Supporting information

Highly selective red- and green-emitting two-photon fluorescent probes for cysteine detection and their bio-imaging in living cells

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Materials

All chemicals used are of analytical grade, and triphenylamine, carbazole and NBS were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Palladium(II) acetate and tri-*o*-tolylphosphine were purchased from J&K Chemical (Beijing, China). GSH, DTT, amino acids, Tris were purchased from Seikagaku Corporation (Japan). The solvents used in the spectral measurement are of chromatographic grade. All spectroscopic measurements of **AM1** and **CA1** were performed in acetonitrile/Tris-HCl buffer solution (tris:10 mM, KCl:100 mM) with pH = 7.27 and v/v of 4:1 and methanol/Tris-HCl buffer solution (tris:10 mM, KCl:100 mM) with pH = 7.00 and v/v of 4:1, respectively. TLC analyses were performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from Qingdao Ocean Chemicals.

Methods

Nuclear magnetic resonance spectra (^{1}H and ^{13}C) were obtained on a Bruker Avanace 300/400 spectrometer. The HRMS spectra were recorded on Agilent Technologies 6510 Q-TOF LC/MS or ThermoFisher LCQ FLEET. The elemental analyses were performed on a Vario EI III instrument.

Spectroscopic Measurements

The UV-visible-near-IR absorption spectra of dilute solutions were recorded on a Cary50 spectrophotometer using a quartz cuvette having 1 cm path length. IR spectra were measured on Nexus 670. One-photon fluorescence spectra were obtained on a HITACH F-4500 spectrofluorimeter equipped with a 450-W Xe lamp. Two-photon ones were measured on a SpectroPro300i and the pump laser beam came from a mode-locked Ti:sapphire laser system at the pulse duration of 200 fs, a repetition rate of 76 MHz (Coherent Mira900-D).

The luminescence quantum yields can be calculated by means of equation (1):

$$\Phi_s = \Phi_r \left(\frac{A_r(\lambda_r)}{A_s(\lambda_s)} \right) \left(\frac{n_s^2}{n_r^2} \right) \frac{F_s}{F_r} \qquad (1)$$

where the subscripts *s* and *r* refer to the sample and the reference materials, respectively. Φ is the quantum yield, *F* is the integrated emission intensity, *A* stands for the absorbance, and *n* is the refractive index. In this paper, the quantum yields were calculated by using coumarin 307 in methanol (Φ_r = 56%) [1] as a standard.

Two-photon absorption (TPA) cross sections have been measured using the two-photon induced fluorescence method, and thus the cross section can be calculated by means of equation (2): [2-4]

$$\delta_s = \delta_r \frac{\Phi_r}{\Phi_s} \frac{c_r}{c_s} \frac{n_r}{n_s} \frac{F_s}{F_r} \qquad (2)$$

where the subscripts *s* and *r* refer to the sample and the reference materials, respectively. The terms *c* and *n* are the concentration and refractive index of the solution, respectively. *F* is two-photon excited fluorescence integral intensity. Φ is the fluorescence quantum yield. TPA quantum yield measurement

is quite difficult as compared to the well-established quantum yield measurement of SPF. So, one might suppose that the two quantum yields are coincidental. δ_r is the TPA cross-section of coumarin 307 in methanol (δ = 27.7 GM) at 800 nm.[5]

Cell Culture and Staining

HeLa cells were grown in H-DMEM (Dulbecco's Modified Eagle's Medium, High Glucose) supplemented with 10% FBS (Fetal Bovine Serum) in a 5% CO_2 incubator at 37 °C. Cells (1×10^5 / mL) were placed on glass coverslips and allowed to adhere for 24 h. For living cells imaging experiment of **AM1** or **CA1**, cells were incubated with 5 μM **AM1** or **CA1** in DMSO-PBS (1:100, v/v, pH 7.4) for 30 min at 37 °C. After rinsing with PBS twice, cells were imaged immediately. For *N*-ethylmaleimide (NEM) treated experiment, HeLa cells were pretreated with a DMSO-PBS (1:49, v/v, pH 7) solution of 5 mM NEM for 30 min in a 5% CO_2 incubator at 37 °C, and then incubated with 5 μM **AM1** or **CA1** in DMSO-PBS (1:100, v/v, pH 7.4) for 30 min at 37 °C. Fluorescence imaging was then carried out after washing cells with the PBS buffer.

Fluorescence Imaging

Confocal fluorescence and two-photon fluorescence imaging were obtained with Olympus FV 300 Laser Confocal Microscope with a 60× water-immersion objective lens (N.A. = 1.25). Emission was collected with a beam splitter DM570 and IF565 nm band pass filter or BA510–540 nm band pass filter combination. In confocal experiment, excitation wavelength was 488 nm from an Ar^+ laser. In two-photon one, excitation wavelengths were 800 nm or 910 nm from a Ti:sapphire femtosecond laser source (Coherent Chamelon Ultra), and the incident power on samples was modified by means of an attenuator and examined with Power Monitor (Coherent). The differential interference contrast (DIC) image was taken with 488 nm Ar^+ ion laser.

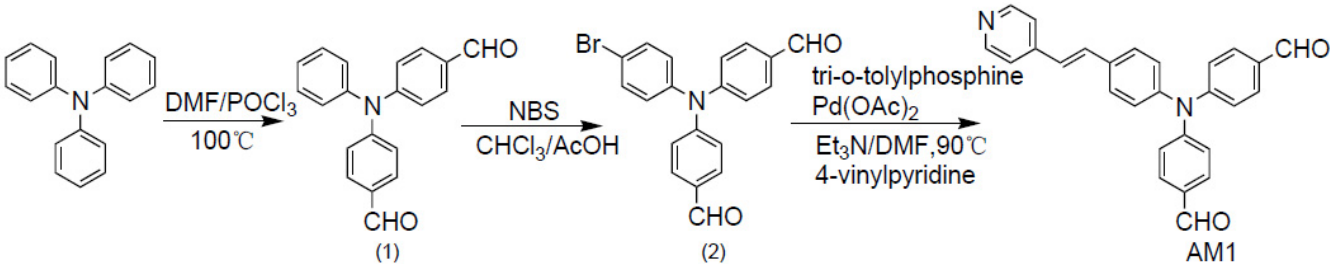
The detection limit of AM1 and CA1 for Cys

To determine the detection limit of **AM1** and **CA1** for Cys, the titrations on absorption, SPEF and TPEF have been carried out (Figure S28). And experimental results show that when the molar ratio between Cys and **AM1**/**CA1** is less than 30, the photophysical properties of the mixture do not change.

When the ratio is 30–60, absorbance, SPEF and TPEF intensity of **AM1**/**CA1** increase fleetly. When the ratio is larger than 60, these optical properties remain stable.

We find, when the molar ratio between Cys and **AM1**/**CA1** is 30-40, the absorbance, SPEF and TPEF intensity of **AM1**/**CA1** are linearly proportional to the concentrations of Cys (from 300 μM to 400 μM). According to their linear fitted results, the detection limit, namely the concentration of Cys in which the S/N equal to 3 [6, 7] can be obtained.

Synthesis of AM1



Scheme S1. The synthetic route of AM1

Synthesis of 4,4'-diformyltriphenylamine (1):

To a mixture of triphenylamine (5.0 g, 20.4 mmol) and dry dimethylformamide (35 mL) at 0 ºC was dropwisely added phosphoryl chloride (19 mL, 203.8 mmol) with stirring. The reaction mixture was stirred at room temperature for 1 h and then mixture was warmed at 100 °C under nitrogen for 8 h. After being cooled to room temperature, the reaction mixture was poured into ice-water, neutralized with NaOH solution and then extracted with CH_2Cl_2. The combined organic phase was washed with water and saturated brine, dried over anhydrous magnesium sulfate overnight. After CH_2C1_2 was removed, the crude product was purified by column chromatography with ethyl acetate/petroleum ether (10:1) as eluent, finally the light-yellow solid was obtained for **1** with a yield of 55%. ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 9.88 (s, 2H), 7.84 (d, J = 8.7, 4H), 7.47 (t, J = 7.8, 2H), 7.31 (t, J = 7.5, 1H), 7.21 (d, J = 8.1, 2H), 7.17 (d, J = 8.4, 4H).

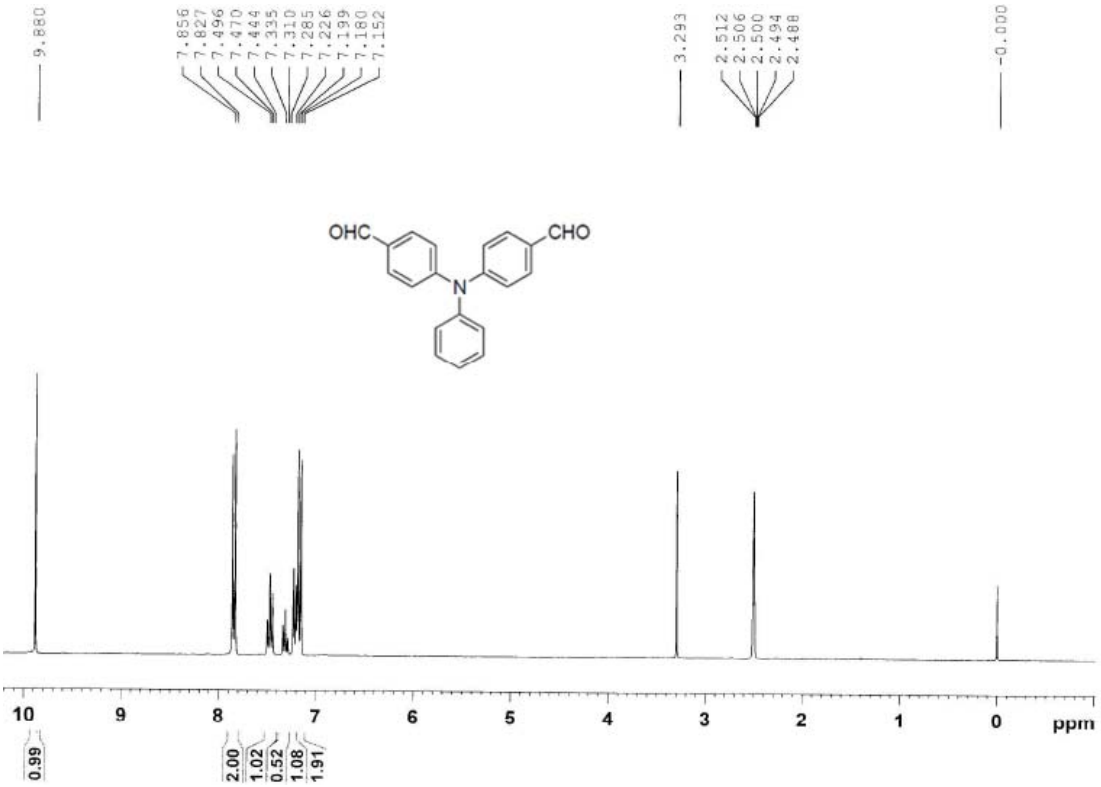


Figure S1. ^{1}H NMR spectrum of compound **1** in DMSO-d_6

Synthesis of (4-bromophenyl)-bis-(4-formylphenyl)amine (2):

To a solution of compound **1** (0.50 g, 1.66 mmol) in chloroform/AcOH (30 ml, v/v, 1:1) was added NBS (0.33 g, 1.85 mmol) at room temperature. The mixture was stirred under argon for 20 h at room temperature and then quenched with water, and the aqueous layer was extracted with CH_2Cl_2. The organic phase was washed with water and saturated brine, dried over anhydrous magnesium sulfate overnight. After CH_2C1_2 was removed, the crude product was purified by column chromatography with ethyl acetate/petroleum ether (10:1) as eluent, finally the yellow solid was obtained for **2** with a yield of 78%. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 9.91 (s, 2H), 7.79 (d, J = 8.56, 4H), 7.50 (d, J = 8.64, 2H), 7.18 (d, J = 8.56, 4H), 7.06 (d, J = 8.64, 2H).

