Electronic Supplementary Material (ESI) for ChemComm.

Derivatizing Nile Red Fluorophores to Quantify the Heterogeneous Polarity upon Protein Aggregation in the Cell.

Rui Sun[a,b], Wang Wan[a], Wenhan Jin[a], Yulong Bai[a,b], Qiuxuan Xia[a,b], Mengdie Wang[a,b], Yanan Huang[a], Lianggang Zeng[a], Jialu Sun[a], Congcong Peng[a,c], Biao Jing[a,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 the Chinese Academy of Sciences, Beijing 100049, China.
c. The Second Hospital of Dalian Medical University, 467 Zhongshan Road, Dalian, 116023, China.

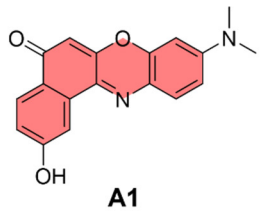
* E-mail: liuyu@dicp.ac.cn

Table of Contents

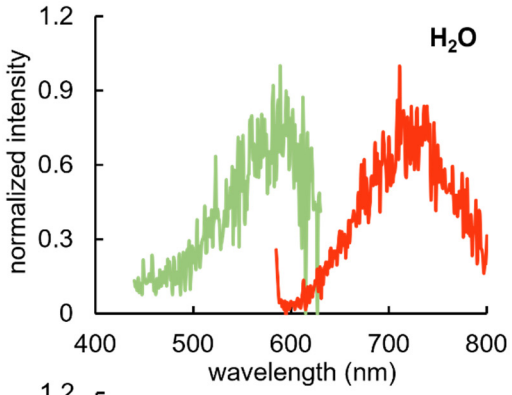
1. Supplemental Figures and Tables

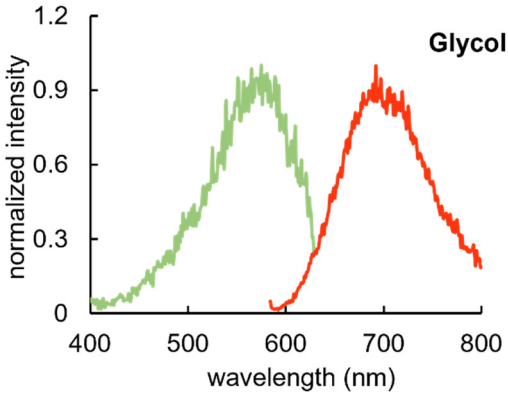
solvent	dielectric constant	A1				A2				A3			
		λ_{abs} (nm)	λ_{ex} (nm)	λ_{em} (nm)	Φ	λ_{abs} (nm)	λ_{ex} (nm)	λ_{em} (nm)	Φ	λ_{abs} (nm)	λ_{ex} (nm)	λ_{em} (nm)	Φ
H_2O	80.3	573	589	711	--	526	593	661	--	564	603	661	--
Glycol	37.0	569	575	692	0.03	562	578	653	0.27	567	580	653	0.26
MeOH	32.7	550	551	691	0.02	543	546	642	0.31	542	551	643	0.34
EtOH	24.5	543	551	678	0.03	537	548	632	0.43	541	551	635	0.45
^{i}PrOH	19.9	540	551	660	0.03	532	547	628	0.45	542	551	629	0.54
^{n}BuOH	17.1	546	548	672	0.03	535	547	628	0.43	538	551	630	0.55
^{t}BuOH	11.4	537	551	652	0.04	529	539	622	0.52	532	538	624	0.56
DCM	8.9	528	537	624	0.31	524	535	621	0.57	532	547	607	0.65
THF	7.6	519	522	609	0.16	517	532	601	0.57	523	533	606	0.61
EA	6.0	515	515	608	0.20	512	523	605	0.65	513	522	602	0.74
Toluene	2.4	515	523	601	0.11	510	525	587	0.52	512	516	589	0.64
Dioxane	2.2	520	522	608	0.13	504	516	589	0.56	514	517	592	0.69
CCl_4	2.2	511	502	604	0.07	508	501	589	0.60	511	503	603	0.70

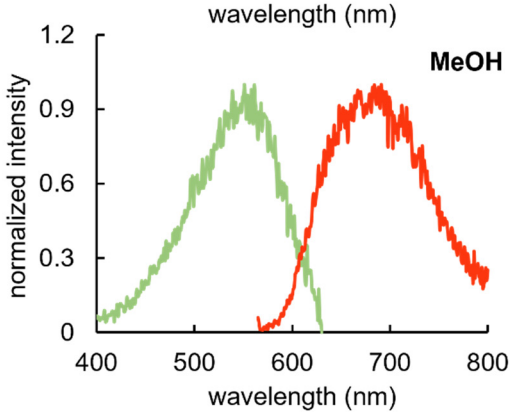
Table S1. Photophysical properties of A1, A2 and A3 across the different solvents. All measurements were carried out using a Tecan Spark Fluorescence Plate Reader in NEST^{TM} 96-Well flat bottom transparent plates. Measurement of quantum yield in different solvents were described in supplementary **Experimental Methods 2.2** using Nile blue (Φ = 0.27 in ethanol) as reference and 500 nm as the excitation wavelength. All measurements were repeated for three times.

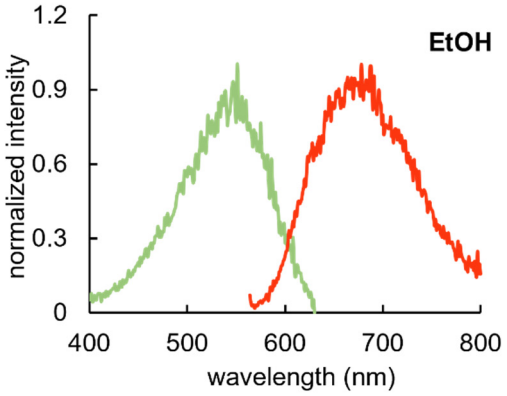

O
O
N
N
OH

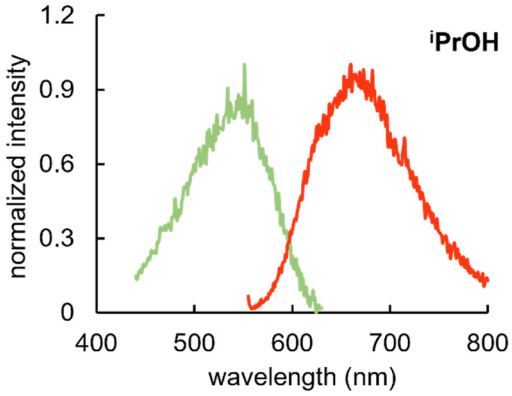
A1

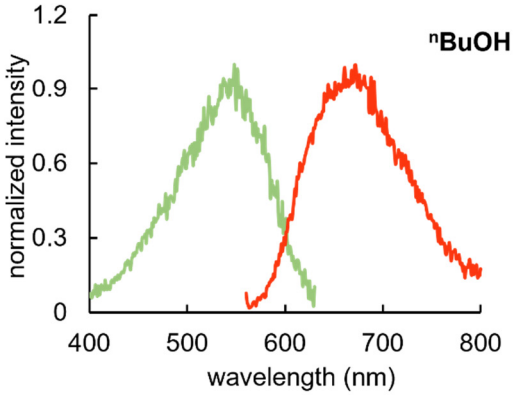

H2O
normalized intensity
wavelength (nm)

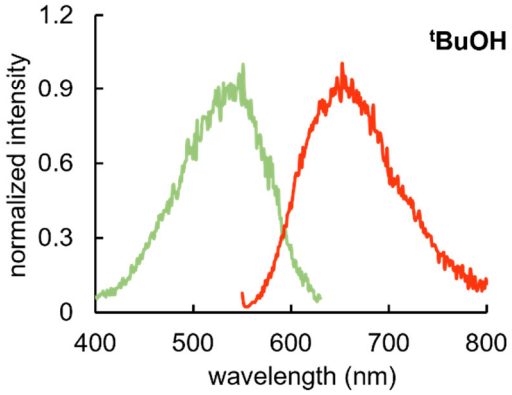

Glycol
normalized intensity
wavelength (nm)


MeOH
normalized intensity
wavelength (nm)


EtOH
normalized intensity
wavelength (nm)


iPrOH
normalized intensity
wavelength (nm)


nBuOH
normalized intensity
wavelength (nm)


tBuOH
normalized intensity
wavelength (nm)

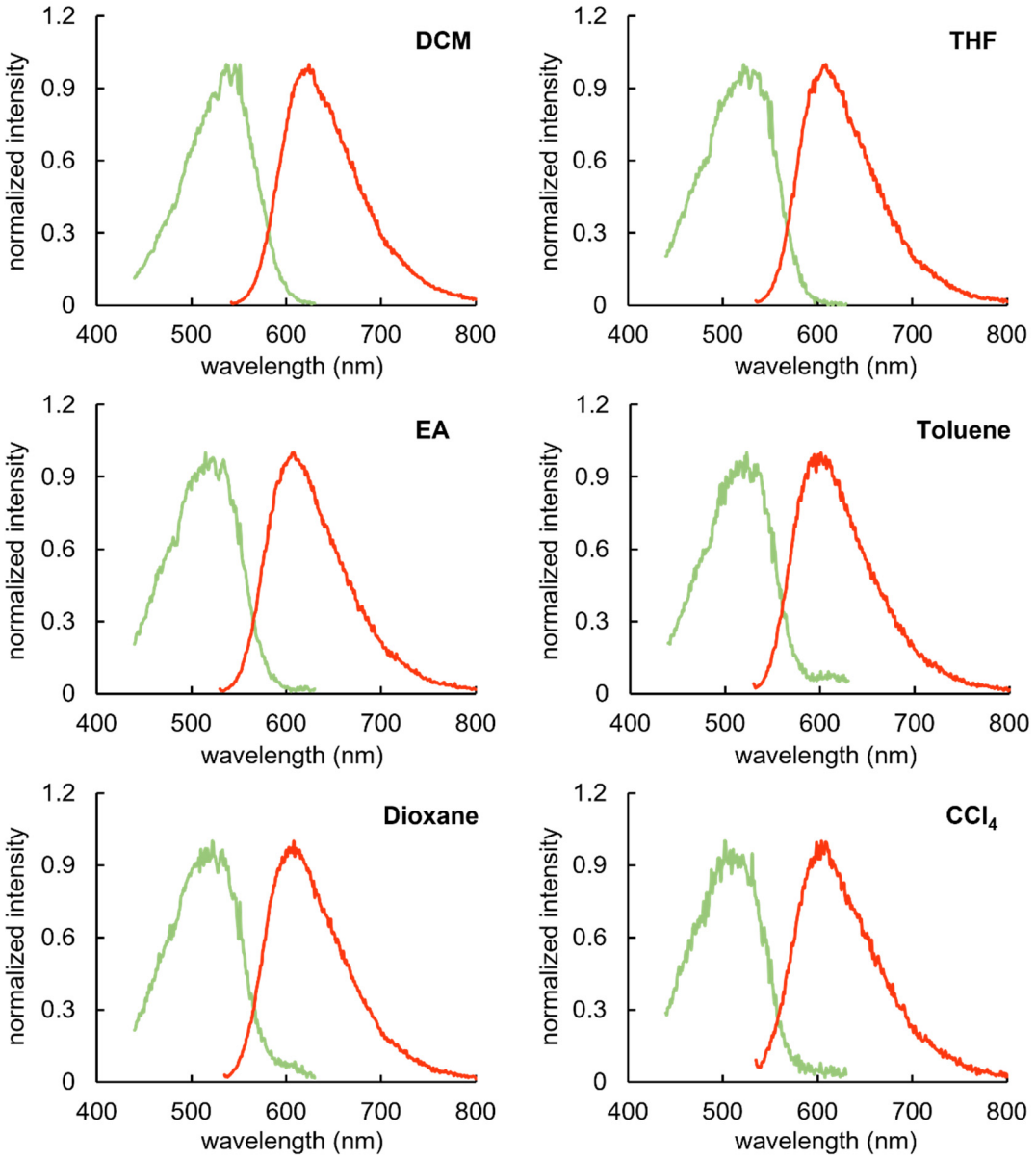
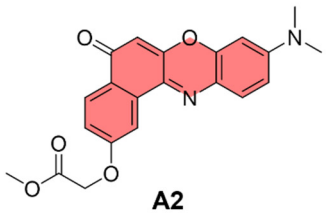
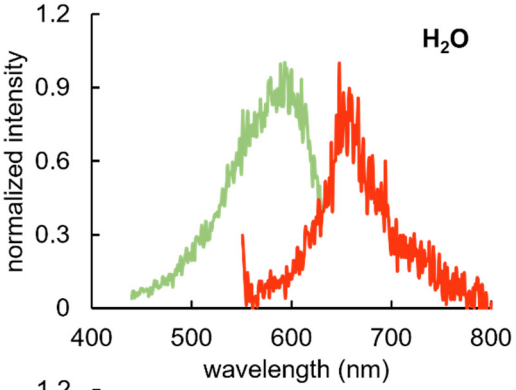
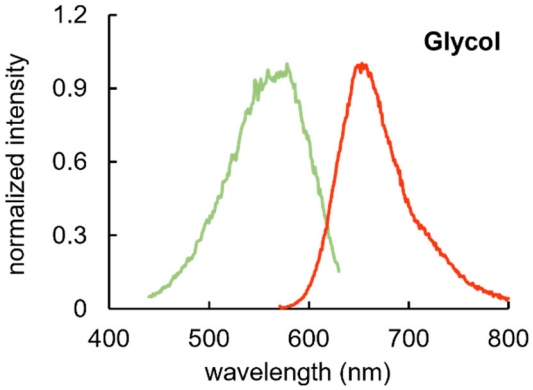


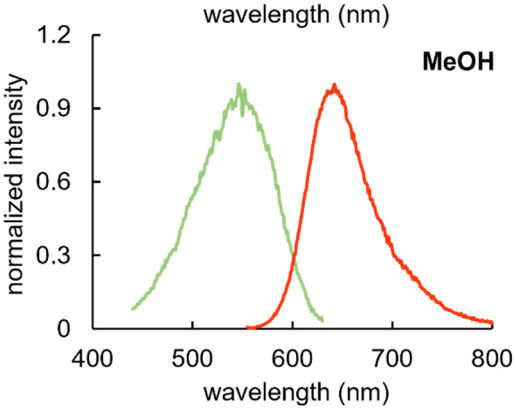
Fig. S1. Normalized fluorescence excitation and emission spectra of A1 across the tested solvents. A1 probe was prepared to 8 μM for excitation scan and 20 μM for emission measurements. All measurements were carried out using Tecan Spark Fluorescence Plate Reader in NEST^{TM} 96-Well flat bottom transparent plates. The experimental procedure followed **Experimental Methods 2.1.** All measurements were repeated for three times.

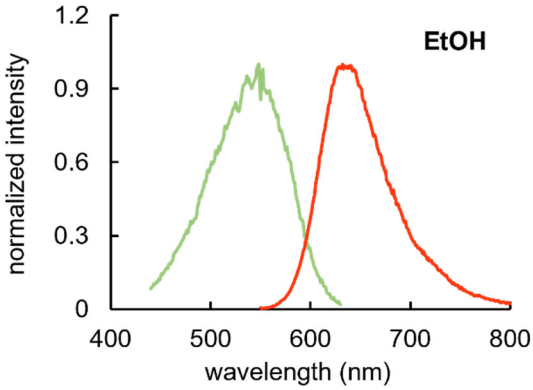

O
O
N
O
O
O

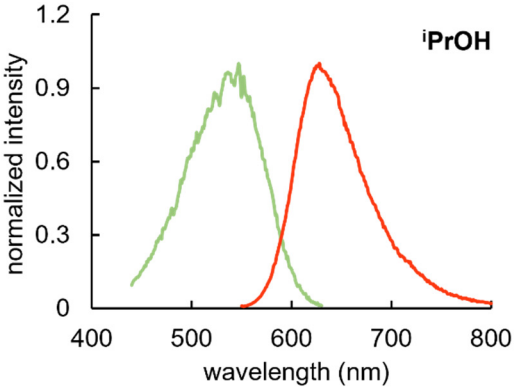
A2

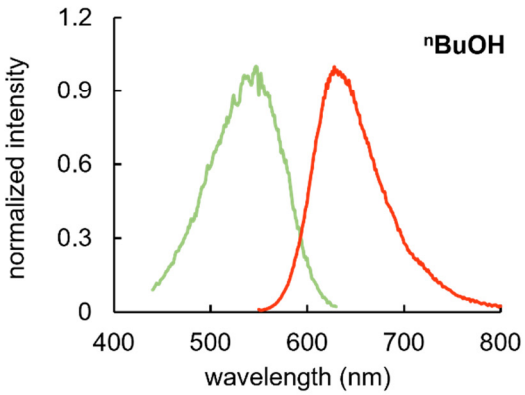

H2O
normalized intensity
wavelength (nm)

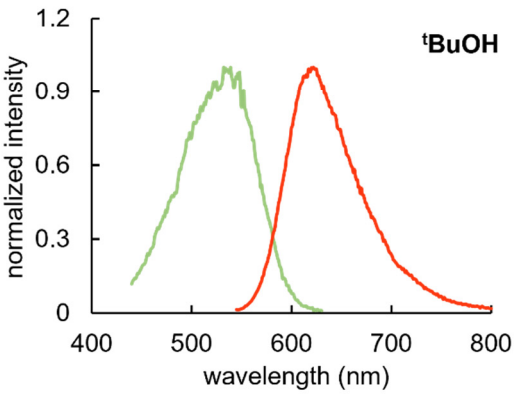

Glycol
normalized intensity
wavelength (nm)


MeOH
normalized intensity
wavelength (nm)


EtOH
normalized intensity
wavelength (nm)


iPrOH
normalized intensity
wavelength (nm)


nBuOH
normalized intensity
wavelength (nm)


tBuOH
normalized intensity
wavelength (nm)

