3β-Hydroxysialic Acid Glycosides. I. Calcium-Binding Ability and Chemical and Enzymatic Stabilities

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Methyl α- and β-glycosides of *N*-acetylneuraminic acid (Neu5Ac) and *N*-acetyl-3β-hydroxyneuraminic acid (Neu5Acβ3OH) (1—4) were prepared to evaluate their calcium-binding ability. (Methyl α-glucopyranosidonyl) α- and β-, and 4-methylumbelliferyl α-glycosides of Neu5Ac and Neu5Acβ3OH (5—10) were also synthesized for the comparison of chemical and enzymatic stabilities, respectively. Methyl β-glycosides of Neu5Ac and Neu5Acβ3OH, 3 and 4, respectively, showed intense calcium-binding abilities, while no such ability was observed in the corresponding α-glycosides, 1 and 2. The Neu5Acβ3OH glycosides, 6, 8, and 10, showed much stronger resistance to acidic hydrolysis and sialidase digestion than the corresponding Neu5Ac glycosides, 5, 7, and 9.

Keywords sialic acid; *N*-acetylneuraminic acid; Neu5Ac; 3β-hydroxysialic acid; glycoside; calcium-binding; hydrolysis; sialidase; chemical stability; enzymatic stability

Sialic acids are aminosugars located at the non-reducing ends of glycoproteins, glycolipids, and polysaccharides.[1-4] These glycoconjugates are believed to be involved in various biological processes such as cell-cell adhesion,[5] recognition,[6] differentiation,[7,8] and transport of ions, amino acids, and viruses through membranes.[1-4] They also play an important role in masking of cancer cell surface and formation of receptors for pathogenic viruses and bacteria.[2]

Because of its great therapeutic potential, *N*-acetylneuraminic acid (Neu5Ac), the most prominent of the sialic acids, has attracted much attention from organic and medicinal chemists. A large number of derivatives of Neu5Ac including natural gangliosides and artificially designed molecules have been synthesized and their biological activities have been evaluated.[9-21] At the same time, modifications of the hydroxyl groups at 2-, 4-, 7-, 8-, and 9-positions[22-28] and the acetamido group at 5-position[29] of Neu5Ac have been studied to pursue the structure–activity relationships in their anabolic and catabolic pathways. Though these studies helped to clarify the function of the each group, they were not necessarily successful in the induction of functions superior to those of the original Neu5Ac.

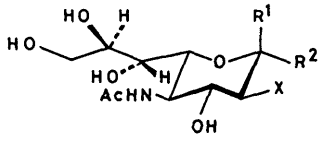
On the other hand, the α-glycosidic bond of Neu5Ac, which is a normal bond pattern of natural products, is known to be sensitive to hydrolysis, particularly in an acidic media at high temperature,[30,31] and also to enzymatic hydrolysis by sialidases.[32,33] These properties may make handling difficult and their application disadvantageous.

In the course of our synthetic studies of gangliosides, we found that glycosides of 3β-hydroxy-*N*-acetylneuraminic acid (Neu5Acβ3OH, Fig. 1, R^1, R^2 = OH, COOH, X = OH) could be relatively easily prepared, and we also noticed that these glycosides were apt to be more stable than those of Neu5Ac.[34,35] This suggests the possibility of Neu5Ac being successively superseded by Neu5Acβ3OH because the latter has the same functional groups and configuration as Neu5Ac except that it has one additional hydroxyl group. Since the detail of chemical and biological properties had not been examined, we decided to pursue the chemistry and properties of Neu5Acβ3OH glycosides.

For the expression of the biological activity, the glycosides of Neu5Acβ3OH should maintain the same conformation as that of Neu5Ac. Determination of the conformation of Neu5Acβ3OH, however, did not appear to be easy, but fortunately it has been shown in NMR study that free Neu5Ac was strongly and preferentially complexed with calcium ion at pH 7 in a molar ratio of 1 : 1.[36] So, if glycosides of Neu5Acβ3OH show the same tendency toward calcium ion as those of Neu5Ac in NMR, it would presumably be rational to say that both compounds have the same conformation.

The present paper describes the calcium-binding abilities of Neu5Ac and Neu5Acβ3OH glycosides. Comparison of the chemical and enzymatic stabilities of the two is also detailed.

Chemistry For the calcium-binding experiment, sodium salts of methyl α- and β-glycosides of Neu5Ac, **1** and **3**, were prepared from the corresponding free glycosides, which were easily obtained from Neu5Ac according to the literature[32]; those of Neu5Acβ3OH, **2** and **4**, were synthesized by glycosidation of the epoxide **11**.[37] The glycosidation of the epoxide **11** with MeOH as a solvent in the presence of Dowex50W-X8 [H^+] resin gave methyl α-glycoside **12** exclusively. Compound **12** was deprotected in the usual manner using Zemplen's deacetylation,[38] saponification, and acidification with [H^+] resin to give the α-glycoside **13**, which was converted to the



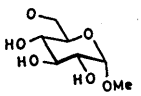
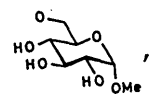
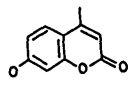
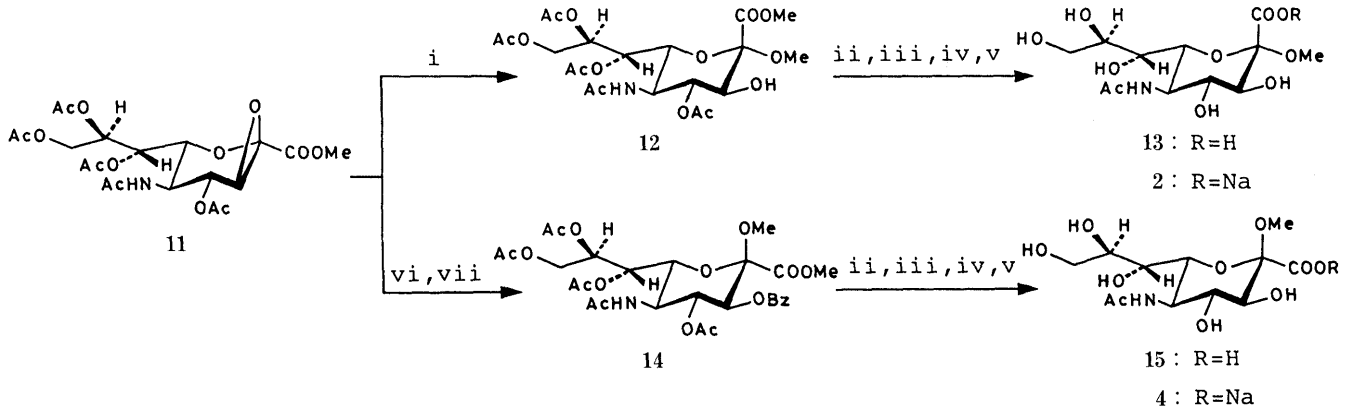
	X=H	X=OH
R^1=COONa, R^2=OCH_3	1	2
R^1=OCH_3, R^2=COONa	3	4
R^1=COOH, R^2= 	5	6
R^1=  , R^2=COOH	7	8
R^1=COOH, R^2= 	9	10

