# A Novel α-2,6-Sialyltransferase: Transfer of Sialic Acid to Fucosyl and Sialyl Trisaccharides

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The substrate specificity and enzymatic sialylation ability of the bacterium α-2,6-sialyltransferase were examined. The enzyme assay displayed a remarkable ability to catalyze sialyl transfer to type-II oligosaccharides possessing fucoside or sialoside at the 2 or 3 position of the terminal galactoside. Enzymatic syntheses were performed in order to confirm the structure of unusual assay products found when using Neu5Ac $\beta$2,3Gal$\beta$1,4Glc and Fuc α1,2Gal$\beta$1,4Glc as the sialyl acceptors. Both sialylation reactions (10 $\mu$mol scales) were run using 83 munits of enzyme, were complete in 2 h, and afforded the sialoside analogues Neu5Ac α2,6(Fuc α1,2) Gal$\beta$1,4Glc (88%) and Neu5Ac α2,6(Neu5Ac $\beta$2,3) Gal$\beta$1,4Glc (92%).

## Introduction

Sialic acid (Neu5Ac) attached to the terminal end of oligosaccharide chains in glycoproteins and glycolipids plays an important role during cell adhesion events.[1] To clarify its role, many different sialosides and their analogues, e.g., sialidase-inhibitors[2] and SLex analogues,[3] have been synthesized. However, chemical preparation of α-sialosides has proven to be extremely difficult. In recent studies, highly efficient sialylation methods have been developed.[4] During the total syntheses of sialyloligosaccharides and their analogues, sialylation is generally performed in a relatively early stage.[5] Therefore, in order to obtain the target compound on a practical scale, large quantities of expensive sialic acid are needed.

In contrast, chemoenzymatic synthesis of sialoside using sialyltransferase[6] is very concise, since the sialylation reaction can be performed as the last step in the synthetic sequence. However, this methodology also has its disadvantages. Application of this methodology is generally limited to natural products or to their close structural analogues due to the strict substrate specificity of sialyltransferases. With respect to rat liver α-2,6- and α-2,3-sialyltransferases, which are the most widely used enzymes in chemoenzymatic synthesis, chemical modification of the peripheral hydroxyl group of the glycosylation position of the sialyl acceptor causes a decrease or complete loss of sialyl transfer activity.[7] Modification of the 4″, 5″, 7″, and 8″ positions of CMP-Neu5Ac also caused the same tendency of the sialyl transfer.[8] This strict substrate specificity has been a hindrance to the enzymatic synthesis of sialyloligosaccharide analogues. In order to synthesize the sialyloligosaccharide analogues on a practical scale using chemoenzymatic methods, novel sialyltransferases having unusual- or broad-substrate specificity[9] are needed.

Recently, we isolated a novel α-2,6-sialyltransferase from a bacterium found in sea water.[10] This enzyme has a unique substrate specificity that should be useful in the syntheses of sialyloligosaccharide analogues. Here

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(1) (a) Schauer, R. *Adv. Carbohydr. Chem. Biochem.* **1982**, *40*, 131−234. (b) Edelman, G. M. *Science* **1983**, *219*, 450−457. (c) Phillips, M. L.; Nudelman, E.; Gaeta, F. C. A.; Perez, M.; Singhal, A. K.; Hakomori, S.; Paulson, J. C. *Science* **1990**, *250*, 1130−1132. (d) Walz, G.; Aruffo, A.; Kolanus, W.; Bevilacqua, M.; Seed, B. *Science* **1990**, *250*, 1132−1135.

(2) (a) Suzuki, Y.; Sato, K.; Kiso, M.; Hasegawa, A. *Glycoconjugate J.* **1990**, *7*, 349−356. (b) Sabesan, S.; Neira, S.; Davidson, F.; Duus, J.; Bock, K. *J. Am. Chem. Soc.* **1994**, *116*, 1616−1634. (c) Sabesan, S.; Neira, S.; Wasserman, Z. *Carbohydr. Res.* **1995**, *267*, 239−261.

