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Neuraminidase Substrate Promiscuity Permits a Mutant *Micromonospora viridifaciens* Enzyme To Synthesize Artificial Carbohydrates

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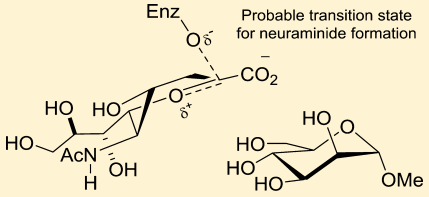
Supporting Information

ABSTRACT: Mutation of the nucleophilic amino acid residue tyrosine to the small nonpolar residue glycine (Y370G) in the active site of *Micromonospora viridifaciens* neuraminidase (*Mv*NA) produces an efficient catalyst for the transfer of *N*-acetylneuraminic acid from an artificial substrate (i.e., phenyl *N*-acetyl-*β*-D-neuraminide) to a sugar acceptor (e.g., D-lactose, D-glucose, D-mannose, D-raffinose, D-allose, or D-fructose) to give *N*-acetyl-*α*-neuraminide coupled carbohydrate products. In addition, this mutant enzyme (*Mv*NA Y370G) catalyzes the transfer of a sugar residue from the artificial substrate 2-fluorophenyl *N*-acetyl-*β*-D-neuraminide to methyl glycopyranoside acceptors. Interestingly, when trans-glycosylation reactions are conducted in aqueous solutions containing 30% (v/v) acetonitrile, the *α*-anomeric acceptors of methyl glucopyranoside and galactopyranoside generate higher product yields than do their corresponding *β*-anomers. Specifically, a 64 h reaction with 2-fluorophenyl *N*-acetyl-*β*-D-neuraminide as the limiting reagent and the acceptors methyl *α*-D-galactopyranoside, methyl *α*-D-glucopyranoside, or methyl *α*-D-mannopyranoside gives trans-glycosylation product yields of 22%, 31%, or 34%, respectively. With methyl *α*-D-galactopyranoside as the acceptor, trans-glycosylations catalyzed by both *Mv*NA Y370G and a 2,6-sialyltransferase yield identical products, which we identified as methyl *N*-acetyl-*α*-D-neuraminyl-(2 → 6)-*α*-D-galactopyranoside. The *Mv*NA Y370G-catalyzed coupling of *N*-acetylneuraminic acid to these three methyl *α*-D-glycopyranoside acceptors is favored by factors of 18−27-fold over the competing hydrolysis reaction. These coupling efficiencies likely arise from nonselective interactions between the acceptor glycopyranoside and *Mv*NA Y370G, which preferentially places a carbohydrate hydroxyl group rather than water in close proximity to the active site where this functionality intercepts the nascent neuraminyl oxacarbenium ion that is formed during cleavage of the glycosidic bond in the aryl *N*-acetyl-*β*-D-neuraminide donor. The ability to transfer *N*-acetylneuraminic acid from a stable and readily accessible donor to acceptor carbohydrates that are not substrates for sialyltransferases is one step on the path for the production of pseudohuman glycoproteins from nonmammalian cell lines.



Sialic acids are a family of structurally diverse nine-carbon keto-sugars typically found as terminal carbohydrate residues of glycoconjugates, a position that impacts key biological recognition events.[1−4] In mammalian systems, sialic acid, also known as *N*-acetylneuraminic acid, is often an important carbohydrate end-cap for modulators of cellular responses in a range of physiological processes, including differentiation, proliferation, and apoptosis.[5−7] Many of these processes are mediated by asialoglycoprotein receptors (ASGPRs) that bind glycoconjugates with carbohydrate structures that terminate in galactose or *N*-acetylgalactosamine residues; if, however, these carbohydrate moieties are capped with sialic acid, then the underlying galactose or *N*-acetylgalactosamine-containing carbohydrate epitopes are bound less tightly to the ASGPRs.[8,9] Interestingly, the rate of glycoprotein clearance from the circulation, a process that is mediated by the ASGPRs of liver cells, depends on the number of appended sialic acid residues and the number of exposed galactose or *N*-acetylgalactosamine moieties.[10−12]

Neuraminidases (sialidases; E.C. 3.2.1.18) are a specific type of glycosyl hydrolase (GH) that acts on sialic acid, removing it from the end-cap position of a glycoconjugate. Given sialic acid's important role in mammalian biological recognition events, it is not surprising that, in addition to neuraminidases from the host cell, bacterial and viral neuraminidases can affect molecular recognition events that impact the organism. During *Streptococcus pneumoniae* infection, for example, a bacterial neuraminidase catalyzes the removal of sialic acid residues from the host organism's blood platelets. This causes the previously protected sugar structures on the surface of platelets to be recognized by Ashwell receptors (a type of ASGRP) in the liver[8,9] and thereby initiates clearance of these platelets from the circulation, an outcome that lessens coagulation during sepsis.[13] This natural system of molecular recognition has implications for the design of pharmaceuticals. In particular, if a recombinant glycoprotein, such as a glycoprotein hormone or therapeutic agent, lacks terminal sialic acid residues on the carbohydrate chains, then that

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recombinant molecule will be cleared more rapidly from the circulation.[9]

The synthesis of glycoconjugate-containing therapeutic enzymes and protein hormones with long serum half-lives remains challenging despite recent advances in recombinant DNA technologies. One approach to lengthening the circulatory half-live of recombinant glycoproteins is to introduce additional asparagine *N*-glycosylation sites within the protein, effectively increasing the sialic acid content of the therapeutic and thus lengthening its circulatory half-life.[14] Alternatively, glycoproteins expressed in systems that synthesize glycosylation structures that are different than those of humans (e.g., yeast and insect cell lines)[15] can be more closely matched to human type *N*-glycosylation patterns by subsequently attaching *N*-acetylneuraminic acid to the termini of the oligosaccharide chains; ideally, this is done using enzymatic methods.[16] This approach has been successful in cases where the acceptor glycoconjugate contains a terminal sugar residue that is recognized by a sialyltransferase[17] or a *trans*-sialidase.[18]

In another approach, neuraminidases, which normally remove terminal *N*-acetylneuraminic acid residues from glycoproteins, can be used to generate sialoglycoproteins by catalyzing the reaction in reverse. For example, wild-type neuraminidases are capable of transferring an *N*-acetylneuraminic acid residue onto an acceptor sugar.[19] The usefulness of this approach is limited, however, because the trans-glycosylation product is also a substrate for the normal hydrolytic reaction, and thus the enzyme degrades the sialylated products as they are produced.[20]

