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Note

Chemo-enzymatic synthesis of the carbohydrate antigen *N*-glycolylneuraminic acid from glucose

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ABSTRACT

N-Glycolylneuraminic acid (Neu5Gc) is a non-human sialic acid, which may play a significant role in human pathologies, such as cancer and vascular disease. Further studies into the role of Neu5Gc in human disease are hindered by limited sources of this carbohydrate. Using a chemo-enzymatic approach, Neu5Gc was accessed in six steps from glucose. The synthesis allows access to gram-scale quantities quickly and economically and produces Neu5Gc in superior quality to commercial sources. Finally, we demonstrate that the synthesized Neu5Gc can be incorporated into the cell glycocalyx of human cells, which do not naturally synthesize this sugar. The synthesis produces Neu5Gc suitable for in vitro or in vivo use.

Sialic acids (sias) are a family of acidic 9-carbon chain carbohydrates typically found in terminal positions on the cell glycocalyx (the vast coating of glycans that covers all cell surfaces).[1] Their biological functions include regulation of processes such as innate immunity, inflammation,[2] cell–cell interactions and neural plasticity. They are also involved in tumour metastasis and pathogen binding. In recent years the significance of this family of glycans to human health and evolution is becoming ever clearer.[3] However, advancements have been hindered by a relative lack of available sias and derivatives thereof with which to conduct these biological studies. The sia *N*-glycolylneuraminic acid (Neu5Gc) is a non-human sialic acid, which is biosynthesized from *N*-acetylneuraminic acid (Neu5Ac) via the enzyme CMP-Neu5Ac hydroxylase (CMAH) encoded by the gene *CMAH*. Approximately 2–3 million years ago the human *CMAH* gene was mutated and its product was no longer able to hydroxylate CMP-Neu5Ac to CMP-Neu5Gc.[4]

Although humans are no longer able to make Neu5Gc, dietary Neu5Gc can still be metabolically incorporated and displayed on the glycocalyx of the human epithelia and associated carcinomas.[5] In principle, Neu5Gc could be an important human-specific 'xeno-autoantigen'. Potential roles in tumourigenesis[6] and vascular pathologies[7] have recently been identified.

To further investigate how dietary Neu5Gc is involved in human-specific disease requires synthetic access to Neu5Gc in large quantities and high purity. Current commercial sources are limited and expensive, contain 1–3% Neu5Ac which can interfere with some biological assays, and their continued support and supply have an uncertain future.[8] We have developed a chemo-enzymatic strategy to access Neu5Gc that is high yielding, suitable for use in cell experiments requiring sterile conditions and allows quick access to gram-scale quantities.

Synthetic methods to access the sias have been reported since the 1980s.[9] Because of the stereochemical considerations, most methodologies take advantage of monosaccharide starting materials. Auge et al.,[9a] synthesized Neu5Gc in milligram scale starting from mannosamine hydrochloride, introducing the glycolyl moiety using a benzyloxyacetic acid derivative followed by hydrogenation to yield 2-deoxy-2-[(hydroxyacetyl)amino]-D-mannopyranose (ManNGc), which was enzymatically converted to Neu5Gc. This is an attractive route for small-scale synthesis, however, the 2-deoxy-2-amino-mannose, although commercially available, is expensive and therefore impractical for use on large scale. Some synthetic methods start from little or no chirality within the starting materials.[9b–d] Kang et al. demonstrated a highly diastereoselective synthesis of Neu5Ac by stereoselective functionalization of olefin starting materials.[9b] Some purely enzymatic synthetic routes have also been described.[10] These use enzymatic conversion of the *manno* derivative, or the *gluco* derivative via a single or multiple enzyme procedure. Wang et al. has recently demonstrated the synthesis of Neu5Ac starting from *N*-acetyl-D-glucosamine, via two immobilized enzymes, *N*-acetyl-D-glucosamine 2-epimerase and *N*-acetyl-D-neuraminic acid aldolase.[10a] In addition, bioreactors may also prove an important method to access neuraminic acids. Feirfort et al. genetically engineered *Escherichia coli* to make excessive quantities of sialylated glycans.[11] Although further development would be required here to isolate the pure sialic acid monomer. Although a large body of work has already been undertaken in the synthetic access of the neuraminic acids (examples are summarized in Refs. 9–11), these studies have not been primarily

