Electronic Supplementary Information

Orchestration of dual cyclization processes and dual quenching mechanisms for enhanced selectivity and drastic fluorescence turn-on detection of cysteine

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General Information

Commercial reagents were purchased from commercial suppliers and used as received, unless otherwise stated.

$^1$H and $^{13}$C NMR spectra were recorded on Bruke DRX 400 (400 MHz). Data for $^1$H are reported as follows: chemical shift (ppm), and multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet). Data for $^{13}$C NMR are reported as ppm. Mass Spectra were obtained from East China University of Science and Technology LC-Mass spectral facility. HPLC-MS was performed using Waters e2695 using gradient elution of MeCN and H$_2$O with a flow rate 0.8 mL/min. UV-Vis spectra were collected on a Shimadzu UV-1800 spectrophotometer. Fluorescence spectra were collected on a FluoroMax-4 (Horiba Scientific) fluorescence spectrophotometer with slit widths were set at 2 nm both for excitation and emission. The pH measurements were carried out with a FE20 plus (Mettler Toledo) pH meter.
Part I: Probe Design:

a) Aldehyde

Fluorophore

Cys/Hcy

imine formation

Fluorophore

S

O

Ar

Fluorophore

S

O

NH

S

CO

2-

O

+  

Fluorophore

O

O

S

H

2

N

CO

2-

NH

2

CO

2-

Fluorophore

H

N

O

CO

2-

SH

transthioesterfication

Michael

Addition

thiol addition to imine

Fluorophore

5-endo-trig for Cys

6-endo-trig for Hcy

b) Acrylate

Fluorophore

Cys/Hcy

Michael

Addition

Fluorophore

S

O

NH

S

CO

2-

OH

Fluorophore

Cys/Hcy

N

S

O

NH

S

CO

2-

SH

7-exo-trig for Cys

8-exo-trig for Hcy

c) Thioester

Fluorophore

transthioesterification

S, S-acyl shift

Fluorophore

5-exo-trig for Cys

6-exo-trig for Hcy

d) Maleimide

Fluorophore

Cys

Michael

Addition

Fluorophore

transcyclization

N, N-acyl shift

Fluorophore

major product

5-exo-trig for Cys

7-exo-trig for Hcy

e) Electron-deficient aromatic halide

Fluorophore

Cys/Hcy

S,S-Ar

intramolecular S,S-Ar

S, N-Ar shift

Fluorophore

5-exo-trig for Cys

6-exo-trig for Hcy

Scheme S1. Aminothiol recognition groups with different cyclization processes.
1) Acrylate-based Cys-selective fluorescent probes (mainly ICT sensing mechanism)

2) Maleimide-based thiol-selective fluorescent probes (mainly PET quenching mechanism)

This work: dual reactive groups with dual ICT and PET quenching effect as well as two cyclization strategies for improved selectivity

Scheme S2. Probe design base on dual cyclization processes and dual PET and ICT quenching mechanisms of the acrylate and the maleimide groups.
Part II Synthetic Procedures and Structural Determinations:

2.1 Synthesis of the probe 1

3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-2-oxo-2H-chromen-7-yl acrylate (1)

A solution of acryloyl chloride (113 μL, 1.4 mmol) in dichloromethane (3 mL) was added dropwise to a solution of compound 5 (193 mg, 0.7 mmol) and triethylamine (700 μL) in dichloromethane (15 mL) in an ice-bath. After stirring for 2 h at room temperature, water (20 mL) was added to the reaction mixture. The organic phase was washed with water, then dried over Na₂SO₄. After the solvent was evaporated under reduced pressure, the crude solid was purified by silica gel column chromatography with petroleum ether / ethyl acetate (1:1, v/v) as the eluent followed by recrystallized from a mixture of dichloromethane and n-hexane to give pure compound 1 as white solid (201 mg, 92% yield). "H NMR (500 MHz, CDCl₃): δ 7.76 (s, 1H), 7.57 (d, J = 8.4 Hz, 1H), 7.25 (s, 1H), 7.18 (d, J = 8.4 Hz, 1H), 6.91 (s, 2H), 6.66 (d, J = 17.3 Hz, 1H), 6.34 (dd, J = 17.3, 10.4 Hz, 1H), 6.10 (d, J = 10.4 Hz, 1H); "C NMR (125 MHz, CDCl₃): δ 168.3 (2C), 163.7, 157.0, 154.3, 154.1, 142.2, 135.1(2C), 134.1, 129.4, 127.3, 119.2, 118.1, 116.1, 110.6. EI-MS: m/z [M]+ calcd for C₁₆H₉NO₆: 311.0430, found: 311.0429.

