Substrate Specificity of the Sialic Acid Biosynthetic Pathway[†]

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Abbreviations

ManNAc, *N*-acetylmannosamine; UDP, uridine diphosphate; UDP-GlcNAc, UDP-*N*-acetylglucosamine; GlcNAc, *N*-acetylglucosamine; CMP, cytidine monophosphate; MAG, myelin-associated glycoprotein; ManLev, *N*-levulinoylmannosamine; PBS, phosphate-buffered saline; FITC, fluoroscein isothiocyanate; EDTA, disodium ethylenediamine tetraacetate; FBS, fetal bovine serum; FAB, fast atom bombardment; ESI-MS, electrospray ionization mass spectrometry; HPAEC, high pH anion exchange chromatography; PAD, pulsed amperometric detector; THF, tetrahydrofuran; tlc, thin layer chromatography; Ac_4ManNAc, peracetylated ManNAc; ManProp, *N*-propanoylmannosamine; Ac_4ManProp, peracetylated ManProp; ManBut, *N*-butanoylmannosamine; Ac_4ManBut, peracetylated ManBut; ManPent, *N*-pentanoylmannosamine; Ac_4ManPent, peracetylated ManPent; ManHex, *N*-hexanoylmannosamine; Ac_4ManHex, peracetylated ManHex; Ac_4ManLev, peracetylated ManLev; DTT, dithiothreitol; MW, molecular weight; kD, kilodalton; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ATP, adenosine triphosphate; PEP, phosphoenolpyruvate; NADH, nicotinamide adenine dinucleotide; HPLC, high pressure liquid chromatography.

Abstract

Unnatural analogs of sialic acid can be delivered to mammalian cell surfaces through the metabolic transformation of unnatural *N*-acetylmannosamine (ManNAc) derivatives. In previous studies, mannosamine analogs bearing simple *N*-acyl groups up to five carbon atoms in length were recognized as substrates by the biosynthetic machinery and transformed into cell-surface sialoglycoconjugates [Keppler, O. T., *et al*. (2001) *Glycobiology 11*, 11R-18R]. Such structural alterations to cell surface glycans can be used to probe carbohydrate-dependent phenomena. This report describes our investigation into the extent of tolerance of the pathway toward additional structural alterations of the *N*-acyl substituent of ManNAc. A panel of analogs with ketone-containing *N*-acyl groups that varied in the length or steric bulk was chemically synthesized and tested for metabolic conversion to cell-surface glycans. We found that extension of the *N*-acyl chain to six, seven, or eight carbon atoms dramatically reduced utilization by the biosynthetic machinery. Likewise, branching from the linear chain reduced metabolic conversion. Quantitation of metabolic intermediates suggested that cellular metabolism is limited by the phosphorylation of the *N*-acylmannosamines by ManNAc 6-kinase in the first step of the pathway. This was confirmed by enzymatic assay of the partially purified enzyme with unnatural substrates. Identification of ManNAc 6-kinase as a bottleneck for unnatural sialic acid biosynthesis provides a target for expanding the metabolic promiscuity of mammalian cells.

The ability to biochemically or genetically manipulate the composition of a cell surface has provided new avenues for fundamental studies of cell-surface recognition. Methods for "cell surface engineering" have also enabled the chemical restructuring of cell surfaces for tailored purposes (*1*). One approach involves the metabolic delivery of unnatural moieties to cell surface glycoconjugates via pathways for oligosaccharide biosynthesis (*2*). An unnatural monosaccharide precursor is "fed" to cells and processed by the biosynthetic machinery into a novel product that is presented on the cell surface. The process requires that biosynthetic enzymes transform the modified substrate with relative efficiency compared to endogenous natural substrates.

The pathway for sialic acid biosynthesis shows tolerance for unnatural substrates and consequently has attracted attention as a vehicle for metabolic cell surface engineering. Shown in Figure 1, sialic acid biosynthesis comprises a chemically and topologically complex set of events. *N*-Acetylmannosamine (ManNAc), the first committed precursor, is either biosynthesized from UDP-*N*-acetylglucosamine (UDP-GlcNAc), obtained from the extracellular environment (*3*), or derived from GlcNAc via the action of GlcNAc 2-epimerase (*4*). ManNAc undergoes a series of enzymatic transformations resulting in the formation of CMP-sialic acid in the nucleus. This activated sugar is then transported to the Golgi compartment where it acts as the donor for glycosylation of an elongating glycan by one of a family of sialyltransferases. The sialylated glycoprotein or glycolipid is then secreted or delivered to the plasma membrane by the secretory machinery.

