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Substrate Recognition of the Membrane-Associated Sialidase NEU3 Requires a Hydrophobic Aglycone

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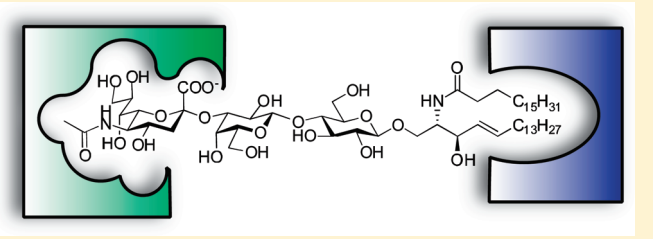
Supporting Information

ABSTRACT: The human neuraminidases (NEU) consist of a family of four isoforms (NEU1−NEU4). Members of this enzyme family are proposed to have important roles in health and disease through regulation of the composition of cellular sialosides. The NEU3 isoform is a membrane-associated enzyme that cleaves glycolipid substrates. However, few reports have examined the substrate specificity of the enzyme for non-natural substrates. We report here a series of 11 synthetic trisaccharides that feature modifications of the aglycone or the Neu5Ac residue of an octyl β-sialyllactoside. The time course of substrate cleavage by NEU3 was monitored using an electrospray ionization mass spectrometry assay to obtain relative rates (k_{rel}). We observed that NEU3 substrate activity was directly dependent upon the hydrophobicity of the aglycone but had no apparent requirement for features of the ceramide headgroup. We also observed that trisaccharides with incorporated azide groups in the Neu5Ac residue at either C9 or the N5-Ac position were substrates, and in the case of the N5-azidoacetyl derivative, the activity was superior to that of GM3. However, the incorporation of larger aryl groups was tolerated only at C9, but not at N5-Ac. We propose a two-site model for enzyme recognition, requiring interaction at both the Neu5Ac residue and the hydrophobic aglycone.



The plasma membrane is a dynamic cellular compartment that is continually remodeled by the action of transport, biosynthesis, and catabolic mechanisms.[1] In recent years, the role of glycolipids in organizing membrane nanostructures, or lipid rafts, has become a major focus of study.[2,3] While the precise details of the lipid raft hypothesis may still be under revision,[4] glycolipids are well-known to participate in receptor signaling and clustering events. As a result of their central role in membrane organization, mechanisms that alter the structure or composition of membrane glycolipids constitute a potential regulatory mechanism.[5]

Biosynthetic mechanisms of glycolipid remodeling are relatively slow; however, catabolic mechanisms, such as hydrolysis of membrane glycolipids, have the potential to affect rapid remodeling. Membrane-associated glycosylhydrolase enzymes may, therefore, play a role in signaling pathways through processing of glycolipids. This hypothesis is especially attractive when one considers that the half-lives of terminal glycan residues, such as sialic acid and fucose, are unusually low relative to those of core residues[6] and that cell surface glycans are known to be sialylated after endocytosis.[7] A number of membrane-associated glycosidase activities are known in mammalian cells, including β-galactosidase,[8] β-glucosidase,[9] α-mannosidase,[10] α-fucosidase,[11] and multiple isoforms of neuraminidase.[12] Among these enzymes, the neuraminidases have attracted attention as participating in a variety of signaling pathways and pathologies, including inflammation, adhesion, and cancer metastasis.[13] Neuraminidase 3 (NEU3) is known as a plasma membrane-associated sialidase, which has a substantial preference for ganglioside substrates over glycoproteins.[14]

Despite its apparent importance in disease and membrane structure, to date the substrate specificity of human NEU3 has not been well-defined. Previous work has primarily examined the specificity of NEU3 using natural ganglioside substrates. A number of groups have observed that the enzyme hydrolyzes α(2→3)-sialosides (e.g., GM3, GD1a, and GT1b).[14−20] Additionally, NEU3 has been found to hydrolyze α(2→8)-sialosides (e.g., GD3, GD1b, and GT1b)[14,17] and may even preferentially cleave this linkage,[21] although some reports contradict this finding.[15] Glycolipids that are α(2→3)-sialylated but contain a branch point at the adjacent galactose residue {GalNAcβ(1→4)[Neu5Acα(2→3)]Galβ(1→4)Glcβ-Cer}, as found in GM1 and GM2, are poor substrates for NEU3.[14,16,17] Reports of synthetic glycan cleavage by human neuraminidase (hNEU) enzymes are limited. Watson et al. found that legionaminic acid derivatives (5,7-diacetamido-3,5,7,9-tetradeoxy-D-glycero-D-galacto-non-2-ulosonic acid) were poor substrates for the human neuraminidase 2 (NEU2).[22] The NEU2 linkage specificity has

Received: March 25, 2011
Revised: June 13, 2011
Published: June 15, 2011

been studied in detail: the enzyme preferentially cleaves α(2→3) linkages but also cleaves α(2→8)- and α(2→6)-sialosides.[23,24]

We recently developed a recombinant expression system for NEU3,[25] and we sought to use this system to explore the substrate tolerance of the enzyme. In particular, we were interested in examining the role of the lipid chain in substrate recognition, as previous reports have suggested that the enzyme has specificity for glycolipid substrates.[14] Additionally, we aimed to test if the active site of the protein could tolerate modifications of the Neu5Ac residue that would allow labeling studies.[26] Herein we report the development of a sensitive electrospray ionization mass spectrometry (ESI-MS) assay for detection of enzyme activity with nanograms of glycolipid analyte. Using a series of synthetic glycolipid analogues, we found that the lipid chain hydrophobicity, but not its identity, was critical for NEU3 recognition. We also determine that modifications of Neu5Ac at the C9 position can be tolerated by the enzyme.