Fig. 1. Neu5Ac and Neu5Acβ3OH Glycosides

corresponding sodium salt **2** by treatment with one equimolar amount of $NaHCO_3$.

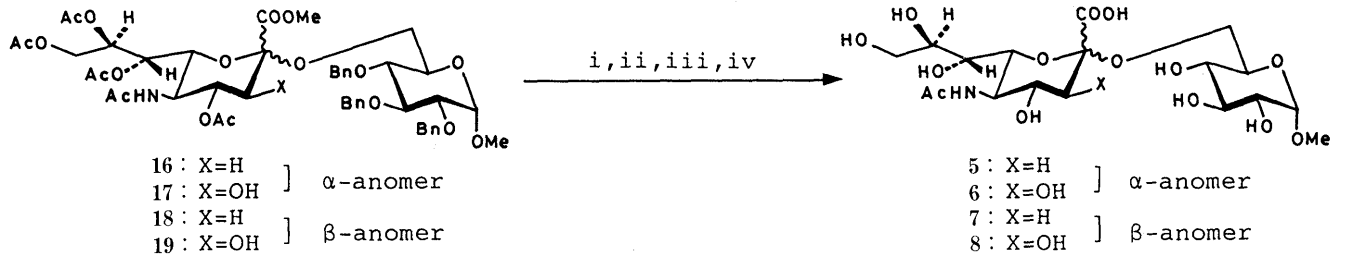
Since the selective preparation of the β-glycoside **4** from the epoxide **11** seemed difficult, **11** was treated with two equivalents of MeOH in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) giving a mixture of methyl α- and β-glycosides in the ratio of *ca.* 6:3.[37] The anomeric mixture obtained was converted to the corresponding benzoates by treatment with benzoyl chloride, which were separable by silica gel column chromatography. The pure β-glycoside **14** was subjected to deprotecting and neutralization processes to give **4** *via* the free acid **15**.

The methyl α- and β-glycosides of Neu5Ac were not suitable for evaluation of the chemical stability because their retention times (t_R) by high performance liquid chromatography (HPLC) were the same as those of Neu5Ac. So, the methyl α-glucopyranoside was chosen as the aglycon of Neu5Ac and Neu5Acβ3OH for HPLC analysis. Thus, α- and β-glycosides of Neu5Ac, **16** and **18**, obtained according to the literature,[39,40] were deprotected by Zemplen's deacetylation, debenzylation (10% Pd–C/H_2), saponification (1 N NaOH), followed by acidification (Dowex50W-X8 [H^+]) to give the desired α-glycoside **5** and β-glucoside **7**, respectively. Similarly, α- and β-glycosides **17** and **19**, having 3β-hydroxyl group



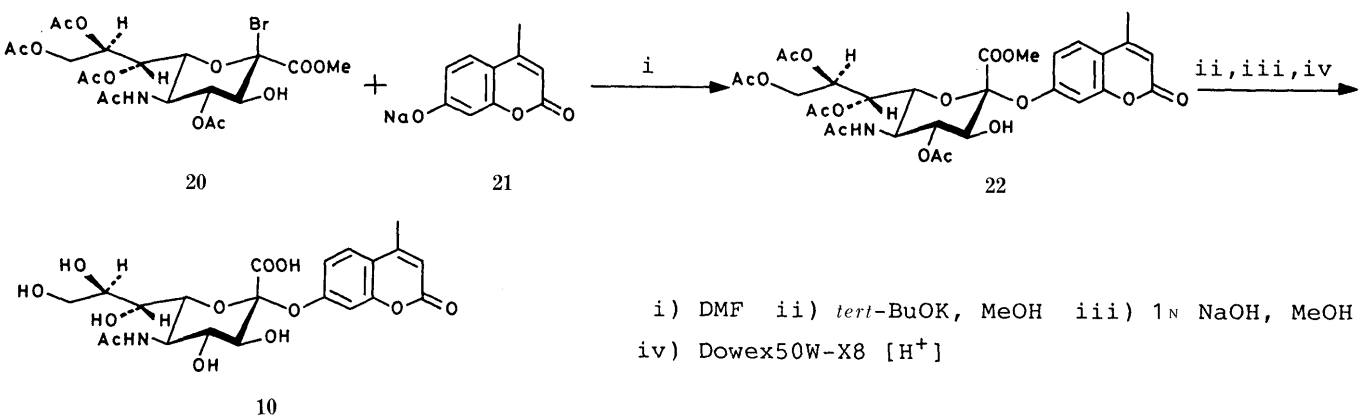
i) MeOH, Dowex50W-X8 [H^+] ii) *tert*-BuOK, MeOH iii) 1 N NaOH, MeOH iv) Dowex50W-X8 [H^+] v) $NaHCO_3$, H_2O vi) MeOH, TMSOTf, $ClCH_2CH_2Cl$ vii) BzCl, pyridine

Chart 1



i) *tert*-BuOK, MeOH ii) 10% Pd-C, H_2, MeOH iii) 1 N NaOH, MeOH v) Dowex50W-X8 [H^+]

Chart 2



i) DMF ii) *tert*-BuOK, MeOH iii) 1 N NaOH, MeOH iv) Dowex50W-X8 [H^+]

Chart 3

in Neu5Ac moiety, were prepared by glycosidation[35] of **20** with the methyl α-glucoside derivative and subjected to the deprotecting steps in the same manner as above to give the α- and β-glycosides, **6** and **8**, respectively. Neu5Acβ3OH, which would be liberated if the glycosides **6** and **8** were hydrolyzed chemically in their ketosidic bonds, was prepared as the standard compound in HPLC by the reported method.[41]

