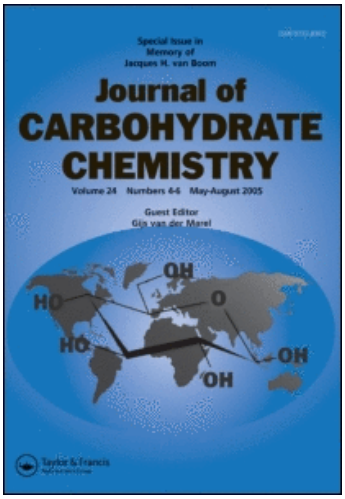

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Cloning, Expression, and Preparative Application of a Mutated, Bifunctional α(2 → 3/8)-Sialyltransferase from *Campylobacter jejuni*

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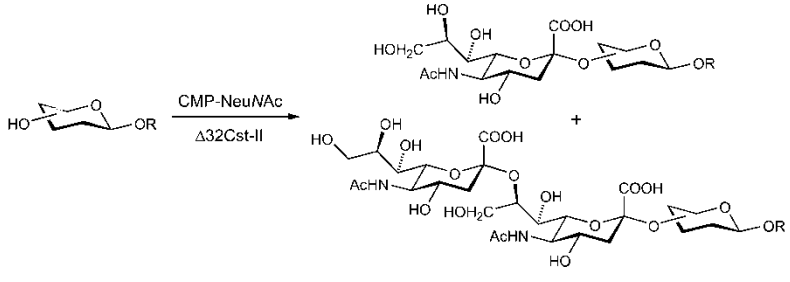
Recombinant, truncated α(2 → 3/8)-sialyltransferase Δ32Cst-II from *Campylobacter jejuni* ATCC43438 and its mutant Δ32Cst-II (S53N177N182) were cloned and overexpressed as His-tagged proteins in *Escherichia coli* on a preparative scale. In order to explore their substrate tolerance as well as their potential for preparative applications, type I [Galβ(1 → 3)Glc*N*Ac (**1a**) and Siaα(2 → 3)Galβ(1 → 3)Glc*N*Ac (**2a**)] and type II substrates [Galβ(1 → 4)Glc*N*Ac (**1b**) and Siaα(2 → 3)Galβ(1 → 4)Glc*N*Ac (**2b**)] were investigated. The wild-type Δ32Cst-II showed α(2 → 8)-activity only with type II substrates, whereas the mutant Δ32Cst-II (S53N177N182) acts as a bifunctional α(2 → 3/8)-sialyltransferase with type I and II acceptor substrates.



Keywords Bifunctional sialyltransferase, *Campylobacter jejuni*, Enzymatic synthesis, Substrate specificity

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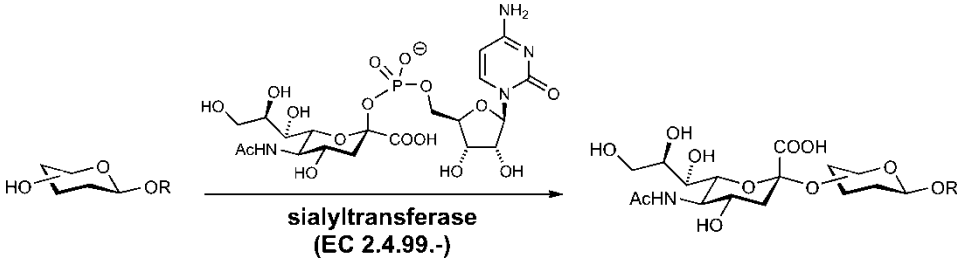
INTRODUCTION

Sialic acid, an essential constituent of glycoproteins and glycolipids, is associated with many important biological functions,[1] and is therefore of considerable therapeutic interest. In order to investigate these functions and to develop novel carbohydrate-based therapeutics, libraries of diversified natural and modified sialooligosaccharides have to be synthesized and screened. Although recent progress facilitates their synthesis, the chemical sialylation reaction still remains difficult because of the lack of a participating group to control the stereochemical outcome and the hindered tertiary anomeric center often leading to unacceptably low chemical yields.[2–5] In particular, the formation of the $\alpha(2 \rightarrow 8)$-linkage between two sialic acid moieties is one of the most difficult reactions in carbohydrate chemistry.[2] A convenient alternative is the use of sialyltransferases (STs) (EC 2.4.99.-),[6,7] which catalyze the α-selective transfer of sialic acid from cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-Neu*N*Ac) to the 3 or 6 position of a terminal galactose residue or to the hydroxyl group in the 8 or 9 position of a terminal sialic acid unit (Sch. 1).

However, the high substrate specificity of mammalian STs limits their application for the synthesis of sialylated oligosaccharide libraries. In addition, the expression of STs in mammalian expression systems is prohibitively expensive. Recently, initial representatives of bacterial STs have been identified.[8–10] They accomplish transformations analogous to mammalian enzymes, and are of high interest because they are available by inexpensive large-scale expression in bacterial systems. Furthermore, initial studies clearly revealed a broader substrate specificity compared to their mammalian counterparts.[9–11]

Only a limited number of microorganisms has been found to express sialylated oligosaccharides on their surface.[12] Among those, only a few are known to be nonpathogenic to vertebrates, such as some *Rhodobacter* species[13] and *(Sino)rhizobium meliloti*.[14] The majority of the microbial strains expressing STs are pathogenic, such as *Neisseria gonorrhoeae*,



Scheme 1: Enzymatic sialylation of carbohydrates using sialyltransferases (STs) and CMP-Neu*N*Ac.