(3) (a) Ichikawa, I.; Lin, Y. C.; Dumas, D. P.; Shen, G. J.; Garcia-Junceda, E.; Williams, M. A.; Bayer, R.; Ketcham, C.; Walker, L. E.; Paulson, J. C.; Wong, C. H. *J. Am. Chem. Soc.* **1992**, *114*, 9283−9298. (b) DeFrees, S. A.; Kosch, W.; Way, W.; Paulson, J. C.; Sabesan, S.; Halcomb, R. L.; Huang, D. H.; Ichikawa, Y.; Wong, C. H. *J. Am. Chem. Soc.* **1995**, *117*, 66−79. (c) Uchiyama, T.; Vassilev, V. P.; Kajimoto, T.; Wong, W.; Huang, H.; Lin, C. C.; Wong, C. H. *Am. Chem. Soc.* **1995**, *117*, 5395−5396. (d) Maeda, H.; Ishida, H.; Kiso, M.; Hasegawa, A. *J. Carbohydr. Chem.* **1995**, *14*, 369−385. (e) Sprengard, U.; Schudok, M.; Schmidt, W.; Kretzschmar, G.; Kunz, H. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 321−324.

(4) (a) Murase, T.; Ishida, H.; Kiso, M.; Hasegawa, A. *Carbohydr. Res.* **1988**, *184*, C1−C4. (b) Ito, Y.; Ogawa, T. *Tetrahedron* **1990**, *46*, 89−102. (c) Okamoto, K.; Goto, T. *Tetrahedron* **1990**, *46*, 5835−5857. (d) Hasegawa, A.; Nagahama, T.; Ohki, H.; Hotta, K.; Ishida, H.; Kiso, M. *J. Carbohydr. Chem.* **1991**, *10*, 493−498. (e) Birberg, W.; Lönn, H. *Tetrahedron Lett.* **1991**, *32*, 7453−7456. (f) Lönn, H.; Stenvall, K. *Tetrahedron Lett.* **1992**, *33*, 115−116. (g) Martin, T.; Schmidt, R. R. *Tetrahedron Lett.* **1992**, *33*, 6123−6126. (h) Kondo, H.; Ichikawa, Y.; Wong, C. H. *J. Am. Chem. Soc.* **1992**, *114*, 8748−8750. (i) Martichonok, V.; Whitesides, G. M. *J. Org. Chem.* **1996**, *61*, 1702−1706.

(5) (a) Murase, T.; Kameyama, A.; Kartha, K. P. R.; Ishida, H.; Kiso, M.; Hasegawa, A. *J. Carbohydr. Chem.* **1989**, *8*, 265−283. (b) Ito, Y.; Numata, M.; Sugimoto, M.; Ogawa, T. *J. Am. Chem. Soc.* **1989**, *111*, 8508−8510. (c) Nicolaou, K. C.; Hummel, C. W.; Iwabuchi, Y. *J. Am. Chem. Soc.* **1992**, *114*, 3126−3128. (d) Hotta, K.; Ishida, H.; Kiso, M.; Hasegawa, A. *J. Carbohydr. Chem.* **1995**, *14*, 491−506. (e) Kameyama, A.; Ehara, T.; Yamada, Y.; Ishida, H.; Kiso, M.; Hasegawa, A. *J. Carbohydr. Chem.* **1995**, *14*, 507−523.

(6) (a) Sabesan, S.; Paulson, J. C. *J. Am. Chem. Soc.* **1986**, *108*, 2068−2080. (b) Thiem, J.; Treder, W. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 1096−1097. (c) Unverzagt, C.; Kunz, H.; Paulson, J. C. *J. Am. Chem. Soc.* **1990**, *112*, 9308−9309. (d) Wong, C. H.; Halcomb, R. L.; Ichikawa, Y.; Kajimoto, T. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 521−546.

(7) (a) Wlasichuk, K. B.; Kashem, M. A.; Nikrad, P. V.; Bird, P.; Jiang, C.; Venot, A. P. *J. Biol. Chem.* **1993**, *268*, 13971−13977. (b) Sabesan, S.; Bock, K.; Paulson, J. C. *Carbohydr. Res.* **1991**, *218*, 27−54.

