Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

SUPPORTING MATERIALS

Protein Labeling and Albumin Binding Characteristics Of The Near-IR Cy7 Fluor, QuatCy

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A. General Experimental Procedures and Methods

General Information

All reagents were purchased at a high commercial quality (typically 97 % or higher) and used without further purification. The compound **A**, **2A** and **3A** were synthesized according to the previously reported procedure. [1, 2] Bruker Avance III was used for recording NMR spectra (400 MHz for ^{1}H, and 100 MHz for ^{13}C) and was calibrated using residual non-deuterated solvent as an internal reference ($CDCl_3$: ^{1}H NMR = 7.24, ^{13}C NMR = 77.0, MeOD: ^{1}H NMR = 3.30, ^{13}C NMR = 49.0, DMSO-d_6: ^{1}H NMR = 2.50, ^{13}C NMR = 39.5). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, dd = double doublet, dt = double triplet, dq = double quartet, m = multiplet, br = broad. Electrospray ionization mass spectrometry (ESI-MS) data were collected on triple-stage quadrupole instrument in a positive mode. LC-MS analyses were collected from Agilent 1260 Infinity Quaternary LC and Agilent 6120 Quadrupole LC/MS modules using Poroshell 120 EC-C18 2.7 µM (4.6 x 50 mm) column in 5-95% CH_3CN/H_2O gradient with 0.1% formic acid over 10 minutes. Prep HPLC was performed on Agilent 1260 Infinity in 30-80% CH_3CN/H_2O gradient with 0.1% TFA over 20 mins. All statistical analyses were carried out by Graphpad Prism version 6.0 (Graphpad Software, La Jolla, CA, USA).

UV-Vis and Fluorescence Analysis

All compounds (**A**-**3A** and **1**-**3**) were prepare in 20 mM in DMSO as a stock solution. Compound **A**-**3A** and **1**-**3** were diluted by in 10 mM PBS buffer pH 7.24 to provide 5 µM final concentration. The absorbance and fluorescence spectra were performed by using a Varian Cary 100 UV-Vis spectrometer and Varian Cary Eclipse fluorescence spectrophotometer, respectively. The excitation wavelength for compounds was set as 730 nm for fluorescence measurement. The data were plotted using GraphPad Prism version 6.0 (GraphPad Software).

Selective Study of QuatCy (1) with Amino Acids in Aqueous Buffer

The solution of QuatCy **1** was diluted in 1 M HEPES buffer pH 8.00 (500 µL) to prepare a final concentration of 200 µM. Then, 300 µM of each amino acids (*N*-acetyl-*L*-Cys, *N*-acetyl-*L*-Tyr, N^{α}-acetyl-*L*-Lys, N^{ε}-acetyl-*L*-Lys, and *L*-proline) was added in the

solution of **1**. The reaction was shaken and incubated at 37 °C for a certain time and followed by HPLC at 600 nm.

Reactive and Competitive Studies of QuatCy (1) with Amino Acids in Aqueous Buffer

For reactive study, solution of QuatCy **1** and MHI-148 **A** was separately diluted in 1 M HEPES buffer pH 8.00 (500 µL) to prepare a final concentration of 200 µM. Then, 300 µM of *N*-acetyl-*L*-cysteine was added. The reaction was shaken and incubated at 37 °C for a certain time and monitored by HPLC at 600 nm. For competitive study, equimolar concentration (200 µM) of each QuatCy **1** and MHI-148 **A** mixed with 300 µM of *N*-acetyl-*L*-cysteine in 1 M HEPES buffer pH 8.00 (500 µL) was incubated and shaken at 37 °C for a certain time up to 6 h. The reaction was followed by HPLC at 600 nm.

UV Absorbance with HSA

1 µM of compounds was mixed with HSA (50 µM) in PBS (pH 7.24) at 37 °C for a certain time. Afterwards, absorbance was determined using a Cary-Varian 100 UV−vis NIR spectrophotometer.

NIR Gel Image Protocol

Different cyanines (20 mM stock in DMSO; 15 µM for HSA and 4 µM for vimentin) were incubated with equimolar concentration of proteins and thiol-blocked proteins in pH 7.24 HEPES buffer (50 mM) at 37 °C. The reaction was monitored up to 6 h. After that crude proteins of each reaction were treated under non-reducing condition and denatured with heating at 95 °C for 10 min. The denatured crude proteins were loaded into 10 or 15% SDS-PAGE for electrophoresis. The gel was washed with deionized water (10 min x 3 times), and analyzed by an Odyssey CLx Li-COR imager to detect the NIR fluorescence.

Preparation of Thiol-Blocked Vimentin and Thiol-Blocked HSA

Thiol-blocked vimentin was prepared in pH 8.0 HEPES buffer (50 mM) by incubating 6-maleimide-hexanoic acid (6-MA, 400 µM) with vimentin (1 µM) at 37 °C for 18 h. The thiol-blocked vimentin solution was directly used without further purification. In case of *Thiol-Blocked HSA* was prepared by reducing disulfide bonds of HSA (15 µM) in pH 8.0 HEPES buffer (50 mM) with incubation of tris(2-carboxyethyl)phosphine) (TCEP, 1.5

mM, 100-fold) for 2 h at 37 °C, and followed by incubation of 6-MA (6 mM) for another 18 h at 37 °C. The thiol-blocked HSA solution was directly used without further purification.

NIR Gel Image of QuatCy 1 and A with Different Type of Proteins

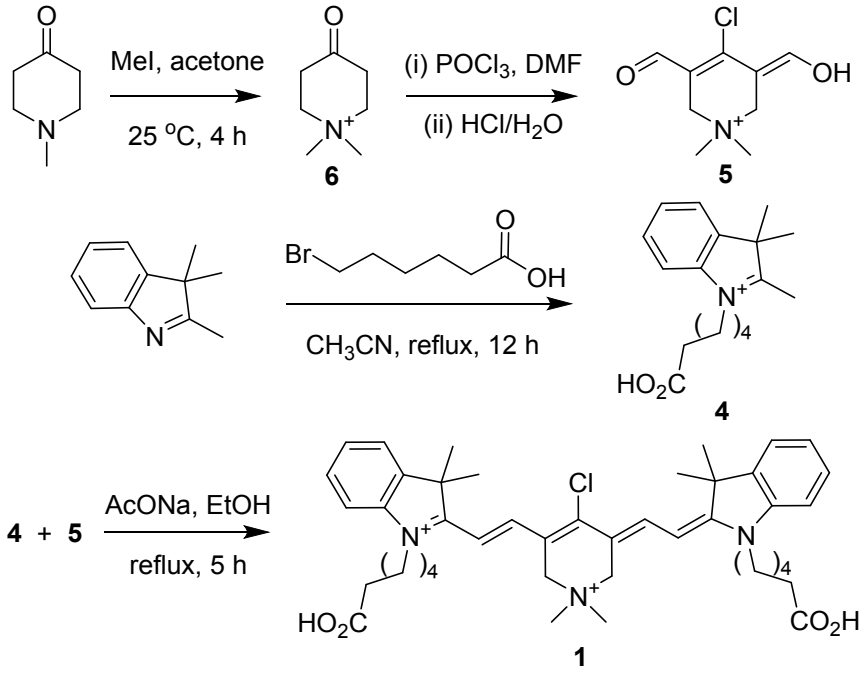
Each of the proteins (4 μM; NEDD8, Ubc12, truncated uPAR (residues 1-281), HSA, PCSK9, EGFR and Src) was incubated with QuatCy **1** (4 μM) in 50 mM HEPES buffer pH 8.0 at 37 °C for 6 h. After that crude proteins of each reaction were treated under non-reducing condition and denatured with heating at 95 °C for 10 min. Each of crude protein samples was loaded into 15% SDS-PAGE for electrophoresis and analyzed by an Odyssey CLx Li-COR imager to detect the NIR fluorescence.

In competition experiment different proteins (Ubc12, PCSK9, Src and vimentin) were mixed with HSA in 1:1 ratio (4 mM each) and incubated with **1** or **A** for 1.5 h at 37 ^{o}C in 50 mM HEPES at pH 8.0.

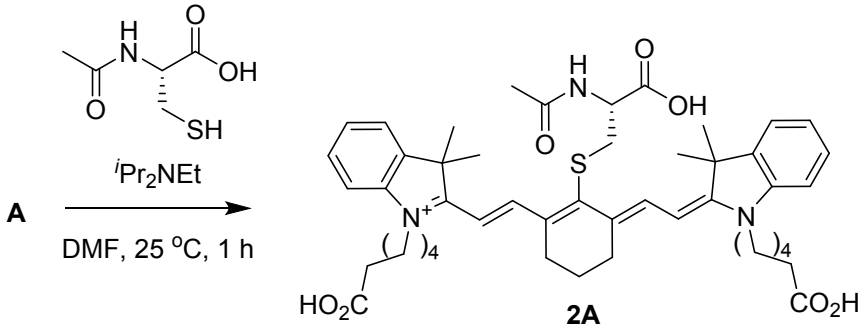
Stability of Vimentin bound QuatCy 1

Vimentin (15 uM) was incubated with QuatCy **1** (10 μM) in 10 mM PBS buffer pH 7.4 at 37 °C for 6 h. Glutathione (GSH) 0.5 mM was added to the protein bound **1** and the stability was measured over time on Agilent HPLC equipped with C4 column.