Neisseria meningitides, or *Campylobacter jejuni*. It has been shown that their surface lipooligosaccharides contain sialooligosaccharides identical to the structures found in mammalian glycolipids. This is presumably a form of molecular mimicry enabling the pathogens to evade the host's immune response.[8,10]

Among the *C. jejuni* genes identified to be involved in the synthesis of ganglioside-related lipooligosaccharides,[15] *cst-II*, which codes for a bifunctional α(2 → 3/8)-ST (EC 2.4.99.-), was found to be responsible for the sialylation of type II [Galβ(1 → 4)Glc*N*Ac] and type III [Galβ(1 → 3)Gal*N*Ac] epitopes. The *cst-II* from *C. jejuni* strain OH4384 was isolated, truncated by deleting the predicted membrane association domain, and cloned into the pET21b expression vector.[15–17] This construct provided an overexpressed recombinant-soluble enzyme (Δ32Cst-II) in high yield. Recently, Blixt et al.[18] evaluated the α(2 → 3)- and α(2 → 8)-activities of the *C. jejuni* OH4384 Δ32Cst-II with several galactosides and type II derivatives. The enzyme showed excellent α(2 → 3)-activity on lactosides and *N*-acetyllactosamines. The α(2 → 8)-activity study revealed that α(2 → 3)- as well as α(2 → 6)-sialylated lactose and *N*-acetyllactosamine derivatives were excellent substrates. In addition, the enzyme was used for the preparative synthesis of the glycan moieties of the gangliosides GD3 and GT3 on a gram scale.[18]

Our investigations are focused on the substrate specificity of sialyltransferases in general and on their preparative use. In order to further elucidate the substrate specificity of the *C. jejuni* Cst-II, we investigated its tolerance toward type I [Galβ(1 → 3)Glc*N*Ac] and type II [Galβ(1 → 4)Glc*N*Ac] saccharides. Recombinant, truncated α(2 → 3/8)-ST Δ32Cst-II from *C. jejuni* strain ATCC43438 and a mutated variant were cloned and overexpressed in preparative amounts as His-tagged proteins in *E. coli*[15] and explored for their enzymatic mono- and bis-sialylation capacity of type I and type II saccharides on a preparative scale.

RESULTS AND DISCUSSION

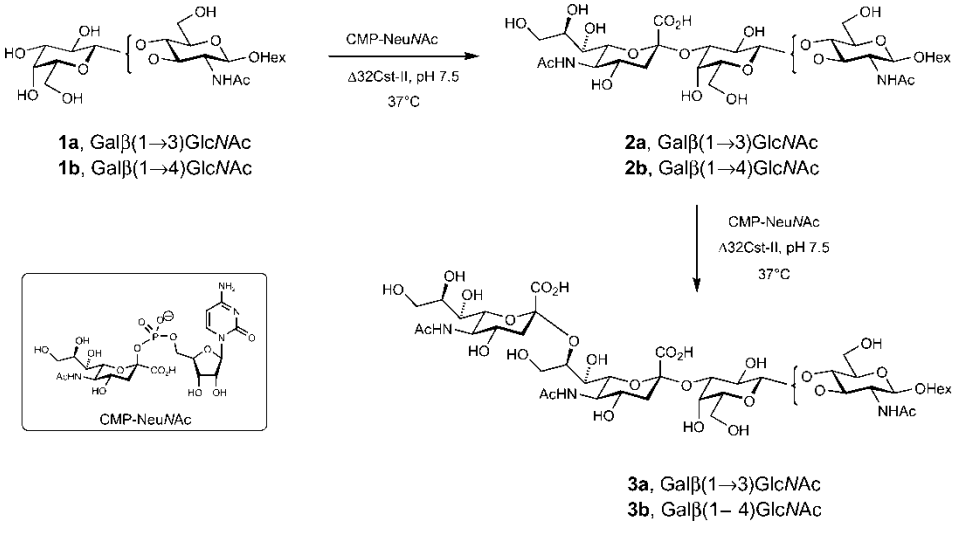
The truncated *cst-II* gene, which lacks 96 nucleotides (32 amino acids of the predicted membrane domain) on the 3′-end, was amplified from the genomic DNA of *C. jejuni* strain ATCC43438. The PCR product was digested with *Nde* I and *Bam*H I and ligated into the pET21b expression vector containing a His_6-tag at the *C*-terminus. For protein expression, the construct was transformed into *E. coli* strain BL21. The cells were induced at 30°C with 1 mM IPTG (isopropyl 1-thio-β-D-galactopyranoside) in LB-medium (Luria Bertani), cultivated for 16 h at the same temperature, collected by centrifugation, and sonicated. The lysate was centrifuged at 4°C and the supernatant loaded on a Ni-NTA FPLC-column. Two washing steps with lysis buffer and

wash buffer were performed before the Δ32Cst-II was eluted with imidazole, and its purity verified by SDS-page and Western blot.

