An Approach to the Precise Chemoenzymatic Synthesis of ^{13}C-Labeled Sialyloligosaccharide on an Intact Glycoprotein: A Novel One-Pot [3-^{13}C]-Labeling Method for Sialic Acid Analogues by Control of the Reversible Aldolase Reaction, Enzymatic Synthesis of [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-(1→4)-GlcNAc-β- Sequence onto Glycoprotein, and Its Conformational Analysis by Developed NMR Techniques

Tatsuo Miyazaki,[†] Hajime Sato,[‡] Tohru Sakakibara,[†] and Yasuhiro Kajihara*,[†]

Contribution from the Graduate School of Integrated Science, and Faculty of Science, Yokohama City University, 22-2, Seto, Kanazawa-ku, Yokohama 236-0027 Japan, and Bruker Japan Co., Ltd. 21-5, Ninomiya, 3-chome, Tsukuba, Ibaraki 305-0051 Japan

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Abstract: A one-pot enzymatic ^{13}C-labeling method for the 3-position of sialic acid (NeuAc) analogues has been developed using NeuAc aldolase, lactate dehydrogenase (LDH), alcohol dehydrogenase (ADH), and nucleotide pyrophosphatase (NPP). This method consists of two steps, the first of which is degradation to 2-acetamido-2-deoxy-D-mannose (ManNAc) analogues. This degradation reaction was accelerated by a cofactor regeneration system which converts pyruvic acid into lactic acid using LDH, ADH, and β-nicotinamide adenine dinucleotide oxidized form (β-NAD$^+$). The second step is condensation of the ManNAc analogue with [3-^{13}C]-pyruvic acid newly added after decomposition of the cofactor by nucleotide pyrophosphatase which play a role like switch to stop conversion of pyruvic acid into lactic acid. Five different NeuAc analogues have been labeled in good yields using this newly developed one-pot enzymatic procedure. Following conversion of [3-^{13}C]-NeuAc to CMP-[3-^{13}C]-NeuAc, enzymatic synthesis of [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-(1→4)-GlcNAc-β-x-ovalbumin (x: hybrid type oligosaccharide) **23** and [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-(1→4)-GlcNAc-β-OMe **26** (sialyl LacNAc) was performed using *bovine* β-1,4-galactosyltransferase and *rat recombinant* α-2,3-sialyltransferase. The ^{1}H chemical shifts of all protons in [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β- on a glycoprotein were assigned by 2D HMQC, 1D HSQC-TOCSY, and the herein described 1D and 2D HSQC-TOCSY-NOESY-TOCSY method. More specifically, the 7-, 8-, and 9-protons of NeuAc could be observed by this HSQC-TOCSY-NOESY-TOCSY method even with only a single ^{13}C atom at the 3-position. In addition, 1D and 2D HMQC-NOESY spectra as well as carbon spin−lattice relaxation times (T_1) were measured to compare the conformational properties and dynamic behavior of the sialylgalactoside as part of the sialyl LacNAc **26** and when bound to a glycoprotein **23**. These analyses suggested that the conformational properties of sialyl LacNAc are similar for both the conjugated and unconjugated forms, and that the torsional angle of the sialyl linkage, i.e., COOH−C2^{NeuAc}−O−C3^{Gal}, is biased toward the anti (−146.7°) conformation. In addition, the flexibility of galactosyl ring when bound to a glycoprotein appears to be significantly restricted by the attachment of NeuAc as compared with unconjugated sialyl LacNAc.

Introduction

Sialic acid occurs frequently as a terminal residue of carbohydrate chains on glycolipids and glycoproteins in cell membranes. The sialic acid residues at the nonreducing end of oligosaccharides are known to be associated with several recognition events in carbohydrate-mediated cell adhesion.[1] Therefore, research into the role of sialic acid is the focus of many research groups around the world.[2] For this purpose, gangliosides, *N*- and *O*-linked type sialyloligosaccharides, and their analogues have been synthesized,[2c−e,h,m,s] and their binding ability during several molecular recognition events have been assayed.[2a,f,g,i−k,n,o,q] Conformational properties as well as the electrostatic nature of the hydroxyl and carboxyl groups are central to the molecular recognition events mediated by binding of sialyloligosaccharide to receptor proteins. However, although the conformational properties[2b,l,p,r] of sialyloligosaccharide analogues of low molecular weight have been reported by many research groups,[2] the conformation and dynamics of sialyloli-

[†] Yokohama City University.
[‡] Bruker Japan Co.

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gosaccharides and their analogues attached to glycoproteins have not yet been fully analyzed.

The purpose of the research described herein is to develop a synthetic technique for preparing sialyloligosaccharides and their analogues on intact glycoproteins with precision and to analyze their conformations, thus providing valuable new information on the conformation and dynamic behavior of these sialyloligosaccharides and their role in binding to receptor proteins.

Recently, enzymatic methods[3] have been used successfully to attach sugar analogues to the nonreducing end of oligosaccharides on glycoproteins.[4] The drawback to this approach is the difficulty of analyzing short sugar analogue sequences of a much larger glycoprotein. Moreover, structural analysis of the nonreducing end of sugar residues on glycoproteins has not been successful using X-ray crystallography,[1j] and it is difficult to identify the sugar protons due to overlap with the resonances of the amino acids using NMR.[5] Therefore, it would be very essential to develop a concise analytic method for sialyloligosaccharide analogues attached to glycoproteins using NMR, just as if structures of oligosaccharides of low molecular weights are analyzed. To address this problem, we developed a concise [3-^{13}C]-labeling method for NeuAc analogues, and reported preliminary results[6] for the synthesis of the ^{13}C-labeled sialyloligosaccharide, 9-deoxy-9-fluoro-[3-^{13}C]-NeuAc-α-(2→6)-[U-^{13}C]-Gal-β-(1→4)-GlcNAc-β- sequence on an intact glycoprotein using a CMP-[3-^{13}C]-NeuAc analogue and UDP-[U-^{13}C]-Glc, and its analysis by NMR measurement.

^{13}C-labeled NeuAc[7] has been utilized for conformational analysis of sialyloligosaccharides on artificial membrane surfaces[7a,b] and TRNOE experiments.[7f] These ^{13}C-labeled NeuAc compounds were synthesized by condensation of ^{13}C-labeled pyruvic acid with an excess of ManNAc.[7a] However, to synthesize ^{13}C-labeled NeuAc analogues, such as the 5-, 7-, 8-, and 9-modified NeuAc, corresponding 2-, 4-, 5-, and 6-modified ManNAc analogues must first be prepared. To date, a large number of NeuAc analogues have been synthesized from NeuAc,[8] the common synthetic route including selective protection of hydroxyl groups has become fairly common, unlike the synthesis of ManNAc analogues. Additionally, the substrate specificity of NeuAc aldolase is flexible to a variety of NeuAc and ManNAc analogues.[3d,9] Using this knowledge, if [3-^{13}C]-NeuAc analogues can be obtained from NeuAc derivatives, these established routes[8] for synthesis of the NeuAc analogues can be practically utilized. The reason the 3-position of NeuAc should be ^{13}C-labeled is that we hope to use novel NMR techniques for observation of the H-3 to H-9 protons. In a previous report, we were not able to observe the H-7 proton of 9-deoxy-9-fluoro-[3-^{13}C]-NeuAc because HMQC-TOCSY development stopped at H-6. Therefore, ^{13}C-labeling of the side chain of NeuAc was thought necessary. However, if a combined

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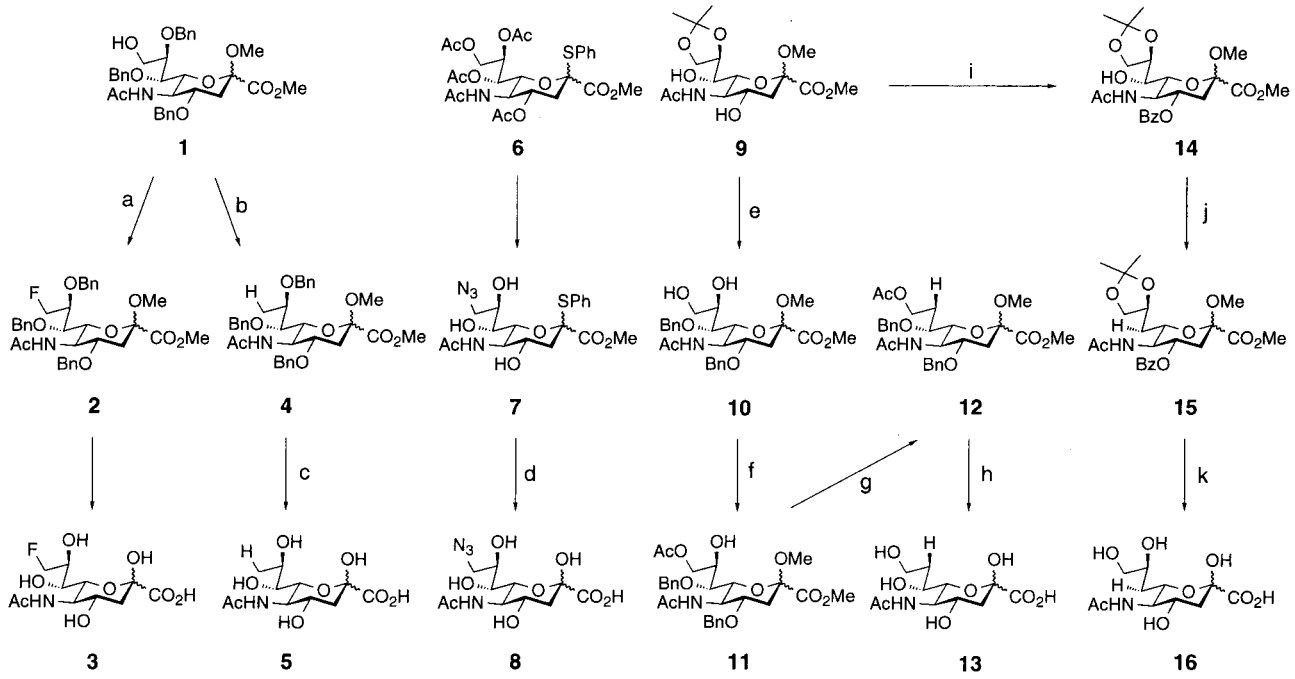
Scheme 1[a]



[a] Reagents: (a) (1) Tf_2O, pyridine, CH_2Cl_2; (2) TASF, CH_2Cl_2, y = 52% (2 steps); (b) (1) phenyl chlorothionoformate, pyridine, CH_2Cl_2; (2) AIBN, *n*-Bu_3SnH, toluene, 80 °C, y = 39% (2 steps); (c) (1) 10% Pd−C, AcOH; (2) 0.3 N NaOH: MeOH = 1:1; (3) 25 mM HCl, Amberlyst 15 (H^+) 80 °C, y = 64% (3 steps); (d) (1) NBS, acetone: H_2O = 9:1; (2) 0.3 N NaOH, y = 52% (2 steps); (e) (1) $Ba(OH)_2 \cdot 8H_2O$, BaO, BnBr, DMF; (2) CH_2N_2; (3) 60% AcOH, 60 °C, y = 62% (3 steps); (f) AcCl, pyridine: CH_2Cl_2 = 1:1, y = 82%; (g) (1) phenyl chlorothionoformate, DMAP, pyridine: CH_2Cl_2 = 1:1; (2) AIBN, *n*-Bu_3SnH, toluene, 100 °C, y = 68% (2 steps); (h) (1) 10% Pd−C, AcOH; (2) 0.3 N NaOH: MeOH = 2:1; (3) 25 mM HCl, Amberlyst 15 (H^+) 85 °C, y = 59% (3 steps); (i) BzCl, pyridine: CH_2Cl_2 = 1:4, y = 68%; (j) (1) 1,1′-thiocarbonyldiimidazole, DMAP, CH_2Cl_2; (2) AIBN, *n*-Bu_3SnH, toluene, 80 °C, y = 80% (2 steps); (k) (1) 60% AcOH, 60 °C; (2) 0.3N NaOH: MeOH = 1:1; (3) 25 mM HCl, Amberlyst 15 (H^+) 80 °C, y = 69% (3 steps).

TOCSY-NOESY-TOSCY[10] and HSQC technique, that is, HSQC-TOCSY-NOESY-TOCSY, could be developed, all protons of the NeuAc from H-3 to H-9 would be measurable even with only a single ^{13}C-atom at 3-position of NeuAc which bound to a glycoprotein. During the synthesis of NeuAc analogues by multistep sequences, ^{13}C-labeling of several carbons of the sugar skeleton would require expensive, time-consuming, advanced techniques. Therefore, the development of such a novel measurement technique promised to enable us to simplify the synthetic routes for ^{13}C-labeling.

In this paper, we would like to describe a novel one-pot [3-^{13}C]-labeling method for NeuAc analogues, the chemoenzymatic synthesis of the [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β- sequence on an intact glycoprotein, and its conformational analysis with a newly developed HSQC-TOCSY-NOESY-TOCSY technique which requires only one ^{13}C-labeled carbon at the 3-position of NeuAc.

Results and Discussion

Chemical Synthesis of NeuAc Analogues. For this study, we prepared five different NeuAc analogues (9-deoxy-9-fluoro-NeuAc **3**, 9-deoxy-NeuAc **5**, 9-deoxy-9-azido-NeuAc **8**, 8-deoxy-NeuAc **13**, 7-deoxy-NeuAc **16**) as shown in Scheme 1. This scheme represents slightly modified routes of several previously reported synthetic routes for NeuAc analogues.[11]

Simple Labeling Method Consisting of Exchange Reaction between a NeuAc Analogue and a [3-^{13}C]-Pyruvic Acid Mediated by NeuAc Aldolase. When aldolase is added to a mixture of a NeuAc analogue and [3-^{13}C]-pyruvic acid, [3-^{13}C]-pyruvic acid should be incorporated into the first three carbons (C1−C2−C3) of the NeuAc analogue since the NeuAc aldolase reaction is reversible. As a simple ^{13}C-labeling method for NeuAc analogues, we first examined this exchange reaction using the following condition: NeuAc analogues (15 μmol), 3 equiv of [3-^{13}C]-pyruvic acid (45 μmol) and 10 U of NeuAc aldolase (Figure 1). The degree of ^{13}C-labeling was determined by measurement of the integration values for the both the H-3 proton of [3-^{13}C]-NeuAc analogue (Figure 2A) and the CH_3-protons of [3-^{13}C]-pyruvic acid (Figure 2B) by ^{1}H NMR. The 7- and 8-deoxy-[3-^{13}C]-NeuAc analogues could not be estimated by comparison of the integration values due to overlap between the H-3^{NeuAc} proton with other protons. Therefore, the degree of mixing between [3-^{13}C]-pyruvic acid and normal pyruvic acid was monitored, and the results are shown in Figure 2B. NeuAc, 9-deoxy-9-fluoro (9-F), 9-azido-9-deoxy (9-N_3), and 9-deoxy-NeuAc analogues quickly achieved equilibrium and were labeled at rates of roughly 75% each (Figure 2A). In contrast, exchange with the 7- and 8-deoxy derivatives proceeded very slowly (Figure 2B). These analogues were labeled at lower rates even after 90 h incubation. After purification on an anion-exchange column, the labeling rate of all NeuAc analogues were determined, and the results are shown in Table 1. The labeling rates ranged from 62 to 77% for the 8-, and 9-modified NeuAc compounds. In contrast, the 7-deoxy-analogue showed only 8%

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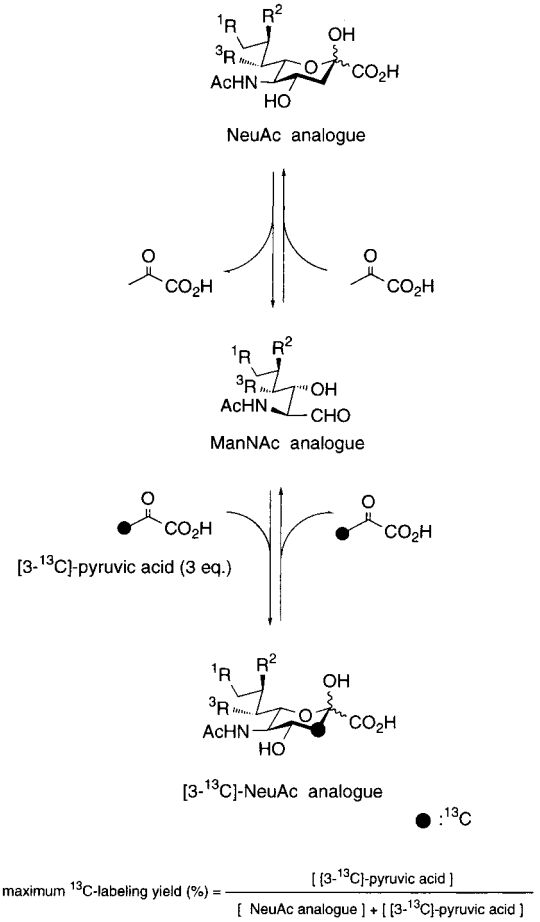

Figure 1. [3-^{13}C]-labeling method for NeuAc analogues by an exchange reaction using NeuAc aldolase.

enrichment. To determine why the labeling rates ranged from 8 to 77%, we measured the K_m and V_{max} values for the aldolase degradation of synthetic NeuAc analogues.[12] As shown in Table 1, NeuAc and the 9-modified analogues which had good labeling rates have small K_m values (2.9−4.6 mM) and higher velocities. In contrast, the 7-deoxy derivative had a large K_m value and lower velocity. Therefore, the labeling rate appears to depend on the V_{max}/K_m values of the NeuAc derivatives. The addition of more enzyme might have improved the labeling rates but would have been impractical for larger scale reactions. Therefore, we concluded that this exchange reaction was not suitable for use as a general [3-^{13}C]-labeling method for NeuAc analogues but was useful for the synthesis of ^{14}C-labeled NeuAc analogues.

