The Hydrolase and Transferase Activity of an Inverting Mutant Sialidase Using Non-natural β-Sialoside Substrates†

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ABSTRACT: The Y370G inverting mutant sialidase from *Micromonospora viridifaciens* possesses β-sialidase activity with phenyl β-sialoside (Ph-βNeuAc) to give α-sialic acid as the first formed product. The derived catalytic rate constants for k_{cat} and k_{cat}/K_m are 13.3 ± 0.3 and (2.9 ± 0.3) × 10^5 M^{-1} s^{-1}, respectively. This enzyme is highly specific for the phenyl substrate, with substituted phenyl and thiophenyl leaving groups having k_{cat} values that are at least 1000-fold lower. In addition, the Y370G mutant can transfer the sialic acid moiety from Ph-βNeuAc to lactose in yields of up to 13%. Greater than 90% of the sialyllactose product formed in the coupling reactions is the α-2,6-isomer. A library encoding 6 × 10^5 different sialidases was constructed by mutating Y370, E260, T309, N310, and N311, residues that include and are proximal the catalytic tyrosine residue. A total of 2628 individuals were screened for hydrolytic activity against 4-nitrophenyl 2-thio-β-sialoside and 4-methylumbelliferyl β-sialoside. However, none of the mutants screened possessed a significant activity against either of the β-sialosides.

Sialic acids are a unique family of nine-carbon keto sugars that are typically found as the terminal sugar, α-linked to various glycoconjugates (*1*, *2*). Unlike other monosaccharides, sialic acids have an extensive range of modifications that are species-specific (*3*). It is this structural diversity and their terminal location that undoubtedly confers sialic acids with such a variety of affects in molecular and cellular recognition, including roles that range from facilitating influenza and cholera pathogenesis (*4*, *5*) to influencing the clearance rate of glycoprotein-based therapeutics (*6*).

Carbohydrate-bound β-sialosides do not occur in nature; indeed, the only reported β-sialoside is the sialyl-transferase donor, CMP-β-D-*N*-acetylneuraminide (CMP-βNeuAc)[1] (*3*), although in the free carbohydrate, βNeuAc is the major stereoisomer in the equilibrium mixture (*7*). All known natural sialidases and *trans*-sialidases act on α-sialosides, which are made by sialyl transferases using the CMP-β donor, to release α-sialic acids as the first product (*8*, *9*). For *exo*-sialidases (*10*, *11*) and *trans*-sialidases (*12*, *13*), a covalently linked β-sialyl intermediate is formed with the tyrosine nucleophile (β-NeuAc-OTyr), an intermediate that is subsequently attacked by an incoming ROH molecule to give the product, αNeuAc-OR (with ROH being water for the hydrolases). The presently accepted mechanism for retaining *exo*-sialidases is shown in Scheme 1 (*10*, *11*, *14*).

It has previously been reported that replacement of the nucleophilic tyrosine in the sialidase from *M. viridifaciens* causes a change in the mechanism (*10*, *15*, *16*). Specifically, the hole created by substitution of the tyrosine with certain smaller residues (see Figure 1 for an example) can allow a water molecule in the enzymatic active site to attack an α-sialoside from the opposite face, thus releasing β-NeuAc as the first product via a mechanism that involves a single inversion of the configuration rather than the two commonly associated with the wild-type retaining sialidases (Scheme 2). These mutant enzymes are not as catalytically efficient as the wild-type, but the level of activity observed is still impressive. What is more intriguing is that this change in the mechanism is not observed by replacing the tyrosine with all 20 amino acids; for example, phenylalanine or glutamate replacement of the tyrosine (Y370F and Y370E) generates less active retaining enzymes, where an alternate residue likely acts as a nucleophile in place of the tyrosine (*16*).

In 2005, Dookhun and Bennet reported a kinetic and product study on the nonenzymatic hydrolysis reactions of a panel of aryl β-sialosides (*17*). In this study, it was shown that between pH values of 5−11 α-sialosides underwent hydrolysis over 100 times faster than the corresponding β-sialoside (*17*).

The present study details an investigation of the ability of inverting Y370 mutants to accommodate more than a simple water molecule in the hole created by replacement of the tyrosine. The objective here is to design mutant inverting sialidases that can hydrolyze these β-sialoside substrates by fitting the leaving group in the hole that is usually occupied by the tyrosinyl ring. Finally, these inverting mutants with β-sialoside activity would ultimately be tested for trans-

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[1] Abbreviations: CMP-βNeuAc, CMP-β-D-*N*-acetylneuraminide; DANA, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid; DCP-βNeuAc, 3,4-dichlorophenyl β-D-*N*-acetylneuraminide; FNP-βNeuAc, 3-fluoro-4-nitrophenyl β-D-*N*-acetylneuraminide; MU-αNeuAc, 4-methylumbelliferyl α-D-*N*-acetylneuraminide; MU-βNeuAc, 4-methylumbelliferyl β-D-*N*-acetylneuraminide; MvNA, *Micromonospora viridifaciens* neuraminidase; Ph-αNeuAc, phenyl α-D-*N*-acetylneuraminide; Ph-βNeuAc, phenyl β-D-*N*-acetylneuraminide; pNP-αNeuAc, *p*-nitrophenyl α-D-*N*-acetylneuraminide; pNP-βNeuAc, *p*-nitrophenyl β-D-*N*-acetylneuraminide; pNP-*S*-βNeuAc, *p*-nitrophenyl 2-thio-β-D-*N*-acetylneuraminide.

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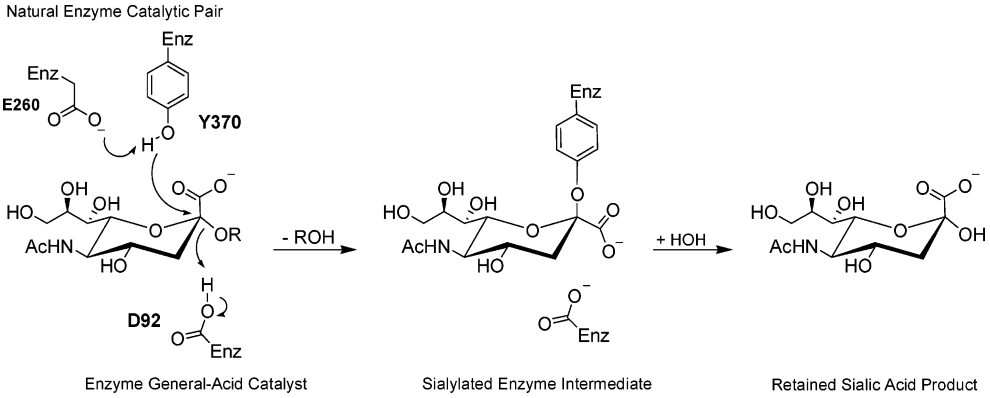
Scheme 1: Mechanism for Wild-Type Sialidases



glycosylation activity, because these mutants are very poor catalysts with the natural, unactivated substrates [i.e., sialyllactose (*10*, *15*)], and would consequently display virtually no product degradation.

MATERIALS AND METHODS

Materials. All chemicals were of analytical grade or better and were purchased from Sigma-Aldrich unless otherwise noted. All restriction endonucleases and DNA-modification enzymes were purchased from New England BioLabs (Beverly, MA). All DNA manipulations were carried out according to standard procedures (*18*). 4-Methylumbelliferyl α-D-*N*-acetylneuraminide (MU-αNeuAc) was purchased from Rose Scientific (Edmonton, Canada).

Substrate Synthesis. Phenyl β-D-*N*-acetylneuraminide (Ph-βNeuAc) and *p*-nitrophenyl β-D-*N*-acetylneuraminide (pNP-βNeuAc) were made according to a literature procedure (*17*). Nuclear magnetic resonance (NMR) spectra were acquired on a Varian Unity-500 spectrometer. Chemical shifts are reported in parts per million downfield from the signal for tetramethylsilane (TMS). The residual signals from deuterated chloroform and 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.

Methyl [4-Methylumbelliferyl (5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulopyranosyl)]onate. To activated 4 Å molecular sieves was added a mixture of 4-methylumbelliferone (893 mg, 5.1 mmol, 5 equiv) and sialosyl fluoride (*19*) (500 mg, 1.0 mmol, 1 equiv), and the resultant mixture was dried under vacuum for 30 min. The mixture was then placed under N_2 atmosphere; dry CH_2Cl_2 (25 mL) was added; and the resultant mixture was stirred for 1 h. Then, BF_3·OEt_2 (0.51 mL, 4.1 mmol, 4 equiv) was added at −20 °C, and the mixture was stirred overnight. The mixture was filtered with the aid of a Celite pad, and the solid residue was washed thoroughly with CH_2Cl_2. The organic layer was washed with saturated $NaHCO_3$ (100 mL), water (100 mL), and brine (100 mL), and the resulting solution was dried over anhydrous Na_2SO_4. A syrup was obtained after evaporation of the solvent, and this material was purified by flash-column chromatography using ethyl acetate/hexane (5:1) as the eluent to afford fully protected 4-methylumbelliferyl β-D-*N*-acetylneuraminide (MU-βNeuAc) as a white powder (219 mg, 33% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 11.78, 1.88, 2.06, 2.13, 2.17 (5× s, 15 H, CH_3), 2.03 (m, 1 H, H3a), 2.40 (s, 3 H, CH_3), 2.67 (dd, 1 H, $J_{3e,3a}$ = 13.0 Hz, $J_{3e,4}$ = 4.5 Hz, H3e), 3.78 (s, 3 H, −OCH_3), 4.04 (dd, 1 H, $J_{6,5}$ = 10.5 Hz, $J_{6,7}$ = 2.0 Hz, H6), 4.14 (dd, 1 H, $J_{9a,9b}$ = 12.5 Hz, $J_{9a,8}$ = 7.0 Hz, H9a), 4.24 (q, 1 H, $J_{5,4} + J_{5,6} + J_{5,NH}$ = 32.0 Hz, H5), 4.67 (dd, 1 H, $J_{9b,9a}$ = 12.5 Hz, $J_{9b,8}$ = 2.5 Hz, H9b), 4.92 (ddd, 1 H, $J_{8,9a}$ = 7.0 Hz, $J_{8,9b}$ = 2.5 Hz, $J_{8,7}$ = 4.5 Hz, H8), 5.25

