Supporting Information

Design and Synthesis of Piperine-based Photoaffinity Probes for Revealing Potential Targets of Piperine in Neurological Disease

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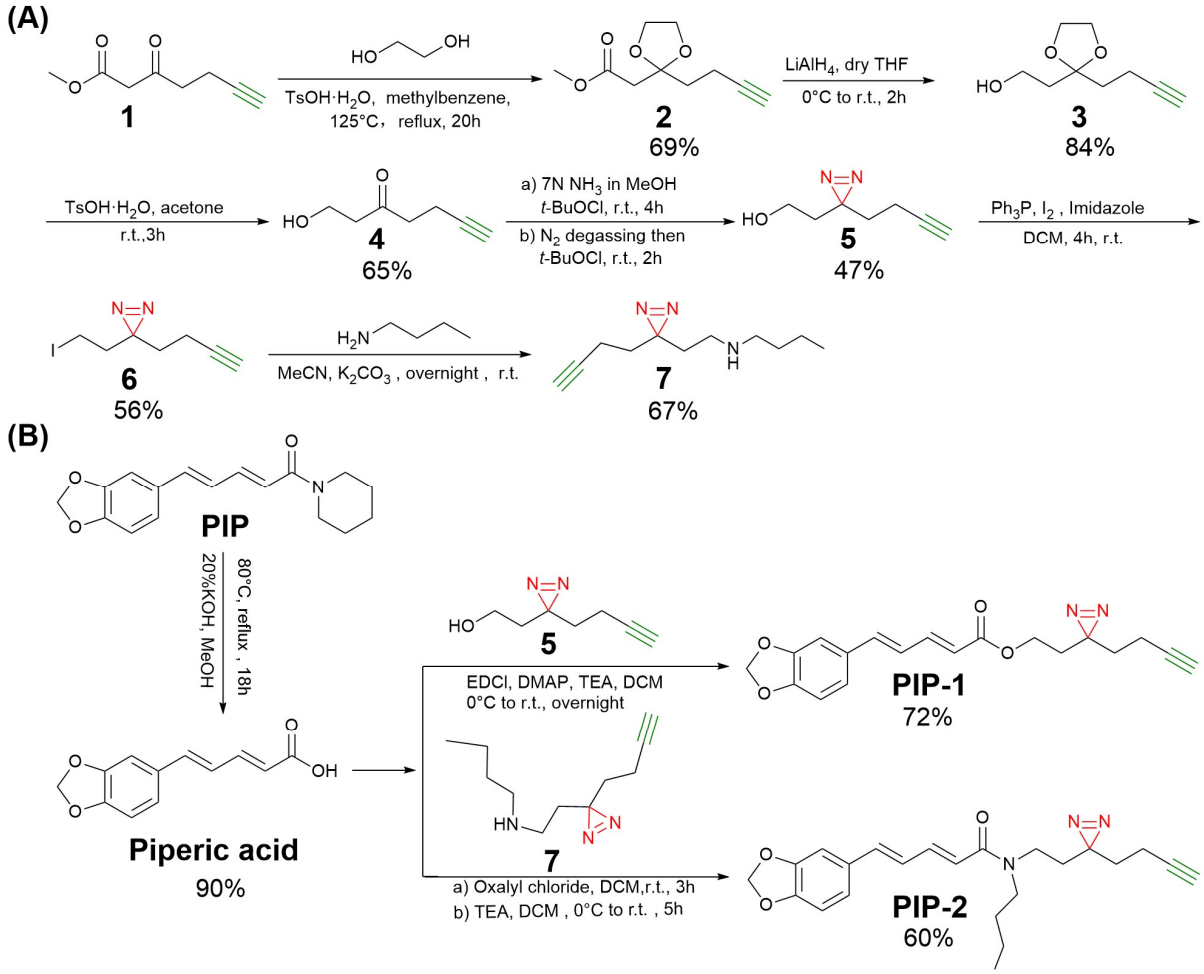
I. General Information

Materials and methods

All small-molecule reactions were performed in oven-dried glassware unless otherwise specified. Analytical thin-layer chromatography was performed using 0.25 mm silica gel 60-F plates and visualized under UV light (254nm) or by staining with a solution of potassium permanganate. Flash chromatography was performed using 200-400 mesh silica gel (Qingdao Ocean Chemical Co., Ltd., Qingdao, China). All reagents were purchased from Bidepharm (Shanghai, China), Di bai (Shanghai, China), Ze sheng (Anhui, China), Macklin (Shanghai, China), ACMEC (Shanghai, China), or Boer (Shanghai, China) without further purification.

^{1}H nuclear magnetic resonance (NMR) spectra (400 MHz) and ^{13}C NMR (400 MHz) were recorded on a Bruker 500/600 MHz NMR instrument with tetramethylsilane used as an internal standard. NMR spectra were recorded at ambient temperature with $CDCl_3$ as the solvent unless otherwise specified. High-resolution ESI mass spectrometry (ESI-HRMS) data were measured using an Agilent 6520B Q-TOF mass spectrometer (Agilent, Inc., USA).

II. Preparation of Compound



Scheme S1. Synthetic route to PIP probes.

Synthesis of methyl 2-(2-(but-3-yn-1-yl)-1,3-dioxolan-2-yl)acetate (2)[1]

Methyl 3-oxohept-6-ynoate (1 mmol, 154.2 mg), TsOH · H_2O (0.05 mmol, 9.5 mg), toluene (10 mL), and ethylene glycol (2 mmol, 124.1 mg) were placed in a 50 mL round flask. The mixture was refluxed at 125 °C for 10 hours. After the reaction, toluene was removed by vacuum decompression. Then, saturated aqueous $NaHCO_3$ (10 mL) was added, and the mixture was extracted with ethyl acetate (3 × 10 mL). The combined organic layers were washed with brine (30 mL), dried over Na_2SO_4, and concentrated under vacuum. The target material was purified by silica gel column chromatography(petroleum ether: ethyl acetate, 20:1) to afford **2**(136.8 mg) as colorless oil, yield: 69%.^{1}H NMR (400 MHz, $CDCl_3$) δ 3.98 – 3.89 (m, 4H),

3.64 (s, 3H), 2.61 (s, 2H), 2.27 – 2.21 (m, 2H), 2.09 – 2.03 (m, 2H), 1.89 (t, J = 2.8 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.68, 108.24, 83.91, 68.22, 65.25, 51.87, 42.52, 36.35, 12.78.

Synthesis of 2-(2-(But-3-yn-1-yl)-1,3-dioxolan-2-yl)ethanol (3)[1]

Compound **2** (198.2 mg, 1 mmol) was added to a mixture of $LiAlH_4$ (56.9 mg, 1.5 mmol) in dry THF (2 mL) at 0 °C. The reaction mixture was stirred for 2 hours at room temperature and then slowly quenched with water (20 mL). The organic layer was separated, and the water layer was extracted with ethyl acetate (3 × 10 mL). The combined organic layers were washed with brine (30 mL), dried over Na_2SO_4, and concentrated under vacuum. The resulting material was purified by silica gel column chromatography (petroleum ether: ethyl acetate, 4:1) to afford **3**(143 mg) as colorless oil, yield: 84%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.99 – 3.90 (m, 4H), 3.72 – 3.64 (m, 2H), 2.61 (s, 1H), 2.27 – 2.16 (m, 2H), 1.95 – 1.81 (m, 5H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 110.99, 84.00, 68.34, 64.97, 58.64, 38.32, 35.93, 13.15.

Synthesis of 1-Hydroxyhept-6-yn-3-one (4)[1]

Compound **3** (170.2 mg, 1 mmol) was dissolved in acetone (2 mL). P-toluenesulfonic acid monohydrate (47.6 mg, 0.25 mmol) was added to the solution, and the resulting mixture was stirred at room temperature for 3 hours. A saturated aqueous solution of $NaHCO_3$ (10 mL) was added to the reaction mixture, which was then extracted with ethyl acetate (3 × 10 mL). The combined organic layers were washed with brine (30 mL), dried over Na_2SO_4, and concentrated under vacuum. The target material was purified by silica gel column chromatography (petroleum ether: ethyl acetate, 1:1) to afford **4**(87 mg) as pale yellow oil, yield: 65%.^{1}H NMR (400 MHz, $CDCl_3$) δ 3.80 (t, J = 5.6 Hz, 2H), 2.65 (m, 5H), 2.40 (td, J = 7.6, 2.8 Hz, 2H), 1.92 (t, J = 2.4 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.63, 108.19, 83.87, 68.18, 65.20, 51.82, 42.48, 36.30, 12.73.

Synthesis of 2-(3-(But-3-yn-1-yl)-3H-diazirin-3-yl)ethanol (5)[2]

Compound **4** (126.2 mg, 1 mmol) in a 7 N solution of NH_3 (10 mmol, 1.4mL) in MeOH (1 mL) was prepared in a dried round-bottom flask equipped with a rubber septum. To this solution, a solution of t-butyl hypochlorite (340 μL, 3 mmol) in t-butanol (1.5 mL) was carefully added. The resulting mixture was stirred for 4 h at room temperature. Subsequently, the excess NH_3 was removed by N_2 degassing for 20 minutes before adding the second portion of t-butyl hypochlorite (170 μL, 1.5 mmol) in t-butanol (1 mL). The mixture was stirred for 2 h at room temperature and then concentrated under reduced pressure. The crude product was diluted with a solution of saturated aqueous $Na_2S_2O_3$ (5 mL) and extracted with ethyl acetate (3 × 10 mL). The combined organic layers were dried over Na_2SO_4 and evaporated under vacuum. Finally, the crude material was purified by silica gel column chromatography (petroleum ether: ethyl acetate, 4:1) to afford **5**(64.9 mg) as yellow oil, yield: 47%. ^{1}H NMR (400 MHz, CDCl3) δ 3.45 (t, J = 6.4 Hz, 2H), 2.10 (brs, 1H), 2.04 – 1.96 (m, 3H), 1.67 (m, 4H). ^{13}C NMR (101 MHz, CDCl3) δ 169.63, 108.19, 83.87, 68.18, 65.20, 51.82, 42.48, 36.30, 12.73.

Synthesis of 3-(but-3-yn-1-yl)-3-(2-iodoethyl)-3H-diazirine (6)[3]

Iodine (305 mg, 1.2 mmol) was added to a solution containing Ph_3P (315 mg, 1.2 mmol) and imidazole (163 mg, 2.4 mmol) in dichloromethane (5 mL) at 0 °C. After stirring for 15 minutes, compound **5** (138 mg, 1.0 mmol) in dichloromethane (1 mL) was added, and the mixture was stirred for 4 hours at room temperature. Then, water (10 mL) was added to the reaction mixture, and the organic layer was separated. The aqueous layer was extracted with dichloromethane (3 × 10 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated. Finally, the crude material was purified by silica gel column chromatography (petroleum ether: ethyl acetate, 20:1) to afford **6**(138.9 mg) as yellow oil, yield: 56%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.89 (t, J = 7.6 Hz, 2H), 2.12 (t, J = 7.6 Hz, 2H), 2.06 – 1.98 (m, 3H),

1.68 (t, J = 7.2 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 82.41, 69.42, 37.47, 31.77, 28.60, 13.23, -3.99.

Synthesis of N-(2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)ethyl)butan-1-aminee (7)[4]

K_2CO_3 (207 mg, 1.5 mmol) was added to a solution of n-butylamine (109.7 mg, 1.5 mmol) in MeCN (3 mL). The resulting suspension was stirred for 10 minutes. Then, compound **6** (248 mg, 1 mmol) was added, and stirring was continued overnight. Afterward, the reaction mixture was filtered to remove undissolved solids and washed multiple times with ethyl acetate. The filtrate was further washed with brine, dried over Na_2SO_4, and evaporated. Finally, the crude material was purified by silica gel column chromatography (petroleum ether: ethyl acetate, 2:1) to afford **7**(234.4 mg) as yellow oil, yield: 72%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.51 (t, J = 7.2 Hz, 2H), 2.36 (t, J = 7.2 Hz, 2H), 2.04 – 1.94 (m, 3H), 1.63 (t, J = 7.2 Hz, 4H), 1.42 (m, 2H), 1.31 (m, 2H), 1.16 (brs, 1H), 0.89 (t, J = 7.6 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 84.56, 68.37, 49.82, 49.70, 32.05, 29.42, 28.42, 26.56, 20.59, 18.44, 14.09. ESI-HRMS found: m/z 194.1652.