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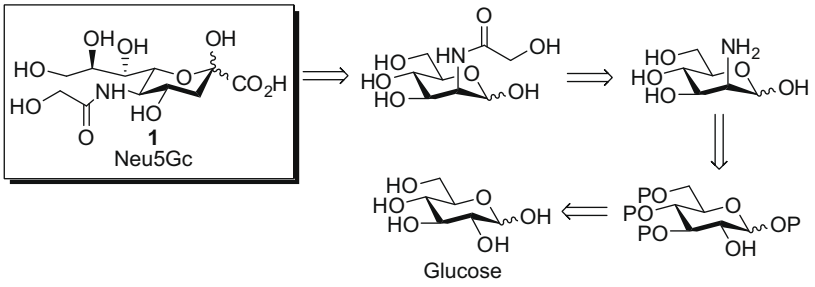


Scheme 1. Retrosynthetic analysis of Neu5Gc synthesis reveals glucose as a suitable starting material. P = protecting group.

concerned with a quick, large-scale, high purity synthesis of the Neu5Gc analogue.

Retrosynthetic analysis of Neu5Gc (Scheme 1) presents a synthetic pathway from the readily available and cheap monosaccharide D-glucose. Orthogonal protection of the pyranose ring followed by triflation prepares the molecule for selective stereochemical inversion to the *manno* stereochemistry. Global deprotection and appending of the glycolyl moiety prepare the molecule for final enzymatic conversion to the product Neu5Gc **1**.

Scheme 2 outlines the synthetic route taken. Reaction of D-glucose with benzyl alcohol in the presence of acetyl chloride produced benzyl D-glucopyranoside **2** in 86% yield, which was further modified to produce the 4,6-benzylidiene derivative **3** in 84% yield, by reaction with benzaldehyde dimethylacetyl and a catalytic amount of (+)-camphor-10-sulfonic acid. Triflation of the 2-OH group of **3** using triflic anhydride at −30 °C[12] followed by displacement with sodium azide selectively inverted the stereochemistry to afford the 2-azido-2-deoxy-mannopyranoside **4** in 72% yield. A global deprotection strategy was envisaged using Pd/C and $H_{2(g)}$, however, initial experiments showed only reduction of the azide moiety to form the amine product (Table 1, entry 1). No reduction of benzyl moieties occurred, thought to be due to inhibition of the catalyst by the amine product (compare Table 1, entries 1 and 2), an observation that has been made previously.[13] It has been demonstrated that this problem can be overcome using large amounts of Pd/C,[14] however, this is only practical for small-scale synthesis (5–10 mg). We initially changed catalyst to the more rigorous Pearlmans catalyst, $Pd(OH)_2/C$, which has been successfully used to reduce more difficult *O*-benzyl moieties. However, this was unsuccessful (Table 1, entry 3). Access to **5** was successfully achieved by addition of 1 M hydrochloric acid to the reaction mixture (Table 1, entry 4), which afforded the product after filtration through Celite in 89% yield. One previous example of this has been demonstrated.[15] Preparation of ManNGc **6** was achieved in 94% yield by reaction with acetoxyacetyl chloride under mild basic conditions and reduced temperature. Final conversion of **6** to the target **1** was achieved using an aldolase isolated from *Pasteurella multocida*, using a previously described method.[16]

