Effect of Neutral Pyridine Leaving Groups on the Mechanisms of Influenza Type A Viral Sialidase-Catalyzed and Spontaneous Hydrolysis Reactions of α-D-*N*-Acetylneuraminides

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Abstract: A reagent panel, comprised of five pyridinium salts of α-D-*N*-acetylneuraminic acid, was synthesized and then used to probe enzymatic (α-sialidase) and nonenzymatic mechanisms of neuraminide hydrolysis. Spontaneous hydrolysis of the pyridinium salts proceeded via two independent pathways, where unassisted C−N bond cleavage was the rate-determining step. Cationic species (i.e., anomeric carboxylate protonated) displayed apparent pK_a values in the range of 0.4−0.7. However, spontaneous hydrolyses of the cationic and zwitterionic species had similar β_{lg} values of -1.22 ± 0.16 and -1.22 ± 0.07, respectively. The results, plus the activation parameters calculated from the hydrolysis of pyridinium α-D-*N*-acetylneuraminide ($\Delta H^{\ddagger} = 112 \pm 2$ kJ mol^{-1} and $\Delta S^{\ddagger} = 28 \pm 4$ J mol^{-1} K^{-1}), strongly suggest that the anomeric carboxylate does not assist in the departure of neutral pyridine leaving groups. Enzymatic hydrolysis was studied using an influenza viral α-sialidase (A/Tokyo/3/67) which was recombinantly expressed using a baculovirus/insect cell expression system. Sialidase protein was purified by a combination of density gradient centrifugation and gel filtration chromatography. Kinetic parameters for the enzymatic hydrolysis of the pyridinium salts were measured at 37 °C and at pH values of 6.0 and 9.5. The β_{lg} values derived for k_{cat}/K_m and k_{cat} were essentially zero, indicating that chemical transformations/events are not rate-determining. Rather, this observation is consistent with a model for α-sialidase-catalyzed hydrolyses (Guo, X.; Laver, W. G.; Vimr, E.; Sinnott, M. L. *J. Am. Chem. Soc.* **1994**, *116*, 5572) in which k_{cat}/K_m is determined by a conformational change of the first-formed Michaelis complex and k_{cat} is determined by the virtual transition state made up of two separate conformational events.

Introduction

This report addresses several critical questions concerning the spontaneous and enzyme-catalyzed hydrolysis reactions of *N*-acetylneuraminides. For example, does the C-1 carboxylate group of the *N*-acetylneuraminides have a catalytic function? Is chemistry or conformational change rate-determining in the enzyme-catalyzed hydrolysis of the *N*-acetylneuraminides?

Carbohydrates have roles in a variety of biologically important processes, including receptor/ligand recognition, intracellular trafficking, and immunity.[1] One of the most biologically important keto-sugars is the nine-carbon *N*-acetylneuraminic acid (also known as sialic acid).[2] *N*-Acetylneuraminides are structurally distinct from glucopyranosides in that they contain tertiary reaction centers (ketose vs aldose) and have an ionizable carboxylic acid group attached to the anomeric center.

Nucleophilic substitution reactions on aldopyranosides containing anionic leaving groups, such as fluoride, react with anionic nucleophiles[3] and water[4] via an "exploded" A_ND_N (S_N2) transition state (TS). Alternatively, aldopyranosides that contain neutral leaving groups react via short-lived nonsolvent equilibrated oxacarbenium ions ($D_N{*}A_N$).[5,6] In the latter case, nucleophilic attack occurs prior to the complete dissociation of the intermediate ion−molecule pair.[6] Such reactivities are a consequence of the short lifetime of the glucosyl oxacarbenium ion, estimated to be on the order of $(1-3) \times 10^{-12}$ s.[6,7]

In contrast to the body of work on aldopyranoside hydrolysis reactions, there are comparatively few mechanistic studies involving *N*-acetylneuraminides. Mechanistic studies have been reported for hydrolysis of aryl α-D-*N*-acetylneuraminides (**1**)[8] and CMP-β-D-*N*-acetylneuraminide (**2**).[9,10] The presence of an ionizable carboxylic acid group in the aryl α-*N*-acetylneuraminides increases the number of hydrolytic pathways relative

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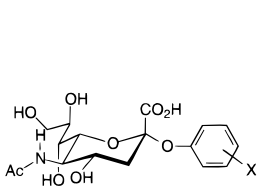

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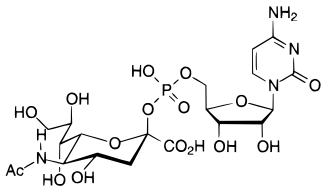

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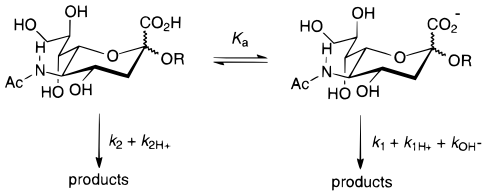
to aryl aldopyranosides. Potential reaction pathways are shown in Scheme 1 as follows: (a) spontaneous reaction of the carboxylate (k_1), (b) acid catalysis of the carboxylate (k_{1H^+}) or the kinetically equivalent spontaneous reaction of the carboxylic acid (k_2), (c) acid catalysis of the carboxylic acid (k_{2H^+}), and (d) base-catalyzed reaction of the carboxylate (k_{OH^-}). Notably, the existence of a second ionic functionality in CMP β-D-*N*-acetylneuraminide (**2**) further increases the number of potential hydrolytic pathways.

