Enzymes in Organic Synthesis, 18‡

CMP-Sialate Synthetase from *Neisseria meningitidis* – Overexpression and Application to the Synthesis of Oligosaccharides Containing Modified Sialic Acids

Marion Knorst, Wolf-Dieter Fessner*

Institut für Organische Chemie der Technischen Universität, Petersenstraße 22, 64287 Darmstadt, Germany
Fax: (+49) 6151–16–6636, e-mail: fessner@tu-darmstadt.de

Received May 14, 2001; Accepted July 4, 2001

Dedicated to Prof. Horst Prinzbach on the occasion of his 70th birthday.

Abstract: The gene *siaB* encoding the CMP-sialate synthetase [EC 2.7.7.43] from *Neisseria meningitidis* serogroup B was subcloned for overexpression in *Escherichia coli* K12 using the expression vector pKK223–3. With the recombinant strain, 7500 U of synthetase could be produced per liter of cell culture on a 10 L-scale (1350 U/g cells; 20 U/mg protein). Purified enzyme was obtained in high yields (>85%) and with a high specific activity (≥134 U/mg protein) by an efficient, two-step scheme consisting of DEAE anion-exchange chromatography and ammonium sulfate precipitation. In contrast to known bacterial CMP-sialate synthetases, this enzyme was found to exhibit a broad substrate tolerance, particularly by accepting C5-modified Neu5Ac derivatives as substrates. This included neuraminic acid *N*-carbamoylated with typical protective groups of different length and bulkiness, an unsaturated acrylamide derivative and the corresponding saturated moiety, as well as the deaminated KDN analogue. Also, the latter structure can be varied by deoxygenation, epimerization at C5 or at the terminal chain, and by shortening the chain length to an octulosonic acid. The high expressivity of the recombinant production clone, the high catalytic efficiency of the enzyme, and its broad substrate tolerance make this synthetase from *N. meningitidis* the preferred catalyst for the enzymatic synthesis of CMP-Neu5Ac and of derivatives modified in the sialic acid moiety. Several CMP-conjugates made available by this procedure could be transferred effectively onto the acceptor *N*-acetyllactosamine using the α-2,6-sialyltransferase from rat liver to generate the corresponding trisaccharides.

Keywords: enzyme catalysis; glycoconjugates; glycosylation; oligosaccharides; sialic acids

Introduction

Sialic acids are key saccharide components on the surface of mammalian cells as constituents of glycoproteins, gangliosides, and related structures, where they are implicated in a variety of physiological and pathological processes (e.g., cell-cell adhesion, inflammation, cell growth regulation, or malignancy).[1] The typical sialic acids are amide derivatives of neuraminic acid (5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid) with *N*-acylation by acetyl (Neu5Ac, **1**), glycolyl, or lactoyl residues being the most prominent members. In bacteria, sialic acids are major constituents of extracellular capsular polysaccharides, e.g., those from meningococci.[2,3,4,5] Deaminated structures such as KDN (2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid) or KDO (2-keto-3-deoxy-D-*manno*-octonic acid) also occur frequently as components of various animal glycoproteins and glycosphingolipids,[6] or of the lipooligosaccharide portion (LOS) of the outer membrane of various Gram-negative bacteria, respectively.[7] Molecular mimicry of sialylated host structures by such bacterial saccharides of the LOS is considered to be a virulence factor of mucosal pathogens, which could use this strategy to evade the immune response against bacterial intruders.[3,8]

‡ Part 17: See J. M. Juarez Ruiz, G. Oßwald, M. Petersen, W.-D. Fessner, *J. Mol. Catal. B: Enzym.* **2001**, *11*, 189–197.

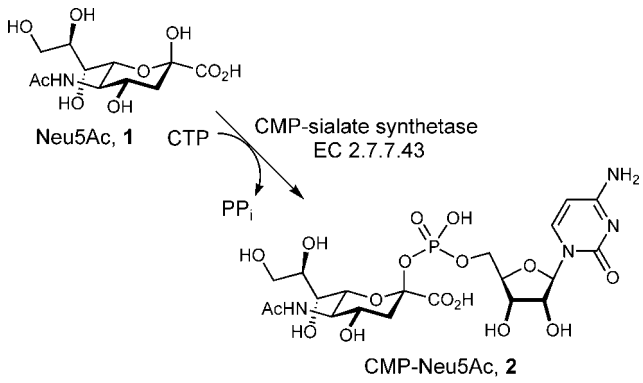
  1615-4150/01/34306–7-698–710 $ 17.50-.50/0

For the investigation of receptor recognition of sialic acids in the search for a rational drug development, biomimetic *neo*-glycoconjugate structures containing either naturally occurring or structurally modified sialic acids constitute both invaluable tools and challenging synthetic targets. The development of efficient and broadly applicable chemoenzymatic methods for the synthesis of such complex structures are essential in developing carbohydrate-based therapeutic agents with novel biological and physiological properties.[9] As an example, KDN ketosonic linkages have been found to impart a unique resistance to KDN glycoconjugates against known sialidases,[10,11] and thus can induce protection against bacterial and viral attacks; KDN capping in oligosaccharides has also been suggested to constitute a stop signal to terminate polysialylation.[10,12]

For the synthesis of complex carbohydrates, methods of chemical synthesis suffer from the need for tedious and expensive protection schemes. Especially in the case of sialoside formation, the known chemical methods are plagued with side reactions, low yields, and poor stereoselectivities.[13] Enzymatic glycosylation based on the use of glycosyltransferases is a practical alternative because these enzymes operate on unprotected sugar substrates in water, they are regio- and stereospecific in the glycoside formation, they are becoming broadly available by gene cloning,[14] and they have lately been shown to be quite tolerant for substrate variations on both the donor and acceptor moieties.[15,16]

Glycosyltransferase-based technology (Leloir pathway) requires activation of the free sialic acid or of synthetic analogues to the corresponding CMP-conjugates at the expense of one CTP equivalent.[17] Given the expense of multi-step chemical syntheses of CMP-Neu5Ac (**2**)[16,18] and its hydrolytic instability, protocols for preparative enzymatic synthesis are highly advantageous. Biosynthetically, the CMP-activation of Neu5Ac is catalyzed by a class of enzymes designated as CMP-sialate synthetase [EC 2.7.7.43]. The enzyme catalyzes a nucleophilic attack of the anomeric oxygen of β-Neu5Ac on the α-phosphate of CTP[19] and requires Mg^{2+} or Mn^{2+} for activity.[17,20] So far, enzymatic procedures for the synthesis of **2** (Equation 1) have been developed to routine application,[21,22,23,24,25,26,27,28] however, these are not generally amenable to the preparative nucleotide activation of modified sialic acids or their structural analogs because of the narrow substrate specificity of known CMP-sialate synthetases, and an often poor access to larger protein quantities.[25,29]

CMP-sialate synthetases have been isolated from various eukaryotic and prokaryotic sources.[17,21,24,27,30,31,32,33] Synthetases purified from different vertebrate tissues are rather unstable and offer only low specific activities (ca. 0.2 U/mg) which



(1)

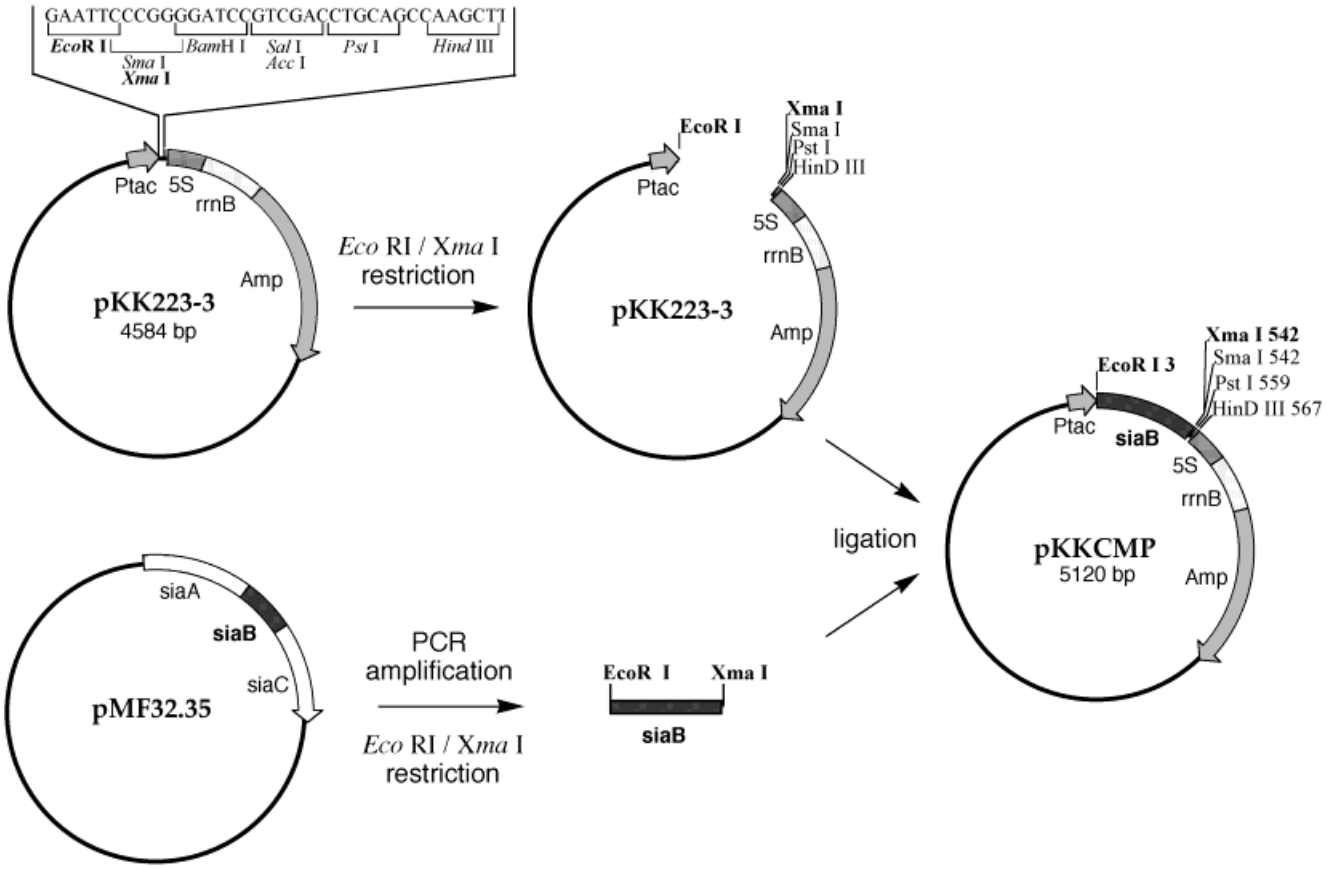
significantly restricts their use for preparative syntheses.[21,23,24,32,34] Several microorganisms have also been shown to contain CMP-sialate synthetase but are unsuited as possible enzyme sources because sialic acid metabolism parallels pathogenic virulence.[17,27,31,33] Therefore, some bacterial CMP-sialate synthetases have been produced from non-pathogenic clones and a few have been tested for their synthetic utility.[2,5,25,26,29,33,35,36,37] So far, only the recombinant enzyme from *E. coli* K1 has been intensively studied, commercialized, and applied in the preparative synthesis of CMP-Neu5Ac.[25,26,29,35] Its main limitations as a practical catalyst remain in its relatively strict substrate specificity for its natural substrate: the enzyme was found to accept only a few C9 substituted derivatives of **1**, while modifications at the acetylamido group (except for a methylcarbamate[25]) were not tolerated.[25,29]