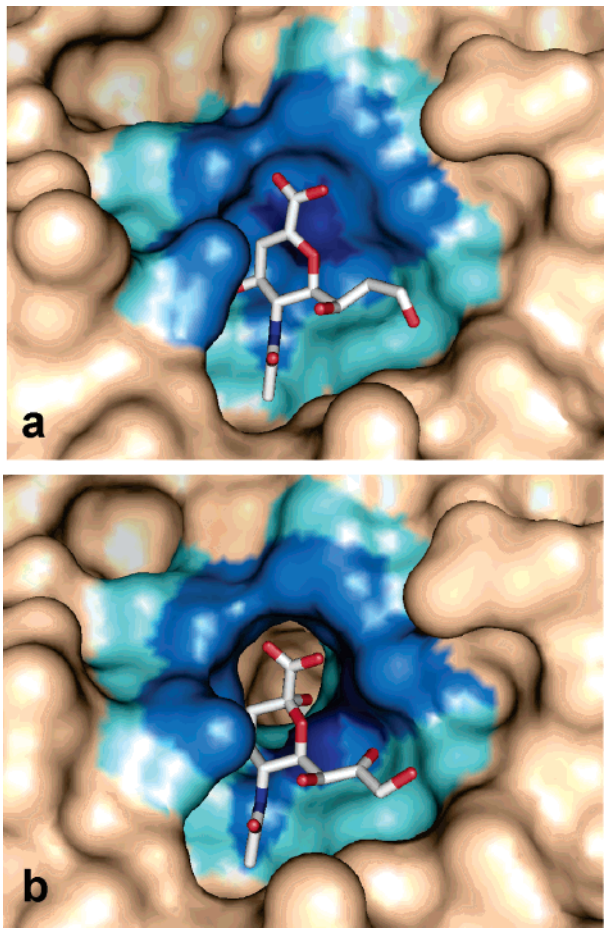
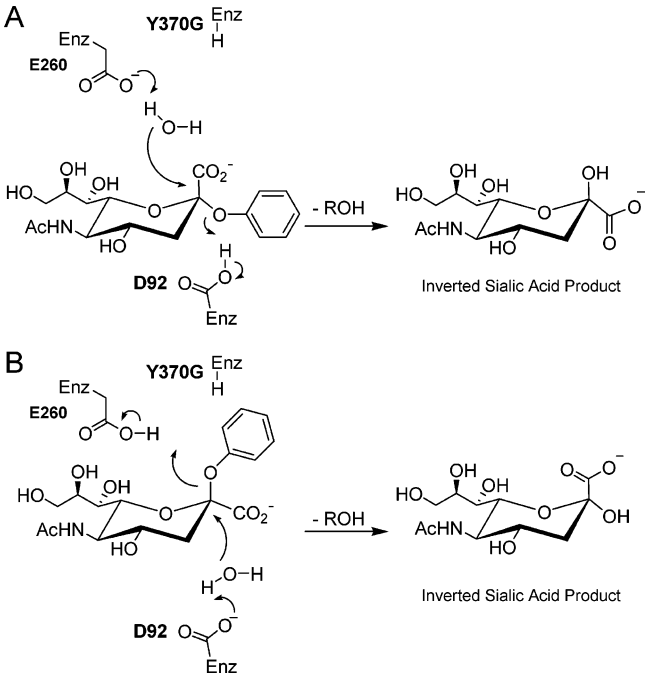


FIGURE 1: Illustration of the hole created by the substitution of Y370 by a glycine residue. (a) Wild-type MvNA structure with DANA bound (PDB code 1EUS). (b) Y370G mutant MvNA structure with βNeuAc bound (PDB code 2BER). Ligand shown as sticks, and protein shown as a surface (wheat). Color divisors are 4, 6, and 8 Å from the C2 atom of the ligand (blue, marine, and cyan, respectively).

Scheme 2: Proposed Mechanism for Y370G-Catalyzed Hydrolysis of (A) Ph-αNeuAc and (B) Ph-*β*NeuAc



(d, 1 H, $J_{NH,5}$ = 10.0 Hz, N−H), 5.35 (dd, 1 H, $J_{7,8}$ = 4.0 Hz, $J_{7,6}$ = 2.0 Hz, H7), 5.48 (td, 1 H, $J_{4,3e}$ = 4.5 Hz, $J_{4,3a}$ = $J_{4,5}$ = 11.0 Hz, H4), 6.18, 6.93, 6.96, 7.50 (4 H, Ar−H). ^{13}C NMR (125 MHz, $CDCl_3$) *δ*: 18.8, 20.9, 21.0, 21.1, 23.3 (6 C, CH_3), 38.6 (C3), 49.2 (C5), 53.8 (−OCH_3), 62.2 (C9), 68.3 (C7), 68.4 (C4), 72.3 (C8), 73.1 (C6), 99.8 (C2), 104.6, 113.6, 113.7, 115.7, 126.4, 152.2, 154.9, 156.8 (8 C, Ar−C), 161.0 (C═O), 167.0 (C1), 170.3, 170.5, 170.7, 171.1, 171.2 (5× C═O). Anal. Calcd for $C_{30}H_{35}NO_{15}$: C, 55.47; H, 5.43; N, 2.16. Found: C, 55.21; H, 5.53; N, 1.99.

4-Methylumbelliferyl (5-acetamido-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulopyranosylonic Acid) (MU-βNeuAc). To a solution of the peracetylated sialoside (70 mg, 0.11 mmol, 1 equiv) in dry methanol (5 mL) was added a methanolic sodium methoxide solution (5 mL) and stirred for 30 min at room temperature. Then, the solution was neutralized by adding Amberlite IR-120 (H^+ form). The resulting solution was filtered, and the resin was washed several times with methanol. The solutions were combined and evaporated to give a solid residue that was subsequently dissolved in a solution of THF/H_2O (3:1, 4 mL), which was maintained at 0 °C. Then, LiOH·H_2O (23 mg, 0.55 mmol, 5 equiv) was added, and the resultant solution was stirred for 15 min. The solution was then neutralized by adding Amberlite IR-120 (H^+ form) and filtered. The resin was washed several times with methanol. The solutions were combined and evaporated to give a solid residue that was purified by flash-column chromatography using ethyl acetate/methanol/water (10:2:1). The fractions that contained the product were combined and concentrated. The concentrated aqueous solution was then lyophilized to obtain MU-*β*NeuAc as a white powder (48 mg, 96% yield). mp decomposed at 147 °C. $[\alpha]_D^{20}$ = −82.0 (*c* 1.13, H_2O). ^{1}H NMR (500 MHz, D_2O) *δ*: 1.87 (dd, 1 H, $J_{3a,3e}$ = 13.5 Hz, $J_{3a,4}$ = 11.5 Hz, H3a), 2.02, 2.41 (2× s, 6 H, CH_3), 2.59 (dd, 1 H, $J_{3e,3a}$ = 13.5 Hz, $J_{3e,4}$ = 5.0 Hz, H3e), 3.49−3.55 (m, 2H, H7, H9a), 3.67 (dd, 1 H, $J_{9b,9a}$ = 12.0 Hz, $J_{9b,8}$ = 3.0 Hz, H9b), 3.73 (ddd, 1 H, $J_{8,7}$ = 8.5 Hz, $J_{8,9a}$ = 5.5 Hz, $J_{8,9b}$ = 3.0 Hz, H8), 3.79 (m, 1 H, H6), 4.03 (t, 1 H, $J_{5,6}$ = $J_{5,4}$ = 10.5 Hz, H5), 4.27 (td, 1 H, $J_{4,3e}$ = 5.0 Hz, $J_{4,3a}$ = $J_{4,5}$ = 11.0 Hz, H4), 6.21, 7.06, 7.09, 7.68 (4 H, Ar−H). ^{13}C NMR (125 MHz, D_2O) *δ*: 18.0, 22.2 (2 C, CH_3), 40.5 (C3), 51.8 (C5), 63.0 (C9), 66.6 (C4), 68.1 (C7), 70.6 (C6), 71.7 (C8), 101.2 (C2), 104.4, 111.1, 114.5, 115.0, 126.5, 153.9, 156.5, 157.6 (8 C, Ar−C), 164.9 (C═O), 175.0 (2 C, C1, C═O). HRMS−FAB (*m*/*z*): [M − H^+] calcd for $C_{21}H_{25}NO_{11}$, 466.1328; found, 466.1358.

