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Supporting Information

Photo-induced and Rapid Labeling of Tetrazine-Bearing Proteins via Cyclopropenone-Caged Bicyclononynes

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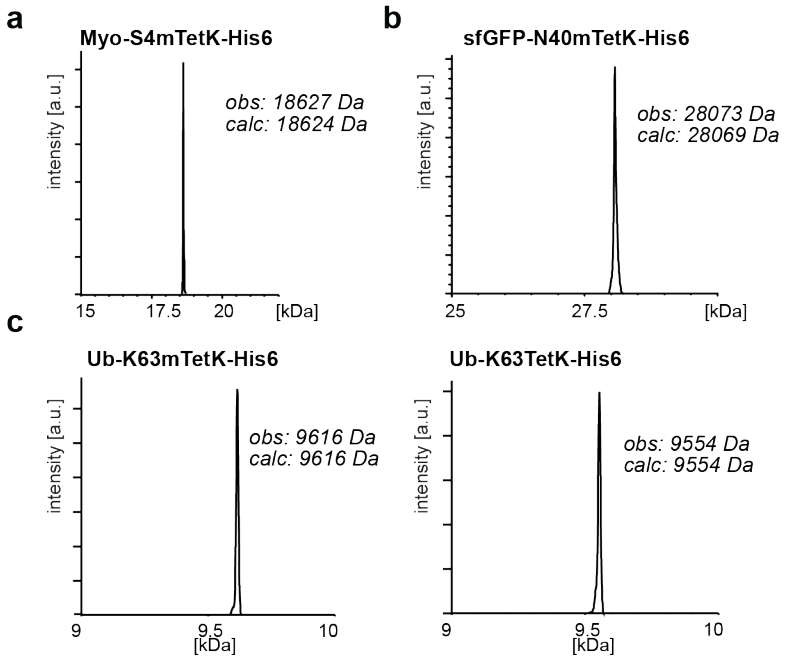
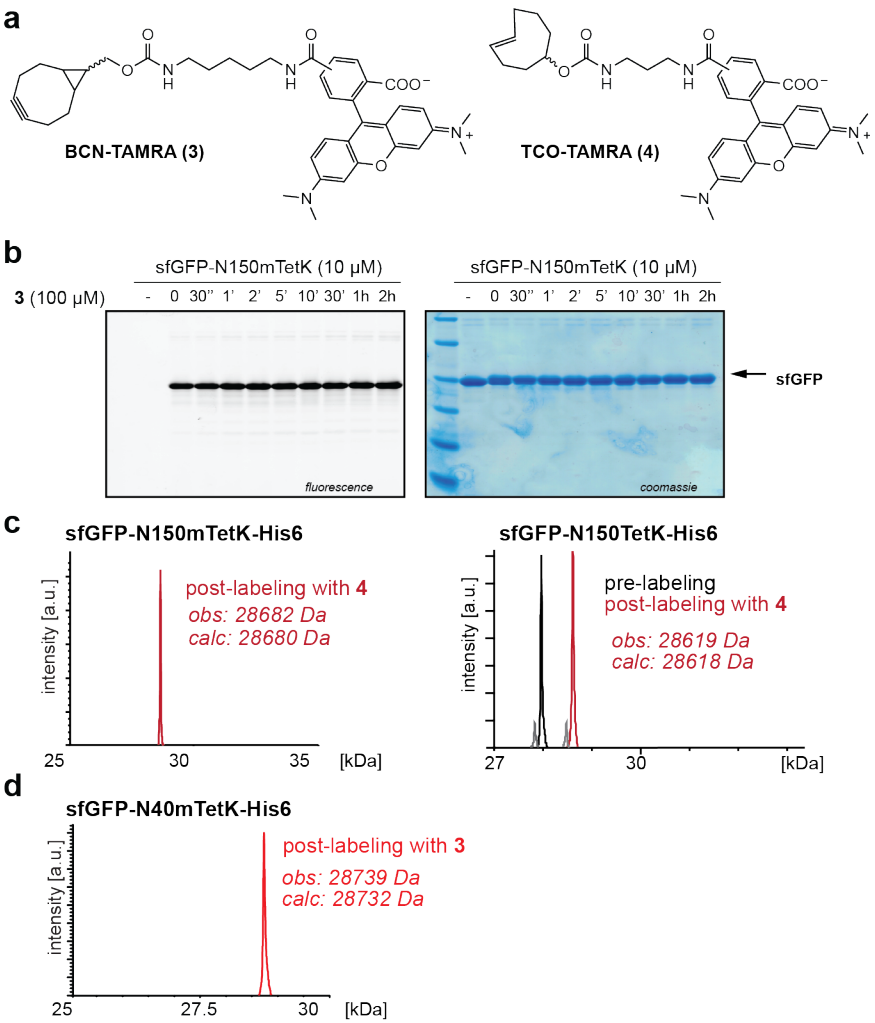
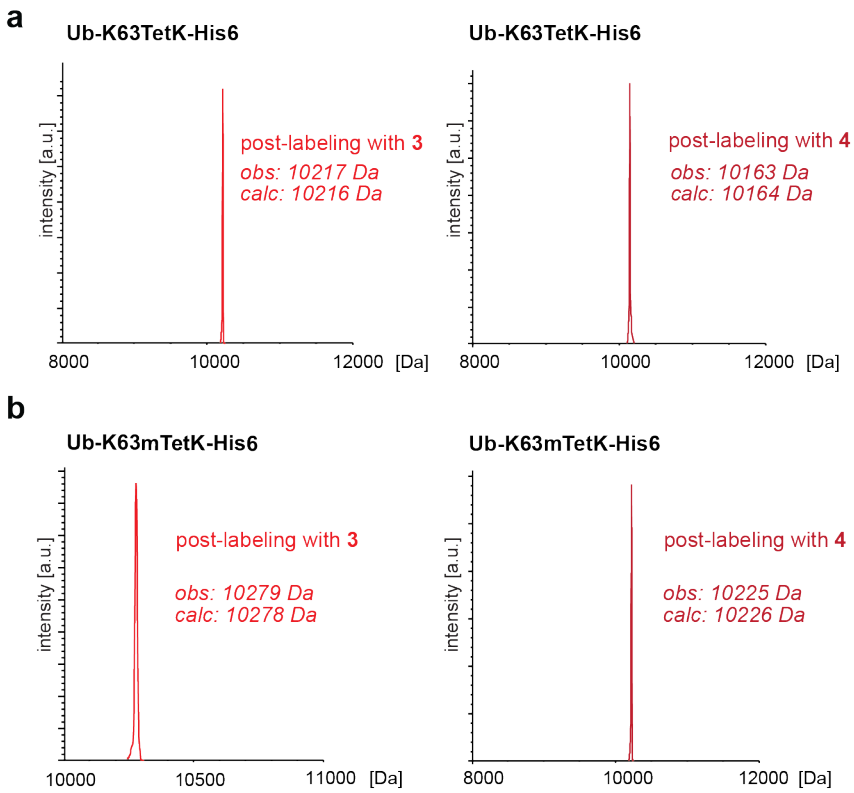
SUPPLEMENTARY FIGURES S1-S24



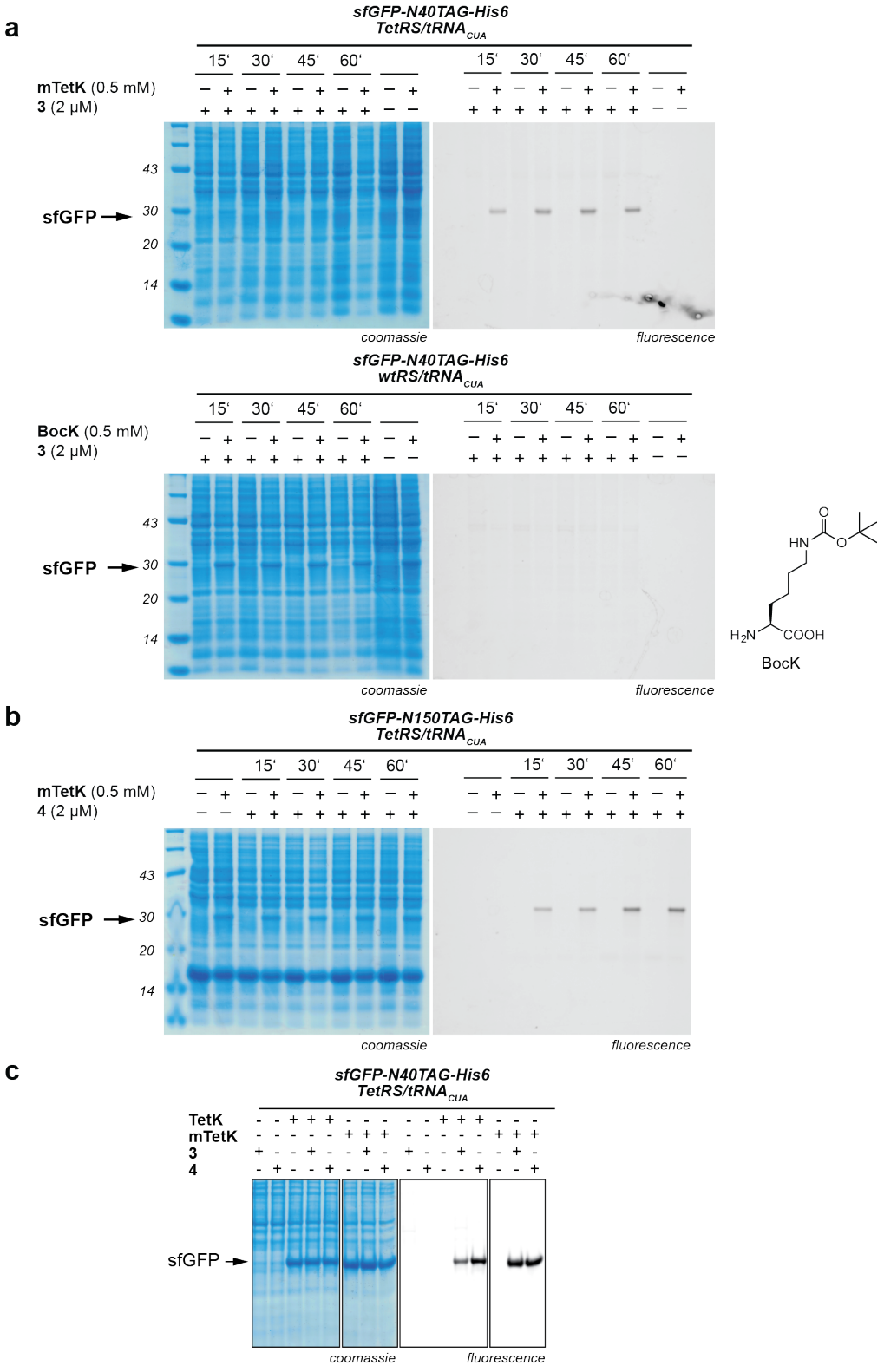
Supplementary Figure S1. Site-specific incorporation of mTetK and TetK into proteins expressed in *E. coli*. a) ESI-MS characterization of site-specific incorporation of mTetK into Myo-S4TAG-His6. b) ESI-MS characterization of site-specific incorporation of mTetK into sfGFP-N40TAG-His6. c) ESI-MS characterization of site-specific incorporation of mTetK into and TetK into Ub-K63TAG-His6



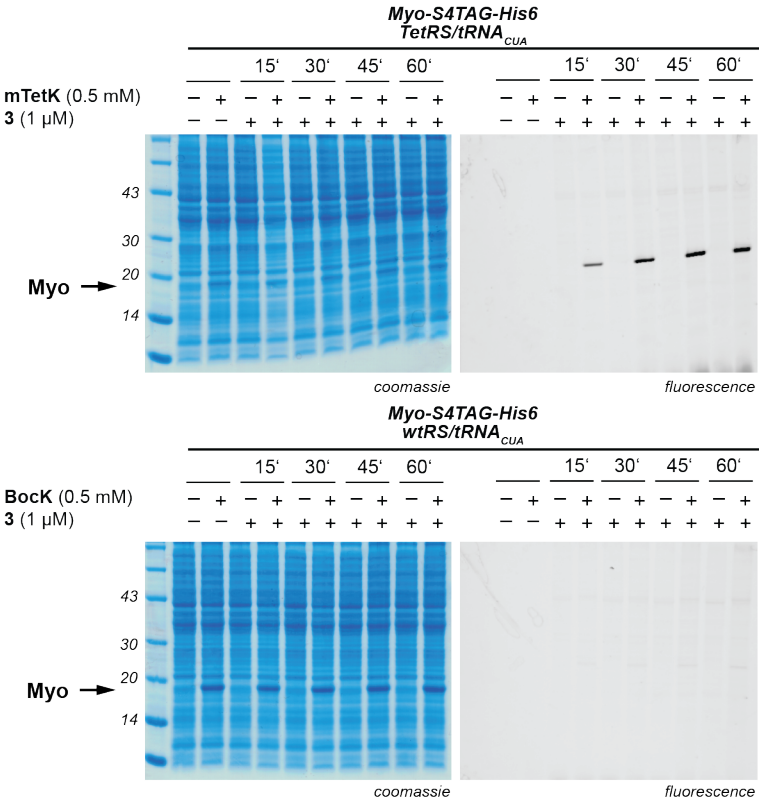
Supplementary Figure S2. Selective and rapid labeling of tetrazine-bearing proteins with BCN- or TCO-fluorophores a) Structures of BCN-TAMRA (**3**) and TCO-TAMRA (**4**). b) SDS-PAGE in gel fluorescence confirms rapid labeling of sfGFP-N150mTetK-His6 with a ten-fold excess of BCN-TAMRA. Samples were incubated for the indicated time points and quenched with a 160-fold excess of 3,6-dipyridyl-1,2,4,5-tetrazine c) ESI-MS characterization confirms quantitative labeling of sfGFP-N150mTetK-His6 and sfGFP-N150TetK-His6 with TCO-TAMRA. d) ESI-MS characterization confirms quantitative labeling of sfGFP-N40mTetK-His6 with BCN-TAMRA. For ESI-MS characterization a 10 µM solution of purified protein was incubated with 100 µM of the respective dye and the mixture analyzed by LC-MS after 15 minutes.



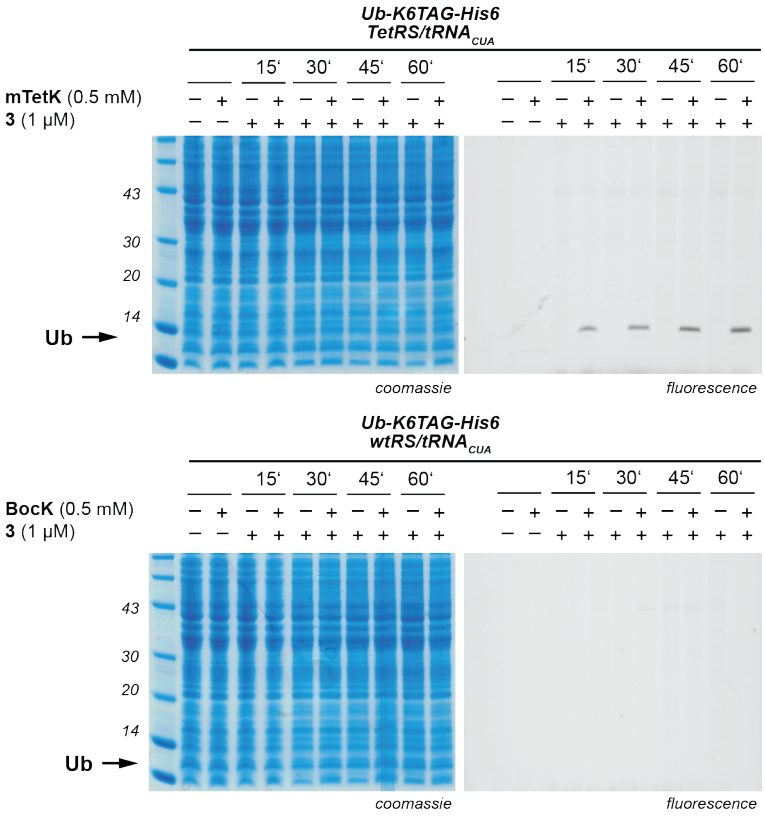
Supplementary Figure S3. Specific labeling of tetrazine-bearing proteins with BCN- or TCO-fluorophores. a) ESI-MS characterization confirms quantitative labeling of Ub-K63TetK-His6 with BCN-TAMRA and TCO-TAMRA. b) ESI-MS characterization confirms quantitative labeling of Ub-K63mTetK-His6 with BCN-TAMRA and TCO-TAMRA. A 10 µM solution of purified protein was incubated with 100 µM of the respective dye and the mixture analyzed by LC-MS after 15 minutes.



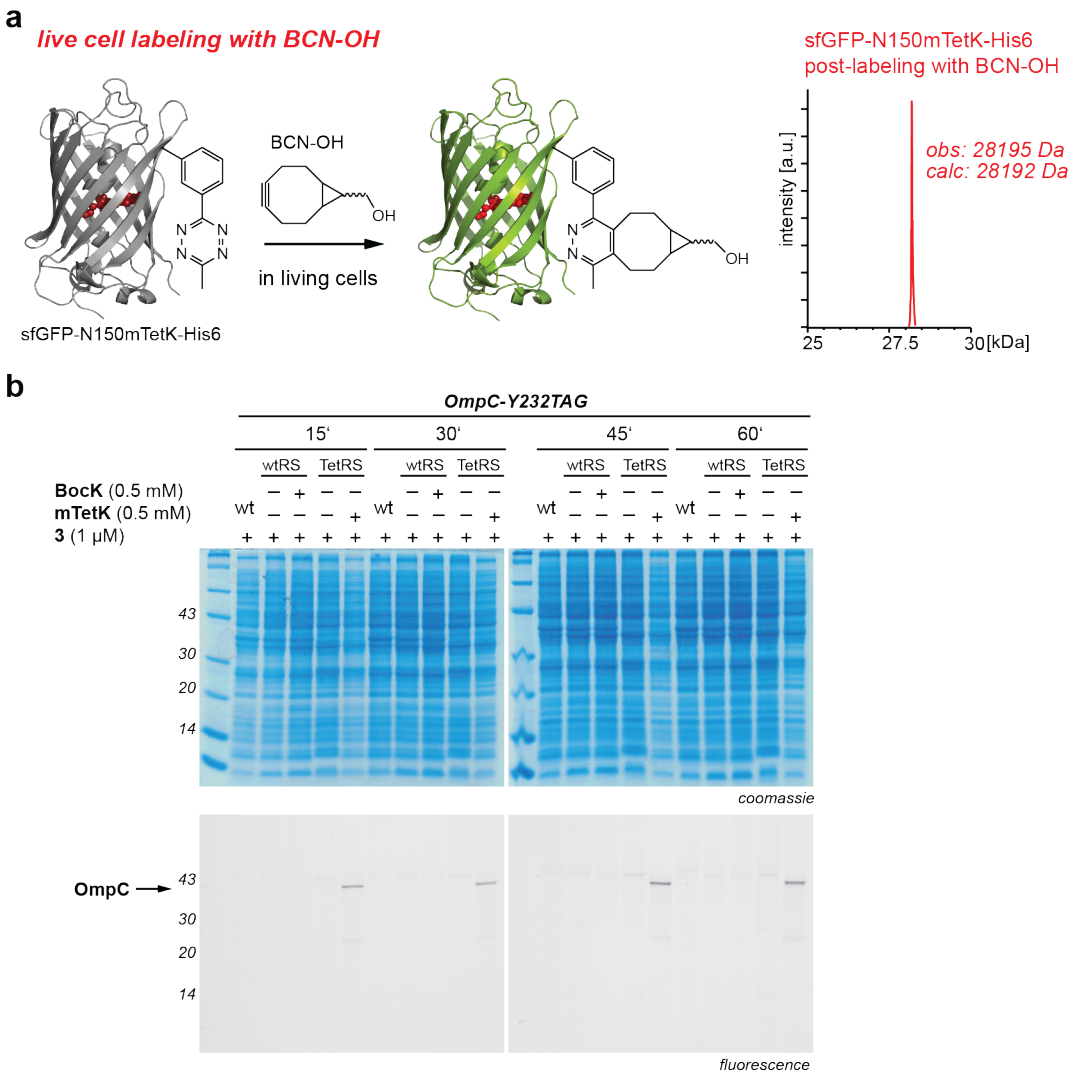
Supplementary Figure S4. Specificity of labeling sfGFP-mTetK and sfGFP-TetK versus the *E. coli* proteome. a) Cells were grown in the presence of 0.5 mM mTetK or TetK and harvested 16 hours after induction of protein expression, washed several times with PBS to remove residual mTetK and treated with 2 µM BCN-TAMRA (**3**) for the indicated time points. Fluorescence scanning of SDS-PAGE gels confirms specific and selective labeling of sfGFP containing mTetK. No unspecific labeling was observed for cellular proteins or for over-expressed sfGFP bearing *N*ε-*tert*-butoxy-carbonyl-L-lysine (BocK) instead of mTetK. b) Same as a) for labeling mTetK in sfGFP-N150TAG-His6 with TCO-TAMRA (**4**). c) Selective labeling of TetK and mTetK-bearing sfGFP in *E. coli* cell lysate. *E. coli* overexpressing sfGFP-N40mTetK or sfGFP-N40TetK were lysed and labeled for 15 min with **3** or **4**.



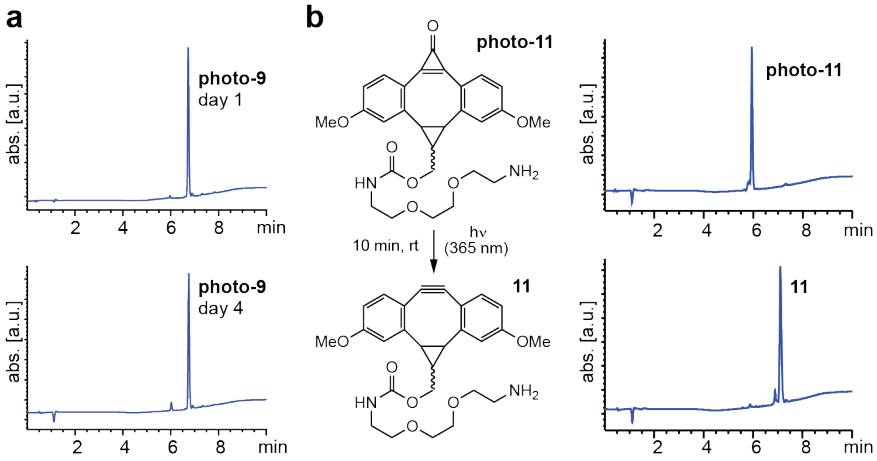
Supplementary Figure S5. Specificity of labeling mTetK in Myo-S4TAG-His6 versus the *E. coli* proteome. a) Cells were grown in the presence of 0.5 mM mTetK and harvested 16 hours after induction of protein expression, washed several times with PBS to remove residual mTetK and treated with 1 µM BCN-TAMRA (**3**) for the indicated time points. Fluorescence scanning of SDS-PAGE gels confirms specific and selective labeling of Myo containing mTetK. No unspecific labeling was observed for cellular proteins or for over-expressed Myo bearing BocK instead of mTetK.



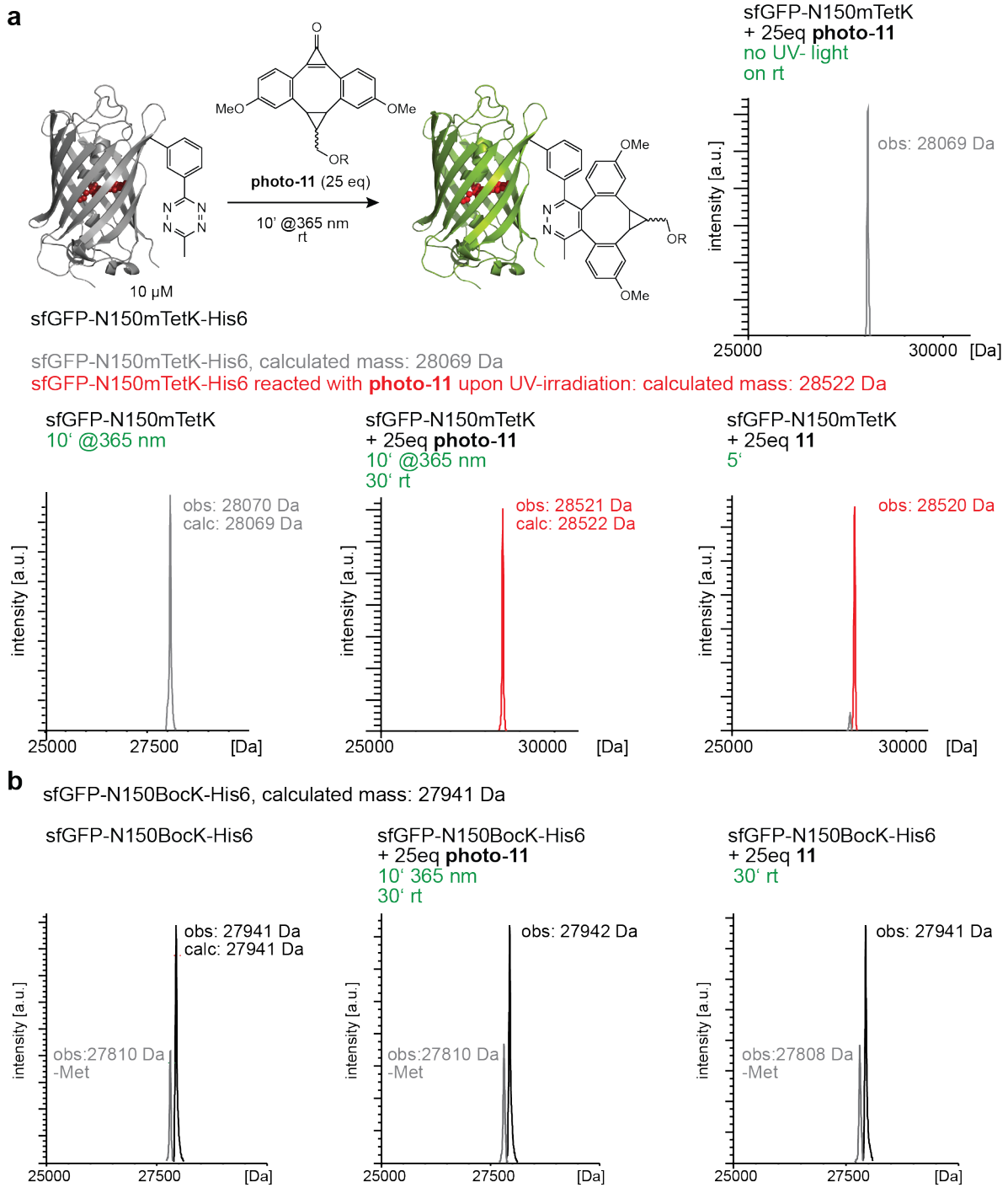
Supplementary Figure S6. Specificity of labeling mTetK in Ub-K6TAG-His6 versus the *E. coli* proteome. a) Cells were grown in the presence of 0.5 mM mTetK and harvested 16 hours after induction of protein expression, washed several times with PBS to remove residual mTetK and treated with 1 µM BCN-TAMRA (**3**) for the indicated time points. Fluorescence scanning of SDS-PAGE gels confirms specific and selective labeling of Ub containing mTetK. No unspecific labeling was observed for cellular proteins or for over-expressed Ub bearing BocK instead of mTetK.