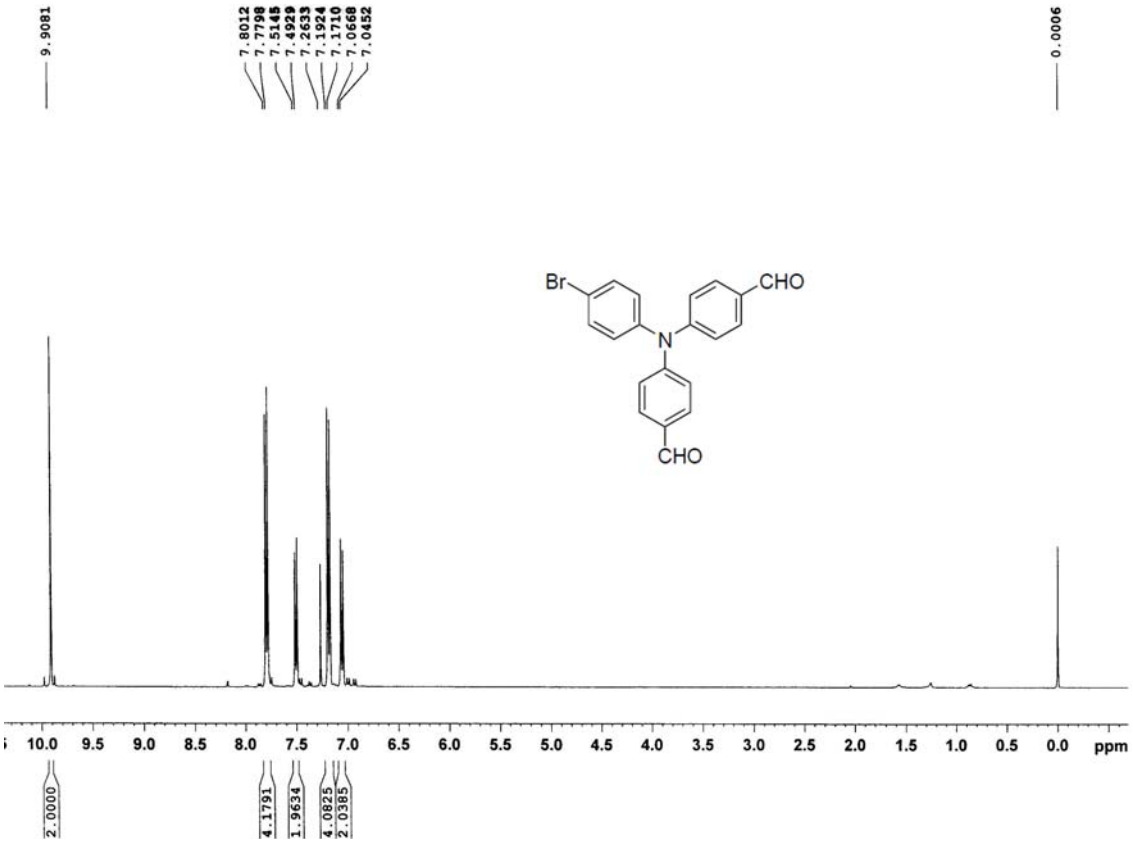


Figure S2. ^{1}H NMR spectrum of compound **2** in $CDCl_3$

Synthesis of (*E*)-4-(4-vinylpyridine)phenyl-*bis*-(4-formylphenyl)amine (AM1):

Compound **2** (0.38 g, 1 mmol), palladium(II) acetate (22.45 mg, 0.1 mmol) and tri-(*o*-tolyl)phosphine (60.87 mg, 0.2 mmol) were introduced into a dry and degassed TEA/DMF (21 mL, 2:1, v/v) mixture, which was stirred under argon for 30 min. 4-Vinylpyridine (0.27 mL, 2.5 mmol) was added and then the reaction mixture was heated up to 130 ºC under argon atmosphere and kept at this temperature in an oil bath for 48 h. The resulting mixture was allowed to cool to room temperature, and TEA was removed *in vacuo*. The crude mixture was diluted with dichloromethane, washed with water and saturated $NaHCO_3$, and dried over anhydrous magnesium sulfate overnight. After concentration, the residue was purified by column chromatography with ethyl acetate/petroleum ether (1:1) as eluent to give the yellow product (**AM1**) with a yield of 40%. IR (cm^{-1}): 1686 ($\upsilon_{C=O}$). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.90 (s, 2H), 8.55 (d, J = 5.92, 2H), 7.87 (d, J = 8.64, 4H), 7.73 (d, J = 8.48, 2H), 7.60 (s, 1H), 7.56 (d, J = 6.12, 2H), 7.27 (s, 1H), 7.22 (d, J = 8.52, 6H). ^{1}H NMR (300 MHz, CD_3OD): δ (ppm) 9.88 (s, 2H), 8.49 (dd,

J = 4.80, 2H), 7.84–7.89 (m, 4H), 7.70 (d, *J* = 8.40, 2H), 7.50–7.60 (m, 3H), 7.17–7.27 (m, 7H). ^{13}C-NMR (300 MHz, DMSO-d_6), δ (ppm) = 192.2, 152.1, 151, 146.2, 145.2, 134.7, 133.0, 132.3, 129.8, 127.6, 127.1, 123.9, 121.8. HRMS (m/z): calcd for $C_{27}H_{20}N_2O_2$: 404.15; found: m/z 405.16 $(M + H)^+$. Anal. calcd for $C_{27}H_{20}N_2O_2$: C, 80.18; H, 4.98; N, 6.93; found: C , 80.45; H, 5.21; N , 6.77.

