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# Brønsted Analysis of an Enzyme-Catalyzed Pseudo-Deglycosylation Reaction: Mechanism of Desialylation in Sialidases[†]

Fahimeh S. Shidmoossavee, Lydia Cheng, Jacqueline N. Watson,[‡] and Andrew J. Bennet*

*Departments of Chemistry and Molecular Biology and Biochemistry, Simon Fraser University, 8888 University Drive, Burnaby, British Columbia V5A 1S6, Canada.* [‡]*Current address: Northern Catalyst Scientific Consulting, 76 North Star Dr., Whitehorse, Yukon, Y1A 0A3, Canada.*

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ABSTRACT: The *Micromonospora viridifaciens* Y370G inverting mutant sialidase has been found to possess $\beta$-sialidase activity with various fluoro-substituted phenyl $\beta$-sialosides. A reagent panel of seven mono- and difluorophenyl $\beta$-D-sialosides was synthesized, and these compounds were used, in conjunction with the parent phenyl $\beta$-D-sialoside, to probe the mechanism of *M. viridifaciens* Y370G mutant sialidase-catalyzed hydrolyses. These hydrolysis reactions mimic the deglycosylation reaction step of the crucial tyrosinyl enzyme-bound intermediate that is formed during the corresponding wild-type sialidase reactions. The derived Brønsted parameter ($\beta_{lg}$) on $k_{cat}/K_m$ is $-0.46 \pm 0.02$ for the four substrates that display significant activity, and these span a range of leaving group abilities (as judged by the p$K_a$ of their conjugate acids being between 7.09 and 9.87). The 4-fluoro, 2,3- and 2,5-difluorosubstrates display a diminished activity, whereas the 3,5-difluoro compound undergoes catalyzed hydrolysis exceedingly slowly. These observations, taken with solvent deuterium kinetic isotope effects ($k_{H_2O}/k_{D_2O}$) on the catalyzed hydrolysis of the 2-fluorophenyl substrate of $0.88 \pm 0.24$ ($k_{cat}/K_m$) and $1.16 \pm 0.12$ ($k_{cat}$) and the poor inhibition shown by phenol ($IC_{50} > 1$ mM), are consistent with glycosidic C−O cleavage being rate determining for both $k_{cat}/K_m$ and $k_{cat}$ with little or no protonation of the departing aryloxide leaving group. The kinetic data reported herein are consistent with rate-limiting glycoside hydrolysis occurring via two distinct transition states that incorporates a nonproductive binding component for the tighter binding substrates.

Glycosyl transferring enzymes are found ubiquitously in all kingdoms of life, and moreover, around 1−3% of the proteins encoded by a typical genome possess such activity (*1*). Glycosyl hydrolases (glycosidases) can be initially subdivided into two families: those in which the product of hydrolysis has either the same (retaining) or the opposite (inverting) configuration at the anomeric center (*2*). With regard to retaining glycosidases, three different mechanisms of action have been reported, and these involve (i) $NAD^+$ dependent oxidation-elimination (*3*); (ii) intramolecular neighboring group assisted catalysis from an acetamido group (*4*); and (iii) nucleophilic catalysis from an enzyme active site residue (*2*). For the latter mechanism, the nucleophile is normally either a glutamate or an aspartate residue (*5*). However, the *exo*-sialidases (*6*, *7*) and *trans*-sialidases (*8*, *9*) use a tyrosine residue as the nucleophile during the transfer of a sialic acid moiety from an α-sialoside onto either a water molecule or a carbohydrate acceptor, respectively. Scheme 1 shows the mechanism by which these two classes of enzymes catalyze the transfer reaction via a covalently linked $\beta$-sialosyl tyrosine intermediate ($\beta$-NeuAc-OTyr), a species that is subsequently attacked by an incoming ROH molecule to give the product, αNeuAc-OR (ROH being water for the hydrolases) (*6*, *7*, *10*).

Many of the traditional tools developed by physical organic chemists (*11*) have been adapted for use in probing the mechanisms of enzyme-catalyzed reactions (*12*). One of the more commonly used protocols for glycosidases and many other enzyme families involves measuring the changes in the enzymatic rate constants as the leaving group ability of the aglycone is varied. Usually, such experiments involve making a series of aryl glycosides and plotting the logarithm of the measured rate constant against the p$K_a$ of the conjugate acid of the leaving group; the slope of such a graph (Brønsted parameter $\beta_{lg}$) can give mechanistic information on the enzymatic glycosylation step. The complementary protocol that involves measuring rate constant changes as the nucleophilicity of the attacking group (Swain-Scott parameter *n*) is varied, an approach that has been used in physical organic studies on glycoside hydrolyses in solution as a means to probe for concerted ($S_N2$, $A_ND_N$) (*13*) or sequential ($S_N1$; $D_N{*}A_N$) (*14*) reactions, cannot be used for those enzyme-catalyzed reactions where the nucleophile is an enzymatic active site residue because of the limited number of substitutions that are possible with the current technology. A recent report detailed an in-depth study on a glycosidase that operates by the substrate-assisted mechanism; in this case, the intramolecular acetamido group's nucleophilicity was attenuated by the incorporation of fluorine atoms (*15*).

In theory, a second Brønsted-type analysis is possible for the deglycosylation reaction if one could vary the leaving group ability of the enzymatic nucleophile; however, to date this approach has not been possible due to the same factors mentioned above for varying the nucleophilicity of an enzymatic group to probe the mechanism of the glycosylation reaction. Previously, it has been reported that mutation of the active site nucleophilic tyrosine in the *M. viridifaciens* sialidase to small

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*To whom all correspondence should be addressed. Tel: (778)782-8814. Fax: (778)782-3765. E-mail: bennet@sfu.ca.


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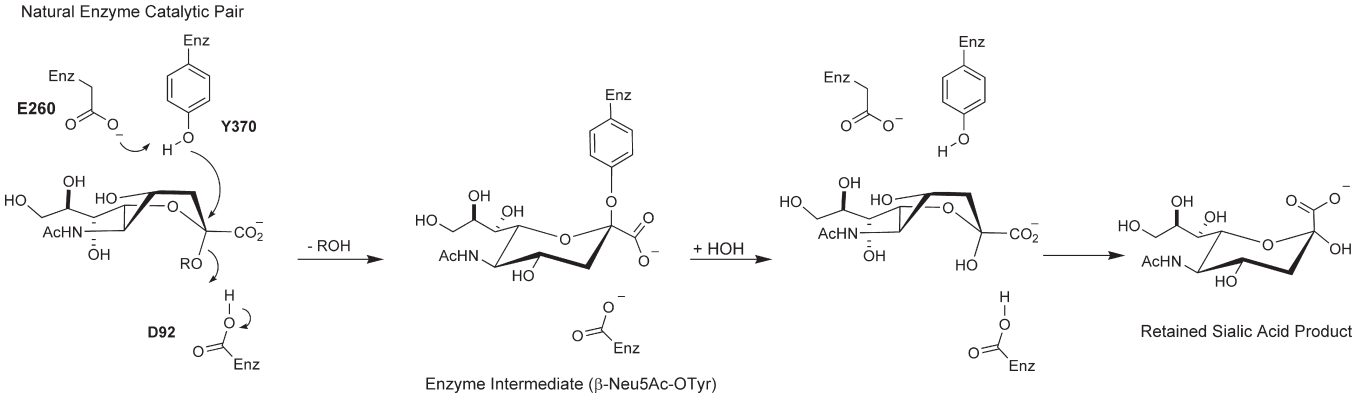
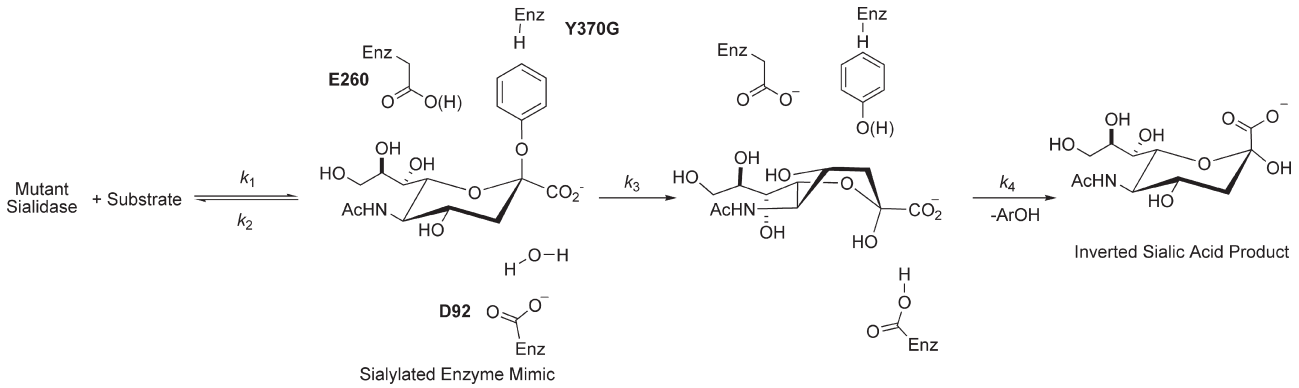
Scheme 1: Mechanism of Wild-Type Sialidase-Catalyzed Hydrolysis of α-Sialosides




Scheme 2: Proposed Mechanism of Y370G Mutant Sialidase-Catalyzed Hydrolysis of Aryl β-Sialosides




amino acid residues, such as glycine, aspartic acid, and serine, causes a change in mechanism from one that occurs with retention of configuration to one where the first-formed product has an inverted anomeric center (*6*, *16*, *17*). The Y370G mutant *M. viridifaciens* sialidase has been shown to be able to hydrolyze the non-natural phenyl β-D-sialoside (*18*), a reaction in which the Michaelis complex mimics the enzymatic tyrosinyl-bound intermediate that is formed in wild-type sialidase-catalyzed reactions (Scheme 2). The present study details a Brønsted-type mechanistic investigation into the Y370G mutant enzyme-catalyzed hydrolysis of fluoro-substituted aryl β-sialosides (**1a**−**h**), a reaction that mimics deglycosylation of the wild-type sialidase tyrosinyl enzyme intermediate.

## MATERIALS AND METHODS

*Materials*. All chemicals were of analytical grade or better and were purchased from Sigma-Aldrich unless noted otherwise.

*Substrate Synthesis*. Ph-βNeuAc[1] and fully protected β-sialosyl fluoride **4** were made according to literature procedures (*19*, *20*). NMR spectra were acquired on either a Bruker 600 or 400 MHz spectrometer. Chemical shifts are reported in parts per million downfield from the signal for TMS. The residual signal from deuterated chloroform and that from external TMS-salt ($D_2O$) were used as $^1$H NMR references; for $^{13}$C NMR spectra, natural abundance signals from $CDCl_3$ and external TMS-salt ($D_2O$) were used as references. Coupling constants (*J*) are given in hertz. Melting points were determined on a Gallenkamp melting-point apparatus and are not corrected. Optical rotations were measured on a Perkin-Elmer 341 polarimeter and are reported in units of deg cm$^2$ g$^{-1}$ (concentrations reported in units of g per 100 mL).

*Methyl* [*2-Fluorophenyl* (*5-Acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulopyranosyl*)]*onate* (***2b***). To activated 3 Å molecular sieves (5 g) was added peracetylated sialosyl fluoride **4** (400 mg, 0.81 mmol), and the mixture was dried under vacuum for 30 min. To this mixture, a solution of 2-fluorophenol (482 mg, 4.30 mmol) in dry $CH_2Cl_2$ (30 mL) was added under a $N_2$ atmosphere, and the resultant mixture was then stirred for 1 h. Following which a solution of $BF_3 \cdot OEt_2$ (0.7 mL, 5.8 mmol) in $CH_2Cl_2$ (4 mL) was added, and the resultant mixture was stirred overnight under a $N_2$ atmosphere at room temperature. The resultant mixture was filtered through Celite and 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 over anhydrous $Na_2SO_4$. After evaporating the solvent under reduced pressure, a yellow syrup was obtained, which crystallized from diethyl ether to give a white powder (187 mg, 40% yield). mp 167−168 °C; $[\alpha]^{20}_D = -57.6$ (0.17, $CH_2Cl_2$). $^1$H NMR (600 MHz, $CDCl_3$) δ: 1.79, 1.87, 2.02, 2.02, 2.13 (5 × s, 15 H, $CH_3$), 2.02 (m, H-3a), 2.71 (dd, 1 H, $J_{3e,3a} = 13.0$, $J_{3e,4} = 5.0$, H-3e), 3.70 (s, 3 H, $OCH_3$), 4.10 (dd, 1 H, $J_{9a,9b} = 12.4$, $J_{9a,8} = 6.6$, H-9a), 4.14 (dd, 1 H, $J_{6,5} = 10.7$, $J_{6,7} = 2.3$, H-6), 4.22 (q, 1 H, $J_{5,4} + J_{5,6} + J_{5,NH} = 31.1$, H-5), 4.61 (dd, 1 H, $J_{9b,9a} = 12.5$, $J_{9b,8} = 2.3$, H-9b), 4.92 (m, 1 H, H-8), 5.27 (d, 1 H, $J_{NH,5} = 10.5$,

[1]Abbreviations: 23DFP-βNeuAc, 2,3-difluorophenyl β-D-*N*-acetylneuraminide; 25DFP-βNeuAc, 2,5-difluorophenyl β-D-*N*-acetylneuraminide; 26DFP-βNeuAc, 2,6-difluorophenyl β-D-*N*-acetylneuraminide; 35DFP-βNeuAc, 3,5-difluorophenyl β-D-*N*-acetylneuraminide; 2FP-βNeuAc, 2-fluorophenyl β-D-*N*-acetylneuraminide; 3FP-βNeuAc, 3-fluorophenyl β-D-*N*-acetylneuraminide; 4FP-βNeuAc, 4-fluorophenyl β-D-*N*-acetylneuraminide; KIE, kinetic isotope effect; MvNA, *Micromonospora viridifaciens* neuraminidase; Neu2en5Ac, 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid; Neu5Ac, *N*-acetylneuraminic acid; Ph-βNeuAc, phenyl β-D-*N*-acetylneuraminide; pNP-αNeuAc, *p*-nitrophenyl α-D-*N*-acetylneuraminide.

