**SUPPLEMENTAL INFORMATION**

# HSA-Lys-161 Covalent Bound Fluorescent Dye for In Vivo Blood Drug Dynamic Imaging and Tumor Mapping

Yongkang Yue,[a] Tingting Zhao,[a] Yuting Wang,[a] Kaiqing Ma,[a] Xingkang Wu,[a] Fangjun Huo,[b] Fangqin Cheng,[c] Caixia Yin*[a]

a) *Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Shanxi laboratory for Yellow River, Institute of Molecular Science, Shanxi University, Taiyuan 030006, China. E-mail: yincx@sxu.edu.cn*
b) *Research Institute of Applied Chemistry, Shanxi University, Taiyuan 030006, China.*
c) *Institute of Resources and Environment Engineering, Shanxi University, Taiyuan, 030006, China*

## Tables of contents

**SUPPLEMENTAL INFORMATION**

## Materials and Methods

**Materials.** Albumin from rat serum, Alpha 1 Antitrypsin from human plasma were purchased from Sigma-Aldrich. Bovine serum albumin was purchased from Innochem. Albumin human was purchased from Psaitong. The 4-12% SDS-PAGE Precast Gel, 8% Native-PAGE Precast Gel, Prestained Color Protein Marker (15-120 kD, 10-170 kD), Coomassie Blue Super-Fast Staining Solution and 12% SDS-PAGE Gel Super-Quick Preparation Kit and the related materials were all purchased from Beyotime. DL-dithiothreitol, iodoacetamide and formic acid were purchased from Sigma (St. Louis, MO, USA), trypsin and chymotrypsin were purchased from Promega (Madison, WI, USA). All chemicals were purchased from commercial suppliers and used without further purification. All solvents were purified prior to use. Distilled water was used after passing through a water ultra-purification system. TLC analysis was performed using precoated silica plates.

Hitachi F−7000 fluorescence spectrophotometer was employed to measure fluorescence spectra. Hitachi U-3900 UV-vis spectrophotometer was employed to measure UV-vis spectra. Shanhai Huamei Experiment Instrument Plants provided a PO-120 quartz cuvette (10 mm). $^{1}$H NMR and $^{13}$C NMR experiments were performed with a BRUKER AVANCE III HD 600 MHz and 151 MHz NMR spectrometer, respectively (Bruker, Billerica, MA). Coupling constants (J values) are reported in hertz. HR-MS determinations were carried out on a Thermo Scientific Q Exactive Instrument. Ultimate 3000 system coupled with a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, USA) with an ESI nano-spray source were used in the protein modification site analysis experiment. The BIO-RAD Mini-PROTEAN Tetra Cell was used in the electrophoresis experiments. The corresponding fluorescent images and Coomassie blue staining images were obtained by a BIO-RAD ChemiDoc XRS+ Imaging System. The *in vivo* imaging experiments and *ex vivo* imaging experiments were performed with a LVIS Lumina LT Series III system. Cell and tissue sections imaging experiments were performed with a Leica DMI8 fluorescence microscope. Microtome Cryostat Microm HM525NX was used to obtain the tissue slice. Male 5-week-old BALB/c-nu mice were purchased from SPF (Beijing) Biotechnology Co., Ltd. Production Permit No.: SCXK (Beijing) 2019-0010.

This study was performed in strict accordance with the Chinese guidelines for the care and use of laboratory animals and was approved by the Institutional Animal Care and Use Committee of Scientific Research in Shanxi University (Taiyuan, China).

**Preparation of solutions of probes and analytes.** Stock solution of the dyes (2 mM) were prepared in DMSO. Stock solutions of other analytes were prepared by direct dissolution in deionized water. All chemicals used were of analytical grade.

**General fluorescence spectra measurements.** The detection experiments were measured in PBS (pH 7.4, 10 mM). The procedure was as follows: into a PBS solution, containing 5 μM SS-1, an analyte sample was added. The process was monitored by fluorescence spectrometer. All of the fluorescent spectra were obtained 10 min after analytes added.

**Cell culture and imaging.** The HeLa cells were grown in 1640 medium supplemented with 12% FBS and 1% antibiotics at 37 °C in humidified environment of 5% $CO_2$. Cells were plated on a 6-well plate with slides and allowed to adhere for 24 h. Before the experiments, cells were washed with PBS 3 times. The fluorescent images were obtained by a Leica DMI8 fluorescence microscope ($\lambda_{ex}$ = 536-556 nm; $\lambda_{em}$ = 560-625 nm).

**In Vivo Fluorescence Imaging.** 100 μL PBS solution containing 200 μM HSA, 100 μM **SS-1** and 0.5 % DMSO was injected intravenously into male 5-week-old BALB/c-nu mice. 100 μL sodium carboxymethyl cellulose suspension containing 2 mg/mL ibuprofen was administrated immediately by gavage. Then, the fluorescent images were taken on an IVIS imaging system (Excitation filter: 500; Emission filter: Cy5.5). Mice without probe injection were used as the control group in the first set of in vivo imaging experiment. Mice injected with the probe but without drug administration were used as the control group in the second set of in vivo imaging experiment.

**SUPPLEMENTAL INFORMATION**

**Ex vivo mouse imaging.** Male 5-week-old BALB/c-nu mice were sacrificed and the vital organs were harvested immediately and imaged by the IVIS imaging system (Excitation filter: 500; Emission filter: Cy5.5). After that, 10 μm fresh slices were obtained and imaged immediately by the Leica DMI8 fluorescence microscope ($\lambda_{ex}$ = 536-556 nm; $\lambda_{em}$ = 560-625 nm). For the tumor imaging experiments, $10^6$ HeLa cells were injected into male 5-week-old BALB/c-nu mouse to obtain xenograft tumor bearing mouse model which was sacrificed at 9-week-old. The tumor was immediately sectioned using surgical scalpel blade for fluorescent imaging.

**In-gel digestion.** Gel pieces were destained in 50 mM $NH_4HCO_3$ in 50% acetonitrile (v/v) until clear. Gel pieces were dehydrated with acetonitrile (100 μL) for 5 min. After the liquid removed, the gel pieces were rehydrated in 10 mM dithiothreitol and incubated at 56 °C for 1 h. Gel pieces were then dehydrated in acetonitrile again. After removing the liquid, gel pieces were rehydrated with 55 mM iodoacetamide and incubated for 45 min at room temperature in the dark. The gel pieces were washed with 50 mM $NH_4HCO_3$ and dehydrated with acetonitrile. Finally, resuspend the gel block with 50 mM ammonium bicarbonate containing 10 ng/μL trypsin, and incubate on ice for 1 h. After removing the excess solution from the sample, the gel block was digested overnight at 37 °C. The peptides after enzymatic hydrolysis were used to extract the gel pieces with 50% acetonitrile/5% formic acid and acetonitrile in turn, and the peptide solution was freeze-dried and resuspended in 2% acetonitrile/0.1% formic acid.

**LC-MS/MS analysis.** The peptides were dissolved in the mobile phase A of liquid chromatography and then separated using the EASY-nLC 1000 ultra-high performance liquid system. Mobile phase A is an aqueous solution containing 0.1% formic acid and 2% acetonitrile; mobile phase B is an aqueous solution containing 0.1% formic acid and 90% acetonitrile. Liquid phase gradient setting: 0-16min, 9%~25% phase B; 16-22min, 25%~40% phase B; 22-26min, 40%~80% phase B; 26-30min, 80% phase B. The flow rate is maintained at 450 nL/min.

The peptides were separated by the ultra-high performance liquid system and injected into the NSI ion source for ionization and then analyzed by Thermo Scientific™Q Exactive mass spectrometry. The ion source voltage was set to 2.2 kV, and the peptide precursor ions and their secondary fragments were detected and analyzed by high-resolution Orbitrap. The scanning range of the primary mass spectrometer was set to 350-1800 m/z, the scanning resolution was set to 70,000; the secondary scanning resolution was set to 17,500. The data acquisition mode uses the data-dependent scanning (DDA) program, that was, after the first-level scan, the first 20 peptide precursor ions with the highest signal intensity were selected to enter the HCD collision cell and use 28% of the fragmentation energy for fragmentation. Same parameters were used for the secondary mass spectrometry analysis. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 15.0 s dynamic exclusion. Automatic gain control (AGC) was set at $5e^4$.

**Data processing.** The resulting MS/MS data were processed using Proteome Discoverer 1.3. Tandem mass spectra were searched against Homo Sapiens (SwissProt, 20366 sequences) data base. Trypsin/p was specified as cleavage enzyme allowing up to 2 missing cleavages. Mass error was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethyl on Cys was specified as fixed modification. Oxidation on Met and protein N-terminal acetylation were specified as variable modification. Peptide confidence was set at high, and peptide ion score was set > 20.

**Modified HSA digestion.** Dissolve 10 mg HSA in 1 mL $ddH_2O$ to obtain the stock solution of HSA. 1 μL **SS-1** (20 mM, DMSO) was added to 250 μL HSA solution. After 10 min reaction, 5 μL mixture was transferred to a 10 kD ultrafiltration tube with the addition of 200 μL $NH_4HCO_3$ solution (50 mM), the obtained solution was centrifuged (12000 rcf) at 4 °C for 10 min which was repeated once. $NH_4HCO_3$ solution (50 mM) was added to make the volume up to 100 μL. Dithiothreitol with a final concentration of 10 mM was then added, and reduced in a 56°C water bath for 1 h. Added iodoacetamide (final concentration 50 mM) to the above mixture and reacted for 40 min in the dark.

**SUPPLEMENTAL INFORMATION**

After centrifugation (12000 rcf) at 4 °C for 10 min, 200 μL $NH_4HCO_3$ solution (50 mM) was added and further centrifuged (12000 rcf) at 4 °C for 10 min which was repeated once. After replacing the cannula, 100 μL $NH_4HCO_3$ solution (50 mM) and trypsin (trypsin/substrate of 1/100) were added to digest at 37 °C for 4 hours. After that, another dose of trypsin (trypsin/substrate of 1/100) was added and the solution was incubated at 37°C overnight. The obtained solution was sequentially centrifuged (12000 rcf) at 4 °C for 10 min, vibrated after addition of 100 μL $ddH_2O$, centrifuged (12000 rcf) at 4 °C for 10 min, and repeated once. After digestion, the peptide was desalted using a self-priming desalting column, and the solvent was evaporated in a vacuum centrifuge at 45 ° C. The peptide was dissolved in the sample solution (0.1% formic acid, 2% acetonitrile), vortexed thoroughly, centrifuged at 13200 rpm for 10 min at 4 ° C, and the supernatant was transferred to the sample tube for mass spectrometry analysis.

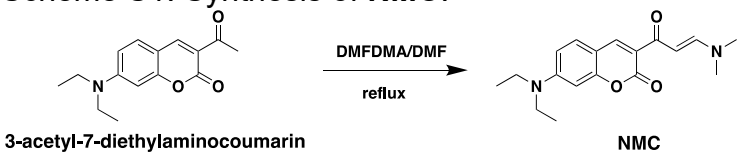
For the synchronous chymotrypsin digestion experiments, similar procedures were performed. 100 mM Tirs-HCl (PH 8.0) was used in this experiment and the dosage of chymotrypsin was 1/40 of the substrate.

**Data analysis.** The raw MS files were analyzed and searched against target protein database based on the species of the samples using Byonic. The parameters were set as follows: the protein modifications were carbamidomethylation (C) (fixed), oxidation (M) (variable), YYK(KR) (+416.1737) (variable), YYK(KR) (+434.1842) (variable); the enzyme specificity was set to chymotrypsin or trypsin; the maximum missed cleavages were set to 3; the precursor ion mass tolerance was set to 20 ppm, and MS/MS tolerance was 0.02 Da. Only high confident identified peptides were chosen for downstream protein identification analysis.

**SUPPLEMENTAL INFORMATION**

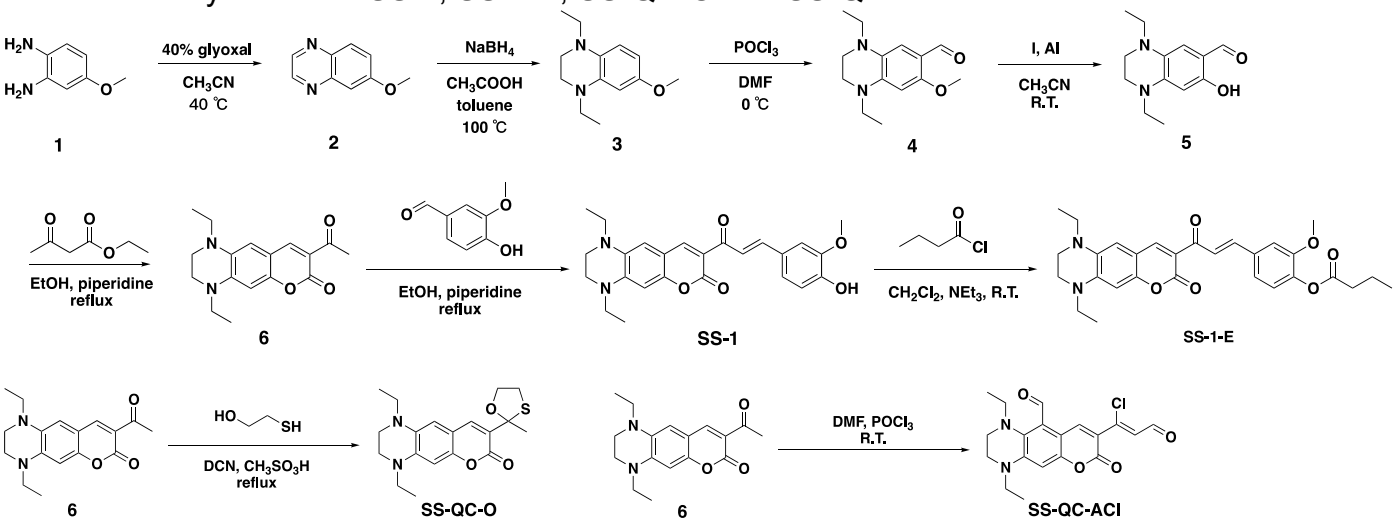
# Synthesis of the probes

Scheme S1. Synthesis of **NMC**.




**NMC**: 3-Acetyl-7-diethylaminocoumarin was synthesized based on our previous report.[1] The following fluorescent dye NMC was then synthesized based on the Batcho-Leimgruber reaction. Upon addition of 2 mmol 3-acetyl-7-diethylaminocoumarin and 6 mmol DMFDMA to 10 mL DMF, the mixture was stirred under reflux for 6 h. The corresponding mixture was poured into ice water after cooling to room temperature. The precipitate was collected and dried under reduced pressure to obtain dye **NMC** with 96% yield. $^{1}$H NMR (600 MHz, DMSO-$d_6$) $\delta$ 8.44 (s, 1H), 7.72 (d, J = 12.4 Hz, 1H), 7.60 (d, J = 8.9 Hz, 1H), 6.73 (dd, J = 9.0, 2.5 Hz, 1H), 6.54 (d, J = 2.4 Hz, 1H), 6.19 (d, J = 12.4 Hz, 1H), 3.46 (q, J = 7.0 Hz, 4H), 3.13 (s, 3H), 2.85 (s, 3H), 1.13 (t, J = 7.0 Hz, 6H). $^{13}$C NMR (151 MHz, DMSO) $\delta$ 181.81, 160.47, 157.77, 154.35, 152.29, 146.19, 131.68, 118.36, 110.01, 108.29, 96.27, 95.00, 44.71, 40.58, 12.82.

Scheme S2. Synthesis of **SS-1**, **SS-1-E**, **SS-QC-O** and **SS-QC-ACl**.




**SS-1**: Compound **6** was synthesized based on the reported procedure with minor modification.[2] The obtained fluorescent dye **6** was condensed with vanilline to produce **SS-1** as the fluorescent dye used for Ibuprofen pharmacokinetics imaging upon assemble with human serum albumin. In details, 0.5 mmol compound **6** and 0.7 mmol vanilline was dissolved in 20 mL EtOH. 3 drops piperidine was then added. The resulting mixture was refluxed for 70 h under Argon atmosphere. After cooling to room temperature, the solvent was removed under reduced pressure and the residue was separated by column chromatography using EtOAc/PE (1/1) as eluent to obtain **SS-1** as a dark red powder. $^{1}$H NMR (600 MHz, Chloroform-*d*) $\delta$ 8.52 (s, 1H), 8.07 (d, J = 15.6 Hz, 1H), 7.78 (d, J = 15.6 Hz, 1H), 7.23 – 7.18 (m, 2H), 6.92 (d, J = 8.6 Hz, 1H), 6.54 (s, 1H), 6.43 (s, 1H), 5.89 (s, 1H), 3.96 (s, 3H), 3.59 – 3.55 (m, 2H), 3.45 (q, J = 7.1 Hz, 2H), 3.36 (q, J = 7.1 Hz, 2H), 3.27 – 3.23 (m, 2H), 1.25 (t, J = 7.1 Hz, 3H), 1.21 (t, J = 7.1 Hz, 3H). $^{13}$C NMR (151 MHz, $CDCl_3$) $\delta$ 186.73, 161.52, 153.39, 148.12, 147.96, 146.70, 143.38, 132.50, 128.26, 124.20, 122.83, 116.37, 114.59, 109.71, 109.35, 107.79, 95.49, 56.06, 47.77, 46.36, 45.46, 44.51, 10.62, 9.70. HR-MS [**SS-1** + H]$^{+}$: m/z Calcd 435.1914, Found 435.1740.