Using site-directed mutagenesis of the *Micromonospora viridifaciens* neuraminidase (*Mv*NA) gene, we produced a mutant neuraminidase that works in the synthetic direction to give modest yields of sialic acid linked to an acceptor molecule. Specifically, we changed the active site nucleophilic tyrosine residue of *Mv*NA to a glycine residue (Y370G) to give a mutant enzyme (*Mv*NA Y370G) that transfers sialic acid from the non-natural substrate phenyl *N*-acetyl-β-D-neuraminides (Ph-βNeu5Ac) to an acceptor to give α-2,6-sialyl-lactose in yields of up to 13%.[21] In contrast to wild-type neuraminidases, this mutant enzyme is an extremely poor catalyst for the hydrolysis of natural *N*-acetyl-α-D-neuraminides,[22] which contain unactivated carbohydrate aglycones, and thus the concentration of the coupled product remains constant.[21]

As a first step toward increasing the yield of coupled products using mutant neuraminidases, we aimed to understand the mechanism of Y370G mutant-catalyzed reactions and then apply that knowledge toward the selection of optimal conditions for trans-glycosylation.[23] Since both academic and industry researchers express proteins using nonmammalian cell systems and recombinant DNA techniques,[24] an approach that produces nonsialylated glycoproteins, we decided to test the ability of our Y370G mutant sialidase to transfer *N*-acetylneuraminyl units onto a variety of carbohydrates. This paper presents a study of the range of acceptor sugars that undergo *Mv*NA Y370G mutant-catalyzed coupling with *N*-acetylneuraminic acid and the identity of the resulting reaction products.

MATERIALS AND METHODS

Chemicals and Reagents. Raffinose, ribose, and *N*-acetylneuraminic acid were purchased from British Drug Houses Ltd., Eastman Kodak Co., and Rose Scientific, respectively. Dithiothreitol was purchased from Bioshop, and cytidine 5′-triphosphate disodium salt (CTP) was purchased from 3B Scientific Corporation. Pyridine and acetic acid were purchased from Anachemia, and sodium acetate and magnesium chloride were purchased from Caledon Laboratories Ltd. All other chemicals, reagents, and enzymes were purchased from Sigma-Aldrich. Ph-βNeu5Ac and 2FPh-βNeu5Ac were synthesized according to literature procedures.[23,25]

Enzymes. *Mv*NA Y370G mutant was expressed and purified as previously reported.[22] *Escherichia coli N*-acetylneuraminic acid (Neu5Ac) aldolase was purchased from Codexis. *Neisseria meningitides* CMP-Neu5Ac synthase,[26] *Photobacterium sp.* JT-ISH-224 α-2,6-sialyltransferase,[27] and *Campylobacter jejuni* Cst-I α-2,3-sialyltransferase[28] were expressed as reported.

Trans-glycosylation Reactions. We added the *N*-acetylneuraminic acid donor Ph-βNeu5Ac (0.31 mg) and different acceptor sugars (0.60−2.38 mg) to reaction tubes containing *Mv*NA Y370G mutant (6.8 μg) in an aqueous acetonitrile (30% v/v) solution (20 μL, pH 5.25, 100 mM acetate buffer). The reaction conditions in each tube were such that the acceptor concentration (200 mM) was five times greater than the donor concentration (40 mM). All coupling reactions and negative control reactions (the reaction mix without enzyme) were incubated at 37 °C overnight. The acceptor sugars tested were D-lactose, D-glucose, D-mannose, D-raffinose, D-allose, D-fructose, L-xylose, D-ribose, D-lyxose, and D-isoascorbic acid. To see whether the reactions produced a coupled product, we took an aliquot (5 μL) from each of the coupling reaction mixtures, heated it at 85 °C for 15 min to inactivate the enzyme, diluted the sample 1/60 in H_2O (v/v), and analyzed the resultant solution by HPLC-PAD (Dionex ICS-3000 HPLC using amperometric detection). Specifically, we injected the sample (10 μL) 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^2). We identified the trans-glycosylation product by comparing the chromatogram with that of the negative control. After new peaks were identified, we treated an aliquot from the coupling reaction with wild type *M. viridifaciens* neuraminidase and reanalyzed to confirm that the new peak disappeared upon neuraminidase treatment, and thus we concluded that it was *N*-acetylneuraminide.

In a second set of trans-glycosylation reactions, we used *Mv*NA Y370G mutant neuraminidase to transfer Neu5Ac from 2FPh-βNeu5Ac (sialyl donor) to four methyl glycoside acceptor sugars: α- and β-D-galactopyranoside and α- and β-D-glucopyranoside. In a typical experiment, we added neuraminyl donor (5.0 mg; 50 mM), acceptor sugar (25.0 mg; 0.51 M), and *Mv*NA Y370G mutant (0.37 mg) to acetate buffer (pH 5.25, 100 mM) containing acetonitrile (30% v/v) in a total volume of 250 μL and incubated at 37 °C for 16 h. We terminated the reactions by adding ethanol to give a final concentration of 70% (v/v) and then precipitated the enzyme by storing the samples at −20 °C for 20 min. The supernatant was separated from the precipitate by centrifugation (13000 rpm for 10 min at 4 °C), evaporated to dryness, and resuspended in D_2O for ^1H NMR spectral analysis.

To identify the major regioisomer formed during the coupling reactions, we increased the concentrations of both acceptor and donor to achieve Y370G mutant enzyme (0.37 mg), 2FPh-NeuAc (25.0 mg; 250 mM), and acceptor (55.0 mg; 1.13 M) in pH 5.25 acetate buffer (100 mM, containing acetonitrile 30% v/v) in a total volume of 250 μL and then incubated the sample at 37 °C for 64 h. After precipitation of the *Mv*NA Y370G mutant (as described above), we analyzed the sample by NMR spectroscopy to determine the coupling to hydrolysis product ratio. Subsequently, we degraded the hydrolysis product (*N*-acetylneuraminic acid) by adding *N*-acetylneuraminic acid