In this paper,[38] we describe the construction of a new and highly efficient heterologous overexpression system for CMP-sialate synthetase from *Neisseria meningitidis* serogroup B in non-pathogenic *E. coli* from which the enzyme can be obtained by a simple, two-step purification scheme for synthetic applications. We also report on the characterization of the recombinant synthetase by molecular weight measurement, kinetic studies, and investigations about its stability and substrate specificity. Its synthetic capacity is demonstrated by its application to the mmol-scale preparation of several CMP-activated sialic acids, and by transfer of the latter to *N*-acetyllactosamine using rat liver α-2,6-sialyltransferase for generation of the respective trisaccharides.

Results and Discussion

Subcloning of the CMP-Sialate Synthetase from *N. meningitidis* Serogroup B

The *N. meningitidis* structural gene *siaB* encoding the CMP-sialate synthetase had been cloned by Frosch et al.[2] and its sequence determined (GeneBank acces-



Scheme 1. Summary of the subcloning strategy for overexpression of the CMP-sialate synthetase from *N. meningitidis*.

sion No. M95053) as part of a 24 kb fragment of chromosomal DNA on plasmid pMF32.35, which contained all genes necessary for the capsular polysaccharide biosynthesis. For the construction of a suitable overexpression system (Scheme 1), the *siaB* gene was amplified from pMF32.35 by the primer-oriented PCR technique. Mutating sequences (Scheme 2) were used to insert two flanking recognition sequences for appropriate restriction; the sense primer introduced an *Eco*RI restriction site immediately upstream of the gene's start codon, and the antisense primer incorporated an *Xma*I restriction site downstream of the stop codon. From the PCR amplification only a single band with the expected molecular weight (710 bp) was observed upon agarose gel electrophoresis, and no further purification was required. After *Eco*RI and *Xma*I digestion, the insert was unidirectionally ligated into the pKK223–3 vector. The vector pKK223–3, containing the strong hybrid *tac* promoter and *rrn*B ribosomal termination for controlled protein expression,[39] was chosen because in our hands it had proved to be highly successful in the overproduction of other enzymes.[40] From experimental variation of the primer design, a 10 bp distance of the ATG start codon from the vector's Shine-Dalgarno sequence was found to be optimal for high protein expression. This construct, designated pKKCMP, was transformed into competent cells of *E. coli* strain JM105. Out of 6 colonies randomly selected from LB-ampicillin plates, 4 carried the de-

5′	*TATGAATTC* - ATGGAAAAAC AAAATATTGC GG 3′		primer CMP5
	*Eco*RI	Start	
5′	*TATTCCCGGG* - TTAGCTTTCC TTGTGATTAA GAATG 3′		primer CMP3
	*Xma*I	Stop	

Scheme 2. Oligonucleotide primers used in the subcloning of CMP-sialate synthetase. Recognition sequences for restriction endonucleases are underlined. Sequence stretches with identity to genetic DNA are indicated in regular font, mutating sequences introduced by PCR extension in italics.

sired functional insert as determined by a 12% SDS-polyacrylamide gel and by enzymatic assay for the desired activity.

Production and Purification of the CMP-Sialate Synthetase

For the production of the CMP-sialate synthetase, the recombinant strain JM105/pKKCMP was grown in a 10-L culture. Production of the enzyme was induced by addition of 0.5 mM IPTG. Analysis of cell extracts indicated a production of 7500 units of synthetase per liter of culture and 1350 U/g wet cell weight, providing the crude enzyme with a specific activity of 20 U/mg protein. Analysis by denaturing SDS polyacrylamide gel electrophoresis showed that fully induced recombinant cells contained about 25% of the CMP-sialate synthetase among total soluble cell protein. Despite the high level of foreign protein expression, no formation of inclusion bodies was detected. Due to its strong prevalence, the recombinant enzyme could be easily purified by anion-exchange chromatography of the crude cell extracts on DEAE-Sepharose followed by ammonium sulfate precipitation from pooled active fractions. As judged by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie staining, the purified synthetase was at least 95% homogenous (Figure 1). From a 10-L culture, purification resulted in about 63 kU with an overall yield of 85%. The results of a typical purification are summarized in Table 1. The purified recombinant *N. meningitidis* enzyme routinely showed a specific activity of better than 130 U/mg. This value is almost 70-fold higher than that achieved with either the native CMP-sialate synthetase from *N. meningitidis* serogroup C[17] or the corresponding recombinant enzyme from *E. coli* K1.[25,26,29] If desired, minor contaminants remaining in the enzyme preparation can be removed by a subsequent gel permeation chromatography (GPC) step on Sephadex G-150 to give homogenous protein, as judged by SDS-PAGE. However, for enzyme preparations to be used in the synthesis of CMP-sialic acids, such an additional purification step was found to be unnecessary.

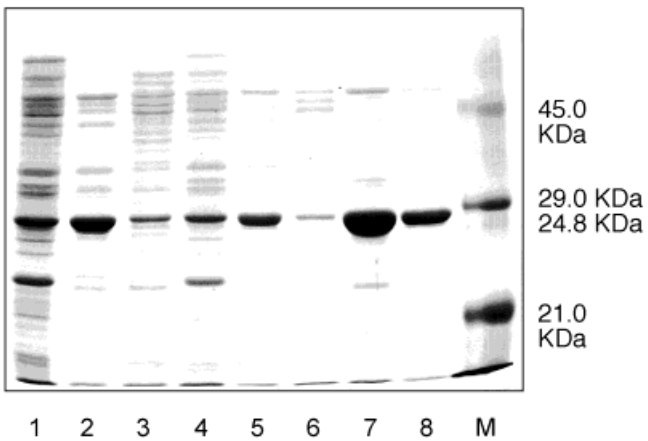


Figure 1. Electrophoretic analysis of the expression of the recombinant CMP-sialate synthetase in *E. coli* by SDS-PAGE separation on a 12% gel. Protein bands were stained by Coomassie blue. Molecular weight markers with the size indicated in kilodaltons to the right. Lanes 1 + 4: crude cell extract of strain JM105/pKKCMP after induction with 0.5 mM IPTG; lanes 2 + 5: purified CMP-sialate synthetase after DEAE chromatography; lanes 3 + 6: low activity chromatographic fractions; lanes 7 + 8: purified CMP-sialate synthetase after ammonium sulfate precipitation; lane M: protein markers.

Characterization of the Purified CMP-Sialate Synthetase

The molecular weight of the native synthetase was determined to be approximately 45.5 kDa by size exclusion chromatography on Sephadex G-150. This is slightly higher than values published for the *N. meningitidis* synthetase,[5,31] but verifies the assumption that in the native state the enzyme associates to form active dimers. Denaturing SDS-PAGE displayed a single band at about 25 kDa (Figure 1) indicating that the enzyme is composed of two subunits apparently identical in molecular weight. This value is in excellent agreement with the molecular mass predicted from the translated DNA sequence (24,895 Da). In fact, it has been determined most recently by X-ray structural analysis that the synthetase indeed forms a functional homodimer in the solid state.[41]

For kinetic studies, the enzymatic activity of a purified sample of CMP-sialate synthetase was determined by assaying for phosphate release in a coupled system with inorganic pyrophosphatase (two-fold stoichiometry) via formation of a phosphate complex to malachite green according to the method of Lanzetta et al.[42] We found that this measurement gives identical results for CMP-sialate synthetase activity,

Table 1. Purification of the recombinant *N. meningitidis* CMP-sialate synthetase expressed in *E. coli*.

