopolymers of *N*-acetylneuraminic acid (Neu5Ac) in α2,8 linkages in mammalian brain tissues.[2] Sialic acid containing structures in eukaryotic systems play important roles in a variety of physiological and pathological processes, including cell–cell interactions, inflammation, fertilization, viral infection, differentiation, malignancies, and cell signaling etc.[1,2] In bacteria, sialic acids are found as components of extracellular capsular polysaccharides and lipooligosaccharides (LOS). These structures are believed to mimic sialylated host cell surface carbohydrates and their presence could account for the ability of the bacteria to evade the host immune defense mechanisms.[3–5]

Glycoconjugates containing either naturally occurring or structurally modified sialic acids are invaluable tools in understanding important biological and physiological properties of sialylated structures. Enzymatic glycosylation employing sialyltransferases overcomes tedious and expensive synthetic scheme of chemical synthesis and produces regio- and stereospecific sialates in a short and economic way. Thus, it has become one of the most practical and efficient methods for production of complex sialates and derivatives. Sialyltransferase-based synthesis requires activation of free sialic acids or their synthetic analogs to form cytidine 5′-monophosphate-sialic acid (CMP–sialic acid) analogs.[6] Biosynthetically, this monosaccharide activation process is catalyzed by a class of enzymes named CMP–sialic acid synthetase (CSS, or sialic acid cytidylyltransferase, EC 2.7.7.43; Scheme 1, Reaction B).[7]

CSS has previously been isolated from eukaryotic sources and bacterial cultures and has been used in the synthesis of CMP–sialic acids.[8–15] The enzyme has also

Keywords: Aldolase; CMP–sialic acid synthetase; CMP–sialic acid; Enzymatic synthesis.
* Corresponding author. Tel.: +1 530 754 6037; fax: +1 530 752 8995; e-mail: chen@chem.ucdavis.edu

0968-0896/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2004.09.030



Scheme 1. One-pot two-enzyme synthesis of CMP–sialic acid derivatives (**3a**–**h**) from ManNAc or mannose derivatives (**1a**–**h**) with a recombinant sialic acid aldolase cloned from *E. coli* K12 and a recombinant microbial CMP–sialic acid synthetase.

been cloned from various eukaryotic and prokaryotic sources.[16–25] The crystal structures of the N-terminal catalytically active domain of a mouse CSS,[26] a full length *Neisseria meningitidis* CSS,[27] and a related CMP–KDO synthetase of *Escherichia coli*[28,29] have been reported. Recombinant CMP–sialic acid synthetases cloned from bacteria sources are effective in obtaining natural and unnatural CMP–sialic acid analogs. The recombinant CSS from *E. coli* K1 is the first commercially available CSS. This enzyme, however, has narrow substrate specificity. The enzyme has been found to accept only a few C-9 substituted derivatives of Neu5Ac.[30,31] Recently, high level expression of *N. meningitidis* CSS has been reported[32,33] and shown to have a broad substrate specificity.[35,36] Both *E. coli* K1 and *N. meningitidis* enzymes have been applied in the enzymatic synthesis of CMP–sialic acid and sialic acid-containing glycoconjugates.[30–40] However, no direct comparison of the substrate specificity of these two enzymes has been reported. CSS has also been isolated[41] and cloned[19] from *Streptococcus agalactiae* (group B streptococci), but its substrate specificity and application in the synthesis of CMP–sialic acid analogs have not been investigated.

Here, we report a study on direct comparison of the ability and efficiency of three recombinant microbial CSSs, cloned from *N. meningitidis* group B, *S. agalactiae* serotype V, and *E. coli* K1, respectively, in the synthesis of CMP–sialic acid analogs in a one-pot two-enzyme reaction system. In this system, the substrates (sialic acid analogs) of the CSS were obtained by condensation reactions of *N*-acetylmannosamine (ManNAc) or mannose (Man) analogs and an excess amount (5 equiv) of pyruvate using a recombinant sialic acid aldolase cloned from *E. coli* K12 in the same reaction mixture. Among the three recombinant CSSs, the *N. meningitidis* synthetase was shown to have the highest expression level, the most flexible substrate specificity, and the highest catalytic efficiency as indicated by quantitative high performance liquid chromatography (HPLC) analysis. A series of structural analogs of CMP–Neu5Ac were then synthesized on a preparative scale from ManNAc/Man analogs, sodium pyruvate, and CTP using the *N. meningitidis* CSS and the recombinant sialic acid aldolase.

2. Results and discussion

2.1. Cloning, expression, and purification of CMP–sialic acid synthetases

Three C-terminal hexahistidine-tagged full length CSSs were cloned in pET vectors (Novagen, Madison, WI) from *N. meningitidis* group B, *S. agalactiae* serotype V, and *E. coli* K1, respectively. The expression of the enzymes in *E. coli* BL21 (DE3) cells was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and carried out in LB rich medium at 20 °C for 20 h with vigorous shaking (250 rpm). The recombinant enzymes were purified using an affinity column packed with nickel–nitrilotriacetic acid–agarose (Ni^{2+}–NTA–agarose) resin, which bind specifically to the C-terminal hexahistidine tag in the recombinant proteins. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) data (Fig. 1) indicated that high level expressions were achieved for both *N. meningitidis* and *S. agalactiae* CSSs with the recombinant CSS presented more than 85% of whole cell proteins (lane 3 in Fig. 1A and B). More interestingly, the *N. meningitidis* CSS was very

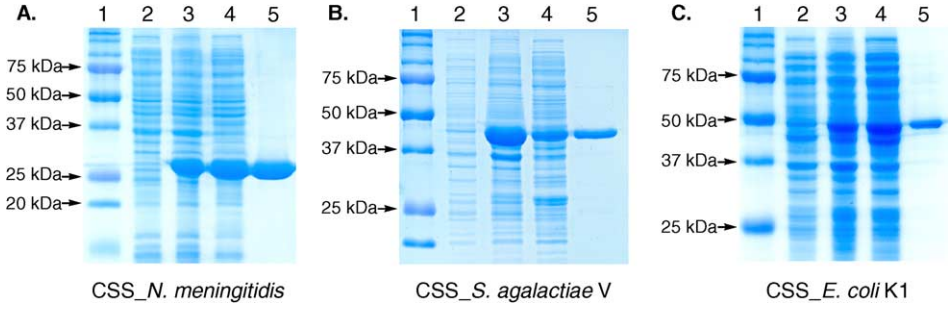


Figure 1. SDS-PAGE (12% Tris–glycine gel) for CSS. Lanes: 1, protein standards; 2, whole cells, before induction; 3, whole cells, after induction; 4, lysate, after induction; 5, CSS purified by Ni^{2+}–NTA column.

soluble and existed in more than 85% of the lysate proteins (lane 4 in Fig. 1A), the soluble portion of total host proteins. However, the *S. agalactiae* CSS presented only about 25% of the lysate proteins (lane 4 in Fig. 1B). The expression level of the *E. coli* CSS (lane 3 in Fig. 1C) was much lower than that of the *N. meningitidis* or the *S. agalactiae* CSS. Nevertheless, a similar percentage of *E. coli* (lane 4 in Fig. 1C) and *S. agalactiae* (lane 4 in Fig. 1B) CSSs were found in the lysate samples. Overall, the highest expression level of the soluble protein was achieved for the *N. meningitidis* CSS. The SDS-PAGE also indicated that one step Ni^{2+}–NTA column purification was very efficient to provide CSSs in over 95% purity (lane 5 in Fig. 1A–C).