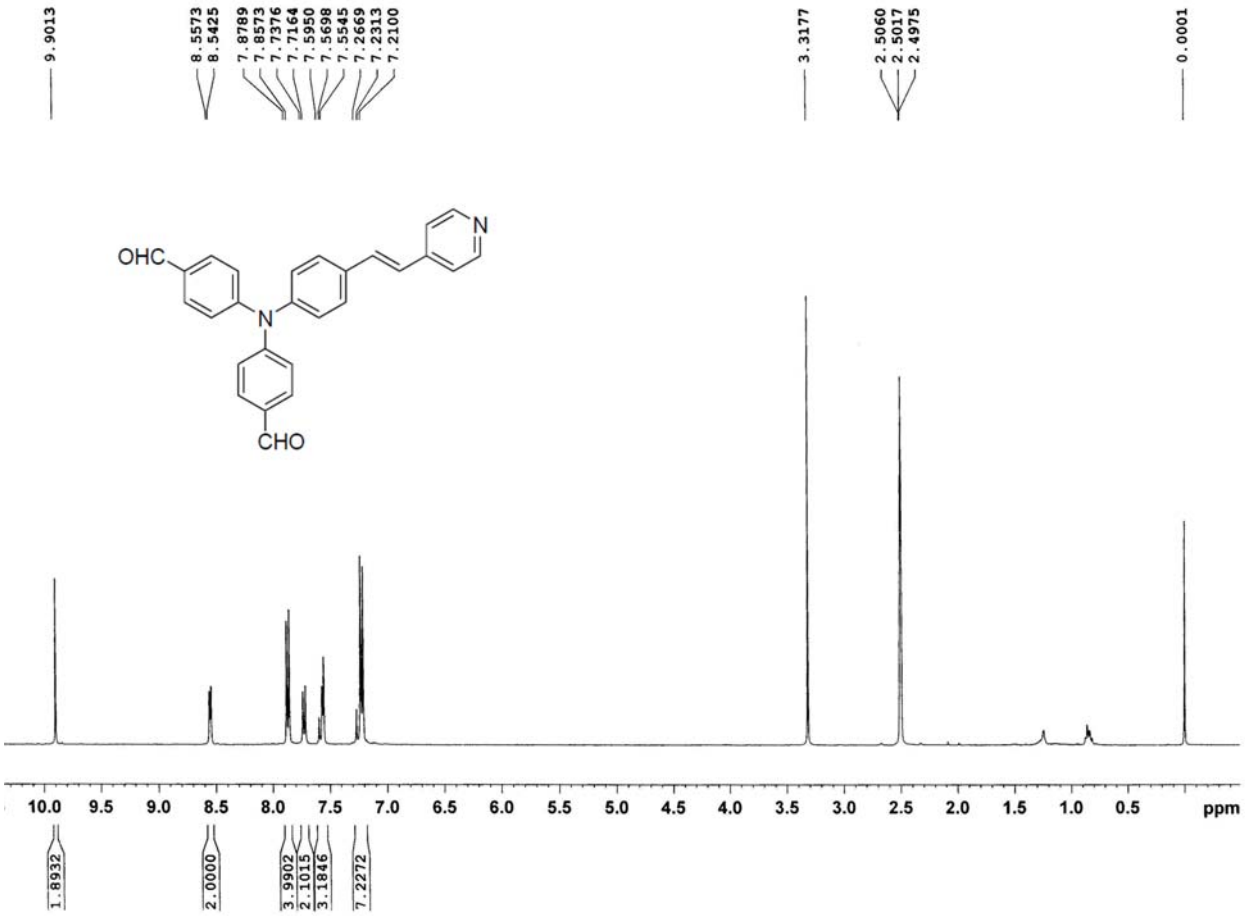


Figure S3. ^{1}H NMR spectrum of **AM1** in DMSO-d_6

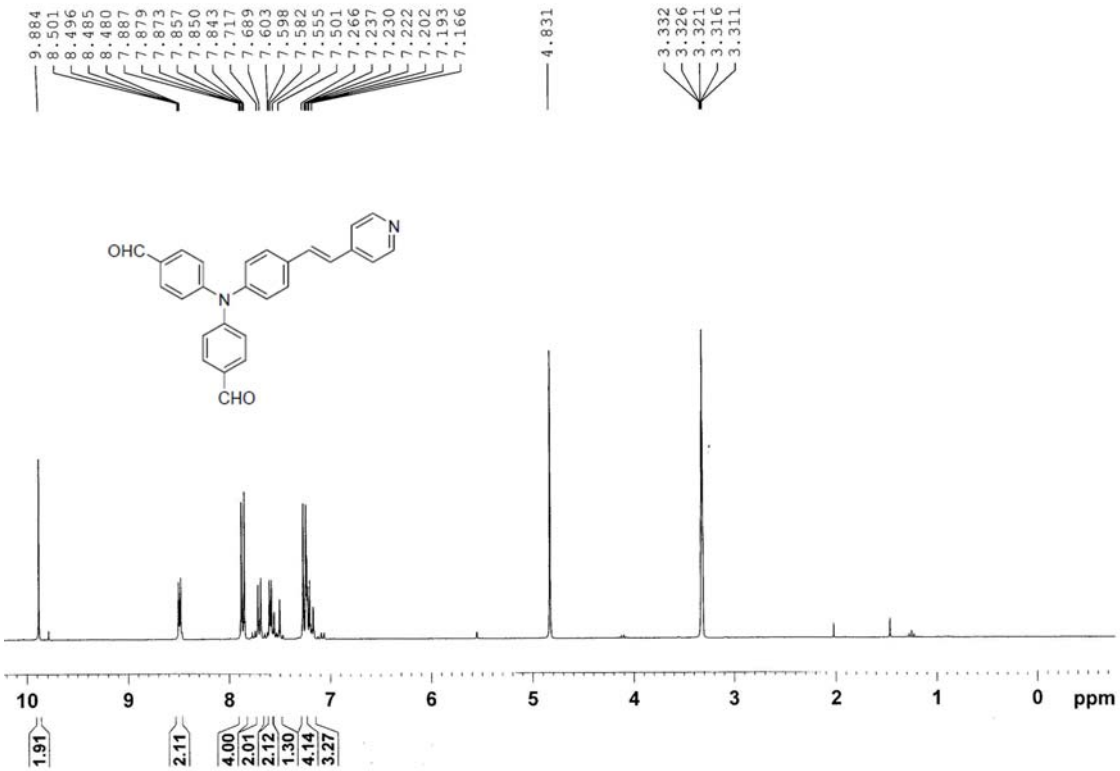


Figure S4. ^{1}H NMR spectrum of **AM1** in CD_3OD

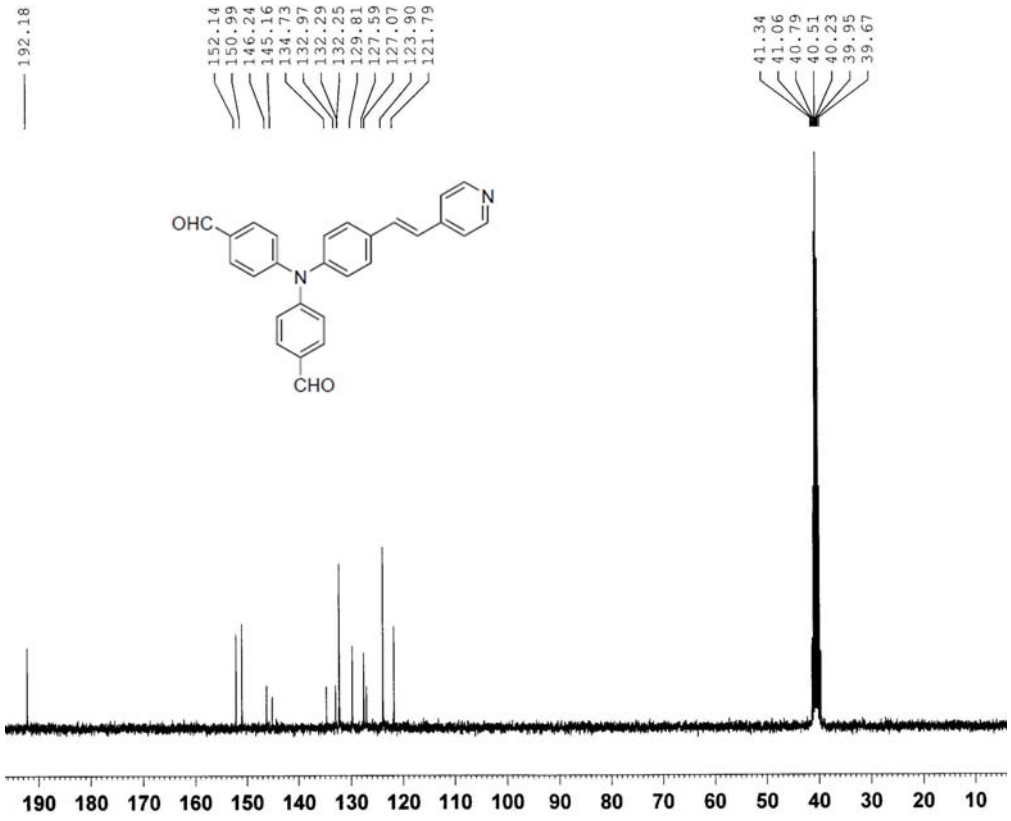


Figure S5. ^{13}C NMR spectrum of **AM1** in DMSO-d_6

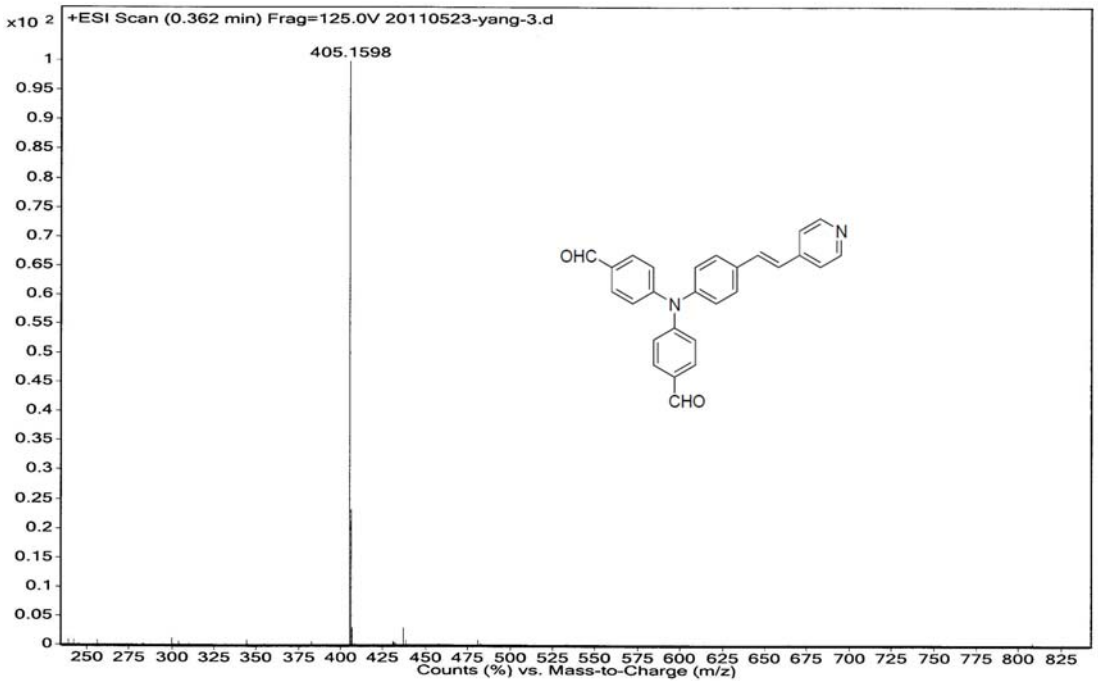


Figure S6. HRMS of **AM1**

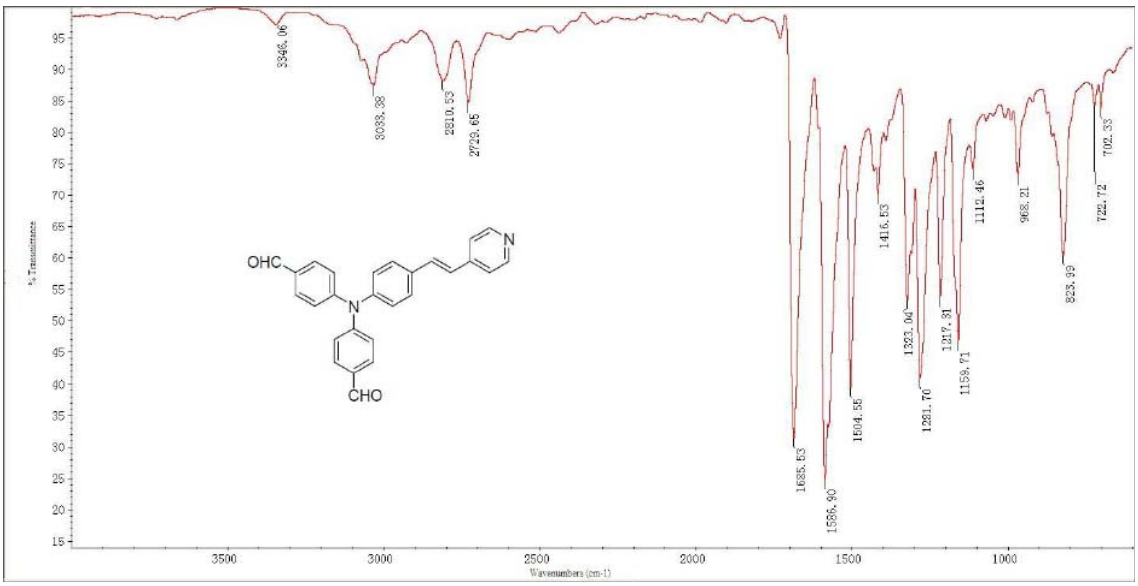
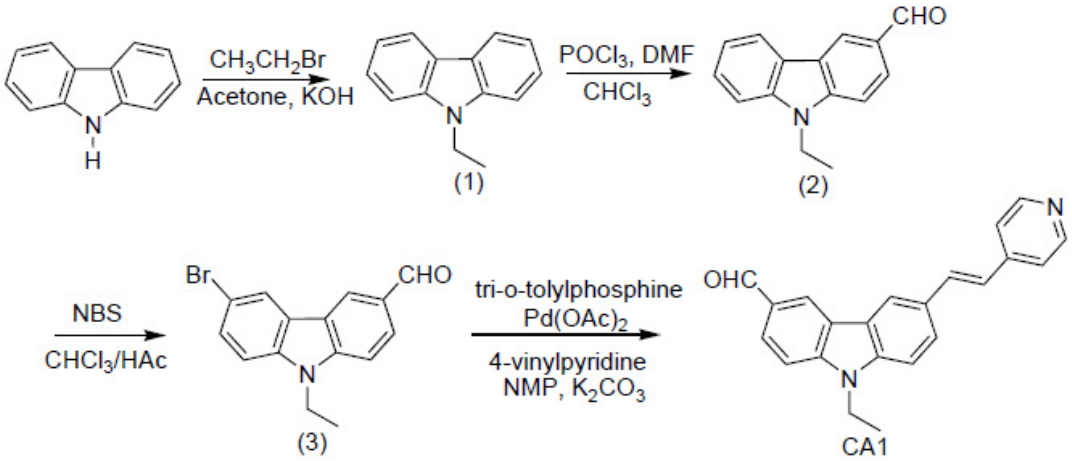


Figure S7. IR spectrum of **AM1**

Synthesis of CA1



Scheme S2. Synthesis of CA1

Synthesis of 9-ethyl-carbazole (1):

KOH (28 g, 500 mmol) was dissolved in acetone (60 mL) and the resulting solution was stirred at room temperature for 30 min. Carbazole (13.2 g, 80 mmol) was then added and reacted for another 4h. The acetone containing bromoethane (9 mL, 120 mmol) was dropped into the above solution and the resulting system was allowed to react overnight. When the reaction mixture was poured into water (1 L), the yellow solid was filtrated. After recrystallization from ethanol, a white solid was obtained with a yield of 83%. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 8.10 (d, J = 7.8 Hz, 2H), 7.39–7.49 (m, 4H), 7.20–7.25 (m, 2H), 4.36 (q, J = 7.2 Hz , 2H), 1.43 (t, J = 7.2 Hz, 3H).

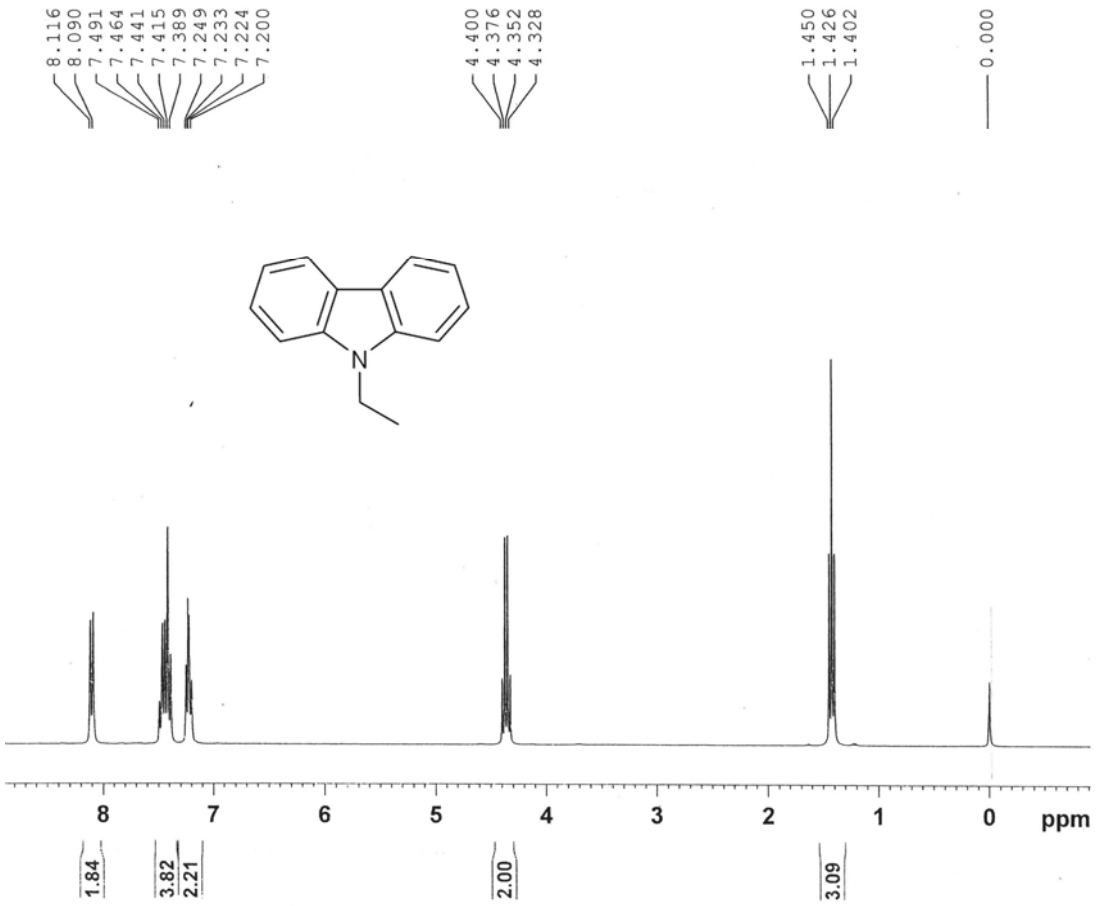


Figure S8. ^{1}H NMR spectrum of compound **1** in $CDCl_3$

Synthesis of 3-formyl-9-ethyl-carbazole (2):

DMF (1.86 mL) was added into a drying round-bottom flask and the system was cooled to 0 °C. A solution of $CHC1_3$ (15 mL) containing compound **1** (4.87 g, 25 mmol) was then added following the addition of redistilled $POC1_3$ (2.3 mL). Then, the solution mixture was refluxed for 16 h. After most of $CHC1_3$ was removed, the residue was poured into ice water and pH was then adjusted to 7–8 by $NaHCO_3$. The water layer was extracted by CH_2C1_2 and the organic layer was washed by water for several times before dried with $MgSO_4$. After CH_2C1_2 was removed, the crude product was purified by column chromatography with ethyl acetate/petroleum ether (10:1, v/v) as eluent, and finally the primrose solid was obtained for **2** (yield: 87%). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 10.10 (s, 1H),

8.61 (s, 1H), 8.16 (d, J = 7.8 Hz, 1H), 8.01 (dd, J_1 = 8.4 Hz, J_2=1.5 Hz, 1H), 7.47–7.57 (m, 3H), 7.26–7.35 (m, 1H), 4.40 (q, J = 7.2 Hz, 2H), 1.47 (t, J = 7.4 Hz, 3H).

