Supporting information

Light-Triggered Release of Lenalidomide with Fluorescent Indication for Inhibition of COX-2 Enzyme Activity in Cancer Cells

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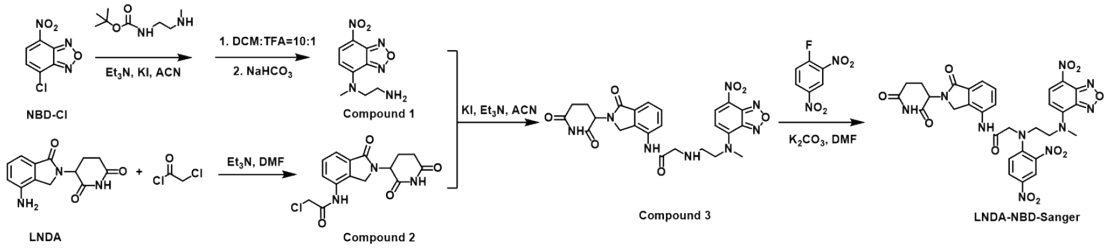
Content

Materials and instruments

All chemicals and reagents were obtained from commercial suppliers and used without further purification. Ultrapure water was used throughout the experiments.^{1}H NMR and ^{13}C NMR spectra were performed on a Bruker 400 MHz and 101 MHz spectrometer, respectively. high-resolution mass spectrometry (HRMS) were analyzed on an Agilent Model 1260 HPLC-6500Q-TOF mass spectrometer. The ultraviolet–visible (UV–Vis) absorption spectra were recorded on an α-1860A UV–Vis Spectrometer. The fluorescence spectra were measured using a Hitachi F-4700 spectrophotometer. All irradiation experiments were performed using an OmniCure LX500 UV LED system (Excelitas Technologies) with a central wavelength of 400 nm and irradiance of 50 mW/cm². All HPLC chromatograms were generated using a Waters Breeze2 1525 EF system, which was equipped with a Poroshell 120 EC-C18 column (3.0 × 50 mm, 2.7 μm), and the detection wavelength was set at 254 nm.

Synthesis of Compounds



Scheme S1. The synthesis route of **LNDA-NBD-Sanger.**

Synthesis of Compound 1

Accurately weighed 4-chloro-7-nitrobenzofurazan (NBD-Cl) (0.93 g, 5.30 mmol), Tert-Butyl 2-(methylamino) ethylcarbamate (1.20 g, 6.00 mmol), and KI (0.50 g, 3.00 mmol) were placed into a 250 mL round-bottom flask, and 100 mL of anhydrous acetonitrile was added to fully dissolve the components. Under nitrogen protection, triethylamine (1.1 mL, 5.96 mmol) was added, and the reaction was stirred at 50 °C for 4 h. The progress of the reaction was monitored by TLC. After the reaction was complete, the solvent was removed under vacuum, and the crude brown oily intermediate product was purified by silica gel column chromatography. After thorough drying, it was dissolved in a mixed solvent of DCM : TFA=10 : 1 and stirred at room temperature for 4 h, with the progress of the reaction continuously monitored by TLC. When the reaction was complete, the solvent was removed under vacuum, and then a certain amount of DCM was added to dissolve it. Then, a prepared saturated $NaHCO_3$ solution was added to the solution, and it was continuously stirred at room temperature until no more bubbles appeared. Afterward, the product was extracted with DCM (3 × 25 mL), dried with $MgSO_4$, and the filtrate was concentrated

under vacuum after filtration. The product was then purified by silica gel column chromatography to yield 0.80 g of dark red solid **Compound 1**, with a yield of 80%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.26 (d, *J* = 9.2 Hz, 1H), 6.27 (d, *J* = 9.4 Hz, 1H), 3.53 (dd, *J* = 16.5, 10.4 Hz, 3H), 3.17 (s, 2H), 2.86 (s, 2H), 2.36 (s, 2H) ; ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm): 158.40, 158.09, 157.78, 137.43, 118.75, 115.76, 99.72, 45.66, 33.73; HRMS: $[M + H]^+$ calcd. for $[C_9H_{11}N_5O_3 + H]^+$: 238.0923; found: 238.0940; $[M + Na]^+$ calcd. for $[C_9H_{11}N_5O_3 + Na]^+$: 260.0737; found: 260.0760.

Synthesis of Compound 2

Accurately weighed **LNDA** (2.00 g, 7.71 mmol) was placed in a 250 mL round-bottom flask, and 150 mL of anhydrous DMF was added. Under nitrogen protection at 0 ℃, triethylamine (1.39 mL, 10.03 mmol) was added using a pipette, and the mixture was stirred. Subsequently, chloroacetyl chloride (0.92 mL, 11.57 mmol) dissolved in anhydrous DMF was slowly added to the reaction mixture via a pressure-equalizing funnel. After the addition was complete, the reaction mixture was allowed to warm to room temperature and stirred for an additional 1 h. During the reaction, a white solid was observed to precipitate from the solution. After further reaction for approximately 1 h, deionized water was added to the solution, resulting in the precipitation of a large amount of gray-white solid. The mixture was stirred for an additional 0.5 h. The solvent was then removed by filtration, yielding a gray-white solid powder, **Compound 2** (2.46 g, 95% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 11.03 (s, 1H), 10.22 (s, 1H), 7.83 (dd, *J* = 7.4, 1.5 Hz, 1H), 7.53 (m, 2H), 5.14 (dd, *J* = 13.3, 5.1 Hz, 1H), 4.38 (m, 4H), 2.91 (ddd, *J* = 17.3, 13.6, 5.4 Hz, 1H), 2.60 (dt, *J* = 16.8, 3.6 Hz, 1H), 2.34 (dd, *J* = 13.1, 4.5 Hz, 1H), 2.02 (m, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm): 172.87, 171.05, 167.68, 165.03, 134.05, 133.05, 132.81, 128.81, 125.46, 119.69, 51.56, 45.29, 43.10, 31.20, 22.65; HRMS: $[M + H]^+$ calcd. for $[C_{15}H_{14}ClN_3O_4 + H]^+$: 336.0733; found: 336.0751; $[M + Na]^+$ calcd. for $[C_{15}H_{14}ClN_3O_4 + Na]^+$: 358.0551; found: 358.0571.