NH), 5.36 (dd, 1 H, $J_{7,8}$ = 4.8, $J_{6,7}$ = 2.3, H-7), 5.45 (td, 1 H, $J_{4,3a}$ = $J_{4,5}$ = 11.1, $J_{3e,4}$ = 4.9, H-4), 6.91−7.01 (m, 3 H, H-4′, H-5′, H-6′), 7.08 (m, 1 H, H-3′). $^{13}$C NMR (150 MHz, $CDCl_3$) $\delta$: 20.80, 20.82 (2C), 20.90, 23.21 (5 × C, $CH_3$), 37.97 (C-3), 49.21 (C-5), 53.22 ($OCH_3$), 62.09 (C-9), 67.92 (C-7), 68.42 (C-4), 71.67 (C-8), 72.72 (C-6), 100.02 (C-2), 116.85 (d, $J_{C,F}$ = 18.5, C-3′), 118.47 (C-5′), 124.02 (d, $J_{C,F}$ = 7.0, C-4′), 124.54 (d, $J_{C,F}$ = 3.9, C-6′), 141.65 (d, $J_{C,F}$ = 10.7, C-1′), 152.99 (d, $J_{C,F}$ = 247.4, C-2′), 166.85 (C-1), 170.07, 170.34, 170.45, 170.53, 170.99 (5 × C═O). Anal. Calcd for $C_{26}H_{32}FNO_{13}$: C, 53.33; H, 5.51; N, 2.39. Found: C, 53.06; H, 5.45; N, 2.22.

*Methyl* [*3-Fluorophenyl* (*5-Acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-*D*-glycero-β-*D*-galacto-non-2-ulopyranosyl*)]*onate* (**2c**). A mixture of activated 3 Å molecular sieves (5 g) and peracetylated sialosyl fluoride **4** (400 mg, 0.81 mmol) was dried under vacuum for 30 min. To the resultant solid, 3-fluorophenol (482 mg, 4.30 mmol) and dry $CH_2Cl_2$ (30 mL) were added under a $N_2$ atmosphere, and the mixture was then stirred for 1 h at rt. Then, $BF_3 \cdot OEt_2$ (0.7 mL, 5.80 mmol) in $CH_2Cl_2$ (4 mL) was added, and the mixture was stirred overnight under a $N_2$ atmosphere at room temperature. The resultant mixture was filtered through Celite and 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 over anhydrous $Na_2SO_4$. After evaporating the solvent under reduced pressure, a yellow syrup was obtained, which crystallized from diethyl ether to give a white powder (280 mg, 60% yield). mp 194−195 °C; $[\alpha]^{20}_D$ = −38.5 (0.12, $CH_2Cl_2$). $^1$H NMR (600 MHz, $CDCl_3$) $\delta$: 1.77, 1.87, 2.03, 2.04, 2.14 (5 × s, 15 H, $CH_3$), 1.98 (t, 1 H, $J_{3a,3e}$ = $J_{3a,4}$ = 11.7, H-3a), 2.62 (dd, 1 H, $J_{3e,3a}$ = 13.0, $J_{3e,4}$ = 5.0, H-3e), 3.73 (s, 3 H, $OCH_3$), 4.04 (dd, 1 H, $J_{6,5}$ = 10.7, $J_{6,7}$ = 2.4, H-6), 4.12 (dd, 1 H, $J_{9a,9b}$ = 12.5, $J_{9a,8}$ = 6.6, H-9a), 4.20 (q, 1 H, $J_{5,4}$ + $J_{5,6}$ + $J_{5,NH}$ = 31.4, H-5), 4.66 (dd, 1 H, $J_{9b,9a}$ = 12.6, $J_{9b,8}$ = 2.4, H-9b), 4.90 (m, H-8), 5.23 (d, 1 H, $J_{NH,5}$ = 10.2, NH), 5.34 (dd, 1 H, $J_{7,8}$ = 4.6, $J_{6,7}$ = 2.3, H-7), 5.44 (td, 1 H, $J_{4,3a}$ = $J_{4,5}$ = 11.1, $J_{4,3e}$ = 4.9, H-4), 6.66−6.77 (m, 3 H, H-2′, H-4′, H-6′), 7.18 (m, 1 H, H-5′). $^{13}$C NMR (150 MHz, $CDCl_3$) $\delta$: 20.66, 20.70, 20.76, 20.87, 23.17 (5 × C, $CH_3$), 38.44 (C-3), 49.16 (C-5), 53.28 ($OCH_3$), 62.01 (C-9), 67.93 (C-7), 68.30 (C-4), 71.88 (C-8), 72.59 (C-6), 99.22 (C-2), 104.50 (d, $J_{C,F}$ = 25.4, C-2′), 109.99 (d, $J_{C,F}$ = 21.1, C-4′), 112.34 (d, $J_{C,F}$ = 2.9, C-6′), 130.79 (d, $J_{C,F}$ = 9.8, C-5′), 154.90 (d, $J_{C,F}$ = 10.8, C-1′), 163.16 (d, $J_{C,F}$ = 247.7, C-3′′), 167.00 (C-1), 170.07, 170.23, 170.45, 170.65, 171.98 (5 × C═O). Anal. Calcd for $C_{26}H_{32}FNO_{13}$: C, 53.33; H, 5.51; N, 2.39. Found: C, 53.26; H, 5.66; N, 2.17.

*Methyl* [*4-Fluorophenyl* (*5-Acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-*D*-glycero-β-*D*-galacto-non-2-ulopyranosyl*)]*onate* (**2d**). To activated 3 Å molecular sieves (5 g) was added peracetylated sialosyl fluoride **4** (400 mg, 0.81 mmol), and the mixture was dried under vacuum for 30 min. To the resultant mixture was added 4-fluorophenol (482 mg, 4.3 mmol) and dry $CH_2Cl_2$ (30 mL) under $N_2$ atmosphere, and the mixture was then stirred for 1 h. Then, $BF_3 \cdot OEt_2$ (0.7 mL, 5.8 mmol) in $CH_2Cl_2$ (4 mL) was added, and the mixture was stirred overnight under $N_2$ atmosphere at room temperature. The resultant mixture was filtered through Celite and 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 over anhydrous $Na_2SO_4$. After evaporating the solvent under reduced pressure, a yellow syrup was obtained, which crystallized from diethyl ether to give a white powder (234 mg, 50% yield). mp 202−203 °C; $[\alpha]^{20}_D$ = −45.2 (0.22, $CH_2Cl_2$). $^1$H NMR (600 MHz, $CDCl_3$) $\delta$: 1.77, 1.87, 1.96, 2.03, 2.14 (5 × s, 15 H, $CH_3$), 1.96 (dd, 1 H, $J_{3a,3e}$ = 13.0, $J_{3a,4}$ = 11.5, H-3a), 2.63 (dd, 1 H, $J_{3e,3a}$ = 13.0, $J_{3e,4}$ = 4.9, H-3e), 3.68 (s, 3 H, $OCH_3$), 4.08 (dd, 1 H, $J_{6,5}$ = 10.7, $J_{6,7}$ = 2.3, H-6), 4.12 (dd, 1 H, $J_{9a,9b}$ = 12.6, $J_{9a,8}$ = 6.4, H-9a), 4.18 (q, 1 H, $J_{5,4}$ + $J_{5,6}$ + $J_{5,NH}$ = 31.1, H-5), 4.62 (dd, 1 H, $J_{9b,9a}$ = 12.5, $J_{9b,8}$ = 2.4, H-9b), 4.94 (ddd, 1 H, $J_{8,9a}$ = 6.4, $J_{8,7}$ = 5.0, $J_{8,9b}$ = 2.4, H-8), 5.22 (d, 1 H, $J_{NH,5}$ = 10.1, NH), 5.36 (dd, 1 H, $J_{7,8}$ = 5.0, $J_{6,7}$ = 2.3, H-7), 5.45 (m, 1 H, H-4), 6.85 (m, 2 H, H-2′, H-6′), 6.87 (m, 2 H, H-3′, H-5′). $^{13}$C NMR (150 MHz, $CDCl_3$) $\delta$: 20.94, 21.00, 21.01, 21.12, 23.43 (5 × C, $CH_3$), 38.67 (C-3), 49.49 (C-5), 53.37 ($OCH_3$), 62.15 (C-9), 68.10 (C-7), 68.61 (C-4), 71.80 (C-8), 72.51 (C-6), 99.63 (C-2), 116.49 (d, $J_{C,F}$ = 22.9, C-3′, C-5′), 118.40 (d, $J_{C,F}$ = 8.2, C-2′, C-6′), 150.14 (s, C-1′), 158.66 (d, $J_{C,F}$ = 242.1, C-4′) 167.30 (C-1), 170.27, 170.45, 170.71, 171.23 (5 × C═O). Anal. Calcd for $C_{26}H_{32}FNO_{13}$: C, 53.33; H, 5.51; N, 2.39. Found: C, 53.09; H, 5.49; N, 2.24.

*Methyl* [*2,5-Difluorophenyl* (*5-Acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-*D*-glycero-β-*D*-galacto-non-2-ulopyranosyl*)]*onate* (**2f**). To activated 3 Å molecular sieves (5 g) was added peracetylated sialosyl fluoride **4** (400 mg, 0.81 mmol), and the resulting solid mixture was dried under vacuum for 30 min. To this mixture, a solution of 2,5-difluorophenol (482 mg, 4.30 mmol) in dry $CH_2Cl_2$ (30 mL) was added under a $N_2$ atmosphere, and the mixture was then stirred for 1 h. Then, $BF_3 \cdot OEt_2$ (0.7 mL, 5.8 mmol) in $CH_2Cl_2$ (4 mL) was added, and the mixture was stirred overnight under a $N_2$ atmosphere at room temperature. The resultant mixture was filtered through Celite and 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 this solution was dried over anhydrous $Na_2SO_4$. After evaporating the solvent under reduced pressure, a yellow syrup was obtained, which crystallized from diethyl ether to give a white powder (157 mg, 33% yield). mp 167−168 °C; $[\alpha]^{20}_D$ = −44.0 (0.10, $CH_2Cl_2$). $^1$H NMR (600 MHz, $CDCl_3$) $\delta$: 1.86, 1.92, 2.02, 2.03, 2.14 (5 × s, 15 H, $CH_3$), 2.03 (dd, 1 H, $J_{3a,3e}$ = 13.1, $J_{3a,4}$ = 11.5, H-3a), 2.71 (dd, 1 H, $J_{3e,3a}$ = 13.3, $J_{3e,4}$ = 5.0, H-3e), 3.77 (s, 3 H, $OCH_3$), 4.07 (m, 2 H, H-6 and H-9a), 4.24 (q, 1 H, $J_{5,4}$ + $J_{5,6}$ + $J_{5,NH}$ = 31.3, H-5), 4.67 (dd, 1 H, $J_{9b,9a}$ = 12.5, $J_{9b,8}$ = 2.3, H-9b), 4.84 (m, 1 H, H-8), 5.31 (d, 1 H, $J_{NH,5}$ = 10.3, NH), 5.32 (dd, 1 H, $J_{7,8}$ = 6.1, $J_{6,7}$ = 2.5, H-7), 5.45 (td, 1 H, $J_{4,3e}$ = 5.0, $J_{4,3a}$ = $J_{4,5}$ = 11.3, H-4), 6.70 (m, 1 H, H-4′), 6.77 (m, 1 H, H-6′), 7.06 (m, 1 H, H-3′). $^{13}$C NMR (150 MHz, $CDCl_3$) $\delta$: 21.08, 21.17, 21.20, 21.27, 23.55 (5 × C, $CH_3$), 38.29 (C-3), 49.46 (C-5), 53.87 ($OCH_3$), 62.36 (C-9), 68.55 (C-7), 68.70 (C-4), 72.87 (C-8), 73.81 (C-6), 100.47 (C-2), 106.45 (d, $J_{C,F}$ = 25.1, C-6′), 110.10 (dd, $J_{C,F}$ = 24.4 and 7.1, C-4′), 117.50 (dd, $J_{C,F}$ = 21.1, $J_{C,F}$ = 9.8, C-3′), 142.24 (m, C-1′), 149.65 (dd, $J_{C,F}$ = 243.9 and 3.2, C-2′), 158.45 (dd, $J_{C,F}$ = 244.3 and 2.3, C-5′), 166.91 (C-1), 170.53, 170.76, 171.14, 171.16, 171.35 (5 × C═O). Anal. Calcd for $C_{26}H_{31}F_2NO_{13}$: C, 51.74; H, 5.18; N, 2.32. Found: C, 51.56; H, 5.13; N, 2.09.

