Supporting Information

for

A Small-Molecule Probe for Monitoring Binding to Prolyl Hydroxylase Domain 2 by Fluorescence Polarisation

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Materials and Instrumentation

Reagents were from commercial sources except where stated. Organic solutions were concentrated using a rotary evaporator (EYELA-N1300) below 55 °C under reduced pressure. Reactions were monitored by thin-layer chromatography (TLC) using 0.25 mm silica gel plates (GF-2.5) and visualised under UV light. Melting points were determined with an MP420 Melting Point System. ^{1}H NMR and ^{13}C NMR spectra were measured using Bruker AV-300/AV-400/AV-500 instruments using deuterated solvents with tetramethylsilane (TMS) as an internal standard. EI-MS and high-resolution mass spectra (HRMS) were recorded using a Waters Q-Tof micro mass spectrometer. Analytical results are within 0.40 % of the theoretical values. The purity (≥ 98 %) of the target probes was validated by the HPLC assays performed using an Agilent C18 (4.6 mm× 150 mm, 3.5 μm) column using a mixture of solvent methanol/water (80:20, v/v) at a flow rate of 0.5 mL/min and peak detection at 365/254 nm under UV. Human embryonic kidney HEK293 cells, human hepatoma Hep3B cells, and human normal liver L02 cell lines were from American Tissue Culture Collection (ATCC, Rockville, MD, USA). PHD2 (181-426), which is reported to be similarly active to the full-length PHD2,[1] was from Nanjing Zoombio biotechnology, and fluorescence assays were recorded by a SpecultraMax GeminiXS (Molecular Devices, Sunnyvale, CA.). Excitation at a wavelength of 485 ± 25 nm and emission at a wavelength of 535 ± 25 nm). Peptides were prepared by solid-phase synthesis with a C-terminal amide.

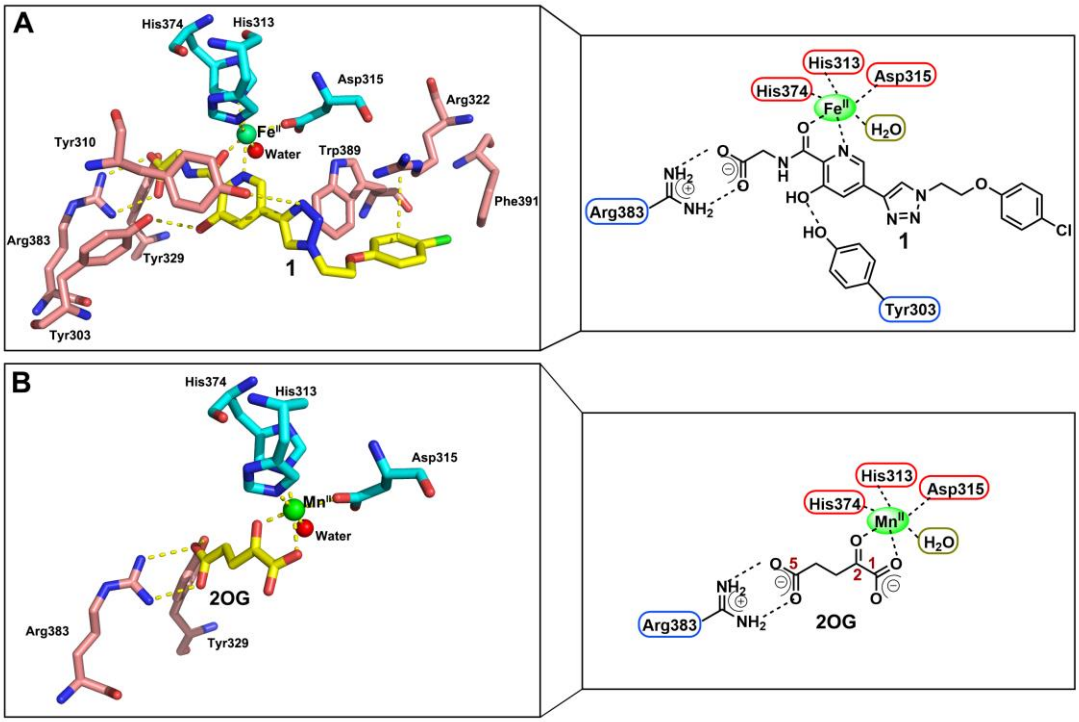


Fig. **S1.** Comparison of binding modes of compound **1** and 2OG at the PHD2 active site. (A) Docked poses of **1** at the active site of PHD2. A predicted binding mode of **1** was obtained through molecular docking using GOLD 5.1 with PDB: 2G19 being used as a structural starting point,[2] and is depicted using PyMOL. Key residues in the binding sites of PHD2 are coloured in pink and cyan (Fe-ligating residues); hydrogen bond and salt bridges are depicted as yellow dashed lines. (B) The binding mode of the cosubstrate 2OG with PHD2 (PDB:5L9B – with Mn substituting for Fe)[3].

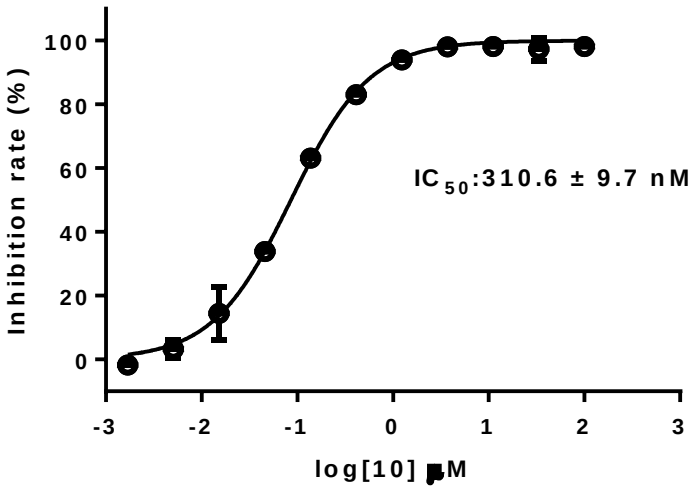


Fig. **S2.** Inhibition of PHD2 by **10**. The IC_{50} value was calculated using GraphPad Prism 7.0.

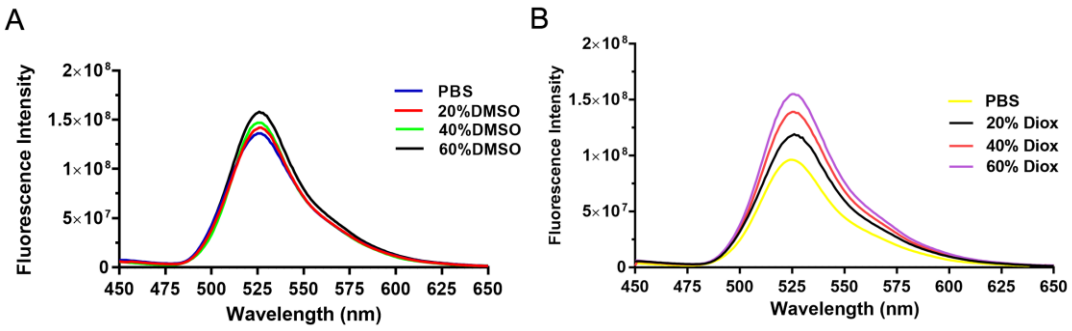


Fig. **S3.** Spectroscopic characteristics of Probe **12.** The influence of the medium polarity on the fluorescence of Probe **12** was investigated by the addition of DMSO (**A**, indicated in % v/v) and dioxane (**B**) in PBS measured at a λ_{max} of 520 nm. These results indicated that Probe **12** has stable spectroscopic properties suited for use in FP assays.

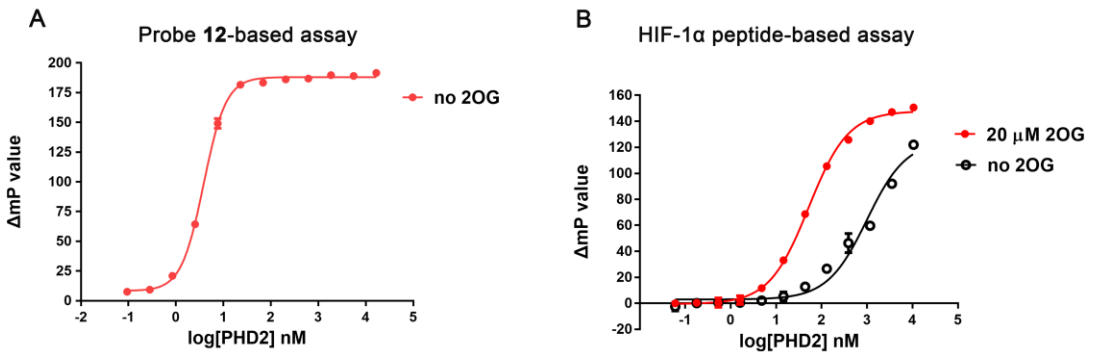


Fig. **S4**. The effect of 2-oxoglutate (2OG) on the Probe **12**-based FP assay and FITC-HIF-1α peptide-based FP assay. (A) The standard binding curve of PHD2 and Probe **12** without 2OG. The results indicate that binding of PHD2 and Probe **12** does not require 2OG. (B) Optimisation of the binding conditions for PHD2 and FITC-HIF-1α peptide (DLDLEMLAPYIPM DDDFQL) with different concentrations of 2OG.[4] The affinity is not strong between PHD2 and FITC-HIF-1α peptide without 2OG. When the 2OG is added, a dramatic increase in fluorescence polarisation is observed, consistent with ordered sequential binding of 2OG then substrate. At a 2OG concentration of 20 μM, binding of PHD2 and FITC-HIF-1α is saturated.

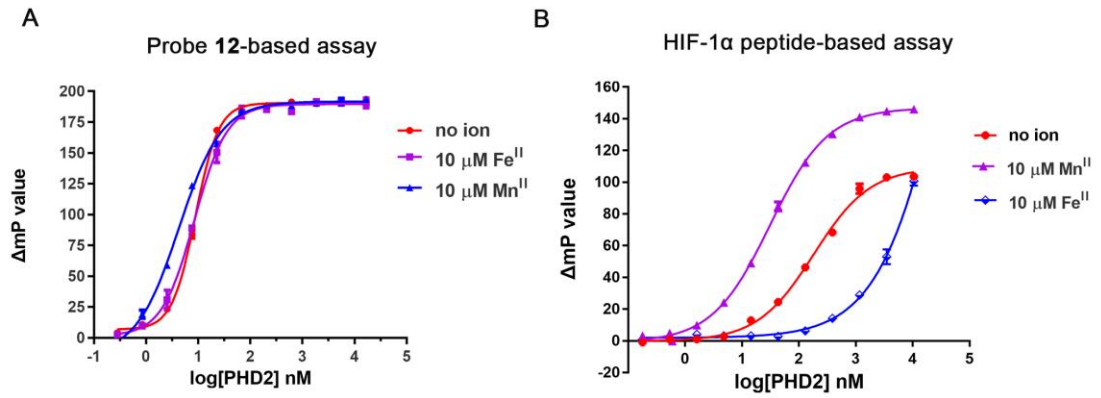


Fig. **S5.** The effect of metal ions on probe binding to PHD2. (A) Investigtions of the binding conditions for PHD2 and Probe **12** with metal chelating ions. The binding affinity remains constant when the enzyme concentration varies between 0 μM and 10 μM; note that it is reported that PHD2 protein can copurify with iron ions.[5] Thus, no additional metal ion was added in the subsequent investigations (B). (B) Optimisation of the binding conditions for PHD2 and FITC-HIF-1α peptide (DLDLEMLAPYIPM DDDFQL) with metal ions and 20 μM 2OG.[4] Binding to PHD2 was also measured with Fe^{II} and no additional metal ion in the assay buffer; in this case, HIF-1α peptide was oxidised to the hydroxylated product as expected, resulting in a substantial decrease in fluorescence polarisation (as anticipated in the presence of 2OG)[4,5]. When no additional metal was added, the binding affinity was also reduced. By contrast, no evidence for turnover was observed with excess Mn^{II}, which was used as a substitute for Fe^{II} in HIF-1α peptide-based binding assays.[4]

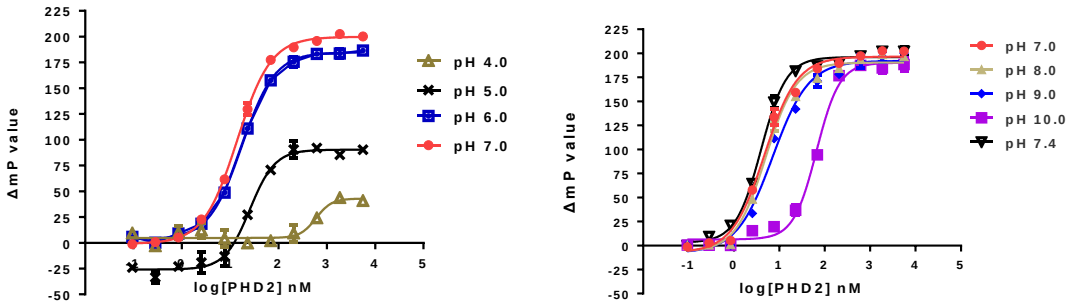


Fig. **S6.** Evaluation of the influence of pH on assay performance. Fluorescence polarisation was measured from pH 4 to pH 10. As shown in the Figure, there is little influence of pH on the binding of PHD2 with Probe **12** at pH 6-9. At pH 5 and 4, the binding decreased, possibly due to an altered protein fold.

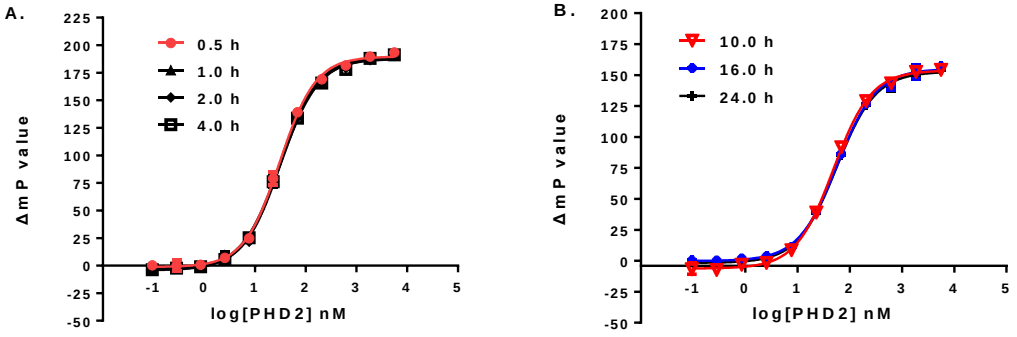


Fig. **S7.** Evaluation of incubation time on the assay performance. Fluorescence polarisation signals were measured at various time points over 24 hours. Once the binding has reached equilibrium (approximately 0.5 h), the signals remained ed stable for at least 24 hours, implying binding of the probe to PHD2 is stable over time.

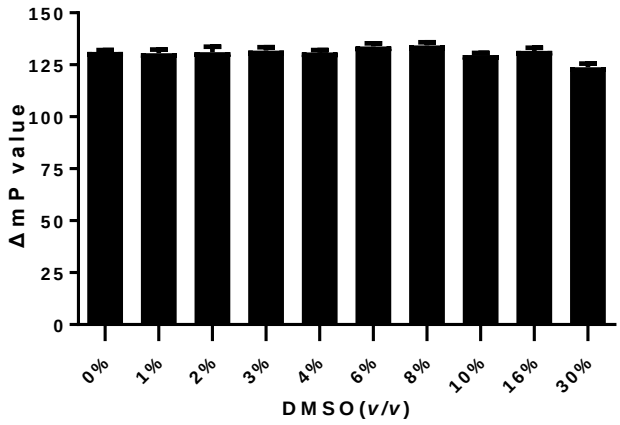


Fig. **S8.** Effect of DMSO on binding experiments. Increasing concentrations of DMSO (1%–30% of assay volume) were added to the reaction mixture containing 30 nM Probe **12** and 20 nM PHD2 (181-426) protein. The reaction mixture was incubated at room temperature for 60 min, and then total fluorescence and fluorescence polarisation measurements were taken.

