Supporting Information

A reaction-based near-infrared fluorescent probe that can visualize endogenous selenocysteine *in vivo* in tumor-bearing mice

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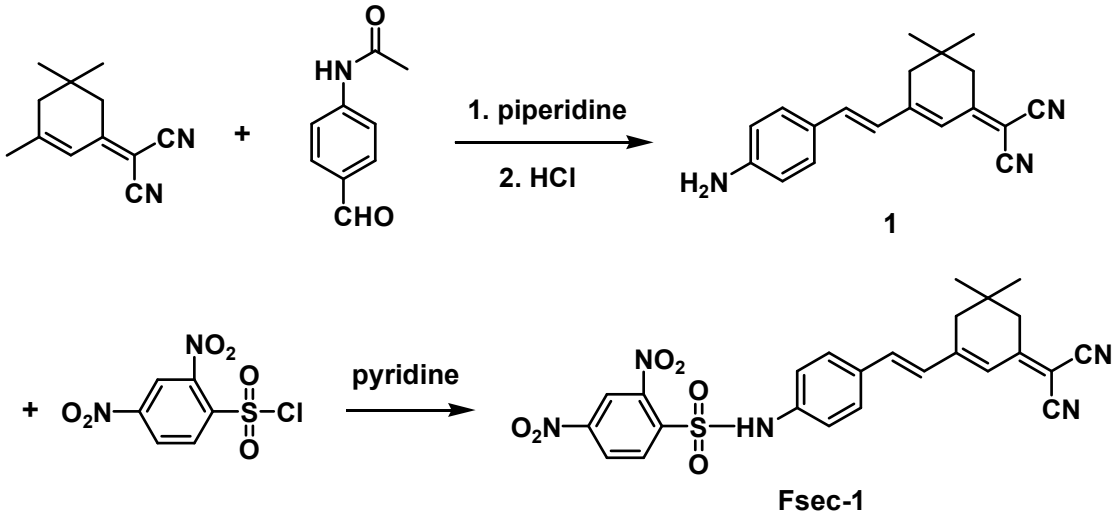
Chemicals and media. Unless noted otherwise, all reagents and solvents used for synthesis were obtained from commercial suppliers and employed without further purification. DMEM media, fetal bovine serum (FBS), penicillin (100 μg/mL) and streptomycin (100 μg/mL) (Life Technologies, CA, USA).

Synthesis and Characterisation of compounds

Scheme S1. Synthesis Route for Fsec-1



Synthesis of 2-[3-[(1E)-2-(4-aminophenyl)ethenyl]-5,5-dimethyl-2-cyclohexen-1-ylidene]-propanedinitrile (1)

To a stirred solution of 2-(3,5,5-trimethylcyclohex-2-enylidene) malononitrile (330 mg, 1.77 mmol) and N-(4-formyl-phenyl) acetamide (289 mg, 1.77 mmol) in dry ethanol (10 mL) was added piperidine (17 μL, 0.17 mmol). The mixture was stirred at 80 ºC for 12 h. Then the solvent was removed under reduced pressure to give a yellow solid. The yellow solid was refluxed in a solution of conc. HCl and ethanol (2:1, 15 mL) for another 2 h. After the reaction completes, the pH of the solution was adjusted to neutral. The precipitate was collected by filtration and then purified by column chromatography on silica gel to afford compound 1 as a red solid (172 mg, yield: 34.0 %). TLC (silica, Hexane: EtOAc, 2:1 v/v): R_f = 0.5; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.34 (d, *J* = 8.0 Hz,

2H), 6.99 (d, J = 16.0 Hz, 1H), 6.76-6.83 (m, 2H), 6.68 (d, J = 8.0 Hz, 2H), 2.57 (s, 2H), 2.44 (s, 2H), 1.06 (s, 6H). HRMS: m/z calcd for compound 1 ($C_{19}H_{18}N_3$, M-H^+) 288.1501; found, 288.1502.

Evidence of mechanism detection. Fsec-1 (7.8 mg, 0.015 mmol) was dissolved in DMSO (15 mL), and then the solution of Sec (0.15 mmol) in PBS buffer (15 mL, 20 mM, pH = 7.4) was added. After stirring at 37 ºC for 30 min, the resultant mixture was extracted by EtOAc. The fluorescent product was thereafter purified by column chromatography and further characterised by HRMS and ^{1}H NMR. The NMR and HRMS spectra of fluorescent product were consistent with those of compound 1, hence the confirmation of the fluorescent product as compound 1.

Quantum Yields. Quantum yields were determined by using rhodamine B as fluorescence standard. The quantum yield was calculated according to the equation[1]: $\Phi_{sample} = \Phi_{standard} (Grad_{sample}/Grad_{standard})(\eta^2_{sample}/\eta^2_{standard})$; where Φ is the quantum yield, $\Phi_{rhodamine} = 0.72$ in methanol, Grad is the slope of the plot of absorbance versus integrated emission intensity, and η is the refractive index of the solvent. Absorbance of sample and standard at their respective excitation wavelengths was kept below 0.05.

Determination of the detection limit. The detection limit was calculated based on previous method.[2] The fluorescence emission spectrum of Fsec-1 without Sec was measured by 10 times and the standard deviation of blank measurement was determined. Then the probe solution was added with Sec of concentration from 0 to 100 μM. A linear regression curve was then achieved according to the fluorescence intensity in the range of Sec from 0 to 1 μM. The detection limit was calculated with the following equation: Detection limit = 3σ/k. Where σ is the standard deviation of blank measurements, k is the slope between the fluorescence intensity ratios versus Sec concentrations.

Preparation of the test solution

(1) Fsec-1 stock solution preparation: Fsec-1 (5.19 mg, 0.01 mmol) was dissolved into DMSO (10 mL) to get 1.0 mM stock solution.

(2) Sec stock solution preparation: Sec was generated in *situ* by mixing equal molar of $(Sec)_2$ with DTT to get 10.0 mM stock solution in PBS buffer, which was then diluted to 1.0 mM and 100 μM solution for general use.

(3) Cys (L-Cysteine) stock solution preparation: Cys (24.2 mg, 0.2 mmol) was dissolved into DI H_2O (10 mL) to get 20.0 mM stock solution, which was then diluted to 1.0 mM solution for general use.

(4) Hcy (Homocysteine) stock solution preparation: Hcy (27.0 mg, 0.2 mmol) was dissolved into DI H_2O (10 mL) to get 20.0 mM stock solution, which was then diluted to 1.0 mM solution for general use.

(5) GSH (Glutathione) stock solution preparation: GSH (61.5 mg, 0.2 mmol) was dissolved into DI H_2O (10 mL) to get 20.0 mM stock solution, which was then diluted to 10 mM solution for general use.

(6) Na_2S stock solution preparation:[3] 5 mg EDTA was dissolved in 10 mL DI H_2O in a 25 mL Schlenk tube. The solution was purged vigorously with nitrogen for 15 min. Then 48 mg sodium sulfide ($Na_2S{\cdot}9H_2O$) was dissolved in the solution under nitrogen. The resulting solution was 20 mM Na_2S, which was then diluted to 1.0 mM-100 μM stock solution for general use.

(7) N-acetyl-L-cysteine (NAC) stock solution preparation: NAC (32.6 mg, 0.2 mmol) was dissolved into DI H_2O (10 mL) to get 20.0 mM stock solution, which was then diluted to 1.0 mM and 100 μM solution for general use.

(8) Stock solutions of other biological analytes, including amino acids such as Ala, Glu, Trp, Met, Tyr, Leu, Val, Ser, Pro, Arg, Gly, Phe, His, Gln, Asn, Ile, Thr; metal salts such as LiCl, NaCl, KCl, $MgCl_2$, $AlCl_3$, $Zn(NO_3)_2$, $Mn(NO_3)_2$, $Co(NO_3)_2$, $Cd(NO_3)_2$, $Ni(NO_3)_2$, $CaCl_2$, $HgCl_2$, $Cu(NO_3)_2$, $FeCl_2$, $FeCl_3$, $AgNO_3$; anions such as NaF, NaCl, KBr, KI, NaAcO, $NaHCO_3$, NaN_3, $NaNO_3$, Na_2SO_4, NaSCN, $Na_2C_2O_4$, $Na_2S_2O_7$, $NaHSO_3$, KCN, NaClO, Na_2HPO_4; reducing agent (DTT, NADH), glucose, ascorbic acid, selenocompounds such as Na_2SeO_3, Na_2Se, Se-methylselenocysteine, selenocystine, selenomethionine; reactive oxygen species such as

H_2O_2, $\cdot OCl^-$, O_2^-, $\cdot OH$, ^tBuOOH; reactive nitrogen species such as NO_2^-, NO, were prepared in DI H_2O.

