Electronic Supplementary Material (ESI) for ChemComm.

Derivatizing Merocyanine Dyes to Balance Their Polarity and Viscosity Sensitivities for Protein Aggregation Detection

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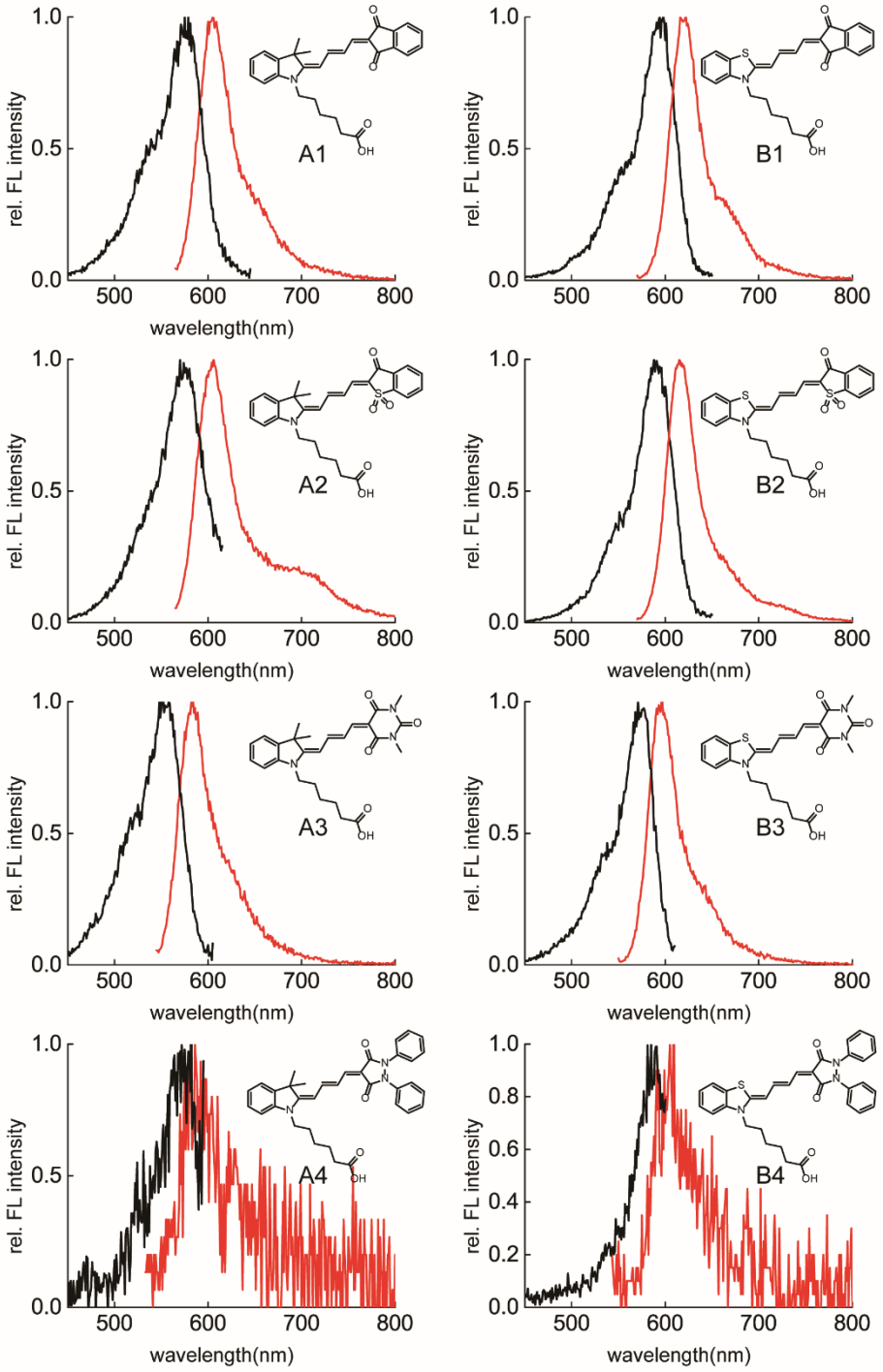


Fig. S1 Normalized emission and excitation spectra of merocyanines in non-polar dioxane. Merocyanines (10 μM) were prepared in dioxane. Spectra were collected with excitation wavelength of 550 nm for A1, 550 nm for A2, 530 nm for A3, 515 nm for A4, 555 nm for B1, 555 nm for B2, 535 nm for B3, 515 nm for B4. Excitation spectra were collected with emission wavelength of 660 nm for A1, 660 nm for A2, 620 nm for A3, 610 nm for A4, 665 nm for B1, 665 nm for B2, 625 nm for B3, 615 nm for B4. Black line represents excitation spectra and red line represents emission spectra. All measurements were carried out using Tecan Spark Fluorescence Plate Reader in BeyoGold™ 96-Well Black Opaque plates.

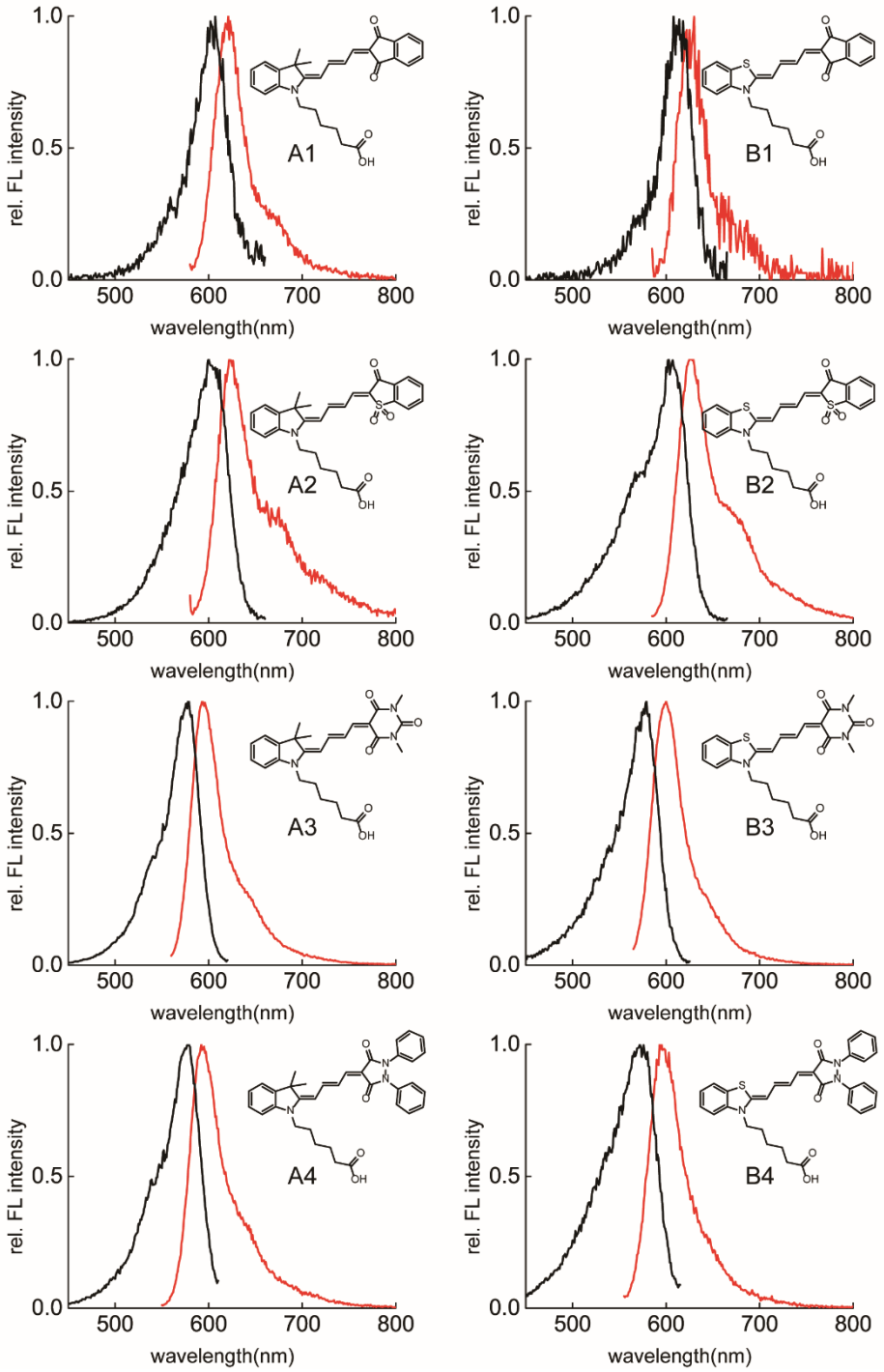


Fig. S2 Normalized emission and excitation spectra of merocyanines in viscous glycerol. Merocyanines (10 μM) were prepared in glycerol. Emission spectra were collected with excitation wavelength of 565 nm for A1, 565 nm for A2, 545 nm for A3, 535 nm for A4, 570 nm for B1, 570 nm for B2, 550 nm for B3, 540 nm for B4. Excitation spectra were collected with emission wavelength of 675 nm for A1, 675 nm for A2, 635 nm for A3, 625 nm for A4, 680 nm for B1, 680 nm for B2, 640 nm for B3, 630 nm for B4. Black line represents excitation spectra and red line represents emission spectra. All measurements were carried out using Tecan Spark Fluorescence Plate Reader in BeyoGold™ 96-Well Black Opaque plates.

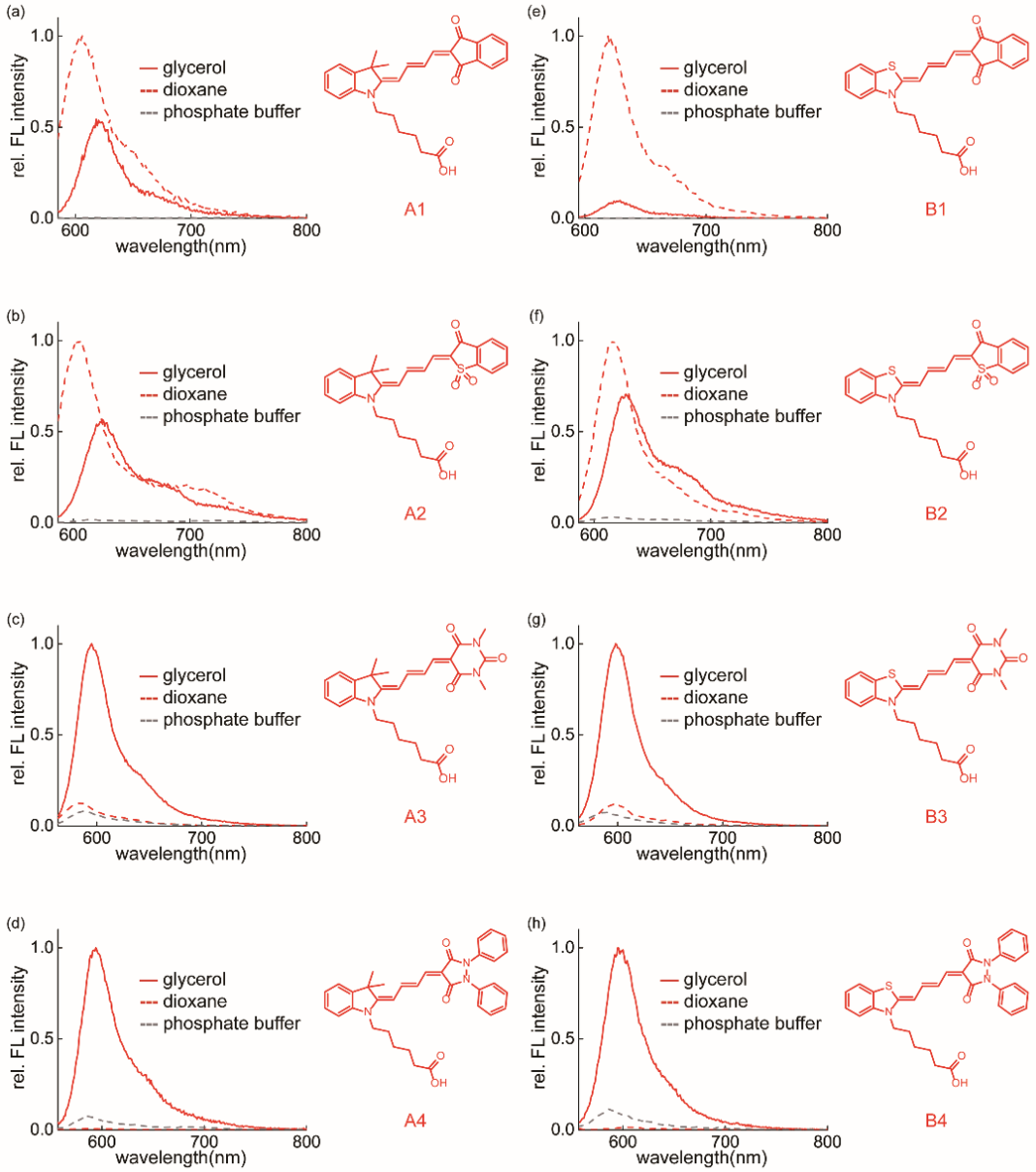


Fig. S3 Merocyanine dyes (10 μM) were dissolved in viscous glycerol, non-polar dioxane and polar phosphate buffer. Fluorescent emission spectra were collected by using 570 nm for A1, 572 nm for A2, 548 nm for A3, 545 nm for A4, 580 nm for B1, 578 nm for B2, 549 nm for B3, 5542 nm for B4 as excitation wavelength. The fluorescent spectra were measured by Tecan Spark Fluorescence Plate Reader using BeyoGold™ 96-Well Black Opaque plates.

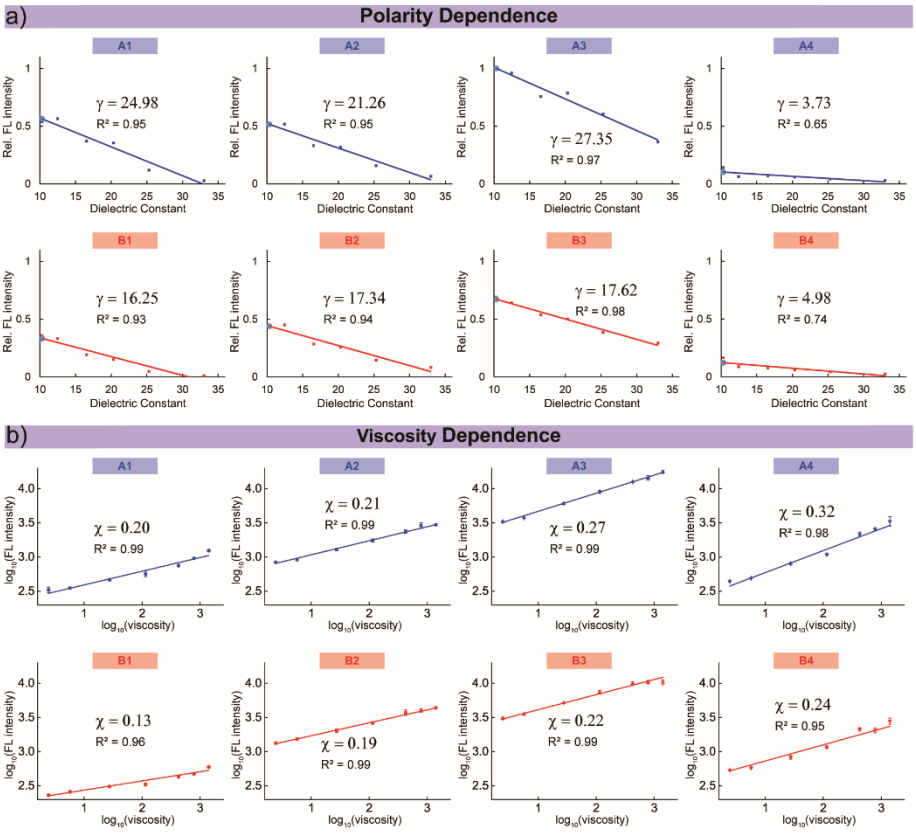


Fig. S4 Modulation of merocyanine's fluorescence properties. (a) Polarity dependence of eight merocyanines. The scatter diagram of each merocyanine showed the relation of relative fluorescence intensity and dielectric constant. γ value was calculated as 1000 times the absolute value of gradient between relative fluorescence intensity and dielectric constants of solvents. (b) Viscosity dependence of eight merocyanines. The scatter diagram of each merocyanine showed the relation of fluorescence intensity and viscosity of solvents. χ value was calculated as the gradient between log of fluorescence intensity and log of viscosity.

