[i]*Supporting Information for:*

A Smart Cascade Theranostic Prodrug System Activated by Hydrogen Peroxide for Podophyllotoxin Delivery

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CONTENTS

1 Materials and Methods

1.1 Reagents and instruments

Dichloromethane (DCM) was distilled from calcium hydride. N-ethyl-N'- (3-dimethylaminopropyl) carodiimide hydrochloride (EDCI), 4-dimethylaminopyridine (DMAP) were purchased from commercial sources and used as received without further purification. Silica-gel plates [GF254, thin-layer chromatography (TLC)] and silica gel (200-300 mesh) were bought from Qingdao Haiyang (Qingdao, China). ^{1}H NMR and ^{13}C NMR spectra were measured using Bruker Advance 400 and 600 NMR spectrometer at ambient temperature, and tetramethylsilane (TMS) was used as a reference. MS spectra recorded on a Shimadzu LCMS-2020 system. HRMS was obtained on an Orbitrap Elite (Thermo Scientific).

1.2 Spectra measurements

Absorption spectra were recorded on UV-vis spectrometer evolution 200 (Thermo Scientific). Fluorescence studies were recorded using an Agilent Cary Eclipse Fluorescence spectrophotometer with the excitation at 431 nm, and the slit width was 5 nm for both excitation and emission. Both the absorption and fluorescence spectra were measured in phosphate buffer solution (PBS, 10 mM, pH 7.4) at 37 ℃. Stock solutions of **CM-PPT** (10 mM) were prepared in DMSO, The final test compound concentration of 10 μM (final DMSO concentration of 50% v/v). Hydrogen peroxide (H_2O_2), hypochlorite (HOCl) and t-BuOOH stock solutions were prepared by dilution of commercial H_2O_2 (30%), available NaOCl solution (14.5% available chlorine) and t-BuOOH (70%) in tri-distilled water. Superoxide anion (O_2^-) was generated from KO_2 (1 mg) in dry DMSO (1 mL).[1] The nitric oxide (NO) was generated from sodium nitroferricyanide dihydrate in tri-distilled water.[2] Hydroxyl radical (•OH) and t-BuOO• was prepared in situ by Fenton reaction from 200 μM H_2O_2 or t-BOOH and $FeSO_4$ (1 mM).[3] $ONOO^-$ was generated from $NaNO_2$ and H_2O_2.[4] Singlet oxygen ($^1O_2$) was prepared in situ by the H_2O_2/HClO (200 μM/200 μM) system in alkaline media.[5] AAPH (2,2'-Azobis(2-methylpropionamidine) dihydrochloride) was dissolved in Tris-HCl (50 mM, pH = 7.4) and incubated for 15 min at 37 ℃.[6] The other amino acid were prepare

in tri-distilled water.

1.3 HPLC Analyses of the Reaction between CM-PPT and H_2O_2

CM-PPT (10 μM) was incubated with H_2O_2 (100 μM) in a DMSO-PBS mixed solvent (50:50, v/v) at 37 ℃. All samples were passed through a 0.22 μm filter, and 10 μL of sample was loaded onto a Shimadzu LCMS-2020 system equipped with a Wondasil C18 Superb reversed-phase column (5 μM, 4.6 × 150 mm). The mixture of methanol and water (75:25, v/v) was used as eluent at the flow rate of 0.6 mL min^{-1}. The detection wavelength for **CM-PPT** was set at 254 nm.

1.4 Cell culture and cytotoxic activity assay

HepG2 cells were seeded in 24-well plates at 1 × 10^4 cells per well in 0.5 mL growth medium and incubated at 37 ℃ for 24 h, and then the cells were poured into three groups. The first group cells were incubated with **CM-PPT** (2 μM) for 1 h and 4 h at 37 ℃. The second group cells were pretreated with H_2O_2 (200 μM) for 30 min, then were incubated with **CM-PPT** (2 μM) for 1 h and 4 h at 37 ℃. The third group cells were pretreated with LPS (1 μg/mL) for 24 h, then were incubated with **CM-PPT** (2 μM) for 1 h and 4 h at 37 ℃. After washing the cells with PBS three times, the bright-field and fluorescence images were acquired with Floid cell imaging station (Life Technology).

The cytotoxicity of PPT and **CM-PPT** was measured by the MTT assay. HepG2 cells (4 × 10^3) were seeded in 96-well plates and allowed to attach for 12 h. Cells then were incubated with varying concentrations of PPT and **CM-PPT** for 48 h. Then the medium was removed, and the same medium (100 μL) containing 0.5 mg/mL MTT was added to each well and incubated for an additional 4 h at 37 ℃. An extraction buffer (100 μL, 10% SDS, 5% isobutanol, 0.1% HCl) was added, and the cells were incubated overnight at 37 ℃. The absorbance was measured at 570 nm using a microplate reader (Thermo Scientific Multiskan GO, Finland). The cell viability was expressed as the percentage of the control (cells without drug treatment).

1.5 Cell-Cycle Assay

Cell-cycle distributions were determined using propidium iodide (PI) staining assay. In brief, HepG2 cells were seeded in 6-well plates (2×10^5 per well) for 8 h and treated with serial concentrations of **CM-PPT** (0 nM, 100 nM, 200 nM, 500 nM) for another 24 h. Then the cells were washed with ice-cold PBS, trypsinized, centrifuged, and fixed with 75% ice-cold ethanol at 4 °C for 2 h. The cell pellets were resuspended in 0.3 mL PBS, centrifuged at 1000g for 3-5 min. Subsequently, the cells were exposed to 0.2 mL PI (50 μg/mL) for 30 min at 37 °C in the dark, respectively. DNA content was confirmed by FCM and analyzed with NovoExpress software. Cells treated with drug-free medium served as negative control.

1.6 Synthetic route of **CN-PPT**

Synthesis of compound 4

4-(diethylamino)-salicylaldehyde (966 mg, 5 mmol) was dissolved in 40 mL acetonitrile, and then (chloromethyl) benzene was added. The reaction system was stirred at 65 ℃ for 10 h under argon protection. After the TLC detection reaction was completed, the solvent was removed by vacuum distillation, and the residue was purified by column chromatography (Petroleum Ether: Ethyl Acetate = 5:1) to give a yellow solid (1.032 g, 73% yield). ^{1}H NMR (600 MHz, DMSO) δ 10.07 (s, 1H), 7.52 (d, *J* = 8.9 Hz, 1H), 7.51 – 7.48 (m, 2H), 7.41 (dd, *J* = 8.4, 6.9 Hz, 2H), 7.35 – 7.32 (m, 1H), 6.37 – 6.33 (m, 1H), 6.22 (d, *J* = 2.3 Hz, 1H), 5.27 (s, 2H), 3.41 (q, *J* = 7.0 Hz, 4H), 1.08 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 185.71, 163.14, 153.99, 137.47, 130.13, 129.01, 128.30, 127.79, 113.92, 104.91, 94.84, 69.80, 44.57, 40.52, 12.87. HRMS (ESI) calculated for $[C_{18}H_{21}NO_2]^+$ $[M+H]^+$ requires m/z= 283.1572, found 284.1649.

Synthesis of compound 5

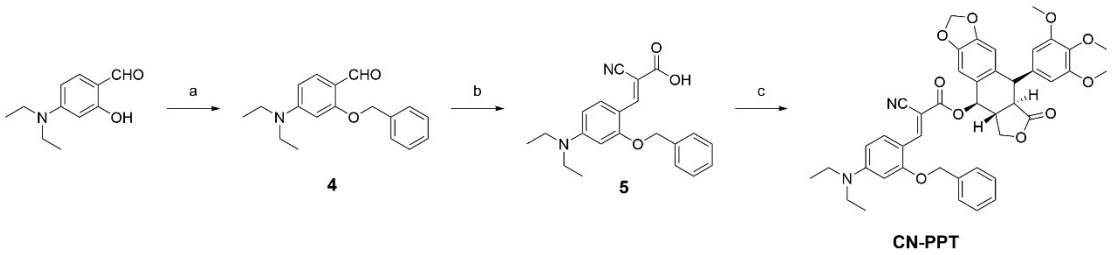
Compound **4** (212 mg, 0.75 mmol) was dissolved in 30 mL ethanol, followed by slow addition of cyanoacetic acid (128 mg, 1.50 mmol), piperidine (0.3 mL) and acetic acid (0.3 mL), respectively. The reaction system was protected by argon gas at 50 ℃ for 10 h. After the TLC detection reaction, the system solvent was removed by rotary steaming. The crude product was purified by column

chromatography on silica gel (Dichloromethane: Methanol = 100:1) to give a yellow solid (196.875 mg, 75% yield). ^{1}H NMR (600 MHz, DMSO) δ 12.99 (s, 1H), 8.51 (s, 1H), 8.23 (d, *J* = 9.3 Hz, 1H), 7.49 – 7.45 (m, 2H), 7.42 (dd, *J* = 8.5, 6.8 Hz, 2H), 7.37 – 7.32 (m, 1H), 6.50 (dd, *J* = 9.3, 2.4 Hz, 1H), 6.26 (d, *J* = 2.4 Hz, 1H), 5.29 (s, 2H), 3.45 (q, *J* = 7.0 Hz, 4H), 1.08 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 165.83, 161.14, 153.87, 146.72, 137.18, 130.45, 129.09, 128.47, 127.92, 119.05, 108.32, 105.95, 95.18, 91.36, 70.22, 44.73, 40.51, 12.95. HRMS (ESI) calculated for $[C_{21}H_{22}N_2O_3]^+$ $[M+Na]^+$ requires m/z= 350.1630, found 373.1522.

