Supporting information for

An ultrasensitive ratiometric fluorescent probe based on ICT-PET-FRET mechanism for quantitative measurement of pH values in endoplasmic reticulum (ER)

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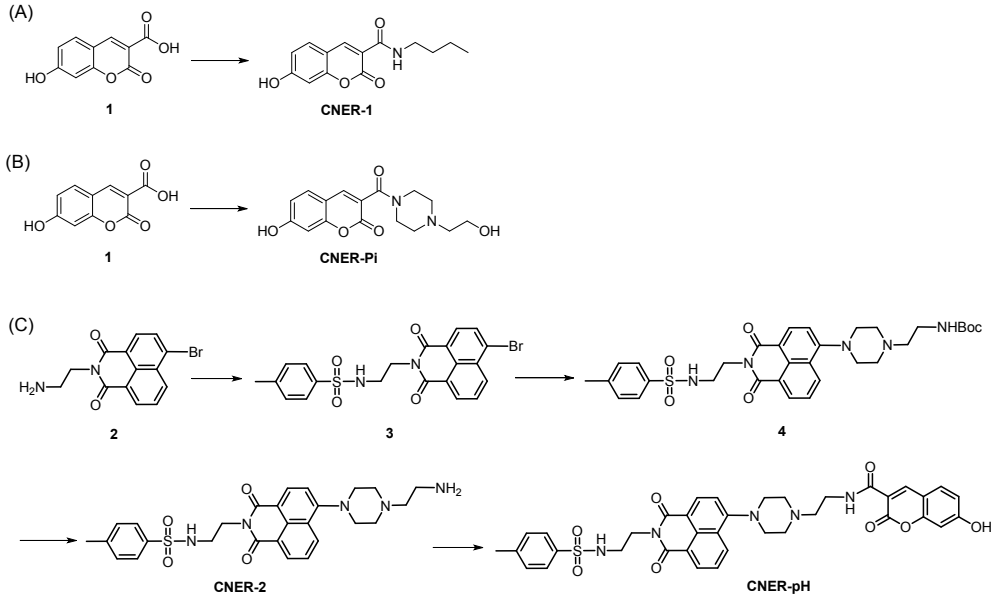
Materials and instruments

All solvents and reagents were commercially available and used without further purification. Doubly distilled water was used in all the experiments. Thin-layer chromatography (TLC) analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200-300), both of which were purchased from the Qingdao Ocean Chemicals. Xanthine oxidase from bovine milk was pruchased Sigma Company. Fluorescence spectra and relative fluorescence intensity were measured with a Hitachi F-4600 spectrofluorimeter with a 10 mm quartz cuvette. UV/vis spectra were obtained with a Shimadzu UV-2700 spectrophotometer. LC-MS were collected using an Agilent 6510 Q-TOF LC/MS. High-resolution mass spectra (HRMS) for the characterization of structures were collected using a Bruker apex-Ultra mass spectrometer (Bruker Daltonics Corp., USA) in electrospray ionization (ESI) mode. ^{1}H and ^{13}C NMR spectra were recorded on an AVANCE III 400 MHz Digital NMR Spectrometer, using tetramethylsilane (TMS) as internal reference. The fluorescence lifetime was detected using a Quantaurus-Tau C11367-34 (Hamamatsu Photonics Co., Ltd, Japan). Flow cytometry data were acquired on ACEA NovoCyte.

Synthesis

The probe and the control compounds were synthesized as following:



(1) Synthesis of compound **CNER-1**

A mixture of 2,4-dihydroxybenzaldehyde (1.38 g, 10 mmol), Meldrum's acid (1.44g, 10 mmol) and ammonium acetate (770 mg, 10 mmol) in 20 mL EtOH was refluxed for 6 h. After cooling to room temperature, the mixture was filtrated and washed with 10 mL EtOH to give compound 1 (1.40 g, 68 %) as white solid. n-Butylamine (73 mg,

1mmol), 7-hydroxy-2-oxo-2*H*-chromene-3-carboxylic acid (206 mg, 1mmol), 1-hydroxybenzotriazole (68 mg, 0.5 mmol) and EDC (382 mg, 2 mmol) in 5 mL DMF was stirred at room temperature. After 10 min, 400 μL N-ethyldiisopropylamine was added and then stirred for 12 h. Then, the solvent was removed and purified by column chromatography (PE: EA = 5:1) to give compound **CNER-1** (177 mg, 68 %). ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.78 (s, 1H), 7.69 (d, J = 8.4 Hz, 1H), 6.89 (dd, 1H), 6.79 (d, J = 2.0 Hz, 1H), 3.42 (t, 2H), 1.62 (m, 2H), 1.45 (m, 2H), 1.31 (s, 1H), 0.98 (t, 3H). ^{13}C NMR (100 MHz, Methanol-d_4) δ 163.46, 162.27, 157.44, 148.11, 131.37, 115.78, 110.40, 102.11, 38.93, 31.18, 19.78, 12.66. MS (ESI) m/z calcd. For $C_{14}H_{15}NO_4$; $[M + H]^+$ 262.1, found 262.2.

(2) Synthesis of compound **CNER-Pi**

7-Hydroxy-2-oxo-2*H*-chromene-3-carboxylic acid (103 mg, 0.5 mmol), 1-(2-hydroxyethyl)piperazine (65 mg, 0.05 mmol), EDC (191 mg, 1 mmol) and HOBT (33.75 mg, 0.025 mmol) was dissolved in 3 ml DMF at room temperature. After 10 min, 100 μL DIEPA was added and the solution was stirred for 5 h. Thereafter, the mixture was dissolved in 20 mL water, and then the crude product was separated and purified by column chromatography (DCM : MeOH = 10:1) to give compound **CNER-Pi** (106 mg, 66.7%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.96 (s, 1H), 7.48 (d, J = 8.8 Hz, 1H), 6.79 (dd, J = 8.4, 2.0 Hz, 1H), 6.67 (d, J = 2.0 Hz, 1H), 3.77 (s, 2H), 3.70 (t, 2H), 3.49 (s, 2H), 2.58 (t, 6H).^{13}C NMR (101 MHz, Methanol-d_4) δ 165.94, 165.25, 159.47, 156.66, 144.38, 130.17, 117.25, 115.13, 110.09, 102.52, 59.68, 58.39. MS (ESI) m/z calcd. for $C_{16}H_{18}N_2O_5$, $[M+H]^+$ 319.1, found 319.2.

(3) Synthesis of compound **3**

(2-aminoethyl)-6-bromo-1H-benzo[de]isoquinoline-1,3(2H)-dione (**2**, 318 mg, 1 mmol) and triethylamine (101.9 mg, 1 mmol) were dissolved in 25 mL CH_2Cl_2, and p-toluenesulfonyl chloride (190 mg, 1 mmol) in 2 mL CH_2Cl_2, was added to the reaction solution dropwise slowly and then the mixture was stirred in ice bath for 3 h. Next, the solvent was removed by evaporation under reduced pressure to give crude product, and then separation and purification of the compound by column chromatography (PE：EA=5:1) to afford the compound 3（813 mg，86%）. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (m, 2H), 8.26 (d, J = 7.6 Hz, 1H), 7.98 (d, J = 7.8 Hz, 1H), 7.79 (t, 1H), 7.52 (d, J = 8.0 Hz, 2H), 6.76 (d, J = 8.0 Hz, 2H), 5.36 (t, 1H), 4.25 (t, 2H), 3.46 (m, 2H), 1.98 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.02, 163.96, 142.69, 137.18, 133.59, 132.25, 131.39, 131.14, 130.70, 130.53, 129.19, 128.91, 128.14, 126.67, 122.58, 121.68, 42.37, 39.28, 21.14. MS (ESI) m/z calcd. for $C_{21}H_{17}BrN_2O_4S$ $[M + H]^+$ 474.3, found 474.0.

