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PAPER

A mechanistic study of sialic acid mutarotation: Implications for mutarotase enzymes†

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The mutarotation of *N*-acetylneuraminic acid (Neu5Ac) proceeds by four kinetically distinct pathways: (i) the acid-catalyzed reaction of neutral Neu5Ac; (ii) the spontaneous reaction of the carboxylic acid (the kinetically equivalent acid-catalyzed reaction on the anion being ruled out by the solvent deuterium kinetic isotope effect of 3.74 ± 0.68); (iii) a spontaneous, water-catalyzed, reaction of the anion; and (iv) a specific-base catalyzed reaction of the anion. The magnitude of the solvent kinetic isotope effect, $k_{H2O}/k_{D2O} = 4.48 \pm 0.74$ is consistent with a ring-opening transition state in which a water molecule is deprotonating the anomeric hydroxyl group in concert with strengthening solvation of the ring oxygen atom. The mechanistic implications for Neu5Ac mutarotases are discussed.

Introduction

Sialic acids are a family of 9-carbon keto sugars found throughout nature.[1] Chemical derivatization of the core sialic acid structure, which includes acetylation, phosphorylation, methylation and sulphonation, increases the biodiversity of this important class of biomolecules.[2] *N*-acetylneuraminic acid (Neu5Ac), the most prevalent and frequently studied member, has been implicated in a number of human pathologies including cancer.[3] Many metastatic cancer cells exhibit hypersialylation of their surface glycoproteins, a modification which is believed to facilitate migration of these cells into the blood stream.[4] Enzymatic degradation of these glycoproteins are frequently used to delineate the role and effect of hypersialylation. *Exo*-sialidases (neuraminidiases) catalyze the hydrolytic cleavage of terminal Neu5Ac residues connected *via* α-(2→3)-, α-(2→6)-, α-(2→8)-glycosidic linkages with net retention of anomeric stereochemistry. The initial product formed, α-Neu5Ac, undergoes spontaneous mutarotation to produce an equilibrium mixture, which at a pH of 8.0 consists of the β-anomer (92.1%), the α-anomer (7.5%) and three open-chain species (a ketone, an enol and a *gem*-diol).[5]

In 1846, Dubrunfaut observed that the specific rotation of aqueous glucose solutions changed as a function of time until a value of +52.7° was established.[6] This process, coined as mutarotation,[7] was attributed to the interconversion between the two anomers of glucopyranose. This reaction involves a key ring-opening event that generates an open-chain carbonyl species followed by subsequent σ-bond rotation of the carbonyl group and ring-closing. Since the initial discovery, the mutarotation of numerous sugars has been studied.[8,9] For many sugars, polarimetry is the analytical method of choice to monitor this reaction because one or both anomers are readily isolable as crystalline solids. In contrast, investigation of Neu5Ac mutarotation has proven to be far more challenging as neither of the anomers have been isolated in pure form from the equilibrium mixture. Friebolin *et al.* were the first to address this problem by treating 2-azido-α-Neu5Ac with an *exo*-sialidase to generate α-Neu5Ac *in situ*.[10,11] Hydrolysis of the substrate and the subsequent mutarotation were monitored by ^{1}H NMR spectroscopy at 5 pD values (1.3, 3.9, 5.4, 6.7 and 11.7). The authors were unable to determine rate constants at pD 1.3 and 11.7 but reported a half-life of 80 min at a pD value of 5.4.[11] However, many mechanistic details of this fundamental process of Neu5Ac mutarotation remain unclear, for instance, what are the respective efficiencies of acid- and base-catalysis? Is there a water-catalyzed process?

Of note, a β-propeller protein, YjhT, from *E. coli* was found recently to possess Neu5Ac mutarotase activity.[12] As such, a mechanistic study on the corresponding non-enzymatic reaction will provide valuable insight as to why nature evolved the specific active site architecture in order to accelerate this spontaneous and facile process. In this report ^{13}C and ^{1}H NMR spectroscopy were used to provide pH and pD-rate profiles for the mutarotation of Neu5Ac, respectively.