In vitro biochemical assays have determined that several enzymes in this pathway can utilize unnatural substrates with elongated *N*-acyl groups, as well as modifications at other positions (*5-12*). Reutter and coworkers exploited the permissivity of these enzymes and transport proteins to express unnatural sialic acids on the surface of living cells (*13*). Cells that were incubated with *N*-propanoyl, *N*-butanoyl or *N*-pentanoylmannosamines generated the corresponding unnatural sialosides on cell-surface glycoconjugates and secreted glycoproteins. This phenomenon was used to alter the proliferation of specific cell types in a mixed population

(*14*) and the susceptibility of cells to viral infection (*15, 16*). More recently, Schnaar and coworkers demonstrated that metabolic conversion of *N*-glycolylmannosamine to the corresponding *N*-glycolyl sialic acid abrogated the binding of myelin-associated glycoprotein (MAG) to neuroblastoma-glioma hybrid cells (*17*).

Our laboratory has extended this substrate-based approach to introduce chemically reactive functional groups into cell surface sialosides. For example, inclusion of a ketone group in the elongated *N*-acyl substituent of a ManNAc analog allows further elaboration of the corresponding cell surface sialic acid by selective chemical reaction with a hydrazide or aminooxy conjugate (*18*). Applications of ketone-based cell surface remodeling include selective delivery of diagnostic or therapeutic agents to cells based on their relative levels of sialic acid (*19*), glycoform remodeling (*20*), and construction of novel viral receptors (*21*). These studies all utilized a single mannosamine analog, *N*-levulinoylmannosamine (ManLev, Figure 2A, compound **3a**). To further expand sialic-acid based cell surface engineering, new chemical coupling partners such as azide-phosphine pairs are being explored (*22*). Still, the extent of unnatural substrate tolerance and the possible preference for some analogs over others have not been examined in detail. Such information is crucial for expanding the applications of sialic acid-based cell surface engineering, and for understanding the cellular mechanisms for substrate selection.

In this study, we addressed the scope and limitations of unnatural substrate tolerance in the sialic acid biosynthetic pathway. A panel of unnatural mannosamine analogs (shown in Figure 2A) was synthesized, each bearing a ketone group on a side-chain that replaces the *N*-acetamido group. In each case, the ketone provided a chemical cell-surface marker for quantifying unnatural sialoside expression. By systematically varying the length or size of the modified *N*-acyl group, we explored the global substrate requirements of the complete pathway in intact cells. In order to dissect the detailed metabolism of these compounds within a cell, radiolabel HPLC, colorimetric and mass spectrometry assays were developed to measure intracellular levels of the metabolites

downstream of ManNAc, namely ManNAc-6-phosphate, sialic acid and CMP-sialic acid, as well as their unnatural analogs. The relative amounts of these metabolic products in cells treated with natural and unnatural substrates were compared in order to identify the bottleneck step for metabolism of unnatural mannosamines. These experiments indicated that the phosphorylation step of the pathway was either inefficient or bypassed; thus, the ManNAc 6-kinase was tested *in vitro* to verify the hypothesis that this enzyme acts as a bottleneck for unnatural sialic acid biosynthesis.

Materials and Methods

Materials

Biotin hydrazide, Dulbecco's PBS, FITC-labeled avidin, sodium azide, trypsin-ETDA, ATP, PEP, NADH, pyruvate kinase, lactate dehydrogenase and penicillin-streptomycin were purchased from Sigma. DTT was from New England Biolabs, RPMI medium 1640 was from Life Technologies, Inc., DME medium was from CellGro and fetal bovine serum (FBS) was from HyClone Laboratory. Hygromycin B (HygB) and geneticin were from Boehringer. All protease inhibitors were from Sigma except Pefabloc®, which was from Boehringer-Mannheim. [1-^{14}C]-mannosamine hydrochloride was purchased from American Radiolabeled Chemicals. All reagents used for chemical synthesis were obtained from commercial suppliers and were used without further purification. Fast atom bombardment (FAB+) mass spectra were obtained at the U. C. Berkeley Mass Spectral Laboratory. Mass spectra of acetylated mannosamines were obtained with a Bruker 3000 electrospray ionization-ion trap instrument in the positive mode by flow-injection in acetonitrile. Mass spectra of sialic acids were obtained by electrospray ionization in the negative mode using ESI-MS (Hewlett Packard 1100 Series MSD). Cell densities were determined using a Coulter Counter-ZM. Flow cytometry analysis was performed on a Coulter EPICS® XL-MCL cytometer using a 488 nm argon laser. At least 10^4 viable cells were analyzed from each sample. High pH anion exchange chromatography (HPAEC) was performed using a

Dionex system equipped with a CarboPac™ PA1 column, a pulsed amperometric detector (PAD) and an in-line flow scintillation counter (Packard, Meriden, CT). The system was run at a flow rate of 1 ml/min. All biological experiments were performed in duplicate.

Cell culture conditions

Jurkat cells were grown in RPMI medium 1640 supplemented with 5% FBS and penicillin/streptomycin. HeLa cells were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin. HL-60 cells were grown in RPMI medium 1640 supplemented with 10% FBS and penicillin/streptomycin.