(4) Synthesis of compound **4**

Compound **3** (473 mg, 1 mmol) and Boc-N-aminoethylpiperazine (229 mg, 1 mmol) were dissolved in 15 mL 2-methoxyethanol and heated to 120℃ under nitrogen for 4 hours. After completion of the reaction, the reaction solution was cooled to room temperature, and then poured into 15 mL distilled water to precipitate yellow solid. Filtration of the mixture to remove solvent, and the obtained solid was purified by column chromatography (CH_2Cl_2：MeOH=30:1) to afford compound 4 (503 mg, 81%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (d, *J* = 7.3 Hz, 1H), 8.43 (t, 2H), 7.77 (m, 1H), 7.57 (d, *J* = 8.2 Hz, 2H), 7.22 (d, *J* = 8.1 Hz, 1H), 6.76 (d, *J* = 8.0 Hz, 2H), 4.26 (m, 2H), 3.45 (m, 2H), 3.35 (s, 6H), 2.83 (s, 4H), 2.65 (t, 2H), 1.97 (s, 3H), 1.49 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.90, 156.01, 142.62, 132.88, 131.42, 129.94, 129.21, 126.78, 126.07, 126.05, 125.83, 115.08, 53.00, 42.74, 39.02, 39.00, 28.45, 21.24, 21.19. MS (ESI) m/z calcd. for $C_{32}H_{39}N_5O_6S$ $[M + H]^+$ 622.2, found 622.3.

(5) Synthesis of compound **CNER-2**

Compound 3 (310 mg, 0.5 mmol) was dissolved in 25 mL CH_2Cl_2, and then 1.5 mL trifluoroacetic acid was added to the mixture dropwise at room temperature for 3 h. After the solvent was distilled off under reduced pressure, and purification of the crude product by column chromatography obtained compound **CNER-2** (216 mg, 83%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.27 (dd, 2H), 8.16 (dd, 1H), 7.58 (t, 1H), 7.49 (d, *J* = 8.4 Hz, 2H), 7.12 (d, *J* = 8.0 Hz, 1H), 6.87 (d, *J* = 8.4 Hz, 2H), 4.10 (t, 2H), 3.26 (m, 6H), 3.16 (t, 2H), 2.82 (m, 6H), 2.03 (s, 3H).^{13}C NMR (100 MHz, Methanol-d_4) δ 164.48, 164.00, 156.15, 142.89, 137.34, 132.37, 130.72, 130.55, 129.42, 129.01, 126.27, 125.59, 125.39, 122.25, 115.39, 114.64, 54.17, 52.61, 44.05, 40.81, 39.13, 36.09, 19.98. HRMS (ESI) m/z calcd. For $C_{27}H_{31}N_5O_4S$; $[M+H]^+$ 522.2170, found 522.2177.

(6) Synthesis of compound **CNER-pH**

Compound **CNER-1** (104mg, 0.2mmol), 7-hydroxy-2-oxo-2*H*-chromene-3-carboxylic acid(41mg, 0.2mmol), HOBT (13.5mg, 0.1mmol), and EDC (76mg, 0.4mmol) in 5 mL DMF was stirred at room temperature for 10 min, then 200 μL DIPEA was added to the reaction mixture and stirred for 5 h. The reaction solution was washed three times by saturated NaCl solution, thereafter the solvent was removed in vacuo to get crude yellow product. The crude product was purified by column chromatography to get compound **CNER-pH** (68 mg, 48%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.08 (s, 1H), 8.92 (t, 1H), 8.81 (s, 1H), 8.44 (q, 2H), 8.35 (d, J = 8.4 Hz, 1H), 7.81 (m, 2H), 7.73 (t, 1H), 7.58 (d, J = 8.4 Hz, 2H), 7.34 (d, J = 8.0 Hz,

1H), 7.23 (d, J = 8.0 Hz, 2H), 6.89 (dd, 1H), 6.79 (d, J = 2.0 Hz, 1H), 4.11 (d, J = 6.2 Hz, 2H), 3.51 (d, J = 5.6 Hz, 2H), 3.28 (s, 4H), 3.04 (dd, 2H), 2.81 (s, 4H), 2.67 (s, 2H), 2.27 (s, 3H). ^{13}C NMR (100MHz, DMSO) δ 164.12, 163.58, 161.91, 161.50, 156.77, 156.04, 148.50, 142.90, 138.11, 132.59, 132.46, 131.03, 130.93, 129.93, 129.73, 126.83, 126.44, 125.78, 123.17, 120.83, 116.17, 115.46, 114.80, 114.06, 111.56, 102.25, 56.61, 53.20, 52.97, 49.07, 36.76, 21.34. HRMS(ESI) m/z calcd. For $C_{37}H_{35}N_5O_8S$; $[M+H]^+$ 710.2279, found 710.2277.

Determination of fluorescence quantum yields

Fluorescence quantum yield was measured by the relative method and using Rhodamine-6G as reference (Φ_f = 0.94 in ethanol). The fluorescence quantum yield was calculated according to the equation: $\Phi_S = \Phi_R*(F_S/F_R)*(A_R/A_S)*(\eta_S/\eta_R)^2$. Here, Φ_S and Φ_R are the quantum yields of sample and reference, F_S and F_R are the integrated emission intensities of the corrected spectra for the sample and reference, A_R and A_S are the absorbance of the reference and sample at the excitation wavelength, η_S/η_R stand for the values of refractive index for the respective solvent used for the sample and reference.

Cell Culture and Fluorescence Imaging

HeLa cells were cultured in modified Eagle's medium supplemented with 10% calf bovine serum in an atmosphere of 5% CO_2 and 95% air at 37 °C. Then cells were seeded into 35 mm glass-bottom culture dishes and cultured for 24 h. For imaging at various pH, the cells were incubated with 5 μM **CNER-pH** for 10 min at 37 °C, then the media was replaced with PBS buffer at various pH. The cells were sequentially incubated with the PBS buffer, 10 μM nigericin and 5 μM monensin for another 30 min, and then imaged using a Nikon A1 MP confocal microscope.

For the intracellular pH calibration, the HeLa cells were incubated with 5 μM **CNER-pH** for 30 min, and then treated with 5 μM nigericin, 5 μM monensin and B-R buffer with different pH for another 30 min. Flow cytometry analysis was then carried out on ACEA NovoCyte. For the quantitative measurement of pH in HeLa cells stimulated by Hcy, tunicamycin or dexamethanose, HeLa cells stained with 5 μM **CNER-pH** for 30 min at 37 °C were washed three times with PBS buffer, and then 50 μM Hcy, 2 μM tunicamycin or 1 μM dexamethanose was added to stimulate cellular pH change. Flow cytometry analysis was then carried out on ACEA NovoCyte.

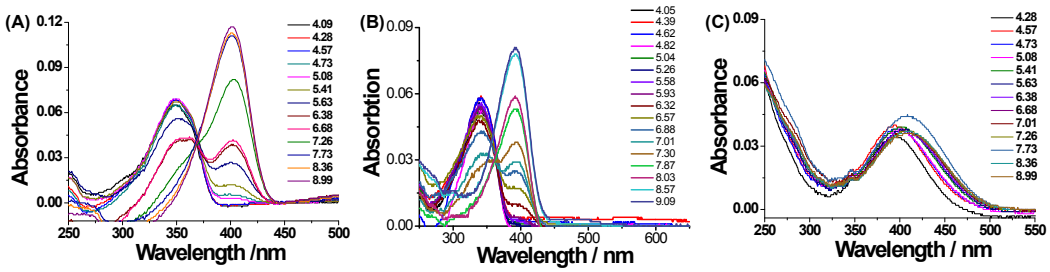


Fig. S1 UV-Vis spectra of 5 μM **CNER-1** (A), **CNER-Pi** (B) and **CNER-2** (C) in B-F buffers (5% EtOH) at various pH.