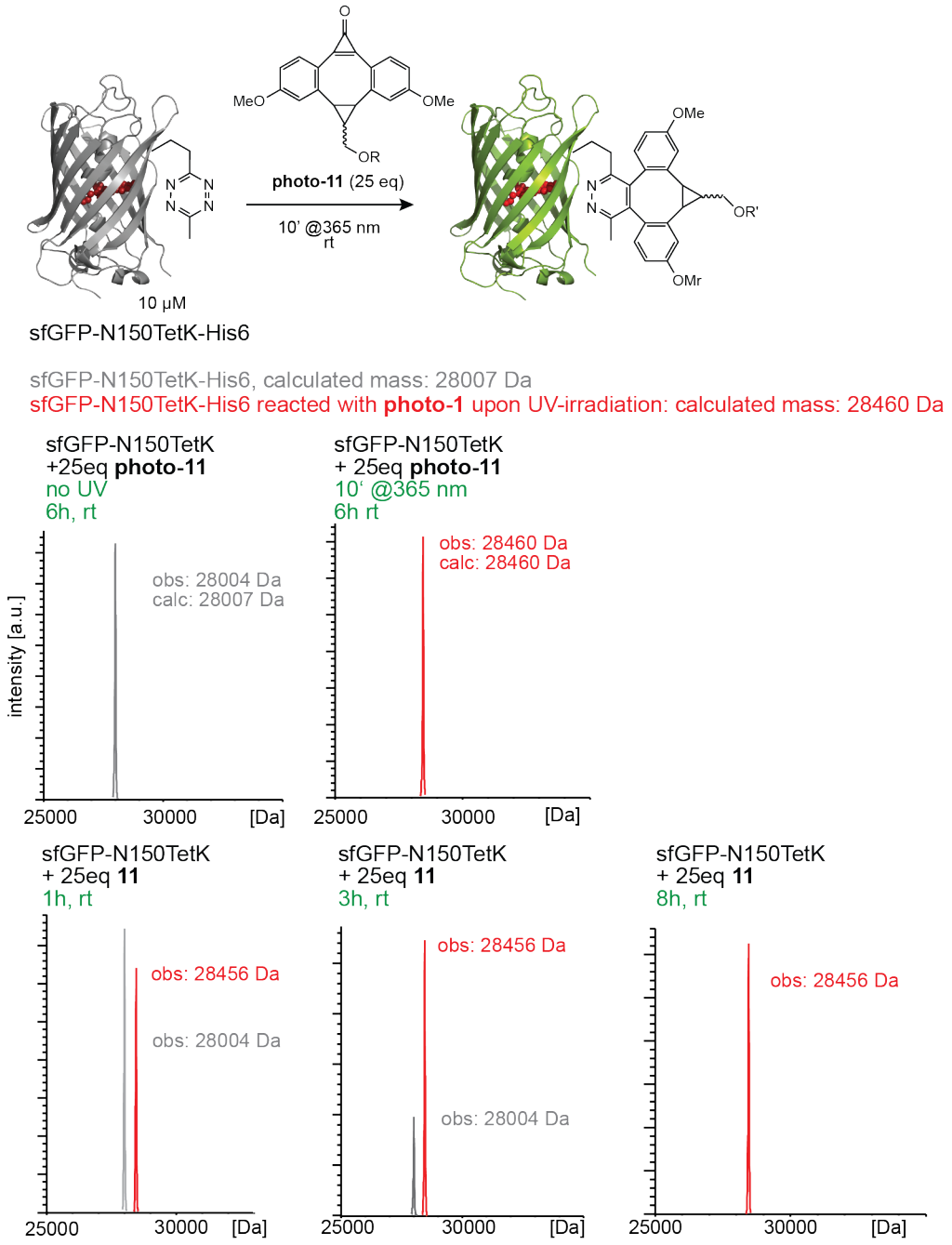
Supplementary Figure S7. Quantitative and selective labeling of mTetK-bearing proteins in living *E. coli.* a) To confirm quantitative *in cellulo* labeling, cells expressing sfGFP-N150mTetK were harvested 16 hours after induction of protein expression and washed several times with PBS to remove residual mTetK and treated with an excess of BCN-OH. After 30 min, labeled sfGFP was purified via Ni-NTA chromatography and subjected to LC-MS characterization, which confirmed fully labeled protein. b) Specificity of labeling mTetK in outer membrane protein OmpC-Y232TAG on the surface of live *E. coli.* Cells were grown in the presence of 0.5 mM mTetK and harvested 16 hours after induction of protein expression, washed with PBS to remove residual mTetK and treated with 1 µM BCN-TAMRA (**3**) for the indicated time points. Fluorescence scanning of SDS-PAGE gels confirms specific and selective labeling of sfGFP containing mTetK. No unspecific labeling was observed for cellular proteins or for over-expressed OmpC bearing BocK instead of mTetK or for over-expressed wt-OmpC.



Supplementary Figure S8. Establishing photo-iEDDAC reactivity. a) **Photo-9** is stable for several days at room temperature. No degradation was observed over several days at room temperature of a 50 µM stock solution of **photo-9** in 8/2 PBS/CH_3CN via LC-MS. b) Structures of **photo-11** and **11** as well as quantitative photodecaging of **photo-11** to **11** as followed by LC-MS.

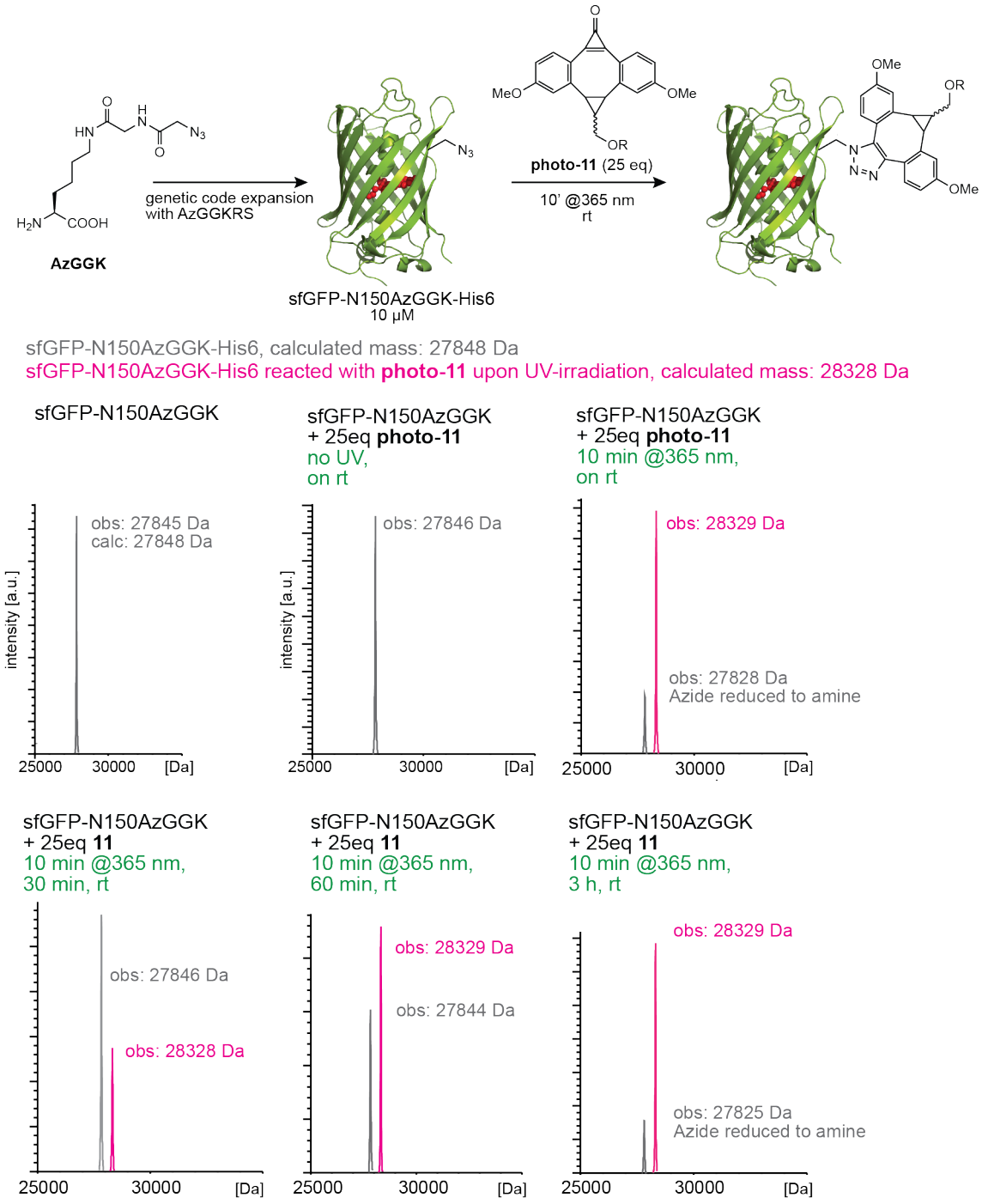


Supplementary Figure S9. Photo-DMBO reacts selectively, quantitatively and rapidly with mTetK-modified proteins. a) Schematics of labeling of sfGFP-N150mTetK-His6 with **photo-11** upon light irradiation and MS-characterization of labeling reaction. Photo-iEDDAC between mTetK and photo-DMBO is selective, rapid (quantitative labeling after 5 min incubation of 10 µM sfGFP-N150mTetK-His6 with 250 µM **11**) and tightly controlled by light. b) A BocK-modified sfGFP mutant is refractory to labeling with **photo-11** and **11**, confirming the selectivity of photo-iEDDAC.
10 µM sfGFP-N150mTetK-His6 or sfGFP-N150BocK-His6 were incubated with a 25-fold excess of **photo-11** or **11**, irradiated with 365 nm where indicated and the samples were analyzed at the indicated time points by LC-MS.

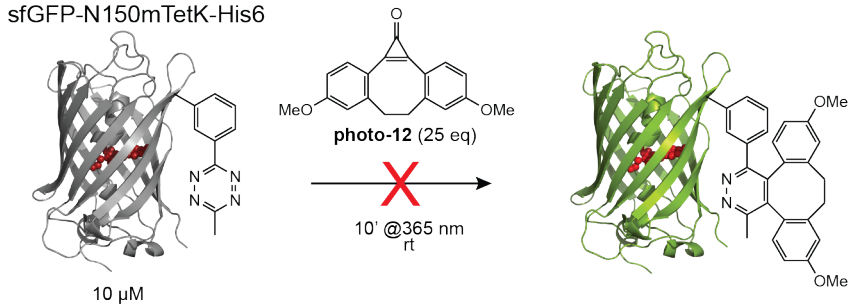


Supplementary Figure S10. Photo-DMBO reacts selectively and quantitatively with TetK-modified proteins. Schematics of labeling of sfGFP-N150TetK-His6 with **photo-11** upon light irradiation and MS-characterization of labeling reaction is shown at the top. Photo-iEDDAC between TetK and photo-DMBO is selective and tightly controlled by light. The reaction is however not as quick as the reaction between **11** or **photo-11** with mTetK-modified proteins and takes several hours to be completed.

10 µM sfGFP-N150TetK-His6 was incubated with a 25-fold excess of **photo-11** or **11**, irradiated with 365 nm where indicated and the samples were analyzed at the indicated time points by LC-MS.



Supplementary Figure S11. Reactivity of photo-DMBO towards azides in photo-SPAAC reactions. AzGGK was incorporated site-specifically into sfGFP[1] and a 10 µM solution of sfGFP-N150AzGGK-His6 was incubated with a 25-fold excess of **photo-11** or **11**, irradiated with 365 nm where indicated and the samples were analyzed at the indicated time points by LC-MS. LS-MS confirms that the reaction between **11** or **photo-11** and azide-modified proteins proceeds considerably slower than cycloaddition with mTetK-modified proteins. After 60 min incubation, approximately 60% of sfGFP-N150AzGGK-His6 have reacted with **11** (the corresponding reaction with sfGFP-N150mTetK was complete within 5 min). Even after overnight incubation a small amount of starting material is left. The mass of this starting material peak corresponds to a sfGFP variant, where the azide group in AzGGK has been reduced to amine through the long incubation at room temperature and is therefore not anymore reactive towards iEDDAC with **11**, highlighting the better suitability of mTetK in photo-iEDDAC for labeling proteins in *E. coli* in a light-dependent fashion.



sfGFP-N150mTetK-His6, calculated mass: 28069 Da

sfGFP-N150mTetK-His6 reacted with **photo-12** upon UV-irradiation, calculated mass: 28305 Da.

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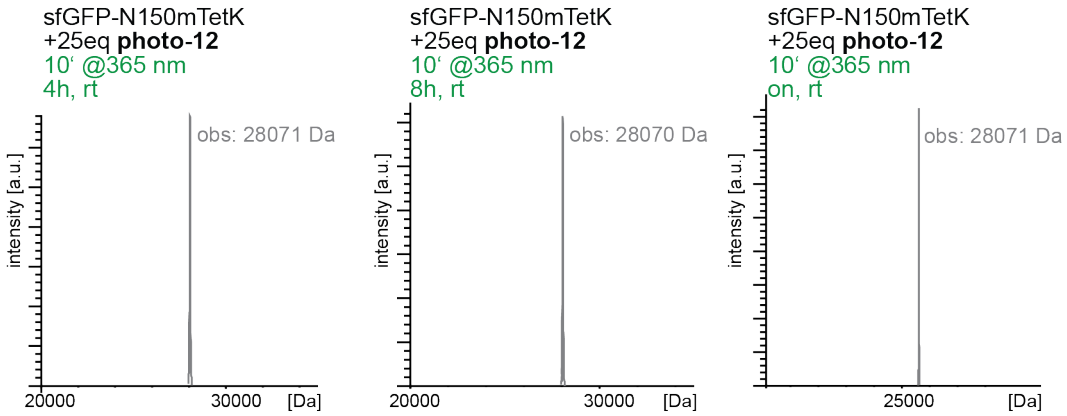
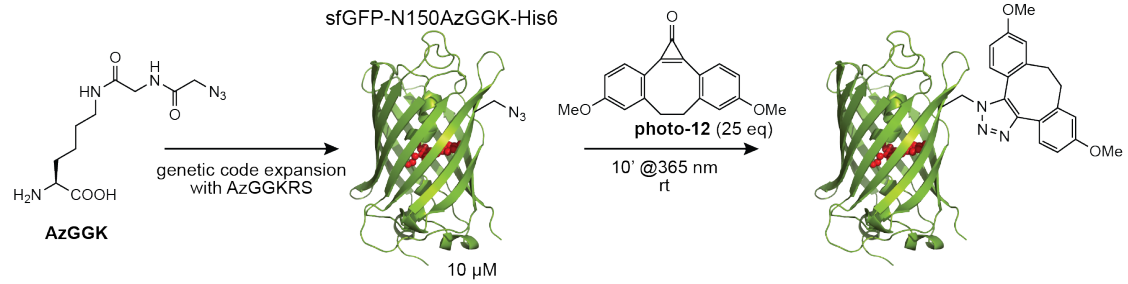
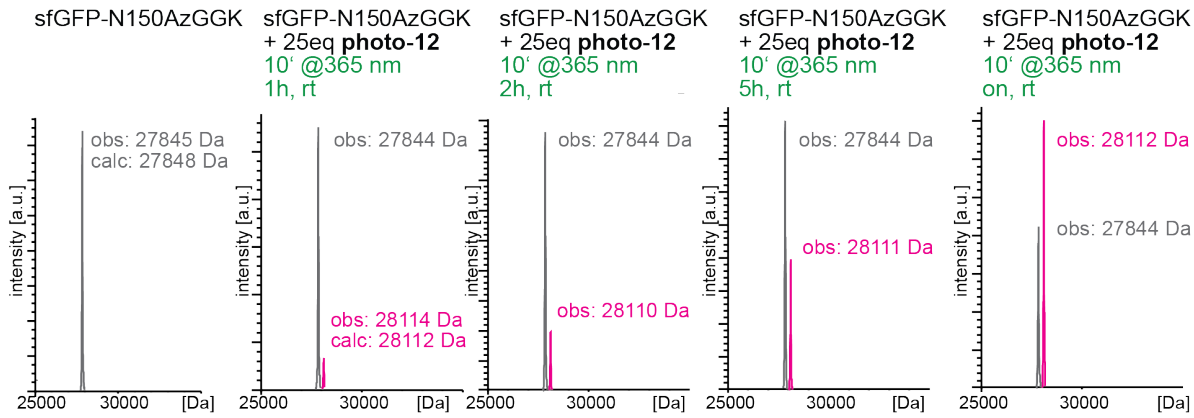


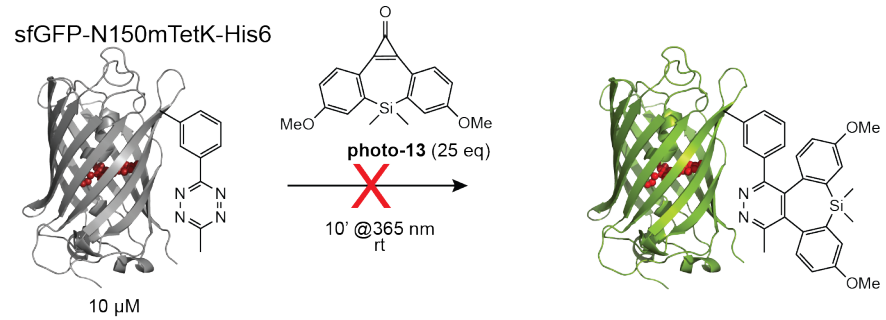
Supplementary Figure S12. Reactivity of **photo-12**[2] towards tetrazines in photo-iEDDAC reactions. sfGFP-N150mTetK (10µM) was incubated with a 25-fold excess of **photo-12**. The mixture was irradiated for 10 min at 365 nm and samples were analyzed by LC-MS at the indicated time points. No cycloaddition product between sfGFP-N150mTetK and **photo-12** could be observed, indicating that **photo-12** containing no extra ring strain on the cyclooctyne ring is unable to undergo an inverse-electron demand Diels-Alder cycloaddition with a mTetK-modified protein.



sfGFP-N150AzGGK-His6, calculated mass: 27848 Da
sfGFP-N150AzGGK-His6 reacted with **photo-12** upon UV-irradiation, calculated mass: 28112 Da (**ca 60% overnight**)



Supplementary Figure S13. Reactivity of **photo-12** towards azides in photo-SPAAC reactions. sfGFP-N150AzGGK (10µM) was incubated with a 25-fold excess of **photo-12**. The mixture was irradiated for 10 min at 365 nm and samples were analyzed by LC-MS at the indicated time points. Overnight incubation under these conditions did not yield quantitatively labeled sfGFP, but ~60% labeled sfGFP, indicating the superior reactivity of the more strained DMBO compounds (e.g. **photo-11**) towards azides and tetrazines.



sfGFP-N150mTetK-His6, calculated mass: 28069 Da

sfGFP-N150mTetK-His6 reacted with **photo-13** upon UV-irradiation, calculated mass: 28335 Da.

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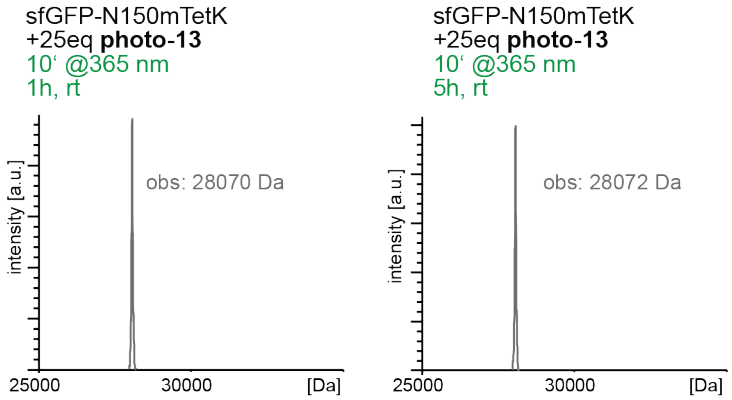
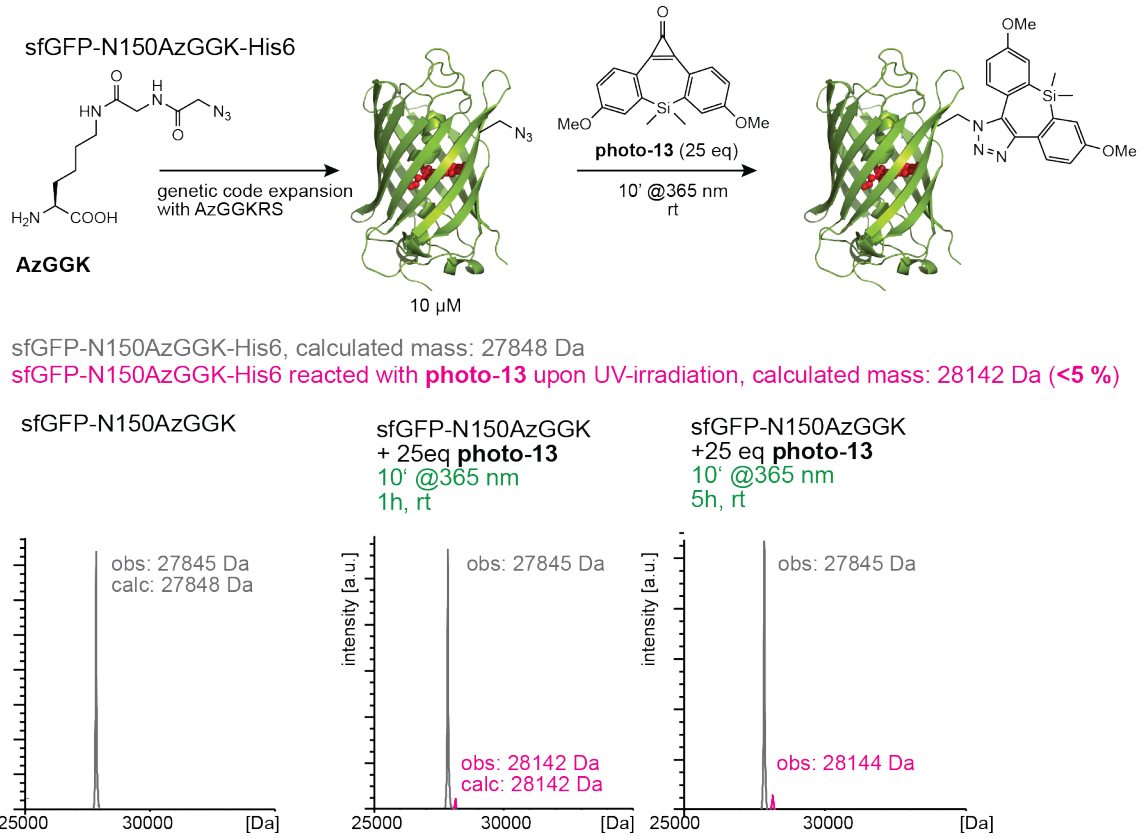
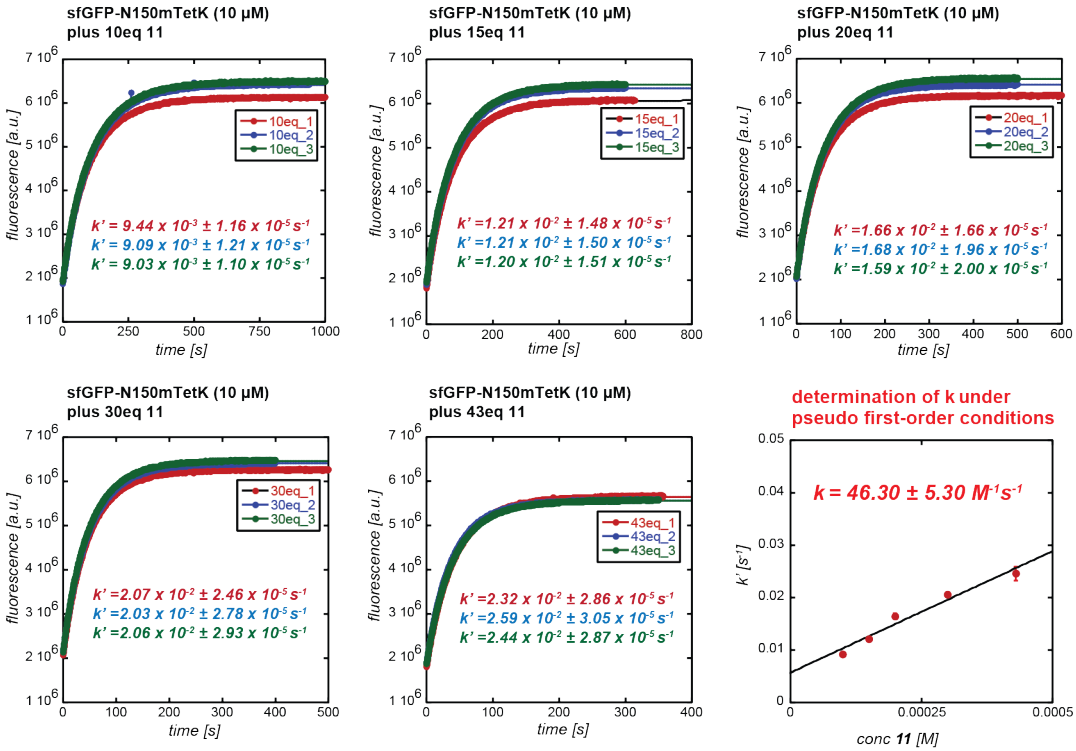


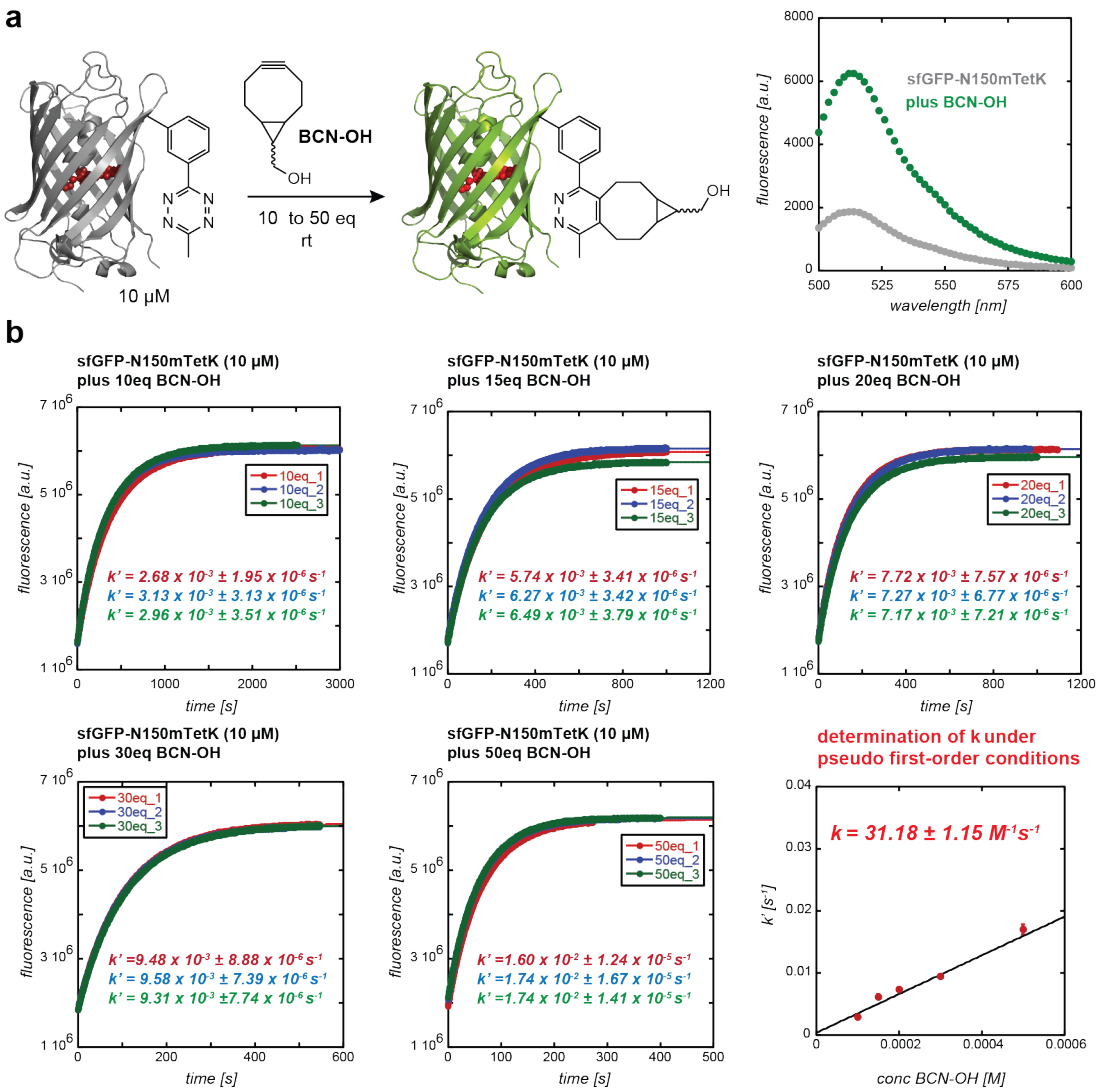
Supplementary Figure S14. Reactivity of **photo-13**[3] towards mTetK in photo-iEDDAC reactions. sfGFP-N150mTetK (10µM) was incubated with a 25-fold excess of **photo-13**. The mixture was irradiated for 10 min at 365 nm and samples were analyzed by LC-MS at the indicated time points. No cycloaddition product between sfGFP-N150mTetK and **photo-13** could be observed even after prolonged incubation.



