Electronic Supporting Information

Diselenide as novel effective fluorescence quenchers to construct a two-photon fluorescent probe for thiols in a mouse stroke model

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CONTENT

1. Experimental Section

1.1 Materials:

All chemicals and reagents were used directly as obtained commercially unless otherwise stated. Solvents were purified and dried by standard procedures before use. MiliQ water was used in preparing aqueous systems0 if necessary, double distilled water was used throughout the other experiments. Dimethyl sulfoxide (DMSO) and esterase from porcine liver were obtained from Sigma-Aldrich (St. Louis, MO, USA). N-Acetylcysteine (NAC) and lipopolysaccharides (LPS) were obtained from Aladdin (Shanghai, China). Other reagents were analytical grade purchased from local supplier.

1.2 Instruments:

^{1}H NMR (400 MHz) and ^{13}C NMR (151 MHz) analyses were performed on a Bruker Advance spectrometer. ESI-MS spectra were recorded on a Bruker Daltonics esquire 6000 mass spectrometer. High-resolution mass spectrometry (HRMS) was performed on an Orbitrap Q Exactive (Thermo Scientific). UV–vis spectra were recorded by a UV–vis spectrometer (Evolution 200, Thermo Scientific). Fluorescence spectroscopic studies were performed using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies). The fluorescence images of the cells were acquired on a Floid cell imaging station (Life Technologies). The fluorescence images the brain of mice were taken with a two-photon fluorescence microscope (Olympus FV1000). All procedures for *in vivo* experiments were carried out in accordance with the institutional guidelines (Guidance of the Care and Use of Laboratory Animals), and all in *vivo* experiments were approved by the Ethics Committee of Lanzhou University, China.

1.3 Spectral measurements:

Stock solution of **AMC**, **AMC-SS**, **AMC-SeSe**, **AFC**, **AFC-SS**, **AFC-SeSe**, **ANC**, **ANC-SS**, **ANC-SeSe**, **ARC**, **ARC-SS**, **ARC-SeSe** (10 mM) was prepared in DMSO. Stock solutions of various amino acids and other analytes were prepared by direct dissolution in deionized water or DMSO. All chemicals used were of analytical grade. The detection experiments were measured in PBS buffer (pH =7.4, 10 mM).

Imaging of probes in Living Cells

Hela cells (2×10^4) were seeded in 12-well plates and incubated for 24 h at 37 °C. To determine the membrane permeability, HeLa cells were treated with **AFC-SeSe** (10 μM), **AFC-SS** (10 μM), **AMC-SeSe** (10 μM) and **AMC-SS** (10 μM) for 30 min or 60 min; Then the cells were washed three times with PBS, followed by imaging under a Floid cell imaging station microscope.

To image probes reacted with intracellular thiols, First, Hela cells were pretreated with NEM (100 μM) for 30 min, following treated with GSH (1 mM), Cys (100 μM) and Hcy (100 μM), respectively, and then incubated with **AFC-SeSe** (10 μM), **AFC-SS** (10 μM) for 30 min, Then the cells were washed three times with PBS, followed by imaging under a Floid cell imaging station microscope.

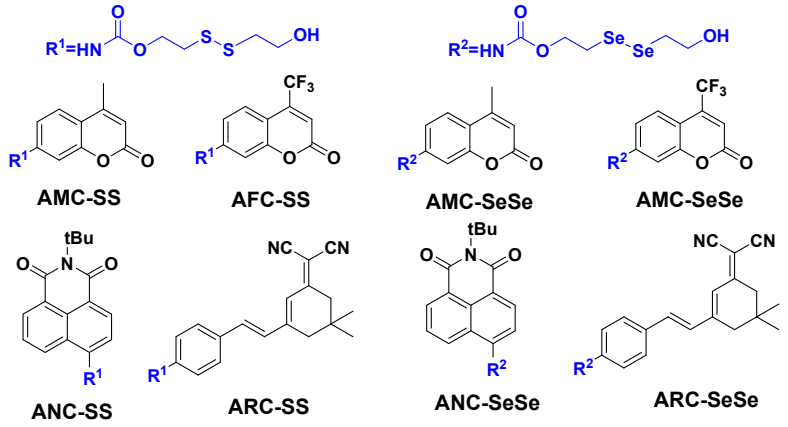
To image probes reacted with intracellular thiols under oxidation condition, First, Hela cells were pretreated with H_2O_2 (100 μM) for 30 min or LPS (100 μM) for 12 h, following treated with NAC (100 μM), respectively, and then incubated with **AFC-SeSe** (10 μM), **AFC-SS** (10 μM) for 30 min, Then the cells were washed three times with PBS, followed by imaging under a Floid cell imaging station microscope.

Two-Photon Imaging of AFC-SeSe and AFC-SS in Mouse Brains.

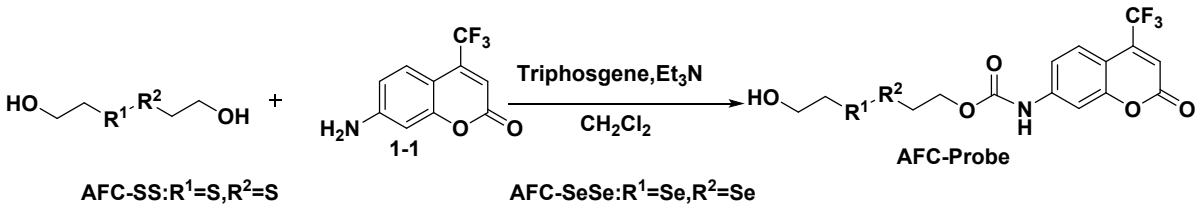
The cerebral tissue underwent incubation with **AFC-SeSe or AFC-SS** via a cranial window positioned above the mouse skull. A φ3 mm area along the mouse skull perimeter was meticulously thinned using a dental drill. Subsequently, the bone flap was delicately removed using curved forceps. Throughout the surgical procedure, a sterilized artificial cortical-spinal solution (ACSF) was intermittently applied to the exposed brain tissue to prevent desiccation, while the animal's body temperature was maintained using a heating pad. Any residual blood was cleaned using a piece of moistened gelfoam. Following this, **AFC-SeSe or AFC-SS** (1 mM, 20 μL) was administered to the brain tissue, and a cover glass (φ4 mm) was affixed to the skull and sealed using dental cement. After a 30-minute incubation period, the mouse was gently repositioned beneath a 25 × water-immersion objective (Olympus FV1000) for intravital imaging. The cortical region was imaged repeatedly through the cranial window, and 1024*1024 image stacks were acquired with a step size of 1 μm. It was important to minimize exposure of the cortex to the laser to mitigate potential tissue

damage. The excitation wavelength for two-photon imaging was set at 800 nm.

2. Synthesis and Characterization:



Scheme S1. Chemical structures of fluorescence probes.



Scheme S2. The synthesis route of probe **AFC-SS and AFC-SeSe.**

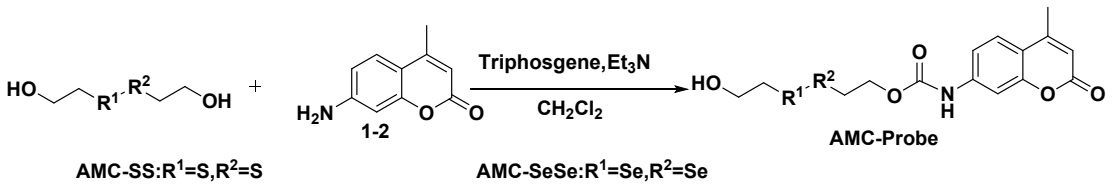
Synthesis details of **AFC-SS**:

Compounds **1-1** (100 mg, 0.37 mmol) was dissolved in DCM (5 mL) and then added to a 50 mL round-bottled flask, and triphosgene (56 mg, 0.19 mmol) was added to the system in batches at 0 ℃ followed with triethylamine (154 μL, 1.11 mmol). After the reaction at room temperature for 30 min, bis (2-hydroxyethyl) disulfide (171 mg, 1.11 mmol) was added to the reaction system, and the reaction continued at room temperature for 6 h. After the reaction, the saturated sodium bicarbonate (5 mL) was added to quench the reaction, the organic phase was separated by DCM (3 x 6 mL), finally the solvent was removed, and yellow solid **AFC-SS** was obtained by column chromatography (PE/EA = 2/1) (36 mg, 42% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.41 (d, J = 13.2 Hz, 1H), 7.67-7.60 (m, 2H), 7.51-7.47 (m, 1H), 6.86 (d, J = 2.5 Hz, 1H), 4.90 (t, J = 5.4 Hz, 1H), 4.38 (t, J = 6.3 Hz, 2H), 3.63 (td, J = 6.4, 5.3 Hz, 2H), 3.04 (t, J = 6.3 Hz, 2H), 2.82 (t, J = 6.4 Hz, 2H).; ^{13}C NMR (151 MHz, DMSO-d_6) δ

159.10, 155.31, 153.48, 144.15, 125.97, 121.24, 115.60, 114.49, 108.09, 105.56, 63.13, 59.85, 41.51, 37.05.; ESI-MS (m/z): calculated for $C_{21}H_{25}N_2O_5S_2$ $[M-H]^+$ 408.01, found 408.05.

Synthesis details of **AFC-SeSe**:

The synthesis route is similar to that of compound **AFC-SS**, and yellow solid **AFC-SeSe** is obtained by column chromatography (PE/EA = 2/1)(35 mg, 39% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.40 (d, J = 17.5 Hz, 1H), 7.70-7.39 (m, 3H), 6.87 (s, 1H), 5.02-4.87 (m, 1H), 4.41 (t, J = 6.8 Hz, 2H), 3.78-3.59 (m, 2H), 3.29-3.19 (m, 2H), 3.15-2.91 (m, 2H).; ^{13}C NMR (151 MHz, DMSO-d_6) δ 158.03, 154.14, 152.39, 143.11, 124.92, 124.72, 107.03, 106.83, 104.52, 104.38, 32.39, 32.11.; calculated for $C_{15}H_{14}F_3NO_5Se_2$ $[M-H]^-$ 503.90, found 503.91.



Scheme S3. The synthesis route of probe **AMC-SS and AMC-SeSe.**

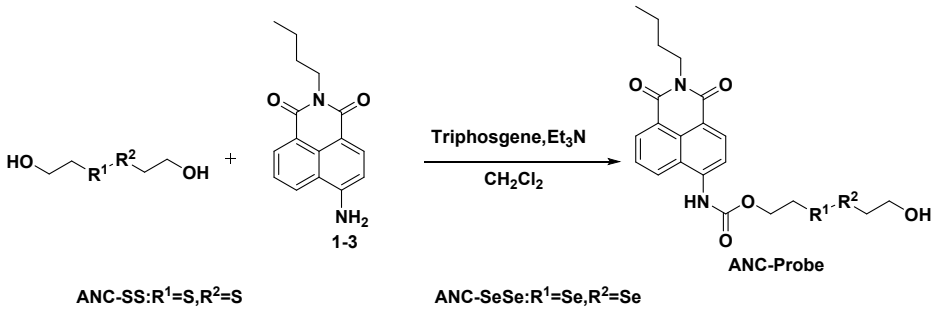
Synthesis details of **AMC-SS**:

The synthesis route is similar to that of compound **AFC-SS**, and yellow solid **AMC-SS** is obtained by column chromatography (PE/EA = 3/1) (47 mg, 51% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.20 (d, J = 14.3 Hz, 1H), 7.65 (d, J = 8.7 Hz, 1H), 7.51 (d, J = 2.0 Hz, 1H), 7.38 (dd, J = 8.7, 2.1 Hz, 1H), 6.18 (dd, J = 17.3, 1.5 Hz, 1H), 4.87 (t, J = 5.5 Hz, 1H), 4.33 (t, J = 6.2 Hz, 2H), 3.60 (q, J = 6.2 Hz, 2H), 3.00 (t, J = 6.3 Hz, 2H), 2.79 (t, J = 6.4 Hz, 2H), 2.32 (dd, J = 15.3, 1.2 Hz, 3H).; ^{13}C NMR (151 MHz, DMSO-d_6) δ 160.49, 154.25, 153.60, 153.51, 143.09, 126.42, 114.84, 114.75, 114.73, 112.39, 104.95, 62.96, 59.87, 41.53, 37.12, 18.45.