*Methyl* [*3,5-Difluorophenyl* (*5-Acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-*D*-glycero-β-*D*-galacto-non-2-ulopyranosyl*)]*onate* (**2h**). To activated 3 Å molecular sieves (5 g) was added peracetylated sialosyl fluoride **4** (400 mg, 0.81 mmol), and the mixture was dried under vacuum for 30 min. To the resulting mixture, 3,5-difluorophenol (560 mg, 4.30 mmol) and dry $CH_2Cl_2$ (30 mL) were added under a $N_2$ atmosphere, and the mixture was then stirred for 1 h. Subsequently, a solution of $BF_3 \cdot OEt_2$ (0.7 mL, 5.81 mmol) in $CH_2Cl_2$ (4 mL) was added, and the mixture was stirred overnight under $N_2$ atmosphere at room temperature. The resultant mixture was filtered through Celite and 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 over anhydrous $Na_2SO_4$. After removal of the solvent under reduced pressure, a yellow syrup was obtained, and this material was crystallized from diethyl ether to give a white powder (241 mg, 50% yield). mp 196−197 °C; $[\alpha]^{20}_D = -44.2$ (0.13, $CH_2Cl_2$). $^1H$ NMR (600 MHz, $CDCl_3$) $\delta$: 1.87, 1.88, 2.03, 2.04, 2.14 (5 × s, 15 H, $CH_3$), 1.98 (dd, 1 H, $J_{3a,3e} = 13.0$, $J_{3a,4} = 11.5$, H-3a), 2.62 (dd, 1H, $J_{3e,3a} = 13.1$, $J_{3e,4} = 5.0$, H-3e), 3.76 (s, 3 H, $OCH_3$), 4.02 (dd, 1 H, $J_{6,5} = 10.6$, $J_{6,7} = 2.4$, H-6), 4.10 (dd, 1 H, $J_{9a,9b} = 12.5$, $J_{9a,8} = 7.3$, H-9a), 4.20 (q, 1 H, $J_{5,4} + J_{5,6} + J_{5,NH} = 31.4$, H-5), 4.69 (dd, 1 H, $J_{9b,9a} = 12.6$, $J_{9b,8} = 2.3$, H-9b), 4.88 (m, 1 H, H-8), 5.24 (d, 1 H, $J_{NH,5} = 10.2$, NH), 5.33 (dd, 1 H, $J_{7,8} = 3.6$, $J_{6,7} = 2.4$, H-7), 5.39 (td, 1 H, $J_{4,3a} = J_{4,5} = 11.2$, $J_{4,3e} = 4.9$, H-4), 6.48−6.56 (m, 3 H, Ar−H). $^{13}C$ NMR (150 MHz, $CDCl_3$) $\delta$: 20.87, 20.93, 20.97, 21.08, 23.39 (5 × C, $CH_3$), 38.54 (C-3), 49.31 (C-5), 53.70 ($OCH_3$), 62.43 (C-9), 68.27 (C-7), 68.39 (C-4), 72.60 (C-8), 73.32 (C-6), 99.08 (t, $J_{C,F} = 25.2$, C-4′), 99.70 (C-2), 100.86 (m, C-2′ and C-6′), 155.58 (C-1′), 163.32 (dd, $J_{C,F} = 248.6$ and 15.7, C-3′ and C-5′), 166.84 (C-1), 170.32, 170.45, 170.82, 170.96, 171.16 (5 × C═O). Anal. Calcd for $C_{26}H_{31}F_2NO_{13}$: C, 51.74; H, 5.18; N, 2.32. Found: C, 51.38; H, 5.36; N, 2.33.

*Methyl* [*2,3-Difluorophenyl* (*5-Acetamido-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulopyranosyl*)]*onate* (***3e***). A mixture of activated 3 Å molecular sieves (12 g) and peracetylated sialosyl fluoride **4** (1.0 g, 2.02 mmol) was dried under vacuum for 30 min. To the resulting solid, 2,3-difluorophenol (1.398 g, 10.75 mmol) and dry $CH_2Cl_2$ (75 mL) were added under a $N_2$ atmosphere, and the mixture was then stirred for 1 h at rt. Then, $BF_3 \cdot OEt_2$ (1.75 mL, 14.5 mmol) in $CH_2Cl_2$ (10 mL) was added, and the mixture was stirred overnight under a $N_2$ atmosphere at room temperature. The resultant mixture was filtered through Celite and washed thoroughly with $CH_2Cl_2$. The combined filtrates were washed with saturated $NaHCO_3$ (375 mL), water (375 mL), and brine (375 mL), and the resulting solution was dried over anhydrous $Na_2SO_4$. After evaporating the solvent under reduced pressure, a yellow syrup containing 26% of Neu2en5Ac was obtained. The syrup was dissolved in dry methanol (60 mL), and a methanolic sodium methoxide solution (22 mL, 11 mmol) was then added at 0 °C. The resulting solution was stirred at 0 °C for 30 min. Subsequently, the solution was then neutralized by the addition of Amberlite IR-120 resin ($H^+$ form). After the removal of the resin, which was washed several times with methanol, the combined solutions were concentrated under reduced pressure to give a yellow syrup that was purified by flash-column chromatography using ethyl acetate/methanol/water (10:3:1 v/v/v). The fractions containing the product were combined and concentrated to give a solid residue (371 mg, 42% yield); mp 71−72 °C; $[\alpha]^{20}_D = -41.7$ (*c* 0.29, $H_2O$). $^1H$ NMR (400 MHz, $D_2O$) $\delta$: 1.85 (dd, 1 H, $J_{3a,3e} = 13.2$ $J_{3a,4} = 11.4$, H-3a), 2.07 (s, 3 H, $CH_3$), 2.73 (dd, 1 H, $J_{3e,3a} = 13.3$, $J_{3e,4} = 4.9$, H-3e), 3.55 (d, 1 H, $J_{7,8} = 9.2$, H-7), 3.67 (m, 2 H, H-9a and H-8), 3.77 (dd, 1 H, $J_{9b,9a} = 11.51$, $J_{9b,8} = 2.13$ H-9b.), 3.82 (s, 3 H, $OCH_3$), 3.93 (d, 1 H, $J_{5,6} = 10.64$, H-6), 4.06 (t, 1 H, $J_{5,6} = J_{5,4} = 10.33$, H-5), 4.34 (td, 1 H, $J_{4,3e} = 4.89$, $J_{4,3a} = J_{4,5} = 11.1$, H-4), 7.02 (m, 3 H, H-4′, H-5′, H-6′). $^{13}C$ NMR (100 MHz, $D_2O$) $\delta$: 22.09 ($CH_3$), 39.87 (C-3), 51.48 (C-5), 54.03 (OCH3), 63.02 (C-9), 65.96 (C-4), 67.77 (C-7), 69.81 (C-8), 71.92 (C-6), 100.37 (C-2), 100.14 (d, $J_{C,F} = 17.46$, C-6′), 113.35 (d, $J_{C,F} = 3.33$, C-5′), 123.35 (dd, $J_{C,F} = 8.4$ and 5.10, C-4′), 141.52 (dd, $J_{C,F} = 244.16$ and $J_{C,F} = 14.69$, C-2′), 142.55 (dd, $J_{C,F} = 7.87$ and $J_{C,F} = 2.86$, C-1′), 151.04 (dd, $J_{C,F} = 245.37$ and $J_{C,F} = 10.92$, C-3′), 169.57, 174.89 (C═O, C-1). HRMS-FAB (*m*/*z*): [M + $H^+$] calcd for $C_{18}H_{24}NO_9F_2$, 436.1419; found, 436.1410.

*Methyl* [*2,6-Difluorophenyl* (*5-Acetamido-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulopyranosyl*)]*onate* (***3g***). To activated 3 Å molecular sieves (12 g), peracetylated sialosyl fluoride **4** (1.0 g, 2.02 mmol) was added, and the resulting mixture was dried under vacuum for 30 min. To this mixture, 2,6-difluorophenol (1.4 g, 10.76 mmol) and dry $CH_2Cl_2$ (75 mL) were added under a $N_2$ atmosphere, and the mixture was then stirred for 1 h at rt. Then, $BF_3 \cdot OEt_2$ (1.75 mL, 14.5 mmol) in $CH_2Cl_2$ (10 mL) was added, and the mixture was stirred overnight under a $N_2$ atmosphere at room temperature. The resultant mixture was filtered through Celite and washed thoroughly with $CH_2Cl_2$. The combined filtrates were washed with saturated $NaHCO_3$ (375 mL), water (375 mL), and brine (375 mL), and the resulting solution was dried over anhydrous $Na_2SO_4$. After evaporating the solvent under reduced pressure, a yellow syrup containing 54% Neu2en5Ac was obtained. The syrup was dissolved in dry methanol (50 mL), and a methanolic sodium methoxide solution (22 mL, 11 mmol) was then added at 0 °C. The resulting solution was stirred at 0 °C for 30 min. Subsequently, the solution was then neutralized by the addition of Amberlite IR-120 resin ($H^+$ form). After filtration of the resin, which was washed several times with methanol, the filtrate was concentrated under reduced pressure to give a yellow syrup that was purified by flash-column chromatography using ethyl acetate/methanol/water (10:3:1 v/v/v). The fractions containing the product were combined and concentrated to give a solid residue (120 mg, 14% yield); mp 68−69 °C; $[\alpha]^{20}_D = -15.6$ (*c* 0.29, $H_2O$). $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$: 2.0 (dd, 1 H, $J_{3a,3e} = 14.13$ $J_{3a,4} = 11.19$, H-3a), 2.06 (s, 3 H, $CH_3$), 2.76 (dd, 1 H, $J_{3e,3a} = 13.83$, $J_{3e,4} = 4.97$, H-3e), 3.48 (m, 3 H, H-9a, H-8 and H-7), 3.67 (dd, 1 H, $J_{9b,9a} = 121.01$, $J_{9b,8} = 2.36$ H-9b.), 3.84 (d, 1 H, $J_{5,6} = 10.62$, H-6), 3.89 (s, 3 H, OCH3), 4.02 (t, 1 H, $J_{5,6} = J_{5,4\,5} = 10.42$, H-5), 4.32 (td, 1 H, $J_{4,3e} = 4.74$, $J_{4,3a} = J_{4,5} = 11$, H-4), 7.07 (m, 2 H, H-3′, H-5′), 7.19 (m, 2 H, H-4′). $^{13}C$ NMR (100 MHz, $D_2O$) $\delta$: 22.06 ($CH_3$), 39.27 (C-3), 51.99 (C-5), 53.86 (OCH3), 62.88 (C-9), 65.67 (C-4), 67.84 (C-7), 70.01 (C-8), 73.25 (C-6), 100.99 (C-2), 112.56 (dd, 2 × C, $J_{C,F} = 17.21$, $J_{C,F} = 5.44$, C-3′ and C-3′), 125.12 (t, $J_{C,F} = 9.58$, C-4′), 128.17 (t, $J_{C,F} = 14.62$, C-1′), 155.10 (dd, 2 × C, $J_{C,F} = 245.78$ and $J_{C,F} = 4.79$, C-2′ and C-6′), 169.70, 174.94 (C═O, C-1). HRMS-FAB (*m*/*z*): [M + $H^+$] calcd for $C_{18}H_{24}NO_9F_2$, 436.1419; found, 436.1409.

*2-Fluorophenyl* (*5-Acetamido-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulopyranosylonic Acid*) (***1b***). Peracetylated sialoside **2b** (153 mg, 0.26 mmol) was dissolved in dry methanol (7.6 mL), and a methanolic sodium methoxide solution (2.7 mL, 1.30 mmol) was then added at 0 °C. The resulting solution was stirred at 0 °C for 30 min, and the solution was then neutralized by adding Amberlite IR-120 resin ($H^+$ form). After filtering and washing the resin several times with methanol, the combined filtrate was concentrated under reduced pressure, and the resulting residue was dissolved in a 3:1 v/v THF/$H_2O$ solution (3 mL). After adding $LiOH \cdot H_2O$ (55 mg, 1.31 mmol), the mixture was stirred at 0 °C for 30 min. Then, the solution was neutralized using Amberlite IR-120 resin ($H^+$ form), and the resin was then filtered and washed thoroughly with methanol. The filtrate was concentrated under reduced pressure to give a solid residue that was purified by flash-column chromatography using ethyl acetate/methanol/water (10:2:1 v/v/v). The fractions that contained the product were combined and evaporated to give a colorless solution (∼ 5 mL). This aqueous solution was then lyophilized to give a white solid (63 mg, 60% yield); mp 182 °C (dec); $[\alpha]^{20}_D = -64.4$ (*c* 0.477, $H_2O$).

$^1$H NMR (600 MHz, $D_2O$) $\delta$: 1.84 (t, 1 H, $J_{3a,3e} = J_{3a,4} = 12.9$, H-3a), 2.05 (s, 3 H, $CH_3$), 2.63 (dd, 1 H, $J_{3e,3a} = 13.2$, $J_{3e,4} = 5.0$, H-3e), 3.45 (d, 1 H, $J_{7,8} = 9.3$, H-7), 3.59 (dd, 1 H, $J_{9a,9b} = 11.9$, $J_{9a,8} = 5.3$, H-9a), 3.65 (m, 1 H, H-8), 3.72 (m, 2 H, H-6, H-9b), 4.02 (t, 1 H, $J_{5,6} = J_{5,4} = 10.3$, H-5), 4.30 (td, 1 H, $J_{4,3e} = 4.9$, $J_{4,3a} = J_{4,5} = 10.6$, H-4), 7.03 (m, 1 H, H-4′), 7.08 (m, 1 H, H-6′), 7.2 (m, 2 H, H-3′ and H-5′). $^{13}$C NMR (150 MHz, $D_2O$) $\delta$: 22.05 ($CH_3$), 40.39 (C-3), 51.72 (C-5), 63.04 (C-9), 66.67 (C-4), 68.13 (C-7), 70.02 (C-8), 72.28 (C-6), 100.71 (C-2), 116.33 (d, $J_{C,F} = 18.2$, C-3′), 117.32 (C-5′), 122.50 (d, $J_{C,F} = 6.8$, C-4′), 124.12 (d, $J_{C,F} = 3.6$, C-6′), 141.75 (d, $J_{C,F} = 10.2$, C-1′), 151.99 (d, $J_{C,F} = 243.0$, C-2′), 174.45, 174.73 (C═O, C-1). HRMS-FAB ($m/z$): [M + H$^+$] calcd for $C_{17}H_{23}NO_9F$, 404.1357; found, 404.1352.