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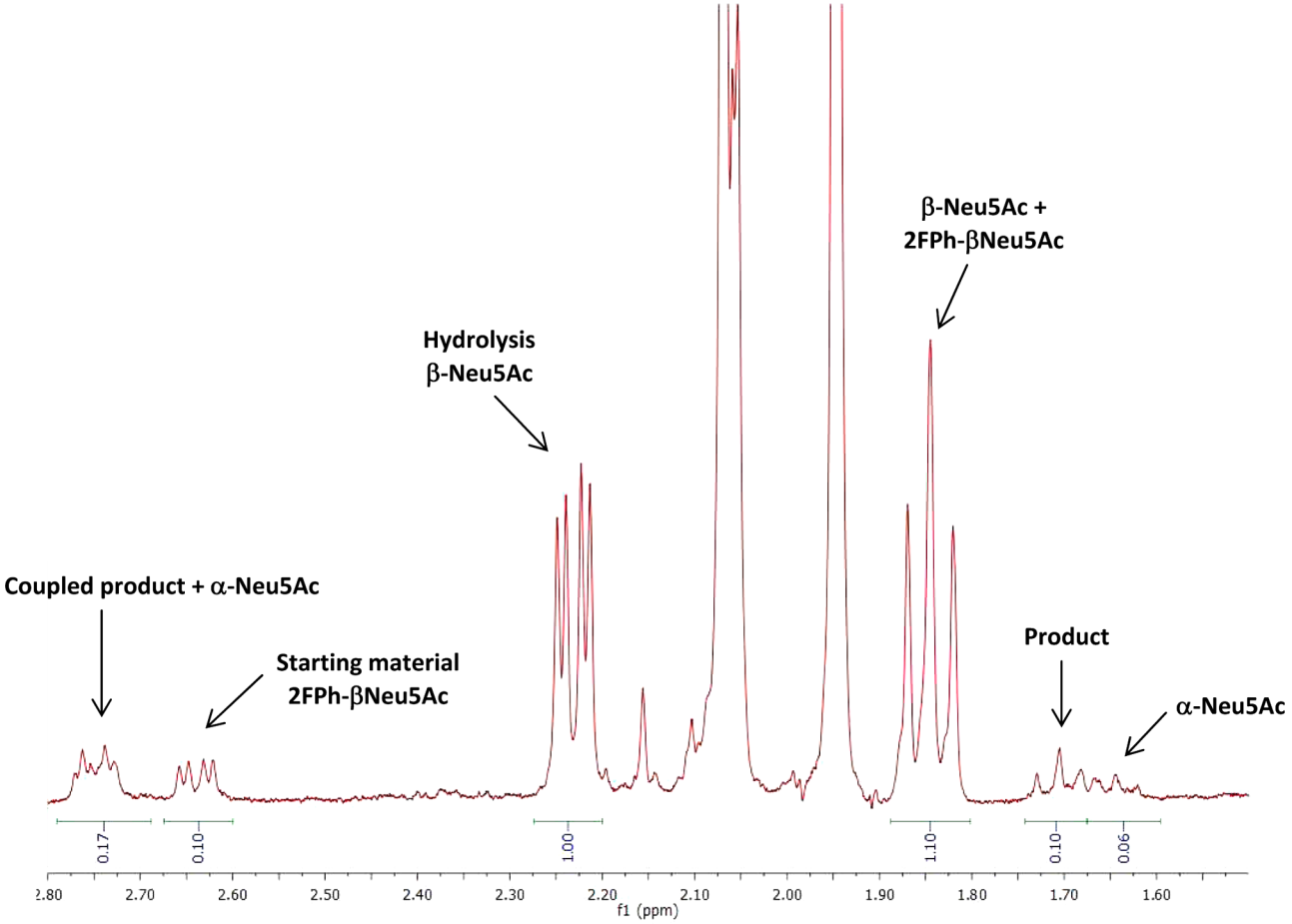

Figure 1. Expansion of the ^{1}H NMR spectra showing the resonances for the various H-3$_{eq}$ and H-3$_{ax}$ protons of the coupling reaction mixture for the *Mv*NA Y370G mutant-catalyzed coupling of 2FPh-*β*Neu5Ac to methyl *α*-D-galactopyranoside acceptor.

aldolase and pyruvate decarboxylase at pH 7.03 (100 mM phosphate buffer) to the sample and incubating at room temperature overnight (16 h). At this time, ^{1}H NMR spectral analysis showed the absence of *N*-acetylneuraminic acid. We then removed the protein by ethanol precipitation and centrifugation (as detailed above). Following evaporation of the supernatant, we resuspended the sample in Milli-Q water and loaded it onto an anion exchange column (DOWEX 1 × 2−200 anion exchange resin−acetate form). The column was washed thoroughly with water, and subsequently *N*-acetylneuraminide products were eluted using a pyridinium acetate buffer (20 mM, pH 5.01). Fractions were collected in 1.5 mL Eppendorf tubes and evaporated to dryness. Based on the acquired ^{1}H NMR spectra for all lyophilized fractions, those containing the purest sample of the major regioisomeric *N*-acetylneuraminide product were combined, and the structure and chemical formula of each product were confirmed by NMR spectroscopy and high resolution mass spectrometry. All NMR peak assignments are based on ^{1}H−^{1}H COSY and ^{1}H−^{13}C HMQC experiments; coupling constants are reported in hertz (Hz). The ESI-MS analyses were performed by the Mass Spectrometry and Proteomics Facility at the University of Notre Dame.

Sialyltransferase-Catalyzed Synthesis. We used a published protocol for the enzyme-catalyzed synthesis of 2,6-*N*-acetylneuraminides with minor modifications.[29] Specifically, we added fresh supernatant (200 *μ*L) from an expression of CMP-Neu5Ac synthase[26] to a reaction medium containing *N*-acetylneuraminic acid (20 mg), CTP (100 mg), $MgCl_2$ (200 mM), and DTT (4 mM) in Tris buffer (0.8 mL; 500 mM; pH 8.01), incubated it for 1.5 h at 37 °C, and centrifuged the sample (5000 rpm) for 5 min at rt. We transferred the supernatant to a clean Eppendorf tube, added methyl *α*-D-galactopyranoside (150 mg), and supernatant (200 *μ*L) from an expression of 2,6-sialyltransferase[27] and then incubated the sample overnight at 37 °C. The progress of the enzymatic reaction was monitored by ^{1}H NMR spectroscopy. Purification and analysis of the trans-glycosylation product was performed as described above. The protocol for synthesis of the 2,3-isomer is given in Supporting Information.

RESULTS

Trans-glycosylation reactions. We investigated the ability of the *Mv*NA Y370G mutant enzyme to use different sugars as acceptors of the *N*-acetylneuraminyl group from the donor Ph-*β*NeuAc; the HPLC-PAD chromatograms from coupling reactions containing D-lactose, D-glucose, D-mannose, D-raffinose, D-allose and D-fructose as acceptors are shown in Figure S1 (Supporting Information). Several acceptor sugars gave rise to a newly observed peak in the HPLC-PAD chromatogram that disappeared if the reaction mixture was subsequently treated with wild-type *Mv*NA (data not shown). In the presence of Ph-*β*NeuAc, the mutant enzyme catalyzed trans-glycosylation with acceptors D-lactose, D-glucose, D-mannose, D-allose, and D-fructose to form single dominant *N*-acetylneuraminyl product peaks (Figure S1, Supporting Information), whereas the acceptor D-raffinose gave two peaks that are

