Diazo Groups Endure Metabolism and Enable Chemoselectivity *in Cellulo*

Kristen A. Andersen, Matthew R. Aronoff, Nicholas A. McGrath, and Ronald T. Raines*

Page	Contents

Materials. Silica gel (40 μm) was from SiliCycle (Québec City, Canada). All reagent-grade materials were from Sigma–Aldrich (St. Louis, MO) and were used without further purification, except for D-mannosamine·HCl which was from CarboSynth (San Diego, CA). Alexa Fluor® reagents, and cell culture medium and supplements were from Invitrogen (Carlsbad, CA).

Instrumentation. ^{1}H, ^{13}C NMR spectra for all compounds were acquired at ambient temperature on Bruker Spectrometers in the National Magnetic Resonance Facility at Madison (NMRFAM) at the University of Wisconsin–Madison operating at 400, 500, or 750 MHz for ^{1}H and 126 or 189 MHz for ^{13}C. Chemical shift data are reported in units of δ (ppm) relative to residual solvent or TMS. Electrospray ionization (ESI) mass spectrometry was performed with a Micromass LCT at the Mass Spectrometry Facility in the Department of Chemistry at the University of Wisconsin–Madison.

Super-resolution structured illumination microscopy (SR-SIM) was performed with an Elyra PS.1 super-resolution system from Zeiss (Oberkochen, Germany). Confocal microscopy was carried out with an Eclipse TE2000-U laser scanning confocal microscope from Nikon (Tokyo, Japan), equipped with an AxioCam digital camera from Zeiss. Flow cytometry was performed at the University of Wisconsin–Madison Carbone Cancer Center Flow Cytometry Facility with a FACS Calibur instrument from BD Biosciences (San Jose, CA). Cytometry data were analyzed by using the program FlowJo 8.7 from Treestar (Ashland, Oregon). Absorbance measurements were made with an infinite M1000 plate reader from Tecan (Männedorf, Switzerland). Immunoblots were imaged on an ImageQuant LAS4000 from GE Healthcare Bio-Sciences (Pittsburgh, PA)**.** Image quantification was performed with the program ImageJ.

Statistics. Calculations were performed with GraphPad Prism version 6 software from GraphPad Software (La Jolla, CA).

Computational Methods. Calculations were performed using Gaussian software (Wallingford, CT). Structures were first constructed and calculated for geometry optimization at routine HF/6-31G basis set. Optimized structures were then subjected to full natural bond order (NBO) computations. Output files were exported to PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC. Solvent-accessible surface areas were discretely approximated using the "get_area" selection command of PyMOL for each of the NBO outputs for the acetamide, diazoacetamide, and the azidoacetamide model compounds.

Solvent-Accessible Surface Area

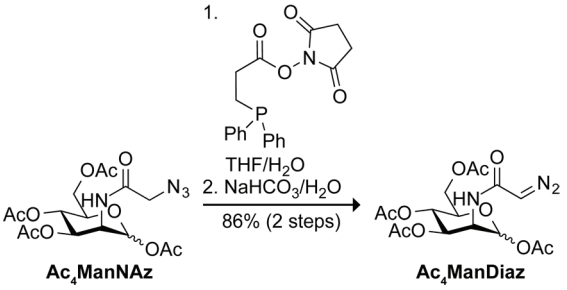
acetamide	85.698 A^2
diazoacetamide	101.109 A^2
azidoacetamide	117.310 A^2

Caution. Diazo compounds can be unstable and thus dangerous. The diazo-containing compounds in our work are, however, stabilized by their conjugation with an acetamido group and do not pose the same dangers as do primary diazoalkanes such as diazomethane. Milligram quantities of diazo compound **2** (R=Bn) were subjected to (1) impact tests, (2) vigorous grinding with a mortar and pestle, and (3) heating to >150 °C, as described previously.[1] None of these procedures led to an explosion. Moreover, diazoacetamide compounds used in this work have been stored as solids at –20 °C for >1 year with no apparent decomposition or deterioration. Nevertheless, caution should be invoked upon handling any diazo compound.

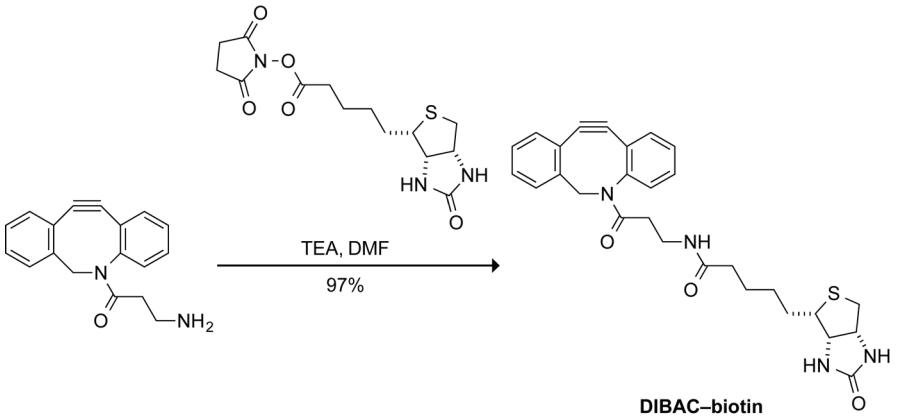
Synthesis. The phrase "concentrated under reduced pressure" refers to the removal of solvents and other volatile materials using a rotary evaporator at water aspirator pressure (<20 torr) while maintaining the water-bath temperature below 40 °C. Residual solvent was

removed from samples at high vacuum (<0.1 torr).

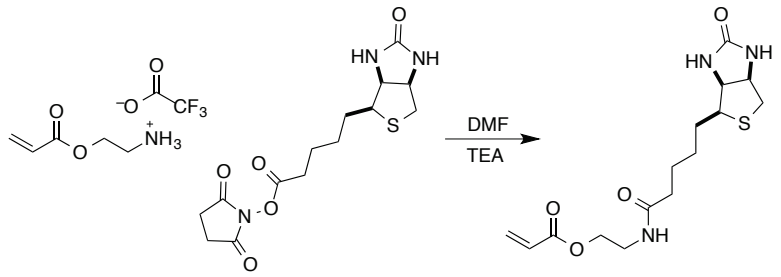
Ac_4ManAc and Ac_4ManNAz were synthesized as reported previously,[2] as was Ac_4ManKyne.[3] *N*-Benzyl-2-azidoacetamide (**1**, R=Bn) and *N*-benzyl-2-diazoacetamide (**2**, R=Bn) were prepared as reported previously.[4,5] The ^{1}H and ^{13}C NMR spectra of diazo compound **2** (R=Bn) were recorded in acetonitrile. ^{1}H NMR (400 MHz, CD_3CN) δ 7.38–7.21 (m, 6H), 6.53 (s, 1H), 5.02 (s, 1H), 4.37 (d, J = 6.1 Hz, 2H). ^{13}C NMR (101 MHz, CD_3CN) δ 166.48, 140.63, 129.50, 128.31, 128.04, 47.47, 43.96. Starting materials for the synthesis of biotinylated compounds (2-(acryloyloxy)ethanaminium trifluoroacetate[6] and diazoacetamide–NHS ester[7] were also prepared as reported previously. NMR spectroscopy and mass spectrometry data matched those in the literature.



Tetra-*O*-acetyl-*N*-2-diazoacetyl-D-mannosamine (Ac_4ManDiaz). Tetra-*O*-acetyl-*N*-2-azidoacetyl-D-mannosamine (Ac_4ManNAz)[2] (285 mg, 0.662 mmol) was dissolved in 90:10 THF/H_2O (5 mL). *N*-Succinimidyl 3-(diphenylphosphino)propionate[3] (247 mg, 0.695 mmol) was added, and the resulting solution was stirred for 5 h. A saturated solution of $NaHCO_3$(aq) (4 mL) was added, and the resulting solution was stirred for an additional 6 h. The reaction mixture was diluted with brine (20 mL) and extracted with dichloromethane (4 × 30 mL), dried over Na_2SO_4(s), and concentrated under reduced pressure to a yellowish solid. The residue was purified by chromatography on silica gel (30:70 EtOAc/hexanes) to provide Ac_4ManDiaz as a yellow solid (236 mg, 86%) (~1:1 mixture of α/β anomers). ^{1}H NMR (750 MHz, CD_3Cl) δ 6.03 (s, 1H), 5.98 (d, J = 9.3 Hz, 1H), 5.86 (s, 1H), 5.73 (d, J = 9.1 Hz, 1H), 5.33 (dd, J = 10.3, 4.5 Hz, 1H), 5.17 (t, J = 10.3 Hz, 1H), 5.11 (t, J = 9.8 Hz, 1H), 5.06 (dd, J = 10.0, 3.9 Hz, 1H), 5.00 (s, 1H), 4.96 (s, 1H), 4.88–4.81 (m, 1H), 4.71 (s, 1H), 4.29 (td, J = 12.1, 5.5 Hz, 2H), 4.09–4.01 (m, 3H), 3.81 (ddd, J = 8.4, 5.3, 2.3 Hz, 1H), 2.18 (s, 3H), 2.12 (s, 3H), 2.09 (s, 6H), 2.06 (s, 6H), 2.03 (s, 6H). ^{13}C NMR (189 MHz, $CDCl_3$) δ 170.64, 170.60, 170.18, 170.10, 169.77, 169.75, 168.45, 168.22, 91.93, 90.82, 73.45, 71.47, 70.16, 69.02, 65.71, 65.44, 62.34, 62.11, 49.77, 47.58, 45.78, 42.22, 20.87, 20.80, 20.77, 20.75, 20.73, 20.66, 20.64. HRMS (ESI) calcd. for $C_{16}H_{21}N_3O_{10}$ $[M + H]^+$ 416.1300, found 416.1310.

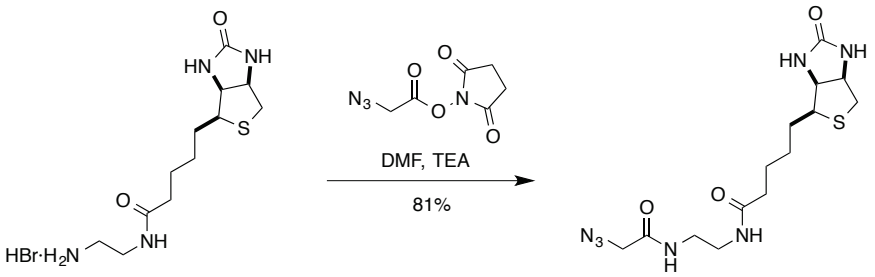


DIBAC–biotin. DIBAC (0.022 g, 0.078 mmol) was dissolved in 1 mL of anhydrous DMF. Triethylamine (0.022 mL, 0.159 mmol) and biotin–NHS (0.03 g, 0.084 mmol) were added, and the resulting solution was stirred for 7 h. The reaction mixture was then concentrated under reduced pressure. The residue was dissolved in dichloromethane (5 mL), washed with brine (2 × 5 mL), dried over Na_2SO_4(s), and concentrated under reduced pressure. The residue was purified by chromatography on silica gel (90:10, DCM/MeOH) to give the DIBAC–biotin conjugate. (0.039 g, 97%). ^{1}H NMR (750 MHz, DMSO-d_6) δ 7.63 (d, J = 7.6 Hz, 1H), 7.59 (td, J = 7.8, 6.1, 3.8 Hz, 2H), 7.51–7.49 (m, 1H), 7.48–7.44 (m, 2H), 7.39 (td, J = 7.5, 1.4 Hz, 1H), 7.35 (t, J = 7.5 Hz, 1H), 7.30 (d, J = 7.5 Hz, 1H), 6.40–6.38 (s, 1H), 6.35 (s, 1H), 5.04 (d, J = 14.2 Hz, 1H), 4.30 (t, J = 6.4 Hz, 1H), 4.10 (dt, J = 5.2, 2.4 Hz, 1H), 3.63 (d, J = 14.2 Hz, 1H), 3.1 –3.02 (m, 2H), 2.94–2.88 (m, 1H), 2.81 (dd, J = 12.5, 5.2 Hz, 1H), 2.57 (d, J = 12.5 Hz, 1H), 2.44–2.38 (m, 1H), 1.91 (t, J = 7.6 Hz, 2H), 1.85–1.79 (m, 1H), 1.55 (m, 1H), 1.44–1.33 (m, 3H), 1.26–1.15 (m, 2H).^{13}C NMR (126 MHz, DMSO) δ 171.91, 170.22, 162.75, 151.45, 148.44, 132.45, 129.59, 129.00, 128.28, 128.13, 127.80, 126.88, 125.27, 122.50, 121.44, 114.34, 108.12, 61.03, 59.21, 55.44, 54.87, 35.03, 34.94, 34.31, 28.19, 28.02, 27.99, 25.16. HRMS (ESI) calcd. for $C_{28}H_{30}N_4O_3S$ $[M + H]^+$ 503.2112, found 503.2105.

