Chemoenzymatic Synthesis of the 9-Deoxy-9-fluoro-[3-^{13}C]-NeuAc-α-(2→6)-[U-^{13}C]-Gal-*β*-Sequence on an Intact Glycoprotein

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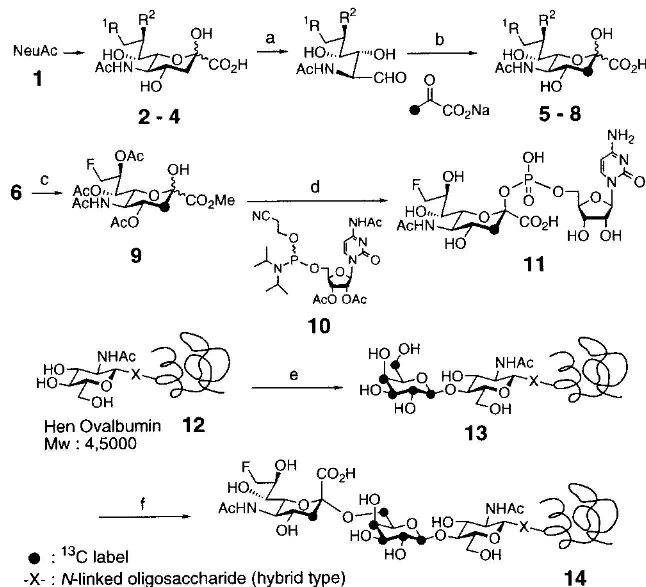
The synthesis of sialyloligosaccharides and their analogues has enabled researchers to study the conformation and the structure−function relationship of these important cellular components.[1] However, the synthesis of sialyloligosaccharide analogues on intact glycoprotein backbones has proven far more difficult than the chemical synthesis of gangliosides and their analogues.[2] If sialyloligosaccharide analogues synthesized on glycoprotein backbones could be easily analyzed by NMR, we would be able to study the dynamics and conformation of sialyloligosaccharide analogues on proteins. Recent research using glycosyltransferases has centered on the transfer of a sugar analogue to the nonreducing terminal of the oligosaccharide of a glycoprotein.[3] However, in most cases, analysis by NMR of the resulting sugar analogue is difficult due to overlap with the proton resonances of the amino acids in the protein.[4,5] We report here a concise ^{13}C-labeling[6] method of a NeuAc analogue, synthesis of the 9-deoxy-9-fluoro-[3-^{13}C]-NeuAc-α-(2→6)-[U-^{13}C]-Gal-*β*- sequence on an intact glycoprotein by sialyl (STase) and galactosyltransferase (GTase), and its structure analysis by NMR.

Since a large number of NeuAc analogues have been synthesized from NeuAc,[7] their synthetic route including selective protection of hydroxyl groups has become fairly common. Therefore, if [3-^{13}C]-NeuAc analogues can be obtained from NeuAc analogues, the published route for the NeuAc analogues will be practically utilized. As is shown in Scheme 1, we performed the aldolase reaction twice: first, degradation[8] of the NeuAc analogue to a ManNAc analogue and, second, condensation of the ManNAc analogue with [3-^{13}C]-pyruvic acid. However, degradation of analogues **2**−**4** did not afford ManNAc analogues in sufficient yields (26−69%) due to the competing reverse reaction. Therefore, the reactions were run in the presence of lactate dehydrogenase in order to push the equilibrium toward the ManNAc analogues. When 1 equiv of *β*-NADH was used, the degradation yields increased to the range 45−98% (Table 1). After purification, ManNAc analogues were condensed with 3 equiv of [3-^{13}C]-pyruvic acid. Among these analogues, the fluoro analogue **6** in which the fluorine atom can be utilized for

Scheme 1[a]



[a] Reagents: (a) aldolase, lactate dehydrogenase, *β*-NADH, yields are summarized in Table 1; (b) aldolase, [3-^{13}C]-sodium pyruvate, yields are summarized in Table 1; (c) (1) Dowex 50W-X8 (H^+), MeOH, (2) $HClO_4$, Ac_2O, $y = 82\%$; (d) (1) 1H-tetrazole, MeCN, (2) *t*-BuOOH, MeCN, (3) DBU, THF, (4) NaOMe, MeOH:H_2O = 1:2, $y = 27\%$; (e) UDP-[U-^{13}C]-glucose, UDP-glucose-4-epimerase, bovine *β*-(1→4)-galactosyltransferase; (f) (1) reaction of bacterium α-(2→6)-sialyltransferase (three times), **11**, (2) *Diplococcus pneumoniae β*-galactosidase.

Table 1. Stable Isotope Labeling of NeuAc Analogues by Use of NeuAc Aldolase[a]

			ManNAc analogue		[3-^{13}C]-Neu5Ac analogue	
substrate	R[1]	R[2]	aldolase (%)[c]	aldolase + LDH[b] (%)[d]	product	(%)[e]
1	OH	OH	69%	90%	**5**	87%
2	F	OH	48%	94%	**6**	91%
3	N_3	OH	60%	98%	**7**	93%
4	OH	H	26%	45%	**8**	90%

[a] Conversion yields were determined by NMR spectra. [b] LDH:lactate dehydrogenase. [c] Reaction used NeuAc analogue (32 mM) and aldolase (3.8 U). [d] Reaction used NeuAc analogue (32 mM), aldolase (3.8 U), and LDH (20 U). [e] Reaction used ManNAc analogue (63 mM) and aldolase (10 U).

