Electronic Supplementary Material (ESI) for Chemical Science.

Quantitative Interrogation of Protein Co-aggregation using Multi-color Fluorogenic Protein Aggregation Sensors

Yulong Bai, [ab] Wang Wan, *[a] Yanan Huang, [a] Wenhan Jin, [a] Haochen Lyu, [a] Qiuxuan Xia, [ab] Xuepeng Dong, [c] Zhenming Gao, [c] Yu Liu *[a]

a. CAS Key Laboratory of Separation Science for Analytical Chemistry Dalian Institute of Chemical Physics, Chinese Academy of Sciences 457 Zhongshan Road, Dalian 116023, China.

b. University of Chinese Academy of Sciences, Beijing 100049, China.

c. The Second Hospital of Dalian Medical University, 467 Zhongshan Road, Dalian, 116044, China.

* E-mail: liuyu@dicp.ac.cn; wanwang@dicp.ac.cn

Table of Contents

Supplemental Figures

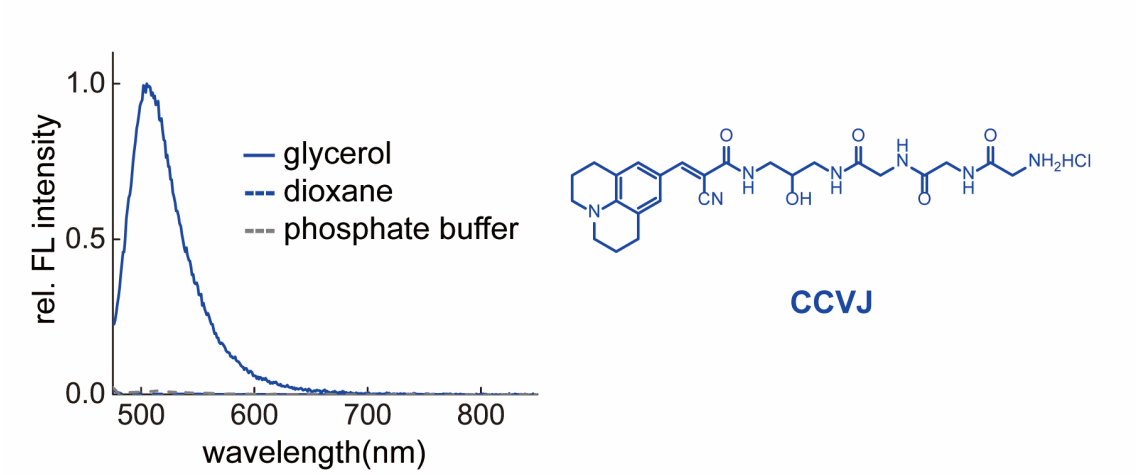


Figure S1. 9-(2-carboxy-2-cyanovinyl) julolidine (CCVJ) is a fluorescent molecular rotor that is sensitive to viscosity. CCVJ (10 µM) was dissolved in glycerol, dioxane and phosphate buffer, and fluorescent emission spectra were collected by using 460 nm as excitation wavelength. CCVJ showed strong fluorescence intensity in glycerol and almost no fluorescence emission in dioxane or phosphate buffer (10 mM sodium phosphate, 100 mM KCl, 1 mM EDTA, pH = 7.40). This result confirmed that the CCVJ is a fluorescent molecular rotor. The fluorescent spectra were measured by Tecan Spark Fluorescence Plate Reader using BeyoGold™ 96-Well Black Opaque plates.

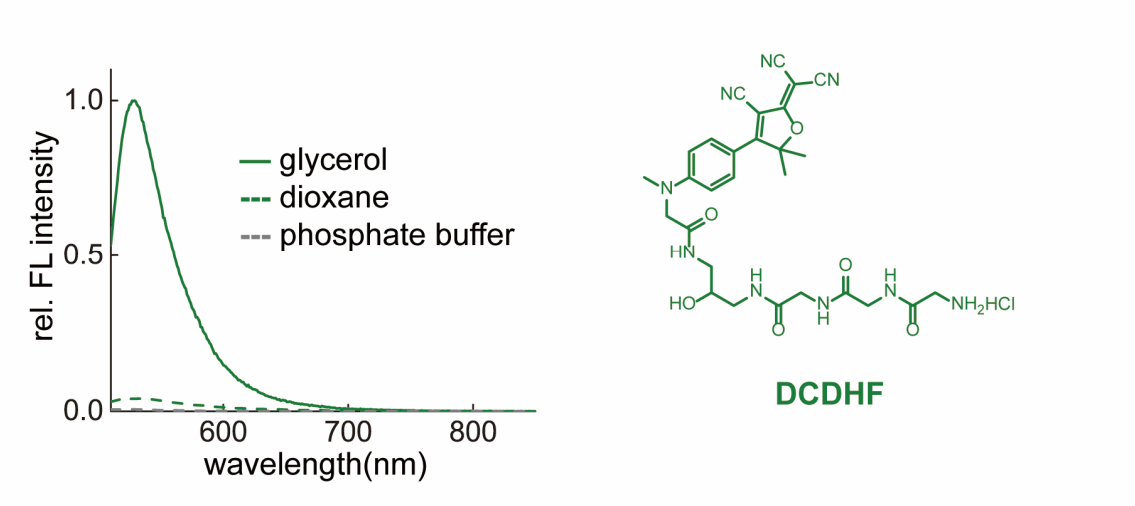


Figure S2. Dicyanomethylenedihydrofuran (DCDHF) is a fluorescent molecular rotor that is sensitive to viscosity. DCDHF (10 μM) was dissolved in glycerol, dioxane and phosphate buffer, and fluorescent emission spectra were collected by using 495 nm as excitation wavelength. DCDHF showed relatively strong emission intensity in glycerol solution, and much weaker emission intensity in dioxane or phosphate buffer (10 mM sodium phosphate, 100 mM KCl, 1 mM EDTA, pH = 7.40). It's confirmed that the DCDHF is a fluorescent molecular rotor. The fluorescent spectra were measured by Tecan Spark Fluorescence Plate Reader using BeyoGold™ 96-Well Black Opaque plates.

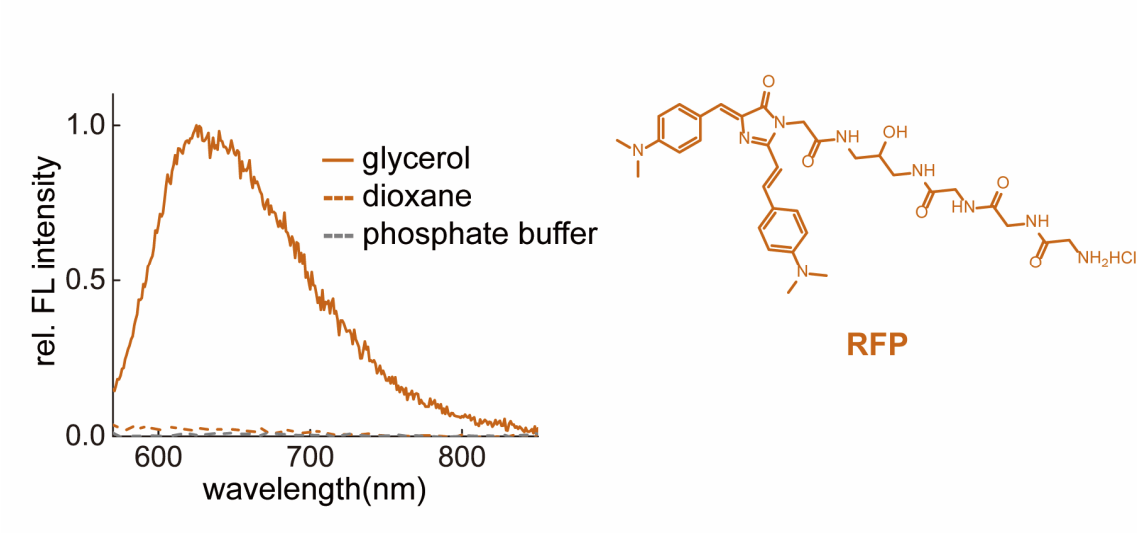


Figure S3. RFP chromophore analogue is a fluorescent molecular rotor that is sensitive to viscosity. RFP (10 μM) was dissolved in glycerol, dioxane and phosphate buffer, and fluorescent emission spectra were collected by using 555 nm as excitation wavelength. RFP showed strong emission intensity in glycerol solution, and much weaker emission intensity in dioxane or phosphate buffer (10 mM sodium phosphate, 100 mM KCl, 1 mM EDTA, pH = 7.40). It's confirmed that RFP probe is a fluorescent molecular rotor. The fluorescent spectra were measured by Tecan Spark Fluorescence Plate Reader using BeyoGold™ 96-Well Black Opaque plates.

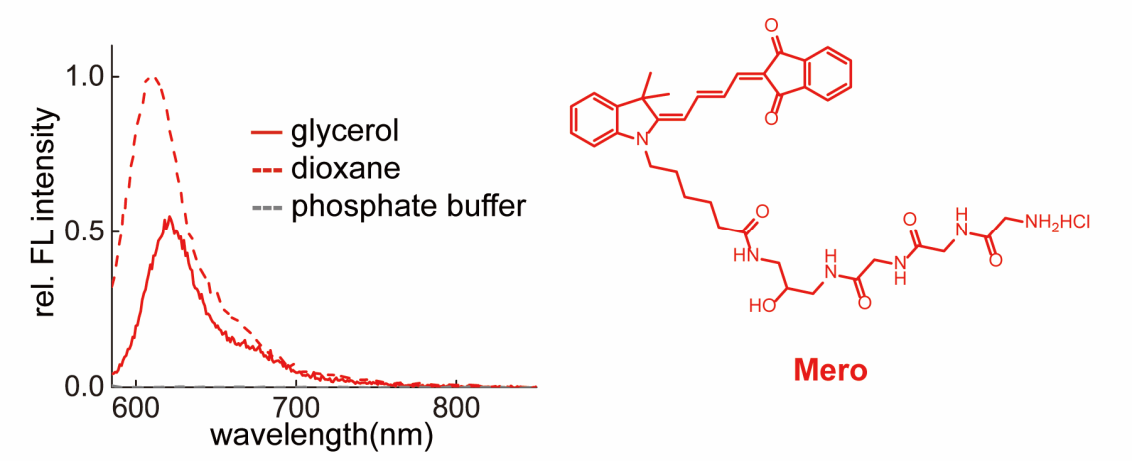


Figure S4. Mero is sensitive to polarity and viscosity, indicating it is both a solvatochromic fluorophore and a fluorescent molecular rotor. Merocyanine (10 µM) was dissolved in glycerol, dioxane and phosphate buffer to collected fluorescent emission spectra by using 570 nm as excitation wavelength. Mero showed relatively strong fluorescence intensity in both glycerol and dioxane, but no emission intensity in phosphate buffer (10 mM sodium phosphate, 100 mM KCl, 1 mM EDTA, pH = 7.40) was observed. This result demonstrated that Mero is both a solvatochromic dye and a fluorescent molecular rotor. But the solvatochromism plays a dominant role in environmental sensitivity of fluorescence intensity as the probe exhibited higher fluorescence in non-polar solvent than in viscous solvent. The fluorescent spectra were measured by Tecan Spark Fluorescence Plate Reader using BeyoGold™ 96-Well Black Opaque plates.

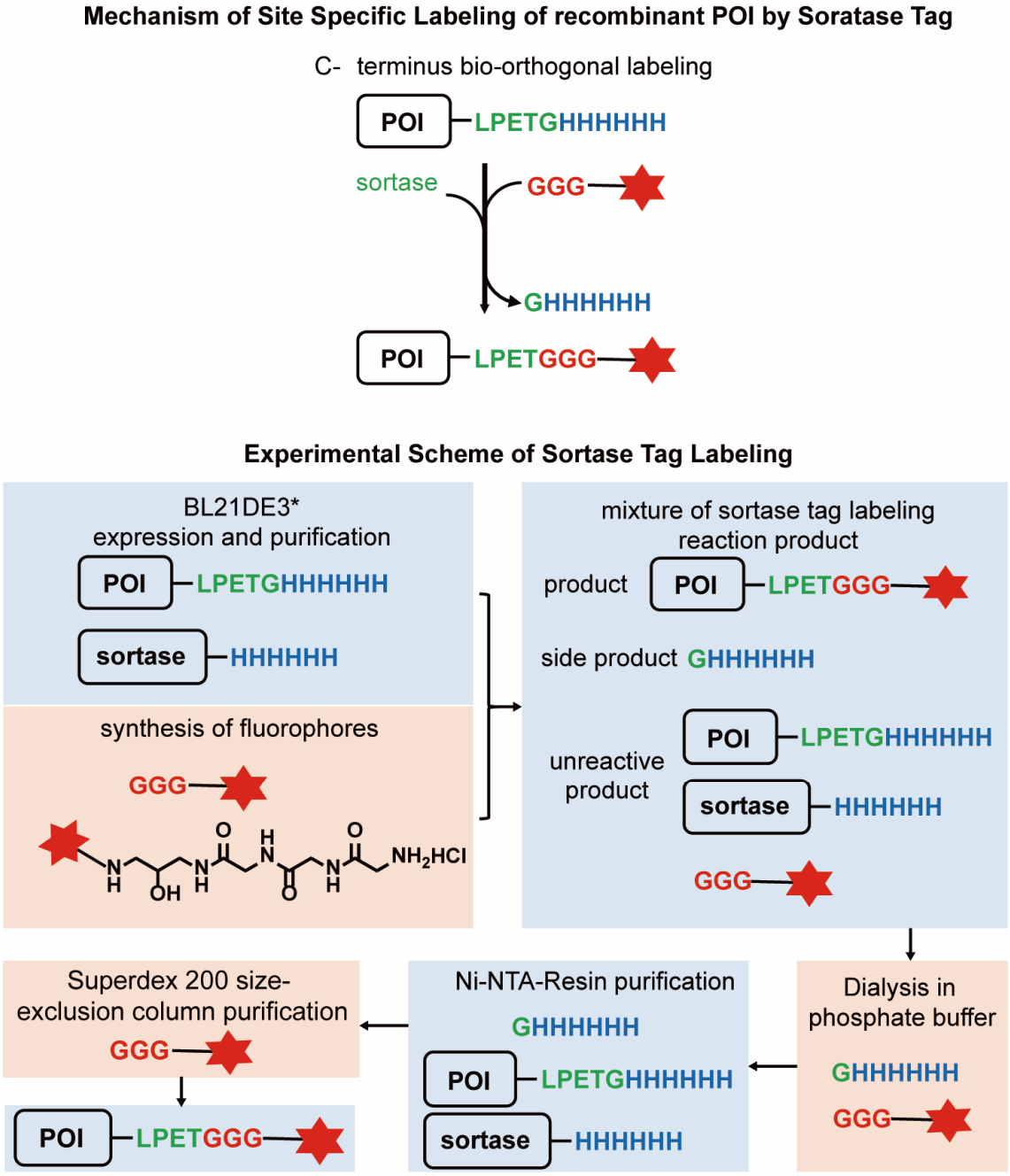


Figure S5. Mechanism and experimental scheme of site-specific labeling of POI with fluorophores via sortase tag mediated protein ligation technology. Experimental details see Experimental Methods 2. Briefly, protein-of-interest and sortase enzyme were prepared and purified in *E. coli* BL21 DE3* cells. Probes containing GGG sequence for sortase ligation were synthesized accordingly. To site specifically modify the C-terminus of a protein-of-interest, the starting materials were prepared as follows: 10X ligation buffer (500 mM Tris-HCl, 1.5 M NaCl, 100 mM $CaCl_2$), sortase (500 μM) in phosphate buffer (10 mM phosphate, 100 mM KCl, 1.0 mM EDTA, pH = 7.40), POIs (500 μM) in phosphate buffer (10 mM phosphate, 100 mM KCl, 1.0 mM EDTA, pH = 7.40), probe stock solution (50 mM) in DMSO and ddH_2O. To 50 mL sterilized reaction vessel, reaction mixture was prepared to the final concentrations as ligation buffer (1×), sortase (50 μM), protein of interest (100 μM), fluorophore (500 μM) and gently mixed well. The reaction mixture was then kept at room temperature over 4 hours. Dialysis of the reaction mixture in phosphate buffer overnight at 4 °C removed the majority of the unreacted probe and side product (GHHHHHH). The labelled protein mixture was further purified through Ni-NTA-Resin purification to remove the intact unlabelled protein-of-interest and sortase enzyme. The desired product was the flow-through from Ni-NTA column. Finally, the labelled POI was then eluted out of the Superdex 200 size-exclusion column to remove residual probes and yield pure fully labelled proteins.

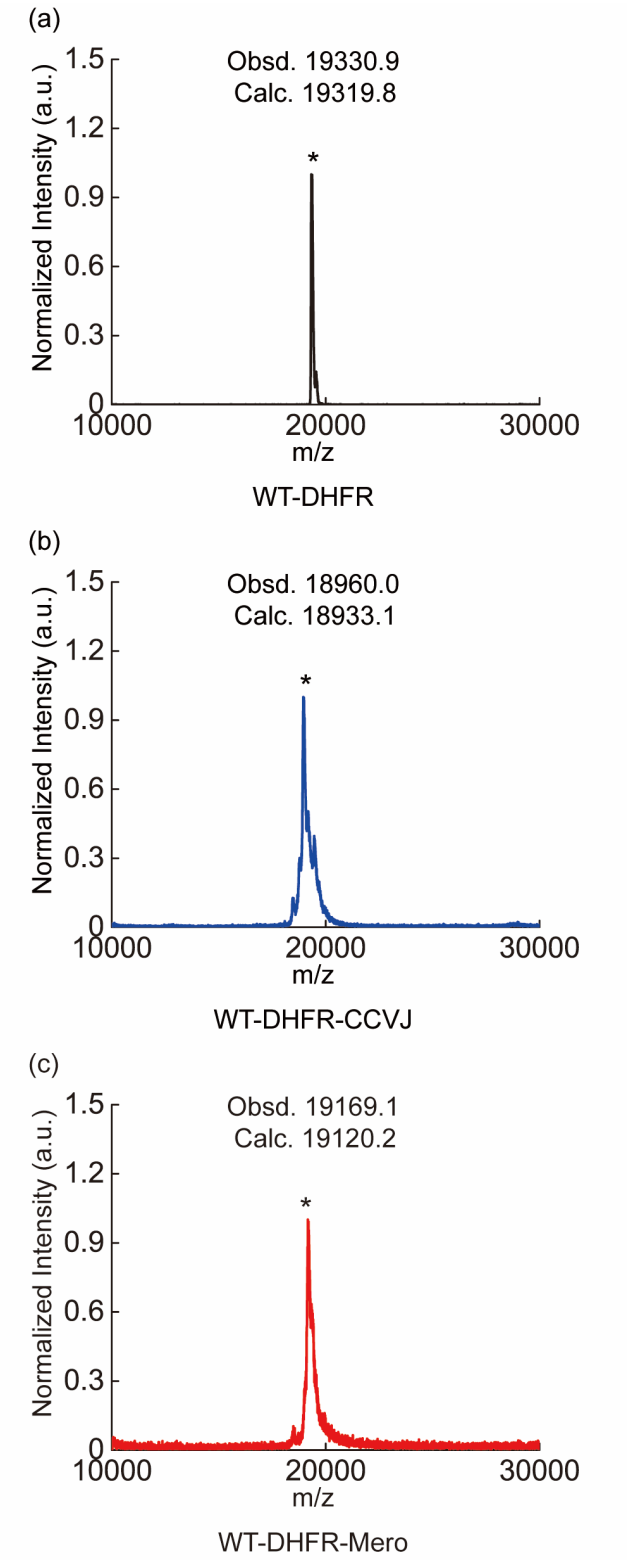


Figure S6. MALDI-TOF mass spectrometry results showed that WT-DHFR was efficiently labelled with CCVJ and Mero probes. (a) MALDI mass spectra of WT-DHFR (19330.9 Da). (b) MALDI mass spectra of WT-DHFR-CCVJ (18960.0 Da). (c) MALDI mass spectra of WT-DHFR-Mero (19169.1 Da). MALDI results indicated satisfactory labeling efficiency of CCVJ and Mero with WT-DHFR using the sortase tag mediated site-specific ligation. Meanwhile, no unmodified peak and other peaks were observed in CCVJ and Mero labelled samples, showing the high purity of the samples we utilized for the experiments throughout this work. Protein concentration is 0.1 mg/mL in double deionized water, sinapic acid was selected as matrix to assist ionization. For each sample, 1.0 μL protein solution and 1.0 μL SA solution were coated together on steel target plate. The measurement was performed on ABI MALDI TOF/TOF 5800.

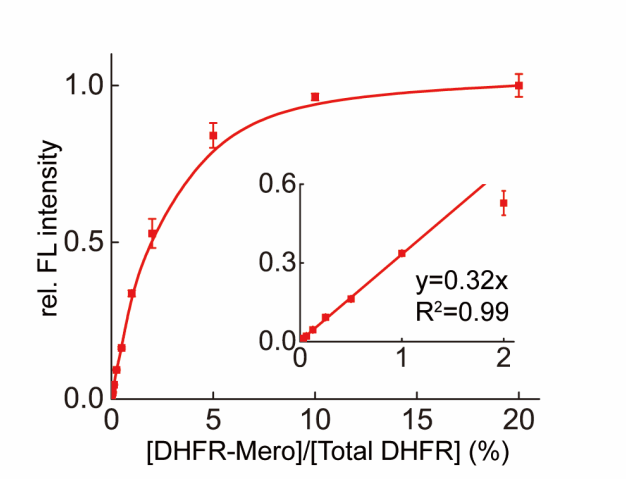


Figure S7. Detection limits and linear range measurement of Mero sensor in detecting WT-DHFR aggregation. WT-DHFR protein (50 μM, 0.63‰-20.00% labelled with Mero) was incubated in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) at 61 °C for 5 min to induce its aggregation. Fluorescence intensity was measured with excitation wavelength at 600 nm. Linear range was determined as 0.63 ‰ to 2.00%. The lowest limit of detection is down to 0.63‰ of labelled protein. These results demonstrated that our method exploring environment-sensitive fluorophore Mero can quantitatively detect protein aggregation using substoichiometric labelled proteins at fairly low concentration (0.0315 μM).

