Supporting Information

Visualizing Peroxynitrite Fluxes in Myocardial Cells by a New Fluorescent Probe Reveals the Protective Effect of Estrogen

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1. Materials and instruments

All chemicals were purchased from Adamas Reagent, Ltd. (China), and analytical grade solvents were used without further purification. All aqueous solutions were prepared using ultrapure water (ultrapure water, 18 MΩ cm^{-1}). MTT was purchased from Sigma Corporation, and column chromatography silica gel (200-300 mesh) was purchased from Qingdao Haiyang Reagent Co., Ltd. RIPA cell lysates, PMSF protease inhibitor, ECL, SDS-PAGE gel preparation kits and Annexin-V, FITC Apoptosis Detection Kitwere purchased from Boster Corporation. Estradiol benzoate (E2), glutathione (GSH), D/L-propargyl glycine and fulvestrant were purchased from Aladdin. H9C2 cells were purchased from Procell Life Science &Technology Co., Ltd. Instruments.

pH measurements were performed with a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China). Absorption spectra were recorded on a UV-1700 spectrophotometer (Shimadzu, Japan). ^{1}H NMR and ^{13}C NMR spectra were taken on a 400 MHz spectrometer (Bruker Co., Ltd., Germany), δ values are in *ppm* relative to TMS. The mass spectra were obtained using the Bruker Maxis ultra-high-resolution-TOF MS system. All fluorescence measurements were carried out at room temperature on an FLS-980 Edinburgh fluorescence spectrometer. Two-photon images were acquired with the Zeiss LSM 880 NLO with a 20× water objective. A Ti: sapphire laser was used to excite the specimen at 800 nm with a laser power of 70 mW. MTT assay was performed using a TRITURUS microplate reader.

Preparation of various interference substances. All reagents were used right after they were ready. Cys, Hcy, GSH and vitamin C (Vc) were all used as received. HS^-, HSO_3^-, SO_4^{2-} and $S_2O_3^{2-}$ were all used as their sodium salt and prepared as the stock solutions. Na^+, K^+, Zn^{2+}, Mg^{2+}, Hg^{2+}, Ca^{2+}, Co^{2+}, Fe^{2+} and Cu^{2+} were all used as their chloride salt and prepared as the stock solutions. All these compounds were commercial available with analytical purity and used directly.

We prepared reactive oxygen species (ROS) as follows[1]:

Peroxynitrite ($ONOO^-$): hydrochloric acid (0.6 M) was added to the mixture of $NaNO_2$ (0.6 M) and H_2O_2 (0.7 M), then NaOH (1.5 M) was added. The resulted faint

yellow solution was split into small aliquots and stored at lower than -20 °C. The concentration of the prepared peroxynitrite was determined by testing the absorption of the solution at 302 nm. The extinction coefficient of $ONOO^-$ solution is 1670 M^{-1} cm^{-1} at 302 nm. C_{ONOO^-} = Abs $_{302nm}$ /1.67 (mM).

Hydroxyl radical (•OH) was prepared by the reaction of Fe^{2+} with H_2O_2 (1:6), and the concentration of •OH is equal to the concentration of Fe^{2+}.

Singlet oxygen ($^1O_2$) was prepared in situ by addition of the H_2O_2 stock solution into a solution containing 10 eq of NaClO.

Superoxide solution ($O_2^{\bullet-}$) was prepared by adding KO_2 to dry dimethylsulfoxide and stirring vigorously for 10 min.

Hypochlorous acid (HClO) was obtained by diluting commercial aqueous solutions. The concentration was determined by measuring the absorbance at 292 nm with a molar extinction coefficient of 391 M^{-1} cm^{-1}.

Hydrogen peroxide (H_2O_2) was diluted appropriately in water. The concentration of H_2O_2 was determined by measuring the absorbance at 240 nm with a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

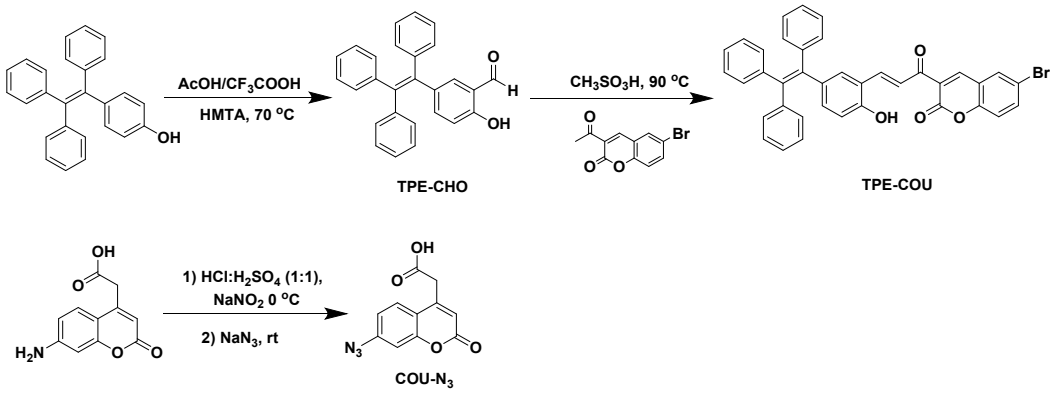
Nitric oxide (NO) was used from a stock solution prepared by sodium nitroprusside.

2. Fluorescence analysis

Fluorescence titration profile of TPE-COU was constructed by mixing TPE-COU (5 μM) with different level of $ONOO^-$ (0-100 μM) in CH_3CN/PBS buffer solution (4:6, v/v, 100 mM, pH=7.4, 37 °C). The measurement was carried out at λex/λem = 490/525 nm. The specificity experiment of TPE-COU toward $ONOO^-$ was carried out by incubation of the probe with $ONOO^-$ and other biorelevant species, including reactive oxygen and nitrogen species (H_2O_2, $^1O_2$, $^{\cdot}OH$, $O_2^{\cdot-}$, $ONOO^-$, and NO), reactive sulfur species (GSH, Cys, Hcy, and H_2S), anions (HSO_3^-, ClO^-, SO_4^{2-}, $S_2O_3^{2-}$), and metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, and Hg^{2+}) in CH_3CN/PBS buffer solution (4:6, v/v,. 100 mM, pH=7.4, 37 °C). A kinetic study of fluorescence responses was performed by incubating the probe (25 μM) with $ONOO^-$ (80 μM) at λex/λem =

490/525 nm.

3. Synthesis and characterization



Scheme S1. Synthesis route of **TPE-COU** and **COU-N_3**

Synthesis of ***TPE-COU***

To a solution of 1-(4-hydroxyphenyl)-1,2,2-tristyrene (1.05 g, 3 mmol) in acetic acid and trifluoroacetic acid was added hexamethylenetetramine (1.26 g, 9 mmol), the mixture was refluxed for 3-4 h, then quenched with water and extracted with dichloromethane. The organic phase was collected and dried with anhydrous Na_2SO_4 to obtained TPE-CHO. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.71 (s, 1H), 10.10 (s, 1H), 7.21 (d, J = 2.3 Hz, 1H), 7.18-7.05 (m, 16H), 6.73 (d, J = 8.6 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 191.15, 159.89, 143.59, 143.57, 143.32, 140.92, 139.75, 139.13, 134.80, 131.19, 131.12, 131.08, 128.46, 128.40, 128.29, 127.17, 127.07, 126.98, 122.25, 117.20.

