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Supporting Information

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Increasing the Efficacy of Bioorthogonal Click Reactions for Bioconjugation: A Comparative Study**

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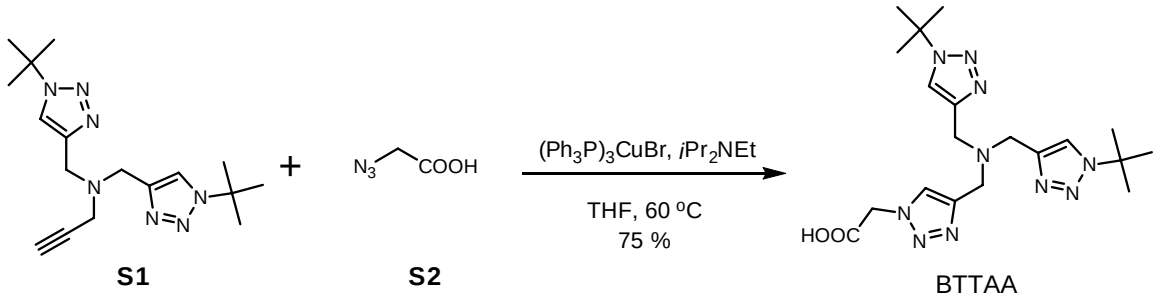
Materials and methods

All chemical reagents and solvents were obtained from Sigma-Aldrich and Acros and used without further purification unless otherwise noted unless otherwise specified. Flash chromatography was performed using a Sorbent 60 Å 230- to 400-mesh silica gel. Analytical thin layer chromatography (TLC) was performed on glass-backed Analtech Uniplate silica gel plates, and compounds were visualized by staining with *p*-anisaldehyde or phosphomolybdic acid or $KMnO_4$ stain. Organic extracts were dried over anhydrous $MgSO_4$ or Na_2SO_4, and the drying agent was removed by gravity filtration. Unless otherwise specified, all solvents were removed under reduced pressure, using a rotary evaporator. Rhodamine-dextran, Alexa Fluor dye-conjugated azides and alkynes were purchased from Invitrogen. Frosted Microscope Slides and wide-bore Pasteur pipets were purchased from Fisher Scientific and 18 × 18 mm square cover glasses were purchased from Corning. Melting point (mp) was taken on a Melt-Temp (Laborotary Device USA) apparatus using a Traceable® digital thermometer without calibration. ^1H chemical shifts (δ) are referenced to residual protic solvent (D_2O, 4.79 ppm) and coupling constants (*J*) are reported in hertz (Hz). ^{13}C NMR spectra were recorded at 150 MHz and proton decoupled. Electrospray ionization mass spectra (ESI-MS) were obtained at the Albert Einstein Laboratory for Macromolecular Analysis and Proteomics. Kinetic measurements using propargyl alcohol and 3-azido-7-hydroxycoumarin as the model system were optimized using a 96-well BioTek Synergy Hybrid Plate Reader. Microinjections were performed using a PV 820 Pneumatic PicoPump (World Precision Instruments) under Nikon SMZ1500 with lens Plan Apo 1× WD70.

Biotin-azide and biotin-alkyne were purchased from Click Chemistry Tools. Alexa Fluor dye conjugates were purchased from Invitrogen. *DC* protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). Horseradish peroxidase-conjugated anti-biotin antibody (HRP-anti-biotin antibody) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). SuperSignal West Pico Chemiluminescent Substrate was obtained from Pierce Biotechnology (Rockford, IL). Protease inhibitor Complete (EDTA-free) was purchased from Roche (Nutley, NJ). *N,N*-bis((1-*tert*-butyl-1*H*-1, 2,3-triazol-4-yl)methyl)prop-2-yn-1-amine (**S1**) was synthesized according to literature procedures.[s1]

Synthesis procedures

Synthesis of 2-(4-((bis((1-*tert*-butyl-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)acetic acid



S1 **S2** BTTAA

To a 100-mL round-bottom flask were added *N,N*-bis((1-*tert*-butyl-1*H*-1, 2,3-triazol-4-yl)methyl)prop-2-yn-1-amine (**S1**) (1.00 g, 0.30 mmol, 1.0 eq) and 2-azidoacetic acid (**S2**) (0.45 g, 0.45 mmol, 1.5 eq) in 30 mL of THF. To the mixture were added *N,N*'-diisopropylethylamine (0.62 g, 0.48 mmol, 1.6 eq) and tris(triphenylphosphine)copper(I) bromide (0.28 g, 0.03 mmol, 10 mol%). The reaction mixture was stirred vigorously at 60 °C overnight under argon. To the reaction mixture were added 20 mL water and ~ 2 g

CupriSorb™. The mixture was stirred for 30 additional min and then filtered. The crude product was concd *in vacuo* and was purified by flash chromatography (400 g silica gel, 40% MeOH in EtOAc with 1% acetic acid, R_f 0.2, $KMnO_4$ stain) to provide 1.09 g of product (yield: 75%) as a white solid. mp 115.5-116.5 °C; ^{1}H NMR (600 MHz, D_2O) δ 8.00 (s, 2H), 7.92 (s, 1H), 5.05 (s, 2H), 3.92 (s, 2H), 3.90 (s, 4H), 1.65 (s, 18H); ^{13}C NMR (150 MHz, D_2O) δ 173.2, 143.0, 142.6, 126.3, 122.8, 60.3, 53.1, 48.0, 47.9, 28.9. HRMS calculated for $C_{19}H_{30}N_{10}O_2$ $[M+H]^+$ 431.2631, found 431.2590.

