

Activity-Based Protein Profiling *in Vivo* Using a Copper(I)-Catalyzed Azide-Alkyne Cycloaddition

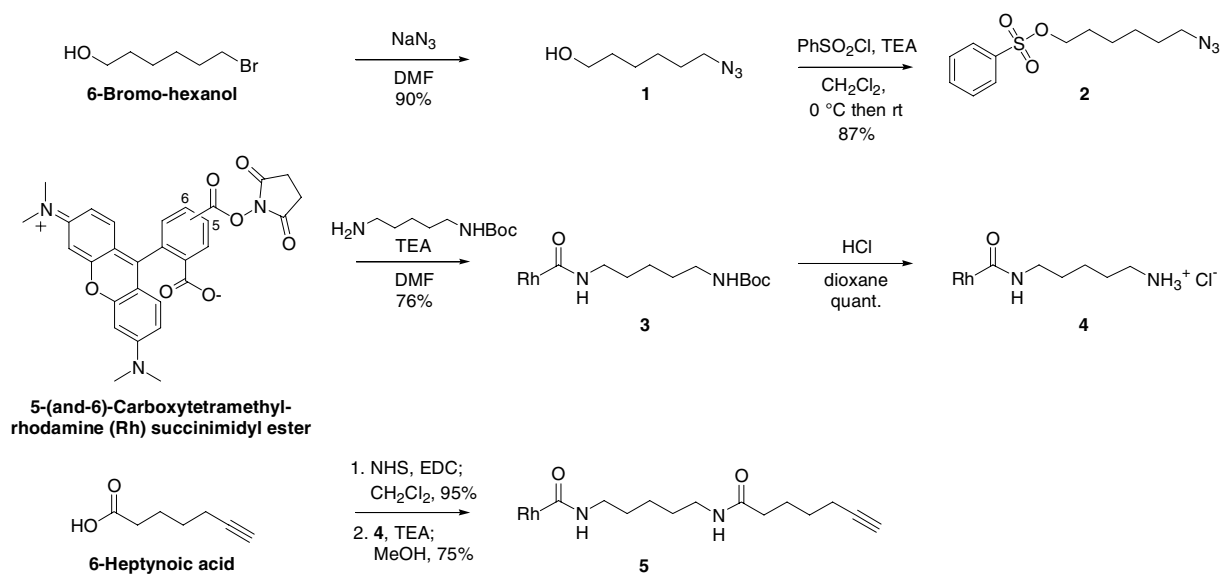
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Supporting Information

A. Materials and Methods.

1. Synthesis of phenylsulfonate azide probe (2) and rhodamine-alkyne reagent (5).

Scheme S1.



Note on synthesis: The order of coupling could be reversed to append Boc-diaminopentane to 6-heptynoic acid before coupling to Rhodamine-SE. This route was not taken because we already had a large amount of 4 on hand.

General Methods. All chemicals were obtained from Aldrich, Acros, Novabiochem, or Molecular Probes, and were used without further purification, except where noted. Triethylamine was distilled over calcium hydride and stored over potassium hydroxide. Solvents were used as received (anhydrous DMF and methanol, Acros sure seal) or passed over an activated alumina column (methylene chloride). All reactions were carried out under an argon atmosphere using oven-baked glassware cooled in a desiccator. Flash silica gel chromatography was performed using 230-400 mesh silica gel 60 or 60-325 mesh neutral alumina, Brockman Activity I. ^1H spectra were recorded on a Bruker AMX-400 or AMX-500 MHz spectrometer. Chemical shifts are reported in δ values relative to tetramethylsilane, and coupling constants (J) are reported in Hz.

6-azido-hexanol 1. To a flask containing 6-bromo-hexanol (600 mg, 3.3 mmol, 1 equiv) was added anhydrous DMF (6.6 mL, 0.5 M) followed by sodium azide (300 mg, 4.6 mmol, 1.4 equiv). After stirring for 12 hrs, the reaction was partitioned between ether (200 mL) and water (200 mL). The organic layer was washed with water (200 mL) and brine (200 mL), and then dried with Na_2SO_4 . The

solvent was removed via rotary evaporation under reduced pressure and dried under high vacuum for 1 hour to give **1** as a light yellow oil (428 mg, 90%). ^1H NMR (CDCl_3) δ 3.60 (t, $J=6.6$, 2H), δ 3.27 (t, $J=7.0$, 2H), δ 2.84 (s, 1H), δ 1.61 (m, 2H), δ 1.56 (m, 2H), δ 1.40 (m, 4H). ESI m/z 166 ($\text{C}_6\text{H}_{13}\text{N}_3\text{O} + \text{Na}^+$ requires 166.0951).