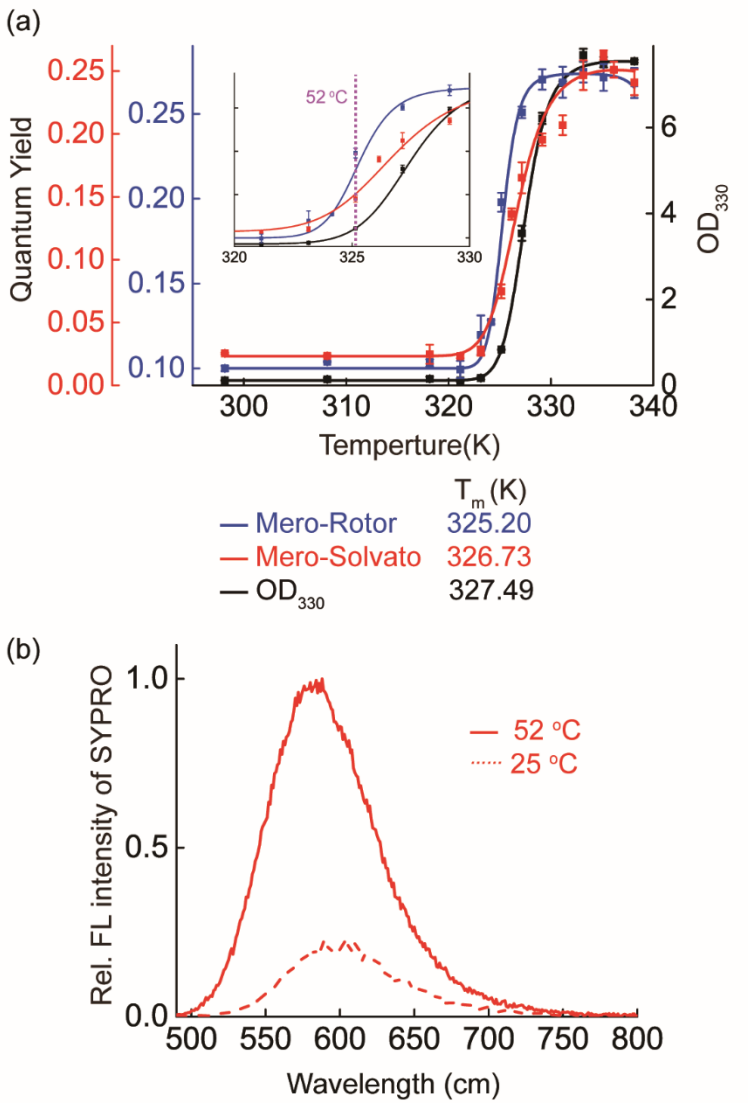


Fig. S5 Mero-Rotor detected early-stage aggregated species (misfolded soluble oligomers), earlier than Mero-Solvato. (a) Thermal shift assay of DHFR measured by Mero-Rotor, Mero-Solvato and OD_{330}. The melting temperature (T_m) was calculated based on the equation as showed in Experimental Method Section. Thermal shift assay of DHFR measured by OD_{330} represented the formation of insoluble protein aggregates. The T_m values of Mero-Rotor and Mero-Solvato were smaller than that of OD_{330}, which indicated the response to early-stage aggregated species (misfolded soluble oligomers) of probes. (b) DHFR formed misfolded oligomers when incubated at 52 °C, which was confirmed by SYPRO® Orange probe, a fluorescent sensor response to early-stage unfolded and misfolded proteins.

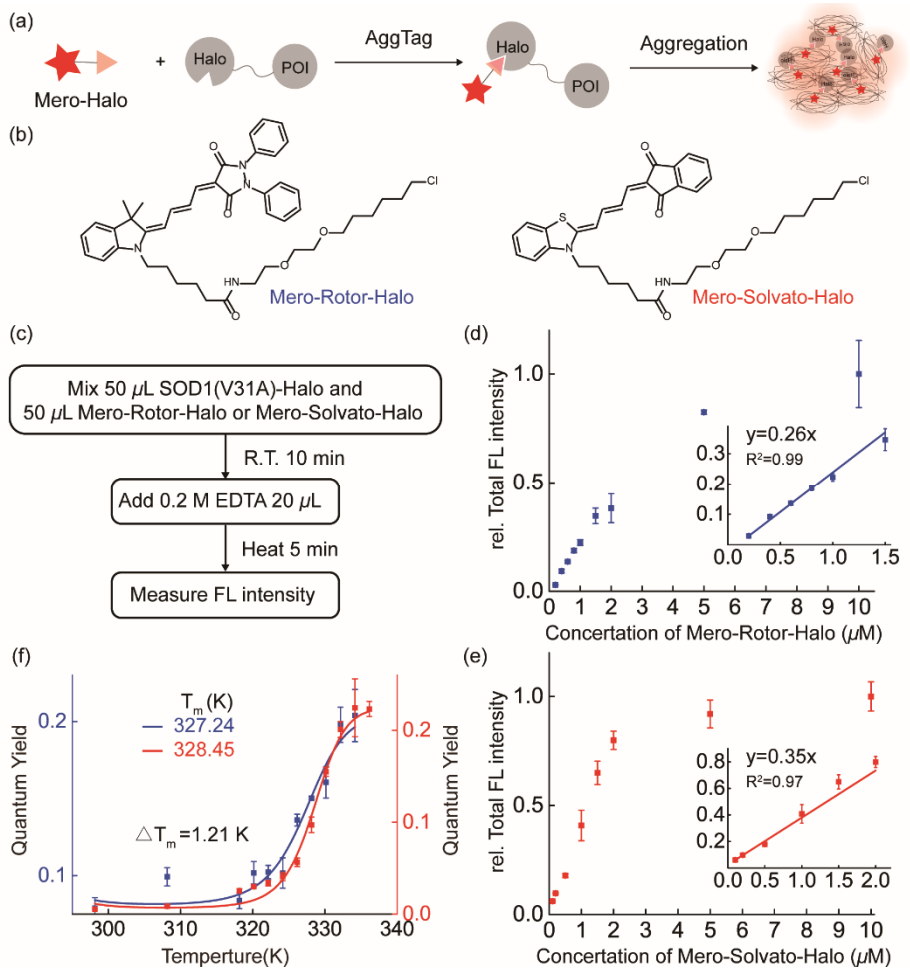


Fig. S6 Monitoring SOD1 (V31A)-Halo protein aggregation using two probes with different micro-environmental sensitivities. (a) Scheme of AggTag method to detect protein aggregation. (b) Structures of Mero-Rotor-Halo and Mero-Solvato-Halo probes. (c) Flow chart of SOD1 (V31A)–Halo protein aggregation experiment. (d) Stoichiometry, linear range, and lowest detection limits of Mero-Rotor-Halo labelled SOD1 (V31A)–Halo in detecting SOD1 (V31A)–Halo aggregation. (e) Stoichiometry, linear range, and lowest detection limits of Mero-Solvato-Halo labelled SOD1 (V31A)–Halo in detecting SOD1 (V31A)–Halo aggregation. (f) Thermal shift assay of SOD1 (V31A)–Halo measured by Mero-Rotor-Halo and Mero-Solvato-Halo probes. The melting temperature (T_m) was calculated based on the equation as showed in supporting information. The earlier fluorescence response by Mero-Rotor-Halo probe (blue curve) than Mero-Solvato-Halo probe in detecting the aggregation of SOD1 proteins highlighted its better sensitivity in detecting early-stage protein aggregation, presumably the misfolded soluble oligomers. These results echoed what was observed and concluded in Fig. 4.

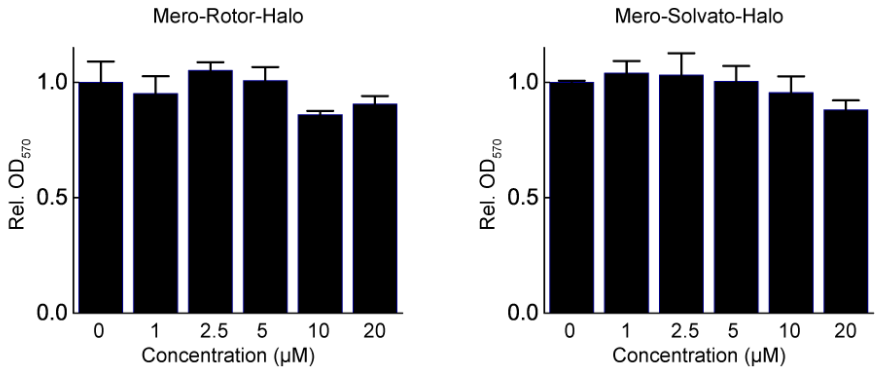


Fig. S7 Mero-Rotor-Halo and Mero-Solvato-Halo showed minimal cytotoxicity to HEK293T cells.

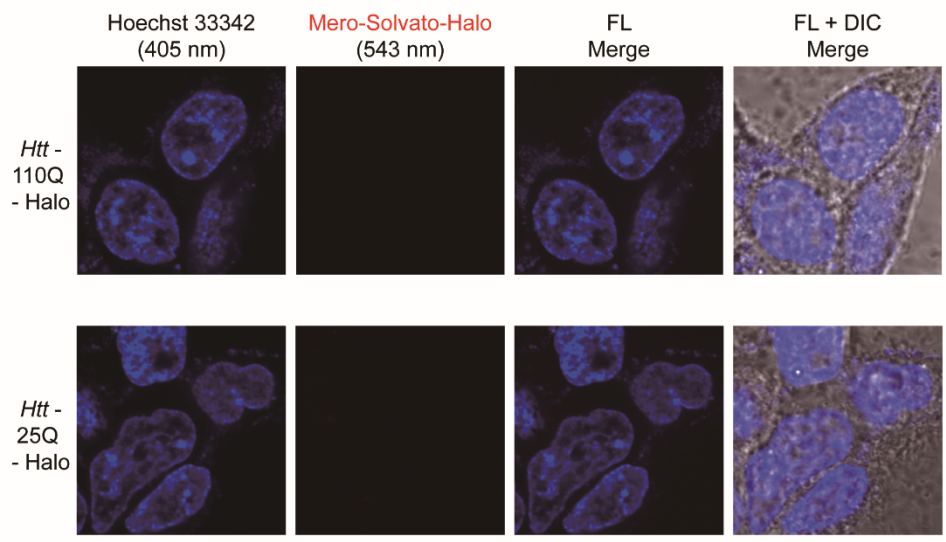


Fig. S8 Mero-Solvato-Halo cannot image insoluble aggregates of *Htt*-110Q-Halo in live HEK293T cells. No punctate fluorescence was found under 543 nm channel.

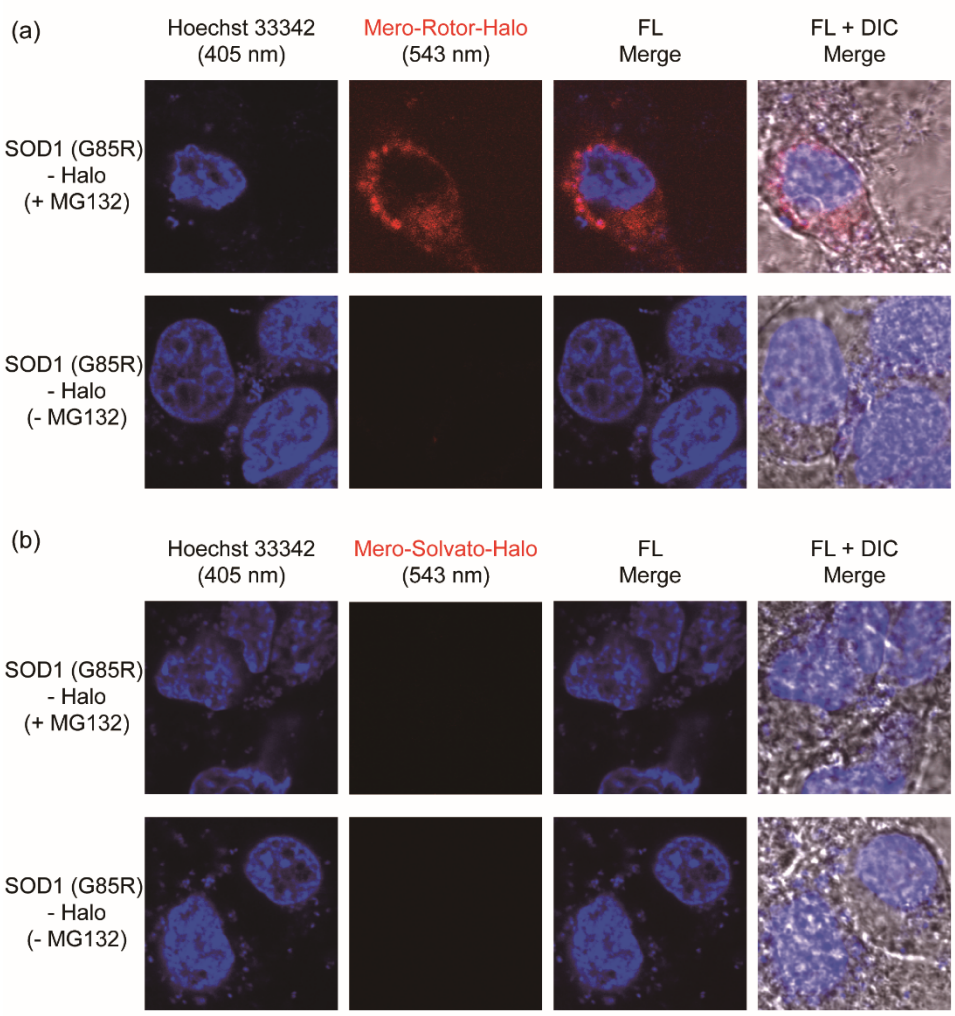


Fig. S9 Detecting SOD1 mutant protein aggregation in live cells using two merocyanine probes with different micro-environmental sensitivities. (a) Mero-Rotor-Halo probe can image the aggregates of SOD1(G85R)-Halo upon inhibition protein degradation in live HEK293T cells. Punctate fluorescence was found under 543 nm channel. (b) Mero-Solvato-Halo cannot image the aggregates of SOD1(G85R)-Halo upon inhibiting protein degradation in live HEK293T cells. No punctate fluorescence was found under 543 nm channel.

Table S1 The photophysical parameters of merocyanine probes in dioxane.[a]

Compound	λ_{ex} (nm)	λ_{em} (nm)	Φ	λ_{abs} (nm)	ε ($M^{-1}cm^{-1}$)[b]
A1	576	605	0.08	575	72683
A2	572	604	0.14	572	91067
A3	558	584	0.05	553	107496
A4	561	588	< 0.01	561	56206
B1	594	619	0.05	594	118376
B2	593	618	0.13	590	110072
B3	571	595	0.04	571	94199
B4	582	604	< 0.01	578	59758

[a] All photophysical parameters in this table were measured in dioxane except extinction coefficient.

[b] Extinction coefficient was measured in methanol. Further details see experimental method.

Table S2 The photophysical parameters of merocyanine probes in glycerol.[a]

Compound	λ_{ex} (nm)	λ_{em} (nm)	Φ	λ_{abs} (nm)	ε ($M^{-1}cm^{-1}$)[b]
A1	600	622	0.03	604	72683
A2	602	627	0.07	603	91067
A3	578	595	0.46	574	107496
A4	575	595	0.12	575	56206
B1	610	627	< 0.01	609	118376
B2	608	626	0.12	605	110072
B3	579	600	0.37	575	94199
B4	572	598	0.13	572	59758

[a] All photophysical parameters in this table were measured in glycerol except extinction coefficient.

[b] Extinction coefficient was measured in methanol. Further details see experimental method.

Experimental Method Section：

1. Plasmids construct and protein purifications.

cDNA gene of WT-DHFR was synthesized by Genescript, Nanjing, China, followed by subcloning into the pET29b$^+$ vector. The C-termini of protein was fused with a LPETGHHHHHH sequence for sortase ligation and His-Tag purification purposes. SOD1 (V31A)-Halo in pET29b$^+$ vector, *Htt*-110Q-Halo and *Htt*-25Q-Halo in pHTC vector were previously reported. They were gifts generously provided by Professor Xin Zhang's group from the Pennsylvania State University, USA.

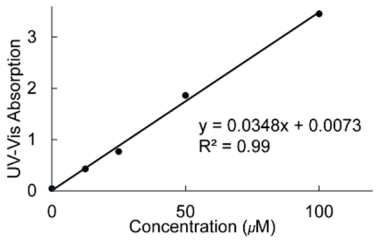
WT-DHFR-His (or SOD1 (V31A) -Halo) plasmid was transformed into BL21 DE3 *E. coli* cells. Cells were grown to OD_{600} at 0.6-0.8 before induced by IPTG (0.5 mM/L) at 37 °C for 4 h (SOD1 (V31A) -Halo at 18 °C overnight). Cultured cells were harvested and resuspended in resuspension buffer (50 mM Tris,100 mM NaCl, pH = 8.00). Cells expressing recombinant proteins were thawed and lysed by sonication at 4 °C. 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 by 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 impurity was identified and purity was estimated to be 98% based on SDS-PAGE electrophoresis analysis.

2. Site-specific labeling and purification (Fig. 3a).

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 ddH2O. 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. Preparation of dye solutions and determination of their concentrations.

Stock solutions of merocyanine dyes were prepared by dissolving in DMSO. We weighed the solid by analytical balance and prepared 10 mM solution in DMSO. Then we used standard curve in quantifying the concentration of dye solutions. For example, we can obtain the precise weight of easily synthesized B1 and measure the linear relationship between UV-Vis absorption and probe concentration of B1 ($y = 0.0348x + 0.0073$). When we prepare the solution of Mero-Solvato (B1-GGG) or Mero-Solvato-Halo (B1-Halo probe), we can quantify their concentrations by the standard curve of B1. In this way, we can determine the concentrations of dye solutions as accurately as possible.



4. Fractionation experiment of aggregated DHFR (Fig. 3d).

WT-DHFR (49 μM) was mixed with Merocyanine-labelled WT-DHFR (1 μM) in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23). Protein aggregation (1 mL) was induced by incubating 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 aggregated sample was then subject to centrifugation at 13,800 × g 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 resuspended 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 followed the **Experimental Method Section 10**.

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.