Results and Discussion

The mechanism of glucose mutarotation in solution has been studied for over 100 years, but it was not until 2006 that experiments were performed to probe which of the two transition states, ring opening of the α- or β- anomer, had the higher free energy.[13] That is, the key intermediate for the interconversion of anomers is an acyclic carbonyl compound (Scheme 1) and that the observed rate constant for mutarotation $k_{obs} = k_\alpha + k_\beta$, where $k_\alpha = (k_1k_2)/(k_{-1} + k_2)$ and $k_\beta = (k_{-1}k_{-2})/(k_{-1} + k_2)$.

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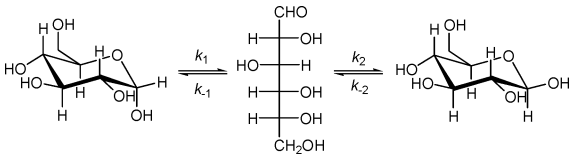


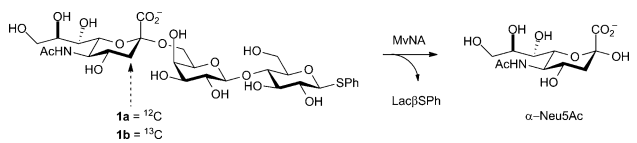
Scheme 1 Mutarotation of glucose involves an open-chain aldehyde intermediate.

In order to delineate the barrier heights for the two ring-closing reactions (k_{-1} and k_2; Scheme 1) a saturation transfer experiment was performed in which the ^{13}C NMR signal for the aldehyde carbon was irradiated and the decrease in intensities of the two hemi-acetal carbons (α- and β-glucopyranose) was monitored.[13]

Therefore, it was decided to perform a similar set of experiments on sialic acid. Previously, Serianni and co-workers tried using this ^{13}C saturation transfer technique[14] with ^{13}C-labeled sialic acid to determine the energy barriers associated with ring-closing of the ketone to either α-Neu5Ac (k_{-1}) or *β*-Neu5Ac (k_2).[15] However, these authors reported that at pH 2.0 and 30 °C, selective irradiation did not affect the C2 signal intensities for either anomer, thus suggesting that the unidirectional rate constants were very small (<0.05 s^{-1}).

In the current study, these same experiments were performed with [C2-^{13}C]-Neu5Ac[16] in order to conduct ^{13}C saturation transfer experiments at pH values of 4.5 and 6.0. Unfortunately, despite the greater sensitivity afforded by a QNP-cyroprobe no ketone C2 signal was observed under these conditions. As a result, the rate constants determined in the current study are given by the equation for approach to equilibrium $k_{obs} = k_\alpha + k_\beta$, where k_{obs} is the observed rate constant for mutarotation and k_α and k_β are the rate constants for conversion of the α-anomer to the β-anomer and for the reverse reaction, respectively.

Scheme 2 illustrates the general protocol used to generate α-Neu5Ac. The substrate Neu5Acα2,6LacβSPh (**1a** or **1b**) is treated with a concentrated solution of *M. viridifaciens* neuraminidase (MvNA) under conditions in which α-Neu5Ac is rapidly generated *in situ*.



Scheme 2 Application of MvNA to generate α-Neu5Ac *in situ*.

MvNA was chosen for the current study, rather than other *exo*-sialidases, because this enzyme displays a very high catalytic efficiency[17,18] and it remains active over a large pH range.[17] Under the reaction conditions employed essentially all of the substrate is hydrolyzed to yield α-Neu5Ac by the time the first NMR spectrum can be acquired.

The subsequent interconversion to *β*-Neu5Ac, the thermodynamically favored species in solution, is monitored by ^1H or ^{13}C NMR spectroscopy. Typical NMR spectra are shown in Fig. 1 and 2 for reactions run in D_2O and H_2O, repectively (full details in experimental section). The calculated rate constants (k_{obs}) and the associated reaction half-lives ($t_{\frac{1}{2}}$) are tabulated in Tables S1 and S2 (ESI†).

