Electronic Supplementary Material (ESI) for Analyst.


# Supporting Information

**Rational Design of a Turn-On Near-Infrared Fluorescence Probes for Highly Sensitive and Selective Monitoring of carboxylesterase 2 in Living Systems**

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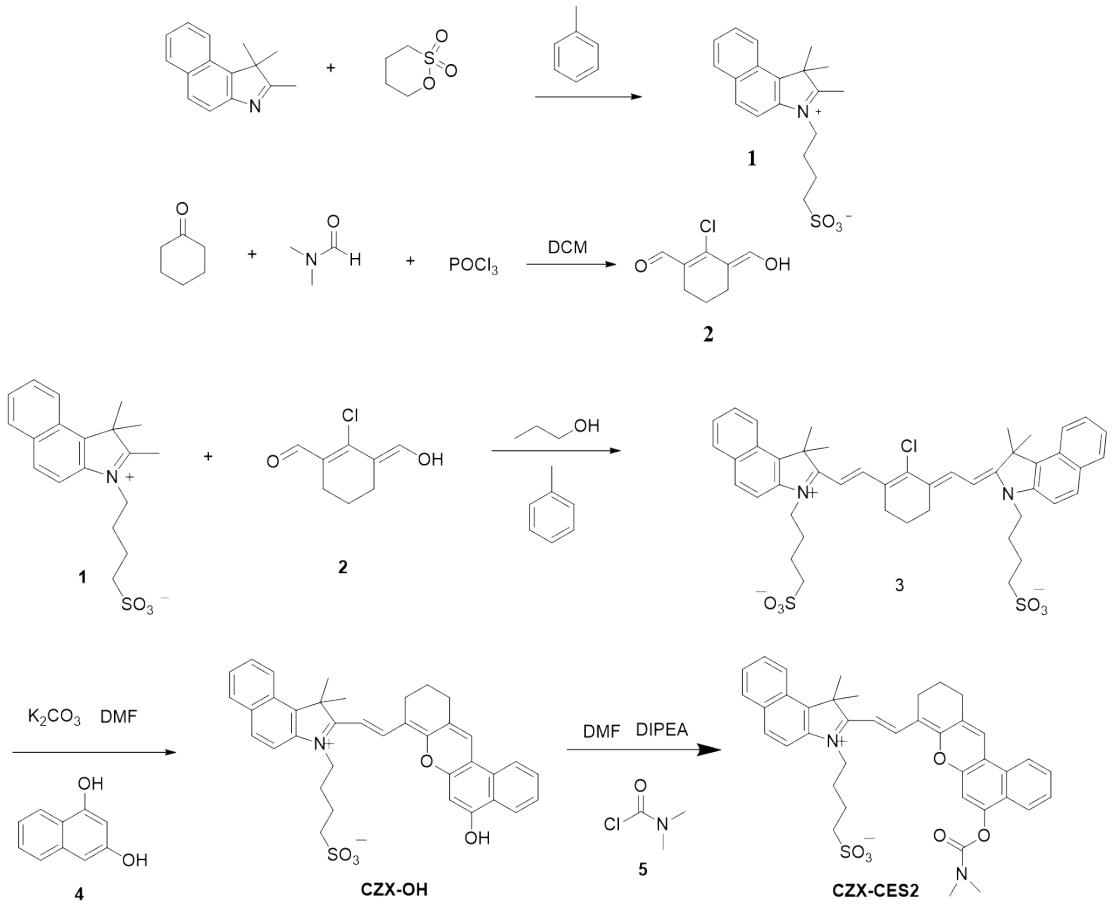
**Contents**

**1.Synthesis of Compounds**




**Scheme S1**. Synthetic scheme for compound **CZX-CES2**

**Synthesis of Compound 1**: Under nitrogen protection, benzindole (2.09 g, 10 mmol), 1,4-butane sultone (4.08 g, 30 mmol) and anhydrous toluene (30 ml) were added to a 100 ml flask, and after stirring for 10 min, the temperature was increased. The reaction was carried out at 120 ℃ for 12 hours. After the reaction was completed, it was cooled to room temperature, and 40 ml of mixed solution (EA/PE=V/V=1:1) was added and stirred thoroughly. After standing for 15 minutes, perform suction filtration. Then it was washed with mixed solution (EA/PE=V/V=1 ： 1) and dried under vacuum at 70 ℃ to obtain 2.896g of gray blue solid with yield of 83.82%. Proceed to the next step without purification.

**Synthesis of Compound 2：** The mixed solution of DMF (10 ml, 129 mmol) and dichloromethane (10 ml) was placed in an ice bath at 0 ℃, and a mixed solution of phosphorus

oxychloride (9 ml, 99.2 mmol) and dichloromethane (10 ml) was added thereto, followed by cyclohexanone (2.5 g, 25 mmol) was slowly added to the mixed system, stirred at 0 ℃ for 0.5 h, then heated to 50 ℃ and refluxed for 5 h. After the reaction was completed, it was cooled at room temperature, and the reaction solution was slowly dropped into 100 ml of stirring ice water to quench the remaining phosphorus oxychloride in the reaction. Filtration and washing with cold water gave 2.6 g of a yellow solid. According to literature reports, this product is not further processed, and directly proceeds to the next step.