5. Heat induced DHFR aggregation and fluorescence measurement.

WT-DHFR protein (49 μM) and Merocyaine-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 65 °C for 5 min. The fluorescent spectra were collected by Tecan Spark Fluorescence Plate Reader using BeyoGold™ 96-Well Black Opaque plates (for Mero-Solvato-labelled proteins: ex. 575 nm, em. 633 nm; for Mero-Rotor-labelled proteins: ex. 535 nm, em. 596 nm).

To measure the linear range and detection limits, freshly purified WT-DHFR protein and Merocyanine-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. Mixing 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 prepared samples were incubated at 65 °C for 5 min to induce DHFR aggregation. For Mero-Solvato-labelled proteins, total fluorescence intensity was measured by exciting aggregated samples at 575 nm and then we collected the fluorescent emission intensity from 590 nm to 850 nm. For Mero-Rotor-labelled proteins, total fluorescence intensity was measured by exciting the samples at 535 nm and collecting the fluorescent emission intensity from 550 nm to 850 nm.

6. DHFR thermal shift assay.

Freshly purified WT-DHFR protein (49 μM) and Mero-Solvato-labelled WT-DHFR (1 μM) were mixed together in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated from 25 °C to 65 °C with 3 °C temperature intervals. The incubated mixtures (100 μL) were pipetted into BeyoGold™ 96-Well Black Opaque plates then we collected the fluorescent emission from 590 nm to 850 nm by using 575 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

Freshly purified WT-DHFR protein (49 μM) and Mero-Rotor-labelled WT-DHFR (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 65 °C with 3 °C temperature intervals. The incubated mixtures (100 μL) were pipetted into BeyoGold™ 96-Well Black Opaque plates then we collected the fluorescent emission from 550 nm to 850 nm by using 535 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

7. Aggregation kinetics of DHFR.

Freshly purified WT-DHFR (49 μM), Mero-Solvato-labelled-WT-DHFR (1 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated at 51 °C. The incubated mixtures (100 μL) were pipetted into BeyoGold™ 96-Well Black Opaque plates then we collected the fluorescent emission from 590 nm to 850 nm by using 575 nm as the excitation wavelength. The fluorescent emission signal was collected every 2 minutes and repeated three times.

Freshly purified WT-DHFR (49 μM), Mero-Rotor-labelled WT-DHFR (1 μM) were mixed in acidic aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) and incubated at 51 °C. The incubated mixtures (100 μL) were pipetted into BeyoGold™ 96-Well Black Opaque plates then we collected the fluorescent emission intensity from 550 nm to 850 nm by using 535 nm as the excitation wavelength. The fluorescent emission was collected every 2 minutes and repeated three times.

8. SOD1(V31A)-Halo thermal shift assay.

50 μL freshly purified SOD1(V31A)-Halo (120 μM) and 50 μL Mero-Solvato-Halo (4.8 μM) were mixed at room temperature for 10 minutes, and then 20 μL EDTA (0.2 *M*) was added to incubate for 5 minutes at different temperatures (25 °C to 63 °C). The incubated mixtures (100 μM) were pipetted into BeyoGold™ 96-Well Black Opaque plates and then we collected the fluorescent emission intensity from 590 nm to 850 nm by using 575 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

50 μL freshly purified SOD1(V31A)-Halo (120 μM) and 50 μL Mero-Rotor-Halo (4.8 μM) were mixed at room temperature for 10 minutes, and then 20 μL EDTA (0.2 M) was added to incubate for 5 minutes at different temperatures (25 °C to 63 °C). The incubated mixtures (100 μM) were pipetted into BeyoGold™ 96-Well Black Opaque plates and then we collected the fluorescent emission intensity from 550 nm to 850 nm by using 535 nm as the excitation wavelength. For each temperature, the experiments were repeated three times.

9. Analysis of thermal shift data [2].

The fluorescence thermal shift assay data from Mero-Solvato (or Mero-Rotor) in different proteins were fitted to Eq. (1) to obtain $\triangle H_u$, $\triangle C_{pu}$, and Tm by nonlinear curve fitting using the program OriginPro 2015:

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

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.

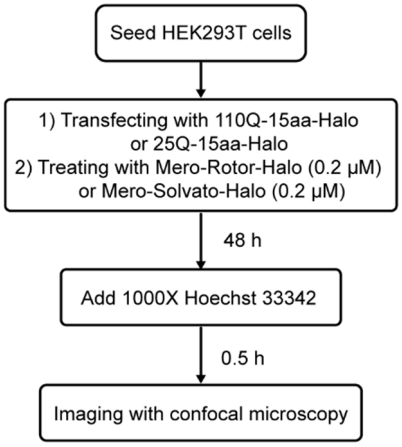
10. SDS-PAGE of insoluble section of transfected cell lysate [3].

HEK293T cells were seeded on 6-Well Cell Culture Plates and transiently transfected when the cell density reached 70%. In 100 μL opti-mem medium, 12 μL of X-tremegene 9 DNA transfection reagent (Roche) was added, and then plasmid DNA (4 μg of DNA for each transfection) and the mixture was fully mixed at room temperature for 20 min. The mixture and probe (TMR-Halo 2 μM) were then dripped into the cell medium. The experimental trial transfected with *Htt*-110Q-Halo was expressed for 36 hours. The controlled trial transfected with *Htt*-25Q-Halo was expressed for 36 hours. Next, 250 μL of RIPA (containing 1 % PMSF) was added into the cells to lyse. The samples were then subjected to centrifugation under 13,800 × g for 30 min at 4 °C. The insoluble fraction was prepared by resuspending the insolube aggregates in 100 μL RIPA (containing 1 % PMSF) and then

80 μL of each sample was finely mixed with 20 μL of 5 × SDS loading buffer. These mixtures were incubated at 95 °C for 5 min, and then loaded onto a 15 % acrylamide to run gel electrophoresis.

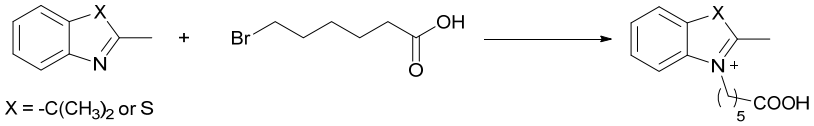
11. Procedure for cell culture, transfection, and confocal fluorescence imaging [4].

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 each transfection) and the mixture was fully mixed at room temperature for 20 min. The transfection mixture and probe (Mero-Solvato-Halo 0.2 μM or Mero-Rotor-Halo 0.2 μM, Fig. S7) were then dripped into the cell medium. The experimental trial transfected with *Htt*-110Q-Halo was expressed for 48 hours, and the controlled trial transfected with *Htt*-25Q-Halo was expressed for 48 hours as well. Hoechst 33342 staining reagent was added into cells 30 minutes before imaging. Confocal fluorescence images were collected by using Olympus FV1000MPE. Blue: 405 nm laser for Hoechst 33342; Red, 543 nm laser for Merocyanine probes. Th procedures was shown as flowchart.



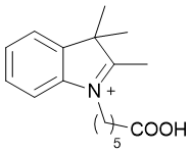
Synthetic Methods and Schemes:

Scheme S1. General methods of the synthesis of Donors.



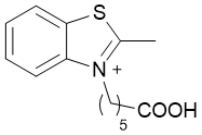
To a solution of acetonitrile (20.0 mL), 2,3,3-trimethylindolenine (3.2 mL, 20.0 mmol, 1.0 eq.), 6-bromohexanoic acid (15.6 g, 80.0 mmol, 4.0 eq.) and catalytic amount of KI (0.1 equiv.) were added, and then refluxed for 50 hours under inert atmosphere to yield dark purple solution. The reaction was monitored by TLC until the aromatic substrate consumed completely. The reaction mixture cooled down to room temperature, and then added it into cold ether (200.0 ml) with vigorous stirring and the precipitation was collected as crude product. The crude product was recrystallized to yield pure desired product.

Compound **Donor 1**



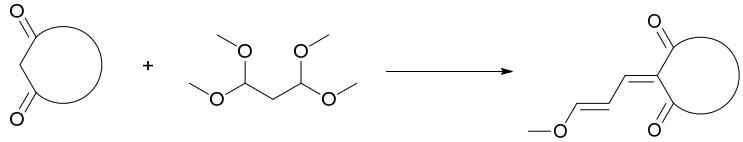
Compound **Donor 1** (86.6 %) was a purple crystal. ^{1}H NMR (400 MHz, DMSO-d^6) δ 7.92 (m, 1H), 7.77 (m, 1H), 7.53 (m, 2H), 4.23 (t, *J* = 7.7 Hz, 2H), 2.79 (s, 3H), 2.14 (t, *J* = 7.2 Hz, 2H), 1.77 (m, 2H), 1.47 (m, 2H), 1.46 (s, 6H), 1.34 (m, 2H) ppm.

Compound **Donor 2**



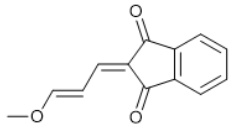
Compound **Donor 2** (52.9 %) was a purple crystal. ^{1}H NMR (400 MHz, DMSO-d^6) δ 8.19 (dd, *J* = 8.1, 1.1 Hz, 1H), 8.05 (d, *J* = 8.4 Hz, 1H), 7.59 (ddd, *J* = 8.5, 7.2, 1.3 Hz, 1H), 7.51 (ddd, *J* = 8.1, 7.2, 1.0 Hz, 1H), 4.44 (m, 2H), 2.95 (s, 3H), 1.94 (t, *J* = 7.2 Hz, 2H), 1.57 (p, *J* = 8.2 Hz, 2H), 1.27 (m, 2H), 1.17 (tt, *J* = 10.7, 5.9 Hz, 2H) ppm.

Scheme S2. General methods of the synthesis of Acceptors.

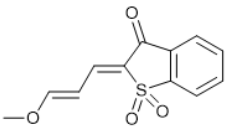


Dione (10.0 mmol) was added into 15 mL heavy-wall pressure tube with 1,1,3,3- tetramethoxypropane (8.2 mL, 50.0 mmol), followed by addition of trifluoroacetic acid (77.0 μL, 1.0 mmol). The reaction vial was screwed cap equipped with a Viton O-ring and stirred under 150 °C for 15 min. The reaction was then gradually cooled down to ambient temperature, and the formed precipitate was filtered off. The residual solid was rinsed with cold hexanes/Et_2O = 3:1. This obtained product was applied to to the next step without further purification.

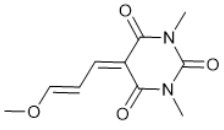
Compound **Acceptor1**



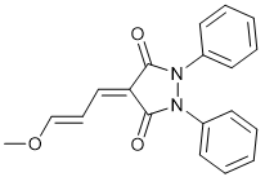
Compound **Acceptor2**



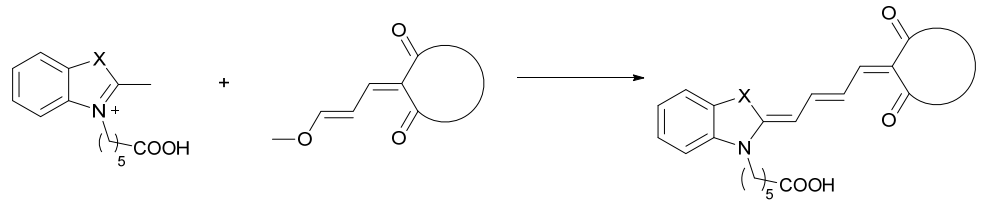
Compound **Acceptor3**



Compound **Acceptor4**

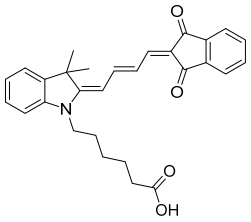


Scheme S3. General methods of the synthesis of Merocyanines [5].



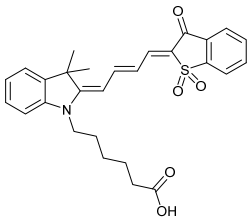
Compound **Donor** (5.0 mmol, 1.0 eq.), Compound **Acceptor** (5.0 mmol, 1.0 eq.) and NaOAc (0.6 g, 7.5 mmol) were mixed tgogether into MeOH/DCM = 1:1 (10.0 mL) in 50 mL round bottom flask. The reaction mixture was refluxed for 3 hours monitored *via* TLC until the substrate consumed completely. Next, the reaction mixture was cooled down to room temperature, and all solvent was removed in vaccum. The residue product was further purified by silica gel flash column chromatography (DCM:MeOH = 10:1) to afford goal product.

Compound **A1**



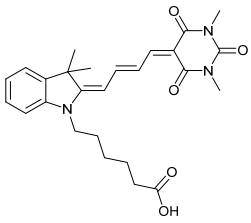
Compound **A1** (55.0 %) was prepared from **Donor 1** and **Acceptor 1**. ^{1}H NMR (400 MHz, DMSO-d^{6}) δ 12.18 – 11.87 (m, 1H), 8.13 (t, *J* = 13.1 Hz, 1H), 7.76 (m, 1H), 7.70 (m, 4H), 7.57 – 7.48 (m, 2H), 7.33 (m, 1H), 7.22 (d, *J* = 7.9 Hz, 1H), 7.13 (m, 1H), 6.22 (d, *J* = 13.4 Hz, 1H), 4.00 (m, 2H), 2.21 (m, 2H), 1.63 (s, 6H), 1.60-1.40 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d^{6}) δ 190.9, 190.6, 174.8, 170.3, 155.6, 146.9, 142.9, 141.6, 140.9, 140.3, 134.2, 134.0 , 128.6, 123.8, 122.6, 121.6, 121.40, 120.0, 117.4, 110.3, 101.0, 48.4, 34.0, 27.9, 26.9, 26.2, 24.8, 23.0. HRMS (ESI^{+}) calcd for $C_{29}H_{29}NO_4$ $[M+H]^+$: 456.2169, found $[M+H]^+$: 456.2172.

Compound **A2**



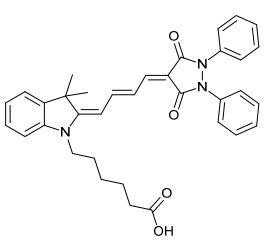
Compound **A2** (44.1 %) was prepared from **Donor 1** and **Acceptor 2**. ^{1}H NMR (400 MHz, DMSO-d^{6}) δ 11.99 (s, 1H), 8.23 (t, *J* = 5.9 Hz,1H), 7.99 (m, 1H), 7.88 (m, 4H), 7.53 (dd, *J* = 17.3, 7.3 Hz, 1H), 7.34 (m, 1H), 7.16 (m, 2H), 6.67 (m, 1H), 6.37 (d, *J* = 13.6 Hz,1H), 4.03 (d, *J* = 7.0 Hz, 2H), 2.20 (t, *J* = 7.2 Hz, 2H), 1.65 (s, 6H), 1.55(m, 2H), 1.39 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d^{6}) δ 176.0, 174.8, 171.9, 170.8, 155.9, 144.2, 143.6, 142.7, 141.2, 135.1, 134.2, 133.7, 128.7, 124.3, 123.6, 122.7, 120.7, 117.6, 116.3, 110.8, 101.5, 48.8, 34.0, 27.8, 27.1, 26.2, 24.8, 21.2. HRMS (ESI^{+}) calcd for $C_{28}H_{29}NO_5S$ $[M+H]^+$: 492.1839, found $[M+H]^+$: 492.1879.

Compound **A3**



Compound **A3** (23.1 %) was prepared from **Donor 1** and **Acceptor 3**. ^{1}H NMR (400 MHz, DMSO-d^{6}) δ 11.98 (s, 1H), 8.11 (dd, *J* = 13.5, 11.5 Hz, 2H), 7.72 (t, *J* = 13.0 Hz, 1H), 7.48 (m, 1H), 7.32 (m, 1H), 7.24 (d, *J* = 7.9 Hz, 1H), 7.13 (t, *J* = 7.4 Hz, 1H), 6.20 (d, *J* = 13.4 Hz, 1H), 3.99 (m, 2H), 3.16 (d, *J* = 4.8 Hz, 6H), 2.20 (t, *J* = 7.2 Hz, 2H), 1.65 (m, 2H), 1.61 (s, 6H), 1.54 (m, 2H), 1.38 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d^{6}) δ 174.8, 171.3, 163.1, 162.3, 157.2, 155.8, 152.0, 142.8, 141.0, 128.6, 124.0, 122.6, 121.4, 110.6, 102.5, 101.3, 48.6, 43.2, 34.0 , 28.3, 27.8, 27.6, 27.0, 26.2, 24.8. HRMS (ESI^{+}) calcd for $C_{26}H_{31}N_3O_5$ $[M+H]^+$: 466.2336, found $[M+H]^+$: 466.2332.

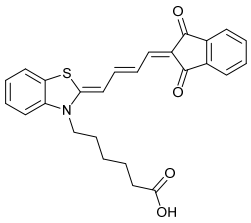
Compound **A4**



Compound **A4** (40.1 %) was prepared from **Donor 1** and **Acceptor 4**. ^{1}H NMR (400 MHz, DMSO-d^{6}) δ 12.00 (s, 1H), 8.03 (d, *J* = 14.4 Hz, 1H), 7.57 (m, 1H), 7.51 (m, 1H), 7.37 (m, 6H), 7.31 (m, 6H), 7.17 (m, 1H), 7.13 (m, 2H), 4.04 (t, *J* = 7.0 Hz, 2H), 2.20 (m, 2H), 1.75 (m, 2H), 1.63 (s, 6H), 1.54 (m, 2H), 1.39 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d^{6}) δ 176.0, 174.8, 174.8, 174.7, 172.50, 172.5, 170.8, 157.1, 151.3, 146.1, 142.4, 141.3, 141.2, 139.2, 139.0 , 129.0, 125.3, 122.8, 122.4, 122.1, 119.7,

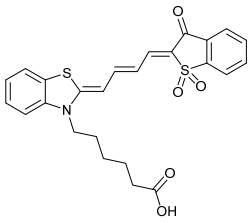
111.6, 111.0, 101.8, 60.2, 55.4, 49.3, 34.0, 28.5, 27.7, 26.3, 24.6, 21.5, 14.5. HRMS (ESI$^+$) calcd for $C_{35}H_{35}N_3O_4$ $[M+H]^+$: 562.2700, found $[M+H]^+$: 562.2751.