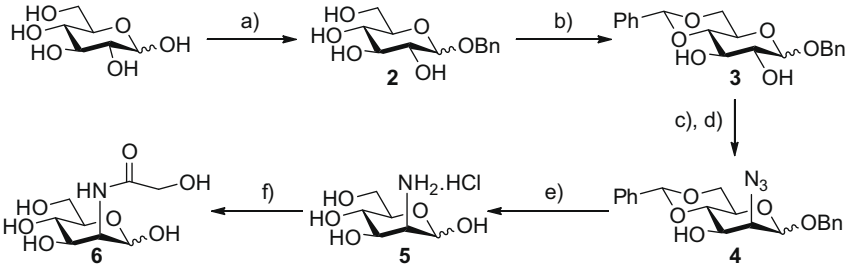
Table 1
O-Linked hydrogenation in the presence of an amine, achieved using $HCl_{(aq)}$ within the reaction mixture

Entry	Starting material	Catalyst	Solvent	Yield[a] (%)
1	4	Pd/C	MeOH	ND
2	3	$Pd(OH)_2/C$	MeOH	100
3	4	$Pd(OH)_2/C$	MeOH	ND
4	4	$Pd(OH)_2/C$	MeOH/$HCl_{(aq)}$	100

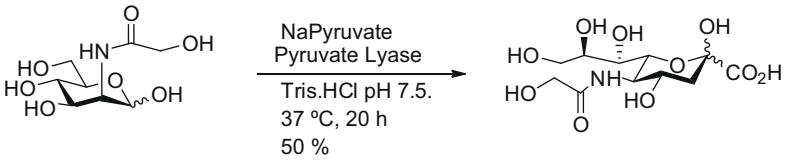
[a] The yield of the fully deprotected product (determined by TLC). ND = not detected.

Conversion of ManNGc to Neu5Gc using this methodology has previously been reported on a milligram scale.[9a,16] Reaction of **6** with the aldolase in the presence of an excess of pyruvate afforded Neu5Gc **1** in 50% yield (Scheme 3). The starting material **6** could be recovered during the purification step (see SI) and used again as a substrate for the aldolase.

Comparison of the commercial Neu5Gc and synthesized versions by amperometric analysis (see SI Figs. S1–3) revealed the methodology produced Neu5Gc which had no trace of Neu5Ac, compared to approximately 3% seen in commercial sources. Although humans can no longer make Neu5Gc, human tissue samples, including carcinomas, have been shown to have significant levels of Neu5Gc on their surface.[17] A likely explanation for this is incorporation from dietary sources such as red meat products. To test whether Neu5Gc could be incorporated into the cell glycocalyx of human cells, human monocytic cell line THP-1 was grown in media containing either Neu5Ac, synthesized Neu5Gc, or neither. After three days the cells were stained with a recently described anti-Neu5Gc IgY[18] and analyzed by flow cytometry (Fig. 1). Neu5Gc fed cells showed incorporation of Neu5Gc into the cell surface (1A). Moreover, the anti-Neu5Gc IgY staining seen in Neu5Gc fed cells could be inhibited by addition of free Neu5Gc. Twenty millimolar free Neu5Gc showed complete inhibition (Fig. 1B). In addition, cells fed with the synthesized Neu5Gc showed comparable cell proliferation to unfed cells (cell proliferation was measured using a cytometer. Cells fed synthesized Neu5Gc, or commercial



Scheme 2. Synthesis of ManNGc. Reagents and conditions: (a) benzyl alcohol, acetyl chloride, 86%; (b) benzyl-dimethylacetyl, (+)-camphor-10-sulfonic acid, acetonitrile, 84%; (c) pyridine, triflic anhydride, dichloromethane; (d) sodium azide, dimethylformamide, 72% (over two steps); (e) $Pd(OH)_2/C_{(cat)}$, $H_{2(g)}$, $HCl_{(aq)}$, methanol, 89%; (f) acetoxyacetyl chloride, sodium bicarbonate, water, 94%.