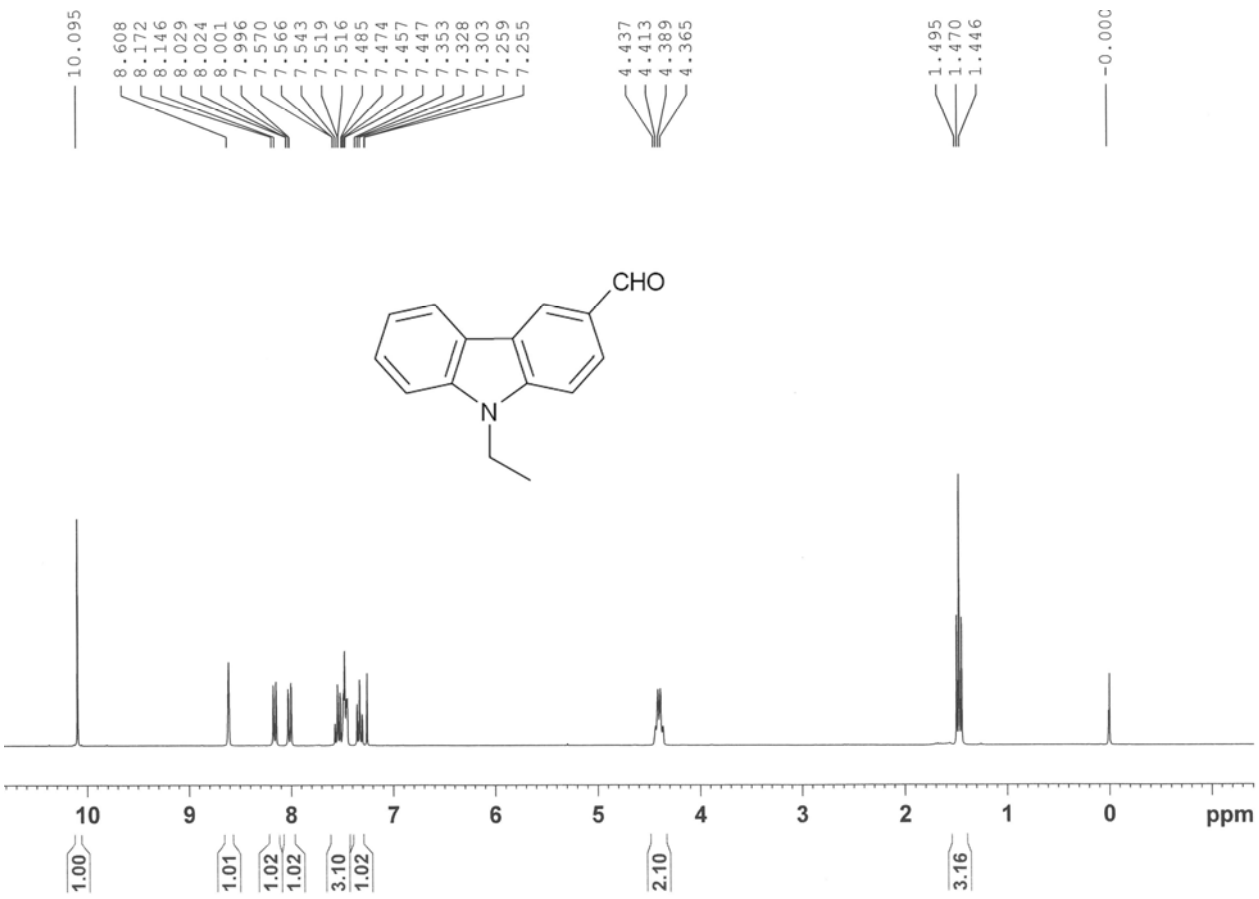


Figure S9. ^{1}H NMR spectrum of compound **2** in $CDCl_3$

Synthesis of 3-formyl-6-bromo-9-ethyl-carbazole (3):

Under the protection of argon, compound **2** (2.2 g, 9.85 mmol) and NBS (1.9 g, 10.67 mmol) were dissolved in the 60 mL of chloroform/AcOH (1:1, v/v) and the resulting solution was allowed to react for 15 h at room temperature. The mixture was poured into water (500 mL) and then extracted by CH_2C1_2. The organic layer was washed by saturated NaCl solution for several times before being dried with $MgSO_4$. After CH_2C1_2 was evaporated, the crude product was purified by column chromatography with ethyl acetate/petroleum ether (10:1, v/v) as eluent, and finally a white solid was obtained for **3** with a yield of 81%. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 10.10 (s, 1H), 8.56 (d, J = 1.2 Hz, 1H), 8.27 (d, J

= 1.8 Hz, 1H), 8.05 (dd, J_1 = 8.6 Hz, J_2 = 1.5 Hz , 1H), 7.62 (dd, J_1 = 8.7 Hz, J_2 = 1.8 Hz, 1H), 7.49 (d, J = 8.4 Hz, 1H), 7.35 (d, J = 8.7 Hz, 1H), 4.4 (q, J = 7.3 Hz, 2H), 1.47 (t, J = 7.4 Hz, 3H).

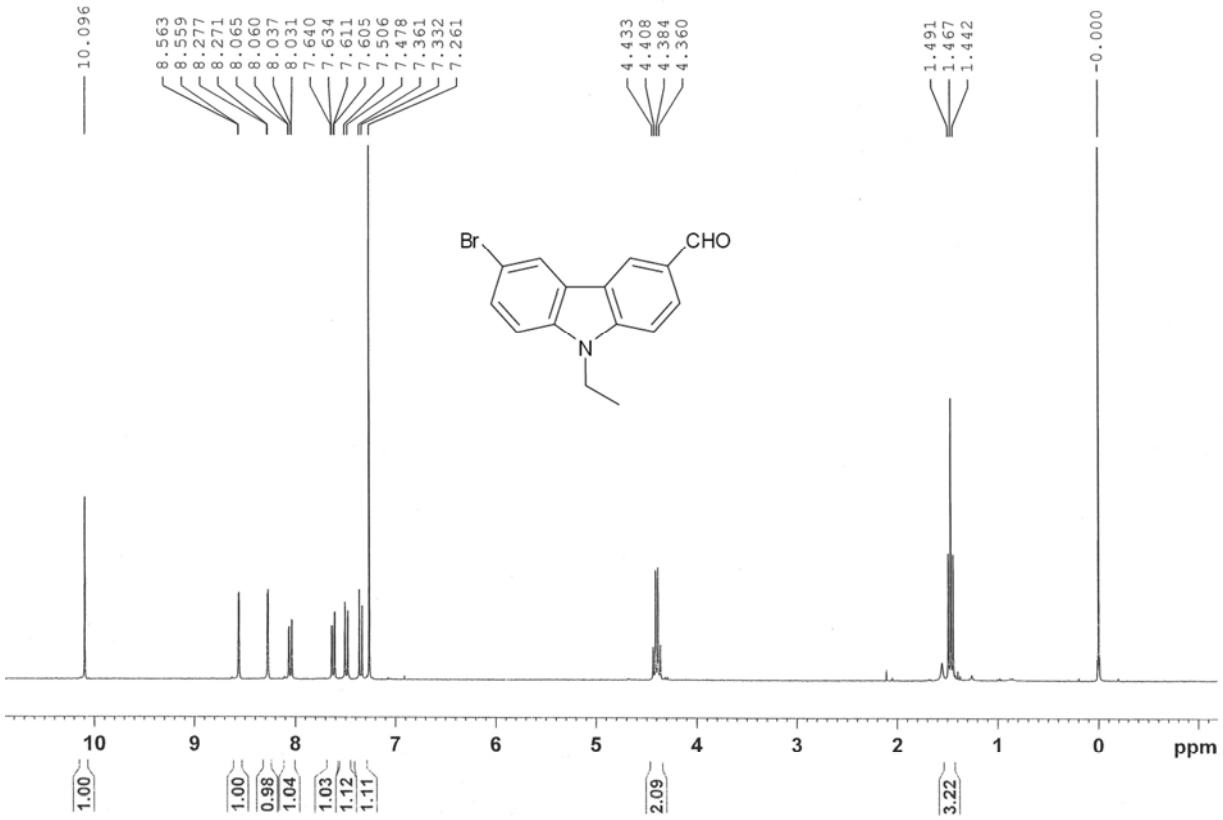


Figure S10. ^{1}H NMR spectrum of compound **3** in $CDCl_3$

Synthesis of 3-aldehyde-6-[2′-*para*-pyridylethenyl-9-ethyl-carbazole] (CA1):

Compound **3** (0.61 g, 2.0 mmol) was added into a flask containing a mixture of palladium(II) acetate (44.9 mg, 0.2 mmol), tri-(*o*-tolyl)phosphine (182.62 mg, 0.6 mmol), K_2CO_3 (2.2 g, 16.0 mmol) and 35 mL of *N*-methyl-2-pyrrolidone (NMP). The 4-vinylpyridine (0.43 mL, 4.0 mmol) was then added after the above mixture was bubbled with argon for 30 min. The system was heated at 130 °C for 36 h under the protection of argon and a dark-red suspension was obtained. The mixture was poured into H_2O (500 mL) and extracted with CH_2Cl_2 after the resulting mixture was cooled to room temperature. The organic phase was separated, dried with $MgSO_4$ and removed by vacuum distillation. The title product was obtained as a yellow solid after the residue was purified by column chromatography with ethyl acetate/petroleum ether (2:1, v/v) as eluent with a yield of 52%. IR (cm^{-1}): 1672 ($\upsilon_{C=O}$), 984 ($\upsilon_{trans\ =C-H}$).

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 10.09 (s, 1H), 8.80 (s, 1H), 8.65 (s, 1H), 8.56 (d, J = 5.6 Hz, 2H), 8.03 (d, J = 8.5 Hz,1H), 7.71–7.88 (m, 4H), 7.58 (d, J = 5.6 Hz, 2H), 7.34 (d, J = 16.4 Hz, 1H), 4.54 (q, J = 7.1 Hz, 2H), 1.37 (t, J = 7.1 Hz, 3H). ^{1}H NMR (400 MHz, CD_3OD): δ (ppm) 10.07 (s, 1H), 8.74 (d, J = 1.1 Hz, 1H), 8.47-8.50 (m, 3H), 8.06 (dd, J_1 = 8.5 Hz, J_2 = 1.5 Hz, 1H), 8.06 (dd, J_1 = 8.6 Hz, J_2 = 1.5 Hz, 1H), 7.68–7.73 (m, 2H), 7.62–7.64 (m, 3H), 7.25 (d, J = 16.3 Hz, 1H), 4.52 (q, J = 7.2 Hz, 2H), 1.47 (t, J = 7.2 Hz, 3H). ^{13}C-NMR (300 MHz, DMSO-d_6, δ, ppm): δ = 192.8, 151.0, 145.7, 144.6, 141.6, 134.6, 129.7, 129.6, 128.1, 127.3, 124.9, 124.8, 123.8, 123.4, 121.6, 120.6, 111.2, 110.9, 38.6, 14.7. HRMS (m/z): M^+calcd for $C_{22}H_{18}N_2O$: 327.15; found, 327.33. Anal. calcd for $C_{22}H_{18}N_2O$: C 80.96, H 5.56, N 8.58; found: C 80.85, H 5.32, N 8.54.