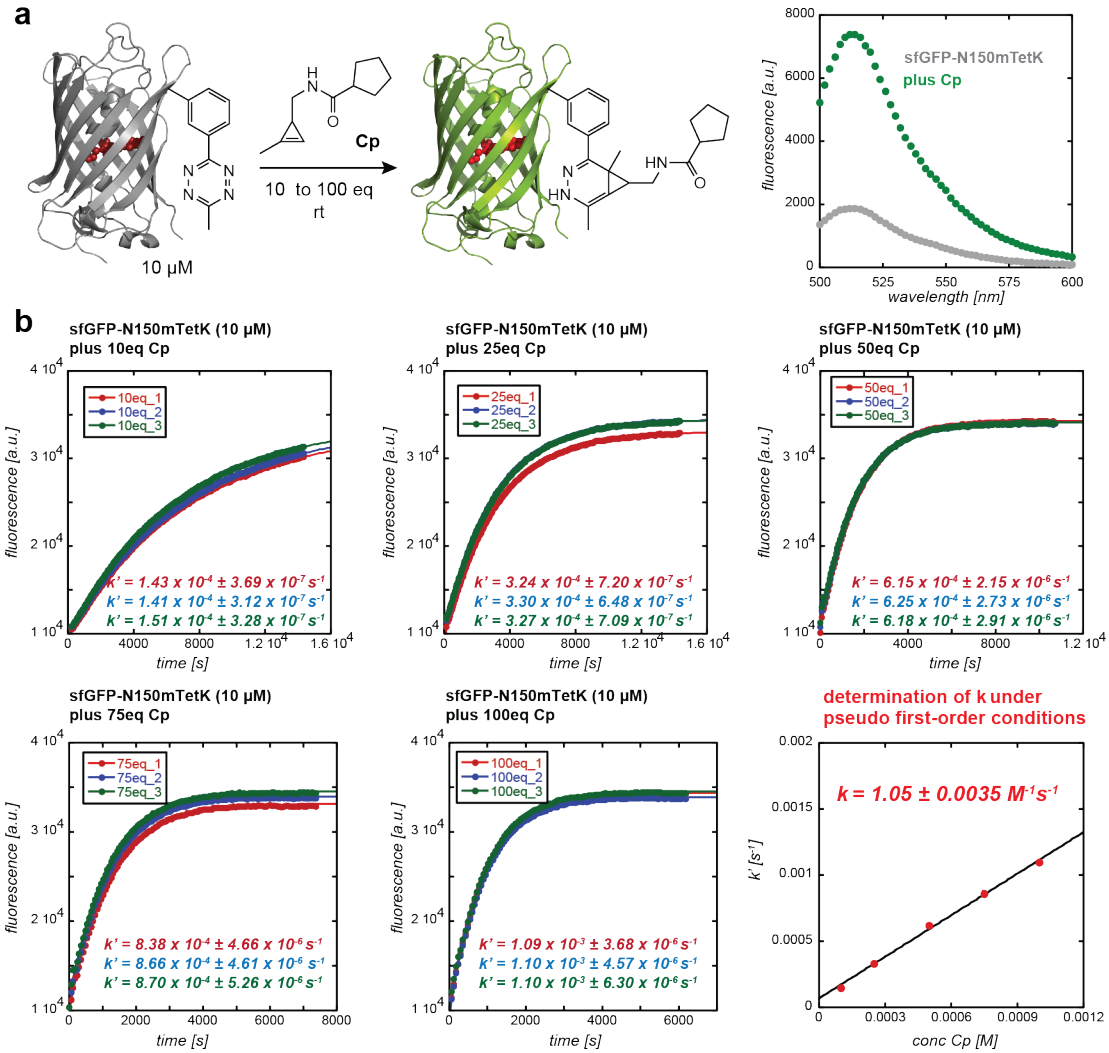
Supplementary Figure S15. Reactivity of **photo-13** towards AzGGK in photo-SPAAC reactions. sfGFP-N150AzGGK (10µM) was incubated with a 25-fold excess of **photo-13**. The mixture was irradiated for 10 min at 365 nm and samples were analyzed by LC-MS at the indicated time points. Only tiny amounts of cycloaddition product (< 5%) could be observed at prolonged incubation times.



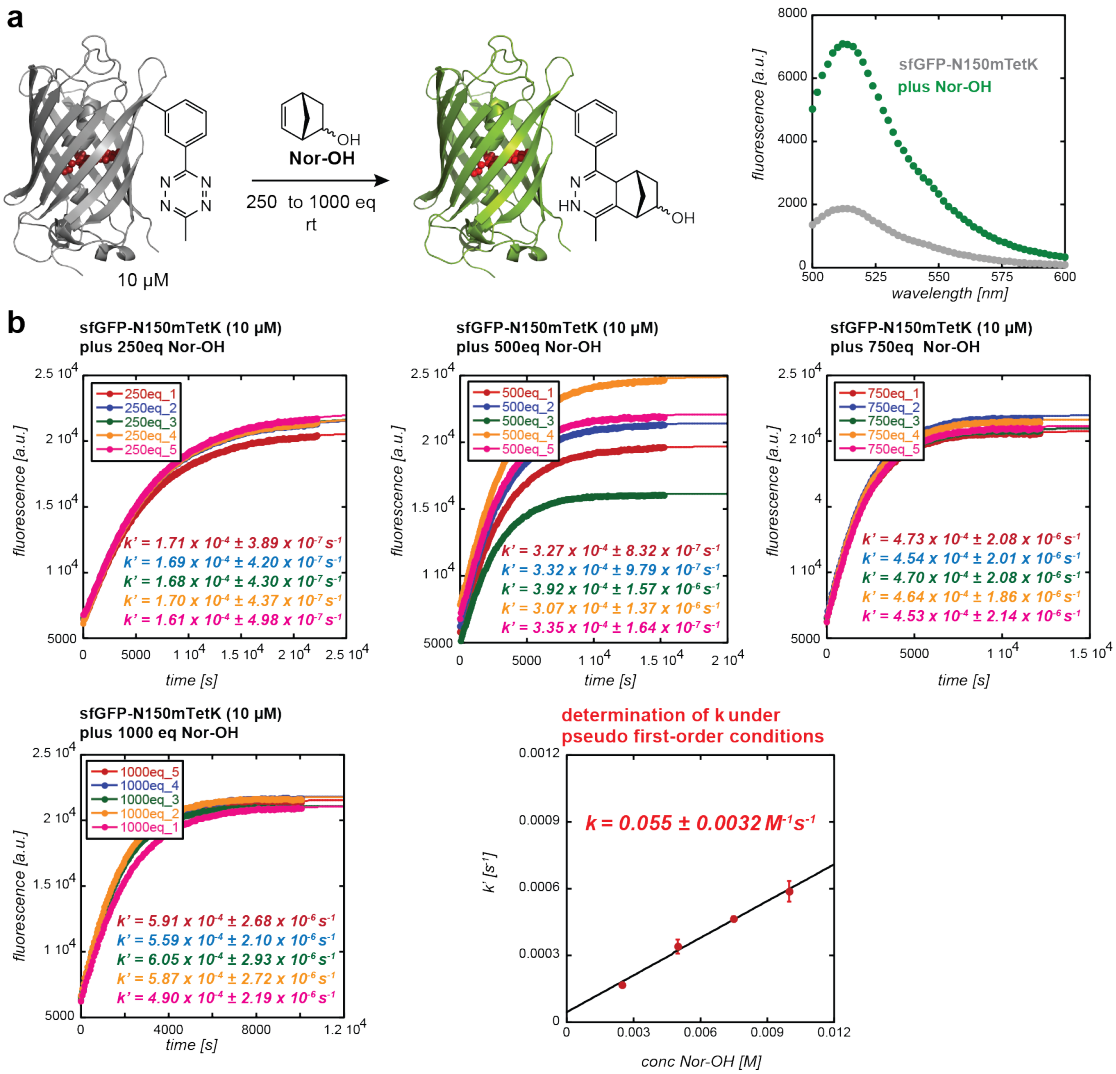
Supplementary Figure S16. Determination of second order rate constant k between sfGFP-N150mTetK-His6 and **11** under pseudo-first order conditions. 10 µM solutions of sfGFP-N150mTetK-His6 in PBS buffer were individually mixed with solutions of **11** in PBS buffer to reach final concentrations of 10 µM sfGFP-N150mTetK-His6 and 100 µM to 430 µM of **11** and the increase of the sfGFP-fluorescence at 508 nm (excitation: 488 nm) was followed over time. By fitting the data to a single exponential equation, k' values were determined. Determined k' values for different concentrations of **11** were plotted against concentration of **11** and subjected to a linear fit. The slope of the plot yields the rate constant k. All experiments were recorded at 25°C. All data processing was performed using Kaleidagraph software (Synergy Software; Reading, UK). Experiments were performed in triplicates.



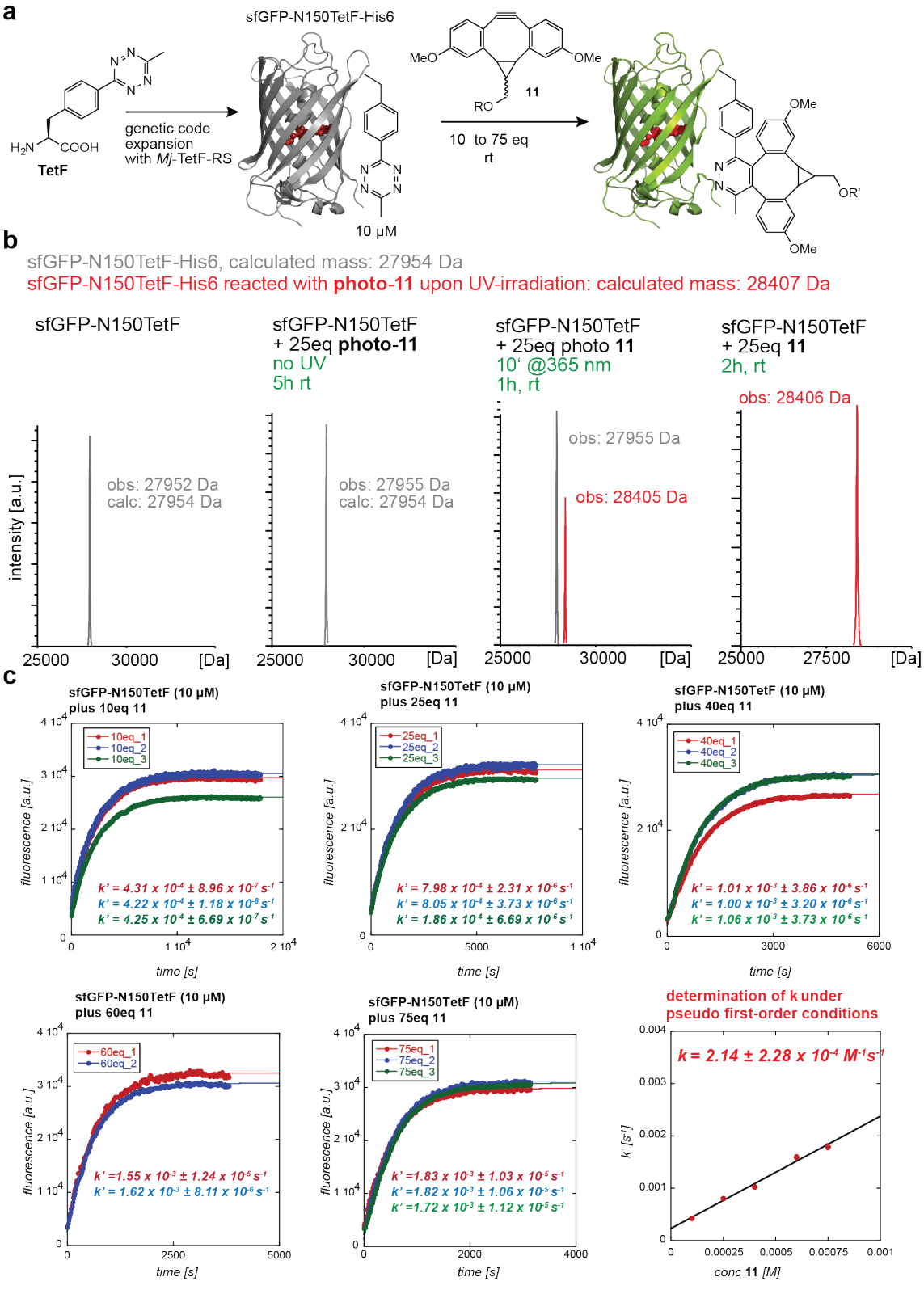
Supplementary Figure S17. Determination of second order rate constant k between sfGFP-N150mTetK-His6 and **BCN-OH** under pseudo-first order conditions. a) Left: schematic illustration of sfGFP-N150mTetK-His6 labeling with **BCN-OH**. Right: wavelength scan showing fluorescence emission (excitation: 460 nm) of sfGFP-N150mTetK-His6 and sfGFP-N150mTetK-His6 reacted with an excess of **BCN-OH**. b) 10 µM solutions of sfGFP-N150mTetK-His6 in PBS buffer were individually mixed with **BCN-OH** solutions to reach final concentrations of 10 µM sfGFP-N150mTetK-His6 and 100 µM to 500 µM of **BCN-OH** in PBS buffer and the increase of the sfGFP-fluorescence at 508 nm (excitation: 488 nm) was followed over time. By fitting the data to a single exponential equation, k' values were determined. Determined k' values for different concentrations of **BCN-OH** were plotted against concentration of **BCN-OH** and subjected to a linear fit. The slope of the plot yields the rate constant k. All experiments were recorded at 25°C. All data processing was performed using Kaleidagraph software (Synergy Software; Reading, UK). Experiments were performed in triplicates.



Supplementary Figure S18. Determination of second order rate constant k between sfGFP-N150mTetK-His6 and **Cp** under pseudo-first order conditions. a) Left: schematic illustration of sfGFP-N150mTetK-His6 labeling with **Cp**. Right: wavelength scan showing fluorescence emission (excitation: 460 nm) of sfGFP-N150mTetK-His6 and sfGFP-N150mTetK-His6 reacted with an excess of **Cp**. b) 10 µM solutions of sfGFP-N150mTetK-His6 in PBS buffer were individually mixed with solutions of Cp to reach final concentrations of 10 µM of sfGFP-N150mTetK-His6 and 100 µM to 1000 µM of **Cp** in PBS buffer and the increase of the sfGFP-fluorescence at 508 nm (excitation: 488 nm) was followed over time. By fitting the data to a single exponential equation, k' values were determined. Determined k' values for different concentrations of **Cp** were plotted against concentration of **Cp** and subjected to a linear fit. The slope of the plot yields the rate constant k. All experiments were recorded at 25°C. All data processing was performed using Kaleidagraph software (Synergy Software; Reading, UK). Experiments were performed in triplicates.

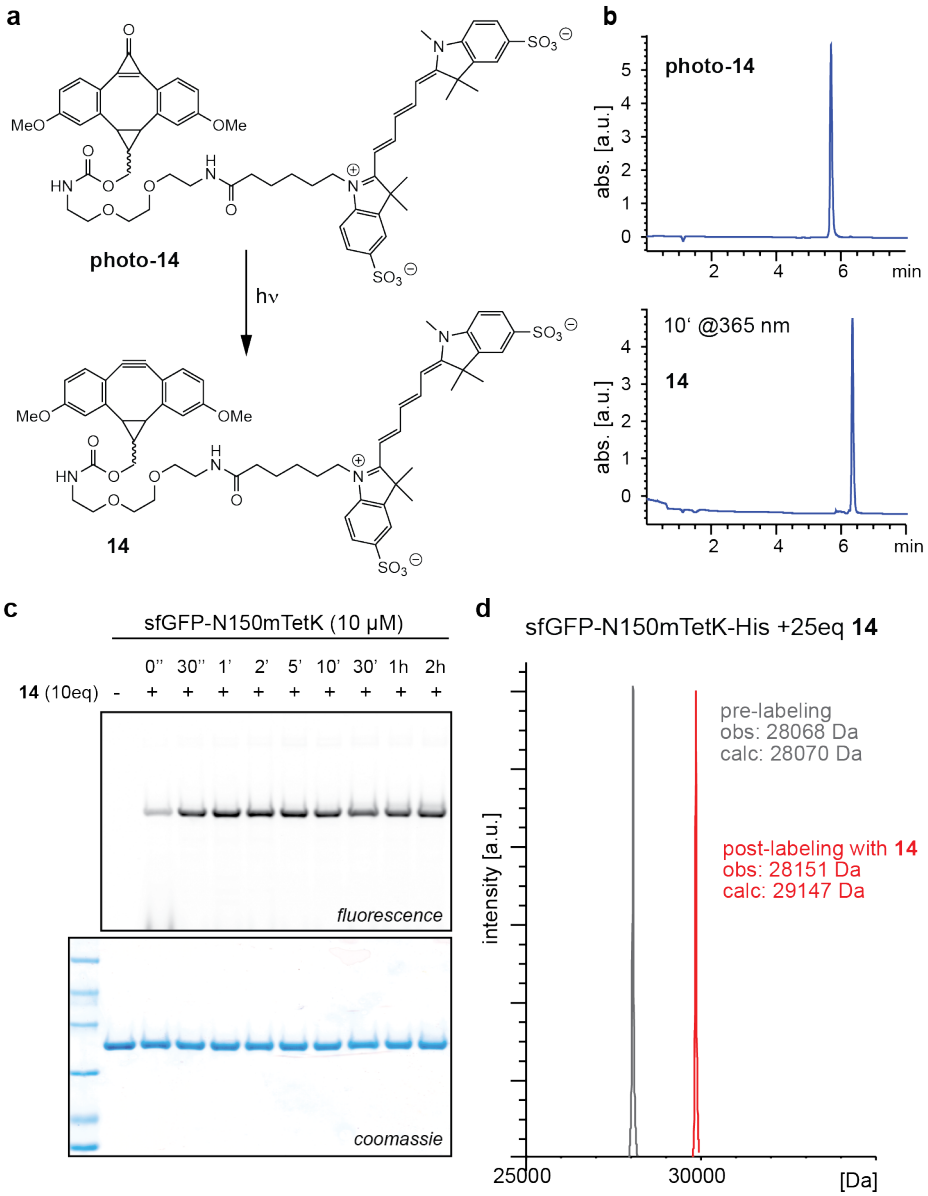


Supplementary Figure S19. Determination of second order rate constant k between sfGFP-N150mTetK-His6 and **Nor-OH** under pseudo-first order conditions. a) left: schematic illustration of sfGFP-N150mTetK-His6 labeling with **Nor-OH**. Right: wavelength scan showing fluorescence emission (excitation: 460 nm) of sfGFP-N150mTetK-His6 and sfGFP-N150mTetK-His6 reacted with an excess of **Nor-OH**. b) 10 µM solutions of sfGFP-N150mTetK-His6 in PBS buffer were individually mixed with solutions of **Nor-OH** to reach final concentrations of 10 µM of sfGFP-N150mTetK-His6 and 2.5 mM to 10 mM of **Nor-OH** in PBS buffer and the increase of the sfGFP-fluorescence at 527 nm (excitation: 480 nm) was followed over time. By fitting the data to a single exponential equation, k' values were determined. Determined k' values for different concentrations of **Nor-OH** were plotted against concentration of **Nor-OH** and subjected to a linear fit. The slope of the plot yields the rate constant k. All experiments were recorded at 25°C. All data processing was performed using Kaleidagraph software (Synergy Software; Reading, UK). Experiments were performed five times.

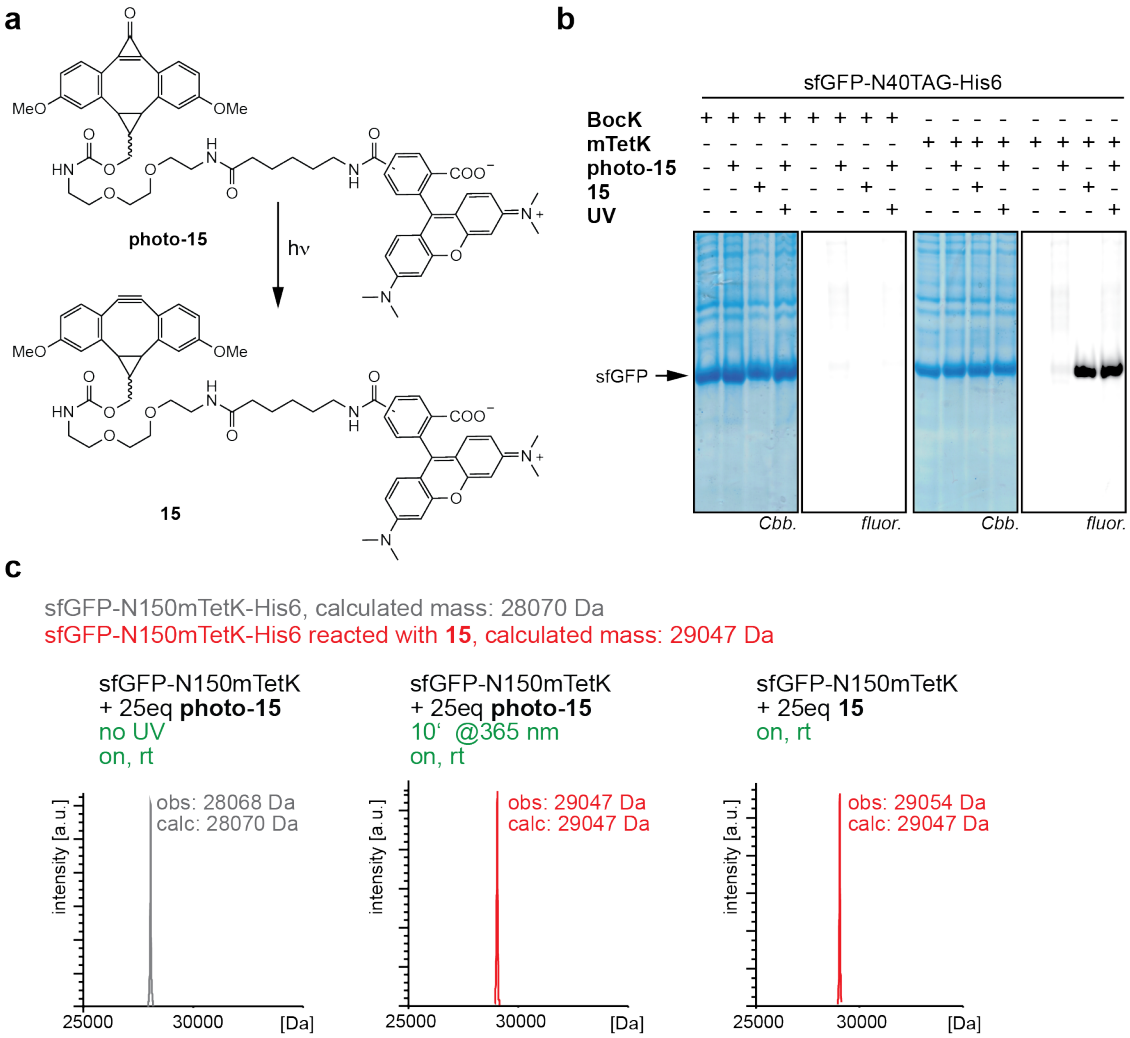


Supplementary Figure S20. Reactivity of amino acid TetF[4] towards photo-DMBO compounds in photo-iEDDAC. a) TetF was incorporated site-specifically into sfGFP by using a described *M. jannaschi* Tyrosyl-tRNA synthetase/tRNA pair (TetF-RS) and sfGFP-N150TetF-His6 was labeled with **11**. b) Labeling of sfGFP-N150TetF-His6 with **photo-11** or **11** was analyzed by LC-MS. Labeling is specific for TetF and only observed when induced by light. c) Determination of second order rate constant k between sfGFP-N150TetF-His6 and **11** under pseudo-first order conditions. 10 µM solutions of sfGFP-N150TetF-His6 in PBS buffer were individually mixed with solutions of **11** to reach final concentrations of 10 µM of sfGFP-N150TetF-His6 and 100 µM to 750 µM of **11** in PBS buffer and the increase of the

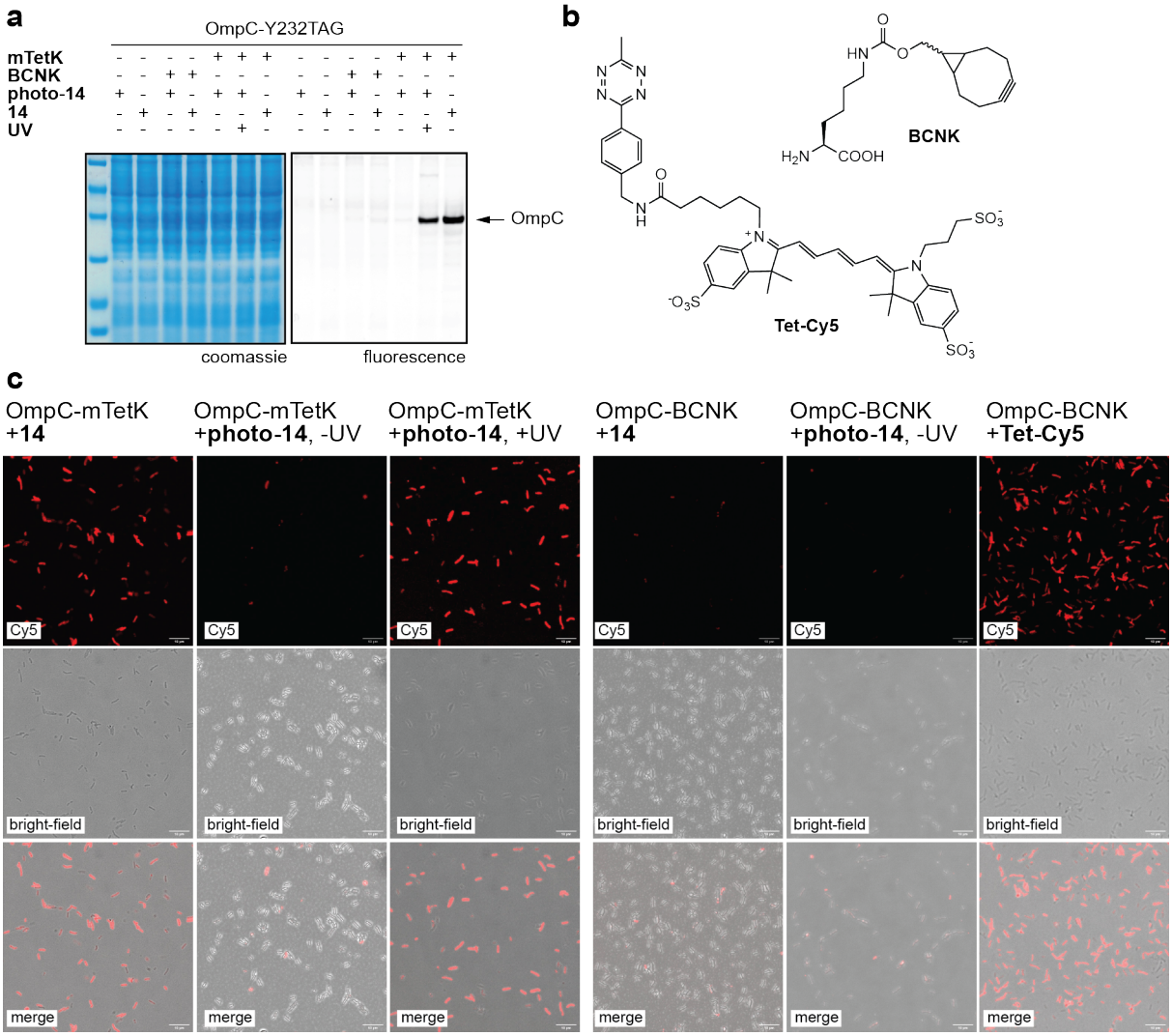
sfGFP-fluorescence at 527 nm (excitation: 480 nm) was followed over time. By fitting the data to a single exponential equation, k' values were determined. Determined k' values for different concentrations of **11** were plotted against concentration of **11** and subjected to a linear fit. The slope of the plot yields the rate constant k. All experiments were recorded at 25°C. All data processing was performed using Kaleidagraph software (Synergy Software; Reading, UK). Experiments were performed in triplicates.



