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Reversible *O*-acetyl migration within the sialic acid side chain and its influence on protein recognition

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Abstract

O-Acetylation is a common naturally occurring modification of carbohydrates and is especially widespread in sialic acids, a family of nine-carbon acidic monosaccharides. *O*-Acetyl migration within the glycerol side chain of mono-*O*-acetylated sialic acid reported previously was from the C7- to C9-hydroxyl group with or without an 8-*O*-acetyl intermediate, which resulted in an equilibrium that favors the formation of the 9-*O*-acetyl sialic acid. Herein, we provide direct experimental evidence demonstrating that *O*-acetyl migration is bidirectional, and the rate of equilibration is influenced predominantly by the pH of the sample. While the *O*-acetyl group on sialic acids and sialoglycans is stable under mildly acidic conditions (pH<5), reversible *O*-acetyl migration occurs readily at neutral pH and becomes more significant when the pH increases to slightly basic. Sialoglycan microarray studies showed that esterase-inactivated porcine torovirus hemagglutinin-esterase bound strongly to sialoglycans containing a more stable 9-*N*-acetylated sialic acid analog, but these compounds were less resistant to periodate oxidation treatment compared to their 9-*O*-acetyl counterparts. Together with prior studies, the results support the possible influence of sialic acid *O*-acetylation and *O*-acetyl migration to host-microbe interactions and potential application of the more stable synthetic *N*-acetyl mimics.

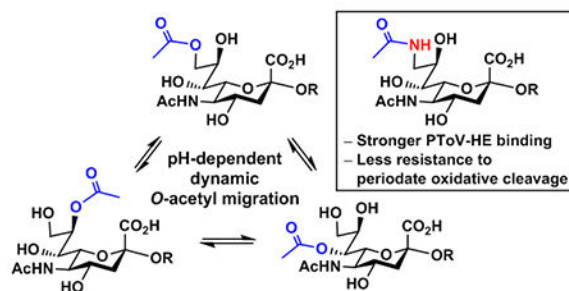
Graphical Abstract

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Supporting Information *Available*: NMR spectra and additional tables and figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

CONFLICT OF INTEREST

The authors declare no competing interests.



Keywords

carbohydrate; O-acetyl; O-acetyl migration; sialic acid; sialoglycan

Sialic acids (Sias) are a family of negatively charged nine-carbon monosaccharides typically found at the non-reducing end of glycans on glycoconjugates of vertebrates. Remarkable structural diversity has been observed for Sias, which include *N*-acetylneuraminic acid (Neu5Ac, **1**, Figure 1), *N*-glycolylneuraminic acid (Neu5Gc), ketodeoxynonulosonic acid (Kdn), and their modified forms.¹⁻⁴ *O*-Acetylation of the hydroxyl groups at C9 (**2**), C8 (**3**), C7 (**4**), and/or C4 is the most common Sia modification.

Numerous biological functions of Sias and their involvement in human health and human diseases have been recognized. Many of these roles can be influenced by Sia *O*-acetylation. For example, while *O*-acetylation of the C9 hydroxyl group (C9-OH) in Sia prevents it from being recognized by hemagglutinins of influenza A and B viruses, it is required for influenza C and D virus hemagglutinin binding.⁵⁻⁹ In addition, 7-*O*-acetylation of Sias in Group B Streptococcal capsular polysaccharides blocks the binding of Siglec-9 to a greater extent than 9-*O*-acetylation of Sias.¹⁰ The biological implications of *O*-acetylation in Sias have been reviewed and summarized.^{1, 2, 11} Although the importance of Sia *O*-acetylation is increasingly recognized, its functional studies have been hampered by the instability of *O*-acetyl (OAc) groups and their propensity to migrate under biological and analytical conditions. OAc groups are especially labile under basic conditions frequently used in procedures to release, purify, and analyze Sias and sialoglycans.¹² While migration of the Sia 4-*O*-acetyl group was not observed,¹³ *O*-acetyl migration within the glycerol side chain of Sia from C7-OH to C9-OH to reach an equilibrium favoring the formation of 9-OAc-Sias was reported and was believed to contribute to the prevalence of 9-OAc-Sias among OAc-Sias in nature.¹²⁻¹⁵ In sialoglycans where the Sia C9-OH position is already occupied, such as in di-*O*-acetylated Sias or α 2-9-linked polysialic acids, *O*-acetyl migration from C7-OH to C8-OH in Sia was also observed.^{13, 14} Studies of sialate:7(9)-*O*-acetyltransferase suggested that Ac was added by the enzyme to the C7-OH of Sia, which then migrated to the C9-OH.¹⁶ An additional Ac could then be added by the enzyme to the C7-OH for the formation of 7,9-di-*O*-acetylated Neu5Ac (Neu5,7,9Ac₃). Further migration of the Ac group from C7-OH to C8-OH followed by the addition of another Ac group to C7-OH led to the formation of Neu5,7,8,9Ac₄.^{13, 16} Alternatively, the hydrolysis of OAc-Sia by sialate-*O*-acetyltransferase-catalyzed reactions was hypothesized to also involve *O*-acetyl migration as a

9-OAc-substrate was the preferred substrate while the OAc group was initially on the C7 of Sia.^{17, 18}

During recent sialoglycan microarray studies, we found that sialoglycans containing 9-*N*-acetyl-Neu5Ac (Neu5Ac9NAc, a stable synthetic mimic of Neu5,9Ac₂) showed stronger binding to a Neu5,9Ac₂-glycan-recognizing protein than their Neu5,9Ac₂-glycan homologues. The enhancement in its binding to Neu5Ac9NAc-glycans exceeded the gain that would be expected from preventing the de-*O*-acetylation of Neu5,9Ac₂, indicating that *O*-acetyl migration of Neu5,9Ac₂ in the sialoglycans may contribute to the weaker binding of the protein to the Neu5,9Ac₂-glycans. To gain additional insight into Sia *O*-acetyl migration and to investigate whether the Ac group at the C9-OH of Sia can undergo reversible migration to the C7-OH and/or C8-OH positions, we improved 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) derivatization and high-performance liquid chromatography analysis (DMB-HPLC) by developing a low-temperature pH-controlled derivatization process to minimize *O*-acetyl migration during analysis. Nuclear magnetic resonance (NMR) studies of Neu5,9Ac₂ and Neu5,9Ac₂-glycan samples at different pH values as well as preparation of deuterium-labeled OAc-Sia and its use for analyzing *O*-acetyl migration at different pHs were carried out. We also performed sialoglycan microarray studies of esterase-inactivated porcine torovirus (PToV) hemagglutinin-esterase (HE) using paired sialoglycan probes containing Neu5,9Ac₂ or its more stable 9-*N*-acetyl analog, Neu5Ac9NAc. The sensitivities of a set of sialoglycans containing Neu5Ac, Neu5,9Ac₂, or Neu5Ac9NAc to periodate oxidation treatment were also compared. All experimental results were consistent with reversible *O*-acetyl migration at neutral or slightly basic conditions. The potential impact of Sia *O*-acetyl migration on other biomolecular recognition and bioprocess regulation events awaits further exploration.

RESULTS AND DISCUSSION

DMB-HPLC sialic acid quantitative assay improvement to minimize *O*-acetyl migration during analysis.

DMB derivatization followed by HPLC analysis (DMB-HPLC) has been commonly used to quantify Sias.¹² Conventional DMB derivatization (50 °C for 2.5 h) was successful for non-*O*-acetylated Sias but led to a significant loss of OAc groups and unwanted *O*-acetyl migration in OAc-Sias. Lowering the reaction temperature to 4 °C significantly decreased the level of de-*O*-acetylation or *O*-acetyl migration, but concomitantly decreased DMB derivatization efficiencies even with extended reaction times (e.g. 48 h) for samples originally prepared in a buffer at neutral or basic pH. To quantify OAc-Sias more accurately with minimal OAc loss or migration while retaining optimal DMB derivatization yields, an improved DMB derivatization method was developed. Controlling the pH during sample preparation for DMB derivatization at 4 °C was critical to achieving optimal yields. Previous low-temperature derivatization procedures used DMB in 0.02 M trifluoroacetic acid.¹⁹ DMB reagent, mixed with the same volume of a sample prepared in water (pH 7.0) or sodium acetate solution (50 mM, pH = 5.0 or 7.0), resulted in samples with varied pH values of 2.7, 4.3, and 4.7, respectively. The pH variation may have caused the inconsistencies in derivatization efficiencies. To maintain a consistent acidic pH range for optimal DMB

derivatization of α -keto acids such as Sias, the DMB reagent was prepared in a high concentration (1.6 M) acetic acid solution. Mixing with the same volume of Sia solution in either water or in a 50 mM sodium acetate solution of pH = 5.0 or 7.0, yielded samples with a steady pH range (3.8–4.1). Improved peak-separation in HPLC-based analysis of DMB-derivatized Sias was further optimized by changing the isocratic conditions (acetonitrile/methanol/water = 8:7:85, v/v) to a linear gradient (acetonitrile/methanol/water from 7:7:86 to 11:7:82, v/v) in a 75-min run.

