Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

Design of a reversible biotin analog and applications in protein labeling, detection, and isolation

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General Materials and Methods

^{1}H-NMR spectra were recorded at room temperature on a Bruker ADVANCE-400 spectrometer with residual proton resonances in deuterated solvents (DMSO-d_6, MeOD-d_4, or $CDCl_3$) as the internal standard. LC/MS spectra were obtained with Waters micromass ZQ instrument. TLC was performed on aluminum-backed plates with silica gel 60 with F_{254} indicator. Visualization was accomplished with UV light (254 nm) or a biotin dip solution (2g of 4-dimethylamino cinnamaldehyde in 100 mL of 6 M HCl and 100 mL of isopropyl alcohol). Flash column chromatography was carried out on silica gel (230-400 mesh). High-performance liquid chromatography (HPLC) purification was carried out on a Waters prep LC instrument equipped with a Waters 2998 photodiode array detector. If not otherwise noted, HPLC solvent A is 25 mM triethylammonium acetate (TEAA) in water and solvent B is MeOH. All moisture- or air-sensitive reactions were carried out under a static argon atmosphere. All commercially available reagents and solvents were used without further purification.

Synthesis

N3′-Ethyl biotin succinimidylester (2)

Biotin **1** (5 g, 20.5 mmol, Aldrich) was suspended in 100 mL anhydrous MeOH. 2 mL of 2 M HCl in MeOH was added and the reaction mixture was stirred (magnetic) under argon at room temperature overnight. The mixture was concentrated and crystallized in MeOH to afford biotin methylester (4.8 g, 18.5 mmol, 90% yield). Biotin methylester (989 mg, 3.83 mmol) in a 50 mL round flask was dried under high vacuum pump overnight, and then dissolved in 10 mL anhydrous pyridine and stirred (magnetic) under argon at room temperature. 4,4'-Dimethoxytrityl chloride (2.59 g, 7.66 mmol) was added at one shot as solid, followed by addition of DMAP (47 mg, 0.38 mmol) at one shot as solid. The reaction mixture was stirred (magnetic) under argon at room temperature for 24 h. Excess of 4,4'-Dimethoxytrityl chloride

was quenched by addition of MeOH (2 mL) by syringe and the reaction mixture was stirred (magnetic) under argon at room temperature for 5 min, and concentrated *in vacuo* using rotary evaporator connected to a high vacuum pump. The mixture was extracted with 60 mL CH_2Cl_2, washed with 30 mL aq. $NaHCO_3$, 30 mL brine, dried over Na_2SO_4, and concentrated *in vacuo* using rotary evaporator connected to a high vacuum pump. The crude mixture was purified by column chromatography on SiO_2 (CH_2Cl_2: MeOH = 100:4) to afford *N1′*-DMTr biotin methylester (1.2 g, 2.14 mmol, 56% yield) as a pale yellow solid. *N1′*-DMTr biotin methylester (320 mg, 0.57 mmol) was dissolved in 4 mL dry DMF and stirred (magnetic) under argon at room temperature. NaH (25 mg, 0.63 mmol, 60 wt%) was added at one shot as solid. After stirring (magnetic) under argon at room temperature for 10 min, CH_3CH_2I (92 μL, 1.1 mmol) was added by syringe. The reaction mixture was stirred (magnetic) under argon at room temperature for 3 h. The mixture was diluted with 40 mL CH_2Cl_2, washed with 30 mL H_2O, 30 mL brine, dried over Na_2SO_4, and concentrated *in vacuo* using rotary evaporator connected to a high vacuum pump, which was directly used for next step. The crude was dissolved in 5 mL of 80% HOAc. The reaction mixture was stirred (magnetic) at room temperature for 20 min at atmosphere. The mixture was extracted with 40 mL CH_2Cl_2, washed with 30 mL H_2O, 30 mL brine, dried over Na_2SO_4, and concentrated *in vacuo* using rotary evaporator connected to a high vacuum pump. The crude mixture was purified by column chromatography on SiO_2 ($CHCl_3$: MeOH = 100:5) to give a white solid. The obtained white solid was dissolved in 3 mL MeOH. 1 M NaOH (3 mL) was added by syringe. The reaction mixture was stirred (magnetic) at room temperature for 1 h at atmosphere. The mixture was concentrated *in vacuo* using rotary evaporator connected to a high vacuum pump, and acidified by 2 M HCl to pH 3.0. The mixture was extracted with CH_2Cl_2 (3 × 30 mL). The combined organic layer was washed with 30 mL brine, dried over Na_2SO_4, and concentrated *in vacuo* using rotary evaporator connected to a high vacuum pump. The crude mixture was purified by column chromatography on SiO_2 ($CHCl_3$: MeOH: HOAc = 10:1:0.1) to afford a white solid. The intermediate (131 mg, 0.48 mmol) was dissolved in 2 mL dry DMF and stirred (magnetic) under argon at room temperature. O-(*N*-Succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU, 175 mg, 0.58 mmol) was added at one shot as solid, followed by addition of dry TEA (134 μL, 0.96 mmol) by syringe. The reaction mixture was stirred (magnetic) under argon at room temperature for 1 h. The mixture was concentrated *in vacuo* using rotary evaporator connected to a high vacuum pump,