**SS-1-E**: The control compound **SS-1-E** was synthesized based on a similar procedure with our previous report.[3] $^{1}$H NMR (400 MHz, Chloroform-*d*) $\delta$ 8.53 (s, 1H), 8.14 (d, *J* = 15.7 Hz, 1H), 7.78

(d, *J* = 15.7 Hz, 1H), 7.28 (s, 1H), 7.04 (d, *J* = 8.0 Hz, 1H), 6.61 (s, 1H), 6.44 (s, 1H), 3.89 (s, 3H), 3.60 (s, 2H), 3.46 (s, 2H), 3.37 (s, 2H), 3.28 (s, 2H), 2.57 (t, *J* = 7.4 Hz, 2H), 1.81 (q, *J* = 7.3 Hz, 2H), 1.24 – 1.19 (m, 6H), 1.06 (t, *J* = 7.4 Hz, 3H). $^{13}$C NMR (151 MHz, $CDCl_3$) $\delta$ 186.63, 171.39, 161.29, 151.37, 148.16, 143.51, 142.20, 141.53, 134.48, 125.41, 123.10, 121.99, 116.41, 111.85, 109.36, 95.66, 55.98, 47.52, 46.32, 45.72, 44.60, 35.91, 18.52, 13.54, 10.63, 9.82.

**SS-QC-O**: Compound **6** (0.3 mmol) and mercaptoethanol (1 mmol) were dissolved in 20 mL DCM. After addition of 50 μL methane sulfonic acid, the mixture was refluxed for 2 h which was then washed with water thrice. The organic layer was dried with anhydrous sodium sulfate and separated by column chromatography using EtOAc/PE (1/3) as eluent to obtain **SS-QC-O** as a yellow powder. $^1$H NMR (600 MHz, Chloroform-*d*) δ 7.62 (s, 1H), 6.54 (s, 1H), 6.46 (s, 1H), 4.40 (dt, *J* = 9.4, 4.8 Hz, 1H), 4.15 (q, *J* = 7.8, 7.2 Hz, 1H), 3.49 (s, 2H), 3.38 (q, *J* = 7.1 Hz, 2H), 3.33 (q, *J* = 7.1 Hz, 2H), 3.28 (s, 2H), 3.18 – 3.10 (m, 1H), 2.97 (dt, *J* = 9.8, 4.8 Hz, 1H), 1.95 (s, 3H), 1.20 (q, *J* = 7.7, 7.1 Hz, 6H). $^{13}$C NMR (151 MHz, $CDCl_3$) $\delta$ 161.03, 139.72, 134.67, 108.67, 96.67, 92.08, 71.18, 45.74, 45.31, 33.36, 10.28, 9.96.

**SS-QC-ACl**: 1 mL $POCl_3$ was added to 1 mL DMF dropwise under stirring in an ice bath and the mixture was further stirred at 0 °C for 15 min. Then compound **6** (0.3 mmol) was added. After 3 h reaction under room temperature, 20 mL water was added to quench the reaction. The pH of the mixture was adjusted with sodium bicarbonate to neutral and extracted with DCM. The organic layer was combined, dried with anhydrous sodium sulfate and separated by column chromatography using EtOAc/PE (1/1) as eluent to obtain **SS-QC-ACl** as a red powder. $^1$H NMR (400 MHz, Chloroform-*d*) $\delta$ 10.33 (s, 1H), 10.30 (d, *J* = 6.9 Hz, 1H), 9.61 (s, 1H), 7.66 (d, *J* = 6.9 Hz, 1H), 6.67 (s, 1H), 3.51 (q, *J* = 7.2 Hz, 2H), 3.43 (t, *J* = 5.2 Hz, 2H), 3.25 (t, *J* = 5.2 Hz, 2H), 3.07 (q, *J* = 7.1 Hz, 2H), 1.33 – 1.27 (m, 6H). $^{13}$C NMR (151 MHz, $CDCl_3$) $\delta$ 192.43, 192.05, 157.73, 153.07, 145.60, 145.21, 142.92, 140.76, 126.99, 123.43, 115.82, 106.33, 98.96, 55.54, 46.47, 43.26, 42.24, 13.70, 10.60.

## Additional figures

Figure S1. Fluorescence responses of dye **NMC** (10 μM) toward various nucleophile reagents (200 μM) and human serum albumin in PBS (pH 7.4, 10 mM). $\lambda_{ex}$ = 470 nm; $\lambda_{em}$ = 485-650 nm; slit 5/5 nm; 600 v.

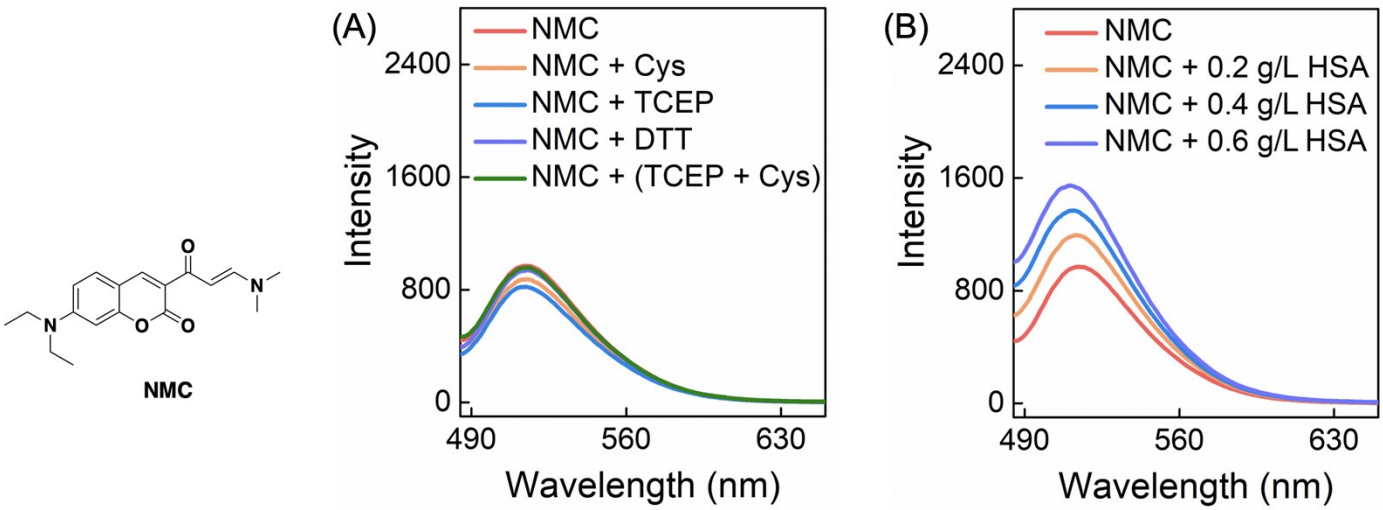
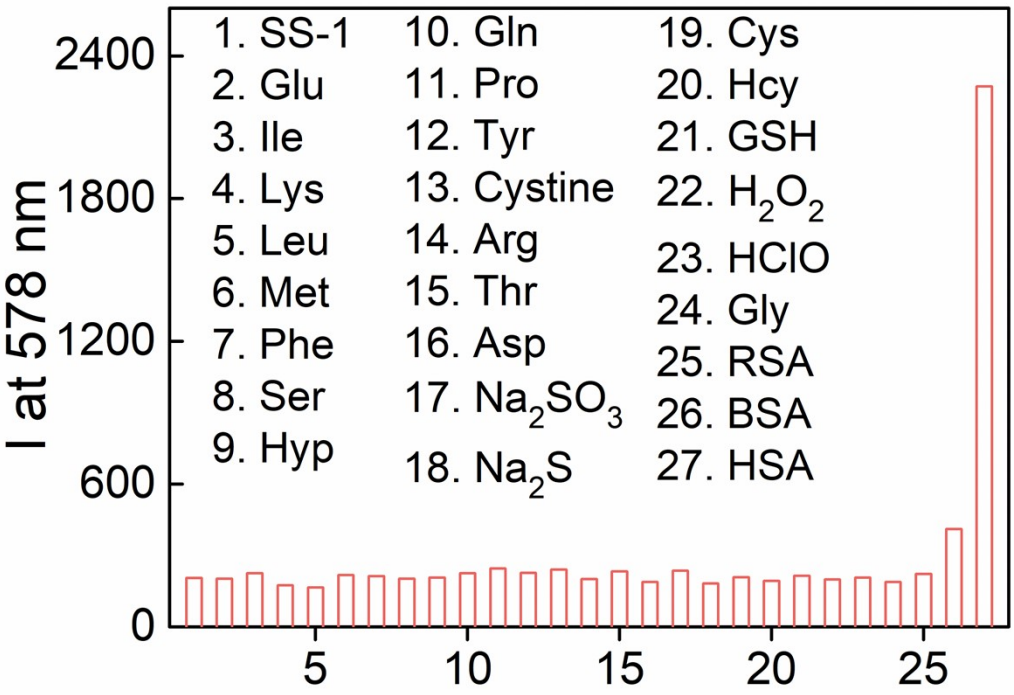



Figure S2. Fluorescence responses of **SS-1** (5 μM) toward various amino acids, nucleophilic anions, oxidative species, rat serum albumin (1 mg/mL), bovine serum albumin (2 mg/mL) and human serum albumin (2 mg/mL) in PBS (pH 7.4, 10 mM). The concentrations of the small molecular analytes used in this experiment were 200 μM except for Cys (1 mM), Hcy (100 μM) and GSH (2 mM). $\lambda_{ex}$ = 505 nm; $\lambda_{em}$ = 525-700 nm; slit 5/5 nm; 700 v.



**SUPPLEMENTAL INFORMATION**

Figure S3. 8% Native-PAGE Precast Gel analysis of human serum stained with **SS-1**. (1) A solution of human serum (10% in PBS) was incubated with (1) or without (2) **SS-1** (20 μM) and analyzed by Native-PAGE gel. The bands highlighted by the red and green boxes were further analyzed by LC-MS.

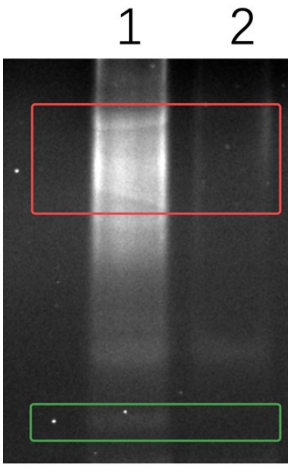
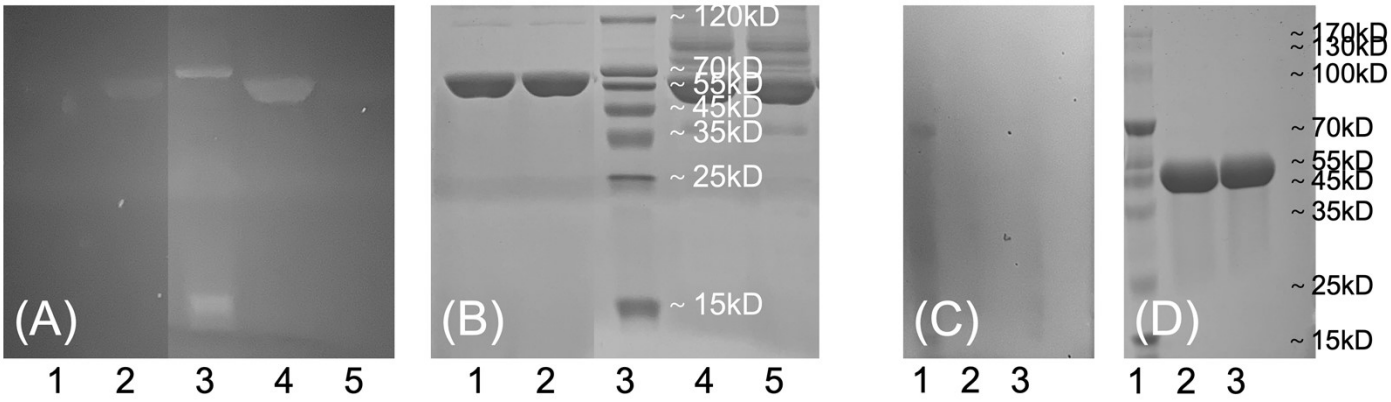
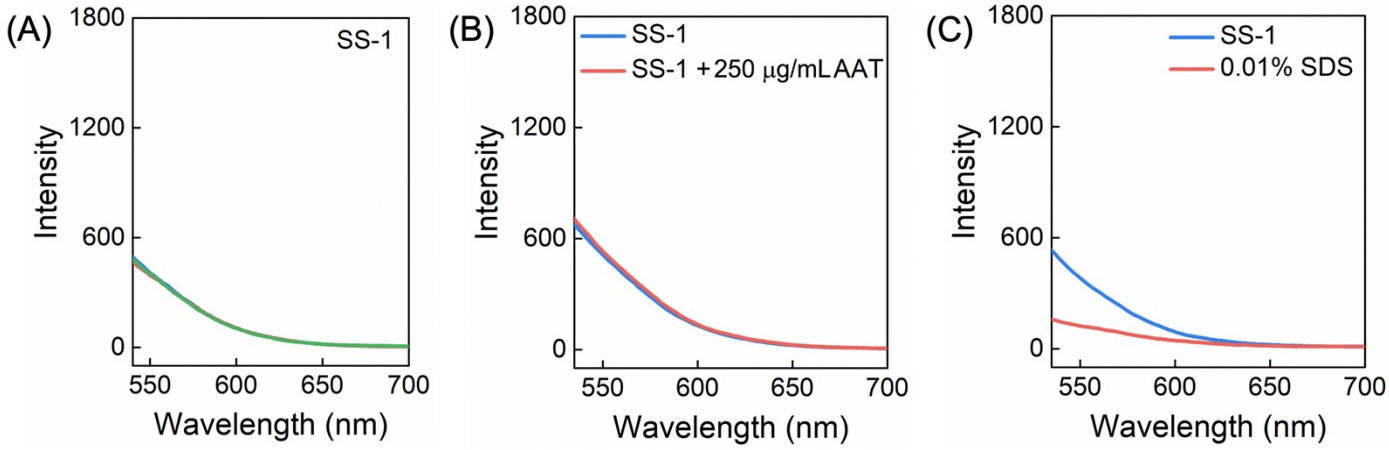



Figure S4. SDS-PAGE Gel analysis of human serum, HSA and alpha-1-antitrypsin stained with **SS-1**. (A) Fluorescence image of (1) 1 mg/mL HSA, (2) 1 mg/mL HSA + 20 μM **SS-1**, (3) marker, (4) 3.3% human serum + 20 μM **SS-1**, (5) 3.3% human serum on a 12% SDS-PAGE Gel. (B) Corresponding bright field image of (A) after further staining with Coomassie Blue Super-Fast Staining Solution. (C) Fluorescence image of (1) marker, (2) 0.6 mg/mL alpha-1-antitrypsin, (3) 0.6 mg/mL alpha-1-antitrypsin + 20 μM **SS-1** on a 4-12% SDS-PAGE Gel. (D) Corresponding bright field image of (C) after further staining with Coomassie Blue Super-Fast Staining Solution.



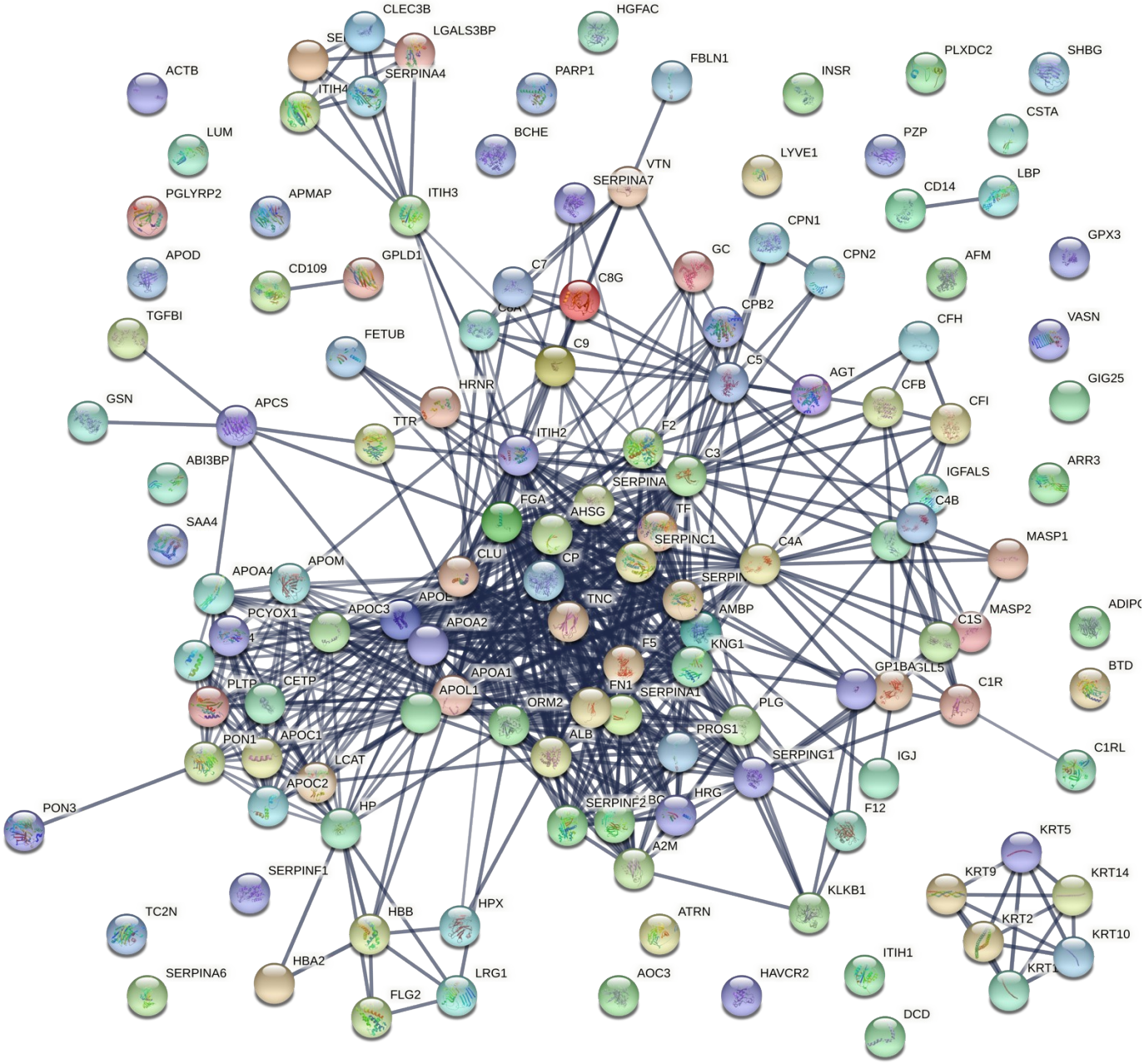
**SUPPLEMENTAL INFORMATION**

Figure S5. Fluorescence responses of **SS-1** (5 μM) toward alpha-1-antitrypsin (250 μg/mL) or 0.01% SDS in PBS (pH 7.4, 10 mM). $\lambda_{ex}$ = 505 nm; $\lambda_{em}$ = 525-700 nm; slit 5/5 nm; 700 v.