TPE-CHO and 3-acetyl-6-bromo-coumarin (266.0 mg, 1.0 mmol) were dissolved in 5.0 mL of concentrated sulfuric acid and then heated at 90 °C for 12 h. After cooling to room temperature, the solution was slowly poured into 50 mL of ice water (precipitated with a yellow solid), the resulted mixture was stirred for 20 min and filtered, the solid was washed with cold water and dried in vacuo to give crude product. The residue was purified by silica gel column chromatography (PET/EtOAc, 40/1) to obtain **TPE-COU** of 160 mg (yield, 46%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.41 (s, 1H), 8.52 (s, 1H), 8.18 (d, J = 2.4 Hz, 1H), 7.85-7.88 (m, 1H), 7.70-7.76 (m, 1H), 7.43-7.45 (d, J = 8.9 Hz, 1H), 7.31-7.35 (d, J = 16.0 Hz, 1H), 6.94-7.18 (m, 15H)

6.84-6.86 (m, 1H), 6.70 (d, J = 8.5 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 158.45, 153.44, 151.00, 144.39, 140.97, 139.44, 136.89, 135.60, 132.77, 132.41, 131.71, 131.59, 129.88, 128.96, 128.80, 127.51, 125.19, 124.41, 121.37, 119.94, 119.09, 117.10, 116.30, 95.08. HRMS-ESI(m/z): calcd for $C_{38}H_{27}BrO4^+$ $[M+H]^+$ = 625.1008/627.0995, found 625.0980/627.0964; $[M+Na]^+$ = 647.0828/649.0815, found 647.0800/649.0797.

Synthesis of ***COU-N$_3$***

To a solution of 2-(7-amino-2-oxo-2*H*-chromen-4-yl)acetic acid (219.90 mg, 1.0 mmol) in water (2 mL) was added the mixture of conc. HCl (1.5 mL) and conc. H_2SO_4 (1.5 mL) at 0 ^{o}C. After 5 min, $NaNO_2$ (0.5 g) in water (5 mL) was added dropwise. The mixture was stirred for 30 min. Then NaN_3 (200 mg) dissolved in water (1 mL) was added to the mixture and stirred 3 h. Ethyl acetate (30 mL) was used to extract the resulted mixture and the combined organic phase was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (DCM/MeOH, 20/1) to obtain **COU-N$_3$** (150 mg, 68%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.71-7.74 (d, J = 8.4 Hz, 1H), 7.18-7.19 (m, 1H), 7.13-7.16 (m, 1H), 6.45 (s, 1H), 3.37 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.01, 159.97, 154.64, 150.15, 143.84, 127.64, 116.57, 116.16, 115.51, 107.43, 37.59; HRMS-ESI(m/z): calcd for $C_{11}H_7N_3O_4$ $[M-H]^-$ = 244.0352; found 244.0345.

4. Cell culture

The rat myocardium cell line (H9C2) were grown in Dulbecco's modified Eagle's medium (DMEM) with 1% 100 U mL^{-1} antibiotics penicillin/streptomycin and 10% fetal bovine serum (FBS) at 37 °C under a humidified atmosphere containing 5% CO_2.

5. OGD/R experiment[2]

Confocal imaging during Oxygen-Glucose Deprivation/Reperfusion (OGD/R). H9C2 cells were washed twice with PBS and then cultured with DMEM without glucose that had been pregassed with 95% N_2/5% CO_2 for 10 min to remove residual oxygen. The dishes were placed into an airtight AnaeroPouch bag, which provided near

anaerobic conditions with an O_2 concentration <1% (monitored with the oxygen indicator) and a CO_2 concentration of about 5% within 1 h of incubation at 37 °C. Cells were exposed to these conditions for 5 h. After this, the cells were washed with PBS twice, and then incubated again in high-glucose DMEM containing **COU-N$_3$** (5 μM) and **TPE-COU** (5 μM) at 37 °C in 95% air/5% CO_2 for another 40 min. Then the medium was removed and the cells were washed with PBS for three times. The cells were excited using a 780 nm laser and the emission was collected between 400-495 nm and 510-580 nm respectively.

