Supporting Information for

A mitochondria-targeted colorimetric and NIR ratiometric fluorescent probe for biothiols with large Stokes shift based on thiol-chromene click reaction

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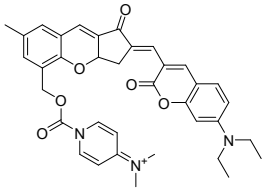
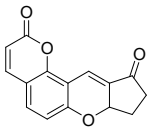
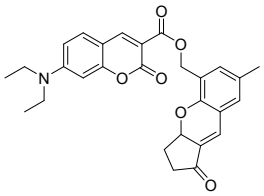
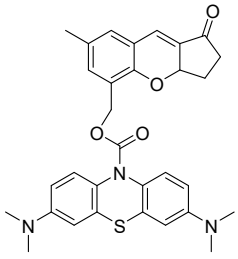
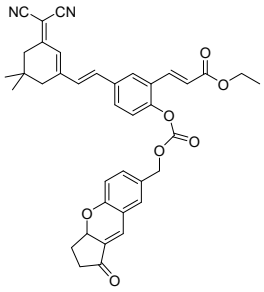
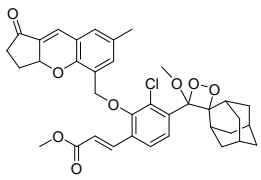
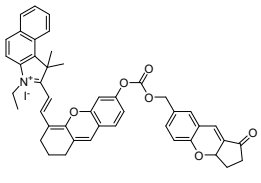
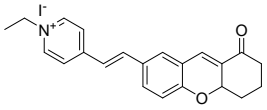
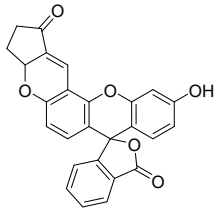
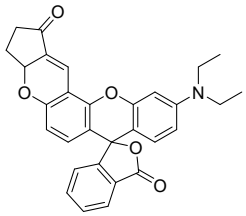
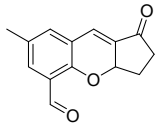
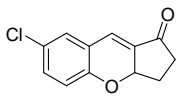
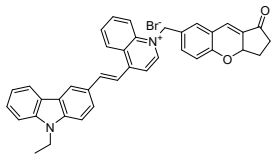
Table S1. Comparison of probes based on thiol-chromene click reaction.

Probes	Solvent	Detection limit	Detection type	Targeting organelle	Ref.
	PBS/DMSO (9:1, pH 7.4)	Cys: 0.49 μM	Turn on Em = 578 nm	Mitochondria	[S1]
	HEPES (10 mM, pH 7.4)	Cys: 0.18 μM GSH: 0.70 μM Hcy: 0.82 μM	Turn on Em = 455 nm	—	[S2]
	PBS (10 mM, 0.5 % DMSO, pH 7.4)	Cys: 76 nM GSH: 152 nM Hcy: 47 nM	Turn on Em = 470 nm for Hcy/Cys Em = 546 nm for GSH	—	[S3]
	DMSO/PBS (v/v, 1:1, pH 7.4)	Cys: 85 nM GSH: 60 nM	Turn on Em = 698 nm	—	[S4]
	PBS (10 mM, 45 % DMSO, pH 7.4)	Cys: 33 nM GSH: 40 nM Hcy: 100 nM	Turn on Em = 705 nm	—	[S5]
	PBS/DMSO 9:1 (v/v, 10 mM, pH 7.4)	Cys: 0.13 μM GSH: 0.18 μM Hcy: 0.16 μM	Turn on Em = 550 nm	—	[S6]

	PBS (10 mM, 30% ethanol, pH 7.4)	Cys: 0.39 μM GSH: 0.59 μM Hcy: 0.54 μM	Turn on Em = 731 nm	Mitochondria	[S7]
	PBS (10 mM, pH 7.4)	GSH: 3.75 μM	Ratiometric Em = 496nm/550 nm	—	[S8]
	HEPES (20 mM, 1% CH_3CN, pH 7.4)	Cys: 50 nM GSH: 53 nM Hcy: 100 nM	Turn on Em = 520 nm	—	[S9]
	HEPES (10 mM, pH 7.4)	Cys: 70 nM GSH: 49 nM Hcy: 62 nM	Turn on Em = 557 nm	—	[S10]
	MSO/HEPES (v/v, 1:1, pH 7.4)	Cys: 64 nM	Turn on Em = 515 nm	—	[S11]
	Tris-HCl (10 mM, 0.15% EtOH, pH 8.0)	—	Turn off Em = 550 nm	—	[S12]
	**PBS (10 mM PBS:DMSO, 1:1, v/v, pH 7.4)**	**Cys: 97 nM** **GSH: 94 nM** **Hcy: 93 nM**	**Ratiometric** Em = 530 nm/650 nm	**Mitochondria**	**This work**

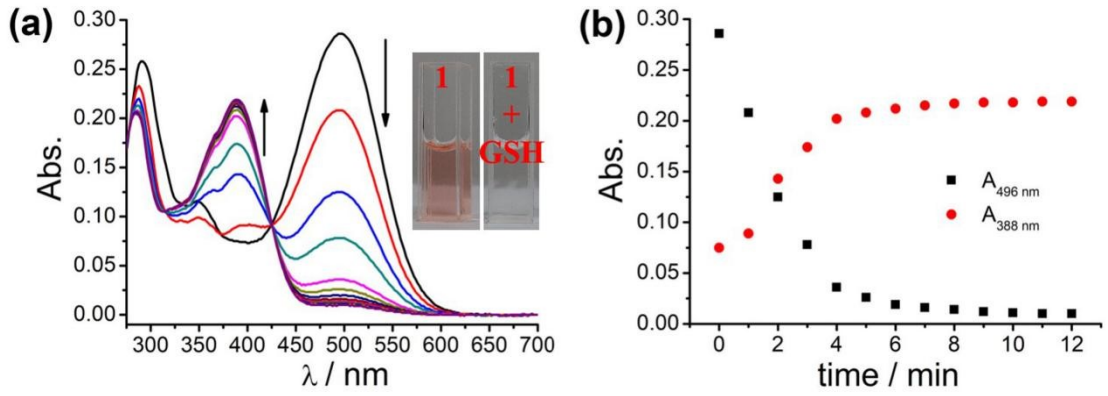


Fig. S1. (a) Absorption spectra of **1** (10 μM) after addition of GSH (30 μM) recorded every 1 min within 12 min. Insets: The visible color of **1** before and after reaction with GSH. (b) Absorbance at 496 nm and 388 nm changes as a function of time. Data were acquired in PBS buffer solution (10 mM PBS:DMSO = 1:1, v/v, pH = 7.4, 25°C).

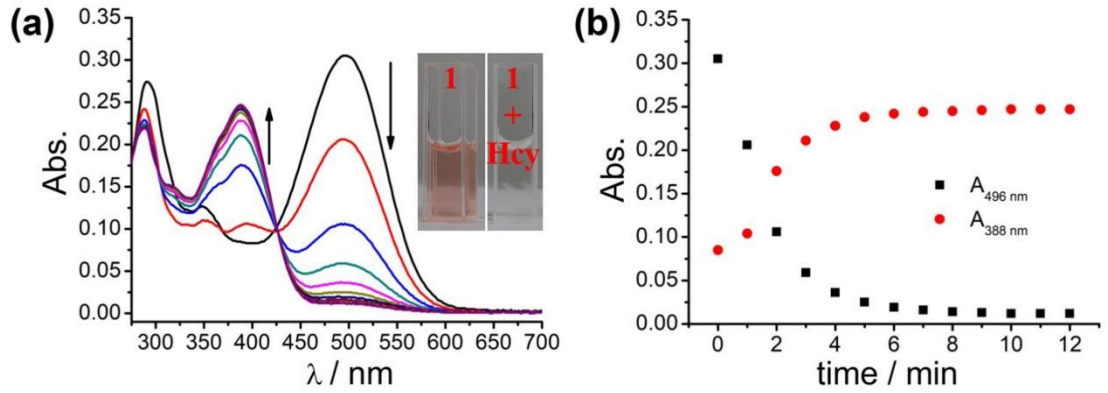


Fig. S2. (a) Absorption spectra of **1** (10 μM) after addition of Hcy (30 μM) recorded every 1 min within 12 min. Insets: The visible color of **1** before and after reaction with Hcy. (b) Absorbance at 496 nm and 388 nm changes as a function of time. Data were acquired in PBS buffer solution (10 mM PBS:DMSO = 1:1, v/v, pH = 7.4, 25°C).