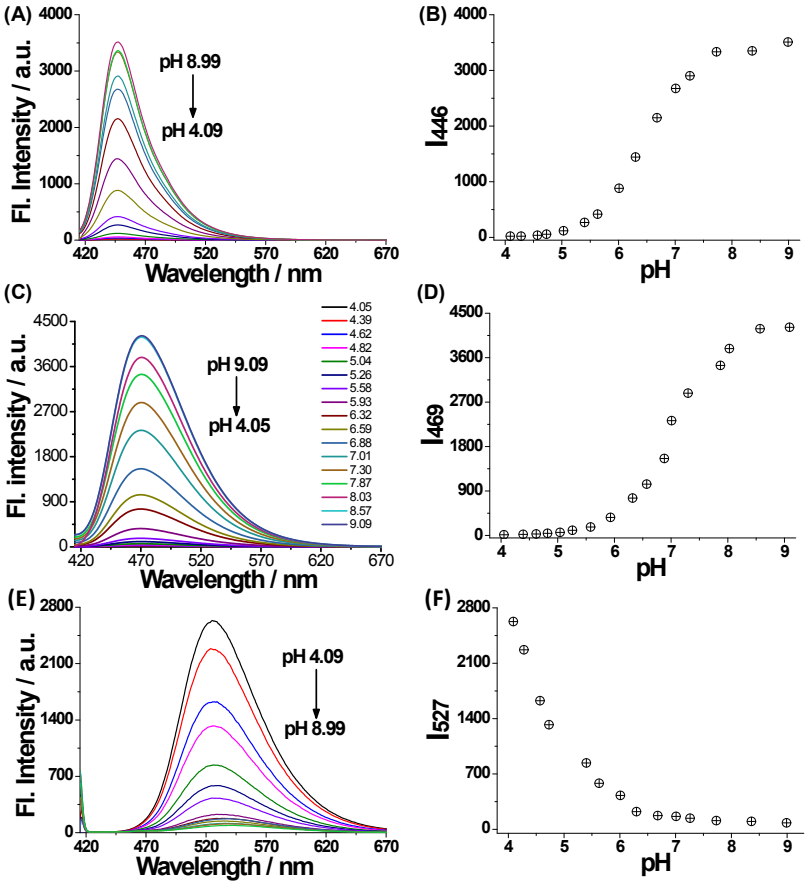


Fig. S2 Fluorescence spectra (A) and fluorescence intensity (B) at 446 nm of 5 μM **CNER-1** in B-F buffers (5% EtOH) at various pH. Fluorescence spectra (B) and fluorescence intensity (C) at 469 nm of 5 μM **CNER-Pi** in B-F buffers (5% EtOH) at various pH. Fluorescence spectra (E) and fluorescence intensity (F) at 446 nm of 5 μM **CNER-2** in B-F buffers (5% EtOH) at various pH. λ_{ex} = 405 nm.

Table S1. Fluorescent quantum yields of the compounds in different solvents.

	CNER-1	CNER-Pi	CNER-2	CNER-pH
MeOH	0.23	0.26	0.25	0.21
EtOH	0.23	0.31	0.39	0.36
CH_3CN	0.31	0.28	0.32	0.31
PBS	0.22	0.28	0.29	0.25
DMF	0.11	0.13	0.35	0.23
DMSO	0.12	0.16	0.31	0.30

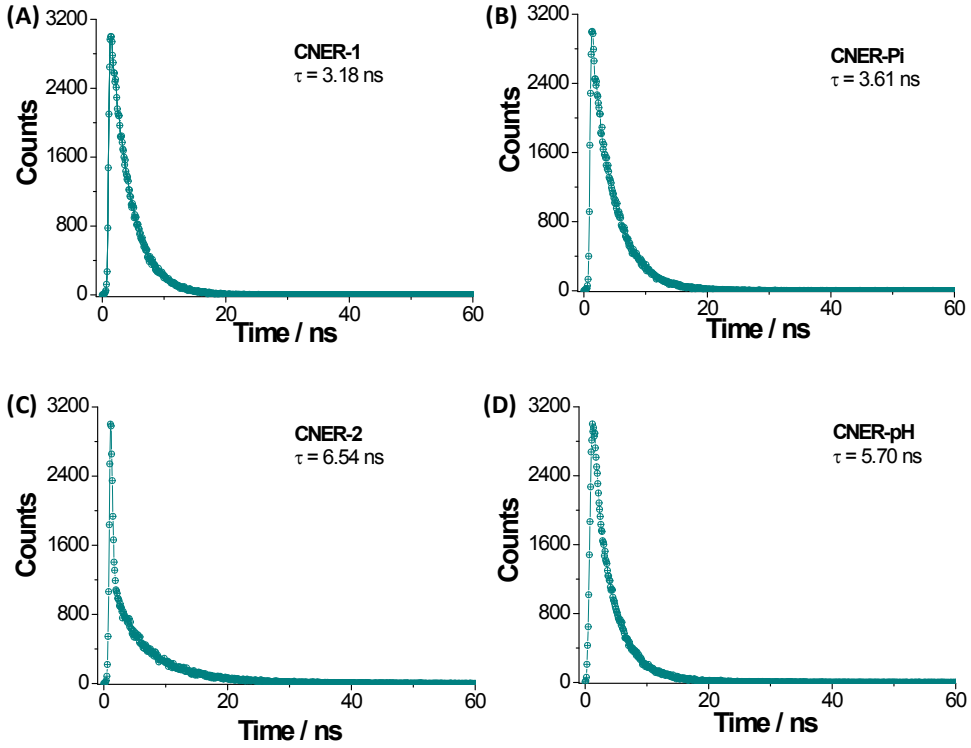


Fig. S3 Fluorescence lifetime of the compounds in EtOH.

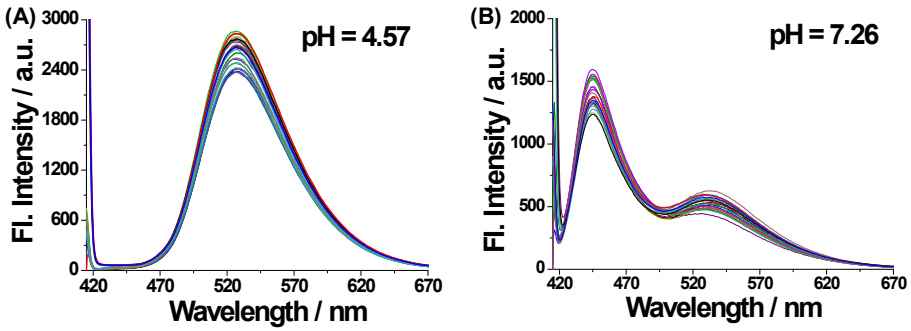


Fig. S4 Fluorescence spectra of 5 μM **CNER-pH** treated with various biologically relevant analytes in B-F buffers (5% EtOH) at pH 4.57 (A) and 7.26 (B). Analytes: Mg^{2+}; Fe^{2+}; Zn^{2+}; Ca^{2+}; Cu^{2+}; Al^{3+}; SO_3^{2-}; S^{2-}; Cys; Hcy; GSH; ClO^-; NO; H_2O_2; glucose; VC. λ_{ex} = 405 nm.

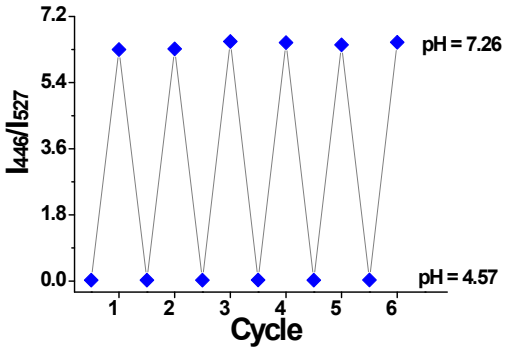


Fig. S5 pH reversibility study of 5 μM **CNER-pH** in B-R buffer solution (5% EtOH) between pH 4.57 and 7.26. λ_{ex} = 405 nm.

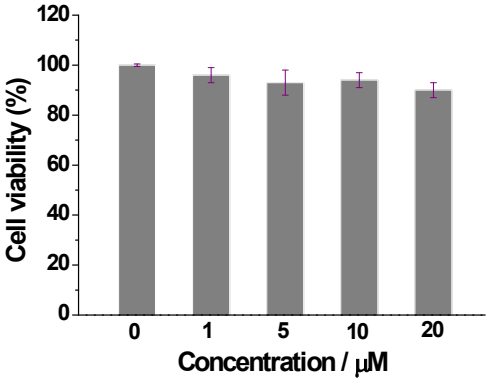


Fig. S6 Survial of HeLa cells in the presence of **CNER-pH** at various concentrations measured using MTT assay.