Unlike the *N. meningitidis* CSS, which contains only 228 amino acid (aa) residues with a molecular weight of about 25 kDa, both *S. agalactiae* (413 aa, 46 kDa) and *E. coli* K1 (419 aa, 49 kDa) CSSs are longer proteins. A recent study indicates that only the N-terminal amino acid residues (1-229 aa) of the *E. coli* CSS are essential for CSS activity.[42] Due to a high sequence homology found in the *N. meningitidis* CSS, and the N-terminus of the *S. agalactiae* and *E. coli* CSSs, only the N-terminus of the *S. agalactiase* CSS is presumably required for the synthetase activity. The lower expression level of the *E. coli* CSS and the lower proportion of the *S. agalactiae* CSS in the lysate compared to those of the *N. meningitidis* CSS indicate that the C-terminal peptide sequences may interfere with the protein expression and solubility.

2.2. Thin layer chromatography (TLC) analysis for substrate specificity assays

D-*N*-Acetylmannosamine (ManNAc) and mannose (Man) analogs were either obtained from commercial sources or synthesized using known methods as described in the experimental section. These ManNAc/Man derivatives were used in TLC analysis to test the substrate flexibility of a recombinant sialic acid aldolase cloned from *E. coli* K12. Sialic acid aldolase catalyzes the reversible condensation of pyruvate and ManNAc to form Neu5Ac with the equilibrium favoring the aldol cleavage (Scheme 1, Reaction A).[43] In agreement with the previous reports,[43] the aldolase was shown to have a flexible acceptor specificity. The recombinant *E. coli* K12 aldolase was very effective in accepting all the ManNAc/Man analogs we tested as the substrates, and produced corresponding Neu5Ac analogs. Pyruvate was used in an excess amount (5 equiv) to drive the aldolase reaction toward the formation of the condensation products. Then, qualitative TLC assays were carried out as an initial screening step to test the feasibility of the synthesis of CMP–sialic acid analogs from pyruvate, CTP, and ManNAc analogs using a combined enzyme system (Scheme 1) containing both the aldolase and a microbial CSS cloned from *E. coli* K1, *N. meningitidis*, or *S. agalactiae*. TLC results provided informative indication of suitable reaction conditions and reaction times. The CSSs were shown to require a pH of 8.5–9.0 for best activity. Although the aldolase worked most efficiently at pH 7.5, it also worked well at pH 8.5–9.0. Therefore, a Tris–HCl buffer of pH 8.8 was used for all assays and syntheses. The optimal reaction time was 2 h. Elongating the incubation time resulted in the breakdown of the CMP–sialic acid products.

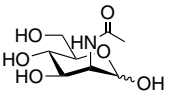
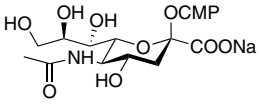
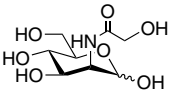
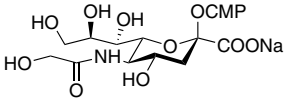
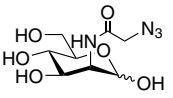
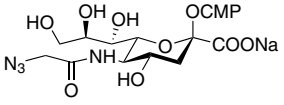
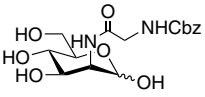
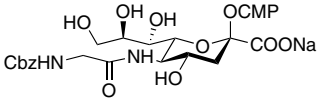
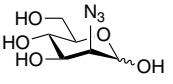
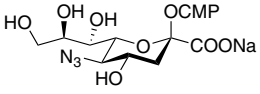
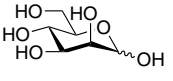
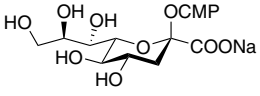
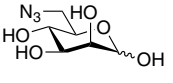
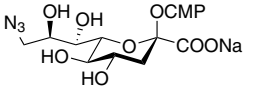
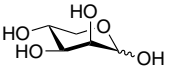
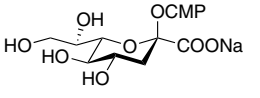
2.3. High performance liquid chromatography (HPLC) analysis for substrate specificity

HPLC analysis was carried out to quantitatively compare the substrate flexibility and reaction efficiency of the *N. meningitidis*, *S. agalactiae*, and *E. coli* CSSs. Reactions were carried out with CTP (10 mM), sodium pyruvate (50 mM), and ManNAc/Man analogs (10 mM) in a Tris–HCl (100 mM, pH 8.8) buffer containing 20 mM of $MgCl_2$. The reaction was stopped at 2 h by adding ice cold acetonitrile.

Various assay methods have been previously reported for CMP–Neu5Ac synthetase. The most frequently used method is the colorimetric periodate-thiobarbituric acid assay developed by Kean and Roseman.[44] However, this method is not suitable for Neu5Ac derivatives that do not participate in the periodate–thiobarbituric acid reaction.[45] An improved ion-pair high-performance liquid chromatographic (HPLC) method with gradient elution[46] overcomes the limitations of the colorimetric method and provides quantitative data. Thus, it was chosen for our studies. CMP–sialic acid analogs were efficiently separated from CTP, CMP, and CDP within 35 min using methanol-pH gradient elution with tetrabutylammonium hydrogensulfate as a pairing agent in a Premier C18 reversed-phase column (5 μ) monitored by a UV detector at 270 nm.