Synthesis of CN-PPT

Compound **5** (35 mg, 0.10 mmol) was dissolved in 5 mL anhydrous CH_2Cl_2, and then N-ethyl-N'- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) (21 mg, 0.11 mmol), and 4-dimethylaminopyridine (DMAP) were added into the solution. The reaction solution was then kept stirring at room temperature for 30 min. Podophyllotoxin (41 mg, 0.10 mmol) was added to the solution. The reaction system was stirred at room temperature overnight. The solvent was removed in vacuo and the crude solid was purified by column chromatography on silica gel (Petroleum Ether: Ethyl Acetate = 2:1) to obtain the product **CN-PPT** (22 mg, 30% yield). ^{1}H NMR (600 MHz, DMSO-d6) δ 8.59 (s, 1H), 8.29 (d, *J* = 9.3 Hz, 1H), 7.43 (s, 2H), 7.32 (s, 3H), 7.02 (s, 1H), 6.66 (s, 1H), 6.55 (d, *J* = 9.3 Hz, 1H), 6.36 (s, 2H), 6.30 (s, 1H), 6.03 (s, 3H), 5.29 (s, 2H), 4.58 (s, 1H), 4.41 (s, 1H), 4.22 (s, 1H), 3.60 (s, 9H), 3.49 (s, 4H), 3.40 (d, *J* = 15.1 Hz, 1H), 2.81 (s, 1H), 1.11 (s, 6H). ^{13}C NMR (151 MHz, DMSO-d6) δ 174.33, 165.07, 161.52, 154.37, 152.54, 147.96, 147.46, 146.86, 137.04, 136.76, 135.92, 133.00, 130.67, 129.03, 128.98, 128.38, 127.69, 118.64, 109.67, 108.47, 108.29, 107.77, 106.34, 101.91, 95.04, 89.68, 74.33, 71.33, 70.23, 60.36, 56.06, 55.39, 44.81, 44.68, 43.34, 38.89, 29.49, 12.99. HRMS (ESI) calculated for $[C_{43}H_{42}N_2O_{10}]^+$ $[M+Na]^+$ requires m/z= 746.2839, found 769.2731.

Scheme S1. Synthesis route of **CN-PPT**.



Reagents and conditions: a. (chloromethyl)benzene, K_2CO_3, CH_3CN, 65 ℃; b. Cyanoacetic acid, Piperidine, AcOH, EtOH, 50 ℃; c. Podophyllotoxin, EDCI, DMAP, DCM, rt.

2 Experiment results

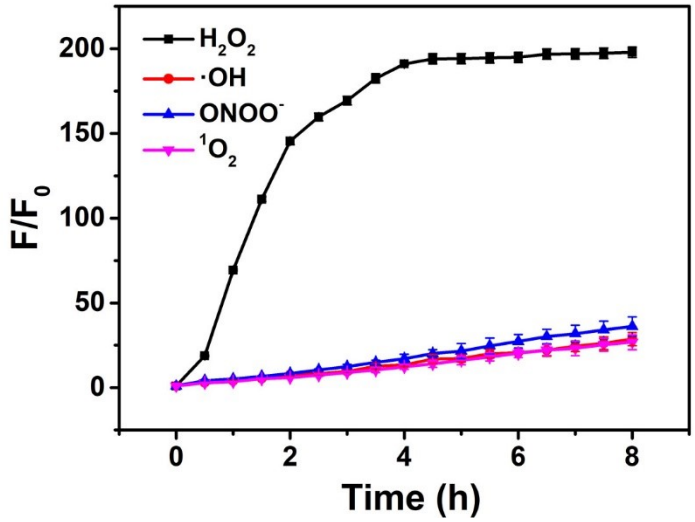


Figure S1. Kinetics of the fluorescence change upon **CM-PPT** responding to H_2O_2, OH, $ONOO^-$ and $^1O_2$. The flod of fluorescence increase at 473 nm was determined after mixing prodrug with H_2O_2, ·OH, $ONOO^-$ and $^1O_2$ at the indicated times in the PBS buffer, pH 7.4 (λ_{ex}=431 nm).

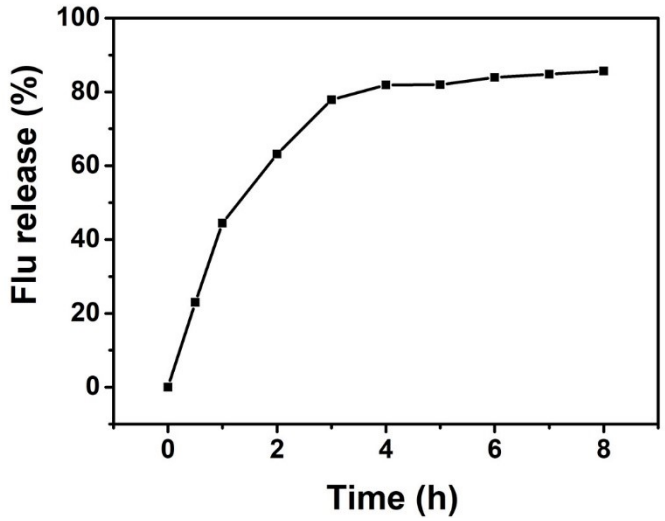


Figure S2. Time-dependent release of compound **3** determined by HPLC.

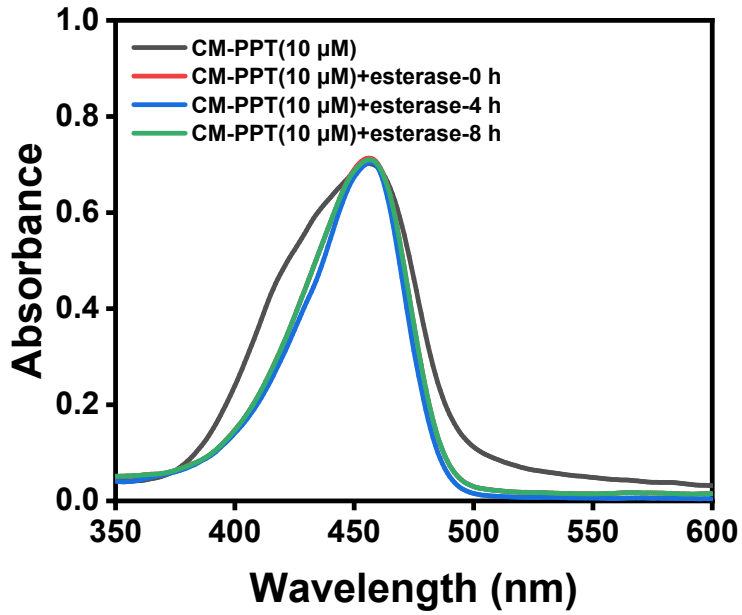


Figure S3. Absorption spectra of **CM-PPT** (10 μM) before and after incubating esterase (0.15 U/mL) for different time (0 h, 4 h, 8 h) in DMSO/PBS (1:1) solution.

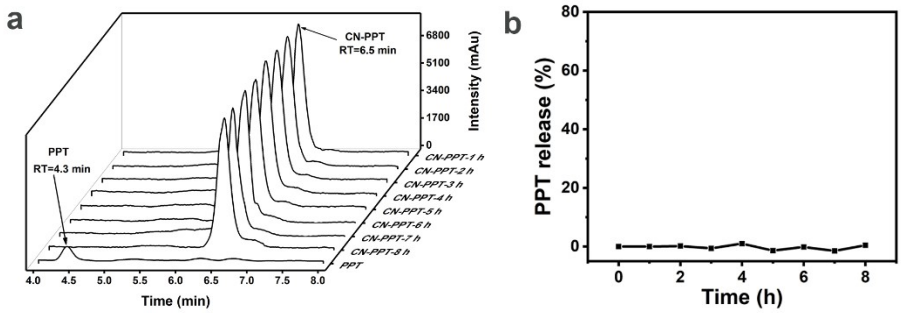


Figure S4 (a) HPLC chromatogram of **CN-PPT** (10 μM) incubated with H_2O_2 (100 μM). Peaks in the chromatograms were detected by monitoring the absorption at 254 nm. (b) Time-dependent release of Podophyllotoxin (PPT) determined by HPLC.

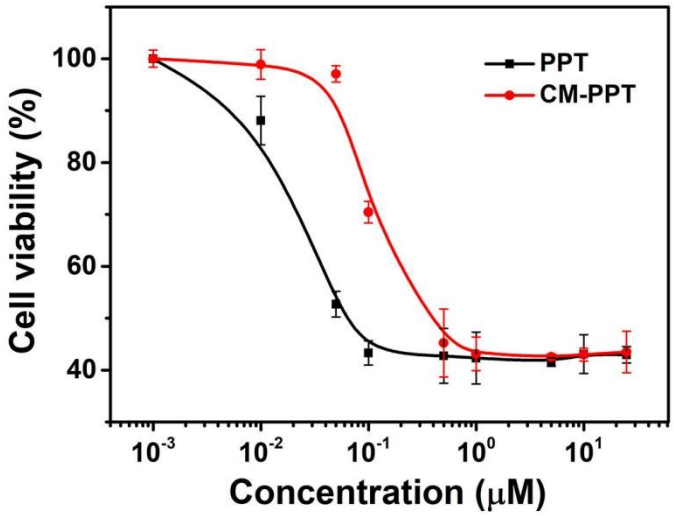


Figure S5. The cytotoxicity of **CM-PPT** and PPT were performed in HepG2 cells by the MTT assay.