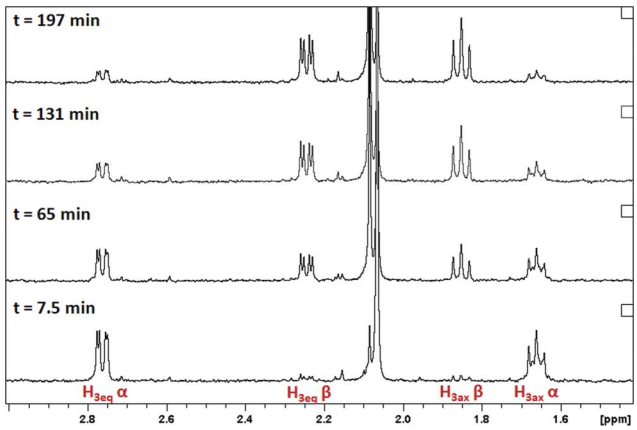


Fig. 1 Representative ^1H NMR spectra acquired during Neu5Ac mutarotation, pD 4.45 (25 mM sodium formate, $I = 0.5$) at 25 °C.

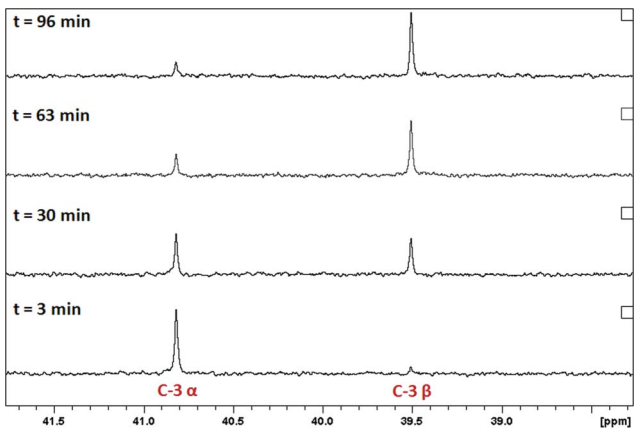
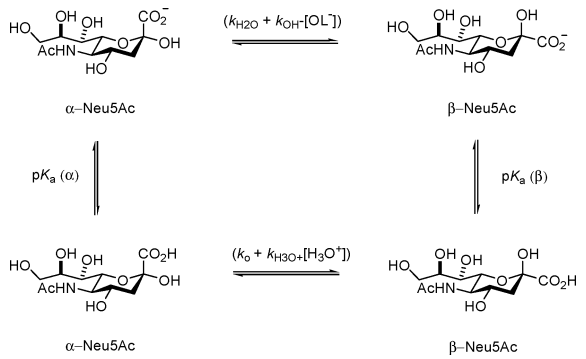


Fig. 2 Typical ^{13}C NMR spectra acquired during Neu5Ac mutarotation, pH 4.45 (25 mM sodium formate, $I = 0.5$) at 25 °C.

Mutarotation of glucose has been shown to be governed by three processes that do not involve buffer catalysis and these are acid- (H_3O^+), base- (HO^-) and water-catalyzed reactions.[19,20] However, because sialic acid contains an ionizable carboxylic acid group it is expected that its mutarotation will involve at the least the following four kinetic terms: (1) an acid-catalyzed reaction of the neutral Neu5Ac species, $k_{L3O}+$; (2) the spontaneous reaction of neutral Neu5Ac or the kinetically equivalent acid-catalyzed reaction of the carboxylate anion, k_0; (3) the spontaneous, water-catalyzed, reaction of the anion, k_{L2O}; and (4) a base-catalyzed reaction of the carboxylate, k_{OL}-. (Scheme 3).