Conflicting evidence has been reported concerning the role of the carboxylate group during nonenzymatic hydrolysis reactions. Ashwell et al. proposed that the C-1 carboxylate group assists nucleophilically in both the general-acid-catalyzed departure of a phenol and the spontaneous departure of a phenolate anion during the hydrolysis reactions of aryl α-D-*N*-acetylneuraminides (**1**).[8] In contrast, Horenstein and co-workers propose that the carboxylate group is coplanar to the oxacarbenium ion at the transition state for hydrolysis of CMP-β-D-*N*-acetylneuraminide (i.e., nucleophilic participation cannot occur).[9,11]

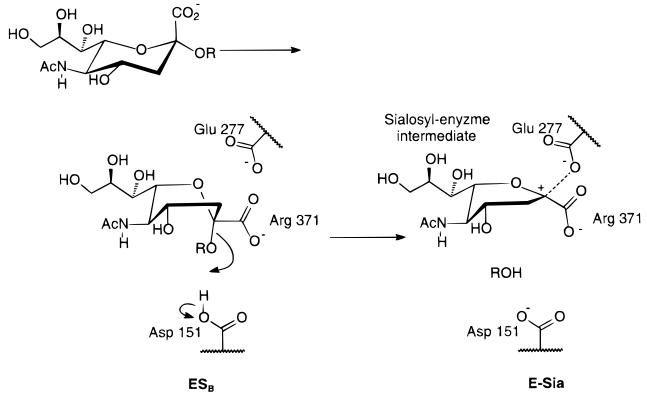
Analysis of enzyme-catalyzed reactions is naturally more complex than that of the corresponding solution-phase reactions. For example, there are two acidic residues (Asp/Glu) present in the active site of the majority of glycosidases; these groups participate during the hydrolysis of carbohydrates. In 1953, Koshland[12] proposed a mechanism for retaining glycosidases which is still generally accepted.[13] One of the two acidic amino acid residues acts as a general-acid catalyst, while the conjugate base of the second residue reacts as a nucleophile.

Sialidases (*N*-acetylneuraminyl glycohydrolase, EC 3.2.1.18) are retaining glycosidases that catalyze the hydrolysis of *N*-acetylneuraminic acid residues α-linked to glycoproteins, glycolipids, and polysaccharides.[14−16] Different hydrolytic mechanisms have been proposed for the sialidase family of enzymes, also involving two acidic active-site residues.[17,18] One mechanism, based largely on X-ray structure determinations on an influenza type A enzyme,[19,20] is proposed in which the α-*N*-acetylneuraminide is bound in a boat conformation (ES_B). Strong electrostatic binding occurs between the anionic carboxylate of the *N*-acetylneuraminide and an arginine residue on the enzyme.

Scheme 1



Scheme 2

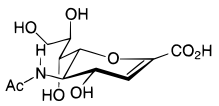


According to this mechanism, an aspartic acid residue then functions as a general-acid catalyst to assist departure of the aglycon (Scheme 2). Consistent with this role of the aspartic acid, evidence for proton transfer exists from a kinetic study on the influenza virus sialidase[21] and from a computational study.[22]

An alternative mechanism for enzyme-catalyzed hydrolysis has been proposed by von Itzstein and co-workers.[18] According to this proposal, water directly attacks an oxacarbenium ion via an internal return mechanism.

Notably, the catalytic roles of essential glutamate and tyrosine residues are not fully described,[23] though it appears that one or both residues stabilizes the transition state either electrostatically or nucleophilically. Thomas et al. concluded that an oxacarbenium ion is a potential intermediate in sialidase-catalyzed hydrolyses; their conclusion was based on a modeling study of the cation bound to the enzyme active site (B/Beijing/1/87).[24]

We have also initiated studies to unravel the complex nature of these hydrolyses. The current investigation simplifies the hydrolysis kinetics through the use of neutral leaving groups that obviate the need for acid catalysis. Five pyridinium α-D-*N*-acetylneuraminides were synthesized and their hydrolyses characterized in both enzymatic (influenza type A/Tokyo/3/67) and nonenzymatic systems.



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Materials and Methods

The buffers used in this study, 2-(*N*-morpholino)ethanesulfonic acid (MES), 3-(*N*-tris(hydroxymethyl)methyl)amino)propanesulfonic acid

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(TAPS), and 2-(*N*-cyclohexyamino)ethanesulfonic acid (CHES) were purchased from Sigma and used without further purification. Pyridine, 3-methoxypyridine, 3-methylpyridine, 4-methylpyridine, and 3,4-dimethylpyridine were purchased from Aldrich and used without further purification. *N*-Acetylneuraminic acid was purchased from Rose Scientific and used without further purification. Hydrochloric acid solutions were made by dilutions of a standard 1.00 M solution (Fischer Scientific). All other salts used in the hydrolysis studies were of analytical grade and were used without further purification. Milli-Q water (18.2 MΩ cm^{-1}) was used for kinetic experiments. NMR spectra were acquired on a Bruker AMX-400 spectrometer. The conditions used for HPLC purification were as follows: C-18 reverse-phase column, 25 × 10 mm, using a flow rate of 5 mL/min.

The protected β-D-*N*-acetylneuraminyl chloride (**5**),[25] (1-pyridinio)-acetate,[26] and 4-nitrophenyl *N*-acetylneuraminide[25a,27] were synthesized according to published procedures. Full experimental details for the synthesis of the parent pyridinium *N*-acetylneuraminide (**3b**) are given below, while the experimental particulars for the synthesis and characterization of **3a**,**c**,**d**,**e** are given in the Supporting Information.[28]

***N*-[Methyl (5-Acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosyl)onate]pyridinium Tetrafluoroborate (4b).** *N*-Acetylneuraminyl chloride **5** (300 mg, 0.59 mmol) and silver tetrafluoroborate (0.14 g, 0.71 mmol) were added with stirring to a cooled solution (−10 °C) of pyridine (1 mL) in anhydrous THF (8 mL). After the solution was stirred at 5 °C for 48 h, the solvent was evaporated under reduced pressure. Methanol (15 mL) was added to the residue, and the resulting mixture was sonicated for 10 min. After sonication the solution was filtered and the methanol removed under reduced pressure to give a pale yellow syrup. The crude product was purified by flash column chromatography (silica gel, 6:1 CH_2Cl_2/MeOH) to give a white solid (0.17 g, 45%). R_f = 0.49 (7:1 CH_2Cl_2/MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.90 (s, 3 H, CH_3), 1.93 (s, 3 H, CH_3), 2.06 (s, 3 H, CH_3), 2.16 (s, 3 H, CH_3), 2.18 (s, 3 H, CH_3), 2.45 (dd, 1 H, $J_{3a,3e}$ = 13.0 Hz, $J_{3a,4}$ = 9.0 Hz, H-3a), 3.77 (dd, 1 H, $J_{3e,3a}$ = 13.0 Hz, $J_{3e,4}$ = 4.6 Hz, H-3e), 3.85 (s, 3 H, OCH_3), 4.06 (dd, 1 H, $J_{9a,9b}$ = 12.0 Hz, $J_{9a,8}$ = 6.5 Hz, H-9a), 4.17 (q, 1 H, $J_{5,4} = J_{5,6} = J_{5,NH}$ = 10.0 Hz, H-5), 4.32 (dd, 1 H, $J_{9b,9a}$ = 12.0 Hz, $J_{9b,8}$ = 3.0 Hz, H-9b), 4.62 (dd, 1 H, $J_{6,7}$ = 2.0 Hz, $J_{6,5}$ = 11.0 Hz, H-6), 5.4−5.48 (m, 2 H, H-4, H-7), 5.51 (ddd, $J_{8,9a}$ = 6.5 Hz, $J_{8,9b}$ = 3.0 Hz, $J_{8,7}$ = 9.0 Hz, H-8), 6.44 (d, 1 H, $J_{NH,H5}$ = 10.0 Hz, NH), 8.28−8.34 (m, 2 H, Ar−H), 8.66−8.71 (m, 1 H, Ar−H), 9.30−9.34 (m, 2 H, Ar−H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.6, 20.8, 20.9, 21.0, 22.9 (CH_3), 36.0 (C3), 47.8 (C5), 55.4 (OCH_3), 62.5 (C9), 66.9 (C7), 67.5 (C8), 68.5 (C4), 94.2 (C2), 128.7 (Ar−C), 141.2 (Ar−C), 148.0 (Ar−C), 164.7, 169.6, 170.0, 170.6, 170.8, 170.9 (C1, C═O).