Acceleration of Degradation Reaction by NeuAc Aldolase. The problem with the [3-^{13}C]-labeling method for NeuAc analogues is that reaction velocities of some analogues are very slow. We then tried to accelerate the aldolase-mediated degradation step. The velocity of the aldolase reaction depends on the quantity of pyruvic acid generated. Therefore, we added lactate dehydrogenase (LDH) and 1 equiv of β-nicotinamide adenine dinucleotide, reduced form (β-NADH), to remove the pyruvic acid, as lactic acid, from the equilibrium mixture. Preliminary results using this approach have been reported for three different analogues, the 9-F-, 9-N_3-, and 8-deoxy-analogues.[6] By this strategy, following degradation of NeuAc to ManNAc, the ManNAc derivatives were isolated and then condensed with [3-^{13}C]-pyruvic acid. Although the velocity of degradation was indeed accelerated under these conditions, the 8-deoxy-NeuAc was degraded to 5-deoxy-ManNAc in 45% yield. Further investigation revealed that the 7- and 8-deoxy NeuAc analogues were smoothly degraded to the corresponding ManNAc analogues after 9 h of incubation with 1.5 equiv of β-NADH. These reactions were monitored by measurement of the remaining NeuAc in the reaction mixture using the thiobarbituric acid

(12) Although the kinetic parameters of these NeuAc analogues were reported, K_m and V_{max} values of 9-F, 9-N_3, 9-deoxy, 8-deoxy, and 7-deoxy NeuAc toward NeuAc aldolase (*E. coli*) had not been measured under the same conditions: (a) Gantt, R.; Millner, S.; Binkley, S. B. *Biochemistry* **1964**, *3*, 1952−1960. (b) Schauer, R.; Stoll, S.; Zbiral, E.; Schreiner, E.; Brandstetter, H.; Vasella, A.; Baumberger, F. *Glycoconjugate J.* **1987**, *4*, 361−369. (c) Aisaka, K.; Igarashi, A.; Yamaguchi, K.; Uwajima, T. *Biochem. J.* **1991**, *276*, 541−546. (d) Zbiral, E.; Kleineidam, R. G.; Schreiner, E.; Hartmann, M.; Christian, R.; Schauer, R. *Biochem. J.* **1992**, *282*, 511−516.

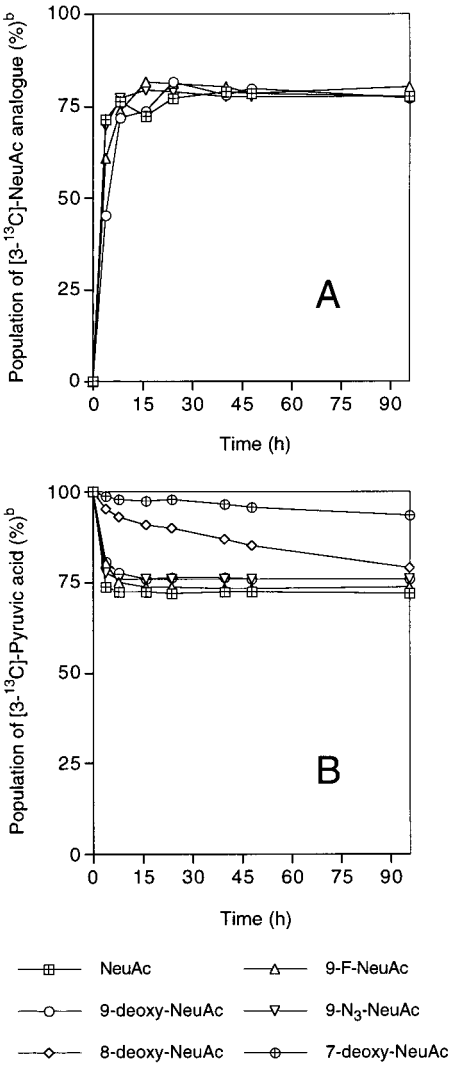

Figure 2. Degree of ^{13}C-labeling in the 3-position of NeuAc analogues[a] and (A) pyruvic acid (B) during exchange reaction. [a]7- and 8-Deoxy-NeuAc are not contained. [b]Populations were estimated using integration of H-3 proton in ^{1}H NMR spectra.

Table 1. Kinetic Parameters for NeuAc Aldolase Reacting with Several NeuAc Analogues in Degradation Reactions

substrate		^{13}C-labeling (%)	K_m (mM)	V_{max} (μmol/min)	V_{max}/K_m
NeuAc		74	4.4	56.0	12.7
9-F-NeuAc	**3**	75	4.6	42.3	9.2
9-deoxy-NeuAc	**5**	75	3.0	41.5	13.8
9-N_3-NeuAc	**8**	77	2.9	43.3	14.9
8-deoxy-NeuAc	**13**	62	19	10.4	0.55
7-deoxy-NeuAc	**16**	8	28	4.8	0.17

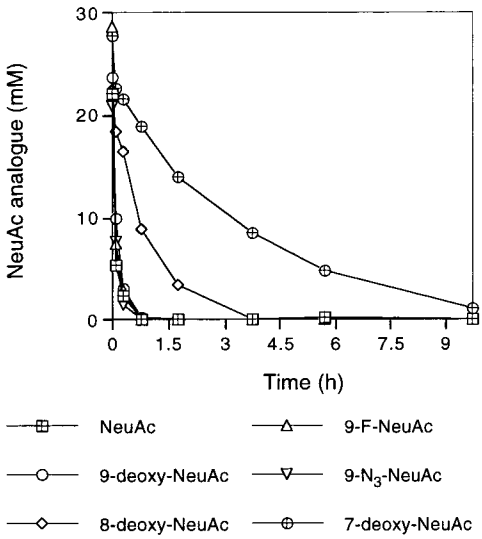

Figure 3. Degradation of NeuAc analogues by use of NeuAc aldolase and LDH (1.5 equiv of β-NADH).

assay[13] (TBA) as shown in Figure 3. These results suggest that the low yield for 8-deoxy-NeuAc under our previous reaction conditions might have arisen from decomposition of β-NADH due to the long reaction time. The accelerated velocity achieved by use of LDH corresponds to the V_{max}/K_m values of NeuAc analogues. Unfortunately, this method also proved to be impractical for ^{13}C-labeling of NeuAc analogues, because the β-NADH complicates the purification of the ManNAc analogues.

^{13}C-Labeling Method for NeuAc Analogues by One-Pot Enzymatic Procedure. The equilibrium bias for the aldolase reaction is determined by the quantity of pyruvic acid in reaction mixture. Therefore, we have developed a novel one-pot labeling method which controls the quantity of pyruvic acid in the reaction mixture as shown in Figure 4. The first step is degradation of the NeuAc analogues accelerated by cofactor regeneration.[3d,14] The second step is recondensation of [3-^{13}C]-pyruvic acid with a ManNAc analogue following inactivation of LDH. The key to this reaction is the use of nucleotide pyrophosphatase (NPP) to remove β-NADH and the oxidized form of β-nicotinamide adenine dinucleotide (β-NAD$^+$). The NPP reaction serves as a switch to stop conversion of pyruvic acid into lactic acid. After decomposition of the cofactor, addition of [3-^{13}C]-pyruvic acid triggers [3-^{13}C]-NeuAc formation. The merits of this strategy are the use of catalytic amounts of β-NADH and the ability to perform this reaction as a one-pot procedure. To determine whether this strategy was viable or not, two points had to be confirmed: (1) how much β-NADH in the cofactor regeneration is needed for degradation of NeuAc, and (2) whether NPP effectively inactivates LDH. To regenerate β-NADH from β-NAD$^+$, we utilized ethanol and alcohol dehydrogenase (ADH) because ethanol and the resulting acetaldehyde are easily removed during the workup.

We first examined degradation of NeuAc by a cofactor regeneration system. As shown in Figure 5, NeuAc was quantitatively degraded to ManNAc using the β-NADH regeneration system (β-NAD$^+$ of 0.01 equiv based on NeuAc). After 1.5 h, the reaction velocity was found to be roughly equal to that when 1.5 equiv of β-NADH is used. We then showed that NPP quantitatively hydrolyzes pyrophosphate bonds. The hydrolysis reaction of the cofactors can be easily monitored by TLC (solvent system: ethyl acetate/MeOH/H_2O = 3/2/1.5; R_f: β-NAD$^+$ = 0.20, β-NADH = 0.45).

On the basis of these results, we attempted a synthesis of [3-^{13}C]-NeuAc analogues by a one-pot enzymatic procedure, and the results are summarized in Figure 6. The reactions were monitored by measuring the quantity of NeuAc analogue by TBA. The degradation reactions were run in solutions containing 30 mg of NeuAc analogue, 30 U of NeuAc aldolase, 150 U of LDH and ADH, 120 μL of EtOH, and 0.01 equiv of β-NAD$^+$ (based on NeuAc). After 20 h, the degradation reaction of the NeuAc analogues using the cofactor regeneration system was virtually complete. Then NPP (2.8 U) was added to the reaction mixture to inactivate the LDH. After hydrolysis of pyrophosphates in β-NADH and β-NAD$^+$, [3-^{13}C]-pyruvic acid was added. The recondensation reaction proceeded immediately and appeared to achieve an equilibrium state after 20 h. After purification of [3-^{13}C]-NeuAc analogues by anion-exchange and gel permeation column chromatography, the isolation yields and ^{13}C-enrichment (%) were determined (Table 2). The yields of the condensation reaction ranged from 55 to 76% and the degree of ^{13}C-labeling ranged from 87 to 97%. In particular, the [3-^{13}C]-7-deoxy-derivative was 91% labeled, dramatically higher than that resulting from the exchange reaction (8%). This procedure provides the desired [3-^{13}C]-labeled NeuAc analogues with an excellent degree of ^{13}C-enrichment. In the condensation reaction, the yield for formation of [3-^{13}C]-NeuAc analogues estimated by TBA (Figure 6), also depended on the V_{max}/K_m values. The NeuAc, 9-deoxy-, 9-N_3-NeuAc having the largest V_{max}/K_m value achieved an equilibrium state in which the concentration of the NeuAc analogue ranged from 10 to 15 mM in the mixture, as shown in Figure 6. In contrast, the 9-F, 8-deoxy, and 7-deoxy analogues having small V_{max}/K_m values achieved equilibrium states in which the concentrations of NeuAc ranged from 15 to 20 mM. These results suggest that good substrates for degradation result in low condensation yields, but poor substrates result in good condensation yields. Although in this experiment we did not use 5-modified NeuAc, this method should also be useful for the synthesis of 5-modified-[3-^{13}C]-NeuAc derivatives.[9h−j] Since this route allows for the preparation of the desired [3-^{13}C]-NeuAc analogues in moderate yields with high degrees of ^{13}C-labeling, we were able to proceed with the synthesis of ^{13}C-labeled sialyloligosaccharide containing glycoproteins.

Synthesis of the [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-x-Ovalbumin 23 and [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-(1→4)-GlcNAc-β-OMe 26. Many examples of recognition events mediated by α-(2→3) sialoside are now known such as those involving SLe^X, GM_1, and GM_4, and many conformational studies of these ligands have been reported.[15] Nevertheless the differences in conformational properties and dynamic behavior between sialosides bound to proteins and unconjugated sialyloligosaccharides is not yet well understood. Therefore, in this study we wanted to synthesize a [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β- sequence at the nonreducing end of a glycoprotein (Scheme 2).

Esteration and selective acetylation[16] of [3-^{13}C]-NeuAc **17** afforded derivative **18**, which was then converted into cytidine 5′-monophospho-[3″-^{13}C]-NeuAc (CMP-[3″-^{13}C]-NeuAc) **20** by

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(14) (a) Mosbach, K.; Larsson, P.-O.; Lowe, C. *Methods Enzymol.* **1976**, *44*, 859−887. (b) Shaked, Z.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 7104−7105. (c) Wong, C.-H.; Whitesides, G. M. *J. Am. Chem. Soc.* **1981**, *103*, 4890−4899. (d) Matos, J. R.; Wong, C.-H. *J. Org. Chem.* **1986**, *51*, 2388−2389. (e) Ichikawa, Y.; Shen, G.-J.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 4698−4700. (f) Ichikawa, Y.; Liu, J. L.-C.; Shen, G.-J.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 6300−6302. (g) Fang, J.-M.; Wong, C.-H. *Synlett* **1994**, 393−402.

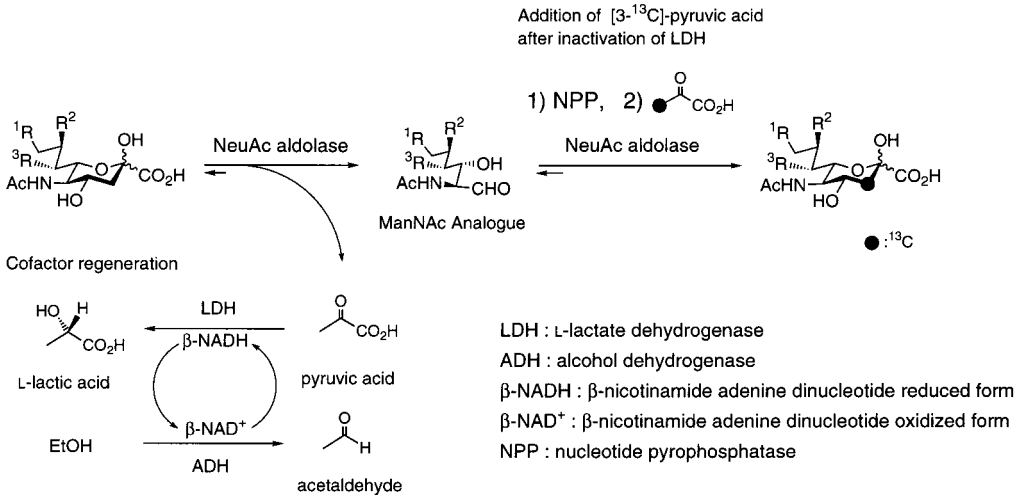

Figure 4. Novel strategy for ^{13}C-labeling method of NeuAc analogues by a one-pot enzymatic procedure.

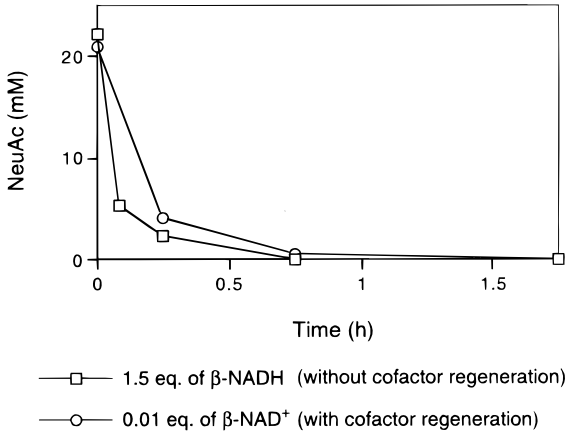

Figure 5. Comparison of degradation of NeuAc analogues using cofactor regeneration and with 1.5 equiv of β-NADH.

a method previously reported by this laboratory.[17] To synthesize the [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β- sequence on a glycoprotein, enzymatic transfer of [U-^{13}C]-Gal and [3-^{13}C]-NeuAc from the corresponding uridine 5′-diphospho-[U-^{13}C]-Glc (UDP-[U-^{13}C]-Glc)[18] and CMP-[3″-^{13}C]-NeuAc **20** was examined using ovalbumin **21**. It is known that *bovine* β-1,4-galactosyltransferase also catalyzes the transfer of glucose from UDP-Glc.[19] Therefore, the use of UDP-[U-^{13}C]-Glc risks formation of a mixture of [U-^{13}C]-Glc-β-(1→4)-GlcNAc and [U-^{13}C]-Gal-β-(1→4)-GlcNAc at the nonreducing end of the glycoprotein. However, in our first trial to transfer the [U-^{13}C]-Gal residue from UDP-[U-^{13}C]-Glc following treatment with UDP-Glc-4-epimerase, no glucosyl linkages were observed in the 2D HMQC spectrum.[6] Furthermore, to obtain a NOE between H-8^{NeuAc} and H-3^{Gal}, which is necessary for evaluation of the population of the syn conformation of the sialyl linkage,[15c] ^{13}C-labeling of the C-3^{Gal} is essential for the HMQC-NOESY measurement. However [U-^{13}C]-Gal is much more difficult to obtain than [U-^{13}C]-Glc. Therefore, we used UDP-[U-^{13}C]-Glc. Since the ovalbumin has only one oligosaccharide chain containing either one or two GlcNAc residues at the nonreducing terminal,[20] this glycoprotein was a good model substrate for our research. Transfer of the [U-^{13}C]-Gal residue proceeded smoothly toward this glycoprotein.[6] However, when the NeuAc was transferred using the *rat recombinant* α-2,3-sialyltransferase, only 50% of the galactoside was estimated to be sialylated according to the 2D HMQC spectrum despite repeated sialylation reactions on this glycoprotein. Therefore, the [U-^{13}C]-Gal which was not sialylated was hydrolyzed by *Diplococcus pneumoniae* β-galactosidase. Consequently the sialoside **23** was obtained in an analytically pure state. We also prepared [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-(1→4)-GlcNAc-β-OMe **26**, to compare its conformational properties and dynamic behavior with those of the glycoprotein.

Assignments of the ^{1}H and ^{13}C Chemical Shifts of the [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β- on an Ovalbumin. A 2D HMQC experiment on [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-x-ovalbumin **23** (x: hybrid type) was performed to analyze the ^{1}H and ^{13}C chemical shifts of the ^{13}C-labeled positions. Slices from the galactosyl part of the 2D HMQC spectrum are shown in Figure 7 d−h. The differences in ^{13}C chemical shift values between galactoside **22** and sialoside **23** were measured in order to determine the sialylated position. The chemical shift of the C-3^{Gal} of galactoside **22** was shifted downfield (2.93 ppm), and those for both C-2^{Gal} and C-4^{Gal} of

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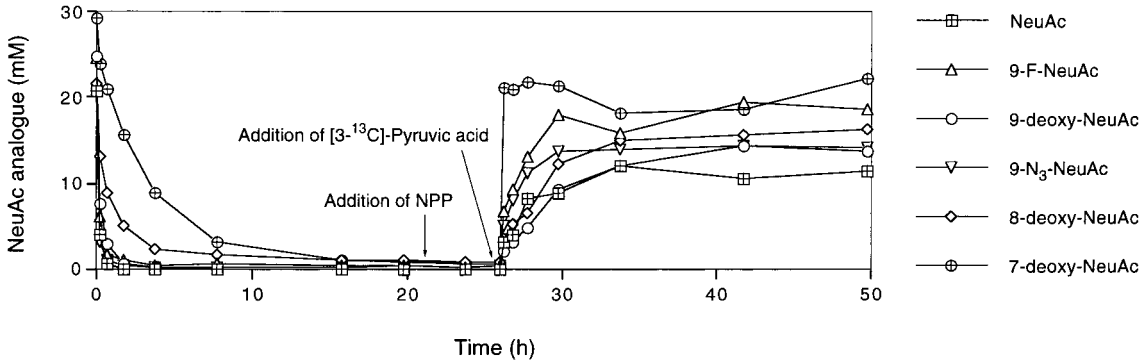

Figure 6. Quantity (mM) of NeuAc analogues in the reaction mixture during a one-pot [3-^{13}C]-labeling method.

