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Supplementary Information

NIR Fluorescent Probe for Accurate Diagnosis of Acute Kidney Injury by Monitoring Leucine Aminopeptidase

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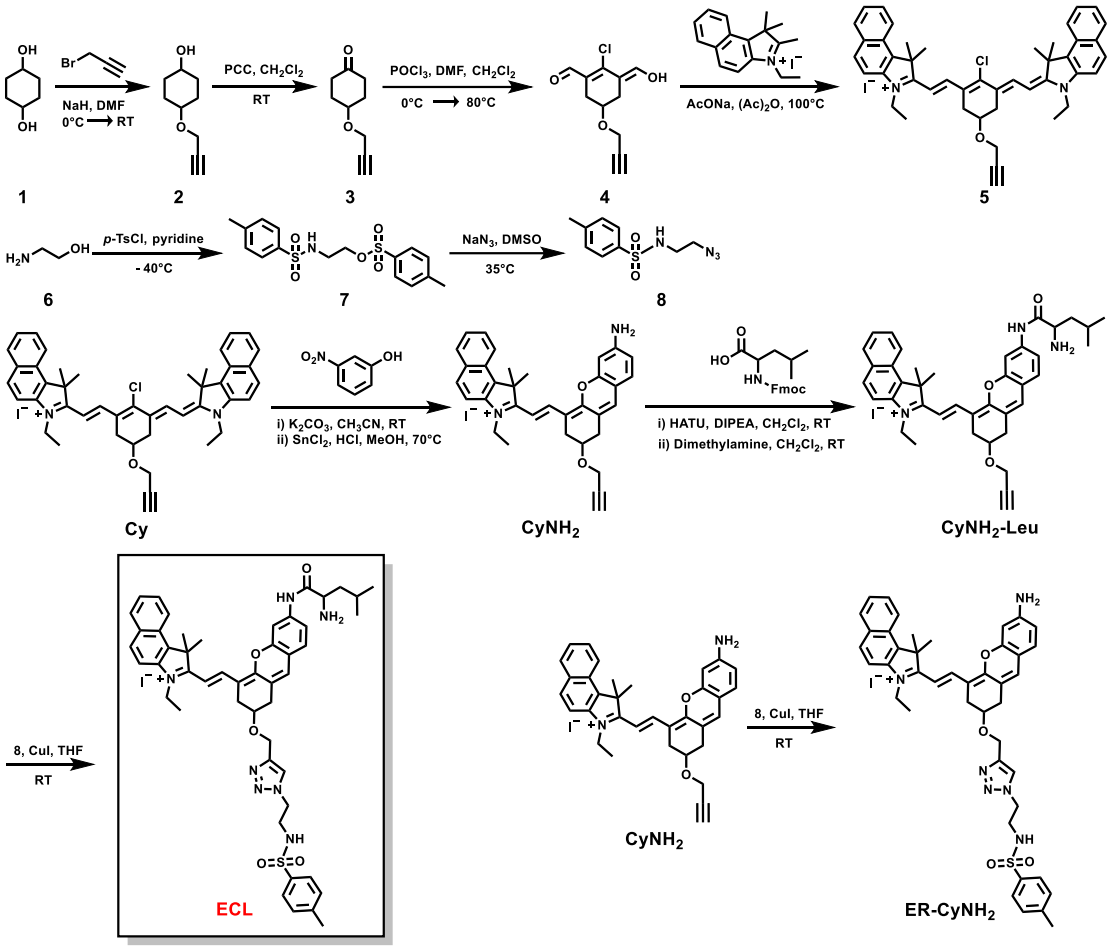
1. Experimental Section

1.1. Apparatus and reagents

All reagents were purchased from commercial suppliers and directly used without further treatment. ^{1}H-NMR and ^{13}C-NMR spectra for all compounds were recorded on a Bruker AV 400 spectrometer. Agilent 1260-6224 TOF LC-MS spectrometer was used to acquire high-resolution mass spectra (HRMS). UV absorption spectra were obtained with SHIMADZU UV-1800 spectrometer. Fluorescence emission spectra were obtained using Agilent Cary Eclipse spectrometer. High-performance liquid chromatograms were acquired on SHIMADZU LC-16 chromatography. Confocal fluorescence imaging was conducted with Zeiss LSM 900 microscope. NIRF imaging in mice was conducted on VISQUE InVivo Smart-LF systems. The stained images with hematoxylin-eosin (H&E) were obtained via Olympus IX51 microscope. The optical density (OD) was recorded on a microplate reader (CMax Plus).

Leucine aminopeptidase (LAP), aprotinin, cellulase, lipase, tyrosinase, β-galactosidase and α-chymotrypsin were purchased from Sigma-Aldrich. Cell counting kit-8 (CCK-8) and hoechst 33342 were purchased from Beyotime Biotechnology. HEK-293T cells were purchased from Servicebio. BALB/c-nu mice were purchased from HYcell Biotechnology.

1.2. Synthesis



Scheme S1. Synthetic route of ECL.

Cy and N-(2-azidoethyl)-4-methylbenzenesulfonamide were synthesized according to the reported protocol.[1-3]

Synthesis of CyNH₂: Resorcinol (860 mg, 6.2 mmol) and K_2CO_3 (855 mg, 6.2 mmol) were stirred in 25 mL CH_3CN for 15 min. Under nitrogen atmosphere, Cy (1.4 g, 1.8 mmol) was slowly added to the above suspension. After stirring at room temperature for 24 h, the reaction mixture was distilled to remove the solvent. The obtained residue was dissolved in CH_2Cl_2, extracted with water, washed with brine and dried over anhydrous Na_2SO_4. After filtering, the filtrate was dried to obtain a solid for standby use. $SnCl_2$ (12.5 g, 65.9 mmol) was dissolved in concentrated HCl (12 mL). Under nitrogen atmosphere, the above solid was dissolved in CH_3OH (50 mL) and added dropwise to the HCl solution of $SnCl_2$. After reacting at 70 ℃ for 24 h,