**Synthesis of Compound 3**: Under the protection of nitrogen, add compound 1 (345mg, 1mmol), compound 2 (86mg, 0.5mmol), n-butanol (30ml) and toluene (15ml) into the drying flask, add into the water separator, gradually raise the temperature to 136 ℃ and react for 13 hours. After the reaction is completed, it is cooled at room temperature, and then the reaction mixture is dropped into a strongly stirred mixed solution (200ml) of ethyl acetate and petroleum ether (EA/PE=V/V=2:1), stirred for 0.5h, filtered, washed three times with ethyl acetate and collected solids, finally 0.24g of metallic luster golden green solid is obtained, with a yield of 45.63%.$^1$H NMR (600 MHz, DMSO-$d_6$) $\delta$8.38 (d, $J$ = 14.1 Hz, 2H), 8.30 (d, $J$ = 8.6 Hz, 2H), 8.08 (dd, $J$ = 13.0, 8.5 Hz, 4H), 7.82 (t, $J$ = 8.0 Hz, 2H), 7.66 (t, $J$ = 7.7 Hz, 2H), 7.53 (q, $J$ = 7.5 Hz, 2H), 6.42 (d, $J$ = 14.2 Hz, 2H), 4.35 (t, $J$ = 7.7 Hz, 4H), 2.78 (t, $J$ = 6.3 Hz, 5H), 1.96 (s, 12H), 1.90 (p, $J$ = 6.7, 5.2 Hz, 6H), 1.83 – 1.75 (m, 6H).

**Synthesis of Compound CZX-OH** :Under nitrogen protection, compound **3** (155 mg, 0.187 mmol), compound **4** (30 mg, 0.187 **mmol**), potassium carbonate (77.53 mg, 0.561 mmol) and DMF (10 ml) were added to a dry flask, and then the temperature was gradually increased to 80 ℃, the reflux reaction was stopped after 6 h, the remaining solvent was removed by rotary evaporation, and then separated and purified by silica gel chromatography (MeOH:DCM=V/V=1:15) to obtain 20 mg of blue-green product with a yield of 28.86%.$^1$H NMR (600 MHz, Methanol-$d_4$) $\delta$ 8.65 (d, $J$ = 14.9 Hz, 1H), 8.27 (d, $J$ = 9.2 Hz, 2H), 8.11 (s, 1H), 7.97 (d, $J$ = 8.7 Hz, 1H), 7.80 – 7.73 (m, 2H), 7.62 (dd, $J$ = 14.2, 8.7 Hz, 1H), 7.55 (d, $J$ = 7.9 Hz, 1H), 7.44 (d, $J$ = 6.5 Hz, 1H), 7.25 (t, $J$ = 7.4 Hz, 1H), 6.47 (d, $J$ = 14.9 Hz, 1H), 6.40 (s, 1H), 5.77 (s, 1H), 4.37 (t, $J$ = 7.6 Hz, 2H), 2.82 (dd, $J$ = 9.5, 5.2 Hz, 4H), 2.09 (t, $J$ = 7.6 Hz, 2H), 1.98 (s, 6H), 0.83 – 0.73 (m, 6H).

**Synthesis of Compound CZX-CES2**: Under nitrogen protection, compound **CZX-OH** (40 mg, 0.052 mmol), compound **5** (24 μl, 0.26 mmol), DIPEA (45.2 μl, 0.26 mmol) and DMF (2 ml) were added to a dry flask, and then gradually The temperature was raised to 50 ℃, the reaction was stopped after 12 h, the remaining solvent was removed by rotary evaporation, and then separated and purified by silica gel column chromatography (MeOH:DCM=V/V=1:10) to obtain 10 mg of green product with a yield of 28.57%. $^1$H NMR (600 MHz, Methanol-$d_4$) $\delta$ 8.63 (d, $J$ = 14.8 Hz, 1H), 8.35 (d, $J$ = 8.4 Hz, 1H), 8.11 (s, 1H), 7.97 (d, $J$ = 8.3 Hz, 1H), 7.66 (t, $J$ = 7.7 Hz, 1H), 7.59 – 7.52 (m, 3H), 7.45 (q, $J$ = 6.7, 5.4 Hz, 4H), 7.36 (t, $J$ = 7.2 Hz, 1H), 6.43 (d, $J$ = 14.8 Hz, 1H), 4.24 (t, $J$ = 7.4 Hz, 2H), 3.21 (t, $J$ = 1.8 Hz, 6H), 2.82 (t, $J$ = 6.3 Hz, 3H), 2.65 (t, $J$ = 6.2 Hz, 2H), 1.94 – 1.86 (m, 2H), 1.74 (s, 6H), 1.19 (d, $J$ = 11.1 Hz, 6H). HRMS (m/z): $[M + H]^+$ calculated for $C_{40}H_{40}N_2O_6S$ 677.8280, found 677.2681.

**2.Determination of the limit of detection**

The detection limit was calculated based on the fluorescence titration. In the absence of CES2, the fluorescence emission spectrum of **CZX-CES2** was measured five times and the standard

deviation of the blank measurement was obtained. To obtain the slope, the fluorescence intensity at 850 nm was plotted as the concentration of CES2. The detection limit is therefore calculated with the following equation:

detection limit = 3σ/k

where σ is the standard deviation of the blank measurement and k is the slope between the fluorescence intensity at 850 nm and the CES2 concentration[1].