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Table 2. ^{1}H and ^{13}C Chemical Shifts and Coupling Constants of Sialoside **14**[a]

		^{1}H	coupling constants (Hz)	^{13}C	$^1J_{C,H}$	NOE
NeuAc	3ax	1.70	$^3J_{H3ax,H4}$ (ca. 14.7)	40.84	129.8	NeuAc-H 4, 5, 6
	3eq	2.66	$^2J_{gem}$ (ca. 14.7)		132.1	
	4	3.64	$^3J_{H4,H5}$ (8.0)			
	5	3.82	$^3J_{H5,H6}$ (10.0)			
	6	3.72	$^3J_{H6,H7}$ (ca. 0.0)[b]			
	8	4.10	$^3J_{F,H8}$ (21.5)			
	9	4.80−4.66	$^2J_{F,H9}$ (46.6)			
Gal	1	4.46		103.64	162.9	Gal-H 3, GlcNAc-H4
	2	3.53		71.49	140.9	Gal-H 3
	3	3.66		72.99	136.5	
	4	3.92		69.13	146.7	Gal-H 3, 5, 6*R*, 6*S*
	5	3.82		74.35	143.8	Gal-H 3, 4, 6*R*, 6*S*
	6*R*	3.98		64.10	152.6	
	6*S*	3.54			152.6	
GlcNAc	4	3.79	$^3J_{H4,H5}$ (8.0)			
			$^3J_{H3,H4}$ (8.0)			

[a] ^{1}H chemical shifts were measured at 298 K (HOD = 4.81 ppm), ^{13}C chemical shifts were measured at 298 K (1,4-dioxane = 67.48 ppm), ^{19}F chemical shift is −234.64 ppm at 298 K (trifluorotoluene = 0.00 ppm); [b] Because HMQC-TOCSY did not show the H-7 resonance from H-6 of NeuAc (spin locking time, 45, 60, 100 ms)

^{1}H−^{19}F HMQC was selected to synthesize CMP-9″-deoxy-9″-fluoro-[3″-^{13}C]-NeuAc (**11**).[9] As shown in Scheme 1, CMP-NeuAc analogue **11** was prepared by a previously reported method.[10]

For synthesis of sialoside **14**, hen ovalbumin **12** was used, because this glycoprotein has only one glycan chain on its backbone.[11] Transfer of the [U-^{13}C]-Gal residue proceeded smoothly toward this glycoprotein. However, when the NeuAc analogue was transferred by repetition of the α-(2→6)-STase reaction,[12] only about 60% of galactoside was estimated to be sialylated according to the HMQC spectrum. Therefore, the [U-^{13}C]-Gal which was not sialylated was subject to digestion by galactosidase. Consequently, the sialoside analogue was obtained in an analytically pure state. To assign chemical shifts and to analyze the conformation, 1D/2D HMQC-TOCSY, 1D/2D ^{1}H−^{19}FHMQC, and 2D HMQC-NOESY analyses were performed, and then the difference in the ^{13}C chemical shifts values between galactoside **13** and sialoside **14** was measured in order to determine the sialylated position.[12] The C-6 and C-5 chemical shifts of galactoside were shifted downfield and upfield, respectively, and the H-6 (*pro-R*) and H-6 (*pro-S*)[13] peaks were also shifted downfield and upfield, respectively. Therefore, the sialylated position was determined to be the 6 position of the galactoside **13**. The changes in the $^1J_{C,H}$ values at the 2, 5, and 6 positions of the galactoside residues in compounds **13** and **14**, suggest that the galactose-ring is strained[14] by sialylation (Table 2 and Table 3 in Supporting Information). Although an NOE between the NeuAc and the galactoside was not observed,[1a,15] based on the NOE data obtained (Table 2) free rotation around the 6 position of the galactoside should result in the gt-rotamer being the predominant orientation. These data are in good agreement with those of the sialyloligosaccharide.[15] Furthermore, there appears to be an NOE between H-1 of the galactoside and H-4[16] of GlcNAc, and the $^1J_{C1,H1}$ value of the galactoside was found to be 162.9 Hz. These data suggest that the galactose is bound to the 4 position of the GlcNAc by a β-bond. The ^{1}H vicinal and geminal coupling constant values observed indicate the ring of NeuAc residue adopts a $_4C^1$ chair form, and the $^3J_{F,H8}$ value (21.5 Hz) suggests that the dihedral angle (9F−9C−8C−8H) adopts an antiperiplanar orientation.[17] Comparison of the hydrogen and carbon chemical shift data between sialoside **14** and sialyl-*N*-acetyl-β-D-lactosaminide,[1a,18] reveals that the chemical shifts are almost identical. These results suggest that conformational properties of sialoside on the protein are similar to those of sialyl-*N*-acetyl-β-D-lactosaminide.[18] Research is in progress to synthesize other ^{13}C-labeled sialyloligosaccharide analogues.

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Supporting Information Available: Synthesis of NeuAc analogues **2**−**9**, **11**, **13**, **14** and its NMR spectra (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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