**Electronic Supplementary Information**

# A PET-based Fluorescent Probe for Monitoring Labile Fe(II) Pools in Macrophage Activations and Ferroptosis

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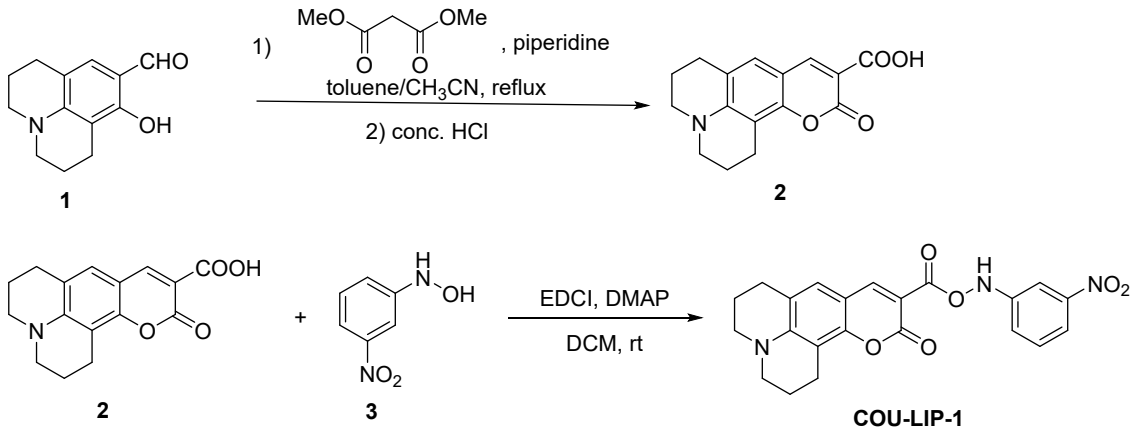
**General Information**

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. 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 4 nm both for excitation and emission unless otherwise stated. The pH measurements were carried out with a FE20 plus (Mettler Toledo) pH meter.

## Part I: Probe Synthesis and Structure Characterizations




**Scheme S1** Synthesis of the probe **COU-LIP-1**.

### Coumarin 343 (2)[1]

To a solution of 9-formal-8-hydroxyjulolidine **1** (1.0 g, 4.6 mmol) in 10 mL $CH_3CN$ and 19 mL toluene, was added dimethyl malonate (1.82 g, 13.8 mmol), followed by addition of piperidine (2.3 mL, 23 mmol) dropwise. The reaction mixture was then heated to reflux for 6 h. The solvent was removed under vacuum, and 17 mL conc. HCl was added and stirred at r. t. for 24 h. The reaction mixture was cooled to 0 $^oC$ and neutralized by addition of aqueous NaOH (2 M) solution, and extracted with 100 mL methylene chloride three times. The combined organic layer was washed with brine 100 mL, dried over anhydrous sodium sulfate, filtered and concentrated. The residue was applied to column chromatograph (silica gel) using a mixture of methylene chloride and methanol (50:1, V/V) as eluent to afford 908 mg (69 % yield) the title coumarin 343 (**2**) as yellow solid. $^1$HNMR (400 MHz, DMSO-$d_6$) δ 8.43 (s, 1H), 7.22 (s, 1H), 3.38-3.30 (m, 4H), 2.76-2.66 (m, 4H), 1.93-1.82 (m, 4H).

### Coumarin 343 3-nitrophenylazanyl ester (COU-LIP-1)

To a solution of Coumarin 343 (256 mg, 0.897 mmol) and *N*-(3-nitrophenyl)hydroxylamine (**3**)[2] (140 mg 0.91 mmol) in 10 mL DCM, was added EDCI (185 mg, 0.963 mmol) and DMAP (45 mg, 0.37 mmol). The reaction mixture was stirred at r. t. for 50 min. The solvent was removed under vacuum. The residue was applied to column chromatograph using a mixed solvent of methylene chloride and methanol (DCM:MeOH=50:1, V/V) as eluent to afford 45 mg (12% yield) of the probe **COU-LIP-1** as a yellow solid. $^1$H NMR (400 MHz, $CDCl_3$) δ 9.22 (s, 1H), 8.41 (s, 1H), 7.99 (t, $J$ = 2.2 Hz, 1H), 7.88 (d, $J$ = 7.8 Hz, 1H), 7.54-7.43 (m, 2H), 7.00 (s, 1H), 3.42-3.34 (m, 4H), 2.89 (t, $J$ = 6.4 Hz, 2H), 2.78 (t, $J$ = 6.3 Hz, 2H), 1,99 (p, $J$ = 6.1 Hz, 4H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 163.7, 157.2, 153.2, 149.9, 149.2, 149.1, 148.4, 130.4, 127.7, 120.1, 119.4, 116.0, 107.9, 107.1, 104.6, 102.8, 49.7, 49.2, 26.8, 20.5, 19.5, 19.5; ESI-HRMS: m/z $[M-H]^-$ calcd for $[C_{22}H_{18}N_3O_6]^-$ 420.1201, found 420.1194.

## Part II: Additional Fluorescence and Absorption Spectroscopy Studies

All aqueous solutions were prepared using double distilled water. **COU-LIP-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 Tris-HCl 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).

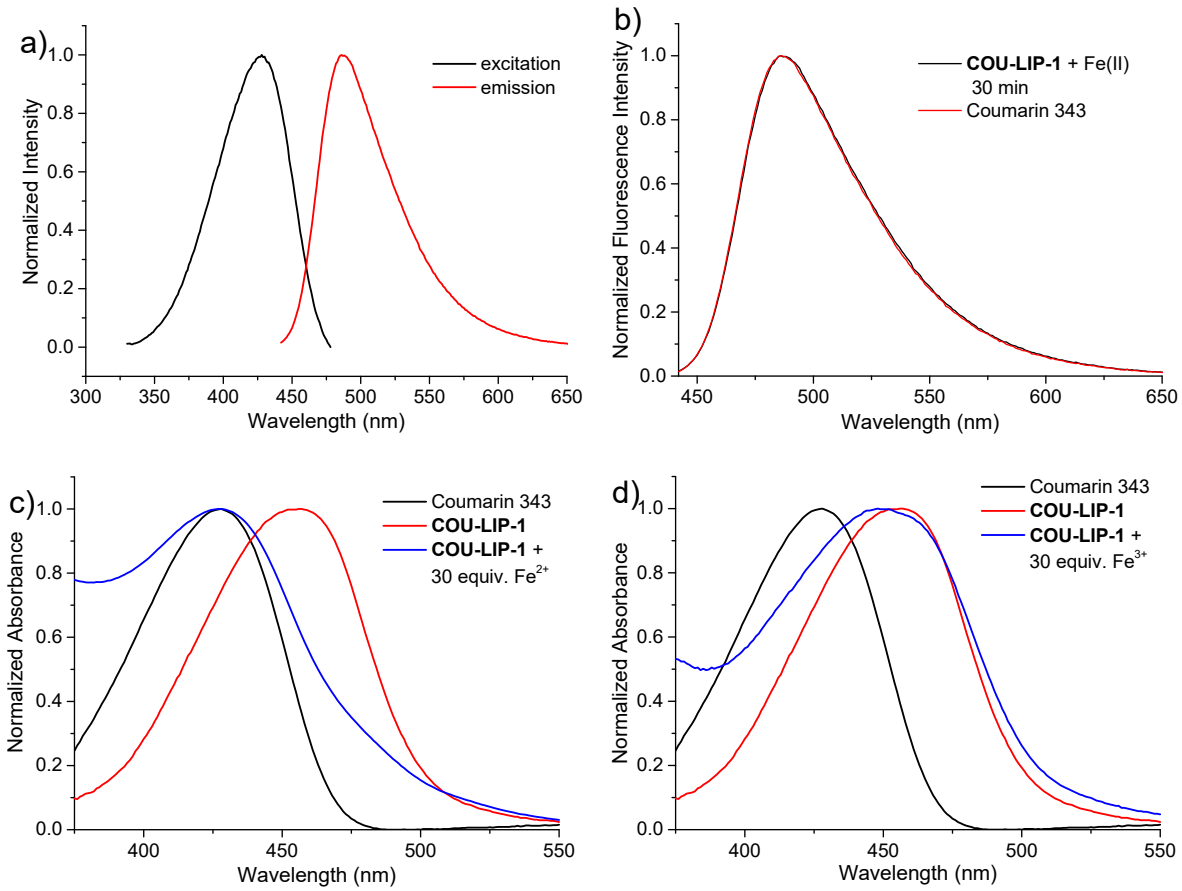



**Figure S1** a) Normalized fluorescence excitation ($\lambda_{em}$ = 488 nm) and emission spectra ($\lambda_{ex}$ = 432 nm) of the probe **COU-LIP-1** (1 μM) upon incubation with 30 equiv. $Fe^{2+}$ for 30 min; b) Overlap of the normalized fluorescence emission of the probe **COU-LIP-1** (1 μM) upon incubation with 30 equiv. $Fe^{2+}$ for 30 min and that of the coumarin 343 (1 μM, $\lambda_{ex}$ = 432 nm); c) Overlap of the normalized absorption spectra of the probe **COU-LIP-1** (5 μM) upon incubation with 30 equiv. $Fe^{2+}$ for 30 min and those of the coumarin 343 and **COU-LIP-1**; d) Overlap of the normalized absorption spectra of the probe **COU-LIP-1** (5 μM) upon incubation with 30 equiv. $Fe^{3+}$ for 30 min and those of the coumarin 343 and **COU-LIP-1**.