**3. Water solubility**

Prepare a water-soluble stock solution of the probe in water. Dilutions were made to produce solutions with concentrations ranging from 50 μM to 1 mM. Measure the UV-Vis spectrum and plot the absorbance at Imax against the concentration. The maximum linear response was used to quantify the solubility limit. The maximum linear response was taken as the solubility limit in PBS, pH7.4, 25°C. Probe has solubility up to 800μM, further concentrations were not tested since higher absorbance values may deviate from linearity due to detection by the spectrophotometer rather than insolubility.

**4. Enzyme kinetic analyses**

Enzyme kinetic parameters were estimated by incubating **CZX-CES2** (0-1 mM) and CES2 (10 μg/mL) in 50 μL of 100 mM PBS (pH 7.4), respectively. Reactions were initiated by adding a range of concentrations of **CZX-CES2** (diluted in PBS) to the enzyme mixture pre-incubated at 37°C. After 30 min of incubation, the reaction was stopped by adding an equal volume of ice-cold acetonitrile[2]. Probe formation was determined by measuring the fluorescence intensity at 850 nm. Kinetic parameters (Km and Vmax) were determined by nonlinear regression analysis using the Michaelis-Menten equation in OriginPro 9 software.

**5. Cytotoxicity assays**

The effect of **CZX-CES2** on cell viability was investigated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2h-tetrazolium bromide (MTT) method. Cells (8 × 103 cells/mL, 100 μL) were seeded in 96-well plates in the corresponding medium containing 10% fetal bovine serum (FBS) and cultured in a 5% CO2 incubator at 37°C for 24 h. Cells were then incubated with different concentrations of **CZX-CES2** (prepared in FBS-free medium, 100 μL) for an additional 24 hours. Subsequently, 0.5 mg/mL MTT was added to the adherent cells and incubated at 37°C for an additional 4 hours. Finally, cells were lysed in DMSO (150 μL/well) and absorbance at 490 nm was measured. Cell viability was calculated as $A/A_0 \times 100\%$ (A and $A_0$ are the absorbance of the experimental group and the control group, respectively)[3].

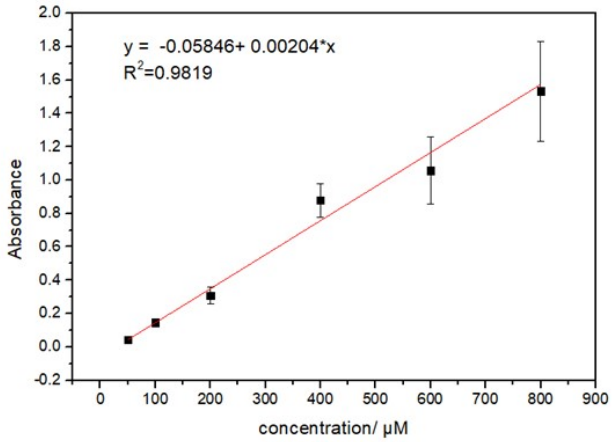
**6. Confocal fluorescence imaging in living HepG2 cells**

HepG2 cells in logarithmic growth phase were seeded in small confocal petri dishes (φ20mm), and each confocal petri dish was seeded with 10,000 HepG2 cells. Cells were cultured overnight in DMEM medium containing 10% fetal bovine serum (37°C, 5% CO2). After the cells are fully attached, wash twice with serum-free DMEM medium to remove non-adherent cells and fetal bovine serum. Adherent cells were then incubated for 30 min at 37°C in a 5% CO2 incubator with/without 100 μM BNPP (prepared in FBS-free medium). Probe **CZX-CES2** stock solution (1 mM) was diluted with FBS-free cell culture medium to a final concentration of 10 μM, then adherent cells were incubated with **CZX-CES2**-containing FBS-free cell culture medium at 37°C reincubate for 30 min under PBS. The residual probe molecules were then washed 3 times with

phosphate buffer, and images were collected using a confocal laser fluorescence microscope (OLYMPUS FV3000).

**7.Molecular docking**

The crystal structure of human CES1 is obtained from PDB (ID: 1MX5), and the crystal structure of CES2 is obtained after using 5fv4 (PDB ID) as a template, using amino acids Nos. 30 to 544 for homologous molding. The molecular docking process is simulated using AutoDockTools-1.5.6, with Discovery Studio 4.5 Client representing the structure of proteins and




ligands. The characteristic region of the protein is set to hotspots for docking simulation, the ligand conformation is scored by energy minimization, and the ligand conformation with the highest score is optimized and matched to the hotspot of CES1 and CES2.

**Fig. S1**† Plot of absorbance at $\lambda_{max}$ versus concentration of probe. Maximum linear response as solubility limit in PBS, pH 7.4, 25°C. The probes are soluble up to 800 μM; more concentrations were not tested as higher absorbance values may deviate from linearity due to spectrophotometric detection rather than insolubility.