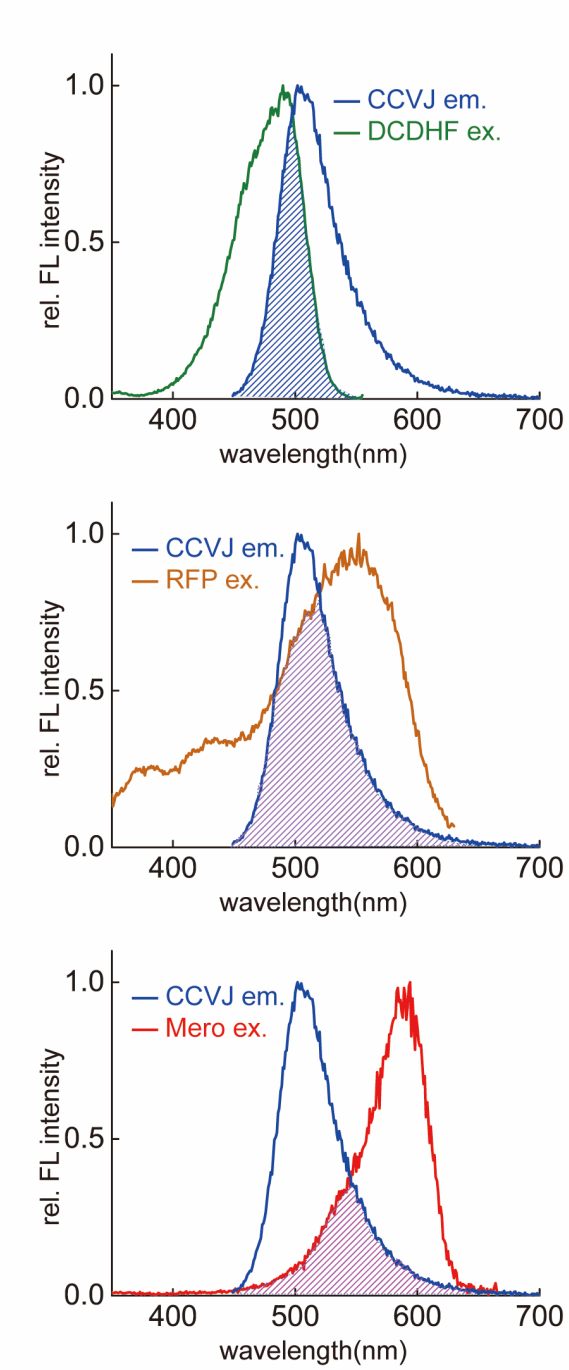


Figure S8. CCVJ/DCDHF and CCVJ/RFP pairs are not suitable for dual-color thermal shift assay due to the significant spectral overlaps, while CCVJ/Mero pair has relatively less spectral overlap**.** Excitation spectra of both RFP and DCDHF overlapped heavily with emission spectrum of CCVJ. However, excitation spectrum of Mero overlapped with emission spectrum of CCVJ much less than RFP and DCDHF. In particular, we utilized 460 nm wavelength to collect the emission intensity of CCVJ, at which Mero was not significantly excited. In addition, as we reduced the stoichiometry of labelled proteins, the CCVJ-Mero pair does not exhibit FRET effect at all (Figure 2f, 2g). No FRET effect ensures accurate reporting on the aggregation behavior when simultaneously detecting two proteins-of-interet. Therefore, CCVJ and Mero pair was selected to monitor dual-protein co-aggregation process.

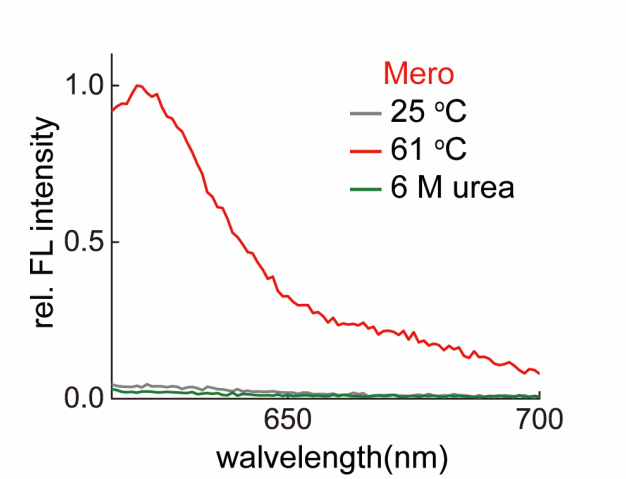


Figure S9. Mero-labelled WT-DHFR was non-fluorescent upon urea induced protein unfolding. Unfolded WT-DHFR (50 uM, 2.0% labelled with Mero) was prepared by incubating with 6 M urea for 12 h at 4 °C and emission spectrum was collected (green line, ex. = 600 nm). As a negative control, WT-DHFR (50 uM, 2.0% labelled with Mero) was incubated in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) for 5min at 25 °C. Emission spectrum was collected (grey line, ex. = 600 nm). As a positive control, WT-DHFR (50 uM, 2.0 % labelled with Mero) was incubated in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) for 5 min at 61 °C to induce protein aggregation and then the emission spectrum was collected (red line, ex. = 600 nm). This result demonstrated unfolded protein is not capable of turning on the fluorescence of Mero.

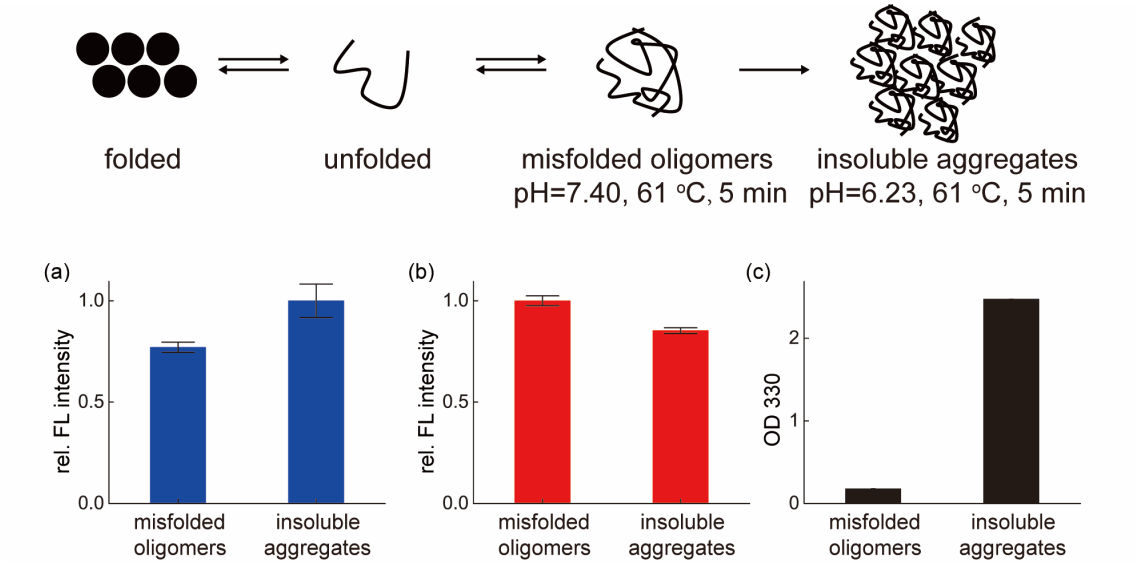


Figure S10. The fluorescence of CCVJ (in blue) and Mero (in red) was turned on as the formation of misfolded soluble oligomeric DHFR and the fluorescence intensity was retained in aggregated DHFR. Misfolded soluble oligomers of WT-DHFR was formed by incubating the protein (50 μM) in heated neutral phosphate buffer (10 mM sodium phosphates,100 mM KCl,1 mM EDTA, acidified by HCl to pH = 7.40, 5 min at 61 °C). Presence of soluble oligomers at this condition was shown by chemical crosslinking experiment in Figure 3e. Insoluble aggregated DHFR (50 μM) was formed in heated acidic aggregation formation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23, 61 °C for 5 min). (a) CCVJ fluorescence was turned on to the similar intensity when DHFR formed soluble oligomers (pH = 7.40, left bar) and insoluble aggregates (pH = 6.23, right bar). (b) Mero fluorescence was turned on to the similar intensity when DHFR formed soluble oligomers (pH = 7.40, left bar) and insoluble aggregates (pH = 6.23, right bar). (c) WT-DHFR formed misfolded oligomers when heated to 61 °C for 5 min at pH = 7.40. At this neutral pH and heated conditions, low turbidity was observed (pH = 7.40, left bar) (error bars), indicating its soluble oligomeric state.

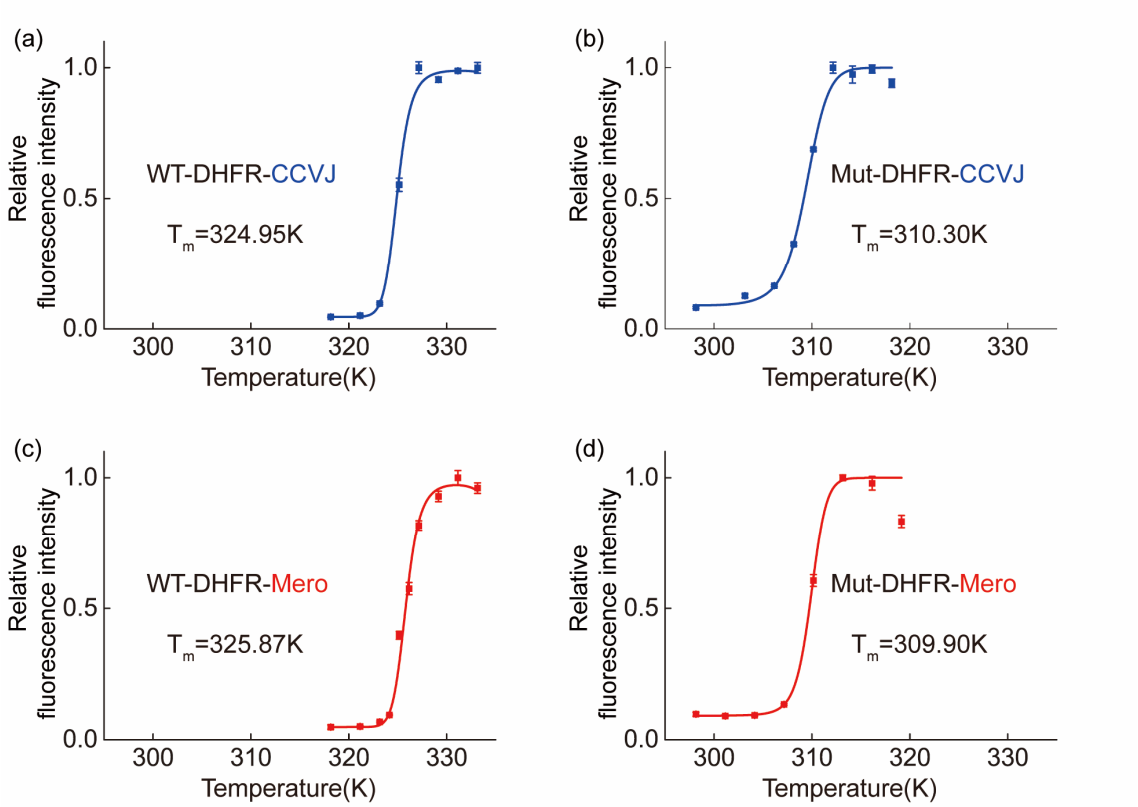


Figure S11. Swapping CCVJ and Mero to conjugate different proteins yields similar melting temperatures (T_m), demonstrating the robustness of this assay in quantitative detection of protein aggregation. WT-DHFR was conjugated with CCVJ (a, 324.95 K) and Mero (c, 325.87 K), resulting in similar T_m values. Mut-DHFR was conjugated with CCVJ (b, 310.30 K) and Mero (d, 309.90 K), resulting in similar T_m values as well. Experimental conditions: (a) For WT-DHFR-CCVJ, freshly purified WT-DHFR protein (49.0 μM) and CCVJ labelled WT-DHFR protein (1.0 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated from 46 °C to 60 °C with 2 °C intervals. The heated mixture was pipetted into BeyoGold™ 96-Well Black Opaque plates and then collected the fluorescence emission intensity at 500 nm by using 460 nm as the excitation wavelength. For each temperature, the experiments were repeated three times. (b) For Mut-DHFR-CCVJ, freshly purified Mut-DHFR protein (49.0 μM) and CCVJ labelled Mut-DHFR protein (1.0 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated from 25 °C to 46 °C with 3 °C intervals. The heated mixture was pipetted into BeyoGold™ 96-Well Black Opaque plates and then collect the fluorescence emission intensity at 500 nm using 460 nm as the excitation wavelength. For each temperature, the experiments were repeated three times. (c) For WT-DHFR-Mero, freshly purified WT-DHFR protein (49.0 μM) and Mero labelled WT-DHFR protein (1.0 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated from 46 °C to 60 °C with 2 °C intervals. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates and then collect the fluorescence emission intensity at 620 nm by using 600 nm as the excitation wavelength. For each temperature, the experiments were repeated three times. (d) For Mut-DHFR-Mero, freshly purified Mut-DHFR protein (49.0 μM) and Mero labelled Mut-DHFR protein (1.0 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated from 25 °C to 46 °C with 3 °C intervals. The heated mixture was pipetted into BeyoGold™ 96-Well Black Opaque plates and then collect the fluorescence emission intensity at 620 nm by using 600 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

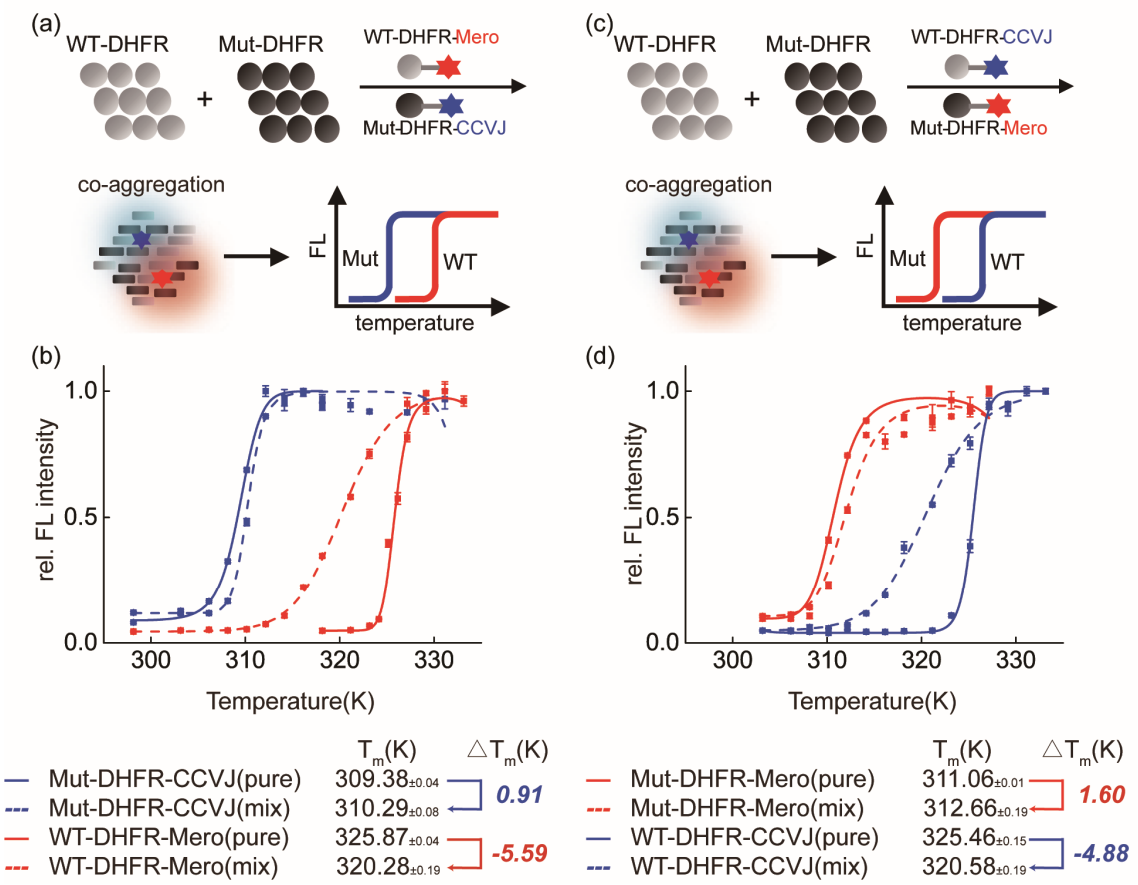


Figure S12. Swapping CCVJ and Mero to conjugate different proteins yields similar melting temperatures (T_m), demonstrating the robustness of this assay in quantitative detection of protein aggregation. WT-DHFR was conjugated with Mero while Mut-DHFR was conjugated with CCVJ, or WT-DHFR was conjugated with CCVJ while Mut-DHFR was conjugated with Mero,resulting in similar conclusion. Experimental conditions: (b) Freshly purified WT-DHFR (24 μM) and Mut-DHFR protein (24 μM), Mero labelled WT-DHFR (1 μM) and CCVJ labelled Mut-DHFR protein (1 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated from 25 °C to 64 °C with 3 °C temperature intervals. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates. We then collected the fluorescence emission intensity at 620 nm using 600 nm as the excitation wavelength for Mero fluorescence, and the fluorescence emission intensity at 500 nm using 460 nm as the excitation wavelength for CCVJ fluorescence. For each temperature, the experiments were repeated three times. (d) Freshly purified WT-DHFR (24 μM) and Mut-DHFR protein (24 μM), CCVJ labelled WT-DHFR (1 μM) and Mero labelled Mut-DHFR protein (1 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated from 25 °C to 64 °C with 3 °C temperature intervals. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates. We then collected the fluorescence emission intensity at 620 nm using 600 nm as the excitation wavelength for Mero fluorescence, and the fluorescence emission intensity at 500 nm using 460 nm as the excitation wavelength for CCVJ fluorescence. For each temperature, the experiments were repeated three times.

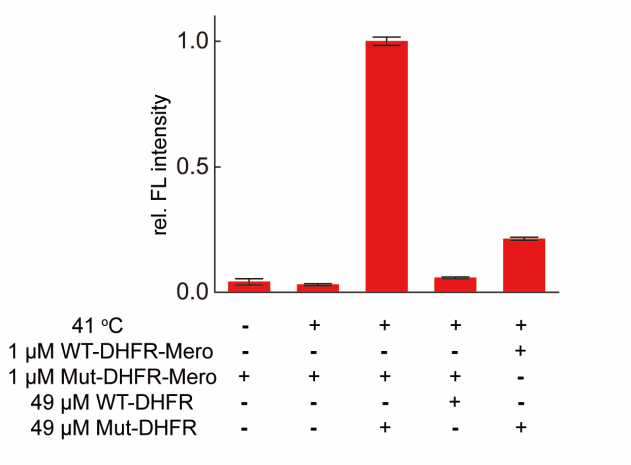


Figure S13. Co-aggregation of unlabelled Mut-DHFR and surrounding labelled Mut-DHFR-Mero is required to fully turn on the fluorescence of Mero. Since we utilized substoichiometric condition to detect protein aggregation, we asked ourselves what are required to turn on Mero's fluorescence. To dissect this mechanism, we aggregated Mut-DHFR protein at 41 °C for 5 min in acidic aggregation buffer whereas WT-DHFR was well folded at this condition. The 1st bar, the 2nd, and the 4th bars showed no fluorescence increase when the substoichiometric Mut-DHFR-Mero was aggregated in the presence of unlabelled and folded WT-DHFR. This result indicated that aggregation of the labelled conjugates itself is not capable of fully turning on the fluorescence. In contrast, when Mut-DHFR-Mero was aggregated in the presence of unlabelled Mut-DHFR (3rd bar), Mero fluorescence was fully turned on. This result indicated that the intermolecular interaction of Mero and surrounding aggregated unlabelled Mut-DHFR is essential to create a non-polar and compact environment to fully turn on Mero's fluorescence. Interestingly, we observed less fluorescence turn-on when WT-DHFR-Mero was folded (5th bar) even unlabelled Mut-DHFR was aggregated. It indicated that surrounding aggregated proteins is not capable of turning on Mero probe on folded proteins. These results together supported that it requires the labelled protein to be misfolded first and then aggregated together with the surrounding unlabelled proteins to fully switch on the fluorescence. In all 5 different conditions, the fluorescence emission intensity was collected at 620 nm by using 600 nm as the excitation wavelength.

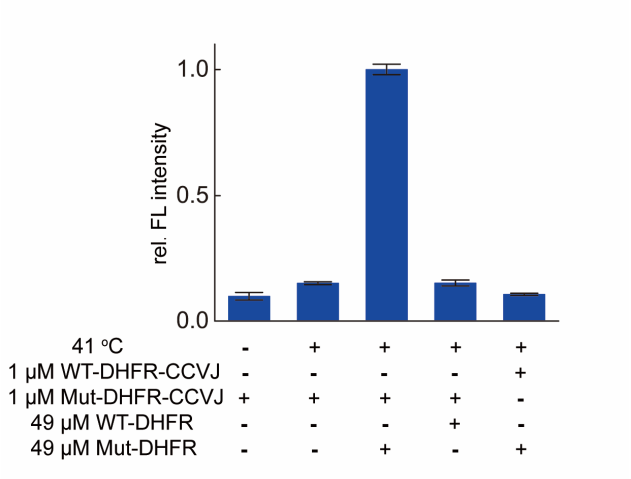


Figure S14. Co-aggregation of unlabelled Mut-DHFR and surrounding labelled Mut-DHFR-CCVJ is required to fully turn on the fluorescence of CCVJ. Experimental details were the same as in Figure S12 and only differed in the fluorophore used. Both Figure S12 and S13 supported the same conclusion. To fully switch on the fluorescence, the labelled protein needs to aggregated together with the surrounding unlabelled proteins.