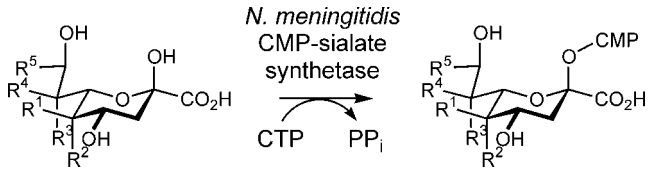
Sample	Volume [mL]	Activity [U/mL]	Specific Activity [U/mg][a]	Purification	Total Activity [U]	Yield [%]
crude extract	1000	75	20	–	75000	100
DEAE-Sepharose	935	87	97	4.85	81345	108
$(NH_4)_2SO_4$	250	254	135	1.39	63555	85

[a] Protein determined by Bio-Rad assay.

but proved much faster and much more sensitive, as compared to the conventional modified thiobarbituric acid method which usually is applied for CMP-sialate synthetase assays.[35] The K_m of the synthetase for **1** was determined to be 0.43 mM and for CTP 0.17 mM at pH 8.5, which roughly corresponds to values reported for a homologous enzyme from *N. meningitidis* serogroup 406Y (0.34 mM for **1**; 0.31 mM for CTP).[36] This indicates that the recombinant *N. meningitidis* enzyme has a significantly higher affinity to its natural substrates than the corresponding commercial enzyme from *E. coli* K1; the latter shows a more than ten-fold higher K_m value for **1** (4.8 mM) and more than six-fold higher for CTP (1.99 mM).[26,29] The purified enzyme was found to be quite stable upon storage, with 80% activity remaining after 18 weeks at 4 °C.

Synthetic Applications

For an evaluation of the substrate specificity of the CMP-NeuAc synthetase, a series of structural analogues of **1** were screened as replacement for the natural substrate under standard assay conditions (Equation 2). The reaction course was controlled by TLC and by monitoring phosphate formation using the Lanzetta method.[42] In this study, mainly analogues of **1** modified at C5[43] were evaluated for potential nucleotide activation because such structural variation had been reported to be unacceptable for the commercial synthetase from *E. coli* K1.[25,29] This included neuraminic acid *N*-carbamoylated with typical protective groups of different length and bulkiness (Table 2, entries 4 – 6), an unsaturated acrylamide derivative and the corresponding saturated moiety (entries 2 + 3), as well as the deaminated KDN analogue (entry 7). Also, the latter structure was varied by deoxygenation (entries 9 + 10), epimerization at C5 or at the terminal chain (entries 10 + 11), and by shortening the chain length to an octulosonic acid (entry 8).



(2)

Rather surprisingly, all of those sugars tested turned out to be suitable substrates for the CMP-sialate synthetase. Thus, as apparent from this limited survey for structural modifications, the *Neisseria* synthetase is highly unusual in its capacity for the binding and conversion of sialic acids that may differ from the natural Neu5Ac substrate in its substitution pattern, stereochemistry, or even deletion of its typical amide functionality. Moreover, combinations of such modifications (entry 10) appeared to be permissible at a relative reaction velocity still remaining in a reasonable range for preparative synthesis. A mammalian CMP-sialate synthetase (prepared from calf brain) had been reported to be reactive with a number of synthetic analogues of **1**, in which not only the terminal hydroxy group at C9 were modified but also the *N*-acetyl group at C5 could be replaced by a corresponding *N*-glycolyl or *N*-lactoyl substitution.[22,44] However, this synthetase showed only very low activity with KDN and its derivatives, suggesting that the presence of an acylamino group at C5 in the nonulosonic acid is essential for catalysis by this enzyme. Thus, the *N. meningitidis* synthetase studied here differs from both *E. coli* K1 and mammalian synthetases in having high catalytic activity also towards those sialic acids that are devoid of an acylamino group at the C5 position.

In order to verify the assay results, most of the substrate analogues were submitted to preparative scale synthesis up to a 1.5-mmol scale for conversion into the corresponding CMP-conjugates to allow product isolation and spectroscopic analysis. Reactions were supplemented with pyrophosphatase to improve conversion rates since the stoichio-

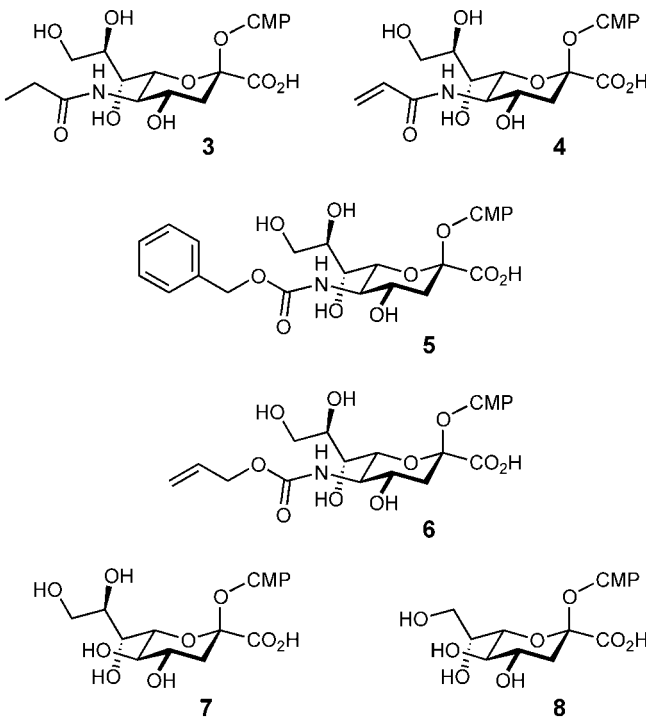
Table 2. Substrate specificity of the recombinant CMP-sialate synthetase from *N. meningitidis* (Equation 2).

Entry	Compound	R^1	R^2	R^3	R^4	R^5	Product	R_f [a]	Yield [%]
1	Neu5Ac	NH(acetyl)	H	OH	H	CH_2OH	**2**	0.67	100
2	Neu5Prop	NH(propanoyl)	H	OH	H	CH_2OH	**3**	0.67	99
3	Neu5Acryl	NH(acryloyl)	H	OH	H	CH_2OH	**4**	0.69	98
4	Neu5Cbz	NHCbz	H	OH	H	CH_2OH	**5**	0.75	93
5	Neu5Alloc	NHAlloc	H	OH	H	CH_2OH	**6**	0.73	100
6	Neu5Boc	NHBoc	H	OH	H	CH_2OH		0.74	n.i.[b]
7	KDN	OH	H	OH	H	CH_2OH	**7**	0.51	80
8	4,6-bis-*epi*-KDO	OH	H	OH	H	H	**8**	0.51	97
9	5-deoxy-KDN	H	H	OH	H	CH_2OH		0.49	n.i.
10	5-deoxy-7-*epi*-KDN	H	H	H	OH	CH_2OH		0.51	n.i.
11	5-*epi*-KDN	H	OH	OH	H	CH_2OH		0.41	n.i.

[a] Silica gel plates; eluent: satd. ammonia/ethanol = 1:1 (v/v).
[b] No product isolation.

metric release of pyrophosphate was found to inhibit the synthesis at increasing concentrations (pyrophosphatase catalyzes the conversion of inorganic pyrophosphate to phosphate, which precipitates from the reaction mixture as insoluble magnesium ammonium salt).

Purified enzyme preparations (≥95% purity) of the *N. meningitidis* CMP-sialate synthetase showed no detectable phosphatase or nucleotidase activity as evidenced by the stoichiometric conversion of a **1**/CTP mixture to the corresponding parent conjugate **2** which, for reference purposes, could be isolated by gel chromatography in practically quantitative yield. In comparison to that, the reported synthesis of **2** with enzyme prepared from calf brain requires the addition of up to 3 mole equivalents of CTP to drive the reaction to completion, due to the presence of phosphatase impurities.[22,23,24] With those sialic acid compounds for which the screening assay had indicated reasonably good reaction velocity, preparative-scale syntheses of corresponding CMP-nonulosonates were carried out successfully to afford the products **3** – **8** (Scheme 3) in 80 – 100% isolated yields. In view of the ready availability of highly active catalyst, larger quantities of the synthetase can be applied if desired, so that for substrate analogues that react more slowly both the reaction time and the extent of spontaneous CTP (or product) hydrolysis can be reduced significantly. Notably, the octosulose CMP-conjugate **8** could be prepared without difficulty, quite unlike CMP-KDO which is unstable in solution ($t_{1/2} \approx$ 34 min at pH 7.5).[45]



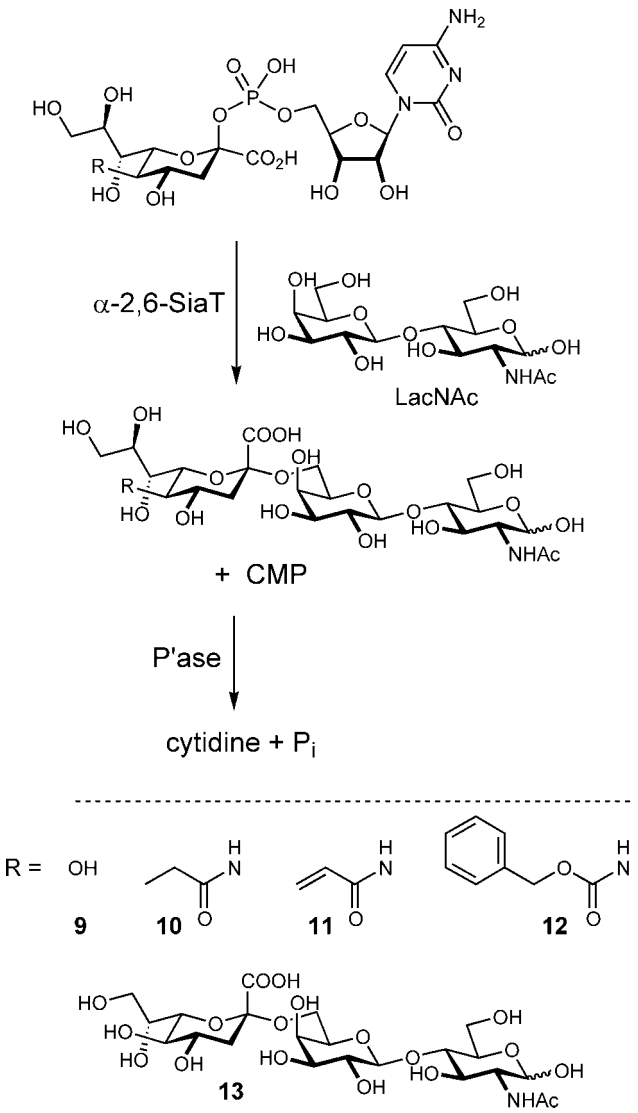
Scheme 3.