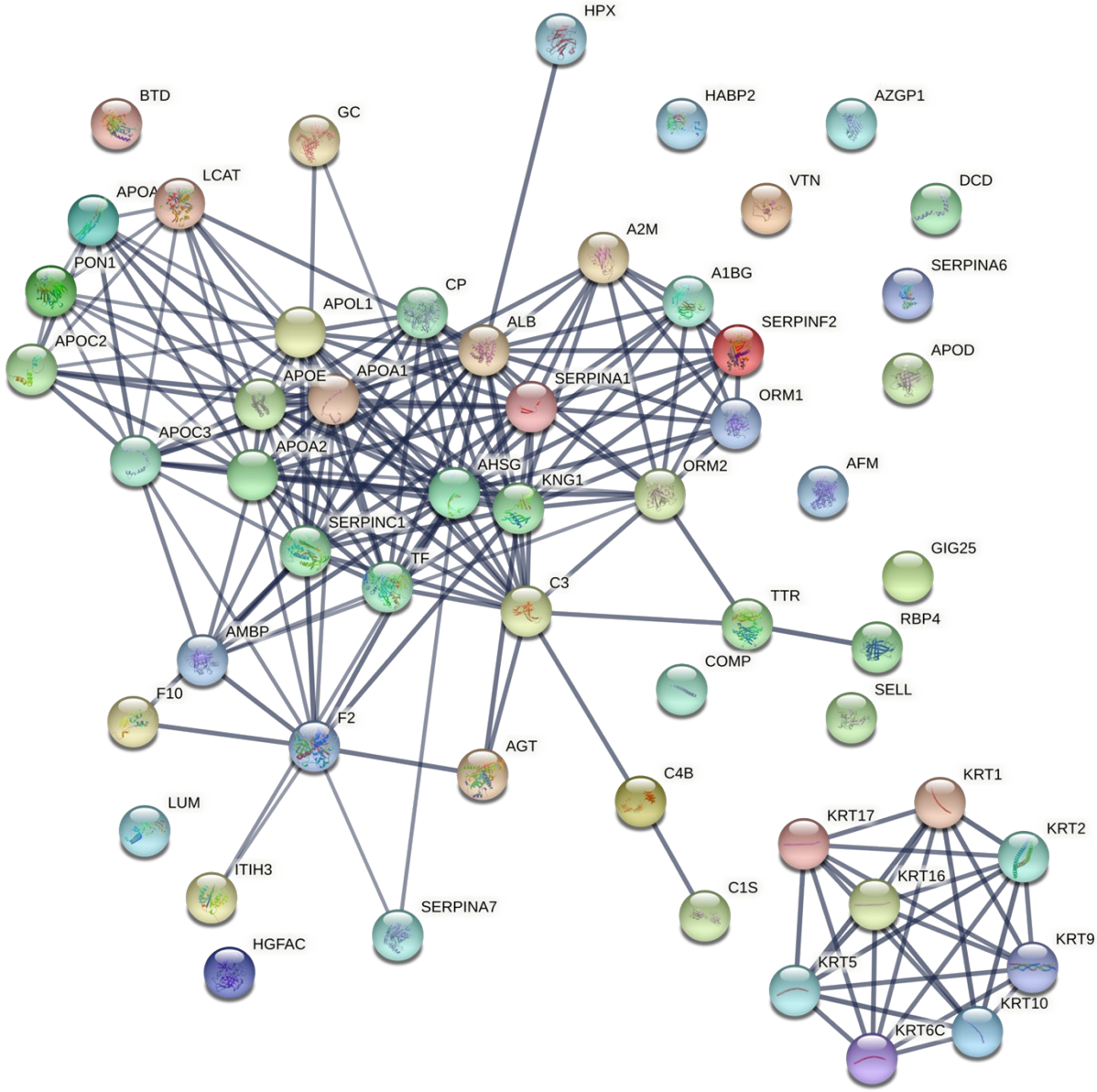


**SUPPLEMENTAL INFORMATION**

Figure S6. Protein interaction analysis by STRING.

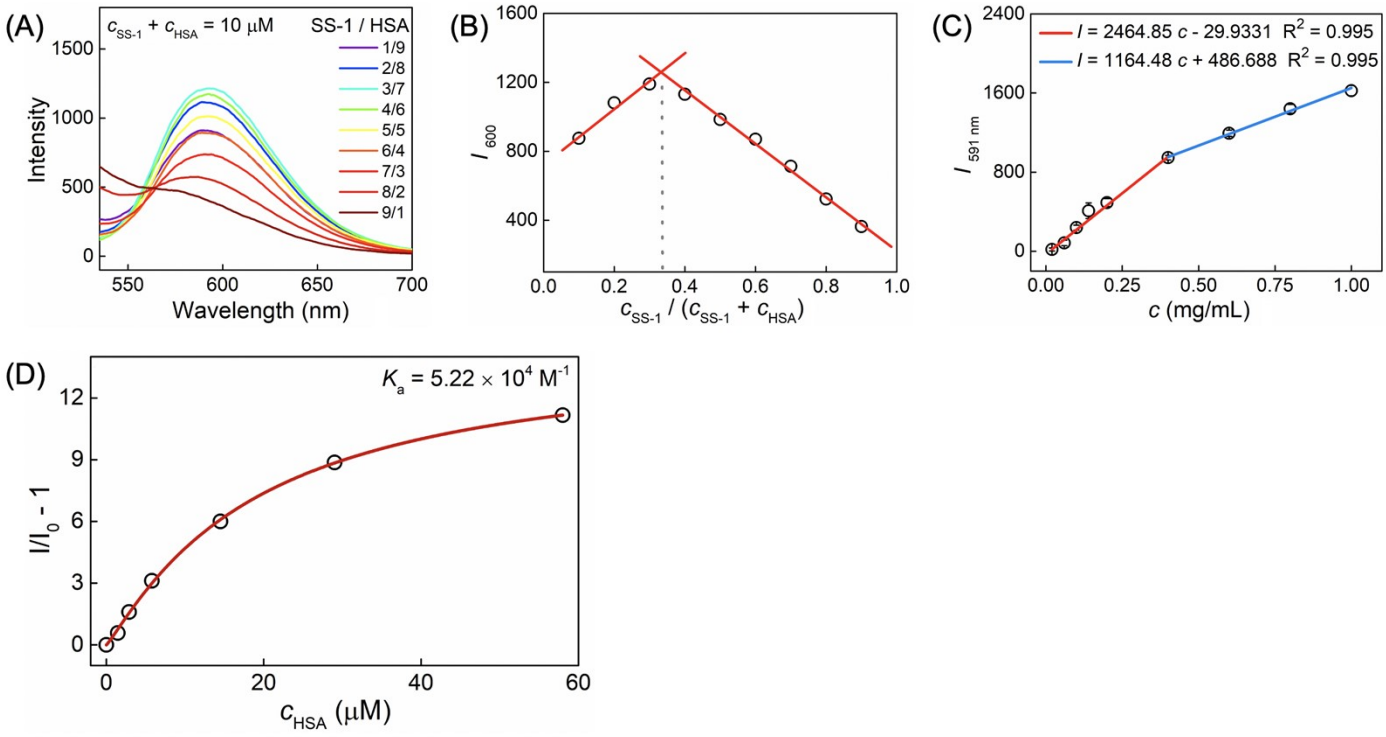


**SUPPLEMENTAL INFORMATION**



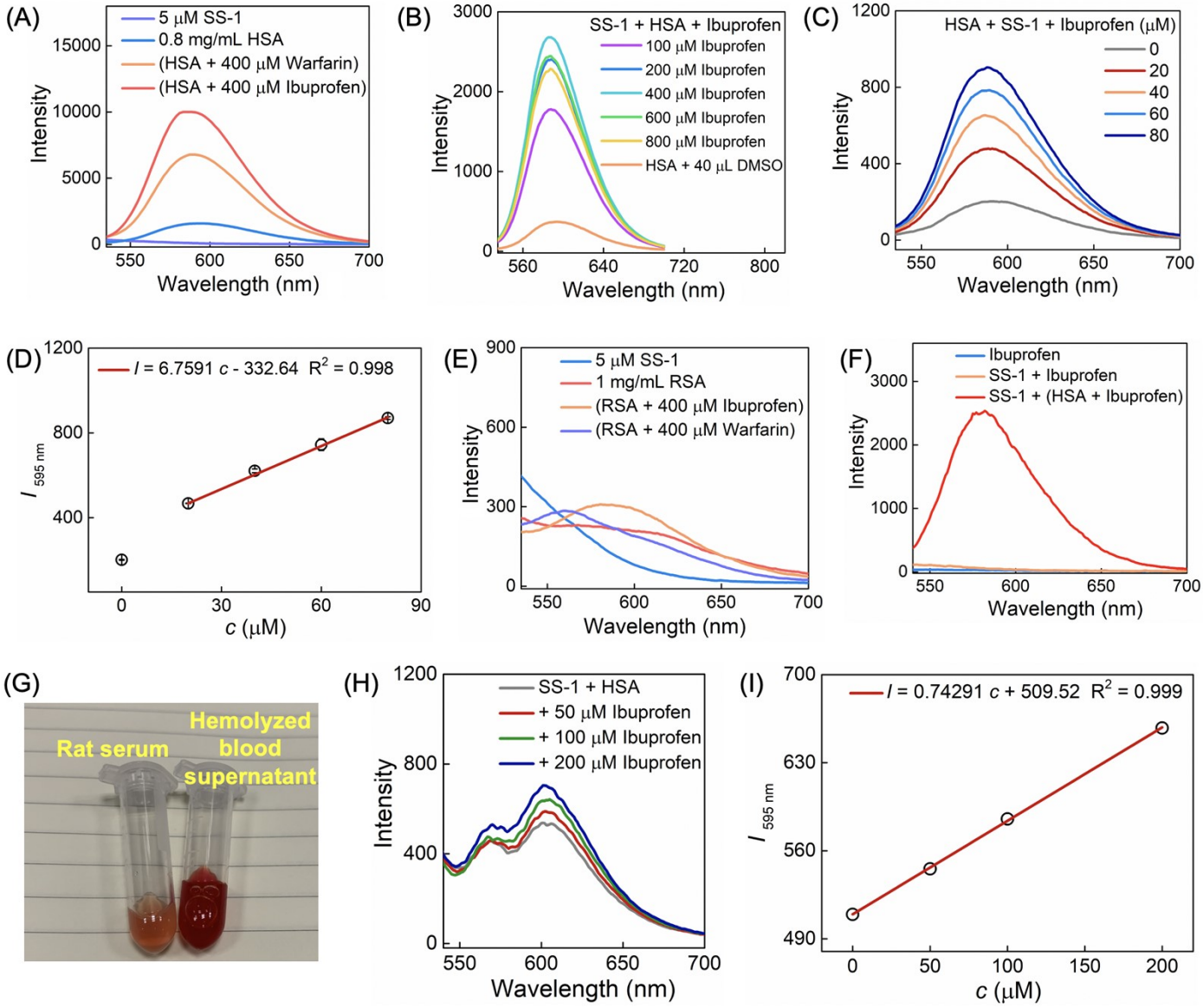
**SUPPLEMENTAL INFORMATION**

Figure S7. Fluorescence spectra (A) and the corresponding intensity analysis (B, l = 600 nm) of the mixture of HSA and **SS-1** with different ratios, the overall concentration of the mixture was 10 μM. (C) Job plots of the HSA concentration (0-1 mg/mL) based linear fluorescent intensity changes in **SS-1** (5 μM) containing PBS solution. (D) $K_a$ evaluation obtained by the area of fluorescence spectra (550-650 nm). Error bars represent standard deviations obtained from three independent experiments. $\lambda_{ex}$ = 505 nm; $\lambda_{em}$ = 525-700 nm; slit 5/5 nm; 700 v.



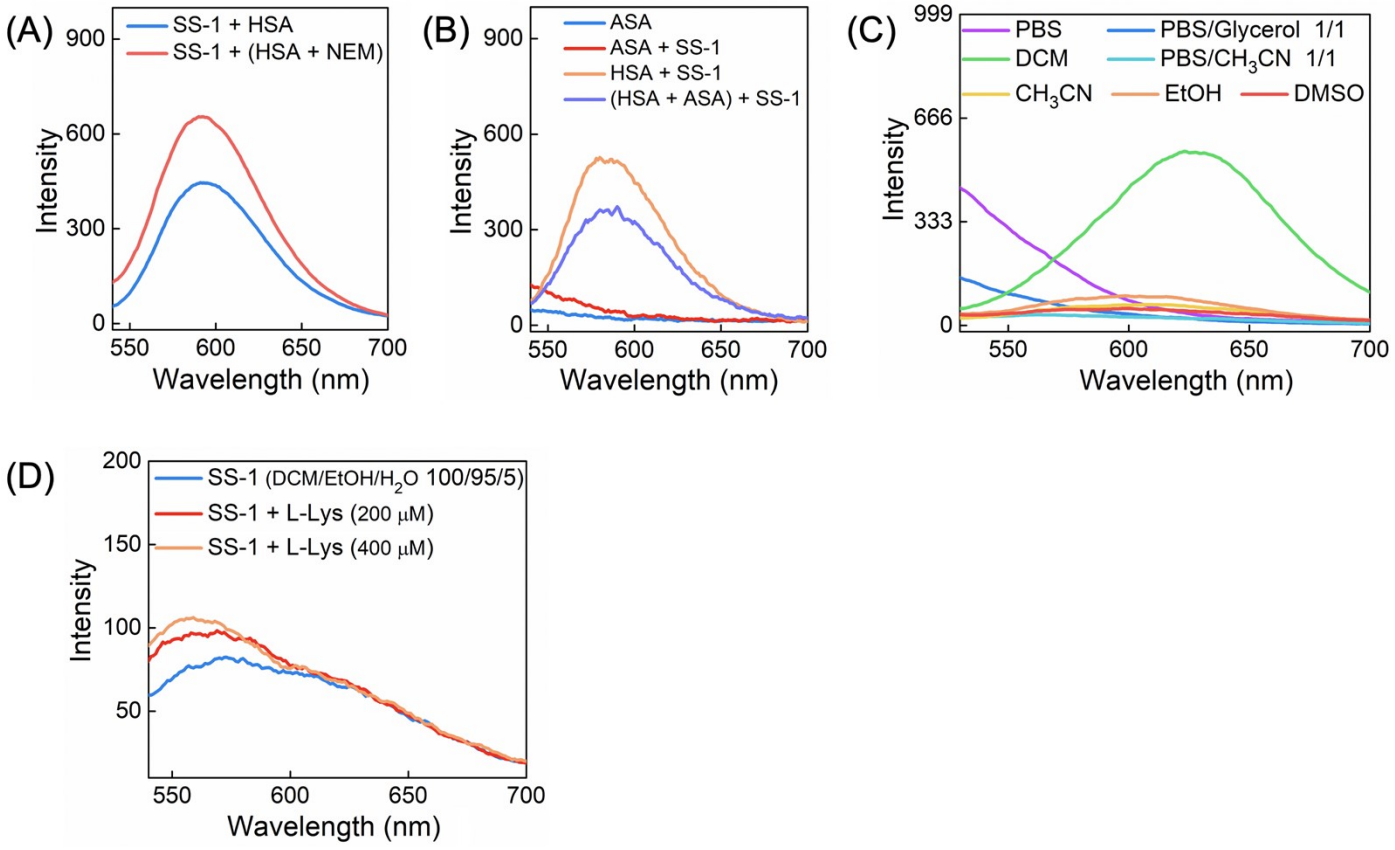
**SUPPLEMENTAL INFORMATION**

Figure S8. (A) Presence of ibuprofen (400 μM) or warfarin (400 μM) in the HSA labeling system induced fluorescent spectra changes. (B, C and D) Ibuprofen concentration dependent fluorescent intensity changes of the mixture containing 1 mg/mL HSA and 5 μM **SS-1**. (E) Fluorescent responses of 5 μM **SS-1** toward RSA (1 mg/mL) and the interference caused by the presence of ibuprofen (400 μM) or warfarin (400 μM). (F) Fluorescent responses of 5 μM **SS-1** toward 400 μM ibuprofen. (G) Hemolyzed rat blood supernatant and rat serum obtained by orbital venous plexus blood collection. (H, I) Fluorescence responses of the complex for ibuprofen in blood/PBS 1/1 system and the corresponding linear relationship. $\lambda_{ex}$ = 505 nm; $\lambda_{em}$ = 525-700 nm; slit 5/5 nm for A and E; slit 5/2.5 nm for B, C, D and F; slit 10/5 nm for H and I; 700 v.