Synthesis of Compound 3

Accurately weighed **Compound 2** (0.50 g, 1.49 mmol), **Compound 1** (0.71 g, 2.98 mmol), and KI (0.32 g, 1.93 mmol) were placed into a 250 mL round-bottom flask, and 100 mL of anhydrous acetonitrile was added to fully dissolve the components. Under nitrogen protection, triethylamine (0.42 mL, 2.98 mmol) was added using a pipette, and the reaction temperature was raised to 80 ℃ for reflux overnight. The progress of the reaction was monitored by TLC. Once the reaction was complete, heating was stopped. The acetonitrile solvent was first removed under vacuum to obtain the crude product, which was then thoroughly dried and purified by silica gel column chromatography, yielding 0.40 g of **Compound 3** with a yield of 50%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.01 (s, 1H), 9.65 (s, 1H), 9.44 (s, 1H), 8.44 (d, *J* = 8.9 Hz, 1H), 7.66 (d, *J* = 7.8 Hz, 1H), 7.46 (dd, *J* = 20.6, 7.5 Hz, 2H), 6.43

(d, *J* = 9.0 Hz, 1H), 5.11 (dd, *J* = 13.3, 5.1 Hz, 1H), 4.31 (s, 2H), 3.64 (s, 2H), 3.30 (s, 2H), 2.94 (dd, *J* = 15.6, 3.5 Hz, 1H), 2.85 (t, *J* = 6.1 Hz, 2H), 2.59 (d, *J* = 17.1 Hz, 1H), 2.41 (s, 3H), 2.34 (dd, *J* = 13.1, 4.3 Hz, 1H), 2.01 (dd, *J* = 9.0, 3.6 Hz, 1H);^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm): 172.84, 170.99, 169.01, 167.70, 145.03, 144.37, 137.73, 134.05, 132.97, 132.64, 128.56, 125.46, 120.78, 119.40, 99.28, 60.78, 54.53, 51.55, 46.36, 42.34, 31.19, 22.52; HRMS: $[M + H]^+$ calcd. for $[C_{24}H_{24}N_8O_7 + H]^+$: 537.1902; found: 537.1846; $[M + Na]^+$ calcd. for $[C_{24}H_{24}N_8O_7 + Na]^+$: 559.1723; found: 559.1666.

Synthesis of LNDA-NBD-Sanger

Accurately weighed **Compound 3** (100.00 mg, 0.18 mmol) and K_2CO_3 (30.00 mg, 0.22 mmol) were placed into a 50 mL round-bottom flask, and 25 mL of anhydrous DMF was added to fully dissolve the components. Then, 2, 4-dinitrofluorobenzene (41.62 mg, 0.22 mmol) was added, and the reaction temperature was raised to 90 ℃. To ensure the safety and smooth progress of the reaction, it was heated under reflux in the dark under nitrogen protection. The progress of the reaction was monitored by TLC. Once the reaction was complete, heating was stopped. The DMF was removed under vacuum to obtain the crude product, which was thoroughly dried and then purified by silica gel column chromatography, yielding 39.00 mg of **LNDA-NBD-Sanger** with a yield of 30%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.01 (s, 1H), 9.59 (s, 1H), 8.92 (d, *J* = 2.6 Hz, 1H), 8.71 (dd, *J* = 8.8, 2.7 Hz, 1H), 8.54 (d, *J* = 8.8 Hz, 1H), 8.27 (d, *J* = 8.8 Hz, 1H), 7.67 (dd, *J* = 7.9, 1.1 Hz, 1H), 7.49 (dd, *J* = 7.6, 1.1 Hz, 1H), 7.43 (t, *J* = 7.6 Hz, 1H), 6.79 (d, *J* = 8.8 Hz, 1H), 5.13 (dd, *J* = 13.3, 5.1 Hz, 1H), 4.30 (d, *J* = 22.4 Hz, 4H), 3.19 (s, 2H), 2.99 (s, 2H), 2.90 (m, 1H), 2.60 (m, 1H), 2.26 (s, 3H), 2.00 (m, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm): 172.85, 171.04, 168.68, 167.69, 146.56, 146.10, 144.63, 144.00, 143.44, 142.91, 135.80, 133.52, 133.08, 132.65, 129.56, 128.55, 124.80, 121.50, 119.31, 106.26, 59.47, 53.24, 52.74, 51.48, 41.53, 31.20, 29.35, 29.03, 22.61, 22.10; HRMS: $[M + H]^+$ calcd. for $[C_{30}H_{26}N_{10}O_{11} + H]^+$: 703.2126; found: 703.1861; $[M + Na]^+$ calcd. for $[C_{30}H_{26}N_{10}O_{11} + Na]^+$: 725.1948; found: 725.1680.

Preparation of Related Solutions

Weigh 7.02 mg of **LNDA-NBD-Sanger** and 2.59 mg of **LNDA**, and dissolve each in 10 mL of DMSO to prepare a 1.0 mM stock solution. 0.5958 g HEPES was added to the volume bottle of 250 mL and proper amount of deionized water was added to dissolve the HEPES solid. Use deionized water to set the volume to the calibration line and shake well. The pH value of the solution was adjusted to 7.4 by trace NaOH solution to obtain 10 mM HEPES buffer solution.

Measurement of Absorption and Fluorescence Spectra

Mix 30 μL of the **LNDA-NBD-Sanger** solution (1.0 mM) and 2970 μL of the HEPES buffer solution (10.0 mM) in a quartz cuvette to prepare a sample with a concentration of 10.0 μM **LNDA-NBD-Sanger-HEPES**. After irradiating the sample with light at a wavelength of 400 nm, record the spectra at different time intervals using a UV-Vis spectrophotometer and a fluorescence spectrometer to monitor the photoreaction of the light-responsive molecule **LNDA-NBD-Sanger**.

HRMS and HPLC Experiments

Mix 300 μL of the **LNDA-NBD-Sanger** solution (1.0 mM) with 700 μL of 50% acetonitrile-water solution to prepare a sample with a concentration of 300 μM. After irradiating the sample with light at a wavelength of 400 nm, record the spectra at different time intervals using HRMS and high-performance liquid chromatography (HPLC) chromatography to monitor the photoreaction of the light-responsive molecule **LNDA-NBD-Sanger**. The analysis conditions for HPLC are outlined in **Table S1**.

The analytical conditions for the molecules **LNDA-NBD-Sanger** and **LNDA** are as follows: The chromatographic column used is Poroshell 120 EC-C18 (3*50 mm, 2.7 Micron) with a column temperature of 35 ℃ and an injection volume of 30 μL. The mobile phase consists of acetonitrile and water, both mixed with 1‰ formic acid.

Table S1. HPLC analysis conditions of **LNDA-NBD-Sanger**.

Time (min)	Flow rate (mL / min)	ACN (%)	H_2O (%)
0	0.5	5	95
13	0.5	100	0
20	0.5	100	0

COX-2 Inhibition Assay

Take 300 μL of **LNDA-NBD-Sanger** solution (1.0 mM) and 700 μL of deionized water to obtain a 300 μM **LNDA-NBD-Sanger** sample for testing. Irradiate the sample with light at a wavelength of 400 nm for 0 min, 5 min, 15 min, 25 min, and 30 min, respectively. Prepare the sample for testing at different irradiation times into solutions with concentrations of 0 nM, 5 nM, 10 nM, 15 nM, 30 nM, 50 nM, 80 nM, and 100 nM. Similarly, prepare eight concentrations of solutions for **LNDA** and Celecoxib as control groups according to the above method.