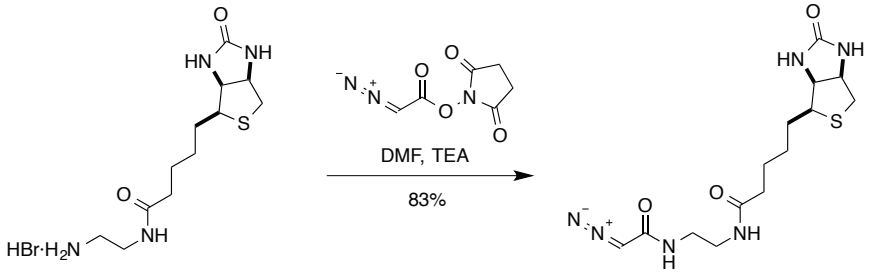


2-Aminoethyl Acrylate–biotin Conjugate (AEB). 2-(Acryloyloxy)ethanaminium trifluoroacetate[6] (0.041 g, 0.176 mmol) was dissolved in 1.5 mL of anhydrous DMF and followed by the addition triethylamine (0.051 mL, 0.44 mmol). Biotin–NHS was added (0.05 g, 0.146 mmol), and the resulting solution was stirred at overnight. The reaction mixture was

concentrated by rotary evaporation, and the residue was purified by chromatography on silica gel (DCM/MeOH 9:1) to give the acrylate–biotin conjugate. (0.049 g, 98%). ^{1}H NMR (500 MHz, MeOD) δ 6.37 (dd, J = 17.3, 1.5 Hz, 1H), 6.14 (dd, J = 17.3, 10.5 Hz, 1H), 5.86 (dd, J = 10.5, 1.5 Hz, 1H), 4.45 (dd, J = 7.8, 4.9 Hz, 1H), 4.26 (dd, J = 7.9, 4.5 Hz, 1H), 4.17 (t, J = 5.4 Hz, 2H), 3.43 (tt, J = 5.5, 3.1 Hz, 2H), 3.14 (d, J = 6.9 Hz, 1H), 2.88 (dd, J = 12.8, 5.0 Hz, 1H), 2.69–2.62 (m, 2H), 2.17 (t, J = 8.1 Hz, 1H), 1.74–1.50 (m, 4H), 1.43–1.35 (m, 2H). ^{13}C NMR (126 MHz, MeOD) δ 177.62, 168.82, 167.38, 133.11, 130.65, 65.41, 64.61, 62.88, 58.26, 42.31, 40.69, 37.94, 30.98, 30.74, 28.10. HRMS (ESI) calcd. for $C_{15}H_{23}N_3O_4S$ [M + H]$^+$ 342.1483, found 342.1486.

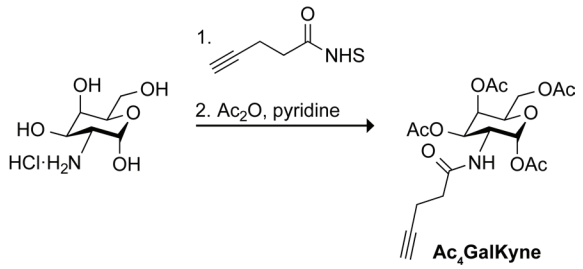


Azidoacetamide–biotin Conjugate (AB). Biotin ethylenediamine hydrobromide (0.040 g, 0.109 mmol) was dissolved in 1 mL of anhydrous DMF followed by triethylamine (0.030 mL, 0.22 mmol). The azidoacetamide–NHS ester (0.023 g, 0.114 mmol) was added, and the resulting solution was stirred overnight. The reaction mixture was concentrated under reduced pressure, and the residue was purified by chromatography on silica gel (DCM/MeOH 9:1) to give the azide–biotin conjugate. (0.033 g, 81%). ^{1}H NMR (500 MHz, MeOD) δ 4.46 (dd, J = 7.8, 4.8 Hz, 1H), 4.28 (dd, J = 7.9, 4.5 Hz, 1H), 3.87 (s, 2H), 3.28 (m, 5H), 2.90 (dd, J = 12.7, 5.0 Hz, 1H), 2.67 (d, J = 12.7 Hz, 1H), 2.17 (td, J = 7.3, 2.3 Hz, 2H), 1.77–1.49 (m, 4H), 1.41 (m, 2H). ^{13}C NMR (126 MHz, MeOD) δ 177.78, 171.78, 167.41, 64.61, 62.90, 58.25, 54.29, 42.31, 41.54, 41.06, 38.05, 31.03, 30.74, 28.02. HRMS (ESI) calcd. for $C_{14}H_{23}N_7O_3S$ [M + H]$^+$ 370.1656, found 370.1658.



Diazoacetamide–biotin Conjugate (DB). Biotin ethylenediamine hydrobromide (0.025 g, 0.068 mmol) was dissolved in 0.7 mL of anhydrous DMF. The resulting solution was cooled to

0 °C and stirred for 15 min before the addition of triethylamine (0.020 mL, 0.143 mmol). After stirring at 0 °C for 15 min, the diazo–NHS ester[6] (0.014 g, 0.0715 mmol) was added, and the resulting mixture was stirred at 0 °C for 30 min before warming to room temperature and stirring for an additional 16 h. The reaction mixture was then concentrated under reduced pressure, and the residue was purified by chromatography on silice gel (DCM/MeOH 9:1) to give the diazo–biotin conjugate (0.020 g, 83%). ^{1}H NMR (500 MHz, MeOD) δ 5.10 (s, 1H), 4.46 (dd, J = 7.8, 4.9 Hz, 1H), 4.27 (dd, J = 7.8, 4.4 Hz, 1H), 3.33–3.28 (m, 1H), 3.24 (m 1H), 2.89 (dd, J = 12.7, 5.0 Hz, 1H), 2.81 (d, J = 16.4 Hz, 1H), 2.70–2.64 (d, J = 12.7 Hz 1H), 2.63 (s, 2H), 2.17 (td, J = 7.3, 1.7 Hz, 2H), 1.75–1.50 (m, 4H), 1.45–1.35 (m, 2H). ^{13}C NMR (126 MHz, MeOD) δ 177.66, 170.20, 167.40, 64.61, 62.90, 58.25, 42.33, 41.65, 38.05, 31.00, 30.74, 28.04, 27.69, 27.54. HRMS (ESI) calcd. for $C_{14}H_{22}N_6O_3S$ $[M + Na]^+$ 377.1367, found 377.1370.



1,3,4,6-Tetra-*O*-acetyl-*N*-4-pentynoyl-D-galactosamine (Ac$_4$GalKyne) was prepared from 2-amino-2-deoxy-D-galactopyranose·HCl by using the procedure reported for 1,3,4,6-tetra-*O*-acetyl-*N*-4-pentynoyl-D-mannosamine (Ac$_4$ManKyne).[3] α/β Anomers ~10:1, α anomer: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.23 (d, J = 3.5 Hz, 1H), 5.75–5.66 (d, J = 8.5 Hz 1H), 5.43 (dd, J = 3.4, 1.3 Hz, 1H), 5.23 (dd, J = 11.6, 3.2 Hz, 1H), 4.82–4.71 (m, 1H), 4.29–4.21 (m, 1H), 4.15–4.04 (m, 2H), 2.50 (td, J = 7.2, 6.4, 2.6 Hz, 2H), 2.36 (tt, J = 7.2, 3.0 Hz, 2H), 2.18 (s, 6H), 2.04 (s, 3H), 2.03 (s, 3H).^{13}C NMR (126 MHz, $CDCl_3$) δ 171.23, 171.21, 170.53, 170.36, 168.94, 91.37, 82.68, 69.69, 68.64, 67.91, 66.75, 61.38, 46.88, 35.31, 21.12, 20.94, 20.83, 20.79, 14.87. HRMS (ESI) calcd. for $C_{19}H_{25}NO_{10}$ $[M+H]^+$ 428.1552, found 428.1541.

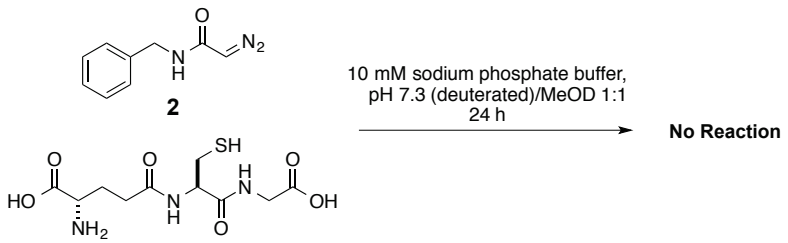
Reaction Rate Constants. NMR spectroscopy was used to determine rate constants for cycloaddition reactions, as described previously.[5] Briefly, an equimolar solution of reactants in CD_3CN were mixed in an NMR tube (final concentration: 0.02 M). The tube was inverted once and inserted into a spectrometer, and scanning was initiated 60 s after the initial mixing. A 16-scan NMR spectrum was acquired every 77 s, and integrations were used to calculate concentrations from the known initial concentrations. The second-order rate constant was then determined from the slope of a plot of $[\text{octyne}]^{-1}$ *vs* time. All NMR kinetics experiments were conducted in triplicate, and the reported rate constants are the mean (± SE).

Table S1. Rate constants ($M^{-1}s^{-1}$) for the reaction of Azide 1 (R=Bn) and Diazo Compound 2 (R=Bn) with Strained Cyclooctynes

	BCN[a]	DIBONE[a]	DIBAC[a]	DIBAC[b]
Azide **1**	0.11 ± 0.02	0.14 ± 0.04	0.23 ± 0.06	0.40 ± 0.09
Diazo Compound **2**	0.08 ± 0.02	0.34 ± 0.01	0.45 ± 0.09	2.6 ± 0.2

[a]In CD_3CN. [b]In CD_3CN/H_2O 1:1.

Assay for the Reactivity of a Diazo Compound with Glutathione



N-Benzyl-2-diazoacetamide (**2**, R=Bn) (1 equiv, 0.066 mmol, 1.16 mg) and reduced glutathione (1 equiv, 0.066 mmol, 2.0 mg) were dissolved in 10 mM sodium phosphate buffer, pH 7.3 (deuterated)/MeOD 1:1 (0.33 mL, pH 7.3). The resulting solution was stirred for 24 h, then analyzed by ^{1}H NMR spectroscopy.

Diazo compound 2: ^{1}H NMR (500 MHz, 10 mM sodium phosphate buffer, pH 7.3 (deuterated)/MeOD 1:1) δ 7.37–7.26 (m, 5H), 4.39 (s, 2H).

GSH: ^{1}H NMR (500 MHz, 10 mM sodium phosphate buffer, pH 7.3 (deuterated)/MeOD 1:1) δ 4.53 (dd, J = 7.2, 5.1 Hz, 1H), 3.79 (s, 2H), 3.71 (t, J = 6.3 Hz, 1H), 2.91 (qd, J = 14.1, 6.2 Hz, 2H), 2.54 (t, J = 7.5 Hz, 2H), 2.14 (q, J = 7.6 Hz, 2H).