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Table 1. Comparison of *trans*-Glycosylation and Hydrolytic Activities of the *Micromonospora viridifaciens* Y370G Mutant Sialidase with Various Carbohydrate Acceptors[a]

	coupling experiment 1[b]			coupling experiment 2[c]		
acceptor	coupling	hydrolysis	starting material	coupling	hydrolysis	starting material
Me α-galactoside	9%	83%	8%	22%	43%	36%
Me β-galactoside	3%	87%	9%			
Me α-glucoside	24%	78%	3%	31%	45%	24%
Me β-glucoside	3%	94%	3%			
Me α-mannoside				34%	44%	22%

[a]Yields do not necessarily add up to 100% due to rounding; estimated errors are 10% of the quoted value. [b]Reaction conditions: Y370G mutant enzyme (0.37 mg), 2FPh-NeuAc (5.0 mg), and acceptor (25.0 mg) in pH 5.25 acetate buffer (100 mM containing acetonitrile 30% v/v, total volume = 250 μL); incubated for 16 h at 37 °C. [c]Reaction conditions: Y370G mutant enzyme (0.37 mg), 2FPh-NeuAc (25.0 mg), and acceptor (55.0 mg) in pH 5.25 acetate buffer (100 mM containing acetonitrile 30% v/v, total volume = 250 μL); incubated for 64 h at 37 °C.

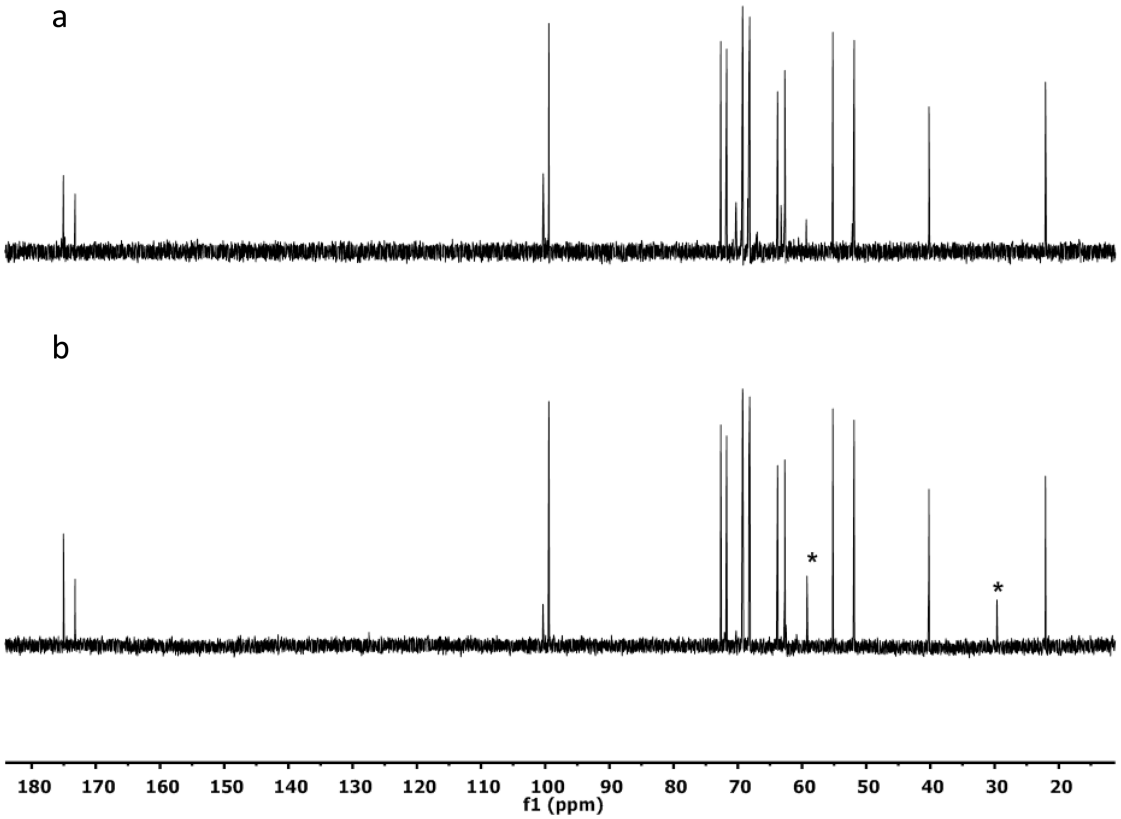

Figure 2. ^{13}C NMR spectra for the coupling reactions of methyl α-D-galactopyranoside to give methyl *N*-acetyl-α-D-neuraminyl-(2 → 6)-α-D-galactopyranoside: (a) reaction catalyzed by *Mv*NA Y370G; (b) reaction catalyzed by *Photobacterium sp.* JT-ISH-224 α-2,6-sialyltransferase using CMP-sialic acid as donor; the two peaks marked by asterisks are impurities.

consistent with two newly formed *N*-acetylneuraminide-containing products. In contrast, the carbohydrate acceptors L-xylose, D-ribose, D-lyxose, and D-isoascorbic acid did not produce any observable product peak in the HPLC-PAD chromatogram.

All of the productive trans-glycosylation acceptor carbohydrates we tested are present in solution as a mixture of pyranose and furanose anomers and as such would require the synthesis of an impracticable number of standard compounds for product identification to proceed via HPLC-PAD chromatography. Instead, we chose to identify the products by NMR spectroscopy, a method with which we could characterize the type of glycosidic linkage formed between sialic acid and commercially available methyl glycopyranosides. Figure 1 shows the ^{1}H NMR spectra we acquired following an overnight incubation of *Mv*NA Y370G mutant (0.51 mg) in a coupling reaction mixture of 2FPh-βNeu5Ac (5.0 mg) and methyl α-D-galactopyranoside (~15 mg). The corresponding NMR spectra for the coupling reactions to methyl β-D-galactopyranoside and methyl α- and β-D-glucopyranosides[30] are shown in Figure S2, Supporting Information, while the spectrum for the sialic acid donor 2FPh-βNeu5Ac is shown in Figure S3 (Supporting Information).

Both the galactoside and the glucoside α-anomers showed a greater propensity for coupling than did their corresponding β-anomers. We calculated the yield of coupled and hydrolysis products by integrating the respective peaks in the ^{1}H NMR spectra and correcting the product integral ($\delta \approx 2.7$) for the small fraction of α-sialic acid that is present at equilibrium[31,32] (Table 1).