Synthesis of piperic acid[5]

Piperine (7 mmoL) was added to a 20% KOH methanol solution (100 mL). The mixture underwent reflux hydrolysis at 80 °C for 18 h, after which methanol was removed by vacuum decompression. Water was added to fully dissolve the solid. Insoluble impurities were eliminated through suction filtration, and the resulting filtrate was collected. The pH value was adjusted to less than 1 by adding HCl. The aqueous solution was then extracted with ethyl acetate, dried with Na_2SO_4, and the solvent was removed under vacuum. Obtained piperic acid (1.3734 g) as yellow solid, yield: 90%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.15 (s, 1H), 7.31 (ddd, J = 15.2, 6.0, 4.2 Hz, 1H), 7.23 (d, J = 1.5 Hz, 1H), 7.03 – 6.94 (m, 3H), 6.92 (dd, J = 8.0, 3.6 Hz, 1H), 6.05 (s, 2H), 5.93 (dd, J = 15.2, 3.6 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ

167.54, 148.06, 147.95, 144.52, 139.71, 130.49, 124.81, 122.99, 121.10, 108.45, 105.69, 101.31.

Synthesis of PIP-1[6]

Compound **5** (157.7 mg, 1.14 mmol) and EDCI (327.8 mg, 1.71 mmol) were dissolved in 10 mL of dichloromethane (DCM) in the dark. Then, DMAP (27.9 mg, 0.228 mmol) and TEA (240 μL, 1.71 mmol) were added. The solution was cooled to 0 °C and stirred for 1 hour. After that, piperic acid (199.5 mg, 0.914 mmol) was added and the cooling bath was removed. The mixture was stirred overnight. The solvent was then removed under reduced pressure, and the residue was purified by by silica gel column chromatography (petroleum ether: ethyl acetate, 20:1) to obtain PIP-1(138.9 mg) as yellow oil, yield: 67%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 (dd, J = 15.2, 10.8 Hz, 1H), 6.98 (d, J = 1.6 Hz, 1H), 6.90 (dd, J = 8.0, 1.6 Hz, 1H), 6.84 – 6.76 (m, 2H), 6.69 (dd, J = 15.6, 10.8 Hz, 1H), 5.97 (s, 2H), 5.94 (d, J = 15.2 Hz, 1H), 4.06 (t, J = 6.4 Hz, 2H), 2.04 (td, J = 7.6, 2.8 Hz, 2H), 1.99 (t, J = 2.4 Hz, 1H), 1.78 (t, J = 6.4 Hz, 2H), 1.69 (t, J = 7.4 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.82, 148.75, 148.42, 145.45, 140.66, 130.63, 124.55, 123.10, 119.89, 108.64, 106.02, 101.50, 82.72, 69.42, 59.07, 32.58, 32.44, 26.50, 13.36. ESI-HRMS found m/z 339.1342.

Synthesis of PIP-2[5]

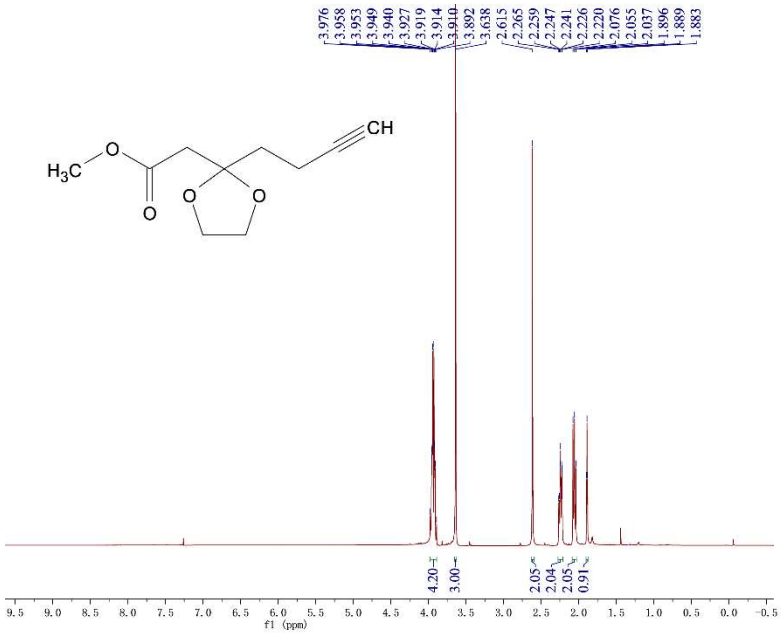
Oxalyl chloride (195 μL, 2.29 mmol) was added to a mixture of piperic acid (50 mg, 0.229 mmol) in CH_2Cl_2 (5 mL), and the mixture was stirred at room temperature for 3 hours. The solvent and excess oxalyl chloride were then evaporated under reduced pressure. The crude acid chloride generated was dissolved in CH_2Cl_2 (2 mL) and added dropwise to a mixture of compound **7** (53.2 mg, 0.275 mmol) and Et_3N (255 μL, 1.328 mmol) in CH_2Cl_2 at 0 °C. The reaction mixture was stirred for 5 hours at room temperature. Ice-water was added to the mixture, which was subsequently extracted with dichloromethane (3 × 10 mL). The organic layer was dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. Finally,

the crude material was purified by silica gel column chromatography (petroleum ether: ethyl acetate, 6:1) to obtain PIP-2(67.7 mg) as yellow oil, yield: 75%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 (dd, J = 14.8, 8.8 Hz, 1H), 6.97 (s, 1H), 6.88 (d, J = 8.0 Hz, 1H), 6.78 – 6.72 (m, 3H), 6.30 (d, J = 14.8 Hz, 1H), 5.95 (s, 2H), 3.32 – 3.23 (m, 4H), 2.06 – 1.96 (m, 3H), 1.69-1.48 (m, 6H), 1.31 (dq, J = 14.4, 7.2 Hz, 2H), 0.93 (t, J = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.58, 148.33, 143.05, 139.06, 130.99, 125.25, 122.79, 119.70, 108.61, 105.81, 101.43, 82.86, 69.31, 48.38, 42.13, 32.20, 31.99, 31.47, 29.81, 27.04, 20.15, 13.94, 13.48. ESI-HRMS found m/z 394.2122.

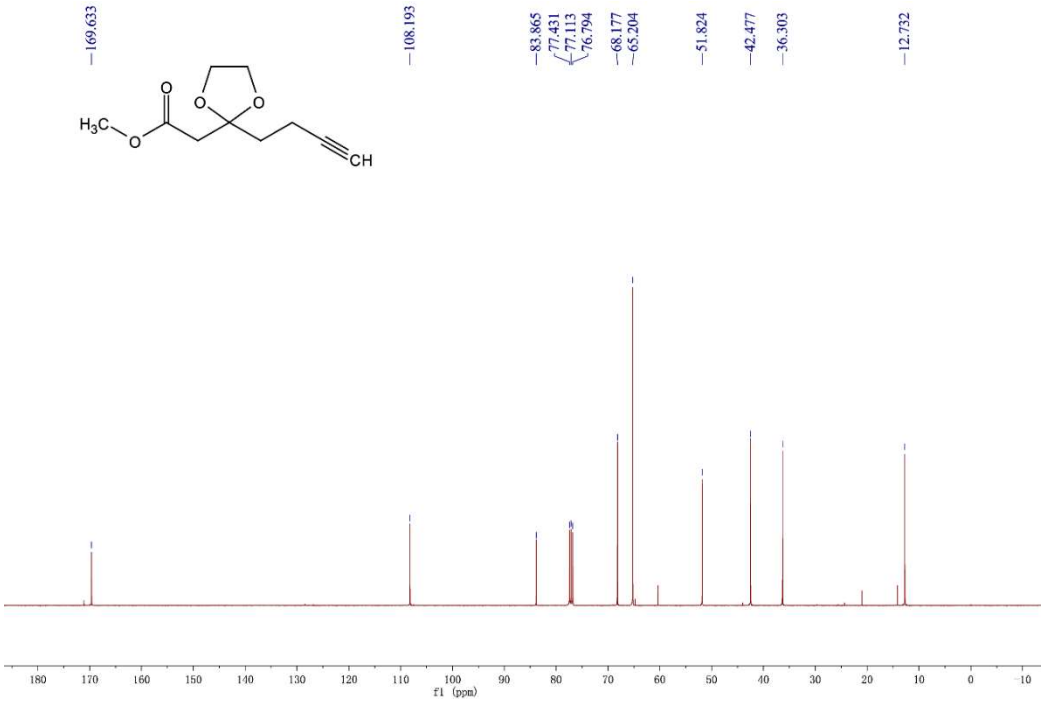
III. Characterization of Compound

Compound NMR spectra

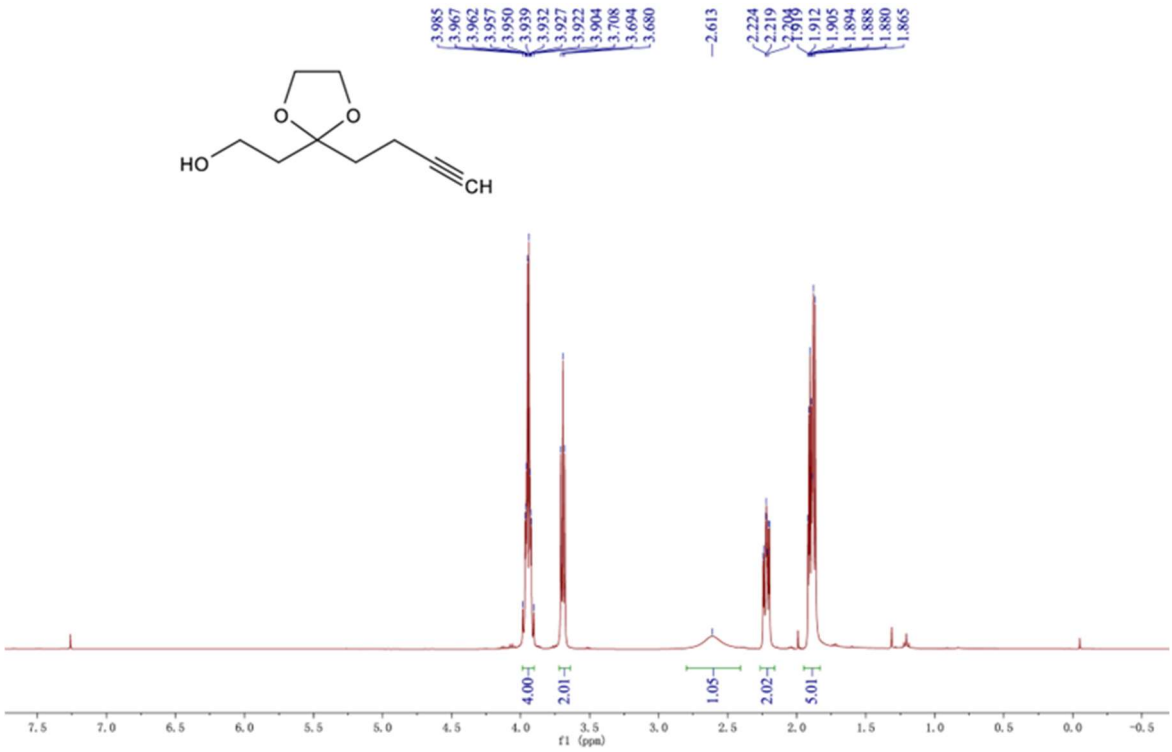
Compound 2 – ^{1}H NMR Spectrum - $CDCl_3$,400 MHz



