Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Information

N-propargyloxycarbamate monosaccharides as metabolic chemical reporters of carbohydrate salvage pathways and protein glycosylation

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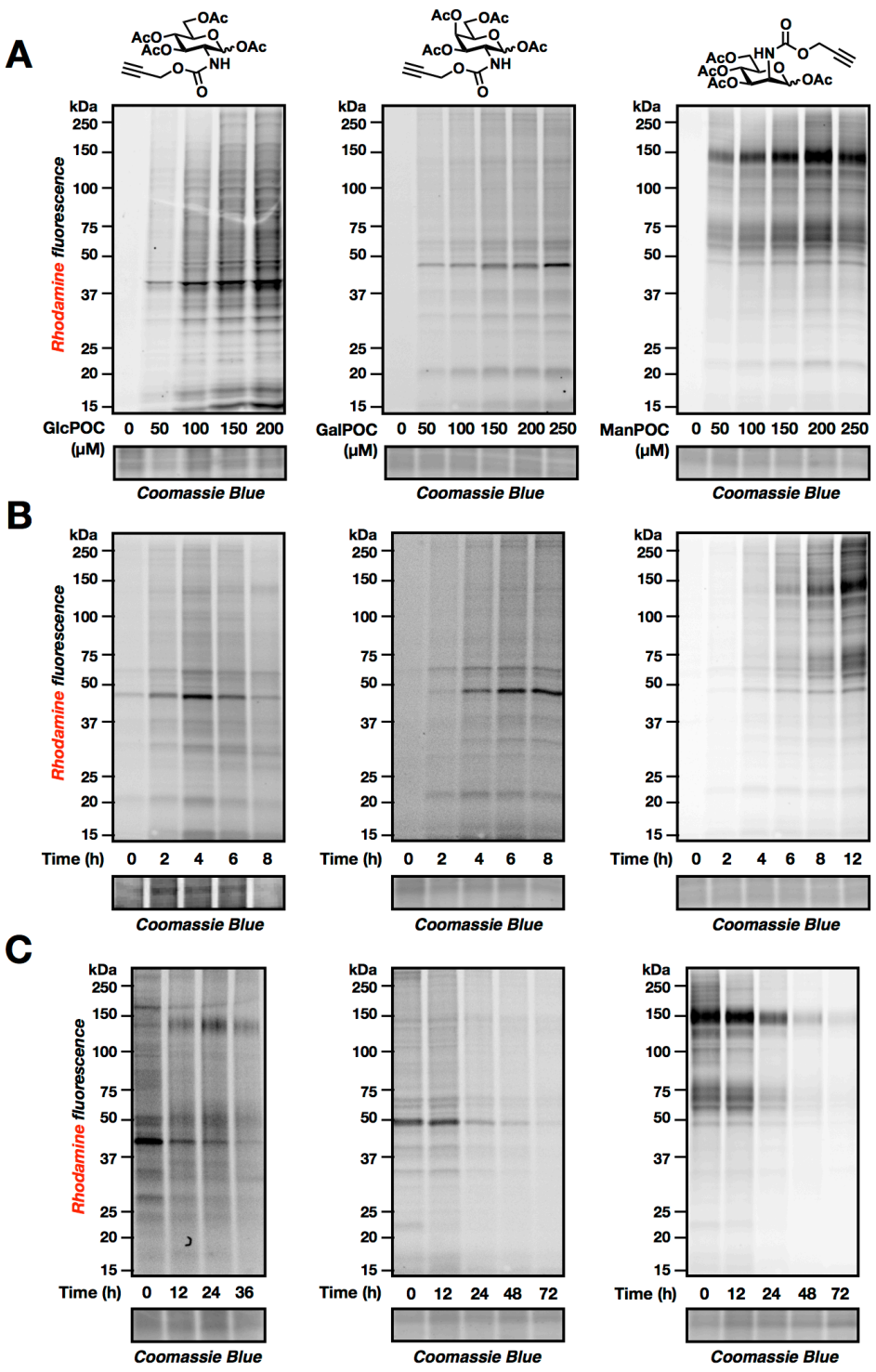