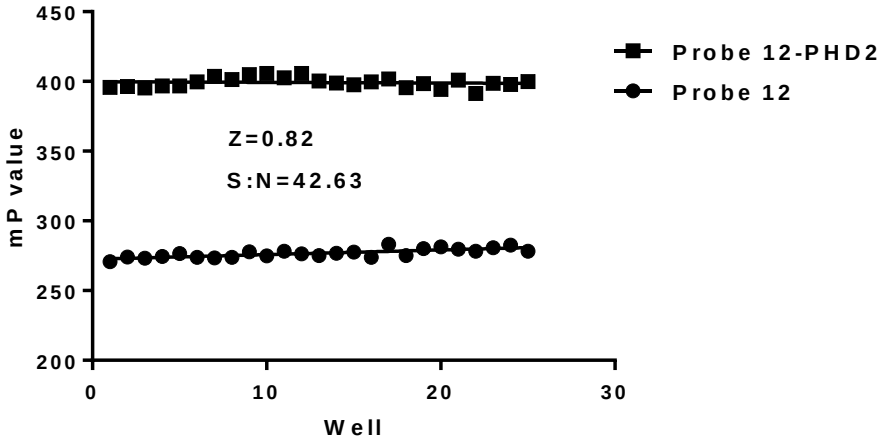


Fig. **S9.** Evaluation of PHD2 fluorescence polarisation assay performance. Z' factor statistical experiments were done to determine assay robustness in a high signal group (Probe **12** bound to PHD2 (181-426) and a low-signal group (Probe **12**) in 30 replicates and experiments were repeated 2 more times on different days. The reported Z′ factor for the assay is an average of three experiments. The Z′ factor was calculated using Equation 1, where σb and σf are the standard deviations of the high- and low-signal groups, respectively, and μb and μf are the means of the high and low signal groups, respectively.

$$Z'=1-3(\sigma b-\sigma f)/|\mu b-\mu f|$$

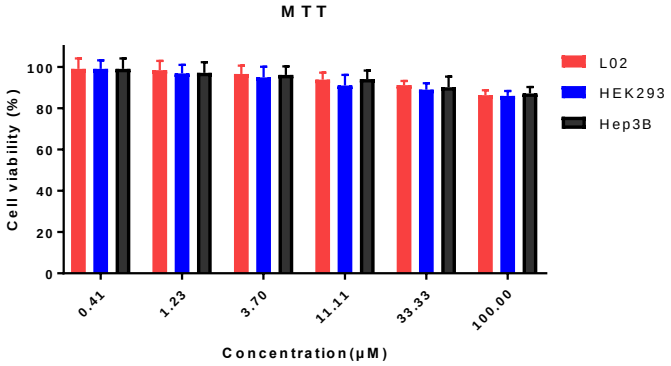


Fig. **S10** Cytotoxicity of Probe **12** toward L02, Hep3B, and HEK293 cells. The results indicate Probe **12** has no significant cytotoxic activity.

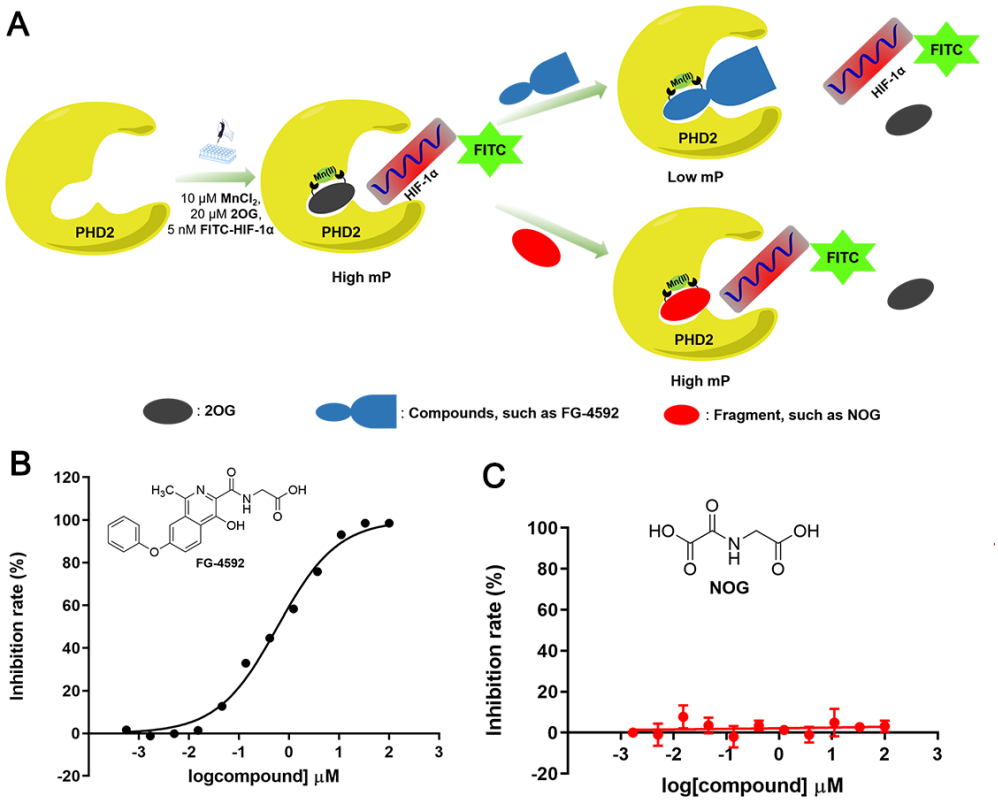
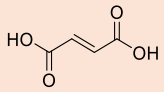
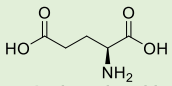
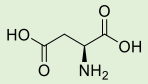
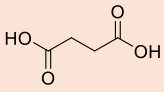
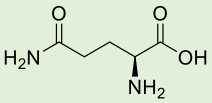
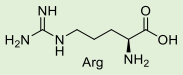
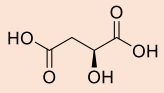
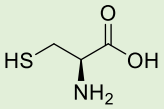
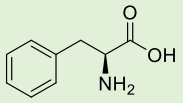
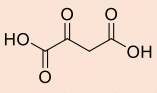
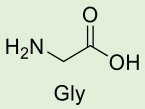
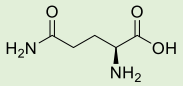
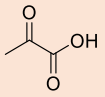
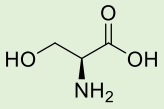
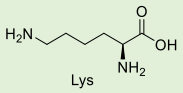
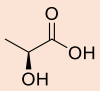
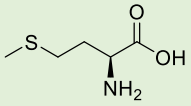
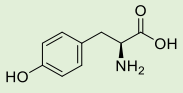
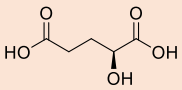
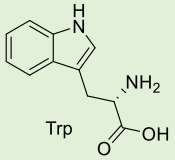
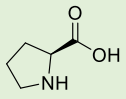
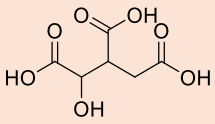
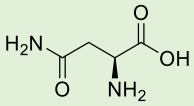
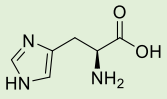
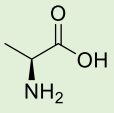
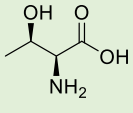
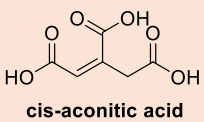
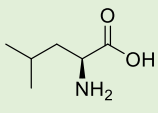
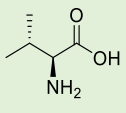


Fig. **S11**. The HIF-1α peptide-based FP assay. (A) Schematic representation of fluorescence polarisation assays used to monitor the interactions between FITC-labelled HIF-1α peptide (DLDLEMLAPYIPM DDDFQL) and PHD2 and displacement of the labelled peptide by small molecules. (B) (C) The HIF-1α peptide-based FP assay is useful for identifying inhibitors like FG-4592 (IC_{50} = 591.4 ± 13 nM)[4], but fails to identify certain types of inhibitor, including fragment-like compounds, e.g. NOG.

Table **S1**. IC_{50} values of fragments, tricarboxylic acid (TAC) intermediates, and natural amino-acids for PHD2 (181-426) using the Probe **12**-based FP assay.

TCA intermediates	IC_{50}	Natural amino-acids	IC_{50}	Natural amino acids	IC_{50}
 fumarate	77.94 ± 1.8 μM	 L-glutamic acid	65.07 ± 4.2 μM	 L-aspartic acid	162.2 ± 4.2 μM
 succinate	64.04 ± 1.4 μM	 Gln	Nd	 Arg	Nd
 L-malic acid	73.79 ± 3.6 μM	 Cys	Nd	 Phe	Nd
 oxaloacetic acid	121.04 ± 5.6 μM	 Gly	Nd	 Gln	Nd
 pyruvic acid	269.12 ± 7.2 μM	 Ser	Nd	 Lys	Nd
 L-lactic acid	306.91 ± 8.3 μM	 Met	Nd	 Tyr	Nd
 L-2-hydroxyglutarate	276.5 ± 22.1 μM	 Trp	Nd	 Pro	Nd
 DL-isocitric acid	Nd	 Asn	Nd	 His	Nd
 citric acid	Nd	 Ala	Nd	 Thr	Nd
 cis-aconitic acid	Nd	 Leu	Nd	 Ile	Nd

Nd: IC_{50} > 500 μM

Molecular docking studies.

A crystal structure of PHD2 (PDB: 2G19)[2] was taken from the Protein Data Bank (PDB). The compounds were imported into BIOVIA Discovery Studio 2019, and the SD conformation was generated by the protocol "Prepare Ligands". Molecular docking was accomplished using GOLD 5.1. The protein was prepared for docking by standard procedures. The conserved active site water molecule HOH601 was retained for docking. Protein residues surrounding the original ligand (4-hydroxy-8-iodoisoquinoline-3-carbonyl)glycine (radius = 8 Å) in the PHD2 active site were defined as the binding site. Docking studies were performed using the standard default setting with 100 GA runs of molecules. For each GA run, a maximum of 125,000 operations was performed. The annealing parameters were used as default. Cut off values of 3.0 Å for hydrogen bonds and 4.0 Å for van der Waals interactions were set. Docking was terminated when the top ten solutions attained root-mean-square deviation (RMSD) values within 1.5 Å.

Fluorescence polarisation competition assays.[4]

FP assays were performed using a SpectraMax Multi-Mode Microplate Reader (Molecular Devices) using the excitation and emission filters appropriate for the fluorophore used in the binding experiment. Fluorescence was recorded by a SpectraMax GeminiXS (Molecular Devices, Sunnyvale, CA. Excitation at the wavelength of 485 ± 25 nm and emission at the wavelength of 535 ± 25 nm).

Fluorescence polarisation experiments were performed in 384-well, flat bottom, black assay plates (#3575, Corning) in a final volume of 60 μL. The final assay buffer contained 30 nM Probe **12**, 20 nM PHD2 (181-426), 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% Tween-20, and less than 1% DMSO (except for DMSO tolerance experiments, in which different DMSO concentrations were used as indicated). All plates measured in fluorescence polarisation assays were incubated for a minimum of 30 min at RT. Polarisation was measured from the top of the well with a SpectraMax GeminiXS plate reader with polarised filters and optical modules for fluorescein (λex = 485 nm ± 25 nm, λem = 535 nm ±25 nm). All measurements were performed in duplicate except Z′ factor determination assays in which 30 replicates were used in each group (Fig. S9). The standard error of the mean (SEM) values, were calculated by dividing the sample standard deviation by the square root of the sample size and are recorded as ± values for IC_{50}.

Dose-dependent experiments were performed in the same manner using at least 10 concentrations of compounds in 3-fold serial dilutions from 100 μM. For each assay, negative controls (equivalent to 0% displacement) contained the fluorescent ligand, PHD2 (181-426), and 0.6 μL buffer, and blank controls contained only the fluorescent

ligand and buffer. The reaction mixtures were incubated at RT for 60 min, then total fluorescence and fluorescence polarisation measurements were taken. The percentage inhibition was calculated using Equation 2 where mP_{free} is the signal for the free probe (blank control) and mP_{bound} is the signal for the bound probe (negative control).

$$\%\ inhibition = 100*(1 - (mP_{bound} - mP) / (mP_{bound} - mP_{free}))$$

The IC_{50} was determined for duplicate measurements by non-linear least-squares analysis using GraphPad Prism 7.0.

Solid Phase Extraction (SPE) Mass Spectrometry (MS) assays for PHD2 and FIH.[6]

Assays were performed in 50 mM Tris-HCl pH 7.5, 50 mM NaCl. Titrations of compounds for IC_{50} determinations (3-fold and 11-point) were performed using an ECHO 550 acoustic dispenser (Labcyte) and dry dispensed into 384-well polypropylene assay plates. The final assay concentration of DMSO was kept constant at 0.5% (v/v). tPHD2 (residues 181-426) or full-length FIH was used at a concentration of 300 nM in the assay buffer; 25 μL of the protein solution was dispensed across each 384-well assay plate. The protein was allowed to equilibrate with compounds for 15 minutes and the enzyme reaction then initiated by dispensing of 25 μL of the substrate (20 μM ferrous iron sulfate, 200 μM L-ascorbic Acid, 10 μM HIF-1α CODD peptide (DLDLEMLAPYIPMDDDFQL) for PHD2 or 10 μM HIF-1α 789-822 peptide (DESGLPQLTSYDCEVNAPIQGSRNL LQGEELLRAL) for FIH, and 20 μM 2OG in the assay buffer). Reactions were allowed to proceed for 15 minutes, then terminated by the addition of 10% (v/v) aqueous formic acid (5 μL). Assay plates were then transferred to a RapidFire RF365 sampling robot (Agilent) connected to an Agilent 6550 quadrupole-time-of-flight (Q-TOF) mass spectrometer. Assay samples were aspirated under vacuum and loaded onto a C4 solid-phase extraction (SPE) cartridge. After loading the C4 SPE cartridge was washed with 0.1% formic acid in water to remove non-volatile buffer salts. The peptide was then eluted from the C4 solid-phase extraction (SPE) cartridge with 85% acetonitrile, 15% water containing 0.1% formic acid into the mass spectrometer. Peptide charge states were monitored in the positive ion mode. Ion chromatogram data were extracted for the +2 charge state and peak area

data integrated using RapidFire Integrator software (Agilent). The % conversion of the peptide substrate to the +16 hydroxylated peptide was calculated using the equation: % conversion = 100 x hydroxylated / (hydroxylated + non-hydroxylated peptide). IC_{50} data were determined from non-linear regression plots using GraphPad prism 7.0. The level of +16 (methionine residue oxidation) as observed in the no enzyme control was around 4-5%. All data were normalised to no enzyme controls.