General procedure for the synthesis of ketone-containing mannosamine derivatives

To a solution of the keto-acid (1.1 eq) in distilled THF (~0.2 M) was added triethylamine (1.1 eq) under nitrogen, and the reaction mixture was stirred. After 10 min, isobutyl chloroformate (1.1 eq) was added dropwise by syringe, during which time a white precipitate formed. The reaction mixture was stirred vigorously for 5 h. The anhydride generated was used directly in the next step.

To a solution of mannosamine hydrochloride (1.0 eq) in water (1/2 volume of previous THF solution) was added triethylamine (1.3 eq). The reaction mixture was stirred for 30 min, and the anhydride solution was added slowly, giving a final reaction mixture in 2:1 THF/H_2O as solvent. The reaction mixture was stirred vigorously for 24 h. The solvent was then removed *in vacuo*, and the resulting foam was passed through cation (BioRad AG50W-X8 pyridine form) and anion (Biorad AGI-X2, acetate form) exchange columns, eluting with water. The resulting yellow amorphous foam was purified by silica gel chromatography, eluting with a gradient of 20:1 to 5:1 $CHCl_3$/CH_3OH, to yield the desired product as a white amorphous foam, which was characterized as a mixture of anomers. Full synthetic methods and characterization are available in the Supplemental Information section.

General procedure for synthesis of N-acyl mannosamine derivatives

To a solution of mannosamine hydrochloride (1.0 eq) in methanol (200 mM) was added a solution of NaOMe in methanol (0.5 M, 1.0 eq), and the reaction mixture was stirred for 1 h. The desired acyl anhydride (1.1 eq) was added to the solution and the reaction mixture was stirred and monitored by tlc. Upon depletion of the starting material, the solvent was removed *in vacuo*, and the material was purified by silica gel chromatography, eluting with EtOAc/hexanes to yield the desired product, which was characterized as a mixture of anomers.

General procedure for synthesis of 1,3,4,6-tetra-O-acetylmannosamine derivatives

N-Acylation with the desired acid or anhydride was performed as described above, but without purification. The solvent was then removed *in vacuo* and the crude *N*-acylmannosamine was dissolved in pyridine (200 mM), to which acetic anhydride (100 mM) was added. The reaction mixture was stirred for 4-10 h. The solvent was then removed *in vacuo* and the residue was dissolved in CH_2Cl_2, washed successively with $CuSO_4$ (0.25 M), sat. $NaHCO_3$ and water, dried over $NaSO_4$ and concentrated. The resulting residue was purified by silica gel chromatography eluting with a gradient of 10:1 to 2:1 hexanes/EtOAc, to yield a white amorphous foam which was characterized as a mixture of anomers.

Synthesis of ^{14}C-labeled acetylated mannosamine derivatives

[1-^{14}C]-Mannosamine hydrochloride (0.9 μmol, 50 μCi) was dissolved in 20 μl of 50 mM NaOMe in MeOH in an eppendorf tube and allowed to react for 2 h. A solution of the desired alkyl anhydride in MeOH (1.1 μmol, 2 μl of a 550 mM solution) was added, and the reaction was allowed to proceed overnight. The reaction was followed by tlc (5:1 $CHCl_3$:MeOH) and the tlc plate was visualized by phosphorimaging. When the reaction was complete, the solvent was evaporated and the residue was purified by silica gel chromatography, eluting with a gradient of

20:1 to 5:1 $CHCl_3$:MeOH. Fractions containing radioactivity at the correct R_F were combined and the solvent was removed. The resulting pure mannosamine derivative was dissolved in 50 µl of pyridine, to which was added 25 µl of acetic anhydride. Progress of the reaction was followed by tlc (EtOAc). Upon completion, the solvents were removed, the residue was dissolved in EtOH, and the yield was determined by scintillation counting of 0.1% of the solution. ^{14}C-labeled compounds were characterized by ESI mass spectroscopy, confirming the absence of contaminating ^{14}C-labeled peracetylated ManNAc (^{14}C-Ac_4ManNAc, compound **9c**) in the unnatural derivatives.

Quantification of cell surface ketones

Cell-surface ketones were quantified after incubating cells with unprotected or peracetylated sugars for two days. Generally, concentrations of 5 mM unprotected sugar or 10-50 µM acetylated sugar were used. The cells were labeled with biotin hydrazide followed by FITC-avidin staining according to a previously published procedure (*18, 20*).

Colorimetric analysis of sialic acids using N-acyl substrates.