(9) Superoxide radicals (O_2^-) were generated according to the previous reported method.[4] $\cdot OH$ was generated by Fenton reaction between Fe^{II}(EDTA) and H_2O_2 quantitively.[5] NO was generated in from of 3-(Aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5, 50 μmol/ml). NO_2^- was generated from $NaNO_2$.

Absorption analyses. UV-Vis absorption spectra were measured at room temperature on a Shimadzu PharmaSpec UV-2401PC UV-Visible spectrophotometer. Fsec-1 probe (DMSO) was diluted to 20 μM by the addition of 10 mM PBS buffer, and then mixed with a certain amount of Sec. The resulting solution was incubated for 20 min prior to measurements, with the mean ± SD expressed (n = 3).

MTT assay. The in vitro cytotoxicity of Fsec-1 and compound 1 were measured using standard MTT assay (Sigma-Aldrich). MCF-7 cells were seeded into 96-well plates at a density of 50,000 cells/well and then maintained at 37 °C in a 5 % CO_2 incubator. The cells were incubated with varying concentrations of Fsec-1 and compound 1 for 1 h, 2 h, 4 h, 6 h, and 12 h, respectively. Cells in culture medium without Fsec-1 and compound 1were used as control group. After the incubation time, 20 μL of MTT dye (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide, 5 mg/ml in phosphate buffered saline), was added to each well, and incubated for an additional 4 h at 37 ºC. Then, the remaining MTT solution was removed, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. The plate was shaken for 10 min and the absorbance was measured at 570 nm on a spectrophotometer. Each sample was performed in triplicate, and the entire experiment was repeated three times. The cell viability of Fsec-1 and compound 1 (20 μM) at 1, 2, 4, 6 and 12 h further demonstrated that the Fsec-1 and 1 are of low toxicity to cultured MCF-7 cells.

References

(1) Williams, A. T. R.; Winfield, S. A.; Miller, J. N. Relative fluorescence quantum yields using a computer-controlled luminescence spectrometer. *Analyst.* **1983**, 108, 1067-1071.

(2) Joshi, B. P.; Park, J.; Lee, W. I.; Lee, K. Ratiometric and turn-on monitoring for heavy and transition metal ions in aqueous solution with a fluorescent peptide sensor. *Talanta.* **2009**, 78, 903-909.

(3) Qian, Y.; Zhang, L.; Ding, S.; Deng, X.; He, C.; Zheng, X. E.; Zhu, H. L.; Zhao, J. A fluorescent probe for rapid detection of hydrogen sulfide in blood plasma and brain tissues in mice. *Chem. Sci.* **2012**, 3, 2920-2923.

(4) Arudi, R.; Allen, A.; Bielski, B. Some observations on the chemistry of KO_2-DMSO solutions. *FEBS. Lett.* **1981**, 135, 265-267.

(5) Halliwell, B.; Gutteridge, J. M. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.* **1986**, 246, 501-514.

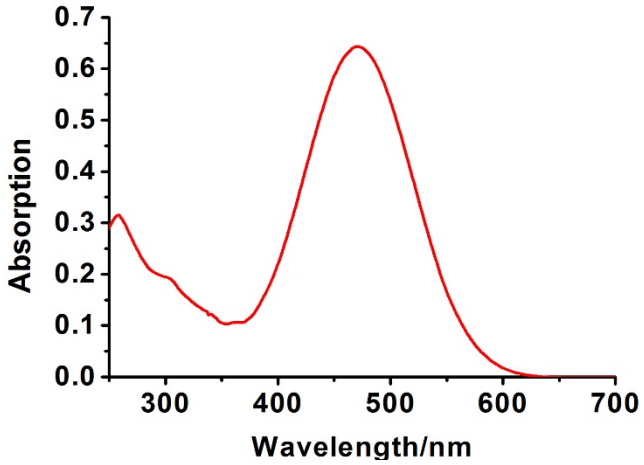


Figure S1. Absorption spectra of Fsec-1 in PBS buffer (10 mM, pH 7.4, 10% DMSO).

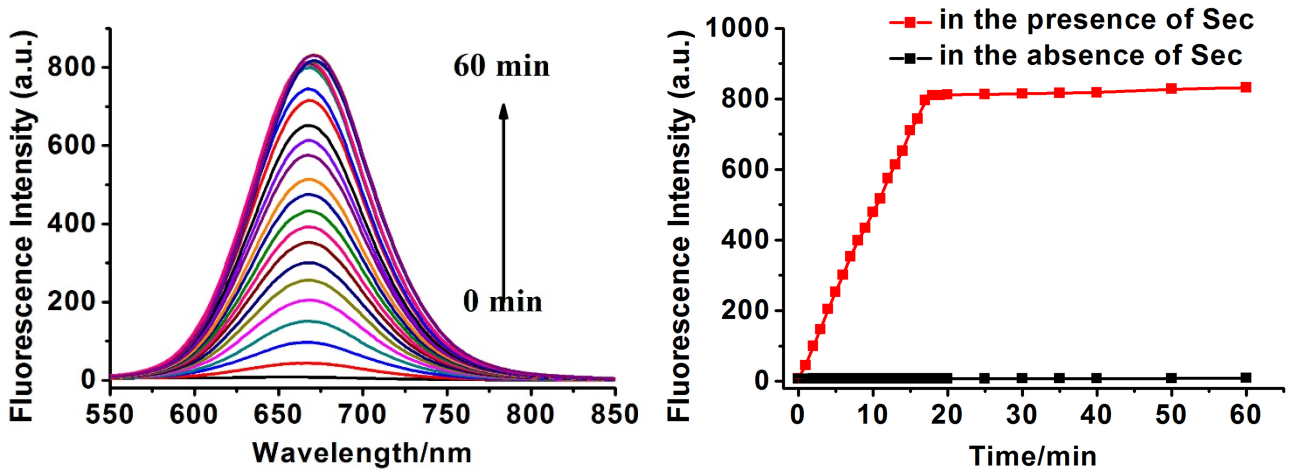


Figure S2. (**A**) Fluorescence spectra of Fsec-1 (10 μM) with Sec (100 μM) in PBS buffer (10 mM, pH 7.4, 10% DMSO) at 37 °C for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50 and 60 min. (**B**) Time profile of Fsec-1 (10 μM) toward Sec (100 μM) in PBS buffer (10 mM, pH 7.4, 10 % DMSO) at 37 °C for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50 and 60 min. Data are presented as the mean ± SD (n = 3).

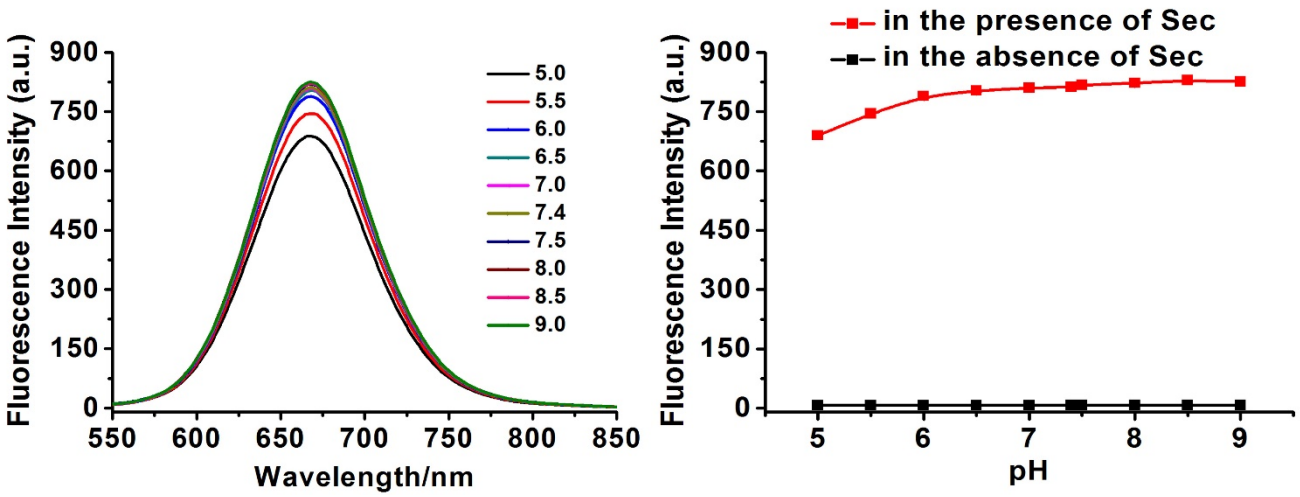


Figure S3. (**A**) Fluorescence spectra of Fsec-1 (10 μM) with Sec (100 μM) in different pH buffer (10 mM, pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.4, 7.5, 8.0, 8.5 and 9.0, 10% DMSO) at 37 °C for 20 min. (**B**) Fluorescence responses of Fsec-1 (10 μM) with Sec (100 μM) in different pH buffer (10 mM, pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.4, 7.5, 8.0, 8.5 and 9.0, 10% DMSO) at 37 °C for 20 min.