Figure 2A shows that DMB-derivatization at 4 °C for 48 h with improved sample preparation conditions better preserved the OAc groups on Sias and reduced de-*O*-acetylation and *O*-acetyl migration compared to the reaction performed at 50 °C for 2 h. Additionally, consistent derivatization efficiencies and results were obtained when the DMB reagent was prepared in acetic acid (1.6 M) and mixed with Sia in the same volume in a cold sodium acetate solution (50 mM) of a different pH (pH 5.0 or pH 7.0) at 4 °C for 48 h (Figure 2B). A Neu5,9Ac₂ sample synthesized by regioselective acetylation of Neu5Ac using trimethyl orthoacetate with purity confirmed by NMR analysis previously²¹ was converted to a mixture containing Neu5Ac (~3%), Neu5,7Ac₂ (~2%), Neu5,8Ac₂ (~30%), and Neu5,9Ac₂ (~65%) after roughly three years of storage as a lyophilized powder at –20 °C.

pH-Dependent reversible *O*-acetyl migration among the hydroxyl groups on the glycerol side chain of Neu5Ac by DMB-HPLC and NMR studies.

The pH-dependent *O*-acetyl migration in OAc-Sias was initially observed for samples prepared from bovine submaxillary mucins by gas liquid chromatograph (GLC) analysis¹² and was later confirmed by more thorough HPLC and ¹H-NMR spectroscopy studies.¹³ As the samples used for the studies had high percentages of 7/8-OAc-Sias, the *O*-acetyl migration observed was from C7-OH or C8-OH to C9-OH. Therefore, *O*-acetyl migration in mono-OAc-Sia was assumed to be unidirectional with a rate dependent on both pH and temperature. Upon reaching the equilibrium after an extended incubation period (>1250 min, 37 °C, pH 7.2), about 10% Neu5,7Ac₂ remained by ¹H-NMR analysis.¹³ Neu5,8Ac₂ was not detected, therefore whether it was involved in the migration was unresolved in previous studies.

Using the synthetic Neu5,9Ac₂ sample that was converted to a mixture of Neu5Ac (~3%), Neu5,7Ac₂ (~2%), Neu5,8Ac₂ (~30%), and Neu5,9Ac₂ (~65%) after approximately three years of storage as a lyophilized powder at –20 °C, we revisited Sia *O*-acetyl migration using the improved DMB-HPLC method described above. When the sample (starting material 1 or **SM1**, Figure 3) was incubated in a sodium acetate solution with a pH in the range of 3.0–8.0 at 37 °C for 2 h, *O*-acetyl migration from C8-OH to C9-OH was clearly observed at pH 5.0, although the degree of migration was larger at pH 7.0. For example, at pH 8.0 where de-*O*-acetylation was less than 1%, Neu5,8Ac₂ decreased from 32.4% to 11.8% while Neu5,9Ac₂ concomitantly increased from 65.6% to 85.1%. A slight increase (2.0% to 3.0%) of Neu5,7Ac₂ was also observed.

In another more freshly purified Neu5,9Ac₂ sample (Starting material 2 or **SM2**), incubation in a sodium acetate solution at pH 8.0 and 37 °C for 2 h increased the percentages of

Neu5,7Ac₂ and Neu5,8Ac₂ from 0.8% to 3.1% and 6.1% to 10.7%, respectively, while the percentage of Neu5,9Ac₂ decreased from 93.1% to 86.2% (Figure 3). The results demonstrated *O*-acetyl migration from C9-OH to C8-OH and C7-OH which is the opposite direction of that reported previously for mono-*O*-acetylated Sias. Similar to **SM1**, *O*-acetyl migration in **SM2**, although in the opposite direction to that observed for **SM1**, occurred at pH 5.0 and at a higher level at pH 7.0. The migration in both directions seen in **SM1** and **SM2** seemed to have reached an equilibrium among Neu5,9Ac₂ (~87%), Neu5,8Ac₂ (~10%), and Neu5,7Ac₂ (3%) at pH 8.0. When the sample pH was lowered to 3.0, no significant *O*-acetyl migration was observed for over 2 h. Similar results were obtained for an OAc-Sia sample (Starting material 3 or **SM3**), which contained 90.1% Neu5,9Ac₂, 8.5% Neu5,8Ac₂, and 1.4% Neu5,7Ac₂ before incubation and a mixture of 88% Neu5,9Ac₂, 10% Neu5,8Ac₂, and 2% Neu5,7Ac₂ was obtained after incubation at pH 7.0 and 37 °C for 2 h.

To determine the experimental errors of the DMB-HPLC assays (e.g. Figure 3), the assays were repeated for both **SM1** and **SM3** in triplicate. The standard deviations of the percentages of individual OAc-Sia determined did not exceed 0.2% (Table S1).

SM3 was also used in a series of two-dimensional NMR experiments including ¹H-¹³C HSQC,²² HSQC-TOCSY,^{23, 24} and long-range (LR)-HSQMBC²⁵ (Figures S1–S2, 37 °C, D₂O, pH* 6.14) to assign the chemical shifts of *O*-acetylated Sias, including the α and β anomers of Neu5,9Ac₂, Neu5,8Ac₂, Neu5,7Ac₂, and Neu5Ac (Table S2). The LR-HSQMBC showed a cross peak at ¹H 4.9 ppm and ¹³C 173.3 ppm, confirming *O*-acetyl at C8 in Neu5,8Ac₂ (Figure S2). Previously, a similar ¹H peak at δ = 5.1 ppm which displayed a doublet of doublets of doublets splitting pattern (ddd) was assigned to the H8 of Neu5,8,9Ac₃ at pH* 7.5.¹³ The assignments were then used to interpret one-dimensional ¹H-NMR spectra of another sample of **SM3** recorded under acidic (pH* 3.5) conditions at t = 0 min and after incubation at 37 °C for 120 min and 12 h. The H9s of Neu5,9Ac₂ were integrated as 1.00 for reference. The ddd peak at δ = 4.8 ppm for the H-8 of Neu5,8Ac₂ and the dd peak at δ = 4.9 for the H-7 of Neu5,7Ac₂ were used for integration to determine the percentages of individual species. Approximately 10% of Neu5,8Ac₂ (δ = 4.95 ppm) and 2.1% of Neu5,7Ac₂ (δ = 5.05 ppm) were detected in the sample at pH* 3.5 at the beginning (t = 0 min), which remained unchanged throughout the NMR experiment (Figure S3A–C). The pH* of the sample was then adjusted to 7.0, and the spectra were recorded again at t = 0 min and after incubation at 37 °C for 120 min and 12 h. After the pH* was changed to 7.0, about 12% of Neu5,8Ac₂ and 3% of Neu5,7Ac₂ were detected at t = 0 min, which remained unchanged during the incubation at 37 °C for 12 h (Figure S3D–F). The results indicated that the sample prepared in an aqueous solution at pH* 7.0 reached an equilibrium for *O*-acetyl migration at the beginning of the NMR experiment and the ratios of OAc-Sias agreed with those obtained by the improved DMB-HPLC method described above.

Confirmation of simultaneous bidirectional *O*-acetyl migration in OAc-Sias by synthesizing a deuterated Neu5,9Ac₂.

To confirm the reversible *O*-acetyl migration in OAc-Sias, Neu5,9Ac₂ with a 9-*O*-deuterated acetyl group (deuterated Neu5,9Ac₂) was synthesized by modifying a lipase-catalyzed *O*-acetylation reaction using deuterated vinyl acetate as the acetylation reagent.²⁶ The

deuterated Neu5,9Ac₂ sample was mixed with non-deuterated Neu5,8Ac₂ and Neu5,9Ac₂, and an OAc-Sia mixture containing 87.3% Neu5,9Ac₂ (64.0% non-deuterated and 23.3% deuterated), 12.2% Neu5,8Ac₂ (9.2% non-deuterated and 3.0% deuterated), and 0.5% Neu5,7Ac₂ (non-deuterated) was obtained, as confirmed by LC-MS (the control sample in Table S3 and Figure S4). The effective separation of Neu5,7Ac₂, Neu5,8Ac₂, and Neu5,9Ac₂ by HPLC and comparison with bovine submaxillary mucin (BSM) Sia standards ensured the correct designation of OAc-Sia species. The differentiation and quantification of the deuterated and non-deuterated forms of Neu5,9Ac₂, Neu5,8Ac₂, and Neu5,7Ac₂ were based on the peaks in the extracted ion current (XIC) chromatograms of the MS. If Sia *O*-acetyl migration were unidirectional from C7 and/or C8 to C9-OH, we would see an unchanged percentage of each OAc-Sia species, or a slight decrease of Neu5,8Ac₂ (both deuterated and non-deuterated) in the given OAc-Sia mixture. However, in a dynamic equilibrium, the deuterated Ac group on deuterated Neu5,9Ac₂ would migrate back to form [8-deuteroacetyl]Neu5,8Ac₂, while the non-deuterated acetyl group on Neu5,8Ac₂ would migrate to C9-OH concurrently. Therefore, we would observe an increase in both [8-deuteroacetyl]Neu5,8Ac₂ and non-deuterated Neu5,9Ac₂.