Protocol for kinetic measurement of CuAAC of 3-azido-7-hydroxycoumarin and propargyl alcohol

Stock Solutions:

$CuSO_4$: 10 mM in water

Ligand: for TBTA 6 mM in 1:4 DMSO/tBuOH, for THPTA, BTTES and BTTAA 20 mM in water

Sodium Ascorbate: 25 mM in water

Azido coumarin: 1 mM in DMSO

Propargyl alcohol: 1.0 mM

Buffer: 0.5 M potassium phosphate, pH 7.0

Final Concentrations:

$CuSO_4$: 50 μM

Ligand: ligand to copper ratio is 6:1

Sodium Ascorabte: 2.5 mM

Azido coumarin: 0.1 mM

Propargyl alcohol: 50 μM

Buffer: 0.1 M potassium phosphate, pH 7.0

Procedure for 200 μL reactions:

In a 96-well fluorescence plate, add the reagents in the order listed below:

1. 40 μL of 0.5 M phosphate buffer, pH 7.0
2. 10 μl of 1.0 mM propargyl alcohol
3. 10 μl of DMSO
4. For $CuSO_4$ and TBTA premix, 11 μl ([Cu] = 0.909 mM). For $CuSO_4$ and THPTA, BTTES, or BTTAA, 4 μl ([Cu] = 2.5 mM)
5. 20 μl of 1.0 mM azido coumarin
6. Add water to 180 μl volume
7. 20 μl of 25 mM sodium ascorbate
8. Read fluorescence (λ_{ex} = 404 nm, λ_{em} = 477 nm, RFU) on Synergy plate reader

Tissue culture/cell growth conditions

Jurkat cells were grown in RPMI 1640 Medium supplemented with 10% FCS (Sigma). HEK-293 cells expressing PD1-Fc were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. All cells were incubated in a 5.0% carbon dioxide, water saturated incubator at 37 °C.

Flow cytometry

Flow cytometry experiments were performed on a Becton Dickinson FACSCalibur flow cytometer using a 488 nm argon laser. At least 20,000 cells were recorded for each sample. Flow cytometry data were analyzed using FlowJo. Mean fluorescence intensity (MFI) was calculated for live cells. Cell viability was determined by gating the sample on basis of forward scatter (to sort by size) and FL3 (to sort by 7-AAD negative). Scatter plots are shown for the total cell population.

Expression and purification of PD1-Fc

HEK-293 cells expressing PD1-Fc fusion protein (created in the Macromolecular Therapeutics Development Facility of Albert Einstein College of Medicine, unpublished) were incubated in DMEM containing 50 μM Ac_4ManNAz. After 4 days the supernatant (150 mL) was collected from confluent cells. Cells and cellular debris were removed from the supernatant by centrifugation (300 × g, 3 min). The supernatant was concentrated to 25 mL using Spin-X UF 20 concentrators, 10k MWCO (Corning). The sample was diluted 1:1 in protein G resin binding/wash buffer (20 mM Na_2HPO_4, 150 mM NaCl, pH 8.0) and incubated overnight at 4 °C with 0.6 mL protein G resin (GenScript). The sample/resin slurry was applied to a pre-equilibrated disposable polypropylene column (Thermo Scientific) and washed with 50 mL G resin binding/wash buffer. Proteins binding to the G resin were eluted with 6 mL elution buffer (0.1 M citric acid, pH 2.0). The eluant was collected in 0.5 mL fractions in tubes containing 1 mL neutralization buffer (1 M Tris, pH 8.0). All fractions were checked to ensure that the final pH was neutral. Protein purity and identity were confirmed by SDS-PAGE of the collected fractions followed by Coomassie Brilliant Blue staining. Staining was achieved by incubating protein gels in coomassie (0.1% coomassie in 50% methanol, 40% H_2O, 10% glacial acetic acid) for 1 hr, followed by overnight incubation in water. Both steps were performed on a mechanical shaker at room temperature. In all fractions the major protein band ran at 65 kDa confirming successful purification of PD1-Fc. Fractions were combined and concentrated using Spin-X UF 6 concentrators, 10k MWCO (Corning) and final protein concentration was determined using the D_C protein assay kit (BIO-RAD).

Labeling of PD1-Fc via the CuAAC and Cu-free click chemistry and detection by Western blot.