The $\alpha(2 \rightarrow 3)$- and $\alpha(2 \rightarrow 8)$-activity of the Δ32Cst-II and its potential for preparative sialylations were investigated using the disaccharides Gal$\beta(1 \rightarrow 3)$Glcβ-OHex (**1a**, type I) and Gal$\beta(1 \rightarrow 4)$Glcβ-OHex (**1b**, type II), as well as the corresponding mono-sialylated trisaccharides **2a** and **2b**. These acceptor substrates were synthesized by chemical and chemoenzymatic methods in analogy to published procedures.[19–21] The lipophilic *n*-hexyl aglycon was used to facilitate purification by reversed-phase chromatography.[22]

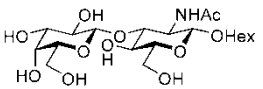
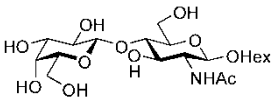
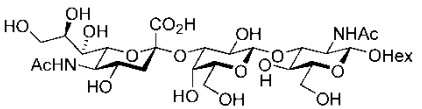
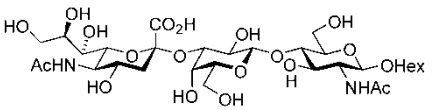
On a preparative scale, acceptors **1** or **2** were incubated with CMP-Neu*N*Ac (2.5 eq for disaccharides **1** and 1.5 eq for trisaccharides **2**) and Δ32Cst-II at 37°C for 3 to 4 days (Sch. 2).[23] Because TLC control after 1 day of incubation indicated incompletion of the sialylation reaction, an additional aliquot of transferase was added. However, when supplementary amounts of Δ32Cst-II and/or CMP-Neu*N*Ac were added at a later time point, the conversion could not be further enhanced. When TLC showed no further improvement in the yield, the mixture was centrifuged, and the supernatant passed through a C_{18} RP column. For the removal of the protein, the nucleoside, and the excess of activated sugar, the column was washed with water. The products were finally eluted with methanol and characterized by ^{1}H and ^{13}C NMR.

The results (Table 1) indicate that both type I and type II disaccharides **1a** and **1b** were accepted by the *C. jejuni* ATCC43438 Δ32Cst-II, but with remarkable differences in specificity. The $\alpha(2 \rightarrow 3)$-activity was significantly higher for



Scheme 2: Enzymatic sialylation of type I and II saccharides using recombinant, bifunctional $\alpha(2 \rightarrow 3/8)$-ST (Δ32Cst-II) from *C. jejuni* ATCC43438.

Table 1: Isolated yields[a] of the enzymatic sialylations of type I and II saccharides with truncated Δ32Cst-II (wild-type and mutant) from *C. jejuni* ATCC43438.

Transferase		Δ32Cst-II (wild-type)			Δ32Cst-II (S53N177N182) (mutant)		
		Isolated yield (% (mg))			Isolated yield (% (mg))		
Entry	**Acceptor**	**1**	**2**	**3**	**1**	**2**	**3**
1	 **1a**	11(1.1)	80(13.0)	—	11 (1.1)	72 (11.7)	15 (3.5)
2	 **1b**	36 (3.6)	45 (7.3)	13 (2.9)	39 (3.9)	42 (6.8)	15 (3.5)
3	 **2a**		98 (9.8)	—		52 (5.2)	48 (6.6)
4	 **2b**		49 (4.9)	48 (6.7)		46 (4.6)	49 (6.8)

[a]Isolated yields after separation by RP-18 chromatography. All reactions were run on a 10-mg scale with 2.5 eq of donor CMP-Neu*N*Ac for disaccharides **1** and 1.5 eq for trisaccharides **2**. Hex = *n*-hexyl.

the type I acceptor **1a** (entry 1) than for the type II acceptor Lac*N*Ac (**1b**, entry 2). For the $\alpha(2 \rightarrow 8)$-activity, a reversed behavior was observed. Whereas the type I sugars **1a** and **2a** (entries 1 & 3) were not $\alpha(2 \rightarrow 8)$-sialylated, the type II substrates **1b** and **2b** (entries 2 & 4) were converted into the corresponding bis-sialylated tetrasaccharide **3b**, although with a much lower rate as described for the *C. jejuni* OH4384 Cst-II.[18]

In 2000, Gilbert et al. isolated the bacterial sialyltransferase *C. jejuni* OH4384 CstII.[15] In 2004, the same authors published the crystal structure of its truncated mutant, OH4384 Δ32Cst-II (Table 2, entry 2). This mutant shows enhanced $\alpha(2 \rightarrow 8)$-specificity with type II and III substrates.[16,17] When cocrystallized with CMP-3-F-Neu*N*Ac, an inert analog of the donor substrate, a detailed view of donor and substrate binding sites was disclosed.[17] A careful analysis suggests that the point mutation of amino acid 53 probably contributes to the stabilization of the enzyme/acceptor substrate complex. In addition, *in silico* analysis of the crystal structure revealed two neutral amino acids, Asn177 and Asn182, at the entrance to the binding site.