Fig. S1. Characterization of *N*-propargyloxycarbamate (Poc) bearing metabolic chemical reporters. A) NIH3T3 cells were treated with the indicated concentrations of Ac_4GlcPoc, Ac_4GalPoc, or Ac_4ManPoc for 16 hours, followed by analysis by in-gel fluorescence scanning. B) NIH3T3 cells were treated with 150 μM Ac_4GlcPoc, Ac_4GalPoc, or Ac_4ManPoc for the indicated times before analysis by in-gel fluorescence scanning. C) NIH3T3 cells were treated with 150 μM Ac_4GlcPoc, Ac_4GalPoc, or Ac_4ManPoc and chased with 150 μM Ac_4GlcNAc, Ac_4GalNAc, or Ac_4ManNAc, respectively, followed by in-gel fluorescence scanning. Coomassie blue staining demonstrates protein loading.

Materials and Methods

All reagents used for chemical synthesis were purchased from Sigma-Aldrich unless otherwise specified and used without further purification. All anhydrous reactions were performed under argon atmosphere. Analytical thin-layer chromatography (TLC) was conducted on EMD Silica Gel 60 F_{254} plates with detection by potassium permanganate ($KMnO_4$), anisaldehyde or UV. For flash chromatography, 60 Å silica gel (EMD) was utilized. ^1H spectra were obtained at 600 MHz or 500 MHz on a Varian VNMRS-600 or AMX-500. Chemical shifts are recorded in ppm (δ) relative to $CHCl_3$ (7.26 ppm) for spectra acquired in $CDCl_3$ or methanol. ^{13}C spectra were obtained at 150 or 125 MHz on the same instruments.

Synthesis of chemical reporters

Known compounds, az-rho, and azido-azo-biotin were synthesized according to literature procedures.[1]

***N*-propargyloxycarbamate-1,3,4,6-tetra-*O*-acetyl-glucosamine (Ac$_4$GlcPoC):** Glucosamine HCl (1 g, 4.6 mmol), and sodium bicarbonate (0.7 g, 7.88 mmol, Mallinckrodt) were dissolved in H_2O (10 mL). To the stirring solution, propargyl chloroformate (679 µL, 6.96 mmol) was added dropwise. The solution was allowed to stir for 16 h at room temperature. The reaction mixture was concentrated and washed with methanol (10 mL), and filtered. Resulting filtrate was concentrated and dissolved in pyridine (10 mL) and stirred. Acetic anhydride (1.8 mL, 19.14 mmol) was then added and allowed to stir for 16 h at room temperature. Purification by silica gel column chromatography (45:55, ethyl acetate:hexanes) afforded the product (853.2 mg, 52% yield) as a white solid. ^1H NMR (600 MHz, $CDCl_3$) δ 5.63 (d, J = 8.3 Hz, 1H), 5.11 (t, J = 9.8 Hz, 1H), 5.05 (t, J = 9.6 Hz, 1H), 4.90 (d, J = 8.1 Hz, 1H), 4.59 (s, 1ifH), 4.22 (dd, J = 12.5, 4.4 Hz, 1H), 4.05 (dd, J = 12.4, 1.7 Hz, 1H), 4.00 (t, J = 6.7 Hz, 1H), 3.87 (dd, J = 19.0, 9.4 Hz, 1H), 3.75 (dd, J = 9.9, 4.6, 2.2 Hz, 1H), 2.39 (s, 1H), 2.06 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.97 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.78, 169.48, 155.00, 92.60, 77.96, 73.00, 72.36, 68.01, 64.51, 61.76, 55.16, 53.07, 21.02, 20.87, 20.78, 20.73.

