Supporting Information

A Carboxylic acid-Functionalized Coumarin-Hemicyanine Fluorescent Dye and Its Application to Construct a Fluorescent Probe for Selective Detection of Cysteine over Homocysteine and Glutathione

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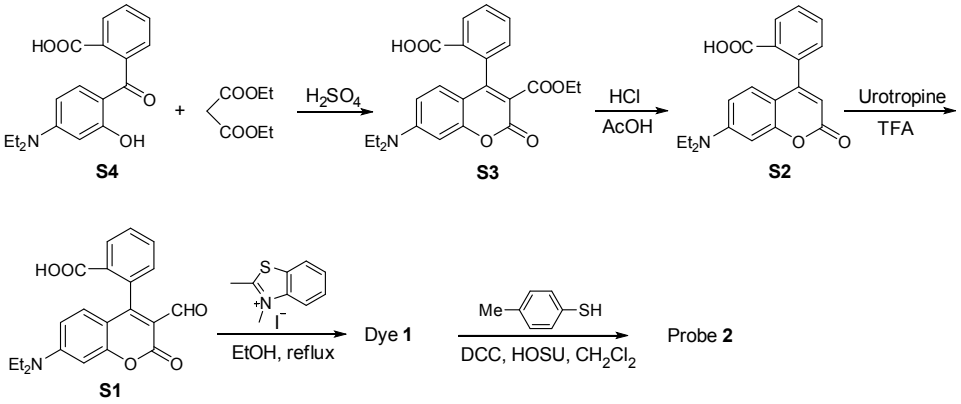
1. General methods

All reagents and solvents were purchased from commercial sources and were of the highest grade. Solvents were dried according to standard procedures. All reactions were magnetically stirred and monitored by thin-layer chromatography (TLC). Flash chromatography was performed using silica gel 60 (200–300 mesh). Absorption spectra were taken on a Varian Carry 4000 spectrophotometer. Fluorescence spectra were taken on Hitachi F-7000 fluorescence spectrometer. The ^{1}H NMR and ^{13}C NMR spectra were recorded at 600 and 150 MHz, respectively. The following abbreviations were used to explain the multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad. High resolution mass spectra were obtained on a Varian QFT-ESI mass spectrometer. Compound **S4** was synthesized according to the reported procedure (Eur. Pat EP511,019).

2. Synthesis



Compound S2. Compound **S4** (0.313 g, 1 mmol) and diethyl malonate (0.327 g, 2 mmol) were dissolved in methanesulfonic acid (10 mL) and stirred at 90 ^{o}C for 24 h. After cooling to room temperature, the solution was poured into ice water (10 g). NaOH solution (40%) was added dropwise to modulate pH of the solution to ~7, the precipitate was filtered, and washed with water to afford crude **S3**, which is directly used without further purification. Then concentrated HCl (5 mL) and glacial acetic acid (5 mL) were added to crude **S3** with stirring for 12 hours under reflux conditions. The solution was cooled to room temperature and poured into 50 mL ice water. NaOH

solution (40%) was added dropwise to modulate pH of the solution to ~7, and a pale precipitate formed immediately. After stirring for 30 min, the precipitate was filtered, washed with water, dried, then purified by column chromatography on silica gel (CH_2Cl_2/MeOH = 25:1, v/v) to give **S2** as yellow solid (0.245 g, 72.7% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 8.10 (d, J = 7.2 Hz, 1H), 7.69 (t, J = 7.8 Hz, 1H),7.61 (t, J = 7.8 Hz, 1H), 7.37 (d, J = 7.2 Hz, 1H), 6.83 (d, J = 9.0 Hz, 1H), 6.60 (m, 2H), 5.88 (s, 1H), 3.46 (q, J = 7.2 Hz, 4H), 1.19 (t, J = 7.2 Hz, 6H); ^{13}C NMR (150 MHz, CD_3OD) δ 166.2, 161.7, 158.9, 153.9, 139.4, 137.5, 134.2, 132.8, 132.3, 131.8, 131.6, 130.3, 111.9, 109.4, 106.6, 99.7, 47.1, 14.2; HRMS: calcd for 338.1387, found 338.1390.

Compound S1. A solution of **S2** (0.337 g, 1 mmol) and urotropine (0.701, 5 mmol) in 8 mL trifluoroacetic acid (TFA) was heated to 120 ºC for 6h in a sealed Teflon tube. The solvent was evaporated under reduced pressure and the residue was then poured into 50 mL of ice water. The mixture was stirred at 60 ºC for 1 h. After cooling, the crude product was extracted with CH_2Cl_2, the organic part was concentrated to dryness and purified by chromatography on silica gel (CH_2Cl_2: CH_3OH = 25:1, v/v) to give **S1** as an orange solid (0.247 g, 67.7% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.21 (d, J = 7.8 Hz, 1H), 7.64 (t, J = 7.2 Hz, 1H), 7.55 (t, J = 7.2 Hz, 1H), 7.11 (d, J = 7.8 Hz, 1H), 6.66(d, J = 9.6 Hz, 1H), 6.51(d, J = 2.4 Hz, 1H), 6.44 (dd, J_1 = 2.4 Hz, J_2 = 9.6 Hz, 1H), 3.43 (q, J = 7.2 Hz, 4H), 1.22 (t, J = 7.2 Hz, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 191.4, 172.3, 164.6, 164.2, 160.5, 155.8, 139.8, 135.8, 134.2, 133.2, 131.7, 131.6, 131.4, 114.2, 112.8, 112.4, 100.0, 49.9, 15.4; HRMS: calcd for 366.1336, found 366.1338.

Dye 1. S1 (0.183 g, 0.5 mmol) and 2,3-dimethylbenzothiazolium iodid (0.146 g, 0.5 mmol) were dissolved in 5 mL EtOH. The reaction mixture was refluxed with stirring for 12 h and then cooled to r.t., the precipitate was collected by filter to give **1** as dark green solid (0.257 g, 80.6% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 8.34 (d, J = 7.8 Hz, 1H), 8.30 (d, J = 15.0 Hz, 1H), 8.07 (d, J = 3.6 Hz, 1H), 8.06 (d, J = 3.6 Hz, 1H), 7.86 (t, J = 7.8 Hz, 1H), 7.80 (m, 2H), 7.67 (t, J = 7.8 Hz, 1H), 7.42 (d, J = 7.2 Hz,

1H), 7.39 (d, J = 15.0 Hz, 1H), 6.81 (d, J = 9.6 Hz, 1H), 6.73 (dd, J_1 = 2.4 Hz, J_2= 9.0 Hz, 1H), 6.66 (s, 1H), 4.19 (s, 3H), 3.57 (q, J = 7.2 Hz, 4H), 1.25 (t, J = 7.2 Hz, 6H); ^{13}C NMR (150 MHz, CD_3OD) δ 175.7, 170.1, 163.1, 156.6, 146.1, 145.1, 137.7, 135.3, 134.2, 133.9, 133.0, 132.9, 132.7, 132.2, 130.9, 130.4, 126.2, 118.6, 114.5, 113.6, 113.2, 112.8, 99.2, 47.7, 37.6, 14.3; HRMS: calcd for 511.1686, found 511.1688.

Probe 2. Dye **1** (0.128 g, 0.2 mmol), N-hydroxysuccinimide (HOSU, 69.1 mg, 0.6 mmol), and DCC (116.5 mg, 0.6 mmol) were dissolved in CH_2Cl_2 (10.0 mL) with stirring at room temperature. After 30 min, triethylamine (one drop) and 4-methylbenzenethiol (0.124 g, 1.0 mmol) were added. The reaction solution was stirred at room temperature overnight, and then concentrated under vacuum. The resulting residue was purified by chromatography on silica gel (CH_2Cl_2: CH_3OH = 40:1, v/v) to give **1** as carmine powder (25 mg, 16.8% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.35 (d, J = 7.8 Hz, 1H), 8.23 (d, J = 9.0 Hz, 1H), 8.18 (d, J = 7.8 Hz, 1H), 8.09 (d, J = 8.4 Hz, 1H), 7.91 (m, 2H), 7.75 (t, J = 7.8 Hz, 1H), 7.63 (t, J = 7.8 Hz, 1H), 7.39 (d, J = 7.2 Hz, 1H), 7.28 (m, 2H), 7.14 (d, J = 2.4 Hz, 3H), 6.85 (d, J = 9.0 Hz, 1H), 6.56 (dd, J_1 = 2.4 Hz, J_2 = 9.0 Hz, 1H), 6.51 (d, J = 2.4 Hz, 1H), 4.27 (s, 3H), 3.49 (q, J = 7.2 Hz, 4H), 2.30 (s, 3H), 1.25 (t, J = 7.2 Hz, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 193.6, 174.6, 163.4, 162.5, 160.0, 156.4, 147.0, 144.7, 143.2, 139.9, 137.4, 136.5, 134.5, 133.9, 133.7, 133.5, 133.1, 132.9, 132.8, 132.2, 131.7, 131.4, 130.1, 127.1, 126.0, 119.4, 114.8, 114.2, 113.5, 113.1, 100.0, 48.3, 39.9, 24.2, 15.5; HRMS: calcd for 617.19271, found 617.19287.