The appropriate volume of a solution of the desired compound (100 mM in ethanol, compounds **9b** – **13b**) was added to 8.0 ml of Jurkat cells at a density of $1x10^6$ cells/ml to give final concentrations of 25, 50, 75, 100, 150 and 200 µM. In addition, equivalent volumes of ethanol were added to cells to serve as negative controls. Approximately every 24 h, 4.0 ml of the cell suspension were removed and replaced with an equal volume of complete medium containing the original concentration of the appropriate substrate. The harvested cells were counted and assayed for sialic acid levels by the periodate-resorcinol assay (*23*). The cells were washed twice with 1.0 ml PBS, pelleted, resuspended in 200 µl PBS and divided into two 100 µl aliquots. Cell lysates were achieved by four freeze/thaw cycles; the lysates were then analyzed by the method of Jourdian (*23*) adapted for a 96-well plate format. Oxidation of each sample was performed at

both 0 °C and 37 °C to give values for total sialic acid or glycoconjugate-bound sialic acid (including CMP-sialic acid), respectively. After 2 d of incubation, the levels of sialic acid in the Jurkat cells reached equilibrium. Aliquots of cells were harvested and assayed daily for a period of two weeks. Control experiments using authentic CMP-sialic acid confirmed that no hydrolysis occurred during either freeze/thaw cycles or the 37 °C oxidation step. Control experiments for the periodate-resorcinol assay confirmed that discrimination between the free and glycoside-bound forms of sialic acid is essentially quantitative.

Preparation of cytosolic extracts for mass spectrometry analysis

Jurkat cells were grown in 10-cm tissue culture plates, seeded at cell densities of approximately 500,000 cells/ml in 20 ml of media. These cultures were supplemented with the appropriate volume of d_3-Ac_4ManNAc (compound **9d**), Ac_4ManProp (**10b**), Ac_4ManBut (**11b**), Ac_4ManPent (**12b**), or Ac_4ManLev (**3b**) as an EtOH solution to give a final concentration of 200 μM. The cells were allowed to incubate under typical growth conditions for 36 h. The cells were counted, washed 3x with PBS and resuspended in 200 μl of H_2O. Lysis of these cells was accomplished by three freeze/thaw cycles. The membrane components were pelleted by centrifugation using a benchtop centrifuge at 20,800 x g for 15 min. The supernatant was transferred to a Centrifree column (Millipore, Bedford, MA) to remove all large proteins and particles. The filtrate was directly analyzed for the presence of sialyl compounds by ESI-MS, eluting with 95% CH_3CN/5% H_2O. When an internal standard was desired, a solution of ^{13}C-NeuAc was added to the sample to give a final concentration of 500 μM before analysis by ESI-MS.

Analysis of cytosolic metabolites produced from radiolabeled substrates.

Jurkat cells were incubated at a density of $1x10^6$ cells/100 μl in a 96-well tissue culture plate with 200 nCi of peracetylated ^{14}C-mannosamine derivatives. Unlabeled substrate was added

in some cases, in varying concentrations up to 200 μM. The addition of cytidine (1 mM) was used in some cases to increase conversion of sialic acid to CMP-sialic acid. Cells were incubated for 20 h, then pelleted and washed twice with 0.5 ml of PBS. Cells were then suspended in 150 μl water and lysed by four freeze/thaw cycles, after which time the samples were maintained at 4 °C. The lysate was cleared of macromolecules using Microcon filters (Millipore), 30,000 and 3,000 kDa cut-off, sequentially. The cleared lysate was dried and dissolved in 80 μl of water, and 20 μl aliquots were analyzed by HPAEC followed by flow scintillation counting. Two different gradients were used to ensure that peaks corresponded to authentic standards. Gradient A: 25 mM NaOH for 10 min; ramp to 45 mM NaOH, 200 mM NaOAc over 15 min; ramp to 140 mM NaOH, 300 mM NaOAc over 15 min and hold for 15 min; ramp to 25 mM NaOH over 5 min and hold for 20 min. Gradient B: ramp from 25 mM NaOH to 50 mM NaOH, 200 mM NaOAc over 10 min; ramp to 140 mM NaOH, 600 mM NaOAc over 5 min and hold 5 min; ramp to 25 mM NaOH over 5 min and hold for 10 min. Peaks were identified by comparing elution times to those of authentic standards. ManNAc, sialic acid and CMP-sialic acid standards were commercially available. ManNAc-6-phosphate was chemo-enyzmatically synthesized using hexokinase (*24*). Peaks were integrated using Flo-ONE analysis software (Packard, Meriden, CT).

In vitro enzyme assay for ManNAc 6-kinase

Human UDP-GlcNAc 2-epimerase/ManNAc 6-kinase was overexpressed in Sf9 cells as previously reported (*25*). The cells (about 100 μl pellet volume) were lysed in 1 ml of 10 mM NaH_2PO_4 with 1 mM EDTA, 1 mM DTT (pH 7.5) and 1 μl of protease inhibitor cocktail (1 mg/ml leupeptin, 2 mg/ml antipain, 20 mg/ml turkey trypsin inhibitor, 10 mg/ml benzamidine, 5 mg/ml Pefabloc®, 2 mg/ml aprotinin) by 20 passages through a narrowbore needle. The cytosol was clarified by centrifugation (16,000 x g for 30 min at 4 ºC). The supernatant was purified on a HiTrap Q Sepharose column (Pharmacia, Sweden) as follows: the column was loaded with the clarified cytosol and washed with 5 ml of 10 mM NaH_2PO_4, 1 mM EDTA, 1 mM DTT (pH 7.5)

and the protein was eluted in the same buffer using a gradient of 0 to 600 mM NaCl over 10 ml (0.5-ml fractions). The desired protein (MW = 75 kD) eluted in approximately 300 mM NaCl and was approximately 90% pure as determined by SDS-PAGE analysis (10% gel, Coomasie stain).