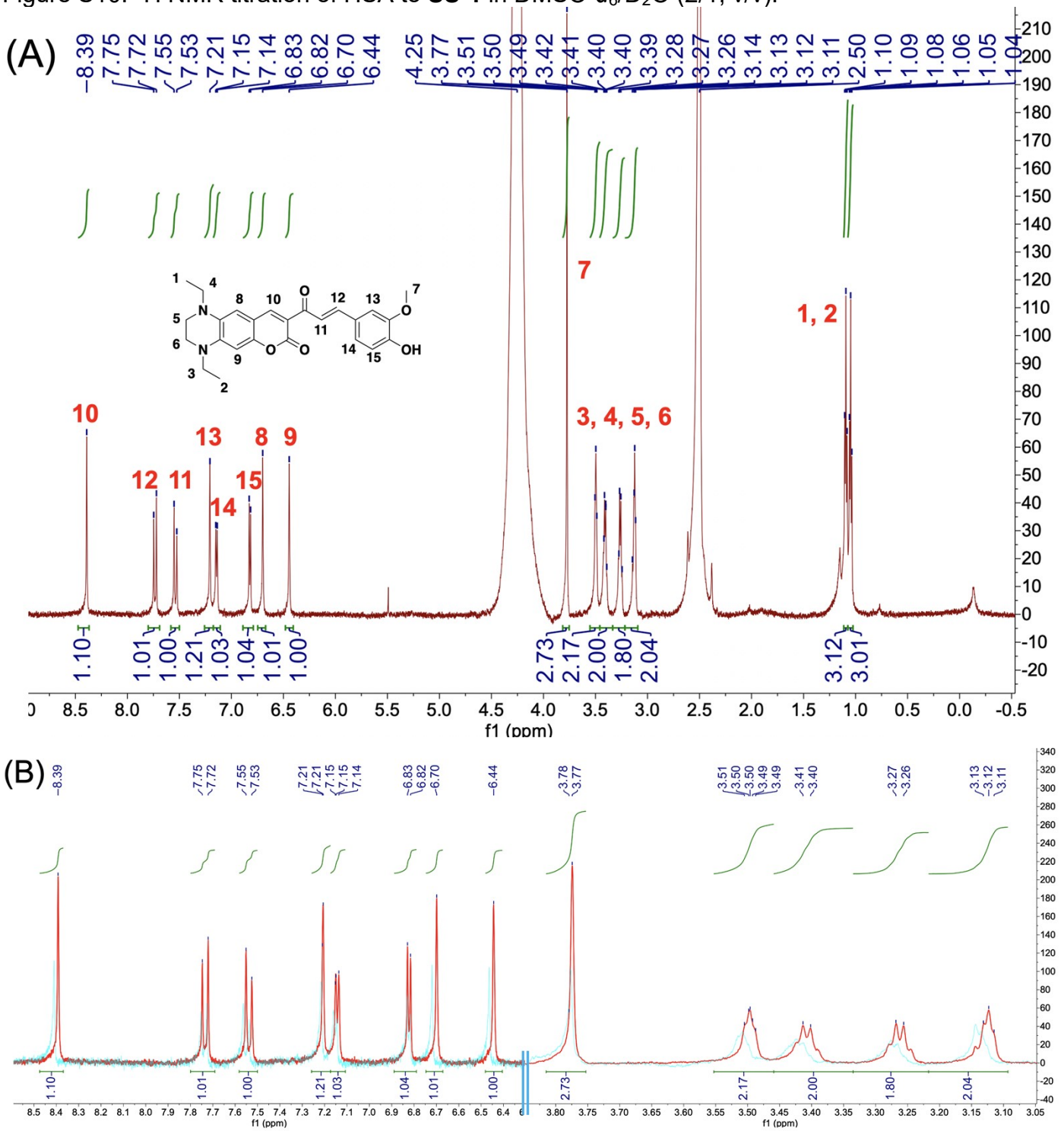
**SUPPLEMENTAL INFORMATION**

Figure S9. Fluorescence modulation of HSA and **SS-1**. (A) Fluorescent spectra changes of 5 μM **SS-1** toward 1 mg/mL HSA in the appearance of N-ethylmaleimide (NEM, 800 μM). (B) Aspirin (ASA, 400 μM) interference of HSA (1 mg/mL) labeling by **SS-1** (5 μM). (C) Fluorescent spectra of 5 μM **SS-1** in various solvents. (D) Fluorescent responses of **SS-1** toward lysine in a mixed solution containing DCM/EtOH/$H_2O$ (100/95/5, v/v/v) which was used to mimic the hydrophobic environment of the protein while ensure the soluble of lysine. $\lambda_{ex}$ = 505 nm; $\lambda_{em}$ = 525-700 nm; slit 5/5 nm for D and D; slit 5/2.5 nm for A and B; 700 v.



**SUPPLEMENTAL INFORMATION**

Figure S10. $^{1}H$ NMR titration of HSA to **SS-1** in DMSO-$d_6$/$D_2O$ (2/1, v/v).



**SUPPLEMENTAL INFORMATION**

Figure S11. Fragment Mass results of **L.KK[+416.174]Y.L** and the corresponding calculated mass data. The signals labeled b1 and y2 were found in the experimental result.

| # | a calc. | b calc. | b-18 calc. | Seq. | y calc. | y++ calc. | # |
|---|---|---|---|---|---|---|---|
| 1 | 101.1073 | **129.1022** | 111.0917 | K | | | 3 |
| 2 | 645.3760 | 673.3709 | 655.3603 | K | **726.3498** | 363.6786 | 2 |
| 3 | | | | Y | 182.0812 | 91.5442 | 1 |

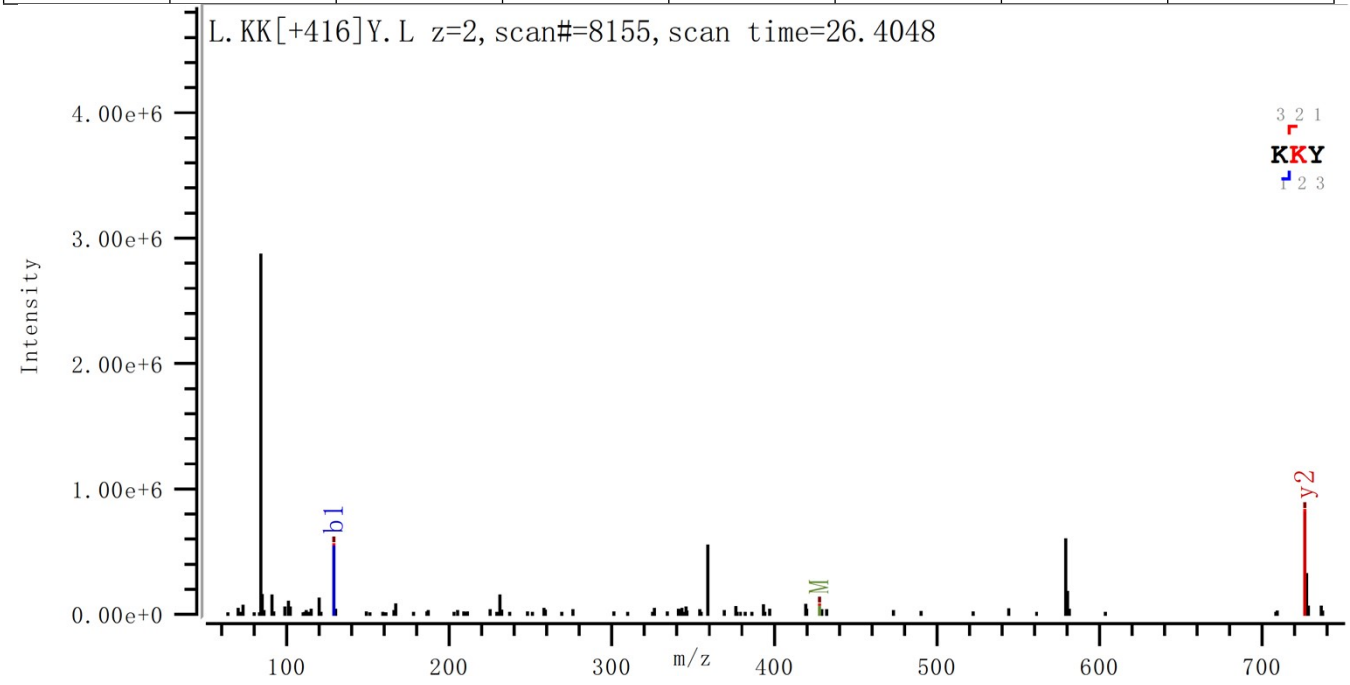
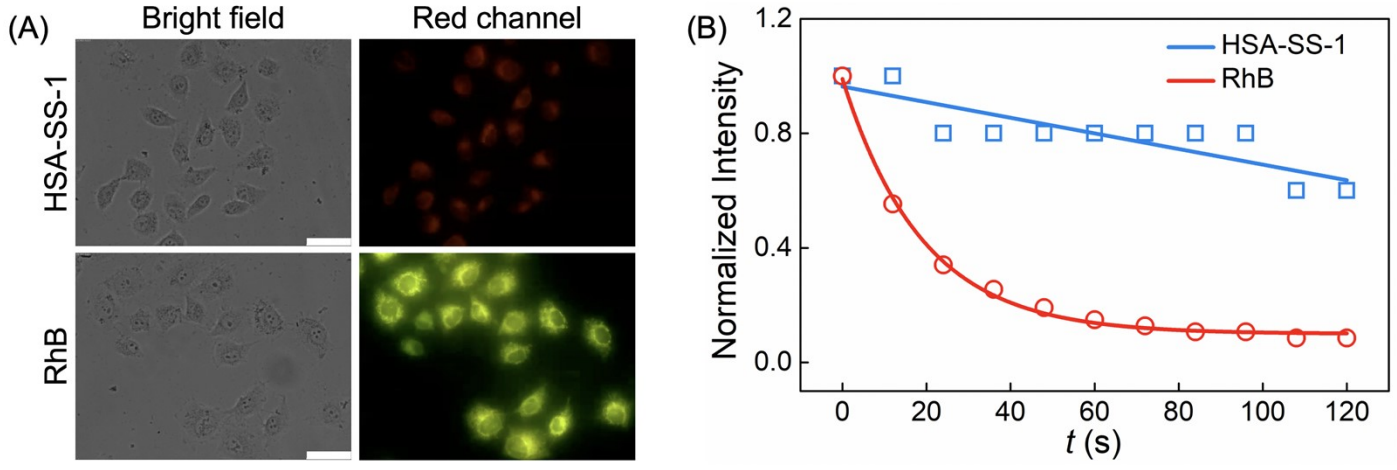



Figure S12. Cellular photo stability testing of HSA-**SS-1** and rhodamine B in HeLa cells upon continuous excitation light irradiation. All of parameters used in the two imaging experiments were exactly the same. The images of the red channel were taken from the original movies. For the HSA-**SS-1** group, HeLa cells preincubated with 1 mg/mL HSA containing incubation media for 1 h were washed with PBS for three times, which were further incubated with 10 μM **SS-1** containing PBS for 10 min. For the rhodamine B group, HeLa cells were incubated with 0.5 μM rhodamine B containing PBS for 15 min, the fluorescent movie were then obtained on a fluorescence microscope. (A) Initial fluorescence and bright field images for the HSA-**SS-1** and rhodamine B, respectively. (B) Normalized fluorescent intensities changes of the two group under light irradiation. $\lambda_{ex}$ = 536-556 nm; $\lambda_{em}$ = 560-625 nm.



**SUPPLEMENTAL INFORMATION**

Figure S13. *Ex vivo* organs fluorescent imaging of mice administrated with HSA-**SS-1** by intravenous injection which were sacrificed at 20 min and 60 min.

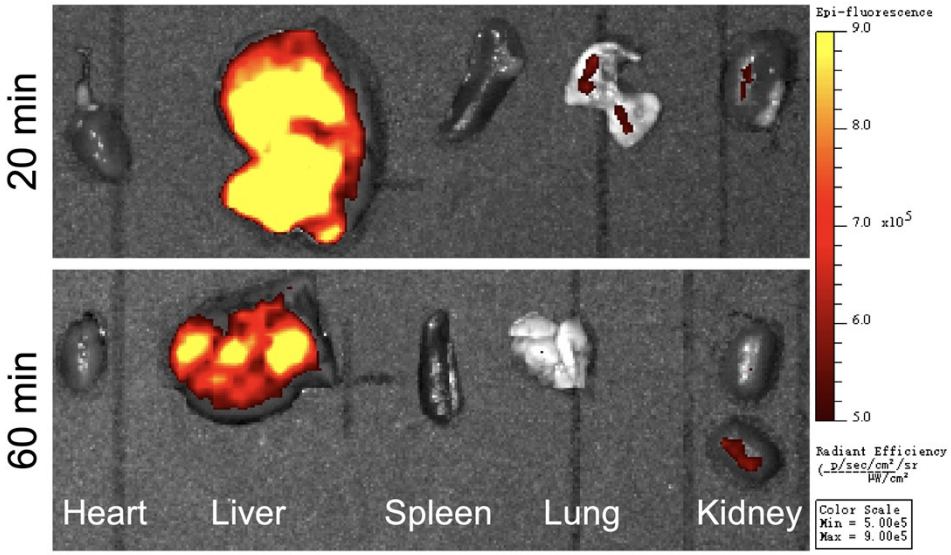
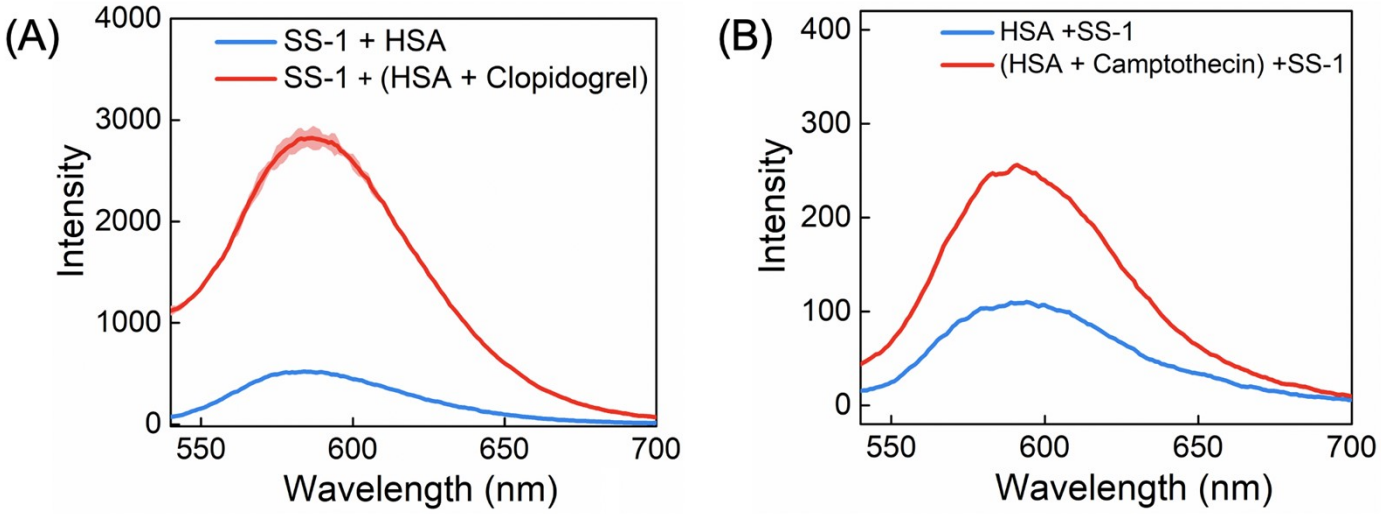



Figure S14. (A) Presence of clopidogrel (400 μM) or (B) camptothecin (400 μM) in the HSA labeling system induced fluorescent spectra changes. $\lambda_{ex}$ = 505 nm; $\lambda_{em}$ = 525-700 nm; slit 5/2.5 nm for A; slit 2.5/2.5 nm for B; 700 v.



**SUPPLEMENTAL INFORMATION**

Figure S15. Time dependent fluorescent images of nude mice administrated with 100 μL PBS solution containing 200 μM HSA, 100 μM **SS-1** and 0.5 % DMSO via intravenous injection. Histogram of the fluorescent intensities in ROI 1 and ROI 2.