The sequences of the bifunctional Δ32Cst-IIs from *C. jejuni* strains OH4384 (Table 2, entry 2) and ATCC43438 (entry 3), the sialyltransferase investigated by us, differ by only eight amino acids (96.9% sequence identity). To improve the $\alpha(2 \rightarrow 8)$-activity of the Δ32Cst-II from strain ATCC43438, the three amino acids positioned in the active site and in its close proximity (Gly53, Asp177, and Arg182) were adjusted by site-directed mutagenesis (Gly53Ser, Asp177Asn, Arg182Asn) (entry 4). Cloning into pET15b (containing a His_6-tag at the *N*-terminus) and then expression in *E. coli* BL21 cells and purification were performed as described for the wild type. The specificity of this enzyme was again evaluated with the acceptor substrates **1** and **2** (see Sch. 2 and Table 1).

This new mutant ATCC43438 Δ32Cst-II (S53N177N182) acts as a bifunctional $\alpha(2 \rightarrow 3/8)$-ST with both type I and type II acceptor substrates. With type I disaccharide **1a** the $\alpha(2 \rightarrow 3)$-activity is similar to the one observed for the wild-type enzyme (Table 1, entry 1). However, the mutant shows additional

Table 2: Relevant differences in the amino acid sequences of Cst-IIs from *C. jejuni* strains OH4384 and ATCC43438.

		Position of amino acid		
Entry	**Transferase Cst-II**	**53**	**177**	**182**
1	OH4384 Cst-II[12,13] (wild type)	Ile	Asn	Asn
2	OH4384 Δ32Cst-II[14] (mutant, truncated)	Ser	Asn	Asn
3	ATCC43438 Δ32Cst-II (wild type, truncated)	Gly	Asp	Arg
4	ATCC43438 Δ32Cst-II (S53N177N182) (mutant, truncated)	Ser	Asn	Asn

α(2 → 8)-activity (Table 1, entries 1 & 3). Surprisingly, with type II substrate **1b** and **2b** (Table 1, entries 2 & 4), no significant change in either the α(2 → 3)- or the α(2 → 8)-activity could be observed.

Interestingly, the conversion rates of the trisaccharides **2a** and **2b** to the bis-sialylated tetrasaccharides **3a** and **3b** did not exceed 50%. Running the reactions with higher amounts of CMP-Neu*N*Ac as well as adding more donor or transferase during the incubation did not influence this outcome. In contrast to findings by Blixt et al.[18] with the *C. jejuni* OH4384 Δ32Cst-II, formation of oligo-sialylated products could not be observed under these conditions.

For a better understanding of these findings various ratios of mono- and bis-sialylated type I tri- and tetrasaccharides **2a** and **3a** were treated with CMP-Neu*N*Ac and mutant Δ32Cst-II (S53N177N182) (Table 3). When the starting material contained 0%, 33% or 50% of tetrasaccharide **3a** (Table 3, entries 1 to 3), the isolated product mixture consisted of a 1 : 1-mixture of tri- and tetrasaccharide. In cases where the portion of bis-sialylated saccharide **3a** in the starting mixture was higher than 50%, no change in the ratio could be observed (Table 3, entries 4 & 5), indicating that the incomplete conversion is not the result of a sialidase activity of the enzyme, but may stem from product inhibition by the tetrasaccharide **3a**.

These results correlate well with data obtained with a metabolically engineered *E. coli* strain overexpressing *C. jejuni* ATCC43438 Cst-II and *N. meningitides* CMP-Neu*N*Ac synthetase (EC 2.7.7.43).[24] This strain allowed the synthesis of the oligosaccharide moiety of the ganglioside GD3 [Neu*N*Acα(2 → 8)Neu*N*Acα(2 → 3)Galβ(1 → 4)Glc] from lactose and sialic acid.[24] It is noteworthy that, similar to the observation in our study, the ATCC43438 Cst-II did not completely convert lactose into the bis-sialylated GD3. Rather, the reaction stopped at a GM3/GD3 ratio of 43 : 57.

In conclusion, we demonstrated that the substrate tolerance of the recombinant, truncated α(2 → 3/8)-sialyltransferase Δ32Cst-II from *C. jejuni*

Table 3: Treatment of different ratios of type I saccharides **2a** and **3a** with mutant Δ32Cst-II (S53N177N182) from *C. jejuni* ATCC43438.

	Ratio of starting materials (%)[a]		Ratio of isolated products (%)[a]	
Entry	Trisaccharide 2a	Tetrasaccharide 3a	Trisaccharide 2a	Tetrasaccharide 3a
1	100	0	52	48
2	67	33	50	50
3	48	52	47	53
4	33	67	33	67
5	0	100	0	100

[a]Isolated yields after separation by RP-18 chromatography. All reactions were run on a 5-mg scale with 1.5 eq of CMP-Neu*N*Ac.