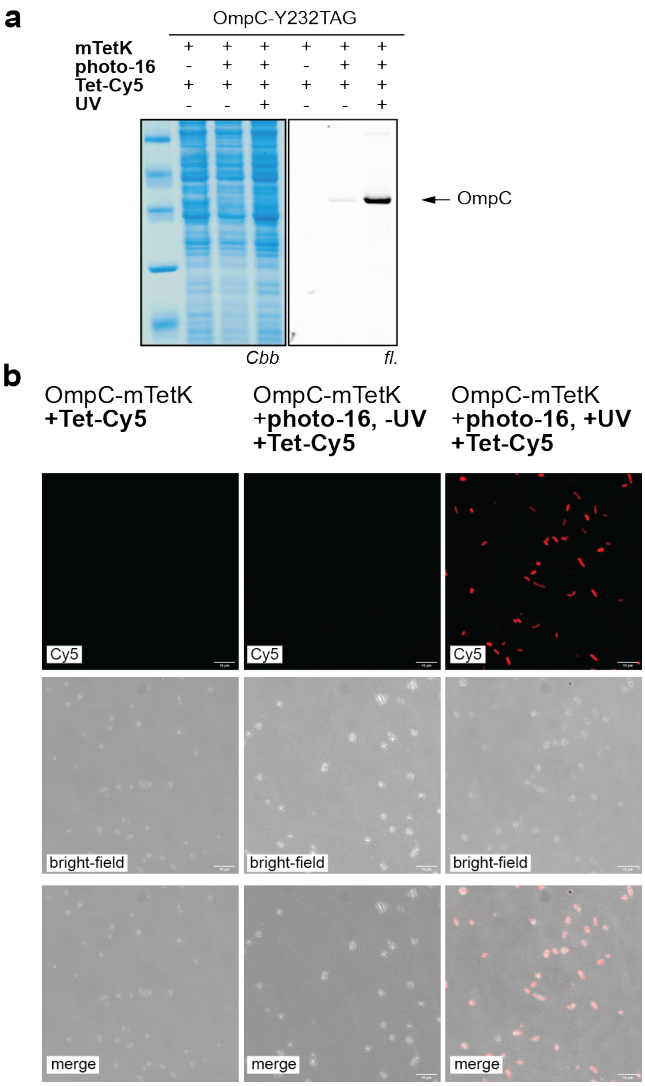
Supplementary Figure S21. Specific and rapid labeling of mTetK-bearing proteins with photo-DMBO fluorophore conjugates. a) Structure of the Cy5 conjugate **photo-14** and **14**. b) LC-MS analysis confirms quantitative photo-decaging of **photo-14** at 365 nm light. c) SDS-PAGE in gel fluorescence confirms rapid labeling of sfGFP-N150mTetK-His6 (10 µM) with a ten-fold excess of **14**. Samples were incubated for the indicated time points and quenched with a 160-fold excess of 3,6-dipyridyl-1,2,4,5-tetrazine. d) LC-MS analysis confirms quantitative labeling of sfGFP-N150mTetK-His6 with **14**.



Supplementary Figure S22. Specific and rapid labeling of mTetK-bearing proteins with photo-DMBO fluorophore conjugates. a) Structure of the TAMRA conjugate **photo-15** and **15**. b) Labeling of mTetK-bearing proteins is specific towards the *E. coli* proteome. Cells were grown in the presence of mTetK and harvested 16 hours after induction of protein expression, washed several times with PBS to remove residual mTetK, lysed and treated with **photo-15** or **15** in the presence or absence of light as indicated. Fluorescence scanning of SDS-PAGE gels confirms specific and selective labeling of sfGFP containing mTetK. Importantly, labeling with **photo-15** is light dependent. No unspecific labeling was observed for cellular proteins or for over-expressed sfGFP bearing BocK instead of mTetK. c) LC-MS analysis confirms quantitative labeling of sfGFP-N150mTetK-His6 with **14** and **photo-14** upon UV-irradiation.



Supplementary Figure S23. Selective and light-induced labeling of mTetK-bearing proteins in living *E. coli.* a) Specificity of labeling mTetK in OmpC-Y232TAG on the surface of live *E. coli.* Cells were grown in the presence of 0.5 mM mTetK and harvested 16 hours after induction of protein expression, washed with PBS to remove residual mTetK and treated with **photo-14** or **14** in the presence or absence of light as indicated. Fluorescence scanning of SDS-PAGE gels confirms specific and selective labeling of sfGFP containing mTetK. No unspecific labeling was observed for over-expressed OmpC bearing BCNK instead of mTetK. b) Structures of BCNK and tetrazine-Cy5 conjugated **Tet-Cy5**. c) Live-cell labeling of *E. coli* expressing OmpC-Y232TAG in the presence of mTetK or BCNK shows light-induced selective labeling of OmpC-Y232mTetK with **photo-14**. Upper panels show red fluorescence in the Cy5 channel, middle panels show bright field images, bottom panels show merged images.



Supplementary Figure S24. Selective and light-induced sequential labeling of OmpC-Y232mTetK on the surface of living *E. coli.* a) Specificity of labeling mTetK in OmpC-Y232TAG on the surface of live *E. coli.* Cells were grown in the presence of 0.5 mM mTetK and harvested 16 hours after induction of protein expression, washed with PBS to remove residual mTetK, treated with **photo-16**, illuminated with UV where indicated to decage photo-DMBO in **photo-16** and reacted with **Tet-Cy5** allowing temporal and spatial control over the labeling reaction. a) In gel fluorescence of SDS-PAGE gels and b) live-cell microscopy imaging. Upper panels show red fluorescence in the Cy5 channel, middle panels show bright field images, bottom panels show merged images.

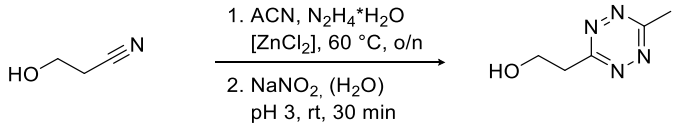
GENERAL METHODS

All solvents and chemical reagents were purchased from Sigma, Carbolution, Acros Organics, Fisher Scientific, Lumiprobe or Jena Bioscience and were used without further purification unless otherwise stated. Flash column chromatography used for product purification was performed on silica gel 60 (230-400 mesh). Thin-layer chromatography (TLC) was performed on Merck Millipore silica gel 60 F-254 plates. The developed silica plates were visualized by UV light (254 nm) and/or staining with potassium permanganate or ninhydrin. Reverse-phase HPLC purification was carried out with a Shimadzu LC-20AT Prominence system. A Phenomenex Luna C18, 5 µm (4.6 x 250 mm) column was used to separate compounds on an analytical scale; a Phenomenex Luna C18, 5 µm (10 x 250 mm) column and a Phenomenex Luna C18, 10 µm (21.2 x 250 mm) for preparative scale runs. Solvents A (MilliQ H_2O+ 0.1 % formic acid) and B (ACN + 0.1 % formic acid) were used without filtration. NMR spectra were recorded on a Bruker 500 UltraShield™ (500 MHz for ^{1}H-NMR, 125 MHz for ^{13}C-NMR) or a AVHD300 (300 MHz for ^{1}H-NMR, 75 MHz for ^{13}C-NMR). Chemical shifts (δ), reported in ppm, are referenced to the residual proton solvent signals (DMSO-d6–2.50 ppm for ^{1}H-NMR and 39.5 ppm for ^{13}C-NMR spectra; $CDCl_3$ – 7.26 ppm ^{1}H-NMR and 77.0 ppm for ^{13}C-NMR spectra). Coupling constants (J) are reported in Hertz (Hz) while peak multiplicities are descripted as follows: s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), dt (doublet of triplets), ddd (doublet of doublet of doublets), m (multiplet), br (broad signal). Small molecule and protein ESI-LC-MS was carried out on an Agilent Technologies 1260 Infinity LC-MS system with a 6310 Quadrupole spectrometer. The solvent system consisted of 0.1 % formic acid in water as buffer A and 0.1 % formic acid in ACN as buffer B. A Phenomenex Jupiter™ C18 column (2 x 150 mm, 5 µm) was used for small molecule separation. The samples were analyzed in both positive and negative mode and followed by UV absorbance at 193, 254 and/or 280 nm. Protein samples were separated on a Phenomenex Jupiter™ C4 column (2 x 150 mm, 5 µm) and analyzed in positive mode, visualized by UV absorbance at 280 nm and protein masses deconvoluted using OpenLab ChemStation Edition Software C.01.07.SR3 [465]. ESI-HRMS of small molecules was performed on a Thermo Fisher Scientific LTQ FT Ultra linear ion trap with a Fourier Transform Ion Cyclotron Resonance (FT-ICR) MS detector. Samples were analyzed in positive or negative mode and visualized with Thermo Xcalibur™ 2.2 SP1 48 Qual Browser. Oligonucleotide primers for Q5™ Site-Directed Mutagenesis were designed with NEBaseChanger v 1.2.8 and purchased at Sigma. 15 % SDS-PAGE (110 V for 15 min, then 200 V for 45 min) was carried out on or a Bolt™ Mini Gel Tank (Invitrogen) system. Gels were stained with Quick Coomassie Stain (Generon). Protein Marker III 6.5-200 kDa (AppliChem), Protein Marker I 14.4 to 116 kDa (peqGOLD, VWR) or Color Prestained Protein Standard, Broad Range 11-245 kDa (NEB) were used as protein markers. DNA concentrations were measured on NanoPhotometer® P330 (Implen) and protein concentrations determined by Bradford assay at 595 nm. A UV Lamp VL-215.L with a wavelength of 365 nm was used to decage cyclopropenone protected compounds to the alkyne. *E. coli* images were taken with Leica SP8 LIGHTNING confocal microscope. The Cy5 fluorescence was excited with a diode laser at 638 nm with a laser power of 2 % and emission captured between 650-700 nm.

CHEMICAL SYNTHESIS

Synthesis of TetK (1)

3-(2-Hydroxyethyl)-6-methyl-1,2,4,5-tetrazine[5]

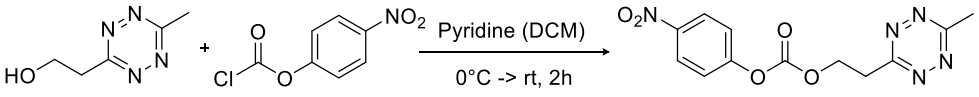


Hydroxypropionitrile (5.0 g, 70.3 mmol, 1.0 eq) was added to a flask containing zinc chloride (4.8 g, 35.2 mmol, 0.5 eq), followed by acetonitrile (37 mL, 7.03 mol, 10 eq) and hydrazine hydrate (90 mL, 1.7 mol, 25 eq). The resulting reaction mixture was stirred at 60 °C for 36 h. Then, sodium nitrite (24.3 g, 0.35 mol, 5.0 eq) dissolved in a minimal amount of water was added and the mixture cooled on ice. 6 M HCl solution (300 mL) was slowly added to acidify the reaction mixture to pH 3. It was stirred for 30 minutes on ice until the formation of nitrous fumes was completed, then the water phase was extracted with EtOAc (5 × 200 mL) until it was not pink anymore. The organic phase was dried over Na_2SO_4 before removing the solvent under reduced pressure. The crude residue was purified by flash chromatography (0-60 % EtOAc/pentane), which resulted the product (1.11 g, 11 %) as a red liquid.

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 4.26 (t, $^3J_{HH}$ = 5.8 Hz, 2H, CH_2OH), 3.57 (t, $^3J_{HH}$ = 5.8 Hz, 2H, CH_2), 3.06 (s, 3H, CH_3).

The characterization is in agreement with the literature.[5]

2-(6-methyl-1,2,4,5-tetrazin-3-yl)ethyl-(4-nitrophenyl)-carbonate

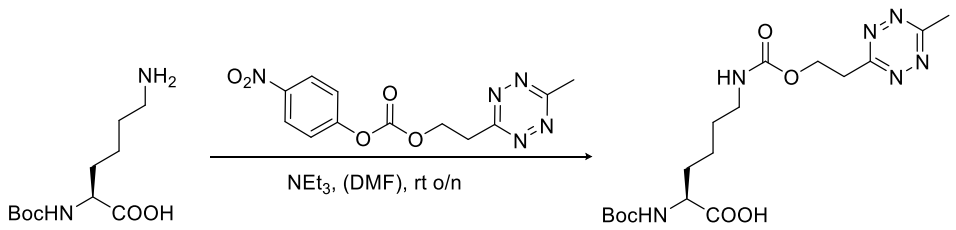


3-(2-Hydroxyethyl)-6-methyl-1,2,4,5-tetrazine (0.11 g, 0.79 mmol, 1.0 eq) was dissolved in DCM (2 mL) and cooled to 0 °C. 4-Nitrophenyl chloroformate (0.35 g, 1.7 mmol, 2.2 eq) was added to the solution in small portions, followed by the addition of pyridine (70 µL, 0.86 mmol 1.1 eq) and then stirred at room temperature for two hours. After removal of the solvent under reduced pressure flash chromatography was used to purify the crude mixture (0-30 % EtOAc/pentane), which resulted the product (0.2 g, 83 %) as a red liquid.

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 8.27 (d, $^3J_{HH}$ = 9.2 Hz, 2H, H_{Ar}), 7.35 (d, $^3J_{HH}$ = 9.2 Hz, 2H, H_{Ar}), 4.98 (t, $^3J_{HH}$ = 6.2 Hz, 2H, CH_2O), 3.78 (t, $^3J_{HH}$ = 6.2 Hz, 2H, CH_2), 3.08 (s, 3H, CH_3).

^{13}C NMR (75 MHz, $CDCl_3$) δ = 168.2, 166.7, 155.4, 152.4, 145.6, 125.4, 121.9, 66.0, 34.2, 21.3.

N^2-(*tert*-butoxycarbonyl)-N^6-((2-(6-methyl-1,2,4,5-tetrazin-3-yl)ethoxy)-carbonyl)-*L*-lysine (BocTetK)



Boc-*L*-lysine (1.84 g, 7.45 mmol, 1.2 eq) was dissolved in DMF (20 mL), then NEt_3 (1 mL, 7.45 mmol, 1.2 eq) and 2-(6-methyl-1,2,4,5-tetrazin-3-yl)ethyl-(4-nitrophenyl)-carbonate (1.90 g, 6.21 mmol, 1.0 eq) were added to the solution, which was stirred at room temperature overnight. Afterwards the solvent was evaporated under reduced pressure and the crude residue purified by flash chromatography (0-10 % MeOH/DCM + 0.1 % AcOH) to result the product as a red oil (1.94 g, 73 %).

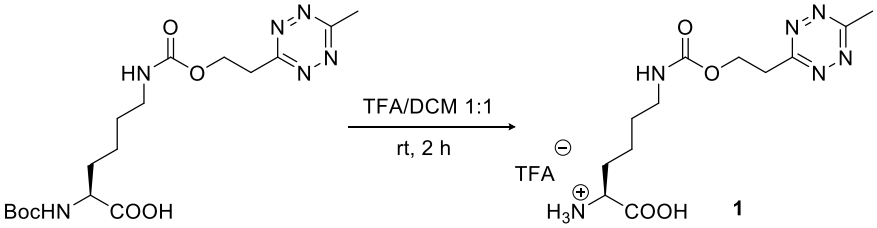
^{1}H-NMR (500 MHz, DMSO-d_6): δ = 7.09 (t, $^3J_{HH}$ = 5.8 Hz, 1H, $NH_{-\varepsilon}$), 6.99 (d, $^3J_{HH}$ = 7.9 Hz, 1H, $NH_{-\alpha}$), 4.49 (t, $^3J_{HH}$ = 6.3 Hz, 2H, CH_2O), 3.76-3.84 (m, 1H, $CH_{-\alpha}$), 3.52 (t, $^3J_{HH}$ = 6.3 Hz, 2H, CH_2), 2.95 (s, 3H, CH_3), 2.84-2.90 (m, 2H, $CH_{2-\varepsilon}$), 1.46-1.64 (m, 2H, $CH_{2-\beta}$), 1.37 (s, 9H, Boc), 1.19-1.36 (m, 4H, $CH_{2-\gamma,\delta}$).

^{13}C-NMR (75 MHz, DMSO-d_6): δ = 174.2, 167.3, 167.1, 155.9, 155.6, 78.0, 61.2, 53.4, 39.9, 34.4, 30.4, 28.9, 28.2, 22.8, 21.1.

MS (ESI), *m/z* calcd for $C_{17}H_{28}N_6O_6$: 412.21, found 435.0 $[M+Na]^+$, 312.8 $[M-Boc+H]^+$, 411.1 $[M-H]^-$.

HRMS (ESI), *m/z* $[M+H]^+$ calcd for $C_{17}H_{28}N_6O_6$: 412.2149, found: 412.2143.

N^6-((2-(6-methyl-1,2,4,5-tetrazin-3-yl)ethoxy)-carbonyl)-*L*-lysine trifluoroacetate (TetK, 1)



BocTetK (1.90 g, 5 mmol, 1.0 eq) was dissolved in 25 mL TFA/DCM (1:1) with a drop of H_2O and stirred at room temperature for two hours. The solvent was evaporated under reduced pressure, the product dissolved in a minimal amount of MeOH and then precipitated from cold Et_2O and subsequently dried to obtain **1** (1.41 g, 75 %) as a pink solid.

^{1}H-NMR (300 MHz, DMSO-d_6): δ = 8.20 (s, 3H, $NH_{-\alpha}$), 7.08 (t, $^3J_{HH}$ = 5.0 Hz, 1H, $NH_{-\varepsilon}$), 4.49 (t, $^3J_{HH}$ = 6.3 Hz, 2H, CH_2O), 3.85-3.87 (m, 1H, $CH_{-\alpha}$), 3.52 (t, $^3J_{HH}$ = 6.3 Hz, 2H, CH_2), 2.95 (s, 3H, CH_3), 2.88-2.91 (m, 2H, $CH_{2-\varepsilon}$), 1.75 (m, 2H, $CH_{2-\beta}$), 1.23-1.35 (m, 4H, $CH_{2-\gamma,\delta}$).

^{13}C-NMR (75 MHz, DMSO-d_6 + TFA): δ = 171.2, 167.5, 167.1, 156.0, 61.3, 52.1, 39.9, 34.4, 29.7, 28.8, 21.8, 20.8.

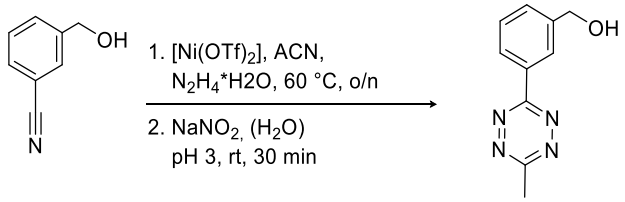
Due to solubility problems of **1** in DMSO-d_6, 10% TFA was added before NMR measurements.

MS (ESI), *m/z* calcd for $C_{12}H_{20}N_6O_4$ 312.15, found 313.2 $[M+H]^+$, 311.1 $[M-H]^-$.

HRMS (ESI), *m/z* $[M+H]^+$ calcd for $C_{12}H_{20}N_6O_4$: 313.1624, found: 313.1618.

Synthesis of mTetK (2)

3-(3-(Hydroxymethyl)phenyl)-6-methyl-1,2,4,5-tetrazine[5]



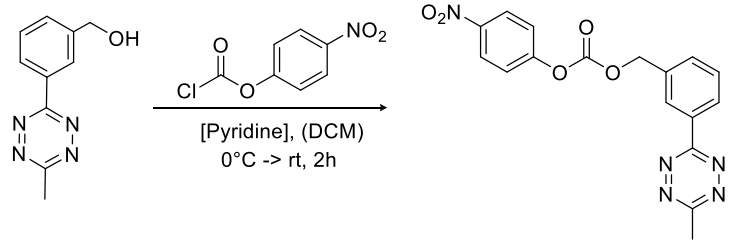
3-(Hydroxymethyl)benzonitrile (1.0 g, 7.5 mmol, 1.0 eq) was combined in a flask with $Ni(OTf)_2$ (1.3 g, 3.8 mmol, 0.5 eq), then ACN (3.9 mL, 75 mmol, 10 eq) and hydrazine hydrate (9.1 mL, 0.19 mol, 25 eq) were added and the reaction heated to 60 °C for 24-36 h. $NaNO_2$ (2.6 g, 38 mmol, 5.0 eq) dissolved in H_2O (10 mL) was added and the reaction mixture cooled in an ice bath before slowly acidifying to pH 3 using a 6 M HCl solution. After stirring for 30 minutes on ice until the formation of nitrous fumes was complete, the reaction was extracted with EtOAc (5 × 80 mL) until it wasn't pink anymore. The organic phase was dried over Na_2SO_4 and the solvent removed under reduced pressure. The crude residue was purified by flash chromatography (0-50 % EtOAc/pentane), which resulted the product (0.56 g, 38 %) as a pink solid.

^{1}H-NMR (300 MHz, DMSO-d_6): δ = 8.45-8.48 (m, 1H, H_{Ar-2}), 8.32-8.37 (m, 1H, H_{Ar-5}), 7.60-7.64 (m, 2H, $H_{Ar-4,6}$), 5.42 (t, $^3J_{HH}$ = 5.8 Hz, 1H, OH), 4.65 (d, $^3J_{HH}$ = 5.8 Hz, 2H, CH_2), 3.00 (s, 3H, CH_3).

MS (ESI), *m/z* calcd for $C_{10}H_{10}N_4O$ 202.09, found 202.7 $[M+H]^+$.

The characterization is in agreement with the literature.[6]

3-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl-(4-nitrophenyl)-carbonate

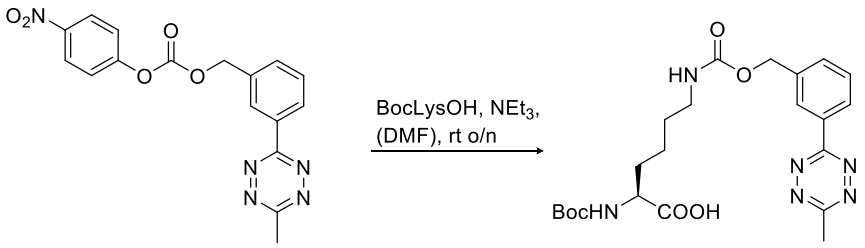


3-(3-(Hydroxymethyl)phenyl)-6-methyl-1,2,4,5-tetrazine (2.2 g, 11 mmol, 1.0 eq) was dissolved in DCM (50 mL) and cooled to 0 °C. 4-Nitrophenyl chloroformate (2.6 g, 13 mmol, 1.2 eq) was added to the solution, followed by the addition of pyridine (0.88 mL, 11 mmol 1.0 eq) and then stirred at room temperature for 2 hours. After removal of the solvent under reduced pressure flash chromatography was used to purify the crude mixture (0-60 % EtOAc/pentane), which resulted the product (3.5 g, 88 %) as a pink solid.

^{1}H-NMR (300 MHz, DMSO-d_6): δ = 8.57-8.60 (m, 1H, H_{Ar-2}), 8.49 (td, $^3J_{HH}$ = 7.7 Hz, $^4J_{HH}$ = 1.5 Hz, 1H, H_{Ar-6}), 8.33 (d, $^3J_{HH}$ = 9.2 Hz, 2H, $H_{Ar-orthoNO2}$), 7.80 (td, $^3J_{HH}$ = 7.7 Hz, $^4J_{HH}$ = 1.5 Hz, 1H, H_{Ar-4}), 7.75 (dd, $^3J_{HH}$ = 7.7, 7.7 Hz, 1H, H_{Ar-5}), 7.60 (d, $^3J_{HH}$ = 9.2 Hz, 2H, $H_{Ar-metaNO2}$), 5.48 (s, 2H, CH_2), 3.02 (s, 3H, CH_3).

^{13}C-NMR (75 MHz, $CDCl_3$): δ = 167.7, 163.8, 155.6, 152.5, 145.6, 135.7, 132.6, 132.5, 130.0, 128.6, 128.0, 125.5, 121.9, 70.5, 21.3.

N^2-(*tert*-butoxycarbonyl)-N^6-(((3-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)oxy)carbonyl)-*L*-lysine (BocmTetK)



Boc-*L*-lysine (0.32 g, 1.3 mmol, 1.2 eq) was dissolved in DMF (3.5 mL), then NEt_3 (0.18 mL, 1.3 mmol, 1.2 eq) and 3-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl-(4-nitrophenyl)-carbonate (0.4 g, 1.1 mmol, 1.0 eq) were added to the solution, which was stirred at room temperature overnight. Afterwards the solvent was evaporated under reduced pressure and the crude residue purified by flash chromatography (0-2 % MeOH/DCM + 0.1 % AcOH) to result the product as a pink solid (0.36 g, 70 %).

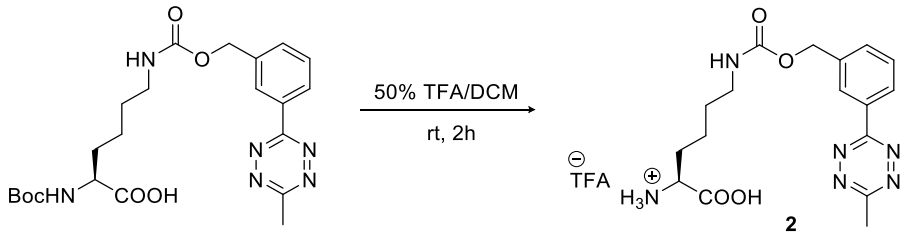
^{1}H-NMR (300 MHz, DMSO-d_6): δ = 8.44-8.47 (m, 1H, H_{Ar-2}) 8.38-8.43 (m, 1H, H_{Ar-5}), 7.62-7.68 (m, 2H, $H_{Ar-4,6}$), 7.35 (t, $^3J_{HH}$ = 5.6 Hz, 1H, NH_ε), 7.00 (d, $^3J_{HH}$ = 8.1 Hz, 1H, NH_α), 5.17 (s, 2H, CH_2), 3.75-3.86 (m, 1H, $CH_{2-\alpha}$), 3.01 (s, 3H, CH_3), 2.94-3.00 (m, 2H, $CH_{2-\varepsilon}$), 1.47-1.69 (m, 2H, $CH_{2-\beta}$), 1.36 (s, 9H, Boc), 1.22-1.54 (m, 4H, $CH_{2-\gamma,\delta}$).

^{13}C-NMR (75 MHz, DMSO-d_6): δ = 174.3, 167.3, 163.2, 156.1, 155.7, 138.8, 132.1, 131.6, 129.6, 126.8, 126.4, 78.0, 64.8, 53.6, 40.2, 30.6, 29.1, 28.3, 23.0, 21.1.

MS (ESI), *m/z* calcd for $C_{22}H_{30}N_6O_6$ 474.22, found 497.0 $[M+Na]^+$, 375.0 $[M-Boc+H]^+$, 473.1 $[M-H]^-$.

HRMS (ESI), *m/z* $[M+H]^+$ calcd for $C_{22}H_{30}N_6O_6$: 475.2305, found: 475.2298.

N^6-(((3-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)oxy)carbonyl)-*L*-lysine trifluoroacetate (mTetK, 2)



BocmTetK (6.8 g, 14.3 mmol, 1.0 eq) was dissolved in 60 mL TFA/DCM (1:1) with a drop of H_2O and stirred at room temperature for two hours. The solvent was evaporated under reduced pressure, the product dissolved in a minimal amount of MeOH and then precipitated from cold Et_2O and subsequently dried to obtain **2** (5.0 g, 75 %) as a pink solid. It was further purified by RP-HPLC (C18, 250 x 21.2 mm, 10 mL/min, 1-80 % ACN in 16 min).