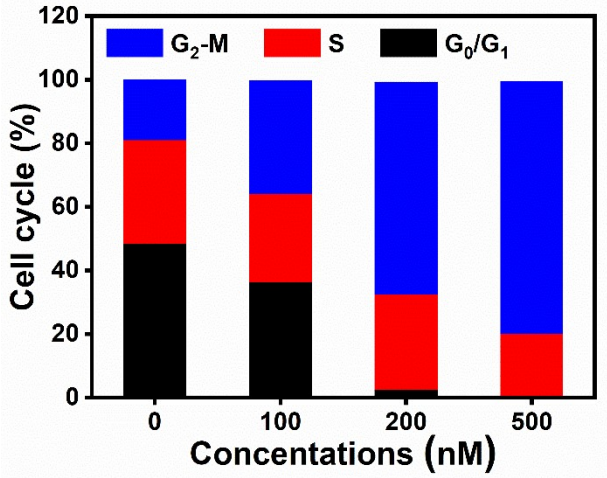


Figure S6. Cell cycle distribution histograms for HepG2 cells treated with **CM-PPT** for 24 h.

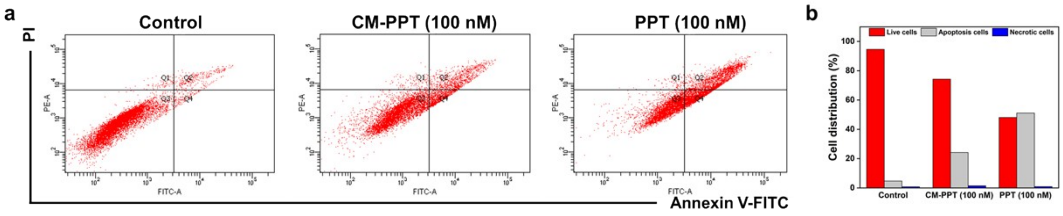


Figure S7. (a) Apoptosis of HepG2 cells treated with PBS, **CM-PPT** (100 nM) or PPT (100 nM)

for 24 h. (b) Quantification data.

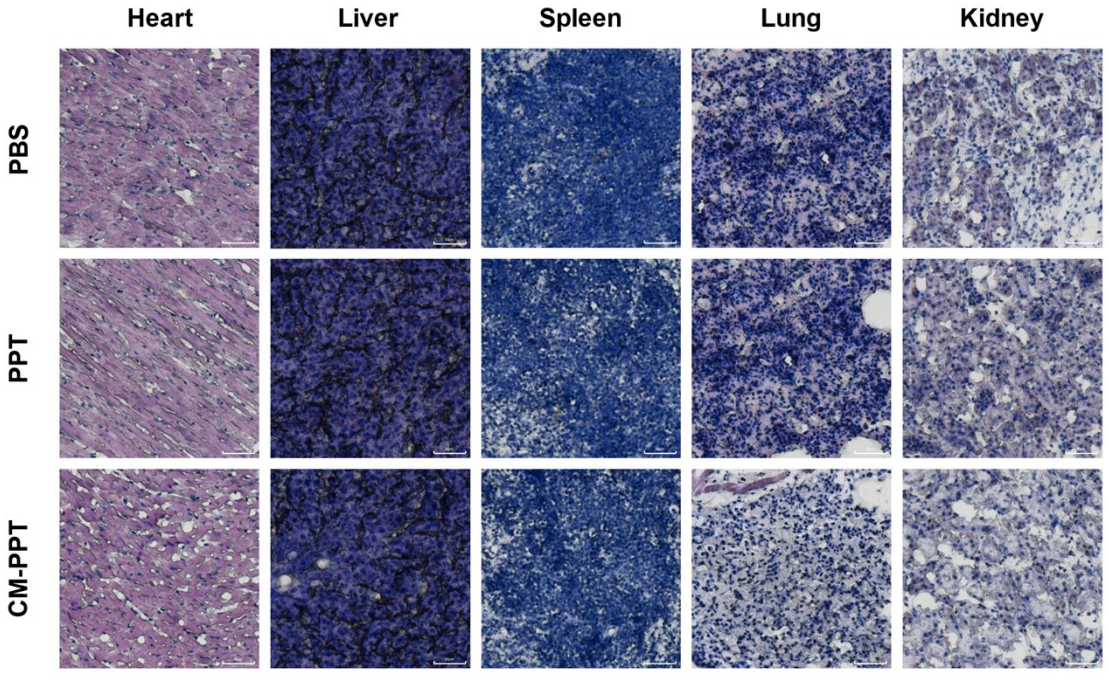


Figure S8. H&E staining of main organs (heart, liver, spleen, lung and kidney) in mice after 14 days of treatment. Scale bar: 50 μm.

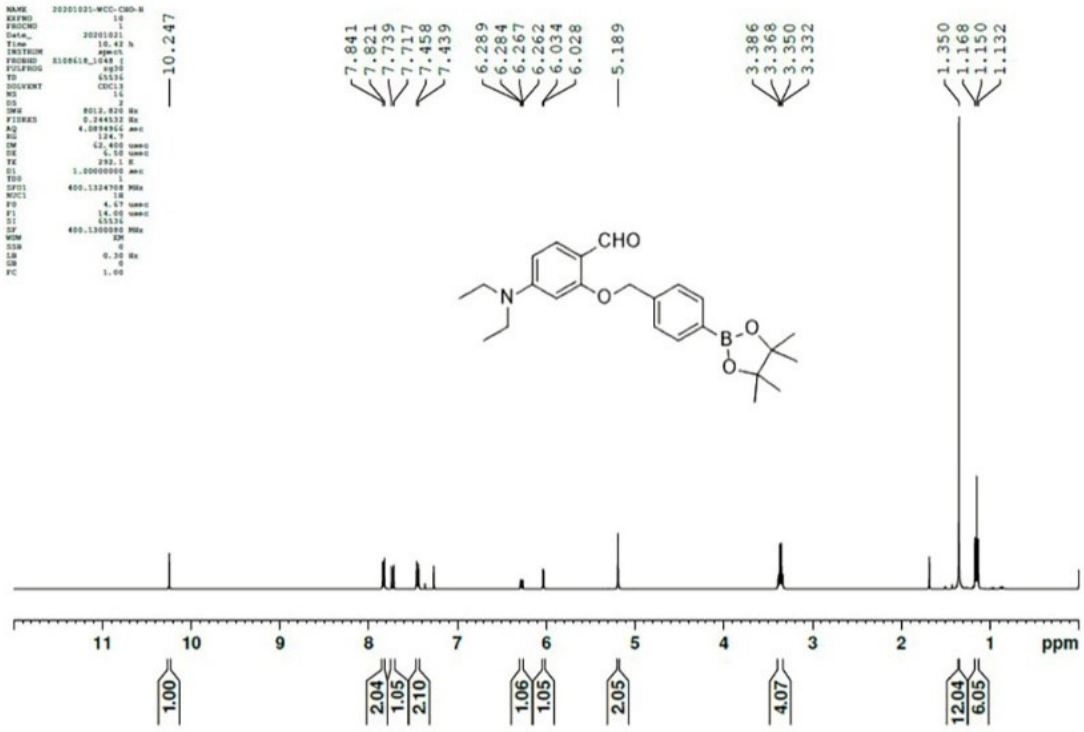


Figure S9. ^{1}H NMR spectrum of compound **1** in $CDCl_3$ (400 MHz).

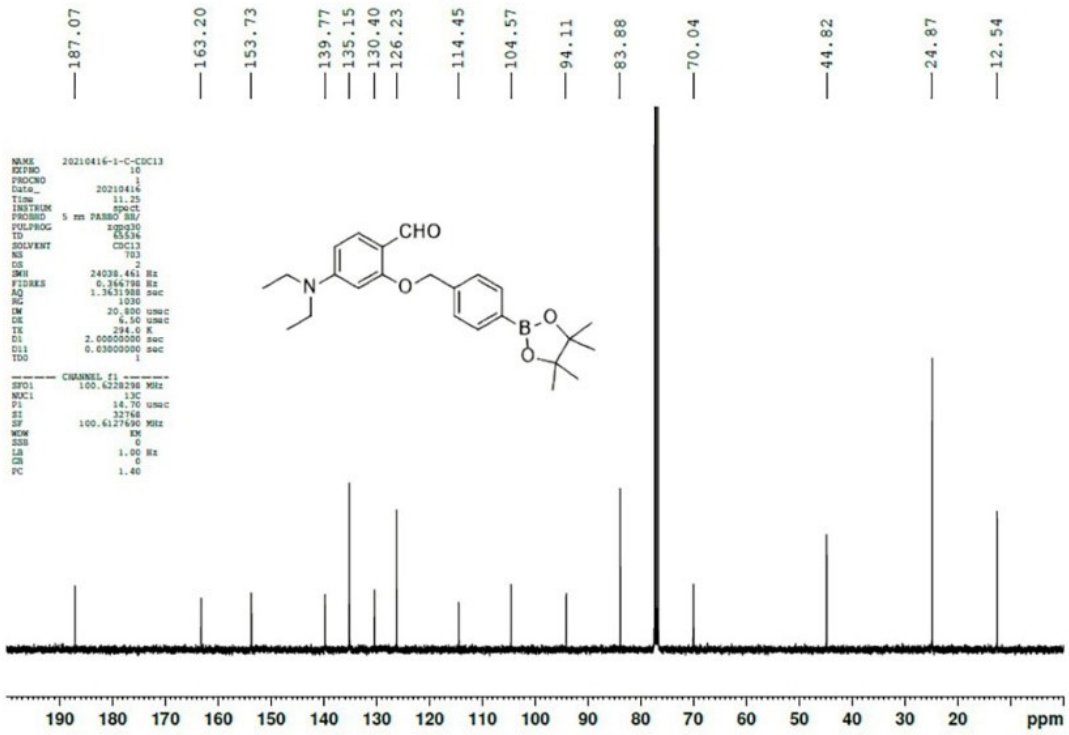


Figure S10. ^{13}C NMR spectrum of compound **1** in $CDCl_3$ (100 MHz).