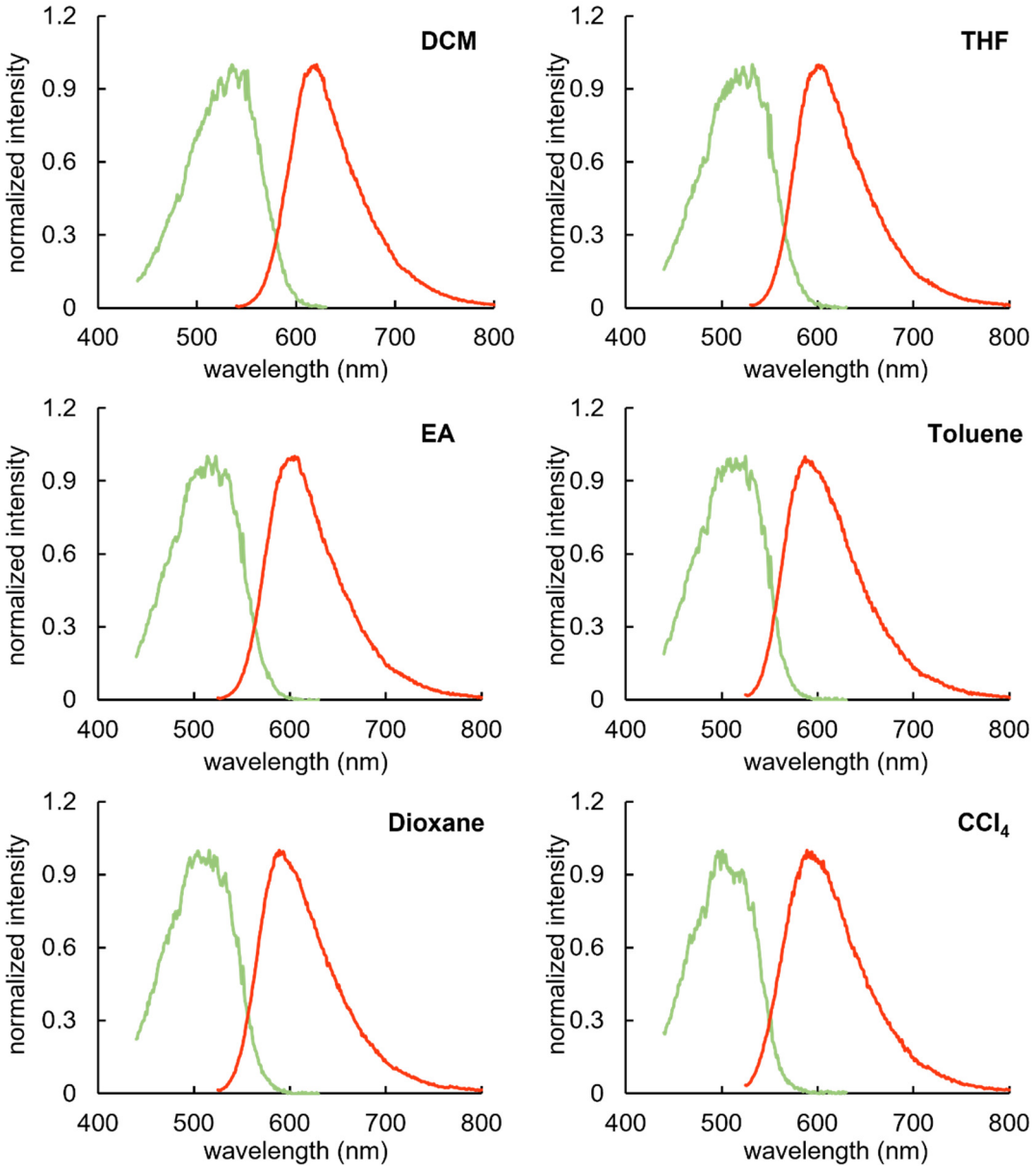
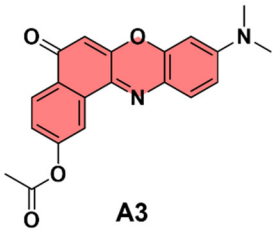
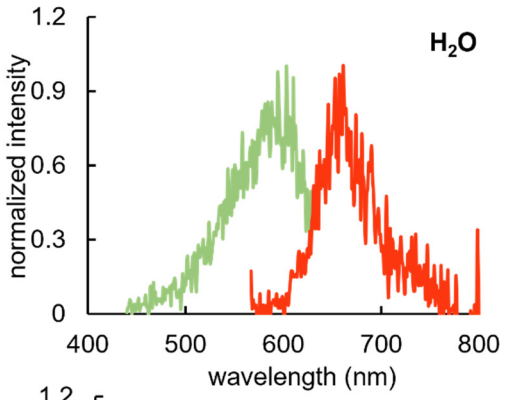
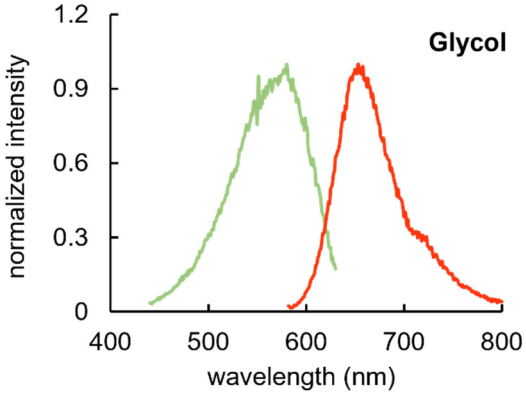
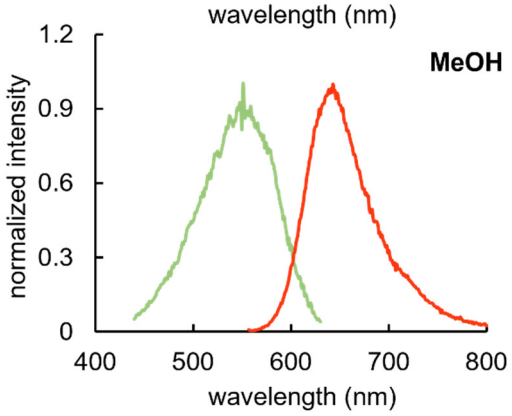


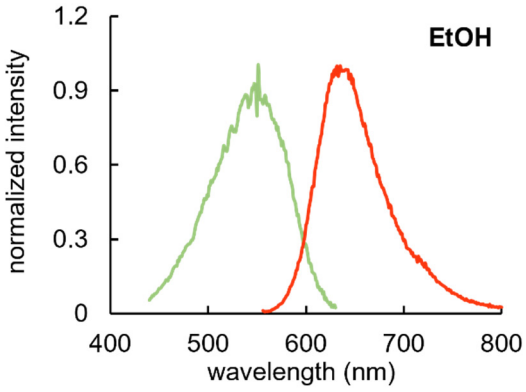
Fig. S2. Normalized fluorescence excitation and emission spectra of A2 across the tested solvents. A2 was prepared to 8 μM for excitation scan and 20 μM for emission measurement. All measurements were carried out using Tecan Spark Fluorescence Plate Reader in NEST^{TM} 96-Well flat bottom transparent plates. The experimental procedure followed **Experimental Methods 2.1.** All measurements were repeated for three times.

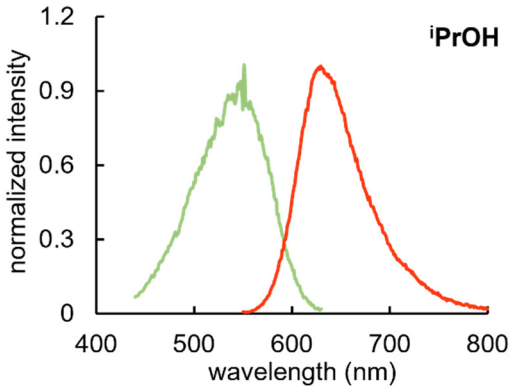

O
O
N
O
O
A3

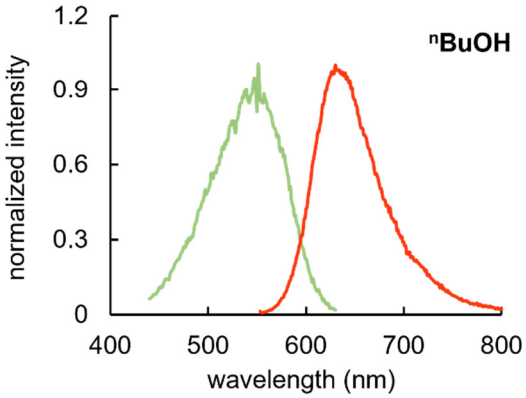

H2O
normalized intensity
1.2
0.9
0.6
0.3
0
400
500
600
700
800
wavelength (nm)

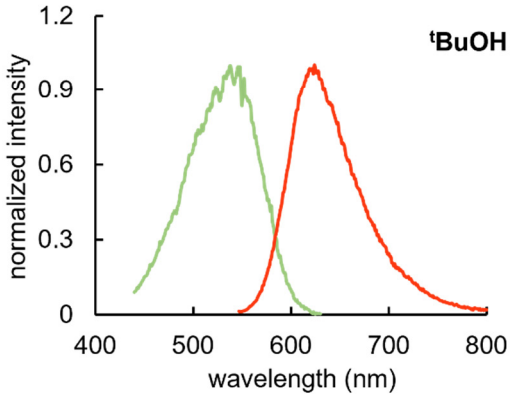

Glycol
normalized intensity
1.2
0.9
0.6
0.3
0
400
500
600
700
800
wavelength (nm)


MeOH
normalized intensity
1.2
0.9
0.6
0.3
0
400
500
600
700
800
wavelength (nm)


EtOH
normalized intensity
1.2
0.9
0.6
0.3
0
400
500
600
700
800
wavelength (nm)


iPrOH
normalized intensity
1.2
0.9
0.6
0.3
0
400
500
600
700
800
wavelength (nm)


nBuOH
normalized intensity
1.2
0.9
0.6
0.3
0
400
500
600
700
800
wavelength (nm)


tBuOH
normalized intensity
1.2
0.9
0.6
0.3
0
400
500
600
700
800
wavelength (nm)

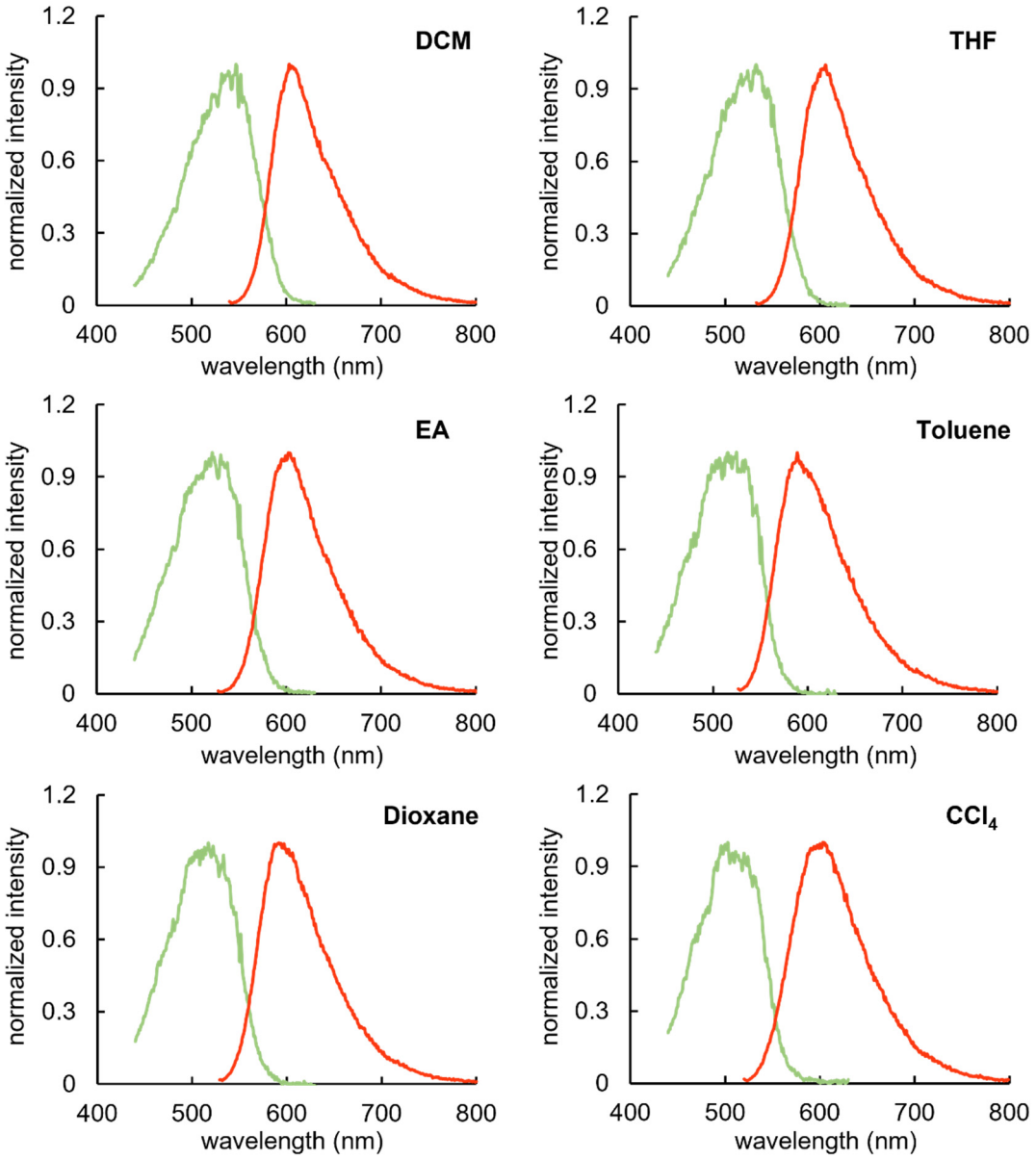


Fig. S3. Normalized fluorescence excitation and emission spectra of A3 across the tested solvents. A3 was prepared to 8 μM for excitation scan and 20 μM for emission scan in different solvents. All measurements were carried out using Tecan Spark Fluorescence Plate Reader in NEST^{TM} 96-Well flat bottom transparent plates. The experimental procedure followed **Experimental Methods 2.1.** All measurements were repeated for three times.

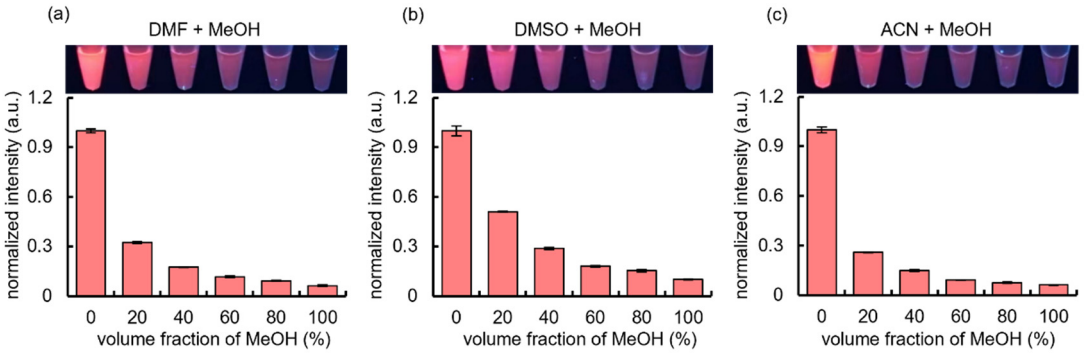


Fig. S4. The fluorescence intensity of A1 decreased with the addition of protic solvent (MeOH) to polar solvents (DMF, DMSO and acetonitrile), confirming the excited state proton transfer process of A1. A1 was prepared to 20 μM for emission scan in all aprotic polar solvents (DMF, DMSO, ACN) with increasing volume of protic MeOH. The maximum λ_{abs} was selected as the λ_{ex}. All measurements were carried out using Tecan Spark Fluorescence Plate Reader in NEST^{TM} 96-Well flat bottom transparent plate. The experimental procedure followed **Experimental Methods 2.2**. All measurements were repeated for three times.

