Supporting information

A ratiometric fluorescent probe for imaging of enzyme dependent hydrogen sulfide variation in mitochondria and in living mice

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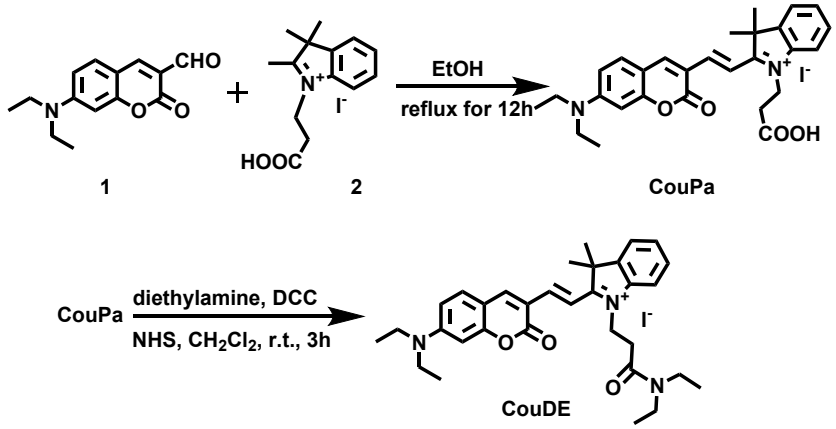
1 Materials and instruments

All chemicals were purchased from Aladdin, Innochem and J&K reagent Co. Ltd. without further purification except otherwise stated. All organic solvents used in synthesis were of analytical grade. DMSO used as probe solvent was spectrum pure. Water was purified by a Milli-Q system. Mito-marker Deep Red 633 was purchased from Thermo Fisher Scientific.

^{1}H-NMR and ^{13}C-NMR spectra were collected on Bruker DRX-300 or DRX-500 spectrometer at 25ºC, and TMS was used as internal standard. Mass spectra were performed on Agilent 6540Q-TOF LC/MS mass spectrometer. Uv-vis absorption spectra were collected on Perkin Elmer Lambda 35 spectrometer, and fluorescent emission spectra were collected on Horiba FluoroMax-4 spectrometer. All pH measurements were made with PHS-3 precise pH-Meter. Cellular imaging was conducted on Zeiss LSM 710 Laser scanning confocal fluorescence microscope. Living animal imaging was performed on PerkinElmer IVIS Lumina K Series III *in vivo* imaging system.

2 Synthesis



Scheme S1 Synthesis route of CouPa and CouDE.

CouPa: Compound 1 and compound 2 were synthesized according to literature.[1] Compound 1 (245 mg, 1.0 mmol) and compound 2 ((359 mg, 1.0 mmol) were stirred and refluxed in ethanol (20 mL) for 12 h. Solvent was removed in vacuum, and residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 20:1 v/v) to give CouPa (234 mg) as a dark blue solid. Yield, 40%. ^{1}H NMR (500 MHz, CD_3OD, δ, ppm): 9.84(s, 1H), 8.50(s,1H), 8.24(d, 1H, J = 12.4 Hz), 7.87(d, 1H, J = 15.8 Hz), 7.69(d, 1H, J = 7.9 Hz), 7.60(d, 1H, J = 7.2 Hz), 7.52- 7.44(m, 3H), 6.80(d, 1H, J = 9.1 Hz), 6.53(s, 1H), 4.65(t, 2H, J = 7.1 Hz), 3.51(q, 4H, J = 7.1 Hz), 2.71(t, 2H, J = 7.1 Hz),1.71(s, 6H), 1.18(t, 6H, J = 7.1 Hz).^{13}C NMR (125 MHz, CD_3OD, δ, ppm): 181.93, 172.06, 160.21, 158.19 154.76, 150.60, 149.84, 143.21, 140.81, 132.52, 128.97, 128.61, 122.56, 114.15, 112.39, 111.31, 110.14, 109.77, 96.45, 51.79, 45.18, 44.36,

42.39, 32.00, 25.95, 22.38, 21.65, 11.54, 11.40. HRMS (positive mode, *m/z*): calcd. 459.2278, found 459.2276 for $[M]^+$.