Some of the substrates showed a noticeable product formation by TLC analysis, however, the resulting conjugates (Table 2, entries 6, 9 – 11) seemed to be so labile that they could not be isolated from preparative-scale reactions for NMR analysis. Generally, nucleotide-activated sialic acids are sensitive to spontaneous hydrolysis even under neutral or alkaline conditions,[46] with particular compounds showing a lability that is several orders of magnitude higher than that of **2**. This reactivity has been suggested to be due to electronic factors and to intramolecular assistance from hydroxy substituents.[45] In such cases, direct coupling of the synthetase-catalyzed CMP activation to the enzymatic sialyl transfer *in situ*[29] may be a viable solution to avoid the need for isolation of highly labile CMP conjugates.

In order to test their suitability for the synthesis of *neo*-sialoconjugates that display a designed sialic acid functionality, five of the enzymatically prepared CMP-conjugates were subjected to an α-2,6-sialyltransferase-catalyzed reaction for sialylation of *N*-acetyllactosamine using the commercial rat liver enzyme. This procedure cleanly generated the corresponding trisaccharides **9** – **13** which were isolated in good yield (37 – 77%) after purification by size exclusion chromatography (Scheme 4, Table 3). Lower than quantitative oligosaccharide yields are due to the limited quantities of the transferase employed in this study owing to its high cost, and therefore reflect incomplete conversions and hydrolytic loss of nucleotide sugars upon extended reaction times; optimization was not pursued. Previous investigations with chemically synthesized CMP-Neu5Cbz **5** and CMP-KDN **7** had already shown that some α-2,3- and α-2,6-sialyltransferases are indeed quite tolerant to structural modification at C5 of the sialic acid component.[12,47] The resultant *neo*-KDN-glycoconjugate **9** contains a terminal KDN-(α–2,6)-Gal-residue, a glycotope that has not yet been described in natural glycoconjugates. The successful synthesis of the KDO-analogue containing trisaccharide **13** further demonstrates that sialic acids with carbon backbones smaller than C_9 can be transferred with similar high efficiency. In conjunction with our improved technique for the rapid stereospecific chemical synthesis of KDN and related sialic acids,[48] a short chemoenzymatic sequence is now at hand which holds promise for the rapid preparation of a variety of designed sialylated oligosaccharides for biological studies.

Remarkably, the acrylamide function (**4**, **11**) survived both the enzyme-catalyzed conversions for nucleotide activation and sialyl transfer. At the outset, this moiety had been introduced for the purpose to incorporate in *neo*-glycoconjugates a highly reactive functionality that structurally mimics the natural acetyl group but may be selectively manipulated after the construction of complex targets by orthogonal chemi-

cal reactions,[49] including the option for polymerization to novel glycopolymers. On the other hand, given the acrylamide group's typical proclivity for a facile Michael-type capture of nucleophiles, this unit was anticipated to possibly react with a side chain nucleophile upon binding to an enzyme active site. Such a bond forming process would essentially be irreversible and would cause an effective inactivation in a suicide-type fashion that would potentially be applicable to any enzyme involved in the metabolism of sialic acid or sialoconjugates. However, neither the activity of the synthetase (**4**) nor that of the transferase (**11**) were apparently affected to a noticeable degree during the binding and conversion of this substrate. In conjunction with the tolerance of the enzymes for large structural variations at this particular substrate position it may be concluded that this partial structure is only very loosely enzyme-bound (if at all) and may rather be oriented to solvent environment. In fact, inspection of the synthetase crystal structure indicates an active site disposition, close to the subunit interface, that is rather open and flexible in the sector that is held responsible for binding of the sialic acid moiety.[41,50]



Scheme 4.

Conclusions

We have developed an efficient method for the heterologous overproduction of CMP-sialate synthetase from *N. meningitidis* serogroup B in *E. coli* providing this activity in much larger quantities than reached from any other known sources of CMP-sialate synthetase reported so far. The synthetase was found to offer an unusual and remarkably broad tolerance for structural modifications of its natural substrate **1**, allowing the substitution of the *N*-acylamino and peripheral hydroxy groups, the epimerization of stereocenters at the pyranose core and at peripheral sites, and the deletion of functionalities, including that of the entire *N*-acylamino substituent being characteristic for the sialic acids. The synthetic utility of the enzyme could be demonstrated by the formation of several new CMP-conjugates that further served in the transfer to an appropriate acceptor substrate in an α-2,6-sialyltransferase-catalyzed reaction. Consequently, it can be used for the synthesis of both natural carbohydrates and synthetic derivatives with novel properties.

The high productivity of the expression system, the simplicity of an effective two-step purification sequence, the enzyme's high specific activity and stability, and especially its broad substrate tolerance make this recombinant CMP-sialate synthetase by far superior to previously investigated enzymes. These characteristics render it the preferred catalyst for the

Table 3. α-2,6-SiaT-catalyzed synthesis of *neo*-sialotrisaccharides containing modified sialic acids.

CMP-Sialate	Product	Constitution	R_f value[a]	Isolated yield
3	**10**	Neu5Prop-α-2,6-LacNAc	0.33	12.9 mg (72%)
4	**11**	Neu5Acryl-α-2,6-LacNAc	0.35	13.7 mg (77%)
5	**12**	Neu5Cbz-α-2,6-LacNAc	0.63	7.4 mg (37%)
7	**9**	KDN-α-2,6-LacNAc	0.33	9.6 mg (58%)
8	**13**	4,6-bis-*epi*-KDO-α-2,6-LacNAc	0.32	9.6 mg (58%)

[a] Silica gel plates; eluent 1-butanol/acetone/acetic acid/water = 35:35:7:23 (v/v/v/v)

enzymatic synthesis of CMP-Neu5Ac and of derivatives modified in the sialic acid moiety. In comparison to the synthetases from *E. coli* K1 and from calf brain as the current standards for *in vitro* syntheses, the *N. meningitidis* enzyme promises to become an overall effective replacement, particularly with regard to potential large-scale applications.[43]

Experimental Section

General Remarks

Plasmid pMF32.35 was provided by Professor M. Frosch, Würzburg. Oligonucleotide primers, pKK223–3 vector, *E. coli* strain JM105, and media for protein chromatography were purchased from Pharmacia Biotech. PCR Core Kit, restriction enzymes, and T4 DNA ligase were obtained from Boehringer Mannheim and used according to the manufacturer's instructions. Other routine cloning operations were performed by the standard procedures.[51] The nuclease (Benzonase, grade I) was purchased from Merck, Darmstadt.

NMR spectra were recorded on Varian VXR 300 or Unity 500 spectrometers; chemical shifts are referenced to internal TSP (0.00 ppm). Mass spectra were recorded on a Finnigan MAT 212 system. Column chromatography was performed on BioGel P-2 Fine (65 ± 20 μm, Bio-Rad), and analytical thin-layer chromatography (TLC) was performed on Merck silica gel plates 60 GF_{254} using anisaldehyde stain for detection. Inorganic pyrophosphatase (PPase; [EC 3.6.1.1]) was from Sigma, calf intestinal alkaline phosphatase (CIAP; [EC 3.1.3.1]) was from Boehringer Mannheim, and α-2,6-sialyltransferase from rat liver (α-2,6-SiaT; [EC 2.4.99.1]) was obtained from Calbiochem. CTP was either purchased from Sigma or prepared from CMP according to published procedures.[23,52] The synthesis of the modified sialic acids will be reported elsewhere.[43]

PCR Amplification

The gene *siaB* contained in the plasmid pMF32.35 was amplified along standard PCR protocols[53] but using mutating oligonucleotide primers as shown in Scheme 2. Amplification was performed in a 20-μL reaction mixture containing 5 ng of plasmid pMF32.35, 375 nM each of primers, 250 μM each of dNTPs, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 50 mM KCl. The reaction mixture was heated for 2 min at 95 °C, then *Taq* DNA polymerase (2.5 U) was added, the solution was overlayed with mineral oil (20 μL) and subjected to 25 – 30 cycles of amplification (Eppendorf Mastercycler 5330). Cycle conditions were set for denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min, and elongation at 72 °C for 2 min (plus 3 sec per cycle). The mineral oil was removed and DNA was collected by phenol/chloroform extraction and ethanol precipitation. Analysis by agarose gel electrophoresis (1.2%) showed the desired DNA insert (710 bp) to be the only detectable PCR product, which was used for the subsequent steps without further purification.

Construction of the Expression Vector pKKCMP

The PCR amplified *siaB* insert was digested by incubation with restriction enzymes *Xma*I and *Eco*RI under standard conditions. A 15-μL reaction mixture containing the DNA insert, 6 mM Tris-HCl (pH 7.5), 50 mM NaCl, 6 mM $MgCl_2$, 1 mM DTT, and *Xma*I (1 μL, 5 U) was incubated at 37 °C. After 4 – 5 h the enzyme was denatured by heating at 65 °C for 15 min. After cooling to ambient temperature, there was added 2.5 μL of water, 1.5 μL of restriction buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 1.0 M NaCl, 10 mM dithioerythritol, pH 7.5), and 1 μL of *Eco*RI (10 U), and the mixture was incubated at 37 °C for another 4 – 5 h. After denaturation by heating at 65 °C for 15 min, the restricted insert was recovered by phenol/chloroform extraction and by precipitation with ethanol (2 volumes) and 3.0 M NaOAc (1/10 vol, pH 5.2). The DNA pellet was taken up with 20 μL Tris-HCl (10 mM, pH 8.0).