***N*-[(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosyl)onate]pyridinium (3b).** Compound **4b** (150 mg, 0.23 mmol) was dissolved in an ice-cold solution of sodium methoxide (10−15 equiv) in anhydrous methanol (5 mL). After the solution had stirred at 0 °C for 2 h, Dowex 50W HCR-W2 resin (H^+ form) was added to neutralized the solution. Following filtration the resin was washed twice with methanol. The combined filtrate and methanol washings were evaporated under reduced pressure, and the resulting residue was dissolved in aqueous 0.1 M NaOH solution (5 mL). After being stirred for 30 min at 0 °C, the mixture was neutralized with Dowex 50W HCR-W2 (H^+) resin, and the resin was removed by filtration. The resin was then washed with water, and the combined aqueous layers were evaporated to dryness under reduced pressure to give a pale yellow solid. The crude product was purified by HPLC using 1.0% (v/v) acetic acid in water as the eluent (retention time = 9.52 min). The combined fractions were collected and lyophilized to give a white solid (26 mg, 30%): $[\alpha]^{20}_D$ = −33.3 (*c* 0.36, H_2O). ^{1}H NMR (400 MHz, D_2O): δ 1.98 (t, 1 H, $J_{3a,3e} = J_{3a,4}$ = 12 Hz, H-3a), 2.32 (s, 3 H, CH_3), 2.36 (dd, 1 H, $J_{3e,3a}$ = 12 Hz, $J_{3e,4}$ = 4 Hz, H-3e), 3.61−3.68 (m, 2 H, H-7, H-9a), 3.85 (dd, 1 H, $J_{9b,9a}$ = 12 Hz, $J_{9b,8}$ = 3 Hz, H-9b), 3.94 (ddd, 1 H, $J_{8,7}$ = 10 Hz, $J_{8,9a}$ = 6 Hz, $J_{8,9b}$ = 3 Hz, H-8), 3.97−4.11 (m, 3 H, H-4, H-5, H-6), 8.08−8.12 (m, 2 H, Ar−H), 8.55−8.61 (m, 1 H, Ar−H), 9.10−9.15 (m, 2 H, Ar−H). ^{13}C NMR (100 MHz, D_2O): δ 24.8 (CH_3), 43.4 (C3), 53.9 (C5), 65.9 (C9), 70.6 (C7), 70.7 (C4), 73.7 (C8), 77.5 (C6), 98.2 (C2), 130.8, 142.5, 149.8 (Ar−C), 171.4 (C1), 177.8 (C═O). HRMS (EI): calcd for $C_{16}H_{23}N_2O_8$ [M + H^+], 371.1454; found, 371.1462.

Hydrolysis Kinetics. The hydrolysis reactions of **3a**−**e** were followed by monitoring absorbance versus time using a Cary-3E UV−vis spectrophotometer equipped with the Cary six-cell Peltier constant-temperature accessory. Hydrolysis reactions were initiated by injection of an aqueous stock solution of the pyridinium salt (25 μL, 45−60 mM) into a cuvette containing 2.0 mL of the required buffer that had been preequilibrated for 20 min at 65 °C.

At all [H_3O^+] the reactions of **3a** and **3c** were monitored at 298 and 275 nm, respectively, while the hydrolysis of **3b** was followed at 268 and 271 nm for reactions at pH values of >4.0 and <4.0, respectively. The hydrolysis reactions of **3d** were monitored at 229 and 265 nm for [H_3O^+] ≥ 0.2 M and [H_3O^+] < 0.2 M, respectively, and the reactions of **3e** were monitored at 235 and 272 nm for [H_3O^+] concentrations ≥0.1 M and <0.1 M, respectively. Clean isosbestic points were observed during the hydrolyses of both **3a** and **3b** at 260.0 and 289.5 nm, and 243.0 and 257.5 nm, respectively. Hydrolysis rate constants for reactions of **3a** and **3b** at all pH values, and those of **3c**−**e** at pH's greater than 4.0, were calculated by using a standard nonlinear least-squares fit of the absorbance versus time data (to 4 halftimes for hydrolysis). Hydrolyses of **3c**−**e** in acidic solutions ([H_3O^+] ≥ 0.01 M) were monitored using an initial rate methodology. Specifically, the change in absorbance was monitored until about 2−3% of the starting material had reacted. The reaction endpoint absorbance was measured, under identical reaction conditions, using an authentic mixture of *N*-acetylneuraminic acid and the corresponding pyridine. Rate constants were then calculated using a standard first-order rate expression.

Product Studies. The UV−vis spectrum (220−350 nm) of a completely hydrolyzed (>10 half-lives) sample of **3a**−**e** (5.0×10^{-5} M) in TAPS buffer (μ = 1.00, KCl) at 65 °C was identical within 0.010−0.025 au with the spectrum of a solution containing *N*-acetylneuraminic acid and the corresponding freshly distilled pyridine (5.0×10^{-5} M).