3. Cell culture and fluorescence imaging

The B16 cell line, HeLa cell line, and human kidney carcinoma cell line 786-O were provided by Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education (China), which were grown in RPMI 1640 and DMEM medium, respectively, supplemented with 10 % FBS (Fetal Bovine Serum) and 1% antibiotics at 37 ºC in humidified environment of 5% CO_2. Cells were plated on 6-

well plate and allowed to adhere for 12 hours. Fluorescence imaging was performed with a LEICA TCS-SP5 Laser Scanning Confocal Microscope. To confirm that dye **1** could specifically stain the mitochondria, B16 cells and HeLa Cells were incubated in a sequence with **1** (4 μM, 20 min) and MitoTracker Red FM (0.15 μM, 20 min) in RPMI 1640 medium at 37 °C. After each treatment, the cells were washed with PBS 3 times. Emission was collected from band path of 500-550 nm upon excitation of MitoTracker Green FM at 488 nm. Emission was collected from band path of 590-750 nm upon excitation of **1** at 543 nm. To confirm that probe **2** could specifically image intracellular Cys, 786-O cells were incubated with **2** (4 μM), or pretreated with 0.5 mM NEM in DMEM medium at 37 °C. After each treatment, the cells were washed with PBS 3 times. Emission was collected at 425-475 nm for blue channel (Ex: 405 nm).

4. Cytotoxicity assays

B16 Cells were grown in RPMI 1640 medium supplemented with 10 % FBS (Fetal Bovine Serum) and 1% antibiotics at 37 ºC in humidified environment of 5% CO_2. Immediately before the experiment, the cells well placed in a 96-well plate, followed by addition of increasing concentrations of dye **1** or probe **2**. The final concentrations of dye **1** or probe **2** were kept from 0 to 10 μM. The cells were then incubated at 37 ºC in an atmosphere of 5% CO_2 and 95% air for 8 h, followed by MTT assays (n= 6). Untreated assay with RPMI 1640 medium (n = 6) was also conducted under the same conditions.

5. Supplementary Data

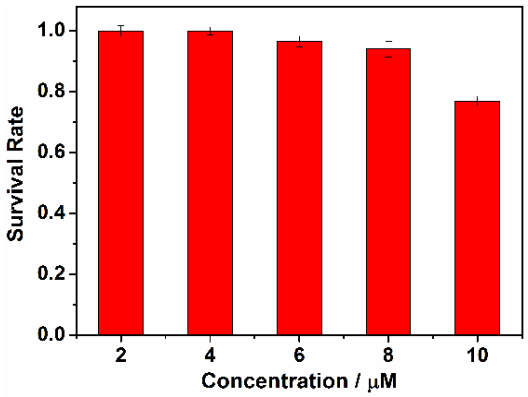


Figure S1. Percentage of viable B16 cells after treatment with indicated concentrations of dye **1** after 8 hours.

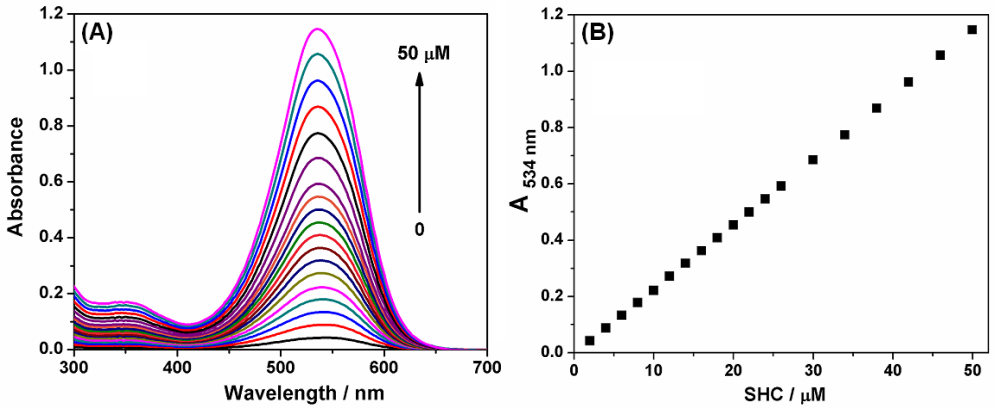


Figure S2. Absorption spectra (A) and corresponding absorption intensities (B) *vs* the varied concentrations of dye **1** in PB buffer (pH 7.4, 20 mM).

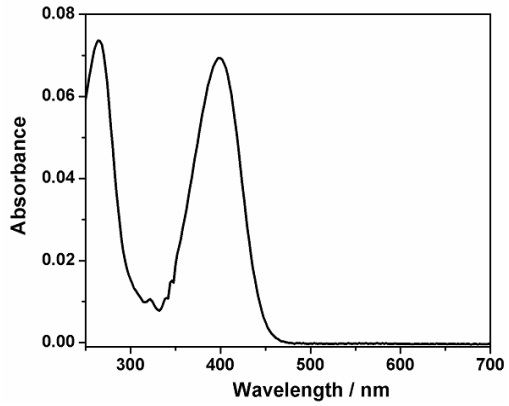


Figure S3. Absorption spectra of the control compound **S2** in PB buffer (pH 7.4, 20 mM, containing 10% DMF).

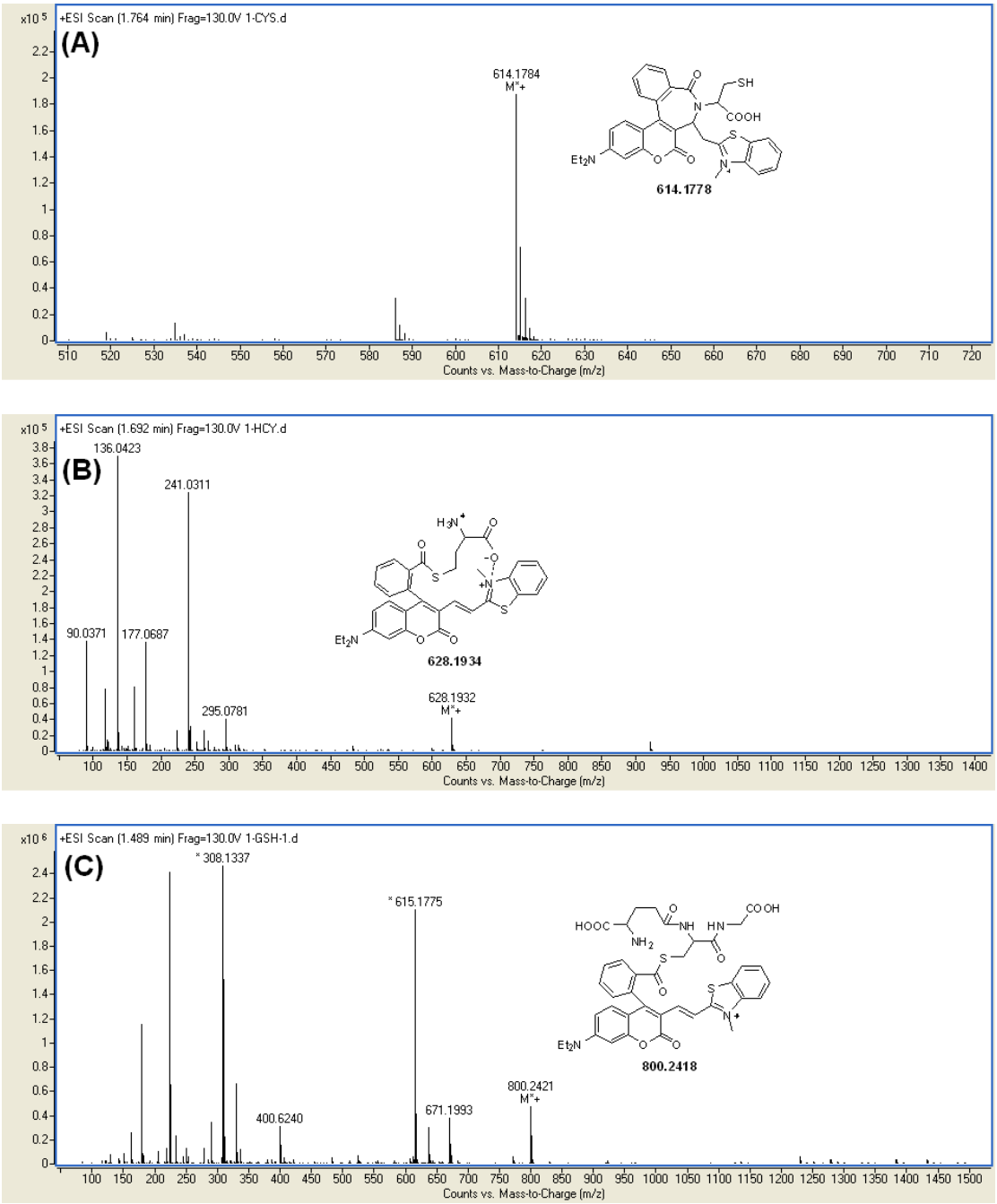


Figure S4. HRMS chart of **2**-Cys (A), **2**-Hcy (B), and 2-GSH (C).