Phenylsulfonic acid-6-azido-hexyl ester 2. To a flask containing **1** (100 mg, 0.7 mmol, 1 equiv) was added anhydrous methylene chloride (4.6 mL, 0.15 M) and triethylamine (295 μL , 2.1 mmol, 3 equiv). The flask was cooled to 0°C and benzenesulfonyl chloride (180 μL , 1.4 mmol, 2 equiv) was added dropwise over the course of 10 min. After an additional 10 min at 0°C , the flask was warmed to room temperature and stirred for 8 hrs. The reaction was then partitioned between ether (50 mL) and water (50 mL), and the organic layer was washed with saturated sodium bicarbonate (2x50 mL), water (3x50 mL), and brine (1x50 mL), and then dried with Na_2SO_4 . The solvent was removed via rotary evaporation under reduced pressure. Column chromatography (0-10% ethyl acetate/hexane) afforded **2** as a yellow oil (172 mg, 87%). ^1H NMR (CDCl_3) δ 7.91 (m, 2H), δ 7.67 (tt, $J=7.5, 1.2$, 1H), δ 7.57 (m, 2H), δ 4.05 (t, $J=6.4$, 2H), δ 3.23 (t, $J=6.8$, 2H), δ 1.66 (m, 2H), δ 1.54 (m, 2H), δ 1.33 (m, 4H). MALDI-FTMS m/z 306.0883 ($\text{C}_{12}\text{H}_{17}\text{N}_3\text{O}_3\text{S} + \text{Na}^+$ requires 306.0883).

Rhodamine-N-Boc-pentylamine (mixed isomers) 3. 5-(and-6)-Carboxytetramethylrhodamine succinimidyl ester (25 mg, 0.047 mmol, 1 equiv) was dissolved in anhydrous DMF (0.4 mL, 0.12 M) and N-Boc-1,5-diaminopentane (23 μL , 0.11 mmol, 2.4 equiv) in DMF (0.1 mL, to give 0.094 M) was added to the reaction. After 1 hr, the solvent was removed via rotary evaporation under reduced pressure, and the crude material was dried under high vacuum for 12 hrs. Column chromatography (neutral alumina, 8% methanol/chloroform) afforded **3** as a dark pink solid (22 mg, 76%). ^1H NMR (CDCl_3) δ 8.43 (s, 1H), δ 8.20 (dd, $J=7.9, 1.5$, 1H), δ 8.07 (dd, $J=8.0, 1.3$, 1H), δ 8.03 (d, $J=7.9$, 1H), δ 7.54 (s, 1H), δ 7.24 (dd, $J=7.9, 0.6$ 1H), δ 6.99 (br s, 1H), δ 6.70 (br s, 1H), δ 6.67 (d, $J=6.2$, 2H), δ 6.64 (d, $J=6.2$, 2H), δ 6.51 (t, $J=2.8$, 4H), δ 6.45 (d, $J=2.3$, 2H), δ 6.43 (d, $J=2.6$, 2H), δ 4.63 (m, 2H), δ 3.49 (m, 2H), δ 3.36 (m, 2H), δ 3.11 (m, 4H), δ 3.02 (s, 24H), δ 1.67 (m, 2H), δ 1.51 (br m, 10H), δ 1.42 (s, 9H), δ 1.41 (s, 9H). MALDI-FTMS m/z 615.3187 ($\text{C}_{35}\text{H}_{42}\text{N}_4\text{O}_6 + \text{H}^+$ requires 615.3177).