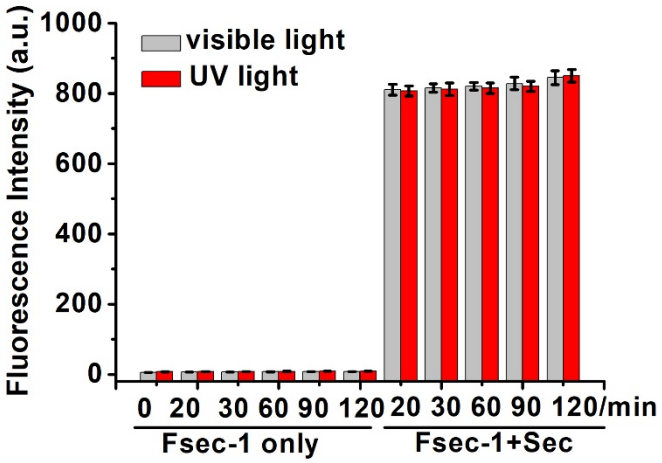


Figure S4. Photostability of Fsec-1 (10 μM) and Fsec (10 μM) +Sec (100 μM) in PBS buffer (10 mM, pH = 7.4, 10 % DMSO) at different times (0, 20, 30, 60, 60 and 120 min) under visible light and UV light.

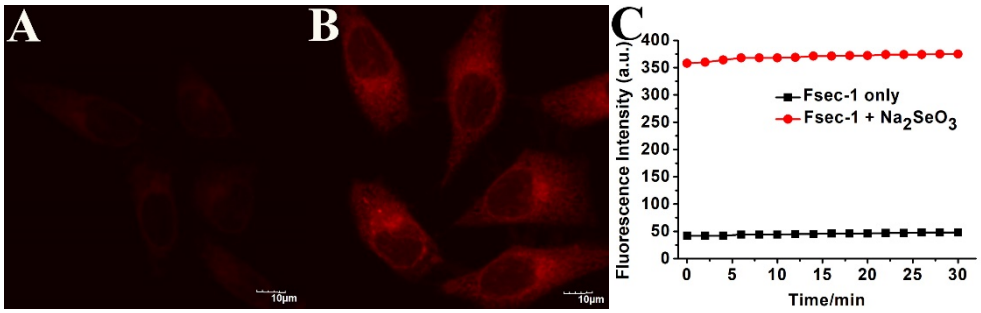


Figure S5. Photostability of Fsec-1 in living cells. Cells were incubated with Fsec-1 (20 μM) alone for 6 h (A). Cells were pretreated with 5 μM Na_2SeO_3 for 6 h and then incubated with Fsec-1 (20 μM) for 20 min (B). The average fluorescence intensity from cells in panels A and B was recorded with 2 min intervals (C).

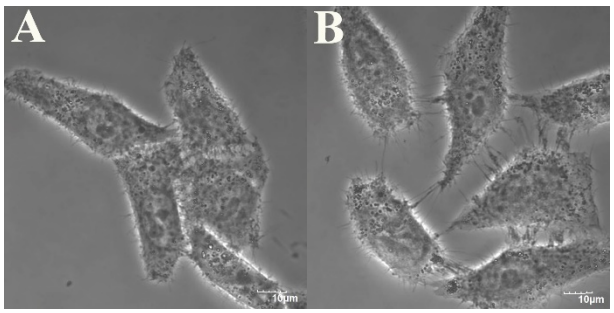


Figure S6. The corresponding bright images of Fig. S5, panels A and B.

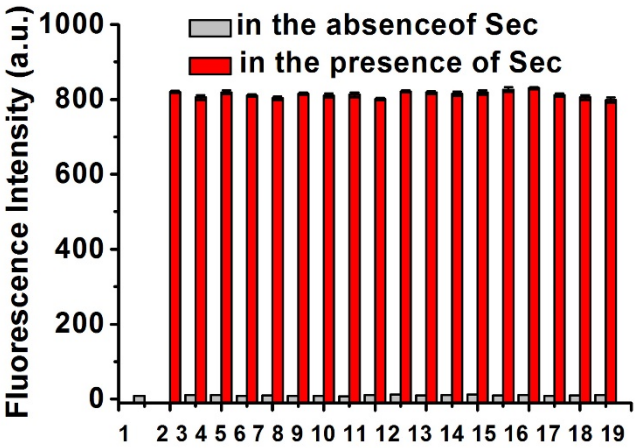


Figure S7. Fluorescence responses of Fsec-1 (10 μM) with Sec (100 μM) and various amino acids (1 mM) in PBS buffer (10 mM, pH 7.4, 10% DMSO) after 20 min of incubation. 1. blank; 2. Sec (100 μ M); 3. Ala; 4. Glu; 5. Trp; 6. Met; 7. Tyr; 8. Leu; 9. Val; 10. Ser; 11. Pro; 12. Arg; 13. Gly; 14. Phe; 15. His; 16. Gln; 17. Asn; 18. Ile; 19. Thr. Red bars represent the subsequent addition of Sec (100 μM) to the mixture. Data are presented as the mean ± SD (n = 3).

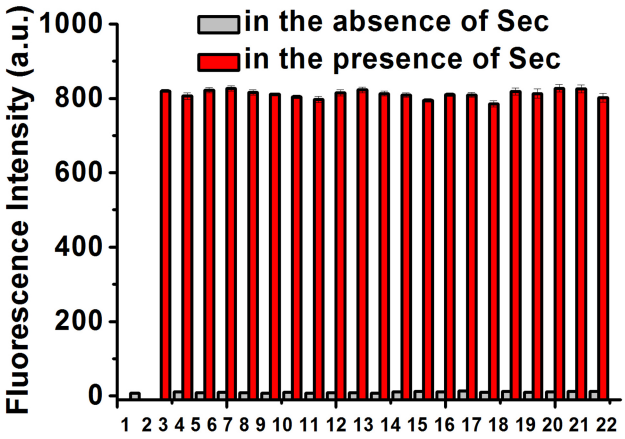


Figure S8. Fluorescence responses of Fsec-1 (10 μM) with Sec (100 μM) and metal ions (1 mM), other agent (1 mM) in PBS buffer (10 mM, pH 7.4, 10% DMSO) after 20 min of incubation. 1. blank; 2. Sec (100 μ M); 3. Li^{+}; 4. Na^{+}; 5. K^{+}; 6. Mg^{2+}; 7. Al^{3+}; 8. Zn^{2+}; 9. Mn^{2+}; 10. Co^{2+}; 11. Cd^{2+}; 12. Ni^{2+}; 13. Ca^{2+}; 14. Hg^{2+}; 15. Cu^{2+}; 16. Fe^{2+}; 17. Fe^{3+}; 18. Ag^{+}; 19. DTT; 20. NADH; 21. glucose; 22. ascorbic acid. Red bars represent the subsequent addition of Sec (100 μM) to the mixture. Data are presented as the mean ± SD (n = 3).

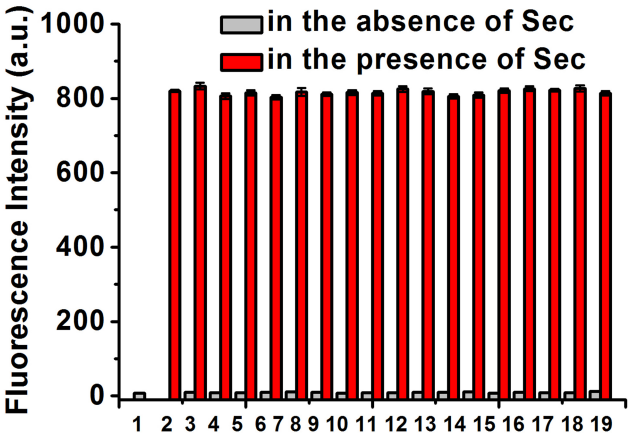


Figure S9. Fluorescence responses of Fsec-1 (10 μM) with Sec (100 μM) and ions (1 mM) in PBS buffer (10 mM, pH 7.4, 10% DMSO) after 20 min of incubation. 1. blank; 2. Sec (100 μ M); 3. F^-; 4. Cl^-; 5. Br^-; 6. I^-; 7. AcO^-; 8. HCO_3^-; 9. N_3^-; 10. NO_3^-; 11. SO_4^{2-}; 12. $S_2O_3^{2-}$; 13. SCN^-; 14. $C_2O_4^{2-}$; 15. $S_2O_7^{2-}$; 16. HSO_3^-; 17. CN^-; 18. ClO^-; 19. HPO_4^{2-}. Red bars represent the subsequent addition of Sec (100 μM) to the mixture. Data are presented as the mean ± SD (n = 3).