(a)

solvent	ε (cm^{-1}•mol^{-1})		
	A1	A2	A3
Glycol	9800	5750	3850
MeOH	12850	7800	5100
EtOH	14100	9000	6100
^{i}PrOH	14900	9250	6400
^{n}BuOH	14550	8850	5800
^{t}BuOH	15300	9250	6000
DCM	17650	10650	7200
THF	17750	10300	7250
EA	19000	11750	8600
Toluene	18150	11100	7900
Dioxane	17600	11550	8100
CCl_4	18550	11550	7950

(b)

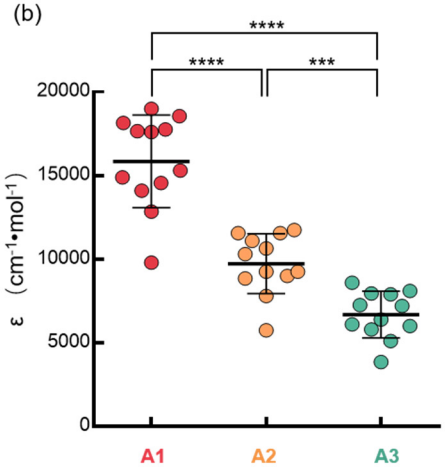


Fig. S5. The extinction coefficient of A1, A2 and A3 (a) and statistic comparation (b). A1, A2 and A3 (1 mM in DMSO) was diluted to 20 μM in listed solvents as testing samples. The measurements were carried out using 1cm quartz cuvette and UV-Vis Spectrometer (METASH UV-5100). The absorbance wavelength was selected as 500 nm, which was same to λ_{ex} of quantum yield measurement. The experimental procedure followed **Experimental Methods 2.3.**

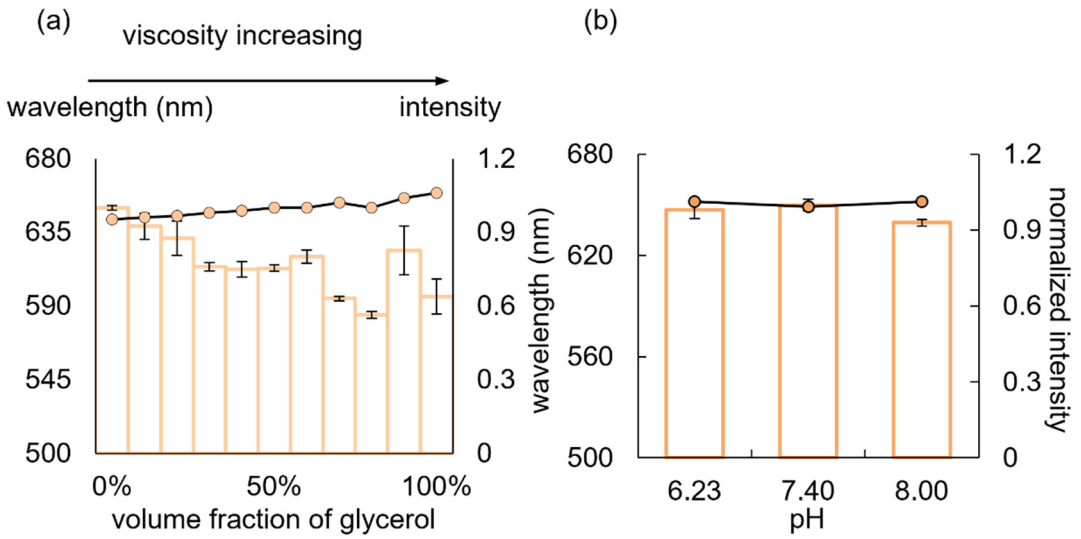


Fig. S6. P1 is not sensitive toward viscosity (a) and pH (b) variation. (a) P1 was prepared as 20 μM in solvents with varying volume fractions of glycerol in methanol. Both intensity and emission wavelength sustained constantly, indicating the insensitivity of P1 toward viscosity. (b) P1 was prepared in buffers with different pH (acidic aggregation buffer-pH 6.24, phosphate buffer-pH 7.40, buffer A-pH 8.00). No obvious fluorescence fluctuation was observed in all buffers, suggesting the insensitivity of P1 to different pH. All measurements were carried out using Tecan Spark Fluorescence Plate Reader in NESTTM 96-Well flat bottom transparent plates. The experiment followed **Experimental Methods 2.5.** All measurements were repeated for three times. Dots represent fluorescence emission wavelength; bars represent fluorescence emission intensity. Error bars represent standard errors (n = 3).

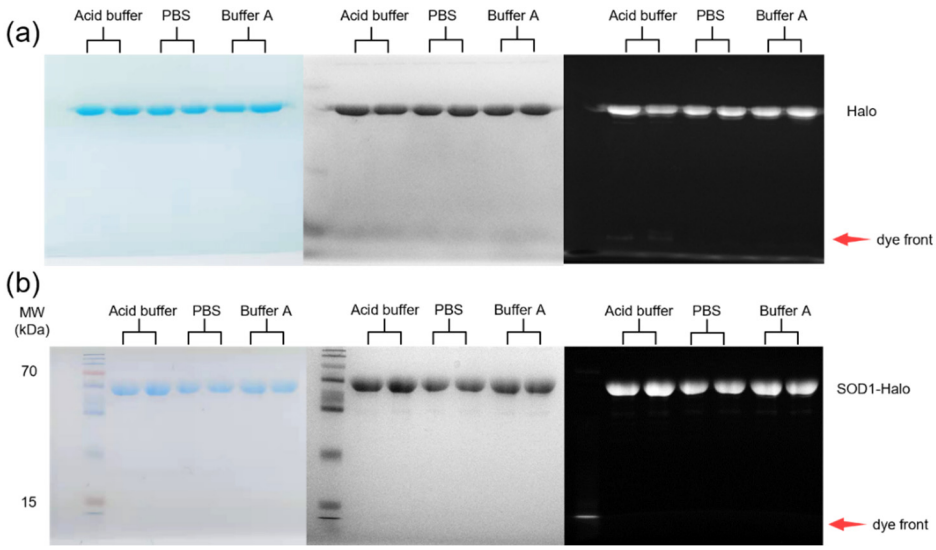


Fig. S7. The covalent conjugations of P1 to (a)WT-Halo and (b)SOD1(V31A)-Halo were visualized *via* SDS-PAGE electrophoresis gel. The experiment followed **Experimental Methods 2.8.** The conjugation of P1 with proteins was performed in the three different buffers: acid aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23), PBS buffer (pH = 7.40), buffer A (50 mM Tris-HCl, 100 mM NaCl, acidified by HCl to pH = 8.00). As shown on SDS-PAGE gels (Fig. S5a and 5b), P1 covalently modified POI (Halo and SOD1(V31A)-Halo). Left: coomassie blue stained SDS-PAGE gels, bright view; Middle: coomassie blue stained SDS-PAGE gels, in gray scale; Right: UV-illuminated SDS-PAGE gels for fluorescence detection.

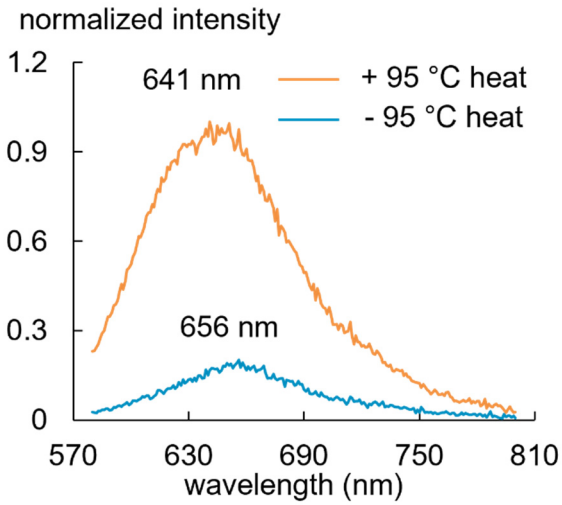


Fig. S8. The A2 probe endows moderate inherent affinity toward aggregated SOD1-Halo. The fluorescent intensity of A2 exhibited 5-fold enhancement upon heat induced aggregation of SOD1. Furthermore, maximal emission wavelength was shifted from 656 nm to 641 nm as well. As A2 probe harbored no HaloTag warhead, it only bound to aggregated proteins non-covalently, suggesting its moderate binding affinity. The experiment followed **Experimental Methods 2.7.** All measurements were repeated for three times.

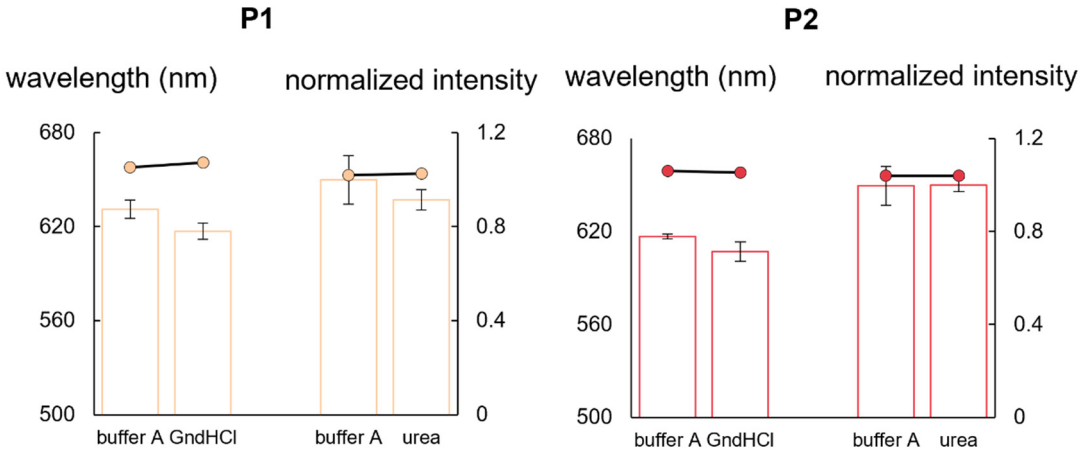


Fig. S9. P1 and P2 exhibited similar fluorescence signal in both folded and unfolded states. For folded fluorophores-labeled SOD1(V31A)-Halo: Protein and probe (P1 or P2) were incubated in buffer A (50 mM Tris-HCl, 100 mM NaCl, acidified by HCl to pH = 8.00) at 30 °C for 5 min. For each unfolded fluorophore-labeled SOD1(V31A)-Halo: Two sets of folded probe conjugated proteins were dissolved in 6 M urea and 6 M Guanidium hydrochloride (Gnd·HCl), respectively. The retained λ_{em} of P1 and P2 in buffer A and Gnd·HCl/urea suggested that P1 and P2 were not sensitive to unfolded proteins. All measurements were carried out using Tecan Spark Fluorescence Plate Reader in NEST^{TM} 96-Well flat bottom transparent plates. The intensity was normalized to 1 against the maximal intensity. All measurements were repeated for three times. The experiment followed **Experimental Methods 2.13.**

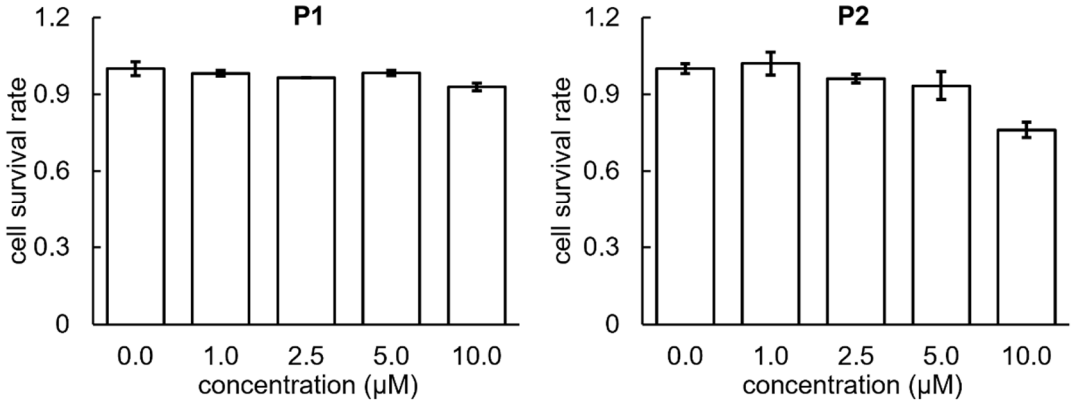


Fig. S10. P1 and P2 showed minimal cytotoxicity to HEK293T cells measured by MTT assay. The experiment followed **Experimental Methods 2.16.** All measurements were repeated for three times.

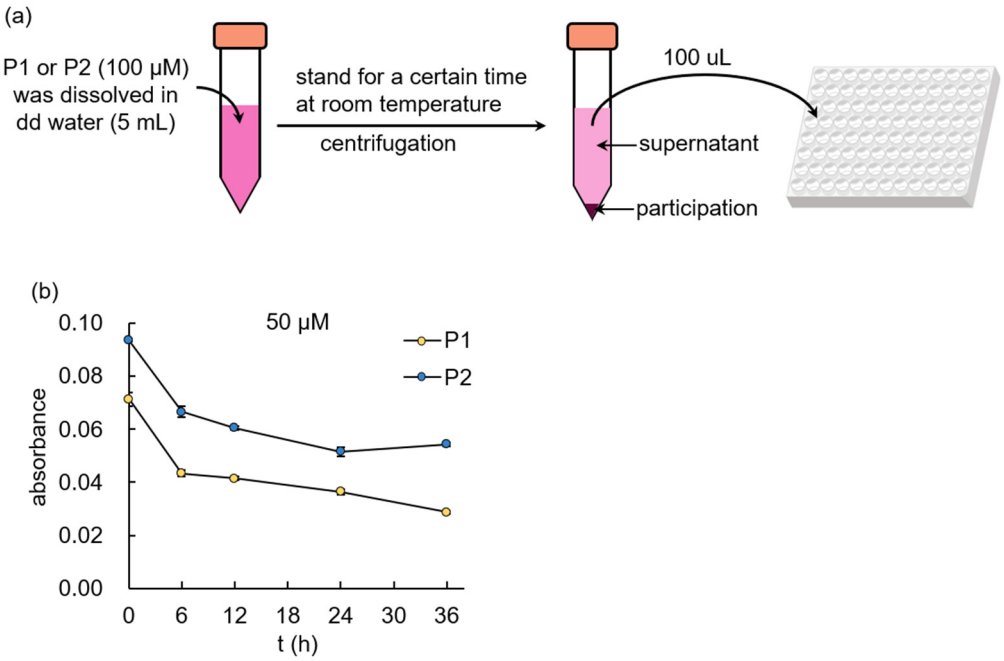


Fig. S11. Resistance to precipitation measured by residual absorbance. (a) P1 and P2 (100 μM) were dissolved in deionized water (5 mL) respectively and kept steadily at room temperature. At indicated time points, clear supernatant after brief centrifugation was pipetted into NEST 96 flat bottom transparent plates with 100 μL solution. The absorption spectra were collected on Tecan Spark Fluorescence Plate Reader. (b) The experiment in Fig 4b was repeated using 50 μM samples to further confirm the better solubility of P2 in water. The experiment followed Experimental Methods 2.15. All measurements were repeated for three times.

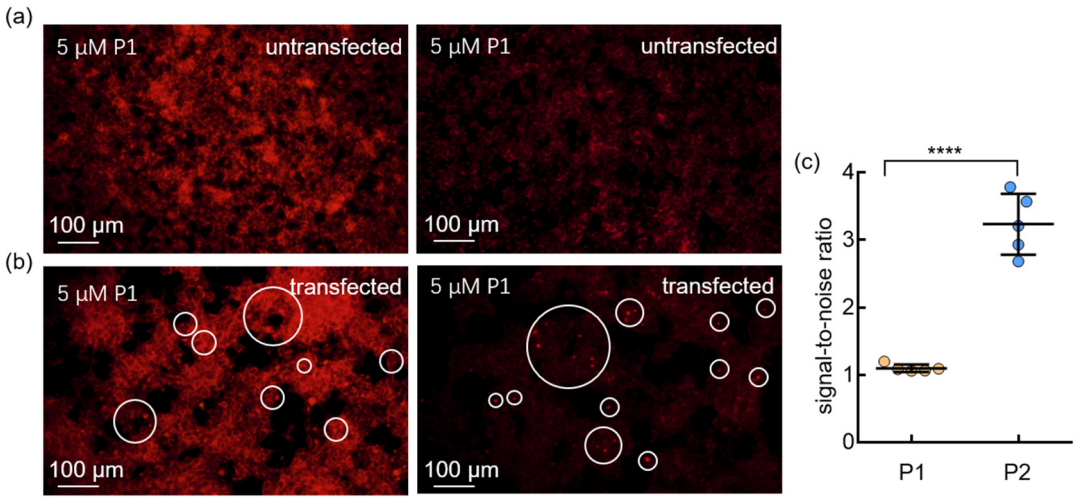


Fig. S12. Quantitative comparison of the signal-to-noise ratio of P1 and P2 in imaging polyQ aggregation in live cells. (a) The untransfected HEK293T cells were stained by P1 and P2 (5 μM). (b) The HEK293T cells were transfected with *Htt*-110Q-Halo in presence of P1 and P2. The signature bright fluorescent puncta were only clearly observed in transfected cells stained with P2 probe. (c) In transfected cells, the signal-to-noise ratios (S/N) of P1 and P2 were quantified and statistically analyzed. The S/N of P2 was obviously higher than P1, indicating the P2 of improved aqueous solubility exhibited much lower background in cellular imaging. The experiment followed **Experimental Methods 2.19.** Images were collected under excitation by green light source (BP 530-550 nm). Statistical analysis was performed using GraphPad Prism software (n = 5, mean ± SD; ****P < 0.0001).

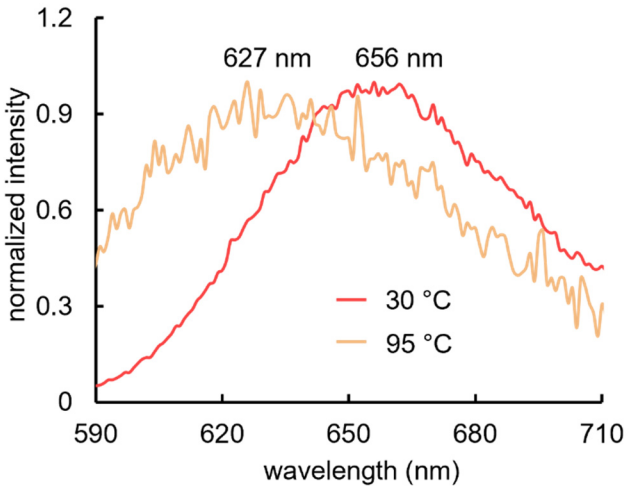


Fig. S13. P2 detected SOD1(V31A) aggregation via wavelength shift. The emission wavelength of SOD1(V31A)-Halo-P2 conjugate displayed 29 nm hypsochromic in from folded protein (red line) to aggregated one (orange line). SOD1(V31A)-Halo (36 μM), P2 (20 μM) and EDTA disodium salt (12.5 mM) was mixed in buffer A (50 mM Tris-HCl, 100 mM NaCl, acidified by HCl to pH = 8.00) and incubated at room temperature for 5 min to measure fluorescence emission wavelength when proteins were folded (red line). The mixture was incubated at 25 °C for 5 min then further heated at 95 °C for 5 min to measure fluorescence emission of aggregated proteins (orange line). The incubated mixtures (100 μL) were pipetted into BeyoGold™ 96-Well bottom transparent plates and λ_{em} was recorded using Tecan Spark Plate Reader (λ_{ex} = 550 nm for sample I and 540 nm for sample II). Error bars: standard error (n = 3). The experiment followed **Experimental Methods 2.13.**

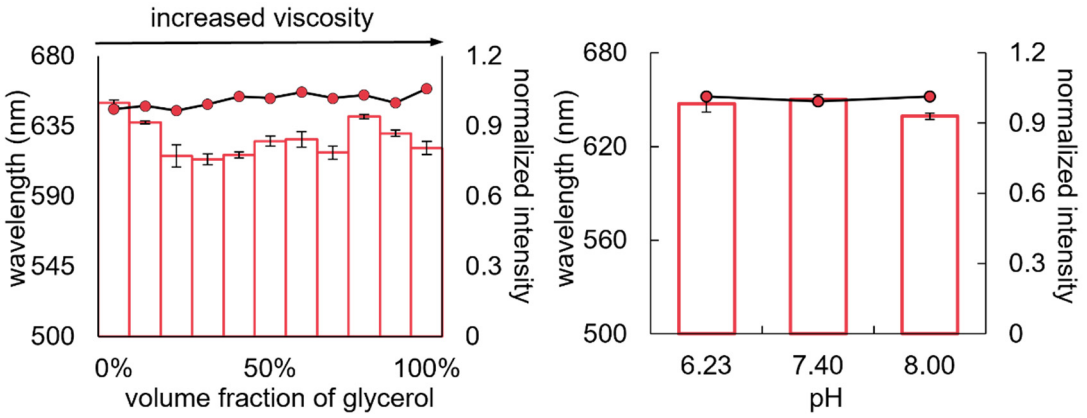


Fig. S14. P2 was not sensitive toward viscosity and pH. **(a)** P2 was prepared as 20 μM in solvents with varying volume fractions of glycerol in methanol. Both intensity and emission wavelength sustained constantly, indicating the insensitivity of P2 toward viscosity. **(b)** P2 was prepared in buffers of different pH (acidic aggregation buffer-pH 6.24, phosphate buffer-pH 7.40, buffer A-pH 8.00). No obvious fluctuation was observed in all pH systems, suggesting the insensitivity of P2 to different pH. All measurements were carried out using Tecan Spark Fluorescence Plate Reader in NEST^{TM} 96-Well flat bottom transparent plates. The experiment followed **Experimental Methods 2.5.** All measurements were repeated for three times. Dots represent fluorescence emission wavelength; bars represent fluorescence emission intensity. Error bars represent standard errors (n = 3).