and extracted with 40 mL CH_2Cl_2, washed with 30 mL H_2O, 30 mL brine, dried over Na_2SO_4, and concentrated *in vacuo* using rotary evaporator connected to a high vacuum pump. The crude mixture was purified by column chromatography on SiO_2 ($CHCl_3$: MeOH = 10:1) to afford **2** (80 mg, 0.22 mmol, 38% yield) as a white solid.

^{1}H-NMR (400 MHz, DMSO-d_6): δ 6.62 **(**s**,** 1H, NH), 4.25 (m, 1H), 4.18 (m, 1H), 3.22 (m, 1H), 2.88 (m, 3H), 2.81 (s, 4H), 2.69 (m, 2H), 2.63 (m, 1H), 1.78-1.40 (m, 6H), 1.0 (t, *J* = 6.8 Hz, 3H).

LC/MS: 370.03$[M+H]^+$.

***N*-(2-(2-(2-Aminoethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-3-ethyl-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (3)**

N3′-Ethyl biotin succinimidylester **2** (200 mg, 0.54 mmol) in 4 mL DMF was added dropwise by syringe to a solution of 2,2′-(ethylenedioxy)bis-(ethylamine) (238 μL, 1.62 mmol, Aldrich) in 6 mL DMF at 0 °C. After addition, the reaction mixture was stirred at 0 °C for 2 h, and then concentrated. The crude was purified by RP-HPLC (Column: Phenomenex Luna C18 250 × 21.2 mm column; Buffer A: 0.1% TFA in H_2O; Buffer B: 0.1% TFA in MeOH; Gradient: 5−95% buffer B over 40 min; Flow rate: 20 mL/min) to afford **3** (163 mg, 0.41 mmol, 75% yield).

^{1}H-NMR (400 MHz, D_2O): δ 4.41 (m, 1H), 4.29 (m, 1H), 3.64 (m, 3H), 3.59 (s, 4H), 3.52 (m, 2H), 3.38 (m, 1H), 3.27 (m, 3H), 3.09 (m, 2H), 2.88 (m, 2H), 2.67 (m, 1H), 2.16 (t, *J* = 7.2 Hz, 3H), 1.57-1.28 (m, 6H), 0.97 (t, *J* = 6.8 Hz, 3H).

LC/MS: 403.22$[M+H]^+$.

