Electronic Supplementary Material (ESI) for ChemComm.
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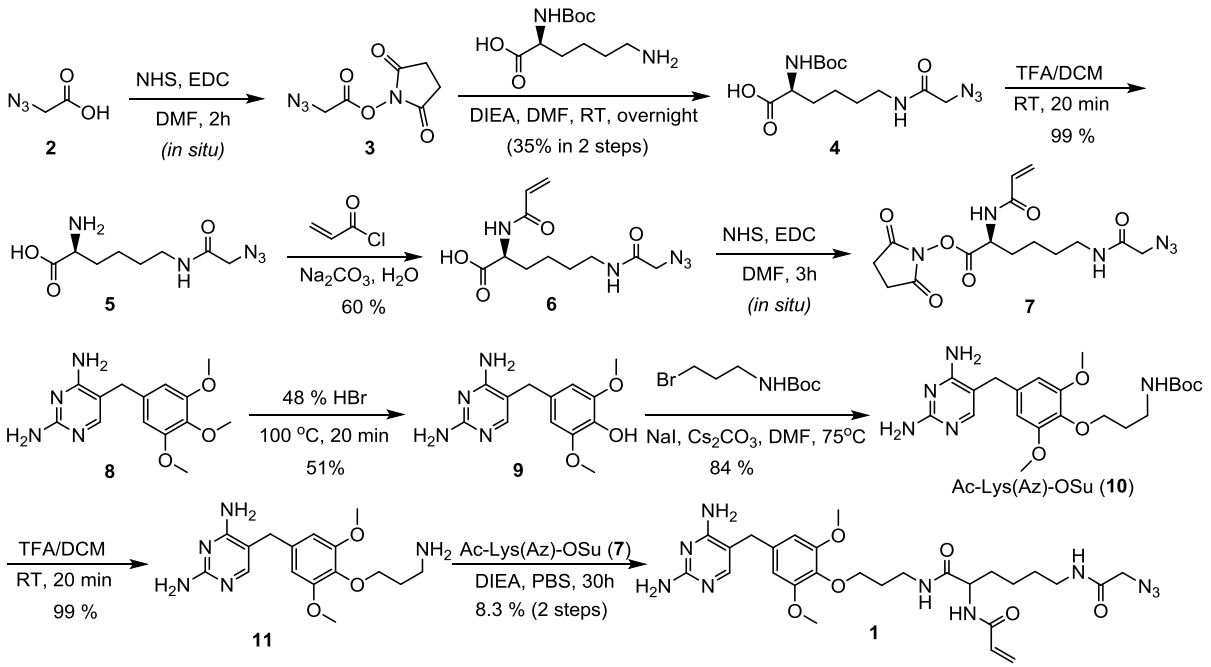
Supporting Information

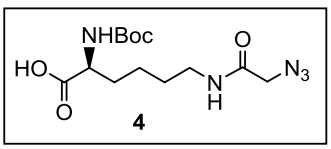
1. GENERAL

Unless otherwise specified, all chemicals were purchased from commercial sources and used without further purification. The DBCO-TAMRA probe was obtained from Jena Bioscience (# CLK-A13). The ^{1}H and ^{13}C NMR spectra were measured on a Bruker 400 MHz or 600 MHz magnetic resonance spectrometer. Data for ^{1}H NMR spectra are reported as follows: Chemical shifts are reported as δ in units of parts per million (ppm); multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), or br (broadened); coupling constants are reported as *J* values in Hertz (Hz); the number of protons (n) for a given resonance is indicated as nH, and is based on the spectra integration values. ESI-MS was performed on an Agilent 1100 series chromatography system equipped with an LCQ ESI mass spectrometer using a C18 column for small molecules and a C4 column for proteins (for the removal of salts and small molecules). High resolution mass spectra (HRMS, 70 eV) were measured on a Thermo Orbitrap coupled to a Thermo Accela HPLC machine and using the electron spray ionization technique (ESI).

2. ORGANIC SYNTHESIS:

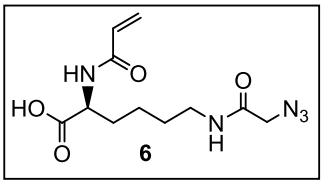
Scheme S1. Synthetic scheme of TMP-AcAz (**1**):



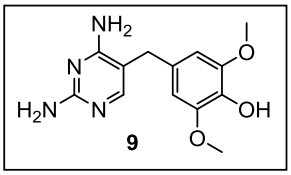


***N*^{6}-(2-Azidoacetyl)-*N*^{2}-(*tert*-butoxycarbonyl)-*L*-lysine** (**4**): This intermediate was prepared from a modified procedure that was reported previously (PCT Int. Appl. 2014, WO 2014044873A1). EDC (998 mg, 5.2 mmol), NHS (598mg, 5.2mg) and DMF were added into a Schlenk RBF equipped with a

stir bar under Ar. Then 2-azidoacetic acid (525 mg, 5.2 mmol) was injected. The resulting reaction solution was stirred at RT for 2 h. This allowed the activation of 2-azidoacetic acid as an NHS ester. Boc-Lys-OH (994 mg, 4.0 mmol) and DIEA (1.96 g, 14.4 mmol) were added and the reaction solution was stirred at room temperature (RT) under Ar for 2.5 h. The reaction mixture was dissolved in EtOAc and then aqueous $NaHSO_4$ was added. The organic layer was separated and the aqueous phase was extracted twice more by EtOAc. All organic layers were combined, washed with brine, dried over anhydrous Na_2SO_4, filtered, concentrated and purified by silica gel chromatography (DCM→ DCM:MeOH 7 → DCM:MeOH 5, stained by ninhydrin) to yield 775 mg (59 %w, rest DMF) of light brownish viscous oil as the product in a yield of 35 %. **^{1}H-NMR** ($CDCl_3$): δ 10.23 (s, br, 1H, -COO*H*), 6.66 (s, 1H, -N*H*), 5.24 (d, *J*=4Hz, 1H, -N*H*), 4.25 (m, 1H), 3.96 (s, 2H), 3.26 (q, *J*=5Hz, 2H), 1.83 (m, 1H), 1.68 (m, 1H), 1.54 (m, 2H), 1.41 (12H); **ESIMS**: $C_{13}H_{23}N_5NaO_5^+$ calcd. 352.16, found 352 $[M+Na]^+$.

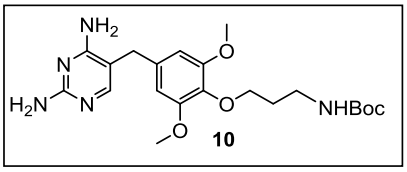


***N*^{2}-Acryloyl-*N*^{6}-(2-azidoacetyl)-*L*-lysine** (**6**): *N*^{6}-(2-Azidoacetyl)-*N*^{2}-(*tert*-butoxycarbonyl)-*L*-lysine (**4**, 775 mg, 59 %w, 1.39 mmol) was dissolved in anhydrous DCM (3.0 ml) and trifluoroacetic acid (TFA, 1.5 ml) was added. The reaction solution was incubated at RT under Ar for 30 min and then concentrated and dried *in vacuo*. The residue was then dissolved in MQ water and washed by DCM (5 x 5 ml) in ice-water bath to remove impurities. The water was removed under high vacuum to quantitatively afford the deprotected intermediate *N*^{6}-(2-Azidoacetyl)-*L*-lysine (**5**, 318 mg, 1.39 mmol). **LC(ESI)MS** (C18, 10-100% MeCN in H_2O, 15 min gradient elution): t_R 1.77 min, *m/z* 230, $C_8H_{16}N_5O_3^+$ $[M+H]^+$. The intermediate **5** (318 mg, 1.39 mmol) was dissolved in H_2O (10 ml) containing Na_2CO_3 (284 mg, 2.69 mmol) and the reaction solution was cooled in an ice-water bath. Acryloyl chloride (132 mg, 119 µL, 1.46 mmol) was added dropwise. The reaction solution was stirred at 0 ^oC under Ar for 30 min. The reaction solution was then acidified by adding $NaHSO_4$ (aq.) and extracted eight times by EtOAc. All organic layers were combined, washed with brine, concentrated and purified via silica gel chromatography ($CHCl_3$ → $CHCl_3$:MeOH 10 → $CHCl_3$:MeOH 5, visualized by UV and ninhydrin) to give 235 mg yellow sticky solid as the product in a yield of 60 %. **^{1}H-NMR** (MeOD): δ 6.34 (dd, J^1=17Hz, J^2=10, 1H), 6.24 (dd, J^1=17Hz, J^2=1.7Hz, 1H), 5.69 (dd, J^1=10Hz, J^2=1.8Hz, 1H), 4.45 (dd, J^1=8.9Hz, J^2=4.9Hz, 1H, -C**H*-), 3.86 (s, 2H), 3.23 (t, *J*=6.8Hz, 2H), 1.90 (m, 1H), 1.76 (m, 1H), 1.56 (m, 2H), 1.45 (m, 2H); **^{13}C-NMR** (MeOD): δ 175.21, 170.06, 168.06, 131.63, 127.26, 53.57, 53.01, 40.12, 32.28, 29.84, 24.19; **HRMS** (ESI): $C_{11}H_{18}N_5O_4^+$ calcd. 284.1353, found 284.1356 $[M+H]^+$.



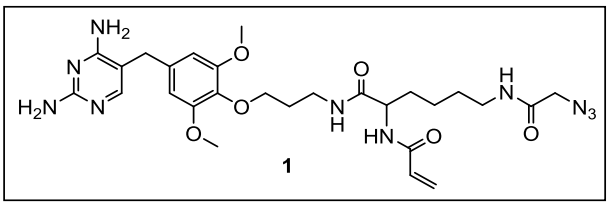
4-((2,4-Diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenol (Dimethoprim, **9**): **9** was obtained via an optimized procedure published recently. Briefly, HBr (con. 47%, 120 mL) was added into a dried 250 ml 2 neck-RBF equipped with a stirrer bar and a condenser. The reaction solution was heated to 90 ^oC under N_2 and trimethoprim (TMP, 10 g, 34 mmol) was added. The reaction mixture was heated to 100 ^oC and stirred for 20 min. Cold 50 % NaOH (aq.) was injected to quench the reaction solution.