SPE-MS assays for KDM4E.[6]

The potency of Probe **12** for KDM4E was assessed by SPE-MS mass spectrometry (RF-MS). The KDM4E RF-MS assay was performed in a 384-well plate format in assay buffer (50 mM MES pH 7.0). All reagent dispenses were performed using a multidrop reagent dispenser (Thermo). Dilutions of compounds were prepared using an ECHO 550 acoustic dispenser (Labcyte) and dry dispensed (250 nL) in duplicate into 384-well polypropylene plates (Greiner Bio-One). KDM4E (300 nM) was dispensed (25 µL) into each well of the plate and allowed to preincubate with Probe **12** for 15 minutes. Reactions were initiated by dispensing of 25 µL of assay buffer containing substrate and cofactors (200 µM LAA, 20 µM FAS, 20 µM 2OG, and 20 µM H3K9Me3 peptide); reactions were allowed to progress for 50 minutes, then stopped by the addition of 5 µL of 10% (v/v) aqueous formic acid. The final assay concentration of DMSO was 0.5% (v/v). Assay plates were transferred to a RapidFire RF365 high throughput sampling robot and samples aspirated under vacuum onto a C4 SPE cartridge. After an aqueous washing step (0.1% v/v formic acid in water) to remove non-volatile buffer components from the C4 SPE, the peptide was eluted from the C4 SPE in an organic wash step (85% v/v aqueous acetonitrile, 0.1% formic acid) into an Agilent 6550 accurate mass Q-TOF. One cycle of sample aspiration, loading, wash, and elution takes approximately 12 seconds. Peptide charge states were monitored in the positive ESI mode with a drying gas temperature of 280 °C, a drying gas flow rate of 13 L/minute, nebuliser gas pressure of 40 PSI, sheath gas temperature of 350 °C, sheath gas flow rate of 12 L/min and a nozzle voltage of 1000V. Ion chromatogram data were extracted for the substrate and

the product methyl marks and peak area data for extracted ion chromatograms were integrated using RapidFire Integrator software (Agilent). % conversion to the dimethyl peptide product was calculated using the equation:

% conversion = 100 x dimethyl / (dimethyl + trimethyl peptide)

IC_{50} values were determined from non-linear regression plots using GraphPad prism 7.0.

AlphaScreen Assays.[7]

All the enzymatic reactions were performed in HEPES buffer assay (50 mM HEPES pH 7.5, BSA (0.1% w/v) with Tween-20 (0.01% v/v)). Reactions were conducted in duplicate at room temperature for 60 min in a 10 μL mixture containing assay buffer, biotinylated histone H3 peptide substrate (30 nM), the demethylase enzyme (KDM3A:0.2 nM, KDM4A:2.0 nM, KDM6B:1.0 nM), ferrous ammonium sulphate (5 μM), 2OG (10 μM), L-ascorbic acid (100 μM), and the potential inhibitor. These 10 μL final volume reactions were carried out using 384-well Optiplates (#3575, Corning). The compounds were first diluted in 100% DMSO with the highest concentration at 10 mM. Enzyme only and blank only wells have a final DMSO concentration of 1% (v/v). From this intermediate step, 3 μL of the compound is added to 4 μL of demethylase enzyme dilution and incubated for 30 min at room temperature. After incubation, 3 μL of the peptide substrate is added. The final DMSO concentration was 1% (v/v). After the enzymatic reactions, 5 μL of anti-mouse acceptor beads and 5 μL of primary antibody were added to the reaction mix. After a brief shaking, the plate was incubated for 30 min. Finally, 10 μL of AlphaScreen Streptavidin-conjugated donor beads were added. After 30 min, the samples were measured in an AlphaScreen microplate reader (EnSpire Alpha 2390 Multilabel Reader, PerkinElmer).

MTT assay.

Hep3B, L02, HEK293 cells were seeded in 96-well plates at a density of 1 x 10^4 cells/mL and allowed to attach overnight. Each compound was added to the wells at concentrations ranging from 1.23 to 100 μM, then they were incubated at 37 °C in a 5% CO_2 atmosphere for 72 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/mL) was added and the cells were incubated for 4 h. The solutions were removed carefully by extraction, and the insoluble MTT formazan was dissolved in 150 μL of DMSO. The absorbance (OD) was read on a plate at 570 nm. All toxicity experiments were repeated with at least three technical replicates. The data were analysed using GraphPad Prism 7.0.

General procedure for the synthesis of intermediates.

Preparation of *N*-(5-(1-(2-(4-chlorophenoxy)ethyl)-1*H*-1,2,3-triazol-4-yl)-3-hydroxy picolinoyl) glycine (1). **1** (308 mg, total yield over two steps: 50%) was prepared from methyl N-(3-hydroxy-5-(trimethylsilyl)ethynyl))picolinoyl)glycinate (**8**, 450 mg, 1.4 mmol) according to the reported method.[8] mp: 232.4-234.1 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ:12.44 (s, 1H), 9.35 (t, J = 6.2 Hz, 1H), 8.94 (s, 1H), 8.72 (d, J = 1.7 Hz, 1H), 7.84 (d, J = 1.8 Hz, 1H), 7.33 (d, J = 8.4 Hz, 2H), 7.00 (d, J = 8.4 Hz, 2H), 4.86 (t, J = 4.9 Hz, 2H), 4.46 (t, J = 4.9 Hz, 2H), 3.99 (d, J = 6.1 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 41.48, 49.89, 66.88, 116.98, 121.47, 124.51, 125.37, 129.74, 130.67, 132.32, 137.27, 143.14, 157.19, 157.79, 169.00, 170.90. HRMS (ESI): calcd for $C_{18}H_{16}ClN_5O_5$ $[M + H]^+$ 418.0913, found 418.0917. HPLC (80 % methanol in water): t_R = 2.546 min, 99.7 %.

Methyl 5-chloro-2-hydroxybenzoate (2). Compound **2** was obtained from Aladdin in the highest purity available and was used as supplied. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.69 (s, 1H), 7.82 (s, 1H), 7.41 (d, J = 8.1 Hz, 1H), 6.95 (d, J = 8.8 Hz, 1H), 3.98 (s, 3H). HRMS (ESI): calcd for $C_8H_7ClO_3$ $[M + H]^+$ 187.0156, found 187.0144.

Methyl 2-(2-bromoethoxy)-5-chlorobenzoate (3). To a solution of methyl 5-chloro-2-hydroxybenzoate **2** (20.0 mmol, 4.6 g) in anhydrous acetonitrile (60 mL) was added potassium carbonate anhydrous (200.0 mmol, 37.4 g) and ethylene dibromide (22.0 mmol, 3.0 g). The reaction mixture was stirred at 70 °C for 12 h under nitrogen, the solution was concentrated under reduced pressure, the crude product was poured into water, then extracted twice with DCM. The organic extractions were combined, dried (sodium sulfate), then concentrated in vacuo. The resulting residue was purified by flash

chromatography (SiO_2, eluent: 1:1 petroleumether/EtOAc) to afford the titled compound **3** as a white solid (4.9 g, 68%). mp: 87.5-89.1 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79 (d, J = 2.7 Hz, 1H), 7.43 (dd, J = 8.8, 2.7 Hz, 1H), 6.93 (d, J = 8.9 Hz, 1H), 4.35 (t, J = 6.4 Hz, 2H), 3.92 (s, 3H), 3.68 (t, J = 6.4 Hz, 2H). HRMS (ESI): calcd for $C_{10}H_{10}BrClO_3$ $[M + H]^+$ 292.9575, found 292.9564.

2-(2-Bromoethoxy)-5-chlorobenzoic acid (4). To a solution of methyl 2-(2-bromoethoxy)-5-chlorobenzoate **3** (10.0 mmol, 2.9 g) in 30 mL anhydrous ethanol and 20 mL water, was added anhydrous potassium hydroxide (200.0 mmol, 37.4 g). The reaction mixture was stirred at 25 °C for 3.0 h, the solution was concentrated under reduced pressure. The crude product was then poured into water and adjusted the pH to about 1 using 3N hydrochloric acid. A large amount of white solid precipitated, which was filtered and dried to give **4** as a white solid (2.4 g. 86.9%). mp: 102.8-105.1 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.97 (s, 1H), 7.62 (d, J = 2.7 Hz, 1H), 7.54 (dd, J = 8.9, 2.7 Hz, 1H), 7.19 (d, J = 8.9 Hz, 1H), 4.47 – 4.30 (m, 2H), 3.85 – 3.71 (m, 2H). HRMS (ESI): calcd for $C_9H_8BrClO_3$ $[M + H]^+$ 278.9418, found 278.9405.

***tert*-Butyl (6-aminohexyl)carbamate (5). 5** was obtained from Aladdin in the highest purity available and used as supplied. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.59 (s, 1H), 3.11 (dd, J = 13.2, 6.5 Hz, 2H), 2.68 (t, J = 6.8 Hz, 2H), 1.46 (d, J = 11.1 Hz, 15H), 1.36 – 1.29 (m, 4H). HRMS (ESI): calcd for $C_{11}H_{24}N_2O_2$ $[M + H]^+$ 217.1911, found 217.1895.

***tert*-Butyl(6-(2-(2-bromoethoxy)-5-chlorobenzamido)hexyl)carbamate (6). 4** (0.78 g, 3.0 mmol), **5** (0.778 g, 3.6 mmol), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.699 g, 4.5 mmol), 1-hydroxybenzotriazole (0.607 g, 4.5 mmol), and

triethylamine (5 mL) were added to DCM (80 mL); the mixture was stirred at room temperature overnight. Then, saturated sodium bicarbonate solution (20 mL) was added to the reaction mixture. The mixture was then extracted with DCM (20 mL×3). The combined organic extractions were dried over anhydrous sodium sulfate and concentrated. The residue was purified by column chromatography (SiO_2, eluent: 5:1 petroleumether/EtOAc) to give **6** as a white solid. (1.1 g, 75%). mp:104.5-105.6 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.19 (d, J = 2.8 Hz, 1H), 7.92 (t, J = 5.3 Hz, 1H), 7.37 (dd, J = 8.8, 2.8 Hz, 1H), 6.84 (d, J = 8.8 Hz, 1H), 4.59 (s, 1H), 4.48 – 4.40 (m, 2H), 3.83 – 3.73 (m, 2H), 3.47 (q, J = 6.9 Hz, 2H), 3.11 (q, J = 6.3 Hz, 2H), 1.66 (p, J = 7.1 Hz, 2H), 1.51 – 1.34 (m, 15H). HRMS (ESI): calcd for $C_{20}H_{30}BrClN_2O_4$ $[M + H]^+$ 477.1150, found 477.1135.

***tert*-Butyl (6-(2-(2-azidoethoxy)-5 chlorobenzamido) hexyl) carbamate (7). 6** (2.1g, 4.0 mmol) in DMF (15 mL) was added dropwise over 10 min to a solution of sodium azide. The mixture was stirred for 10.0 h at 100 ˚C. The reaction was quenched with water (10 mL). The mixture was then extracted with ethyl acetate (10 mL×3). The combined organic extractions were dried over anhydrous sodium sulfate, then concentrated. The residue was purified by column chromatography (SiO_2, eluent: 3:1 petroleumether/EtOAc) to give **7** as a white solid (1.8 g mg, 92.9%). mp: 101.5-103.6 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.12 (t, J = 5.6 Hz, 1H), 7.66 (d, J = 2.8 Hz, 1H), 7.51 (dd, J = 8.9, 2.8 Hz, 1H), 7.20 (d, J = 8.9 Hz, 1H), 6.77 (s, 1H), 4.29 – 4.22 (m, 2H), 3.80 – 3.74 (m, 2H), 3.26 (q, J = 6.8 Hz, 2H), 2.90 (q, J = 6.8 Hz, 2H),

1.50 (m, 2H), 1.39 – 1.25 (m, 15H). HRMS (ESI): calcd for $C_{20}H_{30}ClN_5O_4$ $[M + H]^+$ 440.2059, found 440.2046.

Methyl *N*-(3-hydroxy-5-(trimethylsilyl)ethynyl))-picolinoyl)glycinate (8). 8 (1.2 g, total yield over four steps: 26.2 %) was prepared from 5-bromo-3-nitropicolinonitrile (3.4 g, 15.0 mmol) according to the reported method.[8] mp: 115.4-117. 2 °C ^{1}H NMR (300 MHz, DMSO-d_6) δ: 12.28 (s, 1H), 9.53 (t, J = 6.2 Hz, 1H), 8.23 (d, J = 1.7 Hz, 1H), 7.52 (d, J = 1.6 Hz, 1H), 4.07 (d, J = 6.1 Hz, 2H), 3.67 (s, 3H), 0.26 (s, 9H). m/z (EI-MS): 307.1 [M]+.

Methyl (5-(1-(2-(2-((6-((*tert*-butoxycarbonyl)amino) hexyl)carbamoyl)-4-chlorophenoxy)ethyl)-1*H*-1,2,3-triazol-4-yl)-3-hydroxy picolinoyl)glycinate (9). 7 (1.3 g, 3.0 mmol), **8** (1.0 g, 3.3 mmol), tetra-butylammonium fluoride (1.0 mol/L in THF, 1.56 g, 6.0 mmol), cuprous iodide (0.023 g, 0.12 mmol), and *N*,*N*-diisopropylethylamine (1.2 mL) were added to MeOH (10 mL). The mixture was stirred at 80 ˚C for 5.0 h under N_2. The reaction mixture was then filtered and concentrated. The residue was purified by column chromatography (SiO_2, eluent: 1:1 petroleumether/EtOAc) to give **9** as a white solid (1.8 g, 84.1%). mp: 141.6-143.2 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.33 (s, 1H), 9.51 (t, J = 6.1 Hz, 1H), 8.95 (s, 1H), 8.72 (d, J = 1.6 Hz, 1H), 8.11 (t, J = 5.6 Hz, 1H), 7.83 (d, J = 1.5 Hz, 1H), 7.64 (d, J = 2.7 Hz, 1H), 7.52 (dd, J = 8.8, 2.8 Hz, 1H), 7.22 (d, J = 9.0 Hz, 1H), 6.77 – 6.65 (m, 1H), 4.93 (t, J = 4.9 Hz, 2H), 4.58 (t, J = 4.4 Hz, 2H), 4.10 (d, J = 6.1 Hz, 2H), 3.68 (s, 3H), 3.22 – 3.16 (m, 2H), 2.89 – 2.80 (m, 2H), 1.35 (s, 17H). HRMS (ESI): calcd for $C_{31}H_{40}ClN_7O_8$ $[M + H]^+$ 674.2700, found 674.2685.

(5-(1-(2-(2-((6-((*tert*-Butoxycarbonyl)amino)hexyl)carbamoyl)-4-chlorophenoxy) ethyl)-1*H*-1,2,3-triazol-4-yl)-3-hydroxypicolinoyl)glycine (10). **9** (131.8 mg, 0.2 mmol) was dissolved in THF (10 mL), and 1M LiOH solution (8 mL) was added. The mixture was heated to 30 °C and reacted for 1 h. The reaction mixture was filtered and concentrated to ~6 mL. The residue was neutralized using diluted hydrochloric acid to pH 6. The precipitation was filtered, collected, and then purified by column chromatography (SiO_2, eluent: 1:10 MeOH/DCM) to give **10** as a brown solid (118.7 mg, 92% yield). mp: 139.7-140.2 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.44 (s, 1H), 9.33 (t, J = 6.0 Hz, 1H), 8.96 (s, 1H), 8.71 (s, 1H), 8.12 (t, J = 5.9 Hz, 1H), 7.82 (s, 1H), 7.64 (d, J = 2.7 Hz, 1H), 7.56 – 7.47 (m, 1H), 7.22 (d, J = 8.9 Hz, 1H), 6.71 (s, 1H), 4.93 (t, J = 4.9 Hz, 2H), 4.58 (t, J = 4.6 Hz, 2H), 3.99 (d, J = 6.0 Hz, 2H), 3.20 (d, J = 6.5 Hz, 2H), 2.85 (q, J = 6.0, 5.3 Hz, 2H), 1.35 (m, 17H). HRMS (ESI): calcd for $C_{30}H_{38}ClN_7O_8$ $[M + H]^+$ 660.2543, found 660.2540.