2.2 Synthesis of the reference probe 2a and product 3a
To a solution of probe 1 (31 mg, 0.1 mmol) in THF (6 mL) was added dropwise a solution of 2-aminoethane-1-thiol (8 mg, 0.1 mmol) in MeOH (1 mL) and triethylamine (20 μL). The resulting solution was stirred at room temperature for 5 minutes. The mixture was then condensed in vacuo to remove solvents, and directly purified by silica gel flash column chromatography (petroleum ether / ethyl acetate = 1 / 2) to give 2a as white solid (25 mg, 64% yield). ¹H NMR (400 MHz, DMSO-d₆) δ 9.93 (s, 1H), 8.65 (s, 1H), 8.00 (bs, 1H), 7.76 (d, J = 8.5 Hz, 1H), 7.20 (d, J = 8.5, 1.5 Hz, 1H), 6.57 (d, J = 17.3 Hz, 1H), 6.44 (dd, J = 17.3, 10.4 Hz, 1H), 6.20 (d, J = 10.4 Hz, 1H), 3.92 (t, J = 6.8 Hz, 1H), 3.54-3.38 (m, 2H), 3.07 (dd, J = 15.8, 6.8 Hz, 1H), 2.91(dt, J = 11.5, 3.9 Hz, 1H), 2.85-2.77 (m, 1H), 2.74 (dd, J = 15.8, 6.8 Hz, 1H); ¹³C NMR (125 MHz, DMSO-d₆) δ 170.4, 169.8, 163.9, 157.2, 150.7, 150.0, 134.2, 128.6, 127.4, 124.1, 123.3, 119.0, 117.6, 109.7, 41.7, 37.2, 36.2, 26.5; ESI-HRMS: m/z [M+Na]⁺ calcd for C₁₈H₁₆N₂O₆SNa: 411.0627, found: 411.0622.

To a solution of probe 1 (31 mg, 0.1 mmol) in THF (6 mL) was added dropwise a solution of β-mercaptoethylamine (β-MEA, cysteamine) (16 mg, 0.2 mmol) in MeOH (1 mL) and triethylamine (20 μL). The resulting solution was stirred at room temperature for 5 minutes. The mixture was then condensed in vacuo to remove solvents, and directly purified by silica gel flash column chromatography (petroleum ether / ethyl acetate = 1 / 2) to give 3a as white solid (28 mg, 84% yield). ¹H NMR (400 MHz, DMSO-d₆) δ 10.38 (s, 1H), 9.73 (s, 1H), 8.53 (s, 1H), 7.98 (t, J = 4.2 Hz, 1H), 7.51 (d, J = 8.6 Hz, 1H), 6.79 (dd, J = 8.6, 2.2 Hz, 1H), 6.73 (d, J = 2.2 Hz, 1H), 3.89 (t, J = 6.8 Hz, 1H), 3.53-3.39 (m, 2H), 3.02 (dd, J = 15.7, 6.6 Hz, 1H), 2.90 (dt, J = 12.0, 4.1 Hz, 1H), 2.80 (m, 1H), 2.69 (dd, J = 15.7, 7.0 Hz, 1H); ¹³C NMR (100 MHz, DMSO-d₆) δ 170.0, 169.8, 159.6, 157.8, 151.5, 129.0, 125.7, 120.9, 113.7, 111.4, 102.0, 41.7, 37.1, 36.3, 26.4; ESI-HRMS: m/z [M+Na]⁺ calcd for C₁₅H₁₄N₂O₅SNa: 357.0521, found: 357.0519.

2.3 Synthesis of the reference probe 6

Scheme S5 Synthesis of the reference probe 6.