Compound **B1**



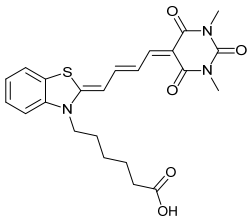
Compound **B1** (36.6 %) was prepared from **Donor 2** and **Acceptor 1**. ^{1}H NMR (400 MHz, DMSO-d^6) δ 12.01 (s, 1H), 7.90 (m, 1H), 7.66 (m, 2H), 7.60 (m, 4H), 7.50 (m, 1H), 7.46 (m, 2H), 7.30 (m, 1H), 6.57 (d, *J* = 12.9 Hz, 1H), 4.27 (t, *J* = 7.6 Hz, 2H), 2.21 (t, *J* = 7.3 Hz, 2H), 1.70 (p, *J* = 7.7 Hz, 2H), 1.56 (m, 2H), 1.42 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d^6) δ 190.8, 190.5, 174.8, 163.7, 154.8, 145.0 , 141.8, 141.6, 140.3, 133.6, 133.4, 128.2, 125.6, 125.0 , 123.2, 121.1, 120.9, 117.8, 115.2, 113.4, 99.8, 46.1, 34.0, 27.5, 26.0, 24.7. HRMS (ESI$^+$) calcd for $C_{26}H_{23}NO_4S$ $[M+H]^+$: 446.1421, found $[M+H]^+$: 446.1421.

Compound **B2**



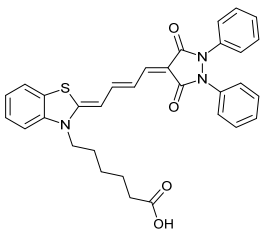
Compound **B2** (18.7 %) was prepared from **Donor 2** and **Acceptor 2**. ^{1}H NMR (400 MHz, DMSO-d^6) δ 12.03 (s, 1H), 7.98 (d, *J* = 8.1 Hz, 1H), 7.93 (d, *J* = 18.9 Hz,1H), 7.82 (m, 4H), 7.70 (m, 2H), 7.54 (t, *J* = 7.9 Hz, 1H), 7.39 (t, *J* = 7.6 Hz, 1H), 6.76 (d, *J* = 13.2 Hz, 1H), 6.62 (d, *J* = 13.3 Hz, 1H), 4.34 (t, *J* = 7.7 Hz, 2H), 2.21 (t, *J* = 8.5 Hz, 2H), 1.72 (m, 2H), 1.56 (m, 2H), 1.43 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d^6) δ 174.9, 141.7, 134.4, 134.1, 133.9, 128.4, 125.5, 123.5, 123.2, 120.4, 114.7, 113.9, 101.0 , 46.5, 40.6, 34.1, 27.7, 26.0, 24.7. HRMS (ESI$^+$) calcd for $C_{25}H_{23}NO_5S$ $[M+H]^+$: 482.1090, found $[M+H]^+$: 482.1117.

Compound **B3**



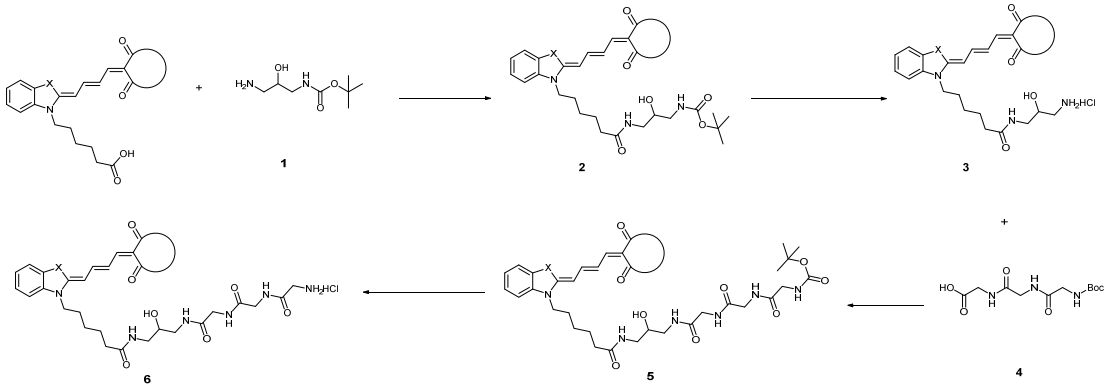
Compound **B3** (18.4 %) was prepared from **Donor 2** and **Acceptor 3**. ^{1}H NMR (400 MHz, DMSO-d^6) δ 11.99 (s, 1H), 7.91 (d, *J* = 19.7 Hz, 1H), 7.66 (m, 4H), 7.50 (t, *J* = 7.8 Hz, 1H), 7.34 (q, *J* = 8.2, 7.5 Hz,1H), 6.57 (m, 1H), 4.30 (t , *J* = 15.4 Hz, 2H), 3.14 (s, 6H), 2.21 (t, *J* = 7.2 Hz, 2H), 1.70 (m, 2H), 1.56 (m, 2H), 1.42 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d^6) δ 174.8, 165.0, 163.1, 162.2, 156.4, 153.2, 152.1, 141.7, 128.2, 125.8, 125.3, 123.3, 118.9, 113.7, 99.5, 55.4, 46.3, 34.0, 28.1, 27.6, 26.0, 24.7. HRMS (ESI$^+$) calcd for $C_{23}H_{25}N_3O_5S$ $[M+H]^+$: 456.1588, found $[M+H]^+$: 456.1593.

Compound **B4**



Compound **B4** (12.7 %) was prepared from **Donor 2** and **Acceptor 4**. ^{1}H NMR (400 MHz, DMSO-d^6) δ 11.99 (s, 1H), 8.01 (t, *J* = 7.9 Hz, 1H), 7.84 (m, 1H), 7.72 (m, 2H), 7.57 (m, 2H), 7.44 (m, 2H), 7.35 (m, 4H), 7.31 (m, 4H), 7.10 (m, 4H), 4.34 (m, 2H), 2.19 (t, *J* = 6.9 Hz, 2H), 1.74 (m, 2H), 1.55 (m, 2H), 1.41 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d^6) δ 169.4, 166.8, 166.1, 143.6, 141.7, 139.3, 132.0, 129.0, 128.9, 128.6, 126.0, 125.5, 125.3, 125.2, 125.0, 123.7, 122.1, 122.0, 114.5, 114.1, 96.4, 94.9, 67.80, 34.2, 29.0, 26.2 , 24.8, 22.9. HRMS (ESI$^+$) calcd for $C_{32}H_{29}N_3O_4S$ $[M+H]^+$: 552.1952, found$[M+H]^+$: 552.1951.

Scheme S4. General methods of connecting Merocyanine with water-soluble and GGG linker [6].



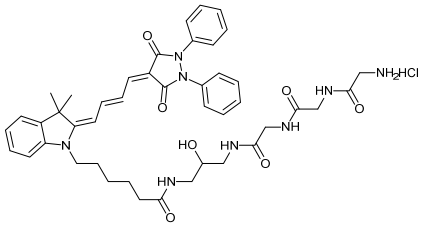
Merocyanine (1.0 eq, 1.0 mmol), Compound **1** (1.0 eq, 1.0 mmol, 190.2 mg), 1-Hydroxybenzotriazole hydrate (HOBt·H_2O) (3.0 eq, 3.0 mmol, 460.0 mg), and triethylamine (3.0 eq, 3.0 mmol, 420.0 μL) were added into 10.0 mL anhydrous DMF, and then N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl) (3.0 eq, 3.0 mmol, 580.0 mg) was introduced into reaction mixture. The reaction was stirred for 12 hours under room temperature in dark, and then quenched by water and extracted with DCM (3 × 200 mL). The combined organic fractions was dried over anhydrous Na_2SO_4 then concentrated *via* rotary evaporator. The product was further purified by silica gel flash column chromatography by using 5% MeOH in DCM as eluent to yield the compound **2**.

To a solution of compound **2** (1.0 eq, 1.0 mmol) in DCM, HCl (4 *M* in dioxane, 10.0 eq, 10.0 mmol) was added dropwise at room temperature with stirring. The reaction was kept stirring in dark and monitored *via* TLC. After all reactant consumed, the solution was dried *in vacuo* to obtain deprotected compound **3** without further purification.

Compound **3** (1.0 eq, 1.0 mmol) was dissolved in 10 mL anhydrous DMF, and then compound **4** (1.0 eq, 1.0 mmol, 289.3 mg), 1-Hydroxybenzotriazole hydrate (HOBt·H_2O) (3.0 eq, 3.0 mmol, 460.0 mg) and triethylamine (3.0 eq, 3.0 mmol, 420.0 μL), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl) (3.0 eq, 3.0 mmol, 580.0 mg) were added into reaction. The reaction mixture was stirred at ambient temperature in dark for 12 hours and then extracted with water (200 mL) and DCM (3 × 200 mL). The combined organic phase was dried over anhydrous Na_2SO_4 and then concentrated *via* rotary evaporator. The crude product was further purified *via* silica gel flash column chromatography by using 5% MeOH in DCM as eluent to obtain the compound **5**.

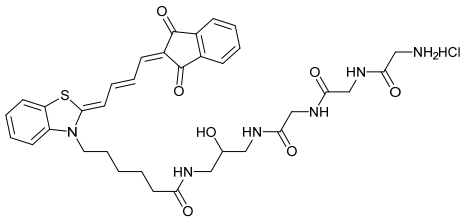
To a solution of compound **5** (1.0 eq, 1.0 mmol) in DCM, HCl (4 *M* in dioxane, 10.0 eq, 10.0 mmol) was added dropwise. The reaction was stirred in dark and monitored *via* TLC. After deprotection completed, the reaction mixture was dried *in vacuo* to yield final product -fluorophore with linker.

Compound **A4-6 (Mero-Rotor)**



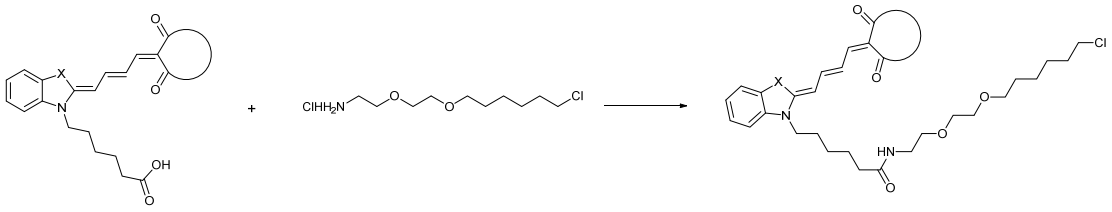
(56.3 %). ^{1}H NMR (700 MHz, DMSO-d^6) δ 8.74 (t, *J* = 5.8 Hz, 1H), 8.33 (t, *J* = 6.0 Hz, 1H), 8.23 (t, *J* = 13.0 Hz, 1H), 7.90 (m, 1H), 7.86 (m, 1H), 7.54 (d, *J* = 7.4 Hz, 1H), 7.42 (t, *J* = 13.1 Hz, 1H), 7.35 (m, 5H), 7.31 (m, 5H), 7.18 (t, *J* = 7.4 Hz, 1H), 7.11 (t, *J* = 7.3 Hz, 2H), 6.28 (d, *J* = 13.6 Hz, 1H), 5.21 (s, 6H), 4.04 (t, *J* = 7.5 Hz, 2H), 3.83 (d, *J* = 5.7 Hz, 2H), 3.69 (d, *J* = 5.9 Hz, 2H), 3.60 (m, 2H), 3.51 (m, 2H), 3.05 (m, 2H), 3.01 (m, 2H), 2.09 (t, *J* = 7.4 Hz, 2H), 1.64 (m, 2H), 1.63 (s, 6H), 1.54 (m, 2H), 1.36 (m, 2H). ^{13}C NMR (176 MHz, DMSO-d^6) δ 172.9, 172.6, 169.3, 169.0, 166.8, 157.2, 151.2, 142.6, 141.3, 139.1, 129.0, 125.3, 124.6, 122.8, 122.1, 119.7, 111.1, 101.9, 99.1, 68.8, 49.0, 43.5, 43.2, 43.2, 42.5, 42.5, 40.6, 35.6, 27.7, 27.1, 26.3, 25.5. HRMS (ESI+) calcd for $C_{44}H_{53}N_8O_7Cl$ [M-Cl]$^+$: 805.4032, found [M-Cl]$^+$: 805.4061.

Compound **B1-6 (Mero-Solvato)**



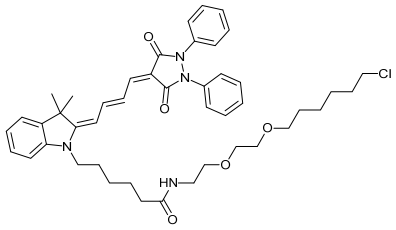
(78.7 %). ^{1}H NMR (700 MHz, DMSO-d^{6}) δ 8.73 (m, 1H), 8.32 (m, 1H), 8.15 (m, 4H), 7.93 (m, 1H), 7.89 (t, *J* = 15.8 Hz,1H), 7.86 (m, 1H), 7.63 (m, 2H), 7.61 (m, 2H), 7.51 (m, 1H), 7.49 (m, 1H), 7.35 (m, 1H), 6.61 (m, 1H), 4.31 (t, *J* = 7.6 Hz, 2H), 3.84 (d, *J* = 5.9 Hz, 2H), 3.70 (d, *J* = 5.9 Hz, 2H), 3.59 (m, 2H), 3.52 (m, 1H), 3.06 (m, 2H), 3.02 (m, 2H), 2.11 (t, *J* = 7.4 Hz, 2H), 1.70 (m, 2H), 1.56 (m, 2H), 1.40 (t, *J* = 15.1 Hz 2H). ^{13}C NMR (176 MHz, DMSO-d^{6}) δ 190.7, 172.9, 169.3, 169.1, 166.8, 163.9, 154.9, 145.0, 141.8, 133.6, 130.1, 128.3, 125.6, 125.1, 123.3, 121.0, 117.8, 115.0, 113.6, 100.0, 72.4, 68.8, 46.2, 43.2, 42.5, 40.6, 35.6, 27.6, 27.0, 26.1, 25.5. HRMS (ESI+) calcd for $C_{35}H_{41}N_6O_7Cl$ $[M\text{-}Cl]^+$: 689.2752, found $[M\text{-}Cl]^+$: 689.2774.

Scheme S5. Methods of connecting Merocyanine with Halo-Tag linker [4].



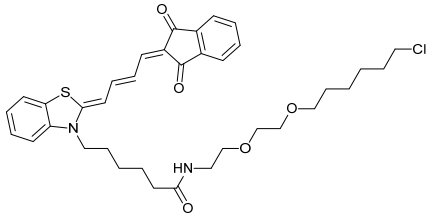
Merocyanine (1.0 eq, 0.1 mmol) was dissolved in 1.0 mL anhydrous DMF, and then Halo-Tag linker (1.0 eq, 0.1 mmol, 26.0 mg), 1-Hydroxybenzotriazole hydrate (HOBt·H_2O) (3.0 eq, 0.3 mmol, 46.0 mg), triethylamine (3.0 eq, 0.3 mmol, 42.0 μL) and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl) (3.0 eq, 0.3 mmol, 58.0 mg) were introduced to the reaction mixture in one portion. The reaction mixture was stirred for 12 hours in dark followed extraction with water (200 mL) and DCM (3 × 200 mL). The combined organic phase was concentrated *in vacuo* and further purified *via* flash chromatography by using 5% MeOH in DCM as eluent to obtain the compound Mero-Halo probes.

Compound **Mero-Rotor-Halo**



(36.5 %). ^{1}H NMR (700 MHz, DMSO-d^{6}) δ 8.23 (t, *J* = 13.1 Hz, 1H), 7.89 (d, *J* = 13.3 Hz, 1H), 7.80 (m, 1H), 7.53 (d, *J* = 7.4 Hz, 1H), 7.42 (m, 1H), 7.35 (m, 4H), 7.31 (m, 4H), 7.28 (m, 1H), 7.18 (t, *J* = 7.3Hz, 1H), 7.11 (m, 2H), 6.27 (d, *J* = 13.5 Hz, 1H), 4.03 (t, *J* = 7.5 Hz, 2H), 3.59 (m, 2H), 3.45 (s, 2H), 3.44 (s, 2H), 3.35 (d, J = 6.2 Hz, 2H), 3.15 (s, 2H), 2.05 (m, 2H), 1.67 (m, 2H), 1.64 (m, 2H), 1.63 (s, 6H), 1.53 (m, 2H), 1.45 (m, 2H), 1.35 (m, 4H), 1.27 (m, 2H). ^{13}C NMR (176 MHz, DMSO-d^{6}) δ 172.4 , 166.8 , 166.1 , 157.2 , 151.2 , 146.1 , 142.6 , 141.3 , 139.2 , 139.1 , 130.1 , 129.00 , 129.0 , 128.7, 125.3, 124.5, 122.8, 133.4, 122.1, 122.1, 119.7, 111.0, 101.9, 99.2, 70.6, 70.0, 69.9, 69.6, 49.0, 45.8, 38.9, 35.5, 32.5, 29.5, 27.7, 26.6, 26.2, 25.4. HRMS (ESI^{+}) calcd for $C_{36}H_{42}N_2O_5SCl$ $[M+H]^+$: 651.2654, found $[M+H]^+$: 651.2655.

