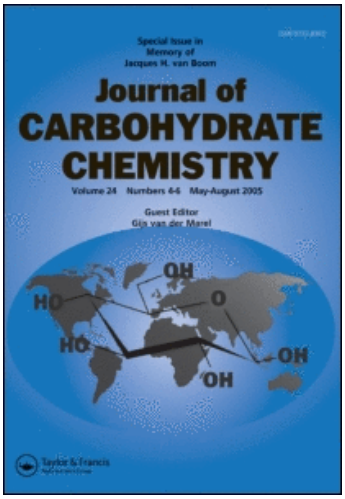

Journal of Carbohydrate Chemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

An α2,3-Sialyltransferase from *Photobacterium* sp. JT-ISH-224 Transfers *N*-Acetylneuraminic Acid to Both the O-2 and O-3' Hydroxyl Groups of Lactose

Toshiki Mine[a]; Hitomi Kajiwara[a]; Takefumi Murase[b]; Yasuhiro Kajihara[c]; Takeshi Yamamoto[a]
[a] Glycotechnology Business Unit, Japan Tobacco Inc., Iwata, Shizuoka, Japan [b] International Graduate School of Arts and Sciences, Yokohama City University, Yokohama, Kanagawa, Japan [c] Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka, Japan

Online publication date: 30 March 2010

To cite this Article Mine, Toshiki , Kajiwara, Hitomi , Murase, Takefumi , Kajihara, Yasuhiro and Yamamoto, Takeshi(2010) 'An α2,3-Sialyltransferase from *Photobacterium* sp. JT-ISH-224 Transfers *N*-Acetylneuraminic Acid to Both the O-2 and O-3' Hydroxyl Groups of Lactose', Journal of Carbohydrate Chemistry, 29: 2, 51 — 60
To link to this Article: DOI: 10.1080/07328300903586422
URL: http://dx.doi.org/10.1080/07328300903586422

Journal of Carbohydrate Chemistry, 29:51–60, 2010

ISSN: 0732-8303 print / 1532-2327 online
DOI: 10.1080/07328300903586422

An α2,3-Sialyltransferase from *Photobacterium* sp. JT-ISH-224 Transfers *N*-Acetylneuraminic Acid to Both the O-2 and O-3′ Hydroxyl Groups of Lactose

Toshiki Mine,[1] Hitomi Kajiwara,[1] Takefumi Murase,[2] Yasuhiro Kajihara,[3] and Takeshi Yamamoto[1]

[1]Glycotechnology Business Unit, Japan Tobacco Inc., 700 Higashibara, Iwata, Shizuoka 438-0802, Japan
[2]International Graduate School of Arts and Sciences, Yokohama City University, 22-2 Seto, Kanazawaku, Yokohama, Kanagawa 236-0027, Japan
[3]Department of Chemistry, Graduate School of Science, Osaka University, 1-1 Machikaneyamacho, Toyonaka, Osaka 560-0043, Japan

We found that α2,3-sialyltransferase from *Photobacterium* sp. JT-ISH-224 produced a regio-mistaken sialyl-transferred by-product. Spectroscopic analysis of the purified by-product indicated that it contained two *N*-acetylneuraminic acids: one attached to the O-3′ hydroxyl group of lactose, and the other attached to the O-2 hydroxyl group of lactose. The relative configuration between the C-1 and C-3 of the α-glucopyranose residue is superimposable with that between C-4 and C-2 of galactopyranoside. Therefore, formation of this by-product, designated 2,3′-disialyllactose, was simply rationalized as a regio-mistaken reaction of bacterial α2,3-sialyltransferase. This finding indicates that this bacterial α2,3-sialyltransferase has a possibility to synthesize several unusual sialosides.