The vector pKK223–3 (1 μg) was digested with *Xma*I and *Eco*RI under the same conditions. The crude digested vector was taken up in 49 μL dephosphorylation buffer (50 mM Tris-HCl, 0.1 mM $ZnCl_2$, 1 mM $MgCl_2$, 1 mM spermidine, pH 9.0) and treated with calf intestinal alkaline phosphatase (1 μL, 0.01 U) at 37 °C for 30 min. The reaction was stopped by addition of 0.6 μL of 0.5 M EDTA solution and heating at 65 °C for 60 min. Plasmid DNA was recovered by phenol/chloroform extraction followed by ethanol precipitation, and the DNA pellet was taken up in 20 μL of Tris-HCl buffer (10 mM, pH 8.0).

The restricted insert (7.5 μL) and the restricted and dephosphorylated vector (0.5 μL) were incubated overnight at 20 °C with T4 DNA ligase (1 μL, 1 U) in 1 μL of the appropriate buffer (660 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM dithioerythritol, 10 mM ATP, pH 7.5). After ligation, competent cells of *E. coli* JM105 strain were transformed with the recombinant plasmid, denominated pKKCMP, and plated onto LB agar plates supplemented with ampicillin (200 mg/L). Out of 6 colonies randomly selected, 4 carried the desired insert as determined by plasmid isolation and restriction analysis, as well as by enzymatic assay for CMP-sialate synthetase activity (after IPTG induction).

Expression and Purification of CMP-Sialate Synthetase

A positive clone was grown aerobically in 200 mL of LB medium supplemented by ampicillin (200 mg/L) at 37 °C in a shake flask at 300 rpm. When the turbidity reached an OD_{600} of 1.0, this was used as a starter culture to inoculate a 10-L-fermentor (Biostat B, B. Braun Biotech) charged with 9.8 L of the same medium. Cells were grown aerobically with stirring at 37 °C to 0.8 – 1.0 OD_{600} when 1.2 g IPTG were added to give a final concentration of 0.5 mM. After a further 8 – 10 h, cells were harvested by continuous centrifugation (Heraeus Biofuge 28 RS; 20,000 *g*; 4 °C) to give a wet cell weight of 56 g. The pellet was resuspended in buffer (20 mM Tris-HCl, 2 mM $MgCl_2$, 20 μM DTT, pH 7.8; 5 mL/g cells), and 10,000 U of Benzonase was added. Cells were disrupted by glass bead milling (Biomatik, cell disintegrator-S), and the extracts were clarified by filtration followed by centrifugation at 24,000 *g* for 60 min at 4 °C. Total crude CMP-sialate synthetase activity, determined by the enzyme assay

described below, amounted to 75,000 U (7,500 U/L of cell culture; 1340 U/g of cells). Protein purification was performed by ion exchange chromatography on DEAE-Sepharose CL-6B, applying a NaCl gradient for protein elution (50 – 300 mM). CMP-sialate synthetase activity eluted around 150 mM, and active fractions were pooled and concentrated by ammonium sulfate precipitation at 50% saturation (total recovered activity 64,000 U; 85%).

Enzyme Activity and Substrate Affinity (K_m Values)

CMP-sialate synthetase activity was assayed by coupling the synthetase reaction to inorganic pyrophosphatase, and either measuring the amount of CMP-Neu5Ac (**2**) by a modified thiobarbituric acid method described by Vann et al.,[55] or by monitoring the production of inorganic phosphate with malachite green reagent. The standard assay mixture contained 0.2 M Tris-HCl (pH 8.5), 20 mM $MgCl_2$, 0.2 mM DTT, 2.8 mM Neu5Ac, 1.65 mM CTP and 2 U of inorganic pyrophosphatase in a final volume of 0.5 mL. After addition of CMP-sialate synthetase and incubation of the reaction mixture at 37 °C, aliquots were removed at 5 min time intervals over 5 – 30 minutes and analyzed according to the published procedures. One unit of synthetase activity is defined as the amount of enzyme required to produce 1 μmol **2** or 1 μmol PP_i (≡ 2 μmol P_i) from CTP and Neu5Ac (**1**) per minute under assay conditions at 37 °C.K_m values of the purified CMP-sialate synthetase were determined by measuring the initial velocities at various concentrations of CTP (0.138 mM – 11.00 mM) and Neu5Ac (0.14 mM – 11.20 mM), and evaluating the accumulated data by computational analysis (EnzymeKinetics v1.1; Trinity Software).

Protein Analysis

Protein concentration was determined by the Bradford method[54] with assay reagents supplied by Bio-Rad, using bovine serum albumin for calibration. Protein purity was analyzed by SDS-PAGE performed according to the method of Laemmli[55] using a 12% polyacrylamide gel.

Molecular Weight Measurement

The molecular weight of the native CMP-sialate synthetase was estimated by gel filtration on a column of Sephadex G-150 (90 × 2.5 cm). Elution of the enzyme was performed with 20 mM Tris-HCl buffer (pH 7.8) containing 0.1 M NaCl, 2 mM $MgCl_2$ and 20 μM DTT. The column was separately calibrated with dextran blue (2 MDa), aldolase (161 kDa), bovine serum albumin (66 kDa), and ATP (605 Da) as molecular weight standards.

Enzyme Stability

The enzyme stability was studied with a sample of CMP-sialate synthetase purified by anion exchange chromatography and ammonium sulfate precipitation, and stored at 4 °C. At weekly intervals, 50 μL aliquots were removed and assayed for remaining activity as described above.

Substrate Specificty

For screening of the substrate specificity of the recombinant synthetase, the natural substrate Neu5Ac was replaced by various sugar derivatives using the standard assay conditions as described above. The progress of the coupling reactions was determined both by measuring the phosphate release using the method of Lanzetta and by determining product formation using TLC analysis. In case of low conversion rates, the reaction was repeated at higher enzyme and/or substrate concentration in order to get unambiguous results.

General Procedure for the Enzymatic CMP-Activation of Neu5Ac and Analogues

All CMP-sialate synthetase catalyzed reactions were performed in 50 mM Tris-HCl buffer (pH 8.5) containing 50 mM $MgCl_2$ and 0.2 mM DTT. After addition of CTP (0.1 – 1.5 mmol) and an equimolar amount of the appropriate neuraminic acid to the buffer solution (2.5 – 6 mL), the pH was readjusted to 8.5 with 2 M NaOH (if necessary). The reaction was initiated by the addition of inorganic pyrophosphatase (5 – 25 U) and CMP-sialate synthetase (50 – 500 U), and allowed to proceed for 2 – 4 h with shaking at ambient temperature. The reaction was monitored by TLC analysis, and the pH was maintained at 8.5 by periodic manual addition of 2 M NaOH. If no further product formation could be detected, enzymes and the resultant precipitate (presumably magnesium-ammonium phosphate formed during the reaction) were removed by membrane filtration (pore size: 0.2 μm). The product was precipitated by the addition of ethanol (9:1 v/v), isolated by centrifugation (10,000 *g*; 10 min), and dried under vacuum. For analytical purification, ethanol precipitation was repeated twice.

Cytidine-5'-monophospho-5-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonic Acid (2)

From Neu5Ac (**1**; 31 mg, 0.1 mmol), CTP (57 mg, 0.1 mmol), PPase (5 U), synthetase (50 U)in 2.5 mL; colorless solid, yield: 75 mg (100%, assuming **2**·Na_2); R_f = 0.67 [saturated NH_3-EtOH, 1:1]; NMR data were in full agreement with published values.[23]

Cytidine-5'-monophospho-*N*-propionyl-5-amino-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonic Acid (3)

From Neu5Prop (162 mg, 0.5 mmol), CTP (286 mg, 0.5 mmol), PPase (15 U), synthetase (130 U) in 4.0 mL; colorless solid; yield: 400 mg (99%, assuming **3**·Na_2); R_f = 0.67 [saturated NH_3-EtOH. 1:1]; ^{1}H NMR (300 MHz; D_2O): δ = 7.94 (d, 1H, *J* = 7.6 Hz, H-6,), 6.12 (d, 1H, *J* = 7.6 Hz, H-5,), 6.00 (d, 1H, *J* = 4.1 Hz, H-1'), 4.42 – 4.24 (m, 5H, ribose), 4.19 (dd, 1H, *J* = 10.4 Hz, H-6), 4.13 (ddd, 1H, *J* = 10.7, 4.6 Hz, H-4), 4.04 – 3.88 (m, 3H), 3.66 (dd, 1H, *J* = 11.5, 6.3 Hz, H-9a), 3.49 (d, 1H, *J* = 9.3 Hz, H-7), 2.53 (dd, 1H, *J* = 12.9, 4.6 Hz, H-3eq), 2.35 (q, 2H, *J* = 7.6 Hz, H-11 Prop), 1.71 (ddd, 1H, *J* = 12.9, 12.2, 5.8 Hz, H-3ax), 1.16 (t, 3H, *J* = 7.6 Hz, H-12 Prop).

Cytidine-5'-monophospho-*N*-acryloyl-5-amino-3,-5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonic Acid (4)

From Neu5Acryl (113 mg, 0.35 mmol), CTP (200 mg, 0.35 mmol), PPase (15 U), synthetase (130 U) in 4.0 mL; colorless solid; yield: 265 mg (98%, assuming **4**·Na_2); R_f = 0.69 [saturated NH_3-EtOH, 1:1]; ^{1}H NMR (300 MHz; D_2O): δ = 7.95 (d, 1H, *J* = 7.6 Hz, H-6,), 6.34 (dd, 1H, *J* = 17.0, 9.3 Hz, H-11 Acryl), 6.25 (dd, 1H, *J* = 17.0, 1.9 Hz, H-12a Acryl), 6.12 (d, 1H, *J* = 7.6 Hz, H-5,), 6.00 (d, 1H, *J* = 4.1 Hz, H-1'), 5.83 (dd, 1H, *J* = 9.3, 1.9 Hz, H-12b Acryl), 4.40 – 4.23 (m, 5H, ribose), 4.23 (dd, 1H, *J* = 10.4 Hz, H-6), 4.17 (ddd, 1H, *J* = 10.7, 4.4 Hz, H-4), 4.10 – 3.87 (m, 3H), 3.64 (dd, 1H, *J* = 11.8, 6.6 Hz, H-9a), 3.48 (d, 1H, *J* = 9.6 Hz, H-7), 2.54 (dd, 1H, *J* = 13.2, 4.4 Hz, H-3eq), 1.71 (ddd, 1H, *J* = 13.2, 12.0, 5.5 Hz, H-3ax).