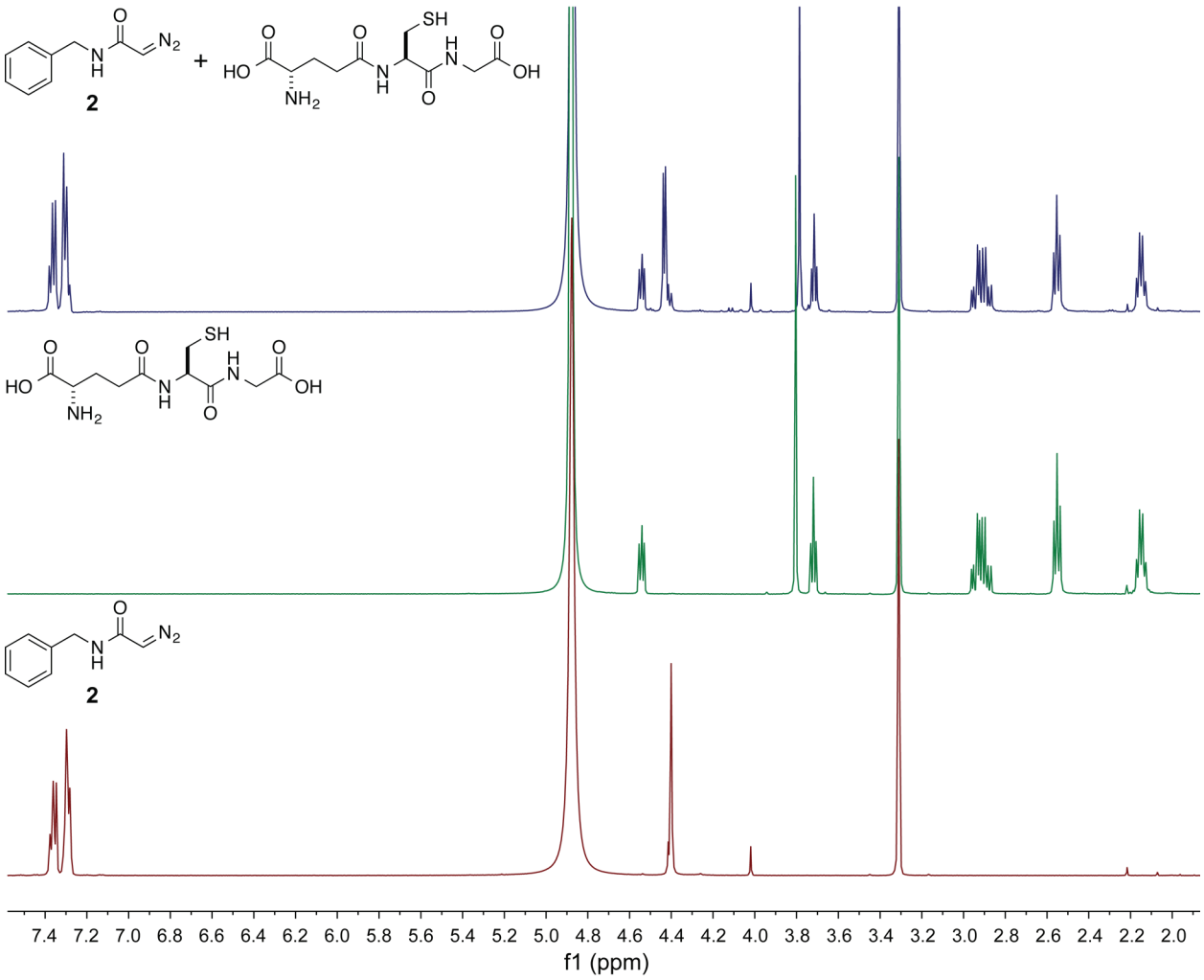


Figure S1. ^{1}H NMR spectra (500 MHz, 10 mM sodium phosphate buffer, pH 7.3 (deuterated)/MeOD 1:1) of *N*-benzyl-2-diazoacetamide (**2**) (bottom, red), reduced glutathione (middle, green), and an equimolar mixture (0.20 M each) after a 24-h incubation in 10 mM sodium phosphate buffer, pH 7.3 (deuterated)/MeOD 1:1 (top, blue). There was no apparent reaction between the glutathione and diazo compound **2**.

General Cell Culture. Cell lines were obtained from American Type Culture Collection (Manassas, VA) and were maintained according to the recommended procedures. Cells were grown in a cell-culture incubator at 37 °C under CO_2(g) (5% v/v) in flat-bottomed culture flasks. Cell medium was supplemented with GIBCO fetal bovine serum (FBS) (10% v/v), penicillin (100 units/mL), and streptomycin (100 μg/mL) in an appropriate cellular medium: Chinese hamster ovary (CHO K1), F12K nutrient medium; HeLa, DMEM; HEK293T, DMEM; and Jurkat, RPMI 1640. Cells were counted with a hemocytometer to determine their seeding density in 12-well plates from Corning Costar (Lowell, MA) or 8-well chambered glass slides from Ibidi (Madison, WI).

Biotin Conjugates to Probe Chemoselectivity. *Microscopy.* CHO K1 cells were seeded at a density of 50,000 cells/dish in 35-mm μ-dish microscopy imaging dishes from Ibidi. Cells were

incubated with 1 mM acrylic ester–biotin, 25 μM biotin–diazo, 25 μM biotin–azide, or DMSO in standard medium for 24–48 h. On the day of an experiment, cells were rinsed twice with wash buffer (DPBS containing 1% v/v FBS) and incubated with avidin–Alexa Fluor488® (20 μg/mL) for 20 min at 4 °C. After treatments, cell nuclei were stained with Hoechst 33342 (2 μg/mL) for 5 min at 37 °C. Cells were then washed twice with wash buffer, and examined using a scanning confocal microscope.

Flow Cytometry. CHO K1 cells were seeded at a density of 50,000 cells/well in 12-well plates. Cells were incubated with 1 mM acrylic ester–biotin, 25 μM biotin–diazo, or 25 μM biotin–azide for 24–48 h. The medium was then removed, and the cells were washed twice with wash buffer (DPBS containing 1% v/v FBS) and incubated with avidin–Alexa Fluor488® (20 μg/mL) for 20 min at 4 °C. After these treatments, cells were rinsed twice with wash buffer and released from the plate with 250 μL of trypsin/EDTA (0.25% w/v). Cells were resuspended in an additional 500 μL of medium and incubated on ice until analyzed by flow cytometry. The fluorescence intensity of at least 20,000 events was then measured by flow cytometry. Alexa Fluor488® was excited with a 488 nm solid- state laser, and the emission was measured through a 530/30 bandpass filter.

Immunoblotting. CHO K1 cells were seeded at a density of 50,000 cells/well in 12-well plates. Cells were incubated in 1 mM acrylic ester–biotin, 25 μM biotin–diazo, 25 μM biotin–azide, or DMSO for 24–48 h. After these treatments, cells were rinsed twice with DPBS and released from the plate with 250 μL of trypsin/EDTA (0.25% w/v). Cells were washed twice, pelletted by centrifugation, and resuspended in DPBS. SDS–PAGE sample buffer was added to cell suspensions, and samples were boiled for 5 min, subjected to brief centrifugation, and analyzed by SDS–PAGE on a 12% w/v gel before being transferrred to a PVDF membrane. Membranes were analyzed for the presence of azido or diazo sugars by immunoblotting with α-biotin (1:1000) from Cell Signaling Technology (Danvers, MA). To control for loading levels, membranes were analyzed for β-actin by immunoblotting with α–β-actin (1:1000) from Cell Signaling Technology.

Metabolism of Ac_4ManDiaz and Ac_4ManNAz by CHO K1 Cells. *Microscopy*. CHO K1 cells were seeded at a density of 100,000 cells/dish in 35-mm μ-dish microscopy imaging dishes from Ibidi, and grown in the presence of Ac_4ManDiaz or Ac_4ManNAz (25 μM) in standard medium for 2 d. On the day of the experiment, the cells were rinsed twice with wash buffer (DPBS containing 1% v/v FBS) and treated with DIBAC–biotin (10 μM) in standard medium for 1 h at 37 °C. Cells were again washed twice, and incubated with avidin–Alexa Fluor488® (20 μg/mL) for 20 min at 4 °C. Cell nuclei were stained with Hoechst 33342 (2 μg/mL) from Invitrogen for 5 min at 37 °C. Cells were again washed twice, and incubated on ice in standard medium until ready to be imaged by super-resolution microscopy.

Cytometry. CHO K1 cells were seeded at a density of 50,000 cells/well in 12-well plates, and grown in the presence of Ac_4ManDiaz (0–50 μM) for 2 days in standard medium. The cells were then rinsed twice with wash buffer (DPBS containing 1% v/v FBS) and treated with DIBAC–biotin (10 μM) in standard medium for 1 h at 37 °C. Cells were again washed twice, and incubated with avidin–Alexa Fluor488® (20 μg/mL) for 20 min at 4 °C. Cells were again washed twice and released from the dish by treatment with trypsin/EDTA (0.25% w/v) for 5 min at 37 °C. Cells were resuspended in standard medium, and incubated on ice. The fluorescence intensity of 20,000 events was then measured by flow cytometry. Alexa Fluor488® was excited with a 488 nm solid-state laser, and the emission was measured through a 530/30 bandpass filter.

Metabolism of Ac_4ManDiaz by Multiple Cell Types. CHO K1, HeLa, HEK293T, and Jurkat cells were seeded at a density of 50,000 cells/well in 12-well plates, and grown in the presence of Ac_4ManDiaz (0–50 μM) for 2 days in standard medium. Cells were then rinsed twice with wash buffer (DPBS containing 1% v/v FBS) and treated with DIBAC–biotin (10 μM) in standard medium for 1 h at 37 °C. Jurkat cells were washed by centrifugation at 1000*g* for 5 min; all other cell types are adherent. Cells were again washed twice, and incubated with avidin–Alexa Fluor488® (20 μg/mL) for 20 min at 4 °C. Cells were washed twice with wash buffer, and adherent cells were released from the dish by incubation with trypsin/EDTA (0.25% w/v) for 5 min at 37 °C. Cells were resuspended in standard medium, and incubated on ice until analysis. The fluorescence intensity of 20,000 events was then measured by flow cytometry. Alexa Fluor488® was excited with a 488 nm solid-state laser, and the emission was measured through a 530/30 bandpass filter.

Neuraminidase and Peptide-*N*-Glycosidase F treatment of CHO K1 cells. CHO K1 cells were treated with Ac_4ManNAc, Ac_4ManNAz, or Ac_4ManDiaz (250 μM) for 3 d. Cells were lifted from the plate with trypsin/EDTA (0.25% w/v), collected by centrifugation, and washed twice with DPBS. Cells were treated with DIBAC–biotin (20 μM) for 2 h at 37 °C. G7 reaction buffer (New England Biolabs) was added, and the suspension was divided into three samples. Neuraminidase (acetyl-neuraminyl hydrolase) from New England Biolabs (Ipswich, MA) was added to one sample; peptide-*N*-glycosidase F (PNGase F) from New England Biolabs was added to another sample, and PBS was added to the remaining sample. Samples were incubated at 37 °C overnight. SDS–PAGE sample buffer was added, and samples were boiled for 5 min, subjected to brief centrifugation, and analyzed by SDS–PAGE on a 12% w/v gel before being transferrred to a PVDF membrane. Membranes were analyzed for the presence of azido or diazo sugars by immunoblotting with α-biotin (1:1000) from Cell Signaling Technology. To control for loading levels, membranes were analyzed for β-actin by immunoblotting with α–β-actin (1:1000) from Cell Signaling Technology.

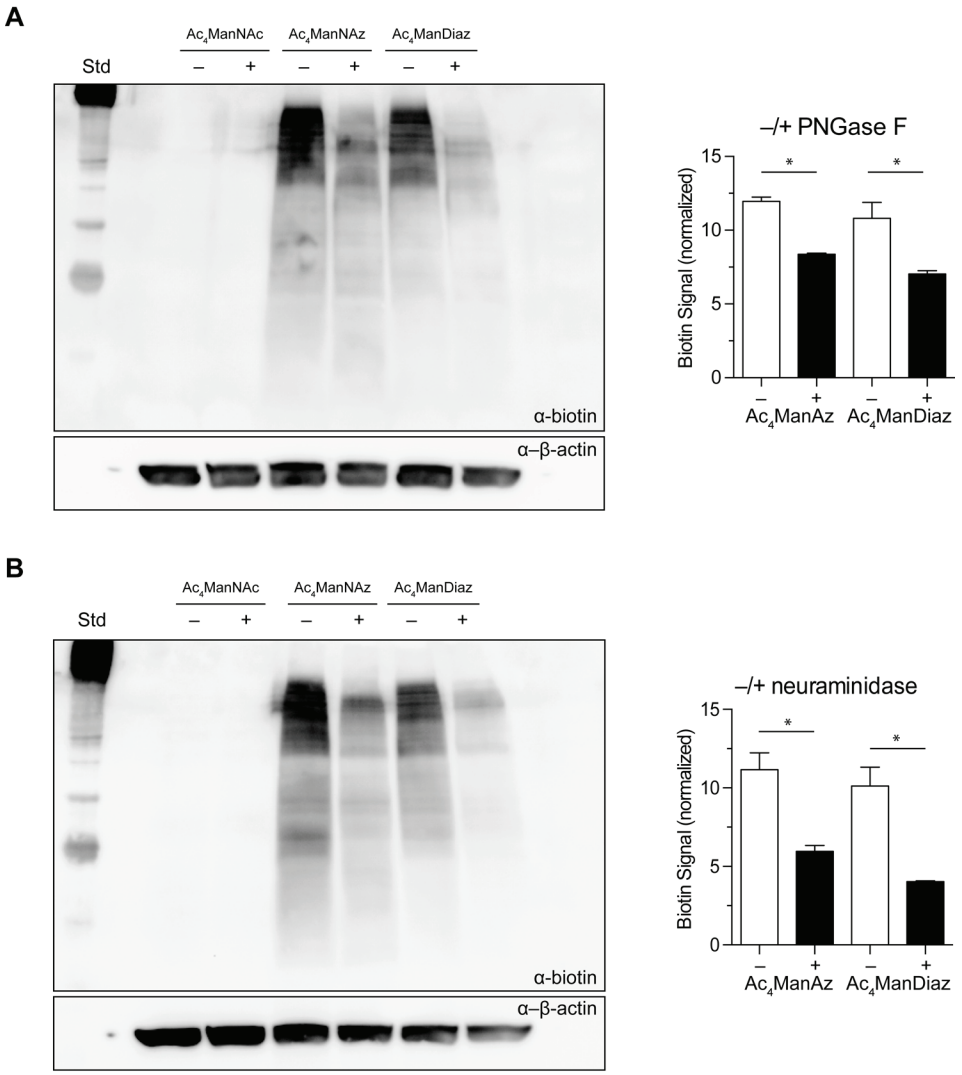


Figure S2. Metabolic incorporation of Ac_4ManDiaz into sialic acid. Immunoblot analysis of cell lysates treated with DIBAC–biotin, after growth in the presence of Ac_4ManNAc, Ac_4ManNAz, or Ac_4ManDiaz (250 μM) for 3 d, and (A) incubation in either PNGase F or buffer, and (B) incubation in either neuraminidase or buffer. Quantification was normalized to actin to control for loading, $p < 0.05$. Quantification of the azido and diazo untreated lanes from all experiments showed that expression of a diazo group from Ac_4ManDiaz is (86 ± 3)% of the azido group from Ac_4ManNAz.