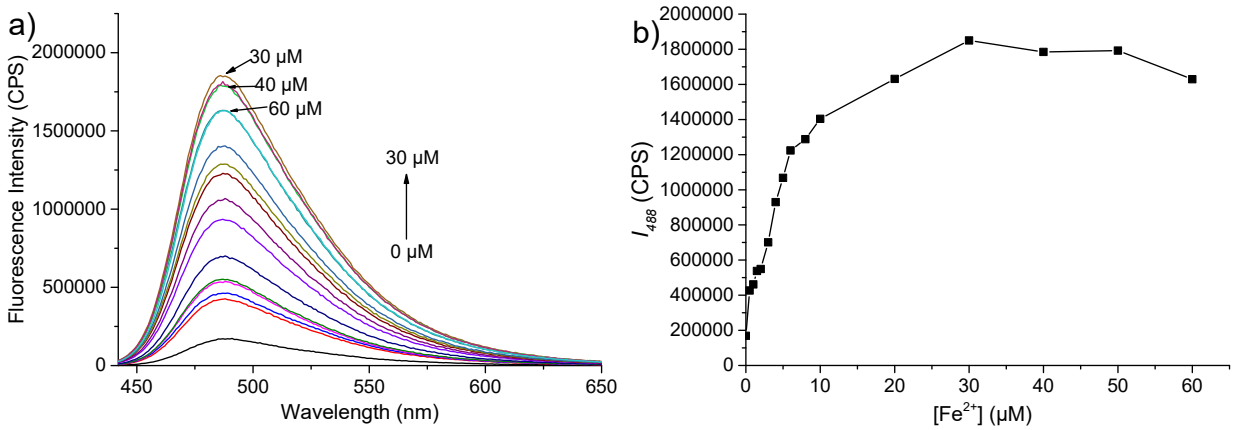



**Figure S2** a) Concentration-dependent fluorescence emission spectra ($\lambda_{ex}$=432 nm) of the probe **COU-LIP-1** (1 μM) upon addition of increasing amount of $Fe^{2+}$ (0-60 μM); b) Fluorescence intensity of the probe **COU-LIP-1** (1 μM) at 488 nm ($\lambda_{ex}$=432 nm) after incubation with $Fe^{2+}$ for 30 min versus [$Fe^{2+}$]. The maximum fluorescence intensity at 488 nm was achieved with addition of 30 μM $Fe^{2+}$.

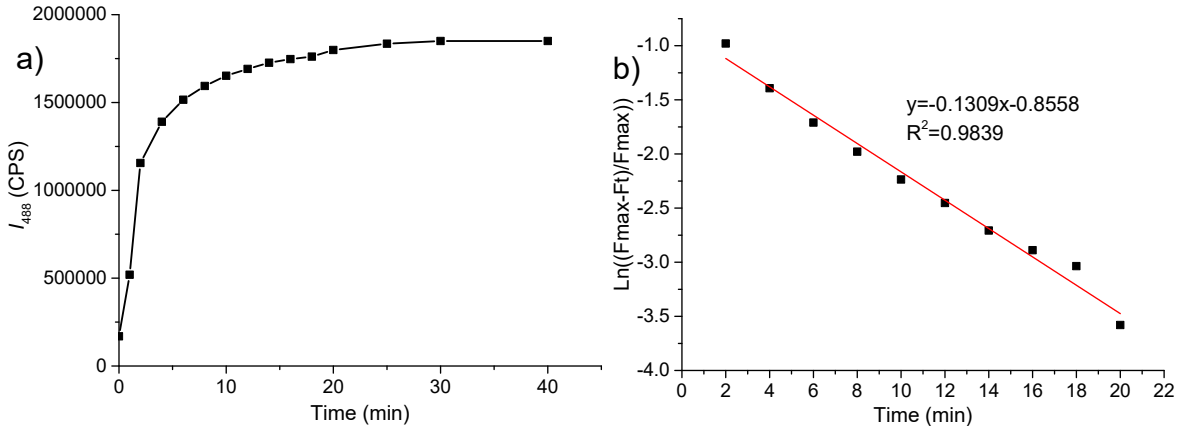



**Figure S3** a) Time-dependent (0 to 30 min) fluorescence intensity of the probe **COU-LIP-1** (1 μM) at 488 nm ($\lambda_{ex}$=432 nm) upon addition with 30 equiv. $Fe^{2+}$; b) Linear regression of Ln( ($F_{max}$-$F_t$)/$F_{max}$) versus incubation time (0 to 20 min). The calculated pseudo first-order reaction kinetic constant was 0.131 $min^{-1}$. The second-order reaction kinetic constant was 72.8 $M^{-1}s^{-1}$.

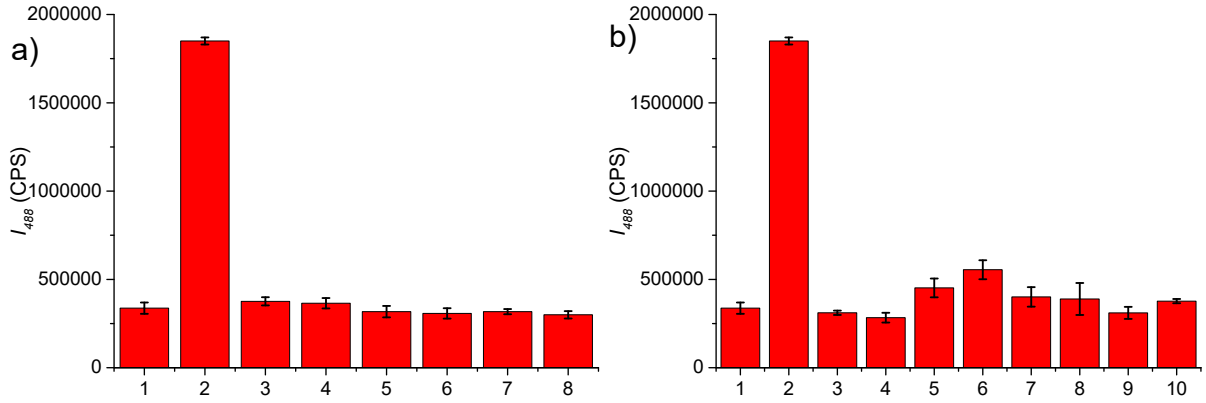



**Figure S4** a) Selectivity studies of the probe **COU-LIP-1** for various ROS (1 μM probe incubation with 30 μM different species for 30 min): 1) probe only, 2) $Fe^{2+}$; 3) TBHP (*t*-butylhydrogenperoxide); 4) $O_2^{\bullet -}$; 5) *t*-BuO$^{\bullet}$; 6) OH$^{\bullet}$; 7) $H_2O_2$; 8) NaClO; b) Selectivity studies of the probe **COU-LIP-1** for various reductive species (1 μM probe incubation with 30 μM different species except for GHS for 30 min): 1) probe only; 2) $Fe^{2+}$; 3) $Na_2S_2O_3$; 4) $Na_2SO_3$ ; 5) $Na_2S$ ; 6) ascorbic acid; (7) Cys; (8) NaSH ; 9) NADH; 10) 5 mM GSH .

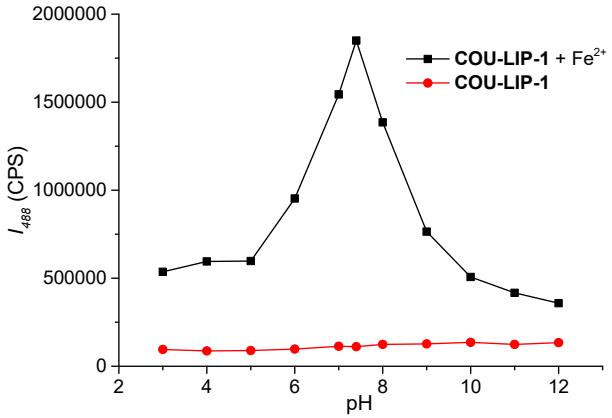



**Figure S5** pH-dependent fluorescence intensity at 488 nm of the probe **COU-LIP-1** (1 μM) incubated with 30 μM $Fe^{2+}$ for 30 min.

**Table S1** Photophysical properties of the probe **COU-LIP-1** and Coumarin 343 in different solvents.

| | **COU-LIP-1** | | | | **Coumarin 343** | | | |
|---|---|---|---|---|---|---|---|---|
| solvent | $\lambda_{max}/\lambda_{em}$ (nm) | $\Delta\lambda$ (nm) | $\varepsilon$ ($\times10^4$ $M^{-1}cm^{-1}$) | $\Phi$ | $\lambda_{max}/\lambda_{em}$ (nm) | $\Delta\lambda$ (nm) | $\varepsilon$ ($\times10^4$ $M^{-1}cm^{-1}$) | $\Phi$ |
| 10mM Tris-HCl | 448/488 | 48 | 3.12 | 0.098 | 429/488 | 56 | 1.8 | 1.28 |
| 1×PBS | 447/488 | 41 | 2.87 | 0.053 | 432/486 | 54 | 1.8 | 1.32 |
| MeOH | 447/482 | 35 | 6.67 | 0.34 | 430/474 | 44 | 2.01 | 1.04 |
| DMF | 440/481 | 41 | 4.45 | 0.0078 | 392/471 | 79 | 1.28 | 0.17 |
| EA | 439/472 | 33 | 6.28 | 0.65 | 440/477 | 37 | 2.74 | 1.13 |
| DCM | 451/477 | 26 | 6.45 | 0.34 | 451/474 | 23 | 3.3 | 0.064 |
| Acetone | 443/481 | 38 | 5.92 | 0.11 | 444/485 | 41 | 2.69 | 0.028 |
| 1,4-Dioxane | 437/467 | 30 | 5.79 | 0.17 | 434/466 | 32 | 2.44 | 1.14 |

$\lambda_{max}$: maximum absorption wavelength; $\lambda_{em}$: maximum emission wavelength; $\varepsilon$: molar extinction coefficient; $\varphi$: quantum yield measured using quinine sulfate in 0.1 M $H_2SO_4$ ($\varphi$=0.58[3]) as the standard. Tris-HCl: 2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride; PBS: phosphate buffer saline; DMF: *N*,*N*-dimethyl formamide; EA: ethyl acetate; DCM: dichloromethane.