Purified recombinant PD1-Fc metabolically labeled with Ac_4ManNAz was diluted in lysis buffer (100 mM Na_3PO_4, 150 mM NaCl, 1% NP-40, pH 7.4) at a concentration of 0.2 mg/mL and reacted with 100 μM biotin-alkyne in a 100 μL reaction containing premixed ligand-$CuSO_4$ complex ([ligand]:[$CuSO_4$] = 2:1, [$CuSO_4$] = 250 μM) and 2.5 mM freshly prepared sodium ascorbate. Ligands used included BTTAA, BTTES, TBTA and THPTA. For Cu-free click chemistry, 0.2 mg/mL purified protein was reacted with or 100 μM BARAC-biotin in a 100 μL reaction. The samples were lightly vortexed and allowed to react for 1 hour (25 °C, 800 rpm in eppendorf Theromomixer R). The samples were resolved on 4-20% Precise^{TM} Protein Gels (Pierce) (6 μg protein/well). The samples were transferred to nitrocellulose, and incubated for 1 hr at room temperature in blocking buffer (5% non-fat milk in 1X TBST (Tris buffered saline with 0.1% Tween-20, pH 7.5)). The blocked membrane was incubated for 1 hour at room temperature with an HRP-anti-biotin antibody (1:100,000 dilution) in blocking buffer, washed with 1X TBST (3×, 15 min/wash) and developed using SuperSignal® West Pico Chemiluminescent Substrate (Pierce). X-OMAT LS film (Kodak) was used to detect the chemiluminescence. Coomassie Blue staining was used to verify equal protein loading.

Metabolic labeling of Jurkat cells and preparation of cell lysate

Jurkat cells were incubated for 3 d in untreated RPMI medium or RPMI medium containing 50 μM Ac_4ManNAz or Ac_4ManNAl. The cells were harvested by

centrifugation (300 × g, 3 min) and homogenized in lysis buffer (100 mM Na_3PO_4, 150 mM NaCl, 1% NP-40, pH 7.4) containing protease inhibitors (Roche *cO*mplete tablets, EDTA-free) by 10 free-thaw cycles. Insoluble debris was removed by centrifugation (10,000 × g, 10 min) and the soluble protein concentration was determined using the D_C protein assay kit (Bio-Rad).

Labeling of sialylated glycoproteins in Jurkat cells lysates via the CuAAC and Cu-free click chemistry and detection by Western blot analysis

Jurkat cell lysates metabolically treated with Ac_4ManNAz or Ac_4ManNAl was diluted in lysis buffer (100 mM Na_3PO_4, 150 mM NaCl, 1% NP-40, pH 7.4) at a concentration of 0.6 mg/mL and reacted with 100 μM biotin-alkyne (for azide-bearing protein) or 100 μM biotin-azide (for alkyne-bearing protein) in a 100 μL reaction containing premixed ligand-$CuSO_4$ complex ([$CuSO_4$] = 250 μM, [ligand]:[$CuSO_4$] = 2:1) and 2.5 mM freshly prepared sodium ascorbate. Ligands used included BTTAA, BTTES, TBTA and THPTA. For Cu-free click chemistry, 0.6 mg/mL cell lysates metabolically treated with Ac_4ManNAz were reacted with 100 μM BARAC-biotin in a 100 μL reaction. The samples were lightly vortexed and allowed to react for 1 hour (25 °C, 800 rpm in eppendorf Theromomixer R). Reacted samples were resolved on 4-20% Precise™ Protein Gels (Pierce) (9 μg sample/well). The samples were transferred to nitrocellulose, and incubated for 1 hr at room temperature in blocking buffer (5% non-fat milk in 1X TBST (Tris buffered saline with 0.1% Tween-20, pH 7.5)). The blocked membrane was incubated for 1 hour at RT with an HRP-anti-biotin antibody (1:100,000 dilution) in blocking buffer, washed with 1X TBST (3×, 15 min/wash) and developed

using SuperSignal® West Pico Chemiluminescent Substrate (Pierce). Coomassie staining was used to verify equal protein loading.

Metabolic labeling of sialylated glycans in Jurkat cells, detection of azido sialic acids via the CuAAC and Cu-free click chemistry, and analyzing by flow cytometry.

Jurkat cells were incubated for 3 days in untreated RPMI or RPMI medium containing 50 μM Ac_4ManNAz. On day three, the cells were harvested and washed 2x with cold labeling buffer (PBS, pH 7.4, 1% FCS), and were aliquoted into a 96-well round bottom tissue culture plate (0.5 million cells in 90 μL/well) (Corning Inc.). For CuAAC, 45 μM biotin-alkyne, ligand-$CuSO_4$ complex ([ligand]:[$CuSO_4$] = 6:1, [$CuSO_4$] = 30-60 μM,) and 2.5 mM sodium ascorbate were added to each well. After reaction for 3 min at room temperature, the reactions were quenched with 1 mM BCS. Ligands used included BTTAA, BTTES, TBTA and THPTA. For copper-free click chemistry, 4.5 μL BARAC-biotin (1 mM in 15% H_2O, 85% DMSO) and 5.5 μL PBS were added to each well and allowed to react for 3 min at room temperature before dilution with 200 μL labeling buffer. Following the reactions, the cells were pelleted, washed 3× with 200 μL cold labeling buffer and resuspended in the same buffer containing 1 μg/mL streptavidin-Alexa Fluor 488 (Invitrogen). The plate was covered with aluminum foil and incubated at 4 °C for 30 min (resuspension once after 15 minutes). The cells were then washed 3× with 200 μL cold labeling buffer and resuspended in 400 μL cold FACS buffer (Hank's Balanced Salt Solution, pH 7.4, 1% FCS, 2 μg/mL 7-AAD, 0.2% NaN_3) for flow cytometry analysis. FACS was performed on a Becton Dickinson FACSCalibur.

