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Unexpected Stability of Aryl β-*N*-Acetylneuraminides in Neutral Solution: Biological Implications for Sialyl Transfer Reactions

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Abstract: A reagent panel comprised of seven aryl β-D-*N*-acetylneuraminides was synthesized and then used to probe the mechanisms of nonenzymatic hydrolysis. These reactions proceeded via four independent pathways: (1) acid-catalyzed hydrolysis of the neutral molecule; (2) acid-catalyzed hydrolysis of the anionic form, or its kinetic equivalent spontaneous hydrolysis of the neutral form; (3) spontaneous hydrolysis of the anionic form; and (4) a base-promoted pathway. The pH-independent spontaneous hydrolysis of 4-nitrophenyl α-D-*N*-acetylneuraminide (**5**) occurs at a rate that is over 100 times faster than that of the corresponding reaction of 4-nitrophenyl β-D-*N*-acetylneuraminide (**4a**). Spontaneous hydrolyses of four aryl β-D-*N*-acetylneuraminides displayed a β_{lg} value of -1.24 ± 0.16 (pH = 8.1, $T = 100$ °C), and at a pH value of 1.0 (50 °C), all seven panel members gave a β_{lg} value of 0.14 ± 0.08. The aqueous ethanolyses of **4a** and **5** gave similar products and displayed sensitivity parameters (m) in a standard Winstein−Grunwald analysis of -0.04 ± 0.01 and $+0.23 \pm 0.02$, respectively. These results, plus the activation parameters calculated for the spontaneous hydrolyses of the anionic forms of **5** ($\Delta H^{\ddagger} = 116 \pm 2$ kJ mol^{-1} and $\Delta S^{\ddagger} = 27 \pm 4$ J mol^{-1} K^{-1}) and **4a** ($\Delta H^{\ddagger} = 138 \pm 3$ kJ mol^{-1} and $\Delta S^{\ddagger} = 59 \pm 8$ J mol^{-1} K^{-1}), are inconsistent with anomeric carboxylate assistance occurring during the hydrolysis reactions, and the likely cause for the enhanced reactivity of **5** in comparison to that of **4a** is an increase in ground-state steric strain.

Introduction

Many specific biological processes are mediated by carbohydrate/protein interactions, examples include receptor/ligand recognition and glycoconjugate intracellular trafficking.[1,2] In many of these biologically important interactions, the sugar component contains *N*-acetylneuraminic acid (sialic acid), a common constituent of the glycolipids and glycoproteins produced by animal cells.[3] Indeed, *N*-acetylneuraminic acid is frequently found at the nonreducing termini of cell surface oligosaccharide moieties,[4] and this prominence is in keeping with its importance in cellular and molecular recognition events.[5]

Several distinct families of sialyl-transferring enzymes are found in nature and these include the following: (a) *exo*-sialidases (*N*-acetylneuraminyl glycohydrolases, EC 3.2.1.18) are retaining glycosidases that remove sialic acid from glycoconjugates;[4,6−10] (b) *trans*-sialidases are retaining enzymes expressed by trypanosomes that enable the parasite to transfer sialic acid from an external source onto its own surface carbohydrates;[11] and (c) sialyltransferases are inverting enzymes that utilize CMP-β-D-*N*-acetylneuraminide (**1**) as the sialyl donor to make α-sialoside linkages.[12] A fourth family, the *endo*-sialidases (EC 3.2.1.129),[13] has been reported, although at the present time, it is not known whether these enzymes operate via a retaining or an inverting mechanism.

Retaining glycosidases generally operate via a process in which an active site carboxylate group acts as the nucleophile in a double displacement mechanism.[14−18] Such a process

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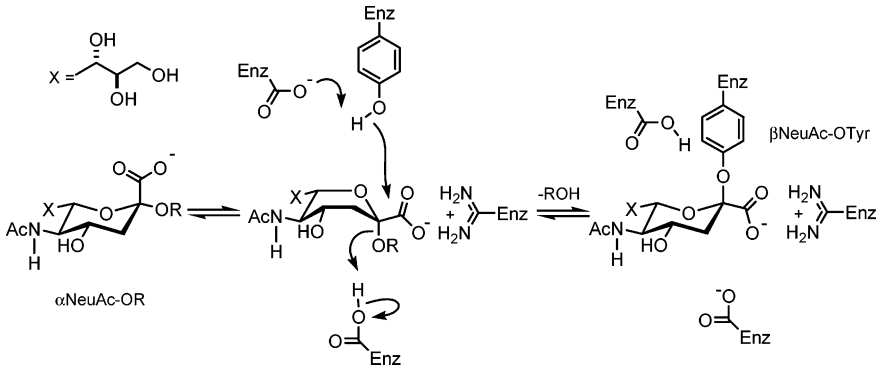
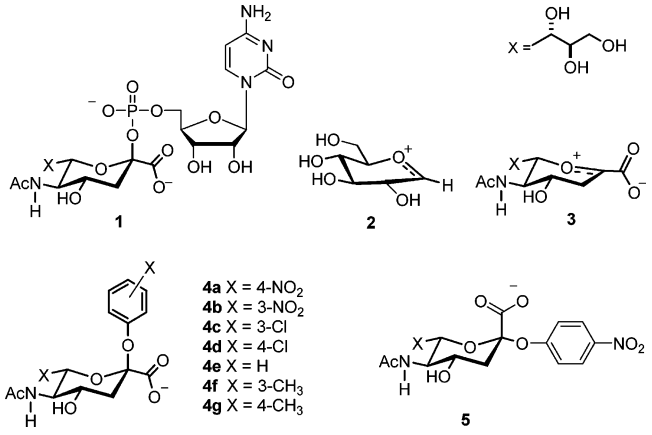
Scheme 1. Mechanism of Glycosylation in Sialidases



generates a covalent acylal enzyme intermediate and thus avoids formation of a reactive high-energy oxacarbenium ion. In aqueous solution, the lifetime of the quintessential carbohydrate oxacarbenium ion, the glucopyranosylium ion **2**, has been estimated to be around $(1-3) \times 10^{-12}$ s.[19,20]

The results contained in recent reports point to the conserved tyrosine residue, rather than the catalytic glutamic acid, as being the nucleophile for both classes of retaining sialyl transferring enzymes, that is, *exo*-sialidases[21] and *trans*-sialidases.[22,23] Thus, the enzymatic mechanism involves formation of an enzyme-bound aryl *β*-*N*-acetylneuraminyl intermediate (Scheme 1, *β*-NeuAc-OTyr).[21] A central feature of this mechanism is that formation of an *N*-acetylneuraminosylium ion (sialyl oxacarbenium ion; **3**) intermediate is avoided, a species that has an estimated lifetime in aqueous solution of $\geq 3 \times 10^{-11}$ s.[24,25]

The present report details a series of kinetic and product studies performed, in aqueous media, on analogues of the sialosyl−enzyme intermediate (Scheme 1, *β*-NeuAc-OTyr), that is, aryl *β*-D-*N*-acetylneuraminides (**4a**−**g**). In addition, a specific comparison is made between **4a** and 4-nitrophenyl α-D-*N*-acetylneuraminide (**5**), the anomer of **4a**. The results from these studies address critical questions concerning the intrinsic reactivity of sialosides. For example, does the C-1 carboxylate group of the *N*-acetylneuraminides function intramolecularly as a nucleophile? Are the spontaneous solvolysis reactions of *N*-acetylneuraminides associative (S_N2) or dissociative (S_N1) in nature?