3,4-Dichlorophenyl (5-acetamido-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulopyranosylonic Acid) (DCP-βNeuAc). To activated 4 Å molecular sieves was added a mixture of 3,4-dichlorophenol (826 mg, 5.1 mmol, 5 equiv) and sialosyl fluoride (*19*) (500 mg, 1.0 mmol, 1 equiv), and the resultant mixture was dried under vacuum for 30 min. The mixture was then placed under N_2 atmosphere; dry CH_2Cl_2 (25 mL) was added; and the resultant mixture was stirred for 1 h. Then, BF_3·OEt_2 (0.51 mL, 4.1 mmol, 4 equiv) was added, and the mixture was stirred overnight at room temperature. Then, the mixture was filtered with the aid of a Celite pad, and the solid residue was washed thoroughly with CH_2Cl_2. The organic layer was washed with saturated $NaHCO_3$ (100 mL), water (100 mL), and brine (100 mL), and the resulting solution was dried over anhydrous Na_2SO_4. A syrup was obtained after evaporation of the solvent, and this material was purified by flash-column chromatography using ethyl acetate/hexane (5:1) as the eluent to afford the peracetylated methyl ester of DCP-*β*NeuAc as well as the product from elimination as an inseparable mixture. To a solution of the above mixture in dry methanol (15 mL) was added a methanolic sodium methoxide solution (15 mL) and stirred for 30 min at room temperature. Then, the solution was neutralized by adding Amberlite IR-120 (H^+ form). The resulting solution was filtered, and the resin was washed several times with methanol. A yellow-color syrup was obtained after evaporation of the solvent, and this material was purified by flash-column chromatography using $CHCl_3$/CH_3OH (5:1) as the eluent to afford the methyl ester of DCP-*β*NeuAc as a white powder (310 mg, 66% over two steps). ^{1}H NMR (500 MHz, D_2O) *δ*: 1.91 (dd, 1 H, $J_{3a,3e}$ = 13.0 Hz, $J_{3a,4}$ = 11.5 Hz, H3a), 2.02 (s, 3 H, CH_3), 2.61 (dd, 1 H, $J_{3e,3a}$ = 13.0 Hz, $J_{3e,4}$ = 4.5 Hz, H3e), 3.50 (m, 1 H, H7), 3.61 (dd, 1 H, $J_{9b,9a}$ = 12.0 Hz, $J_{9b,8}$ = 5.0 Hz, H9a), 3.68 (ddd, 1 H, $J_{8,9a}$ = 5.0 Hz, $J_{8,9b}$ = 2.5 Hz, $J_{8,7}$ = 7.5 Hz, H8), 3.76 (s, 1 H, −OCH_3), 3.74−3.77 (m, 1 H, H9b), 3.85 (m, 1 H, H6), 4.01 (t, 1 H, $J_{5,6}$ = $J_{5,4}$ = 10.5 Hz, H5), 4.24 (td, 1 H, $J_{4,3e}$ = 4.5 Hz, $J_{4,3a}$ = $J_{4,5}$ = 11.0 Hz, H4), 6.98, 7.24, 7.43 (3 H, Ar−H). ^{13}C NMR (125 MHz, D_2O) *δ*: 216 (CH_3), 41.2 (C3), 52.3 (−OCH_3), 52.5 (C5), 63.9 (C9), 66.0 (C4), 69.0 (C7), 70.2 (C8), 72.4 (C6), 100.8 (C2), 117.9, 120.4, 126.0, 130.6, 132.3, 154.1 (6 C, Ar−C), 168.4 (C═O), 173.5 (C1). The methyl ester of DCP-*β*NeuAc (250 mg, 0.54 mmol, 1 equiv) was dissolved in a solution of THF/H_2O (3:1, 20 mL) that was maintained at 0 °C. Then, LiOH·H_2O (113 mg, 2.7 mmol, 5 equiv) was added, and the resultant solution was stirred for 15 min. The solution was then neutralized by adding Amberlite IR-120 (H^+ form) and filtered. The resin was washed several times with methanol. The solutions were combined and evaporated to give a solid residue that was purified by flash-column chromatography using ethyl acetate/methanol/water (10:2:1). The fractions that contained the product were combined and concentrated. The concentrated

aqueous solution was then lyophilized to obtain DCP-βNeuAc as a white powder (170 mg, 70%). mp decomposed at 207 °C. $[\alpha]_D^{20} = -61.4$ (*c* 1.03, H_2O). ^{1}H NMR (500 MHz, D_2O) δ: 1.83 (t, 1 H, $J_{3a,3e} = J_{3a,4} = 13.5$ Hz, H3a), 2.02 (s, 3 H, CH_3), 2.53 (dd, 1 H, $J_{3e,3a} = 13.5$ Hz, $J_{3e,4} = 5.0$ Hz, H3e), 3.49 (dd, 1 H, $J_{9a,9b} = 12.0$ Hz, $J_{9a,8} = 5.5$ Hz, H9a), 3.54 (m, 1 H, H7), 3.61 (dd, 1 H, $J_{9b,9a} = 12.0$ Hz, $J_{9b,8} = 3.0$ Hz, H9b), 3.70−3.75 (m, 2 H, H6, H8), 4.01 (t, 1 H, $J_{5,6} = J_{5,4} = 10.5$ Hz, H5), 4.22 (td, 1 H, $J_{4,3e} = 5.0$ Hz, $J_{4,3a} = J_{4,5} = 11.0$ Hz, H4), 6.98, 7.25, 7.40 (3 H, Ar−H). ^{13}C NMR (125 MHz, D_2O) δ: 22.2 (CH_3), 40.39 (C3), 51.8 (C5), 62.9 (C9), 66.7 (C4), 67.9 (C7), 70.8 (C6), 71.6 (C8), 101.0 (C2), 116.8, 118.7, 124.8, 130.7, 132.0 153.4 (6 C, Ar−C), 174.2 (C═O), 175.0 (C1). HRMS−FAB (*m*/*z*): [M − H^+] calcd for $C_{17}H_{20}NO_9Cl_2$, 452.0521; found, 452.0527.

3-Fluoro-4-nitorophenyl (5-acetamido-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulopyranosylonic Acid) (FNP-βNeuAc). To activated 4 Å molecular sieves was added a mixture of 3-fluoro-4-nitorophenol (796 mg, 5.1 mmol, 5 equiv) and sialosyl fluoride (*19*) (500 mg, 1.0 mmol, 1 equiv), and the resultant mixture was dried under vacuum for 30 min. The mixture was then placed under N_2 atmosphere; dry CH_2Cl_2 (25 mL) was added; and the resultant mixture was stirred for 1 h. Then, $BF_3{\cdot}OEt_2$ (0.51 mL, 4.1 mmol, 4 equiv) was added, and the mixture was stirred overnight at room temperature. Then, the mixture was filtered with the aid of a Celite pad, and the solid residue was washed thoroughly with CH_2Cl_2. The organic layer was washed with saturated $NaHCO_3$ (100 mL), water (100 mL), and brine (100 mL), and the resulting solution was dried over anhydrous Na_2SO_4. A syrup was obtained after evaporation of the solvent, and this material was purified by flash-column chromatography using ethyl acetate/hexane (5:1) as the eluent to afford the product as well as the product from elimination as an inseparable mixture. To a solution of the above mixture in dry methanol (15 mL) was added a methanolic sodium methoxide solution (15 mL) and stirred for 30 min at room temperature. Then, the solution was neutralized by adding Amberlite IR-120 (H^+ form). The resulting solution was filtered, and the resin was washed several times with methanol. A yellow-colored syrup was obtained after evaporation of the solvent, and this material was purified by flash-column chromatography using $CHCl_3/CH_3OH$ (5:1) as the eluent to afford the methyl ester of FNP-βNeuAc as a white powder (180 mg, 38% over two steps). ^{1}H NMR (500 MHz, methanol-d_4) δ: 1.81 (dd, 1 H, $J_{3a,3e} = 13.0$ Hz, $J_{3a,4} = 11.5$ Hz, H3a), 1.96 (s, 3 H, CH_3), 2.55 (dd, 1 H, $J_{3e,3a} = 13.0$ Hz, $J_{3e,4} = 4.5$ Hz, H3e), 3.43 (m, 1H, H7), 3.59 (dd, 1 H, $J_{9b,9a} = 11.5$ Hz, $J_{9b,8} = 5.0$ Hz, H9a), 3.65 (m, 1 H, H8), 3.70 (s, 1 H, $-OCH_3$), 3.75−3.70 (m, 1 H, H9b), 3.85 (m, 1 H, H6), 3.96 (t, 1 H, $J_{5,6} = J_{5,4} = 10.5$ Hz, H5), 4.15 (td, 1 H, $J_{4,3e} = 4.5$ Hz, $J_{4,3a} = J_{4,5} = 10.5$ Hz, H4), 7.00, 8.05 (3 H, Ar−H). The methyl ester of FNP-βNeuAc (150 mg, 0.32 mmol, 1 equiv) was dissolved in a solution of THF/H_2O (3:1, 10 mL) that was maintained at 0 °C. Then, LiOH·H_2O (68 mg, 1.6 mmol, 5 equiv) was added, and the resultant solution was stirred for 15 min. The solution was then neutralized by adding Amberlite IR-120 (H^+ form) and filtered. The resin was washed several times with methanol. The solutions were combined and evaporated to give a solid residue that was purified by flash-column chromatography using ethyl acetate/methanol/water (10:2:1). The fractions that contained the product were combined and concentrated. The concentrated aqueous solution was then lyophilized to obtain a solid (105 mg, 72%). mp decomposed at 140 °C. $[\alpha]_D^{20} = -79.2$ (*c* 1.14, H_2O). ^{1}H NMR (500 MHz, D_2O) δ: 1.86 (dd, 1 H, $J_{3a,3e} = 13.5$ Hz, $J_{3a,4} = 11.5$ Hz, H3a), 2.02 (s, 3 H, CH_3), 2.57 (dd, 1 H, $J_{3e,3a} = 13.5$ Hz, $J_{3e,4} = 5.0$ Hz, H3e), 3.50 (m, 1 H, H7), 3.57 (dd, 1 H, $J_{9a,9b} = 12.0$ Hz, $J_{9a,8} = 5.5$ Hz, H9a), 3.68−3.76 (m, 2 H, H6, H8, H9b), 4.02 (t, 1 H, $J_{5,6} = J_{5,4} = 10.5$ Hz, H5), 4.24 (td, 1 H, $J_{4,3e} = 5.0$ Hz, $J_{4,3a} = J_{4,5} = 11.0$ Hz, H4), 7.01, 8.12 (3 H, Ar−H). ^{13}C NMR (125 MHz, D2O) δ: 22.2 (CH_3), 40.3 (C3), 51.8 (C5), 63.0 (C9), 66.5 (C4), 67.9 (C7), 70.3 (C6), 71.9 (C8), 101.7 (C2), 106.2, 113.0, 128.0, 131.2, 157.9, 160.9 (6 C, Ar−C), 173.6 (C═O), 175.0 (C1). HRMS−FAB (*m*/*z*) [M − H^+] calcd for $C_{17}H_{20}N_2O_{11}F$, 447.1057; found, 447.1067.