sodium carbonate solution was added to the reaction mixture to adjust the pH value to 8.0. After filtering, the filtrate was concentrated under reduced pressure. The obtained residue was dissolved in CH_2Cl_2, extracted with water, washed with brine, dried over anhydrous Na_2SO_4, and filtered. The resulting filtrate was concentrated and purified by silica gel column chromatography (CH_2Cl_2/CH_3OH = 20/1) to obtain $CyNH_2$ (300 mg, 27%) as a dark green solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.77 (d, *J* = 14.4 Hz, 1H), 8.28 (d, *J* = 8.4 Hz, 1H), 8.07 (d, *J* = 8.8 Hz, 1H), 8.04 (d, *J* = 8.4 Hz, 1H), 7.68 (d, *J* = 8.8 Hz, 2H), 7.56 – 7.51 (t, 2H), 7.35 (d, *J* = 9.2 Hz, 1H), 6.77 – 6.72 (m, 2H), 6.33 (d, *J* = 14.4 Hz, 1H), 4.40 (q, *J* = 7.2 Hz, 2H), 4.31 (d, *J* = 2.4 Hz, 2H), 4.25 (q, *J* = 5.2 Hz, 1H), 3.04 - 2.96 (m, 2H), 2.93 (t, *J* = 3.6 Hz, 2H), 2.90 (t, *J* = 2.4 Hz, 1H), 2.06 (s, 6H), 1.51 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 175.9, 162.4, 156.1, 155.1, 143.0, 139.1, 134.3, 132.4, 130.7, 129.9, 129.5, 128.0, 127.6, 125.1, 122.0, 120.1, 114.2, 113.4, 110.8, 110.5, 99.3, 97.6, 79.5, 74.6, 70.1, 55.2, 39.5, 32.9, 26.8, 11.4. HRMS (ESI) m/z calculated for $C_{34}H_{33}N_2O_2^+$ $[M]^+$ 501.25365, found 501.25359.

Synthesis of $CyNH_2$-Leu: Fmoc-L-Leu (70 mg, 0.2 mmol), HATU (76 mg, 0.2 mmol) and DIPEA (26 mg, 0.2 mmol) were stirred in anhydrous CH_2Cl_2 (4 mL) under ice-cooling. After stirring for 30 min, $CyNH_2$ (63 mg, 0.1 mmol) was slowly added to the above solution. The mixture was warmed to room temperature and reacted for 24 h. After that, the solvent was distilled off under reduced pressure, and a crude product was obtained after simple purification by silica gel column chromatography (CH_2Cl_2/CH_3OH = 400/1) and directly used in the next step without further purification. Dimethylamine (2M solution in THF, 0.3 mL) was added dropwise to the solution of the crude product in anhydrous CH_2Cl_2 (2 mL). The mixture was stirred at room temperature for 3 h. After that, the solvent was distilled off under reduced pressure and the resulting residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH = 200/1) to obtain $CyNH_2$-Leu (12 mg, 16%) as a blue solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.74 (d, *J* = 15.2 Hz, 1H), 8.26 (d, *J* = 8.4 Hz, 1H), 8.03 – 7.96 (m, 2H), 7.92 (d, *J* = 6.4 Hz, 1H), 7.71 (t, *J* = 7.6 Hz, 1H), 7.60 – 7.55 (m, 1H), 7.52 (d, *J* = 8.8 Hz, 1H), 7.34 (d, *J* = 8.4 Hz, 1H), 7.28 (d, *J* = 8.4 Hz, 1H), 7.11 (s, 1H), 6.48 (d, *J* = 15.2 Hz, 1H), 4.52-4.41 (m, 2H), 4.28 (d, *J* = 2.4 Hz, 2H), 4.25 - 4.23 (m, 1H), 3.72 - 3.67 (m, 1H), 2.98 – 2.88 (m, 4H), 2.44 (t, *J* = 2.4 Hz, 1H), 2.22 - 2.16 (m, 1H), 2.04 (s, 6H), 1.59 – 1.49 (m, 5H), 1.01 (dd, *J* = 8.8, 6.4 Hz, 6H). ^{13}C

NMR (101 MHz, $CDCl_3$) δ 179.0, 175.8, 159.9, 153.4, 145.7, 141.0, 138.2, 136.6, 133.6, 132.9, 131.4, 130.1, 128.4, 128.0, 127.8, 126.6, 126.2, 122.8, 118.2, 115.3, 111.1, 105.8, 104.1, 79.9, 74.7, 69.8, 64.2, 59.8, 56.3, 52.7, 41.2, 40.4, 34.3, 29.7, 29.3, 25.4, 23.4, 21.6, 13.1. HRMS (ESI) m/z calculated for $C_{40}H_{44}N_3O_3^+$ $[M]^+$ 614.33772, found 614.33728.

Synthesis of ECL: Under nitrogen atmosphere, $CyNH_2$-Leu (44 mg, 0.06 mmol), CuI (22 mg, 0.12 mmol) and N-(2-azidoethyl)-4-methylbenzenesulfonamide (22 mg, 0.09 mmol) were dissolved in 2 mL of anhydrous THF and stirred at room temperature for 12 h. After that, the catalyst was removed by neutral alumina, and the crude product obtained after vacuum distillation was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH = 100/1) to obtain ECL (52 mg, 88%) as a blue solid. ^1H NMR (400 MHz, $CDCl_3$) δ 8.73 (d, *J* = 14.8 Hz, 1H), 8.28 (d, *J* = 8.4 Hz, 1H), 8.01 – 7.94 (m, 2H), 7.91 (s, 1H), 7.87 (s, 1H), 7.69 (d, *J* = 8.4 Hz, 3H), 7.53 (d, *J* = 7.6 Hz, 1H), 7.47 (dd, *J* = 9.2, 4.0 Hz, 2H), 7.24 (d, *J* = 7.2 Hz, 3H), 7.10 (s, 1H), 6.48 (d, *J* = 14.8 Hz, 1H), 4.72 (s, 2H), 4.51 – 4.40 (m, 4H), 4.07 – 3.99 (m, 1H), 3.38 (t, *J* = 5.6 Hz, 2H), 2.91 – 2.81 (m, 4H), 2.35 (s, 3H), 2.02 - 1.95 (m, 7H), 1.85 – 1.77 (m, 2H), 1.57 - 1.50 (m, 4H), 0.99 (dd, *J* = 6.0, 3.2 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.9, 158.8, 147.9, 144.6, 143.6, 142.3, 140.3, 137.3, 135.7, 133.0, 131.7, 130.3, 128.8, 127.4, 126.8, 126.0, 125.4, 124.8, 124.0, 121.8, 117.0, 115.6, 110.3, 110.2, 110.0, 105.1, 102.8, 92.0, 60.5, 53.0, 52.4, 51.6, 49.0, 42.0, 40.0, 32.8, 28.7, 26.7, 26.3, 23.8, 20.5, 20.3, 12.1. HRMS (ESI) m/z calculated for $C_{49}H_{56}N_7O_5S^+$ $[M]^+$ 854.40582, found 854.40416.

