## Supplementary Information

# Hijacking a biosynthetic pathway yields a glycosyltransferase inhibitor within cells

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**Contents of the Supplementary Information.**

**Supplementary Methods**

***Chemoenzymatic synthesis of UDP-5SGlcNAc (4):*** Inorganic pyrophosphatase (PPA), ATP and UTP were purchased from Sigma. GNK, AGM, and AGX1 were buffer exchanged into 50 mM Tris, pH 7.5, 2 mM $MgCl_2$ using PD10 columns (GE Healthcare). GDP-Glc (68 μM) was included as an internal standard for CE analysis. A one-pot reaction consisting of 2.5 mM ATP, 1 mM UTP, 0.6 mM 5SGlcNAc (**3**), 0.4 μM GNK, 2.9 μM AGM, 0.3 μM AGX and 50 mU/mL PPA was incubated for 14 h at 37 °C. The reaction mixture was treated for an additional 4 h at 37 °C with calf alkaline phosphatase (Roche). The reaction was monitored by CE (see below). Epimerization of UDP-5SGlcNAc (**4**) to UDP-5SGalNAc was carried out using UDP-GlcNAc 4-epimerase (14 h at 37 °C).

***Purification of UDP-5SGlcNAc (4):*** Enzymes were removed by passing the mixture through a centrifugal filtration device (10 kDa molecular weight cut-off; Centricon). The filtrate containing the desired product was desalted using a column containing Dowex AG 1-X4 ion exchange resin (BioRad; 7 mL bed volume) converted into the formate form and pre-equilibrated in water. After loading, the column was washed with 10 bed volumes of $H_2O$ and 10 bed volumes of 4 M formic acid, and eluted with 10 bed volumes of 550 mM ammonium formate, pH 4.0[1]. Fractions containing UDP-5SGlcNAc (**4**) were concentrated *in vacuo* and further purified by HPLC on a Hewlett Packard series 1100 instrument, equipped with an Eclipse XDB-C18 (5 μm, 9.4 x 250 mm) column (Agilent Technologies), using ion-paired conditions (adapted from Ref. 2). Compounds were detected by monitoring the UV absorbance at 254 nm and UV-active peaks were assessed for purity by HPLC and CE. Fractions containing the desired product were pooled and lyophilized.

***Capillary electrophoresis (CE):*** CE was performed on a ProteomeLab PA800 (Beckman-Coulter) using fused silica capillaries of 50 μM internal diameter x 44 cm (to detector). The running buffer was 40 mM $Na_2B_4O_7$ (Sigma), pH 9.5, containing 1.0% (w/v) polyethylene

glycol (MW 20,000; Fluka) and was filtered prior to use. The capillary was conditioned by washing with 1 N NaOH (2 min, 20 psi), 18 MΩ $H_2O$ (3 min, 20 psi) and running buffer (5 min, 40 psi). After injecting a short (15 nL) $H_2O$ plug, samples were electrostatically introduced according to an established field-amplified sample injection technique[3]. Electrophoresis was carried out at a constant voltage of 30 kV and capillary temperature of 22 °C. Electropherograms were derived by measuring the absorbance at 254 (+/- 10) nm at a rate of 4 Hz. Peaks were integrated using 32 Karat 5.0 software (Beckman-Coulter) and all peaks were normalized to the GDP-Glc internal standard.

***OGT transfer:*** The ability of OGT (over-expressed and purified as described in Ref. 4) to transfer UDP-5SGlcNAc (**4**) was tested using recombinant nup62 as the acceptor. Assays contained 30 μM nup62, 20 μM UDP-5SGlcNAc (**4**) or UDP-GlcNAc (**2**) and 0.4 μM OGT in phosphate buffered saline (PBS), in a total volume of 100 μL, and were allowed to proceed for an appropriate time between 10 min and 2 h at 37 °C in order to maintain a constant rate without substrate depletion. Reactions were quenched by the addition of an equal volume of ethanol, frozen at -20 °C for 1 h to precipitate proteins and centrifuged at 13,000 rpm for 20 min. 5 μM GDP-Glc, an internal standard, was added prior to freezing. The supernatant was removed and lyophilized. Nucleotides and nucleotide sugars were extracted as described below for the cell lysates, resuspended in 200 μL $H_2O$, and analyzed by CE. Production of UDP was monitored at 254 nm; the concentration was determined from a standard curve of UDP standards, which were prepared in triplicate using the same procedure as for the reactions, at a concentration of between 1 and 20 μM. Controls in the absence of nup62 and OGT were subtracted to account for hydrolysis of the UDP-sugars.

***OGT inhibition:*** The ability of 5SGlcNAc (**3**) and UDP-5SGlcNAc (**4**) to inhibit OGT activity was assessed using radiolabelled UDP-[$^3$H]-GlcNAc (American Radiolabel) as the donor and recombinant nup62 as the acceptor. Assays contained 18 μM of nup62, 1 μM of

UDP-GlcNAc (**2**, constant specific activity of 0.5 Ci/mmol of UDP-[$^3$H]-GlcNAc), 100 nM OGT, and either various concentrations (0-25 μM) of UDP-5SGlcNAc (**4**) or 1 mM 5SGlcNAc (**3**) in PBS. The $V_{max}$ for OGT was determined using high (30, 40 and 50 μM) concentrations of UDP-GlcNAc (**2**, containing 0.033, 0.025, 0.02 Ci/mmol specific activity, respectively). Assays were carried out essentially as described previously[4] and the $K_i$ determined by Dixon analysis.

***Western/lectin blots:*** Samples were electrophoresed through 10% SDS polyacrylamide gels and transferred onto nitrocellulose membranes (Biorad). Membranes were blocked for 1 h in PBS with 0.1% Tween-20 (PBS-T) containing 1% (unless otherwise stated) bovine serum albumin (BSA), and probed with the appropriate primary antibody or lectin in 1% BSA in PBS-T overnight at 4 °C. Blots were washed for 1 h with PBS-T, and blocked for a further 30 min with 1% BSA in PBS-T at room temperature. They were then probed with the appropriate horseradish peroxidise (HRP)-conjugated secondary antibody/probe in 1% BSA in PBS-T at room temperature for 1 h, followed by washing in PBS-T for 1 h. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to CL-XPosure film (Pierce). Densitometry was performed using ImageQuant 5.2 (Molecular Dynamics), and fits to the data were made using GRAFIT[5].

***Antibodies/lectins:*** *O*-GlcNAc levels were assessed primarily using CTD110.6 (Covance), but RL2 (Abcam) and HGAC85 (Abcam) were also tested. Actin was probed using JLA20 (Developmental Studies Hybridoma Bank). OGA levels were assessed using a polyclonal anti-OGA antibody and OGT levels using H-300 (Santa Cruz Biotechnology). nup62 was probed using mAb 414 (Covance) and Sp1 using anti-Sp1 (PEP2) (Santa Cruz Biotechnology). Secondary antibodies employed (HRP-conjugated goat anti-mouse IgM, goat anti-mouse IgG, goat anti-chicken IgY and goat anti-rabbit IgG) were obtained from Santa Cruz Biotechnology. Biotinylated-ConA, PHA-L, SNA, and MAA were purchased from EY

Laboratories, and biotinylated-GNA from Vector Laboratories. HRP-conjugated streptavidin (Pierce) was used as a probe for the biotinylated lectins and for ligating biotin phosphine.

***Immunocytochemistry:*** Following removal of media, cells were fixed in 4% paraformaldehyde at 37 °C for 12 min and washed 3 times with PBS for 5 min each. Cells were permeabilized using 0.3% Triton X-100 in PBS-T at rt for 30 min and blocked with 10% normal goat serum (NGS) and 5% BSA in PBS-T at room temperature for a further 30 min. Cells were probed with primary antibody (CTD110.6) overnight at 4 °C, washed 3 times with PBS for 5 min each, and then incubated with secondary antibody (donkey anti-mouse IgM, conjugated to FITC) in the dark, at room temperature, for 1.5 h. Cells were washed again in PBS (3 times, 5 min each), coverslipped with Vectashield Mounting Medium with DAPI (Vector Laboratories) and mounted onto slides. Cells were imaged using a Leica DM4000B fluorescent microscope with appropriate filter sets for FITC and DAPI.

***Cell growth curves:*** CHO and EMEG$^{+/-}$ cells were seeded at an approximate initial density of 15,000 cells/mL, in a volume of 1 mL, and allowed to settle for 8 hours. Following this they were treated with 50 μM Ac-5SGlcNAc (**5**), 50 μM Ac-GlcNAc (**6**), or vehicle. Cells were then counted and approximately every 24 hours afterwards for 5 days. Each time, cells were washed with PBS then 100 μL trypsin was added and incubated at 37 °C for 3 minutes. Trypsinization was stopped upon the addition of 100 μL media and the cells were resuspended. 10 μL of the cells were added to an appropriate dilution of trypan blue, and counted using a haemocytometer. All measurements were performed in triplicate.

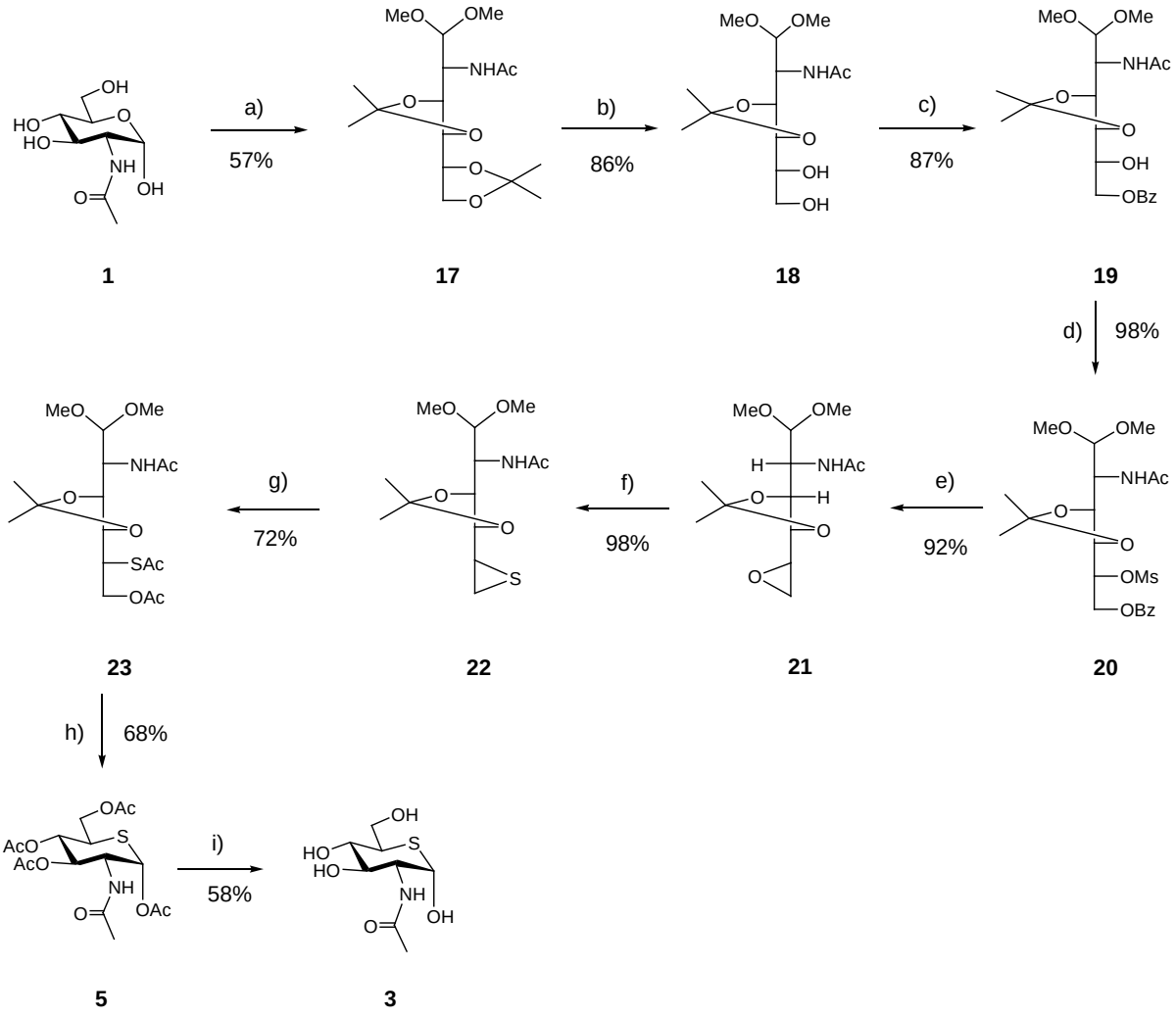
***Click-iT® kit for labelling O-GlcNAc residues:*** The 'Click-iT® *O*-GlcNAc Enzymatic Labeling System' was purchased from Invitrogen, and essentially the manufacturer's protocol was followed with some alterations. nup62 was immunoprecipitated and eluted from the beads in 1% SDS in 20 mM Hepes, pH 7.9, and boiled for 10 min; this was used as the substrate for the chemoenzymatic labeling using UDP-GalNAz and GalT1. Following the

overnight reaction, biotinylated phosphine (in 20% DMF) was added at a final concentration of 50 μM and incubated at 37 °C for 90 min. The reaction was stopped by the addition of SDS-PAGE loading buffer, and the samples were boiled for 10 min. The same procedure as described for the western blots was used to analyze the samples, starting from the second blocking step. Blots were then probed with HRP-conjugated streptavidin (Pierce).

***Extraction of nucleotide sugars from cell lysates:*** COS-7 cells were treated with Ac-5SGlcNAc (**5**, 0 to 1000 μM) for 24 h in triplicate. Cells were washed gently with PBS prior to the addition of 1 mL PBS containing 10 mM EDTA. Cells were incubated at 37 °C for 4 min, the cells scraped from the plate, and pelleted by centrifugation (1000 rpm, 10 min, 4 °C). Cell pellets were immediately lysed by the addition of 750 μL 75% (v/v) ethanol (-20 °C) and sonicated using a W-375 ultrasonic processor (Heat Systems Ultrasonics, Inc.). Insoluble material was removed by centrifugation (14,000 rpm, 15 min, 4 °C) and the supernatant was snap frozen and lyophilized[6,7]. The crude extract was spiked with GDP-Glc (200 pmol) as an internal standard, dissolved in 0.5 mL 18 MΩ $H_2O$ and nucleotide sugars extracted using Envi-Carb graphite solid-phase extraction cartridges (200 mg) (Supelco) as described by Räbinä *et al*[2]. Briefly, Envi-Carb cartridges were conditioned with 80% (v/v) $CH_3CN$ and 20% 0.1% (v/v) TFA (3 mL), followed by 18 MΩ $H_2O$ (6 mL), before samples were applied. After passing the samples through the SPE cartridge, the cartridge was sequentially washed with 2 mL each of 18 MΩ $H_2O$, 25% (v/v) $CH_3CN$, and 50 mM triethylammonium acetate (TEAA), pH 7.0. Sugar nucleotides were eluted with 3 x 1 mL 25% $CH_3CN$ in 50 mM TEAA, pH 7.0, passed through a 0.22 μm filter (Millipore), lyophilized, and stored at -20 °C until analysis. Extracted sugar nucleotides were dissolved in 200 μL 18 MΩ $H_2O$ and an aliquot was diluted 1:4 prior to characterization by CE using a method adapted from Feng *et al*[8].