CouDE: CouPa (200 mg, 0.34 mmol) was dissolved in CH_2Cl_2 (5 mL), then DCC (140 mg, 0.68 mmol) and NHS (78 mg, 0.68 mmol) were added. The mixture was stirred under 0-5°C for 0.5 h. Then ethylamine (45 mg, 1 mmol) in THF solution was added, and the mixture was stirred for another 2.5 h under room temperature. The precipitate was removed and solvent was evaporated in vacuum. Then residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 20:1 v/v) to give CouDE (132 mg) as a dark blue solid. Yield, 50%. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 10.10 (s, 1H), 8.59 (d, J = 15.9 Hz, 1H), 8.16 (d, J = 9.1 Hz, 1H), 7.98 (d, J = 15.9 Hz, 1H), 7.84 (d, J = 7.8 Hz, 1H), 7.62-7.40 (m, 3H), 6.70 (dd, J = 9.1, 2.3 Hz, 1H), 6.46 (d, J = 2.1 Hz, 1H), 5.10 (t, J = 6.0 Hz, 2H), 3.52 (q, J = 7.1 Hz, 4H), 3.40 (q, J = 7.2 Hz, 2H), 3.32 (q, J = 7.1 Hz, 2H), 3.21 (t, J = 6.0 Hz, 2H), 1.84 (s, 6H), 1.29 (t, J = 7.1 Hz, 6H), 1.15 (t, J = 7.1 Hz, 3H), 1.04 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm): 180.92, 167.80, 161.06, 158.77, 154.52, 150.69, 149.28, 142.72, 141.09, 134.41, 129.16, 128.43, 122.16, 114.83, 112.48, 111.00, 110.93, 108.73, 96.73, 77.37, 77.15, 76.95, 76.53, 51.53, 45.56, 43.41, 42.47, 40.52, 32.21, 27.64, 14.44, 12.88, 12.52. HRMS (positive mode, *m/z*):Calcd. 514.3064, found 514.3067 for $[M]^+$.

3 Method

3.1 Quantum Yield Determination

Relative quantum yield of CouPa and CouDE were determined in pure methanol with cresyl violet (Φ = 0.54, λ_{ex}=588 nm) as reference.[2]Absorbance of probes and reference under their respective excitation wavelength was controlled less than 0.1. The quantum yield was calculated following equation:

$$\Phi_u = [(A_s F_u n^2)/ (A_u F_s n_0^2)]\Phi_s$$

Where s stands for sample, n stands for reference, A is the absorbance under 588 nm, F is the corresponding integrated fluorescence intensity, n and n_0 are the solvent refractive indexes of sample and reference, respectively.

3.2 Cell culture and imaging

MCF-7 cells was cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37°C, and Hep3B cells was conducted with the same condition except that FBS content is 15%.

Hypoxia atmosphere was created with an AnaeroPack (Mitsubishi Gas Corp., less than 0.1% O_2).

In the colocalization tests, MCF-7/Hep3B cells were stained at 37°C with probe for 1h and stained with Mito-marker Deep Red 633 for 30 min. Fluorescent signal of probe was collected at 660-750 nm with excitation at 488 nm. Fluorescent signal of Mito-marker was collected at 665-750 nm with excitation at 633 nm.

For H_2S imaging tests, the excitation wavelength was 488 nm, the green channel was collected from 490 nm to 583 nm, and red channel was collected from 592 nm to 758 nm. The ratiometric channel signal was obtained by mediating the green channel signal to the corresponding red channel signal.