Set up control and sample wells in a 96-well black plate, and add 5 μL of COX-2 Probe and 5 μL of COX-2 Substrate working solution to each well, respectively. Subsequently, add the required working solutions and test samples for

the COX-2 inhibition assay in the order of **Table S2**. After mixing, incubate the plate at 37 ℃ in a light-protected environment for 5 min. Then, perform fluorescence measurement and calculate the inhibition rate of COX-2 by **LNDA-NBD-Sanger** before and after irradiation based on its fluorescence intensity at 545 nm (λ_{ex} = 470 nm).

Table S2. The sample addition sequence and dosage in the inhibition experiment of COX-2.

	Blank control group	COX-2 control group	COX-2 inhibitor control group	Sample group
COX-2 Assay Buffer	80 μL	75 μL	75 μL	75 μL
COX-2 Cofactor Solution	5 μL	5 μL	5 μL	5 μL
COX-2 Working Solution	-	5 μL	5 μL	5 μL
Sample solvent*	5 μL	5 μL	-	-
LNDA/Celecoxib	-	-	5 μL	-
Test samples	-	-	-	5 μL

Note: The sample solvent* refers to the solvent used for preparing and diluting the inhibitor to be tested.

Fluorescence Imaging Experiment of LNDA-NBD-Sanger on HeLa Cells

Digest HeLa cells in the logarithmic growth phase with trypsin solution and seed them into a confocal culture dish for 12 h of culture. Then, culture the cells in medium containing **LNDA-NBD-Sanger** (10 μM) for 20 min, and wash off the medium with PBS buffer. Under a fluorescence microscope, irradiate the culture dish with light at a wavelength of 400 nm for different durations and observe and image the cells.

LNDA-NBD-Sanger Cytotoxicity Assay on HeLa Cells

Mix 300 μL of **LNDA-NBD-Sanger** solution (1.0 mM) with 700 μL of deionized water to obtain a 300 μM **LNDA-NBD-Sanger** sample for testing. Irradiate the sample with light at a wavelength of 400 nm for 0 min, 5 min, 15 min, 25 min, and 30 min, respectively. Prepare **LNDA-NBD-Sanger** samples at different irradiation times into solutions with concentrations of 0 μM, 60 μM, 150 μM, and 300 μM. Similarly, prepare **LNDA** solutions at concentrations of 0 μM, 60 μM, 150 μM, and 300 μM from a 1.0 mM **LNDA** solution for backup. Product III was purified by HPLC, and 1.2 mg of Product III was weighed and dissolved in 3 mL of DMSO to prepare a 1.0 mM stock solution. Subsequently, the product III solution was diluted to concentrations of 0 μM, 60 μM, 150 μM, and 300 μM for further use.

Seed HeLa cells in a 96-well plate and culture at 37 ℃ for 24 h. Then, add **LNDA-NBD-Sanger** and **LNDA** solutions

(0 μM, 60 μM, 150 μM, 300 μM) that have been irradiated for different times and continue to culture at 37 ℃ for another 24 h. Afterward, add MTT solution (5 mg/mL) to each well and continue to culture for 4 - 6 h. Measure the optical density of the solution in each well at a wavelength of 490 nm using an enzyme-linked immunosorbent assay reader, and calculate the cell survival rate. Similarly, product III was subjected to the same experimental procedure to determine its cytotoxicity. All experiments were conducted at least three times.

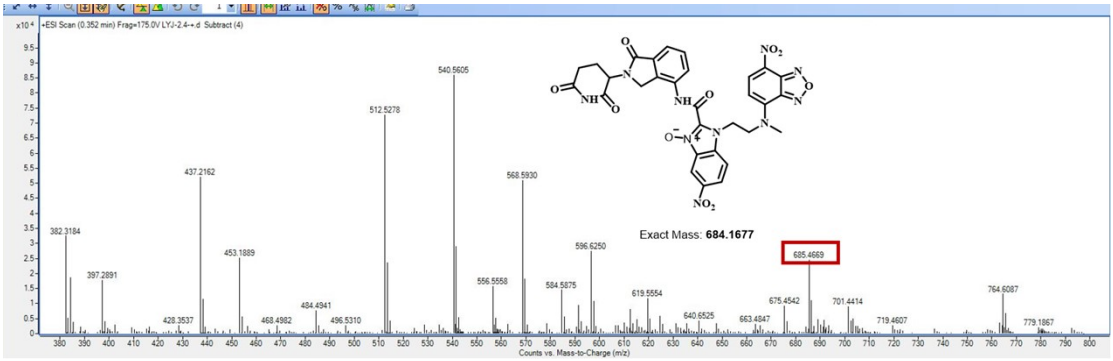


Fig. S1 HRMS Analysis of **Product I** in HPLC.

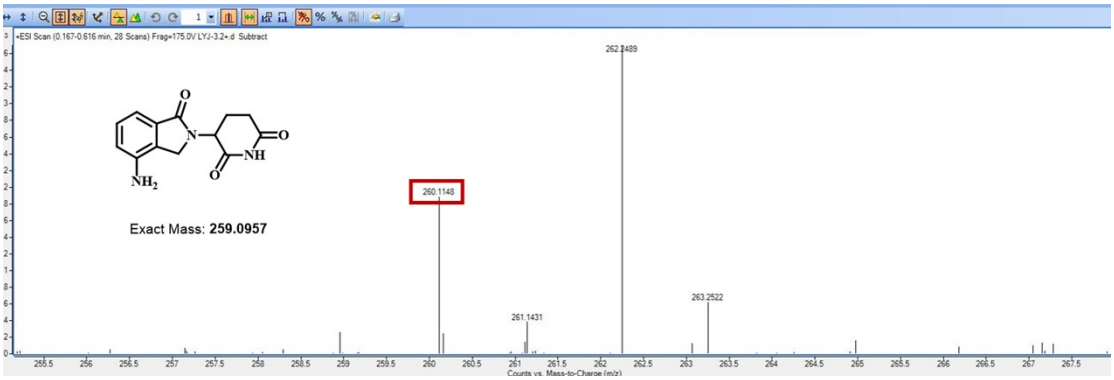


Fig. S2 HRMS Analysis of **Product II (LNDA)** in HPLC.

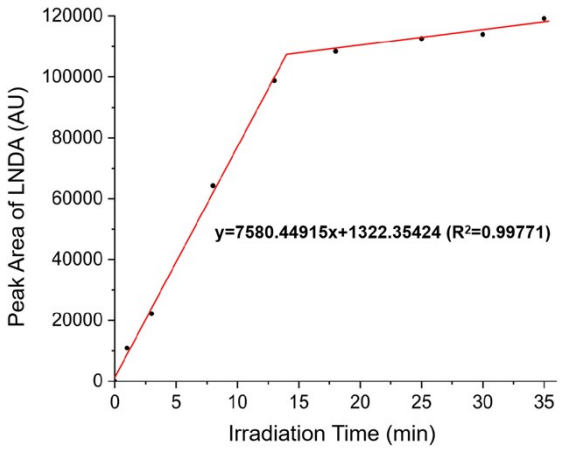


Fig. S3 Time-dependent release of **LNDA** from **LNDA-NBD-Sanger** under 400 nm UV irradiation.