Purified enzyme (10 μl) was added to a preincubated mixture that contained 5 mM desired substrate, 10 mM ATP, 2 mM PEP, 0.2 mM NADH, 2 U of pyruvate kinase and 2 U of lactate dehydrogenase in 60 mM Tris, 10 mM $MgCl_2$, pH 8.1 (total reaction volume = 0.1 ml). The enzymatic reaction was monitored at 340 nm with a SpectraMAX 190 UV/vis microplate reader (Molecular Devices) over 15 min or until the reaction rate began to plateau. Initial rates were determined using SOFTmax® PRO 3.1 software (Molecular Devices).

Results

Synthesis of mannosamine derivatives

We designed a panel of mannosamine analogs, shown in Figure 2A, bearing side chains that are longer, shorter or more sterically demanding than the five-carbon side chain of ManLev (compound **3**). Compound **2** has an acetoacetyl side chain that is one methylene unit shorter than the side chain of ManLev, and compounds **4**-**6** have successively longer side chains of six, seven or eight carbon atoms, respectively. Compounds **7** and **8** have side chains of the same length as found in ManLev, but present greater steric demands due to an added methyl group (**7**) or cyclopentanone ring (**8**). Finally, compound **1** is isosteric with the native substrate ManNAc; substitution of the NH group with a methylene group generates the ketone functionality (*26*). Some compounds were prepared in both free (**a**) and peracetylated (**b**) forms to address possible differences in cellular uptake of the unprotected mannosamine analogs (*vide infra*).

Cellular metabolism of unnatural ketone-containing mannosamine analogs

Jurkat cells were incubated with compounds **2** and **3a** – **8a** (5 mM, 2 days) and evaluated for metabolic incorporation of the corresponding sialic acids into cell-surface glycoconjugates by

staining with biotin hydrazide followed by FITC-avidin (*18*). Analysis of labeled cells by flow cytometry produced the data depicted in Figure 3. Cells treated with compound **3a** (ManLev) showed a high level of fluorescence and were used as a positive control for this experiment. The majority of detectable ketones on Jurkat cells has been previously shown to reside on *N*-linked glycoproteins (*18*).

Compound **4a**, which bears an *N*-acyl group that is one methylene unit longer than the five-carbon side-chain of ManLev, produced drastically reduced fluorescence compared to ManLev. Likewise, treatment of cells with compound **7a**, a methylated version of ManLev, resulted in only background levels of fluorescence. Compound **2a** proved to be unstable in cell culture media, and generated variable ketone expression in cells between 15 and 60% of that observed using ManLev. The remaining compounds (**5, 6** and **8**) produced no detectable ketones on Jurkat cells, as determined by the observation of only background fluorescence levels.

To investigate whether these analogs function as inhibitors rather than substrates of the pathway, we co-incubated ManLev with the eight-carbon analog **6a**. No reduction in ketone expression was observed at concentrations of **6a** that were three-fold higher than ManLev, suggesting that **6a** is not blocking normal metabolic processes (Supporting Information).

The human cell lines HL-60 and HeLa were also incubated with the unnatural mannosamines. The results from these experiments echoed observations from the similar experiment using Jurkat cells (Supporting Information). These results suggest that metabolic discrimination between ManLev and larger, untolerated analogs is a general feature of the human sialic acid biosynthetic machinery. These cell lines exhibit different patterns of glycosylation, including varying levels of α2-3 and α2-6 linked sialic acids as determined by lectin binding studies (Supporting Information). In addition, we have previously demonstrated the incorporation of *N*-levulinoyl sialic acid into CD43 bearing α2-3-linked sialic acids (*27*) and NCAM bearing α2-8-linked sialic acids (*28*), suggesting the corresponding sialyltransferases are similarly

tolerant of the unnatural side chain. Overall, these results suggest that sialyltransferases are relatively uniform in their permissivity toward unnatural CMP-sialic acids.

Peracetylated monosaccharides have been shown to passively diffuse through mammalian cell membranes and undergo subsequent deacetylation by cytosolic or ER esterases (*29*). We hypothesized that any discrimination against unnatural substrates at the level of cellular uptake would be circumvented by their acetylation. We therefore synthesized peracetylated monosaccharides **1** and **3b – 8b** and incubated them with Jurkat cells for two days at concentrations of 50-100 μM. Interestingly, compound **1**, the analog most similar to ManNAc in structure but lacking the hydrogen-bonding capability of the amide NH, was not converted to cell-surface sialosides at a detectable level. Compounds **3b – 8b** were converted into cell-surface sialosides at the same relative level as their unprotected counterparts (Figure 3). Specifically, compound **3b** was metabolized to a significant extent, while the longer or bulkier *N*-acyl analogs were metabolized to a lesser or undetectable extent. Since the mechanism of cellular uptake does not affect the metabolic fate of these derivatives, we conclude that their discrimination occurs at a downstream step in the process.