MATERIALS AND METHODS

General Synthetic Methods. Dry solvents (CH_2Cl_2, MeOH, CH_3CN, and DMF) were purchased from Sigma Aldrich in capped DriSolv bottles and used without purification and stored under argon. GM3 was purchased from Avanti polar lipids and stored under argon at −20 °C. 4MU-NA was purchased from Sigma Aldrich (St. Louis, MO). All reactions were conducted under a stream of argon and monitored by TLC on silica gel G-25 UV254 (0.25 mm). TLC plates were visualized under a UV lamp or dipped in cerium molybdate stain and charred on a heating plate. Flash column chromatography was performed using a Combiflash companion chromatography instrument (Teledyne Isco, Inc., Lincoln, NE) with Redisep and Silicycle flash silica gel columns (40−63 μm). The purification of polar trisaccharides was performed by manual column chromatography with strong anion exchange resin (POROS 50 HQ) and 0.25 M ammonium bicarbonate as eluent and by reversed phase C-18 silica with MeOH and H_2O as eluents. Some of the lipid-modified trisaccharides were purified using Iatrobead silica. Iatrobeads (6RS-8060, 60 μm) were purchased from Shell-USA Inc. NMR experiments were conducted on Varian 400, 500, 600, and 700 MHz instruments. Chemical shifts are reported relative to the deuterated solvent peaks in parts per million. Optical rotations were measured at 21 ± 2 °C at the sodium D line (589 nm). ESI-MS spectra were recorded on samples suspended in solvent with added NaCl. Details of glycolipid substrate synthesis and characterization are included as Supporting Information.

Mass Spectrometry. Electrospray ionization mass spectrometry (ESI-MS) measurements were taken using an Apex-Qe 9.4 T Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS) (Bruker, Billerica, MA) equipped with an external ESI source. ESI was performed using a borosilicate tube (1.0 mm outside diameter, 0.68 mm inside diameter), pulled to ∼5 μm outside diameter at one end using a P-97 micropipet puller (Sutter Instruments, Novato, CA). The electric field required to spray the solution in negative ion mode was established by applying a voltage of approximately −900 V to a platinum wire inserted inside the ESI tip. The solution flow rate was typically ∼20 nL/min. The droplets and gaseous ions produced by ESI were introduced into the mass spectrometer through a glass sampling capillary (0.5 mm inside diameter). Nitrogen gas at a flow rate of 2.0 L/min and 90 °C was used as the drying gas to facilitate the production of gas-phase ions. The capillary entrance

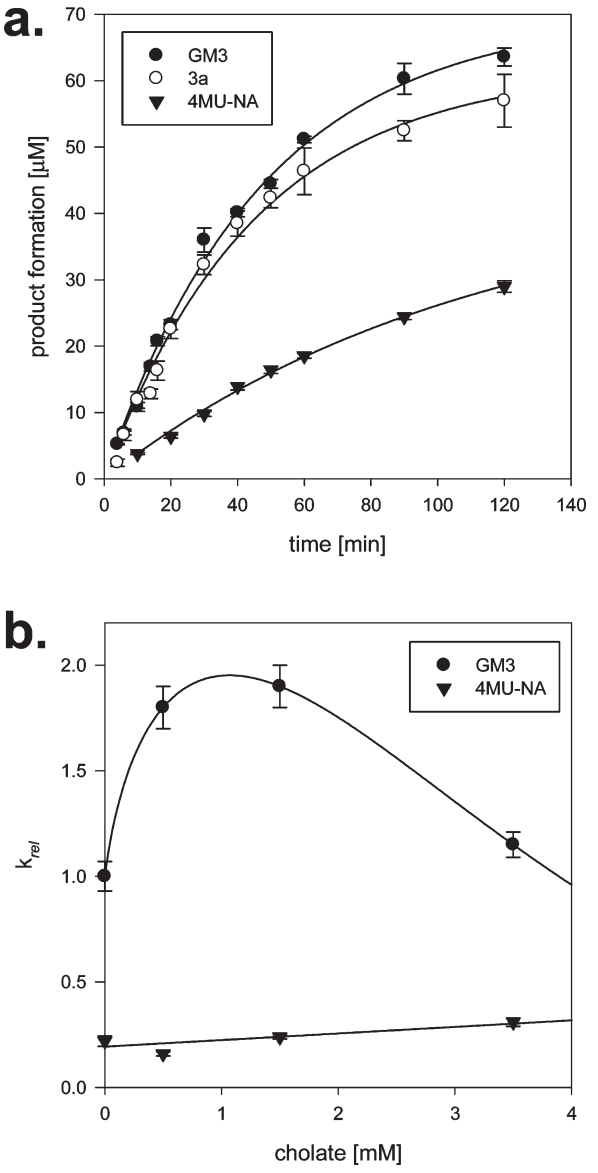


Figure 1. NEU3 cleavage of GM3. (a) Hydrolysis of 4MU-NA, GM3, and **3a** by NEU3 monitored using the ESI-MS assay. Curves were fit to an exponential for the sake of clarity. (b) Relative rates of 4MU-NA and GM3 hydrolysis in the presence of sodium cholate (0−3.5 mM). Initial rates were used to determine k_{rel} (see the Supporting Information). All points represent five replicates, and the error is shown as the standard deviation.

voltage was held at 0 V, and the capillary exit was held at −340 V. A deflector voltage of −250 V was used. Gaseous ions were transmitted through the first funnel and skimmer held at −190 and −20 V, respectively, and then through the second funnel and skimmer held at −7.6 and −4.5 V, respectively. The ions were stored electrodynamically in an rf hexapole for 0.5 s followed by further accumulation in a hexapole collision cell for 0.4 s. Following accumulation, the ions were transferred from the hexapole collision cell and, through a series of ion optics, introduced into the ICR cell. The front and back trapping plates of the cell were maintained at −0.65 and −0.95 V, respectively, throughout the experiment. The typical base pressure for the instrument was $\sim 1 \times 10^{-10}$ mbar. Data acquisition and analysis