*3-Fluorophenyl (5-Acetamido-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulopyranosylonic Acid)* (***1c***). Peracetylated sialoside **2c** (168 mg, 0.29 mmol) was dissolved in dry methanol (8.5 mL), and a methanolic sodium methoxide solution (3 mL, 1.50 mmol) was then added at 0 °C. The resulting solution was stirred at 0 °C for 30 min. Subsequently, the solution was then neutralized by the addition of Amberlite IR-120 resin (H$^+$ form). After filtration of the resin, which was washed several times with methanol, the filtrate was concentrated under reduced pressure. The resultant residue was dissolved in a 3:1 v/v THF/$H_2O$ solution (3.4 mL). After adding LiOH·$H_2O$ (60 mg, 1.42 mmol), the mixture was stirred at 0 °C for 30 min. Then, the solution was neutralized using Amberlite IR-120 resin (H$^+$ form) and then filtered. The resin was then washed thoroughly with methanol, and the combined solution was concentrated under reduced pressure to give a solid residue that was purified by flash-column chromatography using ethyl acetate/methanol/water (10:2:1 v/v/v). The fractions containing the product were combined and concentrated to a volume of approximately 5 mL. This aqueous solution was lyophilized to give a white solid (70 mg, 60% yield); mp 195 °C (dec); $[\alpha]^{20}_D = -42.3$ ($c$ 0.354, $H_2O$). $^1$H NMR (600 MHz, $D_2O$) $\delta$: 1.8 (t, 1 H, $J_{3a,3e} = J_{3a,4} = 12.3$, H-3a), 2.03 (s, 3 H, $CH_3$), 2.54 (dd, 1 H, $J_{3e,3a} = 13.2$, $J_{3e,4} = 5.0$, H-3e), 3.46 (d, 1 H, $J_{7,8} = 9.8$, H-7), 3.59 (dd, 1 H, $J_{9a,9b} = 12.6$, $J_{9a,8} = 5.9$, H-9a), 3.67−3.76 (m, 3 H, H-6, H-8, H-9b), 3.99 (t, 1 H, $J_{5,6} = J_{5,4} = 10.3$, H-5), 4.24 (td, 1 H, $J_{4,3a} = J_{4,5} = 10.7$, $J_{4,3e} = 4.9$, H-4), 6.75−6.89 (m, 3 H, H-2′, H-4′, H-6′), 7.28 (m, 1 H, H-5′). $^{13}$C NMR (150 MHz, $D_2O$) $\delta$: 22.06 ($CH_3$), 40.51 (C-3), 51.76 (C-5), 63.01 (C-9), 66.69 (C-4), 68.09 (C-7), 70.12 (C-8), 71.18 (C-6), 100.57 (C-2), 104.28 (d, $J_{C,F} = 25.4$, C-2′), 108.70 (d, $J_{C,F} = 21.4$, C-4′), 112.37 (d, $J_{C,F} = 2.4$, C-6′), 130.33 (d, $J_{C,F} = 9.9$, C-5′), 155.44 (d, $J_{C,F} = 11.4$, C-1′), 162.84 (d, $J_{C,F} = 242.5$, C-3′), 174.54, 174.72 (C═O, C-1). HRMS-FAB ($m/z$): [M + H$^+$] calcd for $C_{17}H_{23}NO_9F$, 404.1357; found, 404.1365.

*4-Fluorophenyl (5-Acetamido-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulopyranosylonic Acid)* (***1d***). To a solution of protected sialoside **2d** (150 mg, 0.26 mmol) in dry methanol (7.5 mL), a methanolic sodium methoxide solution (2.7 mL, 1.30 mmol) was added at 0 °C. After the resulting solution was stirred at 0 °C for 30 min, it was neutralized by the addition of Amberlite IR-120 resin (H$^+$ form). After removing and washing the resin with methanol, the combined filtrate was concentrated under reduced pressure. The resulting solid residue was dissolved in a 3:1 v/v THF/$H_2O$ solution (3 mL) to which LiOH·$H_2O$ (54 mg, 1.28 mmol) was added, and the mixture was stirred at 0 °C for 30 min. Then, the solution was neutralized using Amberlite IR-120 resin (H$^+$ form) and filtered. The resin was then washed thoroughly with methanol, and the filtrate was concentrated under reduced pressure to give a solid residue that was purified by flash-column chromatography using ethyl acetate/methanol/water (10:2:1 v/v/v). The fractions containing the product were combined and concentrated. The remaining aqueous residue was then lyophilized to give a white solid (53 mg, 50% yield); mp 188 °C (dec); $[\alpha]^{20}_D = -42.9$ ($c$ 0.933, $H_2O$). $^1$H NMR (600 MHz, $D_2O$) $\delta$: 1.80 (t, 1 H, $J_{3a,3e} = J_{3a,4} = 12.5$, H-3a), 2.05 (s, 3 H, $CH_3$), 2.56 (dd, 1 H, $J_{3e,3a} = 13.2$, $J_{3e,4} = 4.9$, H-3e), 3.46 (d, 1 H, $J_{7,6} = 9.3$, H-7), 3.61 (dd, 1 H, $J_{9a,9b} = 11.8$, $J_{9a,8} = 5.3$, H-9a), 3.69 (m, 1 H, H-8), 3.75 (m, 2H, H-6, H-9b), 4.00 (t, 1 H, $J_{5,6} = J_{5,4} = 10.3$, H-5), 4.26 (td, 1 H, $J_{4,3e} = 5.0$, $J_{4,3a} = J_{4,5} = 10.8$, H-4), 7.05 (m, 4 H, Ar−H). $^{13}$C NMR (150 MHz, $D_2O$) $\delta$: 24.60 ($CH_3$), 43.13 (C-3), 54.36 (C-5), 65.61 (C-9), 69.31 (C-4), 70.72 (C-7), 72.60 (C-8), 73.56 (C-6), 102.95 (C-2), 118.15 (d, $J_{C,F} = 23.3$, C-3′, C-5′), 120.32 (d, $J_{C,F} = 8.1$, C-2′, C-6′), 152.83 (d, $J_{C,F} = 2.0$, C-1′), 160.13 (d, $J_{C,F} = 237.0$, C-4′), 177.25, 177.33 (C═O, C-1). HRMS-FAB ($m/z$): [M + H$^+$] calcd for $C_{17}H_{23}NO_9F$, 404.1357; found, 404.1356.

*2,3-Difluorophenyl (5-Acetamido-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulopyranosylonic Acid)* (***1e***). Methyl ester **3e** (371 mg, 0.852 mmol) was dissolved in a 3:1 v/v THF/$H_2O$ solution (11 mL). After adding LiOH·$H_2O$ (194.3 mg, 4.57 mmol) to the solution, the resultant mixture was stirred at 0 °C for 30 min. Then, the solution was neutralized using Amberlite IR-120 resin (H$^+$ form) and filtered. The resin was then washed thoroughly with methanol, and the combined filtrate was concentrated under reduced pressure to give a solid residue. Purification was accomplished using flash-column chromatography with ethyl acetate/methanol/water (10:3:1 v/v/v) as the eluent. The fractions containing the product were combined and evaporated. The remaining aqueous residue lyophilized to give a white solid (250 mg, 70%); mp 176−177 °C. $[\alpha]^{20}_D = -54.1$ ($c$ 0.59, $H_2O$). $^1$H NMR (400 MHz, $D_2O$) $\delta$: 1.86 (dd, 1 H, $J_{3a,3e} = 13.08$, $J_{3a,4} = 11.52$, H-3a), 2.06 (s, 3 H, $CH_3$), 2.64 (dd, 1 H, $J_{3e,3a} = 13.24$, $J_{3e,4} = 4.96$, H-3e), 3.49 (d, 1 H, $J_{7,8} = 9$, H-7), 3.59 (dd, 1 H, $J_{9a,9b} = 11.48$, $J_{9a,8} = 4.94$, H-9a), 3.69 (m, 2 H, H-9b and H-8), 3.77 (d, 1 H, $J_{5,6} = 10.59$, H-6), 4.03 (t, 1 H, $J_{5,6} = J_{5,4} = 10.33$, H-5), 4.34 (td, 1 H, $J_{4,3e} = 4.95$, $J_{4,3a} = J_{4,5} = 11.1$, H-4), 6.99 (m, 3 H, H-4′, H-5′, H-6′). $^{13}$C NMR (100 MHz, $D_2O$) $\delta$: 22.11 ($CH_3$), 40.33 (C-3), 51.72 (C-5), 63.04 (C-9), 66.58 (C-4), 68.04 (C-7), 70.11 (C-8), 71.49 (C-6), 101.21 (C-2), 110.20 (d, $J_{C,F} = 17.29$, C-6′), 112.61 (d, $J_{C,F} = 3.02$, C-5′), 123.23 (dd, $J_{C,F} = 8.32$ and 4.99, C-4′), 141.05 (dd, $J_{C,F} = 244.90$ and $J_{C,F} = 14.56$, C-2′), 143.48 (dd, $J_{C,F} = 7.65$ and $J_{C,F} = 3.01$, C-1′), 151.02 (dd, $J_{C,F} = 244.46$ and $J_{C,F} = 11$, C-3′), 174.08, 174.79 (C═O, C-1). HRMS-FAB ($m/z$): [M + H$^+$] calcd for $C_{17}H_{22}NO_9F_2$, 422.1263; found, 422.1251.

*2,5-Difluorophenyl (5-Acetamido-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulopyranosylonic Acid)* (***1f***). To a solution of peracetylated sialoside **2f** (61 mg, 0.10 mmol) in dry methanol (2.7 mL), a methanolic sodium methoxide solution (1 mL, 0.50 mmol) was added at 0 °C. The resulting solution was stirred at 0 °C for 30 min, after which the solution was neutralized by adding Amberlite IR-120 resin (H$^+$ form). Following the removal of the resin, which was washed several times with methanol, the resulting filtrates were combined and concentrated under reduced pressure to give a solid. This material was dissolved in a 3:1 v/v THF/$H_2O$ solution (1 mL). After adding LiOH·$H_2O$ (20 mg, 0.47 mmol) to the solution, the resultant mixture was stirred at 0 °C for 30 min. Then, the solution was neutralized using Amberlite IR-120 resin (H$^+$ form) and filtered. The resin was then washed thoroughly with methanol, and the combined filtrate was concentrated under reduced pressure to give a solid residue. Purification was accomplished using flash-column chromatography with ethyl acetate/methanol/water (10:3:1 v/v/v) as the eluent. The fractions containing the product

were combined and evaporated. The remaining aqueous residue lyophilized to give a white solid (24 mg, 58% yield); mp 161 °C (dec); $[\alpha]^{20}_D = -62.8$ (*c* 0.17, $H_2O$). $^1$H NMR (600 MHz, $D_2O$) *δ*: 1.85 (t, 1 H, $J_{3a,3e} = J_{3a,4} = 12.2$, H-3a), 2.05 (s, 3 H, $CH_3$), 2.63 (dd, 1 H, $J_{3e,3a} = 13.4$, $J_{3e,4} = 4.6$, H-3e), 3.49 (d, 1 H, $J_{7,8} = 9.5$, H-7), 3.64 (dd, 1 H, $J_{9a,9b} = 12.0$, $J_{9a,8} = 4.9$, H-9a), 3.69 (m, 1 H, H-8), 3.76 (m, 2 H, H-6 and H-9b), 4.03 (t, 1 H, $J_{5,6} = J_{5,4} = 10.2$, H-5), 4.29 (td, 1 H, $J_{4,3a} = J_{4,5} = 10.5$, $J_{4,3e} = 4.9$, H-4), 6.76 (m, 1 H, H-4′), 6.97 (m, 1 H, H-6′), 7.17 (m, 1 H, H-3′). $^{13}$C NMR (150 MHz, $D_2O$) *δ*: 22.06 ($CH_3$), 40.25 (C-3), 51.66 (C-5), 63.01 (C-9), 66.52 (C-4), 67.94 (C-7), 70.01 (C-6), 71.43 (C-8), 101.21 (C-2), 105.11 (d, $J_{C,F} = 28.7$, C-6′), 108.20 (dd, $J_{C,F} = 25.2$ and 7.5, C-4′), 116.46 (dd, $J_{C,F} = 21.1$ and 10.9, C-3′), 142.29 (t, $J_{C,F} = 11.4$, C-1′), 148.38 (d, $J_{C,F} = 237.9$, C-2′), 157.77 (d, $J_{C,F} = 157.8$, C-5′), 173.89, 174.75 (C═O, C-1). HRMS-FAB (*m*/*z*): $[M + H^+]$ calcd for $C_{17}H_{22}NO_9F_2$, 422.1263; found, 422.1256.