^{1}H-NMR (300 MHz, DMSO-d_6 + TFA): δ = 8.42-8.46 (m, 1H, H_{Ar-2}) 8.39-8.41 (m, 1H, H_{Ar-5}), 8.22 (s, 3H, NH_α), 7.64-7.67 (m, 2H, $H_{Ar-4,6}$), 7.34 (t, $^3J_{HH}$ = 5.6 Hz, 1H, NH_ε), 5.17 (s, 2H, CH_2), 3.87-3.89 (m, 1H, $CH_{2-\alpha}$), 2.98-3.02 (m, 5H, CH_3, $CH_{2-\varepsilon}$), 1.72-1.79 (m, 2H, $CH_{2-\beta}$), 1.31-1.43 (m, 4H, $CH_{2-\gamma,\delta}$).

^{13}C-NMR (75 MHz, DMSO-d_6 + TFA): δ = 171.1, 167.3, 163.2, 156.1, 138.8, 132.1, 131.6, 129.6, 126.8, 126.4, 64.8, 52.0, 39.8, 29.7, 28.9, 21.7, 20.9.

Due to solubility problems of **2** in DMSO-d_6, 10% TFA was added before NMR measurements.

MS (ESI), *m/z* calcd for $C_{17}H_{22}N_6O_4$ 374.21, found 375.2 $[M+H]^+$, 373.1 $[M-H]^-$.
HRMS (ESI), *m/z* $[M+H]^+$ calcd for $C_{17}H_{22}N_6O_4$: 375.1781, found: 375.1774.

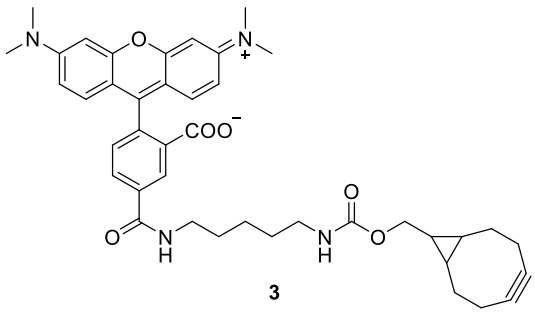
Synthesis of BCN-TAMRA (3)

Bicyclo[6.1.0]non-4-yn-9-ylmethyl (4-nitrophenyl) carbonate (BCN-carbonate)

Bicyclo[6.1.0]non-4-yn-9-ylmethanol (0.1 g, 0.65 mmol, 1.0 eq) was dissolved in 2 mL DCM, then 4-nitrophenylchloroformate (0.16 g, 0.8 mmol, 1.2 eq) and pyridine (54 µL, 0.65 mmol, 1.0 eq) was added and the reaction stirred at room temperature for 2 hours, before the solvent was removed under reduced pressure. The crude residue was purified by flash chromatography to yield the product (0.15 g, 70 %) as yellowish solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.33 – 8.25 (m, 2H), 7.45 – 7.35 (m, 2H), 4.41 (d, ^3J = 8.2 Hz, 2H), 2.42 – 2.18 (m, 6H), 1.70 – 1.57 (m, 2H), 1.49 (d, ^3J = 8.6 Hz, 1H), 1.12 – 1.01 (m, 2H).
The characterization is in agreement with the literature.[7]

5-((5-(((bicyclo[6.1.0]non-4-yn-9-ylmethoxy)carbonyl)amino)pentyl)carbamoyl)-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate (BCN-TAMRA, 3)



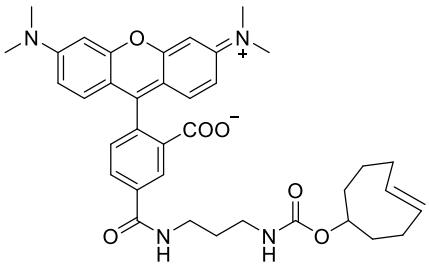
3

TAMRA-cadaverine (5 mg, 9.7 µmol, 1.0 eq) was dissolved in 2 mL DMF, followed by addition of DIPEA (10 µL, 58 µmol, 6.0 eq) and BCN-carbonate (9.2 mg, 29 µmol, 3.0 eq). The resulting reaction was stirred at room temperature overnight, then the solvent was evaporated under reduced pressure and the crude product purified by RP-HPLC (C18, 250 x 10 mm, 4 mL/min, 20-90 % ACN in 15 min) to obtain **3** (4.7 mg, 70 %) as purple solid, which was dissolved in DMSO to make a 2 mM stock solution, which was stored at -20 °C.

MS (ESI), *m/z* calcd for $C_{41}H_{46}N_4O_6$ 690.34, found 691.3 $[M+H]^+$.
HRMS (ESI), *m/z* $[M+H]^+$ calcd for $C_{41}H_{46}N_4O_6$: 691.3496, found: 691.3482.

Synthesis of TCO-TAMRA (4)

Synthesis of (*E*)-5-((3-(((cyclooct-3-en-1-yloxy)carbonyl)amino)propyl)-carbamoyl)-2-(6-(dimethyl-amino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate (TCO-TAMRA, 4)



4

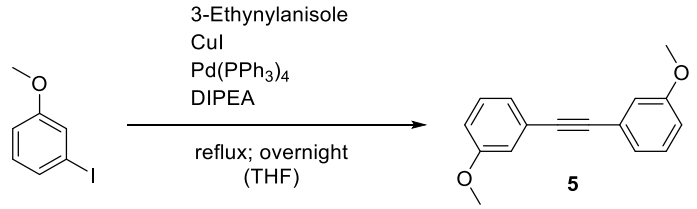
5,6-TAMRA-succinimidyl ester (3 mg, 5.7 µmol, 1.0 eq) was added to a solution of (*E*)-cyclooct-3-en-1-yl (3-aminopropyl)carbamate (3.9 mg, 17 µmol, 3.0 eq) and DIPEA (6 µL, 37 µmol, 6.0 eq) in 2 mL DMF. The resulting reaction was stirred at room temperature overnight, then the solvent was evaporated under reduced pressure and the crude product purified by RP-HPLC (C18, 250 x 10 mm, 4 mL/min, 20-90 % ACN in 15 min) to obtain **4** (1.9 mg, 52 %) as purple solid, which was dissolved in DMSO to make a 2 mM stock solution, which was stored at -20 °C.

MS (ESI), *m/z* calcd for $C_{37}H_{42}N_4O_6$ 638.31, found 639.3 $[M+H]^+$, 637.3 $[M-H]^-$.

HRMS (ESI), *m/z* $[M+H]^+$ calcd for $C_{37}H_{42}N_4O_6$: 639.3183, found: 639.3168.

Synthesis of photo DMBO compounds

Bis(3-methoxyphenyl)acetylene[2]



DIPEA (2.17 mL, 12.7 mmol, 3.0 eq.) was added to a mixture of 3-iodoanisole (509 µL, 4.23 mmol, 1.0 eq.), 3-ethynylanisole (672 µL, 5.08 mmol, 1.2 eq.), copper(I) iodide (81.0 mg; 423 µmol; 0.1 eq.) and tetrakis(triphenylphosphin)palladium(0) (244 mg, 212 µmol, 0.05 eq.) in 21 ml anhydrous THF and the resulting reaction mixture heated to reflux overnight. The reaction was cooled to room temperature and the solvent removed under reduced pressure. The crude product was purified by flash column chromatography (pentane:ethyl acetate = 99:1). The resulting product was further purified by recrystallization. Therefore it was dissolved in minimal amount of DCM, then a tenfold excess of pentane was added and the solution stored at 4 °C overnight. The crystals were washed with cold pentane, dried under reduced pressure and that yielded the acetylene **5** as colorless crystals in 71% yield (0.72 g, 3.02 mmol).

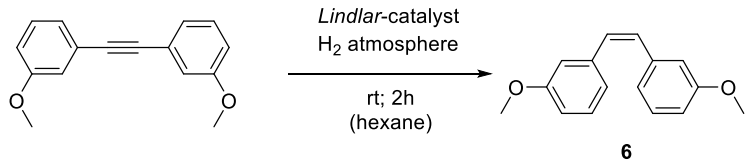
^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.29 – 7.23 (m, 2H), 7.13 (dt, ^3J = 7.6, ^4J = 1.2 Hz, 2H), 7.07 (dd, ^4J = 2.6, 1.4 Hz, 2H), 6.90 (ddd, ^3J = 8.3, ^4J = 2.6, 1.0 Hz, 2H), 3.83 (s, 6H).

MS(ESI), *m/z* $[M+H]^+$ calcd for $C_{16}H_{14}O_2$: 239.10; found: 239.1.

R_f= 0.42 (pentane : ethyl acetate = 98:2)

The characterization is in agreement with the literature.[2]

(Z)-3,3′-Dimethoxystilbene[2]



5 (816 mg, 3.42 mmol, 1.0 eq.) and *Lindlar*-catalyst (163 mg, 20% w/w of **5**) were stirred under H_2 atmosphere for 1.5 hours in 21 mL hexane at rt. After that LCMS showed complete conversion of the starting material, the reaction mixture was filtered and the solvent removed under reduced pressure. The crude product was purified by flash column chromatography (pentane:EtOAc= 98:2). This way **6** was isolated as a mixture with the corresponding alkane in 77% yield as a colorless oil (616 mg, 2.56 mmol). The product was used without further purification.

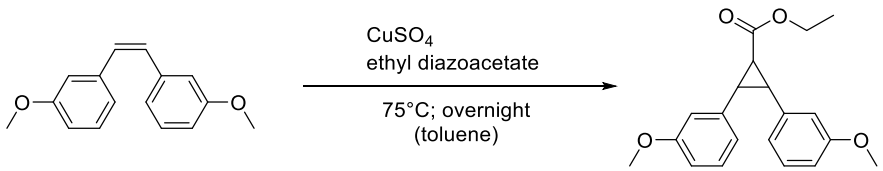
^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.20 – 7.09 (m, 2H), 6.88 – 6.72 (m, 6H), 6.58 (s, 2H), 3.67 (s, 6H).

MS(ESI), *m/z* $[M+H]^+$ calcd for $C_{16}H_{16}O_2$: 241.12; found: 241.1.

R_f= 0.46 (pentane : ethyl acetate = 98:2)

The characterization is in agreement with the literature.[2]

Ethyl 2,3-bis(3-methoxyphenyl)cyclopropane-1-carboxylate[8]



7

Ethyl diazoacetate (1.41 g, 10.5 mmol, 2.5 eq.) was added to a mixture of copper(II) sulfate (40.0 mg, 0.25 mmol, 0.06 eq.) and **6** (1.01 g, 4.2 mmol; 1.0 eq.) in 6.8 mL anhydrous toluene over 4 hours at 75 °C and then further stirred overnight at that temperature. The reaction mixture was filtered and the solvent removed under reduced pressure. The crude product was purified by flash column chromatography (pentane:ethyl acetate = 98:2 → 92:8). This way the unreacted starting material was recovered (roughly 66%) and **7** was isolated as a mixture with diethyl maleate as a yellow oil in 18% yield (0.23 g, 0.71 mmol). The product was used without further purification.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.12 – 7.00 (m, 2H), 6.70 – 6.56 (m, 4H), 6.47 (dd, ^4J = 2.5, 1.6 Hz, 2H), 4.24 (q, ^3J = 7.1 Hz, 2H), 3.63 (s, 6H), 3.02 (d, ^3J = 5.3 Hz, 2H), 2.51 (t, ^3J = 5.3 Hz, 1H), 1.33 (t, ^3J = 7.1 Hz, 3H).

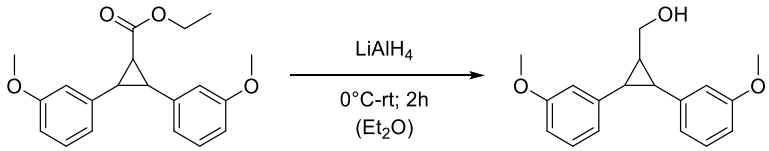
^{13}C NMR (101 MHz, $CDCl_3$) δ = 173.35, 159.38, 137.39, 129.07, 121.54, 114.40, 112.46, 61.14, 55.20, 33.38, 27.70, 14.44.

MS(ESI), *m/z* calcd for $C_{20}H_{22}O_4$: 326.15; found: 327.2 $[M+H]^+$, 281.2 $[M\text{-}OEt]^+$.

HRMS(ESI), *m/z* $[M+H]^+$ calcd for $C_{20}H_{22}O_4$: 327.1596; found: 327.1591.

R_f= 0.57 (pentane : ethyl acetate = 9:1)

(2,3-bis(3-methoxyphenyl)cyclopropyl)methanol[8]



$LiAlH_4$ (1 M in Et_2O, 1.45 ml, 1.45 mmol, 2.0 eq.) was added dropwise to a solution of Ethyl 2,3-bis(3-methoxyphenyl)cyclopropane-1-carboxylate (237 mg, 726 µmol, 1.0 eq.) in 6.9 mL anhydrous diethyl ether at 0 °C. The reaction was stirred at room temperature for 2 hours until TLC showed full conversion of starting material. The reaction was quenched by addition of 3 mL isopropanol and 5 mL water. The resulting suspension was filtered over celite, the organic phase washed with 5 mL brine, dried over Na_2SO_4 and the solvent removed under reduced pressure. The crude product was purified by flash column chromatography (DCM:MeOH = 99:1) and the product isolated as a yellow oil in 68 % yield (140 mg, 494 µmol).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.07 – 6.99 (m, 2H), 6.65 – 6.57 (m, 4H), 6.48 – 6.45 (m, 2H), 3.84 (d, ^3J = 6.5 Hz, 2H), 3.62 (s, 6H), 2.39 (d, ^3J = 5.7 Hz, 2H), 2.11 – 2.01 (m, 1H).

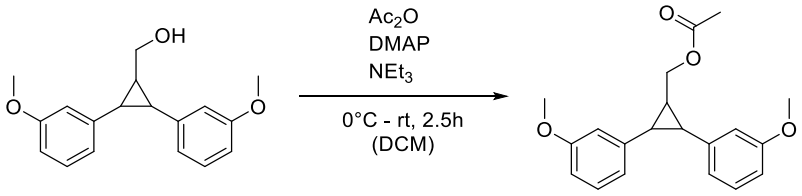
^{13}C NMR (75 MHz, $CDCl_3$) δ = 159.3, 139.2, 128.9, 121.6, 114.5, 111.9, 66.3, 55.2, 29.8, 28.3.

MS(ESI), *m/z* calcd for $C_{18}H_{20}O_3$: 284.14; found: 285.2 $[M+H]^+$, 307.1 $[M+Na]^+$, 267.1 $[M\text{-}OH]^+$.

HRMS(ESI), *m/z* $[M+H]^+$ calcd for $C_{18}H_{20}O_3$: 285.1491; found: 285.1485.

R_f= 0.47 (DCM : MeOH = 98:2)

(2,3-bis(3-methoxyphenyl)cyclopropyl)methyl acetate[9]



8

Acetic anhydride (68.0 µL, 711 µmol, 2.6 eq.) was added to a solution of (2,3-bis(3-methoxyphenyl)cyclopropyl)methanol (79.0 mg, 0.277 mmol, 1.0 eq.), DMAP (2.0 mg, 14.0 mmol, 0.05 eq.) and triethylamine (189 µL, 1.40 mmol, 4.9 eq.) in 2 mL anhydrous DCM at 0 °C. The reaction was stirred for 2 hours at room temperature until TLC showed full conversion of starting material. The organic phase was washed with 2 mL 1 M HCl and 2 mL brine, dried over Na_2SO_4 and the solvent removed under reduced pressure. The crude product was purified by flash column chromatography (pentane:ethyl acetate = 95:5 → 90:10) and **8** isolated as yellow oil in 70% yield (58.0 mg, 178 µmol).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.08 – 7.00 (m, 2H), 6.63 – 652 (m, 4H), 6.47 – 6.43 (m, 2H), 4.26 (d, ^3J = 7.0 Hz, 2H), 3.62 (s, 6H), 2.43 (d, ^3J = 5.6 Hz, 2H), 2.13 – 2.07 (m, 4H).

^{13}C NMR (75 MHz, $CDCl_3$) δ = 171.4, 159.3, 138.9, 128.9, 121.6, 114.5, 111.9, 67.8, 55.2, 30.2, 24.6, 21.2.

MS(ESI), *m/z* calcd for $C_{20}H_{22}O_4$: 326.15; found: 349.1 $[M+Na]^+$, 267.1 $[M-OAc]^+$.

HRMS(ESI), *m/z* $[M+Na]^+$ calcd for $C_{20}H_{22}O_4$: 349.1416; found: 349.1410.

R_f= 0.28 (pentane : ethyl acetate = 9:1)

(3,9-dimethoxy-6-oxo-1,1a,6,10b-tetrahydrodibenzo[a,e]dicyclopropa[c,g][8]annulen-1-yl)methyl acetate (photo-9)[2]

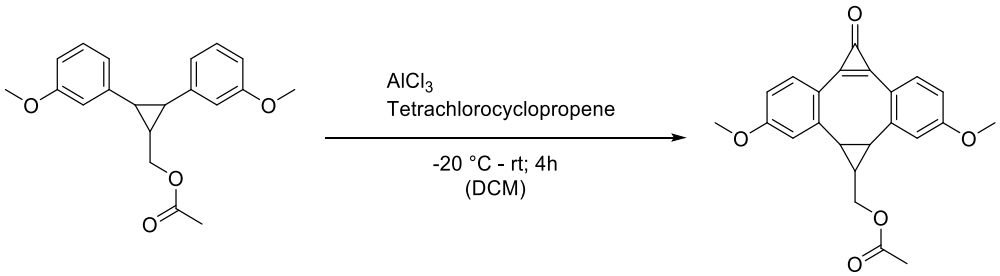


photo-9

In a flame dried flask $AlCl_3$ (147 mg, 1.10 mmol, 3.0 eq.) and tetrachlorocyclopropene (54.0 mL, 441 µmol, 1.2 eq.) were stirred in 5.5 mL anhydrous DCM for 20 minutes at room temperature. The reaction mixture was cooled to -20 °C and a solution of **8** (120 mg, 368 µmol, 1.0 eq.) in 1.8 mL DCM was added dropwise. The reaction was stirred for 1 hour at -20 °C, then warmed to room temperature over 2 hours and the reaction stirred for an additional hour at room temperature. The reaction mixture was quenched with 5 mL water and stirred for 30 minutes. The aqueous phase was extracted with DCM (5 × 5 mL), the combined organic phases washed with 20 ml brine, dried over Na_2SO_4 and the

solvent removed under reduced pressure. The crude product was purified by flash column chromatography (DCM:MeOH = 98:2) and **photo-9** isolated as yellow oil in 55% yield (76.0 mg, 202 µmol).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.70 (d, ^{3}J = 8.5 Hz, 2H), 7.12 (d, ^{4}J = 2.5 Hz, 2H), 6.87 (t, ^{3}J = 8.5, ^{4}J = 2.5 Hz, 2H), 4.38 (d, ^{3}J = 6.9 Hz, 2H), 3.86 (s, 6H), 2.38 (d, ^{3}J = 6.9 Hz, 2H), 2.17 (s, 3H), 2.01 (p, ^{3}J = 6.9 Hz, 1H).

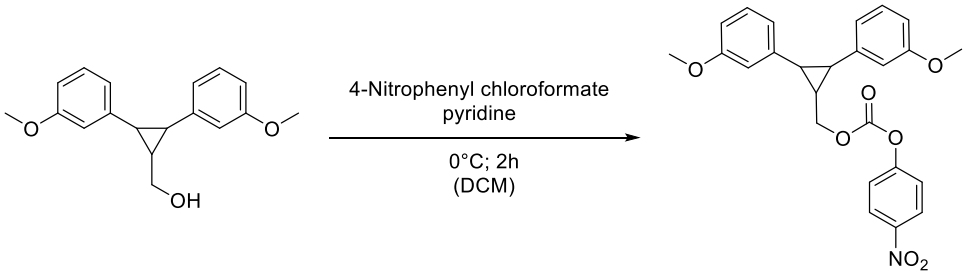
^{13}C NMR (75 MHz, $CDCl_3$) δ = 171.1, 161.7, 153.1, 147.3, 142.3, 133.3, 118.6, 117.6, 112.8, 68.4, 55.6, 31.4, 28.3, 21.2.

MS(ESI), *m/z* calcd for $C_{23}H_{20}O_5$: 376.13; found: 377.1 $[M+H]^+$, 753.2 $[2M+H]^+$.

HRMS(ESI), *m/z* $[M+H]^+$ calcd for $C_{23}H_{20}O_5$: 377.1389; found: 377.1384.

R_f= 0.38 (DCM : MeOH = 98:2)

(2,3-bis(3-methoxyphenyl)cyclopropyl)methyl (4-nitrophenyl) carbonate



(2,3-bis(3-methoxyphenyl)cyclopropyl)methanol (100 mg, 352 µmol, 1.0 eq.) and pyridine (100 µL, 1.23 mmol, 3.5 eq.) were dissolved in 4 mL DCM and 4-nitrophenyl chloroformate (106 mg, 528 µmol, 1.5 eq.) was added at 0 °C in one portion. The reaction was slowly warmed to room temperature and stirred for 2 hours. The solvent was removed under reduced pressure and the crude product purified by column chromatography (pentane:EtOAc = 90:10 → 85:15) to yield the product as a yellow solid in 66% yield (91.3 mg, 203 µmol).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.33 – 8.24 (m, 2H), 7.43 – 7.35 (m, 2H), 7.06 (t, ^{3}J = 7.9 Hz, 2H), 6.65 (ddd, $^{3,4}J$ = 8.2, 2.6, 0.9 Hz, 2H), 6.59 (dt, $^{3,4}J$ = 7.6, 1.6 Hz, 2H), 6.47 (dd, ^{4}J = 2.6, 1.6 Hz, 2H), 4.48 (d, ^{3}J = 7.2 Hz, 2H), 3.63 (s, 6H), 2.53 (d, ^{3}J = 5.6 Hz, 2H), 2.27 – 2.15 (m, 1H).

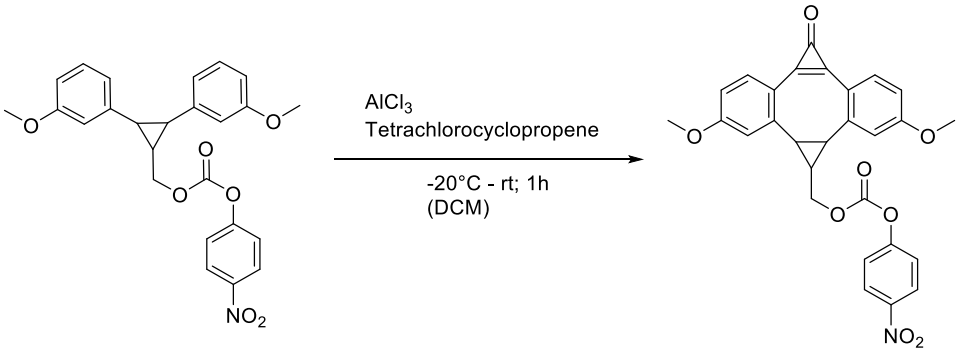
^{13}C NMR (75 MHz, $CDCl_3$) δ = 159.4, 155.7, 152.8, 145.6, 138.2, 129.1, 125.5, 122.0, 121.6, 114.6, 112.0, 72.8, 55.2, 30.2, 24.3.

MS(ESI), *m/z* calcd for $C_{25}H_{23}NO_7$: 449.15; found: 472.1 $[M+Na]^+$.

HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{25}H_{23}NO_7$: 450.1553; found: 450.1546.

R_f= 0.31 (pentane : ethyl acetate = 85:15)

(3,9-dimethoxy-6-oxo-1,1a,6,10b-tetrahydrodibenzo[a,e]dicyclopropa[c,g][8]annulen-1-yl)methyl (4-nitrophenyl) carbonate (p-Nitrophenyl photo DMBO)



In a flame dried flask $AlCl_3$ (42.7 mg, 320 µmol, 2.0 eq.) and tetrachlorocyclopropene (23.6 µL, 192 µmol, 1.2 eq.) were stirred in 3 mL anhydrous DCM for 20 minutes at room temperature. The reaction mixture was cooled to –20 °C and a solution of **7** (72.0 mg, 160 µmol, 1.0 eq.) in 1 mL DCM was added dropwise. The reaction mixture turned dark green and was stirred for 1 hour at –20 °C until TLC showed full conversion. The reaction mixture was then filtered through a small silica column and washed with ethyl acetate until all product was flushed through (R_f= 0,35 see below). The solvent was removed under reduced pressure and the crude product purified by column chromatography (DCM:EtOAC = 100:0 → 9:1 → 8:2 → 7:3) to isolate the product as a yellow solid in 22% yield (17.5 mg, 35 µmol).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.35 – 8.26 (m, 2H), 7.73 (d, ^3J = 8.5 Hz, 2H), 7.46 – 7.35 (m, 2H), 7.14 (d, ^4J = 2.5 Hz, 2H), 6.89 (dd, $^{3,4}J$ = 8.5, 2.6 Hz, 2H), 4.58 (d, ^3J = 7.1 Hz, 2H), 3.87 (s, 6H), 2.49 (d, ^3J = 6.9 Hz, 2H), 2.17 (p, ^3J = 7.0 Hz, 1H).

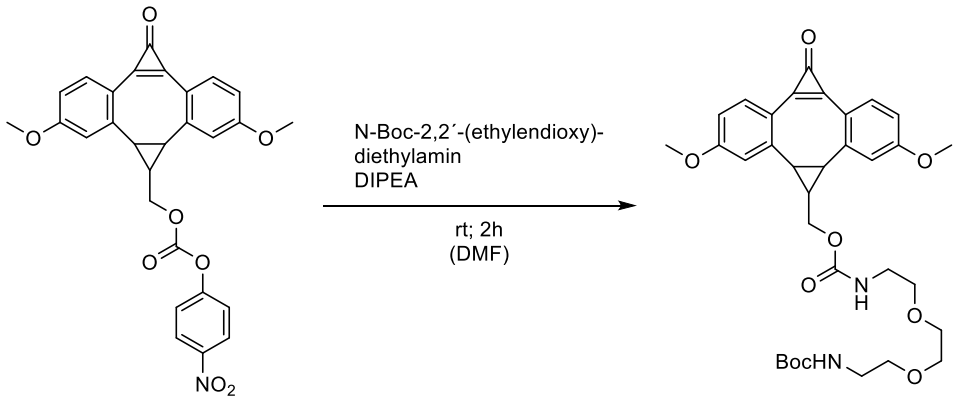
^{13}C NMR (75 MHz, $CDCl_3$) δ = 161.6, 155.6, 152.9, 152.8, 147.7, 145.7, 141.5, 133.4, 125.6, 122.0, 118.9, 117.8, 112.6, 73.4, 55.7, 31.7, 27.6.

MS(ESI) calcd for $C_{28}H_{21}NO_8$: 499.13; found: 500.1 $[M+H]^+$.

HRMS(ESI) $[M+H]^+$ calcd for $C_{28}H_{21}NO_8$: 500.1345; found: 500.1337.

R_f= 0.35 (DCM : ethyl acetate = 2:1)

Boc photo-DMBO amine (Boc-photo-11)



DIPEA (26 µl, 150 µmol, 2.5 eq.) and *N*-Boc- 2,2'-(ethylenedioxy)diethylamine (9.6 µl, 63.1 µmol, 1.05 eq.) were added to a solution of p-Nitrophenyl DMBO (30.0 mg, 60.1 µmol, 1.0 eq.) in 1 mL DMF. The reaction was stirred at room temperature for 2 hours when TLC showed full conversion of the starting material. The solvent was removed under reduced pressure and the crude product purified by HPLC (C18, 250 x 10 mm, 4 mL/min, 20-90 % ACN in 10 min) and lyophilized, which resulted the product as a colorless solid in 49 % yield (18.0 mg, 29.6 µmol).

^{1}H NMR (300 MHz, Chloroform-*d*) δ = 7.66 (d, ^{3}J = 8.5 Hz, 2H), 7.11 (d, ^{4}J = 2.6 Hz, 2H), 6.85 (dd, $^{3,4}J$ = 8.5, 2.6 Hz, 2H), 5.46 (br s, 1H), 5.03 (br s, 1H), 4.37 (d, ^{3}J = 6.6 Hz, 2H), 3.85 (s, 6H), 3.65 – 3.51 (m, 8H), 3.43 (q, ^{3}J = 5.3 Hz, 2H), 3.32 (q, ^{3}J = 5.1 Hz, 2H), 2.37 (d, ^{3}J = 6.9 Hz, 2H), 2.04 (p, ^{3}J = 6.7 Hz 1H), 1.42 (s, 9H).