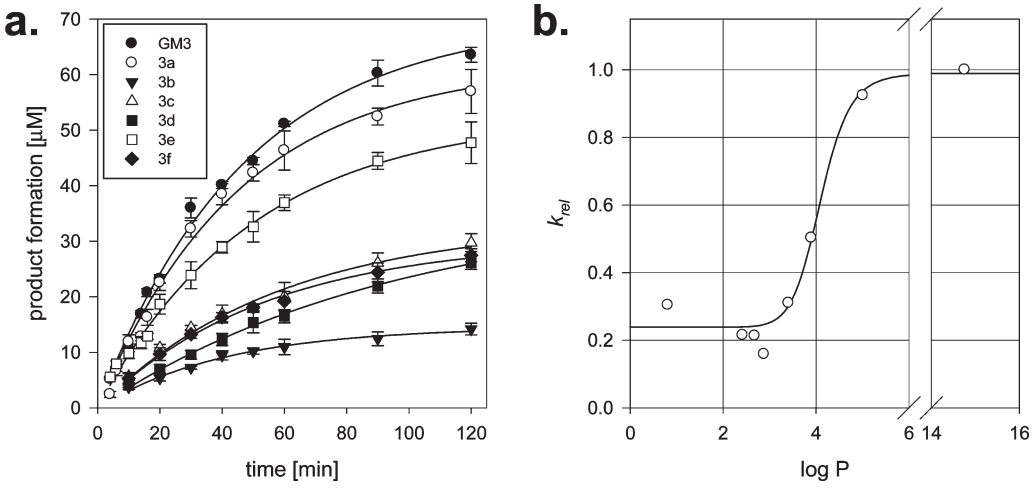


Figure 2. NEU3 cleavage of α(2→3)-sialosides with a modified aglycone. (a) Time course of synthetic glycolipid hydrolysis by NEU3 monitored by ESI-MS. Initial rates were used to determine k_{rel}, and values are listed in Table 1 (see the Supporting Information for expansion plots). All points represent five replicates, and the error is shown as the standard deviation. (b) Relative rates of NEU3 hydrolysis are dependent on the hydrophobicity of the acyl chain. Calculated log P values (octanol−water) of GM3, 4MU-NA, and **3a**−**f** were plotted vs the measured k_{rel} values.

were performed using ApexControl, version 4.0 (Bruker Daltonics). A minimum of 30 transients with 512K data points per transient were used for each acquisition.

Calibration of the ESI-MS Assay. Calibration curves were generated for quantification of product formation.[27−29] The observed signal intensity of the product and an internal standard (I_P and I_{IS}, respectively) were used to determine an intensity ratio (I_P/I_{IS}). Calibration curves were generated by plotting I_P/I_{IS} against a known concentration ratio ([product]/[IS]) to provide a slope, *m*, and intercept, int. The experimental concentration of the product, [P], was then estimated using the following equation:[30]

$$[P] = \left(\frac{I_P}{I_{IS}} - int\right)\frac{[IS]}{m} \quad (1)$$

In reactions of 4MU-NA, GM3, and compounds **3a**−**f** (Figures 1 and 2), we monitored Neu5Ac as the product ion (*m*/*z* 308) with compound **9** as the internal standard (*m*/*z* 333). In reactions of compounds **6**, **8**, **11**, **12**, and **14** (Figure 3), we monitored the formation of octyl β-lactoside product ion (*m*/*z* 453.2) with compound **2d** as an internal standard (*m*/*z* 431.2). Calibration curves for Neu5Ac and octyl β-lactoside are provided as Supporting Information.

Enzyme Kinetics. Purified human NEU3 was prepared as an N-terminal MBP fusion protein (MBP−NEU3) as previously reported.[25] Briefly, the enzyme was expressed in BL21(DE3) pLysS *Escherichia coli* cells as an MBP fusion protein (pMAL-c2x vector). After cell lysis, proteins were purified over an amylose affinity column. The yield of pure protein was 4 mg/L of culture. The major band observed was consistent with the expected molecular mass of the MBP−NEU3 fusion protein (92 kDa). Purified protein was stored at −80 °C (see the Supporting Information). Steady-state kinetic studies were performed for 4MU-NA hydrolysis by NEU3 using ESI-MS. The reactions were prepared by mixing 4MU-NA (25, 50, 75, 100, 150, 200, 250, or 300 μM) and purified MBP−NEU3 (0.83 μM) in 0.1 M ammonium acetate buffer (pH 5.0), with incubation at 37 °C. The reactions were stopped at predetermined periods (16, 20, 24, 28, 32, 36, and 40 min) by adding an aliquot (5 μL) of the reaction mixture to 35 μL of 0.1 M ammonium bicarbonate buffer (pH 10) containing the internal standard (5 μM). The quenched solutions were stored at −20 °C until they were analyzed by ESI-MS. All solutions were diluted (1:8) using Milli-Q water prior to ESI-MS analysis.

For k_{rel} determinations, the reaction mixtures were prepared by mixing the indicated substrate (100 μM) with purified MBP−NEU3 (0.83 μM) in 0.1 M ammonium acetate buffer (pH 5.0) or buffer containing 3.5 mM sodium cholate (Sigma Aldrich), with incubation at 37 °C. The reactions were stopped at predetermined periods (4−120 min) and measured as described above. Rates were determined by fitting the initial linear portion of the curves, with a minimum of four points over 20 min (see the Supporting Information).