Scheme 3. Enzymatic synthesis of Neu5Gc from ManNGc.

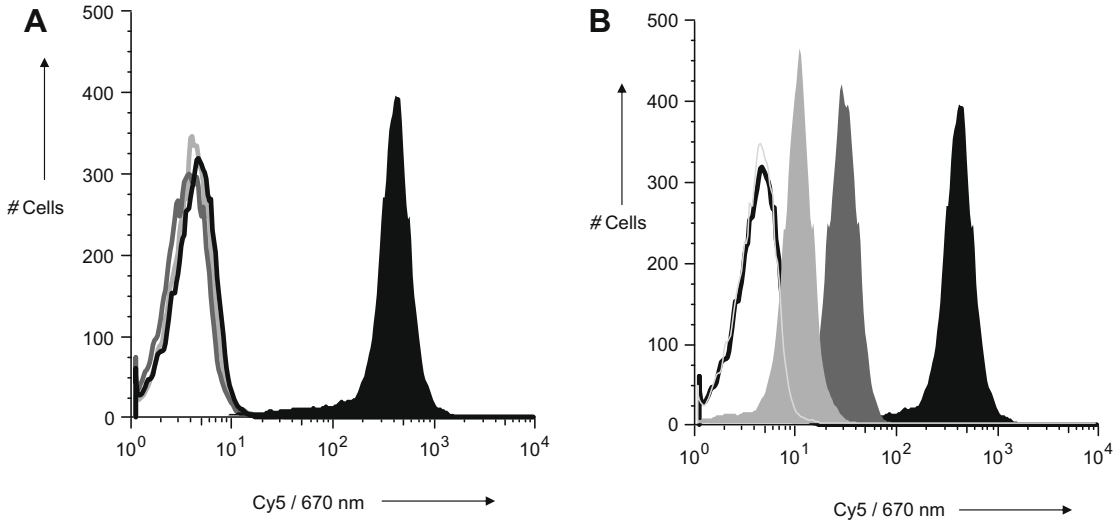


Figure 1. Synthetic Neu5Gc is metabolized by human THP-1 cells and displayed in terminal positions on the cell glycocalyx. (A) Synthesized Neu5Gc can be metabolized and displayed on terminal positions of the cell glycocalyx of human cells. THP-1 cell, a human monocytic cell line that cannot synthesize Neu5Gc, was fed with synthesized Neu5Gc, Neu5Ac, or no additional glycan. After three days the cells were fixed and stained for Neu5Gc using a primary IgY. Open black: IgY isotype control, closed black: Neu5Gc fed, open dark grey: Neu5Ac fed, open light grey: not fed. (B) The binding of anti-Neu5Gc IgY was specific for Neu5Gc. Binding of the IgY to cells fed with Neu5Gc could be blocked with 'free' Neu5Gc. Twenty millimolar 'free' Neu5Gc was sufficient to completely block IgY binding. Solid black: no 'free' Neu5Gc, solid dark grey: 0.2 mM 'free' Neu5Gc, solid light Grey: 2 mM free Neu5Gc, open light grey: 20 mM free Neu5Gc, open black: IgY isotype control.

Neu5Ac proliferated in a comparable manner to unfed cells) indicating that the synthesized material contained no trace amounts of cytotoxic materials.

In summary, we have produced a chemo-enzymatic synthesis of the human carbohydrate antigen Neu5Gc. The synthetic route is quick and allows access to gram-scale quantities in high yield and purity. Given the uncertain future of commercial sources and the known Neu5Ac contamination issue, this methodology provides an alternative means to access this important sugar. We have also demonstrated that the synthesized Neu5Gc is appropriate for use in sterile in vitro conditions and can be loaded into the glycocalyx of human cells in vitro. We have also used it within in vivo models with no reported atypical effects (unpublished data). We envisage that the synthesis is flexible enough to allow a wide range of ManNGc or Neu5Gc derivatives to be made which may prove useful to other areas of glycobiology research.