More than 50 naturally occurring sialic acid derivatives were described.[2] The most abundant forms found in animal glycans were *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc), while deaminoneuraminc acid (KDN) was frequently found in the lipooligosaccharide (LOS) of various Gram-negative bacteria.[1,2] In agreement with previous reports,[34,35] the *N. meningitidis* CSS had a broad substrate specificity. It accepted all the compounds we tested as the substrates, including an eight-carbon sugar 4,6-bis-*epi*-KDO (**2h**), and produced activated CMP–sialic acid derivatives in high yields (Table 1). In comparison, the *E. coli* K1 CSS only recognized the natural substrate Neu5Ac (*N*-acetylneuraminic acid, **2a**) and its hydroxyl- and azido-derivatives of the *N*-acetyl group linked to carbon 5 (*N*-glycolylneuraminic acid, Neu5Gc, **2b**; and *N*-azidoacetylneuraminic acid, $Neu5AcN_3$, **2c**). The activity of the *E. coli* enzyme was also less effective for modified substrates. Only about 57–82% yields were achieved with the same molar concentration of the protein. The substrate specificity of the *S. agalactiae* serotype V CSS was more narrow than that of the *N. meningitidis* CSS but was more flexible than that of the *E. coli* synthetase. It showed very good activities for Neu5Ac and its derivatives, and produced CMP activated Neu5Ac (**2a**), Neu5Gc (**2b**), $Neu5AcN_3$ (**2c**), and Neu5CbzGly (*N*-Cbzglycine neuraminic acid, **2d**) in high yields. It also accepted a KDN derivative $KDN9N_3$ (9-azido-9-deoxy-KDN, **2g**) and synthesized CMP–$KDN9N_3$ (**3g**) in a relatively good yield (80%).

Table 1. Substrate specificity and catalytic efficiency of three recombinant microbial CMP–sialic acid synthetases in a one-pot two-enzyme reaction system[a]

Analogs	**1** Staring sugar	**3** Product	Overall yield (%) (HPLC analysis)			Synthetic yield (%)
			N. meningitidis serotype B	*E. coli* K1	*S. agalactiae* serotype V	*N. meningitidis* serotype B
a			96	98	99	80
b			94	57	81	85
c			95	82	90	85
d			97	<0.1	68	82
e			100	<0.1	<0.1	88
f			99	<0.1	30	90
g			85	<0.1	80	80
h			82	<0.1	<0.1	80

[a] The sialic acid analogs, which are the substrates for the CSS, were generated in situ from pyruvate and ManNAc/Man analogs by a sialic acid aldolase catalyzed condensation reaction.

However, it catalyzed the synthesis of CMP–KDN (**2f**) less efficiently with a low yield of 30%. Neu5N$_3$ (5-azido-5-deoxy-neuraminic acid, **2e**) and the eight-carbon sugar 4,6-bis-*epi*-KDO (**2h**) were not acceptable substrates for the enzyme. These results indicated an important role of an acylamino group at C-5 of the Neu5Ac in *S. agalactiae* CSS activity. The study of the variation of the substrate specificity of different microbial CSSs may help us to understand the mechanism of sialic acid metabolism in bacteria and may help in elucidating the pathogen host-invading mechanism.

CMP–Neu5Ac can also be synthesized from *N*-acetyl-D-glucosamine (GlcNAc) since GlcNAc was able to be epimerized to ManNAc chemically under basic condition.[47] In this situation, ManNAc was generated in situ from GlcNAc in a reversible chemical epimerization, and then reacted with pyruvate and CTP in the one-pot two-enzyme reaction containing both the aldolase and a CSS to produce activated Neu5Ac. However, the yields were relatively low for these reactions, with 15% for the *N. meningitidis* CSS, and 12% for both *E. coli* and *S. agalactiae* CSSs. The low yields may be resulted from the inhibitory effect of the GlcNAc to the CSS.[48]

2.4. Preparative synthesis of CMP–Neu5Ac and analogs

Eight compounds, including CMP–Neu5Ac and its derivatives with substitutions at C-5, C-8, or C-9 at Neu5Ac, were successfully synthesized in a preparative scale (100 mg scale) using the one-pot two-enzyme system with the *N. meningitidis* CSS and the aldolase. The products were purified by anion-exchange chromatography and their structures were confirmed by ^{1}H and ^{13}C NMR spectra, as well as High Resolution Mass Spectra as described in the experimental section. These sugar nucleotide donors are important intermediates for future enzymatic synthesis of sialylated oligosaccharides.

3. Conclusion

The one-pot two-enzyme system containing a recombinant sialic acid aldolase and a microbial CSS, especially the *N. meningitidis* CSS, was efficient in producing CMP–Neu5Ac derivatives from Mannose or ManNAc analogs. The *N. meningitidis* synthetase was shown to have the highest expression level, the most flexible substrate specificity, and the highest catalytic efficiency among three recombinant synthetases. Together with a sialic acid aldolase and a sialyltransferase, the *S. agalactiae* and *N. meningitidis* CSSs could be invaluable tools for large-scale synthesis of biologically and medicinally important sialylated oligosaccharides, polysialic acids, and glycoconjugates.

4. Experimental

4.1. Materials

Vector plasmids pET15b and pET22b(+) were purchased from Novagen (EMD Biosciences, Inc. Madison, WI). Ni^{2+}–NTA agarose (nickel–nitrilotriacetic acid–agarose), QIAprep spin miniprep kit, and QIAEX II gel extraction kit were from Qiagen (Valencia, CA). Herculase enhanced DNA polymerase was from Stratagene (La Jolla, CA). Electrocompetent *E. coli* DH5α cells and chemically competent *E. coli* BL21 (DE3) cells were from Invitrogen (Carlsbad, CA). T4 DNA ligase, 1 kb DNA ladder, and restriction enzymes were from Promega (Madison, WI). BCA protein assay kit was from Pierce Biotechnology, Inc. (Rockford, IL). D-*N*-Acetylmannosamine (ManNAc, **1a**), D-mannose (**1f**), and D-lyxose (**1h**) were purchased from Sigma (St. Louis, MO). All other materials were obtained from commercial suppliers and used as received with or without further purification.