RESULTS AND DISCUSSION

Synthesis of Glycolipids. To explore the recognition of NEU3, we set out to generate a series of substrates containing aglycone or glycone modifications of a known substrate. The ganglioside GM3 [Neu5Ac-α(2→3)-Gal-β(1→4)-Glc-β-Cer] is a native substrate for the enzyme,[14] and we based the design of our ligands on the functionality of this parent compound. We first generated a series of seven glycolipid analogues containing the GM3 trisaccharide and a modified aglycone. To synthesize this series, we used a perbenzoylated lactosyl trichloroacetimidate donor (**SI3**) that was reacted with a series of primary alcohols to form the benzoyllactosides (**1a**−**e**, Scheme SI1 of the Supporting Information).[31] Subsequent deprotection to the lactoside derivatives (**2a**−**e**) provided substrates for an α(2→3)-sialyltransferase. We also prepared a truncated sphingosine analogue (**2f**).[32,33] Sialylation of the terminal galactose residue was performed using a chemoenzymatic strategy to obtain compounds **3a**−**f** (Scheme 1).[34] We used a similar strategy to generate modified sialoside analogues of GM3. Using compound **2a** as the transferase substrate, we used the chemoenzymatic method developed by Chen and co-workers.[34,35] An

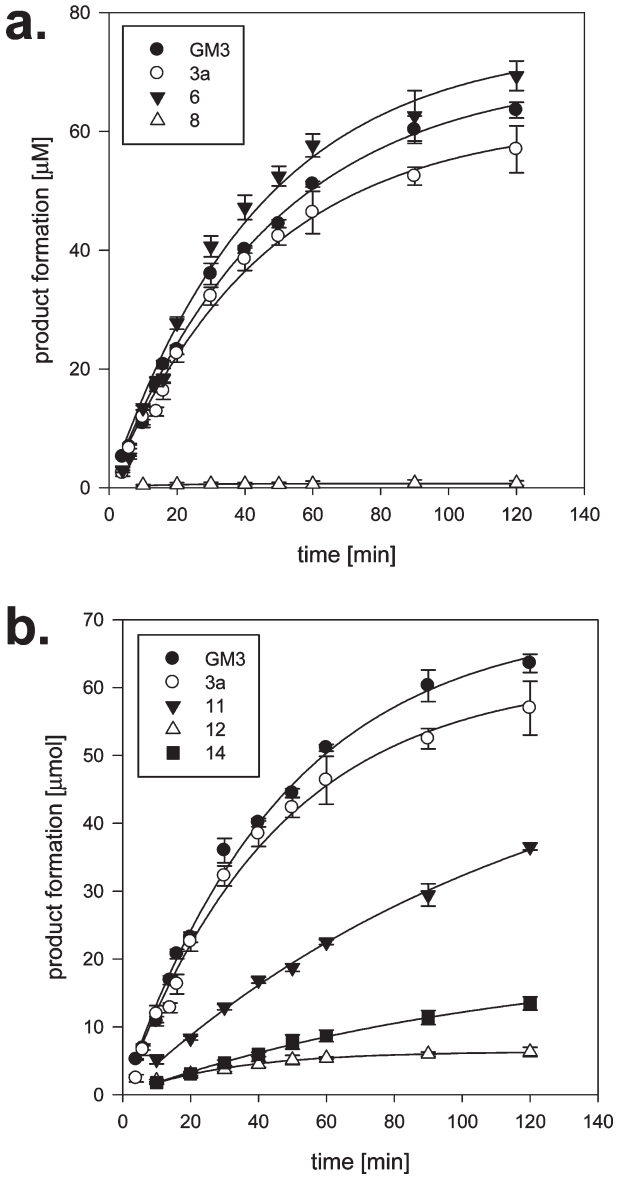


Figure 3. NEU3 cleavage of α(2→3)-sialosides with a modified Neu5Ac. Initial rates of hydrolysis for (a) N5-Ac-modified and (b) C9-modified sialosides were used to determine k_{rel}, and values are listed in Table 1 (see the Supporting Information for expansion plots). All points represent five replicates, and the error is shown as the standard deviation.

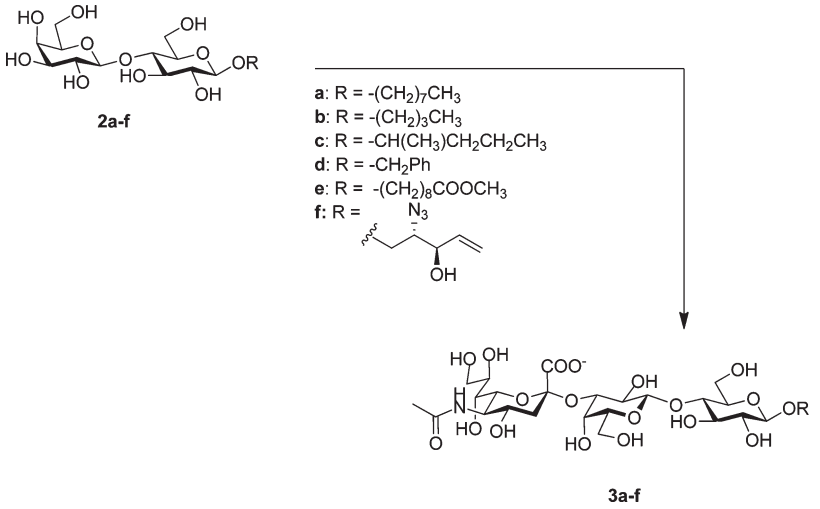
N-azido-acetyl mannosamine (**4**) was converted in situ to the corresponding CMP-sialic acid (**5**) by the action of recombinant sialic acid aldolase and CMP-sialic acid synthetase (CSS) (Scheme 2). The desired trisaccharide (**6**) was then formed by addition of α(2→3)-sialyltransferase. We converted the azidoacetate to a bulkier group via Cu-catalyzed azide−alkyne cycloaddition (CuAAC)[36] with a 1-propargyl-2,4-dinitrophenyl, **7**,[37] to generate compound **8**. We selected a dinitrophenyl derivative as this moiety can often serve as an intramolecular quencher in fluorogenic substrates.[26,38] To introduce modifications of the GM3 trisaccharide at the C9 position, we started from a known 9-azido-9-deoxysialoside (**9**) as the substrate for CSS. The modified CMP-sialic acid derivative (**10**) was generated in situ and reacted with **2a** and an α(2→3)-sialyltransferase to yield trisaccharide **11** (Scheme 3). We found azide **11** unreactive to CuAAC with alkyne **7**, and we were unable to obtain the corresponding triazole. Instead, we reduced the azide to a primary amine (**12**), which was reacted with an activated ester of 2,4-dinitrophenol (**13**)[39] to yield the C9-modified trisaccharide **14**. With three sets of glycolipid analogues in hand, we turned our attention to the recognition of these substrates by NEU3.