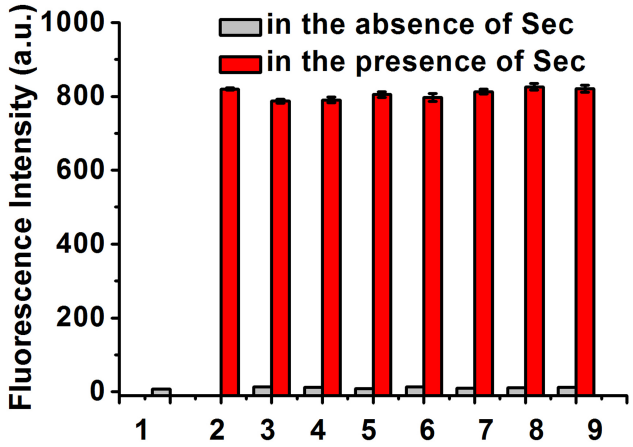


Figure S10. Fluorescence responses of Fsec-1 (10 μM) with Sec (100 μM) and reactive oxygen species, reactive nitrogen species (1 mM) in PBS buffer (10 mM, pH 7.4, 10% DMSO) after 20 min of incubation. 1. blank; 2. Sec (100 μ M); 3. H_2O_2; 4. $\cdot OCl^-$ 5. $O_2^{\cdot-}$; 6. $\cdot OH$; 7. ^tBuOOH; 8. NO; 9. NO_2^-. Red bars represent the subsequent addition of Sec (100 μM) to the mixture. Data are presented as the mean ± SD (n = 3).

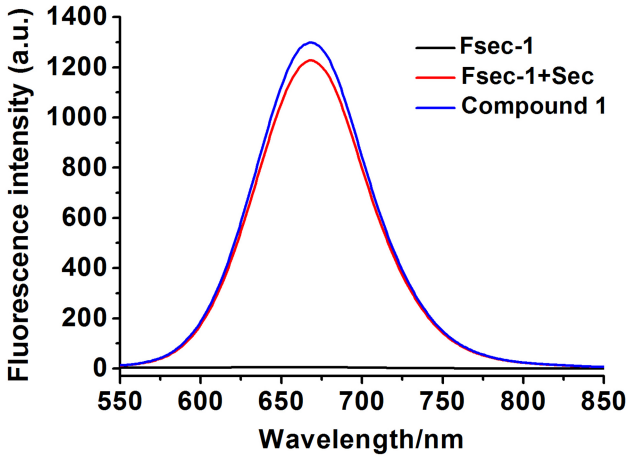


Figure S11. Fluorescence spectra of compound 1, Fsec-1 and Fsec-1 + Sec in PBS buffer (10 mM, pH 7.4, 10% DMSO).

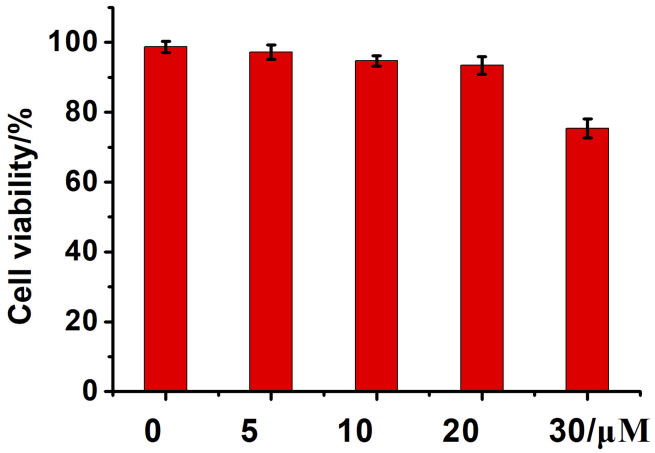


Figure S12. Cell viability of Fsec-1 (0, 5, 10, 20 and 30 μM) at 12 h in MCF-7 cell. Data are presented as the mean ± SD (n = 3).

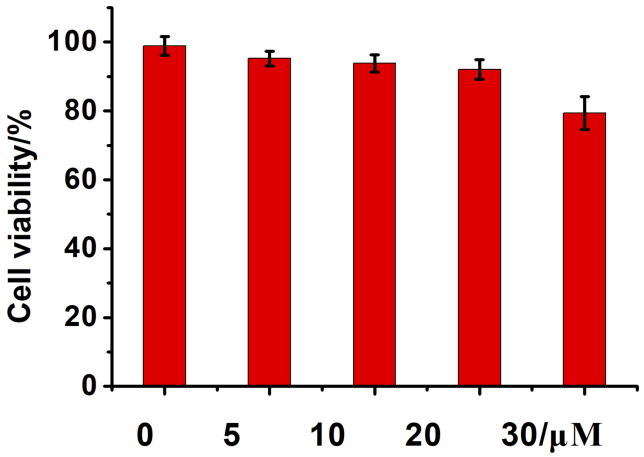


Figure S13. Cell viability of compound-1 (0, 5, 10, 20 and 30 μM) at 12 h in MCF-7 cell. Data are presented as the mean ± SD (n = 3).

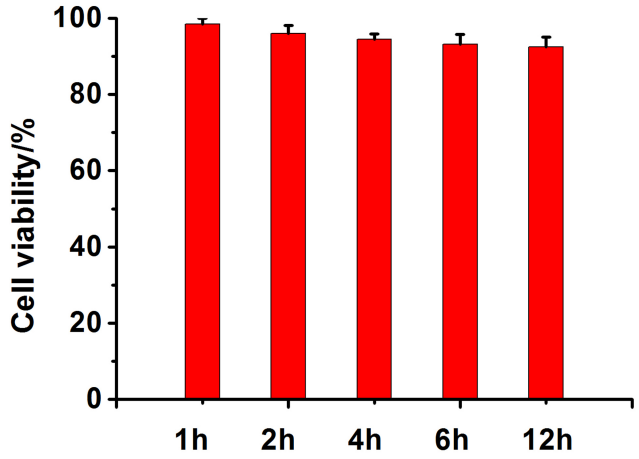


Figure S14. Cell viability of Fsec-1 (20 μM) at different times in MCF-7 cell. Data are presented as the mean ± SD (n = 3).

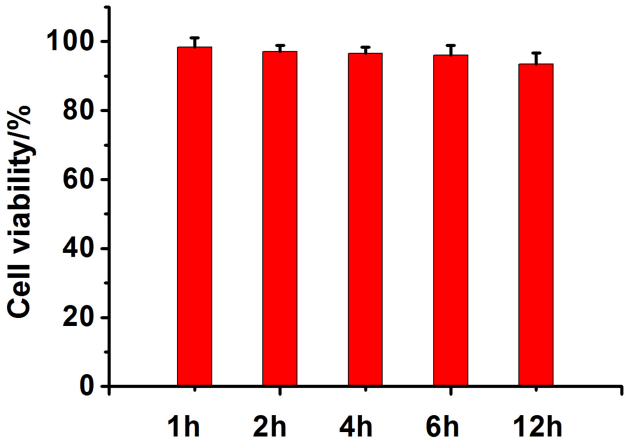


Figure S15. Cell viability of compound 1 (20 μM) at different times in MCF-7 cell. Data are presented as the mean ± SD (n = 3).