Table 2. Results for the ^{13}C-labeling Method of NeuAc Analogues by a One-Pot Enzymatic Procedure

substrate		conversion yield (%)[a] degradation	condens.	isolated yields (%)	^{13}C-labeling (%)[b]
NeuAc		99 (68)[c]	55	52	94 [74][d]
9-F-NeuAc	**3**	98 (48)	75	62	96 [75]
9-deoxy-NeuAc	**5**	99	56	51	96 [75]
9-N_3-NeuAc	**8**	99 (60)	68	54	97 [77]
8-deoxy-NeuAc	**13**	95 (26)	75	76	87 [62]
7-deoxy-NeuAc	**16**	96	76	46	91 [8]

[a] Conversion yields were determined by thiobarbituric acid assay or HPLC. [b] The yields were determined by ^{1}H NMR spectra of reaction mixture. [c] The yields designated in parentheses are degradation yield by use of only NeuAc aldolase.[6] [d] The yields designated in brackets are ^{13}C-labeling yields for the exchange reaction using NeuAc aldolase.

galactoside **22** were shifted upfield (1.55 ppm, 1.05 ppm, respectively) in the ^{13}C NMR spectrum. The H-3^{Gal} peak of galactoside **22** in the ^{1}H NMR spectrum was also shifted downfield (0.47 ppm). Therefore, the sialylated position was determined to be the 3-position of the galactoside **22**.[21] To assign the ^{1}H chemical shifts of other protons on the NeuAc residue, a 1D HSQC-TOCSY experiment with selective excitation of C-3^{NeuAc} was performed, and the spectrum obtained is shown Figure 7a (carbon decoupling was not used during acquisition). Although the H-4^{NeuAc} peak is broad, the ^{1}H chemical shifts of H-4^{NeuAc}, H-5^{NeuAc}, and H-6^{NeuAc}, and the coupling constants ($J_{H4,H5}$, $J_{H5,H6}$) were determined. As shown in Figure 7a, the small coupling constant ($J_{H6,\ H7} < \sim$1.5 Hz) normally found in NeuAc often makes it impossible to observe the correlations between H-6^{NeuAc} and H-7^{NeuAc} in a TOCSY spectrum. Therefore, it is also difficult to assign the H-7, -8, -9, and -9′ protons with one ^{13}C atom at the 3-position of a conjugated sialyloligosaccharide. To overcome the problem, we developed a new measurement technique. Homonuclear TOCSY-NOESY-TOCSY has recently been reported.[10] This measurement technique was used for NeuAc residues of a large molecular weight capsular polysaccharide in order to observe all of the protons of the NeuAc residues. The mechanism of this measurement technique is transfer of H-6 magnetization, detected by the first TOCSY from H-3^{NeuAc}, to H-7 by NOESY and then the magnetization of H-7 is transferred to H-9 by the second TOCSY. If a combined HSQC and TOCSY-NOESY-TOCSY experiment, that is, HSQC-TOCSY-NOESY-TOCSY, could be developed, all protons from H-3 to H-9 of a NeuAc residue could be assigned using one ^{13}C atom at the 3-position of the NeuAc residue, even on an intact glycoprotein. Therefore, we conducted a 1D HSQC-TOCSY-NOESY measurement using several NOESY mixing times (1, 50, 100, 200, 300, 400, and 500 ms). As shown in Figure 8, the NOE between H-6^{NeuAc} and H-7^{NeuAc} clearly increased, depending on the mixing time, and the maximum NOE as an inphase magnetization was observed at τ_m = 300 ms. By this measurement, the H-7^{NeuAc} chemical shift and coupling constant $J_{H7,H8}$ were determined (Figure 7b). Finally, we conducted the 1D HSQC-TOCSY-NOESY-TOCSY experiment. Unfortunately, the H-6 and H-7 peaks overlapped, hindering our ability to selectively excite H-7 in the second TOCSY. As a result the 1D spectrum contained both the desired broad H-7, -8, -9, and -9′ peaks as well as the undesired H-4, -5, and -6 peaks, as shown in Figure 7c. In this measurement, decreasing peak intensity results from two factors: (1) transverse magnetization is used in three of the measurements (i.e., HSQC-TOCSY, TOCSY-NOESY, and NOESY-TOCSY), and (2) selective excitation pulses[22] are used twice. As a result the intensity of the magnetization decreased dramatically. In addition, when the magnetization of H-7 was extracted, peak intensity of its magnetization had decreased to less than 10% of the absolute intensity of H-3 as observed by HSQC. Although we tried to optimize this measurement in order to eliminate the undesired H-4, -5, and -6 peaks, we could not obtain the desired spectrum. However the spectral pattern in the 1D HSQC-TOCSY-NOESY-TOCSY spectrum obtained here was almost identical to that reported for NeuAc in a capsular polysaccharide.[10] Despite this difficulty, assignment of the H-8, -9, and -9′ peaks was roughly accomplished. We then developed a 2D version of the HSQC-TOCSY-NOESY-TOCSY spectrum (Figure 9). In this spectrum the resonance of these cross-peaks could not be improved despite trying several conditions. The desired cross-peaks for H-8, -9, and -9′ were observed clearly in the expected chemical shift ranges and are in good agreement with those of unconjugated sialyl LacNAc **26**. The merit of this 2D measurement is that selective excitation of the H-7 proton is not essential for the last TOCSY pulse sequence. Therefore, the loss of transverse magnetization of H-7 is small compared to the 1D-version. In addition, TOCSY development from H-7 to H-9, -9′ can be easily observed as a well-separated cross-peak from TOCSY development of H-4, -5, and -6. The ^{1}H- and ^{13}C-chemical shifts and the coupling constants for [U-^{13}C]-Gal-β-x-ovalbumin **22**, [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-x-ovalbumin **23**, [U-^{13}C]-Gal-β-(1→4)-GlcNAc-β-OMe (LacNAc) **25**, and [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-(1→4)-GlcNAc-β-OMe (sialyl LacNAc) **26** are summarized in Table 3.

^{13}C Spin−Lattice Relaxation Times of the [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-x-Ovalbumin 23 and [3-^{13}C]-NeuAc-

(21) Bradbury, J. H.; Jenkins, G. A. *Carbohydr. Res.* **1984**, *126*, 125−156.

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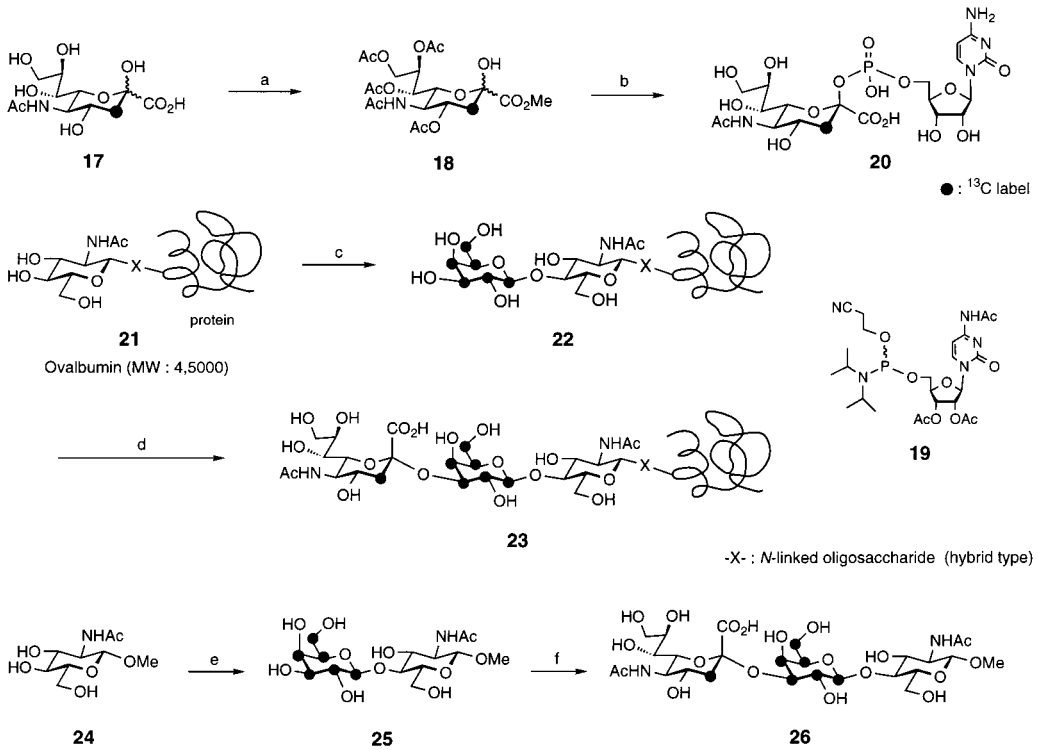
Scheme 2[a]



[a] Reagents: (a) (1) Dowex 50W-X8 (H^+), MeOH; (2) $HClO_4$, Ac_2O, y = 37% (2 steps); (b) (1) 1H-tetrazole, MeCN, **19**; (2) *t*-BuOOH, MeCN; (3) DBU; (4) NaOMe, MeOH: H_2O = 1:2, y = 35% (4 steps); (c) UDP-[U-^{13}C]-glucose, UDP-glucose-4-epimerase, *bovine* β-1,4-galactosyltransferase; (d) (1) *rat recombinant* α-2,3-(*N*)-sialyltransferase, **20**; (2) *D. pneumoniae* β-galactosidase; (e) UDP-[U-^{13}C]-glucose, UDP-glucose-4-epimerase, *bovine* β-1,4-galactosyltransferase, y = 57%; (f) *rat recombinant* α-2,3-(*N*)-sialyltransferase, **20**, y = 30%.

α-(2→3)-[U-^{13}C]-Gal-β-(1→4)-GlcNAc-β-OMe 26. ^{13}C spin−lattice relaxation times (T_1) have often been used to evaluate the molecular dynamics of oligosaccharides.[2i,23] The ^{13}C atom is a suitable nucleus for this purpose since the relaxation time (T_1^{DD}) is dominated by carbon−proton dipolar relaxation for protonated carbons, and since NT_1^{DD} and τ_c are inversely proportional (where N is the number of protons attached to the carbon).[24] Therefore, we measured the T_1^{obsd} and T_1^{DD} of the ^{13}C labeled position in [U-^{13}C]-Gal-β-x-ovalbumin **22**, [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-x-ovalbumin **23**, [U-^{13}C]-Gal-β-(1→4)-GlcNAc-β-OMe **25**, and [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-(1→4)-GlcNAc-β-OMe **26** by the use of 100.61 MHz NMR for ^{13}C nuclei (9.4 T). The T_1 values for [U-^{13}C]-Gal-β-x-ovalbumin **22** were previously reported using a 25 MHz spectrometer.[18a] However we conducted this measurement again because the T_1 value is dependent on the Larmor frequency. All T_1^{obsd} values are averages of experiments performed in triplicate and are summarized in Table 4. In the comparison between [U-^{13}C]-Gal-β-x-ovalbumin **22** and unconjugated LacNAc **25**, the difference of each T_1^{DD} value is small. Attachment of sialic acid to galactoside **25** caused a decrease in the T_1^{DD} value for 230 ms at the 3-position of the galactoside, but the T_1^{DD} of other carbons were only slightly decreased. However, in the case of [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-x-ovalbumin **23**, the T_1^{DD} values of C-2^{Gal}, C-3^{Gal}, and C-4^{Gal} dramatically decreased for 240, 390, and 280 ms, respectively. This phenomena indicates that the flexibility of the galactosyl ring is severely restricted by the attachment of sialic acid to the 3-position as compared with that of the unconjugated sialyl LacNAc **26**. The T_1^{DD}'s of the C-3^{NeuAc} are similar for both sialoside **23** and **26**, and this suggests the flexibility of the NeuAc ring is not affected by the attachment of the glycoprotein.

Conformational Properties of the [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-Ovalbumin 23 and Sialoside 26. To analyze the conformational properties, we measured NOE by both 1D and 2D HMQC-NOESY techniques. In the 2D HMQC-NOESY, we observed NOEs to H-4^{NeuAc}, H-5^{NeuAc}, and H-3^{Gal} from either H-3ax^{NeuAc} or H-3eq^{NeuAc}. We could not determine whether the NOE was derived from H-3ax^{NeuAc} or H-3eq^{NeuAc}, because the 2D HMQC-NOESY shows NOEs to the H-4^{NeuAc}, H-5^{NeuAc}, and H-3^{Gal} at the same chemical shifts as the C-3^{NeuAc} in the F1 dimension (Figure 10f). Therefore, we modified the 1D HMQC-NOESY in order to determine whether the NOE resulted from H-3ax^{NeuAc} or H-3eq^{NeuAc}. We changed the two rectangular pulses (90°) for both proton and carbon to Gaussian and half-Gaussian shaped selective pulses, respectively. The pulse sequence is summarized in the Experimental Section (Figure 13). As shown in Figures 10b and 10d, we have 1D HMQC spectra showing the individual excitations of H-3ax^{NeuAc} and H-3eq^{NeuAc}, respectively. We applied this magnetization to the NOESY pulse sequence. The 1D HMQC-NOESY spectra show that NOEs to H-3eq^{NeuAc}, H-4^{NeuAc}, H-5^{NeuAc}, and H-3^{Gal} resulted from H-3ax^{NeuAc} (Figure 10c). NOEs from H-3eq^{NeuAc} were observed for H-3ax^{NeuAc} and H-4^{NeuAc} (Figure 10e). These NOE patterns are similar to those of unconjugated sialyl LacNAc **26** except for the NOE between H-3^{Gal} and H-8^{NeuAc} (Table 5, H-3^{Gal} excitation). In addition we also observed NOEs from H-1^{Gal} on the protein (Figure 7i). Since chemical shifts of these

(23) (a) Czarniecki, M. F.; Thornton, E. R. *J. Am. Chem. Soc*. **1977**, *99*, 8279−8282. (b) Berman, E.; Allerhand, A. *J. Biol. Chem*. **1980**, *255*, 4407−4410. (c) Rutherford, T. J.; Partridge, J.; Weller, C. T.; Homans, S. W. *Biochemistry* **1993**, *32*, 12715−12724. (d) Poppe, L.; van Halbeek, H.; Acquotti, D.; Sonnino, S. *Biophys. J*. **1994**, *66*, 1642−1652. (e) van Zuylen, C. W. E. M. T.; de Beer, T.; Leeflang, B. R.; Boelens, R.; Kaptein, R.; Kamerling, J. P.; Vliegenthart, J. F. G. *Biochemistry* **1998**, *37*, 1933−1940.

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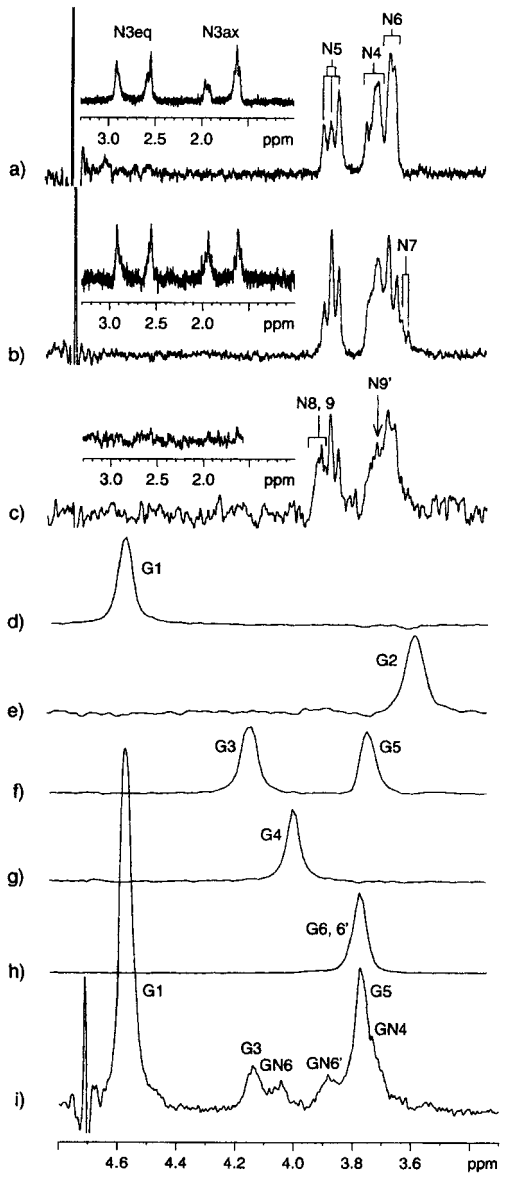

Figure 7. 1D NMR spectra of [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-x-ovalbumin **23**. (a) 1D HSQC-TOCSY obtained with the pulse sequence from Figure 13C. (b) 1D HSQC-TOCSY-NOESY (NOESY mixing time: 300 ms) obtained with the pulse sequence from Figure 13D. (c) 1D HSQC-TOCSY-NOESY-TOCSY (NOESY mixing time 300 ms) obtained with the pulse sequence from Figure 13E. (d) A slice of C-1^{Gal} in 2D HMQC spectrum. (e) A slice of C-2^{Gal} in 2D HMQC spectrum. (f) A slice of C-3^{Gal} and C-5^{Gal} in 2D HMQC spectrum. (g) A slice of C-4^{Gal} in 2D HMQC spectrum. (h) A slice of C-6^{Gal} in 2D HMQC spectrum. (i) A slice of C-1^{Gal} in 2D HMQC-NOESY spectrum (NOESY mixing time: 100 ms). N: NeuAc, G: Gal, GN: GlcNAc. The span of all 1D spectra (a−i) is identical.

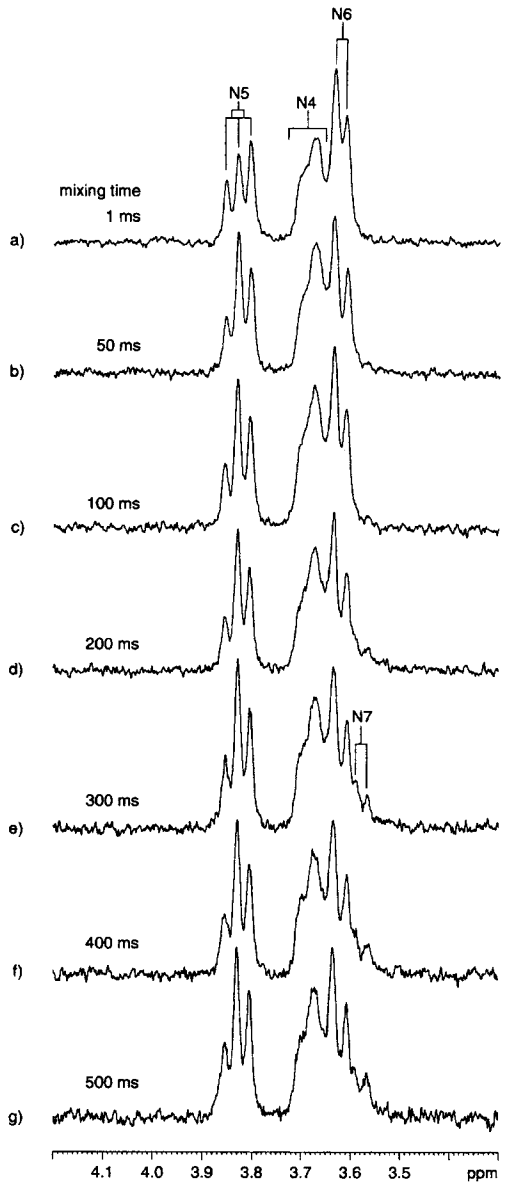

Figure 8. 1D HSQC-TOCSY-NOESY spectra of sialoside **23** with different mixing times.