1.3. Preparation of sample solutions

ECL was dissolved in CH_3CN to obtain a 10 mM stock solution. In selective study, stock solutions of all analytes were prepared in PBS buffer (pH 7.4, 20 mM). All stock solutions and LAP were diluted in PBS buffer to desired concentrations for spectral analysis.

1.4. Determination of the limit of detection

The detection limit was determined from the fluorescence titration assay. The fluorescence intensity of ECL (10 μM) was measured after incubation with various concentrations of LAP. The standard deviation of blank measurement was calculated by the fluorescence intensity of ECL in the absence of LAP. To gain the slop, the

fluorescent intensity at 717 nm was plotted as LAP concentration. The detection limit of ECL toward LAP was calculated by the following equation:

$$\text{Detection limit} = 3\sigma/k \quad \text{(S1)}$$

Where σ was the standard deviation of blank measurement. k was the slope of the plot of fluorescence intensity (717 nm) against LAP concentration.

1.5. Selective study

ECL (10 μM) was incubated with various analytes in PBS buffer (pH 7.4, 20 mM) at 37 ℃ for 30 min. The concentrations of all analytes were as follows: KCl (150 mM); $MgCl_2$ (2.5 mM); NaClO, H_2O_2, Ala, Arg, Glu, Met, Pro (100 μM); Cys, Hcy (1 mM); GSH (10 mM); Sulfatase (180 U/L); Cellulase, lipase, tyrosinase, β-Gal (100 U/L); Aprotinin (50 U/L); α-Chymotrypsin (400 U/L); LAP (25 U/L).

1.6. Enzyme kinetic study

Different concentrations of ECL (1, 1.7, 3.3, 5, 7.7, and 10 mM) were incubated with LAP (25 U/L) in PBS buffer (pH 7.4, 20 mM). The fluorescence intensity at 717 nm was collected every 1 min and recorded continuously for 10 min. The initial enzymatic cleavage rate (v) was calculated, plotted against ECL concentration, and fitted with a Michaelis-Menten curve. The Michaelis constant (K_m) and maximum reaction rate (V_{max}) were calculated according to the Lineweaver-Burk formula.

$$\frac{1}{v} = \frac{K_m}{V_{max} \times [S]} + \frac{1}{V_{max}}$$

Where v represents the initial reaction rate, V_{max} represents the maximum reaction rate, [S] represents the ECL concentration, and K_m represents the Michaelis constant.

1.7. Response mechanism study

ECL (10 μM) were incubated with LAP (25 U/L) in PBS buffer (pH 7.4, 20 mM) at 37 °C for 5 min, followed by high performance liquid chromatography (HPLC) and high resolution mass spectrometry (HRMS) analysis. HPCL chromatograms were acquired with 80% methanol (containing 0.1% HCOOH) and 20% water (containing 0.1% HCOOH) as the eluent and 600 nm as the detection wavelength.

1.8. Cell culture

Human embryonic kidney (HEK-293T) cells were cultured in Dulbecco's

modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptavidin. Cell culture was conducted in a humidified incubator maintaining 5% CO_2 and 95% air at 37 ℃. Cells were for passage when the confluence reached over 80%.

1.9. Cytotoxicity assay

Cells Counting Kit-8 (CCK-8) assay was conducted to evaluate cytotoxicity of ECL against HEK-293T cells. The cells (1×10^4 cells/well) were seeded in 96-well plates and then allowed to adhere for 24 h. After removing the culture medium, the cells were incubated with various concentrations of ECL (0, 2, 5, 10, 15, 20, and 30 μM) at 37 ℃ in an atmosphere of 5% CO_2 for 24 h. The 10% CCK-8 solution prepared with serum-free DMEM was evenly added to each well and incubated for another 4 h. The optical density (OD) of each well was measured at 450 nm using a microplate reader. The cell viability value without ECL treatment was normalized to 100%. The cell viability was calculated by the following equation:

$$\text{Cell viability} = (OD_{sample} - OD_{blank}) / (OD_{control} - OD_{blank}) \quad \text{(S2)}$$

Where OD_{sample} was the optical density of cells incubated with ECL and CCK-8. $OD_{control}$ was the optical density of cells incubated with CCK-8. OD_{blank} was the optical density of CCK-8.

1.10. Confocal fluorescence imaging in cells

HEK-293T cells were seeded in confocal dishes and incubated overnight for cell attachment. After washing off the culture medium, the cells were stained with Hoechst 33342 (5 μg/mL) for 15 min and then incubated with ECL in the complete medium (1 mL). The activity of endogenous LAP was inhibited by bestatin (400 μM). Before imaging, cells were washed with PBS buffer for three times. Confocal fluorescence images of cells were acquired on Laser Scanning Confocal Microscope. The cellular fluorescence intensity was quantified by Image J software. Blue channel: Hoechst 33342, λex = 405 nm and λem = 445 - 490 nm. Red channel: activated ECL. λex = 640 nm and λem = 650 - 700 nm.

1.11. Histological study

Tissues harvested from sacrificed mice were fixed in 4% paraformaldehyde for 24 h. The paraffin sections were obtained after dehydration, embedding in paraffin and slicing. After dewaxing, hematoxylin and eosin (H&E) staining was conducted on the

sections according to the conventional standard method. The stained images were captured under a microscope and processed using CaseViewer software.

1.12. sCr and BUN assay

Blood samples collected from mice were placed at room temperature for 1 h. After coagulation, the blood was centrifuged at 2000 g for 10 min, and the pale yellow supernatant (i.e., serum) was collected and stored at -80 ℃ for later use. According to the instructions of the serum creatinine (sCr) and urea nitrogen (BUN) assay kits, sCr and BUN levels in mouse serum were determined based on the sarcosine oxidase method and urease method, respectively.

1.13. Renal LAP assay

Kidney tissues were rinsed with cooled PBS buffer and cut into small pieces. In a homogenizer, these pieces were mixed with the tissue protein extraction reagent and then homogenized at 0 ℃. The homogenate was transferred to a centrifuge tube and shaken on ice to ensure complete lysis. Subsequently, the homogenate was centrifuged at 12000 g for 5 min at 4 °C and the supernatant was collected. Leucine aminopeptidase (LAP) levels in mouse kidneys were determined based on the L-leucine-4-nitroaniline method according to the instructions of the Elabscience® Leucine Aminopeptidase Colorimetric Assay Kit.

1.14. Establishment of mouse models

All animal experiments were performed in accordance with the Regulation on the Administration of Laboratory Animals of Hubei Province and approved by the Ethics Committee of Hubei University. Female Balb/c-nu mice (5-6 weeks old) were fed in a temperature controlled (22 ± 3 ℃) room with 12 h dark/light cycles.