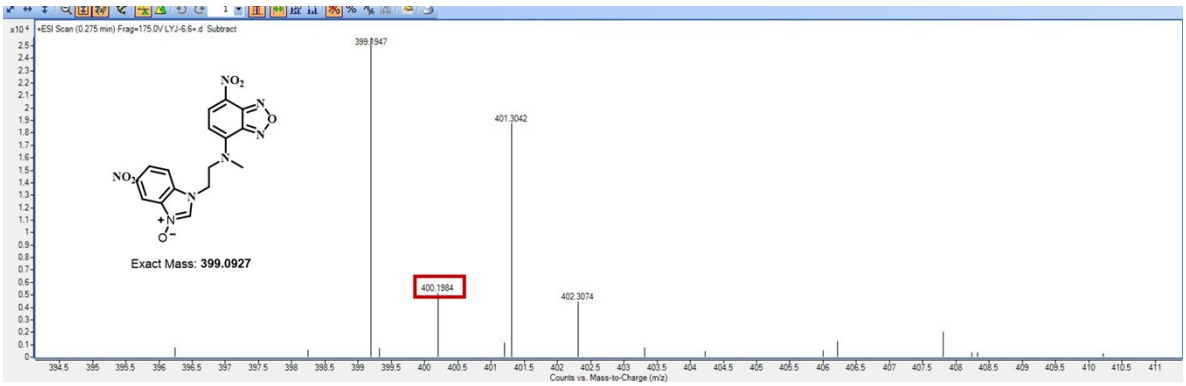


Fig. S4 HRMS Analysis of **Product III** in HPLC.

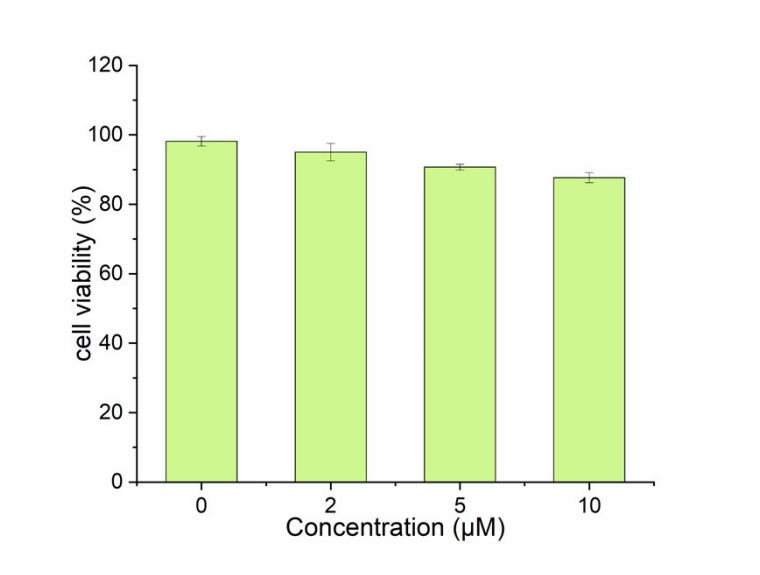


Fig. S5 Cell cytotoxicity assay of **product III** in HeLa cells. Data represent the mean ± SD (n = 3).

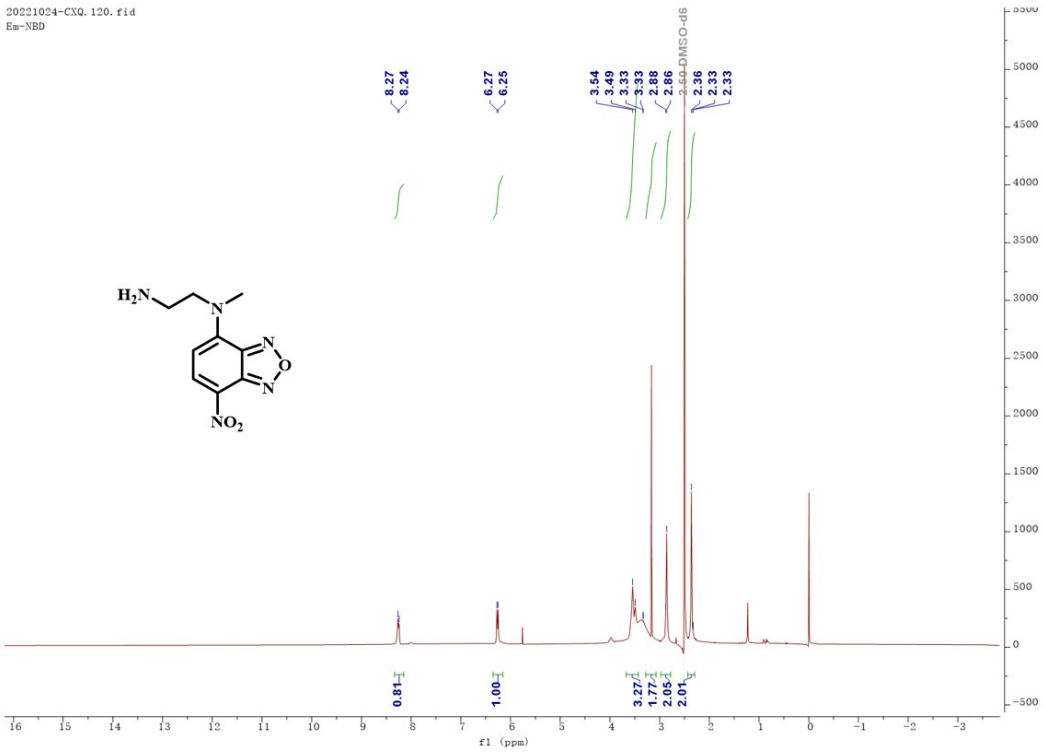


Fig. S6 ^{1}H NMR (400 MHz) spectrum of **Compound 1** in DMSO-d_6.