4.2. Cloning, expression, and purification of a sialic acid aldolase and three CMP–sialic acid synthetases

4.2.1. Cloning of a sialic acid aldolase and three CMP–sialic acid synthetases. The C-terminal His_6-tagged full length sialic acid aldolase (NanA) was cloned in pET15b vector from *E. coli* K12, substrain MG1655 (ATCC#47076). Primers used were: forward primer 5′CATGCCATGGCAACGAATTTACGTGGC3′ (*Nco*I restriction site is underlined) and reverse primer 5′CGCGGATCCTCATTA*GTGATGATGATGATGATG*CCCGCGCTCTTGCATCAACTG3′ (*BamH*I restriction site is underlined, the gene sequence for a hexahistidine-tage is in italics). Three full length C-terminal His_6-tagged CMP–sialic acid synthetases were cloned in a similar method. *N. meningitidis* CSS was cloned in pET22b(+) vector from group B strain MC58 using genomic DNA (ATCC#BAA-335D) as a template. The primers used were: forward primer 5′GATCCATATGGAAAAACAAAATATTGCGG3′ (*Nde*I restriction site is underlined) and reverse primer 5′CCGCTCGAGGCTTTCCTTGTGATTAAG3′ (*Xho*I restriction site is underlined). *S. agalactiae* CSS was cloned in pET15b vector from serotype V strain 2603V/R using genomic DNA (ATCC#BAA-611D) as a template. The primers used were: forward primer 5′CATGCCATGGGCATGAAGCCAATTTGTATTATTCCTGCG3′ (*Nco*I restriction site is underlined) and reverse primer 5′CGCGGATCCTTA*GTGGTGGTGGTGGTGGTG*TAAGGTTTTAACTTCGTCTAC3′ (*BamH*I restriction site is underlined, the gene sequence for a hexahistindine-tag is in italics). The primers for the *E. coli* K1 CSS cloned in pET15b vector were: forward primer 5′CTGTCATGAGAACAAAAATTATTGCG3′ (*BspH*I restriction site is underlined) and reverse primer 5′CGCGGATCCTCA*GTGGTGGTGGTGGTGGTG*TTTAACAATCTCCGCTATTTCG3′ (*BamH*I restriction site is underlined, the gene sequence for a hexahistindine-tag is in italics). Polymerase chain reactions (PCR) for the amplification of the target genes were performed in a 50 μL reaction mixture containing 1 μg of genomic DNA as the template DNA, 1 μM each of forward and reverse primers, 5 μL of 10 × Herculase buffer, 1 mM dNTP mixture, and 5 units (1 μL) of Herculase enhanced DNA polymerase. The reaction mixtures were subjected to 30 cycles of amplifications with an annealing temperature of 50–60 °C. The resulting PCR products were digested with the corresponding restriction enzymes introduced in the primers, purified, and ligated with predigested pET15b or pET22b(+) vector. The ligated product was transformed into electrocompetent *E. coli* DH5α cells. Positive plasmids were selected and subsequently transformed into BL21 (DE3) chemically competent cells. Selected clones were grown for minipreps and characterization by restriction mapping and DNA sequencing.

4.2.2. Expression of the recombinant sialic acid aldolase and CMP–sialic acid synthetases. All plasmid-bearing *E. coli* strains were cultured in LB rich medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) supplemented with 100 μg/mL ampicillin. Generally, overexpression of the target protein was achieved by inducing the *E. coli* culture with 0.1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) at $OD_{600\,nm} = 0.8–1.0$ and incubating at 20 °C for 20 h with vigorous shaking at 250 rpm in a C25KC incubator shaker (New Brunswick Scientific, Edison, NJ).

4.2.3. Purification of the recombinant sialic acid aldolase and CMP–sialic acid synthetases. C-Terminal His_6-tagged target proteins were purified from cell lysate individually. To obtain the cell lysate, cells were harvested by centrifugation at 4000 rpm for 30 min. The cell pellet was resuspended at 20 mL per liter cell culture in lysis buffer (pH 8.0, 100 mM Tris–HCl containing 0.1% Triton X-100). Lysozyme (1 mg/L culture) and DNaseI (50 μg/L culture) were then added to the cell resuspension. The mixture was incubated at 37 °C for 50 min with vigorous shaking. Cell lysate was obtained by centrifugation at 4000 rpm for 60 min to separate the cell lysate from the inclusion bodies and other cellular debris.

The enzyme was purified directly from the lysate using a Ni^{2+}–NTA affinity column, which binds to the C-terminal hexahistidine sequence of the enzymes. All procedures were performed on ice. After loading the cell

lysate, the Ni^{2+} column was washed with six column volumes of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.5), followed by 8 volumes of washing buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.5). The overexpressed protein was eluted with 8 volumes of elution buffer (200 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.5). Detected by a UV–vis spectrometer at 280 nm, fractions containing the purified enzyme were collected and stored at 4 °C. The purified enzyme in elute solution was dialyzed before use.

4.2.4. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed in a 12% Tris–glycine gel using Bio-Rad Mini-protein III cell gel electrophoresis unit (Bio-Rad, Hercules, CA) at DC = 150 V. Bio-Rad Precision Plus Protein Standards (10–250 kDa) were used as molecular weight standards. Gels were stained with Coomassie Blue.

4.2.5. Quantification of purified protein. The concentration of purified enzymes was obtained using a Bicinchoninic acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as a protein standard.

4.3. General method for compound characterization and purification

^1H NMR and ^{13}C NMR spectra were recorded on an Mercury (300 MHz) and an Inova (400 MHz) NMR spectrometers. High resolution mass spectra (FAB) were obtained at the mass spectrometry facility at the University of California, Riverside. Gel filtration chromatography was performed with a column (100 cm × 2.5 cm) packed with BioGel P-2 Fine resins (Bio-Rad, Hercules, CA). Analytical thin-layer chromatography was performed on silica gel 60 F_{254} plates (Sorbent Tech.) using anisaldehyde stain for detection.

4.4. Synthesis of mannose and *N*-acetylmannosamine derivatives

4.4.1. *N*-Glycolyl-D-mannosamine (1b). This compound was synthesized using a reported method.[49] Mannosamine hydrochloride salt (0.32 g, 1.48 mmol) was dissolved in 10 mL of dry MeOH under argon. To this solution was added 0.42 mL of triethylamine. The mixture was stirred for 10 min until the solution turned clear. *N*-hydroxysuccinimidyl glycolate (0.38 g, 1.5 equiv) was then added and the resulting solution was stirred at room temperature overnight. When TLC analysis indicated the disappearance of the starting material, the reaction mixture was concentrated using a rotavapor (Buchi Rotavapor R-205). The product was purified by flash chromatography on silica gel by eluting with $MeOH/CH_2Cl_2$ = 20:1 to 10:1. The product was obtained as a white solid (0.32 g, 91%) after concentration using a rotavapor. ^1H NMR (300 MHz, D_2O): δ 5.00 (s, 0.6H), 4.92 (s, 0.4H), 4.23 (d, 0.4H, J = 3.3 Hz), 4.21 (d, 0.6H, J = 3.6 Hz), 4.05–3.90 (m, 3H), 3.79–3.60 (m, 4H), 3.46 (t, 0.6H, J = 9.6 Hz), 3.36 (t, 0.4H, J = 9.6 Hz).

4.4.2. *N*-Azidoacetyl-D-mannosamine (1c). This compound was synthesized in a similar method described for **1b**. Mannosamine hydrochloride salt (0.5 g, 2.32 mmol) was dissolved in 10 mL of dry MeOH under argon. To this solution was added 1.3 mL of triethylamine, followed by azidoacetic acid NHS ester (0.6 g, 1.3 equiv). The resulting solution was stirred at room temperature for overnight until the completion of the reaction. The reaction mixture was then concentrated and the product was purified by flash chromatography ($MeOH/CH_2Cl_2$ = 20:1 to 10:1) to give a white solid (0.498 g, 82%). ^1H NMR (400 MHz, D_2O): δ 4.97 (s, 0.5H), 4.87 (s, 0.5H), 4.32 (d, 0.5H, J = 4.0 Hz), 4.19 (d, 0.5H, J = 4.4 Hz), 3.94–3.88 (m, 3H), 3.73–3.59 (m, 4H), 3.43 (t, 0.5H, J = 10.0 Hz), 3.33 (t, 0.5H, J = 10.0 Hz).