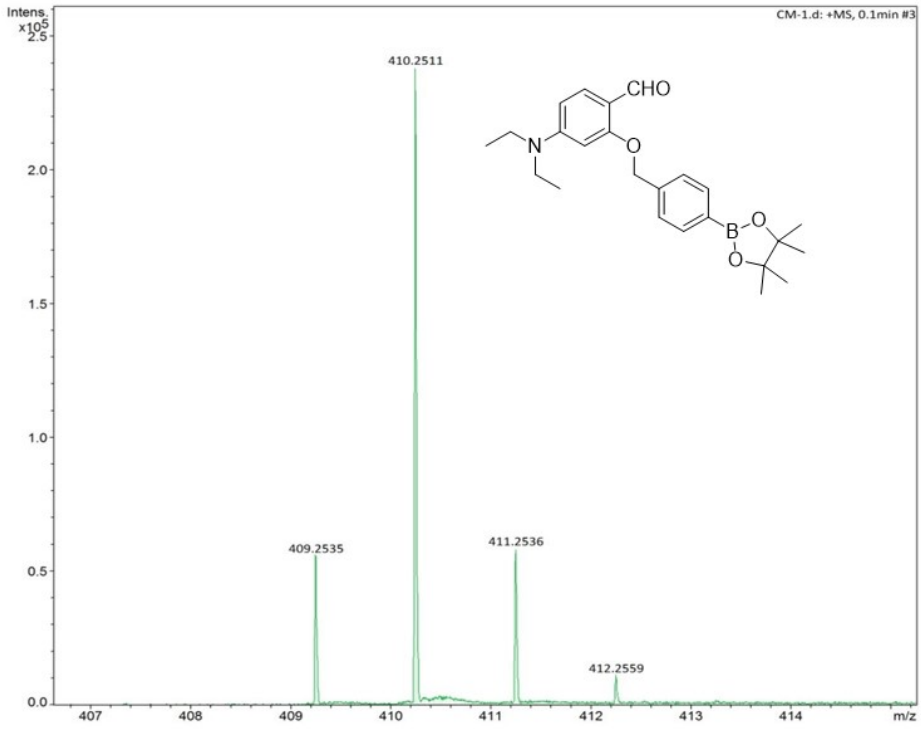


Figure S11. HRMS spectrum of compound **1**.

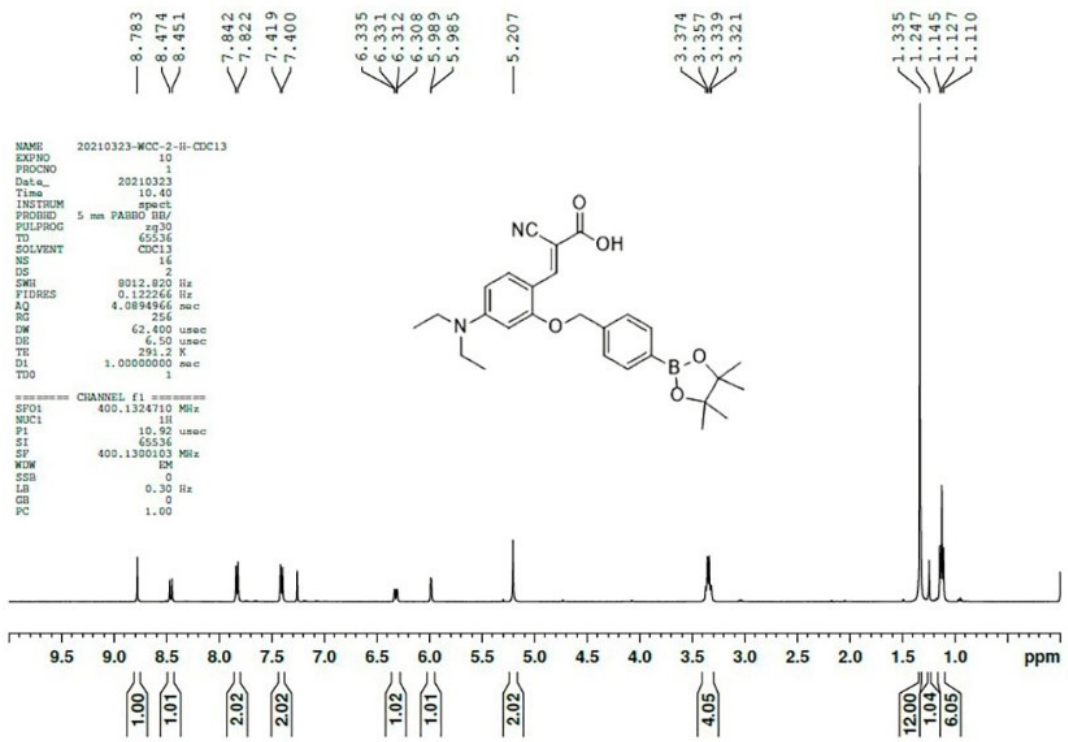


Figure S12. ^{1}H NMR spectrum of compound **2** in $CDCl_3$ (400 MHz).

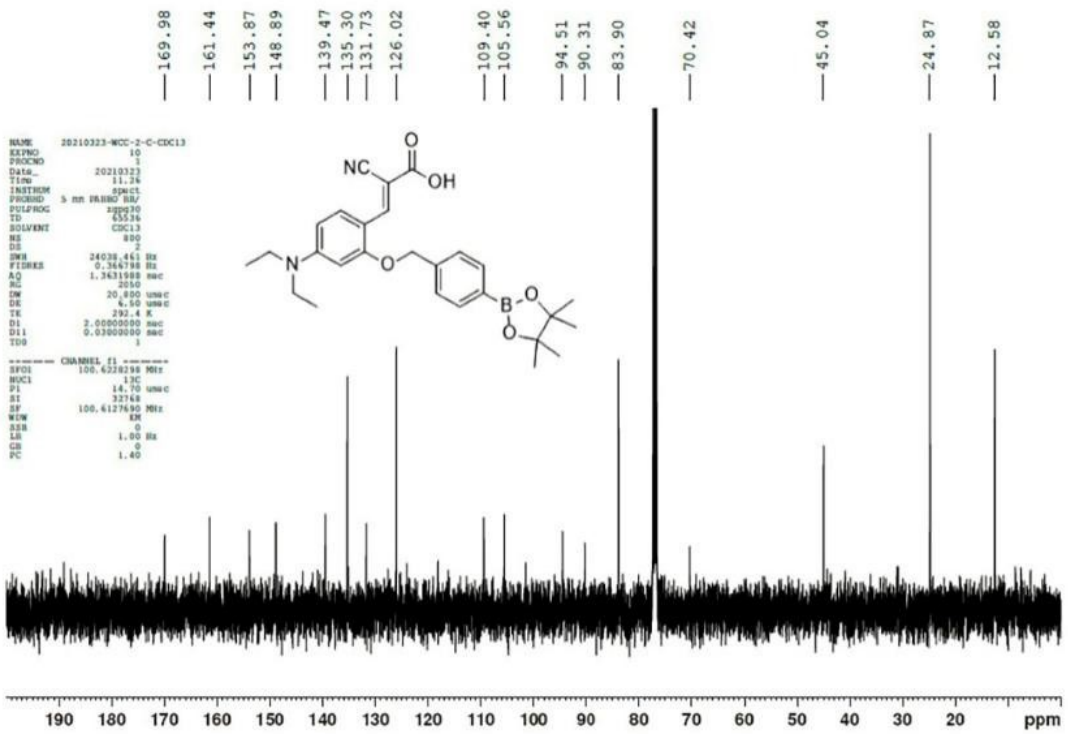


Figure S13. ^{13}C NMR spectrum of compound **2** in $CDCl_3$ (100 MHz).

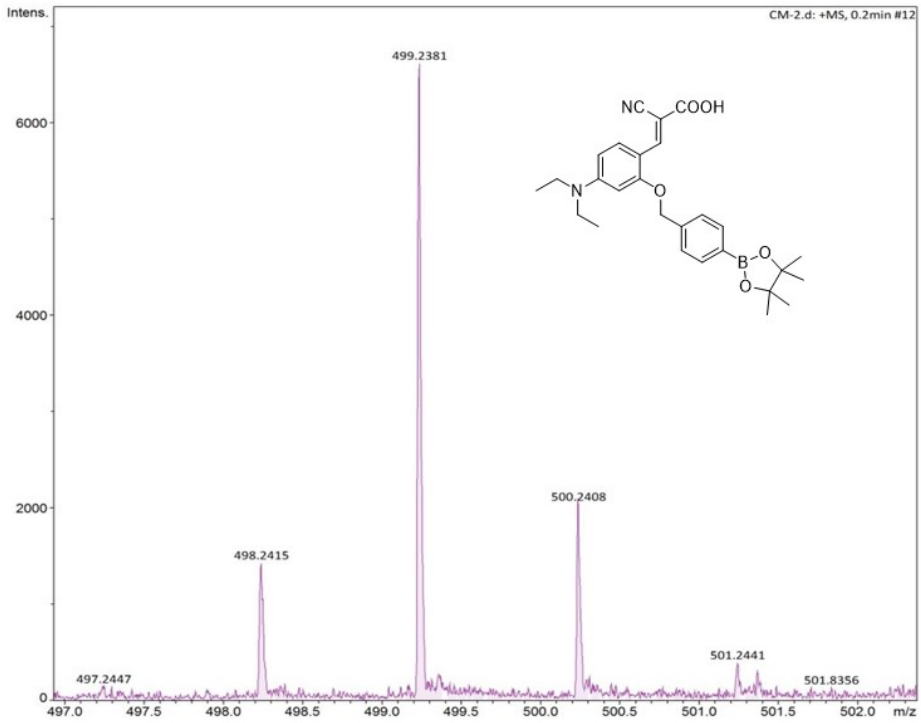


Figure S14. HRMS spectrum of compound **2**.