Trans-glycosylation Product Isolation and Characterization. We used anion exchange chromatography to purify the products of mutant enzyme-catalyzed trans-glycosylation of a *N*-acetylneuraminyl group and the acceptors methyl α-D-

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galactopyranoside, methyl α-D-glucopyranoside, and methyl α-D-mannopyranoside, as well as that of wild-type 2,3- and 2,6-sialyltransferases with CMP-Neu5Ac and methyl α-D-galactopyranoside. Based on the known specificity of sialyltransferases, we assigned methyl *N*-acetyl-α-D-neuraminyl-(2 → 6)-α-D-galactopyranoside as the major transglycosylation product.[19] The ^{1}H and ^{13}C NMR spectra for three products formed in the *Mv*NA Y370G mutant-catalyzed coupling reactions are shown in Supporting Information (Figures S4−S9). All resonance assignments were based on $^{1}H-^{1}H$ COSY and $^{1}H-^{13}C$ HMQC NMR experiments, and all high resolution *m*/*z* ratios (in negative ion mode) for the three purified methyl *N*-acetyl-α-D-neuraminyl-(2 → 6)-α-D-glycopyranosides were in the *m*/*z* range of 484.1639−484.1687, which brackets that expected ($M - H^{+}$; *m*/*z* = 484.1666) for the molecular formula $C_{18}H_{31}NO_{14}$ (Supporting Information).

Based on a series of low intensity signals (Figure S7, Supporting Information), the ^{13}C NMR spectrum for the mutant enzyme mediated coupling of a *N*-acetylneuraminyl glycosidic bond to methyl α-D-galactopyranoside is consistent with the formation of more than one regioisomer in this coupling reaction. The major product is an α-2 → 6 linked sugar as shown by a comparison of the ^{13}C NMR spectra for the products formed by the mutant neuraminidase and the 2,6-sialyltransferase (Figure 2). To determine whether any 2,3-isomeric coupled product, methyl *N*-acetyl-α-D-neuraminyl-(2 → 3)-α-D-galactopyranoside, is formed in the *Mv*NA Y370G mutant-catalyzed reactions, we synthesized this material by using a 2,3-sialyltransferase-catalyzed reaction and compared the ^{1}H NMR spectrum of this product (Figure S15, Supporting Information) with that from the *Mv*NA Y370G mutant catalyzed reaction. On the basis of the integration of the ^{1}H NMR spectrum from the *Mv*NA Y370G mutant reaction mixture with methyl α-D-galactopyranoside as the acceptor, we conclude that, at most, only a small quantity of the 2,3-regioisomer is formed (Supporting Information, Figures S7 and S8a).

For all isolated methyl glycoside coupling products, we acquired $^{1}H-^{13}C$ two-dimensional HMBC correlation spectra, which show two and three bond ^{1}H to ^{13}C correlations, in order to assign the major regioisomer in the coupled product mixtures. In the case of the mannoside coupled product, we observed the presence of cross peaks that are consistent with the major linkage being *N*-acetyl-α-D-neuraminyl-(2 → 6)-α-D-mannopyranoside. However, for the coupling product when methyl α-D-glucopyranoside was the acceptor, we could not unambiguously determine the regiochemistry, but we suggest that it is likely the 2 → 6-isomer. Although this compound has been synthesized,[30] no ^{13}C NMR spectrum was reported so that we could not compare it to our spectrum. Also, we were unable to identify the regiochemistry of minor stereoisomers because of low NMR signal intensities and the complexity of the ^{1}H NMR spectra.

DISCUSSION

Nucleophilic mutant glycosidase enzymes, which are called glycosynthases,[33] are a useful and efficient tool for the synthesis of glycosides.[33−35] Glycosynthases, which typically use a reactive glycosyl fluoride as the carbohydrate donor, have either a nonpolar (typically an alanine) or a hydrogen-bond donor (typically a serine) residue in place of the carboxylate nucleophile (aspartate or glutamate) of the natural enzyme (a glycosidase) from which the glycosynthase was derived. With our mutant neuraminidase, we have both extended the glycosynthase concept to sialidases and broadened the utility of this synthetic approach. In particular, by replacing the *M. viridifaciens* neuraminidase tyrosine nucleophile with a glycine residue, we generated a hole within the active site that can accommodate the phenyl leaving group of our carbohydrate donors.[21,22,36] Moreover, our sialic acid donors have an increased stability relative to the glycosyl fluorides that are typical glycosynthase donor substrates. That is, the half-lives for hydrolysis (at 37 °C) of our sialic acid donors Ph-βNeu5Ac and 2FPh-βNeu5Ac are estimated to be 1500 and 29 years, respectively,[25] while that for the typical glycosynthase donor α-D-glucopyranosyl fluoride is approximately 34 h.[37]

The approach of adding *N*-acetylneuraminic acid residues to carbohydrates using a mutant neuraminidase (glycosynthase) also takes advantage of an important characteristic of the parent enzyme, which functions catabolically as a hydrolase. In general, catabolic enzymes exhibit greater substrate promiscuity than anabolic enzymes. For example, the anabolic sialyltransferases (*N*-acetylneuraminyl transferases; E.C. 2.4.99.X) usually display high selectivities for the neuraminide acceptor (which becomes the aglycone in the product), although a few examples of tolerance with regard to the acceptor carbohydrate have been reported.[38,39] In contrast, wild-type *Mv*NA efficiently catalyzes the removal of Neu5Ac residues irrespective of both the identity of the aglycone and the type of glycosidic linkage.[22,40,41] The promiscuity of wild-type *Mv*NA toward the aglycone structure is preserved in the *Mv*NA Y370G mutant, because this genetically modified enzyme catalyzes trans-glycosylation of *N*-acetylneuraminic acid with a variety of aglycone acceptors, including lactose, glucose, raffinose (β-D-fructofuranosyl α-D-galactopyranosyl-(1 → 6)-α-D-glucopyranoside), allose, and fructose, to give neuraminide products. Moreover, if the wild-type *Mv*NA is added to the mutant enzyme coupling mixtures, it removes these trans-glycosylation products, presumably by hydrolysis. Based on this observation, we conclude that the Y370G-catalyzed trans-glycosylation reactions form novel *N*-acetylneuraminides.

We identified the products of *Mv*NA Y370G-catalyzed coupling of the *N*-acetylneuraminyl group to various carbohydrate acceptors, focusing on methyl glycosides of sugars that yielded a single observable product peak in the HPLC-PAD chromatogram (Figure S1, Supporting Information). We used commercial samples of methyl α- and β-D-glucopyranosides, methyl α- and β-D-galactopyranosides, and methyl α-D-mannopyranoside in the coupling reactions and employed NMR spectroscopy to characterize the reaction products. The regiochemistry of coupling to the methyl galactoside acceptors was confirmed by comparing the results to those from reactions conducted with a wild-type sialyltransferase. Previously we noted that *Mv*NA Y370G preferentially forms 2,6-sialyl lactose during the coupling reaction with Ph-βNeu5Ac and lactose[21] despite the fact that the catalytic efficiency of the wild-type *Mv*NA (k_{cat}/K_{m}) is higher for 2,3-sialyl lactose as substrate.[22] In order to minimize coupling reaction times, we employed the more reactive 2FPh-βNeu5Ac rather than Ph-βNeu5Ac as the *N*-acetylneuraminyl group donor. Specifically, 2FPh-βNeu5Ac has a 25-fold higher rate of turnover in a *Mv*NA Y370G-catalyzed reaction than the parent compound ($k_{cat(2FPh\text{-}\beta Neu5Ac)} \approx 25k_{cat(Ph\text{-}\beta Neu5Ac)}$[21]).