(8) (a) Beau, J. M.; Schauer, R. *Eur. J. Biochem.* **1980**, *106*, 531−540. (b) Conradt, H. S.; Bunsch, A.; Brossmer, R. *FEBS Lett.* **1984**, *170*, 295−300. (c) Higa, H. H.; Paulson, J. C. *J. Biol. Chem.* **1985**, *260*, 8838−8849. (d) Gross, H. J.; Bunsch, A.; Paulson, J. C.; Brossmer, R. *Eur. J. Biochem.* **1987**, *168*, 595−602. (e) Gross, H. J.; Brossmer, R. *Eur. J. Biochem.* **1988**, *177*, 583−589. (f) Kajihara, Y.; Ebata. T.; Koseki, K.; Kodama, H.; Matsushita, H.; Hashimoto, H. *J. Org. Chem.* **1995**, *60*, 5732−5735.

(9) Pelt, J. V.; Dorland, L.; Duran, M.; Hokke, C. H.; Kamerling, J. P.; Vliegenthart, J. F. G. *FEBS Lett.* **1989**, *256*, 179−184.

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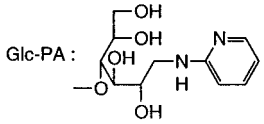
**Table 1. Transfer Assay Using PA-Labeled Oligosaccharides**

| entry | acceptor | transfer yield (%)[a] |
|---|---|---|
| a | Neu5Ac α 2,3 Gal β 1,4 Glc - PA | 28 [b] |
| b | Neu5Gc α 2,3 Gal β 1,4 Glc - PA | 78 [c] |
| c | Neu5Ac α 2,8 Neu5Ac α 2,3 Gal β 1,4 Glc - PA | 76 [c] |
| d | Fuc α 1,2 Gal β 1,4 Glc - PA | 57 [c] |
| e | Gal β 1,4 GlcNAc β 1,3 Gal β 1,4 Glc - PA | 44 [d] |
| f | Gal β 1,4 GlcNAc β 1,3 Gal β 1,4 Glc - PA<br>3<br>\|<br>Fuc α 1 | < 5 [e] |
| g | Gal β 1,3 GalNAc β 1,4 Gal β 1,4 Glc - PA<br>3<br>\|<br>Neu5Ac α 2 | < 5 [e] |
| h | Gal β 1,3 GalNAc β 1,4 Gal β 1,4 Glc - PA<br>3 3<br>\| \|<br>Neu5Ac α 2 Neu5Ac α 2 | < 5 [e] |
| i | Gal β 1,3 GlcNAc β 1,3 Gal β 1,4 Glc - PA<br>4<br>\|<br>Fuc α 1 | < 5 [e] |
| j | Gal β 1,3 GalNAc β 1,4 Gal β 1,4 Glc - PA<br>3<br>\|<br>Neu5Ac α 2,8 Neu5Ac α 2 | < 5 [e] |

[a] The transfer yields were estimated by HPLC.
[b] Incubation time: 4.5 h. [c] Incubation time: 12 h.
[d] Incubation time: 8.0 h. [e] Incubation time: 20 h.

Glc-PA :



we report its unusual substrate specificity toward sialyl acceptors, its kinetic parameters, and a practical enzymatic sialylation reaction in which it catalyzes the transfer of a sialic acid to fucosyl and sialyl trisaccharides in high yields.

## Results

A novel α-2,6-sialyltransferase (EC 2.4.99.1) was isolated from *Photobacterium damsela* 0160 found in sea water and was purified in four steps.[10] The pure enzyme was then used to investigate its substrate specificity. The standard enzyme assay showed that the enzyme catalyzed the transfer of sialic acid to methyl β-D-galactopyranoside ($K_m$ = 174 mM), *N*-acetyllactosamine (= 8.95 mM), and lactose (= 6.82 mM).[10] Mammalian α-2,6-sialyltransferase does not recognize lactose as an acceptor,[11] so the substrate specificity of this enzyme is indeed unique.[12] We next investigated the possibility that this enzyme could catalyze the transfer of sialic acid to other oligosaccharides using pyridylamino (= PA)-labeled oligosaccharides as acceptors. The results are summarized in Table 1. The enzyme catalyzed the transfer of sialic acid to type-II oligosaccharides, Galβ1,4Glc or Galβ1,-4GlcNAc sequence (Table 1, entries a−e) but did not recognize type-I oligosaccharides (Table 1, entries g−j) as sialyl acceptors. Furthermore, the assay showed a remarkable ability to catalyze the sialyl transfer to type-II oligosaccharides possessing a fucoside at the 2 position or a sialoside (sialyl dimer and *N*-glycolylneuraminic acid) at the 3 position of the galactoside (Table 1, entries a−d). The kinetic parameters of four sialyl acceptors were also measured under standard conditions, and the results are shown in Table 2. The assay results showed that the $K_m$ value ranged from 6.8 to 13.6 mM when using the four sialyl acceptors. These parameters suggest that the 2 and 3 hydroxyl groups of the galactoside are not key polar functional groups[13] when the enzyme recognizes the acceptor and are positioned away from the catalytic site.