ATCC43438 could be broadened by three point mutations, offering the highly prospective opportunity to engineer transferases with tailor-made specificities. The mutant Δ32Cst-II (S53N177N182) was produced in preparative amounts and acted as a bifunctional $\alpha(2 \rightarrow 3/8)$-ST not only with the physiological type II substrates, but also with type I saccharides. Thus, the preparative applicability of this transferase has been substantially extended.

EXPERIMENTAL

General Methods

NMR spectra were recorded on a Bruker Avance DMX-500 (500 MHz) spectrometer. Assignment of ^{1}H and ^{13}C NMR spectra was achieved using 2D methods (COSY, HSQC, TOCSY). Chemical shifts are expressed in ppm using residual HDO as reference. Optical rotations were measured using a Perkin-Elmer Polarimeter 341. Low-resolution MS analyses were carried out using a Waters ZQ mass spectrometer. The spectra were recorded in positive ESI mode. Reactions were monitored by TLC (DCM/MeOH/H_2O 10 : 4 : 0.8) using glass plates coated with silica gel 60 F_{254} (Merck) and visualized by using UV light and by charring with a molybdate solution (a 0.02 M solution of ammonium cerium sulfate dihydrate and ammonium molybdate tetrahydrate in aqueous 10% H_2SO_4). Column chromatography was performed on RP-18 (Waters cartridges C_{18} Plus).

Production of Sialyltransferase Δ32Cst-II

The *cst-II* gene was amplified from the genomic DNA of *C. jejuni* strain ATCC 43438 using specific primers derived from the DNA sequence (Accession number AAK91725). The PCR product was digested with *Nde* I and *Bam*H I and ligated into pET21b expression vector (Qiagen, Switzerland). The construct containing a His_6-tag at the *C*-terminus was then transformed into *E. coli* strain DH5α, amplified, and verified by restriction control and double-strand DNA sequencing (Microsynth, Switzerland). For protein expression, the construct was transformed into *E. coli* strain BL21 (DE3). A 50 mL of LB medium[25] containing 150 μg/mL ampicillin was inoculated with 1 mL overnight culture and grown at 37°C and 300 rpm. At an OD_{600} of 0.6 to 0.8, the cells were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside, 500 μL of an 100 mM aqueous stock solution) and further cultivated for 16 h at 30°C. The cells were collected by centrifugation and sonicated in lysis buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, and 10 mM imidazole. The lysate was centrifuged for 1 h at 21,000 rpm and 4°C and filtrated (0.22 μm). After loading the supernatant (4 mL) on a Ni-NTA column (10 mL; Qiagen, Switzerland) attached to a

BioLogic FPLC system (BioRad, Switzerland), two washing steps were performed. For the first 10 column volumes of lysis buffer and for the second 10 column volumes of wash buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl and 30 mM imidazole were used. The enzyme was eluted with five column volumes of elution buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, and 500 mM imidazole. The purity of the collected fractions was verified by SDS-PAGE and Western blot, and the samples were stored at 4°C.

The mutant Δ32Cst-II (S53N177N182) was generated by site-directed mutagenesis (Gly53Ser, Asp177Asn, Arg182Asn). Cloning into pET15b (containing a His_6-tag at the *N*-terminus), expression in BL21 (DE3) cells, and purification were performed as described for the wild-type.

Sialyltransferase Enzyme Assay

The reactions were conducted in a total volume of 20 μL containing 50 mM MES (pH 7.5), 0.3% Triton X-100, 10 mM $MgCl_2$, 1 mM CMP-Neu*N*Ac, 60,000 dpm CMP-[^{14}C]-Neu*N*Ac (287 mCi/mmol, Amersham-Pharmacia, Switzerland), 3 μg BSA (Sigma, Switzerland), 1 U of calf intestine alkaline phosphatase (CIAP; Roche, Switzerland), 600 μM type I acceptor substrate **1a**, and 1 μL of the purified transferase. After 1 h incubation at 37°C, the reaction was stopped by the addition of 1 mL water and loaded onto a Sep-Pak cartridge C_{18} Plus (360 mg, 0.7 mL; Waters, Switzerland). The cartridge was washed with bidistilled water (2 × 5 mL) and eluted with methanol (6 mL). The transfer rate of [^{14}C]-Neu*N*Ac onto the acceptor substrate was determined by liquid scintillation counting. The activity of the enzyme was calculated as percentage of radioactivity counted in the methanol fraction.