Cell Growth Assay

Jurkat cells were cultured in RPMI medium or RPMI medium containing 50 μM Ac_4ManNAz. After 3 days the cells were harvested by centrifugation (300 × g, 3 min) and washed twice with 10 mL of PBS (1% FCS). Following the washes the cells were resuspended in the wash buffer at a concentration of 1 M viable cells per 90 μL. Cells were reacted with biotin-alkyne (100 μM), premixed ligand-$CuSO_4$ complex ([ligand]:[$CuSO_4$] = 6:1, [$CuSO_4$] = 30 or 50 μM) and 2.5 mM freshly prepared sodium ascorbate. Ligands used included BTTAA, BTTES, TBTA and THPTA. As a positive apoptosis control, cells cultured in the presence of Ac_4ManNAz were reacted with 30 or 50 μM $CuSO_4$ and 2.5 mM sodium ascorbate in the absence of ligand. After 3 minutes all reactions were quenched with 1 mM BCS. Unreacted cells cultured in the absence and presence of Ac_4ManNAz were included as negative controls. All cells were washed twice with 0.5 mL PBS (1% FCS) and once with 0.5 mL RPMI medium. After the washes, cells were resuspended in 1 mL medium and total cell counts were determined. Approximately 70% of cells were recovered. Cells were seeded into 6-well plates at a concentration of 600,000 cells per well. Cells were incubated at 37 ° C and viable cells were counted every 24 hours for 4 d using the Trypan blue dye exclusion method.

Metabolic labeling of zebrafish embryos by microinjection with Ac_4GalNAz and detection by the CuAAC and copper-free click chemistry.

Zebrafish embryos at the one-cell stage were dechorionated by 1 mg/mL Pronease E; followed with microinjection of 2 nL of 12.5 mM Ac_4GalNAz and rhodamine-dextran (5% w/v) as tracer in 0.2 M KCl. After injection, embryos were cultured in E3 embryo

medium at 28 °C. When embryos reached 24 hours post fertilization (hpf), reactions were set up for either the CuAAC or with BARAC-based Cu-free click chemistry. For CuAAC, 94 μL E3 embryo medium was added to each well of a 1% agarose-coated 96-well plate, followed by addition of 2 μL biotin-alkyne (from a 2.5 mM stock in H_2O), 2 μL BTTAA-$CuSO_4$ complex ([BTTAA]:[$CuSO_4$] = 6:1, [Cu]= 45 μM). Embryos were then transferred into these wells for less than five embryos per well using a fire-polished glass Pasteur pipette. The solutions were gently shaken, and freshly prepared sodium ascorbate (2.5 mM from 100 mM stock in embryo medium) was added to initiate the click reactions. After 5 min, the reaction was quenched with 1 mM BCS and diluted immediately with 100 μL embryo medium. For copper-free click chemistry, embryos were transferred into the well containing 95 μL E3 embryo medium, followed by addition of 5 μL BARAC-biotin (1 mM in 15% H_2O, 85% DMSO). After 5 min, the reaction was diluted by adding 200 μL embryo medium. After reaction, embryos labeled with biotin were washed 2 × with 15 mL embryo medium and then incubated with Alexa Fluor-488 streptavidin (1 μg/mL, Invitrogen) for 30 minutes in the dark. These embryos were washed 3 × again with 15 mL embryo medium and anesthetized with 0.2% (w/v) Tricaine in embryo medium. After mounting on a 35 mm glass bottom dishes (MatTek) in 1.2% ultralow melting point agarose, the embryos were ready for imaging by Leica confocal microscopy SP5. Composite figures are prepared using ImageJ.

Metabolic labeling of fucosylated glycans in zebrafish embryos by microinjection with GDP-FucAl and detection by the CuAAC.