^{13}C NMR (75 MHz, $CDCl_3$) δ = 161.5, 156.7, 156.1, 153.1, 147.6, 142.4, 133.1, 118.7, 117.8, 112.6, 77.4, 70.5, 70.4, 70.4, 70.3, 69.1, 55.6, 41.1, 40.5, 31.4, 28.7, 28.5.

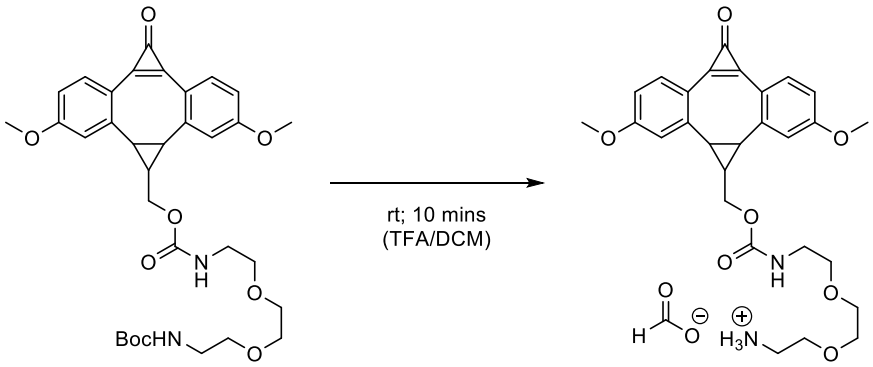
MS(ESI), *m/z* calcd for $C_{33}H_{40}N_2O_9$: 608.27; found: 609.3 $[M+H]^+$, 509.3 $[M-Boc+H]^+$.

HRMS(ESI), *m/z* $[M+H]^+$ calcd for $C_{33}H_{40}N_2O_9$: 609.2812; found: 609.2805.

R_f= 0.33 (DCM : MeOH = 96:4).

Boc-photo-**11** was stored at -20 °C and freshly deprotected with TFA when the following compounds were synthesized.

photo-DMBO amine trifluoroacetate (photo-11)



Boc-protected **photo-11** was dissolved in 2 mL DCM and 0.2 mL trifluoroacetic acid was added at room temperature. The reaction was stirred for 10 minutes and the solvent removed under reduced pressure. The crude product was purified by HPLC (C18, 250 x 10 mm, 4 mL/min, 20-70 % ACN in 10 min) and lyophilized, which resulted the product as the formic acid salt as a light yellow solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.55 (d, ^{3}J = 8.5 Hz, 2H), 7.37 (t, ^{3}J = 5.7 Hz, 1H), 7.23 (d, ^{4}J = 2.5 Hz, 2H), 6.98 (dd, $^{3,4}J$ = 8.5, 2.6 Hz, 2H), 4.25 (d, ^{2}J = 6.7 Hz, 2H), 3.84 (s, 6H), 3.56 – 3.49 (m, 6H), 3.42 (t, ^{3}J = 6.0 Hz, 2H), 3.16 (q, ^{3}J = 5.7 Hz, 2H), 2.88 (t, ^{3}J = 5.2 Hz, 2H), 2.46 (d, ^{3}J = 7.0 Hz, 2H), 2.08 (p, ^{3}J = 6.6 Hz, 1H).

^{13}C NMR (101 MHz, DMSO) δ = 161.1, 156.5, 151.2, 146.9, 142.8, 132.6, 118.5, 116.5, 113.0, 69.6, 69.4, 69.2, 67.6, 67.6, 55.6, 40.2, 38.8, 31.2, 28.7.

MS(ESI), *m/z* $[M+H]^+$ calcd for $C_{28}H_{32}N_2O_7$: 509.23; found: 509.2.

HRMS(ESI), *m/z* $[M+H]^+$ calcd for $C_{28}H_{32}N_2O_7$: 508.2288; found: 509.2280.

photo-DMBO-Cy5 (photo-14)

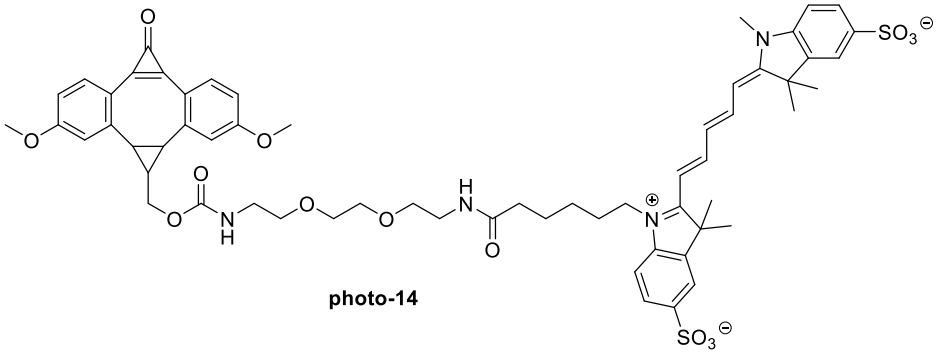


photo-14

DIPEA (2.2 µl, 12.9 µmol, 4.0 eq.) and sulfo-Cy5 NHS ester (2.5 mg, 3.2 µmol, 1.0 eq.) in 0.1 mL DMF were added to a solution of **photo-11** (3.0 mg, 4.8 µmol, 1.5 eq.) in 0.5 mL DMF. The resulting reaction mixture was stirred at room temperature for 1.5 hours until analysis via LC-MS showed full consumption of the NHS-fluorophore. The solvent was removed under reduced pressure and the crude product purified by HPLC (C18, 250 x 10 mm, 4 mL/min, 20-70 % ACN in 25 min) and lyophilized, which resulted the product as a blue solid in 69% yield (2.5 mg, 2.2 µmol).

HRMS(ESI), *m/z* $[M]^-$ calcd for $C_{60}H_{67}N_4O_{14}S_2$: 1131.4095; found: 1131.4078.

photo-DMBO-TAMRA (photo-15)

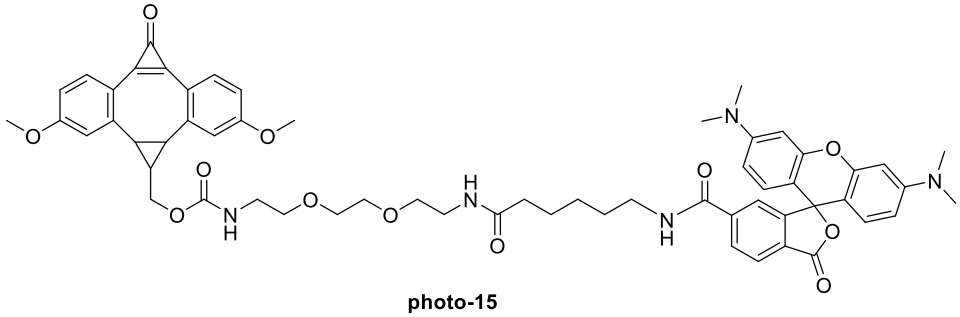


photo-15

To a solution of **photo-11** (2.3 mg, 3.7 µmol, 1.5 eq.) in 0.5 mL DMF was added DIPEA (3.2 µL, 19 µmol, 5.0 eq.) and 5(6)-TAMRA-X NHS ester (1.6 mg, 2.5 µmol, 1.0 eq.) in 0.1 mL DMF. The reaction was stirred at room temperature overnight. The solvent was removed under reduced pressure and the crude product purified by HPLC (C18, 250 x 10 mm, 4 mL/min, 30-70 % ACN in 15 min) and lyophilized, which yielded the product as purple solid in 24% yield (0.6 mg, 0.58 µmol).

HRMS(ESI), *m/z* $[M+H]^+$ calcd for $C_{59}H_{63}N_5O_{12}$: 1034.4551; found: 1034.4547.

photo-DMBO-BCN (photo-16)

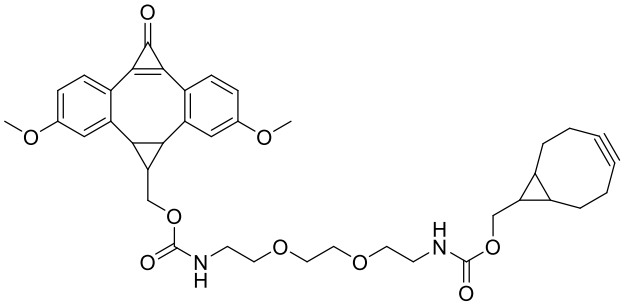


photo-16

To a solution of **photo-11** (4.8 mg, 9.4 µmol, 1.10 eq.) in 0.5 mL DMF DIPEA (4.5 µL, 25.7 µmol, 3.00 eq.) and BCN-NHS ester (2.5 mg, 8.6 mg, 1.00 eq.) were added and the reaction stirred for 3 hours at room temperature. The solvent was removed under reduced pressure, the crude product purified by HPLC (C18, 250 x 10 mm, 4 mL/min, 20-90 % ACN in 10 min) and lyophilized, and the product was isolated as colourless solid in 34% yield (2.0 mg, 2.92 µmol).

^{1}H NMR (400 MHz, Chloroform-*d*) δ = 7.67 (d, ^{3}J = 8.5 Hz, 2H), 7.12 (d, ^{4}J = 2.3 Hz, 2H), 6.85 (dd, $^{3,4}J$ = 8.5, 2.5 Hz, 2H), 5.45 (s, 1H), 5.24 (s, 1H), 4.37 (d, ^{3}J = 6.6 Hz, 2H), 4.13 (d, ^{3}J = 8.0 Hz, 2H), 3.86 (s, 6H), 3.66 – 3.55 (m, 8H), 3.44 (q, ^{3}J = 5.2 Hz, 2H), 3.38 (q, ^{3}J = 5.3 Hz, 2H), 2.38 (d, ^{3}J = 7.0 Hz, 2H), 2.31 – 2.15 (m, 6H), 2.09 – 2.00 (m, 1H), 1.57 – 1.49 (m, 2H), 1.38 – 1.28 (m, 1H), 0.96 – 0.84 (m, 2H).

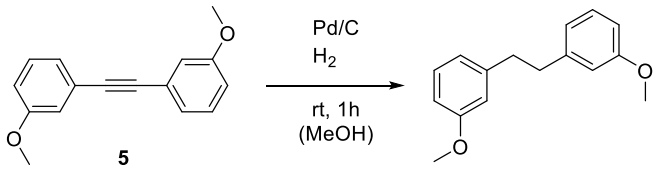
^{13}C NMR (126 MHz, $CDCl_3$) δ = 161.5, 156.9, 156.6, 153.1, 147.6, 142.4, 133.2, 118.8, 117.7, 112.5, 98.9, 70.5, 70.4, 70.4, 70.3, 68.7, 62.9, 55.6, 41.1, 40.9, 31.4, 29.1, 28.6, 21.6, 20.2, 17.9.

MS(ESI), *m/z* $[M+H]^+$ for $C_{39}H_{44}N_2O_9$: 685.31; found: 685.3.

HRMS(ESI), *m/z* $[M+H]^+$ for $C_{39}H_{44}N_2O_9$: 685.3125; found: 685.3117.

Synthesis of photo-12

1,2-bis(3-methoxyphenyl)ethane



1,2-bis(3-methoxyphenyl)ethyne (266 mg, 1.12 mmol, 1.0 eq.) and palladium on charcoal (53.2 mg, 20 wt%) were added to 15 mL methanol. The reaction was then stirred for 1.5 hours under H_2-atmosphere. The reaction mixture was filtered and the solvent removed under reduced pressure. The crude product was purified by column chromatography (pentane:EtOAc = 98:2) and the product isolated as colorless oil in 82% yield (219 mg, 904 µmol).

^{1}H NMR (300 MHz, Chloroform-*d*) δ = 7.20 (td, ^3J = 7.5, ^4J = 1.2 Hz, 2H), 6.82 – 6.72 (m, 6H), 3.79 (s, 6H), 2.90 (s, 4H).

MS(ESI), *m/z* calculated for $C_{16}H_{18}O_2$: 242.13; found: 243.1 $[M+H]^+$.

The characterization is in agreement with the literature.[10]

4,9-dimethoxy-6,7-dihydro-1H-dibenzo[a,e]cyclopropa[c][8]annulen-1-one[2]

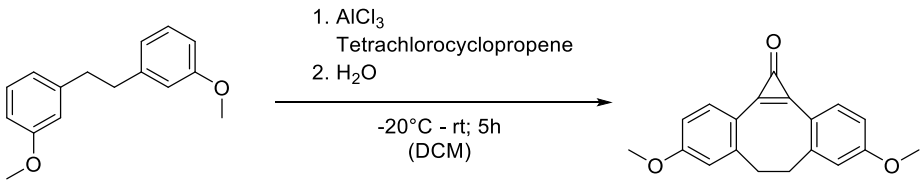


photo-12

In a flame dried flask $AlCl_3$ (361 mg, 2.71 mmol, 3.0 eq.) and tetrachlorocyclopropene (133 µL, 1.08 mmol, 1.2 eq.) were stirred in 5.5 mL anhydrous DCM for 20 minutes at room temperature. The reaction mixture was cooled to –20 °C and a solution of 1,2-bis(3-methoxyphenyl)ethane (219 mg, 904 µmol, 1.0 eq.) in 1.8 mL DCM was added dropwise. The reaction was stirred for 1 hour at –20 °C, then warmed to room temperature over 2 h and stirred for an additional hour at room temperature. The reaction was quenched with 7 mL water and stirred for 30 minutes. The aqueous phase was extracted with DCM (4 × 5 mL), the combined organic phases washed with 20 ml brine, dried over Na_2SO_4 and the solvent removed under reduced pressure. The crude product was purified by flash column chromatography (DCM:MeOH = 98:2) and **photo-12** isolated as a yellow solid in 26% yield (68.0 mg, 233 µmol).

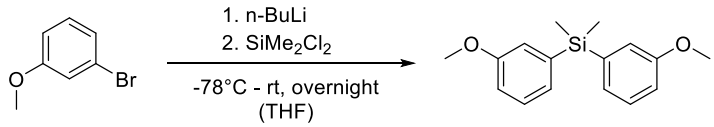
^{1}H NMR (500 MHz, Chloroform-*d*) δ = 8.07 (d, ^3J = 8.3 Hz, 2H), 6.99 – 6.86 (m, 4H), 3.90 (s, 6H), 3.37 (d, ^3J = 10.7 Hz, 2H), 2.66 (d, ^3J = 10.7 Hz, 2H).

MS(ESI), *m/z* calculated for $C_{19}H_{16}O_3$: 292,11; found: 293.1 $[M+H]^+$

The characterization is in agreement with the literature.[11]

Synthesis of photo-13

Bis(3-methoxyphenyl)dimethylsilane[3]



A solution of 3-bromoanisole (3.00 g, 16.0 mmol, 1.96 eq.) in tetrahydrofuran (60 ml) was cooled to −78 °C and n-BuLi (2.5 M in n-hexane, 6.6 mL, 16.5 mmol, 2.02 eq.) was added dropwise over a 15 minutes period. The resulting mixture was stirred at −78 °C for 90 minutes. Dichlorodimethylsilane (1.06 g, 8.18 mmol, 1.0 eq.) was then added dropwise, the mixture warmed to room temperature and stirred overnight. A saturated solution of NH_4Cl (1 mL) and water (5 mL) were added, the mixture was extracted with diethyl ether (3 × 6 mL) and the organic layer was dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the crude product was purified by column chromatography (pentane:EtOAc = 98:2) to isolate the product as colorless oil in 55% yield (1.21 g, 4.44 mmol).

^{1}H NMR (500 MHz, Chloroform-*d*) δ = 7.29 (t, ^3J = 7.7 Hz, 2H), 7.10 (d, ^3J = 7.2 Hz, 2H), 7.06 (d, ^4J = 2.5 Hz, 2H), 6.90 (dd, $^{3,4}J$ = 8.2, 2.3 Hz, 2H), 3.79 (s, 6H), 0.54 (s, 6H).

MS(ESI), *m/z* calculated for $C_{16}H_{20}O_2Si$: 272,12; found: 273.1 $[M+H]^+$.

The characterization is in agreement with the literature.[3]

4,8-dimethoxy-6,6-dimethyldibenzo[b,f]cyclopropa[d]silepin-1(6H)-one[3]

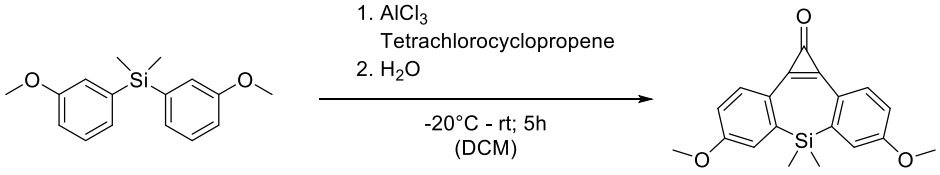


photo-13

In a flame dried flask $AlCl_3$ (367 mg, 2.75 mmol, 2.5 eq.) and tetrachlorocyclopropene (162 µL, 1.32 mmol, 1.20 eq.) were stirred in 5.5 mL anhydrous DCM for 20 minutes at room temperature. The reaction mixture was cooled to –20 °C and a solution of bis(3-methoxyphenyl)dimethylsilane (300 mg, 1.10 mmol, 1.00 eq.) in 1.8 mL DCM was added dropwise. The reaction was warmed to room temperature and stirred for 4 hours. The reaction was quenched by addition of 10 g ice, the organic phase was separated and the aqueous phase extracted with DCM (3 × 6 mL). The combined organic phases were washed with 15 mL brine, dried over Na_2SO_4 and the solvent removed under reduced pressure. The crude product was purified by flash column chromatography (DCM:EtOAc = 1:1) and **photo-13** isolated as brown solid in 12% yield (41.5 mg, 128 µmol).

^{1}H NMR (300 MHz, Chloroform-*d*) δ = 7.90 (d, ^3J = 8.5 Hz, 2H), 7.25 (d, ^4J = 2.6 Hz, 2H), 7.04 (dd, $^{3,4}J$ = 8.5, 2.6 Hz, 2H), 3.91 (s, 6H), 0.49 (s, 6H).

MS(ESI), *m/z* calculated for $C_{19}H_{18}O_3Si$: 322,10; found: 323.1 $[M+H]^+$.

The characterization is in agreement with the literature.[3]

BIOCHEMICAL METHODS

Cloning of expression plasmids

pPylT_sfGFP-N40TAG-His6 was derived from pPylT_sfGFP-N150TAG-His6 by two sequential steps using the Q5™ Site-Directed Mutagenesis Kit (New England Biolabs) to first introduce TAG150N to yield sfGFP wt, followed by mutagenesis of N40 to an amber stop codon. pPylT_OmpC-232TAG-His6[12] was constructed from pPylT_sfGFP-N150TAG-His6 by introduction of the OmpC gene instead of sfGFP. Therefore, the OmpC gene was PCR amplified from *E. coli* genome with primer pair P1 and cloned into linearized pPylT backbone (using PCR with primer pair P2) using Gibson Assembly (NEBuilder® HiFi DNA AssemblyMaster Mix, New England Biolabs). Amber stop codon at position Y232 was introduced into OmpC-His6 (pPylT_OmpC-His6) Q5™ Site-Directed Mutagenesis Kit (New England Biolabs). Primer sequences can be found in Supplementary Tables S2 and S3.

Protein expression and purification

To express proteins with incorporated unnatural amino acids (uAA) TetK (**1**), mTetK (**2**) and BCNK, *E. coli* DH10B cells were co-transformed with pBK_TetRS and pPylT_POI-His6. Cells were rescued in 1 ml of SOC media for 1 h at 37 ºC, before incubation (16 h, 37 °C, 200 rpm) in 50 ml of 2xYT containing ampicillin (100 µg/mL) and tetracycline (17 µg/mL). The overnight culture was diluted in 50 mL fresh 2xYT supplemented with ampicillin (100 µg/mL) and tetracycline (17 µg/mL) to an OD_{600} = 0.05 and incubated at 37 ºC. At OD_{600} = 0.3 to 0.4, a solution of uAA (100 mM stock in H_2O or DMSO with 200 mM TFA (**1** and **2**) or 50 mM in DMSO/100 mM NaOH (BCNK)) was added to a final concentration of 0.5-2 mM. Protein expression was induced at OD_{600} = 0.6 to 0.7 by the addition of arabinose to a final concentration of 0.02 %. After overnight incubation at 37 °C cells were harvested by centrifugation, flash frozen and stored at -80 °C until required. For protein purification, cell pellets were thawed on ice and resuspended in lysis buffer (20 mM Tris pH 8.0, 30 mM imidazole pH 8.0, 300 mM NaCl, 0.175 mg/mL PMSF, 0.1 mg/mL DNase I and one cOmplete™ protease inhibitor tablet (Roche)). The cell suspension was incubated on ice for 30 min and sonicated with cooling in an ice-water bath. The lysed cells were centrifuged (20 min, 14.000 × g, 4 °C). The resulting supernatant was added to Ni^{2+}-NTA beads (Jena Bioscience) and the mixture was incubated with agitation for 1 h at 4 ºC. After incubation, beads were transferred to a gravity flow column and washed with 10 CV wash buffer (20 mM Tris pH 8.0, 30 mM imidazole pH 8.0 and 300 mM NaCl). The protein was eluted with wash buffer supplemented with 300 mM imidazole pH 8.0. Fractions containing the protein were pooled, concentrated and rebuffered in wash buffer without imidazole (20 mM Tris pH 8.0 and 300 mM NaCl) with an Amicon Ultra-0.5 10 kDa MWCO centrifugal filter device (Millipore). Purified proteins were analyzed by 15 % SDS-PAGE and their mass confirmed by mass spectrometry (see Supplementary Information). For the incorporation of BocK *E. coli* cells were co-transformed with pBK_wtRS instead of pBK_TetRS. Yields of purified sfGFP variants were up to 80 mg/L.

Labeling of proteins *in vitro* for MS analysis

His6-tag purified POI_uAA was diluted with MQ H_2O or PBS to a final concentration of 10 µM in a MS Vial with 0.1 mL micro insert. Small molecules probes were added in a 10 to 100-fold excess and 1-2 µL protein samples analyzed via LC-MS (C4, 5-55 % B in 5 min) at certain time points (e.g. 30 min, 1, 2, 5 and 24 h).

Labeling of proteins *in vitro* with 3 or 14 for SDS PAGE fluorescence imaging

Purified sfGFP-N150mTetK was diluted in MQ water to a final concentration of 10 µM in a volume of 100 µL in a 1.5 mL Eppendorf tube. **3** or **14** (final concentration 100 µM) were added in 10-fold excess and the reaction followed over time. Therefore, 8 µL samples were taken at the following time points: 0, 30 s, 1, 2, 5, 10, 30, 60 and 120 min and the fluorophore quenched with 4 µL dipyridyl-1,2,4,5-tetrazine (50 mM). 3 µL 4x SDS loading buffer were added, the samples cooked at 95 °C for 10 min and the whole samples loaded onto an SDS-PAGE. After separation labeling was visualized by fluorescence using an ImageQuant LAS 4000 (**3**: ex. 520 nm, 575DF20 Cy3 filter; **14**: ex. 630 nm, R670 Cy5 filter), followed by staining with Quick Coomassie Stain (Serva) and destaining in deionized water.

Lysate labeling with 3 and 4

5 mL of *E. coli* culture expressing POI (sfGFP-NXTAG-His6, X = 40 or 150; Myo-S4TAG-His6 or Ub-K6TAG-His6) without uAA, with BocK, BCNK, TetK or mTetK (**2**) were harvested, washed 3x 10 % (v/v) DMSO/PBS and then sonicated in 5 mL lysis buffer (20 mM Tris pH 8.0, 30 mM imidazole pH 8.0, 300 mM NaCl, 0.175 mg/mL PMSF, 0.1 mg/mL DNase I and one cOmplete™ protease inhibitor tablet (Roche)). The lysed cells were centrifuged (20 min, 14.000 × g, 4 °C) and the cleared lysate was flash frozen and stored at -80 °C for labeling experiments.

Cleared *E.coli* lysate (adjusted to the same OD_{600}) was thawed on ice and 50 µL taken for labeling experiments. **3** or **4** were added to a final concentration of 2-4 µM and the reaction performed at 37 °C for 1 h at 350 rpm or samples were taken at different time points for time-dependent labeling: 15 min, 30 min, 45 min and 60 min. 16 µL 4x SDS loading buffer were added and the samples heated at 95 °C for 10 min. 14 µL samples were analyzed by a 15 % SDS PAGE. Labeling was visualized by excitation of TAMRA in-gel fluorescence at 520 nm utilizing an ImageQuant LAS 4000 with a 575DF20 Cy3 filter, followed by staining with Quick Coomassie Stain and destaining in deionized water.

sfGFP-N40 lysate labeling with 14 or 15

5 mL of *E. coli* culture expressing sfGFP-N40TAG without uAA, with BocK, BCNK or mTetK were harvested, washed 3x 10 % (v/v) DMSO/PBS and then sonicated in 5 mL lysis buffer (20 mM Tris pH 8.0, 30 mM imidazole pH 8.0, 300 mM NaCl, 0.175 mg/mL PMSF, 0.1 mg/mL DNase I and one cOmplete™ protease inhibitor tablet (Roche)). The lysed cells were centrifuged (20 min, 14.000 × g, 4 °C) and the cleared lysate was flash frozen and stored at - 80 °C for labeling experiments.

Cleared *E.coli* lysate (adjusted to the same OD_{600}) was thawed on ice. 45 µL lysate was mixed with 20 % DMSO (final concentration) and **photo-14** and **14** added to a final concentration of 10 µM

(**photo-15** and **15**: 50 µM). The reaction was induced at 365 nm with UV Lamp VL-215.L for 30 min and then incubated at 37 °C for 1 h at 350 rpm. The reaction was quenched with 5 µL BCN-OH (100 mM) for 15 min and then 18 µL 4x SDS loading buffer were added and the samples heated at 95 °C for 10 min. 15 µL samples were analyzed by a 15 % SDS PAGE. Labeling was visualized by fluorescence using an ImageQuant LAS 4000 (**15**: ex. 520 nm, 575DF20 Cy3 filter; **14**: ex. 630 nm, R670 Cy5 filter), followed by staining with Quick Coomassie Stain and destaining in deionized water.

Labeling of OmpC-Y232mTetK on live *E. coli* with 3 and 4

E. coli cultures expressing OmpC-Y232TAG without uAA, with BocK or mTetK were harvested in 0.5-1 mL samples (pellet resuspended in 1 mL ≙ OD_{600} = 2), pelleted at 4000 g for 3 min and the pellet washed 3x with 1 mL 10 % (v/v) DMSO/PBS buffer (resuspension followed by centrifuged at 4000 g for 3 min). The pellets were resuspended in 100 µL 20 % (v/v) DMSO/PBS, 5 µM **3** added and reacted at 37 °C for 60 min (or samples taken at different time points: 15 min, 30 min, 45 min and 60 min). Samples were diluted with 1 mL 10 % (v/v) DMSO/PBS buffer and pelleted at 4000 g for 3 min and washed 2x with 10 % (v/v) DMSO/PBS buffer. The reaction was quenched for 30 min by resuspending the pellets with 10 µL dipyridyl-1,2,4,5-tetrazine (50 mM) diluted in 40 µL PBS. The pellets were then washed 3x with 10 % DMBO/PBS and the samples resuspended in 1x SDS loading buffer (100 µL for a pellet of 1 mL OD_{600} = 1), cooked for 10 min at 95 °C and centrifuged (17.000 x g, 10 min). 15 µL sample was separated via SDS-PAGE and labeling was visualized by excitation of TAMRA fluorescence at 520 nm utilizing an ImageQuant LAS 4000 with a 575DF20 Cy3 filter, followed by staining with Quick Coomassie Stain and destaining in deionized water.