2,5-Dioxopyrrolidin-1-yl-21-((3aS,4S,6aR)-3-ethyl-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-17-oxo-4,7,10,13-tetraoxa-16-azahenicosan-1-oate (4)

N3′-Ethyl biotin succinimidylester **2** (43 mg, 0.12 mmol) was dissolved in 1 mL DMF and stirred (magnetic) under argon at room temperature. Amino-dPEG$_4$-acid (31 mg, 0.12 mmol) and Et_3N (33 μL, 0.23 mmol) in 0.3 mL H_2O were added by pipette to above solution. The reaction mixture was stirred (magnetic) under argon at room temperature for 2 h. The mixture was concentrated *in vacuo* using rotary evaporator connected to a high vacuum pump. The crude was directly used for next step. The crude intermediate was dissolved in 2 mL dry DMF and stirred (magnetic) under argon at room temperature. TSTU (43mg, 0.14 mmol) was added at one shot as solid, followed by addition of dry TEA (33 μL, 0.24 mmol) by syringe. The reaction mixture was stirred (magnetic) under argon at room temperature for 1 h. The mixture was concentrated *in vacuo* using rotary evaporator connected to a high vacuum pump. The residue was dissolved in 30 mL CH_2Cl_2, washed with 30 mL of 0.1 M HCl, 30 mL brine, dried over Na_2SO_4, and concentrated *in vacuo* using rotary evaporator connected to a high vacuum pump. The crude mixture was purified by column chromatography on SiO_2 ($CHCl_3$: MeOH = 8:1) to afford **4** (48 mg, 0.078 mmol, 65% yield) as a clear oil.

^{1}H-NMR (400 MHz, DMSO-d$_6$): δ 7.85 **(**s**,** 1H, NH), 6.61 **(**s**,** 1H, NH), 4.26 (m, 1H), 4.17 (m, 1H), 3.72 (m, 1H), 3.62 (m, 2H), 3.53 (m, 14H), 3.38 (m, 2H), 3.19 (m, 4H), 2.87 (m, 2H), 2.82 (s, 4H), 2.07 (t, *J* = 7.6 Hz, 2H), 1.65-1.30 (m, 6H), 1.0 (t, *J* = 6.8 Hz, 3H).

LC/MS: 617.19 $[M+H]^+$.

Triethylammonium-2-(6-amino-3-iminio-4,5-disulfonato-3H-xanthen-9-yl)-5-(((S)-23-carboxy-1-((3aS,4S,6aR)-3-ethyl-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-5,21-dioxo-9,12,15,18-tetraoxa-6,22-diazaheptacosan-27-yl)carbamoyl)benzoate (5)

Compund **4** (21 mg, 0.034 mmol) was dissolved in 5 mL dry CH_2Cl_2 and stirred (magnetic) under argon at room temperature. H-Lys(Boc)-OBu^t·HCl (11.5 mg, 0.034 mmol, Advanced ChemTech) and TEA (14 μL, 0.1 mmol) were added. The reaction mixture was stirred (magnetic) under argon at room temperature for 2 h. The mixture was diluted with 40 mL of CH_2Cl_2, and washed with 30 mL of 0.1 M HCl, 30 mL brine, dried over Na_2SO_4, and concentrated *in vacuo* using rotary evaporator. The crude mixture was purified by column chromatography on SiO_2 ($CHCl_3$: MeOH = 10:1~8:1). The intermediate was dissolved in 4 mL of 1/1 TFA/ CH_2Cl_2. The reaction mixture was stirred (magnetic) at room temperature for 2 h. The mixture was concentrated *in vacuo* using rotary evaporator, and the residue was precipitated in ethyl ether (15 mL), and dried *in vacuo* and used directly for next step. The intermediate (18 mg, 0.028 mmol) was dissolved in 2 mL dry DMF and stirred (magnetic) under argon at room temperature. Alexa Fluor 488-5TFP (25 mg, 0.028 mmol) and TEA (12 μL, 0.08 mmol) were added. The reaction mixture was stirred (magnetic) under argon at room temperature for 2 h. Ethyl ether (30 mL) was added to the reaction mixture, and the resulting precipitate was collected by centrifuge, and washed with ethyl acetate (10 mL), acetone (10 mL), and dissolved in 1 mL H_2O. The crude was purified by RP-HPLC (Column: Phenomenex Luna C18 250 × 21.2 mm column; Buffer A: 25 mM TEAA in H_2O; Buffer B: MeOH; Gradient: 5−95% buffer B over 40 min; Flow rate: 20 mL/min) to afford **5** (32 mg, 0.023 mmol, 68% yield).