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Experimental Section

Materials and Methods. The buffers 2-(*N*-morpholino)ethanesulfonic acid (MES), *N*-tris[hydroxymethyl]methyl-3-amino-propanesulfonic acid (TAPS), and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) were purchased from Sigma and used without further purification. *N*-Acetylneuraminic acid was purchased from Rose Scientific and used without further purification. Milli-Q water (18.2 MΩ cm^{-1}) was used for kinetic and product study experiments. Perchloric acid and sodium hydroxide solutions were made by dilutions of standardized 1.00 M solutions. All other salts used in the hydrolysis runs were of analytical grade and were used without purification. CH_2Cl_2 was dried by distillation from CaH_2. NMR spectra were acquired on a Bruker AMX-400 spectrometer.

Synthesis of Aryl *β*-D-*N*-Acetylneuraminide. The protected *β*-D-*N*-acetylneuraminyl fluoride **6**[26] and 4-nitrophenyl α-D-*N*-acetylneuraminide **5**[27,28] were synthesized according to published procedures. Full experimental details for the synthesis of 4-nitrophenyl *β*-D-*N*-acetylneuraminide (**4a**) are given below, while the experimental particulars for the synthesis and characterization of **4b**−**g** are given in the accompanying Supporting Information.

Methyl [4-Nitrophenyl(5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-*β*-D-*galacto*-non-2-ulopyranosyl)]onate (7a). To dried powdered 4 Å molecular sieves was added a mixture of 4-nitrophenol (580 mg, 4.17 mmol) and sialosyl fluoride **6** (400 mg, 0.83 mmol), and the resultant solid mixture was maintained under vacuum (6 mmHg) for 30 min. The solid mixture was then placed under a N_2 atmosphere, and dry CH_2Cl_2 (30 mL) was added via a syringe; this mixture was stirred for 1 h. Then a solution of $BF_3{\cdot}OEt_2$ (0.72 mL, 0.83 mmol) in CH_2Cl_2 (4 mL) was added, and the mixture was stirred overnight at room temperature. The mixture was filtered, and the solid residue was washed thoroughly with CH_2Cl_2. The combined filtrates were washed with saturated $NaHCO_3$ (150 mL), water (150 mL), and brine (150 mL), and the resulting solution was dried Na_2SO_4. A pale yellow syrup was obtained after evaporation of the solvent, and this material was crystallized from Et_2O to give a white powder (196 mg, 39% yield), mp 187−189 °C. $[\alpha]^{20}_D = -77.8$ ($c = 1.26$, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.75, 1.89, 2.06, 2.08, 2.17 (5 × s, 15 H, CH_3), 2.03 (m, 1 H, H-3a), 2.68 (dd, 1 H, $J_{3e,3a} = 12.9$ Hz, $J_{3e,4} = 5.0$ Hz, H-3e), 3.76 (s, 3 H, OCH_3), 4.04 (dd, 1 H, $J_{6,5} = 10.4$ Hz, $J_{6,7} = 2.4$ Hz, H-6), 4.12 (dd, 1 H, $J_{9a,9b} =$

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12.5 Hz, $J_{9a,8}$ = 6.8 Hz, H-9a), 4.20 (q, 1 H, $J_{5,4} + J_{5,6} + J_{5,NH}$ = 31.2 Hz, H-5), 4.64 (ddd, 1 H, $J_{9b,8}$ = 2.7 Hz, H-9b), 4.90 (ddd, 1 H, $J_{8,7}$ = 8.7, H-8), 5.26 (d, 1 H, $J_{NH,5}$ = 10.3 Hz, NH), 5.35 (dd, 1 H, H-7), 5.48 (td, 1 H, $J_{4,3a} + J_{4,5}$ = 21.8 Hz, $J_{4,3e}$ = 5.0 Hz, H-4), 7.08−7.15 (m, 2 H, Ar−H), 8.12−8.22 (m, 2 H, Ar−H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 20.6. 20.7, 20.8, 23.1 (CH_3), 38.5 (C-3), 49.1 (C-5), 53.5 (OCH_3), 61.8 (C-9), 67.8 (C-4, C-7), 71.5 (C-8), 72.9 (C-6), 99.7 (C-2), 116.8, 125.9, 143.1, 158.8 (Ar−C), 170.0, 170.2, 170.3, 170.5, 170.9 (C═O, C-1). Anal. Calcd for $C_{26}H_{32}N_2O_{15}$: C, 50.98; H, 5.27; N, 4.57. Found: C, 50.75; H, 5.44; N, 4.34.