NOEs do not overlap with protons of the NeuAc residue or the galactoside but are identical to the chemical shifts of H-4^{GlcNAc}, H-6^{GlcNAc}, and H-6′^{GlcNAc} of **26**, we assigned the observed NOEs from H-1^{Gal} are to be H-4^{GlcNAc}, H-6^{GlcNAc}, and H-6′^{GlcNAc}. All observed NOEs and build up rate curves for H-3ax^{NeuAc} of conjugated form **23** and unconjugated **26** are summarized in Table 5 and Figure 11, respectively.

Conformational properties of the common NeuAc-α-(2→3)-Gal-β- sequence in oligosaccharides have been reported by several research groups.[15] In GM_1, the sialyl linkage adopts a near anti conformation due to steric hindrance of neighboring branched disaccharide chain, Gal-β-(1→3)-GalNAc.[15d] Other sialyl linkages which have been found exist in a variety of syn: anti ratios. For example, NeuAc-α-(2→3)-Gal-α,β-OH,[15c] NeuAc-α-(2→3)-Gal-β-(1→4)-GlcNAc-β-Asn,[15b] and NeuAc-α-(2→3)-Gal-β-(1→4)-GlcNAc-β-$(CH_2)_8$COOMe,[15f] exist in syn:anti populations of 1:1, 3:1, and 0:1, respectively. Therefore, the conformation of the α-(2→3)-sialyl linkage appears to have no fixed standard conformation, and to be heavily dependent on the aglycons at the reducing end of the galactoside and GlcNAc. It is also reported that the NOE between H-3^{Gal} and H-8^{NeuAc}, and between H-3^{Gal} and H-3ax^{NeuAc} are caused by syn (i.e., COOH−C2−O−C3 ≅ −85°) and anti (i.e., COOH−C2−O−C3 ≅ −153°) conformers of the sialyl linkage, respectively.[15c] Therefore, these NOEs are essential to an evaluation of syn: anti populations. For NeuAc-α-(2→3)-Gal-β-(1→4)-GlcNAc-β-OMe **26**, we observed two typical NOEs, between H-3^{Gal} and H-8^{NeuAc} and between H-3^{Gal} and H-3ax^{NeuAc} (Table 5). However the intensity of the NOE between H-3^{Gal} and H-8^{NeuAc} was found to be very small in comparison with that between H-3^{Gal} and H-3ax^{NeuAc}. The ratio of these NOE volumes (H-3^{Gal}/H-8^{NeuAc}: H-3^{Gal}/H-3ax^{NeuAc}) in **26** was estimated to be <1:8 by the use of a build-up rate curve (NOESY mixing time: 100 ms, data

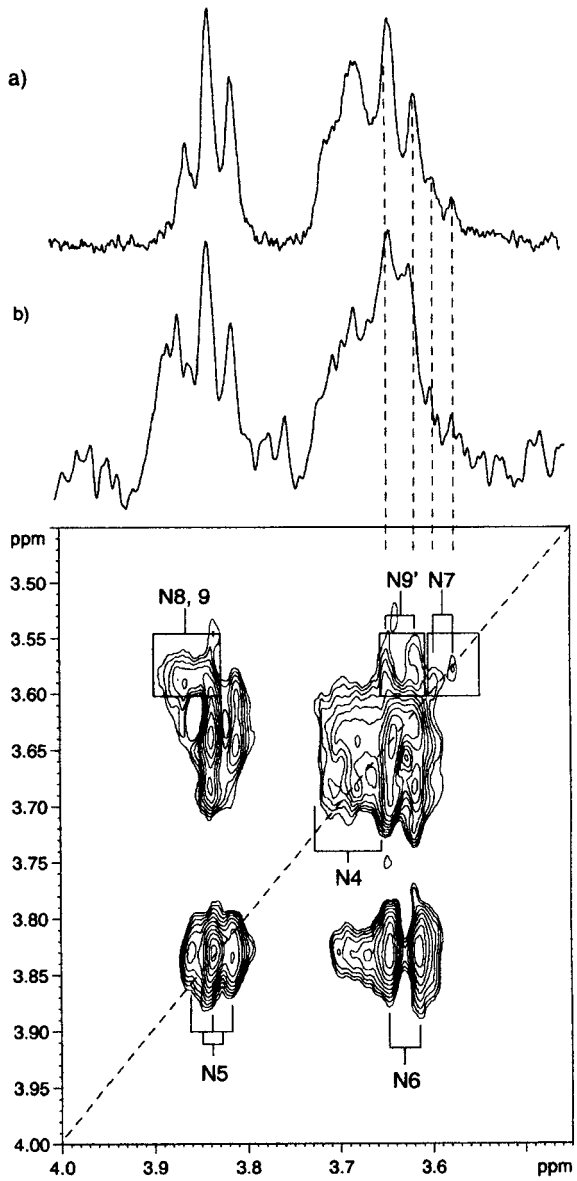

Figure 9. 2D HSQC-TOCSY-NOESY-TOCSY spectrum of sialoside **23**. (a) 1D HSQC-TOCSY-NOESY (Figure 8e). (b) 1D HSQC-TOCSY-NOESY-TOCSY (Figure 7c). Dashed line in the 2D spectrum shows diagnal peaks.

are not shown). Therefore, these results suggest that conformation of unconjugated sialyl LacNAc **26** is biased to the anti conformation. On the other hand, an NOE between H-3^{Gal} and H-8^{NeuAc} was not observed in the conjugated sialylgalactoside **23** under various experimental conditions (for example, using shorter or longer mixing times or increasing the scan time), but an NOE between H-3^{Gal} and H-3ax^{NeuAc} was clearly observed. These results suggest that the sialyl linkage of the glycoprotein also adopts a near anti conformation. The distance between H-3ax^{NeuAc} and H-3^{Gal} was then estimated according to a reported procedure.[25] As shown in Figure 11, since both the NOE build up curves for H-3ax^{NeuAc} excited (A: conjugated, B: unconjugated) are not saturated until at 50 ms of mixing time, peak volume of H-3eq^{NeuAc} and H-3^{Gal} at 50 ms were used to estimate the length (standard NOE H-3ax^{NeuAc}/H-3eq^{NeuAc}: 1.77 Å). The estimated distance between H-3^{Gal} and H-3ax^{NeuAc} was 2.7 Å (conjugated) and 3.0 Å (unconjugated). However, the distance between H-3^{Gal} and H-3ax^{NeuAc} in the conjugated form is found to be lengthy compared with those for the sialyl linkages which adopt the anti conformation.[15f] There are two possible reasons for this. In the first case the population of anti conformer is slightly decreased due to equilibrium between syn and anti conformations. In the latter case the major conformation is the anti conformation in which distance between H-3^{Gal} and H-3ax^{NeuAc} is about 2.7 Å. However, since we could not prove this, we simulated an average 3D structure of sialyl LacNAc in which the distance between H-3^{Gal} and H-3ax^{NeuAc} is constrained to 2.7 Å. In addition, since the NOEs to H-4^{GlcNAc}, H-6^{GlcNAc}, and H-6′^{GlcNAc} from H-1^{Gal} are observed, the distance between proton pairs (H-6^{GlcNAc}, H-1^{Gal}) and (H-6′^{GlcNAc}, H-1^{Gal}) can be estimated to be 3.0 and 3.0 Å, respectively, and these values were also used as constraints for simulation. The global minimum structure (COOH−C2^{NeuAc}−O−C3^{Gal} = −146.7°, C2^{NeuAc}−O−C3^{Gal}−H3^{Gal} = −25.6°, H1^{Gal}−C1^{Gal}−O−C4^{GlcNAc} = 51.3°, C1^{Gal}−O−C4^{GlcNAc}−H4^{GlcNAc} = −7.4°), as shown in Figure 12, was in good agreement with those of the previously reported unconjugated sialyl LacNAc.[15d,f]

These results suggest that the conformational properties of sialyloligosaccharides are not influenced by attachment to the glycoprotein, but that the flexibility of the sugar backbone is decreased. In our case, since the sialyloligosaccharide may be exposed on protein surface, the conformational properties are not very different from those of the unconjugated form. We were able to obtain insight into the conformational properties on a large molecular glycoprotein (>40 kDa) for the first time, and we believed this methodology may be applied to different conjugated sialyloligosaccharide−protein combinations.

Conclusions

In summary, we have described a concise ^{13}C-labeling method for 5-, 7-, 8-, and 9-modified NeuAc analogues using a one-pot enzymatic procedure. This procedure is based on biasing the equilibrium in the reversible aldolase reaction using cofactor regeneration and reaction with a nucleotide pyrophosphatase. This procedure successfully afforded five different [3-^{13}C]-NeuAc analogues in which the degree of ^{13}C-labeling is 87% or greater. This strategy enabled us to synthesize a [3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-*β*- sequence by the use of glycosyltransferases on an intact glycoprotein. We also analyzed the conformational properties of sialoside **23** on a glycoprotein using several standard NMR techniques and developed a new procedure, 1D HSQC-TOCSY-NOESY-TOCSY, for this purpose. The ^{13}C-labeled sialoside **23** bound to a glycoprotein gave us highly useful information, namely, that the major conformation of sialyl linkage is anti. We were able to demonstrate that a combined chemical, enzymatic, and NMR technique allows for the precise synthesis of a sialylgalactoside on an intact glycoprotein. In addition, with only minimal ^{13}C-enrichment (the 3-position of NeuAc residue and a [U-^{13}C]-galactoside), we were able to analyze the conformation of such glycoprotein-bound sialylgalactosides, as if we had been dealing with a much smaller molecular weight sialyloligosaccharide.

Experimental Section

NMR spectra were recorded with JEOL EX-270 or Bruker AVANCE 400 instruments. The chemical shifts of ^{1}H NMR are presented in ppm and referenced to tetramethylsilane (δ = 0.00 ppm) in $CDCl_3$, HOD (δ = 4.69 ppm) in D_2O, and HOD (δ = 4.69 ppm) in CD_3OD as an internal standard. The chemical shifts of ^{13}C NMR spectra are expressed in ppm and referenced to $CDCl_3$ (δ = 77.00 ppm) in $CDCl_3$, 1,4-dioxane (δ = 67.17 ppm, external) in D_2O, and CD_3OD (δ = 49.80 ppm) in CD_3OD. Thin-layer chromatography (TLC) was used DC-Platten

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Table 3. ^{1}H and ^{13}C NMR Data of **22**, **23**, **25**, and **26**

residue	δ^a	(J^a)	**22**	**25**	**23**	**26**
α-D-NeuAc	H-3ax	($^3J_{H3ax,H3eq}$)			1.78 (10.8)	1.77 (12.4)
	H-3eq	($^3J_{H3ax,H4}$)			2.75 (10.8)	2.73 (12.4)
	H-4	($^2J_{H3eq,H4}$)			3.67	3.67 (4.6)
	H-5	($^3J_{H4,H5}$)			3.83 (10.0)	3.82 (9.9)
	H-6	($^3J_{H5,H6}$)			3.62 (10.0)	3.62 (10.4)
	H-7	($^3J_{H6,H7}$)			3.58 (<1.5)[b]	3.57 (1.4)
	H-8	($^3J_{H7,H8}$)			3.90−3.83 (8.4)	3.86 (8.7)
	H-9	($^2J_{H8,H9}$)			3.90−3.83	3.85 (2.5)
	H-9′	($^2J_{H9',H9}$)			3.63 (11.3)	3.62 (11.8)
		($^3J_{H8,H9'}$)				(5.9)
	NAc					2.01
	C-3	($^1J_{C3,H3ax}$)			40.09 (128.4)	40.04 (130.2)
		($^1J_{C3,H3eq}$)			(134.3)	(134.9)
	C-4					68.27
	C-5					52.20
	C-6					73.13
	C-7					68.37
	C-8					72.11
	C-9					63.05
	NAc					22.58
β-D-Gal	H-1	($^3J_{H1,H2}$)	4.43	4.44 (7.8)	4.53	4.52 (7.8)
	H-2		3.52	3.51	3.56	3.54
	H-3		3.63	3.64	4.10	4.08
	H-4		3.89	3.90	3.95	3.93
	H-5		3.70	3.70	3.70	3.69
	H-6,6′		3.80−3.66	3.77−3.67	3.81−3.67	3.73−3.67
	C-1	($^1J_{C1,C2}$)	103.33 (45.5)	103.33 (45.9)	103.04 (47.9)	103.01 (47.1)
	C-2	($^1J_{C2,C3}$)	71.33 (39.3)	71.37 (39.7)	69.78 (40.3)	69.77 (40.3)
	C-3	($^1J_{C3,C4}$)	72.95 (38.5)	72.98 (38.7)	75.88 (38.4)	75.90 (38.4)
	C-4	($^1J_{C4,C4}$)	68.95 (38.5)	68.96 (38.6)	67.90 (38.4)	67.90 (38.4)
	C-5	($^1J_{C5,C6}$)	75.76 (44.5)	75.78 (44.5)	75.56 (44.7)	75.57 (44.3)
	C-6		61.42	61.44	61.43	61.43
β-D-GlcNAc	H-1	($^3J_{H1,H2}$)		4.43 (7.6)		4.43 (7.8)
	H-2			3.69		3.76−3.65
	H-3			3.76−3.65		3.76−3.65
	H-4			3.67	3.69	3.67
	H-5	($^3J_{H5,H6}$)		3.55 (2.2)		3.58 (2.1)
	H-6	($^3J_{H5,H6'}$)		3.98 (5.2)	4.03	3.99 (5.7)
	H-6′	($^2J_{H6,H6'}$)		3.81 (12.3)	3.85	3.82 (12.1)
	H-NAc			2.01		2.01
	H-OMe			3.48		3.48
	C-1			102.31		102.80
	C-2			55.42		55.40
	C-3			73.02		73.07
	C-4			79.09		78.58
	C-5			75.46		75.41
	C-6			60.63		60.65
	C-OMe			57.53		57.52
	C-NAc			22.61		22.58

[a] The chemical shifts and coupling constants were presented in ppm and Hz, respectively. ^{1}H chemical shifts were measured at 303 K (internal standard: HOD = 4.69 ppm), ^{13}C chemical shifts were measured at 303 K (external standard: 1,4-dioxane = 67.17 ppm). [b] The HSQC-TOCSY did not show a H-7^{NeuAc} resonance from H-6^{NeuAc}.

Table 4. Relaxation Parameters T_1^{obsd}, NOE, and T_1^{DD} for ^{13}C Nuclei in D_2O Solution at 303 K[a]

		22			**25**			**23**			**26**		
		T_1^{obsd} (s)	NOE (1+η)	T_1^{DD} (s)	T_1^{obsd} (s)	NOE (1+η)	T_1^{DD} (s)	T_1^{obsd} (s)	NOE (1+η)	T_1^{DD} (s)	T_1^{obsd} (s)	NOE (1+η)	T_1^{DD} (s)
NeuAc	C-3	-	-	-	-	-	-	0.15	2.1	0.27	0.18	2.3	0.28
Gal	C-1	0.34	1.9	0.75	0.54	2.4	0.77	0.28	1.8	0.70	0.32	2.0	0.64
	C-2	0.34	1.9	0.75	0.53	2.5	0.70	0.28	2.1	0.51	0.31	1.9	0.68
	C-3	0.34	1.9	0.75	0.53	2.4	0.75	0.20	2.1	0.36	0.26	2.0	0.52
	C-4	0.33	1.9	0.73	0.44	2.4	0.62	0.25	2.1	0.45	0.28	1.8	0.70
	C-5	0.32	2.0	0.64	0.53	2.4	0.75	0.28	2.0	0.56	0.32	2.1	0.58
	C-6	0.22	2.3	0.34	0.36	2.6	0.45	0.20	2.1	0.36	0.24	2.4	0.34
ave.		0.32	2.0	0.66	0.49	2.5	0.67	0.25	2.0	0.49	0.29	2.0	0.58

[a] T_1^{DD} (s) = $(1.988/\eta)T_1^{obsd}$ (s).

Kieselgel 60 F_{254} (Merck). Column chromatography was carried out on Merck Silica gel 60 of 230−400 mesh.

Materials. The solvents and reagents were purified according to standard procedures. *N*-acetyl-neuraminic acid aldolase (NeuAc aldolase, EC 4.1.3.3) was obtained from TOYOBO Co., Ltd. Lactate dehydrogenase (LDH, EC 1.1.1.27) and β-nicotinamide adenine dinucleotide oxidized form (β-NAD^+) were from Oriental Yeast Co., Ltd. [3-^{13}C]-Sodium pyruvate (99.1% ^{13}C-labeled) was obtained from

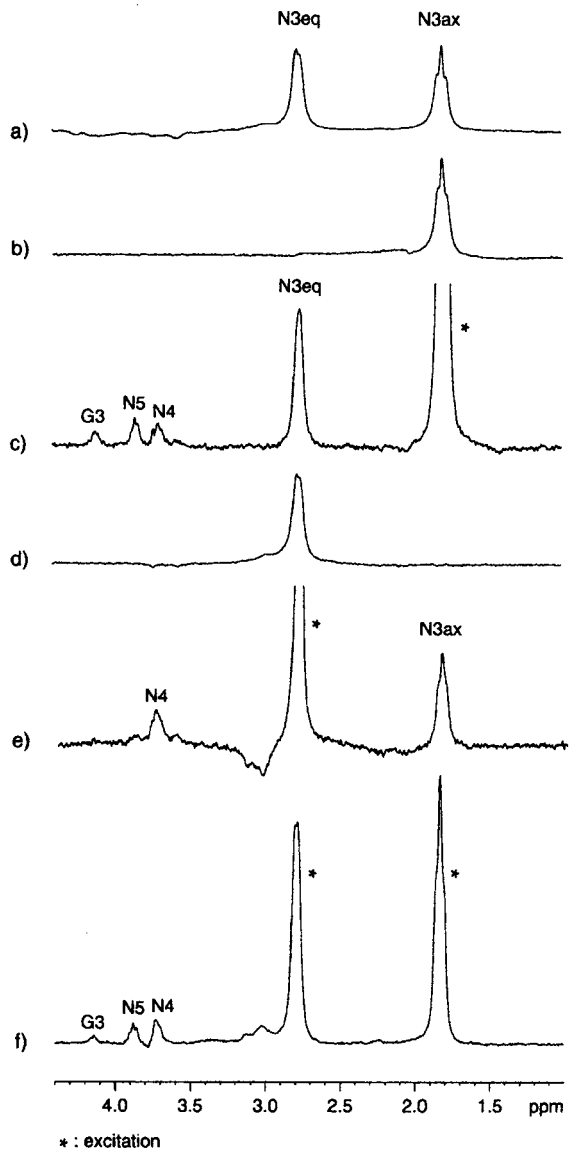

Figure 10. 1D HMQC-NOESY spectrum of sialoside **23** with shaped pulses for both H-3ax^{NeuAc} (or H-3eq^{NeuAc}) and C-3^{NeuAc}. (a) Standard 1D HMQC spectrum[6] with selective excitation for C-3^{NeuAc}. (b) 1D HMQC (selective excitation for H-3ax^{NeuAc}) using modified pulse sequence from Figure 13A. (c) 1D HMQC-NOESY (selective excitation for H-3ax^{NeuAc}) using modified pulse sequence from Figure 13B. (d) 1D HMQC (selective excitation for H-3eq^{NeuAc}) using the modified pulse sequence from Figure 13A. (e) 1D HMQC-NOESY (selective excitation for H-3eq^{NeuAc}) using modified pulse sequence from Figure 13B. (f) Slice of C-3^{NeuAc} resonance in 2D HMQC-NOESY spectrum.