*2,6-Difluorophenyl (5-Acetamido-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulopyranosylonic Acid)* (***1g***). Methyl ester **3g** (120 mg, 0.275 mmol) was dissolved in a 3:1 v/v THF/$H_2O$ solution (4 mL). After adding $LiOH \cdot H_2O$ (62.8 mg, 1.47 mmol) to the solution, the resultant mixture was stirred at 0 °C for 30 min. Then, the solution was neutralized using Amberlite IR-120 resin ($H^+$ form) and filtered. The resin was then washed thoroughly with methanol, and the combined filtrate was concentrated under reduced pressure to give a solid residue. Purification was accomplished using flash-column chromatography with ethyl acetate/methanol/water (10:3:1 v/v/v) as the eluent. The fractions containing the product were combined and evaporated. The remaining aqueous residue lyophilized to give a white solid (48 mg, 41%. mp 141−142 °C. $[\alpha]^{20}_D = -28.47$ (*c* 0.28, $H_2O$). $^1$H NMR (400 MHz, $CDCl_3$) *δ*: 1.88 (dd, 1 H, $J_{3a,3e} = 13.30$ $J_{3a,4} = 11.45$, H-3a), 2.05 (s, 3 H, $CH_3$), 2.69 (dd, 1 H, $J_{3e,3a} = 13.39$, $J_{3e,4} = 4.86$, H-3e), 3.48 (d, 1 H, H-7), 3.40 (m, 2 H, H-9a, H-8), 3.64 (m, 2 H, H-6, H-9b), 3.99 (t, 1 H, $J_{5,6} = J_{5,4} = 10.42$, H-5), 4.31 (td, 1 H, $J_{4,3e} = 4.83$, $J_{4,3a} = J_{4,5} = 11$, H-4), 7.07 (m, 3 H, H-3′, H-4′, H-5′). $^{13}$C NMR (100 MHz, $D_2O$) *δ*: 22.09 ($CH_3$), 40.22 (C-3), 51.67 (C-5), 62.98 (C-9), 66.40 (C-4), 68.13 (C-7), 70.22 (C-8), 72.81 (C-6), 102.32 (C-2), 112.40 (dd, 2 × C, $J_{C,F} = 16.94$, $J_{C,F} = 5.58$, C-3′ and C-3′), 123.41 (t, $J_{C,F} = 9.73$, C-4′), 129.21 (t, $J_{C,F} = 14.25$, C-1′), 154.72 (dd, 2 × C, $J_{C,F} = 245.22$ and $J_{C,F} = 5.81$, C-2′ and C-6′), 174.61, 174.82 (C═O, C-1). HRMS-FAB (*m*/*z*): $[M + H^+]$ calcd for $C_{17}H_{22}NO_9F_2$, 422.1263; found, 422.1253.

*3,5-Difluorophenyl (5-Acetamido-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulopyranosylonic Acid)* (***1h***). Protected sialoside **2h** (206 mg, 0.34 mmol) was dissolved in dry methanol (9.5 mL), and a methanolic sodium methoxide solution (3.5 mL, 1.70 mmol) was then added at 0 °C. The resulting solution was stirred at 0 °C for 30 min. Then, the solution was neutralized by the addition of Amberlite IR-120 resin ($H^+$ form), and the resin, which was removed by filtration, was then washed several times with methanol. The combined filtrate was concentrated under reduced pressure, and the resulting residue was dissolved in a 3:1 v/v THF/$H_2O$ solution (3.8 mL). After adding $LiOH \cdot H_2O$ (68 mg, 1.60 mmol), the mixture was stirred at 0 °C for 30 min. Then, the solution was neutralized using Amberlite IR-120 resin ($H^+$ form) and filtered. The resin was then washed thoroughly with methanol, and the filtrate was concentrated under reduced pressure to give a solid residue which was purified by flash-column chromatography using ethyl acetate/methanol/water (10:2:1 v/v/v). The fractions containing the product were combined and concentrated. The remaining aqueous residue was then lyophilized to give a white solid (70 mg, 60% yield); mp 180 °C (dec); $[\alpha]^{20}_D = -88.7$ (*c* 0.248, $H_2O$). $^1$H NMR (150 MHz, $D_2O$) *δ*: 1.82 (t, 1 H, $J_{3a,3e} = J_{3a,4} = 12.3$, H-3a), 2.05 (s, 3 H, $CH_3$), 2.56 (dd, 1 H, $J_{3e,3a} = 13.2$, $J_{3e,4} = 4.9$, H-3e), 3.49 (d, 1 H, $J_{7,8} = 9.3$, H-7), 3.65 (dd, 1 H, $J_{9a,9b} = 12.1$, $J_{9a,8} = 4.9$, H-9a), 3.73 (m, 1 H, H-8), 3.77 (m, 2 H, H-6, H-9b), 4.00 (t, 1 H, $J_{5,6} = J_{5,4} = 10.2$, H-5), 4.24 (td, 1 H, $J_{4,3a} = J_{4,5} = 10.7$, $J_{4,3e} = 4.8$, H-4), 6.62 (m, 1 H, H-4′), 6.68 (m, 2 H, H-2′ and H-6′). $^{13}$C NMR (150 MHz, $D_2O$) *δ*: 22.07 ($CH_3$), 40.41 (C-3), 51.70 (C-5), 63.06 (C-9), 66.59 (C-4), 68.03 (C-7), 69.95 (C-8), 71.25 (C-6), 97.38 (t, $J_{C,F} = 27.2$, C-4′), 100.42 (m, C-2′ and C-6′), 100.95 (C-2), 156.04 (t, $J_{C,F} = 14.1$, C-1′), 162.90 (dd, $J_{C,F} = 244.3$ and 15.1, C-3′ and C-5′), 174.27, 174.91 (C═O, C-1). HRMS-FAB (*m*/*z*): $[M + H^+]$ calcd for $C_{17}H_{22}NO_9F_2$, 422.1263; found, 422.1253.

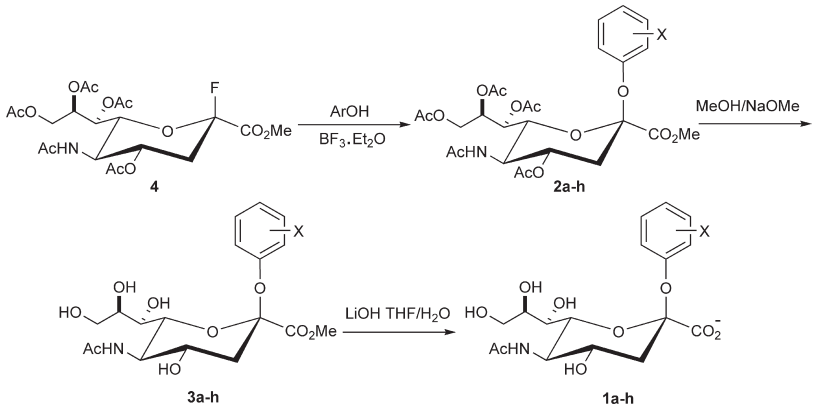
*MvNA Active Site Construct*. Plasmid pJW-OSH-Y370g, encoding the full-length Y370G mutant sialidase (*17*, *18*), was used as the template DNA. A portion of the sialidase gene encoding the MvNA active site domain (up to amino acid residue G401 (*21*)) was amplified by PCR, incorporating a new Hind III site to allow read-through for the expression of a C-terminal linker and $His_6$-tag. The amplification reaction was carried out in 10% DMSO, using the forward primer, Eco*′ (*17*), and the reverse primer, GGHin-R′ (5′-CCC AAG CTT CAG CCA GGC GAG GTT G-3′) to produce a 1,205 bp fragment. The isolated fragment was purified, digested with EcoR I and Hind III, and then ligated into a similarly digested and dephosphorylated pJW-OSH vector and propagated in *Escherichia coli*. Plasmid DNA was isolated from a single colony and the resultant 6,526 bp plasmid, designated as pJW*β*-Y370g, was verified by restriction digests, and DNA sequencing using primer MV1396R′ (5′-CCA GCC CGG GTC CGG GG-3′) and the T7 Terminator primer (Novagen) to ensure that spurious mutations had not occurred during DNA manipulations.

*Y370G Expression and Purification*. Expression was performed as reported previously (*6*). At 41 h postinduction, the 35 mL culture was harvested by setting on ice for 20 min prior to removing the cells by centrifugation at 3,700*g* for 15 min. The sialidase active site-$His_6$ protein was purified using agarose Ni-NTA resin (2 mL, Qiagen), $(NH_4)SO_4$ precipitation, and dialysis, as described in a previous report (*17*). Purity was assessed by SDS−PAGE with Coomassie staining, and total protein concentration was determined by Bradford assay using bovine serum albumin as the standard. Aliquots of the purified enzyme were stored at −80 °C. *N*-Terminal Edman sequencing was performed on an ABI Procise 494 sequencer (Alphalyse Inc.).

*Product Studies*. $^1$H NMR spectroscopy (600 MHz) was employed to identify the products of the enzyme-catalyzed reactions (*6*). Reactions were carried out in 0.6 mL (25 °C, 10 mM tartrate buffer at pD 5.5), and $^1$H NMR spectra were recorded at various time intervals. Also, a solution of each substrate (1 mM), buffered to a pH value of 5.25, was incubated with enzyme for either 24 h (substrates **1b**, **1c**, **1e**, **1f**, and **1g**) or 48 h (substrates **1d** and **1h**), and then an aliquot was diluted (10−fold), and the UV−vis spectrum (250−400 nm) was recorded and compared to a spectrum of the appropriate phenol at the same pH and concentration. Of note, even after 48 h, substrate **1h** had not been completely hydrolyzed.

*Enzyme Kinetics*. The enzyme-catalyzed hydrolysis reactions were monitored using a HPLC protocol. Specifically, each 0.5 mL reaction was performed at 37 °C by equilibrating the buffer, substrate, and internal standard (4-nitrophenyl *β*-D-glucopyranoside, 0.01 mM) for 1 min before the addition of enzyme stock solution containing BSA (final concentration of BSA in the

Scheme 3: Synthetic Route to Make Aryl $\beta$-Sialoside Substrates




reaction mixture = 0.01% w/v). Reactions were terminated at five different time points by adding an aliquot of the reaction mixture (100 $\mu$L) to ethanol (300 $\mu$L). Protein precipitation occurred when the ethanolic solutions were cooled to −20 °C for 20 min. Following centrifugation (20 min), the supernatants were evaporated to dryness. The resultant residues were dissolved in milli-Q water (100 $\mu$L), and HPLC analysis was performed on a gradient pump system from Agilent Technologies using a Phenomenex C18 column 150 × 60 mm (3 $\mu$m particle size). The two mobile phase solutions were water containing 0.1% formic acid (solvent A) and acetonitrile (solvent B). Isocratic mobile phases were delivered at 1.0 mL/min, 10 $\mu$L sample aliquots were injected onto the column, and the column temperature was 25 °C. The decrease in substrate concentration was monitored as a function of reaction time using a standard integration protocol. Substrate and internal standard retention times were 2.73 min for Ph-$\beta$NeuAc (eluting with 20% B and followed at $\lambda$ = 270 nm); 3.50 min for 2FP-$\beta$NeuAc (15% B, $\lambda$ = 270 nm); 2.87 min for 3FP-$\beta$NeuAc (20% B, $\lambda$ = 270 nm); 3.02 min for 4FP-$\beta$NeuAc (20% B, $\lambda$ = 270 nm); 2.35 min for 23DFP-$\beta$NeuAc (30% B, $\lambda$ = 254 nm); 2.81 min for 25DFP-$\beta$NeuAc (20% B, $\lambda$ = 270 nm); 2.52 min for 26DFP-$\beta$NeuAc (20% B, $\lambda$ = 260 nm); 3.15 min for 35DFP-$\beta$NeuAc (20% B, $\lambda$ = 270 nm); and 6.20, 4.00, and 3.00 min for 4-nitrophenyl $\beta$-D-glucopyranoside eluting with 15% B, 20% B, or 30% B, respectively. Michaelis−Menten parameters were calculated by nonlinear least-squares fitting of a minimum of five initial rate data, which spanned a substrate concentration of at least $K_m$/4 to 4$K_m$, to the standard Michaelis−Menten equation (Prism). Estimates of $k_{cat}$ for the two slowest substrates, 4FP-$\beta$NeuAc and 35DFP-$\beta$NeuAc, were made using $^1$H NMR spectroscopy. Specifically, under identical conditions where the substrate concentration (~5 mM) was much greater than $K_d$, the hydrolysis of 2FP-$\beta$Neu5Ac, 4FP-$\beta$Neu5Ac, and 35DFP-$\beta$Neu5Ac were monitored by $^1$H NMR spectroscopy. Solvent deuterium KIEs were measured using identical ratios of buffer components in both $H_2O$ and $D_2O$.

*Measurement of Phenol p$K_a$ Values*. During neutralization of a solution of the appropriate phenol (0.01 M, 50 mL, $I$ = 0.05 M NaCl), by the addition of aliquots of a NaOH solution (0.5 mL; 0.05 M), pH values were automatically recorded using a standard pH titrator assembly. The phenolic p$K_a$ values were calculated by fitting the measured pH versus addition data to a standard titration curve (Prism).

*Measurement of pH-Rate Profiles*. To determine the effect of pH on catalysis, kinetics parameters were measured over a pH range of 3.8−6.6. The buffers used were NaOAc-HOAc (pH range 3.8−5.7) and 2-(*N*-morpholino)ethanesulfonic acid (MES-NaOH) (pH range 5.6−6.6). The substrate for this experiment was 2FP-$\beta$NeuAc. Separate Michaelis−Menten plots were obtained for each of the five buffers according to the HPLC procedure described above.

*Measurement of Dissociation Constants*. To determine the dissociation constants for the slow substrates (4FP-$\beta$NeuAc and 35DFP-$\beta$NeuAc) and a known neuraminidase inhibitor (Neu2en5Ac), standard $IC_{50}$ assays were performed using pNP-αNeu5Ac at a single concentration, and the $IC_{50}$ value was corrected to give $K_d$ values (*22*). For the reaction product phenol, three separate assays were performed: (i) an $IC_{50}$ experiment with 2FP-$\beta$Neu5Ac as substrate; (ii) a time-dependent inhibition assay with 2FP-$\beta$Neu5Ac as substrate; and (iii) an activation assay with pNP-αNeu5Ac to probe whether bound phenol could accelerate the production of 4-nitrophenol. This last experiment was also performed with 4-fluorophenol and 3,5-difluorophenol.