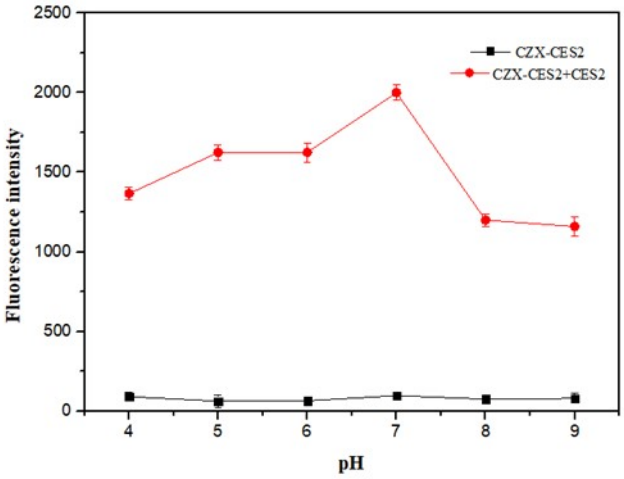



**Fig. S2**† The stability of probe **CZX-CES2** under various pH conditions.

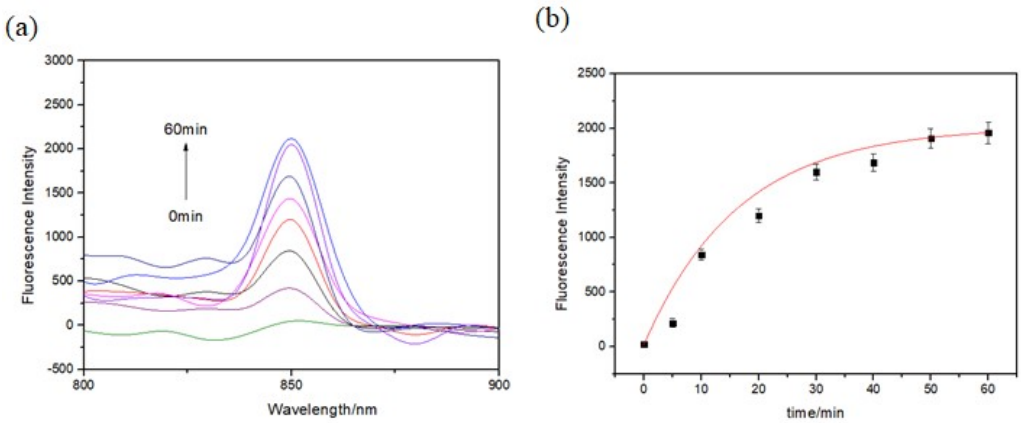



**Fig. S3**†. (a) Fluorescence spectra of **CZX-CES2** (10 μM) incubated with CES2(10 μg/mL) in PBS (containing less than 1% DMSO) at 37 °C for (0-60) min. (b) The nonlinear relationship between fluorescence intensity and time(0-60min). The experiments were conducted in triplicate, and the data were obtained as mean (±SD). $\lambda_{ex}$= 675 nm.

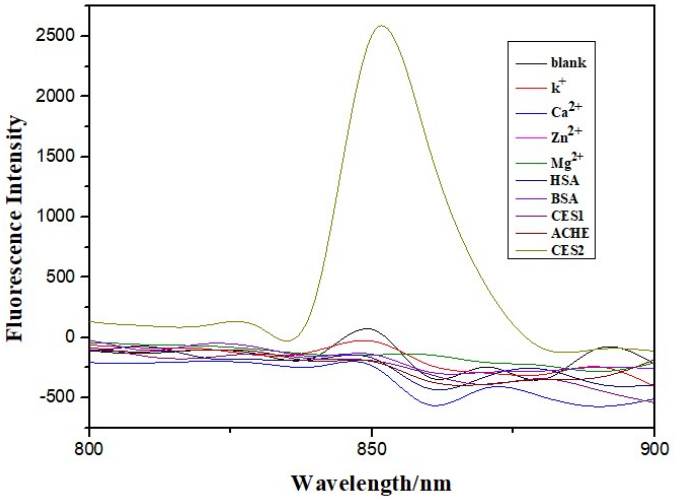



**Fig. S4†.** Fluorescence intensity of **CZX-CES2** (10 μM) to several species in PBS (pH 7.4) at 37 °C for 30 min.

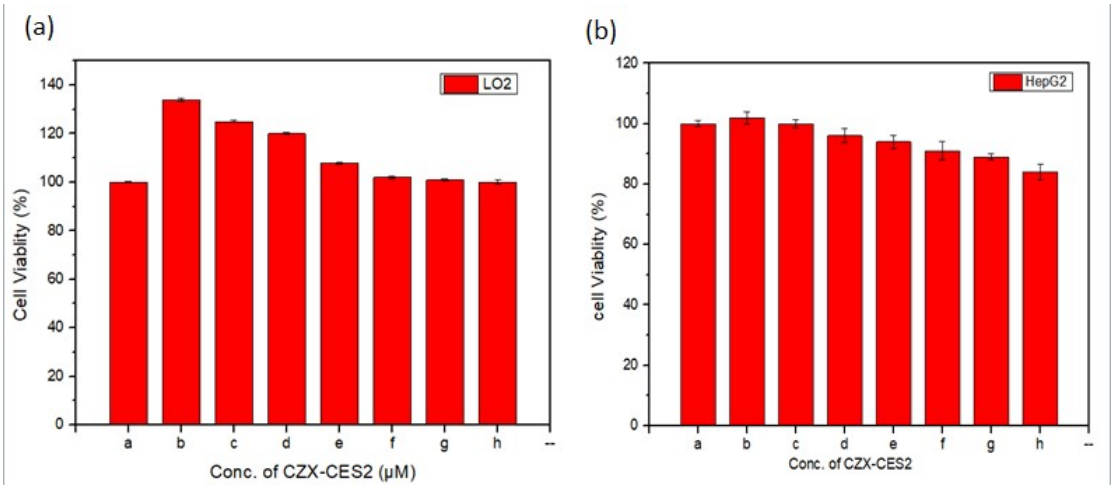



**Fig. S5†** Cell viability of a) LO2 cells; b) Hep G2 cells was determined by MTT assay in the presence of different concentrations of **CZX-CES2** (a-f :0-10 μM). Experiments were repeated three times and data are shown as mean (±S.D.)