Our results suggest that for both methyl glucopyranosides and galactopyranosides it is the α-anomer that is the better acceptor, because it produces correspondingly higher coupling yields (Table 1, coupling experiment 1). This result is surprising, because the most common aglycone found in *N*-acetylneuraminides is a β-galactoside linkage that often is present in sialylated glycoconjugates as a lactose or a *N*-acetyllactosamine

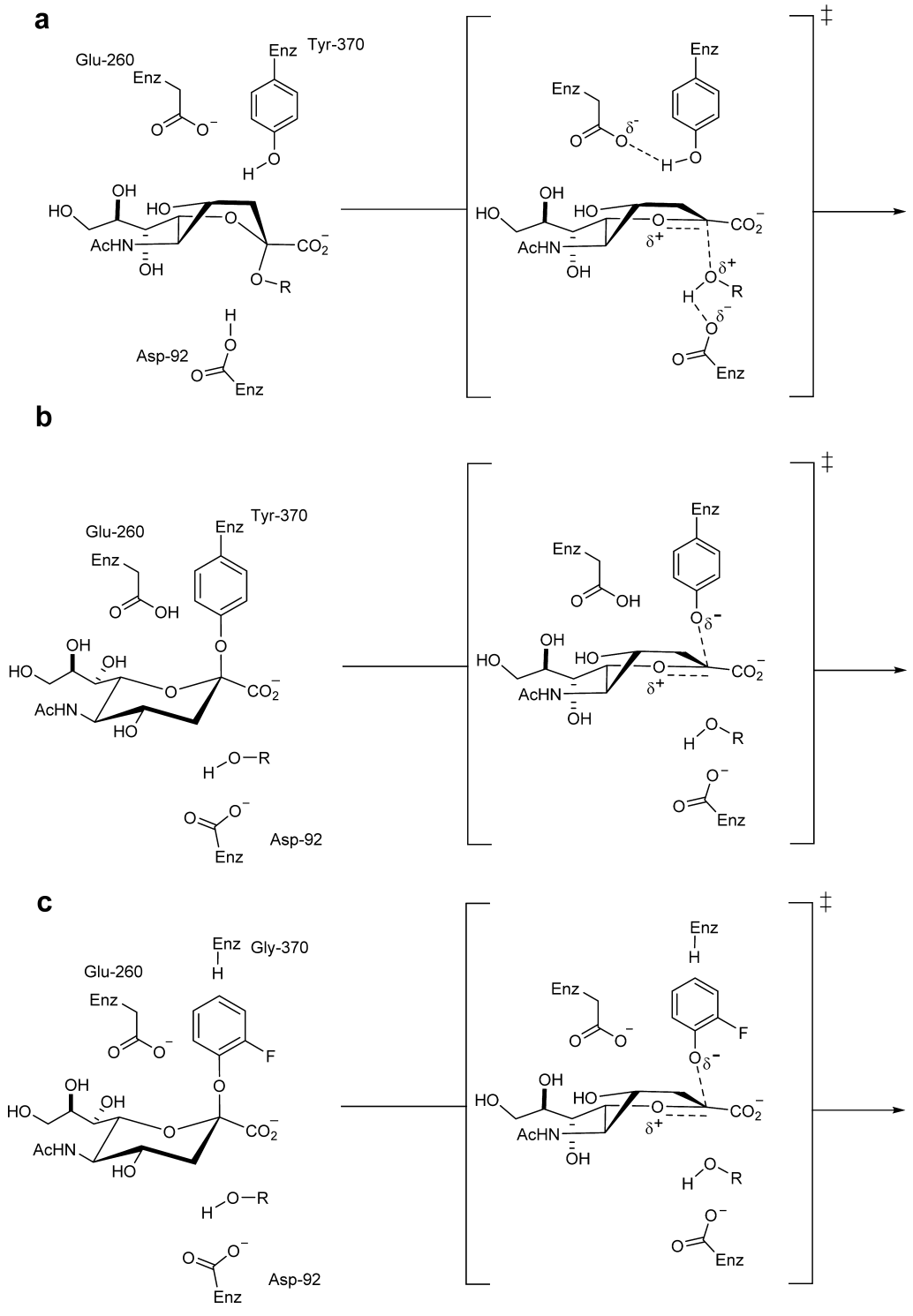

Figure 3. Proposed transition states for (a) glycosylation of wild-type *Mv*NA in which cleavage of the anomeric C−O bond is occurring with little or no nucleophilic assistance from the enzymatic tyrosine residue, (b) deglycosylation of wild-type *Mv*NA in which cleavage of the tyrosinyl-bound intermediate occurs with little or no nucleophilic assistance, and (c) trans-glycosylation for *Mv*NA Y370G catalysis in which cleavage of the anomeric C−O bond to the fluorophenyl leaving group is occurring with little or no nucleophilic assistance from nonselectively bound sugars (R = carbohydrate) or solvent waters (R = H).

constituent.[42,43] The nonspecific binding of methyl α-glycopyranoside acceptors to the active site of *Mv*NA Y370G during catalysis results in a selection factor for coupling over hydrolysis of 18−27 (see Supporting Information for the calculations).

The regioisomers form simultaneously from the E/S complex (E/βNeu5Ac-OAr in Figure 3c). If the E/S complex is a good mimic of the naturally occurring sialosyl-enzyme intermediate (Figure 3b), then the relative proportions of 2,3- and 2,6-products can be combined with the relative rates for *Mv*NA-catalyzed hydrolysis of 2,3- and 2,6-*N*-acetyl-α-D-neuraminyl lactose regioisomers[22] to give an estimate of the free energy difference between these regioisomers.[44] For example, had we

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detected an estimated 10% of the 2,3-isomer in the presence of the 2,6-isomer as reaction products (Figures S7 and S8a, Supporting Information) and used the Haldane equation[44] to calculate a free energy difference between the two regioisomers of >9 kJ mol^{-1}. However, in a previous report, we suggested that *Mv*NA Y370G-mediated catalysis proceeds via a transition state that involves no general acid catalysis (Figure 3c), a situation that occurs because the catalytic glutamate is likely to be deprotonated in the mutant enzyme ground state.[23] In contrast, the transition state for the *Mv*NA-catalyzed glycosylation has an addition proton that bridges between the nucleophilic tyrosine and the general base glutamate[45,46] (Figure 3a).