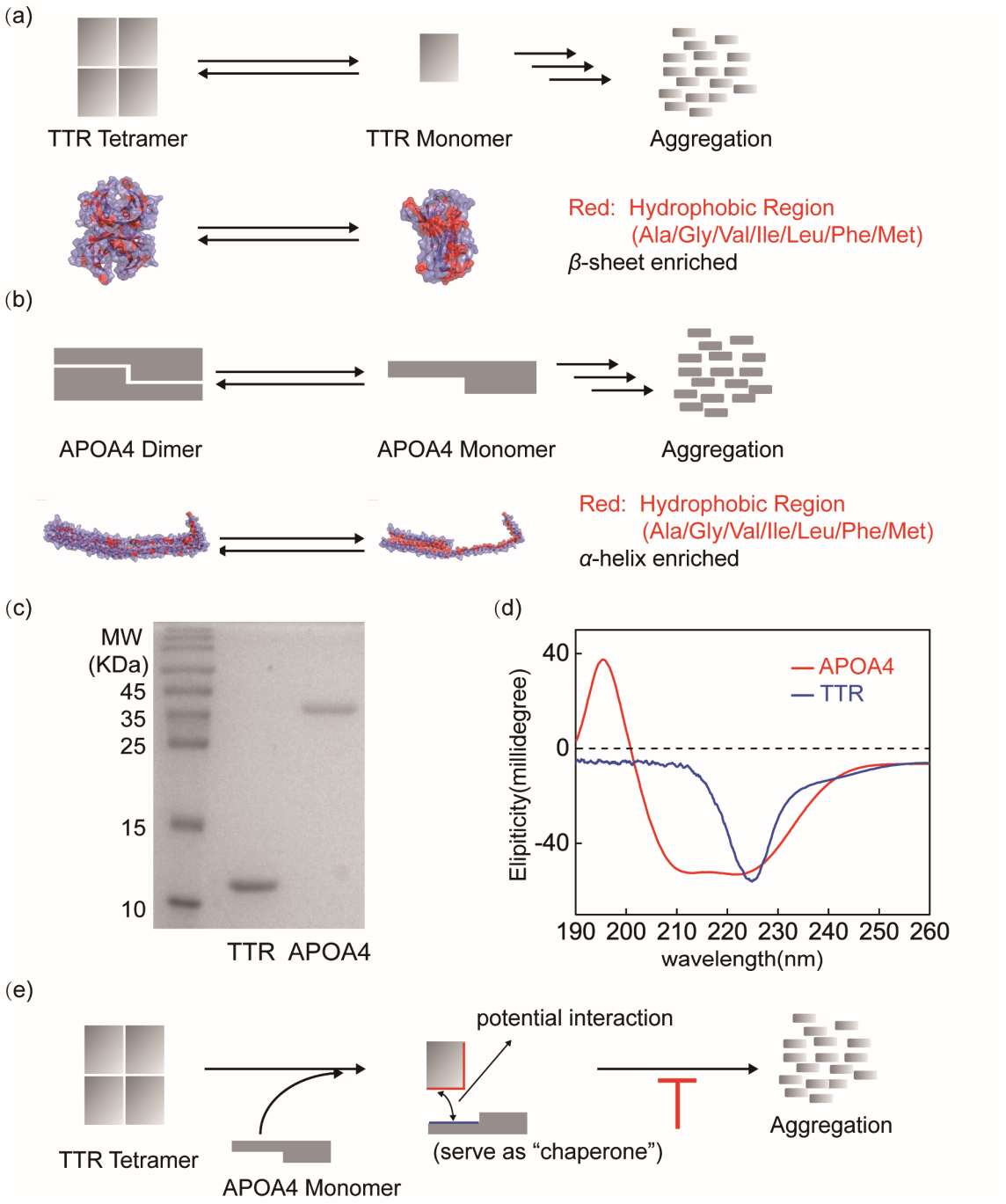


Figure S15. (a) & (b) Structural analysis of transthyretin (TTR) and apolipoprotein A-IV (APOA4). TTR-L55P structure (PDB: 3DK0) and APOA4 structure (PDB: 3S84) were chosen from RCSB Protein Data Bank. TTR is a β-sheet enriched tetrameric protein, whose tetramer dissociation is the rate-limiting step of protein misfolding and amyloid formation. APOA4 protein is an α-helix enriched protein in both monomeric and dimeric forms. Hydrophobic residues (Ala/Gly/Val/Ile/Leu/Phe/Met) were highlighted in red color, showing the dimer or tetramer interphase of APOA4 and TTR is exclusively hydrophobic. (c) Purity of recombinant TTR and APOA4. (d) Far UV Circular Dichroism Spectroscopy analysis of TTR and APOA4 showed that TTR is a β-sheet enriched protein and APOA4 protein is an α-helix enriched protein. (e) Proposed model of APOA4 serving as a pathological chaperone that may potentially interact with TTR upon its misfolding and aggregation.

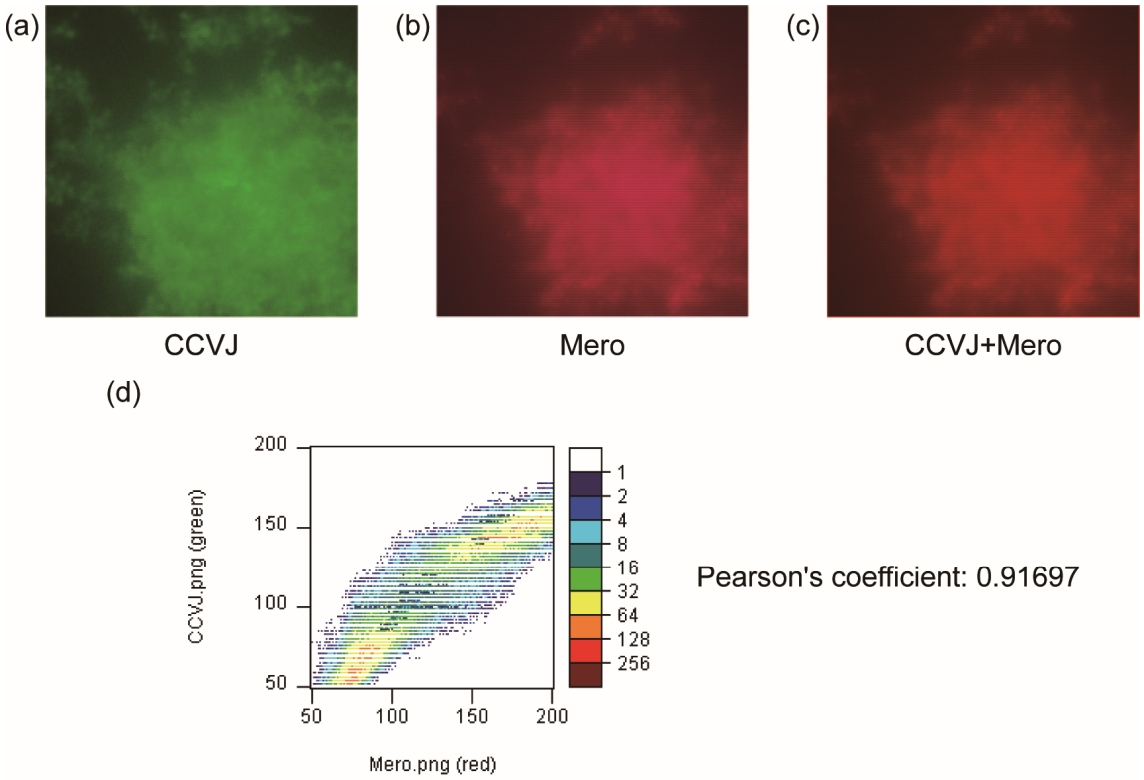


Figure S16. Colocalization of CCVJ and Mero fluorescence in the mixture of aggregated TTR(L55P)-CCVJ and APOA4-Mero, supporting the co-aggregation of these two proteins.

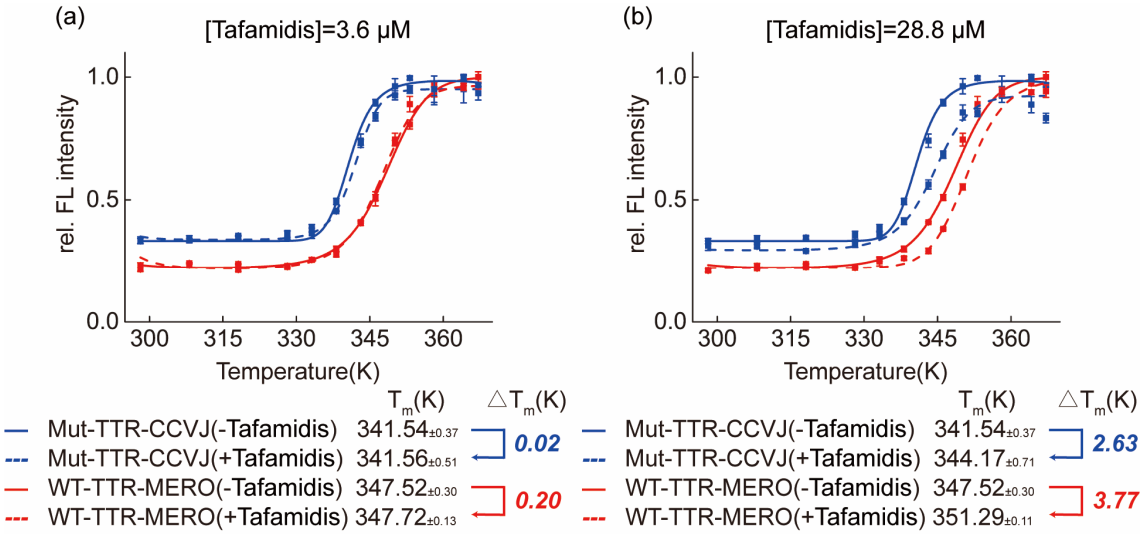


Figure S17. Quantitative evaluation of the thermodynamic stabilization effect on the heterozygous mixture of wild type (WT) and mutant (Mut) transthyretin (TTR) upon binding to the kinetic stabilizer Tafamidis. Thermodynamic stabilization effect of both WT- and Mut-TTR by Tafamidis at substoichiometric (a) and stoichiometric (b) concentrations was quantified. To investigate the differential effect of Tafamidis toward stabilizing WT-TTR and Mut-TTR protein in a heterozygous system, TTR mixture (14.4 μM in total) containing WT-TTR (6.912 μM), Mut-TTR (6.912 μM), Mut-TTR-CCVJ (0.288 μM) and WT-TTR-Mero (0.288 μM) after subunit exchange were prepared in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 4.40). Tafamidis at different concentration (3.6 μM or 28.8 μM) was introduced into the TTR mixture followed by the same aggregation and fluorescence measurements protocol in Figure 5. $\triangle T_m$ describes the extent of thermodynamic stabilization of TTR by Tafamidis. Compared to lower concentration of tafamidis (3.6 μM), higher concentration of tafamidis (28.8 μM) displayed much better stabilization effect for both WT- and Mut-TTR proteins. As a kinetic stabilizer, it is not surprise to see less stabilization effect compared to what we observed in the TMP stabilization of DHFR scenario. Because Tafamidis only stabilizes the dissociation of the tetramer of TTR, its effect on the thermodynamic stability of monomeric TTR is minimal.

Experimental Method Section

1. Plasmids construct and protein purifications.

cDNA gene of WT-DHFR (*Ec*DHFR), Mut-DHFR (M42T:H114R *Ec*DHFR), *h*DHFR, WT-TTR and Mut-TTR (L55P), APOA4 were synthesized by Genescript, Nanjing, China, followed by subcloning into the pET29b+ vector. The C-termini of these four proteins were fused with a LPETGHHHHHH sequence for sortase ligation and His-Tag purification purposes. SOD1 (V31A) -Halo and SOD1 (G85R) -Halo in pHTC vector and 97Q-SNAPf in pcDNA3.1 vector were previously reported. They were gifts generously provided by Professor XinZhang's group from the Pennsylvania State University, USA.

WT-DHFR-His (or Mut-DHFR-His, or *h*DHFR-His, or WT-TTR-His, or Mut-TTR-His, or APOA4) plasmid was transformed into BL21 DE3 *E. coli* cells. Cells were grown to OD_{600} at 0.6-0.8 before induced by IPTG (0.5 mM/L) at 37 °C for 4 h (Mut-DHFR and *h*DHFR at 30 °C for 4 h). Cultured cells were harvested and resuspended in resuspension buffer (50 mM Tris,100 mM NaCl, pH = 8.00). Cells expressing recombinant proteins were thawed and lysed by sonication at 4 ºC. Lysed cells were centrifuged for 30 min at 16,000 rpm. The supernatant was collected and loaded onto a 10 mL Ni-NTA column and washed with buffer A (50 mM Tris,100 mM NaCl, pH = 8.00). The proteins were then eluted by gradient addition of buffer B (50 mM Tris • HCl, 100 mM NaCl, 500 mM imidazole, pH = 8.00). The protein fractions were identified by SDS-PAGE analysis, pooled, and concentrated. The proteins were further purified using a 120 mL Superdex 200 size-exclusion column in phosphate buffer (10 mM sodium phosphates,100 mM KCl,1 mM EDTA, pH = 7.40). The protein containing fractions were identified by SDS-PAGE gel analysis, pooled, and concentrated. No significant impurities were identified and purity was estimated to be 98% based on SDS-PAGE electrophoresis analysis.

2. Site-specific labeling and purification (Figure S5)

Protein of interest was labelled by using sortase mediated ligation protocols.[1] Probes were modified with three glycine (GGG) repeat moiety via chemical synthesis (Synthetic Method). Starting materials were readily prepared including 10× ligation buffer (500 mM Tris-HCl, 1.5 M NaCl, 100 mM $CaCl_2$, working concentration is 1X), sortase in phosphate buffer (10 mM phosphate, 100 mM KCl, 1.0 mM EDTA, pH = 7.40), protein in phosphate buffer (10 mM sodium phosphates,100 mM KCl,1 mM EDTA, pH = 7.40), stock solution (50 mM in DMSO) and ddH_2O. To 50 mL sterilized reaction vessel ligation buffer (1×), sortase (50 μM), protein of interest (100 μM), fluorophore (500 μM) was finely mixed, and then kept at room temperature over 4 hours. Dialysis of the reaction mixture in phosphate buffer overnight at 4 °C removed the unreacted probes and by-products. The labelled protein was purified through Ni-NTA-Resin purification to remove the unlabelled proteins and sortase enzymes, and then further purified using Superdex 200 size-exclusion column to yield pure fully labelled proteins.

3. Fractionation experiment of aggregated DHFR (Figure 2c).

WT-DHFR (49 μM) was mixed with CCVJ/DCDHF/RFP/Mero labelled WT-DHFR (1 μM) respectively in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23). Protein aggregation (1 mL) was induced by heating the sample at 61 °C for 5 min. The total fraction of the aggregated sample (100 μL) was pipetted out to measure its fluorescence intensity as the total sample (T). The sample was then subject to centrifugation at maximum speed for 30 min at 4 °C. The soluble fraction (100 μL) was sampled to measure its fluorescence intensity as the soluble fraction (S). The insoluble fraction (100 μL) was prepared by resuspending the DHFR protein in aggregation buffer (900 μL) as the insoluble fraction (I). Fluorescence intensity was measured by Tecan Spark Fluorescence Plate Reader by using BeyoGold™ 96-Well Black Opaque plates. The total, soluble, and insoluble samples after measurement of fluorescence were further prepared for SDS-PAGE electrophoresis.

To image the fluorescent aggregates of DHFR proteins, 5 μL of the resuspended insoluble sample was placed on an imaging glass slide and covered with a glass coverslip, followed by nail polish sealing. The sample slides were imaged on an Olympus® IX73 Research Inverted Microscope using respective filters.

4. Heat induced DHFR aggregation and fluorescence measurement.

WT-DHFR protein (49 μM) and labelled WT-DHFR protein (1 μM, 2%) were mixed together in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23). The mixture was aliquoted into two samples. The first one was incubated at ambient temperature (25 °C), and the other one was incubated in PCR thermal cycler at 61 °C for 5 min. The fluorescent spectra were measured by Tecan Spark Fluorescence Plate Reader using BeyoGold™ 96-Well Black Opaque plates (for CCVJ, ex. 460 nm, em. 500 nm; for DCDHF, ex. 495 nm, em. 526 nm; for RFP, ex. 555 nm, em. 605 nm; for Mero, ex. 600 nm, em. 620 nm).

To measure the linear range and detection limits, freshly purified WT-DHFR protein and labelled WT-DHFR protein in phosphate buffer (10 mM phosphate, 100 mM KCl, 1 mM EDTA, acidified by saturated HCl to pH = 7.40) were prepared. Mix these two proteins with varying

concentration of labelled protein (0.01%-5%) in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23). The samples were heated at 61 °C for 5 min to induced DHFR aggregation. Fluorescence intensity was measured by exciting the samples at 460 nm and collecting the fluorescent emission intensity at 500 nm for CCVJ. Fluorescence intensity was measured by exciting the samples at 600 nm and collecting the fluorescent emission intensity at 620 nm for Mero.

5. Chemical crosslinking to identify the presence of soluble oligomers[2] (Figure 3e).

SDS-PAGE gel of chemically crosslinked DHFR soluble oligomers was visualized by Coomassie blue staining. Two folding states of WT-DHFR have been captured under different conditions. 1) Folded state was prepared in pH = 7.40 (10 mM sodium phosphate, 100 mM KCl, 1 mM EDTA, pH = 7.40) at 25°C; 2) Misfolded state was captured in pH = 7.40 neutral buffer (10 mM sodium phosphate, 100 mM KCl,1 mM EDTA, pH = 7.40) after incubated at 64 °C for 5 min; 100 μL of each prepared samples were treated with 5 μL 30 mM DSS (disuccinimidyl suberate) at 25°C for 15 min, and then added 2 μL 1 M Tris-HCl buffer (pH = 7.40) to quench the crosslinking reaction. The well mixed samples were incubated at 25°C for 15 min followed by incubating with 25 μL 5X loading buffer (2.0 % sodium dodecyl sulfate, 0.1 % Bromophenol blue) at 95 °C for 5 min. The samples were loaded on to a 12 % acrylamide SDS-PAGE gel for electrophoresis analysis. To visualize the presence of crosslinked soluble oligomers, the gel was stained with Coomassie blue. BeyoTime Protein Marker served as the protein molecular weight ladder on the SDS-PAGE gel.

6. DHFR thermal shift assay.

Freshly purified WT-DHFR or Mut-DHFR protein (49 μM) and CCVJ labelled WT-DHFR or Mut-DHFR protein (1 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated from 25 °C to 64 °C with 3 °C temperature intervals. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates then collect the fluorescent emission intensity at 500 nm using 460 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

Freshly purified WT-DHFR or Mut-DHFR protein (49 μM) and Mero labelled WT-DHFR or Mut-DHFR protein (1 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated from 25 °C to 64 °C with 3 °C temperature intervals. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates then collect the fluorescent emission intensity at 620 nm using 600 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

Freshly purified WT-DHFR or Mut-DHFR protein (50 μM) and SYPRO® (2X) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated from 25 °C to 64 °C with 3 °C temperature intervals. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates then collect the fluorescent emission intensity at 580 nm using 470 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

Freshly purified WT-DHFR or Mut-DHFR protein (50 μM) and PROTEOSTAT® (0.1X) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated from 25 °C to 64 °C with 3 °C temperature intervals. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates then collect the fluorescent emission intensity at 580 nm using 470 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

To investigate TMP stabilizing effect on Mut-DHFR protein (49 μM), CCVJ labelled Mut-DHFR protein (1 μM) and various concentration of TMP (working concentrations were 10 μM, 25 μM, 50 μM and 100 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) followed by the same protocol for non TMP treated samples. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates then collect the fluorescent emission intensity at 500 nm using 460 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

7. Dual-color thermal shift assay in DHFR.

Freshly purified WT-DHFR (24 μM) and Mut-DHFR protein (24 μM), Mero labelled WT-DHFR (1 μM) and CCVJ labelled Mut-DHFR protein (1 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated from 25 °C to 64 °C with 3 °C temperature intervals. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates. We then collected the fluorescence emission intensity at 620 nm using 600 nm as the excitation wavelength for Mero fluorescence, and the fluorescence emission intensity at 500 nm using 460 nm as the excitation wavelength for CCVJ fluorescence. For each temperature, the experiments were repeated three times.