4-Nitrophenyl 5-acetamido-3,5-dideoxy-2-thio-D-glycero-β-D-galacto-2-nonulopyranosidonic Acid (pNP-S-βNeuAc). To a stirred solution of sialosyl acetate (*20*) (1.79 g, 3.35 mmol) and 4-nitrothiophenol (2.81 mg, 14.4 mmol, 80% purity) in dichloromethane (30 mL) at 0 °C was added boron trifluoride etherate (1.82 mL, 14.4 mmol), and the reaction mixture was allowed to warm slowly to room temperature. After 16 h, the reaction mixture was poured into a saturated aqueous solution of sodium bicarbonate (100 mL) and diluted with dichloromethane (250 mL). The organic layer was separated, washed with a saturated aqueous solution of sodium bicarbonate (5 × 100 mL), dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure to afford a bright orange solid. Purification by silica-gel chromatography using ethyl acetate as an eluent afforded the peracetylated thiosialoside as a pale-yellow foam (1.40 g, 66%, $\beta/\alpha = 92{:}8$). Recrystallization from ethyl acetate/hexanes afforded a portion of pure peracetylated methyl ester (519 mg). ^{1}H NMR ($CDCl_3$) δ: 1.90 (s, 3 H, Ac), 2.00 (s, 3 H, Ac), 2.03 (s, 3 H, Ac), 2.04 (s, 3 H, Ac), 2.12 (s, 3 H, Ac), 2.19 (dd, 1 H, $J_{3ax,3eq} = 14.0$ Hz, $J_{3ax,4} = 11.6$ Hz, H3ax), 2.70 (dd, 1 H, $J_{3eq,4} = 4.8$ Hz, H3eq), 3.69 (s, 3 H, Me), 4.09 (dd, 1 H, $J_{9a,9b} = 12.6$ Hz, $J_{9a,8} = 8.8$ Hz, H9a), 4.12 (d, 1 H, $J_{5,6} = 7.1$ Hz, H5), 4.48 (dd, 1 H, $J_{9b,8} = 2.5$ Hz, H9b), 4.50 (dd, 1 H, $J_{8,7} = 5.5$ Hz, H8), 4.75−4.79 (m, 1 H, H7), 5.32 (d, 1 H, $J_{NH,5} = 10.2$ Hz, NH), 5.33−5.40 (ddd, 1 H, H4), 5.39−5.41 (m, 1 H, H6), 7.64 (d, 2 H, $J = 8.9$ Hz, Ar−H), 8.17 (d, 2 H, Ar−H). To a stirred solution of the peracetylated methyl ester (510 mg, 0.811 mmol) in methanol (40 mL) at 0 °C was added a solution of sodium methoxide [prepared from sodium metal (57 mg, 2.5 mmol) and methanol (10 mL)]. After 1 h, the reaction mixture was neutralized with Amberlite 120(H^+) resin, filtered, and concentrated under reduced pressure to afford an orange foam. Purification by silica-gel chromatography using chloroform/methanol (from 8:1 to 4:1) as an eluent afforded the methyl ester (278 mg, 74%) as a white solid. ^{1}H NMR (CD_3OD) δ: 2.03 (t, 1 H, $J_{3ax,4} + J_{3ax,3eq} = 25.5$ Hz, H3ax), 2.04 (s, 3 H, Ac), 2.72 (dd, 1 H, $J_{3eq,3ax} = 13.8$ Hz, $J_{3eq,4} = 4.7$ Hz, H3eq), 3.53 (s, 3 H, Me), 3.58 (d, 1 H, $J_{7,8} = 9.5$ Hz, H7), 3.68 (dd, 1 H, $J_{9a,H9a} = 11.3$ Hz, $J_{9a,8} = 5.6$ Hz, H9a), 3.79 (ddd, 1 H, $J_{8,9b} = 2.9$ Hz, H8), 3.86 (dd, 1 H, H9b), 3.92 (t, 1 H, $J_{5,6} + J_{5,4} = 20.5$ Hz, H5), 4.11 (ddd, 1 H, $J_{4,5}$

= 10.1 Hz, H4), 4.47 (d, 1 H, $J_{6,5}$ = 10.5 Hz, H6), 7.84 (d, 2 H, J = 8.9 Hz, Ar−H), 8.20 (d, 2 H, Ar−H). A solution of the methyl ester (278 mg, 0.604 mmol) and lithium hydroxide monohydrate (75 mg, 1.8 mmol) in tetrahydrofuran/water (20 mL, 3:1, v/v) was stirred at 0 °C for 1.5 h. The reaction mixture was neutralized with Amberlite 120-(H^+) resin, filtered, and concentrated under reduced pressure to afford the required 2-thiosialoside (245 mg, 91%) as a pale-yellow solid. mp decomposed at 193 °C. $[\alpha]_D^{20}$ = −14.5 (*c* 0.227, ethanol). ^{1}H NMR (D_2O) δ: 2.04 (s, 3 H, Ac), 2.05 (t, 1 H, $J_{3eq,3ax} + J_{3eq,4}$ = 25.6 Hz, H3eq), 2.69 (dd, 1 H, $J_{3eq,3ax}$ = 13.7 Hz, $J_{3a,4}$ = 4.7 Hz, H3eq), 3.56 (d, 1 H, $J_{7,8}$ = 9.5 Hz, H7), 3.64 (dd, 1 H, $J_{9a,9b}$ = 11.8 Hz, $J_{9a,8}$ = 5.3 Hz, H9a), 3.71−3.76 (m, 1 H, H8), 3.78 (dd, 1 H, $J_{9b,8}$ = 2.6 Hz, H9b), 3.94 (t, 1 H, $J_{5,6} + J_{5,4}$ = 20.5 Hz, H5), 4.16 (ddd, 1 H, $J_{4,5}$ = 10.2 Hz, H4), 4.34 (d, 1 H, $J_{6,5}$ = 10.6 Hz, H6), 7.67 (d, 2 H, J = 9.0 Hz, Ar−H), 8.17 (d, 2 H, Ar−H). ^{13}C NMR (D_2O) δ: 22.3 (Ac), 41.0 (C3), 52.3 (C5), 63.2 (C9), 67.2 (C4), 68.3 (C7), 70.2 (C8), 72.2 (C6), 91.1 (C2), 124.0 (Ar), 132.5 (Ar), 141.3 (Ar), 147.1 (Ar), 173.1 (C1), 174.9 (Ac). Anal. Calcd for $C_{17}H_{22}N_2O_{10}S$: C, 45.74; H, 4.97; N, 6.27. Found: C, 45.50; H, 5.23; N, 6.38.

Phenyl 5-Acetamido-3,5-dideoxy-2-thio-D-glycero-β-D-galacto-2-nonulopyranosidonic Acid (Ph-S-βNeuAc). To a stirred solution of sialosyl acetate (*20*) (1.59 g, 2.99 mmol) and thiophenol (1.32 mL, 12.8 mmol) in dichloromethane (30 mL) at 0 °C was added boron trifluoride etherate (1.62 mL, 12.8 mmol), and the reaction mixture was allowed to slowly warm to room temperature. After 16 h, the reaction mixture was poured into a saturated aqueous solution of sodium bicarbonate (100 mL) and diluted with dichloromethane (250 mL). The organic layer was separated, then washed with a saturated aqueous solution of sodium bicarbonate (3 × 100 mL), dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure to afford the peracetylated thiosialoside (*21*) (2.34 g, β/α = 83:17) as a colorless oil, which was used without further purification. ^{1}H NMR ($CDCl_3$) δ: 1.91 (s, 3 H, Ac), 1.96 (s, 3 H, Ac), 2.04 (s, 3 H, Ac), 2.07 (s, 3 H, Ac), 2.11 (s, 3 H, Ac), 2.14 (t, 1 H, $J_{3a,3e} + J_{3a,4}$ = 26.9 Hz, H3a), 2.67 (dd, 1 H, $J_{3e,3a}$ = 13.8 Hz, $J_{3e,4}$ = 4.8 Hz, H3e), 3.60 (s, 3 H, Me), 4.00 (dd, 1 H, $J_{9a,9b}$ = 12.2 Hz, $J_{9a,8}$ = 8.5 Hz, H9a), 4.13 (q, 1 H, $J_{5,6} + J_{5,4} + J_{5,NH}$ = 31.2 Hz, H5), 4.48 (dd, 1 H, $J_{9b,8}$ = 2.1 Hz, H9b), 4.60 (dd, 1 H, $J_{6,7}$ = 2.5 Hz, $J_{6,5}$ = 10.5 Hz, H6), 4.93−4.97 (m, 1 H, H8), 5.33 (d, 1 H, $J_{NH,5}$ = 10.2 Hz, NH), 5.34−5.41 (m, 1 H, H4), 5.45 (t, 1 H, $J_{7,6} + J_{7,8}$ = 4.8 Hz, H7), 7.31−7.38 (m, 3 H, Ar−H), 7.45 (d, 2 H, J = 8.4 Hz, Ar−H). To a stirred solution of the peracetylated sialoside (2.34 g, β/α = 83:17) in methanol (150 mL) at 0 °C was added a solution of sodium methoxide [prepared from sodium metal (0.58 g, 25 mmol) and methanol (30 mL)]. After 1 h, the reaction mixture was neutralized with Amberlite 120(H^+) resin, filtered, and concentrated under reduced pressure to afford a colorless oil. Purification by silica-gel chromatography using chloroform/methanol (from 8:1 to 4:1) as an eluent afforded the methyl ester (767 mg, 62% over 2 steps, β/α = 92:8) as a white solid. ^{1}H NMR (CD_3OD) δ: 1.96 (dd, 1 H, $J_{3a,3e}$ = 13.6 Hz, $J_{3a,4}$ = 11.7 Hz, H3a), 2.03 (s, 3 H, Ac), 2.69 (dd, 1 H, $J_{3e,4}$ = 4.7 Hz, H3e), 3.49 (s, 3 H, Me), 3.56 (d, 1 H, $J_{7,8}$ = 8.8 Hz, H7), 3.67 (dd, 1 H, $J_{9a,9b}$ = 11.0 Hz, $J_{9a,8}$ = 5.1 Hz, H9a), 3.79 (ddd, 1 H, $J_{8,9b}$ = 3.0 Hz, H8), 3.82 (dd, 1 H, H9b), 3.90 (t, 1 H, $J_{5,6} + J_{5,4}$ = 20.6 Hz, H5), 4.10 (ddd, 1 H, $J_{4,5}$ = 10.0 Hz, H4), 4.51 (dd, 1 H, $J_{6,7}$ = 0.9 Hz, $J_{6,5}$ = 10.5 Hz, H6), 7.32−7.39 (m, 3 H, Ar−H), 7.58−7.61 (m, 2 H, Ar−H). A solution of the methyl ester (760 mg, 1.83 mmol, β/α = 92:8) and lithium hydroxide monohydrate (208 mg, 4.95 mmol) in tetrahydrofuran/water (40 mL, 3:1) was stirred at 0 °C for 1.25 h and then at room temperature for 15 min. The reaction mixture was neutralized with Amberlite 120(H^+) resin, filtered, and concentrated under reduced pressure to afford a colorless solid. Purification by silica-gel chromatography using ethyl acetate/methanol/water (from 20:2:1 to 10:2:1) as an eluent afforded, first, a mixture (453 mg, 62%, β/α = 90:10) of the required 2-thiosialoside and the α-anomer as a white solid. Continued elution then afforded the pure 2-thiosialoside (201 mg, 27%) as a white solid. mp decomposed at 185 °C. $[\alpha]_D^{20}$ = −16.4 (*c* 0.333, ethanol). ^{1}H NMR (D_2O) δ: 2.02 (dd, 1 H, $J_{3ax,3eq}$ = 13.6 Hz, $J_{3eq,4}$ = 11.8 Hz, H3eq), 2.06 (s, 3 H, Ac), 2.67 (dd, 1 H, $J_{3a,4}$ = 4.7 Hz, H3eq), 3.58 (d, 1 H, $J_{7,8}$ = 8.8 Hz, H7), 3.61 (dd, 1 H, $J_{9a,9b}$ = 12.2 Hz, $J_{9a,8}$ = 6.0 Hz, H9a), 3.73−3.78 (m, 2 H, H8 + H9b), 3.90 (t, 1 H, $J_{5,6} + J_{5,4}$ = 20.5 Hz, H5), 4.16 (ddd, 1 H, $J_{4,5}$ = 10.1 Hz, H4), 4.43 (d, 1 H, $J_{6,5}$ = 10.5 Hz, H6), 7.35−7.43 (m, 3 H, Ar−H), 7.52−7.55 (m, 2 H, Ar−H). ^{13}C NMR (D_2O) δ: 22.3 (Ac), 40.6 (C3), 52.4 (C5), 63.2 (C9), 67.3 (C4), 68.6 (C7), 70.2 (C8), 71.7 (C6), 90.7 (C2), 129.3 (Ar), 129.4 (Ar), 130.2 (Ar), 134.6 (Ar), 173.4 (C1), 174.9 (Ac). Anal. Calcd for $C_{17}H_{23}NO_8S$: C, 50.86; H, 5.77; N, 3.49. Found: C, 50.82; H, 6.01; N, 3.30.

