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Supporting Information for

Novel photoactivatable substrates for *Renilla* luciferase *in vitro* and *in vivo*

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Supplementary Experimental Procedures

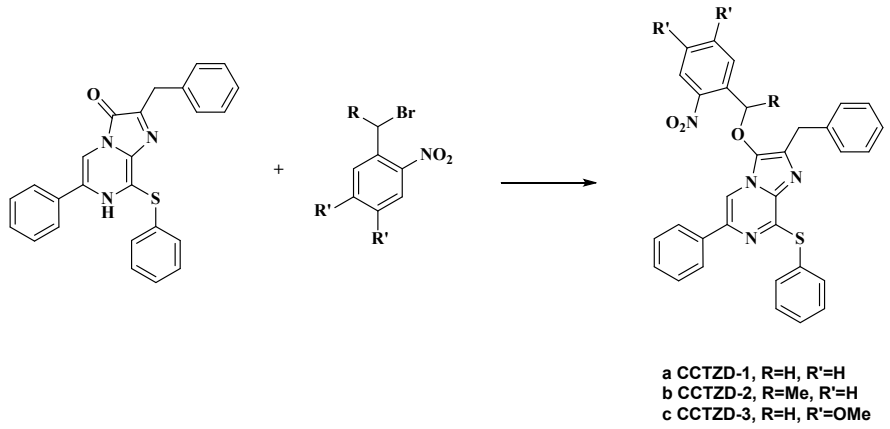
Materials

All chemicals were purchased from commercial suppliers and used without further purification. Ultrapure water was purified with a Mill-Q filtration system. ES-2-Rluc cells (a human ovarian cancer cell line stably expressing *Renilla* luciferase) were purchased from Shanghai BioDiagnosis Co., Ltd. Female nude mice (BALB/cA-nu) were purchased from the Animal Center of China Academy of Medical Sciences (Beijing, China).

Instruments

NMR spectral data were obtained for ^{1}H NMR and ^{13}C NMR at 400 MHz and 100 MHz, respectively. Chemical shifts are reported in ppm with the solvent resonance in DMSO-d_6 solution. ESI-HRMS spectral data were obtained by using a Water SYNAPTG2 mass spectrometer. High pressure liquid chromatography (HPLC) spectra and the purity of the compounds were determined by analytical reverse-phase HPLC (Agilent, 1200 Infinity) on a Phenomenex C-$_{18}$ column (250 × 4.6 mm). The pH test was performed using a pH-meter (OHAUS, STARTER3100). Melting points were measured using a Mel-Temp apparatus and were not corrected. Photoirradiation experiments *in vitro* were carried out with handheld UV lamp (36 W, 365 nm). Photoirradiation experiments *in vivo* were carried out with UV lamp (Thorlabs, M365FP1, 365 nm). Luminescence imaging was recorded using an IVIS Kinetic imaging system (Caliper Life Sciences, USA) equipped with a cooled charge-coupled device (CCD) camera. Circular specified regions of interest (ROIs) were drawn over the areas, and the total flux was calculated using the Living Image software version 4.0 (Caliper Life Sciences).

Synthesis and structural characterization of caged luciferin derivatives.



Scheme S1. Scheme of synthesis of caged luciferin derivatives.

2-benzyl-3-((2-nitrobenzyl)oxy)-6-phenyl-8-(phenylthio)imidazo[1,2-a]pyrazine (CCTZD-1).