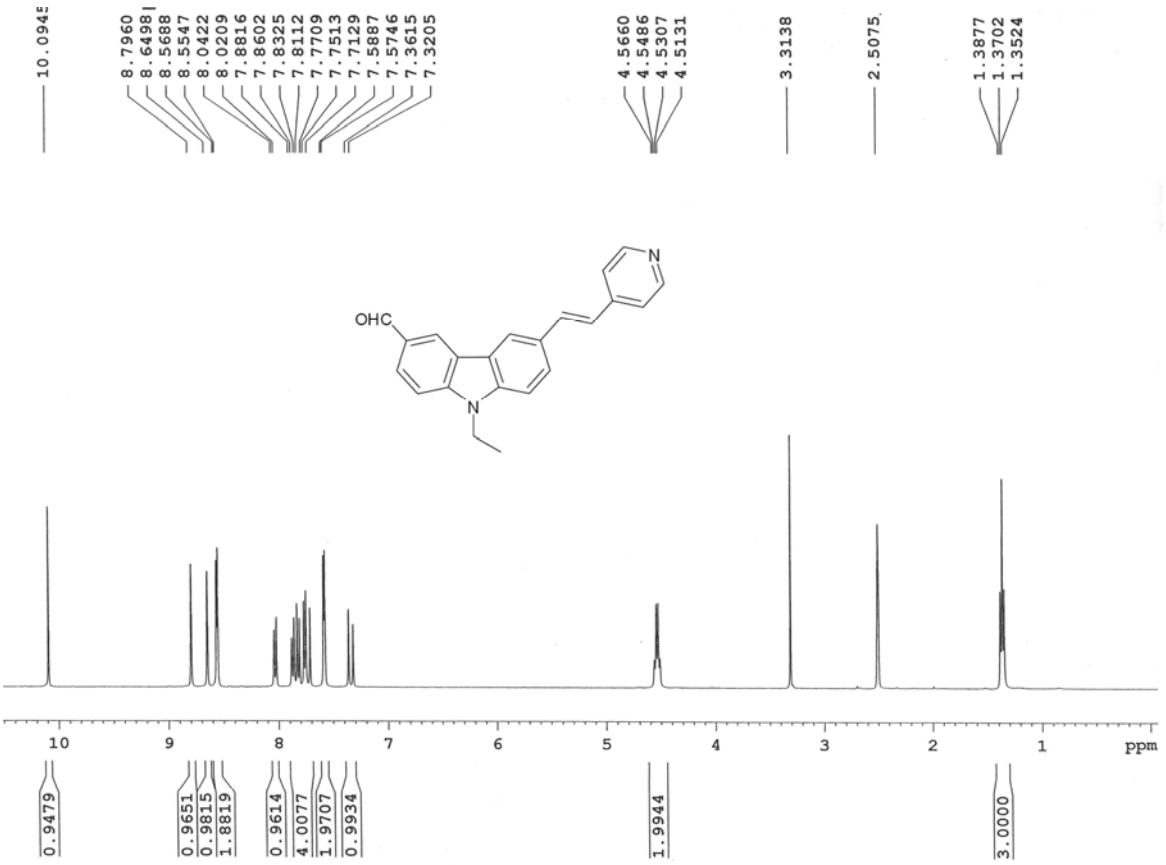


Figure S11. ^{1}H NMR spectrum of **CA1** in DMSO-d_6

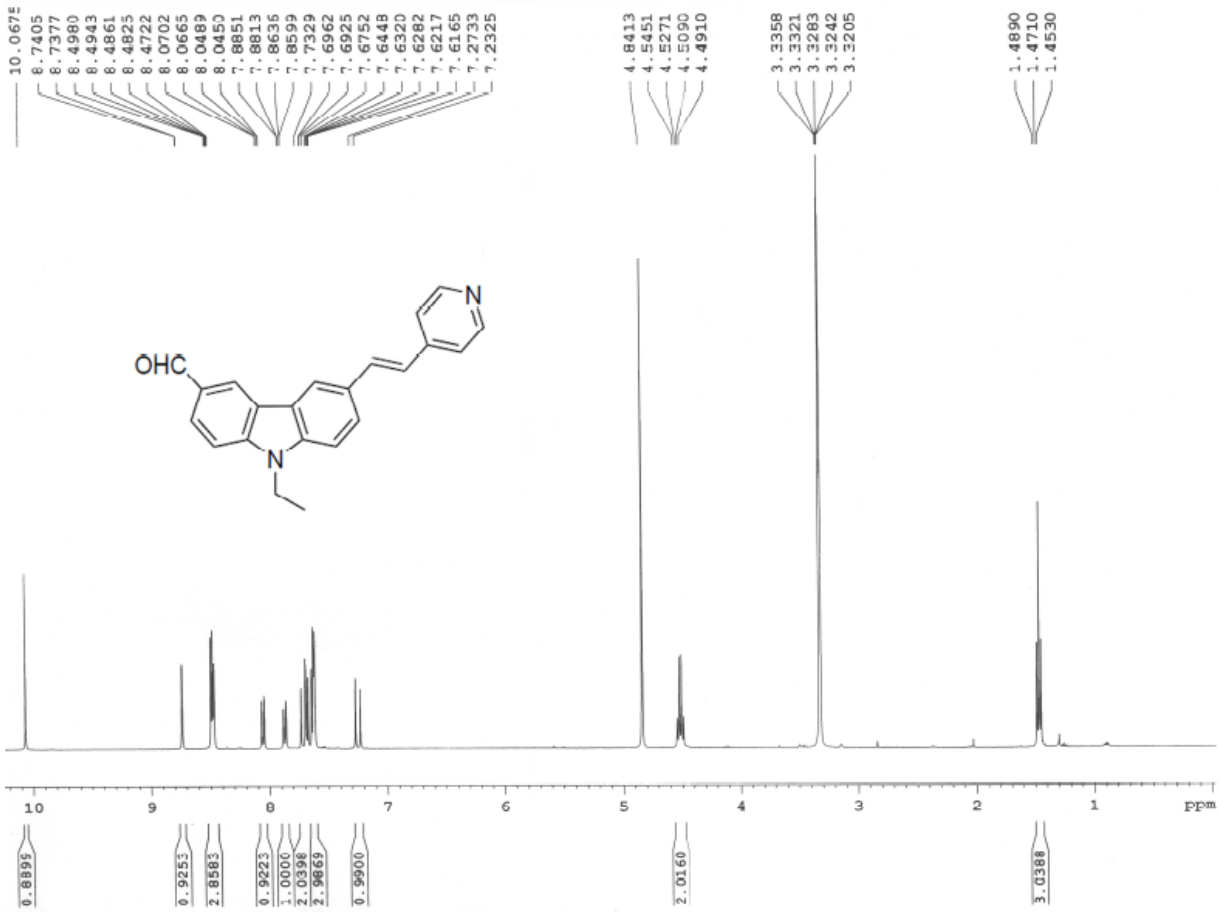


Figure S12. ^{1}H NMR spectrum of **CA1** in CD_3OD

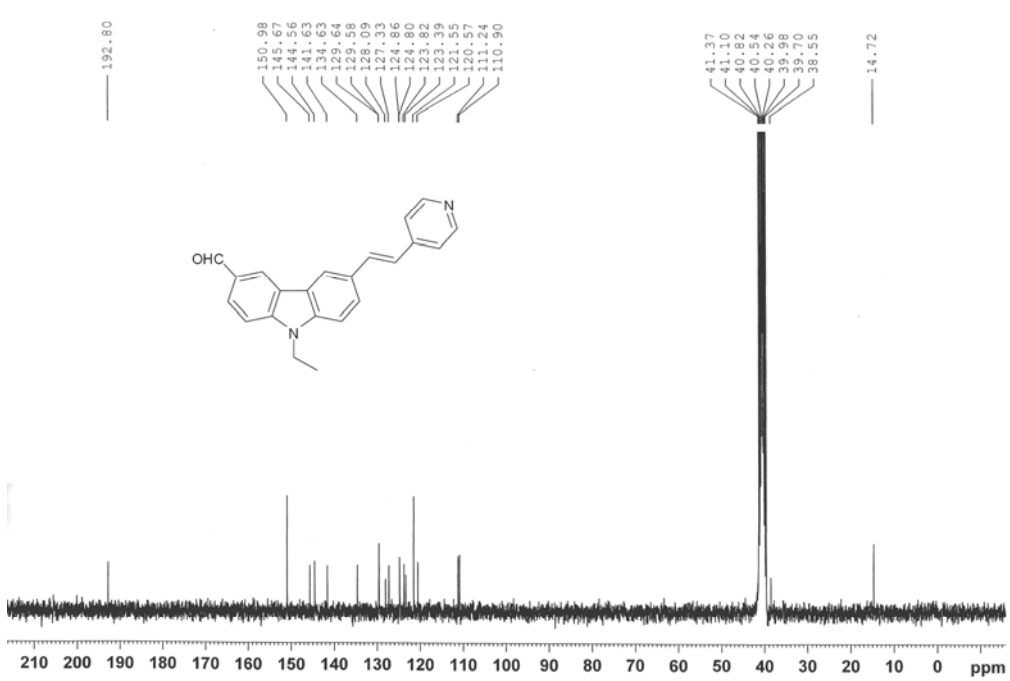


Figure S13. ^{13}C NMR spectrum of **CA1** in DMSO-d_6

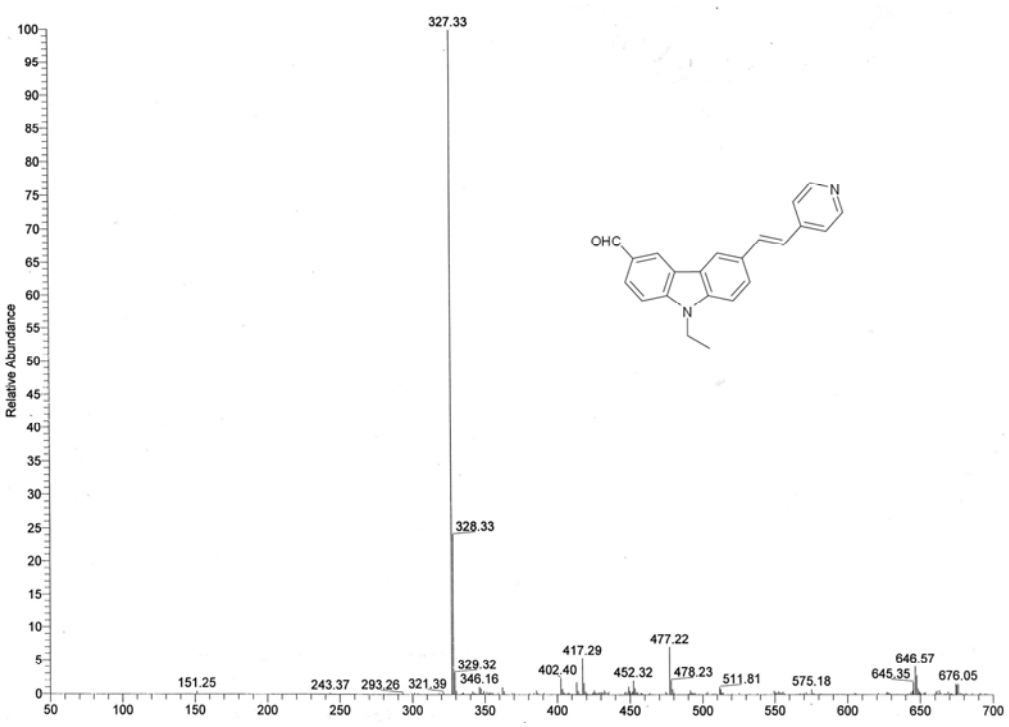


Figure S14. HRMS of **CA1**

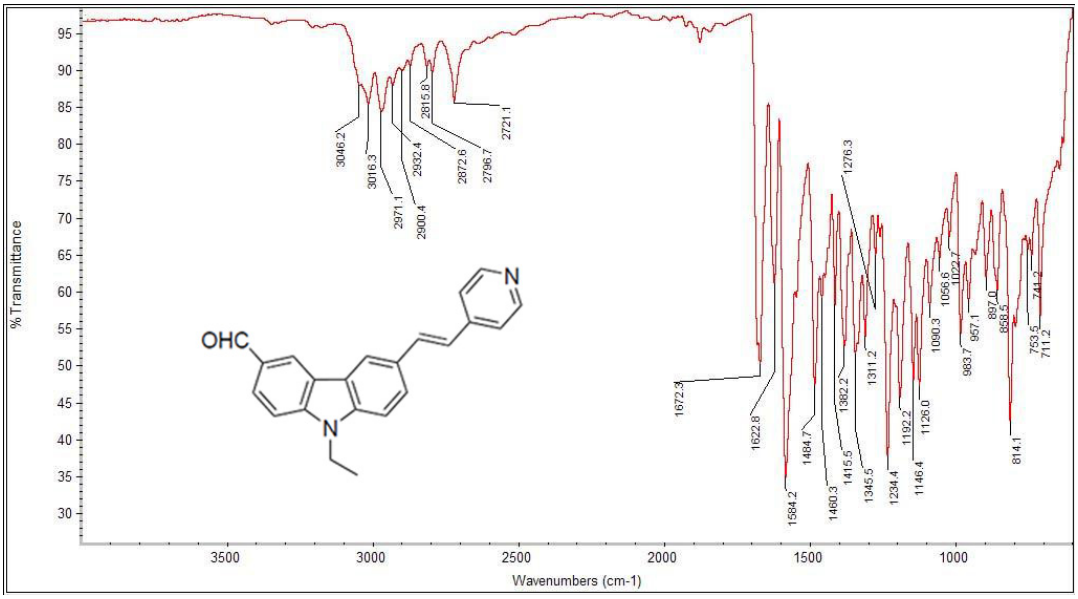


Figure S15. IR spectrum of **CA1**

Synthesis of AM1+Cys and CA1+Cys

AM1+Cys: A mixture of **AM1** (110.1 mg, 0.25 mmol) and L-cysteine (Cys, 121.2 mg, 1 mmol) in ethanol (60 mL) was stirred at room temperature for three days. The ethanol was concentrated in *vacuo* , and then the solid was collected, washed with ethanol and dried to afford the thiazolidine.

CA1+Cys: A mixture of **CA1** (81.5 mg, 0.25mmol) and L-cysteine (Cys, 61 mg, 0.5 mmol) in ethanol (70 mL) was stirred at room temperature for three days, and the flocculent solid was collected, filtered, washed with ethanol and dried to afford the thiazolidine.

In the ESI mass spectrum, the isolated **AM1**+Cys thiazolidine displayed strong peaks. The molecular ion peaks at m/z = 611.1771 and 306.0923 corresponding to $(M + H)^+$ and $(M + 2H)^{2+}/2$ (calculated value 610.1708) were clearly observed. In addition, the peak of **AM1** was not present, indicating **AM1** has almost completed the reaction with Cys. Meanwhile, the strong peaks at m/z = 430.1576 $(M + H)^+$ and 215.5832 $(M + 2H)^{2+}/2$ (calculated value 429.1511) are typical of **CA1**+Cys thiazolidine. The fragment peak at m/z = 327.1491 $(M + H)^+$ (calculated value 326.1419) corresponding to **CA1** indicates that **CA1** has no complete reaction with Cys. Mass spectral analysis has shown a good evidence for the thiazolidine formation. The result also confirms that thiazolidine is formed by the interaction of aldehyde with Cys [8].

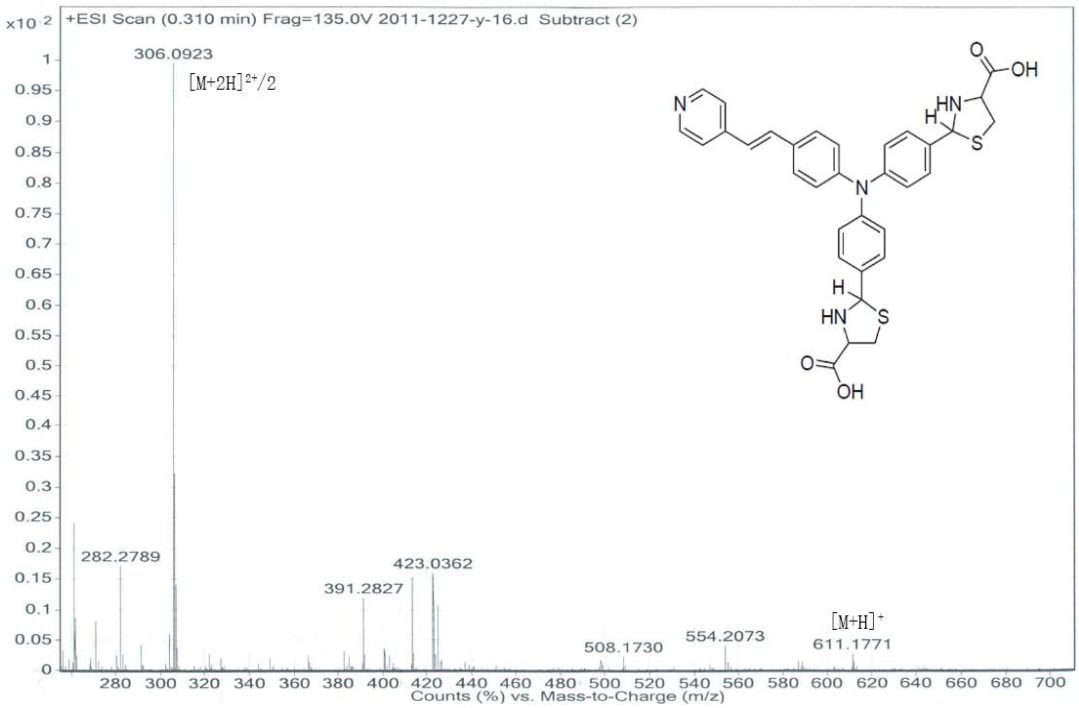


Figure S16. The mass spectrum of the isolated **AM1** + Cys thiazolidine.