As shown in Figure 4, the net percent increase in deuterated Neu5,8Ac₂ by incubation at 7.0 or 8.0 (from 3.0% to 4.6% and 5.1%, respectively) clearly demonstrated the migration of the deuterated Ac group from C9-OH to C8-OH (Table S3). The net percent decrease in non-deuterated Neu5,8Ac₂ under the same conditions (from 9.2% to 5.1% at pH 7.0, to 4.4% at pH 8.0) indicated the migration of non-deuterated Neu5,8Ac₂ from C8-OH to C9-OH. Furthermore, the fact that 0.3% deuterated Neu5,7Ac₂, which was absent in the control, was detected at pH 8.0 together with the consistent results in Figure 3, suggests the involvement of the C7-OH position in the bidirectional *O*-acetyl migration.

To further investigate the participation of C7-OH in OAc-Sia *O*-acetyl migration, the deuterated Neu5,9Ac₂ sample was mixed with non-deuterated Neu5,7Ac₂ and Neu5,9Ac₂, and a mixture of 69.9% Neu5,9Ac₂ (56.0% deuterated and 13.9% non-deuterated), 3.7% Neu5,8Ac₂ (2.3% deuterated and 1.4% non-deuterated), and 26.4% Neu5,7Ac₂ (all non-deuterated) was obtained (the control sample in Table S4). *O*-Acetyl migration was significant at pH 7.0 and 8.0 (Figure 5 and Table S4). The net percent increases in deuterated Neu5,7Ac₂ and deuterated Neu5,8Ac₂ by incubation at pH 7.0 or 8.0 demonstrated migration of the deuterated Ac from C9-OH to C7-OH and C8-OH. The net percent decrease of non-deuterated Neu5,7Ac₂ and the net percent increases of non-deuterated Neu5,8Ac₂ and Neu5,9Ac₂ clearly demonstrated the migration of Ac from C7-OH to C8-OH and C9-OH.

To determine the experimental errors in the LC-MS assays (e.g. Figures 4 and 5), the study was repeated for **SM3** in triplicate. The standard deviations of the percentages of individual OAc-Sia determined ranged between 0.02–0.09% (Table S5).

9-OAc in Neu5,9Ac₂-containing sialoglycans can also migrate to C8-OH and C7-OH in a pH-dependent manner.

Using two structurally defined synthetic sialoglycans Neu5,9Ac₂α3Galβ3GlcNAcβProNH₂ and Neu5,9Ac₂α3Galβ4GlcNAcβProNH₂, pH-dependent *O*-acetyl migration in

glycosidically bound OAc-Sias was also analyzed. After the sialoglycans were incubated at various pHs in a water bath at 37 °C for 2 h, the terminal Sias were quickly released by incubating each sample with 10 mU of *Arthrobacter ureafaciens* sialidase for 10 min before low temperature DMB-HPLC analysis of OAc-Sia composition. The purpose of the relatively short incubation time with the sialidase was to minimize *O*-acetyl migration during sample analysis. The increase in pH accelerated migration of the Ac group from C9-OH to C8-OH in Sia (Figure 6). Neu5,8Ac₂ increased from 1.7% at pH 5.0 to 8.0% at pH 8.0 in Neu5,9Ac₂α3Galβ3GlcNAcβProNH₂. For Neu5,9Ac₂α3Galβ4GlcNAcβProNH₂, Neu5,8Ac₂ increased from 0.4% at pH 5.0 to 7.0% at pH 8.0. A similar increase in %Neu5,8Ac₂ was observed for Sias on bovine submaxillary mucin (from 0.2% at pH 5.0 to 4.9% at pH 8.0) although the results were complicated by the accompanying loss of di-OAc-Sias (Figure S5).

The pH-dependent *O*-acetyl migration from C9-OH to C8 and C7-OH in sialoglycans, such as Neu5,9Ac₂α3Galβ4GlcβProN₃, was also confirmed by NMR studies at pH* 3.5 and pH* 8.0. In this study, one of the two diastereotopic protons on C9 of Neu5,9Ac₂ (dd, δ 4.25 ppm), the proton on C8 of Neu5,8Ac₂ (ddd, δ 5.2 ppm), and the proton on C7 of Neu5,7Ac₂ (dd, δ 5.1 ppm)^{27, 28} were used for peak integration, the total area under which was normalized to 1.00 (Figure S6). The integrals of the H3-equatorial and H3 axial signals were also used as references as the *O*-acetyl substitution on C9-OH, C8-OH, or C7-OH of Neu5Ac in sialoglycans only had minimal influence on the chemical shift of H3 in Neu5Ac.²⁹ Incubating the sample in D₂O at pH* 3.5 for 2 h (entries 1 and 2) did not change the ratios of OAc-Sias in the sialoglycans (~92% Neu5,9Ac₂-glycan, 5.8–5.9% Neu5,8Ac₂-glycan, and 2.5% Neu5,7Ac₂-glycan, Table 1). The presence of Neu5,8Ac₂-glycan and Neu5,7Ac₂-glycan in the initial time point (entry 1) at t = 0 may be due to *O*-acetyl migration that occurred during sample preparation. The ratio of different OAc-Sias in the sialoglycans changed quickly to 83% Neu5,9Ac₂-glycan, 7.6% Neu5,8Ac₂-glycan, and 9.2% Neu5,7Ac₂-glycan when the pH* of the sample was adjusted to 8.0 (entry 3 in Table 1, Figure S6C). The ratio remained constant during a 2 h incubation at pH* 8.0 and 37 °C (entry 4 in Table 1, Figure S6D), indicating that an equilibrium was established shortly after the pH* was adjusted to 8.0, before the first NMR spectrum was taken at t = 0 min. Changing the pH* of the sample back to 3.5 (entry 5 in Table 1, Figure S6E) did not alter the ratio of OAc-Sias in the sialoglycans significantly, even after a 3-day incubation at 37 °C (entry 6 in Table 1, Figure S6F). This is consistent with the observations for unbound OAc-Sia monosaccharides described above. Adjusting the pH* of the sample again to pH* 8.0 (entry 7 in Table 1, Figure S6G) did not significantly alter the ratio of glycosidically bound OAc-Sias as the equilibrium was reached previously. The ratio remained unchanged after an additional 12 h at 37 °C (entry 8 in Table 1, Figure S6H).

A Neu5,9Ac₂-binding protein, PToV HE, shows stronger binding to immobilized sialoglycans containing Neu5Ac9NAc than those containing Neu5,9Ac₂.

Our previous glycan microarray study with a matching set of α2–3-linked sialyl lactosides (Siaα3Galβ4GlcβProNH₂) containing a terminal Neu5Ac, Neu5,9Ac₂, or its more stable *N*-acetyl analog Neu5Ac9NAc showed that PToV HE recognizes both Neu5,9Ac₂-glycan and its Neu5Ac9NAc-analog but not Neu5Ac-glycan.³⁰ Using a library of α2–3- and

α 2–6-linked synthetic sialoglycans containing different internal glycans and the matching terminal Neu5,9Ac₂ and its Neu5Ac9NAc analog,³¹ sialoglycan microarray studies showed that the esterase-inactivated PToV HE bound to Neu5Ac9NAc-glycans with much stronger signals than Neu5,9Ac₂-glycans (Figure 7). Strong binding signals were also shown for other Neu5Ac9NAc-glycans with unmatched internal glycan structures (the last six bars shown in Figure 7). The lack of binding to Neu5Ac-glycans observed previously was also confirmed, consistent with the report of PToV HE as a Neu5,9Ac₂-recognizing probe.³² The lower binding to Neu5,9Ac₂-glycans, partly due to the lower stability of the 9-OAc group in Neu5,9Ac₂-glycans compared to the 9-*N*-acetyl group in Neu5Ac9NAc-glycans, may be explained by the *O*-acetyl migration in Neu5,9Ac₂-glycans. Our study also indicates that the terminal Sia is the main recognition component of PToV HE while the sialyl linkage and the internal glycan structures do not contribute significantly to binding. The study also supports the role of Neu5Ac9NAc-glycans as useful stable mimics of Neu5,9Ac₂-glycans and potent probes for research.

Neu5Ac9NAc-glycans are more sensitive to periodate oxidative cleavage than Neu5,9Ac₂-glycans.