Mouse model of acute kidney injury: Healthy mice were randomly divided into five groups and injected intraperitoneally with equal doses of cisplatin (20 mg/kg, 100 μL). The next experiment was performed at 0, 12, 24, 48, or 72 h after administration.

Mouse model of breast cancer chemotherapy: 4T1 cells were inoculated into the right armpit of mice at a density of 1×10^6 cells/mouse. After 14 days of feeding, the tumor-bearing mice were randomly divided into four groups and began to receive cisplatin treatment (0 h). The mice in the control group did not receive any treatment, the mice in group I were intraperitoneally injected with cisplatin five times (once every 12 hours, 3 mg/kg for each time), the mice in group II were intraperitoneally

injected with cisplatin three times (once every 24 hours, 5 mg/kg for each time), and the mice in group III were intraperitoneally injected with cisplatin once (15 mg/kg). The next experiment was performed 72 hours after the first dose.

1.15. Fluorescence imaging in mice

Fluorescence images were captured from mice following isoflurane anesthesia using an InVivo Smart-LF system with Cy5.5 filter and HyperRed lamp (excitation at 630 - 680 nm and emission at 690 - 740 nm). Fluorescence intensity was quantified by CleVue software.

1.16. Statistical analysis

The data were expressed as mean ± SD (n = 3). Statistical differences between two groups were tested with a two-tailed Student's t-test and more than three groups were determined by one-way ANOVA or two-way ANOVA with Tukey post-hoc test. For all tests, p values less than 0.05 were considered statistically significant. p values were denoted by (n.s.) for not significant, (*) for $p < 0.05$, (**) for $p < 0.01$, (***) for $p < 0.001$, and (****) for $p < 0.0001$. All statistical calculations were performed using GraphPad Prism 8.0.

2. Supplementary Data and Discussion

2.1. Temperature effects

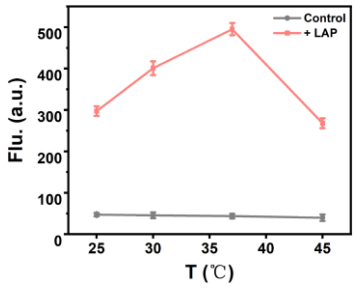


Figure S1. Temperature-dependent fluorescence intensity change (717 nm) of ECL (10 μM) in the absence or presence of LAP (25 U/L). λ_{ex} = 680 nm.

2.2. Response mechanism

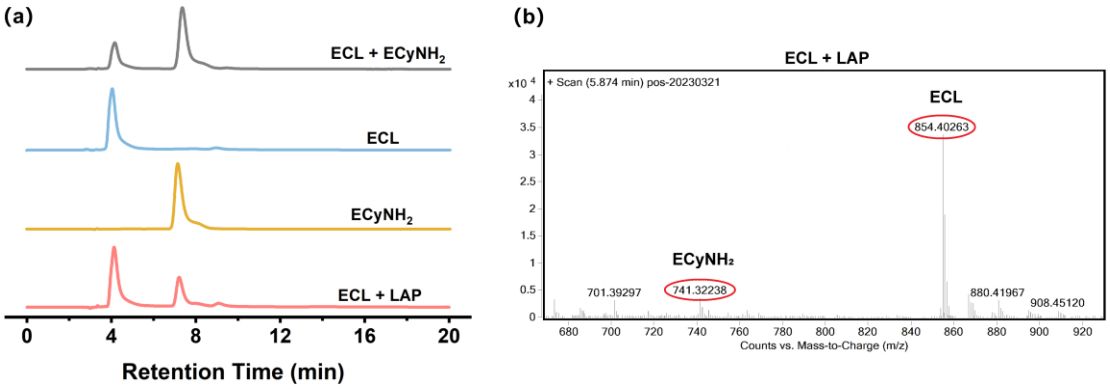


Figure S2. (a) HPLC chromatograms of ECL + $ECyNH_2$, ECL, $ECyNH_2$, and ECL + LAP. (b) HRMS spectrum of ECL in the presence of LAP.

2.3. Cytotoxicity

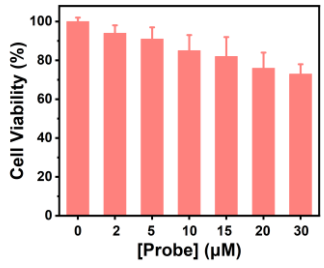


Figure S3. Cytotoxicity assays of ECL at various concentrations (0, 2, 5, 10, 15, 20, and 30 μM) in HEK-293T cells.

2.4. Cell imaging

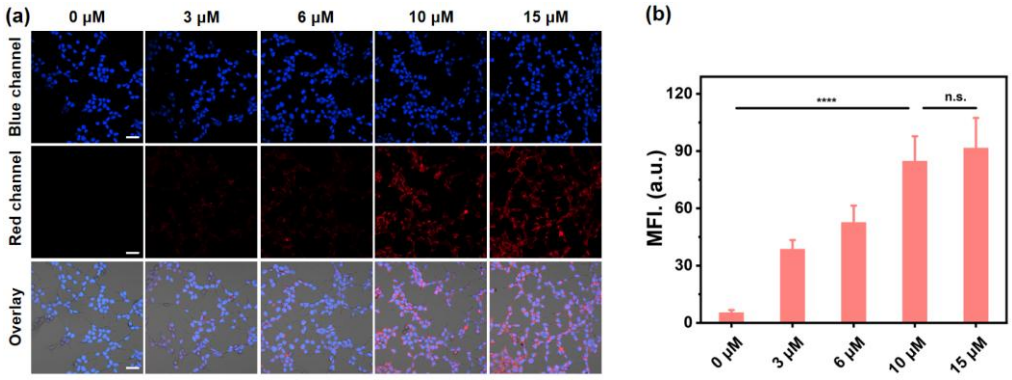


Figure S4. Concentration-dependent fluorescence imaging of ECL in living cells. (a) Confocal fluorescence images of HEK-293T cells incubated with ECL at various concentrations (0, 3, 6, 10, and 15 μM) for 30 min. Scale bar: 40 μm. (b) Mean fluorescence intensities (MFI.) from the red channel in (a). Blue channel: Hoechst 33342, λ_{ex} = 405 nm and λ_{em} = 445 - 490 nm. Red channel: activated ECL. λ_{ex} = 640 nm and λ_{em} = 650 - 700 nm.