6. Reaction mechanism by MS

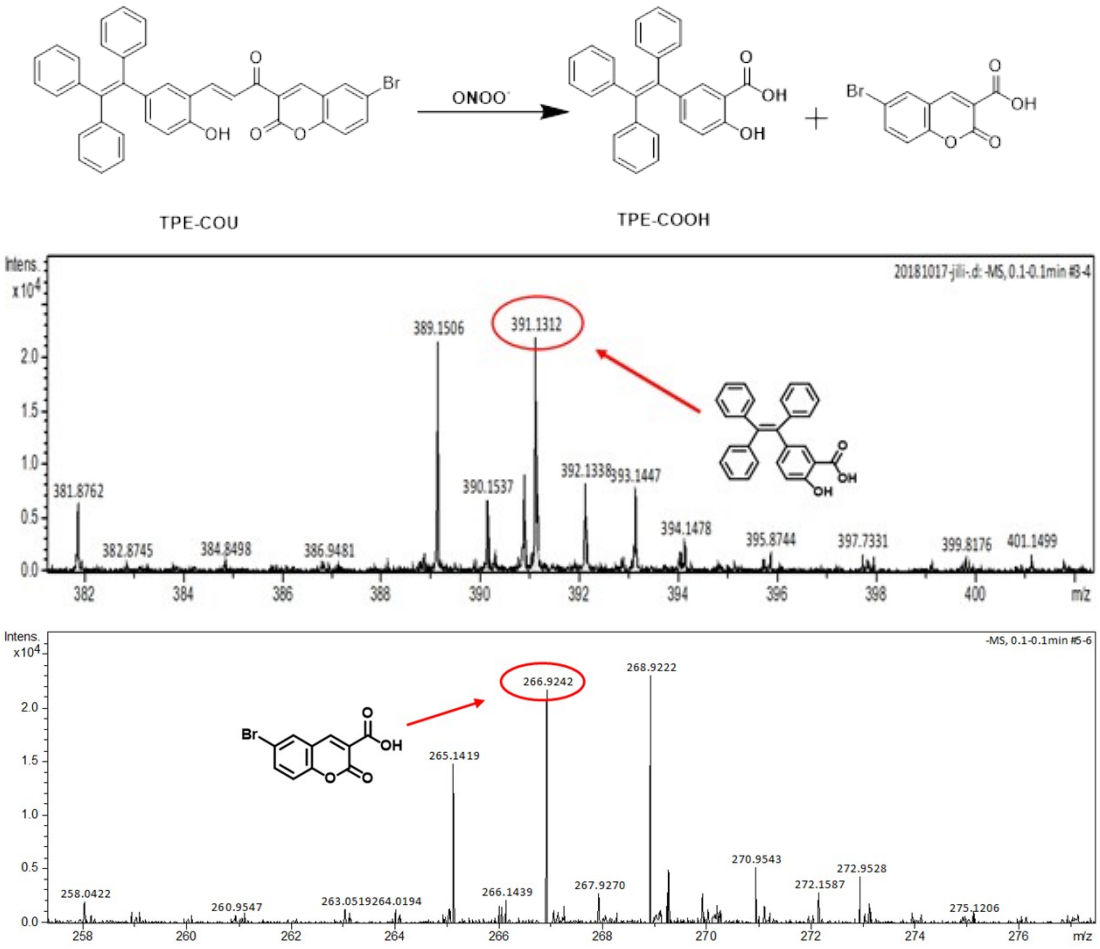


Figure S1. The HRMS spectra of the solution containing **TPE-COU** (10 μM) and $ONOO^-$ (80 μM).

7. Selectivity of TPE-COU

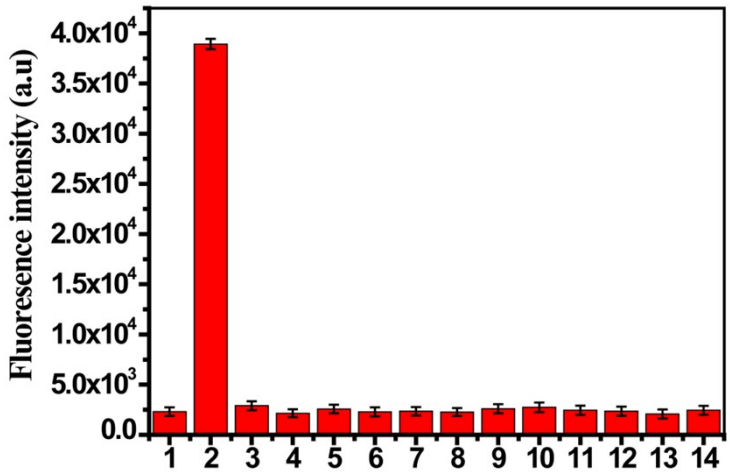


Figure S2. Fluorescence of **TPE-COU** (5.0 μM) in the presence of $ONOO^-$ and various substances: 1. Control, 2. $ONOO^-$ (10 μM), 3. Vc (5.0 mM), 4. Cu^{2+} (0.2 mM), 5. Mg^{2+} (0.2 mM), 6. Hg^{2+} (0.2 mM), 7. Zn^{2+} (0.2 mM), 8. Na^+ (0.2 mM), 9. K^+ (0.2 mM), 10. SO_4^{2-} (0.2 mM), 11. $S_2O_3^{2-}$ (0.2 mM), 12. Fe^{2+} (0.2 mM), 13. Co^{2+} (0.2 mM), 14. Ca^{2+} (0.2 mM).

8. Effect of pH

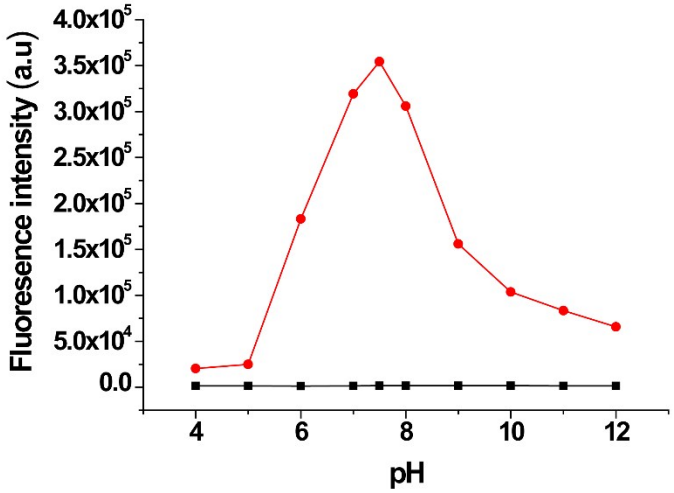


Figure S3. The effects of pH on the fluorescence intensity of **TPE-COU** (5.0 μM) in the absence (black) and presence (red) of $ONOO^-$ (50 μM) at room temperature.

9. Absorption spectra

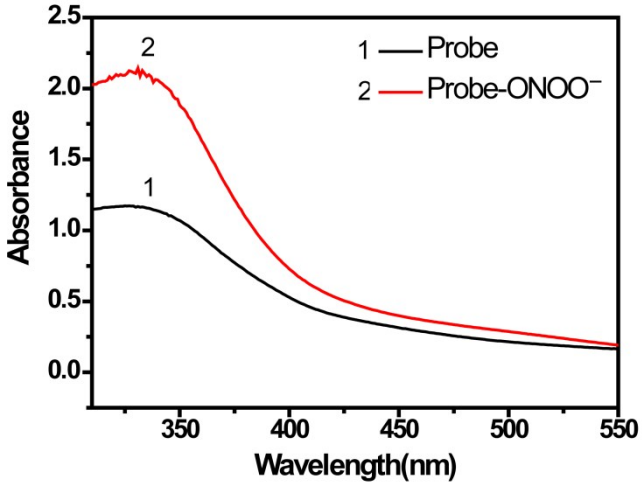


Figure S4. The absorption spectra of **TPE-COU** (5 μM) in CH_3CN/PBS buffer (4:6, v/v, 100 mM, pH 7.4) before (black) and after (red) the addition of 100 μM $ONOO^-$.

10. MTT assay

The cytotoxicity of **TPE-COU** was tested in H9C2 cells using a standard MTT assay. The IC_{50} value was calculated according to the method of Huber and Koella.[2] It was displayed that (Fig. S5 and S6) the value of IC_{50} was 278.5 μM for **TPE-COU** and 236.8 μM **COU-N_3** respectively, which indicated the good biocompatibility for cell imaging.

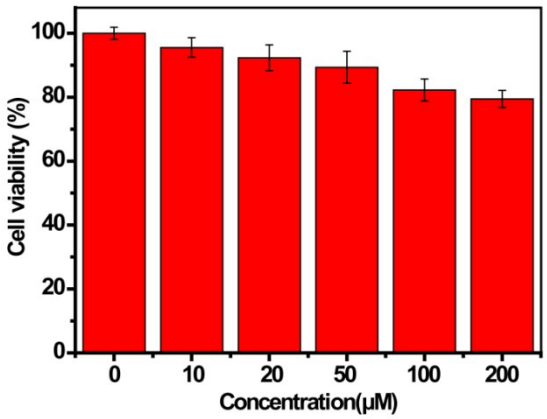


Figure S5. The MTT assay of H9C2 cells with different concentrations of **TPE-COU** (0 μM, 10 μM, 20 μM, 50 μM, 100 μM, 200 μM).