Mass Analysis of Sialic acids from Biological Samples. Sialic acids were isolated for analysis by using a method reported by Hackenberger and coworkers.[8] CHO K1 cells were seeded into 6-well plates and treated with Ac_4ManDiaz or Ac_4ManNAz (250 μM) for 3 d. Cells were released from the dish by incubation with trypsin/EDTA (0.25% w/v) for 5 min at 37 °C. Cells were washed twice in DPBS. The cells were resuspended in 200 μL of H_2O, and 500 μL of 3 M acetic acid was added. The resulting suspension was incubated at 90 °C for 90 min before being cooled to 0 °C for 15 min and neutralized by the addition of 650 μL of 3% v/v NH_3 for

30 min at room temperature. Samples were lyophilized, and resuspended in 500 µL of EtOH/H_2O 4:1. Samples were mixed with a vortex mixer and subjected to centrifugation at 10,000*g* for 2 min. The supernatant was lyophilized. The residue was suspended in 300 µL of EtOH/MeOH 2:1, and the resulting suspension was mixed with a vortex mixer and subjected to centrifugation at 10,000*g* for 2 min. The supernatant was lyophilized. The residue was dissolved in PBS, and enriched for biotin–linked sugars by purification over SoftLink™ Soft Release Avidin Resin from Promega. The biotin–linked sugars were eluted from the resin with 3 M acetic acid, and the eluent was concentrated by lyophilization. Enriched isolates were dissolved in 100 µL of MeOH, and 25 µL was injected directly onto a Shimadzu 2010A single quadrupole mass analyzer LCMS in single-ion monitoring mode with a scanning width of 0.9 *m/z* so as to observe the sialic acid conjugates in the presence of excess DIBAC–biotin.

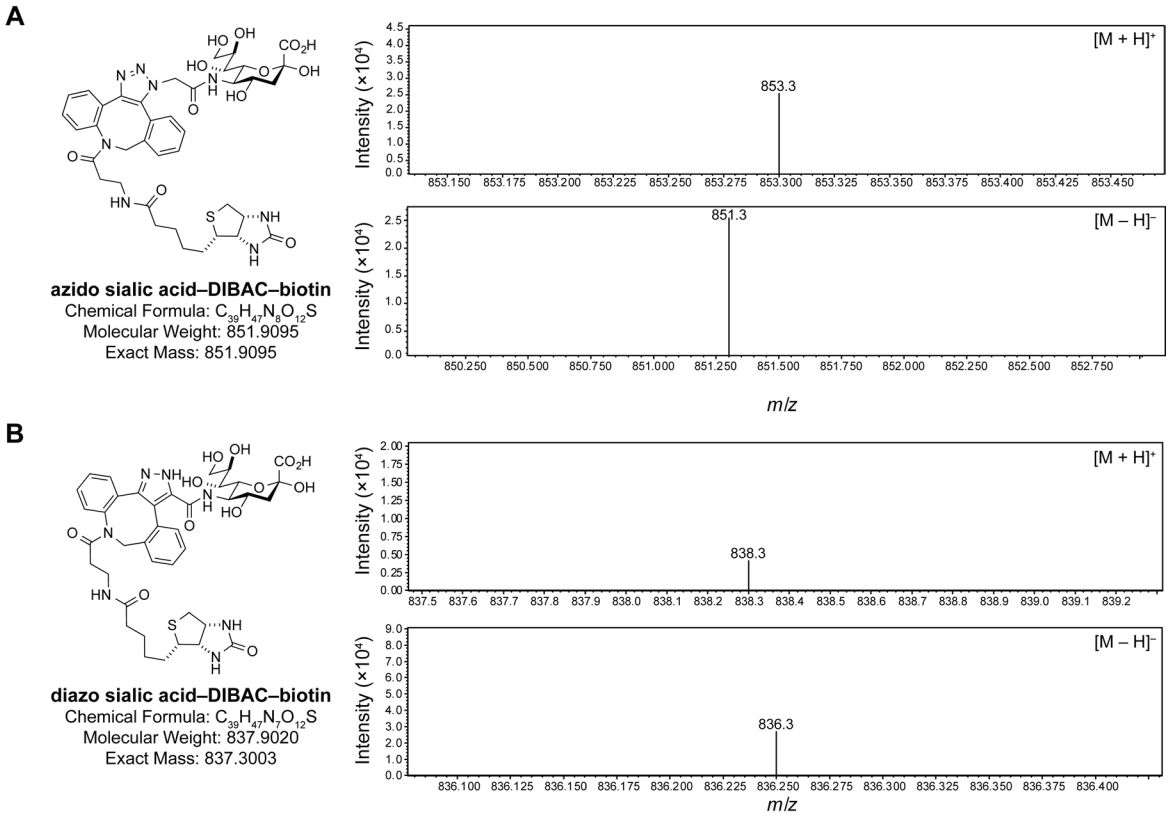


Figure S3. Detection of sialic acid derivatives generated *in cellulo* from (A) Ac_4ManNAz and (B) Ac_4ManDiaz. Positive and negative ions were observed in independent LC-LRMS (ESI) analyses after cycloaddition with DIBAC–biotin and product isolation.

Optimal Labeling of CHO K1 Cells with Ac_4ManDiaz. *Increasing Amounts of DIBAC–Biotin*. CHO K1 cells were seeded at a density of 50,000 cells/well in 12-well plates, and grown in the presence of Ac_4ManDiaz (25 µM) for 2 days in standard medium. The cells were then rinsed twice with wash buffer (DPBS containing 1% v/v FBS) and treated with DIBAC–biotin (0–20 µM) in standard medium for 1 h at 37 °C. Cells were again washed twice, and incubated with avidin–Alexa Fluor488® (20 µg/mL) for 20 min at 4 °C. Cells were washed twice with

wash buffer, and released from the dish by incubation with trypsin/EDTA (0.25% w/v) for 5 min at 37 °C. Cells were resuspended in standard medium, and incubated on ice. The fluorescence intensity of 20,000 events was then measured by flow cytometry. The fluorescence intensity of 20,000 events was then measured by flow cytometry. Alexa Fluor488® was excited with a 488 nm solid-state laser, and the emission was measured through a 530/30 bandpass filter.

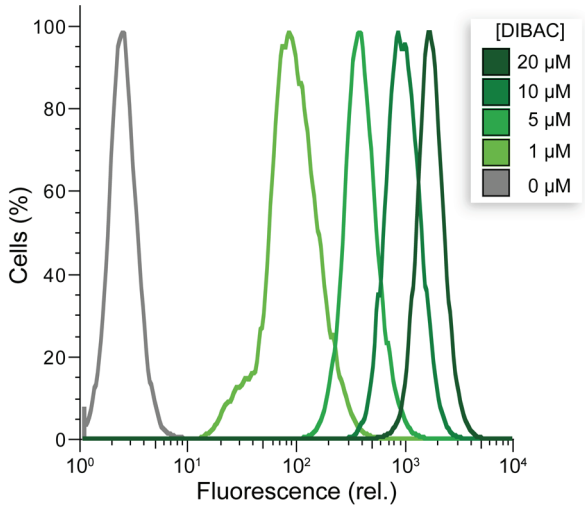


Figure S4. Histogram of CHO K1 cells grown in medium containing Ac_4ManDiaz (25 μM) and treated with increasing amounts of DIBAC–biotin and avidin–Alexa Fluor488®. Data were acquired by flow cytometry. At 20 μM, DIBAC–biotin begins to cause cytotoxicity.

Increasing Incubation Times with DIBAC–Biotin. CHO K1 cells were seeded at a density of 50,000 cells/well in 12-well plates, and grown in the presence of Ac_4ManDiaz (25 μM) for 2 days in standard medium. The cells were then rinsed twice with wash buffer (DPBS containing 1% v/v FBS) and treated with DIBAC–biotin (10 μM) in standard medium for 0–120 min at 37 °C. Cells were again washed twice, and incubated with avidin–Alexa Fluor488® (20 μg/mL) for 20 min at 4 °C. Cells were washed twice with wash buffer, and released from the dish by incubation with trypsin/EDTA (0.25% w/v) for 5 min at 37 °C. Cells were resuspended in standard medium, and incubated on ice. The fluorescence intensity of 20,000 events was then measured by flow cytometry. The fluorescence intensity of 20,000 events was then measured by flow cytometry. Alexa Fluor488® was excited with a 488 nm solid-state laser, and the emission was measured through a 530/30 bandpass filter.

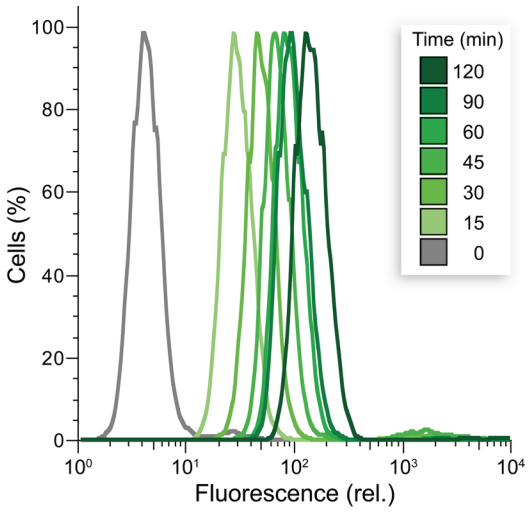


Figure S5. Histogram of CHO K1 cells grown in medium containing Ac_4ManDiaz (25 μM) and treated with increasing amounts of DIBAC–biotin and avidin–Alexa Fluor488®. Data were acquired by flow cytometry. At times >60 min, DIBAC–biotin begins to cause cytotoxicity.

Cytotoxicity of Ac_4ManDiaz and Ac_4ManNAz for CHO K1 Cells. The cytotoxicity of Ac_4ManDiaz and Ac_4ManNAz was determined with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) from Promega (Madison, WI). Briefly, CHO K1 cells were plated at a concentration of 5000 cells/well in a clear 96-well plate. Cells were allowed to adhere for 4 h. The medium was removed, and varying concentrations of either Ac_4ManDiaz or Ac_4ManNAz in medium was added. Cells were incubated at 37 °C for 24 h. The medium was removed, and cells were washed with DPBS. The MTS reagent was added at a ratio of 1:5, and cells were incubated at 37 °C for 2 h before measuring the absorbance at 490 nm. Reported data are the average of three measurements for each concentration, and the entire experiment was repeated in triplicate. The percentage of viable cells was determined by normalizing to a PBS control (100% viable), and a H_2O_2 control (0% viable). The data were fitted by nonlinear regression analysis to obtain values of LD_{50}.

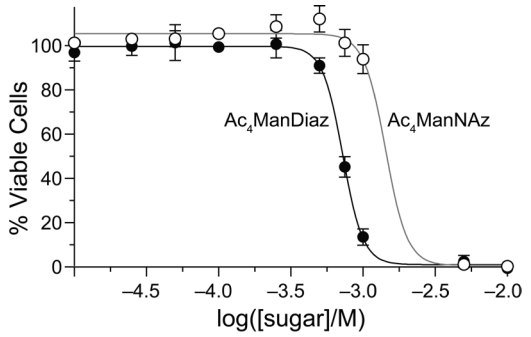


Figure S6. Graph of the cytotoxicity of Ac_4ManDiaz and Ac_4ManNAz (10 μM–10 mM) for CHO K1 cells. LD_{50} values are (0.7 ± 0.1) mM for Ac_4ManDiaz and (1.4 ± 0.1) mM for Ac_4ManNAz.