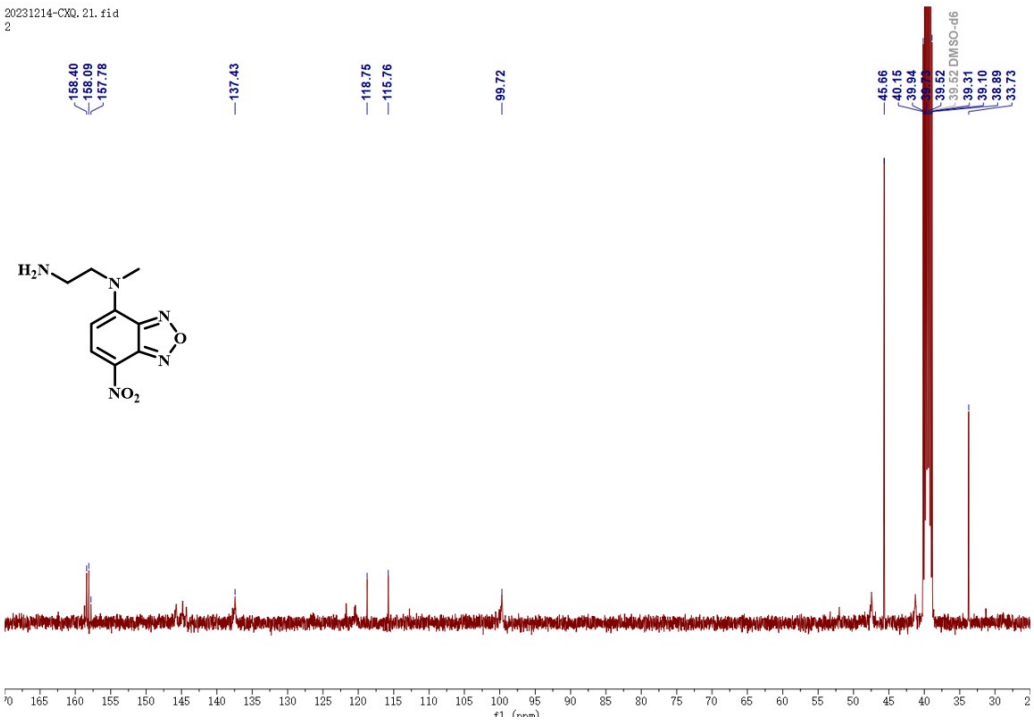


Fig. S7 ^{13}C NMR (400 MHz) spectrum of **Compound 1** in DMSO-d_6.

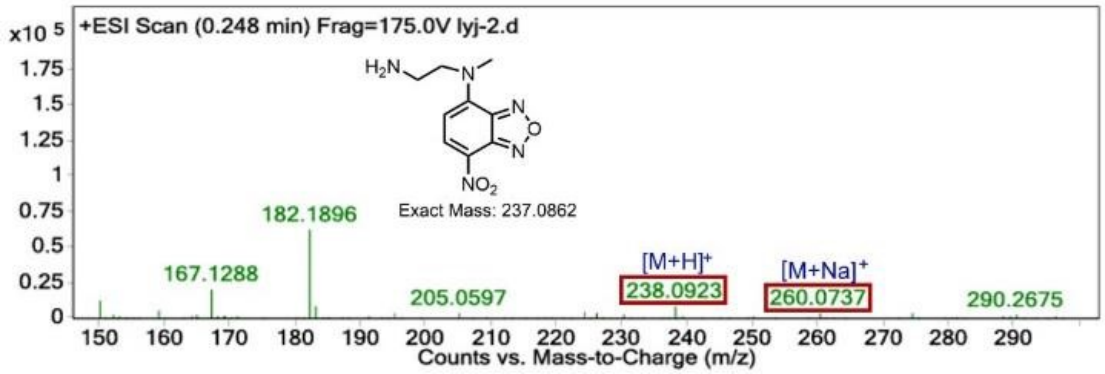


Fig. S8 HRMS of **Compound 1**: 238.0923 $[M + H]^+$, 260.0737 $[M + Na]^+$.

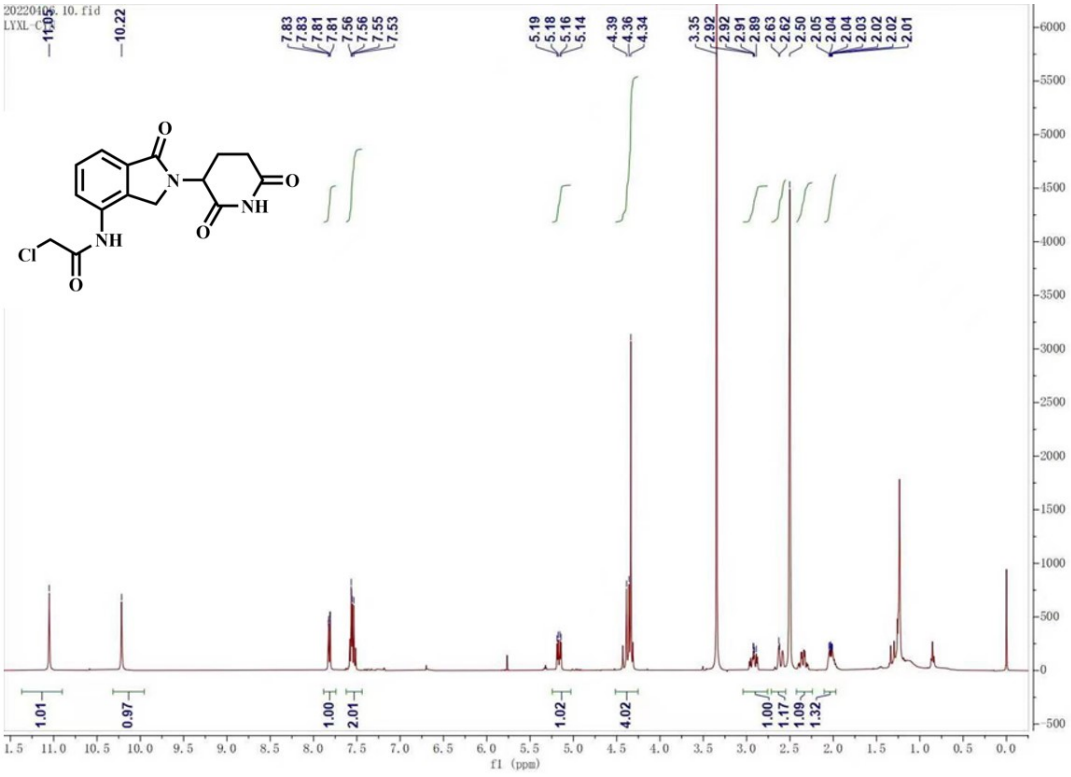


Fig. S9 ^{1}H NMR (400 MHz) spectrum of **Compound 2** in DMSO-d_6.