## Part III: HPLC-MS Studies of COU-LIP-1's Reaction with $Fe^{2+}$

To 990 μL 10 mM Tris-HCl buffer was added 10 μL stock solution of **COU-LIP-1** (10 mM) in DMSO, was added 5 μL (ferrous ammonium sulfate, FAS) 100 mM $Fe^{2+}$ (5 equiv.) solution in distilled water. The reaction mixture was stirred at room temperature. At the time-point of 1, 5, 10, 20 min, the reaction solution was extracted with ethyl acetate. The organic phase was separated, dried in vaccuo, redissolved in 1 mL methanol, and filtered through a 0.22 μm micro-syringe filter (13 mm), and an aliquot of 10 μL was taken for HPLC-MS analysis.

Similarly, to 990 μL 10 mM Tris-HCl buffer was added 10 μL stock solution of **COU-LIP-1** (10 mM) in DMSO, was added 5 μL (ferric percholorate, $Fe(ClO_4)_3$) 100 mM $Fe^{3+}$ (5 equiv.) solution in distilled water. The reaction mixture was stirred at room temperature. At the time-point of 20 min, the reaction solution was extracted with ethyl acetate. The organic phase was separated, dried in vaccuo, redissolved in 1 mL methanol, and filtered through a 0.22 μm micro-syringe filter (13 mm), and an aliquot of 10 μL was taken for HPLC-MS analysis.

HPLC-MS analysis was performed on Thermo Scientific with Q-Exactive plus Mass Spectrometry equipped with Hypersil Gold C18 (Thermo Scientific) 3 μm, 100 mm×2.1mm column. All the samples were eluted using a gradient mixture 65:35 $CH_3CN$: $H_2O$ containing 0.1 % formic acid at a flow rate of 0.3 mL/min in 10 min. MS was detected in ESI positive mode. The m/z ranges of 138.6-139.6, 285.6-286.6, and 421.6-422.6 were extracted as LC peaks of 3-nitroaniline, Coumarin 343, and the probe, respectively.

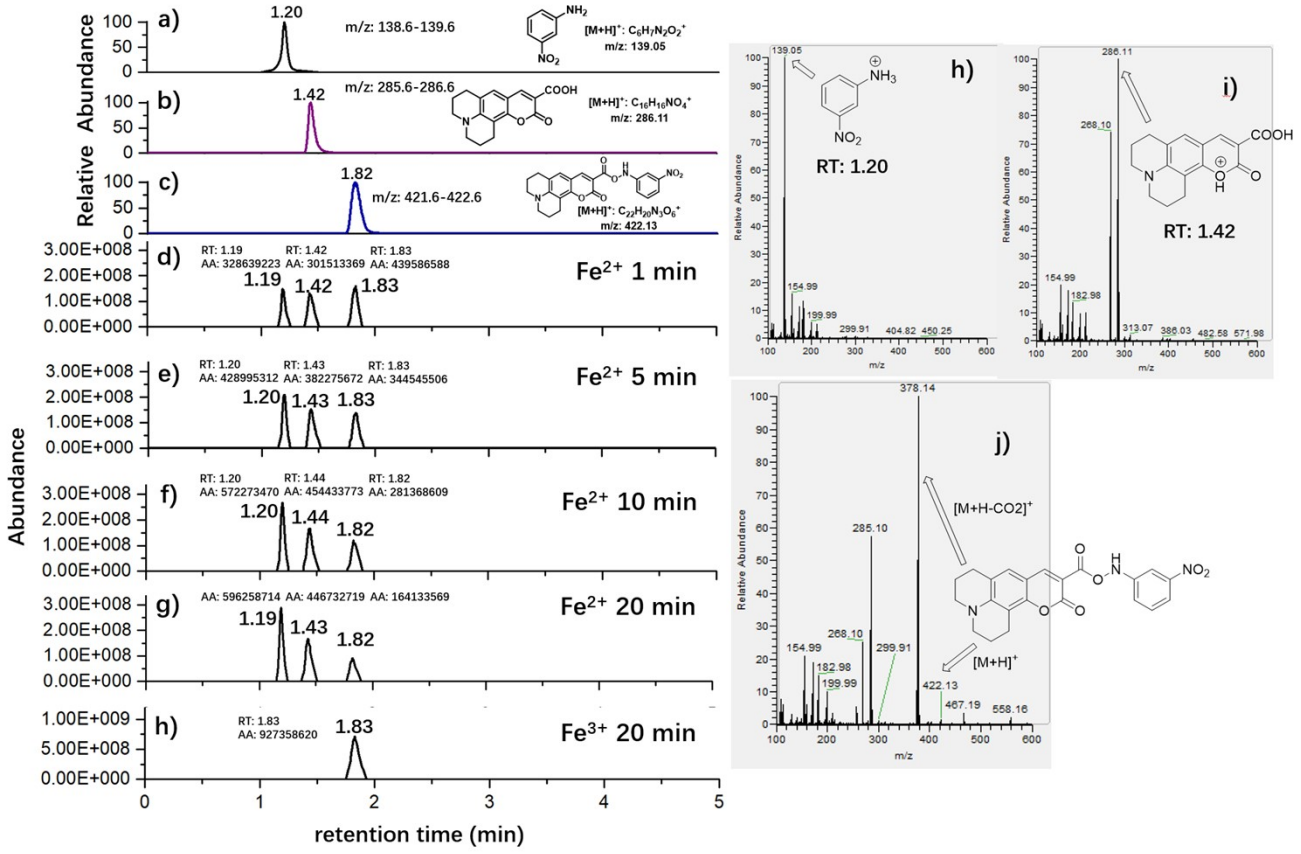



**Figure S6** Time-dependent HPLC-MS analysis of the reaction between the probe **COU-LIP-1** and 5 equiv. $Fe^{2+}$. a-c) HPLC-MS analysis of the standard samples: a) 3-nitroaniline; b) Coumarin 343; c) **COU-LIP-1**; d) HPLC-MS analysis of the reaction mixture at the time point of 1 min; e) HPLC-MS analysis of the reaction mixture at the time point of 5 min; f) HPLC-MS analysis of the reaction mixture at the time point of 10 min; g) HPLC-MS analysis of the reaction mixture at the time point of 20 min; g-i) Typical MS spectra of the corresponding HPLC peaks at retention time 1.20, 1.42, and 1.82 min. (RT means retention time; AA means the integration of a peak around a specific retention time)

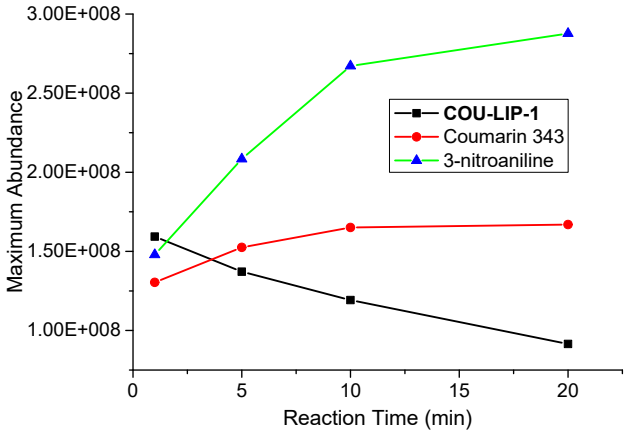



**Figure S7** Plot of the change of the maximum abundance of the **COU-LIP-1** peak at retention time 1.82 min, the maximum abundance of the Coumarin 343 peak at retention time 1.42 min, and the maximum abundance of the 3-nitroaniline peak at retention time 1.20 min versus the reaction time at

1, 5, 10, 15 and 20 min.

**Part IV: Theoretical Calculations**

Density functional theory (DFT) and time-dependent DFT (TD-DFT) were employed to rationalize the working principle of this probe. Geometry optimizations in the ground state were carried out with the M06-2X[4] functionals in combination with the def2SVP basis set[5] in water. Frequency analysis was performed to confirm that we have obtained stable structures on the potential energy surfaces. When the solvent effect (in water) was applicable, it was accounted for using the solvation model based on the density (SMD) model.[6] All DFT/TD-DFT calculations were carried out with *Gaussian 16A*. [7]

**4.1 Verification of PET quenching mechanism**

The calculation shows that the $S_1$ photoexcitation of probe **COU-LIP-1** mainly corresponds to the transition from HOMO to LUMO+1, which is centralized at the coumarin moiety. And between these two molecular orbitals, one additional molecular orbital (LUMO) is present, and this orbital is localized at the 3-nitrophenyl fragment. The energy levels of these orbitals satisfy the requirements of d-PET, where the coumarin fluorophore will donate an electron to the nitro-benzene fragment (from LUMO+1 to LUMO) upon photoexcitation.[8] This transition forms a dark electron-transfer state, rending the probe non-emissive. Upon the cleavage of the nitro-phenyl quencher by $Fe^{2+}$, PET process would be inhibited, thus turning on bright emissions of the Coumarin 343 fluorophore.