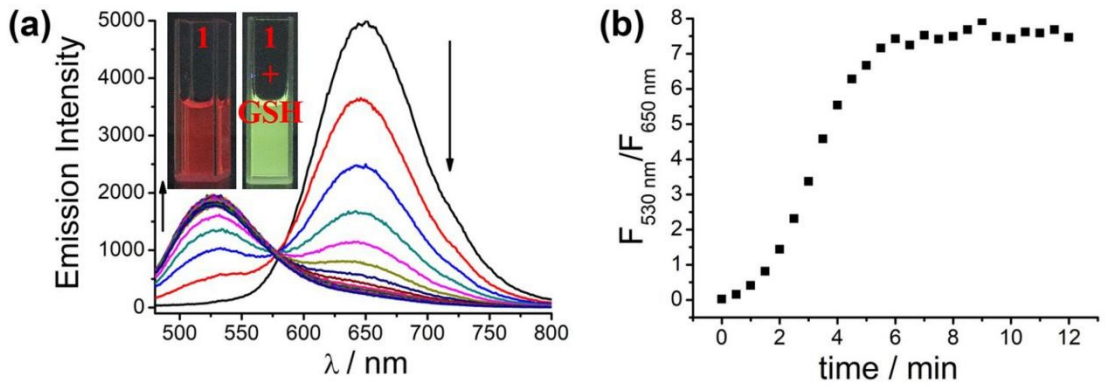


Fig. S3. (a) Fluorescence spectra of **1** (10 μM) after addition of GSH (30 μM) recorded every 1 min within 12 min. Insets: The fluorescence color of **1** before and after reaction with GSH under a 365 nm UV lamp. (d) The fluorescence intensity ratio of $F_{530\ nm}/F_{650\ nm}$ changes as a function of time. Data were acquired in PBS buffer solution (10 mM PBS:DMSO = 1:1, v/v, pH = 7.4, 25°C, λ_{ex} = 458 nm).

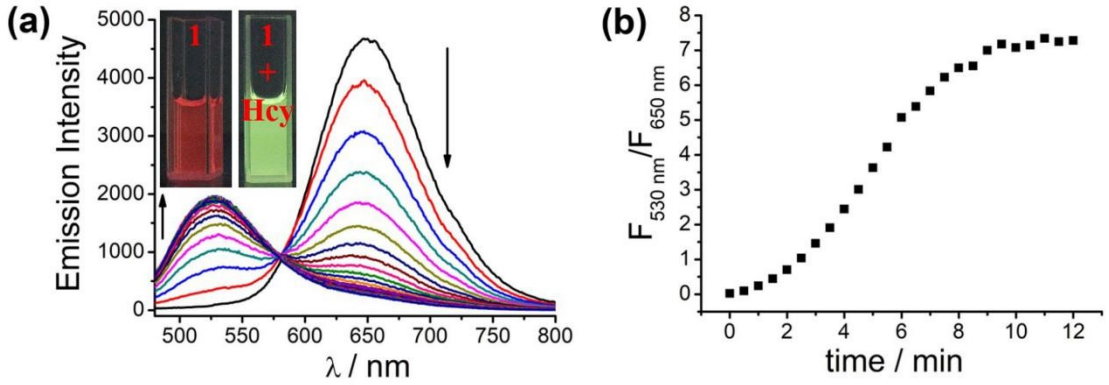


Fig. S4. (a) Fluorescence spectra of **1** (10 μM) after addition of Hcy (30 μM) recorded every 1 min within 12 min. Insets: The fluorescence color of **1** before and after reaction with Hcy under a 365 nm UV lamp. (d) The fluorescence intensity ratio of $F_{530\ nm}/F_{650\ nm}$ changes as a function of time. Data were acquired in PBS buffer solution (10 mM PBS:DMSO = 1:1, v/v, pH = 7.4, 25°C, λ_{ex} = 458 nm).

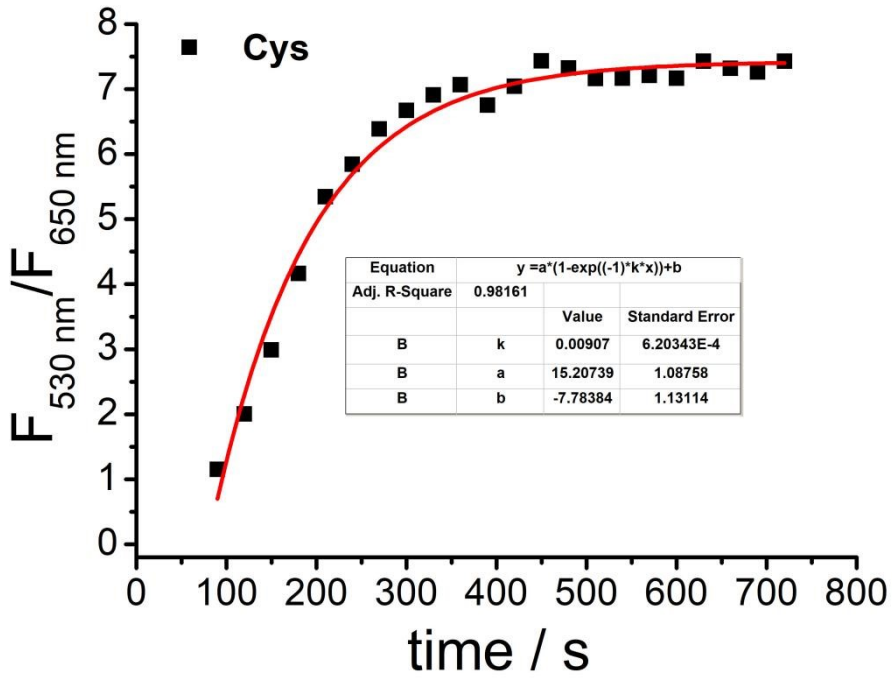


Fig. S5. Kinetic plot of fluorescence intensity ratio ($F_{530\ nm}/F_{650\ nm}$) of the pseudo-first order reaction of **1** (10 μM) to Cys (30 μM), using excitation wavelength at 458 nm. The slope of the plot corresponds to the observed reaction rate of $\mathbf{9.07 \times 10^{-3}\ s^{-1}}$.

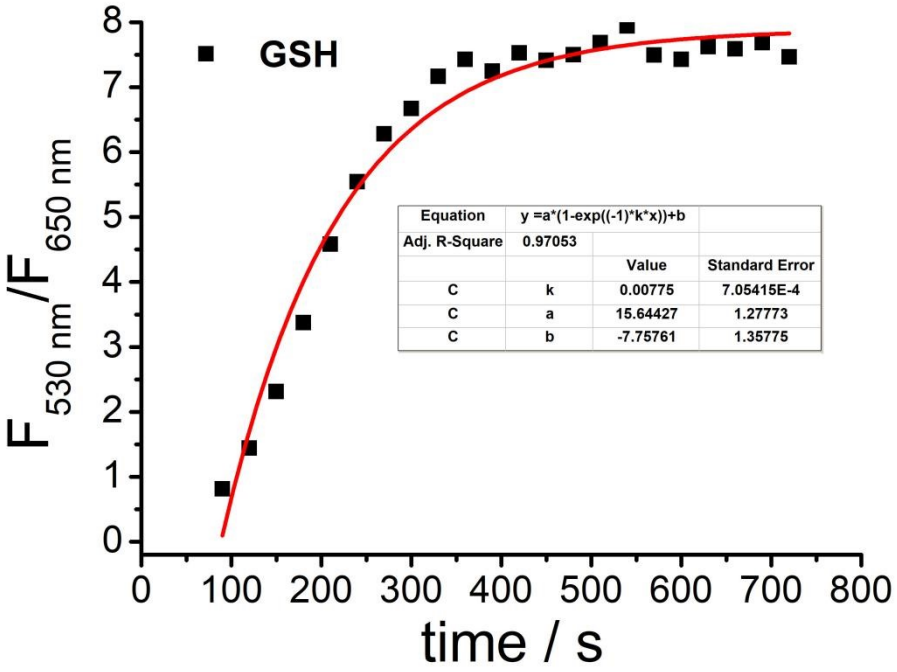


Fig. S6. Kinetic plot of fluorescence intensity ratio ($F_{530\ nm}/F_{650\ nm}$) of the pseudo-first order reaction of **1** (10 μM) to GSH (30 μM), using excitation wavelength at 458 nm. The slope of the plot corresponds to the observed reaction rate of $\mathbf{7.75 \times 10^{-3}\ s^{-1}}$.

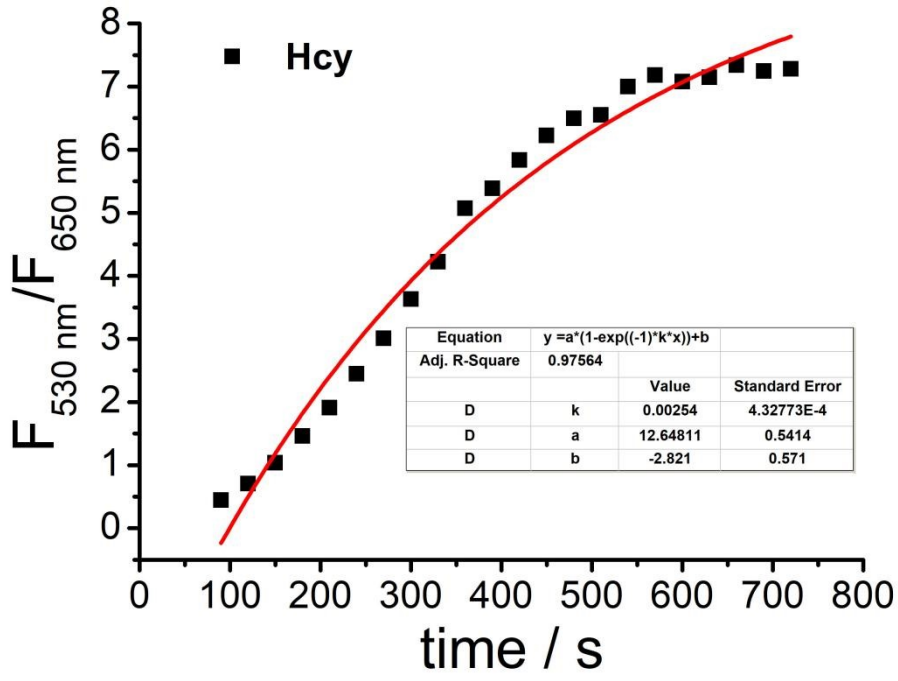


Fig. S7. Kinetic plot of fluorescence intensity ratio ($F_{530\ nm}/F_{650\ nm}$) of the pseudo-first order reaction of **1** (10 μM) to Hcy (30 μM), using excitation wavelength at 458 nm. The slope of the plot corresponds to the observed reaction rate of $\mathbf{2.54 \times 10^{-3}\ s^{-1}}$.