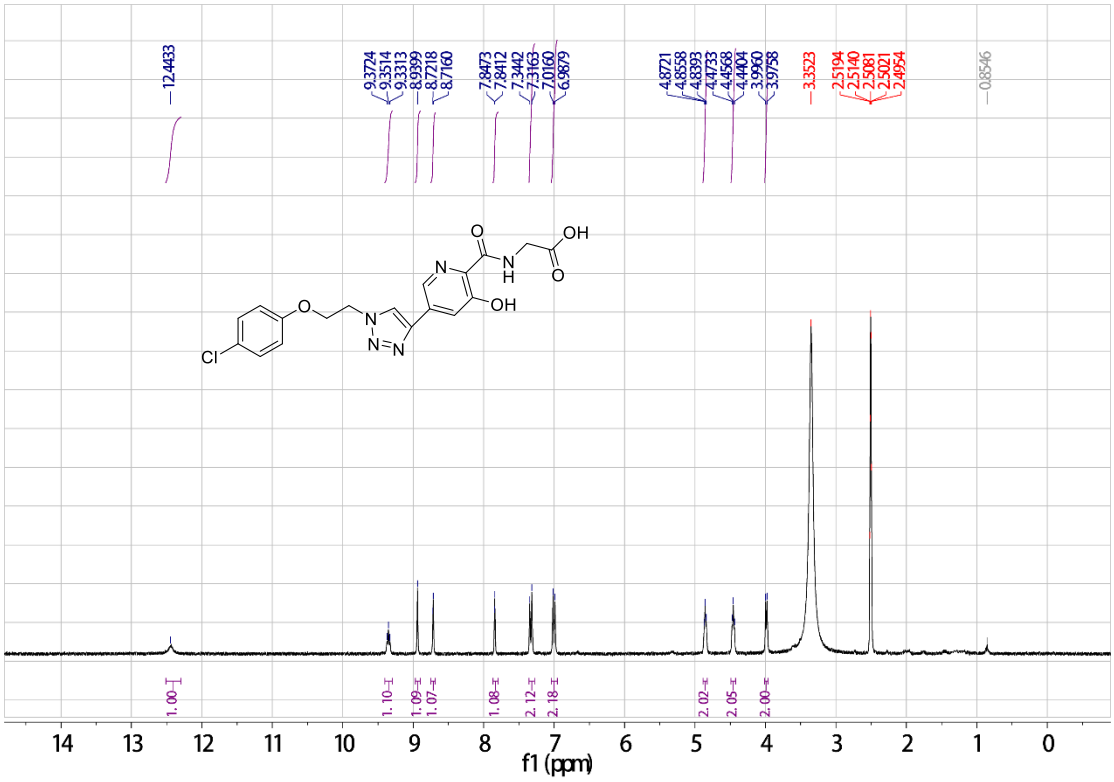
Methyl(5-(1-(2-(2-((6-aminohexyl)carbamoyl)-4-chlorophenoxy)ethyl)-1*H*-1,2,3-triazol-4-yl)-3-hydroxypicolinoyl)glycinate (11). A stirred solution of **9** (330.5 mg, 0.5 mmol) in dry DCM (5 mL) was treated dropwise with CF_3COOH (1 mL). After stirring at room temperature for 3 h, the reaction mixture was concentrated in vacuo. The solid residue was taken up with water, cooled in an ice bath, treated with a 3 M solution of NaOH (20 mL), and extracted with DCM (3 × 5 mL). The combined organic phases were washed with brine (5 mL), dried (Na_2SO_4), filtered, then concentrated in vacuo to give **11** as brown solid (207.6 mg, 60%). mp: 125.6-126.8 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.75 (s, 1H), 8.24 (s, 1H), 7.95 (s, 1H), 7.71–7.68 (m, 1H), 7.59–

7.49 (m, 2H), 7.45 (s, 1H), 7.26 (d, *J* = 8.7 Hz, 1H), 4.94 (s, 2H), 4.62 (s, 2H), 4.14 (s, 2H), 3.68 (s, 3H), 3.20 (m, 2H), 2.75 (t, *J* = 6.6 Hz, 2H), 1.49 (t, *J* = 5.0 Hz, 2H), 1.40 (t, *J* = 5.0 Hz, 2H), 1.29 (m, 4H), 1.22 (q, *J* = 10 Hz, 5 Hz, 2H). HRMS (ESI): calcd for $C_{26}H_{32}ClN_7O_6$ $[M+H]^+$ 574.2175, found 574.2158.

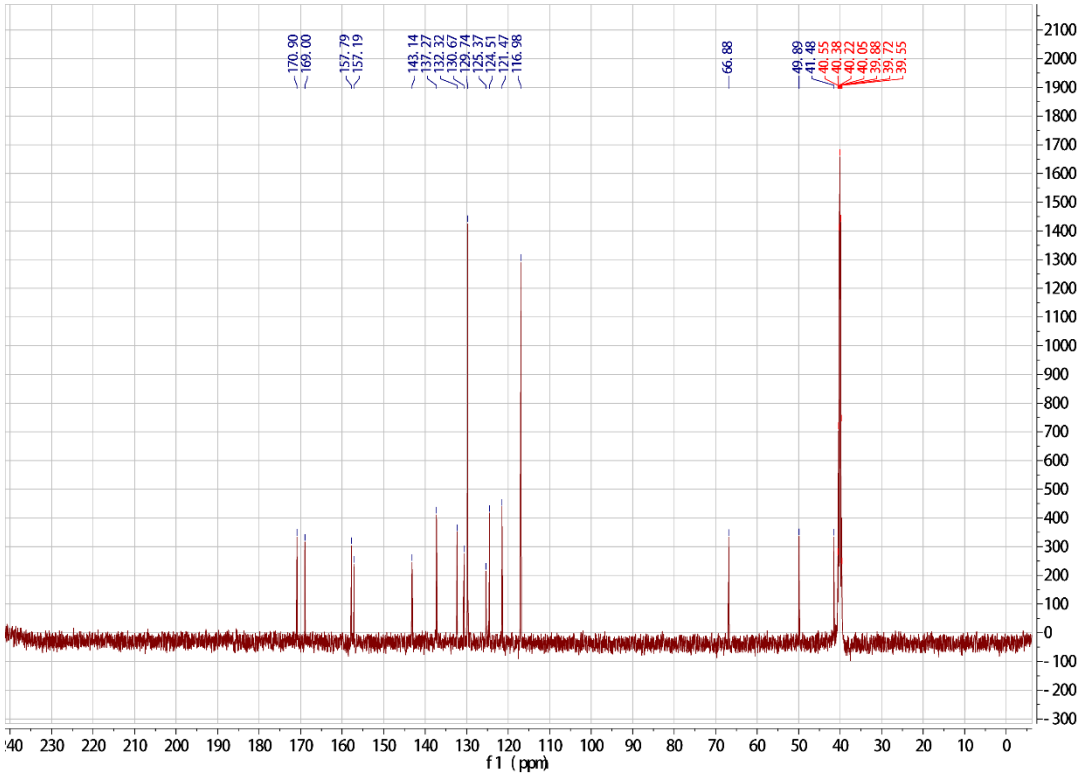
(5-(1-(2-(4-Chloro-2-((6-(5-((3aS,4S,6aR)-2-oxohexahydro-1*H*-thieno[3,4-*d*] imidazole-4-yl)pentanamido)hexyl)carbamoyl)phenoxy)ethyl)-1*H*-1,2,3-triazol-4-yl)-3-hydroxypicolinoyl)glycine (Probe **12**). To a solution of **11** (58.0 mg, 0.1mmol) in dry DMF (2 mL) was added dropwise a solution of fluorescein isothiocyanate (FITC) (38.9 mg, 0.1 mmol) in dry DMF (1 mL), TBTU (48.2 mg, 0.15 mmol), and DIPEA (94 μL, 0.2 mmol). After 3 h, the reaction mixture dropped into ice water (3 mL). The resultant precipitate was filtered and collected. The crude product was dissolved in THF (6 mL). 1M LiOH solution (4 mL) was added and the mixture was stirred for 4 h at the room temperature. The residue was neutralized by dilute hydrochloric acid to pH 2. The resultant precipitate was filtered, collected, and then recrystallized by DCM to give Probe **12** as a yellow solid (39.0 mg, 41%). mp: 176.1-179.5 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.43 (bs, 1H), 10.23 (s, 2H), 9.37 (s, 1H), 8.97 (s, 1H), 8.72 (s, 1H), 8.46 – 8.17 (m, 2H), 8.14 (s, 1H), 7.84 (s, 1H), 7.78 (s, 1H), 7.65 (s, 1H), 7.51 (d, *J* = 8.5 Hz, 1H), 7.20 (m, 2H), 6.76 (s, 2H), 6.60 (m, 4H), 4.94 (s, 2H), 4.59 (s, 2H), 4.01 (s, 2H), 3.47 (s, 2H), 3.23 (s, 2H), 1.47 (m, 4H), 1.25 (m, 4H).^{13}C NMR (100 MHz, DMSO-d_6) δ 180.82, 170.98, 169.15, 169.05, 164.02, 160.14, 160.08, 157.80, 154.52, 152.47, 152.42, 143.22, 137.26, 137.23, 132.34, 131.88, 131.81, 130.64, 130.09, 129.58, 129.55, 126.07, 125.42, 124.63, 121.47, 115.42, 113.45, 113.17, 110.31,

102.72, 67.61, 49.72, 44.11, 41.16, 29.46, 29.33, 28.76, 27.42, 26.68, 25.99. HRMS (ESI): calcd for $C_{46}H_{41}ClN_8O_{11}S$, $[M+H]^+$, 949.2377, found 949.2375. (80% methanol in water): t_R = 1.616 min, 98.88%.

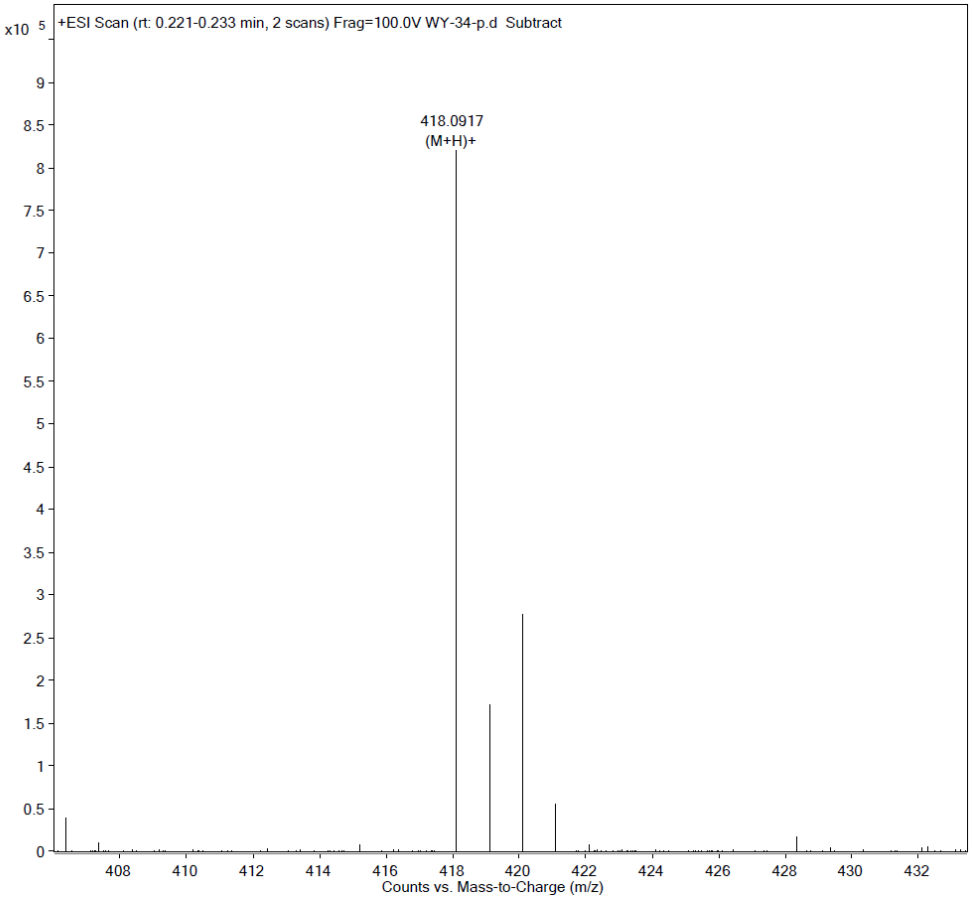
Spectra (^{1}H NMR, ^{13}C NMR, and HRMS) of key intermediates and target compounds