Compound **Mero-Solvato-Halo**

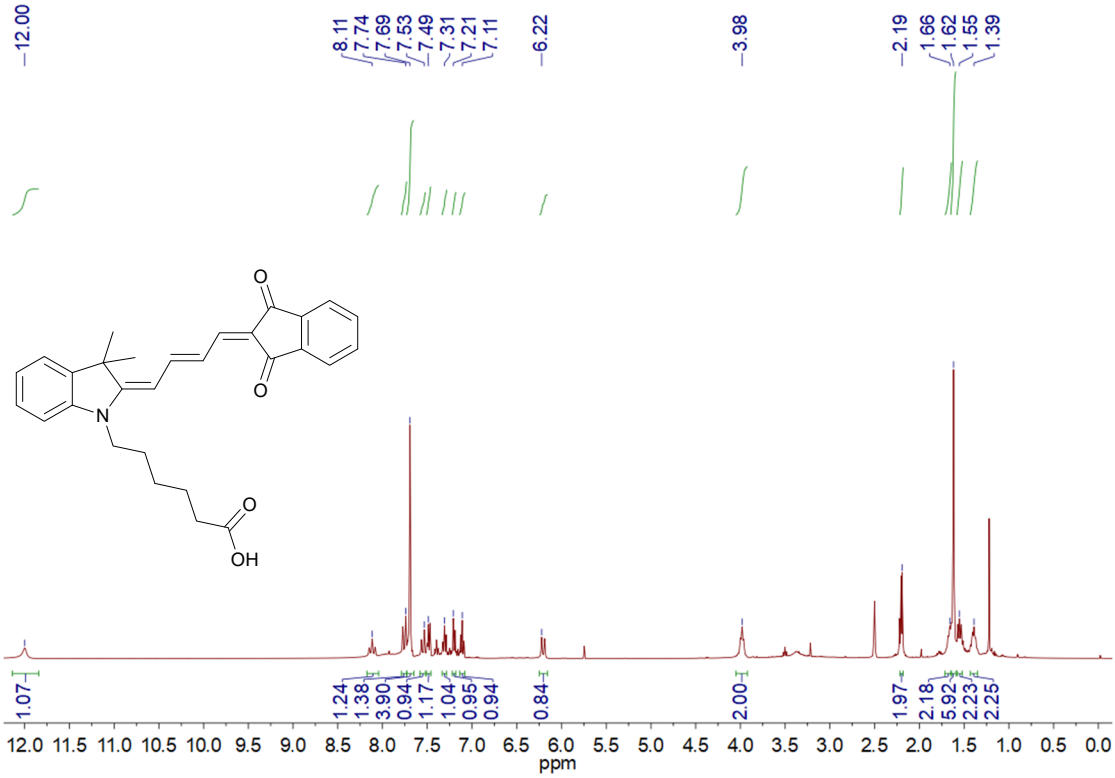


(42.8 %). ^{1}H NMR (700 MHz, DMSO-d^{6}) δ 7.92 (d, *J* = 7.9 Hz, 1H), 7.83 (t, *J* = 5.7 Hz, 1H), 7.71 (t, *J* = 12.3 Hz, 1H), 7.64 (m, 2H), 7.61 (m, 2H), 7.49 (m, 2H), 7.47 (m, 1H), 7.34 (t, *J* = 7.6 Hz, 1H), 6.60 (d, *J* = 12.9 Hz, 1H), 4.30 (t, *J* = 7.8 Hz, 2H), 3.59 (m, 2H), 3.48 (m, 2H), 3.44 (m, 2H), 3.37 (t, *J* = 5.9 Hz, 2H), 3.33 (t, *J*

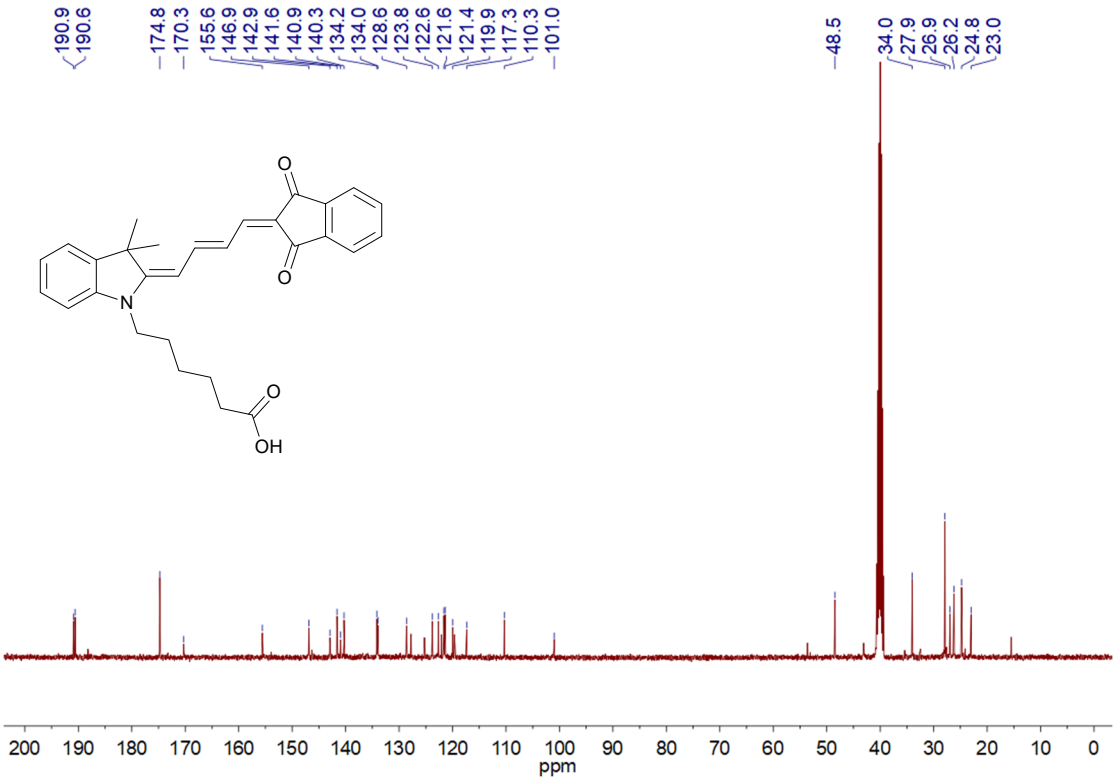
= 6.6 Hz, 2H), 3.18 (q, *J* = 5.9 Hz, 2H), 2.08 (t, *J* = 7.3 Hz, 2H), 1.70 (m, 2H), 1.67 (m, 2H), 1.56 (t, *J* = 7.5 Hz, 2H), 1.45 (m, 2H), 1.40 (m, 2H), 1.34 (m, 2H), 1.26 (m, 2H). ^{13}C NMR (176 MHz, DMSO-d^{6}) δ 190.8, 190.5, 172.4, 163.8, 154.8, 145.0, 141.6, 140.3, 133.7, 130.1, 128.2, 125.6, 125.0, 123.3, 120.9, 117.8, 115.2, 113.5, 99.9, 70.6, 70.0, 69.9, 69.6, 46.2, 45.8, 40.5, 38.9, 35.5, 32.5, 29.5, 27.5, 26.6, 26.0, 25.4. HRMS (ESI^{+}) calcd for $C_{45}H_{55}N_4O_5Cl$ $[M+H]^+$: 767.3934, found $[M+H]^+$: 767.3936.

NMR Spectra

A1:

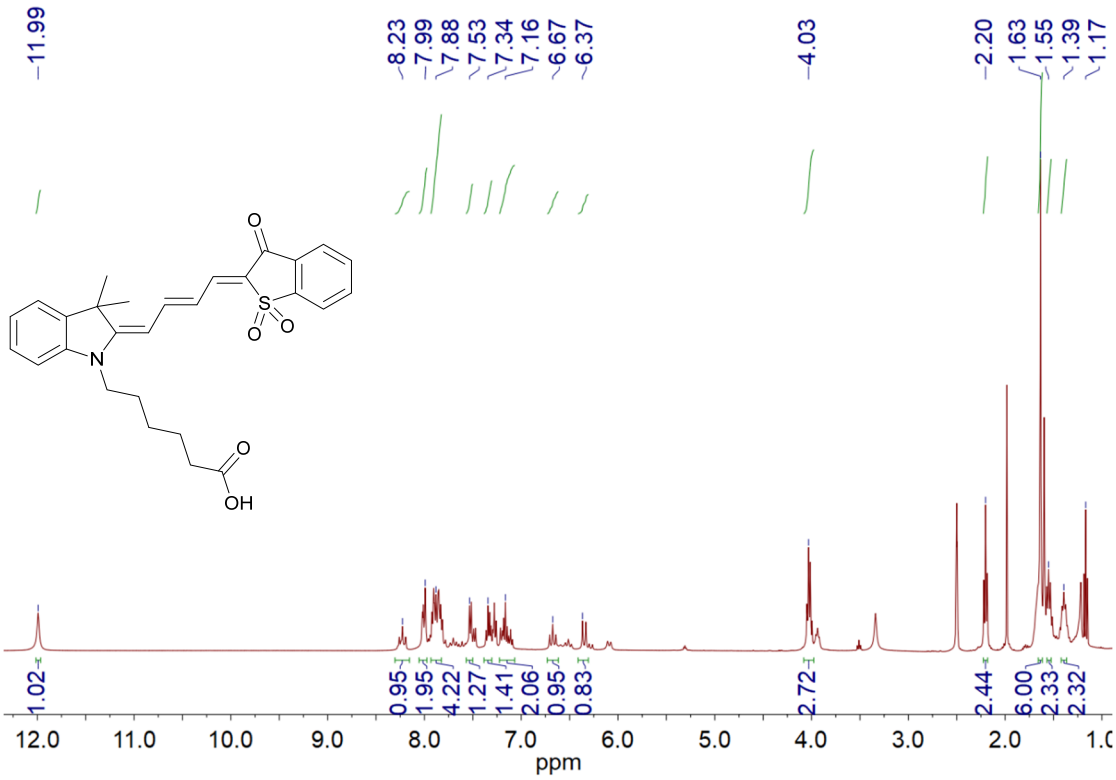


^{1}H-NMR spectrum of **A1** (DMSO-d^{6})

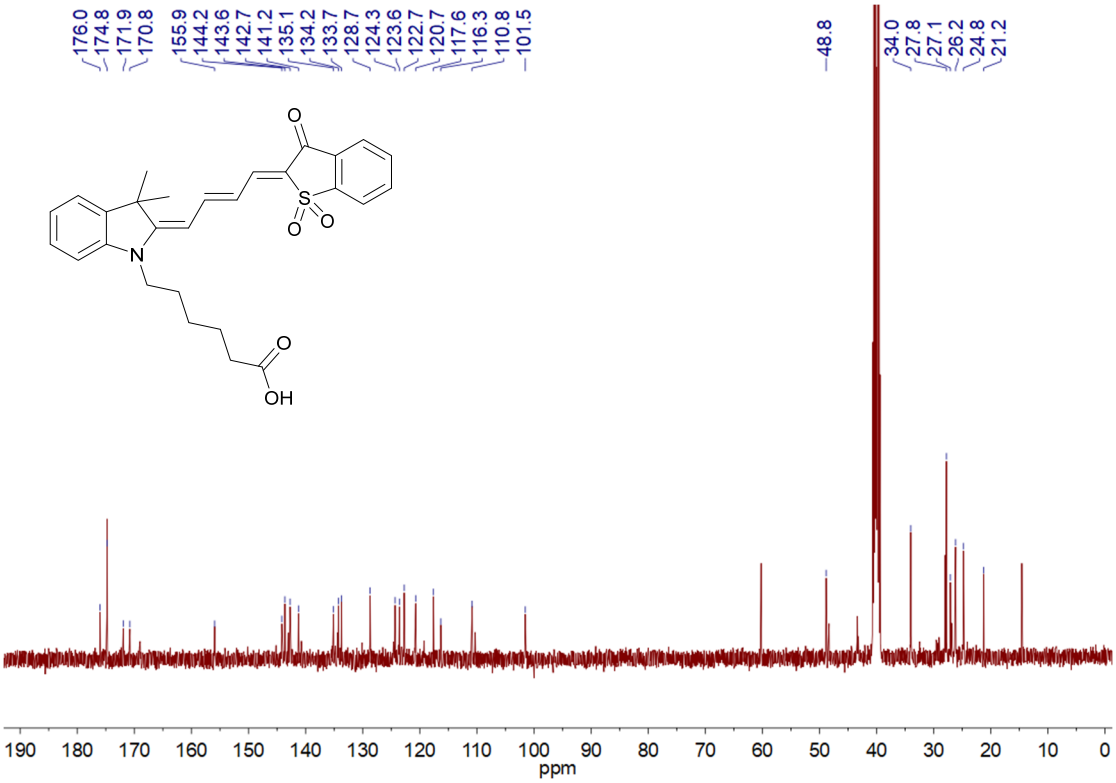


^{13}C-NMR spectrum of **A1** (DMSO-d^{6})

A2:

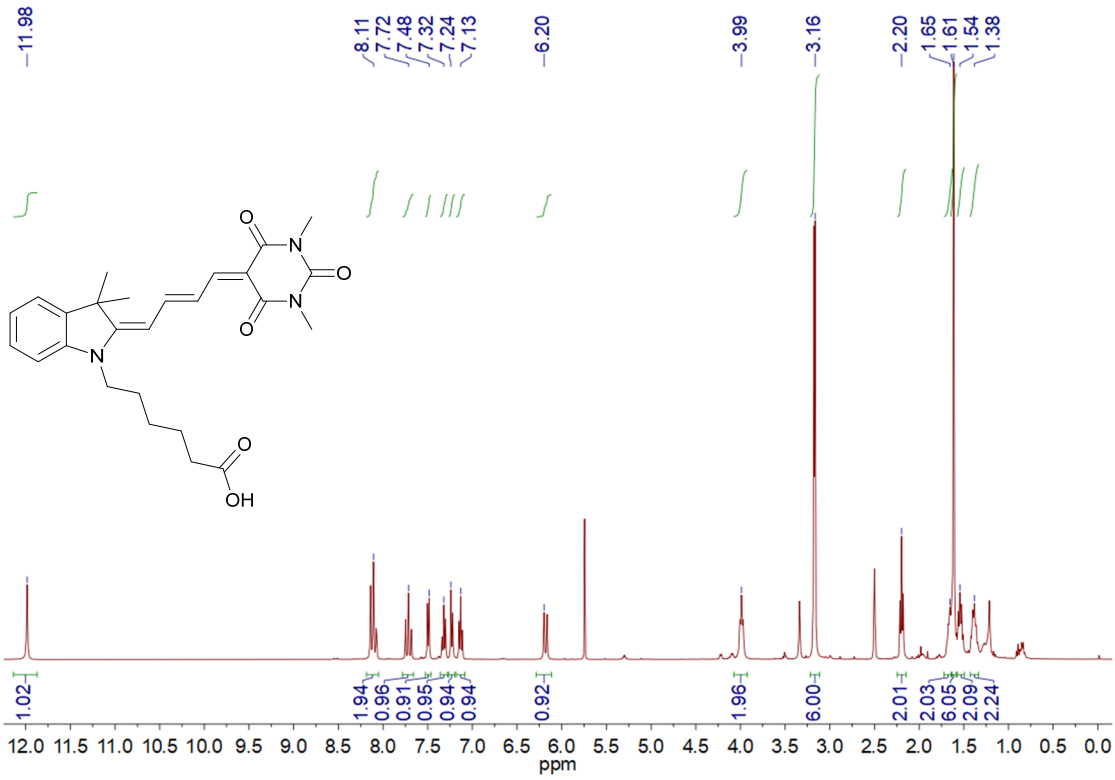


^{1}H-NMR spectrum of **A2** (DMSO-d^{6})