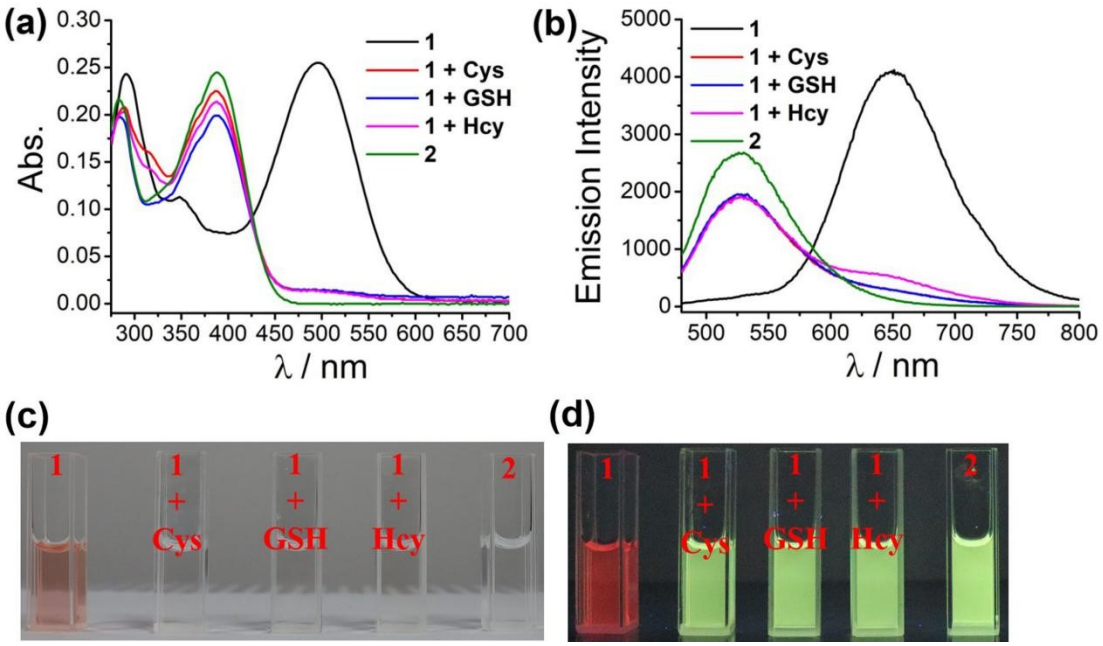


Fig. S8. (a) Absorption spectra of **1** (10 μM) (black line) before and after reaction with Cys (30 μM) (red line), GSH (30 μM) (blue line), Hcy (30 μM) (pink line) respectively, as well as **2** (10 μM) (green line) in PBS buffer solution (10 mM PBS:DMSO = 1:1, v/v, pH = 7.4, 25°C). (b) Fluorescence spectra of **1** (10 μM) (black line) before and after reaction with Cys (30 μM) (red line), GSH (30 μM) (blue line), Hcy (30 μM) (pink line) respectively, as well as **2** (10 μM) (green line) in PBS buffer solution (10 mM PBS:DMSO = 1:1, v/v, pH = 7.4, 25°C, λ_{ex} = 458 nm). (c) The visible color of **1** before and after reaction with Cys, GSH, Hcy respectively, as well as **2** in PBS buffer solution (10 mM PBS:DMSO = 1:1, v/v, pH = 7.4, 25°C). (d) The fluorescence color of **1** before and after reaction with Cys, GSH, Hcy respectively, as well as **2** in PBS buffer solution (10 mM PBS:DMSO = 1:1, v/v, pH = 7.4, 25°C) under a 365 nm UV lamp.

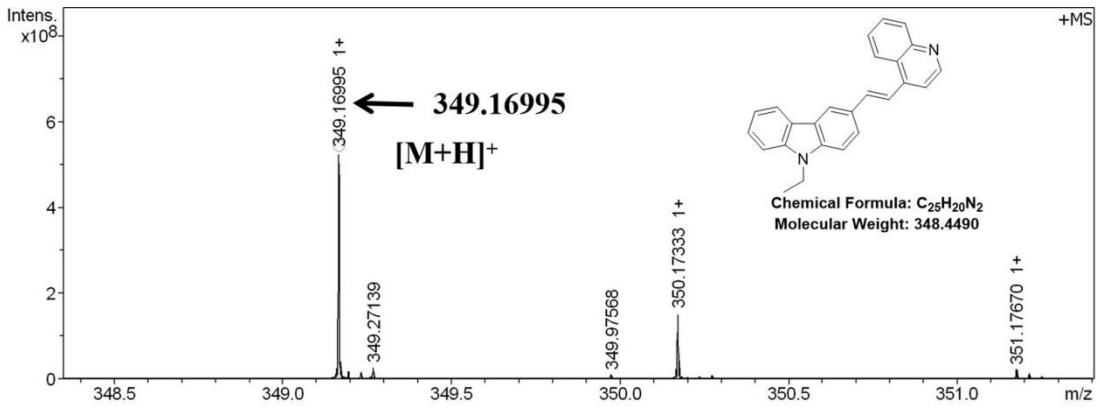


Fig. S9. HRMS spectrum of **1** after addition of Cys.

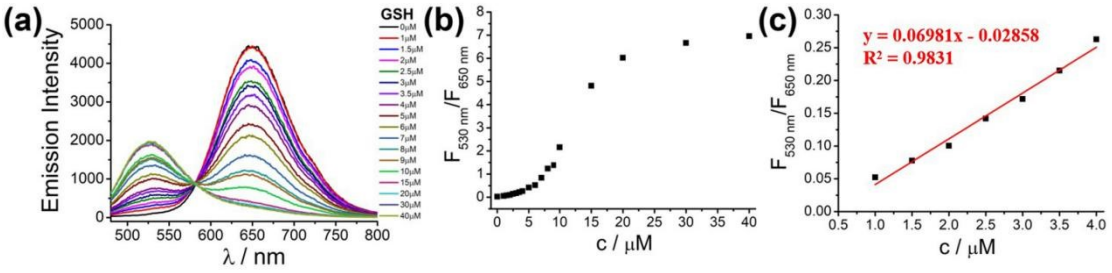


Fig. S10. (a) Fluorescence spectra of **1** (10 μM) after addition of various concentrations of GSH (0, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30 and 40 μM). (b) The ratios of fluorescence intensities ($F_{530\ nm}/F_{650\ nm}$) as a function of the GSH concentration. (c) The linear relationship between the ratios of fluorescence intensities ($F_{530\ nm}/F_{650\ nm}$) and the GSH concentration in the range of 1 - 4 μM. Data were acquired after addition of various concentrations of GSH in PBS buffer solution (10 mM PBS:DMSO = 1:1, v/v, pH = 7.4, 25°C, λ_{ex} = 458 nm).

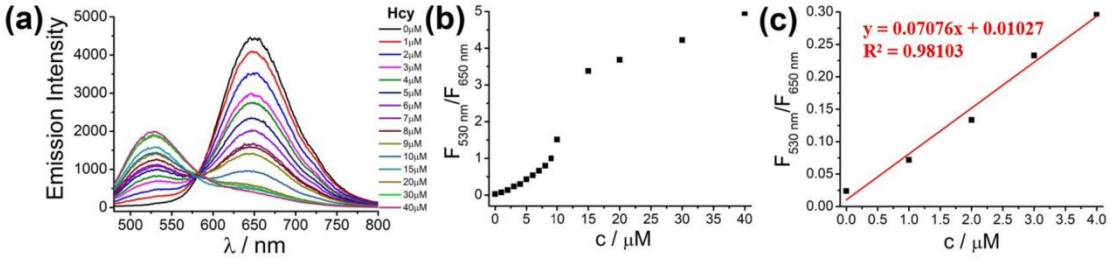


Fig. S11. (a) Fluorescence spectra of **1** (10 μM) after addition of various concentrations of Hcy (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30 and 40 μM). (b) The ratios of fluorescence intensities ($F_{530\ nm}/F_{650\ nm}$) as a function of the Hcy concentration. (c) The linear relationship between the ratios of fluorescence intensities ($F_{530\ nm}/F_{650\ nm}$) and the Hcy concentration in the range of 0 - 4 μM. Data were acquired after addition of various concentrations of Hcy in PBS buffer solution (10 mM PBS:DMSO = 1:1, v/v, pH = 7.4, 25°C, λ_{ex} = 458 nm).