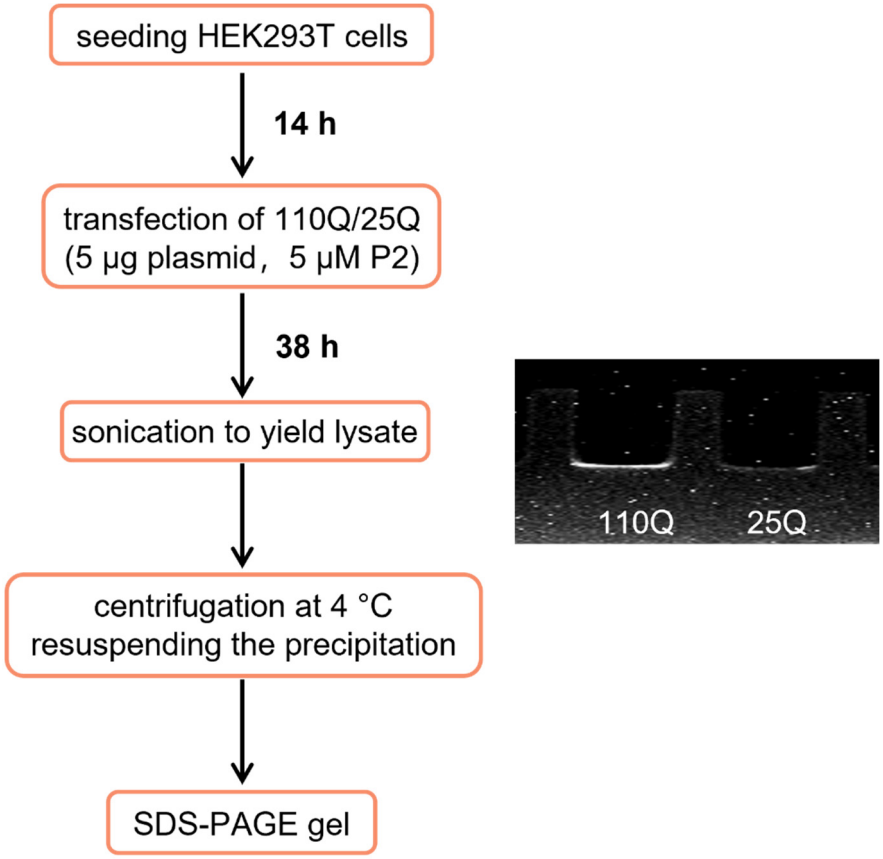


Fig. S15. P2 probe highlighted the formation of insoluble aggregation in cells expressing *Htt*-110Q. As shown in right image, the cells expressing *Htt*-110Q exhibited bright fluorescence signal in insoluble fraction, and no obvious fluorescence signal observed in cells expressing *Htt*-25Q. The insoluble aggregates of 110Q were SDS resistant, did not penetrate into the SDS-PAGE gel and stayed in the load well. The experiment followed **Experimental Methods 2.17.**

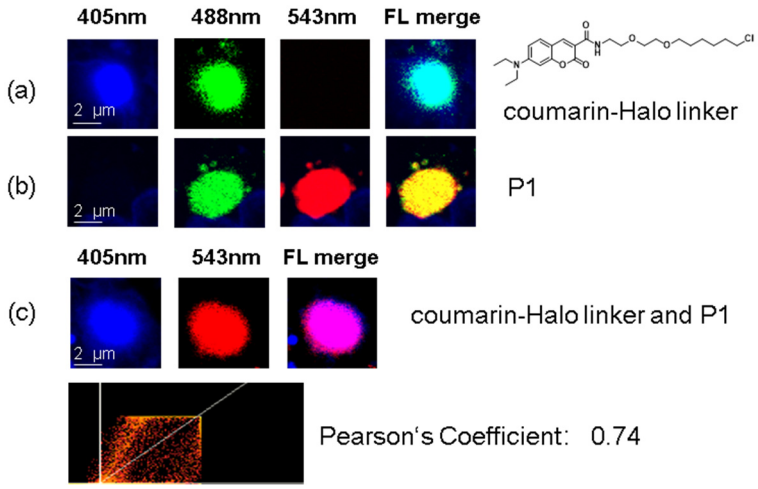


Fig. S16. Co-localization imaging of P1 and coumarin-Halo linker to prove the bioorthogonal conjugation of P1 with POI (Halo-tagged *Htt*-110Q) in HEK293T cells. (a) *Htt*-110Q stained by coumarin-Halo linker probe. As shown in Fig. S16a, we chose coumarin-Halo linker as a control, which labeled the POI specifically. (b) *Htt*-110Q stained by P1. The puncta of *Htt*-110Q showed fluorescence signal under 488 nm and 543 nm excitation wavelengths. (c) *Htt*-110Q stained by coumarin-Halo linker and P1 together. The merged fluorescence signal indicated P1 and coumarin labeled at the same location. The Pearson's coefficient (0.74) was generated from ImageJ. Blue: 405 nm laser for coumarin-Halo linker; Green: 488 nm laser for coumarin-Halo linker probe and P1; Red: 543 nm laser for P1. The experiment followed **Experimental Methods 2.18.**

Note: The power of 488 nm laser used here was doubled than it used in **Fig. S18, Fig. S19 and Fig. S21** to prove both of coumarin-Halo linker and P1 could be excited by it.

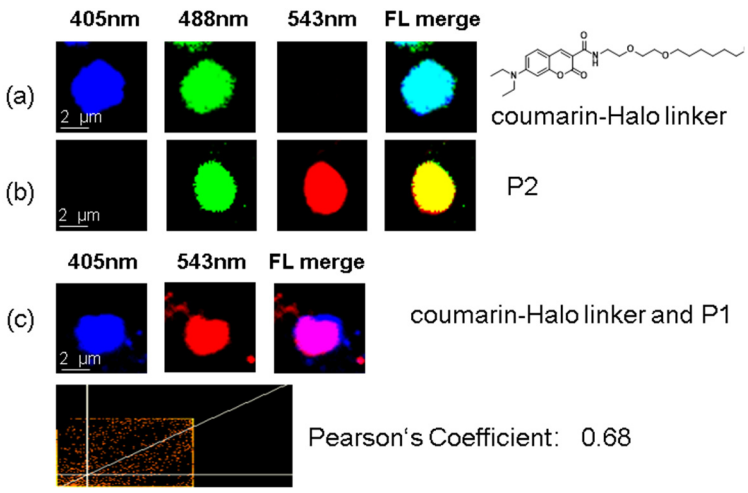


Fig. S17. Colocalization imaging of P2 and coumarin-Halo linker to prove the bioorthogonal conjugation of P2 and POI (Halo-tagged *Htt*-110Q) in HEK293T cells. (a) *Htt*-110Q stained by coumarin-Halo linker probe. As shown in Fig. S17a, we chose coumarin-Halo linker as a control, which labeled the POI specifically. (b) *Htt*-110Q stained by P2. The puncta of *Htt*-110Q showed fluorescence signal under 488 nm and 543 nm excitation wavelengths. (c) *Htt*-110Q stained by coumarin-Halo linker probe and P2 together. The merged fluorescence signal indicated P2 and coumarin labeled at the same location. The Pearson's coefficient (0.68) was generated from ImageJ. Blue: 405 nm laser for coumarin-Halo linker; Green: 488 nm laser for coumarin-Halo linker and P2; Red: 543 nm laser for P2. The experiment followed **Experimental Methods 2.18.**

Note: The power of 488 nm laser used here was doubled than it used in **Fig. S18, Fig. S19 and Fig. S21** to prove both of coumarin-Halo linker and P2 could be excited by it.

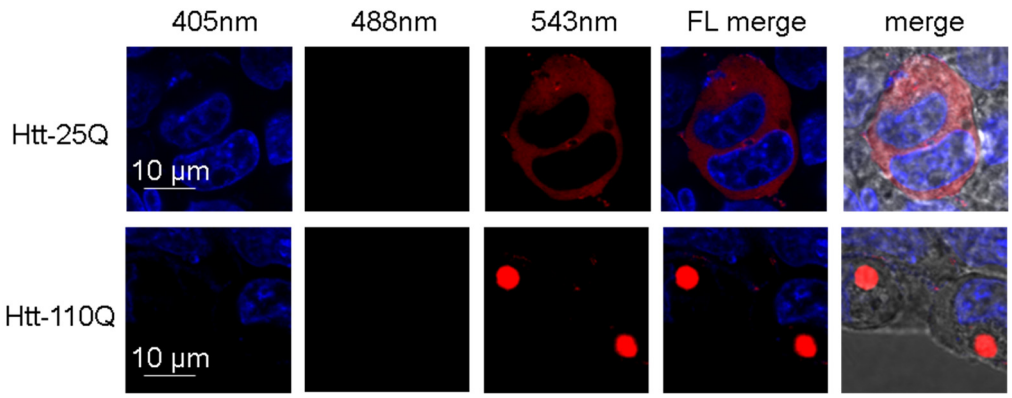
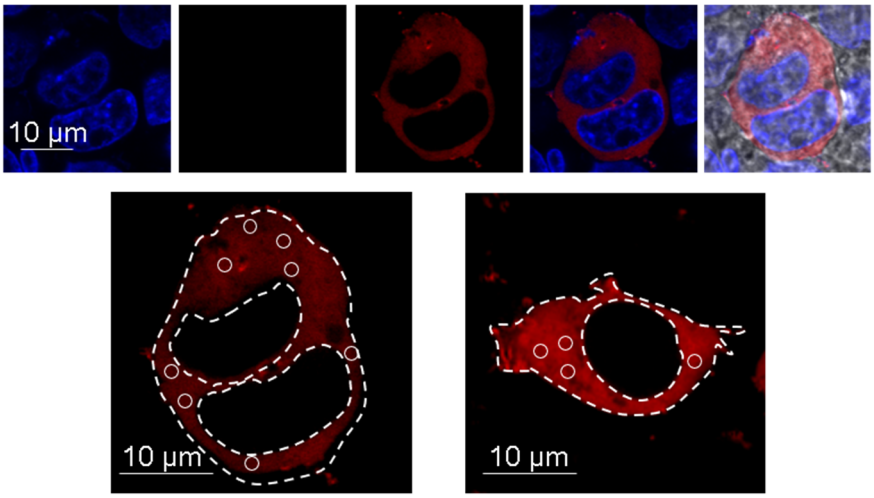
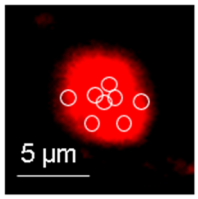


Fig. S18. Folded *Htt*-25Q-Halo and aggregate *Htt*-110Q-Halo were visualized by P2 probe. Upper panel: the diffuse fluorescence of P2 was observed in cells expressing folded 25Q-Halo. Lower panel: Large bright puncta was observed in cells expressing aggregated 110Q-Halo. Blue: 405 nm laser for Hoechst 33342; Red: 543 nm laser for P2. The experiment followed **Experimental Methods 2.20.**

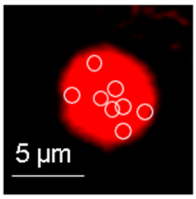


λ_{em1} (nm)	λ_{em2} (nm)	λ_{em3} (nm)
626	626	626
626	626	627
628	627	626
625	627	626

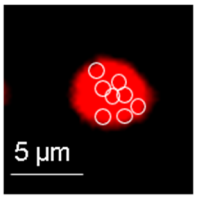
Fig. S19. λ-Deconvoluted fluorescence imaging of folded *Htt*-25Q-Halo in cytoplasmic condition. The measured areas were highlighted by white circles, and emission wavelength of all selected areas was concluded as a table in Fig. S15. Blue: 405 nm laser for Hoechst 33342; Red: 543 nm laser for P2. The experiment followed **Experimental Methods 2.20.**



shell	core
618 nm	616 nm
615 nm	613 nm
616 nm	614 nm
619 nm	616 nm



shell	core
615 nm	613 nm
617 nm	614 nm
616 nm	614 nm
616 nm	616 nm



shell	core
618 nm	615 nm
617 nm	614 nm
618 nm	613 nm
616 nm	616 nm

Fig. S20. λ-Deconvoluted fluorescence imaging of 110Q-Halo showed structural heterogeneity of the polarity inside aggregated protein. The measured regions were highlighted by white circles, and results of emission were listed under corresponding images. More blue-shifted wavelength of P2 was observed in the core region of aggregates than outer shell, indicating more compacted structure and less polar environment inside aggregated proteins in live cells. Blue: 405 nm laser for Hoechst 33342; Red:543 nm laser for P2. The experiment followed **Experimental Methods 2.20.**

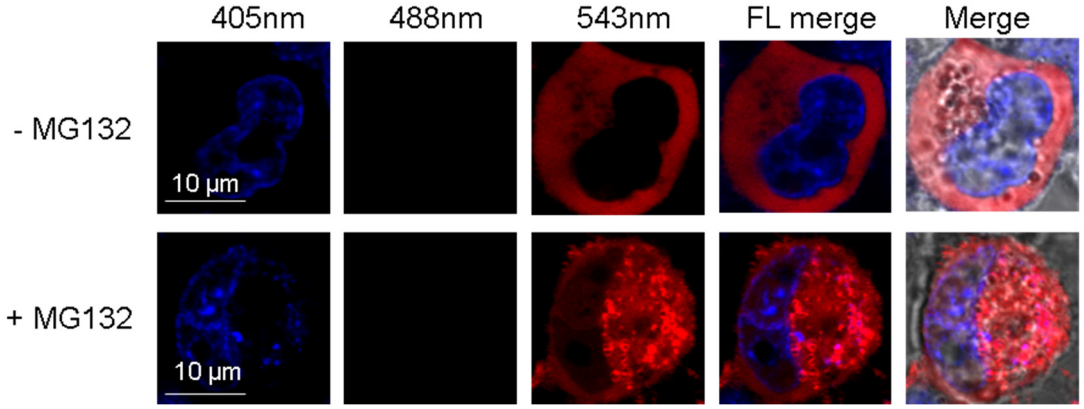
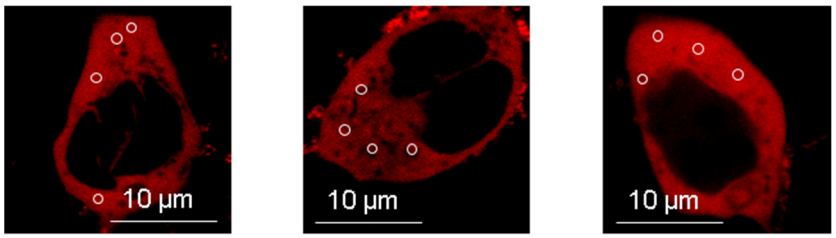
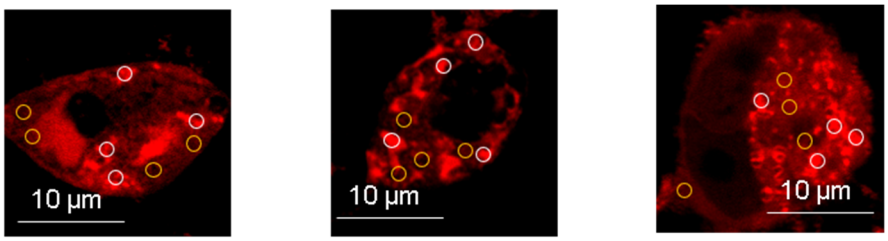


Fig. S21. Confocal images of SOD1(G85R)-Halo with and without MG132 treatment. As shown in upper panel, SOD1(G85R)-Halo displayed diffuse signal without MG132. In the lower panel, both small speckle-like puncta and diffuse fluorescence were observed in cell after aggregation induced by MG132. Blue: 405 nm laser for Hoechst 33342; Red: 543 nm laser for P2. The experiment followed **Experimental Methods 2.20.**



λ_{em1} (nm)	λ_{em2} (nm)	λ_{em3} (nm)
623	621	620
624	620	620
624	622	624
622	622	624

Fig. S22. λ-Deconvoluted fluorescence imaging of folded SOD1(G85R)-Halo in cytoplasmic condition. The measured regions were highlighted by white circles, and emission wavelength of all selected areas was concluded as a table in Fig. S15. Blue: 405 nm laser for Hoechst 33342; Red: 543 nm laser for P2. The experiment followed **Experimental Methods 2.20.**



diffuse (nm)	speckle (nm)	diffuse (nm)	speckle (nm)	diffuse (nm)	speckle (nm)
615	611	615	612	615	612
615	613	617	614	618	612
616	612	615	611	618	609
616	610	616	614	616	610

Fig. S23. λ-Deconvoluted fluorescence imaging to compared polarity inside soluble oligomers and insoluble aggregated SOD1(G85R)-Halo upon treatment of MG132. Both aggregated and diffuse fluorescence regions have been picked. The measured regions were highlighted by white circles, and results of emission wavelength were listed under corresponding images. Blue: 405 nm laser for Hoechst 33342; Red: 543 nm laser for P2. The experiment followed **Experimental Methods 2.20.**

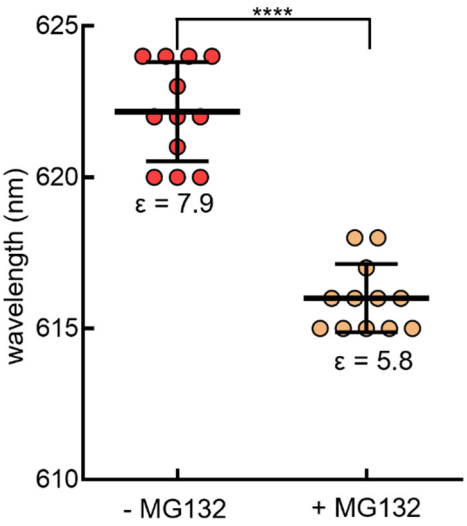


Fig. S24. Detailed quantification of polarity within folded and aggregated SOD1 proteins in HEK293T cells via λ-imaging method. Quantitative comparison of the folded (-MG132) and aggregated (+MG132) SOD1-G85R-Halo in live cells. As shown in statistical analysis, aggregated SOD1 exhibited hypsochromic shift of emission wavelength, indicating lower polarity inside aggregation. Statistical analysis was performed using GraphPad Prism software (n = 12, mean ± SD, ****P < 0.0001)

2. Experimental Methods.

2.1. Measurements of fluorescence spectra and solvatochromic properties (Fig. 2 b-e, Fig. 4 e, Fig. S1-S3, Table S1)

For the measurement of solvatochromism, A1, A2, A3 and 3' (the initial ester of P2) from a 1 mM DMSO stock solution was diluted to 20 μM in anhydrous solvents as listed in **Table 1**. 100 μL of each sample was pipetted into a BeyoGold™ 96-Well Black Opaque plate. Fluorescence spectra were collected using a Tecan Spark Fluorescence Plate Reader. Maximal λ_{abs} was selected as excitation wavelength. Each spectrum was normalized against its maximal fluorescence intensity. All measurements were repeated three times.

