Supplementary information

Design, synthesis and biological evaluation of *2H*-[1,4]oxazino [2,3-*f*]quinazolin derivatives as potential EGFR inhibitors for Non-small cell lung cancer

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1. General information

The reagents were purchased and used without further purification. Column chromatography was performed on a 300-400 mesh silica gel column. The ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker 500 MHz Avance spectrometer with TMS and solvent signals allotted as internal standards. The chemical shifts are reported in ppm (δ). Splitting patterns are described as below: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets), ddd (three of doublets). ESI-MS spectra were obtained on an Esquire 6000 Mass Spectrometer. HRMS data were measured using a Bruker APEX IV Fourier transform ion cyclotron resonance mass spectrometer. The purity of the final compounds was determined with an Agilent 1260 HPLC system with a Agilent Zorbax Extend C-18 column, UV detector at 245 nm, with the purity of all compounds being higher than 95%. NCI-H1975 (NSCLC, EGFR L858R/T790M), NCI-H1563 (NSCLC, EGFR wild type) and 16HBE (human bronchial epithelial cells) cells were obtained from ATCC. The cells were maintained at 37 ºC in a 5% CO_2 incubator in RPMI 1640 (Invitrogen, CA, USA) containing 10% fetal bovine serum (FBS, Biochrom, AG).

2. Biological evaluation

2.1. MTT assay

The effects of indicated agents on the cell viability of H1975, H1563 and 16HBE cells were investigated by MTT assay. Exponentially growing cells were seeded at a density of 2 × 10^4 cells/mL into 96-well plates and cultured. After 12 h of incubation, 100 μL of medium was removed, and 100 μL of sample solution with different concentrations of inhibitor was added and then the cells were incubated for 48h. Control cells were treated with vehicle alone. During the last 4 h of incubation, the cells were exposed to methyl thiazolyl tetrazolium (MTT) assay (KeyGEN Biotech, Nanjing, China) solution (5 mg/mL, 20 mL per well). The generated formazan crystals were dissolved in 150 mL of dimethyl sulfoxide (DMSO), and the absorbance was measured on a microplate reader (Thermo Multiskan MK3, MA, USA) at a wavelength of 490 nm. The data was calculated using SPSS 17.0 software (SPSS, IL, USA) to determine the IC_{50} values of the target compounds.

H1975 cells were plated into 96-well plates at a density of 2 × 10^3 cells per well. The cell viability was also measured by the MTT assay, cells were treated with indicated concentrations of compound **4a** (0.5 μM) at 0, 12, 24, 36, 48 and 60 h, control cells were treated with vehicle alone. Absorbance was measured on a microplate reader (Thermo Multiskan MK3, MA, USA) at a wavelength of 490 nm. The results were expressed as means ± SD.

2.2. Colony formation assay

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H1975 and H1563 cells were seeded into 6-well plate at a density of 500 cells per well. H1975 cells were treated with indicated concentrations of compound **4a** and erlotinib (0.5 μM) for 12 h, respectively. H1563 cells were treated with indicated concentrations of compound **4a** (0.5 μM) for 12 h. The medium was then replaced with drug free medium and cells were further incubated for about 7 days. Cells were fixed with 75% ethanol for 30 min and stained with 0.2% crystal violet. The number of clones was then counted. Each experiment was repeated in triplicate.

2.3. Transwell assay

Migration: 3×10^4 H1975 and H1563 cells, were respectively suspended in 200 μl serum-free RPMI 1640 medium and added to the upper chamber of 8.0 μm pore size transwell apparatus (COSTAR transwell, Corning Incorporated, MA, USA). H1975 cells were treated with indicated concentrations of compound **4a** and erlotinib (0.5 μM). H1563 cells were treated with indicated concentrations of compound **4a** (0.5 μM). Cells that migrated to the lower surface of the membrane were stained with crystal violet and counted in three independent high-power fields (×100) after incubation for 20 hours.