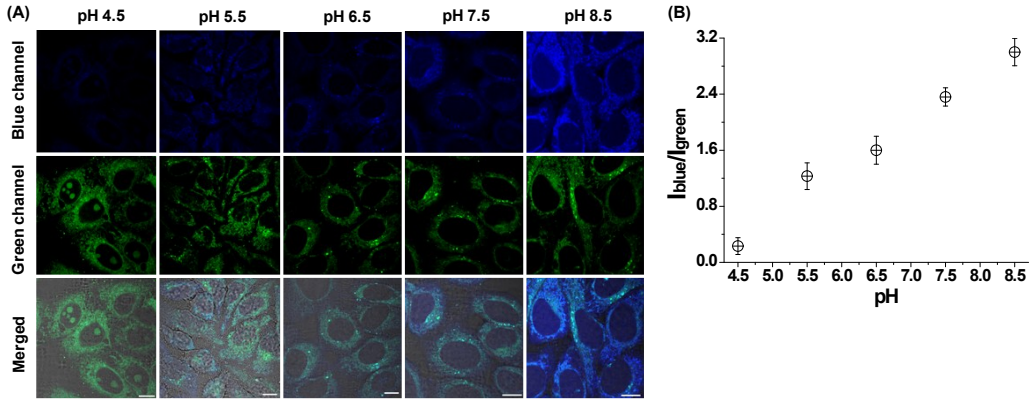


Fig. S7 (A) Fluorescence images for 5 μM **CNER-pH** at various pH. Blue channel (425-475 nm), Green channel (500-550 nm), λ_{ex} = 405 nm. (B) Quantified the ratio (I_{blue}/I_{green}) of fluorescence intensity between blue channel and green channel at various pH was analyzed using ImageJ. Error bars are ± SD, n = 3.

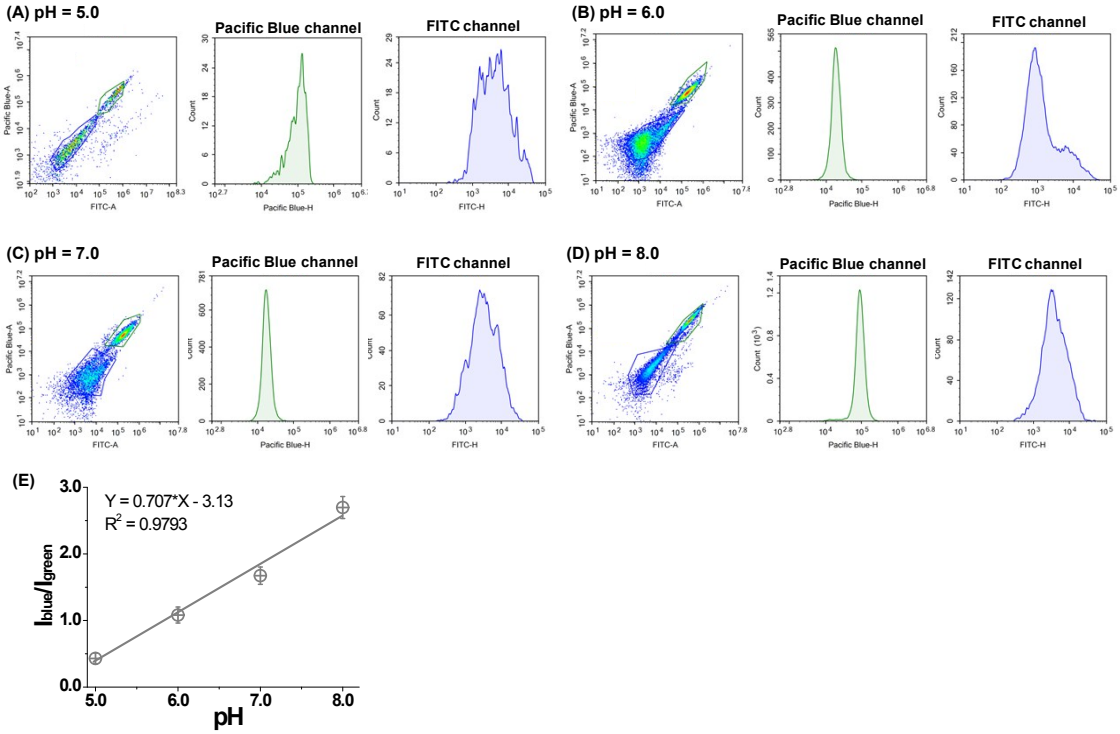


Fig. S8 Flow cytometry analysis of the HeLa cells incubated with 5 μM **CNER-pH** for 30 min, and then treated with 5 μM nigericin, 5 μM monensin and B-R buffer with pH 5.0 (A), pH 6.0 (B), pH 7.0 (C) and pH 8.0 (D) for another 30 min. Pacific Blue channel (Blue channel): λ_{ex} = 387 nm; λ_{em} = 445 nm. FITC channel (Green channel): λ_{ex} = 455 nm; λ_{em} = 530 nm. (E) The average ratio (I_{blue}/I_{green}) values in panels A-D. Error bars are ± SD, n = 3.

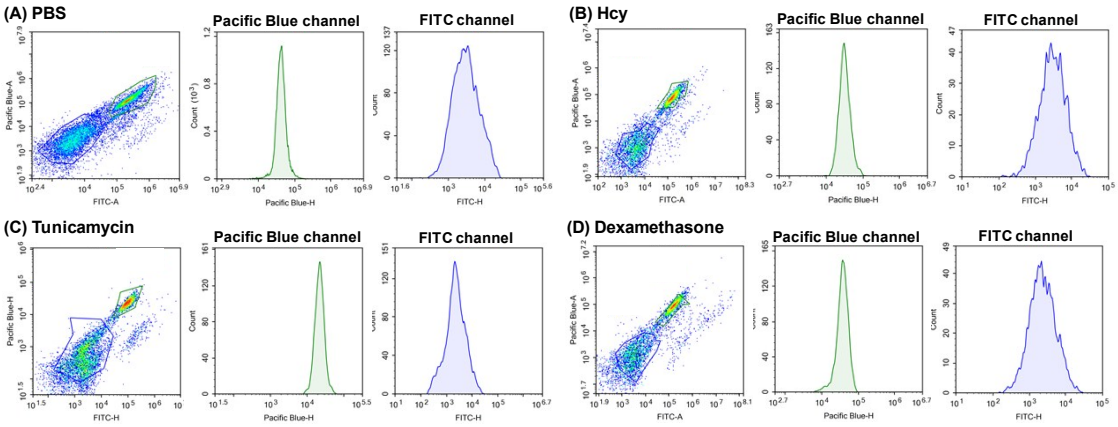


Fig. S9 Flow cytometry analysis of the HeLa cells incubated with 5 μM **CNER-pH** for 30 min, and then treated with PBS buffer (A), 50 μM Hcy (B), 2 μM tunicamycin (C) or 1 μM dexamethanose (D) 30 min. Pacific Blue channel (Blue channel): λ_{ex} = 387 nm; λ_{em} = 445 nm. FITC channel (Green channel): λ_{ex} = 455 nm; λ_{em} = 530 nm.

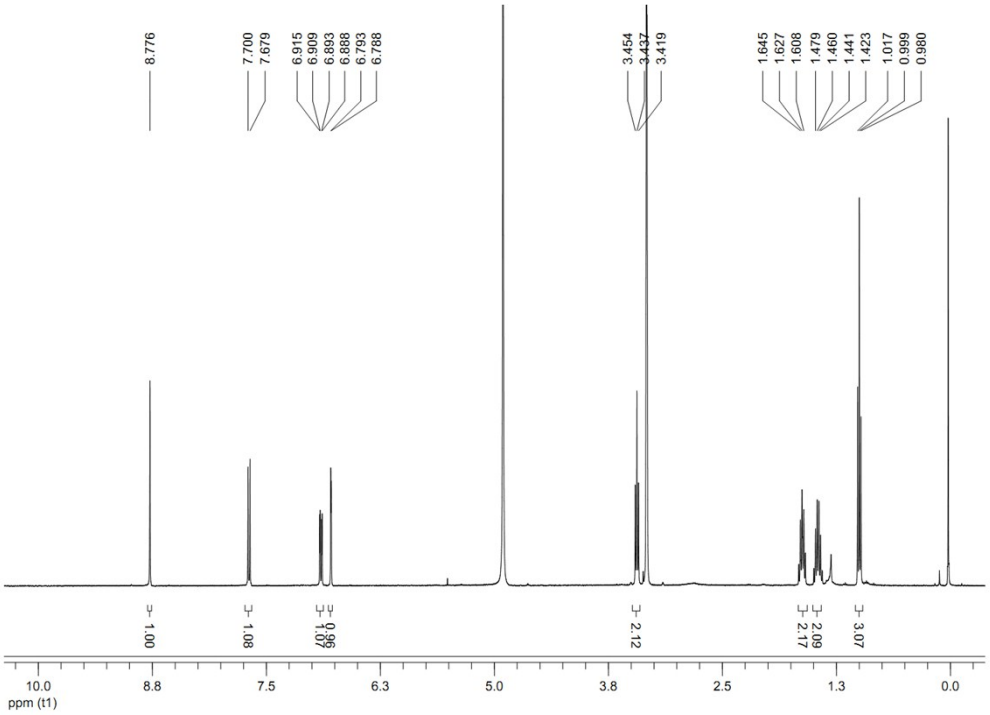


Fig. S10 ^{1}H NMR spectrum of **CNER-1** (Methanol-d_4).

