SUPPORTING INFORMATION

Expanding the Scope of Alkyne-Mediated Bioconjugations Utilizing Unnatural Amino Acids

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EXPERIMENTAL SECTION

General

All solvents and reagents, including the AlexaFluor 488 Alkyne, were obtained from Sigma Aldrich or Acros Organics and used without further purification. Plasmids were provided by the laboratory of Dr. Peter Schultz at The Scripps Research Institute. Reactions were conducted under ambient atmosphere with solvents directly from the manufacturer without further purification. All proteins were purified according to manufacturer's protocols using a Qiagen Ni-NTA Quik Spin Kit. Compound purities were assessed by NMR and found to be 90% or greater for all compounds. All NMRs were acquired on an Agilent Technologies 400 MHz NMR. MS analysis was conducted on a Thermo Finnigan LCQ Deca Quadropole Ion Trap via direct injection of samples at 100 μM in a 1:1 H_2O/MeOH solution. Unless indicated otherwise, all solutions were prepared in deionized water (pH ~ 7).

Synthesis of *p*-bromopropargyloxyphenylalanine (*p*BrPrF). *p*-Propargyloxyphenylalanine (*p*PrF, 0.050g, 0.150 mmol) was synthesized according to the literature[1], and dissolved in acetone (5 mL). This solution was then transferred to a vial containing $AgNO_3$ (0.03g, 0.015 mmol) and N-bromosuccinamide (NBS, 0.030g, 0.165 mmol). The mixture was allowed to stir for 3 hours at room temperature, after which it was diluted with hexanes (10 mL) and the resulting crystals were filtered off. The filtrate was then evaporated under reduced pressure and

purified via flash chromatography (5:1 hexanes/ethyl acetate) yielding the desired product as a white crystal (0.042 g, 0.102 mmol, 68% yield). The product was then subjected to 1M LiOH in dioxanes (500 μL each) on ice, and then stirred for two hours at room temperature. The dioxanes were subsequently removed by rotatory evaporation. Additional deionized water (1 mL) was then added to the product, and this solution was then brought to a pH = 4 with 6 N HCl. The solution was extracted in ethyl acetate, dried with $MgSO_4$, filtered, and evaporated under pressure to dryness. A 1 mL solution 2% trifluoroacetic acid (TFA) in dimethylene chloride (DCM) was then added to the vial, and the mixture was allowed to stir at room temperature for 1 hour, prior to evaporation under reduced pressure to yield the final product as a yellowish crystal (0.030g, 0.101 mmol, 99% yield, 67% overall yield). ^{1}H NMR (400 MHz, CD_3OD): δ 7.19 (d, J =8 Hz, 2H), 6.93 (d, J = 8 Hz, 2H), 4.91 (s, 1 H), 4.14 (m 1 H), 3.06 (m, 2 H). ^{13}C NMR (400 MHz, CD_3OD): δ 168.4, 155.7, 128.6, 125.4, 113.5, 73.4, 54.4, 52.3, 45.4, 25.4; MS: calcd for $C_{12}H_{13}BrNO_3$: 299.14, found 298.1 and 300.1.

Synthesis of *p*-bromoethynylphenylalanine (*p*BrEtF). *p*-ethynylphenylalanine (*p*EtF, 0.052g, 0.171 mmol) was synthesized according to the literature protocol[2], and dissolved in acetone (5 mL). This solution was then transferred to a vial containing $AgNO_3$ (~0.03g, 0.0171 mmol) and N-bromosuccinamide (NBS, 0.034g, 0.189 mmol). The mixture was allowed to stir for 3 hours at room temperature, after which it was diluted with hexanes (10 mL) and the resulting crystals were filtered off. The filtrate was then evaporated under reduced pressure and purified via flash chromatography (3:1 hexanes/ethyl acetate) yielding the desired product as a yellow crystal (0.024 g, 0.063 mmol, 38% yield). The product was then subjected to 1M LiOH in dioxanes (500 μL each) on ice, and then stirred for two hours at room temperature. The dioxanes were subsequently removed by rotatory evaporation. Additional deionized water (1 mL) was then