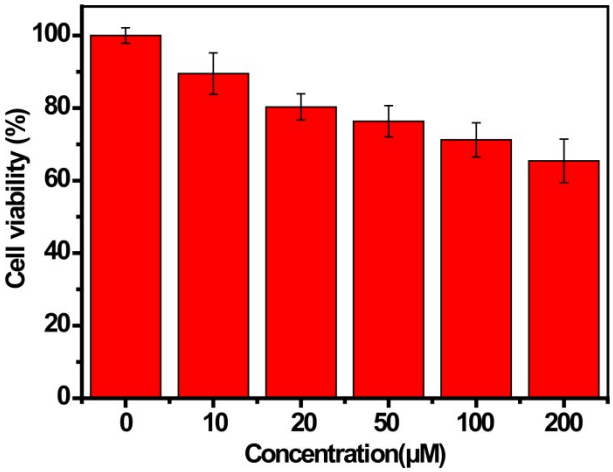


Figure S6. The MTT assay of H9C2 cells with different concentrations of **COU-N_3** (0 μM, 10 μM, 20 μM ,50 μM, 100 μM, 200 μM).

11. Live cell imaging

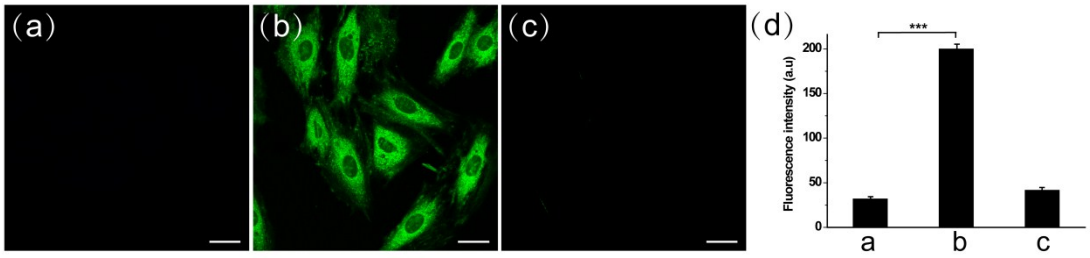


Figure S7. TP images of endogenous $ONOO^-$ in H9C2 cells during OGD/R. (a): The H9C2 cells were incubated with **TPE-COU** (5.0 μM) for 40 min. (the control group); (b): The OGD/R H9C2 cells were incubated with **TPE-COU** (5.0 μM) for 40 min. (c): The OGD/R H9C2 cells were treated with uric acid (100 μM, 3 h) before incubated with **TPE-COU** (5.0 μM) for 40 min. (d) Relative TP fluorescence intensity in (a), (b) and (c). The TP excitation wavelength was 780nm, the emission was collected at 510-580 nm. Significant difference: ***p <0.001. Scale bars: 20 μm.

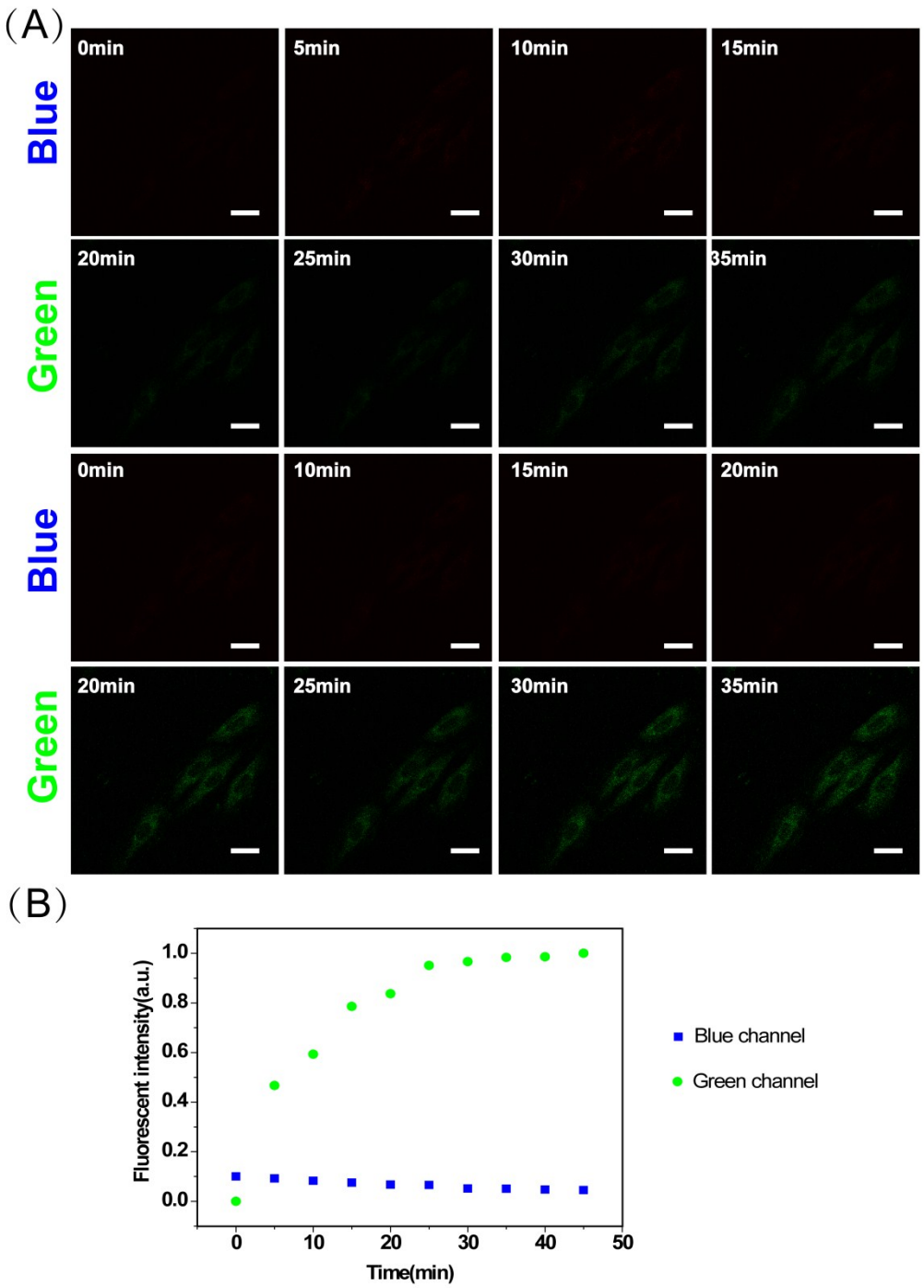


Figure S8. Time-dependent TP images of endogenous $ONOO^-$ and H_2S with dual channels in H9C2 cells during OGD/R. (A) The images were captured as soon as **COU-N_3** (5 μM) and **TPE-COU** (5 μM) were added. (B) The simultaneous readout of blue and green channel every 5 min. The images were collected at 400-495 nm (blue) and 510-580 nm (green) with excitation at 780 nm. Interval time = 5 min. Scale bar = 20 μm.

