

A novel red AIE fluorescent probe for ratiometric detection of carbon monoxide *in vitro* and *in vivo*

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Material and methods

Reagents.

Unless otherwise stated, all reagents and chemicals purchased from suppliers were not further purified and all glassware was dried before use. NMR spectra were measured on a Bruker AVANCE III HD 400 MHz and Bruker AVANCE III HD 500 MHz spectrometer with tetramethylsilane (TMS) as the internal standard. UV-vis absorption spectra were performed on a UV-2550 scanning spectrophotometer (Shimadzu, Japan). Fluorescent spectra were recorded on a Hitachi F-2700 equipped with a 1 cm quartz cell. Dynamic light scattering measurements were performed at 25 °C on Zestier Nano ZS (Malvern Instruments Ltd, UK). Tricarbonylchloro (glycinato) ruthenium (II) (CORM-3) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis).

Quantitative calculation method

Both Density Functional Theory (DFT) and Time-Dependent Density Functional Theory (TDDFT) were calculated using Gaussian 16 ^[1]. Electron hole analysis used mutiwn program ^[2], and Orbit diagram visualization used VMD ^[3].

General Procedure for Fluorescence Measurement

We firstly prepared a 1 mM probe stock solution using DMSO. Then, 1 mM PdCl₂ solution and CORM-3 solution were pre-pared in deionized water, respectively. Then different solutions were added according to different measurement systems, so that the THBTA-CO concentration in the solution was 10 μM, the PdCl₂ concentration was 10 μM, and the solutions with different CORM-3 concentrations. And the total volume of the solution is 1 mL, the ratio of DMSO: PBS is 1: 99. In the selectivity study, we firstly used deionized water to con-figure each analyte to a solubility of 10 mM, and the final detection concentration is 100 μM. All samples were measured after incubation at 37 °C for 30 min and then the fluorescence spectra were recorded by using 410 nm as the excitation wavelength.

Cell Incubation and Fluorescence Image

Human breast cancer MCF-7 cells, HepG2 cells were purchased from Institute of Basic Medical Sciences (IBMS) of the Chinese Academy of Medical Sciences. The

cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) at 37 °C in a 5% CO₂ atmosphere.

In the imaging experiment of exogenous CO, only the probe and Pd²⁺ (both at a concentration of 10 µM) were added to the control group and incubated for 30 minutes, and added to incubate with cells. The test group firstly added CORM-3 (10 µM) and incubated with cells for 30 minutes, then added probe and Pd²⁺ (both at a concentration of 10 µM) to incubate for 30 minutes, and then used for fluorescence imaging. In heme-induced endogenous CO imaging, heme (100 µM) was incubated with cells at 37 °C for 0 h, 4 h, and 8 h, respectively, and then probes were added and Pd²⁺ (both at a concentration of 10 µM) were incubated for 30 minutes before imaging. In the endogenous CO imaging experiment under LPS-induced oxidative stress, we incubated cells with different concentrations of LPS (0, 0.5, 1 µg / mL) for 12 hours, and then added probes co-incubated with Pd²⁺ after 30 minutes, then performed imaging experiments by a confocal fluorescence microscope.

Fluorescence Image in mice

Five-week-old BALB/c female mice (13-16 g in weight) were purchased from The Medical Experimental Animal Center of Central South University (Changsha, China), and all animal experimental procedures were performed according to the Guideline for Animal experimentation with the approval of the animal care committee of Central South University. A model of LPS-induced inflammation was constructed by subcutaneously injecting LPS (100 µL LPS (0.5 mg/mL in PBS)) into mice once a day for three consecutive days for high endogenous CO expression.

Experimental section

Synthesis of THBTA-CO.

Synthesis of compound **1**: A solution of 2-aminothiophenol (4 mL, 4 mmol) and 5-bromosalicylaldehyde (800 mg, 4 mmol) in EtOH (10 mL), aqueous H₂O₂ (30%, 12.0

mmol) and aqueous HCl (37%, 8.5 mmol) was stirred for 6 h at ambient temperature. The solution was quenched by 10 mL H₂O. The precipitated compound was filtered through a filter paper and washed with EtOH (2 × 10 mL). Then, the obtained product was dried under vacuum and recrystallized from EtOH to afford the desired product as a white solid (850 mg, 70% yield). ¹H NMR (CDCl₃, 400 MHz) δ (ppm): 12.556 (s, 1H), 7.946 (q, 2H, 8.1 Hz), 7.770 (s, 1H), 7.461 (m, 3H), 6.989 (d, 1H, 4.2 Hz). ¹³C NMR (CDCl₃, 100 MHz): 167.62, 156.89, 151.50, 135.16, 132.48, 130.33, 126.78, 125.80, 122.23, 121.47, 119.67, 118.18, 110.89.

Synthesis of compound **2**: Compound **1** (850 mg, 2.8 mmol), hexamethylenetetramine (840 mg, 6 mmol), and trifluoroacetic acid (10 mL) were mixed in a r. b. flask. The mixture was allowed to reflux overnight. Then, the mixture was cooled, and the solution was neutralized with aqueous NaOH solution. The precipitated mass was collected through filtration on a filter paper under suction and washed with water (3 × 10 mL). After drying under vacuum, compound **2** (550 mg) was obtained in 60% yield, and had the following spectral properties. ¹H-NMR (CDCl₃, 400 MHz) δ (ppm): 12.578 (s, 1 H), 10.479 (s, 1H), 7.943 (m, 2H), 7.782 (s, 1H), 7.470 (m, 2H), 7.003 (d, 1H, J = 6 Hz). ¹³C NMR (CDCl₃, 100 MHz): 188.90, 165.63, 159.29, 151.20, 127.10, 126.86, 126.29, 125.88, 125.48, 122.54, 122.29, 121.64, 120.60, 119.72.