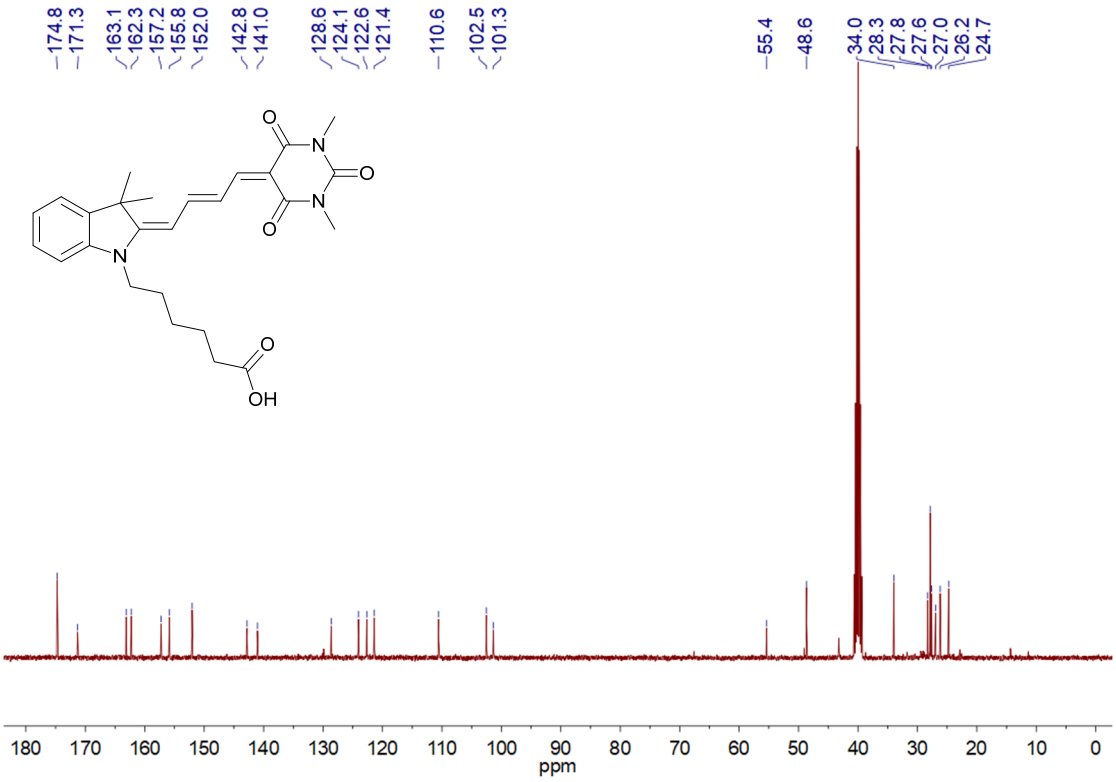


^{13}C-NMR spectrum of **A2** (DMSO-d^{6})

A3:

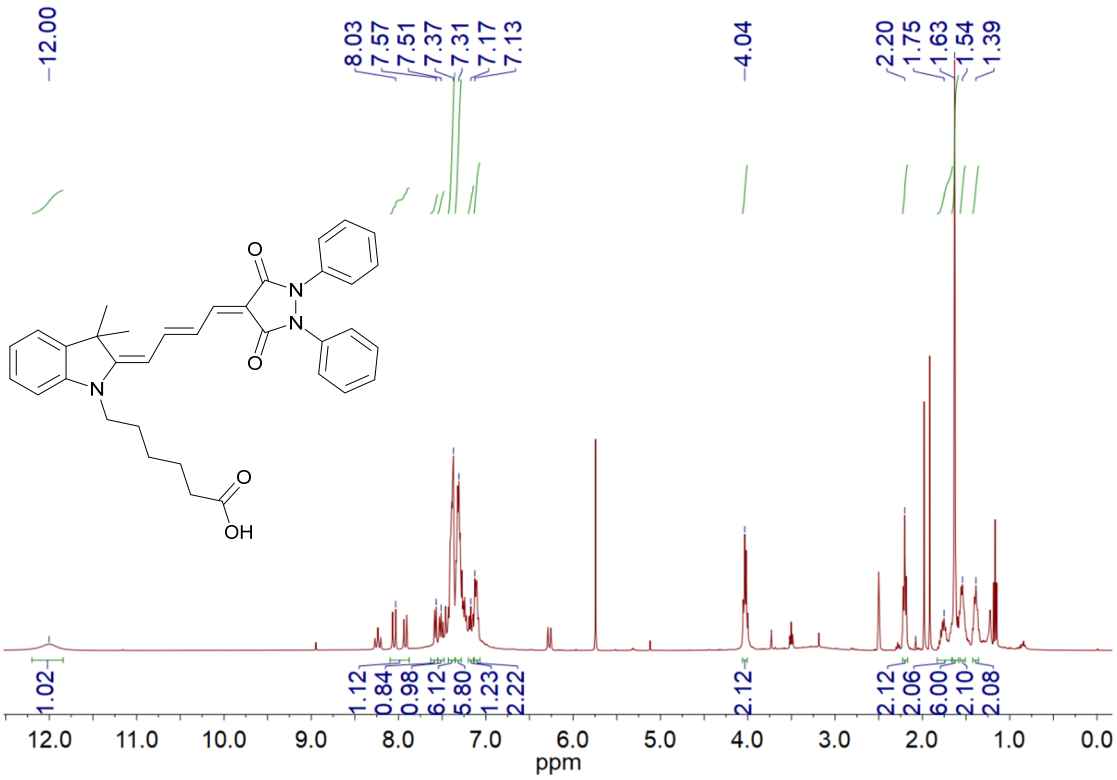


^{1}H-NMR spectrum of **A3** (DMSO-d^{6})

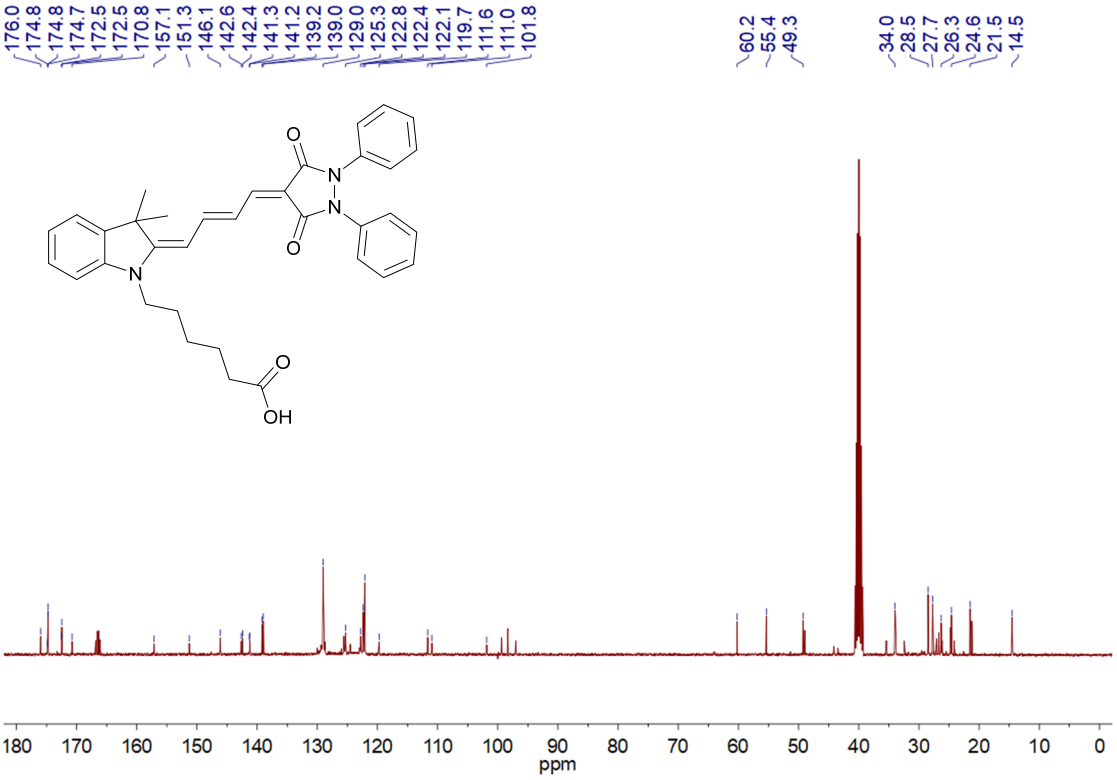


^{13}C-NMR spectrum of **A3** (DMSO-d^{6})

A4:

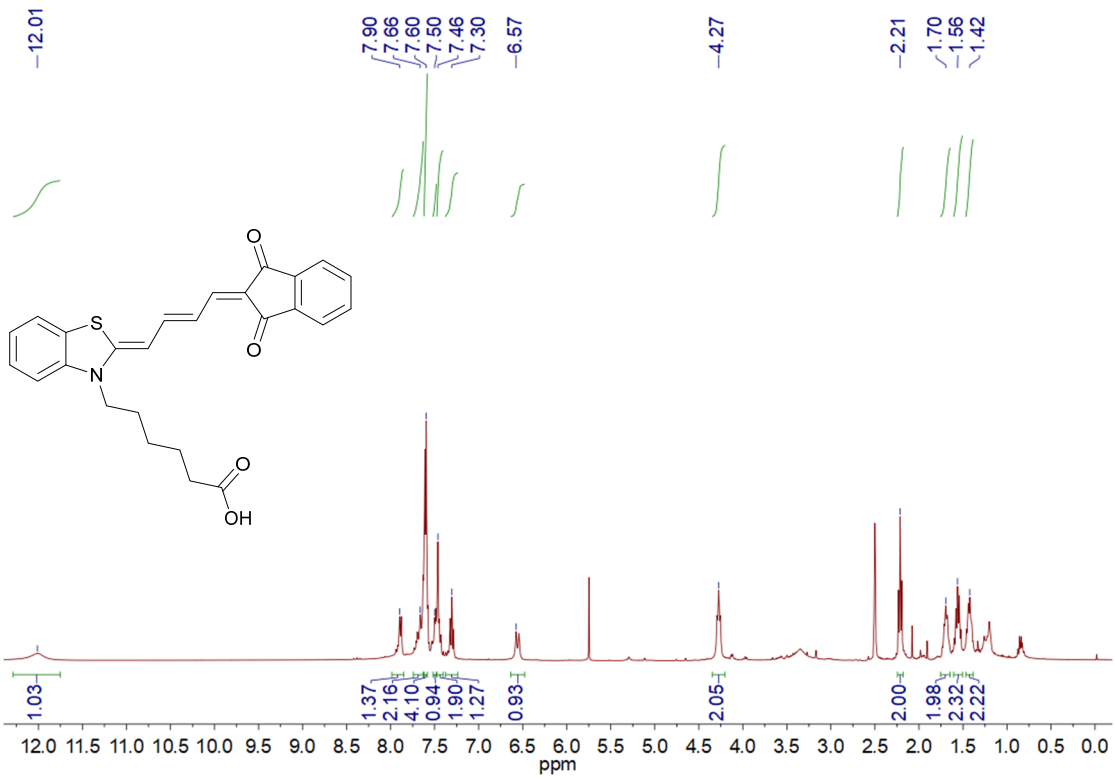


^{1}H-NMR spectrum of **A4** (DMSO-d^{6})

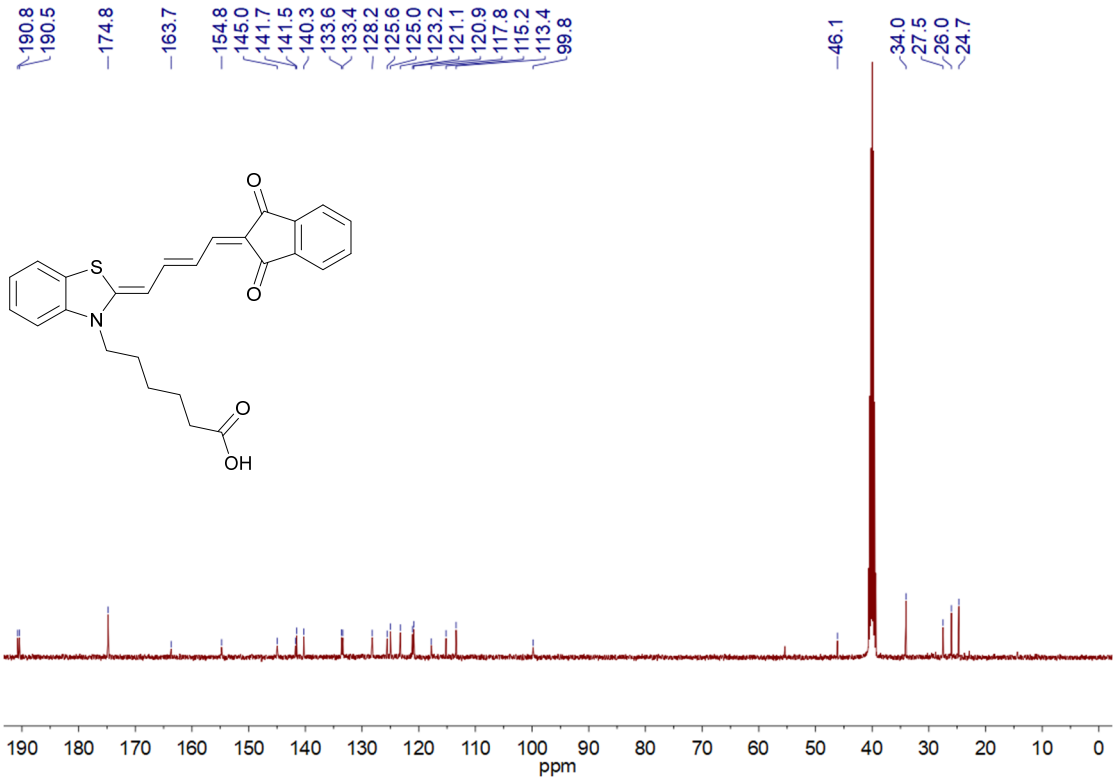


^{13}C-NMR spectrum of **A4** (DMSO-d^{6})

B1:

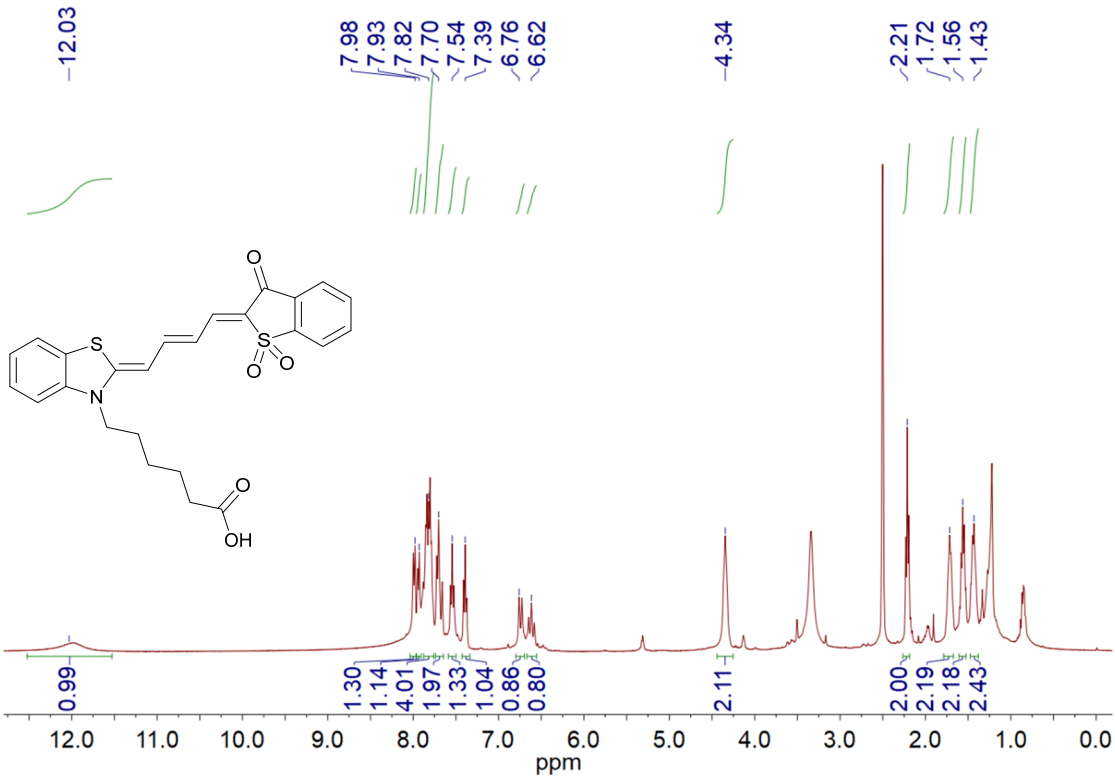


^{1}H-NMR spectrum of **B1** (DMSO-d^{6})

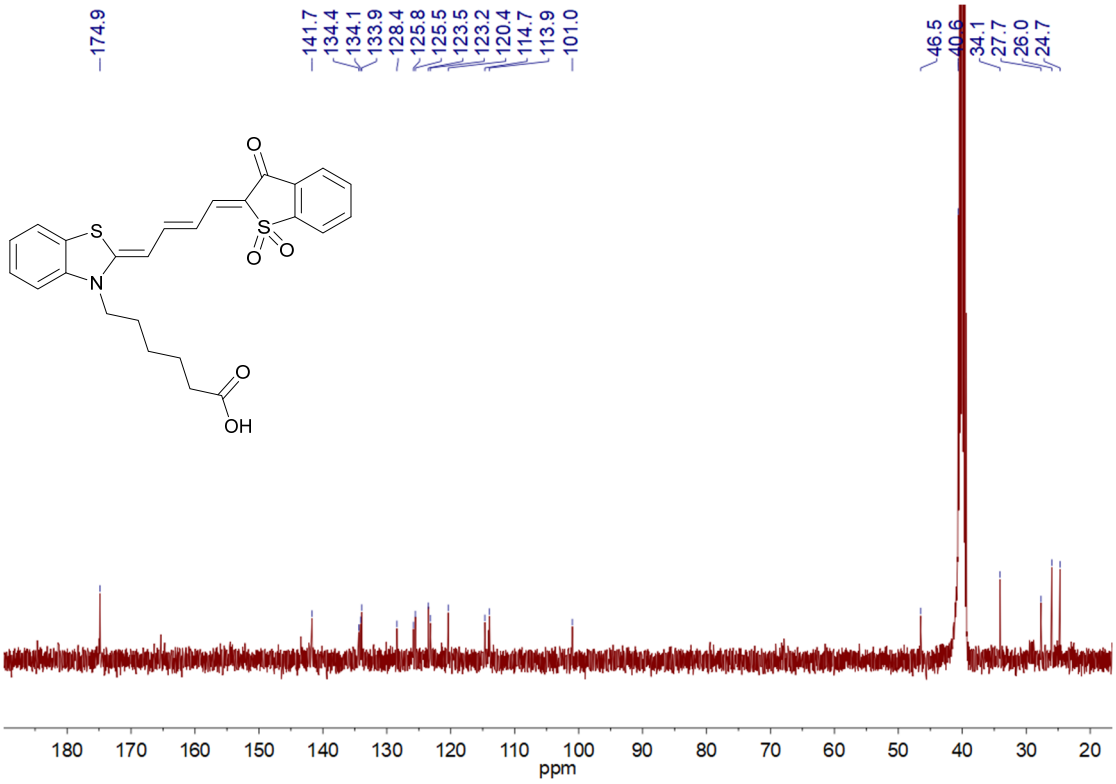


^{13}C-NMR spectrum of **B1** (DMSO-d^{6})