Freshly purified WT-DHFR (25 μM), Mut-DHFR protein (25 μM) and SYPRO® (2X) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated from 25 °C to 64 °C with 3 °C temperature intervals. The heated mixtures were

pipetted into BeyoGold™ 96-Well Black Opaque plates then collect the fluorescent emission intensity at 580 nm using 470 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

Freshly purified WT-DHFR (25 μM), Mut-DHFR protein (25 μM) and PROTEOSTAT® (0.1X) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated from 25 °C to 64 °C with 3 °C temperature intervals. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates then collect the fluorescent emission intensity at 580 nm using 470 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

Freshly purified WT-DHFR, Mut-DHFR protein (25 μM), SYPRO® (2X) and PROTEOSTAT® (0.1X) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated from 25 °C to 64 °C with 3 °C temperature intervals. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates then collect the fluorescent emission intensity at 580 nm using 470 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

To test the differential stabilizing effect of TMP at different concentration on WT-DHFR and Mut-DHFR protein in this heterozygous system, WT-DHFR protein (24 μM) and Mut-DHFR protein (24 μM), CCVJ labelled Mut-DHFR protein (1 μM), Mero labelled WT-DHFR (1 μM) and various concentration of TMP (working concentration was 25 μM and 100 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) followed by the same protocol for non TMP treated samples. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates then collect the fluorescence emission intensity at 620 nm using 600 nm as the excitation wavelength and the fluorescence emission intensity at 500 nm using 460 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

To test the differential stabilizing effect of TMP at different concentration on WT-DHFR and Mut-DHFR protein in this heterozygous system, WT-DHFR protein (25 μM) and Mut-DHFR protein (25 μM), SYPRO® Orange (2X) and TMP (100 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) followed by the same protocol for non TMP treated samples. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates then collect the fluorescence emission intensity at 580 nm using 470 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

To test different drugs' selectivity towards *Ec*DHFR and *h*DHFR protein in a mixed system, *Ec*DHFR protein (24 μM) and *h*DHFR protein (24 μM), CCVJ labelled *h*DHFR protein (1 μM), Mero labelled *Ec*DHFR (1 μM) and various concentration of drugs (working concentration was 25 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) followed by the same protocol for non TMP treated samples. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates then collect the fluorescence emission intensity at 620 nm using 600 nm as the excitation wavelength and the fluorescence emission intensity at 500 nm using 460 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

8. Dual-color thermal shift assay in APOA4 and TTR.

Freshly purified Mut-TTR (7.056 μM), APOA4 (8.722 μM), CCVJ labelled Mut-TTR (0.144 μM) and Mero labelled APOA4 (0.178 μM) (in total APOA4 0.4mg/mL, Mut-TTR 0.4mg/mL) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 4.40) and incubated from 25 °C to 99 °C. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates then collect the fluorescence emission intensity at 620 nm using 600 nm as the excitation wavelength and the fluorescence emission intensity at 500 nm using 460 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

Freshly purified WT-TTR (6.912 μM), Mut-TTR protein (6.912 μM), Mero labelled WT-TTR (0.288 μM) and CCVJ labelled Mut-TTR (0.288 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 4.40) and incubated from 25 °C to 94 °C. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates then collect the fluorescence emission intensity at 620 nm using 600 nm as the excitation wavelength and the fluorescence emission intensity at 500 nm using 460 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

To test tafamidis' stabilization effect on WT-TTR and Mut-TTR protein in a heterozygous system, WT-TTR protein (6.912 μM) and Mut-TTR protein (6.912 μM), CCVJ labelled Mut-TTR protein (0.288 μM), Mero labelled WT-TTR (0.288 μM) and various concentration of tafamidis (working concentration was 3.6 μM and 28.8 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 4.40) followed by the same protocol for non tafamidis treated samples. The heated mixtures were pipetted into BeyoGold™ 96-Well Black Opaque plates then collect the fluorescence emission intensity at 620 nm using 600 nm as the excitation wavelength and the fluorescence emission intensity at 500 nm using 460 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

9. Analysis of thermal shift data.[3]

The fluorescence imaging data from CCVJ (or Mero) in different proteins were fitted to Eq. (1) to obtain $\triangle H_u$, $\triangle C_{pu}$, and T_m by nonlinear curve fitting using the program OriginPro 2015:

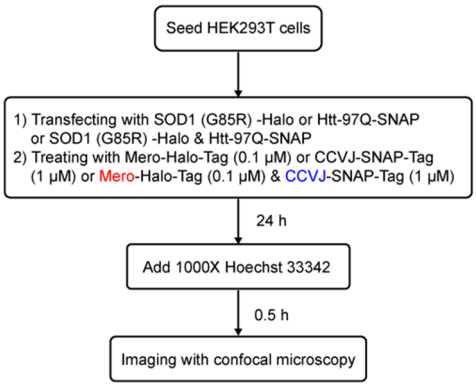
$$F(T) = F(post) + \frac{[F(pre) - F(post)}{1 + \exp\{\frac{-\triangle H_u}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right) + \frac{\triangle C_{pu}}{R}\left[\ln\left(\frac{T}{T_m}\right) + \frac{T}{T_m} - 1\right]\}} \quad (1)$$

where F(T) is the fluorescence intensity at temperature T; T_m is the midpoint temperature of the protein aggregation transition, F(pre) and F(post) are the pre transitional and post transitional fluorescence intensities,

respectively, R is the gas constant, $\triangle H_u$ is the enthalpy of protein aggregation, and $\triangle C_{pu}$ is the heat capacity change upon protein aggregation.

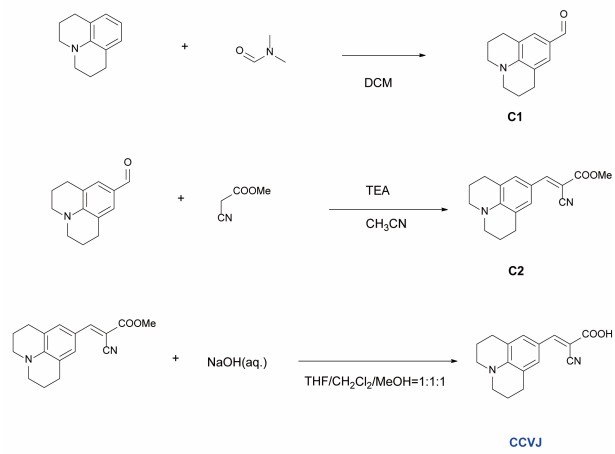
10. Procedure for cell culture, transfection of cells, and cell imaging experiments.[4]

HEK293T cells were seeded on 35 mm confocal culture dishes and transiently transfected when the cell density reached 70%. In 100 μL opti-mem medium, 3 μL of X-tremegene 9 DNA transfection reagent (Roche) was added, and then plasmid DNA (1 μg of DNA for single transfection, and 0.5 μg of each DNA for co-transfection) and probe (Mero-Halo 0.1 μM, CCVJ-SNAP 1 μM) were added, and the mixture was fully mixed at room temperature for 20 min. The mixture was then dripped into the cell medium. In the experimental group with Mero-Halo-Tag, MG132 (2.5 μM) was added after protein expression for 24 hours, and then it was expressed for another 24 hours. The control group was left untreated. For the CCVJ-SNAP-Tag, the experimental group was transfected, and the control group was not transfected and only added the probe for 24h. A 1000x 33342 dye was added to the cells 30 minutes before imaging. Images were collected using Olympus FV1000MPE. Blue: 405 nm laser, Hoechst 33342; Green:488 nm laser, CCVJ-SNAP probe; Red, 543 nm laser, Mero-Halo probe. Flowchart of this procedures showed as follows.

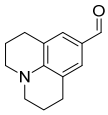


Synthetic Methods and Schemes

Scheme S1. Synthesis of **CCVJ.**[5]

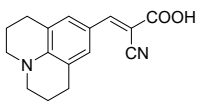


Compound **C1**



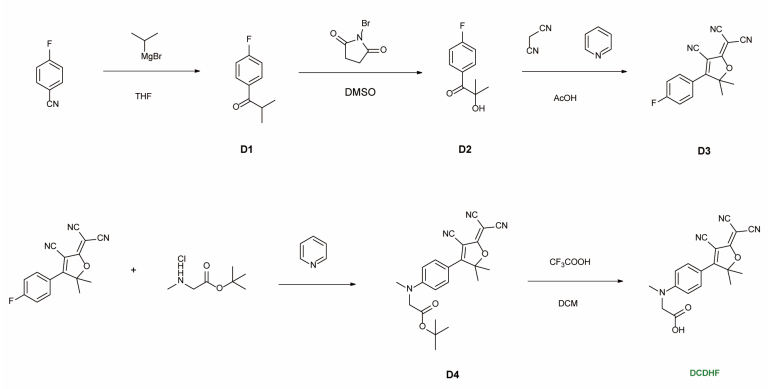
$POCl_3$ (1.2 eq, 6.34 mmol) was added dropwise to a solution of julolidine (1.0 eq, 5.8mmol) and dimethylformamide (1.1 eq, 6.2 mmol) in anhydrous dichloromethane (10 mL) under argon. After overnight stirring at RT, the reaction was quenched with 10 mL of 1 M NaOH solution. The aqueous layer was extracted with EtOAc. The organic fractions were combined and dried over $MgSO_4$. After concentration the crude product was purified by flash column chromatography (SiO_2, 20% EtOAc: hexanes) to give the desired product **C1** (700 mg, 60.0%), which was used directly in the next step. ^{1}H NMR (400 MHz, DMSO-d^6) δ 9.50 (s, 1H), 7.21 (s, 2H), 3.27 (t, J = 5.8 Hz, 4H), 2.70 (t, J = 6.3 Hz, 4H), 1.86 (p, J = 6.1 Hz, 4H).

Compound **CCVJ**

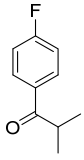


Compound **C1** (700 mg, 3.5 mmol, 1.0 eq.), cyanoacetic acid (595 mg, 7.0 mmol, 2.0 eq.), and piperidine (298 mg, 3.5 mmol, 1.0 eq.) was dissolved in 20 mL of CH_3CN and heated to reflux for 2 h. The reaction mixture was cooled to RT and concentrated under vacuum. The residue was dissolved in water and acidified to pH = 1 with 1 M HCl (aq.). The crude precipitate was collected by filtering the mixture over a celite pad. The desired product was isolated via recrystallization from ethanol, yielding an orange solid (376 mg, 40.0%). ^{1}H NMR (400 MHz, DMSO-d^6) δ 13.03 (s, 1H), 7.86 (s, 1H), 7.49 (s, 2H), 3.33 (d, J = 5.6 Hz, 4H), 2.66 (t, J = 6.2 Hz, 4H), 1.91 – 1.80 (m, 4H).

Scheme S2. Synthesis of **DCDHF.**



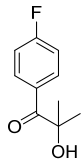
Compound **D1**[6]



A brownish solution of isopropyl magnesium bromide (3.0 eq., 1 M in 2-methyl THF, 15 mmol) was cannulated into 0 ºC solution of 4-fluorobenzonitrile (1.0 eq., 5 mmol) in approximately 10 mL of THF. Copper bromide (20 mg) was added against an Ar counterflow and the resulting brown solution was refluxed for 1 hour under Ar over which time a color change to black was observed. The solution was then cooled to 0ºC and water (8 mL) was added slowly. Once the vigorous reaction ceased, a 1M solution of sulfuric acid (50 mL) was added causing a color change to yellow/green. The resulting solution was heated to reflux for 1 hour following

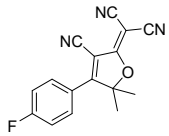
which time it was cooled to room temperature, basified with 2 M NaOH until pH = 9-10. Extraction with ethyl acetate (3 × 100 mL), dried over $MgSO_4$ and the volatiles removed to yield a pale yellow liquid (1.772 g, 70.0 %). ^{1}H NMR (400 MHz, Chloroform-d) δ 8.04 – 7.94 (m, 2H), 7.18 – 7.08 (m, 2H), 3.52 (p, J = 6.8 Hz, 1H), 1.22 (d, J = 6.9 Hz, 6H).

Compound **D2**[7]



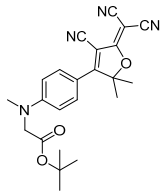
Compound **D1** (1.0 eq., 10 mmol), NBS (20 mol%, 2 mmol), and DMSO (20 mL) and a stir bar were added to a 50 mL reaction tube under air. The mixture was stirred at 100 ºC for 24 hours as monitored by TLC. After cooling down to room temperature, the solution was diluted with ethyl acetate (200 mL) and washed with water (100 mL), extracted with ethyl acetate (3×150 mL), and evaporated under vacuum. The crude reaction mixture was purified by column chromatography on silica gel (eluent: petroleum ether / ethyl acetate = 20:1) to get the desired product **D2** (1.341 g, 73.6%). ^{1}H NMR (400 MHz, Chloroform-d) δ 8.16 – 8.06 (m, 2H), 7.19 – 7.09 (m, 2H), 4.00 – 3.85 (m, 1H), 1.62 (s, 6H).

Compound **D3**[8]



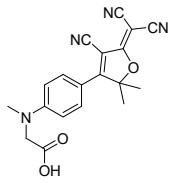
A mixture of compound **D2** (1.0 eq., 11.4 mmol), malononitrile (4.4 eq., 50.16 mmol), pyridine (23 ml) and 10 drops of acetic acid was stirred at 40 ºC for 2 days. Pyridine was removed under vacuum and the residue was dissolved in ethyl acetate and washed with 5% hydrochloric acid, water, brine and dried with anhydrous MgSO4. Solvent was removed by rotary evaporation and the residue was purified by silica gel chromatography with a hexane: ethyl acetate mixture (10:1 to 1:1) as eluents to give a purple solid (772 mg, 24.0%). ^{1}H NMR (400 MHz, DMSO-d6) δ 7.91 (dd, J = 8.7, 5.2 Hz, 2H), 7.53 (t, J = 8.7 Hz, 2H), 1.75 (s, 6H).

Compound **D4**



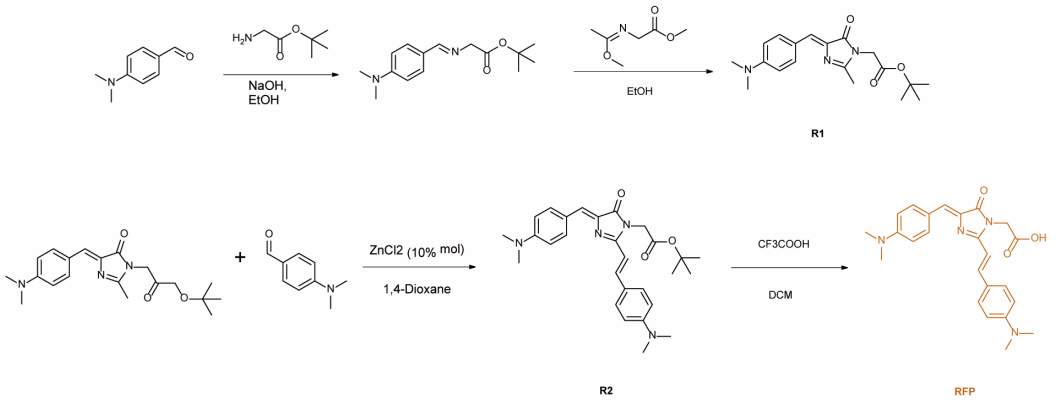
A mixture of compound **D3** (1.0 eq., 1.0 mmol), tert-butyl methyl glycinate hydrochloride (15.0 eq., 15.0 mmol) and pyridine (5 ml) was stirred at 30 ºC for 4 days. Pyridine was removed under vacuum and the residue was dissolved in DCM and washed with PBS many times and dried with anhydrous MgSO4. Solvent was removed by rotary evaporation and the residue was purified by silica gel chromatography with a hexane: ethyl acetate mixture (2:1 to 1:1) as eluents to give a orange solid (193 mg, 47.6%). ^{1}H NMR (400 MHz, DMSO-d^6) δ 8.06 (d, J = 9.0 Hz, 2H), 6.91 (d, J = 9.0 Hz, 2H), 4.36 (s, 2H), 3.16 (s, 3H), 1.82 (s, 6H), 1.42 (d, J = 1.7 Hz, 9H).

Compound **DCDHF**

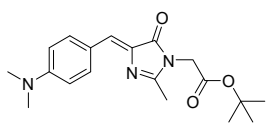


To a solution of compound **D4** (1.0 eq, 0.15 mmol) in DCM, trifluoroacetic acid (15 eq, 0.76 mmol) was added slowly, the reaction was stirred overnight. The product was concentrated by vacuum evaporation and without further purification to next step.

Scheme S3. Synthesis of **RFP**.[2]



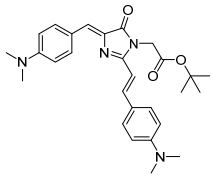
Compound **R1**



4-Dimethylaminobenzaldehyde (1.0 eq, 10.0 mmol), primary amine hydrochloride (1.1 eq, 11.0 mmol) were placed in 100 mL round bottom with 40 mL anhydrous ethanol under protection of Ar atmosphere. NaOH (1.1 eq, 11.0 mmol) was required. The reaction mixture was kept stirring at room temperature overnight, and then the freshly prepared imidate (1.1 eq, 10.0 mmol) intermediate was added quantitatively. After stirred at room temperature overnight, the reaction

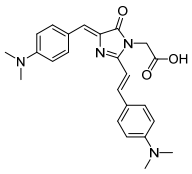
was quenched by distilled water and then extracted with 50 mL DCM three times. The organic phase was combined and washed with brine, and then dried over anhydrous Na_2SO_4. DCM was removed in vacuum and the crude residual was purified through flash silica gel chromatography to yield precursor as bright yellow crystal (1.374 g, 40.0%). ^{1}H NMR (400 MHz, DMSO-d^6) δ 8.08 (d, J = 8.6 Hz, 2H), 6.92 (s, 1H), 6.76 (d, J = 8.8 Hz, 2H), 4.37 (s, 2H), 3.02 (s, 6H), 2.26 (s, 3H), 1.44 (s, 9H).

Compound **R2**



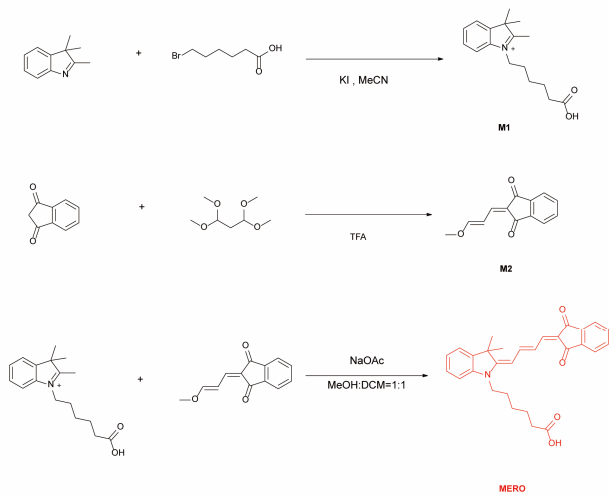
To the 15 mL heavy-wall pressure tube compound **R1** (1.0 eq, 2.0 mmol), 4-dimethylaminobenzaldehyde (10.0 eq, 20.0 mmol) were dissolved in 3 mL anhydrous 1,4-dioxane under argon atmosphere. After added $ZnCl_2$ solution (0.1 eq, 0.2 mL, 1.0 M in THF), the reaction vessel was sealed up with Teflon screw cap with Viton O-ring and then kept heating in oil bath at 95°C overnight. After cooling down to ambient temperature, the reaction mixture was poured into 200 mL DCM. The organic mixture was washed with sat. NaCl and DI water three times. The crude product was concentrated by vacuum evaporation and purified via silica gel chromatography to yield desired product as dark red solid (376 mg, 41.7%). ^{1}H NMR (400 MHz, Chloroform-d, 25 °C): δ 8.09 (d, J = 8 Hz, 2H), 7.89 (d, J = 16 Hz, 1H), 7.40 (d, J = 8 Hz, 2H), 7.01 (s,1H), 6.63 (m, 4H), 6.33 (d, J = 16 Hz, 1H), 4,35 (s, 2H), 2.94 (br, d, 12H), 1.37 (s, 9H).

Compound **RFP**

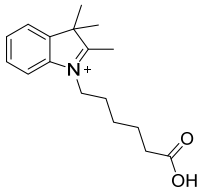


To a solution of compound **R2** (1.0 eq, 0.4 mmol) in DCM, trifluoroacetic acid (10 eq, 4 mmol) was added slowly, the reaction was stirred overnight. The product was concentrated by vacuum evaporation and without further purified to next step.

Scheme S4. Synthesis of **Mero.**[9]

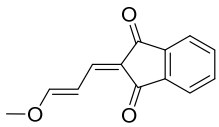


Compound **M1**



To a solution of acetonitrile (20 mL), 2,3,3-trimethylindolenine (3.21 mL, 20mmol, 1.0 eq.), 6-bromohexanoic acid (15.6 g, 80 mmol, 4.0 eq.) and KI (0.1 g) were added, then refluxed for about 50 hours and yielded an dark purple colored solution. TLC monitored, until the substrate was left a little, and the product was major (R_f=0.2 (DCM:MeOH=10:1)). Let the reaction cool down, dropwise added the solution to ice ether (200ml) and stirred with grass bar. Put the beaker to the refrigerator overnight and get the recrystallization product. At last filter and get compound **M1** (6.14g, 86.6%) as a purple crystal. ^{1}H NMR (400 MHz, DMSO-d^6) δ 8.03 – 7.93 (m, 1H), 7.85 (dd, J = 5.9, 2.9 Hz, 1H), 7.68 – 7.59 (m, 2H), 4.46 (t, J = 7.7 Hz, 2H), 2.84 (s, 3H), 2.23 (t, J = 7.2 Hz, 2H), 1.85 (p, J = 7.8 Hz, 2H), 1.54 (s, 6H), 1.50 (m, 2H), 1.43 (m, 2H).

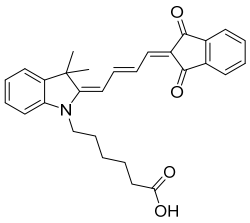
Compound **M2**



1,3-Indandione (0.146 g, 1.00 mmol) was added to a small microwave vial with 1,1,3,3- tetramethoxypropane (0.823 mL, 5.00 mmol), followed by addition of trifluoroacetic acid (7.7 μL, 0.10 mmol). The vial was capped and heated to 150 °C for 15 min. The reaction was cooled and the precipitate was filtered and rinsed with ice chilled 3:1

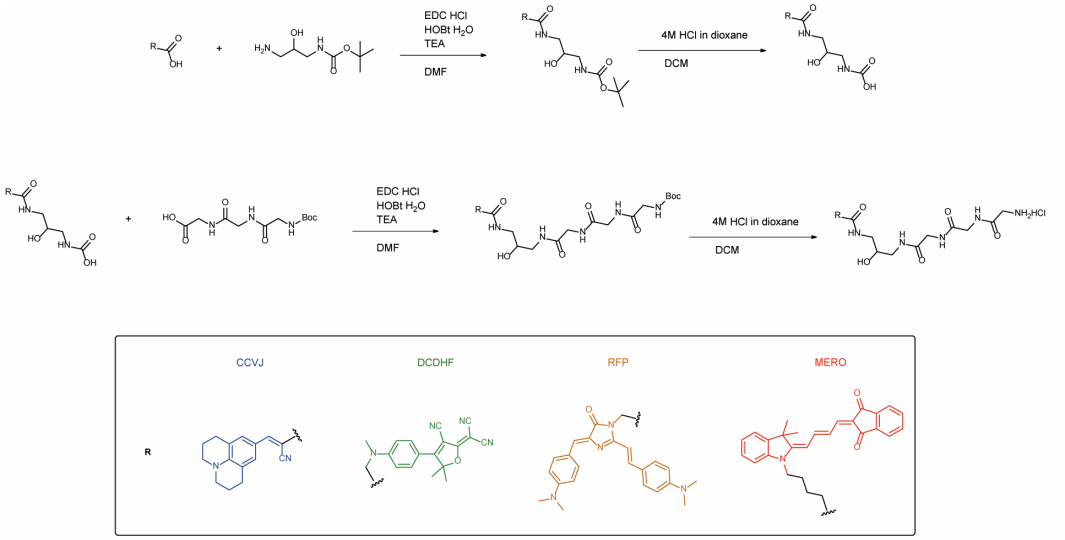
hexanes/Et_2O. Isolated 0.158 g (74.0 %) brown solid. ^1H NMR (400 MHz, DMSO-d^6) δ 7.96 (d, J = 12.0 Hz, 2H), 7.90 – 7.84 (m, 3H), 7.65 (d, J = 12.4 Hz, 1H), 7.13 (t, J = 12.2 Hz, 1H), 3.93 (s, 3H).