Identification of the carbohydrate-based hydrolysis product was accomplished by ^{1}H NMR analysis. Specifically, the *N*-acetylneuraminide was dissolved in phosphate buffer (10 mM, μ = 1.00 M KCl, pH 8.0), and the solution was then heated at 65 °C for a period of time corresponding to 10 half-lives for hydrolysis. The reaction mixture was then evaporated to dryness, and the resultant residue was subjected to two rounds of D_2O exchange (1.5 mL) followed by lyophilization to afford a colorless solid. The ^{1}H NMR spectrum of the hydrolysis product was identical to a spectrum of authentic *N*-acetylneuraminic acid.

The hydrolyses of **3a** and **3b** afford a small amount of an unidentified product (∼2%), as shown by the appearance of a broad singlet peak at 5.88 ppm in the ^{1}H NMR spectra. The hydrolyses of **3c**−**e** also give rise to a second minor product (∼5%), as shown by the appearance in the ^{1}H NMR spectra of a singlet at a chemical shift of 5.65. However, neither of these two minor products was the corresponding unsaturated sugar (i.e., glycal **6**) that would result from an elimination reaction.

Enzymatic Materials and Methods—Wild-Type Sialidase Gene. All restriction endonucleases and DNA modification enzymes were purchased from Gibco BRL or New England BioLabs Inc. (Beverly, MA). All DNA manipulations were carried out according to standard protocols.[29] The BS-TokNA plasmid, containing the wild-type sialidase gene, was a generous gift from Dr. G. Air (University of Oklahoma). As described by Lentz et al., the gene was cloned into the *Sal*I site of

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(28) We were unable to acquire a ^{13}C NMR spectrum for **3a** because of this compound's significant rate of hydrolysis at ambient temperatures. A consequence of this reactivity can be seen in the emergence of peaks corresponding to 3-methoxypyridine in the ^{1}H NMR spectrum (δ).

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the vector Bluescript KS (Stratagene, La Jolla, CA) in an orientation to allow expression from the T7 promoter.[30,31] The NA gene was excised by *Sal*I digestion from the BS-TokNA plasmid, gel-purified, and treated with T4 DNA polymerase to form blunt ends. The 1.5 kb fragment containing the NA gene was then ligated into *Sma*I-digested pUC19 (New England BioLabs Inc.) The new plasmid construct, pJW1, was propagated in *Escherichia coli* DH5α before further manipulation.[32]

Insertion of NA into pVL1392 Shuttle Vector. The sialidase gene was excised from pJW1 by *Eco*RI/*Bam*HI digestion. The 1.5 kb fragment was gel-purified and ligated into *Eco*RI/*Bam*HI-digested vector pVL1392 (PharMingen, San Diego, CA) to create the 11.1 kb plasmid pJW2. DNA sequence analysis (University of Calgary) using the dideoxy chain termination method[33] was used to verify accurate cloning and to ensure that spurious mutations had not occurred during DNA manipulation.

Cells and Viruses. *Trichoplusia ni* insect cells were cultured in EX-CELL 405 medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (JRH Biosciences) according to standard procedures.[34] Recombinant baculovirus stocks were produced by cotransfecting 5 μg of the pJW2 recombinant shuttle vector with 1 μg of BaculoGold AcNPV DNA (PharMingen). Single clones were isolated by plaque assays.[35] High-titer virus stocks were produced by three rounds of virus amplification[34] at a multiplicity of infection (MOI) of 0.1.

Expression of NA. *T. ni* cells (2×10^6 cells mL^{-1}) were infected with recombinant baculovirus at a MOI of 10 using serum-free medium, supplemented with 1% antibiotic/antimycotic (Gibco BRL). Infected cells were incubated at 27 °C in 1-L spinner flasks until levels of active NA had reached a plateau, typically 60−65 h postinfection.

Sialidase Assay. The fluorometric substrate 4-methylumbelliferyl α-D-*N*-acetylneuraminide (MUNANA) (Sigma Chemical Co.) was used to monitor sialidase activity. Based on the method of Potier et al.,[36] 1−10 μL of sample was typically used in a 50-μL reaction volume containing 200 mM NaAc, pH 6.0, 2 mM $CaCl_2$, and 0.4 mM MUNANA. Following a 30-min incubation at 37 °C, the enzymatic reaction was stopped by the addition of 250 μL of 133 mM glycine, pH 10.7, 60 mM NaCl, and 83 mM $NaHCO_3$. A standard curve for the measurement of product was prepared using 4-methylumbelliferone. The fluorescence of standards and unknown samples was determined using an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

Purification of Sialidase. *T. ni* cells were removed from the culture medium by centrifugation. Eight-hundred-milliliter volumes of medium were centrifuged at 10000*g* for 15 min. Recombinant virus particles (decorated with NA) were isolated from the culture supernatant by a second centrifugation at 40000*g* for 30 min at room temperature. Virus pellets were resuspended in 50 mL of buffer A (50 mM HEPES, pH 7.3, 150 mM NaCl, and 2 mM $CaCl_2$). Active sialidase heads were cleaved from the baculovirus particles by Pronase digestion as described by Laver,[37] using 0.05 mg/mL Pronase (Calbiochem, La Jolla, CA) at 37 °C for 16 h. Virus particles were separated from NA by centrifugation as before. To separate NA and Pronase, supernatants were loaded onto a 10−25% sucrose gradient and centrifuged in an SW28 rotor at 28 000 rpm for 29 h at 5 °C. Fractions containing NA but not containing Pronase were pooled, concentrated, and exchanged into buffer B (50 mM HEPES, pH 8, 150 mM NaCl, and 2 mM $CaCl_2$) using ultrafiltration (30 kDa cutoff, Amicon). Concentrated 1-mL samples were then loaded onto a Sephacryl-200-HR (Sigma Chemical Co.) gel filtration column, equilibrated with buffer B. The purity of the active fractions was assessed by SDS−PAGE and silver staining. Aliquots of the pure enzyme were then stored frozen at −80 °C until needed.