Enzyme Kinetics and Product Studies. Kinetic experiments and NMR-based product studies were carried out according to protocols described for the wild-type enzyme (*10*). The Y370 mutant sialidase stocks and reaction buffers were identical to those reported previously (*10*, *15*, *16*). The enzyme-catalyzed hydrolysis reactions were monitored using a Cary 3E UV−vis spectrophotometer equipped with the Cary six-cell Peltier constant-temperature accessory (see the Supporting Information for wavelengths and $\Delta\epsilon$ values for each substrate). Michaelis−Menten parameters were calculated using a standard nonlinear least-squares fit of the rate versus substrate concentration data (GraFit). The parameter k_{cat}/K_m was calculated from the values calculated for k_{cat} and K_d. Binding constants for substrates with very low activity were determined by a competitive inhibition assay using MU-αNeuAc as the substrate.

Trans-glycosylation Reactions. Using Ph-βNeuAc as the sialyl donor and lactose as the acceptor sugar, the Y370G mutant sialidase was assessed for its ability to form sialyllactose. Initial reactions were performed using 200 mM lactose, 40 mM Ph-βNeuAc, and 5 × 10^{-6} M enzyme at pH 5.25 and 37 °C and then monitored over time. The effects of varying the acceptor concentration (40 mM donor and 0−200 mM acceptor) as well as the donor concentration (25−100 mM donor and 200 mM acceptor) were investigated. To optimize these reactions, the effects of varying the temperature (17−57 °C), pH (3.8−8.9), and organic solvent concentration (acetonitrile, 0−30%, v/v) were also compared. At each time point, a 5 μL aliquot was removed from the reaction and heated at 85 °C for 15 min to inactivate the enzyme. For product analysis, the 5 μL samples were diluted $^1/_{60}$ in H_2O and then analyzed using a Dionex ICS-

3000 HPLC system equipped with an amperomeric detector. Using a protocol modified from literature procedures (*22*−*28*), samples (10 μL volumes) were injected at a flow rate of 0.5 mL/min with isocratic elution (0.15 M NaOH and 0.015 M NaOAc) onto a Carbopac PA20 column (3 × 150 mm). Standards and unknowns were analyzed in duplicate runs. The concentration of Ph-βNeuAc, NeuAc, and sialyl-lactose were determined using standard curves generated for each of these compounds (with a standard curve range of 10^{-7}−10^{-3} M). The yields for trans-glycosylation reactions were calculated by dividing the sialyl-lactose concentration by the sum of the sialyl products (NeuAc and sialyl-lactose). The progress of the reaction was calculated by dividing the sum of the sialyl products by the sum of all sialyl species (Ph-βNeuAc, NeuAc, and sialyl-lactose).

E_5TNNY_{15} Library Construction. Residue E260 was mutated to alanine, aspartate, cysteine, and histidine, in four separate mutagenesis experiments, using the strand-overlap extension method (see the Supporting Information for primer sequences). The pJW-HS plasmid was used as the template for the sialidase gene (*10*). Substitution of the E260 codon was achieved by making the mutations in both strands in two separate PCR experiments. The front of the gene was amplified using the Nhe*′ primer with the reverse mutagenic primer (i.e., E260A-R′) to give a 0.79 kb product. The back portion of the gene was amplified using the forward mutagenic primer (i.e., E260A-F′) with the reverse primer MV1987-R′ to produce a 0.40 kb fragment. These two purified fragments had an overlapping sequence at the site of mutation and were subsequently used to prime each other in an extension reaction of 1 cycle. The full 1.2 kb mutant gene was then amplified with the outer Nhe*′ and MV1987-R′ primers. After digestion with *Bam* HI and *Msc* I, the purified 0.4 bp product was ligated into similarly digested and dephosphorylated pJW-HS vector and propagated in *Escherichia coli*. Restriction and DNA sequence analysis was used to confirm the E260 mutations, as well as to ensure that spurious mutations had not occurred during DNA manipulation. Primer Y370A-R′ was used for sequencing.

To create a restricted library of E260 and Y370 mutants, the individual E260 and Y370 mutant plasmids were mixed in equal proportion and then recombined to create the double-mutant library E_5Y_{15}, which encodes for 5 possible residues at E260 (A, C, D, E, and H) and 15 amino acids at position Y370 (all 20, except C, K, V, W, and Y). Specifically, the 5 E260 mutant plasmids were mixed equally and then digested with *Age* I and *Msc* I to give a 794 bp product. The 15 different Y370 mutant plasmids in pJW-OSH (*16*) were mixed in equal ratio, then digested similarly, and dephosphorylated to yield a 6471 bp fragment. The purified E260 and Y370 fragments were ligated and propagated in *E. coli*. Approximately 600 transformants were combined and grown to produce the plasmid library E_5Y_{15}, which contains 75 members in approximately equal ratio.

Using the E_5Y_{15} plasmid library (in pJW-OSH) as the template, the codons for residues T309, N310, and N311 were completely randomized using saturation mutagenesis (NNK substitutions encoding 32 possible codons at each of the 3 positions). The front of the gene was amplified using the EcoR*′ primer with the reverse mutagenic primer TNN-R′ to give a 0.94 kb product. The back portion of the gene was amplified using the forward mutagenic primer TNN-F′ with primer TerHin′ to give a 1.0 kb product. The two purified fragments with an overlapping sequence at the site of mutation were subsequently used to prime each other in an extension reaction of 1 cycle. The full 1.9 kb product was then amplified with the outer primers Eco*′ and TerHin′. After digestion with *Age* I and *Kpn* I, the 1.0 kb product was ligated to similarly digested and dephosphorylated vector pJW-OSH (6.2 kb). The final plasmid library, E_5TNNY_{15}, which contains 2.5×10^6 possible mutant clones ($5 \times 32 \times 32 \times 32 \times 15$), encodes for 6.0×10^5 different proteins.

Library Expression. Using the ligation mixture, 1−50 ng of DNA was used to transform BL21(λDE3)Gold ultracompetent cells (Stratagene). Transformations were carried out according to the instructions of the manufacturer, except that the heat-shock step was performed for 35 s to give more transformants and the rescue step involved no more than 45 min to minimize the redundancy of individual clones in the library. Colonies were picked to 200 μL of Luria broth containing kanamycin in 96-well plates and covered with an airpore (Qiagen) membrane. Cells were grown at 37 °C and 260 rpm for 3−4 h. A 130 μL aliquot of the cultures was transferred to another plate containing glycerol for long-term storage of a master plate at −80 °C. To the remaining 70 μL of culture, 200 μL of TB/Kan (12 g of bactotryptone, 24 g of yeast extract, 4 mL of glycerol, and 100 mL of K phosphate in 1 L containing 50 μg/mL kanamycin) was added and returned to incubation for another 3−4 h. Plates were cooled to room temperature before induction with 1 mM IPTG and then incubated at room temperature and 260 rpm. Cells were harvested 40 h postinduction by centrifugation for 10 min at 3700*g*. The expression supernatants were removed and stored at −20 °C until needed for screening. A number of individual crude supernatants were analyzed by SDS−PAGE to compare the variation in expression levels between individual mutants in the library.