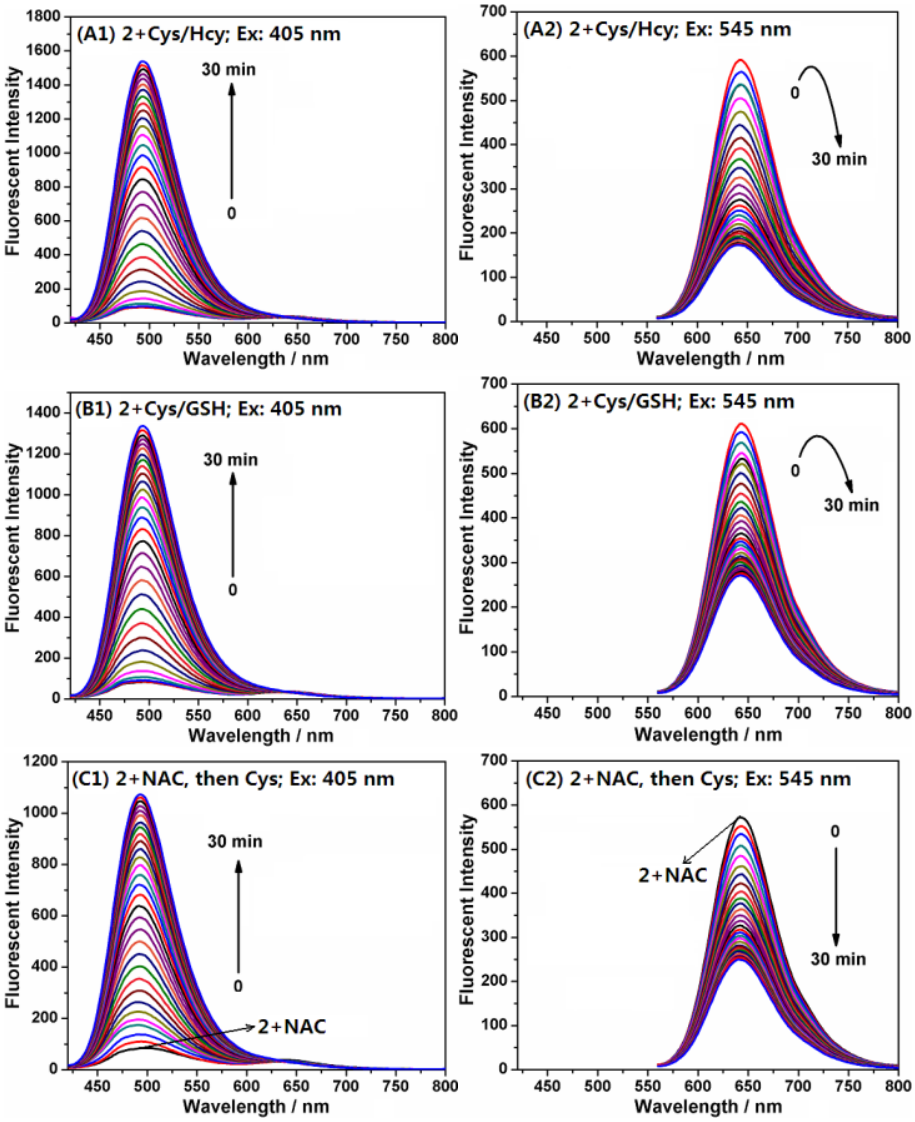


Figure S5. Time dependent fluorescence spectra of probe **2** (4 μM) treated with the mixture of Cys/Hcy (A1, A2) and Cys/GSH (B1, B2), respectively. Time dependent fluorescence spectra of probe **2** pre-treated with NAC for 30 min, then with Cys (C1, C2). The amount for each thiol is 20 equiv. Conditions: PB buffer (pH 7.4, 20 mM, containing 10% DMF); 37 ^{o}C; Ex = 405 nm for A1–C1; Ex = 545 nm for A2–C2; Slit: 5/10 nm; voltage: 600V.

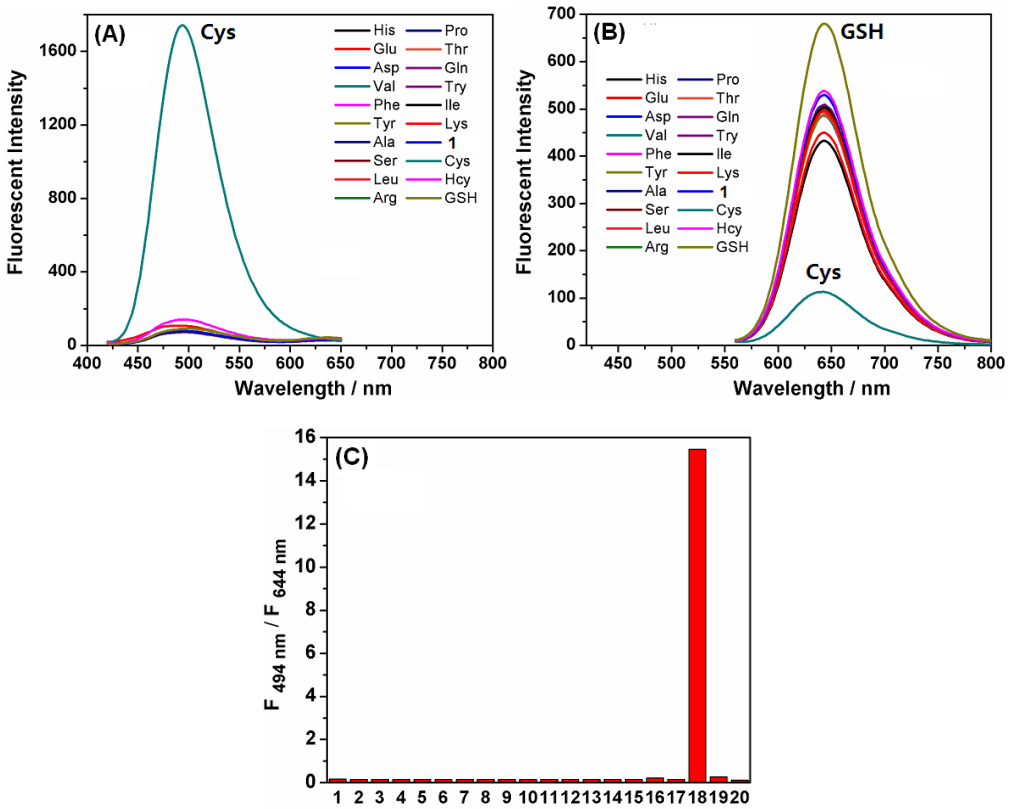


Figure S6. Fluorescence intensity changes of **2** (4 μM) upon addition of 20 equiv of various amino acids in PB buffer (pH 7.4, 20 mM, containing 10% DMF) at 37 °C. (1) **2** only, (2) His, (3) Glu, (4) Asp, (5) Val, (6) Phe, (7) Tyr, (8) Ala, (9) Ser, (10) Leu, (11) Arg, (12) Pro, (13) Thr, (14) Gln, (15) Try, (16) Ile, (17) Lys, (18) Cys, (19) Hcy, and (20) GSH. Each spectrum was recorded after 40 min. (A) λ_{ex} = 405 nm. (B) λ_{ex} = 545 nm. (C) The corresponding column charts. Slits: 5/10 nm. Voltage: 600V.