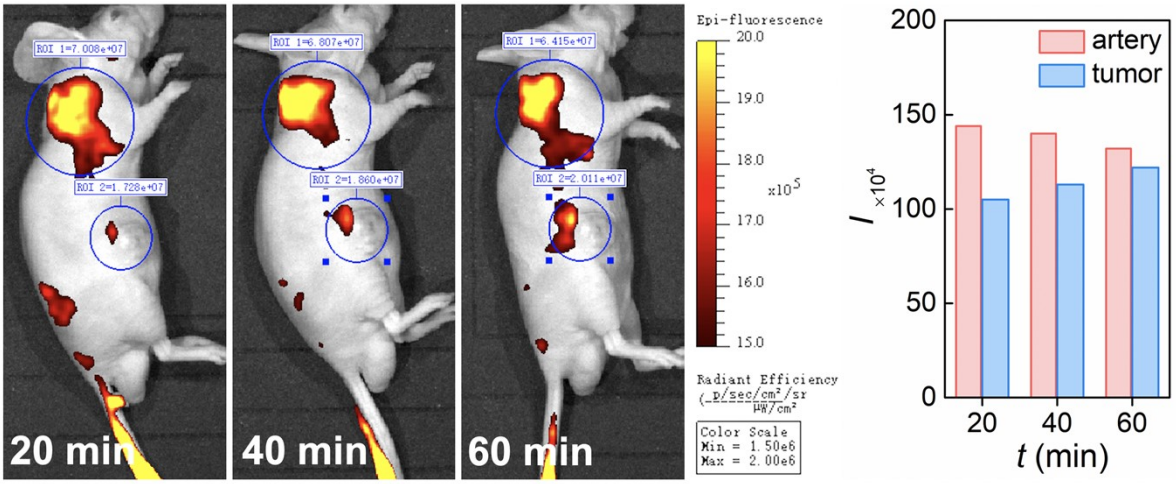
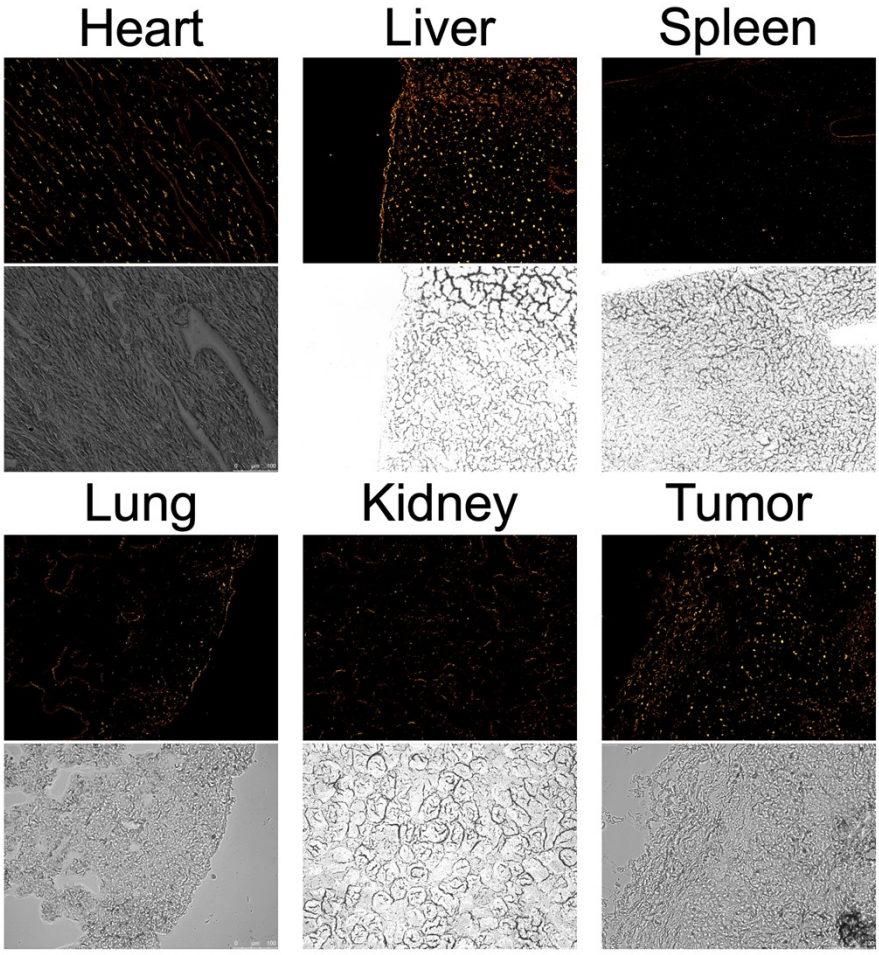



Figure S16. Fluorescent imaging of the tissue slices obtained from the corresponding organs. $\lambda_{ex}$ = 536-556 nm; $\lambda_{em}$ = 560-625 nm.



**SUPPLEMENTAL INFORMATION**

# Additional tables

Table S1. Protein analysis of the Native-PAGE Precast Gel pieces in the red circle.

| Accession | Protein names | Gene names | MW [kDa] | Protein score | Sequence coverage (%) | # Unique Peptides | # Peptides | # PSMs |
|---|---|---|---|---|---|---|---|---|
| P01024 | Complement C3 | C3 | 187.03 | 9739.89 | 59.95 | 85 | 85 | 235 |
| P01023 | Alpha-2-macroglobulin | A2M | 163.19 | 4341.60 | 42.27 | 40 | 47 | 109 |
| P00450 | Ceruloplasmin | CP | 122.13 | 4003.68 | 37.37 | 36 | 36 | 87 |
| P02787 | Serotransferrin | TF | 77.01 | 3439.42 | 31.38 | 22 | 22 | 94 |
| P0C0L5 | Complement C4-B | C4B | 192.63 | 3156.34 | 37.67 | 3 | 52 | 71 |
| P0C0L4 | Complement C4-A | C4A | 192.66 | 3036.99 | 36.35 | 2 | 51 | 69 |
| P19827 | Inter-alpha-trypsin inhibitor heavy chain H1 | ITIH1 | 101.33 | 2041.03 | 35.89 | 23 | 23 | 47 |
| P02647 | Apolipoprotein A-I | APOA1 | 30.76 | 1912.94 | 76.03 | 26 | 26 | 51 |
| P02751 | Fibronectin | FN1 | 272.15 | 1664.69 | 16.79 | 29 | 29 | 39 |
| P19823 | Inter-alpha-trypsin inhibitor heavy chain H2 | ITIH2 | 106.40 | 1627.88 | 34.78 | 29 | 29 | 46 |
| P35527 | Keratin, type I cytoskeletal 9 | KRT9 | 62.03 | 1563.28 | 55.86 | 22 | 23 | 34 |
| Q14624 | Inter-alpha-trypsin inhibitor heavy chain H4 | ITIH4 | 103.29 | 1476.56 | 35.27 | 24 | 24 | 34 |
| P06396 | Gelsolin | GSN | 85.64 | 1400.11 | 37.08 | 23 | 23 | 34 |
| P04264 | Keratin, type II cytoskeletal 1 | KRT1 | 66.00 | 1135.31 | 44.41 | 19 | 22 | 26 |
| P01008 | Antithrombin-III | SERPINC1 | 52.57 | 1132.95 | 41.16 | 18 | 18 | 27 |
| P02768 | Serum albumin | ALB | 69.32 | 1132.73 | 25.45 | 18 | 18 | 34 |
| P01009 | Alpha-1-antitrypsin | SERPINA1 | 46.71 | 1130.51 | 52.15 | 20 | 20 | 30 |
| P06727 | Apolipoprotein A-IV | APOA4 | 45.37 | 1030.74 | 45.96 | 18 | 18 | 26 |
| P02790 | Hemopexin | HPX | 51.64 | 959.20 | 24.68 | 9 | 9 | 26 |
| P20742 | Pregnancy zone protein | PZP | 163.76 | 958.61 | 19.03 | 15 | 22 | 27 |
| Q96PD5 | N-acetylmuramoyl-L-alanine amidase | PGLYRP2 | 62.18 | 935.95 | 34.72 | 10 | 10 | 18 |
| P29622 | Kallistatin | SERPINA4 | 48.51 | 922.31 | 44.03 | 17 | 17 | 24 |
| P06681 | Complement C2 | C2 | 83.21 | 887.37 | 22.47 | 17 | 17 | 23 |
| P10909 | Clusterin | CLU | 52.46 | 873.74 | 27.39 | 13 | 13 | 22 |
| P01011 | Alpha-1-antichymotrypsin | SERPINA3 | 47.62 | 869.71 | 33.10 | 11 | 11 | 21 |
| P05546 | Heparin cofactor 2 | SERPIND1 | 57.03 | 864.01 | 28.26 | 11 | 11 | 21 |
| P02649 | Apolipoprotein E | APOE | 36.13 | 859.66 | 49.84 | 15 | 15 | 19 |
| P08603 | Complement factor H | CFH | 139.00 | 816.50 | 12.92 | 14 | 14 | 22 |
| P13645 | Keratin, type I cytoskeletal 10 | KRT10 | 58.79 | 793.70 | 29.28 | 15 | 17 | 19 |
| P09871 | Complement C1s subcomponent | C1S | 76.63 | 765.21 | 24.13 | 14 | 15 | 22 |
| P00751 | Complement factor B | CFB | 85.48 | 752.73 | 21.47 | 16 | 16 | 20 |
| P05155 | Plasma protease C1 inhibitor | SERPING1 | 55.12 | 730.32 | 22.00 | 11 | 11 | 18 |
| P01019 | Angiotensinogen | AGT | 53.12 | 694.52 | 30.72 | 10 | 10 | 13 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| P02748 | Complement component C9 | C9 | 63.13 | 680.45 | 24.51 | 13 | 13 | 16 |
| P35908 | Keratin, type II cytoskeletal 2 epidermal | KRT2 | 65.39 | 606.06 | 29.89 | 10 | 15 | 15 |
| P00736 | Complement C1r subcomponent | C1R | 80.07 | 583.66 | 16.88 | 7 | 8 | 12 |
| P04004 | Vitronectin | VTN | 54.27 | 554.48 | 19.67 | 6 | 6 | 13 |
| P01876 | Immunoglobulin heavy constant alpha 1 | IGHA1 | 37.63 | 526.40 | 20.96 | 3 | 7 | 13 |
| P05156 | Complement factor I | CFI | 65.71 | 507.94 | 17.32 | 8 | 8 | 12 |
| P01857 | Immunoglobulin heavy constant gamma 1 | IGHG1 | 36.08 | 482.04 | 27.88 | 3 | 6 | 11 |

Table S2. Protein analysis of the Native-PAGE Precast Gel pieces in the green circle.

| Accession | Protein names | Gene names | MW [kDa] | Protein score | Sequence coverage (%) | # Unique Peptides | # Peptides | # PSMs |
|---|---|---|---|---|---|---|---|---|
| P01009 | Alpha-1-antitrypsin | SERPINA1 | 46.71 | 3238.03 | 56.94 | 24 | 24 | 106 |
| P02768 | Serum albumin | ALB | 69.32 | 2068.41 | 30.21 | 25 | 25 | 73 |
| P04264 | Keratin, type II cytoskeletal 1 | KRT1 | 66.00 | 1088.74 | 30.90 | 16 | 19 | 27 |
| P35527 | Keratin, type I cytoskeletal 9 | KRT9 | 62.03 | 897.75 | 43.50 | 18 | 19 | 21 |
| P13645 | Keratin, type I cytoskeletal 10 | KRT10 | 58.79 | 823.58 | 27.40 | 14 | 17 | 20 |
| P01024 | Complement C3 | C3 | 187.03 | 793.19 | 8.48 | 10 | 10 | 19 |
| P01019 | Angiotensinogen | AGT | 53.12 | 792.45 | 36.70 | 12 | 12 | 19 |
| P02647 | Apolipoprotein A-I | APOA1 | 30.76 | 669.93 | 56.93 | 17 | 17 | 20 |
| P35908 | Keratin, type II cytoskeletal 2 epidermal | KRT2 | 65.39 | 637.50 | 30.36 | 11 | 15 | 15 |
| P0C0L5 | Complement C4-B | C4B | 192.63 | 590.72 | 10.21 | 12 | 12 | 15 |
| P01011 | Alpha-1-antichymotrypsin | SERPINA3 | 47.62 | 490.08 | 27.90 | 10 | 10 | 15 |
| P43652 | Afamin | AFM | 69.02 | 486.95 | 20.87 | 12 | 12 | 17 |
| P02765 | Alpha-2-HS-glycoprotein | AHSG | 39.32 | 469.99 | 10.08 | 3 | 3 | 10 |
| P04217 | Alpha-1B-glycoprotein | A1BG | 54.22 | 453.20 | 23.03 | 9 | 9 | 13 |
| P00450 | Ceruloplasmin | CP | 122.13 | 441.69 | 13.24 | 11 | 11 | 11 |
| P01042 | Kininogen-1 | KNG1 | 71.91 | 425.10 | 12.73 | 8 | 8 | 12 |
| P08185 | Corticosteroid-binding globulin | SERPINA6 | 45.11 | 402.93 | 24.20 | 7 | 7 | 10 |
| P08779 | Keratin, type I cytoskeletal 16 | KRT16 | 51.24 | 349.98 | 17.34 | 5 | 8 | 10 |
| P05543 | Thyroxine-binding globulin | SERPINA7 | 46.29 | 305.78 | 18.07 | 6 | 6 | 7 |
| P02774 | Vitamin D-binding protein | GC | 52.88 | 293.27 | 7.81 | 5 | 5 | 7 |
| P13647 | Keratin, type II cytoskeletal 5 | KRT5 | 62.34 | 276.94 | 12.03 | 4 | 8 | 8 |
| P01008 | Antithrombin-III | SERPINC1 | 52.57 | 243.33 | 16.38 | 7 | 7 | 7 |
| P25311 | Zinc-alpha-2-glycoprotein | AZGP1 | 34.24 | 220.38 | 20.47 | 4 | 4 | 5 |
| P04180 | Phosphatidylcholine-sterol acyltransferase | LCAT | 49.55 | 217.67 | 12.50 | 5 | 5 | 5 |
| P48668 | Keratin, type II cytoskeletal 6C | KRT6C | 59.99 | 214.31 | 10.28 | 3 | 7 | 7 |
| P04004 | Vitronectin | VTN | 54.27 | 197.46 | 19.25 | 6 | 6 | 7 |
| P02656 | Apolipoprotein C-III | APOC3 | 10.85 | 189.05 | 27.27 | 2 | 2 | 3 |

**SUPPLEMENTAL INFORMATION**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| P27169 | Serum paraoxonase/arylesterase 1 | PON1 | 39.71 | 188.42 | 18.31 | 5 | 5 | 6 |
| P02787 | Serotransferrin | TF | 77.01 | 180.35 | 14.61 | 5 | 5 | 5 |
| Q04695 | Keratin, type I cytoskeletal 17 | KRT17 | 48.08 | 177.08 | 11.34 | 3 | 5 | 5 |

Table S3. Protein identification by enzyme digestion experiment.