Sialidase cleaves α-glycosidically bound Neu5Ac from oligosaccharides, glycoproteins, and glycolipids. For the detection and assays of sialidase activity, the artificial fluorogenic substrate, 4-methyl-2-oxo-2*H*-1-benzopyran-7-yl (4-methylumbelliferyl = 4MU-) 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosidonic acid (**9**, 4MU-Neu5Ac) is usually used because of its high sensitivity and ease of preparation.[42-47] We adopted the same method. This compound **9** was prepared according to the literature and 4MU derivative **10** was synthesized as

TABLE I. ^{1}H-NMR Data for Neu5Ac Moiety

Compd.	Chemical shifts (δ)														
	H-3ax	H-3eq	H-4	H-5	H-6	H-7	H-8	H-9	H-9′	NH	Me ester OBz		OMe	OH-3	O–Ac, N–Ac
1[a]	1.55	2.64	3.60	3.73	3.63	3.51	3.80	3.55	3.82				3.27		1.96
2[a]	3.41		3.53	3.87	3.65	3.47	3.82	3.57	3.79				3.34		1.96
3[a]	1.60	2.29	3.96	3.83	3.74	3.49	3.84	3.62	3.80				3.15		2.00
4[a]	3.50		3.77	4.00	3.77	3.47	3.83	3.62	3.80				3.26		2.01
5[b]	1.79	2.72	c)	c)	c)	c)	c)	c)	c)						2.03
6[b]	c)		c)	c)	c)	c)	c)	c)	c)						2.03
7[b]	1.77	2.46	c)	c)	c)	c)	c)	c)	c)						2.05
8[b]	3.84		3.90	4.06	3.98	3.54	3.83	c)	c)						2.05
10[b]	3.84		3.89	4.13	4.42	3.57	3.83	3.63	3.84						2.06
13[b]	3.63		c)	3.97	4.03	3.53	c)	c)	c)				2.03		3.44
14[d]	5.42		5.54	4.38	4.03	5.41	5.24	4.11	4.74	5.45	3.72	7.4—8.0	3.41		1.90, 1.90, 2.03, 2.09, 2.23
15[b]	3.61		3.82	4.05	3.86	3.54	c)	3.65	c)				3.36		2.03
22[d]	4.07		5.29	4.21	4.77	5.30	5.37	4.10	4.32	5.67	3.76			3.55	1.93, 2.05, 2.09, 2.11, 2.14

Compd.	Coupling constants (Hz)										
	$J_{3ax,4}$	$J_{3eq,4}$	$J_{3ax,3eq}$	$J_{4,5}$	$J_{5,6}$	$J_{5,NH}$	$J_{6,7}$	$J_{7,8}$	$J_{8,9}$	$J_{8,9'}$	$J_{9,9'}$	$J_{OH,3ax}$
1[a]	12.0	4.6	12.5	10.0	10.1		1.6	9.1	6.4	2.2	11.8	
2[a]	9.8			10.3	10.6		1.7	9.4	6.4	2.6	12.1	
3[a]	11.4	5.0	13.2	10.0	10.4		1.0	9.2	5.7	2.7	11.7	
4[a]	9.7			10.4	10.3		0.9	9.5	5.9	2.3	12.2	
5[b]	12.4	4.5	12.6	c)	c)		c)	c)	c)	c)	c)	
6[b]	c)			c)	c)		c)	c)	c)	c)	c)	
7[b]	11.4	4.9	13.1	c)	c)		c)	c)	c)	c)	c)	
8[b]	9.8			10.3	10.6		0.0	9.1	c)	c)	c)	
10[b]	9.5			9.4	10.7		1.3	9.0	6.7	c)	12.6	
13[b]	9.7			c)	10.7		1.1	8.9	c)	c)	c)	
14[d]	9.9			10.2	10.6	10.0	2.2	4.4	7.4	2.8	12.5	
15[b]	9.3			10.2	10.8		0.8	9.2	6.4	c)	12.7	
22[d]	9.8			9.7	10.8	10.0	1.6	7.9	5.9	2.7	12.7	5.5

a) Measured in D_2O (external TMS). *b*) Measured in D_2O (TSP). *c*) Not assigned owing to the complexity of the spectrum. *d*) Measured in $CDCl_3$.

TABLE II. ^{1}H-NMR Data for Other Moieties

Compd.	Chemical shifts (δ) and coupling constants (Hz) for Glc moiety														
	H-1	H-2	H-3	H-4	H-5	H-6	H-6′	OMe	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{5,6'}$	$J_{6,6'}$
5[a]	4.77	b)	b)	b)	b)	b)	b)	3.40	3.7	b)	b)	b)	b)	b)	b)
6[a]	4.78	3.56	3.64	3.47	3.76	3.98		3.41	3.7	9.8	9.2	9.3	b)	b)	b)
7[a]	4.79	b)	b)	b)	b)	b)	b)	3.42	3.7	b)	b)	b)	b)	b)	b)
8[a]	4.81	3.61	b)	b)	3.77	3.85	3.92	3.41	3.8	8.9	b)	b)	3.5	1.3	10.6

Compd.	Chemical shifts (δ) and coupling constants (Hz) for 4MU moiety							
	H-3	H-5	H-6	H-8	CH_3	$J_{3,Me}$	$J_{5,6}$	$J_{6,8}$
10[a]	6.23	7.70	7.18	7.15	2.41	1.0	8.8	2.3
22[c]	6.20	7.52	7.06	7.12	2.42	1.0	8.8	2.4

a) Measured in D_2O (TSP). *b*) Not assigned owing to the complexity of the spectrum. *c*) Measured in $CDCl_3$.

follows. The bromo-hydrin **20** was condensed with the sodium salt of 4MU (**21**) in *N*,*N*-dimethylformamide (DMF) to give an α-glycoside **22** exclusively in 84% yield. The structure was confirmed by ^{1}H-NMR spectrum in which $J_{7,8}$ and $\Delta\delta$ |H-9′-H-9| values were 7.9 Hz and 0.22 ppm, respectively. This fact supports α-configuration from the empirical rule for determination of the anomeric position.[35] The conversion of **22** to the desired compound **10** was carried out by the usual deprotecting methods.