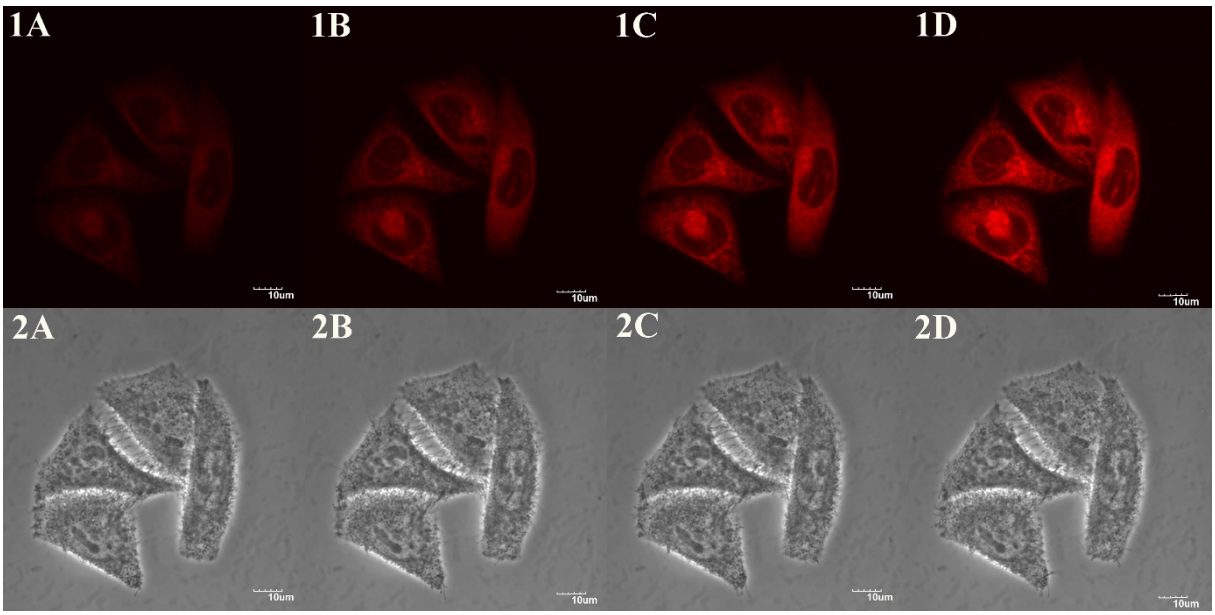


Figure S16. Confocal fluorescence imaging of exogenous Sec in living MCF-7 cells at different times. Cells were pretreated with 5 μM $(Sec)_2$ for 6 h, then incubated with Fesc-1 (20 μ M) for 5 min (1A), 10 min (1B), 15 min (1C) and 20 min (1D). The corresponding bright images of panels 1A, 1B, 1C and 1D. Scale bars = 10 μ m.

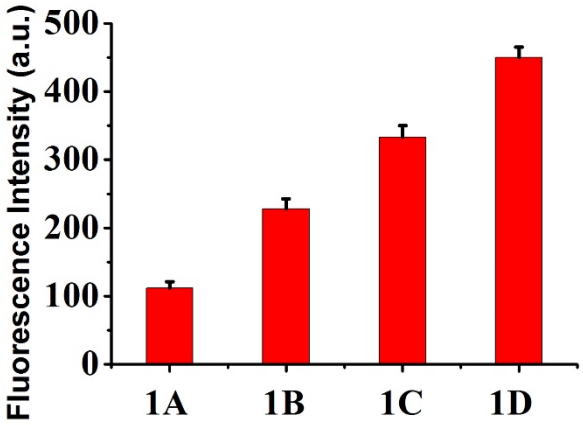


Figure S17. The average fluorescence intensity of the cells in Fig S16. Data are presented as the mean ± SD (n = 3).

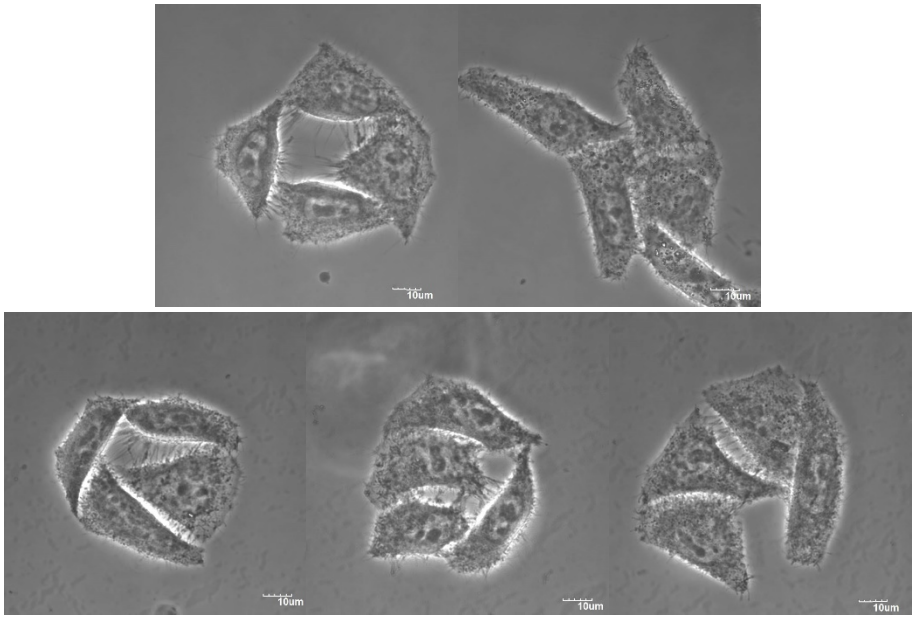
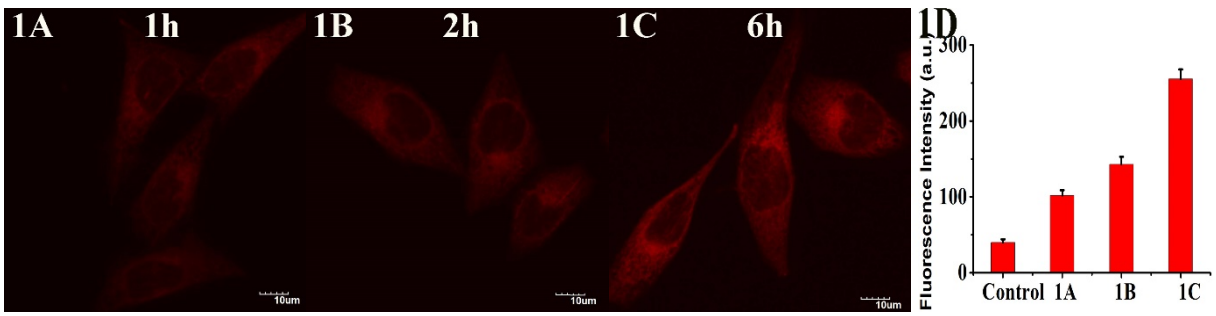


Figure S18. The corresponding bright images of Fig. 3, panels 1A, 1B, 2A, 2B and 2C.



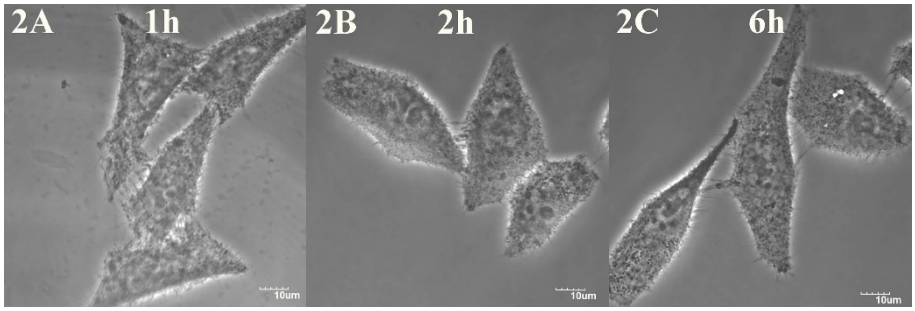


Figure S19. Confocal fluorescence imaging of endogenous Sec in living MCF-7 cells using Fsec-1. Cells were pretreated with 2 μM Na_2SeO_3 for 1 h (**1A**), 2 h (**1B**) and 6 h (**1C**), then incubated with Fesc-1 (20 μM) for 20 min. The average fluorescence intensity of the above images (**1D**). The corresponding bright images of panels 1A, 1B and 1C. Scale bars = 10 μm. Data are presented as the mean ± SD (*n* = 3).