***N*-propargyloxycarbamate-1,3,4,6-tetra-*O*-acetyl-galactosamine (Ac$_4$GalPoC):** Galactosamine HCl (100 mg, 0.46 mmol, Carbosynth), and sodium bicarbonate (133 mg, 1.58 mmol, Mallinckrodt) were dissolved in H_2O (2.3 mL) and 1,4-dioxane (4 mL). To the stirring solution, propargyl chloroformate (68 µL, 0.7 mmol) was added dropwise. The solution was allowed to stir for 16 h at room temperature. The reaction mixture was concentrated and washed with methanol (10 mL), and filtered. Resulting filtrate was concentrated and purified by silica gel column chromatography (20:80, methanol, methylene chloride). Resulting product was dissolved in pyridine (704 µL, 8.7 mmol) and stirred. Acetic anhydride (328 µL, 3.48 mmol) was then added and allowed to stir for 16 h at room temperature. Reaction mixture was concentrated and pyridine was removed. Extracted with 1 M HCl, saturated sodium bicarbonate, water and brine. Purification by silica gel column chromatography (45:55, ethyl acetate:hexanes) afforded the product (118 mg, 79% yield) as a white solid. ^1H NMR (500 MHz, $CDCl_3$) δ 6.22 (d, 1H), 5.41 (d, J = 2.6 Hz, 1H), 5.17 (dd, J = 11.6, 2.9 Hz, 1H), 4.88 (d, J = 9.7 Hz, 1H), 4.73 – 4.58 (m, 2H), 4.41 (td, J = 11.4, 3.5 Hz, 1H), 4.22 (dd, J = 13.5, 6.7 Hz, 1H), 4.10 – 4.02 (m, 2H), 2.48 (t, J = 2.0

Hz, 1H), 2.16 (s, 6H), 2.01 (s, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.93, 170.47, 170.27, 169.96, 168.93, 155.01, 91.45, 75.16, 68.67, 68.09, 66.83, 61.35, 53.14, 48.92, 21.03, 20.81, 20.76, 20.74.

***N*-propargyloxycarbamate-1,3,4,6-tetra-*O*-acetyl-manosamine (Ac_4ManPoC):** Mannosamine HCl (100 mg, 0.46 mmol. Carbosynth), and sodium bicarbonate (133 mg, 1.58 mmol, Mallinckrodt) were dissolved in H_2O (2.3 mL) and 1,4-dioxane (4 mL). To the stirring solution, propargyl chloroformate (68 µL, 0.7 mmol) was added dropwise. The solution was allowed to stir for 16 h at room temperature. The reaction mixture was concentrated and washed with methanol (10 mL), and filtered. Resulting filtrate was concentrated and purified by silica gel column chromatography (15:85, methanol, methylene chloride). Resulting product was dissolved in pyridine (671 µL, 8.3 mmol) and stirred. Acetic anhydride (314 µL, 3.3 mmol) and DMAP (0.4 mg, 0.003 mmol) were then added and allowed to stir for 16 h at room temperature. Reaction mixture was concentrated and pyridine was removed. Extracted with 1 M HCl, saturated sodium bicarbonate, water and brine. Purification by silica gel column chromatography (50:50, ethyl acetate:hexanes) afforded the product (123.5 mg, 86% yield) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.03 (d, J = 1.6 Hz, 1H), 5.28 – 5.17 (m, 2H), 4.97 (dd, J = 9.8, 3.9 Hz, 1H), 4.69 – 4.60 (m, 1H), 4.28 (dd, J = 8.5, 3.4 Hz, 1H), 4.23 – 4.15 (m, 2H), 4.07 – 3.93 (m, 3H), 2.44 (t, J = 2.2 Hz, 1H), 2.04 (s, 6H), 1.99 (s, 6H).^{13}C NMR (125 MHz, $CDCl_3$) δ 170.66, 170.07, 169.59, 168.09, 155.06, 91.70, 75.10, 73.34, 70.17, 69.00, 65.27, 62.00, 53.03, 51.27, 20.83, 20.71, 20.60.