Synthesis details of **AMC-SeSe**:

The synthesis route is similar to that of compound **AFC-SS**, and yellow solid **AMC-SeSe** is obtained by column chromatography (PE/EA = 2/1) (34 mg, 29% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.24 (s, 1H), 7.68 (d, J = 9.0 Hz, 1H), 7.60-7.47 (m, 1H), 7.45-7.33 (m, 1H), 6.23 (s, 1H), 5.02-4.91 (m, 1H), 4.38 (s, 2H), 3.75-

3.63 (m, 2H), 3.30-2.94 (m, 4H), 2.37 (s, 3H).; ^{13}C NMR (151 MHz, DMSO-d_6) δ 160.51, 154.27, 153.66, 153.41, 143.07, 126.49, 126.30, 114.65, 112.40, 104.85, 64.88, 61.59, 33.44, 27.73, 18.47.; calculated for $C_{15}H_{14}F_3NO_5Se_2$ $[M-H]^-$ 449.94, found 449.90.



Scheme S4. The synthesis route of probe **ANC-SS and ANC-SeSe.**

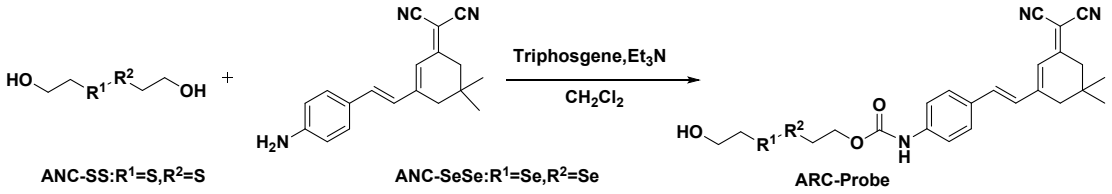
Synthesis details of **ANC-SS**:

The synthesis route is similar to that of compound **AFC-SS**, and yellow solid **ANC-SS** is obtained by column chromatography (PE/EA = 2/1) (47 mg, 39% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.37 (s, 1H), 8.69 (d, J = 8.5 Hz, 1H), 8.48 (dd, J = 19.6, 7.8 Hz, 2H), 8.16 (d, J = 8.3 Hz, 1H), 7.83 (t, J = 7.9 Hz, 1H), 4.94 (s, 1H), 4.46 (t, J = 6.5 Hz, 2H), 4.03 (t, J = 7.5 Hz, 2H), 3.66 (d, J = 6.6 Hz, 2H), 3.11 (t, J = 6.5 Hz, 2H), 2.86 (t, J = 6.4 Hz, 2H), 1.61 (p, J = 7.3 Hz, 2H), 1.35 (q, J = 7.5 Hz, 2H), 0.93 (t, J = 7.4 Hz, 3H).; ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.92, 163.37, 154.35, 141.08, 132.09, 132.00, 131.37, 129.81, 128.77, 126.86, 124.43, 122.67, 118.97, 117.64, 63.46, 59.88, 41.53, 37.14, 30.14, 20.28, 14.21.

Synthesis details of **ANC-SeSe**:

The synthesis route is similar to that of compound **AFC-SS**, and yellow solid **ANC-SeSe** is obtained by column chromatography (PE/EA = 2/1) (27 mg, 19% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.34 (s, 1H), 8.71 (d, J = 8.5 Hz, 1H), 8.49 (dd, J = 20.4, 7.7 Hz, 2H), 8.17 (d, J = 8.2 Hz, 1H), 7.84 (dd, J = 8.6, 7.2 Hz, 1H), 5.00 (s, 1H), 4.47 (t, J = 7.0 Hz, 2H), 4.04 (t, J = 7.5 Hz, 2H), 3.68 (t, J = 6.8 Hz, 2H), 3.27 (t, J = 7.0 Hz, 2H), 3.09 (t, J = 6.8 Hz, 2H), 1.61 (dd, J = 8.5, 6.5 Hz, 2H), 1.37-1.33 (m, 2H), 0.93 (s, 3H).; ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.95, 163.41, 154.33, 141.15, 132.11, 131.39, 129.86, 128.81, 126.88, 124.50, 122.70, 119.02, 117.65, 65.35, 61.72,

33.48, 33.19, 30.15, 22.56, 20.28, 14.18.; calculated for $C_{22}H_{23}N_2O_5Se_2$ $[M-H]^-$ 543.00, found 543.01.



Scheme S5. The synthesis route of probe **ARC-SS and ARC-SeSe.**

Synthesis details of **ARC-SS**:

The synthesis route is similar to that of compound **AFC-SS**, and red solid **ARC-SS** is obtained by column chromatography (PE/EA = 2/1) (36 mg, 42% yield). ^{1}H NMR (400 MHz, $CDCl_3$-d_4) δ 7.48-7.42 (m, 4H), 7.10 (s, 1H), 7.03-6.88 (m, 2H), 6.81 (s, 1H), 4.46 (t, J = 6.0 Hz, 2H), 3.92 (t, J = 6.0 Hz, 2H), 3.00 (t, J = 6.4 Hz, 2H), 2.93 (t, J = 6.0 Hz, 2H), 2.59 (s, 2H), 2.45 (s, 2H), 1.07 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$-d_4) δ 169.31, 154.02, 152.91, 139.13, 136.42, 130.95, 128.64, 127.92, 123.23, 118.75, 113.66, 112.82, 78.18, 63.21, 60.3, 43.0, 41.6, 39.1, 37.51, 32.03, 28.04.

Synthesis details of **ARC-SeSe**:

The synthesis route is similar to that of compound **AFC-SS**, and red gelatinous substance **ARC-SeSe** is obtained by column chromatography (PE/EA = 2/1) (16 mg, 12% yield). ^{1}H NMR (400 MHz, $CDCl_3$-d_4) δ 7.45 (d, J = 3.6 Hz, 4H), 7.01 (d, J = 16.0 Hz, 1H), 6.89 (d, J = 15.8 Hz, 1H), 6.81 (s, 1H), 4.54 (t, J = 23.5, 6.7 Hz, 4H), 3.30 (t, J = 77.0, 6.7 Hz, 4H), 2.60 (s, 2H), 2.46 (s, 2H), 1.08 (s, 6H).; ^{13}C NMR (151 MHz, $CDCl_3$-d_4) δ 169.24, 153.95, 136.35, 130.99, 129.66, 128.60, 128.57, 128.06, 127.69, 123.24, 118.87, 113.56, 112.84, 64.59, 43.00, 39.22, 32.05, 29.71, 28.12, 28.04.; calculated for $C_{15}H_{14}F_3NO_5Se_2$ $[M-H]^-$ 562.03, found 562.08.

3. Additional Test data:

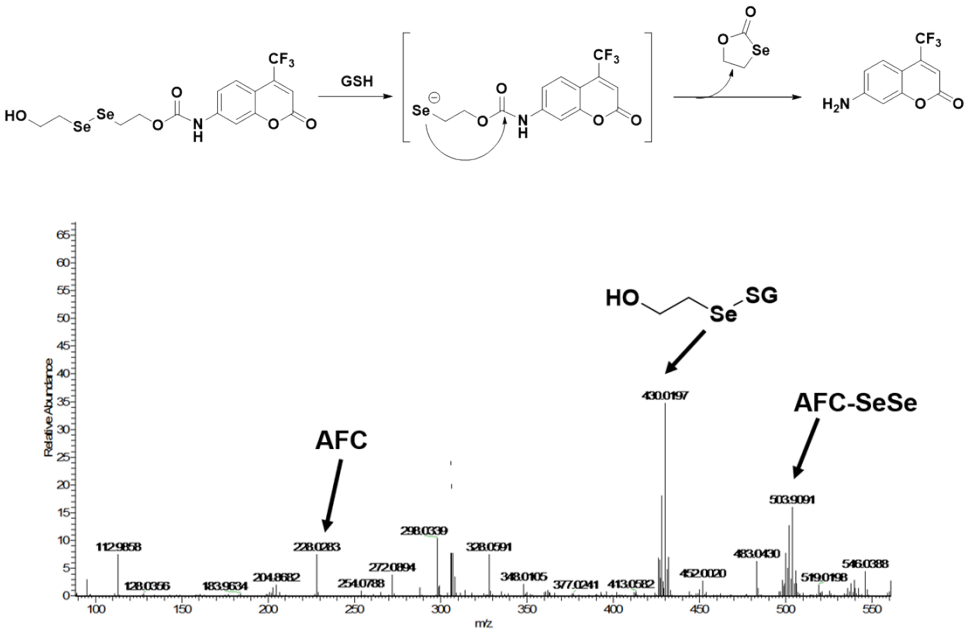


Figure S1. The mass after **AFC-SeSe** reacted with GSH.

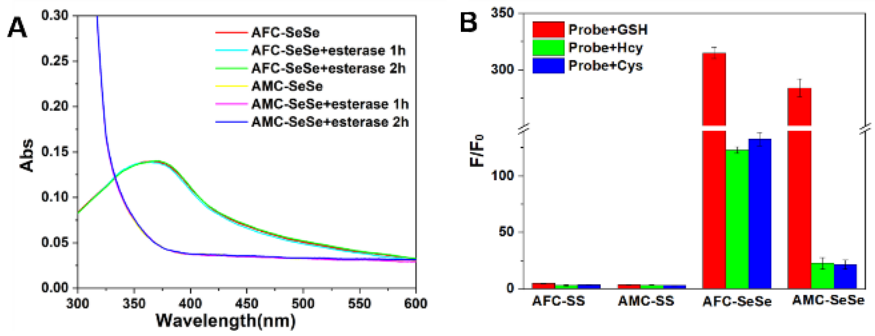


Figure S2. UV-vis absorption and fluorescence response of **AFC-SeSe** and **AMC-SeSe**. (A) UV-vis absorption spectra of **AFC-SeSe** and **AMC-SeSe** incubated with esterase (0.15 U/mL) in PBS buffer (10 mM, pH 7.4) at 37°C. (B) Fluorescence response of **AFC-SeSe** and **AFC-SS** ($\lambda_{ex}/\lambda_{em}$ = 368/490 nm), and **AMC-SeSe** and **AMC-SS** ($\lambda_{ex}/\lambda_{em}$ = 330/440 nm) towards GSH (1 mM), Hcy (100 μM), and Cys (100 μM) for 20 min in PBS buffer (10 mM, pH 7.4) at 37°C.

Table S1 Fluorescence response of probe with thiols for 20 min

Probe	GSH(F/F_0)	Hcy(F/F_0)	Cys(F/F_0)
AFS-SS	4.67	3.03	3.73
AMC-SS	3.49	3.34	3.07
★**AFC-SeSe**	315.41	123.1	132.7
★**AMC-SeSe**	284.12	22.71	21.5

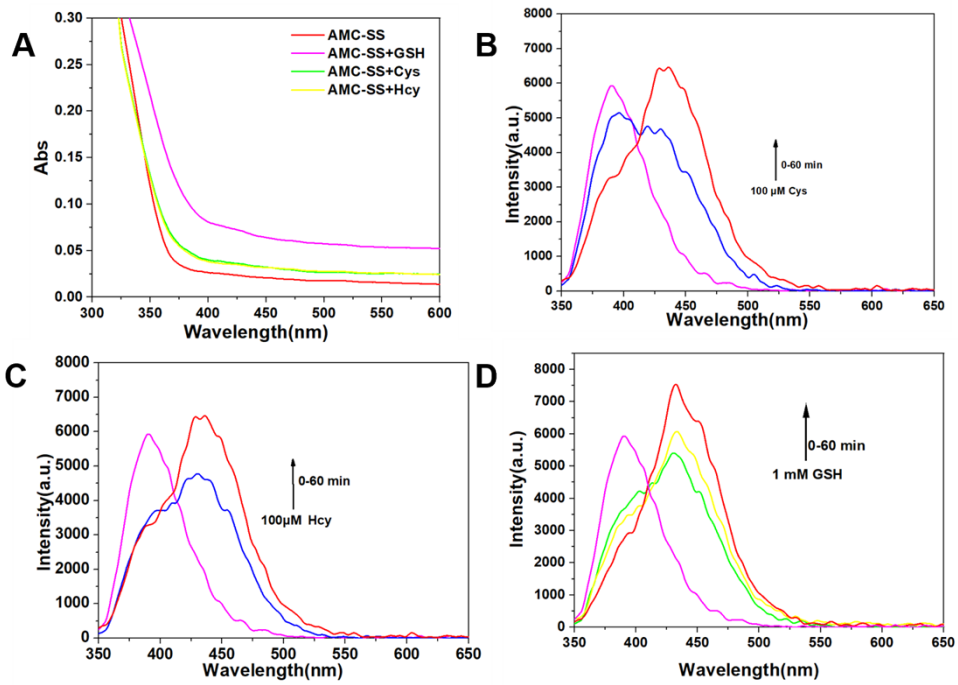


Figure S3. (A) UV-vis absorption of **AMC-SS** incubated with GSH (1 mM), Hcy (100 μM), Cys (100 μM) for 60 min in PBS buffer (10 mM, pH 7.4) at 37 ℃.; (B-D) Fluorescence response of **AMC-SS** towards GSH (1 mM), Hcy (100 μM), Cys (100 μM) for 60 min in PBS buffer (10 mM, pH 7.4) at 37 ℃ (λ_{ex} =368 nm).