***Synthesis of Ac-5SGlcNAc (5) and 5SGlcNAc (3).***

Synthesis of Ac-5SGlcNAc (**5**) and 5SGlcNAc (**3**) was carried out following literature procedures described in two papers with some modifications[1,2]. The synthetic scheme is outlined in Scheme 1 and the high field NMR data are reported since these were not previously reported in some cases. Details of the modified procedures and greater experimental details are also provided.




***Supplementary Scheme 1.*** Synthesis of Ac-5SGlcNAc (**5**) and 5SGlcNAc (**3**). a) 2,2-Dimethoxypropane, TsOH, dioxane, 80 °C, 24 h. b) 80% AcOH, 40 °C, 2 h. c) BzCl, py, -20 °C, 6h. d) MsCl, pyridine, 0 °C, 2 h. e) NaOMe, chloroform, -20→0 °C, 2 h. f) Thiourea, methanol, 60 °C, 2 h. g) Potassium acetate, AcOH, $Ac_2O$, 160 °C, 20 h. h) 1) 10:1 AcOH:10 M HCl, 40 °C, 24 h; 2) $Ac_2O$, py, 0 °C, 12 h. i) 1) MeOH, NaOMe, rt, 1 h; 2) AcOH, MeOH.

*2-Acetamido-2-deoxy-3,4,5,6-di-O-isopropylidine-aldehydo-D-glucose dimethyl acetal* (**17**)[9] – 15.0 g (67.8 mmol) of **1** was suspended in 1,4 dioxane (150 mL) at 80 °C. 2,2-dimethoxypropane (60 mL) was added to the suspension, together with 2.25 g (11.8 mmol) of *p*-toluene sulphonic acid. The reaction was stirred for 24 hours at 80 °C. The mixture was cooled and subsequently treated with enough potassium carbonate to obtain a neutral solution. The reaction was filtered and directly concentrated under high vacuum. The concentrated crude reaction was subjected to flash column silica chromatography using a solvent system of 1-2 % methanol in chloroform, affording the title compound as a caramel coloured viscous syrup (13.4 g, 57%). $^{1}$H-NMR (400 MHz, $CDCl_3$) $\delta$ (ppm) 5.86 (d, $J$ = 10.0 Hz, 1H), 4.45 (ddd, $J$ = 9.60, 6.00, 1.20 Hz, 1H), 4.35 (d, $J$ = 6.00 Hz, 1H), 4.23 (dd, $J$ = 8.00, 1.20 Hz, 1H), 4.14-4.04 (m, 2H), 3.95 (dd, $J$ = 8.40, 4.40 Hz, 1H), 3.61 ( t, $J$ = 8.00 Hz, 1H), 3.40 (s, 3H), 3.36 (s, 3H), 2.01 (s, 3H), 1.46 (s, 3H), 1.38 (s, 3H), 1.36 (s, 3H), 1.33 (s, 3H); $^{13}$C-NMR (100 MHz, $CDCl_3$) $\delta$ (ppm) 169.58, 109.93, 109.46, 103.46, 77.90, 77.60, 67.41, 54.68, 53.55, 49.05, 26.99, 26.93, 26.38, 25.24, 23.36.

*2-Acetamido-2-deoxy-3,4-O-isopropylidine-aldehydo-D-glucose dimethyl acetal* (**18**)[9] – 10.35 g (29.80 mmol) of **17** was dissolved in a solution of 80% acetic acid in water, and stirred at 40 °C for 2 hours. Upon completion of the reaction, the mixture was concentrated under high vacuum in small aliquots until the whole reaction mixture was concentrated to afford a thick oil. The reaction was dried directly under high vacuum until the acetic acid was removed. The crude reaction mixture was purified *via* flash column silica chromatography using a solvent system of increasing polarity, beginning at 7% methanol and increasing to 10% methanol in chloroform. The pure fractions were concentrated and dried on the high vacuum pump to afford the title compound as a yellow syrup. (8.55 g, 86 %). $^{1}$H-NMR (500 MHz,

$CDCl_3$) $\delta$ (ppm) 6.32 (d, $J$ = 9.67 Hz, 1H), 4.42 (d, $J$ = 6.98 Hz, 1H), 4.34 (t, $J$ = 9.68 Hz, 1H), 4.23 (d, $J$ = 9.48 Hz, 1H), 3.73 (dd, $J$ =11.18, 3.08 Hz, 1H), 3.60 (m, 3H), 3.36 (s, 3H), 3.27 (s, 3H), 2.02 (s, 3H), 1.33 (s, 3H); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$ (ppm) 171.23, 109.08, 102.64, 78.02, 76.21, 73.45, 64.17, 54.61, 52.48, 50.04, 26.96, 23.19.

*2-Acetamido-6-O-benzoyl-2-deoxy-3,4-O-isopropylidene-aldehydo-D-glucose dimethyl acetal* (**19**)[10] **–** To a stirred solution of **18** (7.70 g, 22.2 mmol) in pyridine, dry benzoyl chloride (3.14 g, 22.2 mmol) was added at -20 °C. The mixture was stirred for 6 hours between -10 to -5 °C. Upon reaction completion, the mixture was extracted with chloroform. The extract was washed two times with 2 M HCl, saturated sodium carbonate, and water. Each aqueous layer was back extracted with chloroform. The combined organic layers were dried with magnesium sulphate, filtered, and concentrated. The concentrated crude reaction mixture was purified *via* flash column silica chromatography using a solvent system of 1% methanol in chloroform with increasing polarity to 2% methanol in chloroform. The pure fractions were concentrated, dried, and then crystallized from ethyl acetate and hexanes to afford the title compound as white needles (7.68 g, 87 %). $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$ (ppm) 8.11 (dd, $J$ = 8.45, 1.33 Hz, 2H), 7.53 (t, $J$ = 7.49 Hz, 1H), 7.41 (t, $J$ = 8.18 Hz, 2H), 4.45 (m, 4H), 4.24 (d, $J$ = 10.1 Hz, 1H), 3.90 (m, 1H), 3.71 (t, $J$ = 8.71 Hz, 1H), 3.36 (s, 3H), 3.26 (s, 3H), 2.03 (s, 3H), 1.35 (s, 3H), 1.35 (s, 3H), 1.32 (s, 3H); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$ (ppm) 171.20, 166.62, 132.99, 129.82, 128.29, 109.07, 102.16, 79.03, 75.38, 71.62, 66.83, 54.67, 51.95, 50.36, 26.95, 26.93, 23.12.

*2-Acetamido-6-O-benzoyl-2-deoxy-3,4-O-isopropylidine-5-O-mesyl-aldehydo-D-glucose dimethyl acetal* (**20**)[10] – 6.52 g (16.4 mmol) of **19** was cooled to 0 °C in pyridine (14 mL). To this solution mesyl chloride (2.50 g, 20.8 mmol) was added. The reaction was kept at 0 °C

until judged complete (2 hours). The mixture was concentrated under high vacuum. The remaining residue was extracted with chloroform, and successively washed two times with 2 M HCl, saturated sodium carbonate solution and water. Each aqueous layer was back extracted with chloroform. All organic layers were combined, dried with magnesium sulphate, concentrated *in vacuo*, and pumped under high vacuum until residual pyridine was removed. The crude reaction mixture was purified *via* flash column silica chromatography in a solvent system of increasing polarity of 1-2% methanol in chloroform. Pure fractions were concentrated and dried on the high vacuum pump, giving rise to pure product as an amorphous white solid (7.64 g, 98 %). $^{1}$H-NMR (500 MHz, $CDCl_3$) $\delta$ (ppm) 8.12 (d, $J$ = 7.11 Hz, 1H), 7.60 (t, $J$ = 7.44 Hz, 1H), 7.48 (t, $J$ =8.12 Hz, 2H), 5.84 (d, $J$ =9.66 Hz, 1H), 5.14 (m, 1H), 4.80 (dd, $J$ =12.54, 2.64 Hz, 1H), 4.49 (dd, $J$ = 12.54, 7.10 Hz, 1H), 4.45 (m, 2H), 4.39 (m, 1H), 4.00 (dd, $J$ = 7.94, 6.23 Hz, 1H), 3.45 (s, 3H), 3.34 (s, 3H), 3.16 (s, 3H), 2.07 (s, 3H), 1.45 (s, 3H), 1.43 (s, 3H); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$ (ppm) 170.07, 166.10, 133.29, 129.91, 129.50, 128.48, 110.39, 103.18, 78.40, 76.78, 75.65, 63.55, 55.78, 52.79, 49.69, 38.89, 26.99, 26.90, 23.36.

*2-Acetamido-5,6-anhydro-2-deoxy-3,4-O-isopropylidene-aldehydo-L-idose dimethyl acetal* (**21**)[10] **–** To a solution of **20** (6.23 g, 13.1 mmol) in dry chloroform (62 mL) was added drop-wise at -20 °C a fresh solution of sodium methoxide (0.50 g Na in 20 mL of methanol). The reaction mixture was kept between -10 °C and 0 °C until the reaction was complete (5 hours). Upon completion, the reaction was treated with 102 mL of additional methanol. The reaction was neutralized with $H^+$ amberlite ion exchange resin (IR-120) until the reaction mixture reached a pH of 7. The resin was filtered and washed with additional methanol. The filtered reaction mixture was concentrated *in vacuo* and the remaining material triturated with ether and hexanes to afford a pure white amorphous solid (3.51 g, 92 %). $^{1}$H-NMR (500 MHz,

$CDCl_3$) $\delta$ (ppm) 5.91 (d, $J$ = 9.83 Hz, 1H), 4.37 (d, $J$ = 7.06 Hz, 1H), 4.18 (m, 2H), 3.36 (s, 3H), 3.35 (m, 1H), 3.25 (s, 3H), 2.98 (m, 1H), 2.80 (m, 1H), 2.75 (dd, $J$ = 5.13, 4.17 Hz, 1H), 1.97 (s, 3H), 1.35 (s, 3H), 1.32 (s, 3H); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$ (ppm) 170.03, 109.79, 102.72, 78.36, 76.00, 55.43, 52.20, 50.92, 48.67, 43.75, 26.88, 26.76, 23.17.

*2-Acetamido-2,5,6-trideoxy-5,6-epithio-3,4-O-isopropylidine-aldehydo-D-glucose dimethyl acetal* (**22**)[10] – To a solution of **21** (3.65 g, 12.64 mmol) in dry methanol (78 mL), was added thiourea (2.88 g, 37.87 mmol), and the mixture was stirred at 60 °C. Upon completion, the reaction mixture was cooled to room temperature and then concentrated *in vacuo*. The title compound was purified using a solvent system of 2% methanol and chloroform (3.77 g, 98 %). $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$ (ppm) 5.85 (d, $J$ = 9.43 Hz, 1H), 4.33 (m, 3H), 3.40 (s, 3H), 3.36 (s, 3H), 3.26 (t, $J$ = 7.94 Hz, 1H), 2.92 (m, 1H), 2.51 (d, $J$ = 6.03 Hz, 1H), 2.26 (d, $J$ = 5.19 Hz, 1H), 1.99 (s, 3H), 1.44 (s, 3H), 1.39 (s, 3H); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$ (ppm) 169.67, 109.95, 103.38, 81.91, 78.22, 55.38, 53.56, 49.39, 33.92, 27.04, 27.03, 22.96.

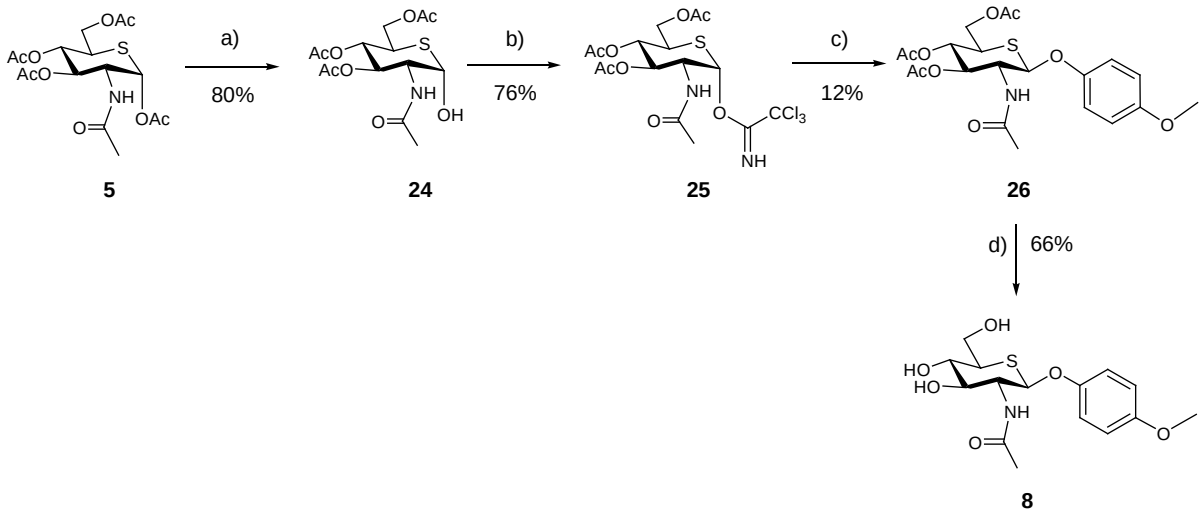
*2-Acetamido-6-O-acetyl-5-S-acetyl-2-deoxy-3,4-O-isopropylidine-5-thio-aldehydo-D-glucose dimethyl acetal* (**23**)[10]**.** A solution of **22** (1.02 g, 3.34 mmol), acetic anhydride (31 mL), potassium acetate (1.53 g, 15.6 mmol), and acetic acid (5 mL) was brought to reflux at 160 °C with stirring for 20 hours. After completion of the reaction, the mixture was cooled to room temperature and evaporated under high vaccum. The remaining residue was extracted with chloroform and successively washed with 2 M HCl, saturated sodium bicarbonate, and brine. Each aqueous layer was back extracted with chloroform. The pooled organic layers were dried with magnesium sulphate, and evaporated to a dark syrup. Flash column chromatography using a solvent system of 70% ethyl acetate/30% hexanes furnished the title compound as a white crystalline solid (0.980 g, 72 %).$^1$H-NMR (500 MHz, $CDCl_3$) $\delta$ (ppm)

5.83 (d, *J* = 9.69 Hz, 1H), 4.37 (m, 2H), 4.32 (d, *J* = 6.77 Hz, 1H), 4.26 (dd, *J* = 11.48, 5.11 Hz, 1H), 4.16 (d, *J* = 7.52 Hz, 1H), 3.96 (m, 1H), 3.82 (t, *J* = 7.90 Hz, 1H), 3.37 (s, 3H), 3.30 (s, 3H), 2.36 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 1.42 (s, 3H), 1.39 (s, 3H); $^{13}$C-NMR (100 MHz, $CDCl_3$) $\delta$ (ppm) 193.76, 170.51, 169.74, 109.77, 103.08,, 76.62, 63.78, 55.38, 52.57, 50.27, 44.94, 30.46, 27.03, 26.80, 23.27, 20.66.