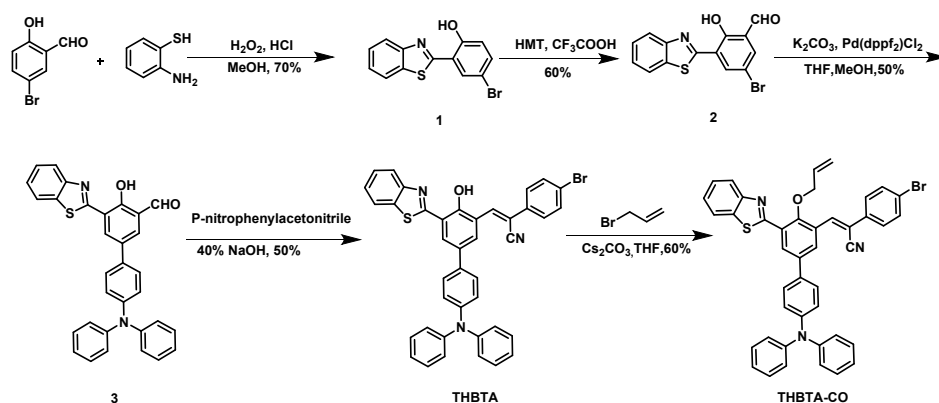
Synthesis of compound **3**: Compound **2** (550 mg, 1.6 mmol) and 4-(Diphenylamino) phenylboronic acid (580 mg, 2 mmol) were added to a three-necked flask, then

vacuumed and protected by N₂. After that, 10 ml of methanol and 5 ml of THF were added to dissolve the compounds. Finally, 20 mg of catalyst [1,1'-Bis(diphenylphosphino) ferrocene] dichloropalladium (II) was added and stirred at 90 °C for 6 h. After cooled to room temperature, the system was concentrated under reduced pressure, and the crude product was separated and purified on a chromatographic column to obtain a yellow powder product **3** (426 mg, yield, 50%). ¹H NMR (500 MHz, CDCl₃) δ = 10.56 (s, 1H), 8.40 (s, 1H), 8.12 (d, J=2.3, 2H), 7.97 (d, J=7.9, 1H), 7.58 (s, 1H), 7.54 (d, J=8.5, 2H), 7.49 (d, J=7.3, 1H), 7.29 (t, J=7.9, 4H), 7.18 (d, J=8.6, 2H), 7.15 (d, J=7.6, 4H), 7.06 (t, J=7.3, 2H). ¹³C NMR (125 MHz, CDCl₃) δ = 190.43, 173.41, 159.50, 152.16, 151.47, 147.62, 147.49, 139.31, 135.89, 132.52, 132.32, 131.18, 129.39, 127.42, 126.98, 126.01, 124.81, 124.58, 123.82, 123.20, 122.46, 121.66.

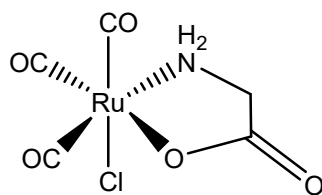
Synthesis of compound **THBTA**: Dissolve compound **3**(425 mg 0.85mmol) and p-bromophenylacetonitrile (200 mg, 1mmol) in ethanol, add 1 mL of 40% soluble NaOH, and stir at room temperature for 4 hours. When the reaction is complete, adjust the pH to neutral conditions, extract with EA, combine the organic layers, dry the organic layers with anhydrous magnesium sulfate, and obtain crude products by vacuum distillation and the crude product was separated and purified on a chromatographic column to obtain a yellow powder product **THBTA** (286 mg, yield, 50%). ¹H NMR (500 MHz, CDCl₃) δ = 8.53 (s, 1H), 8.20 (s, 1H), 8.02 (d, J=8.1, 1H), 7.95 (d, J=7.5, 2H), 7.62 (dd, J=18.1, 8.6, 4H), 7.55 (d, J=8.3, 2H), 7.47 (t, J=7.7, 2H), 7.32 – 7.27 (m,

4H), 7.19 (d, J=8.5, 2H), 7.16 (d, J=7.8, 4H), 7.05 (t, J=7.6, 2H).

Synthesis of compound **THBTA-CO**: 100 mg of **THBTA** was dissolved in THF, Cs₂CO₃ was added as a base and stirred at 50 °C for 30 minutes, 100 mg of propene bromide was added, and the reaction was stirred at 70 °C under reflux for 4 hours. Then, the reaction solution was spin-dried, and the product was further processed through a silica gel column with PE/EA (10:1, v/v) as the eluent to afford a yellow solid. ¹H NMR (500 MHz, CDCl₃) δ = 8.77 (s, 1H), 8.51 (s, 1H), 8.18 (d, J=8.1, 1H), 8.01 (s, 1H), 7.99 (d, J=7.9, 1H), 7.71 – 7.60 (m, 6H), 7.56 (t, J=7.5, 1H), 7.46 (t, J=7.5, 1H), 7.31 (t, J=7.8, 5H), 7.21 (d, J=8.4, 2H), 7.18 (d, J=7.8, 4H), 7.08 (t, J=7.2, 2H), 6.13 (ddd, J=22.0, 10.6, 5.3, 1H), 5.47 (d, J=16.9, 1H), 5.36 (d, J=10.5, 1H), 4.51 (d, J=5.4, 2H). ¹³C NMR (125 MHz, CDCl₃) δ = 154.90, 154.57, 147.83, 147.76, 147.55, 147.45, 139.65, 137.95, 137.22, 136.90, 132.44, 132.40, 130.35, 130.07, 129.75, 129.35, 128.66, 128.38, 128.03, 127.55, 124.63, 124.58, 123.75, 123.63, 123.21, 123.15, 121.57, 121.52, 119.56, 119.12, 53.43.



Scheme S1. The synthesis route of **THBTA** and **THBTA-CO**



Scheme S2. Chemical structure formula of CORM-3.

Cell cytotoxic assays

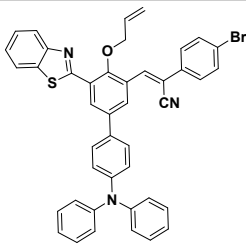
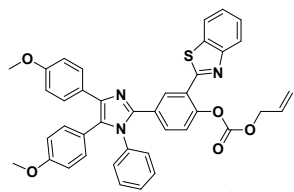
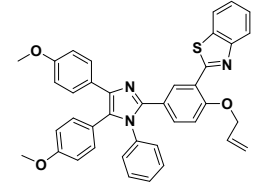
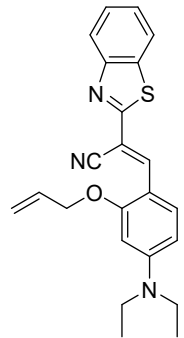
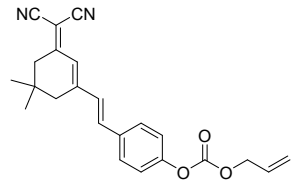
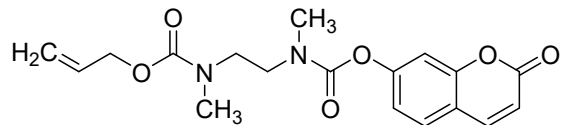
The cells were inoculated into petri dishes with DMEM, 10% fetal bovine serum, 1% penicillin and 1% streptomycin. After inoculating HepG2 cells in a 96-well plate for 24 hours, they were exposed to probe **THBTA** of different concentrations (0-20 μM) for 24 hours, 20 μL of MTT solution was added and incubated for 6 h, the supernatant was aspirated, and 150 μL of DMSO was added to dissolve the purple crystals. Measure the absorbance at 490 nm with a microplate reader.