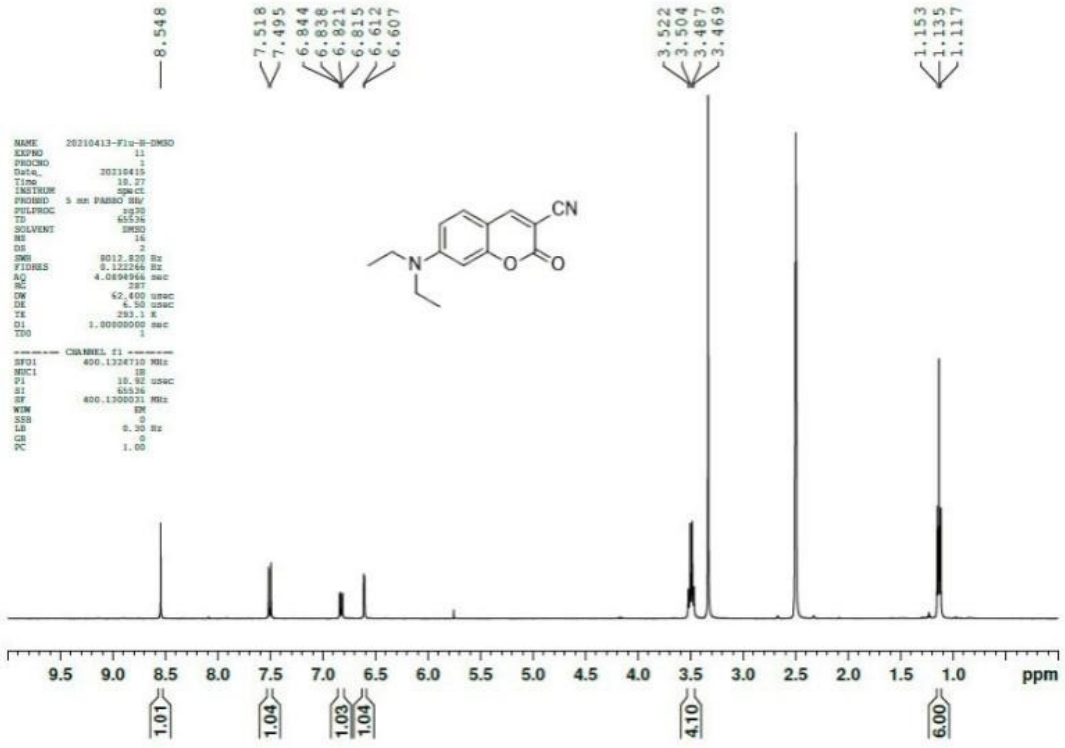


Figure S15. ^{1}H NMR spectrum of compound **3** in DMSO-d_6 (400 MHz).

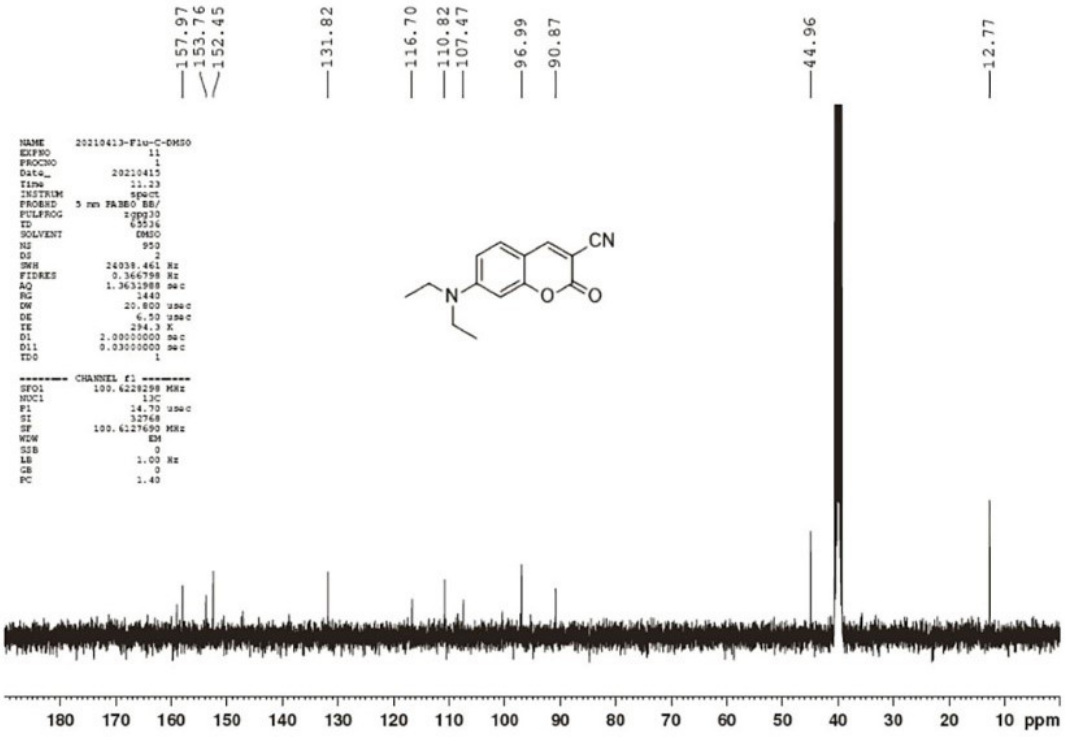


Figure S16. ^{13}C NMR spectrum of compound **3** in DMSO-d_6 (100 MHz).

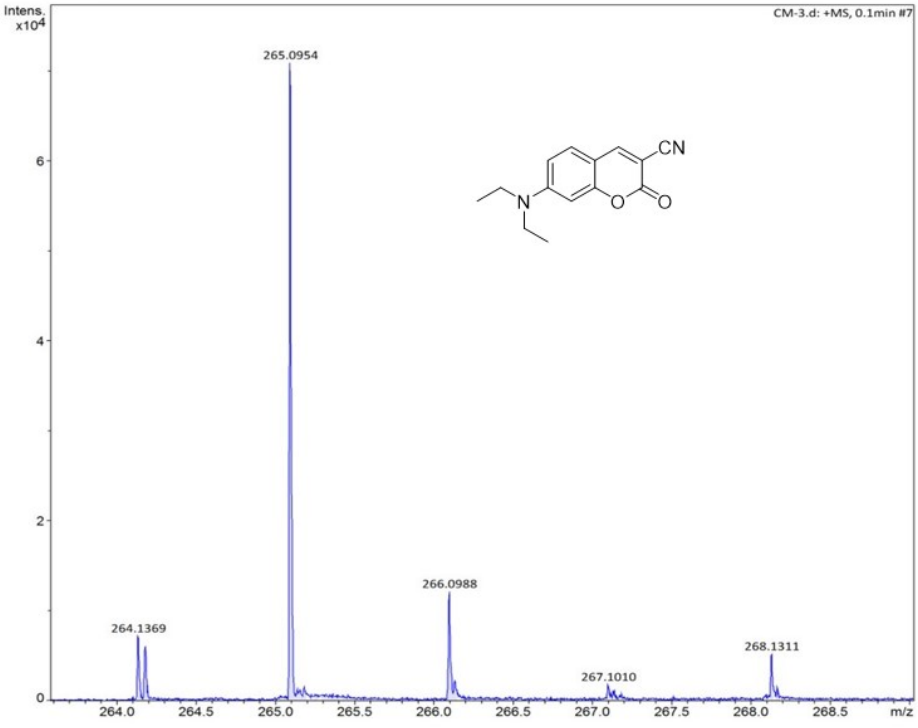


Figure S17. HRMS spectrum of compound **3**.

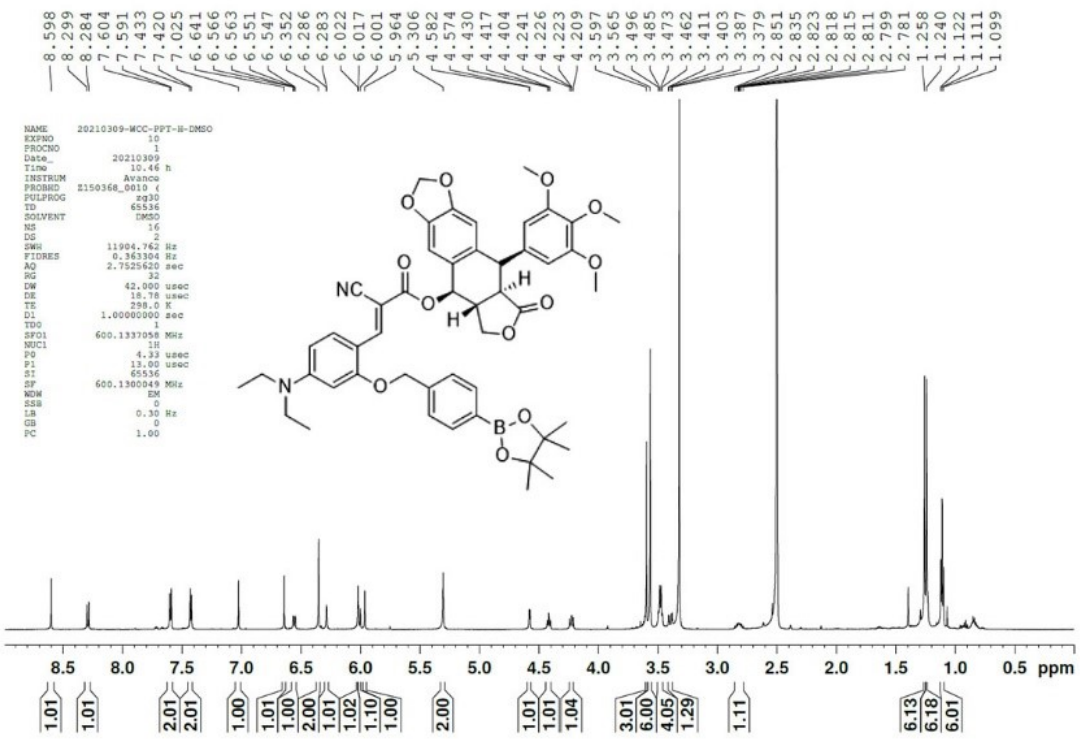


Figure S18. ^{1}H NMR spectrum of **CM-PPT** in DMSO-d_6 (600 MHz).