## RESULTS

*Synthesis*. The panel of fluorinated phenyl $\beta$-sialosides were synthesized by following the literature procedure as reported by Dookhun et al (*19*) (Scheme 3). In order to ensure that measured kinetic parameters are as accurate as possible, it is essential to ensure that the synthetic substrates are free of contaminating inhibitors. Thus, fully protected Neu2en5Ac, which is invariably produced during glycosylation, was removed from the majority of protected sialosides by recrystallization from diethyl ether. However, for the syntheses of 23DFP-$\beta$-NeuAc and 26DFP-$\beta$-NeuAc the methyl ester of Neu2en5Ac was separated from the methyl esters of 23DFP-$\beta$NeuAc and 26DFP-$\beta$NeuAc by flash column chromatography. Presented in the Supporting Information section are the $^1$H and $^{13}$C NMR spectra of all new substrates (**1b**−**1h**).

*Cloning, Expression and Purification of Y370G Mutant Sialidase*. The full-sized Y370G mutant *M. viridifaciens* sialidase, which comprises three separate domains (*16*, *21*), has been shown previously to hydrolyze phenyl $\beta$-sialoside, a process that occurs with an inversion of anomeric configuration (*18*). In order to eliminate any possibility of kinetic complications being caused by binding of either substrate or reaction products to the hydrophobic surface of the lectin domain in the full-sized sialidase (*21*), it was decided to express the active site domain only, and to measure kinetic parameters on this single domain protein. Successful plasmid construction was confirmed by DNA sequencing. The expression profile showed sialidase activity similar to that of the full-size Y370G sialidase reported previously (*16*). The protein was purified by Ni-NTA affinity chromatography in a manner similar to that used to purify the full-size enzyme with a C-terminal $His_6$-tag.

SDS−PAGE analysis revealed that the purified product actually contained two closely running bands; the lower molecular weight band had higher intensity, corresponding to the predicted size of the active site with the C-terminal $His_6$-tag. The two bands were submitted for *N*-terminal Edman sequencing (6 rounds): the upper (larger) band did not produce good quality signals, and the deduced sequence did not appear to match the sialidase fusion protein, suggesting that the upper band is a contaminant that coeluted from the affinity column with the lower molecular weight band. The lower (smaller) band *N*-terminal sequence analysis exhibited a very strong signal that contained two superimposed sequences, IAGAPV and GAPVPP. These two sequences correspond to the *N*-terminal cleavage sites for recombinant MvNA proteins that have been reported previously (*6*, *23*). Shown in Figure S1 (Supporting Information) is a picture of the SDS−PAGE gel for the recombinant active site domain Y370G mutant sialidase.

*Enzyme Kinetics and Product Studies*. Enzyme kinetic parameters were evaluated using an enzyme stock containing both coeluting bands under the assumption that the contaminant protein did not possess β-sialidase activity, an activity that is unknown in nature, and that the two amino acid differences at the *N*-terminus in the lower band do not affect the catalytic activity. The reaction products from the active site Y370G mutant sialidase-catalyzed hydrolysis of aryl β-sialosides were shown to be sialic acid and phenol by NMR spectroscopic analysis. Also, the phenol absorption band in the UV−vis spectrum of the product mixture was of equal intensity, within experimental error, to that of an identical concentration of free phenol. Thus, chromatographic purification of the final products on silica gel did not lead to any silica gel contamination.

Because of the generally small absorbance changes that occur during the catalyzed hydrolyses of these β-sialosides, resulting from the absence of an auxochromic group in the fluoroaryl substrates, a HPLC analytical method had to be developed in order to monitor the reaction progress. For the two slowest substrates (4FP-βNeu5Ac and 35DFP-βNeu5A) full Michaelis−Menten curves could not be obtained because of the difficulty of measuring hydrolysis rates at low substrate concentration. Therefore, estimates for the relative $k_{cat}$ values were made by noting the fraction of reaction for 2FP-βNeu5Ac, 4FP-βNeu5Ac, and 35DFP-βNeu5Ac at single time points under saturating conditions. Using identical high concentrations of enzyme, we observed that 33% of 2FP-βNeu5Ac (4.77 mM) had reacted after 3 min, while 6 h was needed for 25% hydrolysis of 4FP-βNeu5Ac (4.90 mM), and after 3 days, 16% of 35DFP-βNeu5Ac (4.90 mM) had hydrolyzed. Michaelis−Menten kinetic parameters for the active site Y370G mutant sialidase with the eight aryl β-sialosides are listed in Table 1.

Shown in Figures 1 and 2 are Brønsted plots for the two catalytic constants $k_{cat}/K_m$ and $k_{cat}$. Substitution at the para position with fluorine (4FP-βNeuAc) results in a lower enzymatic activity, while all difluorinated substrates with the exception of the 2,6-isomer also display low activities (Table 1). The variations of the kinetic parameters $k_{cat}/K_m$ and $k_{cat}$ for the Y370G-catalyzed hydrolysis of 2-fluorophenyl β-sialoside as a function of pH are listed in Table 2.

The solvent deuterium kinetic isotope effects on the Y370G mutant-catalyzed hydrolysis of 2FP-βNeuAc were measured by maintaining identical ratios of the two buffer components in $H_2O$ and $D_2O$ as suggested by Schowen (*24*). Thus, at an acetate to acetic acid ratio of 3.46 and with a total buffer concentration of 0.1 M, the following kinetic parameters were measured: $k_{cat}$ ($H_2O$) = $2.29 \pm 0.19\ s^{-1}$ and $k_{cat}$ ($D_2O$) = $2.23 \pm 0.26\ s^{-1}$; therefore, $k_{H_2O}/k_{D_2O} = 1.03 \pm 0.15$, while $k_{cat}/K_m$ ($H_2O$) = $(1.42 \pm 0.30) \times 10^4\ M^{-1}\ s^{-1}$ and $k_{cat}/K_m$ ($D_2O$) = $(1.60 \pm 0.54) \times 10^4\ M^{-1}\ s^{-1}$ so that $k_{H_2O}/k_{D_2O} = 0.89 \pm 0.35$. A second independent set of measurements using a different batch of enzyme gave the following values for the solvent deuterium KIE: $k_{H_2O}/k_{D_2O} = 1.36 \pm 0.19$ ($k_{cat}$) and $0.88 \pm 0.33$ ($k_{cat}/K_m$). The weighted averages for these solvent KIE values are $k_{H_2O}/k_{D_2O} = 1.16 \pm 0.12$ ($k_{cat}$) and $0.88 \pm 0.24$ ($k_{cat}/K_m$) (*25*).

The binding of phenol to the Y370G mutant was probed using a standard $IC_{50}$ inhibition assay with 2FP-βNeu5Ac as the substrate. At a phenol concentration of 1 mM, the rate for the mutant sialidase-catalyzed hydrolysis of 2FP-βNeu5Ac (100 μM ≈ $K_m/2$)

Table 1: Michaelis−Menten Kinetic Parameters of the Y370G Mutant Sialidase Catalyzed Hydrolysis of Fluoro-Substituted Ph-βNeuAc at 37 °C and pH 5.25

| substrate | p$K_a$ (ArOH)[a] | rel ($k_{cat}$) | rel ($k_{cat}/K_m$) | $K_m$ (μM) |
|---|---|---|---|---|
| Ph-βNeuAc[b] | 9.87 | 1.00[c] | 1.00[d] | 33 ± 5 |
| 4FP-βNeuAc | 9.72 | 0.16[e] | 0.14[e] | 39 ± 3[f] |
| 3FP-βNeuAc | 9.03 | 14.4 ± 1.5 | 2.2 ± 0.6 | 220 ± 60 |
| 2FP-βNeuAc | 8.49 | 25.2 ± 0.8 | 4.6 ± 0.6 | 182 ± 18 |
| 35DFP-βNeuAc | 8.26 | $8.7 \times 10^{-4}$[e] | $1.1 \times 10^{-4}$[e] | 270 ± 70[f] |
| 23DFP-βNeuAc | 7.65 | 8.77 ± 0.34 | 1.48 ± 0.16 | 200 ± 20 |
| 25DFP-βNeuAc | 7.63 | 2.58 ± 0.10 | 1.3 ± 0.2 | 61 ± 9 |
| 26DFP-βNeuAc | 7.09 | 21.0 ± 0.8 | 18.5 ± 2.7 | 38 ± 5 |

[a]Ionic strength = 0.05 M (NaCl). [b]Reported values for the full-sized *M. viridifaciens* Y370G mutant sialidase are $k_{cat} = 13.3 \pm 0.3\ s^{-1}$ and $k_{cat}/K_m = (2.9 \pm 0.3) \times 10^5\ M^{-1}\ s^{-1}$ (*18*). [c]Calculated value for the truncated *M. viridifaciens* Y370G mutant sialidase used in this study assuming 100% activity, $k_{cat} = 0.110 \pm 0.004\ s^{-1}$. [d]Calculated value for the truncated *M. viridifaciens* Y370G mutant sialidase used in this study assuming 100% activity, $k_{cat}/K_m = (3.3 \pm 0.5) \times 10^3\ M^{-1}\ s^{-1}$. [e]Value was estimated using NMR spectroscopy; see experimental details ($T = 25$ °C); estimated error ±10%. [f]Calculated according to ref *22* using the measured $IC_{50}$ value for the inhibition Y370G-catalyzed hydrolysis of pNP-αNeuAc (expt conc = 50 μM; $K_m = 65.8$ μM).

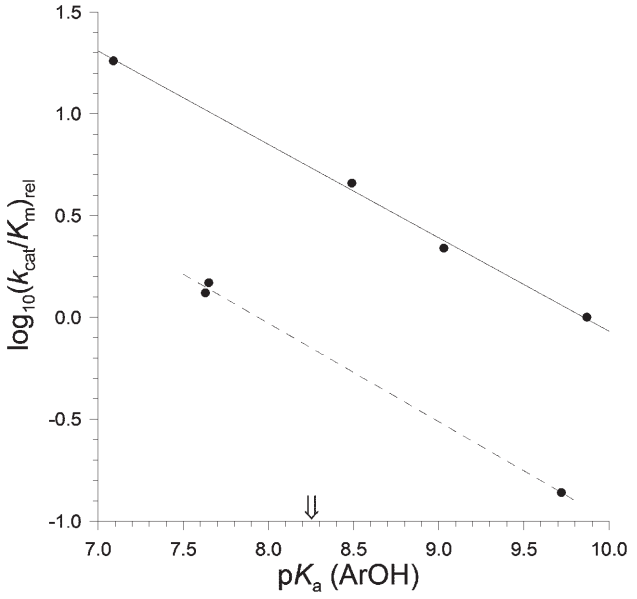



FIGURE 1: Effect of leaving group ability on $k_{cat}/K_m$ relative to that for the parent phenyl β-sialoside for the Y370G mutant sialidase. Leaving group ability represented as p$K_a$ (ArOH) is as follows: 2,6-difluorophenol (7.09); 2,5-difluorophenol (7.63); 2,3-difluorophenol (7.65); 3,5-difluorophenol (8.26); 2-fluorophenol (8.49); 3-fluorophenol (9.03); 2-fluorophenol (9.72); and phenol (9.87). The lines shown are the best linear fits to two data subsets (see Discussion). The point for 3,5-difluorophenyl β-sialoside is not shown because of the extremely low activity of this substrate with the mutant sialidase; rather, the p$K_a$ of 3,5-difluorophenol is indicated on the *x*-axis by an arrow.

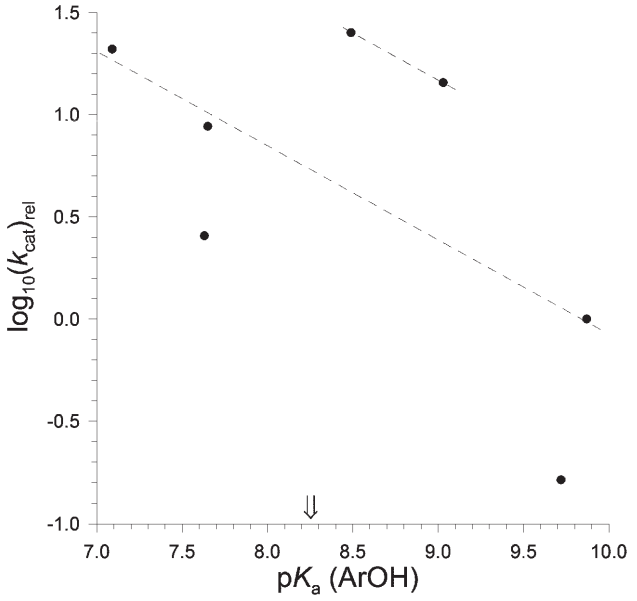



FIGURE 2: Effect of leaving group ability on $k_{cat}$ relative to that for the parent phenyl $\beta$-sialoside for the Y370G mutant sialidase. Leaving group ability represented as p$K_a$ (ArOH) is as follows: 2,6-difluorophenol (7.09); 2,5-difluorophenol (7.63); 2,3-difluorophenol (7.65); 3,5-difluorophenol (8.26); 2-fluorophenol (8.49); 3-fluorophenol (9.03); 2-fluorophenol (9.72); and phenol (9.87). The drawn lines have $\beta_{lg}$ values of $-0.46$ (see Discussion). The point for 3,5-difluorophenyl $\beta$-sialoside is not shown because of the extremely low activity of this substrate with the mutant sialidase; rather, the p$K_a$ of 3,5-difluorophenol is indicated on the $x$-axis by an arrow.