Under argon, to a solution of a mixture containing coelenterazine derivative (200 mg, 0.5 mmol) and 1-(bromomethyl)-2-nitrobenzene (864 mg, 4.0 mmol) in anhydrous DMF (5 mL) was added K_2CO_3 (69 mg, 0.05 mmol), and then stirred at 30 °C. After 1 h, the mixture was added to water and the product was extracted with ethyl acetate. The organic layer was dried with $MgSO_4$ and concentrated in vacuo. The crude product was further purified by chromatography on silica gel as eluent to give 100 mg of white solid. Yield: 36.7 %. Melting point: 128 °C -130 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.54 (s, 1H), 8.16 (d, *J* = 7.8 Hz, 1H), 7.93 (d, *J* = 7.6 Hz, 1H), 7.85 (t, *J* = 7.4 Hz, 1H), 7.71 (ddd, *J* = 14.8, 12.4, 7.3 Hz, 5H), 7.59 – 7.53 (m, 3H), 7.32 (p, *J* = 6.0 Hz, 3H), 7.24 (q, *J* = 7.8 Hz, 4H), 7.17 (t, *J* = 6.6 Hz, 1H), 5.54 (s, 2H), 4.00 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 150.52, 147.86, 139.53, 137.48, 137.06, 136.34, 136.23, 136.23, 134.68, 133.20, 131.91, 130.61, 130.21, 130.14, 129.95, 129.65, 129.65, 129.07, 129.07, 129.07, 128.99, 128.86, 128.81,128.81, 128.81, 127.61, 126.63, 126.04, 125.26, 109.48, 73.54, 32.82. MS (ESI): m/z calcd for Chemical Formula: $[C_{32}H_{25}N_4O_3S]^+$ 545.1647, found 545.1646$[M+H]^+$.

Compounds CCTZD-2 and CCTZD-3 were synthesized similarly as CCTZD-1.

2-benzyl-3-(1-(2-nitrophenyl)ethoxy)-6-phenyl-8-(phenylthio)imidazo[1,2-a]pyrazine (CCTZD-2): Yield: 24.5 %. Melting point: 146 °C -148 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.39 (s, 1H), 8.26 (d, *J* = 6.9 Hz, 1H), 7.95 (dd, *J* = 8.2, 1.0 Hz, 1H), 7.87 (t, *J* = 7.6 Hz, 1H), 7.73 – 7.67 (m, 4H), 7.64 – 7.58 (m, 1H), 7.57 – 7.52 (m, 3H), 7.38 – 7.30 (m, 3H), 7.27 (t, *J* = 7.2 Hz, 2H), 7.19 (dd, *J* = 8.8, 7.4 Hz, 3H), 5.84 (q, *J* = 6.2 Hz, 1H), 3.95 (s, 2H), 1.73 (d, *J* = 6.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 150.55, 148.22, 139.31, 137.02, 136.63, 136.31, 136.25, 136.25, 134.35, 133.18, 130.24, 130.20, 129.95, 129.63, 129.63, 129.22, 129.00, 128.93, 128.93, 128.93, 128.88, 128.78, 128.78, 128.78, 127.55, 126.61, 126.02, 126.02, 124.61, 109.29, 78.74, 32.82, 22.81. MS (ESI): m/z calcd for Chemical Formula: $[C_{33}H_{27}N_4O_3S]^+$ 559.1798, found 559.1798$[M+H]^+$.

2-benzyl-3-((4,5-dimethoxy-2-nitrobenzyl)oxy)-6-phenyl-8-(phenylthio)imidazo[1,2-a]pyrazine (CCTZD-3): Yield: 20.5 %. Melting point: 133 °C -135 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.52 (s, 1H), 7.84 – 7.64 (m, 5H), 7.57 (d, *J* = 1.7 Hz, 3H), 7.46 – 7.28 (m, 4H), 7.20 (dd, *J* = 25.6, 6.8 Hz, 4H), 5.52 (s, 2H), 4.02 (d, *J* = 12.6 Hz, 2H), 3.87 (d, *J* = 4.3 Hz, 5H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 153.58, 150.46, 148.78, 140.36, 139.53, 137.58, 136.99, 136.35, 136.22, 136.22, 133.19, 130.11, 129.94, 129.65, 129.65, 128.97, 128.97, 128.97, 128.86, 128.75, 128.75, 128.75, 127.62, 126.59, 126.45, 126.00, 126.00, 112.60, 109.52, 108.62, 73.77, 56.79, 56.60, 32.82. MS (ESI): m/z calcd for Chemical Formula: $[C_{34}H_{29}N_4O_5S]^+$ 605.1859, found 605.1859$[M+H]^+$.

Bioluminescence measurements with *Renilla* luciferase.