Keywords 2,3′-Disialyllactose; Regio-mistaken reaction; Sialyltransferase

Abbreviations

CMP-Neu5Ac, cytidine-5′-monophospho-*N*-acetylneuraminic acid; COSY, correlation spectroscopy; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylgluco-

Received November 9, 2009; accepted December 30, 2009.
Address correspondence to Toshiki Mine, Glycotechnology Business Unit, Japan Tobacco Inc., 700 Higashibara, Iwata, Shizuoka 438-0802, Japan. E-mail: toshiki.mine@jt.com; or Takeshi Yamamoto, Glycotechnology Business Unit, Japan Tobacco Inc., 700 Higashibara, Iwata, Shizuoka 438-0802, Japan. E-mail: takeshi.yamamoto@jt.com

samine; HMBC, hetero-nuclear multiple quantum coherence spectrum; HOHAHA, homo-nuclear hartmann-harn spectrum; HPLC, high-performance liquid chromatography; HSQC, hetero-nuclear single quantum coherence spectrum; Neu5Ac, *N*-acetylneuraminic acid; NMR, nuclear magnetic resonance; TLC, thin layer chromatography; UDP, uridine diphosphate.

INTRODUCTION

Sialic acids are an important component of carbohydrate chains and are usually linked to the terminal positions of the carbohydrate moiety of glycoconjugates such as glycoproteins and glycolipids.[1,2] Many reports have indicated that sialylglycoconjugates play significant roles in many biological processes, including immunological responses, viral infections, cell–cell recognition, and inflammation.[3,4]

Sialylglycoconjugates are formed *in vivo* by specific sialyltransferases. All sialyltransferases use cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) as the common donor substrate. Sialyltransferases have been cloned from various sources, including mammalian organs, bacteria, and viruses.[5,6] It has been reported that sialyltransferases of bacterial origin have acceptor substrate specificity that is much broader than that of their mammalian counterparts.[6,7] Furthermore, several methods for the mass production of bacterial sialyltransferases have been reported.[8,9] From these points, it is thought that bacterial enzymes will be a valuable tool for the preparation of many kinds of sialosides.[7,10] As described earlier, the sialylated carbohydrate chains of glycoconjugates play significant roles in many biological processes. Thus, preparation of sialosides is indispensable to investigate their function in detail.

The crystal structures of six sialyltransferases have been reported so far.[11–16] These sialyltransferases are all of bacterial origin. The first structure described was that of the bifunctional enzyme α2,3-/α2,8-sialyltransferase (CstII) from *Campylobacter jejuni* OH4384 in complex with a substrate analog.[11] The second was that of the multifunctional enzyme α2,3-sialyltransferase (Δ24PmST1) from *Pasteurella multocida* strain P-1059 in the presence and in the absence of CMP.[12] This enzyme shows α2,3-sialyltransferase activity, α2,6-sialyltransferase activity, sialidase activity, and *trans*-sialidase activity.[17] The third structure described was that of monofunctional α2,3-sialyltransferase (CstI) from *C. jejuni* in apo form and substrate analog-bound form.[13] The fourth was that of monofunctional α2,6-sialyltransferase (Δ16pspST6) from a *Photobacterium* sp. in complex with CMP as a donor product and lactose as the acceptor substrate.[14] The fifth was that of α2,3-sialyltransferase from *P. multocida* in complex with CMP-3FNeuAc and lactose.[15] The sixth was that of α2,3-sialyltransferase (ΔNpp23ST) from *Photobacterium phosphoreum* in complex with CMP.[16]

Those crystal studies also discussed the substrate binding sites and the catalytic mechanism of the enzymes.

Previously, we reported the cloning α2,3-sialyltransferase from *Photobacterium* sp. JT-ISH-224 and the characterization of an N-terminus truncated enzyme.[10] Hereafter we designate this truncated enzyme as Δ23pspST3. While using Δ23pspST3 to produce 3′-sialyllactose, we found a by-product in the enzymatic reaction mixture. In this by-product, sialic acid was incorporated at an unusual position through regio-mistaken transfer. In addition to the knowledge provided by x-ray analysis data, this regio-mistaken transfer provides insight into the mode of binding with acceptors in bacterial sialyltransferase. We report herein on the structure of the by-product. The results of this study open the possibility of using this marine bacterial α2,3-sialyltransferase to produce sialosides of unusual structure.