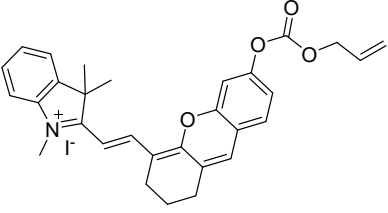
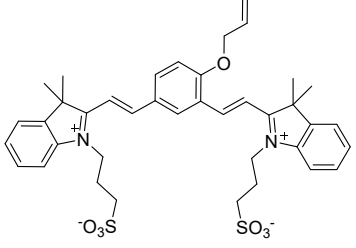
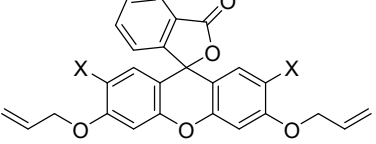
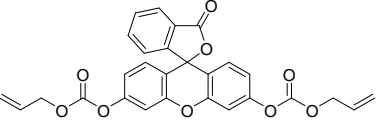
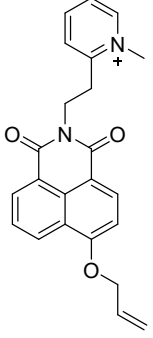
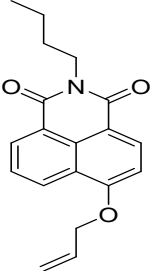
Cell culture and imaging

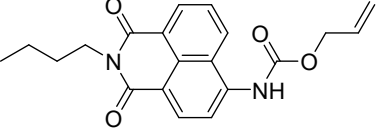
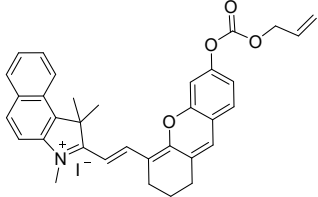
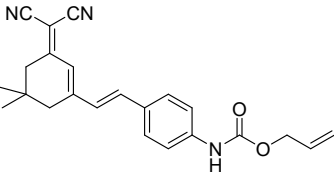
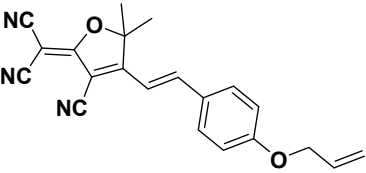
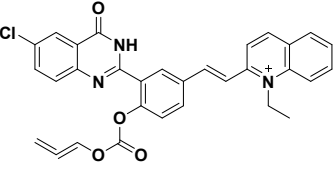
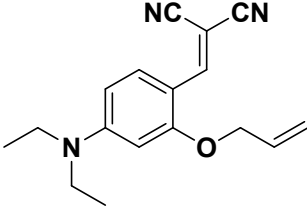
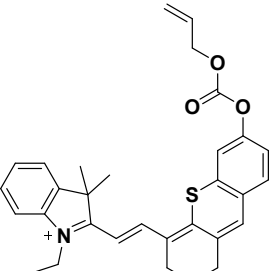
MCF-7 cells were cultured at 37 $^{\circ}\text{C}$ in a DMEM high-glycemic medium containing 10% (V / V) fetal bovine serum. Firstly, after the probe **THBTA-CO** and the cultivated MCF-7 were incubated for 30 minutes, the phosphate buffer was used to wash 3 times to remove the extracellular culture medium and the extracellular **THBTA-CO**. After that, different concentrations of CORM-3 (20 μM , 100 μM) and (PdCl_2 10 μM) were incubated with the pretreated cells for 30 minutes, and then washed with phosphate buffer three times in order to wash off excess CORM-3 outside the cells. In addition, after pretreating the cells with Pd^{2+} (10 μM) for 30 min, then treating the cells with different concentrations of HEME (100 μM) for 0 h, 4 h, 8 h, and finally co-incubating the pretreated cells with **THBTA-CO** for 30 min. Leica TCS SP8 (MP+X) confocal laser scanning microscope (Leica, Germany), objective lens 40 times (excitation

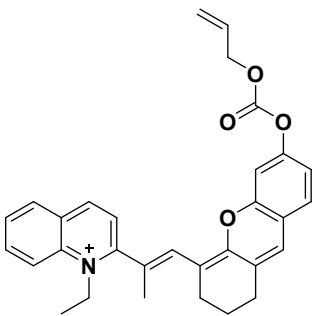
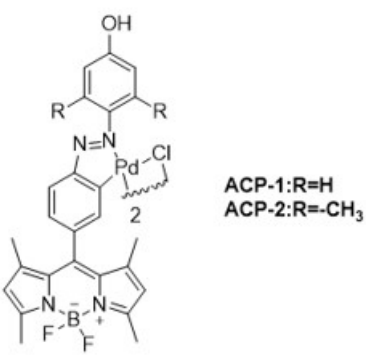
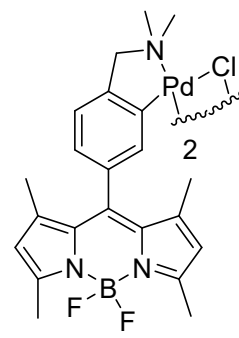
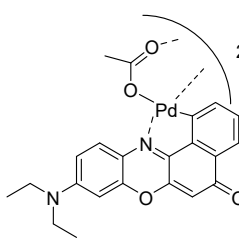
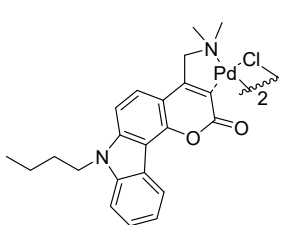
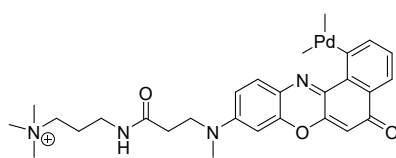
wavelength 410 nm) is used to collect fluorescent cell images.

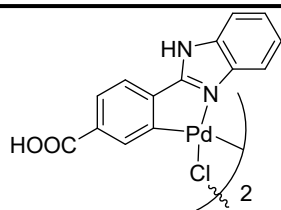
Table S1 Comparison of several fluorescent probes for CO detection

Probe Structures	AIE or ACQ	Response time	$\lambda_{\text{ex}}/\lambda_{\text{em}}$	LOD	Imaging application	Ref.
	Yes	~30 min	410 nm/ 635 nm	23 nM	Cells and living mice (Endogenous CO)	This work
	Yes	~30 min	340nm/ 552 nm;	47.5 nM;	Living cells	4
			350nm/ 552 nm	32.2 nM		
	Yes	20 min	465 nm/ 560 nm	30.8 nM	cells/mice	5
	No	15 min	550 nm/ 671 nm	38 nM	Cell and living mice	6
	No	>30 min	340 nm/ 460 nm	8.49 nM	cells	7