Cytidine-5'-monophospho-*N*-benzyloxycarbonyl-5-amino-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonic Acid (5)

From Neu5Cbz (201 mg, 0.5 mmol), CTP (286 mg, 0.5 mmol), PPase (10 U), synthetase (200 U) in 8.0 mL; colorless solid; yield: 391 mg (93%, assuming **5**·Na_2); R_f = 0.75 [saturated NH_3-EtOH, 1:1], 0.46 [1-butanol-acetone-acetic acid-water, 35:35:7:23]; ^{1}H NMR (300 MHz; D_2O): δ = 7.96 (d, 1H, *J* = 7.6 Hz, H-6,), 7.48 – 7.40 (m, 5H, H_{ar} Cbz), 6.11 (d, 1H, *J* = 7.6 Hz, H-5,), 5.99 (d, 1H, *J* = 4.4 Hz, H-1'), 5.18 (d, 1H, *J* = 12.5 Hz, H-11a Cbz), 5.11 (d, 1H, *J* = 12.5 Hz, H-11b Cbz), 4.39 – 4.22 (m, 5H, ribose), 4.15 (dd, 1H, *J* = 10.4 Hz, H-6), 4.07 (ddd, 1H, *J* = 10.6, 4.7 Hz, H-4), 3.98 – 3.64 (m, 3H), 3.59 (dd, 1H, *J* = 11.8, 6.9 Hz, H-9a), 3.48 (d, 1H, *J* = 9.6 Hz, H-7), 2.50 (dd, 1H, *J* = 12.9, 4.7 Hz, H-3eq), 1.66 (ddd, 1H, *J* = 12.9, 11.7, 5.5 Hz, H-3ax).

Cytidine-5'-monophospho-*N*-allyloxycarbonyl-5-amino-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonic Acid (6)

From Neu5Alloc (246 mg, 0.7 mmol), CTP (400 mg, 0.7 mmol), PPase (15 U), synthetase (130 U) in 4.0 mL; colorless solid; yield: 524 mg (100%, assuming **6**·Na_2); R_f = 0.73 [saturated NH_3-EtOH, 1:1], 0.31 [1-butanol-acetone-acetic acid-water, 35:35:7:23]; ^{1}H NMR (300 MHz; D_2O): δ = 7.94 (d, 1H, *J* = 7.4 Hz, H-6,), 6.12 (d, 1H, *J* = 7.4 Hz, H-5,), 6.07–5.91 (m, 2H, H-12 Alloc, H-1'), 5.35 (dd, 1H, *J* = 17.5, 1.3 Hz, H-13a Alloc), 5.27 (dd, 1H, *J* = 10.7, 1.3 Hz, H-13b Alloc), 4.68 – 4.52 (m, 2H, H-11 Alloc), 4.40 – 4.23 (m, 5H, ribose), 4.22 – 3.62 (m, 6H), 3.59 (d, 1H, *J* = 9.6 Hz, H-7), 2.50 (dd, 1H, *J* = 4.7 Hz, H-3eq), 1.65 (ddd, 1H, H-3ax).

Cytidine-5'-monophospho-3-deoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonic Acid (7)

From KDN (402 mg, 1.5 mmol), CTP (858 mg, 1.5 mmol), PPase (25 U), synthetase (530 U) in 15.0 mL; colorless solid; yield: 783 mg (80%, assuming **7**·Na_2); R_f = 0.51 [saturated NH_3-EtOH, 1:1]; ^{1}H NMR (300 MHz; D_2O): δ = n.n (d, 1H, *J* = 7.6 Hz, H-6,), 6.13 (d, 1H, *J* = 7.6 Hz, H-5,), 6.00 (d, 1H, *J* = 4.4 Hz, H-1'), 4.40 – 4.22 (m, 5H, ribose), 4.13–3.66 (m, 6H), 3.61 (dd, 1H, *J* = 9.6 Hz, H-5), 2.45 (dd, 1H, *J* = 13.2, 5.0 Hz, H-3eq), 1.63 (ddd, 1H, *J* = 13.2, 12.3, 5.7 Hz, H-3ax).

Cytidine-5'-monophospho-3-deoxy-β-D-*galacto*-2-octulopyranosonic Acid (8)

From 4,6-bis-*epi*-KDO (238 mg, 1.0 mmol), CTP (572 mg, 1.0 mmol), PPase (10 U), synthetase (530 U) in 6.0 mL; colorless solid; yield: 702 mg (97%, assuming **8**·Na_2); R_f = 0.51 [saturated NH_3-EtOH, 1:1]; ^{1}H NMR (300 MHz; D_2O): δ = 7.97 (d, 1H, *J* = 7.7 Hz, H-6,), 6.12 (d, 1H, *J* = 7.7 Hz, H-5,), 6.00 (d, 1H, *J* = 4.1 Hz, H-1'), 4.40 – 4.21 (m, 5H, ribose), 4.07 – 3.55 (m, 6H), 2.44 (dd, 1H, *J* = 13.1, 4.9 Hz, H-3eq), 1.62 (ddd, 1H, *J* = 13.1, 12.5, 5.8 Hz, H-3ax).

General Procedure for Enzymatic Transfer of Sialic Acid

A 10 mg portion (26 μmol) of *N*-acetyllactosamine was dissolved in 2 mL of sodium cacodylate (50 mM, pH 7.8) containing 2 mg of bovine serum albumin, 5 μmol of $MnCl_2$, 15 μmol of NaN_3, 30 – 40 μmol of the appropriate CMP-sialic acid, 25 – 35 mU of α-2,6-SiaT, and 15 U of CIAP. The reaction mixture was incubated at 25 °C with analysis for conversion by TLC, and the pH was maintained at 7.8 by periodic addition of 0.5 M NaOH. If no further product formation could be detected, enzyme and salts precipitated during the reaction were removed by membrane filtration (pore size: 0.2 μm), and the reaction mixture was concentrated by lyophilization. The sialoside was isolated by gel chromatography on a BioGel P-2 column (50 × 2.5 cm) using water as eluent. Fractions containing the product were pooled and lyophilized.

3-Deoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic Acid-(2,6)-β-D-galactopyranosyl-(1,4)-2-acetamido-2-deoxy-D-glucopyranose (9)

From **7** using 23 mU SiaT, 4 d; colorless solid; yield: 9.6 mg (58%); R_f = 0.33 [1-butanol-acetone-acetic acid-water, 35:35:7:23]; ^{1}H NMR (500 MHz; D_2O): δ = 5.20 (d, 0.6H, *J* = 3.1 Hz, H-1α), 4.75 (d, 0.4H, *J* = 7.9 Hz, H-1β), 4.45 (2d, 1H, *J* = 7.9 Hz, H-1'), 4.02 – 3.46 (m, 19H), 2.63 (dd, 1H, *J* = 12.2, 4.6 Hz, H-3,eq), 2.08 (s, 3H, CH_3), 1.66 (dd, 1H, *J* = 12.2 Hz, H-3,ax); ^{13}C NMR (75 MHz; D_2O): δ = 177.50 (NAc β), 177.27 (NAc α), 176.61 (C-1,), 106.22 (C-1'), 102.96 (C-2,), 97.56 (C-1β), 93.50 (C-1α), 83.37/83.22 (C-4), 77.51, 76.69, 76.42, 75.24, 75.07, 73.60, 73.04 (C-5,), 72.92, 72.19, 71.39 (C-7,), 71.04 (C-4,), 66.57 (C-6'), 65.57 (C-9,), 63.15 / 62.97 (C-6), 58.95 (C-2β), 56.45 (C-2α), 42.68 (C-3,), 25.22 (CH_3 β), 24.92 (CH3 α); MS: *m/z* (SIMS, Gl/Thgl) = 634.2 (0.7) $[M + H]^-$, 633.3 (1.4) $[M]^-$, 632.2 (1.2) $[M - H]^-$

N-Propionyl-5-amino-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic Acid-(2,6)-β-D-galactopyranosyl-(1,4)-2-acetamido-2-deoxy-D-glucopyranose (10)

From **3** using 23 mU SiaT, 5 d; colorless foam; yield: 12.9 mg (72%); R_f = 0.33 [1-butanol-acetone-acetic acid-water, 35:35:7:23]; ^{1}H NMR (500 MHz; D_2O): δ = 5.21 (d, 0.6H, *J* = 2.8 Hz, H-1α), 4.76 (d, 0.4H, *J* = 8.2 Hz, H-1β), 4.46 (2d, 1H, *J* = 7.9 Hz, H-1'), 4.03 – 3.51 (m, 19H), 2.68 (dd, 1H, *J* = 12.2, 4.6 Hz, H-3,eq), 2.31 (q, 2H, *J* = 7.6 Hz, H-11 Prop), 2.08 (s,

3H, CH_3), 1.73 (dd, 1H, *J* = 12.2 Hz, H-3,ax), 1.12 (t, 3H, *J* = 7.6 Hz, H-12 Prop); ^{13}C NMR (75 MHz; D_2O): δ = 181.82 (NAc,), 177.62 (NAc β), 177.36 (NAc α), 176.47 (C-1,), 106.37 (C-1'), 103.06 (C-2,), 97.58 (C-1β), 93.46 (C-1α), 83.78 / 83.55 (C-4), 77.43 (C-5β), 76.58, 75.47, 75.31, 74.59, 73.64, 72.89, 72.21, 71.30 (C-7,), 71.01 (C-4,), 66.26 (C-6'), 65.51 (C-9,), 63.24 / 63.05 (C-6), 58.86 (C-2β), 56.32 (C-2α), 54.65 (C-5,), 43.02 (C-3,) , 32.14 (C-11 Prop), 25.18 (CH_3 β), 24.88 (CH_3 α), 12.41 (C-12 Prop); MS: *m/z* (SIMS, Gl/Thgl) = 688.1 (1.0) $[M]^-$, 687.1 (1.4) $[M - H]^-$, 305.2 (100) $[Neu5Prop - H_2O]^-$.