Periodate oxidative cleavage of C-C bonds with vicinal hydroxyl groups such as those in carbohydrates is well known.³³ Selective cleavage of the C-C bonds in the glycerol chain of Sia in sialoglycans to generate an aldehyde group at C7 without affecting other sugars can be achieved by using low concentrations of periodate. The strategy has been successfully used for selective labeling of the sialoglycoproteins on living cell surfaces.^{34–37}

We found that mild periodate treatment could differentiate the matching set of Sia α 3Gal β 3(Fuca4)GlcNAc β ProNH₂ containing a terminal Neu5Ac, Neu5,9Ac₂ or its more stable *N*-acetyl analog Neu5Ac9NAc.³⁸ Among these, the Neu5Ac-glycan was the most sensitive toward periodate treatment. Incubation of samples with 6 mM NaIO₄ at 20 °C for 30–120 min followed by reduction with 30 mM NaBH₄ at 20 °C for 20 min, 2 M AcOH release of the Sia at 80 °C for 1 h, DMB derivatization, and HPLC analysis showed that the Neu5Ac in the Neu5Ac-glycan ($m/z = 876.34$) (Figure S8A) was completely converted to its C7-aldehyde derivative by periodate oxidative cleavage in 30 min. Consistent with a previous report,³⁹ the DMB derivatized C7-Neu5Ac derivative was eluted right after Neu5Ac as shown in the HPLC chromatogram (Figure 8A). The formation of the C7-aldehyde derivative of the sialoglycan after periodate oxidative cleavage was also validated by LC-MS analysis of its borohydride reduced product ($m/z = 818.34$, Figure S8B). Interestingly, Neu5Ac9NAc-glycan (Figure 8B) was less resistant to the periodate oxidative cleavage at C7 than its Neu5,9Ac₂-glycan counterpart (Figure 8C). While 74.2% of the Neu5,9Ac₂-glycan was retained after 2 h of periodate treatment (Figure S9B) of a sample originally containing 84.6% Neu5,9Ac₂-glycan and 15.4% de-*O*-acetylated Neu5Ac-glycan (the OAc loss occurred during storage), only 62.8% of the Neu5Ac9NAc-glycan was retained from a pure starting material under the same conditions (Figure S9A). The C7-derivative ($m/z = 818.34$, Figure S10B) formed from the periodate-treated Neu5Ac9NAc-glycan followed by reduction ($m/z = 917.37$, Figure S10A) was the same as that from the periodate-treated Neu5Ac-glycan followed by reduction ($m/z = 818.34$, Figure S8B). Another experiment using a matching set of Sia α 3Gal β 4Glc β ProNH₂ under the same

periodate treatment and downstream assay conditions also showed higher sensitivity of Neu5Ac9NAc-glycan than Neu5,9Ac₂-glycan. As shown in Figure S11 by DMB-HPLC analysis, while 26.2% Neu5,9Ac₂-glycan remained after 2 h of periodate treatment of an original sample containing 39.4% Neu5,9Ac₂-glycan, 62.8% of Neu5Ac9NAc-glycan remained from a pure sample to the same treatment. The data strongly suggest that *O*-acetyl migration may play a role in the resistance of Neu5,9Ac₂ to periodate oxidative cleavage. It was shown previously that amino group substitution of one of the vicinal diols activated the periodate oxidative cleavage of the corresponding C-C bond and *N*-acetylation greatly reduced the reactivity.⁴⁰ Nevertheless, the difference of C9-NAc and C9-OAc on the influence of periodate oxidative cleavage of the diol-containing C7-C8 bond is unclear.

CONCLUSIONS

In summary, we developed an improved DMB-HPLC method with DMB derivatization carried out at a consistent acidic pH range (3.8–4.1) and a low temperature (4 °C) that allowed quantification of Neu5Ac and its mono-*O*-acetylated derivatives with minimal *O*-acetyl migration or de-*O*-acetylation. Advanced NMR methods (i.e. LR-HSQMBC, etc.)²⁴ and equipment (cryoprobe, higher fields) used here enabled us to obtain data for lower abundant species that were not observed previously, such as Neu5,8Ac₂.¹³ In addition to the previous observation of *O*-acetyl migration in Sia from C7-OH to C9-OH, we provide direct evidence here that *O*-acetyl group migration in free and glycosidically-bound Sia occurs in all directions among C7-OH, C8-OH, and C9-OH in a pH-dependent manner. While *O*-acetyl migration was minimal at pH lower than 5.0, it increased with increasing pH. The synthetic monosaccharide and/or sialoglycan samples with or without deuterium-labeling containing varied percentages of Neu5,9Ac₂, Neu5,8Ac₂, and Neu5,7Ac₂ were critical for the observation of bi-directional *O*-acetyl migration. *O*-Acetyl migration may play a role in the higher resistance of Neu5,9Ac₂-glycans to periodate oxidative cleavage compared to their Neu5Ac9NAc-glycan counterpart. It may also influence the interaction of Neu5,9Ac₂-glycans with sialoglycan-binding proteins such as a Neu5,9Ac₂-recognizing esterase-inactivated porcine torovirus hemagglutinin-esterase shown here which had a stronger binding to Neu5Ac9NAc-glycans than their Neu5,9Ac₂-glycan counterparts. Further investigations are needed to fully understand the biological implications of bidirectional *O*-acetyl migration in Sia and sialoglycans.

METHODS

***O*-Acetylated Sia monosaccharide preparation.**

Trimethyl orthoacetate (0.165 mL, 1.30 mmol) and *p*-TsOH·H₂O (10 mg) were added to a solution of Neu5Ac (0.2 g, 0.65 mmol) in DMSO (2 mL). The reaction mixture was stirred for 1 h at room temperature, and then applied to an anion exchange resin column chromatograph (Dowex 1-X8, HCO₂⁻ form). The column was washed with water and the product was eluted with 1 M of formic acid. The fractions containing Neu5,9Ac₂ were combined and concentrated. The resulting material was loaded on a Bio-gel P-2 fine column to obtain the pure product. This compound was stored as the acid form for years which produced significant amount of Neu5,8Ac₂ (Starting material 1 or **SM1**). Starting material

2 (**SM2**) was obtained by releasing from a Neu5,9Ac₂-containing glycan with 10 mU of *Arthrobacter ureafaciens* sialidase (AUS) in 50 mM sodium acetate (pH 5.5). To obtain Starting material 3 (**SM3**), **SM1** was dissolved in H₂O (1 mL) and purified by HPLC using a reverse-phase C18 column (Phenomenex, 21.2 × 250 mm) with a flow rate of 10 mL min⁻¹ and a gradient elution of 0–100% acetonitrile in water over 20 minutes. Mobile phase A: water; Mobile phase B: acetonitrile; Gradient: 0% B for 3 min, 0% to 100% B over 12 min, 100% B for 2 min, then 100% to 0% B over 3 min. HPLC eluents were monitored by absorption at 210 nm, and glycan-containing fractions were analyzed by TLC and MS. The fractions containing the pure product were collected and lyophilized to obtain pure Neu5,9Ac₂. Neu5,7Ac₂ was obtained by releasing from the hyper-*O*-acetylated capsular polysaccharides (CPS) of the group B streptococcus COH1 NeuA +N301A NeuA strain. The culture and releasing conditions were the same as described previously.¹⁸ For lipase-catalyzed synthesis of [9-deuteroacetyl]Neu5,9Ac₂, deuterated vinyl acetate (0.1 mL, 99.2%-D6, CDN Isotopes, Lot. DE169) was added with 50 μL of Neu5Ac in pyridine (50 mM), 0.35 mL acetonitrile and 1 mg lipase (from *Pseudomonas* sp., Type XIII, 26 units mg⁻¹). The reaction was carried out at 45 °C for 3 days. The organic solvent was removed by nitrogen blow down. The product was then dissolved in ice-cold water and passed through 10 kDa Amicon filter to remove the lipase. The flow-through was lyophilized. Deuterated Neu5,9Ac₂ product was confirmed and quantified by DMB-HPLC and LC-MS as described above (Figure S7).

Sialoglycans microarray study.

The production of the Neu5,9Ac₂-recognizing probe by use of ectodomains of the Hemagglutinin-Esterase (HE) of porcine torovirus was described in previous work.⁴¹ In this work, an Avi-tag was inserted between the Fc domain and 6X-His and biotinylated according to the suggestions by manufacturers (NEB, Avidity). Biotinylated proteins were concentrated, and buffer exchanged to PBS. The sialoglycan microarray experiment was performed following the earlier reported literature with a slight modification.^{42, 43} Chemoenzymatically synthesized sialoglycans were quantitated utilizing an improved low temperature DMB-HPLC method described before. Then a 10 mM stock solution was prepared (in water) and further diluted to 100 μM in 300 mM Na-phosphate buffer (pH 8.4). Diluted sialoglycans were printed in quadruplets on NHS-functionalized glass slides (PolyAn 3D-NHS; catalog# PO-10400401) using an ArrayIt SpotBot® Extreme instrument, dried in the printing chamber and then blocked using 0.05 M ethanolamine solution in 0.1 M Tris-HCl (pH 9.0). After washing with warm Milli-Q water and centrifuge drying, the slides were stored at 4 °C, under dark in a vacuum chamber. On the day of the experiment, the slides were fitted in a multiwell microarray hybridization cassette (ArrayIt, CA) to divide into 8 wells. To each well 400 μL of Ovalbumin (1% w/v, PBS) was added and rehydrated for one hour in a humid chamber with gentle shaking. The solution was discarded followed by the addition of a 400 μL solution of the biotinylated PToV B-subunit (30 μg mL⁻¹ dissolved in the same blocking buffer) in the individual well. The slides were then incubated for 2 h at ambient temperature with gentle shaking followed by washing with PBS-Tween (0.1% v/v) and PBS. The wells were then treated with Cy3-conjugated streptavidin as a secondary antibody (1:500 dilution in PBS). The slides were then incubated at ambient temperature for 1 hour in a dark and humid chamber with gentle shaking. The slides were

then washed, dried, and scanned using a Genepix 4000B scanner (Molecular Devices Corp., Union City, CA) at a wavelength of 532 nm. Data analysis was performed using the Genepix Pro 7.3 software (Molecular Devices Corp., Union City, CA).

DMB-HPLC analysis.