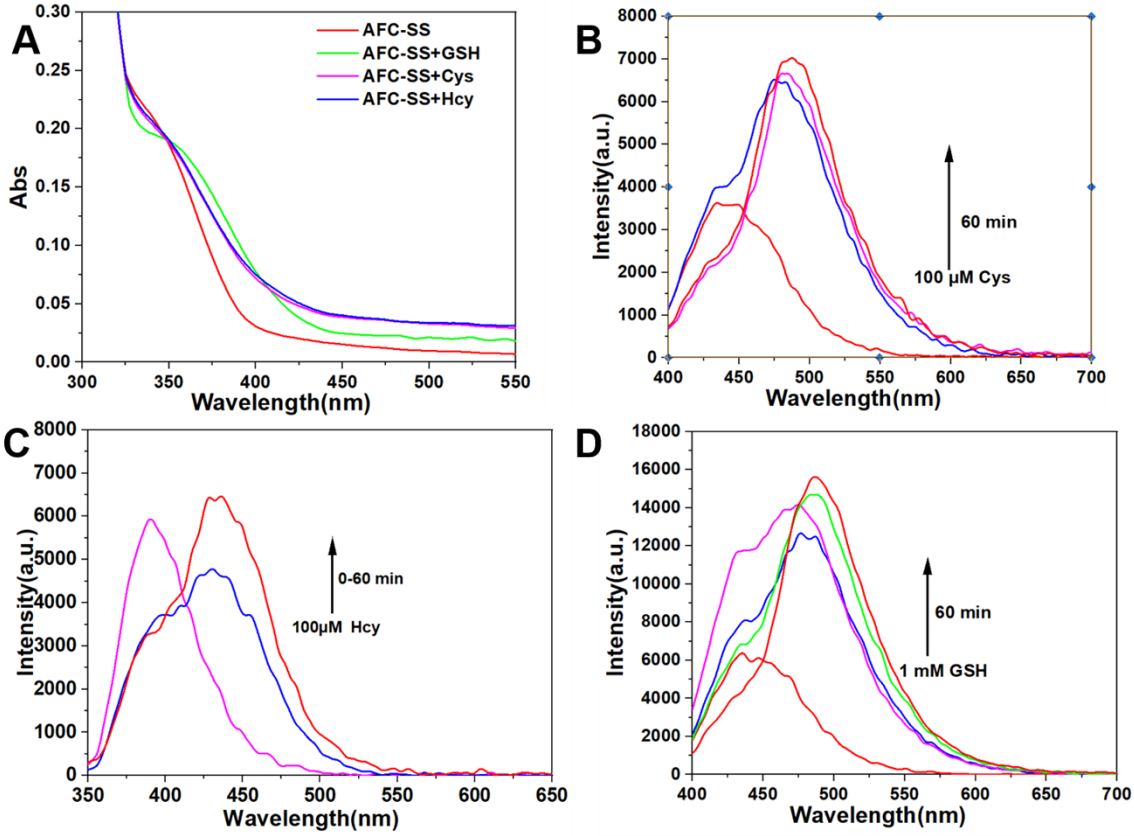


Figure S4. (A) UV-vis absorption of **AFC-SS** incubated with GSH (1 mM), Hcy (100 μM), Cys (100 μM) for 60 min in PBS buffer (10 mM, pH 7.4) at 37 ℃.; (B-D) Fluorescence response of

AFC-SS towards GSH (1 mM), Hcy (100 μM), Cys (100 μM) for 60 min in PBS buffer (10 mM, pH 7.4) at 37 ℃ (λ_{ex} =368 nm).

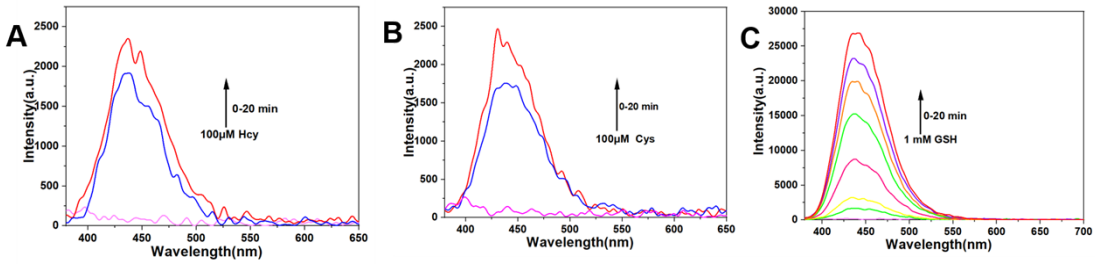


Figure S5. (A-C) Fluorescence response of **AMC-SeSe** towards GSH (1 mM), Hcy (100 μM), Cys (100 μM) for 20 min in PBS buffer (10 mM, pH 7.4) at 37 ℃ (λ_{ex} =330 nm).

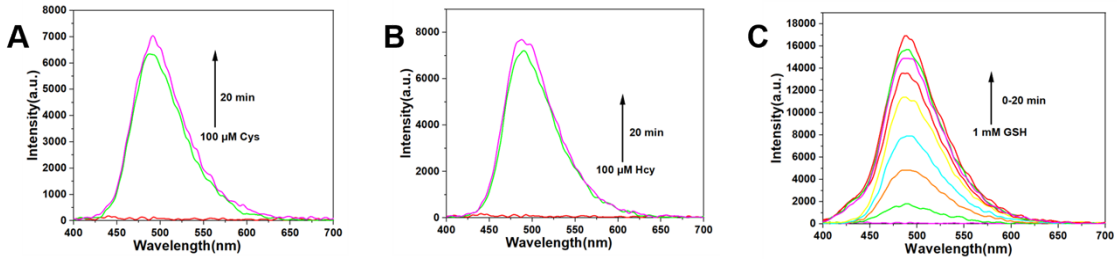


Figure S6. (A-C) Fluorescence response of **AFC-SeSe** towards GSH (1 mM), Hcy (100 μM), Cys (100 μM) for 20 min in PBS buffer (10 mM, pH 7.4) at 37 ℃ (λ_{ex} =368 nm).

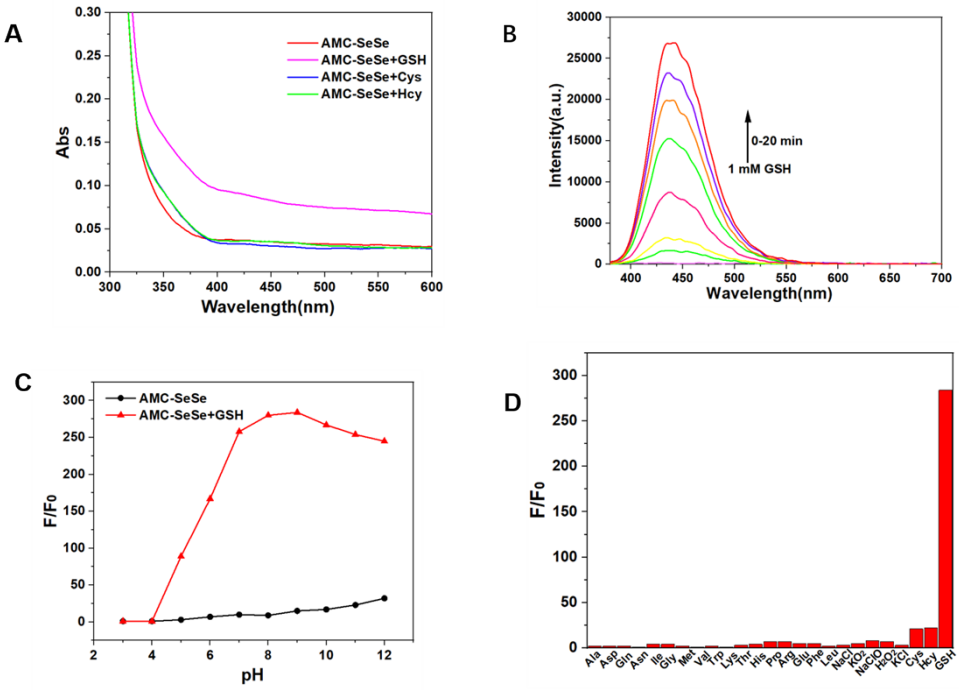


Figure S7. Optical test of **AMC-SeSe** (A) UV-vis absorption of **AMC-SeSe** towars GSH (1 mM), Hcy (100 μM), Cys (100 μM) for 20 min in PBS buffer (10 mM, pH 7.4) at 37 ℃， respectively; (B) Fluorescence response of **AMC-SeSe** ($\lambda_{ex}/\lambda_{em}$=368 /490 nm) towars GSH (1 mM), for 20 min in PBS buffer (10 mM, pH 7.4) at 37 ℃; (C) Fluorescence intensity of **AMC-SeSe** (λ_{em}=490 nm)

before and after treated GSH (1 mM) under the different pH (3.0-11.0) at 37 ℃,; (D) Fluorescence intensity of **AMC-SeSe** (λ_{em}=490 nm) after treated with various amino acids and ionic compounds (100 μM) for 20 min in PBS buffer (10 mM, pH 7.4) at 37 ℃, including Ala, Asp, Gln, Asn, Ile, Gly, Met, Val, Trp, Lys, Thr, His, Pro, Arg, Glu, Phe, Leu, NaCl, KO_2, NaClO, H_2O_2, KCl, Hcy(100 μM), Cys(100 μM), GSH (1 mM).

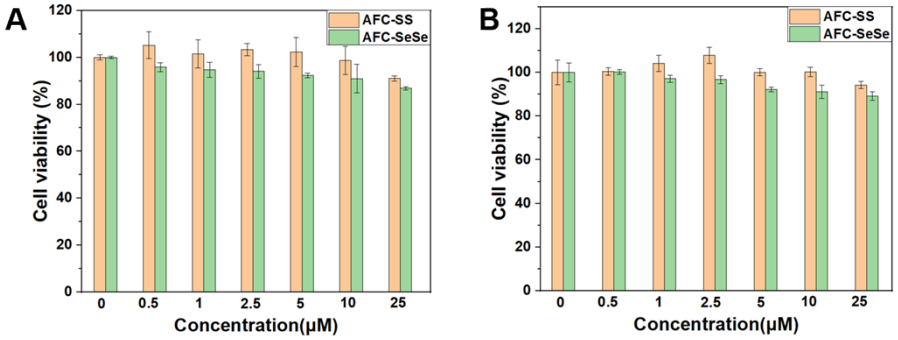


Figure S8. HeLa cells (A) and HeG2 cells (B) were treated with varying concentration of **AFC-SS** and **AFC-SeSe** for 12 h, and the cell viability was determined by CCK-8 assay.

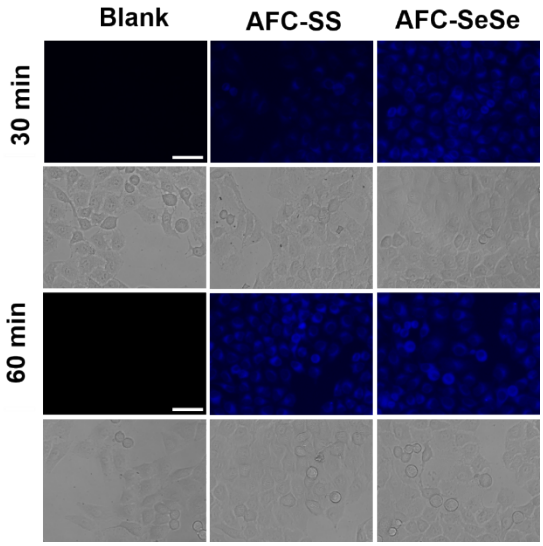


Figure S9. Response of probes to intracellular thiols. HeLa cells were treated with **AFC-SeSe** (10 μM), **AFC-SS** (10 μM) for 30 or 60 min. Scale bars: 20 μm.