These caged coelenterazine derivatives at same concentrations (60 μM) were assayed in 100 μL of Tris-HCl buffer (50 mM, pH 7.4). After UV irradiation at 365 nm (0, 0.5, 1, 2, 4, 6, 8, 10, 12 min), then 100 μL of Tris-HCl buffer containing 2 μg/mL *Renilla* luciferase was added and bioluminescent signals were detected at once with an exposure time of 1 min. All of the experiments were performed in triplicate. Similarly, these caged coelenterazine derivatives at various concentrations (0, 3.75, 7.5, 15, 30, 60, 120 μM) were assayed in 100 μL of Tris-HCl buffer (50 mM, pH 7.4). After UV irradiation at 365 nm (6 min), then 100 μL of Tris-HCl buffer containing 2 μg/mL. *Renilla* luciferase was added and bioluminescent signals were detected at once with an exposure time of 1 min. Fig. S1 is a schematic diagram of bioluminescence imaging. All of the experiments were performed in triplicate.

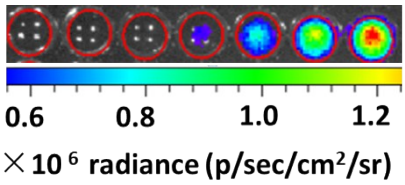


Fig. S1 The bioluminescence imaging of CCTZD-2 (various concentrations).

Metal ion effects.

Luminescence assays were performed with a cooled charge-coupled device (CCD) camera. Various metal ions, including Na^+, K^+, Fe^{3+}, Al^{3+}, Pa^{2+}, Ba^{2+}, Fe^{2+}, Sn^{2+}, Ca^{2+} , were dissolved in 50 mM Tris-HCl buffer (pH = 7.4) up to a concentration of 1 mM. Then *Renilla* luciferase (2 μg/mL) and the caged luciferin derivatives (120 μM) were also diluted with the same Tris-HCl buffer. The detailed procedure is as follows: a volume of 50 μL of caged luciferin derivatives was added to an equivalent volume of various ions and incubated at 25 °C for 30 min in black 96-well plates. After UV irradiation at 365 nm and addition 100 μL of *Renilla* luciferase, the bioluminescent signal was immediately recorded with an exposure time of 1 min. As a blank control, Tris-HCl buffer without metal ions was added under the same experimental conditions. The experiment was carried out three times with similar results.

Cytotoxicity test.

We used the way of Cell Counting Kit-8 to investigate the cell viability. After the ES-2-Rluc cells were incubated in 96-well plates for 24 h, the culture medium was removed and serial dilutions of these caged luciferins (0, 9.38, 18.75, 37.5, 75, 150, 300, 600 μM) in complete medium were added. After 14 h incubation and 12 min UV irradiation at 365 nm to the caged substrates, the solution of CCK-8 was added. Finally, a microplate reader was used to record the absorbance signal at 450 nm. There was no obvious cytotoxicity observed, demonstrating that these compounds have good biocompatibility with living cells (Fig. S3).

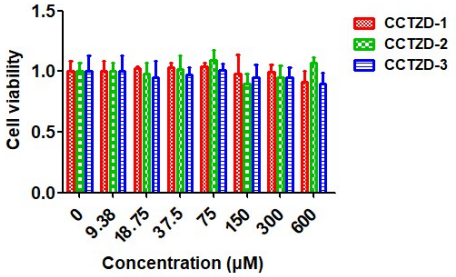


Fig. S2 Cell viability of compounds CCTZD-1, CCTZD-2 and CCTZD-3.

Bioluminescence imaging in cells.

The ES-2-Rluc cells were cultured in HIGH DMEM supplemented with 10 % fetal bovine serum (FBS) and 0.5 μg/mL puromycin at 37 °C. ES-2-Rluc cells were passed and plated (4×10^4 cells per well) in 96-well plates and cultured 24 h. Then the medium were removed and 60 μM of the three caged coelenterazine derivatives in buffer were added. Then, the solution was removed after incubating 1 h and ES-2-Rluc cells were treated with some normal saline. After different time of UV irradiation at 365 nm (0, 0.5, 1, 2, 4, 6, 8, 10, 12 min), and then the bioluminescence was measured at once and the bioluminescence intensity was collected. Fig. S2 is a schematic diagram of bioluminescence imaging. Similarly, at various concentration of the three caged coelenterazine derivatives (1.875, 3.75, 7.5, 15, 30, 60 μM) were also incubated with ES-2-Rluc cells for 1 h. Then normal saline was added after removing the buffer. After 6 min of UV light irradiation, the bioluminescence was measured at once with an exposure of 5 min and the bioluminescence intensity was collected.