3.3 *In vivo* imaging

All animal tests were conducted in compliance with the relevant laws and institutional guidelines for the Care and Use of Research Animals. Nude mice and HCT116 tumour-bearing mice were supplied by Model Animal Research Center of Nanjing University. Female nude mice aged 6 weeks were subcutaneously injected HCT116 cells to implant HCT116 tumor, whose volume was allowed to grow between 100-150 mm^3 in three weeks.

For exogenous H_2S imaging, CouDE (2 μM, 50 μL) was injected besides two legs of a nude mouse subcutaneously, then NaHS (100 μM, 25 μL) and equal saline was injected into the right and left side, respectively.

For endogenous H_2S imaging, 100 μL saline, saline containing Cys (400 μM) and saline containing AOAA (400 μM) were injected into the tumors of test mice respectively. After 30 min, 50 μL CouDE (2 μM) was injected into the three tumors.

4 Figures

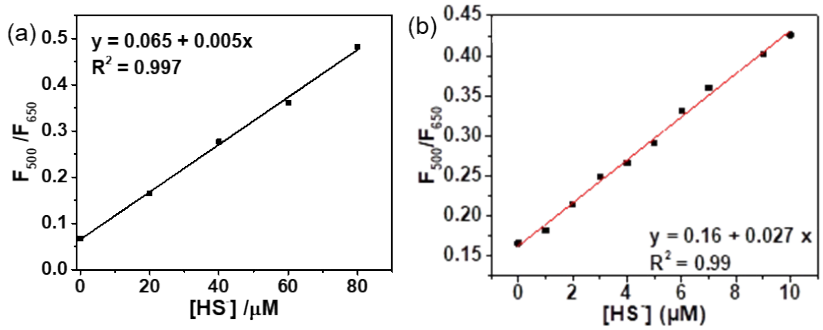


Fig. S1 The linear-relationship of HS^- concentration (a: 0-80 μM, b: 0-10 μM) versus fluorescent intensity ratio (F_{500}/F_{650}) of 10 μM CouDE in HEPES buffer (20 mM, pH 7.4, 10% DMSO, v/v). λ_{ex}, 460 nm.

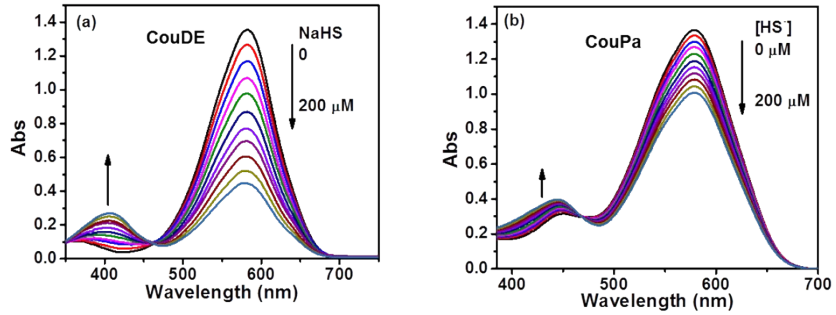


Fig. S2 Absorption spectra of 10 μM CouDE (a) and CouPa (b) in HEPES buffer (20 mM, pH 7.4, 10% DMSO, v/v) when 0-200 μM NaHS was added.

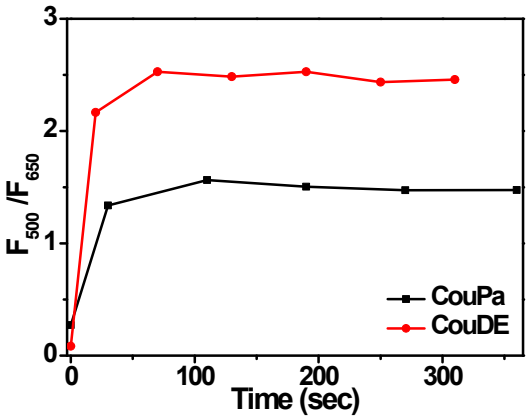


Fig. S3 Time-dependent changes of F_{500}/F_{650} of 10 μM CouPa and CouDE in HEPES buffer solution (20 mM, pH 7.4, 10 % DMSO, v/v) after adding 200 μM NaHS. λ_{ex}, 460 nm.