Dual-Color Labeling of Mannosamine in the Glycocalyx. *Microscopy*. CHO K1 cells were seeded at a density of 100,000 cells/dish in 35-mm μ-dish microscopy imaging dishes from Ibidi, and grown in the presence of a peracetylated sugar (25 μM) in standard medium for 2 d. On the day of an experiment, cells were rinsed twice with wash buffer (DPBS containing 1% v/v FBS).

Alkyne treatment. Cells were treated with medium containing DIBAC–biotin (10 μM) for 1 h at 37 °C. Cells were rinsed twice with wash buffer (DPBS containing 1% v/v FBS), and incubated with avidin–Alexa Fluor594® (20 μg/mL) for 20 min at 4 °C.

Azide treatment. Cells were treated with medium containing picolyl azide–Alexa Fluor647® (5 μM), $CuSO_4$ (50 μM), THPTA (250 μM), and sodium ascorbate (2.5 mM) for 5 min at room temperature.[6]

In one experiment, cells were treated with azide and then alkyne; in another experiment, cells were treated with alkyne and then azide. After treatments, cell nuclei were stained with Hoechst 33342 (2 μg/mL) for 5 min at 37 °C. Cells were then washed twice with wash buffer, and examined using a scanning confocal microscope.

Cytometry. Two days prior to an experiment, CHO K1 cells were seeded in 12-well plates at 1×10^5 cells/well, and a sugar was added to a final concentration of 25 μM. On the day of an experiment, cells were rinsed twice with wash buffer (DPBS containing 1% v/v FBS).

Alkyne treatment. Cells were treated with medium containing DIBAC–biotin (10 μM) for 1 h at 37 °C. Cells were rinsed twice with wash buffer (DPBS containing 1% v/v FBS), and incubated with avidin–Alexa Fluor647® (20 μg/mL) for 20 min at 4 °C.

Azide treatment. Cells were treated with medium containing picolyl azide–Alexa Fluor647® (5 μM), $CuSO_4$ (50 μM), THPTA (250 μM), and sodium ascorbate (2.5 mM) for 5 min at room temperature.[8]

In one experiment, cells were treated with azide and then alkyne; in another experiment, cells were treated with alkyne and then azide. After these treatments, cells were rinsed twice with wash buffer (DPBS containing 1% v/v FBS) and released from the plate with 250 μL of trypsin/EDTA (0.25% w/v). Cells were resuspended in an additional 500 μL of medium and incubated on ice until analyzed by flow cytometry. The fluorescence intensity of at least 20,000 events was measured by flow cytometry. Alexa Fluor488® was excited with a 488 nm solid-state laser, and the emission was measured through a 530/30 bandpass filter. Alexa Fluor647® was excited with a 633 nm solid-state laser and the emission was collected with a 661/16 bandpass filter.

Dual-Color Labeling of Mannosamine and Galactosamine in the Glycocalyx. *Microscopy*. CHO K1 cells were seeded at a density of 100,000 cells/dish in 35-mm μ-dish microscopy imaging dishes from Ibidi, and grown in the presence of a peracetylated sugar (25 μM) in standard medium for 2 d. On the day of an experiment, cells were rinsed twice with wash buffer (DPBS containing 1% v/v FBS).

Alkyne treatment. Cells were treated with medium containing DIBAC–biotin (10 μM) for 1 h at 37 °C. Cells were rinsed twice with wash buffer (DPBS containing 1% v/v FBS), and incubated with avidin–Alexa Fluor594® (20 μg/mL) for 20 min at 4 °C.

Azide treatment. Cells were treated with medium containing picolyl azide–Alexa Fluor647® (5 μM), $CuSO_4$ (50 μM), THPTA (250 μM), and sodium ascorbate (2.5 mM) for 5 min at room temperature.[9]

After treatments, cell nuclei were stained with Hoechst 33342 (2 μg/mL) for 5 min at 37 °C. Cells were then washed twice with wash buffer, and examined using a scanning confocal microscope.

Cytometry. Two days prior to an experiment, CHO K1 cells were seeded in 12-well plates at 1×10^5 cells/well, and a sugar was added to a final concentration of 25 μM. On the day of an experiment, cells were rinsed twice with wash buffer (DPBS containing 1% v/v FBS).

Alkyne treatment. Cells were treated with medium containing DIBAC–biotin (10 μM) for 1 h at 37 °C. Cells were rinsed twice with wash buffer (DPBS containing 1% v/v FBS), and incubated with avidin–Alexa Fluor647® (20 μg/mL) for 20 min at 4 °C.

Azide treatment. Cells were treated with medium containing picolyl azide–Alexa Fluor647® (5 μM), $CuSO_4$ (50 μM), THPTA (250 μM), and sodium ascorbate (2.5 mM) for 5 min at room temperature.[8]

Cells were treated with alkyne for 60 min and then azide for 5 min. After these treatments, cells were rinsed twice with wash buffer (DPBS containing 1% v/v FBS) and released from the plate with 250 μL of trypsin/EDTA (0.25% w/v). Cells were resuspended in an additional 500 μL of medium and incubated on ice until analyzed by flow cytometry. The fluorescence intensity of at least 20,000 events was measured by flow cytometry. Alexa Fluor488® was excited with a 488 nm solid-state laser, and the emission was measured through a 530/30 bandpass filter. Alexa Fluor647® was excited with a 633 nm solid-state laser, and the emission was collected with a 661/16 bandpass filter.

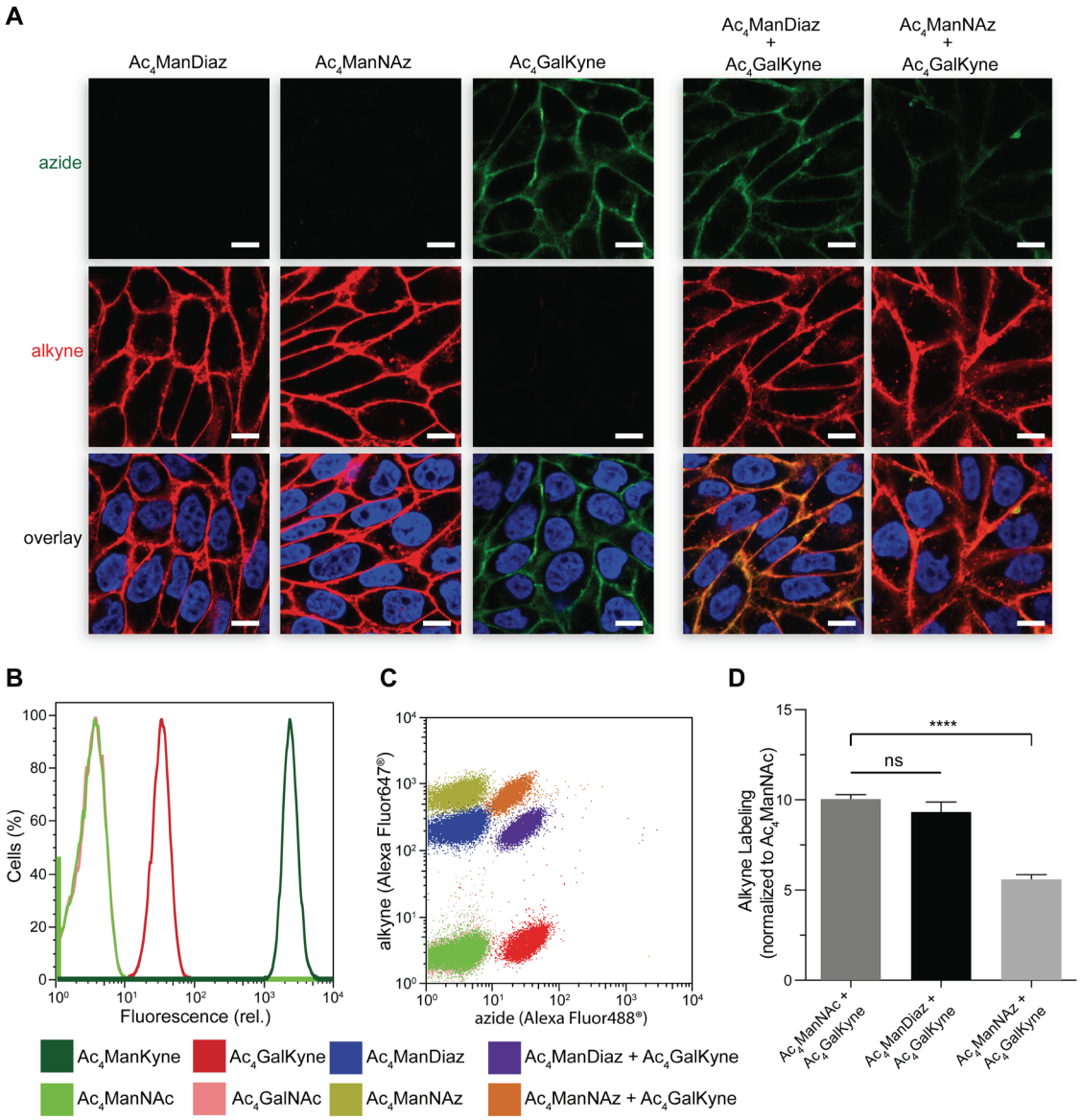


Figure S7. Dual labeling of mammalian cells grown with derivatives of *N*-acetylmannosamine and *N*-acetylgalactosamine. (A) Images of CHO K1 cells grown in medium containing *N*-acetylmannosamine or *N*-acetylgalactosamine derivatives (25 μM) for 2 d, then labeled by cycloaddition with an alkyne (red) and azide (green), and visualized with confocal microscopy. Scale bars: 5 μm. (B) Histogram showing the lesser labeling of cells exposed to Ac_4GalKyne than Ac_4ManKyne as measured by flow cytometry. (C) Plot demonstrating the dual labeling of cells that had metabolized Ac_4GalKyne and either Ac_4ManDiaz or Ac_4ManNAz as measured by flow cytometry. (D) Quantification of the level of Ac_4GalKyne labeling obtained in cells grown with derivatives of *N*-acetylmannosamine, $p < 0.0001$.

References

(1) Goddard-Borger, E. D.; Stick R. V. *Org. Lett.* **2007**, *9*, 3797–3800.

(2) Laughlin, S. T.; Bertozzi, C. R. *Nat. Protoc.*, **2007**, *11*, 2930–2944.

(3) Hsu, T.-L.: Hanson, S. R.: Kishikawa, K.; Wang, S.-K.; Sawa, M.; Wong, C.-H., *Proc. Natl. Acad. Sci. USA,* **2007,** *104*, 2614–2619.

(4) Myers, E. L.; Raines, R. T. *Angew. Chem., Int. Ed*., **2009**, *48*, 2359–2363.

(5) McGrath, N. A.; Raines, R. T. *Chem. Sci.,* **2012**, *3*, 3237–3240.

(6) Rodrigues, D. C; Bader, R. A.; Hasenwinkel, J. M. *Polymer*, **2011**, *52*, 2505–2513.

(7) (a) Mukherjee, M.; Gupta, A. K.; Lu, Z.; Zhang, Y.; Wulff, W. D. *J. Org. Chem.* **2010**, *75*, 5643–5660. (b) Ouihia, A.; René, L.; Guilhem, J.; Pascard, C.; Badet, B. *J. Org. Chem.* **1993**, *58*, 1641–1642.

(8) Möller, H.; Böhrsch, V.; Bentrop, J.; Bender, J.; Hinderlich, S.; Hackenberger, C. P. R. *Angew. Chem., Int. Ed*. **2012,** *51*, 5986–5990.

(9) Uttamapinant, C.; Tangpeerachaikul, A.; Grecian, S.; Clarke, S.; Singh, U.; Slade, P.; Gee, K. R.; Ting, A. Y. *Angew. Chem., Int. Ed*. **2012,** *51*, 5852–5856.