	No	3 min	670 nm/ 714 nm	3.2 nM	Cells and in living mice	8
	No	~10 min	592 nm/ 715 nm	15.8 nM	Cells/mice	9
 Probe 1 (X = H) Probe 2 (X = Cl)	No	>20 min	X=H (490nm/ 516 nm) X=Cl (493 nm/527 nm)	37 nM	cells	10
	No	> 15 min	490 nm/ 516 nm	37 nM	cells	11
	No	40 min	430 nm/ 556 nm, 466 nm	37.2 nM	cells	12
	No	20 min	430 nm/ 545 nm	17.9 nM	Living cells	13

	No	20 min	420 nm/ 545 nm	58 nM	Living cells	14
	No	5.3 min	690 nm / 736 nm	0.17 μM	Cells & tissue and mouse	15
	No	>30 min	440 nm/ 592 nm	61 nM	cells	16
	No	10 min	470 nm/ 620 nm	12 μM	cells	17
	No	1.5 min	560 nm/ 650 nm	35 nM	Cells and zebrafish	18
	No	20 min	450 nm/ 470 nm	4.5 nM	Cells and zebrafish	19
	No	25 min	730 nm/ 785 nm	250 nM	Cells and mice	20

	No	5 min	730 nm/ 780 nm	0.82 μM	Cells and mice	21
 ACP-1:R=H ACP-2:R=CH ₃	No	> 30 min	498 nm/ 512 nm	0.72 μM	cells	22
	No	60 min	475 nm/ 503 nm	1 μM	cells	23
	No	60 min	580 nm/ 660 nm	50 nM	Cell, zebrafish and mouse tissues and living mice	24
	No	40 min	374 nm/ 477 nm	0.653 μM	cell lines and tissues,	25
	No	30 min	543 nm/ 650 nm	0.23 μM	cell lines and tissues, living mice models	26



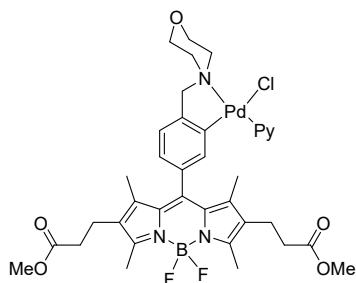
No

20 min

320 nm/
415 nm~0.06
μM

cell

27



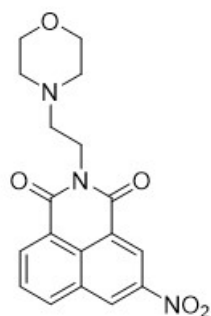
No

60 min

515 nm/
537 nmNo
given

Cell/fruit fly

28



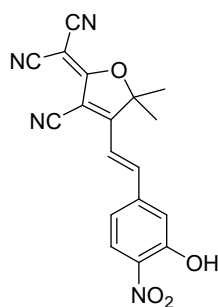
No

45 min

440 nm/
525 nm0.60
μM

cell

29



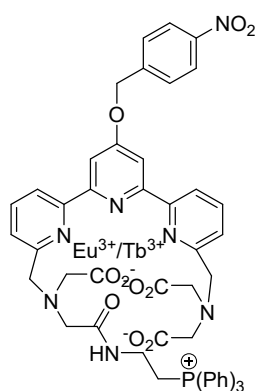
No

40 min

600 nm/
665 nmabout
6.1 nM

cell/zebrafish

30

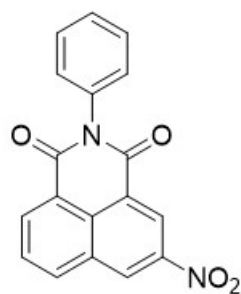


No

60 min

330 nm/
540 nm or
610 nm0.44
μMCell/ D.
magna/mouse

31



No	45 min	440 nm/ 522 nm	123 nM	Living cells	32
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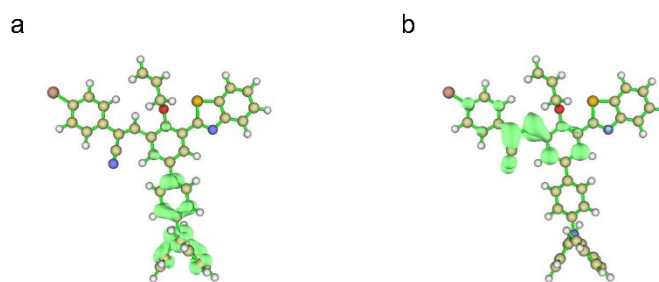


Figure. S1 Electron-hole analysis diagram of **THBTA-CO**, (a) represents hole diagram, (b) represents electron diagram

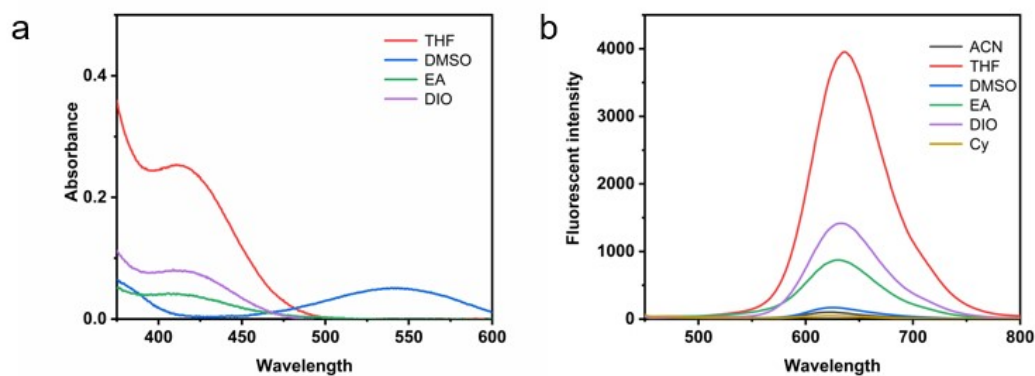


Fig. S2 (a) UV-Vis Absorption Spectroscopy of **THBTA** (20 μ M) in various organic solvents. (b) Fluorescence emission spectra of **THBTA** (20 μ M) in various organic solvents.

solvents.

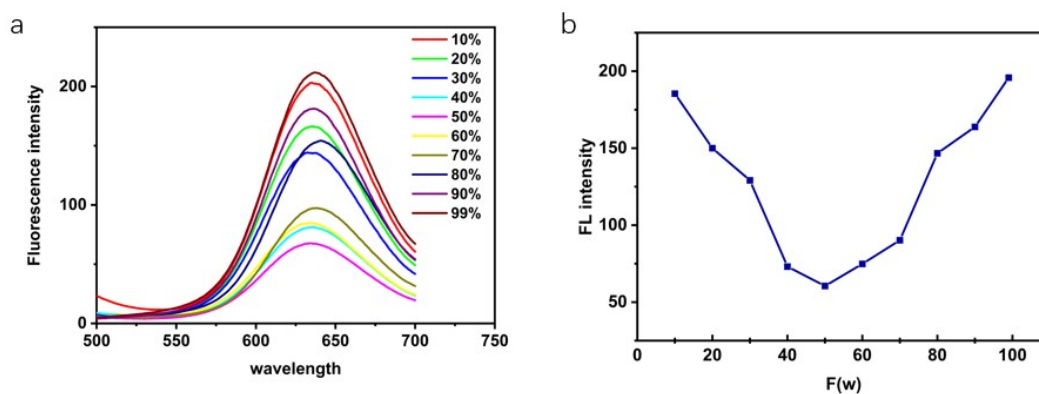


Fig. S3 (a) Fluorescence spectra of **THBTA** (10 μM) in mixtures of DMSO/water with different water fraction (fw). (b) Fluorescence emission at 630 nm of **THBTA** (10 μM) in mixtures of DMSO/water.