**Table 2. Kinetics Parameters of Type-II Tri- and Disaccharides**

| acceptor | $K_m$ (mM) | $V_{max}$ (relative) |
|---|---|---|
| Gal β-1,4-Glc | 6.8 | 66 |
| Gal β-1,4-GlcNAc | 9.0 | 100 |
| Neu5Ac α-2,3-Gal β-1,4-Glc | 8.1 | 94 |
| Fuc α-1,2-Gal β-1,4-Glc | 13.6 | 44 |

In order to confirm the structure of the assay products and investigate whether the enzyme can be used for the practical enzymatic syntheses of sialyloligosaccharide analogues, enzymatic syntheses were examined using Neu5Ac β2,3Galβ1,4Glc[14] and Fuc α1,2Galβ1,4Glc[15] as the sialyl acceptors. When 83 munits of the enzyme were used, the sialyl transfer reactions (10 μmol scales) were complete in 2 h. Purification was performed using previously reported methods[6] and provided the sialoside analogues **1** (88%) and **2** (92%) (Scheme 1).

In order to determine the structure of these products, modern NMR techniques at 500 MHz were carried out. The $^1H$ and $^{13}C$ chemical shifts obtaining from 2D-TOCSY,[16] HMQC,[17] and HMBC[18] spectra are summarized in Table 3. The sialylated position was identified by use of the differences in the $^{13}C$ chemical shift values observed between the products and the starting materials.[6a] The C-6 chemical shifts of the galactoside in both sialosides **1** and **2** were shifted downfield by 2.3 ppm, and the C-5 chemical shifts were shifted upfield by 1.9 ppm compared to the $^{13}C$ chemical shifts of the corresponding sialyl acceptors.[19,20] Furthermore, in the HMBC spectra, the correlation between the C-2 of Neu5Ac and the H-6 of Gal were observed in each product.

In summary, we have described the substrate specificity of a novel α-2,6-sialyltransferase and the enzymatic