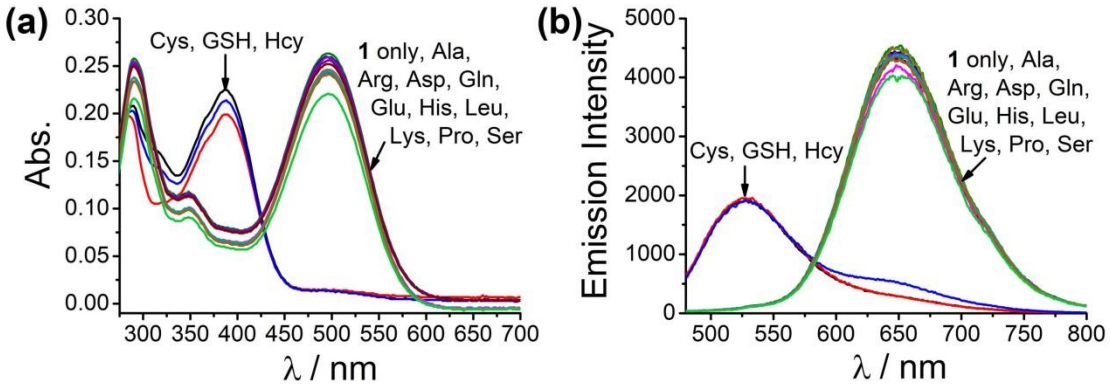


Fig. S12. (a) Absorption spectra of **1** (10 μM) after addition of various analytes (30 μM) in PBS buffer solution (10 mM PBS:DMSO = 1:1, v/v, pH = 7.4, 25°C). (b) Fluorescence spectra of **1** (10 μM) after addition of various analytes (30 μM) in PBS buffer solution (10 mM PBS:DMSO = 1:1, v/v, pH = 7.4, 25°C, λ_{ex} = 458 nm).

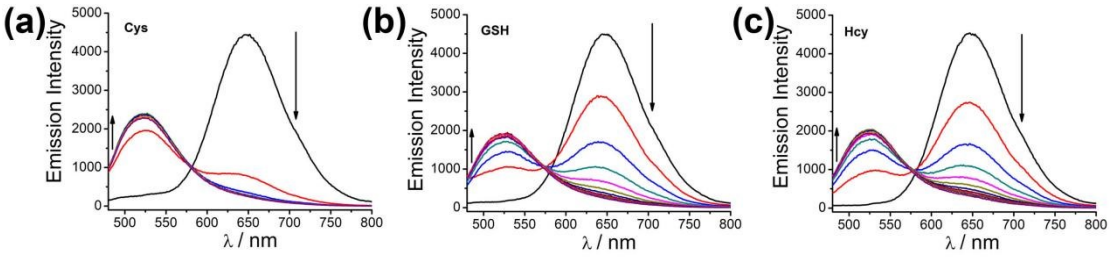


Fig. S13. Fluorescence spectra of **1** (10 μM) after addition of the physiological concentration of three biothiols: (a) Cys (100 μM), (b) GSH (1 mM) and (c) Hcy (10 μM). Data were acquired in PBS buffer solution (10 mM PBS:DMSO = 1:1, v/v, pH = 7.4, 25°C, λ_{ex} = 458 nm) every 1 min within 12 min.

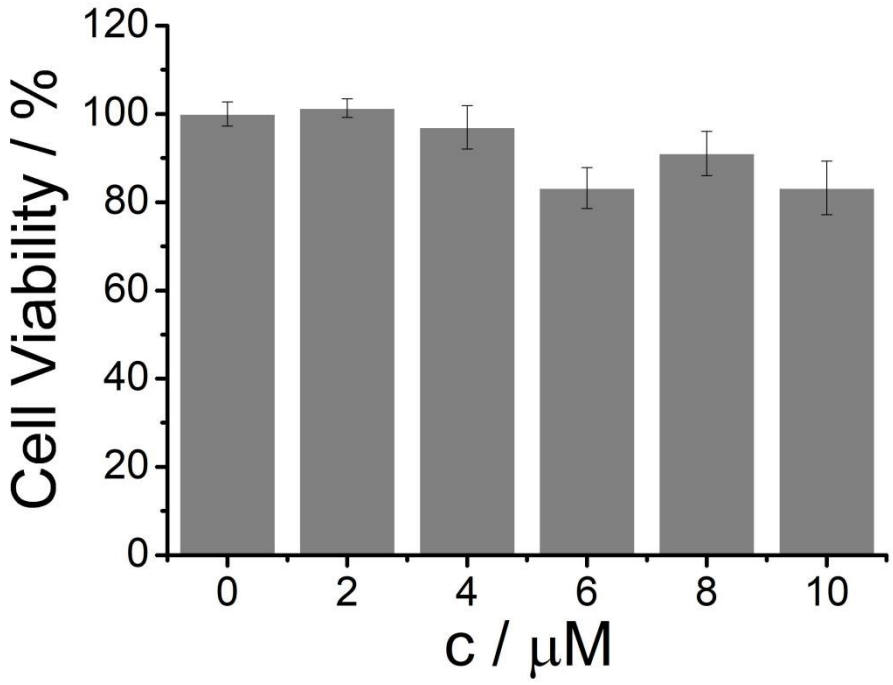


Fig. S14. CCK-8 assay of HeLa cells in the presence of various concentrations of **1** (0, 2, 4, 6, 8, 10 μM) for 12 h at 37°C.

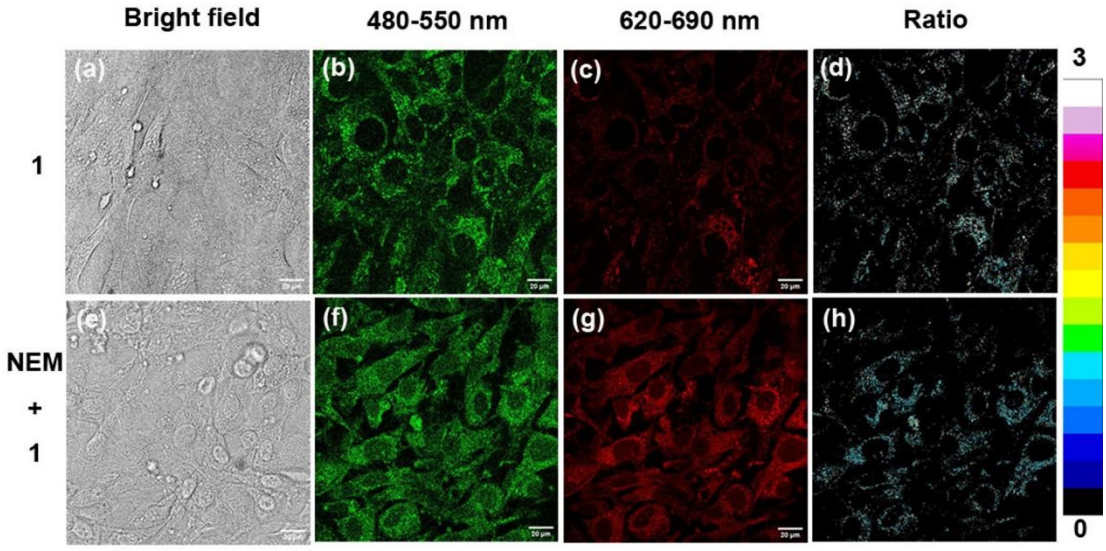


Fig. S15. Fluorescence imaging of biothiols in NIH 3T3 cells with **1**. (a), (b), (c) and (d) Cells were stained with **1** (5 μM) at 37°C for 30 min. (e), (f), (g) and (h) Cells were pretreated with NEM (1 mM) at 37°C for 30 min, then incubated with **1** (5 μM) at 37°C for 30 min. From left to right: Bright field, $Em_{480\text{-}550\ nm}$, $Em_{620\text{-}690\ nm}$, Ratio images of $Em_{480\text{-}550\ nm}/Em_{620\text{-}690\ nm}$. Scale bar: 20 μm, raito bar: 0 - 3, λ_{ex} = 458 nm.