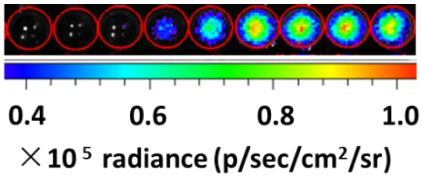


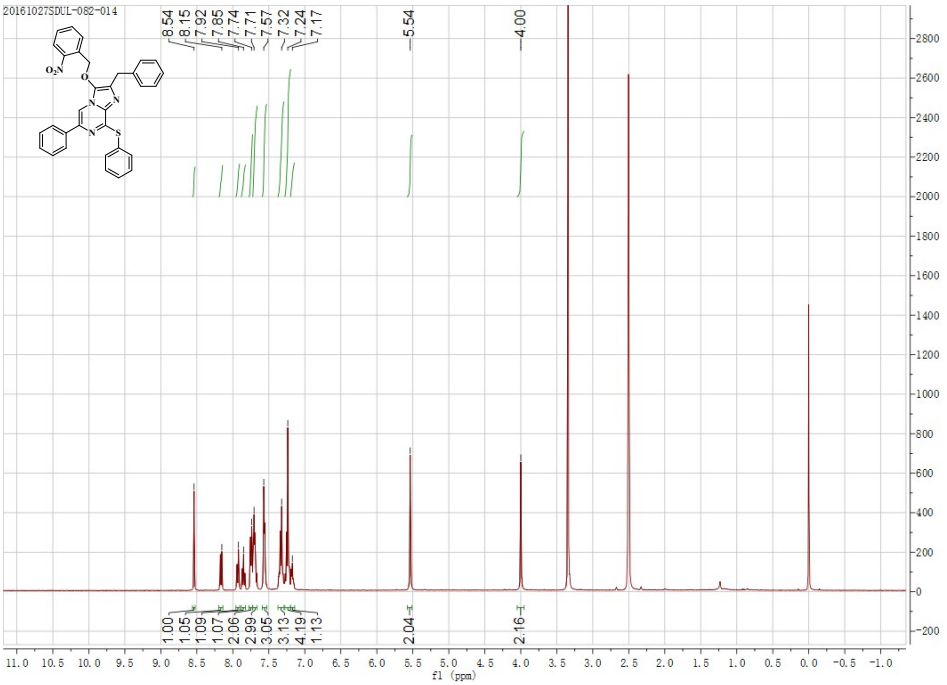
Fig. S3 The imaging of bioluminescence intensities of CCTZD-2 with different time of UV irradiation.

In vivo Bioluminescence Imaging.