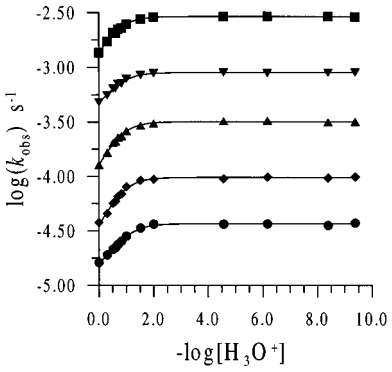
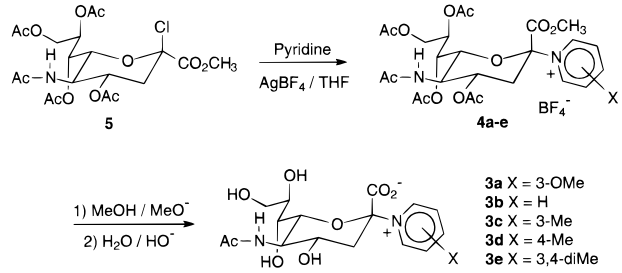


Figure 1. Plots of log(k_{obs}) versus −log [H_3O^+] for the hydrolysis of five α-D-*N*-acetylneuraminyl pyridinium salts, $T = 65$ °C: ■, **3a**; ▼, **3b**; ▲, **3c**; ◆, **3d**; and ●, **3e**. Error limits are encompassed within the symbol diameter. The included lines are the best nonlinear fits of the kinetic data to eq 1.

Scheme 3



Enzyme Kinetics. Michaelis−Menten parameters were measured by standardizing enzyme activity on 4-nitrophenyl α-D-*N*-acetylneuraminide to obtain relative k_{cat} values for the pyridinium *N*-acetylneuraminide substrates. Kinetic measurements at pH 6.0 were performed in 50 mM sodium acetate, 0.1 mM $CaCl_2$, 0.32 mM $MgCl_2$, and 60 mM NaCl.[21] Measurements at pH 9.5 were performed in 50 mM glycine, 0.1 mM $CaCl_2$, 0.32 mM $MgCl_2$, and 60 mM NaCl.[21] Initial rate measurements were made using a Varian Cary 3E UV−vis spectrophotometer equipped with a Peltier temperature controller. The progress of the reactions was continuously monitored for 10−20 min at 37 °C, and the derived initial velocities were corrected for background hydrolysis. Each 400-μL reaction was performed by equilibrating the buffer and substrate in the cell block for 3−5 min prior to the addition of enzyme (50 μL). Kinetic parameters were determined from eight initial rate measurements at substrate concentrations ranging from $K_m/3$ to $3K_m$. Extinction coefficient differences were calculated by measuring the absorbances of the pyridinium *N*-acetylneuraminide substrate and those of the products, *N*-acetylneuraminic acid and the corresponding pyridine. The kinetic rate data were fitted to the Michaelis−Menten equation using a standard nonlinear least-squares program (Grafit).

Results

The *N*-(α-D-*N*-acetylneuraminyl)pyridinium compounds were prepared from a readily made and fully protected β-D-*N*-acetylneuraminyl chloride (**5**)[25] according to the synthetic route outlined in Scheme 3. To some extent dehydrohalogenation of the tertiary β-D-*N*-acetylneuraminyl chloride (**5**) was anticipated, generating an α,β-unsaturated ester product. Nevertheless, the silver-promoted reaction of **5** with various pyridines generated the desired salts in reasonable yields (40−60%).

Spontaneous Hydrolysis. Figure 1 presents the observed rate constants (k_{obs}) obtained as a function of [H_3O^+] for the

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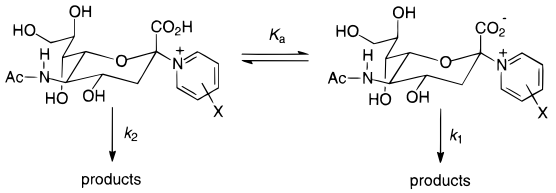
(31) Nuss, J. M.; Air, G. M.*Virology* **1991**, *183*, 496−504.
(32) A gift from Dr. R. Kay, Terry Fox Laboratories, Vancouver, Canada.
(33) Sanger, F.; Nicklen, S.; Coulson, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5463−5467.
(34) Summers, M. D.; Smith, G. E. Texas Agricultural Experiment Station Bulletin No. 1555, 1987.
(35) O'Reilly, D. R.; Miller, L. K.; Luckow, V. A. *Baculovirus Expression Vectors: A Laboratory Manual*; W. H. Freeman and Co.: New York, 1994.
(36) Potier, M.; Mameli, L.; Belisle, M.; Dallaire, L.; Melancon, S. B. *Anal. Biochem.* **1979**, *94*, 287−296.
(37) Laver, W. G. *Virology* **1978**, *86*, 78−87.

Scheme 4



hydrolyses of the five salts (**3a**−**e**). Individual rate constants are given in Table S1 of the Supporting Information. The observed rate constants for **3a**−**e** were fit to eq 1, where k_1 and k_2 are the first-order rate constants for the hydrolysis of the zwitterion and cation, respectively, and K_a is the apparent ionization constant of the carboxylic acid group (Scheme 4).

$$k_{obs} = \frac{k_1 K_a + k_2[H_3O^+]}{K_a + [H_3O^+]} \quad (1)$$

The observed rate constants were determined up to an acid concentration of 1.0 M; beyond this concentration of acid, (1) the ionic strength is no longer constant and (2) the sialic acid product becomes increasingly unstable, thus making it difficult to measure accurate kinetics. Therefore, the values for k_2 were extrapolated since the plateau at high $[H_3O^+]$ was not achieved. At one extreme k_2 is indistinguishable from zero and the observed fall-off in rate constant is caused solely by titration to an inactive form. To discount this possibility, the data were also fit to a model in which $k_2 = 0$ (eq 1). Shown in Figures S1−S5 (Supporting Information) are the residuals of the fits to the unconstrained and the constrained model ($k_2 = 0$). It is evident that the residuals are unevenly distributed about zero for the constrained fit. The data-fitting exercise allows us to conclude that there are two independent pathways for the hydrolysis of the pyridinium *N*-acetylneuraminides, and these pathways are reasonably described by the parameters (k_1, k_2, and pK_a) listed in Table 1. Also given in Table 1 are the pK_a's for the conjugate acids of the pyridine leaving groups.[38]

Figure 2 displays the Brønsted plots, log(k_1) and log(k_2) versus the pK_a of the conjugate acid of the leaving group for the spontaneous reactions of **3a**−**e**. The derived β_{lg} values for these reactions are -1.22 ± 0.07 and -1.22 ± 0.16 for the zwitterionic (k_1) and the cationic (k_2) forms, respectively.