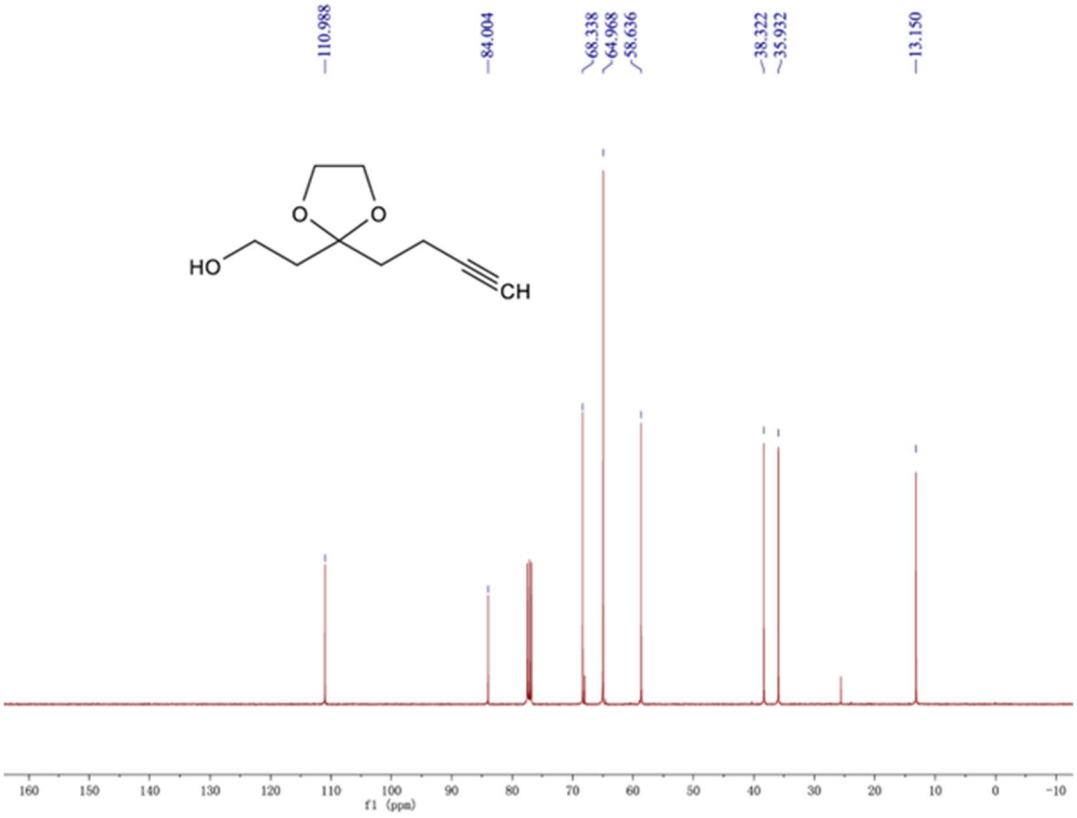
Compound 2 – ^{13}C NMR Spectrum - $CDCl_3$,400 MHz



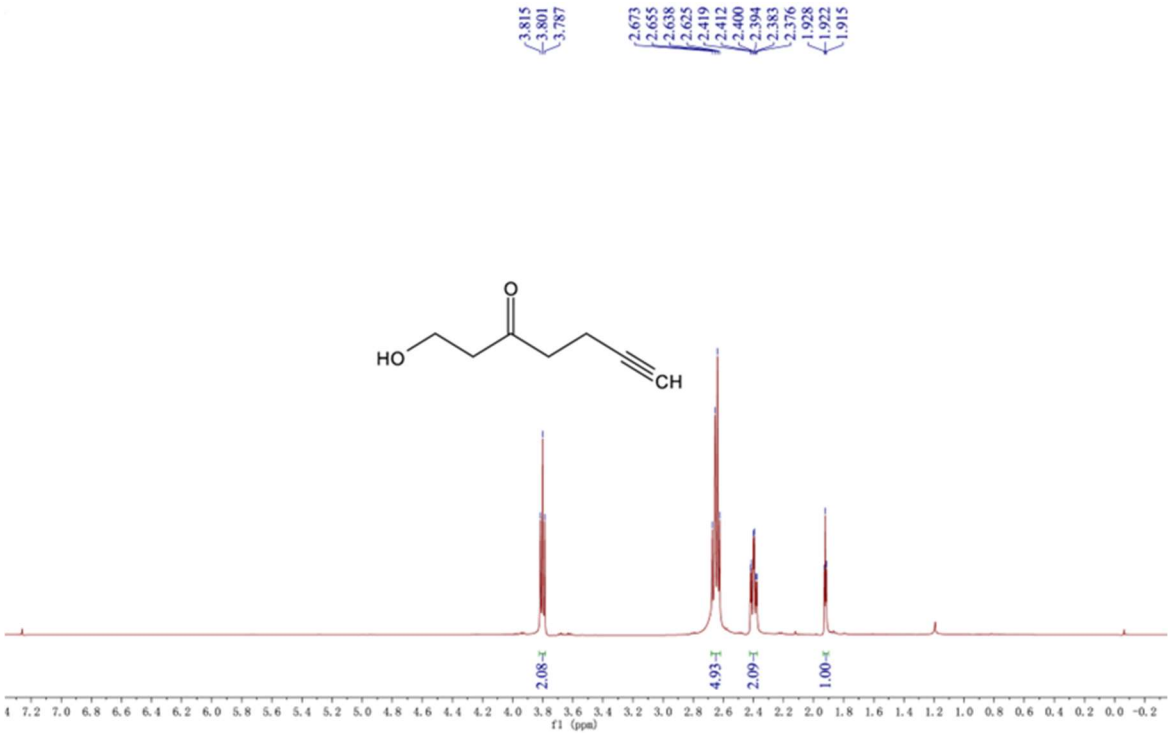
Compound 3 – ^{1}H NMR Spectrum - $CDCl_3$,400 MHz



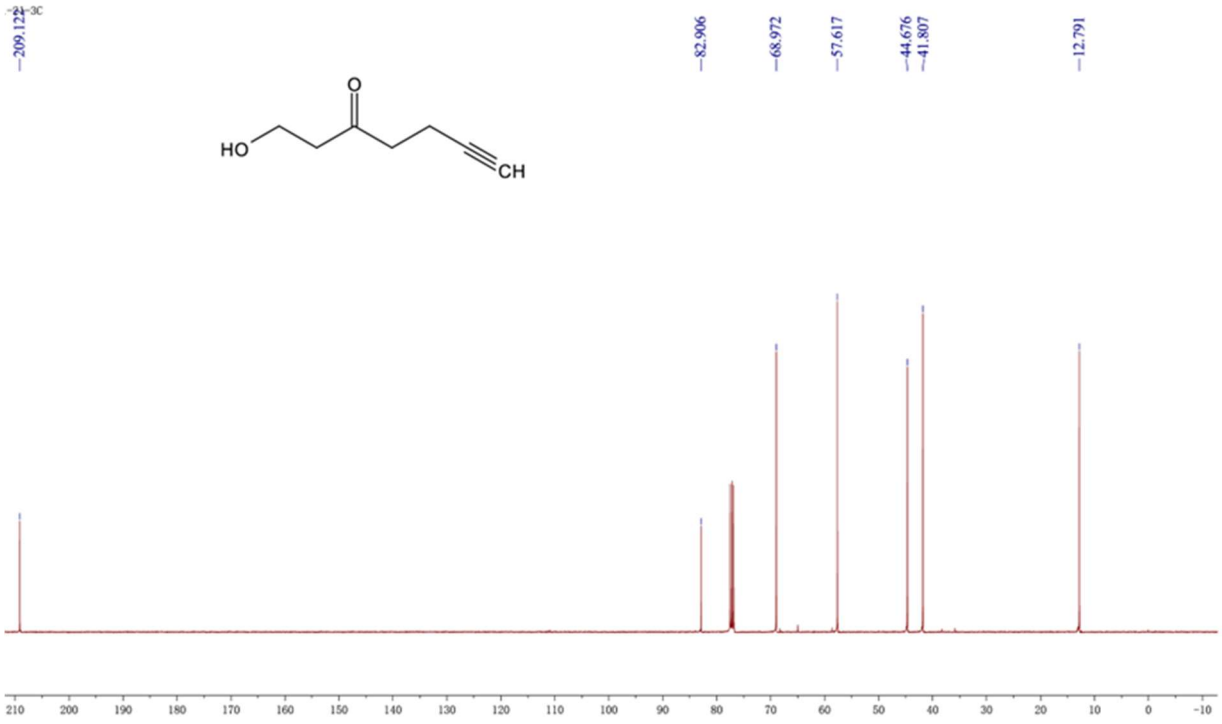
Compound 3 – ^{13}C NMR Spectrum - $CDCl_3$,400 MHz



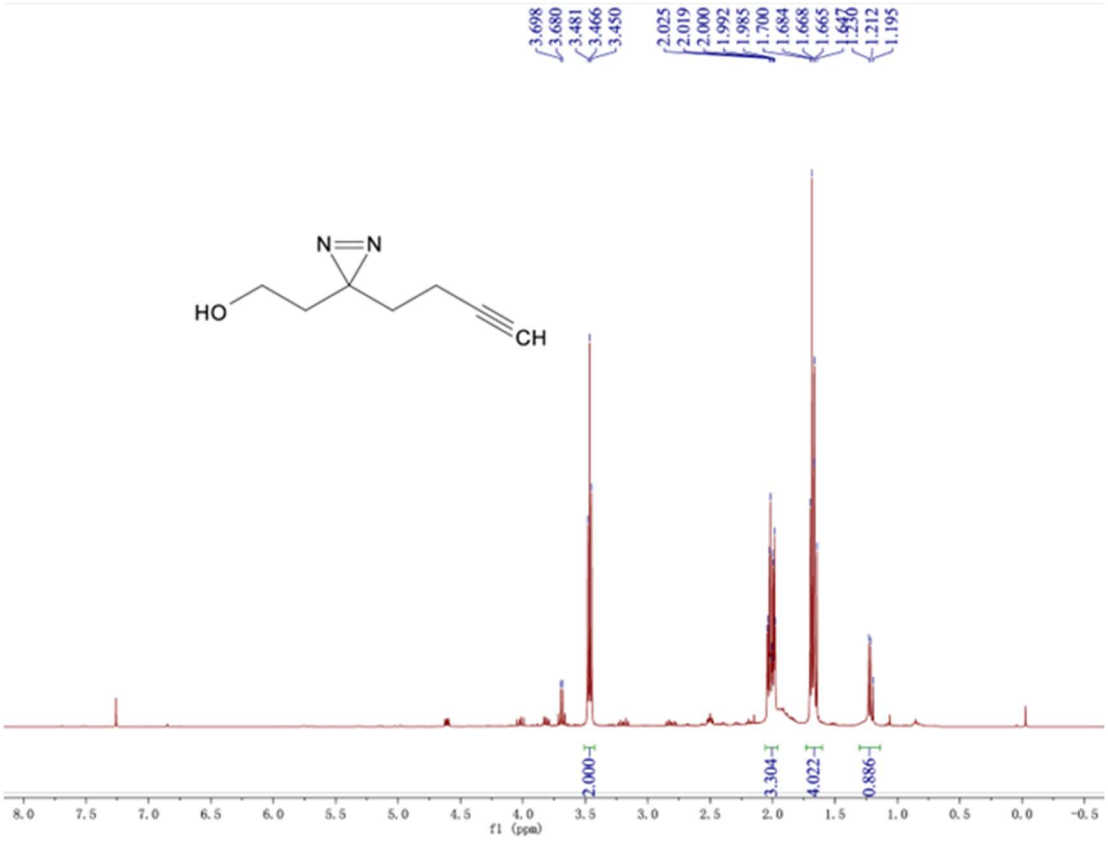
Compound 4 – ^{1}H NMR Spectrum - $CDCl_3$,400 MHz