B. Synthetic Scheme



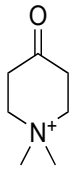
Scheme S1. Preparation of compound **1**.



Scheme S2. Preparation of compound **2A**.[1]

C. Synthetic Procedure and Characterizations

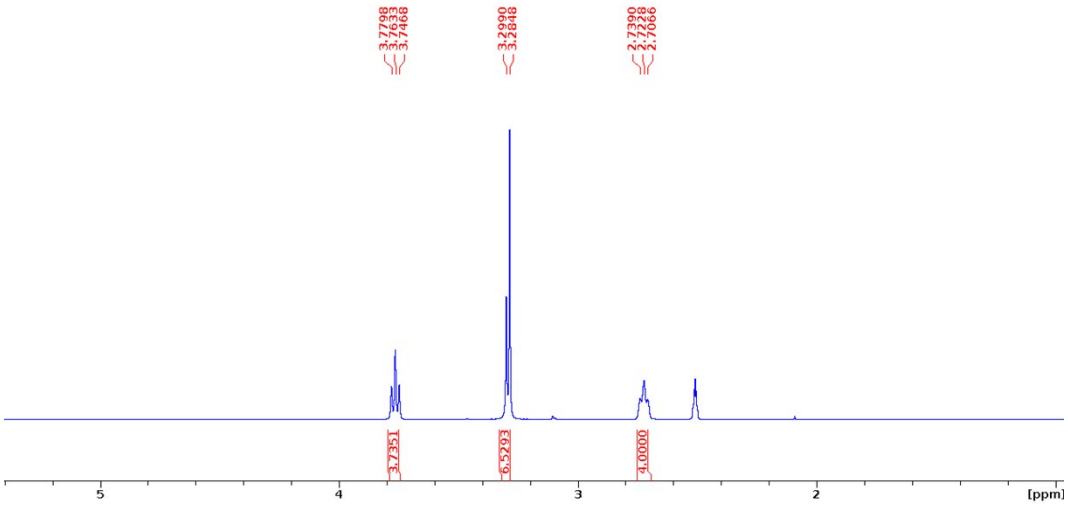
Synthesis of 1,1-dimethyl-4-oxopiperidin-1-ium iodide (**6**)



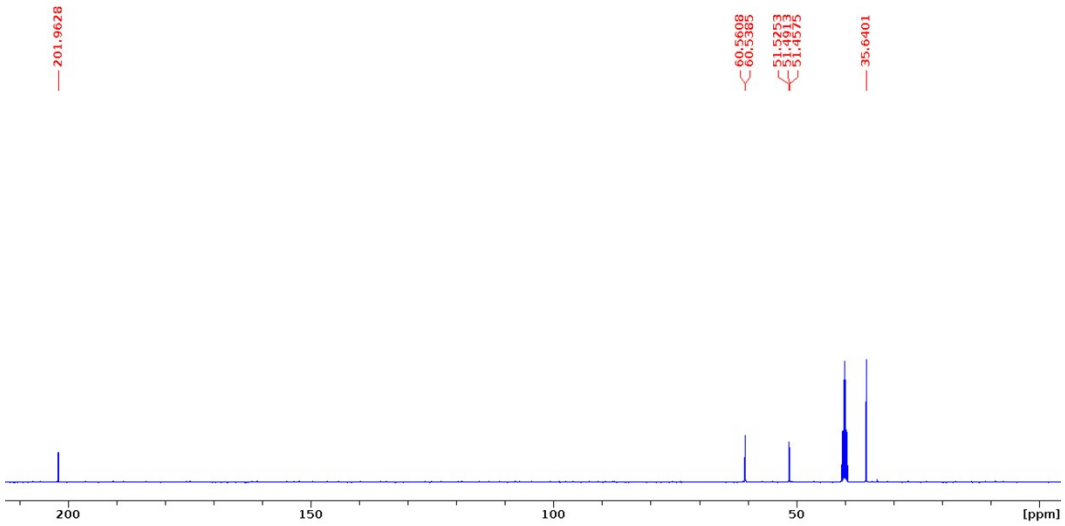
Ammonium salt compound was synthesized according to the previously reported procedure.[3] 1-methyl-4-piperidone (50.0 g, 0.44 mol) was dissolved in acetone (700 mL) and cooled down to 0 °C. Methyl iodide (60.0 mL, 0.96 mol) was slowly added into the solution over 10 minutes. Then, the mixture was stirred at room temperature for 4 h. Subsequently, the white precipitate was filtered and washed with cold acetone (200 mL) to give the white solid (110 g, 98% yield).

^{1}H NMR (400 MHz, DMSO-d6) δ 2.72 (t, *J* = 6.5 Hz, 4H), 3.30 (s, 6H), 3.76 (t, *J* = 6.6 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d6) δ 202.0, 60.6 (2C), 51.5(3C), 35.6.

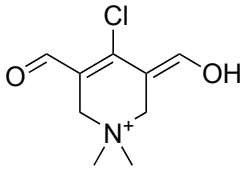
^{1}H NMR spectrum:



^{13}C NMR spectrum:



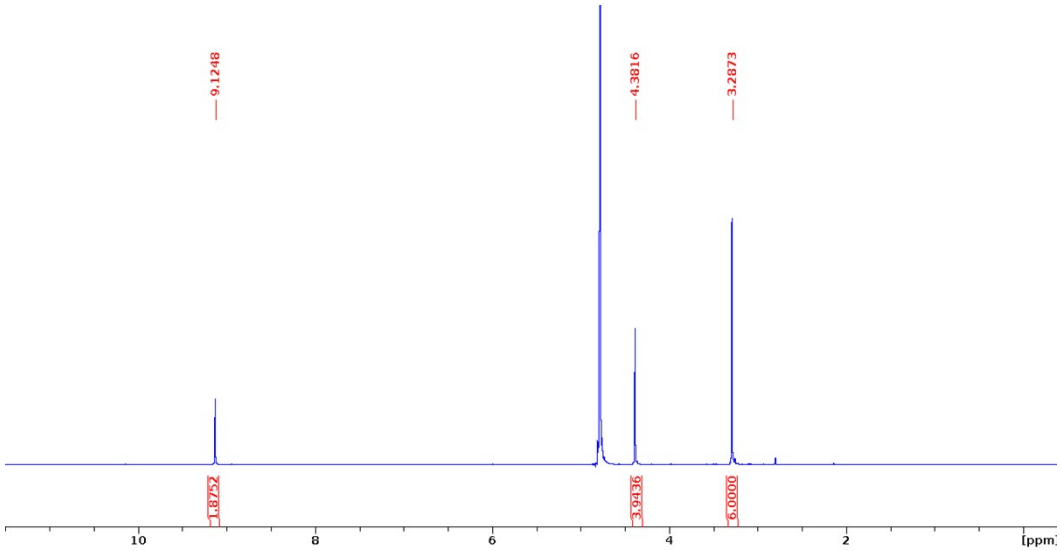
Synthesis of (*E*)-4-chloro-5-formyl-3-(hydroxymethylene)-1,1-dimethyl-1,2,3,6-tetrahydropyridin-1-ium (**5**)