The reaction solution was cooled to RT and then kept at 4 °C overnight to allow crystallization. The mixture was subjected to suction filtration and was further washed twice by ice-cold water to give 15.72 g crude product. This crude product was recrystallized in 83 ml MQ water again and cooled to RT, then at +4 °C overnight to give 9.3 g of recrystalized product. This product was subjected to 2nd recrystallization in 61 ml of MQ water to give 7.16 g of 2nd recrystallized product. This was subjected to 3rd recrystallization in 58 ml of MQ water, giving 5.08 g crystals. Dried in vacuum to yield 4.89 g colorless crystals as the purified dimethoprim (**9**) with high purity (51 % yield). **^{1}H-NMR** (DMSO-d^6): δ 7.47 (s, br, 2H, -N$\underline{H}_2$), 7.38 (s, 1H), 6.99 (s, br, 2H, -N$\underline{H}_2$), 6.54 (s, 2H), 3.72 (s, 6H), 3.53 (s, 2H), 3.47 (s, 1H, -O$\underline{H}$); **ESIMS**: *m/z* 277.27, $C_{13}H_{17}N_4O_3^+$ $[M+H]^+$. The characterization data is in agreement with data published previously.[1, 2]



10

***tert*-Butyl (3-(4-((2,4-diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)propyl)carbamate** (TMP-Pr-NHBoc, **10**): This was synthesized using a modified procedure reported previously. Dimethoprim (276 mg, 1.0 mmol), 3-(N-Boc-amino)propyl bromide (250 mg, 1.05 mmol), NaI (150 mg, 1.0 mmol) and Cs_2CO_3 (678 mg, 2.1 mmol) were combined in a 2 neck RBF equipped with a stir bar. Anhydrous DMF (10 ml, 0.1 M) was injected. The resulting reaction suspension was heated up to 75 °C and stirred for 2h. DMF was removed under high vacuum and the product was suspended in DCM and directly subjected to silica gel chromatography purification (DCM:MeOH 8, Rf 0.3) to yield 342 mg light yellowish solid as the product TMP-Pr-NHBoc (**10**) in a yield of 84 %. **^{1}H-NMR** (MeOD): δ 7.51 (s, 1H), 6.53 (s, 2H), 3.95 (t, *J*=5.9Hz, 2H), 3.77 (s, 6H), 3.64 (s, 2H), 3.28 (t, *J*=6.5Hz, 2H)), 1.83 (m, 3H), 1.44 (s, 9H); **ESIMS**: *m/z* 433.93, $C_{21}H_{32}N_5O_5^+$ $[M+H]^+$. The result is in agreement with previously published data.[3]

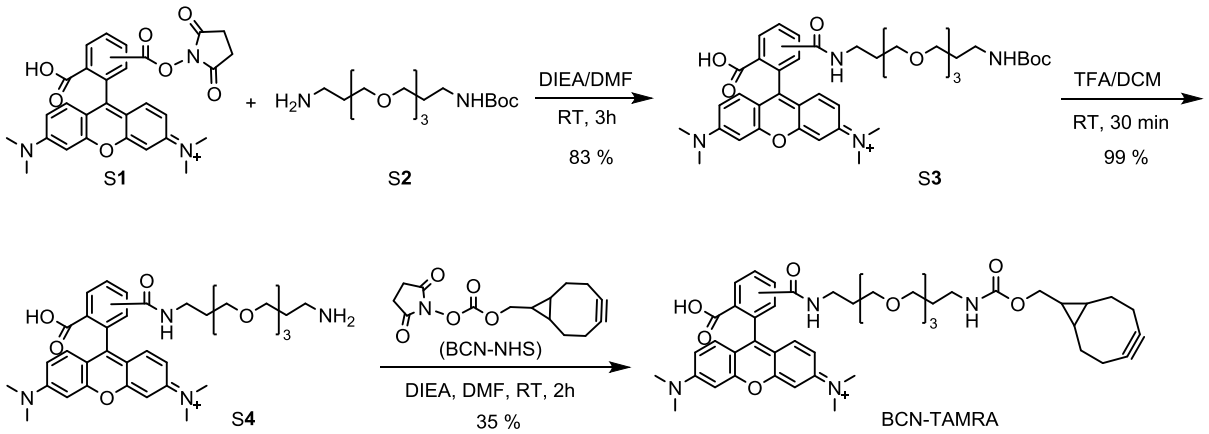


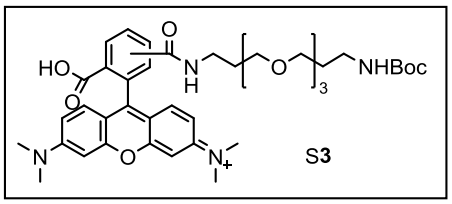
1

TMP-AcAz (**1**): TMP-Pr-NHBoc (**10**, 342 mg, 0.79 mmol) was dissolved in anhydrous DCM (2 ml) and TFA (1 ml) was injected. The resultant reaction solution was incubated at RT under Ar for 20 min. TFA and DCM was removed under high vacuum to give 395 mg (67 %w, rest TFA) of yellowish solid as the deprotected TMP-Pr-NH_2·nTFA (**11**) intermediate in a quantitative yield (*m/z* 334.27, $[M+H]^+$).[3] In parallel, NHS (17.3 mg, 0.15 mmol), EDC (29 mg, 0.15 mmol) and *ε*-azido-*α*-acryloyl-lysine (**6**, 28.5 mg, 0.1 mmol) were combined in a Schlenk flask equipped with a stirrer bar under Ar. DMF (1 ml) was injected. The resulting reaction solution was stirred at RT under Ar for 3 h to yield the NHS ester intermediate (*m/z* 403.3 $[M+Na]^+$). TMP-Pr-NH_2·nTFA (49 mg, 0.1 mmol) was added and PBS (10 ×, 1 ml) and DIEA (49.4 µl, 0.289 mmol) were injected. The reaction solution was stirred under Ar at RT overnight. Solvent was removed under high vacuum and the residue was participated in Na_2CO_3 (sat.)/EtOAc. The organic layer was separated and the aqueous phase was extracted four additional times by EtOAc. All organic layers were combined, washed with brine, dried over anhydrous Na_2SO_4, filtered, concentrated and purified via silica gel chromatography ($CHCl_3$ → $CHCl_3$:MeOH 7 →

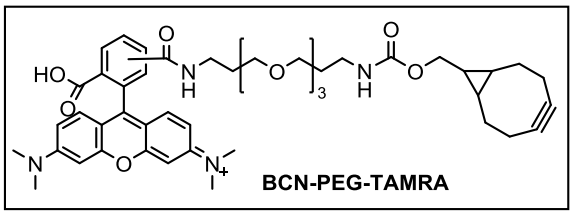
$CHCl_3$:MeOH 5) to give a white solid as the product (5.0 mg) in a yield of 8.3 %. **^{1}H-NMR** (MeOD): δ 7.51 (s, 1H), 6.53 (s, 2H), 6.29-15 (m, 2H), 5.60 (dd, J^1 =9.84 Hz, J^2=2.12 Hz, 1H), 4.34 (dd, J^1=8.6Hz, J^2=5.56Hz, 1H), 3.96 (t, J=4.96Hz, 2H), 3.84 (s, 2H, -C$\underline{H_2}$N$_3$), 3.79 (s, 6H), 3.64 (s, 2H), 3.49-39 (m, 2H), 3.19 (t, J=6.88Hz, 2H), 1.87 (m, 3H), 1.68 (m, 1H), 1.52 (m, 2H), 1.38 (m, 2H); **ESIMS**: *m/z* 621.27 $[M+Na]^+$; **HRMS** (ESI): $C_{27}H_{39}O_6N_{10}^+$, calcd. 599.3045, found 599.3053, $[M+H]^+$.

Scheme S2. Synthetic scheme toward BCN-TAMRA probe:





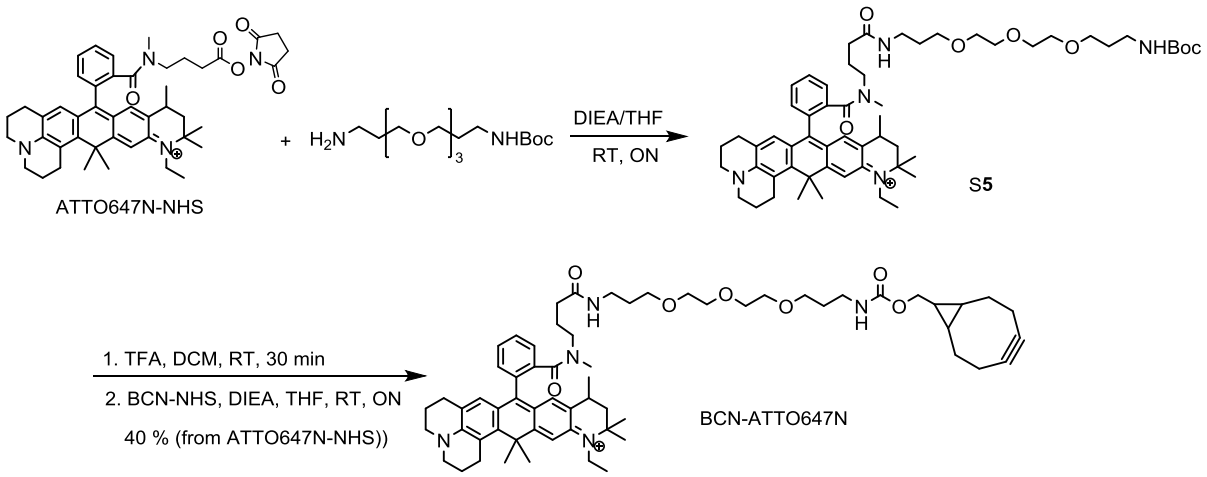
5/6-TAMRA-PEG$_4$-NHBoc (**S3**): 5/6-TAMRA-NHS (10.4 mg, 0.02 mmol) was dissovled in DMF (0.4 ml) in an Eppendorf tube. BocNH-PEG$_4$-NH$_2$ (4.5 mg, 0.021 mmol) and DIEA (6.5 mg, 0.05 mmol) were injected. The Eppendorf tube was wrapped with aluminum and shaken at RT for 3 h. Solvents were removed under high vacuum and purified via silica gel chromatography (DCM→ $CHCl_3$:MeOH 5→$CHCl_3$:MeOH 4) to give 12 mg dark red solid as the product in a yield of 84 %. **^{1}H-NMR** (MeOD): δ 8.15 (d, J=8.1Hz, 0.5H), 8.06 (dd, J^1=12.44Hz, J^2=8.28Hz, 1H), 7.98 (s, 0.5H), 7.71 (s, 0.5H), 7.35 (d, J=7.88Hz, 0.5H), 7.27-7.23 (m, 2H), 7.00 (d, J=9.32Hz, 2H), 6.91 (s, 2H), 3.70-3.62 (m, 5H), 3.60-3.43 (m, 9H), 3.27 (s, 12H), 3.11 (m, 2H), 1.95 (m, 1H), 1.87 (m, 1H), 1.70 (m, 2H), 1.42 (s, 9H); **ESIMS**: *m/z* 733.5, $C_{40}H_{53}N_4O_9^+$ $[M]^+$.

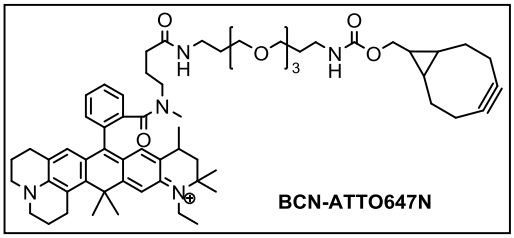


BCN-PEG-TAMRA: 5/6-TAMRA-PEG$_4$-NHBoc (11.5 mg, 0.016 mmol) was dissolved in DCM (1.0 ml) and TFA (0.5 ml) was added. The resulting reaction solution was incubated under Ar at RT for 30 min. DCM and TFA were removed under high vacuum to quantitatively afford the deprotected 5/6-

TAMRA-PEG$_3$-NH$_2$ (S**4**, 16.4 mg, 0.016 mmol, 60 %w) intermediate as dark red solid (*m/z* 656.60, [M+Na]$^+$). Then DMF (0.31 ml) and DIEA (14.4 mg, 0.0237 mmol) were injected and BCN-NHS (6.9 mg, 0.024 mmol) was added. The resulting reaction suspension was shaken at RT for 2 h. DMF and DIEA were removed under high vacuum and the residue was purified via silica gel chromatography ($CHCl_3$→$CHCl_3$:MeOH 15 → $CHCl_3$:MeOH 8 → $CHCl_3$:MeOH 5 → $CHCl_3$:MeOH 4). The crude product was further purified via prepHPLC (C18, Ø10mm, 10-100 % MeCN (TFA-free) in H_2O (TFA-free), 15 min gradient elution, t_R 6.5-7.5 min, *m/z* 809.93 M$^+$) to give 4.5 mg dark violet solid as the product in a yield of 35%. **^{1}H-NMR** (MeOD): δ 8.21 (d, *J*=8.2Hz, 0.5H), 8.11 (d, *J*=7.96Hz, 1H), 7.73 (s, 0.5Hz), 7.41 (d, *J*=7.9Hz, 1H), 7.23 (dd, J^1=9.2Hz, J^2=8.0Hz, 2H), 7.03 (dd, J^1=9.48Hz, J^2=2.24Hz, 2H), 6.94 (d, *J*=2.1Hz, 2H), 4.09 (2H), 3.65 (m, 5H), 3.57 (m, 5H), 3.52-3.45 (m, 4H), 3.29 (s, 12H), 3.17 (m, 2H), 2.27-2.12 (m, 6H), 1.95 (m, 1H), 1.87 (m, 1H), 1.72 (m, 2H), 1.56 (m, 2H), 1.35 (m, 2H), 0.92 (m, 1H); **HRMS** (ESI): $C_{46}H_{57}O_9N_4^+$, calcd. 809.4120, found 809.4137, M$^+$.

Scheme S3. Synthetic scheme toward BCN-ATTO647N probe:





BCN-ATTO647N: A solution of BocNH-PEG$_4$-NH$_2$ (1.04 mg, 3.3 µmol) and DIEA (0.438 mg, 0.59 µl, 3.4 µmol) in anhydrous THF (63 µl) was added into a small Eppendorf tube containing ATTO647N-NHS (2.5 mg, 3.1 µmol). The reaction vial was shaken at RT overnight and then the reaction solution was participated in EtOAc/Na_2CO_3 (sat.). The ester layer was separated and the aqueous phase was extracted three additional times by EtOAc. All organic layers were combined, washed with brine, concentrated and dried *in vacuo*. DCM (0.4 ml) and TFA (0.2 ml) were added to the residue and then incubated at RT under N_2 for 30 min. DCM and TFA were removed in vacuo and the residue was dissolved THF (63 µl). BCN-NHS (1.2 mg, 4.14 µmol) and DIEA (1.64 mg, 2.21 µl, 12.7 µmol) were added. The reaction mixture was shaken at RT overnight and then participated in EtOAc/Na_2CO_3 (sat.). The organic layer was separated and the aqueous phase was extracted twice more by EtOAc.

All organic layers were combined, washed with brine, concentrated and dried in vacuo. The residue was dissolved in ~0.9 ml of HPLC grade MeCN, filtered and purified by preparative HPLC (C18, 10-100 % MeCN (TFA-free)/H_2O (TFA-free), 15 min gradient elution, t_R 8.48 min) to give a blue colored solid as BCN-ATTO647N in a yield of 40 %. **^{1}H-NMR** (MeOD, 600 MHz): δ 7.69 (m, 2H), 7.60 (m, 1H), 7.40 (m, 1H), 6.92 (s, 1H), 6.82 (d, J=7.44Hz, 1H), 6.77 (m, 1H), 4.59 (s, 2H, BCN-C$\underline{H_2}$O-), 4.12 (d, J=7.98Hz, 1H), 3.82 (m, 1H), 3.7-3.4 (m, 15H), 3.18 (m, 4H), 3.10 (m, 1H), 2.89 (d, 3H), 2.76 (m, 1H), 2.66 (s, 6H), 2.63 (m, 1H), 2.58 (m, 1H), 2.23 (m, 5H), 2.2-2.1 (m, 3H), 2.05 (t, J=5.34Hz, 2H), 2.0-1.55 (m, 13H), 1.47 (s, 3H), 1.48-1.25 (m, 10H), 1.03 (dd, J^1=12.12Hz, J^2=6.48Hz, 2H), 0.91 (m, 3H); **LC-MS**(ESI): $C_{63}H_{86}N_5O_7^+$ calcd. 1024.7, found 1024.9 M^+, t_R 5.57 min (LC condition: C18, 10-100 % MeCN/H_2O, 15 min gradient elution).

3. METHODS

Cloning. HA and fluorescent protein vectors were commercially available from Clontech. Fragments of interest were amplified by PCR from plasmids containing the desired genes. The PCR products were gel purified, digested with restriction enzymes, and ligated into one of the fluorescent protein vectors or the pET19 vector precut with the same enzymes. Multiple fragments were assembled by stepwise cloning. A plasmid encoding eDHFR was kindly provided by James C. Hu (Texas A&M University, TX). eDHFR mutants were generated by site-directed mutagenesis from the wild type eDHFR (eDHFR_WT).

Protein expression and purification. The pET19-eDHFR and mutants plasmids were used to transform *E. coli* BL21(DE3) cells and transformants were selected on ampicillin (50 mg/L) agar plates. A single colony was used to inoculate 5 ml of LB medium containing 125 mg/L ampicillin and this culture was grown overnight at 37°C. This pre-culture was used to inoculate 2 L of fresh LB medium (containing 125 mg/L ampicillin) and this new culture was incubated at 37°C until the absorbance at 600 nm (OD_{600}) reached 0.5-0.7. IPTG was added to a final concentration of 0.5 mM and overnight induction was performed at 20°C. Cells were harvested by centrifugation and washed once with PBS. The bacterial pellet was resuspended in lysis buffer (50 mM sodium phosphate, pH 7.5, 0.3 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM β-mercaptoethanol) and cells were lysed by passing them twice through a Microfluidizer (Microfluidics). The lysate was cleared by ultracentrifugation (35,000 *g*, 30 min, 4 °C). The supernatant of the cell lysate was filtered through a 0.2 µm ZapCap filter (Nalgene). Then the supernatant was loaded onto a Ni-NTA column. The proteins were eluted using a gradient of imidazole and were further purified by size exclusion chromatography.

Cell culture and transfection. HeLa cells were maintained at 37°C under 5 % CO_2 in minimum essential medium Eagle (MEME, # M4655, Sigma) supplemented with 10 % fetal bovine serum (FBS), 1.1 % non-essential amino acid (NEAA, 100 ×), 1.1 % sodium pyruvate, 1 % Glutamax, and 1 % penicillin-streptamycin (100 ×). For transient transfection, DNA (1 µg) was dissolved in 100 µl gibco opti-MEM (REF: 31985-062, Life technologies) and incubated with 1.2 µl X-treme GENE HP DNA transfection reagent (REF: 06366236001, Roche) for 15-30 min. Then, take 50 µl to each well (ibidi µ-Slide 4 Well chamber, # 80422) seeding 0.1-0.2 × 10^5 cells in 500 µl complete MEME for 22-24 h.