Analysis of NEU3 Kinetics Using ESI-MS. We required a sensitive assay that could be used to measure the kinetics of NEU3 hydrolysis for our synthetic α(2→3)-sialosides. We initially investigated the use of a TLC-based assay for the purpose of observing the formation of the octyl β-lactoside product after cleavage of the glycosidic bond of sialic acid. Experiments with **3a** and **6** confirmed that the compounds were substrates for the enzyme, while no cleavage of **8** was observed (see the Supporting Information). We also found that the detection limits of this assay precluded measurement of the initial rates of cleavage. Thus, we concluded that the sensitivity of the assay was too low to provide quantitative kinetic data without consuming large amounts of substrate. We turned our attention to developing a more sensitive assay using ESI-MS.

Electrospray ionization mass spectrometry has been previously applied to the study of enzyme kinetics.[40] A number of groups have employed ESI-MS to study enzymes, including glutathione *S*-transferase,[41] hexokinase,[42] phosphoglucomutase,[43] sulfotransferases,[29,44] and others.[28,45,46] To implement a quantitative kinetic assay with ESI-MS, we chose to use an internal standard to correct for any issues relating to ion response factors. We first generated calibration curves for internal standards of similar mass, charge, and functionality to the expected products of NEU3 hydrolysis of compounds related to **3a** (Neu5Ac and octyl β-lactoside); we selected a 9-azido-9-deoxyneuraminic acid (**9**) and octyl β-lactoside (**2a**) as the internal standards (see Materials and Methods). To test the accuracy of the NEU3 ESI-MS assay, we determined the steady-state kinetics of the fluorogenic substrate, 4MU-NA, by acquiring initial rates of hydrolysis (v_0) over a range of substrate concentrations (see the Supporting Information). We obtained a K_m of 39 ± 7 μM, which was in close agreement with solution determinations using fluorescence (K_m = 45 ± 3 μM).[25] After confirming that the ESI-MS assay could provide accurate kinetic measurements, we chose to analyze the activity of the remaining substrates through the use of relative rates (k_{rel}) based on the initial velocity, v_0, of product formation. Analysis based on k_{rel} values has been used in a variety of enzymatic systems,[47,48] including neuraminidases.[14,49] This approach relies on pseudo-first-order conditions under which all parameters are kept constant between samples, in particular the enzyme and initial substrate concentrations.

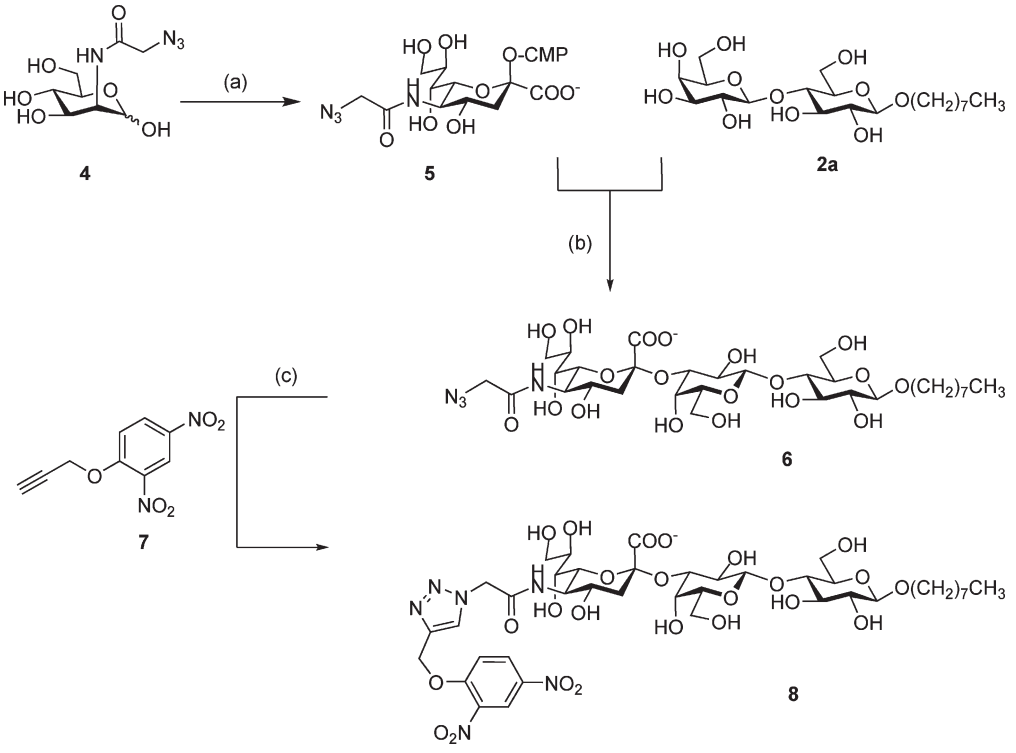
Although 4MU-NA is a substrate for the enzyme, we also examined a native substrate of NEU3, the ganglioside GM3 (Figure 1a). As expected, GM3 was a much better substrate for NEU3, with an approximately 4.5-fold increase in relative rate over that of 4MU-NA (Table 1).[14,17] For comparison, we normalized all rates to that of GM3 (k_{rel} = 1.00 ± 0.04). Previous reports have suggested that NEU3 activity is sensitive to the presence of surfactants,[15] and gangliosides typically have remarkably low critical micelle concentrations.[50] In our studies of purified NEU3, we have previously seen minor effects on NEU3 activity with 4MU-NA as a substrate.[25] Using the ESI-MS assay, we confirmed that, indeed, the presence of sodium cholate (0.5−1.5 mM) increased the relative rates of reaction for GM3 but had an only minor effect on 4MU-NA (Figure 1b).