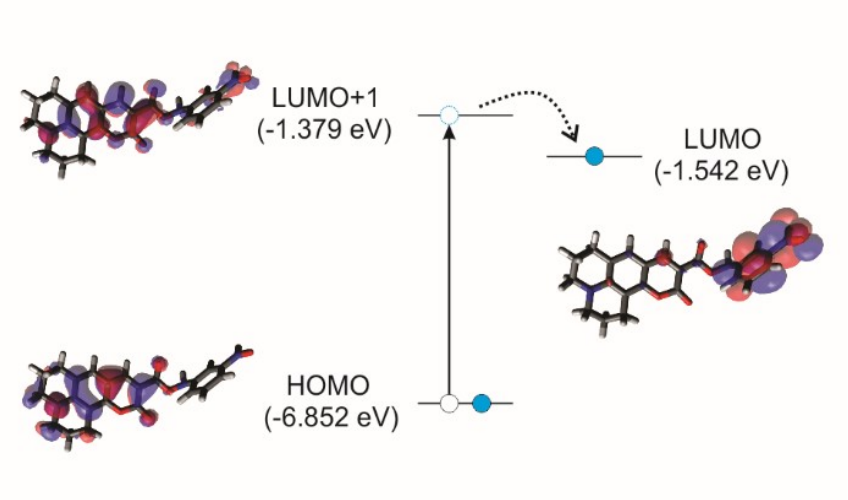



**Figure S8** Calculated frontier molecular orbitals and their corresponding energy levels of the probe in the ground state in water, at the M06-2X/def2-SVP level of theory.

### 4.2 UV-Vis absorption and emission wavelength calculations

The UV-vis absorption and emission wavelengths of **COU-LIP-1** and Coumarin 343 were calculated. As Coumarin 343 was deprotonated in Tris-HCl buffer (pH = 7.4), the deprotonated Coumarin 343 was also modelled. The calculation results confirmed a significant blue shift from **COU-LIP-1** to deprotonated Coumarin 343 as shown in the **Figure S9** below. This blue shift is consistent with experimental observations (See **Table S1**).

The difference between the experimental and calculated values of peak wavelengths is due to the high Hartree–Fock (HF) exchange contributions in the employed functionals (including M06-2X and ωB97-XD). These high HF contributions will result in an over-estimation of the excitation/de-excitation energies (or an underestimation of the UV-Vis absorption and emission wavelengths).[9] However, these two functionals are recommended in the modeling of fluorophores with significant charge transfer (or electron transfer) upon photoexcitation, such as in **COU-LIP-1**, because they provide accurate descriptions of the relative differences of the excitation energy.[8, 9]

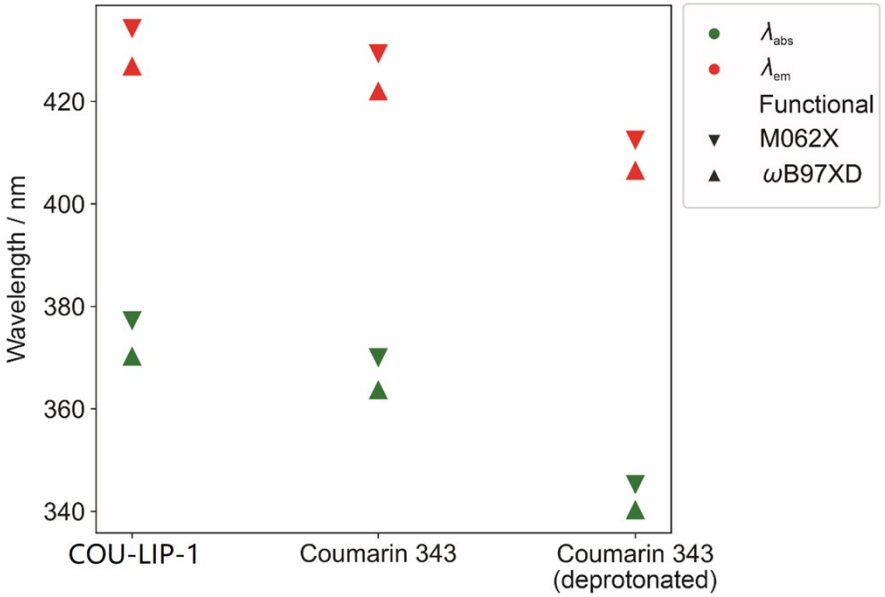



**Figure S9** Calculated UV-Vis absorption ($\lambda_{abs}$) and emission ($\lambda_{em}$) wavelengths of **COU-LIP-1**, Coumarin 343, and deprotonated Coumarin 343 in water.

### 4.3 Rationalization of blue-shift of UV-Vis absorption spectra in detection of $Fe^{2+}$

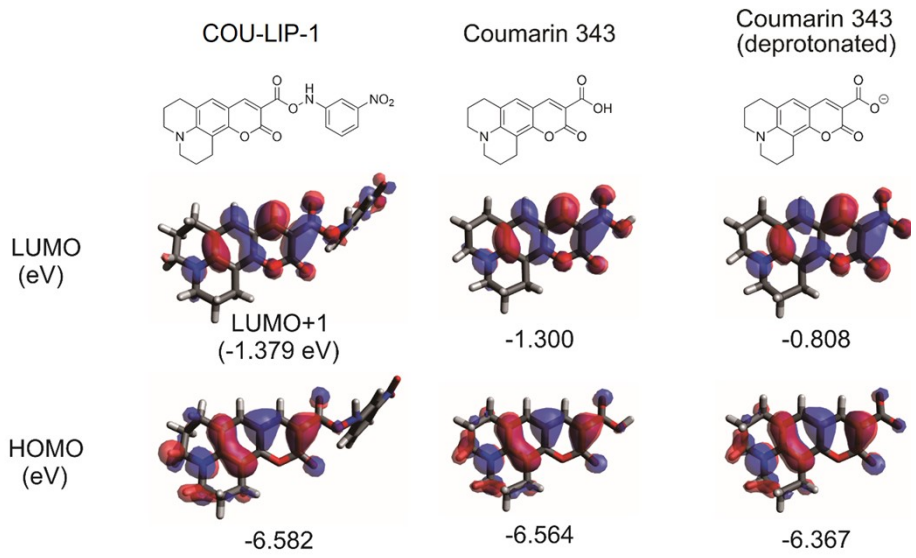



**Figure S10** Calculated frontier molecular orbitals and their corresponding energy levels of the molecules in the ground state in water, at the M06-2X/def2-SVP level of theory. Note that the transition of the first absorption band of **COU-LIP-1** involves HOMO and LUMO+1, instead of HOMO-LUMO transitions in (deprotonated) Coumarin 343.

**Part V: Detection of $Fe^{2+}$ ion in Live Cells**

**Cell culture:** The HT-1080 and murine macrophage RAW264.7 cell lines were kindly provided by Stem Cell Bank, Chinese Academy of Sciences. HT-1080 cells were maintained in DMEM medium (Adamas) supplied with 10% Fetal Bovine Serum (FBS, Sangon Biotech), 1% Penicillin-Streptomycin (Gibco) at 37 °C in a humidified atmosphere containing 95% air and 5% $CO_2$. RAW264.7 cells were maintained in RPMI media (Adamas) supplied with 10% Fetal Bovine Serum (FBS, Sangon Biotech), 1% Penicillin-Streptomycin (Gibco) at 37 °C in a humidified atmosphere containing 95% air and 5% $CO_2$.

**5.1 Cytotoxicity assay of the COU-LIP-1 for HT-1080 cells**

Cell growth assay was performed using Cell Counting Kit-8 (Elabscience Biotechnology). Cell were seeded in 96-well plates with an initial seeding density of 10000 cells per well. Solutions of **COU-LIP-1** (0, 5, 10, 20 and 50 μM) were added into the wells and incubated for 8 h at 37°C. 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfobenzene)-2H-tetrazole monosodium salt (CCK8, 10 μL) was then added into each well for further incubation 1 h. Absorbance was read by a microplate reader (BioTek Synergy Lx) at wavelength λ=450 nm.

Cell Viability (% of control) = (Medicated cells OD / Control cells OD) * 100%

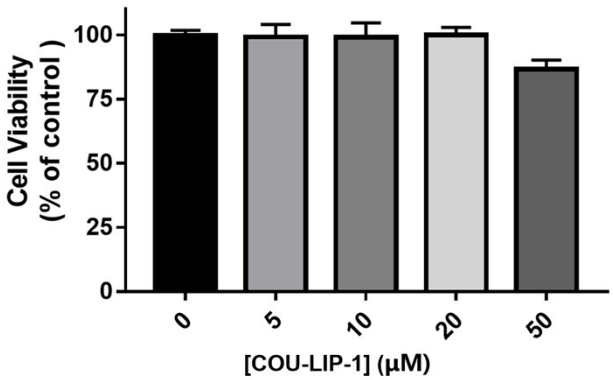



**Figure S11** Cell viabilities of **COU-LIP-1** at various concentrations for HT-1080 cells after 8 h incubation.

**5.2 Confocal fluorescence microscope cell imaging experiments of COU-LIP-1 for externally supplemented $Fe^{2+}$**

HT-1080 cells were seeded in a T-25 cell culture flask in DMEM medium with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin at 37 ˚C for 48 hours. One day before the experiment, HT-1080 cells were transferred to Nunc 35 mm glass-bottom cell culture dishes (Thermo Scientific) to allow the cells to adhere. The cells were incubated in the presence of 100 μM ferrous ammonium sulphate hexahydrate (FAS, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$) at 37 ˚C for 30 min. Then, the cells were washed with Hank's balanced salt solution (HBSS) and then incubated with 10 μM **COU-LIP-1** in the presence or absence of 1 mM 2,2'-dipyridyl (Bpy) in PBS buffer for 30 min. After incubation, the cells were washed with HBSS and then imaged on a Leica TCS SP8 equipped with 40× objective lens and PMT gain of 800 ($\lambda_{ex/em}$ = 458/488±20 nm). The results were shown in **Figure S12**.

