SUPPLEMENTARY MATERIAL

Selective Fluorescent Detection of Cysteine over Homocysteine and Glutathione by a Simple Probe

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1. General information

All the solvents were purified and dried according to general methods. ¹H NMR spectra were recorded on a Bruker AVIII-400 MHz spectrometer. Chemical shifts (in ppm) were determined by reference to the residual solvent peak (Acetone-d₆: 2.05 ppm; CCl₃D: 7.27 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constants (Hz) and integration. ¹³C NMR spectra were recorded on the same NMR spectrometer. Chemical shifts (in ppm) were determined by reference to the residual solvent peak (Acetone-d₆: 28.95 ppm; CCl₃D: 77.05 ppm). High resolution mass spectra (HRMS) were measured with Thermo (Orbitrap Elite). Absorption spectra were measured using a Thermo (BioMate 3S) UV/Vis spectrophotometer. Fluorescence measurements were carried out with an F97pro fluorospectrophotometer.

2. Synthesis of probe 1

![Chemical Structure](image)

7-Hydroxy-4-methyl-3-phenyl-2H-chromen-2-one (2) A mixture of 2,4-dihydroxy-acetophenone (1.52 g, 10 mmol) and phenyl-acetyl chloride (4.4 mL, 30 mmol) was dissolved in acetone (30 mL) in the presence of K₂CO₃ (4.15 g, 30 mmol). Next, the mixture was heated to reflux for 30 h. K₂CO₃ was filtered and solvent was evaporated. The residue was purified by column chromatography (silica gel; petroleum ether / ethyl acetate 4/1) to provide the 7-hydroxy-4-methyl-3-phenyl-2H-chromen-2-one (yield: 40%). ¹H-NMR (400 MHz, Acetone-d₆): δ = 2.25 (s, 3 H), 6.78 (d, J = 2.4 Hz, 1 H), 6.90 (dd, J = 2.4, 8.8 Hz, 1 H), 7.34-7.31 (m, 2 H), 7.40-7.36 (m, 1 H), 7.47-7.43 (m, 2 H), 7.68 (d, J = 8.8 Hz, 1 H), 9.35 (s, 1 H) ppm; ¹³C-NMR (100 MHz, Acetone-d₆): δ = 15.8, 102.2, 112.7, 113.3, 123.5, 127.0, 127.6, 128.0, 130.4, 135.5, 147.8, 154.5, 160.2, 160.6 ppm.

4-Methyl-2-oxo-3-phenyl-2H-chromen-7-yl acrylate (Probe 1) Dichloromethane (30 mL) was added to the mixture of potassium carbonate (0.33 g, 2.4 mmol) and compound 2 (0.5 g, 2 mmol). After the reaction was stirred for 0.5 h, acryloyl chloride (0.22 g, 2.4 mmol) was added dropwise to the above solution at 0 °C, and the mixture continues to react for 12 h. The reaction was quenched with water and the mixture was extracted by ethyl acetate and dried by sodium sulfate. The solvent was evaporated and the residue was purified by column chromatography (silica gel;
petroleum ether / ethyl acetate 4/1) to provide the 4-methyl-2-oxo-3-phenyl-2H-chromen-7-yl acrylate (Probe 1) (yield: 60%).\textsuperscript{1}H-NMR (400 MHz, CCl$_3$D): $\delta = 2.33$ (s, 3 H), 6.09 (dd, $J = 1.2, 6.8$ Hz, 1 H), 6.36 (dd, $J = 10.4, 17.2$ Hz, 2 H), 6.68 (dd, $J = 1.2, 17.2$ Hz, 1 H), 7.17 (dd, $J = 2.4, 8.8$ Hz, 1 H), 7.33-7.31 (m, 2 H), 7.43-7.41 (m, 1 H), 7.49-7.46 (m, 2 H), 7.71 (d, $J = 8.8$ Hz, 1 H) ppm; \textsuperscript{13}C-NMR (100 MHz, CCl$_3$D): $\delta = 16.8, 110.2, 118.1, 118.5, 126.0, 126.9, 127.4, 128.3, 128.5, 130.0, 133.7, 134.2, 147.3, 152.6, 153.3, 160.8, 163.9$ ppm; HRMS (ESI) m/z calcd for C$_{19}$H$_{14}$O$_4$(M+H): 307.0965. Found: 307.0958, error: 2.3 ppm.

3. Quantum Yields

Quantum yields were detected using quinine sulfate as a standard. The quantum yield was calculated according to the equation: \( \Phi_{\text{sample}} = \Phi_{\text{standard}} \times \left( \frac{I_{\text{sample}}}{I_{\text{standard}}} \right) \times \left( \frac{A_{\text{standard}}}{A_{\text{sample}}} \right) \); where \( \Phi \) is the quantum yield, \( \Phi_{\text{standard}} = 0.55 \) in 0.5 M H$_2$SO$_4$; \( I_{\text{sample}} \) and \( I_{\text{standard}} \) are the integrated fluorescence intensities of the sample and the standard, \( A_{\text{sample}} \) and \( A_{\text{standard}} \) are the optical densities, at the excitation wavelength, of the sample and the standard, respectively.