added to the product, and this solution was then brought to a pH = 4 with 6 N HCl. The solution was extracted in ethyl acetate, dried with $MgSO_4$, filtered, and evaporated under pressure to dryness. A 1 mL solution 2% trifluoroacetic acid (TFA) in dimethylene chloride (DCM) was then added to the vial, and the mixture was allowed to stir at room temperature for 1 hour, prior to evaporation under reduced pressure to yield the final product as a yellowish crystal (0.015g, 0.056 mmol, 89% yield, 34% overall yield). ^{1}H NMR (400 MHz, CD_3OD): δ 7.35 (d, J = 8 Hz, 2H), 7.21 (d, J = 8 Hz, 2H), 4.34 (m, 1H), 3.16 (m, 2H), 2.90 (m, 2H). ^{13}C NMR (400 MHz, CD_3OD): δ 173.63, 138.42, 132.16, 131.4, 129.2, 79.2, 54.6, 37.2, 27.22; MS: calcd for $C_{11}H_{11}BrNO_2$: 269.12, found 268.1 and 270.1.

Expression of UAA-containing GFP-151[2]

Escherichia coli BL21(DE3) cells were co-transformed with a pET-GFP-TAG-151 plasmid (0.5 µL) and a pEVOL-*p*CNF plasmid (0.5 µL) using an Eppendorf electroporator. Cells were then plated on LB-agar plates supplemented with ampicillin (50 mg/mL) and chloramphenicol (34 mg/mL) and grown at 37℃. After 16 h, a single colony was selected and used to inoculate LB media (4 mL) supplemented with ampicillin and chloramphenicol. The culture was grown to confluence at 37℃ over 16 h. This culture was then used to initiate an expression culture in LB media (20 mL) at OD_{600} = 0.1, then incubated at 37℃ until an OD_{600} ~0.7 was obtained. At this point, the cells were then pelleted at 4℃ at 5000rpm for 10 mins. The supernatant was removed and the cells were gently resuspended in 4 mL of LB media. Ampicillin and chloramphenicol (4 µL each), 1M IPTG (4 µL), 20% arabinose (4 µL), and 100mM of the UAA (40 µL) were then added. Induced cells were grown for an additional 16 h at 37℃, then harvested via centrifugation (10 min at 5000 rpm). The media was decanted and the cell pellet was placed in the -80℃ freezer for 20 min. Purification of the UAA-containing GFP was then performed using commercially

available Ni-NTA spin columns according to the manufacturer's protocol. Protein yield and purity was then assessed by SDS-PAGE and spectrophotometrically by Nanodrop spectrophotometry. Protein was then transferred into phosphate-buffered saline solution (PBS, pH = 7) using 10k MWCO spin columns prior to use in the biological Cadiot-Chodkiewicz coupling.

General Protocol for the Biological Cadiot-Chodkiewicz Coupling

To a sterile 1.5 mL Eppendorf tube, the following was added in order: 10 µL of bromo-alkyne UAA-containing GFP_{151} (~0.5 mg/mL), 5 µL of a vigorously shaken CuI solution (50 mM), 2 µL of trimethylamine (TEA, 50 mM), 5 µL of 488-Alexafluor alkyne (1 mM in DMSO), and 3 µL of PBS (pH = 7). Control reactions were set up in the absence of CuI and TEA and with the addition of a total of 10 µL of PBS. Reactions were performed at various temperatures and times as indicated in the main manuscript, with the 4℃ for 6 hr variant being the ideal reaction conditions.

General Protocol for the Biological Glaser-Hay Coupling

To a sterile 1.5 mL Eppendorf tube, the following was added in order: 10 µL of termainl alkyne UAA-containing GFP_{151} (~0.5 mg/mL), 5 µL of a vigorously shaken CuI solution (500 mM), 2 µL of trimethylethylenediamine (TMEDA, 500 mM), 5 µL of 488-Alexafluor alkyne (1 mM in DMSO), and 3 µL of PBS (pH = 7). Control reactions were set up in the absence of CuI and TMEDA and with the addition of a total of 10 µL of PBS. Reactions were performed at 4℃ for various times as indicated in the main manuscript.