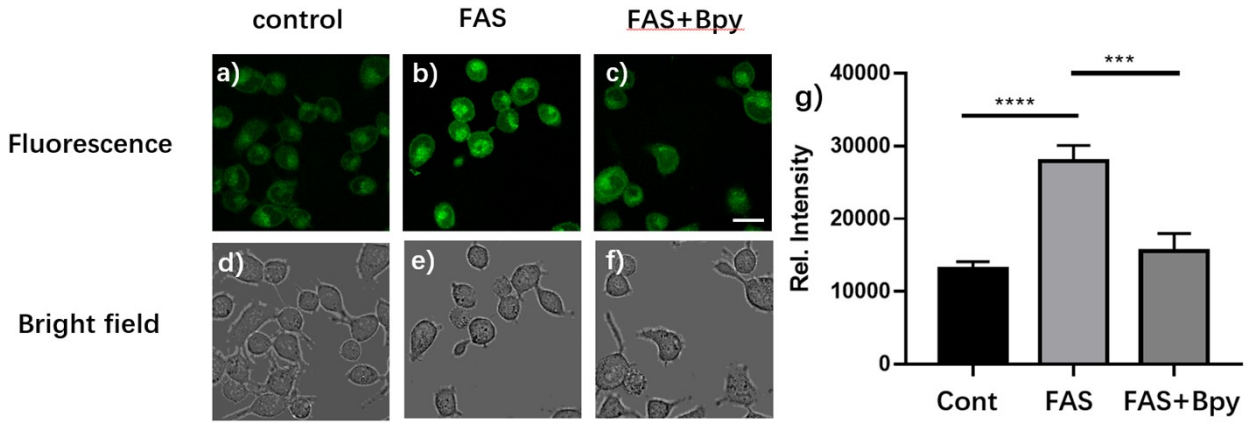



**Figure S12** a-c) Confocal fluorescence images of HT-1080 cells: a) Cells were incubated in normal media for 30 min, and then incubated in media containing 10 μM **COU-LIP-1** for 30 min; b) Cells were incubated in 100 μM FAS for 30 min, and then incubated in media containing 10 μM **COU-LIP-1** for 30 min; c) Cells were imaged in 100 μM FAS for 30 min, and then incubated in media containing 10 μM **COU-LIP-1** and 1 mM Bpy for 30 min; d-j) Corresponding bright field images; h) Relative intensities of the fluorescence images. Cell images were captured on a Leica TCS with $\lambda_{ex/em}$ = 458/488±20 nm nm, scale bar = 20 μm, Error bars represent the standard deviations of three replicates; ****P ⩽ 0.0001, ***P ⩽ 0.001.

### 5.3 Confocal fluorescence microscope cell imaging experiments of COU-LIP-1 for erastin-induced ferroptosis in HT-1080 cells

HT-1080 cells were seeded in a T-25 cell culture flask in DMEM medium with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin at 37 ˚C for 48 hours. One day before the experiment, cells were transferred to Nunc 35 mm glass-bottom cell culture dishes (Thermo Scientific) to allow the cells to adhere. For imaging the ferroptosis process, HT-1080 cells were treated with 10 μM erastin (diluted from 10 mM stock in DMSO with the cell culture media) in the presence or absence of 10 μM Fer-1 at 37 °C for the indicated times (0, 4, and 8 h). Then, the cells were washed with HBSS and then incubatedwith 10 μM **COU-LIP-1** in PBS buffer for 30 min. After incubation, the cells were washed with HBSS and then imaged on a Leica TCS SP8 equipped with 40× objective lens and PMT gain of 800 ($\lambda_{ex/em}$ = 458/488±20 nm). The results were shown in Fig. 3a-j.

### 5.4 Time-dependent flow cytometry studies of COU-LIP-1 for erastin-induced ferroptosis in HT-1080 cells

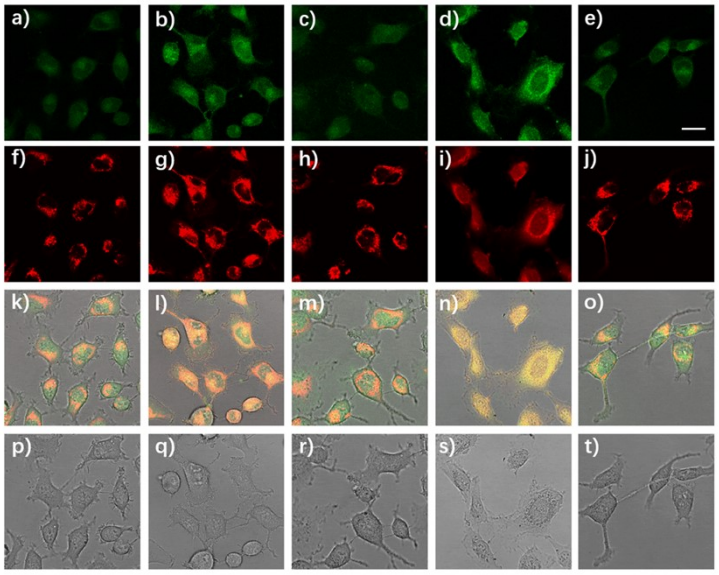
HT-1080 cells were seeded in a T-25 cell culture flask in DMEM medium with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin at 37 ˚C for 48 hours. One day before the experiment, cells were transferred to a Nunc 6-well plate (Thermo Scientific) to allow the cells to adhere. For studying the ferroptosis process, HT-1080 cells were treated with 10 μM erastin (diluted from 10 mM stock in DMSO with the cell culture media) in the presence or absence of 10 μM Fer-1 at 37 °C for the indicated times (0, 4, and 8 h) and then for further probe incubation and flow cytometry analysis. The results were shown in Fig. 3h.

**Standard procedure for probe incubation and cytometry analysis:** The cells were washed with HBSS and then incubated with 10 μM **COU-LIP-1** in PBS buffer for 30 min. After treatment, cells were washed with 1×PBS and trypsinized with 1mL trypsin for 3 min. Afterwards cells were washed with HBSS, and re-suspended by mild centrifugation at 350×g for 5 min, neutralized with 1×PBS. Then use ice buckets for constant cells cooling at 4 ˚C while keeping all cell-containing tubes in dark. Finally, the cells were analyzed with a flow cytometer (Beckman Instruments, USA, $\lambda_{ex/em}$ = 405/525 nm).

### 5.5 Confocal fluorescence imaging studies of COU-LIP-1 and Mitotracker Red for erastin-induced ferroptosis in HT-1080 cells

HT-1080 cells were seeded in a T-25 cell culture flask in DMEM medium with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin at 37 ˚C for 48 hours. One day before the experiment, cells were transferred to Nunc 35 mm glass-bottom cell culture dishes (Thermo Scientific) to allow the cells to adhere. For colocalization imaging studies of the ferroptosis process, HT-1080 cells were treated with 10 μM erastin (diluted from 10 mM stock in DMSO with the cell culture media) in the presence or absence of 10 μM Fer-1 at 37 °C for the indicated times (0, 4, and 8 h). Then, the cells were washed with HBSS and then incubated with 10 μM **COU-LIP-1** in PBS buffer for 20 min, and then 100 nM MitoTracker Red was added and incubated for further 10 min, the cells were washed with HBSS and

then imaged on a Leica TCS SP8 equipped with 40× objective lens and PMT gain of 800 ($\lambda_{ex/em}$ = 458/488±20 nm, $\lambda_{ex/em}$ = 561/599±20 nm). Pearson's colocalization coefficients were analyzed with Image J software. The results were shown in Fig. S13.




| erastin | 0h | 4h | 4h | 8h | 8h |
|---|---|---|---|---|---|
| Fer-1 | - | - | + | - | + |
| Pearson coefficient | 0.73 | 0.88 | 0.74 | 0.98 | 0.33 |

**Figure S13** Colocalization fluorescence imaging studies of **COU-LIP-1** and Mitotracker Red for erastin-induced ferroptosis in HT-1080 cells: a, f, k, p) Cells were incubated in normal media and then incubated in media containing 10 μM **COU-LIP-1** for 20 min (total 30 min) and then 100 nM MitoTracker Red added for additional 10 min; b, g, f, q) Cells were incubated in 10 μM erastin for 4 h, and then incubated in media containing 10 μM **COU-LIP-1** for 20 min (total 30 min) and then 100 nM MitoTracker Red added for additional 10 min; c, h, m, r) Cells were incubated in 10 μM erastin in the presence of 10 μM Fer-1 for 4 h, and then incubated in media containing 10 μM **COU-LIP-1** for 20 min and then 100 nM MitoTracker Red added for additional 10 min; d, i, n, s) Cells were incubated in 10 μM erastin for 8 h, and then incubated in media containing 10 μM **COU-LIP-1** for 20 min and then 100 nM MitoTracker Red added for additional10 min; e, j, o, t) Cells were incubated in 10 μM erastin in the presence of 10 μM Fer-1 for 8 h, and then incubated in media containing 10 μM **COU-LIP-1** for 20 min and then 100 nM MitoTracker Red added for additional 10 min. Cell images were captured on a Leica TCS with 40× objective lens and PMT gain of 800 ($\lambda_{ex/em}$ = 458/488±20 nm, $\lambda_{ex/em}$ = 561/599±20 nm), scale bar = 20 μm.

### 5.6 Cytotoxicity assay of the COU-LIP-1 for RAW 264.7 cells

Cell growth assay was performed using Cell Counting Kit-8 (Elabscience Biotechnology). Cell were seeded in 96-well plates with an initial seeding density of 10000 cells per well. Solutions of **COU-LIP-1** (0, 5, 10, 20 and 50 μM) were added into the wells and incubated for 8 h at 37°C. 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfobenzene)-2H-tetrazole monosodium salt (CCK8, 10 μL) was then added into each well for further incubation 1 h. Absorbance was read by a microplate reader (BioTek Synergy Lx) at wavelength λ=450 nm.