4. Analysis method of pseudo-first-order rate constant

Based on the large excess of Cys, Hcy, or GSH (150 equiv.) over probe 1 (10 \( \mu \)M), the pseudo-first-order rate constants was detected in aqueous buffered solution (pH 7.4 PBS, containing 10% CH$_3$CN) at room temperature. The pseudo-first-order rate constant \( k' \) was calculated on the basis of below equation: \( \ln \left[ \frac{F_{\text{max}} - F_t}{F_{\text{max}}} \right] = -k't \) Where \( F_t \) and \( F_{\text{max}} \) denote the fluorescence intensities at 455 nm at time \( t \) and the maximum value acquired when the reaction was completed, respectively, and \( k' \) is the pseudo-first-order rate constant.

5. HPLC analysis methods

The mixture of probe 1 and Cys was analyzed by a PE HPLC system consisting of Quaternary HPLC pump and UV-detector. C$^{18}$ column (4.6 x 150 mm) was used with a mobile phase of acetonitrile at a flow rate of 1 mL/min. The run was set as follows. Flow rate of 0.3 mL/min (1 min), flow rate of 0.4 mL/min (1.5 min), flow rate of 0.5 mL/min (1.5 min), flow rate of 0.6 mL/min (16 min). Compound 2, mixture of probe 1 and Cys showed a retention time of 3.514 and 3.507 min, respectively.

6. Cytotoxicity of probe 1 in Hela cells

We evaluated the cytotoxicity of probe 1 by classic MTT method in Hela cells. HeLa cells (5×10$^4$/mL) were seeded in 96-well flat microtiter plates for 24 h, and then incubated with assigned concentrations of probe 1 with 24 h. Thereafter, culture medium was discarded, and100 \( \mu \)L mixture of medium and MTT(10:1 v/v) was added to each well with incubation for 4 h at 37 °C in the dark. The culture medium was
then removed and the addition of 100 μL DMSO was added. The absorbance was read at 490 nm by Thermo (Multiskan MK3) microplate reader. The percentage of cell viability was calculated compared with control wells designated as 100% viable cells.

7. Bioimaging of probe 1 in HeLa cells

First, HeLa cells (5×10⁴/mL) were seeded in 6-well flat microtiter plates for adherence within 24 h. Then the cells were incubated with probe 1 for 1 h, and washed with PBS for 3 times. On the other hand, in the N-ethyl maleimide (NEM) experiment, cells were pre-incubated with NEM (1 mM) for 30 min. Biological imaging was obtained after cells were then washed with PBS. Fluorescence images of probe 1 were recorded by a fluorescence microscope.

8. Figures

![Absorbance spectrum of probe 1](image)

**Fig. S1** Time-dependent Uv-vis absorbance response of probe 1 (10 μM) in the presence of Cys in aqueous buffered solution (pH 7.4 PBS, containing 10% CH₃CN) at room temperature. The absorbance was gradually increased at 380 nm with the time extension.
Fig. S2 Fluorescence intensity response of probe 1 (10 μM) upon addition of Cys in aqueous buffered solution (pH 7.4 PBS, containing 10% CH₃CN) at room temperature, λex = 380 nm.

![Graph showing fluorescence intensity response of probe 1 upon addition of Cys](image)

Fig. S3 The analysis of pseudo-first-order rate constant \( k' \) in aqueous buffered solution (pH 7.4 PBS, containing 10% CH₃CN) at room temperature, λex = 380 nm. Each experiment was performed in triplicate.

![Graph showing analysis of pseudo-first-order rate constant](image)

Fig. S4 pH-Dependent fluorescent intensity changes of probe 1 (10 μM) in the presence and absence of Cys at room temperature, λex = 380 nm.

![Graph showing pH-dependent fluorescent intensity changes](image)

Fig. S5 High resolution mass spectra of the mixture of probe 1 and Cys in aqueous buffered solution (pH 7.4 PBS, containing 10% CH₃CN) at room temperature.

![Graph showing high resolution mass spectra](image)
Fig. S6 Fluorescence intensity analysis of probe 1 (10 μM) with incubation of calf serum in PBS (pH 7.4) at 37 °C for 10 min.

Fig. S7 The cell viability analysis of probe 1 in Hela cells.

9. $^1$H NMR, $^{13}$C NMR and HRMS

9.1 $^1$H NMR and $^{13}$C NMR of compound 2
9.2 $^1$H NMR, $^{13}$C NMR and HRMS of probe 1