Scheme 1. Synthesis of Lipid-Modified Trisaccharides[a]



[a] Conditions for the sialyl transferase reaction were as follows: sialic acid, CTP, $MgCl_2$, DTT, CMP-sialic acid synthetase, alkaline phosphatase, and α-2,3-sialyltransferase. Yields ranged between 11 and 86%.

Scheme 2. Synthesis of N5-Modified Trisaccharides[a]



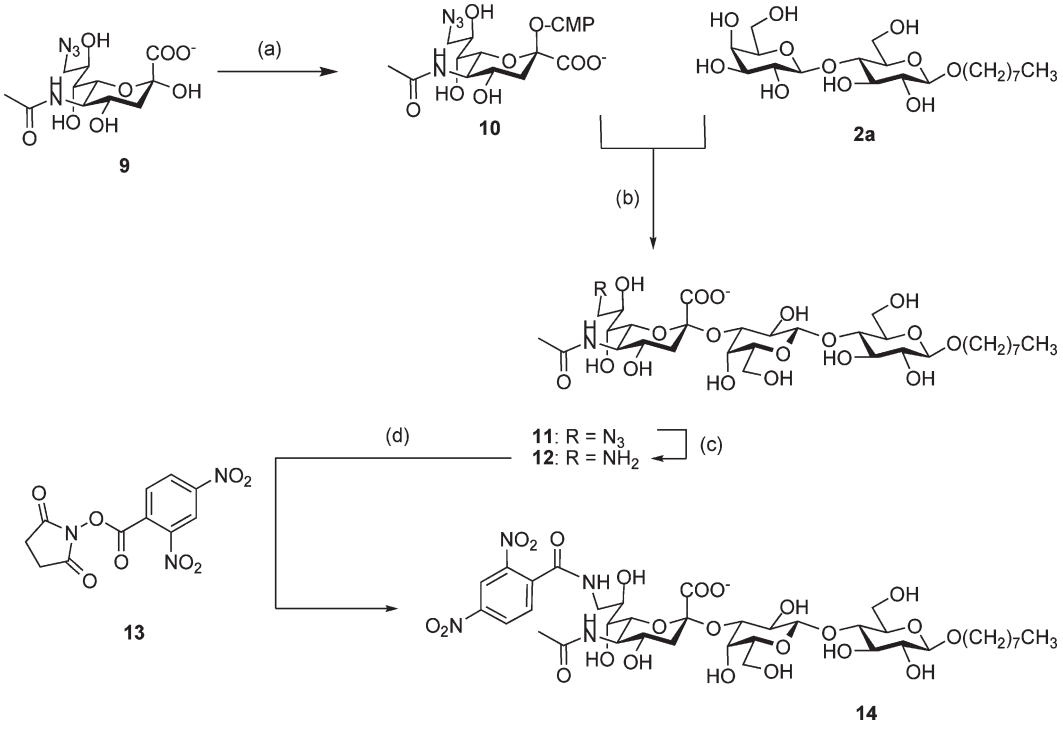
[a] Conditions: (a) sialic acid aldolase, pyruvate, CTP, $MgCl_2$; (b) CMP-Neu5Ac synthetase, CTP, α(2→3)-sialyltransferase, alkaline phosphatase, DTT, $MgCl_2$, 54%; (c) CuI, diisopropyl ethyl amine, 26%.

These data are typical for neuraminidase cleavage of ganglioside substrates, which often have enhanced activity in mixed micelles.[23,51,52] Interestingly, we found that the activity of an octyl β-sialyllactoside (**3a**; $k_{rel} = 0.93 \pm 0.03$) was similar to that of GM3. This finding prompted us to investigate the role of the lipid aglycone in the recognition of substrate by NEU3.

NEU3 Cleavage of α(2→3)-Sialosides with Modified Aglycone. To examine the role of the lipid aglycone, we determined the rates of hydrolysis for a series of six sialyllactosides (**3a**−**f**) relative to that of GM3 (Figure 2a). We found that truncation of the octyl chain in **3a** to a butyl chain (**3b**; $k_{rel} = 0.16 \pm 0.01$) resulted in a large reduction in the rate of hydrolysis. Increasing the bulk of the lipid with a branching methyl group doubled the activity (**3c**; $k_{rel} = 0.31 \pm 0.02$), while attaching a benzyl group in place of the butyl chain showed a smaller increase (**3d**; $k_{rel} = 0.22 \pm 0.01$). We then tested a long hydrophobic chain that included a

dx.doi.org/10.1021/bi200449j |*Biochemistry* 2011, 50, 6753–6762

Scheme 3. Synthesis of C9-Modified Trisaccharides[a]