Compound **Mero**



Compound **M1** (1.873 g, 5.00 mmol, 1.0 eq.), Compound **M2** (1.098 g, 5.00 mmol, 1.0 eq.), and NaOAc (0.600 g, 7.50 mmol) were dissolved in 1:1 MeOH/DCM (10 mL). Then reflux for about 3 hours and TLC monitor,until the substrate was left a little, and the product was major, Rf=0.4(DCM:MeOH=10:1). After the reaction was cooled , the solvent was removed and the residue was purified by column chromatography on silica gel (DCM:MeOH = 10:1) to afford compound **Mero** (1.250 g, 55.0%). ^1H NMR (400 MHz, DMSO-d^6) δ 12.18 – 11.87 (m, 1H), 8.13 (t, J = 13.1 Hz, 1H), 7.76 (d, J = 13.1 Hz, 1H), 7.70 (q, J = 2.7, 2.1 Hz, 4H), 7.57 – 7.48 (m, 2H), 7.33 (t, J = 7.7 Hz, 1H), 7.22 (d, J = 7.9 Hz, 1H), 7.13 (t, J = 7.4 Hz, 1H), 6.22 (d, J = 13.3 Hz, 1H), 4.00 (t, J = 7.3 Hz, 2H), 2.21 (t, J = 7.3 Hz, 2H), 1.63 (s, 6H), 1.60-1.40 (m, 6H).

Scheme S5. General methods of connecting fluorophore with water-soluble and GGG linker.



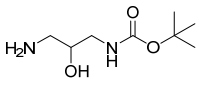
Fluorophore (1.0 eq, 1 mmol) was dissolved in 10 mL anhydrous DMF. Compound **1** was added into the reaction solution (1.0 eq, 1 mmol, 190.24 mg). was added into the reaction solution. 1-Hydroxybenzotriazole hydrate (HOBt•H2O) (3.0 eq, 3 mmol, 460 mg) and triethylamine (3.0 eq, 3 mmol, 420 μL) were introduced to the reaction mixture in one portion. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC•HCl) (3.0 eq, 3 mmol, 580 mg) was added in one portion. The reaction was stirred for 3 h, protected from light. The reaction mixture was quenched by water and extracted with DCM. The organic fraction was dried in vacuo. The product was further purified by flash chromatography using 5% MeOH in DCM as eluent and get the compound **2**.

To a solution of compound **2** (1.0 eq, 1 mmol) in DCM, HCl (4 M in dioxane, 10.0 eq, 10 mmol) was added dropwise. The reaction was stirred, protected from light and monitored via TLC. The solution was dried in vacuo and get compound **3** without further purification.

Compound **3** (1.0 eq, 1 mmol) was dissolved in 10 mL anhydrous DMF. Compound **4** was added into the reaction solution (1 eq, 1 mmol, 289.29 mg). was added into the reaction solution. 1-Hydroxybenzotriazole hydrate (HOBt•H_2O) (3.0 eq, 3 mmol, 460 mg) and triethylamine (3.0 eq, 3 mmol, 420 μL) were introduced to the reaction mixture in one portion. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC•HCl) (3.0 eq, 3 mmol, 580 mg) was added in one portion. The reaction was stirred for 3 h, protected from light. The reaction mixture was quenched by water and extracted with DCM. The organic fraction was dried in vacuo. The product was further purified by flash chromatography using 5% MeOH in DCM as eluent and get the compound **5**.

To a solution of compound **2** (1.0 eq, 1 mmol) in DCM, HCl (4 M in dioxane, 10.0 eq, 10 mmol) was added dropwise. The reaction was stirred, protected from light and monitored via TLC. The solution was dried in vacuo and get the final product **6**-fluorophore with linker.

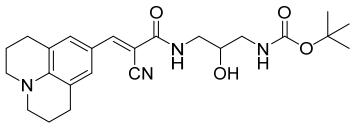
Compound **1**



A solution of 1,3-diamino-2-hydroxypropane (3.0 eq., 55.5 mmol) in anhydrous acetonitrile (50 mL) was maintained at room temperature and treated with a solution of ditert-butyl dicarbonate (1.0 eq., 18.5 mmol) in anhydrous acetonitrile (20 mL) over a 2-hour period with vigorous stirring. The subsequent suspension was left to stir overnight. After a period of 18 hours, the reaction mixture was concentrated and the residue re-dissolved in brine (50 mL). The pH was adjusted to 5 by treatment with HCl (1 M), washed with dichloromethane (3 × 50 mL) and made basic (pH = 12) by the addition of NaOH solution (2.5 M). The

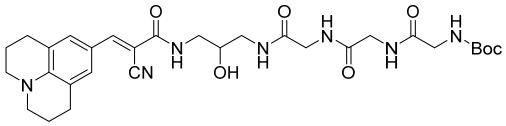
product was extracted using copious amounts of CHCl3 (5 × 100 mL), dried over MgSO4, filtered and concentrated to yield 1 as a white solid (1.230 g, 34.0%). ^{1}H NMR (400 MHz, Chloroform-d) δ 5.21 (t, J = 6.0 Hz, 1H), 3.62 (tt, J = 7.5, 4.1 Hz, 1H), 3.26 (s, 1H), 3.08 (dt, J = 13.2, 6.0 Hz, 1H), 2.87 – 2.56 (m, 2H), 2.34 (s, 3H), 1.44 (s, 9H).

Compound **CCVJ-2**



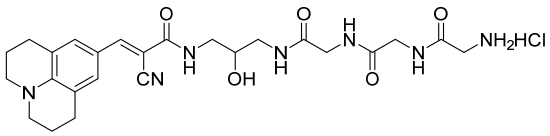
215 mg (49.0 %). ^{1}H NMR (400 MHz, DMSO-d^{6}) δ 7.83 (s, 1H), 7.76 (t, J = 5.8 Hz, 1H), 7.43 (s, 2H), 6.70 (t, J = 6.0 Hz, 1H), 4.94 (d, J = 5.1 Hz, 1H), 3.58 (p, J = 5.7 Hz, 1H), 3.28 – 3.06 (m, 2H), 2.93 (ddd, J = 15.8, 13.3, 7.1 Hz, 2H), 2.67 (t, J = 6.2 Hz, 4H), 1.87 (p, J = 6.2 Hz, 4H), 1.38 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d^{6}) δ 162.99, 150.80, 147.11, 130.68, 120.87, 94.88, 78.14, 68.86, 55.38, 49.78, 44.54, 44.09, 28.70, 27.55, 21.12. HRMS: calcd for [M+H]$^{+}$ 441.2496, found 441.2504.

Compound **CCVJ-5**



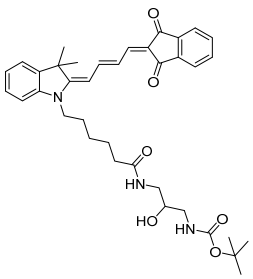
288 mg (72.4%). ^{1}H NMR (400 MHz, DMSO-d^{6}) δ 8.12 (t, J = 5.8 Hz, 1H), 8.06 (t, J = 5.8 Hz, 1H), 7.87 – 7.77 (m, 2H), 7.43 (s, 2H), 7.00 (t, J = 6.0 Hz, 1H), 5.04 (d, J = 4.9 Hz, 1H), 3.76 (d, J = 5.6 Hz, 2H), 3.71 (d, J = 5.8 Hz, 2H), 3.63 (d, J = 5.4 Hz, 1H), 3.58 (d, J = 6.0 Hz, 2H), 3.20 (p, J = 6.8, 5.9 Hz, 2H), 3.08 (dp, J = 25.8, 6.9, 6.0 Hz, 2H), 2.68 (t, J = 6.2 Hz, 4H), 1.87 (p, J = 6.1 Hz, 4H), 1.38 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d^{6}) δ 170.31, 169.58, 169.47, 163.07, 156.29, 150.83, 130.70, 120.86, 119.05, 118.07, 110.25, 94.83, 78.61, 68.60, 49.78, 43.97, 43.81, 43.16, 42.55, 42.50, 28.64, 27.55, 21.11. HRMS: calcd for [M+H]$^{+}$ 612.3140, found 612.3141.

Compound **CCVJ-6**



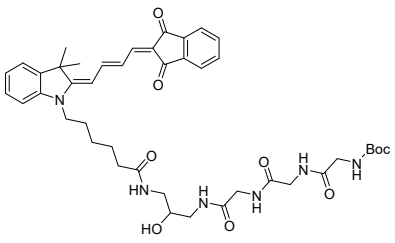
133 mg (97.0%). ^{1}H NMR (400 MHz, DMSO-d^{6}) δ 9.40 (d, J = 5.8 Hz, 1H), 8.70 – 8.58 (m, 2H), 8.24 (dt, J = 11.4, 5.8 Hz, 1H), 7.91 (t, J = 7.5 Hz, 1H), 7.85 (q, J = 5.6 Hz, 1H), 7.79 (d, J = 2.8 Hz, 1H), 7.51 – 7.28 (m, 2H), 4.89 (s, 1H), 3.77 (d, J = 5.6 Hz, 2H), 3.65 (t, J = 5.6 Hz, 2H), 3.53 (d, J = 5.5 Hz, 2H), 3.48 (s, 4H), 3.06 (ddt, J = 43.9, 15.4, 6.8 Hz, 4H), 2.59 (t, J = 6.2 Hz, 2H), 1.78 (p, J = 6.4 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d^{6}) δ 169.53, 169.49, 169.15, 169.12, 166.89, 163.20, 150.61, 150.17, 148.54, 147.92, 147.08, 133.10, 133.07, 131.77, 130.67, 129.96, 127.64, 123.13, 120.84, 118.06, 111.10, 110.24, 95.03, 68.55, 68.38, 68.28, 66.81, 56.98, 49.78, 44.35, 43.97, 43.24, 42.58, 42.52, 40.66, 27.54, 26.83, 24.87, 21.11, 20.48. HRMS: calcd for [M+H]$^{+}$ 512.2616, found 512.2616.

Compound **Mero-2**



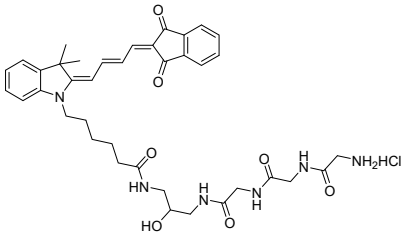
544 mg (86.0%). ^{1}H NMR (400 MHz, DMSO-d^{6}) δ 8.12 (t, J = 13.1 Hz, 1H), 7.95(s, 1H), 7.74 (d, 2H), 7.65 (m, 4H), 7.58 – 7.47 (m, 2H), 7.33 (t, J = 7.6 Hz, 1H), 7.22 (d, J = 7.9 Hz, 1H), 7.13 (t, J = 7.4 Hz, 1H), 6.61 (t, J = 6.0 Hz, 1H), 6.21 (d, J = 13.4 Hz, 1H), 4.83 (d, J = 5.0 Hz, 1H), 3.99 (t, J = 7.2 Hz, 2H), 3.46 (q, J = 5.7 Hz, 1H), 3.00 – 2.80 (m, 2H), 2.89 (m, 2H), 2.09 (t, J = 7.4 Hz, 2H), 1.63 (s, 6H), 1.55 (p, J = 7.6, 7.0 Hz, 2H), 1.36 (s, 9H). ^{13}C NMR (101 MHz, DMSO) δ 172.80, 162.75, 156.13, 155.63, 146.88, 141.60, 140.28, 134.18, 133.98, 128.64, 123.78, 122.65, 121.61, 121.39, 119.95, 117.27, 110.35, 101.02, 78.08, 69.24, 48.46, 44.55, 43.18, 36.24, 35.58, 31.23, 28.68, 27.88, 26.94, 26.28, 25.53. HRMS: calcd for [M+H]$^{+}$ 628.3381, found 628.3390.

Compound **Mero-5**



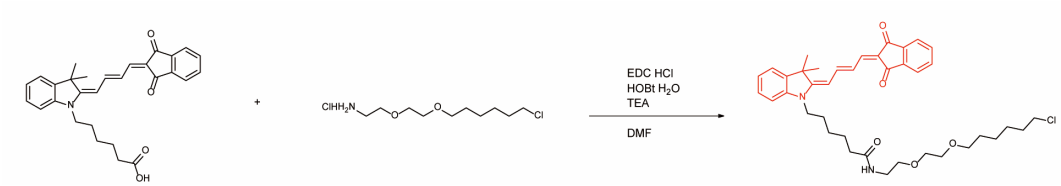
213 mg (67.0%). ^{1}H NMR (400 MHz, DMSO-d^{6}) δ 8.12 (m, 1H), 8.06 (m, 1H), 7.94 (m, 2H), 7.77 (m, 4H), 7.58 – 7.47 (m, 2H), 7.36 (dt, J = 19.4, 7.7 Hz, 1H), 7.22 (d, J = 7.9 Hz, 1H), 7.13 (t, J = 7.4 Hz, 1H), 7.00 (t, J = 6.1 Hz, 1H), 6.22 (d, J = 13.3 Hz, 1H), 4.94 (d, J = 4.9 Hz, 1H), 3.99 (t, J = 7.4 Hz, 2H), 3.72 (dd, J = 23.6, 5.7 Hz, 4H), 3.58 (d, J = 6.0 Hz, 2H), 3.51 (s, 2H), 3.04 (t, J = 5.4 Hz, 4H), 2.11 (t, J = 7.5 Hz, 2H), 1.67 (d, J = 7.3 Hz, 2H), 1.63 (s, 6H), 1.55 (q, J = 7.3 Hz, 2H), 1.37 (s, 9H). ^{13}C NMR (101 MHz, DMSO) δ 190.90, 172.97, 170.31, 169.60, 169.28, 156.29, 146.87, 142.94, 141.59, 134.18, 128.66, 127.23, 124.69, 123.81, 122.65, 121.61, 121.40, 119.93, 119.45, 117.24, 110.37, 110.23, 101.02, 78.61, 68.84, 48.47, 43.81, 43.12, 42.56, 35.61, 28.63, 27.88, 26.96, 26.31, 25.57. HRMS: calcd for [M+H]$^{+}$ 799.4025, found 799.4029.

Compound **Mero-6**

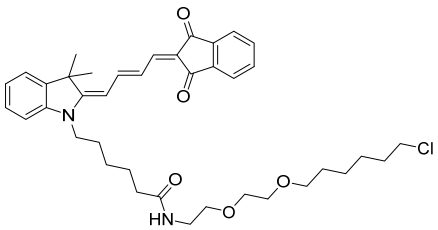


104 mg (97.0%). ^{1}H NMR (400 MHz, DMSO-d^{6}) δ 8.67 (d, J = 5.7 Hz, 1H), 8.18 (t, J = 5.0 Hz, 1H), 7.88 (t, J = 13.0 Hz, 1H), 7.69 (t, J = 5.1 Hz, 1H), 7.58 – 7.53 (m, 1H), 7.48 – 7.44 (m, 4H), 7.33 – 7.29 (m, 1H), 7.26 (d, J = 7.2 Hz, 1H), 7.17 (ddd, J = 8.2, 6.9, 1.0 Hz, 1H), 7.09 (d, J = 7.6 Hz, 1H), 7.00 (d, J = 7.9 Hz, 1H), 6.89 (t, J = 7.3 Hz, 1H), 5.99 (d, J = 13.3 Hz, 1H), 3.77 (t, J = 7.4 Hz, 2H), 3.62 (d, J = 5.6 Hz, 2H), 3.49 (dd, J = 4.9, 3.0 Hz, 2H), 3.41 – 3.39 (m, 2H), 2.83 (dd, J = 15.7, 8.8 Hz, 4H), 1.91 (t, J = 7.5 Hz, 2H), 1.44 (d, J = 7.3 Hz, 2H), 1.35 (d, J = 7.4 Hz, 2H), 1.18 – 1.12 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d^{6}) δ 190.74, 173.05, 170.33, 169.36, 169.14, 166.90, 155.57, 146.79, 143.22, 142.90, 140.90, 134.06, 128.66, 127.57, 124.84, 123.81, 122.61, 121.49, 119.92, 119.43, 117.24, 110.41, 110.33, 101.01, 68.76, 66.79, 55.39, 48.46, 43.23, 42.64, 42.55, 40.67, 35.57, 31.14, 27.87, 26.94, 26.30, 25.57. HRMS: calcd for [M+H]$^{+}$ 699.3501, found 699.3501.

Scheme S6. Methods of connecting Mero with Halo-Tag linker.[4]

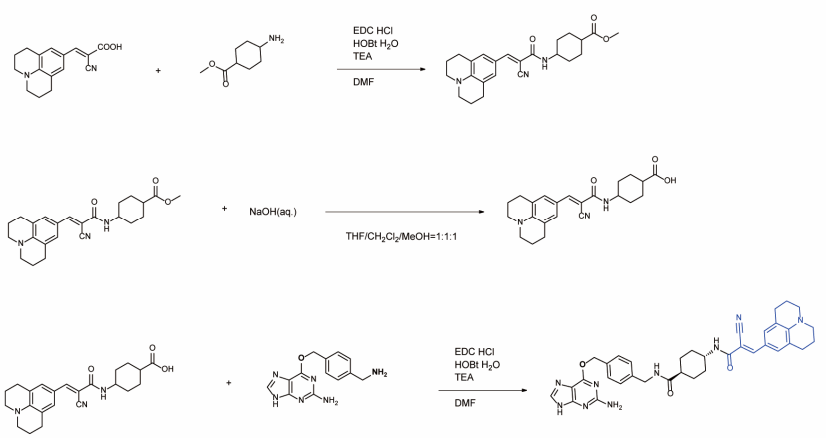


Compound **Mero-Halo**

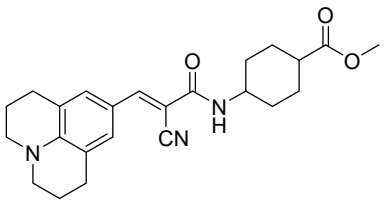


Mero (1.0 eq, 0.1 mmol) was dissolved in 1 mL anhydrous DMF. Halo-tag was added into the reaction solution (1.0 eq, 0.1 mmol, 26 mg). 1-Hydroxybenzotriazole hydrate (HOBt•H_2O) (3 eq, 0.3 mmol, 46 mg) and triethylamine (3.0 eq, 0.3 mmol, 42 μL) were introduced to the reaction mixture in one portion. N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC•HCl) (3.0 eq, 0.3 mmol, 58 mg) was added in one portion. The reaction was stirred for 3 h, protected from light. The reaction mixture was quenched by water and extracted with DCM. The organic fraction was dried in vacuo. The product was further purified by flash chromatography using 5% MeOH in DCM as eluent and get the compound Mero-Halo (54 mg, 81.0%). ^{1}H NMR (400 MHz, DMSO-d^{6}) δ 8.12 (t, J = 13.1 Hz, 1H), 7.81 (t, J = 5.9 Hz, 1H), 7.75 (d, J = 13.1 Hz, 1H), 7.72 – 7.65 (m, 4H), 7.54 (d, J = 13.0 Hz, 1H), 7.51 – 7.47 (m, 1H), 7.32 (td, J = 7.7, 1.2 Hz, 1H), 7.20 (d, J = 7.9 Hz, 1H), 7.12 (t, J = 7.4 Hz, 1H), 6.20 (d, J = 13.3 Hz, 1H), 3.98 (t, J = 7.4 Hz, 2H), 3.58 (t, J = 6.6 Hz, 2H), 3.47 – 3.42 (m, 4H), 3.37 (d, J = 6.2 Hz, 2H), 3.32 (d, J = 6.8 Hz, 2H), 3.17 (t, J = 5.8 Hz, 2H), 2.06 (t, J = 7.3 Hz, 2H), 1.72 – 1.67 (m, 2H), 1.66 – 1.64 (m, 2H), 1.54 (t, J = 7.5 Hz, 2H), 1.44 (dd, J = 7.9, 6.4 Hz, 2H), 1.37 – 1.32 (m, 4H), 1.27 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d^{6}) δ 190.87, 190.61, 172.42, 170.31, 155.59, 146.87, 142.94, 141.61, 140.94, 140.29, 134.17, 133.98, 128.62, 123.77, 122.64, 121.61, 121.38, 119.95, 117.32, 110.34, 101.00, 70.62, 70.02, 69.89, 69.87, 69.62, 48.46, 45.79, 43.11, 38.89, 35.55, 32.47, 29.50, 27.89, 27.02, 26.92, 26.57, 26.22, 25.47, 25.37. HRMS: calcd for [M+H]$^{+}$ 661.3403, found 661.3408.