RESULTS AND DISCUSSION

During the course of purifying 3′-sialyllactose, we found a by-product in the enzymatic reaction mixture. Analysis by HPLC revealed that retention time of the by-product was different from that of 3′-sialyllactose (by-product, 29.5 minutes; 3′-sialyllactose, 21.8 minutes). Furthermore, analysis by thin layer chromatography (TLC) suggested that the by-product was a disialylated saccharide. Each compound was then purified further by size-exclusion column chromatography; 140.3 mg of 3′-sialyllactose and 9.3 mg of by-product were isolated. The yield of 3′-sialyllactose and the by-product based on the donor substrate (353 μmol) was 62.1% and 5.6%, respectively. After this purification, we analyzed the by-product by NMR and mass spectrometry to determine its structure. The mass spectrum of the by-product showed an $(M\text{-}H)^+$ ion peak at 923.5. Because the expected mass of lactose having two *N*-acetylneuraminic acids in its structure is 924.3, this result strongly indicated that the by-product possessed two *N*-acetylneuraminic acids. Next, we analyzed the by-product by ^{1}H-NMR spectroscopy. Interestingly, in the ^{1}H-NMR spectrum of this by-product (Fig 1) we observed no glucose residue β-anomer signals (for example, δ 3.2 ppm for Glc β H-2, δ 4.6 ppm for Glc β H-1, and so on, as were observed for 3′,6′-disialyllactose; Kajihara et al.[18]); furthermore, the H-2 chemical shift of Glc was shifted downfield by 0.3 ppm compared to that of 3′-sialyllactose, indicating that this by-product consisted only of the α-form. The ^{1}H and ^{13}C chemical shifts obtained from HSQC, HMBC, HOHAHA, and COSY spectra are summarized in Table 1. The sialylated position was identified from the differences in the ^{13}C chemical shift values observed between the by-product and 3′,6′-sialyllactose.[18] The C-2 chemical shift of the glucose of the by-product was downfield, and we observed a correlation between the C-2 of Neu5Ac and the H-2 of Glc, and a correlation between the C-2 of Neu5Ac and the H-3 of