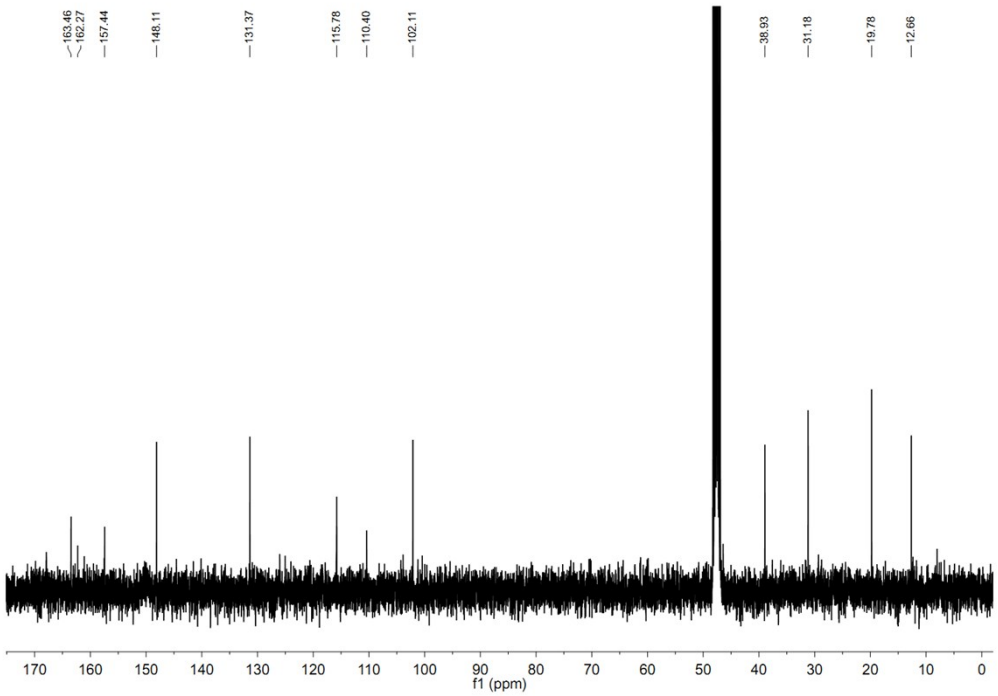


Fig. S11 ^{13}C NMR spectrum of **CNER-1** (Methanol-d_4).

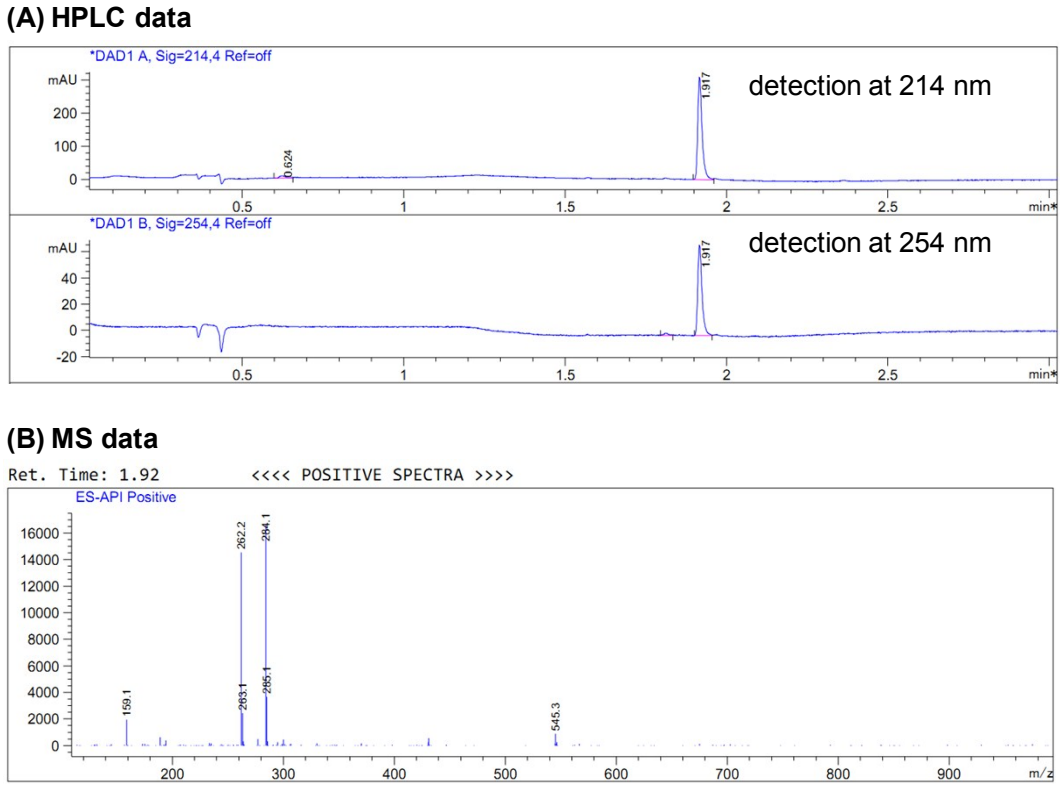


Fig. S12 LC-MS data of **CNER-1**.

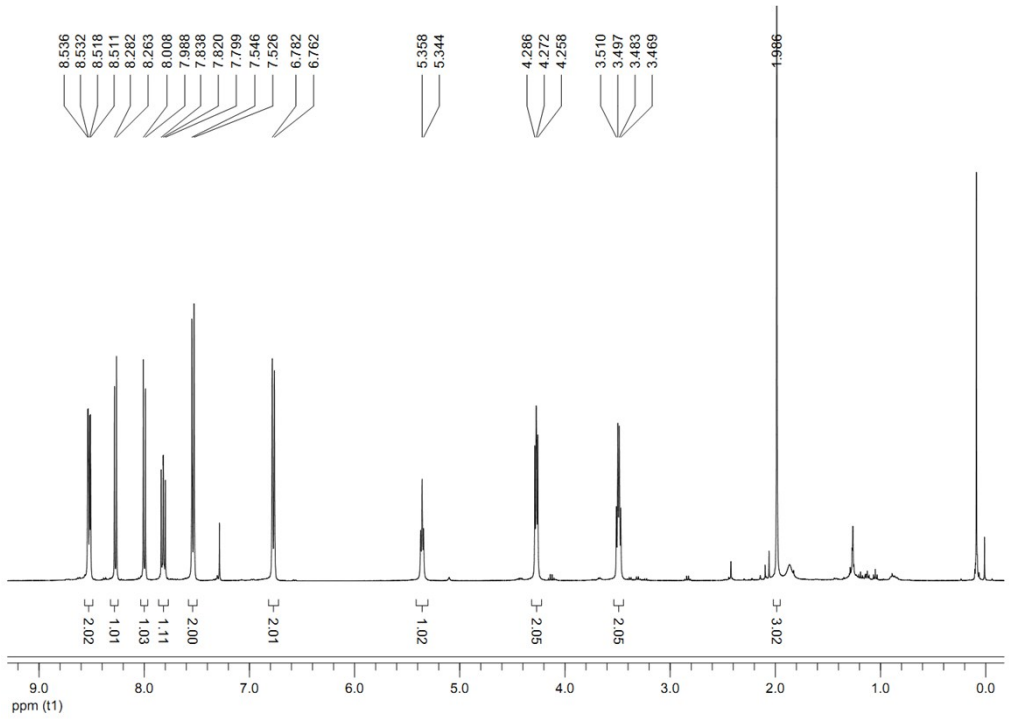


Fig. S13 ^{1}H NMR spectrum of compound **3** ($CDCl_3$).