Invasion: the upper chamber was coated with a layer of extracellular matrix. H1975 and H1563 cells (1×10^5) were seeded in the upper chamber of a transwell apparatus coated with Matrigel (BD Biosciences, CA, USA). H1975 cells were treated with the indicated concentrations of compound **4a** and erlotinib (0.5 μM). H1563 cells were treated with indicated concentrations of compound **4a** (0.5 μM). Cells that invaded to the lower membrane surface were stained with crystal violet and counted in three independent high-power fields (×100) after incubation for 24 hours.

2.4. Wound healing assay

The cancer cells were cultured in 6-well plates at 37℃ for 48 h. Wounds were created in the cell monolayer and washed with PBS to remove cell debris, then the H1975 cells were treated with compound **4a** and erlotinib (0.5μM), H1563 cells were treated with indicated concentrations of compound **4a** (0.5μM). Cellular motility was monitored and photographed. Microscope images of the scratched cultures were captured at the beginning and at various intervals thereafter. The distance travelled from the initial scratch site was measured, and the migration distance was quantified.

2.5. Cell Cycle Analysis

The H1975 and H1563 cells were incubated at a density of approximately 2×10^5 cells/well in 6-well plates, cells were serum starved for 12 hours for synchronisation and then re-stimulated with 10% FBS. H1975 cells were treated with indicated concentrations of compound **4a** and erlotinib (0.5 μM) for 24 hours, H1563 cells were treated with indicated concentrations of compound **4a** (0.5 μM) for 24 hours. Cells were fixed with 70% ethanol and processed for cell cycle detection using the Cell Cycle Detection Kit (KeyGen Biotech, Nanjing, China). Cells were then sorted by a FACSCalibur (BD Biosciences, San Jose, CA) and analysed by the Modfit software (Verity Software House, ME, USA).

2.6.Protein preparation and western blot

Protein samples from H1975 cells and H1975 cells treated by compound **4a** were collected and Western blots were performed. Antibodies were diluted according to the manufacturer·s instructions. Primary antibodies included MMP2 (Cat: BS1236, Bioworld Tech, MN, USA), MMP9 (Cat: BS1241, Bioworld Tech, MN, USA), cyclin D1 (Proteintech, IL, USA), cyclin E1 (Proteintech, IL, USA) and GAPDH (Cat: AF0003, Beyotime Biotech, Jiangsu, China).

2.7. EGFR inhibitory assay

Kinase-Glo luminescent kinase assay is a homogeneous non-radioactive method for determining the activity of purified kinases by quantifying the amount of ATP remaining in solution following a kinase reaction. Target compounds and positive compound gefitinib were dissolved in DMSO as 10 mM stock solution, then dilute it to 100 μM with DMSO and transferred to the dose plate. The compounds was serially diluted with DMSO in 5-fold. Then each concentration was diluted 10-fold with reaction buffer (containing 25 mM HEPES, 10 mM $MgCl_2$, 100 μg/mL BSA, 0.01% TritonX-100, 2.5 mM DTT and adjusted pH to 7.4) to obtain a 10× final concentration. Transfer compounds with its concentration ranging from 10 μM to 0.0006 μM to assay plate for EGFR activity test with a volume of 1 μL/well. For HPE (hundred percent effect: No kinase and no compound, but containing ATP, substrate and 1% DMSO) and ZPE (zero percent effect: No compound but containing kinase, ATP, substrate and 1% DMSO) well, dilute 2 μL DMSO 10-fold with reaction buffer to obtain 10% DMSO solution. Then transfer it to the assay plat, 1 μL/well. Procedure for kinase reaction is: 1) Add 10× compound to the assay plate in a 384-well plate layout, 1 μL/well. For the HPE and ZPE wells, equal volume (1 μL/well) of 10% DMSO was added to the 384-well assay plate; 2) Add 2.5× kinase EGFR into the assay plate as 384-well plate layout, 4 μL/well. For HPE wells, an equal volume (4 μL/well) of assay buffer was added to the 384-well assay plate; 3) Centrifuge the assay plate with 1000 rpm for 1 min to mix them; 4) Pre-incubate the assay plate

at 30℃ for 30 min; 5) Mix equal volume of 4× ATP and 4× substrate to obtain 2× ATP-substrate mixtures; 6) Add 2× ATP-substrate mixture to the assay plate, 5μL/well; 7) Centrifuge the assay plate at 1000 rpm for 1 min to mix; 8) Incubate the plate for one hour at 30 ºC; 9) Kinase glo plus was added to each well (10 μL/well), and then incubated the assay plate for 20 min at 27 ºC; 10) Read luminescence signal with Envision. The raw data were analysed by Prism 5.0 and the inhibitory rate was calculated by the following formula: Compound inhibitory rate = ("compound" reading-ZPE)/(HPE-ZPE)*100%.