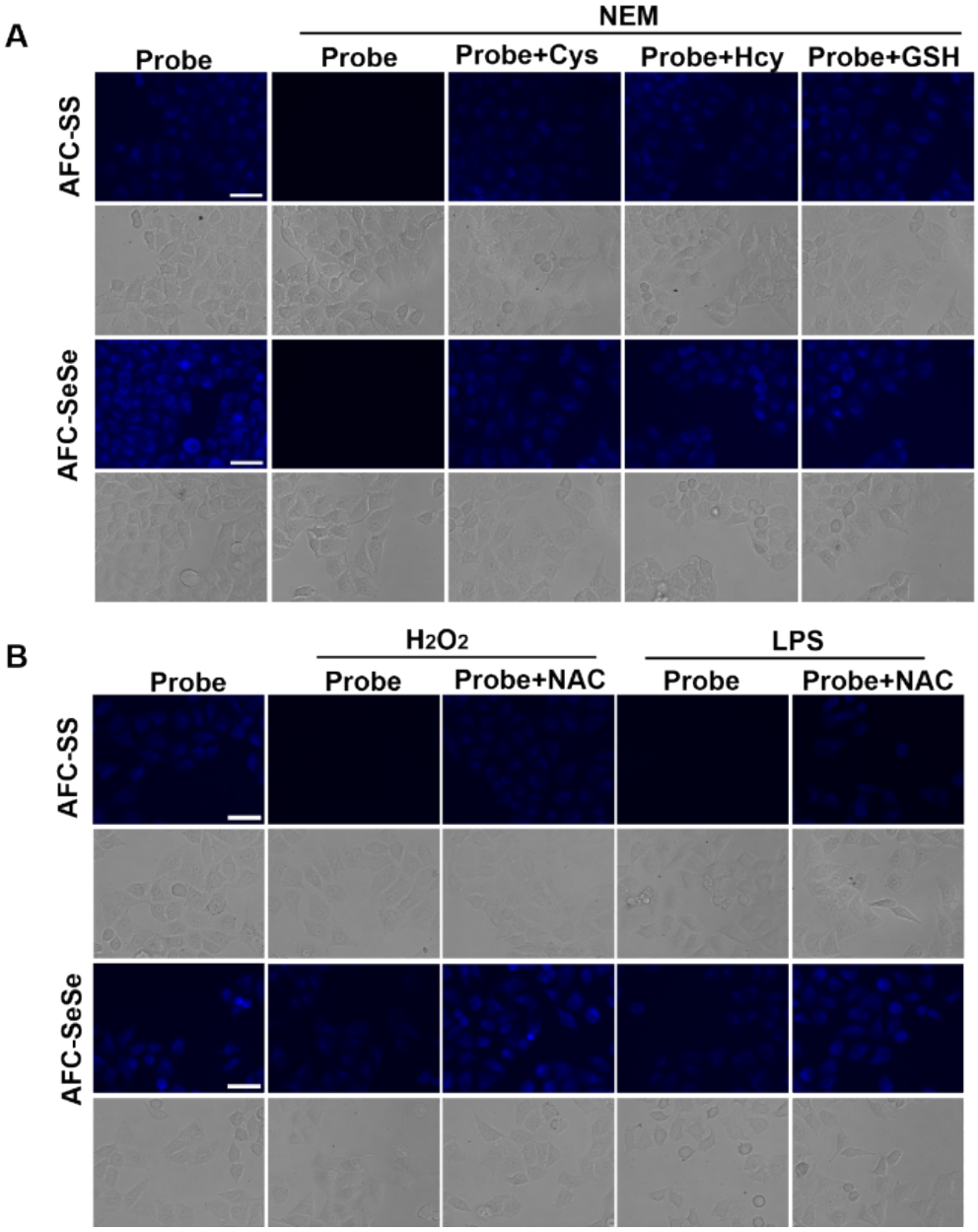


Figure S10. Response of probes to intracellular thiols under different stimuli. (A) HeLa cells were incubated with or without NEM (100 μM) and then treated with GSH (1 mM), Hcy (100 μM), or Cys (100 μM) for 20 min, followed by treatment with **AFC-SeSe** (10 μM) or **AFC-SS** (10 μM) for 30 min. (B) HeLa cells were treated with H_2O_2 (100 μM) for 30 min or LPS (100 μM) for 12 hours, followed by incubation with NAC (100 μM) for 30 min, and then treated with **AFC-SeSe** (10 μM) or **AFC-SS** (10 μM) for 30 min. Scale bars: 20 μm.

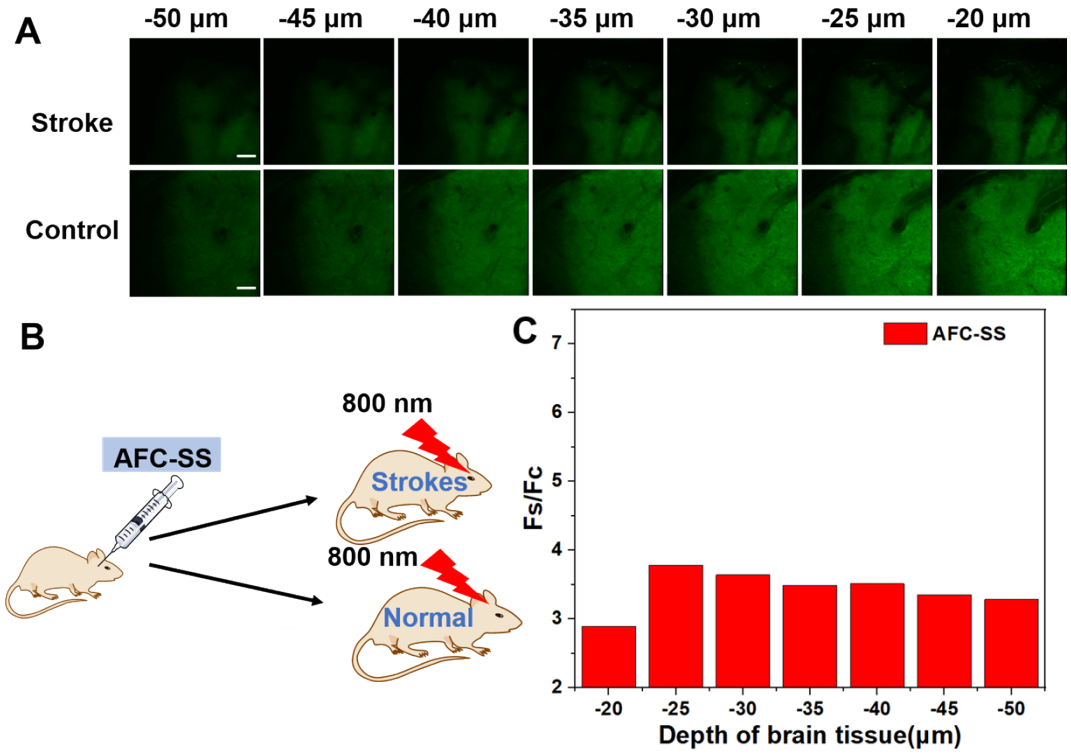


Figure S11. Two-photon fluorescence imaging of **AFC-SS**. (A) Two-photon fluorescence images of **AFC-SS** in the brains of healthy and stroke mice, respectively; (B) The diagram of mouse brain tissue imaging; (C) Comparison of fluorescence intensity at different depths of brain tissue. Fs represents the fluorescence intensity of the tissue of stroke mice; Fc represents the fluorescence intensity of the tissue of healthy mice; λ_{ex}=800 nm, Scale bars: 100 μm.

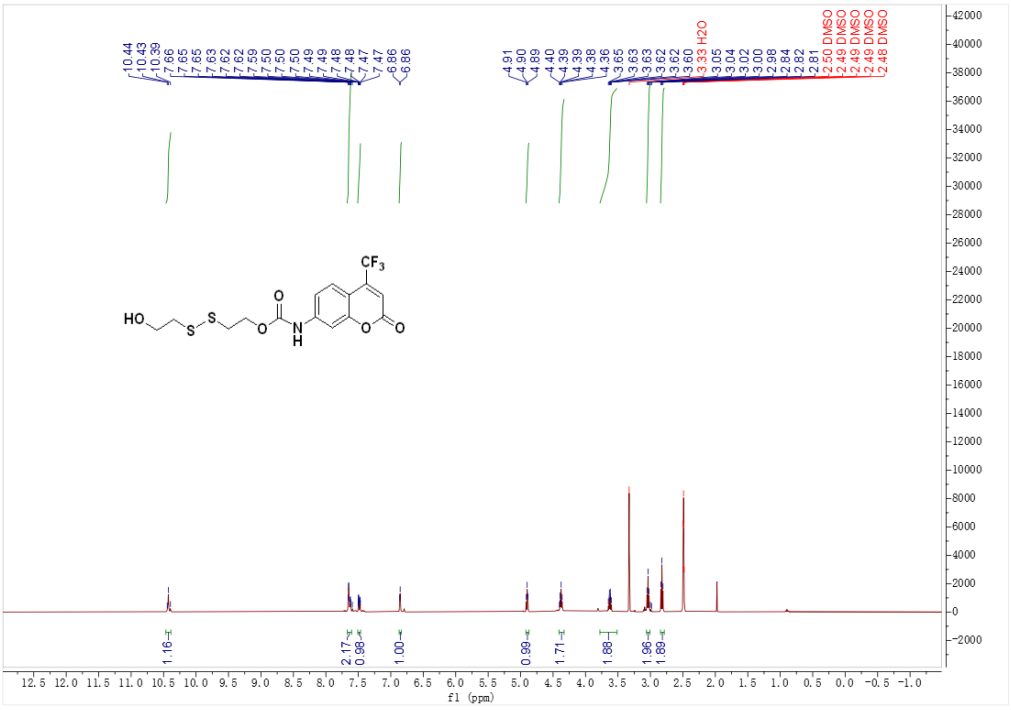


Figure S12. ^{1}H NMR Spectra of **AFC-SS** in DMSO-d_6 (400 MHz).

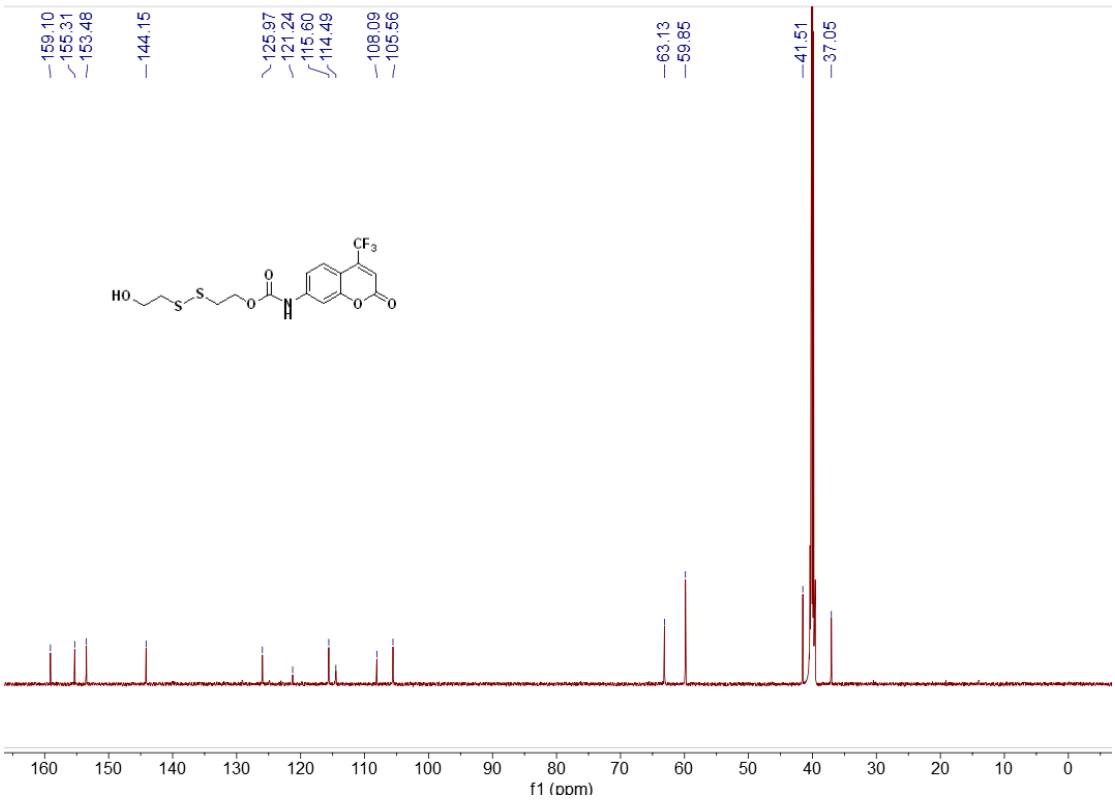


Figure S13. ^{13}C NMR Spectra of **AFC-SS** in DMSO-d_6 (151 MHz).