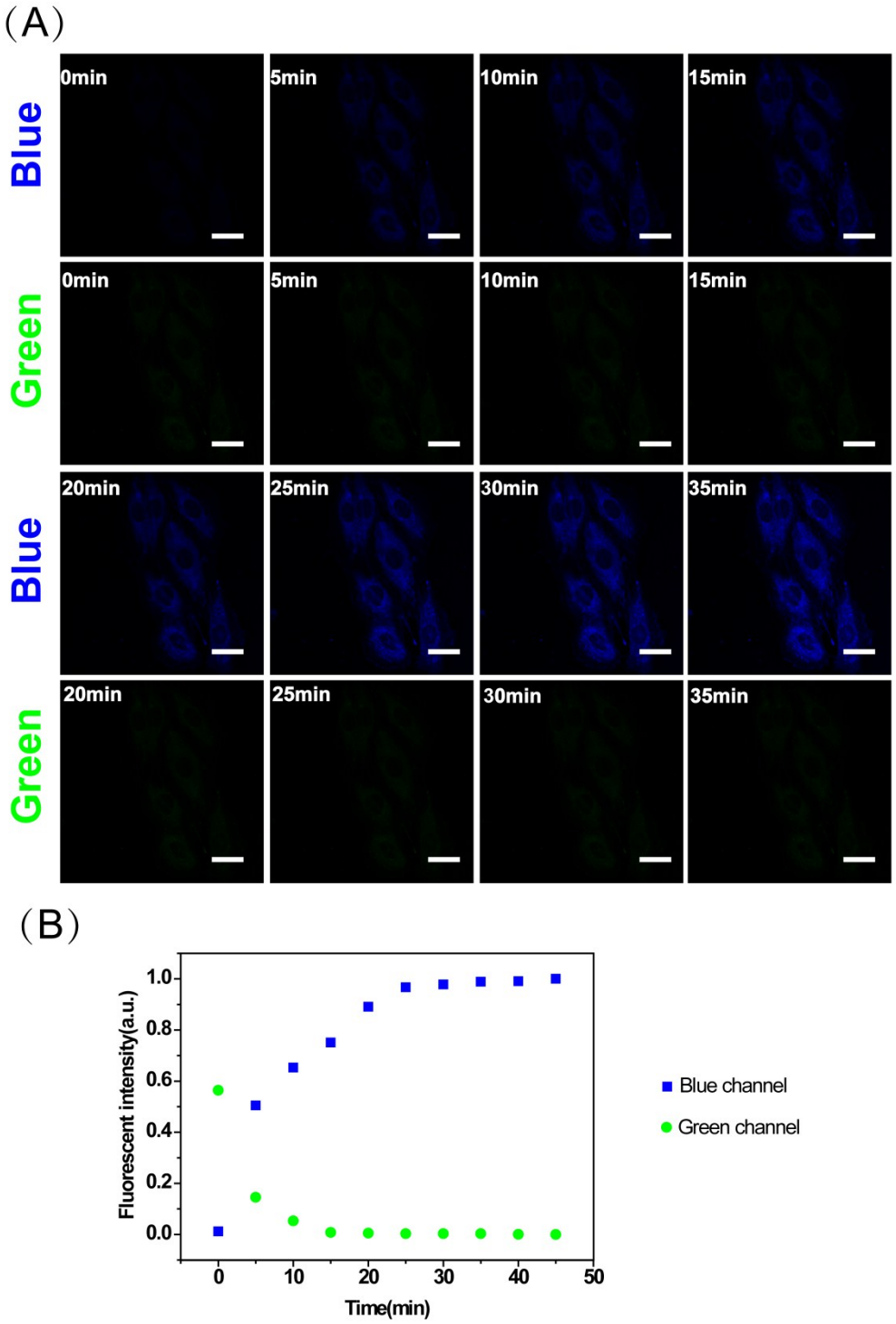


Figure S9. Time-dependent TP images of endogenous $ONOO^-$ and H_2S with dual channels in H9C2 cells co-incubated with E2 during OGD/R. (A) The images were captured as soon as **COU-N_3** (5 μM) and **TPE-COU** (5 μM) were added. (B) The simultaneous readout of blue and green channel every 5 min. The images were collected at 400-495 nm (blue) and 510-580 nm (green) with excitation at 780

nm. Scale bar = 20 μm.