Table 5. Intra- and Inter-residual ^{1}H/^{1}H NOE in Sialoside **23** and **26**

	excitation	**23**	**26**
NeuAc	H-3ax	NeuAc-H4, 5, **Gal-H3**	NeuAc-H4, 5, **Gal-H3**
	H-3eq	NeuAc-H4	NeuAc-H4
Gal	H-1	Gal-H3, 5, **GlcNAc-H4** **GlcNAc-H6, 6′**	Gal-H3, 5, **GlcNAc-H4** GlcNAc-H6,6′
	H-2	Gal-H1	Gal-H1
	H3	Gal-H1, 4	Gal-H1, 4, **NeuAc H8**
	H-4	Gal-H3, 6	Gal-H3, 6
	H-5	Gal-H3	Gal-H3
	H-6/6′	Gal-H4	Gal-H4

ISOTEC INC. *Rat recombinant* α-2,3-(*N*)-sialyltransferase (EC 2.4.99.5) was from CALBIOCHEM. *Calf intestinal* alkaline phosphotase (CIAP, EC 3.1.3.1) and *Diplococcus pneumoniae* β-galactosidase (EC 3.2.1.23)

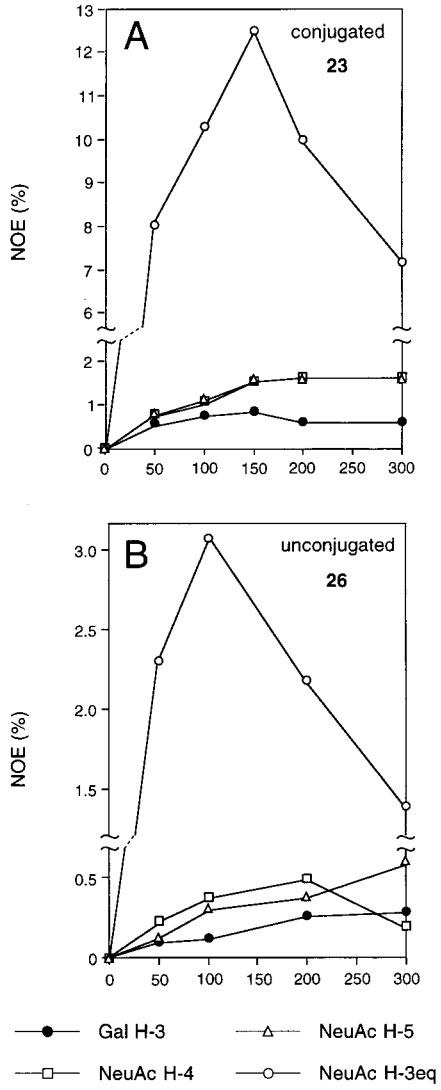

Figure 11. NOE build up curves caused by excitation of H-3ax^{NeuAc} in conjugated **23** and unconjugated **26** by several mixing times.

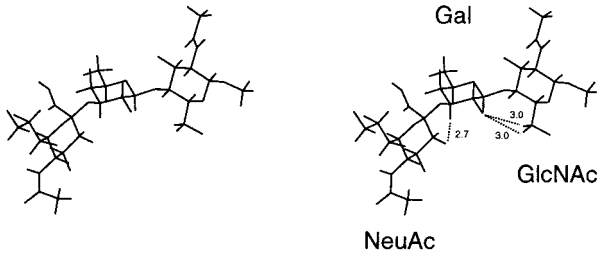

Figure 12. The global minimum energy conformation of conjugated sialoside **23**.

were from Boehriger-Mannheim. Albumin, *chicken egg* (ovalbumin), alcohol dehydrogenase (ADH, EC 1.1.1.1), nucleotide pyrophosphatase (NPP, EC 3.6.1.9), β-nicotinamide adenine dinucleotide reduced form (β-NADH), uridine 5′-diphosphoglucose 4-epimerase (EC 5.1.3.2), and *bovine* β-1,4-galactosyltransferase (EC 2.4.1.22) were purchased from Sigma. All other biochemical and chemical reagents were obtained from Sigma and Aldrich, unless otherwise indicated.

Methyl (Methyl 5-Acetamido-4,7,8-tri-*O*-benzyl-3,5,9-trideoxy-9-fluoro-α,β-D-glycero-D-galacto-2-nonulopyranosid) Onate (2). 9-Deoxy-9-fluoro-NeuAc **3** was synthesized by reported method[11a] except for fluorination. To a cold (−10 °C) solution of compound **1**[11a] (α:β = 1:24, 1.46 g, 2.4 mmol) in CH_2Cl_2 (26.7 mL) and pyridine (0.6 mL) was added trifluoromethanesulfonic anhydride (1.0 mL, 5.9 mmol) dropwise, and then the mixture was allowed to warm to room

temperature. After 45 min, the mixture was diluted with CH_2Cl_2 and washed sequentially with 0.2 M HCl and water. The organic phase was dried with $MgSO_4$ and then concentrated in vacuo. The residue was dried under vacuum (15 Pa) for 1 h. To a solution of this residue in CH_2Cl_2 (12.6 mL) was added tris(dimethylamino)sulfonium difluorotrimethylsilicate (TASF, 1.32 g, 4.8 mmol) at −15 °C, and then the mixture was allowed to warm to room temperature. After 12 h, the mixture was diluted with CH_2Cl_2, washed with water, and dried with $MgSO_4$. After concentration in vacuo, purification of the residue on a silica gel column chromatography (toluene:ethyl acetate = 3:1) gave **2** (α:β = 1:24, 757 mg, 52%, 2 steps) as an amorphous mass; The physical data were identical to the reported data.[11a] Conversion of **2** into **3** was then performed with reported procedure.[11a]

Methyl (Methyl 5-Acetamido-4,7,8-tri-*O*-benzyl-3,5,9-trideoxy-α,β-D-glycero-D-galacto-2-nonulopyranosid) Onate (4). To a solution of **1** (α:β = 1:15, 637 mg, 1.05 mmol) in pyridine (5 mL) and CH_2Cl_2 (5 mL) was added phenyl chlorothionoformate (360 μL, 2.6 mmol) at room temperature, and the mixture was stirred for 2 h at room temperature. After addition of MeOH (1 mL), the mixture was concentrated. The residue was diluted with ethyl acetate and washed sequentially with saturated $CuSO_4$, water (×2), aqueous $NaHCO_3$, and saturated NaCl. After drying with $MgSO_4$, the organic phase was concentrated. The residue was dissolved in toluene (55 mL), and to this solution were added *n*-tributyltin hydride (2.8 mL, 10.4 mmol) and 2,2′-azobisisobutyronitrile (AIBN, 52 mg, 0.32 mmol). The mixture was stirred for 1 h at 80 °C and then concentrated in vacuo. Purification of the residue on a silica gel column chromatography (toluene:ethyl acetate = 2:1) gave **4** (α:β = 1:24, 240 mg, 39%, 2 steps) as an amorphous mass; ^{1}H NMR ($CDCl_3$) δ 7.50−7.20 (m, 15H, Ar), 4.76 (s, 2H, C$\underline{H}_2$Ph), 4.71 (bd, 1H, $J_{5,NH}$ 8.6 Hz, N$\underline{H}$Ac), 4.64, 4.60, 4.51, 4.37 (each d, each 1H, each *J* 11.6 Hz, 12.2 Hz, 11.6 Hz, 12.2 Hz, C$\underline{H}_2$Ph), 4.16 (ddd, 1H, $J_{3a,4}$ 10.6 Hz, $J_{3e,4}$ 4.6 Hz, $J_{4,5}$ 10.6 Hz, H-4), 4.14 (dd, 1H, $J_{5,6}$ 10.6 Hz, $J_{6,7}$ 1.3 Hz, H-6), 3.87 (dq, 1H, $J_{7,8}$ 4.6 Hz, $J_{8,9}$ 5.9 Hz, H-8), 3.76 (s, 3H, COOMe), 3.66 (bdd, 1H, $J_{6,7}$ 1.3 Hz, $J_{7,8}$ 4.6 Hz, H-7), 3.64 (ddd, 1H, $J_{5,NH}$ 8.6 Hz, $J_{4,5}$ 10.6 Hz, $J_{5,6}$ 10.6 Hz, H-5), 3.13 (s, 3H, OMe), 2.54 (dd, 1H, $J_{3e,4}$ 4.6 Hz, J_{gem} 13.2 Hz, H-3e), 1.69 (s, 3H, NH$\underline{Ac}$), 1.69 (dd, 1H, $J_{3a,4}$ 10.6, J_{gem} 13.2 Hz, H-3a), 1.46 (d, 3H, $J_{8,9}$ 5.9 Hz, H-9); ^{13}C NMR ($CDCl_3$) δ 170.20, 168.37, 138.65, 138.54, 138.44, 128.99, 128.37, 128.35, 127.79, 127.77, 127.66, 127.48, 127.46, 99.00 (C-2), 77.42 (C-7), 76.86 (C-8), 73.19 (2C, C-4, C$\underline{H}_2$Ph), 71.37 (C-6), 71.01, 70.51 (C$\underline{H}_2$Ph × 2), 52.82 (C-5), 52.47 (C$\underline{O}$OMe), 50.81 (OMe), 37.52 (C-3), 23.59 (CO$\underline{C}$H$_3$), 15.94 (C-9); HRMS calcd for $C_{34}H_{41}NO_8$ ($M + Na^+$) 614.2730, found 614.2742.

5-Acetamido-3,5,9-trideoxy-D-galacto-2-nonulopyranosonic Acid (5). A solution of **4** (α:β = 1:24, 187 mg, 0.32 mmol) in acetic acid (3.5 mL) was stirred under hydrogen atmosphere in the presence of 10% Pd−C (187 mg) for 6 h. After filtration with Celite 545, the filtrate was concentrated. The acetic acid in the residue was removed by coevaporation with water in vacuo. The mixture was dissolved in a solution of 0.3 M NaOH (7.5 mL) and MeOH (7.5 mL) and kept for 40 min at room temperature. The solution was made neutral with IR120 (H^+) resin, and the mixture was filtered. After concentration, the residue was dissolved in aqueous 0.025 M HCl (15 mL), and to this solution was added Amberlyst 15 (wet) ion-exchange resin (1.1 g). Then the mixture was stirred at 80 °C. After 4 h, the mixture was evaporated to dryness and coevaporated with water (×3). The residue was dissolved in water and passed through a gel permeation column chromatography (Sephadex G-15, water). Fractions containing NeuAc derivative were pooled and then the solution was lyophilized to give **5** (α:β = 1:24, 78 mg, 64%, 3 steps); The physical data were identical to the reported data.[26]

5-Acetamido-9-azido-3,5,9-trideoxy-D-glycero-D-galacto-2-nonulopyranosonic Acid (8). Azido derivative **7** was prepared from known compound **6** by 3 steps with reported procedure.[11e] To a solution of this phenyl thioglycoside **7**[11e] (α:β = 1:2, 233 mg, 0.53 mmol) in acetone (4.8 mL) and H_2O (0.5 mL) was added *N*-bromosuccinimide (282 mg, 1.58 mmol) at room temperature.[27] After 30 min, the mixture was neutralized by the addition of triethylamine. After concentration, the mixture was dissolved in a solution of 0.3 M NaOH (14 mL) and kept for 10 min at room temperature. The solution was made neutral with IR120 (H^+) resin, and the mixture was filtered. After concentration, purification of the residue on a gel permeation column chromatography (Sephadex G-15, water) and subsequent lyophilization afforded **8** (α:β = 1:15, 92 mg, 52%, 2 steps) as an amorphous mass; The physical data were identical to the reported data.[11d,28]

Methyl (Methyl 5-Acetamido-4,7-di-*O*-benzyl-3,5-dideoxy-α,β-D-glycero-D-galacto-2-nonulopyranosid) Onate (10). To a solution of acetonide **9**[11b] (α:β = 1:15, 3.2 g, 8.48 mmol) in dry *N*,*N*′-dimethylformamide (43 mL) was added BaO (9.3 g, 60.65 mmol), $Ba(OH)_2 \cdot 8H_2O$ (2.4 g, 7.61 mmol), and benzyl bromide (10 mL, 84.1 mmol), and this mixture was stirred at room temperature. After 12 h, the mixture was diluted with $CHCl_3$ and washed with aqueous 1% formic acid and water. After drying with $MgSO_4$, the organic solution was evaporated. The residue was dissolved in ethanol (25 mL) and benzene (50 mL), and then to this mixture was added an ethereal solution of diazomethane (42.5 mmol). The mixture was stirred for 10 min at room temperature. After addition of acetic acid (12 mL), the solution was concentrated in vacuo. The residue was dissolved in aqueous 60% acetic acid (50 mL), and this mixture was stirred for 12 h at 60 °C. After concentration of this mixture, purification of the residue on a silica gel column chromatography (ethyl acetate:MeOH = 15:1) gave **10** (α:β = 1:24, 2.7 g, 62%, 3 steps) as an amorphous mass; ^{1}H NMR (D_2O) δ 7.48−7.32 (m, 10H, Ar), 4.65, 4.59, 4.54, 4.44 (each d, each 1H, each *J* 11.9 Hz, 9.9 Hz, 9.9 Hz, 11.9 Hz, C$\underline{H}_2$-Ph), 4.08 (dd, 1H, $J_{4,5}$ 10.6 Hz, $J_{5,6}$ 10.6 Hz, H-5), 3.91 (bd, 1H, $J_{5,6}$ 10.6 Hz, H-6), 3.91−3.82 (m, 2H, H-9a, H-8), 3.81 (m, 1H, $J_{3a,4}$ 11.2 Hz, $J_{3e,4}$ 4.6 Hz, $J_{4,5}$ 10.6 Hz, H-4), 3.79 (s, 3H, COOMe), 3.71 (m, 1H, H-9b), 3.70 (bd, 1H, $J_{7,8}$ 9.2 Hz, H-7), 3.21 (s, 3H, OMe), 2.51 (dd, 1H, $J_{3e,4}$ 4.6 Hz, J_{gem} 13.2 Hz, H-3e), 1.93 (s, 3H, NH$\underline{Ac}$), 1.68 (dd, 1H, $J_{3a,4}$ 11.2, J_{gem} 13.2 Hz, H-3a); ^{13}C NMR (D_2O) δ 174.32, 170.69, 137.90, 137.27, 129.36, 129.20, 129.13, 129.02, 128.98, 128.71, 99.66 (C-2), 76.50 (C-7), 75.33, 74.61 (C-4), 71.76, 71.02 (C-6), 70.30 (C-8), 63.13 (C-9), 53.94 (COO$\underline{Me}$), 51.50 (OMe), 50.28 (C-5), 37.13 (C-3), 22.73 (CO$\underline{C}$H$_3$); HRMS calcd for $C_{27}H_{35}NO_9$ ($M + Na^+$) 540.2210, found 540.2207.

Methyl (Methyl 5-Acetamido-9-*O*-acetyl-4,7-di-*O*-benzyl-3,5-dideoxy-α,β-D-glycero-D-galacto-2-nonulopyranosid) Onate (11). To a solution of **10** (α:β = 1:24, 398 mg, 0.77 mmol) in pyridine (4 mL) and CH_2Cl_2 (2.4 mL) was added a solution of acetyl chloride (109 μL, 1.53 mmol) in CH_2Cl_2 (1.6 mL) at −45 °C, and the mixture was stirred for 10 min at −45 °C. After addition of MeOH (1 mL), the solution was concentrated in vacuo. Purification of the residue on a silica gel column chromatography (ethyl acetate:MeOH = 20:1) gave **11** (α:β = 1:24, 354 mg, 82%) as an amorphous mass; ^{1}H NMR ($CDCl_3$) δ 7.44−7.25 (m, 10H, Ar), 4.96 (d, 1H, $J_{5,NH}$ 7.9 Hz, N$\underline{H}$Ac), 4.75 ∼ 4.59 (m, 3H, C$\underline{H}_2$Ph), 4.48 (m, 1H, H-9a), 4.38 (d, 1H, C$\underline{H}_2$Ph), 4.26−4.15 (m, 3H, H-6, H-9b, H-8), 4.06 (ddd, 1H, $J_{3a,4}$ 10.6 Hz, $J_{3e,4}$ 4.6 Hz, $J_{4,5}$ 9.9 Hz, H-4), 3.83 (m, 1H, H-5), 3.78 (s, 3H, COOMe), 3.61 (dd, 1H, $J_{6,7}$ 2.0 Hz, $J_{7,8}$ 5.3 Hz, H-7), 3.25 (s, 3H, OMe), 2.57 (dd, 1H, $J_{3e,4}$ 4.6 Hz, J_{gem} 13.2 Hz, H-3e), 2.07 (s, 3H, Ac), 1.74 (s, 3H, Ac), 1.70 (dd, 1H, $J_{3a,4}$ 10.6 Hz, J_{gem} 13.2 Hz, H-3a); ^{13}C NMR ($CDCl_3$) δ 171.37, 170.31, 168.12, 138.23, 137.92, 128.75, 128.43, 128.39, 127.92, 127.78, 127.71, 99.08 (C-2), 75.35 (C-7), 73.19 (C-4), 73.05, 70.89 (2C, C-6, C$\underline{H}_2$Ph), 70.06 (C-8), 66.47 (C-9), 52.60 (COO$\underline{Me}$), 51.97 (C-5), 51.25 (OMe), 37.31 (C-3), 23.47, 20.86 (CO$\underline{C}$H$_3$ × 2); Anal. Calcd for $C_{29}H_{37}NO_{10}$: C, 62.24; H, 6.66, N, 2.50. Found: C, 61.94; H, 6.65; N, 2.79.