N-Acryloyl-5-amino-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic Acid-(2,6)-β-D-galactopyranosyl-(1,4)-2-acetamido-2-deoxy-D-glucopyranose (11)

From **4** using 35 mU SiaT, 6 d; colorless solid; yield: 13.7 mg (77%); R_f = 0.35 [1-butanol-acetone-acetic acid-water, 35:35:7:23]; ^{1}H NMR (500 MHz; D_2O): δ = 6.29 (dd, 1H, *J* = 17.1, 9.8 Hz, H-11 Acryl), 6.22 (dd, 1H, *J* = 17.1, 1.8 Hz, H-12a Acryl), 5.80 (dd, 1H, *J* = 9.8, 1.8 Hz, H-12b Acryl), 5.21 (d, 0.5H, *J* = 2.8 Hz, H-1α), 4.77 (d, 0.5H, *J* = 8.2 Hz, H-1β), 4.47 (2d, 1H, *J* = 7.9 Hz, H-1'), 4.06 – 3.52 (m, 19H), 2.70 (dd, 1H, *J* = 12.5, 4.6 Hz, H-3,eq), 2.09 (s, 3H, CH_3), 1.75 (dd, 1H, *J* = 12.2 Hz, H-3,ax); ^{13}C NMR (75 MHz; D_2O): δ = 177.65 (NAc β), 177.40 (NAc α), 176.47 (C-1,), 172.23 (NAc,), 132.57 (C-11 Acryl), 130.80 (C-12 Acryl), 106.39 (C-1'), 103.11 (C-2,), 97.58 (C-1β), 93.48 (C-1α), 83.79/83.55 (C-4), 77.44 (C-5β), 76.59, 75.45, 75.29, 74.62, 73.63, 72.88, 72.23, 71.34 (C-7,), 71.12 (C-4,), 66.29 (C-6'), 65.56 (C-9,), 63.06 (C-6), 58.84 (C-2β), 56.32 (C-2α), 54.84 (C-5,), 42.95 (C-3,) , 25.20 (CH_3 β), 24.89 (CH_3 α); MS: *m/z* (SIMS, Gl/Thgl) = 650.2 (0.4) $[M - 2H_2O]^-$.

N-Benzyloxycarbonyl-5-amino-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic Acid-(2,6)-β-D-galactopyranosyl-(1,4)-2-acetamido-2-deoxy-D-glucopyranose (12)

From **5** using 35 mU SiaT, 9 d; colorless solid; yield: 7.4 mg (37%); R_f = 0.63 [1-butanol-acetone-acetic acid-water, 35:35:7:23]; ^{1}H NMR (500 MHz; D_2O): δ = 7.48 – 7.39 (m, 5H, H_{ar} Cbz), 5.21 (d, 0.4H, *J* = 1.8 Hz, H-1α), 5.16 (d, 1H, *J* = 12.5 Hz, H-11b Cbz), 5.11 (d, 1H, *J* = 12.5 Hz, H-11a Cbz), 4.77 (d, 0.6H, H-1β), 4.46 (2d, 1H, *J* = 7.9 Hz, H-1'), 4.02 – 3.80 (m, 9H), 3.75–3.52 (m, 10H), 2.66 (dd, 1H, *J* = 12.5, 4.6 Hz, H-3,eq), 2.06 (s, 3H, CH_3), 1.71 (dd, 1H, *J* = 12.2 Hz, H-3,ax); ^{13}C NMR (75 MHz; D_2O): δ = 177.87 (NAc β), 177.42 (NAc α), 176.49 (C-1,), 161.43 (NAc,), 139.41 (C-1_{ar} Cbz), 131.70/130.56 (2 C-2_{ar}, 2 C-3_{ar} Cbz), 131.29 (C-4_{ar} Cbz), 106.37 (C-1'), 103.07 (C-2,), 97.60 (C-1β), 93.48 (C-1α), 83.72/83.51 (C-4), 77.27 (C-5β), 76.60, 75.54, 75.31, 74.81, 73.62, 72.92, 72.23, 71.36 (C-7,), 69.93 (C-4,), 66.30 (C-6'), 65.68 (C-9,), 63.24/63.06 (C-6), 58.88 (C-2β), 56.15 (C-2α), 55.63 (C-5,), 42.99 (C-3,), 25.21 (CH_3 β), 24.89 (CH_3 α); MS: *m/z* (SIMS, DTE/DTT/Sul) = 795.1 (1.6) $[M + 4Li + H]^+$, 786.2 (0.5) $[M + 3Li - H]^+$, 780.1 (2.6) $[M + 2Li]^+$, 779.2 (5.6) $[M + 2Li - H]^+$, 183.0 (100) $[Gal + 3Li - H_2O]^+$.

3-Deoxy-α-D-*galacto*-2-octulopyranosylonic acid-(2,6)-β-D-galactopyranosyl-(1,4)-2-acetamido-2-deoxy-D-glucopyranose (13)

From **8** using 35 mU SiaT, 9 d; colorless solid; yield: 9.0 mg (57%); R_f = 0.32 [1-butanol-acetone-acetic acid-water, 35:35:7:23]; ^{1}H NMR (500 MHz; D_2O): δ = 5.21 (d, 0.6H, *J* = 2.7 Hz, H-1α), 4.75 (d, 0.4H, *J* = 7.9 Hz, H-1β), 4.45 (2d, 1H, *J* = 7.9 Hz, H-1'), 4.01 – 3.45 (m, 19H), 2.58 (dd, 1H, *J* = 12.5, 4.6 Hz, H-3,eq), 2.08 (s, 3H, CH_3), 1.63 (dd, 1H, *J* = 12.2 Hz, H-3,); ^{13}C NMR (75 MHz; D_2O): δ = 177.52 (NAc β), 177.29 (NAc α), 176.64 (C-1,), 106.23 (C-1'), 103.20 (C-2,), 97.59 (C-1β), 93.49 (C-1α), 83.40/83.23 (C-4), 77.51, 77.10, 76.72, 75.39, 75.26, 73.63, 73.12 (C-5,), 72.94, 72.71, 72.22, 71.78, 71.38, 66.44, 66.18, 63.00 (C-6), 58.94 (C-2β), 56.44 (C-2α), 42.71 (C-3,), 25.23 (CH_3 β), 24.94 (CH_3 α); MS: *m/z* (SIMS, Gl/Thgl) = 618.2 (0.4) $[M + 2Li + H]^-$, 617.2 (1.2) $[M + 2Li]^-$, 616.2 (0.3) $[M + 2Li - H]^-$.

Acknowledgements

We thank Prof. M. Frosch for generously providing us with plasmid pMF32.35. We are also indebted to Prof. U. Kragl for a sample of KDN. This work has been supported by the Deutsche Forschungsgemeinschaft (grant SFB 380-B25) and the Fonds der Chemischen Industrie.

References and Notes

[1] (a) A. Varki, *Glycobiology* **1993**, *3*, 97–130; (b) S. Rosenberg (Ed.), *Biology of the Sialic Acids,* Plenum Press, New York, **1995**; (c) G. Reuter, H. J. Gabius, *Biol. Chem. Hoppe Seyler* **1996**, *377*, 325–342.

[2] M. Frosch, C. Weisgerber, T. F. Meyer, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 1669–1673.

[3] D. Bitter-Suermann in *Polysialic Acid*, (Eds.: J. Roth, U. Rutishauser, F. A. Troy), Birkhäuser Verlag, Basel, **1993**, pp. 11–24.

[4] M. Frosch, U. Edwards in *Polysialic Acid*, (Eds.: J. Roth, U. Rutishauser, F. A. Troy), Birkhäuser Verlag, Basel, **1993**, pp. 49–57.

[5] S. Ganguli, G. Zapata, T. Wallis, C. Reid, G. Boulnois, W. F. Vann, I. S. Roberts, *J. Bacteriol.* **1994**, *176*, 4583–4589.

[6] (a) S. Inoue, K. Kitajima, Y. Inoue, *J Biol Chem* **1996**, *271*, 24341–24344; (b) M. Ziak, B. X. Qu, X. L. Zuo, C. Zuber, A. Kanamori, K. Kitajima, S. Inoue, Y. Inuoe, J. Roth, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 2759–2763.

[7] F. M. Unger, *Adv. Carbohydr. Chem. Biochem.* **1981**, *38*, 323–88.

[8] (a) K. N. Timmis, G. J. Boulnois, D. Bitter-Suermann, F. C. Cabello, *Curr. Top. Microbiol. Immunol.* **1985**, *118*, 197–218; (b) R. E. Mandrell, M. A. Apicella, *Immunobiol.* **1993**, *187*, 382–402; (c) A. P. Moran, M. M. Prendergast, B. J. Appelmelk, *FEMS Immunol. Med. Microbiol.* **1996**, *16*, 105–115; (d) D. W. Hood, K. Makepeace, M. E. Deadman, R. F. Rest, P. Thibault, A.