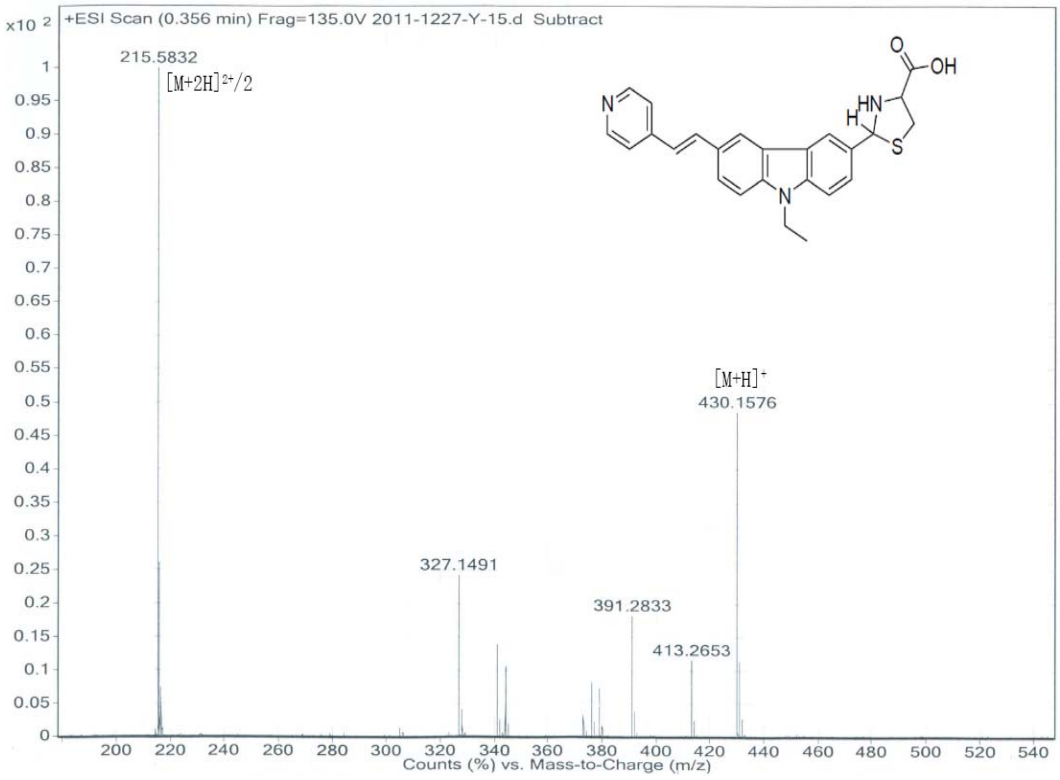


Figure S17. The mass spectrum of the isolated **CA1** + Cys thiazolidine.

^{1}H NMR of AM1 and CA1 after sensing of Cys

The formation of thiazolidine between **AM1** or **CA1** and Cys can be explained by the ^{1}H NMR method. In deuterated methanol, after the addition of Cys, the proton peaks of aldehyde of **AM1** at 9.88 ppm disappear, on the other hand, two new peaks emerge at 5.88 and 5.79 ppm in the **AM1**+**Cys** mixture, which can be assigned to the methane protons. Similarly, for **CA1** and **CA1**+**Cys**, the proton peaks of aldehyde at 10.06 ppm disappear, and those at 6.08 and 5.98 ppm become apparent. These results are consistent with the previous studies [9].

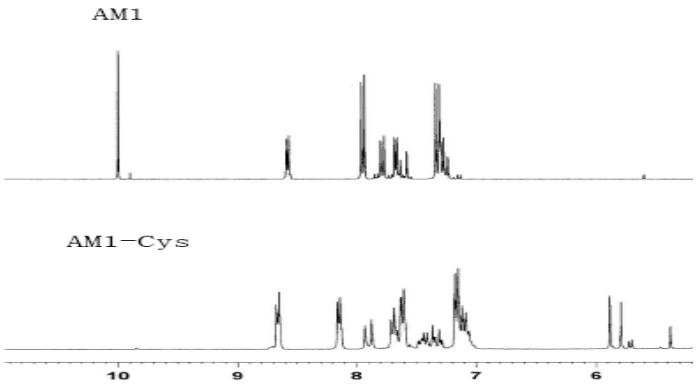


Figure S18. ^{1}H NMR spectrum of **AM1** in the absence (top) and presence (bottom) of Cys in deuterated methanol.

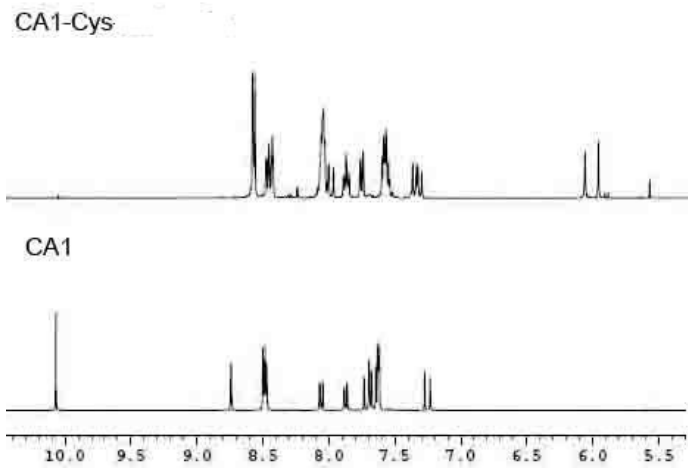


Figure S19. ^{1}H NMR spectrum of **CA1** in the presence (top) and absence (bottom) of Cys in deuterated methanol.

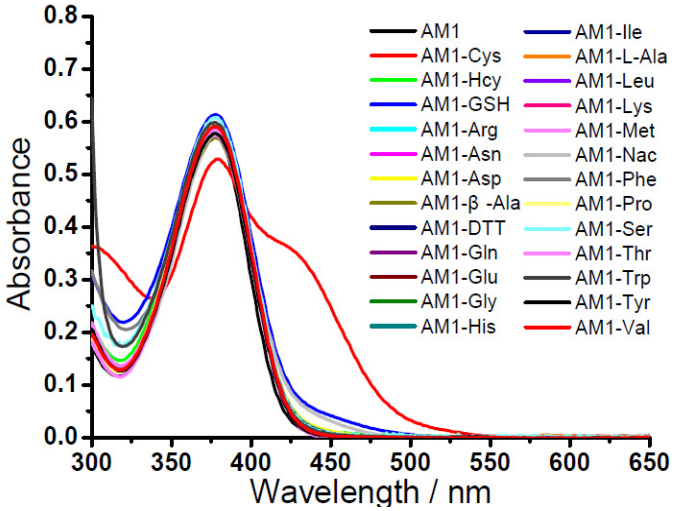


Figure S20a. Absorption spectra for **AM1** (10 μM) in the absence and presence of various biologically relevant analytes (600 μM) in acetonitrile-Tris/HCl buffer solution (Tris:10 mM, KCl: 100 mM) with pH: 7.27 and v/v of 4:1 at room temperature.

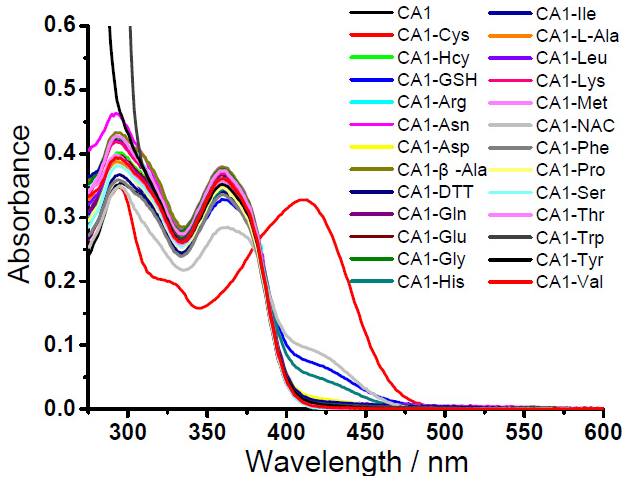


Figure S20b. Absorption spectra for **CA1** (10 μM) in the absence and presence of various biologically relevant analytes (600 μM) in methanol-Tris/HCl buffer solution (Tris:10 mM, KCl: 100 mM) with pH: 7.00 and v/v of 4:1 at room temperature.

Figure S21. The colors of **AM1** (10 μM) in the absence and presence of different analytes (600 μM) under natural light in the same acetonitrile-Tris/HCl buffer solution at room temperature. From left to right, then top to bottom: 1-**AM1**, 2:+Cys, 3:+Hcy, 4:+GSH, 5:+Arg, 6:+Asn, 7:+Asp, 8:+β-ala, 9:+DTT, 10:+Gln, 11:+Glu, 12:+Gly, 13:+His, 14:+Ile, 15:+L-ala, 16:+Leu, 17:+Lys, 18:+Met, 19:+NAC, 20:+Phe, 21:+Pro, 22:+Ser, 23:+Thr, 24:+Trp, 25:+Tyr, 26:+Val.

Figure S22. The colors of **CA1** (10 μM) in the absence and presence of different analytes (600 μM) under natural light in the same methanol-Tris/HCl buffer solution at room temperature. From left to right, then top to bottom: 1:**CA1**, 2:+Cys, 3:+Hcy, 4:+GSH, 5:+Arg, 6:+Asn, 7:+Asp, 8:+β-ala, 9:+DTT, 10:+Gln, 11:+Glu, 12:+Gly, 13:+His, 14:+Ile, 15:+L-ala, 16:+Leu, 17:+Lys, 18:+Met, 19:+NAC, 20:+Phe, 21:+Pro, 22:+Ser, 23:+Thr, 24:+Trp, 25:+Tyr, 26:+Val.

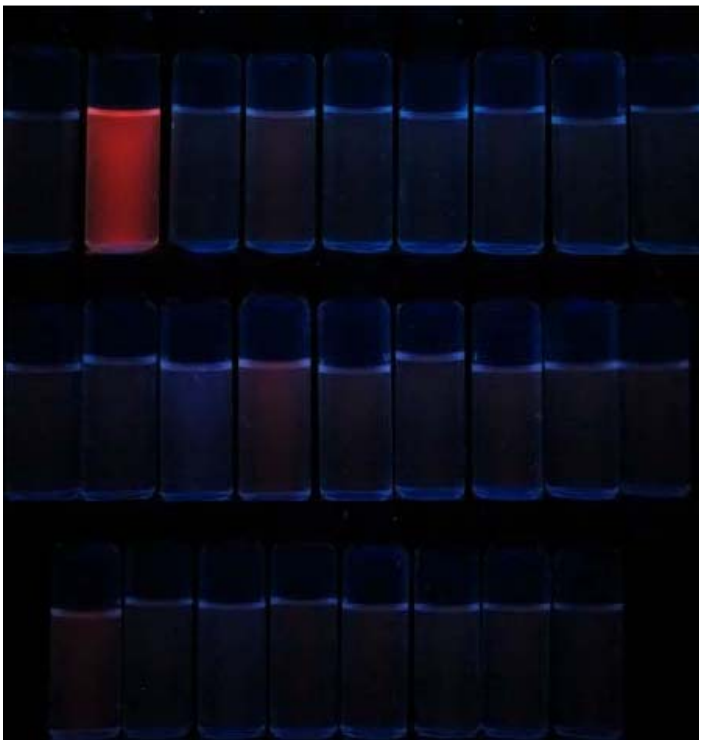


Figure S23. The colors of **AM1** (10 μM) in the absence and presence of different analytes (600 μM) under UV light (λ_{ex} = 365 nm) in the same acetonitrile-Tris/HCl buffer solution at room temperature. From left to right, then top to bottom: 1-**AM1**, 2:+Cys, 3:+Hcy, 4:+GSH, 5:+Arg, 6:+Asn, 7:+Asp, 8:+β-ala, 9:+DTT, 10:+Gln, 11:+Glu, 12:+Gly, 13:+His, 14:+Ile, 15:+L-ala, 16:+Leu, 17:+Lys, 18:+Met, 19:+NAC, 20:+Phe, 21:+Pro, 22:+Ser, 23:+Thr, 24:+Trp, 25:+Tyr, 26:+Val.