B2:

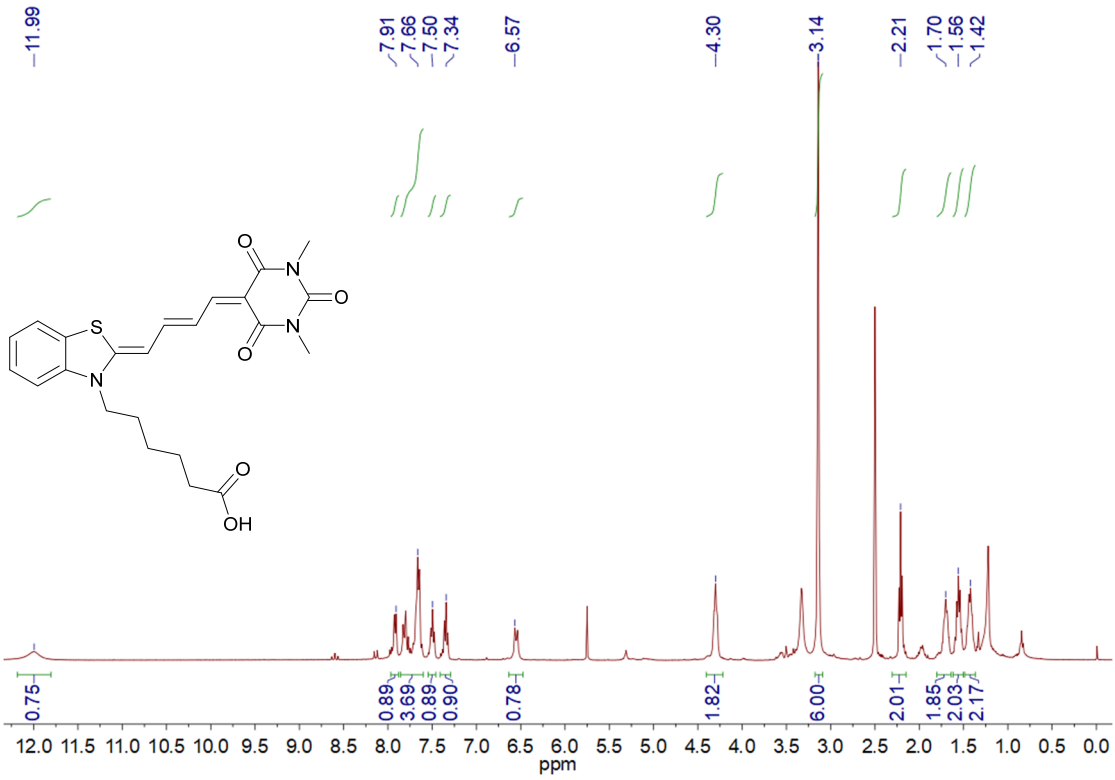


^{1}H-NMR spectrum of **B2** (DMSO-d^{6})

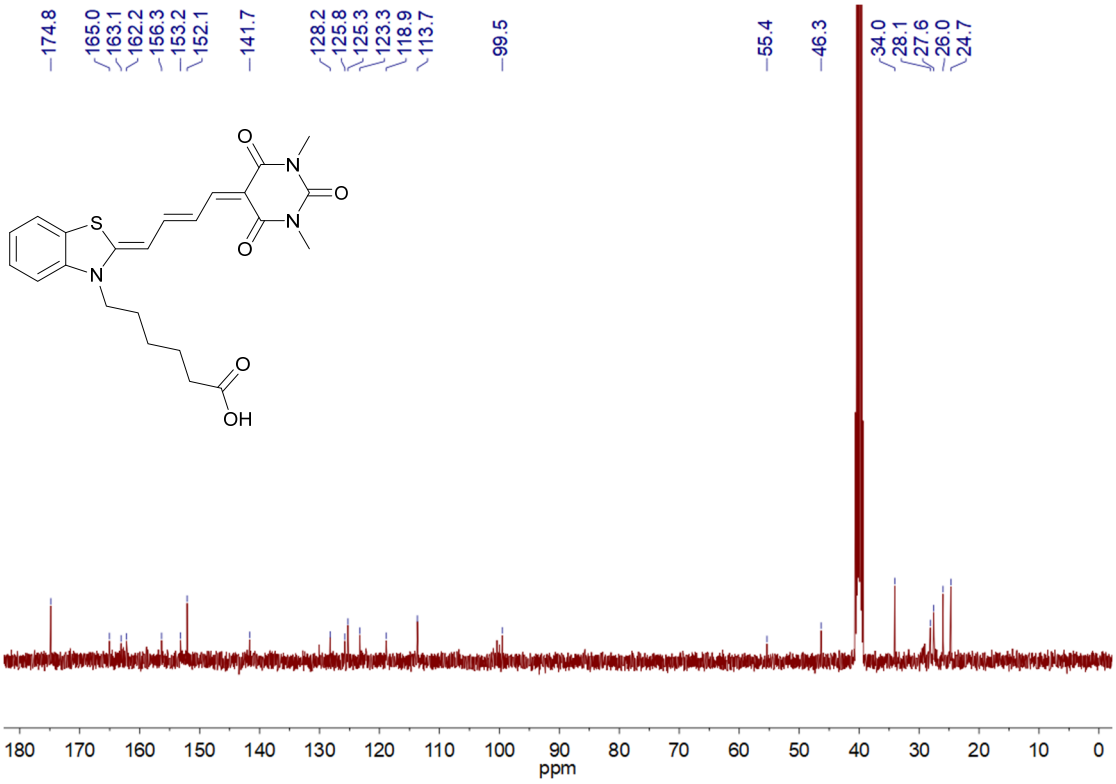


^{13}C-NMR spectrum of **B2** (DMSO-d^{6})

B3:

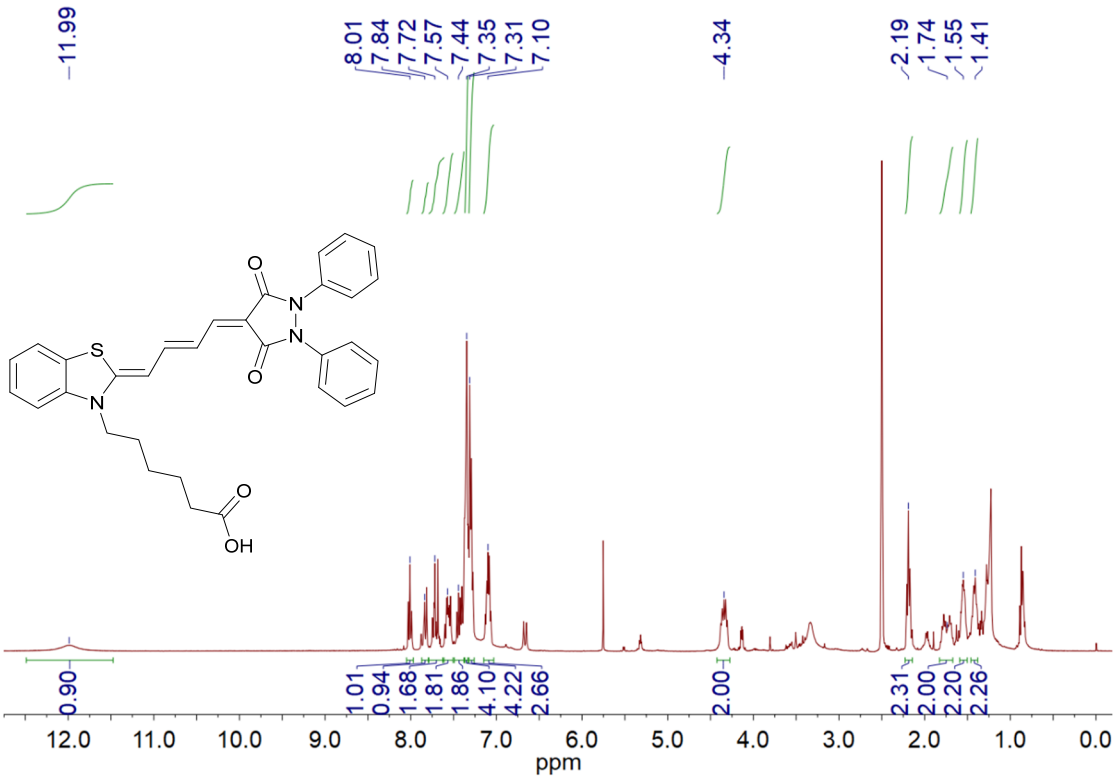


^{1}H-NMR spectrum of **B3** (DMSO-d^{6})

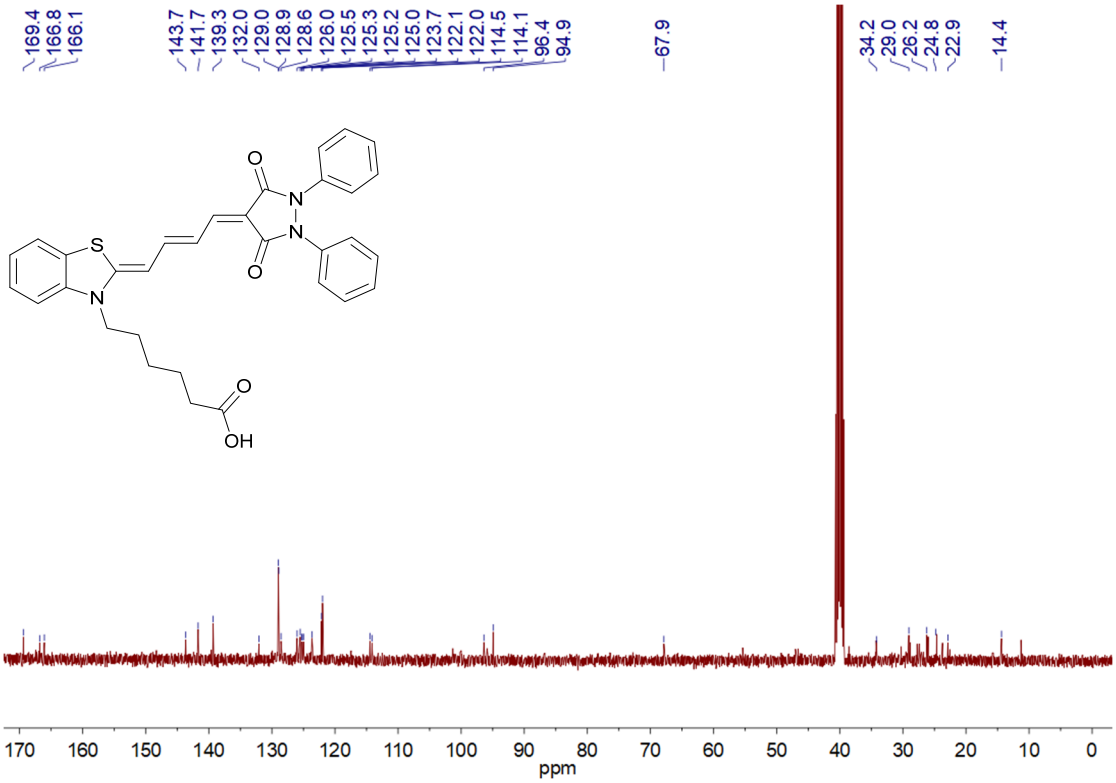


^{13}C-NMR spectrum of **B3** (DMSO-d^{6})

B4:

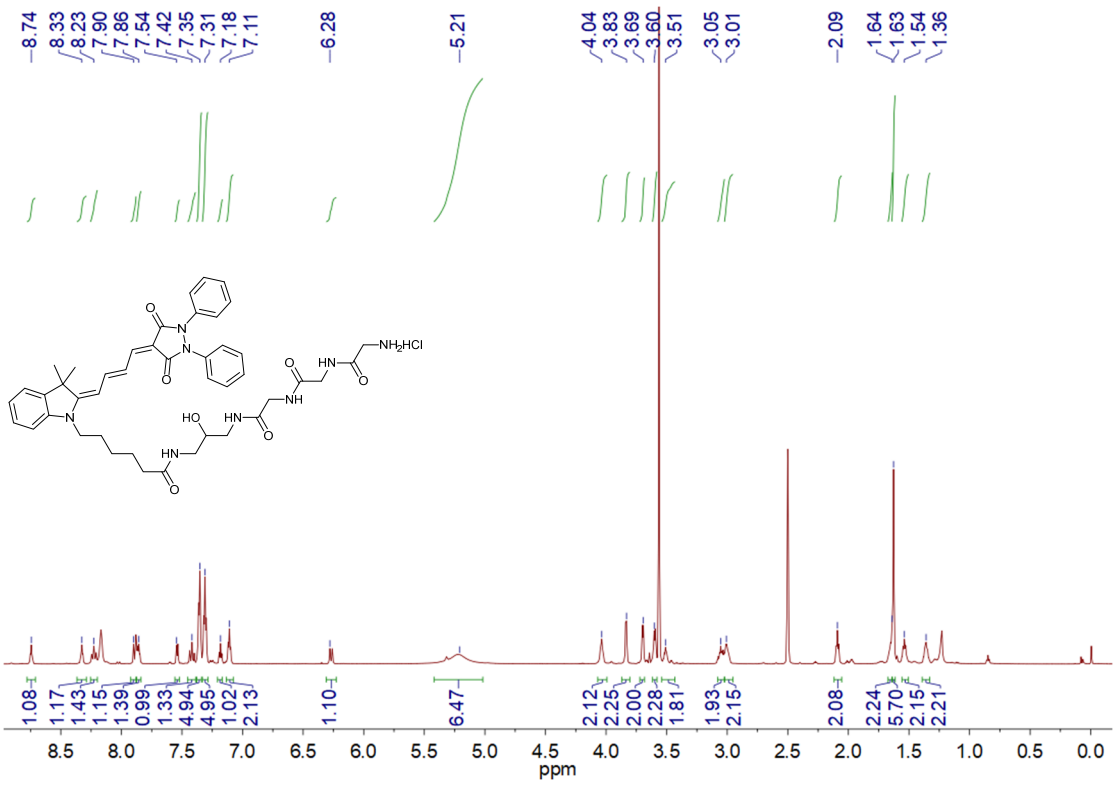


^{1}H-NMR spectrum of **B4** (DMSO-d^{6})

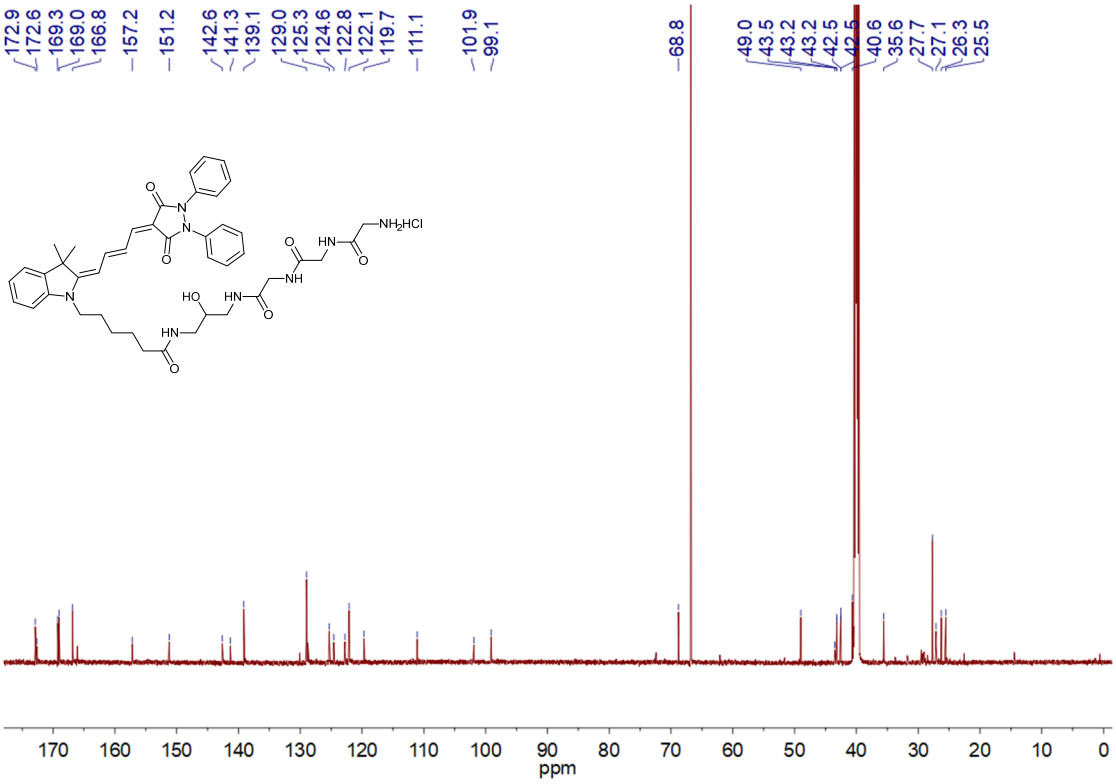


^{13}C-NMR spectrum of **B4** (DMSO-d^{6})