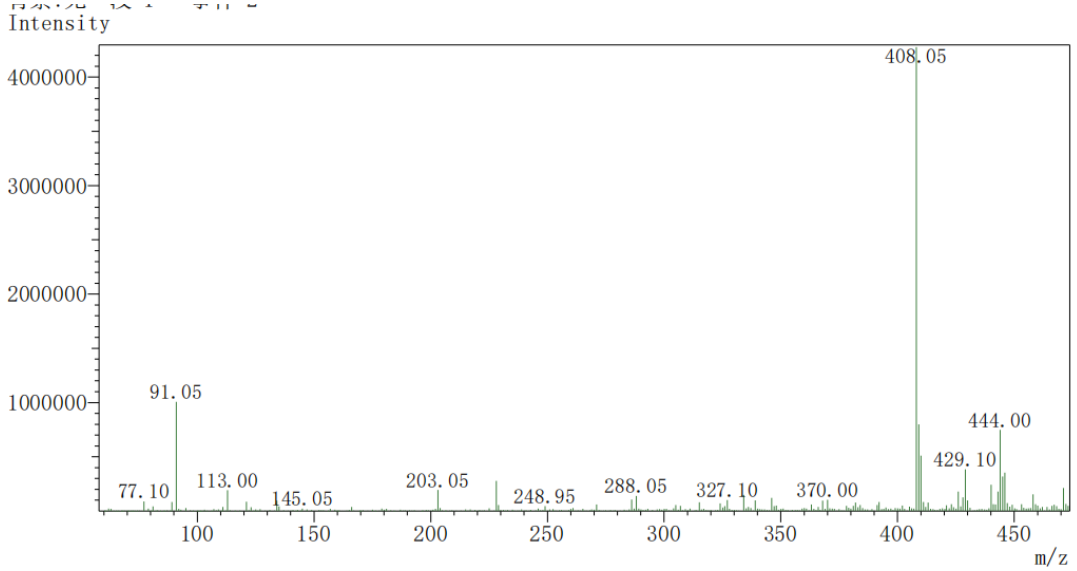


Figure S14. MS Spectra of **AFC-SS**.

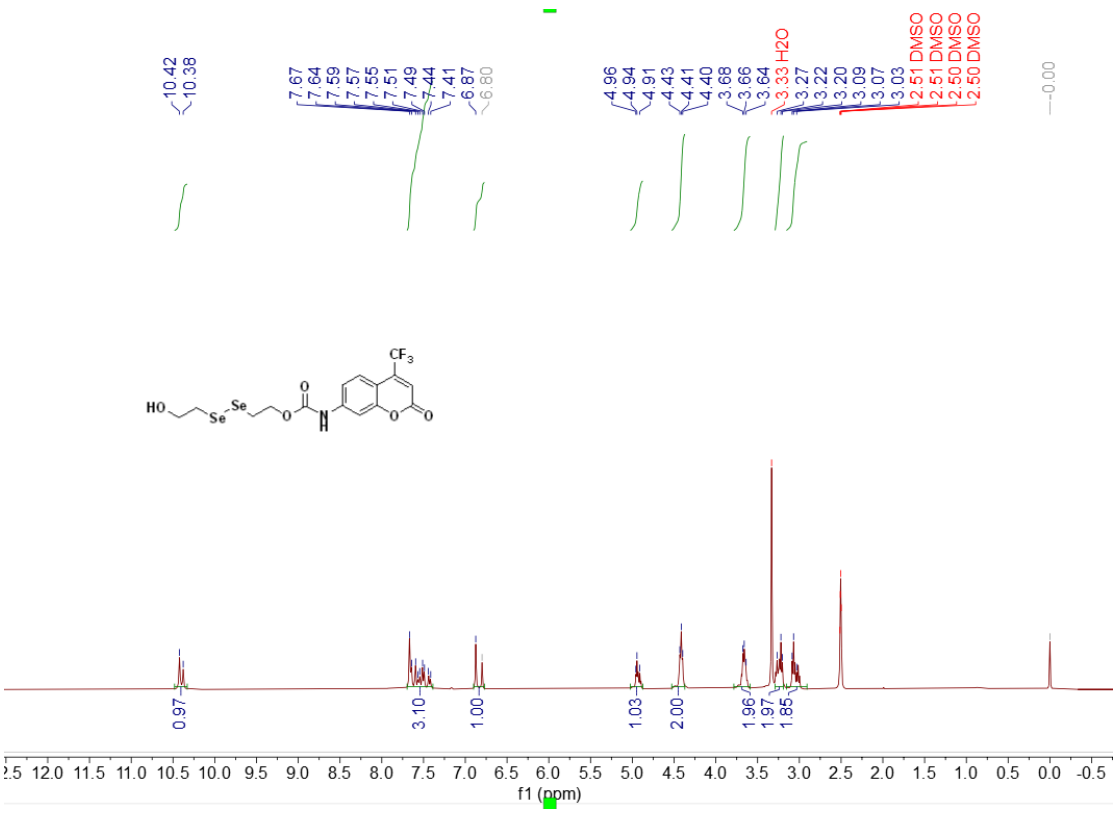


Figure S15. ^{1}H NMR Spectra of **AFC-SeSe** in DMSO-d_6 (400 MHz).

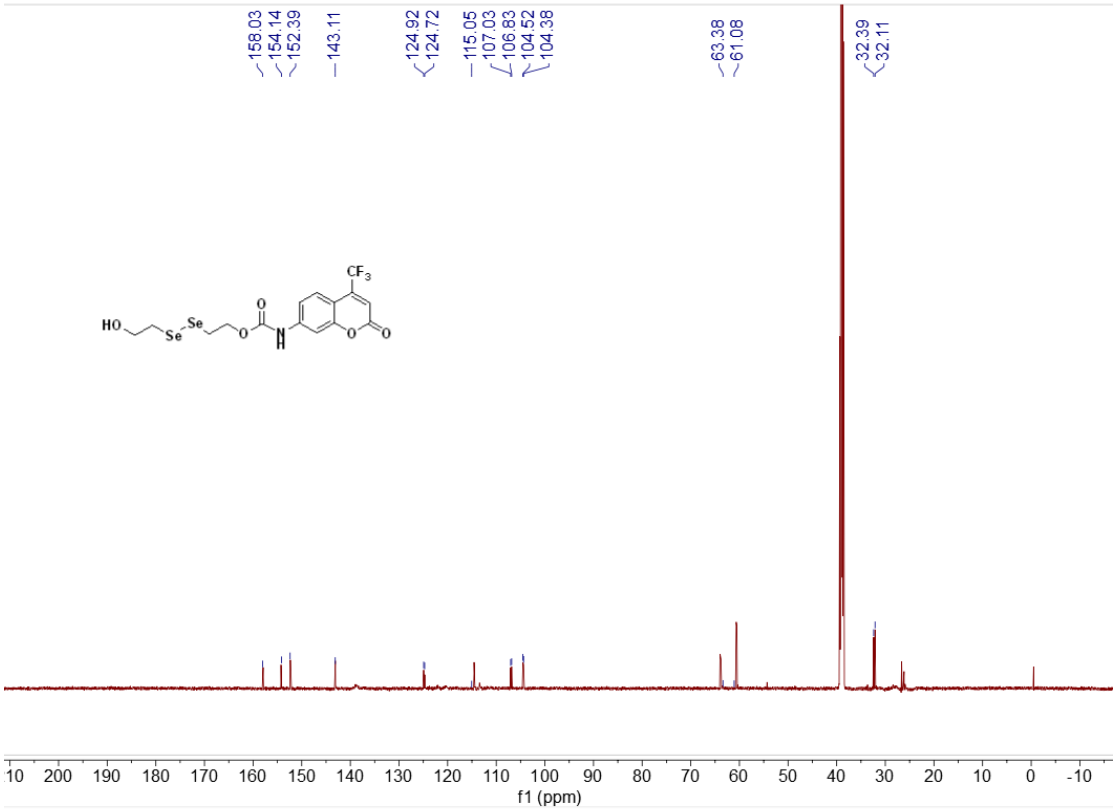


Figure S16. ^{13}C NMR Spectra of **AFC-SeSe** in DMSO-d_6 (151 MHz).

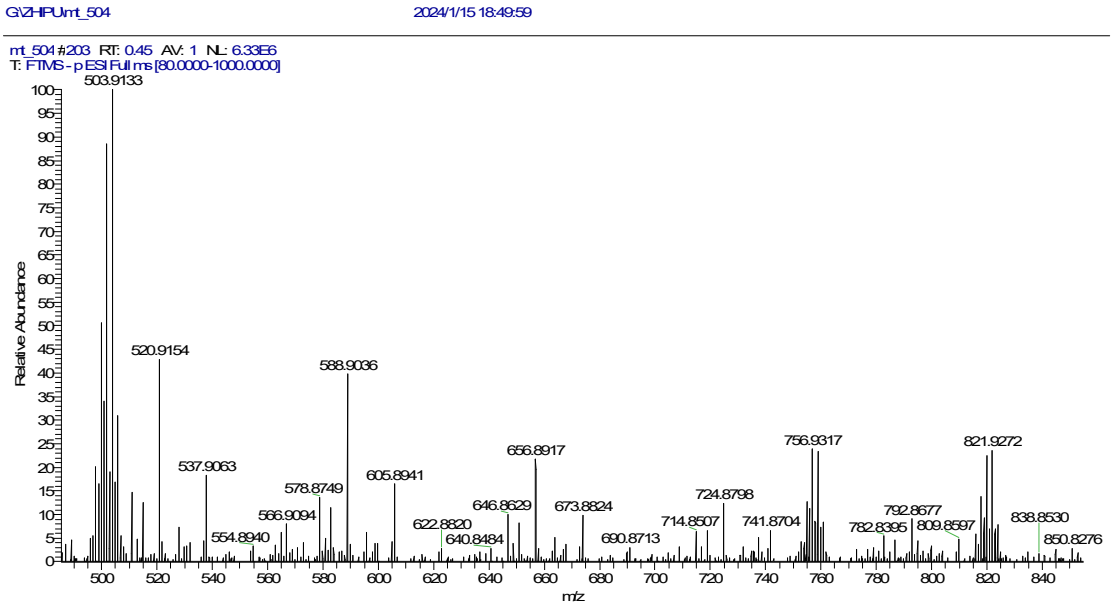


Figure S17. HR-MS Spectra of **AFC-SeSe**.