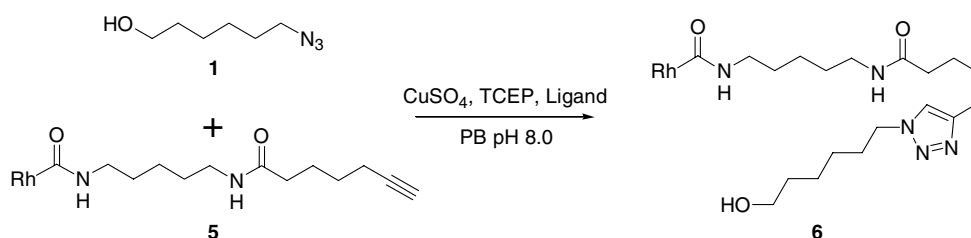
Rhodamine-pentylamine HCl salt (mixed isomers) 4. 4.0 N HCl in dioxane (1.5 mL) was added to a vial containing **3** (19.2 mg, 0.031 mmol). After stirring 4 hrs, the solvent was evaporated under a stream of nitrogen and residue dried under high vacuum 12 hrs to give **4** (16 mg, quant.), which was used directly in the next reaction. ^1H NMR (methanol- d_4) δ 8.78 (s, 1H), δ 8.41 (d, $J=7.9$, 1H), δ 8.30 (d, $J=7.3$, 1H), δ 8.23 (d, $J=8.5$, 1H), δ 7.84 (s, 1H), δ 7.54 (d, $J=7.6$, 1H), δ 7.14 (d, $J=9.4$, 4H), δ 7.06 (d, $J=9.1$, 4H), δ 6.96 (s, 4H), δ 3.74 (m, 2H), δ 3.66 (m, 2H), δ 3.50 (m, 2H), δ 3.42 (m, 2H), δ 3.30 (s, 24H), δ 2.96 (m, 4H), δ 1.76 (m, 8H), δ 1.52 (m, 4H). MALDI-FTMS m/z 515.2634 ($\text{C}_{30}\text{H}_{34}\text{N}_4\text{O}_4 + \text{H}^+$ requires 515.2653).

Rhodamine-alkyne (mixed isomers) 5. 6-heptynoic acid (100 mg, 0.79 mmol, 1 equiv) was dissolved in anhydrous methylene chloride (13 mL, 0.06 M). To the reaction flask was added N-hydroxysuccinimide (230 mg, 2 mmol, 2.5 equiv) followed by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 230 mg, 1.2 mmol, 1.5 equiv). The reaction was stirred for 12 hrs, at which point saturated sodium bicarbonate (50 mL) was added to the reaction flask, and the aqueous layer was extracted with ether (50 mL). The organic layer was washed with water (50 mL) and brine (50 mL), and then dried with Na_2SO_4 . The solvent was removed via rotary evaporation under reduced pressure, and the white solid dried under high vacuum for 1 hr to give 6-heptynoic acid succinimidyl ester (168 mg,

95%, pure by NMR), which was used directly in the next reaction. Rhodamine-pentylamine HCl salt **4** (16 mg, 0.031 mmol, 1 equiv) was dissolved in anhydrous methanol (1.6 mL, 0.02 M) and divided into two equal portions. To each reaction was added triethylamine (6.6 μ L, 0.047 mmol, 3 equiv). After 15 min, 6-heptynoic acid succinimidyl ester (9 mg, 0.040 mmol, 2.6 equiv) was added to each reaction. After 1 hour, the solvent was removed under a stream of nitrogen and the crude material dried under high vacuum for 2 hrs. Column chromatography of the combined reactions (neutral alumina, 2-10% methanol/chloroform) afforded **5** as a dark pink solid (14.6 mg, 75% one step). ^1H NMR (CDCl_3) δ 8.48 (d, $J=1.2$, 1H), δ 8.21 (dd, $J=8.1$, 1.8, 1H), δ 8.06 (dd, $J=7.9$, 1.2, 1H), δ 8.01 (d, $J=8.2$, 1H), δ 7.55 (s, 1H), δ 7.23 (d, $J=8.2$ 1H), δ 7.16 (m, 1H), δ 6.72 (m, 1H), δ 6.61 (d, $J=2.6$, 2H), δ 6.59 (d, $J=2.6$, 2H), δ 6.48 (t, $J=3.2$, 4H), δ 6.41 (t, $J=2.0$, 2H), δ 6.39 (t, $J=2.2$, 2H), δ 5.86 (br m, 1H), δ 5.83 (br m, 1H), δ 3.47 (q, $J=6.5$, 2H), δ 3.34 (q, $J=6.5$, 2H), δ 3.21 (p, $J=6.5$, 4H), δ 2.99 (s, 12H), δ 2.98 (s, 12H), δ 2.16 (m, 8H), δ 1.94 (t, $J=2.6$, 1H), δ 1.91 (t, $J=2.6$, 1H), δ 1.68 (m, 8H), δ 1.50 (m, 8H), δ 1.40 (m, 2H), δ 1.33 (m, 2H). MALDI-FTMS m/z 623.3219 ($\text{C}_{37}\text{H}_{42}\text{N}_4\text{O}_5 + \text{H}^+$ requires 623.3228).