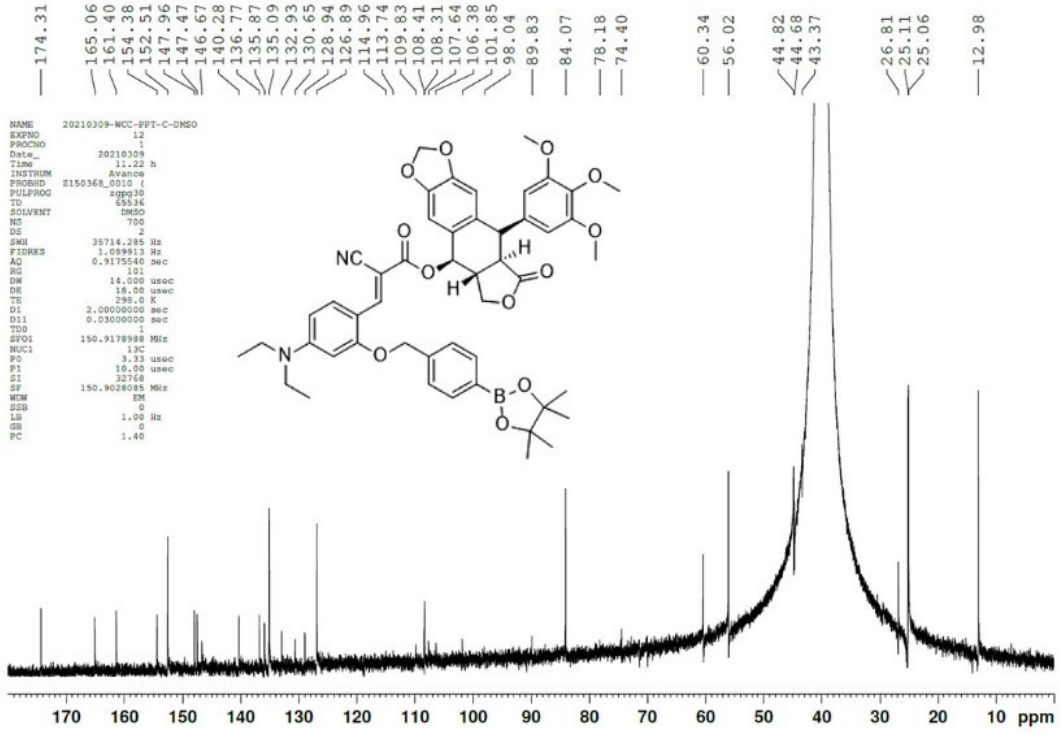


Figure S19. ^{13}C NMR spectrum of **CM-PPT** in DMSO-d_6 (150 MHz).

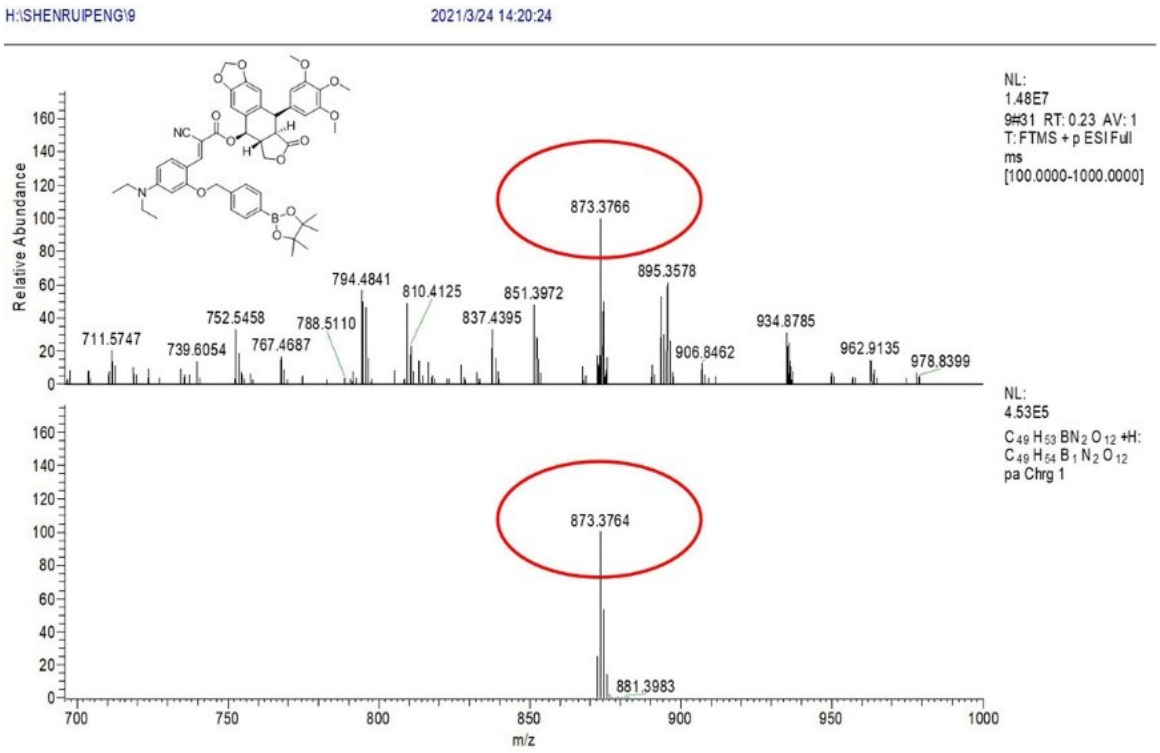


Figure S20. HRMS spectrum of **CM-PPT**.

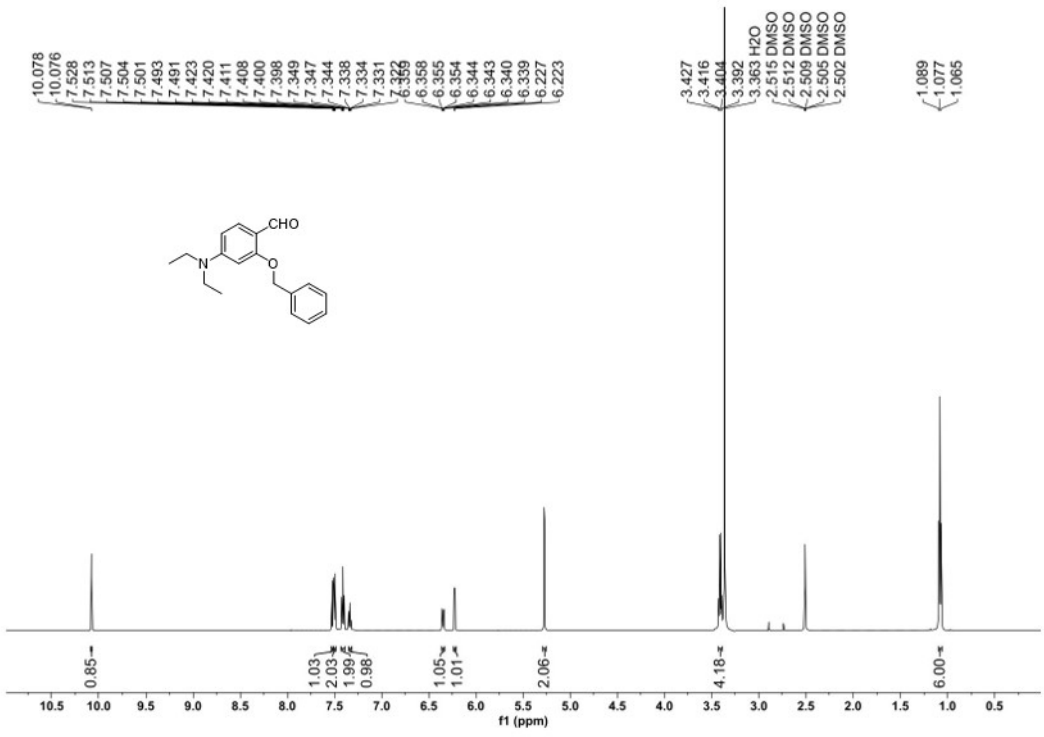


Figure S21. ^{1}H NMR spectrum of compound **4** in DMSO-d_6 (600 MHz).