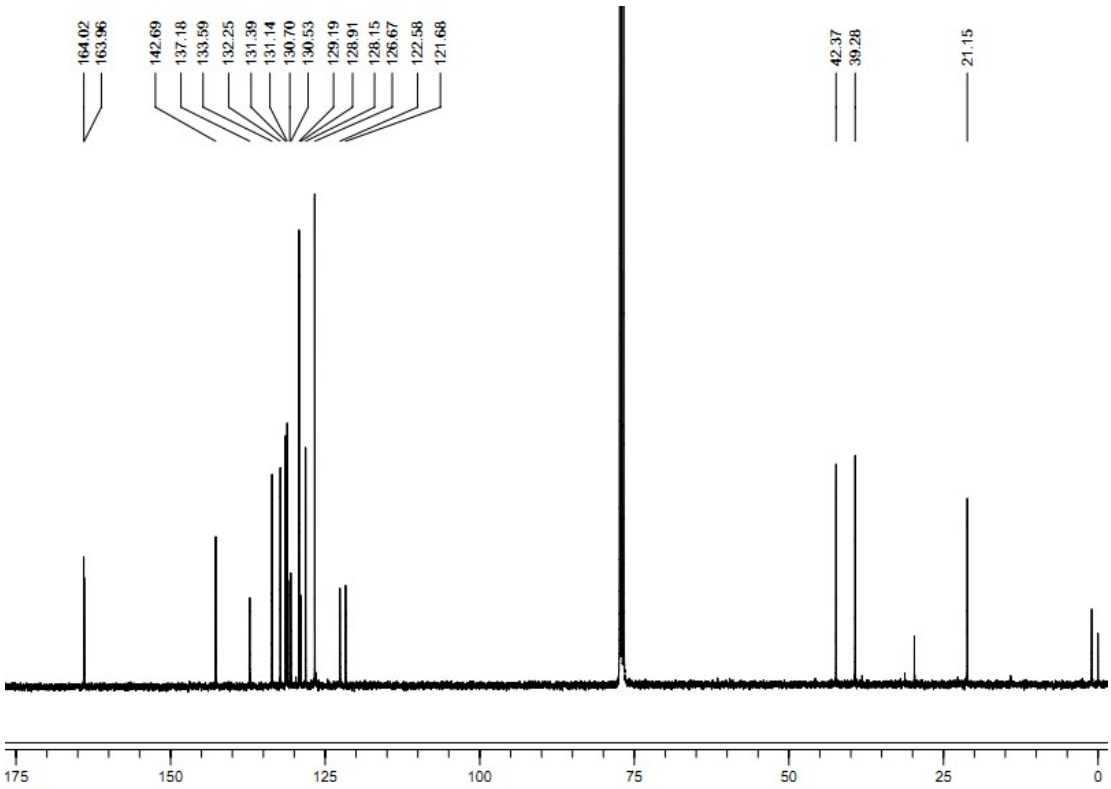


Fig. S14 ^{13}C NMR spectrum of compound **3** ($CDCl_3$).

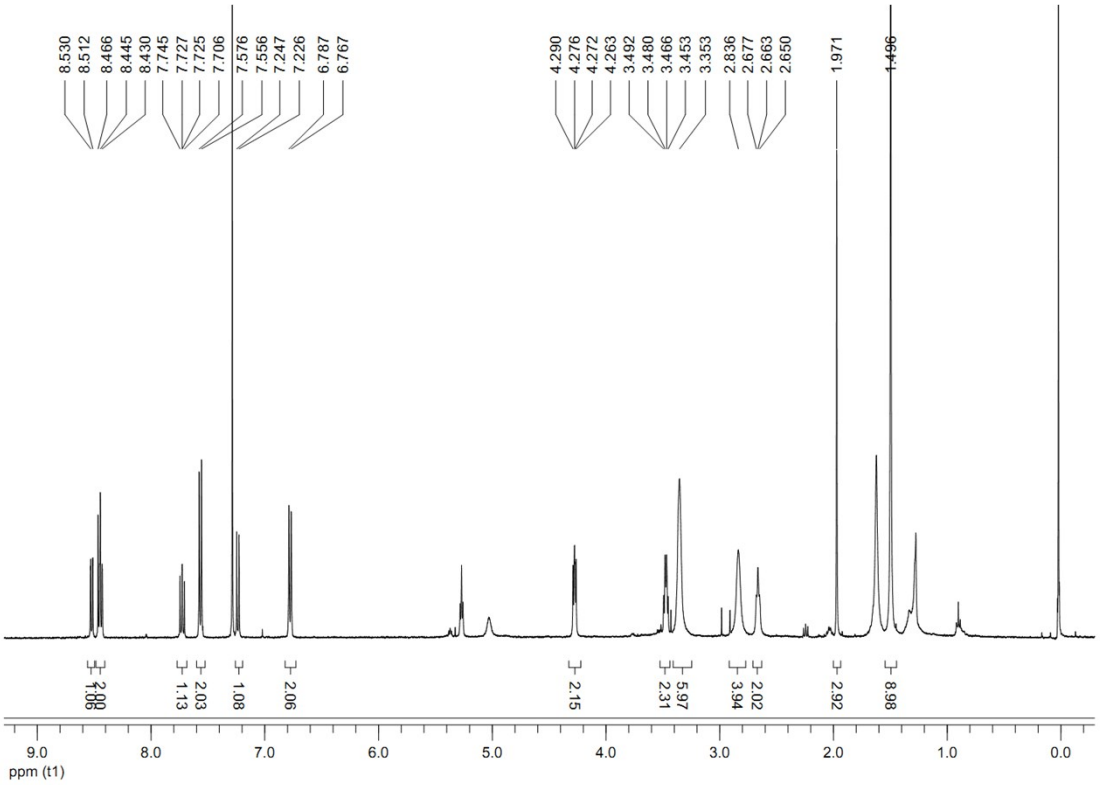


Fig. S15 ^{1}H NMR spectrum of compound **4** ($CDCl_3$).

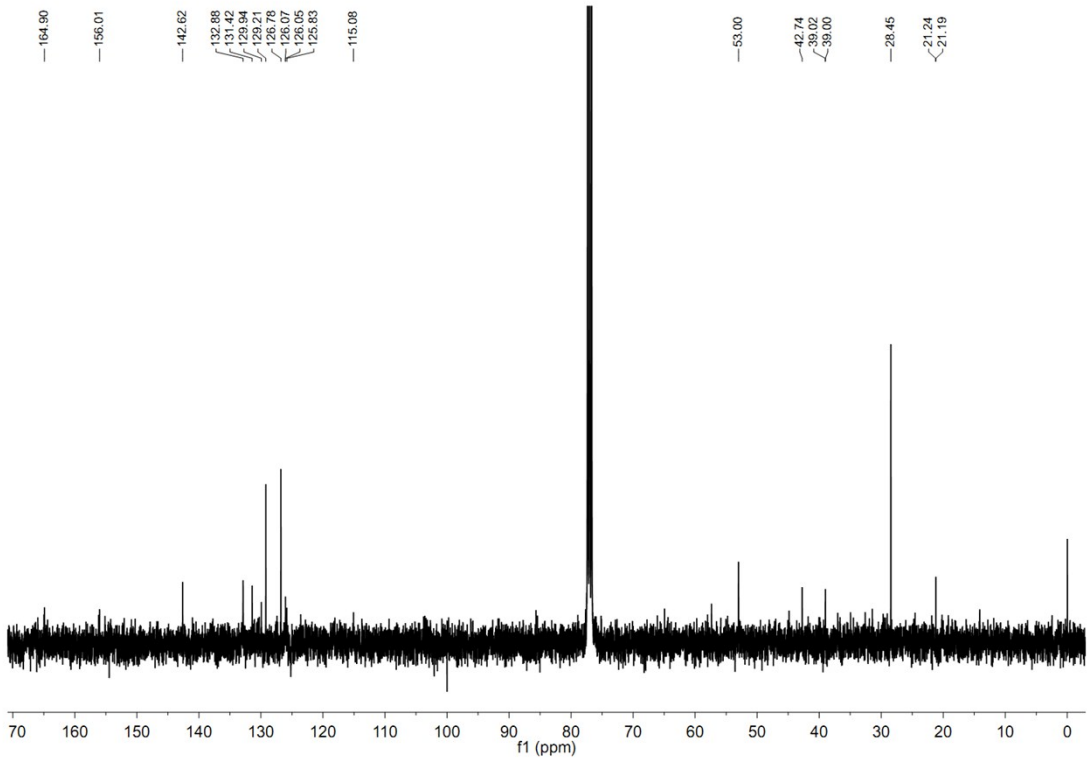


Fig. S16 ^{13}C NMR spectrum of compound **4** ($CDCl_3$).