To investigate the effect of sulfonamide groups on cell membrane permeability, HEK-293T cells were incubated with a sulfonamide-containing fluorophore (ER-$CyNH_2$) or its sulfonamide-free counterpart ($CyNH_2$), followed by comparison of their intracellular fluorescence intensities. As anticipated, HEK-293T cells treated with ER-$CyNH_2$ exhibited significantly stronger fluorescence signals than those treated with $CyNH_2$ (Figure S5). This finding demonstrates that the incorporation of sulfonamide groups enhances the membrane permeability of the probe.

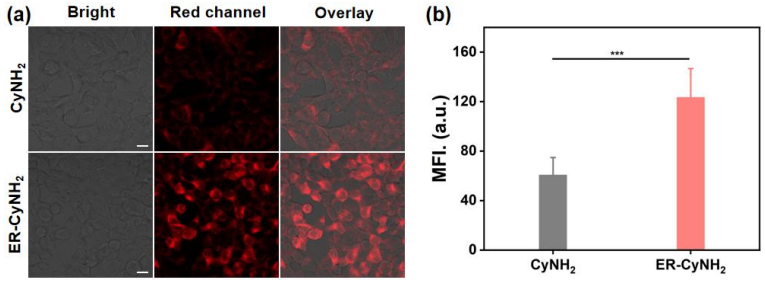


Figure S5. Fluorescence imaging of $CyNH_2$ (without sulfonamide groups) and ER-$CyNH_2$ (with sulfonamide groups) in living cells. (a) Confocal fluorescence images of HEK-293T cells incubated with $CyNH_2$ (10 μM) or ER-$CyNH_2$ (10 μM) for 60 min. Scale bar: 80 μm. (b) Mean fluorescence intensities (MFI.) from the red channel in (a). Red channel: λ_{ex} = 640 nm and λ_{em} = 650 - 700 nm.

2.5. Biocompatibility

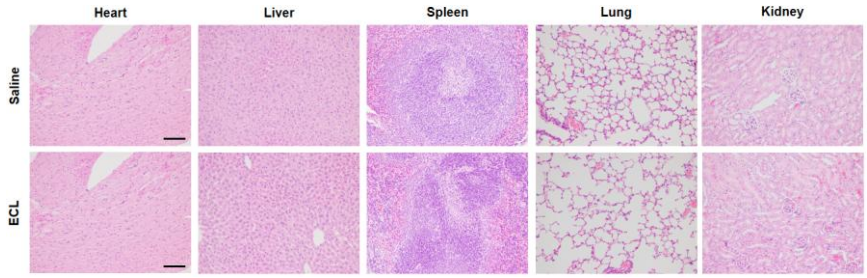


Figure S6. H&E staining of major organ harvest from healthy mice after intravenous injection of saline (100 μL) or ECL (500 μM, 100 μL). Scale bar: 100 μm.

3. Compound Characterization

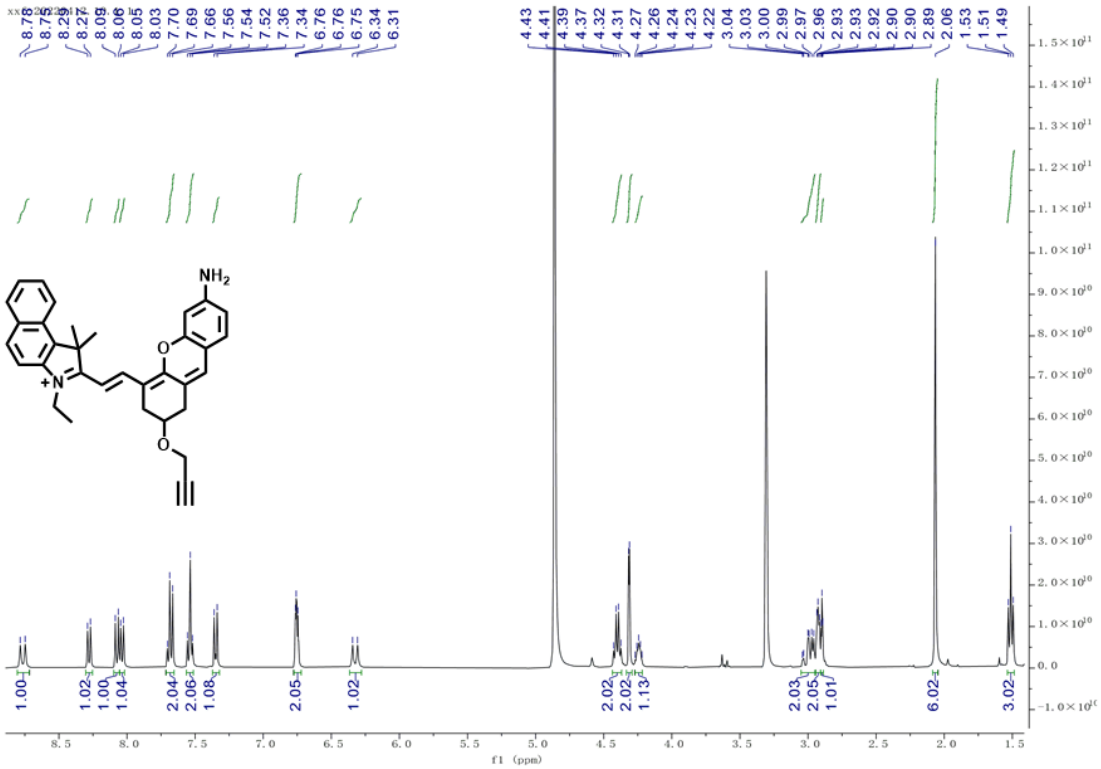


Figure S7. ^{1}H-NMR spectrum of $CyNH_2$.