1. Experimental

1.1. General methods

'Petrol' refers to the fraction of petroleum ether in the boiling range of 35–60 °C. 'Brine' refers to a saturated aqueous solution of sodium chloride. Proton nuclear magnetic resonance (δ_H) was recorded on a Jeol ECA 500 (500 MHz). Five hundred megahertz spectra were assigned using COSY. All chemical shifts are quoted on the δ-scale in ppm, using residual solvent as the internal standard. Low resolution mass spectra were recorded on a Micromass Platform 1 spectrometer using electron spray (ES) ionization with MeOH as carrier solvent. Flash column chromatography was performed using sorbsil C60 40/60 silica gel. Thin-layer chromatography (TLC) was performed using Merck Kieselgel 60F254 pre-coated aluminium-backed plates. Plates were visualized using UV Lamp (λ_{max} = 354 or 365 nm) or 5% sulfuric acid in MeOH. CH_2Cl_2 was dried over molecular beads (8–12 mesh). Other anhydrous solvents were purchased from Fluka and Sigma.

1.2. Benzyl D-glucopyranoside (2)[19]

Glucose (68 g, 0.38 mmol) was slurried in benzyl alcohol (400 mL, 3.7 mol). Acetyl chloride (35 mL, 0.45 mmol) was added slowly over 10 min. Reaction mixture was heated to 50 °C and stirred for 48 h, after which TLC (EtOAc/MeOH 9:1) showed significant consumption of starting material (R_f 0.0) and formation of the product (R_f 0.4). The reaction mixture was dried by vacuum distillation (110 °C). The resulting crude product **2** was recrystallized from acetonitrile to yield a mixture of anomers as a white amorphous material (88 g, 86%).

^{1}H NMR (CD_3OD, 500 MHz) (assigned for the major anomer; alpha) δ = 3.23–3.36 (m, 2H, H-6, H-6′), 3.39 (dd, 1H, $J_{1,2}$ 3.7 Hz, $J_{2,3}$ 9.7 Hz, H-2), 3.64–3.69 (m, 2H, H-3, H-5), 3.76 (dd, J 2.3 Hz, J 11.8 Hz, 1H, H-4), 4.52 (d, 1H, $J_{a,b}$ 11.7 Hz, PhH$_a$H$_b$O), 4.74 (d, 1H, $J_{a,b}$ 11.7 Hz, PhH$_a$H$_b$O), 4.86 (d, 1H, $J_{1,2}$ 3.7 Hz, H-1), 7.23–7.41 (m, 5H, Ph). m/z (ESI$^+$) 288 (M+NH$_4^+$), 100%), 293 (M+Na$^+$, 35%). HRMS m/z (ES$^-$) Calcd for $C_{13}H_{18}O_6$ (M−H) 269.1020. Found 269.1019.

1.3. Benzyl 4-6-*O*-(phenylmethylene)-D-glucopyranoside (3)[20]

1-Benzyl-D-glucopyranoside (17 g, 63 mmol) was slurried in acetonitrile (150 mL) and benzyl-dimethylacetal (40.5 g,

0.26 mol). (+)-Camphor-10-sulfonic acid (154 mg, 0.67 mmol) was added and the reaction mixture was stirred at room temperature for three hours after which TLC (100% EtOAc) showed complete consumption of the starting material (R_f 0.3) and the formation of the product (R_f 0.8). The reaction was quenched with Et_3N (1.8 mL, 17.8 mmol) and the reaction mixture was dried under vacuum. The crude product was purified by flash silica chromatography (Petrol/EtOAc 1:1) to yield the product **3** as a white gum (18.9 g, 84%).