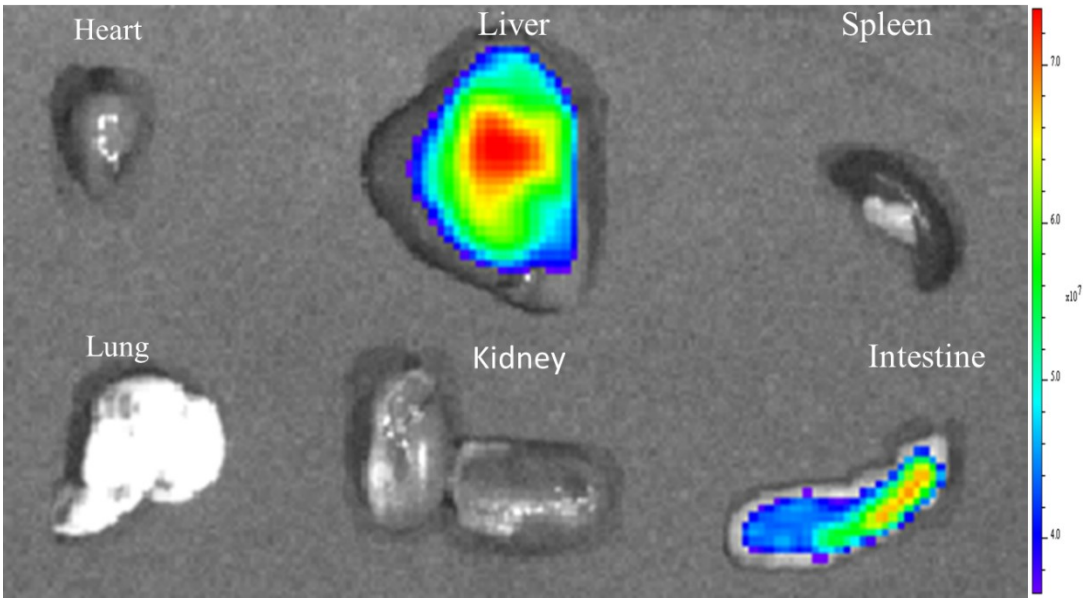



**Fig. S6†** The fluorescence imaging of ex vivo dissected organs after incubating with CZX-CES2 for 90min.

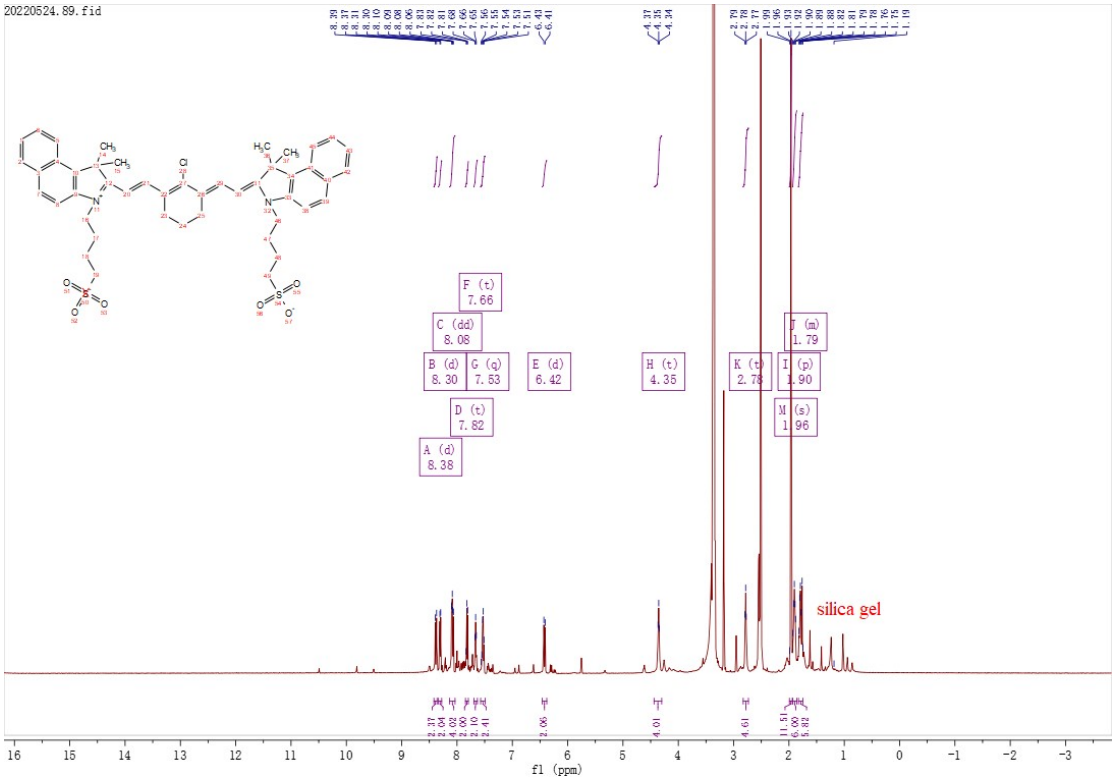



**Fig. S7†** $^{1}$H-NMR spectrum of **3** (600 MHz, 298 