DNA Sequencing of Individuals from the Library. A 10 μL volume of culture was removed from the master plate stocks, added to 200 μL of LB/Kan media in 96-well plates, and incubated for 2.5 h at 37 °C and 260 rpm. These cultures were then used to seed 5 mL of media in 14 mL culture tubes. After an overnight incubation, plasmid DNA was purified (Qiaspin Kit, Qiagen). DNA sequencing was performed on 26 random individuals using the MV1987-R′ primer.

Library Screening. A 6 μL aliquot from the crude supernatant plates was combined in groups of 12 to make screening plates to economize the amount of substrates required for the screens. The mixed crude supernatants were assayed in 150 μL reactions in the screen plates with 500 mM acetate buffer at pH 5.25 and 0.2 mM pNP-*S*-βNeuAc. Samples were incubated at 37 °C and monitored at 410 nm using a SoftMax plate reader at time 0, 0.5, 1, 1.5, 2 h, and overnight. Wells with significant increases in absorbance over the background were identified. In a subsequent, smaller screening assay, the groups of 12 individuals from each "active" well were assayed individually to decipher which clone(s) gave rise to the apparent activity. Positive hits were identified, and cultures were grown up again from the master plate stocks for DNA sequencing (as described above) to look for a consensus sequence.

A second screen of the crude supernatants was performed as described above, using 1 mM MU-βNeuAc and 500 mM

Table 1: Relative Activity of Various Inverting Y370 Mutant Sialidases with 0.5 mM Ph-βNeuAc at 37 °C and pH 5.25

mutant	relative rate (%)	mutant	relative rate (%)
Y370A	4	Y370N	9
Y370D	<0.2	Y370T	9
Y370G	100		

Table 2: Michaelis−Menten Kinetic Parameters of the Y370G Mutant Sialidase Using Ph-αNeuAc and Ph-βNeuAc at 37 °C and pH 5.25

substrate	k_{cat} (s^{-1})	$K_{m\ app}$ (M)	k_{cat}/K_m ($M^{-1}\ s^{-1}$)
Ph-αNeuAc	0.82 ± 0.04	$(2.3 \pm 0.3) \times 10^{-5}$	$(3.6 \pm 0.7) \times 10^4$
Ph-βNeuAc	13.3 ± 0.3	$(4.5 \pm 0.4) \times 10^{-5}$	$(2.9 \pm 0.3) \times 10^5$
fold difference (β/α)	16	n/a	8

acetate buffer in a 100 μL reaction. Samples were incubated at room temperature and monitored continuously for 30 min using a Cary Eclipse fluorescence plate reader (excitation wavelength of 365 nm and emission wavelength of 450 nm). Hits were identified and sequenced as above.

RESULTS

Synthesis. The synthetic routes chosen are standard procedures; however, to ensure the anomeric purity of the β-sialosides, which when made contain small quantities of the α-anomer, the compounds were treated with wild-type sialidase to hydrolyze the contaminating α-anomer (see Figure S3 in the Supporting Information). After lyophilization of the cleansed β-anomer, the resultant solid residue was purified by flash-column chromatography using ethyl acetate/methanol/water (10:2:1).

Enzyme Kinetics and Product Studies. A series of Y370 mutants previously shown to operate with inversion of the configuration with an α-sialoside substrate (*10*, *15*, *16*) were compared to investigate the effect of the size of the hole upon turnover of the β-sialoside, Ph-βNeuAc (see Table 1). Next, a comparison of the activity of the parent aryl α- and β-sialoside was performed using the most active mutant (Y370G). Michaelis−Menten kinetic parameters for the mutant Y370G with phenyl α-D-*N*-acetylneuraminide (Ph-αNeuAc) and Ph-βNeuAc are shown in Table 2.

The stereochemical outcome for the Y370G-catalyzed hydrolysis of Ph-βNeuAc was determined using NMR techniques, and a graphical representation of the time-course reaction is shown in Figure 2 (see Figure S4 in the Supporting Information for NMR spectra). The variation of the kinetic parameters k_{cat} and k_{cat}/K_m for the Y370G-catalyzed hydrolysis of Ph-βNeuAc as a function of pH is shown in Figure 3. A panel of β-sialosides (Scheme 3) was used to probe the effect of the leaving-group structure and reactivity on k_{cat} and k_{cat}/K_m for Y370G. As shown in Table S4 in the Supporting Information, only the unsubstituted phenyl substrate was efficiently turned over by the mutant Y370G.

Trans-glycosylation Reactions. Investigation of the ability of mutant Y370G to catalyze trans-glycosylation reactions, to transfer sialic acid to lactose, yielded both hydrolysis (NeuAc) and coupled products (sialyl-lactose), with the α-2,6 isomer of sialyl-lactose forming at a ratio of approximately

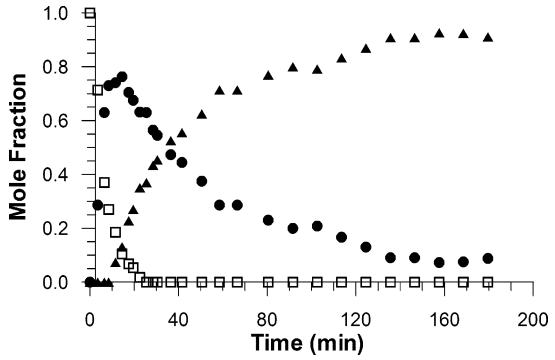


FIGURE 2: Mole fractions of the substrate and products monitored over time for Y370G-catalyzed hydrolysis of 12 mM Ph-βNeuAc in 10 mM tartrate buffer at pD 5.2 and room temperature using 0.44 mg/mL enzyme. Values were calculated using the relative integrals of the H_{3eq} signals of Ph-βNeuAc (□), αNeuAc (●), and βNeuAc (▲).

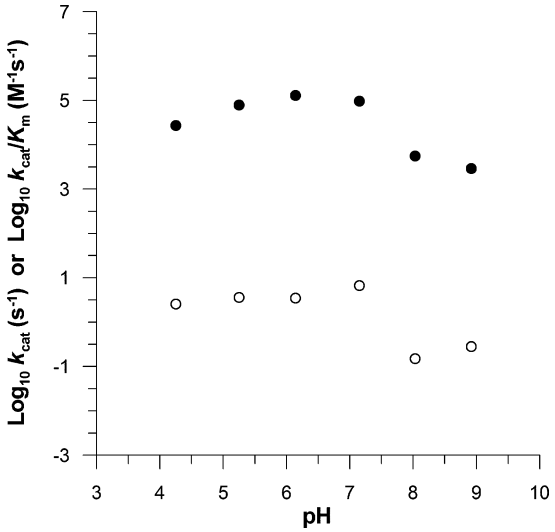
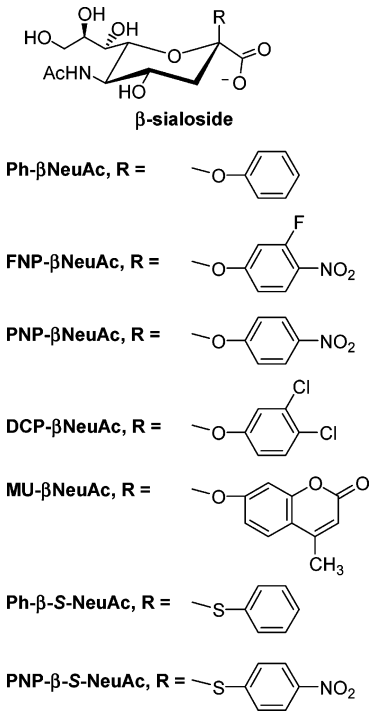


FIGURE 3: Effect of pH on kinetic parameters k_{cat} (○) and k_{cat}/K_M (●) for Y370G-catalyzed hydrolysis of Ph-βNeuAc at 37 °C.

9:1 over the α-2,3 isomer. As illustrated in Figure 4, the initial yield of sialyl-lactose (α-2,3 plus α-2,6 divided by the total sialyl product concentration) was 6% and no product degradation was observed over time. The two reaction conditions that improved the yield most significantly were the concentration of the acceptor (Figure 5) and the presence of acetonitrile (Figure 6). The variation of the donor concentration, pH, and temperature was also optimized, yet these parameters did not significantly improve the yields (Figures S5−S7 in the Supporting Information). The final optimized yield was 13%.

E_5TNNY_{15} Library. A total of 2628 individual clones, representing 1% of the library, were isolated and expressed on a 0.1 mL scale in 96-well format. SDS−PAGE analysis of crude supernatants from 31 individuals revealed an estimated expression success rate of 55% (data not shown). There was no apparent trend observed between specific mutations and the lack of expression. Assessment of the genetic diversity of the E_5TNNY_{15} plasmid library was performed by sequencing randomly selected individuals. Shown in Table S5 in the Supporting Information is a summary of the sequence data comparing the expected mutation distribution to that observed from a random sampling of 28 (see Table S6 in the Supporting Information

Scheme 3: Substrate Structures



for individual sequences). As expected, there were some clones with the T309, N310, and N311 codons unchanged (19%), as well as the appearance of a stop codon (3% chance from each NNK) and a small degree of redundant sequences (8%, likely related to the rescue incubation time following transformation). Notably, there were no mutations present in the random sampling that were not anticipated. Given the expected mutational distribution and the sample size examined (n), it was determined that the E_5TNNY_{15} library was sufficiently diverse and unbiased to go ahead with screening, because 70% of the members in the library definitely contained mutations to all five residues.