An added complication is that mutarotation involves two separate ionization events that are associated with the pK_a values of the two anomers. However, given that the reported pK_a values of α-Neu5Ac and β-Neu5Ac in H_2O are identical within experimental error,[5] a single pK_a value was used when deriving the kinetic expression (eqn (1)).

$$\log(k_{obs}) = \log\left[\frac{k_{L^+}[L^+]}{1+\frac{K_a}{[L^+]}} + \frac{k_0}{1+\frac{K_a}{[L^+]}} + \frac{k_{L_2O}}{1+\frac{[L^+]}{K_a}} + \frac{k_{LO^-}K_w}{[L^+]\left(1+\frac{[L^+]}{K_a}\right)}\right] \quad (1)$$



Scheme 3 Mechanistic Scheme for the Mutaroration of Sialic Acid.

Fig. 3 displays the logarithms of the pseudo-first-order rate constants for Neu5Ac mutarotation as a function of pH and pD at 25 °C. The dotted lines represent the least-squares best fit to eqn (1) where L denotes either deuterium or protium. The D_2O autoprotolysis constant, $K_{w(DOD)}$, at 25 °C is 1.352×10^{-15}.[21] In order to fit the kinetic data in H_2O, it was necessary to constrain the pK_a value to that reported in the literature (2.55[5]) since reliable data could not be obtained for solutions more acidic than those at a pH value of 2.75. Consequently, the rate constant for the acid-catalyzed reaction of the neutral molecule, k_{H3O+}, could not be determined.

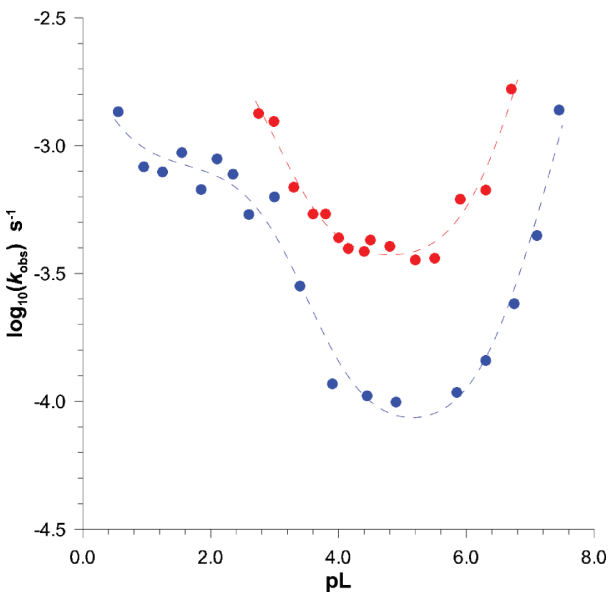


Fig. 3 Plot of log(k_{obs}) values *versus* pH (red circles) and pD (blue symbols) for Neu5Ac mutarotation at 25 °C. The lines shown are the best non-linear least-squares fits to eqn (2).

The pK_a in D_2O derived from eqn (1) is 2.98 ± 0.15. The higher pK_a in D_2O is consistent with the expected decrease in acidity of a carboxylic acid in D_2O.[22] Moreover, it is evident from Fig. 3 that Neu5Ac mutarotation is, as expected, governed by four kinetic processes. All of the derived rate constants from the non-linear least-squares fits to eqn (1) are listed in Table 1. Of note, all rate constants, with the exception of the D_3O^+ catalyzed reaction (k_{L3O+}, Table 1), are defined with errors of approximately 10% or less. As a check on the sensitivity of the derived kinetic parameters the rate constants for mutarotation in D_2O were fit to eqn (1) with the value of k_{L3O+} set to zero, and it can be seen that the parameters are not changed dramatically (Table 1, footnote a).