Analysis of the hydrolytic products for **3a**−**e** showed that the major carbohydrate formed (≥95%) is *N*-acetylneuraminic acid, thus indicating that these hydrolysis reactions involve heterolytic C−N bond cleavages.

The rate of **3b** hydrolysis (Table S2, Supporting Information) was shown to be independent of acetate concentration; therefore, buffer catalysis is not a factor. The first-order rate constants for the hydrolysis of the zwitterionic form of **3b** were also measured as a function of temperature (Table S3, Supporting Information), and the corresponding Eyring plot is shown in Figure 3. The activation parameters derived for the spontaneous hydrolysis of **3b** are $\Delta H^{\ddagger} = 112 \pm 2$ kJ mol^{-1} and $\Delta S^{\ddagger} = 28 \pm 4$ J mol^{-1} K^{-1}.

Enzyme-Catalyzed Hydrolysis. Sialidase concentrations were normalized using a 4-nitrophenyl α-D-*N*-acetylneuraminide substrate, thereby making possible a direct comparison of the enzyme-catalyzed hydrolyses of pyridinium α-D-*N*-acetylneuraminides with the published values for aryl α-D-*N*-acetylneuraminides.[21] The kinetic parameters for viral sialidase-catalyzed hydrolyses are listed in Tables 2 and 3 in terms of relative k_{cat} and absolute K_m values.

The absolute values for k_{cat} and k_{cat}/K_m on the enzyme-catalyzed hydrolyses of pyridinium α-D-*N*-acetylneuraminides are shown in Figure 4. The assumption used in deriving these values is that the recombinant and nonrecombinant forms of the enzyme have the same absolute k_{cat} value.

Despite several attempts, it was not possible to derive kinetic parameters for the hydrolysis of 3,4-dimethylpyridinium (**3e**) at pH 6.0. Hydrolysis of this compound produced too small a change in absorbance ($\Delta\epsilon$, Table 2) to allow the reproducible measurement of α-sialidase-catalyzed kinetic parameters. Also, accurate parameters could not be derived for enzyme-catalyzed hydrolysis of **3a** because the rate of spontaneous hydrolysis was significantly faster than the rate of enzymatic hydrolysis. At a pH of 9.5, the β_{lg} values for sialidase-catalyzed hydrolyses of the series **3b**−**e** were $+0.17 \pm 0.27$ and 0.00 ± 0.12 for k_{cat} and k_{cat}/K_m, respectively.

Discussion

Aryl *N*-acetylneuraminides exist in different protonation states. Therefore, these carbohydrates are potentially hydrolyzed by different independent pathways (Scheme 1),[8] and consequently the catalytic function of the anomeric carboxylic acid group is obscured.

Typically, the accurate description of catalyzed and uncatalyzed reaction mechanisms necessitates kinetic analysis at many different pH's. However, substrates with positively charged leaving groups are able to simplify many aspects of the hydrolysis kinetics, because acid-catalyzed pathways are no longer relevant. Furthermore, such substrates may be used to dissect the catalytic machinery of sialidases by negating the catalytic role of one of the two active-site carboxylic acid residues.

Acidity of Pyridinium *N*-Acetylneuraminides. Although the calculated pK_a's for the cations of **3a**−**e** (Table 1) might appear to be low, these values are not unexpected. The measured pK_a of (1-pyridino)acetic acid ($Py^+CH_2CO_2H$) is 1.72 ± 0.01 (μ = 1, KCl; T = 65 °C). When this value is adjusted to account for the inductive effect of the ring oxygen atom (methoxyacetic acid is 1.18 pK_a units more acidic than acetic acid),[39] the pK_a for **3b** is expected to be ∼0.5 (μ = 1, KCl; T = 65 °C), which is in good agreement with the calculated value of 0.46 (Table 1).

Spontaneous Hydrolysis Reactions—Role of the C-1 Carboxylate Group. The observed rate constants for hydrolysis of pyridinium α-D-*N*-acetylneuraminides (**3a**−**e**) are consistent with spontaneous hydrolyses of both the zwitterion, k_1, and the cation, k_2; the pK_a of the cation is in the range of 0.4−0.7 (Scheme 4).

In principle, the carboxylic acid group could exert an effect on the reactivity of α-D-*N*-acetylneuraminides via an intramolecular nucleophilic attack to form a transient α-lactone.[8] However, the carboxylate group does not appear to play the role of a nucleophile during the C−N bond cleavage reactions for the following reasons: (1) the rate differences between the reactions of the carboxylate (k_1) and carboxylic acid (k_2) only differ by approximately a factor of 3 (Table 1), whereas, with the comparable acetate/acetic acid pair, a rate enhancement of at least 10^4-fold is anticipated;[41] (2) the β_{lg} values for the two processes (k_1 and k_2) are identical, and therefore consistent with late TSs having similar degrees of C−N bond cleavage, (i.e., both processes display characteristics

(38) Perrin, D. D. *Dissociation Constants of Organic Bases in Aqueous Solution*; Butterworth: London, 1965.

(39) pK_a(AcOH) = 4.75; pK_a($MeOCH_2CO_2H$) = 3.57, ref 40.

Table 1. Calculated Rate Constants for the Spontaneous Hydrolysis of the Zwitterionic (k_1) and Cationic (k_2) Forms of the Pyridinium Salts (**3a**−**e**) at 65 °C (μ = 1.0, KCl)

leaving group	pK_a (Py−H$^+$)[a]	10^5k_1 (s^{-1})	10^5k_2 (s^{-1})	pK_a (app)
3-methoxypyridine	4.91	291 ± 1	69 ± 9	0.36 ± 0.04
pyridine	5.22	89.8 ± 0.4	32.9 ± 2.4	0.46 ± 0.04
3-methylpyridine	5.63	31.8 ± 0.2	6.4 ± 1.0	0.47 ± 0.04
4-methylpyridine	5.98	9.79 ± 0.08	1.86 ± 0.37	0.55 ± 0.05
3,4-dimethylpyridine	6.46	3.68 ± 0.03	1.23 ± 0.10	0.74 ± 0.05

[a] Values taken from ref 38.