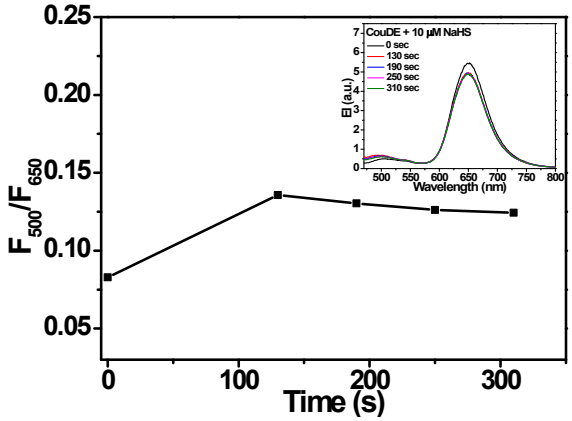


Fig. S4 Time-dependent changes of F_{500}/F_{650} of 10 μM CouDE in HEPES buffer solution (20 mM, pH 7.4, 10 % DMSO, v/v) after adding 10 μM NaHS. (Inset): corresponding emission spectra changes of CouDE. λ_{ex}, 460 nm.

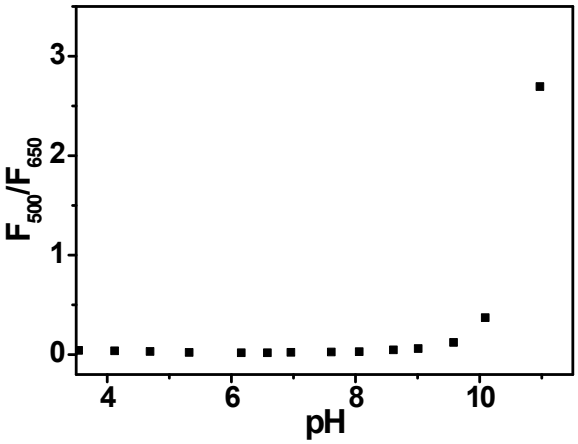


Fig. S5 Stability of the fluorescent intensity ratio (F_{500}/F_{650}) of 10 μM CouDE under physiological pH. pH of the solution was adjusted by HCl and NaOH. λ_{ex}, 460 nm.

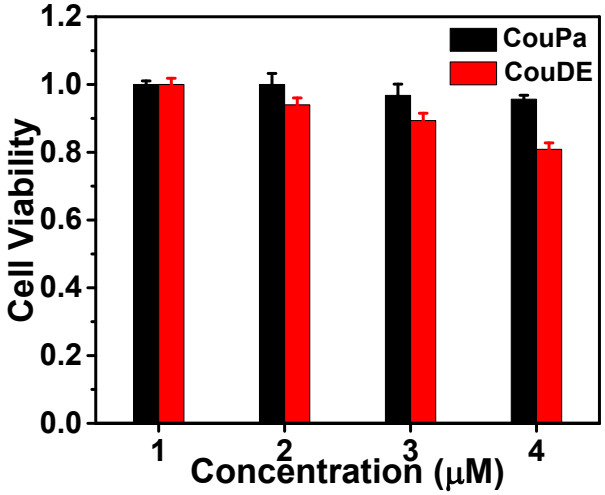


Fig. S6 Cell viability of MCF-7 cells incubated by CouPa or CouDE at 37°C for 2h.