General Procedure for the Synthesis of Tri- and Tetrasaccharides 2 and 3

Acceptors **1** or **2** (10 mg, 21.4 μmol for **1**, 13.2 μmol for **2**), CMP-Neu*N*Ac (34.9 mg, 53.5 μmol, 2.5 eq for bis-sialylations of **1**; 12.9 mg, 19.8 μmol, 1.5 eq for mono-sialylations of **2**), and BSA (0.9 mg) were dissolved in MES-buffer (50 mM, pH 7.5, 6 mL) containing $MgCl_2$ (10 mM) and Triton X-100 (0.3%). The mixture was briefly vortexed and then incubated at 37°C with CIAP (2 μL, 1000 U/L) and Δ32Cst-II [500 μL; 21.6 U/L for wild-type Δ32Cst-II, 3.5 U/L for mutant Δ32Cst-II (S53N177N182)]. Because TLC control after 1 day indicated incomplete sialylation, an additional aliquot of Δ32Cst-II (200 μL) was added, and the incubation was continued for 2 to 3 days. The turbid solution was centrifuged, and the supernatant was passed over a RP-18 column (1.08 g, 2.1 mL), which was washed with water before the

crude product was eluted with MeOH. After evaporation of the solvent, the residue was purified by chromatography on RP-18 (H_2O/MeOH, gradient 1 : 0 to 1 : 1) to yield sialylated saccharides and starting materials as colorless powders after a final lyophilization from water. For results see Table 1.

Enzymatic Sialylation of Mixtures of Trisaccharide 2a and Tetrasaccharide 3a

Different ratios of **2a** and **3a** (total 6.6 μmol, see Table 3) were dissolved in MES-buffer (50 mM, pH 7.5, 3 mL) containing $MgCl_2$ (10 mM) and Triton X-100 (0.3%). CMP-Neu*N*Ac (6.5 mg, 9.9 μmol) and BSA (0.5 mg) were added. The mixture was briefly vortexed and then incubated at 37°C with CIAP (2 μL) and mutant Δ32Cst-II (S53N177N182) (250 μL, 3.5 U/L). Because TLC control after 1 day indicated incomplete sialylation, an additional aliquot of transferase (100 μL) was added, and the incubation was continued for 2 to 3 days. The turbid solution was centrifuged, and the supernatant was passed over a RP-18 column, which was washed with water before the crude product was eluted with MeOH. After evaporation of the solvent, the residue was chromatographed on RP-18 (H_2O/MeOH, gradient 1 : 0 to 1 : 1) to yield saccharides **2a** and **3a** as colorless powders after a final lyophilization from water. For results see Table 3.

Analytical Data

Hexyl (5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2 → 3)-β-D-galactopyranosyl-(1 → 3)-2-acetamido-2-deoxy-β-D-glucopyranoside (2a). $[\alpha]_D$ −12.3 ($c = 0.50$, H_2O); ^1H NMR (500 MHz, D_2O): δ 0.79 (t, $J = 7.0$ Hz, 3H, Hexyl-H6), 1.12–1.24 (m, 6H, Hexyl-H3, H4, H5), 1.47 (m, 2H, Hexyl-H2), 1.83 (t, $J = 12.1$ Hz, 1H, Sia-H3a), 2.02, 2.06 (2s, 6H, $2COCH_3$), 2.77 (dd, $J = 4.2$, 12.1 Hz, 1H, Sia-H3b), 3.33 (m, 1H, Glc*N*Ac-H5), 3.45 (m, 1H, Glc*N*Ac-H4), 3.50–3.58 (m, 4H, Gal-H2, Gal-H5, Sia-H6, Sia-H7), 3.62–3.66 (m, 3H, Gal-H6a, Sia-H9a, Hexyl-H1a), 3.70–3.76 (m, 4H, Glc*N*Ac-H6a, Gal-H6b, Sia-H4, Sia-H5), 3.81–3.90 (m, 6H, Glc*N*Ac-H2, Glc*N*Ac-H3, Glc*N*Ac-H6b, Sia-H8, Sia-H9b, Hexyl-H1b), 3.95 (d, $J = 3.3$ Hz, 1H, Gal-H4), 3.97 (dd, $J = 3.5$, 9.5 Hz, 1H, Gal-H3), 4.42 (d, $J = 7.4$ Hz, 1H, Gal-H1), 4.49 (d, $J = 7.9$ Hz, 1H, Glc*N*Ac-H1); ^{13}C NMR (125 MHz, D_2O): δ 13.4Hexyl-C6), 21.9 (Hexyl-C5), 22.4, 22.8 ($2COCH_3$), 25.9, 29.3, 31.8 (Hexyl-C2, C3, C4), 40.8 (Sia-C3), 53.5 (Sia-C5), 55.9 (Glc*N*Ac-C2), 62.6 (Glc*N*Ac-C6), 62.8 (Gal-C6), 64.2 (Sia-C9), 68.8 (Hexyl-C1), 69.3 (Sia-C4), 69.4 (Gal-C4), 69.8 (Sia-C7), 70.4 (Gal-C2), 70.7 (Glc*N*Ac-C4), 72.7 (Sia-C8), 74.5 (Sia-C6), 76.9 (Gal-C5), 77.2 (Gal-C3), 77.5 (Glc*N*Ac-C5), 83.7 (Glc*N*Ac-C3), 101.0 (Sia-C2), 101.8 (Glc*N*Ac-C1), 104.3

(Gal-C1), 174.9, 175.7, 175.8 (3CO); ESI-MS Calcd for $C_{31}H_{55}N_2O_{19}$ $[M + H]^+$: 759.3. Found: 759.4.