If the *Mv*NA Y370G catalyzed coupling reactions proceed via transition states as depicted in Figure 3c (R = H or a sugar) then the indiscriminate nature of these trans-glycosylation reactions must arise from nonselective binding interactions between the acceptor sugar and the enzymatic active site. Therefore, increasing coupling efficiency and selectivity in these trans-glycosylation reactions only requires increasing the carbohydrate binding efficiency close to the catalytic center; this increase could be accomplished by the mutation of proximal active site amino acid residues.

CONCLUSION

A nucleophile deficient mutant of the neuraminidase from *Micromonospora viridifaciens* catalyzes *N*-acetylneuraminyl transfer reactions for which it displays a high level of promiscuity toward carbohydrate acceptors and an accompanying low selectivity toward water. The formation of products that originate from the attack of a carbohydrate primary hydroxyl group on the nascent *N*-acetylneuraminyl cation are preferred to those initiated by the sterically more encumbered pyranosyl ring secondary ring alcohol functionalities. To increase coupling efficiency, we suggest that mutation of amino acid residues that are close to the catalytic site is required in order to incorporate a binding site for the carbohydrate acceptor.

ASSOCIATED CONTENT

Supporting Information

General methods and NMR characterization data, HPLC-PAD chromatograms of *Mv*NA Y370G mutant-catalyzed trans-glycosylation reactions, ^{1}H NMR spectra of *Mv*NA Y370G mutant-catalyzed reaction mixtures for methyl β-D-galactopyranoside and methyl α- and β-D-glucopyranoside couplings, ^{1}H NMR spectra of 2FPh-βNeu5Ac, ^{1}H NMR spectra of *Mv*NA Y370G mutant-catalyzed reaction mixtures for methyl α-D-galactopyranoside, α-D-glucopyranoside, and α-D-mannopyranoside coupling reactions, NMR spectroscopic characterization data for and ^{1}H and ^{13}C spectra of three methyl *N*-acetyl-α-D-neuraminyl-(2 → 6)-α-D-glycopyranosides, and the ^{1}H and ^{13}C spectra of methyl *N*-acetyl-α-D-neuraminyl-(2 → 3)-α-D-galactopyranoside. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

HOAc, acetic acid; Neu5Ac, *N*-acetylneuraminic acid; Tris, tris(hydroxymethyl)aminomethane; *Mv*NA, *Micromonospora viridifaciens* neuraminidase; *Mv*NA Y370G, *Micromonospora viridifaciens* Y370G mutant neuraminidase; Ph-βNeu5Ac, phenyl *N*-acetyl-β-D-neuraminide; 2FPh-βNeu5Ac, 2-fluorophenyl *N*-acetyl-β-D-neuraminide; CTP, cytidine 5′-triphosphate; DTT, dithiothreitol; rt, room temperature; ESI-MS, electrospray ionization mass spectrometry; ^{1}H−^{1}H COSY, homonuclear correlation spectroscopy; ^{1}H−^{13}C HSQC, heteronuclear single-quantum coherence spectroscopy; ^{1}H−^{13}C HMBC, heteronuclear multiple-bond correlation spectroscopy; HPLC-PAD, high performance liquid chromatography with pulsed amperometric detection; CMP-Neu5Ac, cytidine 5′-(5-acetamido-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulopyranosylonic acid monophosphate); ASGPR, asialoglycoprotein receptor

REFERENCES

(1) Angata, T., and Varki, A. (2002) Chemical diversity in the sialic acids and related α-keto acids: An evolutionary perspective. *Chem. Rev. 102*, 439−470.

(2) Miyagi, T., Takahashi, K., Hata, K., Shiozaki, K., and Yamaguchi, K. (2012) Sialidase significance for cancer progression. *Glycoconjugate J. 29*, 567−577.

(3) Schauer, R. (2000) Achievements and challenges of sialic acid research. *Glycoconjugate J. 17*, 485−499.

(4) Taylor, G. (1996) Sialidases: Structures, biological significance and therapeutic potential. *Curr. Opin. Struct. Biol. 6*, 830−837.

(5) Schauer, R. (2009) Sialic acids as regulators of molecular and cellular interactions. *Curr. Opin. Struct. Biol. 19*, 507−514.

(6) Monti, E., Bonten, E., D'Azzo, A., Bresciani, R., Venerando, B., Borsani, G., Schauer, R., and Tettamanti, G. (2010) Sialidases in vertebrates: A family of enzymes tailored for several cell functions. *Adv. Carbohydr. Chem. Biochem. 64*, 403−479.

(7) Varki, A., and Gagneux, P. (2012) Multifarious roles of sialic acids in immunity. *Ann. N.Y. Acad. Sci. 1253*, 16−36.

(8) Ashwell, G., and Harford, J. (1982) Carbohydrate-specific receptors of the liver. *Annu. Rev. Biochem. 51*, 531−554.

(9) Drickamer, K. (1991) Clearing up glycoprotein hormones. *Cell 67*, 1029−1032.

(10) Mortensen, B., and Huseby, N. E. (1997) Clearance of circulating γ-glutamyltransferase by the asialoglycoprotein receptor. Enzyme forms with different sialic acid content are eliminated at different clearance rates and without apparent desialylation. *Clin. Chim. Acta 258*, 47−58.

(11) Basset, C., Devauchelle, V., Durand, V., Jamin, C., Pennec, Y. L., Youinou, P., and Dueymes, M. (1999) Glycosylation of immunoglobulin A influences its receptor binding. *Scand J. Immunol 50*, 572−579.

(12) Kronman, C., Velan, B., Marcus, D., Ordentlich, A., Reuveny, S., and Shafferman, A. (1995) Involvement of oligomerization, *N*-glycosylation and sialylation in the clearance of cholinesterases from the circulation. *Biochem. J. 311*, 959−967.

(13) Grewal, P. K., Uchiyama, S., Ditto, D., Varki, N., Le, D. T., Nizet, V., and Marth, J. D. (2008) The Ashwell receptor mitigates the lethal coagulopathy of sepsis. *Nat. Med. 14*, 648−655.

(14) Elliott, S., et al. (2003) Enhancement of therapeutic protein in vivo activities through glycoengineering. *Nat. Biotechnol. 21*, 414−421.

(15) Hamilton, S. R., et al. (2003) Production of complex human glycoproteins in yeast. *Science 301*, 1244−1246.