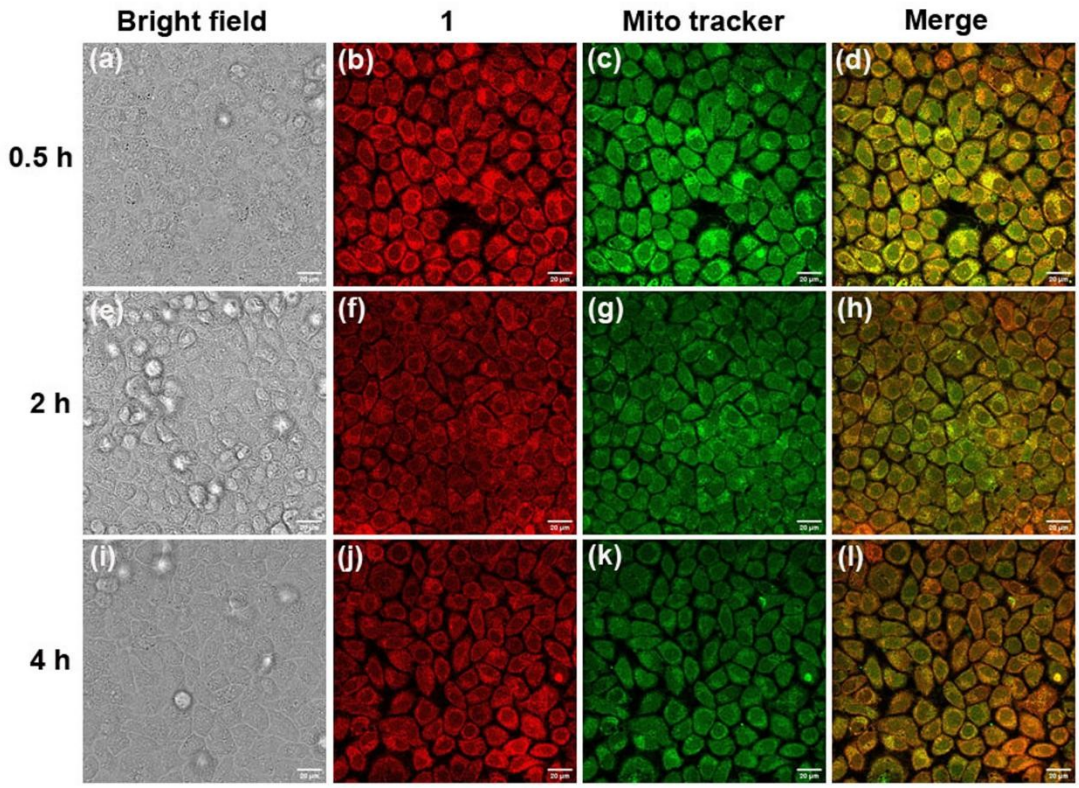


Fig. S16. Fluorescence imaging of HeLa cells co-stained with **1** and Mito-Tracker Green. (a), (e) and (i) Bright-field image; Cells were pretreated with NEM (1 mM) at 37°C for 30 min, then incubated with **1** (5 μM) at 37°C for 0.5 h (b), 2 h (f) and 4 h (j), emission intensities were collected in an optical window 620 - 690 nm, λ_{ex} = 458 nm; Cells were co-stained with Mito tracker Green (500 nM) at 37°C for 0.5 h (c), 2 h (g) and 4 h (k), emission intensities were collected in an optical window 495 - 530 nm, λ_{ex} = 488 nm; (d), (h) and (l) Overlay image of (b) and (c), (f) and (g), (j) and (k), respectively. Scale bar: 20 μm.

Determination of the limit of detection (LOD)

The limit of detection was calculated based on the fluorescence titration. A linear regression curve was fitted according to the fluorescence intensity ratio ($F_{530\ nm}/F_{650\ nm}$) as a function of biothiols (Cys, GSH and Hcy) concentration, and the slope (k) of the curve was obtained. The emission spectrum of **1** (10 μM) in PBS buffer solution (10 mM PBS:DMSO = 1:1, v/v, pH = 7.4, 25°C, λ_{ex} = 458 nm) was collected for 30 times and the standard deviation of blank measurements (δ) was determined. The limit of detection was calculated using the equation.

Limit of detection (LOD) = 3δ/k

Where δ is the standard deviation of the blank measurements; k is the slope of the fluorescence intensity ratio ($F_{530\ nm}/F_{650\ nm}$) versus Cys, GSH and Hcy concentration.

Determination of quantum yields

Fluorescence quantum yields of **1** and **2** were measured using rhodamine B (Φ_f = 0.69 in ethanol) [S13] and fluorescein (Φ_f = 0.85 in 0.1 M NaOH) [S14] as the standards, respectively. The quantum yields of **1** and **2** are calculated according to following equation.

$\Phi_x = \Phi_s(A_s S_x \eta_x^2)/(A_x S_s \eta_s^2)$

where Φ is the fluorescence quantum yield, A is the absorbance at the excitation wavelength, S is the area under the corrected emission curve, and η is the refractive index of the solvent used. Subscripts x and s refer to the unknown and the standard, respectively.

References

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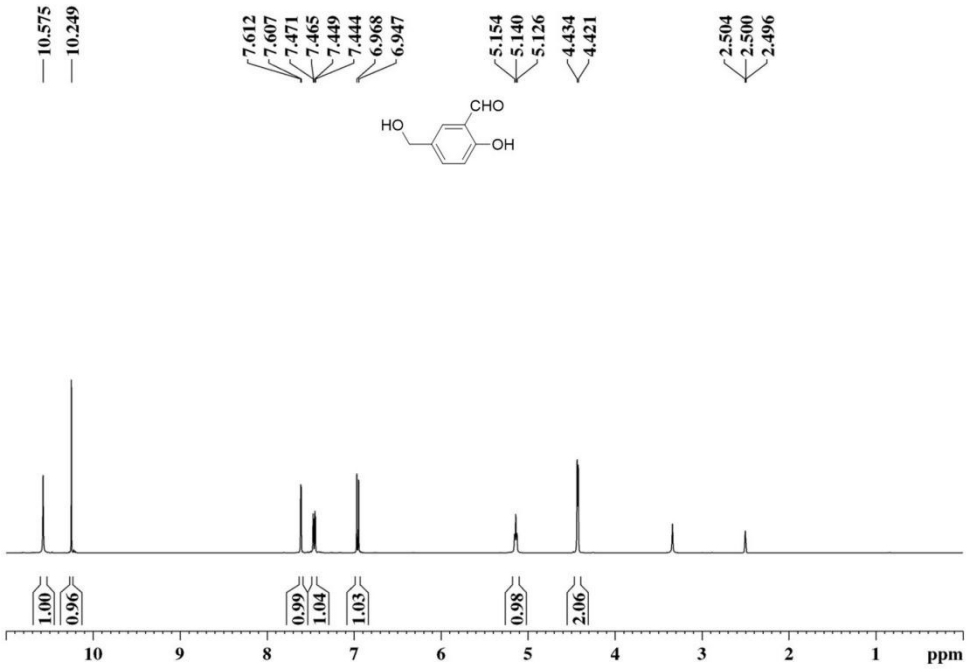
NMR Spectra


