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Fluorogenic sialic acid glycosides for quantification of sialidase activity upon unnatural substrates

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ABSTRACT

Herein we report the synthesis of *N*-acetyl neuraminic acid derivatives as 4-methylumbelliferyl glycosides and their use in fluorometrically quantifying human and bacterial sialidase activity and substrate specificities. We found that sialidases in the human promyelocytic leukemic cell line HL60 were able to cleave sialic acid substrates with fluorinated C-5 modifications, in some cases to a greater degree than the natural *N*-acetyl functionality. Human sialidases isoforms were also able to cleave unnatural substrates with bulky and hydrophobic C-5 modifications. In contrast, we found that a bacterial sialidase isolated from *Clostridium perfringens* to be less tolerant of sialic acid derivatization at this position, with virtually no cleavage of these glycosides observed. From our results, we conclude that human sialidase activity is a significant factor in sialic acid metabolic glycoengineering efforts utilizing unnatural sialic acid derivatives. Our fluorogenic probes have enabled further understanding of the activities and substrate specificities of human sialidases in a cellular context.

In this work, we report a synthesis of sialic acid, a biologically-important monosaccharide, derivatized as a 4-methylumbelliferyl glycoside with various substituents at C-5. Our approach makes use of an *N*-*tert*-butoxycarbonyl-neuraminic acid intermediate, which provides access to many different fluorogenic glycosides. We demonstrate the utility of our methylumbelliferyl glycosides by quantifying the activity and specificity of human and bacterial sialidases, which are enzymes critical to glycome regulation.

Sialic acids (neuraminic acids) are a family of nine-carbon monosaccharide most commonly occurring on the termini of oligosaccharides in the glycome. Their presence on cell surfaces is critical to cell–cell interactions, antigen presentation, and normal tissue development in mammals.[1] In bacteria and other organisms, sialic acids are critical for pathogenesis[2] and bacterial nutrition.[1,3] Sialic acids are also important therapeutic targets against cancer, as the amount of *N*-acetyl neuraminic acid presented on mammalian plasma membranes increases upon malignant transformation of tissues and correlates with their metastatic potential.[2,4,5]

The amount of sialic acids incorporated into glycoconjugates within the cell is regulated by the expression levels and activities of sialyltransferases and sialidases.[6,7] Sialyltransferases are critical enzymes in the Golgi responsible for catalyzing the formation of a glycosidic linkage between sialic acid and a nascent oligosaccharide on a glycoprotein, glycolipid or other glycosylated molecule. Biosynthetic enzymatic pathways involving sialyltransferases are utilized in metabolic engineering to incorporate synthetic monosaccharides in a manner resembling the natural substrate.[8,9]

Metabolic engineering efforts have installed bioorthogonal functional groups such as thiols, azides, and ketones into the glycome of various cell lines. These varied sialic acid derivatives, modified at C-5, have been successfully exploited for glycan imaging,[6,10] drug delivery[11,12] and glycomics studies.[9,13–15] Our laboratory has explored the incorporation of fluorinated sialic acids in this manner to modulate noncovalent cell–cell interactions such as cell adhesion. In some cases, the fluorinated analogues are presented on the cell surface to an even greater extent than the natural sialic acids.[16]

The activity of sialyltransferases toward natural and unnatural substrates, however, is simply one factor determining sialic acid

Abbreviations: 4MU, 4-methylumbelliferyl; TFA, trifluoroacetic acid; *t*-Boc, *tert*-butoxycarbonyl; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; DANA, 2-dehydroxy-2-deoxyneuraminic acid.