3. Docking study

The molecular docking of compound **4a** into the three-dimensional EGFR complex structure (PDB code: 4G5P, downloaded from the PDB) was performed using CDocker. Unwanted water and ligands were removed by the DS4.0. The structures of the molecules were drawn by Gaussian 03 software, then optimized the molecules to the minimum energy conformation used the semi-empirical AM1 method. Docking procedure was performed by AutoDock 4.0 software with the help of Autodock Tools.

4. The ^{1}H NMR, ^{13}C NMR and HRMS spectra of the target compounds

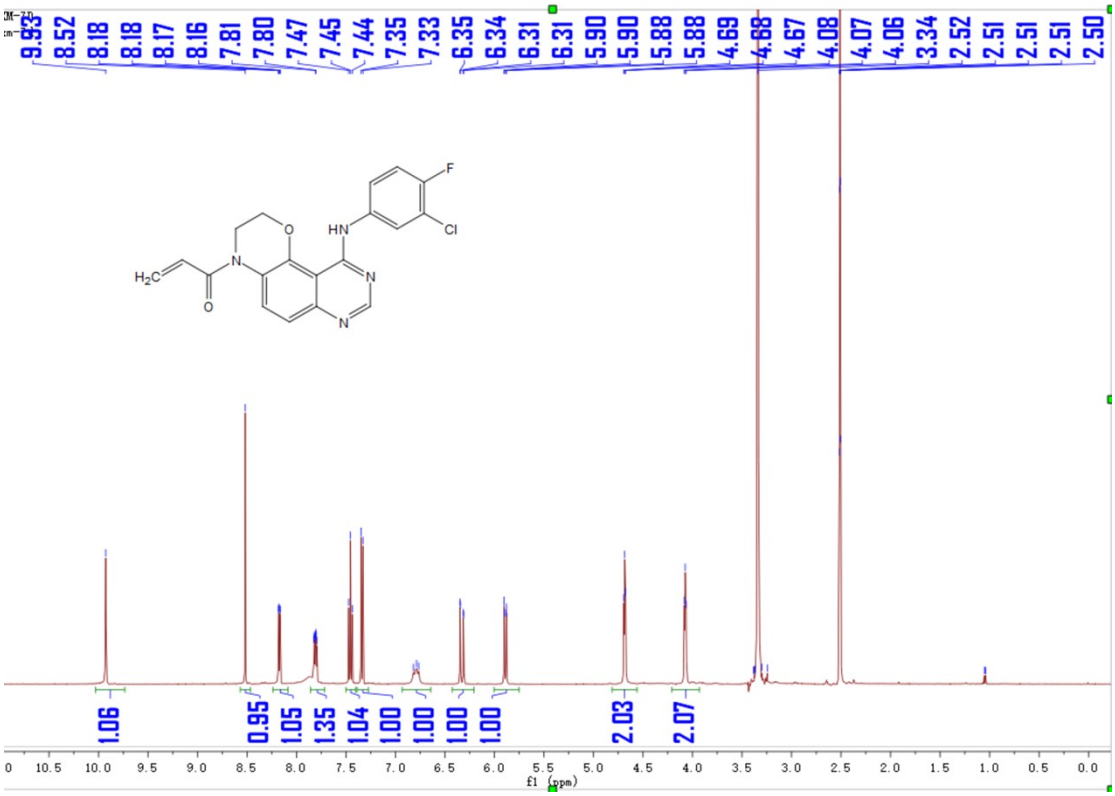


Fig. S1. ^{1}H NMR spectrum of compound **4a**

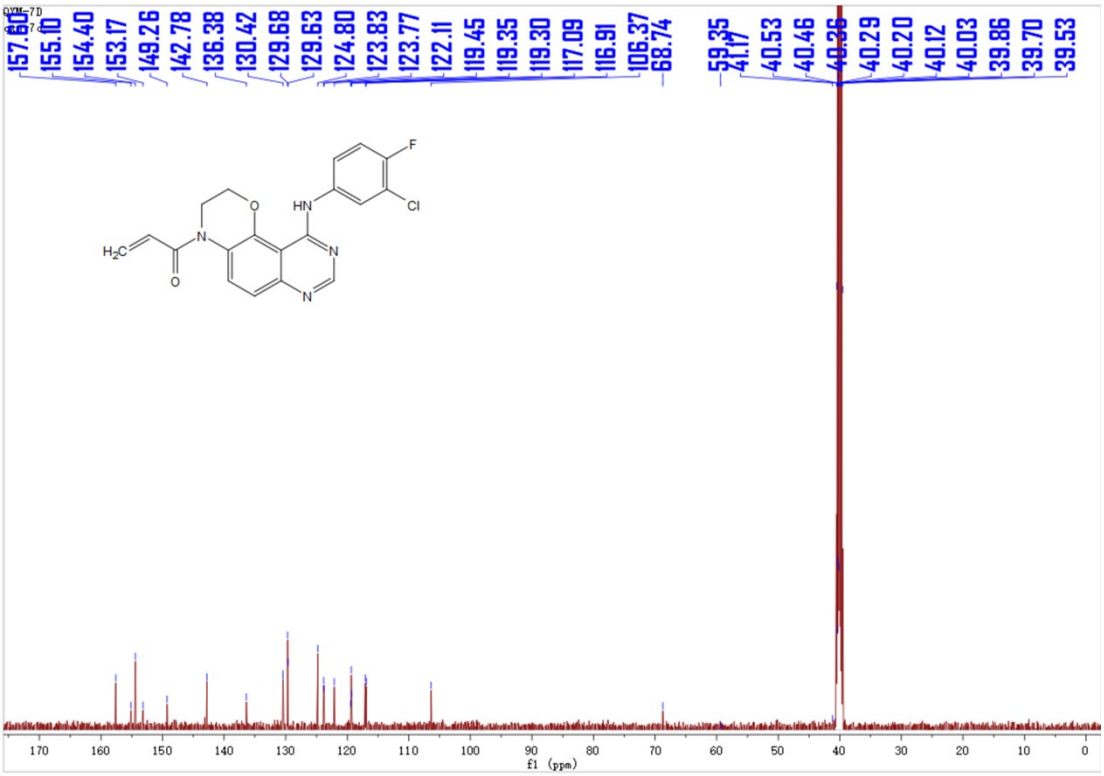


Fig. S2 ^{13}C NMR spectrum of compound **4a**

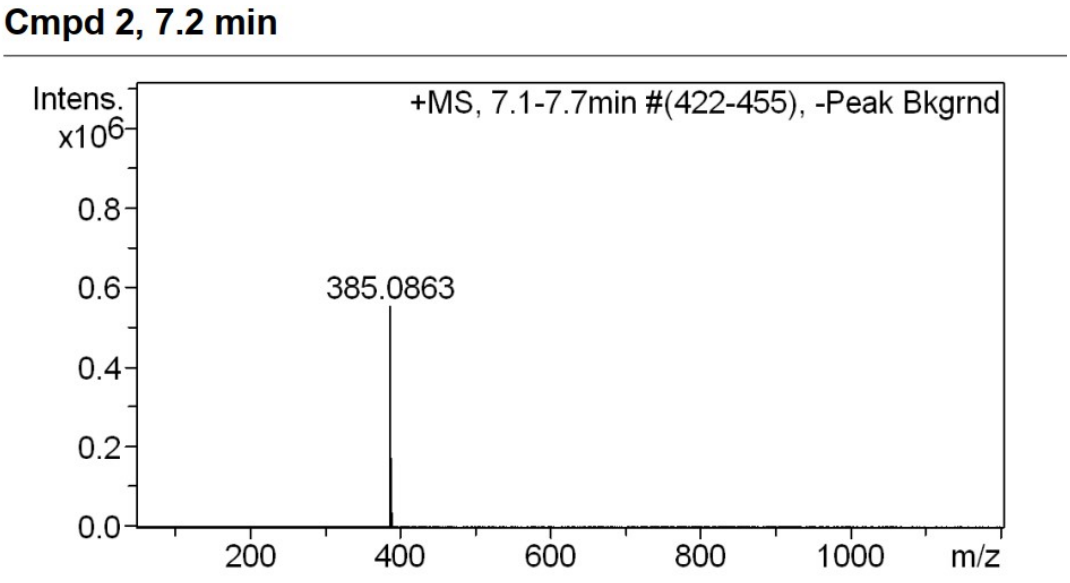


Fig. S3 HRMS spectrum of compound **4a**