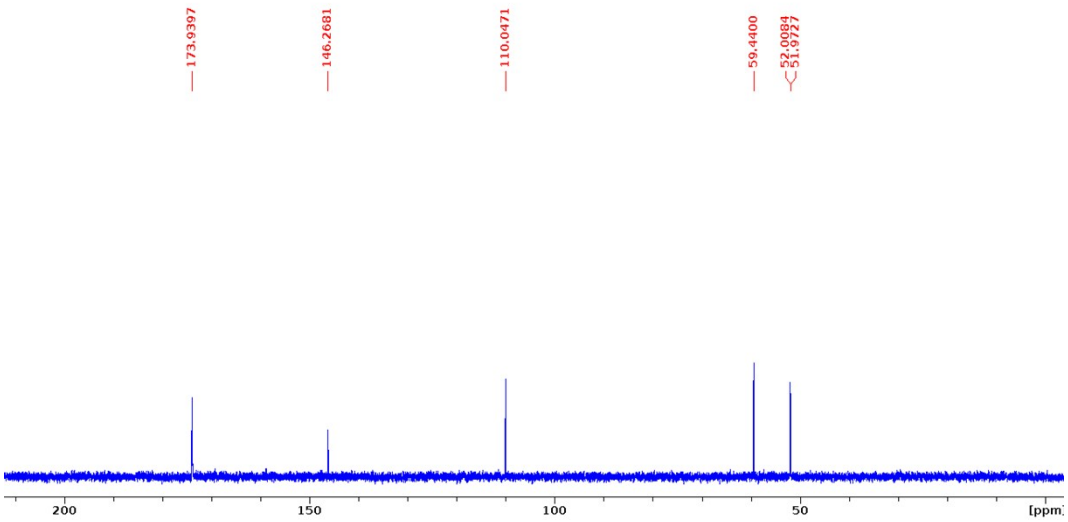
Compound **5** was prepared according to the literature method with some modifications.[4] DMF (9.00 mL, 0.12 mol) was cooled down at 0 ºC. Then, $POCl_3$ (5.50 mL, 0.06 mol) was slowly added and stirred at 0 ºC for 30 min. The 1,1-dimethyl-4-oxopiperidin-1-ium iodide (5.00 g, 0.02 mol) was added. The mixture solution was heated at 80 ºC for 3 h. After cooling down, the hydrochloric acid solution (20% v/v, 30.0 mL) was dropwise added and stirred at room temperature for 1 h. After that the mixture solution was sonicated for 20 min and allowed to stand in freezer overnight (-20 ºC). Consequently, the product was precipitated and filtered to obtain the dark yellow solid (1.45 g, 31% yield).

^{1}H NMR (400 MHz, D_2O) δ 9.12 (s, 2H), 4.38 (s, 4H), 3.29 (s, 6H); ^{13}C NMR (100 MHz, D_2O) δ 173.9, 146.3, 110.1, 59.4, 52.0, 52.0; HRMS (ESI) m/z for $C_9H_{13}ClNO_2^+$ calculated: 202.0629; found 202.0627.

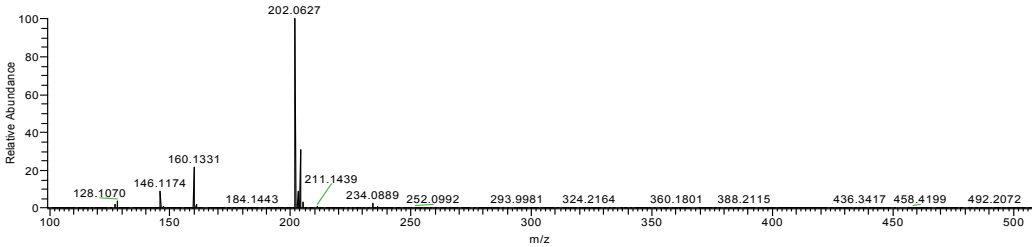
^{1}H NMR spectrum:



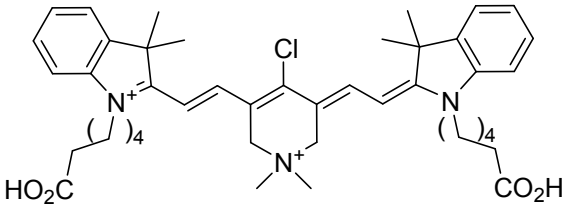
^{13}C NMR spectrum:



HRMS (ESI)



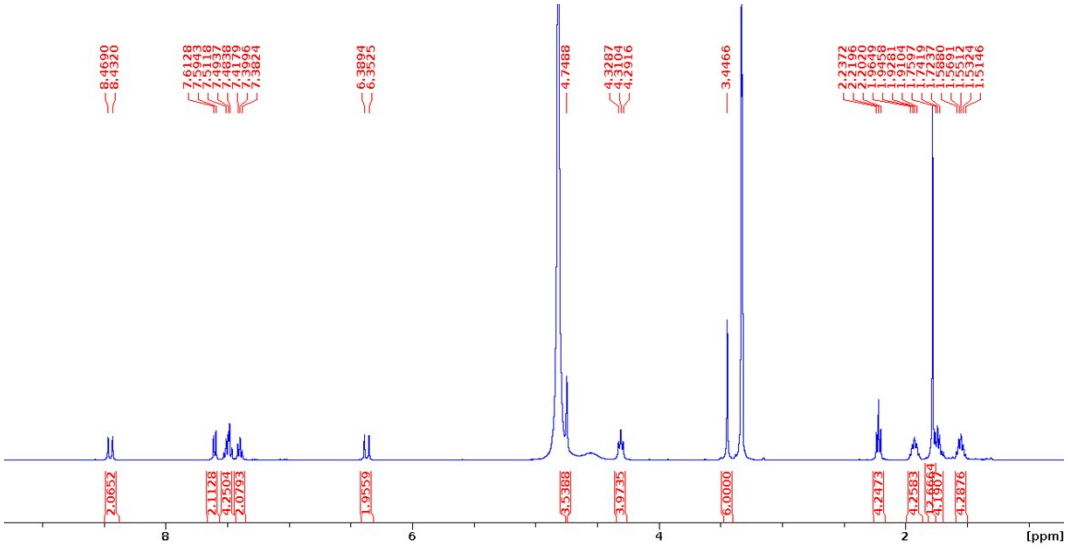
Synthesis of 1-(2-carboxyethyl)-2-((*E*)-2-((*E*)-5-(2-((*E*)-1-(2-carboxyethyl)-3,3-dimethylindolin-2-ylidene)ethylidene)-4-chloro-1,1-dimethyl-1,2,5,6-tetrahydropyridin-1-ium-3-yl)vinyl)-3,3-dimethyl-3*H*-indol-1-ium (**1**)



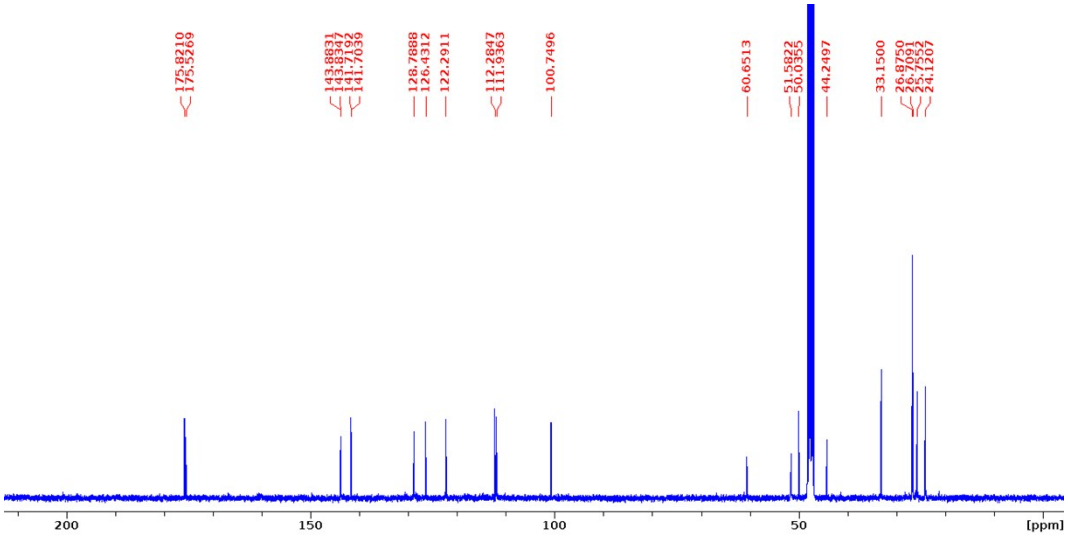
Compounds **4** (2.20 g, 6.23 mmol) which was synthesized according to the previously reported procedure[5] and sodium acetate (0.51 g, 6.22 mmol) were dissolved in absolute ethanol (100 mL) and stirred at room temperature for 30 min. Compound **5** (0.70 g, 2.94 mmol) was added to the solution. The solution was refluxed for 5 h. The solvent was removed by using a rotovap. The crude material was purified by preparative reverse-phase HPLC (30-90% CH_3CN/H_2O containing 0.1% TFA). The purified product was lyophilized to yield the green solid (1.60 g, 76% yield).