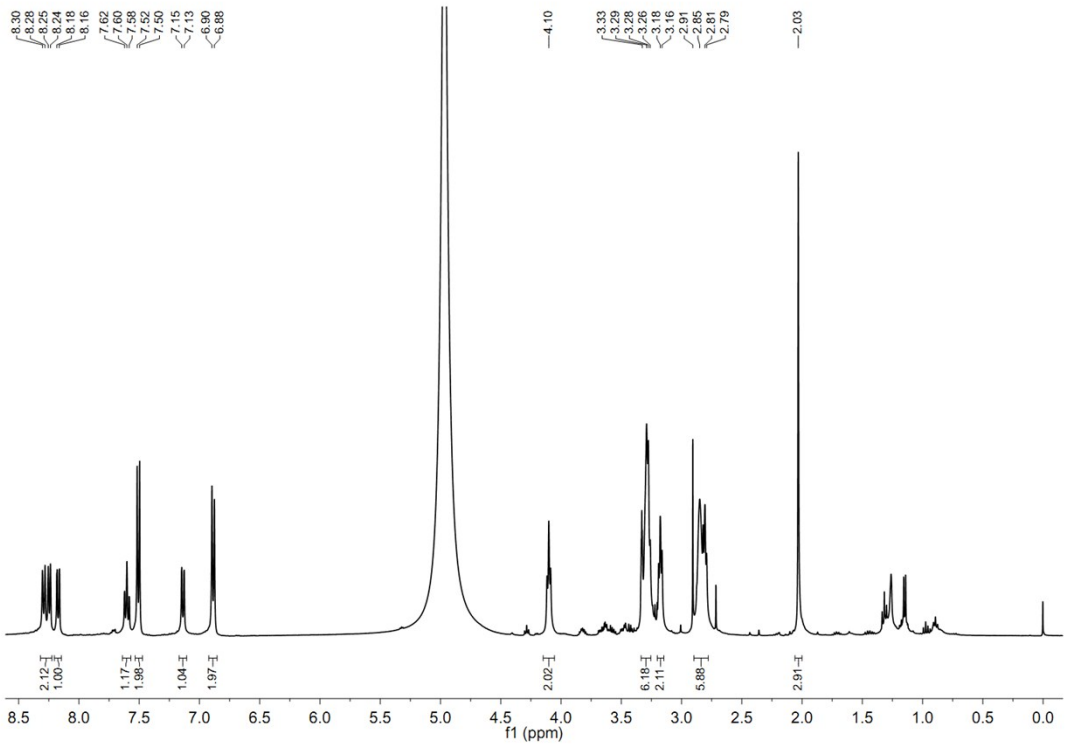


Fig. S17 ^{1}H NMR spectrum of **CNER-2** (Methanol-d_4).

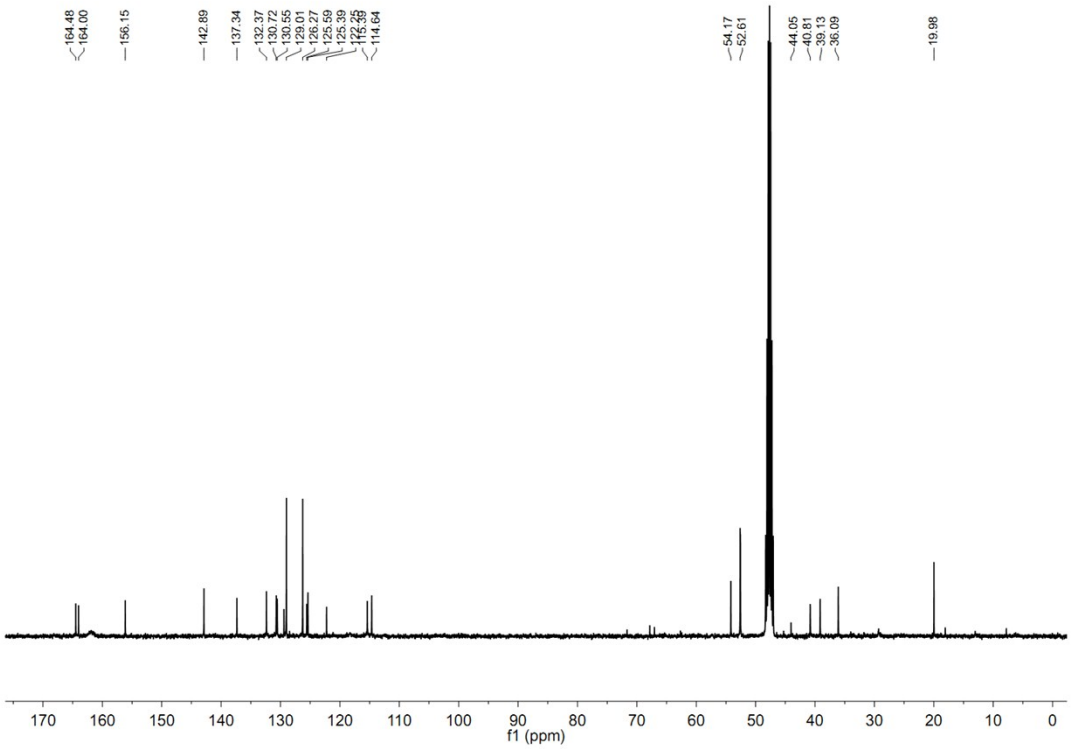
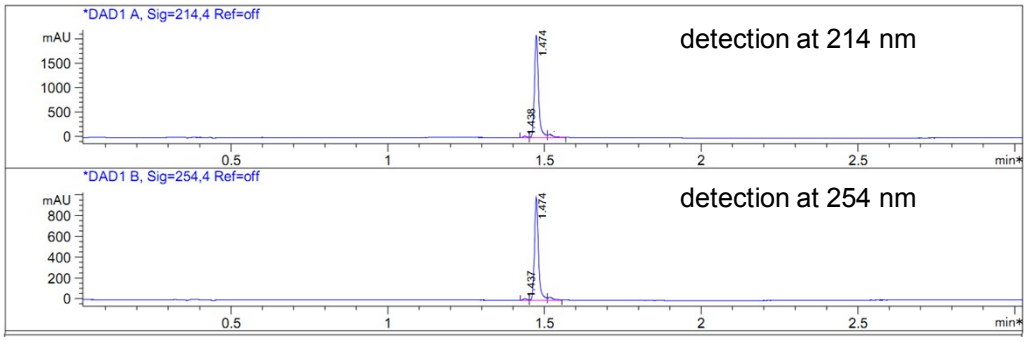


Fig. S18 ^{13}C NMR spectrum of **CNER-2** (Methanol-d_4).

(A) HPLC data



(B) MS data

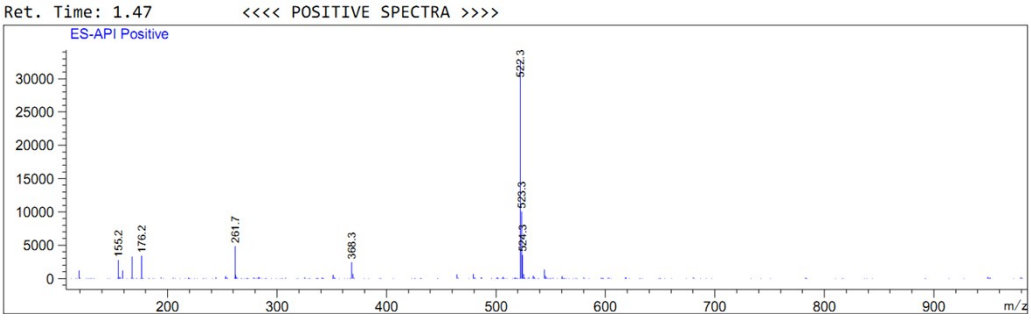


Fig. S19 LC-MS data of **CNER-2**.