A solution of acetyl chloride (15 μL, 0.21 mmol) in dichloromethane (1 mL) was added dropwise to a solution of compound 5 (28 mg, 0.10 mmol) and triethylamine (75 μL, 0.54 mmol) in dichloromethane (2 mL) in an ice-bath. After stirring for 2 h at room temperature, water (5 mL) was added to the reaction mixture. The organic phase was washed with water, then dried over anhydrous Na₂SO₄. After the solvent was evaporated under reduced pressure, the crude solid was purified by silica gel column chromatography using petroleum ether / ethyl acetate (1:1, v/v) as the eluent followed by recrystallization from a mixture of dichloromethane and n-hexane to give compound 6 as white solid (23 mg, 76% yield). ¹H NMR (400 MHz, CDCl₃) δ 7.74 (s, 1H), 7.55 (d, J = 8.5 Hz, 1H), 7.19 (d, J = 2.0 Hz, 1H), 7.13 (dd, J = 8.5, 2.0 Hz, 1H), 6.90 (s, 2H), 2.35 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 168.6, 168.3(2C), 157.0, 154.2, 154.1, 142.2, 135.1(2C), 129.4, 119.2, 118.0, 116.1, 110.6, 21.3. ESI-HRMS: m/z [M+Na]⁺ calcd for C₁₅H₉NO₅Na: 322.0328, found: 322.0321.
2.4 Synthesis of the reference probe 7

A solution of 3-amino-7-methoxy-2H-chromen-2-one (8) (prepared according to the literature) (76 mg, 0.40 mmol) and maleic anhydride (49 mg, 0.50 mmol) in glacial acetic acid (5 mL) was stirred at room temperature overnight. After filtration, the yellow solid was washed by ethyl acetate to give compound 9 which was used without further purification for the next step.

The acid 9 was suspended in acetic anhydride (6 mL), and anhydrous sodium acetate (41 mg, 0.50 mmol) was added. The resulting mixture was stirred at 75 °C for 3 hours and then cooled to room temperature. The solvent was then removed in vacuo, and the residue was directly purified by silica gel flash column chromatography (petroleum ether / dichloromethane = 1 / 2) to give compound 7 as pale yellow solid (65 mg, 60% yield). 

\[ ^1H\text{ NMR (400 MHz, CDCl}_3\right) \delta 7.69 \text{ (s, 1H), 7.42 (d, } J = 8.6 \text{ Hz, 1H), 6.90 (dd, } J = 8.6, 2.3 \text{ Hz, 1H), 6.89 (s, 2H), 6.86 (d, } J = 2.3 \text{ Hz, 1H), 3.89 (s, 3H).} \]

\[ ^13C\text{ NMR (100 MHz, CDCl}_3\right) \delta 168.7 \text{ (2C), 163.9, 157.8, 155.7, 143.1, 135.0 (2C), 129.6, 115.1, 113.6, 111.9, 100.9, 56.1.} \]

ESI-HRMS: m/z [M+H]^+ calcd for C_{14}H_{10}NO_5: 272.0559, found 272.0558.
Part III: Fluorescence and UV-vis Spectroscopy Studies

3.1 Spectroscopic materials
All aqueous solutions were prepared using double distilled water. Enantiomerically pure natural amino acids were used in all experiments, except for Hcy. All solutions of the biological analytes were freshly prepared (100 µM in distilled water, unless otherwise stated) and used within 12 hours. Probe 1 stock solution (1 mM in dry DMSO) was prepared and stored at -20 °C. All fluorescence and absorption spectroscopic measurements were performed in 10 mM phosphate buffer pH = 7.4 at 25 °C unless otherwise stated. Samples for absorption and fluorescence measurements were contained in 1 cm×1 cm quartz cuvettes (3.5 mL volume).

3.2 Fluorescence titration experiment for determination of the suitable concentration of probe 1 and Cys for further fluorescence studies.
Since the fluorimeter has a linear detection range between 0~ 2000000 cps, we mandatory chose 1 µM probe 1 for in vitro fluorescence studies. Fluorescence titration experiment of 1 µM of probe 1 with increasing amount of Cys determined that a minimum 40 µM of Cys was required for generating a maximum fluorescence response in 30 mins (Figure S1).