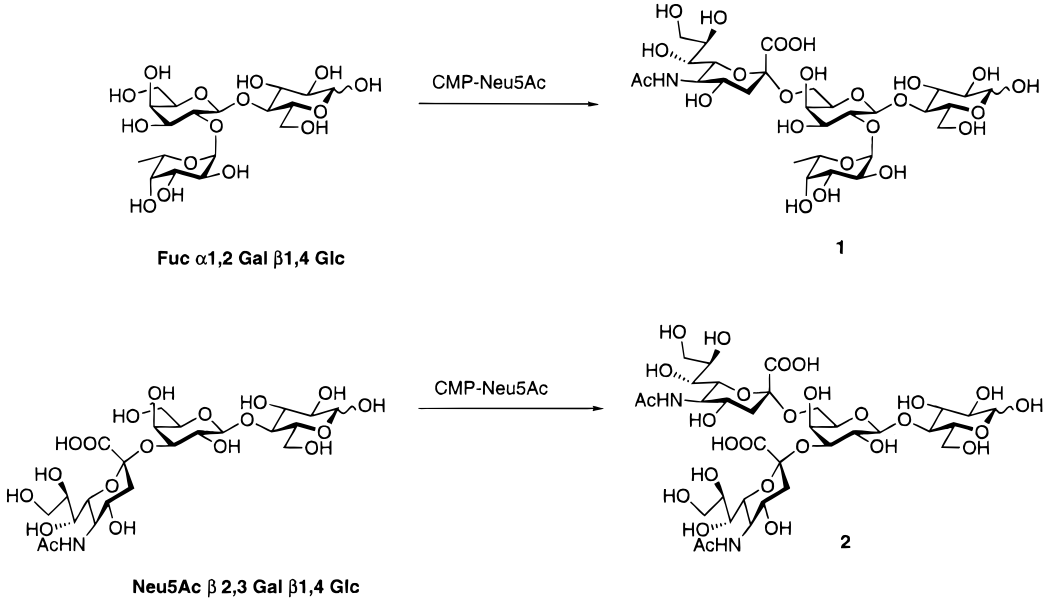
(10) Yamamoto, T.; Nakashizuka, M.; Kodama, H.; Kajihara, Y.; Terada, I. *J. Biochem.* **1996**, *120*, 104−110.
(11) Weinstein, J.; Souza-e-Silva, U.; Paulson, J. C. *J. Biol. Chem.* **1982**, *257*, 13845−13853.
(12) The enzyme assay revealed that the bacterium α-2,6-sialyltransferase catalyzes the transfer of sialic acid to a variety of monosaccharides. The relative transfer rate compared to lactose (100%) is as follows: methyl α-D-galactopyranoside (3.0%), methyl β-D-galactopyranoside (9.2%), methyl α-D-glucopyranoside (0.5%), methyl β-D-glucopyranoside (0.6%), methyl α-D-mannopyranoside (0.5%), methyl β-D-mannopyranoside (0.6%), and methyl α-D-*N*-acetylgalactosaminide (3.0%).
(13) (a) Lemieux, R. U. *Chem. Soc. Rev.* **1989**, *18*, 347−374. (b) Du, M. H.; Spohr, U.; Lemieux, R. U. *Glycoconjugate J.* **1994**, *11*, 443−461.
(14) The sialyl acceptor, Neu5Ac β2,3Galβ1,4Glc, was purified with silica gel column chromatography (*n*-propanol: water = 2:1) from purchased sialyllactose (Sigma: Neu5Ac β2,3Galβ1,4Glc and Neu5Ac β2,6Galβ1,4Glc mixture).
(15) The sialyl acceptor, Fuc α1,2Galβ1,4Glc, was kindly provided by Prof. H. Hashimoto (Tokyo Institute of Technology).
(16) (a) Braunschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521−528. (b) Davis, D. G.; Bax, A. *J. Am. Chem. Soc.* **1985**, *107*, 2821−2823.
(17) Bax, A.; Subramanian, S. *J. Magn. Reson.* **1986**, *67*, 565−569.
(18) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093−2094.
(19) (a) Fuc α1,2Galβ1,4Glc: Jenkins, G. A.; Bradbury, J. H.; Messer, M.; Trifonoff, E. *Carbohydr. Res.* **1984**, *126*, 157−161. (b) The $^{13}C$ NMR data of Neu5Ac β2,3Galβ1,4Glc were reported in ref 6a.
(20) The $^{13}C$ chemical shifts of galactoside in Fuc α1,2Galβ1,4Glc and Neu5Ac β2,3Galβ1,4Glc are C1 (101.1), C2 (77.1), C3 (74.4), C4 (70.0), C5 (**76.0**), C6 (**61.9**) and C1 (103.17), C2 (69.86), C3 (76.03), C4 (68.00), C5 (**75.65**), C6 (**61.50**), respectively. The boldface values were shifted by the sialylation.