Zebrafish embryos at the one-cell stage were dechorionated by 1 mg/mL Pronase E; followed with microinjection of 1 nL of a 20 mM solution of GDP-FucAl and rhodamine-dextran (5% w/v) as a tracer in 0.2 M KCl. In the negative control group, GDP-FucAl was replaced with GDP-fucose in injection solution. The embryos were then cultured in E3 embryo medium at 28 °C. When embryos reached 10 hours post fertilization, reactions were set up for the BTTES-Cu(I) or BTTAA-Cu(I) catalyzed click chemistry. 92 μL E3 embryo medium was added to each well of a 1% agarose-coated 96-well plate, followed by addition of 4 μL Alexa Fluor 488 azide (from a 2.5 mM stock in H_2O, Invitrogen), 2 μL BTTES or BTTAA-$CuSO_4$ complex ([ligand]:[$CuSO_4$] = 6:1, [Cu] = 40 μM). Embryos were then transferred into these wells for less than five embryos per well using a fire-polished glass Pasteur pipette. The solutions were gently shaken, and freshly prepared sodium ascorbate (2.5 mM from 100 mM stock in embryo medium) was added to initiate the click reactions. After 3 min, the reaction was quenched with 1 mM BCS and diluted immediately with 100 μL embryo medium. The treated embryos were washed 3 × with 15 mL embryo medium followed by mounting on a 35 mm glass bottom dishes (MatTek) in 1.2% ultralow melting point agarose. All embryo images are acquired by Leica confocal microscopy SP5, and composite figures are prepared using ImageJ.

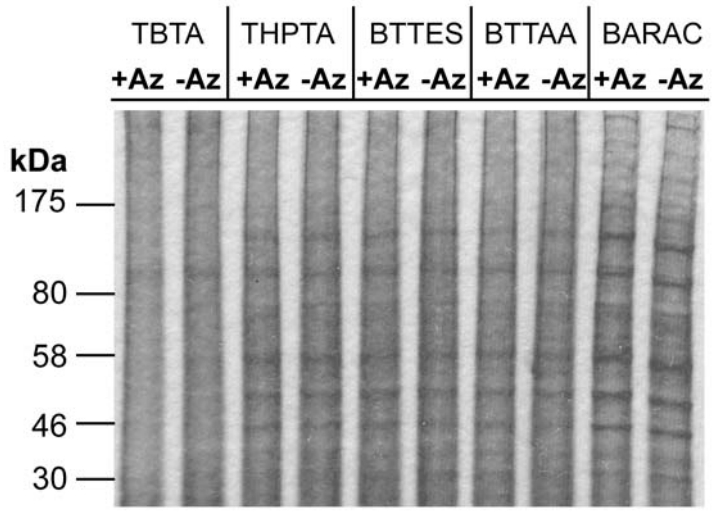


Figure S1: Coomassie staining of a duplicate protein gel for Ac_4ManNAz-treated or untreated Jurkat cell lysates.

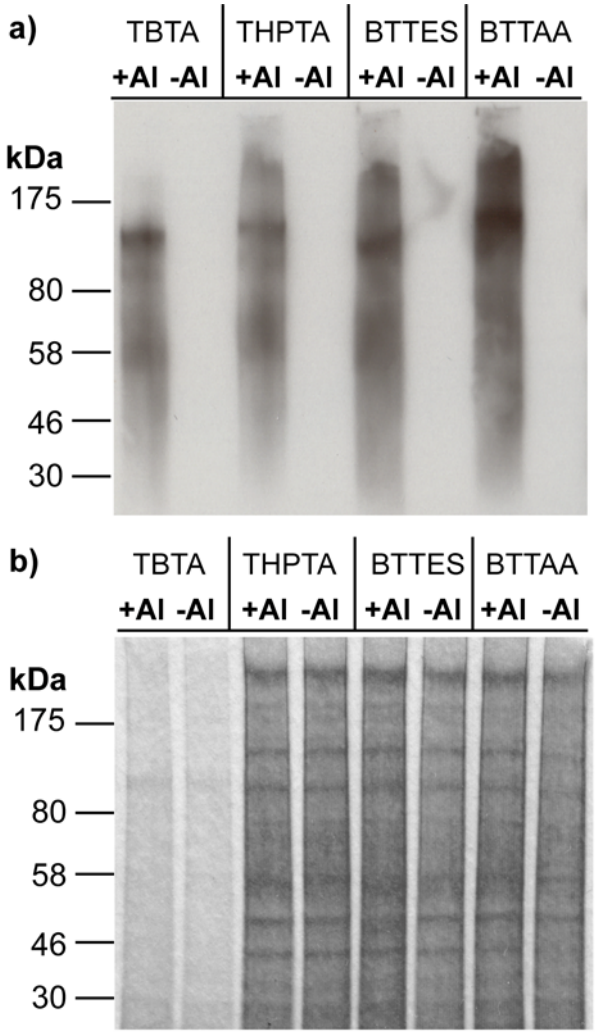


Figure S2: Comparison of the efficiency of ligand-accelerated CuAAC in labeling crude cell lysates. a) Western blot analysis of Ac_4ManNAl-treated or untreated Jurkat cell lysates. Reaction conditions for CuAAC: biotin-azide (100 μM) in the presence of sodium ascorbate (2.5 mM), $CuSO_4$ (250 μM) premixed with various tristriazolylamino ligands (500 μM). Reactions were allowed to proceed for 1 h at room temperature, and analyzed by Western blot using an HRP-conjugated anti-biotin antibody. (b) Total protein loading was confirmed by Coomassie staining.