2.2. Measurement of the fluorescent intensity of P1 among solvents with different volume fraction of MeOH (Fig. S4)

A1 (1 mM in DMSO) was diluted to 20 μM into solvents with different volume fraction of MeOH (0%, 20%, 40%, 60%, 80%, 100%) in DMF, DMSO and ACN. 100 μL of each sample was pipetted into a BeyoGold™ 96-Well Black Opaque plate. Maximum of λ_{abs} was selected as the λ_{ex}. All measurements were carried out using Tecan Spark Fluorescence Plate Reader in NEST™ 96-Well flat bottom transparent plates and repeated for three times.

2.3. Measurement of the extinction coefficient of A1, A2 and A3. (Fig. S5)

A1, A2 and A3 (1 mM in DMSO) was diluted to 20 μM into the tested solvents respectively. The absorbance of background was deducted by the same solvent with 20 μL DMSO. The measurements were carried out using 1cm quartz cuvette and UV-Vis Spectrometer (METASH UV-5100) at 500 nm. The extinction coefficient (ε) was calculated according to the Beer-Lambert Law:

$$A = \varepsilon bc$$

2.4. Measurement of quantum yield (Table S1)

Quantum yield measurement followed the previous published protocol [1]. Nile blue (Φ = 0.27 in ethanol) was selected as the reference to measure the quantum yield of A1, A2 and A3 in all solvents. Nile blue was diluted with ethanol to 1, 2, 4, 8 μM to measure absorbance and emission intensity, and A1, A2 and A3 were diluted to the same concentrations as Nile blue.

UV-vis absorption and fluorescence emission spectra were obtained by using Tecan Spark Plate Reader with NEST 96 flat bottom transparent plate. All emission spectra were measured by applying 500 nm as excitation wavelength.

The quantum yield was calculated based on the equation.

$$\Phi = \Phi_{ST} \left(\frac{\mathrm{Grad_X}}{\mathrm{Grad_{ST}}}\right) \times \left(\frac{\eta_X}{\eta_{ST}}\right)^2$$

The $\mathrm{Grad_x}$ and $\mathrm{Grad_{ST}}$ were obtained by its integrated fluorescence emission peak area/absorbance respectively. η is the refractive index of solvent at 25 °C. All of the absorbance and fluorescent background was deducted for the correspondent solvents.

2.5. Confirmation of insensitivity of P1 and P2 toward viscosity and pH (Fig. S6, Fig. S14)

P1 and P2 from 1 mM DMSO stock solutions were diluted to 20 μM in solvents with different volume fraction of glycerol and MeOH. 100 μL of each solution was pipetted into a BeyoGold™ 96-Well bottom transparent plate. Fluorescence spectra were collected using a Tecan Spark Fluorescence Plate Reader. Maximal λ_{ex} was picked as the maximum of λ_{abs}. Each spectrum was normalized against its maximal fluorescence intensity. And all measurements were repeated for three times and averaged.

Purified SOD1(V31A)-Halo (36 μM), P1 (20 μM), EDTA disodium salt (12.5 mM) were mixed with buffer A (50 mM Tris-HCl, 100 mM NaCl, acidified by HCl to pH = 8.00), PBS (pH = 7.40), acid aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) respectively and incubated at room temperature for 5 min. 100 μL of each solution was pipetted into a BeyoGold™ 96-Well bottom transparent plate. Fluorescence spectra were collected using a Tecan Spark Fluorescence Plate Reader. Maximal λ_{abs} was selected as excitation wavelength. Each spectrum was normalized against its maximal fluorescence intensity. And all measurements were repeated for three times.

2.6. Plasmids construct and protein purifications

Genes of SOD1(V31A)-Halo and *E. coli* wild type HaloTag were first codon optimized, synthesized by GenScript, Nanjing, China, and sub-cloned into pET-29b(+) vectors. All proteins were cloned with His-tag at the C-termini for easy purification purpose for convenient purification.

Protein plasmids were transformed into BL21 DE3 *E. coli* cells. Cells were grown to OD_{600} in the range of 0.6-0.8 before induced by IPTG (0.5 mM) at 18 °C for 16 h (SOD1(V31A)-Halo (WT-HaloTag at 37 °C for 4 h). Cultured cells were harvested and resuspended in buffer A (50 mM Tris•HCl,100 mM NaCl, pH = 8.0). Cells expressing recombinant proteins were thawed and lysed by sonication under the iced condition. Lysed cells were centrifuged for 30 min at 30,700 × g. The supernatant was collected and loaded into a 10 mL Ni-NTA column, and then eluted with buffer A (50 mM Tris•HCl,100 mM NaCl, pH = 8.0). Proteins were then eluted by gradient increase of imidazole-containing Buffer B (50 mM Tris • HCl 100 mM NaCl, 500 mM imidazole, pH = 8). The protein fractions were identified by SDS-PAGE analysis, pooled, and concentrated. Proteins purified by Ni-NTA column 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, acidified by HCl to pH = 7.40). The protein containing fractions were identified by SDS-PAGE gel analysis then pooled and concentrated. No significant impurities were identified and the purity was estimated to be 98% based on SDS-PAGE gel.

2.7. Evaluating the inherent affinity of A2 to POI (Fig. S8)

Purified SOD1(V31A)-Halo (36 μM), A2 (20 μM) and EDTA disodium salt (12.5 mM) was mixed with buffer A. The mixtures were incubated at room temperature for 5 min (sample I) and then incubated at 95 °C for another 5 min (sample II). The incubated mixtures (100 μL) were pipetted into BeyoGold™ 96-Well bottom transparent plates. Fluorescent intensity and λ_{em} were recorded using Tecan Spark Plate Reader (λ_{ex} = 550 nm for sample I and 565 nm for sample II). Error bars: standard error (n = 3).

2.8. Covalent conjugation between P1 and POI visualized via SDS-PAGE electrophoresis (Fig. S7)

Purified SOD1(V31A)-Halo (50 μM), P1 (25 μM) were mixed with buffer A (50 mM Tris-HCl, 100 mM NaCl, acidified by HCl to pH = 8.00), 1× PBS buffer (pH = 7.40), acid aggregation buffer (NaOAc 200 mM, KCl 100 mM, acidified by AcOH to pH = 6.23) respectively and incubated at room temperature for 5 min. Next, 40 μL conjugated mixture and 10 μL 5× loading buffer were mixed and heated at 95°C

for 5 min. The prepared samples were loaded into 12% acrylamide SDS-PAGE gel, and the samples were analysis under 200 mV voltage.

2.9. Temperature-dependent and time-dependent aggregation of SOD1(V31A)-Halo monitoring via λ_{em} of P1 (Fig. 3 d, f)

To measure the fluorescence thermal shift curve, purified SOD1(V31A)-Halo (36 μM), P1 (20 μM), EDTA disodium salt (12.5 mM) were mixed with buffer A (50 mM Tris-HCl, 100 mM NaCl, acidified by HCl to pH = 8.00) to 150 μL. The mixture was incubated at ambient temperature for 5 min, and then incubated at varying temperatures for 5 min. The prepared mixtures (100 μL) were pipetted into BeyoGold™ 96-Well bottom transparent plates and λ_{em} were recorded using Tecan Spark Plate Reader (λ_{ex} = 580 nm). Error bars: standard error (n = 3).

To measure the time-dependent protein aggregation, purified SOD1(V31A)-Halo (36 μM) P1 (20 μM) and EDTA disodium salt (12.5 mM) were mixed with buffer A (50 mM Tris-HCl, 100 mM NaCl, acidified by HCl to pH = 8.00) to 150 μL and incubated at room temperature for 5 min and then incubated under constant temperatures (53 °C and 60 °C) for different time period. The incubated mixtures (100 μL) were pipetted into BeyoGold™ 96-Well bottom transparent plates and λ_{em} were recorded using Tecan Spark Plate Reader (λ_{ex} = 580 nm). Error bars: standard error (n = 3).

2.10. Analysis of thermal shift data[1]

The fluorescence thermal shift assay data from P1 were fitted to Eq. (1) to obtain ΔH_u, ΔC_{pu}, and T_m by nonlinear curve fitting using the program OriginPro 2015:

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

where F(T) is the fluorescence intensity at certain 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; ΔH_u is the enthalpy of protein aggregation; ΔC_{pu} is the heat capacity change upon protein aggregation.

2.11. Monitoring degradation process using P1 probe (Fig. 3e)

Purified SOD1(V31A)-Halo (36 μM), P1 (20 μM), EDTA disodium salt (12.5 mM) were mixed with buffer A (50 mM Tris-HCl, 100 mM NaCl, acidified by HCl to pH = 8.00) and incubated under room temperature for 5 min. Then the mixture was incubated at 95 °C for 5 min to get aggregated. The mixture was cooled down to 30 °C, and then 0.01 mg/L Proteinase K was added. The incubated mixtures (100 μL) were pipetted into BeyoGold™ 96-Well bottom transparent plates. λ_{em} of sample was recorded every 5 min until a plateau by using Tecan Spark Plate Reader (λ_{ex} = 580 nm). Error bars: standard error (n = 3).

2.12. Identification of protein aggregation via λ_{em} shift (Fig. 3c, Fig. S13)

P1: Purified SOD1(V31A)-Halo (36 μM), P1 (20 μM) and EDTA disodium salt (12.5 mM) was mixed with buffer A (50 mM Tris-HCl, 100 mM NaCl, acidified by HCl to pH = 8.00) and incubated at room temperature (sample I) and then at 95 °C (sample II) for 5 min. The incubated mixtures (100 μL) were pipetted into BeyoGold™ 96-Well bottom transparent plates and λ_{em} was recorded using Tecan

Spark Plate Reader (λ_{ex} = 580 nm for sample I and 550 nm for sample II). All measurements were repeated for 3 times.

P2: Purified SOD1(V31A)-Halo (36 μM), P2 (20 μM) and EDTA disodium salt (12.5 mM) was mixed with buffer A (50 mM Tris-HCl, 100 mM NaCl, acidified by HCl to pH = 8.00) and incubated at room temperature (sample I) and then at 95 °C (sample II) for 5 min. The incubated mixtures (100 μL) were pipetted into BeyoGold™ 96-Well bottom transparent plates and λ_{em} was recorded using Tecan Spark Plate Reader (λ_{ex} = 550 nm for sample I and 540 nm for sample II). All measurements were repeated for 3 times.

2.13. Comparison of folded and unfolded proteins via λ_{em} of conjugated solvatochromic probes (Fig. 3h, Fig. S9)

Purified SOD1(V31A)-Halo (36 μM) and probes (P1 or P2 20 μM) were mixed in 6 M urea (or Gnd·HCl) and buffer A (50 mM Tris-HCl, 100 mM NaCl, acidified by HCl to pH = 8.00) (control), respectively. The mixtures were incubated at 4 °C for 24 h. The incubated mixtures (100 μL) were then pipetted into BeyoGold™ 96-Well bottom transparent plates and λ_{em} was recorded using Tecan Spark Plate Reader (λ_{ex} = 580 nm for P1 and 550 nm for P2). Error bars: standard error (n = 3).

2.14. Aggregation monitored by OD_{330} turbidity assay (Fig. 3i)

Purified SOD1(V31A)-Halo (36 μM) and EDTA disodium salt (12.5 mM) was mixed with buffer A (50 mM Tris-HCl, 100 mM NaCl, acidified by HCl to pH = 8.00) and incubated at different temperature (30°C, 40°C, 48°C, 57°C, 66°C, 73.5°C, 75°C, 78°C, 85°C, 91°C, 93°C) for 5 min. Turbidity was recorded at 330 nm using a Metash-5100 UV-vis spectrophotometer. Error bars: standard error (n = 3).

2.15. Resistance to precipitation from H_2O (Fig. 4b, Fig. S11)

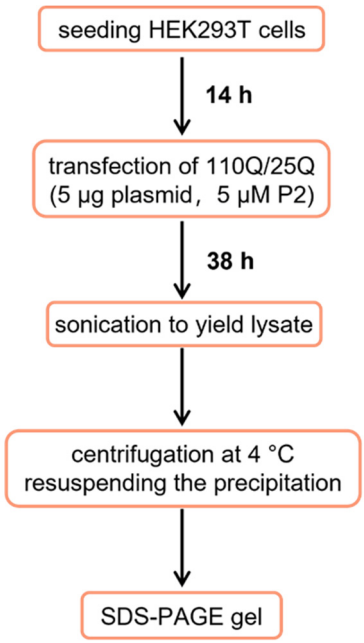
P1 and P2 (100 μM) were dissolved in deionized water (5 mL) and kept at room temperature. At indicated time points, clear supernatant was pipetted into NEST 96 flat bottom transparent plates. The absorption spectra were collected on Tecan Spark Fluorescence Plate Reader. For each time point, the experiment was repeated three times.

The concentration of P1 and P2 were halved and repeat the experiment.

2.16. MTT assay for P1 and P2 (Fig. S10)

The effect of P1-Halo probe on the cell viability was determined by MTT assay. The HEK293T cells ($1X10^4$/well in 200 μL medium) were seeded into 96-well plate and incubated for 24 h. After treatment with P1 or P2 (0.0, 1.0, 2.5, 5.0, 10.0 μM) for 24 h, the viability of the HEK293T cells was detected with MTT. 20 μl of MTT solution (5 mg/ml in PBS (pH = 7.40)) was added to each well, and the mixtures were incubated for 4 h at 37 °C, 5% CO_2. Next, removed the medium containing MTT and added 150 μL DMSO to each well then shook for 10 min to make the crystal dissolve completely. The OD_{492} value of each sample was measured on a Tecan Spark Fluorescence Plate Reader. Error bars: standard error (n = 3).

2.17. SDS-PAGE of insoluble section of transfected cell lysate (Fig. S15)



HEK293T cells were seeded on 6-Well Cell Culture Plates and transiently transfected when the cell density reached 70%. In 250 μL opti-mem medium, 4 μL of lip3000 and plasmid DNA of *Htt*-25Q or *Htt*-110Q (5 μg of DNA for each transfection) was fully mixed at room temperature for 5 min (mixture I). In 250 μL opti-mem medium, 10 μL of P3000 as fully mixed at room temperature for 5 min (mixture II). Then mixture I and II were fully mixed at room temperature for 20 min to obtain mixture III. The mixture III and P2 probe (5 μM) were then dripped into the cell medium and allowed expressing for 38 h. The transfected cells were collected under 200 × g for 5 min at room temperature. The supernatant was abandoned, and the precipitation was re-suspended with 1 mL PBS (pH = 7.40). The resuspension was treated with sonication (30 W, 5min, repeated 4 times) to yield cell lysate. The cell lysate was centrifugated under 14000 × g to isolate insoluble part. The separated precipitation was re-suspended with 1 mL PBS (pH = 7.40) and 250 μL 5 × loading buffer. Finally, the resuspension was loaded into a 15 % acrylamide SDS-PAGE gel to run gel electrophoresis.

2.18. Procedure for cell culture, transfection of cells, and cell imaging experiments. (Fig. S16- S17)

HEK293T cells were seeded on 35 mm confocal culture dishes and transiently transfected when the cell density reached 70%.

For Fig. S16:

(a): In 100 μL opti-mem medium, 6 μL of X-tremegene 9 DNA transfection reagent (Roche) was added, and then plasmid of *Htt*-110Q (1 μg) and 1 μM coumarin-Halo linker were added. and the mixture was fully mixed at room temperature for 25 min in dark.