^{1}H NMR ($CDCl_3$, 500 MHz) (assigned for the major anomer; alpha) δ = 3.49 (at, 1H, *J* 9.5 Hz, H-4), 3.63 (dd, 1H, $J_{1,2}$ 4.0 Hz, $J_{2,3}$ 9.2 Hz, H-2), 3.71 (at, 1H, *J* 10.4 Hz, H-6′), 3.83 (atd, 1H, *J* 5.2 Hz, 10.0 Hz, H-5), 3.95 (at, 1H, *J* 9.2, H-3), 4.21 (dd, 1H, $J_{5,6'}$ 4.3 Hz, $J_{6,6'}$ 10.3 Hz, H-6′), 4.56 (d, 1H, $J_{a,b}$ 11.8 Hz, PhH$_a$*H*$_b$O), 4.67 (d, 1H, $J_{a,b}$ 11.7 Hz, Ph*H*$_a$H$_b$O), 5.01 (d, 1H, $J_{1,2}$ 4.0 Hz, H-1), 5.51 (s, 1H, PhC*H*), 7.30–7.40 (m, 10H, 2 Ph). *m/z* (ESI$^+$) 359 (M+H$^+$, 70%), 376 (M+NH$_4^+$, 70%). HRMS *m/z* (ES$^+$) Calcd for $C_{20}H_{22}O_6$ (M+H$^+$) 359.1411. Found 359.1413.

1.4. Benzyl 2-azido-2-deoxy-4,6-*O*-(phenylmethylene)-D-mannopyranoside (4)[21]

Pyridine (8.8 g, 111.6 mmol) was dissolved in dry CH_2Cl_2 (300 mL) followed by addition of trifluoromethanesulfonic anhydride (8.6 g, 30.7 mmol) and cooled to −30 °C. 1-Benzyl, 4-6-*O*-(phenylmethylene)-D-glucopyranoside (10 g, 27.9 mmol) was dissolved in CH_2Cl_2 (250 mL) and added dropwise over 30 min under anhydrous conditions to the cooled reaction mixture. After 2 h TLC (Petrol/EtOAc 4:1) showed almost complete consumption of the starting material (R_f 0.0) and the formation of the unisolated intermediate (R_f 0.7). The reaction was quenched with brine and the organic layer was dried with sodium sulfate, filtered and concentrated under vacuum (<30 °C). The crude intermediate (oil) was dissolved in dry dimethylformamide (500 mL) and sodium azide (5.4 g, 83.7 mmol) was added to the solution. The reaction mixture was heated to 50 °C and stirred under argon for 16 h, after which time TLC (Petrol/EtOAc 10%) showed the formation of the product (R_f 0.4). The reaction was quenched with excess water and the product was extracted with diethyl ether, dried over magnesium sulfate, filtered and concentrated under vacuum. The crude product was purified by flash silica column chromatography (Petrol/EtOAc 9:1) to yield the product **4** as a clear/colourless oil (7.7 g, 72%).

^{1}H NMR (assigned for the major anomer; alpha) ($CDCl_3$, 500 MHz) δ = 3.81 (m, 2H, H-5, H-6′), 3.91 (at, 1H, *J* 9.5 Hz, H-4), 4.02 (dd, 1H, $J_{1,2}$ 1.4 Hz, $J_{2,3}$ 4.0 Hz, H-2), 4.22 (m, 1H, H-6), 4.32 (dd, 1H, $J_{2,3}$ 3.9 Hz, $J_{3,4}$ 9.6 Hz, H-3), 4.50 (d, 1H, $J_{a,b}$ 11.7 Hz, PhCH$_a$*H*$_b$O), 4.70 (d, 1H, $J_{a,b}$ 11.7 Hz, PhC*H*$_a$H$_b$O), 4.88 (d, 1H, $J_{1,2}$ 1.3 Hz, H-1), 5.58 (s, 1H, PhC*H*), 7.30–7.40 (m, 10H, 2 Ph). *m/z* (ESI$^+$) 384 (M+H$^+$, 100%), 401 (M+NH$_4^+$, 65%), 406 (M+Na$^+$, 35%).