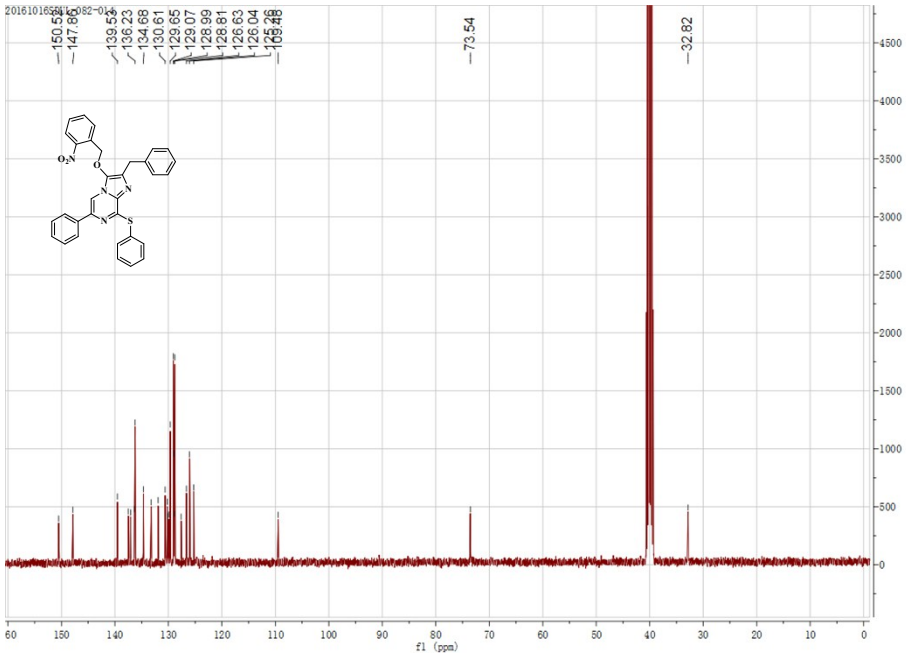
All animal studies were approved by the Ethics Committee and IACUC of QiLu Health Science Centre, Shandong University and were conducted in compliance with European guidelines for the care and use of laboratory animals. The subcutaneous xenotransplanted tumor model in nude mice was established following a reported approach. 5-Week-old female nude mice (BALB/cA-nu) were purchased and group-housed in a room maintained at 25 °C with 40–60 % humidity. To construct an ovarian cancer mouse model, ES-2-Rluc cells (1×10^6) were implanted subcutaneously in the right armpit region of each mouse. When the solid tumours grew to reach a 1.2 cm diameter about 4 weeks and we mashed them into a homogenate. By grafting roughly the same amounts of the tumour homogenate, the new tumour xenografts of nude mice were obtained. After 3 weeks later, two nude mice were injected with 4 mM (100 μL) of caged coelenterazine derivative 1 (CCTZD-1) in the tumour site. And one of them served as a control without UV,

another was treated with 5 minutes UV irradiation at 365 nm. Then bioluminescent imaging was immediately performed and we made a total of three times. Compounds CCTZD-2 and CCTZD-3 were applied with the same operation.

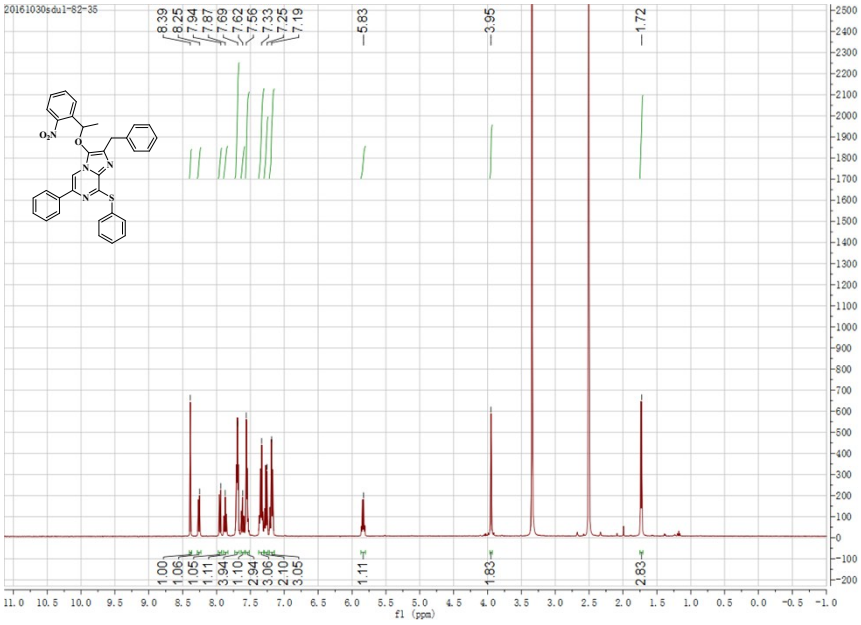
^{1}H NMR, ^{13}C NMR and ESI-HRMS for compounds