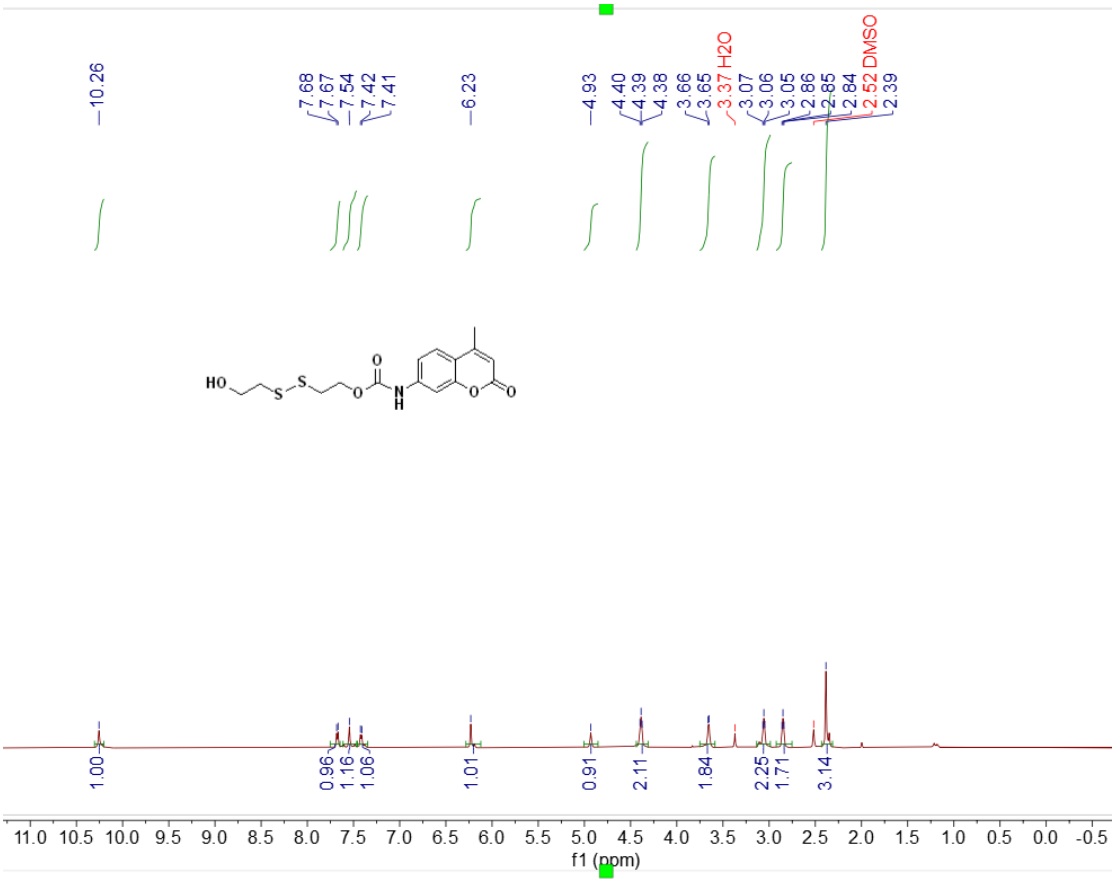


Figure S18. ^{1}H NMR Spectra of **AMC-SS** in DMSO-d_6 (400 MHz).

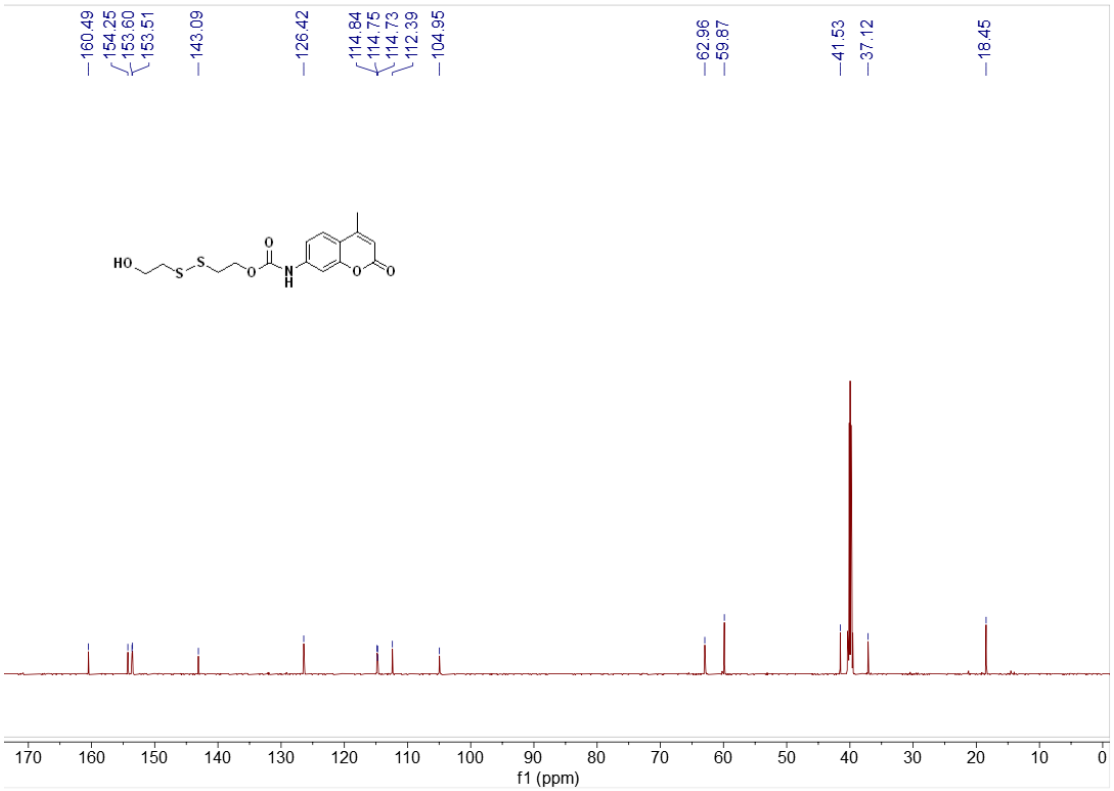


Figure S19. ^{13}C NMR Spectra of **AMC-SS** in DMSO-d_6 (151 MHz).

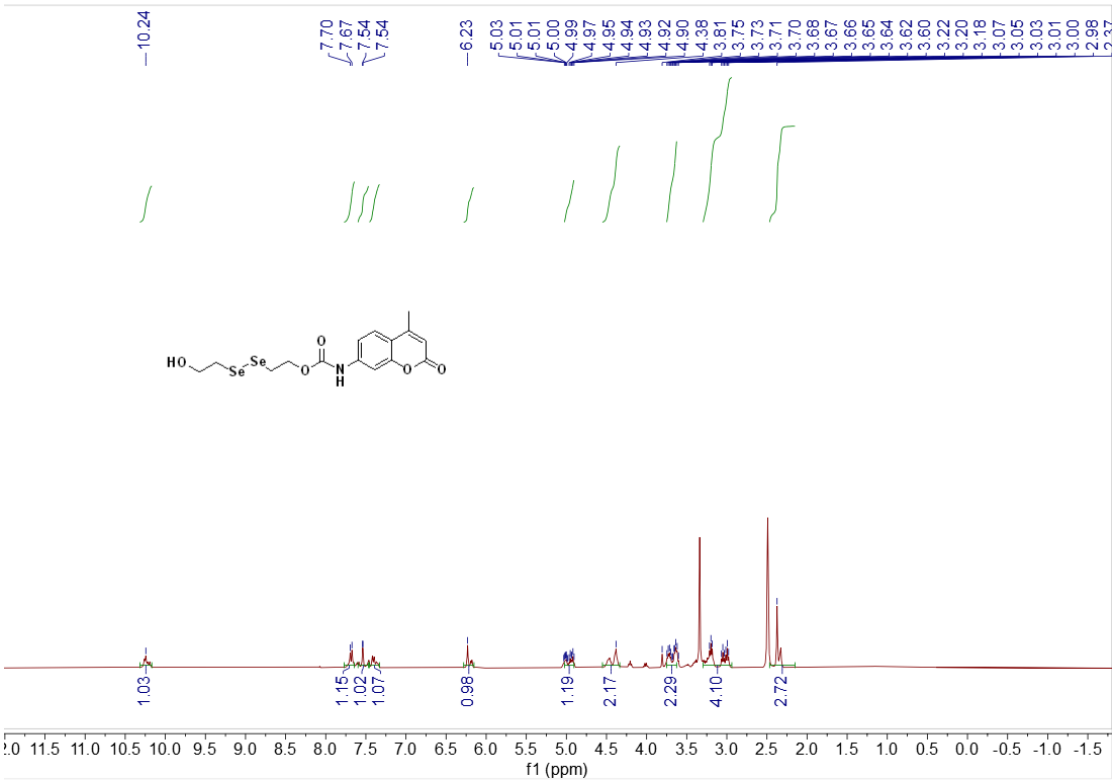


Figure S20. ^{1}H NMR Spectra of **AMC-SeSe** in DMSO-d_6 (400 MHz).

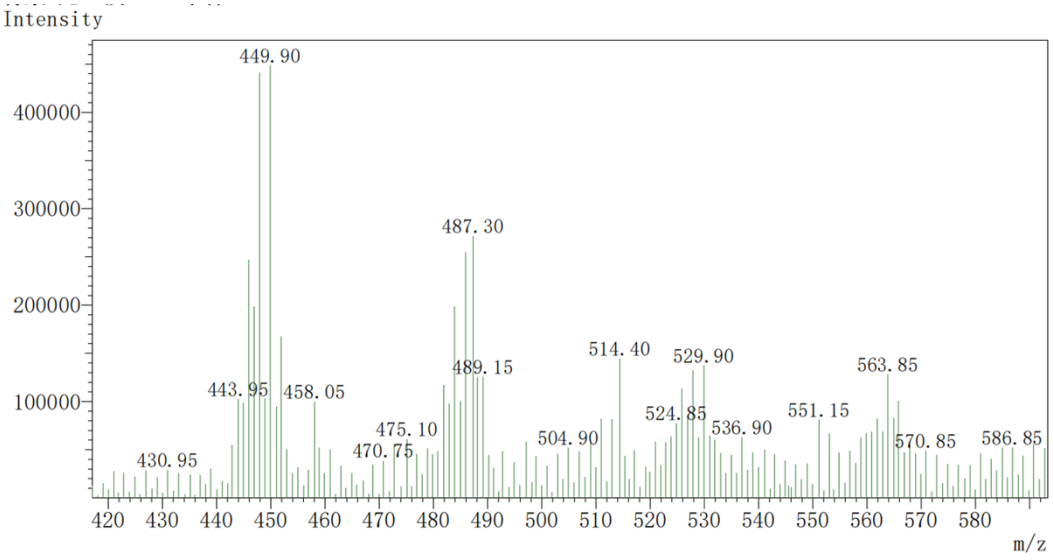


Figure S21. MS Spectra of **AMC-SeSe**.

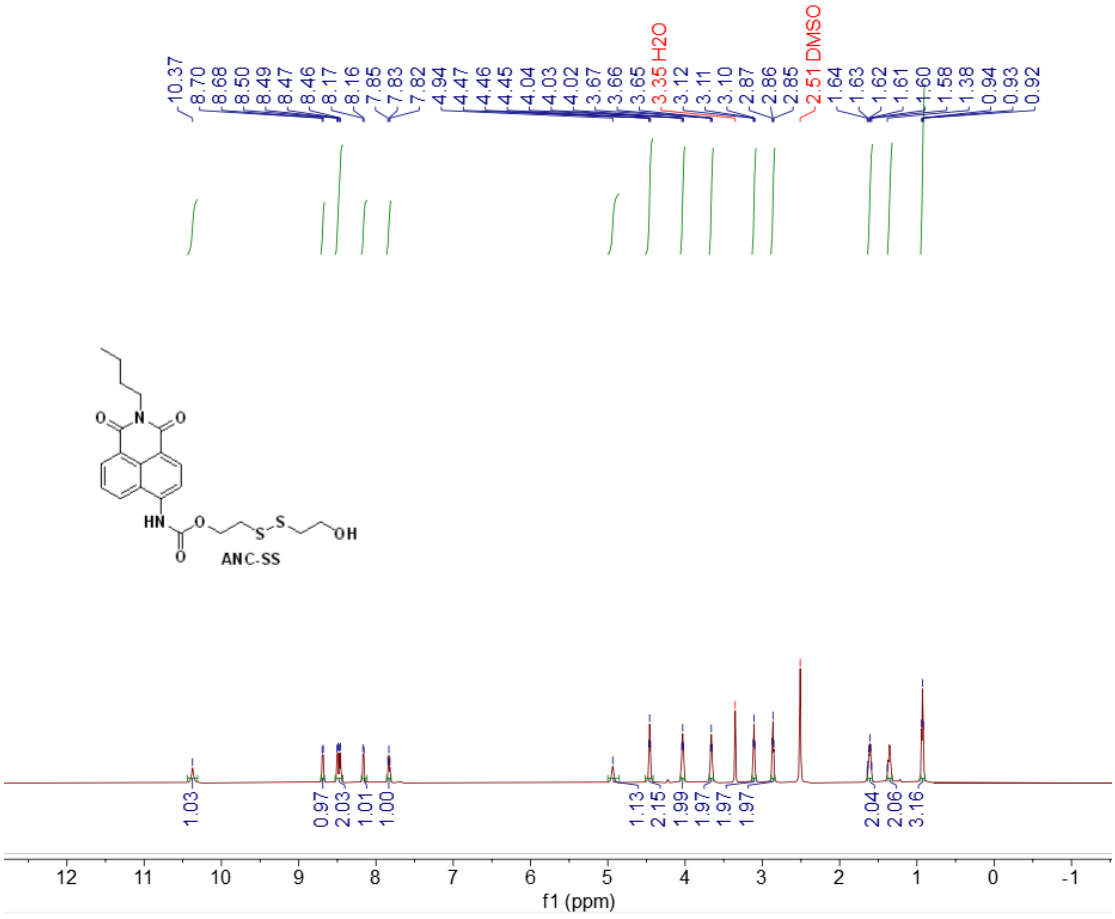


Figure S22. ^{1}H NMR Spectra of **ANC-SS** in DMSO-d_6 (400 MHz).