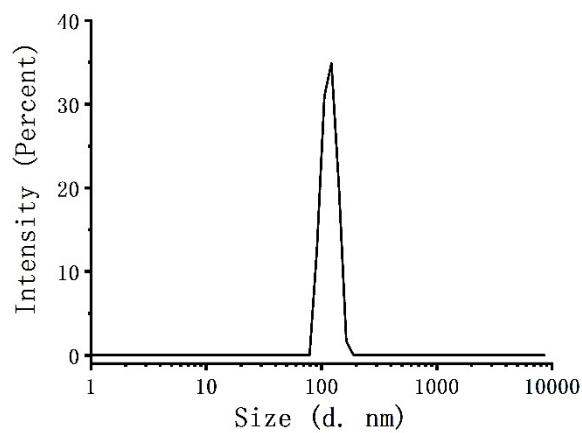


Figure S4. Particle size of **THBTA** in water ratio DMSO (99:1) system measured by DLS.

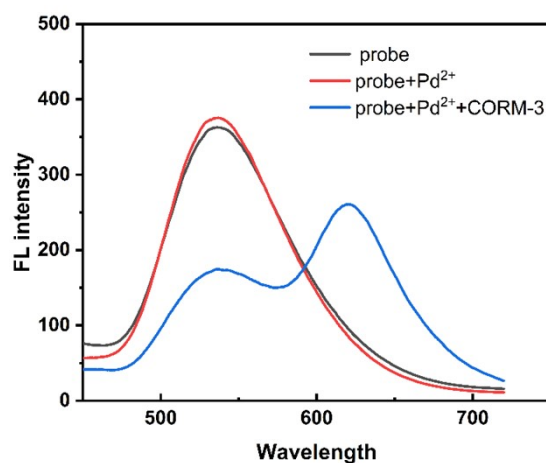


Figure S5. PL spectra of **THBTA-CO** (10 μM), **THBTA-CO** + PdCl_2 (10 μM) and **THBTA-CO** + PdCl_2 (10 μM) + CORM-3 at 37 $^\circ\text{C}$ for 30 min in water solution (pH 7.40).

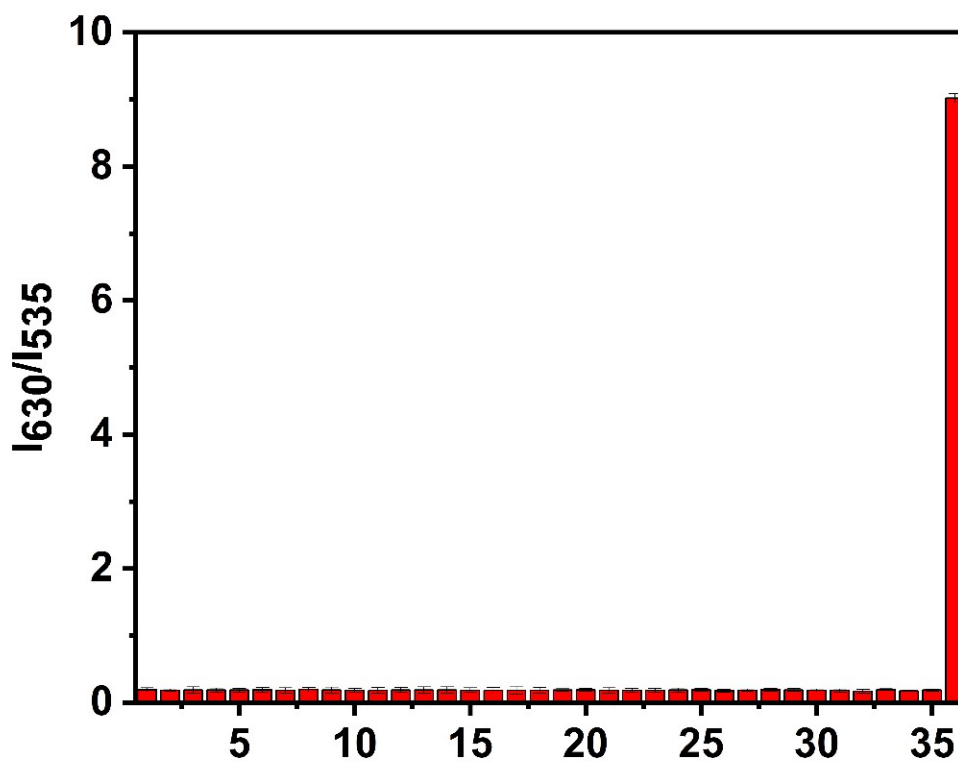


Figure S6. Fluorescence intensity ratio (F630/F535) of the probe system (THBTA-CO + PdCl₂, 10 μM each) for various analytes. 1, Blank; 2, GSH; 3, Cys; 4, Hcy; 5, H₂S; 6, Cu²⁺; 7, K⁺; 8, Ag⁺; 9, Zn²⁺; 10, Ba²⁺; 11, Fe²⁺; 12, Fe³⁺; 13, Mg²⁺; 14, Na⁺; 15, Hg²⁺; 16, Co²⁺; 17, Cd²⁺; 18, Ni²⁺; 19, Pb²⁺; 20, Al³⁺; 21, Ca²⁺; 22, Mn²⁺; 23, Cl⁻; 24, Br⁻; 25, I⁻; 26, NO³⁻; 27, NO²⁻; 28, H₂PO₄⁻; 29, ClO⁻; 30, SO₄²⁻; 31, SO₃²⁻; 32, PO₄³⁻; 33, CO₃²⁻; 34, Leu; 35, Trp; 36, CORM-3 ;. The concentration of each analyte is 100 μM except that CORM-3 is 40 μM.