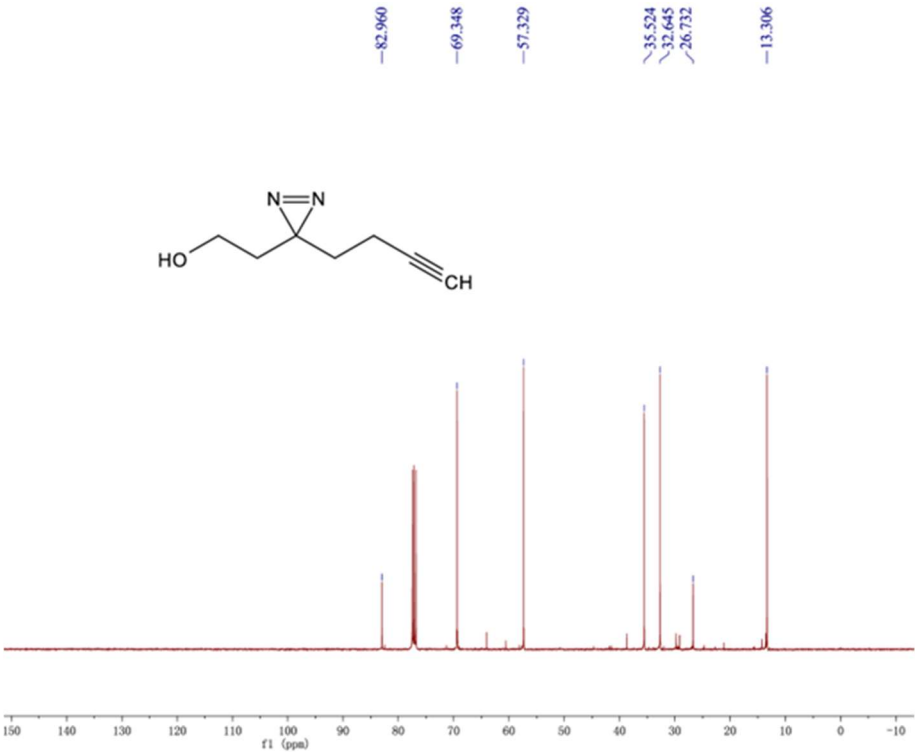
Compound 4 – ^{13}C NMR Spectrum - $CDCl_3$,400 MHz



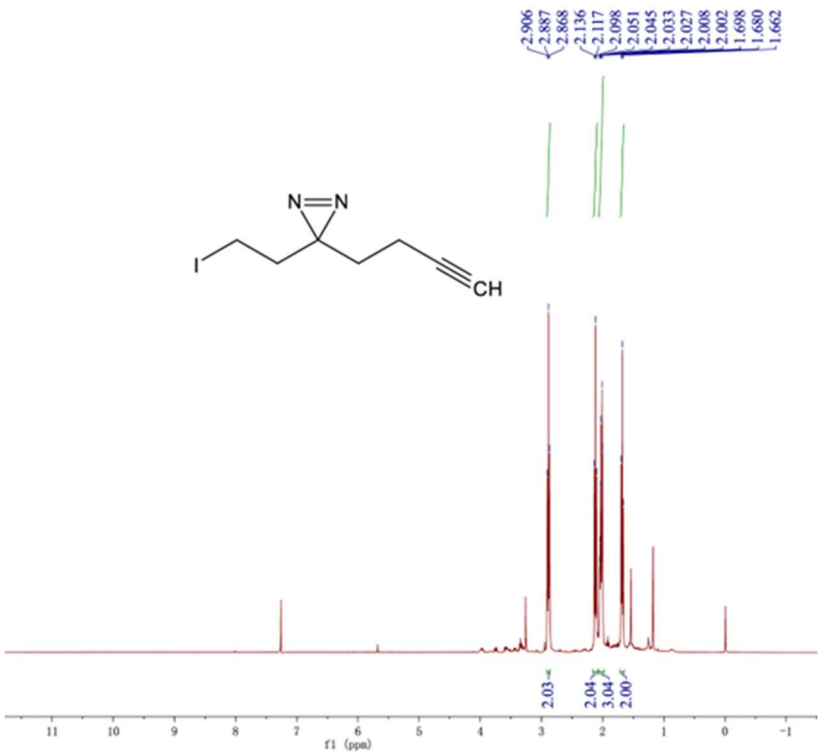
Compound 5 – ^{1}H NMR Spectrum - $CDCl_3$,400 MHz



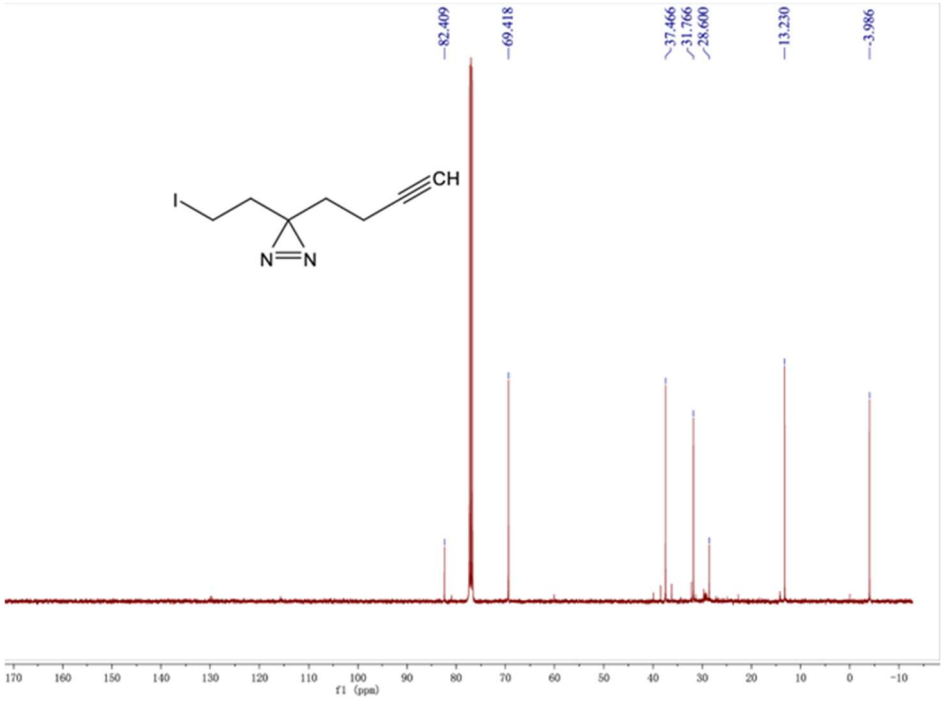
Compound 5 – ^{13}C NMR Spectrum - $CDCl_3$,400 MHz



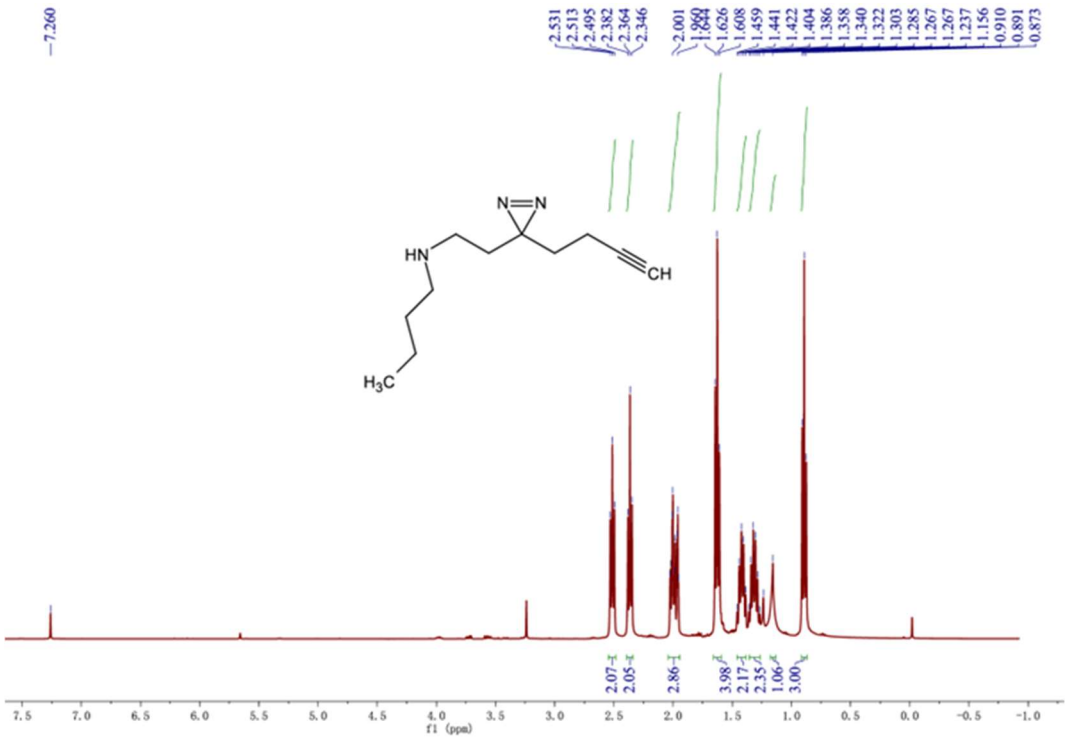
Compound 6 – ^{1}H NMR Spectrum - $CDCl_3$,400 MHz



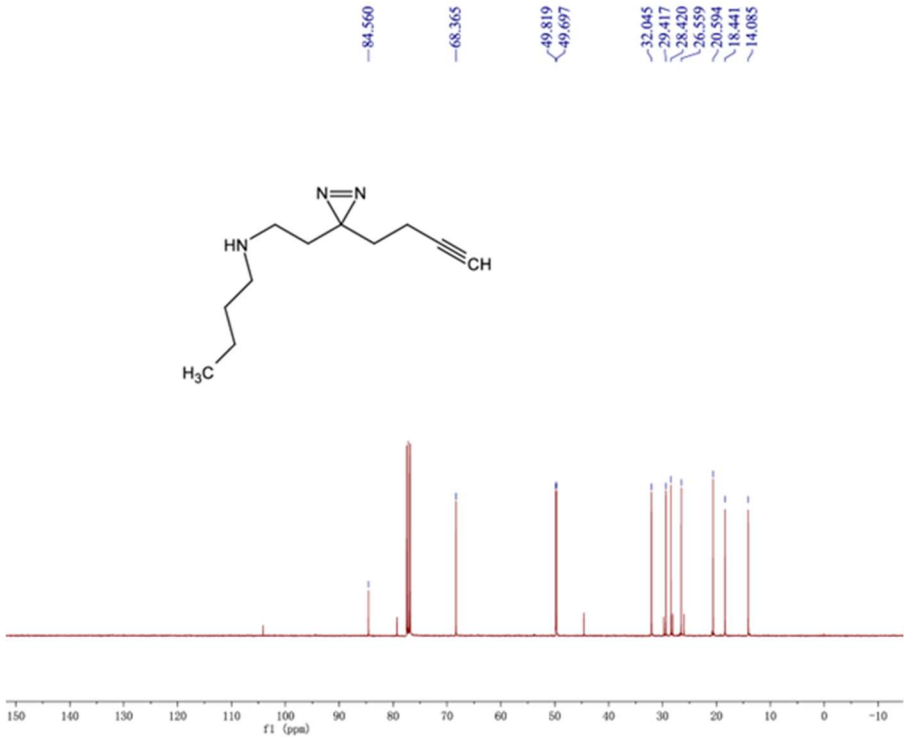
Compound 6 – ^{13}C NMR Spectrum - $CDCl_3$,400 MHz