The rate constants extracted from the fit of k_{obs} to eqn (1) are for the approach to equilibrium for each individual kinetic term. Given that the equilibrium constant ($K_{eq} = k_\beta/k_\alpha$) between the two anomers is 15.7 and 12.3 for the neutral and anionic forms, respectively,[15] the values for the unidirectional rate constants are simply calculated. For example, $k_{\beta(HO-)} = 12.3/13.3 \times k_{OL}$- and $k_{\alpha(HO-)} = 1.0/13.3 \times k_{OL}$-.

Of note, the water-catalyzed mutarotation of sialic acid ($k_{H2O} = 3.42 \times 10^{-4}$ s^{-1}, Table 1) is about five times faster than that for glucose ($k_0 \approx 6.95 \times 10^{-5}$ s^{-1}).[19,23] When comparing the second-order rate constants for acid- and base-catalyzed Neu5Ac mutarotation in D_2O, it is apparent that the base process is more efficient by a factor of almost 2×10^7. The corresponding rate constants for acid and base-catalyzed glucose mutarotation in H_2O are 9.90×10^{-3} M^{-1} s^{-1} and 374 M^{-1} s^{-1}, respectively.[19] Thus suggesting that acid-catalysis is moderately more efficient for glucose, whereas base is a more efficient catalyst for Neu5Ac mutarotation. With regard to buffer catalysis, in all cases, the base form is more efficient than the acid form (data not shown). However, the extent of catalysis was unexpectedly small. For instance, at pH 4.15 increasing the buffer concentration from 25 mM to 100 mM sodium formate only increases the rate constant by 3.5%.

The solvent kinetic isotope effect (KIE) for "water-catalyzed" Neu5Ac mutarotation is 4.48 ± 0.74 (Table 1) a value which is similar to that reported for glucose mutarotation. (k_{H2O}/k_{D2O} = 3.77 ± 0.11).[20] *Ab initio* calculations, which included a single water molecule, performed for the glucose mutarotation TSs were consistent with general base catalysis by the water molecule giving rise to an open-chain aldehyde.[13] In solution, the incorporation of secondary solvation effects by a separate water molecule H-bonded to the ring oxygen atom are probably involved.[24] Based on the similarity of these two solvent KIE values analogous transition state structures are likely for sialic acid mutarotation where the

Table 1 Calculated rate constants and pK_a values for Neu5Ac mutarotation in H_2O and D_2O solutions at 25 °C[a]

Kinetic parameter	H_2O	D_2O[a]	KIE
pK_a	2.55[b]	2.98 ± 0.15	—
k_{L3O+}	N.D.[c]	$(1.36 \pm 0.92) \times 10^{-3}$ M^{-1}s^{-1}	N.D.[c]
k_0	$(3.12 \pm 0.28) \times 10^{-3}$ s^{-1}	$(8.37 \pm 0.94) \times 10^{-4}$ s^{-1}	3.73 ± 0.54
k_{L2O}	$(3.42 \pm 0.19) \times 10^{-4}$ s^{-1}	$(7.63 \pm 1.17) \times 10^{-5}$ s^{-1}	4.48 ± 0.73
k_{OL}-	$(2.32 \pm 0.28) \times 10^4$ M^{-1}s^{-1}	$(2.63 \pm 0.36) \times 10^4$ M^{-1}s^{-1}	0.88 ± 0.16

[a] The fit of kinetic data to eqn (2) with k_{L3O+} set to zero gives the following results: pK_a = 2.88 ± 0.13; $k_0 = (9.42 \pm 0.82) \times 10^{-4}$ s^{-1}; $k_{L2O} = (7.77 \pm 1.21) \times 10^{-5}$ s^{-1}; and k_{OL}- = $(2.61 \pm 0.38) \times 10^4$ M^{-1}s^{-1}. [b] pK_a value for H_2O fit was constrained to 2.55 which represents the average value of the two anomers. [c] N.D. = not determinable.

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hemi-ketal proton is being transferred to a catalytic water at the same time as solvation of the ring oxygen atom strengthens.