^{1}H NMR (400 MHz, MeOD) δ 8.45 (d, *J* = 14.8 Hz, 2H), 7.60 (d, *J* = 7.4 Hz, 2H), 7.51-7.48 (m, 4H), 7.40 (t, *J* = 7.1 Hz, 2H), 6.37 (d, *J* = 14.8 Hz, 2H), 4.75 (s, 4H), 4.31 (t, *J* = 7.4 Hz, 4H), 3.45 (s, 6H), 2.22 (t, *J* = 7.0 Hz, 4H), 1.96-1.91 (m, 4H), 1.78 (s, 12H), 1.76-1.72 (m, 4H), 1.59-1.51 (m, 4H); ^{13}C NMR (100 MHz, MeOD) δ 175.8, 175.5, 143.9, 143.8, 141.7 (2C), 128.8, 126.4, 122.3, 112.3, 111.9, 100.7, 60.7, 51.6, 50.0, 44.2, 33.2, 26.9, 26.7, 25.8, 24.1; HRMS (ESI) m/z for $C_{43}H_{56}ClN_3O_4^{2+}$ calculated: 356.6974; found 356.6968.

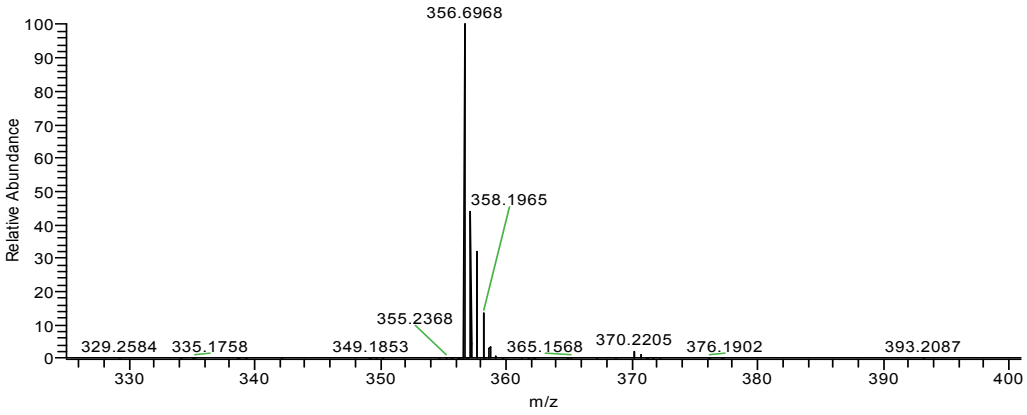
^{1}H NMR spectrum:



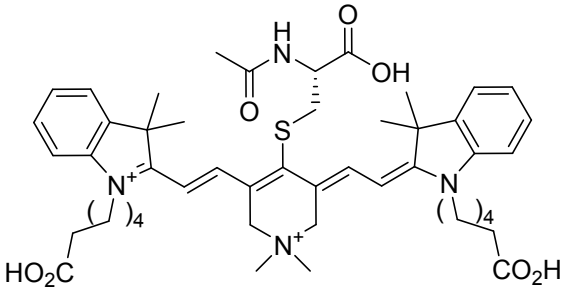
^{13}C NMR spectrum:



HRMS (ESI)



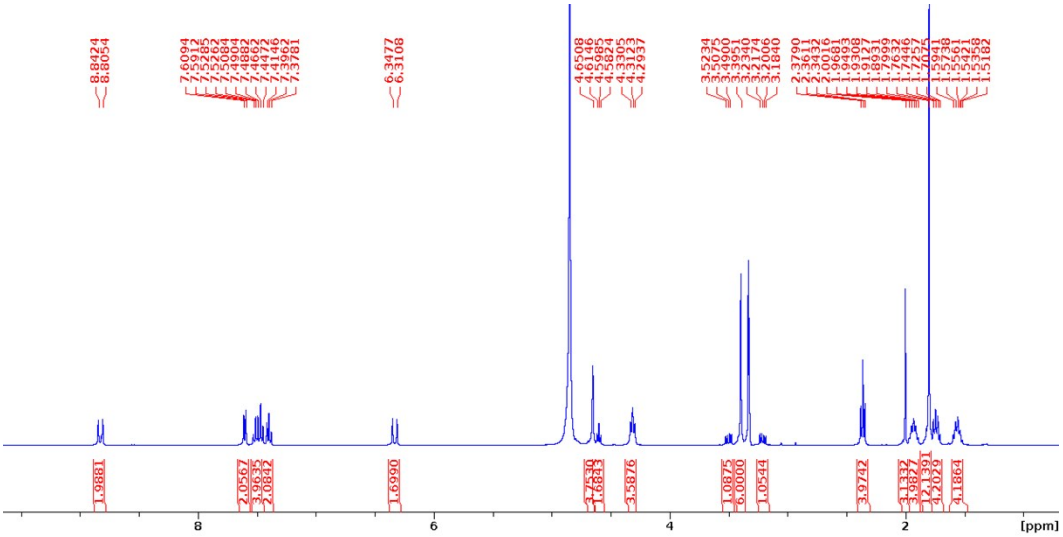
Synthesis of 2-((*E*)-2-((*E*)-4-(((*R*)-2-acetamido-2-carboxyethyl)thio)-5-(2-((*E*)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)ethylidene)-1,1-dimethyl-1,2,5,6-tetrahydropyridin-1-ium-3-yl)vinyl)-1-(5-carboxypentyl)-3,3-dimethyl-3*H*-indol-1-ium (**2**)



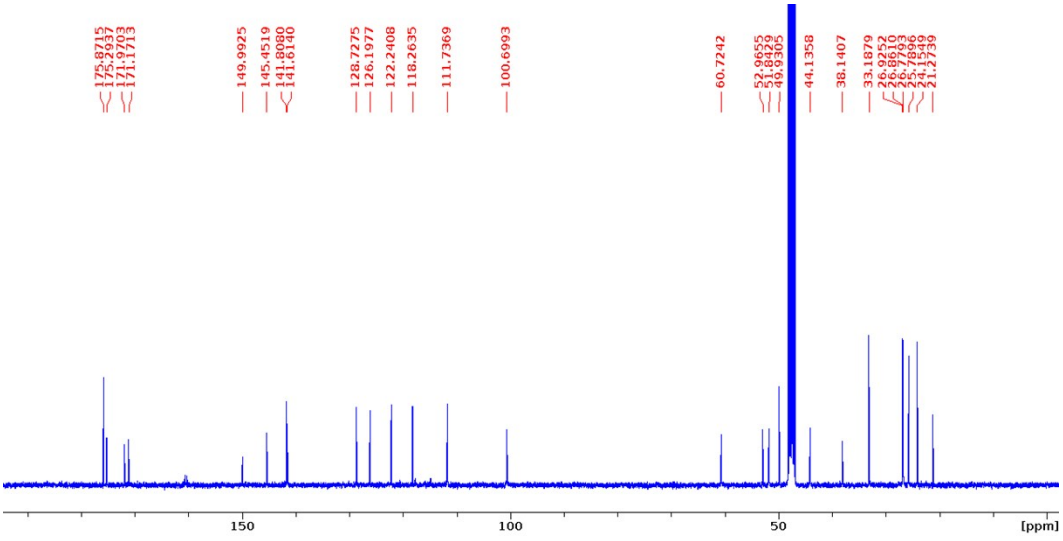
Compound **1** (20.0 mg, 0.028 mmol), DIPEA (7.50 μL, 0.043 mmol) were dissolved in 0.5 mL dry DMF. *N*-acetyl-*L*-Cysteine (7.00 mg, 0.043 mmol) was added afterwards and stirred at 25 °C for 1 h. Solvent was removed and the crude was purified by preparative reverse-phase HPLC (30-80% CH_3CN/H_2O containing 0.1% TFA) to get the desired product as green solid (19.0 mg, 81% yield).

^{1}H NMR (400 MHz, MeOD) δ 8.82 (d, *J* = 14.8 Hz, 2H), 7.60 (d, *J* = 7.3 Hz, 2H), 7.53-7.45 (m, 4H), 7.40 (t, *J* = 7.3 Hz, 2H), 6.33 (d, *J* = 14.8 Hz, 2H), 4.65 (s, 4H), 4.60 (t, *J* = 6.4 Hz, 1H), 4.31 (t, *J* = 7.4 Hz, 4H), 3.50 (dd, *J* = 13.4, 7.0 Hz, 1H), 3.40 (s, 6H), 3.21 (dd, *J* = 13.4, 6.6 Hz, 1H), 2.36 (t, *J* = 7.2 Hz, 4H), 2.00 (s, 3H), 1.97 –1.89 (m, 4H), 1.80 (s, 12H), 1.76-1.70 (m, 4H), 1.59-1.52 (m, 4H); ^{13}C NMR (100 MHz, MeOD) δ 175.9, 175.3, 172.0, 171.2, 150.0, 145.5, 141.8, 141.6, 128.7, 126.2, 122.2, 118.3, 111.7, 100.7, 60.7, 53.0, 51.8, 49.9, 44.1, 38.1, 33.2, 26.9 (2C), 26.8, 25.8, 24.2, 21.3; HRMS (ESI) m/z for $C_{48}H_{64}N_4O_7S^{2+}$ calculated: 420.7259; found 420.7262.