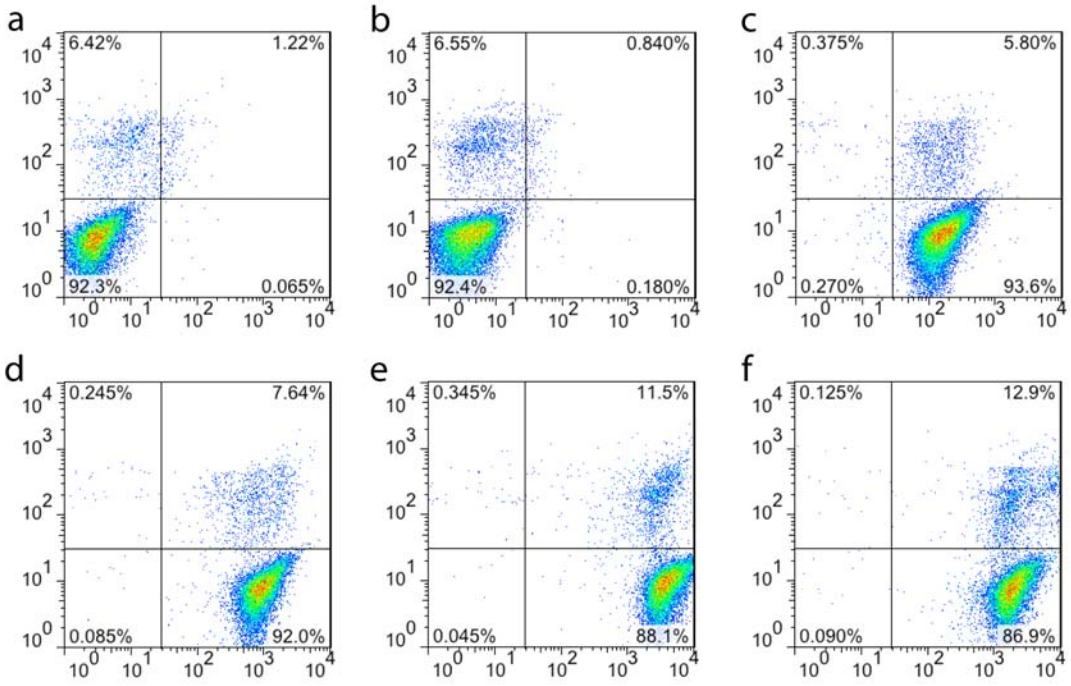


Figure S3: Representative FL3 *vs.* FL1 scatter plots for the labeling experiments described in **Fig. 2**. In all plots, the x-axis shows the degree of cell-surface sialic acid labeling as measured by Alexa Fluor 488 fluorescence (FL1) and the y-axis shows the degree of cell-nucleus labeling as measured by 7-AAD fluoresence (FL3). Jurkat cells were cultured in medium supplemented with 50 μM Ac_4ManNAz. After 3 days, cells were untreated (**a),** labeled with 45 μM biotin-alkyne in the presence of TBTA (**b**), THPTA (**c**), BTTES (**d**), or BTTAA (**e**) in 6:1 ligand–$CuSO_4$ complex ([Cu] = 30 μM) and 2.5 mM sodium ascorbate or labeled with 45 μM BARAC (**f).** The reactions were quenched with BCS (**b-e**) or an excess volume of PBS (**f**) and probed with streptavidin-Alexa Fluor 488. Cells were then treated with 7-AAD and analyzed by flow cytometry. In control experiments (cells cultured in the absence of Ac4ManNAz, but subjected to the CuAAC and BARAC-based copper-free click chemistry), only background fluorescence was observed as described previously in references 12 and 16.

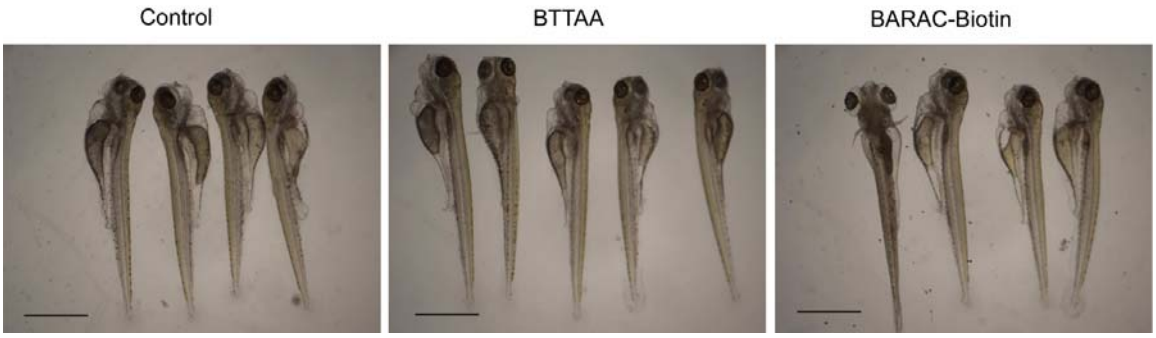


Figure S4: No developmental defects were observed for the zebrafish embryos treated with Ac_4GalNAz through five days post click reactions ([Cu] = 45 μM, ([biotin-alkyne] = 50 μM, [BARAC-biotin] = 50 μM). Each experimental group consisted of 30 embryos. Control group consists of embryos microinjected with Ac_4GalNAz, but untreated with click reactions. Scale bar: 500 μm.