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presentation on glycans. Recent studies have begun to elucidate the catabolic contribution of sialidase remodeling of glyconjugates after their display on the glycocalyx. Sialidases remove α-glycosidically linked sialic acids from oligosaccharides and have been found to modulate protein recruitment,[17] cell–cell interactions,[18] cell adhesion and migration,[7,19] cell differentiation,[20] cell signaling,[21,22] cell receptor activity,[23] and apoptosis signaling.[22,24]

Although sialidases have been well-characterized in viruses and bacteria, the expression levels, localizations, and activities of sialidase isoforms NEU1–4 in healthy and diseased human tissues have only recently been examined.[25] The ability of these sialidases to remodel unnatural sialic acids incorporated within the glycome is unknown, and their substrate specificities toward unnatural sugars could be a significant factor in the efficacy of glycocalyx functionalization efforts. Further, viral neuraminidases share significant sequence homology with human sialidases and there is great interest in developing strategies to determine substrate specificities of human sialidases to inform selective inhibitor design studies.[26] New probes are needed to enhance understanding of sialidase activity in disease states and their role in glycoengineered sialic acid presentation on cell surfaces.

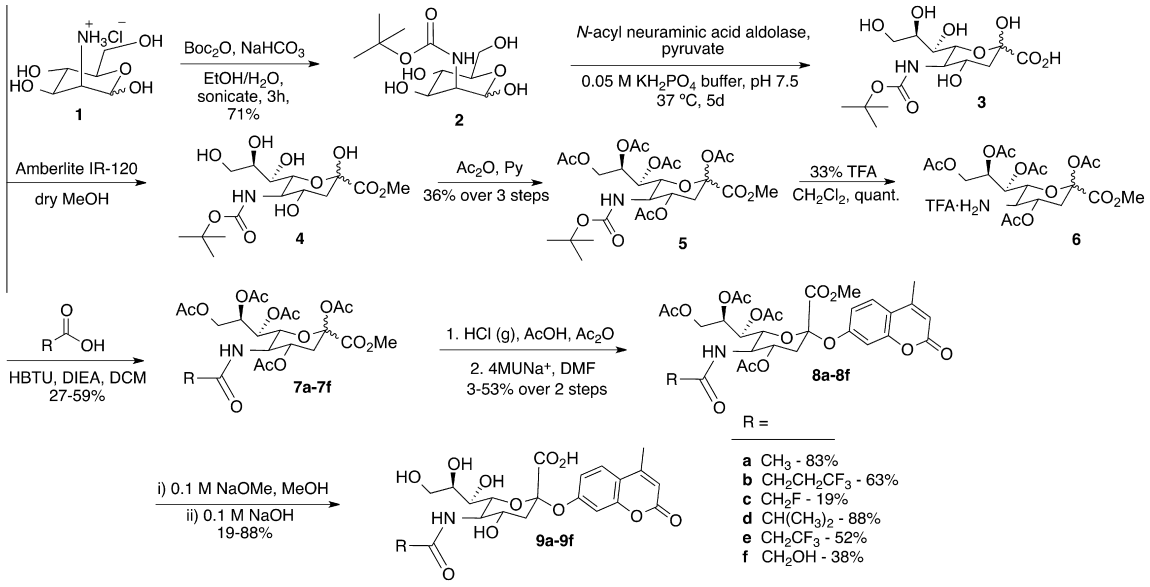
To this end, we describe herein the synthesis of a panel of α-ketosides consisting of C5-modified sialic acids linked to a 4-methylumbelliferyl aglycon, and their use as probes of sialidase activity in the human leukemic cell line HL60. We wished to explore how modifications at the C-5 position could perturb the critical interactions known between the *N*-acyl moiety of sialic acid and conserved residues interacting with this moiety within sialidase binding pockets. Previously, we observed that fluorination of the glycocalyx by incorporation of fluorinated sialic acids **7b** and **7e** (Scheme 1) perturbed protein–protein interactions involved in cellular adhesion behaviors, an effect we attributed to the simultaneous hydrophobic and lipophilic behavior of organic fluorine. In this present work, we installed side chains of varying lengths and degrees of fluorination to determine how fluorination of glycoconjugates affects sialidase activity on HL60 cell surfaces.

