Chemo-enzymatic synthesis of the carbohydrate antigen N-glycoly neuraminic acid from glucose

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N-Glycoly neuraminic 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.

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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-glycoly neuraminic acid (Neu5Gc) is a non-human sialic acid, which is biosynthesized from N-acetylenuraminic 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 tumourigenesis6 and vascular pathologies7 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 benzylxyoxyacetic acid derivative followed by hydrogenation to yield 2-deoxy-2-[(hydroxyacetyl)amino]-o-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 manifold 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-o-glucosamine, via two immobilized enzymes, N-acetyl-o-glucosamine 2-epimerase and N-acetyl-o-neuraminic acid aldolase.10b 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
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 α-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 α-glucose with benzyl alcohol in the presence of acetyl chloride produced benzyl α-glucopyranoside 2 in 86% yield, which was further modified to produce the 4,6-benzylidene derivative 3 in 84% yield, by reaction with benzaldehyde dimethylacetyl chloride, sodium bicarbonate, water, 94%.

Conversion of ManNGc to Neu5Gc using this methodology has previously been reported on a milligram scale. 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. 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 glyocalyx 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 IgY15 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 showed incorporation of Neu5Gc into the cell surface.

Table 1

<table>
<thead>
<tr>
<th>Entry</th>
<th>Starting material</th>
<th>Catalyst</th>
<th>Solvent</th>
<th>Yield% (ND)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>Pd/C</td>
<td>MeOH</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>Pd(OH)_{2}/C</td>
<td>MeOH</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>Pd(OH)_{2}/C</td>
<td>MeOH</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>Pd(OH)_{2}/C</td>
<td>MeOH/HCl (aq)</td>
<td>100</td>
</tr>
</tbody>
</table>

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

Scheme 1. Retrosynthetic analysis of Neu5Gc synthesis reveals glucose as a suitable starting material. P = protecting group.
Neu5Ac proliferated in a comparable manner to un-fed 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 (\(\ddagger\)) was recorded on a Jeol ECA 500 (500 MHz). Five hundred megahertz of sodium chloride. Proton nuclear magnetic resonance (\(\ddagger\)) range of 35–60

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 monocyctic 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.

Figure 3. Enzymatic synthesis of Neu5Gc from ManNGc.
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 (Rf 0.3) and the formation of the product (Rf 0.8). The reaction was quenched with Et3N (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%).

1H NMR (CDCl3, 500 MHz) (assigned for the major anomer; alpha) δ = 3.49 (at, 1H, J = 11.7 Hz, H-5), 3.71 (at, 1H, J = 10.0 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 = 4.3 Hz, J = 10.3 Hz, H-6), 4.56 (d, 1H, J = 11.8 Hz, PhH2O), 4.67 (d, 1H, J = 11.7 Hz, PhH2O), 5.01 (d, 1H, J = 4.0 Hz, H-1), 5.51 (s, 1H, PhCH), 7.30–7.40 (m, 10H, 2 PhH) m/z (ESI+) 359 (M+H+), 70% 376 (M+Na+, 70%). HRMS m/z (ESI+) Calcd for C30H2O6Na (M+Na+) 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 CH2Cl2 (300 mL) followed by addition of trifluoromethanesulfonic anhydride (8.6 g, 30.7 mmol) and cooled to –30 °C. 1-Benzyl-4,6-dideoxy-4,6-O-(phenylmethylene)-d-glucopyranose (10 g, 27.9 mmol) was dissolved in dry dimethylformamide (500 mL) and stirred under argon for 40 min. Sodium azide (5.4 g, 83.7 mmol) was added to the solution. The reaction mixture was diluted and concentrated under vacuum (<30 °C) and the starting material (40%, 65%), 406 (M+Na+), 359 (M+H+) as a white gum (1.0 g, 94%).

1H NMR (CDCl3, 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 = 5.2 Hz, J = 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, COCH2OH), 4.91 (d, 1H, J = 14.0 Hz, H-1), m/z (ESI+) 260 (M+Na+, 100%). HRMS m/z (ESI+) Calcd for C18H13NO5Na (M+Na+) 260.0741. Found 260.0742.

1.7. N-Glycopolymannosamine acid (3)20

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 (PmNaA), 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 through 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/clearless eluent was concentrated under vacuum to yield the product as a white gum (1.6 g, 50%).

1H NMR (D2O, 500 MHz) (assigned for the major anomer) δ = 1.73 (t, 1H, J = 11.7 Hz, H-3), 2.17 (dd, 1H, J = 4.9 Hz, J = 13.2 Hz, H-3), 3.38 (t, 1H, J = 9.2 Hz, H-7), 3.46 (dd, 1H, J = 6.3 Hz, J = 12.0 Hz, H-9), 3.59–3.62 (m, 1H, H-8), 3.68 (dd, 1H, J = 2.5 Hz, J = 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, COCH2OH), m/z (ESI+) 324 (M+H+, 100%).
pellet was resuspended in 100 μL of either affinity-purified chicken anti-Neu5Gc antibody 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, 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.

References