4-Nitrophenyl (5-Acetamido-3,5-dideoxy-D-*glycero*-β-D-*galacto*-non-2-ulopyranosylonic Acid) (4a). To a solution of **7a** (50 mg, 0.08 mmol) in anhydrous methanol (5 mL) was added a methanolic sodium methoxide solution (5 equiv), and this mixture solution was stirred at 0 °C for 15 min. Dowex 50W HCR-W2(H^+) cation exchange resin (prewashed with methanol) was added to neutralize the solution. After removal of the resin by filtration, it was washed several times with methanol. The combined solvent was evaporated under reduced pressure, and the resultant residue was dissolved in 3:1 v/v THF/water (2 mL) at 0 °C. To this solution was added $LiOH\cdot H_2O$ (16 mg, 2.5 equiv). After stirring at 0 °C for 25 min, the mixture was neutralized with Dowex 50W HCR-W2(H^+) resin. Following removal of the resin, the filtrate was concentrated under reduced pressure. The remaining aqueous solution was then lyophilized to give a white solid (33 mg, 92%). $[\alpha]^{20}_D$= −86.9 (c = 1.22, H_2O). ^{1}H NMR (400 MHz, D_2O) δ: 1.85 (t, 1 H, $J_{3e,3a} + J_{3a,4}$ = 24.5 Hz, H-3a), 2.04 (s, 3 H, CH_3), 2.60 (dd, 1 H, $J_{3e,3a}$ = 12.9 Hz, $J_{3e,4}$ = 5.0 Hz, H-3e), 3.45 (d, 1 H, $J_{7,8}$ = 9.0 Hz, H-7), 3.60 (dd, 1 H, $J_{9a,9b}$ = 11.8 Hz, $J_{9a,8}$ = 5.2 Hz, H-9a), 3.64−3.77 (m, 4 H, NH, H-6, H-8, H-9b), 4.00 (t, 1 H, $J_{5,4} + J_{5,6}$ = 20.8 Hz, H-5), 4.25 (td, 1 H, $J_{4,3a} + J_{4,5}$ = 21.8 Hz, $J_{4,3e}$ = 5.0 Hz, H-4), 6.85−7.21 (m, 2 H, Ar−H), 8.20−8.38 (m, 2 H, Ar−H). ^{13}C NMR (100 MHz, D_2O) δ: 19.0 (CH_3), 37.5 (C-3), 48.6 (C-5), 60.3 (C-9), 63.4 (C-4), 65.2 (C-7), 66.8 (C-8), 68.5 (C-6), 98.1 (C-2), 113.8, 122.7, 138.9, 156.8 (Ar−C), 170.3, 171.7. HRMS(FAB) m/z ($M - H^+$), $C_{17}H_{21}N_2O_{11}$ requires 419.1145, found 429.1156.

4-Nitrophenyl ((3*R*)-5-Acetamido-3-*deuterio*-3,5-dideoxy-D-*glycero*-β-D-*galacto*-non-2-ulopyranosylonic Acid) (4a-^{2}H). Deuterium was incorporated stereospecifically on C-3 in *N*-acetylneuraminic acid according to a published procedure.[29] Following the base-catalyzed exchange reaction, 4-nitrophenyl ((3*R*)-5-acetamido-3-*deuterio*-3,5-dideoxy-D-*glycero*-β-D-*galacto*-non-2-ulopyranosylonic acid) was made using the same procedure as outlined above. The NMR spectrum of **4a-^{2}H** was identical to that of the unlabeled compound (**4a**) except for the absence of the apparent triplet at 1.85 ppm and the presence of a doublet at 2.60 ppm instead of a pair of doublets.

4-Nitrophenyl (5-Acetamido-3,5-dideoxy-D-*glycero*-α- and β-D-*galacto*-non-2-ulopyranosylonic ($^{18}O_1$)-Acid) (4a-^{18}O and 5-^{18}O). Oxygen-18 was incorporated into the respective carboxylate groups by performing the final hydrolytic deprotection of the respective methyl esters in THF/$H_2{}^{18}O$ (3:1 v/v, 5 mL) containing $LiOH\cdot H_2O$ (3 equiv) at 0 °C for 30 min.

Hydrolysis Kinetics. The hydrolysis reactions were monitored by measuring absorbance versus time data using a Cary-3E UV−vis spectrophotometer equipped with the Cary Six-Cell Peltier constant temperature accessory. The buffers and their associated pH ranges used in this study were: malonic acid, 2.10−3.19; succinic acid, 3.76−4.02; acetic acid, 4.46−5.36; *N*-methylmorpholinoethanesulfoninc acid (MES), 5.50−6.30; *N*-[tris(hydroxymethyl)methyl]-3-aminopropane-1-sulfonic acid (TAPS), 7.00−8.20; and 3-(cyclohexylamino)propane-1-sulfonic acid (CAPS), 10.00−11.13. Buffer pH values were measured at room temperature, and no temperature corrections were applied. For solution where $[H^+] > 0.01$ M, perchloric acid−sodium perchlorate was used, and when $[OH^-] > 0.01$ M, sodium hydroxide−sodium perchlorate was used. Ionic strength was maintained at 0.3 M with sodium perchlorate except when $[H^+]$ or $[OH^-] > 0.3$ M.

The hydrolysis reactions of **4a** were monitored using three different protocols. For pH values less than 4.50 and greater than 11.20, a methanol stock of **4a** (50 μL, 2.3 μM) was injected into a cuvette containing 3 mL of the required buffer that had been pre-equilibrated for 10 min at 50 °C. The absorbance versus time data were monitored for 3 half-lives at 340 and 408 nm for the low and high pH ranges, respectively. Rate constants were calculated by fitting the absorbance versus time data to a standard first-order rate equation using the nonlinear least-squares routine computer program, Grafit.

For pH values between 4.50 and 6.33, a discontinuous assay method was used. A solution of **4a** (4 mg) in 20 mL of the required buffer was aliquoted into glass ampules (1 mL of solution) which were then sealed. The ampules were placed in a water bath held at 50 °C. Ampules were taken out of the water bath at regular intervals, and the reaction was quenched by cooling to 0 °C. To determine the reaction end point, two ampules were heated at 100 °C for 2 days. A portion of the solution from each ampule (200 μL) was added to pH 10.0 CAPS buffer (0.3 M, 400 μL), and the absorbance of the resultant mixture was measured at 408 nM. These reactions were monitored in this fashion until they were about 5% complete.

For pH values between 6.40 and 11.20, the hydrolysis of **4a** was followed by observing the change in absorbance at 408 nm at 50 °C until about 3−4% of the hydrolytic reaction had occurred. Subsequently, the reaction end-points were determined by increasing the cell-block temperature to 75 °C and monitoring the absorbance until the reaction was complete. Rate constants for the reactions performed between pH values of 4.50 and 11.20 were calculated using the equation $\ln([A]_t/[A]_0) = -k \times t$. The hydrolysis reactions of **5** were monitored using identical protocols.

Solvolysis Kinetics. The aqueous ethanolyses of **4a** and **5** were monitored in a manner similar to that of the corresponding hydrolysis reactions. Specifically, reactions were followed at 408 nm in 15 mM phosphate buffer (Na_2HPO_4:NaH_2PO_4 = 2:1; I = 35 mM) at 75 °C.

Brønsted Plots. For the acid-catalyzed region (pH = 1.00), the reactions were monitored directly by following the change in absorbance versus time. Absorbance values were recorded at wavelengths of 340 and 270 nm for **4b** and **4e**, respectively, while for all other substrates (**4c**, **4d**, **4f**, and **4g**), the wavelength utilized was 280 nm. In the spontaneous hydrolysis regime, the hydrolyses of **4b**−**d** were monitored at a pH of 8.08 using ampules as outlined above. The ampules were maintained at a temperature of 100 °C by placing them in a "boiler" in which they were immersed in the vapor generated from a boiling water bath. Ampules were taken out at regular intervals, and the reactions were quenched by cooling the ampule in an ice/water bath. These reactions were generally monitored for a time period that corresponds to about 2−3 half-lives for hydrolysis. The rate constant for hydrolysis of **4a** under these conditions (pH 8.08, T = 100 °C) was estimated by extrapolation of rate constant data measured at 50, 65, 75, and 85 °C.