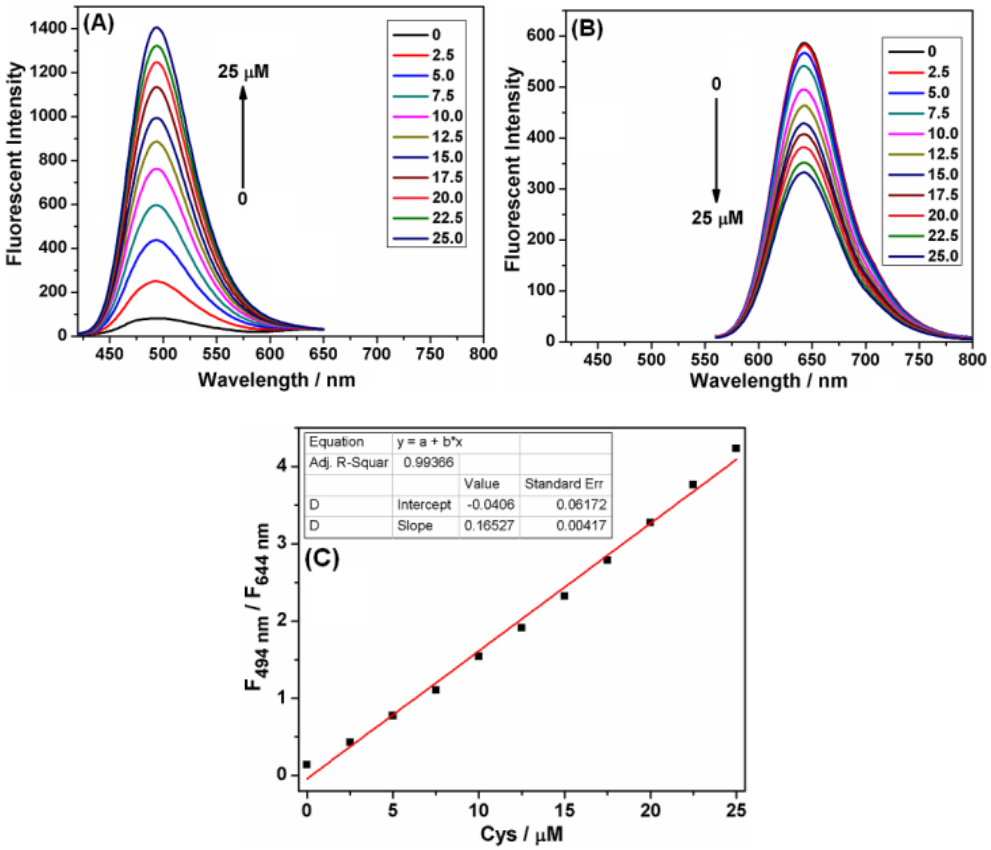


Figure S7. Fluorescence spectral of **2** (4 μM) upon addition of Cys (0–25 μM) in PB buffer (pH 7.4, 20 mM, containing 10% DMF) at 37 °C. Each spectrum was recorded after 40 min. (A) λ_{ex} = 405 nm. (B) λ_{ex} = 545 nm. (C) The linear calibration graph of the ratiometric responses (I_{494}/I_{644}) to Cys concentrations from 0 μM to 25 μM. Slits: 5/10 nm. Voltage: 600V.

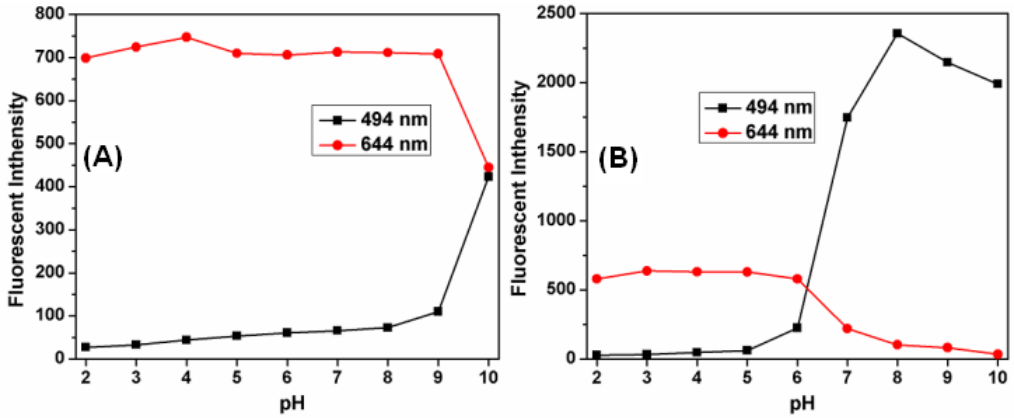


Figure S8. Changes in fluorescence intensity of **2** (4 μM) in B-R buffer at 37 °C measured in the absence (A) or presence (B) of 20 equiv of Cys as a function of pH.

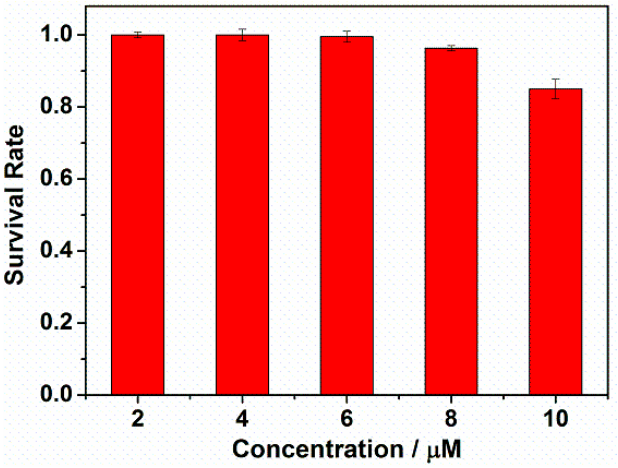


Figure S9. Percentage of viable B16 cells after treatment with indicated concentrations of probe **2** after 8 hours.

6. ^{1}H NMR, ^{13}C NMR and HRMS charts of S2, S1, 1, and 2.

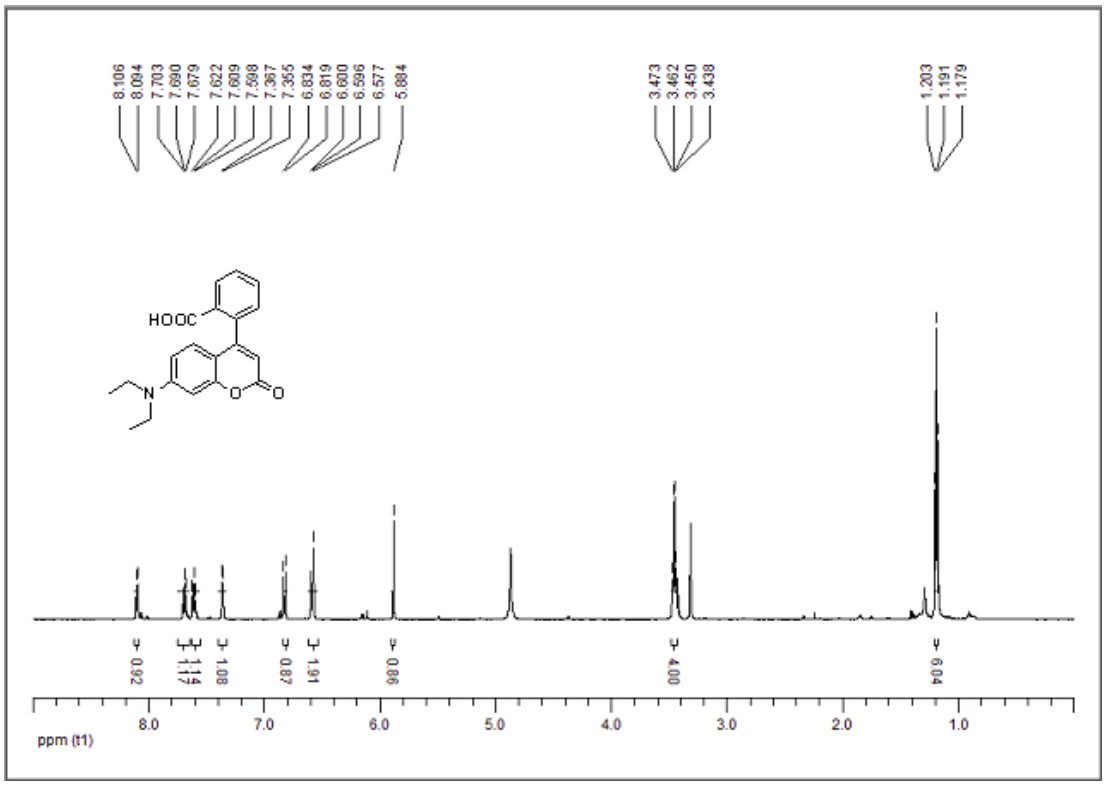


Figure S10 ^{1}H NMR chart of compound **S2** (600 MHz, CD_3OD).