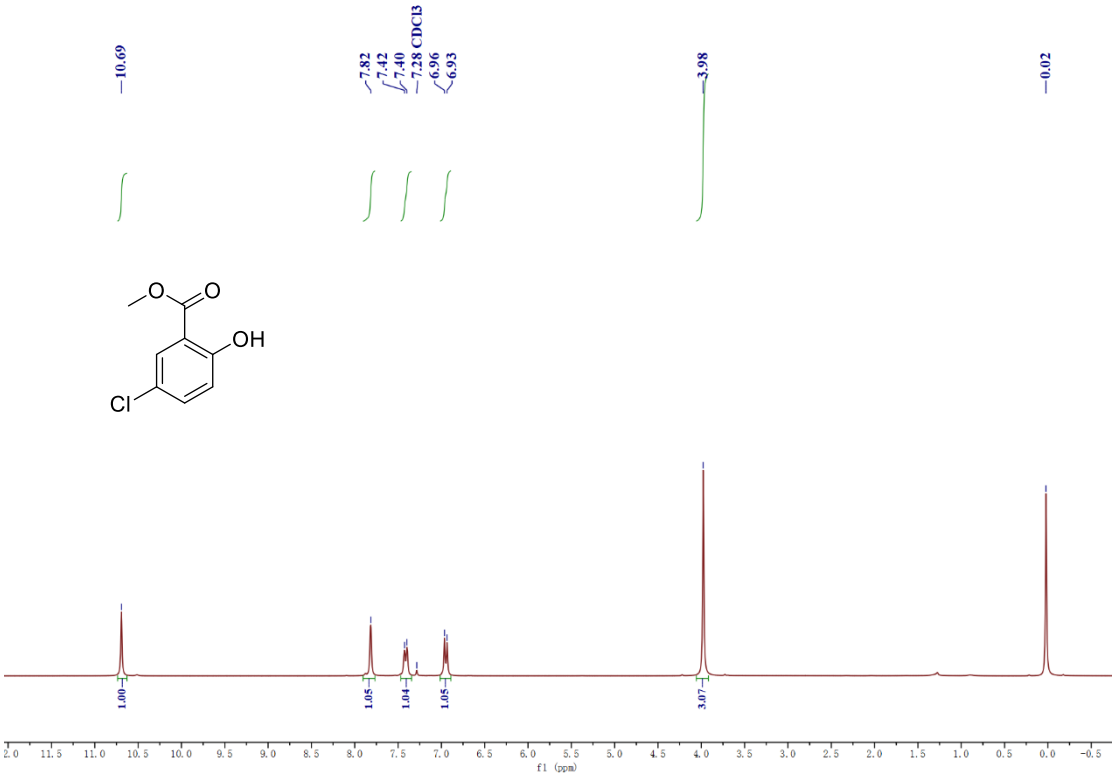
^{1}H NMR (300 MHz) of **1** in DMSO



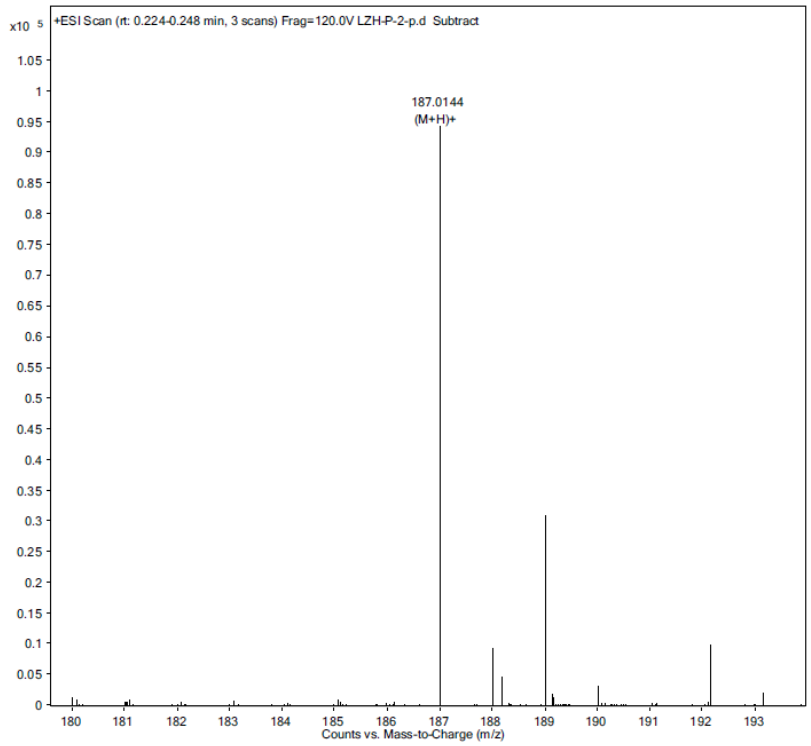
^{13}C NMR (75 MHz) of **1** in DMSO



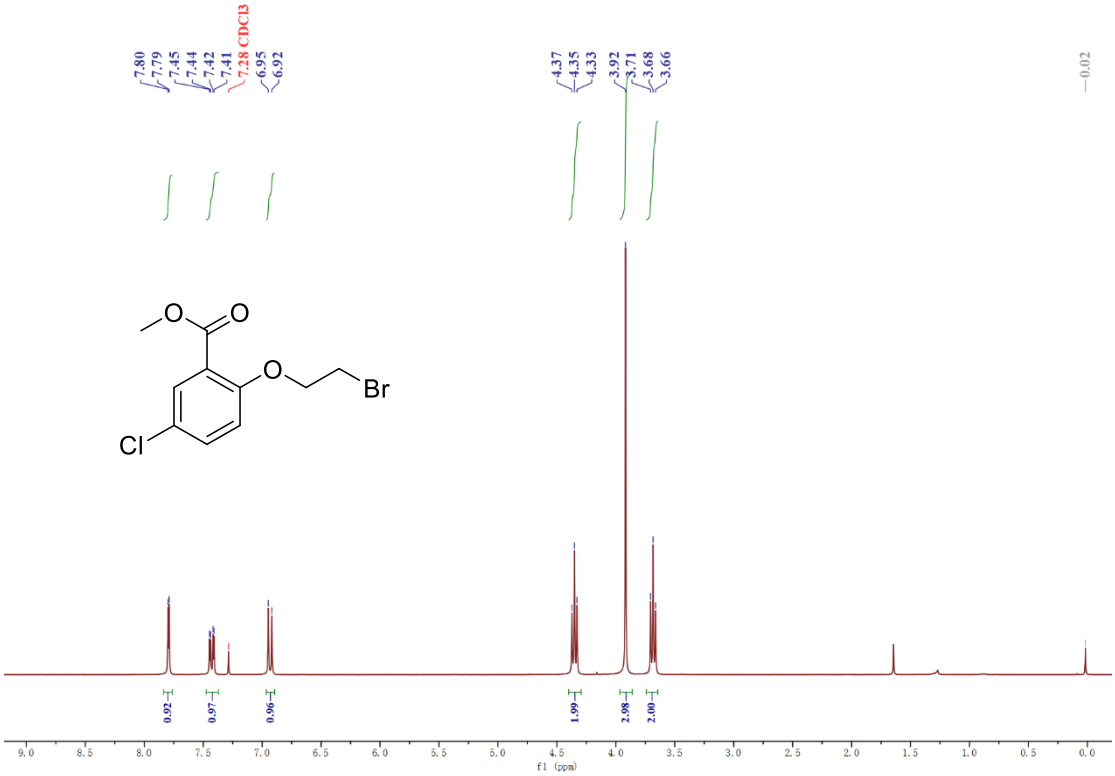
HR-MS (ESI)



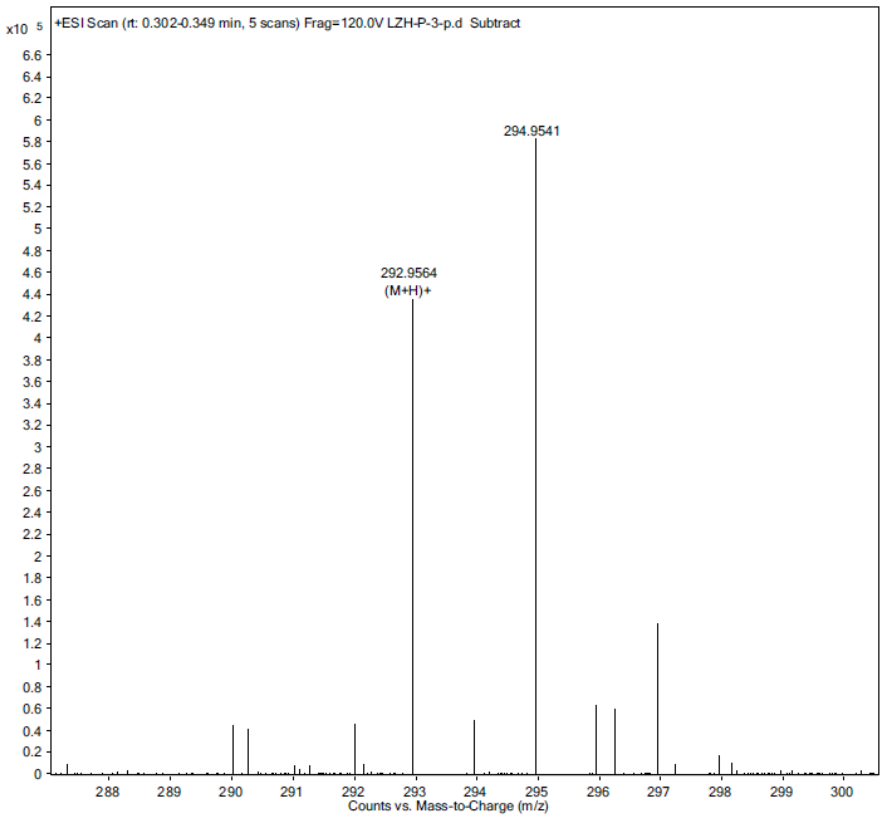
^{1}H NMR (300 MHz) of **2** in $CDCl_3$



HR-MS (ESI)



^{1}H NMR (300 MHz) of **3** in $CDCl_3$



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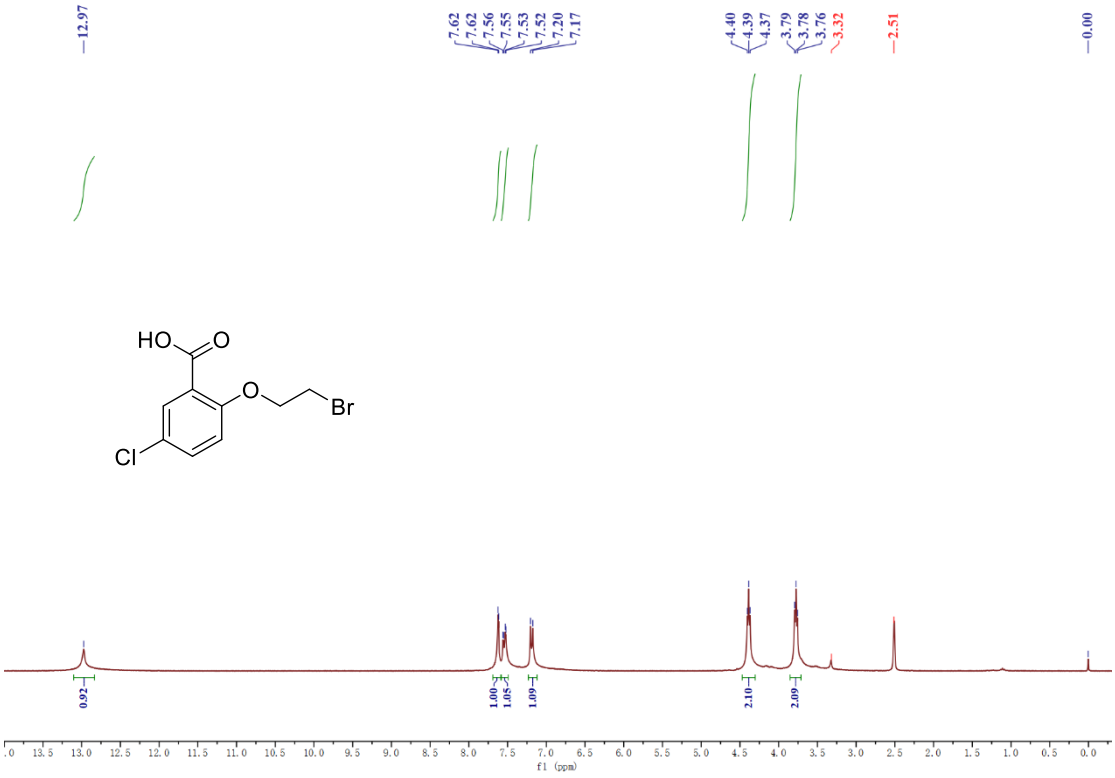
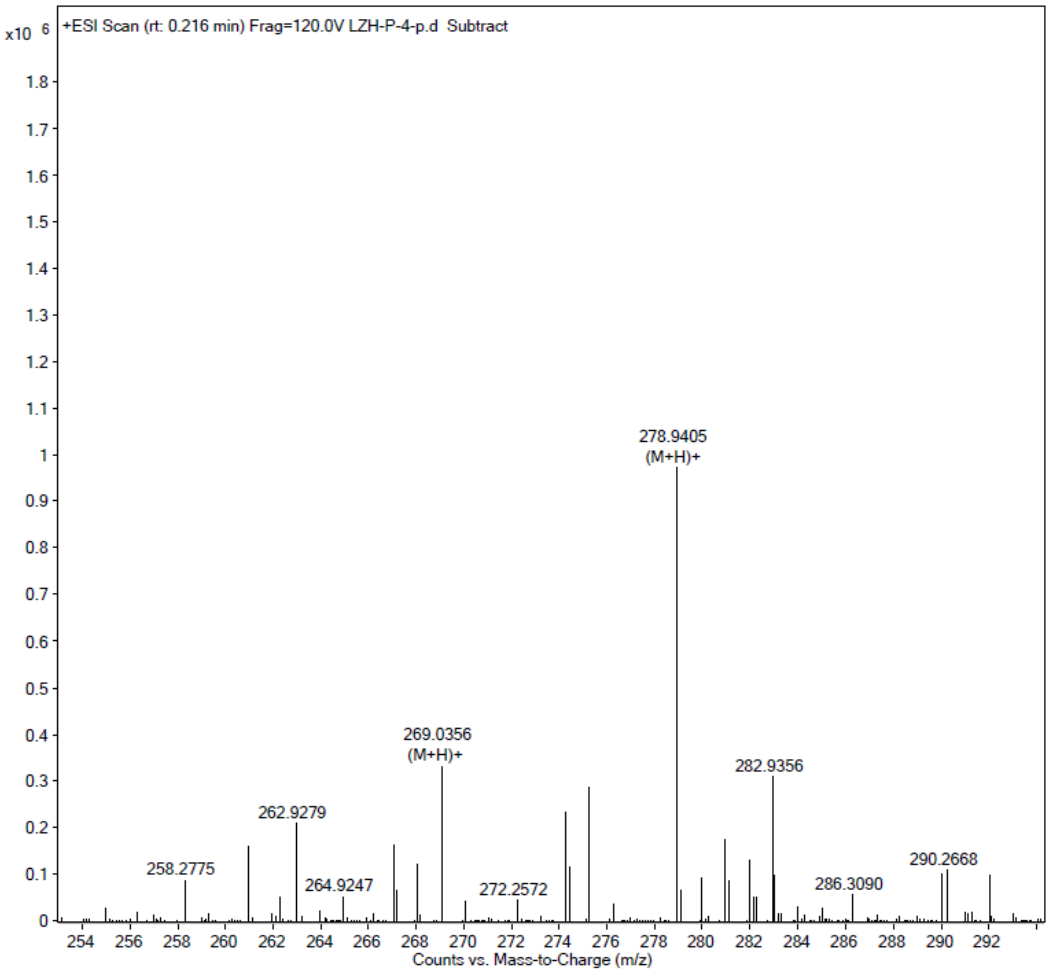
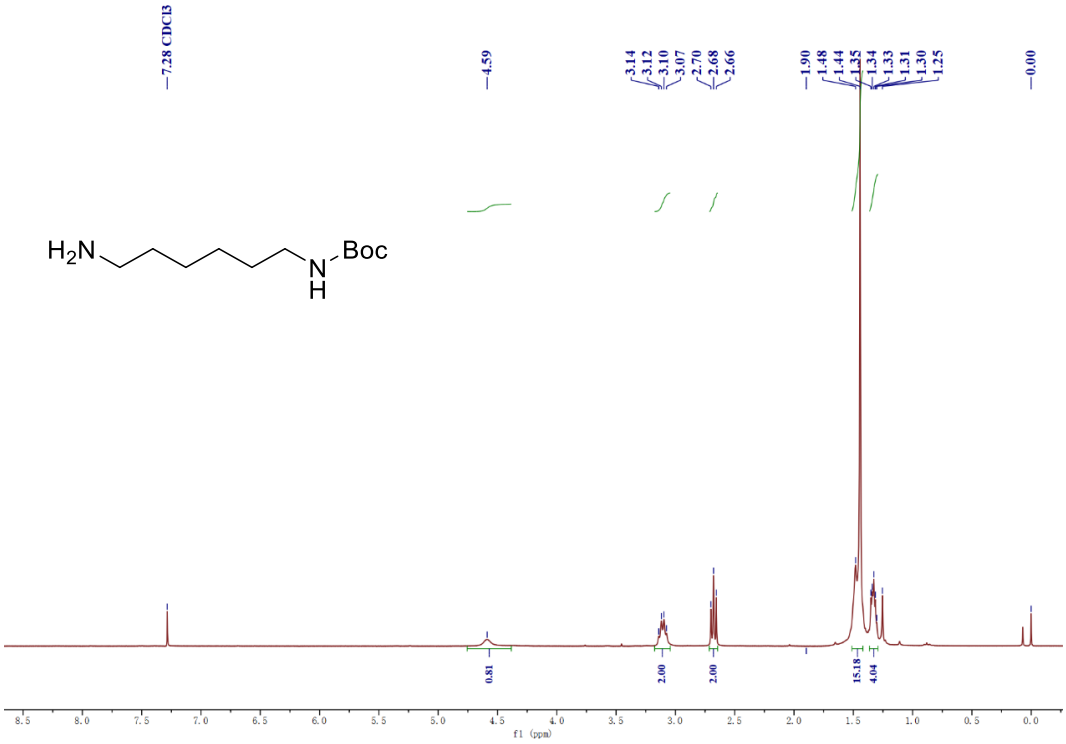