**Table 3. $^{1}H$ and $^{13}C$ Chemical Shift Assignments of 1 and 2**

| | NeuAc | | Gal | | Glc | | Fuc | |
|---|---|---|---|---|---|---|---|---|
| carbon | H | C | H | C | H | C | H | C |
| | | | | Compound **1**[a] | | | | |
| C1 | | 174.12 | 4.51 | 101.09 | β 4.62 | β 96.51 | 5.33 | 99.92 |
| | | | | | α 5.22 | α 92.55 | | |
| C2 | | 101.09 | 3.66 | 76.53 | β 3.32 | β 74.66 | 3.79 | 68.88 |
| | | | | | α 3.62 | α 72.01 | | |
| C3 | 1.71 | 40.82 | 3.87 | 74.17 | β 3.58 | β 75.05 | 3.78 | 70.29 |
| | 2.72 | | | | α 3.79 | α 74.17 | | |
| C4 | 3.66 | 69.06 | 3.91 | 69.79 | β 3.71 | β 77.70 | 3.81 | 72.49 |
| | | | | | α 3.79 | α 77.59 | | |
| C5 | 3.85 | 52.49 | 3.78 | 74.17 | β 3.49 | β 75.98 | 4.24 | 67.61 |
| | | | | | α 3.69 | α 71.03 | 4.21 | |
| C6 | 3.72 | 73.30 | 3.94 | 64.20 | β 3.93, 3.76 | β 61.12 | 1.22 | 15.97 |
| | | | 3.64 | | β 3.91, 3.79 | α 60.96 | | |
| C7 | 3.56 | 68.88 | | | | | | |
| C8 | 3.88 | 72.40 | | | | | | |
| C9 | 3.88 | 63.36 | | | | | | |
| | 3.64 | | | | | | | |
| $CH_3$ | 2.02 | 22.77 | | | | | | |
| C=O | | 175.63 | | | | | | |
| | | | | Compound **2**[b] | | | | |
| C1 | | 173.92 | 4.58 | 103.18 | β 4.74 | β 95.90 | | |
| | | 173.92 | | | α 5.29 | α 92.05 | | |
| C2 | | 100.46 | 3.63 | 68.59 | β 3.38 | β 73.95 | | |
| | | 100.17 | | | α 3.68 | α 71.28 | | |
| C3 | 1.81, 2.78 | 40.25 | 4.20 | 75.51 | β 3.72 | β 74.88 | | |
| | 1.87, 2.82 | 39.71 | | | α 3.94 | α 71.86 | | |
| C4 | 3.72, 3.70 | 68.59 | 4.04 | 67.82 | β 3.71 | β 80.00 | | |
| | | 68.35 | | | α 3.73 | α 79.88 | | |
| C5 | 3.92, 3.94 | 52.00 | 3.89 | 73.72 | β 3.70 | β 74.83 | | |
| | | 51.89 | | | α 4.05 | α 70.16 | | |
| C6 | 3.80, 3.72 | 73.11 | 4.04 | 63.74 | β 4.04, 3.89 | β 60.54 | | |
| | | 72.73 | 3.66 | | α 4.04, 3.96 | α 60.36 | | |
| C7 | 3.63, 3.67 | 69.47 | | | | | | |
| | | 68.59 | | | | | | |
| C8 | 3.97, 3.97 | 71.86 | | | | | | |
| | | 71.86 | | | | | | |
| C9 | 3.96, 3.72 | 62.90 | | | | | | |
| | 3.96, 3.72 | 62.84 | | | | | | |
| $CH_3$ | 2.10, 2.10 | 22.27 | | | | | | |
| | | 22.27 | | | | | | |
| C=O | | 175.10 | | | | | | |
| | | 175.10 | | | | | | |

[a] The chemical shifts of $^{1}H$ and $^{13}C$ NMR are expressed relative to internal HOD = 4.81 ppm (298 K) and external 1,4 dioxane = 67.4 ppm (298 K), respectively.[19a] [b] The chemical shifts of $^{1}H$ and $^{13}C$ NMR are expressed relative to internal HOD = 4.81 ppm (304 K) and external 1,4 dioxane = 66.88 ppm (304 K), respectively.[6a]

**Scheme 1**



syntheses of sialyloligosaccharide analogues. The assay indicated that the enzyme recognizes type-II oligosaccharides in which the nonreducing end-galactoside can be modified at either the 2 or 3 position with a large substitutent, such as a fucoside or sialoside. Since this bacteria can be easily cultivated, large quantities of this enzyme should be available for use as a synthetic catalyst. Isolation of our novel sialyltransferase makes possible the chemoenzymatic syntheses of sialyloligosaccharide analogues that previously could not be synthesized using rat liver α-2,6-sialyltransferase and also suggests the likely the presence of other novel sialyltransferases that could be used to synthesize different sialoside analogues. An investigation of the substrate specificity of this enzyme toward CMP-Neu5Ac is in progress.

## Experimental Section

**Transfer Assay Using Pyridylaminated Oligosaccharides.** To study the substrate specificity of a novel α-2,6-sialyltransferase, enzymatic reaction was carried out with pyridylaminated oligosaccharides that were purchased from TAKARA Co. An assay solution containing the CMP-Neu5Ac (143 pmol), each pyridylaminated oligosaccharide (50 pmol), and purified sialyltransferase (0.1 mU) in sodium cacodylate buffer (20 mM, pH 5.0, total volume 25 μL) containing 0.02% Triton X-100 was incubated 30 °C for 20 h. After the reaction, each mixture was analyzed by HPLC using TAKARA PALPAK type R (0.46 × 25 cm) as an analytical column. The mixture was applied to the column equilibrated with 100 mM acetic acid−triethylamine buffer (pH 5.2) containing 0.15% *n*-butanol. Pyridylaminated oligosaccharides (nonreacted acceptor and product) were eluted using *n*-butanol in the same buffer. The concentration of *n*-butanol was increased linearly from 0.15% to 0.5% (0−35 min). Pyridylaminated oligosaccharides were detected by fluorescence (excitation, 320 nm; emission, 400 nm). The column temperature was 40 °C, and the flow rate was 1.0 mL/min.