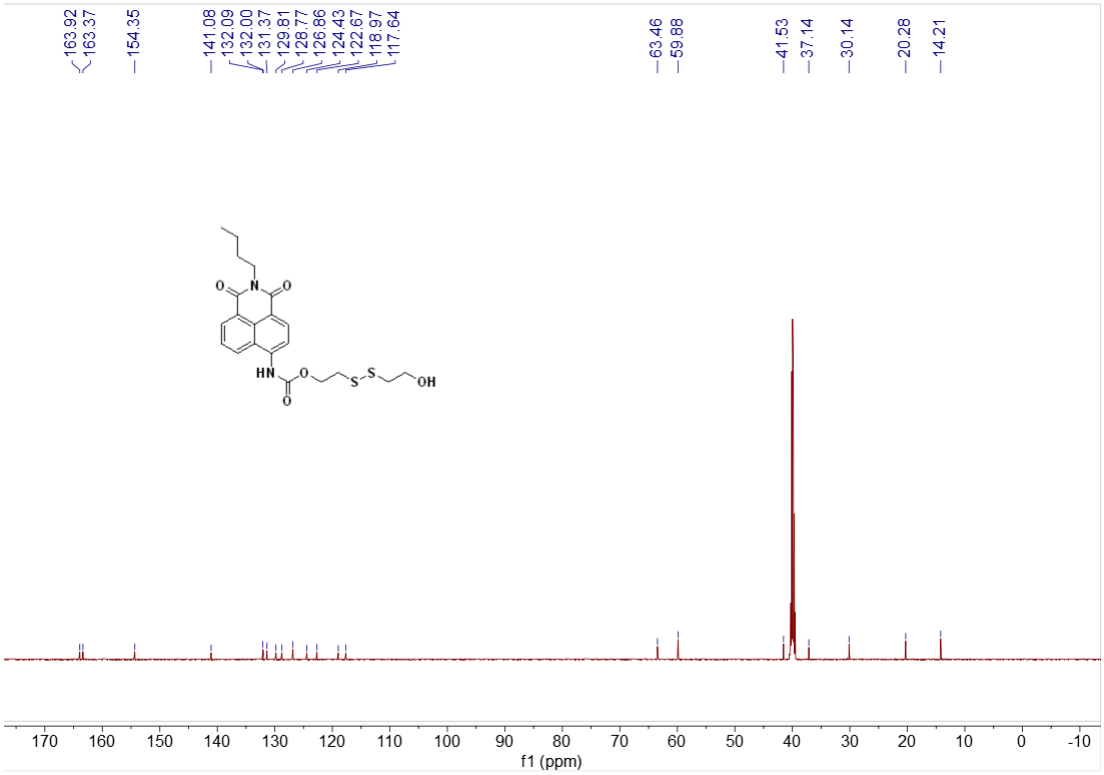


Figure S23. ^{13}C NMR Spectra of **ANC-SS** in DMSO-d_6 (151 MHz).

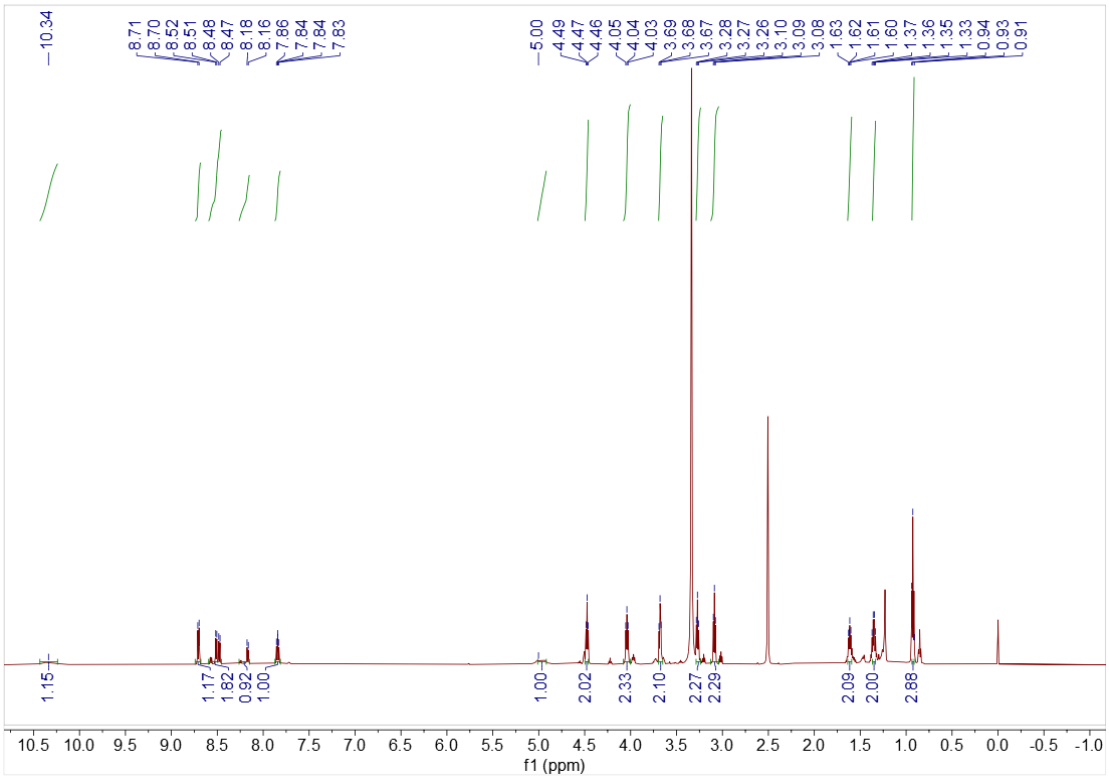


Figure S24. ^{1}H NMR Spectra of **ANC-SeSe** in DMSO-d_6 (400 MHz).

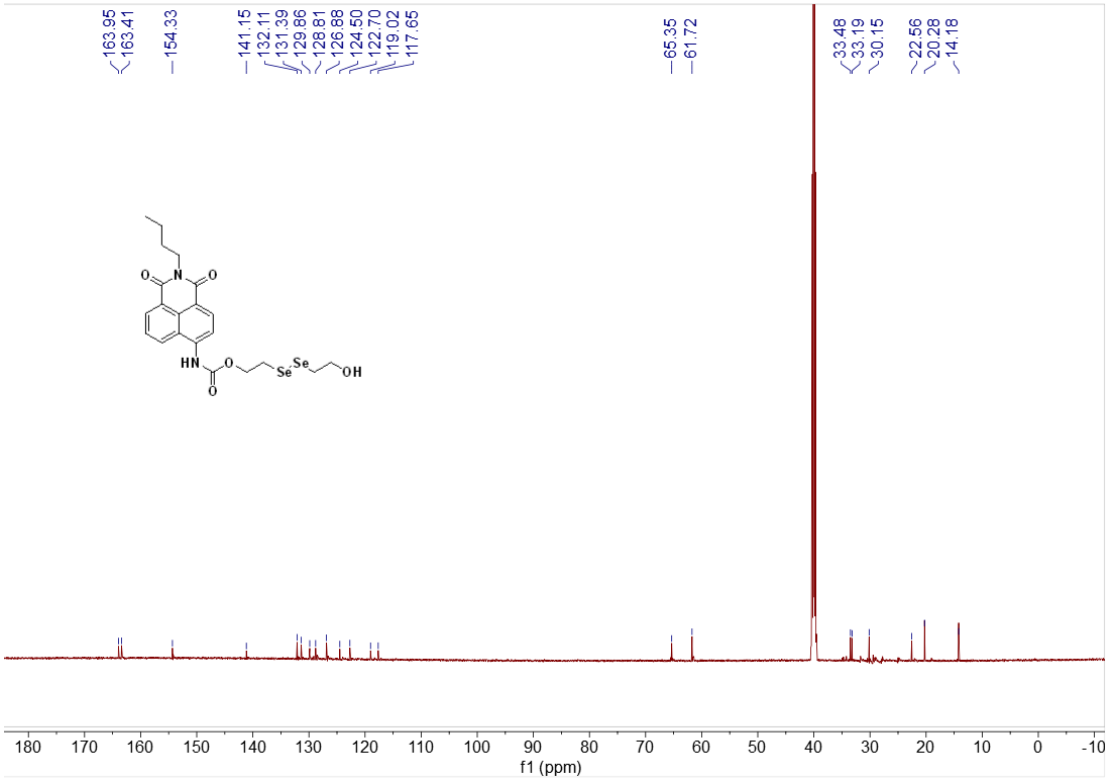


Figure S25. ^{13}C NMR Spectra of **ANC-SeSe** in DMSO-d_6 (151 MHz).

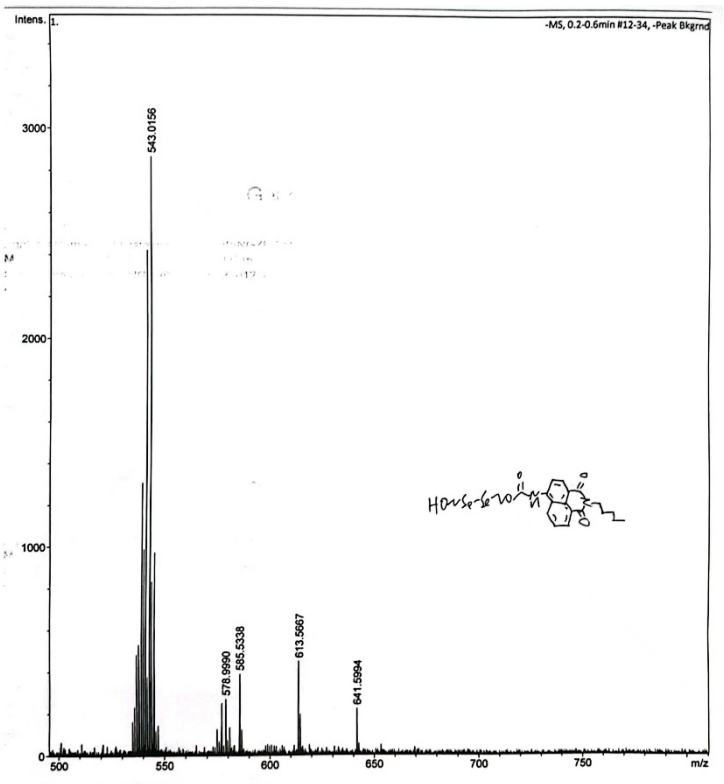


Figure S26. MS Spectra of **AFC-SeSe**.