Cell Viability (% of control) = (Medicated cells OD / Control cells OD) * 100%

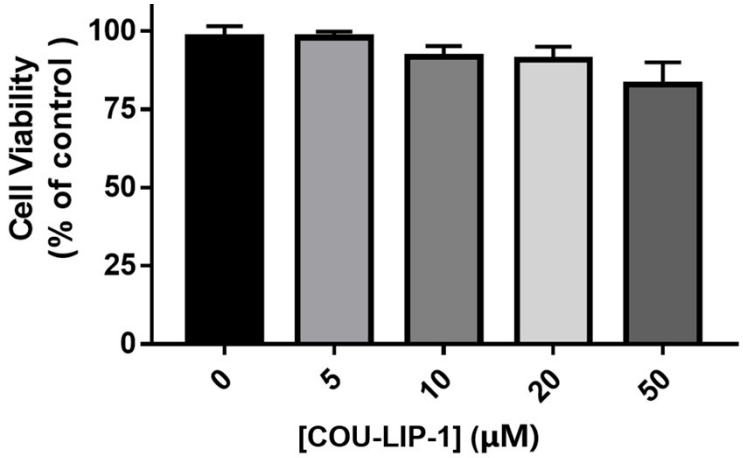



**Figure S14** Cell viabilities of **COU-LIP-1** at various concentrations for RAW 264.7 cells after 8 h incubation.

### 5.7 Flow cytometry studies of COU-LIP-1 for comparison of labile $Fe^{2+}$ levels in different state of RAW 264.7 cells

RAW264.7 cells were maintained in RPMI media (Adamas) supplied with 10% Fetal Bovine Serum (FBS, Sangon Biotech), 1% Penicillin-Streptomycin (Gibco) at 37 °C in a humidified atmosphere containing 95% air and 5% $CO_2$, and were considered as M0 cells for further probe incubation and flow cytometry analysis.

RAW 264.7 cells were seeded in a T-25 cell culture flask in RPMI medium with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin at 37 ˚C for 48 hours. One day before the experiment, cells were transferred to a 35mm tissue culture dishes (Thermo Scientific) to allow the cells to adhere. Cells were incubated with lipopolysaccharide (LPS) which was diluted in cell culture medium and added to the wells in concentration of 100 ng/mL for 16 h. The cells were considered as M1 cells for further probe incubation and flow cytometry analysis.

RAW 264.7 cells were seeded in a T-25 cell culture flask in RPMI medium with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin at 37 ˚C for 48 hours. One day before the experiment, cells were transferred to a 35mm tissue culture dishes (Thermo Scientific) to allow the cells to adhere. Cells were incubated with interleukin 4 (IL-4) which was diluted in cell culture medium and added to the wells in concentration of 20 ng/mL for 72 h. The resulting cells were considered as M2 cells for further probe incubation and flow cytometry analysis.

The results were shown in Fig. 4a

### 5.8 Confocal fluorescence microscope cell imaging experiments of the COU-LIP-1 for $Fe^{2+}$ during the LPS-induced activation

RAW 264.7 cells were seeded in a T-25 cell culture flask in RPMI medium with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin at 37 ˚C for 48 hours. One day before the experiment, RAW264.7 cells cells were transferred to 3 Nunc 35 mm glass-bottom cell culture dishes (Thermo Scientific) to allow the cells to adhere. Cells were incubated with Lipopolysaccharide (LPS) which was diluted in cell culture medium and added to the wells in concentration of 100 ng/mL for the indicated times (0, 4 h, 8 h and 16 h). Then, the cells were washed with HBSS and then incubated with **COU-LIP-1** (10 μM from 10 mM stock in DMSO) in PBS buffer for 30 min. After incubation, the cells were washed with HBSS and then imaged. Cells were imaged on a Leica TCS SP8 equipped with 40× objective lens and PMT gain of 800 ($\lambda_{ex/em}$ = 458/488±20 nm). The results were shown in **Figure S15**.

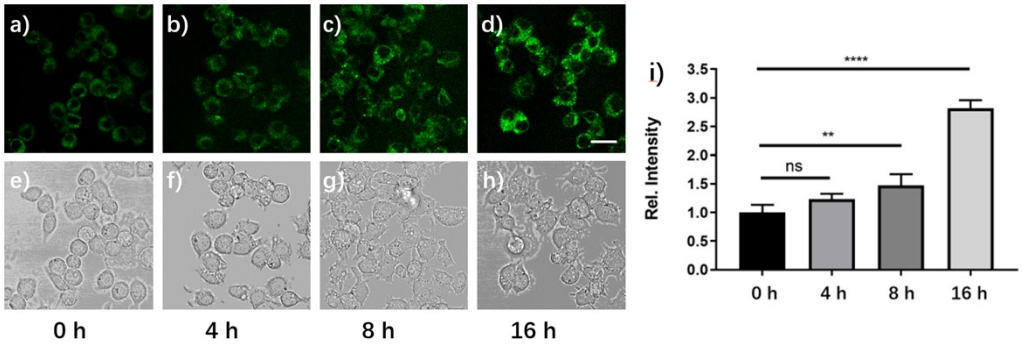



**Figure S15** a-d) Confocal fluorescence images of RAW 264.7 cells: a) Cells were pretreated with 100 ng/mL LPS for 0 h (a), 4 h (b), 8 h (c), 16 h (d) and then were incubated in 10 μM **COU-LIP-1** for 30 min; e-h) Corresponding bright field images. Cell images were captured on a Leica TCS with $\lambda_{ex/em}$ = 458/488±20 nm (scale bar = 20 μm); i) Corresponding relative fluorescence intensities of the RAW 264.7 cells in the fluorescence images. Error bars represent the standard deviations of three replicates;

****P ⩽ 0.0001, **P ⩽ 0.01, ns: no significant difference.

### 5.9 Time-dependent flow cytometry studies of COU-LIP-1 for labile $Fe^{2+}$ levels in LPS-induced activation to form RAW 264.7-M1 cells

RAW 264.7 cells were seeded in a T-25 cell culture flask in RPMI medium with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin at 37 ˚C for 48 hours. One day before the experiment, cells were transferred to a Nunc 6-well plate (Thermo Scientific) to allow the cells to adhere. Cells were incubated with LPS (100 ng/mL) for the indicated time (0, 4, 8, and16 h). The cells were applied to the standard procedure for probe incubation and cytometry analysis (See ESI 5.4 for more details).

The results were shown in Fig. 4b

### 5.10 Time-dependent flow cytometry studies of COU-LIP-1 for erastin-induced ferroptosis in RAW 264.7 -M0 cells

RAW264.7 cells were maintained in RPMI media (Adamas) supplied with 10% Fetal Bovine Serum (FBS, Sangon Biotech), 1% Penicillin-Streptomycin (Gibco) at 37 °C in a humidified atmosphere containing 95% air and 5% $CO_2$ for 48 hours. The cells were in M0 state and considered as RAW 264.7-M0 cells. One day before the experiment, cells were transferred to a Nunc 6-well plate (Thermo Scientific) to allow the cells to adhere. For studying the ferroptosis process, RAW 264. 7 cells were treated with 10 μM erastin (diluted from 10 mM stock in DMSO with the cell culture media) in the presence or absence of 10 μM Fer-1 at 37 °C for the indicated times (0, 4, and 8 h) and then applied to the standard procedure for probe incubation and cytometry analysis (See SI 5.4 for more details).

The results were shown in Fig. 4c.

### 5.11 Time-dependent flow cytometry studies of COU-LIP-1 for erastin-induced ferroptosis in RAW 264.7-M1 cells

RAW 264.7 cells were seeded in a T-25 cell culture flask in RPMI medium with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin at 37 ˚C for 48 hours. One day before the experiment, cells were transferred to a Nunc 6-well plate (Thermo Scientific) to allow the cells to adhere. Cells were incubated with lipopolysaccharide (LPS) which was diluted in cell culture medium and added to the wells in concentration of 100 ng/mL for 16 h. The cells were considered as RAW 264.7-M1 cells.

One day before the experiment, the RAW 264.7-M1 cells were transferred to a 35 mm tissue culture dishes (Thermo Scientific) to allow the cells to adhere. For studying the ferroptosis process, RAW 264. 7-M1 cells were treated with 10 μM erastin (diluted from 10 mM stock in DMSO with the cell culture media) in the presence or absence of 10 μM Fer-1 at 37 °C for the indicated times (0, 4, and 8 h) and then applied to the standard procedure for probe incubation and cytometry analysis (See SI 5.4 for more details).

The results were shown in Fig. 4d.

### 5.12 Confocal fluorescence microscope cell imaging experiments of COU-LIP-1 for erastin-induced ferroptosis in RAW 264.7-M1 cells

RAW 264.7 cells were seeded in a T-25 cell culture flask in RPMI medium with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin at 37 ˚C for 48 hours. One day before the experiment, RAW264.7 cells were transferred to Nunc 35 mm glass-bottom cell culture dishes (Thermo Scientific) to allow the cells to adhere. Cells were incubated with lipopolysaccharide (LPS) which was diluted in cell culture medium and added to the wells to a final concentration of 100 ng/mL for 16 h to afford RAW 264.7-M1 cells.

For imaging the ferroptosis process, RAW 264.7-M1 cells were treated with 10 μM erastin in the presence or absence of 10 μM Fer-1 at 37 °C for the indicated times (0, 4 and 8 h). Then, the cells were washed with HBSS and then incubated with 10 μM **COU-LIP-1** in PBS buffer for 30 min. After incubation, the cells were washed with HBSS and then imaged on a Leica TCS SP8 equipped with 40× objective lens and PMT gain of 800 ($\lambda_{ex/em}$ = 458/488±20 nm). The results were shown in **Figure S16**.