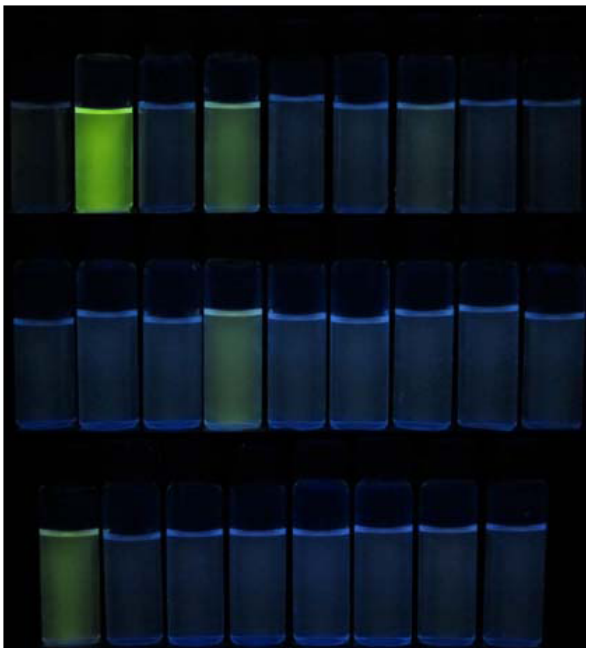


Figure S24. The colors of **CA1** (10 μM) in the absence and presence of different analytes (600 μM) under UV light (λ_{ex} = 365 nm) in the same methanol-Tris/HCl buffer solution at room temperature. From left to right, then top to bottom: 1:**CA1**, 2:+Cys, 3:+Hcy, 4:+GSH, 5:+Arg, 6:+Asn, 7:+Asp, 8:+β-ala, 9:+DTT, 10:+Gln, 11:+Glu, 12:+Gly, 13:+His, 14:+Ile, 15:+L-ala, 16:+Leu, 17:+Lys, 18:+Met, 19:+NAC, 20:+Phe, 21:+Pro, 22:+Ser, 23:+Thr, 24:+Trp, 25:+Tyr, 26:+Val.

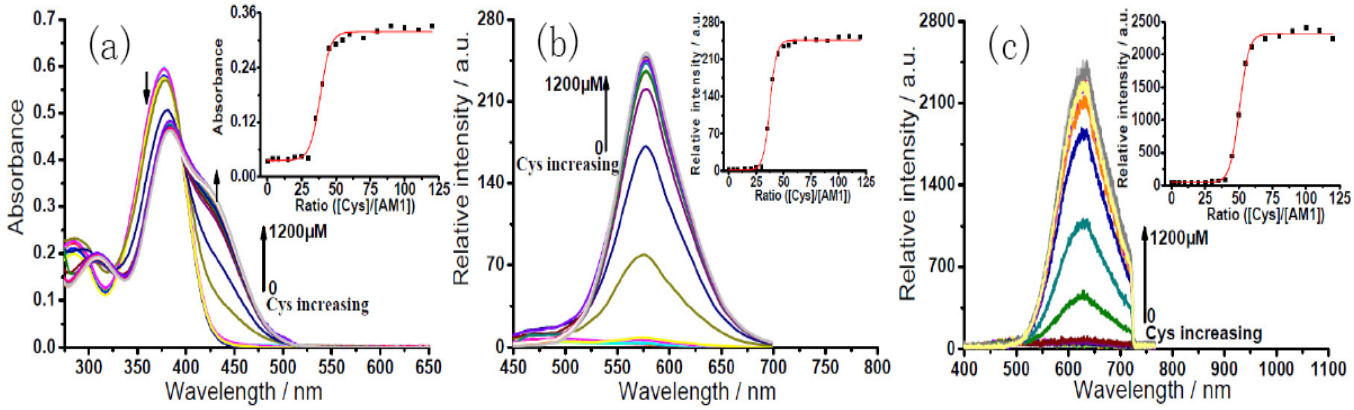
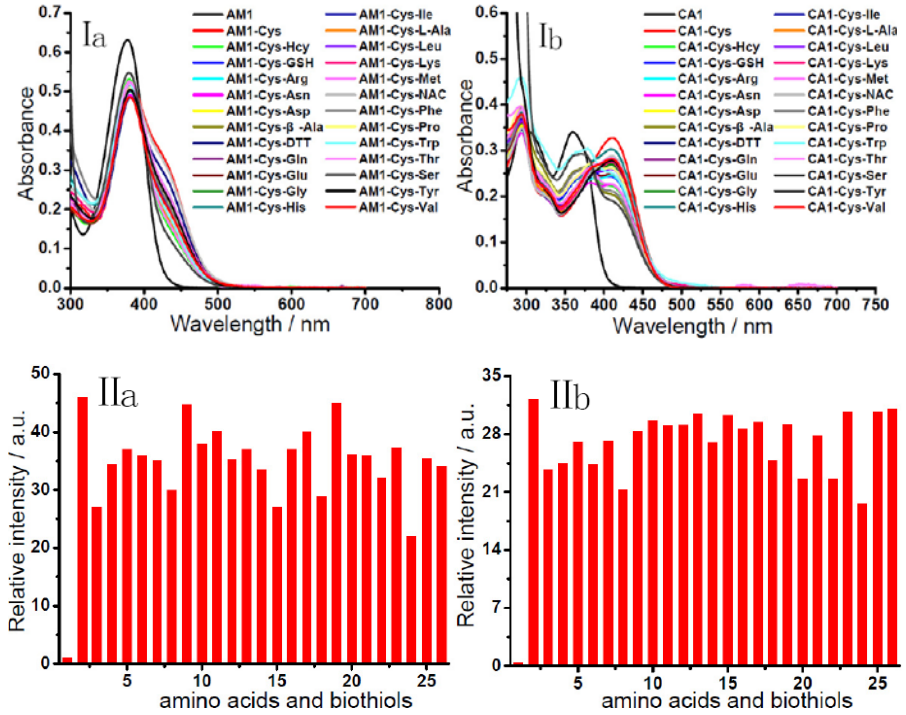


Figure S25. (a) Absorption, (b) One-photon and (c) Two-photon fluorescence titration spectra of **AM1** (10 μM) with addition of Cys (0–1200 μM) in acetonitrile-Tris buffer solution at room temperature. Excitation wavelength, b: 427 nm, c: 800 nm. Inset: the absorbance and fluorescence intensity changes of **AM1** upon addition of Cys. a: absorbance at 427 nm, b: fluorescent intensity at 577 nm, c: fluorescent intensity at 630 nm.



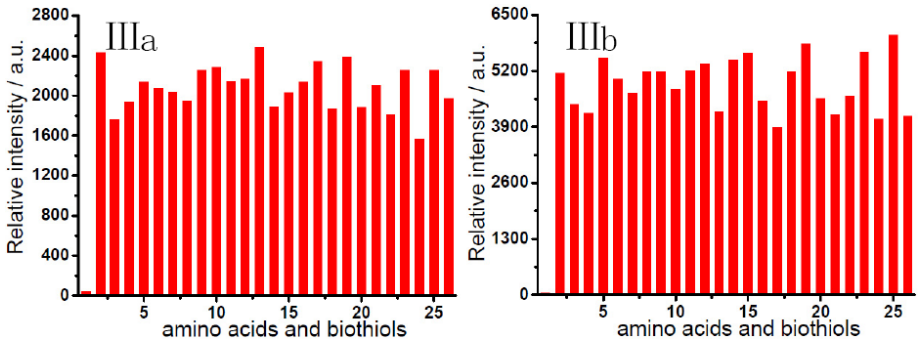


Figure S26. Absorption (I), one (II) and two-photon (III) fluorescence responses of **AM1** (a) (10 μM) in acetonitrile-Tris/HCl buffer solution and **CA1** (b) (10 μM) in methanol-Tris/HCl buffer solution to various bioanalytes (600 μM) in the presence of Cys at room temperature. From left to right: 1:**AM1** or **CA1**, 2:+Cys, 3:+Cys+Hcy, 4:+Cys+GSH, 5:+Cys+Arg, 6:+Cys+Asn, 7:+Cys+Asp, 8:+Cys+β-ala, 9:+Cys+DTT, 10:+Cys+Gln, 11:+Cys+Glu, 12:+Cys+Gly, 13:+Cys+His, 14:+Cys+Ile, 15:+Cys+L-ala, 16:+Cys+Leu, 17:+Cys+Lys, 18:+Cys+Met, 19:+Cys+NAC, 20:+Cys+Phe, 21:+Cys+Pro, 22:+Cys+Ser, 23:+Cys+Thr, 24:+Cys+Trp, 25:+Cys+Tyr, 26:+Cys+Val. Excitation wavelength: SPEF, 488 nm; TPEF: 800 nm for **AM1**, 910 nm for **CA1**.

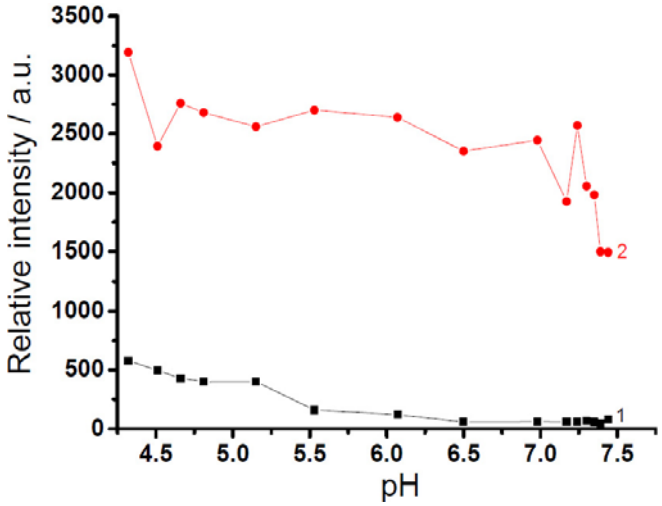


Figure S27. TPEF intensity of **AM1** (10 μM) and **AM1** (10 μM) + Cys (600 μM) in acetonitrile-Tris/HCl buffer solution at different pH value. Excitation wavelength: 800 nm.

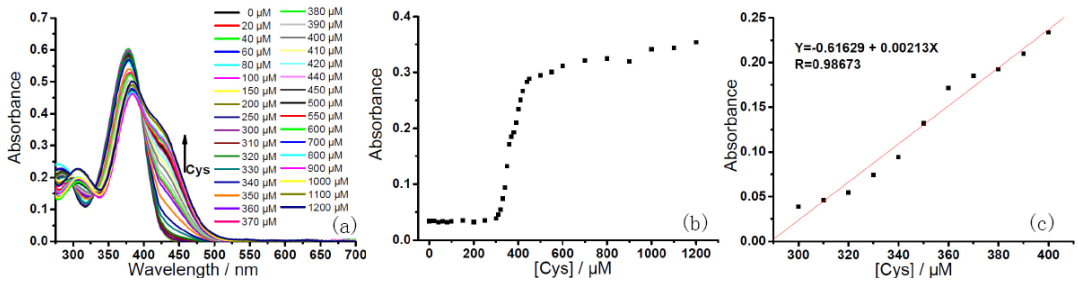


Figure S28a. (a) Absorption titration spectra of **AM1** (10 μM) in acetonitrile-Tris buffer solution at room temperature, (b) Absorbance change at λ_{max}=427 nm in various concentrations of Cys, (c) Plot of absorbance as a function of Cys concentration (300-400 μM). The detection limit of Cys is 337 μM.

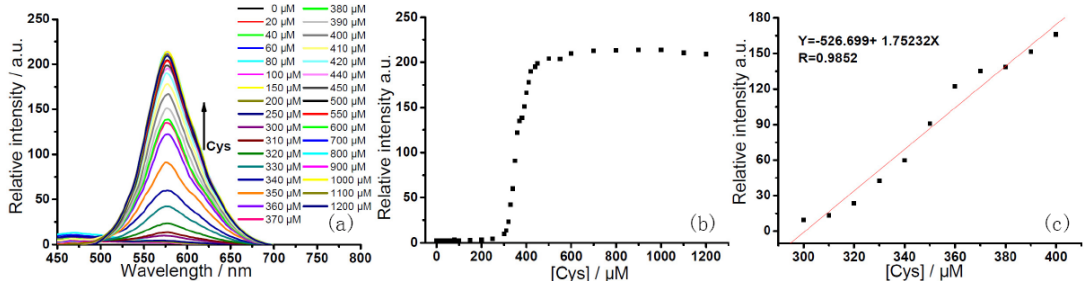


Figure S28b. (a) One-photon fluorescence titration spectra of **AM1** (10 μM) in acetonitrile-Tris buffer solution at room temperature, excitation wavelength: 427 nm. (b) Fluorescence intensity change at 577 nm in various concentrations of Cys. (c) Plot of fluorescence intensity as a function of Cys concentration (300-400 μM). The detection limit of Cys is 304 μM.