4.4.3. *N*-Cbz-glycine-D-mannosamine (1d). This compound was synthesized in a similar method described for **1b** and **1c**. Mannosamine hydrochloride salt (2.0 g, 9.27 mmol) was dissolved in 20 mL of dry MeOH under argon. To this solution was added 1.3 mL of triethylamine. After stirring the reaction mixture for 10 min, *N*-Cbz-glycine *N*-hydroxysuccinimide ester (3.40 g, 1.2 equiv) was added, and the reaction continued with stirring at room temperature overnight. The reaction mixture was concentrated and the product was purified by flash chromatography ($MeOH/CH_2Cl_2$ = 20:1 to 10:1) to give a white solid (3.02 g, 88%). ^1H NMR (400 MHz, CD_3OD): δ 7.35–7.27 (m, 5H), 5.05 (s, 2H), 4.44 (d, 0.3H, J = 4.2 Hz), 4.32–4.30 (d, 0.7H, J = 4.5 Hz), 4.04–4.0 (dd, 1H, J = 4.6 and 9.7 Hz), 3.88 (s, 2H), 3.86–3.68 (m, 4H), 3.62–3.57 (t, 0.7H, J = 9.5 Hz), 3.50–3.46 (t, 0.3H, J = 9.5 Hz).

4.4.4. 2-Azido-2-deoxy-D-mannose (1e). This compound was synthesized from 2-amino-2-deoxy-D-mannopyranose hydrochloride with trifluoromethylsulfonyl azide as reported.[50] An aqueous solution (8 mL) of NaN_3 (3.5 g, 54 mmol) was mixed with CH_2Cl_2 (4 mL) and cooled to 0 °C in an ice-water bath. To the vigorously stirred mixture was added Tf_2O (2.45 g, 1.51 mL, 9 mmol) in droplets. After stirring for an additional 2 h at 0 °C, the organic phase was separated and collected. The aqueous phase was extracted with CH_2Cl_2 (30 mL) twice. The organic phases containing triflic azide were combined and washed with saturated aqueous Na_2CO_3 by extraction. This freshly prepared triflic azide solution was added immediately to a solution prepared by adding mannosamine · HCl (0.45 g, 2.1 mmol), K_2CO_3 (0.48 g), and $CuSO_4 \cdot 5H_2O$ (8 mg) sequentially to a water (8 mL) and MeOH (15 mL) mixture. Additional MeOH was added until the solution became clear. The reaction mixture was then stirred overnight at room temperature. The solution was concentrated and the product was purified by flash chromatography (CH_2Cl_2/MeOH = 10:1 to 5:1) to give a yellow syrup, which was dissolved in MeOH and decolorized by activated charcoal. After filtration, the solution was concentrated to give a white solid (0.37 g, 86%). ^1H NMR (400 MHz, CD_3OD): δ 5.09 (d, 0.5H, J = 1.6 Hz), 4.89 (d, 0.5H, J = 1.2 Hz), 4.00 (dd, 0.5H, J = 5.6 and 9.6 Hz), 3.84–3.56 (m, 4.5H), 3.44 (t, 0.5H, J = 9.6 Hz), 3.20 (m, 0.5H).

4.4.5. 6-Azido-6-deoxy-D-mannose (1g). This compound was prepared from 6-toluenesulfonate-D-mannopyranose and lithium azide.[51] A solution of *p*-toluenesulfonyl chloride (2.83 g, 14.8 mmol) in dry pyridine (10 mL) was added in droplets to a solution of D-(+)-mannose (2.06 g, 11.4 mmol) dissolved in dry pyridine in an ice-water bath. The mixture was stirred at 0 °C for 2 h and the reaction was stopped by the addition of MeOH (10 mL). The mixture was concentrated and the product was purified by flash chromatography (CH_2Cl_2/MeOH = 10:1) to give a yellow syrup (6-*O*-tosyl-D-mannopyranose) (3.44 g, 90%). To a solution of 6-*O*-tosyl-D-mannopyranose (610 mg, 1.82 mmol) in *N*,*N*-dimethylformamide (10 mL) was added lithium azide (in 20% water, 1.31 g, 3 equiv). The mixture was stirred at 65 °C for 12 h. The solution was concentrated and the product was purified by a flash chromatography (CH_2Cl_2/MeOH = 10:1 to 5:1) to give a yellow syrup (265 mg, 71%), which was then decolorized by activated charcoal. ^{1}H NMR (400 MHz, D_2O): δ 5.01 (s, 0.6H), 4.74 (s, 0.4H), 3.78–3.56 (m, 3H), 3.52–3.30 (m, 3H). ^{13}C NMR (100 MHz, D_2O, α anomer only): δ 94.3 (C-1), 71.2, 70.7, 70.2, 67.7, 51.2 (C-6).

4.5. TLC and HPLC analyses for the production of CMP–sialic acid analogs

4.5.1. General reaction procedures. All one-pot two-enzymatic reactions catalyzed by an aldolase and a CMP–Neu5Ac synthetase were performed in a Tris–HCl buffer (100 mM, pH 8.8) containing $MgCl_2$ (20 mM), mannose or ManNAc derivatives (10 mM), sodium pyruvate (50 mM), and CTP (10 mM). After the addition of the sialic acid aldolase and a CMP–sialic acid synthetase, the reaction was allowed to proceed for 2–5 h at 37 °C. The reaction was monitored by TLC analysis until no increased product formation could be detected. The precipitates in the reaction mixture were removed by centrifugation and then filtration (0.2 μm syringe filter). Aliquots of 10 μL clear solution were injected for HPLC analysis.

4.5.2. HPLC conditions. HPLC analysis was carried out using a reverse phase Premier C18 column (250 × 4.6 mm ID, 5 μm, particle size) protected with a C18 guard column cartridge in a Shimadzu LC-2010A system equipped with a membrane on-line degasser, a temperature control unit (maintained at 30 °C throughout the experiment), and a UV–vis detector. The compounds were monitored at 270 nm and the system was controlled via Shimadzu EZStrat v7.2 SP1 chromatography software. Buffer A (0.1 M potassium phosphate buffer supplemented with 8 mM tetrabutylammonium hydrogensulfate, pH 5.3) and buffer B (70% buffer A plus 30% methanol, pH 5.9) were prepared and used in two gradient conditions for separation of different compounds. Gradient condition 1 was: 100% buffer A for 2.5 min, 0–40% buffer B for 14 min, 40–100% buffer B for 1 min, 100% buffer B for 6 min, 100–0% buffer B for 1 min, followed by an equilibration phase of 100% buffer A for 8 min. Gradient condition 2 was: 100% buffer A for 2.5 min, 0–40% buffer B for 7.5 min, 40–100% buffer B for 1 min, 100% buffer B for 4 min, 100–0% buffer B for 1 min, followed by an equilibration phase of 100% buffer A for 4 min. Flow-rate was maintained at 1 mL/min for all separations. Compounds **3a**, **3b**, **3c**, **3f**, **3g**, **3h** were analyzed by gradient condition 2, **3d** and **3e** by gradient condition 1.