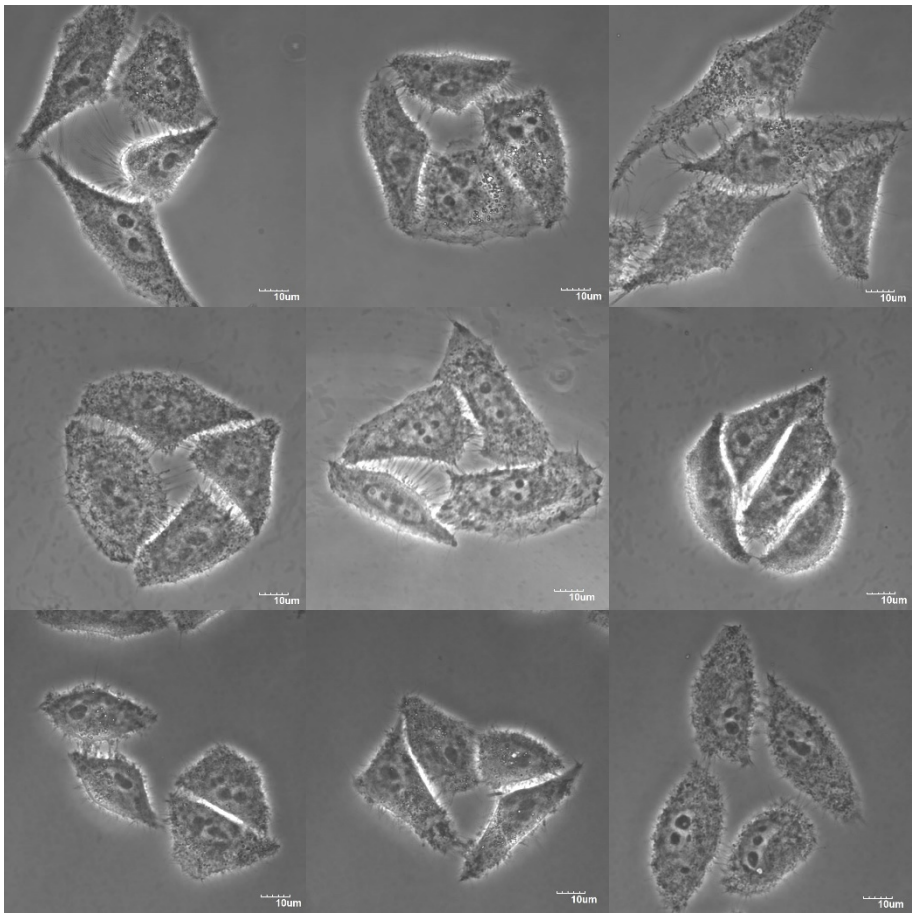
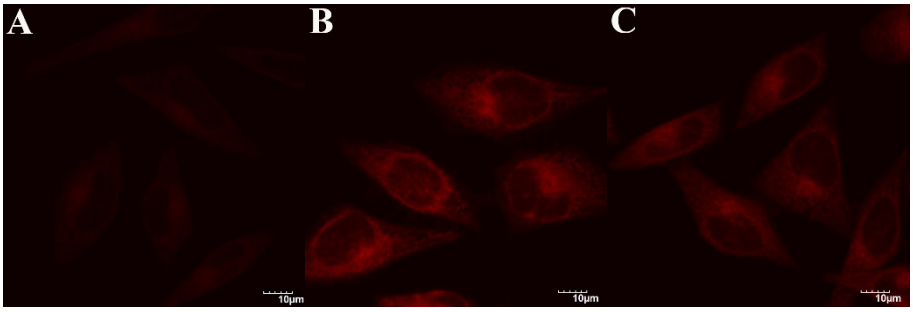


Figure S20. The corresponding bright images of Fig. 4, panels 1A, 1B, 1C, 2A, 2B, 2C, 3A, 3B and 3C.



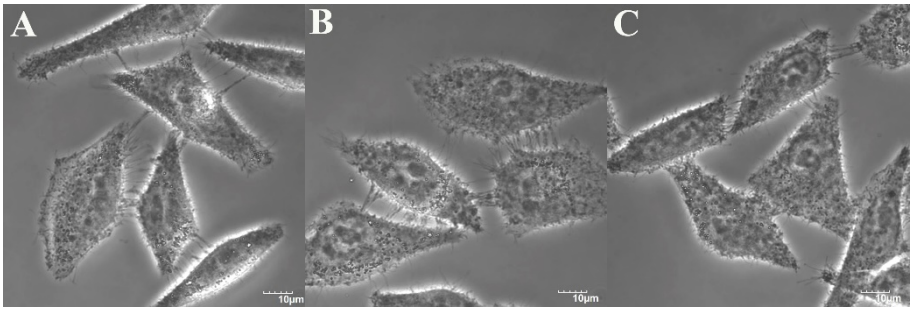


Figure S21. Confocal fluorescence imaging of exogenous and endogenous Sec in live MCF-7 cells using Fsec-1 (2.5 μM). Cells were incubated with Fsec-1 (2.5 μM) alone for 6 h (A). The cells were pretreated with 5 μM $(Sec)_2$ for 6 h (B) and then incubated with Fsec-1 (2.5 μ M) for 20 min. Cells were pretreated with 5 μM Na_2SeO_3 for 6 h (C) and then incubated with Fsec-1 (2.5 μM) for 20 min. Scale bars = 10 μm.

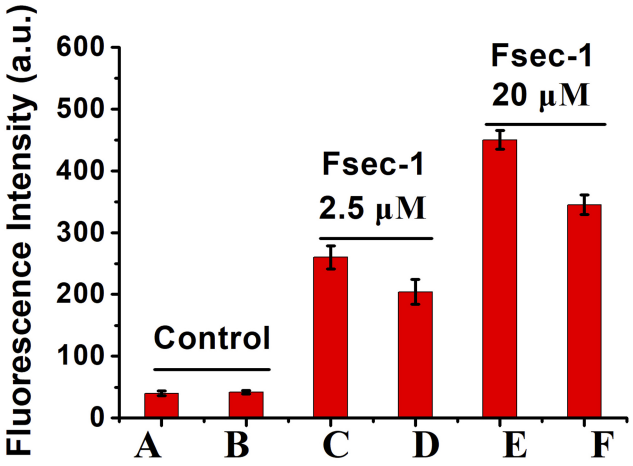
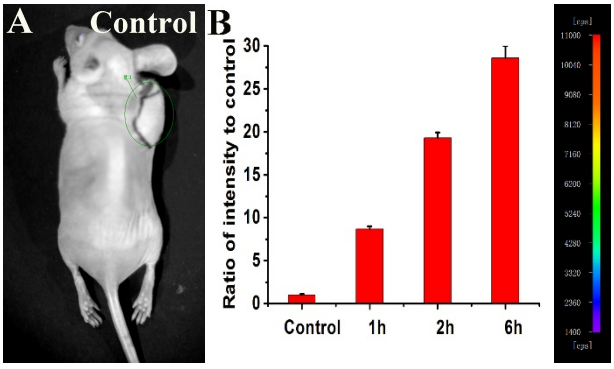


Figure S22. Average fluorescence intensity of the cell images. Cells were incubated with Fsec-1 (2.5 μM) alone for 6 h (A). Cells were incubated with Fsec-1 (20 μM) alone for 6 h (B). The cells were pretreated with 5 μM $(Sec)_2$ for 6 h (C) and then incubated with Fsec-1 (2.5 μM) for 20 min. Cells were pretreated with 5 μM Na_2SeO_3 for 6 h (D) and then incubated with Fsec-1 (2.5 μM) for 20 min. The cells were pretreated with 5 μM $(Sec)_2$ for 6 h (C) and then incubated with Fsec-1 (20 μM) for 20 min. Cells were pretreated with 5 μM Na_2SeO_3 for 6 h (D) and then incubated with Fsec-1 (20 μM) for 20 min.



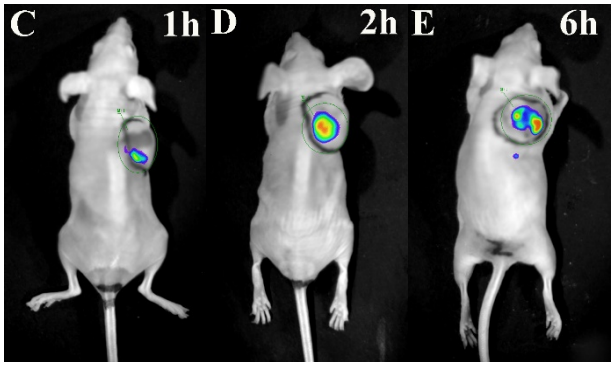


Figure S23. Fluorescence images of Sec in tumor-bearing mice. The mice were intratumorally injected with the probe Fsec-1 (1 μM, in 100 μL saline, 1 % DMSO, 1 % tween 80) as the control group (**A**). Quantification of the fluorescence emission intensities from the tumor area of the mice of the all groups (**B**). The mice were intratumorally injected with Na_2SeO_3 (1 μM, in 100 μL saline)), followed by intratumorally injection of Fsec-1 (1 μM, in 100 μL saline, 1% DMSO, 1% tween 80) after 1 h (**C**), 2 h (**D**), and 6 h (**E**). Data are presented as the mean ± SD (n = 3).