*2-Acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-5-thio-α-D-glucopyranose* (**5**)[10] **–** In a solution of 10:1 acetic acid: 2 M HCl (77 mL), **23** (2.57 g, 6.31 mmol) was dissolved and the mixture was warmed to 40 °C for 48 hours, under an atmosphere of nitrogen. The reaction mixture turned a bright pink colour. Upon completion of the reaction, the mixture was concentrated without heating under high vacuum. The resulting dark brown residue was dried thoroughly under high vacuum until residual acid was removed and a syrup was obtained. The syrup was dissolved in acetic anhydride (13 mL) and pyridine (51 mL) and stirred overnight at 0 °C. Upon completion of the reaction, the mixture was concentrated under high vacuum without heating. The resulting syrup was subjected to flash column silica chromatography using a solvent system of 2-3% methanol in chloroform to give the title compound as an off-white solid that upon crystallization from ethyl acetate and hexanes gave rise to pure white needles (1.74 g, 68 % over two steps). $^{1}$H-NMR (500 MHz, $CDCl_3$) $\delta$ (ppm) 5.92 (d, *J* = 3.04 Hz, 1H), 5.70 (d, *J* = 8.86 Hz, 1H), 5.37 (dd, *J* =10.74, 9.64 Hz, 1H), 5.16 (dd, *J* = 10.89, 9.70 Hz, 1H), 4.63 (m, 1H), 4.34 (dd, *J* = 12.11, 4.95 Hz, 1H), 4.03 (dd, *J* =12.11, 3.15 Hz, 1H), 3.50 (ddd, *J* = 10.8, 4.80, 3.20, 1H), 2.17 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.90 (s, 3H); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$ (ppm) 171.73, 170.60, 169.52, 169.16, 168.77, 72.79, 71.78, 71.47, 61.10, 55.20, 39.75, 23.08, 21.14, 20.65, 20.53.

*2-Acetamido-2-deoxy-5-thio-α-D-glucopyranose* (**3**)[10] **–** In a solution of methanol (1.9 mL), 0.050 g (0.123 mmol) of **5** was dissolved and a catalytic amount of sodium methoxide (enough to raise the pH of the reaction solution to between 9 and 10) was added. The reaction was followed closely by TLC and was found to be complete after 1 hour at room temperature. The reaction was subsequently neutralized by the drop-wise addition of a dilute solution of acetic acid in methanol (pH 4) until the reaction solution was neutralized to pH 7. The reaction mixture was concentrated without heating under high vacuum. The final product was isolated by flash silica column chromatography using a solvent system of 12:2:1 ethyl acetate:methanol:water as a white solid (0.017 g, 58 %).$^{1}$H-NMR (600 MHz, MeOH-$d_4$) δ (ppm) 4.89 (d, *J* = 3.17 Hz, 1H), 4.07 (dd, *J* =10.50, 2.82 Hz, 1H), 3.87-3.79 (m, 2H), 3.65 (dd, *J* = 10.46, 8.82 Hz, 1H), 3.55 (dd, *J* = 10.37, 8.93 Hz, 1H), 3.25-3.22 (m, 1H), 1.96 (s, 3H); $^{13}$C-NMR (150 MHz, MeOH-$d_4$) *δ* (ppm) 173.35, 76.88, 73.52, 73.50, 62.65, 60.01, 44.89, 22.72.

**Synthesis of *p*MP-5SGlcNAc (8).**




***Supplementary Scheme 2.*** Synthesis of *p*MP-5SGlcNAc (**8**). a) Hydrazine acetate, DMF, rt, 6 h. b) Trichloroacetonitrile, DBU (cat.), DCM, -20 °C→room temperature, 1h. c) *p*-Methoxyphenol, $BF_3OEt_2$ (cat.), DCM, -20 °C→room temperature, 1h. d) 1) NaOMe (cat.) MeOH, room temperature, 0.5 h; 2) AcOH, MeOH.

*2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-5-thio-α-D-glucopyranose* (**24**) – Anomeric deacetylation was effected in a manner similar to that reported previously for different saccharides[11]. To a solution of **5** (0.200 g, 0.49 mmol) in DMF (1 mL), stirring at room temperature, hydrazine acetate (0.050 g, 0.54 mmol) was added. The mixture was left to stir until all starting material was consumed (6 hours). The mixture was concentrated under high vacuum, and co-evaporated twice with toluene, without heating. The resulting residue was taken up in ethyl acetate and washed two times with saturated sodium bicarbonate, water (until a neutral pH was obtained), and brine. Each aqueous layer was back extracted with ethyl acetate. The pooled organic layers were dried using magnesium sulphate, filtered, and concentrated *in vacuo*. The residue was subjected to flash column silica chromatography using a solvent system of 70% ethyl acetate, 30% hexanes, and subsequently concentrated

under high vacuum to afford the title compound as a white solid. Crystallization from ethyl acetate and hexanes afforded a white powder (0.144 g, 80 %).$^{1}$H-NMR (600 MHz, $CDCl_3$) $\delta$ (ppm) 6.14 (d, $J$ = 9.31 Hz, 1H), 5.36-5.27 (m, 2H), 5.03 (d, $J$ = 2.74 Hz, 1H), 4.55-4.51 (m, 1H), 4.36 (dd, $J$ = 11.99, 5.04 Hz, 1H), 4.10 (dd, $J$ = 12.00, 3.39 Hz, 1H), 3.67-3.64 (m, 1H), 2.08 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.97 (s, 3H); $^{13}$C-NMR (150 MHz, $CDCl_3$) δ (ppm) 171.31, 170.78, 169.97, 169.39, 72.78, 72.14, 71.69, 61.58, 55.42, 38.67, 23.19, 20.68, 20.64, 20.57. HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{14}H_{21}NO_8S$: 364.1066; found 364.1070.

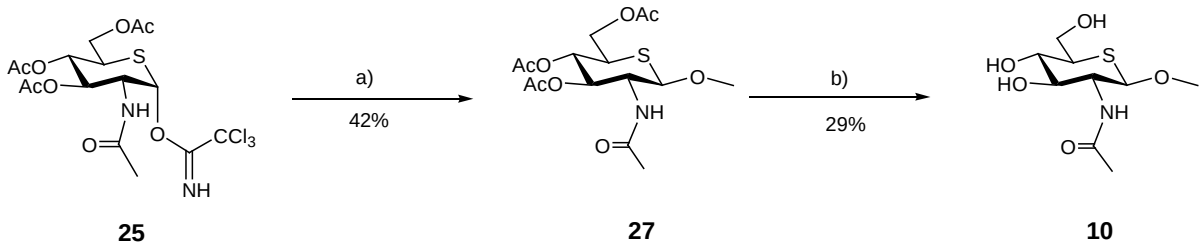
*2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-5-thio-α-D-glucopyranosyl trichloroacetimidate* (**25**) – Preparation of the trichloroacetmidiate donor was carried out by modification of previously described procedures used for different saccharides[12-15]. **24** was dissolved in dichloromethane at 0 °C, and to this solution was added trichloroacetonitrile (0.181 g, 1.27 mmol) and a catalytic amount of DBU (~1 drop). The reaction was allowed to stir at 0 °C until completion (0.5 hours). The mixture was diluted with benzene and concentrated under high vacuum to yield a residue. The crude mixture was subjected to flash column silica chromatography using a solvent system of 60% ethyl acetate in hexanes. The desired material was isolated as a clear oil, which upon standing, solidified to give a white amorphous solid (0.086 g, 76 %).$^{1}$H-NMR (600 MHz, $CDCl_3$) $\delta$ (ppm) 8.81 (s, 1H), 6.13 (d, $J$ = 3.00 Hz, 1H), 5.82 (d, $J$ = 8.80 Hz, 1H), 5.43 (dd, $J$ = 10.68, 9.79 Hz, 1H), 5.27 (dd, $J$ = 10.68, 9.78 Hz, 1H), 4.73-4.69 (m, 1H), 4.35 (dd, $J$ =12.14, 4.98 Hz, 1H), 4.11 (dd, $J$ = 12.11, 3.13 Hz, 1H), 4.07-4.05 (m, 1H), 2.05 (s, 3H), 2.05 (s, 3H), 2.05 (s, 3H), 1.91 (s, 3H); $^{13}$C-NMR (150 MHz, $CDCl_3$) $\delta$ (ppm) 170.42, 170.40, 169.51, 169.12, 159.83, 90.70, 77.73, 71.60, 71.22, 60.90, 55.71, 40.05, 22.98, 20.54, 20.52, 20.43. HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{16}H_{121}N_2O_8SCl_3$: 507.0162; found 507.0173.

*para-Methoxyphenyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-5-thio-β-D-glucopyranoside* (**26**) **–** Glycosylation was effected by adaptation of reported methods used for glycosylation of trichloroacetimidate donors of other saccharides[13,15]. A solution of **25** (0.050 g, 0.100 mmol) in dichloromethane (0.77 mL) and *p*-methoxyphenol (0.025 g, 0.20 mmol) was stirred at -20 °C. To this solution a catalytic amount of boron trifluoroetherate (~ 1 drop) was added, and the reaction was immediately allowed to warm to room temperature. The reaction was stirred for 1 h, after which time the reaction was judged complete. Two equivalents of triethylamine (0.026 mL) were then added to the reaction mixture. The mixture was concentrated under high vacuum without heating. Flash column silica chromatography was carried out to isolate the products and the $^{1}$H NMR spectrum revealed a mixture of two products; the α- and β-anomers in a ratio of 70% α and 30% β. The two anomers were separated *via* flash column chromatography, using a solvent system of 60% ethyl acetate in hexanes, to afford the pure β-anomer (0.056 g, 12 %), and α-anomer (0.096 g, 21 %). (β-anomer) $^{1}$H-NMR (600 MHz, $CDCl_3$) $\delta$ (ppm) 6.95 (d, $J$ = 9.07 Hz, 2H), 6.83 (d, $J$ = 9.09 Hz, 2H), 5.95 (d, $J$ = 8.82 Hz, 1H), 5.38 (t, $J$ = 7.44 Hz, 1H), 5.20 (d, $J$ = 6.33 Hz, 1H), 5.11 (t, J = 7.36 Hz, 1H), 4.61-4.58 (m, 1H), 4.40-4.33 (m, 2H), 3.70 (s, 3H), 3.20 (dd, $J$ = 13.23, 6.23 Hz, 1H), 2.08 (s, 3H), 2.07 (s, 3H), 2.05 (s, 3H), 1.97 (s, 3H); $^{13}$C-NMR (150 MHz, $CDCl_3$) $\delta$ (ppm) 170.52, 170.12, 169.67, 168.92, 155.41, 150.43, 118.17, 114.68, 80.53, 71.01, 69.13, 63.40, 55.63, 40.57, 29.68, 23.38, 20.77, 20.69, 20.57. HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{21}H_{27}N_2O_9S$: 470.1485; found 470.1478.

*para-Methoxyphenyl 2-acetamido-2-deoxy-5-thio-β-D-glucopyranoside* (**8**) – To a solution of **26** (0.0062 g, 0.013 mmol) in methanol (0.25 mL) was added a catalytic amount of sodium methoxide (enough to bring the reaction solution to pH 10). The reaction was allowed to stir at room temperature. After twenty minutes a white solid began to precipitate out of solution.

The reaction had reached completion after 30 minutes. The reaction was first diluted with additional methanol (5 mL), and subsequently brought to a neutral pH by the drop-wise addition of a dilute mixture of acetic acid in methanol (pH 4). The reaction was concentrated under high vacuum without heating. The title compound was isolated by precipitation from ethanol and ether as a fine white powder (0.0033 g, 66 %). $^{1}$H-NMR (600 MHz, MeOH-$d_4$) $\delta$ (ppm) 6.96 (d, $J$ =9.16 Hz, 2H), 6.81 (d, $J$ = 9.16 Hz, 2H), 5.07 (d, $J$ = 9.29 Hz, 1H), 4.16 (t, $J$ = 9.60Hz, 1H), 3.93 (dd, $J$ = 11.44, 3.73 Hz,1H), 3.75 (dd, $J$ = 11.44, 6.47 Hz, 1H), 3.72 (s, 3H), 3.57 (dd, $J$ = 10.07, 8.95 Hz, 1H), 3.38 (dd, $J$ = 9.89, 8.91 Hz, 1H), 2.89-2.85 (m, 1H), 1.93 (s, 3H); $^{13}$C-NMR (150 MHz, $D_2O$) $\delta$ (ppm) 173.69, 155.72, 153.24, 118.96, 115.59, 82.24, 76.53, 75.78, 62.69, 61.32, 55.10, 47.53, 23.03; HRMS ($m/z$): $[M+H]^+$ calcd for $C_{15}H_{21}NO_6S$: 344.1168; found 344.1157.

***Synthesis of Me-5SGlcNAc (10).***




**25** **27** **10**

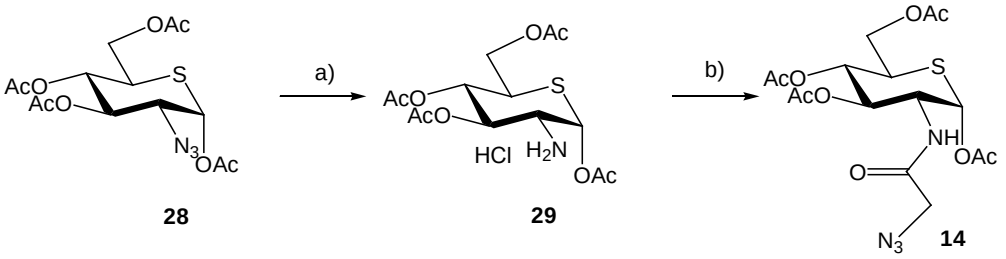
***Supplementary Scheme 3.*** Synthesis of Me-5SGlcNAc (**10**). a) MeOH (2 eq), $BF_3OEt_2$ (cat.), DCM, -20 °C→room temperature. b) 1) NaOMe (cat.), MeOH, rt; 2) AcOH, MeOH.

*Methyl 2-acetamido-3,4,6-tri*-O-acetyl-*2-deoxy-5-thio-β-D-glucopyranoside* – (**27**). **25** (0.050 g, 0.11 mmol) was dissolved in dry dichloromethane (0.76 mL). Two equivalents of dry methanol (0.009 mL, 0.211 mmol) were added to the solution at -20°C and then a catalytic amount of boron trifluoroethyletherate was added, after which the reaction mixture was allowed to warm to room temperature. Once the reaction was judged complete, two equivalents of triethylamine (0.03 mL) were added. The reaction was concentrated under high vacuum and the residue was immediately subjected to silica gel column chromatography using a solvent system of ethyl acetate. The ratio of α- to β-anomers was found to be 1:1 by $^1$H NMR. The desired β-anomer was isolated through a second purification using flash column chromatography in ethyl acetate in order to separate the two anomers. The title compound was isolated as a white powder (0.0154 g , 42%). (β-anomer) $^1$H-NMR (600 MHz, MeOH-$d_4$) $\delta$ (ppm) 5.17 (t, $J$ = 9.74 Hz, 1H), 5.02 (t, $J$ = 9.53 Hz, 1H), 4.60 (d, $J$ = 8.76 Hz, 1H), 4.34 (dd, $J$ = 11.86, 5.23 Hz, 1H), 4.22 (t, $J$ = 9.21 Hz, 1H), 4.10 (dd, $J$ = 11.82, 3.87 Hz, 1H), 3.45 (s, 3H), 3.28-3.26 (m, 1H), 2.02 (s, 3H), 1.97 (s, 3H), 1.96 (s, 3H), 1.89 (s, 3H); $^{13}$C-NMR (150 MHz, MeOH-$d_4$) $\delta$ (ppm) 173.37, 172.26, 171.57, 171.32, 84.41,74.83,

73.41,63.64, 59.21, 58.65, 41.91, 22.72, 20.64, 20.61, 20.54. HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{15}H_{23}NO_8S$: 378.1223; found 378.1214.