4.6. Preparative synthesis of CMP–sialic acid analogs

4.6.1. General reaction procedures. The preparative synthesis of CMP–sialic acid analogs was carried out in a Tris–HCl buffer (100 mM, pH 8.8) containing $MgCl_2$ (20 mM), mannose or ManNAc derivative (50–100 mg), sodium pyruvate (5 equiv), CTP (1 equiv), the recombinant sialic acid aldolase, and the *N. meningitidis* CMP–Neu5Ac synthetase. The reaction mixture was incubated at 37 °C for 2–5 h in an incubator with shaking (140 rpm). The reaction was stopped by addition of MeOH when TLC (elution system: EtOH/1 M NH_4HCO_3; 7:3 by volume) analysis indicated reaction completion. The precipitates in the reaction mixture were removed by centrifugation. The supernatant was concentrated and purified by a gel filtration column packed with BioGel P-2 resin.

4.6.2. Cytidine-5′-monophospho-5-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (3a). HPLC retention time: 6.63 min. ^{1}H NMR (400 MHz, D_2O): δ 7.87 (d, 1H, J = 7.6 Hz, H-6 of cytidine), 6.25 (d, 1H, J = 7.6 Hz, H-5 of cytidine), 5.91 (d, 1H, J = 4.4 Hz, H-1 of ribose), 4.26–4.20 (m, 2H, H-2 of ribose, H-3 of ribose), 4.17–4.10 (m, 3H, H-5a, H-5b, H-4 of ribose), 4.09–3.98 (m, 2H), 3.90–3.80 (m, 3H), 3.59–3.40 (m, 2H), 2.42 (dd, 1H, J = 4.8, 13.2 Hz), 1.98 (s, 3H), 1.60 (dt, 1H, J = 5.6,12.6 Hz); ^{13}C (100 MHz): δ 174.9, 174.6, 166.3, 157.9, 141.7, 100.2, 96.8, 89.3, 83.0, 74.4, 71.9, 69.7, 69.2, 68.9, 67.0, 65.1, 63.1, 51.9, 41.3, 22.3. HRMS (FAB): $C_{20}H_{29}N_4O_{16}PNa$ (M^{2-} + Na^+), calcd 635.1214, found 635.1191.

4.6.3. Cytidine-5′-monophospho-5-(2-hydroxy)-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (3b). HPLC retention time: 5.69 min. ^{1}H NMR (400 MHz, D_2O): δ 7.79 (d, 1H, J = 7.6 Hz, H-6 of cytidine), 5.95 (d, 1H, J = 7.6 Hz, H-5 of cytidine), 5.81 (d, 1H, J = 4.4 Hz, H-1 of ribose), 4.19–4.14 (m, 2H, H-2 of ribose, H-3 of ribose), 4.13–4.04 (m, 3H, H-5a, H-5b, H-4 of ribose), 4.03–3.98 (m, 1H), 3.97 (s, 2H), 3.86–3.70 (m, 3H), 3.48–3.41 (m, 2H), 3.28 (d, 1H, J = 9.6 Hz, H-7″), 2.34 (dd, 1H, J = 4.8, 13.2 Hz), 1.51 (dt, 1H, J = 5.6,12.6 Hz); ^{13}C (100 MHz): δ 175.7, 174.5, 166.2, 157.8, 141.7, 100.2, 96.8, 89.3, 83.0, 74.4, 71.7, 69.7, 68.9, 66.7, 65.0, 63.0, 61.2, 59.5, 51.6, 41.3. HRMS (FAB): $C_{20}H_{29}N_4O_{17}PNa$ (M^{2-} + Na^+), calcd 651.1163, found 651.1126.

4.6.4. Cytidine-5′-monophospho-5-azidoacetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (3c). HPLC retention time: 14.48 min. ^{1}H NMR (400 MHz, D_2O): δ 7.70 (d, 1H, J = 7.6 Hz, H-6 of cytidine), 5.87 (d, 1H, J = 7.2 Hz, H-5 of cytidine), 5.73 (d, 1H, J = 4.0 Hz, H-1 of ribose), 4.15–4.05 (m, 2H, H-2 of ribose, H-3 of ribose), 4.05–4.01 (m, 3H, H-5a, H-5b, H-4 of ribose), 3.90–3.52 (m, 5H), 3.85 (s, 2H),

3.41–3.36 (m, 1H), 3.22 (d, 1H, $J = 9.2$ Hz, H-7″), 2.22 (dd, 1H, $J = 4.8$, 13.6 Hz), 1.42 (dt, 1H, $J = 6.0$,12.8 Hz); ^{13}C (100 MHz): δ 174.7, 169.4, 166.2, 157.8, 141.7, 100.1, 96.7, 89.4, 83.0, 74.3, 73.8, 71.9, 69.8, 69.5, 67.9, 65.0, 63.1, 59.6, 48.9, 41.0. HRMS (FAB): $C_{20}H_{29}N_7O_{16}P$ ($M^{2-} + H^+$), calcd 654.1408, found 654.1421.

4.6.5. Cytidine-5′-monophospho-5-(*N*-benzyloxycarboxamido)glycylamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (3d). HPLC retention time: 27.76 min. ^{1}H NMR (300 MHz, D_2O) δ 7.77 (d, 1H, $J = 7.5$ Hz, H-6 of cytidine), 7.24 (m, 5H), 5.92 (d, 1H, $J = 7.68$ Hz, H-5 of cytidine), 5.79 (d, 1H, $J = 4.2$ Hz, H-1 of ribose), 4.96 (s, 2H), 4.16–4.05 (m, 5H, H-2 of ribose, H-3 of ribose, H-5a, H-5b, H-4 of ribose), 4.03–3.14 (m, 9H), 2.31 (dd, 1H), 1.51 (dt, 1H); ^{13}C (100 MHz): δ 174.5, 166.3, 158.7, 157.9, 141.7, 136.3, 128.9, 128.5, 127.9, 100.2, 96.7, 89.3, 82.9, 74.4, 72.2, 71.7, 69.8, 69.4, 68.9, 67.5, 64.9, 63.1, 52.0, 43.8, 41.0. HRMS (FAB): $C_{28}H_{37}N_5O_{18}P$ ($M^{2-} + H^+$), calcd 762.1871, found 762.1777.