(b): In 100 μL opti-mem medium, 6 μL of X-tremegene 9 DNA transfection reagent (Roche) was added, and then plasmid of *Htt*-110Q (1 μg) and 1 μM P1 were added. and the mixture was fully mixed at room temperature for 25 min in dark.

(c): In 100 μL opti-mem medium, 6 μL of X-tremegene 9 DNA transfection reagent (Roche) was added, and then plasmid of *Htt*-110Q (2 μg) and 1 μM coumarin-Halo linker and 1μM P1 were added. and the mixture was fully mixed at room temperature for 25 min in dark.

For Fig. S17:

(a): In 100 μL opti-mem medium, 6 μL of X-tremegene 9 DNA transfection reagent (Roche) was added, and then plasmid of *Htt*-110Q (1 μg) and 1 μM coumarin-Halo linker were added. The mixture was fully mixed at room temperature for 25 min in dark.

(b): In 100 μL opti-mem medium, 6 μL of X-tremegene 9 DNA transfection reagent (Roche) was added, and then plasmid of *Htt*-110Q (1 μg) and 1 μM P2 were added. The mixture was fully mixed at room temperature for 25 min in dark.

(c): In 100 μL opti-mem medium, 6 μL of X-tremegene 9 DNA transfection reagent (Roche) was added, and then plasmid of *Htt*-110Q (2 μg) and 1 μM coumarin-Halo linker and 1 μM P2 were added. The mixture was fully mixed at room temperature for 25 min in dark.

Each abovementioned mixture was then dripped into the cell medium and expressed for 30 hours. Hoechst 33342 (1000×) was added to the cell medium 30 min before imaging. Images were collected using Olympus FV1000MPE. Blue: 405 nm laser, Hoechst 33342 and coumarin-Halo linker; Green: 488 nm laser, coumarin-Halo linker and P1; Red: 543 nm laser, P1 and P2.

The Pearson's coefficient was generated by ImageJ.

Note: The power of 488 nm laser used in **Fig. S16, Fig. S17** was doubled than in **Fig. S18, Fig. S19** to prove that both coumarin-Halo linker and P1 were able to be excited by 488 nm laser.

2.19. Fluorescence images to detect the background of probes in HEK293T cells (Fig. 4d, Fig. S12)

HEK293T cells were seeded at 30% confluency in 96-well plate. Cells were grown in DMEM media supplemented with 10% FBS and Penicillin-Streptomycin antibiotics until they reached 80% confluency. 5 μM Probe (P1 or P2) was introduced into the media and incubated with cells for 24 h. Fluorescence images were taken using Olympus® IX73 Research Inverted Microscope.

The signal-to-noise ratio was calculated by ImageJ.

2.20. Procedure for cell culture, transfection, and confocal fluorescence imaging (Fig. 4f-g, Fig. S18-S23)

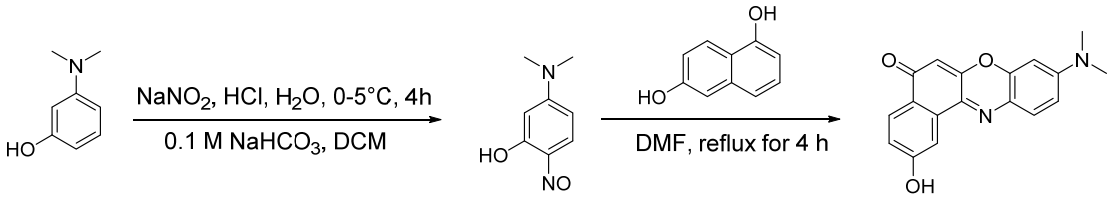
HEK293T cells were seeded on 35 mm confocal culture dishes and transiently transfected when the cell density reached 70 %.

For *Htt*-25Q and *Htt*-110Q: In 100 μL opti-mem medium, 6 μL of X-tremegene 9 DNA transfection reagent (Roche) was added, and then plasmid DNA of *Htt*-25Q or *Htt*-110Q (1 μg of DNA for each transfection) and the mixture was fully mixed at room temperature for 25 min in dark. The transfection mixture and probe P2 (1 μM) were then dripped into the cell medium. The experimental trial transfected with *Htt*-110Q-Halo was expressed for 30 hours, and the controlled trial transfected with *Htt*-25Q-Halo was expressed for 30 hours as well.

For SOD1-(G85R)-Halo ± MG132: In 100 μL opti-mem medium, 3 μL of X-treme Gene 9 DNA transfection reagent (Roche) was added, and then plasmid DNA (1 μg of DNA of SOD1-(G85R)-Halo), and the mixture was fully mixed at room temperature for 25 min. The mixture was then dripped into the cell medium, followed by addition of P2 (1 μM). Treated cells were incubated at 37 °C for protein expression. For the experimental, MG132 (2.5 μM) was added after protein expression for 24 hours, and then it was expressed for 24 more hours. The control group was incubated for the same total period (48 h) in the absence of MG132.

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 P2.

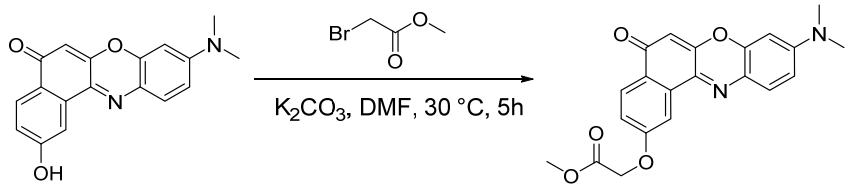
3. General Synthetic Procedures and Spectra [2,3].



1 2

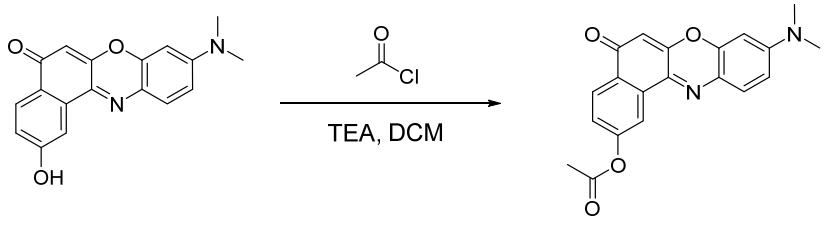
***Synthesis of compound 1*:** Followed previous work, dimethylaminophenol (2.5 g, 18.20 mmol) was dissolved in a mixture of 15 mL of HCl (conc.) and 2 mL of distilled water. The obtained solution was cooled to 0 °C under stirring and an aqueous solution of $NaNO_2$ (1.3 g, 18.20 mmol) was added. The reaction mixture was stirred for 4 h at 0–5 °C. The precipitated solid was isolated as a red-brown solid. The hydrochloride salt was subsequently converted to the free amine by diluting in 200 mL 0.1 M $NaHCO_3$ solution and extracting with DCM (3 × 200 mL). The organic fractions were combined and dried over anhydrous sodium sulfate. All solvent was removed under vacuum to obtain a brick-red solid (1.10 g, 36.4 %). ^{1}H-NMR (700 MHz, $(CD_3)_2SO$) δ 7.33 (d, J = 9.8 Hz, 1H), 6.97 (dd, J = 9.8 Hz, 2.5Hz, 1H), 7.76 (d, J = 2.5 Hz, 1H), 3.35 (s, 3H), 3.21 (s, 3H) ppm. HRMS (m/z) Anal. Calc'd for $C_8H_{10}N_2O_2$ $(M+H)^+$: 167.0815, Found $(M+H)^+$: 167.0831.

***Synthesis of compound 2 (A1)*:** Followed previous work, 1-Hydroxynaphthalene (0.8 g, 5.00 mmol) and compound 1 (1.0 g, 6.00 mmol) were dissolved in 100 mL anhydrous DMF and refluxed for 4 h. The solvent was then evaporated under reduced pressure and the yielded crude product was purified via flash chromatography (ethyl acetate/i-PrOH = 2: 1) to yield a reddish black powder (0.2 g, 13.0 %). ^{1}H-NMR (700 MHz, $(CD_3)_2SO$) δ 7.55 (d, J = 9.0 Hz, 1H), 6.88 (m, 1H), 6.72 (s, 1H), 6.39 (s, 1H), 6.28 (m, 3H), 3.06 (s, 6H) ppm. HRMS (m/z) Anal. Calc'd for $C_{18}H_{14}N_2O_3$ $(M+H)^+$: 307.1077, Found $(M+H)^+$: 307.1106.



2 3

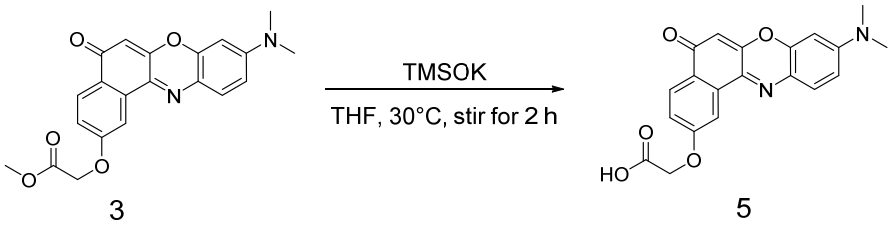
***Synthesis of compound 3 (A2)*:** Followed previous work, A mixture of compound 2 (100 mg, 0.33 mmol), potassium carbonate (450 mg, 3.26 mmol) and Methyl bromoacetate (152 μL, 1.63 mmol) in 40 mL anhydrous DMF was stirred under 30 °C for 5 h. Excess of potassium carbonate was washed off by DCM/H_2O and the organic solvent was removed under reduced pressure. The crude product was purified by column chromatography (DCM/MeOH = 10:1) to yield a reddish black powder (42 mg, 34.3 %). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ 8.10 (d, J = 8.7 Hz, 1H), 7.95 (s, 1H), 7.67 (d, J = 9.0 Hz, 1H), 7.35 (d, J = 8.7 Hz, 1H), 6.86 (d, J = 9.0 Hz, 1H), 6.67 (s, 1H), 6.23 (s,



2 4

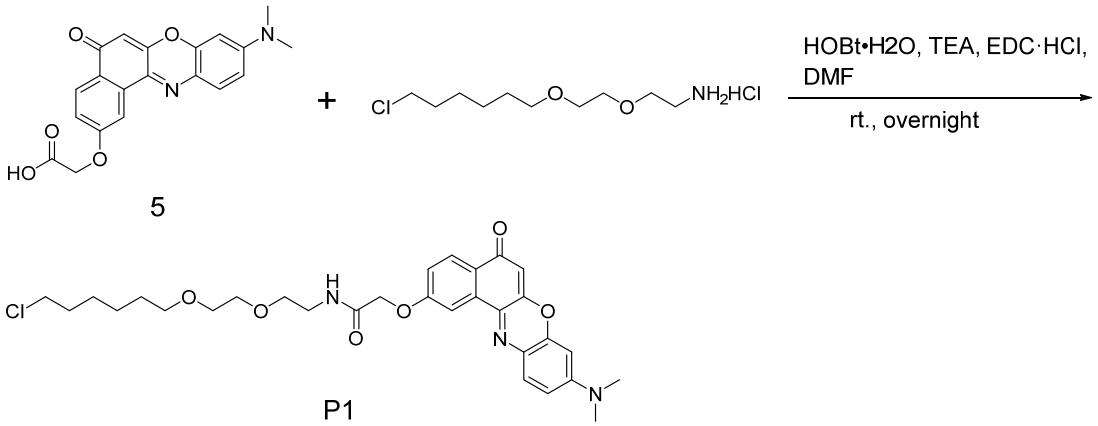
1H), 5.06 (s, 2H), 3.79 (s, 3H), 3.12 (s, 3H) ppm. HRMS (m/z) Anal. Calc'd for $C_{21}H_{18}N_2O_5$ $(M+H)^+$: 379.1288, Found $(M+H)^+$: 379.1290.

***Synthesis of compound 4 (A3)*:** Followed previous work, The compound 2 (48 mg, 0.16 mmol), acetyl chloride (16.7 μL, 0.24 mmol), and triethylamine (33 μL, 0.24 mmol) were dissolved in 10 mL anhydrous DCM at 0 °C. After stirring for 0.5 h, the mixture was warmed to room temperature and stirred for another 1 h. All solvent was evaporated via vacuum evaporation, and the crude product was washed by DCM/H_2O twice. Finally, the crude product was purified by column chromatography (DCM/MeOH = 10: 1) to yield a reddish black solid (7.0 mg, 12.7 %). ^{1}H-NMR (700 MHz, $(CD_3)_2SO$) δ 8.29 (s, 1H), 8.22 (d, J = 8.5 Hz, 1H), 7.70 (d, J = 9.0 Hz, 1H), 7.53 (dd, J = 8.5 Hz, 2.5 Hz, 1H), 6.92 (dd, J = 9.0 Hz, 2.5 Hz, 1H), 6.75 (s, 1H), 6.34 (s, 1H), 3.16 (s, 6H), 2.40 (s, 3H) ppm. HRMS (m/z) Anal. Calc'd for $C_{20}H_{16}N_2O_4$ $(M+H)^+$: 349.1183, Found $(M+H)^+$: 349.1185.



3 5

***Synthesis of compound 5*:** Followed previous work, The compound 3 (100 mg, 0.26 mmol) and potassium trimethylsilanolate (102mg, 0.79 mmol) were stirred in 20 mL anhydrous THF for 2 h under 30 °C. Then the solution was filtered, and the residue was washed by 20 mL cold THF. The residue was dissolved in 50 mL H_2O as a brown solution. The 1 M HCl was added to the aqueous solution to pH about 2~3, and then the product was extracted with DCM (3 × 200 mL). The combined DCM was dried over anhydrous Na_2SO_4 and then removed under reduced pressure to yield a red brown solid (24 mg, 25.4 %) without any further purification.

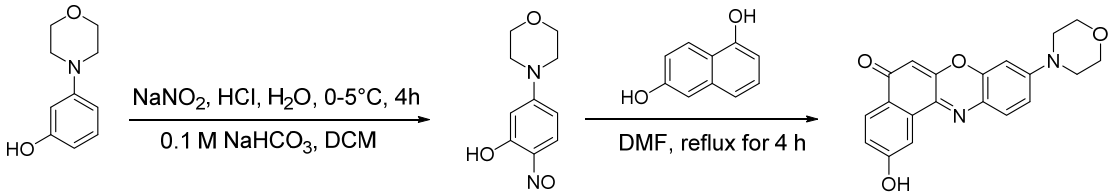


5

P1

Synthesis of compound 6 (P1) **:** The compound **5** (24 mg, 0.07 mmol) was dissolved in 1 mL anhydrous DMF. Halo linker (35 mg, 0.13 mmol), 1-Hydroxybenzotriazole hydrate (HOBt•H_2O) (31 mg, 0.20 mmol) and triethylamine (29 μL, 0.20 mmol) were introduced to the reaction mixture in one portion. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC•HCl) (39 mg, 0.20 mmol) was then added. The reaction mixture was stirred overnight under room temperature in dark. The reaction mixture was poured into 200 mL deionized water to quench reaction and extracted with 200 mL DCM. The organic fraction was dried over anhydrous Na_2SO_4 and

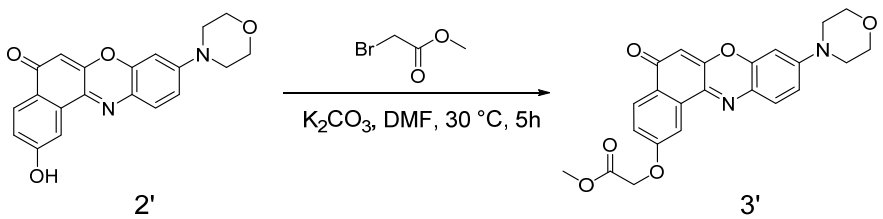
evaporated in vacuo. The crude product was further purified via flash chromatography (DCM/ ethyl acetate = 1: 1) to yield a red brown solid (15 mg, 40.1 %). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ 8.31 (t, *J* = 5.8 Hz, 1H), 8.12 (d, *J* = 8.5 Hz, 1H), 8.04 (s, 1H), 7.68 (m, 1H), 7.38 (d, *J* = 8.5 Hz, 1H), 6.89 (m, 1H), 6.72 (s, 1H), 6.25 (s, 1H), 4.73 (s, 2H), 3.63 (m, 3H), 3.52 (m, 5H), 3.47 (m, 3H), 3.14 (s, 6H), 1.71 (m, 2.34H), 1.52 (m, 4.14H), 1.39 (m, 2.23H) ppm. ^{13}C-NMR (176 MHz, $(CD_3)_2SO$) δ 182.4, 168.2, 161.2, 155.1, 154.0, 152.8, 147.6, 139.6, 133.3, 131.5, 128.2, 126.4, 125.1, 118.8, 111.3, 107.7, 104.8, 97.5, 71.1, 70.5, 70.3, 69.8, 68.0, 46.3, 39.3, 33.0, 30.00, 27.1, 25.8, 23.0, 14.9 ppm. HRMS (m/z) Anal. Calc'd for $C_{30}H_{36}ClN_3O_6$ $(M+H)^+$: 570.2365, Found $(M+H)^+$: 570.2316.



1' 2'

***Synthesis of compound 1'*:** $C_{10}H_{13}NO_2$ (3.0 g, 16.70 mmol) was dissolved in a mixture of 15 mL of concentrated HCl and 2 mL of distilled water. The obtained solution was cooled to 0 °C under stirring and an aqueous solution of $NaNO_2$ (1.2 g, 16.70 mmol) was added dropwise. The reaction mixture was stirred for 4 h under 0–5 °C. The precipitated solid was collected as orange solid. The hydrochloride salt was subsequently converted to the free amine by mixing in 200 mL 0.1 M $NaHCO_3$ solution and extracting with DCM (3 × 20 mL). The organic fractions were combined and dried over anhydrous Na_2SO_4. All solvent was evaporated in vacuum to yield brick-red solid as product (1.8 g, 51.5 %). ^{1}H-NMR (700 MHz, $(CD_3)_2SO$) δ 7.39 (s, 1H), 7.08 (s, 1H), 6.02 (s, 1H), 3.74 (s, 4H), 3.38 (s, 4H) ppm. HRMS (m/z) Anal. Calc'd for $C_{10}H_{12}N_2O_3$ $(M+H)^+$: 209.0921, Found $(M+H)^+$: 209.0933.