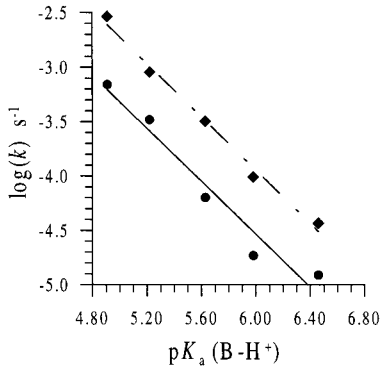


Figure 2. Brønsted plots for the hydrolyses of the five pyridinium α-D-*N*-acetylneuraminides, T = 65 °C: ◆ (k_1), hydrolysis of the zwitterion; and ● (k_2), hydrolysis of the cation. The included lines are the best linear fits to the data.

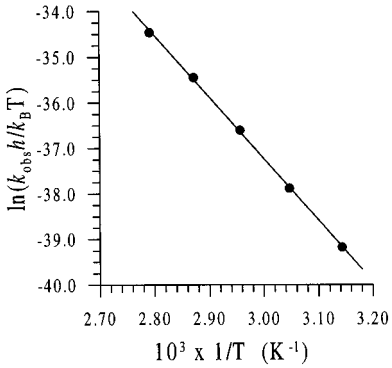


Figure 3. Eyring plot for the hydrolysis of the zwitterionic form of pyridinium α-D-*N*-acetylneuraminide.

Table 2. Michaelis−Menten Parameters for the Influenza Type A Sialidase-Catalyzed Hydrolysis of Pyridinium α-D-*N*-Acetylneuraminides, at pH 6.0 and 37 °C.

	$\Delta\epsilon$ (cm^{-1} M^{-1})	wavelength (nm)	rel k_{cat}[a]	K_m (μM)
3b	−3450	261	0.140 ± 0.007	72 ± 12
3c	−2600	275	0.0214± 0.0020	16 ± 6
3d	−3640	256	0.385 ± 0.022	234 ± 28
3e	−1400	275		

[a] Reported k_{cat} values are relative to that measured for 4-nitrophenyl α-D-*N*-acetylneuraminide, pH 6.0, at 37 °C.

of S_N1 reactions); and (3) the large positive $\Delta S^{\ddagger}$ value for the hydrolysis of the zwitterion (k_1) is not consistent with a highly ordered TS such as would be required for an intramolecular nucleophilic attack.

Lifetime of the *N*-Acetylneuraminyl Oxacarbenium Ion. The influence of the carboxylate group on the lifetime of an oxacarbenium ion can be estimated by a kinetic comparison of the *N*-acetylneuraminyl and the 2-deoxyglucopyranosyl systems.[6] Specifically, the uncatalyzed hydrolysis for the zwitterionic form of **3b** was compared to the 2-deoxyglucosyl isoquinolinium salt **7**. The comparison between **3b** and **7** is valid because (1) the pyridinium ring in both compounds is located in an equatorial position, (2) both compounds have ground-state chair conformations, (3) the pK_a's for the conjugate acids

Table 3. Michaelis−Menten Parameters for the Influenza Type A Sialidase-Catalyzed Hydrolysis of Pyridinium α-D-*N*-Acetylneuraminides, at pH 9.5 and 37 °C.

	$\Delta\epsilon$ (cm^{-1} M^{-1})	wavelength (nm)	rel k_{cat}[a]	K_m (μM)
3b	−3470	261	0.151 ± 0.012	216 ± 44
3c	−3610	275	0.0675 ± 0.0067	138 ± 37
3d	−3690	256	0.215 ± 0.023	450 ± 120
3e	−2180	264	0.178 ± 0.026	261 ± 87

[a] Reported k_{cat} values are relative to that measured for 4-nitrophenyl α-D-*N*-acetylneuraminide, pH 6.0, at 37 °C.

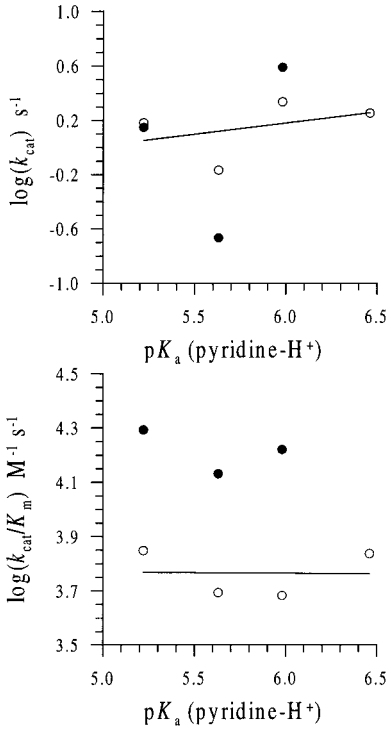
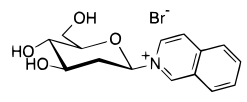


Figure 4. Plots of log(k_{cat}) and log(k_{cat}/K_m) versus pK_a(B−H$^+$) for α-sialidase-catalyzed hydrolysis of four pyridinium α-D-*N*-acetylneuraminides, pH 6.0 (●) and pH 9.5 (○), at T = 37 °C. The included lines are the best linear fits to the pH 9.5 data.



7

(40) Serjeant, E. P.; Dempsey, B. *Ionisation Constants of Organic Acids in Aqueous Solution*; Pergamon Press: Oxford, 1979.

(41) Acetic acid is about 100-fold less nucleophilic than water on the basis of N_{OTs} values, while water is about 500-fold less nucleophilic than acetate on the basis of Swain−Scott n values, ref 42.

of pyridine and isoquinoline are similar,[38] and (4) any structural differences are remote from the anomeric center.

In a previous study, Huang et al. correlated the lifetimes of the 2-deoxyglucosyl and the glucosyl cations with their rates of formation.[6a] To wit, a 680-fold change in reactivity was associated with a 4-fold change in cation lifetime.[6a] In the present study, addition of an anomeric CO_2^- group enhances the reactivity of the pyridinium salt by approximately 200-fold relative to that of the 2-deoxy compound **7**. This rate enhancement was estimated from the pH-independent rate constants for the hydrolyses of **7** ($\mu = 2.0$ M, $NaClO_4$)[6a] and **3b** ($\mu = 1.0$ M, KCl), adjusted by a factor of $^1/_3$ to account for the differences in ionic strength and added salt.[6a] Hence, the zwitterionic form of the *N*-acetylneuraminyl oxacarbenium ion is estimated to have a lifetime of approximately 3 times longer than that of the corresponding 2-deoxyglucosyl cation (i.e., 3×10^{-11} s).[6] This figure is in agreement with the estimate of $\geq 3 \times 10^{-11}$ s made by Horenstein and Bruner for the lifetime of the *N*-acetylneuraminyl oxacarbenium ion.[10]

The cationic form of **3b** reacts more rapidly than **7** despite the presence of the electron-withdrawing carboxylic acid group. For example, Amyes et al. noted that addition of an ester group in 4-methoxybenzyl derivatives caused a 40-fold decrease in rate.[43] The approximately 60-fold faster reaction of the protonated form of **3b** relative to that of **7** may be explained by a steric argument. Specifically, the ground state for the pyridinium *N*-acetylneuraminides contains severe 1,3-diaxial interactions that are relieved upon approach to the reaction TSs; therefore, in the current example, any electronic factors are outweighed.