We functionalized our series of sialic acid derivatives with a 4-methylumbelliferyl (4MU) group[6] at C-2; this moiety is minimally fluorescent when coupled to sialic acid through a glycosidic linkage and becomes fluorescent when the glycosidic bond is hydrolyzed, releasing free 4-methylumbelliferone.[8] 4MU ketosides of sialic acid have been used previously to monitor fluorometrically the hydrolysis of sialic acids glycosides by sialidases.[6,11] All four known isoforms of sialidases in humans cleave the 4MU glycoside of *N*-acetyl neuraminic acid in vivo and in vitro, albeit with varying activity.[13–15] To the best of our knowledge, this work represents the first report of the installation and utility of the 4MU moiety onto neuraminic acids derivatized at C-5 and the first report of 4MU glycosides containing fluorine.

To synthesize these probes (Scheme 1), we condensed *N*-*t*-boc-mannosamine (**2**) with pyruvate in the presence of sialic acid aldolase[27] to produce *N*-*t*-boc-neuraminic acid (**3**). Methyl esterification and acetylation of all hydroxyl groups gave **5**, an easily accessible precursor for a variety of *N*-acyl neuraminic acid derivatives. Removal of the *t*-Boc group in **5** with 33% TFA gave amine **6** quantitatively[28] that was used in HBTU-mediated couplings with appropriate carboxylic acids to afford sialic acid derivatives **7b**–**f**. The peracetylated methyl ester of *N*-acetyl neuraminic acid, **7a**, was synthesized as previously reported.[29,30] To access the umbelliferyl glycosides, **7a**–**f** were converted to the glycosyl chloride by treatment with hydrogen chloride,[11,31] then allowed to react with the sodium salt of 4-methylumbelliferone, to yield 4MU-glycosides **9a**–**f** in modest yields from **7** (48–65%.)

Our initial biochemical studies were directed towards measuring the endogenous sialidase activity in the HL60 cell line against 2-(4-methylumbelliferyl)-5-*N*-acetyl neuraminic acid (**9a**). We chose this cell line because it is commonly used in metabolic glycoengineering and is reported to express sialidases NEU1, NEU2, and NEU3.[2,32,33] To assess endogenous activity, HL60 cells suspended in buffer were treated with **9a** and allowed to react at 37 °C for 2 h. The reaction was stopped by the addition of glycine buffer at pH 10.7, and fluorescence was read immediately. An increase in fluorescence due to released 4MU would indicate the presence of sialidase activity. Sialidase activity in whole HL60 cells has been measured previously at 0.2 units,[32] with a unit defined as the amount of enzyme required to release one nmol of 4MU per 10^6 cells. Our results are consistent with this value, with 0.1–0.2 units detected on HL60 cells using probe **9a** (Fig. 1).

Next we wanted to assess the degree to which sialidases expressed by HL60 would cleave unnatural sialic acid derivatives **9b**–**f** in culture. We conducted the whole cell assay as above and compared the increase in fluorescence over background to that of



Scheme 1. Synthesis of 4-methylumbelliferyl glycosides of *N*-acyl neuraminic acids.