1.5. 2-Amino-2-deoxy-D-mannose hydrochloride (5)

1-Benzyl-2-azido-2-deoxy-4,6-*O*-(phenylmethylene)-D-mannopyranoside (3.3 g, 8.6 mmol) was dissolved in MeOH (100 mL) and 2 M HCl (aq) (8.6 mL, 17.2 mmol of HCl) was added. $Pd(OH)_2/C$ (825 mg) was added to the reaction mixture. The reaction mixture was saturated with H_2 (g) and stirred for 48 h under H_2 (g) after which time TLC (EtOAc/MeOH 7:3) showed complete consumption of the starting material (R_f 1.0) and the formation of the product (R_f 0.3). The reaction mixture was filtered through Celite and washed with MeOH. The clear/colourless solution was concentrated under vacuum. The product was dissolved in water (50 mL) and washed with Et_2O (3 × 50 mL). The aqueous layer was dried under vacuum to yield the product **5** as a clear/colourless gum (1.6 g, 89%).

^{1}H NMR (D_2O, 500 MHz) (assigned for the major anomer) δ = 3.31 (ddd, 1H, $J_{5,6'}$ 2.3 Hz, $J_{5,6}$ 5.7 Hz, $J_{4,5}$ 10.0 Hz, H-5), 3.38 (at, 1H, *J* 9.5 Hz, H-4), 3.56 (dd, 1H, $J_{1,2}$ 1.4 Hz, $J_{2,3}$ 4.6 Hz, H-2), 3.60 (dd, 1H, $J_{5,6}$ 5.3 Hz, $J_{6,6'}$ 12.3 Hz, H-6), 3.75 (dd, 1H, $J_{5,6'}$ 2.3 Hz, $J_{6,6'}$ 12.3 Hz, H-6′), 3.83 (dd, 1H, $J_{2,3}$ 4.9, $J_{3,4}$ 9.4, H-3), 5.01 (d, 1H, $J_{1,2}$ 1.8 Hz, H-1). *m/z* (ESI$^+$) 180 (M+H$^+$, 90%). HRMS *m/z* (ES$^+$) Calcd for $C_6H_{14}NO_5$ (M+H$^+$) 180.0866. Found 180.0865. Spectral data were in agreement with a commercial standard (mannosamine standard purchased from Sigma).

1.6. 2-Deoxy-2-[(hydroxyacetyl)amino]-D-Mannose (6)[9j]

2-Amino-2-deoxy-D-mannopyranoside (1.4 g, 6.5 mmol) was dissolved in water (60 mL) with sodium bicarbonate (10.8 g, 130 mmol) and cooled in an ice bath. Acetoxyacetyl chloride (4.2 mL, 39 mmol) was added slowly to the reaction mixture. After 30 min TLC (EtOAc/MeOH 7:3) showed complete consumption of the starting material (R_f 0.0) and the formation of the product (R_f 0.4). The reaction mixture was neutralized with mixed bed resin and concentrated under vacuum to yield **6** as a white gum (1.0 g, 94%).

^{1}H NMR (D_2O, 500 MHz) (assigned for the major anomer) δ = 3.30 (m, 1H, H-5), 3.45 (at, 1H, *J* 10.0 Hz, H-4), 3.58 (dd, 1H, $J_{5,6}$ 5.2 Hz, $J_{6,6'}$ 12.0 Hz, H-6), 3.69–3.73 (m, 2H, H-2, H-6′), 3.78 (m, 1H, H-3), 4.02 (s, 2H, COC*H*$_2$OH), 4.91 (d, 1H, $J_{1,2}$ 1.4 Hz, H-1). *m/z* (ESI$^+$) 260 (M+Na$^+$, 100%). HRMS *m/z* (ES$^+$) Calcd for $C_8H_{15}NO_7$Na (M+Na$^+$) 260.0741. Found 260.0742.