4.6.6. Cytidine-5′-monophospho-5-azido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (3e). HPLC retention time: 20.02 min. ^{1}H NMR (400 MHz, D_2O) δ 7.77 (d, 1H, $J = 7.6$ Hz, H-6 of cytidine), 5.94 (d, 1H, $J = 7.6$ Hz, H-5 of cytidine), 5.80 (d, 1H, $J = 4.0$ Hz, H-1 of ribose), 4.17 (t, 1H, $J = 4.0$ Hz, H-2 of ribose), 4.13 (t, 1H, $J = 4.0$ Hz, H-3 of ribose), 4.09–4.04 (m, 3H, H-5a, H-5b, H-4 of ribose), 3.97–3.85 (m, 2H), 3.81–3.70 (m, 2H), 3.53–3.48 (m, 1H), 3.45–3.34 (m, 2H), 2.28 (1H, dd, $J = 4.8$, 13.2 Hz), 1.50 (td, 1H, $J = 6.0$ and 12.8 Hz); ^{13}C (100 MHz): δ 174.4, 166.2, 157.8, 141.7, 100.1, 96.7, 89.4, 83.0, 74.3, 72.0, 69.8, 69.5, 69.4, 67.9, 65.0, 63.1, 59.6, 41.4. HRMS (FAB): $C_{18}H_{25}N_6O_{15}PNa$ ($M^{2-} + Na^+$), calcd 619.1013, found 619.0986.

4.6.7. Cytidine-5′-monophospho-3-deoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (3f). HPLC retention time: 5.42 min. ^{1}H NMR (400 MHz, D_2O) δ 7.75 (d, 1H, $J = 7.6$ Hz, H-6 of cytidine), 5.90 (d, 1H, $J = 7.6$ Hz, H-5 of cytidine), 5.77 (d, 1H, $J = 4.0$ Hz, H-1 of ribose), 4.16–4.08 (m, 2H, H-2 of ribose, H-3 of ribose), 4.05–4.01 (m, 3H, H-5a, H-5b, H-4 of ribose), 3.90–3.62 (m, 4H), 3.59–3.35 (m, 3H), 2.22 (dd, 1H, $J = 4.8$, 13.2 Hz), 1.40 (dt, 1H, $J = 6.0$,12.8 Hz); ^{13}C (100 MHz): δ 174.7, 166.1, 157.7, 141.7, 100.2, 96.7, 89.4, 83.0, 74.3, 73.0, 69.9, 69.4, 68.6, 68.4, 64.9, 63.1, 59.5, 41.0. HRMS (FAB): $C_{18}H_{27}N_3O_{16}P$ ($M^{2-} + H^+$), calcd 572.1129, found 572.1152.

4.6.8. Cytidine-5′-monophospho-9-azido-3,9-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (3g). HPLC retention time: 16.74 min. ^{1}H NMR (400 MHz, D_2O) δ 7.76 (d, 1H, $J = 7.6$ Hz, H-6 of cytidine), 5.90 (d, 1H, $J = 7.6$ Hz, H-5 of cytidine), 5.80 (d, 1H, $J = 4.0$ Hz, H-1 of ribose), 4.20–4.00 (m, 5H, H-2 of ribose, H-3 of ribose, H-5a, H-5b, H-4 of ribose), 3.98–3.35 (m, 7H), 2.22 (dd, 1H, $J = 4.8$, 13.2 Hz), 1.40 (dt, 1H, $J = 6.0$,12.8 Hz). HRMS (FAB): $C_{18}H_{25}N_6O_{15}PNa_3$ ($M^{2-} + 3Na^+$), calcd 665.0809, found 665.0807.

4.6.9. Cytidine-5′-monophospho-3-deoxy-β-D-*galacto*-2-octulopyranosonic acid (3h). HPLC retention time: 5.94 min. ^{1}H NMR (400 MHz, D_2O) δ 7.81 (d, 1H, $J = 7.6$ Hz, H-6 of cytidine), 5.96 (d, 1H, $J = 7.2$ Hz, H-5 of cytidine), 5.83 (d, 1H, $J = 3.6$ Hz, H-1 of ribose), 4.20–4.14 (m, 2H, H-2 of ribose, H-3 of ribose), 4.14–4.05 (m, 3H, H-5a, H-5b, H-4 of ribose), 3.90–3.80 (m, 3H), 3.71 (d, 1H, $J = 8.8$ Hz), 3.67–3.63 (m, 1H), 3.52–3.48 (m, 1H), 2.27 (dd, 1H, $J = 4.8$, 13.6 Hz), 1.46 (dt, 1H, $J = 6.0$,12.8 Hz); ^{13}C (100 MHz): δ 174.8, 166.3, 157.9, 141.7, 100.2, 96.7, 89.3, 83.0, 74.4, 73.6, 69.4, 68.4, 64.8, 63.1, 62.1, 59.8, 41.0. HRMS (FAB): $C_{17}H_{24}N_3O_{15}PNa$ ($M^{2-} + Na^+$), calcd 564.0843, found 564.0867.

Acknowledgements

This work was supported by New Faculty Research Grant and start-up funds from the University of California at Davis.