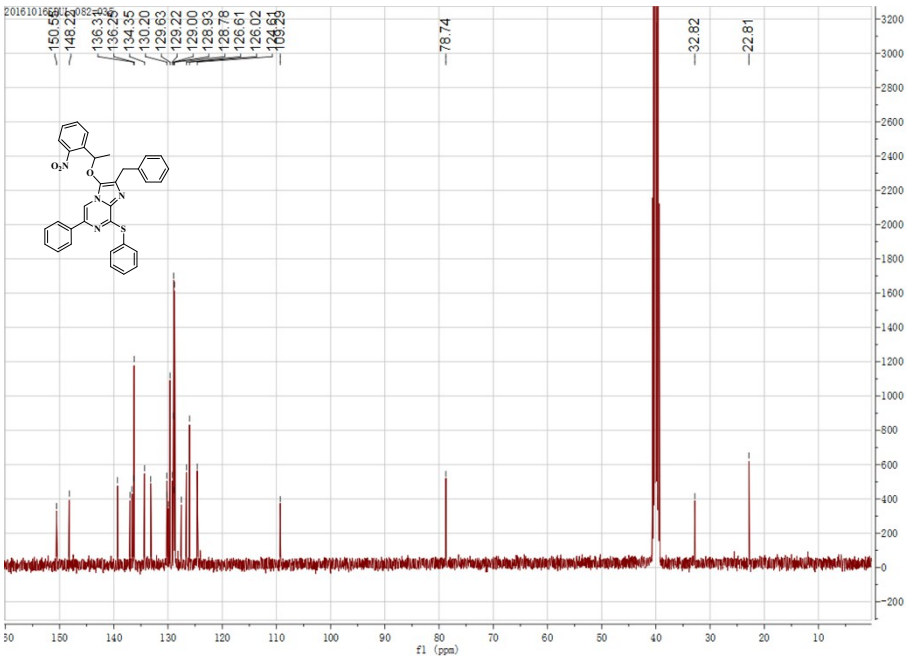
^{1}H NMR spectrum of CCTZD-1



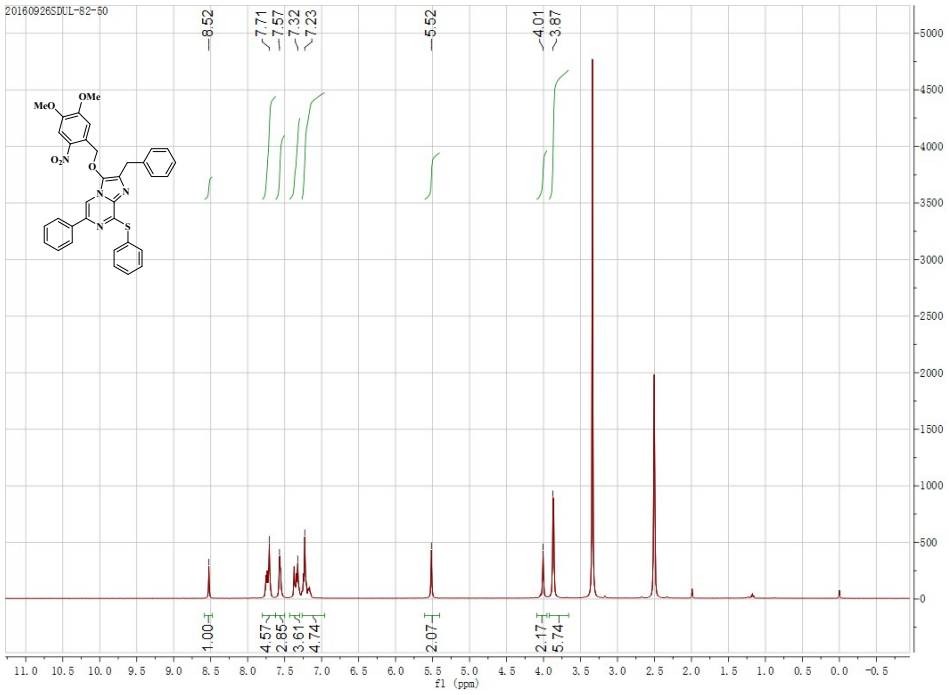
^{13}C NMR spectrum of CCTZD-1



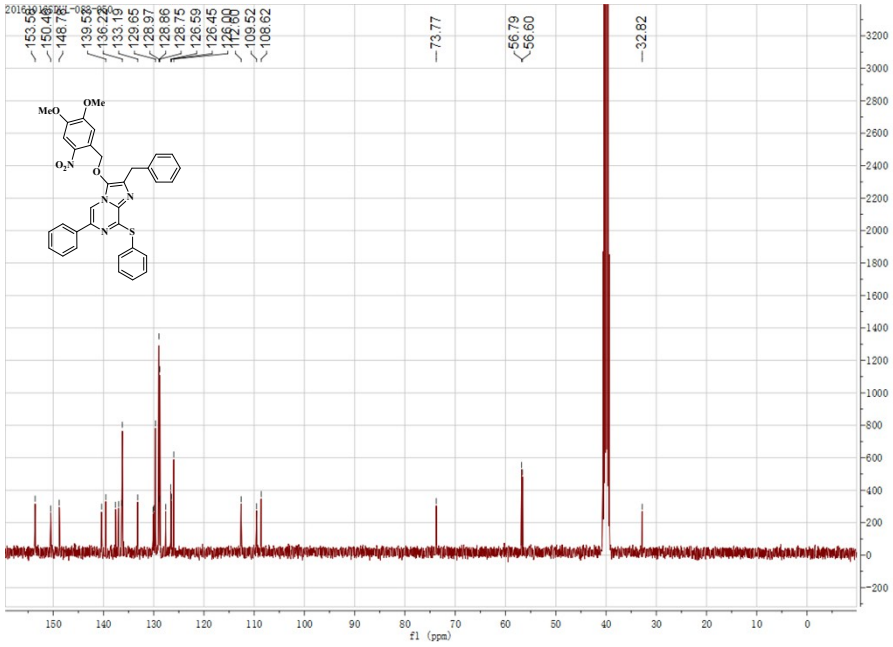