Methyl (Methyl 5-Acetamido-9-*O*-acetyl-4,7-di-*O*-benzyl-3,5,8-trideoxy-α,β-D-galacto-2-nonulopyranosid) Onate (12). To a solution of **11** (α:β = 1:24, 945 mg, 1.69 mmol) in pyridine (8.5 mL) and CH_2Cl_2 (8.5 mL) was added 4-dimethylaminopyridine (104 mg, 0.85 mmol) and phenyl chlorothionoformate (1.2 mL, 8.7 mmol), and the mixture was stirred for 3 h at room temperature. After addition of MeOH (3 mL), the mixture was concentrated. The residue was dissolved in ethyl acetate and washed with saturated NaCl. After drying with $MgSO_4$, the organic phase was concentrated in vacuo. The residue was dissolved

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in toluene (70 mL), and to this solution were added *n*-tributyltin hydride (4.5 mL, 16.8 mmol) and AIBN (83 mg, 0.51 mmol). The mixture was stirred for 30 min at 100 °C and then concentrated in vacuo. Purification of this residue on a silica gel column chromatography (toluene:ethyl acetate = 1:1) gave **12** (α:β = 1:24, 709 mg, 68%, 2 steps) as an amorphous mass; ^{1}H NMR ($CDCl_3$) δ 7.39−7.24 (m, 10H, Ar), 4.88 (bd, 1H, NHAc), 4.66, 4.61, 4.52, 4.39 (each d, each 1H, *J* 11.9 Hz, CH_2Ph), 4.33 ∼ 4.12 (m, 2H, H-9a, H-9b), 4.11 (ddd, 1H, $J_{3a,4}$ 11.2 Hz, $J_{3e,4}$ 4.9 Hz, $J_{4,5}$ 9.9 Hz, H-4), 3.96 (dd, 1H, $J_{6,7}$ 2.3 Hz, $J_{5,6}$ 10.2 Hz, H-6), 3.79 (s, 3H, COOMe), 3.73−3.62 (m, 2H, H-5, H-7), 3.23 (s, 3H, OMe), 2.55 (dd, 1H, $J_{3e,4}$ 4.9 Hz, J_{gem} 13.2 Hz, H-3e), 2.13−2.03 (m, 2H, H-8a, 8b) 2.02 (s, 3H, NHAc), 1.71 (dd, 1H, $J_{3a,4}$ 11.2 Hz, J_{gem} 13.2 Hz, H-3a); ^{13}C NMR ($CDCl_3$) δ 171.01, 170.10, 168.32, 138.38, 138.28, 128.61, 128.48, 128.39, 127.91, 127.78, 127.69, 99.10 (C-2), 73.26 (2C, C-7, C-4), 71.93 (C-6), 71.84, 71.11, 61.46 (C-9), 52.78 (C-5), 52.54 (COOMe), 50.93 (OMe), 37.72 (C-3), 29.31 (C-8), 23.52, 20.95 ($COCH_3$ × 2); Anal. Calcd for $C_{29}H_{37}NO_9$: C, 64.07; H, 6.86, N, 2.58. Found: C, 63.91; H, 6.58; N, 2.86.

5-Acetamido-3,5,8-trideoxy-D-galacto-2-nonulopyranosonic Acid (13). A solution of **12** (α:β = 1:24, 607 mg, 1.12 mmol) in acetic acid (16 mL) was stirred under hydrogen atmosphere in the presence of 10% Pd−C (598 mg) for 2 h. After filtration with Celite 545, the filtrate was concentrated. The acetic acid in the residue was removed by coevaporation with water in vacuo. The mixture was dissolved in 0.3 M NaOH (7.4 mL) and MeOH (3.8 mL), and kept for 1 h at room temperature. The solution was made neutral with IR120 (H^+) resin, and the mixture was filtered. After concentration, the residue was dissolved in aqueous 0.025 M HCl (38 mL), and to this solution was added Amberlyst 15 (wet) ion-exchange resin (1.9 g). Then the mixture was stirred at 85 °C. After 4 h, the mixture was evaporated to dryness and coevaporated with water (×3). The residue was dissolved in water and passed through a column of anion-exchange resin (AG1-X8, HCO_2^- form). After eluting of impurities by water, derivative **13** was eluted by aqueous 1 M formic acid, and the eluant was evaporated. The formic acid in the residue was removed by coevaporation with water in vacuo. The residue was lyophilized to give **13** (α:β = 1:24, 193 mg, 59%, 3 steps); The physical data were identical to the reported data.[26,29]

Methyl (Methyl 5-Acetamido-4-*O*-benzoyl-3,5-dideoxy-8,9-*O*-isopropylidene-α,β-D-glycero-D-galacto-2-nonulopyranosid) Onate (14). To a solution of acetonide **9**[11b] (α:β = 1:15, 1.0 g, 2.8 mmol) in pyridine (6 mL) and CH_2Cl_2 (24 mL) was added benzoyl chloride (974 μL, 8.4 mmol) dropwise at − 5 °C. The mixture was stirred for 1 h at 0 °C, and then to this mixture was added MeOH (200 μL). After concentration, purification of the residue on a silica gel column chromatography (toluene:ethyl acetate = 2:1) gave **14** (α:β = 1:32, 908 mg, 68%) as an amorphous mass; ^{1}H NMR (CD_3OD) δ 8.10−7.40 (m, 5H, Ar), 5.49 (ddd, 1H, $J_{3a,4}$ 11.2 Hz, $J_{3e,4}$ 5.3 Hz, $J_{4,5}$ 10.6 Hz, H-4), 4.39 (dd, 1H, $J_{4,5}$ 10.6 Hz, $J_{5,6}$ 10.6 Hz, H-5), 4.32 (ddd, 1H, $J_{7,8}$ 8.6 Hz, $J_{8,9a}$ 5.9 Hz, $J_{8,9b}$ 5.9 Hz, H-8), 4.16 (dd, 1H, $J_{8,9a}$ 5.9 Hz, J_{gem} 8.3 Hz, H-9a), 4.00 (dd, 1H, $J_{8,9b}$ 5.9 Hz, J_{gem} 8.3 Hz, H-9b), 3.98 (dd, 1H, $J_{5,6}$ 10.6 Hz, $J_{6,7}$ 1.3 Hz, H-6), 3.84 (s, 3H, COOMe), 3.57 (dd, 1H, $J_{6,7}$ 1.3 Hz, $J_{7,8}$ 8.6 Hz, H-7), 3.35 (s, 3H, OMe), 2.63 (dd, 1H, $J_{3e,4}$ 5.3 Hz, J_{gem} 12.9 Hz, H-3e), 1.91 (dd, 1H, $J_{3a,4}$ 11.2 Hz, J_{gem} 12.9 Hz, H-3a), 1.89 (s, 3H, NHAc), 1.40, 1.33 (each s, each 3H, Me × 2); ^{13}C NMR (CD_3OD) δ 174.65, 171.00, 168.15, 135.23, 131.81, 131.45, 130.39, 111.17, 100.96 (C-2), 76.72 (C-8), 73.34 (C-6), 72.46 (C-4), 72.10 (C-7), 69.42 (C-9), 54.11 (COOMe), 52.46 (OMe), 51.09 (C-5), 39.25 (C-3), 28.09, 26.44 (Me × 2), 23.42 ($COCH_3$); Anal. Calcd for $C_{23}H_{31}NO_{10}$: C, 57.37; H, 6.49, N, 2.91. Found: C, 57.08; H, 6.60; N, 3.05.

Methyl (Methyl 5-Acetamido-4-*O*-benzoyl-3,5,7-trideoxy-8,9-*O*-isopropylidene-α,β-D-glycero-D-galacto-2-nonulopyranosid) Onate (15). To a solution of **14** (α:β = 1:32, 144 mg, 0.30 mmol) in CH_2Cl_2 (6 mL) was added 4-dimethylaminopyridine (18 mg, 0.15 mmol) and 1,1′-thiocarbonyldiimidazole (160 mg, 0.90 mmol) at room temperature, and the mixture was stirred for 12 h under the reflux condition. After addition of MeOH (100 μL), the mixture was diluted with ethyl acetate and washed with water (×2). After drying with $MgSO_4$, the mixture was concentrated. The residue was dissolved in toluene (23 mL), and to this solution were added *n*-tributyltin hydride (0.4 mL, 1.49 mmol) and AIBN (15 mg, 0.09 mmol). The mixture was stirred for 30 min at 80 °C and then concentrated. Purification of the residue on a silica gel column chromatography (toluene:ethyl acetate = 1:1) gave **15** (α:β = 1:19, 111 mg, 80%, 2 steps) as an amorphous mass; ^{1}H NMR ($CDCl_3$) δ 8.00−7.35 (m, 5H, Ar), 5.64 (d, $J_{5,NH}$ 9.4 Hz, 1H, NHAc), 5.46 (ddd, 1H, $J_{3a,4}$ 11.1 Hz, $J_{3e,4}$ 5.1 Hz, $J_{4,5}$ 11.1 Hz, H-4), 4.35 (m, 1H, H-8), 4.12 (ddd, 1H, $J_{5,NH}$ 9.4 Hz, $J_{4,5}$ 11.1 Hz, $J_{5,6}$ 10.3 Hz, H-5), 4.10 (dd, 1H, $J_{8,9a}$ 5.9 Hz, J_{gem} 7.8 Hz, H-9a), 3.85 ∼ 3.75 (m, 4H, COOMe, H-6), 3.56 (dd, 1H, $J_{8,9b}$ 7.8 Hz, J_{gem} 7.8 Hz, H-9b), 3.31 (s, 3H, OMe), 2.55 (dd, 1H, $J_{3e,4}$ 5.1 Hz, J_{gem} 12.7 Hz, H-3e), 2.01 (dd, 1H, $J_{3a,4}$ 11.1 Hz, J_{gem} 12.7 Hz, H-3a), 1.95−1.82 (m, 5H, NHAc, H-7a, H-7b); ^{13}C NMR ($CDCl_3$) δ 170.41, 168.09, 166.81, 133.38, 129.72, 129.38, 128.46, 108.53, 98.38 (C-2), 72.53 (C-8), 70.38 (C-6), 69.75 (C-9), 69.51 (C-4), 53.98 (C-5), 52.64 (COOMe), 50.92 (OMe), 37.75 (C-3), 35.71 (C-7), 27.07, 25.76 (Me × 2), 23.23 ($COCH_3$); HRMS calcd for $C_{23}H_{31}NO_9$ (M + H^+) 466.2077, found 466.2065.

5-Acetamido-3,5,7-trideoxy-D-galacto-2-nonulopyranosonic Acid (16). Acetonide **15** was (α:β = 1:19, 303 mg, 0.65 mmol) dissolved in aqueous 60% acetic acid (6.2 mL), and this mixture was stirred for 30 min at 60 °C. After concentration, the mixture was dissolved in a solution of 0.3 M NaOH (15 mL) and MeOH (15 mL), and kept for 10 min at room temperature. The solution was made neutral with IR120 (H^+) resin, and the mixture was filtered. After concentration of the filtrate, the residue was dissolved in aqueous 0.025 M HCl (38 mL), and then to this solution was added Amberlyst 15 (wet) ion-exchange resin (3.0 g). The mixture was stirred for 4 h at 80 °C. After filtration of the reaction mixture, the filtrate was evaporated to dryness and coevaporated with water (×3). The residue was dissolved in water and passed through a gel permeation column chromatography (Sephadex G-15, water). Fractions containing NeuAc derivative were pooled, and the resulting solution was lyophilized to give **16** (α:β = 1:11, 132 mg, 69%, 3 steps); the physical data were identical to the reported data.[28]

Monitoring of [3-^{13}C]-Labeling for NeuAc Analogues by Exchange Reaction of NeuAc Aldolase. NeuAc analogue (15 μmol) was dissolved in a solution of phosphate buffer (150 mM, pH 7.0, total 500 μL) containing NaN_3 (0.1 mg), NeuAc aldolase (10 U), [3-^{13}C]-sodium pyruvate (45 μmol), and D_2O (20 μL). The reaction was carried out in NMR sample tube. The quantity of [3-^{13}C]-NeuAc analogue and [3-^{13}C]-pyruvic acid were determined by ^{1}H NMR measurement (monitoring times: 0, 4, 8, 16, 24, 40, 48, and 96 h, scans: 256, temp. 30 °C). After 96 h, the reaction mixture was passed through a column of anion-exchange resin (AG1-X8, HCO_2^- form). The impurities was removed by eluting with water, and then NeuAc analogue was eluted with aqueous 1 M formic acid. A solution containing [3-^{13}C]-NeuAc analogue was concentrated. The formic acid in the residue was removed by coevaporation with water in vacuo. ^{13}C-labeling yield was determined by ^{1}H NMR and was summarized in Table 1. NeuAc (4.6 mg, 14.9 μmol), 9-deoxy-9-fluoro-NeuAc **3** (4.7 mg, 15.1 μmol) 9-deoxy-NeuAc **5** (4.4 mg, 15.0 μmol), 9-azido-9-deoxy-NeuAc **8** (5.4 mg, 15.1 μmol), 8-deoxy-NeuAc **13** (4.4 mg, 15.0 μmol), and 7-deoxy-NeuAc **16** (4.4 mg, 15.0 μmol) were examined by this exchange reaction with the conditions described above.

Kinetic Measurements toward NeuAc Aldolase. The assay used was based on the method described.[12] NeuAc analogues or NeuAc were incubated in a solution of phosphate buffer (50 mM, pH 7.0, total volume 80 μL) containing 0.2 mM β-NADH, 25 U of LDH, and 4 mU of NeuAc aldolase. In the case of 8-deoxy-NeuAc **13** and 7-deoxy-NeuAc **16**, 20 mU of NeuAc aldolase was used. The mixture was incubated until less 1% consumption of starting material at 37 °C. The amount of pyruvic acid released in the assay mixture was estimated by measurement of decreasing of absorbance at 340 nm. All assays were performed in duplicate with five concentrations of NeuAc substrate (NeuAc and 9-modified NeuAc: 1.25, 2.5, 5.0, 7.5, and 10 mM; 8-deoxy-NeuAc: 2.5, 5.0, 7.5, 10, and 15 mM, 7-deoxy-NeuAc: 5.0, 7.5, 10, 12.5, and 15 mM). The kinetic parameters K_m and V_{max} values were obtained from the Lineweaver−Burk plots.

Degradation Reaction by the Use of NeuAc Aldolase and Lactate Dehydrogenase. NeuAc analogue (30 mM) was dissolved in a solution of phosphate buffer (0.5 M, pH 7.5) containing BSA (0.1 mg), NaN_3 (0.1 mg), NeuAc aldolase (3 U), β-NADH (45 mmol), and LDH (9.3 U), and the mixture was incubated at 37 °C. The quantity of NeuAc

(29) David, S.; Malleron, A.; Cavayé, B. *New J. Chem.* **1992**, *16*, 751−755.

analogue in the reaction mixture was determined by thiobarbituric acid assay[13] (TBA, monitoring times: 0, 5, 15, and 45 min and 1.75, 3.75, 5.75, and 9.75 h). 7-Deoxy-NeuAc **16** could not be assayed by TBA because both 4-deoxy-ManNAc formed and 7-deoxy-NeuAc were positive in this assay. Therefore, the quantity of 7-deoxy-NeuAc was determined by HPLC equipping a column chromatography of aminopropyl (0.4 cm × 25 cm, Asahipak, NH_2-P50), which was run with a mixture of CH_3CN/15 mM KH_2PO_4 = 7/4. 7-Deoxy-NeuAc was detected at 200 nm, and its retention time was found to be 14.3 min. NeuAc (1.9 mg, 6.1 μmol), 9-deoxy-9-fluoro-NeuAc **3** (1.5 mg, 4.8 μmol), 9-deoxy-NeuAc **5** (1.5 mg, 5.1 μmol), 9-azido-9-deoxy-NeuAc **8** (1.2 mg, 3.3 μmol), 8-deoxy-NeuAc **13** (1.0 mg, 3.4 μmol), and 7-deoxy-NeuAc **16** (2.0 mg, 6.8 μmol) were examined by this degradation reaction with the conditions described above. Enzyme assay suggested that all degradation yields of NeuAc and NeuAc analogues **3**, **5**, **8**, **13**, and **16** were quantitative.

Typical Procedure of a One-Pot Enzymatic ^{13}C-Labeling Method. NeuAc analogue (30 mM) was dissolved in a solution of phosphate buffer (50 mM, pH 7.5) containing BSA (5 mg), NaN_3 (5 mg), NeuAc aldolase (30 U), β-NAD^+ (0.3 mM), LDH (150 U), ADH (150 U), and EtOH (120 μL, 2.1 mmol), and then the pH was adjusted to 7.5. The mixture was incubated at room temperature for 20 h. After completion of the degradation reaction (ascertained by the TBA), to this mixture was added NPP (2.8 U) and $MgCl_2$ (6.0 mg). The mixture was incubated at room temperature for 6 h. This NPP reaction was monitored by TLC (solvent system: ethyl acetate/MeOH/H_2O = 3/2/1.5; R_f β-NAD^+ = 0.20, β-NADH = 0.45). After completion of the degradation of β-NADH and β-NAD^+, to this solution was added [3-^{13}C]-sodium pyruvate (3 equiv toward NeuAc analogue), and the mixture was incubated at room temperature for 24 h. After lyophilization, the residue was passed through a column of anion-exchange resin (AG1-X8, HCO_2^- form). After elution of impurities by water, ^{13}C-labeled NeuAc analogue was eluted with aqueous 1 M formic acid, and the eluant was evaporated. The formic acid in the solution was removed by coevaporation with water. Purification of the residue on a gel permeation column chromatography (Sephadex G-15, H_2O) afforded [3-^{13}C]-NeuAc analogue. The quantity of NeuAc analogue during the reaction was monitored by TBA (monitoring time: 0, 15, 45 min and 1.75, 3.75, 7.75, 15.75, 19.75, 23.75, 26.05, 26.80, 27.80, 29.80, 33.80, 41.80, and 49.80 h). In the case of 7-deoxy-NeuAc **16**, the compound was monitored by HPLC. These results of monitoring are shown in Figure 6. NeuAc (30.9 mg, 0.1 mmol), 9-deoxy-9-fluoro-NeuAc **3** (31 mg, 0.10 mmol), 9-deoxy-NeuAc **5** (30.5 mg, 0.1 mmol), 9-azido-9-deoxy-NeuAc **8** (30.1 mg, 0.09 mmol), 8-deoxy-NeuAc **13** (31.3 mg, 0.11 mmol), and 7-deoxy-NeuAc **16** (33.2 mg, 0.11 mmol) were examined by this one-pot ^{13}C-labeling method with condition described above. The isolated yields and ^{13}C-labeling yields are summarized in Table 2.