Martin, J. C. Richards, E. R. Moxon, *Mol. Microbiol.* **1999**, *33*, 679–692.
[9] C. H. Wong, R. L. Halcomb, Y. Ichikawa, T. Kajimoto, *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 412–432 and 521–546.
[10] D. Nadano, M. Iwasaki, S. Endo, K. Kitajima, S. Inoue, Y. Inoue, *J. Biol. Chem.* **1986**, *261*, 11550–11557.
[11] Y. T. Li, J. A. Yuziuk, S. C. Li, A. Nematalla, A. Hasegawa, M. Kimura, H. Nakagawa, *Arch. Biochem. Biophys.* **1994**, *310*, 243–246.
[12] T. Angata, T. Matsuda, K. Kitajima, *Glycobiology* **1998**, *8*, 277–284.
[13] (a) K. Okamoto, T. Goto, *Tetrahedron* **1990**, *46*, 5835–5857; (b) M. P. DeNinno, *Synthesis* **1991**, 583–593.
[14] M. Fukuda, M. F. A. Bierhuizen, J. Nakayama, *Glycobiology* **1996**, *6*, 683–689.
[15] R. Öhrlein, *Top. Curr. Chem.* **1998**, *200*, 227–254.
[16] G. Dufner, R. Schworer, B. Muller, R. R. Schmidt, *Eur. J. Org. Chem.* **2000**, 1467–1482.
[17] L. Warren, R. S. Blacklow, *J. Biol. Chem.* **1962**, *237*, 3527–3534.
[18] (a) K. Ikeda, Y. Nagao, K. Achiwa, *Carbohydr. Res.* **1992**, *224*, 123–131; (b) H. Kondo, Y. Ichikawa, C. H. Wong, *J. Am. Chem. Soc.* **1992**, *114*, 8748–8750; (c) S. Makino, Y. Ueno, M. Ishikawa, Y. Hayakawa, T. Hata, *Tetrahedron Lett.* **1993**, *34*, 2775–2778; (d) T. J. Martin, R. R. Schmidt, *Tetrahedron Lett.* **1993**, *34*, 1765–1768; (e) Y. Kajihara, T. Ebata, K. Koseki, H. Kodama, H. Matsushita, H. Hashimoto, *J. Org. Chem.* **1995**, *60*, 5732–5735; (f) M. D. Chappell, R. L. Halcomb, *Tetrahedron* **1997**, *53*, 11109–11120.
[19] M. G. Ambrose, S. J. Freese, M. S. Reinhold, T. G. Warner, W. F. Vann, *Biochemistry* **1992**, *31*, 775–780.
[20] E. L. Kean, *Glycobiology* **1991**, *1*, 441–447.
[21] J. Haverkamp, J. M. Beau, R. Schauer, *Hoppe Seylers Z. Physiol. Chem.* **1979**, *360*, 159–166.
[22] C. Augé, C. Gautheron, *Tetrahedron Lett.* **1988**, *29*, 789–790.
[23] E. S. Simon, M. D. Bednarski, G. M. Whitesides, *J. Am. Chem. Soc.* **1988**, *110*, 7159–7163.
[24] J. Thiem, P. Stangier, *Liebigs Ann. Chem* **1990**, *11*, 1101–1105.
[25] S. L. Shames, E. S. Simon, C. W. Christopher, W. Schmid, G. M. Whitesides, L. L. Yang, *Glycobiology* **1991**, *1*, 187–191.
[26] G. J. Shen, J. L. C. Liu, C. H. Wong, *Biocatalysis* **1992**, *6*, 31–42.
[27] M. Kittelmann, T. Klein, U. Kragl, C. Wandrey, O. Ghisalba, *Appl. Microbiol. Biotechnol.* **1995**, *44*, 59–67.
[28] U. Kragl, T. Klein, D. Vasic-Racki, M. Kittelmann, O. Ghisalba, C. Wandrey, *Ann. New York Acad. Sci.* **1996**, *799*, 577–583.
[29] J. L. C. Liu, G. J. Shen, Y. Ichikawa, J. F. Rutan, G. Zapata, W. F. Vann, C.-H. Wong, *J. Am. Chem. Soc.* **1992**, *114*, 3901–3910.
[30] E. L. Kean, S. Roseman, *J. Biol. Chem.* **1966**, *241*, 5643–5650.
[31] R. Brossmer, U. Rose, D. Kasper, T. L. Smith, H. Grasmuk, F. M. Unger, *Biochem. Biophys. Res. Commun.* **1980**, *96*, 1282–1289.
[32] (a) H. H. Higa, J. C. Paulson, *J. Biol. Chem.* **1985**, *260*, 8838–8849; (b) T. Terada, S. Kitazume, K. Kitajima, S. Inoue, F. Ito, F. A. Troy, Y. Inoue, *J. Biol. Chem.* **1993**, *268*, 2640–2648.
[33] R. F. Haft, M. R. Wessels, M. F. Mebane, N. Conaty, C. E. Rubens, *Mol. Microbiol.* **1996**, *19*, 555–563.
[34] (a) K. G. I. Nilsson, B. M. E. Gudmundsson, *Methods Mol. Cell. Biol.* **1990**, *1*, 195–202; (b) L. B. Rodriguez-Aparicio, J. M. Luengo, C. Gonzalez-Clemente, A. Reglero, *J. Biol. Chem.* **1992**, *267*, 9257–9263; (c) T. Schmelter, S. Ivanov, M. Wember, P. Stangier, J. Thiem, R. Schauer, *Biol. Chem. Hoppe Seyler* **1993**, *374*, 337–342; (d) A. K. Munster, M. Eckhardt, B. Potvin, M. Muhlenhoff, P. Stanley, R. Gerardy-Schahn, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 9140–9145.
[35] W. F. Vann, R. P. Silver, C. Abeijon, K. Chang, W. Aaronson, A. Sutton, C. W. Finn, W. Lindner, M. Kotsatos, *J. Biol. Chem.* **1987**, *262*, 17556–17562.
[36] M. Gilbert, D. C. Watson, W. W. Wakarchuk, *Biotechnol. Lett.* **1997**, *19*, 417–420.
[37] (a) G. Zapata, W. F. Vann, W. Aaronson, M. S. Lewis, M. Moos, *J. Biol. Chem.* **1989**, *264*, 14769–14774; (b) M. V. Tullius, R. S. Munson, J. Wang, B. W. Gibson, *J. Biol. Chem.* **1996**, *271*, 15373–15380; (c) M. Gilbert, R. Bayer, A.-M. Cunningham, S. DeFrees, Y. Gao, D. C. Watson, N. M. Young, W. W. Wakarchuk, *Nat. Biotechnol.* **1998**, *16*, 769–772.
[38] For a preliminary report, see: W.-D. Fessner, M. Knorst, *Ger. Offen*, DE 19913206, **1999**; *Chem. Abstr.* **1999**, *131*, 271018.
[39] (a) J. Brosius, A. Ullrich, M. A. Raker, A. Gray, T. J. Dull, R. R. Gutell, H. F. Noller, *Plasmid* **1981**, *6*, 112–118; (b) J. Brosius, A. Holy, *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 6929–6933.
[40] (a) W.-D. Fessner, G. Sinerius, A. Schneider, M. Dreyer, G. E. Schulz, J. Badia, J. Aguilar, *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 555–558; (b) M. T. Zannetti, C. Walter, M. Knorst, W. D. Fessner, *Chemistry Eur. J.* **1999**, *5*, 1882–1890.
[41] S. C. Mosimann, M. Gilbert, D. Dombrowski, R. To, W. Wakarchuk, N. C. J. Strynadka, *J. Biol. Chem.* **2001**, *276*, 8190–8196.
[42] P. A. Lanzetta, L. J. Alvarez, P. S. Reinach, O. A. Candia, *Anal. Biochem.* **1979**, *100*, 95–97.
[43] The sialic acid synthetase from *Neisseria meningitidis* described in this paper is now commercially available from Calbiochem (#233266). Details for the synthesis of the modified sialic acids employed in this work, using a novel sialic acid synthase, will be published separately: M. Knorst, W.-D. Fessner, manuscript in preparation.
[44] (a) H. J. Gross, R. Brossmer, *Glycoconjugate J.* **1995**, *12*, 739–746; (b) T. Terada, K. Kitajima, S. Inoue, K. Koppert, R. Brossmer, Y. Inoue, *Eur. J. Biochem.* **1996**, *236*, 852–855.
[45] (a) T. Sugai, C.-H. Lin, G.-J. Shen, C.-H. Wong, *Bioorg. Med. Chem.* **1995**, *3*, 313–320; (b) C.-H. Lin, B. W. Murray, I. R. Ollmann, C.-H. Wong, *Biochemistry* **1997**, *36*, 780–785.
[46] J. M. Beau, R. Schauer, J. Haverkamp, J. P. Kamerling,

L. Dorland, J. F. Vliegenthart, *Eur. J. Biochem.* **1984**, *140*, 203–208.
[47] (a) M. D. Chappell, R. L. Halcomb, *J. Am. Chem. Soc.* **1997**, *119*, 3393–3394; (b) A. Lubineau, V. Somme, C. Auge, *J. Mol. Catal. B: Enzym* **1998**, *5*, 235–240.
[48] M. Warwel, W.-D. Fessner, *Synlett* **2000**, 865–867.
[49] K. J. Yarema, L. K. Mahal, R. E. Bruehl, E. C. Rodriguez, C. R. Bertozzi, *J. Biol. Chem.* **1998**, *273*, 31168–31179.
[50] A. K. Datta, J. C. Paulson, *J. Biol. Chem.* **1995**, *270*, 1497–1500.
[51] J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular cloning: A laboratory manual,* 2nd edn., Cold Spring Harbor Laboratory Press, New York, **1989**.
[52] E. S. Simon, M. D. Bednarski, G. M. Whitesides, *Tetrahedron Lett.* **1988**, *29*, 1123–1126.
[53] H. G. Gassen, G. E. Sachse, A. Schulte, *PCR. Grundlagen und Anwendungen der Polymerase-Kettenreaktion*, Georg Fischer, Stuttgart, **1994**.
[54] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248–254.
[55] U. K. Laemmli, *Nature* **1970**, *227*, 680–685.