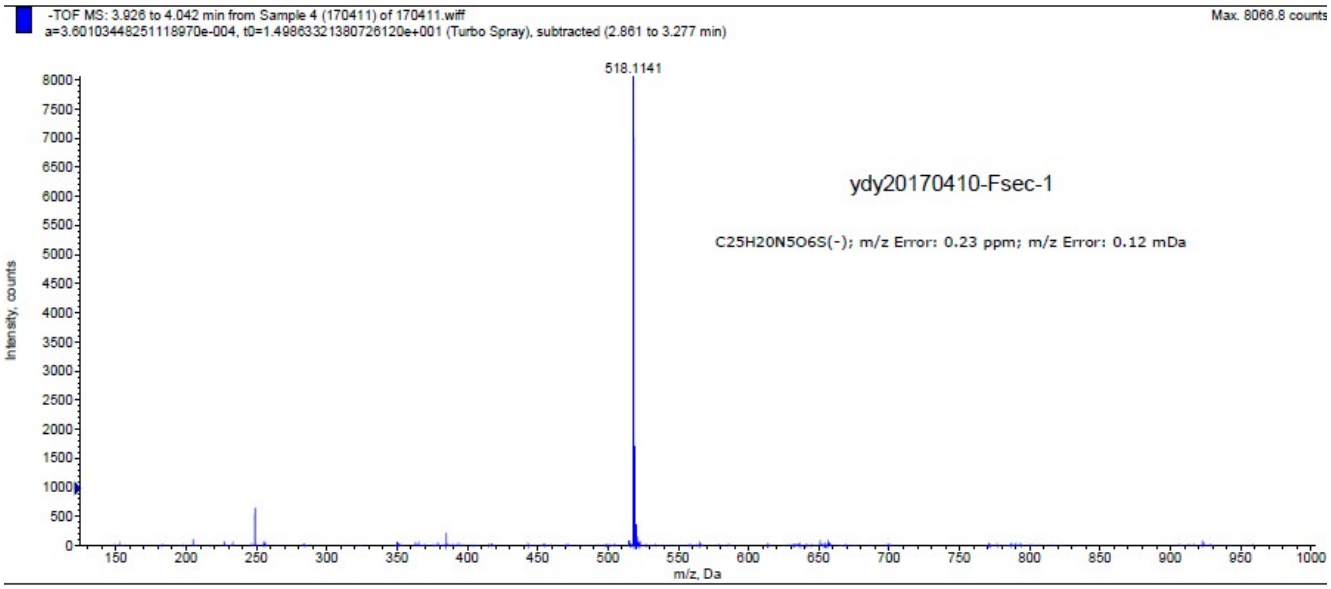
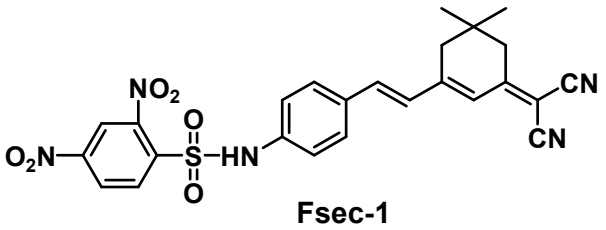


Figure S24. HR-MS identification of Fsec-1 (calculated for $C_{25}H_{20}N_5O_6S$ $(M-H)^+$ 518.1135; found 518.1141).



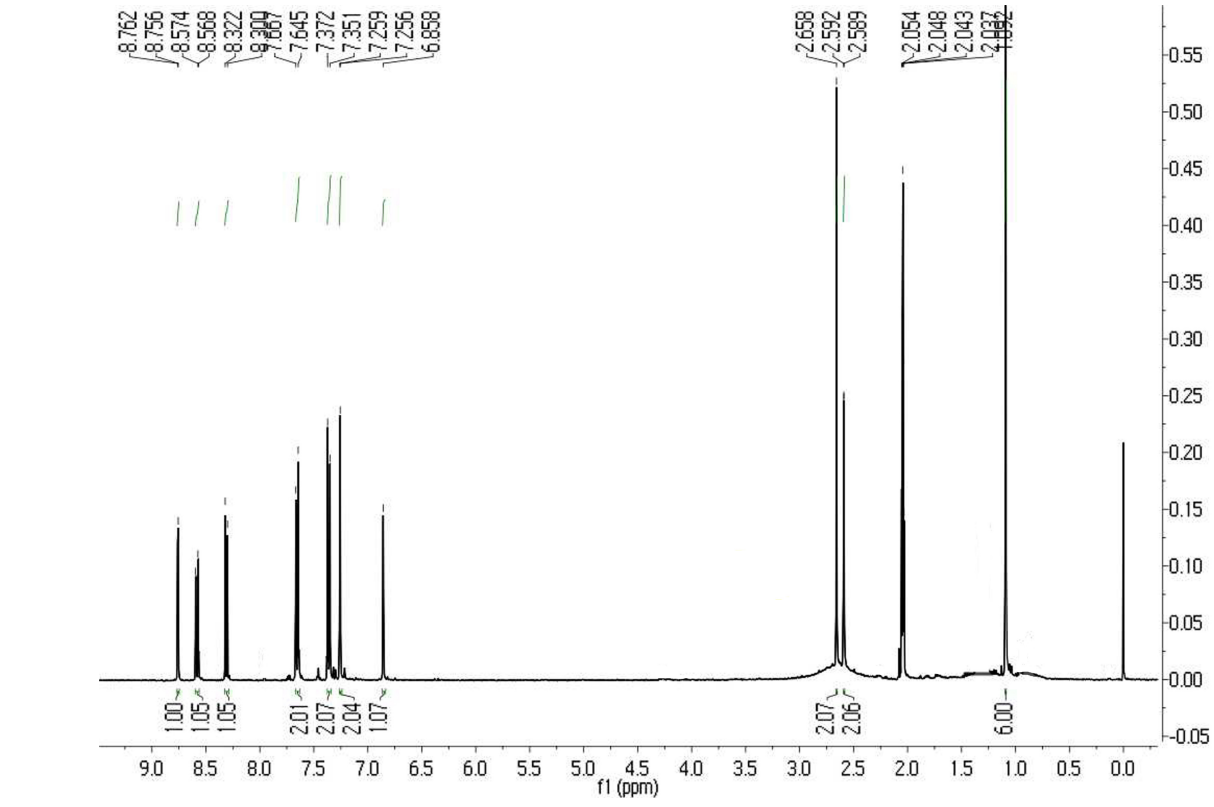


Figure S25. ^{1}H NMR spectra of Fsec-1.

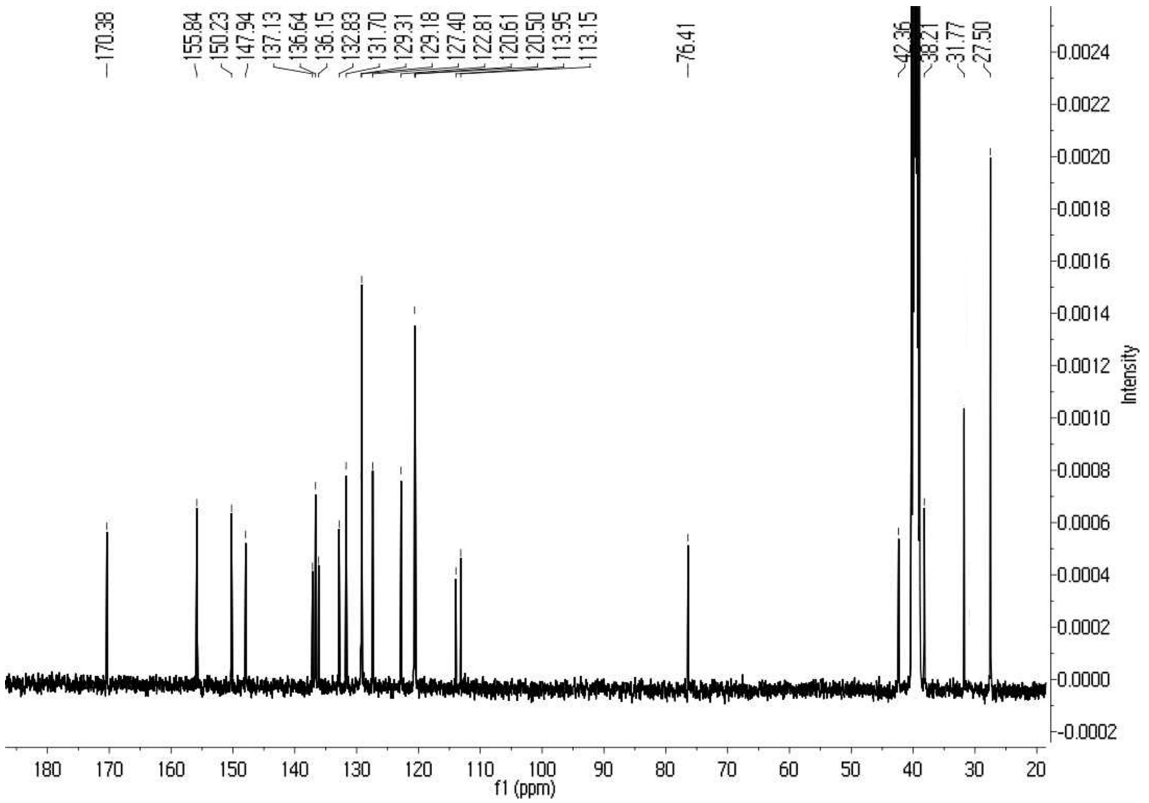


Figure S26. ^{13}C NMR spectra of Fsec-1.