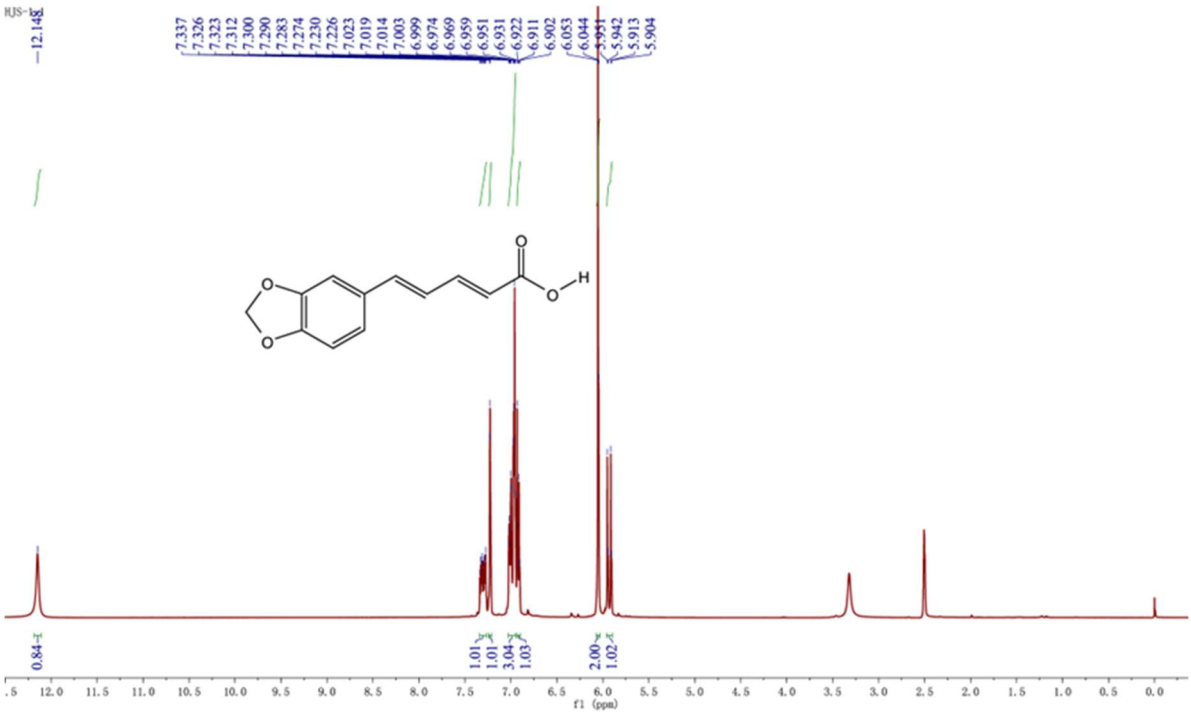
Compound 7 – ^{1}H NMR Spectrum - $CDCl_3$,400 MHz



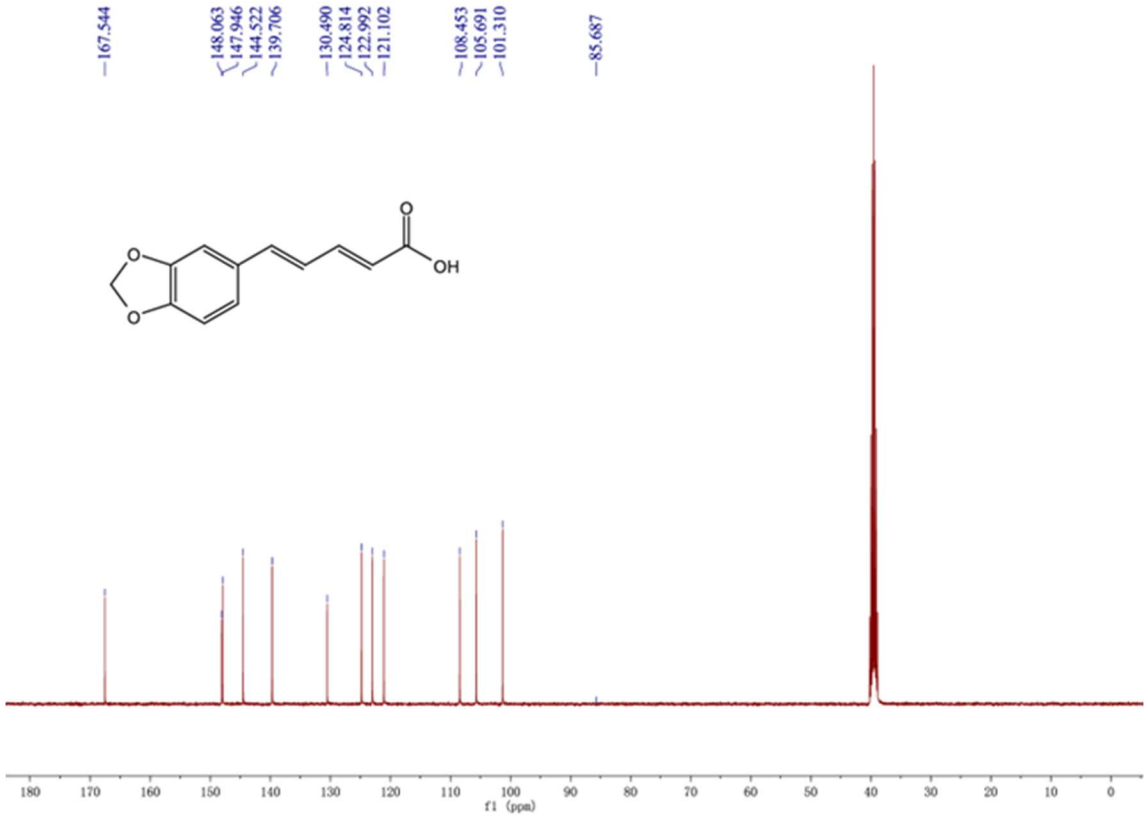
Compound 7 – ^{13}C NMR Spectrum - $CDCl_3$,400 MHz



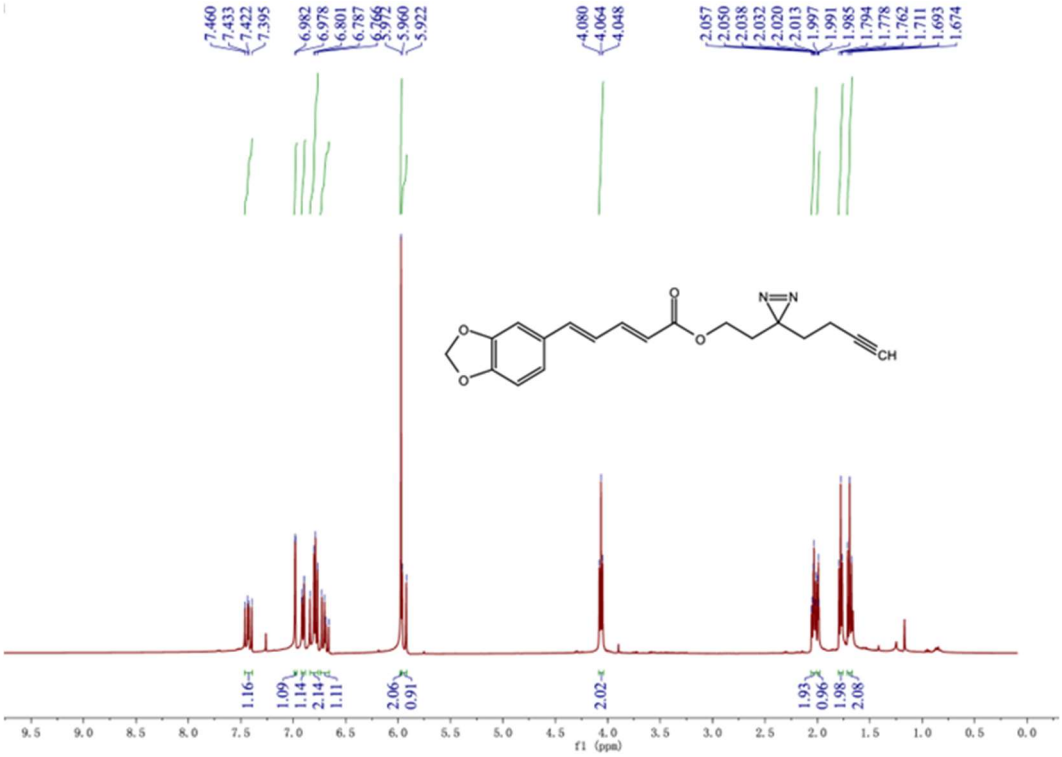
Piperic acid – ^{1}H NMR Spectrum - DMSO-d$_6$,400 MHz



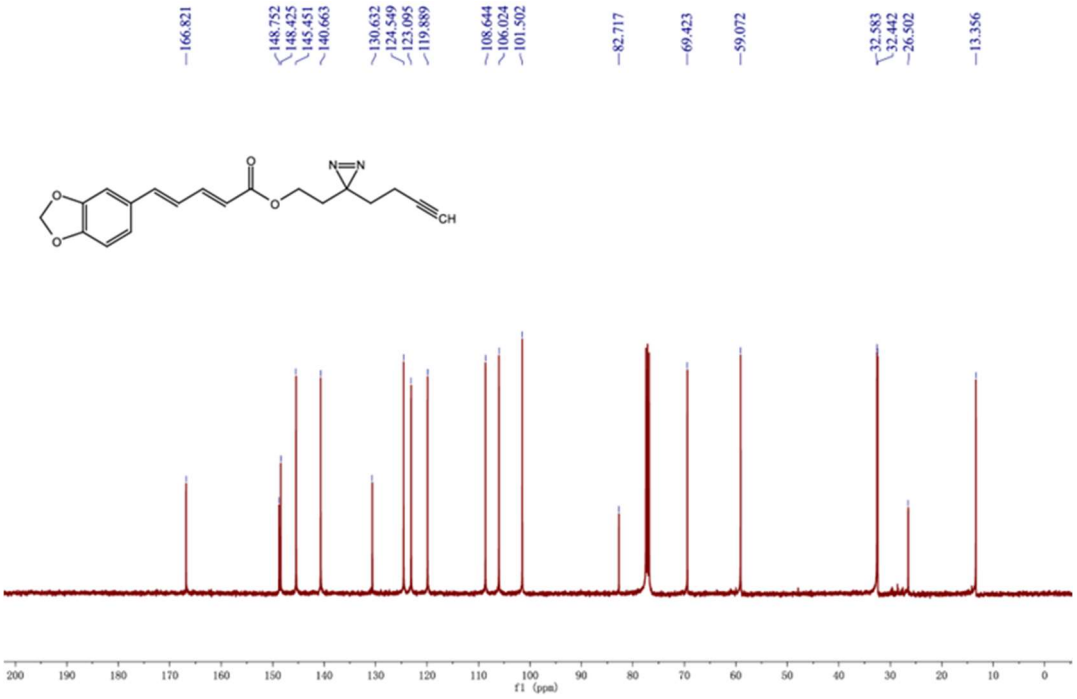
Piperic acid – ^{13}C NMR Spectrum - DMSO-d$_6$,400 MHz