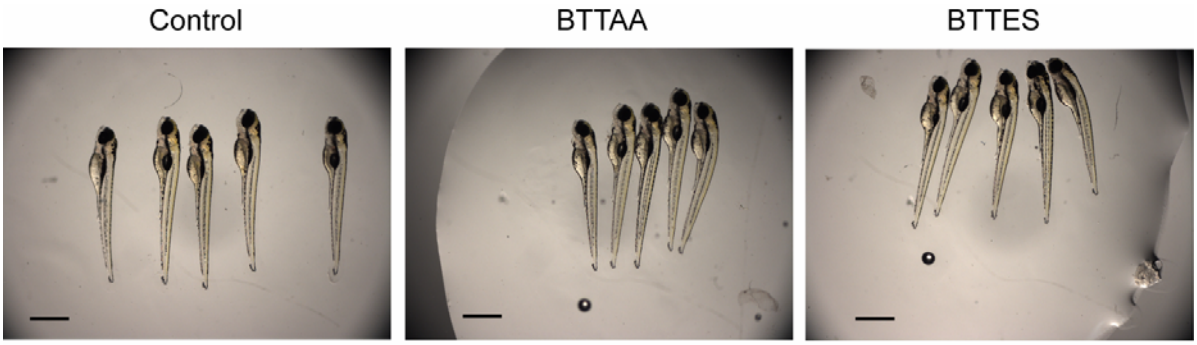


Figure S5: No developmental defects were observed for the zebrafish embryos treated with GDP-FucAl through five days post click reactions ([Cu] = 40 μM). Each experimental group consisted of 30 embryos. Control group consists of embryos microinjected with GDP-FucAl, but untreated with click reactions. Scale bar: 500 μm.

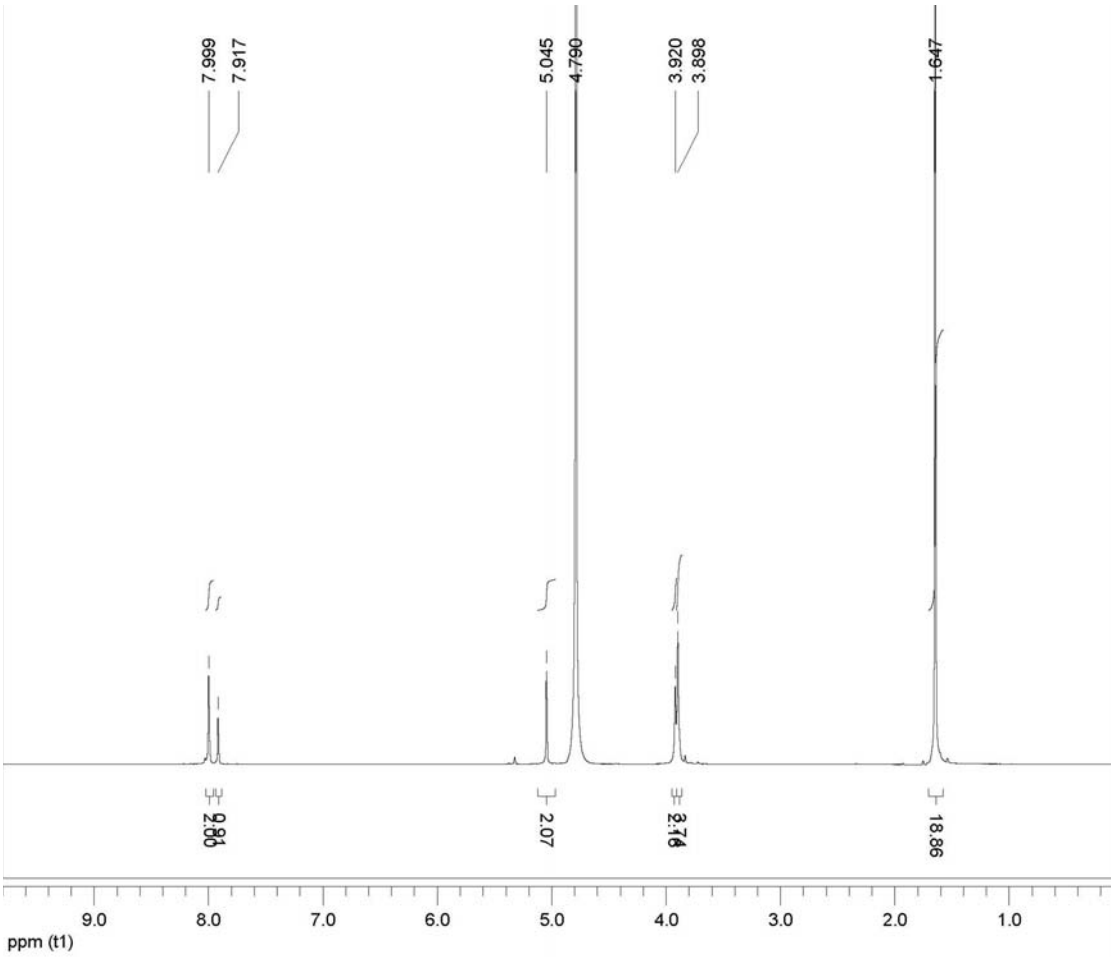


Figure S6: ^{1}H NMR spectrum of BTTAA (D_2O, 298 K).