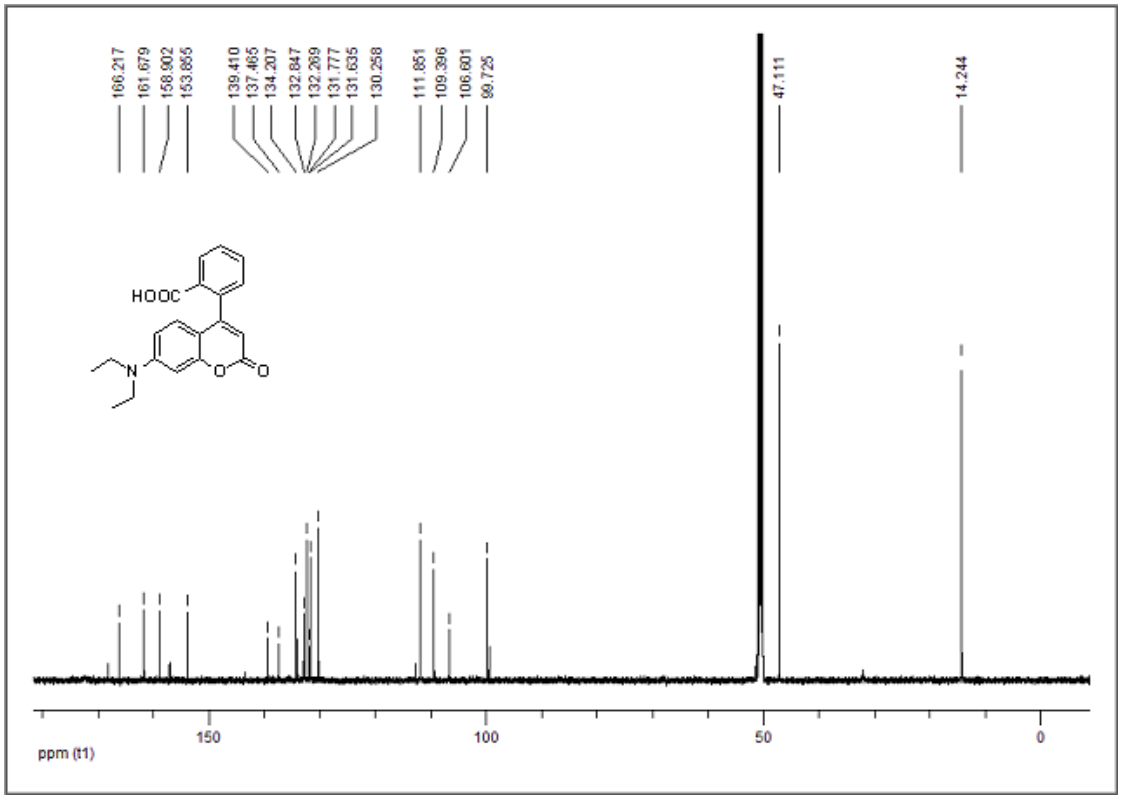


Figure S11 ^{13}C NMR chart of compound **S2** (150 MHz, CD_3OD).

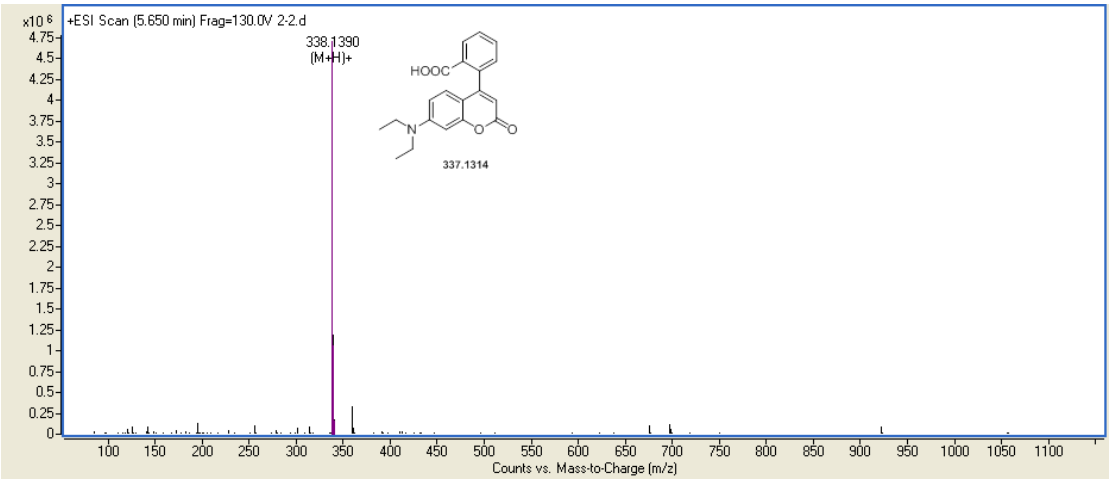


Figure S12 HRMS chart of compound **S2**.

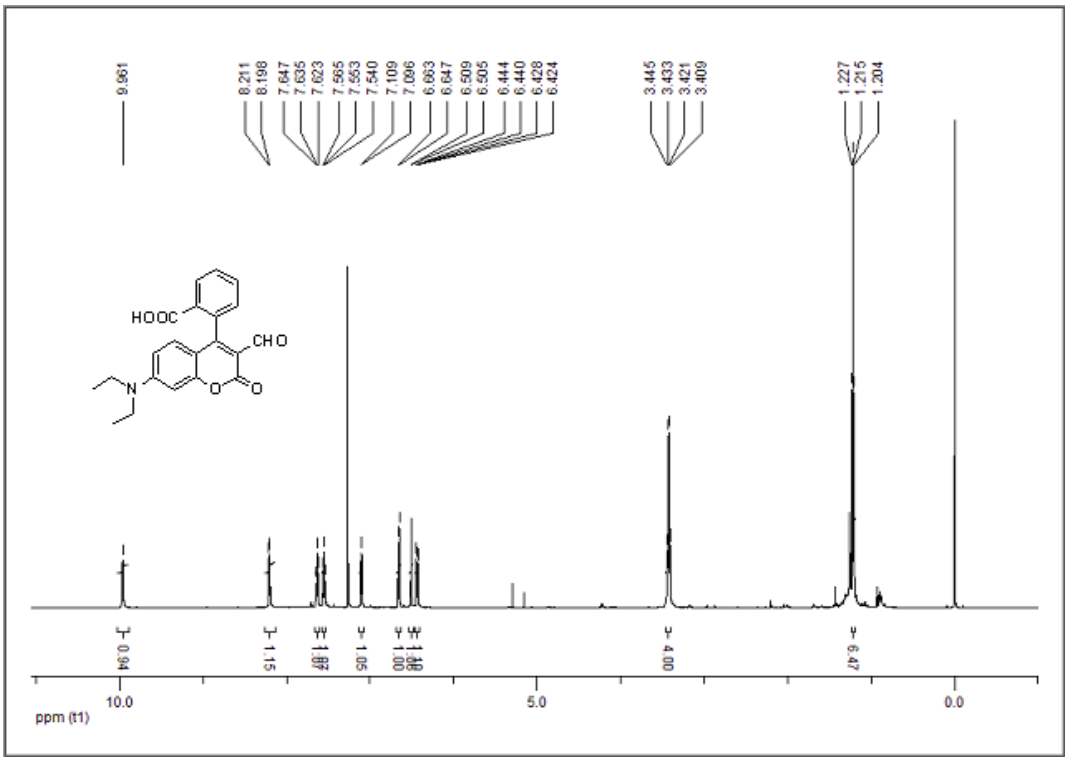


Figure S13 ^{1}H NMR chart of compound **S1** (600 MHz, $CDCl_3$).

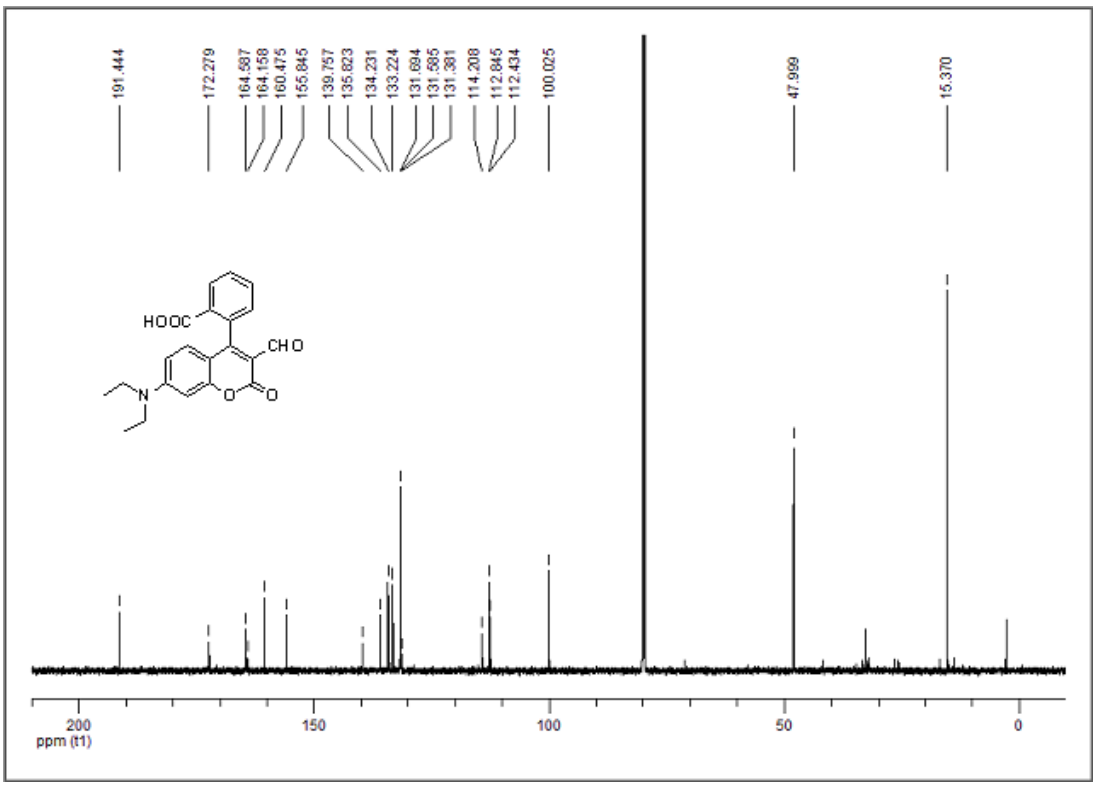


Figure S14 ^{13}C NMR chart of compound **S1** (150 MHz, $CDCl_3$).