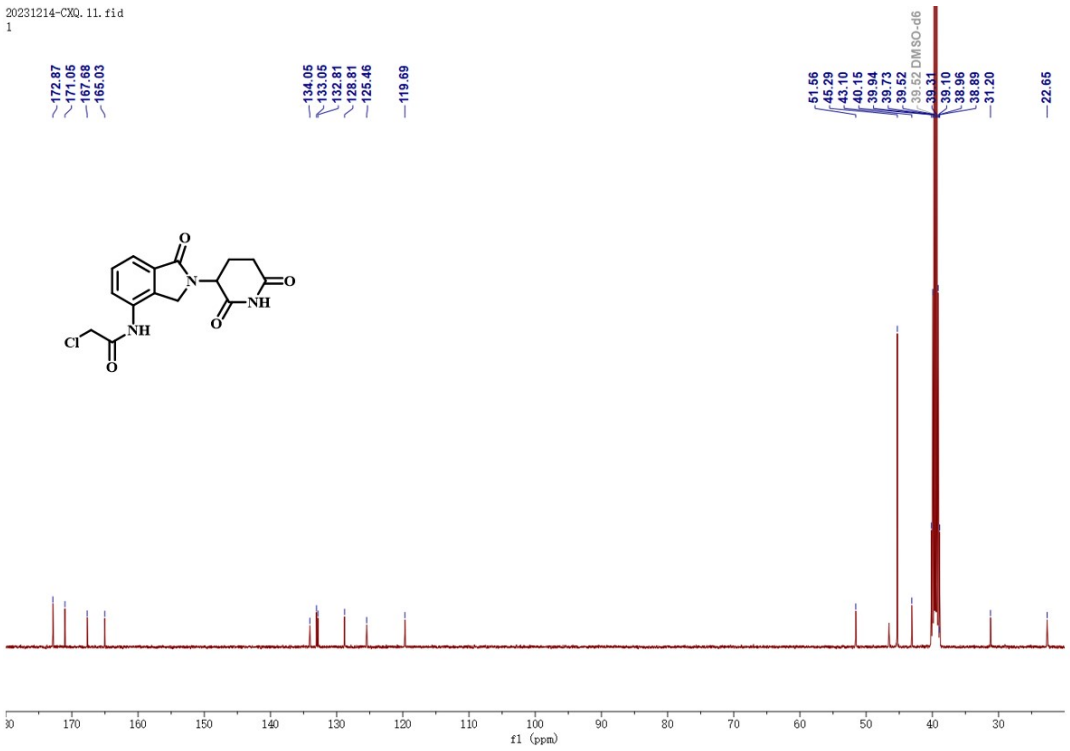


Fig. S10 ^{13}C NMR (400 MHz) spectrum of **Compound 2** in DMSO-d_6.

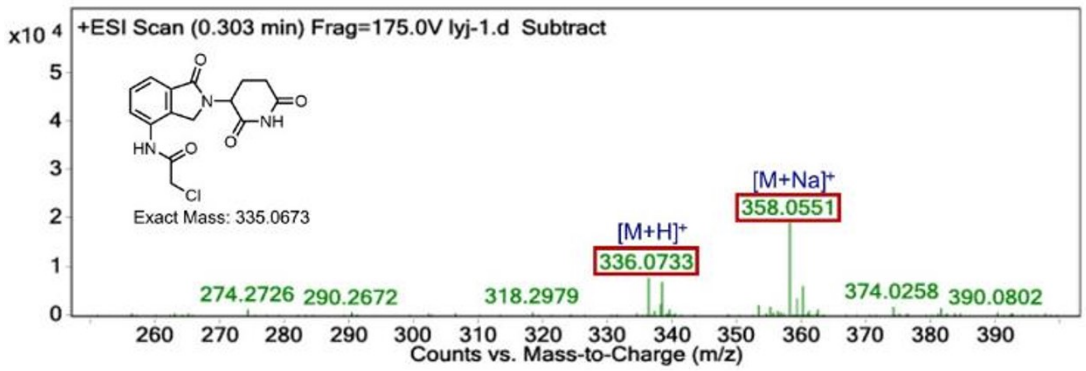


Fig. S11 HRMS of **Compound 2**: 336.0733 $[M + H]^+$, 358.0551 $[M + Na]^+$.

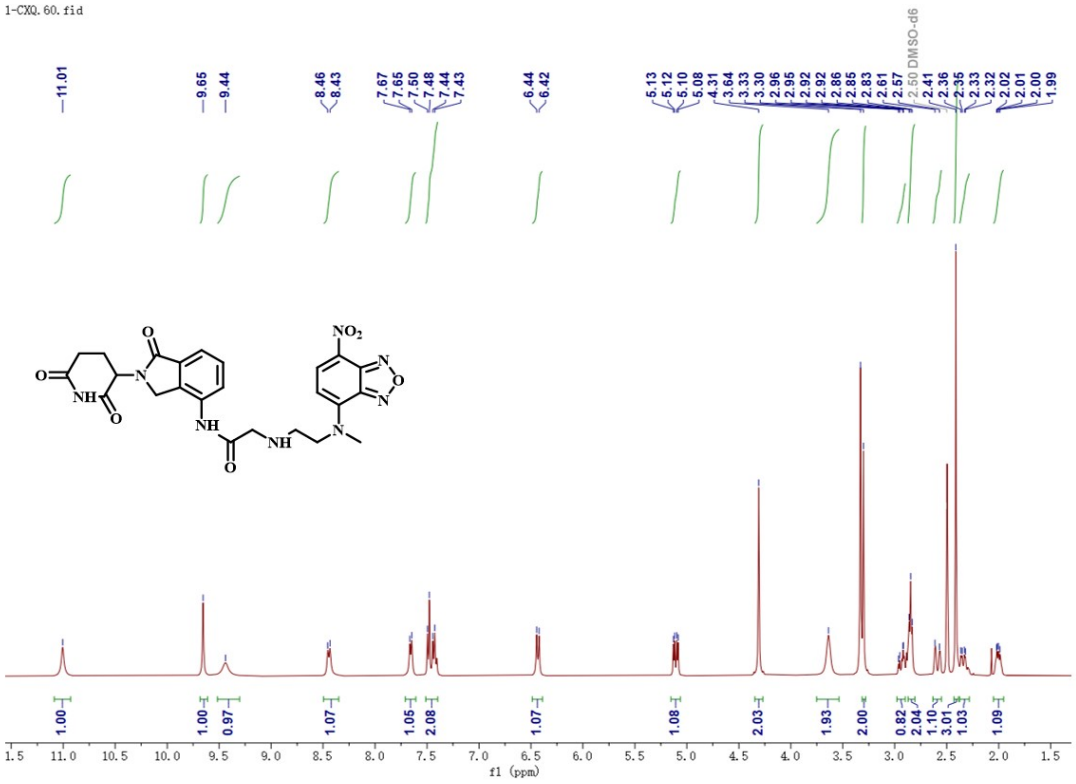


Fig. S12 ^{1}H NMR (400 MHz) spectrum of **Compound 3** in DMSO-d_6.