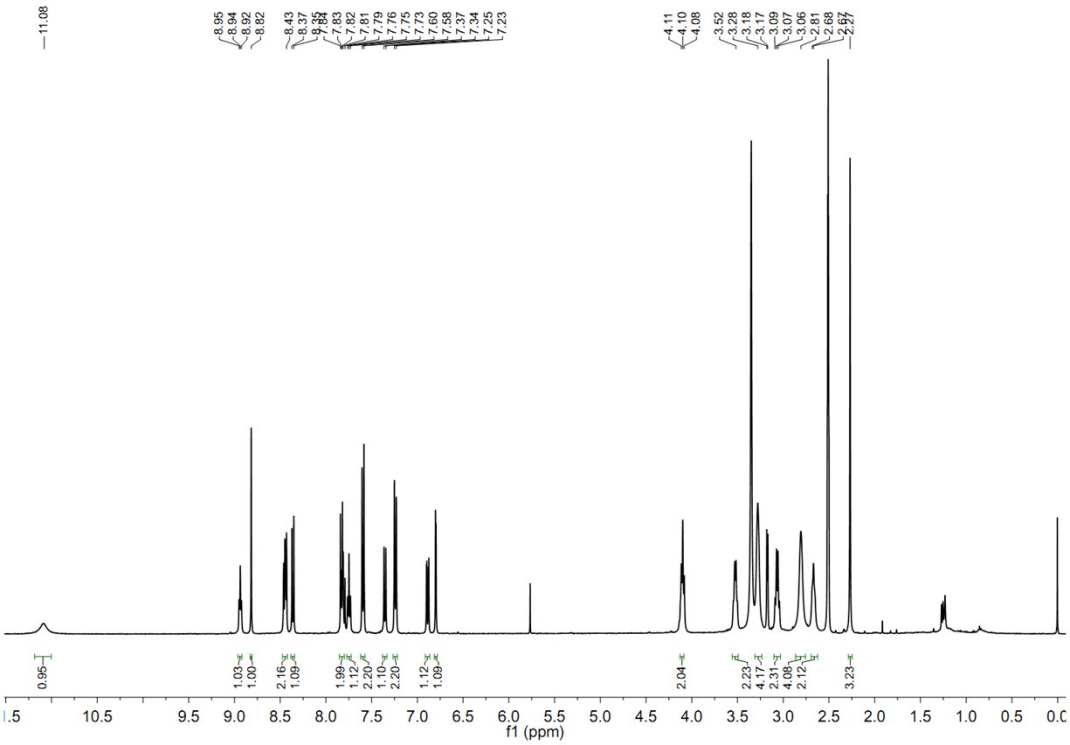


Fig. S20 ^{1}H NMR spectrum of **CNER-pH** (DMSO-d_6).

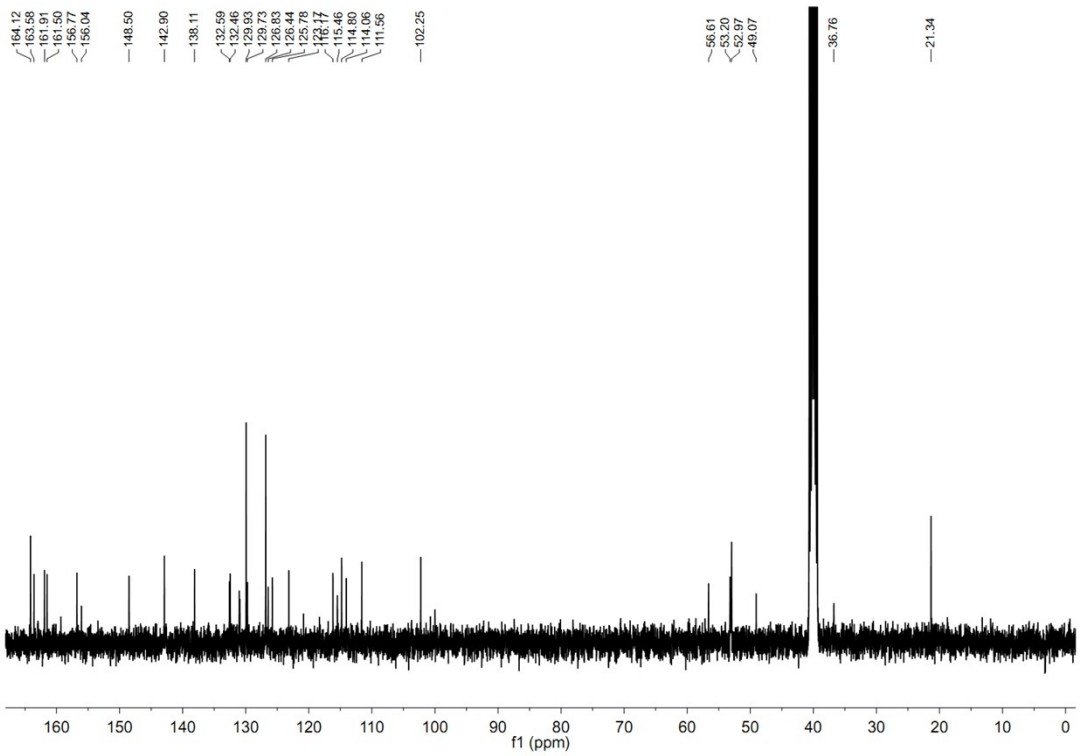


Fig. S21 ^{13}C NMR spectrum of **CNER-pH** (DMSO-d_6).

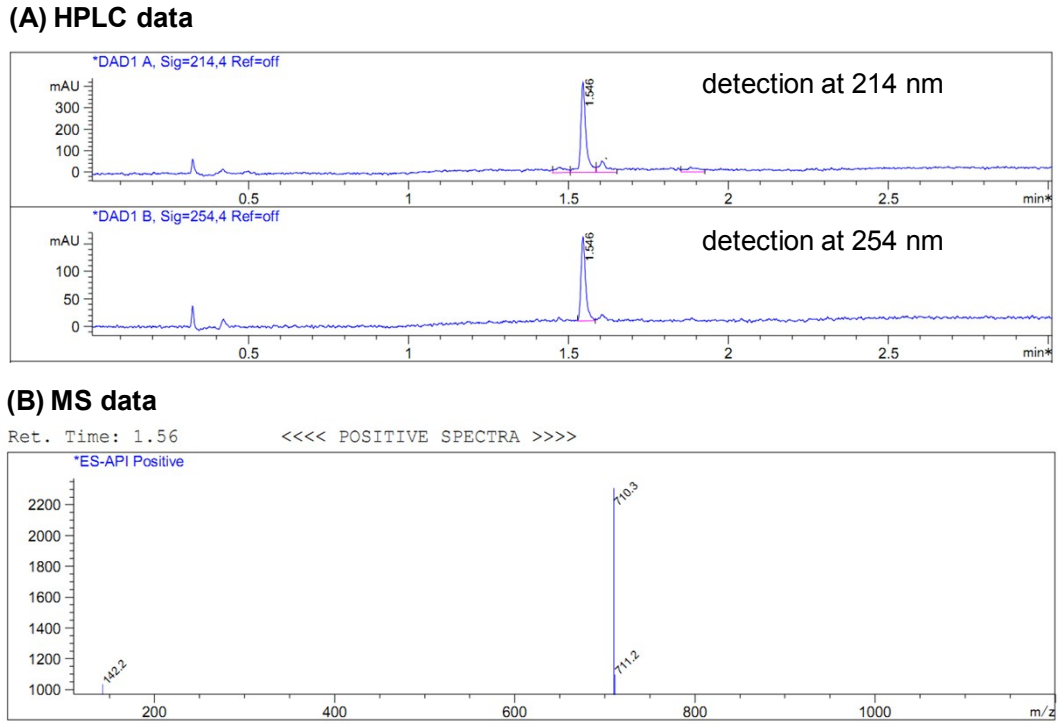


Fig. S22 LC-MS data of **CNER-pH**.