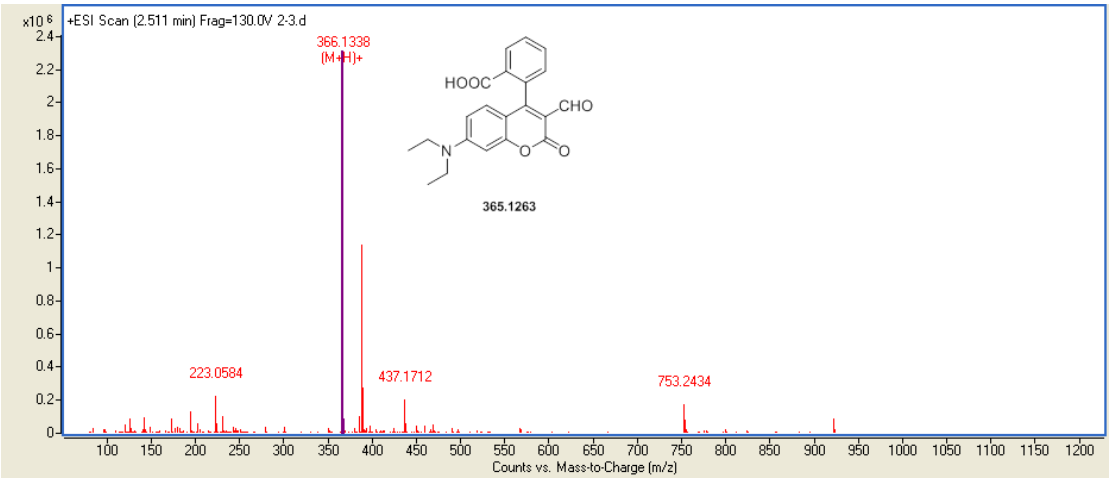


Figure S15 HRMS chart of compound **S1**.

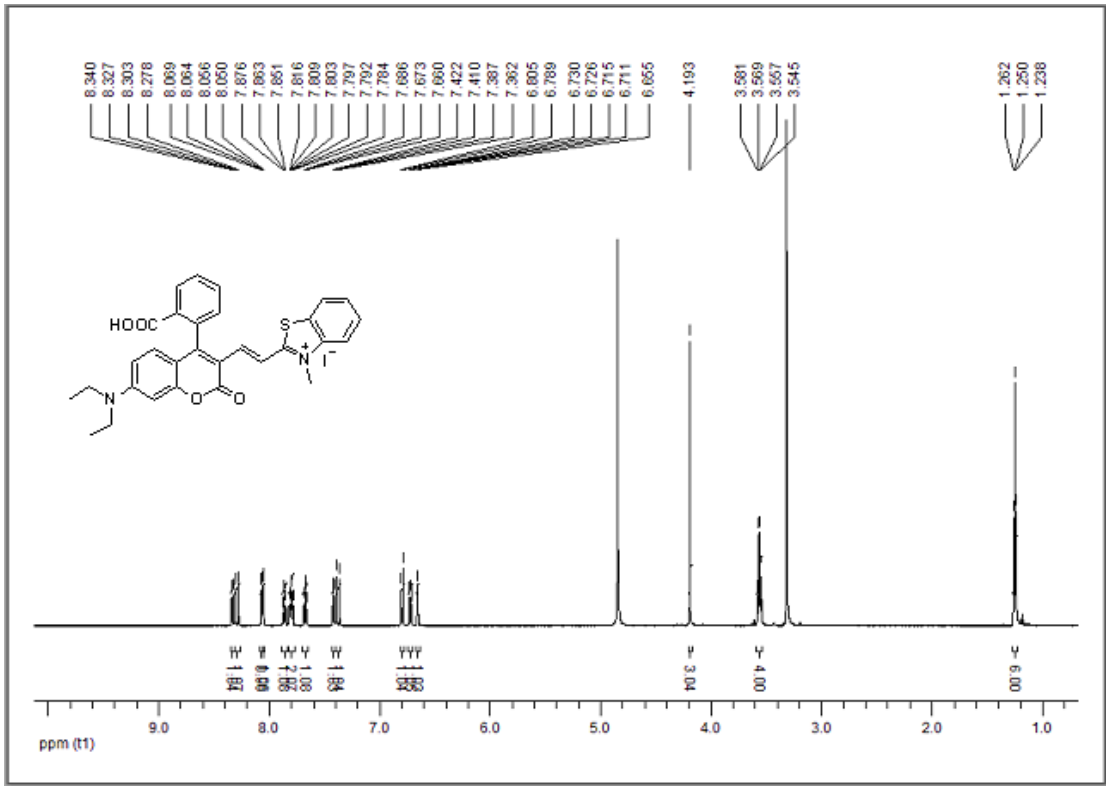


Figure S16 ^{1}H NMR chart of dye **1** (600 MHz, CD_3OD).

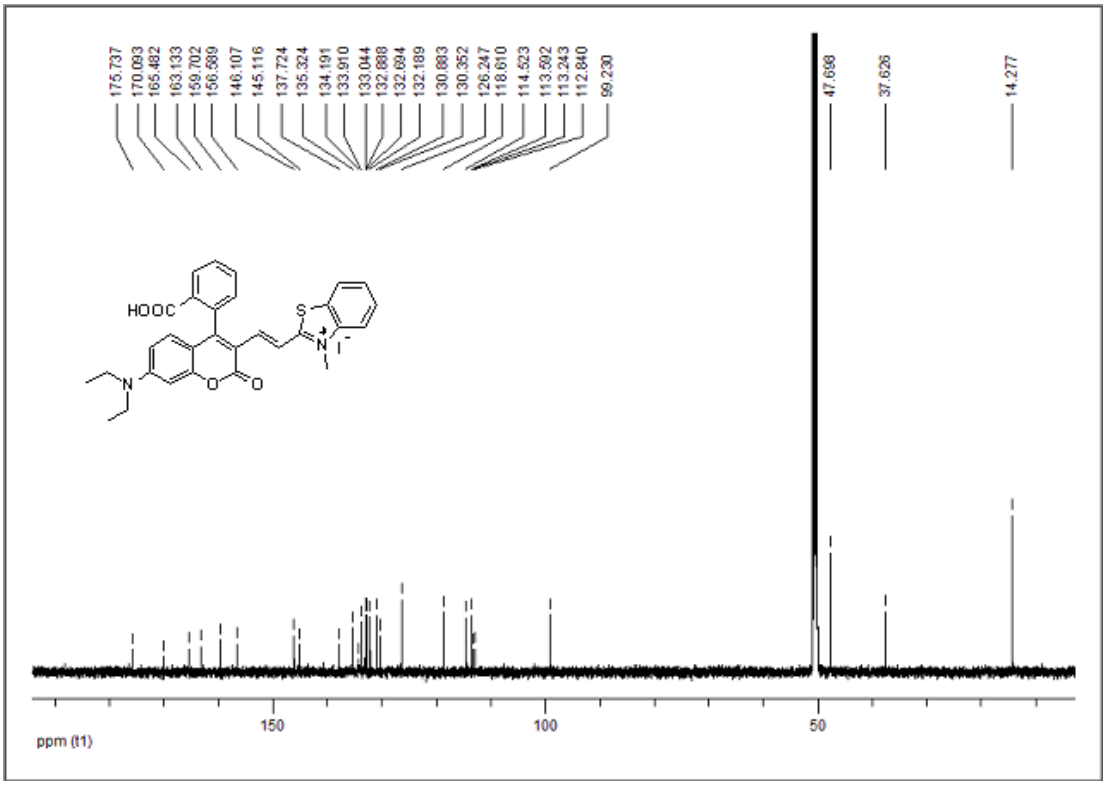


Figure S17 ^{13}C NMR chart of dye **1** (150 MHz, CD_3OD).