Results and Discussion

It was found that sialic acid (Neu5Ac) formed the complex with calcium ion (Ca^{2+}) in a molar ratio of 1 : 1. The glycerol side chain participated in the complexation. In the complexation, the minor α-anomer (*ca.* 9%) was converted to the β-anomer through equilibrium.[36] In ^{1}H-NMR study, we showed that the side chain of fully acetylated Neu5Acβ3OH glycosides had the same conformation as those of acetylated Neu5Ac glycosides.[35,40]

It seemed quite reasonable to think that the non-protected Neu5Acβ3OH glycosides also had the same conformation as Neu5Ac glycosides, but there was no evidence to this fact. To confirm this, we decided to use the calcium binding ability mentioned above. The Ca^{2+}-induced change in ^{1}H and ^{13}C chemical shifts and ^{1}H–^{1}H coupling constants of the α- and β-glycosides of both Neu5Acβ3OH and Neu5Ac were examined in the presence of one equimolar amount of Ca^{2+}. The results are summarized in Fig. 2. The apparent difference was observed between the methyl α-glycosides **1** and **2** and the β-glycosides **3** and **4**. In the β-glycosides, **3** and **4**, the large deviation of chemical shifts and coupling constants of specific positions, *i.e.*, H-7, -9, and 9′- and C-8, -7, -9, and -2 in ^{1}H and ^{13}C chemical shifts, respectively and $J_{7,8}$, $J_{6,7}$, and $J_{8,9}$, values in coupling constant, were induced by the addition of Ca^{2+}. However, such phenomena were not observed in α-glycosides, **1** and **2**. This means that the α-glycosides could hardly bind with Ca^{2+}, whereas the β-glycosides formed the Ca^{2+} complexes regardless of the existence of the 3β-hydroxyl group in Neu5Ac moiety. These facts seem to support the assumption that the α- and β-glycosides of Neu5Acβ3OH have, respectively, the same conformation as the α- and β-glycosides of Neu5Ac.

Interestingly, 5-acetamido-2,3,5-trideoxy-D-*glycero*-D-*galacto*-non-2-enopyranosonic acid (Neu5Ac2en) whose carboxyl group is directed in the plane of the 2,3-double bond showed intermediate values in the three parameters mentioned above.[48] This means that in order to bind strongly with Ca^{2+} the axial glycosidic bond and equatorial carboxyl group on the pyran ring are required.

For examination of the chemical stability, the separation of Neu5Ac glycosides and Neu5Ac in HPLC was first confirmed according to the method developed by Ogura *et al.*[30,31,49,50] When a mixture of **5** and Neu5Ac, or **6** and Neu5Acβ3OH was injected a good separation was observed as shown in Fig. 3. A similar HPLC pattern was obtained in the β-glycosides, **7** and **8**.[51] By measuring the peak height of each glycoside during the time course of reaction, the degree of hydrolysis in water or 0.01 N sulfuric acid was estimated and plotted as shown in Fig. 4. The

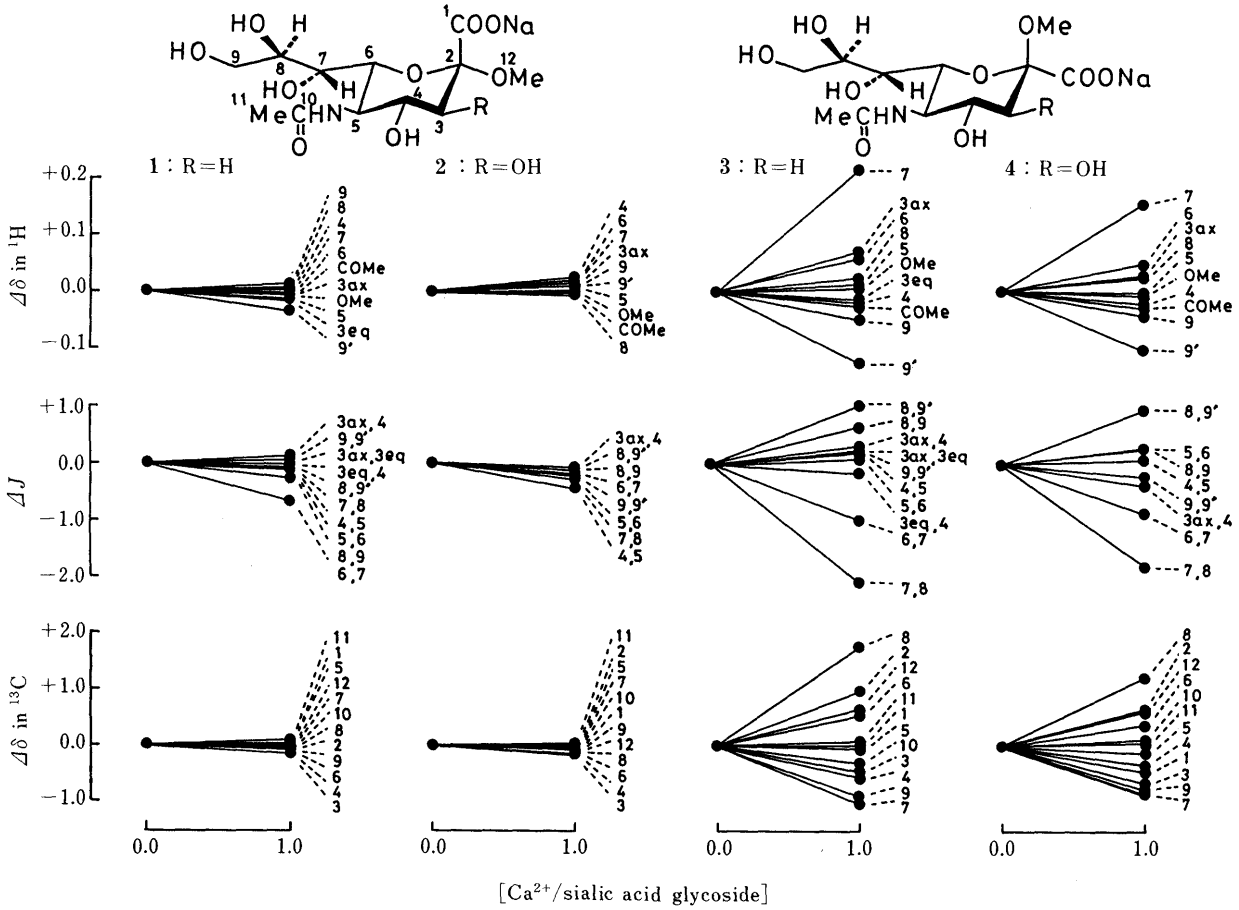


Fig. 2. The Ca^{2+}-Induced Changes of Chemical Shifts (ppm) and Coupling Constants (Hz) in NMR Spectra