10.575
10.249
7.612
7.607
7.471
7.465
7.449
7.444
6.968
6.947
5.154
5.140
5.126
4.434
4.421
2.504
2.500
2.496
CHO
HO
OH
1.00
0.96
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1.03
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2
1
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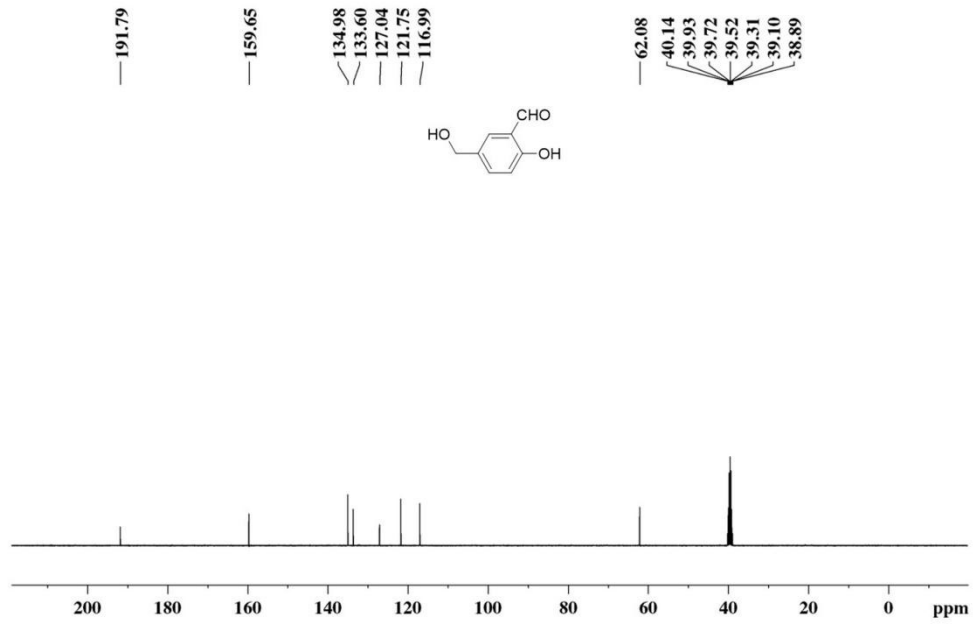
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HO
OH
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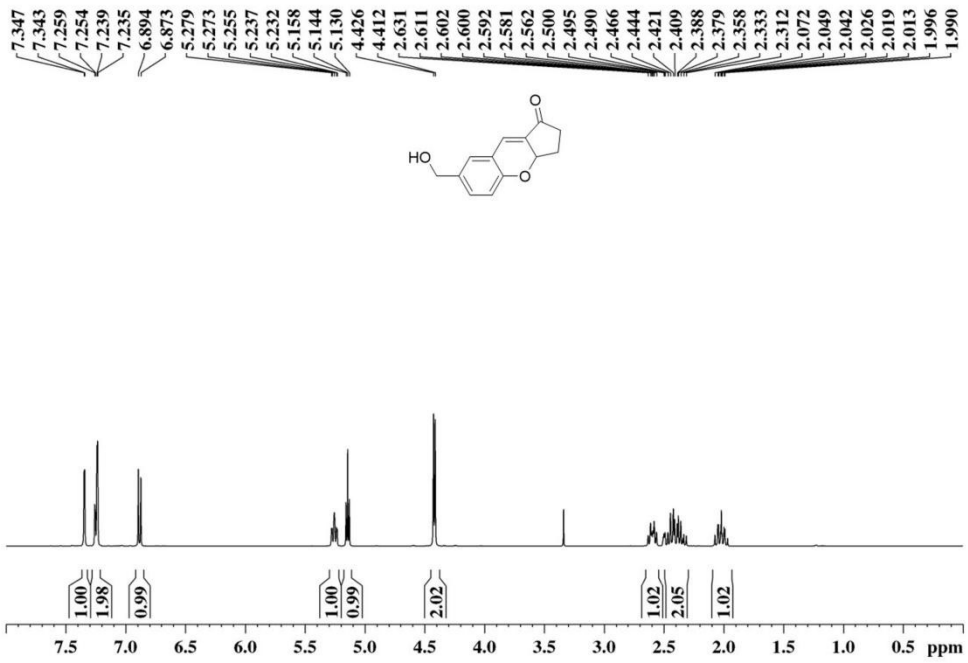
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5.158
5.144
5.130
4.426
4.412
2.631
2.611
2.602
2.600
2.592
2.581
2.562
2.500
2.495
2.490
2.466
2.444
2.421
2.409
2.388
2.379
2.358
2.333
2.312
2.072
2.049
2.042
2.026
2.019
2.013
1.996
1.990
HO
O
O
1.00
1.98
0.99
1.00
0.99
2.02
1.02
2.05
1.02
7.5
7.0
6.5
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3.0
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2.0
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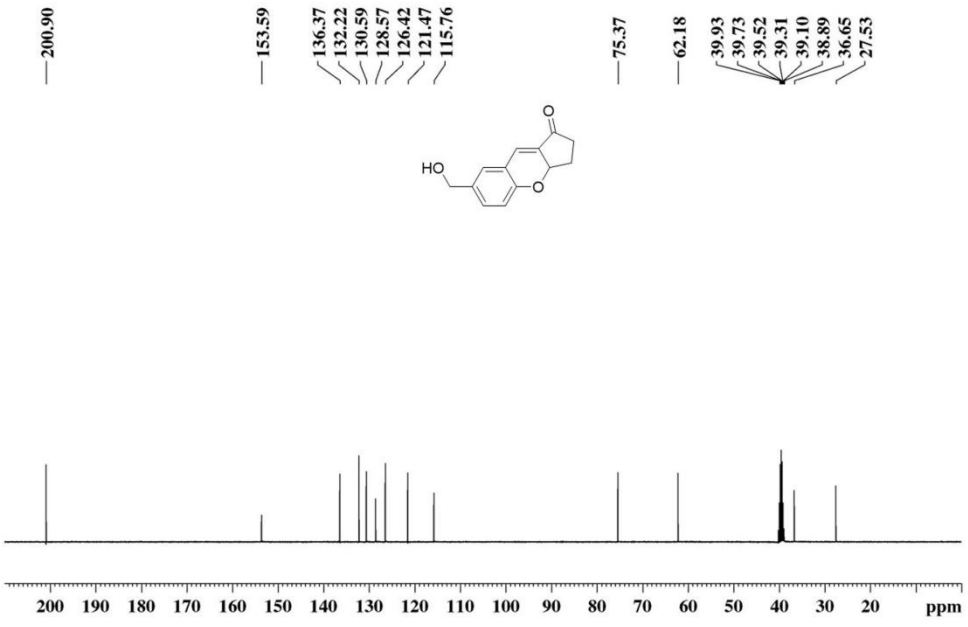
1.0
0.5
ppm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121.47
115.76
75.37
62.18
39.93
39.73
39.52
39.31
39.10
38.89
36.65
27.53
HO
O
O
200
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180
170
160
150
140
130
120
110
100
90
80
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40
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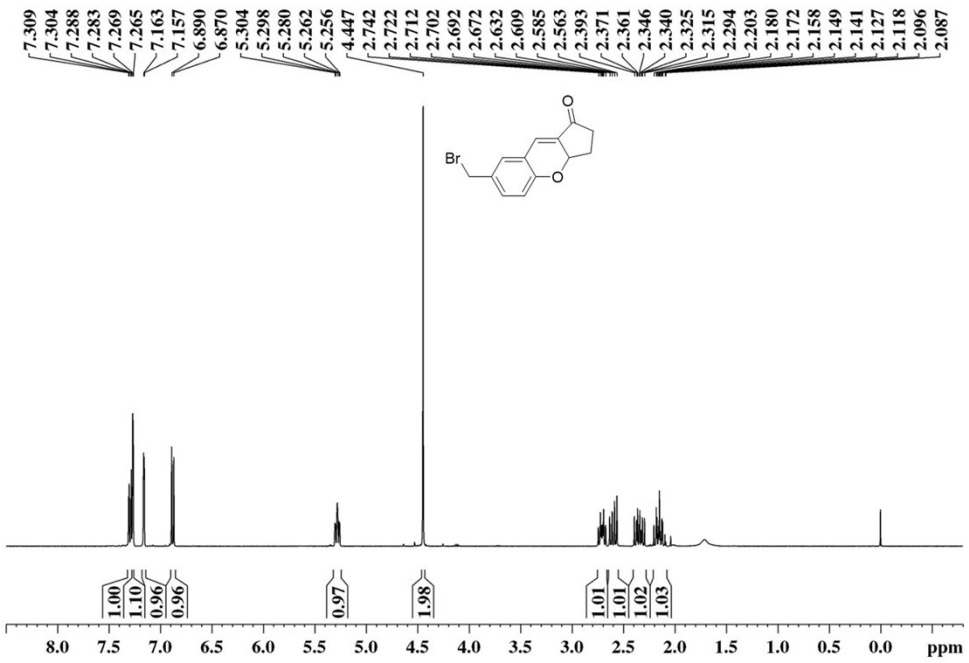
20
ppm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7.283
7.269
7.265
7.163
7.157
6.890
6.870
5.304
5.298
5.280
5.262
5.256
4.447
2.742
2.722
2.712
2.702
2.692
2.672
2.632
2.609
2.585
2.563
2.393
2.371
2.361
2.346
2.340
2.325
2.315
2.294
2.203
2.180
2.172
2.158
2.149
2.141
2.127
2.118
2.096
2.087
O
Br
O
1.00
1.10
0.96
0.96
0.97
1.98
1.01
1.01
1.02
1.03
8.0
7.5
7.0
6.5
6.0
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4.0
3.5
3.0
2.5
2.0
1.5
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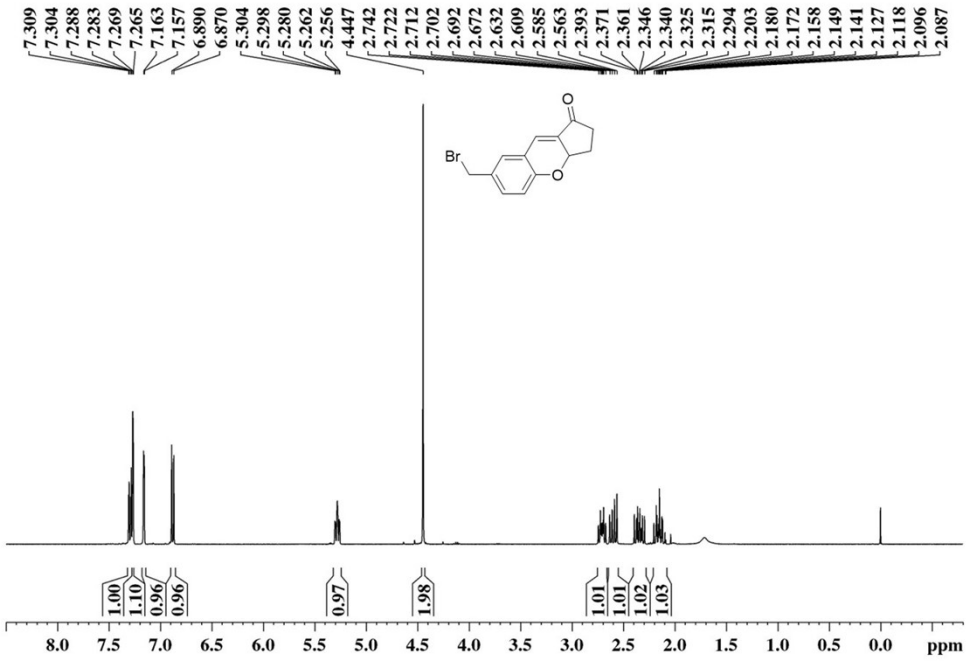
0.0
ppm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4.447
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2.702
2.692
2.672
2.632
2.609
2.585
2.563
2.393
2.371
2.361
2.346
2.340
2.325
2.315
2.294
2.203
2.180
2.172
2.158
2.149
2.141
2.127
2.118
2.096
2.087
O
Br
O
1.00
1.10
0.96
0.96
0.97
1.98
1.01
1.01
1.02
1.03
8.0
7.5
7.0
6.5
6.0
5.5
5.0
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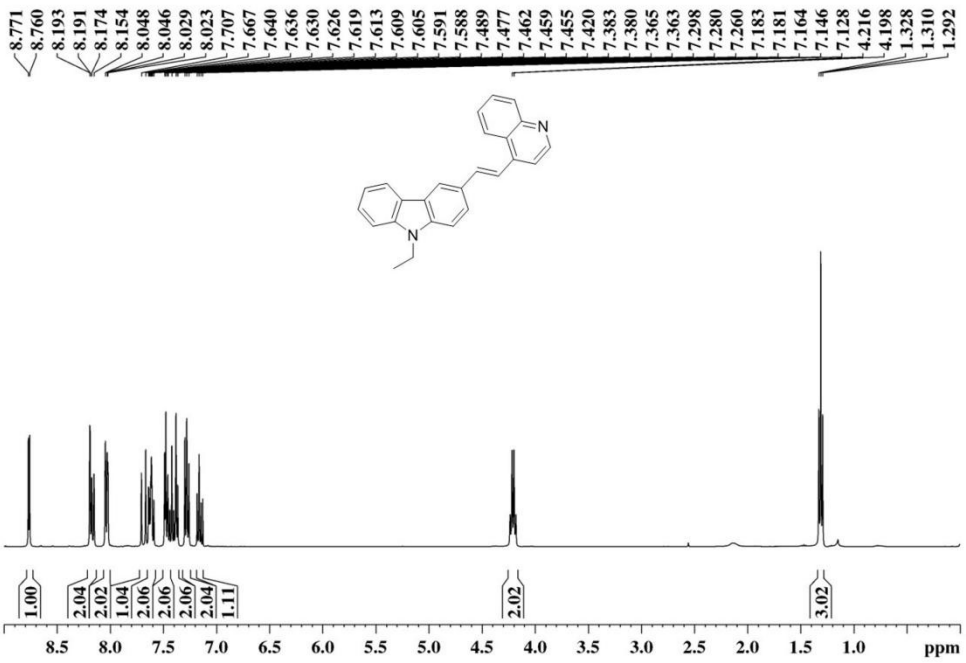
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ppm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8.154
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8.046
8.029
8.023
7.707
7.667
7.640
7.636
7.630
7.626
7.619
7.613
7.609
7.605
7.591
7.588
7.489
7.477
7.462
7.459
7.455
7.420
7.383
7.380
7.365
7.363
7.298
7.280
7.260
7.183
7.181
7.164
7.146
7.128
4.216
4.198
1.328
1.310
1.292
N
N
1.00
2.04
2.02
1.04
2.06
2.06
2.06
2.04
1.11
2.02
3.02
8.5
8.0
7.5
7.0
6.5
6.0
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5.0
4.5
4.0
3.5
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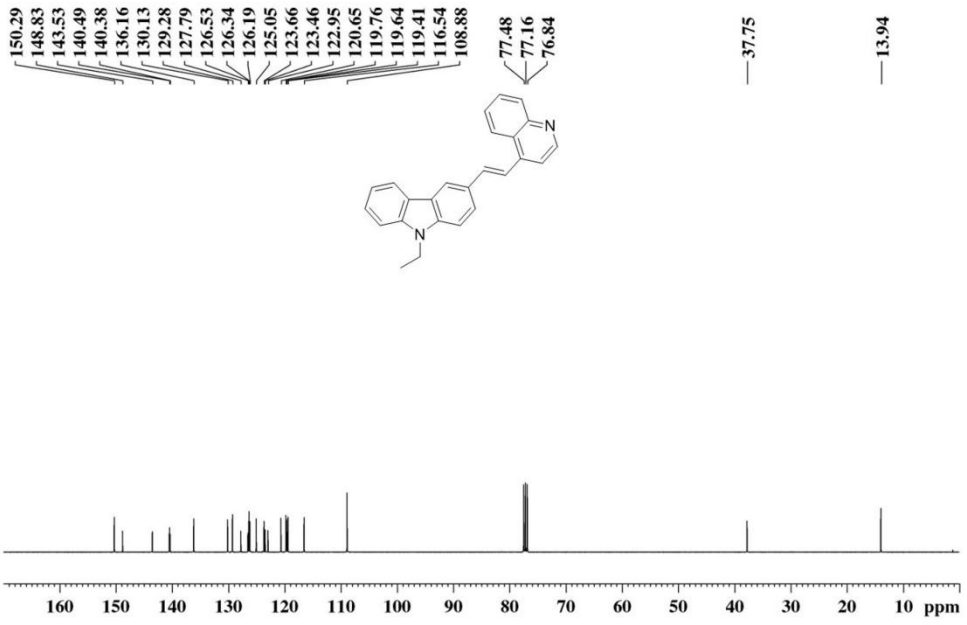
2.5
2.0
1.5
1.0
ppm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143.53
140.49
140.38
136.16
130.13
129.28
127.79
126.53
126.34
126.19
125.05
123.66
123.46
122.95
120.65
119.76
119.64
119.41
116.54
108.88
77.48
77.16
76.84
37.75
13.94
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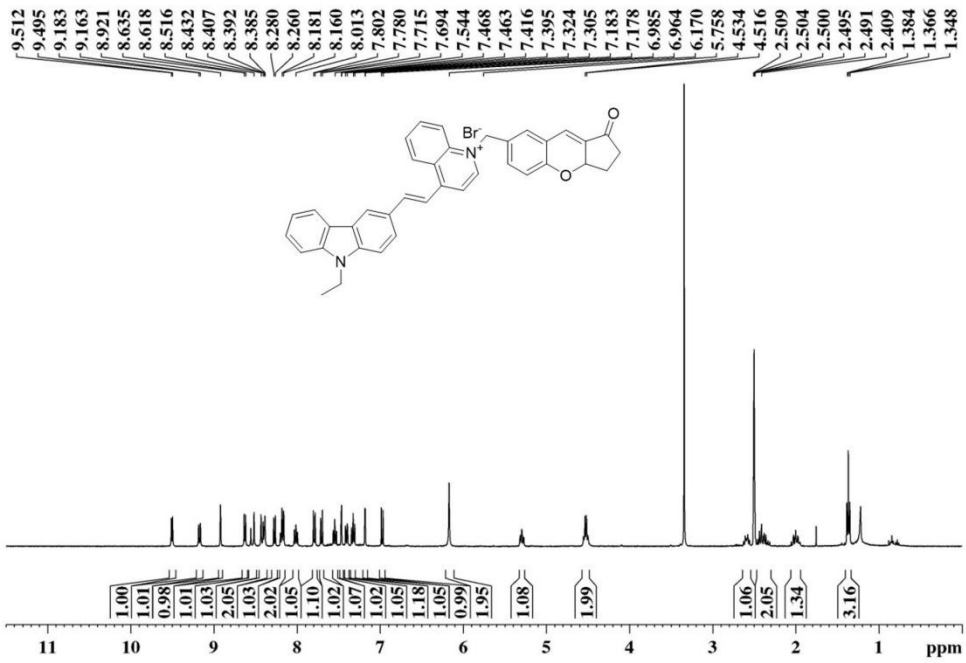
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm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9.495
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9.163
8.921
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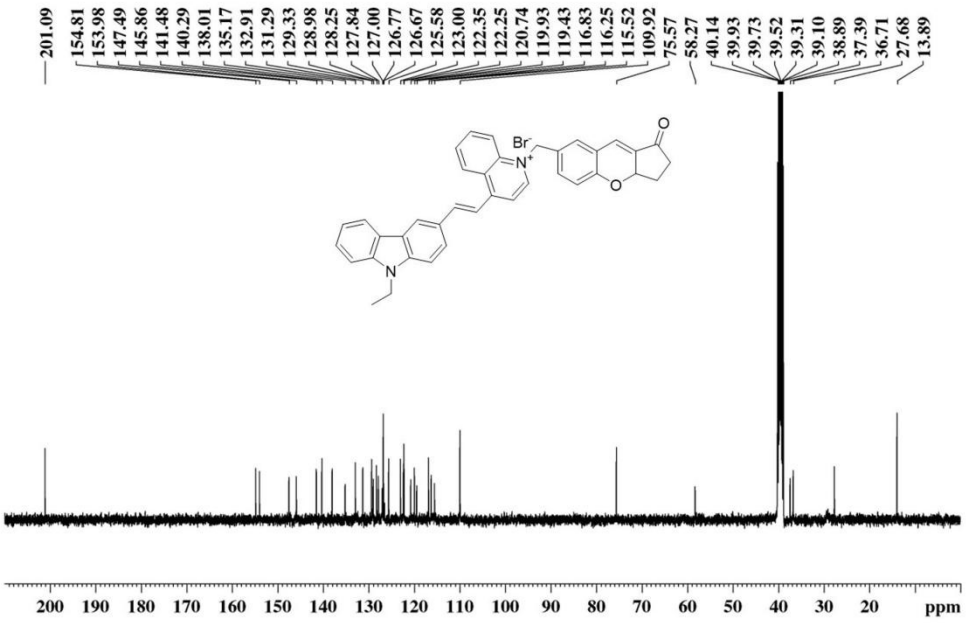
8.516
8.432
8.407
8.392
8.385
8.280
8.260
8.181
8.160
8.013
7.802
7.780
7.715
7.694
7.544
7.468
7.463
7.416
7.395
7.324
7.305
7.183
7.178
6.985
6.964
6.170
5.758
4.534
4.516
2.509
2.504
2.500
2.495
2.491
2.409
1.384
1.366
1.348
Br-
N+
O
O
N
1.00
1.01
0.98
1.01
1.03
2.05
1.03
2.02
1.05
1.10
1.02
1.07
1.02
1.05
1.18
1.05
0.99
1.95
1.08
1.99
1.06
2.05
1.34
3.16
11
10
9
8
7
6
5
4
3
2
1
ppm