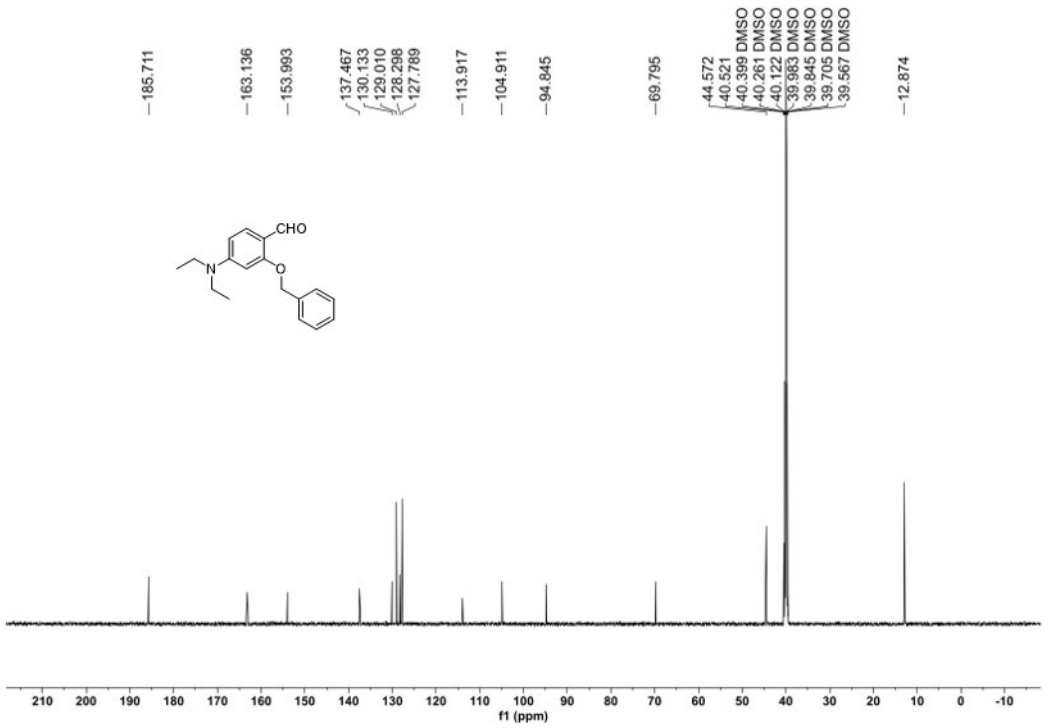


Figure S22. ^{13}C NMR spectrum of compound **4** in DMSO-d_6 (150 MHz).

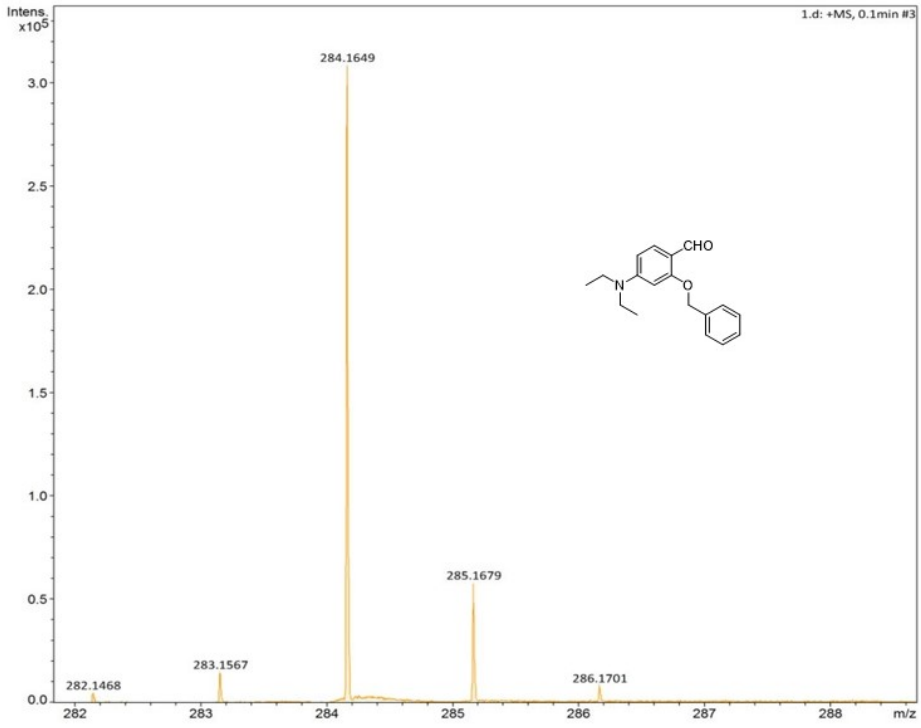


Figure S23. HRMS spectrum of compound **4**.

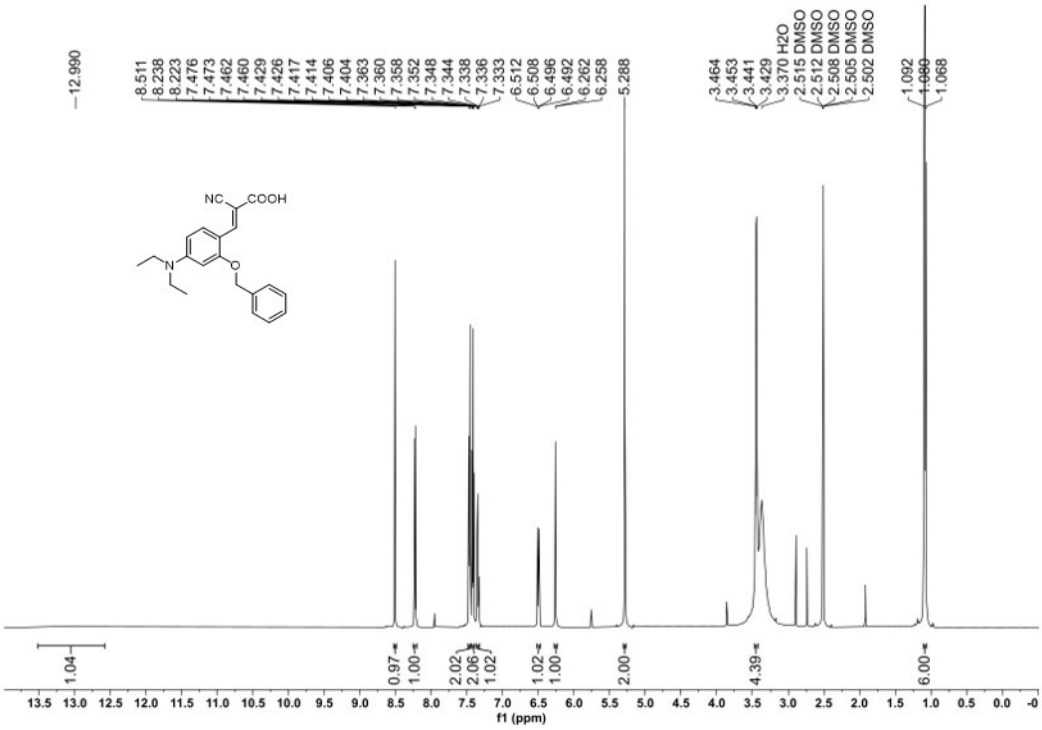


Figure S24. ^{1}H NMR spectrum of compound **5** in DMSO-d_6 (600 MHz).

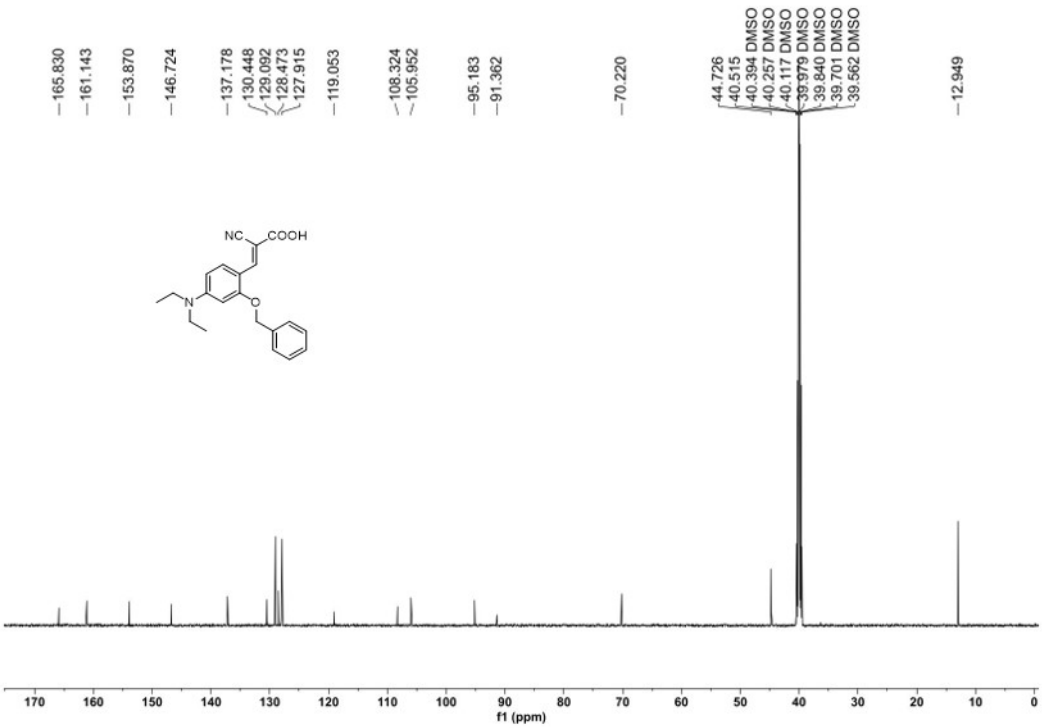


Figure S25. ^{13}C NMR spectrum of compound **5** in DMSO-d_6 (150 MHz).