The 2628 crude expression supernatants were screened for activity with the MU-βNeuAc using 1 mM substrate in pH 5.25 buffer at room temperature. The first screen (of samples combined in groups of 12) resulted in no hits, with activity significantly over the background rate (S/N of >2). However, 5 groups displayed rates that were slightly greater than the background and were subsequently assayed individually in a second screen. The individual screening of these 5 groups of 12 identified 14 potential hits that stood out slightly above the background hydrolysis rate. Again, the difference was only minor, and even after 60 min, there was only one clone that actually had a rate greater than 2-fold over the background. DNA sequencing was performed on these 14 clones to determine whether there was any consensus sequence within this group upon which we could build a secondary library (see Table S7 in the Supporting Information).

Another screen of the E_5TNNY_{15} library was performed using the thio substrate pNP-*S*-βNeuAc (0.2 mM substrate in pH 5.25 buffer at room temperature). The initial screen (of samples combined in groups of 12) resulted in 9 groups with potential activity above the background rate. As a negative control, a clone containing a stop codon was also included in the experiment, which also gave a small signal, suggesting that these signals were very close to the detection

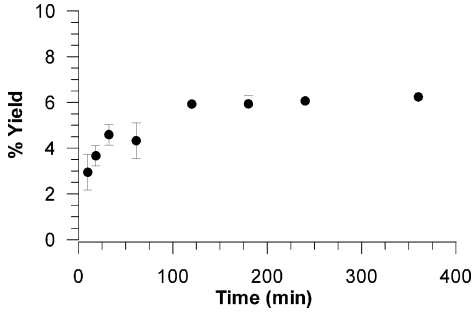


FIGURE 4: Effect of the incubation time on the yield of sialyl-lactose. Conditions: 40 mM Ph-βNeuAc (donor) and 200 mM lactose (acceptor) at pH 5.25 and 37 °C. The reaction of the donor was complete by 240 min.

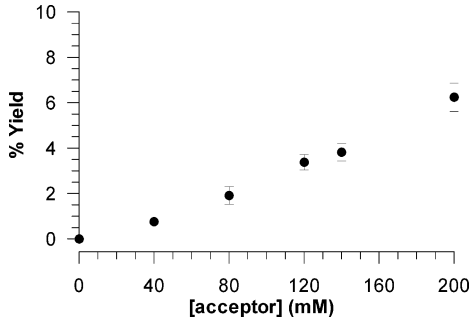


FIGURE 5: Effect of the acceptor sugar concentration on the yield of sialyl-lactose. Conditions: 40 mM donor and 0−200 mM acceptor at pH 5.25 and 37 °C. The timepoint shown is at the end of the reaction (i.e., no donor remaining).

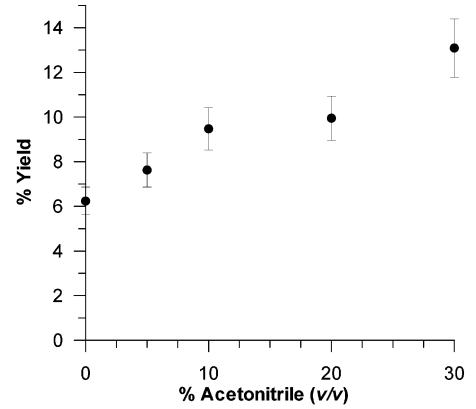


FIGURE 6: Effect of the acetonitrile concentration on the yield of sialyl-lactose. Conditions: 40 mM donor and 200 mM acceptor at pH 5.25, 37 °C, and 0−30% acetonitrile (v/v). The timepoint shown is at 75% completion (i.e., 25% donor remaining).

limits of the assay. Nonetheless, the potential hits within these 9 groups of 12 were deciphered in a second screen of the individual clones in those 9 groups. The sensitivity of the second screen was improved by working at 1.7 mM substrate and 37 °C. This resulted in a group of 15 individual clones with notable or slight activity over the background, and these were then sequenced to look for a consensus (see Table S8 in the Supporting Information).

DISCUSSION

β-Sialidase Activity of Mutant Y370G. The Y370G mutant, which has the largest hole created by replacing the nucleophilic tyrosine with a glycine residue, showed the highest activity toward the Ph-βNeuAc substrate (Table 1). In comparison to the natural substrate analogue Ph-αNeuAc,

the Y370G mutant displays a greater activity with the Ph-βNeuAc substrate (Table 2). Given that β-sialosides are kinetically more stable than the corresponding α-anomers (*17*), a more accurate comparison for the relative specificities of the Y370G mutant is to compare the respective catalytic proficiencies [$(k_{cat}/K_m)/k_{uncat}$] for hydrolysis of the two anomers. For the spontaneous hydrolyses of *p*-nitrophenyl α-D-*N*-acetylneuraminide (pNP-αNeuAc) and pNP-βNeuAc, the k_{uncat} value for pNP-αNeuAc at 37 °C is more than 100-fold larger than that for pNP-βNeuAc (*17*). Assuming that a similar difference would be observed for the parent phenyl sialosides, then the mutant Y370G sialidase exhibits a catalytic proficiency of approximately 900-fold larger with Ph-βNeuAc than with Ph-αNeuAc.

The initial product of the Y370G-catalyzed hydrolysis of Ph-βNeuAc is α-NeuAc, which subsequently undergoes mutarotation to give mainly the β-anomer at equilibrium (Figure 2). Therefore, Y370G catalyzes the hydrolysis of Ph-βNeuAc with an inversion of the anomeric configuration (Scheme 2). This is conceptually similar to the hydrolysis of aryl α-sialosides by Y370G, which also proceeds with inversion, albeit to release β-NeuAc as the first product (*10*, *15*). Of note, the Y370G mutant enzyme displays a high specificity for the Ph-βNeuAc substrate, such that even the addition of a nitro group to the aryl ring profoundly reduces enzymatic activity. Also, this enzyme does not hydrate 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (DANA) (*15*), and thus, it can be concluded that any addition−elimination mechanisms are precluded for this enzyme.

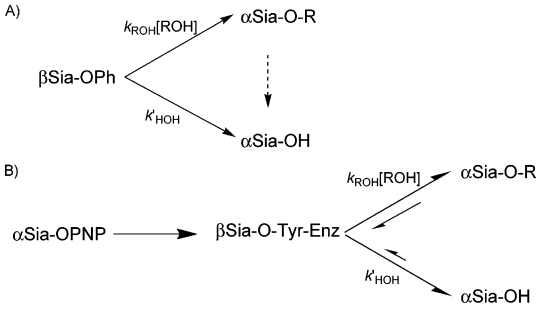
pH Rate Profile. The pH rate profile for the catalyzed hydrolysis of Ph-βNeuAc is relatively flat, with the enzymatic activity only dropping noticeably at pH values greater than 8. A likely cause is that at high pH values Glu-260 cannot assist the departure of the phenyl leaving group because it is probably deprotonated.

Effect of the Leaving Group on the Catalytic Activity. On the basis of these measurements of the Y370G sialidase with Ph-βNeuAc, it was decided to characterize the hydrolytic activity of the Y370G mutant with a series of β-sialoside substrates. Of note, none of the other β-sialosides, except with the possible exception of FNP-βNeuAc, showed any signs of activity with the Y370G mutant enzyme (Table S4 in the Supporting Information). Indeed, only Ph-βNeuAc bound to the Y370G sialidase with a submillimolar dissociation constant. Clearly, the hole in the Y370G mutant is able to differentiate exquisitely between similarly sized aglycones, e.g., thiophenyl versus phenyl.

Sialyl-transferase Activity of the Mutant Y370G Enzyme. A notable activity of sialidases, which has been utilized for the synthesis of sialosides (*23*, *29*−*33*), is that of transferring a sialic acid onto an acceptor sugar, i.e., acting as a *trans*-sialidase. The major difference between sialidases and *trans*-sialidases is the partitioning ratio of the sialyl−enzyme intermediate between the attack by water (hydrolysis) and the attack by a sugar hydroxyl group (trans-glycosylation).

The sialyl-transfer reactions catalyzed by the Y370G mutant are shown in Scheme 4. The following observations are consistent with this picture: (i) the ratio of hydrolysis to coupling is independent of the concentration of the sialyl donor (Ph-βNeuAc), within experimental error (Figure S5 in the Supporting Information). That is, the ratio k_{ROH}[ROH] to k'_{HOH} does not vary with the concentration of Ph-βNeuAc;

Scheme 4: Hydrolysis versus Trans-glycosylation Pathways for (A) Y370G *M. viridifaciens* Sialidases and (B) Wild-Type Enzyme



(ii) the amount of coupling varies as a function of the acceptor (ROH) concentration (Figure 5); and (iii) the addition of acetonitrile leads to an increase in the coupled product as a result of lowering the activity of water (Figure 6). Other parameters that were investigated were the maximal *trans*-sialidase activity of the Y370G enzyme, which mainly gives the 2,6-coupled product at various pH values and temperatures, at a pH of 5.25 and temperature of 37 °C (Figures S6 and S7 in the Supporting Information). To date, the maximal yield of the coupled product (13%) obtained in this system occurs in the presence of 30% acetonitrile.

Comparison of Trans-glycosylation Activity with That of Wild-Type Sialidases. Shown in Table 3 are the pertinent kinetic parameters for the *Vibrio cholerae* sialidase. This sialidase has the highest reported trans-glycosylation activity (*33*, *34*). However, the ratio of k_{cat} values for this sialidase with the donor substrate and the product of trans-glycosylation is around 40. As a consequence, product degradation is problematic with this system, which can be partially negated by immobilization of the enzyme (*34*). As can be seen from the data in Table S6 in the Supporting Information, mutation of the tyrosine residue in concert with changing the donor substrate results in a dramatic improvement of the *Micromonospora viridifaciens* neuraminidase (MvNA) enzyme as a trans-glycosylation catalyst. Also of note, is that the current donor substrate is essentially infinitely stable under the reaction conditions, which is not the case for pNP-αNeuAc (Table 3). Another advantage of the current system is that product degradation by the Y370G mutant enzyme is over 1000 times slower than with the *V. cholerae* enzyme; therefore, the reactions can be allowed to proceed to completion.