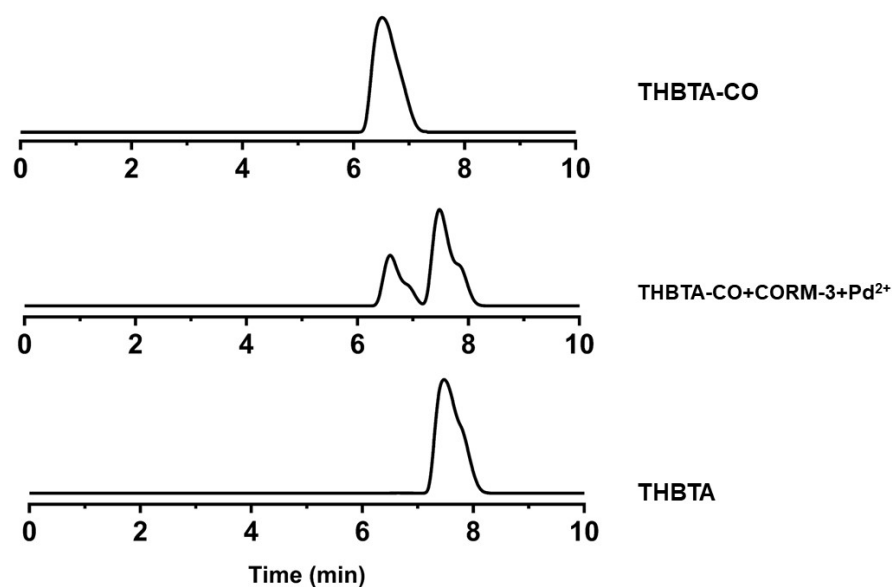


Figure S7. HPLC analysis of THBTA-CO and THBTA-CO with PdCl₂ upon the addition of 10 μM CORM-3 and THBTA. The HPLC mobile phase: solvent A (CH₃CN) and solvent B (water) CH₃CN-H₂O=90:10, v/v; flow rate: 1 mL/min, 37 °C, monitored at 360 nm.

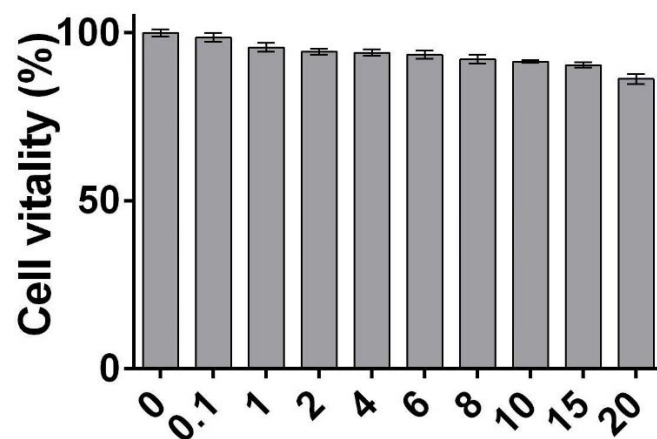


Figure. S8 Cytotoxicity effects of sensor **THBTA** at varied concentrations (0 - 20 μ M) on the viability of MCF-7 cells.

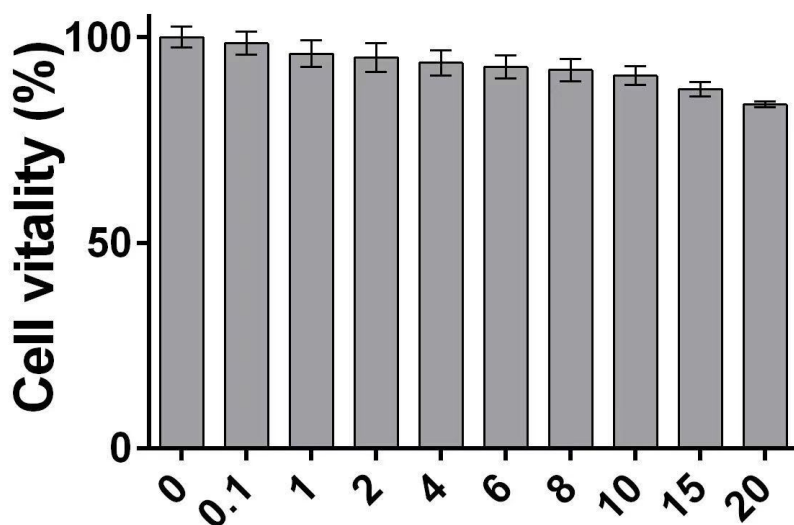


Figure. S9 Cytotoxicity effects of sensor **THBTA-CO** at varied concentrations (0 - 20 μ M) on the viability of MCF-7 cells.

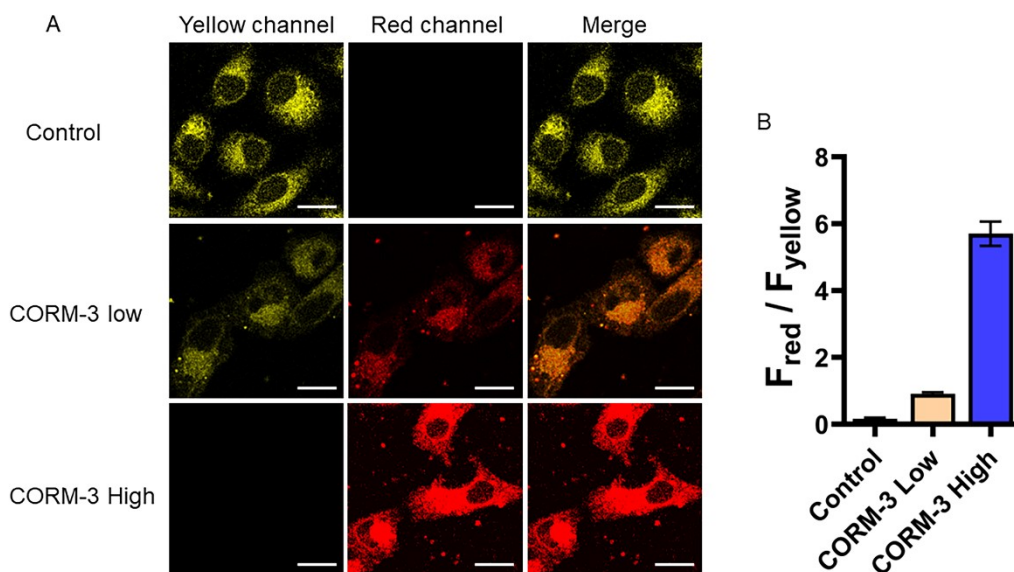


Figure. S10 Fluorescence images for exogenous CO in HepG2 cells by the probe system (THBTA-CO + PdCl₂, 10 μM each). In the control group, cells were cultured with the probe system (THBTA-CO + PdCl₂, 10 μM each) for 30 min. In the low-concentration CORM-3 group, the concentration of CORM-3 was 20 μM, the probe system was 10 μM, and the incubation time was 30 minutes. In the group with high-concentration CORM-3, the concentration of CORM-3 was 100 μM, the probe system was 10 μM and the incubation time was 30 minutes. Scale bar: 10 μm.(B) Fluorescence ratios (red/yellow) of the corresponding fluorescence images in panel.