Cell culture

NIH3T3 cells were cultured in high glucose DMEM media (Cellgro) with 10% fetal calf serum (FCS, Cellgro) and were maintained in a humidified incubator at 37 °C and 5.0% CO_2.

Metabolic labeling

To cells at 80-85% confluency, high glucose media containing PoC analog (1,000 x stock in DMSO), or DMSO vehicle was added as indicated. For chase experiments, media was supplemented with 150 µM GlcNAc, GalNAc or ManNAc.

Preparation of NP-40-soluble lysates

The cells were collected by scraping and pelleted by centrifugation at at 4 °C for 2 min at 2,000 x g, followed by washing with PBS (1 mL) two times. Cell pellets were then resuspended and lysed in 75 µl of 1% NP-40 lysis buffer [1% NP-40, 150 mM NaCl, 50 mM triethanolamine (TEA) pH 7.4] with Complete Mini protease inhibitor cocktail (Roche Biosciences) for 15 min and followed by centrifugation at 4 °C for 10 min at 10,000 x g. The resulting supernatant (soluble cell lysate) was collected and separated to determine protein concentration via BCA assay (Pierce, ThermoScientific).

Cu(I)-catalyzed [3 + 2] azide-alkyne cycloaddition (CuAAC)

Soluable cell lysate (200 µg) was diluted with cold 1% NP-40 lysis buffer to a concentration of 1 µg/µL. Newly made click chemistry cocktail (12 µL) was added to each sample [azido-rhodamine tag (100 µM, 10 mM stock solution in DMSO); tris(2-carboxyethyl)phosphine hydro-

chloride (TCEP) (1 mM, 50 mM freshly prepared stock solution in water); tris[(1-benzyl-1-*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) (100 μM, 10 mM stock solution in DMSO); $CuSO_4 \cdot 5H_2O$ (1 mM, 50 mM freshly prepared stock solution in water) for a total reaction volume of 200 μL. The reaction was gently vortexed and allowed to sit at room temperature for 1 h. Upon completion, 1 mL of ice cold methanol was added to the reaction, and proteins were precipitated at -20 °C for 2 h. The reactions were then centrifuged at 4 °C for 10 min at 10,000 x g. The supernatant was removed, the pellet was allowed to air dry for 5 min, and then 50 μL 4% SDS buffer (4% SDS, 150 mM NaCl, 50 mM TEA pH 7.4) was added to each sample. The mixture was sonicated in a bath sonicator to ensure complete dissolution, and 50 μL of 2x loading buffer (20% glycerol, 0.2% bromophenol blue, 1.4% β-mercaptoethanol) was then added. The samples were boiled for 5 min at 98 °C, and 40 μg of protein was then loaded per lane for SDS-PAGE separation (Any kD Criterion Gel, Bio-Rad).

In-gel Fluorescence Scanning

The gel was scanned on a Molecular Imager FX (Bio-Rad) using a 580 nm laser for excitation and a 620 nm bandpass filter for detection.