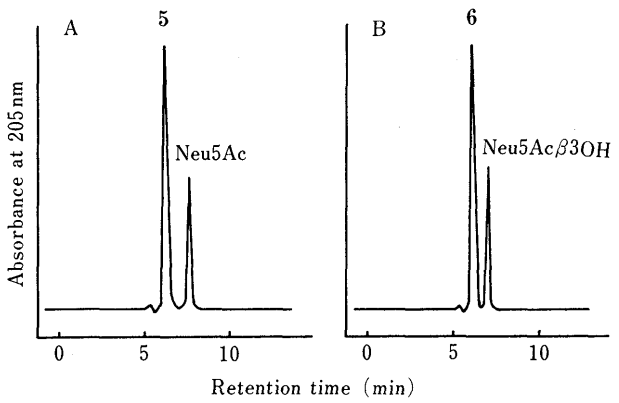


Fig. 3. HPLC Pattern of the Mixture of Glycoside (1.25 μg) and Sialic Acid (0.4 μg)

A, **5**+Neu5Ac; B, **6**+Neu5Acβ3OH.

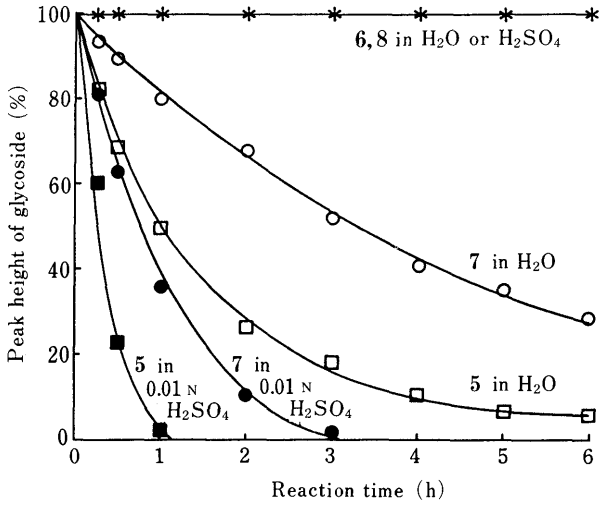


Fig. 4. Chemical Stabilities of α- and β-Glycosides at 80 °C

α-glycoside **5** was hydrolyzed faster than the β-glycoside **7** in both media. The rate of hydrolysis of Neu5Ac glycosides in an acidic solution was faster than that in water. On the other hand, the glycosides **6** and **8** were not damaged by hydrolytic attack after 6 h reaction, even in diluted sulfuric acid. These results show that the ketosidic bond of Neu5Acβ3OH glycoside resists acid hydrolysis more strongly than that of Neu5Ac glycoside. Therefore, Neu5Acβ3OH glycosides can be handled without difficulty unless they have other sensitive or unstable parts.

Sialidase is known as an enzyme selectively hydrolyzing the α-glycosidic bond of sialic acid; but the behavior of sialidase toward the 3β-substituted Neu5Ac glycoside has not yet been studied. Therefore, it is interesting and important to investigate the stabilities of Neu5Acβ3OH glycosides against sialidases in order to know the structure of the substrate recognition part of sialidase as well. Then, the stabilities of α-glycosidic bonds were checked using the artificial substrates, 4MU-Neu5Ac (**9**) and 4MU-Neu5Acβ3OH (**10**). Considering the difference of substrate specificity, four sialidases derived from *Arthrobacter ureafaciens*, *Clostridium pefringens*, *Vibro cholerae*, and bovine brain as that of a mammal were employed for this assay. First, **9** was subjected to sialidase digestion and 4MU liberated was quantified by its fluorescence intensity (Table III). When both **9** and **10** were incubated together with four sialidases, respectively, the quantities of free 4MU were close to those obtained from the digestion of **9** with sialidases. On the other hand, when **10** alone was subjected to the digestion test, the detected quantities of 4MU were very small or negligible. These results indicate that the ketosidic bond of Neu5Acβ3OH glycoside strongly resists sialidase digestion and also that Neu5Acβ3OH glycoside does not inhibit the activity of sialidases.

Table III. Stabilities of 4MU-Neu5Ac (**9**) and 4MU-Neu5Acβ3OH (**10**) against Various Sialidases

Origin of sialidase	Substrate (0.07 mM)[a]		4MU liberated (nmol)
	4MU-Neu5Ac (**9**)	4MU-Neu5Acβ3OH (**10**)	
Arthrobacter ureafaciens	+	−	15.8
	+	+	15.1
	−	+	0.4
Clostridium pefringens	+	−	19.7
	+	+	19.1
	−	+	0.5
Vibrio cholerae	+	−	17.0
	+	+	16.0
	−	+	0.9
Bovine brain	+	−	6.6
	+	+	7.4
	−	+	0.5

a) Each substrate was used as an ammonium salt. The symbols, + and − mean addition and non-addition, respectively.

Conclusion

For studies on calcium-binding ability and chemical and enzymatic stabilities of 3β-hydroxy-Neu5Ac glycosides, methyl, methyl α-glucopyranosidonyl, and 4MU glycosides were synthesized. The results obtained from the calcium-binding evaluation by ^{1}H- and ^{13}C-NMR measurements, the rates of hydrolysis in the acidic solution, and the digestion using four sialidases clearly showed that Neu5Acβ3OH had the same conformation as Neu5Ac and that its glycosidic bond was more stable. Therefore, if the biologically active glycosides of Neu5Acβ3OH can be synthesized[52] their glycosidic bonds will be little hydrolyzed chemically and enzymatically, and also there well be no induction of sialidaise.