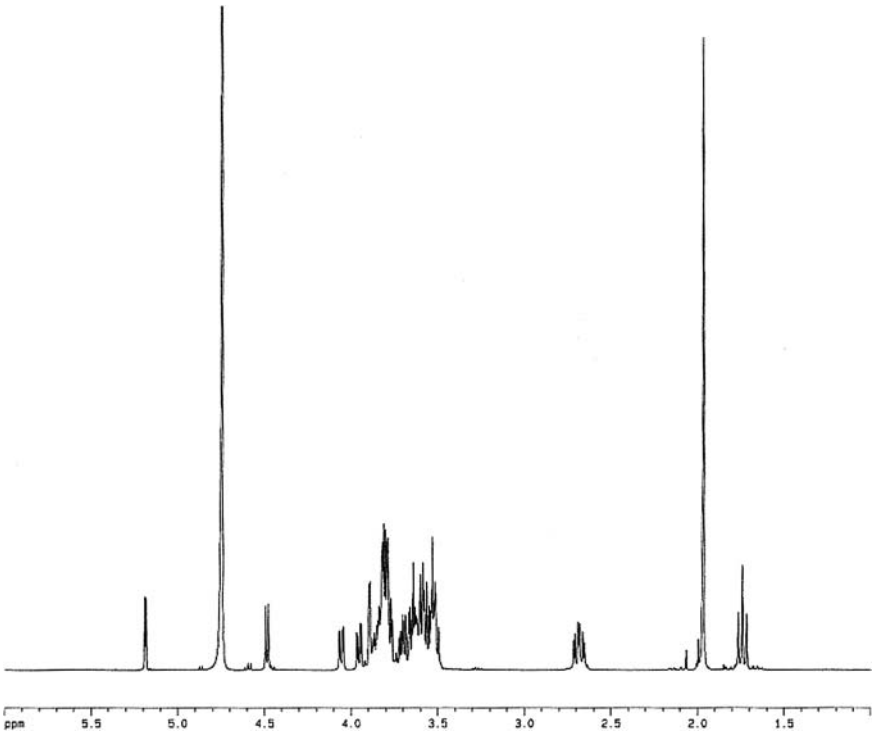

Figure 1: ^{1}H-NMR spectrum of the purified by-product. The analysis conditions are described in Materials and Methods. The chemical shift is expressed relative to internal HOD (4.75 ppm).

Gal (Fig 2). From these results, it was clear that a Neu5Ac was attached to the O-3′ hydroxyl group and that another Neu5Ac was attached to the O-2 hydroxyl group of lactose. The structure of the by-product, designated 2,3′-disialyllactose, is shown in Figure 3.

Although ours is the first report regarding a regio-mistaken reaction by sialyltransferase, a similar type of mistaken glycosyltransferase reaction was reported by Nishida et al. for galactosyltransferase.[19] Nishida et al. reported that although galactosyltransferase prepared from bovine milk usually transfers the galactose residue of UDP-galactose to the OH-4 positions of glucose and *N*-acetylglucosamine (GlcNAc), the galactosyltransferase also catalyzed the transfer of βGal from UDP-galactose to the anomeric position of *N*-acetylkanosamine (Glc3NAc). In this case, the stereochemistry of the β-anomer of Glc3NAc from C-1 to C-4 is identical to that of C-4 to C-1 of GlcNAc. Thus, galactosyltransferase recognizes Glc3NAc as an acceptor substrate and transfers galactose to the β-anomeric position of the Glc3NAc, which corresponds to C-4 of GlcNAc.[19] With lactose, which was the acceptor substrate used in our current study, the stereochemistry of α-glucose from C-1 to C-3 is superimposable with that of C-4 to C-2 of galactose. This fact strongly indicates

Table 1: Assignments of ^{1}H and ^{13}C chemical shift and coupling constant (*J*) of the purified by-product

	Neu5Ac			Gal			Glc		
	^{1}H	*J* (Hz)	^{13}C	^{1}H	*J* (Hz)	^{13}C	^{1}H	*J* (Hz)	^{13}C
1			176.3	4.49	d, 7.85	104.9	5.19 (α)	d, 3.53	93.1
2			102.5	3.52		71.8	3.95 (α)	dd, 9.82, 3.53	75.9
2			102.2						
3ax	1.75	dd, 12.63	41.7	4.06	dd, 9.90, 3.06	77.9	3.79 (α)		72.1
3ax			42.1						
3eq	2.67	dd, 12.63, 4.65	41.7						
3eq	2.70	dd, 12.49, 4.54	42.1						
4	3.62		71.0	3.90		69.9	3.69 (α)		80.1
4			70.8						
5	3.79		54.1	3.65		77.6	3.89 (α)		72.4
6	3.56		75.3	3.72		63.5	3.82 (α)		62.4
6			75.2	3.65					
7	3.53		70.6						
7			70.5						
8	3.85		74.2						
8	3.82		74.2						
9	3.58		65.0						

The chemical shifts of ^{1}H and ^{13}C NMR are expressed relative to internal HOD = 4.75 ppm (298 K) and external 1,4-dioxane = 69.1 ppm (298 K), respectively.

that the marine bacterial α2,3-sialyltransferase recognized the C-1 to C-3 part of the α-glucose as an acceptor substrate site and transferred Neu5Ac from CMP-Neu5Ac to the O-2 hydroxyl group of α-glucose. In the case of β-glucose, the configuration of the hydroxyl groups from C-1 to C-3 is not superimposable with that of C-4 to C-2 of galactose.