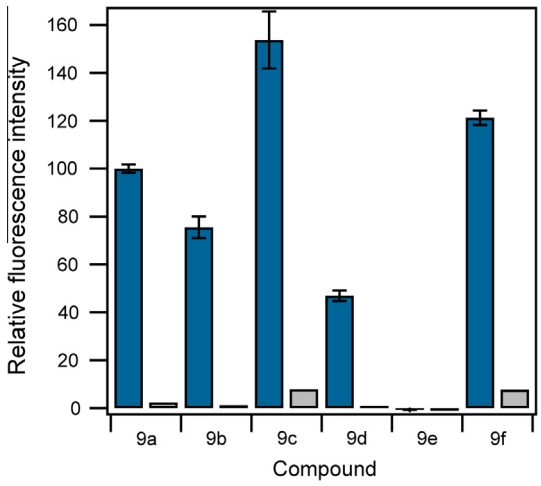


Figure 1. Activity of endogenously-expressed sialidases in HL60 against *N*-acyl neuraminic acid glycosides **9a–f**. Whole HL60 cells in acetate buffer (pH 4.5) were treated for 2 h with 0.125 mM of each compound (blue bars) at 37 °C. Grey bars represent the same reaction in the presence of 0.1 mM of sialidase inhibitor DANA. The pH of each reaction was adjusted to 10.7 with glycine buffer and fluorescence was read with excitation wavelength of 365 nm and emission wavelength of 450 nm. Error bars are the standard deviations. All experiments were performed in at least biological triplicate and are representative of multiple independent trials.

natural substrate **9a**. Previously, Li et al. reported a slight increase in the activity of sialidase NEU2 against *p*-nitrophenol constructs displaying the *N*-glycolyl and *N*-azidoacetyl functionalities on sialic acid.[34] We hypothesized that a similar trend may hold for the collective activities of cell-surface sialidases on our compound **9f**. In addition, we anticipated a decrease in the cleavage activity upon derivatives **9b**, **9c**, and **9e** as a result of fluorination, steric bulk or a combination thereof.

The results are shown in Figure 1. As predicted the *N*-glycolyl compound **9f** showed 20% greater activity than **9a**. We also observed 50% more hydrolysis relative to the natural sugar for compound **9c** in which one hydrogen is replaced by fluorine. However, when a trifluoromethyl group is present distal to the carbonyl, as in **9b** and **9e**, we observed a decrease in the ability of cell-surface enzymes to cleave these constructs. In the case of the *N*-isobutyryl neuraminic acid glycoside (**9d**), we observed a 60% reduction in hydrolysis compared to **9a**, possibly due to the increase in steric bulk and hydrophobicity. We note the surprising result obtained in the presence of compound **9e**, wherein we observed negligible cleavage of the glycoside. The mechanism of action of this behavior is currently under investigation in our laboratory.

In order to rule out hydrolysis mechanisms independent of sialidase activity as a significant source of fluorescence measured, whole cells in acetate buffer were treated with **9a–f** in the presence of 0.1 mM 2-dehydroxy-2-deoxyneuraminic acid (DANA), a non-specific sialidase inhibitor.[13,26] In every case, we observed nearly complete abrogation of cleavage of the 4MU glycosides by the cells in the presence of DANA (Fig. 1). This strongly indicates that the hydrolysis observed for **9a–f** by HL60 cells was due to sialidase activity. Based on the known cellular localization of sialidase isoforms, we postulate that these compounds are primarily acted upon by sialidase isoforms NEU1 and/or NEU3. Our results suggest that these enzymes are likely capable of cleaving unnatural sialic acids from cell surface glycoconjugates to varying degrees, including those with longer *N*-acyl modifications.

We do not expect ionic and polyhydroxylated compounds **9** to be cell permeable and thus accessible to cleavage by sialidase isoforms localized to the cell interior. To confirm this expectation, cells incubated with **9a** for 2 h were washed thoroughly to remove any free 4MU generated from enzymatic activity. The cells were resuspended in cell lysis buffer and the fluorescence intensity of the resulting lysate read. The fluorescence intensity of the washed cell samples was equal to that of empty wells (without cells or compound), indicating that all conjugates (cleaved or uncleaved) had been removed from the sample (Fig. S1). From this result, we conclude that neither the glycosides nor 4MU had diffused across the membrane during the incubation time of these assays, and that that the observed hydrolysis of the glycosides was likely the result of activity of sialidase isoforms associated with the cell membrane. Therefore, our results demonstrate the utility of 4MU sialosides as probes of cell-surface localized sialidase activity toward unnatural sialic acids.