The KIE for process (2) is 3.74 ± 0.68, a value which is comparable in magnitude to the water-catalyzed reaction of the carboxylate, and this observation points to the likelihood of a very similar TS for this pathway. Since acid catalysis has been shown to be poor this process corresponds to the spontaneous reaction of neutral Neu5Ac rather than acid-catalyzed reaction of the carboxylate anion. Of note, the rate of mutarotation is approximately 10 times faster on the CO_2H form than on the carboxylate species, an observation that is consistent with water-catalyzed mutarotation not involving participation of the carboxylate group. Thus, it can be concluded that deprotonation of the anomeric hydroxyl group, which places a partial negative charge adjacent to a negatively charged carboxylate introduces an unfavorable interaction that does not occur during mutarotation of the carboxylic acid species.

With regard to the base-catalyzed reaction, the solvent deuterium KIE is 0.88 ± 0.17. This effect can be dissected in terms of fractionation factor analysis (φ).[25] According to eqn (2), the equilibrium isotope effect (EIE) K_D/K_H is equal to the multiple of all product fractionation factors divided by those in the reactant.[25]

$$\frac{K_D}{K_H} = \frac{\prod_{i}^{\text{product}} \phi_i}{\prod_{i}^{\text{reactant}} \phi_i} \qquad (2)$$

Illustrated in Fig. 4 are the reactant and product states expected for hemi-ketal deprotonation by hydroxide ion. Displayed in blue are three hydrogenic sites, φ = 0.70, that are hydrogen bonding to a hydroxide ion (φ = 1.25, green),[26] and highlighted in red is the anomeric hydroxyl proton, φ = 1.00. For a fully transferred proton, the resultant anion forms three hydrogen bonds. If this anion is fully solvated, the fractionation factors for each site are expected to be similar to those for *tert*-butoxide (φ = 0.76).[27]

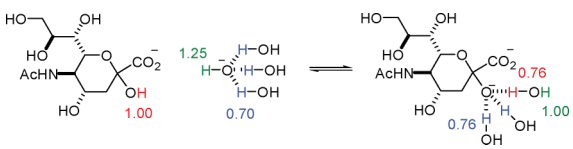

Fig. 4 Proposed equilibrium formation and fractionation factors for the specific-base promoted formation of the hemi-ketal anion, (coloured H's have indicated fractionation factors; all other H's are unity).

The calculated EIE for this deprotonation event is 0.98 (K_D/K_H = $(0.76)^3/(0.70)^3 \times 1.25$). Therefore, the measured solvent deuterium KIE is consistent with a TS for base-catalyzed mutarotation that entails reversible formation of the hemi-ketal anion (specific-base catalysis) followed by a TS that involves ring-opening. On the other hand, a general-base catalyzed mechanism for hydroxide catalysis would be expected to exhibit a large normal ($k_H/k_D > 2$).[28]

A few comments are warranted on the similarities and differences between glucose and sialic acid mutarotation. It has been known for over 80 years that mutarotation of glucose occurs with general-base catalysis that exhibits a Brønsted β coefficient of ≈0.4,[23] and in the ring-opening direction (k_1 and k_{-2}, Scheme 1) catalysis involves reversible deprotonation of the hemi-acetal proton followed by general-acid catalyzed ring opening.[20,29] On the other hand, only very weak catalysis was observed for sialic acid mutarotation using carboxylate anions. This dichotomy between these two mutarotation reactions likely results from two factors: i) the spontaneous mutarotation of sialic acid is faster than that of glucose, a factor that makes the observation of a general-catalysis experimentally more difficult; and ii) the sialic acid hemi-ketal mutarotation reaction requires less general catalysis because the ring-opening reaction, which involves a release of steric strain, would be expected to have an earlier TS than that for the hemi-acetal glucopyranose.

Finally, with regard to the recently discovered β-propeller protein YjhT that displays Neu5Ac mutarotase activity, mutagenesis studies showed that two active site residues, E209A and R215A, displayed a marked decrease in mutarotase activity.[12] Given the conclusion noted above, it is likely that the arginine residue accelerates mutarotation by forming a salt-bridge with the carboxylate group and thus reducing its effective charge (*cf.* k_0 and k_{H2O}, Table 1). Also, it can be predicted that the glutamate residue expedites mutarotation by acting as a general-base catalyst by facilitating deprotonation of the anomeric hydroxyl group.