Although the reason why the by-product is fixed to α-anomer is still unclear, we speculate that after transferring Neu5Ac to the O-2 hydroxyl group of α-glucose, the hydrogen bond between the carboxyl group of Neu5Ac and the O-1 hydroxyl group of α-glucose might immediately form and stabilize the molecule.

Δ23pspST3 used in this study was a truncated recombinant enzyme cloned from *Photobacterium* sp. JT-ISH-224. This enzyme was classified according to amino acid sequence similarities into Glycosyltransferase Family 80 (GT80) in the CAZy database.[20] Of the six sialyltransferases whose crystal structures have been determined so far, four are classified into GT80. These four are the multifunctional α2,3-sialyltransferase (Δ24PmST1) from *P. multocida*

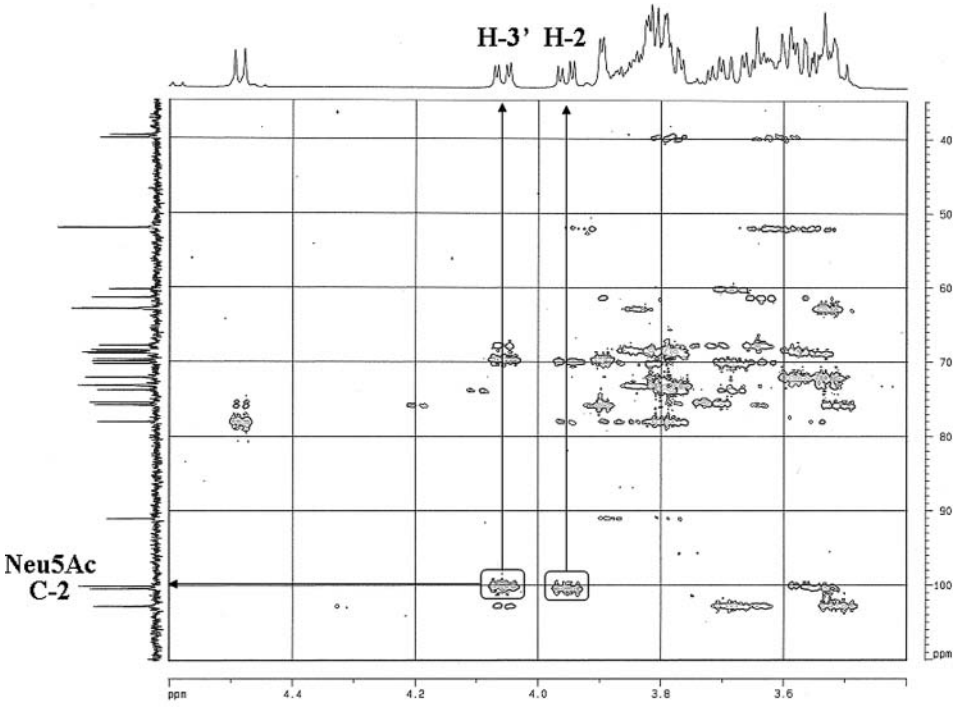

Figure 2: HMBC spectrum of the purified by-product. The analysis conditions are described in Materials and Methods.

strain P-1059,[12] the α2,6-sialyltransferase (Δ16pspST6) from a *Photobacterium* sp.,[14] the α2,3-sialyltransferase from *P. multocida,*[15] and the α2,3-sialyltransferase (ΔNpp23ST3) from *P. phosphoreum.*[16] Although the crystal structure of the α2,3-sialyltransferase cloned from *Photobacterium* sp. JT-ISH-224 has not yet been solved, the ternary structure of the enzyme seems to closely resemble that of Δ24PmST1 owing to their close similarities in terms of amino acid sequence (29% identity). In the crystal structure of Δ24PmST1 in complex with CMP and lactose, the lactose was recognized by five amino acid residues, Trp270, Asp141, His112, Asn85, and Met144.[21] Each of those residues contacted only the galactose part of the lactose. However,