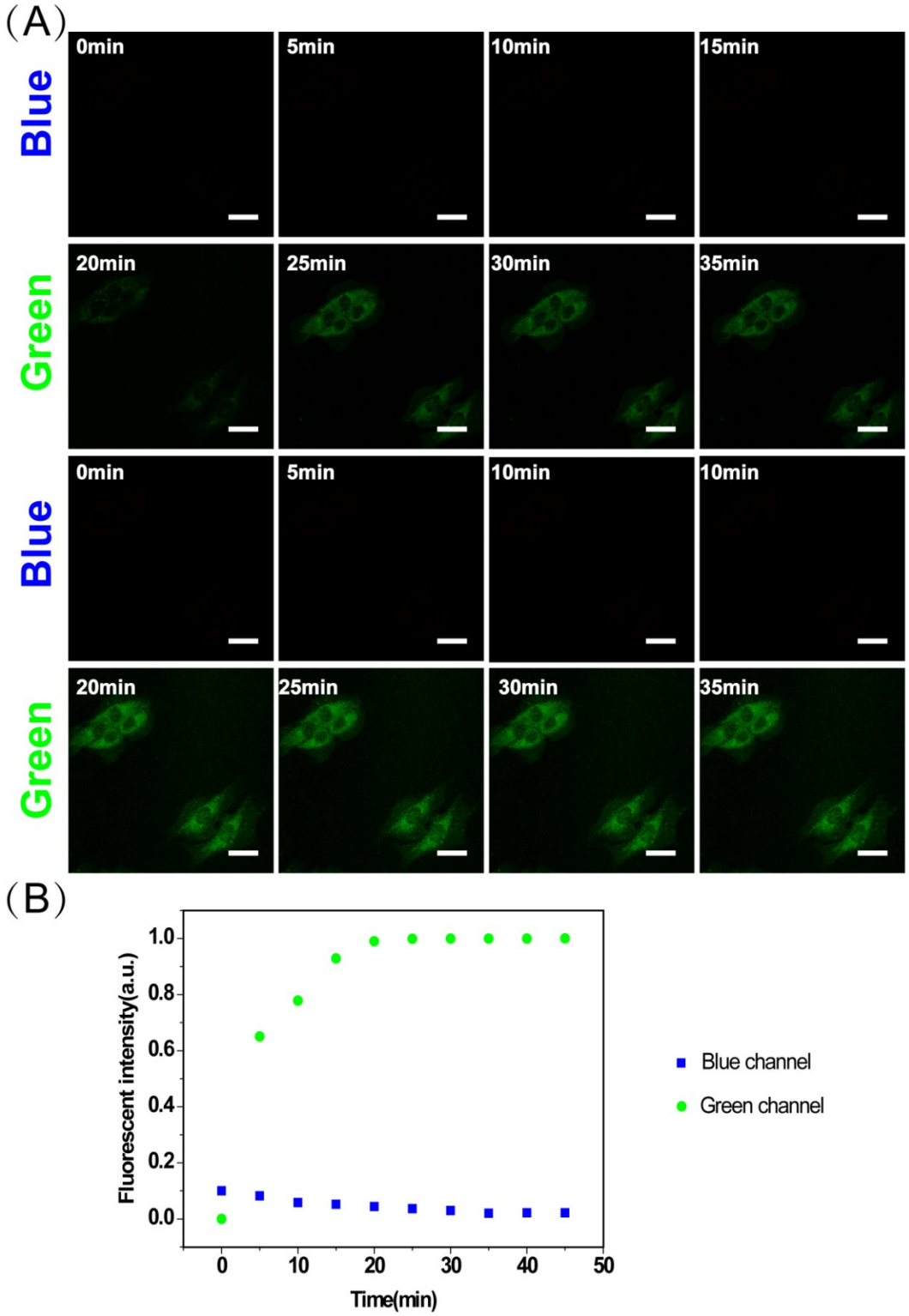


Figure S10. Time-dependent TP images of endogenous $ONOO^-$ and H_2S with dual channels in H9C2 cells co-incubated with E2 and fulvestrant during OGD/R. (A) The

images were captured as soon as **COU-N_3** (5 μM) and **TPE-COU** (5 μM) were added. (B) The simultaneous readout of blue and green channel every 5 min. The images were collected at 400-495 nm (blue) and 510-580 nm (green) with excitation at 780 nm. Scale bar = 20 μm.

12. RT-PCR measurement

RT-PCR Analysis: The total RNA from H9C2 cells was isolated using Promega Eastep Super total RNA extraction kit. First strand cDNA was prepared by reverse transcription in ABI PRISM 7000 sequence detection system using NovoScript Plus All-in-one 1st Strand cDNA Synthesis SuperMix Kit. Real-time PCR was performed in LineGene 9620 (Bioer, Binjiang, China) using NovoScript SYBR One-Step qRT-PCR Kit. Briefly, all PCRs were performed in a volume of 20 μL, using EU 8 tube strip (0.2 mL). The cycling was conducted at 42 °C for 30 min and 94 °C 5min, then followed by 40 cycles of 95 °C for 15 s and at 60 °C for 1 min. The primers of rat CSE gene were 5'CCGATGACCTCAACGAACG3' (forward) and 5'GAGACGGTAGCCCAGGATAA3' (reverse). The primers of *β*-actin were purchased from Sangon Biotech, which produce a product of 295 bp. Relative mRNA quantification was calculated by using the arithmetic formula $2^{-\Delta\Delta CT}$, where CT is the difference between the threshold cycle of a given target cDNA and an endogenous reference *β*-actin cDNA.

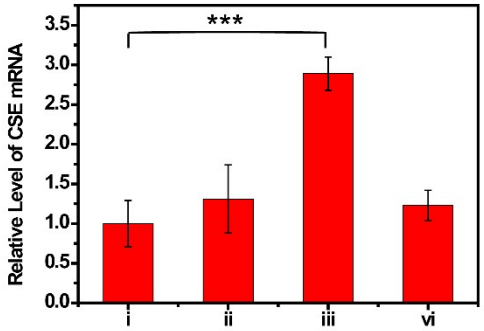


Figure S11 Relative CSE mRNA expression in H9C2 cells under different treatments. i: control; ii: OGD/R; iii: OGD/R+E2; iv: OGD/R+ fulvestrant+E2. ***: $P < 0.001$.