Hexyl (5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2 → 3)-β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside (2b). $[\alpha]_D$ −15.9 ($c = 0.50$, H_2O); ^1H NMR (500 MHz, D_2O): δ 0.78 (t, $J = 7.0$ Hz, 3H, Hexyl-H6), 1.13–1.26 (m, 6H, Hexyl-H3, H4, H5), 1.45 (m, 2H, Hexyl-H2), 1.81 (t, $J = 12.0$ Hz, 1H, Sia-H3a), 2.02, 2.05 (2s, 6H, $2COCH_3$), 2.75 (dd, $J = 4.6$, 12.3 Hz, 1H, Sia-H3b), 3.55 (m, 1H, Gal-H2), 3.59–3.61 (m, 2H, Glc*N*Ac-H5, Sia-H7), 3.62–3.66 (m, 2H, Sia-H6, Sia-H9a), 3.68 (m, 1H, Sia-H4), 3.70–3.74 (m, 5H, Glc*N*Ac-H4, Gal-H5, Gal-H6, Hexyl-H1a), 3.78 (m, 1H, Glc*N*Ac-H2), 3.83–3.88 (m, 4H, Glc*N*Ac-H3, Glc*N*Ac-H6a, Sia-H5, Sia-H9b), 3.92 (m, 1H, Sia-H8), 3.96 (d, $J = 3.1$ Hz, 1H, Gal-H4), 3.99–4.02 (m, 2H, Glc*N*Ac-H6b, Hexyl-H1b), 4.11 (dd, $J = 3.1$, 9.9 Hz, 1H, Gal-H3), 4.55 (d, $J = 7.8$ Hz, 1H, Gal-H1), 4.60 (d, $J = 8.3$ Hz, 1H, Glc*N*Ac-H1); ^{13}C NMR (125 MHz, D_2O): δ 13.2Hexyl-C6), 21.8 (Hexyl-C5), 22.5, 22.7 ($2COCH_3$), 25.7, 29.5, 31.6 (Hexyl-C2, C3, C4), 40.4 (Sia-C3), 52.4 (Sia-C5), 55.8 (Glc*N*Ac-C2), 60.8 (Glc*N*Ac-C6), 61.8 (Gal-C6), 63.3 (Sia-C9), 68.2 (Gal-C4), 68.6 (Hexyl-C1), 68.8 (Sia-C7), 69.1 (Sia-C4), 70.1 (Gal-C2), 72.5 (Sia-C8), 73.2 (Glc*N*Ac-C3), 73.6 (Sia-C6), 75.5 (Glc*N*Ac-C5), 75.9 (Gal-C5), 76.2 (Gal-C3), 79.0 (Glc*N*Ac-C4), 100.6 (Sia-C2), 100.8 (Glc*N*Ac-C1), 103.3 (Gal-C1), 174.8, 175.5, 175.9 (3CO); ESI-MS Calcd for $C_{31}H_{55}N_2O_{19}$ $[M + H]^+$: 759.3. Found: 759.5.

Hexyl (5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2 → 8)-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2 → 3)-β-D-galactopyranosyl-(1 → 3)-2-acetamido-2-deoxy-β-D-glucopyranoside (3a). $[\alpha]_D$ −8.1 ($c = 0.25$, H_2O); ^1H NMR (500 MHz, D_2O): δ 0.78 (t, $J = 7.0$ Hz, 3H, Hexyl-H6), 1.10–1.25 (m, 6H, Hexyl-H3, H4, H5), 1.45 (m, 2H, Hexyl-H2), 1.75 (t, $J = 12.0$ Hz, 1H, Sia′-H3a), 1.78 (t, $J = 12.2$ Hz, 1H, Sia-H3a), 2.02, 2.03, 2.07 (3s, 9H, $3COCH_3$), 2.67 (dd, $J = 4.1$, 12.2 Hz, 1H, Sia-H3b), 2.76 (dd, $J = 4.0$, 12.0 Hz, 1H, Sia′-H3b), 3.34 (m, 1H, Glc*N*Ac-H5), 3.44 (m, 1H, Glc*N*Ac-H4), 3.50–3.61 (m, 5H, Gal-H2, Gal-H5, Sia-H4, Sia′-H6, Sia′-H7), 3.63–3.66 (m, 5H, Gal-H6a, Sia-H6, Sia′-H4, Sia′-H9a, Hexyl-H1a), 3.70–3.76 (m, 3H, Glc*N*Ac-H6a, Gal-H6b, Sia-H9a), 3.81–3.91 (m, 9H, Glc*N*Ac-H2, Glc*N*Ac-H3, Glc*N*Ac-H6b, Sia-H5, Sia-H7, Sia′-H5, Sia′-H8, Sia′-H9b, Hexyl-H1b), 3.98 (d, $J = 3.3$ Hz, 1H, Gal-H4), 4.05 (dd, $J = 3.4$, 9.8 Hz, 1H, Gal-H3), 4.15 (m, 1H, Sia-H8), 4.18 (m, 1H, Sia-H9b), 4.46 (d, $J = 7.5$ Hz, 1H, Gal-H1), 4.49 (d, $J = 8.0$ Hz, 1H, Glc*N*Ac-H1); ^{13}C NMR (125 MHz, D_2O): δ 13.5Hexyl-C6), 21.7 (Hexyl-C5), 22.2, 23.0, 23.1 ($3COCH_3$), 26.0, 29.1, 31.8 (Hexyl-C2, C3, C4), 40.4 (Sia-C3), 41.3 (Sia′-C3), 53.1 (2C, Sia-C5, Sia′-C5), 55.7 (Glc*N*Ac-C2), 62.4 (Sia-C9), 62.6 (Glc*N*Ac-C6), 62.9 (Gal-C6), 63.4 (Sia′-C9), 68.7 (Hexyl-C1),