^{1}H-NMR (400 MHz, D_2O): δ 8.20 (d, *J* = 1.6 Hz, 1H), 7.88 (dd, *J* = 1.6, 8.0 Hz, 1H), 7.29 (d, *J* = 8.0 Hz, 1H), 7.10 (d, *J* = 9.2 Hz, 2H), 6.85 (d, *J* = 9.2 Hz, 2H), 4.38 (m, 1H), 4.26 (m, 1H), 4.19 (m, 1H), 3.65 (m, 2H), 3.53 (s, 12H), 3.47 (m, 2H), 3.35 (m, 3H), 3.23 (m, 3H), 3.06 (m, 18H), 2.82 (m, 2H), 2.62 (m, 1H), 2.45 (m, 2H), 2.11 (t, *J* = 7.2 Hz, 3H), 1.77-1.22 (m, 12H), 1.15 (m, 27H), 0.97 (t, *J* = 6.8 Hz, 3H).

LC/MS: 1164.57 $[M+H]^+$.

Triethylammonium-2-(6-amino-3-iminio-4,5-disulfonato-3H-xanthen-9-yl)-5-(((S)-1-((3aS,4S,6aR)-3-ethyl-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-5,21-dioxo-23-(prop-2-yn-1-ylcarbamoyl)-9,12,15,18-tetraoxa-6,22-diazaheptacosan-27-yl)carbamoyl)benzoate (6)

Compound **5** (8 mg, 0.006 mmol) was dissolved in 2 mL dry DMF and stirred (magnetic) under argon at 0 °C. TSTU (2 mg, 0.006 mmol) and DMAP (2 mg) were added. The reaction mixture was stirred at 0 °C for 2 h. Propargylamine (50 μL) was added. The reaction mixture was stirred at 0 °C for another 15 min. Ethyl ether (20 mL) was added to the reaction mixture, and the resulting precipitate was collected by centrifuge, and washed with ethyl acetate (10 mL), acetone (10 mL), and dissolved in 1 mL H_2O. The crude was purified by RP-HPLC (Column: Phenomenex Luna C18 250 × 21.2 mm column; Buffer A: 25 mM TEAA in H_2O; Buffer B: MeOH; Gradient: 30−60% buffer B over 40 min; Flow rate: 20 mL/min) to afford **6** (6 mg, 0.004 mmol, 73% yield).

^{1}H-NMR (400 MHz, D_2O): δ 8.21 (d, *J* = 1.6 Hz, 1H), 7.88 (dd, *J* = 1.6, 8.0 Hz, 1H), 7.28 (d, *J* = 8.0 Hz, 1H), 7.09 (d, *J* = 9.2 Hz, 2H), 6.85 (d, *J* = 9.2 Hz, 2H), 4.38 (m, 1H), 4.25 (m, 1H), 4.19 (m, 1H), 3.86 (d, *J* = 3.2 Hz, 2H), 3.67 (m, 2H), 3.55 (s, 12H), 3.49 (m, 2H), 3.38 (m, 3H), 3.25 (m, 3H), 3.05 (m, 18H), 3.0 (m, 1H), 2.86 (m, 2H), 2.63 (m, 1H), 2.48 (m, 2H), 2.13 (t, *J* = 7.2 Hz, 3H), 1.77-1.22 (m, 12H), 1.15 (m, 27H), 0.96 (t, *J* = 6.8 Hz, 3H).

HRMS (ESI-TOF) $[M+H]^+$ calcd for $C_{53}H_{69}N_8O_{18}S_3$ 1201.3886, found 1201.3880.