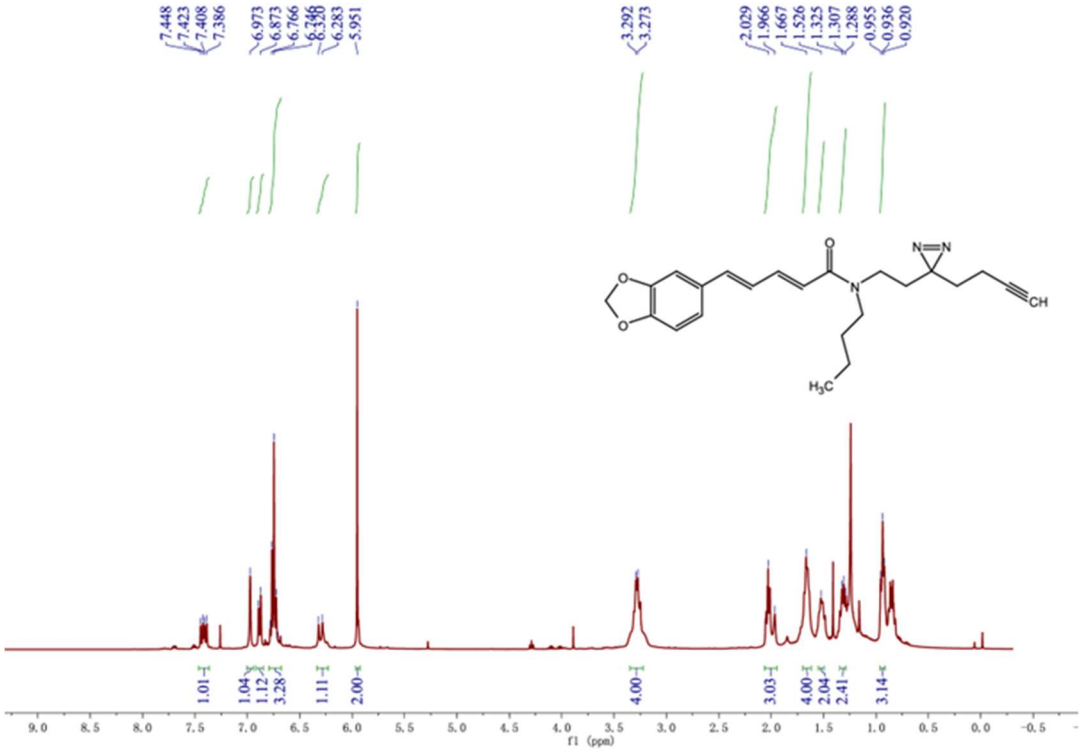
PIP-1 – ^{1}H NMR Spectrum - $CDCl_3$,400 MHz



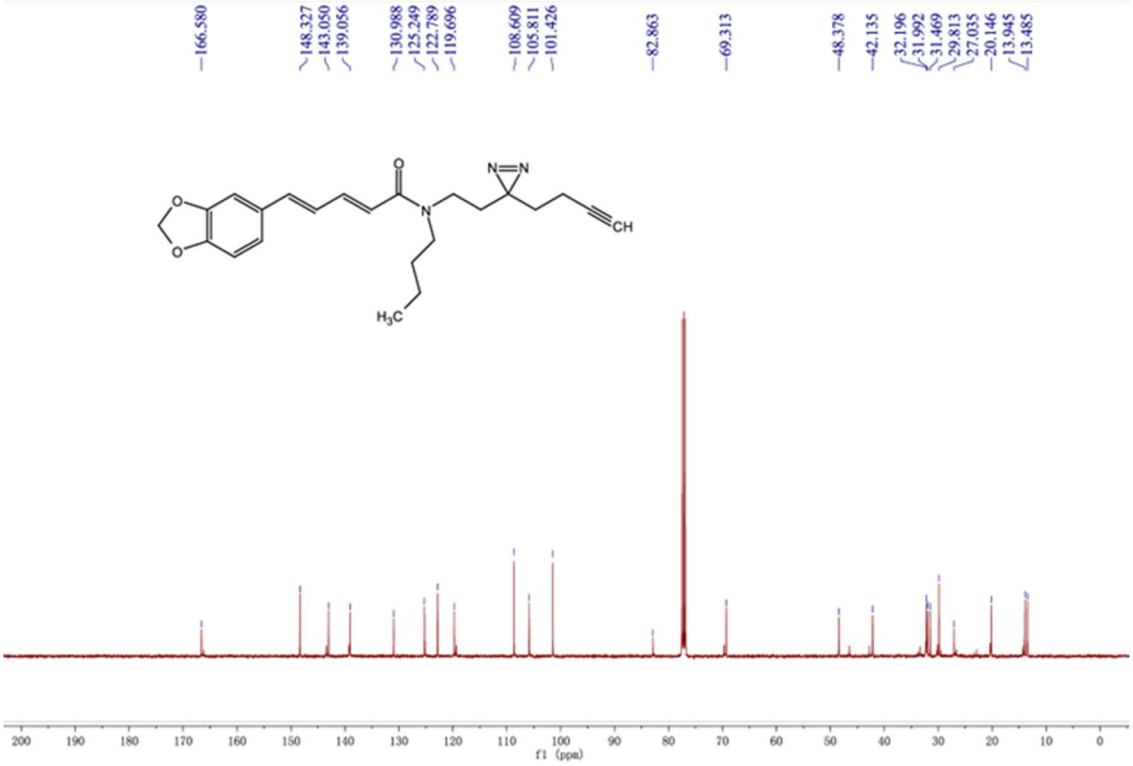
PIP-1 – ^{13}C NMR Spectrum - $CDCl_3$,400 MHz



PIP-2 – ^{1}H NMR Spectrum - $CDCl_3$,400 MHz



PIP-2 – ^{13}C NMR Spectrum - $CDCl_3$,400 MHz



HPLC profiles of compound

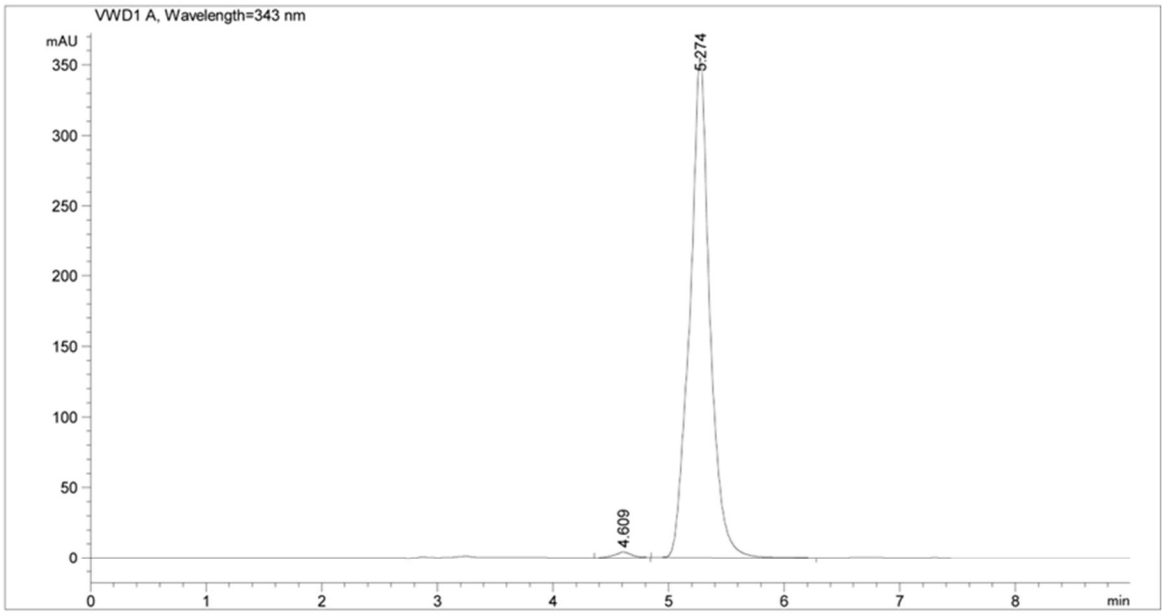
Compounds was evaluated for their purity was analyzed with HPLC method as follows. The purity for compounds was > 97%.

HPLC Analysis Method：

Mobile Phase	MeOH : H_2O (85 : 15)
Column	Express C_{18} 200*4.6 mm, 5μm
Column Temperature	30 °C
Flow Rate	0.8 mL/min
Detector	343 nm
Diluent	MeOH

HPLC porfile of PIP

Sample name： PIP



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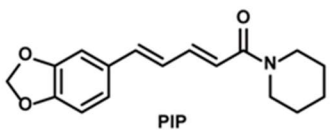
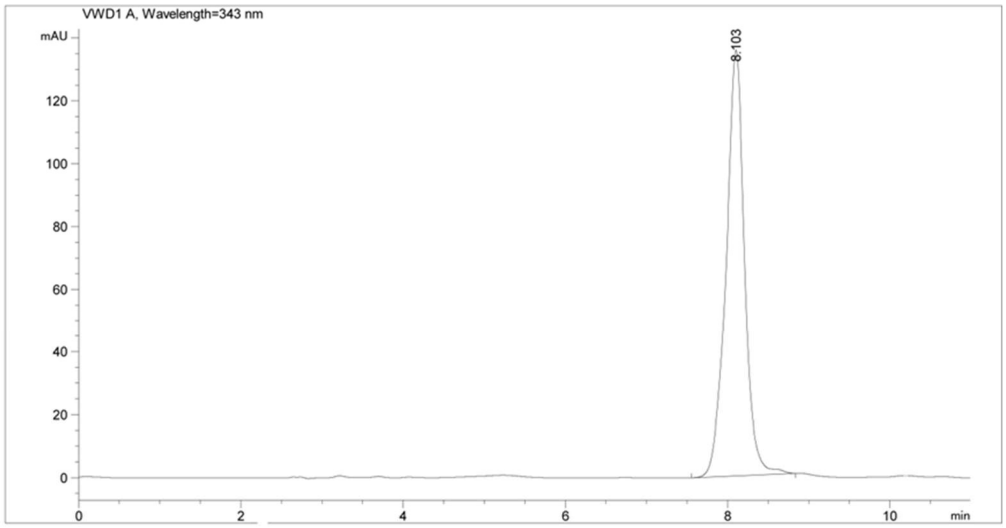


PIP

HPLC porfile of PIP-1

Sample name： PIP-1



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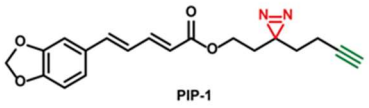
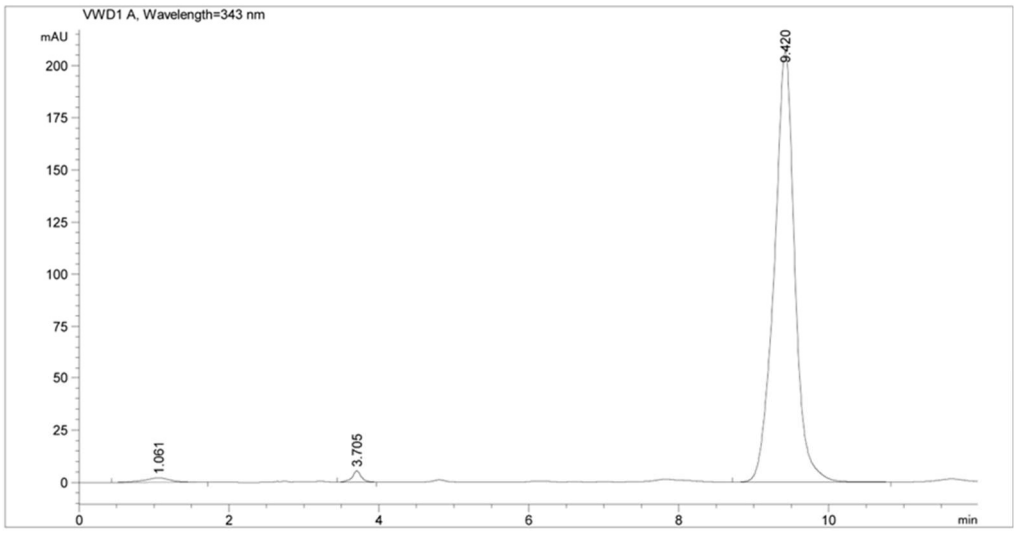


PIP-1

HPLC porfile of PIP-2

Sample name： PIP-2



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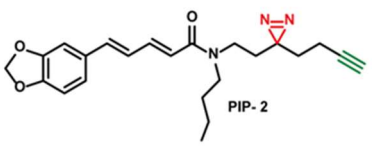
2	3.705	BB	0.1193	44.17208	5.36611	1.0961
3	9.420	BB	0.2801	3933.37402	206.83191	97.6042
Total				4029.92465	214.29961	



PIP- 2

IV. Biological Experiments

Antibodies and Reagents

Rhodamine-N_3 (760765) and biotin-N_3 (762024) were purchased from Sigma-Aldrich. Streptavidin magnetic beads were acquired from MedChemExpress (America). Anti-TGFβ1 was procured from Wanleibio (China).