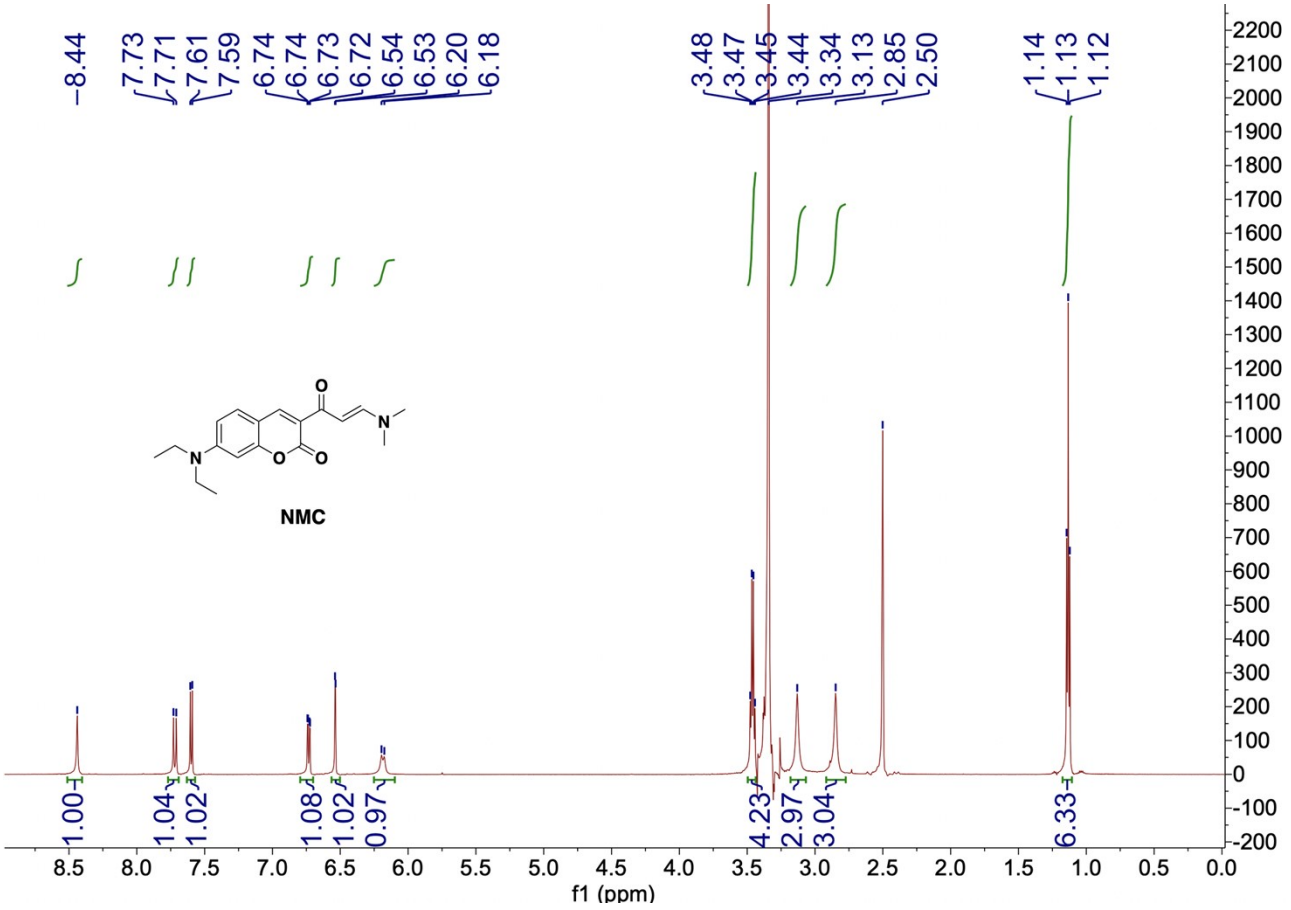
| Sample ID | Enzyme | Protein IDs | Log Prob | Total intensity | # of spectra | Coverage (%) | # AA's in protein |
|---|---|---|---|---|---|---|---|
| HSA | Trypsin | P02768 | 613.31 | 2563374158.6 | 102 | 86.37 | 609 |
| | Chymotrypsin | P02768 | 392.15 | 2417327878.7 | 161 | 92.61 | 609 |

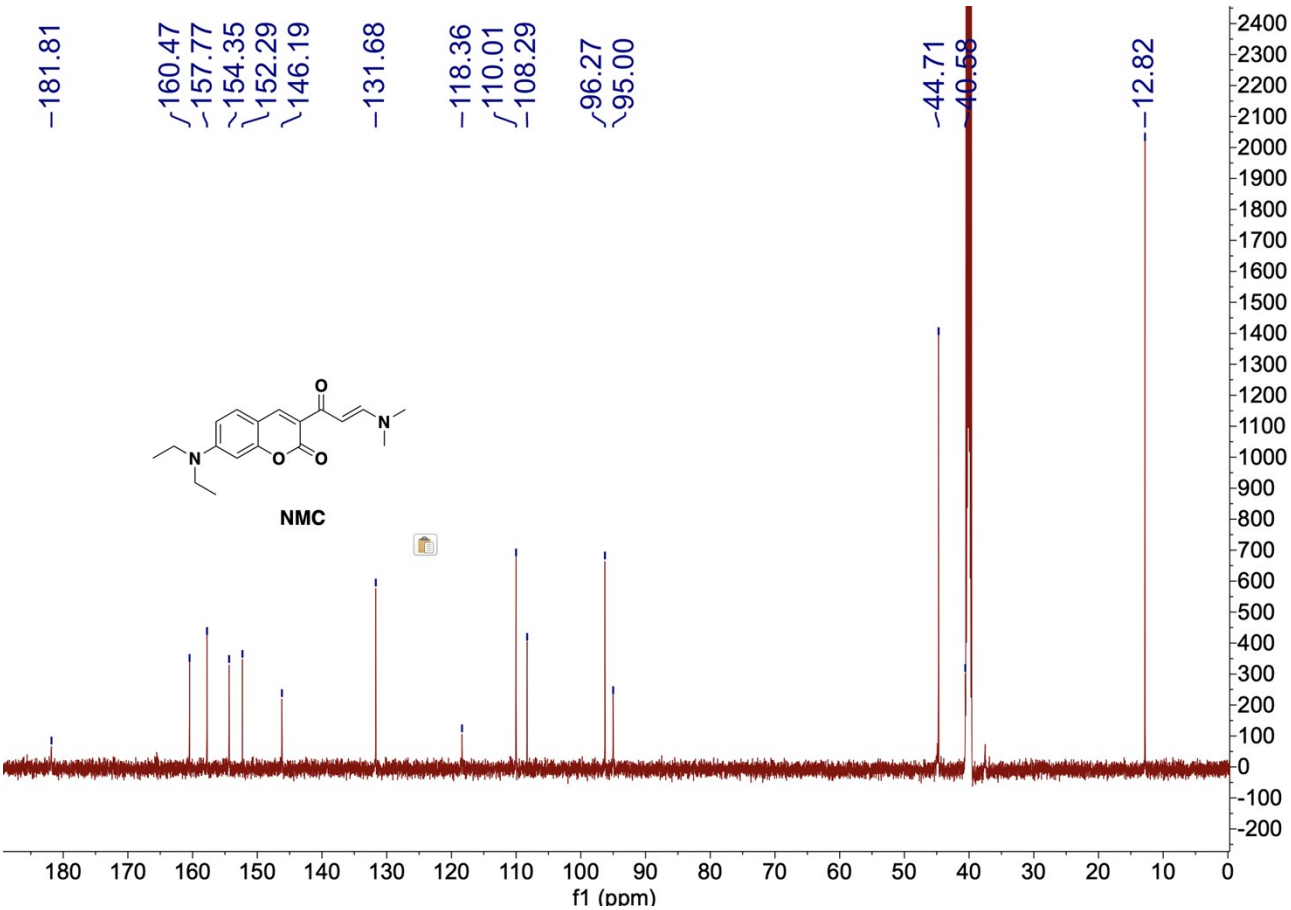
Table S4. Peptide mass spectra of **SS-1** modified HSA. The amino acids of interest in the sequence of HSA that are the same as BSA but different from RSA are marked in red.

| | Position | Peptide <Protein Metrics Confidential> | Modification Type(s) | Observed m/z | z | Observed (M+H) | Calc. mass (M+H) | Mass error (ppm) | Starting position | Score | Scan Time | Intensity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Chymotrypsin | 161 | L.KK[+416.174]Y.L | K[+416] | 427.732 | 2 | 854.457 | 854.445 | 14.0 | 160 | 103.3 | 26.4048 | 143020000 |
| | 19 | Y.SR[+416.174]GVF.R | R[+416] | 491.251 | 2 | 981.495 | 981.483 | 12.3 | 18 | 97.6 | 29.4086 | 1175700 |
| | 524 | Y.VPK[+416.174]EF.N | K[+416] | 518.262 | 2 | 1035.518 | 1035.519 | -1.1 | 522 | 89.9 | 15.432 | 6235300 |
| | 452 | L.VEVSR[+416.174]NL.G | R[+416] | 616.810 | 2 | 1232.613 | 1232.631 | -14.7 | 448 | 74.7 | 15.4171 | 11312000 |
| | 138 | L.PR[+416.174]L.V | R[+416] | 401.213 | 2 | 801.420 | 801.430 | -12.4 | 137 | 71.3 | 11.9504 | 3591600 |
| | 434 | L.VR[+434.184]Y.T | R[+434] | 436.222 | 2 | 871.436 | 871.435 | 1.7 | 433 | 52.9 | 35.675 | 8052200 |
| | 138 | L.PR[+434.184]L.V | R[+434] | 410.229 | 2 | 819.451 | 819.440 | 13.0 | 137 | 48.8 | 26.1631 | 7118000 |
| | 434 | L.LVR[+434.184]Y.T | R[+434] | 492.755 | 2 | 984.503 | 984.519 | -16.4 | 432 | 41.5 | 23.256 | 0 |
| | 183 | L.LFFAK[+416.174]RY.K | K[+416] | 680.863 | 2 | 1360.719 | 1360.709 | 7.5 | 179 | 41.5 | 41.2275 | 9074500 |
| | 168 | Y.EIAR[+434.184]RHPYF.Y | R[+434] | 811.919 | 2 | 1622.831 | 1622.812 | 11.9 | 165 | 32.3 | 38.5851 | 9984600 |
| | 168 | L.YEIAR[+416.174]RHPYF.Y | R[+416] | 442.726 | 4 | 1767.883 | 1767.864 | 10.5 | 164 | 22.2 | 21.3624 | 17065000 |
| | 360 | Y.AR[+434.184]RHPDY.S | R[+434] | 674.834 | 2 | 1348.660 | 1348.643 | 12.5 | 359 | 17.8 | 59.2793 | 41180000 |
| | 205 | L.PK[+416.174]L.D | K[+416] | 387.211 | 2 | 773.415 | 773.423 | -11.4 | 204 | 14.5 | 10.4921 | 793300 |
| | 413 | L.IK[+434.184]QNC[+57.021]EL.F | C[+57], K[+434] | 669.811 | 2 | 1338.615 | 1338.640 | -18.6 | 412 | 10.4 | 14.7076 | 11763000 |
| | | | | | | | | | | | | |
| Trypsin | 219 | K.ASSAK[+416.174]QR.L | K[+416] | 582.297 | 2 | 1163.587 | 1163.585 | 1.7 | 215 | 98.4 | 20.6137 | 11725000 |
| | 141 | R.LVR[+434.184].P | R[+434] | 411.235 | 2 | 821.463 | 821.456 | 9.2 | 139 | 82.5 | 21.7968 | 49301000 |
| | 223 | R.LK[+434.184].C | K[+434] | 347.698 | 2 | 694.388 | 694.381 | 9.6 | 222 | 48.5 | 11.5212 | 27439000 |
| | 210 | K.LDELR[+434.184].D | R[+434] | 540.269 | 2 | 1079.531 | 1079.541 | -9.4 | 206 | 37.0 | 22.9142 | 30591000 |
| | 560 | K.HK[+416.174]PK.A | K[+416] | 463.260 | 2 | 925.512 | 925.493 | 20.0 | 559 | 19.1 | 16.9511 | 5024600 |
| | 468 | K.HPEAK[+434.184]R.M | K[+434] | 586.304 | 2 | 1171.600 | 1171.590 | 9.1 | 464 | 2.4 | 35.2349 | 77037000 |