Confocal fluorescence microscopy. Approximately 22-24 hours after transfection, imaging was performed in complete DMEM (imaging MEM) without phenol red (P04-01163, PAN GmbH) by using an inverted confocal microscope Leica TCS SP5 AOBS equipped with a 63×/1.4 HCX Plan Apo oil

immersion lens and a temperature-controlled hood at 37 °C with 5 % CO_2. In the tagging step, TMP-AcAz (50 μM) in complete MEME was added and the cell culture was incubated for 2 h at 37 °C followed by washing with PBS buffer twice. In the labeling step, BCN-TAMRA (50 μM) or DBCO-TAMRA (50 μM) probe in complete MEME was added and the cell culture was incubated at 37 °C for 2 h, then thoroughly washed several times by PBS buffer and imaged in imaging MEM. EGFP was excited with white laser at 480 nm (PMT: 490-545 nm) while TAMRA was excited at 555 nm (PMT: 565-700 nm) with 80 MHz repetition. Alternatively, EGFP can be excited by Argon (Ar) 476 nm laser (PMT: 485-541 nm) while TAMRA can be excited by DPSS 561 nm laser (PMT: 571-720 nm).

Time-domain FLIM. Fluorescence lifetime images were acquired using the same confocal laser scanning microscope (Leica TCS SP5) equipped with a time-correlated single-photon counting module (LSM Upgrade Kit, Picoquant). For detection of EGFP, the sample was excited using a 488 nm white laser (LDH 470, Picoquant) at 40MHz repetition (PMT 495-520 nm) for 3 min. Fluorescence signal was collected through an oil immersion objective (63×/1.4 HCX Plan Apo) and spectrally filtered using a narrow-band emission filter (HQ 525/15, Chroma). Photons were detected using a single-photon counting avalanche photodiode (PDM Series, MPD) and timed using a single-photon counting module (PicoHarp 300, Picoquant).

In-gel fluorescence. In-gel fluorescence images were recorded by Fujifilm FLA-5000 Imaging Reader or Bio-Rad Gel Doc™ EZ imager. Afterwards, the fluorescence intensity of each protein gel band was quantified by AIDA Image Analyzer (1D evaluation).

Kinetics of affinity conjugation in the first "tagging" step. For the two-step determination of the tagging kinetics of eDHFR_N23C/L28C and eDHFR_L28C, the protein (50 μM) in PBS buffer (pH 7.4) in the presence of dithioerythritol (DTE, 100 μM), w/o adding NADPH (50 μM), was incubated with TMP-AcAz (100 μM) 37 °C. At different time intervals, reaction (10 μL each aliquot) was quenched in 30 μL of 1.33 × SDS sample loading buffer (DTT-free) which contains extra 8 M urea, 25 mM TMP, 2.67 mM *N*-2-(2-aminoethyl)maleimide (NAM) and 267 μM DBCO-TMR (finally 6 M Urea, 200 μM DBCO-TMR and 2 mM NAM in 1x loading buffer are ready for gel-electrophoresis). The quenched aliquots were kept at RT overnight allowing the compete labeling of the "tagged" azido group on the protein. Finally, the reaction aliquots were subjected to SDS-PAGE gel (18 %) electrophoresis and analyzed via in-gel fluorescence. The integrated fluorescence intensity (F) at each time point was plotted against time (t/min). The time-course of reaction was fitted to single exponential equation to give the first order reaction rate constant k^1:

$$F = F_{max}(1 - e^{-kt})$$

Subsequently, the respective reaction half live $t_{1/2}$ can be derived via $t_{1/2} = (\ln 2)/k^1$.

Kinetics of cycloaddition in the second "labeling" step. For the determination of the BCN-TAMRA labeling kinetics, eDHFR_L28C-Az was first prepared via the quantitative reaction between eDHFR_L28C and TMP-AcAz. Afterwards, eDHFR_L28C-Az (50 μM) was treated with BCN-TAMRA (200 μM) at 37 °C in PBS buffer. At different time intervals, aliquote (5 μL) was taken and quenched in 45 μl SDS sample loading buffer, which contains extra 100 mM 2-azidoacetic acid. The protein aliquots were analyzed via in-gel fluorescence where the fluorescence intensity of each protein gel band was determined using AIDA Image Analyzer. Given the parameters: [A] = [eDHFR_L28C-Az],

$[A]_o$=50 µM; [B]=[BCN-TAMRA], $[B]_o$=200 µM; F=integrated fluorescence intensity which is proportional to the product: $[A]_o$-[A]. Given the integrated second order rate law equation:

$$\ln \frac{[B][A]_o}{[A][B]_o} = k([B]_o - [A]_o)t$$

It can be derived to: $F = F_{max}[1\text{-}0.75/(e^{3[A]_o kt} - 0.25)]$ where F_{max} = maximal fluorescence intensity. The fluorescence intensity (F) at each time point is plotted against time (t) and fitted to the equation using SigmaPlot to provide the second order reaction kinetic constant k^2 = 0.97 ± 0.9 M^{-1} s^{-1}.

4. SUPPORTING FIGURES

ESI-MS spectra to monitor conjugation reaction of TMP-AcAz with eDHFR mutants.

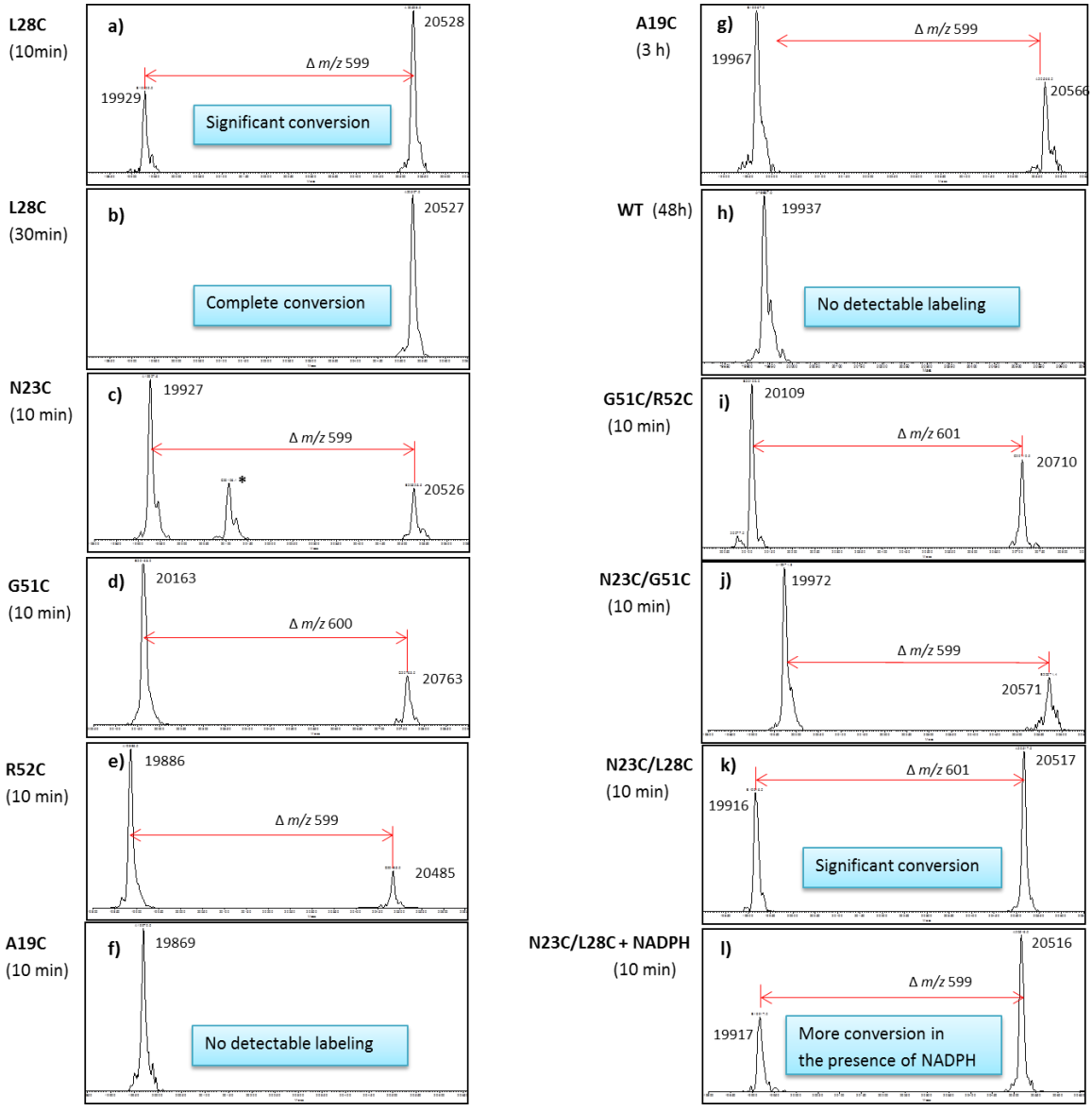


Figure S1. MagTran deconvoluted ESI-MS spectra of eDHFR mutants (5 µM) after reaction with TMP-AcAz (10 µM) for a given time period in the presence of DTE (10 µM). At specified time points, 24 µl of the reaction aliquots were quenched in 48 µl of ESI-MS quench buffer (20 % glycerol + 0.3% TFA).

Note: *this peak (*m/z* 20106) could be an isoform of eDHFR_N23C, because the corresponding labeled product (*m/z* 20525, Δ *m/z* 599) was also detected, suggesting no loss of reactivity to TMP-AcAz.