FIGURES

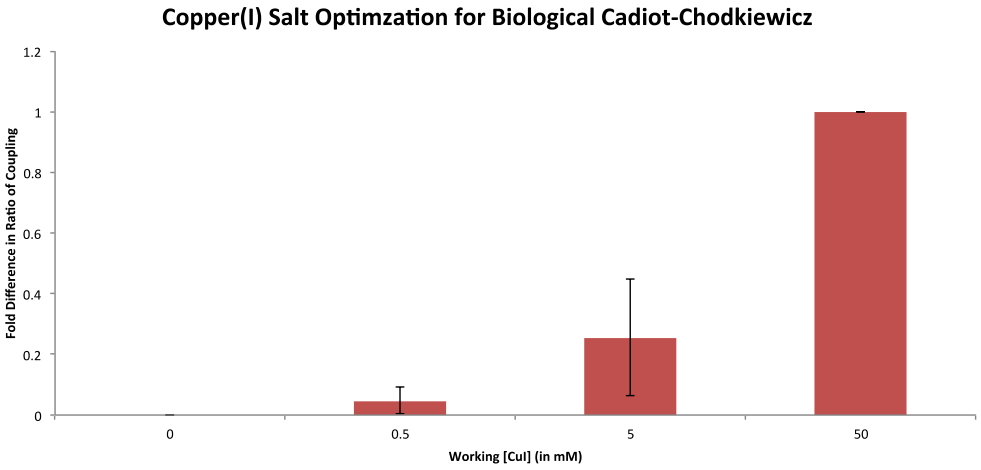

Figure 1. Data for Cu(I) concentration series. Data for coupling of alkyne fluorophore to pBrPrF-containing GFP151 were normalized to the best performing CuI catalyst concentration (50 mM) in order to visualize fold difference in the coupling efficiency.

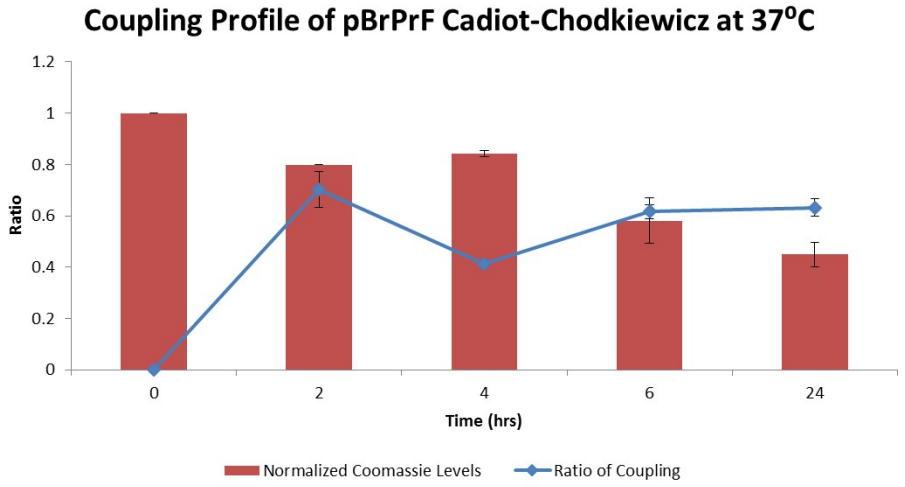

Figure 2. Reaction profile of the pBrPrF Cadiot-Chodkiewicz at 37°C over a 24 hr time period. Following analysis via SDS-PAGE and staining with coomassie blue, protein levels were normalized to the 0 time point control, when no protein degradation could have occurred. The protein levels indicate protein degradation occurs for the 37°C biological Cadiot-Chodkiewicz, especially at higher time points. Analysis of coupling efficiency was determined by taking the ratio of fluorescence to coomassie staining for each time point (with background fluorescence removed prior to performing the calculation).