Fluorescent Capture and Release Assay

100 μL of M280 Streptavidin Dynabeads (10 mg/mL, Invitrogen) was transferred to a 1.5 mL centrifuge tube, and washed with 500 μL PBS three times. 500 μL of *N3′*-Ethyl biotin-AF488 conjugate **5** (0.2 μM in PBS buffer, pH 7.4) was added and mixed under rolling and tilting at room temperature for 25 minutes. The tube was placed on the magnet for 1 minute, to concentrate the magnetic beads on the magnet. The supernatant was carefully removed and saved for measuring the capture efficiency, by measuring the fluorescent intensity of the supernatant solution. The tube was removed from the magnet, and 500 μL PBS was added, then the beads were resuspended by gentle pipetting 5 times. The tube was placed on the magnet for 1 minute. The supernatant was carefully removed. The PBS washing was repeated one more time. The tube was removed from the magnet and the beads were carefully resuspended in 500 μL of 2 mM

biotin in PBS (pH 7.4), with mixing under rolling and tilting at room temperature for 5 minutes. The tube was placed on the magnet for 1 minute. The supernatant was carefully removed and saved for measuring the release efficiency, by measuring the fluorescent intensity of the supernatant solution.

Kinetic Measurement of Rate Constants

All interaction analyses were conducted at 25 ºC in kinetic buffer (PBS, 0.1% BSA, 0.05% Tween-20, pH 7.2). The amine reactive biosensor tips from ForteBio were prewet for 20 min in water prior to use. The prepared samples and buffers were added to a 96-well microplate at 200 µL per well according the following sample layout, and the entire 96-well plate was agitated at 1000 rpm. Eight biosensor tips were performed the kinetic assay in parallel. Each amine reactive biosensor tip was equilibrated in MES buffer for 5 min, then activated with EDC/NHS for 5 min. The activated biosensor tip was labeled with 2 mM *N3′*-ethyl biotin **3** for 10 min, then quenched with 1 M ethanolamine for 5 min. The *N3′*-ethyl biotin **3** immobilized biosensor tip was incubated in kinetic buffer for 5 min to generate baseline, then incubated with 2 µg/mL streptavidin in kinetic buffer or kinetic buffer (control) at 1000 rpm for 10 min to generate association curve, followed by incubation with kinetic buffer or 2 mM biotin in kinetic buffer at 1000 rpm for 10 min to generate dissociation curve. The ForteBio Data Analysis Octet software was used to process the curve by subtracting sample curve from reference curve, and the kinetic data was calculated by fitting curve.

	1	2	3	4	5	6	7
A	MES	EDC/NHS	Ligand	Quench	Buffer	Streptavidin	Buffer
B	MES	EDC/NHS	Ligand	Quench	Buffer	Streptavidin	Buffer
C	MES	EDC/NHS	Ligand	Quench	Buffer	Buffer	Buffer
D	MES	EDC/NHS	Ligand	Quench	Buffer	Buffer	Buffer
E	MES	EDC/NHS	Ligand	Quench	Buffer	Streptavidin	2 mM Biotin
F	MES	EDC/NHS	Ligand	Quench	Buffer	Streptavidin	2 mM Biotin
G	MES	EDC/NHS	Ligand	Quench	Buffer	Buffer	2 mM Biotin
H	MES	EDC/NHS	Ligand	Quench	Buffer	Buffer	2 mM Biotin
Assay Definition	**Baseline**	**Activation**	**Loading**	**Quench**	**Baseline**	**Association**	**Dissociation**
Assay Time (s)	**300**	**300**	**600**	**300**	**300**	**500**	**500**