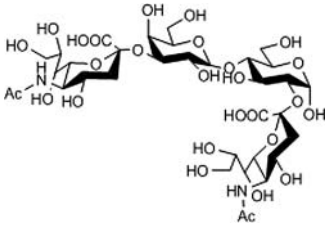

Figure 3: Structure of the by-product.

in Δ23pspST3, only one of those amino acid residues, Asp141, is conserved (as Asp125). Pair-wise alignment Δ24PmST1 and Δ23pspST3 show that the amino acid residues corresponding to the Trp270, His112, Asn85, and Met144 of Δ24PmST1 are all substituted or deleted in Δ23pspST3. We are presently conducting a number of studies, including a mutational study and a structural study of the enzyme, to clarify the mechanisms of acceptor substrate recognition and binding of this enzyme.

The results obtained in this study indicate the possibility for production of several sialosides having unique structures. Although the acceptor substrate binding mechanism of this enzyme is still unclear, compounds having a stereochemical structure similar to that of α-glucose from the C-1 to C-3 part are likely to be candidate acceptor substrates for this enzyme. We are currently investigating the possibility of synthesizing unique sialosides by means of this bacterial sialyltransferase.

MATERIALS AND METHODS

Materials

CMP-Neu5Ac was purchased from Japan Food and Liquor Alliance (Kyoto, Japan); lactose was from Wako Pure Chemical Industries (Osaka, Japan); AG 1-X2 was from Bio-Rad Laboratories (Hercules, CA, USA); Sephadex G-15 was from GE Healthcare Bioscience (Tokyo, Japan); TLC plates were from Merck (Darmstadt, Germany). All other reagents were of commercially available analytical grade.

Preparation of recombinant α2,3-sialyltransferase from *Photobacterium* sp. JT-ISH-224

The recombinant α2,3-sialyltransferase used in this study was prepared using the methods reported by Tsukamoto et al.[22] One unit (U) of sialyltransferase was defined as the amount of enzyme required to transfer 1 μmol of Neu5Ac from CMP-Neu5Ac to lactose in 1 min.

Enzymatic Synthesis of Sialyloligosaccharides

The enzymatic synthesis was performed using purified recombinant α2,3-sialyltransferase. The reaction mixture was composed of 100 mg of lactose (280 μmol), 250 mg of CMP-Neu5Ac (353 μmol), and 5.05 U of purified α2,3-sialyltransferase from *Photobacterium* sp. JT-ISH-224 in 0.5 mL of 100 mM Bis-Tris buffer (pH 6.0), and was incubated at 30°C for 2 h.

Purification of Sialyloligosaccharides

The enzymatic reaction products, including the main product and the by-product, were purified according to the method of Sabesan and Paulson[23] with a slight modification. After the reaction, the reaction mixture was diluted with 10 mL of deionized water and introduced into an Econo column (1.0 cm × 10 cm, Bio-Rad Laboratories) containing AG 1-X2 ion-exchange resin (phosphate form, 200–400 mesh). The column was washed with deionized water, and then the product was eluted twice with 10 mL each of 5, 10, 50, 100, 500, or 1000 mM potassium phosphate buffer (pH 6.8). An aliquot of each eluted fraction was analyzed by TLC, as described later, and the fractions containing glycosidic Neu5Ac were evaporated to dry residues. Each of the dry residues was dissolved in 2.5 mL of deionized water and then loaded onto a Sephadex G-15 column (1.6 cm × 70 cm). The product was eluted with deionized water. The fractions containing glycosidic Neu5Ac were pooled and evaporated to dryness.