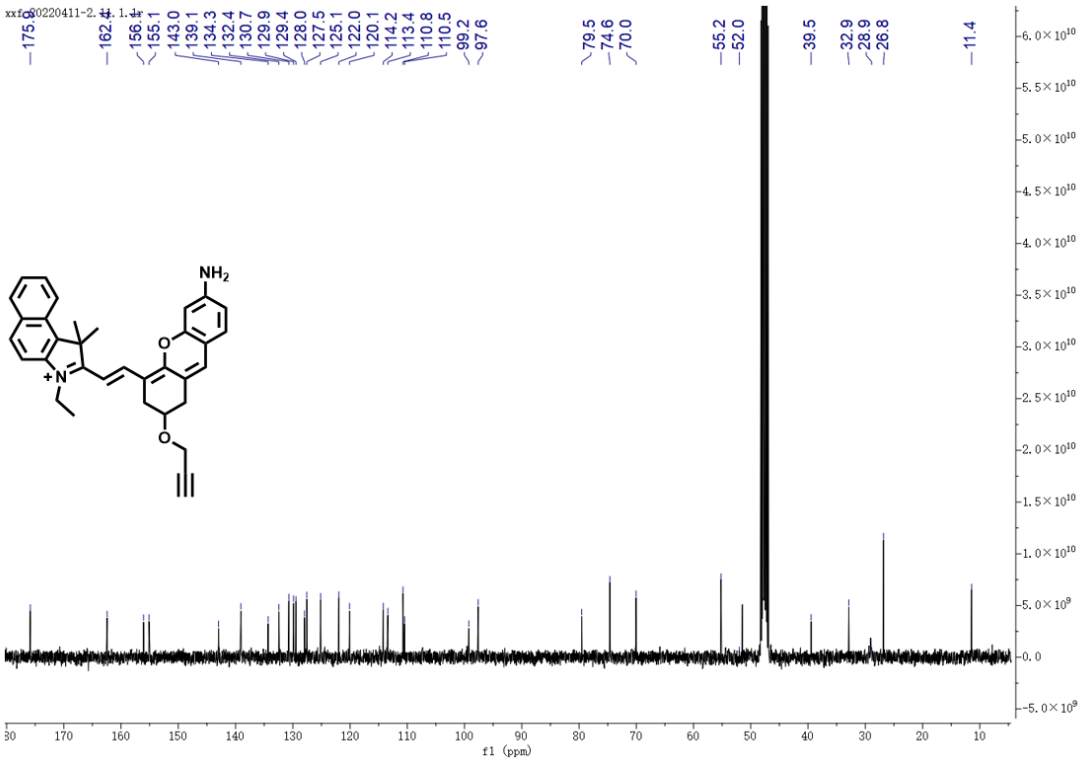


Figure S8. ^{13}C-NMR spectrum of $CyNH_2$.

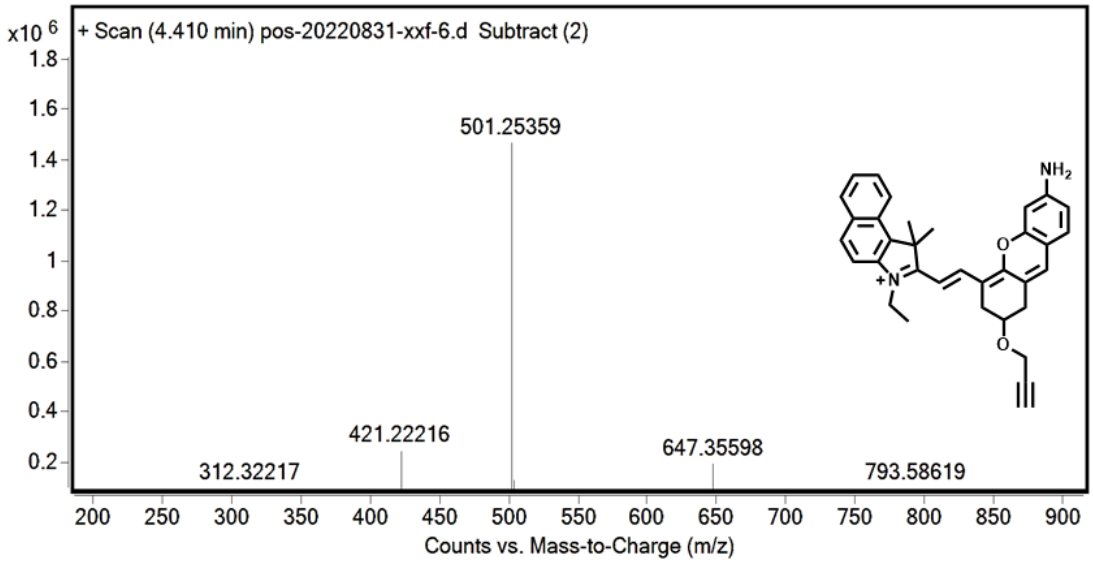


Figure S9. HRMS spectrum of $CyNH_2$.

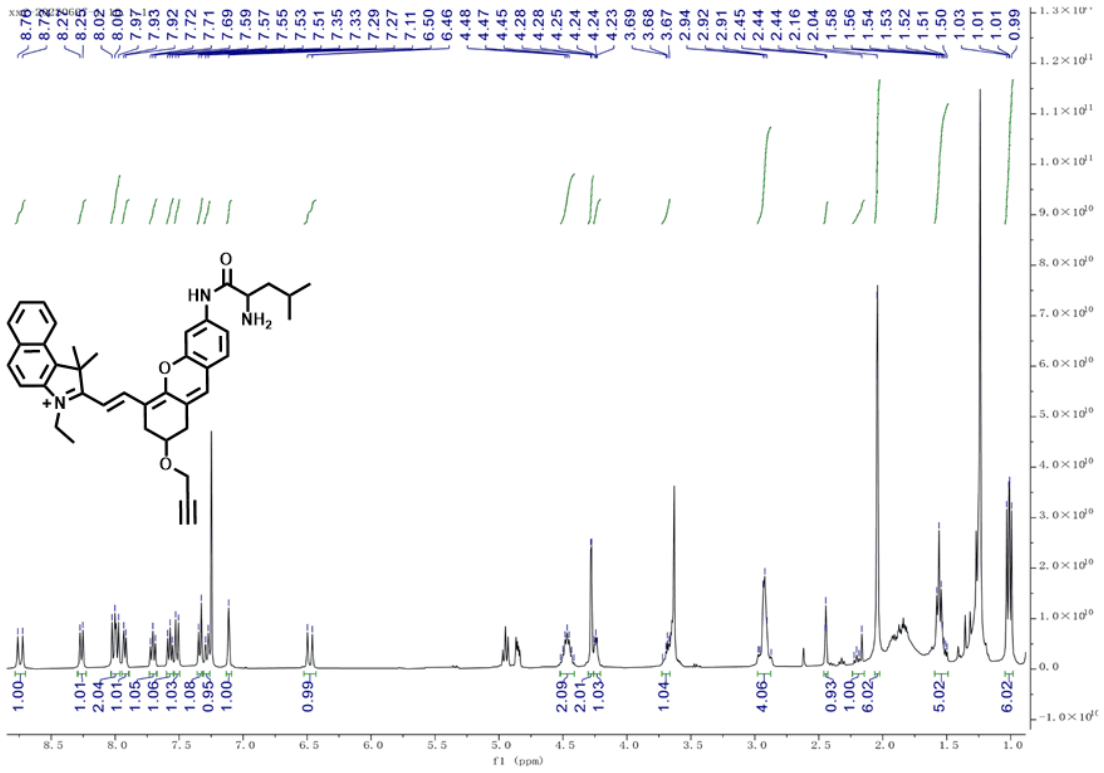


Figure S10. ^{1}H-NMR spectrum of $CyNH_2$-Leu.