13. NMR spectra

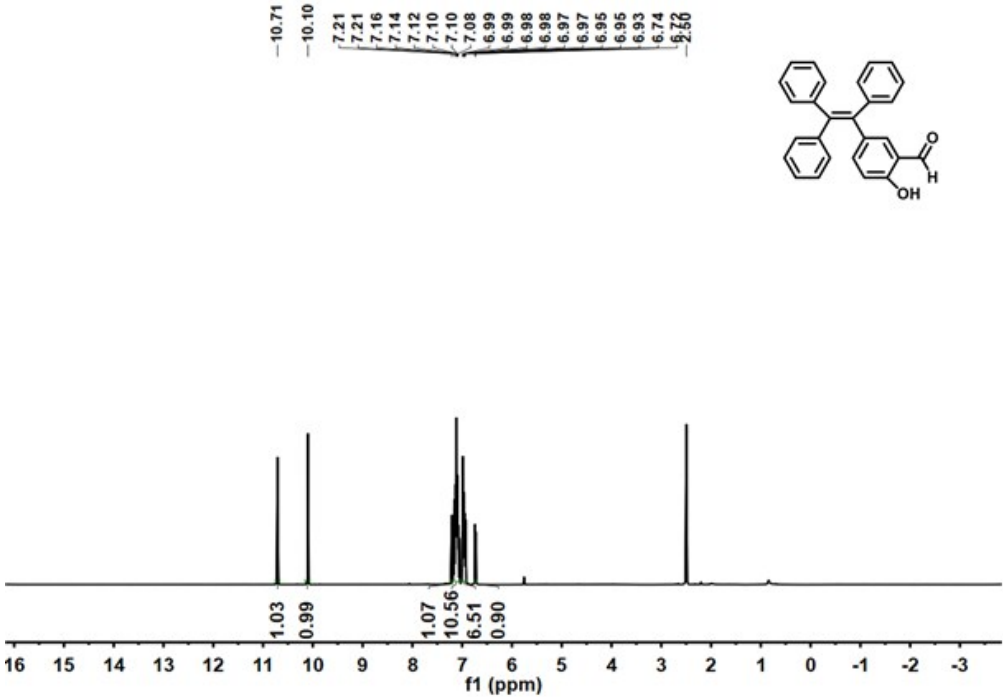


Figure S12 ^{1}H NMR spectrum of TPE-CHO

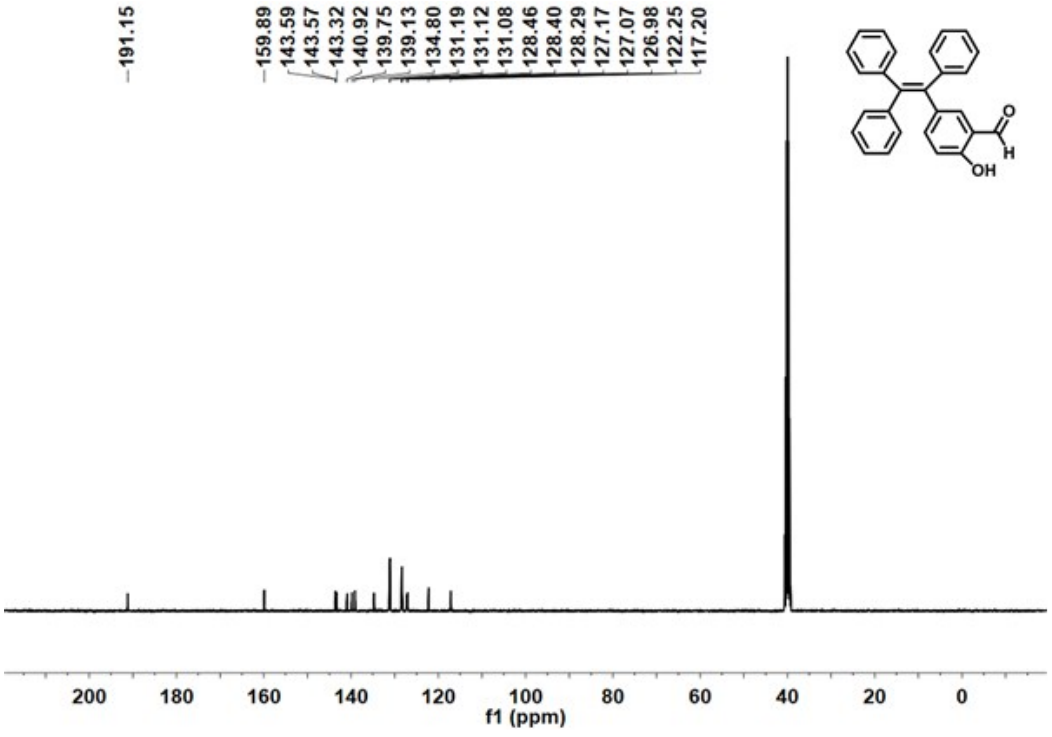


Figure S13. ^{13}C NMR spectrum of TPE-CHO

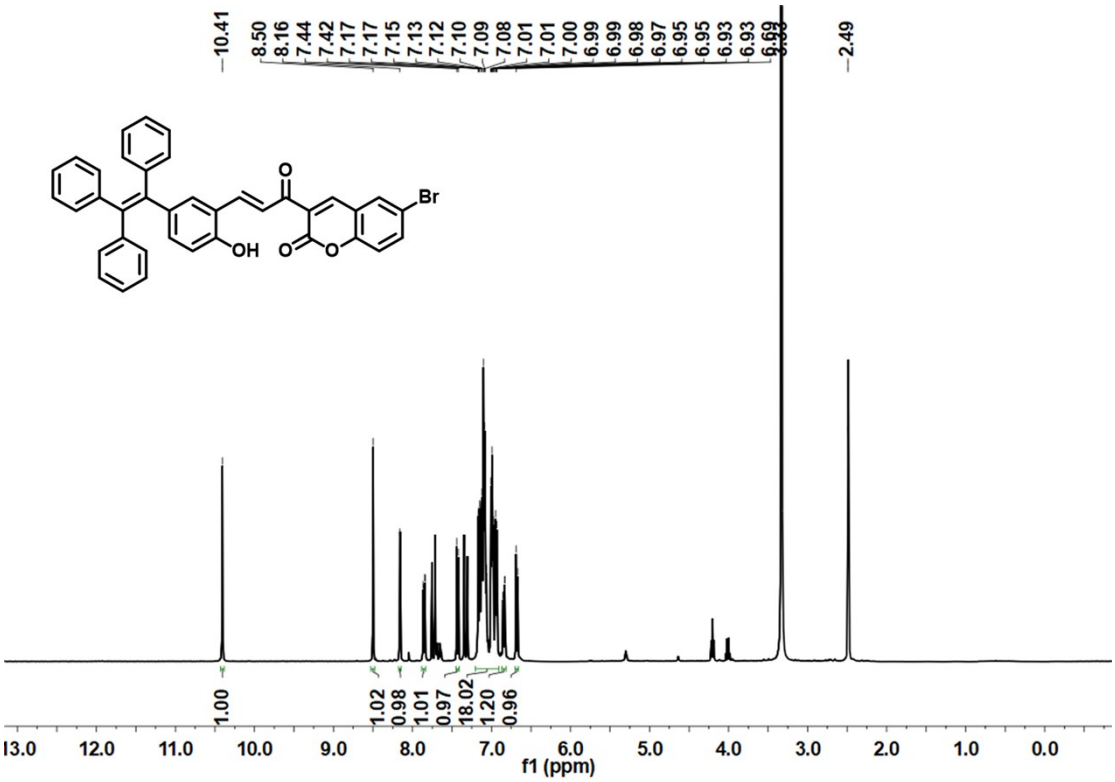


Figure S14. ^{1}H NMR spectrum of **TPE-COU**.

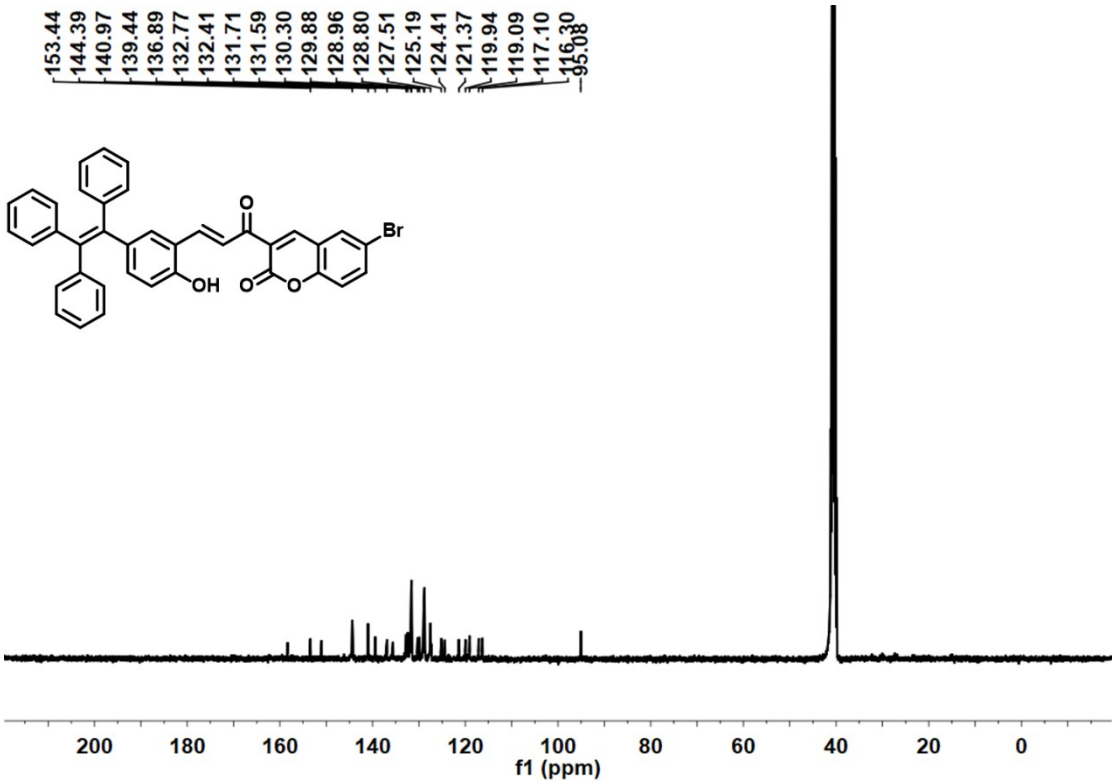


Figure S15. ^{13}C NMR spectrum of **TPE-COU**.