Experimental

Melting points were determined with a Yamato MP-21 melting point apparatus and are uncorrected. Elemental analyses were done on a Perkin-Elmer 240C elemental analyzer. Infrared spectra (IR), ultraviolet spectra (UV), and fluorescence spectra were recorded on a Hitachi 260-30, Shimadzu UV-240 UV-visible, and Hitachi 650-40 fluorescence spectrophotometers, respectively. ^{1}H-NMR spectra were obtained with a Bruker AM-400 instrument. Chemical shifts (δ) were expressed in parts per million from tetramethylsilane (TMS) in chloroform-*d* ($CDCl_3$) or 3-(trimethylsilyl)propionic-2,3,3,3-d_4 acid sodium salt (TSP) in deuterium oxide (D_2O) as an internal standard except for the calcium-binding assay. The NMR measurements in the calcium-binding studies were perfomed in a coaxially mounted 5-mm tube using an external TMS. Assignments were confirmed using ^{1}H–^{1}H decoupling, ^{13}C–^{1}H correlation, and *J* resolution techniques. Mass spectra (MS) were obtained on a Hitachi M80B spectrometer by the secondary ion (SI) method using glycerol matrix. Optical rotations were measured with a JASCO DIP-140 digital

polarimeter at 25 °C.

Precoated silica gel glass sheets (Merck silica gel, Art 5715) were used for analytical thin layer chromatography (TLC) and Merck silica gel 60, Art 7734, for column chromatography. Detection on TLC was carried out effectively by UV light or by dipping into the 5% ethanol solution of phosphomolybdic acid or 4% ethanol solution of sulfuric acid followed by charring on a hot plate.

Bacterial sialidases from *Arthrobacter ureafaciens* and *Clostridium pefringens* were purchased from Boehringer-Mannheim-Yamanouchi, and that from *Vibrio cholerae* was obtained from Sigma. Nonidet P-40 was purchased from Nacalai tesque (Japan).

Bovine brain was obtained from the Nishiwaki slaughter house (Hyogo, Japan) within 30 min after death of the animal, washed with saline, and stored at −80 °C until use. All other chemicals were of guaranteed reagent grade commercially available unless otherwise stated.

Sodium (Methyl 5-Acetamido-5-deoxy-α-D-*erythro*-L-*gluco*-2-nonulopyranosid)onate (2) To a solution of the methyl glycoside **12** (750 mg, 1.44 mmol), obtained quantitatively from the epoxide **11**,[37] in abs. MeOH (10 ml) was added a catalytic amount of *tert*-BuOK. The mixture was stirred for 1 h at room temperature under argon atmosphere and deionized with Dowex50W-X8 [H^+] resin. The resin was filtered and washed with MeOH. The combined filtrate and washings were condensed *in vacuo* to give a syrup, which was treated with 1N NaOH (5 ml) in MeOH (15 ml) for 2 h at room temperature. After the reaction mixture was acidified with Dowex50W-X8 [H^+] resin at 0 °C, the resin was removed by filtration. The filtrate was concentrated under reduce pressure to afford a residue, which was crystallized from ethanol to give methyl 5-acetamido-5-deoxy-α-D-*erythro*-L-*gluco*-2-nonulopyranosidonic acid (**13**) (450 mg, 92%) as white crystals. *Rf* 0.20 (*n*-BuOH–AcOH–H_2O, 3 : 1 : 1). SI-MS *m/z*: 340 $(M+H)^+$, 362 $(M+Na)^+$. mp 194—196 °C (dec). $[\alpha]_D$ −45.0° (*c* = 1.1, H_2O). IR (KBr): 3400 (OH, NH), 1700 (COOH), 1575 (amide I), 1250, 1065, 980 cm^{-1}. *Anal.* Calcd for $C_{12}H_{21}NO_{10}$: C, 42.48; H, 6.24; N, 4.13. Found: C, 42.56; H, 6.13; N, 4.40. ^{1}H-NMR data are given in Table I.

The compound **13** (400 mg, 1.18 mmol) was dissolved in water (5 ml) and an aqueous solution of $NaHCO_3$ (99 mg, 1.18 mmol) was added. The mixture was condensed and dried to give the Na salt, **2** (410 mg, 96%) as a white powder. *Rf* 0.14 (*n*-BuOH–AcOH–H_2O, 3 : 1 : 1). SI-MS *m/z*: 362 $(M+H)^+$. $[\alpha]_D$ −34.1° (*c* = 1.5, H_2O). IR (KBr): 3400 (OH, NH), 1630 (COO^-, amide I), 1555 (amide II), 1400, 1075 cm^{-1}. *Anal.* Calcd for $C_{12}H_{20}NNaO_{10}$: C, 39.89; H, 5.58; N, 3.88. Found; C, 39.95; H, 5.81; N, 3.55. ^{1}H-NMR data are given in Table I.

Sodium (Methyl 5-Acetamido-5-deoxy-α-D-*erythro*-L-*gluco*-2-nonulopyranosid)onate (4) An anomeric mixture of the methyl glycoside (1.3 g, 2.5 mmol), obtained from **11** and MeOH (2 molar eq) in the presence of TMSOTf,[37] was benzoylated with benzoyl chloride (0.35 ml, 3.0 mmol) in pyridine (15 ml) for 30 min at room temperature under argon atmosphere. The reaction mixture was condensed under reduced pressure and the residue was partitioned between EtOAc and water. The EtOAc layer was washed with sat. $NaHCO_3$ and brine and dried to give an oil, which was purified by silica gel column chromatography (benzene–acetone, 5 : 1—3 : 1) to give a crystalline mass. Recrystallization twice from ether–EtOAc gave methyl (methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3-*O*-benzoyl-5-deoxy-β-D-*erythro*-L-*gluco*-2-nonulopyranosid)onate (**14**) (580 mg, 37%) as white crystals. *Rf* 0.27 (benzene–acetone, 3 : 1). SI-MS *m/z*: 626 $(M+H)^+$. mp 97—99 °C. $[\alpha]_D$ −36.0° (*c* = 1.3, $CHCl_3$). IR (KBr): 3470 (NH), 1750 (ester), 1660 (amide I), 1540 (amide II), 1372, 1222, 1115, 1030, 718 cm^{-1}. *Anal.* Calcd for $C_{28}H_{35}NO_{15}$: C, 53.76; H, 5.64; N, 2.24. Found: C, 53.51; H, 5.88, N, 2.01. ^{1}H-NMR data are given in Table I.