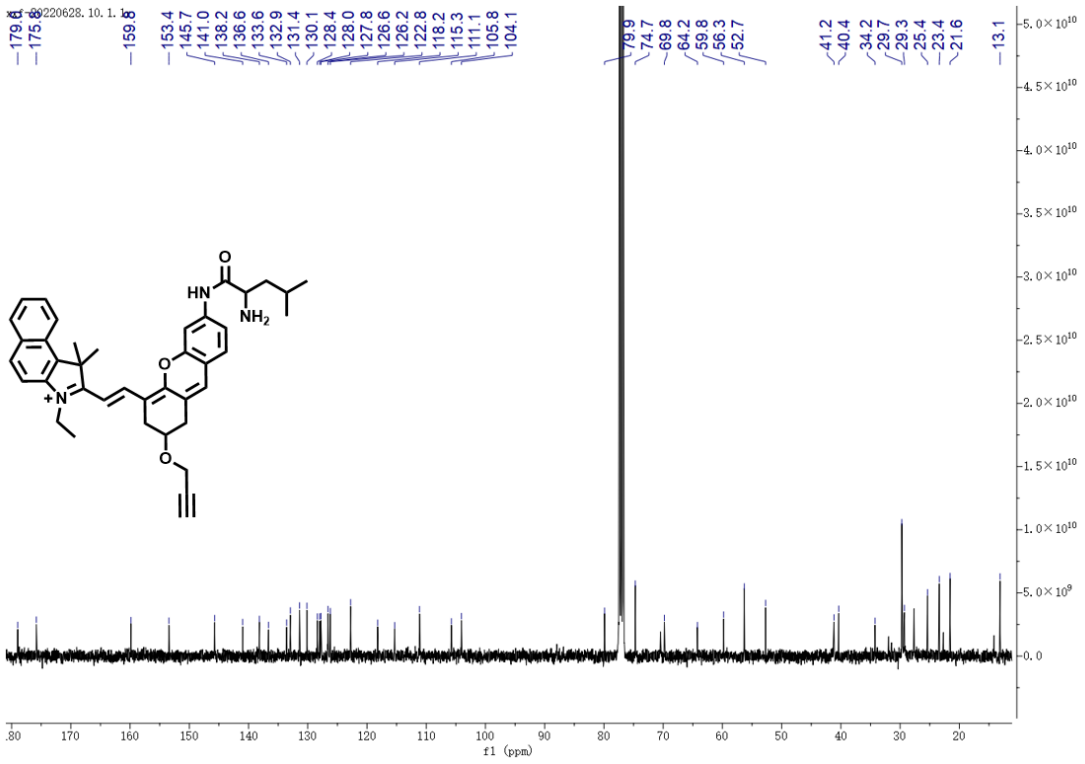


Figure S11. ^{13}C-NMR spectrum of CyNH$_2$-Leu.

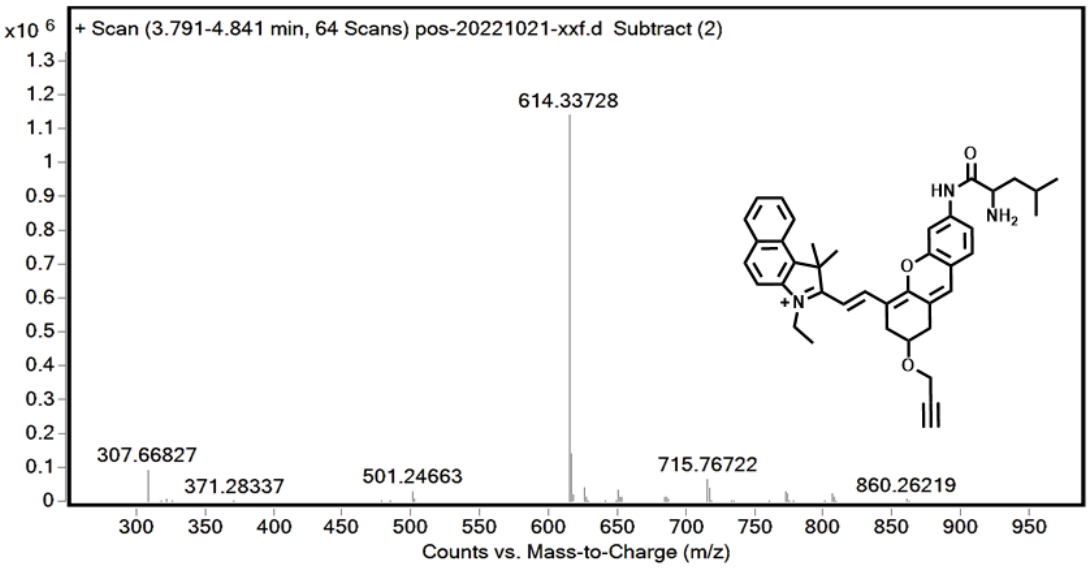


Figure S12. HRMS spectrum of CyNH$_2$-Leu.