Product Studies. The hydrolyses of **4a** and **5** (3 mg) in ethanol/water mixtures (2 mL) containing 3 equiv of *N*-methylmorpholine were performed in sealed glass ampules at 75 °C. The reactions were allowed to proceed for about 9 half-lives. The resultant solution was lyophilized, and the solid residue was analyzed using ^{1}H NMR spectroscopy.

^{18}O Exchange Experiments. ^{18}O-Incorporation into the 4-nitrophenol product was monitored for the base-promoted reactions of **4a** and **5** at $[OH^-]$ = 1.0 M. Specifically, an approximate 1:1 $H_2{}^{16}O/H_2{}^{18}O$ mixture was made by adding equal volumes of $H_2{}^{18}O$ (95.1 atom % ^{18}O; Marshall Isotopes Ltd., batch number 020414nw) and freshly made-up aqueous NaOH (2.0 M). To this aqueous media (0.4 mL) was added either **4a** or **5** (2 mg, 4.6 μmol), and the resulting solutions were maintained at 50 °C for approximately 5 half-lives for hydrolysis. Following neutralization with an excess of malonate buffer (0.3 M, 5 mL, pH 2.69), the resultant aqueous media were extracted with distilled dichloromethane (2 × 2 mL). The combined organic extracts

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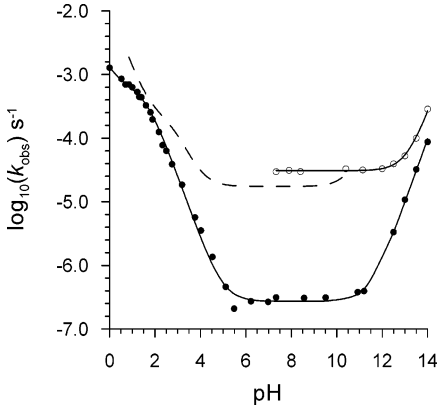
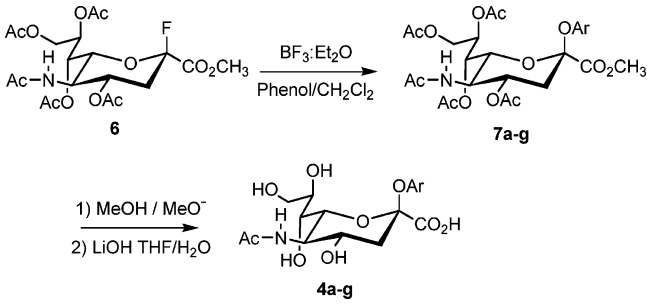


Figure 1. Plot of $\log(k_{obs})$ versus $-\log[H_3O^+]$ for the hydrolyses of 4-nitrophenyl α-D-*N*-acetylneuraminide (○) and 4-nitrophenyl β-D-*N*-acetylneuraminide (●), $T = 50$ °C. The included solid lines are the best nonlinear fits of the kinetic data to eq 1 or eq 2. The dashed line is generated from kinetic rate constants reported for the hydrolysis of 4-nitrophenyl α-D-*N*-acetylneuraminides, $T = 50$ °C (ref 29).

Scheme 2



were then dried (Na_2SO_4), and following removal of the solvent under reduced pressure, the resulting solid residues were analyzed by standard EI mass spectrometry. The atom % ^{18}O in the isolated 4-nitrophenol was calculated by the least-squares method of Brauman[30] using the isotopic peak distribution of the molecular ion observed in the mass spectrum.

Similarly, any ^{18}O-incorporation into the 4-nitrophenol product from the anomeric carboxylate group for the reactions of **4a-^{18}O** and **5-^{18}O** in water at a pH value of 14.0 ($[OH^-] = 1.0$ M) and in aqueous ethanol (0, 50, and 100% v/v containing 3 equiv of *N*-methylmorpholine) was monitored using an analogous protocol to that reported above.

Results

Aryl β-D-*N*-acetylneuraminides were prepared from the readily made and fully protected β-D-*N*-acetylneuraminyl fluoride (**6**)[26] according to the synthetic route outlined in Scheme 2. The coupling reactions that give the protected aryl β-sialosides (**7a**−**g**) also yielded an appreciable quantity of an elimination product (the α,β-unsaturated ester).

pH Rate Profile. Figure 1 presents the logarithms of the measured rate constants (k_{obs}) as a function of pH for the hydrolyses of 4-nitrophenyl β-D-*N*-acetylneuraminide (**4a**) at 50 °C. Individual rate constants are given in Table S1 of Supporting Information. The observed rate constants for **4a** were fit to eq 1, where K_a is the ionization constant of **4a**, while k_{H^+}, k_0, k_{sp}, and $k_{OH^-}K_w$ are the rate constants for (i) the acid-catalyzed reaction of the neutral molecule; (ii) the acid-catalyzed reaction of the carboxylate form, or its kinetic equivalent, the spontaneous reaction of the neutral molecule; (iii) the spontaneous reaction of the anion; and (iv) a base-promoted reaction, respectively.

Table 1. Calculated pK_a Values and Rate Constants for the Hydrolyses of 4-nitrophenyl α- and β-D-*N*-acetylneuraminides (**5** and **4a**, respectively) at 50 °C ($\mu = 0.3$, $NaClO_4$)

parameter	**4a**[a]	**5**[a]
pK_a	1.58 ± 0.07	2.85[b]
k_{H^+}	$(7.0 \pm 2.6) \times 10^{-4}$ M^{-1} s^{-1}	$(1.18 \times 10^{-2}$ M^{-1} $s^{-1})$[b]
k_0	$(6.7 \pm 0.8) \times 10^{-4}$ s^{-1}	$(2.27 \times 10^{-4}$ $s^{-1})$[b]
k_{sp}	$(2.72 \pm 0.17) \times 10^{-7}$ s^{-1}	$(3.08 \pm 0.06) \times 10^{-5}$ s^{-1} $(1.74 \times 10^{-5}$ $s^{-1})$[b]
$k_{OH^-}K_W$	$(9.7 \pm 0.8) \times 10^{-19}$ M s^{-1}	$(2.38 \pm 0.09) \times 10^{-18}$ M s^{-1}

[a] This work unless stated otherwise. [b] Values taken from ref 29.

$$\log(k_{obs}) = \log\{k_{H^+}[H^+]/(1 + K_a/[H^+]) + k_0/(1 + K_a/[H^+]) + k_{sp}/(1 + [H^+]/K_a) + k_{OH^-}K_W/[H^+](1 + [H^+]/K_a)\} \quad (1)$$

Also, shown in Figure 1 are recently measured rate constants for pH values between 7.3 and 14.0 and the best-fit line for the previously reported kinetic data (pH values 0.8−10.0) for hydrolysis of 4-nitrophenyl α-D-*N*-acetylneuraminide (**5**). The newly measured rate constants for **5** (Table S2, Supporting Information) were fit to eq 2, where k_{sp} and $k_{OH^-}K_w$ represent the same rate constants detailed above.

$$\log(k_{obs}) = \log\{k_{sp} + k_{OH^-}K_W/[H^+]\} \quad (2)$$

Listed in Table 1 are the so-derived values for the kinetic parameters.