![Fluorescence spectra](image)

**Figure S1** Fluorescence spectra ($\lambda_{ex}$=340 nm) of probe 1 (1 µM) towards increasing concentration of Cys (0, 5, 10, 15, 20, 25, 30, 35, 40, 50 µM). (All measurement was taken in 10 mM PBS buffer (pH 7.4) at 25 °C with $\lambda_{ex}$=340 nm and incubation time = 30 min).
3.3 Time-dependent fluorescence emission studies

(a) Time-dependent (0 to 30 min) fluorescence intensity of probe 1 (1 µM) and probe 1 (1 µM) upon addition of 40 equiv. of Cys, Hcy, GSH. NAC at 476 nm.

(b) Time-dependent (0 to 60 min) fluorescence intensity of probe 1 (1 µM) and probe 1 (1 µM) upon addition of 40 equiv. of Cys, Hcy, GSH. NAC at 476 nm.

(c) Time-dependent fluorescence intensity of compound 2a (1 µM) and compound 2a (1 µM) upon addition of 40 equiv. of Cys, Hcy, GSH. NAC at 476 nm.

(d) Time-dependent fluorescence intensity of compound 6 (1 µM) and compound 6 (1 µM) upon addition of 40 equiv. of Cys, Hcy, GSH. NAC at 476 nm.

(e) Time-dependent fluorescence intensity of compound 7 (1 µM) and compound 7 (1 µM) upon addition of 40 equiv. of Cys, Hcy, GSH. NAC at 476 nm. (All measurement was taken in 10 mM PBS buffer, pH=7.4 at 25 °C with λ<sub>ex</sub>=340 nm).

Figure S2
3.4 Normalized fluorescence excitation and emission spectra of probe 1 upon incubation of Cys.

![Normalized fluorescence excitation and emission spectra of probe 1 upon incubation of Cys](image)

**Figure S3** Normalized fluorescence excitation ($\lambda_{em} = 476$ nm) and emission ($\lambda_{ex} = 340$ nm) spectra of probe 1 (1 $\mu$M) in the presence of Cys (40 $\mu$M) in PBS buffer (10 mM, pH 7.4).

3.5 Comparison of normalized fluorescence emission spectrum of probe 1 after incubation with Cys with normalized fluorescence emission compound 3a.

![Normalized fluorescence emission spectra of probe 1 with Cys and compound 3a](image)

**Figure S4** Normalized fluorescence emission ($\lambda_{ex} = 340$ nm) spectra of probe 1 (1 $\mu$M) upon treatment of Cys (40 $\mu$M) and the compound 3a (1 $\mu$M). (All measurements were done in 10 mM PBS buffer (pH 7.4) at 25 °C with incubation time = 30 min).
3.6 Determination of detection limit of probe 1 towards Cys

The detection limit of probe 1 (1 µM) towards Cys was derived from the fluorescence titration experiment (476 nm, excited at 340 nm) with increasing amount of Cys (0-5.0 µM) as show in Figure S5. An excellent linear relationship (R² = 0.9944) was obtained at Cys concentrations between 0 and 5 µM. And then the fluorescence intensity data of free probe 1 (1 µM) at 476 nm were collected ten times as the blank measurements for calculation of the standard deviation. The detection limit was calculated using the following the equation: detection limit = 3σbi/m, where σbi is the standard deviation of the blank measurements (σbi = 183.11); m is the slope obtained from linear regression of fluorescence intensity at 476 nm versus Cys concentration. The detection limit was calculated to be 13.7 nM at S/N = 3.

![Figure S5](image)

**Figure S5** A linear relationship between fluorescence intensity at 476 nm and Cys concentration for probe 1 (incubation time = 30 min at 25 °C).