Table 2: Michaelis−Menten Kinetic Parameters of the Y370G Mutant Sialidase-Catalyzed Hydrolysis of 2-Fluorophenyl $\beta$-Sialoside as a Function of pH at 37 °C

| pH | rel ($k_{cat}$) | rel ($k_{cat}/K_m$) |
|---|---|---|
| 3.83 | 4.73 ± 0.14 | 3.48 ± 0.32 |
| 4.25 | 1.45 ± 0.13 | 1.18 ± 0.30 |
| 5.25 | 1.00[a] | 1.00[a] |
| 6.08 | 0.86 ± 0.02 | 0.70 ± 0.04 |
| 6.59 | 0.67 ± 0.03 | 0.70 ± 0.11 |

[a]Calculated value for the truncated *M. viridifaciens* Y370G mutant sialidase used in this study assuming 100% activity, $k_{cat} = 2.77 \pm 0.09\ s^{-1}$ and $k_{cat}/K_m = (1.51 \pm 0.16) \times 10^4\ M^{-1}\ s^{-1}$.

decreased by approximately 35%. Also, it was shown that this inhibition is not time dependent (slow binding of phenol to the active site pocket) by measuring the inhibition of 2FP-$\beta$Neu5Ac (50 $\mu$M) hydrolysis after incubation of up to 30 min with phenol (100 $\mu$M). The dissociation constants for the two slowest substrates (4FP-$\beta$NeuAc and 35DFP-$\beta$NeuAc) and the unsaturated acid Neu2en5Ac were estimated, by using a similar inhibition protocol with pNP-$\alpha$Neu5Ac as the substrate, to be 39, 270, and 13 $\mu$M, respectively. The Y370G mutant-catalyzed rate of hydrolysis for pNP-$\alpha$Neu5Ac did not display any significant deviation as a function of added phenol, 4-fluorophenol, or 3,5-difluorophenol. In addition, during the Y370G mutant-catalyzed hydrolysis of pNP-$\alpha$Neu5Ac (10.3 mM), which was monitored by $^1$H NMR spectroscopy, in the presence of 4-fluorophenol (10.0 mM) no visible sign (< 2%) of transfer product (4FP-$\beta$NeuAc) could be seen in the final spectrum.

## DISCUSSION

*$\beta$-Sialidase Activity of Mutant Y370G*. As reported earlier for phenyl $\beta$-sialoside with the full-sized Y370G mutant sialidase (*18*), the initial product formed in the reactions catalyzed by the active site domain Y370G mutant sialidase of all fluorinated aryl $\beta$-sialoside substrates, which undergo rapid reaction, is $\alpha$-NeuAc, and this material undergoes a subsequently facile water-catalyzed mutarotation to predominantly give the $\beta$-anomer at equilibrium (data not shown) (*26*). Thus, the minimal mechanism for the Y370G mutant sialidase-catalyzed hydrolysis of aryl $\beta$-sialosides is shown in Scheme 2. Here, the first formed Michaelis complex, in which the aryl ring is bound into the active site hole (*16*), undergoes glycosidic bond cleavage ($k_3$) to give a ternary complex from which dissociation of the $\alpha$-sialic acid and phenol(ate) products ($k_4$), presumably in a stepwise manner, results in the regeneration of active inverting enzyme that is ready to perform another catalytic cycle.

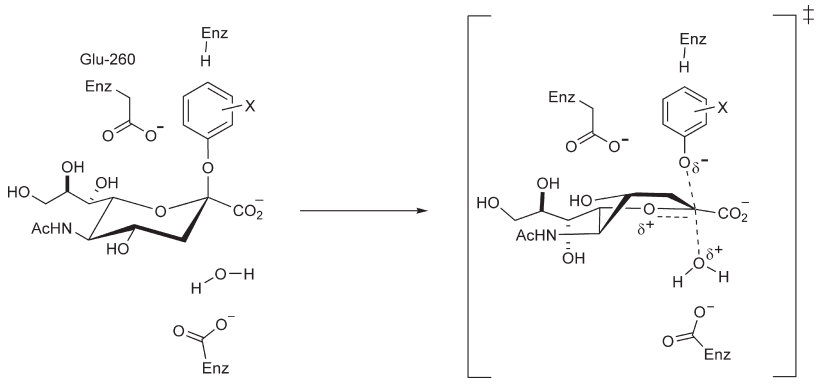
The reported catalytic constants for the full-sized Y370G mutant sialidase (*18*) are about a hundred times larger than those measured with the truncated enzyme, which was used in this study in order to avoid kinetic complications that might arise from binding of the unnatural $\beta$-substrates to the lectin domain of the full length enzyme. The size of the sialidase naturally produced by *M. viridifaciens* depends on the sialoside source that induces expression, where columinic acid gives rise to the single domain enzyme, and milk casein results in the production of the three domain protein (*27*). When expressed in *S. lividans*, the single domain *M. viridifaciens* sialidase displays about 55−60% of the activity of the full-length enzyme (*23*, *27*). Of note, the absolute magnitude of all kinetic parameters reported in the current study and in earlier investigations (*18*, *23*) relies on the assumption that all of the purified, expressed protein is pure and catalytically active. The *N*-terminal sequence results for the truncated *M. viridifaciens* mutant sialidase shows that the enzyme preparation contains a coeluting contaminant protein as well as the Y370G mutant enzyme, a factor that is at least partially responsible for the lower activity of the current enzyme preparation. That being said, the hydrolytic activity of the current Y370G mutant sialidase on six out of the eight substrates studied ($k_{cat}/K_m \geq 3 \times 10^3\ M^{-1}\ s^{-1}$), a value which is greater than that for the *V. cholerae* sialidase-catalyzed hydrolysis of phenyl $\alpha$-sialoside (*28*), suggests that its mechanism of action is a valid model for the deglycoslyation reaction of sialidase enzymes.

The hole created by mutation of the active site tyrosine residue in the single domain construct is able to accommodate a number of p$K_a$ perturbing fluorine for hydrogen atom substitutions, which are often considered to be approximately sterically neutral (Table 1). However, such replacements can result in a catastrophic loss of catalytic activity as observed for the 3,5-difluorophenyl substrate (35DFP-$\beta$Neu5Ac) which reacts about 3 orders of magnitude more slowly than the parent compound (Ph-$\beta$Neu5Ac) despite possessing a better leaving group.

It is important to remember, that unlike other reported Brønsted correlation studies on wild-type glycosidases the current Y370G mutant enzyme possesses a specific pocket in which the substrate's aryl group binds. However, given that this inverting enzyme has not been subject to evolutionary pressure it seems prudent to postulate the simplest possible kinetic scheme (Scheme 2). Thus, the plausible rate determining steps for $k_{cat}/K_m$ are either binding ($k_1$) or glycosidic cleavage ($k_3$), while $k_{cat}$ might be limited by $k_3$ or product release ($k_4$).

*Effect of Leaving Group on the Catalytic Activity*. The magnitude of $k_{cat}/K_m$ values measured for the mutant enzyme-catalyzed hydrolysis of the non-natural aryl $\beta$-sialoside substrates, which are several orders of magnitude below the diffusion

Scheme 4: Proposed Mechanism for the Y370G Mutant-Catalyzed Hydrolysis of Aryl $\beta$-Sialosides




limit (*12*), suggests that $k_3$ is the likely rate determining step. Indeed, the data displayed in Figure 1 suggests two separate Brønsted correlations with the calculated $\beta_{lg}$ value on $k_{cat}/K_m$ for the four most active substrates of $-0.46 \pm 0.02$ and that for the three other active substrates being $-0.48 \pm 0.02$ (Figure 1 solid and dashed lines, respectively). Even though fitting three data points to a line is not optimal, the identical $\beta_{lg}$ values for both fits suggests that the degree of productive binding is important in this system (*29*). With regard to binding, $K_m$ (Scheme 2: $k_2/k_1$) values for the catalyzed hydrolysis reactions can be grouped into two subsets, $K_m \approx 40$ and 200 $\mu$M. These binding interactions between the Y370G mutant sialidase pocket and the aryl groups of the substrates may be productive, and if expressed at both the Michaelis complex and the transition state for the catalyzed cleavage of **1a**−**h**, then an increase in $k_{cat}/K_m$ will result (*29*). That is, the $k_{cat}/K_m$ data is consistent with two separate transition states, which have similar degrees of glycosidic bond cleavage that likely have different productive binding modes for the variously substituted aromatic rings in the active site pocket. The $\beta_{lg}$ values when taken with the measured solvent kinetic isotope effect for 2FP-$\beta$NeuAc catalyzed hydrolysis, a value that is indistinguishable from unity ($0.88 \pm 0.24$), and the $\beta_{lg}$ value of $-1.24$ for the spontaneous hydrolysis of aryl $\beta$-sialosides (pH = 8.1, $T$ = 100 °C) (*19*) suggests that the rate-determining step for the Y370G mutant-catalyzed reaction is glycosidic bond cleavage that occurs with no significant general-acid catalysis. Also, the flatness of the $k_{cat}/K_m$ pH-rate profiles for the catalyzed hydrolyses of 2FP-$\beta$NeuAc (this work) between pH values of 4.2−6.6 (Table 2) and that reported for Ph-$\beta$NeuAc (full-sized Y370G mutant enzyme (*18*)) suggest that no catalytically important protonation change occurs over this pH range.

With regard to the Brønsted plot for $k_{cat}$ (Figure 2), given that sialic acid only weakly binds to wild-type sialidases, a mimic for the initial ternary product complex, and phenol is only a weak inhibitor of the Y370G variant sialidase (35% inhibition at 1 mM), it appears implausible that product release (Scheme 2: $k_4$) limits $k_{cat}$ because combining the $k_{cat}$ value (0.11 $s^{-1}$) with the estimated $K_i$ value for phenol binding (1.2 mM (*22*)) would require an exceptionally slow on rate for phenol binding of $\sim 10^2$ $M^{-1}$ $s^{-1}$ ($= k_{cat}/K_i$). Thus, it can be concluded that both $k_{cat}$ and $k_{cat}/K_m$ are limited by glycosidic bond cleavage ($k_3$) and that the Brønsted plot for $k_{cat}$ should show $\beta_{lg}$ correlations similar to those measured on $k_{cat}/K_m$ (two such lines of slope $-0.46$ are drawn in Figure 2). The apparent scatter in $k_{cat}$ values likely arises from nonproductive binding, which is expressed at the Michaelis complex but not the transition state (*29*). That is, the requisite loss of binding energy on progressing from the Michaelis complex to transition state results in decreasing $k_{cat}$ values but does not affect $k_{cat}/K_m$ (*29*). As noted above for Michaelis complex formation, $K_m$ values can be subdivided into two groups and that, based on the kinetic data, the tight binders, such as Ph-$\beta$NeuAc and 26DFP-$\beta$NeuAc (**1a** and **1g**), likely exhibit a nonproductive binding component during catalysis.

Indeed, with the exception of the worst substrate (35DFP-$\beta$NeuAc), which surely binds unproductively to the mutant sialidase, all $k_{cat}/K_m$ and $k_{cat}$ data are consistent with rate-limiting glycoside hydrolysis occurring via two energetically distinct transition states that may incorporate a nonproductive binding component.

*Mechanism of Action*. On the basis of the solvent KIE on $k_{cat}/K_m$ ($k_{H_2O}/k_{D_2O} = 0.88 \pm 0.24$) and the pH-rate profile data (pH values $\geq$4.25), it is likely that at the transition state, for the desialylation mimic reaction, the phenolate leaving group is departing with little or no general-acid-catalyzed assistance from either of the two residues that are within H-bonding distance to its oxygen atom (Scheme 4).

These two residues are the active site Glu-260 and Arg-276. Figure 3 shows this diad, and the various close O−O and N−O contacts to the tyrosinyl oxygen atom in the wild-type sialidase structure that contains the presumed transition state analogue inhibitor Neu2en5Ac (*21*).

If this wild-type structure reasonably mimics the transition state for the Y370G mutant-catalyzed hydrolysis of aryl $\beta$-sialosides (Scheme 4), then the close proximity of the nascent phenolate and glutamate residues may result in the deprotonation of arginine-276. At the moment, it is impossible to conclude whether this catalyzed hydrolysis reaction proceeds either via a discrete oxacarbenium ion ($D_N{}^*A_N$; $S_N1$) or by direct displacement ($A_ND_N$; $S_N2$). However, if the water nucleophile has started to form a bond to the anomeric carbon center, then this attack must be occurring with no general-base catalysis ($k_{H_2O}/k_{D_2O} = 0.88 \pm 0.24$). Of note, an inverse solvent deuterium KIE would be consistent with an uncatalyzed water attack at the reaction transition state (*30*). Unfortunately, the precision of the HPLC monitored substrate depletion kinetics is inadequate to allow such a conclusion to be made.

Listed in Table 3 are a catalog of reported Brønsted $\beta_{lg}$ values on $k_{cat}$ and $k_{cat}/K_m$ for a series of sialidases.