Scheme S7. Methods of connecting CCVJ with SNAP-Tag linker.[4]

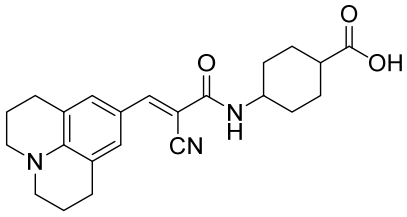


Compound **CCVJ-7a**



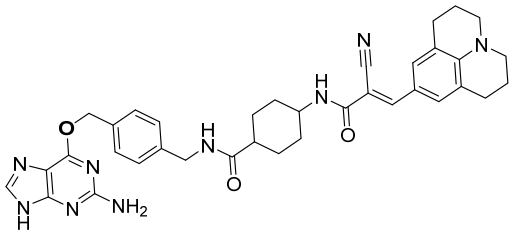
CCVJ (1.0 eq, 1 mmol) was dissolved in 1 mL anhydrous DMF. methyl 4-aminocyclohexane-1-carboxylate was added into the reaction solution (5.0 eq, 5 mmol, 1 mL). 1-Hydroxybenzotriazole hydrate (HOBt•H_2O) (2.0 eq, 2 mmol, 300 mg) and triethylamine (4.0 eq, 4 mmol, 560 μL) were introduced to the reaction mixture in one portion. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC•HCl) (2.0 eq, 2 mmol, 385 mg) was added in one portion. The reaction was stirred overnight, protected from light. The reaction mixture was quenched by water and extracted with DCM. The organic fraction was dried in vacuo. The product was further purified by flash chromatography using 1% MeOH in DCM as eluent and get the compound **CCVJ-7a** (230 mg, 56.0%). ^{1}H NMR (400 MHz, DMSO-d^6) δ 7.77 (s, 1H), 7.73 (d, J = 7.9 Hz, 1H), 7.42 (s, 2H), 3.64 (m, 1H), 3.60 (s, 3H), 3.32 – 3.27 (m, 4H), 2.67 (t, J = 6.2 Hz, 4H), 2.25 (m, 1H), 1.88 (dddd, J = 18.9, 13.3, 8.4, 4.8 Hz, 8H), 1.40 (td, J = 11.1, 9.2, 4.3 Hz, 4H). ^{13}C NMR (400 MHz, DMSO-d^6) δ 175.58, 162.26, 150.30, 130.56, 120.83, 118.12, 95.85, 51.81, 49.76, 48.61, 42.01, 31.38, 28.14, 27.54, 21.14. HRMS: calcd for [M+H]$^+$ 408.2217, found 408.2290.

Compound **CCVJ-7b**



Potassium hydroxide (28 mg, 1 mmol) was added to a solution of compound **CCVJ-7a** (40 mg, 0.1 mmol) in a mixture of THF/MeOH/water (3:2:1, 4 mL). The mixture was stirred for 2 h at room temperature. After complete deprotection, the mixture was diluted with sodium acetate buffer (pH = 4). The aqueous layer was extracted with EtOAc, and the organic fractions were combined. The combined extracts were washed with a saturated aqueous NaCl solution, dried over Na_2SO_4, filtered, and concentrated in vacuo to yield the compound **CCVJ-7b**. ^{1}H NMR (400 MHz, DMSO-d^6) δ 12.06 (s, 1H), 7.77 (s, 1H), 7.72 (d, J = 7.9 Hz, 1H), 7.41 (s, 2H), 2.13 (m, 1H)3.29 (t, J = 6.1 Hz, 4H), 2.67 (t, J = 6.1 Hz, 4H), 2.13 (ddd, J = 12.2, 8.1, 4.0 Hz, 1H), 1.84 (dt, J = 17.7, 5.4 Hz, 8H), 1.36 (t, J = 9.8 Hz, 4H).

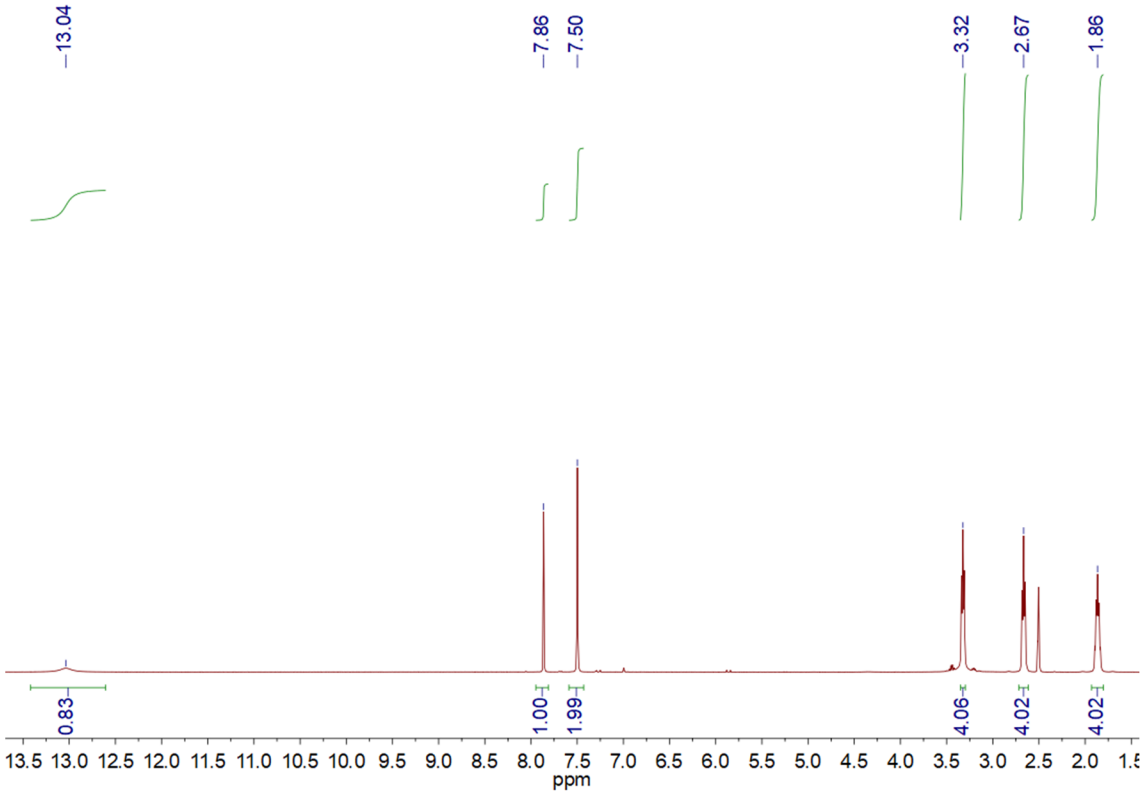
Compound **CCVJ-SNAP**



CCVJ-7b (1 eq, 0.1 mmol) was dissolved in 1 mL anhydrous DMF. SNAP-Tag was added into the reaction solution (1.0 eq, 0.1 mmol, 27 mg). 1-Hydroxybenzotriazole hydrate (HOBt•H_2O) (2.0 eq, 0.2 mmol, 31 mg) and triethylamine (4.0 eq, 0.4 mmol, 56 μL) were introduced to the reaction mixture in one portion. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC•HCl) (2.0 eq, 0.2 mmol, 39 mg) was added in one portion. The reaction was stirred overnight, protected from light. The reaction mixture was poured into EtOAc and extracted with water. The insoluble fraction was dried in vacuo. The product was further purified by flash chromatography using 10% MeOH in DCM as eluent and get the compound **CCVJ-SNAP**. ^{1}H NMR (700 MHz, DMSO-d^6) δ 8.42 (d, J = 6.6 Hz, 1H), 7.92 (d, J = 7.9 Hz, 1H), 7.87 (s, 1H), 7.44 (d, J = 7.8 Hz, 2H), 7.42 (s, 2H), 7.24 (d, J = 7.8 Hz, 2H), 6.28 (s, 2H), 5.44 (s, 2H), 4.24 (d, J = 5.9 Hz, 2H), 3.64 – 3.59 (m, 1H), 3.28 (t, J = 5.8 Hz, 4H), 2.66 (t, J = 6.3 Hz, 4H), 2.15 (td, J = 11.2, 9.7, 5.5 Hz, 1H), 1.85 (q, J = 6.0 Hz, 4H), 1.81 – 1.77 (m, 4H), 1.45 – 1.37 (m, 4H). ^{13}C NMR (176 MHz, DMSO-d6) δ 175.29, 162.32, 150.14, 146.90, 140.15, 130.54, 130.11, 128.98, 127.54, 120.79, 120.39, 119.01, 118.16, 96.12, 49.75, 48.79, 45.61, 43.57, 42.04, 31.74, 31.67, 29.48, 29.28, 29.03, 28.85, 27.55, 27.01, 21.14, 14.42. HRMS: calcd for [M+H]$^+$ 646.3249, found 646.3305.

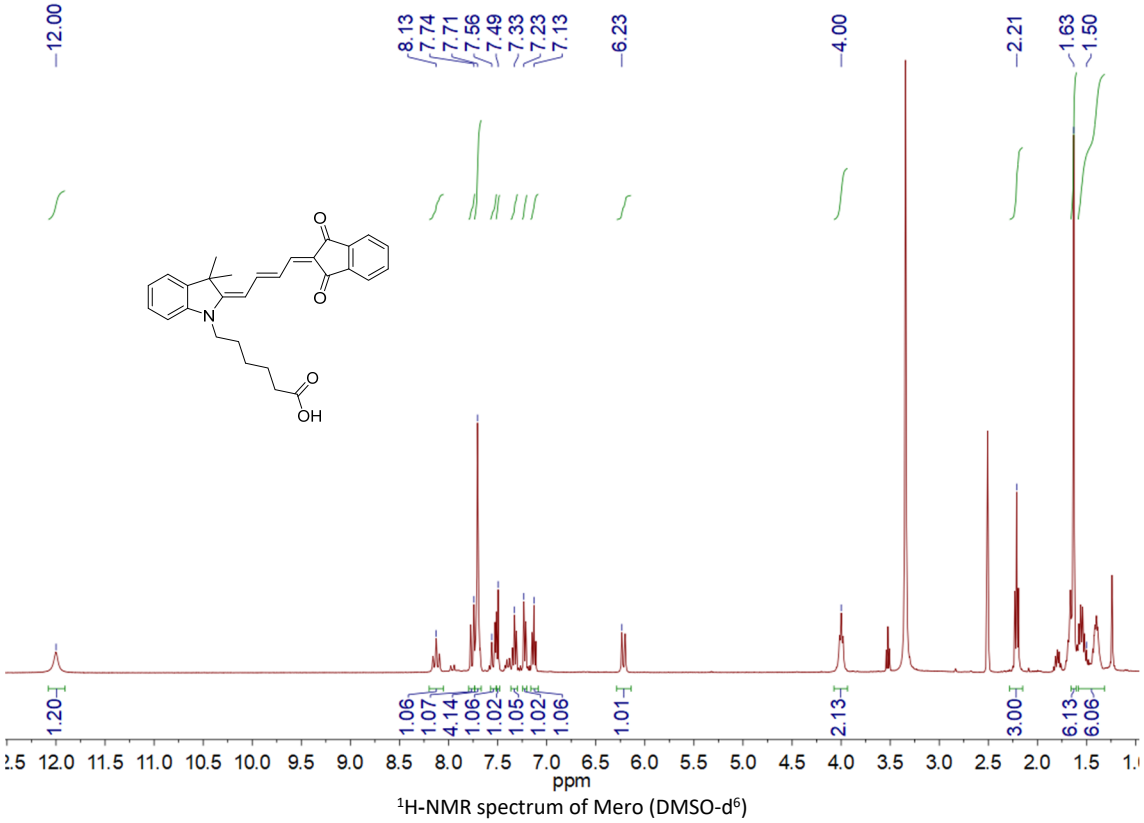
NMR Spectra

Compound **CCVJ**



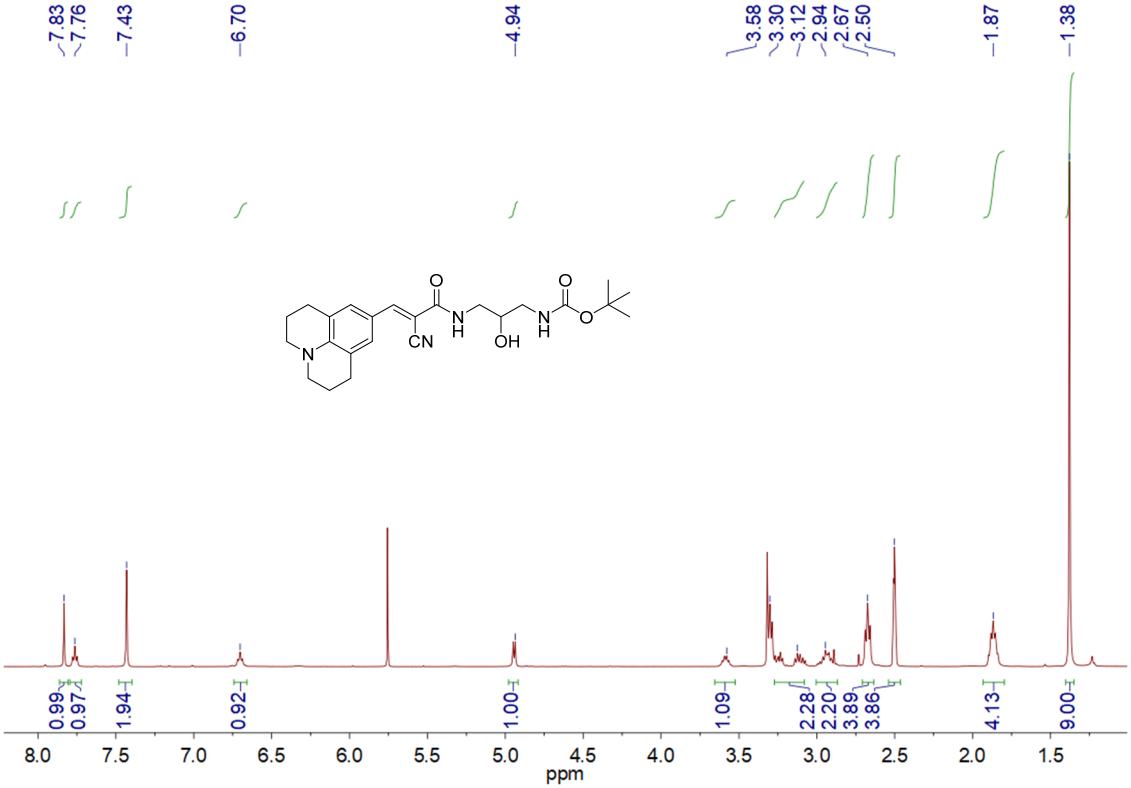
^{1}H-NMR spectrum of CCVJ (DMSO-d^6)

Compound **Mero**

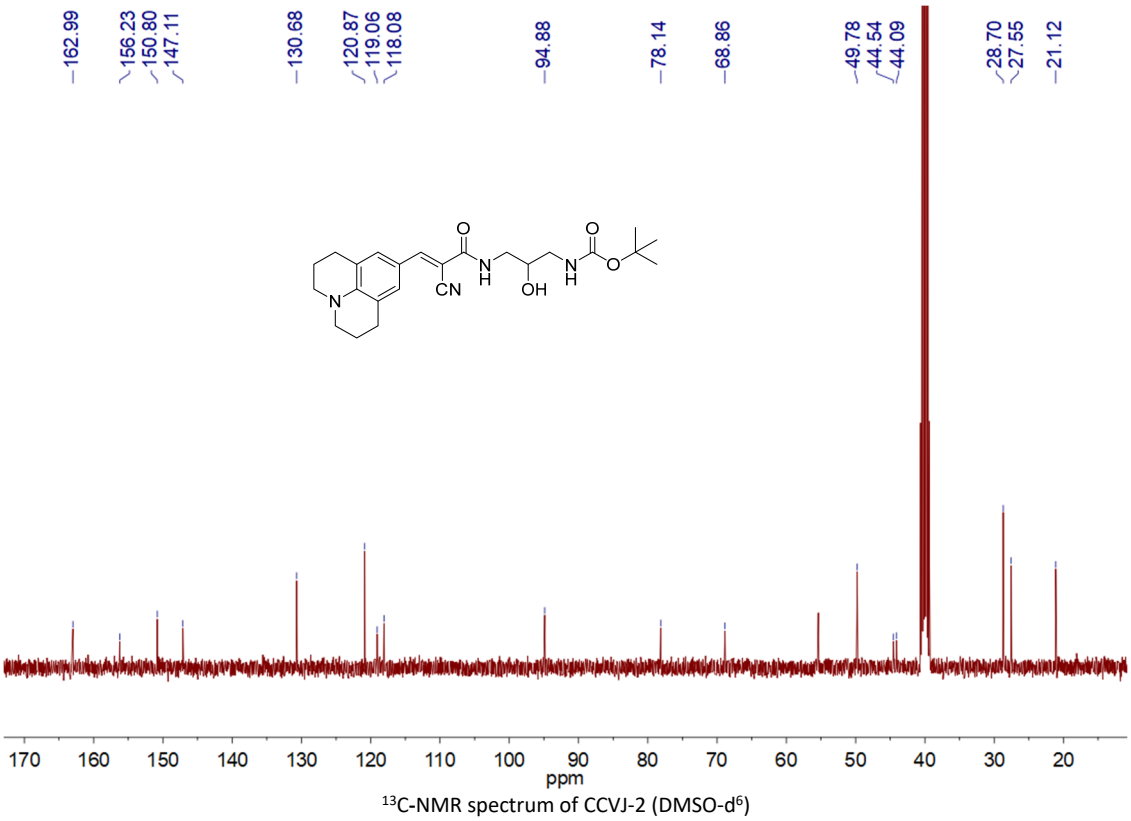


^{1}H-NMR spectrum of Mero (DMSO-d^6)

Compound **CCVJ-2**

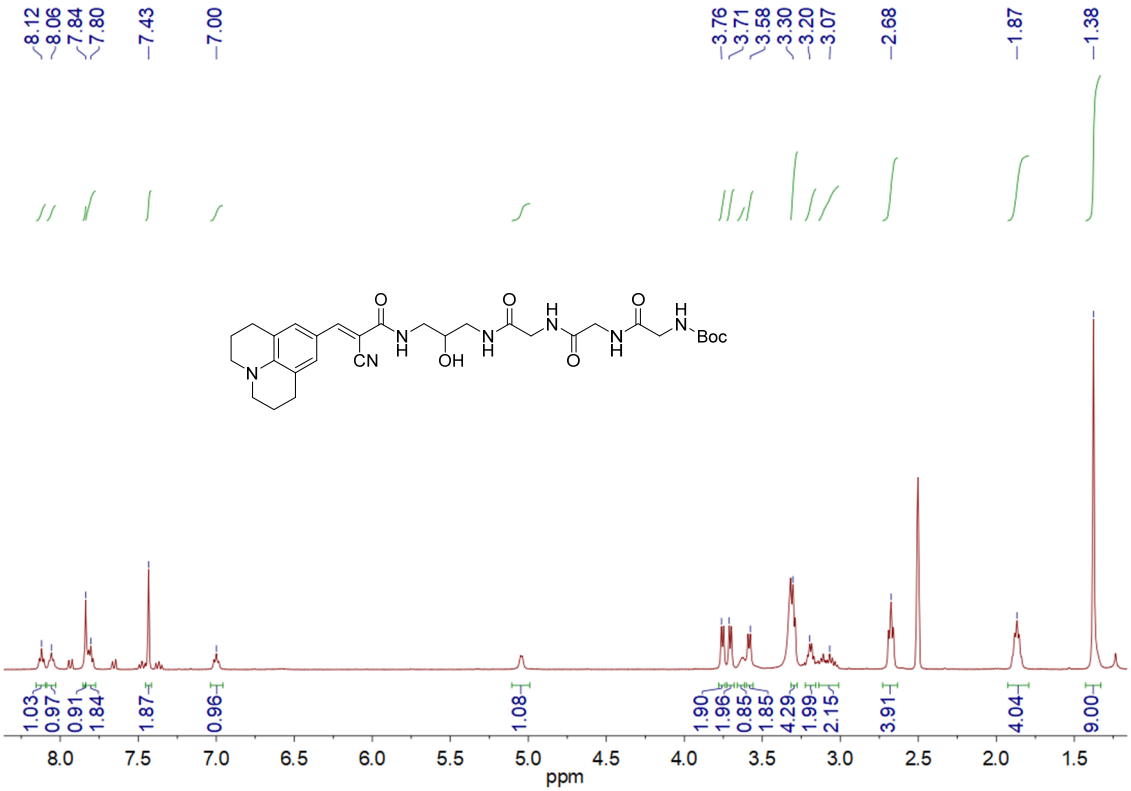


^{1}H-NMR spectrum of CCVJ-2 (DMSO-d^{6})

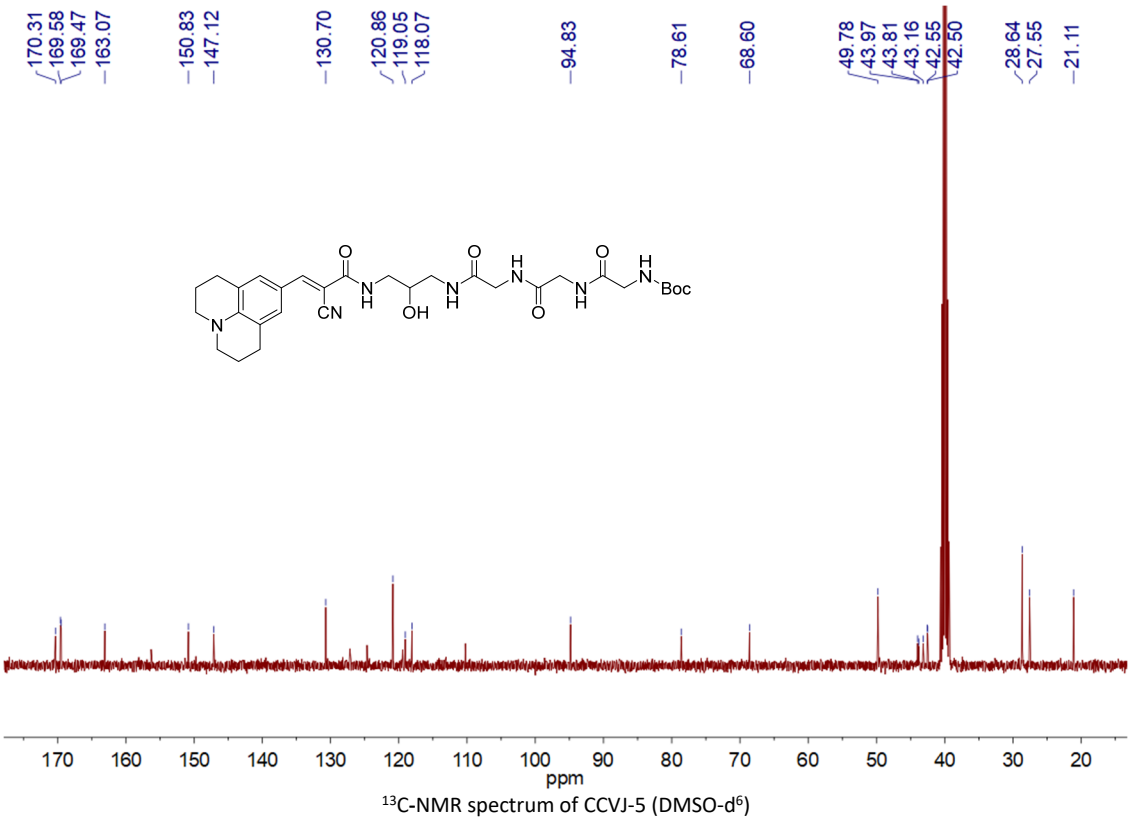


^{13}C-NMR spectrum of CCVJ-2 (DMSO-d^{6})