Figure 2 displays the Brønsted plot, $\log(k_{obs})$ versus the pK_a of the conjugate acid of the leaving group for the spontaneous reactions of **4a**−**d** measured in the pH-independent domain at 100 °C (Table S3, Supporting Information). The derived β_{lg} value for this reaction is -1.24 ± 0.16. Similarly, a Brønsted plot of $\log(k_{obs})$ for the hydrolyses of **4a**−**g** measured at a pH value of 1.0 and a temperature of 50 °C gave a slope of 0.14 ± 0.08 (Figure 2; Table S4, Supporting Information).

The pseudo-first-order rate constants for the spontaneous reactions of the anions of **4a** and **5** were also measured as a function of temperature (Tables S5 and S6, Supporting Information), and the corresponding Eyring plot is shown in Figure 3. The activation parameters derived for the spontaneous hydrolysis of **4a** and **5** are $\Delta H^{\ddagger} = 138 \pm 3$ kJ mol^{-1} and $\Delta S^{\ddagger} = 59 \pm 8$ J mol^{-1} K^{-1}, and $\Delta H^{\ddagger} = 116 \pm 2$ kJ mol^{-1} and $\Delta S^{\ddagger} = 27 \pm 4$ J mol^{-1} K^{-1}, respectively.

To probe for intramolecular nucleophilic attack on the *ipso*-carbon of the aromatic ring either by the anomeric carboxylate or directly by hydroxide ion, oxygen-18 exchange experiments were performed. The 4-nitrophenol isolated from the base-promoted reactions of **4a** and **5** in ^{18}O-water (≈50 atom %) contained approximately 2.1 atom % ^{18}O after time periods of 20 and 6 h at 50 °C, respectively. While the 4-nitrophenol isolated from the spontaneous ethanolysis and base-promoted reactions of **4a**-$^{18}O_1$ and **5**-$^{18}O_1$, it showed no incorporation of ^{18}O into the phenol.

To check for the possibility of a base-promoted E2 elimination mechanism, the β-secondary deuterium kinetic isotope effect (β-SDKIE) for the reactions of **4a** and its axial 3-*deuterio*-labeled isotopomer (**4a**-^{2}**H**) were evaluated in NaOH (1 M) at

(30) Brauman, J. I. *Anal. Chem.* **1967**, *38*, 607−610.

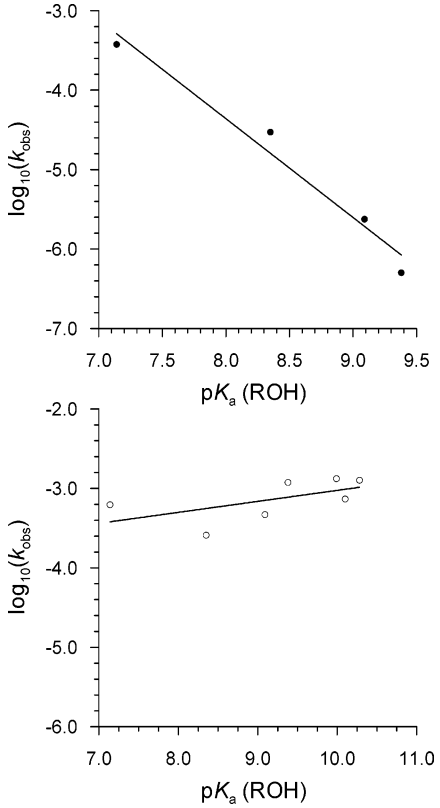


Figure 2. Dependence of the observed rate constants (k_{obs}) for hydrolysis of aryl β-D-*N*-acetylneuraminides on the pK_a of the parent phenol: pH 1.0 at 50 °C (○) and pH 8.08 at 100 °C (●). The lines shown are the best linear least-squares fits through the data points.

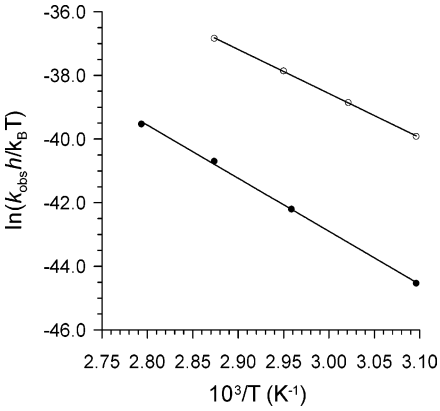


Figure 3. Eyring plot for the spontaneous hydrolyses of the anions of 4-nitrophenyl α-D-*N*-acetylneuraminide (○) and 4-nitrophenyl β-D-*N*-acetylneuraminide (●). The lines shown are the best linear least-squares fits through the data points.

50 °C. Under these conditions, the measured β-SDKIE (k_H/k_D) was 1.096 ± 0.025.

To probe the effect of solvent polarity on the spontaneous rate constant (k_{sp}), **4a** and **5** were solvolyzed in various aqueous ethanol mixtures. The standard Grunwald−Winstein eq 3 is commonly used to analyze solvent effects on $D_N + A_N$ (S_N1) substitution reactions, where the Y_X parameter is a measure of the solvent's ionizing power (leaving group = X) and *m* is a sensitivity variable.[31]

$$\log(k_{obs}/k_0) = mY_X \quad (3)$$

Thus, when the kinetic data for the aqueous ethanolyses of **4a** and **5**, which are given in Tables S7 and S8 (Supporting Information), are fit to eq 3 using Y_{pic} values,[32−34] the derived *m* values for the reactions of **4a** and **5** are −0.04 ± 0.01 and +0.23 ± 0.02, respectively. Figure 4 displays these Grunwald−Winstein plots for the solvolyses of **4a** and **5**. To minimize differential solvation effects on the leaving group,[31] the Y_{pic} scale was chosen because the picrate leaving group in the reference reaction, the solvolysis of 1-adamantyl picrate (25 °C),[33] is structurally similar to the nucleofuge for the spontaneous solvolysis reactions under study (i.e., 4-nitrophenolate).