*Methyl 2-acetamido-2-deoxy-5-thio-β-D-glucopyranoside* (**10**) – **27** (0.0088 g, 0.023 mmol) was dissolved in methanol (0.35 mL) at room temperature. To this solution a catalytic amount of sodium methoxide was added (the pH of the reaction solution was 8-9). The reaction was followed closely by TLC and upon completion was neutralized (to pH 7) using a dilute solution of acetic acid in methanol (pH 4). The neutralized mixture was concentrated under high vacuum without heating. The desired product was purified by silica gel column chromatography using a solvent system of 15% methanol in chloroform. The pure fractions were concentrated *in vacuo* and the residue precipitated from ethanol by addition of ether to afford the title compound as a white residue (1.68 mg, 29%). $^1$H-NMR (600 MHz, MeOH-$d_4$) $\delta$ (ppm) 4.55 (s, 1H), 4.42 (d, $J$ = 8.89 Hz, 1H), 3.97 (t, $J$ = 9.26 Hz, 1H), 3.93 (dd, $J$ = 11.35, 3.93 Hz, 1H), 3.72 (dd, $J$ = 11.35, 6.59 Hz, 1H), 3.51 (t, $J$ = 8.95 Hz, 1H), 3.41 (s, 3H), 3.26 (dd, J = 9.53, 8.90 Hz, 1H), 2.76-2.73 (m, 1H), 1.95 (s, 3H). $^{13}$C-NMR (150 MHz, MeOH-$d_4$) $\delta$ (ppm) 173.79, 84.76, 76.89, 75.63, 63.13, 60.96, 58.36, 47.48, 22.96. HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_9H_{17}NO_5S$: 252.0906; found 252.0913.

***Synthesis of Ac-5SGlcNAz (14)***




***Supplementary Scheme 4.*** Synthesis of Ac-5SGlcNAz (**14**). a) $H_2$, $PtO_2$, MeOH/HCl/$H_2O$, r.t., 2 h; b) Azidoacetic acid (3.0 eq.), HBTU (3.8 eq.), DIPEA (5.9 eq), DCM, 0 °C → r.t., overnight, 30% over 2 steps.

*1,3,4,6-Tetra-O-acetyl-2-azido-2-deoxy-5-thio-α-D-glucopyranoside* (**28**) – Synthesized from D-glucofurano-3,6-lactone using literature procedures with minor modifications in 13 steps[16-18]. Careful flash silica gel column chromatography using a gradient of hexanes:ethyl actetate in a ratio of 5:1 to 4:1 to 3:1, followed by preparative thin layer chromatography using a solvent system of hexanes:ethyl acetate in a ratio of 4:1 yielded pure α-anomer as a colourless syrup (for which the characterisation is described) and impure β-anomer which was not used further. $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$ (ppm) 6.10 (d, $J$ = 3.0 Hz, 1H), 5.40 (t, $J$ = 10.0 Hz, 1H), 5.32 (t, $J$ = 10.0 Hz, 1H), 4.39 (dd, $J$ = 12.0, 5.0 Hz, 1H), 4.03 (dd, $J$ = 12.0, 3.0 Hz, 1H), 3.88 (dd, $J$ = 10.0, 3.0 Hz, 1H), 3.56 (ddd, $J$ = 11.0, 5.0, 3.0 Hz, 1H), 2.19 (s, 3H), 2.11 (s, 3H), 2.07 (s, 3H), 2.05 (s, 3H); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$ (ppm) 170.45, 169.71, 169.51, 168.67, 71.74, 71.70, 64.88, 60.88, 39.80, 20.98, 20.60, 20.59, 20.50. HRMS (*m*/*z*): $[M+NH_4]^+$ calcd for $C_{16}H_{26}NO_{10}S$: 424.1272; found 424.1267. $[M+Na]^+$ calcd for $C_{16}H_{22}NaO_{10}S$: 429.0286; found 429.0280.

*1,3,4,6-Tetra-O-acetyl-2-azidoacetamido-2-deoxy-5-thio-α-D-glucopyranose* (**14**) – 2-Azido sugar **28** (42 mg, 0.11 mmol) and $PtO_2$ (22 mg, 0.097 mmol) were suspended in MeOH (5.0

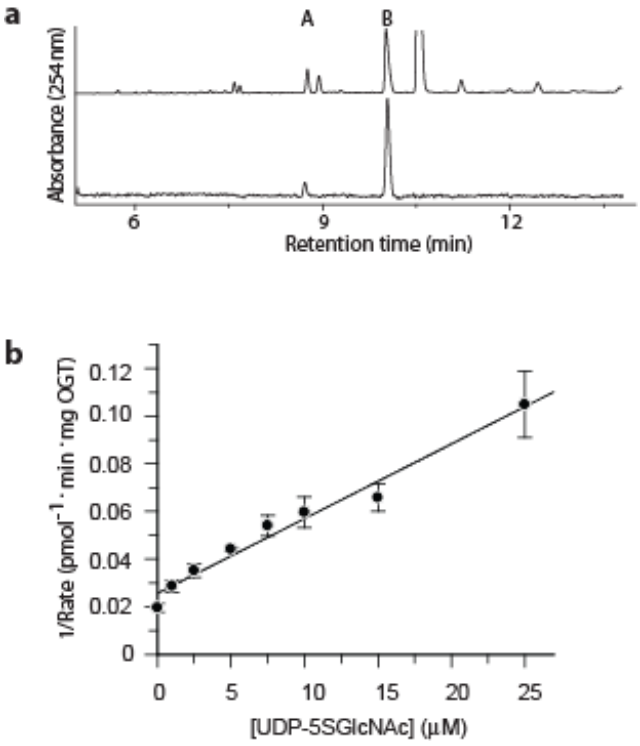
mL) and 1 N HCl (aq., 1.0 mL) was added. The resulting reaction mixture was stirred under an atmosphere of $H_2$ (1 atm) at r.t. for 2 hours and then filtered through Celite. The filtrate was concentrated under reduced vacuum, the residue was co-evaporated with toluene (2 x 5.0 mL), and was then dried under high vacuum for 2 hours to afford crude intermediate 2-amino-1,3,4,6-tetra-*O*-acetyl-5-thio-alpha-α-D-glucopyranose hydrochloride (**29**) as a brown syrup (39 mg). This material was used in the next step directly without purification. To a solution of **29** (39 mg, 0.11 mmol) in dry $CH_2Cl_2$ (4.0 mL), was added HBTU (140 mg, 0.37 mmol), azidoacetic acid[19] (30 mg, 0.30 mmol), and then DIPEA (0.10 mL, 0.58 mmol) was added drop-wise at 0 °C. The resultant reaction mixture was stirred overnight before being quenched by addition of sat. $NaHCO_3$ (aq.) (10 mL), and the reaction mixture was extracted with $CH_2Cl_2$ (3 x 10 mL). The combined organic extracts were washed with brine (1 x 10 mL) and dried ($MgSO_4$). After filtration and concentration under reduced pressure, the residue was purified by flash column silica chromatography (hexanes:ethyl acetate, 3:1 to 2:1 then 1:1), followed by preparative TLC ($CH_2Cl_2$:MeOH, 20:1) to afford a pale yellow syrup (13 mg, 30 %). $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$ (ppm) 6.57 (brd, $J$ = 8.5 Hz, 1H, N*H*), 6.00 (d, $J$ = 2.5 Hz, 1H, H-1), 5.40 (t, $J$ = 10.0 Hz, 1H, H-4), 5.24 (t, $J$ = 10.0 Hz, 1H, H-3), 4.61 (m, 1H, H-2), 4.37 (dd, $J$ = 12.0, 5.0 Hz, 1H, H-6), 4.05 (dd, $J$ = 12.0, 3.0 Hz, 1H, H-6'), 3.92 (s, 2H, $N_3CH_2$), 3.51 (ddd, $J$ = 10.5, 5.0, 3.0 Hz, 1H, H-5), 2.21 (s, 3H), 2.08 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$ (ppm) 171.43, 170.53, 169.17, 168.83, 168.51, 72.20 (C-1), 71.34 (C-4), 71.30 (C-3), 61.02 (C-6), 55.42 (C-2), 52.47 ($N_3CH_2$), 39.82 (C-5), 21.04, 20.63, 20.59, 20.52. HRMS (*m*/*z*): $[M+H]^+$ calcd for $C_{16}H_{23}N_4O_9S$: 447.1180; found 447.1180. $[M+NH_4]^+$ calcd for $C_{16}H_{26}N_5O_9S$: 464.1446; found 464.1440. $[M+Na]^+$ calcd for $C_{16}H_{22}N_4NaO_9S$: 469.1000; found 469.0999.

**Characterization of UDP-5SGlcNAc (4).**

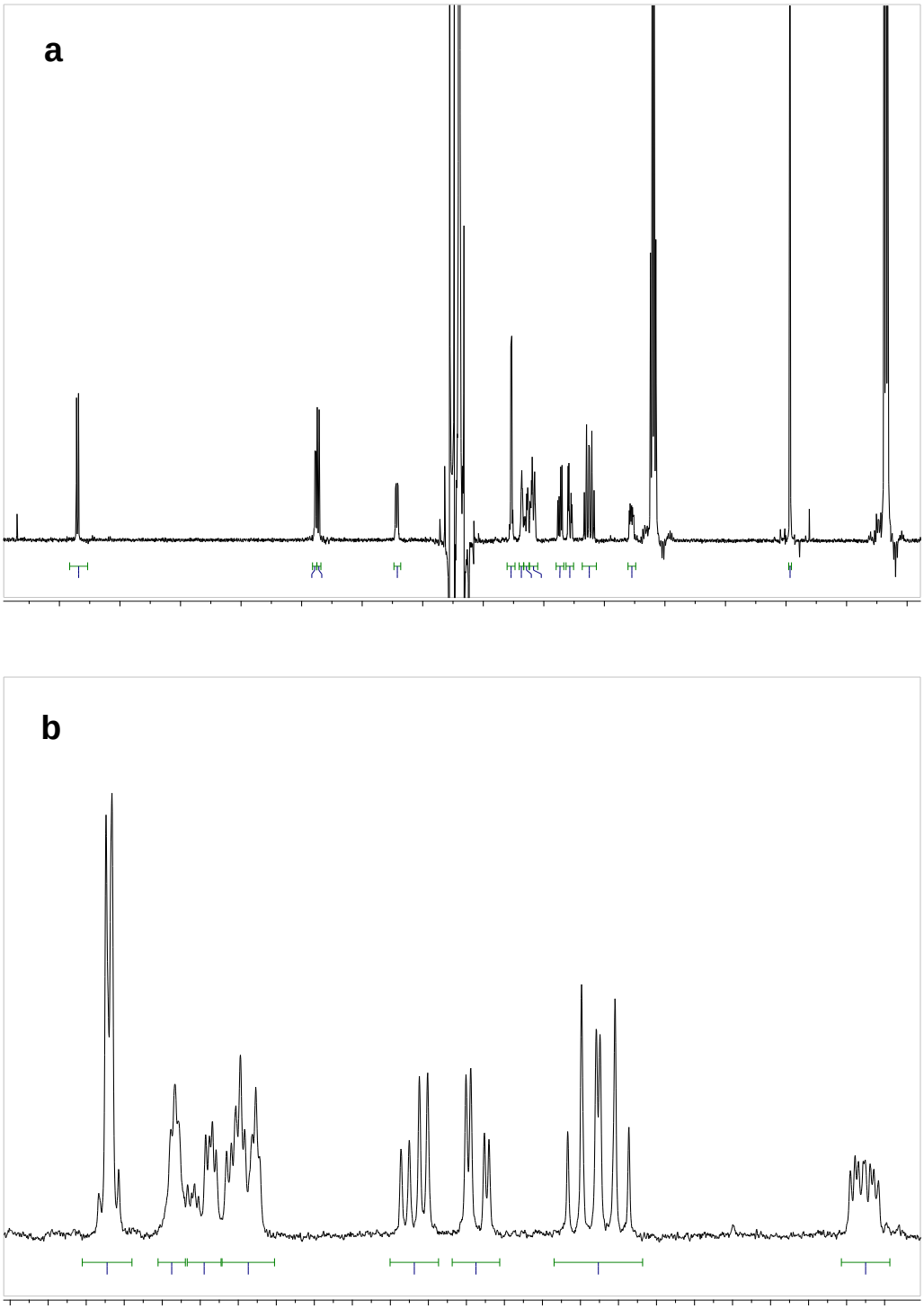
*5'-(2-acetamido-2-deoxy-5-thio-α-D-glucopyranosyl)diphosphate triethylammonium salt (**4**)* - The chemoenzymatic synthesis was carried out as described in the methods section. $^{1}$H NMR (600 MHz, $D_2O$): $\delta$ (ppm) 2.08 (s, 3H, NH(CO)C$H_3$), 3.38 (ddd, 1H, $J_{5",6a"}$ = 5.4 Hz, $J_{5",6b"}$ = 3.0 Hz, H-5"), 3.71 (dd, 1H, $J_{4",5"}$ = 10.2 Hz, H-4"), 3.75 (dd, 1H, $J_{3",2"}$ = 9.6 Hz, $J_{3",4"}$ = 9.0 Hz, H-3"), 3.89 (dd, 1H, $J_{6b",6a"}$ = 12.0 Hz, H-6b"), 3.97 (dd, 1H, H-6a"), 4.18-4.21 (m, 1H, H-2"), H-5b' = 4.21-4.24 (m, 1H, H-5b'), H-5a' = 4.24-4.27 (m, 1H, H-5a'), 4.28-4.30 (m, 1H, H-4'), 4.36-4.38 (m, 2H, H-2',3'), 5.32 (dd, 1H, $J_{1",2"}$ = 2.4 Hz, $J_{1",P}$ = 7.8 Hz, H-1"), 5.97 (d, 1H, H-5), 6.00 (d, 1H, $J_{1',2'}$ = 4.2 Hz, H-1'), 7.96 (d, 1H, $J_{6,5}$ = 7.8 Hz, H-6); $^{13}$C NMR (150 MHz, $D_2O$): $\delta$ (ppm) 22.06 (NH(CO)$C$H$_3$), 43.59 (C-5"), 57.74 (C-2"), 59.91 (C-6"), 64.89 (C-5'), 69.61 (C-3'), 72.03 (C-3"), 73.62 (C-4"), 73.75 (C-2'), 76.62 (C-1"), 83.17 (C-4'), 88.39 (C-1'), 102.63 (C-5), 141.63 (C-6), 151.78 (C-2), 166.20 (C-4), 169.13 (NH($C$O)CH$_3$). $^{31}$P NMR (242 MHz, $D_2O$): $\delta$ (ppm) -10.66 (d, $J_{p,p}$ = 21.8 Hz), -12.09 (d). HRMS (*m*/*z*): [M-H]$^-$ calcd for $C_{17}H_{26}N_3O_{16}P_2S$: 622.0519, found 622.0514.