References and notes

1. Schauer, R. *Glycoconjugate J.* **2000**, *17*, 485.
2. Angata, T.; Varki, A. *Chem. Rev.* **2002**, *102*, 439.
3. Mandrell, R. E.; Apicella, M. A. *Immunobiology* **1993**, *187*, 382.
4. Moran, A. P.; Prendergast, M. M.; Appelmelk, B. J. *FEMS Immunol. Med. Microbiol.* **1996**, *16*, 105.
5. Jennings, H. J. *Curr. Top. Microbiol. Immunol.* **1990**, *150*, 97.
6. Chappell, M. D.; Halcomb, R. L. *J. Am. Chem. Soc.* **1997**, *119*, 3393.
7. Kean, E. L. *Glycobiology* **1991**, *1*, 441.
8. Higa, H. H.; Paulson, J. C. *J. Biol. Chem.* **1985**, *260*, 8838.
9. Kittelmann, M.; Klein, T.; Kragl, U.; Wandrey, C.; Ghisalba, O. *Ann. N.Y. Acad. Sci.* **1992**, *672*, 444.
10. Schmelter, T.; Ivanov, S.; Wember, M.; Stangier, P.; Thiem, J.; Schauer, R. *Biol. Chem. H–S* **1993**, *374*, 337.
11. Vionnet, J.; Concepcion, N.; Warner, T.; Zapata, G.; Hanover, J.; Vann, W. F. *Glycobiology* **1999**, *9*, 481.
12. Kean, E. L.; Roseman, S. *J. Biol. Chem.* **1966**, *241*, 56430.
13. Schauer, R.; Haverkamp, J.; Ehrlich, K. *H–S Z. Physiol. Chem.* **1980**, *361*, 641.
14. Vann, W. F.; Silver, R. P.; Abeijon, C.; Chang, K.; Aaronson, W.; Sutton, A.; Finn, C. W.; Lindner, W.; Kotsatos, M. *J. Biol. Chem.* **1987**, *262*, 17556.
15. Warren, L.; Blacklow, R. S. *J. Biol. Chem.* **1962**, *237*, 3527.
16. Zapata, G.; Vann, W. F.; Aaronson, W.; Lewis, M. S.; Moos, M. *J. Biol. Chem.* **1989**, *264*, 14769.
17. Edwards, U.; Frosch, M. *FEMS Microbiol. Lett.* **1992**, *96*, 161.
18. Ganguli, S.; Zapata, G.; Wallis, T.; Reid, C.; Boulnois, G.; Vann, W. F.; Roberts, I. S. *J. Bacteriol.* **1994**, *176*, 4583.
19. Haft, R. F.; Wessels, M. R.; Mebane, M. F.; Conaty, N.; Rubens, C. E. *Mol. Microbiol.* **1996**, *19*, 555.
20. Guerry, P.; Doig, P.; Alm, R. A.; Burr, D. H.; Kinsella, N.; Trust, T. J. *Mol. Microbiol.* **1996**, *19*, 369.
21. Tullius, M. V.; Munson, R. S., Jr.; Wang, J.; Gibson, B. W. *J. Biol. Chem.* **1996**, *271*, 15373.
22. Gilbert, M.; Watson, D. C.; Wakarchuk, W. W. *Biotechnol. Lett.* **1997**, *19*, 417.
23. Luneberg, E.; Zetzmann, N.; Alber, D.; Knirel, Y. A.; Kooistra, O.; Zahringer, U.; Frosch, M. *Int. J. Med. Microbiol.* **2000**, *290*, 37.

24. Xia, G.; Han, X.; Ding, H.; Yang, S.; Zhang, S.; Jin, C. *J. Mol. Catal. B—Enzym.* **2000**, *10*, 199.
25. Nakata, D.; Munster, A. K.; Gerardy-Schahn, R.; Aoki, N.; Matsuda, T.; Kitajima, K. *Glycobiology* **2001**, *11*, f685.
26. Krapp, S.; Munster-Kuhnel, A. K.; Kaiser, J. T.; Huber, R.; Tiralongo, J.; Gerardy-Schahn, R.; Jacob, U. *J. Mol. Biol.* **2003**, *334*, 625.
27. Mosimann, S. C.; Gilbert, M.; Dombroswki, D.; To, R.; Wakarchuk, W.; Strynadka, N. C. *J. Biol. Chem.* **2001**, *276*, 8190.
28. Jelakovic, S.; Jann, K.; Schulz, G. E. *FEBS Lett.* **1996**, *391*, 157.
29. Jelakovic, S.; Schulz, G. E. *J. Mol. Biol.* **2001**, *312*, 143.
30. Shames, S. L.; Simon, S. L.; Christopher, C. W.; Schmid, W.; Whitesides, G. M.; Yang, L. L. *Glycobiology* **1991**, *1*, 187.
31. Shen, G. J.; Liu, J. L. C.; Wong, C.-H. *Biocatalysis* **1992**, *6*, 31.
32. Liu, J. L. C.; Shen, G. J.; Ichikawa, Y.; Rutan, J. F.; Zapata, G.; Van, W. F.; Wong, C.-H. *J. Am. Chem. Soc.* **1992**, *114*, 3901.
33. Karwaski, M. F.; Wakarchuk, W. W.; Gilbert, M. *Protein Expres. Purif.* **2002**, *25*, 237.
34. Knorst, M.; Fessner, W. D. *Adv. Synth. Catal.* **2001**, *343*, 698.
35. Blixt, O.; Paulson, J. C. *Adv. Synth. Catal.* **2003**, *345*, 687.
36. Gilbert, M.; Bayer, R.; Cunningham, A. M.; DeFrees, S.; Gao, Y.; Watson, D. C.; Young, N. M.; Wakarchuk, W. W. *Nat. Biotechnol.* **1998**, *16*, 769.
37. Kittelmann, M.; Klein, T.; Kragl, U.; Wandrey, C.; Ghisalba, O. *Appl. Microbiol. Biotechnol.* **1995**, *44*, 59.
38. Lee, S. G.; Lee, J. O.; Yi, J. K.; Kim, B. G. *Biotechnol. Bioeng.* **2002**, *80*, 516.
39. Martin, R.; Witte, K. L.; Wong, C.-H. *Bioorg. Med. Chem.* **1998**, *6*, 1283.
40. Nahalka, J.; Wu, B.; Shao, J.; Gemeiner, P.; Wang, P. G. *Biotechnol. Appl. Biochem.* **2004**, *40*, 101.
41. Haft, R. F.; Wessels, M. R. *J. Bacteriol.* **1994**, *176*, 7372.
42. Liu, G.; Jin, C. *J. Biol. Chem.* **2004**, *279*, 17738.
43. Wong, C.-H.; Whitesides, G. M. In *Enzymes in Synthetic Organic Chemistry*; Wong, C.-H., Whitesides, G. M., Eds.; Elsevier Science: Oxford, 1994; Vol. 12, pp 215–219.
44. Kean, E. L.; Roseman, S. In *Methods in Enzymology*; Neufeld, E. F., Ginsburg, V., Eds.; Academic: New York, 1966; Vol. 8, pp 208–215.
45. Petrie, C. R., 3rd; Korytnyk, W. *Anal. Biochem.* **1983**, *131*, 153.
46. Ryll, T.; Wagner, R. *J. Chromatogr.* **1991**, *570*, 77.
47. Simon, E. S.; Bednarski, M. D.; Whitesides, G. M. *J. Am. Chem. Soc.* **1988**, *110*, 7159.
48. Kuboki, A.; Okazaki, H.; Sugai, T.; Ohta, H. *Tetrahedron* **1997**, *53*, 2387.
49. Choi, S. K.; Lee, S.; Whitesides, G. M. *J. Org. Chem.* **1996**, *61*, 8739.
50. Izumi, M.; Shen, G. J.; Wacowich-Sgarbi, S.; Nakatani, T.; Plettenburg, O.; Wong, C.-H. *J. Am. Chem. Soc.* **2001**, *123*, 10909.
51. Saxon, E.; Luchansky, S. J.; Hang, H. C.; Yu, C.; Lee, S. C.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2002**, *124*, 14893.