ESI-MS spectra of a mixture of eDHFR_L28C and eDHFR_L28C-Az at different molar ratios.

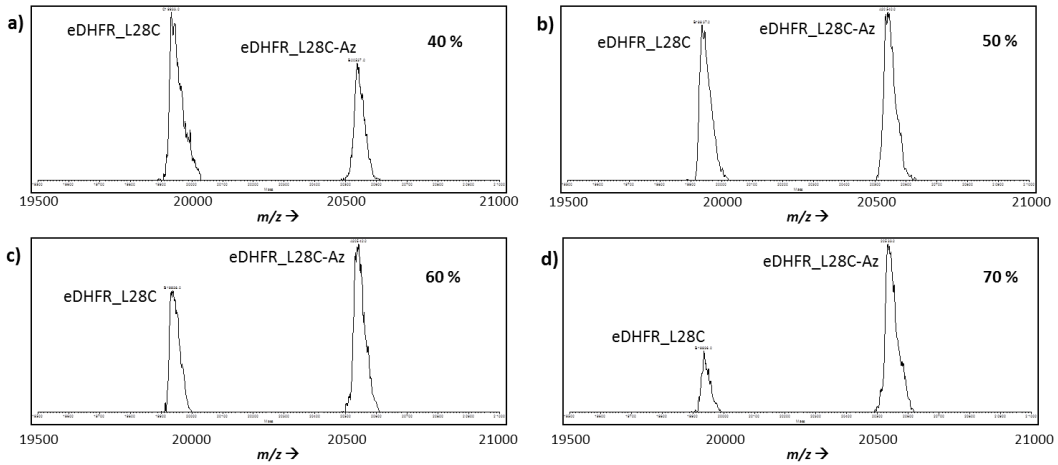


Figure S2. The ESI-MS spectra of a mixture of eDHFR_L28C and eDHFR_L28C-Az containing 40 %mol eDHFR_L28C-Az (a), 50 %mol eDHFR_L28C-Az (b), 60 %mol eDHFR_L28C-Az (c) and 70 %mol eDHFR_L28C-Az. By comparing the ESI-MS spectra of eDHFR_L28C labeled by TMP-AcAz for 10 min (Figure S1a), the conversion of eDHFR_L28C to eDHFR_L28C-Az at 10 min is estimated to be 60-70 % yield.

ESIMS spectra of eDHFR_L28C-Az before and after labeling by BCN-TAMRA for 3 hours.

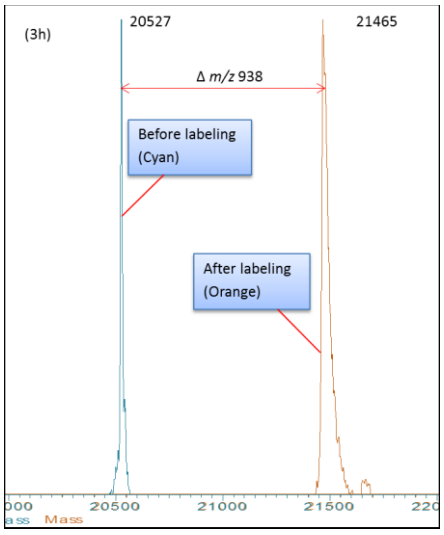


Figure S3. ESIMS spectra of eDHFR_L28C-Az (50 µM) before (cyan, *m/z* 20527) and after (orange, m/z 21465, Δ *m/z* 938) labeling by 200 µM DBCO-TAMRA (Exact Mass: 937) for only 3h revealed a complete labeling. This suggested that overnight incubation of the reaction of eDHFR_L28C-Az (50 µM) with TAMRA-Az (100 µM) in the presence of 200 µM BCN-TAMRA can resulted in quantitative incorporation of the DBCO-TAMRA into the protein.

In-gel fluorescence images for determining the tagging kinetics.

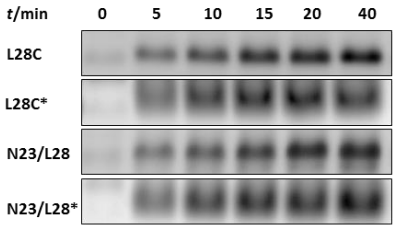


Figure S4. The in-gel fluorescence images for the two-step determination of the tagging kinetics of eDHFR_N23C/L28C and eDHFR_L28C. Note: * in the presence of 50 μM NADPH.

Labeling of non-transfected live Hela cells by TMP-AcAz/BCN-TAMRA.

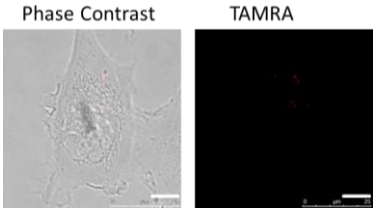


Figure S5. Labeling of non-transfected live Hela cells by TMP-AcAz/BCN-TAMRA showed no specific labeling.

Labeling of eDHFR_N23C/L28C-NLS and EGFP-eDHFR_L28C-CENPA without tagging by TMP-AcAz.

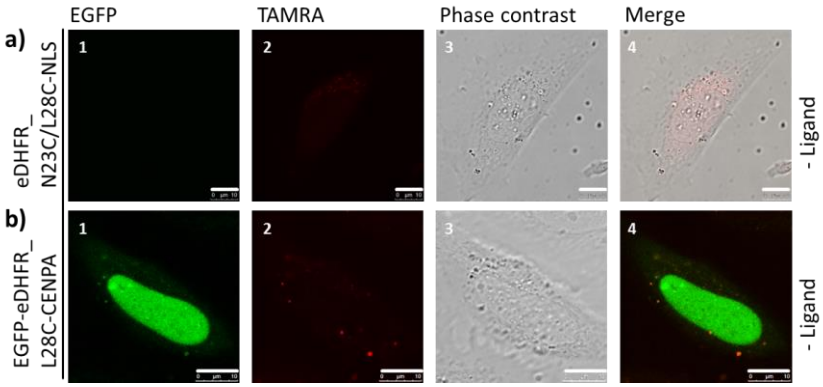


Figure S6. a) BCN-TAMRA (50 μM) treated Hela cells transiently expressing eDHFR_N23C/L28C-NLS and b) BCN-TAMRA (15 μM) treated Hela cells transiently expressing EGFP-eDHFR_L28C-CENPA without tagging by TMP-AcAz ligand showed no specific labeling at nucleus. Scale bar: 10 μm.

Labeling of eDHFR-N23C/L28C-CAAX by TMP-AcAz/BCN-TAMRA pair at the plasma membrane.

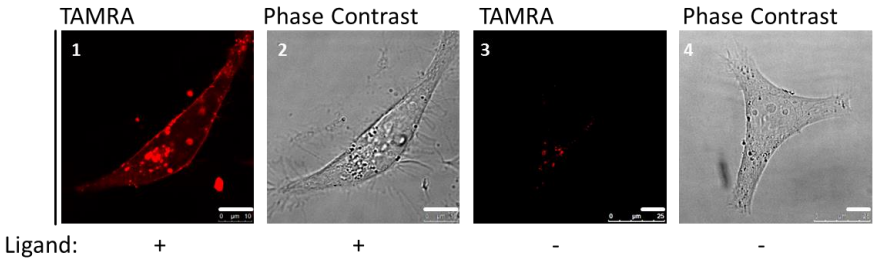


Figure S7. Clear staining of protein at plasma membrane was observed (#1) with the respective phase contrast image provided (#2). If no TMP-AcAz ligand is added, no specific labeling at the plasma membrane could be detected (#3); the corresponding phase contrast image was also given (#4). Scale bar: 10 µM.

Labeling of EGFP-eDHFR-N23C/L28C-NLS by TMP-AcAz/DBCO-TAMRA pair at the nucleus.

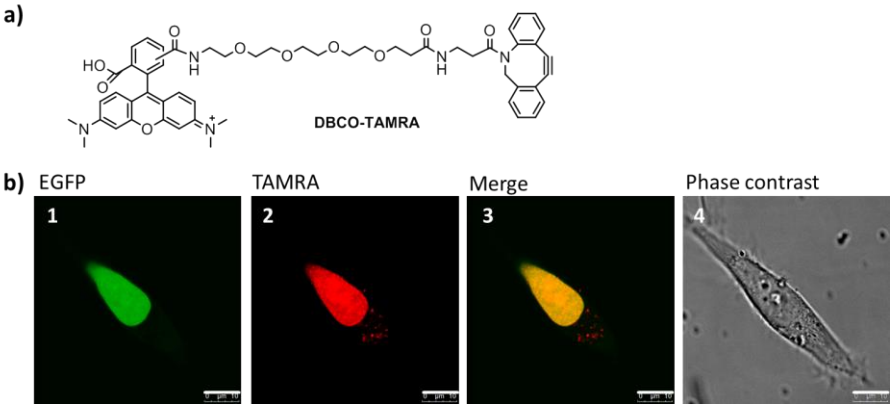


Figure S8. The DBCO-TAMRA probe can also be employed for successful labeling of protein based on our *in vivo* "Tagging-then-Labeling" platform. a) The chemical structure of DBCO-TAMRA probe (Jena Bioscience, # CLK-A131). b) Clear staining of protein at nucleus was observed (b-2) with the respective EGFP channel (b-1), merged image (b-3) and the phase contrast image (b-4) provided. Scale bar: 10 µM.

Labeling of EGFP-eDHFR-N23C/L28C-NLS by TMP-AcAz/BCN-ATTO647N pair at the nucleus.