^{1}H NMR spectrum of CCTZD-2



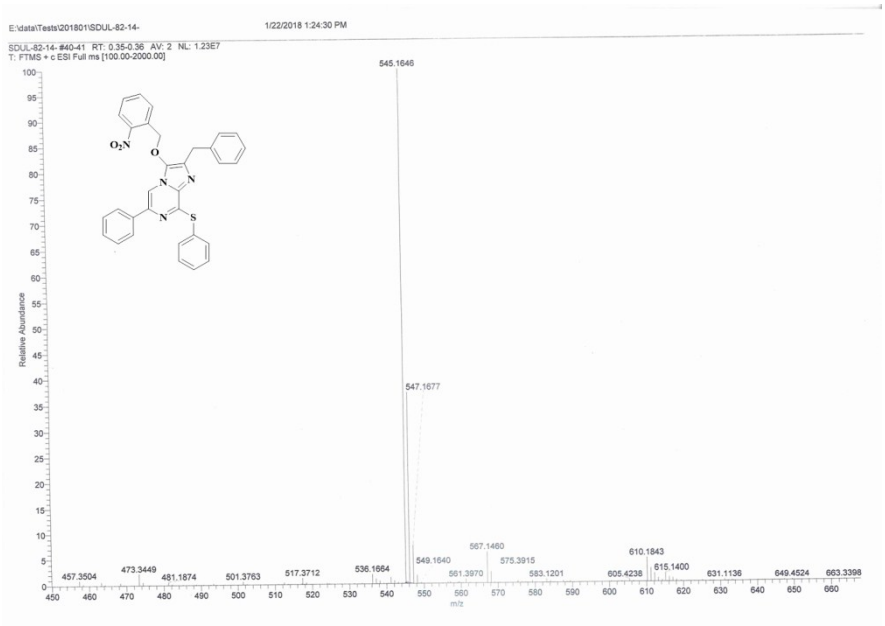
^{13}C NMR spectrum of CCTZD-2



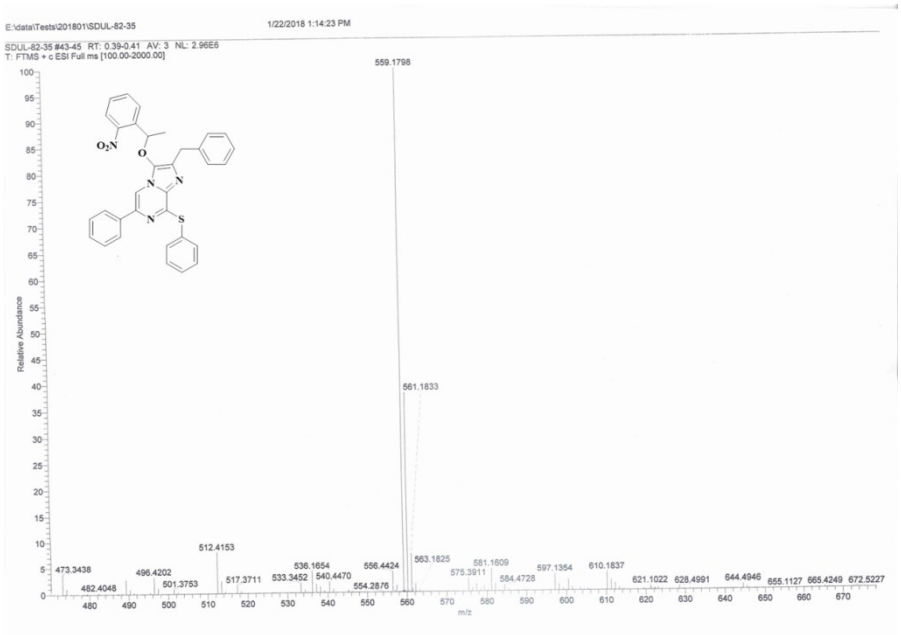