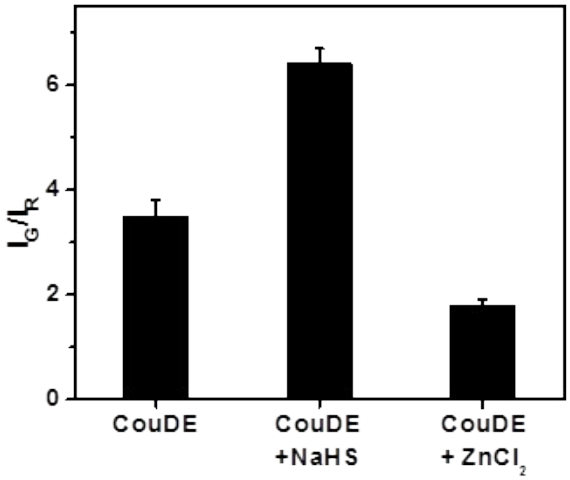


Fig. S7 Quantification of fluorescence intensity ratio I_G/I_R of MCF-7 cells incubated with CouDE, NaHS, $ZnCl_2$ corresponding to Figure 3. n=3.

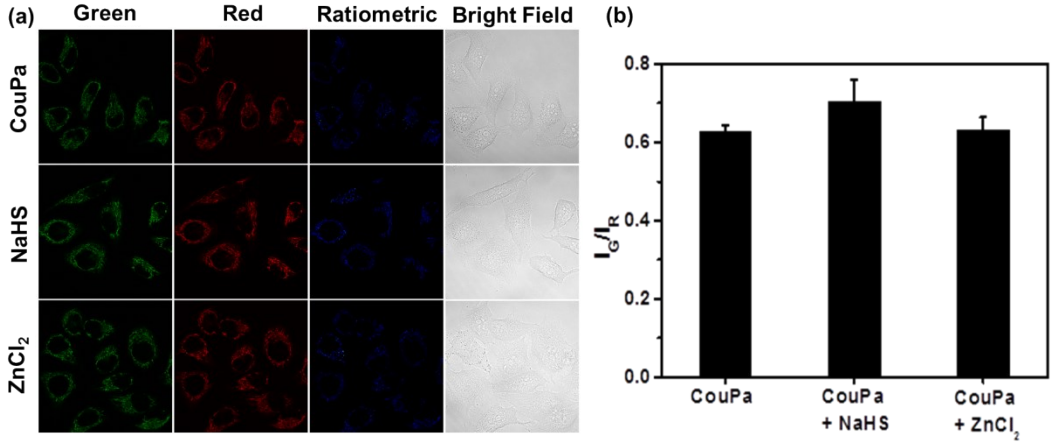


Fig. S8 (a) Fluorescence confocal images of MCF-7 cells incubated with CouPa and excited at 488 nm. (CouPa): cells stained with 2 μM CouPa at 37°C for 1 h. (NaHS): cells stained with CouPa for 1 h and then treated with 50 μM NaHS at 37°C for 30 min. ($ZnCl_2$): cells pre-treated with 1 mM $ZnCl_2$ for 10 min, then incubated by CouPa for 1 h. Images were collected from 490-583 nm for green channel, from 592-758 nm for red channel. Ratiometric images were obtained by mediating green channel with the

corresponding red channel. (b) Average area intensity in ratiometric channel of MCF-7 cells corresponding to (a) images. n=3.

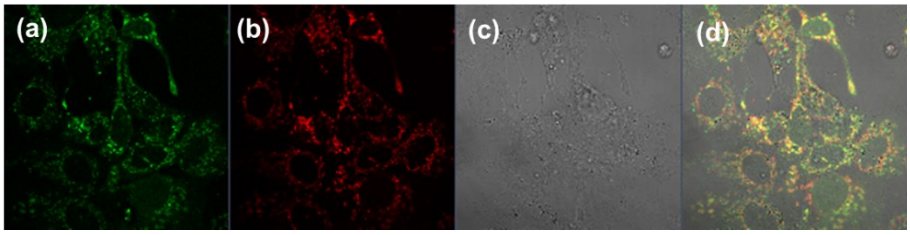


Fig. S9 Fluorescence confocal images of Hep3B cells costained with CouDE (2 μM, 37°C, 1 h) and Mito-marker Deep Red 633 (0.5 μM, 37°C, 30 min). (a): Pseudo green-colour images of CouDE. λ_{ex}= 488 nm, λ_{em}= 660-750 nm. (b): Pseudo red-colour images of Mito-marker, λ_{ex}= 633 nm, λ_{em}= 665-750 nm. (c) Bright field image. (d): Overlay images of a and b.