Cell culture

CTX-TNA2 cells were obtained from iCell Bioscience Inc (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle medium (DMEM; Wuhan, China) supplemented with 1% L-Glutamine, 1% Penicillin-Streptomycin and 10% heat-inactivated fetal bovine serum (FBS; Hangzhou, China) at 37 °C with 5% CO_2.

Evaluation of anti-epileptic effect of PIP probes in PTZ induced acute epileptic zebrafish model

The anti-epileptic effect of PIP probes was evaluated in a zebrafish model with PTZ-induced acute epileptic seizures, following previously described methods.[7,8] Zebrafish embryos obtained from adult zebrafish (Wild type AB strain) were raised at a constant temperature of 28 °C under continuous light conditions in E_3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $MgSO_4$, and 0.33 mM $CaCl_2$). Zebrafish larvae at 7 days post-fertilization (dpf) were divided into 11 groups (n = 8 per group) and transferred into 96-well plates. The normal group was incubated in 100 μL of E_3 media, while the other groups were incubated in E_3 media with the addition of 5 mM PTZ to induce acute seizures. Among the groups with seizure induction, the PTZ group received no further treatment, while the other 9 groups were treated with PIP or PIP probes at concentrations of 10, 50, or 100 μg/mL separately in PTZ-containing E_3 media. Zebrafish larvae were allowed to habituate for 10 minutes after being transferred into a dark

chamber with a temperature control unit and an automated tracking device using the Daniovision observation chamber (Noldus Information and Technology, Wageningen, The Netherlands). The locomotor activities of the zebrafish were then analyzed by comparing the distance moved within 20 minutes among the different groups using EthoVision XT11.5 software (Noldus Image Analysis program, Wageningen, The Netherlands). The distance moved by zebrafish larvae in the groups treated with PIP probes was compared with that of the PTZ group to evaluate the anti-seizure effect of the PIP probe.

Cell viability assay of PIP and PIP-1/2 probe

Cytotoxicity assays were conducted using CTX-TNA2 cells and the CCK-8 assay. CTX-TNA2 cells were seeded into a 96-well plate with a density of 8×10^3 cells per well and incubated for 24 hours. PIP and PIP-1/2 in DMSO were then added to the cells at final concentrations of 0.1, 1, 2.5, 5, 10, 25, 50, and 100 μM, followed by an additional 24-hour incubation period. Subsequently, 10 μL of CCK-8 reagent was added to each well and incubated for 1 hour. The absorbance was measured at 450 nm using a plate reader (PERLONG; Beijing, China). The cell viability rate was determined using the formula $V_R = (A - A_0)/ (A_s - A_0) \times 100\%$, where A represents the absorbance of the experimental group, A_s represents the absorbance of the control group (with DMSO as the control), and A_0 represents the absorbance of the blank group (without cells).

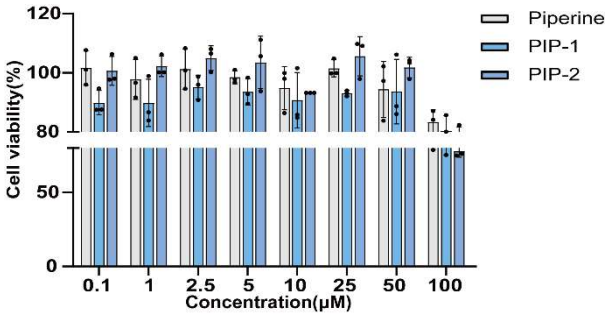
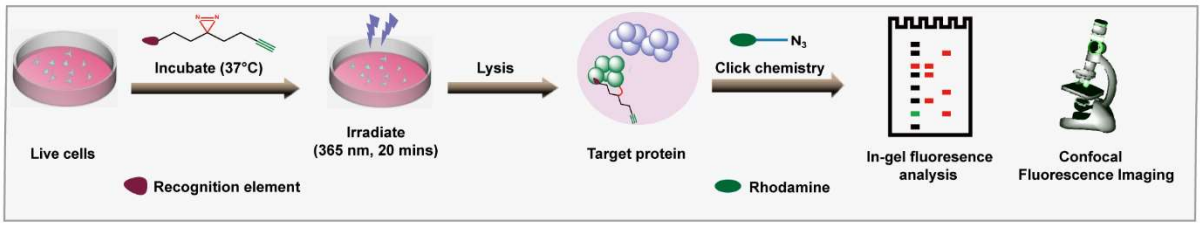


Fig. S1 Effect of PIP and PIP probes on CTX-TNA2 cell viability.



Scheme S2. Probe in-situ labeling workflow diagram.

SDS-PAGE Visualization and Target Identification of PIP-1/2

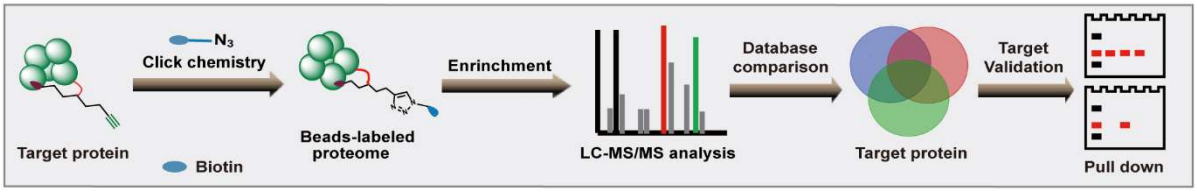
The procedures were performed based on previously published protocols with some modifications.[9-11] Cells were grown to 80−90% confluency in 6-well plates. The medium was removed, and cells were washed twice with cold PBS and then treated with 1 mL DMEM containing probe in the presence or absence of excess PIP (diluted from DMSO stocks whereby DMSO never exceeded 1% in the final solution). The cells were then incubated at 37 °C/5% CO_2 for 3 hours. After incubation, the medium was discarded and the cells were washed twice with cold PBS to remove excess probe. Subsequently, the six-well plates were irradiated with UV light (365 nm wavelength) for 20 minutes. To prevent exothermic damage to the proteins, the plates were placed on ice during the UV irradiation. After UV irradiation, cellular proteins were extracted by lysing the cells in each well. The lysate was prepared by adding 1% v/v of 100 × protease inhibitor (PMSF) to the normal RIPA lysis buffer before cell lysis. Then, 200 μL of this lysate was introduced into each well on ice, and the cells in the six-well plate were scraped with a hanging spoon and lysed for 30 minutes. The lysate was centrifuged at 13,000 rpm for 5 minutes at 4 °C to discard insoluble impurities and retain the protein supernatant. The protein concentration of each sample was measured using a BCA kit (Solebaol). The protein solution was diluted to a concentration of 1 mg/mL with PBS for subsequent click reaction. For each experimental group, 46 μL of the diluted protein solution was taken and the corresponding click-reaction reagent was added. The click-reaction reagent consisted of a DMSO stock solution of 2.5 mM TBTA (final concentration of 0.05 mM), a ddH_2O stock

solution of 25 mM TCEP (final concentration of 0.5 mM), a ddH_2O stock solution of 25 mM $CuSO_4$ (final concentration of 2.5 mM), and a 2.5 mM Rhodamine-azide DMSO stock solution (final concentration of 0.05 mM). The volumes of the four stock solutions added were 1 μL multiplied by the number of experimental group samples. After mixing, each sample was supplemented with 4 μL of click cocktail reagent for the click reaction. The mixture was then placed in a shaker at room temperature (200 rpm) for 2 hours. Next, the samples were added to 0.5 mL of pre-cooled acetone and cooled at -20 °C for 1 hour. The precipitated proteins were collected by centrifugation (13000 rpm × 10 minutes at 4 °C), and the supernatant was discarded. The pellet was then washed with 500 μL of prechilled methanol before being redissolved in 1 × loading buffer and boiling at 100 °C for 5 minutes. Finally, protein isolation was performed using SDS-PAGE (12.5% gel) electrophoresis, and protein labeled fluorescence imaging was detected using the Tanon Multi 5600 molecular imager.

Confocal Fluorescence Imaging

To demonstrate the utility of the cell-permeable probes for imaging potential cellular targets, we conducted fluorescence microscopy using similar procedures as previously reported.[9-12] CTX-TNA2 cells were seeded in glass bottom dishes and grown until they reached 60-70% confluency. The cells were then treated with 2 mL of DMEM containing probes at different concentrations (10 μM final concentration), with or without PIP (10 ×) as a competitor. DMSO was used as a control. After incubating for 3 hours at 37 °C, the medium was removed and the cells were washed twice with PBS. Subsequently, the samples were exposed to UV irradiation (365 nm UV lamp) for 20 minutes on ice (for PIP-1 and PIP-2 treated samples). Following this, the cells were fixed with 4% formaldehyde in PBS for 20 minutes at room temperature, washed three times with cold PBS, and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. Next, the cells were treated with a freshly premixed click chemistry

reaction solution in a 200 μL volume (50 μM Rhodamine-N_3 from a 2.5 mM stock solution in DMSO, 50 μM TBTA from a 2.5 mM freshly prepared stock solution in DMSO, 0.5 mM TCEP from a 25 mM freshly prepared stock solution in deionized water, and 0.5 mM $CuSO_4$ from a 25 mM freshly prepared stock solution in deionized water) for 2 h at room temperature with vigorous shaking (120 rpm). The cells were washed three times with PBS and 0.1% Tween-20 in PBS for 10 minutes. Finally, the cells were stained with Hoechst (10 μg/mL, ready to use) for 5 minutes at room temperature, followed by three washes with PBS. Imaging was performed using the Nikon A1R HD25 confocal microscope system.



Scheme S3. Workflow of the photoaffinity labeling (PAL) employed for target profiling.

Pull-down assay and LC-MS/MS analysis and Western Blot Analysis

The general pull-down procedure was performed according to previously reported methods, with the following optimizations.[9-11] Cells were treated with 10 μM PIP-2, DMSO, or a combination of PIP-2 and 10 × PIP (DMSO should not exceed 1% in the final solution). After 5 hours of incubation at 37 °C/5% CO_2, the medium was aspirated, and cells were gently washed twice with PBS to remove excess probe. Subsequently, UV irradiation (365 nm UV lamp) was conducted for 20 minutes on ice. Protein extraction was performed using the same protocol as the labeling experiments mentioned above. The extracted proteins (250 μL protein solution for each sample) were subjected to Cu^+ catalyzed click reaction. This reaction involved incubating the proteins with TBTA (25 mM DMSO stock solution, 10 μL, 100 μM final concentration), TCEP (25 mM H_2O stock solution, 10 μL, 0.5 mM final concentration), $CuSO_4$

(25 mM H_2O stock solution, 10 μL, 0.5 mM final concentration), and biotin azide (10 μL, 2.5 mM DMSO stock solution, 100 μM final concentration) at room temperature in a shaker (150 rpm) for 2 hours. The samples were then washed sequentially with precooled acetone and methanol. The protein samples were dissolved in 500 μl PBST solution (containing 0.05% Tween-20) and subjected to simple ultrasonic treatment. The supernatant was collected for further experiments. The sample solution was incubated with pre-washed Streptavidin beads in PBST at room temperature for 1 hour. Magnetic separation was performed, and the magnetic beads were washed 5 times with PBST. Subsequently, an appropriate amount of RIPA cell lysate was added and boiled at 100 °C for 5 minutes. This step released the proteins bound to the magnetic beads into the buffer solution. The retained protein buffer was used for the preparation of biomass spectrometry samples and Western blot experiments.

The protein samples obtained from the experimental procedure described above were utilized for SDS-PAGE experiments. Subsequently, the gel was subjected to silver staining using a rapid silver-staining kit (Biyun Tian). The silver-stained protein bands were then excised and sent to Shanghai Aqi Biotechnology Co.