A substantial practical benefit of the peracetylated sugars is that these protected mannosamines can be utilized at concentrations approximately 200-fold less than the free sugars while producing similar levels of cell-surface ketones (*30*). Since this effect was seen for all mannosamines tested thus far, in most subsequent experiments the more efficient peracetylated mannosamines were used for cellular assays.

Colorimetric analysis of cellular sialic acids produced from N-acyl mannosamine analogs

In order to determine whether unnatural mannosamines were being converted to sialic acids inside the cells, we analyzed the intracellular concentrations of total (reducing and non-reducing) and glycoside-bound (non-reducing only) sialic acids using cells treated with different substrates. The two forms of sialic acid can be discriminated by the periodate-resorcinol assay;

oxidation at 0 ºC measures total sialic acid, and oxidation at 37 ºC measures glycoside-bound sialic acid. The concentration of free (reducing) sialic acid can be determined by subtracting the latter from the former. The results of periodate-resorcinol assays are shown in Figure 4.

We observed a substrate concentration-dependent increase in total cellular sialic acid levels that was inversely correlated with the length of the substrate's *N*-acyl side chain (Figure 4A). Not surprisingly, the natural substrate Ac_4ManNAc (compound **9b**) produced the largest increase in total sialic acid levels, although Ac_4ManProp (**10b**) also supported significant levels of sialic acid production. Ac_4ManBut (**11b**) generated an intermediate response, while Ac_4ManPent (**12b**) and Ac_4ManHex (**13b**) produced sialic acid levels that were only marginally above background. Figure 4B shows that in ethanol-treated control cells (as well as untreated cells, not shown), there is virtually no sialic acid in the free monosaccharide form. Upon incubation with Ac_4ManNAc (or analogs), typically 20 to 25% of the increase in total sialic acid can be attributed to CMP-sialic acid and downstream glycoconjugates. Therefore, the bulk of the signal represented in Figure 4A is due to sialic acid or sialic acid 9-phosphate present in the free monosaccharide form of the sugar. These results suggest that while Ac_4ManProp (**10b**) is converted to the corresponding sialic acid at near-native levels, the longer side chain analogs (**11b – 13b**) are metabolized with lower efficiency.

Mass spectral analysis and quantification of cytosolic sialic acids

To definitively prove the identity of the sialic acids derived from unnatural substrates, we performed mass spectrometry analysis of the cytosolic components of the cell. Jurkat cells were incubated with the acetylated mannosamine derivatives, harvested and their cytosolic components were prepared for mass spectral analysis. In a control experiment, Jurkat cells were treated with d_3-Ac_4ManNAc (**9d**). As previously mentioned, Jurkat cells do not store large amounts of free sialic acid, as seen from the lack of a peak corresponding to a molecular weight of 308.3 in untreated cells (Figure 5A). Cells incubated with d_3-Ac_4ManNAc however, show the

corresponding mass peak for d_3-NeuAc at 311.3 (Figure 5B), which can be clearly distinguished from endogenous sialic acid. Corresponding sialic acid peaks for cells incubated with Ac_4ManProp (**10b**), Ac_4ManBut (**11b**), and Ac_4ManPent (**12b**) were also detected at 322.3 (Figure 5C), 336.3 (Figure 5D), and 350.3 (Figure 5E), respectively. When Jurkat cells were incubated with Ac_4ManLev (**3b**) at 200 μM, no mass signal at 364.3 could be detected (Figure 5F). In order to compare unnatural sialic acid levels across the substrate panel, an internal standard (^{13}C-NeuAc, *m/z* at 309.3) was added to the sample during analysis. Such comparisons revealed that the amount of sialic acid analog within the cell decreased as the alkyl chain length on the *N*-acyl position of mannosamine increased. The absence of a detectable peak for unnatural sialic acid in ManLev-treated cells suggests that the ketone group further diminishes conversion to sialic acid compared to the methylene analog ManPent.

Analysis of cytosolic mannosamine metabolites using ^{14}C-Ac_4ManNAc

In order to dissect all intermediates in the biosynthetic pathway, we developed an HPAEC assay that quantified the products from radiolabeled mannosamine analogs. Initially, Jurkat cells were treated with ^{14}C-Ac_4ManNAc (**9c**) along with various concentrations of unlabeled Ac_4ManNAc, with or without added cytidine. Figure 6A shows HPAEC traces that clearly define each intermediate along the pathway, with the exception of sialic acid-9-phosphate, which is presumably present in quantities too minute to be detected. Furthermore, the peak area ratios varied with added unlabeled substrate and added cytidine in a predictable fashion, thereby assuring that the assay is sensitive enough to sense changes in the flux of the pathway. In order to determine the length of incubation that would result in optimal conversion of radiolabeled substrates, a time course was performed using compound **9c** (Figure 6B). This experiment indicated that, at least for Ac_4ManNAc, conversion of exogenous peracetylated substrate to cytosolic intermediates was optimal between 20 and 24 hours.