## Supplementary Results

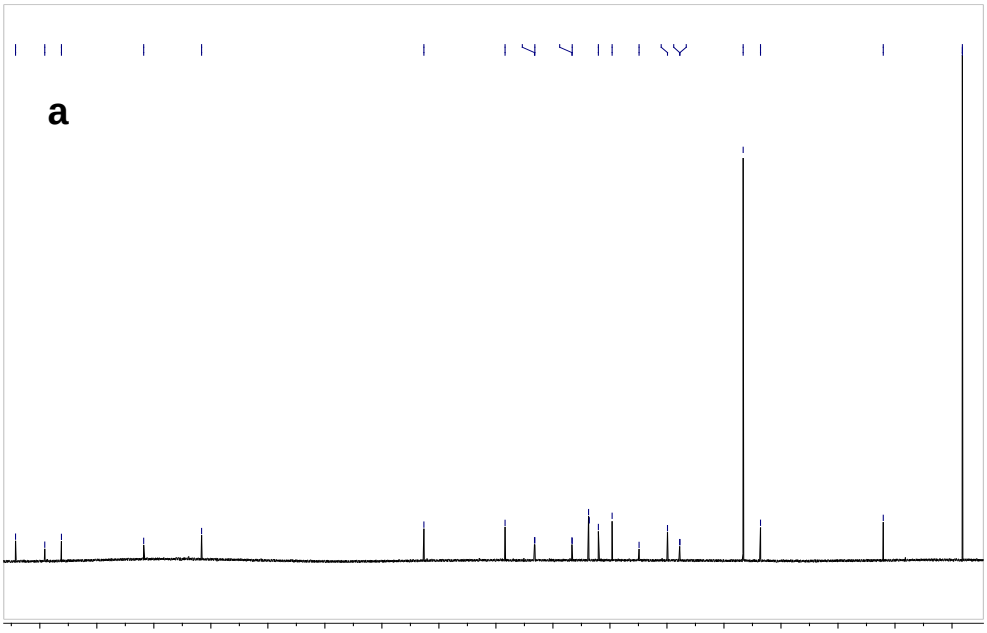
## Supplementary Figure 1

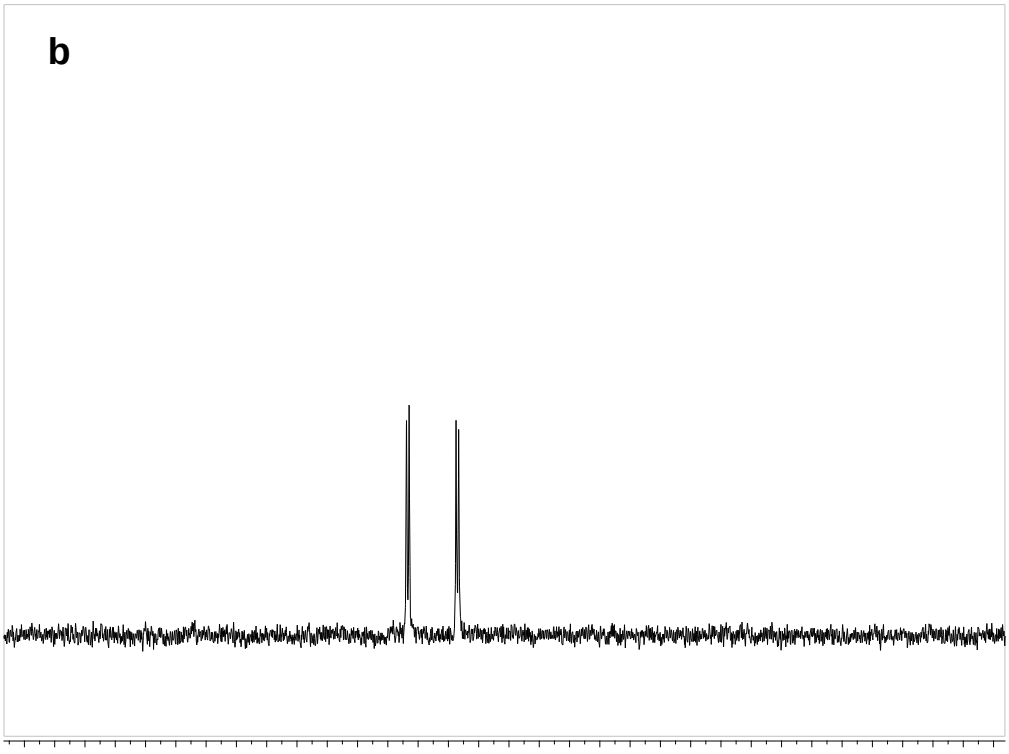


**Supplementary Figure 2**

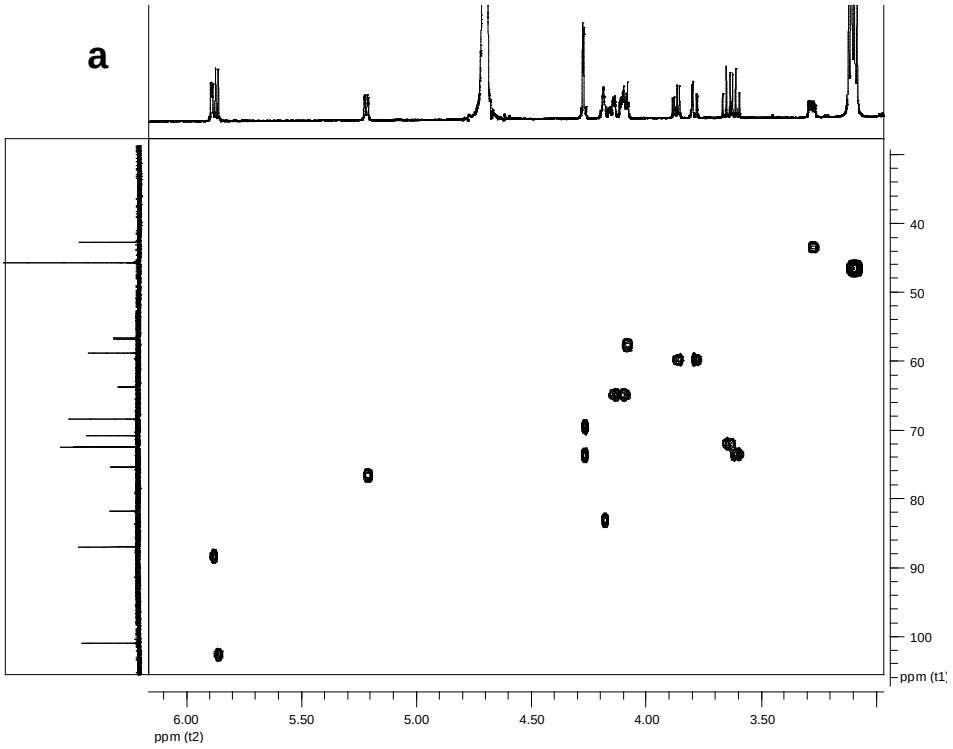


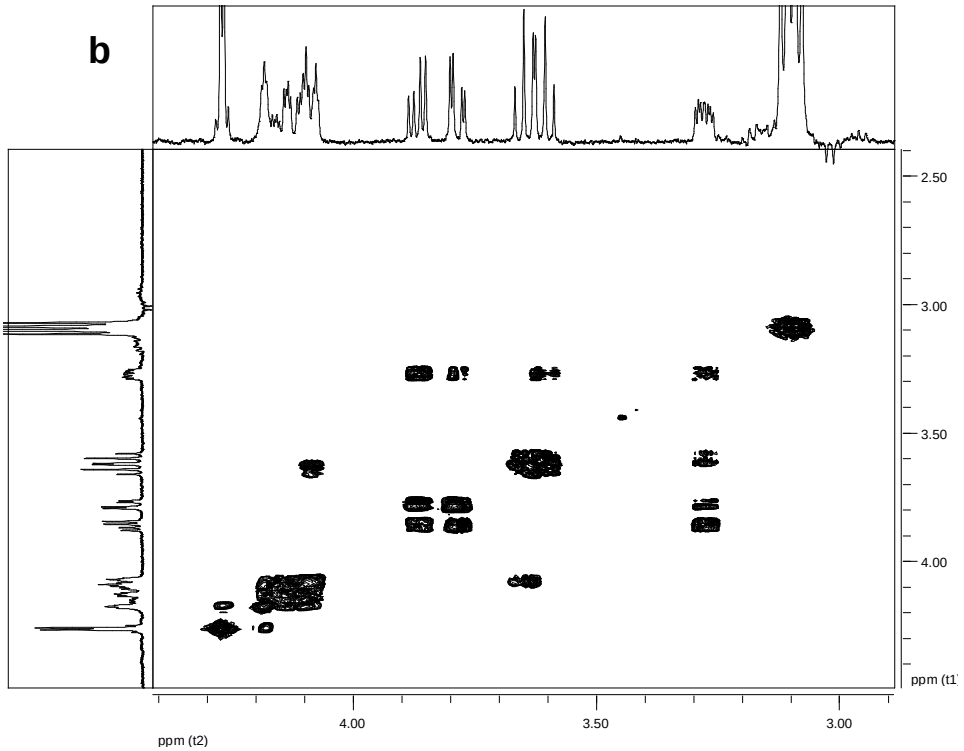
**Supplementary Figure 3**



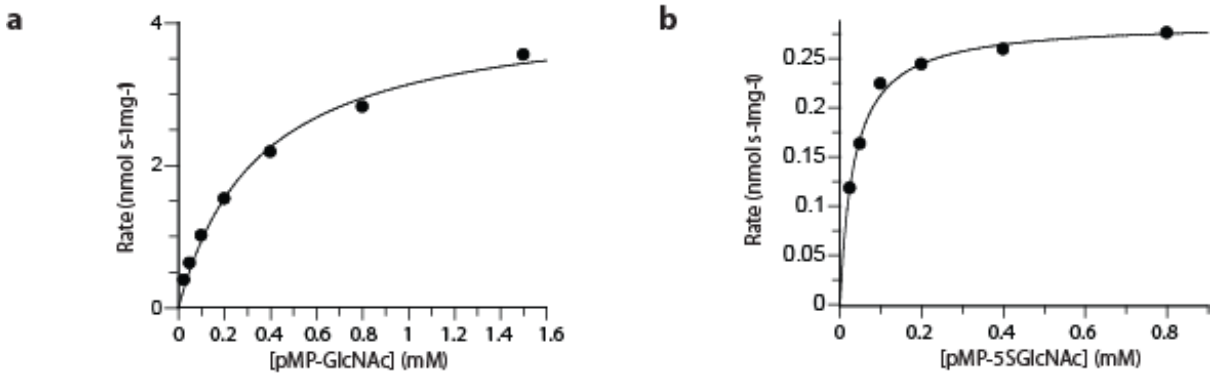



**Supplementary Figure 4**

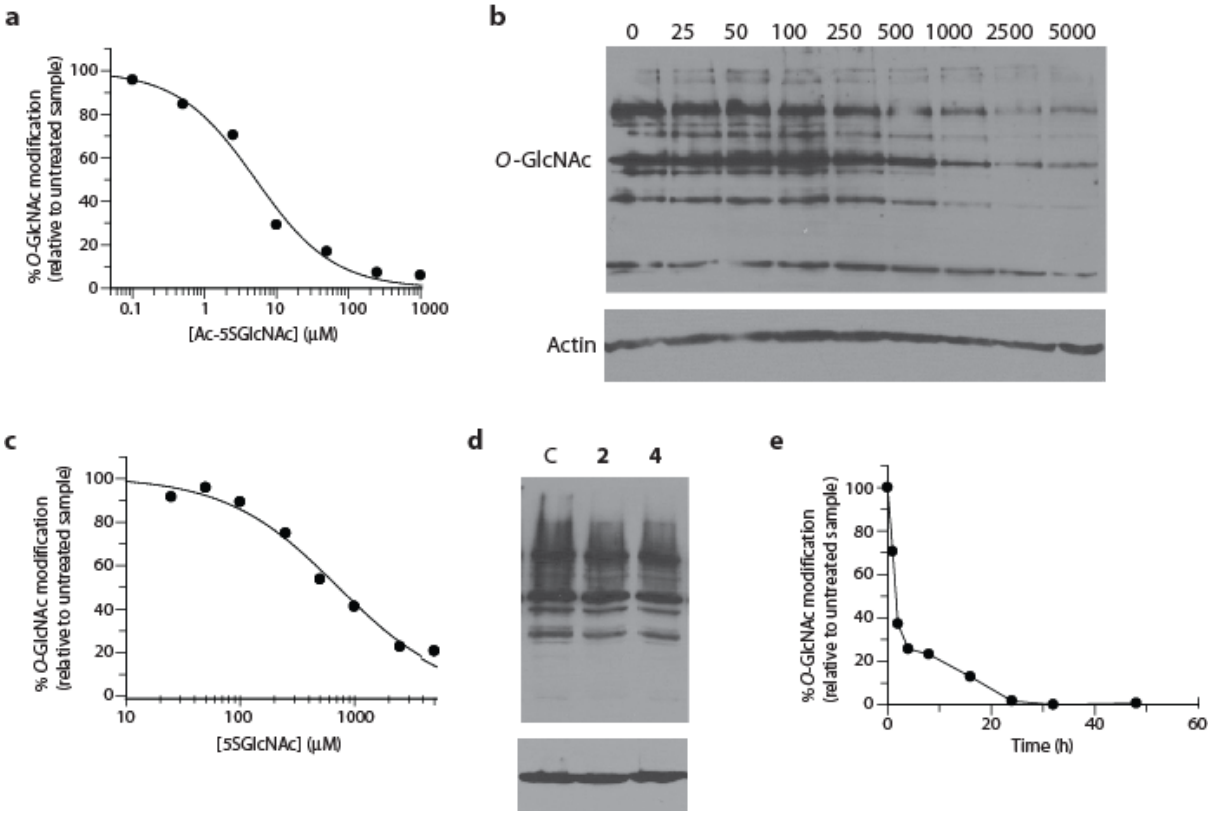


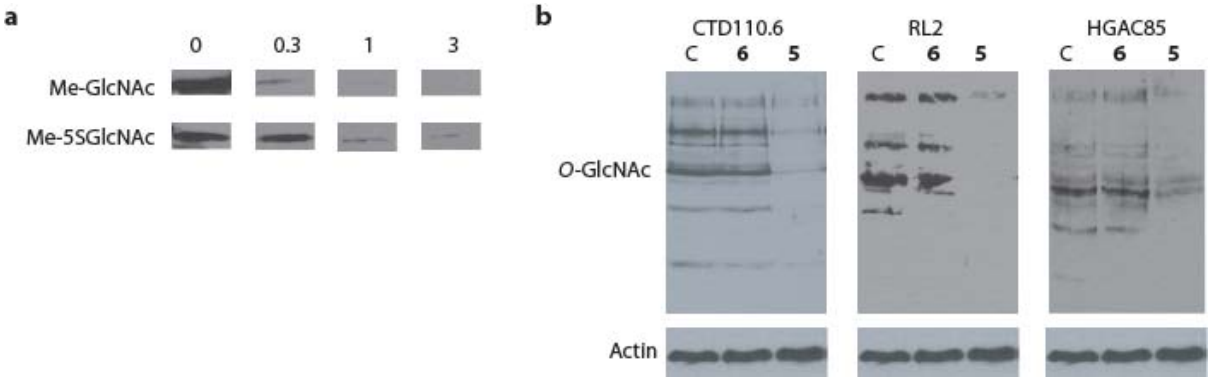
**Supplementary Figure 5**



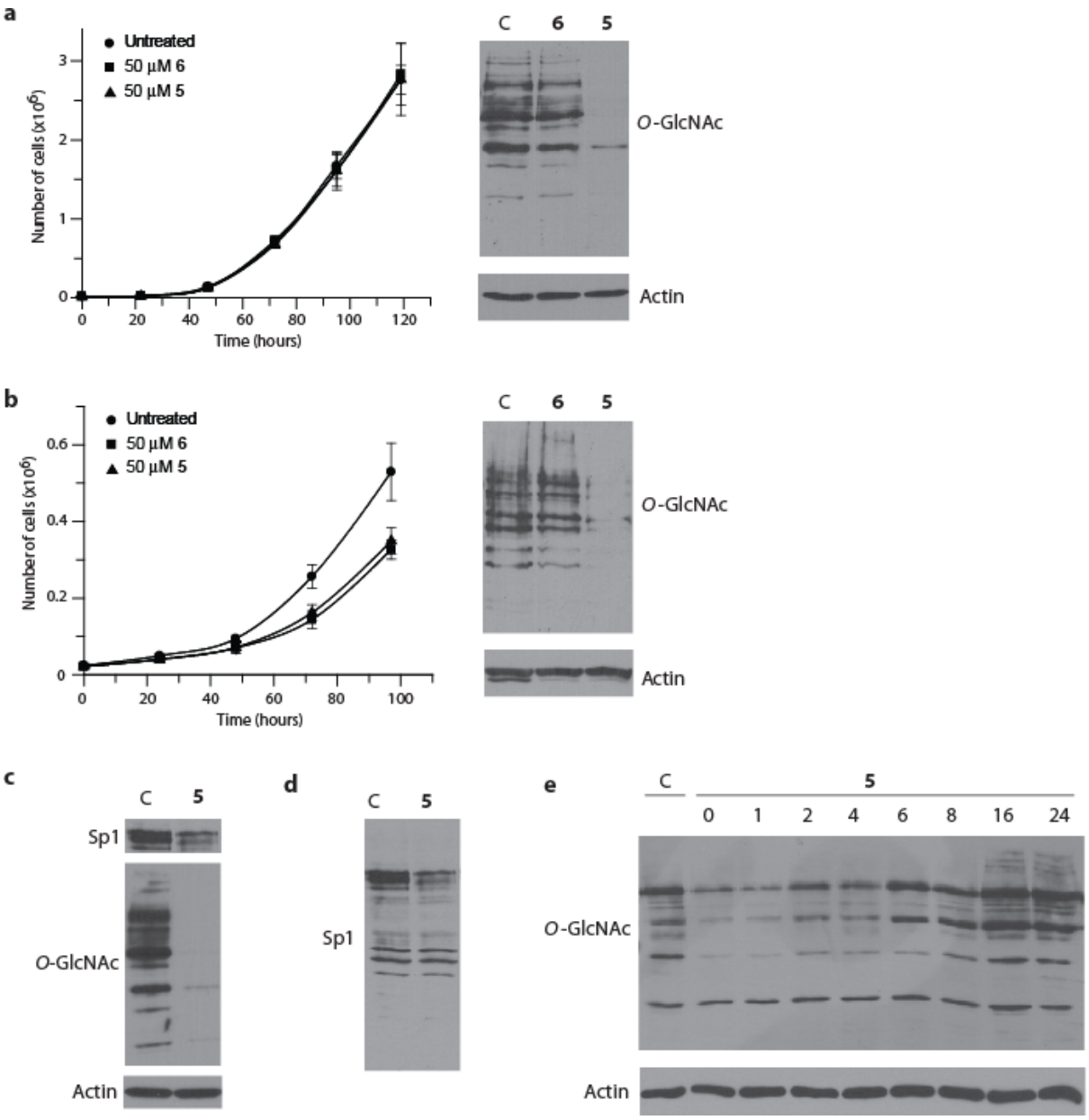
**Supplementary Figure 6**



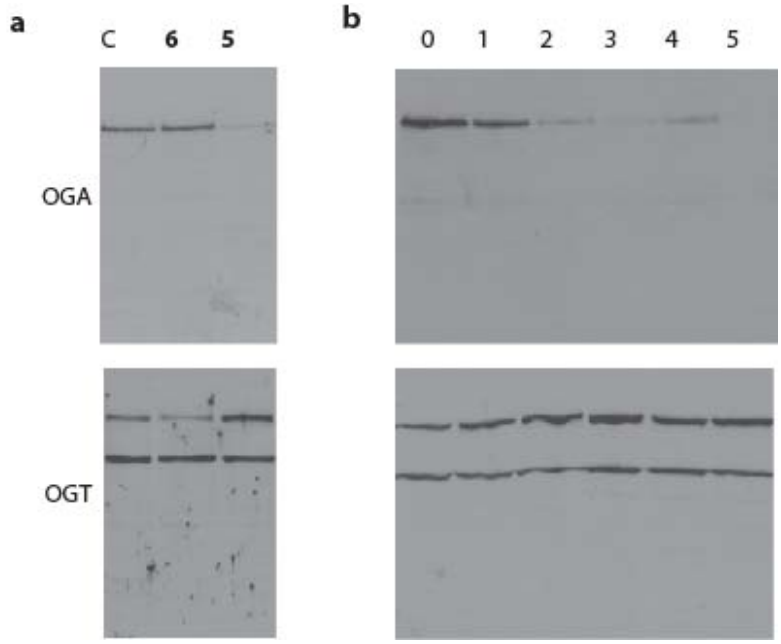
**Supplementary Figure 7**



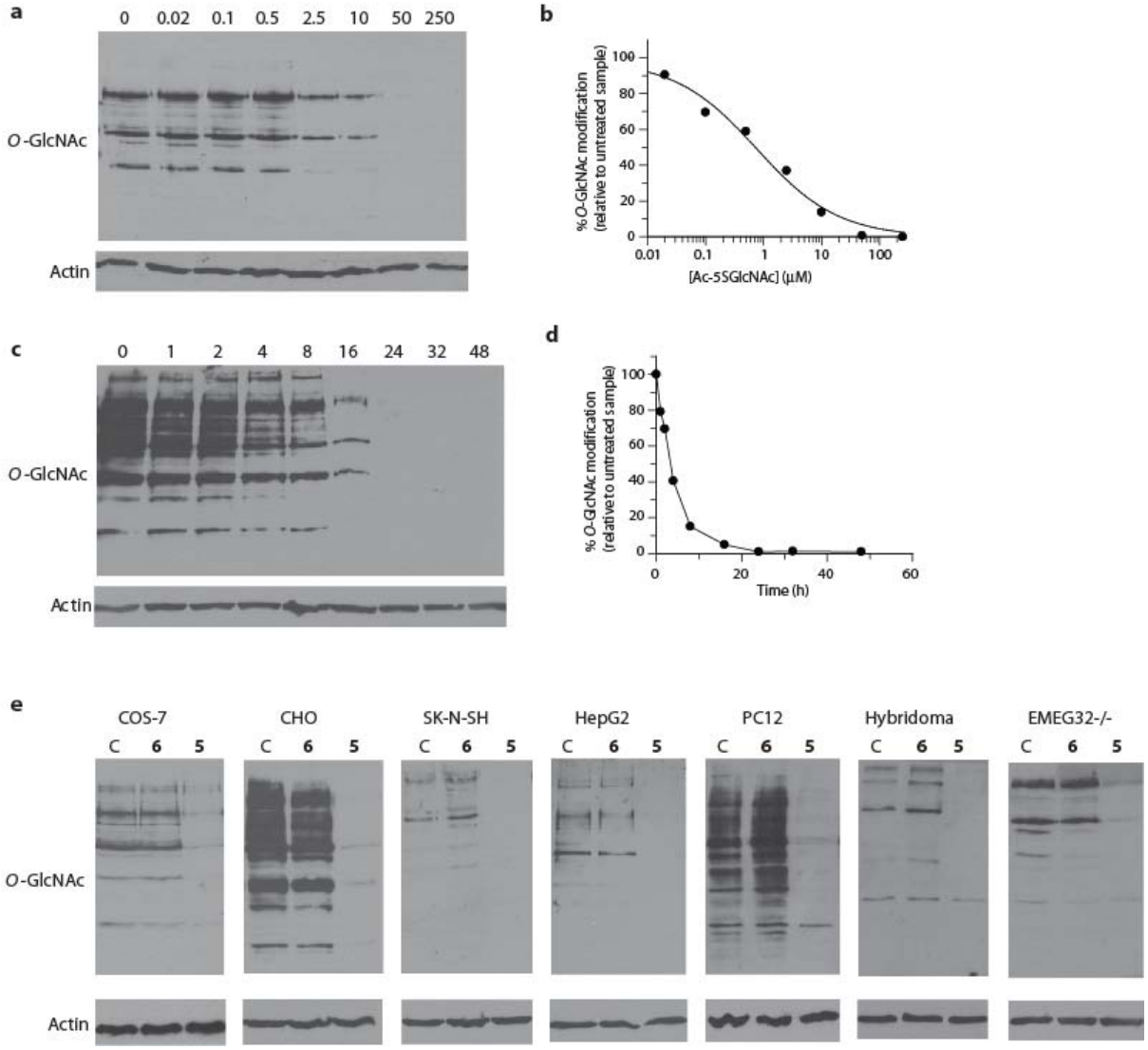
**Supplementary Figure 8**



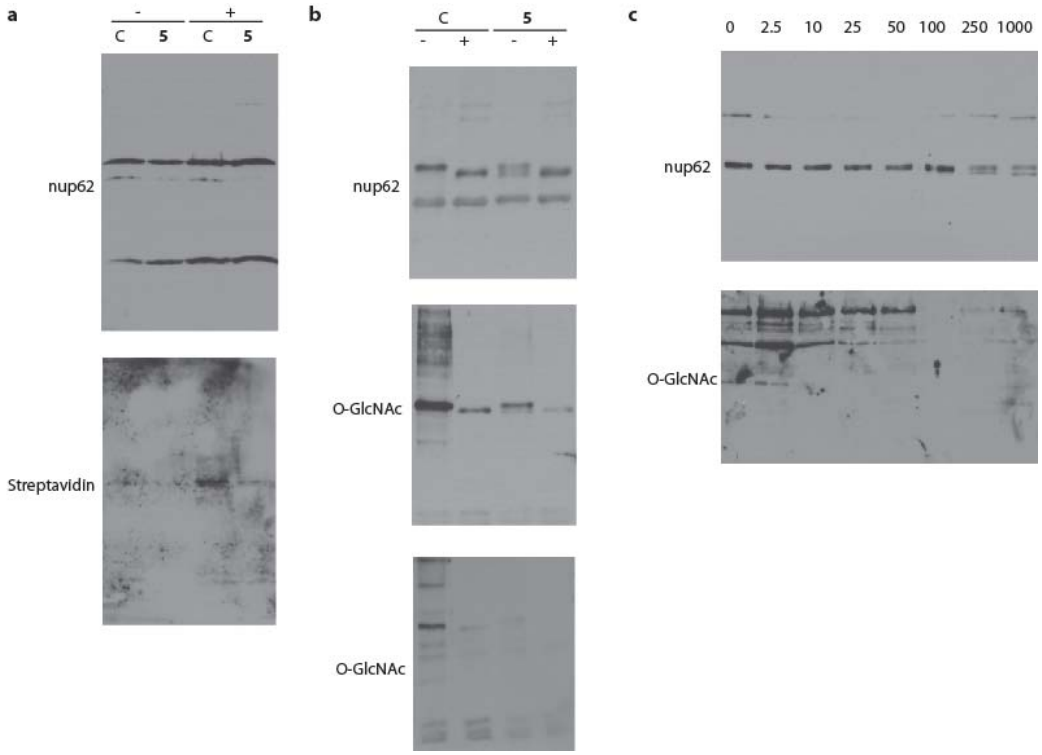
**Supplementary Figure 9**



**Supplementary Figure 10**

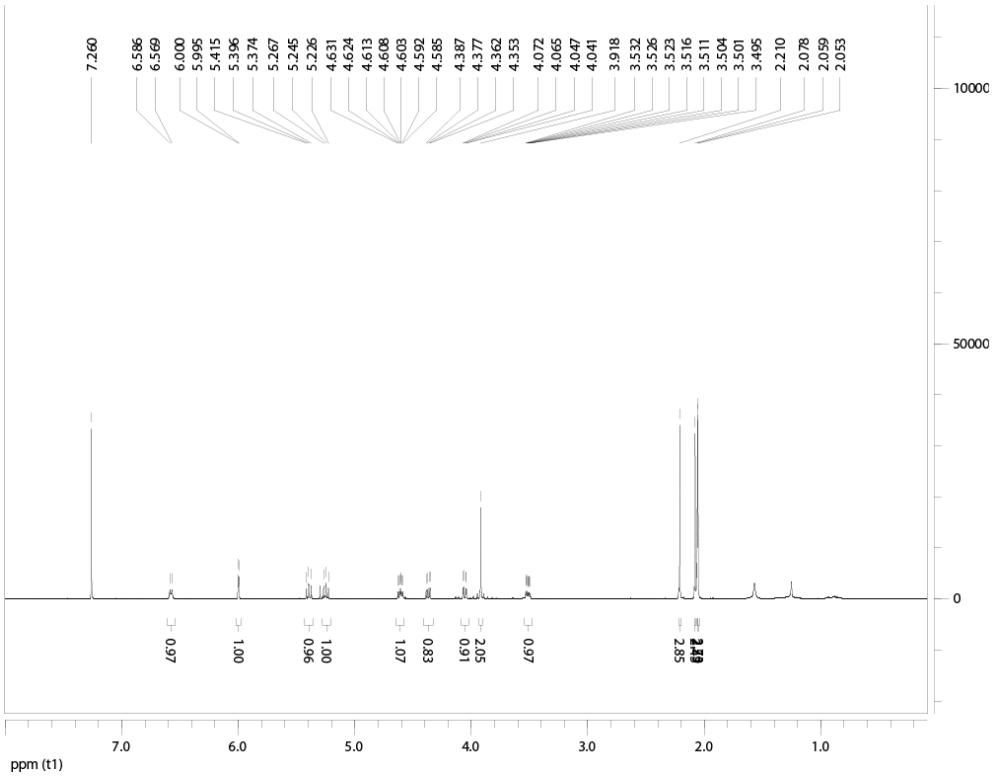


**Supplementary Figure 11**

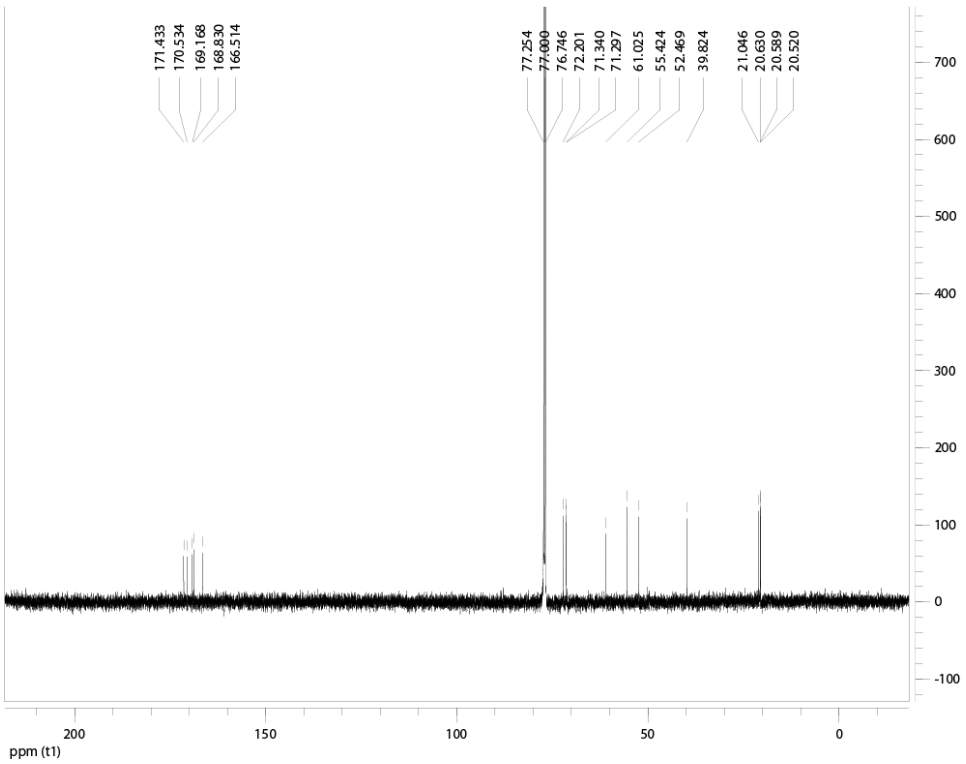


## Supplementary Figure 12

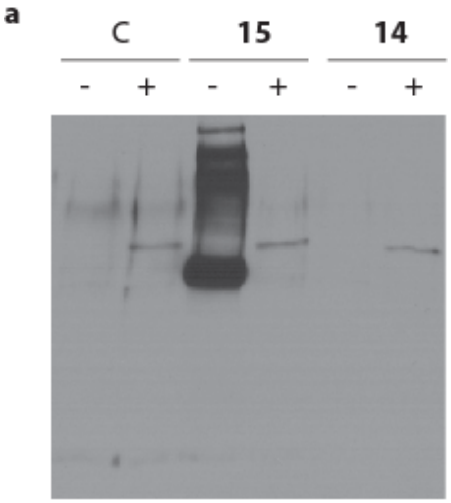
**a**



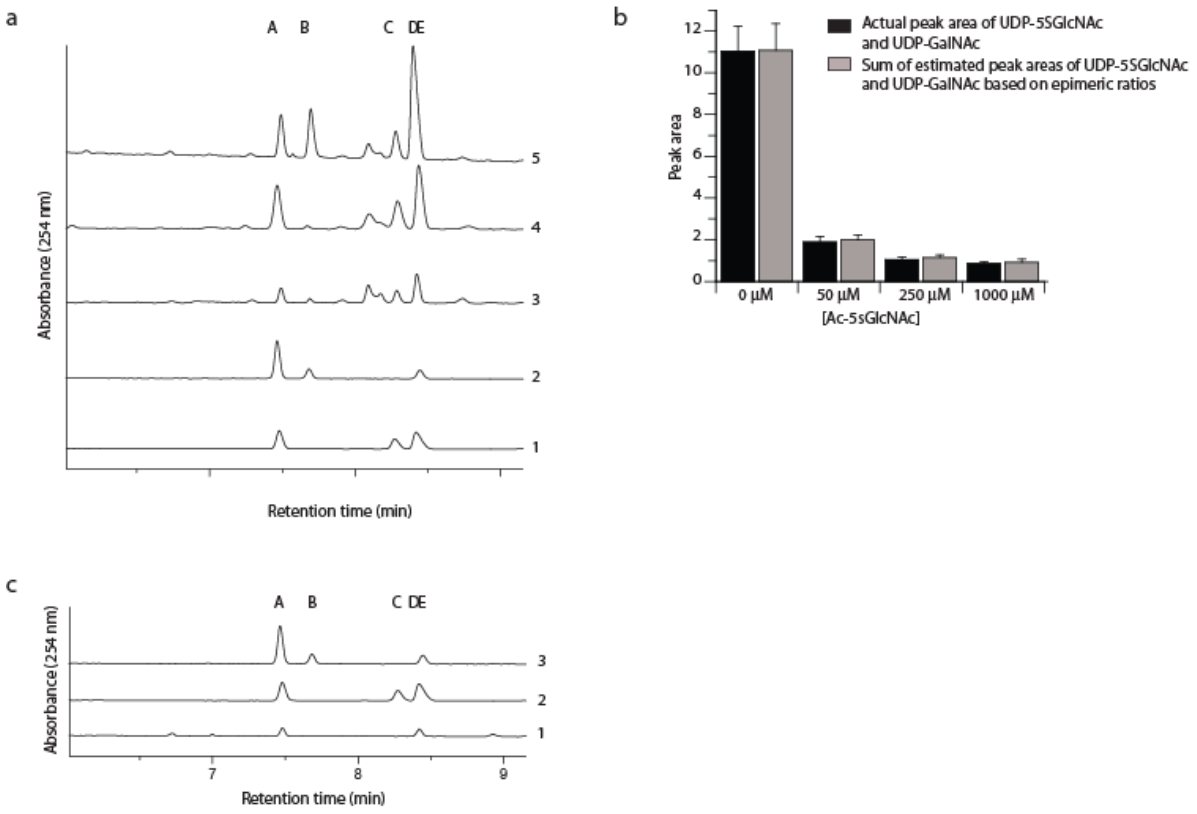

**b**



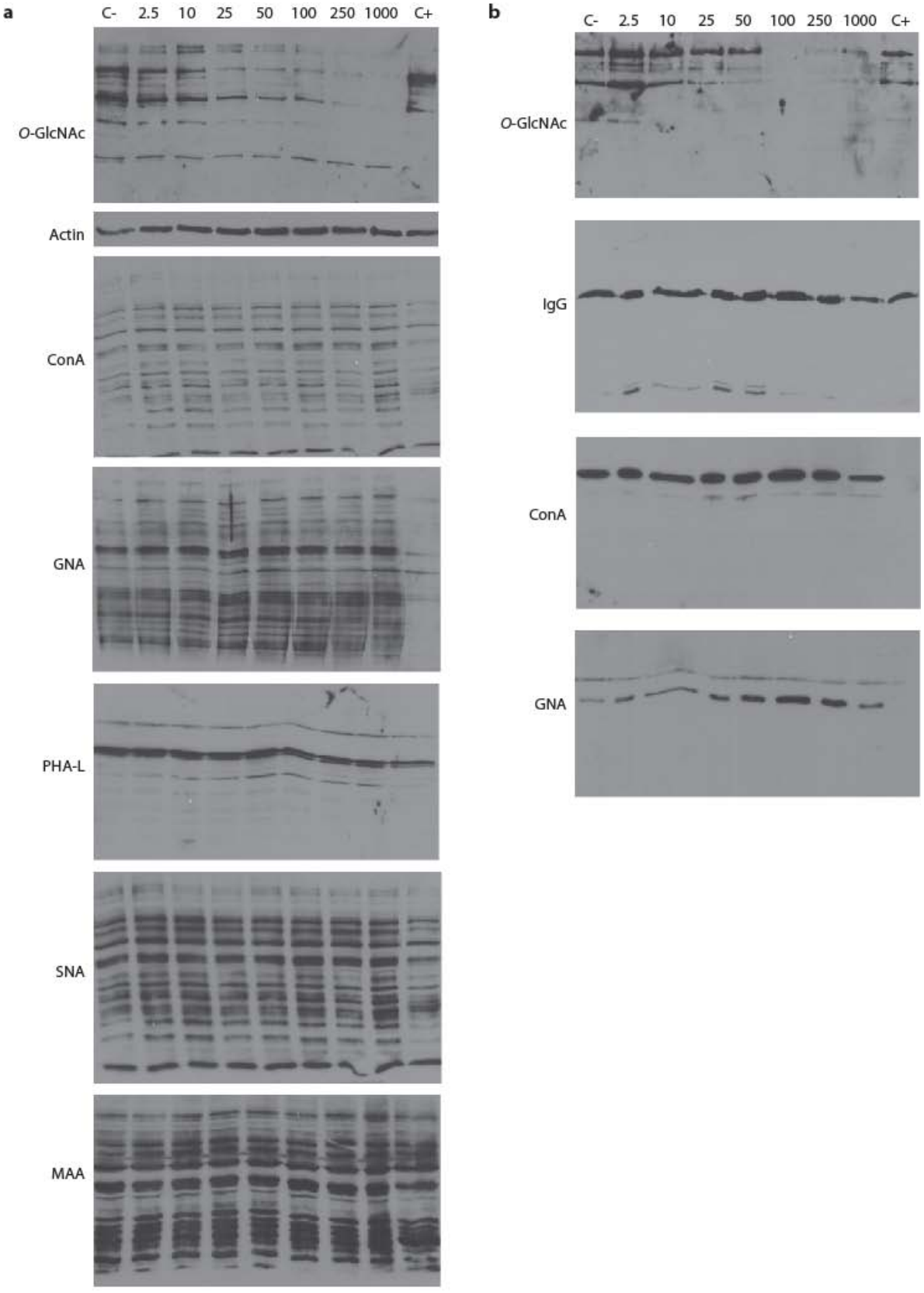
**Supplementary Figure 13**



**Supplementary Figure 14**



**Supplementary Figure 15**



**Supplementary Figure Legends**

**Supplementary Figure 1. *In vitro* characterization of the ability of the human enzymes of the HBP to convert 5SGlcNAc (3) to UDP-5SGlcNAc (4) and *in vitro* evaluation of UDP-5SGlcNAc (4) as an inhibitor of OGT.