Compound **CCVJ-5**

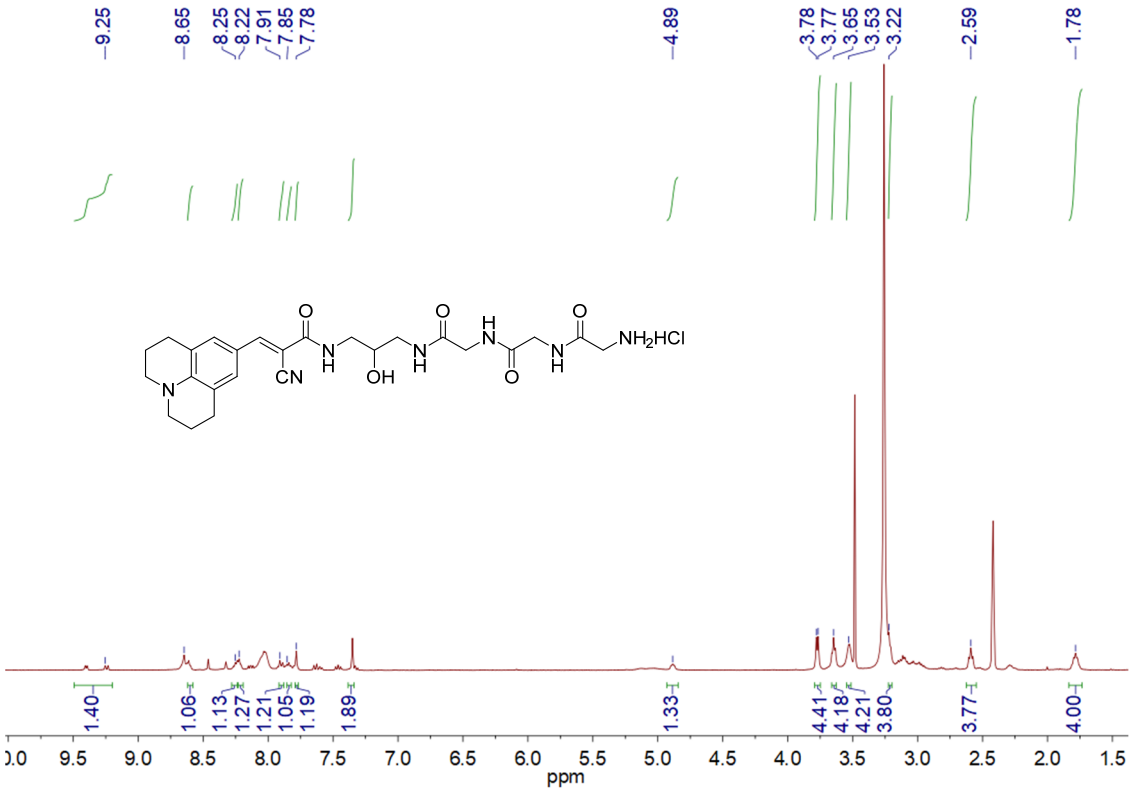


^{1}H-NMR spectrum of CCVJ-5 (DMSO-d^{6})

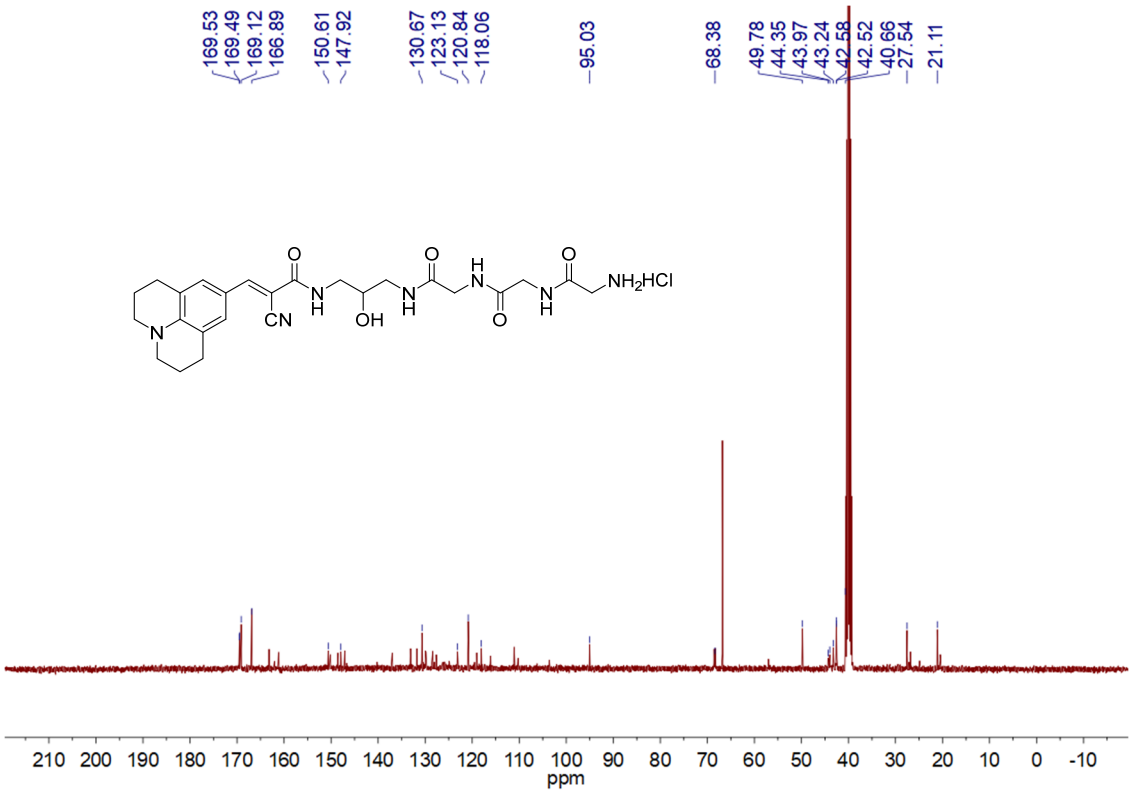


^{13}C-NMR spectrum of CCVJ-5 (DMSO-d^{6})

Compound **CCVJ-6**

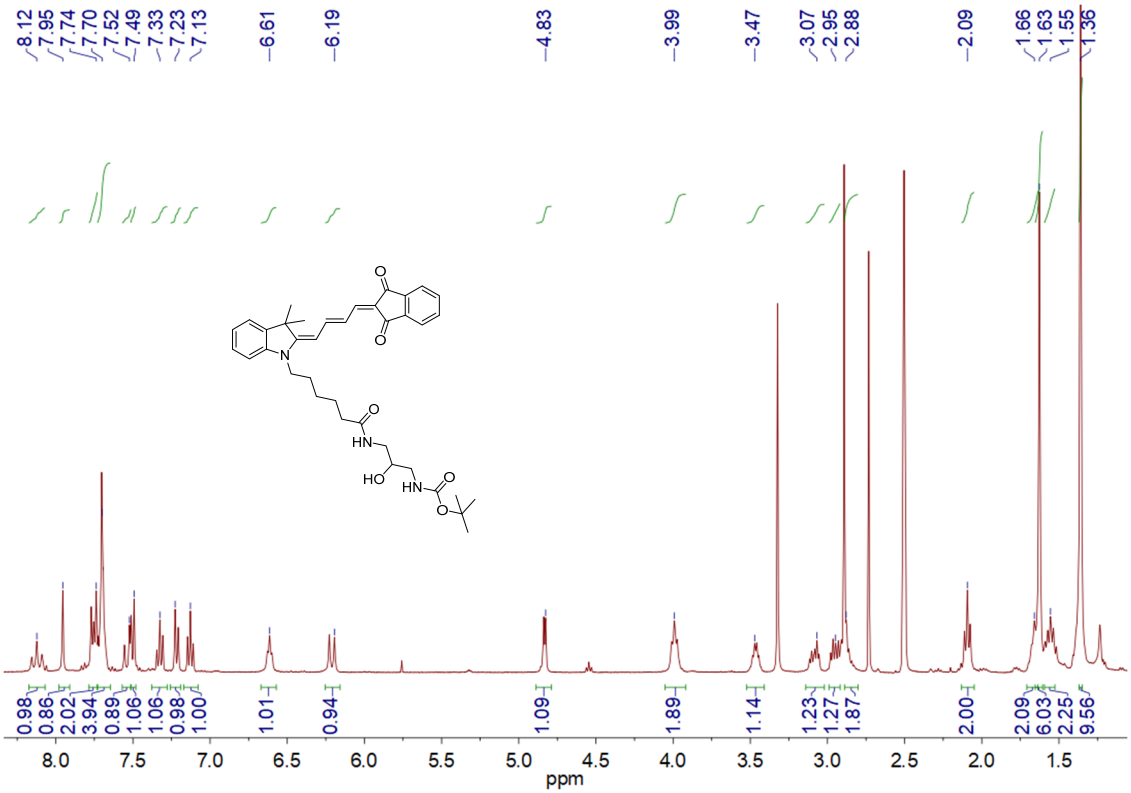


^{1}H-NMR spectrum of CCVJ-6 (DMSO-d^{6})

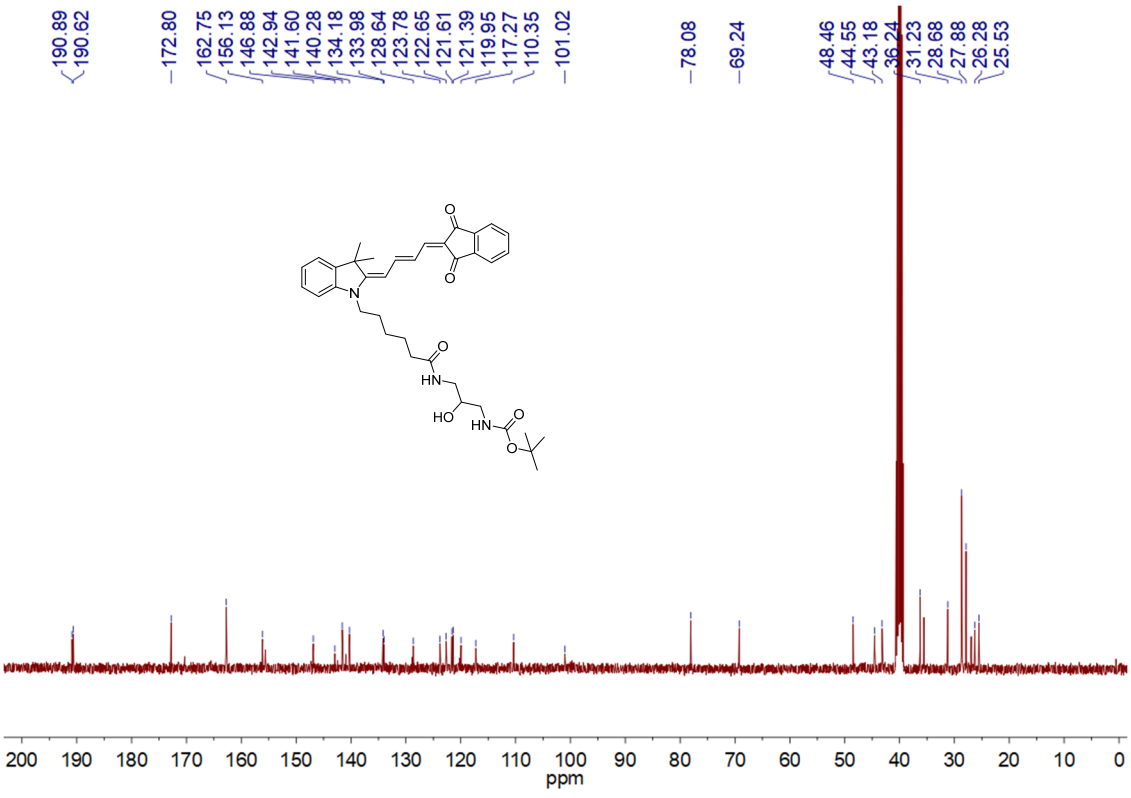


^{13}C-NMR spectrum of CCVJ-6 (DMSO-d^{6})

Compound **Mero-2**

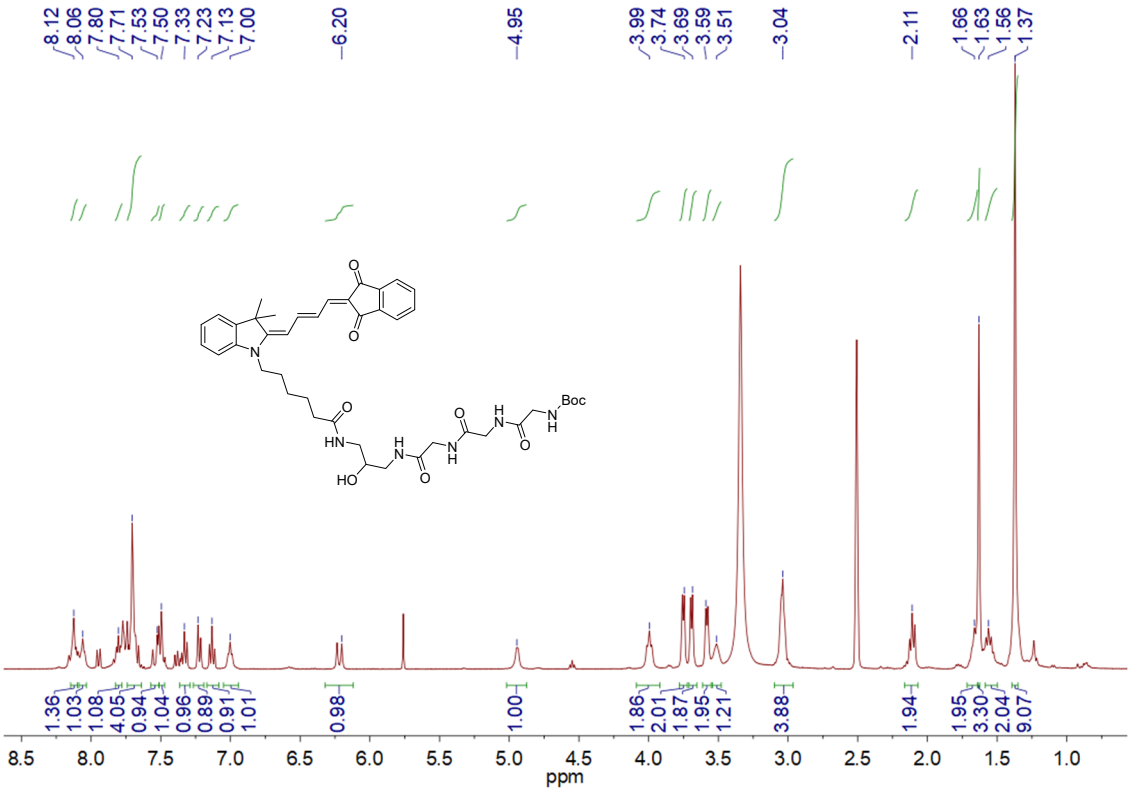


^{1}H-NMR spectrum of Mero-2 (DMSO-d^{6})

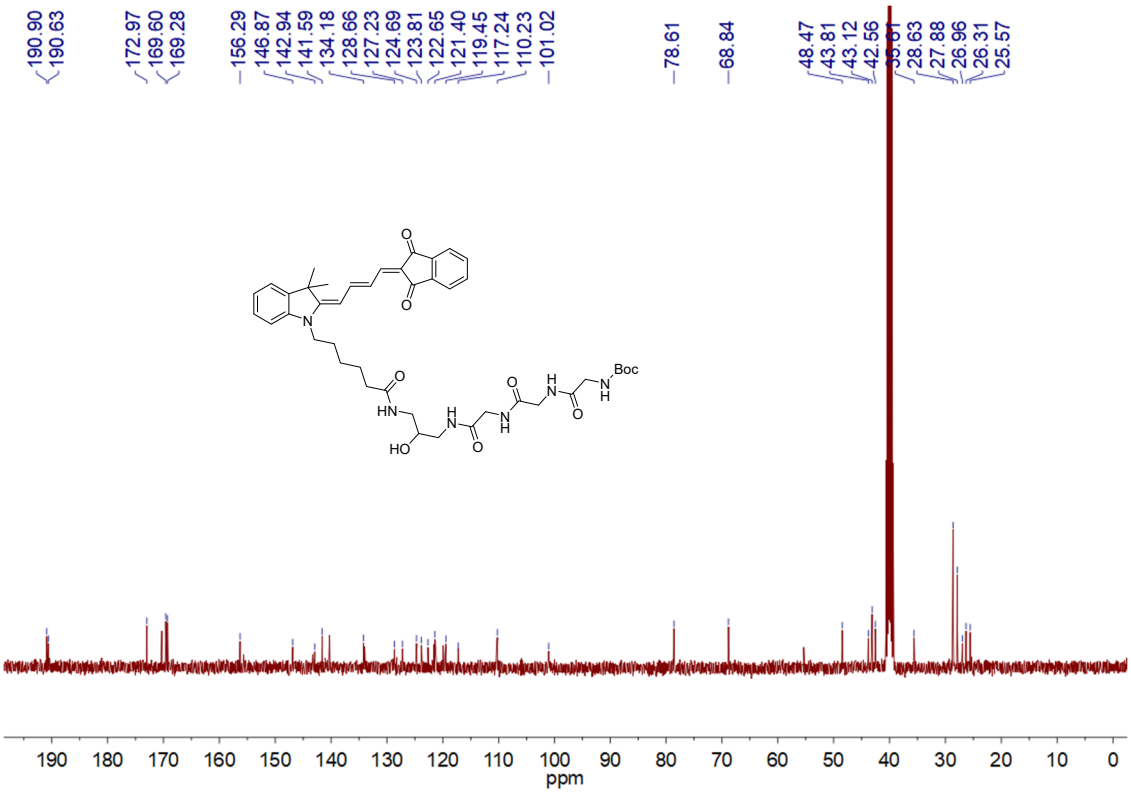


^{13}C-NMR spectrum of Mero-2 (DMSO-d^{6})

Compound **Mero-5**

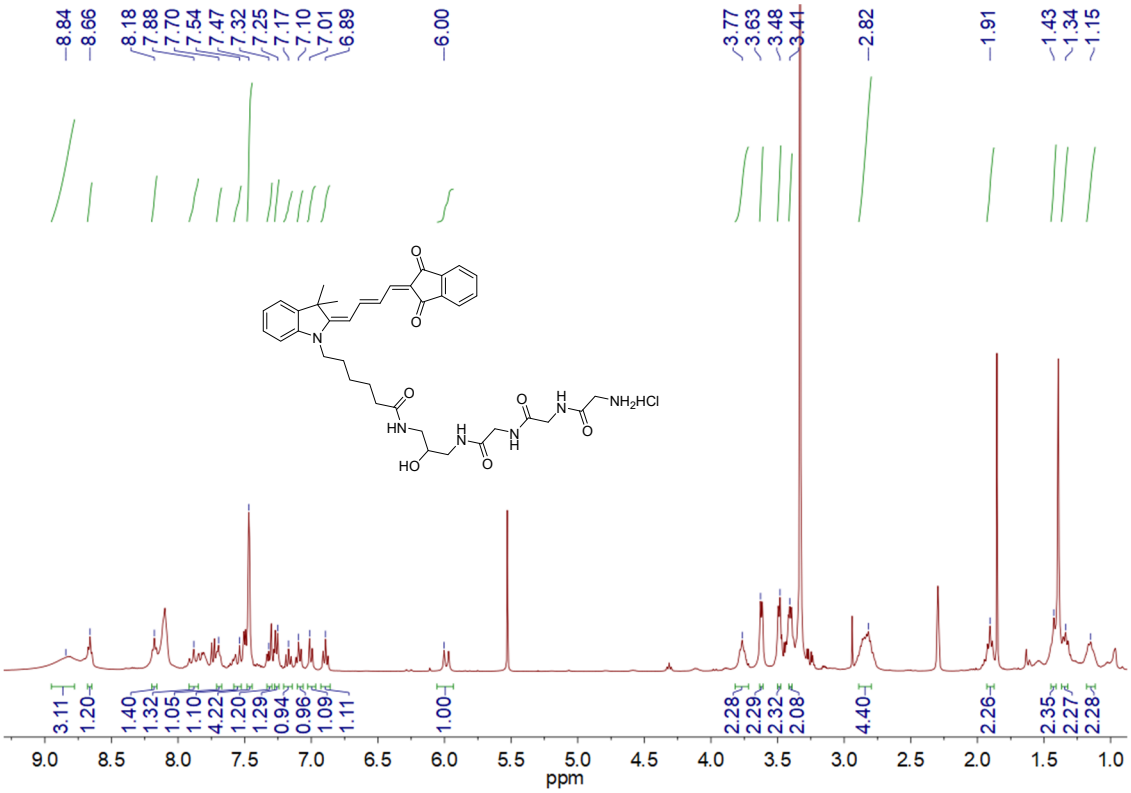


^{1}H-NMR spectrum of Mero-5 (DMSO-d^{6})