(16) Hollister, J. R., and Jarvis, D. L. (2001) Engineering lepidopteran insect cells for sialoglycoprotein production by genetic transformation

with mammalian 1,4- galactosyltransferase and 2,6-sialyltransferase genes. *Glycobiology 11*, 1−9.
(17) Sato, M., Sadamoto, R., Niikura, K., Monde, K., Kondo, H., and Nishimura, S. I. (2004) Site-specific introduction of sialic acid into insulin. *Angew. Chem., Int. Ed. 43*, 1516−1520.
(18) Marcha, I., Cerutti, M., Mir, A. M., Juliant, S., Devauchefle, G., Cacan, R., and Verbert, A. (2001) Expression of a membrane-bound form of *Trypanosoma cruzi* trans-sialidase in baculovirus-infected insect cells: A potential tool for sialylation of glycoproteins produced in the baculovirus-insect cells system. *Glycobiology 11*, 593−603.
(19) Schmidt, D., Sauerbrei, B., and Thiem, J. (2000) Chemoenzymatic synthesis of sialyl oligosaccharides with sialidases employing transglycosidation methodology. *J. Org. Chem. 65*, 8518−8526.
(20) Ajisaka, K., Fujimoto, H., and Isomura, M. (1994) Regioselective transglycosylation in the synthesis of oligosaccharides: Comparison of β-galactosidases and sialidases of various origins. *Carbohydr. Res. 259*, 103−115.
(21) 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.
(22) 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.
(23) Shidmoossavee, F. S., Cheng, L., Watson, J. N., and Bennet, A. J. (2010) Brønsted analysis of an enzyme-catalyzed pseudo-deglycosylation reaction: Mechanism of desialylation in sialidases. *Biochemistry 49*, 6473−6484.
(24) Cregg, J. M., Cereghino, J. L., Shi, J. Y., and Higgins, D. R. (2000) Recombinant protein expression in *Pichia pastoris*. *Mol. Biotechnol. 16*, 23−52.
(25) 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.
(26) Karwaski, M. F., Wakarchuk, W. W., and Gilbert, M. (2002) High-level expression of recombinant *Neisseria* CMP-sialic acid synthetase in *Escherichia coli*. *Protein Expression Purif. 25*, 237−240.
(27) Kakuta, Y., Okino, N., Kajiwara, H., Ichikawa, M., Takakura, Y., Ito, M., and Yamamoto, T. (2008) Crystal structure of vibrionaceae *photobacterium* sp JT-ISH-224 α2,6-sialyltransferase in a ternary complex with donor product CMP and acceptor substrate lactose: Catalytic mechanism and substrate recognition. *Glycobiology 18*, 66−73.
(28) Pukin, A. V., et al. (2008) GM3, GM2 and GM1 mimics designed for biosensing: Chemoenzymatic synthesis, target affinities and 900 MHz NMR analysis. *Carbohydr. Res. 343*, 636−650.
(29) Chan, J., Lu, A., and Bennet, A. J. (2011) Turnover is rate-limited by deglycosylation for *Micromonospora viridifaciens* sialidase-catalyzed hydrolyses: Conformational implications for the Michaelis complex. *J. Am. Chem. Soc. 133*, 1877−1884.
(30) Okamoto, K., Hasegawa, T., Toyomaki, Y., Yamakawa, M., and Okukado, N. (1992) 3β-Hydroxysialic acid glycosides. 1. Calcium-binding ability and chemical and enzymatic stabilities. *Chem. Pharm. Bull. 40*, 2728−2734.
(31) 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.
(32) Chan, J., Sandhu, G., and Bennet, A. J. (2011) A mechanistic study of sialic acid mutarotation: Implications for mutarotase enzymes. *Org. Biomol. Chem. 9*, 4818−4822.
(33) Mackenzie, L. F., Wang, Q., Warren, R. A. J., and Withers, S. G. (1998) Glycosynthases: Mutant glycosidases for oligosaccharide synthesis. *J. Am. Chem. Soc. 120*, 5583−5584.
(34) Williams, S. J., and Withers, S. G. (2002) Glycosynthases: Mutant glycosidases for glycoside synthesis. *Aust. J. Chem. 55*, 3−12.
(35) Rakić, B., and Withers, S. G. (2009) Recent developments in glycoside synthesis with glycosynthases and thioglycoligases. *Aust. J. Chem. 62*, 510−520.
(36) 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.
(37) Zhang, Y., Bommuswamy, J., and Sinnott, M. L. (1994) Kinetic isotope effect study of transition states for the hydrolyses of α- and β-glucopyranosyl fluorides. *J. Am. Chem. Soc. 116*, 7557−7563.
(38) van Dorst, J. A. L. M., Tikkanen, J. M., Krezdorn, C. H., Streiff, M. B., Berger, E. G., VanKuik, J. A., Kamerling, J. P., and Vliegenthart, J. F. G. (1996) Exploring the substrate specificities of α-2,6- and α-2,3-sialyltransferases using synthetic acceptor analogues. *Eur. J. Biochem. 242*, 674−681.
(39) Van Pelt, J., Dorland, L., Duran, M., Hokke, C. H., Kamerling, J. P., and Vliegenthart, J. F. G. (1990) Sialyl-α-2−6-mannosyl-β-1−4-*N*-acetylglucosamine, a novel compound occurring in urine of patients with β-mannosidosis. *J. Biol. Chem. 265*, 19685−19689.
(40) Narine, A. A., Watson, J. N., and Bennet, A. J. (2006) Mechanistic requirements for efficient enzyme-catalyzed hydrolysis of thiosialosides. *Biochemistry 45*, 9319−9326.
(41) Aisaka, K., Igarashi, A., and Uwajima, T. (1991) Purification, crystallization, and characterization of neuraminidase from *Micromonospora viridifaciens*. *Agric. Biol. Chem. 55*, 997−1004.
(42) Varki, A. (1997) Sialic acids as ligands in recognition phenomena. *FASEB J. 11*, 248−255.
(43) Varki, A. (2007) Glycan-based interactions involving vertebrate sialic-acid-recognizing proteins. *Nature 446*, 1023−1029.
(44) Segel, I. H. (1975) *Enzyme kinetics: Behavior and analysis of rapid equilibrium and steady state enzyme systems*, Wiley, New York.
(45) Chan, J., Watson, J. N., Lu, A., Borgford, T. J., and Bennet, A. J. (2012) Bacterial and viral sialidases: Contribution of the conserved active site glutamate to catalysis. *Biochemistry 50*, 433−441.
(46) Chan, J., Lewis, A. R., Indurugalla, D., Schur, M., Wakarchuk, W., and Bennet, A. J. (2012) Transition state analysis of *Vibrio cholerae* sialidase-catalyzed hydrolyses of natural substrate analogues. *J. Am. Chem. Soc. 134*, 3748−3757.