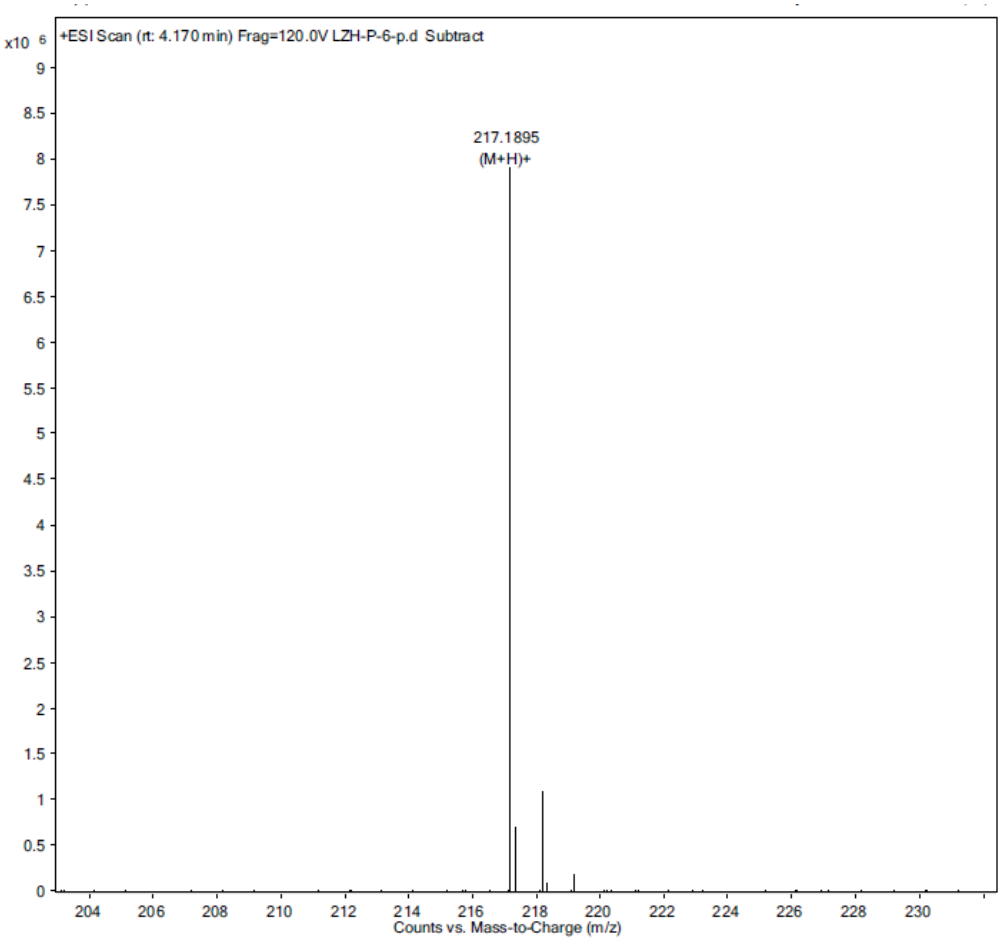
^{1}H NMR (300 MHz) of **4** in DMSO



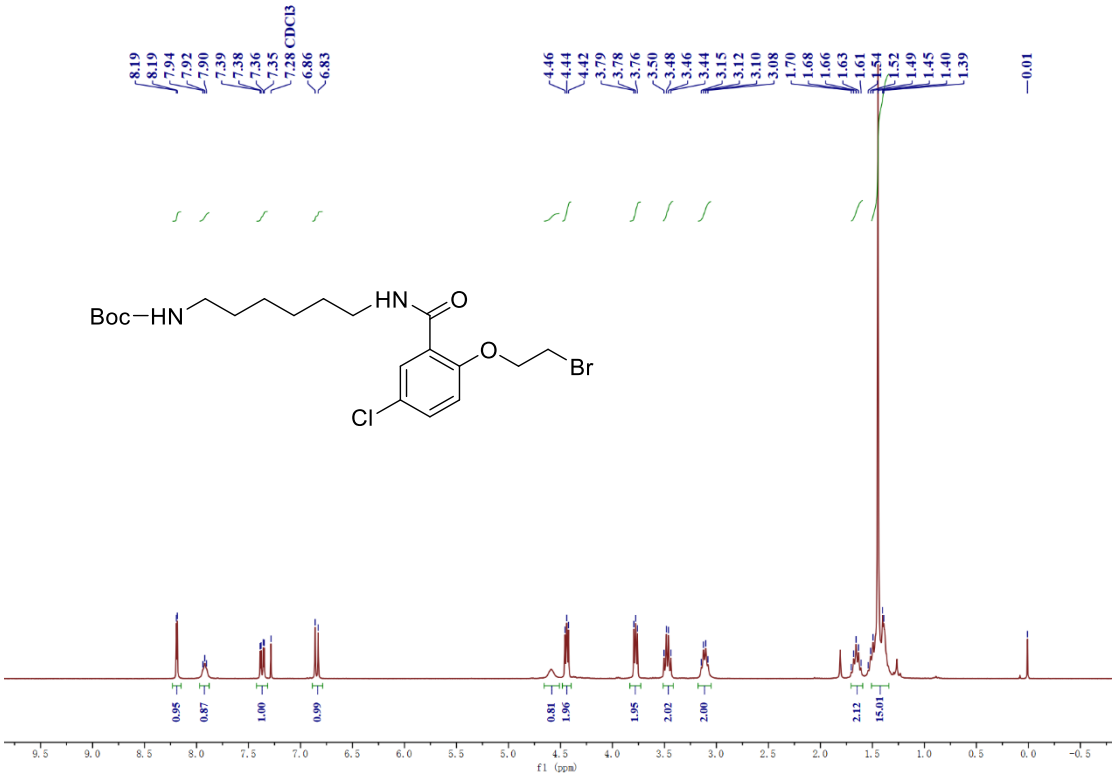
HR-MS (ESI)



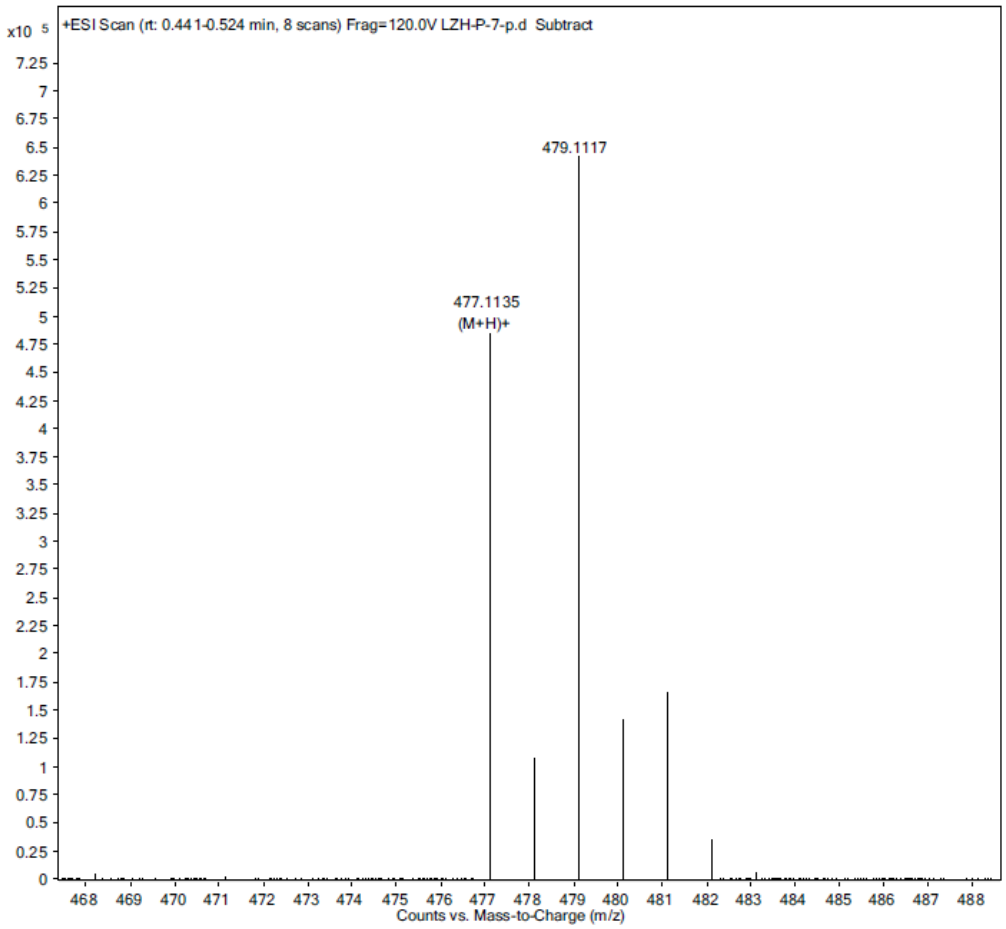
^{1}H NMR (300 MHz) of **5** in $CDCl_3$



HR-MS (ESI)



^{1}H NMR (300 MHz) of **6** in $CDCl_3$



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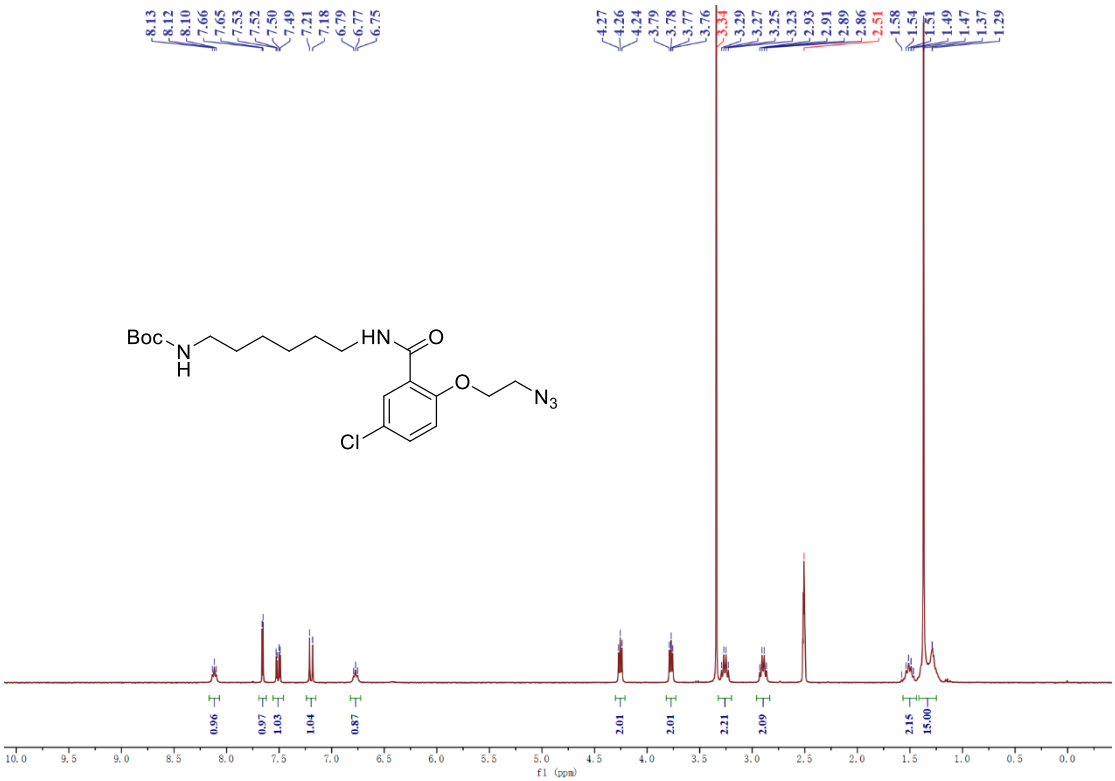
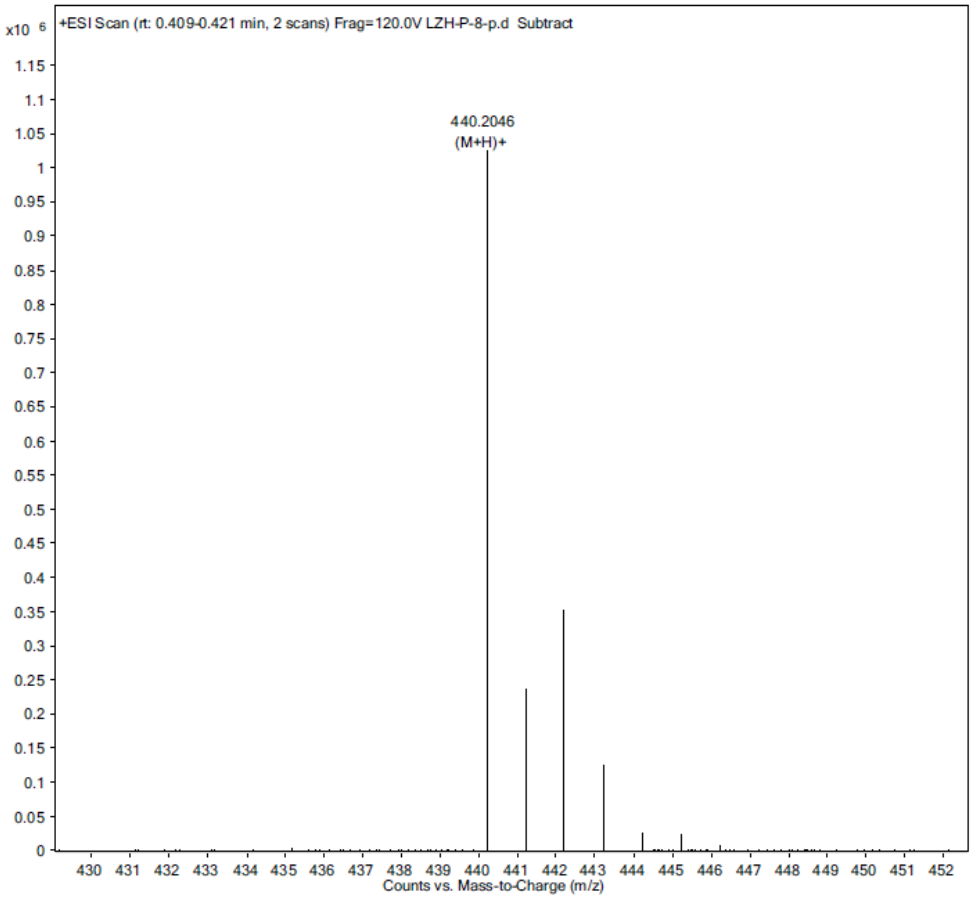
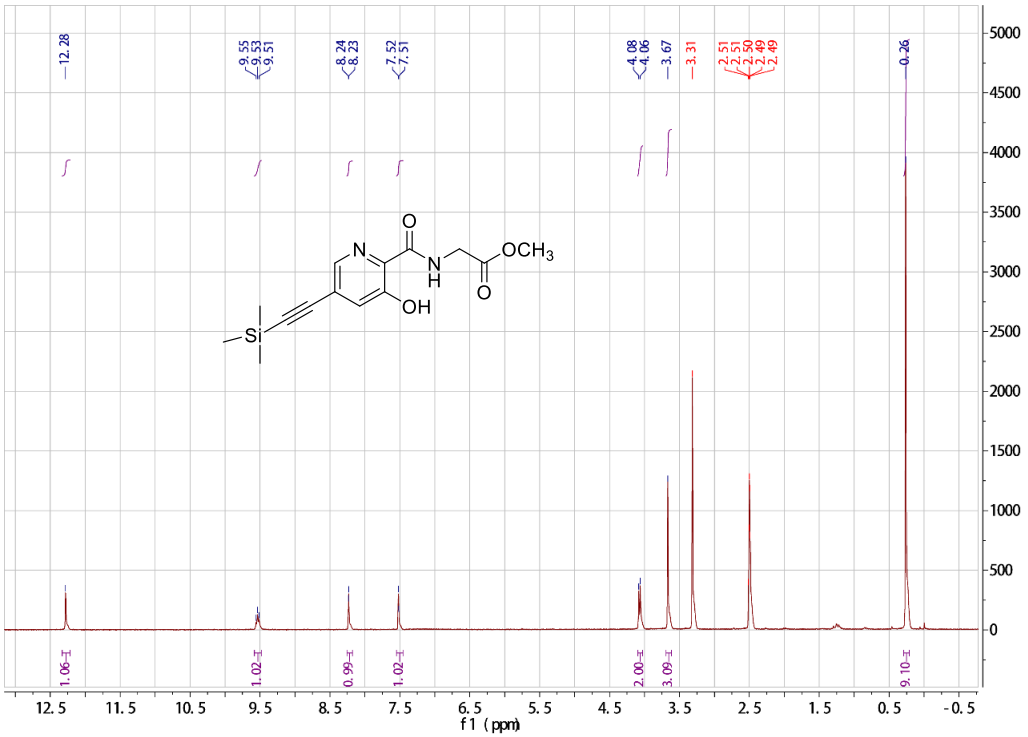