3.7 Time-dependent UV-Vis spectra of probe 1 with N-acetyl cysteine (NAC)

![Figure S6](image)

**Figure S6.** Time-dependent absorption spectra of probe 1 (50 µM) upon addition of NAC (2 mM) (All measurements were taken in 10 mM PBS buffer solution at pH = 7.4 with incubation time of 30 min).
3.8 Time-dependent UV-vis spectra of reference probe 2a with NAC

**Figure S7.** Time-dependent absorption spectra of the reference probe 2a (50 µM) upon addition of NAC (2 mM) (All measurements were taken in 10 mM PBS buffer solution at pH = 7.4 with incubation time of 30 min).
3.9 Comparison of UV-vis spectra of probe 1 with 40 equiv. of Cys at 4 min with NAC at extended time as well as the reference probe 2a with NAC at extended time

The purpose of comparison of UV-vis spectra was to determine the intermediate formed at the time point of 4 min when probe 1 reacted with 40 equiv. of Cys. Due to the reported pKa value of the thiol group of Cys and NAC were 8.22 and 9.5, respectively, the reaction of thiol-Michael addition of Cys should be faster than NAC at pH=7.4. Therefore, NAC needs significant more reaction time for thiol-Michael addition to complete.

Scheme S7 The reaction of probe 1 and probe 2a with NAC and potential intermediates of probe 1 with Cys at time point of 4 min.

Figure S8. Comparison of UV-Vis spectra of probe 1 with 40 equiv. of Cys at 4 min (black line) with NAC at extended time (red line) as well as the reference probe 2a with NAC at extended time (blue line). From the fact that the black line more resembled to the red line, we expected the intermediate was structurally close to the product 10 in Scheme S7.
3.10 Determination of fluorescence quantum yields of 1, 2a, and 3a

The quantum yield of 1, 2a and 3a were determined according to the literature. Quinine sulfate with 0.1 M H₂SO₄ was chosen as standard (ϕ = 0.557, λₑₓ = 350 nm). The quantum yields of 1, 2a, and 3a were measured in distilled water with 5% DMSO, λₑₓ = 350 nm. Fluorescence quantum yields were obtained with the following equation. Where ϕ is quantum yield; I is integrated area under the corrected emission spectra; A is absorbance at the excitation wavelength; η is the refractive index of the solution; the subscripts s and b refer to the sample and the standard, respectively.

$$\phi_s = \frac{\phi_b I_s A_b \eta_s}{I_b A_s \eta_b}$$

Figure S9 Fluorescence quantum yields of 1, 2a, and 3a.

3.11 Selectivity studies.

Figure S10 Fluorescence response of probe 1 (1 µM) at 476 nm (λₑₓ = 340 nm) toward various species in PBS buffer (10 mM, pH 7.4) including 40 equiv. aminothiols (Cys, Hcy, GSH), 400 equiv. different amino acids (Gly, Lys, Arg, His, Tyr, AsP, Thr, Trp, Glu, Pro), 400 equiv. metal ions (K⁺, Ca²⁺, Na⁺, Mg²⁺, Zn²⁺, Fe³⁺), 400 equiv. H₂O₂, and 400 equiv. glucose. The control group was the probe 1 (1 µM) alone.
3.12 pH-dependence studies.

Figure S11 pH-dependent fluorescence intensity at 476 nm ($\lambda_{ex} = 340$ nm) for probe 1 (1 µM), reference product 3a, and probe 1 after incubation with 40 equiv. of Cys at pH 7.4 for 30 min at various pHs from 4-10.
Part IV: HPLC-MS and $^1$HNMR Studies of probe 1 with Cys-OMe

4.1 HPLC-MS studies of probe 1 incubated with cysteine methyl ester

The solution of probe 1 (50 μM) was incubated with or without L-Cys-OMe.HCl (2 mM) in a mixture of MeCN and PBS buffer (10 mM, pH 7.4, 2:8 v/v) for 30 min. After reaction, the solution was filtered through a syringe filter before analyzed by HPLC-MS.

Scheme S8 Model reaction of probe 1 with cysteine methyl ester.

Figure S12 LC-MS profiles of a) probe 1, b) probe 1 with L-Cys-OMe, incubation time = 30 min. (LC peaks were monitored by UV absorption at 254 nm and MS was detected in ESI positive mode.)
4.2 $^1$HNMR studies of probe 1 incubated with cysteine methyl ester

Figure S13 a) $^1$HNMR spectrum of 3b containing a mixture of two diastereomers in a ratio about 3:2 (integration ratio from the peak at δ 8.07 to the peak at 8.16); b) comparison of aromatic region (δ 8.4-6.0) of $^1$HNMR spectrum of 3b with that of the probe 1; c) comparison of aliphatic region (δ 4.6-2.6) of $^1$HNMR spectrum of 3b with that of the Cys-OMe.
Part V: Biological Applications

Cell culture: HeLa cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Glutamine, maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO₂.