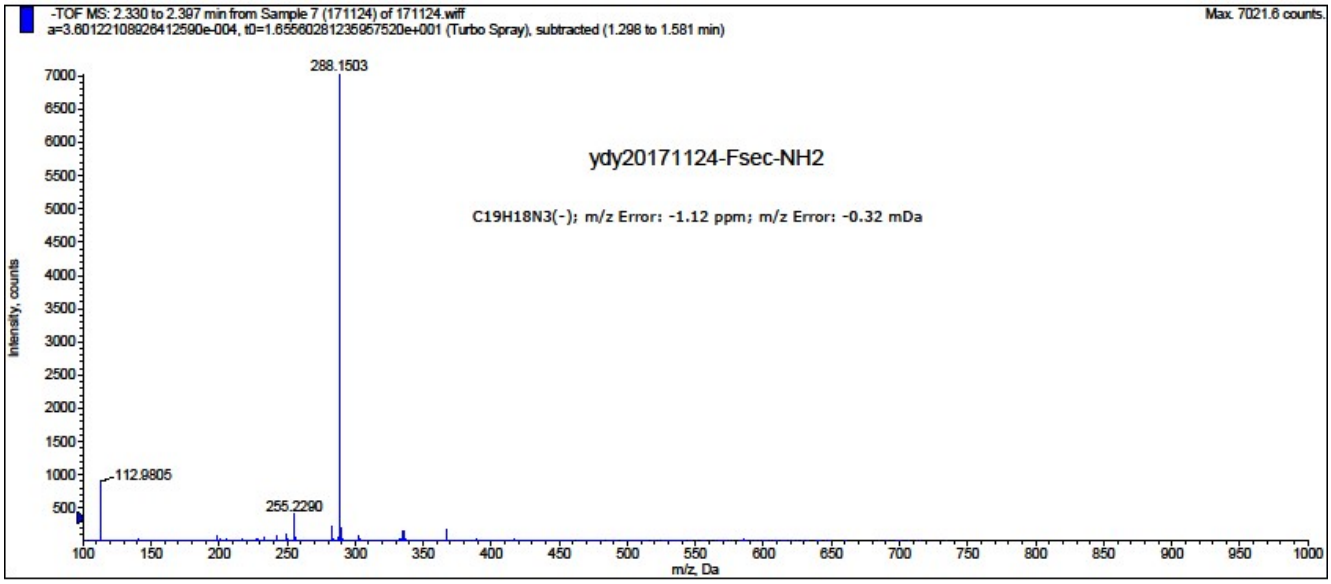


Figure S27. HR-MS identification of compound 1 (calculated for $C_{19}H_{18}N_3$, $(M-H)^+$ 288.1501; found, 288.1503).

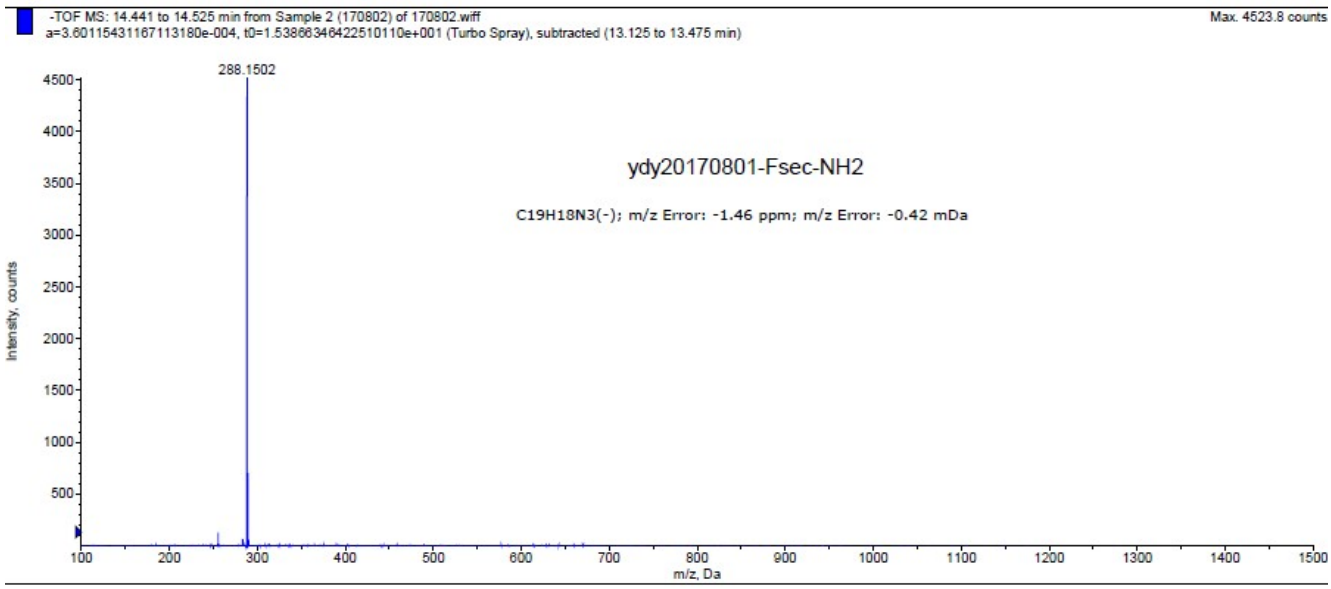
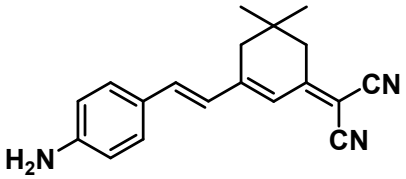


Figure S28. HR-MS identification of isolated fluorescent product of Fsec-1 + Sec (calculated for $C_{19}H_{18}N_3$, $(M-H)^+$ 288.1501; found, 288.1502).



1

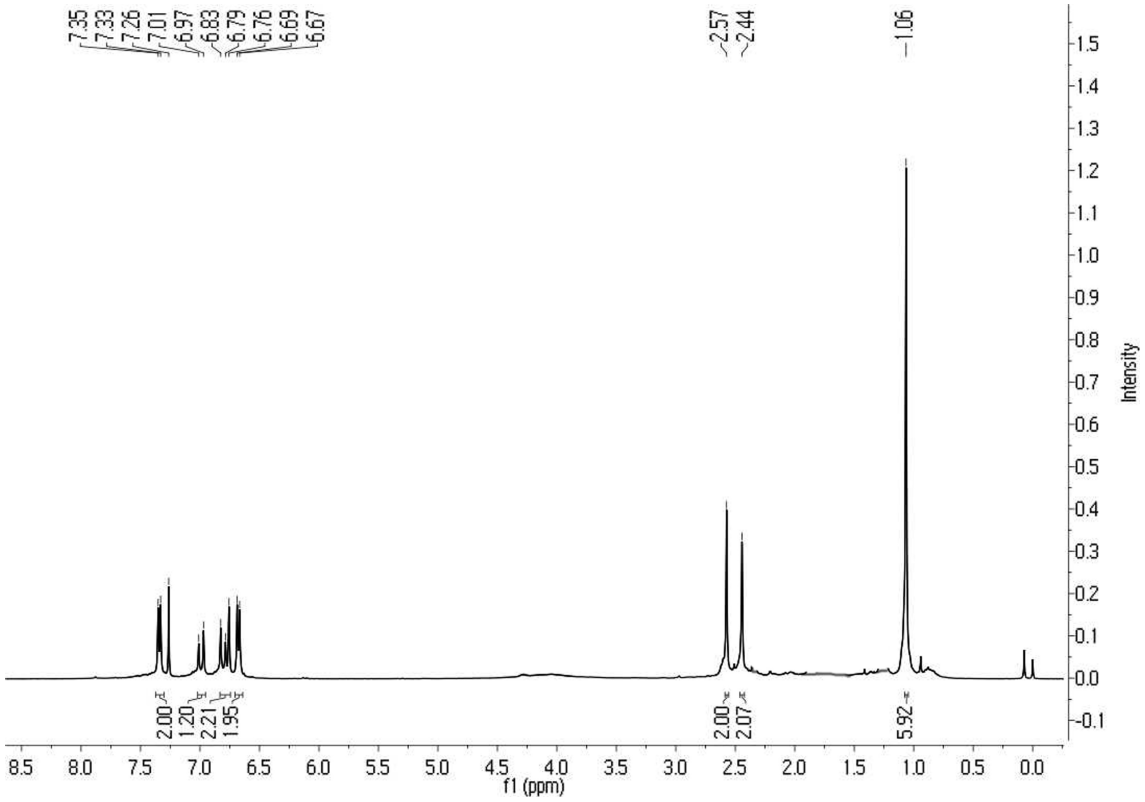


Figure S29. ^1H NMR spectra of the isolated fluorescent product of Fsec-1 + Sec in $CDCl_3$.