K, DMSO-d6)

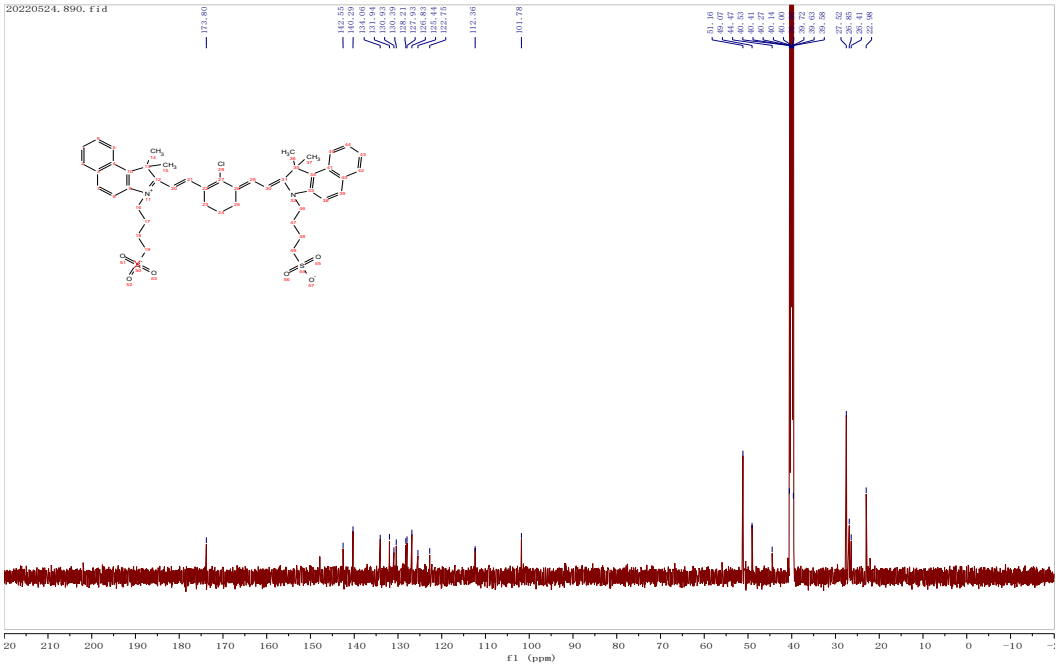



**Fig. S8†** $^{13}$C NMR spectrum of **3** (151 MHz, 298 K, DMSO-d6)

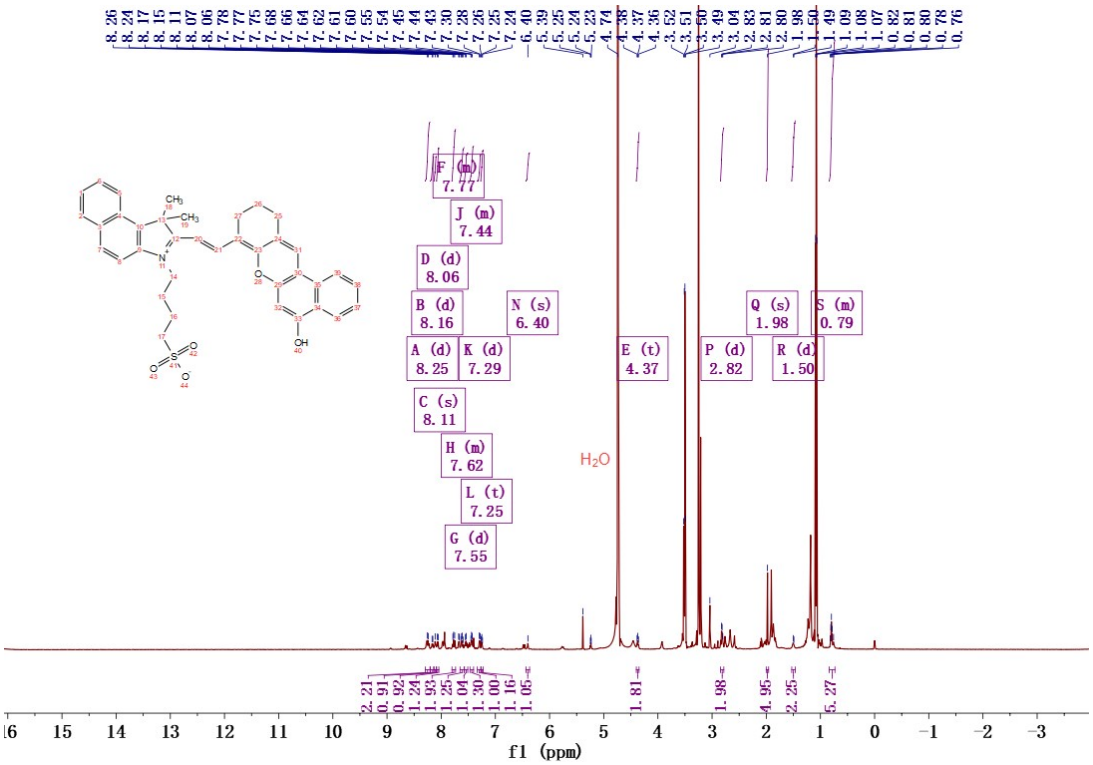



**Fig. S9†** $^{1}$H-NMR spectrum of **CZX-OH** (600 MHz, 298 K, Methanol-$d_4$)

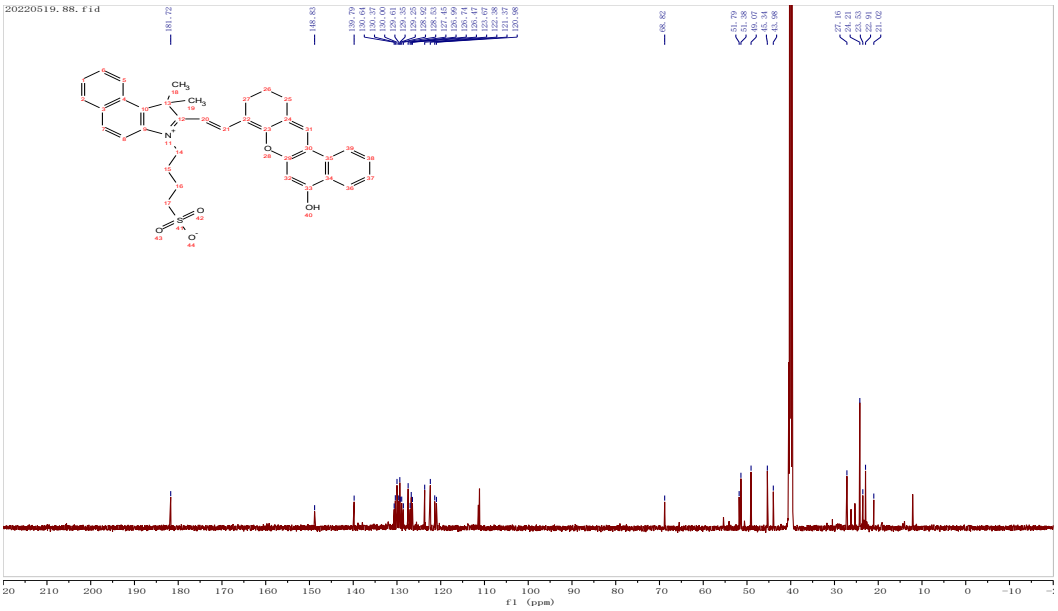