2. Model cycloaddition reaction between rhodamine-alkyne (**5**) and 6-azido hexanol (**1**) to confirm triazole formation.

Scheme S2



Rhodamine-triazole-alcohol 6. In 100 μL of 8.8 mM sodium/potassium phosphate-buffer pH 8.0, rhodamine-alkyne **5** (4 mM) and 6-azido hexanol **1** (4 mM) were allowed to react in the presence of CuSO_4 (1 mM), tris(carboxyethyl)phosphine (1 mM), and ligand (2 mM, see Scheme 1 for structure). After 12 hrs, a sample was submitted for mass analysis. MALDI-FTMS m/z 766.4294 ($\text{C}_{43}\text{H}_{55}\text{N}_7\text{O}_6 + \text{H}^+$ requires 766.4286).

3. Proteome sample preparation, azide probe labeling, cycloaddition reaction, and detection.

Preparation of mouse tissue soluble proteomes. Mouse tissues were Dounce-homogenized in 8.8 mM sodium/potassium phosphate buffer pH 8.0 (PB), and the soluble fraction was isolated using high speed centrifugation to remove the heavy and light membrane components: 22,000g (30 min, pellet = heavy membrane fraction) and 100,000g (60 min, pellet = light membrane, supernatant = soluble fraction). Prepared soluble proteome samples were stored at -80°C until use.

Preparation of cell line soluble proteomes. Cell lines were grown to 80% confluency in RPMI-1640 medium (Invitrogen) containing 10% FCS. Cells were harvested, sonicated, Dounce homogenized in PB, and centrifuged at 100,000g (45 min) to isolate the soluble fraction (supernatant). Prepared samples were stored at -80°C until use.

Procedure for in vitro labeling of proteins. Proteome samples (40 μ L of 2.5 mg/mL protein in PB) were treated with 2.5 μ M PS-N₃ (100 μ M stock in *t*-butanol) for 30-60 min at room temperature. For heated controls, proteome samples were heated at 90 °C for five minutes prior to addition of PS-N₃.

Procedure for in vivo labeling of GSTO 1-1 in COS cells. Cell lines were grown to 80% confluency in RPMI-1640 medium (Invitrogen) containing 10% FCS. Cell cultures were treated with 10 μ M PS-N₃ (25 mM stock in DMSO) for 1 hour, then washed (5 x 5 mL Dulbecco’s phosphate buffered saline, Invitrogen), and prepared as described to isolate the soluble proteome. For the PS-oct competition experiment, cells were treated with 100 μ M PS-oct (50 mM stock in DMSO) for 1 hr prior to addition of PS-N₃.

Procedure for in vivo labeling of ECH-1 in mice. Mice were given intraperitoneal (i.p.) injections of 0, 10, or 20 mg/kg PS-N₃ [in vehicle (18:1:1 saline:emulphor:EtOH)]. After 1 hour, the mice were anesthetized in O₂/CO₂ mixture, sacrificed by decapitation, and heart tissue collected, frozen in dry ice (30 min), and processed as described to isolate the soluble proteome.

General procedures for rhodamine-alkyne reaction, protein electrophoresis, and in-gel fluorescence scanning. Following incubation with the azide probe (see above), 20¹ or 50 μ M² rhodamine-alkyne (1 or 2.5 mM stock in 1:9 ethanol/water) was added to each protein sample. Under a stream of argon,³ 1 mM TCEP (50 mM stock in water), 1 mM CuSO₄ (50 mM stock in water), and 2 mM ligand (20 mM stock in 4:1 *t*-butanol/water⁴), were added to each proteome sample.⁵ Each sample was then gently vortexed and allowed to react at room temperature for 1 hr. The samples were then briefly centrifuged (5900g, 4 min) to pellet the precipitated protein and remove excess reagents⁶, and the supernatant was discarded. 50 μ L (1 volume) of PB and 50 μ L (1 volume) of standard 2xSDS-PAGE loading buffer (reducing) were added to the protein pellet, and the samples were sonicated to solubilize proteins. Proteins were then separated by SDS-PAGE (30 μ g of protein/gel lane), and visualized in-gel using a Hitachi FMBio Iie flatbed laser-induced fluorescence scanner (MiraiBio, Alameda, CA). Labeled proteins were quantified by measuring integrated band intensities (normalized for volume). For all quantification experiments, n = 3 per data point, and error represents standard deviation of the average of these values.