Analysis of Sialyloligosaccharides

Sialyloligosaccharides formed by the enzymatic reaction were analyzed by TLC or HPLC (Shimadzu, Kyoto, Japan) with a TSK-gel amide-80 column (4.6 mm × 25 cm; Tosoh, Tokyo, Japan). In the case of TLC analysis, a small amount of the enzymatic reaction mixture was applied to a precoated silica gel plate (60 F254, Merck) and developed with a solvent consisting of 2-propanol/acetic acid/water (3:2:1). For visualization of the organic compounds, the plate was dipped into a solution of 5% sulfuric acid in ethanol and then heated. HPLC analyses were performed according to the method described by Endo et al.[24] The purified products were analyzed by ESI-MASS spectroscopy as in a previous report[10] and by NMR spectroscopy using a Bruker DMX-500 spectrometer (Bruker Billerica, MA, USA) at 298 K in D_2O. The ^1H–^{13}C single-bond and multibond correlation spectra were measured by use of the HSQC, HMBC, HOHAHA, and COSY methods with the standard Bruker programs. The molecular mass of the by-product was determined by mass spectrometry.

ACKNOWLEDGEMENTS

We thank Mr. Masashi Mizutani, R&D Group, Product Quality Research Division, Japan Tobacco Inc., for conducting the NMR spectroscopy.

REFERENCES

1. Angata, T.; Varki, A. Chemical diversity in the sialic acids and related α-keto acids: an evolutionary perspective. *Chem. Rev.* **2002**, *102*, 439–469.

2. Vimr, E.R.; Kalivoda, K.A.; Denzo, E.L.; Steenbergen, S.M. Diversity of microbial sialic acid metabolism. *Microbiol. Mol. Biol. R.* **2004**, *68*, 132–153.

3. Varki, A. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* **1993**, *3*, 97–130.

4. Gagneux, P.; Vark, A. Evolutionary considerations in relating oligosaccharide diversity to biological function. *Glycobiology* **1999**, *9*, 747–755.

5. Taniguchi, N.; Honke, K.; Fukuda, M. *Handbook of Glycosyltransferases and Related Genes*. 1st Edition, Springer-Verlag Tokyo, Tokyo, 2002, pp. 267–356.

6. Yamamoto, T.; Takakura, Y.; Tsukamoto, H. Bacterial sialyltransferase. *Trends Glycosci. Glycotechnol.* **2006**, *18*, 253–265.

7. Izumi, M.; Wong, C-H. Microbial sialyltransferases for carbohydrate synthesis. *Trends Glycosci. Glycotechnol.* **2001**, *13*, 345–360.

8. Takakura, Y.; Tsukamoto, H.; Yamamoto, T. Molecular cloning, expression and properties of an α/β-galactoside α2,3-sialyltransferase from *Vibrio* sp. JT-FAJ-16. *J. Biochem. (Tokyo)*, **2007**, *142*, 403–412.

9. Yamamoto, T.; Hamada, Y.; Ichikawa, M.; Kajiwara, H.; Mine, T.; Tsukamoto, H.; Takakura, Y. A β-galactoside α2,6-sialyltransferase produced by a marine bacterium, *Photobacterium leiognathi* JT–SHIZ-145, is active at pH 8. *Glycobiology* **2007**, *17*, 1167–1174.

10. Tsukamoto, H.; Takakura, Y.; Yamamoto, T. Purification, cloning and expression of an α-/β-galactoside α2,3-sialyltransferase from a luminous marine bacterium, *Photobacterium phosphoreum*. *J. Biol. Chem.* **2007**, *282*, 29794–29802.

11. Chiu, C.P.; Watts, A.G.; Lairson, L.L.; Gilbert, M.; Lim, D.; Wakarchuk, W.W.; Withers, S.G.; Strynadka, N.C. Structural analysis of the sialyltransferase CstII from *Campylobacter jejuni* in complex with a substrate analog. *Nat. Struct. Mol. Biol.* **2004**, *11*, 163–170.

12. Ni, L.; Sun, M.; Yu, H.; Chokhawala, H.; Chen, X.; Fisher, A.J. Cytidine 5′-monophosphate (CMP)-induced structural changes in a multifunctional sialyltransferase from *Pasteurella multocida*. *Biochemistry* **2006**, *45*, 2139–2148.