NMR Spectra

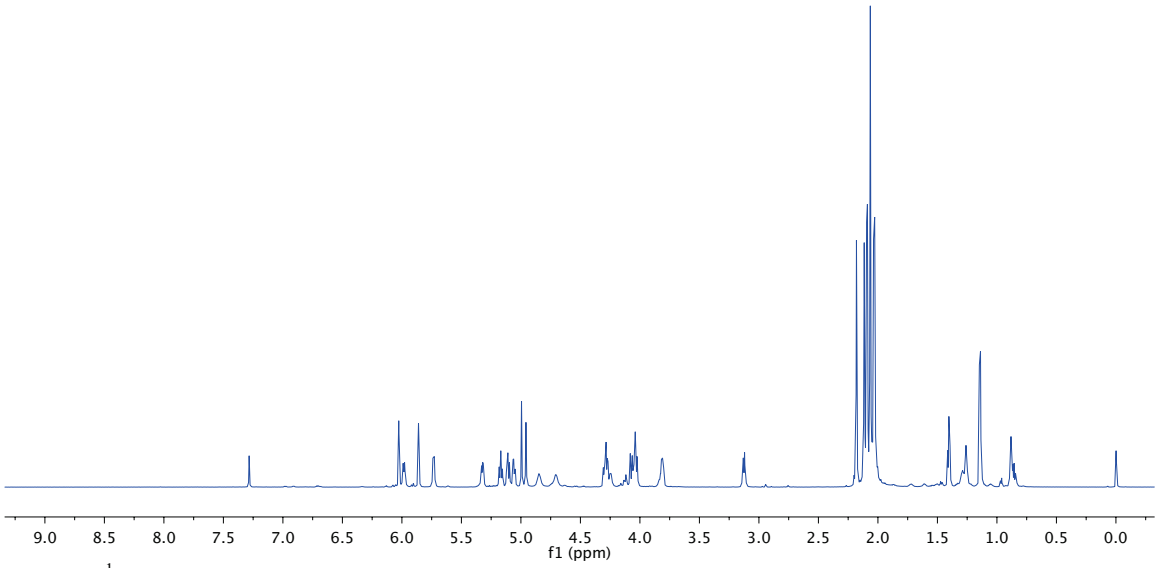


Figure S8. ^{1}H NMR spectrum of **tetra-*O*-acetyl-*N*-2-azidoacetyl-D-mannosamine (Ac$_4$ManDiaz)** (mixed anomers) in $CDCl_3$ (750 MHz)

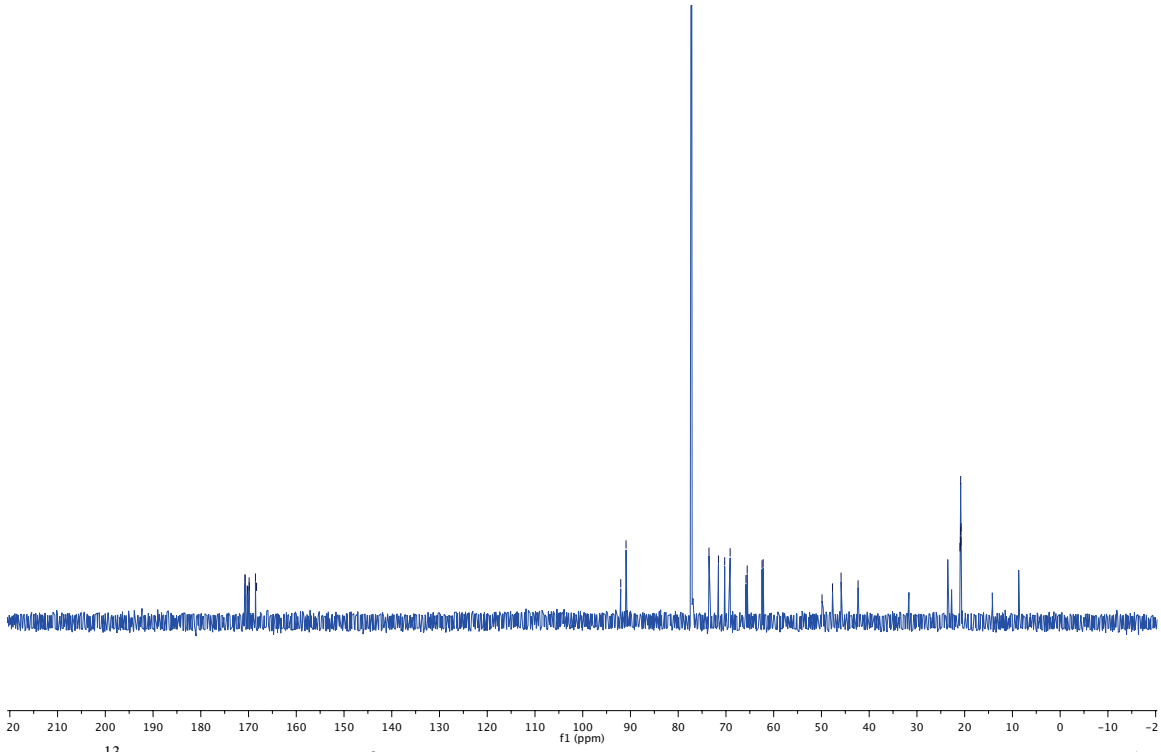


Figure S9. ^{13}C NMR spectrum of **tetra-*O*-acetyl-*N*-2-azidoacetyl-D-mannosamine (Ac$_4$ManDiaz)** (mixed anomers) in $CDCl_3$ (189 MHz)

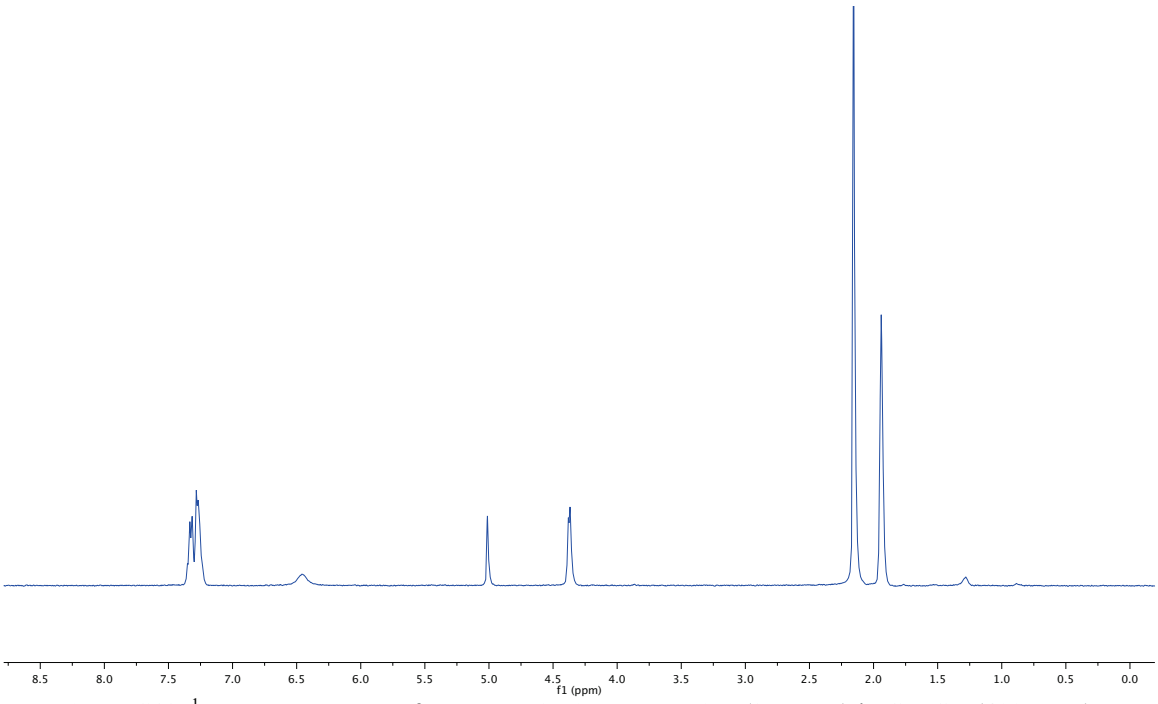


Figure S10. ^{1}H NMR spectrum of ***N*-benzyl-2-diazoacetamide** (**2**, R=Bn) in CD_3CN (400 MHz).

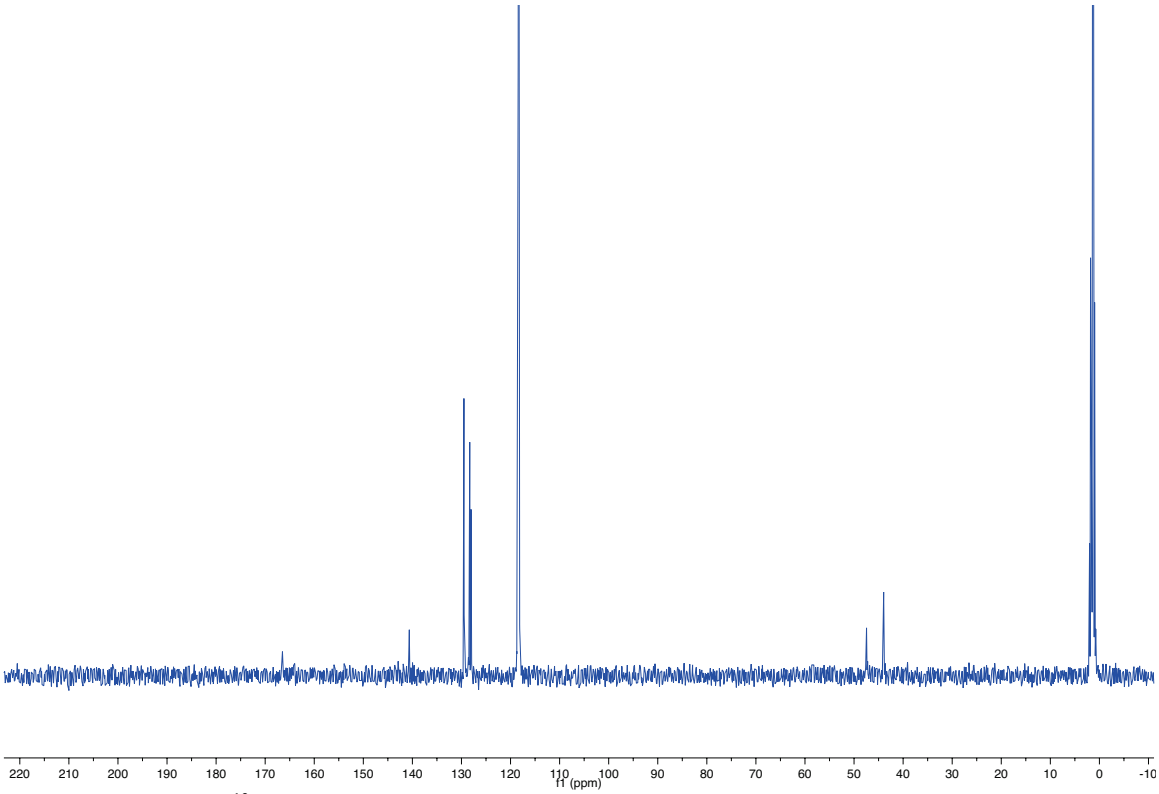


Figure S11. ^{13}C NMR spectrum of ***N*-benzyl-2-diazoacetamide** (**2**, R=Bn) in CD_3CN (101 MHz).

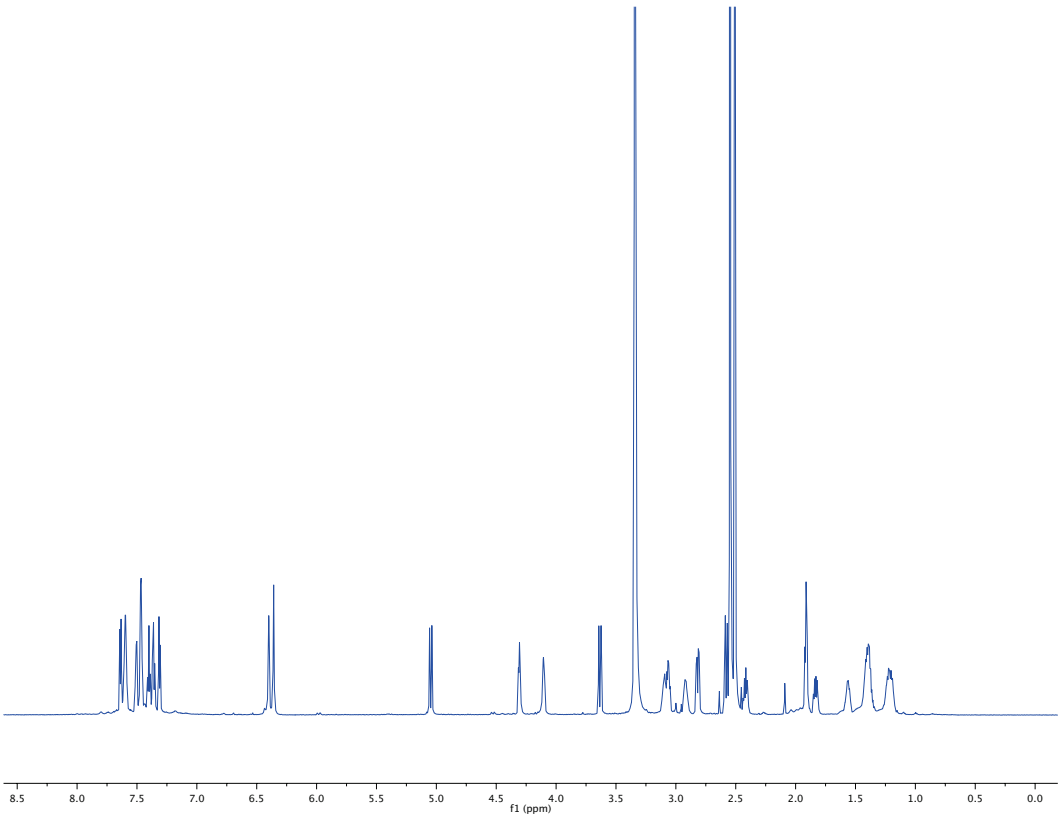


Figure S12. ^{1}H NMR spectrum of **DIBAC–biotin** in DMSO (750 MHz).