Directed Evolution of a β-Sialidase. On the basis of the β-sialidase activity of the Y370G mutant and the observation that other Y370 inverting mutants also displayed this novel catalytic function (Table 1), it was decided to try and enhance this activity using a directed evolution approach. Specifically, the size and shape of the hole created by the removal of the tyrosine was modified to create a library of sialidase mutants that could be screened for enhanced hydrolytic proficiencies. The two substrates chosen for the screen, MU-βNeuAc and pNP-*S*-βNeuAc, were poor substrates for the Y370G mutant (Table S4 in the Supporting Information) but, upon hydrolysis, would be easy to detect because of the large associated changes in fluorescence and/or absorbance.

A library was designed on the basis of the X-ray crystal structure of the Y370G mutant with β-NeuAc bound to its active site (PDB code 2BER) (*15*). When the space that is

Table 3: Comparison of Hydrolytic Activity of Various Sialidases with an Activated and a Natural Substrate

sialidase	donor	donor k_{cat} (s^{-1})	donor $t_{1/2}$[a]	α-2,6-sialyl-lactose k_{cat} (s^{-1})	k_{cat} ratio (donor/product)
V. cholerae	pNP-αNeuAc	107[b]	6 h[c]	2.4[d]	40
				4.9[e]	22
M. viridifaciens	pNP-αNeuAc	145[f]	6 h[c]	207[f]	0.7
M. viridifaciens Y370G	Ph-βNeuAc	13	11 000 years[c,g]	0.012[h]	1080

[a] Half-life for spontaneous hydrolysis at pH 7 and 37 °C. [b] From ref *36*. [c] From ref *17*. [d] Value obtained by extrapolating the kinetic data given in ref *36*. [e] Converted from v_{max} data taken from ref *37*. [f] From ref *10*. [g] Value obtained by extrapolating the kinetic data given in ref *17*. [h] From ref *15*.

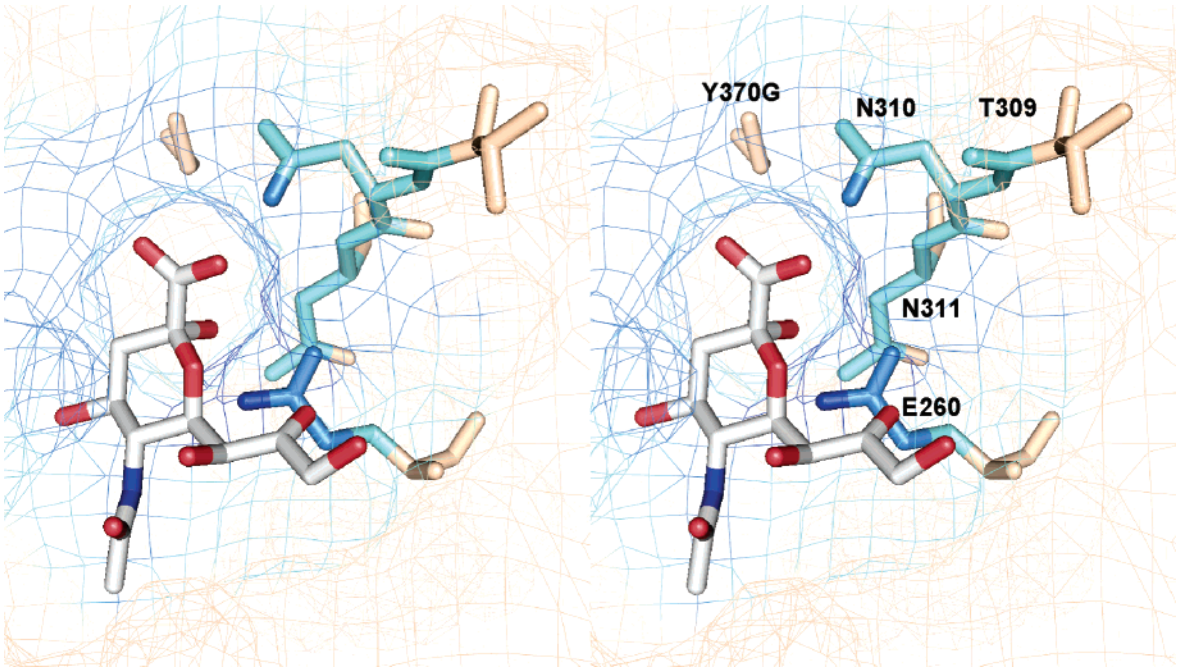


FIGURE 7: Stereoview of the active site of Y370G mutant MvNA. Residues within 8 Å of the oxygen atom on C2 in βNeuAc are displayed as sticks. Atoms closest are shown in blue. The arginine triad has been removed for clarity, as well as residues located in front of the sugar ring.

proximal to the C2 oxygen atom of the bound β-NeuAc is mapped out, four residues (in addition to the Y370 mutation site) are readily identified that are within 8 Å of this atom: E260, T309, N310, and N311 (see Figure 7). Other residues that are within this radius include the conserved arginine triad, which is involved in several binding interactions with the carboxylate group of the substrate, as well as residues located on the front face of the sugar ring, such as the catalytic general-acid residue, D92. These other residues were not considered for mutation because of either their critical roles in binding or their location on the opposite face of the ring to the tyrosine mutation site.

Because a number of Y370 mutants had already been made and characterized individually, it was known that many of these were inverting sialidases (*10*, *16*). Also, one cannot easily predict whether a retaining Y370 mutant (i.e., Y370F) might be able to accommodate a water molecule following mutagenesis of neighboring residues and result in its transformation into an inverting enzyme. Therefore, a group of 15 amino acids at position 370 were used in the library, but the wild-type tyrosine was specifically excluded. In the wild-type enzyme, E260 is thought to act as a general base to assist the nucleophilic tyrosine residue, so that mutating E260 may have a detrimental effect on enzyme activity. Consequently, the variation of amino acids at position E260 was restricted to five residues: E, A, C, D, and H. Finally, the TNN loop (309−311) has no associated catalytic function, and substitution could potentially open up the hole to accommodate much larger aglycones, such as the chosen 4-methylumbelliferyl and 4-nitrothiophenyl. Hence, these three codons were completely randomized using NNK saturation mutagenesis to make all 20 amino acids available at these three positions. The advantages of this library design are that (i) there is no wild-type Y370 present, (ii) the Y370 and E260 mutations were specifically chosen and of equal ratio, and (iii) the introduction of stop codons was minimized by only using NNK substitutions in the codons encoding the TNN loop (309−311).

Before screens for activity could be performed, the library was assessed for mutational diversity as well as the success rate of mutant sialidase expression. The sequencing of 28 random individuals confirmed that the mutational distribution within the library was as expected and that at least 70% of the clones in the library had different mutations at each of the five chosen residues. Roughly one-half of the mutants in the library actually led to expression of a sialidase protein, thus suggesting that multiple mutations to these five residues are tolerated reasonably well and that sufficient sialidase protein would be present for the screen. Nevertheless, because of the approximately 50% expression success rate, individuals were pooled together in groups of 12 for initial screenings, to economize on the substrate.

The screen of 1% of the members from the E_5TNNY_{15} library for activity against MU-βNeuAc did not yield any

hits that possessed an activity greater than the Y370G mutant. To ensure that no active mutants were being overlooked, 14 individuals with rates slightly greater than the background rate were sequenced nonetheless. However, as shown in Table S7 in the Supporting Information, there were three mutants in this group of possible hits that actually contained stop codons in the TNN (309−311) region. This suggested that the apparent low activity was likely due to noise rather than any significant catalysis. It is interesting, however, to compare the distribution of mutations from the MU-βNeuAc screen with those obtained from the random sampling (Tables S5 and S7 in the Supporting Information). While the numbers of clones are relatively small ($n \leq 28$), there are a few noticeable differences. For example, the library contains about 20% E260A mutants, although not a single individual with this mutation appeared in the group of 14 potential hits. Also, about 20% of the members in the library had no mutations to the TNN loop (that is, Y370 and E260/Y370 mutants), yet among the group of 14 individuals sequenced from the MU-βNeuAc screen, this number is increases to 50%. However, it is difficult to discern whether any significance can be attached to these observations given that two stop codons emerged from this screen. In other words, no convincing data emerged to support the creation of a second library based on these possible trends.

The second substrate used to screen the E_5TNNY_{15} library was a 2-thio sialoside, pNP-*S*-βNeuAc, a substrate for which acid catalysis is likely not required (*35*). The screen with pNP-*S*-βNeuAc did not yield any hits that possessed an activity greater than that of the Y370G mutant enzyme. Again, 15 individuals with rates that were greater than the background (negative control) were sequenced. As was the case in the first screen, another potential hit turned out to contain a stop codon (see Table S8 in the Supporting Information). Also, this individual appeared to be one of the "more active" mutants, thus suggesting that the detection limit for activity had been reached. A comparison of the distribution of mutations from this screen and the random sampling (Tables S5 and S8 in the Supporting Information) showed that in this case no obvious differences existed between these two groups. Therefore, given the lack of positive hits, the appearance of a stop codon in the group of possible hits, and the complete lack of any mutational trends within that group, there was no basis on which to continue this series of experiments with this particular library.

CONCLUSIONS

The Y370G inverting mutant enzyme possesses both α- and β-sialidase activity with phenyl sialosides and displays greater specificity for Ph-βNeuAc. With the leaving group in the hole created by replacing Y370 with a glycine residue, this mutant is also capable of catalyzing trans-glycosylation reactions to form sialyl-lactose without measurable product degradation. To accommodate different sialoside leaving groups, expansion of the hole was investigated by screening a sialidase library consisting of multiple mutations to residues that are proximal to Y370. However, a better β-sialidase mutant has yet to be discovered.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

DNA oligonucleotide sequences, detection wavelengths and $\Delta\epsilon$ values, kinetic data for Y370G activity with other β-sialosides, translations for randomly selected E_5TNNY_{15} library individuals, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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