1.7. *N*-Glycolylneuraminic acid (1)[9a]

2-Deoxy-2-[(hydroxyacetyl)amino]-D-mannopyranose (2.4 g, 9.68 mmol), sodium pyruvate (24.8 g, 48.34 mmol) and pyruvate lyase (1553 μL, 33.76 mg/mL, from *P. multocida* (PmNanA), plasmid gifted from Dr. Xi Chen) were dissolved in Tris–HCl (524 mL, 100 mM) and pH was confirmed to be 7.5. The reaction mixture was incubated at 37 °C with shaking for 20 h. TBA analysis was used to confirm formation of the product, and predicted a 50% conversion. The reaction solution was passed through a Dowex-50 column (diameter = 2.5 cm, height = 15 cm). The column was washed with water (5 × 75 mL). The combined eluent and washes were passed though an AG 1 × 8 ion exchange column (diameter = 2.5 cm, height = 100 cm). The column was washed with 10 mM formic acid (7 × 500 mL). The product was eluted from the column with 1 M formic acid (approx 1 L). The clear/colourless eluent was concentrated under vacuum to yield the product as a white gum (1.6 g, 50%).

^{1}H NMR (D_2O, 500 MHz) (assigned for the major anomer) δ = 1.73 (t, 1H, *J* 11.7 Hz, H-3), 2.17 (dd, 1H, $J_{3',4}$ 4.9 Hz, $J_{3,3'}$ 13.2 Hz, H-3′), 3.38 (t, 1H, *J* 9.2 Hz, H-7), 3.46 (dd, 1H, $J_{8,9}$ 6.3 Hz, $J_{9,9'}$ 12.0 Hz, H-9), 3.59–3.62 (m, 1H, H-8), 3.68 (dd, 1H, $J_{8,9'}$ 2.5 Hz, $J_{9,9'}$ 11.7 Hz, H-9′), 3.85 (t, 1H, *J* 10.3 Hz, H-5), 3.98–4.07 (m, 2H, H-4, H-6), 4.00 (s, 2H, COC*H*$_2$OH). *m/z* (ESI$^+$) 324 (M−H$^+$, 100%).

1.8. Neu5Gc/Neu5Ac feeding

THP-1 cells were grown in Dulbecco's Modified Eagle's Medium with 5% human serum. Freshly passaged cells were incubated with 1.9 mM Neu5Gc or Neu5Ac, or just media for three days. Cells were lifted from culture flasks using 2 mM ethylenediaminetetraacetic acid (EDTA, 10–15 min, rt) and immediately pelleted (centrifuged 1400 rpm) and the supernatant was removed. The cell pellet was resuspended in 3% paraformaldehyde (20 min, rt). After fixing, cells were pelleted, then resuspended in 1% fish gelatin (sigma) in phosphate-buffered saline (PBS) (blocking buffer). Once prepared 400,000 cells were used for each staining reaction at 4 °C. The cell

pellet was resuspended in 100 μL of either affinity-purified chicken anti-Neu5Gc antibody[17] or a control chicken IgY (Jackson ImmunoResearch, West Grove, PA) diluted 1:5000 in blocking buffer and incubated for 20 min at 4 °C. Cells were pelleted, then washed with 500 μL of blocking buffer and pelleted. Cells were resuspended in 100 μL Cy5-conjugated Donkey-anti-chicken IgY (Jackson ImmunoResearch, West Grove, PA), diluted 1:1000 in blocking buffer, incubated for 20 min at 4 °C in the dark, then washed as mentioned above. The stained cells were resuspended in 500 μL PBS, the data collected on a FACSCalibur (BD Biosciences Immunocytometry Systems, San Jose, CA) and analyzed with Flowjo software (Tree Star, Ashlan, OR). Competition assays were done by addition of 'free' Neu5Gc during the primary antibody staining step.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2010.04.003.

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