Labeling of OmpC-232mTetK on live *E. coli* with photo-14

E. coli cultures expressing OmpC-Y232TAG without uAA, with BocK, BCNK or mTetK were harvested in 0.5-1 mL samples (pellet resuspended in 1 mL ≙ OD_{600} = 2), pelleted at 4000 g for 3 min and the pellet washed 3x with 1 mL 10 % (v/v) DMSO/PBS buffer (resuspension followed by centrifuged at 4000 g for 3 min). The pellets were resuspended in 100 µL 20 % (v/v) DMSO/PBS and 5 µM **photo-14** or **14** added. The reaction was induced at 365 nm with UV Lamp VL-215.L for 30 min on ice and then incubated at 37 °C and 350 rpm for 60 min. Samples were diluted with 1 mL 10 % (v/v) DMSO/PBS buffer and pelleted at 4000 g for 3 min and washed 2x with 10 % (v/v) DMSO/PBS buffer. The reaction was quenched for 30 min by resuspending the pellet in 10 µL dipyridyl-1,2,4,5-tetrazine (50 mM) diluted in 40 µL PBS, followed by washing the pellets 3x with 10 % DMBO/PBS.

For confocal microscopy 10 µL culture with an OD_{600} = 1 were mixed with 10 µL 1 % (w/v) agarose in MQ water and 10 µL mixture mounted between a coverslip and a microscopy slide.

For SDS-PAGE fluorescence imaging pellets were resuspended in 1x SDS (100 µL for a pellet of 1 mL OD_{600} = 1), cooked for 10 min at 95 °C and centrifuged for 10 min at 17.000 x g. 15 µL samples were separated via SDS-PAGE. Labeling was visualized by excitation of Cy5 fluorescence at 630 nm

and utilizing an ImageQuant LAS 4000 with a R670 Cy5 filter, followed by staining with Quick Coomassie Stain and destaining in deionized water.

Sequential labeling of OmpC232mTetK on live *E. coli* with photo-16, followed by Tet-Cy5

E. coli cultures expressing OmpC-Y232mTetK were harvested in 0.5-1 mL samples (pellet resuspended in 1 mL ≙ OD_{600} = 2), pelleted at 4000 g for 3 min and the pellet washed 3x with 1 mL 10 % (v/v) DMSO/PBS buffer (resuspension followed by centrifuged at 4000 g for 3 min). The pellets were resuspended in 100 µL 20 % (v/v) DMSO/PBS and 50 µM **photo-16** or no compound added. The reaction was carried out at 37 °C and 350 rpm for 60 min. Samples were diluted with 1 mL 10 % (v/v) DMSO/PBS buffer and pelleted at 4000 g for 3 min and washed 2x with 10 % (v/v) DMSO/PBS buffer. The reaction was quenched for 30 min by resuspending the pellets in 10 µL dipyridyl-1,2,4,5-tetrazine (10 mM) diluted in 40 µL PBS. Pellets were then washed 3x with 10 % DMBO/PBS and resuspended in 100 µL 20 % (v/v) DMSO/PBS and 5 µM Tet-Cy5 added. The reaction was induced at 365 nm with UV Lamp VL-215.L for 60 min on ice and then incubated at 37 °C and 350 rpm for 60 min. Samples were diluted with 1 mL 10 % (v/v) DMSO/PBS buffer and pelleted at 4000 g for 3 min and washed 2x with 10 % (v/v) DMSO/PBS buffer. Reaction was quenched by resuspending the pellets in 5 µL BCN-alcohol (100 mM) diluted in 45 µL PBS for 30 min, then washed 3x with 10 % DMBO/PBS.

For confocal microscopy 10 µL culture with an OD_{600} = 1 were mixed with 10 µL 1 % (w/v) agarose in MQ water and 10 µL mixture mounted between a coverslip and a microscopy slide.

For SDS-PAGE fluorescence imaging pellets were resuspended in 1x SDS (100 µL for a pellet of 1 mL OD_{600} = 1), cooked for 10 min at 95 °C and centrifuged for 10 min at 17.000 x g. 15 µL samples separated via SDS-PAGE. Labeling was visualized by excitation of Cy5 fluorescence at 630 nm and utilizing an ImageQuant LAS 4000 with a R670 Cy5 filter, followed by staining with Quick Coomassie Stain and destaining in deionized water.

Kinetic measurements

Fluorimeter

Rate constants k between sfGFP150mTetK and BCN-alcohol or **11** were measured under pseudo first order conditions with an excess of BCN-alcohol (10, 15, 20, 30, 50 or 75-fold) or **11** (10, 15, 20, 30 or 50-fold) in PBS by following exponential increase of GFP fluorescence over time on a Horiba Jobin Yvon Fluoromax-4 in a Quartz cuvette with a stirrer at 25 °C. The final protein concentration of purified sfGF150mTetK was 10 µM in a final volume of 1 mL. GFP fluorescence was excited at 488 nm (slit 1 nm) and measured at 506 nm (slit 2 nm). Data points were taken every 0.25-1 s for 300-5000 s and measured as triplicates. The observed k' was plotted against the concentration of BCN-alcohol or **11** to obtain the rate constant k from the slope of the plot. All data processing was performed using Kaleidagraph software (Synergy Software; Reading, UK).

Tecan Reader

Rate constants k between sfGFP150mTetK and Cp or Nor-OH, as well as sfGFP150TetF and **11** were measured under pseudo first order conditions with an excess of cyclopropene (10, 25, 50, 75 or 100-fold), norbornenol (100, 250, 500, 750 or 1000-fold) or **11** (10, 25, 40, 60 and 75-fold) in water or PBS by following exponential increase of GFP fluorescence over time on a Tecan Spark 10M Microplate reader. The final protein concentration of purified sfGF150mTetK or sfGFP150TetF was 10 µM in a final volume of 100 µL in a 96 well plate. The plate was shaken (amplitude 3.5) during measurements and GFP fluorescence excited at 480 nm and measured at 527 nm. Data points were taken as triplicates per well every 60 s for 6-8 h at 25 °C. Biological replicas were measured in triplicates and the mean of the observed k' was plotted against the concentration of cyclopropane, norbornene or **11** to obtain the rate constant k from the slope of the plot. All data processing was performed using Kaleidagraph software (Synergy Software; Reading, UK).

SUPPLEMENTARY TABLES AND NOTES

Supplementary Table S1: Plasmids for bacterial expression

Plasmid	Purpose
pBK_TetRS	*M. barkeri* TetRS under constitutive glnS promotor for incorporation of TetK, mTetK and BCNK
pBK_wtRS	*M. barkeri* wt PylRS under constitutive glnS promotor for incorporation of BocK
pPylT_sfGFP-N150TAG-His6	sfGFP-N150TAG-His6 under arabinose promotor in-frame with a C-terminal His6-tag and a *M. barkeri* $tRNA_{CUA}$ copy under constitutive lpp promotor
pPylT_sfGFP-N40TAG-His6	sfGFP-N40TAG-His6 under arabinose promotor in-frame with a C-terminal His6-tag and a *M. barkeri* $tRNA_{CUA}$ copy under constitutive lpp promotor
pPylT_Myo-S4TAG-His6	Myo-S4TAG-His6 under arabinose promotor in-frame with a C-terminal His6-tag and a *M. barkeri* $tRNA_{CUA}$ copy under constitutive lpp promotor
pPylT_Ub-K6TAG-His6	Ub-K6TAG-His6 under arabinose promotor in-frame with a C-terminal His6-tag and a *M. barkeri* $tRNA_{CUA}$ copy under constitutive lpp promotor
pPylT_Ub-K63TAG-His6	Ub-K63TAG-His6 under arabinose promotor in-frame with a C-terminal His6-tag and a *M. barkeri* $tRNA_{CUA}$ copy under constitutive lpp promotor
pPylT_Omp-CY232TAG-His6	Omp-CY232TAG-His6 under arabinose promotor in-frame with a C-terminal His6-tag and a *M. barkeri* $tRNA_{CUA}$ copy under constitutive lpp promotor

Supplementary Table S2: Primer sequences used for construct cloning

Primer		Sequence
P1	Fw	5’ ATGAAAGTTAAAGTACTGTCCCTCCTGG 3’
	Rv	5’ GAACTGGTAAACCAGACCCAG 3’
P2	Fw	5’ ccaggagggacagtactttaactttcatGGTTAATTCCTCCTGTTAGCC 3’
	Rv	5’ ctgggtctggtttaccagttcCATCATCATCATCATCATTGAGTTTAAACG 3’

Supplementary Table S3: Primer sequences used for Q5™ Site-Directed Mutagenesis

Primer		Sequence
sfGFPTAG150N	Fw	5’ CAACAGCCATAATGTGTATATTACC 3’
	Rv	5’ AAATTATATTCCAGTTTATGACC 3’

sfGFPN40TAG	Fw	5’ TGATGCGACCTAGGGTAAACTGACCC 3’
	Rv	5’ CCTTCGCCTTCGCCACGA 3’
OmpCY232TAG	Fw	5’ CACCGCTGCTTAGATCGGTAACG 3’
	Rv	5’ TTCTGAGCATCAGTACGTTTGG 3’

Supplementary Note 1–Amino Acid Sequences

TetRS

MDKKPLDVLISATGLWMSRTGTLHKIKHHEVSRSKIYIEMACGDHLVVNNSRSCRTARAFRHHKYRKT
CKRCRVSGEDINNFLTRSTESKNSVKVRVVSAPKVKKAMPKSVSRAPKPLENSVGAKASTNTSRSVP
SPAKSTPNSSVPASAPAPSLTRSQLDRVEALLSPEDKISLNMAKPFRELEPELVTRRKNDFQRLYTND
REDYLGKLERDITKFFVDRGFLEIKSPILIPAEYVERMGINNDTELSKQIFRVDKNLCLRPMLAPTLGNYL
RKLDRILPGPIKIFEVGPCYRKESDGKEHLEEFTMVNFVQMGSGCTRENLEALIKEFLDYLEIDFEIVGD
SCMVYGDTLDIMHGDLELSSAVVGPVSLDREWGIDKPWIGAGFGLERLLKVMHGFKNIKRASRSESY
YNGISTNL*

sfGFP-N150TAG-His6

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sfGFP-N40TAG-His6

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QSVLSKDPNEKRDHMVLLEFVTAAGITHGMDELYKGSHHHHHH*

Myoglobin-K4TAG-His6

MVL*EGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFDRFKHLKTEAEMKASEDLKKHGVT
VLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHPGDFGADAQGAMNKALELF
RKDIAAKYKELGYQGGSGHHHHHH*

Ubiquitin-K6TAG-His6

MQIFV*TLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVL
RLRGGHHHHHH*

Ubiquitin-K63TAG-His6

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQ*ESTLHLVL
RLRGGHHHHHH*

OmpC-Y232TAG-His6

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QNGDGVGGSITYDYEGFGIGGAISSSKRTDAQNTAA*IGNGDRAETYTGGLKYDANNIYLAAQYTQTY
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NMSTYVDYKINLLDDNQFTRDAGINTDNIVALGLVYQFHHHHHH*

AUTHOR CONTRIBUTIONS

K.L. conceived the research plan and experimental strategy. S.V.M synthesized UAAs, performed all experiments in bacteria, including cloning, expression, purification of proteins and labeling in live bacteria. AM synthesized all photo-DMBO compounds. M.-K.v.W. helped with labeling experiments on purified proteins and live bacteria. M.-L.J. helped with live cell labeling and microscopy. All authors analyzed data and K.L. wrote the paper with input from the other authors.

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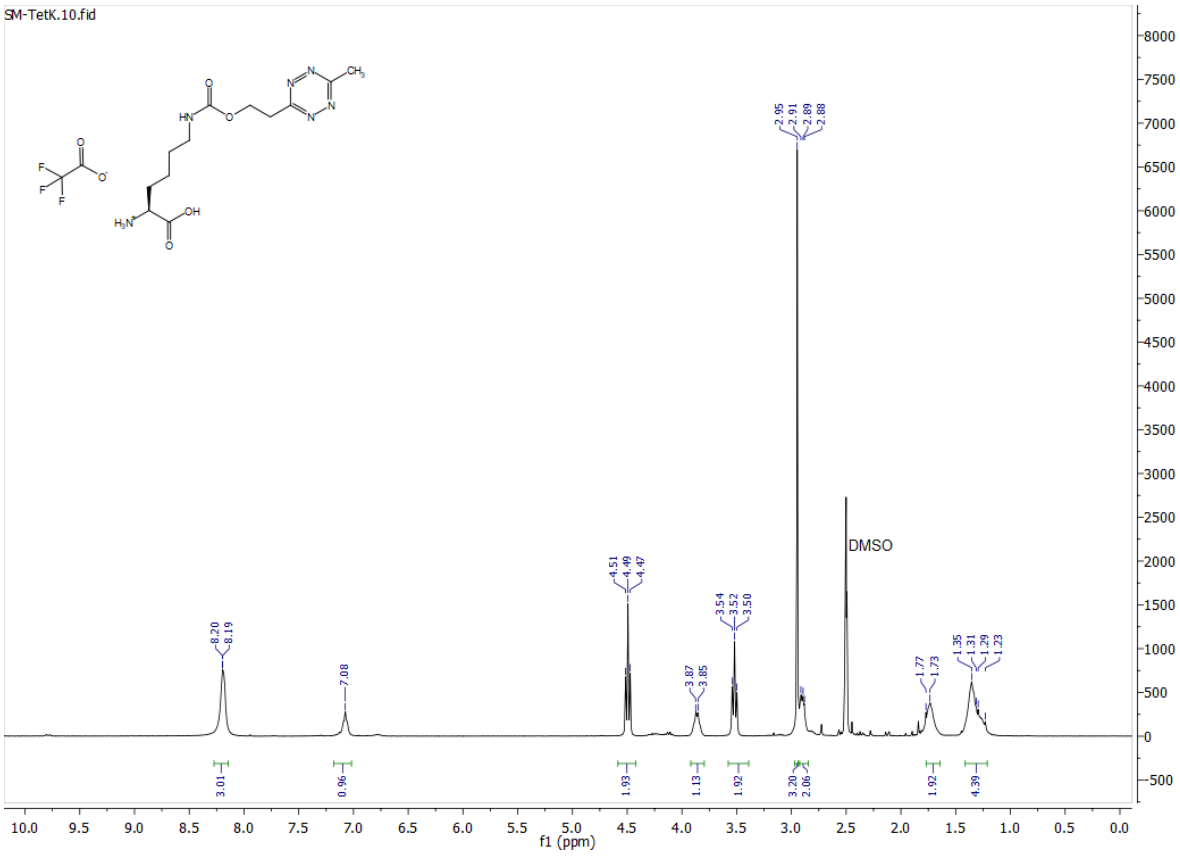
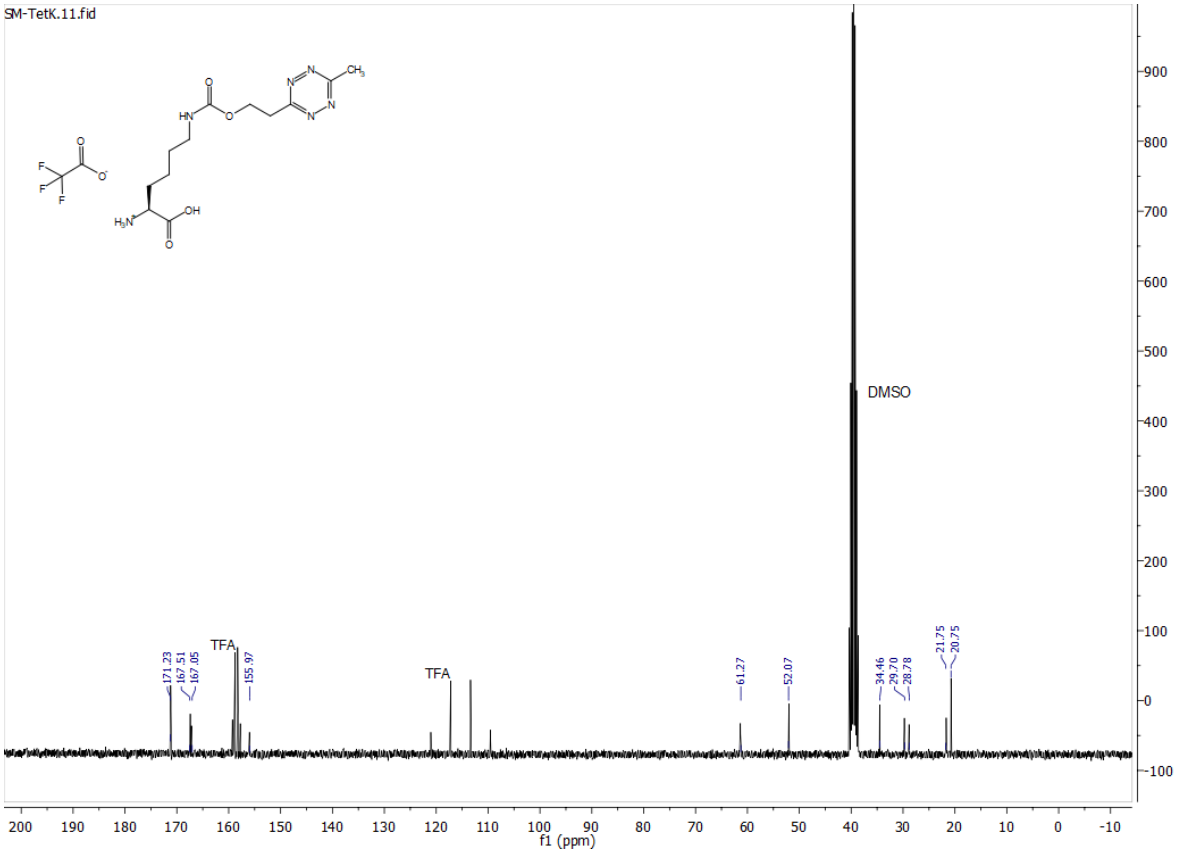
Appendix