Biotin Enrichment

NIH3T3 cell pellets labeled with GlcPoC, GalPoC, ManPoC (150 μM) or DMSO were resuspended in 13 μL H_2O, and 25 μL 0.05% SDS buffer (0.05% SDS, 10 mM TEA pH 7.4, 150 mM $MgCl_2$) with Complete Mini protease inhibitor cocktail (Roche Biosciences). To this was added 1 μL Benzonase (Sigma), and the cells were incubated on ice for 30 min. At this time, 4% SDS buffer (100 μL) was added, and the cells were briefly sonicated in a bath sonicator and collected by centrifugation at 20,000 x g for 10 min at 15 °C. Protein concentration was normalized by BCA assay (Pierce, ThermoScientific) to 1 mg/mL (1 mg total cell lysate). The appropriate amount of click chemistry cocktail was added and the reaction was allowed to proceed for 1 h, after which time 10 volumes of ice-cold methanol were added. Precipitation proceeded 2 hours at -20 °C. Precipitated proteins were centrifuged at 5,200 x g for 30 min at 0 °C and washed 3x with 10 mL ice-cold MeOH, with resuspension of the pellet each time. The pellet was then air-dried for 1 h. To capture the biotinylated proteins by streptavidin beads, the air-dried protein pellet was resuspended in 400 μL of resuspension buffer (6 M urea, 2 M thiourea, 10 mM HEPES pH 8.0) by bath sonication. Samples were then transferred to 2 mL dolphin-nosed tubes containing streptavidin beads () that were pre-washed beads were washed 2x with PBS (1 ml) and 1x with resuspension buffer (2,000 x g, 2 min). Samples were then incubated on a rotator for 2 h. Beads were washed 2x with resuspension buffer, 2x in PBS (1 ml) and 2x with 1% SDS in PBS buffer (2,000 x g, 2 min). Beads were then incubated in 25 μL of sodium dithionite solution (1% SDS, 25 mM sodium dithionite) for 30 min at room temperature to elute captured proteins. The beads were centrifuged for 2 min at 2,000 x g and the eluent collected. The elution step was repeated, and the eluents combined. Protein was precipitated in ice cold methanol (1 mL) overnight in -20 °C. Protein was collected by centrifugation (10 min, 10,000 x g, 4 °C), and the pellet was allowed to air dry for 5 min, and then 30 μL 4% SDS buffer (4% SDS, 150 mM NaCl, 50 mM TEA pH 7.4) was added to each sample. The mixture was sonicated in a bath sonicator to ensure complete dissolution, and 30 μL of 2x loading buffer (20% glycerol, 0.2% bromophenol blue,

1.4% β-mercaptoethanol) was then added. The samples were boiled for 5 min at 98 °C, and 60 µg of protein was then loaded per lane for SDS-PAGE separation (Any kD Criterion Gel, Bio-Rad).

Western Blotting

Proteins were separated by SDS-PAGE before being transferred to PVDF membrane (Bio-Rad) using standard western blotting procedures. Briefly, all western blots were blocked in TBST (0.1% Tween-20, 150 mM NaCl, 10mM Tris pH 8.0) containing 5% non-fat milk for 1 h at rt. They were then incubated with the appropriate primary antibody in blocking buffer overnight at 4 °C. The anti-NEDD4 WW2 antibody (Millipore) was used at a 1:10,000 dilution. The blots were then washed three times in TBST and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h in blocking buffer at RT. HRP-conjugated anti-mouse and anti-human antibodies (Jackson ImmunoResearch) were used at 1:10,000 dilutions. After being washed three more times with TBST, the blots were developed using ECL reagents (Bio-Rad) and the ChemiDoc XRS+ molecular imager (Bio-Rad).

Flow Cytometry

NIH3T3 cells were treated with GlcPoC, GalPoC, ManPoC (150 µM) or DMSO for 16 h. Cells were collected and washed 2X with cold PBS and fixed with 3.7% PFA in PBS for 10 min. Cells were then washed 1x with 2% FCS in PBS and permeabilized (0.1% Triton X-100 in PBS for 10 min at room temperature). Cell were washed with PBS and blocked for 10 min with 2% FCS in PBS. Cells were resuspended in 100 µL PBS that contained 100 µM Az-Rho, 1 mM TCEP, 100 µM TBTA and 1 mM $CuSO_4 \bullet 5H_2O$. Samples were incubated in the dark for 1 h and washed 5x with 1% Tween-20 and 0.5 mM EDTA in PBS and 1x with 2% FCS in PBS. Flow cytometry analysis was then performed on a Beckman Coulter LSR II at USC Flow Cytometry Core.

Bibliography

1. G. Charron, M. M. Zhang, J. S., Yount, J. Wilson, A. S. Raghavan, E. Shamir, H. C. Hang, *J Am Chem Soc*, 2009, **131**, 4967-4975.