Sias were derivatized in a DMB reagent buffer containing a final concentration of 7 mM DMB, 9 mM sodium hydrosulfite and 0.5 M of 2-mercaptoethanol added with either trifluoroacetic acid (20 mM) or acetic acid (0.8 M). The derivatized samples were analyzed on a LaChrom Elite HPLC (Hitachi) system with a Phenomenex Gemini C18 column (5 μ m, 250 mm \times 4.6 mm) at room temperature. A gradient elution (water:acetonitrile:methanol = 86:7:7 \rightarrow 82:11:7) was used. The eluted analytes were detected with a fluorescence detector at excitation 373 nm and emission 448 nm. The data collection time was extended to 75 min. For migration studies, the OAcSia monosaccharides or glycans were dissolved in a sodium acetate solution (50 mM) at various pH (3.0–8.0). The starting material was diluted in the same volume of water as a control. All the samples were incubated in a 37 $^{\circ}$ C water bath for 2 h. The Sias were released from glycans by adding 10 mU of AUS and incubated at 37 $^{\circ}$ C for 10 min. The samples were then cooled on ice and derivatized with the DMB reagent containing acetic acid followed by HPLC analysis. The designation of Sia species was based on retention times compared to the Sia standards from bovine submaxillary mucin. The experiments were repeated three times showing similar results. For periodate treatment study, 50 μ M sialoglycans was prepared in PBS (pH adjusted to 6.5) from a 10 mM stock. The sialoglycans were treated with 6 mM sodium periodate (2 M stock) at 20 $^{\circ}$ C for 2 h in the dark. Aliquots were taken every 30 min and the generated aldehyde was reduced by the addition of sodium borohydride (1 M stock) to a final concentration of 30 mM at 20 $^{\circ}$ C for 20 min. The Sias and C7-analogues were released with 80 $^{\circ}$ C for 60 min. The sample was derivatized with DMB reagent containing acetic acid followed by HPLC analysis. The relative peak area ratio of the C7-Neu5Ac analog to the non-periodate treated Neu5Ac was applied to all samples for the calculation of the molar percentage of each Sia species.

NMR analysis of O-acetylated Sia monosaccharides.

Two samples of different concentrations and pHs were prepared. The first sample was prepared by dissolving 15 mg of O-acetylated Sia in 500 μ L D₂O (80 mM). Its pH* was adjusted to 3.5 (monitored by a micro-pH meter) using HCl in D₂O solutions (100 mM, 10 mM, and 1 mM stock solutions prepared from 1 M HCl in H₂O by dilution with D₂O).^{44, 45} It was then subjected to one-dimensional ¹H-NMR experiments performed on an 800 MHz Bruker Avance III spectrometer at the University of California, Davis. ¹H-NMR spectra were recorded at the beginning (t = 0 min), every 10 min over the first 2 h, and at the end of the incubation period (12 h) at 310.15 K and pH* = 3.5. The sample pH* was checked again using a micro-pH meter at the end of the incubation and was shown to be stable. The pH* of this sample was adjusted to 7.0 using NaOH in D₂O solutions (100 mM, 10 mM, and 1 mM stock solutions prepared by dissolving NaOH in D₂O followed by serial dilution with D₂O) and the sample NMR experiments were carried out with pH* rechecked at the end of the incubation. NMR data were processed and analyzed with MestreNova 12.0.4 software. Assignments of OAcSia in the ¹H-NMR study were verified by ¹H-¹³C HSQC,²² HSQC-TOCSY,²⁴ and LR-HSQMBC²⁵ experiments. For these experiments, a

second sample was prepared by dissolving 20 mg of *O*-acetylated Sia in 450 μ L D₂O (127 mM) at pH* 6.14. The 2D NMR experiments were carried out on a Bruker 700 MHz NMR AV III HD instrument equipped with a triple resonance, xyz gradient TCI cryoprobe™ at the FDA. Two-dimensional NMR spectra were acquired using Bruker's Topspin 3.6.1 software (<http://www.bruker.com>). The carrier frequency, spectral window, and number of points for the direct and indirect dimensions were set to 4.7 ppm, 60 ppm; 10 ppm, 86 ppm; and 2048, 1024 respectively for the ¹H-¹³C HSQC, HSQC-TOCSY, and LR-HSQMBC experiments. A DIPSI-2 mixing sequence with mixing times of 30 ms and 60 ms were used for the ¹H-¹³C HSQC-TOCSY. The INEPT delay in the LR-HSQMBC was optimized for a 6 Hz long-range ¹H-¹³C coupling constant. A multiplicity edited ¹H-¹³C HSQC⁴⁶ was taken with 1024 points in the direct dimension and 2048 points in the indirect dimension using a non-uniform sampling data collection strategy in the indirect dimension with 50% of the points being collected based on the schedules from the Wagner group.⁴⁷ 2D NMR data were processed with NMRpipe,⁴⁸ using a square cosine bell to process both the direct and indirect dimensions. The direct dimension was referenced based on the DSS ¹H signal, and ¹³C was referenced indirectly from the absolute DSS ¹H frequency. Non-uniformly sampled multiplicity edited ¹H-¹³C HSQC data were reconstructed using the SMILE reconstruction algorithm.⁴⁹

NMR analysis of *O*-acetylated sialoglycans.

¹H-NMR experiments of pH*-dependent *O*-acetyl migration of *O*-acetylated sialoglycans were performed on an 800 MHz Bruker Avance III spectrometer at the University of California, Davis. The sample was prepared by dissolving 10 mg of Neu5,9Ac₂α3Galβ4GlcβProN₃ in 500 μ L D₂O (26 mM). Sample pH* was adjusted to 3.5 by adding HCl in D₂O. It was incubated at 310.15 K for 2 h followed by adjusting the pH* to 8.0 by adding NaOH in D₂O and incubation at 310.15 K for 2 h. The pH* of the same sample was then adjusted back to 3.5. It was incubated at 310.15 K for 3 days followed by adjusting the pH* to 8.0 and incubated at 310.15 K for 12 h. ¹H-NMR spectra were recorded at 0 min and at the end of each incubation period. Water suppression was applied when the spectra were recorded. ¹H-NMR data were processed and analyzed with TopSpin 3.6.1 software.

LC-MS analysis.

All mass spectrometry analyses were performed using an LTQ-XL Orbitrap Discovery (Thermo Scientific) mass spectrometer in the negative mode. LC profiling of Sia was done on a C18 column using an ion-pairing reagent in running buffer. The running buffer constitutes of dibutyl amine (DBA, 5 mM), acetic acid (8 mM), and methanol (70%). For the migration assay, deuterated OAc-Sia (almost all Neu5,9Ac₂) was mixed with non-deuterated OAc-Sia to obtain a starting mixture containing desired percentage of each OAcSia species (around 1 mM). The starting mixture (1.5 μ L) was added to 18.5 μ L of 50 mM sodium acetate with a pH value in the range of 5.0–8.0 and incubated at 37 °C. Then 4 μ L of the mixture was injected for LC-MS analysis. For the periodate treatment study, 50 mM of sialoglycans was prepared in PBS (pH adjusted to 6.5) from a 10 mM stock. The sialoglycans were treated with 6 mM sodium periodate (2 M stock) at 20 °C for 2 h in the dark. The resulting aldehyde was reduced by the addition of NaBH₄ (1 M stock) to a final

concentration of 30 mM at 20 °C for 20 min. The pH was adjusted to around 7.0 with 2 M acetic acid before injection for LC-MS analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Ac	acetyl group
AUS	<i>Arthrobacter ureafaciens</i> sialidase
BSM	bovine submaxillary mucin
CPS	capsular polysaccharide
DMB	1,2-diamino-4,5-methylenedioxybenzene
ESI	electrospray ionization
HE	hemagglutinin-esterase
HPLC	high performance liquid chromatography
LC-ESI-MS	liquid chromatography-electrospray ionization-mass spectrometry
MS	mass spectrometry
Neu5Ac	<i>N</i> -acetylneuraminic acid
Neu5,9Ac₂	9- <i>O</i> -acetyl- <i>N</i> -acetylneuraminic acid
Neu5,8Ac₂	8- <i>O</i> -acetyl- <i>N</i> -acetylneuraminic acid
Neu5,7Ac₂	7- <i>O</i> -acetyl- <i>N</i> -acetylneuraminic acid
Neu5Ac9NAc	9-acetamido-9-deoxy- <i>N</i> -acetylneuraminic acid
NMR	nuclear magnetic resonance
OAc	<i>O</i> -acetyl group
PToV	esterase-inactivated porcine torovirus
Sia	sialic acid
XIC	extracted ion current

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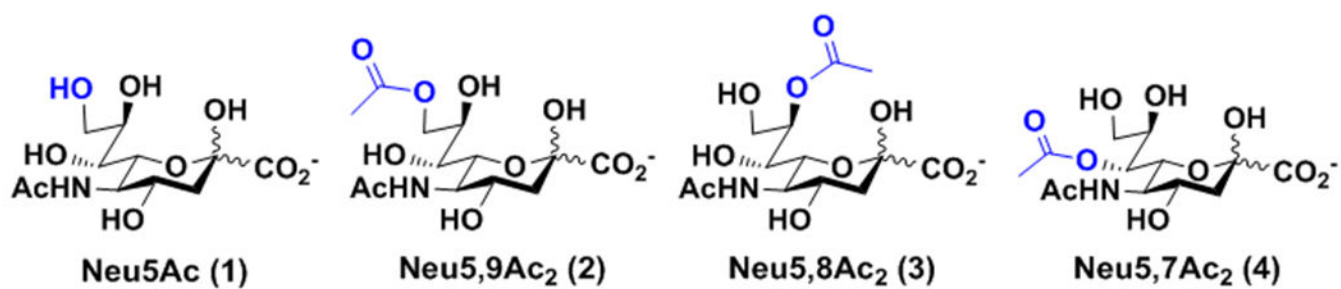


Figure 1. Structures of *N*-acetylneuraminic acid (Neu5Ac, **1**) and its 9-*O*-, 8-*O*-, and 7-*O*-acetyl forms (**2–4**).