To ensure that the ethyl *N*-acetylneuraminide products did not undergo any subsequent acid-catalyzed reactions, all solvolytic product studies were performed in the presence of 3 molar equiv of *N*-methylmorpholine. Given in Table 2 are the measured percentages of the products, which were identified by standard ^{1}H NMR spectroscopic analysis, that were formed during the solvolysis of **4a** and **5**.

Discussion

First, a comment is warranted on the difference between the rate constant for spontaneous hydrolysis of **5** reported by Ashwell et al.[29] and that obtained in the current study (k_{sp}; Table 1). In the current study, no temperature corrections were applied to the buffer pH values (measured at 25 °C), yet all of the computed rate constants for hydrolysis of **5** (up to [NaOH] = 0.01 M) were identical within experimental error (Table S2, Supporting Information). Also, the rate constants measured in TAPS buffer (0.3 M, pH ∼ 7.9) with and without ionic strength adjustment are identical within experimental error (Table S2). Therefore, neither solution acidity nor ionic strength differences can account for the variation in spontaneous rate constant noted above. The most likely explanation is that a temperature difference existed between the two data sets. Indeed, given the activation parameters reported above, an imbalance of 4 °C, between the two studies, is sufficient to rationalize a rate difference of 1.7-fold (Table 1). It is, nevertheless, logical to compare the newly acquired rate data for hydrolysis of **5** with that evaluated for **4a** using the same instrumental setup.

Spontaneous Hydrolytic Reactions. Remarkably, the spontaneous hydrolysis of **5**, the thermodynamically less stable diastereomer, occurs at a rate that is over 100 times faster than that of the corresponding reaction of **4a**. The enhanced reactivity of the α-anomer is not caused by intramolecular nucleophilic catalysis by the C-1 carboxylate group occurring during its spontaneous hydrolysis for the following reasons: (i) the respective β_{lg} values on k_{sp} for the α- and β-anomers of -1.3_2 (60 °C)[29] and −1.24 ± 0.16 (100 °C) are indistinguishable, thus

(31) Bentley, T. W.; Llewellyn, G. *Prog. Phys. Org. Chem.* **1990**, *17*, 121−158.
(32) Bentley, T. W.; Carter, G. E. *J. Am. Chem. Soc.* **1982**, *104*, 5741−5747.
(33) Bentley, T. W.; Roberts, K. *J. Org. Chem.* **1985**, *50*, 4821−4828.
(34) The Y_{pic} values for water, 10% EtOH, and 60% EtOH were estimated from the linear correlation (r = 0.9981) between Y_{pic} values (ref 33) and Y_{Cl} (ref 32).
(35) Horenstein, B. A. *J. Am. Chem. Soc.* **1997**, *119*, 1101−1107.
(36) Chou, D. T. H.; Watson, J. N.; Scholte, A. A.; Borgford, T. J.; Bennet, A. J. *J. Am. Chem. Soc.* **2000**, *122*, 8357−8364.
(37) Chandrasekhar, S.; Kirby, A. J. *J. Chem. Soc., Chem. Commun.* **1978**, 171−172.
(38) Chandrasekhar, S.; Kirby, A. J.; Martin, R. J. *J. Chem. Soc., Perkin Trans. 2* **1983**, 1619−1626.
(39) Wolfenden, R.; Lu, X.; Young, G. *J. Am. Chem. Soc.* **1998**, *120*, 6814−6815.

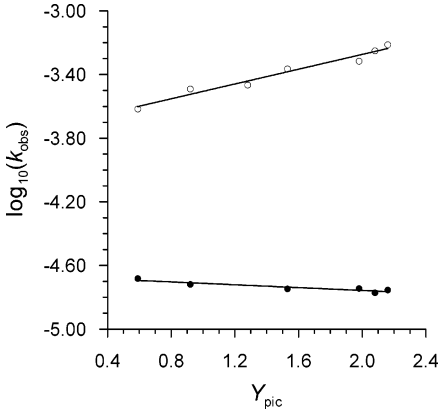
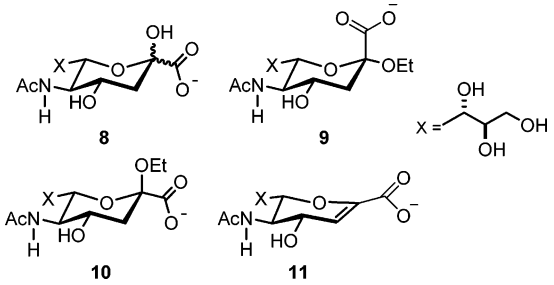


Figure 4. Plot of Grunwald−Winstein correlation for the aqueous ethanolysis of **4a** (●) and **5** (○) versus Y_{pic} at $T = 75$ °C. The lines shown are the best linear least-squares fits through the data points.

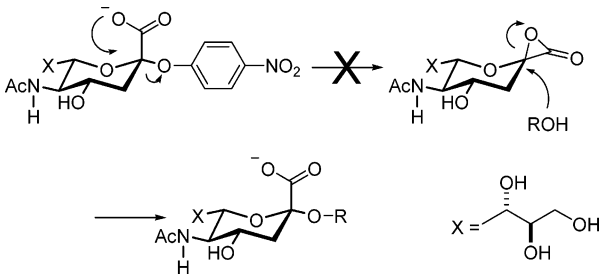
Table 2. Observed Product Percentages Formed during the Solvolysis of **4a** and **5** in Aqueous Ethanol (v/v) Solvent Mixtures at 75 °C[a,b]

substrate	EtOH%	NeuAc (**8**)	Et-αNeuAc (**9**)	Et-βNeuAc (**10**)	glycal (**11**)	unknown
4a[c]	100		9	73	9	10
5	100		10	90	tr[d]	tr[d]
5	50	65	6	19	3	7

[a] All solvents contained 3 molar equiv of *N*-methylmorpholine. [b] Estimated errors ±5%. [c] No values given for solvolysis of **4a** in 50% v/v EtOH:H_2O because extensive decomposition occurs under these conditions. [d] tr = trace.