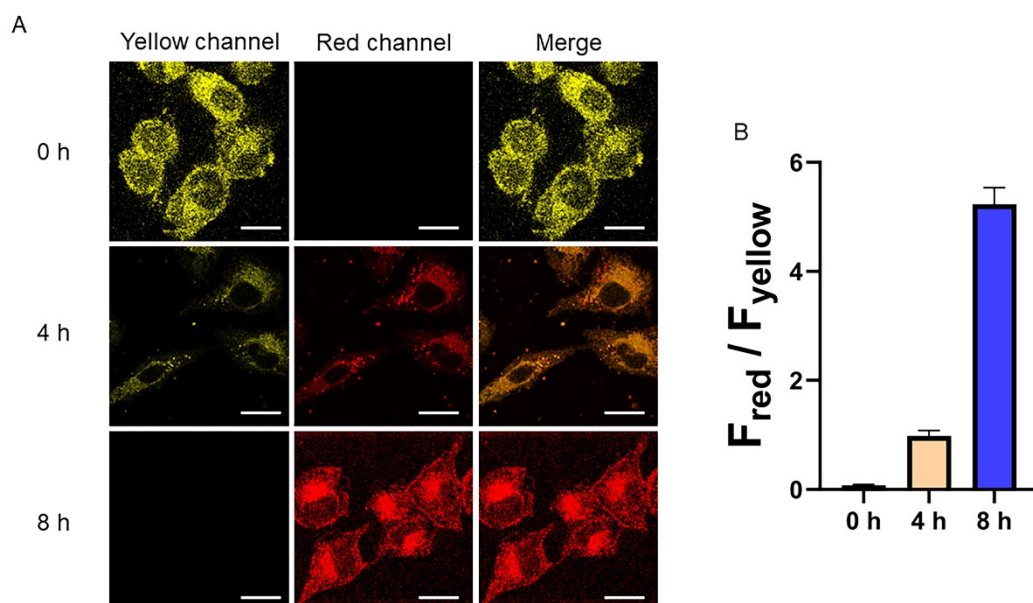


Figure S11 Fluorescence images of endogenous CO produced via heme stimulation in HepG2 cells. (A) Cells pretreated with 100 μM heme for different times (0, 4, 8 h) and then cultured with the probe system (THBTA-CO + PdCl₂, 10 μM each) for 30 min. (Scale bar: 10 μm) (B) Fluorescence ratios (red/yellow) of the corresponding fluorescence images in panel.

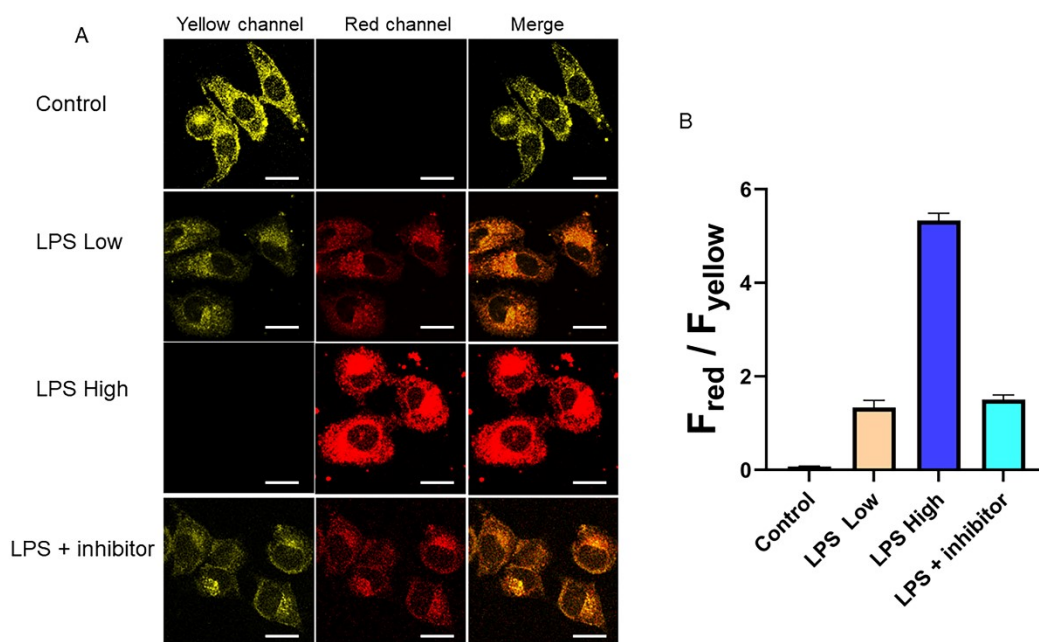


Figure S12 Fluorescence images of CO produced by LPS stimulation in HepG2 cells. The concentration of the probe system (THBTA-CO + PdCl₂) was 10 μM , the concentration of LPS was 0, 0.5, and 1 $\mu\text{g} / \text{mL}$, respectively. The concentration of LPS in the ZnPP group was 1 $\mu\text{g}/\text{mL}$, and the concentration of ZnPP was 10 μM . The incubation time for the above imaging was 12 hours. (Scale bar: 10 μm .) (B) Fluorescence ratios (red/yellow) of the corresponding fluorescence images in panel.