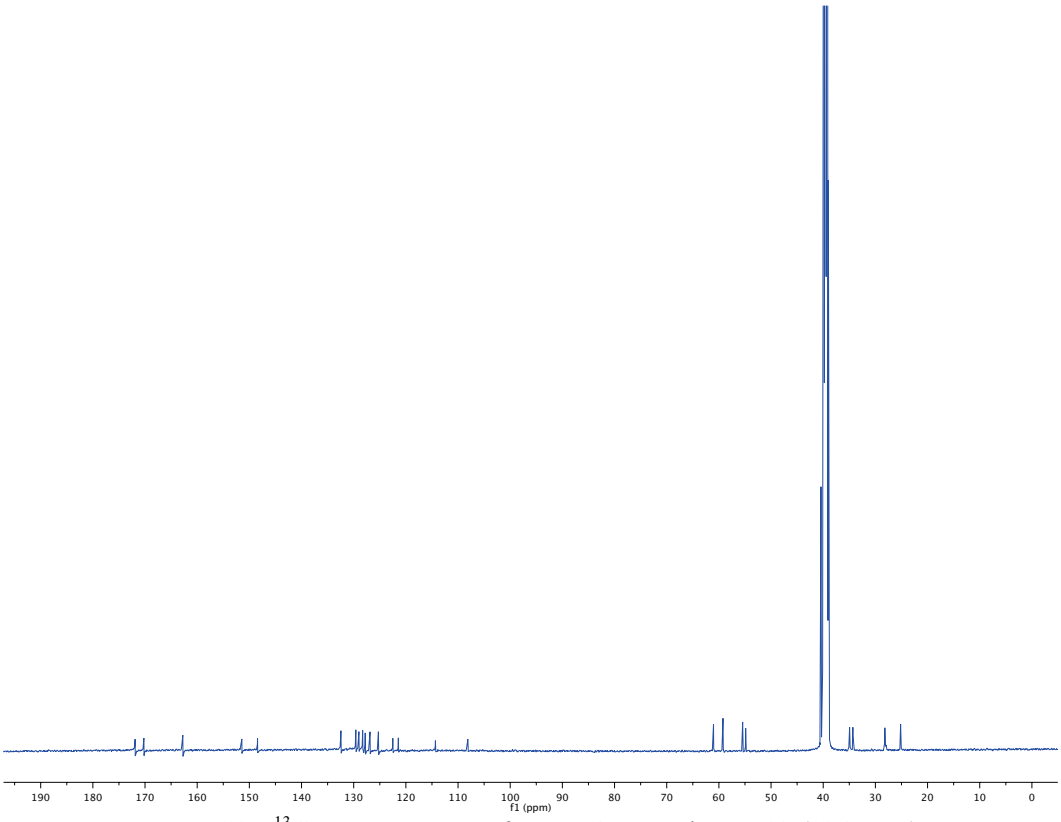


Figure S13. ^{13}C NMR spectrum of **DIBAC–biotin** in DMSO (126 MHz).

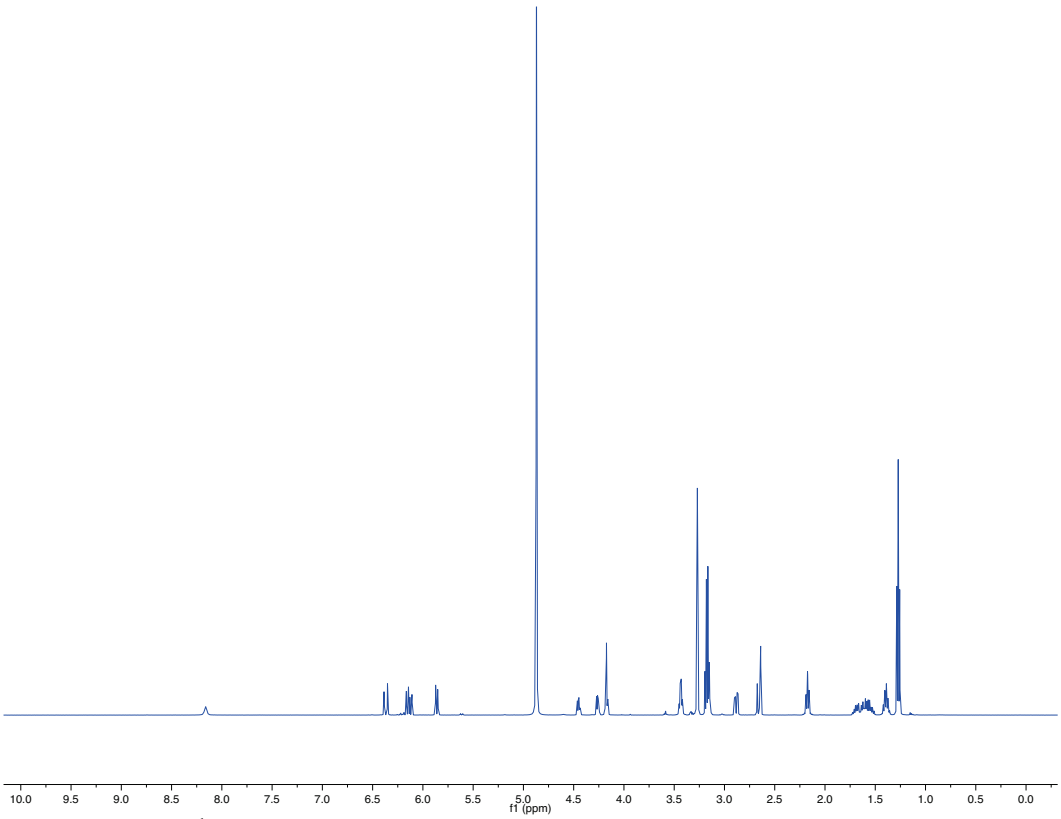


Figure S14. ^{1}H NMR spectrum of **2-aminoethyl acrylate–biotin** in MeOD (500 MHz).

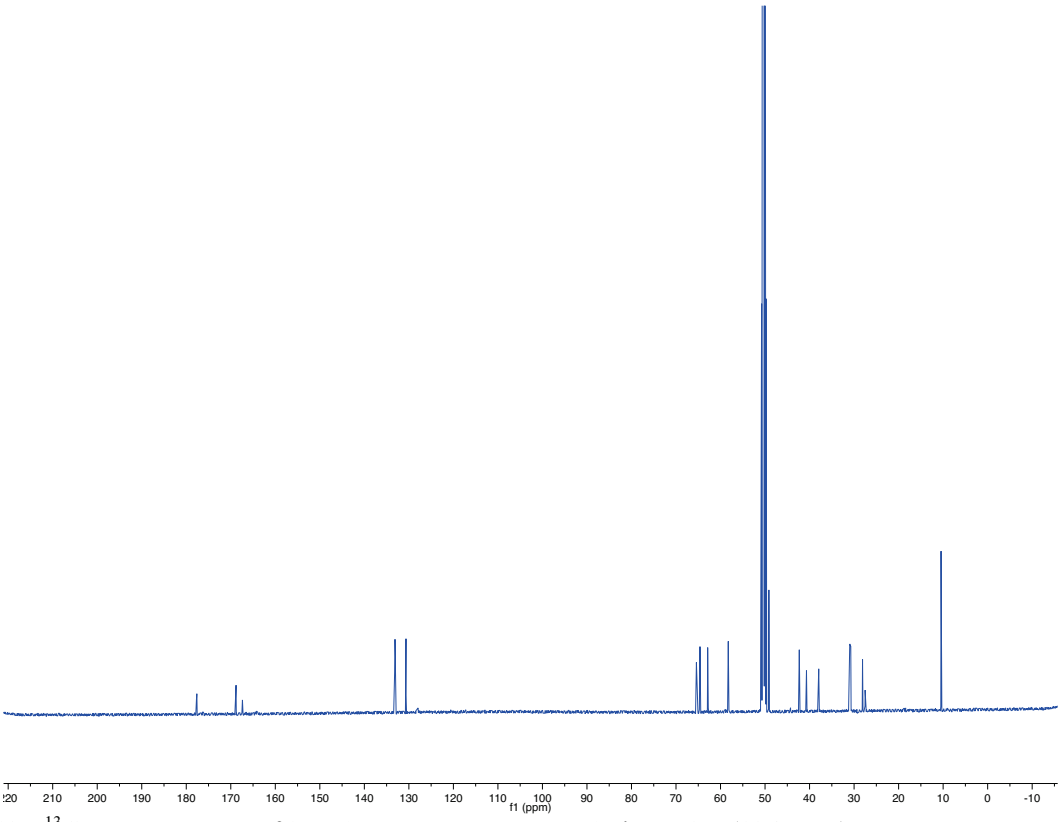


Figure S15. ^{13}C NMR spectrum of **2-aminoethyl acrylate–biotin** in MeOD (126 MHz).

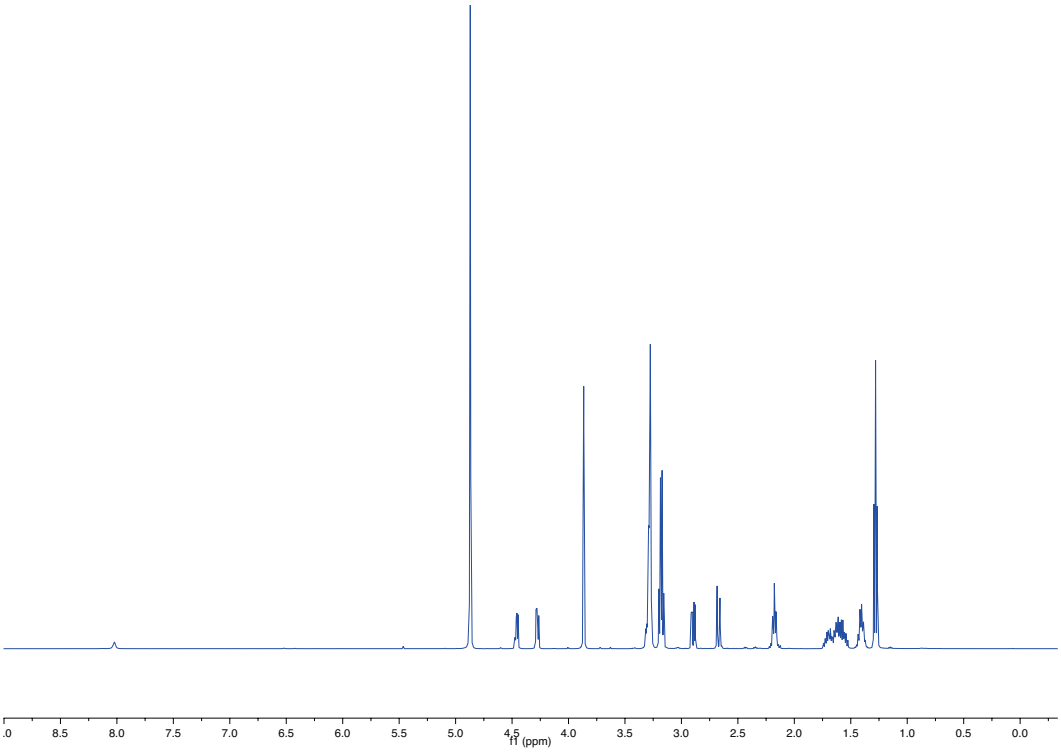


Figure S16. ^{1}H NMR spectrum of **azide–ethylenediamine–biotin conjugate** (500 MHz).