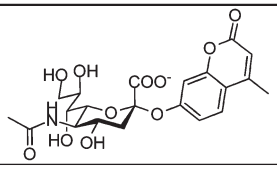
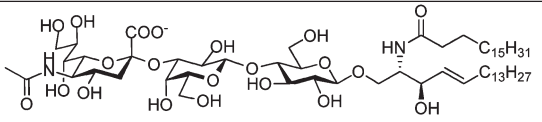
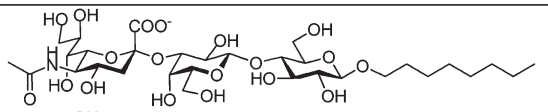
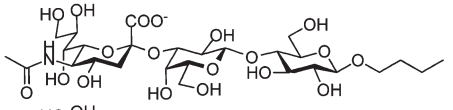
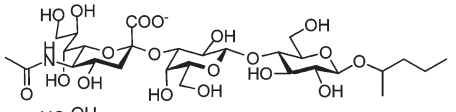
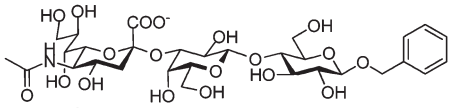
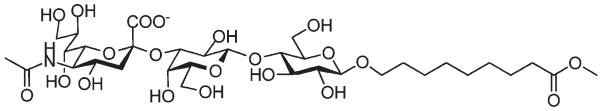
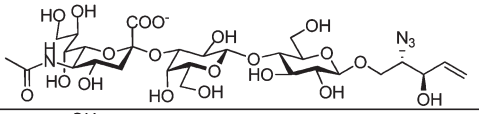
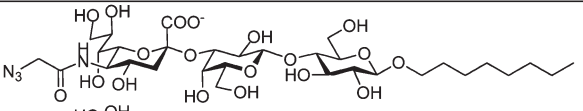
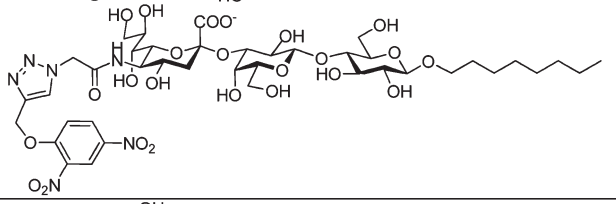
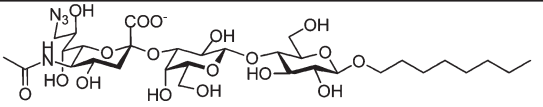
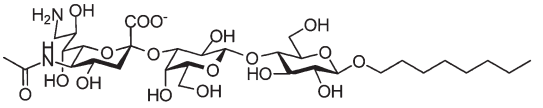
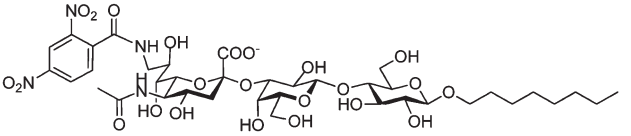
[a] Conditions: (a) CMP-Neu5Ac synthetase, CTP; (b) α(2→3)-sialyltransferase, alkaline phosphatase, DTT, $MgCl_2$, 34%; (c) Pd/C, H_2O, AcOH, 86%; (d) $NaHCO_3$, dioxane/water, 30%.

methyl ester and found this substrate had a relative rate approximately half that of GM3 (**3e**; k_{rel} = 0.51 ± 0.02). Finally, we tested if the presentation of the amino and hydroxyl groups of the core sphingolipid were important to NEU3 recognition using a truncated sphingosine analogue as the aglycone of sialyllactose, **3f**. Although the truncated sphingosine derivative (**3f**; k_{rel} = 0.31 ± 0.02) was more active than the butyl derivative, **3b**, it had activity comparable to that of the 2-pentyl derivative, **3c**. On the basis of these results, we considered that enzyme activity appeared to track with the hydrophobicity of the aglycone, rather than specific functional groups. When we compared the calculated octanol–water partition coefficients (log P) of each of the aglycone alkyl groups, we observed a clear relation between increased hydrophobicity of the alkyl chain and improved enzyme activity (Figure 2b).[53] Glycolipids with an alkyl chain for which log P > 4 (GM3 and **3a**) were excellent substrates, while those for which log P < 3 were poor substrates. We do not expect that the observed dependence on the lipid chain is due to aggregation of the substrate, as the concentration used (100 μM) is well below the critical micelle concentration of octylglycoside.[54]

NEU3 Cleavage of α(2→3)-Sialosides with Modified Neu5Ac. Fluorogenic substrates present an attractive strategy for probing the activity of glycosidase enzymes.[55] However, the design of substrates with appended chromophores requires some foreknowledge of the enzyme tolerance for substrate modifications. There are no reports of NEU3 hydrolysis with substrates containing modified Neu5Ac residues; therefore, we designed two series of compounds to explore the tolerance of the enzyme for these types of modifications. We designed our substrates to allow the incorporation of new functional groups at either the N5-Ac or C9 position of sialic acid using a chemoenzymatic approach (vide supra). Modification of the N5-Ac group to azidoacetate resulted in a moderate improvement in hydrolysis by NEU3 relative to that of GM3 (**6**; k_{rel} = 1.07 ± 0.04). This observation is consistent with our previous study of NEU3 inhibitors, where we observed improved NEU3 inhibitory potency for compounds that contained an N5-azido modification.[56] We note that this finding has subsequently been confirmed in substrates and inhibitors of NEU2.[24] Using the ESI-MS assay, we observed that NEU3 did not tolerate the presence of larger groups, such as a triazole, at the N5-Ac position; this modification led to almost undetectable substrate activity (**8**; k_{rel} = 0.003 ± 0.001). This is consistent with the homology model of NEU3, which predicts a small hydrophobic pocket for the *N*-acyl side chain.[25] We also examined modifications of the C9 position of the Neu5Ac residue. We found that the C9-azido was a moderate substrate (**11**; k_{rel} = 0.29 ± 0.01); however, reduction to the C9-amino analogue led to a dramatic decrease in the rate of NEU3 cleavage (**12**; k_{rel} = 0.06 ± 0.01). We ascribe this decrease in activity to the protonation state of the amine group under assay conditions. Interestingly, we found that coupling of the C9-amido derivative, **12**, to 2,4-dinitrobenzoic acid (**14**) resulted in increased activity (k_{rel} = 0.12 ± 0.01). We have previously suggested that the binding pocket for the glycerol side chain is relatively large in NEU3 and can accommodate larger groups.[56] Confirmation of this finding with NEU3 substrates suggests that incorporation of fluorophores, quenchers, or other labels into glycolipid substrates for NEU3 could be possible at C9. However, modification of the N5-Ac position would likely obscure enzyme activity.