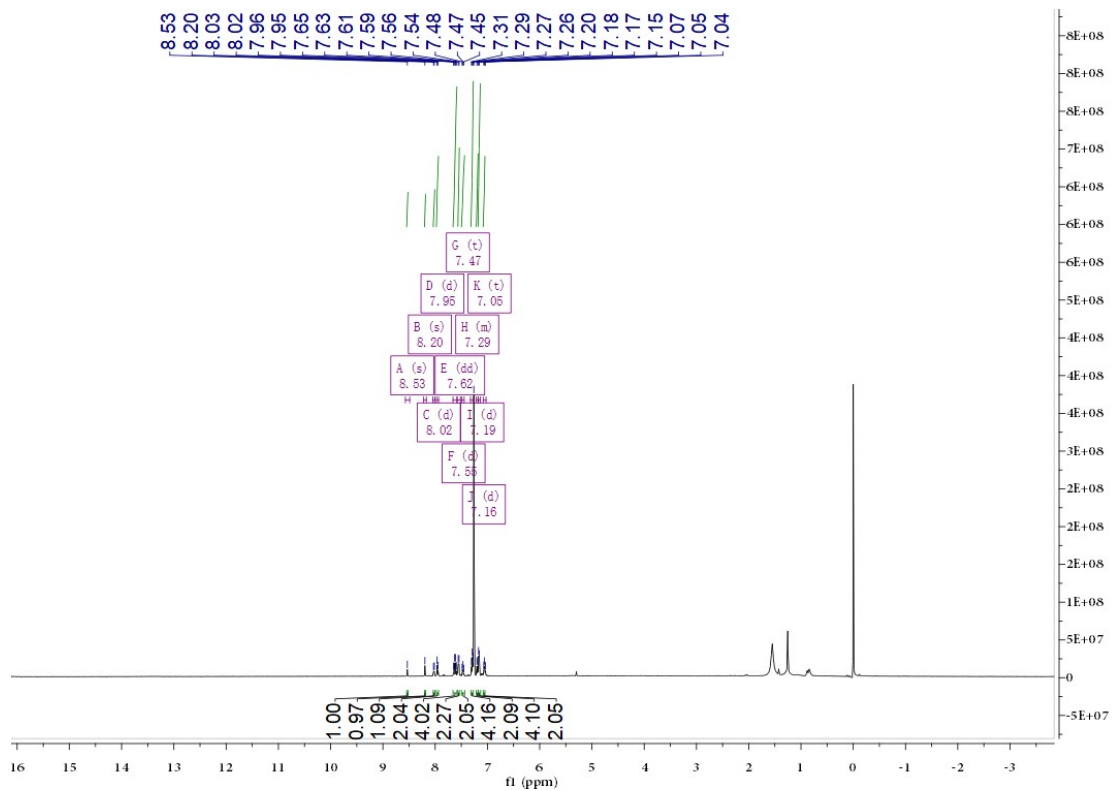


Figure. S15. $^1\text{H-NMR}$ spectrum of THBTA in CDCl_3 .

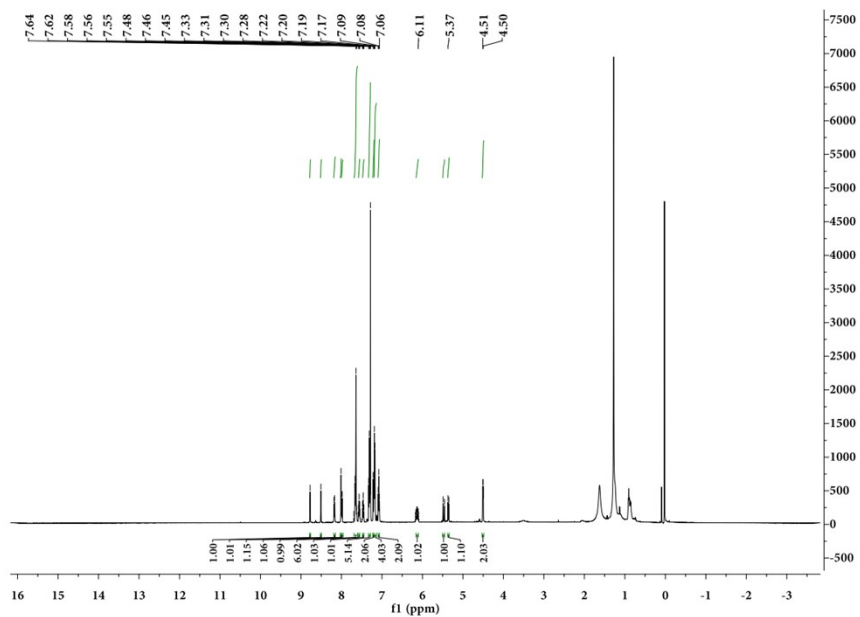


Figure. S16. ^1H -NMR spectrum of THBTA-CO in CDCl_3 .

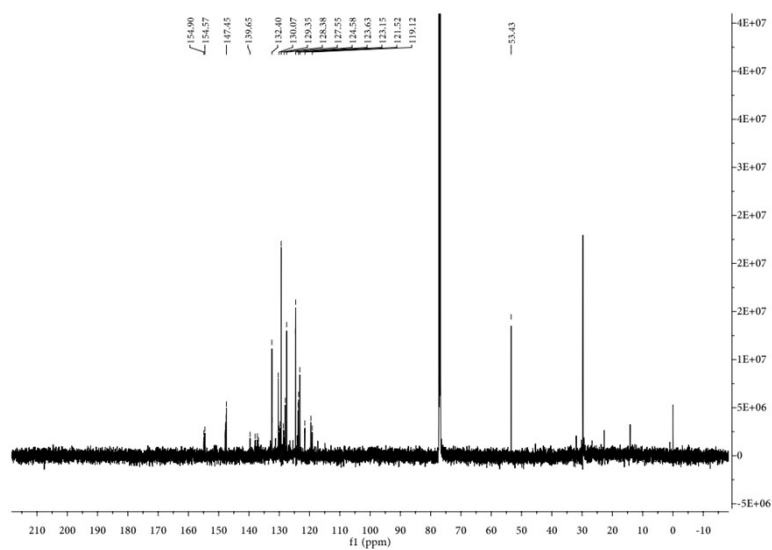


Figure. S17 ^{13}C -NMR spectrum of THBTA-CO in CDCl_3 .

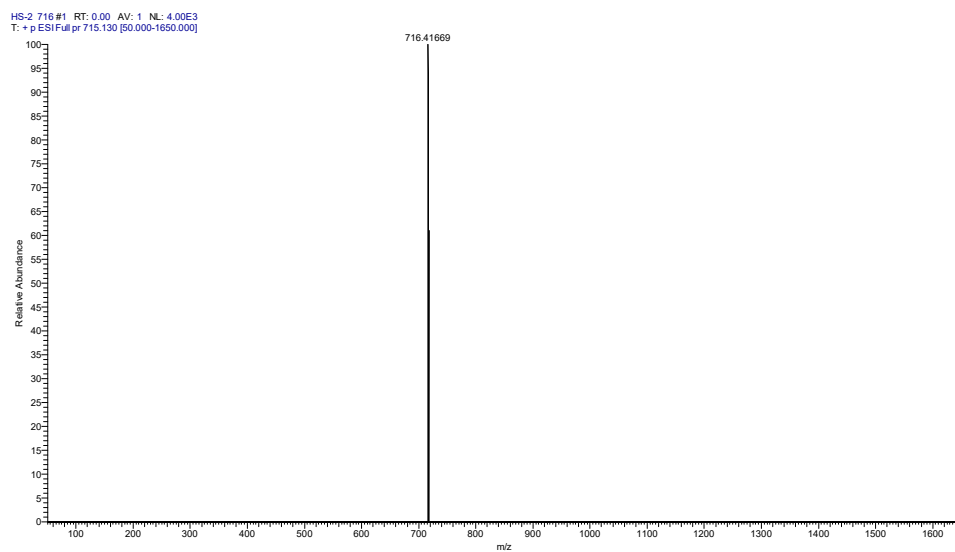


Figure. S18 HRMS spectrum of THBTA-CO.

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