For each sample, 200 ng of total peptides were separated and analyzed with a nano-UPLC (nanoElute2) coupled to a timsTOF Pro2 instrument (Bruker) with a nano-electrospray ion source. Separation was performed using a reversed-phase column (PePSep C18, 1.9 μm, 75 μm×15 cm, Bruker, Germany). Mobile phases were H_2O with 0.1% FA (phase A) and ACN with 0.1% FA (phase B). Separation of sample was executed with a 30 min gradient at 300 nL/min flow rate. Gradient B: 2% for 0 min, 2-22% for 20 min, 22-37% for 4 min, 37-80% for 2 min, 80% for 4 min.

The mass spectrometer adopts DDA PaSEF mode for DDA data acquisition, and the scanning range is from 100 to 1700 m/z for MS1. During PASEF MS/MS scanning, the impact energy increases linearly with ion mobility, from 20 eV (1/K0 = 0.6 Vs/cm2) to 59 eV (1/K0 =

1.6 Vs/cm2).

The raw MS files were processed using SpectroMine software (4.2.230428.52329) and the built-in Pulsar search engine. MS spectra lists were searched against their specieslevel UniProt FASTA databases (uniprot_Homo sapiens_9606_reviewed_2023_09.fasta), Carbamidomethyl [C] as a fixed modification, Oxidation (M) and Acetyl (Protein Nterm) as variable modifications. Trypsin was used as proteases. A maximum of 2 missed cleavage(s) was allowed. The false discovery rate (FDR) was set to 0.01 for both PSM and peptide levels. Peptide identification was performed with an initial precursor mass deviation of up to 20 ppm . All the other parameters were reserved as default.

Protein lists of identified targets

Table S1-S3. Full lists of protein targets of the control, PIP-2 and compete groups in CTX-TNA2 cells.

(Please see accompanying EXCEL file)

Bioinformation analysis

The subcellular localization of PIP-interacting proteins was analyzed using LocTree3[13]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was analyzed through the online database (http://rest.kegg.jp) to obtain the signaling pathways involved in the occurrence and development of the disease. Statistical significance was set at a P value< 0.05. The enrichment bubble chart was drawn using the R package “ggplot2”.

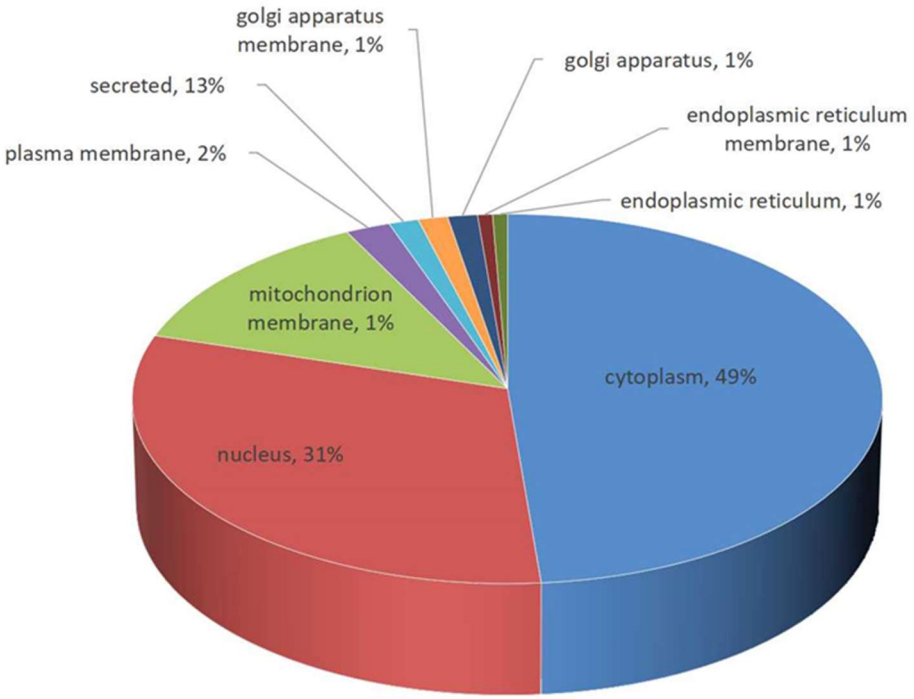


Fig. S2 Subcellular localization.

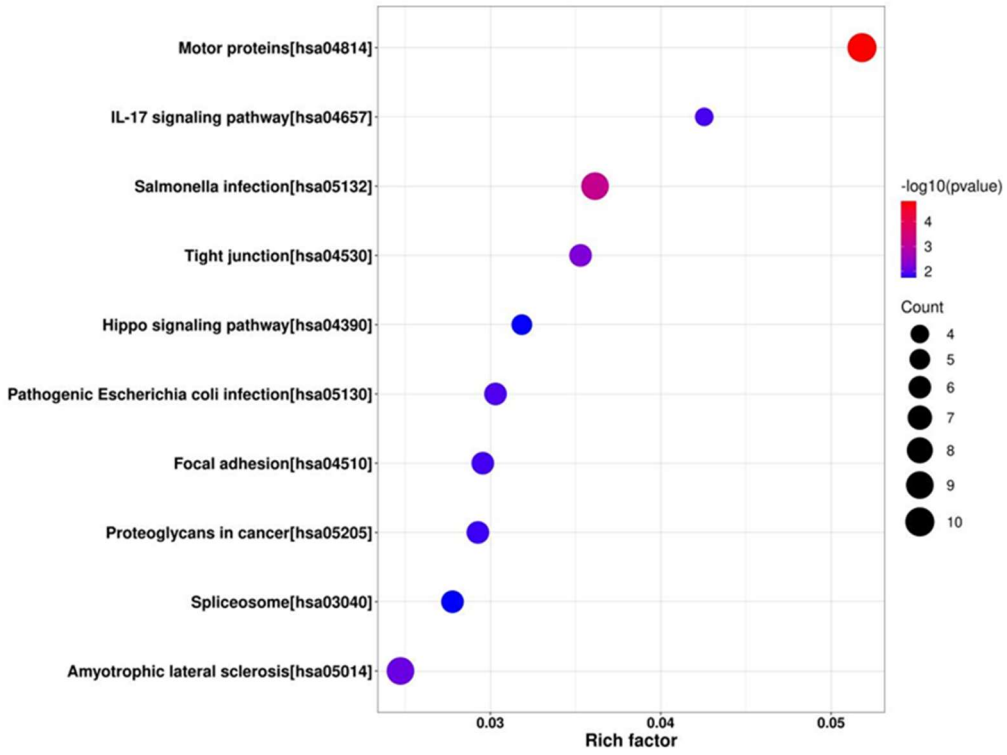


Fig. S3 Bubble charts of KEGG pathway analysis. Note: pathway (Y axis), gene ratio (X axis), p value (colour) and gene counts (size).

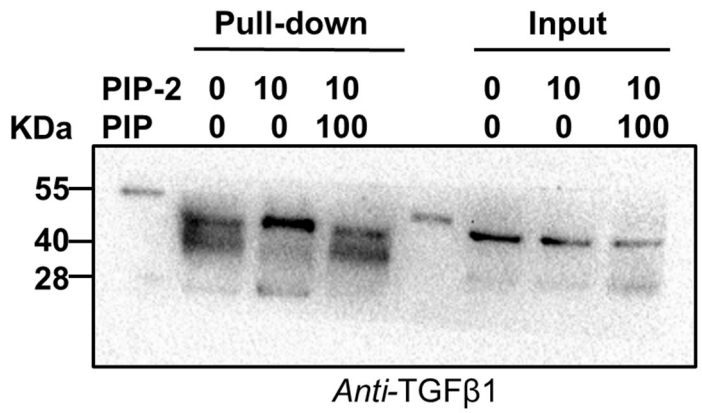
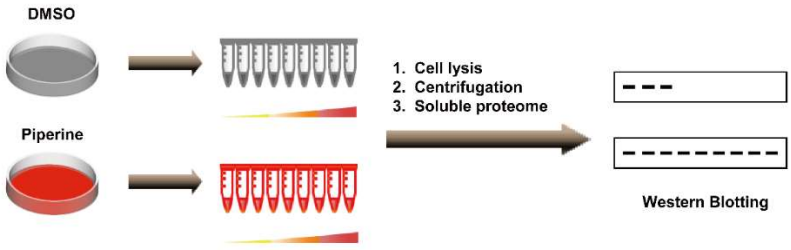


Fig. S4 Validation of the target protein TGFβ-1 by Pull-down/WB.

Scheme S4. Workflow of cellular thermal shift analysis (CETSA).



Cellular thermal shift assay (CETSA)

The procedures were conducted following previously published protocols with some modifications.[14] In brief, CTX-TNA2 cells were cultured in a 10 cm dish until they reached 80-90% confluence. The cells were then treated with 10 μM PIP and incubated at 37 °C/5% CO_2 for 3 hours. A negative control was included using the same volume of DMSO in another 10cm dish. Afterward, the medium was removed and the cells were washed twice with 4mL of PBS to discard excess PIP. Subsequently, the cells were digested with 1mL of Trypsin-EDTA solution and collected by centrifugation at 1000 × rpm for 5 minutes. The cell pellets were resuspended in 1.2 mL of PBS and divided into 1.5mL EP tubes. The tubes were then heated on a metal heater at a specified temperature (40 ~ 64 °C) for 3 minutes. NP-40 cell lysate, containing 1 × protease inhibitor, was added and kept on ice for 20 minutes. The resulting

lysate was homogenized by ultrasonic treatment (100 w, ice bath, 2 seconds of ultrasonic treatment followed by 2 seconds of suspension, repeated 5 times) and centrifuged at 13000 rmp at 4 °C for 10 minutes to remove insoluble impurities and retain the protein solution. From each experimental group, an appropriate amount of diluted protein solution was taken and the loading buffer was added proportionally. The mixture was then boiled in a metal bath at 100 °C for 5 minutes. The samples were stored in a -80 °C refrigerator until western blot analysis.

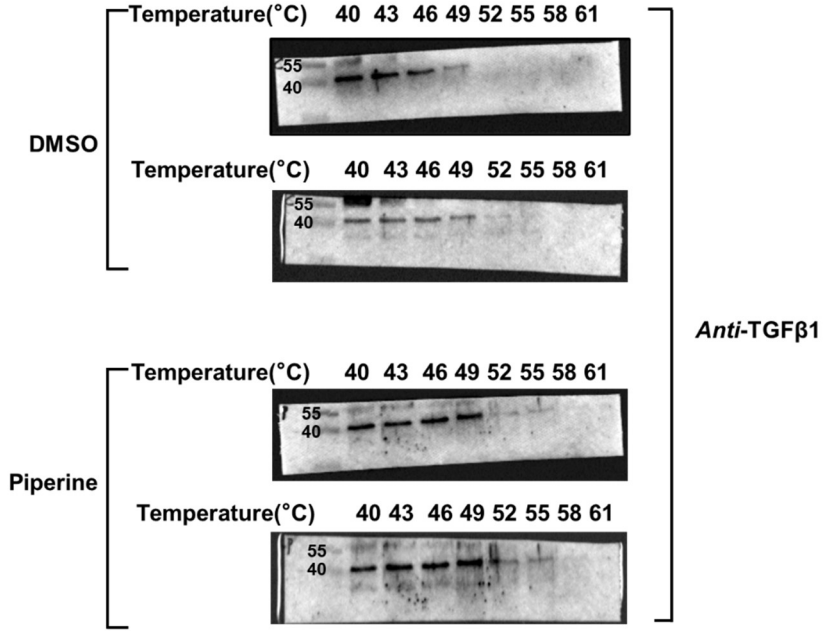


Fig. S5 CETSA was used to evaluate the binding between PIP and TGFβ1 in thermodynamic levels (n=2).

Statistical analysis

Data was presented as the form of means ± SD and One-way ANOVA was applied for statistical analysis. The group differences were considered significantly with p values less than 0.05 (*$p < 0.05$, **$p < 0.005$, ****$p < 0.0001$).

VI. References

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