Analysis of cytosolic unnatural mannosamine metabolites using ^{14}C-labeled N-acyl substrates

A panel of unnatural ^{14}C-labeled *N*-acyl mannosamines was used in metabolic radiolabeling experiments as described above to determine the efficiency of transformation of each unnatural mannosamine to its downstream products, as compared to the natural substrate. Figure 7A shows the HPAEC/flow scintillation traces generated using this substrate panel. The retention times of peaks changed slightly depending on the substrate used, due to the increased hydrophobicity of the *N*-acyl chains. Substrates without ketones (**9b – 13b**) were used in these experiments due to the instability of ketone-containing sugars under the conditions of HPAEC.

The results of two experiments performed with different substrate concentrations are shown in Figures 7B and 7C. Compared to the natural substrate, ^{14}C-Ac$_4$ManNAc (**9c**), the unnatural substrates showed decreased conversion of *N*-acylmannosamine to the corresponding sialic acid. This effect correlates directly with the length of the *N*-acyl chain (Figure 7C). At 20 μM substrate, the ratio of sialic acid to CMP-sialic acid appeared to hold constant at about 2:1 in cells treated with ^{14}C-Ac$_4$ManNAc and ^{14}C-Ac$_4$ManProp (compound **10c**). However, the ratio of ManX (where X indicates the unnatural *N*-acyl side chain) to ManX-6-phosphate changed drastically with even minor modification to the substrate. For example, in Figure 7B, there were approximately equal levels of ManNAc and ManNAc-6-phosphate in cells treated with the natural substrate (**9b/c**), but treatment with ^{14}C-Ac$_4$ManProp (**10c**) yielded no ManProp-6-phosphate within the limits of detection, even though cytosolic ManProp accumulated to a level about six-fold higher than that seen with the natural substrate (Figure 7B).

In order to determine whether unnatural mannosamines or their metabolites may act as inhibitors of natural sialoside biosynthesis, we utilized a modified radiolabel HPAEC assay. Jurkat cells were treated with ^{14}C-Ac$_4$ManNAc (compound **9c**) as a tracer for natural sialoside synthesis, and unlabeled unnatural mannosamine compounds (**10b – 13b**) were added at 20 μM. The results of HPAEC/flow scintillation analysis are shown in Figure 8. As expected, cells treated

with Ac_4ManNAc exhibited increased transformation of ManNAc-6-phosphate to sialic acid compared to untreated cells. Cells treated with Ac_4ManProp (**10b**) showed a response intermediate between untreated and Ac_4ManNAc-treated cells; potentiation of the pathway resulted in an increased level of sialic acid. In contrast, the longer analogs (compounds **11b – 13b**) showed neither inhibition nor potentiation of the natural pathway.

In vitro assay of ManNAc 6-kinase activity using unnatural substrates

Since it appeared from cell-based assays that unnatural mannosamine derivatives were not being efficiently phosphorylated in the first step of the biosynthetic pathway, we performed *in vitro* assays with the enzyme responsible for this activity, ManNAc 6-kinase (*31*). The enzyme exists as part of a bifunctional protein that catalyzes the formation of ManNAc from UDP-GlcNAc and its subsequent phosphorylation. The kinase also acts on exogenously added ManNAc (*25*). Using the partially purified human enzyme that had been overexpressed in Sf9 insect cells, we tested the panel of mannosamine derivatives as substrates in a coupled enzyme spectrophotometric assay (*25*). The screen was performed using ATP at a concentration of 10 mM and the desired substrate at 5 mM. As shown in Figure 9, the enzyme showed reduced activity toward unnatural substrates. ManProp and ManLev were phosphorylated at an initial rate about 6-fold lower than the natural substrate, and ManBut and ManPent were not measurably turned over by the enzyme. The activity of the partially purified enzyme toward GlcNAc was also tested to confirm the absence of other contaminating sugar kinases, i.e. GlcNAc kinase. Clearly, this initial step of the sialic acid pathway is quite restrictive with respect to unnatural substrates.

Discussion

In this study we probed the unnatural substrate tolerance of the sialoside biosynthetic pathway toward C-2 modifications of ManNAc. In an initial cell-based assay, which exploits a uniquely reactive ketone group within cell-surface metabolic products, substrate tolerance of the

entire pathway could be probed without perturbing the subcellular organization of its components. In contrast to conventional biochemical assays for substrate recognition, this approach tests all biosynthetic enzymes at once, along with intracellular transporters, and lends itself to high-throughput analysis by flow cytometry.