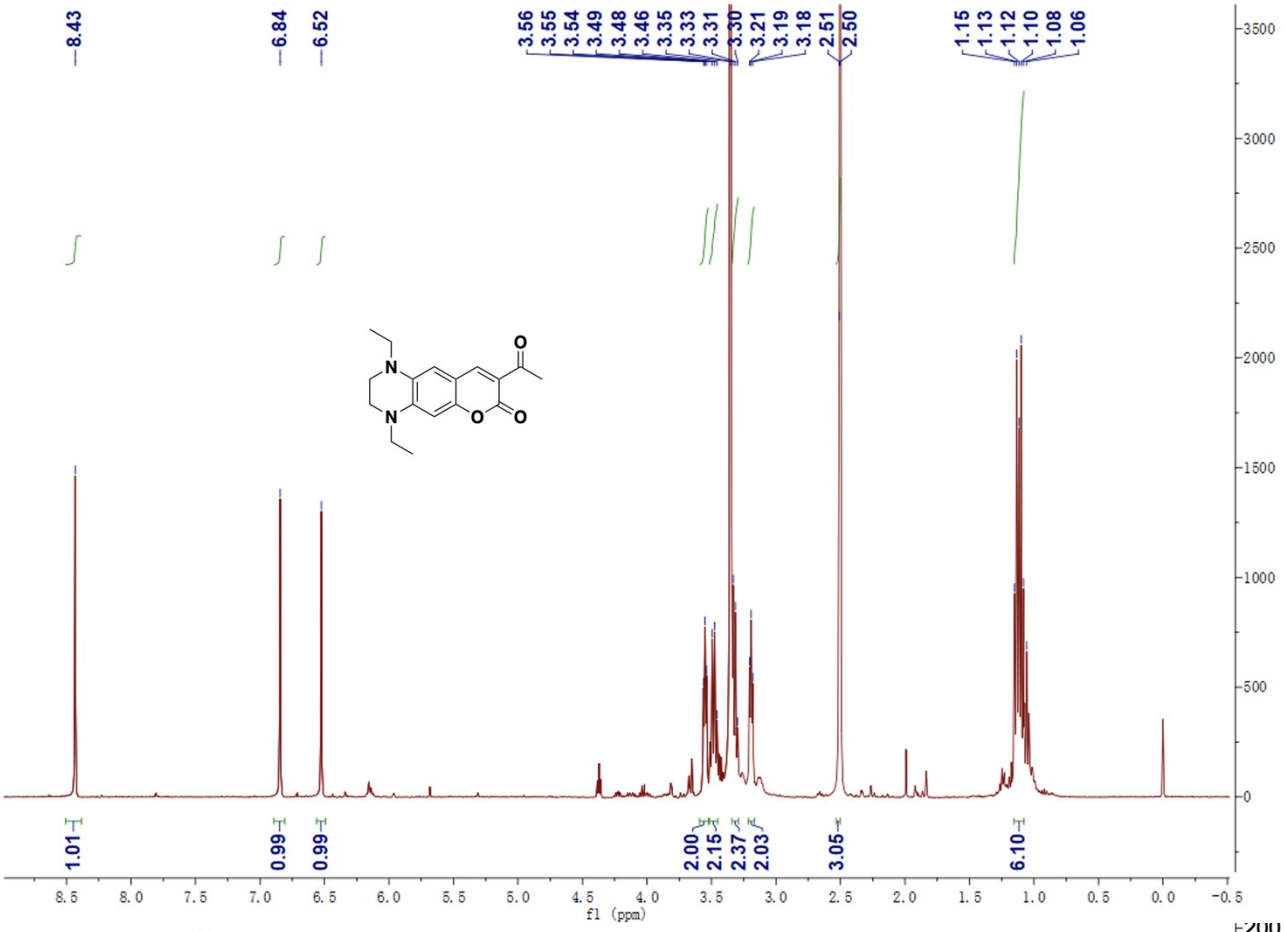
SUPPLEMENTAL INFORMATION

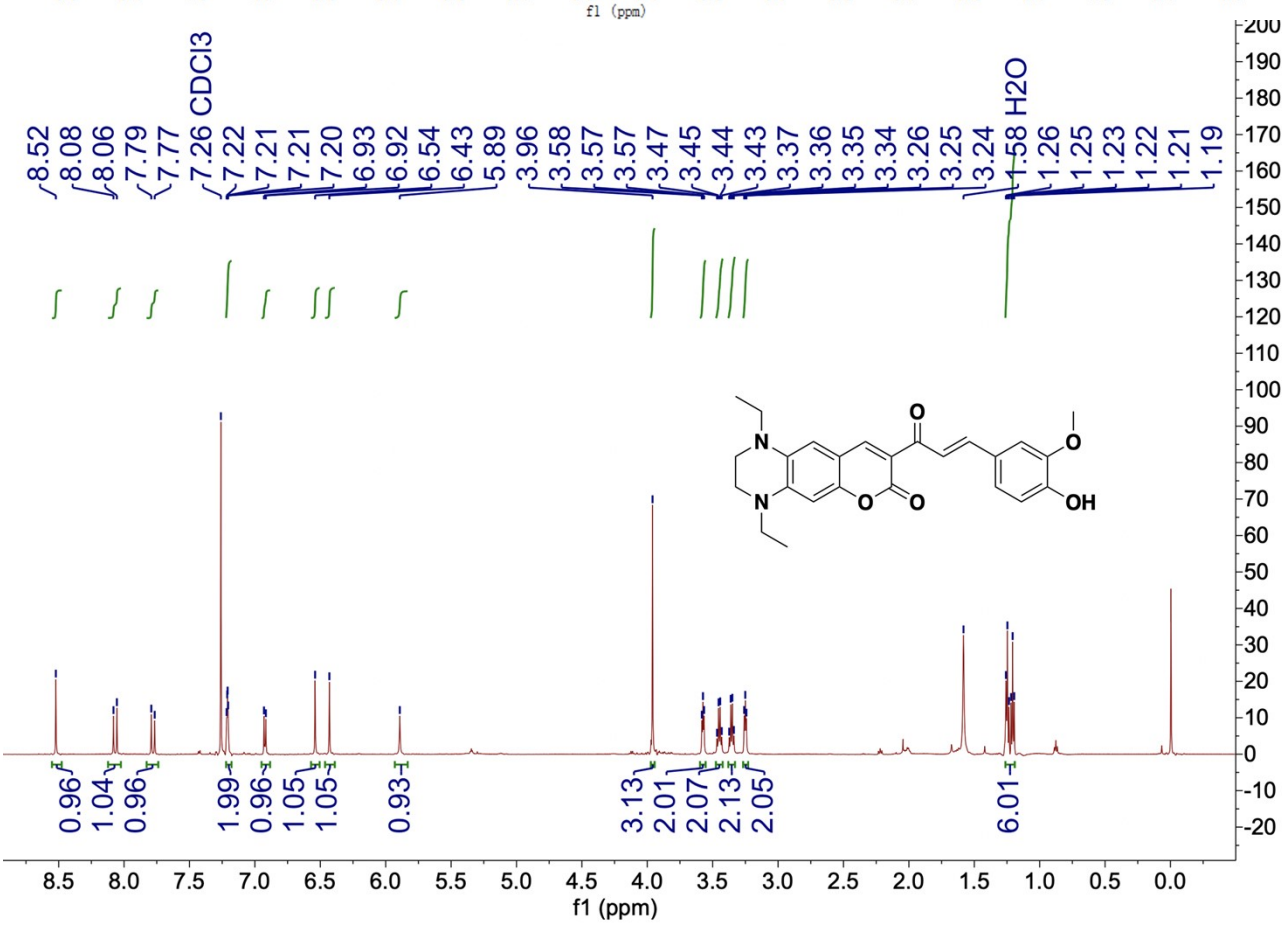
# Characterization



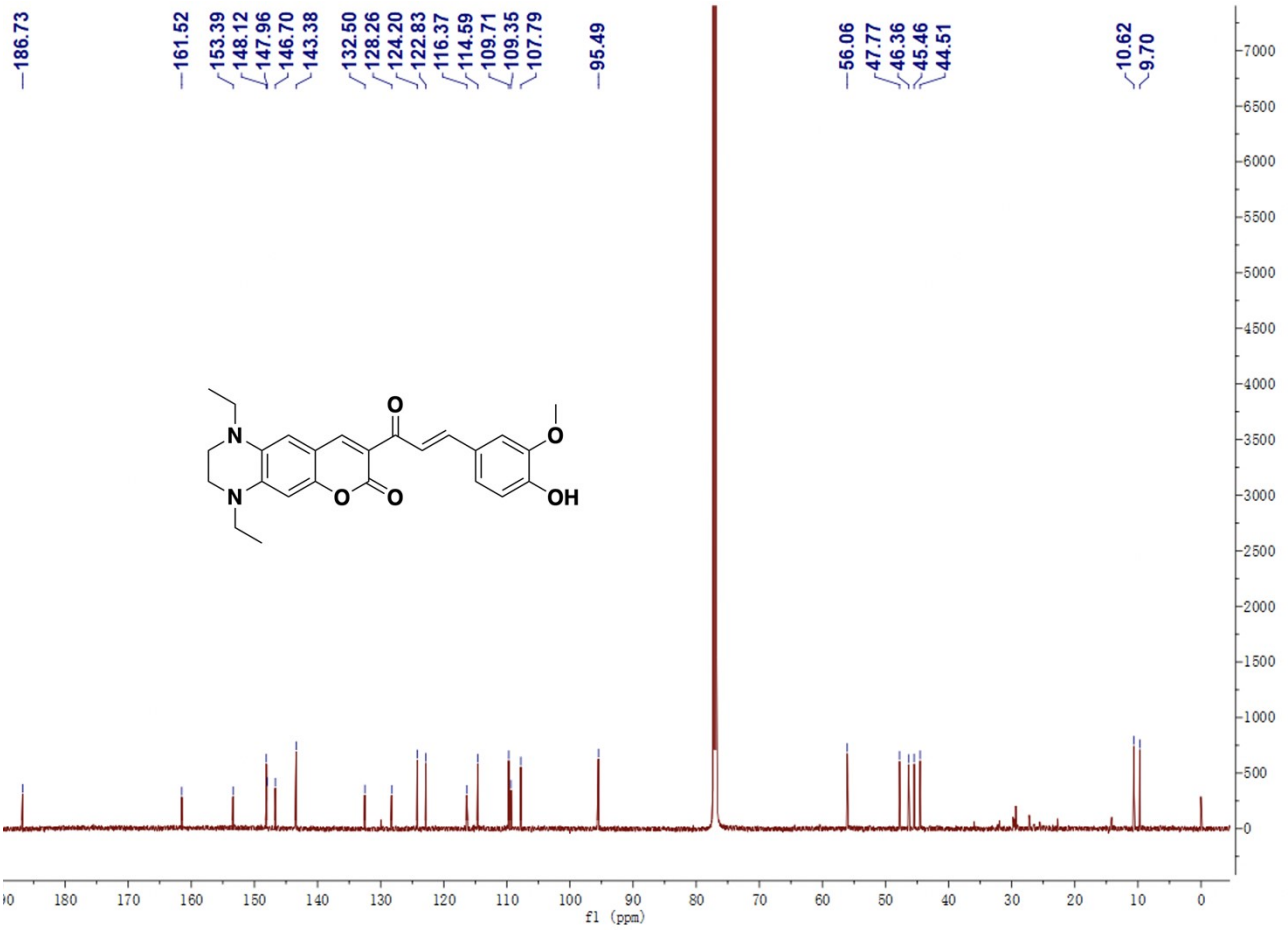



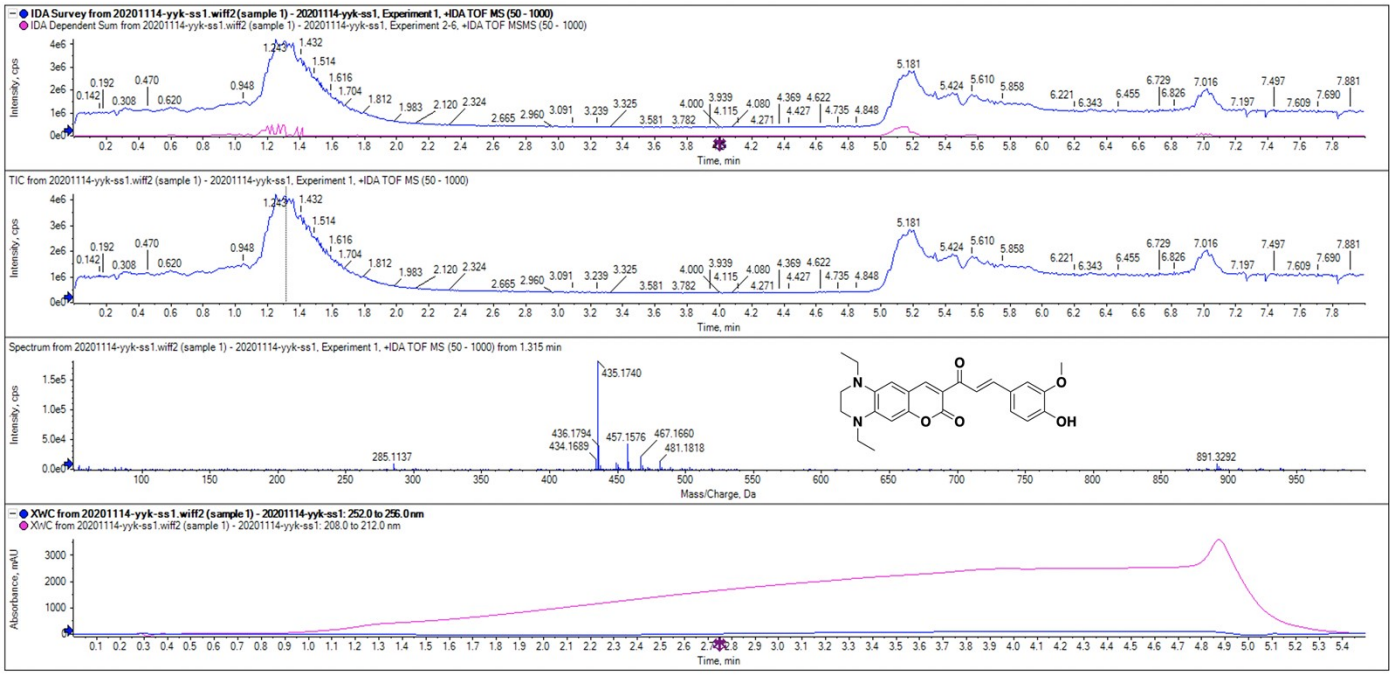
**SUPPLEMENTAL INFORMATION**



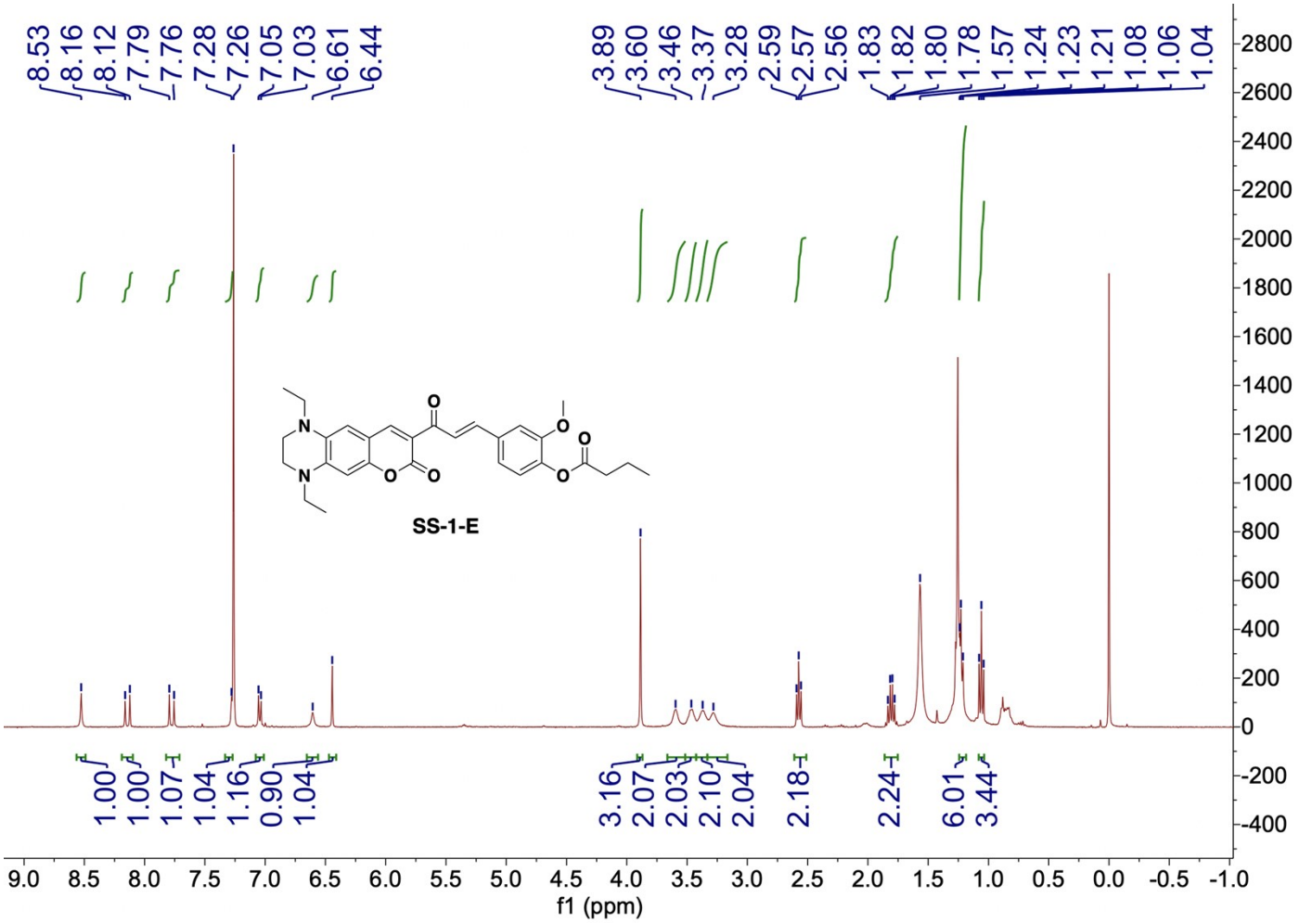



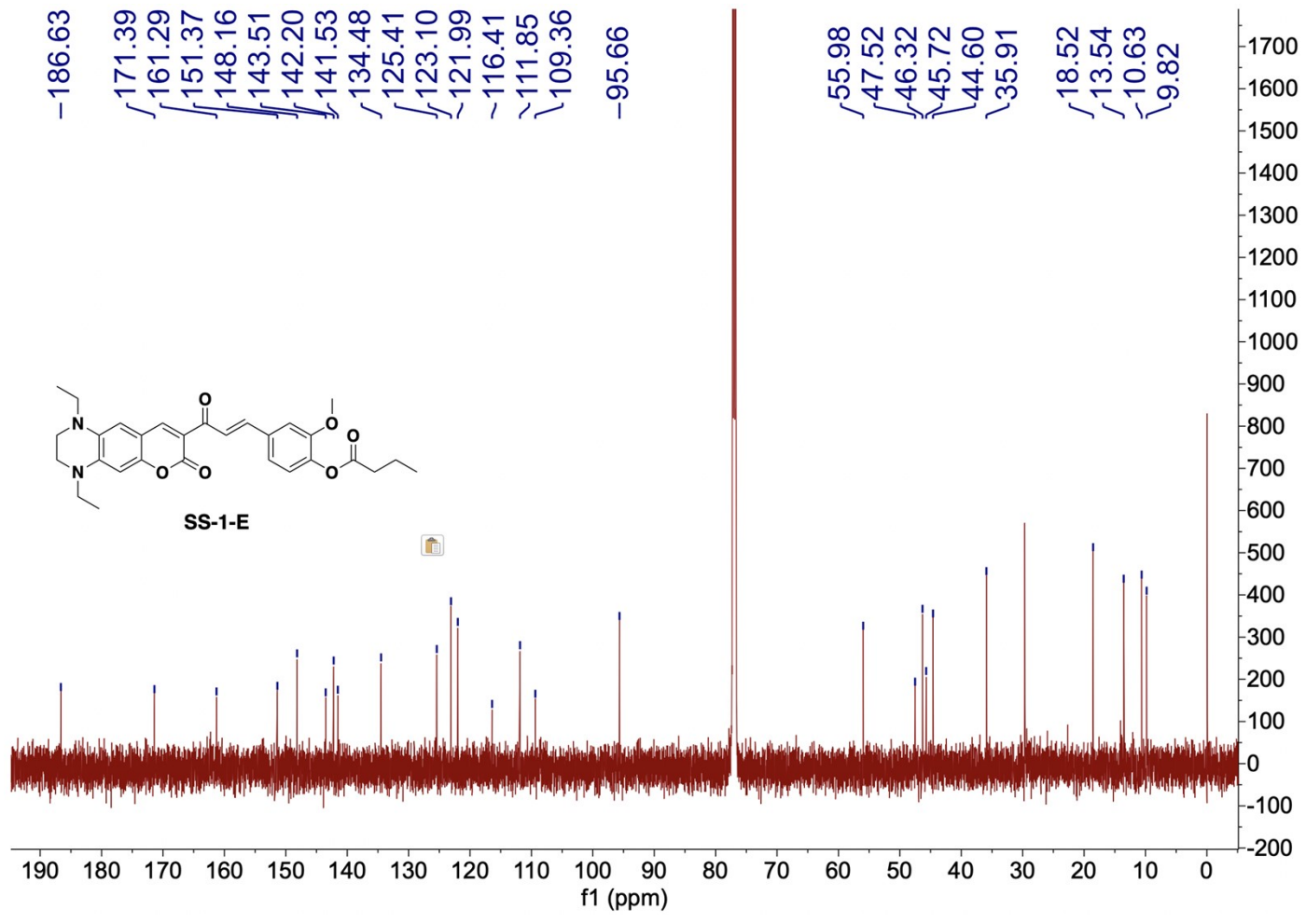
## SUPPLEMENTAL INFORMATION



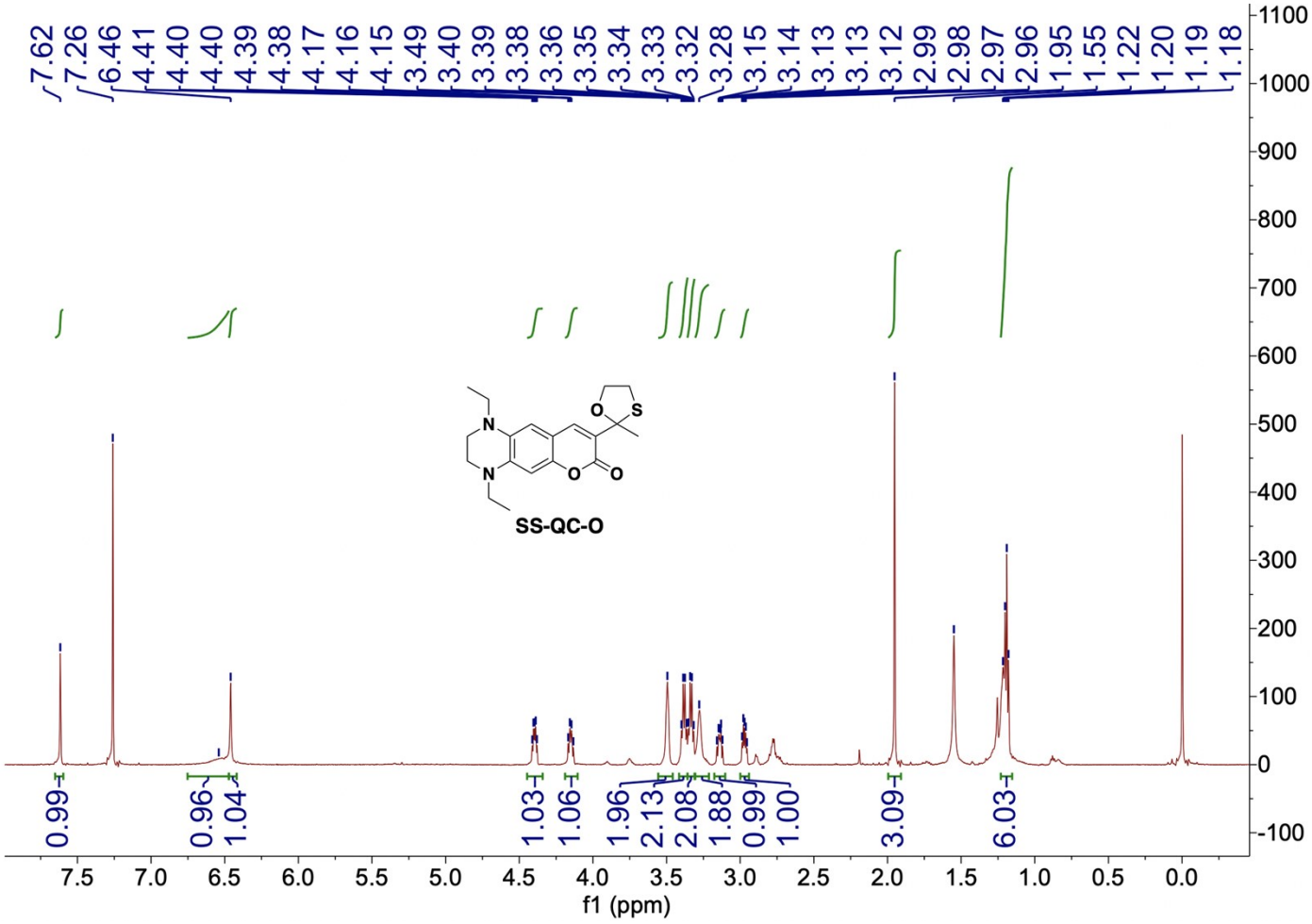



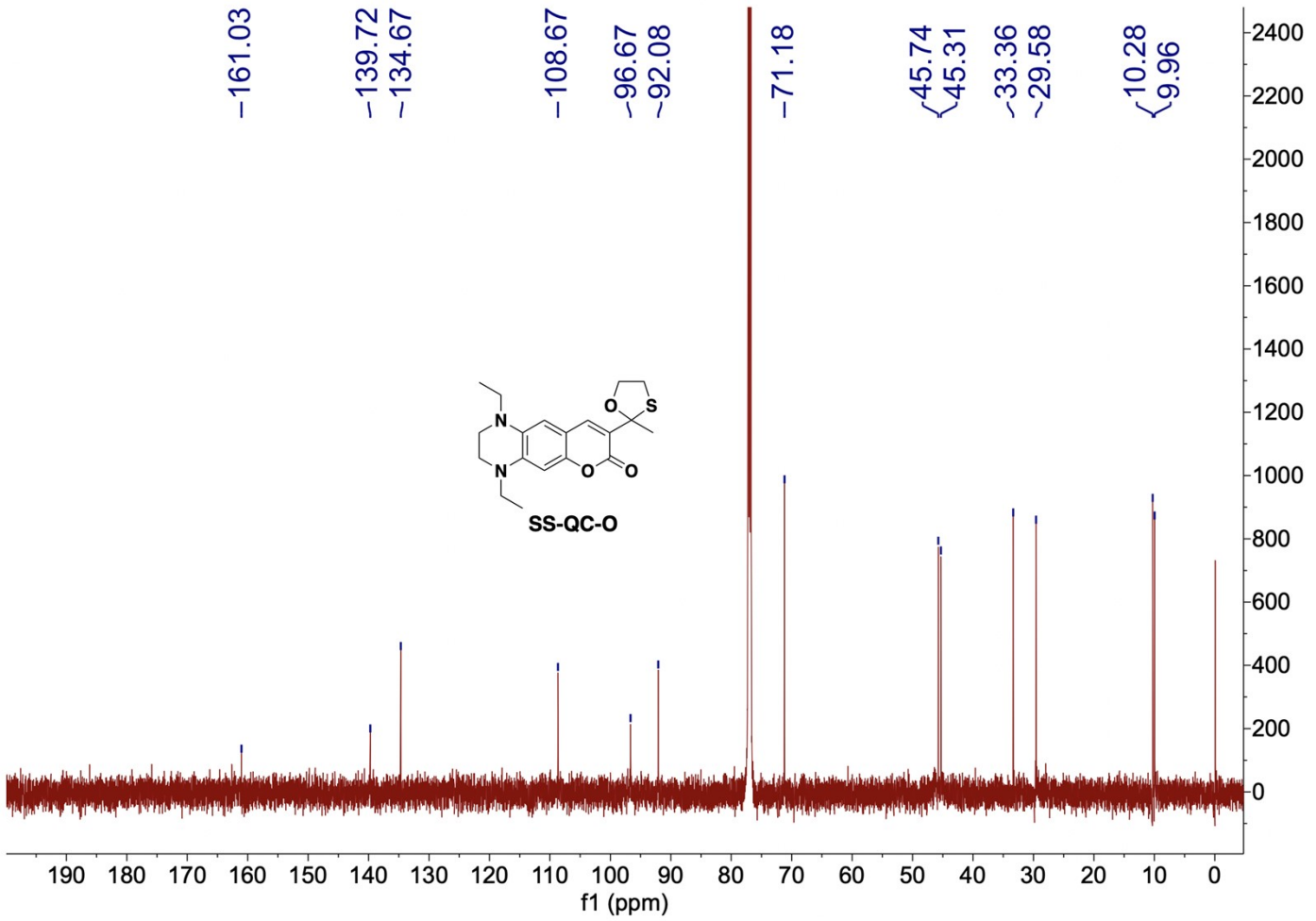
**SUPPLEMENTAL INFORMATION**



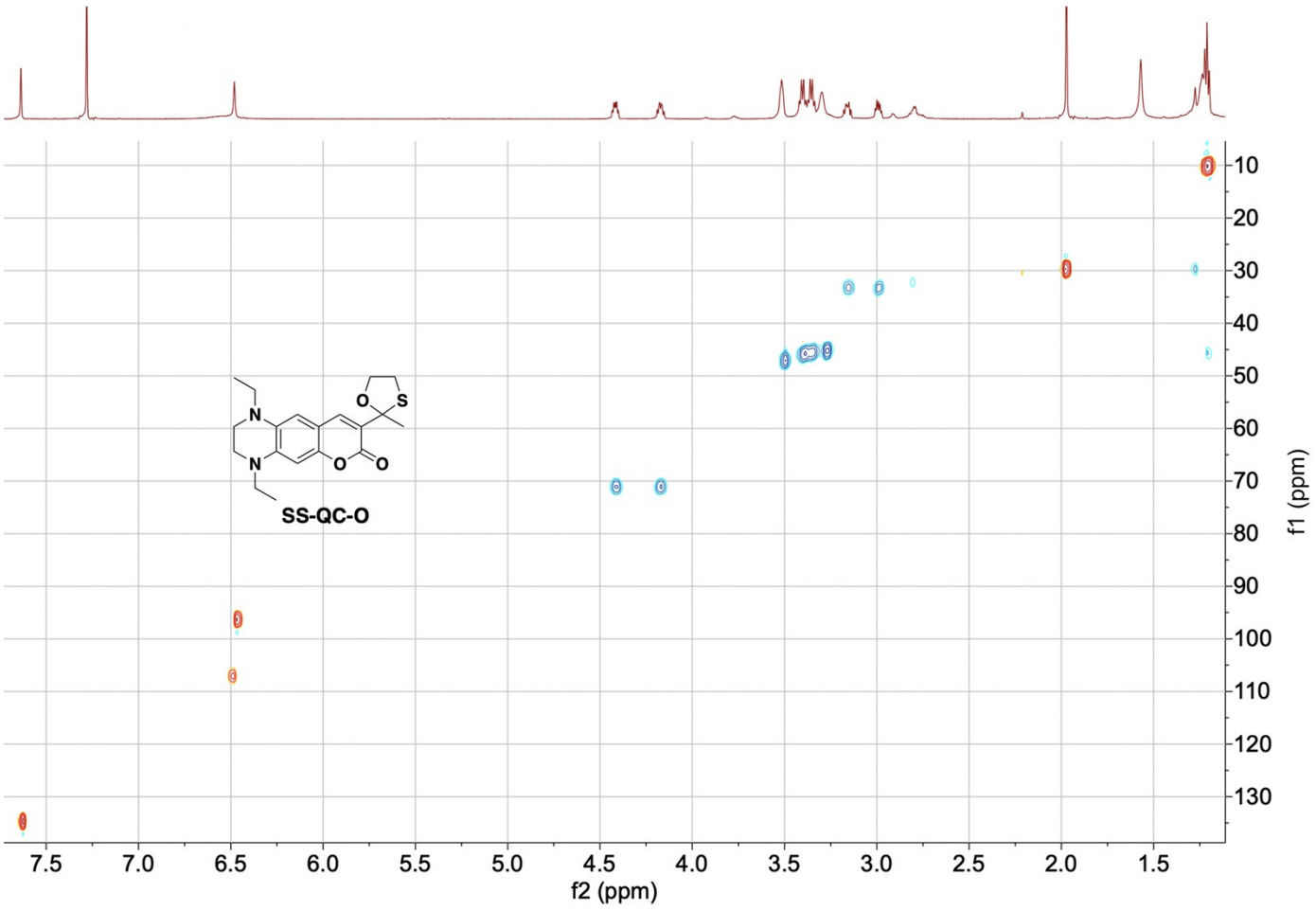



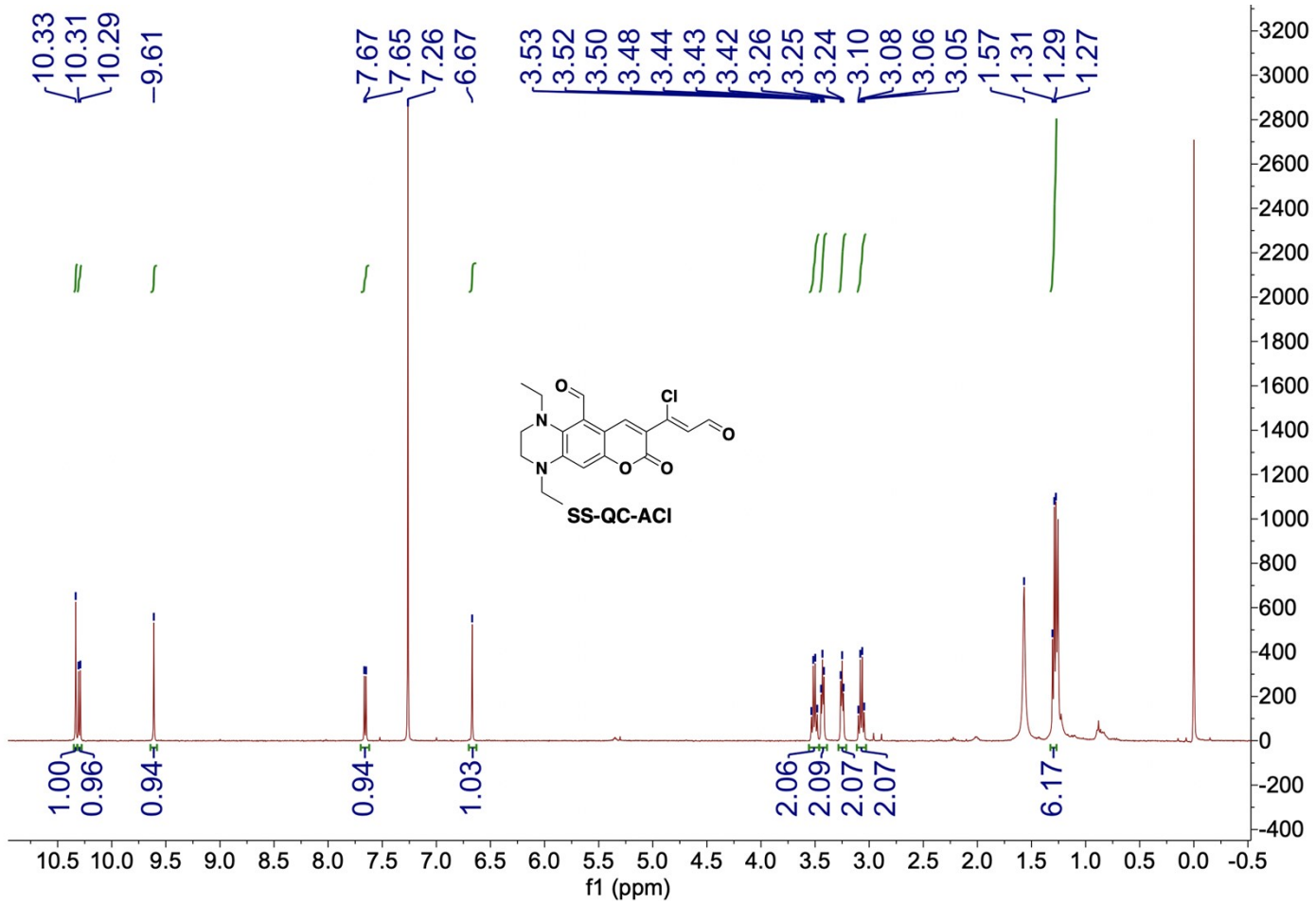
**SUPPLEMENTAL INFORMATION**



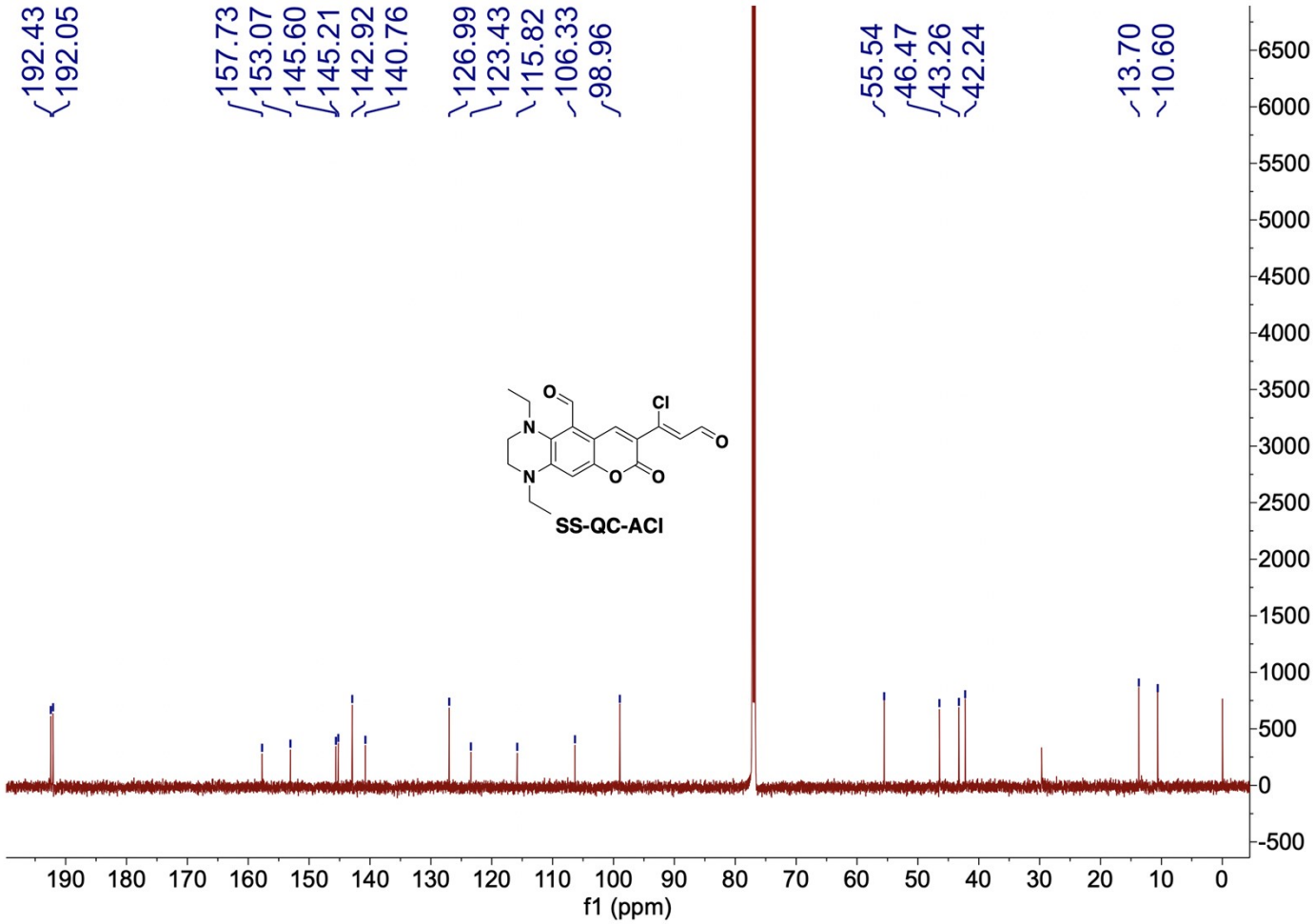



**SUPPLEMENTAL INFORMATION**



**SUPPLEMENTAL INFORMATION**



## Reference

1. Yue, Y., Huo, F., Li, X., Wen, Y., Yi, T., Salamanca, J., Escobedo, J.O., Strongin, R.M., and Yin, C. (2017). PH-dependent fluorescent probe that can be tuned for cysteine or homocysteine. Org. Lett. *19*, 82–85.
2. Jagtap, A.R., Satam, V.S., Rajule, R.N., and Kanetkar, V.R. (2009). The synthesis and characterization of novel coumarin dyes derived from 1,4-diethyl-1,2,3,4-tetrahydro-7-hydroxyquinoxalin-6-carboxaldehyde. Dye. Pigment. *82*, 84–89.
3. Yue, Y., Huo, F., Pei, X., Wang, Y., and Yin, C. (2020). Fluorescent Imaging of Resveratrol Induced Subcellular Cysteine Up-Regulation. Anal. Chem. *92*, 6598–6603.