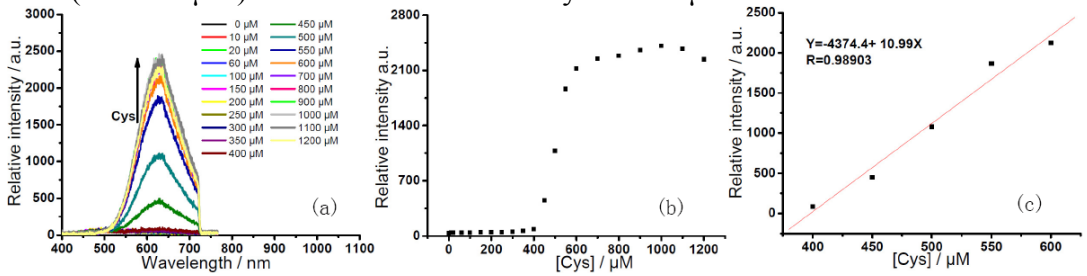


Figure S28c. (a) Two-photon fluorescence titration spectra of **AM1** (10 μM) in acetonitrile-Tris buffer solution at room temperature, Excitation wavelength: 800 nm. (b) Fluorescence intensity change at 630 nm in various concentrations of Cys. (c) Plot of fluorescence intensity as a function of Cys concentration (400-600 μM). The detection limit of Cys is 408 μM.

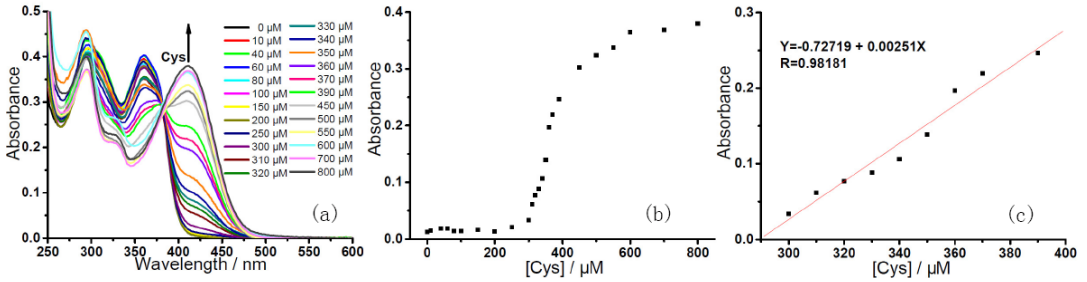


Figure S28d. (a) Absorption titration spectra of **CA1** (10 μM) in methanol-Tris buffer solution at room temperature, (b) Absorbance change at λ_{max}=410 nm in various concentrations of Cys, (c) Plot of absorbance as a function of Cys concentration (300-400 μM). The detection limit of Cys is 305 μM.

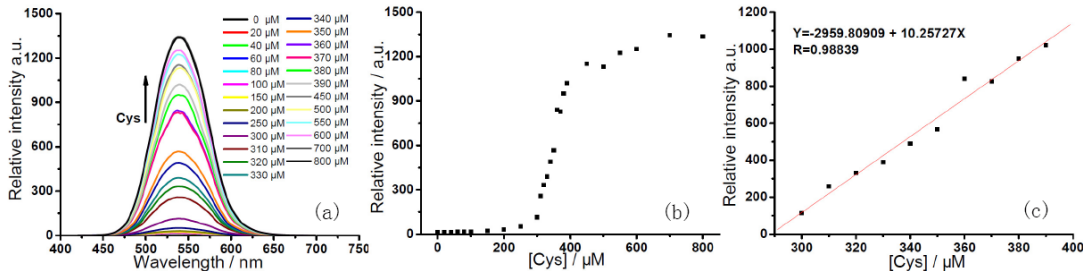


Figure S28e. (a) One-photon fluorescence titration spectra of **CA1** (10 μM) in methanol-Tris buffer solution at room temperature, Excitation wavelength: 410 nm. (b) Fluorescence intensity change at 540 nm in various concentrations of Cys. (c) Plot of fluorescence intensity as a function of Cys concentration (300-400 μM). The detection limit of Cys is 293 μM.

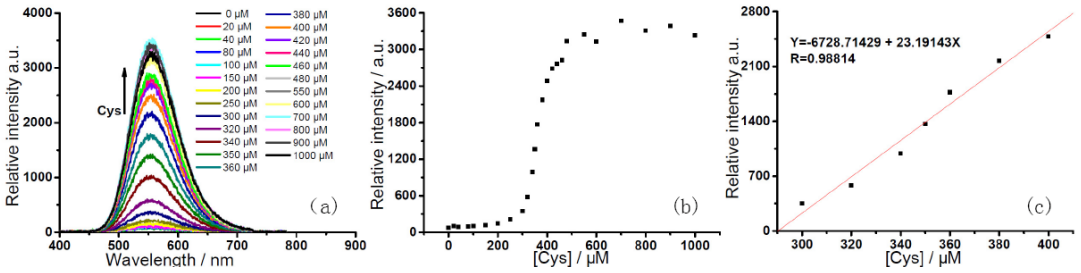


Figure S28f. (a) Two-photon fluorescence titration spectra of **CA1** (10 μM) in methanol-Tris buffer solution at room temperature, Excitation wavelength: 800 nm. (b) Fluorescence intensity change at 555 nm in various concentrations of Cys. (c) Plot of fluorescence intensity as a function of Cys concentration (300-400 μM). The detection limit of Cys is 300 μM.

Figure S28g. The photos of **AM1** (10 μM) in presence of Cys with various concentrations (from left to light: 0 μM, 150 μM, 300 μM, 330 μM, 340 μM, 350 μM, 370 μM, 600 μM) under natural light in acetonitrile-Tris/HCl buffer solution (Tris:10 mM, KCl: 100 mM) with pH: 7.27 and v/v of 4:1 at room temperature.

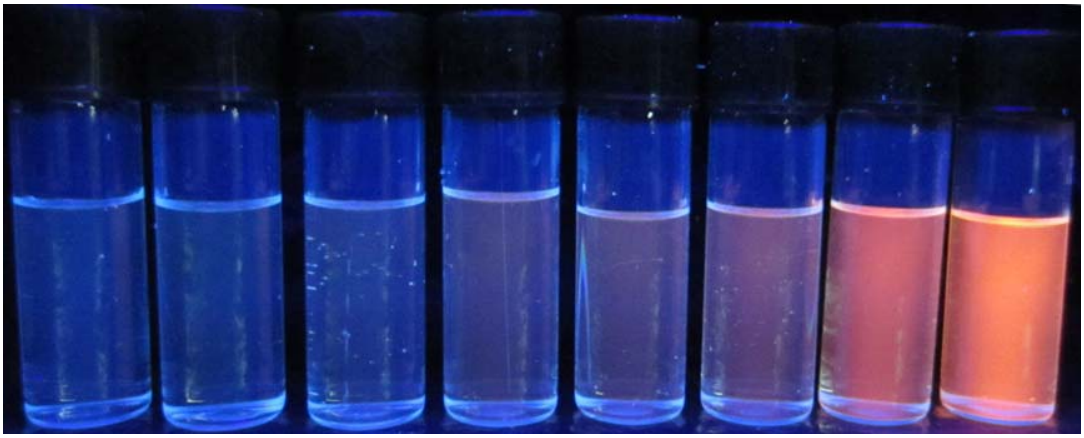


Figure S28h. The photos of **AM1** (10 μM) in presence of Cys with various concentrations (from left to light: 0 μM, 150 μM, 300 μM, 330 μM, 340 μM, 350 μM, 370 μM, 600 μM) under UV light (λ_{ex} = 365 nm) in acetonitrile-Tris/HCl buffer solution (Tris:10 mM, KCl: 100 mM) with pH: 7.27 and v/v of 4:1 at room temperature.

Figure S28i. The photos of **CA1** (10 μM) in presence of Cys with various concentrations (from left to light: 0 μM, 150 μM, 250 μM, 300 μM, 320 μM, 340 μM, 360 μM, 600 μM) under natural light in methanol-Tris/HCl buffer solution (Tris:10 mM, KCl: 100 mM) with pH: 7.00 and v/v of 4:1 at room temperature.

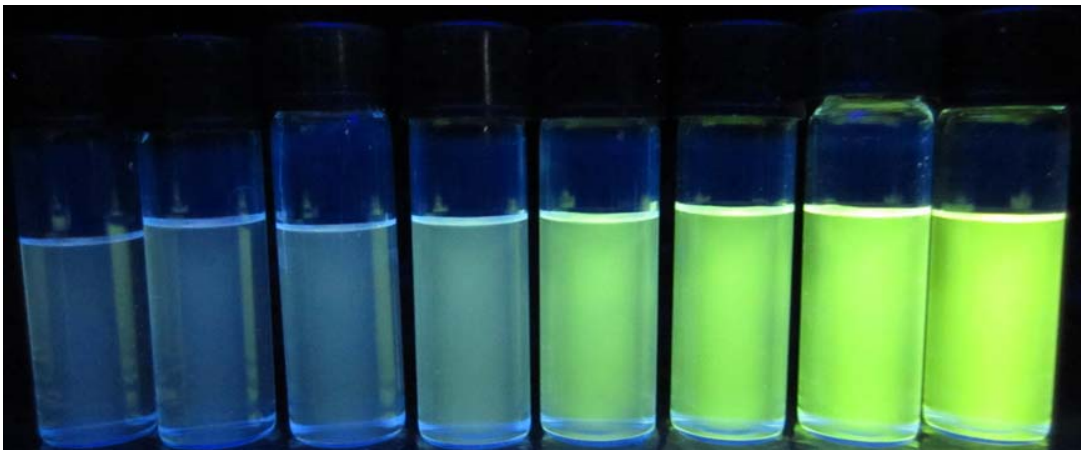


Figure S28j. The photos of **CA1** (10 μM) in presence of Cys with various concentrations (from left to light: 0 μM, 150 μM, 250 μM, 300 μM, 320 μM, 340 μM, 360 μM, 600 μM) under UV light (λ_{ex} = 365 nm) in methanol-Tris/HCl buffer solution (Tris:10 mM, KCl: 100 mM) with pH: 7.00 and v/v of 4:1 at room temperature.

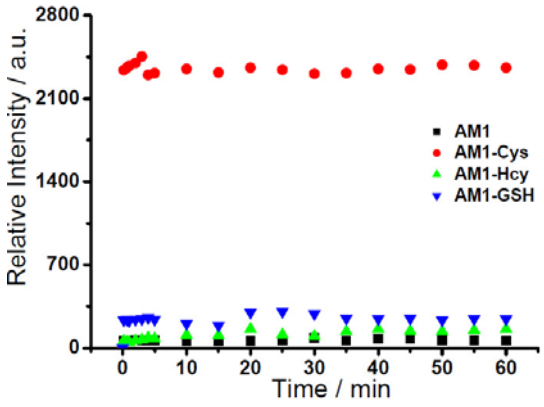


Figure S29. TPEF intensity changes of **AM1** (10 μM), **AM1** (10 μM) +Cys (600 μM), **AM1** (10 μM) +Hcy (600 μM), and **AM1** (10 μM) + GSH (600 μM) in acetonitrile-Tris/HCl buffer solution at different time. Excitation wavelength: 800 nm.

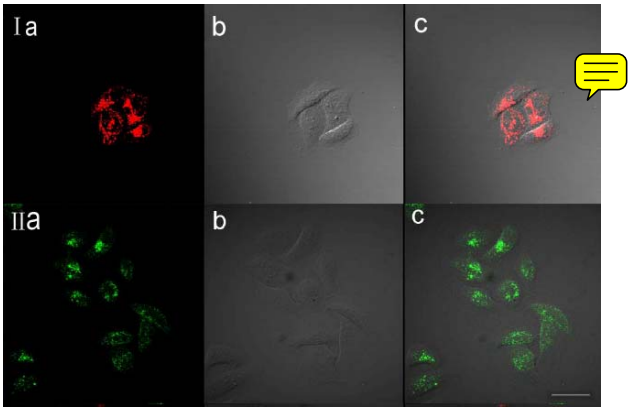


Figure S30. Confocal fluorescence images of **AM1** or **CA1**. (a) Fluorescence imaging, (b) DIC pictures, (c) overlay images of a and b. Detection wavelengths, (I): ＞565 nm. (II): 510–540 nm. Scale bar = 50 μm.

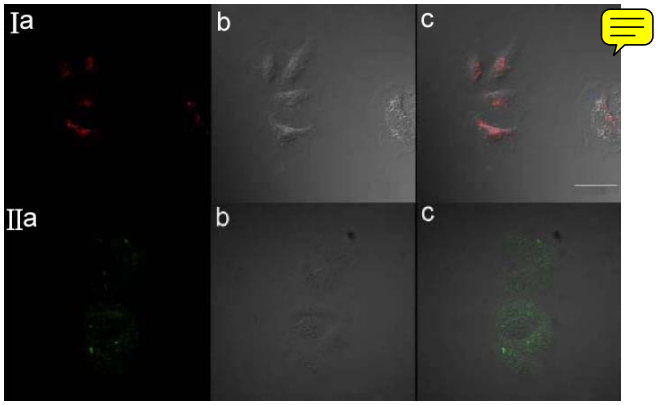


Figure S31. Confocal fluorescence images of HeLa cells treated with 5 mM *N*-ethylmaleimide for 0.5 h at 37 °C and then incubated with 5 μM **AM1** (I) or **CA1** (II) for 0.5 h at 37 °C. Excitation wavelength: 488 nm. (a) Fluorescence imaging, (b) DIC pictures; (c) overlay images of panels a and b. Detection wavelength, (I): ＞565 nm; (II): 510–540 nm. bar = 50 μm.

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