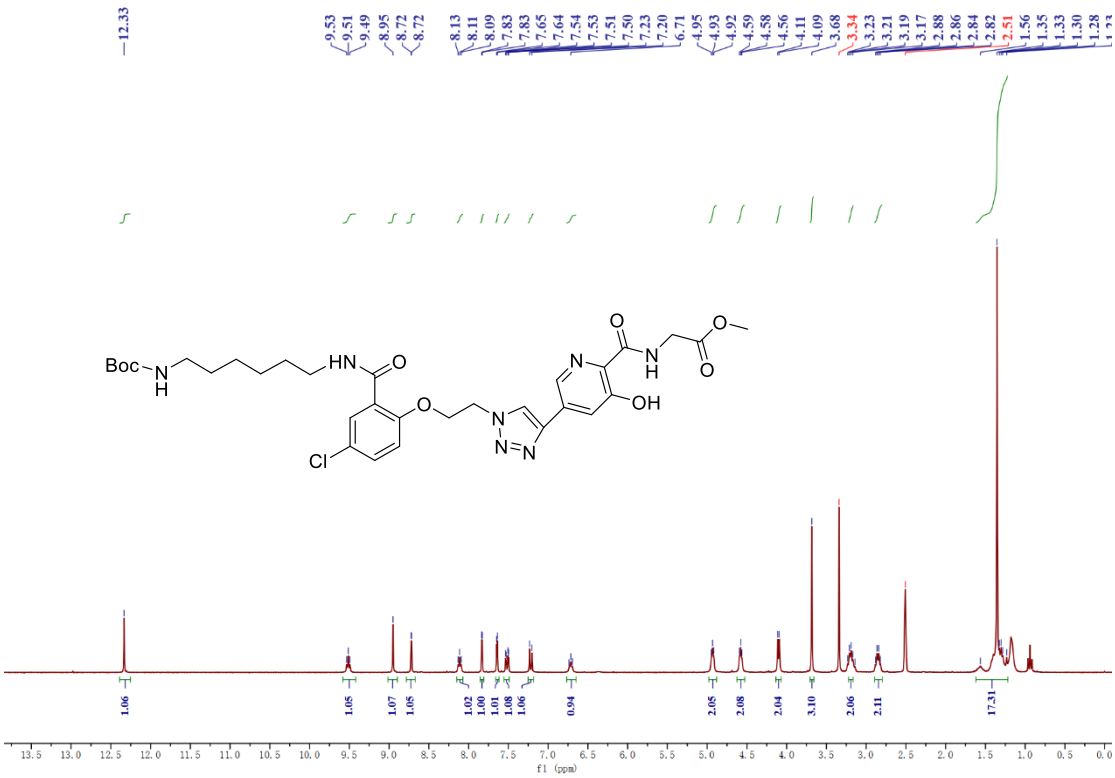
^{1}H NMR (300 MHz) of **7** in DMSO



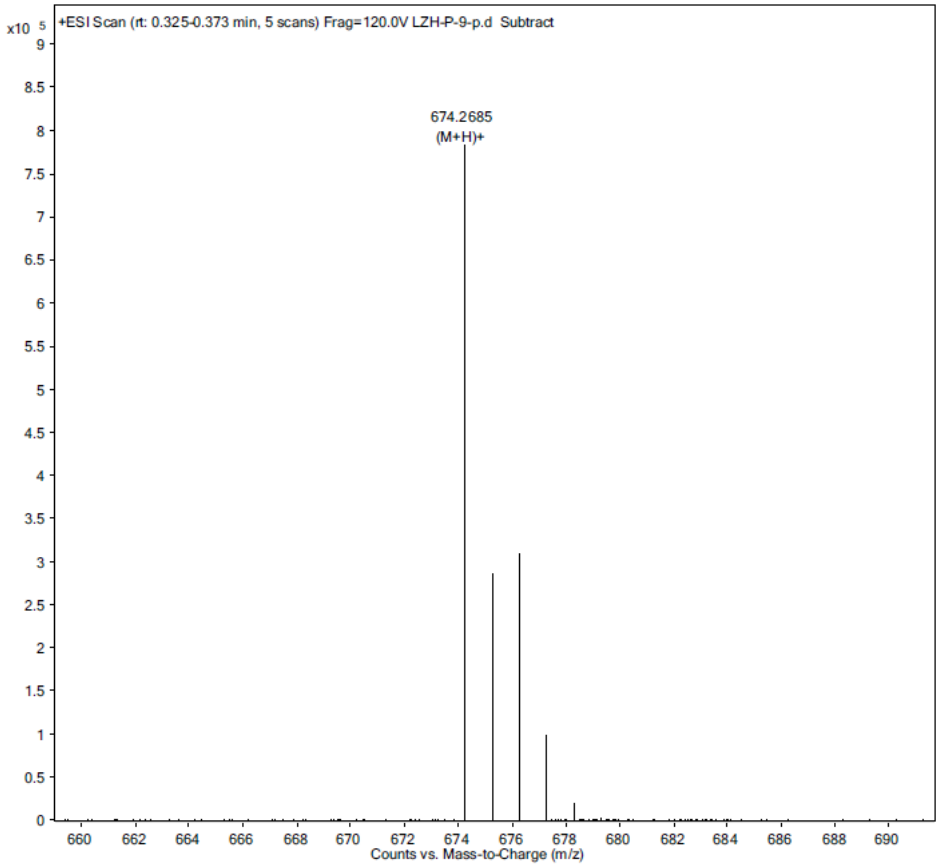
HR-MS (ESI)



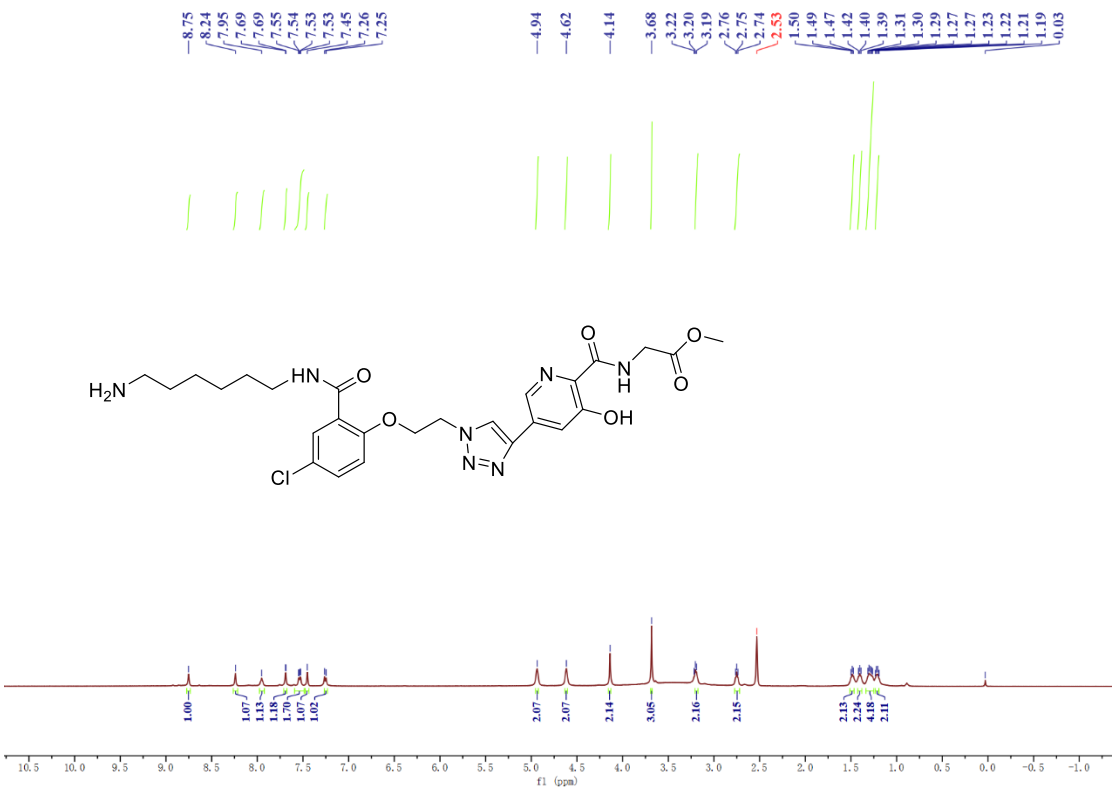
^{1}H NMR (300 MHz) of **8** in DMSO



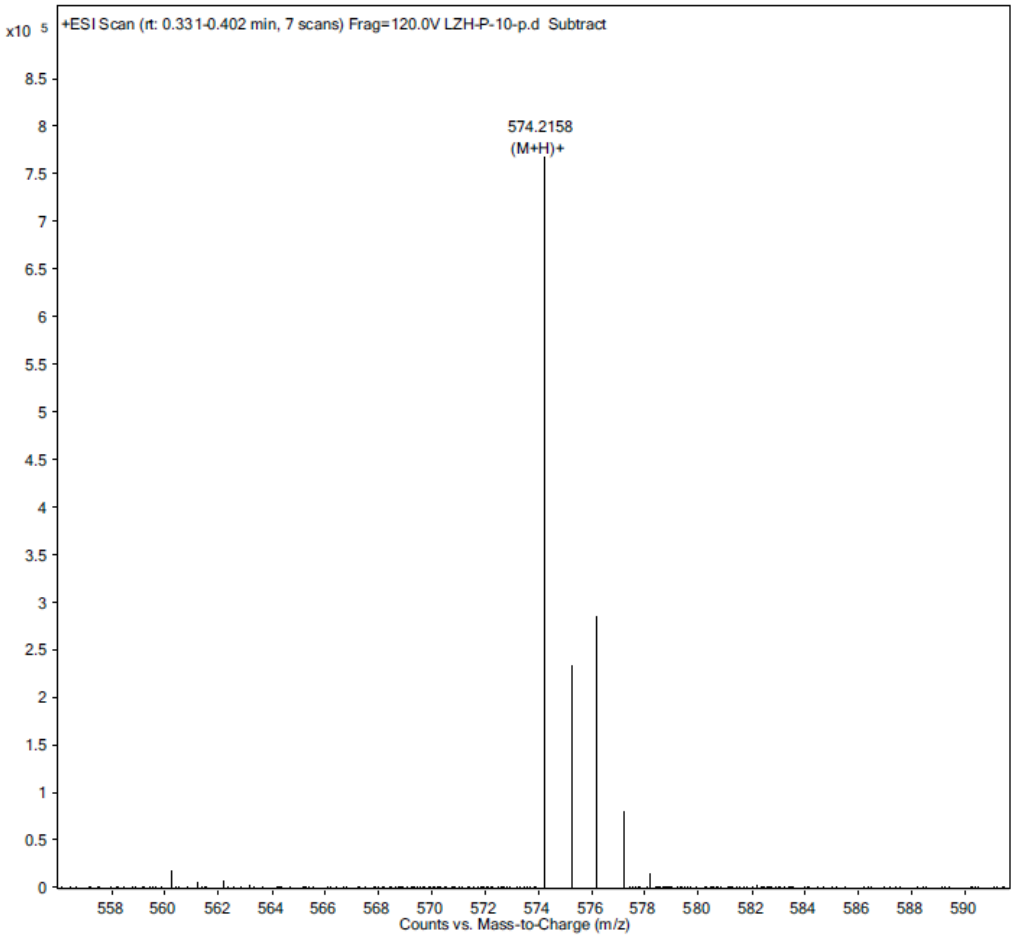
^{1}H NMR (300 MHz) of **9** in DMSO



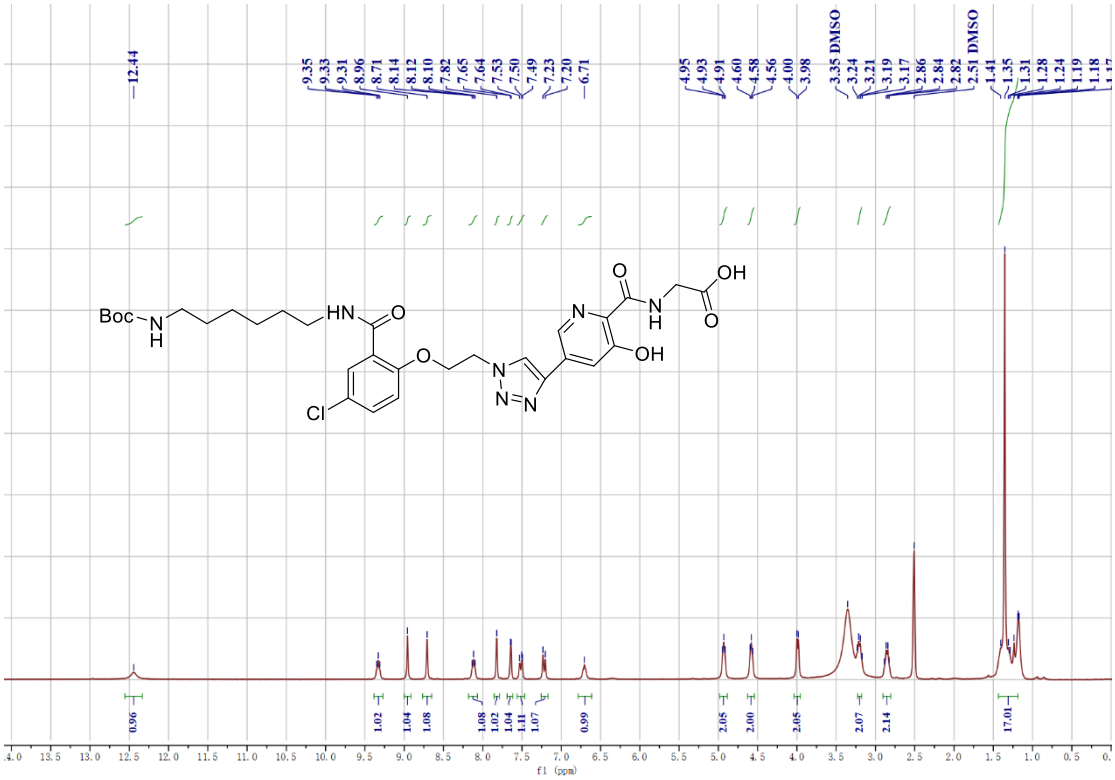
HR-MS (ESI)