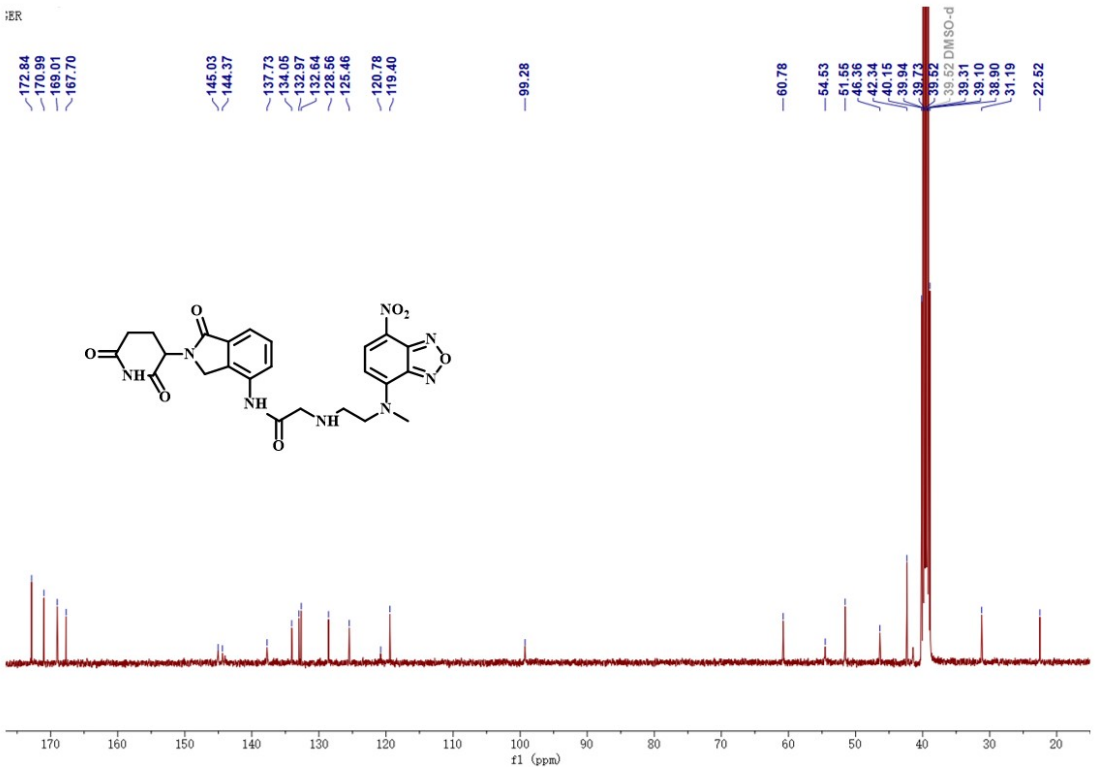


Fig. S13 ^{13}C NMR (400 MHz) spectrum of **Compound 3** in DMSO-d_6.

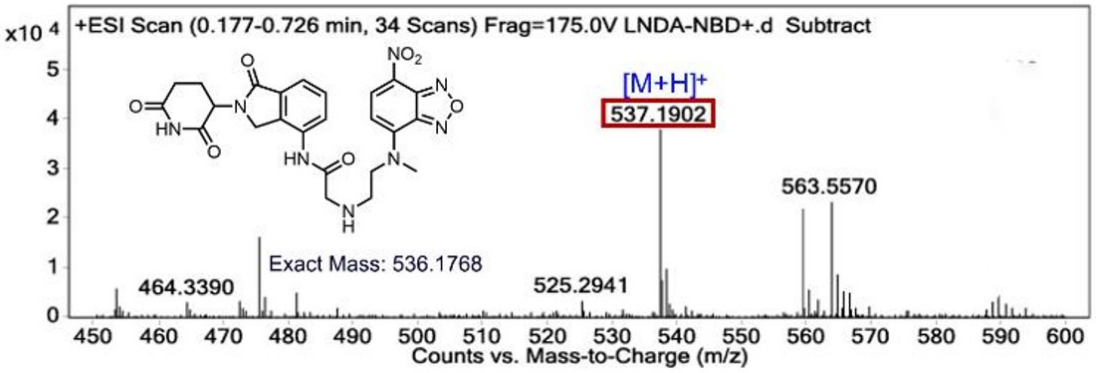


Fig. S14 HRMS of **Compound 3**: 537.1902 $[M + H]^+$.

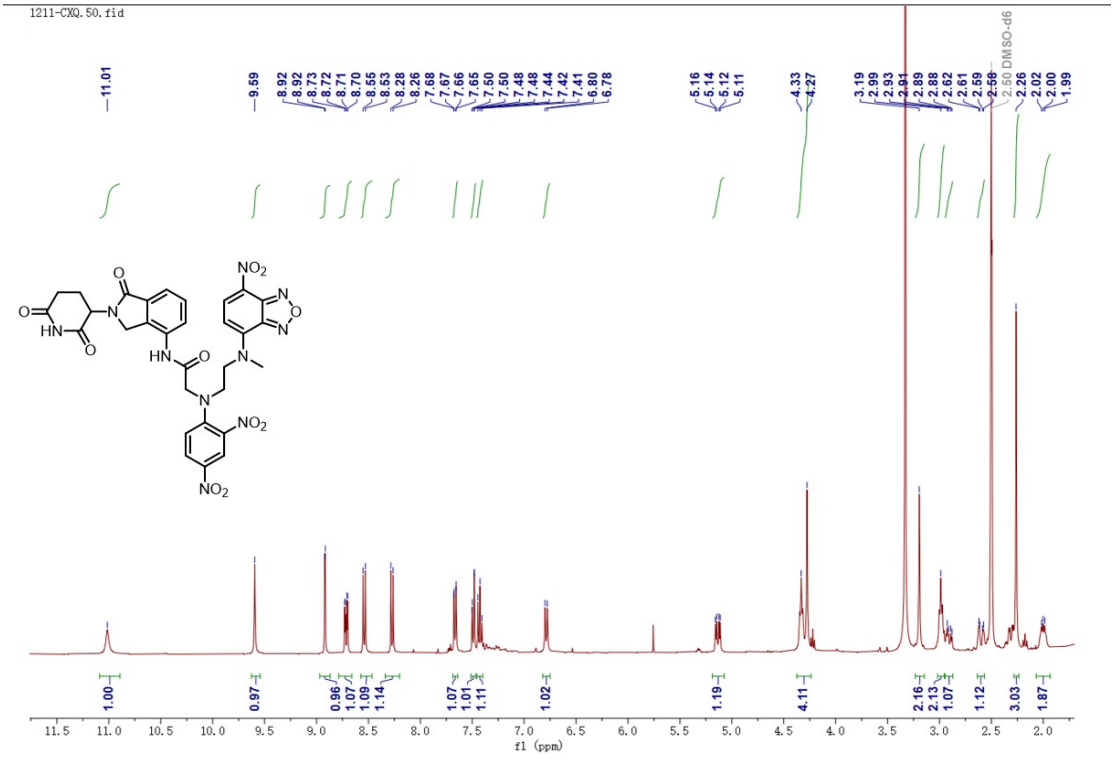


Fig. S15 ^{1}H NMR (400 MHz) spectrum of **LNDA-NBD-Sanger** in DMSO-d_6.