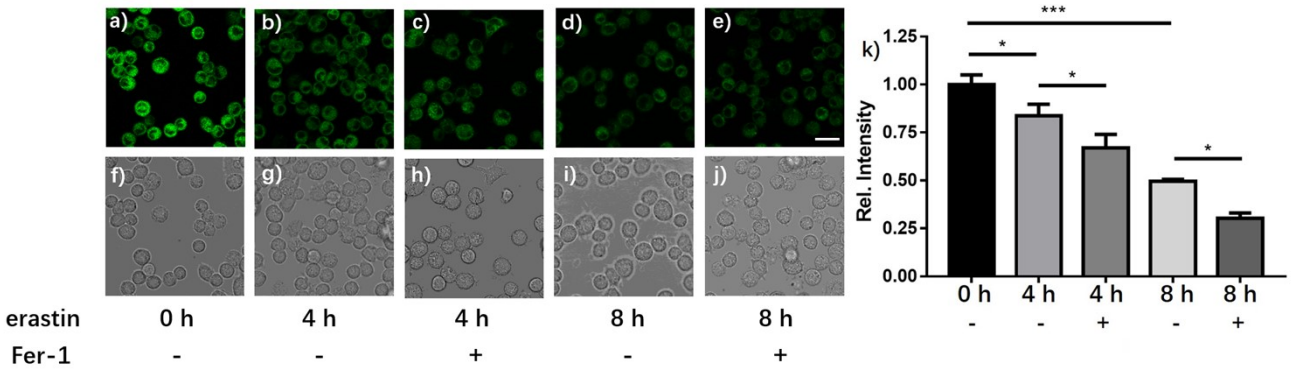



**Figure S16** a-e) Confocal fluorescence images of RAW 264.7-M1 cells: a) Cells were pretreated with 100 ng/mL LPS for 16 h, and then treated with 10 μM erastin in the presence or absence of 10 μM Fer-1 at 37 °C for the indicated times (0, 4 and 8 h): a) control LPS activated RAW 264.7-M1 cells; b) RAW 264.7-M1 cells pretreated with erastin for 4 h; c) RAW 264.7-M1 cells pretreated with both erastin and Fer-1 for 4 h; d) RAW 264.7-M1 cells pretreated with erastin for 8 h; c) RAW 264.7-M1 cells pretreated with both erastin and Fer-1 for 8 h; e-h) Corresponding bright field images. Cell images were captured on a Leica TCS with $\lambda_{ex/em}$ = 458/488±20 nm (scale bar = 20 μm); k) Corresponding relative fluorescence intensities of the RAW 264.7-M1 cells in the fluorescence images. Error bars represent the standard deviations of three replicates; ***P ≤ 0.001, *P ≤ 0.05.

### 5.13 Confocal fluorescence imaging studies of COU-LIP-1 and Mitotracker Red for erastin-induced ferroptosis in RAW 264.7-M1 cells

RAW 264.7 cells were seeded in a T-25 cell culture flask in RPMI medium with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin at 37 ˚C for 48 hours. One day before the experiment,

RAW264.7 cells were transferred to Nunc 35 mm glass-bottom cell culture dishes (Thermo Scientific) to allow the cells to adhere. Cells were incubated with lipopolysaccharide (LPS) which was diluted in cell culture medium and added to the wells to a final concentration of 100 ng/mL for 16 h to afford RAW 264.7-M1 cells.

For imaging the ferroptosis process, RAW 264.7-M1 cells were treated with 10 μM erastin in the presence or absence of 10 μM Fer-1 at 37 °C for the indicated times (0, 4 and 8 h). Then, the cells were washed with HBSS and then incubated with 10 μM **COU-LIP-1** in PBS buffer for 20 min, and then 100 nM MitoTracker Red was added and incubated for additional 10 min. The cells were washed with HBSS and then imaged on a Leica TCS SP8 equipped with 40× objective lens and PMT gain of 800 ($\lambda_{ex/em}$ = 458/488±20 nm , $\lambda_{ex/em}$ = 561/599±20 nm). Pearson's colocalization coefficients were analyzed with Image J software. The results were shown in **Figure S17**.

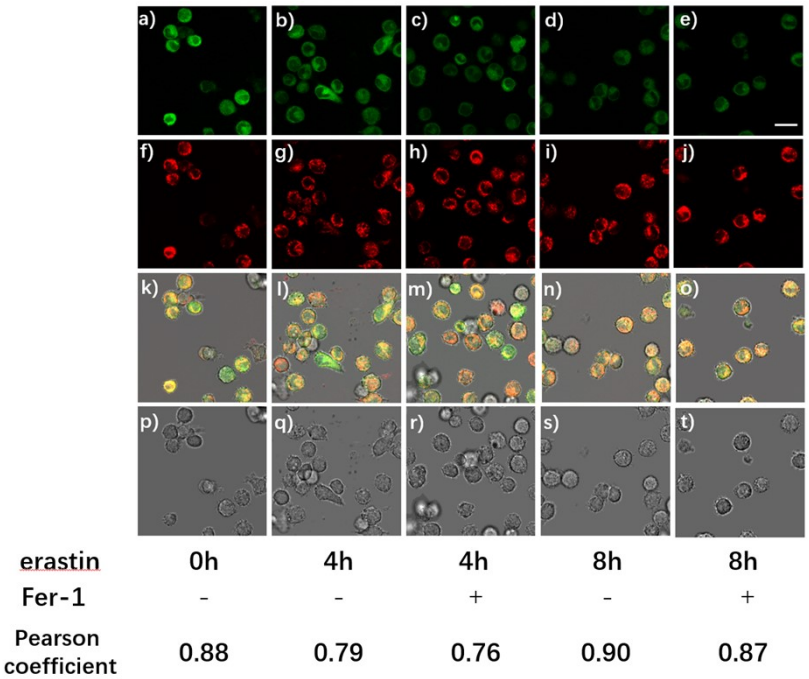



**Figure S17** Colocalization fluorescence imaging studies of **COU-LIP-1** and Mitotracker Red for erastin-induced ferroptosis in RAW 264.7-M1 cells: a, f, k, p) Cells were incubated in normal media and then incubated in media containing 10 μM **COU-LIP-1** for 20 min (total 30 min) and then 100 nM MitoTracker Red added for additional 10 min; b, g, f, q) Cells were incubated in 10 μM erastin for 4 h, and then incubated in media containing 10 μM **COU-LIP-1** for 20 min (total 30 min) and then 100 nM MitoTracker Red added for additional 10 min; c, h, m, r) Cells were incubated in 10 μM erastin in the presence of 10 μM Fer-1 for 4 h, and then incubated in media containing 10 μM **COU-LIP-1** for 20 min (total 30 min) and then 100 nM MitoTracker Red added for additional 10 min; d, i, n, s) Cells were incubated in 10 μM erastin for 8 h, and then incubated in media containing 10 μM **COU-LIP-1** for 20 min (total 30 min) and then 100 nM MitoTracker Red added for additional 10 min; e, j, o, t) Cells were incubated in 10 μM erastin in the presence of 10 μM Fer-1 for 8 h, and then incubated in media containing 10 μM **COU-LIP-1** for 20 min (total 30 min) and then 100 nM MitoTracker Red added for additional 10 min. Cell images were captured on a Leica TCS with 40× objective lens and PMT gain of 800 ($\lambda_{ex/em}$ = 458/488±20 nm , $\lambda_{ex/em}$ = 561/599±20 nm), scale bar = 20 μm.