^{1}H NMR spectrum of CCTZD-3



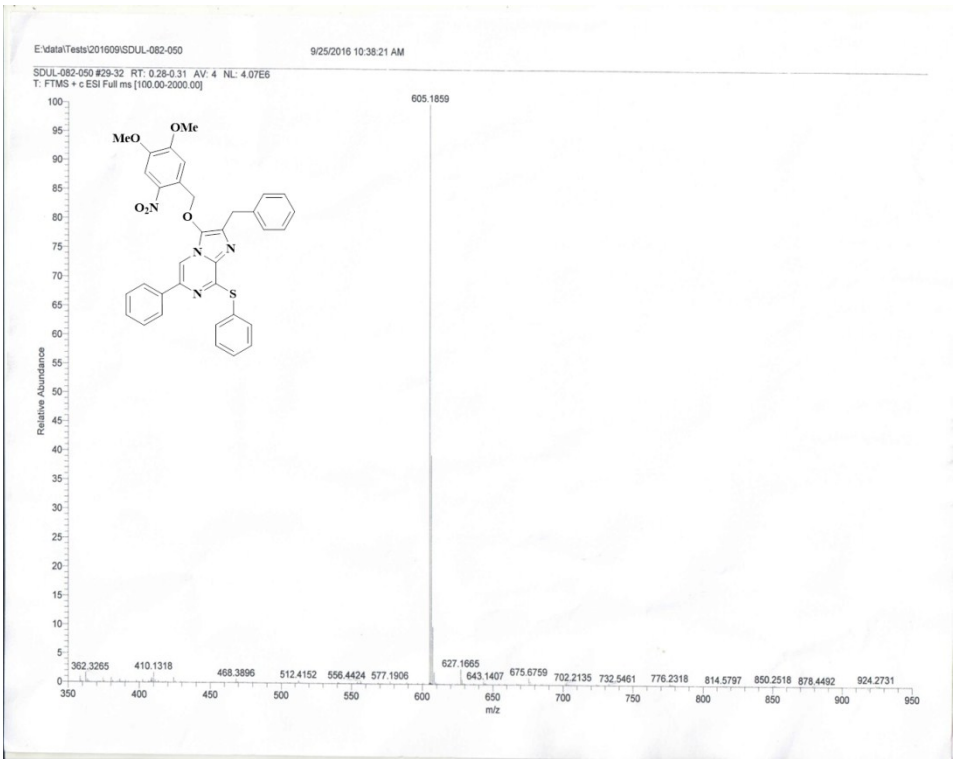
^{13}C NMR spectrum of CCTZD-3



HRMS spectrum of CCTZD-1



HRMS spectrum of CCTZD-2



HRMS spectrum of CCTZD-3