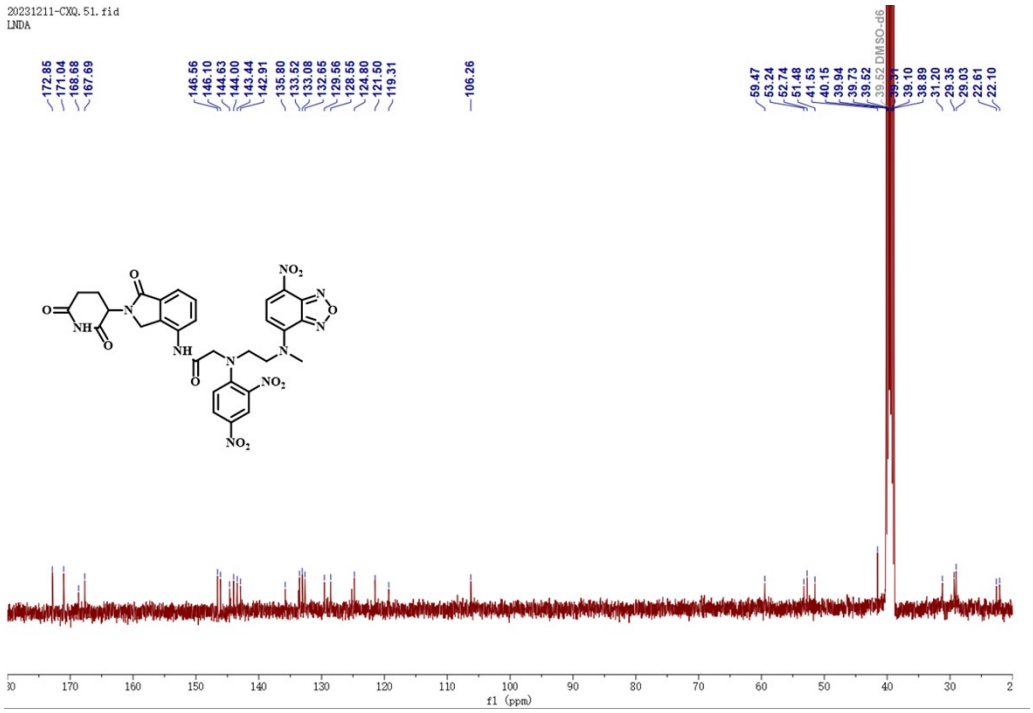


Fig. S16 ^{13}C NMR (400 MHz) spectrum of **LNDA-NBD-Sanger** in DMSO-d_6.

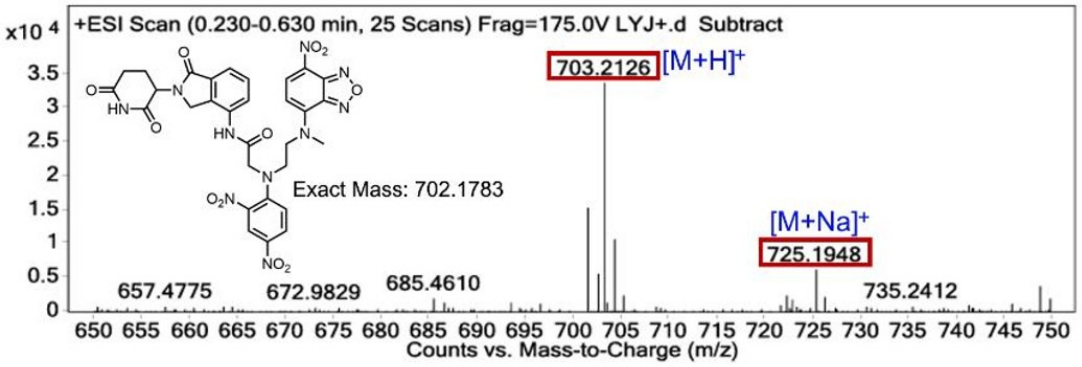
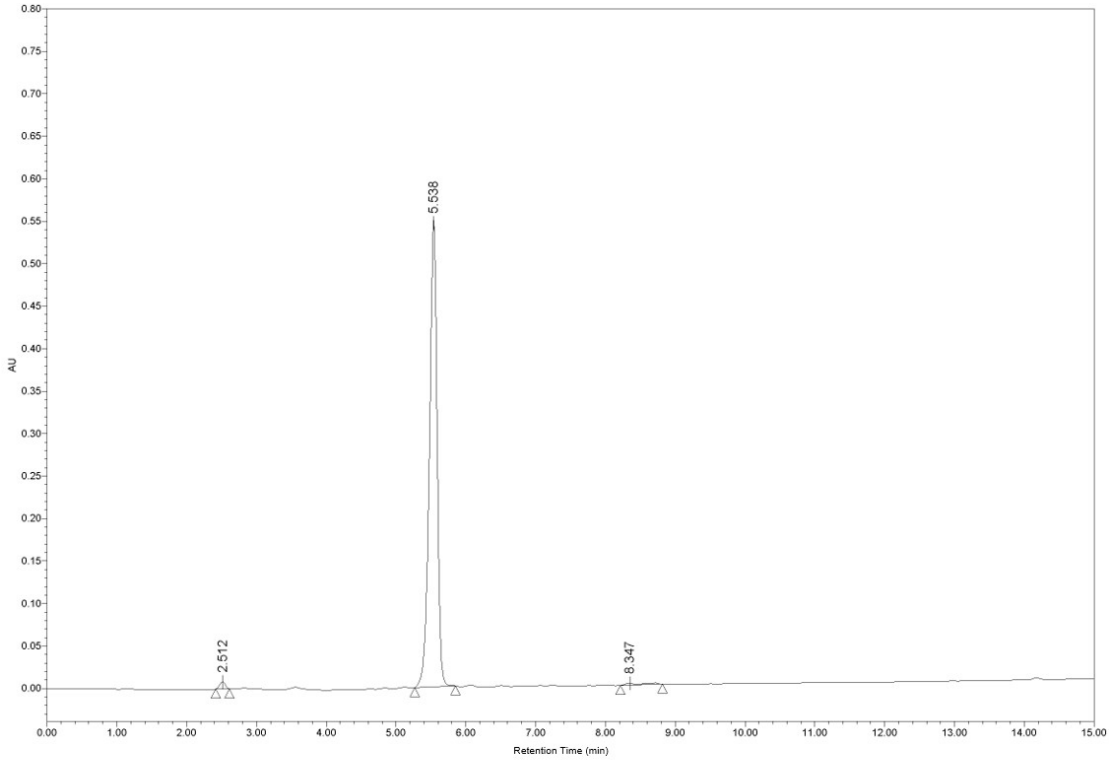


Fig. S17 HRMS of **LNDA-NBD-Sanger**: 703.2126 $[M + H]^+$, 725.1948 $[M + Na]^+$.



	Name	Retention Time (min)	Area (μV·s)	% Area	Integration Type
1		2.512	48068	1.105	bb
2		5.538	4258661	97.898	bb
3		8.347	43382	0.997	bb

Fig. S18 Analysis of the HPLC peak of **LNDA-NBD-Sanger.**