**Fig. S10†** $^{13}$C NMR spectrum of cmpound **CZX-OH**(151 MHz, 298 K, DMSO-d6)

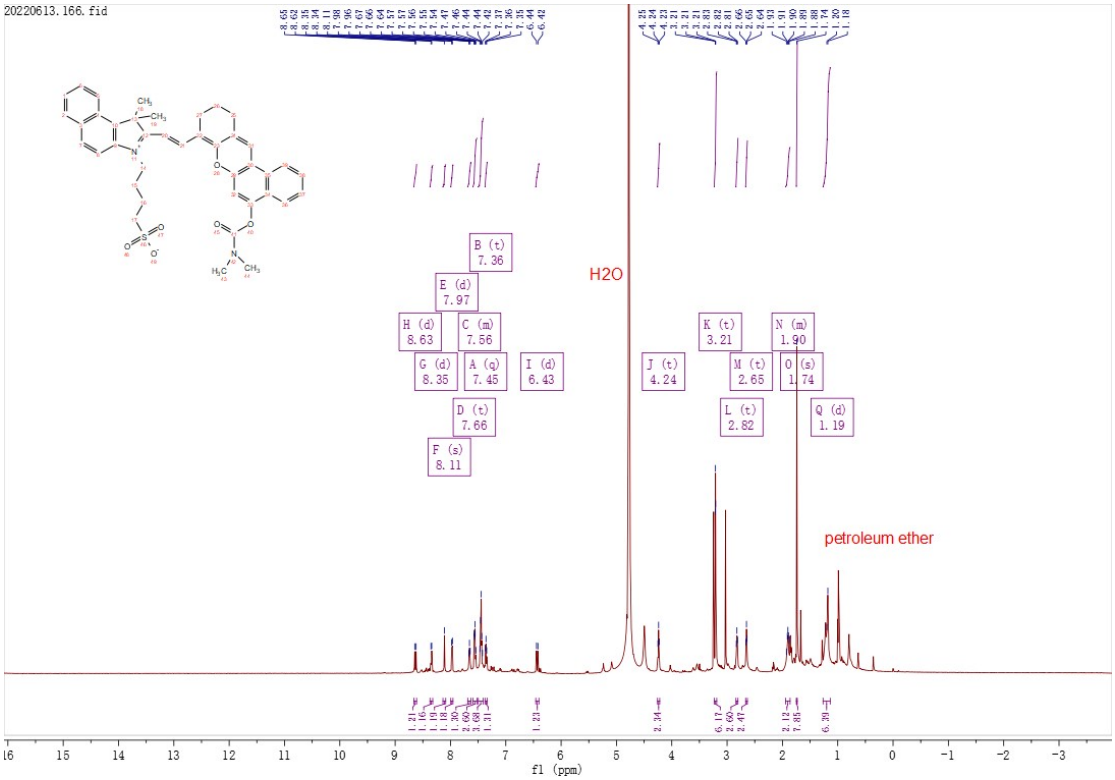



**Fig. S11†** $^{1}$H-NMR spectrum of **CZX-CES2** (600 MHz, 298 K, Methanol-$d_4$)

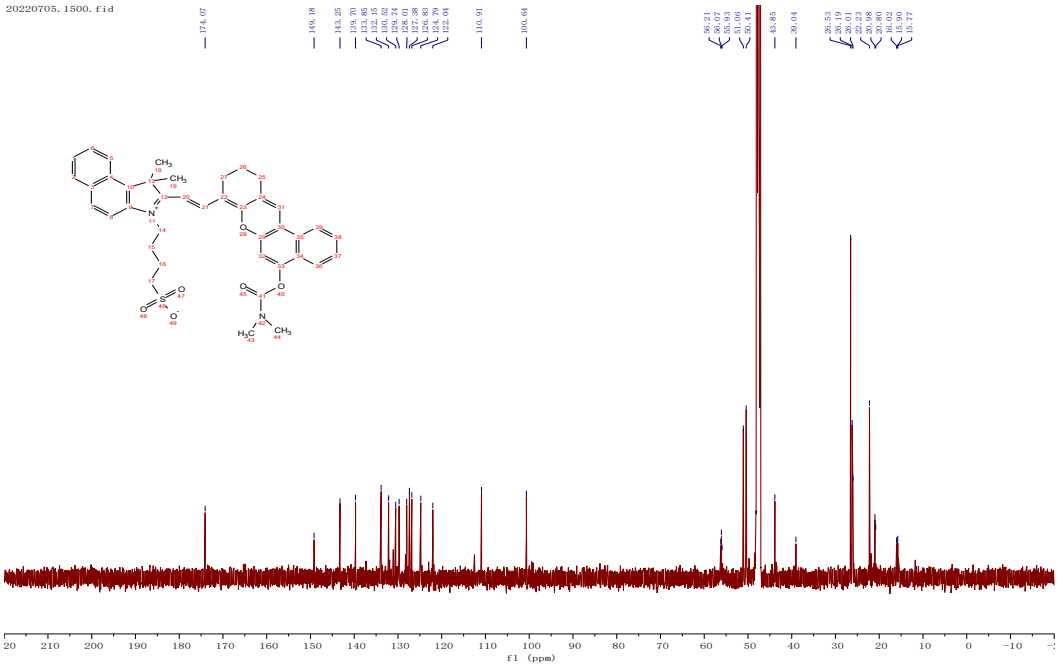