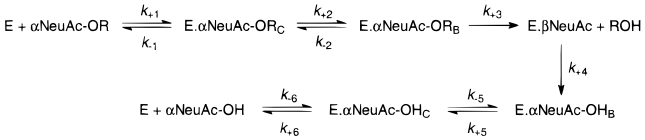
Influenza Virus Type A (Tokyo 3/67) α-Sialidase-Catalyzed Hydrolyses. A long-term objective is to produce a complete description of the enzyme-catalyzed hydrolysis of *N*-acetylneuraminides, dissecting the contributions to catalysis of individual residues in the sialidase active site. This objective will be accomplished by a combination of studies involving site-directed mutagenesis on a viral sialidase and the use of novel substrates.

Recombinant influenza virus sialidase was expressed using a baculovirus/insect cell system and then purified. The use of a recombinant enzyme will facilitate future studies of catalysis because the enzyme is amenable to site-directed mutagenesis. Purification of the recombinant enzyme was similar to that of the natural sialidase; however, it should be noted that the enzyme is a glycoprotein and the insect cell expression system does not glycosylate proteins in an identical manner to that of animal cells. A K_m value of 93 ± 13 μM was observed for the recombinant sialidase-catalyzed hydrolysis of 4-nitrophenyl α-D-*N*-acetylneuraminide. This value is lower than that reported for a similar enzyme isolated directly from a viral strain.[21] It is not known whether the difference reflects a strain-to-strain variation in the activity of the sialidases or rather reflects the difference in protein glycosylation.

In 1994, Guo et al. proposed a mechanistic scheme for sialidase-catalyzed hydrolyses which contains two important features.[21] First, the Michaelis complex undergoes a conformational change that results in a chair-to-boat transition of the substrate, and second a similar conformational change from a boat to a chair occurs for the product Michaelis complex (Scheme 5).

Guo et al. propose that a conjoint change of the initially formed enzyme−product complex limits k_{cat} for the sialidase-catalyzed hydrolyses of aryl α-D-*N*-acetylneuraminides (k_5, Scheme 5). However, both the initial conformational change (k_2) and cleavage of the glycosidic C−O bond (k_3) are partially rate-determining for k_{cat}/K_m in these sialidase-catalyzed reactions (Scheme 5).[21]

Scheme 5



As a first step in dissecting the individual roles of catalytic residues, hydrolysis of the pyridinium salts (**3a**−**e**) was studied. There is no evidence in the current study for rate-determining glycosidic C−N cleavage at either pH 6.0 or 9.5 (Figure 4). Given that the absolute values for k_{cat}/K_m are around 10^4 M^{-1} s^{-1}, it is unlikely that substrate binding is rate-determining for the catalyzed hydrolyses of **3a**−**e**. However, a rate-determining conformational change is consistent with previously observed rates for conformational changes on other enzyme systems.[44,45] In the current example a conjoint enzyme−substrate conformational change will result in the pyridinium moiety being placed in a thermodynamically unfavorable pseudoaxial orientation. Also, it is reasonable to expect that the conjoint change will be slower for the pyridinium substrates than for the aryl neuraminides. However, once the Michaelis complex completes its transition (k_2, Scheme 5), then C−N bond cleavage is facile and not rate-determining for k_{cat}/K_m. The results shown in Figure 4 for the correlation of k_{cat} and the pyridinium pK_a's suggest that chemistry is not rate-limiting; thus, it is proposed that k_{cat} is determined by a virtual transition state comprised of the two required conformational changes (k_2 and k_5, Scheme 5).

We conclude that there is a fine balance between the rates of conformational changes on the enzyme and the rate of C−X bond cleavage reactions. Therefore, subtle changes in leaving group chemistry can alter the various rate-determining steps.

Conclusions

In conclusion, the pyridinium α-D-*N*-acetylneuraminides effectively simplified interpretation of spontaneous and enzymatic hydrolysis kinetics. Therefore, spontaneous hydrolyses were observed to proceed via two independent pathways where C−N bond cleavage was unassisted and rate-determining. Moreover, the anomeric carboxylate does not nucleophilically assist in the departure of the neutral leaving group during hydrolysis of zwitterionic species, as previously conjectured.[8]

In the case of influenza α-sialidase, the pyridinium α-D-*N*-acetylneuraminides were used to show that k_{cat}/K_m is determined by a conformational change of the first-formed Michaelis complex, whereas k_{cat} is a virtual transition state made up of two separate conformational events.

Acknowledgment. The authors gratefully acknowledge the Natural Sciences and Engineering Research Council of Canada for financial support of this work. The authors also thank Dr. David McGillivray for acquiring the HRMS. J.N.W. thanks BC

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(44) Sawicki, C. A.; Gibson, Q. H. *J. Biol. Chem.* **1977**, *252*, 5783−5788.

(45) Eckfeldt, J.; Hammes, G. G.; Mohr, S. C.; Wu, C. *Biochemistry* **1970**, *9*, 3353−3362.

Science Council for receipt of a GREAT scholarship. We also thank Professor Derek Horton for his help with IUPAC nomenclature of carbohydrates.

Supporting Information Available: Experimental details for the synthesis of **3a**,**c**,**d**,**e**; ^{1}H NMR spectra for **3a**−**e**, and ^{13}C NMR spectra for **3b**−**e**; tables of observed rate constants for the hydrolysis reactions of **3a**−**e** at 65 °C, and **3b** as a function of both temperature and sodium acetate concentration; plots of the residuals from the constrained and unconstrained fits to eq 1 of the k_{obs} versus −log [H_3O^+] data for the hydrolyses of **3a**−**e** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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