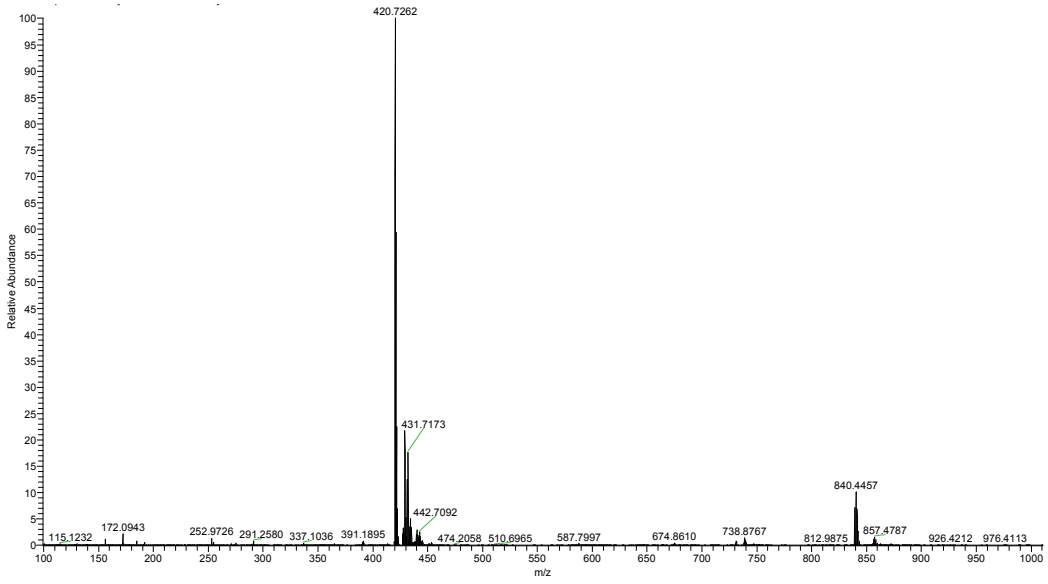
^{1}H NMR spectrum:



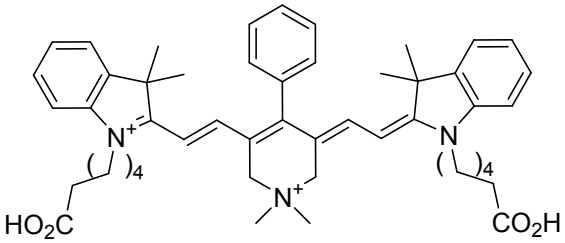
^{13}C NMR spectrum:



HRMS (ESI)



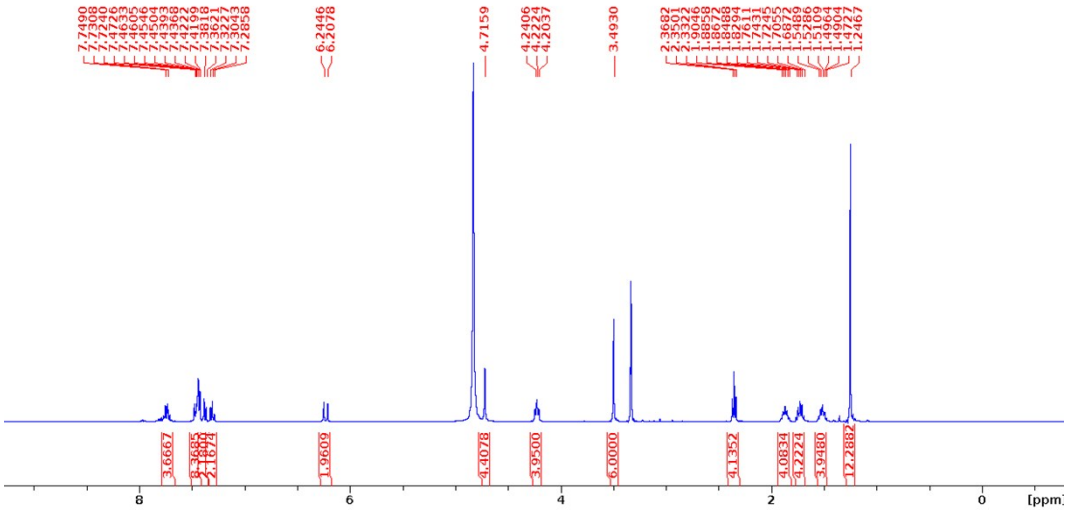
Synthesis of 1-(5-carboxypentyl)-2-((*E*)-2-((*Z*)-5-(2-((*E*)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)ethylidene)-1,1-dimethyl-4-phenyl-1,2,5,6-tetrahydropyridin-1-ium-3-yl)vinyl)-3,3-dimethyl-3*H*-indol-1-ium (**3**)



Compound **1** (20.0 mg, 0.028 mmol), phenylboronic acid (6.80 mg, 0.056 mmol) were added in 3 mL of degassed and deionized water under argon atmosphere. $Pd(PPh_3)_4$ (2.0 mg) was added and heat at 95 °C for 18 h. The crude was filtered and purified by preparative reverse-phase HPLC (30-80% CH_3CN/H_2O containing 0
.1% TFA) to get the desired product as solid (20 mg, 95% yield).

^{1}H NMR (400 MHz, MeOD) δ 7.78-7.69 (m, 3H), 7.48-7.41 (m, 8H), 7.37 (d, *J* = 7.9 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 2H), 6.22 (d, *J* = 14.7 Hz, 2H), 4.72 (s, 4H), 4.22 (t, *J* = 7.4 Hz, 4H), 3.49 (s, 6H), 2.35 (t, *J* = 7.2 Hz, 4H), 1.87 (quint, *J* = 7.5 Hz, 4H), 1.72 (quint, *J* = 7.4 Hz, 4H), 1.51 (quint, *J* = 7.6 Hz, 4H), 1.25 (s, 12H); ^{13}C NMR (100 MHz, MeOD) δ 175.8, 174.7, 155.8, 147.4, 141.7, 141.2, 135.7, 134.5, 130.2, 129.2, 128.6, 125.9, 122.1, 116.4, 111.4, 99.4, 59.9, 51.6, 49.3, 43.9, 33.2, 26.7, 26.4, 25.7, 24.1; HRMS (ESI) m/z for $C_{49}H_{61}N_3O_4^{2+}$ calculated: 377.7326; found 377.7326.

^{1}H NMR spectrum:



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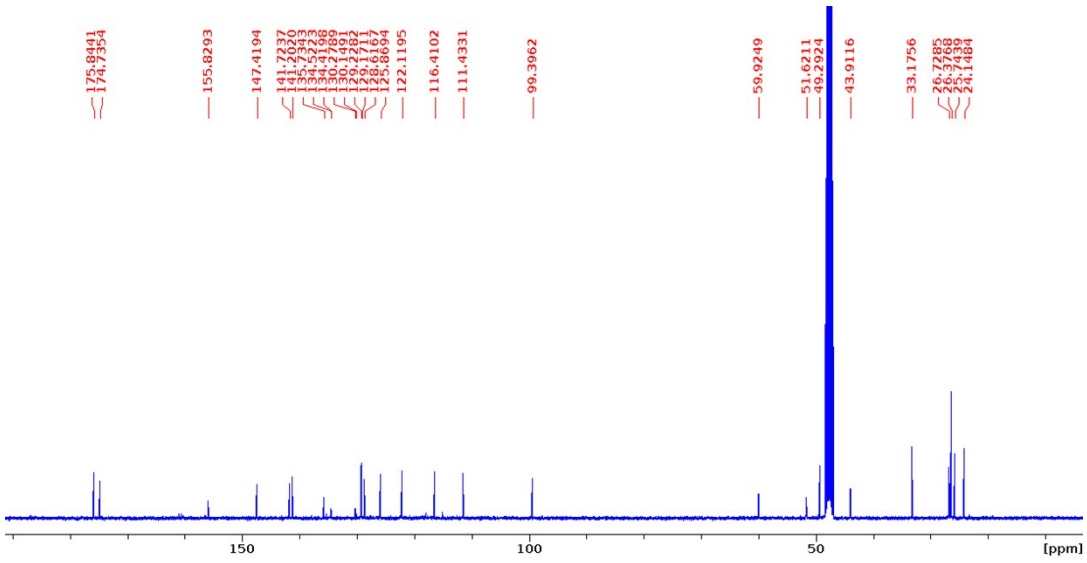
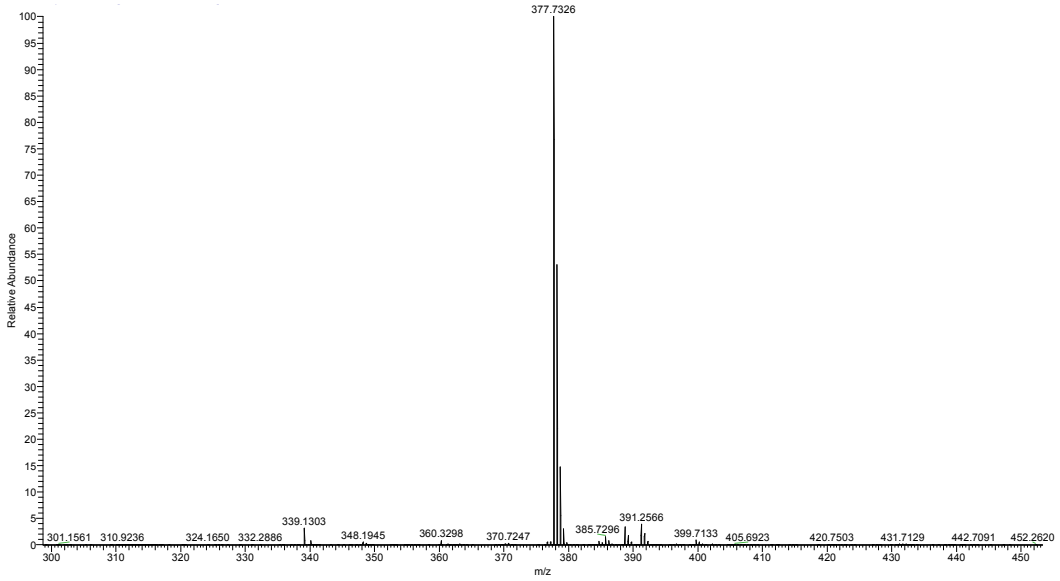