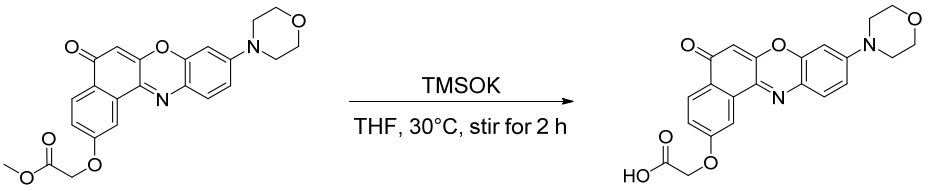
***Synthesis of compound 2'*:** 1-Hydroxynaphthalene (480 mg, 3.00 mmol) and 1' (647 mg, 3.60 mmol) were dissolved in 50 mL of DMF and refluxed for 4 h with Argon protection. The solvent was evaporated under reduced pressure and the crude solid was purified by flash chromatography (Ethyl Acetate/MeOH = 15: 1) to obtain pure product as brown-black powder (209 mg, 20.0 %). ^{1}H-NMR (700 MHz, $(CD_3)_2SO$) δ 7.60 (m, 1H), 7.12 (m, 1H), 7.01 (s, 1H), 6.43 (s, 2H), 6.39 (s, 1H), 6.32 (s, 1H), 3.79 (m, 4H), 3.37 (m, 4H) ppm. HRMS (m/z) Anal. Calc'd for $C_{20}H_{16}N_2O_4$ $(M+H)^+$: 349.1183, Found $(M+H)^+$: 349.1174.



2' 3'

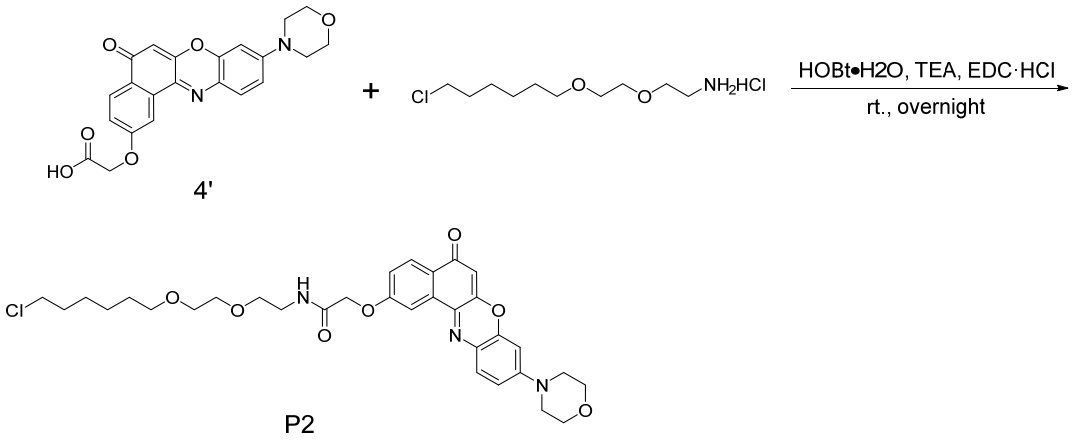
***Synthesis of compound 3'*:** A mixture of the naphthol (100 mg, 0.29 mmol), potassium carbonate (397 mg, 2.87 mmol) and ethyl 6-bromohexanoate (134 μL, 1.44 mmol) in 40 mL DMF was incubated under 30 °C for 5 h. The reaction mixture was washed with DCM/H_2O, and DCM

fractions were combined and dried with anhydrous Na_2SO_4. The DCM was removed in vacuum to yield crude product and then further purified by column chromatography (DCM/MeOH = 15:1) to yield desired product as red brown solid (41 mg, 34.3 %). ^{1}H-NMR (700 MHz, $(CD_3)_2SO$) δ 8.11 (m, 1H), 7.99 (s, 1H), 7.73 (dd, *J* = 9.2 Hz, 2.6 Hz, 1H), 7.39 (m, 1H), 7.11 (d, *J* = 9.2 Hz, 1H), 7.00 (s, 1H), 6.28 (s, 1H), 5.08 (s, 2H), 3.75 (m, 4H), 3.50 (t, 4H) ppm. HRMS (m/z) Anal. Calc'd for $C_{23}H_{20}N_2O_6$ $(M+H)^+$: 421.1394, Found $(M+H)^+$: 421.1383.



3' 4'

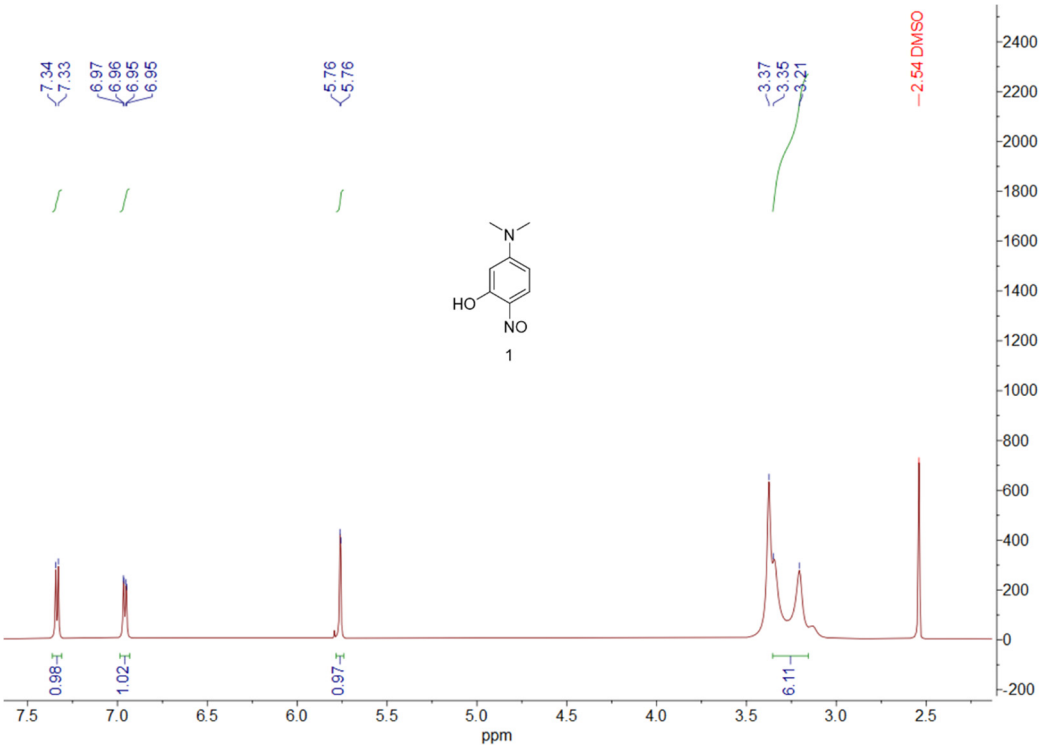
Synthesis of compound 4' **:** The compound 3' (41 mg, 0.13 mmol) and potassium trimethylsilanolate (51 mg, 0.40 mmol) were stirred in 20 mL anhydrous THF for 2 h under 30 °C. The reaction mixture was filtered, and the residue solid was washed with 20 mL cold THF. The residual solid was then dissolved in 50 mL H_2O and acidified with 1 M HCl to pH = 2~3. The liquid mixture was extracted with 200 mL DCM and dried with Na_2SO_4. The solvent was removed under reduced pressure to yield a red brown solid (23 mg, 57.0 %) without any further purification.



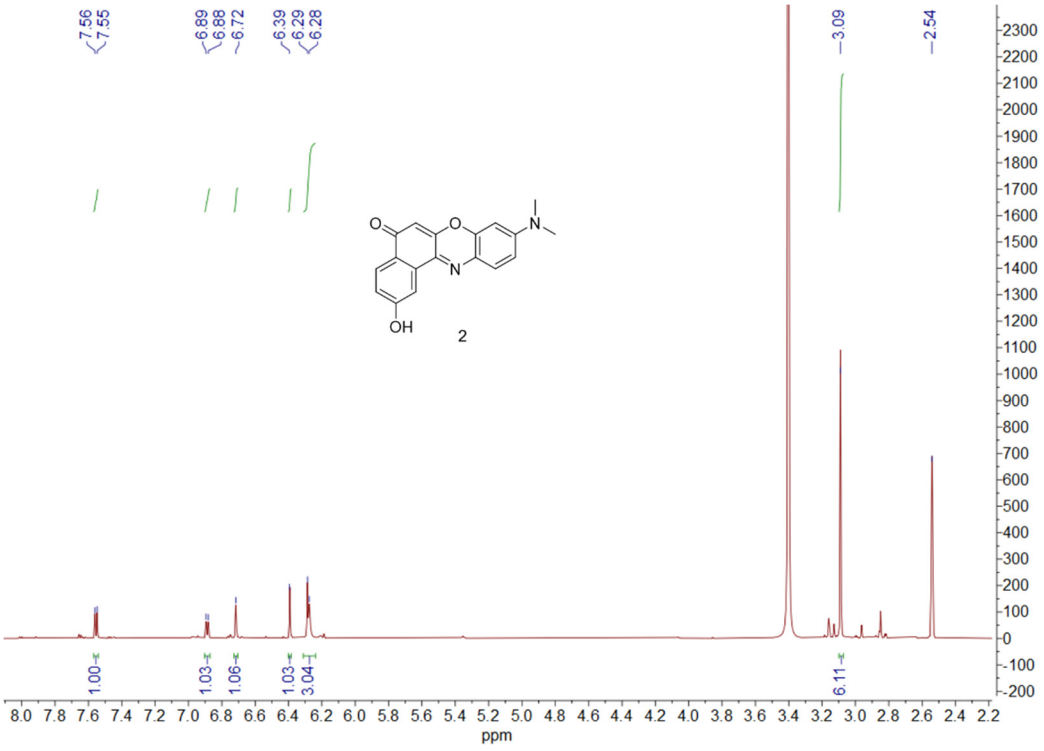
4' P2

Synthesis of compound 5'(P2) **:** The acid 4' (23 mg, 0.06 mmol) was dissolved in 1 mL anhydrous DMF. Halo linker (29 mg, 0.11 mmol), 1-Hydroxybenzotriazole hydrate (HOBt•H_2O) (26 mg, 0.169 mmol) and triethylamine (24 μL, 0.17 mmol) were introduced to the reaction mixture together. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC•HCl) (32.4 mg, 0.169mmol, 3 e.q.) was then added in one portion. The reaction mixture was stirred overnight under room temperature in dark. The reaction mixture was quenched with 100 mL deionized water and then extracted with 300 mL DCM. The organic fraction was dried in vacuo and the crude product was further purified by flash column chromatography (DCM/MeOH = 15:1) to yield red brown solid as final product (13 mg, 33.5 %). ^{1}H-NMR (700 MHz, $(CD_3)_2SO$) δ 8.31 (m, 1H), 8.11 (d, J = 8.7 Hz, 1H), 8.04 (d, J = 2.6 Hz, 1H), 7.69 (d, J = 9.0 Hz, 1H), 7.39 (dd, J = 8.7 Hz, 2.6 Hz, 1H), 7.09 (dd, J = 9.0 Hz, 2.6 Hz, 1H), 6.98 (d, J = 2.6 Hz, 1H), 6.23 (s, 1H), 4.73 (s, 2H), 3.79 (m, 4.42H), 3.62 (m, 2H), 3.53 (m, 4.33H), 3.47 (m, 6H), 3.36 (m, 3.31H), 1.71 (m, 2H), 1.50 (m, 2H), 1.39 (m,

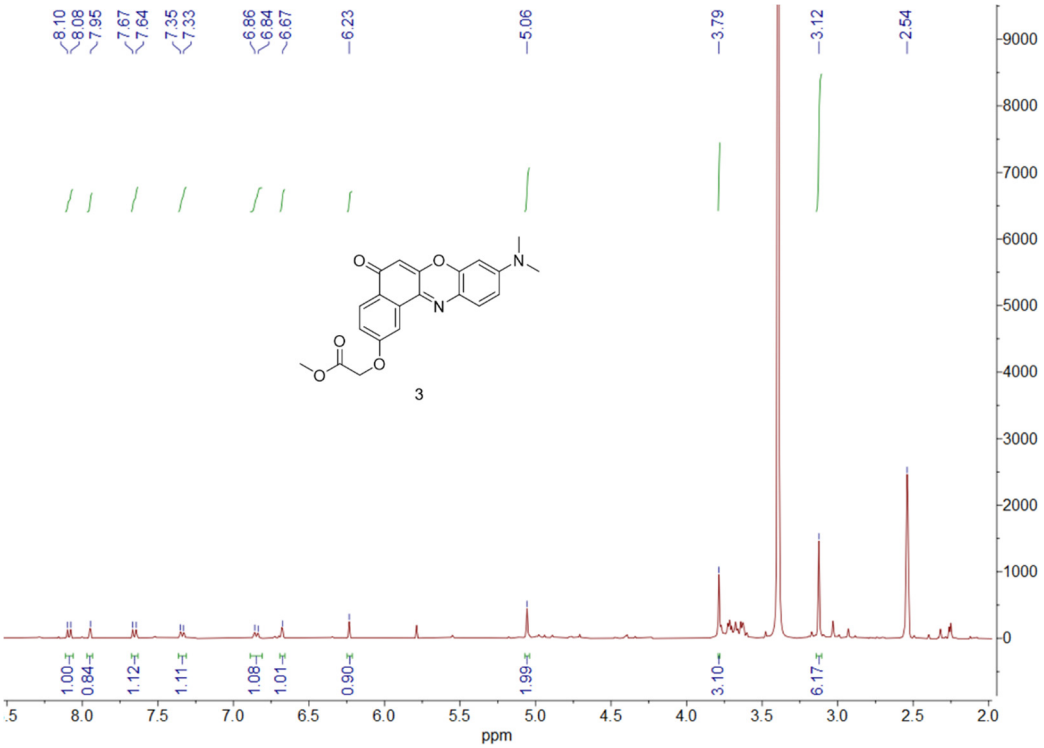
2.43H), 1.31 (m, 2.34H) ppm. ^{13}C-NMR (176 MHz, $(CD_3)_2SO$) δ 182.51, 168.09, 161.29, 154.37, 152.61, 146.77, 141.57, 134.21, 131.33, 128.29, 126.41, 126.12, 119.12, 112.59, 108.69, 105.68, 99.87, 71.13, 70.50, 70.33, 69.78, 67.96, 66.69, 47.74, 46.27, 40.95, 39.27, 32.94, 29.98, 27.05, 25.84 ppm. HRMS (m/z) Anal. Calc'd for $C_{32}H_{38}ClN_3O_7$ (M+H) $^+$: 612.2471, Found (M+H) $^+$: 612.2468.



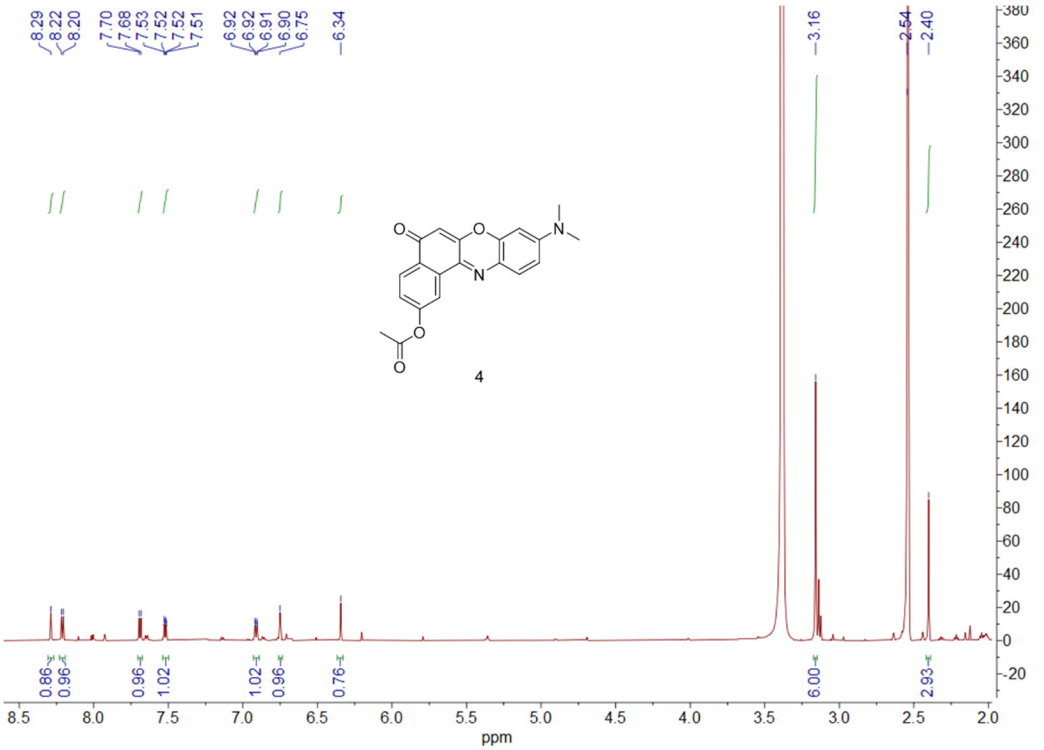
^{1}H NMR spectrum of **1** ($(CD_3)_2SO$)