[3-^{13}C]-5-Acetamido-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosonic Acid ([3-^{13}C]-NeuAc). α:β = 1:15; ^{1}H NMR (D_2O) δ 4.12 (m, 1H, $J_{3a,4}$ 11.2 Hz, $J_{3e,4}$ 4.6 Hz, $J_{4,5}$ 9.9 Hz, H-4), 4.10 (bd, 1H, $J_{5,6}$ 9.9 Hz, H-6), 4.00 (dd, 1H, $J_{4,5}$ 9.9 Hz, $J_{5,6}$ 9.9 Hz, H-5), 3.92 (dd, 1H, $J_{8,9a}$ 2.6 Hz, J_{gem} 11.2 Hz, H-9a), 3.84 (ddd, 1H, $J_{7,8}$ 8.6 Hz, $J_{8,9a}$ 5.9 Hz, $J_{8,9b}$ 2.6 Hz, H-8), 3.70 (dd, 1H, $J_{8,9b}$ 5.9 Hz, J_{gem} 11.2 Hz, H-9b), 3.63 (bd, 1H, $J_{7,8}$ 8.6 Hz, H-7), 2.35 (ddd, 1H, $J_{3e,4}$ 4.6 Hz, J_{gem} 12.5 Hz, $J_{3e,C3}$ 133.3 Hz, H-3e), 2.13 (s, 3H, NHAc), 1.94 (ddd, 1H, $J_{3a,4}$ 11.2 Hz, J_{gem} 12.5, $J_{3a,C3}$ 130.0 Hz, H-3a); ^{13}C NMR (D_2O) δ 175.71, 175.27, 96.56 (d, $J_{C2,C3}$ 41.2 Hz, C-2), 70.97 (C-8), 70.86 (C-6), 68.67 (C-7), 67.35 (d, $J_{C3,C4}$ 35.5 Hz, C-4), 63.51 (C-9), 52.58 (C-5), 39.57 (C-3), 22.53 ($COCH_3$); HRMS calcd for $C_{10}{}^{13}CH_{19}NO_9$ (M + H^+) 311.1172, found 311.1185.

[3-^{13}C]-5-Acetamido-3,5,9-trideoxy-9-fluoro-D-glycero-D-galacto-2-nonulo-pyranosonic Acid ([3-^{13}C]-9-deoxy-9-fluoro-NeuAc). α:β = 1:19; ^{1}H NMR (D_2O) δ 4.74 (dd, 2H, $J_{8,9}$ 3.3 Hz, $J_{9,F}$ 46.1 Hz, H-9), 4.12 (m, 1H, $J_{3a,4}$ 11.2 Hz, $J_{3e,4}$ 4.6 Hz, $J_{4,5}$ 9.7 Hz, H-4), 4.08 (bd, 1H, $J_{5,6}$ 9.9 Hz, H-6), 4.01 (dd, 1H, $J_{4,5}$ 9.7 Hz, $J_{5,6}$ 9.9 Hz, H-5), 4.00 (tdd, 1H, $J_{7,8}$ 9.2 Hz, $J_{8,9}$ 3.3 Hz, $J_{8,F}$ 27.7 Hz, H-8), 3.71 (bd, 1H, $J_{7,8}$ 9.2 Hz, H-7), 2.32 (ddd, 1H, $J_{3e,4}$ 4.6 Hz, J_{gem} 12.5 Hz, $J_{3e,C3}$ 132.6 Hz, H-3e), 2.13 (s, 3H, NH*Ac*), 1.93 (ddd, 1H, $J_{3a,4}$ 11.2 Hz, J_{gem} 12.5 Hz, $J_{3a,C3}$ 131.3 Hz, H-3a); ^{13}C NMR (D_2O) δ 175.23, 173.66, 95.74 (d, $J_{C2,C3}$ 40.6 Hz, C-2), 85.56 (d, $J_{C9,F}$ 164.9 Hz, C-9), 70.69 (C-6), 69.14 (d, $J_{C8,F}$ 17.7 Hz, C-8), 67.58 (d, $J_{C7,F}$ 7.2 Hz, C-7), 67.15 (d, $J_{C3,C4}$ 35.9 Hz, C-4), 52.46 (C-5), 39.26 (C-3), 22.51 ($COCH_3$); HRMS calcd for $C_{10}{}^{13}CH_{18}FNO_8$ (M + H^+) 313.1128, found 313.1150.

[3-^{13}C]-5-Acetamido-3,5,9-trideoxy-D-galacto-2-nonulopyranosonic Acid ([3-^{13}C]-9-deoxy-NeuAc). α:β = 1:15; ^{1}H NMR (D_2O) δ 4.08 (m, 1H, $J_{3a,4}$ 12.8 Hz, $J_{3e,4}$ 4.7 Hz, $J_{4,5}$ 10.1 Hz, H-4), 4.08 (bd, 1H, $J_{5,6}$ 10.3 Hz, H-6), 3.99 (dd, 1H, $J_{4,5}$ 10.1 Hz, $J_{5,6}$ 10.3 Hz, H-5), 3.92 (dq, 1H, $J_{7,8}$ 8.3 Hz, $J_{8,9}$ 6.4 Hz, H-8), 3.39 (bd, 1H, $J_{7,8}$ 8.3 Hz, H-7), 2.28 (ddd, 1H, $J_{3e,4}$ 4.7 Hz, J_{gem} 12.8 Hz, $J_{3e,C3}$ 132.3 Hz, H-3e), 2.13 (s, 3H, NHAc), 1.91 (ddd, 1H, $J_{3a,4}$ 12.8 Hz, J_{gem} 12.8 Hz, $J_{3a,C3}$ 129.1 Hz, H-3a), 1.32 (d, 3H, $J_{8,9}$ 6.4 Hz, H-9); ^{13}C NMR (D_2O) δ 177.07, 175.11, 96.79 (d, $J_{C2,C3}$ 41.2 Hz, C-2), 73.17 (C-7), 70.61 (C-6), 67.49 (d, $J_{C3,C4}$ 35.3 Hz, C-4), 67.06 (C-8), 52.89 (C-5), 39.78 (C-3), 22.55 ($COCH_3$), 19.73 (C-9); HRMS calcd for $C_{10}{}^{13}CH_{19}NO_8$ (M + Na^+) 317.1042, found 317.1032.

[3-^{13}C]-5-Acetamido-9-azido-3,5,9-trideoxy-D-glycero-D-galacto-2-nonulo-pyranosonic Acid ([3-^{13}C]-9-azido-9-deoxy-NeuAc). α:β = 1:15; ^{1}H NMR (D_2O) δ 4.13 (m, 1H, $J_{3a,4}$ 11.9 Hz, $J_{3e,4}$ 4.6 Hz, $J_{4,5}$ 9.9 Hz, H-4), 4.12 (bd, 1H, $J_{5,6}$ 10.6 Hz, H-6), 4.00 (dd, 1H, $J_{4,5}$ 9.9 Hz, $J_{5,6}$ 10.6 Hz, H-5), 3.99 (ddd, 1H, $J_{7,8}$ 9.2 Hz, $J_{8,9a}$ 5.9 Hz, $J_{8,9b}$ 2.6 Hz, H-8), 3.70 (dd, 1H, $J_{8,9a}$ 2.6 Hz, J_{gem} 13.2 Hz, H-9a), 3.64 (bd, 1H, $J_{7,8}$ 9.2 Hz, H-7), 3.55 (dd, 1H, $J_{8,9b}$ 5.9 Hz, J_{gem} 13.2 Hz, H-9b), 2.36 (ddd, 1H, $J_{3e,4}$ 4.6 Hz, J_{gem} 13.2 Hz, $J_{3e,C3}$ 133.3 Hz, H-3e), 2.14 (s, 3H, NHAc), 1.95 (ddd, 1H, $J_{3a,4}$ 11.9 Hz, J_{gem} 13.2 Hz, $J_{3a,C3}$ 129.3 Hz, H-3a); ^{13}C NMR (D_2O) δ 175.27, 174.23, 95.92 (d, $J_{C2,C3}$ 41.6 Hz, C-2), 70.65 (C-6), 69.45 (C-8), 69.19 (C-7), 67.21 (d, $J_{C3,C4}$ 35.8 Hz, C-4), 54.30 (C-9), 52.56 (C-5), 39.37 (C-3), 22.52 ($COCH_3$); HRMS calcd for $C_{10}{}^{13}CH_{18}N_4O_8$ (M + H^+) 336.1236, found 336.1242.

[3-^{13}C]-5-Acetamido-3,5,8-trideoxy-D-galacto-2-nonulopyranosonic Acid ([3-^{13}C]-8-deoxy-NeuAc). α:β = 1:24; ^{1}H NMR (D_2O) δ 4.08 (m, 1H, $J_{3a,4}$ 11.9 Hz, $J_{3e,4}$ 4.6 Hz, $J_{4,5}$ 9.9 Hz, H-4), 3.96 (dd, 1H, $J_{4,5}$ 9.9 Hz, $J_{5,6}$ 10.6 Hz, H-5), 3.90 (ddd, 1H, $J_{6,7}$ 1.3 Hz, $J_{7,8a}$ 8.6 Hz, $J_{7,8b}$ 4.6 Hz, H-7), 3.77 (dd, 2H, $J_{8a,9}$ 6.6 Hz, $J_{8b,9}$ 6.6 Hz, H-9), 3.70 (dd, 1H, $J_{5,6}$ 10.6 Hz, $J_{6,7}$ 1.3 Hz, H-6), 2.32 (ddd, 1H, $J_{3e,4}$ 4.6 Hz, J_{gem} 12.5 Hz, $J_{3e,C3}$ 132.6 Hz, H-3e), 2.14 (s, 3H, NHAc), 1.99 (tdd, 1H, $J_{7,8a}$ 8.6 Hz, J_{gem} 14.5 Hz, $J_{8a,9}$ 6.6 Hz, H-8a), 1.91 (ddd, 1H, $J_{3a,4}$ 11.9 Hz, J_{gem} 12.5 Hz, $J_{3a,C3}$ 129.3 Hz, H-3a), 1.78 (tdd, 1H, $J_{7,8b}$ 4.6 Hz, J_{gem} 14.5 Hz, $J_{8b,9}$ 6.6 Hz, H-8b); ^{13}C NMR (D_2O) δ 175.39, 173.81, 95.65 (d, $J_{C2,C3}$ 41.2 Hz, C-2), 74.18 (C-6), 67.08 (d, $J_{C3,C4}$ 35.7 Hz, C-4), 65.88 (C-7) 58.97 (C-9), 53.07 (C-5), 39.39 (C-3), 35.33 (C-8), 22.48 ($COCH_3$); HRMS calcd for $C_{10}{}^{13}CH_{19}NO_8$ (M + H^+) 295.1223, found 295.1221.

[3-^{13}C]-5-Acetamido-3,5,7-trideoxy-D-galacto-2-nonulopyranosonic Acid ([3-^{13}C]-7-deoxy-NeuAc). α:β = 1:11; ^{1}H NMR (D_2O) δ 4.07 (ddd, 1H, $J_{5,6}$ 10.0 Hz, $J_{6,7a}$ 2.7 Hz, $J_{6,7b}$ 10.0 Hz, H-6), 4.06 (m, 1H, H-4), 3.93 (dddd, 1H, $J_{7a,8}$ 6.4 Hz, $J_{7b,8}$ 6.4 Hz, $J_{8,9a}$ 3.8 Hz, $J_{8,9b}$ 6.8 Hz, H-8), 3.68 (dd, 1H, $J_{4,5}$ 10.0 Hz, $J_{5,6}$ 10.0 Hz, H-5), 3.65 (dd, 1H, $J_{8,9a}$ 3.8 Hz, J_{gem} 11.7 Hz, H-9a), 3.52 (dd, 1H, $J_{8,9b}$ 6.8 Hz, J_{gem} 11.7 Hz, H-9b), 2.39 (ddd, 1H, $J_{3e,4}$ 4.9 Hz, J_{gem} 13.1 Hz, $J_{3e,C3}$ 133.4 Hz, H-3e), 2.16 (s, 3H, NHAc), 1.97 (ddd, 1H, $J_{3a,4}$ 12.5 Hz, J_{gem} 13.1 Hz, $J_{3a,C3}$ 129.7 Hz, H-3a), 1.78−1.63 (m, 2H, H-7a, H-7b); ^{13}C NMR (D_2O) δ 175.17, 173.65, 95.39 (d, $J_{C2,C3}$ 41.7 Hz, C-2), 69.31 (C-6), 68.29 (C-8), 66.99 (d, $J_{C3,C4}$ 35.7 Hz, C-4), 66.20 (C-9), 56.65 (C-5), 39.39 (C-3), 34.80 (C-7), 22.58 ($COCH_3$); HRMS calcd for $C_{10}{}^{13}CH_{19}NO_8$ (M + H^+) 295.1223, found 295.1225.

[3-^{13}C]-Methyl (5-Acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-α,β-D-glycero-D-galacto-2-nonulopyranosonic Acid (18). A mixture of [3-^{13}C]-NeuAc **17** (α:β = 1:15, 184 mg, 0.59 mmol) and dry Dowex 50W-X8 (H^+, 500 mg) in anhydrous MeOH (40 mL) was stirred for 3 h at room temperature to give the methyl ester (165 mg). This methyl ester was used in the next step without purification. To a solution of the methyl ester in acetic anhydride (1.5 mL) was added aqueous 60% $HClO_4$:Ac_2O = 1:9 (33 μL) at 0 °C.[16] The mixture was stirred for 6 h at 10 °C. The acetate derivative was diluted with ethyl acetate, and the organic solution was washed with water and then saturated aqueous $NaHCO_3$. After drying with $MgSO_4$, the solution was evaporated. Purification of the residue on silica gel column chromatography (ethyl acetate) gave **18** (α:β = 1:13; 109 mg, 37%, 2 steps) as an amorphous mass; ^{1}H NMR ($CDCl_3$) δ 5.69 (bd, 1H, $J_{5,NH}$ 9.5, NHAc), 5.35 (dd, 1H, $J_{6,7}$ 1.8 Hz, $J_{7,8}$ 5.6 Hz, H-7), 5.24 (ddd, 1H, $J_{7,8}$ 5.6 Hz, $J_{8,9a}$ 2.5 Hz, $J_{8,9b}$ 7.5 Hz, H-8) 5.27−5.17 (m, 1H, $J_{3a,4}$ 11.4 Hz, $J_{3e,4}$ 5.5 Hz,

$J_{4,5}$ 10.0 Hz, H-4), 4.50 (dd, 1H, $J_{8,9a}$ 2.5 Hz, J_{gem} 12.3 Hz, H-9a), 4.22 (bdd, 1H, $J_{5,6}$ 10.2 Hz, $J_{6,7}$ 1.8 Hz, H-6), 4.16 (ddd, 1H, $J_{4,5}$ 10.0 Hz, $J_{5,6}$ 10.2 Hz, $J_{5,NH}$ 9.5 Hz, H-5), 4.02 (dd, 1H, $J_{8,9b}$ 7.5 Hz, J_{gem} 12.3 Hz, H-9b), 3.86 (s, 3H, COOMe), 2.26 (ddd, 1H, $J_{3a,4}$ 11.4 Hz, J_{gem} 12.7 Hz, $J_{3a,C3}$ 127.7 Hz, H-3a) 2.19 (ddd, 1H, $J_{3e,4}$ 5.5 Hz, J_{gem} 12.7 Hz, $J_{3e,C3}$ 134.1 Hz, H-3e), 2.14, 2.09, 2.03, 2.02, 1.90 (each s, each 3H, NHAc, Ac × 4); ^{13}C NMR ($CDCl_3$) δ 170.98, 170.88, 170.82, 170.31, 170.20, 169.07, 94.85 (d, $J_{C2,C3}$ 43.0 Hz, C-2), 71.22 (C-8), 71.02 (C-6), 69.31 (d, $J_{C3,C4}$ 36.4 Hz, C-4), 68.01 (C-7), 62.65 (C-9), 53.45 (COOMe), 49.52 (C-5), 36.11 (C-3), 23.12, 20.99, 20.88, 20.76, 20.76 (NHAc, Ac × 4); HRMS calcd for $C_{19}{}^{13}CH_{29}NO_{13}$ ($M + H^+$) 493.1751, found 493.1760.

CMP-[3″-^{13}C]-NeuAc (20). The acetate derivative **18** (α:β = 1:13, 86.5 mg, 0.176 mmol) and cytidine 5′-*O*-amidite **19**[17] (350 mg, 0.614 mmol) were separately dried by coevaporating (×3) with dry benzene. They were then combined in freshly prepared dry MeCN (4 mL). To this mixture was added 1H-tetrazole (61.5 mg, 0.88 mmol) at −30 °C under an argon atmosphere. After 5 min, the ice bath was removed. The mixture was further stirred for 2 h at room temperature, and then the mixture was diluted with ethyl acetate. The organic phase was washed with saturated aqueous $NaHCO_3$ (×2), dried with $MgSO_4$, and evaporated. This phosphite derivative was used in the next steps without purification. To a solution of phosphite in dry MeCN (4 mL) was added *t*-BuOOH (2.5 M in toluene, 1.2 mL, 3.0 mmol), and the mixture was stirred at room temperature for 1 h. After addition of dimethyl sulfide (220 μL, 3.0 mM), to the mixture was added 1,8-diazabicyclo-[5.4.0]-7-undecene (40 μL, 0.27 mmol). The mixture was stirred at room temperature. After 5 min, to this mixture were added NaOMe (190 mg, 3.5 mmol) and MeOH:H_2O = 1:2 (6 mL). After 20 h, the mixture was washed with CH_2Cl_2 (×2), and the water layer was evaporated at 25 °C. Purification of the residue on a silica gel column chromatography (*n*-PrOH:20 mM NH_4OH = 3:1) and a gel permeation column chromatography (Sephadex G-15, 1 cm × 60 cm, 20 mM NH_4OH) gave CMP-[3″- ^{13}C]-NeuAc **20** (37 mg, 35%, 4 steps) as an amorphous mass; ^{1}H NMR (D_2O, 50 mM ND_4CO_3) δ 8.04 (d, 1H, $J_{5,6}$ 7.6 Hz, H-6), 6.20 (d, 1H, $J_{5,6}$ 7.6 Hz, H-5), 6.07 (d, 1H, $J_{1',2'}$ 4.5 Hz, H-1′), 4.44 ∼ 4.28 (m, 5H, H-2′, 3′. 4′, 5a′, 5b′), 4.22 (dd, 1H, $J_{5'',6''}$ 10.4 Hz, $J_{6'',7''}$ 0.9 Hz, H-6″), 4.20−4.06 (m, 1H, H-4″), 4.05−3.96 (m, 1H, H-5″, H-8″), 3.96 (dd, 1H, $J_{9a'',8''}$ 2.5 Hz, J_{gem} 11.8 Hz, H-9a″), 3.70 (dd, 1H, $J_{9b'',8''}$ 6.4 Hz, J_{gem} 11.8 Hz, H-9b″), 3.53 (dd, 1H, $J_{6'',7''}$ 0.9 Hz, $J_{7'',8''}$ 9.6 Hz, H-7″), 2.57 (ddd, 1H, $J_{3e'',4''}$ 4.7 Hz, J_{gem} 13.3 Hz, $J_{3e'',C3''}$ 135.5 Hz, H-3e″), 2.13 (s, 3H, NHAc), 1.72 (dddd, 1H, $J_{3a'',4''}$ 12.1 Hz, $J_{3a'',P}$ 5.7 Hz, J_{gem} 13.3 Hz, $J_{3a'',C3''}$ 128.0 Hz, H-3a″); HRMS calcd for $C_{19}{}^{13}$-$CH_{29}N_4Na_2O_{16}P$ ($M + Na^+$) 682.1044, found 682.1005.