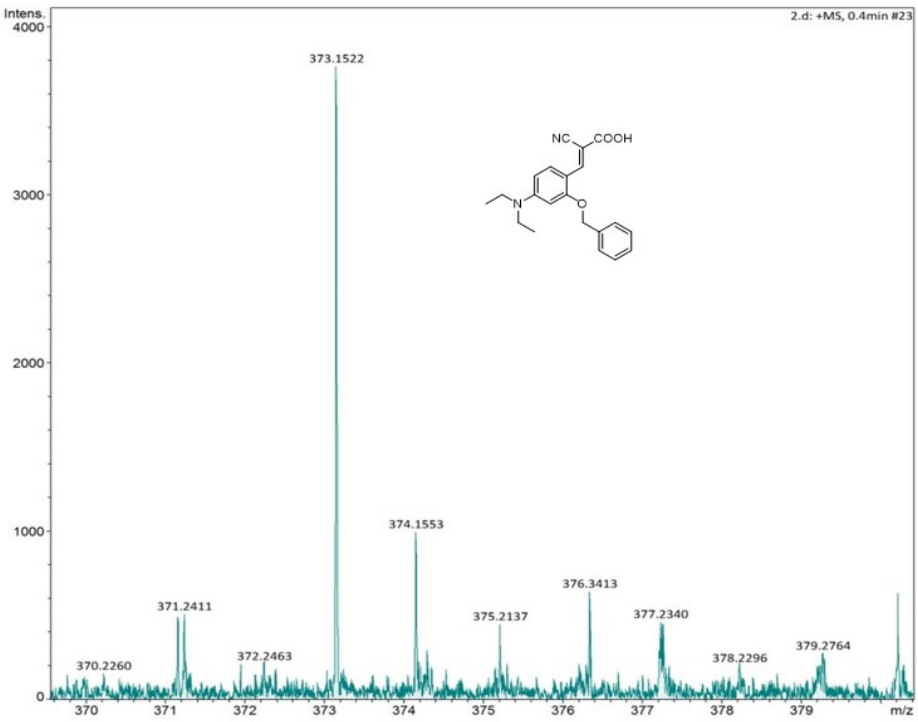


Figure S26. HRMS spectrum of compound **5**.

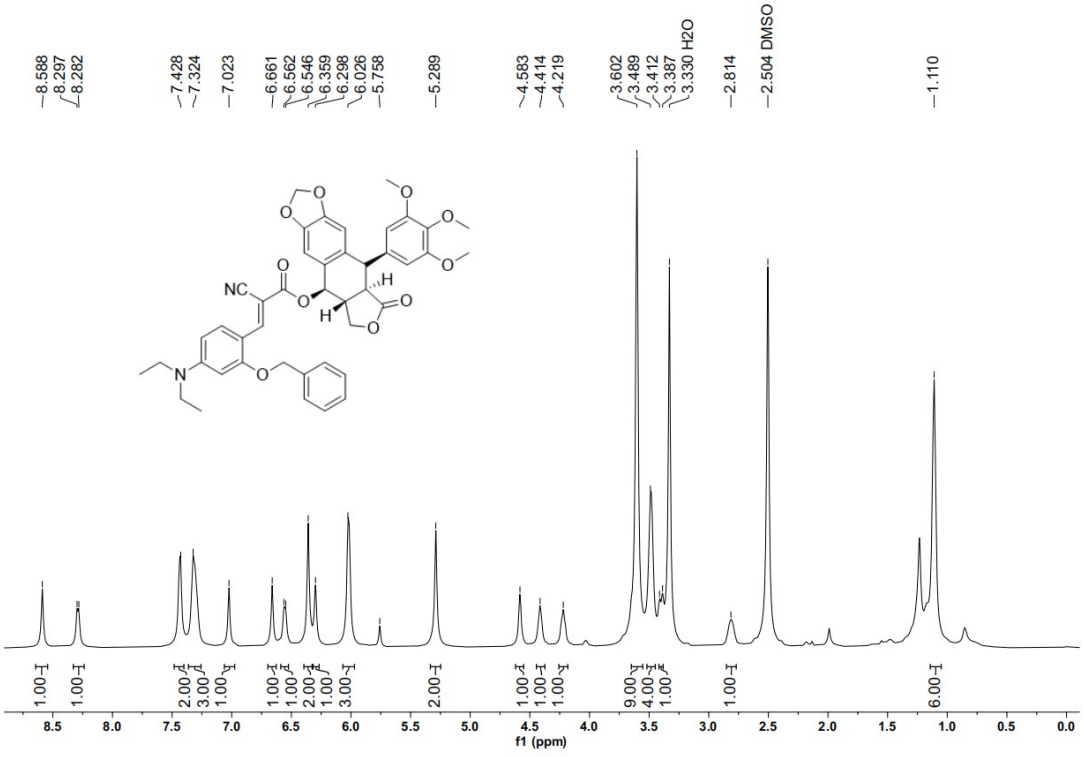


Figure S27. ^{1}H NMR spectrum of **CN-PPT** in DMSO-d_6 (600 MHz).

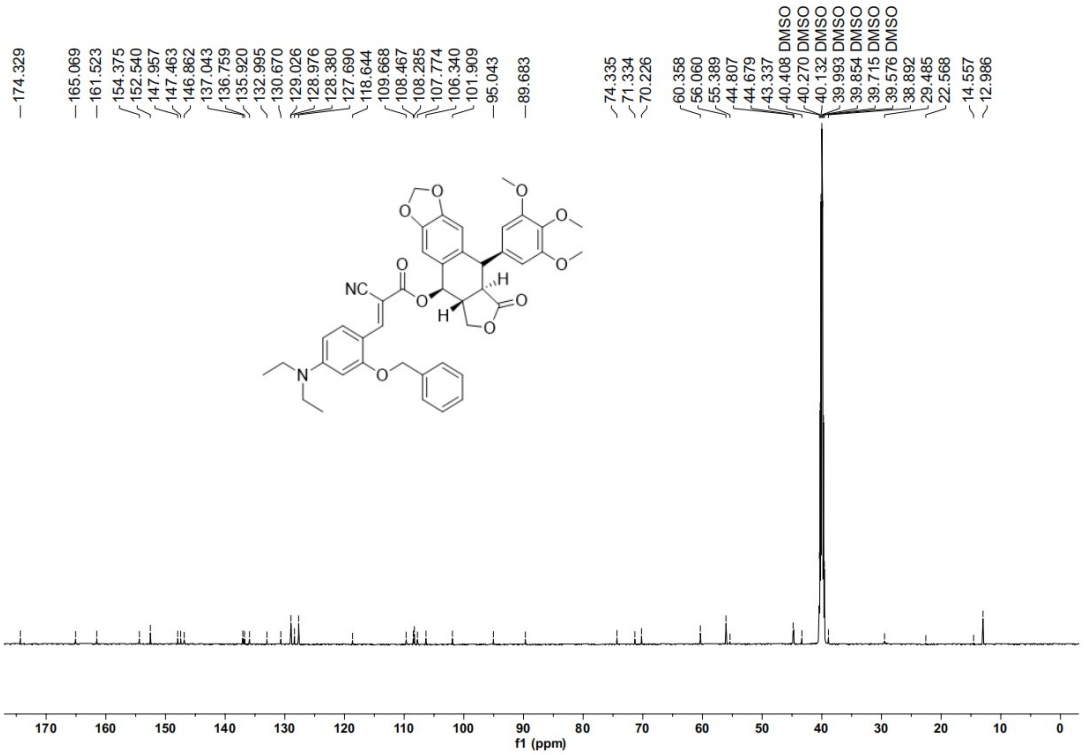


Figure S28. ^{13}C NMR spectrum of **CN-PPT** in DMSO-d_6 (150 MHz).

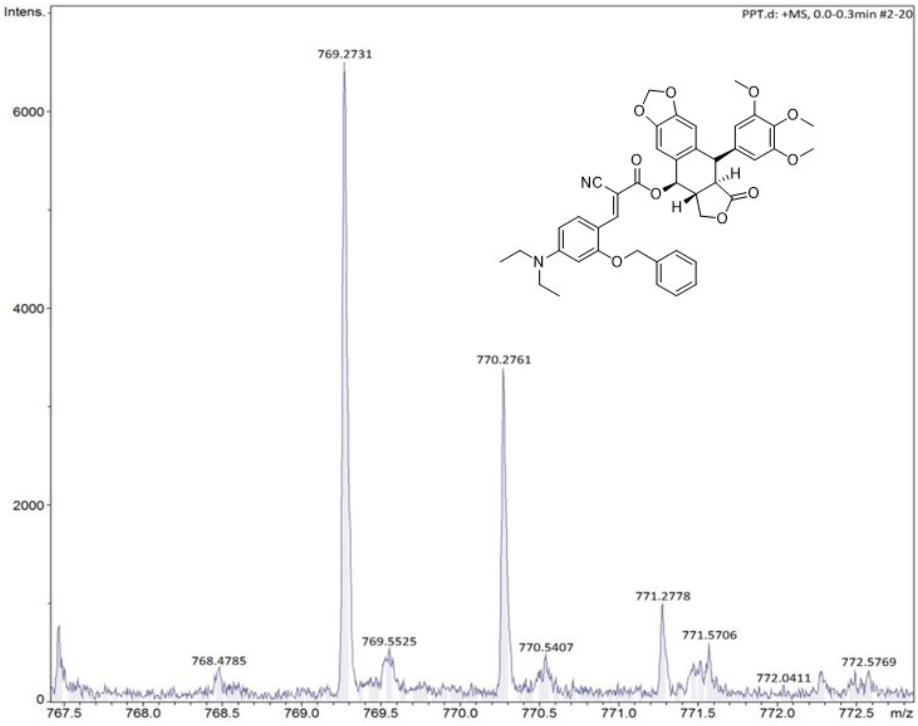


Figure S29. HRMS spectrum of **CN-PPT**.

3 References

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