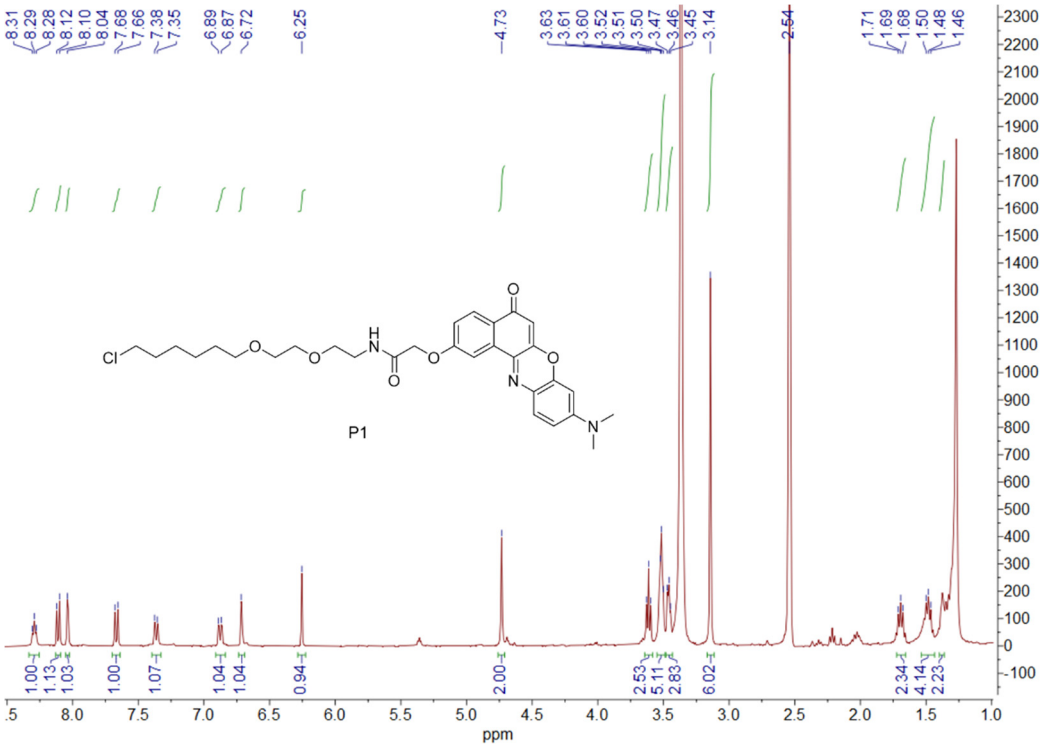
^{1}H NMR spectrum of **2** ($(CD_3)_2SO$)



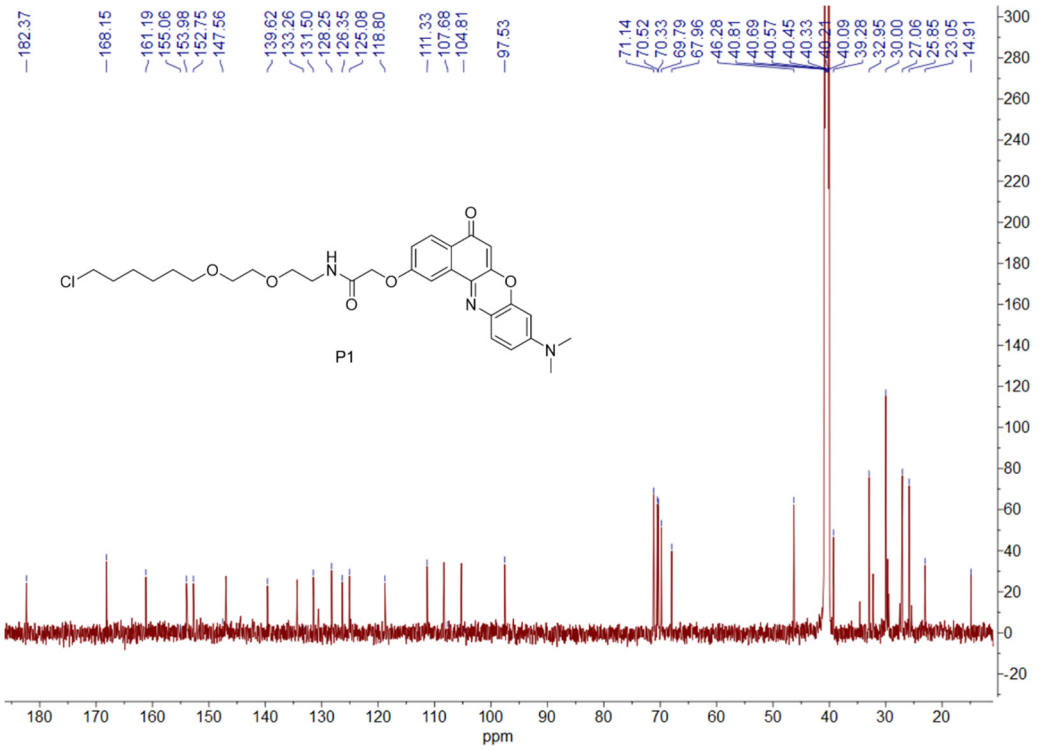
^{1}H NMR spectrum of **3** ($(CD_3)_2SO$)



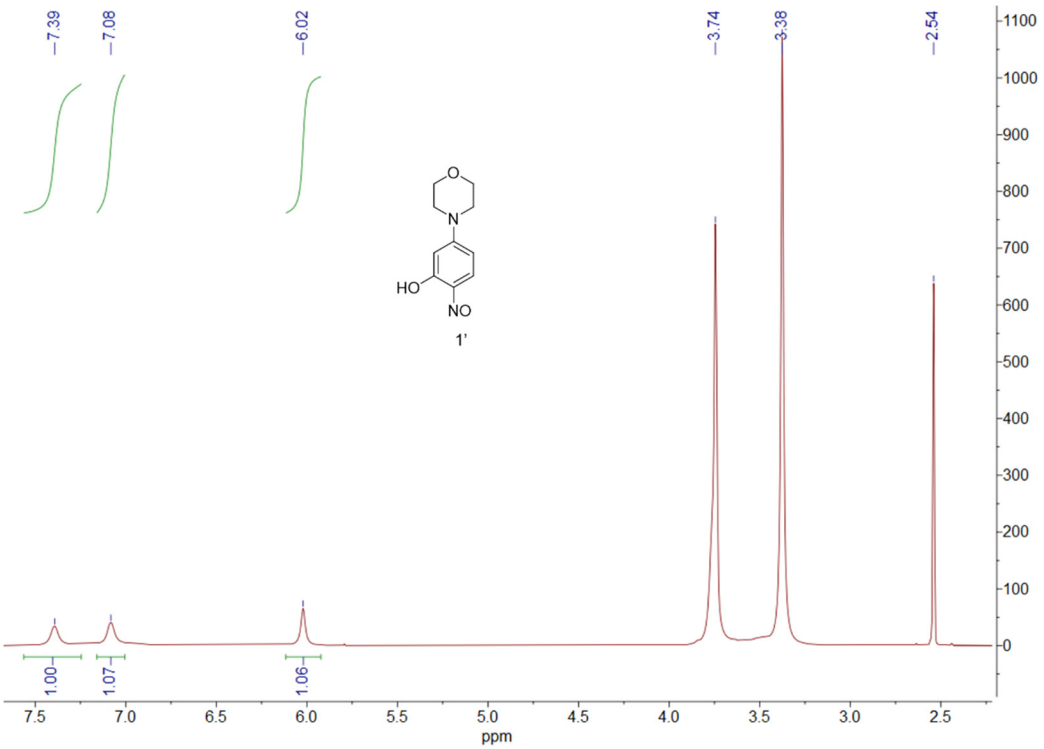
^{1}H NMR spectrum of **4** ($(CD_3)_2SO$)



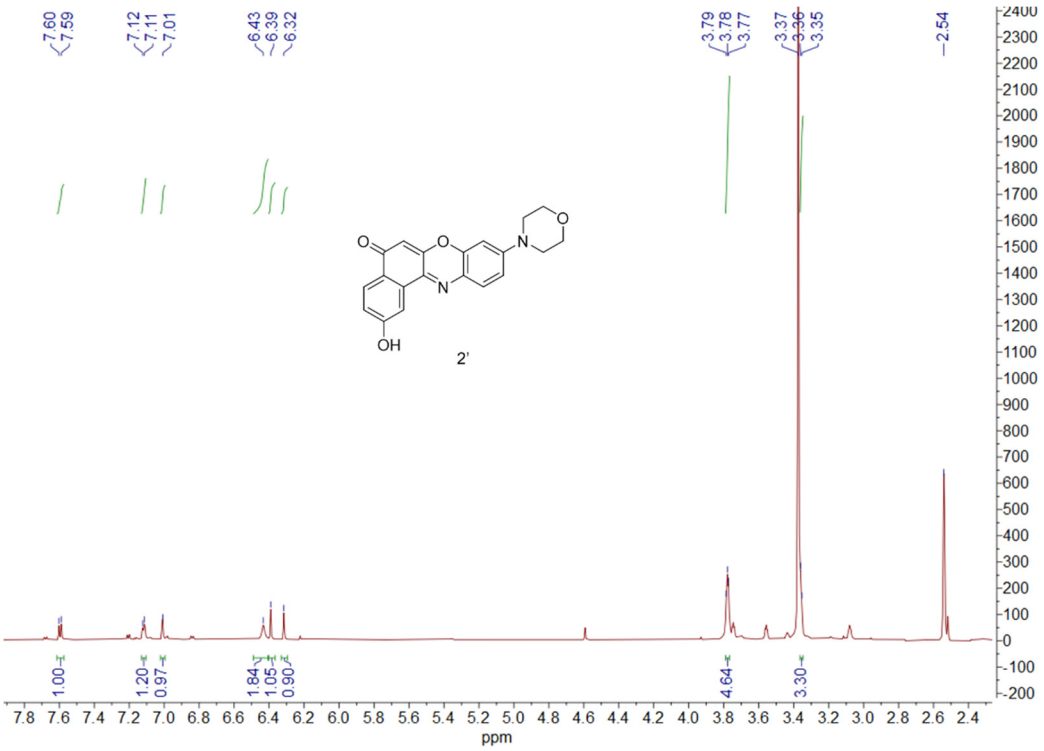
^{1}H NMR spectrum of **P1** ($(CD_3)_2SO$)



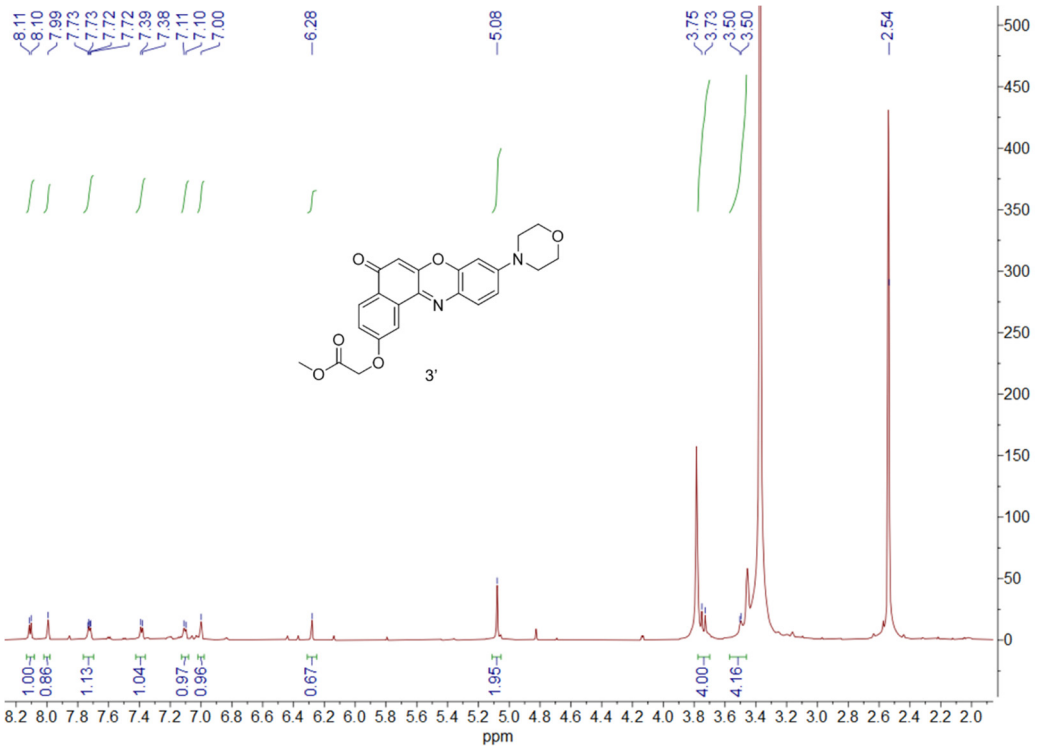
^{13}C NMR spectrum of **P1** ($(CD_3)_2SO$)



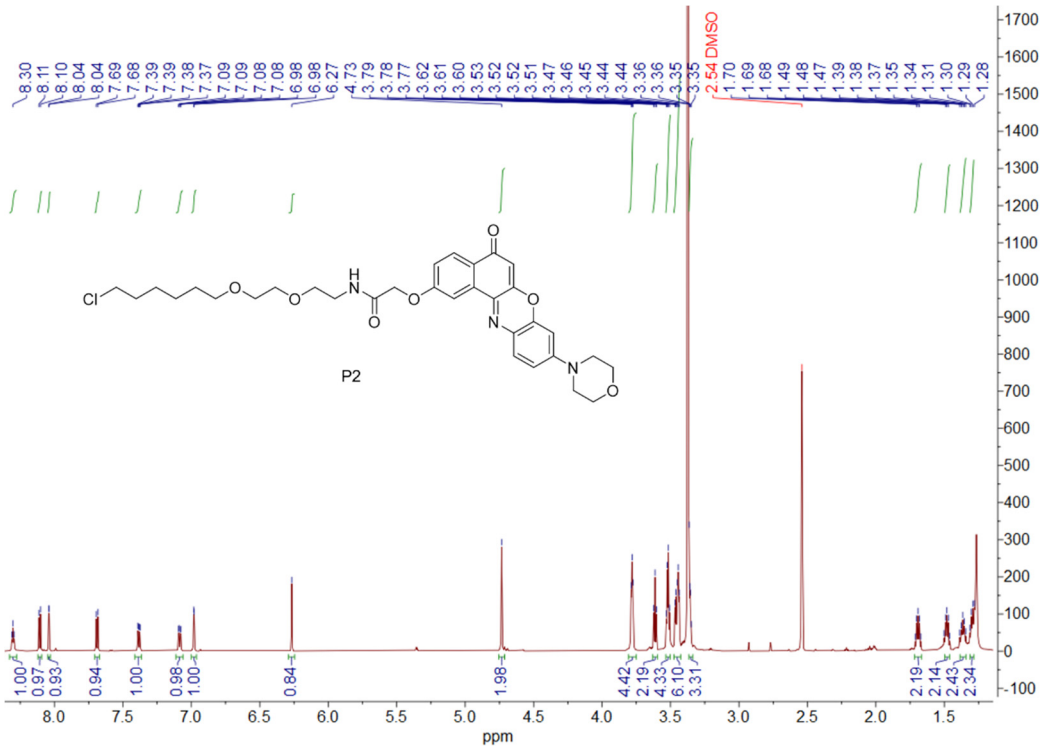
^{1}H NMR spectrum of **1'** ($(CD_3)_2SO$)



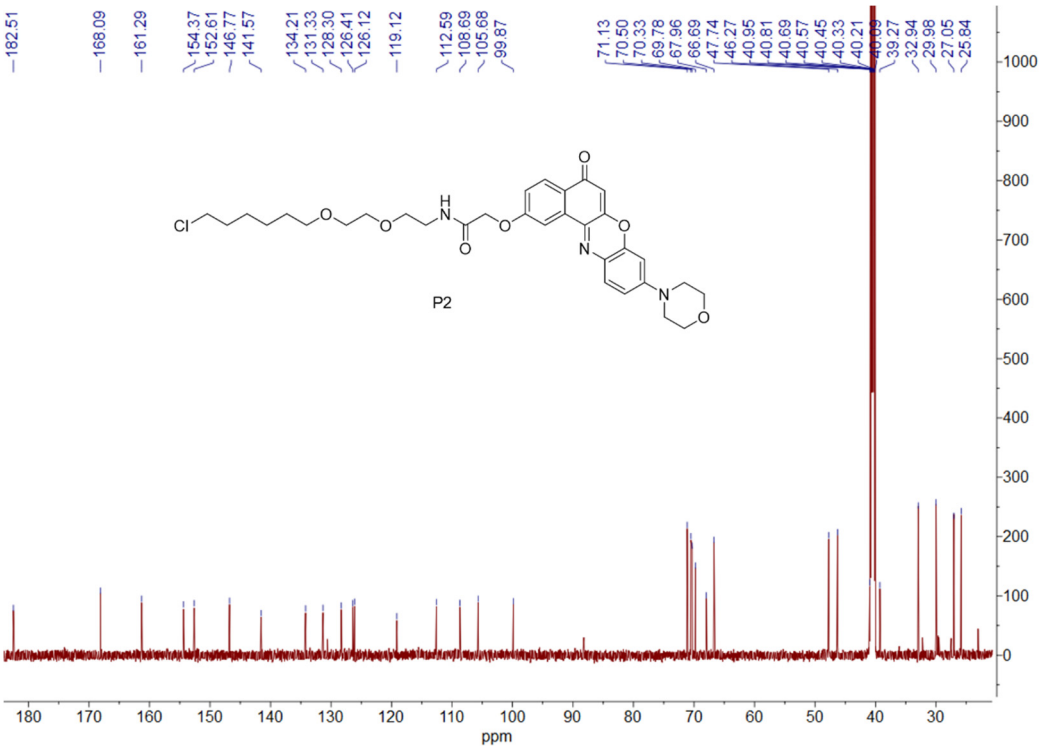
^{1}H NMR spectrum of **2'** ($(CD_3)_2SO$)



^{1}H NMR spectrum of **3'** ($(CD_3)_2SO$)



^{1}H NMR spectrum of **P2** ($(CD_3)_2SO$)



^{13}C NMR spectrum of **P2** ($(CD_3)_2SO$)

4. Supplementary References

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

[2] M. S. J. Briggs, I. Bruce, J. N. Miller, C. J. Moody, A. C. Simmonds and E. S. *J. Chem. Soc., Perkin Trans*. **1997,** 1, 1051-1058.

[3] M. Hornum*, M. W. Mulberg, M. Szomek, P. Reinholdt, J. R. Brewer, D. Wüstner, J. Kongsted, and P. Nielsen. *J. Org. Chem.* **2021**, 86, 1471–1488.