201.09
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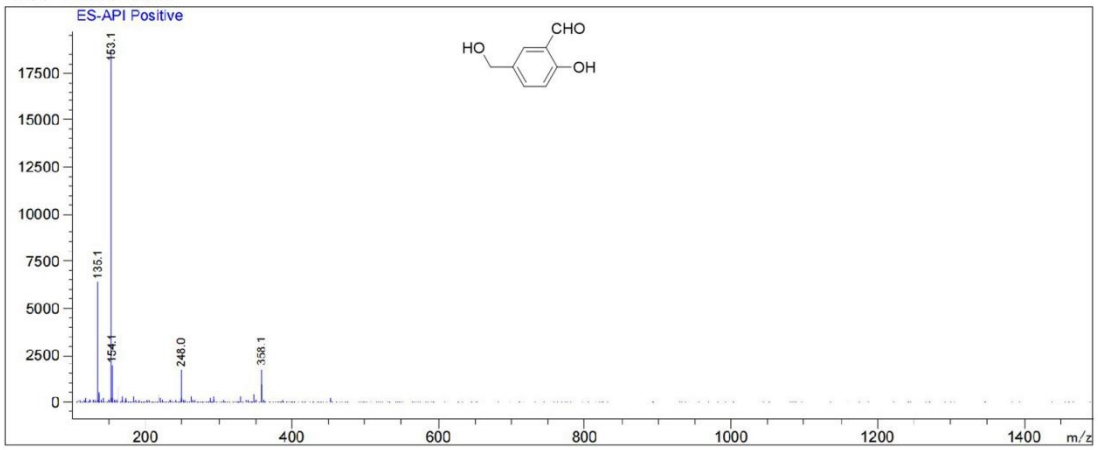
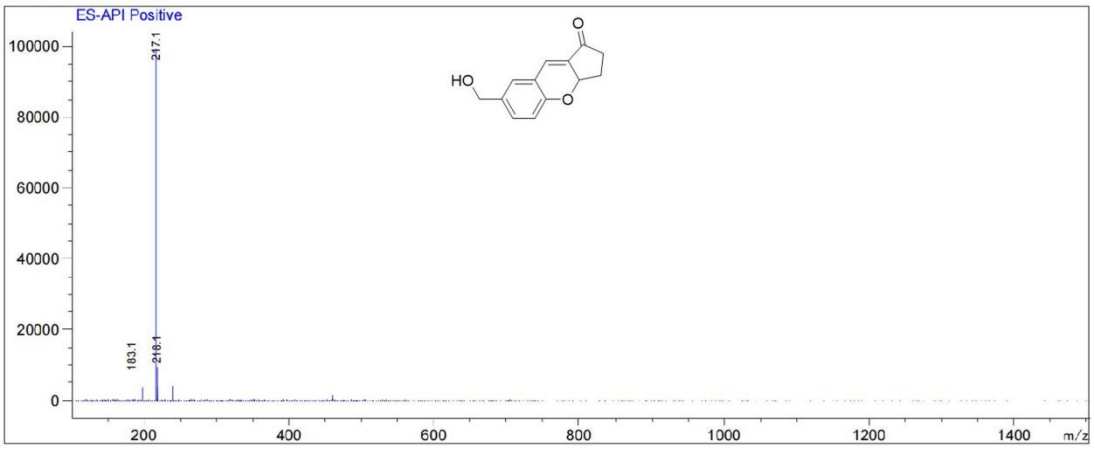
141.48
140.29
138.01
135.17
132.91
131.29
129.33
128.98
128.25
127.84
127.00
126.77
126.67
125.58
123.00
122.35
122.25
120.74
119.93
119.43
116.83
116.25
115.52
109.92
75.57
58.27
40.14
39.93
39.73
39.52
39.31
39.10
38.89
37.39
36.71
27.68
13.89
Br-
N+
O
O
N
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm