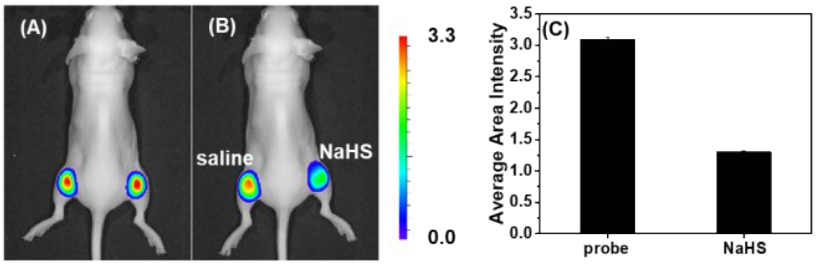


Fig. S10 (A-B) Exogenous H_2S sensing images in mice. 50 μL CouDE (2 μM) was subcutaneously injected besides two back legs (A), then 25 μL NaHS (100 μM) was injected into the right side and equal saline into the left side, respectively (B); (C) Quantification of the two area fluorescence intensity in (B) image (n=3).

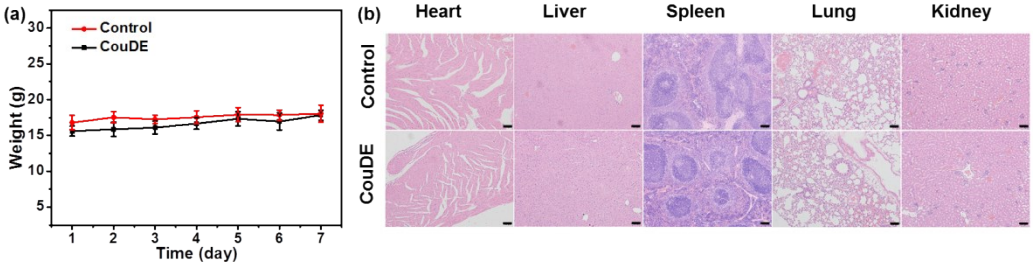


Fig. S11 Control: mice injected 50 μL saline. CouDE: mice injected 50 μL 2 μM CouDE. Body weight changes (a) and H&E staining of organs (b) of mice treated with saline or CouDE. Scale bar: 100 μm. n=3.

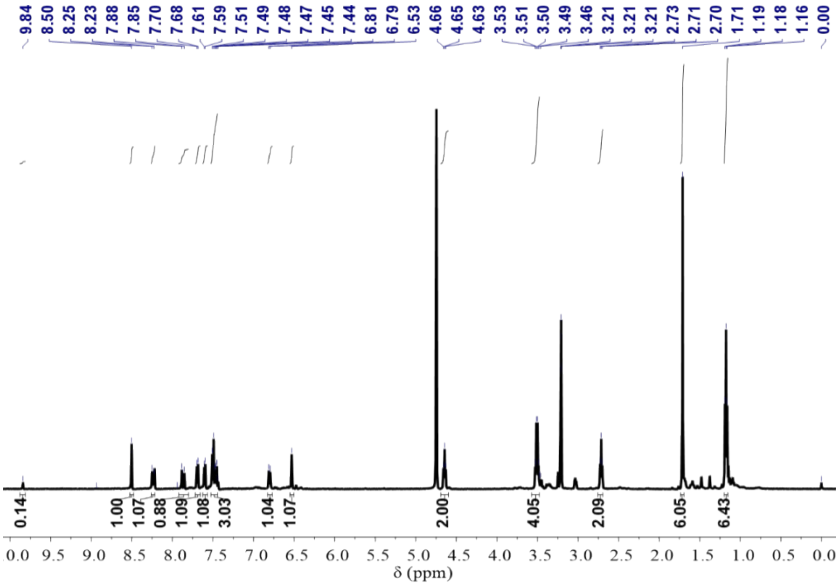


Fig. S12 The ^{1}H NMR spectrum of CouPa in CD_3OD.