[U-^{13}C]-Gal-β-x-Ovalbumin (22). The transfer reaction was performed in a solution of HEPES buffer (100 mM, pH 7.0, 1.0 mL) containing UDP-[U-^{13}C]-Glc[18] (6.0 mg, 10.1 μmol), ovalbumin **21** (153 mg, 3.4 nmol), UDP-glucose-4-epimerase (200 mU), *bovine* β-1,4-galactosyltransferase (100 mU), $MnCl_2$ (50 mM), CIAP (50 U), and the mixture was incubated at 37 °C for 24 h. The mixture was then passed through Molcut-L filter (Millipore, 10 000 cut). The residue in the filter was further washed with water (2 mL × 5) and then analyzed by NMR. The NMR data are summarized in Table 3.

[3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-x-Ovalbumin (23). The transfer reaction was performed in a solution of sodium cacodylate buffer (50 mM, pH 6.0, 1.0 mL) containing CMP-[3″-^{13}C]-NeuAc **20** (2.1 mg, 3.4 μmol), [U-^{13}C]-Gal-ovalbumin **22** (46.5 mg), CIAP (125U), *rat recombinant* α-2,3-(*N*)-sialyltransferase (50 mU), and the mixture was incubated at 37 °C for 16 h. The mixture was passed through Molcut-L filter (UFP1LGCBK, Millipore Ltd., 10 000 cut). This transfer reaction was repeated for more two times. The 50% of galactoside was estimated to be sialylated by HMQC spectrum. To this mixture of asialo- and sialoglycoprotein in a solution of sodium cacodylate buffer (50 mM, 500 μL, pH 6.0) was added NaN_3 (1.0 mg) and *D. pneumoniae* β-galactosidase (50 mU). The mixture was incubated at 30 °C for 24 h. The mixture was passed through Molcut-L filter (Millipore, 10 000 cut). The residue in the filter was washed with water (500 μL × 5) and then analyzed by NMR. The NMR data are summarized in Table 3.

[U-^{13}C]-Gal-β-(1→4)-GlcNAc-β-OMe (25). The transfer reaction was performed in a solution of HEPES buffer (100 mM, pH 7.0, 500 μL) containing UDP-[U-^{13}C]-Glc (15.0 mg, 25.2 μmol), methyl 2-acetamido-2-deoxy-β-D-glucoside **24** (5.0 mg, 21.3 μmol), UDP-glucose-4-epimerase (540 mU), *bovine* β-1,4-galactosyltransferase (270 mU), $MnCl_2$ (50 mM), CIAP (50 U). The mixture was incubated at 37 °C for 36 h. Purification of the mixture on a silica gel column chromatography (ethyl acetate:MeOH = 3:2) gave **25** (4.7 mg, 57%); The NMR data (Table 3) are identical to unlabeled Gal-β-(1→4)-GlcNAc-β-OMe.[30]

[3-^{13}C]-NeuAc-α-(2→3)-[U-^{13}C]-Gal-β-(1→4)-GlcNAc-β-OMe (26). The transfer reaction was performed in a solution of sodium cacodylate buffer (50 mM, pH 6.0, 360 μL) containing CMP-[3″-^{13}C]-NeuAc **20** (3.6 mg, 5.8 μmol), [U-^{13}C]-Gal-β-(1→4)-GlcNAc-β-OMe **25** (2.1 mg), CIAP (25U), *rat recombinant* α-2,3-(N)-sialyltransferase (50 mU), and the mixture was incubated at 37 °C for 24 h. The mixture was passed through a gel permeation column chromatography (Sephadex G-15, water). Then the residue was purified by HPLC equipping a column chromatography of aminopropyl (0.4 cm × 25 cm, Asahipak, NH_2−P50. retention time = 11.0 min), which was run with a mixture of CH_3CN/15 mM KH_2PO_4 = 7/3. Further HPLC purification using a gel permeation column chromatography (0.8 cm × 50 cm, Asahipak, GS-320, H_2O, retention time = 29.6 min) gave **26** (1.1 mg, 30%); The NMR data (Table 3) are identical to unlabeled NeuAc-α-(2→3)-Gal-β-(1→4)-GlcNAc-β-OMe.[30]

NMR Methods for Conformational Analysis. For NMR studies, the glycoprotein (40−50 mg) which was dialyzed with Molcut-L filter was dissolved in 300 μL of D_2O. All NMR experiments were run on a Bruker AVANCE 400 instrument. The sample was set to 303 K. Two-dimensional ^{1}H−^{13}C HMQC, HMQC-TOCSY, and HMQC-NOESY spectra were measured by use of pulse programs in the Bruker standard library (invbtp, invbmltp, and invbnotp, respectively). During acquisition, GARP decoupling was performed toward ^{13}C (F1 dimension). Pulse sequences of 1D HMQC (selective excitation of H-3ax^{NeuAc} or H-3eq^{NeuAc}), 1D HMQC-NOESY(selective excitation of H-3ax^{NeuAc} or H-3eq^{NeuAc}), 1D HSQC-TOCSY-NOESY, and 1D HSQC-TOCSY-NOESY-TOCSY were made with combined Bruker standard programs and a pulse sequence of TOCSY-NOESY-TOCSY[10] modified as shown in Figure 13. The narrow bars, wide bars, and wide bars having diagonal lines represent 90°, 180°, and trim pulse, respectively. The trim pulse at the end of MLEV17 was set for 2.5 ms, and the other trim pulse was set for 1 ms. (A) 1D HMQC experiment with Gaussian (90°, 7 ms) and half-Gaussian (90°, 1 ms) selective pulses for H-3ax^{NeuAc} (or H-3eq^{NeuAc}) and C-3^{NeuAc}, respectively. The following delay time and phase cycling were applied: $d1$ (2.5 s), $d2$ (3.84 ms), $d3$ (484 ms), $d4$ (336 μs), $d5$ (3 μs); $\phi1 = x$, $\phi2 = -x$, $\phi3 = x$, $\phi4 = x$, $\phi5 = 8x$, $8(-x)$, $\phi6 = x, y, -x, -y$, $\phi7 = 4x, 4(-x)$, receiver phase = $x, -y, -x, y, -x, y, x, -y$. (B) 1D HMQC-NOESY experiment with Gaussian (90°, 7 ms) and half-Gaussian (90°, 1 ms) selective pulses for H-3ax^{NeuAc} (or H-3eq^{NeuAc}) and C-3^{NeuAc}, respectively. The following delay time and phase cycling were applied: $d1$ (2.5 s), $d2$ (3.84 ms), $d3$ (484 ms), $d4$ (336 μs), $d5$ (3 μs), $d6$ (mixing time); $\phi1 = 16x, 16(-x)$, $\phi2 = -x$, $\phi3 = x$, $\phi4 = x$, $\phi5 = 32x, 32(-x)$, $\phi6 = x, -x$, $\phi7 = 2x, 2(-x)$, $\phi8 = x$, $\phi9 = 4x, 4y, 4(-x), 4(-y)$, $\phi10 = x$, receiver phase = $x, 2(-x), x, y, 2(-y), y, -x, 2x, -x, -y, 2y, -y, -x, 2(x), -x, -y, 2y, -y, x, 2(-x), x, y, 2(-y), y$. (C) 1D HSQC-TOCSY experiment. Dulations for E-BURP (90°) for C3^{NeuAc} selective pulses[31] was 4 ms. Spin locking (MLEV17) time was 50 ms. The following delay time and phase cycling were applied: $d1$ (1 s), $d2$ (1.6 ms), $d3$ (3 μs), $d4$ (1.1 ms), $d5$ (3 μs), $d6$ (100 μs), $d7$ (26 μs), $d8$ (3 μs), $d9$ (1.05 ms); $\phi1 = x$, $\phi2 = x$, $\phi3 = y$, $\phi4 = 2x, 2(-x)$, $\phi5 = x$, $\phi6 = x, -x$, $\phi7 = y, -y$, $\phi8 = x$, $\phi9 = -y$, $\phi10 = x$, $\phi11 = 4x, 4(x)$, $\phi12 = x$, $\phi13 = x$, $\phi14 = y$, $\phi15 = x$, $\phi26 = y$, receiver phase = $x, -x, x, -x, -x, x, -x, x$. The following gradient ratios were calculated and then used: $gz1$:$gz2$:$gz3$ = 40:15:10.05. (D) 1D HSQC-TOCSY-NOESY experiment. Dulations for E-BURP (90°) for C-3^{NeuAc} selective pulses[31] was 4 ms. The following delay time and phase cycling were applied: $d1$ (1 s), $d2$ (1.6 ms), $d3$ (3 μs), $d4$ (1.1 ms), $d5$ (3 μs), $d6$ (100 μs), $d7$ (26 μs), $d8$ (3 μs), $d9$

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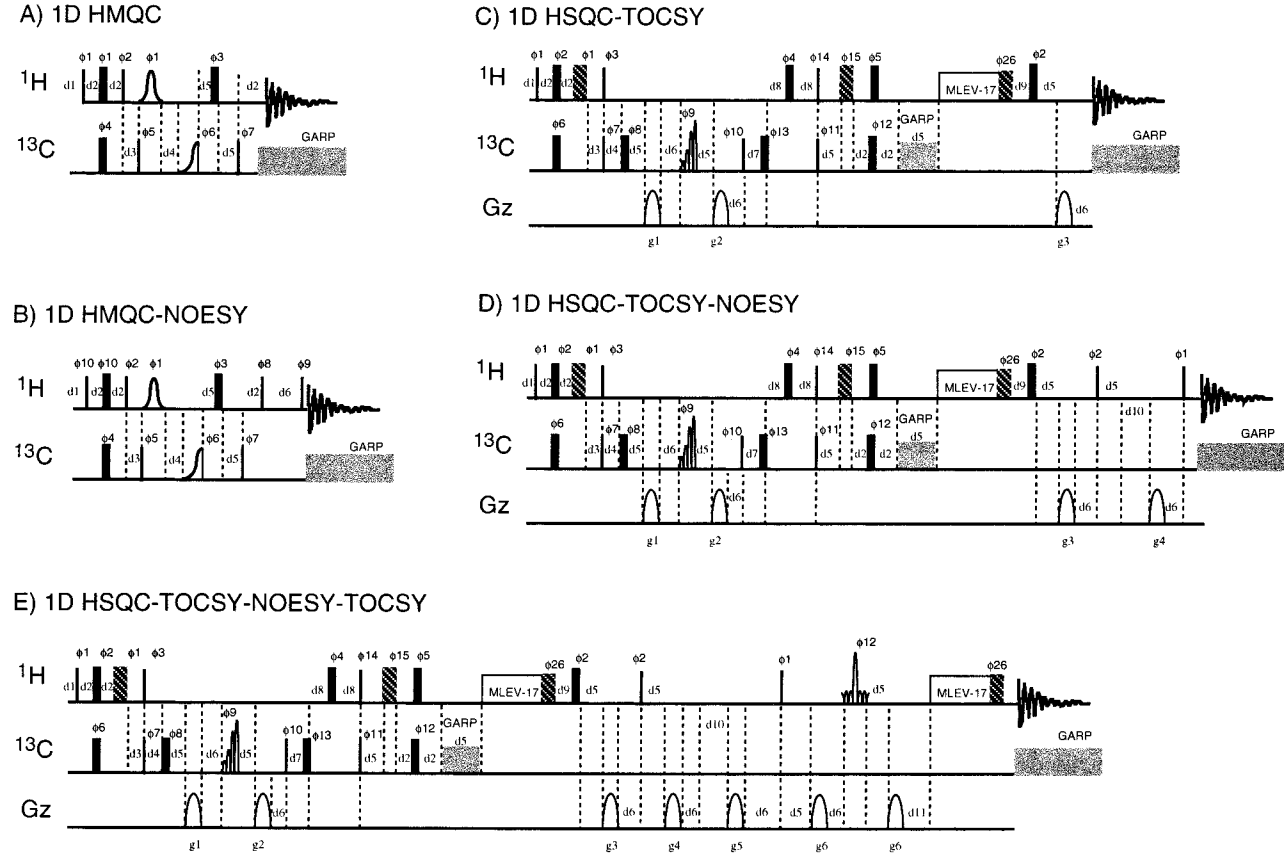

Figure 13. Pulse sequences of modified 1D HMQC, HMQC-NOESY, 1D HSQC-TOCSY, 1D HSQC-TOCSY-NOESY, and 1D HSQC-TOCSY-NOESY-TOCSY.

(1.05 ms), *d*10 (mixing time); $\phi1 = x$, $\phi2 = x$, $\phi3 = y$, $\phi4 = 2x$, $2(-x)$, $\phi5 = x$, $\phi6 = x$, $-x$, $\phi7 = y$, $-y$, $\phi8 = x$, $\phi9 = -y$, $\phi10 = x$, $\phi11 = 4x$, $4(x)$, $\phi12 = x$, $\phi13 = x$, $\phi14 = y$, $\phi15 = x$, $\phi26 = y$, receiver phase = x, $-x$, x, $-x$, $-x$, x, $-x$, x. The following gradient ration were calculated and then used: *gz*1:*gz*2:*gz*3:*gz*4 = 40:15:10.05:−55. (E) 1D HSQC-TOCSY-NOESY-TOCSY experiment. Dulations for RE-BURP (90°) for H-7^{NeuAc}, and E-BURP (90°) for C-3^{NeuAc} selective pulses[31] were 50 ms and 4 ms, respectively. Both spin locking (MLEV17) times were 50 ms.[32] The following delay time and phase cycling were applied: *d*1 (1 s), *d*2 (1.6 ms), *d*3 (3 μs), *d*4 (1.1 ms), *d*5 (3 μs), *d*6 (100 μs), *d*7 (26 μs), *d*8 (3 μs), *d*9 (1.05 ms), *d*10 (mixing time), *d*11 (104 μs); $\phi1 = x$, $\phi2 = x$, $\phi3 = y$, $\phi4 = 2x$, $2(-x)$, $\phi5 = x$, $\phi6 = x$, $-x$, $\phi7 = y$, $-y$, $\phi8 = x$, $\phi9 = -y$, $\phi10 = x$, $\phi11 = 4x$, $4(x)$, $\phi12 = x$, $\phi13 = x$, $\phi14 = y$, $\phi15 = x$, $\phi26 = y$, receiver phase = x, $-x$, x, $-x$, $-x$, x, $-x$, x. The followin gradient ratio were calculated and then used: *gz*1:*gz*2:*gz*3:*gz*4:*gz*5:*gz*6 = 40:15:10.05: −55:32:22. For 2D HSQC-TOCSY-NOESY-TOCSY, the last TOCSY was developped to the second dimension by TPPI mode.

^{13}C Spin−Lattice Relaxation Time (T_1). T_1 measurements were carried out by using the inversion−recovery technique and the T_1^{obsd} values were determined from sixteen delays (τ = 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55, 0.60, 0.65, 0.70, 0.80, 0.90, and 1.00 s) and a fully relaxed spectrum (with delay time between 180° and 90° pulses = $T_1 \times 5$ (s), spectral width = 260 ppm, pulse width (90°) = 13 μs, data size = 32K points) using the fit equation of $I[\tau] = A \exp^{(-\tau/T1)}$ ($I[\tau]$: peak intensity of when delay was used τ s). NOEs ($\eta + 1$) were measured from absolute integrated intensities using the gated decoupling technique.[24b] T_1^{DD} values were calculated from the experimentally determined T_1^{obsd} and η values using the equation $T_1^{DD} = 1.988\ T_1^{obsd}/\eta$.[24]

Computational Methods. Molecular mechanics calculations were performed using MacroModel 5.5,[33] and the force field used was AMBER*[34] which was modified to contain substructure parameters for sialic acid according to A. Bernardi et al.[15k] Conformational analysis of NeuAc-α-(2→3)-Gal-β-(1→4)-GlcNAc-β- sequence was performed in GB/SA[35] water using the Monte Carlo procedure. In this conformational search, aglycon at the reducing end was used methyl glycoside. We searched the lowest-energy conformation satisfying three distance derived by NOE experiments (H-3$_{ax}^{NeuAc}$/H-3^{Gal} = 2.7 ± 0.0 Å, H-1^{Gal}/H-6^{GlcNAc} = 3.0 ± 0.3 Å, H-1^{Gal}/H-6′^{GlcNAc} = 3.0 ± 0.3 Å). These distance constraints were applied with the force constant of 250 kJ/(mol·Å^2). The Monte Carlo simulation was set to vary all dihedral angles except H6^{NeuAc}−C6^{NeuAc}−C7^{NeuAc}−H7^{NeuAc} (−72°), H7^{NeuAc}−C7^{NeuAc}−C8^{NeuAc}−H8^{NeuAc} (−158°), H2^{GlcNAc}−C2^{GlcNAc}−N2^{GlcNAc}−NH^{GlcNAc} (180°), and H5^{NeuAc}−C5^{NeuAc}−N5^{NeuAc}−NH^{NeuAc} (180°). With default force constants, these four dihedral angles estimated by coupling constants (Table 3) were constrained. The Monte Carlo search was conducted with a total 19 000 search steps using TNCG minimization to gradient convergence (<0.05 kJ/mol). A total 2276 unique conformations were saved. The lowest-energy conformer was found three times and there were 14 additional conformaers within 2.0 kcal/mol. The lowest-energy conformer is shown in Figure 12.

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