** (a) *In vitro* enzymatic synthesis of UDP-5SGlcNAc (**4**) catalyzed by the human enzymes of the HBP monitored by capillary electrophoresis at 254 nm. Upper trace, crude reaction mixture; lower trace, UDP-5SGlcNAc (**4**) following ion exchange and HPLC purification. Peak A, GDP-Glc (internal standard spiked into samples prior to analysis); peak B, UDP-5SGlcNAc (**4**). (b) Inhibition by UDP-5SGlcNAc (**4**) of OGT-catalyzed transfer of *O*-GlcNAc onto nup62.

**Supplementary Figure 2. $^{1}$H NMR spectrum of UDP-5SGlcNAc (4).** (a) $^{1}$H-NMR spectrum of UDP-5SGlcNAc (**4**). Peaks at 8.35, 3.10 and 1.18 ppm arise from the triethylammonium counterion. (b) Expansion of $^{1}$H NMR spectrum of UDP-5SGlcNAc (**4**) covering the region from 4.40 to 3.20 ppm.

**Supplementary Figure 3. NMR spectra of UDP-5SGlcNAc (4).** (a) $^{13}C\{^{1}H\}$ NMR spectrum of UDP-5SGlcNAc (**4**). Peaks at 8.1 ($CH_3$) and 46.6 ($CH_2$) are derived from the triethylammonium cation. The peak at 174.2 ppm is from the carbonyl of the acetyl group. (b) $^{31}P\{^{1}H\}$ NMR spectrum of UDP-5SGlcNAc (**4**) referenced to 85% $H_3PO_4$ at 0 ppm.

**Supplementary Figure 4**. **Two dimensional NMR spectra of UDP-5SGlcNAc (4).** (a) HMQC spectrum and (b) COSY spectrum.

**Supplementary Figure 5. Processing of oxygen- and thio-containing glycosides by OGA.** (a) Michaelis-Menten kinetics for OGA hydrolysis of *p*MP-GlcNAc (**7**) performed at 37 °C. The fit gives a $k_{cat}$ of 14.3 nmol $s^{-1}$ $mg^{-1}$ and a $K_M$ of 350 μM. (b) Michaelis-Menten kinetics for OGA hydrolysis of *p*MP-5SGlcNAc (**8**) performed at 37 °C. The fit gives a $k_{cat}$ of 0.28 nmol $s^{-1}$ $mg^{-1}$ and a $K_M$ of 35 μM.

**Supplementary Figure 6. Effects of treating cells with 5SGlcNAc (3), UDP-5SGlcNAc (4) and Ac-5SGlcNAc (5).