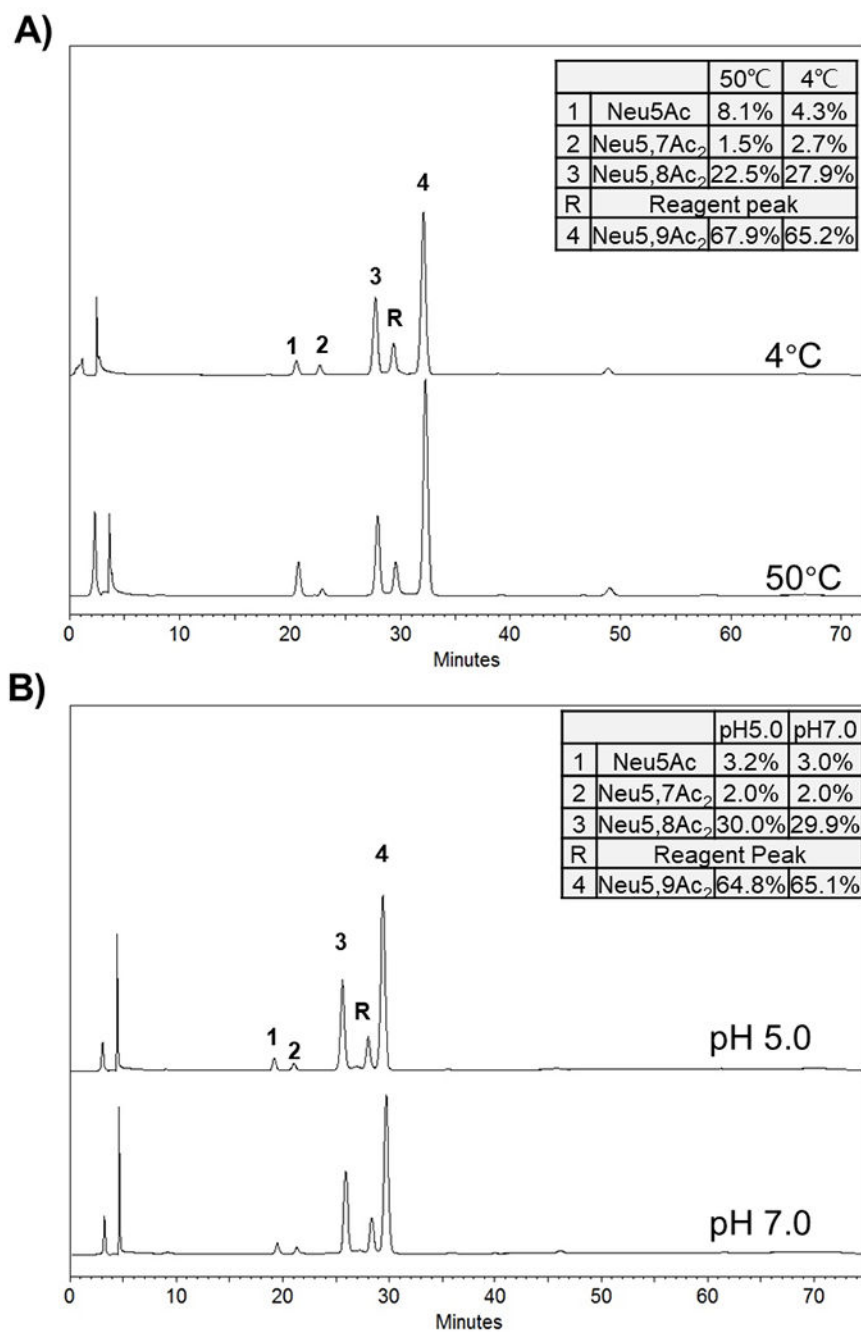


Figure 2. Comparison of DMB derivatization-HPLC analysis methods for quantification of OAc-Sias.

(A) HPLC chromatograms of a DMB-derivatized water-dissolved OAc-Sia sample at 4 °C for 48 h (top panel) and at 50 °C for 2 h (bottom panel). (B) HPLC chromatograms of a DMB-derivatized OAc-Sia sample (at 4 °C for 48 h) by mixing the DMB reagent in acetic acid (1.6 M) with the same volume of the OAc-Sia sample in a cold sodium acetate solution (50 mM) with a pH of 5.0 (top panel) or 7.0 (bottom panel). Sias from bovine submaxillary

mucin (BSM) previously studied by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS)²⁰ were used as the standards.

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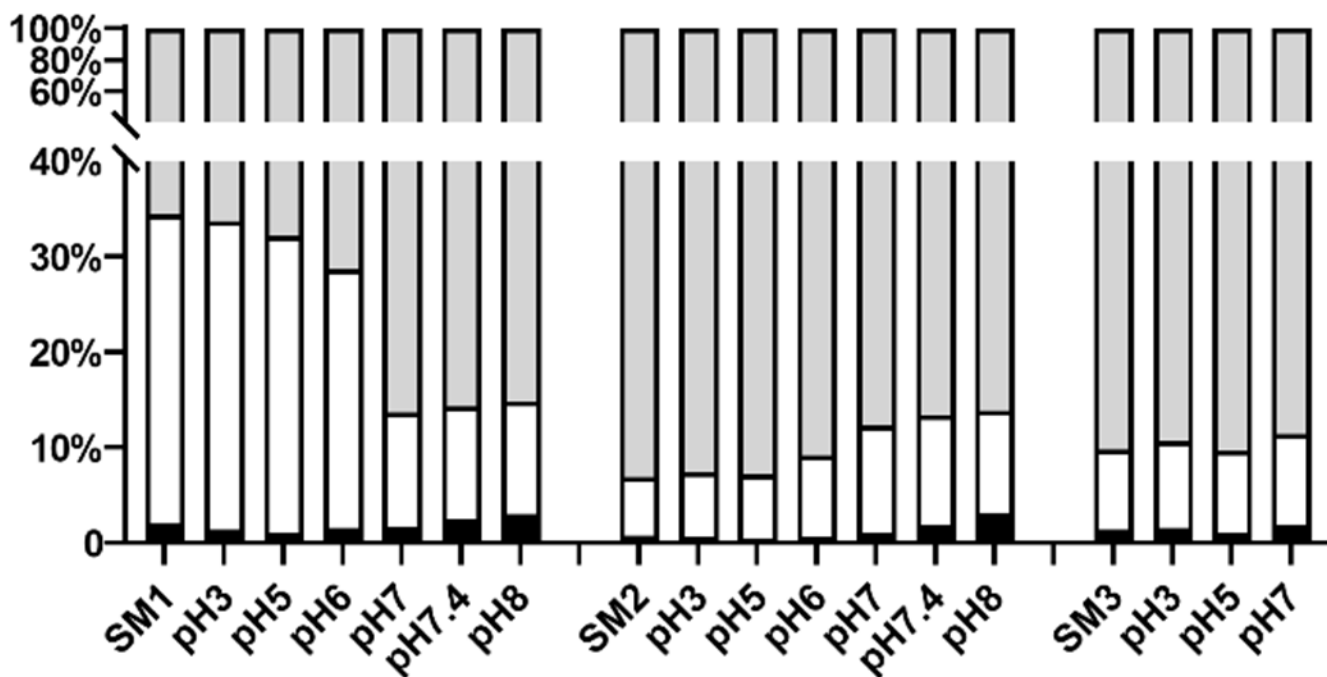


Figure 3. *O*-Acetyl migration in OAc-Sia monosaccharide samples.

DMB-HPLC analysis of *O*-acetyl migration with three OAc-Sia monosaccharide samples, each containing different amounts of Neu5,7Ac₂ (black columns), Neu5,8Ac₂ (white columns), and Neu5,9Ac₂ (gray columns). OAc-Sia samples were incubated at different pHs at 37 °C for 2 h. Before incubation, starting material 1 (SM1) contains 2.0% of Neu5,7Ac₂, 32.4% of Neu5,8Ac₂ and 65.6% of Neu5,9Ac₂; starting material 2 (SM2) contains 0.8% of Neu5,7Ac₂, 6.1% of Neu5,8Ac₂ and 93.1% of Neu5,9Ac₂; starting material 3 (SM3) contains 1.4% of Neu5,7Ac₂, 8.5% of Neu5,8Ac₂ and 90.1% of Neu5,9Ac₂.