**Fig. S12†** $^{13}C$ NMR spectrum of **CZX-CES2**(151 MHz, 298 K,MeOD)

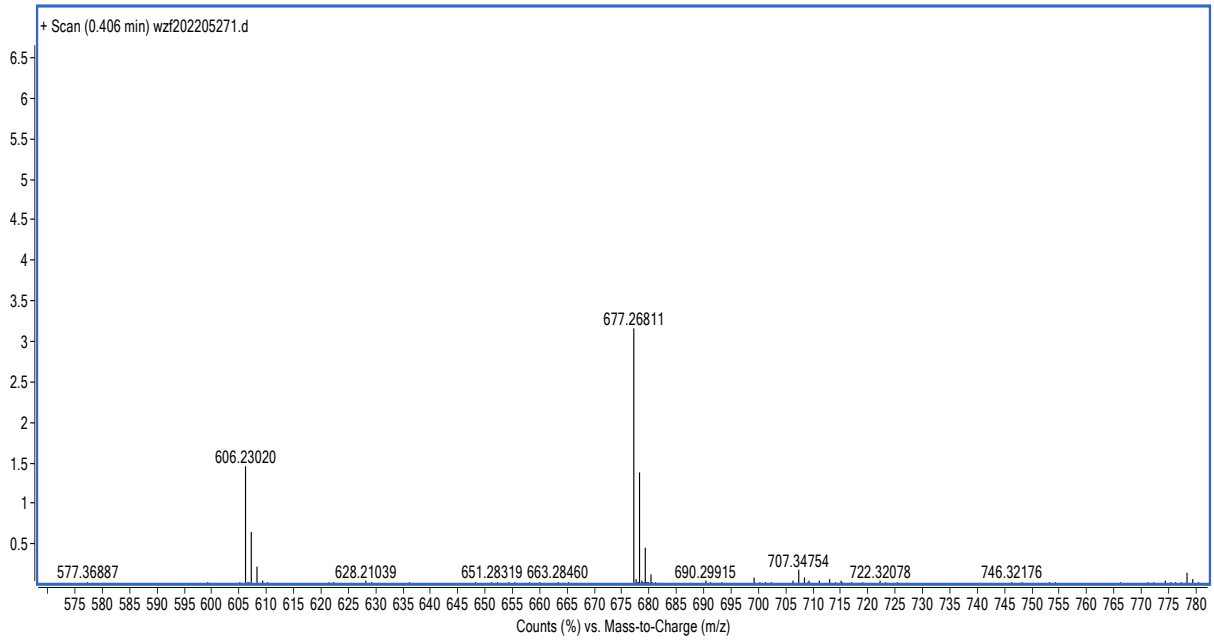



**Fig. S13†** HRMS of **CZX-CES2**.

## 8. References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