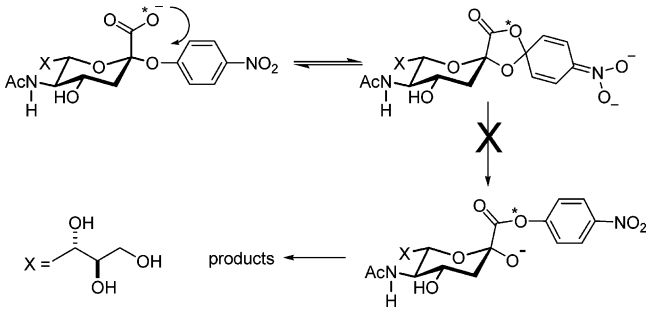


Scheme 3



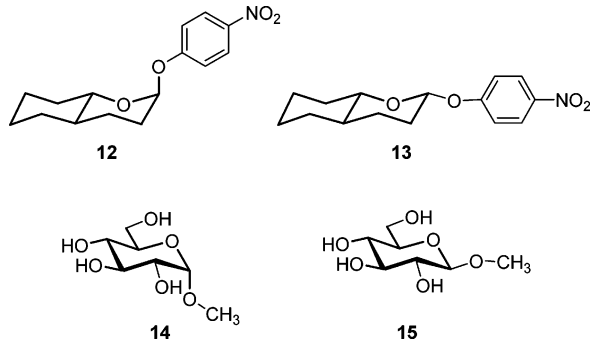
suggesting similarly late transition states for both anomers; and (ii) both retained and inverted substitution products are formed during the aqueous ethanolyses of **4a** and **5** (Table 2), an observation that is inconsistent with intramolecular nucleophilic participation because such reactions should yield retained ethyl *N*-acetylneuraminide as the only glycosidic product (Scheme 3). Therefore, at the present time, no evidence exists for the occurrence of intramolecular assistance during the hydrolysis reactions of *N*-acetylneuraminides, a conclusion that other workers have also made.[35,36] Furthermore, given that no isotopic enrichment was observed in the 4-nitrophenol product when the

Scheme 4



carboxylate groups in both **4a** and **5** were labeled, it is also possible to rule out a mechanism involving an intramolecular nucleophilic attack on the *ipso*-carbon that would result in formation of a transient 4-nitrophenyl ester (Scheme 4).

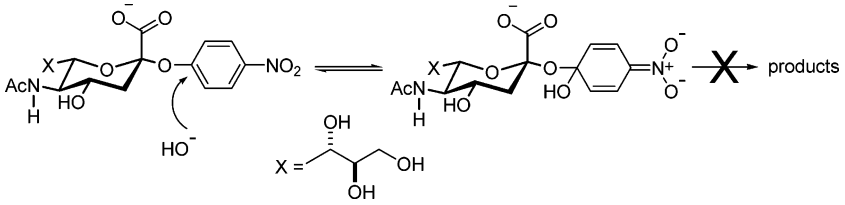
The likely cause for the large difference in the rate of spontaneous hydrolysis of **4a** and **5** is steric relief of ground state strain caused by the anomeric carboxylate group, an effect that is absent in the hydrolysis reactions of comparable acetals. Specifically, the rate ratios for the spontaneous hydrolyses of the two 4-nitrophenyl *trans*-decalin acetals **12** and **13** (3.6-fold at 39 °C[37,38]) and the two anomers of methyl glucopyranoside **14** and **15** (2.5-fold at 25 °C[39]) are considerably smaller than the value measured for the ketals **4a** and **5** (~110-fold at 50 °C). In these two cases, the axial acetal (**12** or **14**), which is the thermodynamically more stable diastereomer, reacts slower than the corresponding equatorial acetal (**13** or **15**). That is, replacement of an equatorial hydrogen atom by a carboxylate group (cf. **12** and **4a**) introduces less ground-state steric strain into the molecule than does the same substitution in an axial position (cf. **13** and **5**).



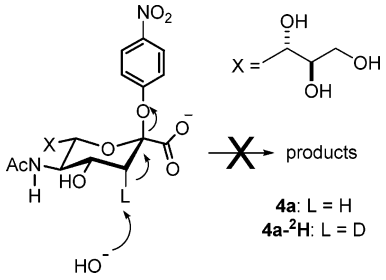
Another consequence of the greater ground state steric compression in these compounds is that the carboxylic acid group in β-*N*-acetylneuraminides is more acidic than the same group in α-*N*-acetylneuraminides; this presumably results from the greater ease of solvation of an equatorial carboxylate in comparison to an axial carboxylate. Thus, the calculated pK_a value of the equatorial carboxylic acid in **4a** is 1.58 (Table 1), a value that is 1.27 units more acidic than the corresponding ionization associated with the axial CO_2H group in **5**.[29] A similar trend in acidity constants has been reported for the cis- and trans-isomers of 4-*tert*-butyl cyclohexanecarboxylic acid, where in 50% aqueous ethanol, the equatorial carboxylic acid (trans) is more acidic than the axial isomer by 0.46 pK_a units.[40]

(40) Hoefnagel, A. J.; Wepster, B. M. *Recl. Trav. Chim. Pays-Bas* **1990**, *109*, 455−462.

Scheme 5



Scheme 6



Furthermore, the activation parameters for the spontaneous hydrolysis of **4a** and **5** are $\Delta H^{\ddagger} = 138 \pm 3$ kJ mol^{-1} and $\Delta S^{\ddagger} = 59 \pm 8$ J mol^{-1} K^{-1}, and $\Delta H^{\ddagger} = 116 \pm 2$ kJ mol^{-1} and $\Delta S^{\ddagger} = 27 \pm 4$ J mol^{-1} K^{-1}, respectively. The large positive activation entropies are consistent with these spontaneous hydrolysis reactions proceeding via dissociative transition states. Moreover, the more positive activation entropy for the β-anomer (**4a**) is consistent with the less sterically congested equatorial carboxylate being solvated to a greater extent in the ground state than is the case for the α-anomer (**5**), and that solvation of these two anionic groups becomes similar at their respective hydrolytic transition states.

Sialosyl Oxacarbenium Ion Lifetime. It is likely that the sialosyl oxacarbenium ion (**3**) possesses a lifetime close to that required for a cationic intermediate to become solvent-equilibrated for the following reasons: (1) both anomers solvolyze in 100% ethanol to give very similar products (Table 2), thus suggesting that these reactions proceed via similar oxacarbenium ion-like intermediates; and (2) more inverted product is obtained during the aqueous methanolysis of CMP β-D-*N*-acetylneuraminide (**1**),[24,25] a compound in which the leaving group is more basic than 4-nitrophenoxide. It is well-known that reactions of glycosides in which the leaving group is a nondelocalized anion, fluoride,[41,42] are more prone to react via an A_ND_N (S_N2) mechanism than are the corresponding spontaneous reactions when the leaving group is a resonance-stabilized anion, such as 2,4-nitrophenoxide.[43]

Given that intramolecular nucleophilic participation by the anomeric carboxylate group has been ruled out, the low *m* values obtained for the solvolyses of **4a** and **5** (−0.04 and +0.23, respectively) are consistent with the occurrence of charge delocalization at their individual zwitterionic transition states. Indeed, the interaction between positive and negative charges at the zwitterionic transition states is the probable origin for the observed low *m* values,[44] which are much lower than the recently reported value of +0.66 (Y_{OTs} scale) for the spontaneous solvolysis of 2-(4-nitrophenoxy)tetrahydropyran.[45]

Base-Promoted Reaction. The results from the ^{18}O-incorporation experiments (in ^{18}O-H_2O) rule out the possibility that the base-promoted reactions of **4a** and **5** occur by nucleophilic attack of hydroxide ion on the *ipso*-carbon of the 4-nitrophenyl ring (Scheme 5).