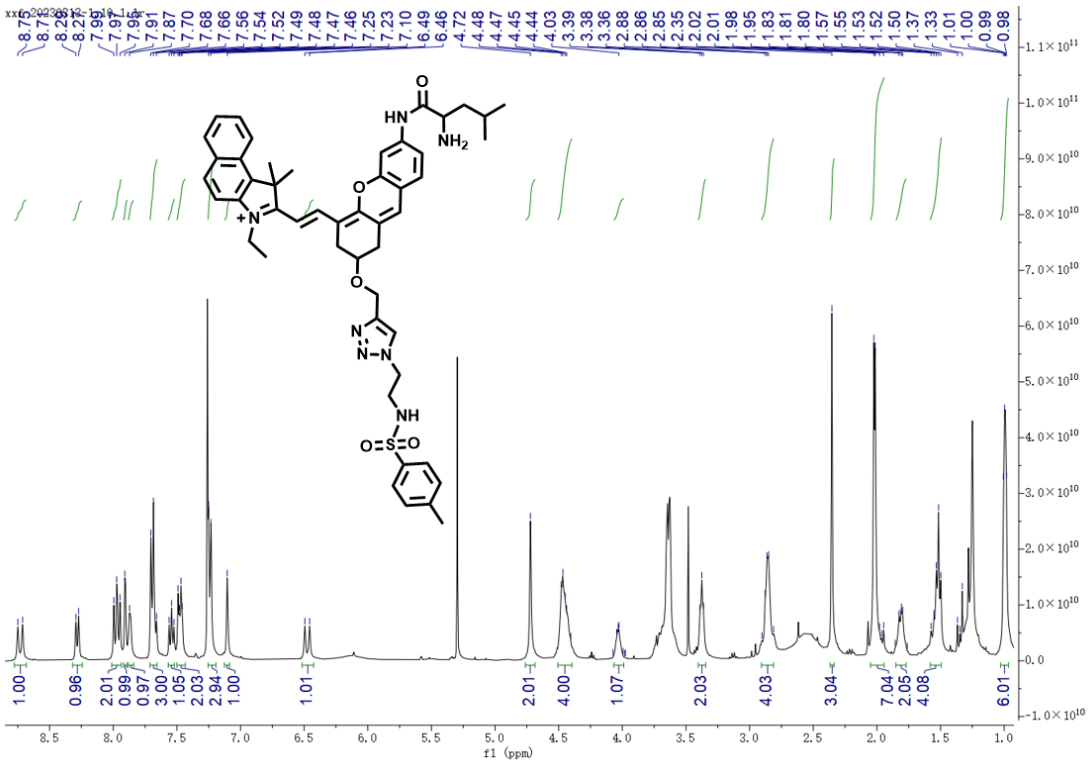


Figure S13. ^{1}H-NMR spectrum of ECL.

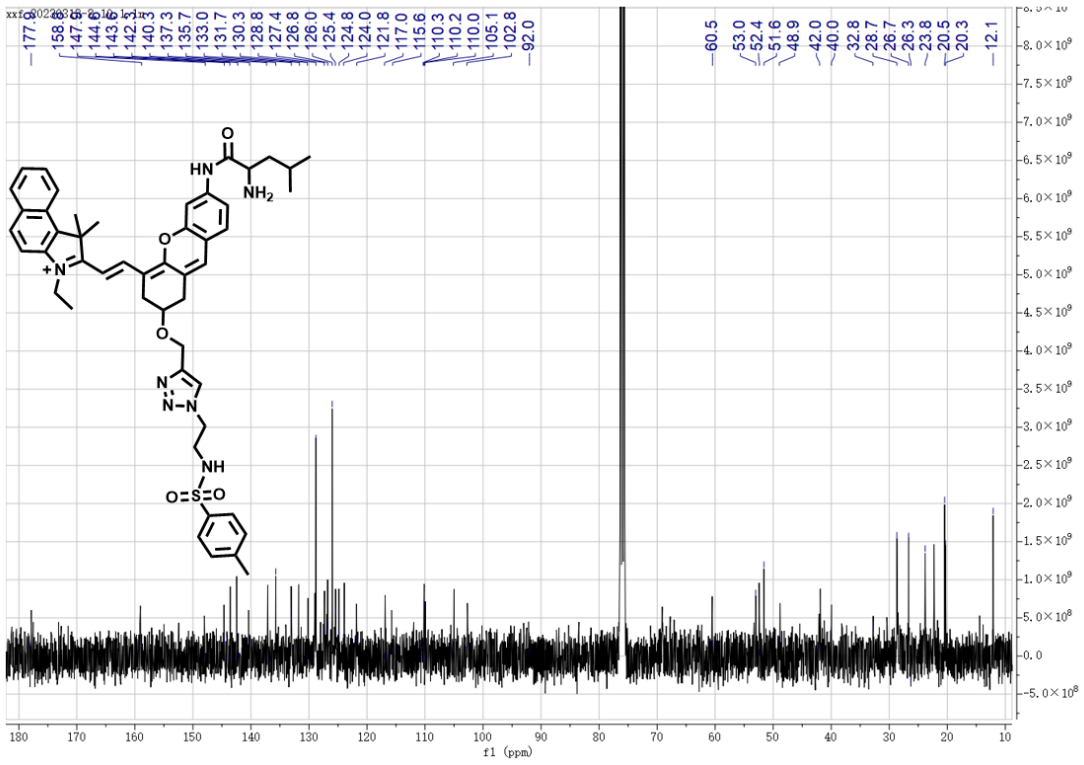


Figure S14. ^{13}C-NMR spectrum of ECL.

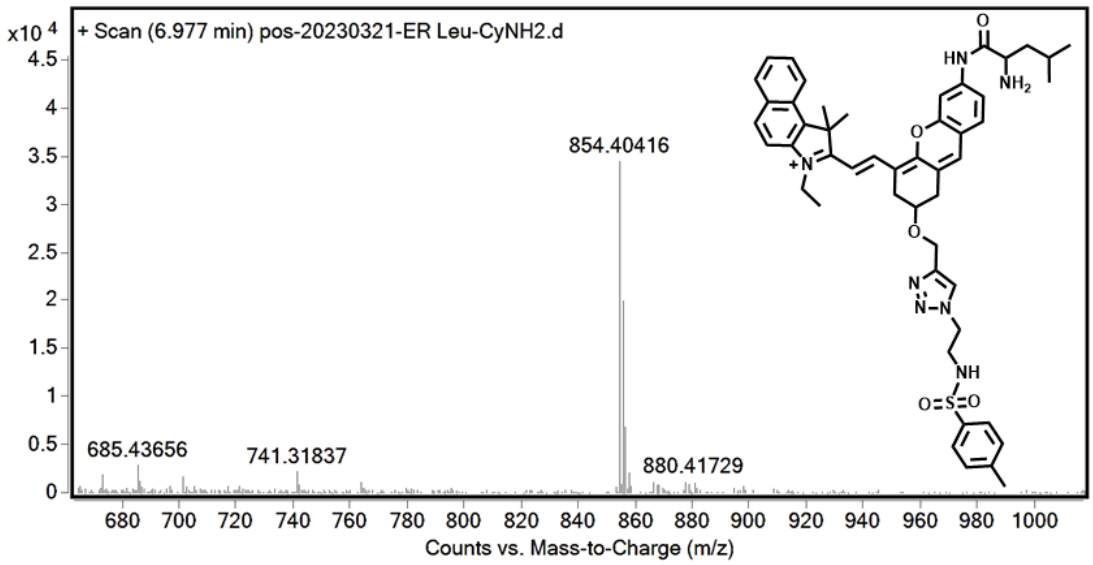


Figure S15. HRMS spectrum of ECL.

4. References

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