Although the current study involves an artificial catalyst, which hydrolyzes unnatural $\beta$-sialosidic linkages, some of the mechanistic conclusions are remarkably similar to those from studies on natural sialidases: an observation that in itself suggests that the multidimensional free energy surface for sialyl transfer reactions is intrinsically flat in the vicinity of the enzymatic transition state(s). For example, the Y370G mutant *M. viridifaciens* enzyme does

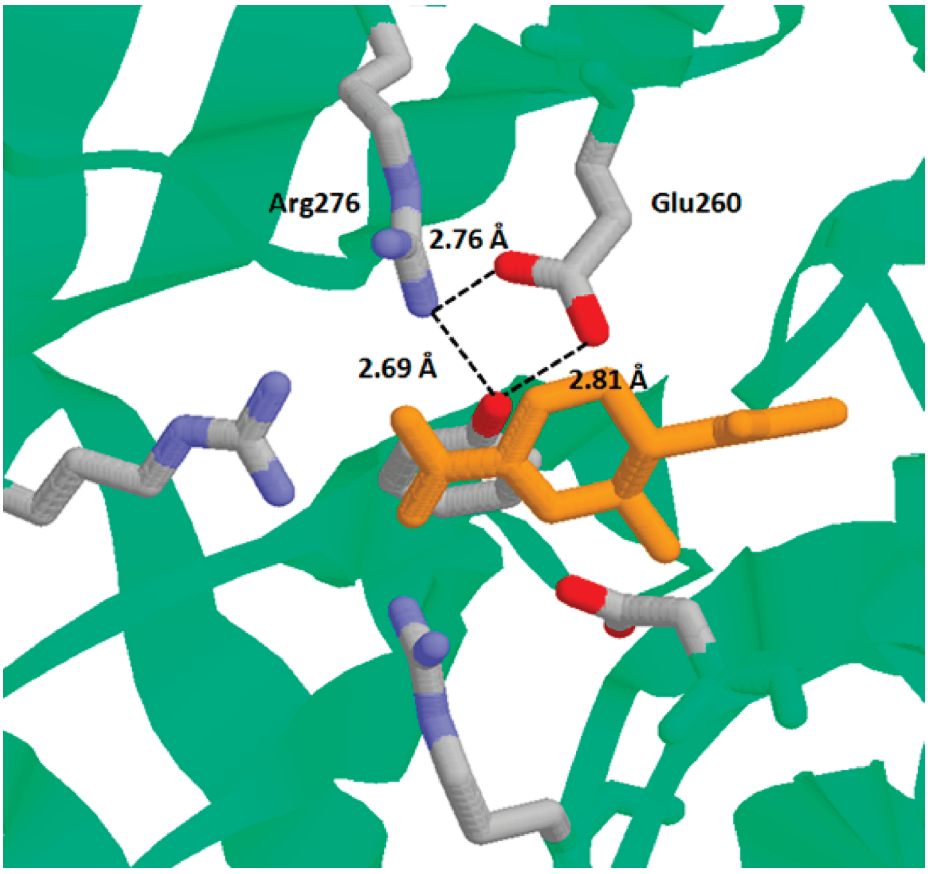



FIGURE 3: Illustration of a possible product complex formed during the Y370G mutant sialidase-catalyzed reactions of aryl $\beta$-sialosides. Wild-type MvNA structure (blue-green ribbon) with Neu2en5Ac (orange) bound (PDB code 1EUS); both the methylene carbon of the wild-type Y370 residue (to show a bound phenol) and the glycerol side chain of Neu2en5Ac have been removed for clarity. Also shown are the short O−O and O−N distances taken from the wild-type MvNA structure. The three arginine residues shown make up the strictly conserved triad that is found in all known *exo*-sialidases.

Table 3: Brønsted $\beta_{lg}$ Values for Sialidase-Catalyzed Reactions

| enzyme source | $\beta_{lg}$ value ($k_{cat}/K_m$) | $\beta_{lg}$ value ($k_{cat}$) | p$K_a$ (ROH)[a] | ref |
|---|---|---|---|---|
| *Vibrio cholerae* | −0.73 ($r$ = −0.93) | −0.25 ($r$ = −0.95) | 7.2−9.9 | *28* |
| influenza A[b] | −0.45 ($r$ = −0.93) | −0.11 ($r$ = −0.81) | 7.2−9.9 | *32* |
| *Salmonella typhimurium* | −0.80 ($r$ = +0.96) | −0.53 ($r$ = −0.95) | 7.2−9.9 | *32* |
| *Micromonospora viridifaciens* | −0.30 ± 0.04 | +0.02 ± 0.03 | 7.2−13.8 | *6* |
| *M. viridifaciens* − Y370G mutant[c] | −0.80 ± 0.08 | −0.63 ± 0.05 | 7.2−13.6 | *16* |
| *M. viridifaciens* − Y370G mutant[d] | −0.46 ± 0.02 | | 7.1−9.9 | this study |

[a]Range of p$K_a$ values for the conjugate acids of the leaving groups used in the correlation. [b]Subtype N2. [c]Catalyzed hydrolysis of α-sialosides with inversion of configuration. [d]Catalyzed hydrolysis of aryl $\beta$-sialosides with inversion of configuration.

not need proton catalysis to hydrolyze aryl $\beta$-sialosides, and the *Vibrio cholerae* sialidase-catalyzed reactions of α-sialosides also occur with little or no protonation of the leaving group, even for carbohydrate-based aglycones (*31*). Such a conclusion may be a consequence of the active sites of sialidases containing a large number of acid/base residues, which likely results in substantial electrostatic stabilization of the various enzymatic transition states. For example, within 7.5 Å of the nucleophilic oxygen (Tyr-370) in the *M. viridifaciens* enzyme there are 5 acidic (3 Asp +2 Glu), 4 basic (4 Arg), 3 hydroxylic (2 Ser +1 Thr), 2 amidic (2 Asn), and 2 hydrophobic (1 Ile +1 Phe) residues (*21*).

## CONCLUSIONS

The Y370G inverting mutant enzyme possesses $\beta$-sialidase activity with several fluorophenyl sialosides. The rate-determining step on $k_{cat}/K_m$ and $k_{cat}$ involves glycosidic bond cleavage that occurs with little or no general-acid catalysis. The enzyme-catalyzed transfer reactions of sialosides and presumably those of other ketoaldonic acid-based carbohydrates appear to be mechanistically distinct from the majority of aldopyranoside processing enzymes that involve nucleophilic reactions at the anomeric center in that glycosidic bond cleavage, even with natural, unactivated, leaving groups, occurs in advance of rather than concerted with the associated general-acid catalytic event.

## ACKNOWLEDGMENT

We thank the two anonymous referees for their many helpful comments concerning this manuscript.

## SUPPORTING INFORMATION AVAILABLE

A SDS−PAGE gel for the recombinant active site domain Y370G mutant sialidase and $^1$H and $^{13}$C NMR spectra for all

new aryl β-D-sialosides (**1b**−**1h**). This material is available free of charge via the Internet at http://pubs.acs.org.

## REFERENCES


1. Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009) The Carbohydrate-Active enZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res. 37*, D233–D238.
2. Davies, G., Sinnott, M. L., Withers, S. G. (1998) Glycosyl Transfer, in Comprehensive Biological Catalysis (Sinnott, M. L., Ed.) pp 119−209, Academic Press, San Diego, CA.
3. Yip, V. L. Y., and Withers, S. G. (2006) Mechanistic analysis of the unusual redox-elimination sequence employed by *Thermotoga maritima* BgIT: A 6-phospho-beta-glucosidase from glycoside hydrolase family 4. *Biochemistry 45*, 571–580.
4. Tews, I., vanScheltinga, A. C. T., Perrakis, A., Wilson, K. S., and Dijkstra, B. W. (1997) Substrate-assisted catalysis unifies two families of chitinolytic enzymes. *J. Am. Chem. Soc. 119*, 7954–7959.
5. Vocadlo, D. J., and Davies, G. J. (2008) Mechanistic insights into glycosidase chemistry. *Curr. Opin. Chem. Biol. 12*, 539–555.
6. Watson, J. N., Dookhun, V., Borgford, T. J., and Bennet, A. J. (2003) Mutagenesis of the conserved active-site tyrosine changes a retaining sialidase into an inverting sialidase. *Biochemistry 42*, 12682–12690.
7. Watts, A. G., Oppezzo, P., Withers, S. G., Alzari, P. M., and Buschiazzo, A. (2006) Structural and kinetic analysis of two covalent sialosyl-enzyme intermediates on *Trypanosoma rangeli* sialidase. *J. Biol. Chem. 281*, 4149–4155.
8. Amaya, M. F., Watts, A. G., Damager, T., Wehenkel, A., Nguyen, T., Buschiazzo, A., Paris, G., Frasch, A. C., Withers, S. G., and Alzari, P. M. (2004) Structural insights into the catalytic mechanism of *Trypanosoma cruzi trans*-sialidase. *Structure 12*, 775–784.
9. Watts, A. G., Damager, I., Amaya, M. L., Buschiazzo, A., Alzari, P., Frasch, A. C., and Withers, S. G. (2003) *Trypanosoma cruzi trans*-sialidase operates through a covalent sialyl-enzyme intermediate: Tyrosine is the catalytic nucleophile. *J. Am. Chem. Soc. 125*, 7532–7533.
10. Watson, J. N., Newstead, S., Dookhun, V., Taylor, G., and Bennet, A. J. (2004) Contribution of the active site aspartic acid to catalysis in the bacterial neuraminidase from *Micromonospora viridifaciens*. *FEBS Lett. 577*, 265–269.
11. Lowry, T. H., and Richardson, K. S. (1987) Mechanism and Theory in Organic Chemistry, 3rd ed., Harper & Row, New York.
12. Fersht, A. (1985) Enzyme Structure and Mechanism, 2nd ed., W.H. Freeman, New York.
13. Banait, N. S., and Jencks, W. P. (1991) Reactions of anionic nucleophiles with α-D-glucopyranosyl fluoride in aqueous solution through a concerted, $A_ND_N$ ($S_N2$) mechanism. *J. Am. Chem. Soc. 113*, 7951–7958.
14. Huang, X., Surry, C., Hiebert, T., and Bennet, A. J. (1995) The hydrolysis of 2-deoxy-β-D-glucopyranosyl pyridinium salts. *J. Am. Chem. Soc. 117*, 10614–10621.
15. Greig, I. R., Macauley, M. S., Williams, I. H., and Vocadlo, D. J. (2009) Probing synergy between two catalytic strategies in the glycoside hydrolase *O*-GlcNAcase using multiple linear free energy relationships. *J. Am. Chem. Soc. 131*, 13415–13422.
16. Newstead, S., Watson, J. N., Knoll, T. L., Bennet, A. J., and Taylor, G. (2005) Structure and mechanism of action of an inverting mutant sialidase. *Biochemistry 44*, 9117–9122.
17. Watson, J. N., Newstead, S., Narine, A., Taylor, G., and Bennet, A. J. (2005) Two nucleophilic mutants of the *Micromonospora viridifaciens* sialidase operate with retention of configuration via two different mechanisms. *ChemBioChem 6*, 1999–2004.
18. Watson, J. N., Indurugalla, D., Cheng, L., Narine, A. A., and Bennet, A. J. (2006) The hydrolase and transferase activity of an inverting mutant sialidase using non-natural β-sialoside substrates. *Biochemistry 45*, 13264–13275.
19. Dookhun, V., and Bennet, A. J. (2005) Unexpected stability of aryl β-*N*-acetylneuraminides in neutral solution: Biological implications for sialyl transfer reactions. *J. Am. Chem. Soc. 127*, 7458–7465.
20. Sharma, M. N., and Eby, R. (1984) Synthesis and conformational studies of 2-β-chloro, 2-α-fluoro, and 2-β-fluoro derivatives of 2-deoxy-*N*-acetylneuraminic acid. *Carbohydr. Res. 127*, 201–210.
21. Gaskell, A., Crennell, S., and Taylor, G. (1995) The three domains of a bacterial sialidase: A β-propeller, an immunoglobulin module and a galactose-binding jellyroll. *Structure 3*, 1197–1205.
22. Segel, I. H. (1975) Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems, Wiley, New York.
23. Sakurada, K., Ohta, T., and Hasegawa, M. (1992) Cloning, expression, and characterization of the *Micromonospora viridifiaciens* neuraminidase gene in *Streptomyces lividans*. *J. Bacteriol. 174*, 6896–6903.
24. Schowen, R. L. (1977) Solvent Isotope Effects on Enzymic Reactions, in Isotope Effects on Enzyme-Catalyzed Reactions (Cleland, W. W., O'Leary, M. H., and Northrop, D. B., Eds.) pp 64−99, University Park Press, Baltimore, MD.
25. Taylor, J. R. (1982) An Introduction to Error Analysis: The Study of Uncertainties in Physical Measurements, University Science Books, Mill Valley, CA.
26. Klepach, T., Carmichael, I., and Serianni, A. S. (2008) $^{13}$C-Labeled *N*-acetyl-neuraminic acid in aqueous solution: Detection and quantification of acyclic keto, keto hydrate, and enol forms by $^{13}$C NMR spectroscopy. *J. Am. Chem. Soc. 130*, 11892–11900.
27. Aisaka, K., Igarashi, A., and Uwajima, T. (1991) Purification, crystallization, and characterization of neuraminidase from *Micromonospora viridifaciens*. *Agric. Biol. Chem. 55*, 997–1004.
28. Guo, X., and Sinnott, M. L. (1993) A kinetic-isotope-effect study of catalysis by *Vibrio cholerae* neuraminidase. *Biochem. J. 294*, 653–656.
29. Richard, J. P., Westerfeld, J. G., and Lin, S. (1995) Structure−reactivity relationships for β-galactosidase (*Escherichic coli*, *lac Z*). 1. Brønsted parameters for cleavage of alkyl β-D-galactopyranosides. *Biochemistry 34*, 11703–11712.
30. Alvarez, F. J., Schowen, R. L. (1987) Mechanistic Deductions from Solvent Isotope Effects, in Secondary and Solvent Isotope Effects: Isotopes in Organic Chemistry, pp 1−60, Elsevier, Amsterdam, The Netherlands.
31. Chan, J., Lewis, A. R., Gilbert, M., Karwaski, M. F., and Bennet, A. J. (2010) A direct NMR method for the measurement of competitive kinetic isotope effects. *Nat. Chem. Biol. 6*, 405–407.
32. Guo, X., Laver, W. G., Vimr, E., and Sinnott, M. L. (1994) Catalysis by two sialidases with the same protein fold but different stereochemical courses: A mechanistic comparison of the enzymes from influenza A virus and *Salmonella typhimurium*. *J. Am. Chem. Soc. 116*, 5572–5578.