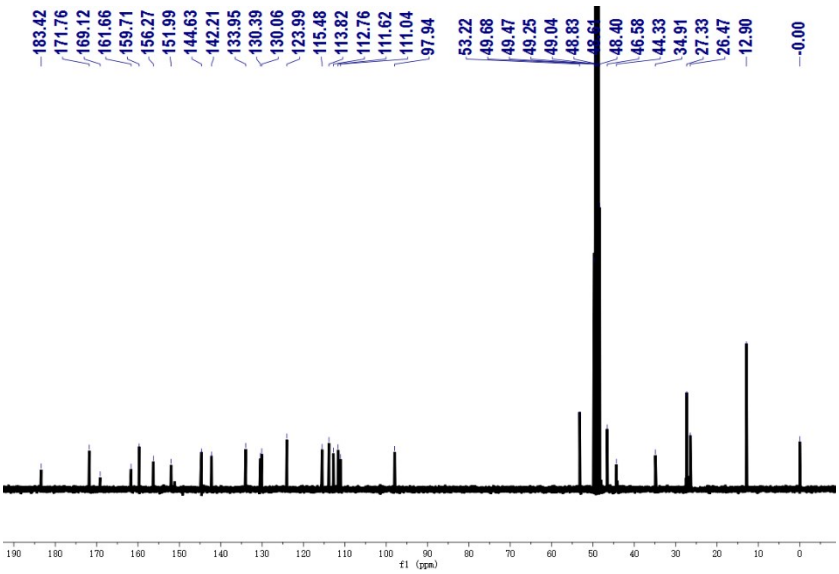


Fig. S13 The ^{13}C NMR spectrum of CouPa in CD_3OD.

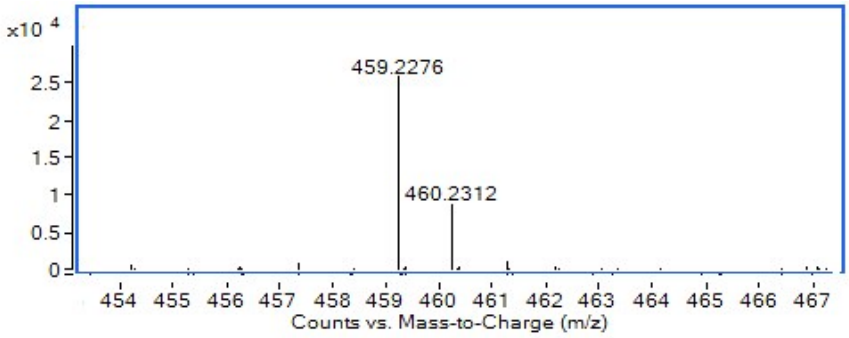


Fig. S14 The high resolution mass spectrum (HRMS) of CouPa.