69.0 (2C, Sia-C4, Sia′-C7), 69.2 (Gal-C4), 69.5 (Sia′-C4), 70.1 (Sia-C7), 70.3 (Gal-C2), 70.7 (Glc*N*Ac-C4), 72.8 (Sia′-C8), 73.6 (Sia′-C6), 74.8 (Sia-C6), 76.3 (Gal-C5), 76.9 (Gal-C3), 77.3 (Glc*N*Ac-C5), 78.9 (Sia-C8), 83.6 (Glc*N*Ac-C3), 100.5, 100.9 (Sia-C2, Sia′-C2), 101.9 (Glc*N*Ac-C1), 103.6 (Gal-C1), 174.7, 175.7, 175.7, 175.9 (5C, 5CO); ESI-MS Calcd for $C_{42}H_{72}N_3O_{27}$ $[M+H]^+$: 1050.4; Found: 1050.4.

Hexyl (5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2 → 8)-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2 → 3)-β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside (3b). $[\alpha]_D$ − 9.5 ($c = 0.25$, H_2O); ^1H NMR (500 MHz, D_2O): δ 0.77 (t, $J = 7.1$ Hz, 3H, Hexyl-H6), 1.12–1.23 (m, 6H, Hexyl-H3, H4, H5), 1.44 (m, 2H, Hexyl-H2), 1.77 (m, 2H, Sia-H3a, Sia′-H3a), 2.02, 2.05, 2.07 (3s, 9H, $3COCH_3$), 2.65 (dd, $J = 4.7$, 12.2 Hz, 1H, Sia-H3b), 2.78 (dd, $J = 4.5$, 12.0 Hz, 1H, Sia′-H3b), 3.54 (m, 1H, Gal-H2), 3.58–3.61 (m, 4H, Glc*N*Ac-H5, Sia-H4, Sia′-H6, Sia′-H7), 3.64–3.67 (m, 3H, Sia-H6, Sia′-H4, Sia′-H9a), 3.70–3.75 (m, 5H, Glc*N*Ac-H4, Gal-H5, Gal-H6, Sia-H9a, Hexyl-H1a), 3.79 (m, 1H, Glc*N*Ac-H2), 3.82–3.88 (m, 6H, Glc*N*Ac-H3, Glc*N*Ac-H6a, Sia-H5, Sia-H7, Sia′-H5, Sia′-H9b), 3.92 (m, 1H, Sia′-H8), 3.95 (d, $J = 3.2$ Hz, 1H, Gal-H4), 3.99–4.02 (m, 2H, Glc*N*Ac-H6b, Hexyl-H1b), 4.11 (dd, $J = 3.2$, 9.8 Hz, 1H, Gal-H3), 4.13 (m, 1H, Sia-H8), 4.19 (m, 1H, Sia-H9b), 4.57 (d, $J = 7.9$ Hz, 1H, Gal-H1), 4.61 (d, $J = 8.2$ Hz, 1H, Glc*N*Ac-H1); ^{13}C NMR (125 MHz, D_2O): δ 13.3Hexyl-C6), 21.8 (Hexyl-C5), 22.1, 22.8, 23.0 ($3COCH_3$), 25.5, 29.4, 31.6 (Hexyl-C2, C3, C4), 40.2 (Sia-C3), 41.4 (Sia′-C3), 53.0, 53.1 (Sia-C5, Sia′-C5), 55.7 (Glc*N*Ac-C2), 61.0 (Glc*N*Ac-C6), 61.9 (Gal-C6), 62.2 (Sia-C9), 63.5 (Sia′-C9), 68.4 (Gal-C4), 68.7 (Hexyl-C1), 68.9 (2C, Sia-C4, Sia′-C7), 69.6 (Sia′-C4), 70.1 (2C, Gal-C2, Sia-C7), 72.6 (Sia′-C8), 73.3 (Glc*N*Ac-C3), 73.7 (Sia′-C6), 74.8 (Sia-C6), 75.8 (Glc*N*Ac-C5), 76.0 (Gal-C5), 76.2 (Gal-C3), 78.7 (Sia-C8), 79.1 (Glc*N*Ac-C4), 100.3, 100.7 (Sia-C2, Sia′-C2), 100.9 (Glc*N*Ac-C1), 103.3 (Gal-C1), 174.5, 175.5, 175.6, 175.9 (5C, 5CO); ESI-MS Calcd for $C_{42}H_{72}N_3O_{27}$ $[M+H]^+$: 1050.4. Found: 1050.6.

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