**Enzyme Assay of Four Kinds of Type-II Oligosaccharide Acceptors.** Apparent kinetic parameters of sialyltransferase toward sialyl acceptor were examined under the following conditions. An assay solution containing CMP-[4,5,6,7,8,9-$^{14}$C]-Neu5Ac (642 cpm/nmol, 70 nmol), the various concentrations of oligosaccharides, and purified sialyltransferase (3 mU) in sodium cacodylate buffer (20 mM, pH 5.0, total volume 25 μL) containing 0.02% Triton X-100 was incubated 30 °C for 3 min. The reaction was followed up to 15% consumption of CMP-Neu5Ac. The $^{14}$C-labeled sialyloligosaccharide was isolated by passage of the mixture diluted with 1 mL of 5 mM sodium phosphate (pH 6.8) through a Pasteur pipette column of Dowex 1-X8 ($PO_4^{2-}$) and further eluted by 1 mL of 5 mM sodium phosphate. The $^{14}$C-labeled sialyloligosaccharide was measured by scintillation counting. The assay was performed in duplicate, and $K_m$ and $V_{max}$ were determined using a Lineweaver−Burk plot.

**Enzymatic Synthesis of Sialyloligosaccharides 1.** A solution containing the CMP-Neu5Ac (9.8 mg, 15.4 μmol), Fuc α1,2Galβ1,4Glc (5.0 mg, 10.2 μmol), BSA (2 mg), alkalinephosphatase (25 u), and purified sialyltransferase (83 mu) in sodium cacodylate buffer (50 mM, pH 6.0, 300 μL) was incubated at 37 °C for 2 h. The mixture was directly loaded onto a column of anion-exchange resin (1 × 5 cm, Dowex 1-X8, formate form). The sialyloligosaccharide was eluted with a gradient of $NH_4HCO_3$ solution (0−400 mM). The sialyloligosaccharide-containing fractions were concentrated, and product was further purified with a column of Sephadex G-15 (1 × 50 cm, water) to give **1** (6.3 mg, 88%): HRMS[21] calcd for $C_{29}H_{49}NO_{23}Na$ ($M + Na^+$) 802.2593, found 802.2631.

**Enzymatic Synthesis of Sialyloligosaccharides 2.** Enzymatic synthesis of disialyloligosaccharide with CMP-Neu5Ac (9.0 mg, 14.2 μmol), Neu5Ac β2,3Galβ1,4Glc (6.0 mg, 9.5 μmol), BSA (2 mg), alkalinephosphatase (25 u), and purified sialyltransferase (83 mu) in sodium cacodylate buffer (50 mM, pH 6.0, 300 μL) was examined in the same manner in the preparation of **1** to give **2** (8.0 mg, 92%): HRMS[21] calcd for $C_{34}H_{56}N_2O_{27}Na$ ($M + Na^+$) 947.2969, found 947.2799.

**NMR Measurement.** Proton and carbon NMR spectra in $D_2O$ were assigned by 1D and 2D techniques using Bruker DMX-500. All spectra were measured with an inverse triple probe. TOCSY spectra were recorded using the MLEV-17 pulse sequence with a total spin locking time of 100 ms. The data were transformed as a 4 K × 1 K matrix with π/2 phase-shifted square sine-bell apodization applied in both dimensions. The $^1$H−$^{13}$C one-bond and multibond shift correlation spectra were measured by use of the HMQC and HMBC methods, respectively, with standard Bruker programs.

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**Supporting Information Available:** Copies of $^1$H, $^{13}$C, HOHAHA, HMQC, and HMBC spectra of **1** and **2** and HPLC profile of transfer assay using pyridylaminated oligosaccharides (15 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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(21) The high-resolution mass spectra were recorded on a Shimadzu-Kratos concept-IIH instrument under FAB conditions.