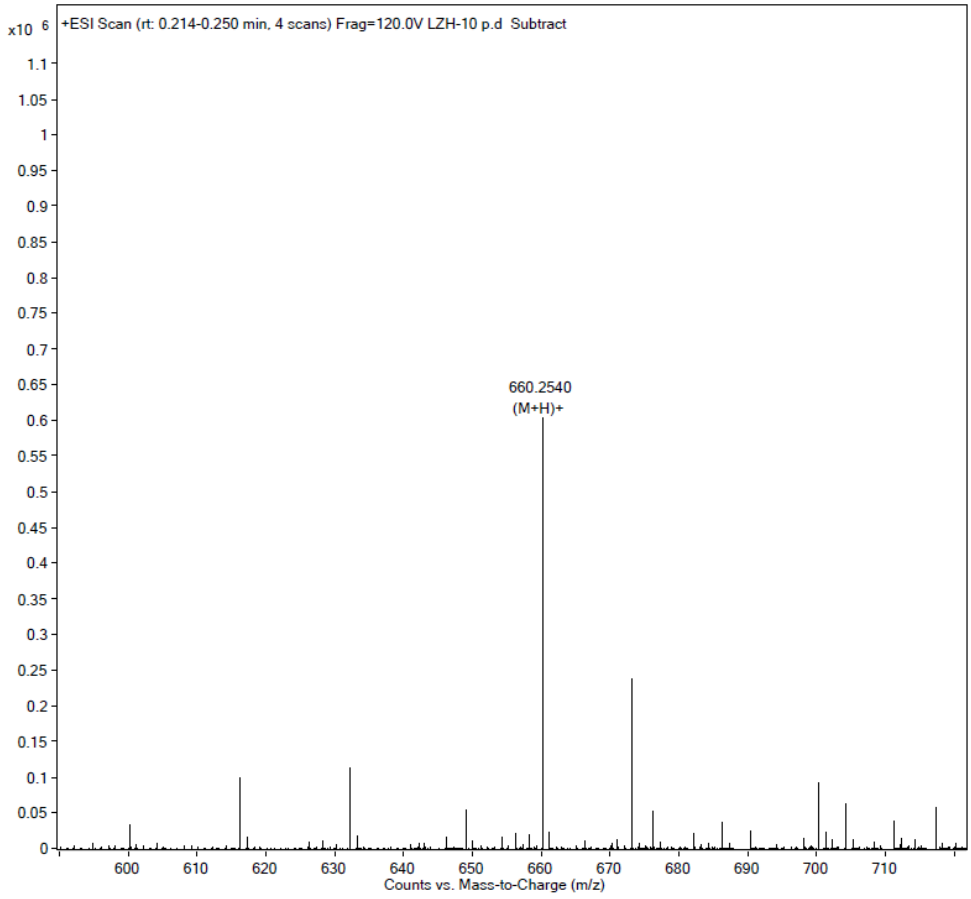
^{1}H NMR (500 MHz) of **11** in DMSO



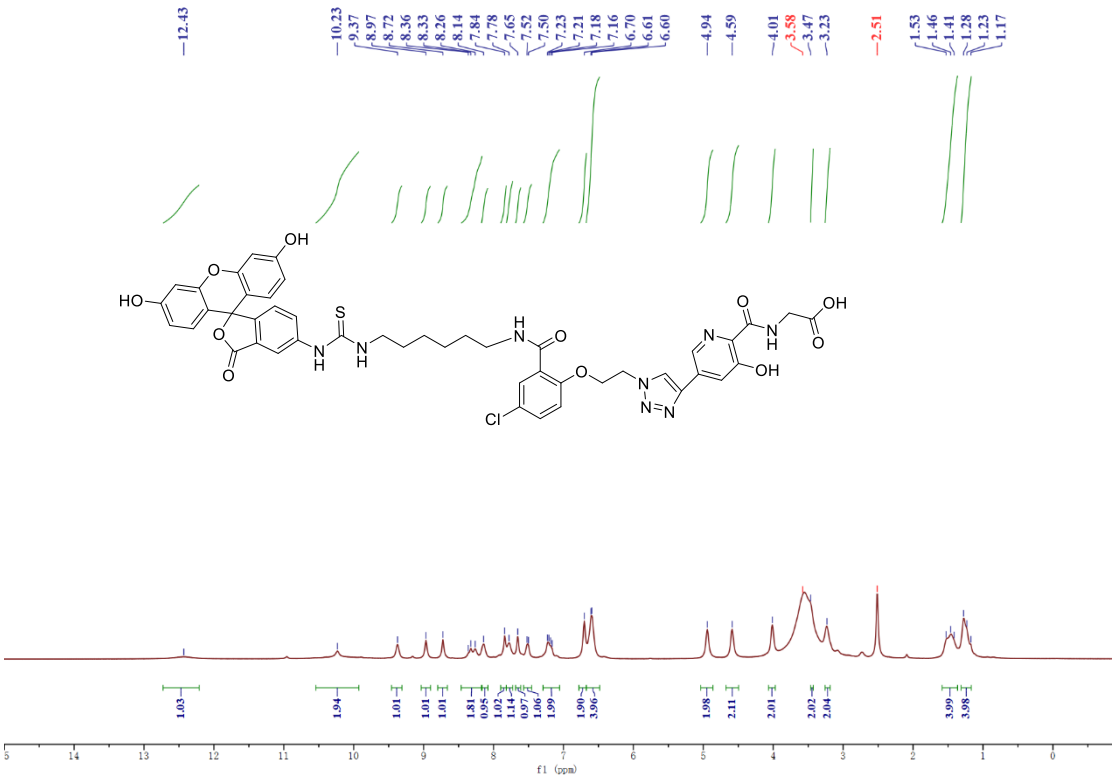
HR-MS (ESI)



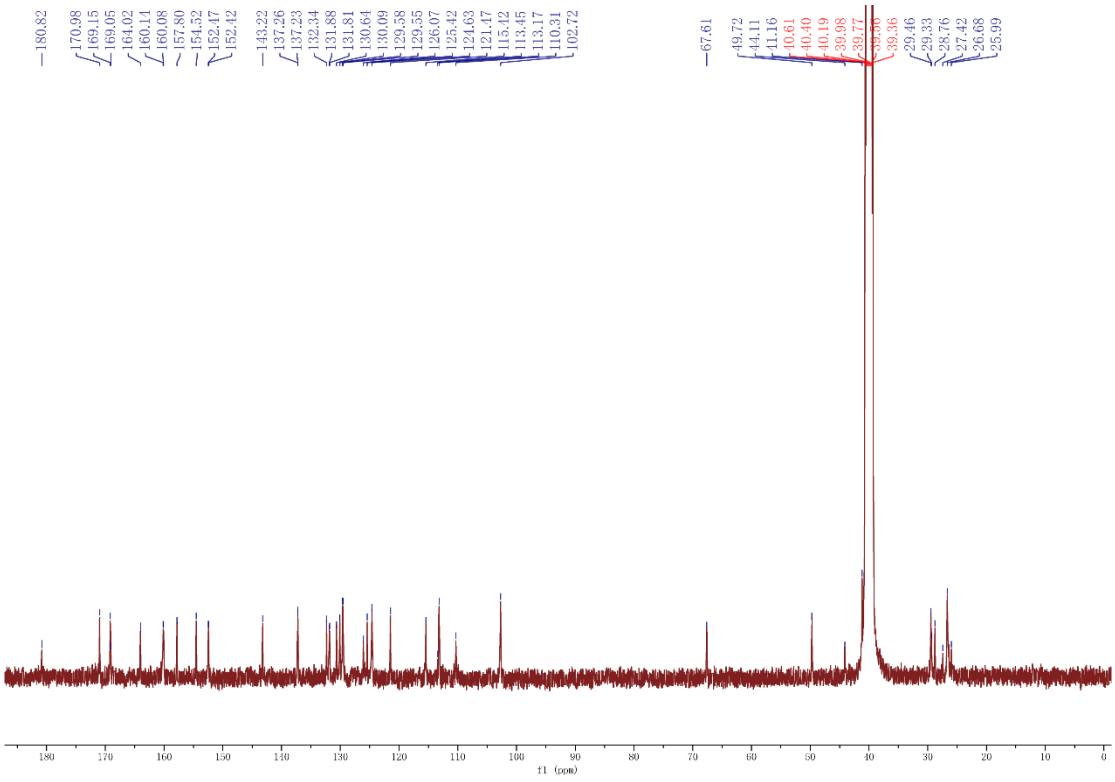
^{1}H NMR (300 MHz) of **10** in DMSO



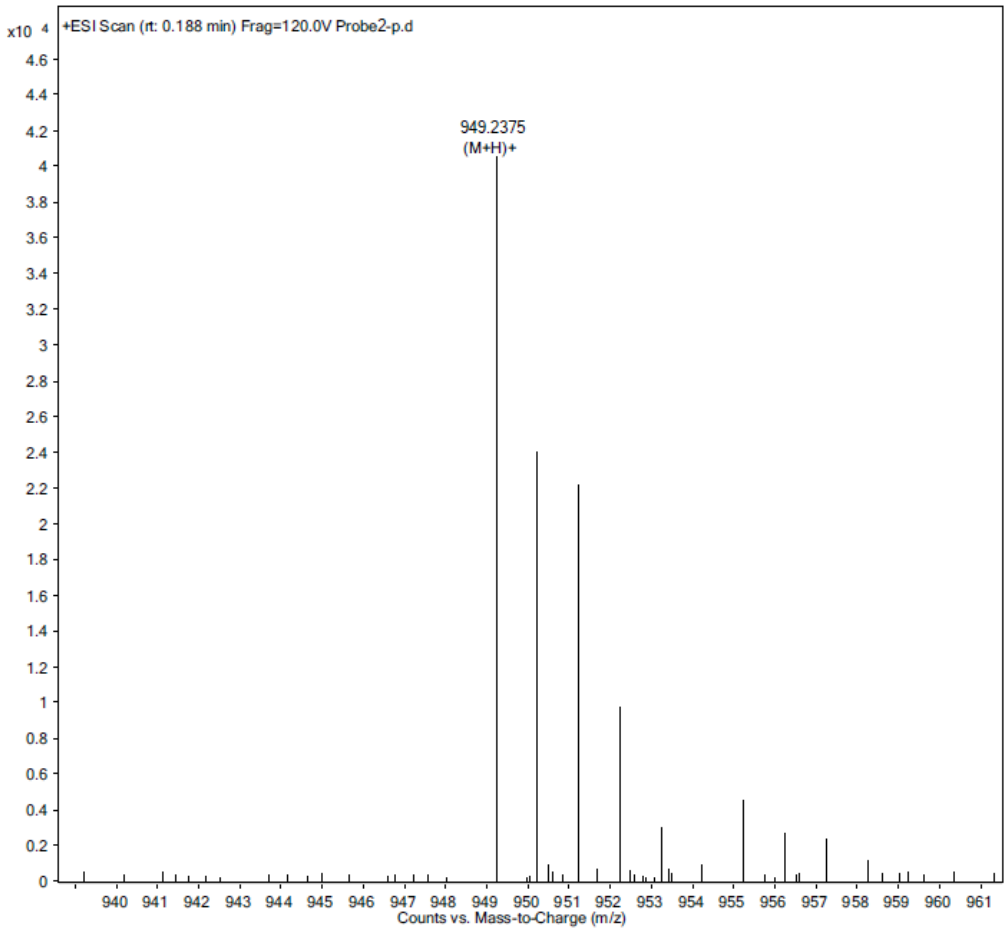
HR-MS (ESI)



^{1}H NMR (400 MHz) of **Probe 12** in DMSO



^{13}C NMR (100 MHz) of **Probe 12** in DMSO

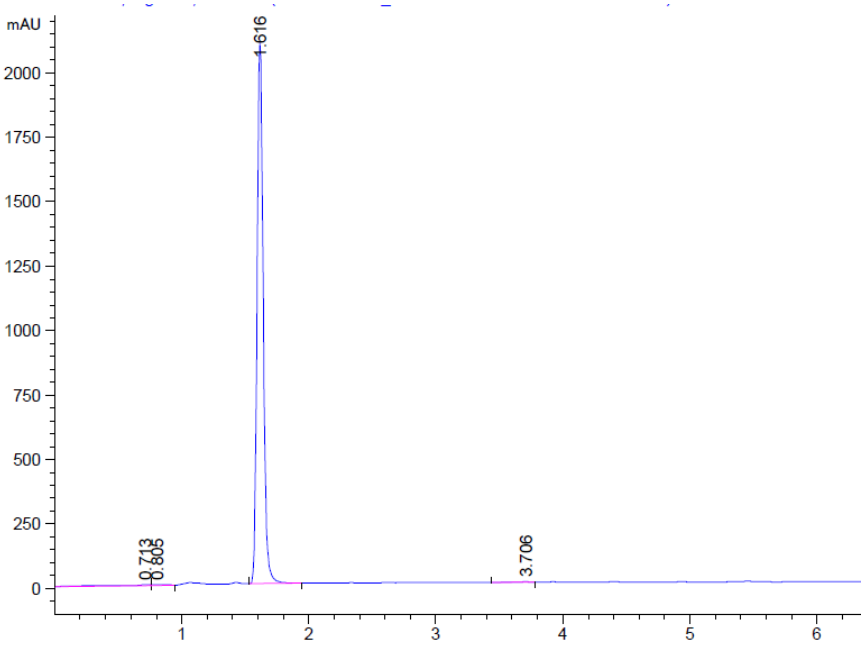

+ESI Scan (rt: 0.188 min) Frag=120.0V Probe2-p.d
x10 4
949.2375
(M+H)+
Counts vs. Mass-to-Charge (m/z)

HR-MS (ESI)

HPLC spectra assessment of purity for target compounds.

The purities of all final compounds were 98% or higher as determined by HPLC analysis. The HPLC assays were performed on an Agilent C18 (4.6 mm× 150 mm, 3.5 μm) column using a mixture of solvent methanol/water (80:20, v/v) at a flow rate of 0.5 mL/min and peak detection at 365 or 254 nm under UV.

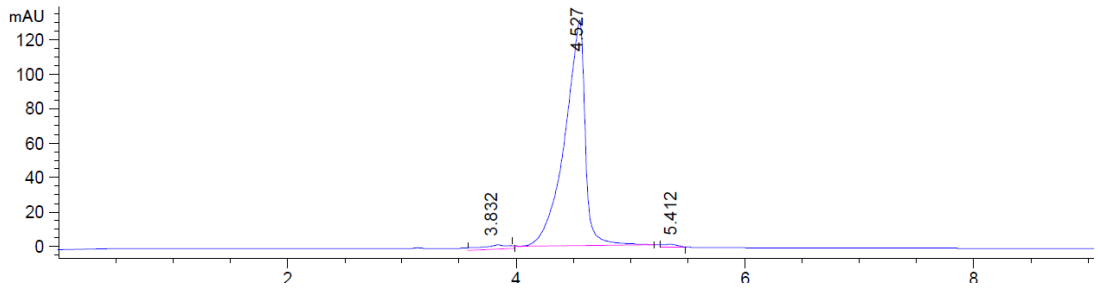
Probe **12** (98.88 %)



信号 1: DAD1 A, Sig=254,4 Ref=off

峰 #	保留时间 [min]	类型	峰宽 [min]	峰面积 [mAU*s]	峰高 [mAU]	峰面积 %
1	0.713	BV	0.1561	41.31433	3.44069	0.5748
2	0.805	VB	0.1175	24.29682	2.90777	0.3380
3	1.616	VB	0.0535	7107.96924	2101.62964	98.8858
4	3.706	BV	0.0866	14.47939	2.39441	0.2014
总量 :				7188.05977	2110.37252	

Compound **1** (98.40 %)



信号 2: DAD1 B, Sig=365,4 Ref=400,100

峰 #	保留时间 [min]	类型	峰宽 [min]	峰面积 [mAU*s]	峰高 [mAU]	峰面积 %
1	3.832	BV	0.0956	9.96711	1.21678	0.6163
2	4.527	BB	0.1862	1600.73042	128.86866	98.4076
3	5.412	VV	0.0763	15.93450	1.93679	0.9793
总量：				1626.63202	132.02223	

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