A solution of **14** (450 mg, 0.72 mmol) and *tert*-BuOK (cat. amount) in abs. MeOH (10 ml) was stirred for 1 h at room temperature under argon atmosphere and deionized with Dowex50W-X8 [H^+] resin. After filtration, the filtrate was condensed to give a methyl ester, which was powdered from MeOH–ether and washed well with ether. The methyl ester was hydrolyzed with 1N NaOH (2.5 ml) in MeOH (7 ml) for 1 h at room temperature. The reaction mixture was treated with Dowex50W-X8 [H^+] resin and after filtration, the filtrate was evaporated *in vacuo* to give **15** (230 mg, 94%) as an amorphous powder. *Rf* 0.15 (*n*-BuOH–AcOH–H_2O, 3 : 1 : 1). SI-MS *m/z*: 340 $(M+H)^+$, 362 $(M+Na)^+$. $[\alpha]_D$ −59.5° (*c* = 1.1, H_2O). IR (KBr): 3400 (OH, NH), 1725 (COOH), 1630 (amide I), 1550 (amide II), 1172, 1080 cm^{-1}. *Anal.* Calcd for $C_{12}H_{21}NO_{10}$: C, 42.48; H, 6.24; N, 4.13. Found: C, 42.68; H, 6.56; N, 4.10. ^{1}H-NMR data are given in Table I.

Na salt **4** was prepared in a similar manner as described for **2** in quantitative yield. *Rf* 0.19 (*n*-BuOH–AcOH–H_2O, 3 : 1 : 1). SI-MS *m/z*: 362 $(M+H)^+$. $[\alpha]_D$ −60.4° (*c* = 1.5, H_2O). IR (KBr): 3400 (OH, NH), 1630 (COO^-, amide I), 1560 (amide II), 1400, 1172, 1075 cm^{-1}. ^{1}H-NMR data are given in Table I.

Methyl 6-*O*-(5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic Acid)-α-D-glucopyranoside (5) To a solution of **16**[34] (700 mg, 0.75 mmol) in abs. MeOH (70 ml) was added *tert*-BuOK (cat. amount) under argon atmosphere. The mixture was stirred for 1 h at room temperature, cooled to 0 °C, and neutralized with Dowex50W-X8 [H^+] resin. After removal of the resin by filtration, the filtrate was evaporated to give a residue, which was triturated with hexane–ether (1 : 1) to give the de-*O*-acetylated compound as a white powder.

A mixture of the above powder, 10% Pd–C (400 mg), and abs. MeOH (60 ml) was vigorously stirred under hydrogen atmosphere for 2 d at room temperature. The catalyst was removed by filtration and washed well with MeOH. The combined filtrates and washings were evaporated *in vacuo* to give the methyl ester, which was hydrolyzed with 1N NaOH (4 ml) and MeOH (40 ml) for 1 h at room temperature. The mixture was cooled to 0 °C and acidified with Dowex50W-X8 [H^+] resin. The resin was filtered and the filtrate was evaporated and triturated with methanol-ether to give **5** (305 mg, 84%) as an amorphous powder. *Rf* 0.14 (*n*-BuOH–AcOH–H_2O, 3 : 1 : 1). SI-MS *m/z*: 486 $(M+H)^+$, 508 $(M+Na)^+$. $[\alpha]_D$ +47.3° (*c* = 1.4, H_2O). IR (KBr): 3370 (OH, NH), 1720 (COOH), 1635 (amide I), 1550 (amide II), 1440, 1380, 1190, 1030 cm^{-1}. *Anal.* Calcd for $C_{18}H_{31}NO_{14}$: C, 44.54; H, 6.44; N, 2.89. Found: C, 44.25; H, 6.19; N, 2.67. ^{1}H-NMR data are given in Tables I and II.

Methyl 6-*O*-(5-Acetamido-5-deoxy-α-D-*erythro*-L-*gluco*-2-nonulopyranosylonic Acid)-α-D-glucopyranoside (6) Deprotection of **17**[35] (1.5 g, 1.57 mmol) was carried out in the same manner as described above to give **6** (705 mg, 89%) as an amorphous powder. *Rf* 0.11 (*n*-BuOH–AcOH–H_2O, 3 : 1 : 1). SI-MS *m/z*: 502 $(M+H)^+$. $[\alpha]_D$ +26.8° (*c* = 1.1, H_2O). IR (KBr): 3350 (OH, NH), 1735 (COOH), 1640 (amide I), 1555 (amide II), 1370, 1030 cm^{-1}. *Anal.* Calcd for $C_{18}H_{31}NO_{15}\cdot 3H_2O$: C, 38.92; H, 6.71; N, 2.52. Found: C, 38.70; H, 6.55; N, 2.73. ^{1}H-NMR data are given in Tables I and II.

Methyl 6-*O*-(5-Acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosylonic Acid)-α-D-glucopyranoside (7) Deprotection of **18**[34] (800 mg, 0.85 mmol) was carried out in the same manner as described above to give **7** (310 mg, 75%) as an amorphous powder. *Rf* 0.10 (*n*-BuOH–AcOH–H_2O, 3 : 1 : 1). SI-MS *m/z*: 486 $(M+H)^+$, 508 $(M+Na)^+$. $[\alpha]_D$ +40.4° (*c* = 1.2, H_2O). IR (KBr): 3400 (OH, NH), 1730 (COOH), 1630 (amide I), 1545 (amide II), 1370, 1023 cm^{-1}. *Anal.* Calcd for $C_{18}H_{31}NO_{14}$: C, 44.54; H, 6.44; N, 2.89. Found: C, 44.50; H, 6.09; N, 2.98. ^{1}H-NMR data are given in Tables I and II.

Methyl 6-*O*-(5-Acetamido-5-deoxy-β-D-*erythro*-L-*gluco*-2-nonulopyranosylonic Acid)-α-D-glucopyranoside (8) Deprotection of **19**[35] (1.0 g, 1.05 mmol) was carried out in the same manner as described above to give **8** (435 mg, 82%) as an amorphous powder. *Rf* 0.06 (*n*-BuOH–AcOH–H_2O, 3 : 1 : 1). mp. 79—81 °C. SI-MS *m/z*: 502 $(M+H)^+$, 524 $(M+Na)^+$. $[\alpha]_D$ +17.7° (*c* = 1.1, H_2O). IR (KBr): 3400 (OH, NH), 1730 cm^{-1}. *Anal.* Calcd for $C_{18}H_{31}NO_{15}\cdot 2H_2O$: C, 40.22; H, 6.50; N, 2.61. Found: C, 40.67; H, 6.96; N, 2.41. ^{1}H-NMR data are given in Tables I and II.