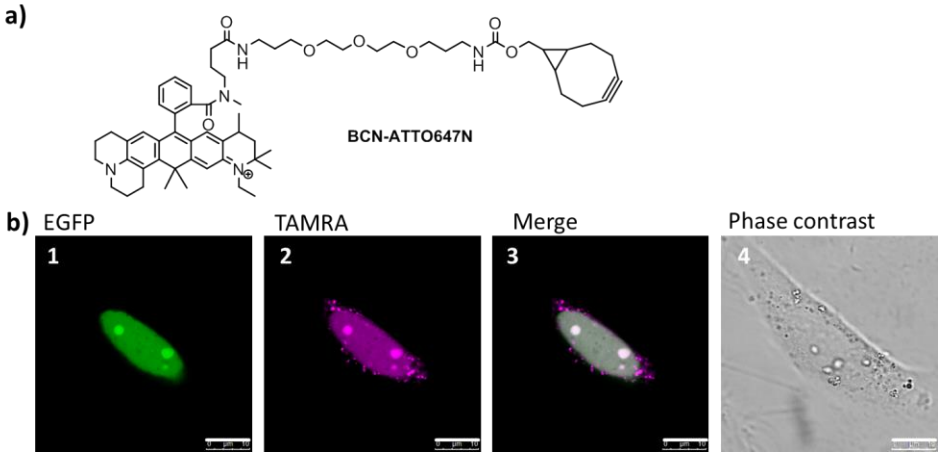


Figure S9. a) The chemical structure of BCN-ATTO647N probe. b) Clear staining of protein at nucleus was observed (b-2) with the respective EGFP channel (b-1), merged image (b-3) and the phase contrast image (b-4) provided. In this labeling study, a reduced concentration of BCN-ATTO647N at 15 µM was used while other parameters were kept the same. For confocal imaging, EGFP was excited by Ar 488 nm laser with PMT 498-600 nm while ATTO647N was excited by Helium-Neon (HeNe) 633 nm red laser with PMT 643-788 nm. Scale bar: 10 µM.

Modeling of a FRET sensor for Rab GTPase labeled by TMP-AcAz/BCN-TAMRA

The size of eDHFR(18 kDa) is smaller that GFP (21 kDa). The principle for designing FRET sensor using eDHFR-GFP pair should be similar as fluorescent protein pairs, which have been extensively demonstrated before.[4] Typically, FRET can occur if the distance between the donor molecule and the acceptor molecule is within 10 nm. In order to estimate if the chemical labeling system can be applied to construct a FRET sensor, we performed a molecular modeling experiment using Molecular Operation Environment (MOE) software.

We inserted a G-protein between eDHFR-GFP pair, i.e. EGFP-Rab1-eDHFR_L28C, where Rab1 GTPase (PDB: 2FOL) is the POI which is fused with an EGFP (PDB: 4N3D) and eDHFR (PDB: 1RF1) at the N- and C- terminus, respectively. This is a typical setup for a GTPase sensor using FP FRET pair. The fusion was energy minimized to obtain the model (Figure S10).

In this model, the distance between EGFP chromophore and TAMRA is estimated to be around 3.6 nm. If we consider the flexibility of the linker, the maximal distance between EGFP chromophore and TAMRA would be 7.1 nm which is still within the effective FRET distance. In this case, addition of a POI between two fluorophores will only lead to a maximal increase of distance of 1.7 nm. Therefore, it is conceivable that the chemical labeling system can be applied to visualize intracellular FRET on proteins.

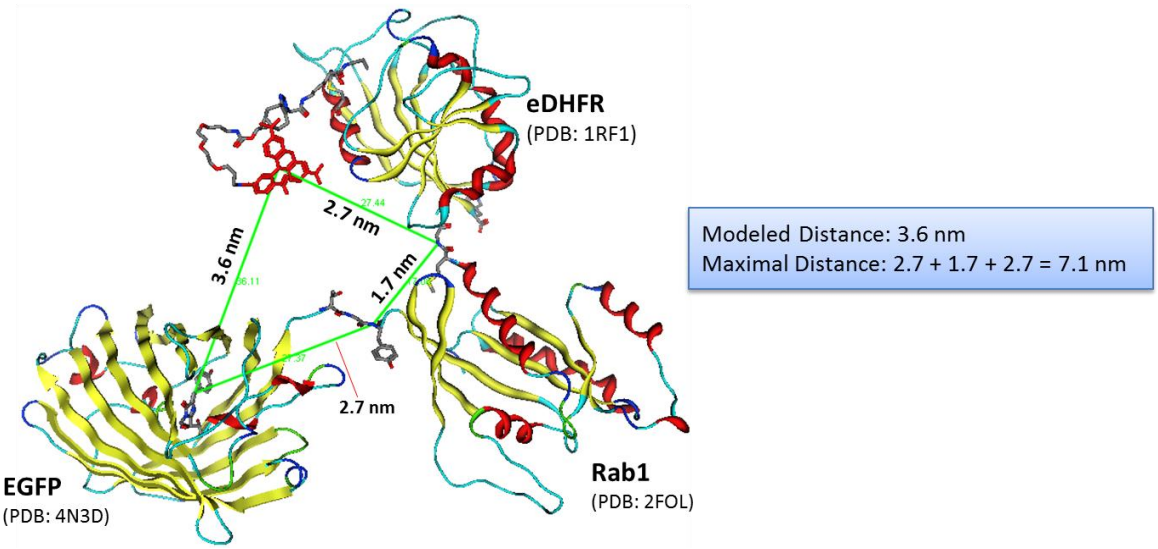
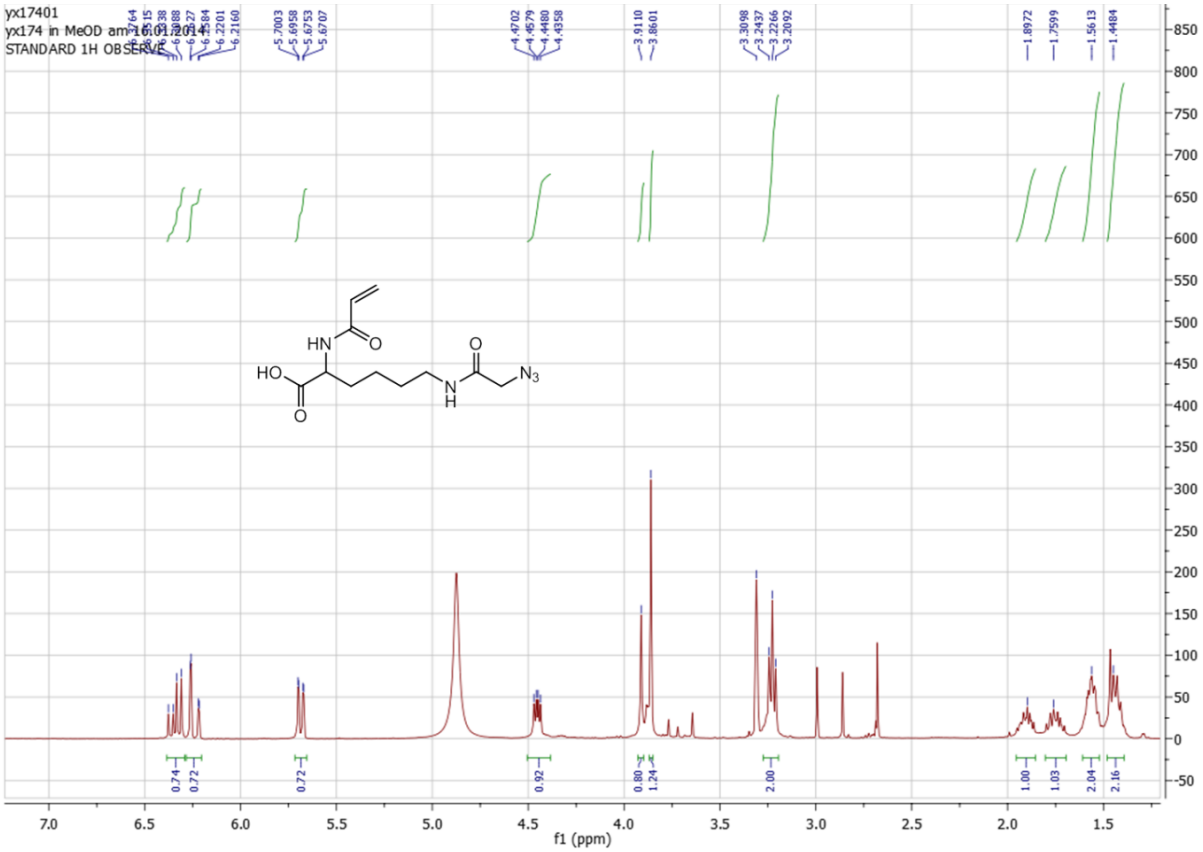