To explore the substrate specificity of a bacterial sialidase, we also assayed a commonly employed neuraminidase isolated from *Clostridium perfringens* [EC 3.2.1.18] for in vitro activity against our modified sialic acids. Many bacterial strains do not have innate biosynthetic pathways for sialic acids and scavenge them from the host.[35] As a result, unnatural sialic acid substrates are readily incorporated by the natural metabolism of many bacterial strains, even in the presence of excess *N*-acetyl neuraminic acid.[36] However, the activity of *C. perfringens* sialidase towards sialosides other than *N*-acetyl or *N*-glycolyl has not been previously reported.

Accordingly, we measured the enzymatic activity of *C. perfringens* sialidase using compounds **9a–f** as fluorogenic substrates. Using 0.05 mU sialidase from *C. perfringens*, we found the cleavage activity against glycosides **9b–f** relative to **9a** by the bacterial enzyme to be significantly lower than that observed in our mammalian whole-cell assay (Fig. 2). Only glycolyl analog **9f** and monofluorinated analog **9c** were cleaved by *C. perfringens* sialidase in our assay with fluorescence intensities of only 6% of that observed for the natural ligand **9a**. It is interesting to note that constructs that were cleaved relatively well by HL60 sialidases, such as **9b**, were not cleaved at all by this enzyme, indicating greater permissivity in the mammalian sialidase pockets than anticipated. Our results suggest that sialidase-catalyzed removal of unnatural sialic acids from metabolically engineered bacterial glycomes

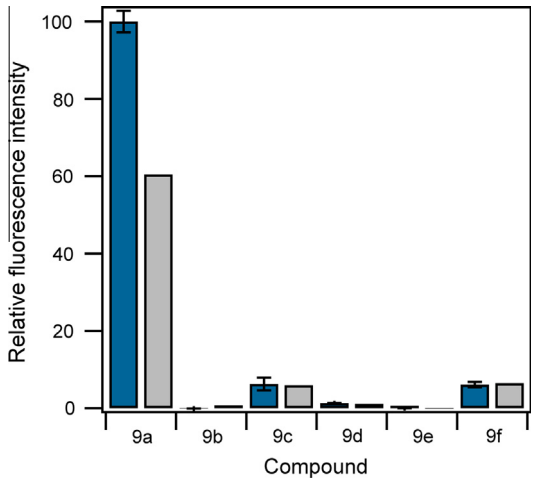


Figure 2. Activity of bacterial sialidase against *N*-acyl neuraminic acid glycosides **9a–f**. Purified recombinant sialidase from *C. perfringens* (0.05 mU) in acetate buffer (pH 4.5) was treated for 1 h with 0.125 mM of each construct (blue bars) at 37 °C. Grey bars represent the same reaction in the presence of 0.1 mM of sialidase inhibitor DANA. The pH of each reaction was adjusted to 10.7 with glycine buffer and fluorescence was read with excitation wavelength of 365 nm and emission wavelength of 450 nm. Error bars are the standard deviations. All experiments were performed in at least biological triplicate and are representative of multiple independent trials.