Methyl (4-Methyl-2-oxo-2*H*-1-benzopyran-7-yl 5-Acetamido-4,7,8,9-tetra-*O*-acetyl-5-deoxy-α-D-*erythro*-L-*gluco*-2-nonulopyranosid)onate (22) A mixture of **20**[35] (500 mg, 0.88 mmol), **21** (350 mg, 1.77 mmol), and dry DMF (5 ml) was stirred for 1 h at room temperature under argon atmosphere. The solvent was removed *in vacuo* and the residue was partitioned between chloroform and water. The chloroform layer was separated, washed with water and brine, dried (Na_2SO_4), and evaporated to give an oil, which was chromatographed on a silica gel column (benzene–acetone, 2 : 1—1 : 1) and triturated with hexane–EtOAc to give **22** (490 mg, 84%) as a white powder. *Rf* 0.44 (benzene–acetone, 1 : 1). SI-MS *m/z*: 666 $(M+H)^+$. $[\alpha]_D$ +43.9° (*c* = 1.0, $CHCl_3$). IR (KBr): 3370 (OH), 1742 (ester), 1660 (amide I), 1607, 1543 (amide II), 1364, 1230, 1062, 1030, 852 cm^{-1}. *Anal.* Calcd for $C_{30}H_{35}NO_{16}$: C, 54.13; H, 5.30; N, 2.10. Found: C, 54.06; H, 5.68; N, 2.13. ^{1}H-NMR data are given in Tables I and II.

4-Methyl-2-oxo-2*H*-1-benzopyran-7-yl 5-Acetamido-5-deoxy-α-D-*erythro*-L-*gluco*-2-nonulopyranosidonic Acid (10) To a solution of **22** (390 mg, 0.59 mmol) in abs. MeOH (10 ml) was added *tert*-BuOK (cat. amount) and the mixture was stirred for 0.5 h at room temperature under argon atmosphere. After removal of the solvent, the residue was diluted with water (30 ml) and treated with 1N NaOH (0.3 ml) for 0.5 h at room temperature. The mixture was cooled to 0 °C and acidified with Dowex50W-X8 [H^+] resin. The resin was filtered and washed well with

50% ethanol, and the combined filtrate and washings were condensed to give a solid, which was recrystallized from ether-ethanol to give **10** (255 mg, 90%) as white crystals. *Rf* 0.38 (*n*-BuOH–AcOH–H_2O, 3 : 1 : 1). mp. 145—147 °C. SI-MS *m/z*: 484 $(M+H)^+$. $[\alpha]_D$ +43.2° (c = 1.1, H_2O). IR (KBr): 3400 (OH, NH), 1720 (COOH), 1700 (CO), 1603, 1552 (amide II), 1380, 1360, 1275, 1068, 1015 cm^{-1}. *Anal.* Calcd for $C_{21}H_{25}NO_{12}$: C, 52.17; H, 5.21; N, 2.90. Found: C, 52.29; H, 4.88; N, 2.56. ^{1}H-NMR data are given in Tables I and II.

For the sialidase assay above crystals were further purified on a Bio-Gel P2 column by eluting with 0.01 N ammonia to give the ammonium salt of **12**. *Anal.* Calcd for $C_{21}H_{25}NO_{12}NH_3 \cdot 1.9H_2O$: C, 47.17; H, 6.00; N, 5.24. Found: C, 47.47; H, 6.08; N, 4.95.

NMR Measurement for Calcium-Binding Aassay The glycoside, **1**, **2**, **3**, or **4** (20 mg) was dissolved in D_2O (0.25 ml) in a 5 mm NMR tube. After addition of calcium chloride dihydrate (one equimolar amount) as a Ca^{2+} source, the inner tube containing TMS in $CDCl_3$ was coaxially mounted and the resulting double tube was set to measure at 27 °C. Through the ^{1}H- and ^{13}C-NMR experiments the same sample was used to avoid any influence of concentration.

Hydrolysis of 5, 6, 7, and 8, and HPLC Analysis The glycosides, **5**, **6**, **7**, and **8** were hydrolyzed at 80 °C in water or 0.01 N sulfuric acid at a concentration of 50 μg/ml. During hydrolysis, each 50 μl of the reaction mixture was analyzed by HPLC using a ULTRON PS-80H strong cation exchange resin column (8.0 i.d. × 300 mm) designed for organic acid analysis at 50 °C. A mobile phase of 60% $HClO_4$–H_2O (1.8 ml/1 l) was used at a flow-rate of 0.8 ml/min. The column eluent was monitored with a UV detector at 205 nm (JASCO, UVIDEC-100-VI). The peak height was automatically calculated by a data station for HPLC (JASCO, DS-L300).

Preparation of Sialidase from Bovine Brain Preparation of bovine gray matter sialidase was based primarily on the method of Schengrund *et al.*[53] Bovine gray matter was homogenized in four volumes of chilled Tris–HCl buffer (5 mM, pH 7.4) for 30 s (Polytron®, Kinematica, Switzerland), and centrifuged at 100000 × g for 60 min (4 °C). The pellet was suspended in 2.5 volumes of the same buffer and Nonidet P-40 was added to a final concentration of 1%. After stirring for 30 min on an ice-water bath, followed by centrifugation at 20000 × g for 60 min (4 °C), the supernatant was used as the enzyme solution of bovine gray matter sialidase without further purification.

Sialidase Assay[53,54] Stabilities of the ammonium salts of 4MU-Neu5Ac (**9**) and 4MU-Neu5Acβ3OH (**10**) against various sialidases were compared fluorometrically. The standard mixture for the bacterial sialidase assay contained 58 mM acetate buffer (pH 4.8), 0.07 mM 4MU-Neu5Ac ammonium salt and/or 4MU-Neu5Acβ3OH ammonium salt and enzyme solution in a final volume of 0.3 ml. Reaction was performed at 37 °C for 25 min and quenched by the addition of 1 ml 133 mM glycine buffer (pH 10.4) containing 60 mM NaCl and 42 mM Na_2CO_3. Free 4-methylumbelliferon was determined in a spectrofluorometer using excitation light at 370 nm and fluorescence emission at 450 nm.

The standard mixture for the bovine brain sialidase assay, on the other hand, contained the same components as those of bacterial sialidases described above except for pH (3.9) and incubation period (2 h).

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