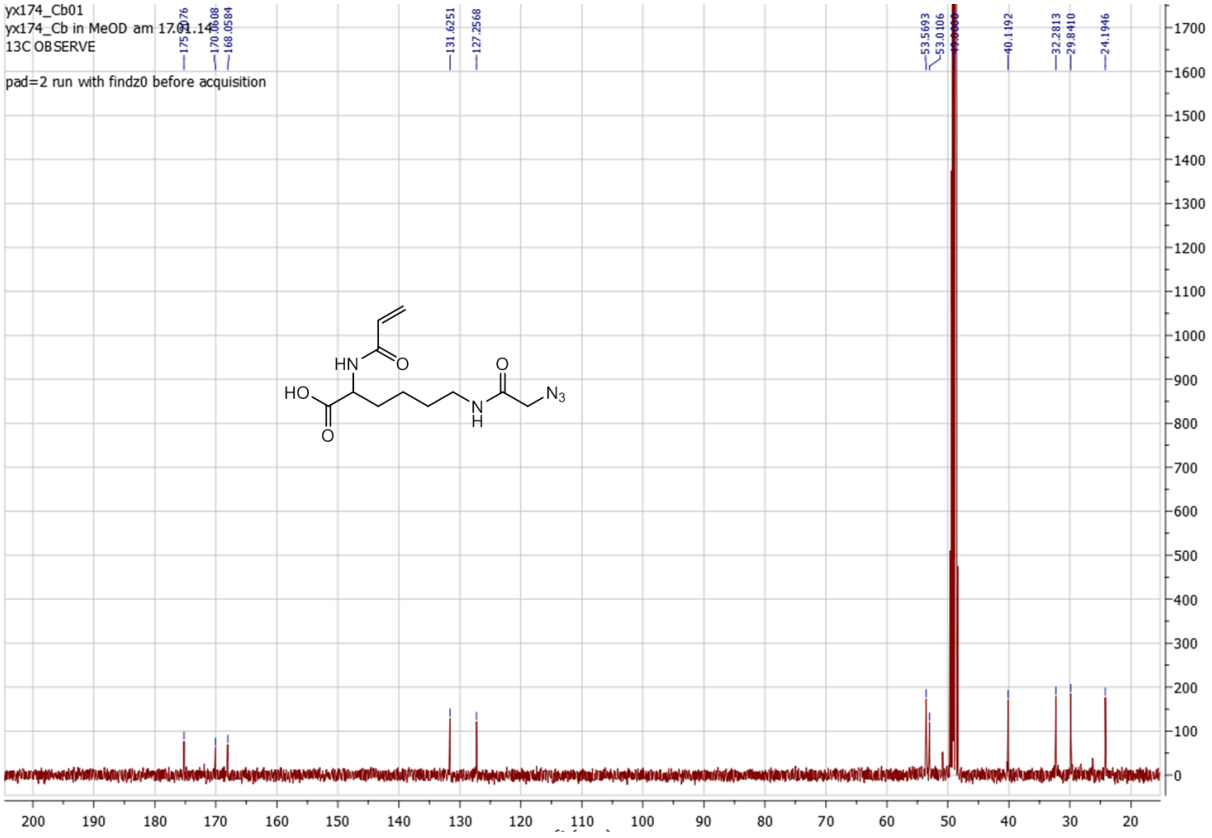
Figure S10: Molecular modeling of EGFP-Rab1-eDHFR-TAMRA revealing the possible distance between TAMRA and EGFP.

NMR SPECTRA:

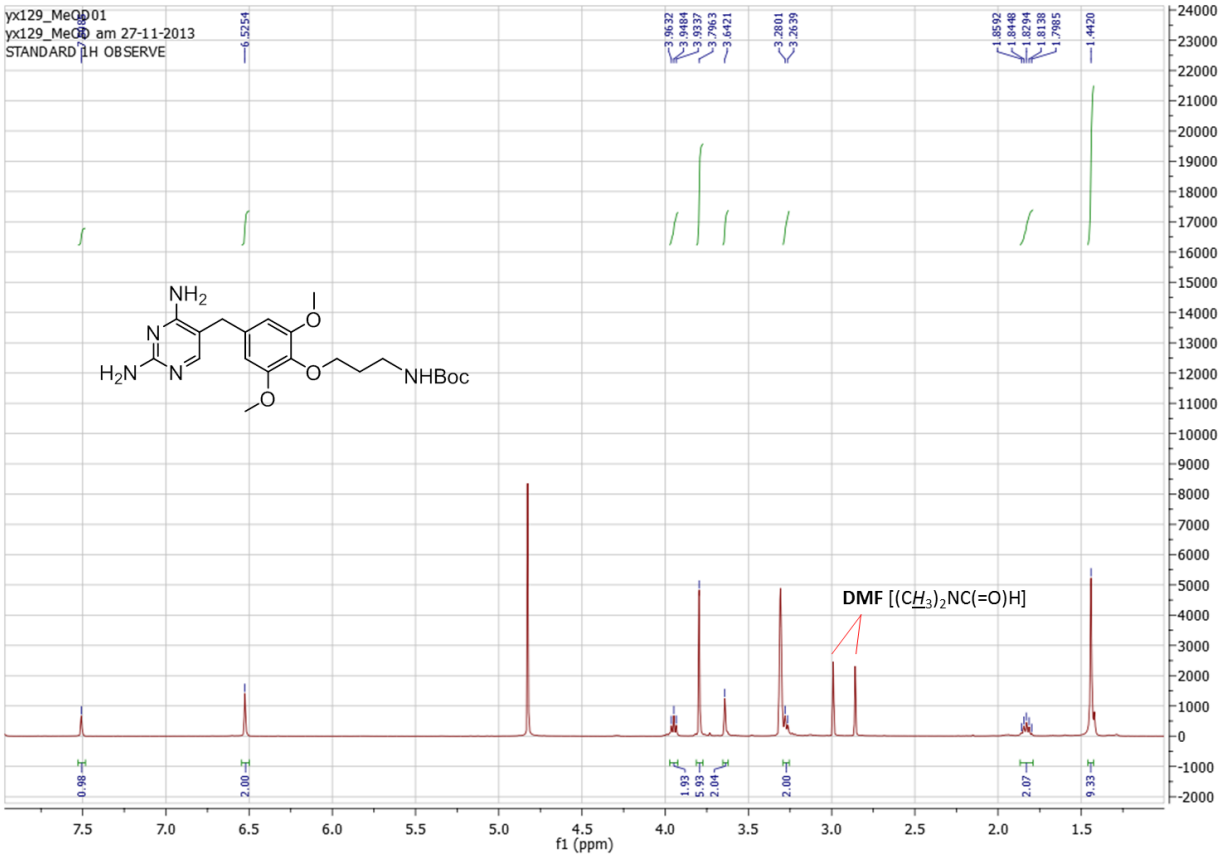
^{1}**H-NMR** (MeOD): N^{2}-Acryloyl-N^{6}-(2-azidoacetyl)-*L*-lysine (**6**)



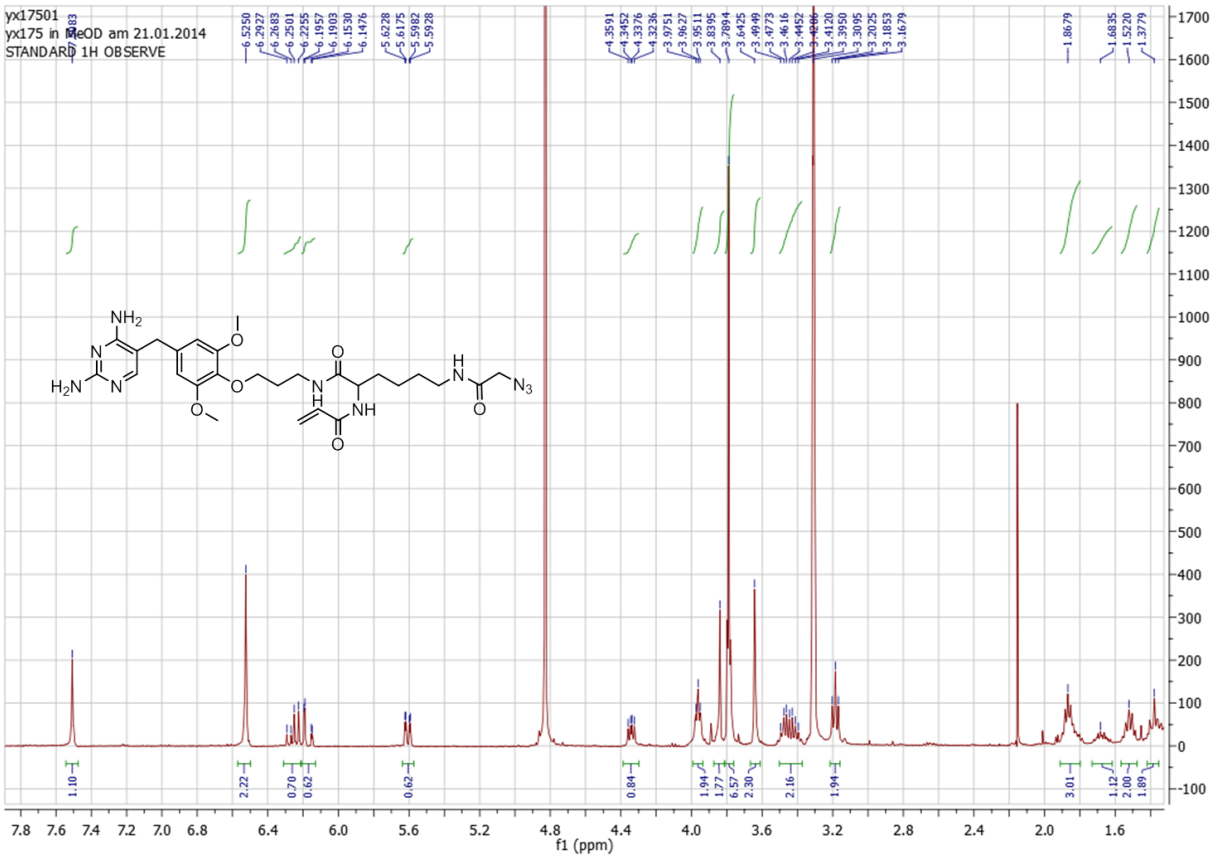
^{13}C-NMR (MeOD): N^2-Acryloyl-N^6-(2-azidoacetyl)-*L*-lysine (**6**)