** (a) Densitometry analysis from western blots (converted to a % *O*-GlcNAc modification (corrected for actin levels) relative to the untreated control sample) of COS-7 cell lysates following Ac-5SGlcNAc (**5**) administration at different doses (0-1000 μM) for 24 h (Figure 2a), which yields an $EC_{50}$ value of 5 μM. (b) Western blots of COS-7 cell lysates following 5SGlcNAc (**3**) administration at different doses (0-5000 μM) for 24 h. Upper panel, probed with anti-*O*-GlcNAc antibody (CTD110.6); lower panel, probed with anti-actin antibody. (c) Densitometry analysis from western blots of COS-7 cell lysates following 5SGlcNAc (**3**) administration at different doses (0-5000 μM) for 24 h (panel b), which yields an $EC_{50}$ value of 700 μM. (d) Western blots of COS-7 cell lysates following either vehicle, 50 μM UDP-GlcNAc (**2**), or 50 μM UDP-5SGlcNAc (**4**) treatment for 24 h. Upper panel, probed with anti-*O*-GlcNAc antibody (CTD110.6); lower panel, probed with anti-actin antibody. (e) Densitometry analysis from western blots of COS-7 cell lysates following Ac-5SGlcNAc (**5**) administration at 50 μM for various times (Figure 2b).

**Supplementary Figure 7. Probing the effectiveness of the anti-*O*-GlcNAc antibodies.** (a) Western blots of recombinantly modified p62 protein, probed with the anti-*O*-GlcNAc antibody CTD110.6 in the presence of increasing concentrations (in mM) of either Me-GlcNAc (**9**) or Me-5SGlcNAc (**10**). (b) Western blots of COS-7 cell lysates following no (C),

Ac-GlcNAc (**6**) or Ac-5SGlcNAc (**5**) administration at 50 μM for 24 h. Probed with different anti-*O*-GlcNAc antibodies: CTD110.6, RL2 and HGAC85, from left to right.