HRMS (ESI)


377.7326
339.1303
391.2566
301.1561
310.9236
324.1650
332.2886
348.1945
360.3298
370.7247
385.7296
399.7133
405.6923
420.7503
431.7129
442.7091
452.2620
Relative Abundance
m/z

D. Table and Figures

Table S1. Maximum excitation and emission wavelength of **A**-**3A** and **1**-**3**.

compound	λ_{abs} (nm)	λ_{em} (nm)
A	775	800
2A	784	806
3A	755	775
1	745	765
2	757	782
3	730	750

Table S2. Photophysical properties of compound **1**, **2**, **A** and **2A**.

compound	λ_{abs} (nm)	λ_{em} (nm)	$\Delta\lambda^a$	ε_{max} ($cm^{-1}M^{-1}$)	Φ	brightness (ε_{max} x Φ)
1	745	768	23	192460	0.051	9901
2	755	778	23	132396	0.016	2118
A	775	799	24	149940	0.025	3689
2A	783	807	24	113698	0.010	1137

[a]Stokes' shifts of **1**, **2**, **A** and **2A**. [b]Fluorescence quantum yield were performed using A (Φ = 0.13 in DMSO; λ_{ex} 730 nm) as a standard.

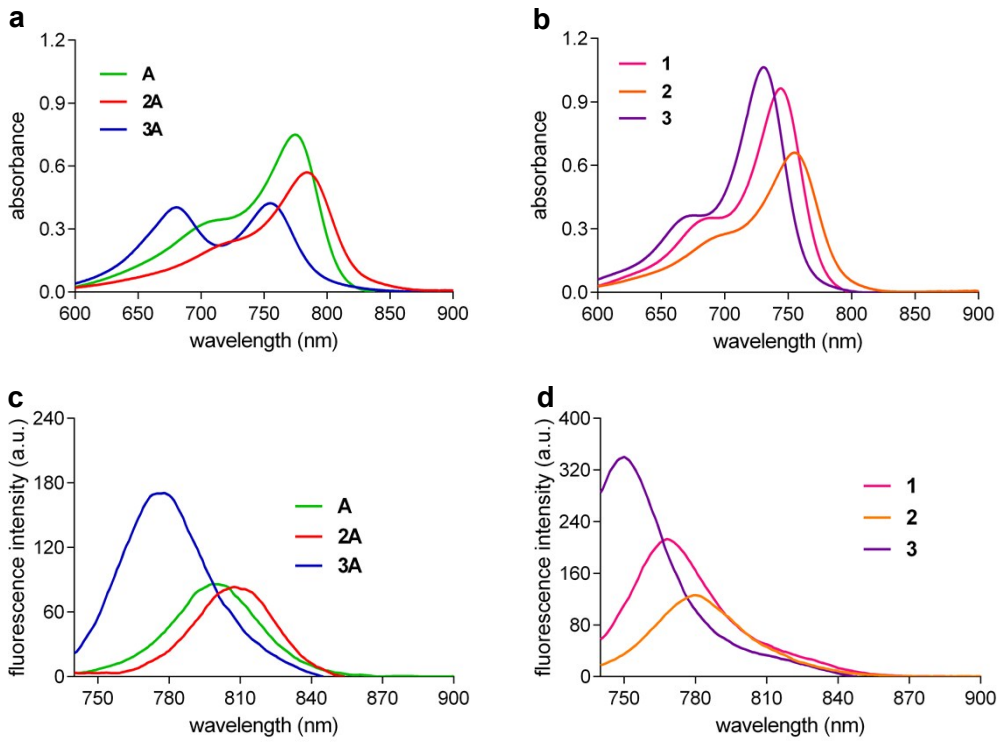


Figure S1. Absorbance and fluorescence spectra of compounds **A-3A** and **1-3** (5 μM, 37 °C) in 10 mM Phosphate Buffer Saline (PBS) pH 7.24.

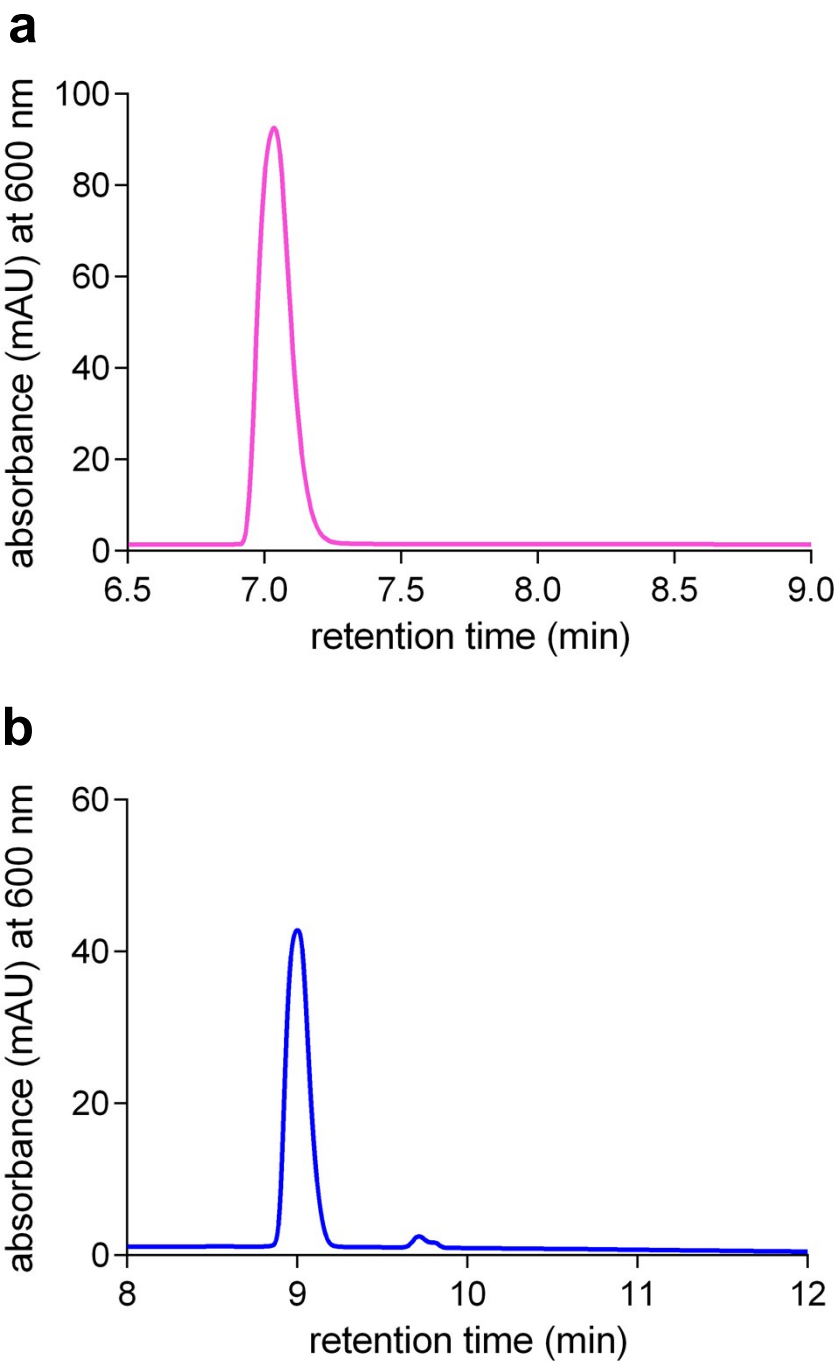


Figure S2. High-performance liquid chromatography (HPLC) analysis of: **a** Cys-adduct **2** and **b** Cys-adduct **2A**. Cys-adduct **2** and **2A** provide the retention time around 7 and 9 min, respectively.