Conclusions

Mutarotation of *N*-acetylneuraminic acid (Neu5Ac) proceeds by four kinetically distinct pathways: (i) the acid-catalyzed reaction of neutral Neu5Ac; (ii) the spontaneous reaction of the carboxylic acid, which involves water acting as a general base; (iii) a spontaneous, water-catalyzed, reaction of the anion; and (iv) a specific-base catalyzed reaction of the carboxylate to give a dianion, which undergoes rate-determining ring opening.

Experimental

Neu5Acα2,6LacβSPh (**1a**) and its ^{13}C-labeled isotopomer (**1b**) were prepared chemoenzymatically as described previously.[16] The *exo*-sialidase (MvNA) from the soil bacterium *M. viridifaciens* was used to hydrolyze the above substrates.[17] Prior to use, MvNA was dialyzed into non-buffered D_2O.

The effect of pH and pD on mutarotation, were determined by performing kinetic measurements over a range of 2.75–6.70 and 0.55–7.45, respectively. The H_2O buffers (I = 0.5, KCl) used were NaH_2PO_4-H_3PO_4 (pH range 2.75–2.99), HCO_2Na-HCO_2H (pH range 3.30–4.50), NaOAc-HOAc (pH range 3.60–5.20) and 2-(*N*-morpholino)ethanesulfonic acid (MES-NaOH) (pH range 5.50–6.70). The D_2O buffers (I = 0.5, KCl) used were NaD_2PO_4-D_3PO_4 (pD range 2.10–3.00), HCO_2Na-HCO_2D (pD range 3.40–4.90), MES-NaOD (pD range 5.85–6.75) and 3-(*N*-morpholino)propanesulfonic acid (MOPS-NaOD) (pD range 7.10–7.45). For pD 0.55–1.85, kinetic measurements were performed using solutions of DCl (I = 0.5, KCl). The pD was measured using a standard pH electrode and applying the following conversion, pD = pH reading + 0.4.[30] The extent of buffer catalysis was probed by measuring the rate of mutarotation at various concentrations and extrapolating to zero buffer concentration.

^1H NMR spectra for experiments performed in D_2O were acquired on a Bruker AVANCE II 600 MHz spectrometer equipped with a 5 mm TCI cryoprobe. ^{13}C NMR spectra for experiments

performed in H_2O were acquired on a Bruker AVANCE II 600 MHz spectrometer equipped with a 5 mm QNP cryoprobe. Quantitative, proton-decoupled ^{13}C NMR spectra were acquired using an inverse-gated pulse sequence to eliminate nOe enhancements.[31] The spectral width was 240 ppm, transmitter frequency was set to 100 ppm. Proton decoupling was performed using the WALTZ-16 decoupling sequence[32] ^1H pulses of 100 μs, 1H transmitter set to 4.00 ppm. ^{13}C T1 values for C3 of α-Neu5Ac (135 ms) and β-Neu5Ac (142 ms) were in good agreement with reported literature values.[32] ^{13}C T1 values were measured using a standard inversion recovery pulse sequence. A typical ^1H NMR experiment involves rapidly hydrolyzing **1a** (~1.0 mg in 600 μL of deuterated buffer) with a concentrated solution of MvNA. The H-3 axial signals corresponding to α-Neu5Ac (~1.66 ppm) and β-Neu5Ac (~1.85 ppm) were integrated and normalized throughout the time-course of the experiment. Similarly, ^{13}C NMR spectroscopy was used for experiments performed in H_2O. Upon hydrolysis of **1b**, the C-3 signals of α-Neu5Ac (~40.8 ppm) and β-Neu5Ac (~39.5 ppm) were processed as described above.

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