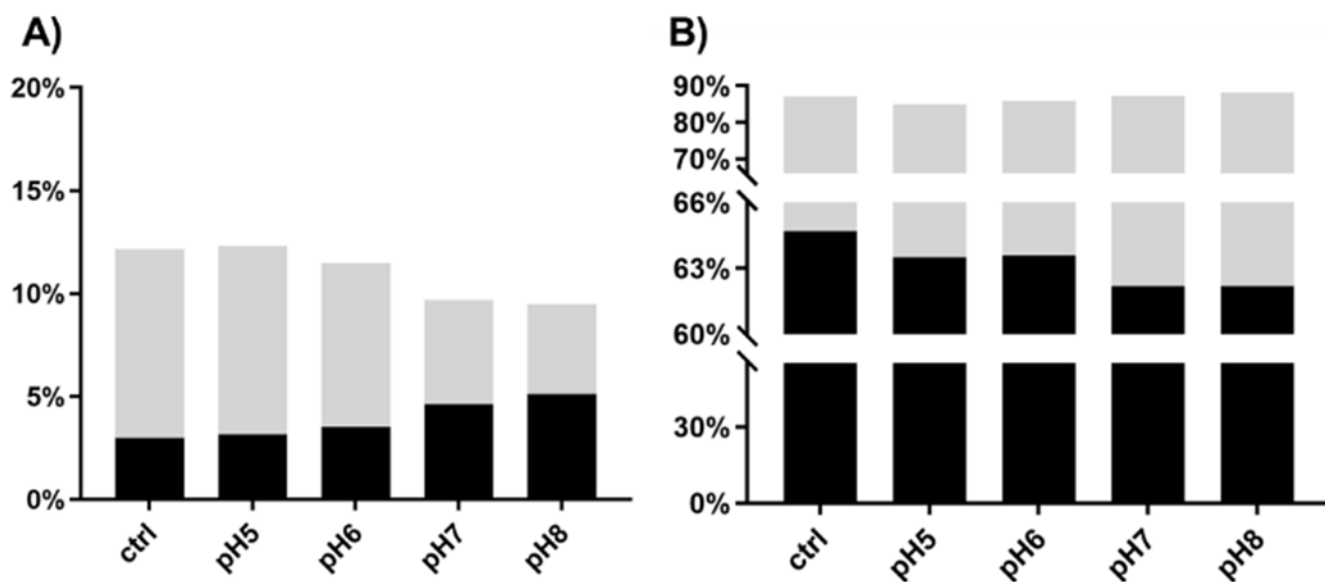


Figure 4. Concurrent interconversion between Neu5,8Ac₂ and Neu5,9Ac₂ at neutral or slightly basic pH.

A [9-deuteroacetyl]Neu5,9Ac₂ sample was mixed with non-deuterated Neu5,8Ac₂/Neu5,9Ac₂ and incubated at various pHs at 37 °C for 30 min followed by LC-MS analysis. The starting mixture without incubation was used as a control (ctrl). The percentage of deuterated and non-deuterated Neu5,8Ac₂ (**A**) and Neu5,9Ac₂ (**B**) was calculated based on the corresponding peak areas in the XIC chromatogram. Deuterated samples are shown in black columns and non-deuterated samples are shown in gray columns.

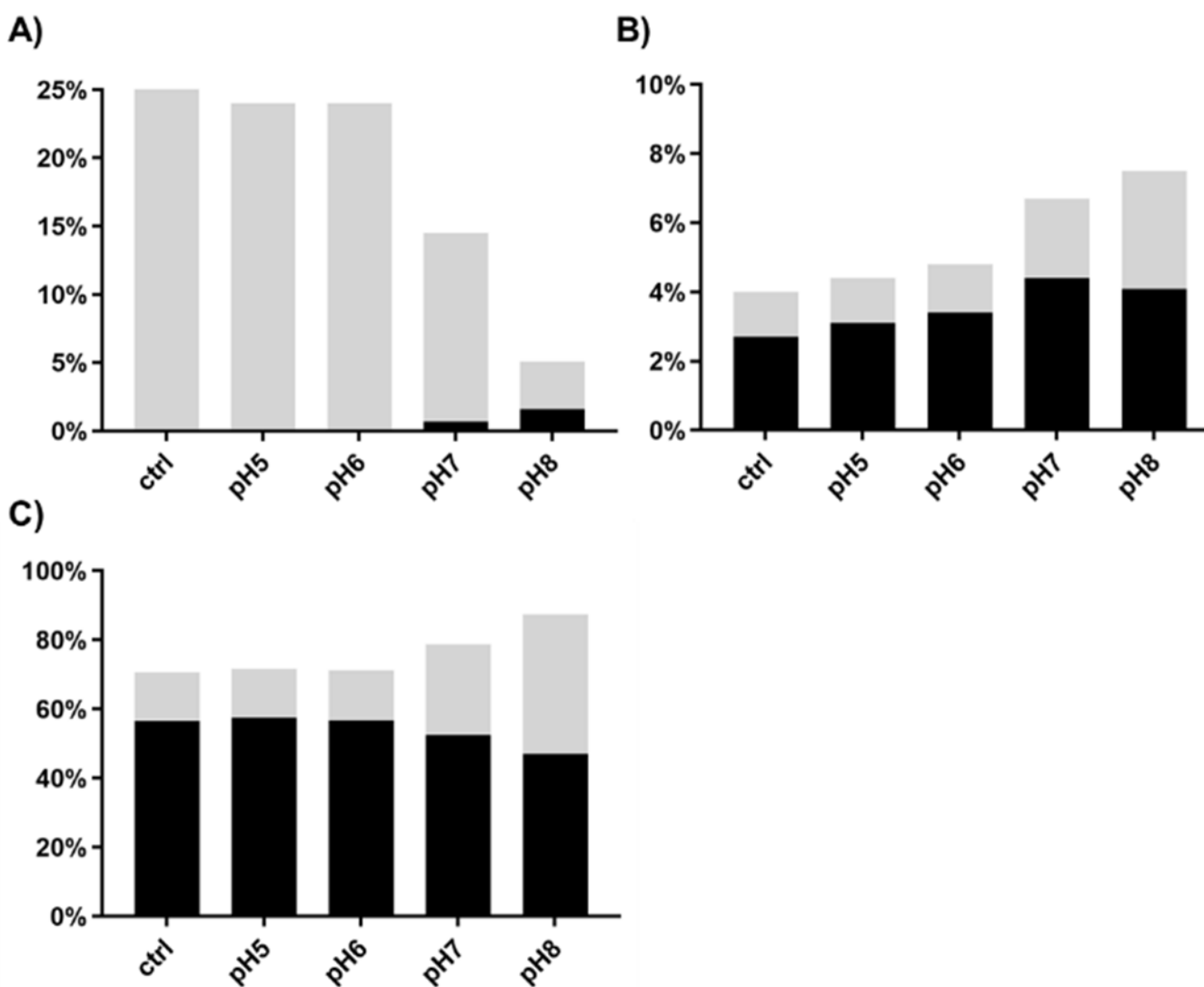


Figure 5. Concurrent interconversion between Neu5,7Ac₂ and Neu5,9Ac₂ at neutral or slightly basic pH.

A [9-deuteroacetyl]Neu5,9Ac₂ was mixed with non-deuterated Neu5,7Ac₂/Neu5,9Ac₂ and incubated at various pHs at 37 °C for 2 h followed by LC-MS analysis. The starting mixture without incubation was used as a control. The percentages of deuterated and non-deuterated Neu5,7Ac₂ (A), Neu5,8Ac₂ (B), and Neu5,9Ac₂ (C) were calculated based on the corresponding peak areas in the XIC chromatogram. Deuterated samples are shown in black columns and non-deuterated samples are shown in gray columns.

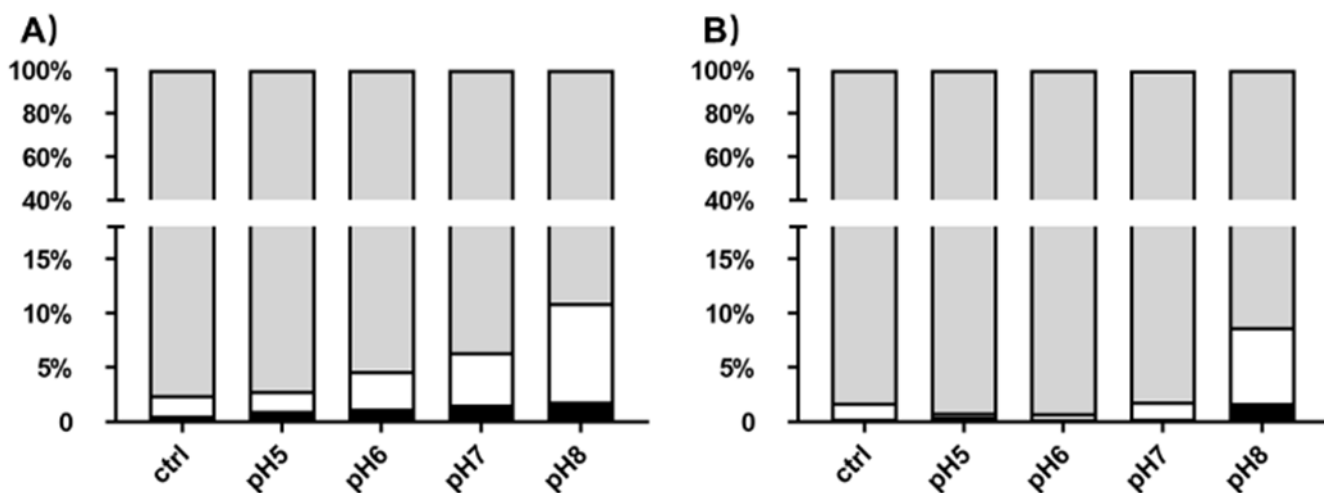


Figure 6.
O-Acetyl migration on OAc-Sia-containing sialoglycans.
Neu5,9Ac₂α3Galβ3GlcNAcβProNH₂ (**A**) and Neu5,9Ac₂α3Galβ4GlcNAcβProNH₂ (**B**) at a different pH by comparing the composition of Neu5,9Ac₂ (gray columns), Neu5,8Ac₂ (white columns), and Neu5,7Ac₂ (black columns) released by sialidase treatment before the low temperature DMB-HPLC analysis.