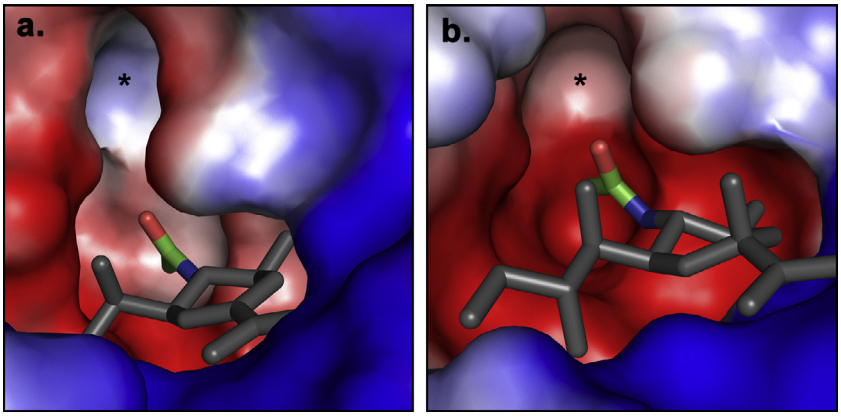


Figure 3. Active site topology of human and bacterial sialidases. (a) The active site of human NEU2 contains two deep pockets that accommodate the *N*-acetyl moiety of sialic acid. The enzyme is shown in complex with DANA, rendered grey except for the *N*-acetyl moiety which is colored (PDB: 1VCU).[37] Active site residues form a pocket of positive potential (indicated by an asterisk) having critical interactions with the carbonyl oxygen (shown in red) and a neutral surface making hydrophobic contacts with the methyl group (shown in green); (b) the active site of sialidase from *C. perfringens* also contains a pocket for the *N*-acetyl moiety. The enzyme is shown in complex with *N*-acetyl neuraminic acid, rendered grey except for the *N*-acetyl moiety which is colored (PDB: 2BF6).[3] This pocket appears to be shallower and significantly more negative than that of the mammalian enzyme, suggesting that sialic acids with larger C5 substituents may not be able to stably bind in the active site. Active site residues having critical interactions with the carbonyl oxygen (shown in red) are indicated by an asterisk.

may not be as significant a factor in sialome remodeling as in mammalian systems, possibly resulting in longer lifetimes of unnatural moieties in bacterial glycoconjugates.

In this work, we observed directly that human cell surface sialidases cleave a variety of C5-modified sialic acids, even with relatively large substituents (Fig. 1). While there are no structures available of NEU1 or NEU3, examination of the active site of NEU2 bound to an inhibitor[37] reveals a possible reason. NEU2 is reported to have 38% sequence homology to plasma membrane-associated isoform NEU3.[15] The methyl group of the *N*-acetyl moiety of sialic acid makes contacts with a region of neutral potential in the binding pocket of NEU2 and the amide carbonyl occupies a larger pocket of positive potential (Fig. 3a). We hypothesize that rotation of the *N*-acyl moiety could occur within the binding pockets of human sialidases, resulting in an acyl group occupying the larger positively-charged pocket and the carbonyl interacting with the smaller neutral surface. This may explain the relatively higher activity of the enzyme toward probes **9c** and **9f**, whose larger, electron-rich side chains might better bind the positively-charged region. In the case of the probes with larger, less polar *N*-acyl side chains **9b**, **9d**, and **9e**, the proposed rotamers may produce a poor Coulombic match with the positive pocket in the enzyme binding site and thus exhibit relatively less cleavage. An increase in cleavage activity of sialic acids bearing *N*-azidoacetyl and *N*-glycolyl moieties by human sialidase isoforms, which could adopt the putative rotamer conformation, has been reported.[14,34]

The *C. perfringens* sialidase active site[3] (Fig. 3b) has a shallow negatively-charged pocket for the *N*-acetyl moiety of sialic acid. We hypothesize that electron rich and/or bulky modifications on C5 of sialic acids may not be able to stably bind in this enzyme pocket. This may result in the stringency observed for the *C. perfringens* sialidase in our experiments.

In conclusion, we have synthesized a series of *N*-acyl neuraminic acids as 4-methylumbelliferyl glycosides and demonstrated their application as probes of sialidase substrate specificity and activity at the cell surface. We showed that fluorinated sialic acids can be cleaved from glycoconjugates by human sialidases, confirming that removal by sialidases must be considered as a prominent factor in metabolic engineering efforts. These probes now enable us to examine substrate specificities of these enzymes via a simple fluorogenic assay—a straightforward alternative to mass spectrometric detection of sialidase activity. Future work will characterize enzymatic action of individual human neuraminidase isoforms against our constructs in vitro and in other cellular contexts.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.03.076.

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