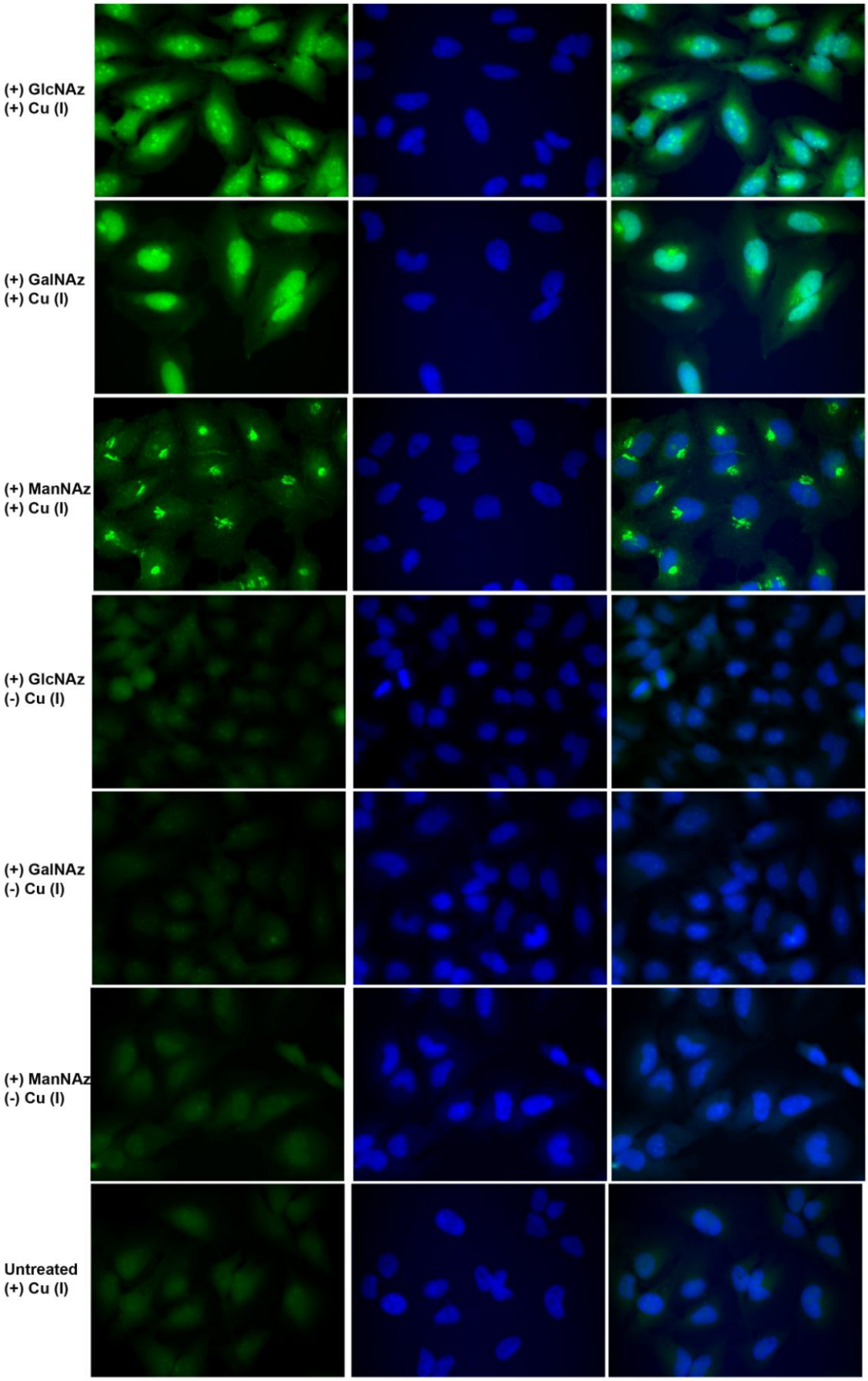
Kinetic buffer: PBS, 0.1% BSA, 0.05% Tween-20, 4 mM azide, pH 7.2.
MES: 100 mM, pH 5.0; EDC: 0.4 M in H_2O; NHS: 0.1 M in H_2O; and mix at 1:1 ratio right before testing.

Ligand: 2 mM *N3′*-ethyl biotin **3** in H_2O, pH 8.5; Quench: 1 M ethanolamine, pH 8.5; Streptavidin: 2 μg/mL in kinetic buffer.