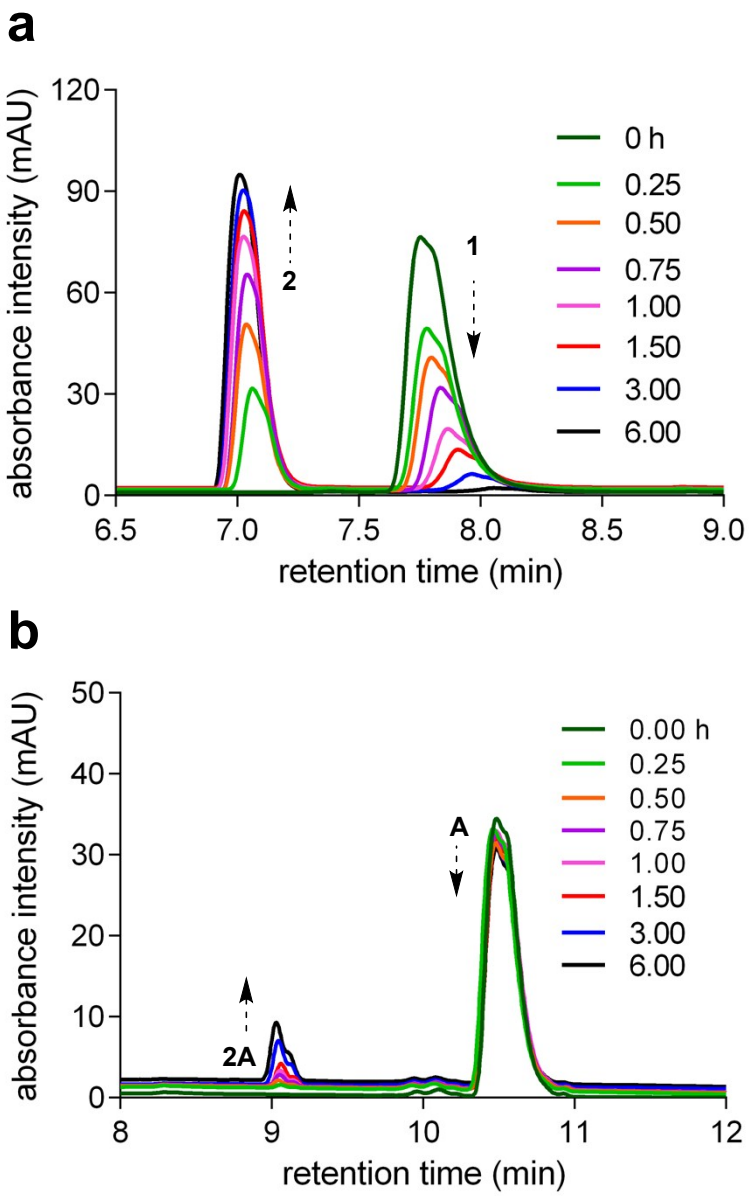


Figure S3. HPLC analyses for reactive studies of 300 μM of *N*-acetyl-*L*-Cys mixed with 200 μM of: **a** QuatCy **1** and **b** MHI-148 **A** in 1 M HEPES buffer pH 8.0 incubating at 37 °C, where only a peak corresponding to the Cys-adduct **2** and Cys-adduct **2A** formed.

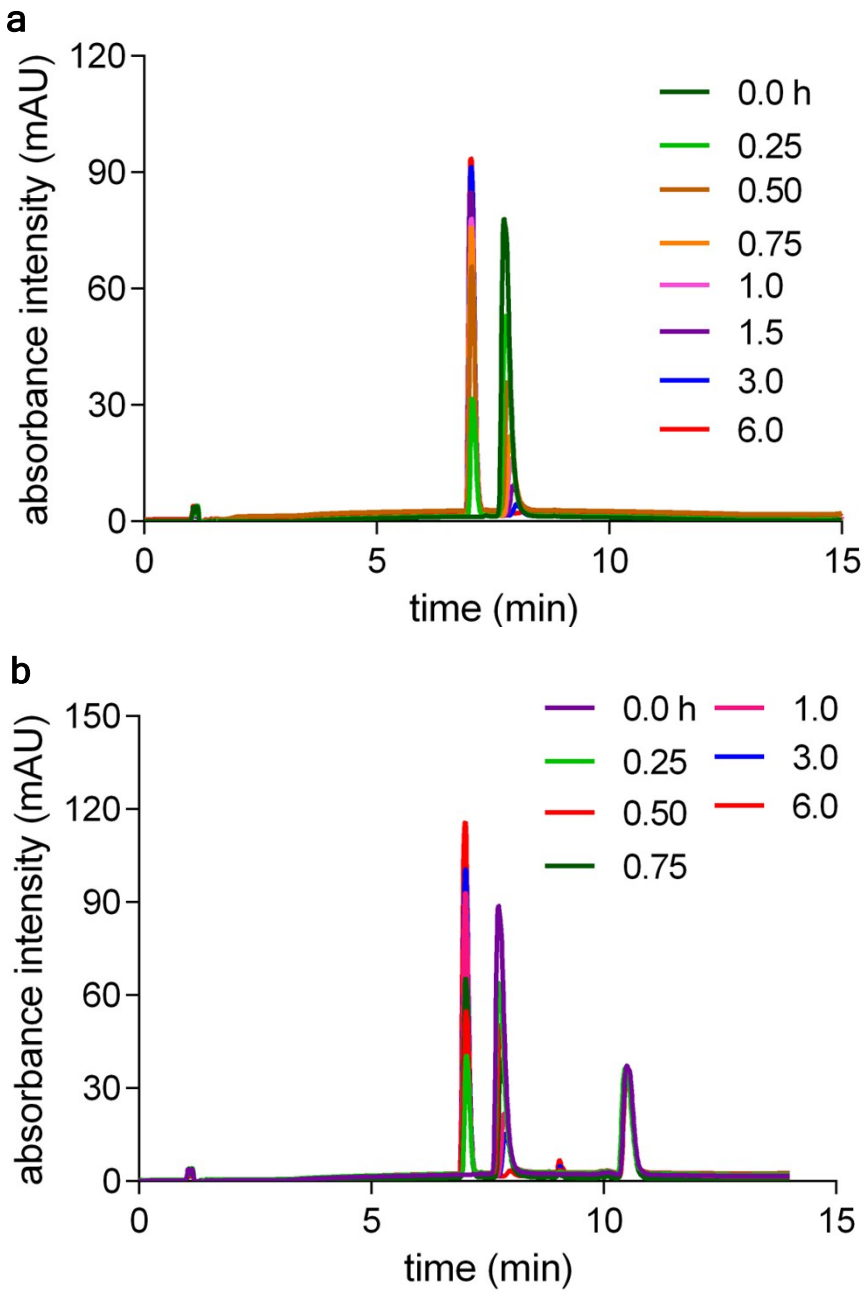


Figure S4. Complete HPLC spectra corresponding to **Fig 1** in the text.

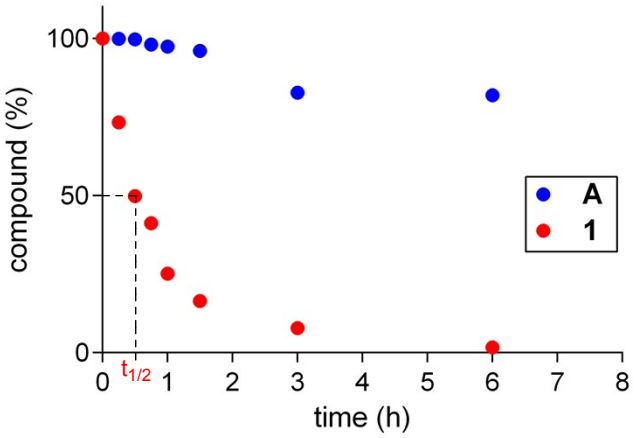


Figure S5. The comparison of rate reactivity for compounds **A** and **1** (200 μM) with *N*-acetyl-*L*-Cys (300 μM) in 1 M HEPES buffer pH 8.0 at 37 °C.