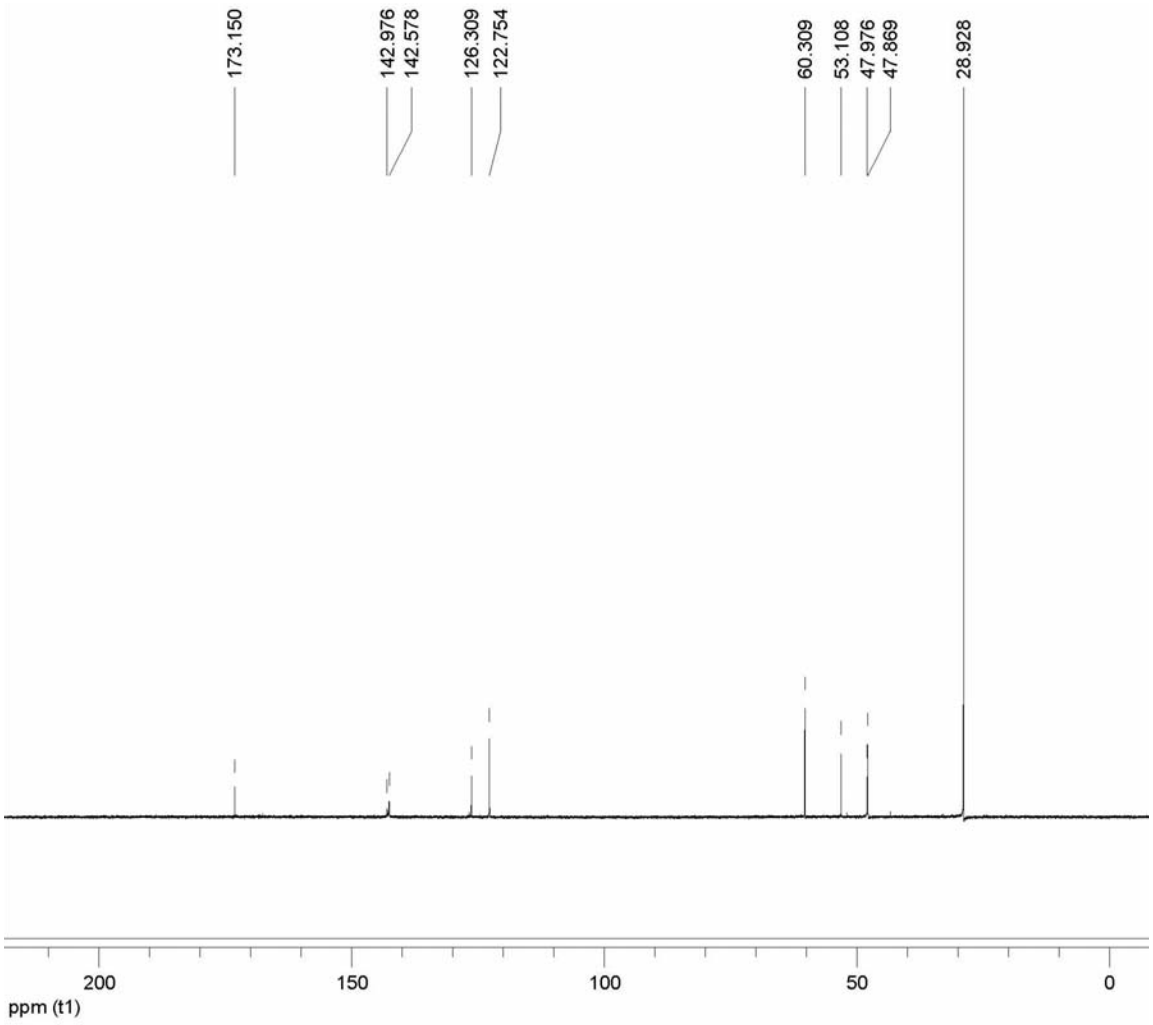


Figure S7: ^{13}C NMR spectrum of BTTAA (D_2O, 298 K).

Reference:

S1. Soriano Del Amo, D.; Wang, W.; Jiang, H.; Besanceney, C.; Yan, A. C.; Levy, M.; Liu, Y.; Marlow, F. L.; Wu, P., *J. Am. Chem. Soc.* **2010,** 132, 16893-16899.