Mero-Rotor:

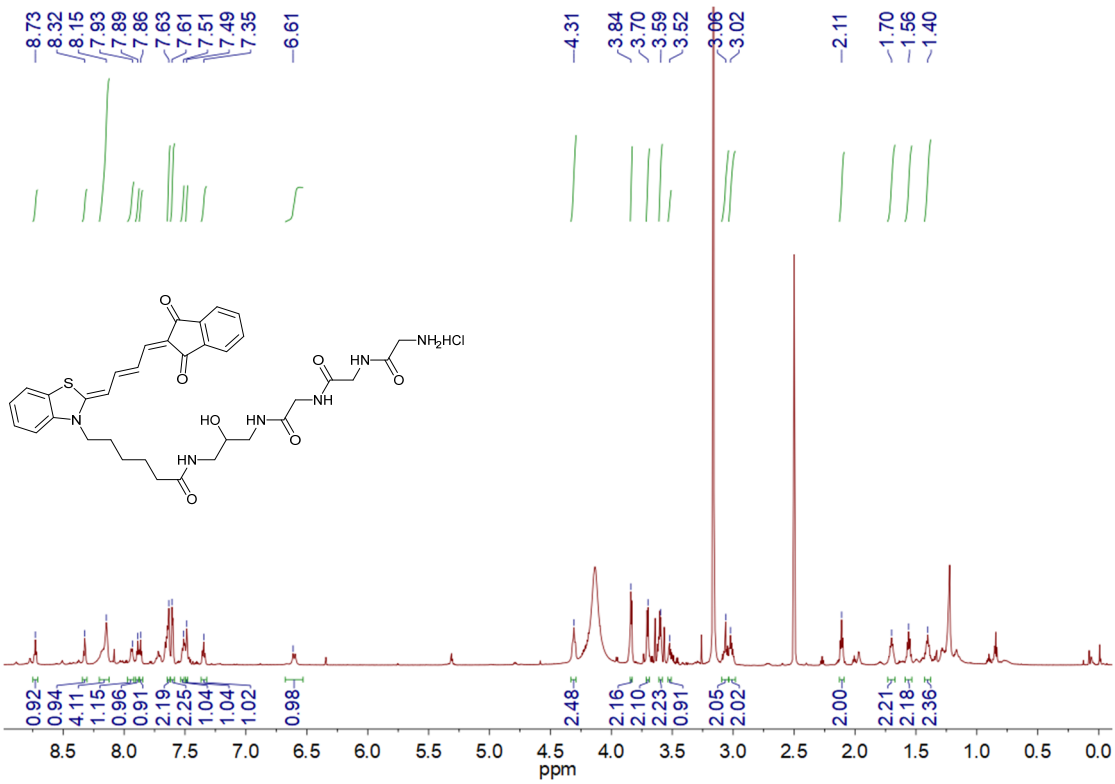


^{1}H-NMR spectrum of **Mero-Rotor** (DMSO-d^{6})

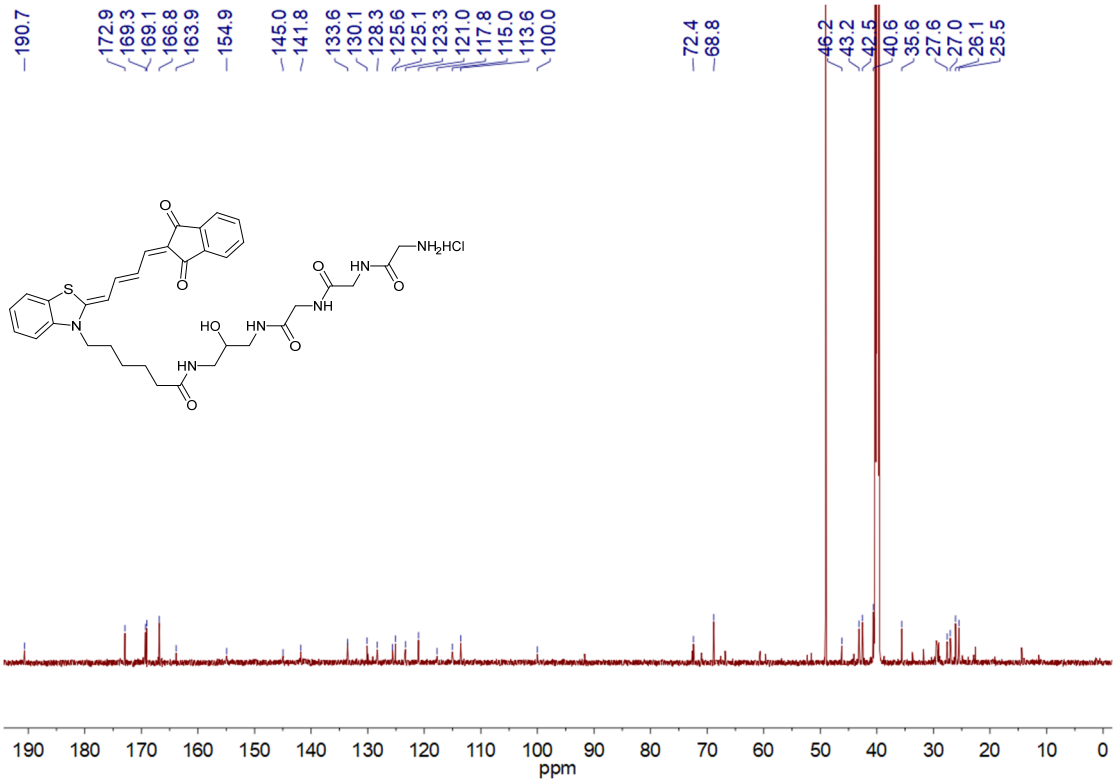


^{13}C-NMR spectrum of **Mero-Rotor** (DMSO-d^{6})

Mero-Solvato:

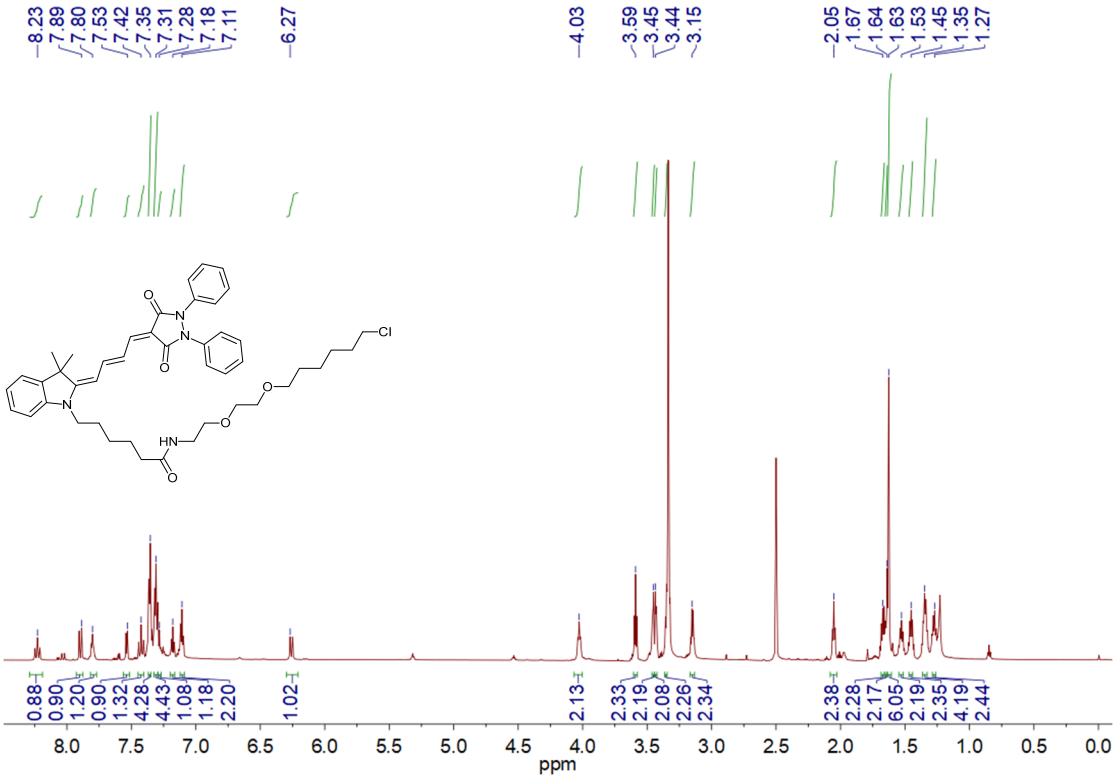


^{1}H-NMR spectrum of **Mero-Solvato** (DMSO-d^{6})

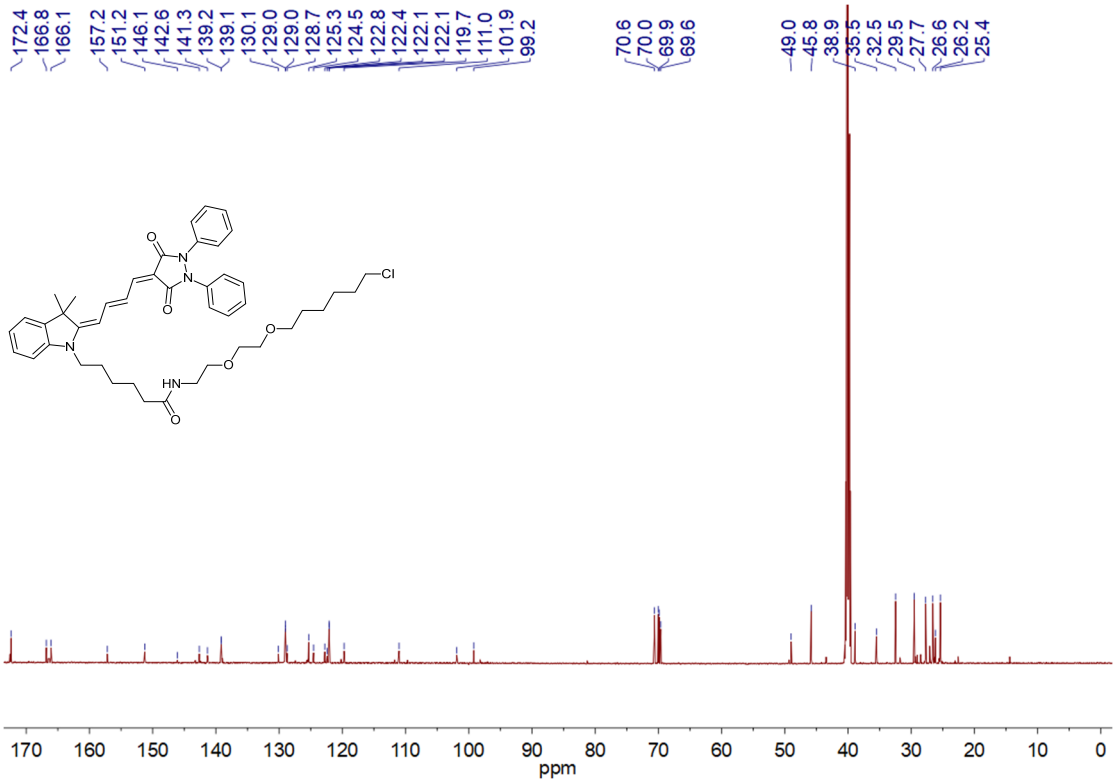


^{13}C-NMR spectrum of **Mero-Solvato** (DMSO-d^{6})

Mero-Rotor-Halo:

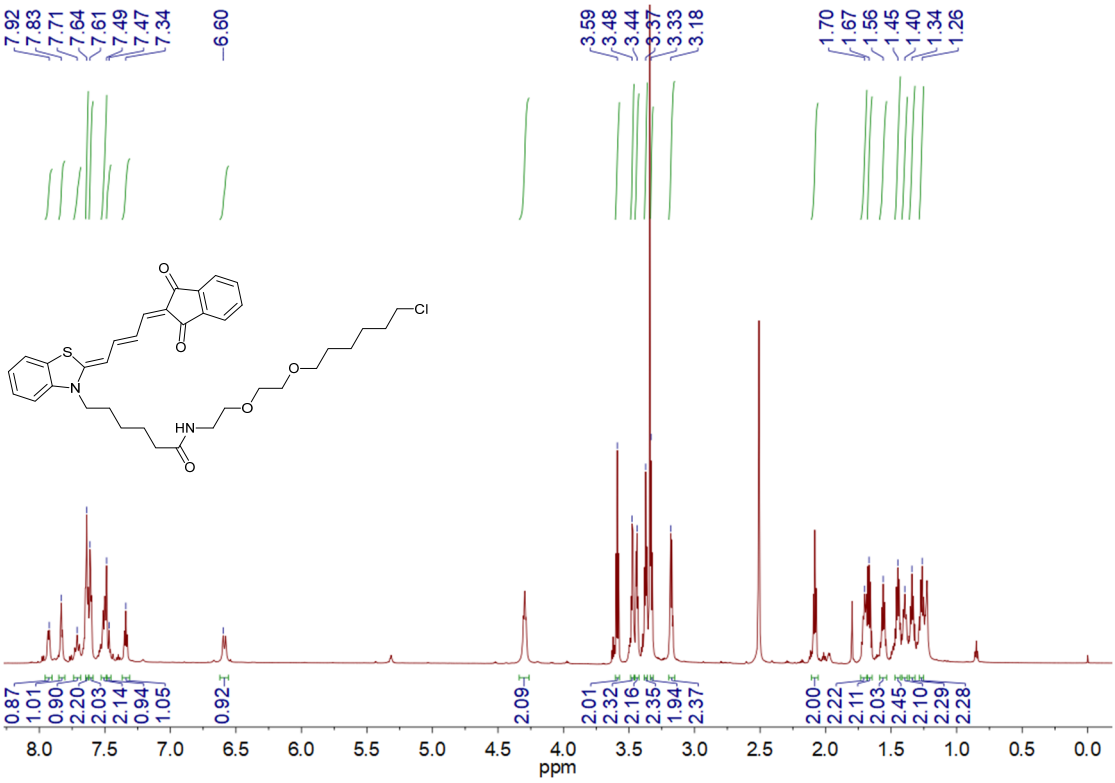


^{1}H-NMR spectrum of **Mero-Rotor-Halo** (DMSO-d^{6})

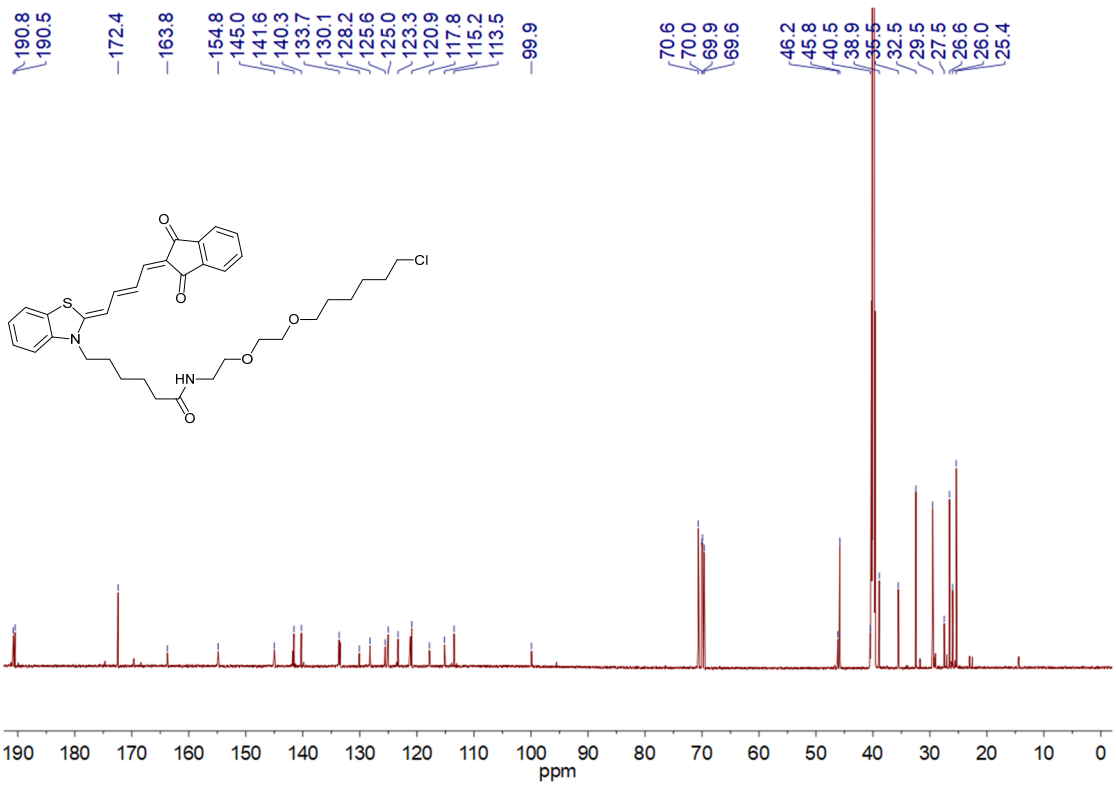


^{13}C-NMR spectrum of **Mero-Rotor-Halo** (DMSO-d^{6})

Mero-Solvato-Halo:



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



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

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

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