Footnotes:

1 For *in vitro*-labeled proteins and labeling of GSTO 1-1 in living cells.

2 For *in vivo*-labeled ECH-1.

3 In initial experiments, the cycloaddition reaction was run for 8-16 hrs. Under these conditions, we found that excluding potential oxidants (O₂, DMSO) was important for the success of the reaction; however, when the reaction is run under optimized conditions (1 hr) such precautions are not required.

4 Stock solution was vortexed just prior to addition to homogenize the suspension.

5 Total volume of 50 μ L, giving 2 mg/ml [protein] and 2 μ M [PS-N₃].

6. In initial experiments using higher concentrations of rhodamine-alkyne (>100 μ M), we observed that gel image quality could be significantly improved by removing excess reagents; however, improvement is minimal when lower concentrations of rhodamine-alkyne (20-50 μ M) are used.

Procedure for protein labeling with PS-Rh. Proteome samples (50 μ L of 2 mg/mL protein in PB) were treated with 5 μ M PS-Rh (250 μ M stock in DMSO) for 1 hour. For competition experiments, proteome samples were treated with 10-50 μ M PS-N₃ (0.5-2.5 mM stock solutions in *t*-butanol) for 30 minutes prior to addition of PS-Rh. At the conclusion of the experiment, 50 μ L (1 volume) of standard 2xSDS-PAGE loading buffer (reducing) was added to the sample. Gel electrophoresis and fluorescence visualization were carried out as described in the above section.

B. Characterization of Cycloaddition Reaction.

Figure S1. GSTO 1-1 labeling intensity as a function of time.

Reaction conditions: GSTO 1-1-transfected COS cell extracts (2 mg/mL protein) were treated with 2.5 μM PS-N₃ (30 minutes) followed by 20 μM rhodamine-alkyne for 0 to 60 minutes. 2xSDS-PAGE loading buffer (reducing) containing 4 mM 5-hexynol was added to quench the reactions, the reaction mixtures were run on an SDS-PAGE gel and quantified by in-gel fluorescence scanning (n = 3 per data point, error represents standard deviation of the average of these values).

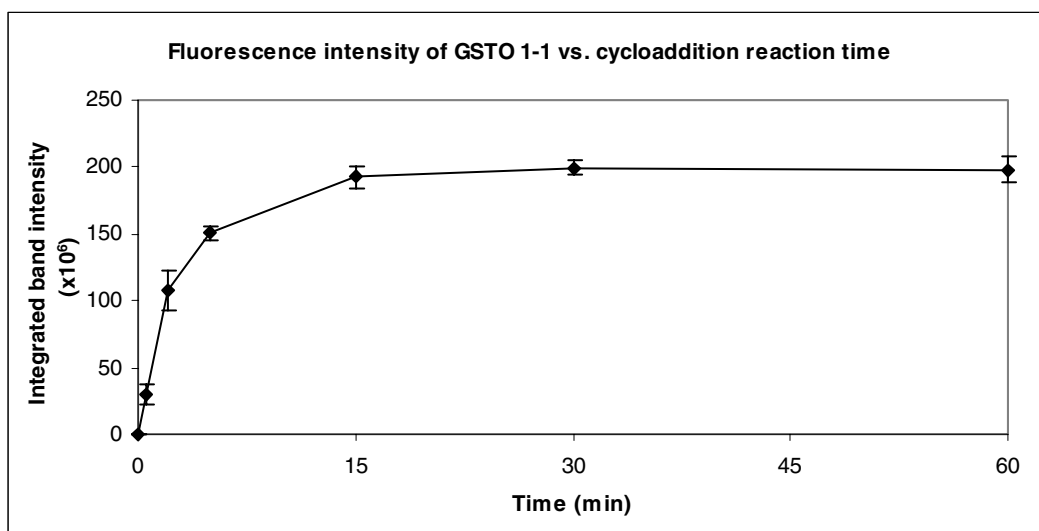


Figure S2. GSTO 1-1 labeling intensity as a function of rhodamine-alkyne concentration.