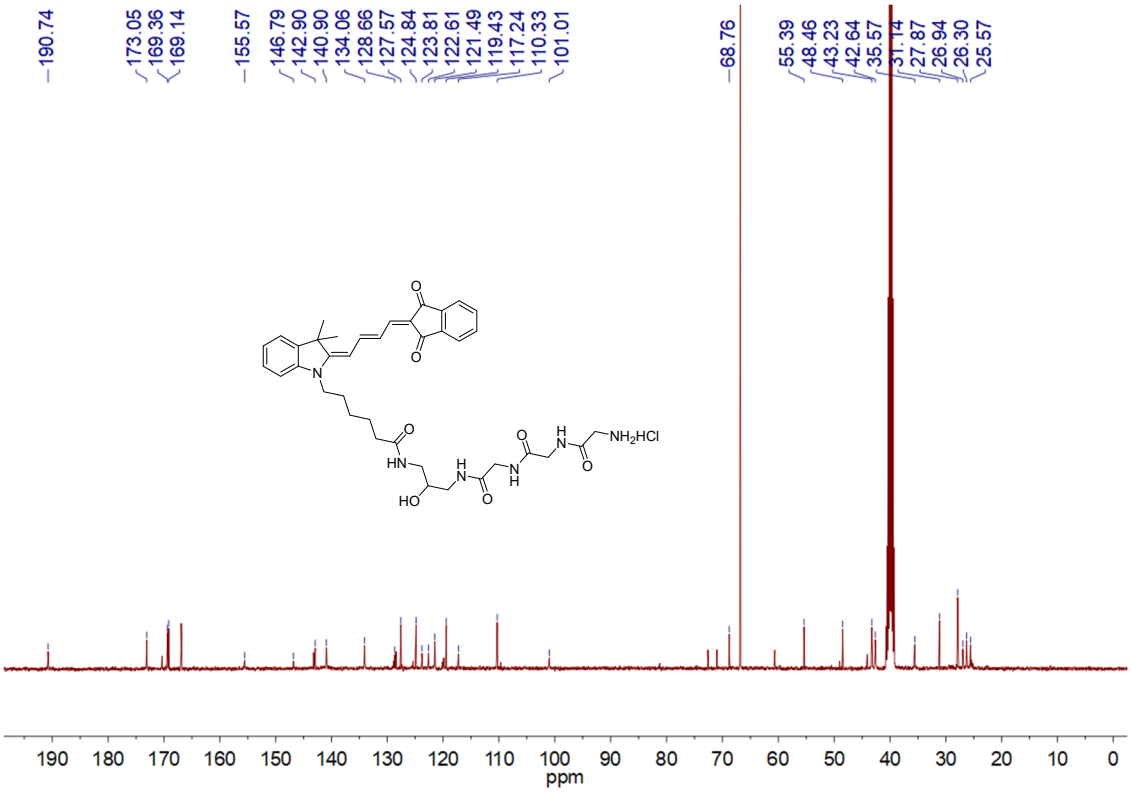


^{13}C-NMR spectrum of Mero-5 (DMSO-d^{6})

Compound **Mero-6**

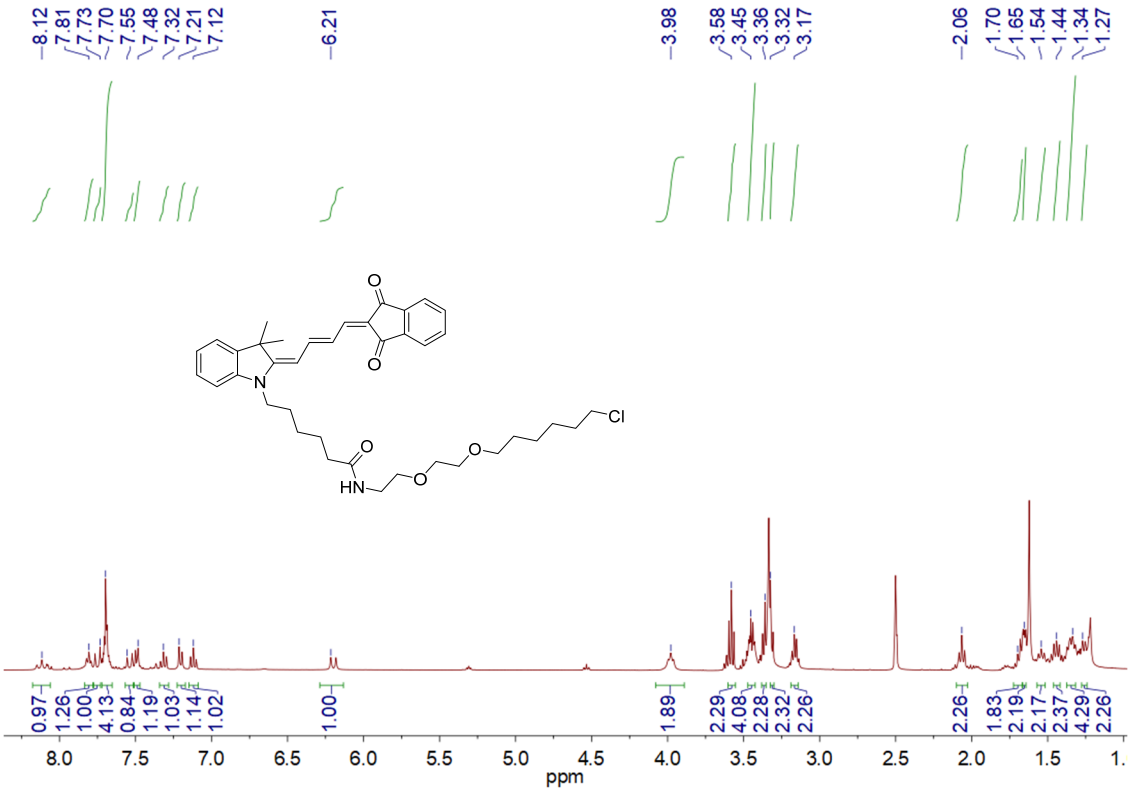


^{1}H-NMR spectrum of Mero-6 (DMSO-d^{6})

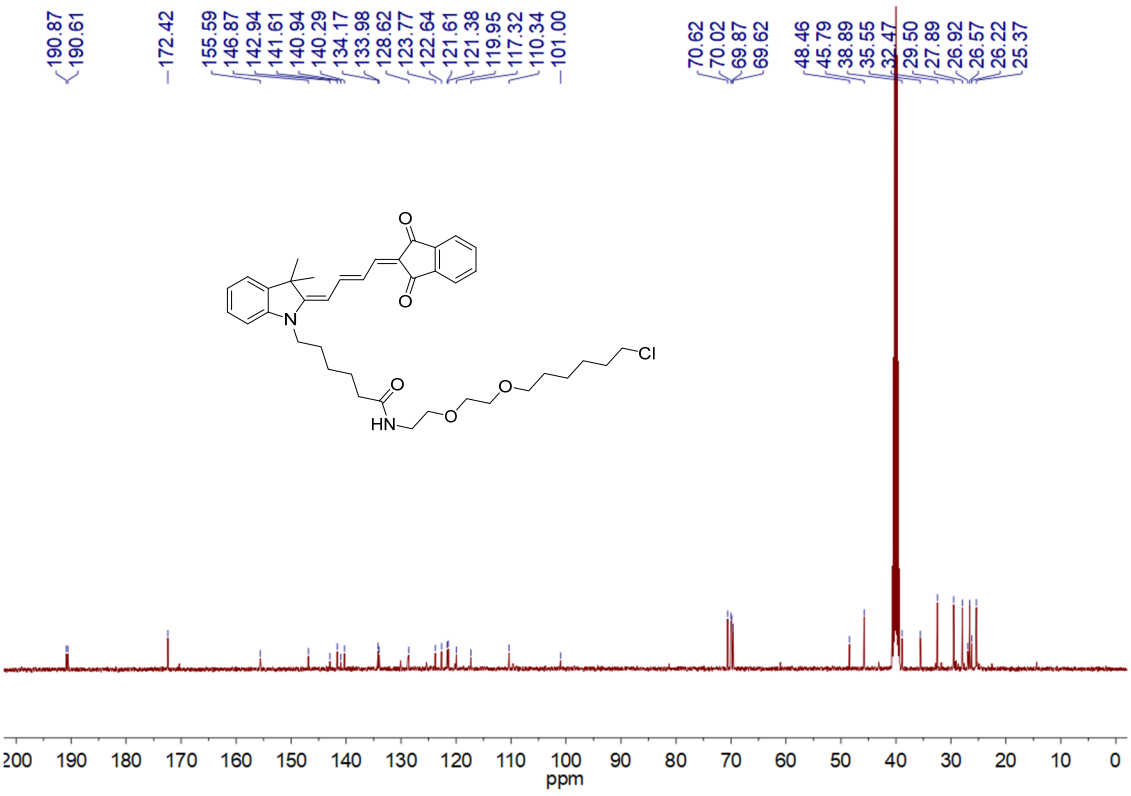


^{13}C-NMR spectrum of Mero-6 (DMSO-d^{6})

Compound **Mero-Halo**

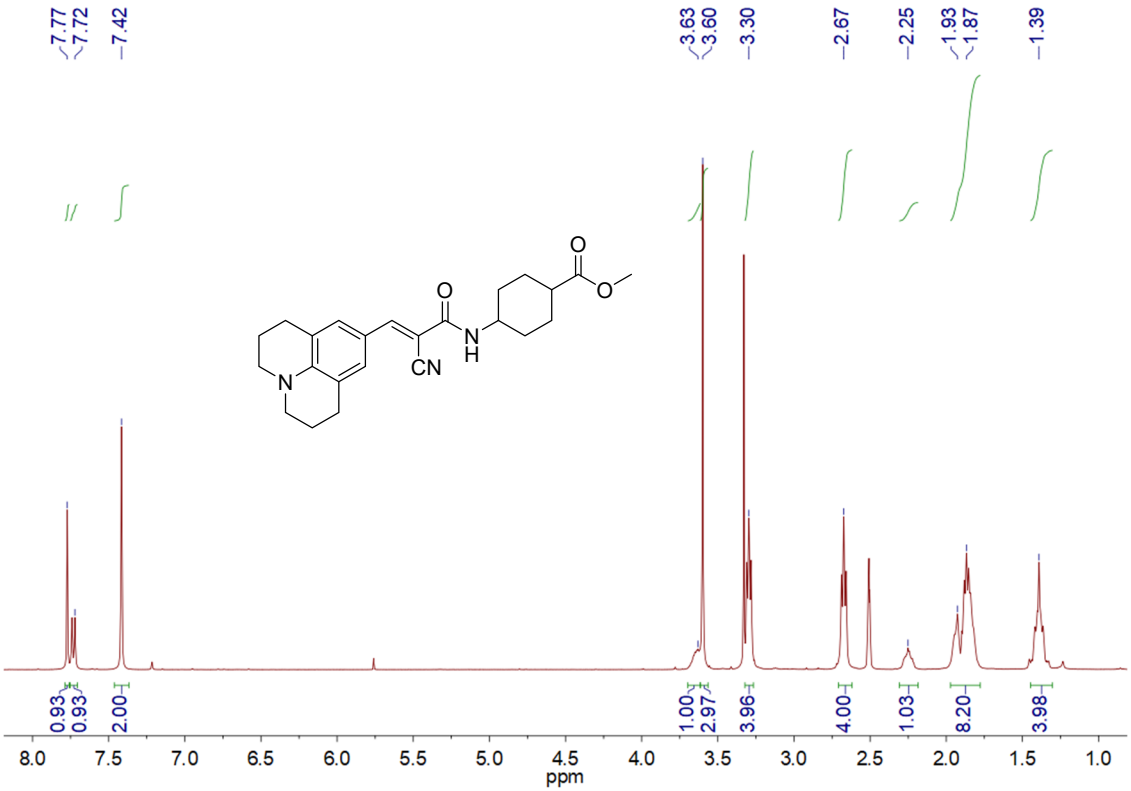


^{1}H-NMR spectrum of Mero-Halo (DMSO-d^{6})

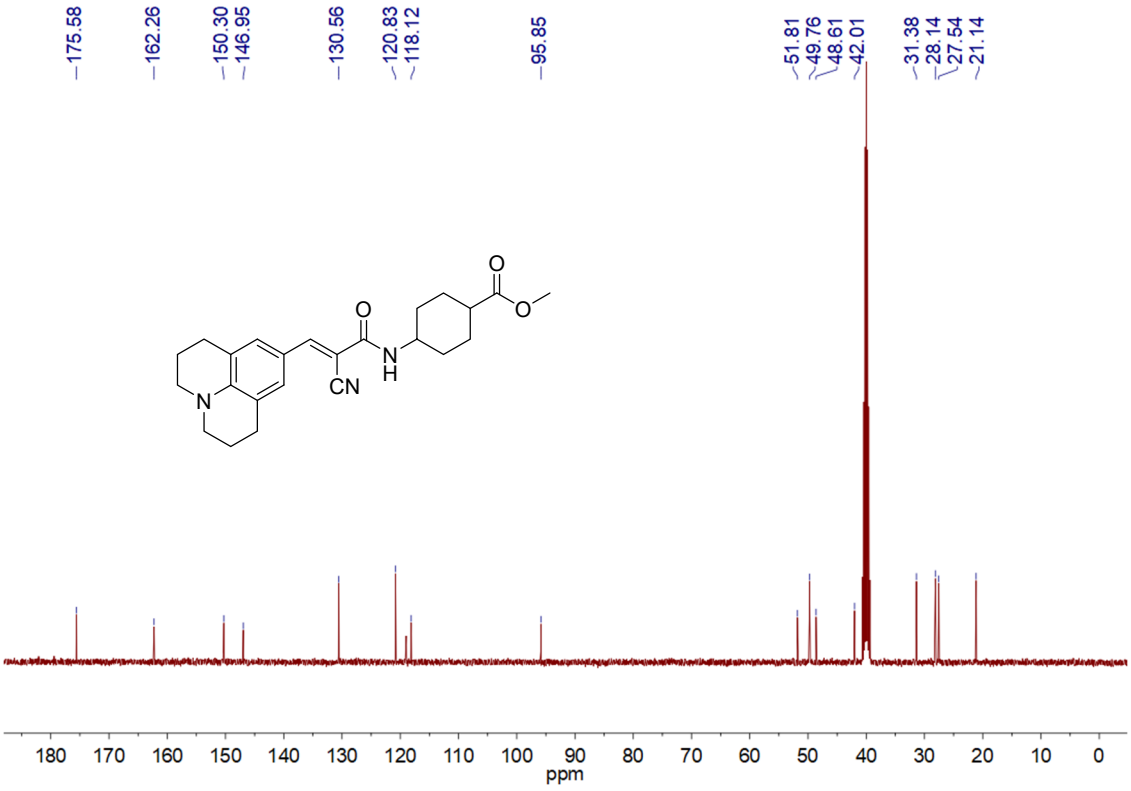


^{13}C-NMR spectrum of Mero-Halo (DMSO-d^{6})

Compound **CCVJ-7a**

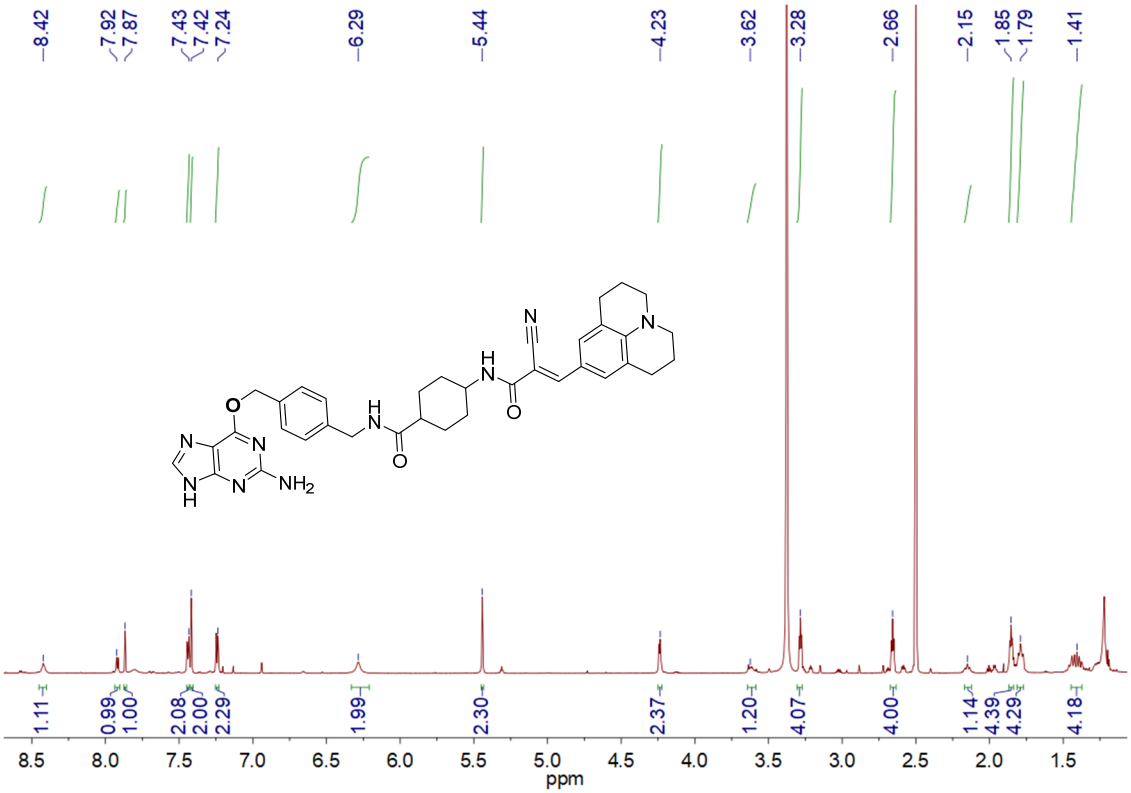


^{1}H-NMR spectrum of CCVJ-7a (DMSO-d^{6})

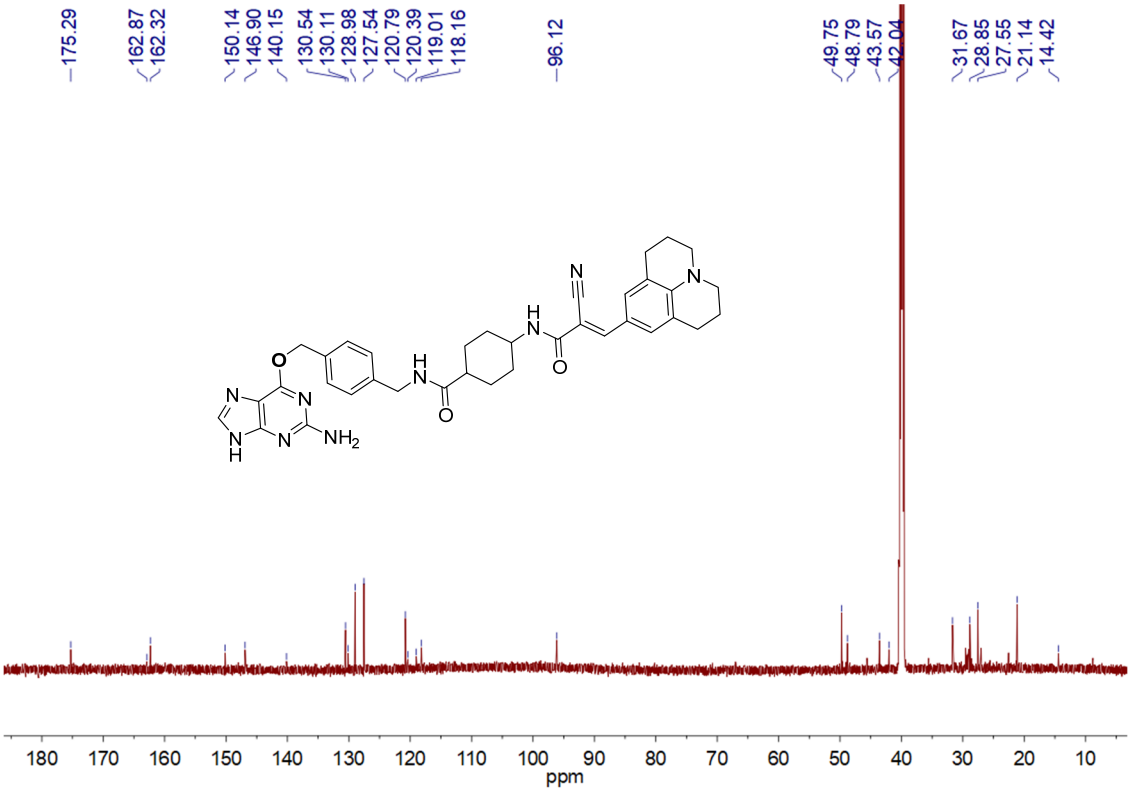


^{13}C-NMR spectrum of CCVJ-7a (DMSO-d^{6})

Compound **CCVJ-SNAP**



^{1}H-NMR spectrum of CCVJ-SNAP (DMSO-d^{6})



^{13}C-NMR spectrum of CCVJ-SNAP (DMSO-d^{6})

References

[1] C. P. Guimaraes, M. D. Witte, C. S. Theile, G. Bozkurt, L. Kundrat, A. E. M. Blom, H. L. Ploegh, *Nat. Protoc.* 2013, **8**, 1787-1799.

[2] Y. Liu, C. H. Wolstenholme, G. C. Carter, H. Liu, H. Hu, L. S. Grainger, K. Miao, M. Fares, C. A. Hoelzel, H. P. Yennawar, G. Ning, M. Du, L. Bai, X. Li, X. Zhang, *J. Am. Chem. Soc.* 2018, **140**, 7381-7384.

[3] M. C. Lo, A. Aulabaugh, G. Jin, R. Cowling, J. Bard, M. Malamas, G. Ellestad, *Anal. Biochem.* 2004, **332**, 153-159.

[4] K. H. Jung, S. F. Kim, Y. Liu, X. Zhang, *Chembiochem* 2019, **20**, 1078-1087.

[5] M. Fares, Y. Li, Y. Liu, K. Miao, Z. Gao, Y. Zhai, X. Zhang, *Bioconjug. Chem.* 2018, **29**, 215-224.

[6] S. Monfette, Z. R. Turner, S. P. Semproni, P. J. Chirik, *J. Am. Chem. Soc.* 2012, **134**, 4561-4564.

[7] Y. F. Liang, K. Wu, S. Song, X. Y. Li, X. Q. Huang, N. Jiao, *Org. Lett.* 2015, **17**, 876-879.

[8] S. Y. Nishimura, S. J. Lord, L. O. Klein, K. A. Willets, M. He, Z. Lu, R. J. Twieg, W. E. Moerner, *J. Phys. Chem. B* 2006, **110**, 8151-8157.

[9] C. J. MacNevin, D. Gremyachinskiy, C. W. Hsu, L. Li, M. Rougie, T. T. Davis, K. M. Hahn, *Bioconjug. Chem.* 2013, **24**, 215-223.