Kinetic assay sample layout

Protein Labeling and Detection in Cells

HeLa cells were cultured on glass coverslips in 10 mL Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) at four 100mm tissue culture dishes. To each tissue culture dish, 10 μL of Ac_4GlcNAz (100 mM in DMSO), Ac_4GalNAz (100 mM in DMSO), Ac_4ManNAz (100 mM in DMSO), and DMSO were added, respectively. The dishes were incubated at 37 ºC humidified incubator with 5% CO_2 for 24 h. After incubation, the media was removed, and the HeLa cells were washed with 10 mL DPBS with 1% FBS twice. Then, the HeLa cells were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature and washed with 10 mL DPBS with 1% FBS once. The HeLa cells were permeabilized with 0.5% Triton X-100 in PBS for 15 min at room temperature, then washed with 10 mL DPBS with 1% FBS once. The HeLa cells were labeled with 10 μM of *N3′*-ethyl biotin alkyne **6** in the presence of 100 μM $CuSO_4$, 500 μM THPTA, and 2.5 mM sodium ascorbate in DPBS with 1% FBS at room temperature for 30 min. For control experiment, the HeLa cells were also labeled with 10 μM of *N3′*-ethyl biotin alkyne **6** without $CuSO_4$. After labeling, the HeLa cells were washed again with DPBS with 1% FBS twice, and incubated with a nuclear stain Hoechst 33342 (1:5000 dilution) in DPBS with 1% FBS for 10 min. The cells were washed with DPBS with 1% FBS three times, and eH_2O once. After the coverslips were air-dried, the coverslips were mounted in Cytoseal onto microscopy slides. Fluorescent images were captured on a Zeiss Axioskop 2 fluorescence microscope with 40× objective equipped with a Hamamatsu ORCA-ER CCD camera using excitation and emission filters from Omega Optical. The nuclear stain Hoechst 33342 was imaged using a 365 ± 5 nm band-pass filter for excitation and a 400 ± 5 nm cutoff filter for emission. The Alexa Fluor 488 fluorophore was imaged using a 480 ± 10 nm band-pass filter for excitation and a 510 ± 10 nm band-pass filter for emission. Exposure times of 20 and 50 ms, for the nuclear stain and Alexa Fluor 488 dye channels, respectively, were used for image collection. All raw images were processed using Slidebook software with identical leveling.



Protein Isolation

Jurkat cells were cultured in 100 mL RPMI 1640 media supplemented with 10% FBS at two T175 tissue culture flasks. When cell density reached ~10 × 10^6 cells /mL, 100 μL of Ac_4GlcNAz (100 mM in DMSO) and DMSO (control) were added, respectively. The flasks were incubated at 37 ºC humidified incubator with 5% CO_2 for 24 h. After incubation, the Jurkat cells were collected by centrifugation at 600 ×g for 5 min, and washed with PBS five times. The cell pellet was suspended in PBS to adjust the cell density at 50 × 10^6 cells /mL, and transferred 1 mL each to 1.5 mL tube. The cells were collected again by centrifugation at 600 ×g for 5 min. Then, 1 mL of 0.5% SDS/PBS was added to each tube, and lysated by sonication. The cell lysate (500 μL, Ac_4GlcNAz treated or untreated) were incubated with 50 μM of *N3′*-ethyl biotin alkyne **6** or biotin alkyne **7** in the presence of 0.5 mM $CuSO_4$, 2.5 mM THPTA, and 5 mM sodium ascorbate in 0.5% SDS/PBS at room temperature for 1 h, respectively. For control experiment, the cell lysate were also incubated with 50 μM of *N3′*-ethyl biotin alkyne **6** or biotin alkyne **7** without $CuSO_4$, respectively. After incubation, 500 μL MeOH and 150 μL $CHCl_3$ were added to each tube. The tube was vortexed and centrifuged at 10,000 rpm for 4 min. The solution was removed and the pellet was washed with MeOH five times, then resolubilized in 500 μL of 0.5% SDS/PBS. 200 μL of Streptavidin agarose (Invitrogen) was added to each tube and incubated at room temperature for 1 h. The aragose beads were transferred to 2 mL spin column (Bio-Rad), and washed with 2 mL TEST (10 mM Tris, 1 mM EDTA, 1 M NaCl, 0.1% Tween-20, pH 7.4) five times. The *N3′*-ethyl biotin-glycoprotein conjugate bound streptavidin agarose column was eluted with 10 mM HCl in H_2O (1 mL × 3). The streptavidin agarose containing biotin-glycoprotein conjugate was transferred to a 1.5 mL tube, and 1 mL of 1% SDS in H_2O was added and heated at 95 ºC for 5 min. The eluate was collected and dried in a speed vac.