MS Spectra

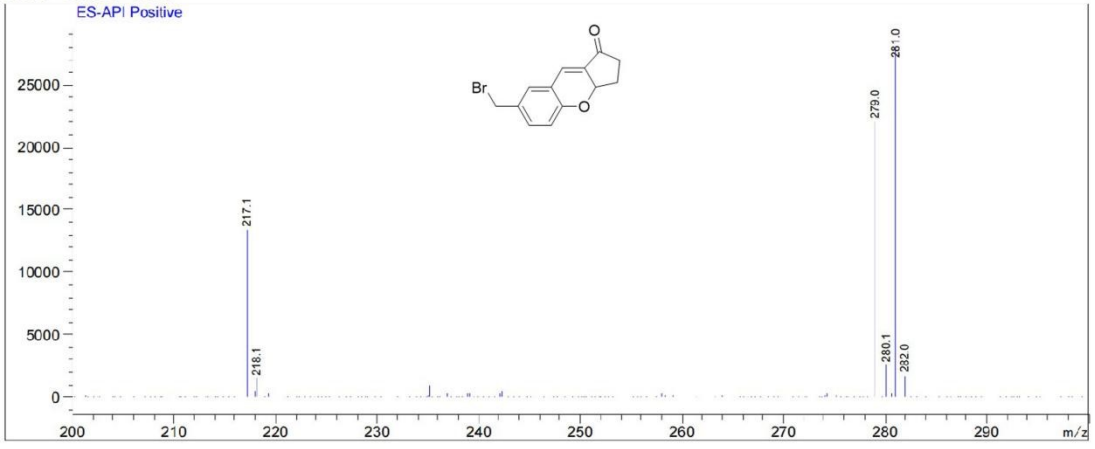
Ret. Time: 1.22



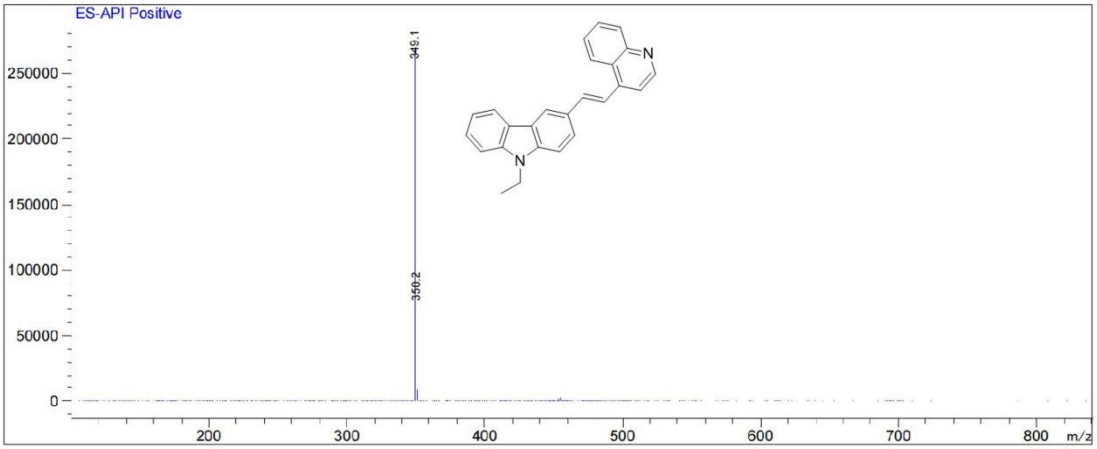
Ret. Time: 2.89



Ret. Time: 4.52



Ret. Time: 4.85


ES-API Positive
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350.2
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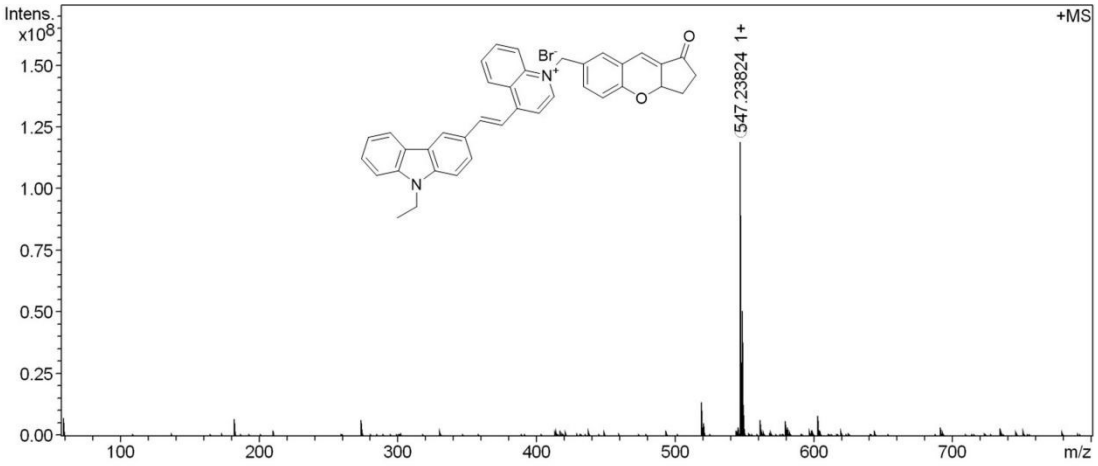
200000
150000
100000
50000
0
200
300
400
500
600
700
800
m/z
N


Intens.
x10^8
1.50
1.25
1.00
0.75
0.50
0.25
0.00
100
200
300
400
500
600
700
m/z
+MS
547.23824 1+
Br
N+
O
N