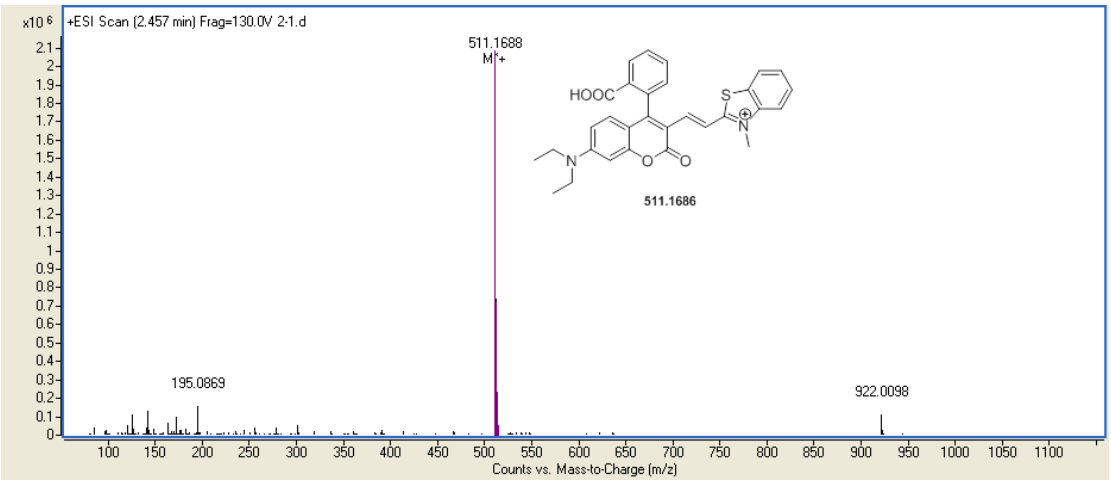


Figure S18 HRMS chart of dye **1**.

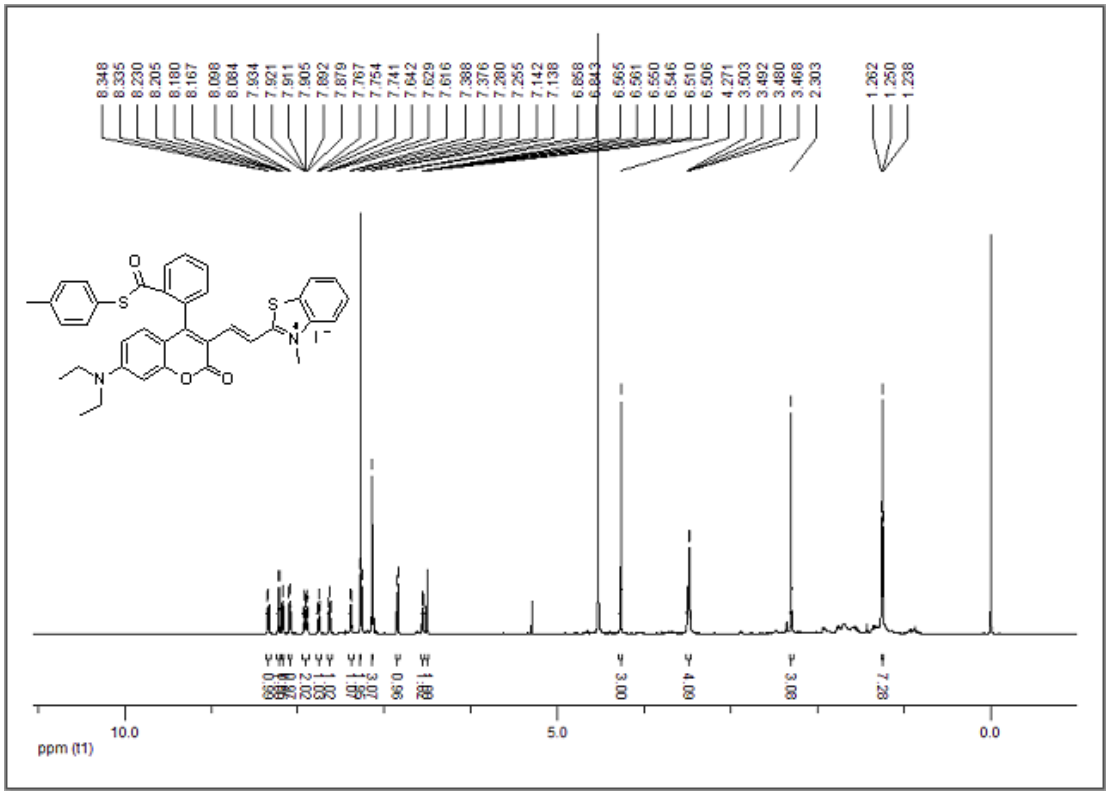


Figure S19 ^{1}H NMR chart of probe **2** (600 MHz, $CDCl_3$).

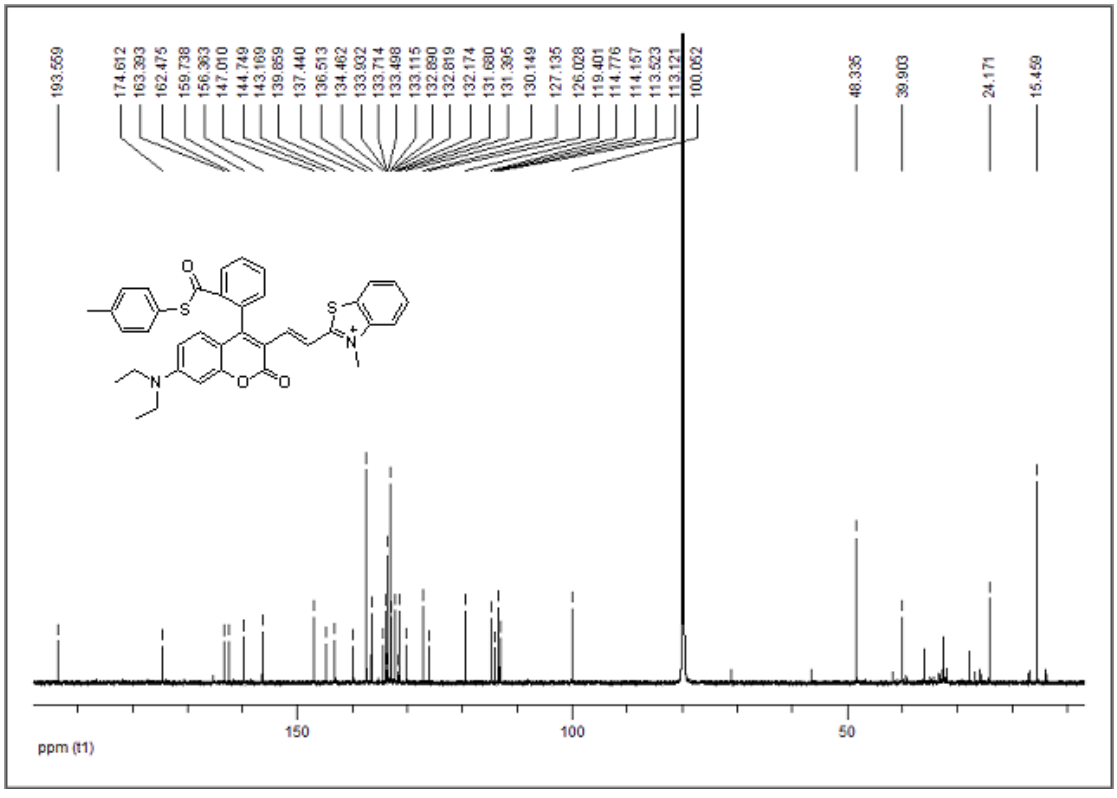


Figure S20 ^{13}C NMR chart of probe **2** (150 MHz, $CDCl_3$).

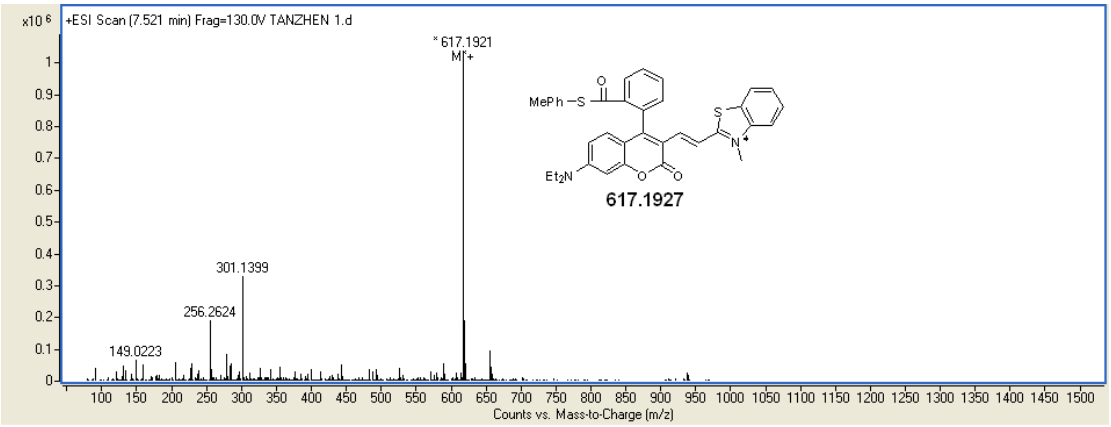


Figure S21. HRMS chart of probe **2**.