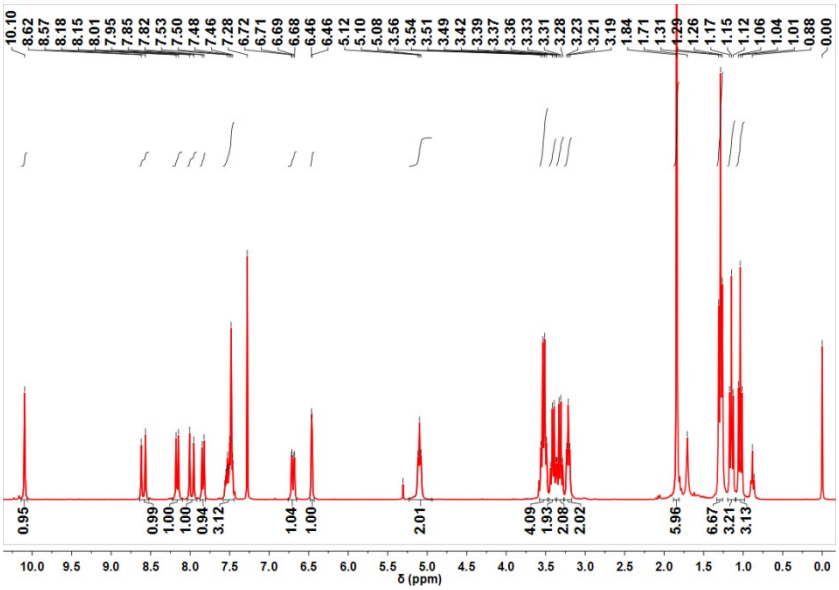


Fig. S15 The ^{1}H NMR spectrum of CouDE in $CDCl_3$.

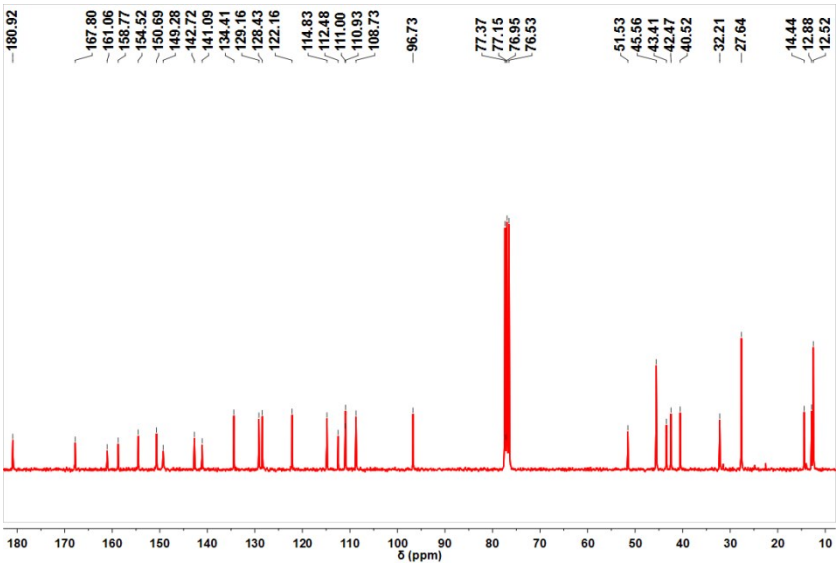


Fig. S16 The ^{13}C NMR spectrum of CouDE in $CDCl_3$.

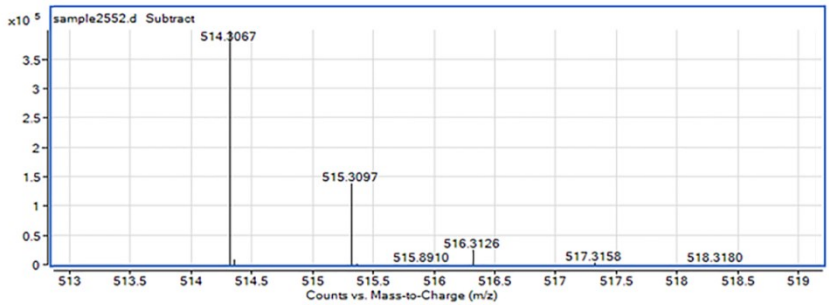


Fig. S17 The high resolution mass spectrum (HRMS) of CouDE.

5 Reference

(a) D. Oushiki, H. Kojima, T. Terai, M. Arita, K. Hanaoka, Y. Urano, T. Nagano, *J. Am. Chem. Soc.,* 2010, **132**, 2795; (b) D. Liu, W. Chen, K. Sun, K. Deng, W. Zhang, Z. Wang, X. Jiang, *Angew. Chem. Int. Ed.,* 2011, **50**, 4103.

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