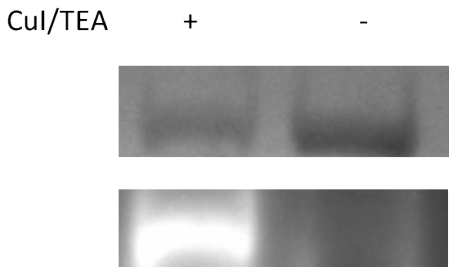

Figure 3. Initial biological Cadiot-Chodkiewicz on pBrEtF-containing GFP151. The initial aromatic attempt of the Cadiot-Chodkiewicz in the presence of 5 mM of CuI and TEA was successful, as fluorescence is only observed in the presence of the catalyst system.

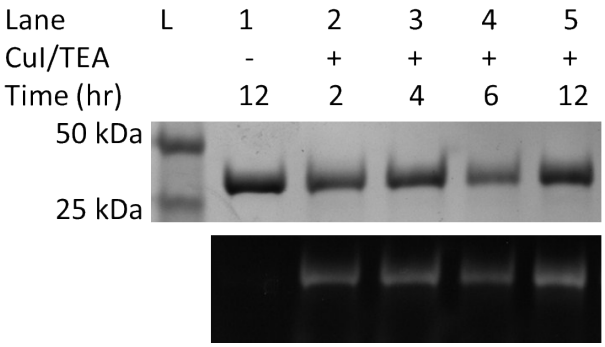

Figure 4. Cadiot-Chodkiewicz bioconjugation timecourse at 4°C. After running on SDS-PAGE, successful conjugation is visualized by the presence of a fluorescent band only when exposed to the CuI/TEA catalyst system (lanes 2-5, bottom gel). Furthermore, minimal protein degradation is detected, as coomassie stain levels remain constant from the control over the course of the 12 hr time course (lanes 1-5, top gel).

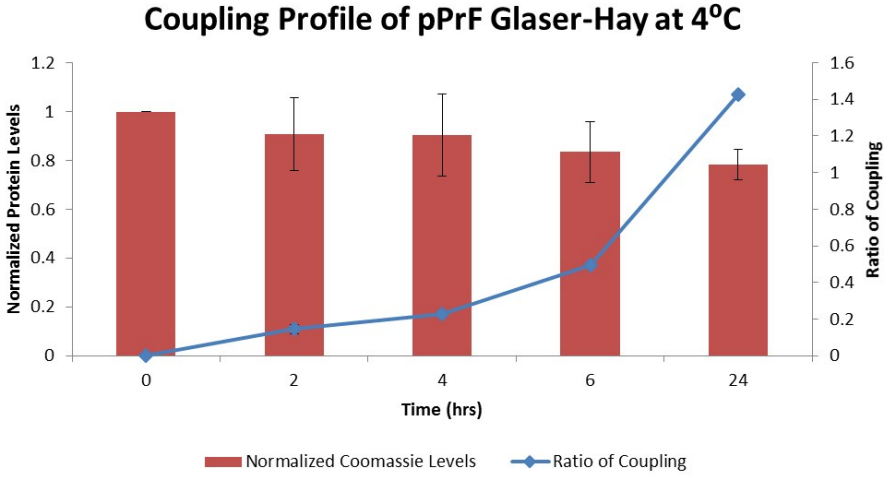

Figure 5. Reaction profile of the pPrF Glaser-Hay at 37°C over a 24 hr time period. Following analysis via SDS-PAGE and staining with coomassie blue, protein levels were normalized to the 0 time point control, when no protein degradation could have occurred. The protein levels indicate protein degradation occurs for the 4°C biological Glaser-Hay, especially at higher time points. Analysis of coupling efficiency was determined by taking the ratio of fluorescence to coomassie staining for each time point (with background fluorescence removed prior to performing the calculation).

REFERENCES

1. Deiters, A. & Schultz, P. G. In vivo incorporation of an alkyne into proteins in Escherichia coli. *Bioorganic Med. Chem. Lett.* **15,** 1521–1524 (2005).

2. Lim, S. I., Mizuta, Y., Takasu, A., Kim, Y. H. & Kwon, I. Site-specific bioconjugation of a murine dihydrofolate reductase enzyme by copper(I)-catalyzed azide-alkyne cycloaddition with retained activity. *PLoS One* **9,** (2014).