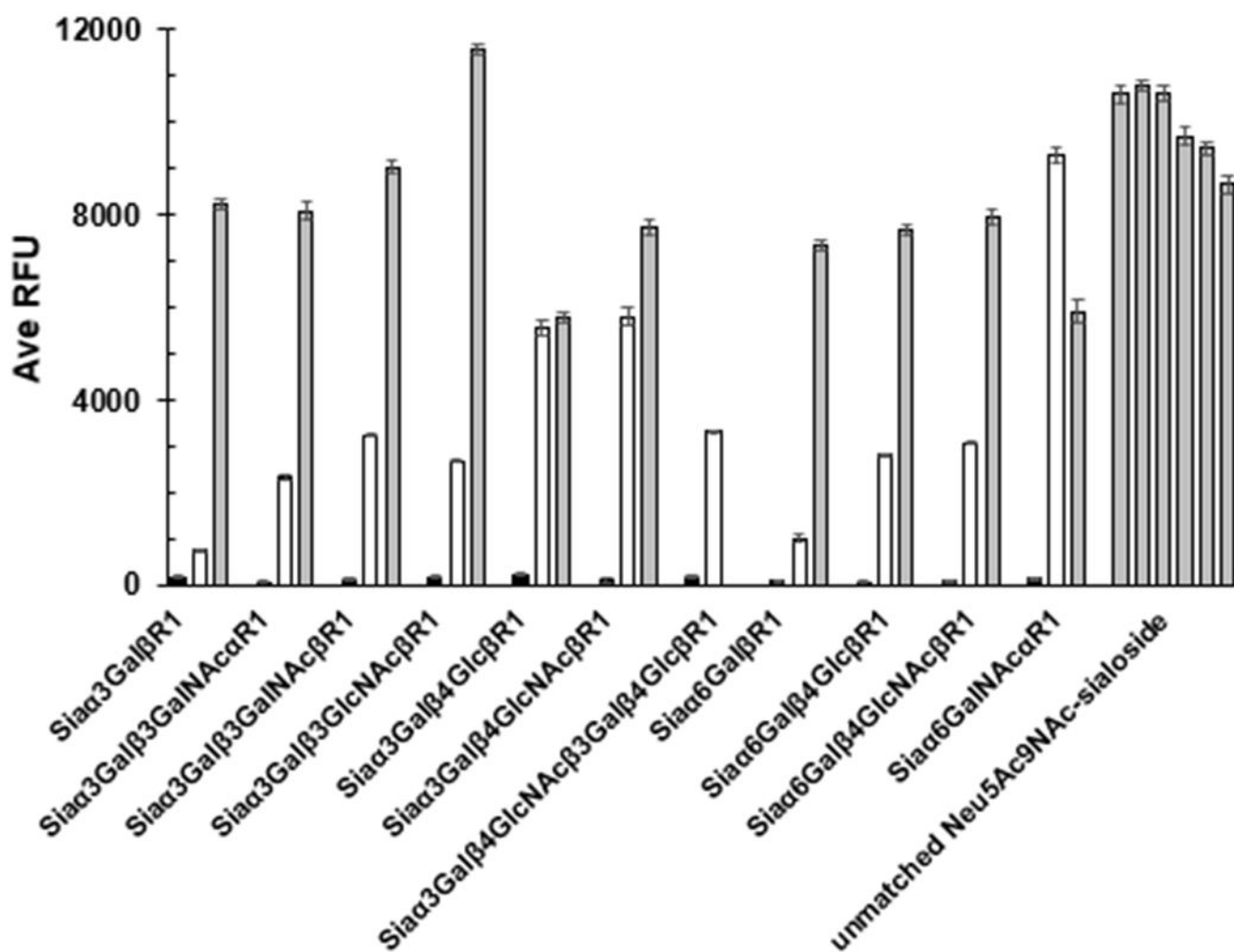


Figure 7. Sialoglycan microarray binding studies of PToV HE toward Neu5Ac, Neu5,9Ac₂, and Neu5Ac9NAc-containing glycans. Neu5Ac (black columns), Neu5,9Ac₂ (white columns), and Neu5Ac9NAc-glycans (gray columns) with matched underlying structures were compared. Neu5Ac9NAc-glycans that did not have comparable Neu5Ac or Neu5,9Ac₂-glycans with matching underlying structures were grouped together and plotted on the right side of the chart. Biotinylated-PToV HE ($30 \mu\text{g mL}^{-1}$) was applied to all glycans.

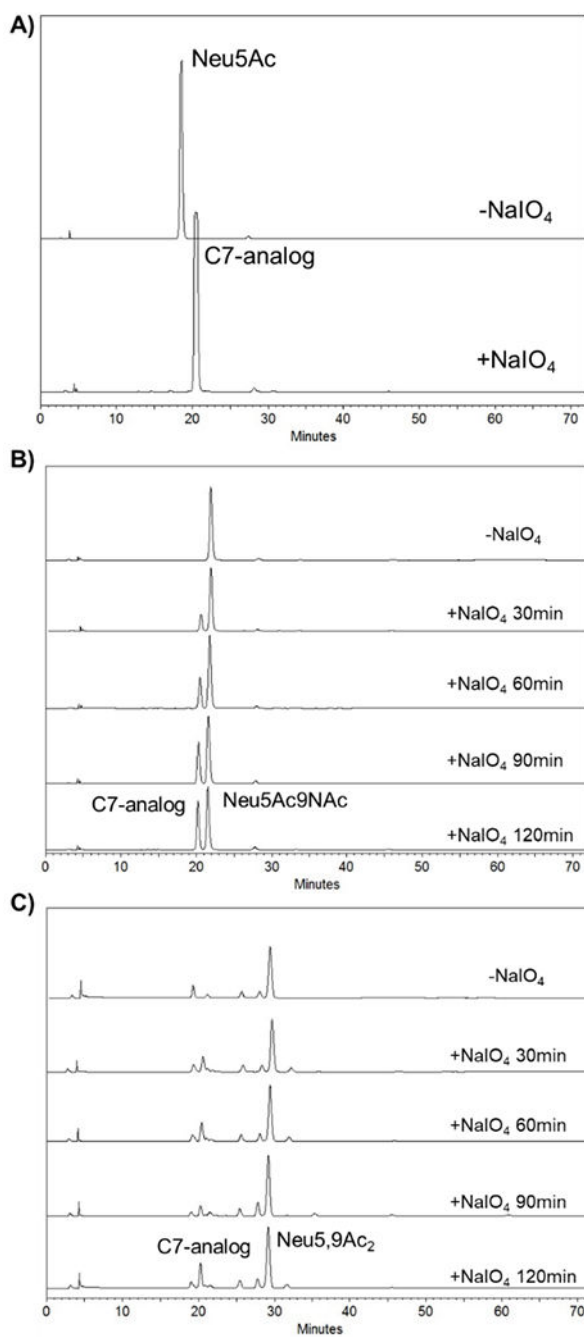


Figure 8. DMB-HPLC assay results of sialoglycan Sia α 3Gal β 3(Fuca4)GlcNAc β ProNH₂ containing Neu5Ac (A), Neu5Ac9NAc (B), or Neu5,9Ac₂ (C) treated with 6 mM NaIO₄ at pH 6.5, 20 °C, for 30–120 min, followed by sodium borohydride (30 mM) reduction at 20 °C for 20 min, and acetic acid (2 M) releasing of Sias at 80 °C for 1 h.

Table 1.

pH*-Dependent *O*-acetyl migration in Neu5,9Ac₂α3Galβ4GlcβProN₃ observed by percent changes in individual mono-*O*-acetyl Sia species by NMR studies (the corresponding NMR spectra are in Figure S6).

No.	pH* (time)	Neu5,9Ac ₂	Neu5,8Ac ₂	Neu5,7Ac ₂	Total
1	3.5 (t = 0)	0.9160	0.0589	0.0250	1.00
2	3.5 (t = 2 h)	0.9175	0.0579	0.0246	1.00
3	8.0 (t = 0)	0.8325	0.0758	0.0917	1.00
4	8.0 (t = 2 h)	0.8335	0.0758	0.0907	1.00
5	3.5 (t = 0)	0.8322	0.0760	0.0919	1.00
6	3.5 (t = 3 d)	0.8339	0.0746	0.0915	1.00
7	8.0 (t = 0)	0.8274	0.0766	0.0925	1.00
8	8.0 (t = 12 h)	0.8314	0.0763	0.0923	1.00