7. The ground state geometry optimization of intermediate M1:

Standard orientation:

Center Number	Atomic Number	Atomic Type	Coordinates (Angstroms) X	Y	Z
1	6	0	-5.181260	0.480030	0.941037
2	6	0	-5.674910	-0.549814	0.086303
3	6	0	-4.722220	-1.442343	-0.452405
4	6	0	-3.380656	-1.326406	-0.121055
5	6	0	-2.886232	-0.331801	0.744100
6	6	0	-3.840418	0.572590	1.256576
7	8	0	-2.537556	-2.234588	-0.685632
8	6	0	-1.164353	-2.242530	-0.469840
9	6	0	-0.621007	-1.242741	0.469068
10	6	0	-1.474417	-0.305719	1.022947
11	8	0	-0.534591	-3.078461	-1.075849
12	6	0	1.785099	-1.869013	0.078771
13	6	0	0.812756	-1.195747	0.727094
14	7	0	-7.025611	-0.676026	-0.189526
15	6	0	3.216182	-1.661635	0.340324
16	7	0	4.062090	-1.396590	-0.705517

17	6	0	5.254484	-0.765791	-0.351096
18	6	0	5.264069	-0.351652	0.993061
19	16	0	3.751515	-0.775205	1.783907
20	6	0	3.796994	-1.967328	-2.026909
21	6	0	6.358779	-0.499459	-1.166213
22	6	0	7.454484	0.163513	-0.615017
23	6	0	7.463003	0.561727	0.726043
24	6	0	6.361371	0.304615	1.542709
25	6	0	-0.985236	0.710384	2.013812
26	6	0	-1.086463	0.329997	3.361465
27	6	0	-0.712036	1.182598	4.396608
28	6	0	-0.233492	2.458443	4.100033
29	6	0	-0.129074	2.856464	2.774360
30	6	0	-0.491790	2.004185	1.712466
31	6	0	-7.945245	0.354562	0.315457
32	6	0	-7.464315	-1.547235	-1.282959
33	6	0	-9.430930	0.024819	0.185144
34	6	0	-7.270216	-0.950879	-2.683069
35	1	0	-5.851873	1.220175	1.354021
36	1	0	-4.988864	-2.240457	-1.130279
37	1	0	-3.511968	1.369599	1.914735
38	1	0	1.538380	-2.575922	-0.699939
39	1	0	1.105668	-0.483657	1.492706
40	1	0	3.544788	-3.023795	-1.902745
41	1	0	4.696239	-1.893755	-2.633971
42	1	0	2.991498	-1.423154	-2.525720
43	1	0	6.370420	-0.793620	-2.208649
44	1	0	8.316256	0.367904	-1.242609
45	1	0	8.328895	1.071270	1.136326
46	1	0	6.358618	0.606592	2.585069
47	1	0	-0.795598	0.849166	5.426515
48	1	0	0.060548	3.136717	4.894819
49	1	0	0.243105	3.841917	2.521941
50	1	0	-7.746000	1.323417	-0.170306
51	1	0	-7.733895	0.491149	1.380532

52	1	0	-8.516291	-1.785610	-1.124232
53	1	0	-6.939150	-2.501878	-1.198992
54	1	0	-10.001944	0.811837	0.686645
55	1	0	-9.678275	-0.925046	0.668578
56	1	0	-9.771834	-0.011628	-0.853004
57	1	0	-7.607455	-1.658531	-3.447553
58	1	0	-6.218571	-0.719772	-2.873691
59	1	0	-7.846070	-0.027195	-2.801058
60	6	0	-0.265337	2.610395	0.352258
61	8	0	0.041897	3.793176	0.246110
62	16	0	-0.376543	1.569099	-1.102014
63	6	0	0.015975	2.778506	-2.433178
64	1	0	-0.444170	3.734939	-2.178184
65	1	0	-0.479269	2.353942	-3.308092
66	6	0	1.510667	2.954069	-2.745511
67	1	0	1.566439	3.467014	-3.714787
68	7	0	2.284705	3.715223	-1.749263
69	1	0	3.443056	2.307676	-1.835766
70	1	0	2.536540	4.632035	-2.103098
71	6	0	2.189279	1.578163	-2.974964
72	8	0	3.359450	1.458158	-2.347590
73	8	0	1.703344	0.714699	-3.672487
74	1	0	1.735309	3.858231	-0.903276
75	1	0	-1.466415	-0.660800	3.589362
76	17	0	3.653674	-3.893704	0.834330

Rotational constants (GHZ): 0.1140525 0.0378687 0.0368821

8. The ground state geometry optimization of 2-Hcy:

Standard orientation:

Center Number	Atomic Number	Atomic Type	Coordinates (Angstroms) X	Y	Z

1	6	0	-5.487395	0.334681	0.830669
2	6	0	-5.888708	-0.782925	0.050910
3	6	0	-4.882784	-1.568324	-0.541815
4	6	0	-3.557053	-1.233253	-0.374031
5	6	0	-3.146624	-0.119904	0.385934
6	6	0	-4.162568	0.654399	0.976096
7	8	0	-2.650980	-2.031855	-0.977284
8	6	0	-1.290820	-1.844879	-0.873329
9	6	0	-0.831139	-0.746506	-0.033015
10	6	0	-1.765285	0.121298	0.534210
11	8	0	-0.587322	-2.616202	-1.490775
12	6	0	1.589080	-1.342736	-0.296858
13	6	0	0.560934	-0.599011	0.211265
14	7	0	-7.207099	-1.079700	-0.114090
15	6	0	2.906305	-1.155646	0.150826
16	7	0	3.987923	-1.759645	-0.352396
17	6	0	5.188037	-1.328863	0.197707
18	6	0	5.002413	-0.406350	1.229907
19	16	0	3.304427	-0.154215	1.523005
20	6	0	3.917006	-2.489674	-1.599045
21	6	0	6.468642	-1.686746	-0.203333
22	6	0	7.542201	-1.106771	0.447366
23	6	0	7.355611	-0.191678	1.487704
24	6	0	6.079196	0.165803	1.893343
25	6	0	-1.279661	1.272769	1.321783
26	6	0	-1.231356	2.570593	0.785464
27	6	0	-0.656301	3.609183	1.520992
28	6	0	-0.142553	3.378054	2.788981
29	6	0	-0.190209	2.096818	3.326769
30	6	0	-0.748295	1.056535	2.594821
31	6	0	-1.877187	2.880125	-0.512444
32	8	0	-3.047337	2.638887	-0.736169
33	16	0	-0.962012	3.745332	-1.770999
34	6	0	0.796315	3.567534	-1.371720

35	6	0	1.377036	2.224430	-1.734680
36	6	0	2.887378	2.254823	-1.734565
37	6	0	3.473100	0.937049	-2.242527
38	7	0	3.559393	2.552045	-0.479731
39	8	0	4.663332	0.698279	-1.714901
40	8	0	2.914939	0.210930	-3.039338
41	6	0	-8.190121	-0.335629	0.648645
42	6	0	-7.585495	-2.279723	-0.818329
43	6	0	-9.619717	-0.729413	0.389223
44	6	0	-7.452990	-3.531318	0.020845
45	1	0	-6.239204	0.962816	1.312615
46	1	0	-5.103711	-2.458247	-1.135529
47	1	0	-3.869971	1.535241	1.556387
48	1	0	1.369352	-2.107913	-1.050840
49	1	0	0.816272	0.198122	0.929129
50	1	0	3.787611	-1.764604	-2.427929
51	1	0	3.080098	-3.198784	-1.544711
52	1	0	4.822751	-3.102056	-1.684561
53	1	0	6.612735	-2.428040	-0.993027
54	1	0	8.557808	-1.383767	0.148864
55	1	0	8.223445	0.243466	1.992723
56	1	0	5.922346	0.874865	2.712076
57	1	0	-0.650956	4.617403	1.087509
58	1	0	0.289845	4.203642	3.363644
59	1	0	0.212888	1.901389	4.325898
60	1	0	-0.768109	0.039564	3.003405
61	1	0	0.963644	3.810210	-0.306075
62	1	0	1.275867	4.370530	-1.964734
63	1	0	1.051397	1.900926	-2.739481
64	1	0	1.013442	1.437648	-1.041392
65	1	0	3.221484	3.021413	-2.465967
66	1	0	4.737853	1.451403	-1.020333
67	1	0	3.120590	2.045290	0.306040
68	1	0	3.597370	3.550570	-0.262104
69	1	0	-8.071637	0.741886	0.418491

70	1	0	-7.972375	-0.439385	1.733416
71	1	0	-6.971086	-2.350819	-1.734152
72	1	0	-8.622148	-2.161356	-1.171394
73	1	0	-10.276845	-0.112240	1.021736
74	1	0	-9.818331	-1.784848	0.640950
75	1	0	-9.918201	-0.557351	-0.658066
76	1	0	-7.714997	-4.429176	-0.561985
77	1	0	-8.119592	-3.487811	0.899251
78	1	0	-6.420557	-3.649582	0.390362
79	17	0	4.512810	-4.118000	0.848962

Rotational constants (GHZ): 0.1169062 0.0365294 0.0323173