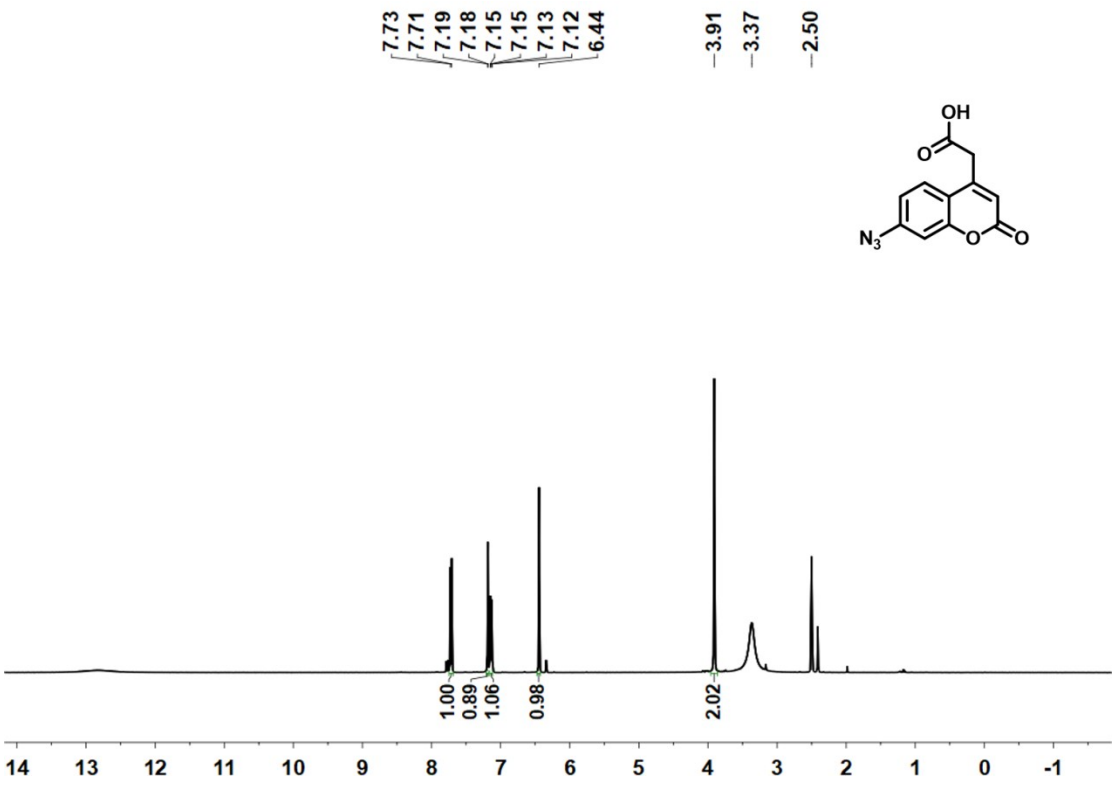


Figure S16. ^{1}H NMR spectrum of **COU-N_3**

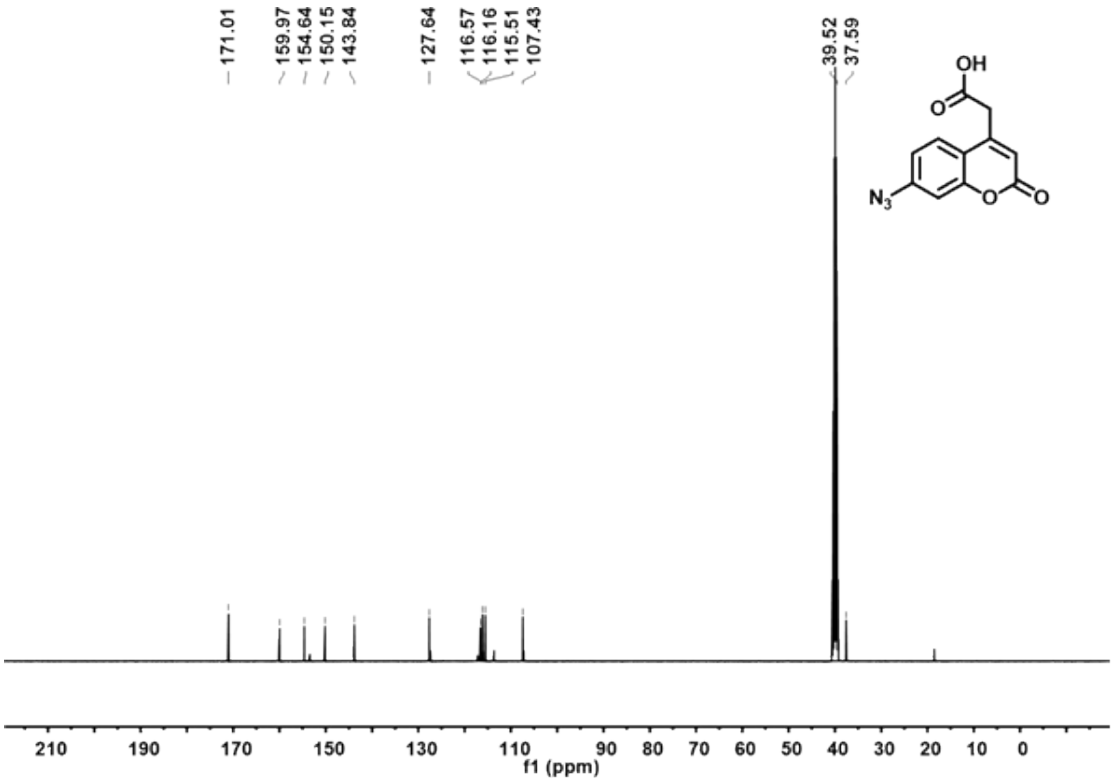


Figure S17. ^{13}C NMR spectrum of **COU-N_3**

References

1. X. Jiao, Y. Xiao, Y. Li, M. Liang, X. Xie, X. Wang and B. Tang, *Anal. Chem*., 2018, **90**, 7510.

2. X. Xie, X. Yang, T. Wu, Y. Li, M. Li, Q. Tan, X. Wang, B. Tang, *Anal. Chem*., 2016, **88**, 8019.

3. T. Bulus and A. B. Ahmed, Determination of IC_{50} and IC_{90} Values of Ethanolic Extracts of Some Medicinal Plants against Trypanosoma brucei. 2016; Vol. 7.