13. Chiu, C.P.; Lairson, L.L.; Gilbert, M.; Wakarchuk, W.W.; Withers, S.G.; Strynadka, N.C. Structural analysis of the α-2,3-sialyltransferase Cst-I from *Campylobacter jejuni* in apo and substrate-analogue bound forms. *Biochemistry* **2007**, *46*, 7196–7204.

14. Kakuta, Y.; Okino, N.; Kajiwara, H.; Ichikawa, M.; Takakura, Y.; Ito, M.; Yamamoto, T. Crystal structure of *Vibrionaceae Photobacterium* sp. JT-ISH-224 α2,6-sialyltransferase in a ternary complex with donor product CMP and acceptor substrate lactose: catalytic mechanism and substrate recognition. *Glycobiology* **2008**, *18*, 66–73.

15. Kim, D.U.; Yoo, J.H.; Lee, Y.J.; Kim, K.S.; Cho, H.S. Structural analysis of sialyltransferase PM0188 from *Pasteurella multocida* complexed with donor analogue and acceptor sugar. *BMB Report* **2007**, *41*, 48–54.

16. Iwatani, T.; Okino, N.; Sakakura, M.; Kajiwara, H.; Takakura, Y.; Kimura, M.; Ito, M.; Yamamoto, T.; Kakuta, Y. Crystal structure of α/β-galactoside α2,3-sialyltransferase from a luminous marine bacterium, *Photobacterium phosphoreum*. *FEBS Lett.* **2009**, *583*, 2083–2087.

17. Yu, H.; Chokhawala, H.; Karpel, R.; Yu, H.; Wu, B.; Zhang, J.; Zhang, Y.; Jia, Q.; Chen, X. A multifunctional *Pasteurella multocida* sialyltransferase: a powerful tool for the synthesis of sialoside libraries. *J. Am. Chem. Soc.* **2005**, *127*, 17618–17619.

18. Kajihara, Y.; Yamamoto, T.; Nagae, H.; Nakashizuka, M.; Sakakibara, T.; Terada, I. A novel α-2,6-sialyltransferase: transfer of sialic acid to fucosyl and sialyl trisaccharides. *J. Org. Chem.* **1996**, *61*, 8632–8635.

19. Nishida, Y.; Wiemann, T.; Sinnwell, V.; Thiem, J. A new type of galactosyltransferase reaction: transfer of galactose to the anomeric position of N-acetylkanosamine. *J. Am. Chem. Soc.* **1993**, *115*, 2536–2537.

20. Coutinho, P.M.; Deleury, E.; Davies, G.J.; Henrissat, B. An evolving hierarchical family classification for glycosyltransferases, *J. Mol. Biol.* **2003**, *328*, 307–317.

21. Ni, L.; Chokhawala, H.; Cao, H.; Henning, R.; Ng, L.; Huang, S.; Yu, H.; Chen, X.; Fisher, A.J. Crystal structures of *Pasteurella multocida* sialyltransferase complexes with acceptor and donor analogues reveal substrate binding sites and catalytic mechanism. *Biochemistry* **2007**, *46*, 6288–6298.

22. Tsukamoto, H.; Takakura, Y.; Mine, T.; Yamamoto, T. *Photobacterium* sp. JT-ISH-224 produces two sialyltransferases. α-/β-galactoside α2,3-sialyltransferase and β-galactoside α2,6-sialyltransferase *J. Biochem. (Tokyo)* **2008**, *143*, 187–197.

23. Sabesan, S.; Paulson, J.C. Combined chemical and enzymatic synthesis of sialyloligosaccharides and characterization by 500 MHz ^{1}H and ^{13}C NMR spectroscopy. *J. Am. Chem. Soc.* **1986**, *108*, 2068–2080.

24. Endo, S.; Morita, M.; Ueno, M.; Maeda, T.; Terabayashi, T. Fluorescent labeling of a carboxyl group of sialic acid for MALDI-MS analysis of sialyloligosaccharides and ganglioside. *Biochem. Biophys. Res. Commun.* **2009**, *378*, 890–894.