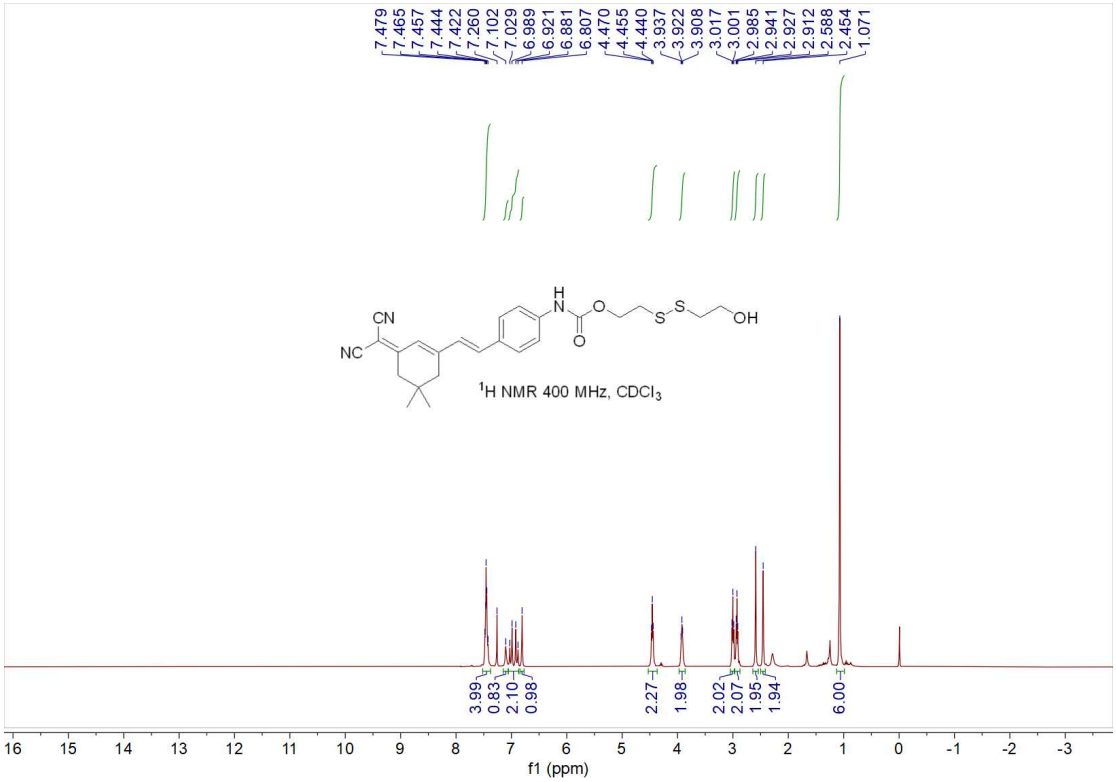


Figure S27. ^{1}H NMR Spectra of **ARC-SS** in $CDCl_3$-d_4 (400 MHz).

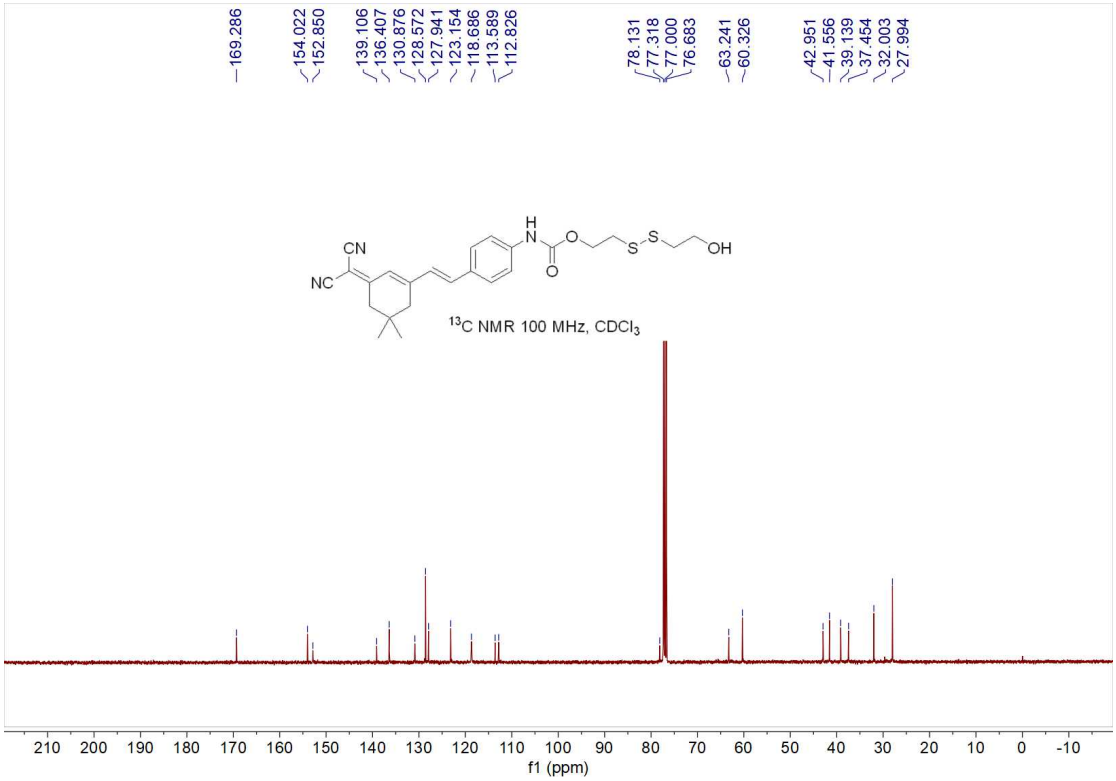


Figure S28. ^{13}C NMR Spectra of **ARC-SS** in $CDCl_3$-d_4 (151 MHz).

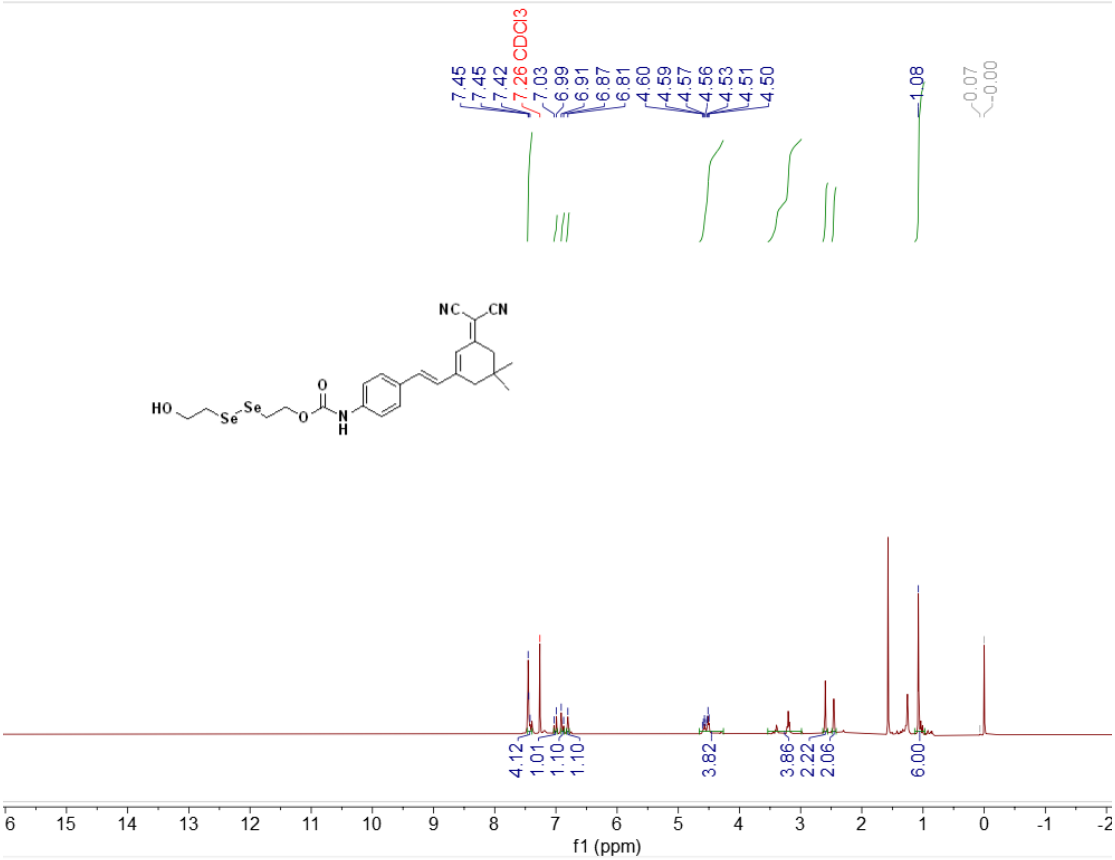


Figure S29. ^{1}H NMR Spectra of **ARC-SeSe** in $CDCl_3$-d_4 (400 MHz).

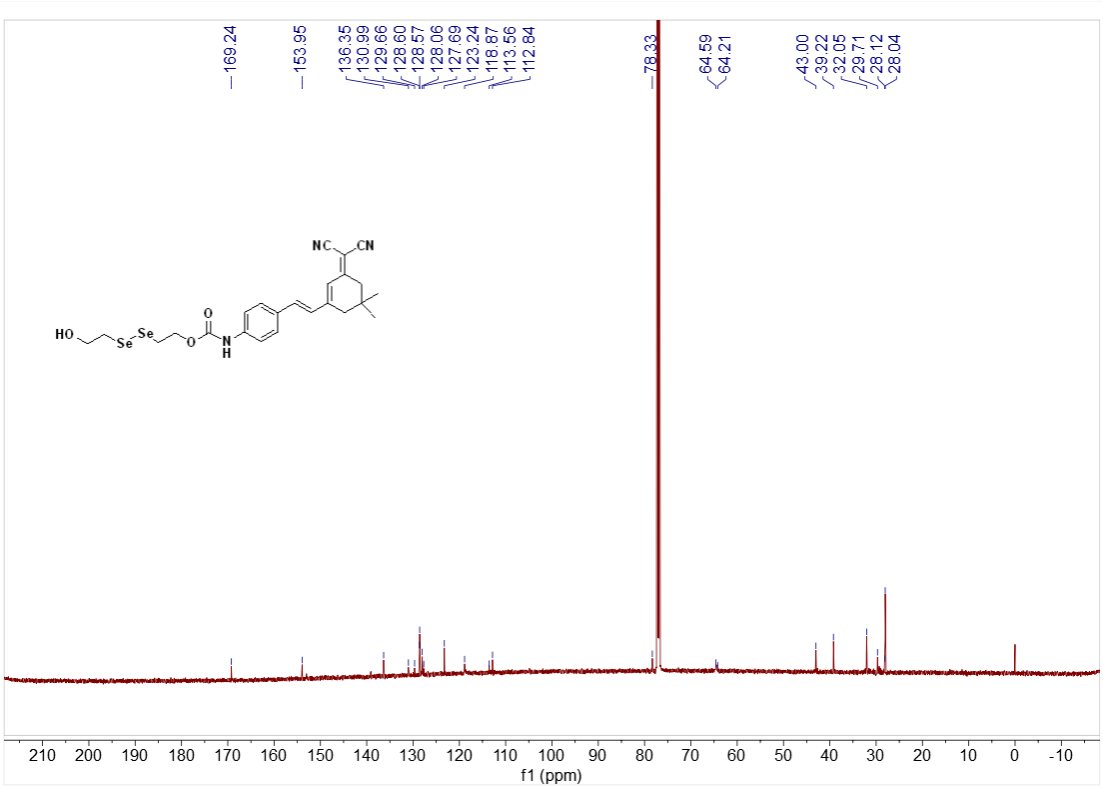


Figure S30. ^{13}C NMR Spectra of **ARC-SeSe** in $CDCl_3$-d_4 (151 MHz).

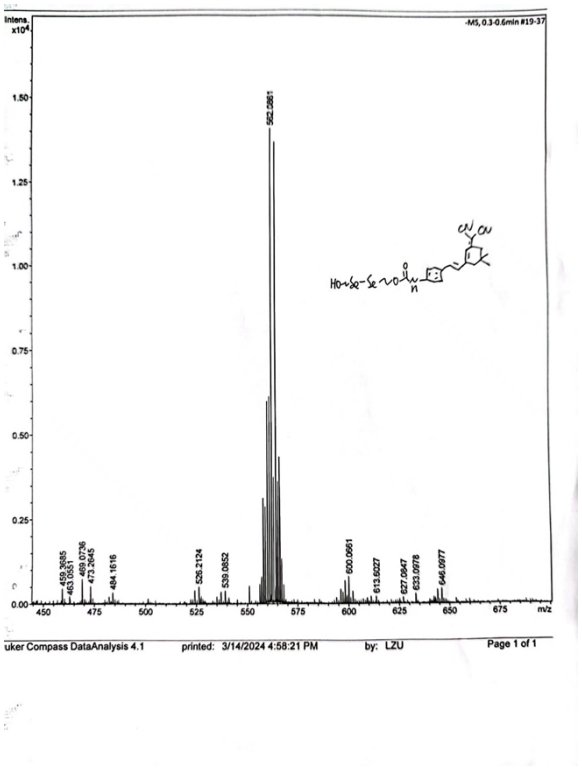


Figure S31. MS Spectra of **ARC-SeSe**.