5.1 Cytotoxicity assay of the probe 1

Cell survival was evaluated by the MTS assay (CellTiter 96 AQueous One Solution Reagent), based on the conversion of a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS), to a colored formazan product by living cells. Absorbance was read by a microplate reader (Molecular Devices SpectraMax I3) at 490 nm. The quantity of formazan product, as measured by the amount of absorbance, was directly proportional to the metabolic activity of viable cells in the culture.

Cell viability (% of control) = (OD\sub{EG} - OD\sub{ZG})/(OD\sub{CG} - OD\sub{ZG}) * 100%

IC\textsubscript{50} value was calculated using GraphPad Prism software based on the cell viability data at different concentrations.

![Figure S14. Cell viabilities of probe 1 at various concentrations for HeLa Cells after 24 h incubation.](image)

5.2 Fluorescence microscopy experiments

HeLa cells were seeded in a 6-well glass tissue culture dish at the cell culture facility of East China University of Science and Technology (ECUST) and cultured in DMEM medium (with phenol red, Gibco/Invitrogen) with 10% Fetal Bovine Serum and 1% Glutamine at 37 °C for 24 hours. Before incubation with probe 1 and other reagents, the upper medium in the culture dish was removed, and the cultured cells were washed with PBS buffer three times. The first group cells were first incubated in a solution of N-ethylmaleimide (1 mM in distilled water) for 1h to reduce the concentration of all thiols including cysteine, then washed with PBS buffer three times before incubated with probe 1 in
DMEM medium (20 μM, containing 2‰ DMSO, prepared from 10 mM stock solution in DMSO) at 37 °C for 1 h. The second to fourth group cells were first pretreated with 1 mM N-ethylmaleimide for 1h, then washed with PBS buffer three times and pretreated with 500 μM Cys, Hcy and GSH for 30 min respectively, finally washed with PBS buffer three times again and incubated with probe 1 solution following the same procedure described above for 1 h. Fluorescence images was obtained using an Olympus IX51 equipped with a Xenon lamp and Olympus digital camera.

In another studies, HepG2 cells were used instead of HeLa cells, and the concentration of N-ethylmaleimide 500 μM was used for cell pretreatment, while all the other conditions were kept the same.

Figure S15. Fluorescence images (e-h) collected at 430-495 nm (blue to cyan-blue, λex = 400 nm) and the corresponding bright field view (a-d) of HepG2 cells after different treatment. a) and e) were pretreated with 500 μM N-ethylmaleimide before incubation with the probe 1 for 1 h. (b, f), (c, g), and (d, h) were first pretreated with 500 μM N-ethylmaleimide for 1 h, then pretreated with 500 μM Cys, Hcy, and GSH for 30 min respectively, finally incubated with 20 μM probe 1 for 1 h (scale bar = 100
Part VI: NMR and HRMS Data
Figure S16. $^1$H NMR, $^{13}$C NMR, and HRMS of probe 1.
(2D-NOESY spectrum: the cross-peaks (labelled red) between the proton on the 3'-position and one of two-protons on the 6'-position supporting the formation of six-membered ring)
**Figure S17.** $^1$H NMR, $^{13}$C NMR, 2D-COSY, 2D-NOESY, and HRMS of the compound 3a.
(2D-NOESY spectrum: the cross-peaks (labelled red) between the proton on the 3'-position and one of two-protons on the 6'-position supporting the formation of six-membered ring)\(^7\)

**Figure S18.** \(^1\)H NMR, \(^13\)C NMR, 2D-COSY, 2D-NOESY, and HRMS of 2a.
Figure S19. $^1$H NMR, $^{13}$C NMR, and HRMS of the reference probe 6.
Figure S20. $^1$H NMR, $^{13}$C NMR, and HRMS of the reference probe 7.

References