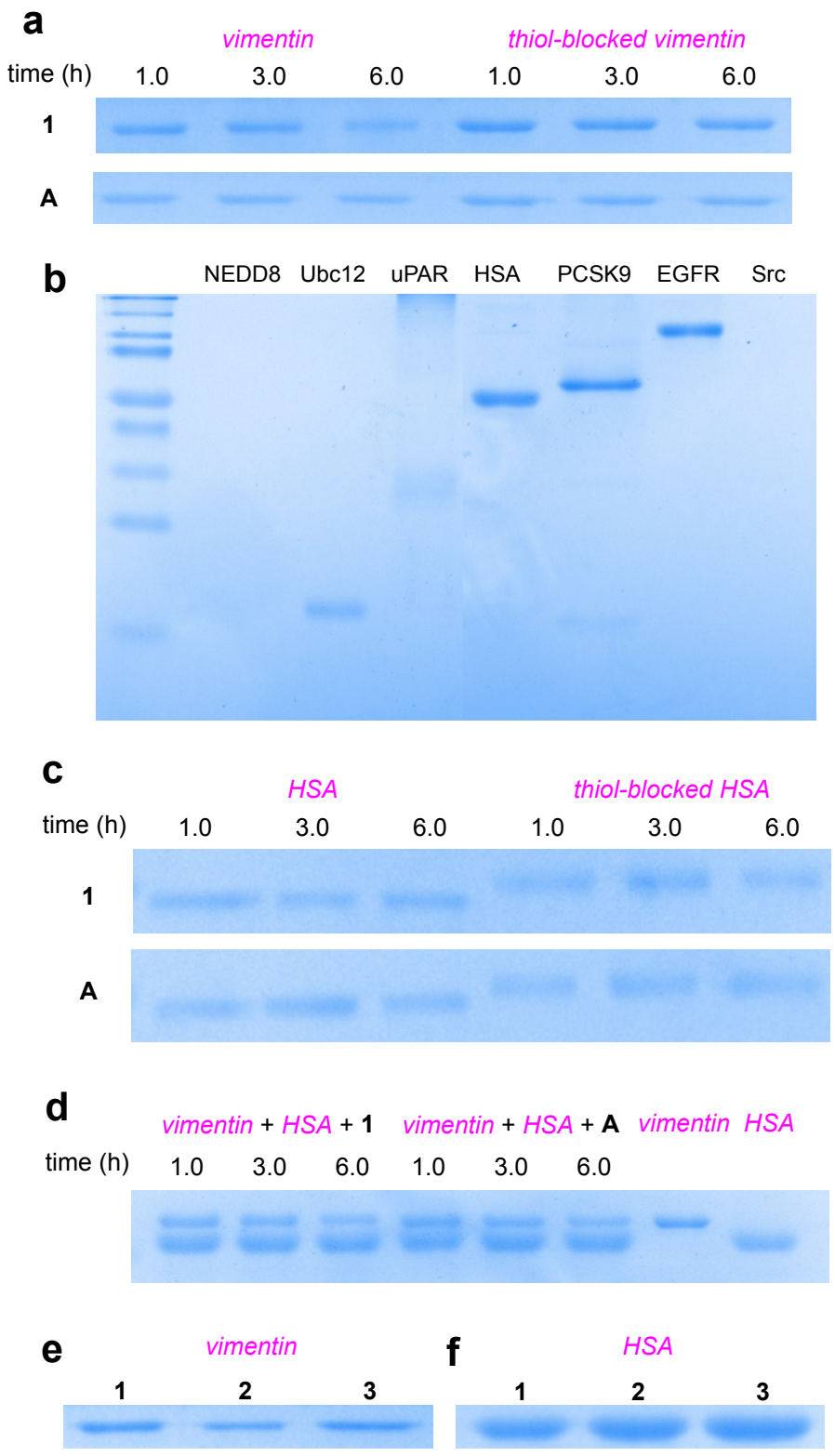


Figure S5. CBB-G250 staining of SDS-PAGE gel referred to Fig 2b.

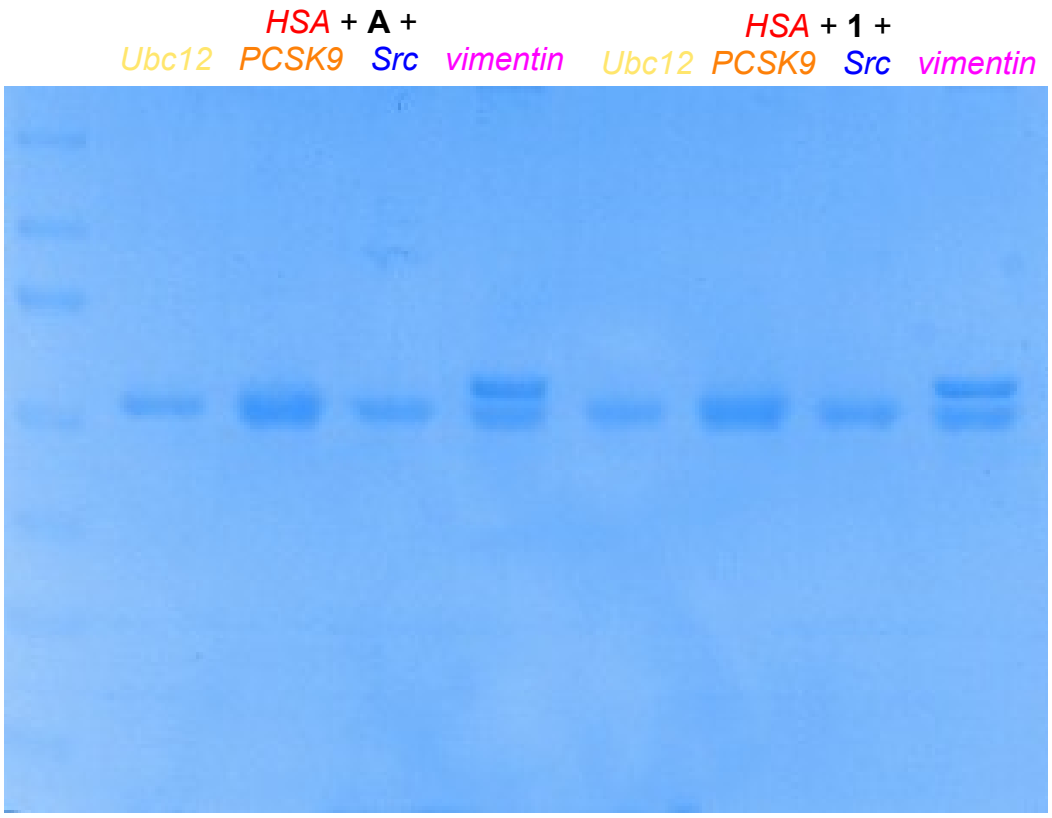


Figure S6. CBB-G250 staining of SDS-PAGE gel referred to Fig 2e.

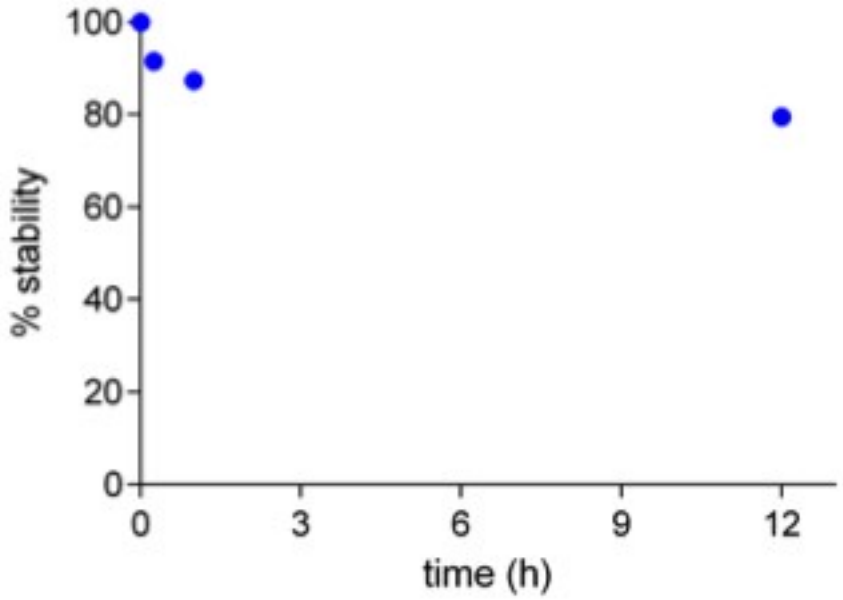


Figure S7. Stability of vimentin bound **1** (10 μM) in the presence of GSH (0.5 mM) over 12 h was monitored by HPLC at 750 nm. Area under the peak was ploted as a function of time.

E. References

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