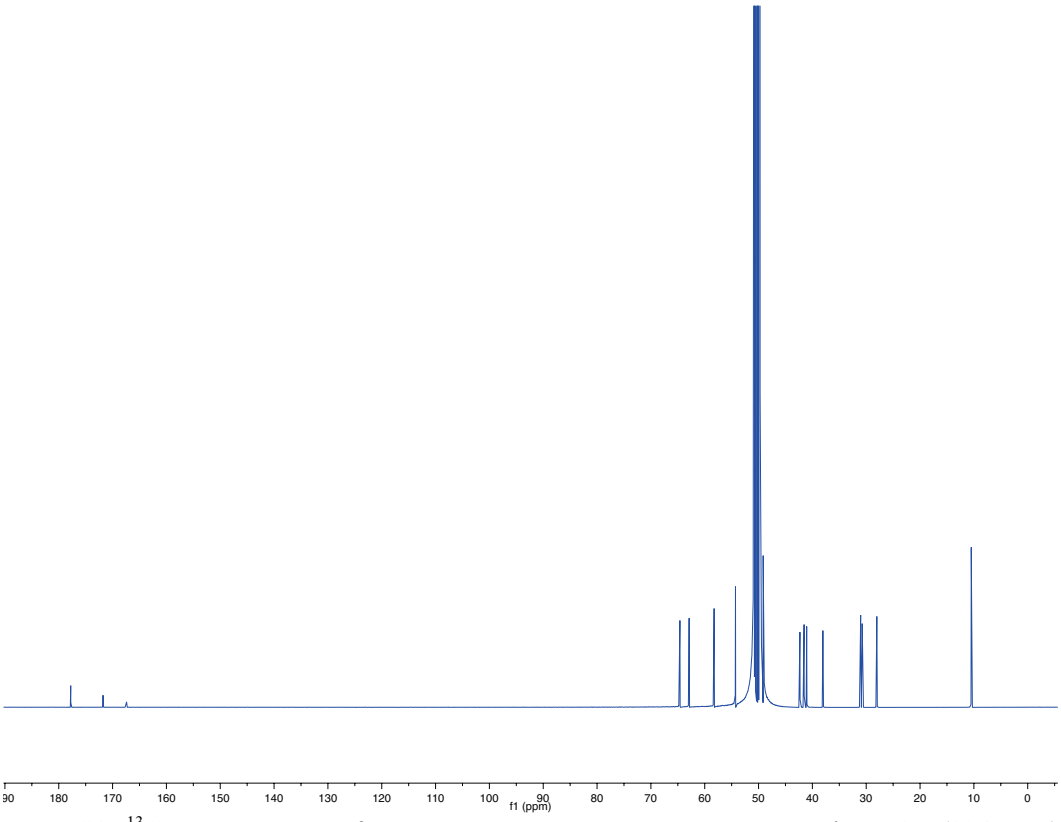


Figure S17. ^{13}C NMR spectrum of **azide–ethylenediamine–biotin conjugate** in MeOD (126 MHz).

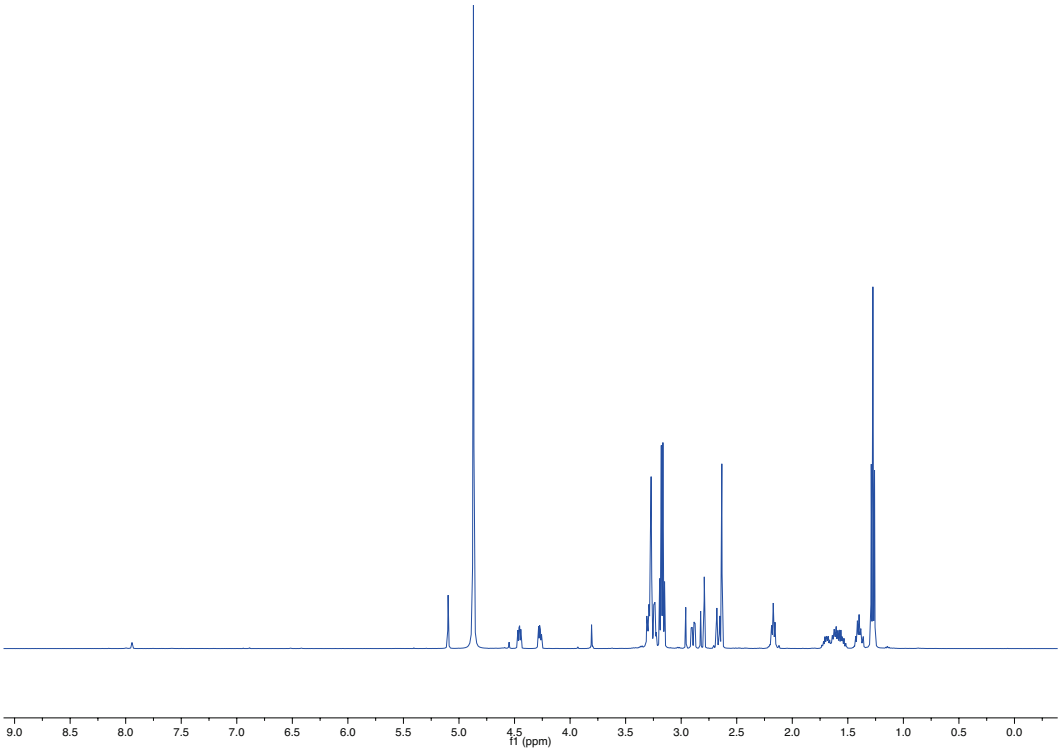


Figure S18. ^{1}H NMR spectrum of **diazo-ethylenediamine–biotin conjugate** in MeOD (500 MHz).

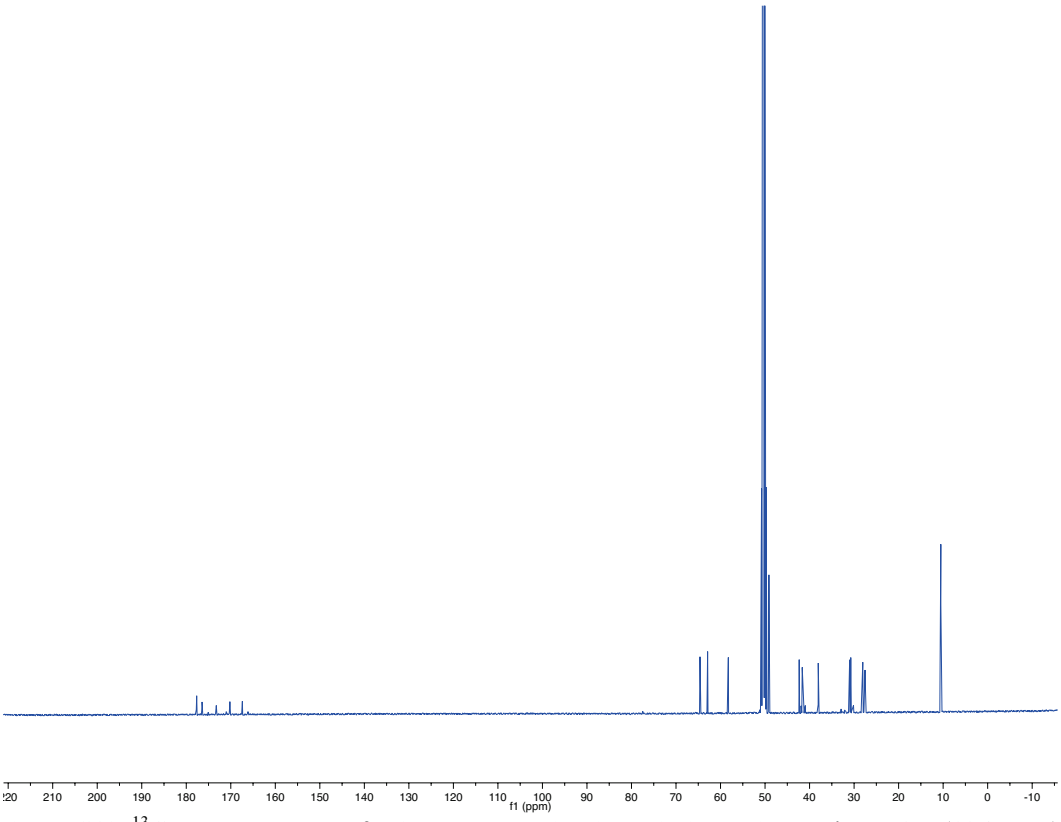


Figure S19. ^{13}C NMR spectrum of **azide-ethylenediamine–biotin conjugate** in MeOD (126 MHz).

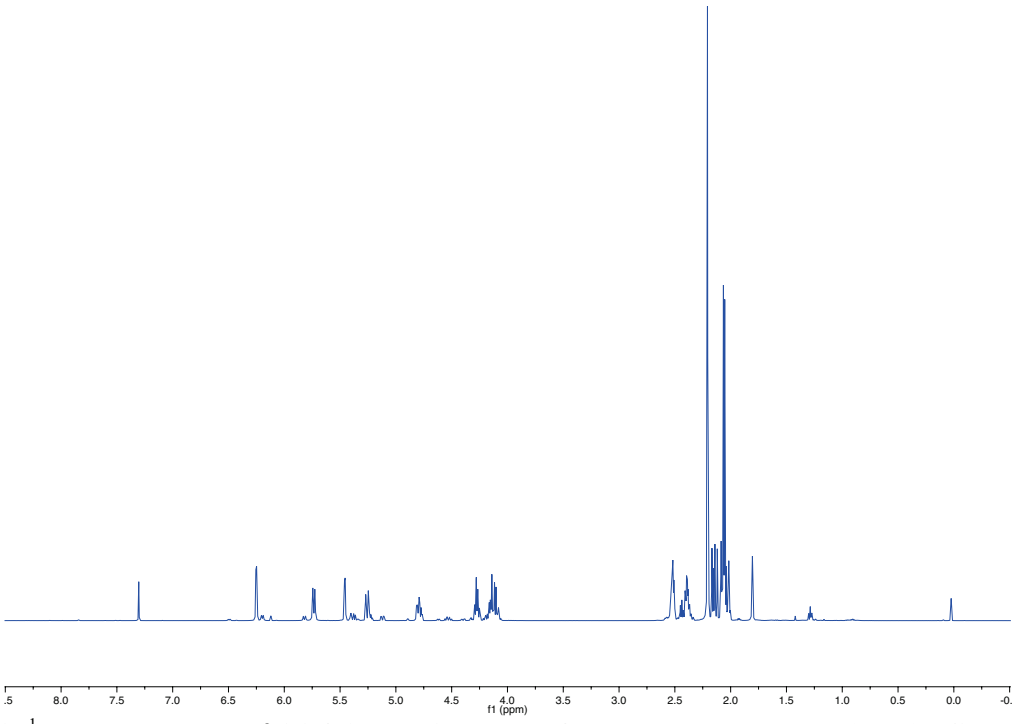


Figure S20. ^{1}H NMR spectrum of **1,3,4,6-tetra-*O*-acetyl-*N*-4-pentynoyl-D-galactosamine (Ac$_4$GalKyne)** mixed anomers in $CDCl_3$ (500 MHz).

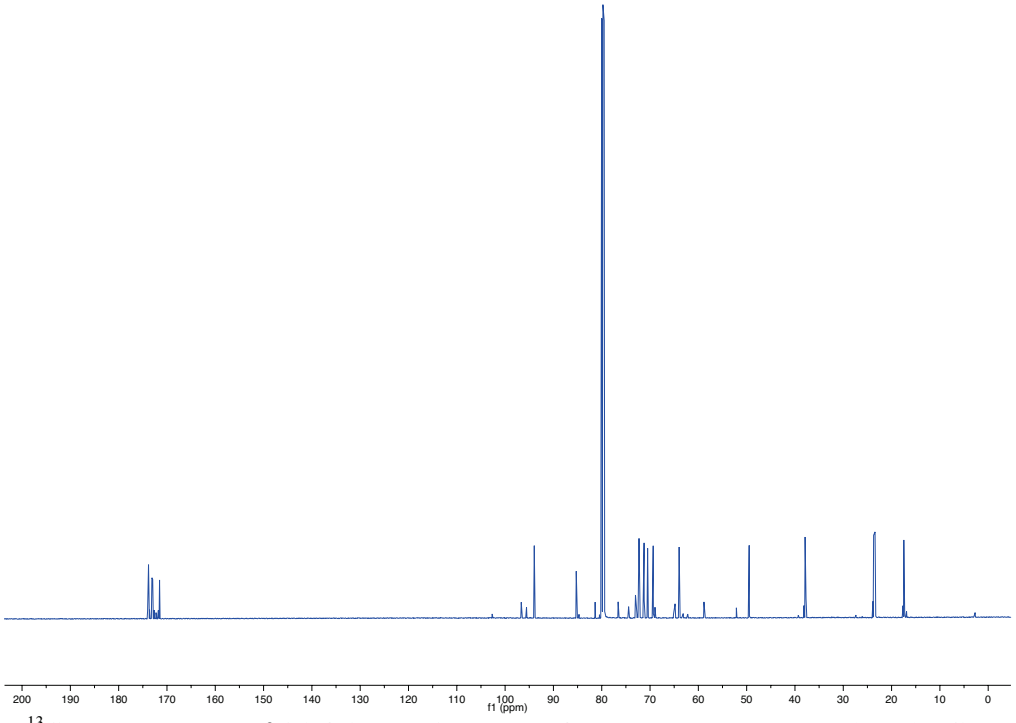


Figure S17. ^{13}C NMR spectrum of **1,3,4,6-tetra-*O*-acetyl-*N*-4-pentynoyl-D-galactosamine (Ac$_4$GalKyne)** mixed anomers in $CDCl_3$ (126 MHz).