**Supplementary Figure 8. Effect of Ac-5SGlcNAc (5) on cell growth and Sp1 levels and testing the reversibility of Ac-5SGlcNAc (5) treatment.** (a) Cell proliferation curves over the course of 5 days for CHO cells following no (circles), 50 μM Ac-GlcNAc (**6**) (squares) or 50 μM Ac-5SGlcNAc (**5**) (triangles) treatment. The error bars indicate the deviation from the mean for triplicate measurements. The western blots for the last time point indicate *O*-GlcNAc levels are still significantly decreased in cells treated with Ac-5SGlcNAc (**5**). Upper panel, probed with anti-*O*-GlcNAc antibody (CTD110.6); lower panel, probed with anti-actin antibody. (b) Cell proliferation curves over the course of 5 days for EMEG heterozygous mouse embryonic fibroblast cells following no (circles), 50 μM Ac-GlcNAc (**6**) (squares) or 50 μM Ac-5SGlcNAc (**5**) (triangles) treatment. The error bars indicate the deviation from the mean for triplicate measurements. The western blots for the last time point indicate *O*-GlcNAc levels are still significantly decreased in cells treated with Ac-5SGlcNAc (**5**). Upper panel, probed with anti-*O*-GlcNAc antibody (CTD110.6); lower panel, probed with anti-actin antibody. (c) Western blots of CHO cell lysates following no (C) or Ac-5SGlcNAc (**5**) administration at 50 μM for 4 days. Probed with (from top to bottom) anti-Sp1 antibody, anti *O*-GlcNAc (CTD110.6) antibody and anti-actin antibody. Full blot of the anti-Sp1 is shown in panel (d). (d) Western blots of CHO cell lysates following no (C) or Ac-5SGlcNAc (**5**) administration at 50 μM for 4 days. The full blot probed with anti-Sp1 antibody is shown. (e) Western blots of COS-7 cell lysates following no (C) or 50 μM Ac-5SGlcNAc (**5**) treatment for 24 h. Following treatment, the media was replaced with no inhibitor, and cells were harvested at the time points indicated (between 0 and 24 h). Upper panel, probed with anti-*O*-GlcNAc antibody (CTD110.6); lower panel, probed with anti-actin antibody.

**Supplementary Figure 9. Full versions of blots shown in Figure 2; Ac-5SGlcNAc (5) acts in cells to decrease OGA levels and increase OGT levels in a compensatory manner.** (a) Full western blots of COS-7 cell lysates administered specified agents at 50 μM for 24 h; vehicle only (C), Ac-GlcNAc (**6**) or Ac-5SGlcNAc (**5**). Upper panel, probed with anti-OGA antibody; lower panel, probed with anti-OGT antibody. (b) Full western blots of COS-7 cell lysates following Ac-5SGlcNAc (**5**) administration at 50 μM for different amounts of time. Upper panel, probed with anti-OGA antibody; lower panel, probed with anti-OGT antibody.

**Supplementary Figure 10. Effect of treating CHO cells and other cells lines with Ac-5SGlcNAc (5).** (a) Western blots of CHO cell lysates following Ac-5SGlcNAc (**5**) administration at different doses (0-250 μM) for 24 h. Upper panel, probed with anti-*O*-GlcNAc antibody (CTD110.6); lower panel, probed with anti-actin antibody. (b) Densitometry analysis of the western blots shown in panel a, converted to a % *O*-GlcNAc modification (corrected for actin levels) relative to the untreated control sample) of as a function of dose; this gives an $EC_{50}$ of 0.8 μM. (c) Western blots of CHO cell lysates following Ac-5SGlcNAc (**5**) administration at 50 μM for different amounts of time (shown in hours). Upper panel, probed with anti-*O*-GlcNAc antibody (CTD110.6); lower panel, probed with anti-actin antibody. (d) Densitometry analysis of the western blots shown in panel c, converted to a % *O*-GlcNAc modification (corrected for actin levels) relative to the untreated control sample as a function of time. (e) Western blots of cell lysates from different cell lines following no (C), Ac-GlcNAc (**6**) or Ac-5SGlcNAc (**5**) administration at 50 μM (or 100 μM for PC12 cells) for 24 h. Upper panel, probed with anti-*O*-GlcNAc antibody (CTD110.6); lower panel, anti-actin antibody. Cell lines used: COS-7 (African green monkey kidney cell line), CHO (Chinese hamster ovary cell line), SK-N-SH (human neuroblastoma cell line), HepG2 (human liver carcinoma cell line), PC12 (rat adrenal medulla pheochromocytoma cell

line, which terminally differentiate upon nerve growth factor treatment), mouse hybridoma cell line and EMEG32$^{-/-}$ (mouse embryonic fibroblasts deficient in glucosamine-6-phosphate acetyltransferase (GAT, shown in Figure 1b)).

**Supplementary Figure 11. Full versions of blots shown in Figure 3; evaluation of the effects of Ac-5SGlcNAc treatment of cells on the *O*-GlcNAc modification state of nup62.** (a) Full western blots of immunoprecipitated nup62 from cell lysates following vehicle (C) or 250 μM Ac-5SGlcNAc (**5**) treatment for 24 h. Following immunoprecipitation, nup62 was incubated with UDP-GalNAz in the absence (-) or presence (+) of GalT1 and chemoselectively labelled. Upper panel, probed with anti-nup62 antibody; lower panel, probed with streptavidin. (b) Full western blots of immunoprecipitated nup62 from cell lysates following vehicle (C) or 250 μM Ac-5SGlcNAc (**5**) treatment for 24 h. Following immunoprecipitation, nup62 was incubated with buffer (-) or with *Bt*GH84 (+) for 2 h to remove *O*-GlcNAc. Upper panel, probed with anti-nup62 antibody; lower panel, probed with anti-*O*-GlcNAc antibody (CTD110.6) (long exposure at top and short exposure below). (c) Full western blots of COS-7 cell lysates following Ac-5SGlcNAc (**5**) administration at different doses (0-1000 μM) for 24 h. Upper panel, probed with anti-nup62 antibody; lower panel, probed with anti-*O*-GlcNAc antibody (CTD110.6).

**Supplementary Figure 12. NMR spectra of Ac-5SGlcNAz (14).** (a) $^{1}$H-NMR spectrum of Ac-5SGlcNAz (**14**). (b) $^{13}$C-NMR spectrum of Ac-5SGlcNAz (**14**). Both were taken in $CDCl_3$.

**Supplementary Figure 13. Full version of blot shown in Figure 4c; metabolic feeding of Ac-5SGlcNAz (14) to cells and subsequent chemoselecctive ligation demonstrates there**

**is no accumulation of 5SGlcNAz (13) on nup62.** Full western blot of immunoprecipitated nup62 from cell lysates following vehicle (C), 50 μM Ac-GlcNAz (**15**) or Ac-5SGlcNAz (**14**) treatment for 24 h. Following immunoprecipitation, nup62 was incubated with buffer (-) or with *Bt*GH84 (+) for 2 h to remove *O*-GlcNAc, and then underwent the Staudinger ligation with biotin phosphine. Blots are probed with streptavidin-HRP.

**Supplementary Figure 14. Analysis of nucleotide sugars by capillary electrophoresis and the effects of treating cells with Ac-5SGlcNAc (5) on nucleotide sugar pools.** (a) UDP-sugar analysis by CE to validate identity of peaks from cells; trace shows absorbance at 254 nm as a function of retention time. Run 1, UDP-5SGlcNAc and UDP-5SGalNAc standards; run 2, UDP-GlcNAc and UDP-GalNAc standards; run 3, UDP-sugars extracted from COS-7 cells following treatment with 250 μM Ac-5SGlcNAc for 24 h; run 4, sample from run 3 spiked with standards from run 1; run 5, sample from run 3 spiked with standards from run 2. (A) GDP-Glc (internal standard); (B) UDP-GlcNAc (**2**); (C) UDP-5SGalNAc; (D) UDP-5SGlcNAc (**4**); (E) UDP-GalNAc. (b) UDP-5SGlcNAc (**4**) and UDP-Gal co-eluted during CE analysis. The contribution from each molecule to the peak could be calculated based on the concentration of UDP-GlcNAc (**2**) and UDP-5SGal, and the epimeric ratio determined from the standards of 2.1:1 GlcNAc:GalNAc. This graph shows a comparison of the total peak area compared to the sum of the estimated peak areas from the epimeric ratios for each concentration of Ac-5SGlcNAc (**5**) administered to cells. (c) Monitoring UDP-5SGlcNAc (**4**) and UDP-5SGalNAc *in vitro* by capillary electrophoresis; trace shows absorbance at 254 nm as a function of retention time. Run 1, UDP-5SGlcNAc (**4**); run 2, UDP-5SGlcNAc (**4**) treatment with UDP-GlcNAc 4-epimerase; run 3, UDP-GlcNAc (**2**) and UDP-GalNAc standards. (A) GDP-Glc (internal standard); (B) UDP-GlcNAc (**2**); (C) UDP-5SGalNAc; (D) UDP-5SGlcNAc (**4**); (E) UDP-GalNAc.

**Supplementary Figure 15. Effect of Ac-GlcNAc (5) treatment on cell surface glycosylation and *N*-glycosylation of a secreted IgG.** (a) Full western blots of COS-7 cell lysates following Ac-5SGlcNAc (**5**) administration at different doses (0-1000 μM) for 24 h. C+ denotes untreated cell lysate incubated with PNGase F and C- denotes untreated cell lysate incubated with vehicle. Blots are probed with (from top to bottom) anti-*O*-GlcNAc antibody (CTD110.6), anti-actin antibody, ConA lectin (recognizes α-D-mannose, α-D-glucose and branched mannose), GNA lectin (recognizes mannose), PHA-L (recognizes complex branched chain oligosaccharide structure), SNA lectin (recognizes sialic acid α(2,6)Gal/GalNAc) and MAA lectin (recognizes sialic acid α(2,3)Gal). (b) Full western blots of mouse hybridoma cell lysates (*O*-GlcNAc) and immunoprecipitated mouse hybridoma antibody (IgG, ConA and GNA) following administration of Ac-5SGlcNAc (**5**) at different doses (0-1000 μM) for 24 h. C+, untreated cell lysate incubated with PNGase F; C-, untreated cell lysate incubated with vehicle. Blots are probed with (from top to bottom) anti-*O*-GlcNAc antibody (CTD110.6), anti-IgG antibody, ConA lectin and GNA lectin.

**References:**


1. Leiting, B., Pryor, K.D., Eveland, S.S. & Anderson, M.S. One-day enzymatic synthesis and purification of UDP-N-[1-$^{14}$C]acetyl-glucosamine. *Anal Biochem* **256**, 185-191 (1998).
2. Rabina, J. *et al.* Analysis of nucleotide sugars from cell lysates by ion-pair solid-phase extraction and reversed-phase high-performance liquid chromatography. *Glycoconj J* **18**, 799-805 (2001).
3. Chien, R.L. & Burgi, D.S. Field-amplified polarity-switching sample injection in high-performance capillary electrophoresis. *J Chromatogr* **559**, 153-161 (1991).
4. Martinez-Fleites, C. *et al.* Structure of an O-GlcNAc transferase homolog provides insight into intracellular glycosylation. *Nat Struct Mol Biol* **15**, 764-765 (2008).
5. Leatherbarrow, R.J. *GraFit Version 5*, (Erithacus Software Ltd., Horley, UK, 2001).
6. Tomiya, N., Ailor, E., Lawrence, S.M., Betenbaugh, M.J. & Lee, Y.C. Determination of nucleotides and sugar nucleotides involved in protein glycosylation by high-performance anion-exchange chromatography: sugar nucleotide contents in cultured insect cells and mammalian cells. *Anal Biochem* **293**, 129-137 (2001).
7. Turnock, D.C. & Ferguson, M.A. Sugar nucleotide pools of *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major*. *Eukaryot Cell* **6**, 1450-1463 (2007).
8. Feng, H.T., Wong, N., Wee, S. & Lee, M.M. Simultaneous determination of 19 intracellular nucleotides and nucleotide sugars in Chinese Hamster ovary cells by capillary electrophoresis. *J Chromatogr B Analyt Technol Biomed Life Sci* **870**, 131-134 (2008).
9. Hasegawa, A. & Kiso, M. A simple preparation of 2-(acylamino)-2-deoxy-3,4:5,6-di-O-isopropyladine-*aldehydo*-D-glucose dimethyl and dibenzylacetal. *Carbohydr Res* **79**, 265-270 (1980).

10. Tanahashi, E., Kiso, T. & Hasegawa, A. A facile synthesis of 2-acetamido-2-deoxy-5-thio-D-glucopyranose. *Carbohydr Res* **117**, 304-308 (1983).

11. Damager, I., Numao, S., Chen, H., Brayer, G.D. & Withers, S.G. Synthesis and characterisation of novel chromogenic substrates for human pancreatic alpha-amylase. *Carbohydr Res* **339**, 1727-1737 (2004).

12. Baudry, M., Barberousse, V., Descotes, G., Pires, J. & Praly, J.P. Synthetic studies in the 5-thio-D-xylopyranose series part 2: Coupling of 5-thio-D-xylopyranosyl donors with electron-rich aryl moieties: Access to C-aryl 5-thio-D-xylopyranosides. *Tet Lett* **54**, 7447-7456 (1998).

13. Hashimoto, H. & Izumi, M. Efficient and stereoselective 1,2-cis glycoside formation of 5-thioaldopyranoses: glycosylation with peracetylated 5-thio-D-arabinopyranosyl and 5-thio-L-fucopyranosyl trichloroacetimidates. *Tet Lett* **34**, 4949-4952 (1993).

14. Tsuruta, O., Yuasa, H., Hashimoto, H., Kurono, S. & Yazawa, S. Affinity of 5-thio-L-fucose-containing Lewis X (LeX) trisaccharide analogs to anti-LeX monoclonal antibody. *Bioorg Med Chem Lett* **9**, 1019-1022 (1999).

15. Yuasa, H., Suga, Y. & Hashimoto, H. Glycosidation reactions of 5-thioxylpyranosyl Donors. *Lett Org Chem* **5**, 429-431 (2008).

16. Driguez, H. & Henrissat, B. A novel synthesis of 5-thio-D-glucose. *Tet Lett* **22**, 5061-5062 (1981).

17. Tsuda, Y. *et al.* Thio Sugars. I. Radical promoted thione-thiol rearrangement of cyclic thionocarbonates: synthesis of 5-thioglucose. *Chem Pharm Bull* **44**, 1465-1475 (1996).

18. Csuk, R. & Glanzer, B.I. A short synthesis of 2-acetimido-2-deoxy-5-thio-D-glucose and D-mannose from 5-thio-glucal. *J Chem Soc Chem Comm*, 343-344 (1986).

19. Taggi, A.E. *et al.* The development of the first catalyzed reaction of ketenes and imines: catalytic, asymmetric synthesis of beta-lactams. *J Am Chem Soc* **124**, 6626-6635 (2002).