## Part VIII: NMR and HRMS Data

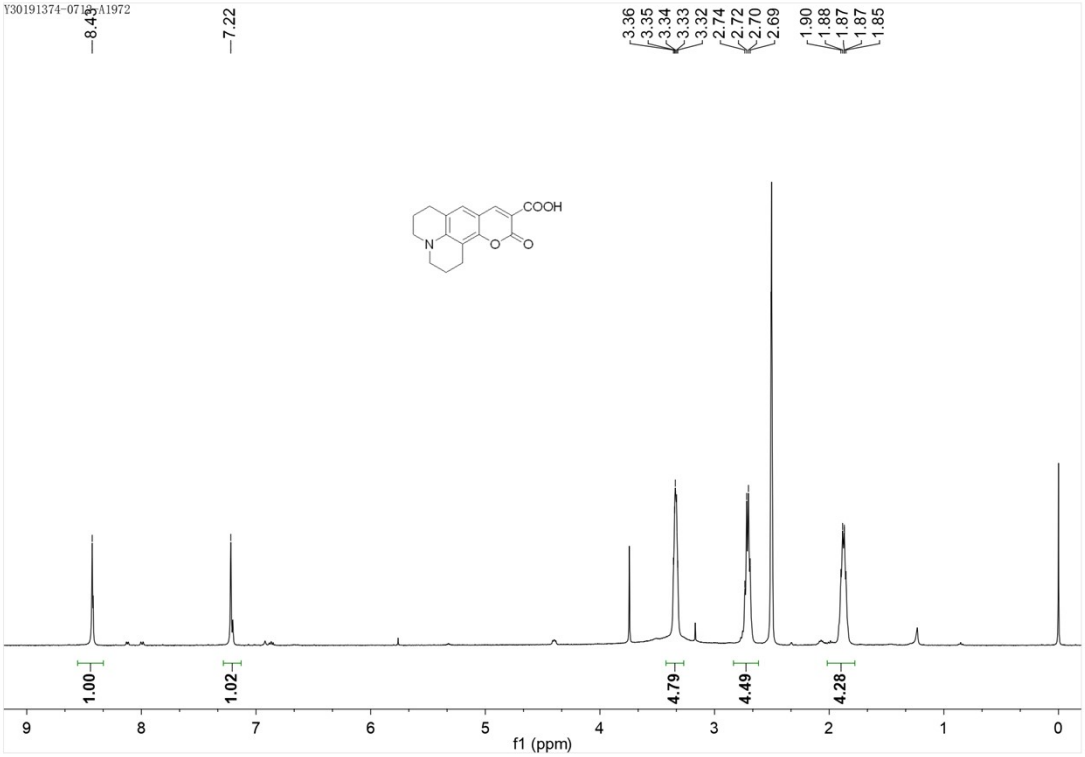
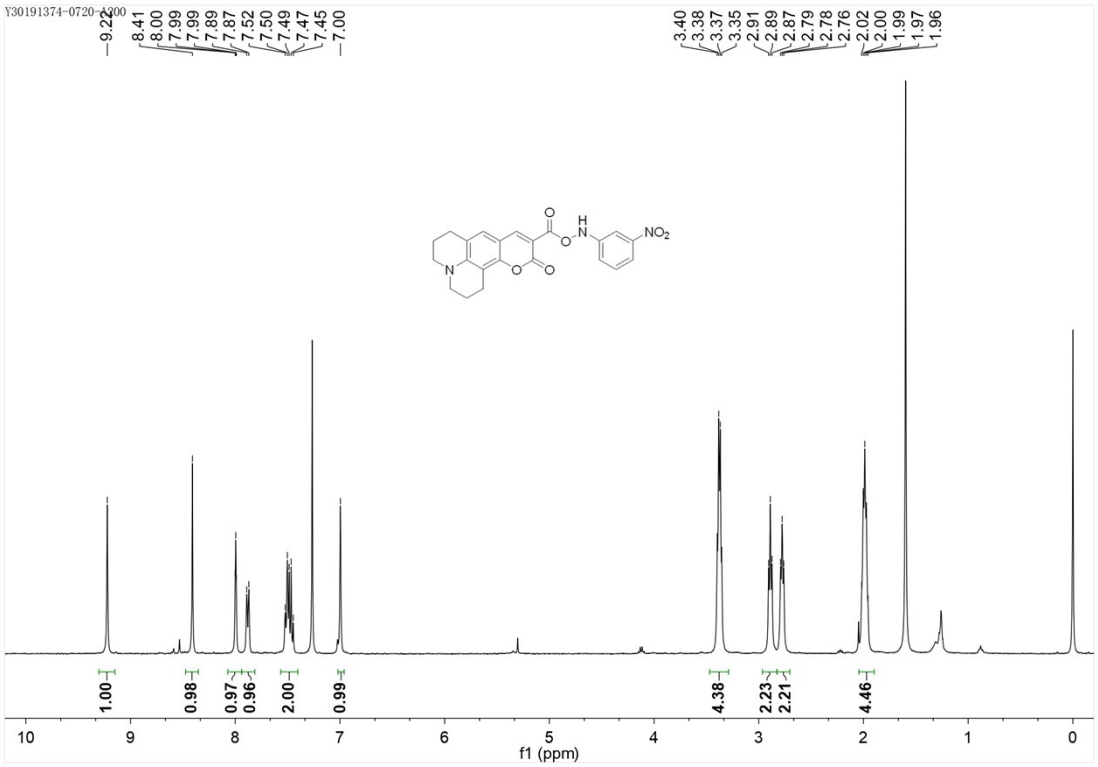



**Figure S18** $^{1}$H NMR of the Coumarin 343.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8.41
8.00
7.99
7.99
7.89
7.87
7.52
7.50
7.49
7.47
7.45
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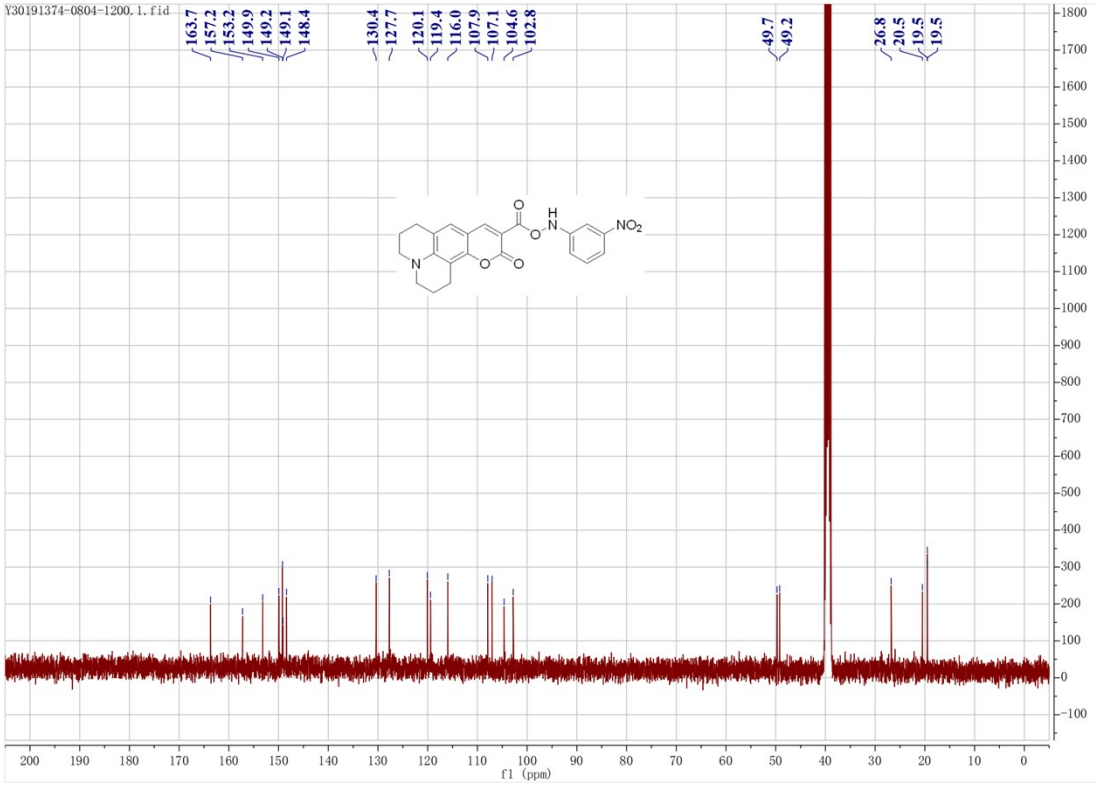
3.40
3.38
3.37
3.35
2.91
2.89
2.87
2.79
2.78
2.76
2.02
2.00
1.99
1.97
1.96
1.00
0.98
0.97
0.96
2.00
0.99
4.38
2.23
2.21
4.46
f1 (ppm)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153.2
149.9
149.2
149.1
148.4
130.4
127.7
120.1
119.4
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49.7
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20.5
19.5
19.5
f1 (ppm)

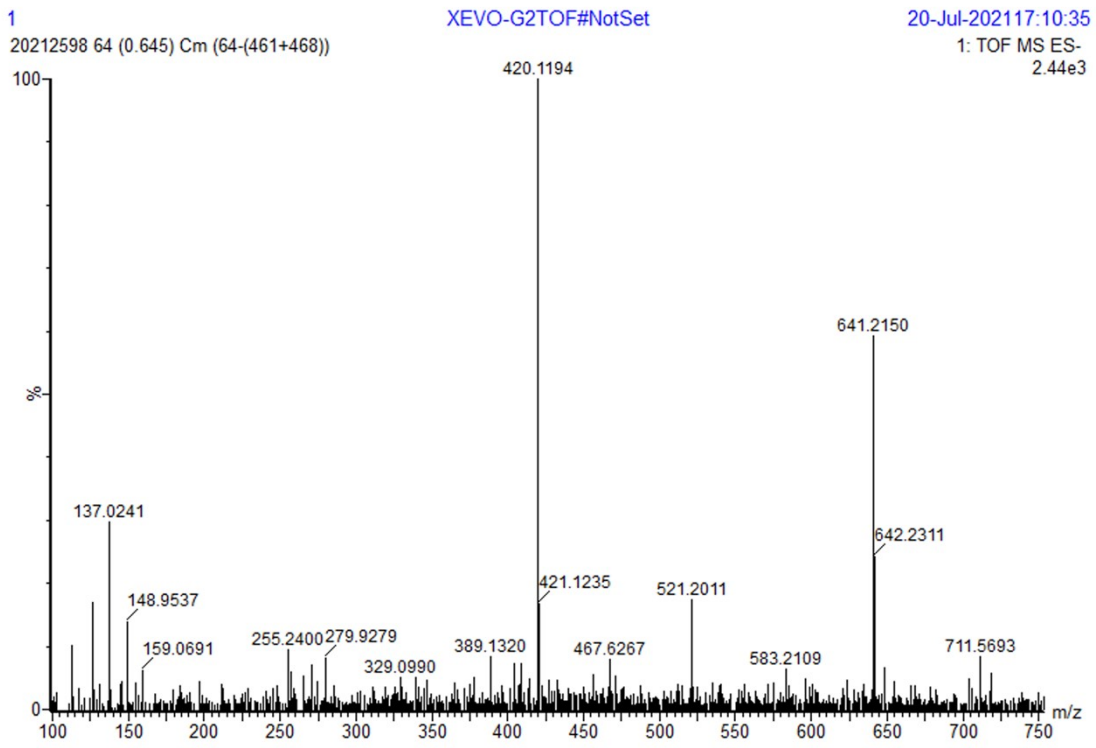


**Figure S19** $^{1}$H NMR, $^{13}$C NMR, and HRMS of the probe **COU-LIP-1**.

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