Indeed, the small amount of ^{18}O-exchange measured in the current system is consistent with hydroxide attacking the liberated 4-nitrophenoxide.[46] In addition, the possibility that the base-promoted reaction involves an E2 elimination mechanism is precluded, at least in the case of **4a**, because the observed deuterium KIE ($k_H/k_D = 1.097$) for this reaction is too small to be associated with a primary KIE for a standard a trans-diaxial elimination reaction (Scheme 6).

It appears that the base-promoted reactions of **4a** and **5** do not involve initial reaction at either the anomeric carbon or the *ipso*-carbon, but rather probably involve reaction at a remote center, as proposed by Sinnott and co-workers.[29]

Low pH Regime. In the low pH domain, the rate constants for hydrolysis of **4a** display an inflection point, which presumably is associated with protonation of its carboxylate group. The calculated pK_a value of this equatorial carboxylic acid is 1.58 (Table 1), a value that is 1.27 units more acidic than the corresponding ionization associated with the axial CO_2H group in **5**.[29] A similar trend in acidity constants has been reported for the cis- and trans-isomers of 4-*tert*-butyl cyclohexanecarboxylic acid, where in 50% aqueous ethanol, the equatorial carboxylic acid (trans) is more acidic than the axial isomer by 0.46 pK_a units.[40]

The second-order rate constant, k_H+, which refers to the acid-catalyzed hydrolysis of the neutral *N*-acetylneuraminide, is approximately 17-fold larger for the reactions of **5** than for the corresponding reactions of **4a** (Table 1). Of note, this ratio of second-order rate constants is larger than that normally associated with the specific acid-catalyzed hydrolysis of glycosides, an observation that is consistent with relief of additional steric crowding of the axial carboxylic acid group in the α-anomer (vide supra).

The kinetic process (k_0, Table 1) that is associated with either the acid-catalyzed reaction of the carboxylate form or the kinetic equivalent the spontaneous reaction of the neutral molecule is calculated to be faster for the β-anomer. However, in the pH rate profiles for both **4a** and **5**, it is evident that for both compounds, only small deviations from linearity are discernible, and this observation makes a thorough mechanistic analysis of this difference impracticable.

Biological Implications. Clearly, the natural leaving group for sialyl transferases, cytidine monophosphate (CMP), is a good

(41) Banait, N. S.; Jencks, W. P. *J. Am. Chem. Soc.* **1991**, *113*, 7951−7958.
(42) Zhang, Y.; Bommuswamy, J.; Sinnott, M. L. *J. Am. Chem. Soc.* **1994**, *116*, 7557−7563.
(43) Namchuk, M. N.; McCarter, J. D.; Becalski, A.; Andrews, T.; Withers, S. G. *J. Am. Chem. Soc.* **2000**, *122*, 1270−1277.
(44) The *m* values obtained in fits against Y_{OTs} for the solvolyses of **4a** and **5** are −0.02 and +0.11, respectively.
(45) Ahmad, I. A.; Birkby, S. L.; Bullen, C. A.; Groves, P. D.; Lankau, T.; Lee, W. H.; Maskill, H.; Miatt, P. C.; Menneer, I. D.; Shaw, K. *J. Phys. Org. Chem.* **2004**, *17*, 560−566.
(46) Hengge, A. C. *J. Am. Chem. Soc.* **1992**, *114*, 2747−2748.

leaving group. Given the above analysis, one would expect CMP α-D-*N*-acetylneuraminide to react about 100-fold faster than the natural substrate CMP β-D-*N*-acetylneuraminide (**1**), a compound for which the pseudo-first-order rate constant for hydrolysis (k_{hyd}) at pH values of 5.0, 6.0, and 7.0 is calculated to be 1.51×10^{-4}, 2.48×10^{-5}, and 1.15×10^{-5} s^{-1}, respectively.[24] Therefore, assuming a factor of 100 for the increased reactivity of the α-anomer relative to that of the natural β-anomer, the expected half-time ($t_{1/2}$) for the spontaneous hydrolysis of CMP α-D-*N*-acetylneuraminide at pH values of 5.0, 6.0, and 7.0 are around 0.75, 4.5, and 10 min, respectively. This time interval is likely too short to allow for efficient transport of CMP α-D-*N*-acetylneuraminide from where it would be synthesized, if it were the donor sugar instead of its β-anomer, (nucleus) to the site where sialyl transfer occurs (golgi).[47]

In addition, the rate constant for the spontaneous hydrolysis of CMP β-D-*N*-acetylneuraminide (**1**) at 37 °C is 1×10^{-5} s^{-1},[24] a value that is approximately 270 times larger than that extrapolated for 4-nitrophenyl β-D-*N*-acetylneuraminide (**4a**) of 3.7×10^{-8} s^{-1}. On the basis of the pK_a for CMP of 6.43[48] and the β_{lg} value for the spontaneous hydrolyses of aryl β-D-*N*-acetylneuraminide given above, it can be calculated that CMP β-D-*N*-acetylneuraminide would be expected to hydrolyze about 8−10-fold faster than **4a**. The discrepancy between the experimental and the calculated values likely results from an electrostatic destabilization of the CMP β-D-*N*-acetylneuraminide ground state that is induced by the close proximity of two negative charges.

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Supporting Information Available: Experimental details for the synthesis of aryl β-D-*N*-acetylneuraminides: 3-nitrophenyl (**4b**), 4-chlorophenyl (**4c**), 3-chlorophenyl (**4d**), phenyl (**4e**), 4-methylphenyl (**4f**), and 3-methylphenyl (**4g**). Tables of observed rate constants for the solvolysis reactions of 4-nitrophenyl α- and β-D-*N*-acetylneuraminide (**5** and **4a**). Also included are tables of observed rate constants for the hydrolysis reactions of the seven aryl β-D-*N*-acetylneuraminides at pH values of 1.00 and 8.08. This material is available free of charge via the Internet at http://pubs.acs.org.

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(47) Murrell, M. F.; Yarema, K. J.; Levchenko, A. *ChemBioChem* **2004**, *5*, 1334−1347.
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