^{1}H and ^{13}C NMR spectra

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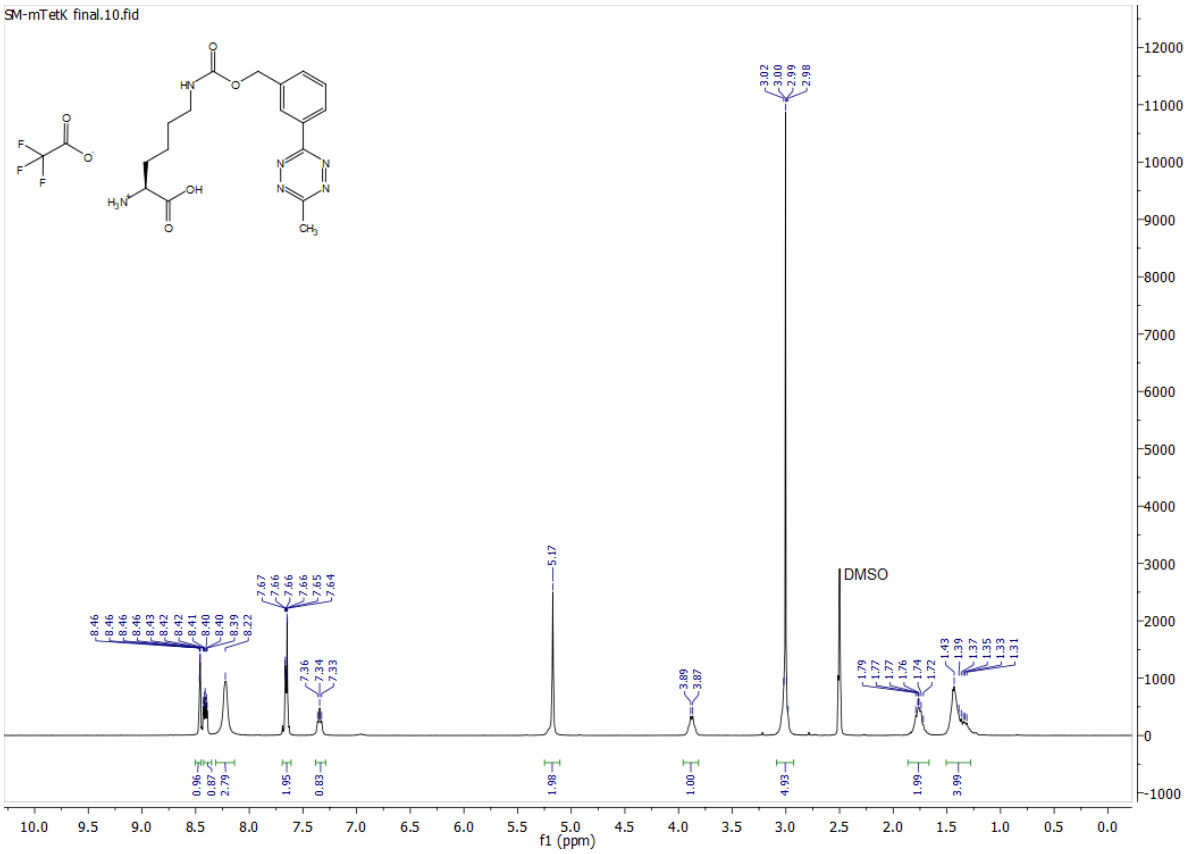
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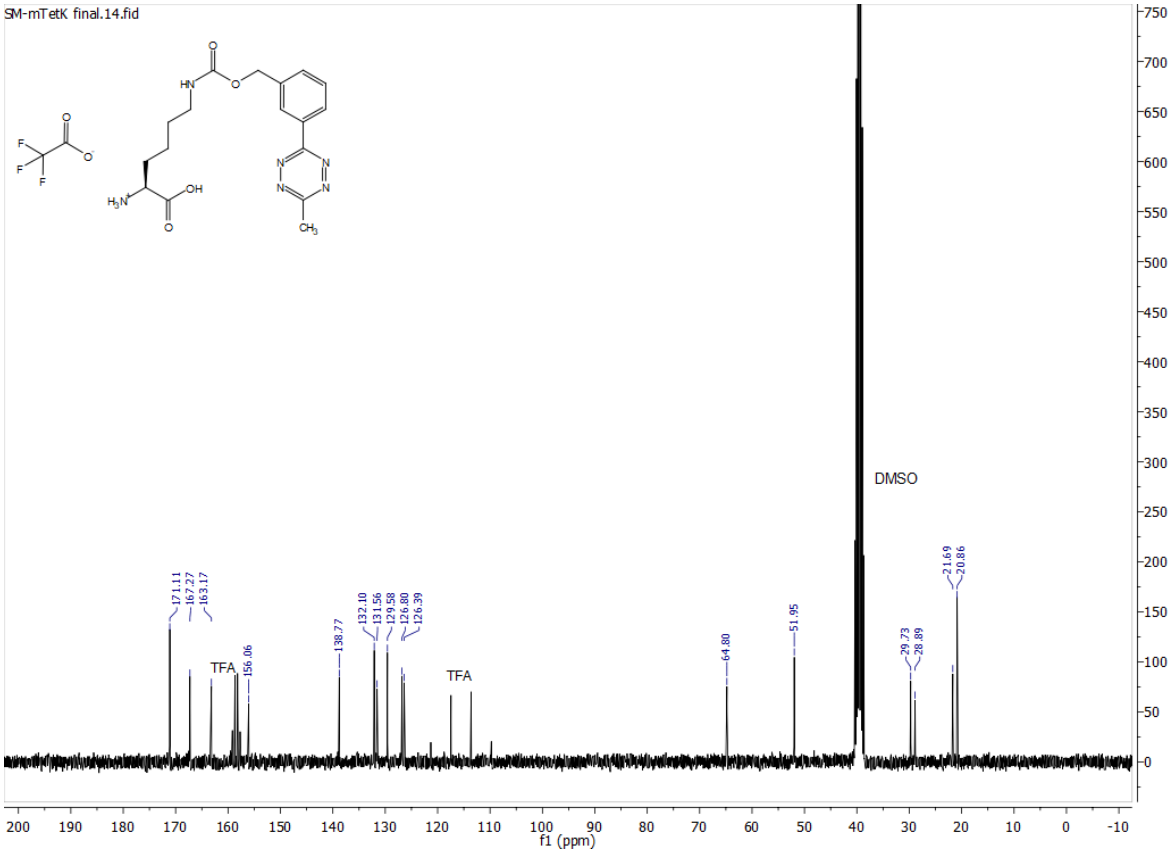
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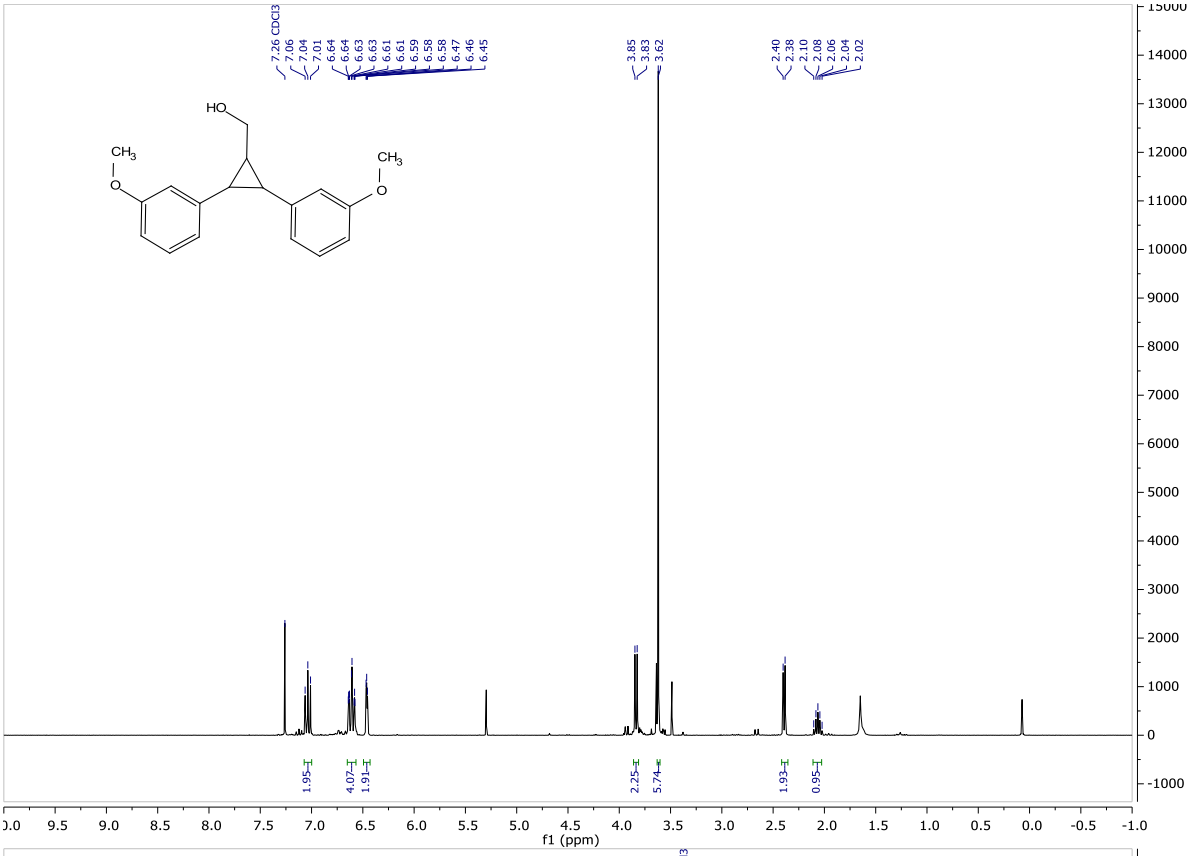
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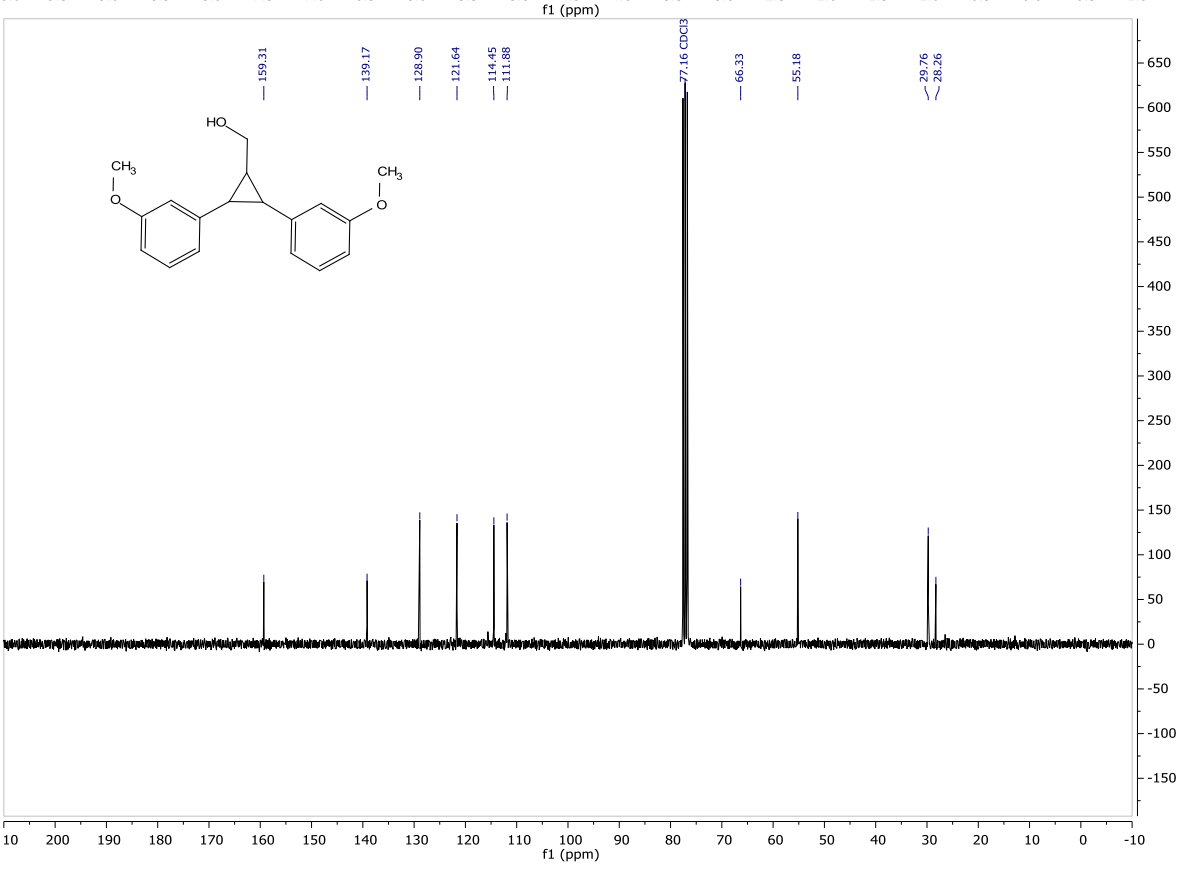
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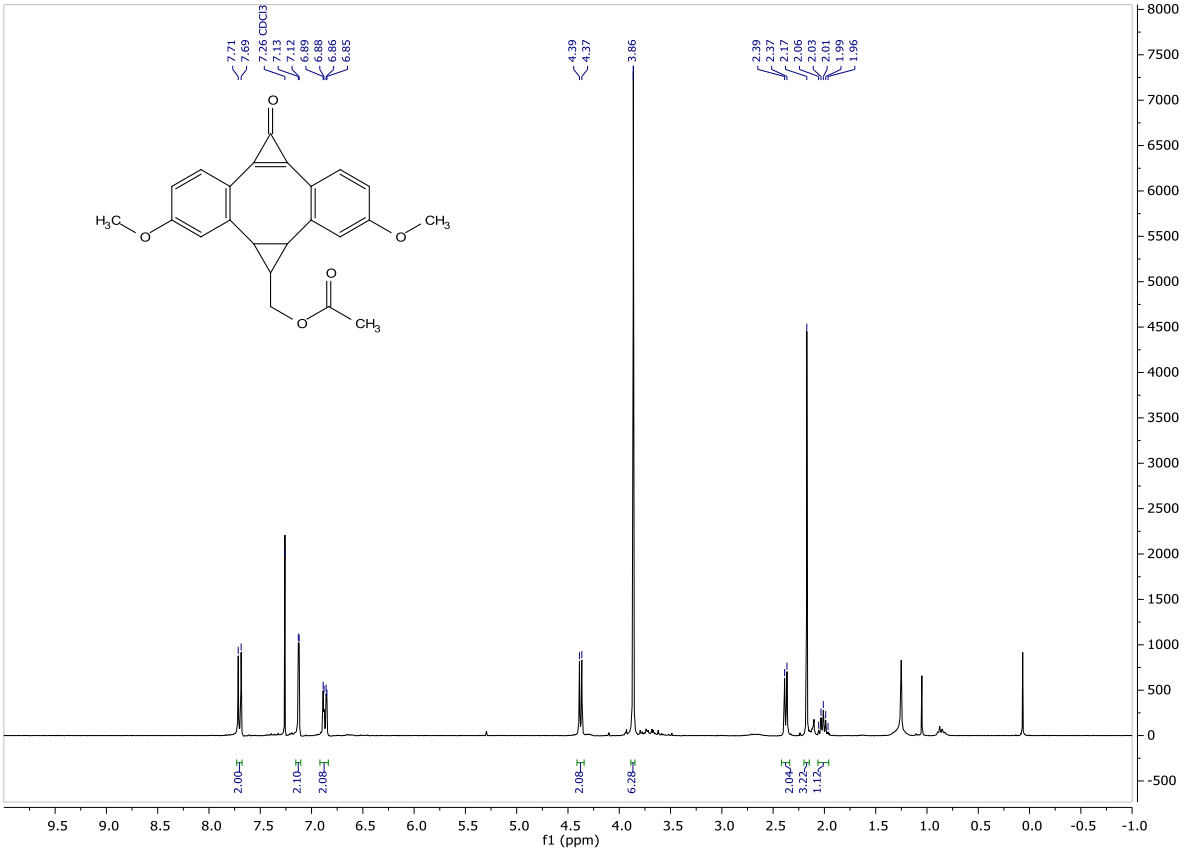
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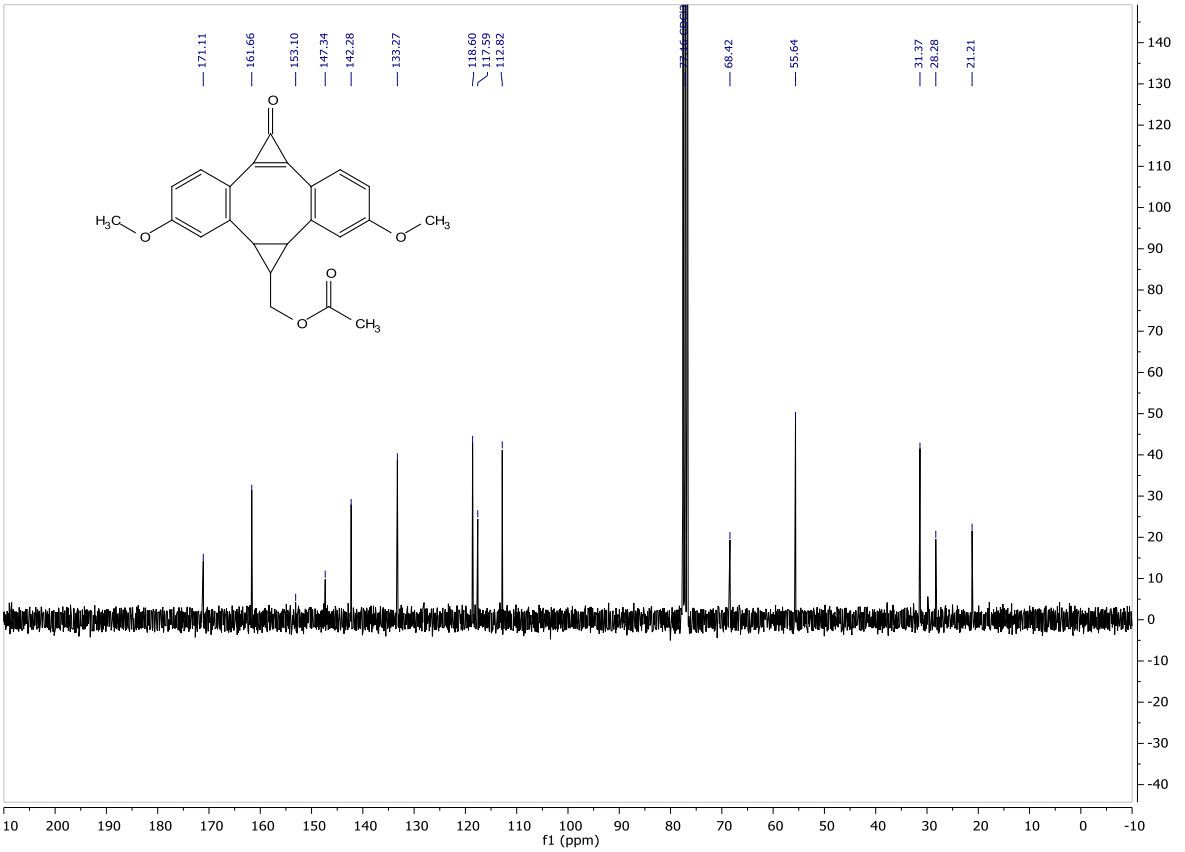
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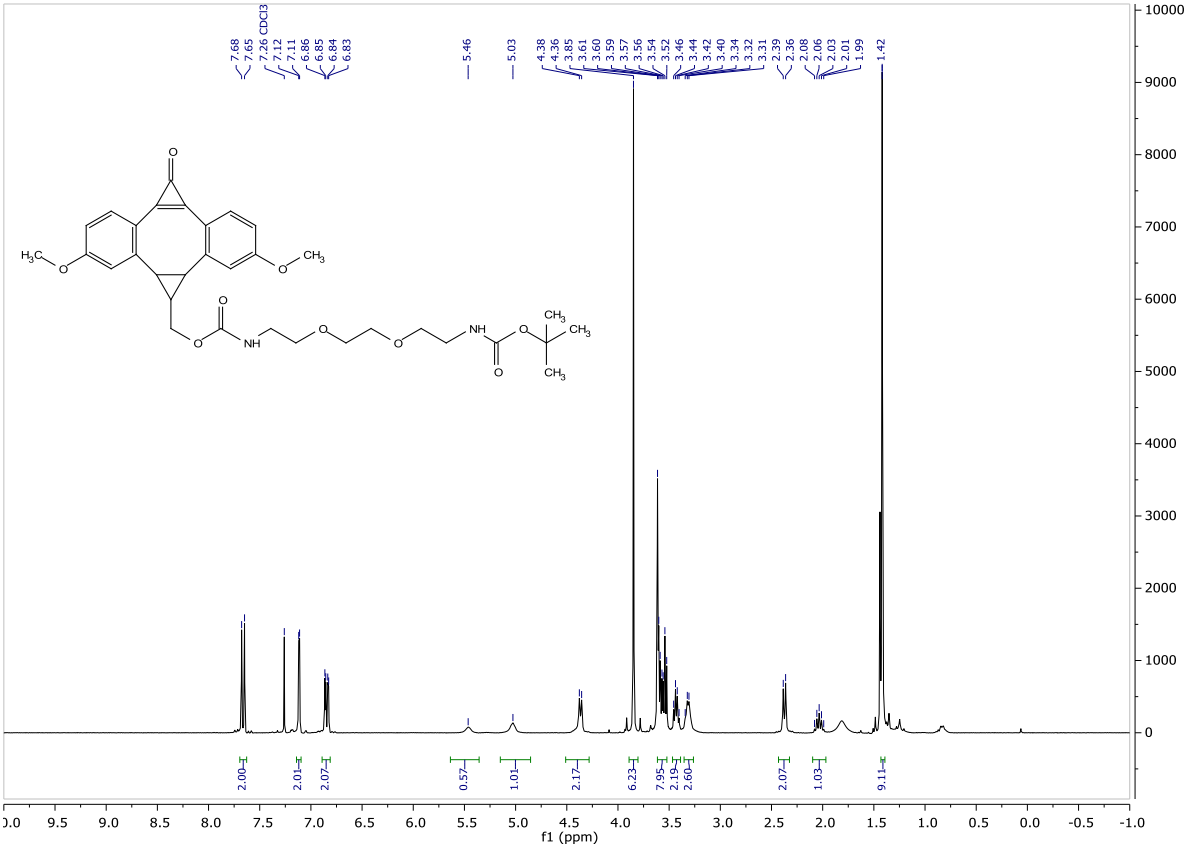
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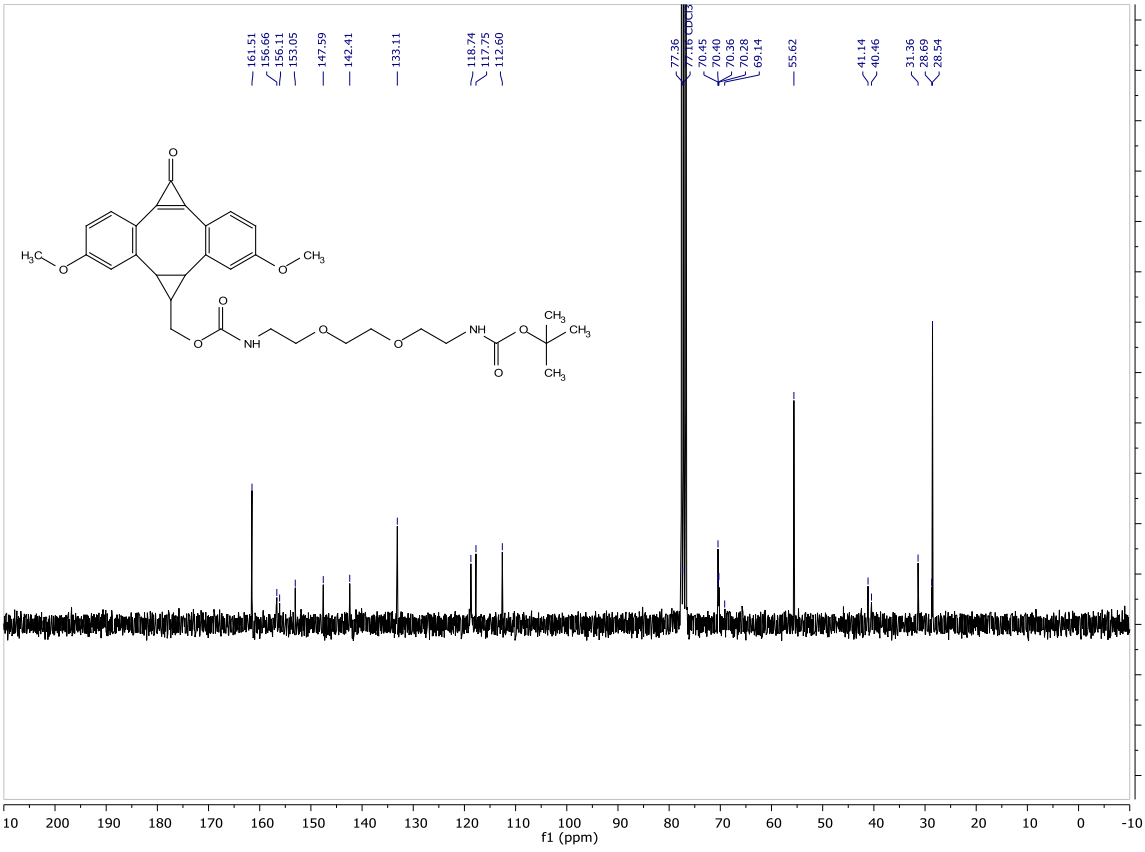
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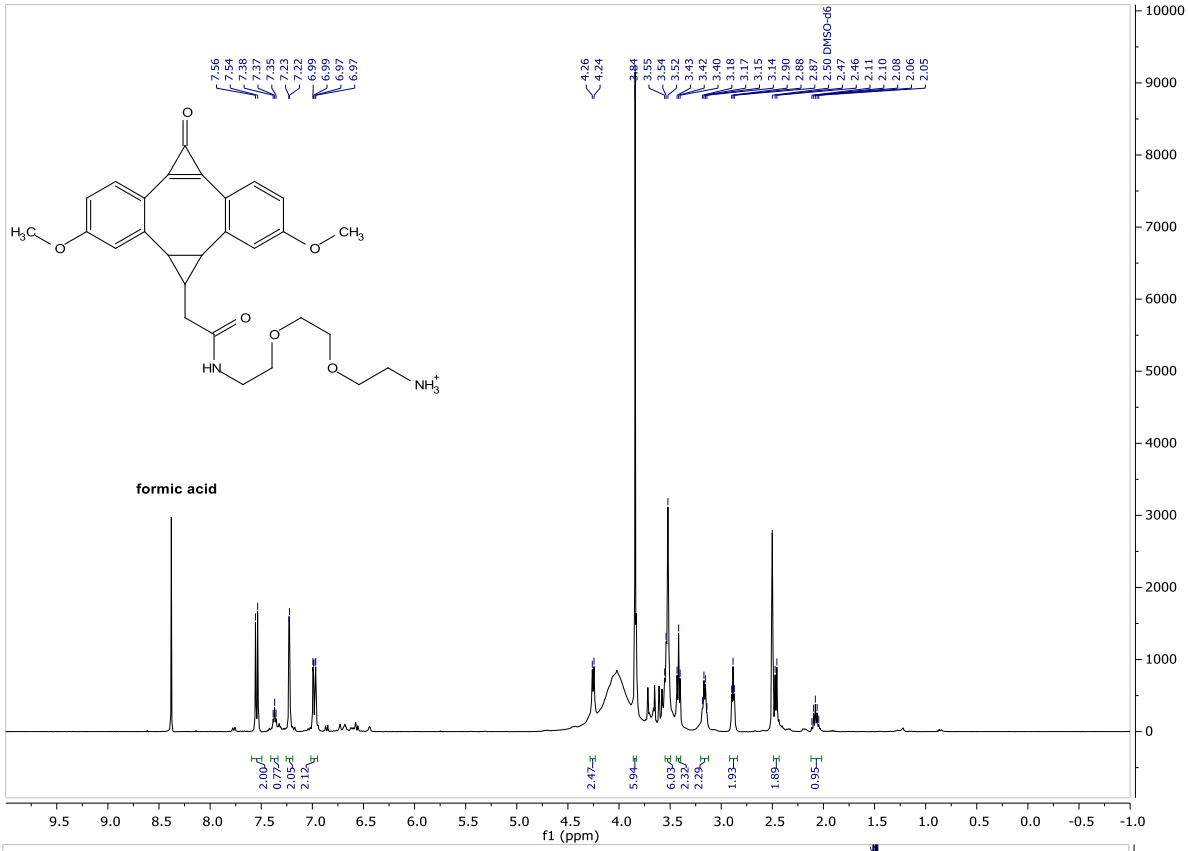
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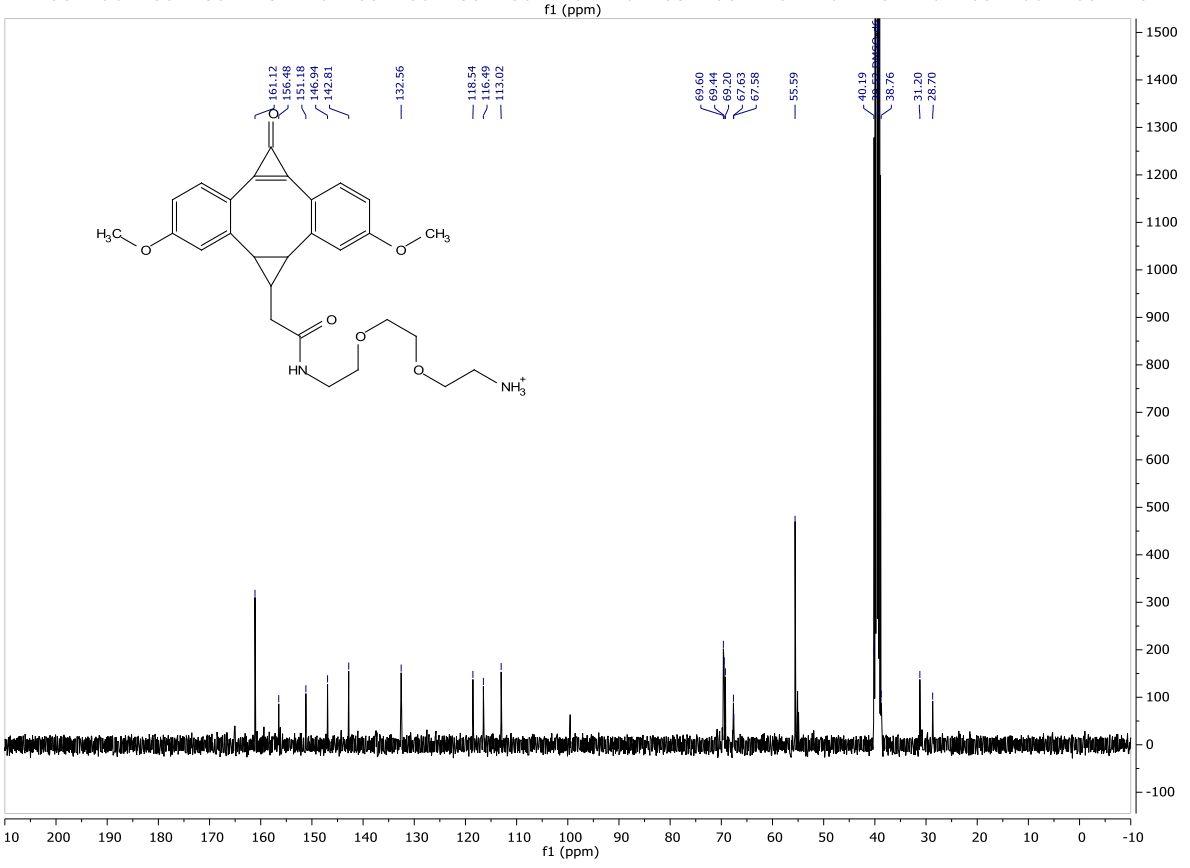
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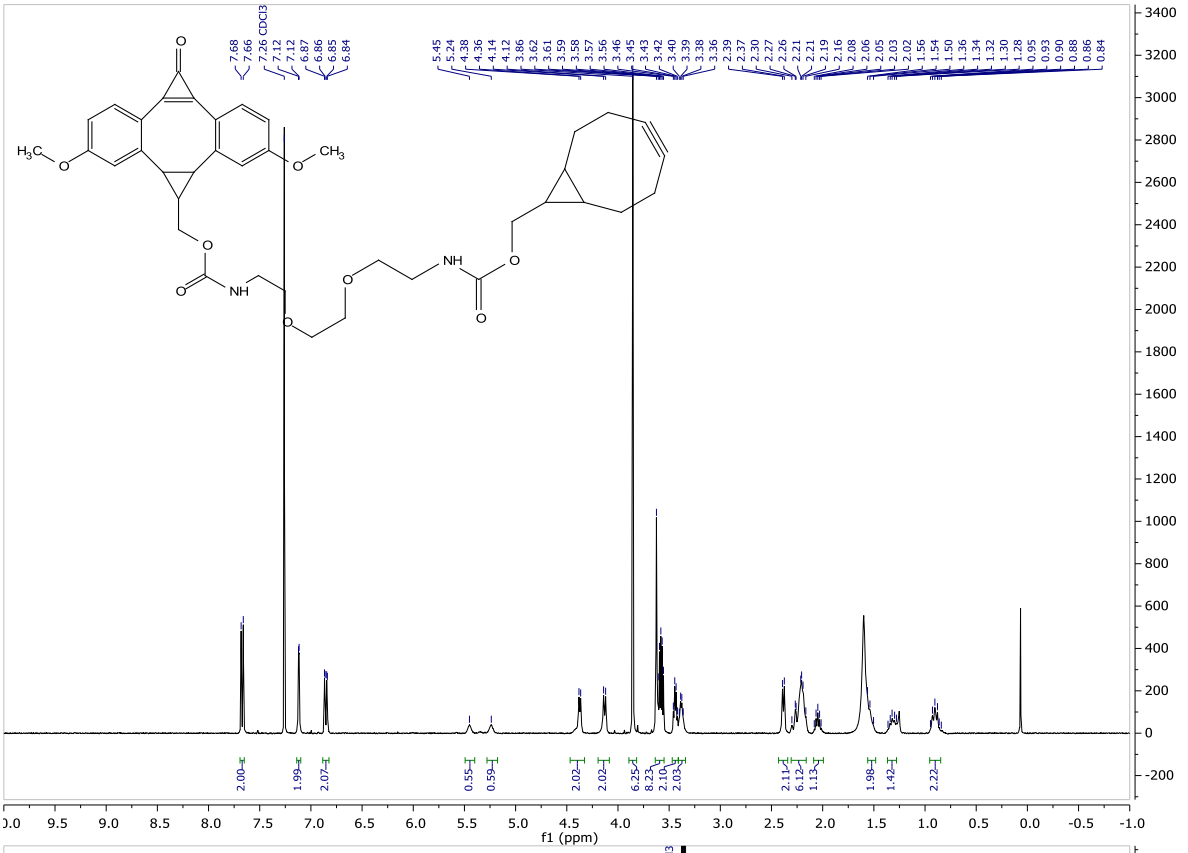
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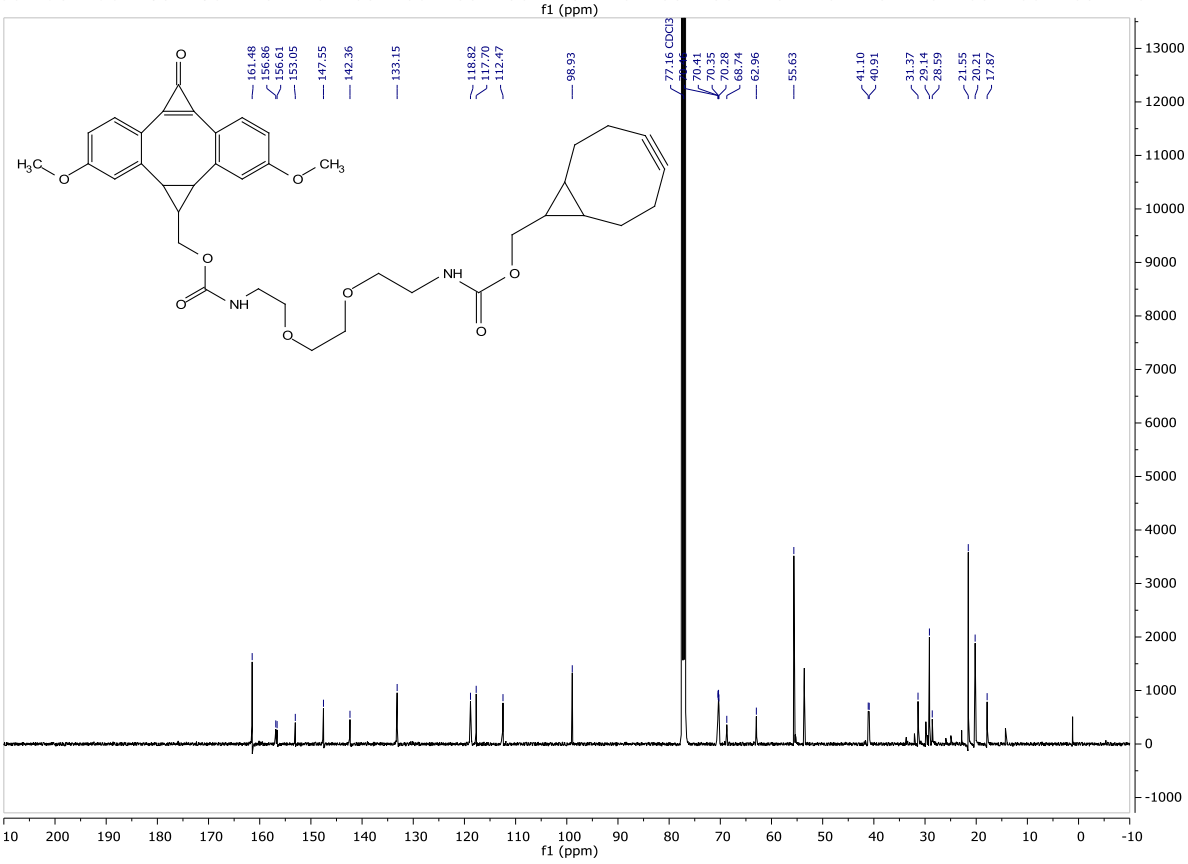
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