Although the very earliest studies of NEU3 identified the enzyme's clear preference for glycolipids, ours is the first study to examine the role of the lipid in substrate recognition.[14] We observed a direct dependence upon the hydrophobicity of the aglycone, where increased hydrophobicity resulted in increased NEU3 activity. Although we cannot rule out the possibility that

Table 1. Rates of Substrate Hydrolysis by NEU3 As Determined by the ESI-MS Assay

Cmpd	*Structure*	v_0 [$\mu M\ min^{-1}$]	k_{rel}	log P [a]
4MU-NA		0.25 ± 0.02	0.21 ± 0.02	2.42
GM3		1.20 ± 0.03	1.00 ± 0.04	14.76
3a		1.11 ± 0.03	0.93 ± 0.03	5.01
3b		0.19 ± 0.01	0.16 ± 0.01	2.88
3c		0.37 ± 0.02	0.31 ± 0.02	3.41
3d		0.26 ± 0.01	0.22 ± 0.01	2.68
3e		0.61 ± 0.02	0.51 ± 0.02	3.90
3f		0.37 ± 0.02	0.31 ± 0.02	0.81
6		1.28 ± 0.03	1.07 ± 0.04	5.01
8		0.003 ± 0.001	0.003 ± 0.001	5.01
11		0.35 ± 0.01	0.29 ± 0.01	5.01
12		0.08 ± 0.01	0.06 ± 0.01	5.01
14		0.14 ± 0.01	0.12 ± 0.01	5.01

[a] Log P values were calculated only for the aglycone portion of the substrate.

dx.doi.org/10.1021/bi200449j |*Biochemistry* 2011, 50, 6753–6762

this dependence is due to the formation of micelles by the substrate, assays were conducted at concentrations of substrate that are expected to be below the CMC for octyl glycosides, which we expect to be the lower limit for the compounds in the lipid series **3a**−**f**.[54] Additionally, substrates that included features of the sphingosine headgroup (**3f**) did not show improvement over similar length alkyl chains (**3c**), and replacement of the ceramide moiety (GM3) with an octyl chain (**3a**) showed an only minor loss of activity. Together, these data are consistent with a model of NEU3 recognition that includes at least two sites of recognition. In addition to the glycone recognition site that has been proposed by homology,[25] a second hydrophobic subsite must recognize general hydrophobic properties of the substrate. Human β-glucosidases are known to have a similar dependence on glycolipid hydrophobicity but in contrast are proposed to exploit a three-site binding mode that includes a site that recognizes sphingosine headgroup features.[57,58] Our data do not exclude the possibility that other elements of the substrate, such as the intervening lactoside, are recognized by the binding site.

Our understanding of the specific function of the hNEU family members in health and disease continues to improve. Biological studies of these enzymes are difficult without biochemical characterization of their substrate specificity or access to specific inhibitors and probes.[12] It is interesting to note that this family of human enzymes shares similar features with the viral neuraminidase enzymes (vNEU), an important target for antiviral therapeutics.[59] The increased level of use of vNEU inhibitors to combat influenza outbreaks has led to concerns of potential side effects as both enzymes share a similar mechanism.[60,61] Some experimental evidence has suggested that polymorphism in hNEU isoforms could contribute to side effects of antivirals.[62] As a result, there is growing interest in improving our understanding of the substrate recognition[25,61] and inhibition of the hNEU isoforms.[56,63−65] This study is the first to systematically explore the substrate specificity of NEU3, and future studies of hNEU may help identify features of the enzymes that could be used to develop specific inhibitors[56] and identify strategies that avoid unwanted off-target effects.[65] Future work could also address the role of activator proteins that may improve the activity of purified human sialidases.[66,67]

Our analysis of NEU3 hydrolysis was made possible by the recombinant production of the enzyme and the development of a sensitive assay for detection of substrate hydrolysis. Standard TLC assays can require large amounts of material, and the generally low signal-to-noise ratio of these measurements may obscure important results. In contrast, the use of ESI-MS for monitoring the course of enzyme kinetics allowed us to conduct the analysis with smaller amounts of the substrate and to quantitatively observe the cleavage of even weak substrates. Moreover, the ESI-MS assay distinguishes products from substrates by their mass-to-charge ratio (m/z), allowing the detection of labeled or modified substrates.

CONCLUSIONS

Membrane glycosyl hydrolases are important regulators of membrane composition; however, little is known about substrate recognition in this family of enzymes. We have developed a sensitive kinetic assay based on ESI-MS to allow studies of synthetic glycolipid substrates. Using a series of synthetic trisaccharides and natural substrates, we conclude that NEU3 requires a hydrophobic aglycone for substrate recognition. More hydrophobic groups (log P > 4) were excellent substrates (k_{rel} > 0.9). We find no evidence that features of the ceramide headgroup are recognized by the enzyme. Finally, we observed that modifications of the α(2→3)-Neu5Ac residue with an azide at C9 or N5-Ac remained substrates. However, larger modifications at N5-Ac resulted in inactive substrates (k_{rel} < 0.1). In at least one trisaccharide, we were able to introduce a large aryl group at C9 and maintain moderate substrate activity (**14**; $k_{rel} = 0.12 \pm 0.01$), suggesting that this position could be exploited in the design of labeled substrates.

ASSOCIATED CONTENT

Supporting Information. Supplementary data is available, including synthetic protocols, compound characterization, glycolipid TLC assays, ESI-MS calibration curves, and additional kinetic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding Sources

We acknowledge support of this work from the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants program (C.W.C., J.S.K., and D.R.B.) and the Alberta Ingenuity Centre for Carbohydrate Science (AICCS).

ACKNOWLEDGMENT

The plasmid encoding the *E. coli* sialic acid aldolase was a kind gift of Dr. X. Chen (University of California, Davis, CA).

ABBREVIATIONS

4MU-NA, 4-methylumbelliferone-5-*N*-acetylneuraminic acid; CSS, CMP-sialic acid synthetase; CuAAC, Cu-catalyzed azide−alkyne cycloaddition; ESI-MS, electrospray ionization mass spectroscopy; FT-ICR MS, Fourier transform ion cyclotron resonance mass spectroscopy; hNEU, human neuraminidase; NEU2, neuraminidase 2; NEU3, neuraminidase 3; Neu5Ac, 5-*N*-acetylneuraminic acid; vNEU, viral neuraminidase.

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