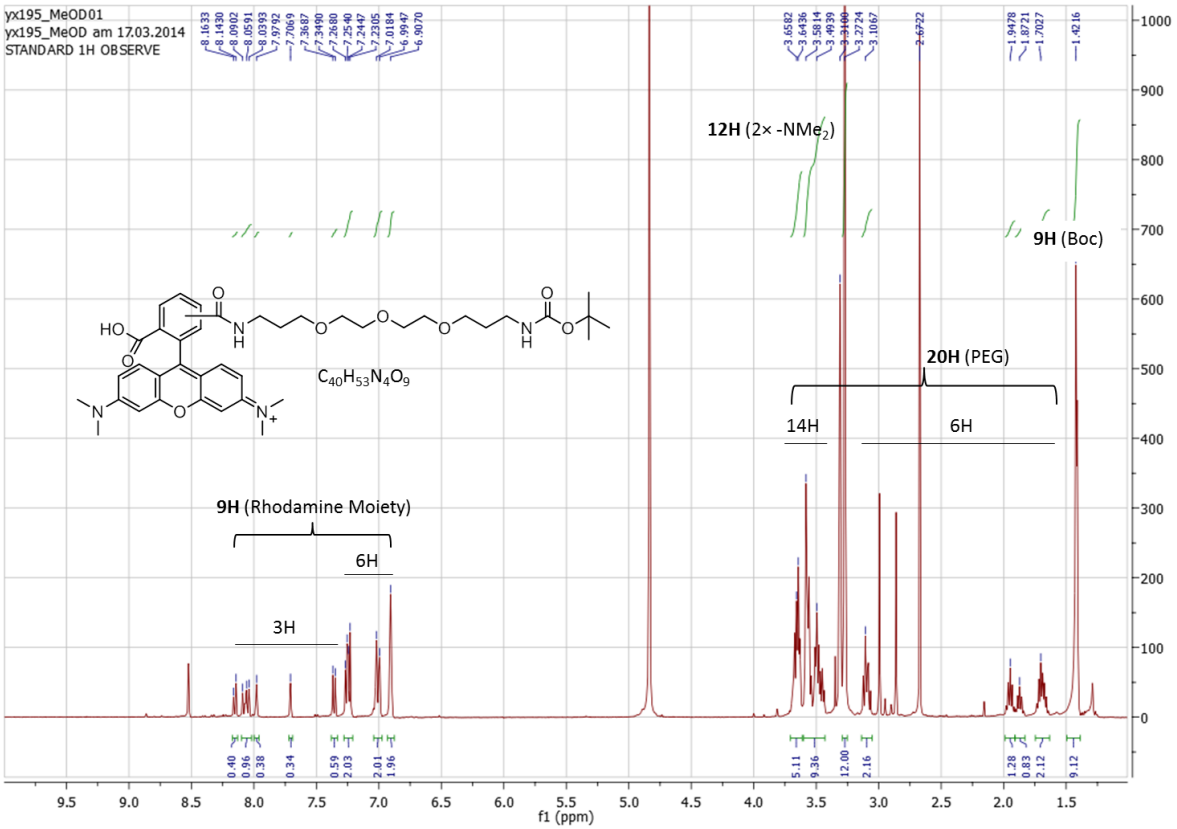
^{1}H-NMR (MeOD): TMP-Pr-NHBoc (**10**)



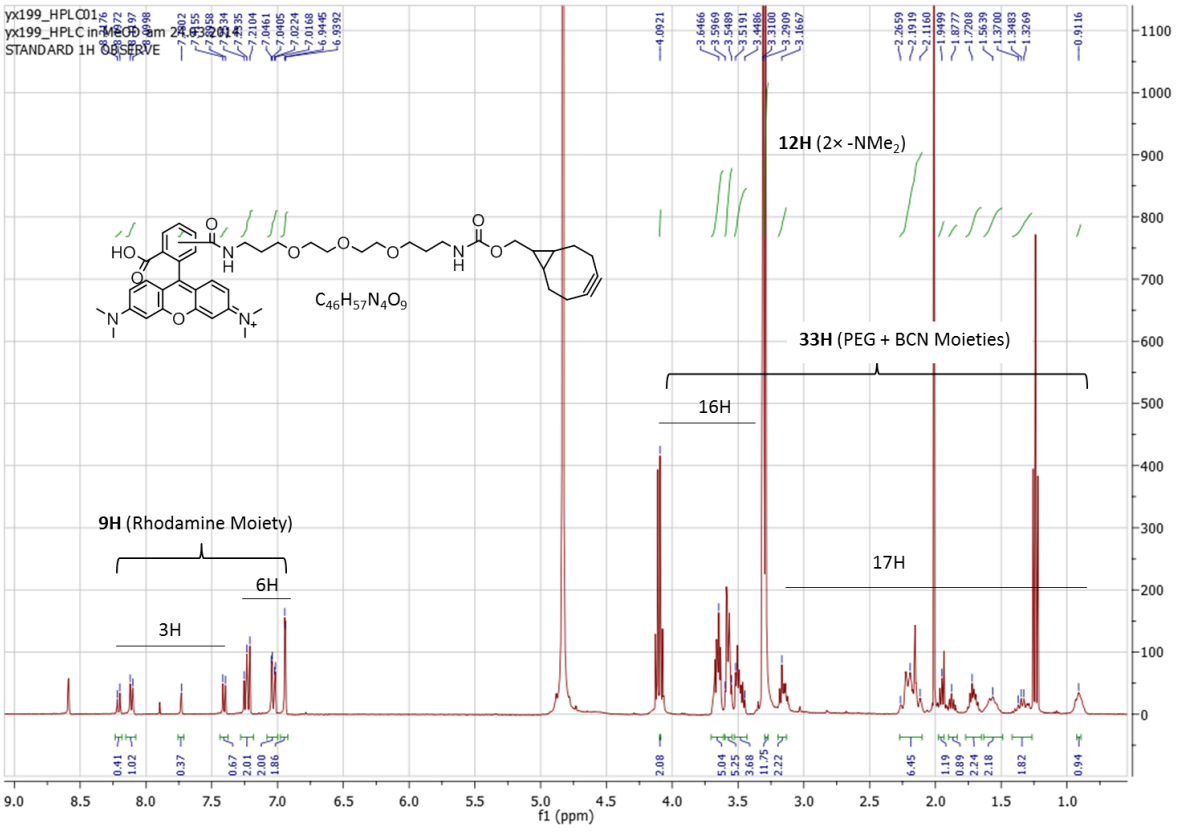
^{1}H-NMR (MeOD): TMP-AcAz (**1**)



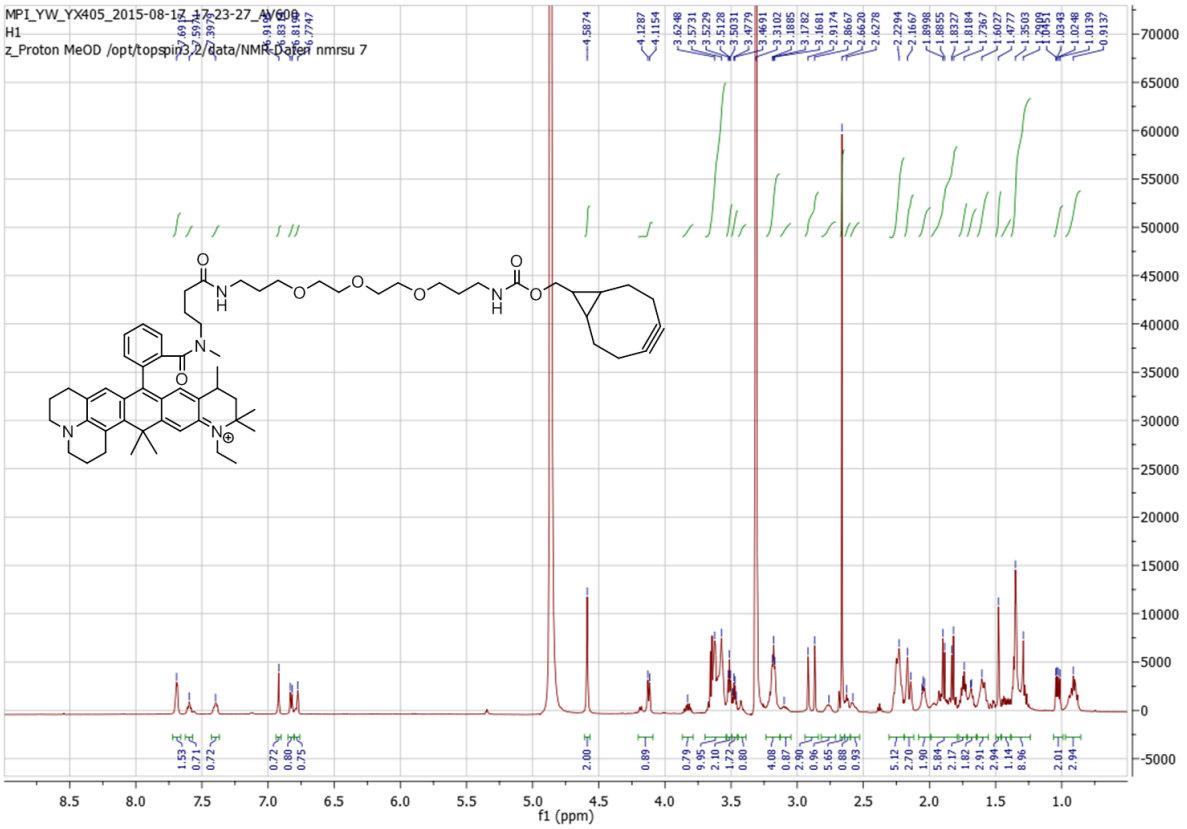
^{1}H-NMR (MeOD): 5/6-TAMRA-PEG$_3$-NHBoc (**S3**):



^{1}H-NMR (MeOD): BCN-TAMRA



^{1}H-NMR (MeOD): BCN-ATTO647N



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2. W. Liu, F. Li, X. Chen, J. Hou, L. Yi and Y. W. Wu, *Journal of the American Chemical Society*, 2014, **136**, 4468-4471.
3. Z. Chen, C. Jing, S. S. Gallagher, M. P. Sheetz and V. W. Cornish, *Journal of the American Chemical Society*, 2012, **134**, 13692-13699.
4. N. Mochizuki, S. Yamashita, K. Kurokawa, Y. Ohba, T. Nagai, A. Miyawaki, and M. Matsuda, *Nature*, 2001**, 411**, 1065-1068.