We found that substitution of the amide group at C-2 of ManNAc with a ketone, as in compound **1**, disrupted processing of this compound by the sialoside biosynthetic pathway. This could be attributed to subtle changes in structure or the loss of key hydrogen bonding interactions, preventing binding to an active site or catalytic activity. The amide group at C-2 is therefore a critical determinant that cannot be replaced in an unnatural sialic acid precursor.

A major finding of these initial experiments is that the sialic acid biosynthetic pathway is highly sensitive to elongation of the side chain beyond five carbon atoms. Extension of the chain to six, seven or eight carbon atoms caused a precipitous drop in the efficiency (compared to smaller *N*-acyl groups) of metabolism to cell surface sialosides in all cell lines tested. Moreover, subtle increases in steric bulk, such as methylation or addition of a ring, also compromised metabolic conversion to the cell surface product in all cell lines tested. Thus, one or more enzymes or transporters in the pathway are unable to transform the larger analogs. The initial step, uptake of the analogs from the media, was ruled out as a point of stringency or "bottleneck" since peracetylated derivatives were metabolized to sialosides at the same relative levels as the free sugars. Since exclusion of the longer *N*-acyl chains was largely independent of the type of sialic acid linkages found on the cells, one or more steps prior to the sialyltransferases is most likely responsible for the initial metabolic blockade.

To explore the intracellular basis for the differential cell surface expression of various unnatural sialosides, we developed colorimetric, mass spectral and radiolabel HPLC assays using whole-cell lysates to quantify metabolic intermediates. These assays were applied to determine which biosynthetic step or steps are responsible for the discrimination between substrates observed by flow cytometry. Using a colorimetric assay to detect total cellular or non-reducing

sialic acid, we confirmed that the amount of detectable sialic acid decreases with increasing chain length of the mannosamine substrate. Mass spectral analysis corroborated these results. Interestingly, two substrates of identical side chain length can have different metabolic conversion efficiencies. Ac_4ManLev (**3b**), bearing a five-carbon ketone-containing side chain, produced sialic acid levels that were below the mass spectrometry detection limit, while the similar compound Ac_4ManPent (**12b**), lacking a ketone, generated a small but detectable amount of the corresponding sialic acid.

We used a radiolabel HPAEC assay to measure the relative levels of multiple intermediates of the pathway at once. The observation of a build-up of any individual intermediate derived from an unnatural mannosamine indirectly implicates the previous enzymatic step as a bottleneck for unnatural sialoside biosynthesis. Results generated using the ^{14}C-labeled panel, compounds **9c** – **13c**, as well as *in vitro* enzyme assays indicated that ManNAc 6-kinase is intolerant of even modest changes in substrate structure such that unnatural products cannot accumulate to detectable levels. However, cells fed unnatural compounds 10, 11 and 12 generated detectable amounts of sialic acid. It is possible that *N*-acylmannosamines are converted directly into sialic acids by sialic acid-9-phosphate synthase, bypassing the phosphorylation step entirely. However, previous *in vitro* assays did not detect any synthase activity on unphosphorylated ManNAc (*32*). Sialic acid aldolase, which normally operates in the direction of sialic acid decomposition, might also be capable of converting unnatural mannosamines into sialic acids under the right cellular conditions. Indeed, a bacterial homologue has been shown to be quite permissive with respect to the *N*-acyl position of the substrate (*33*). Additionally, GlcNAc 6-kinase can utilize ManNAc as a substrate, albeit with 100-fold lower efficiency than GlcNAc, providing an alternative route to ManNAc-6-phosphate from ManNAc (*34*).

Using unnatural substrates, we did not observe the accumulation of ManX-6-phosphate in cells. Thus, any ManX-6-phosphate that is generated by the action of either ManNAc or GlcNAc 6-kinase must be immediately converted to sialic acid, suggesting that the two intervening

enzymes (sialic acid 9-phosphate synthase and sialic acid 9-phosphate phosphatase) are relatively more permissive.

In conclusion, we have identified ManNAc 6-kinase as a bottleneck step in the biosynthesis of unnatural sialic acids. This enzyme is therefore an attractive target for genetic engineering with the goals of relaxing substrate specificity and expanding the scope of sialic acid-based cell surface engineering. We think it likely that enzymes further downstream are also contributing to unnatural substrate discrimination; it is possible that multiple bottlenecks exist. Further biochemical and metabolic experiments should identify additional enzyme targets for genetic engineering.

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Supporting Information Available

Complete synthetic methods and characterization for compounds **1** (including intermediates), **2, 3a – 8a, 10b – 13b, 9c – 13c** and ManNAc-6-phosphate, biological assay data from competition flow cytometry experiments and analysis of HeLa and HL-60 cells by ketone-specific and lectin-based flow cytometry. This material is available free of charge via the Internet at http://pubs.acs.org.

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