Reaction conditions: GSTO 1-1-transfected COS cell extracts (2 mg/mL protein) were treated with 2.5 μM PS-N₃ (30 minutes) followed by 0 to 250 μM rhodamine-alkyne for 1 hour. 2xSDS-PAGE loading buffer (reducing) was added to each sample, and the reaction mixtures were run on an SDS-PAGE gel and quantified by in-gel fluorescence scanning. (n = 3 per data point, error represents standard deviation of the average of these values).

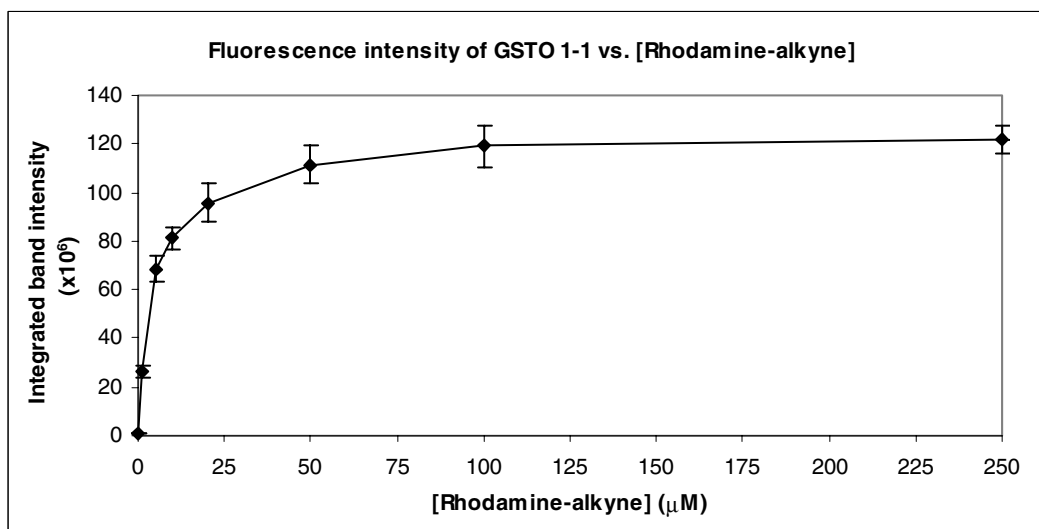
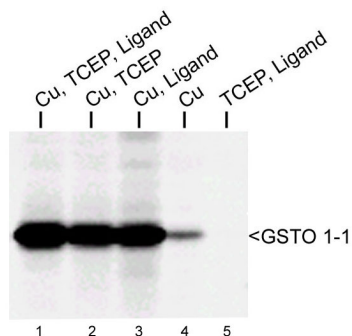


Figure S3. GSTO 1-1 labeling intensity using various combinations of Cu, TCEP, and ligand.

Reaction conditions: GSTO 1-1-transfected COS cell extracts (2 mg/mL protein) were treated with 2.5 μ M PS-N₃ (30 minutes) followed by 20 μ M rhodamine-alkyne (1 hour) using reagents indicated (1 mM CuSO₄, 1 mM TCEP, 2 mM Ligand).

**Figure S4. Activity-based labeling and background labeling are dependent upon PS-N₃.**

Cycloaddition-based ABPP analysis of the MDA-MB-231 cellular proteome in the presence (lane 2) or absence (lane 3) of PS-N₃. Activity-based labeling of GSTO 1-1 is only observed in the presence of PS-N₃ and is heat-sensitive (lane 1). Background labeling is also mostly dependent on PS-N₃ (compare signal intensities of lanes 2 and 3). Reaction conditions: MDA-MB-231 cell extracts (2 mg/mL protein) were treated with 2.5 μ M PS-N₃ (30 minutes) as indicated, followed by the cycloaddition reaction (1 mM CuSO₄, 1 mM TCEP, 2 mM Ligand) with or without 20 μ M rhodamine-alkyne (1 hour). For the heated control, proteome sample was heated at 90 °C for five minutes prior to addition of PS-N₃.