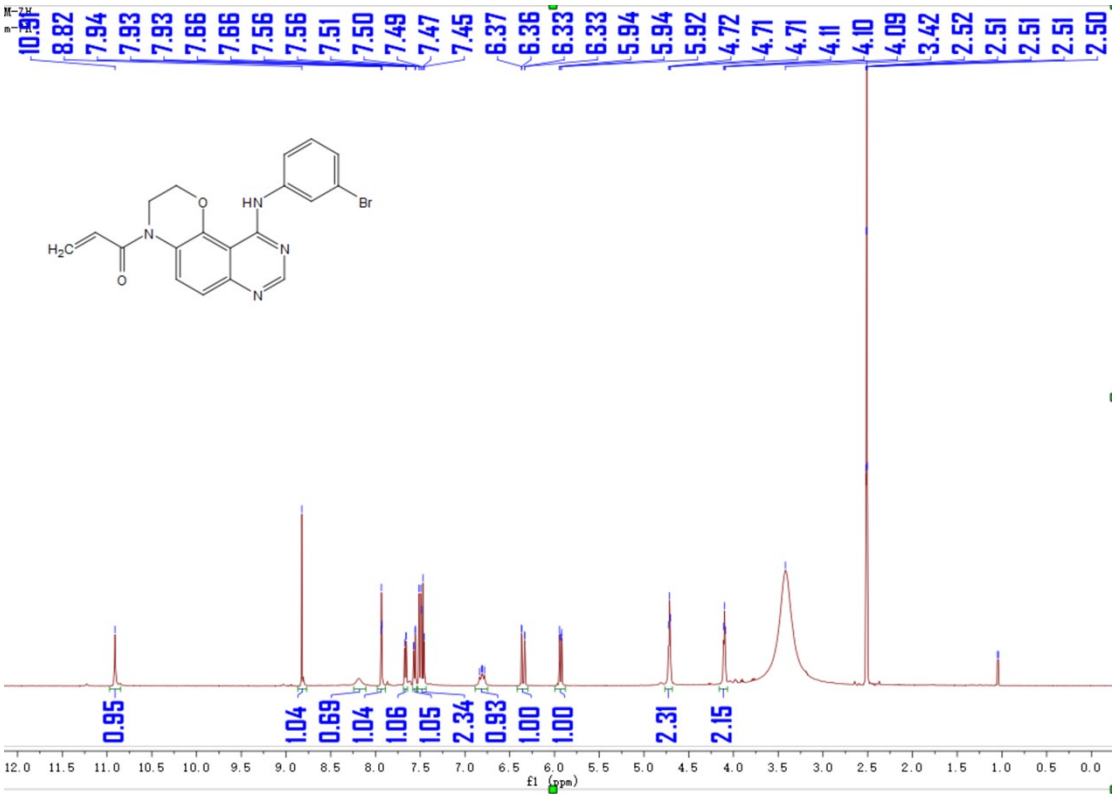


Fig. S4. ^{1}H NMR spectrum of compound **4b**

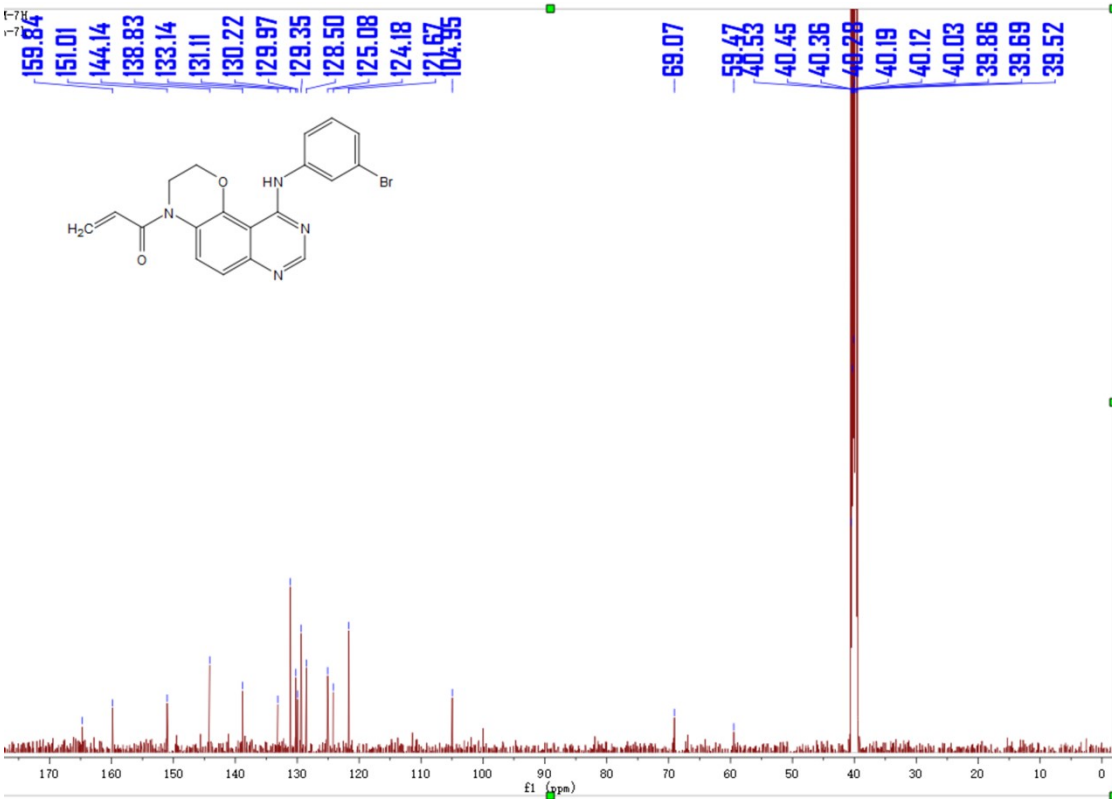


Fig. S5 ^{13}C NMR spectrum of compound **4b**

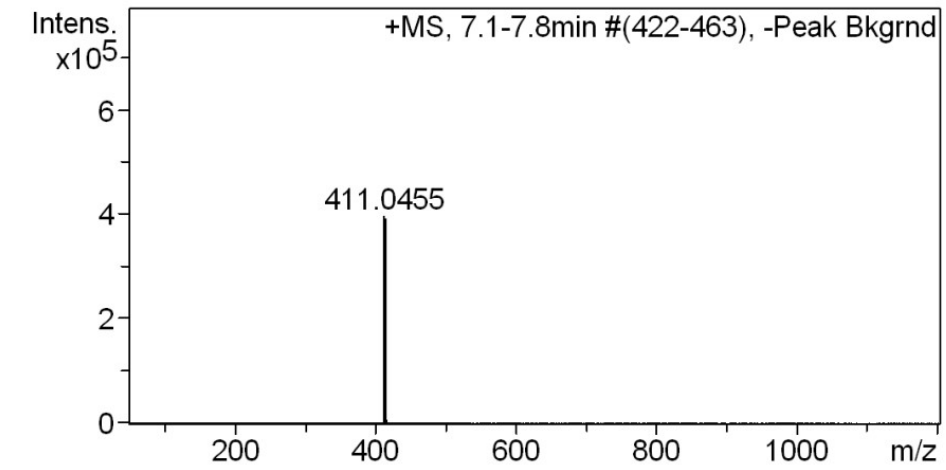


Fig. S6 HRMS spectrum of compound **4b**

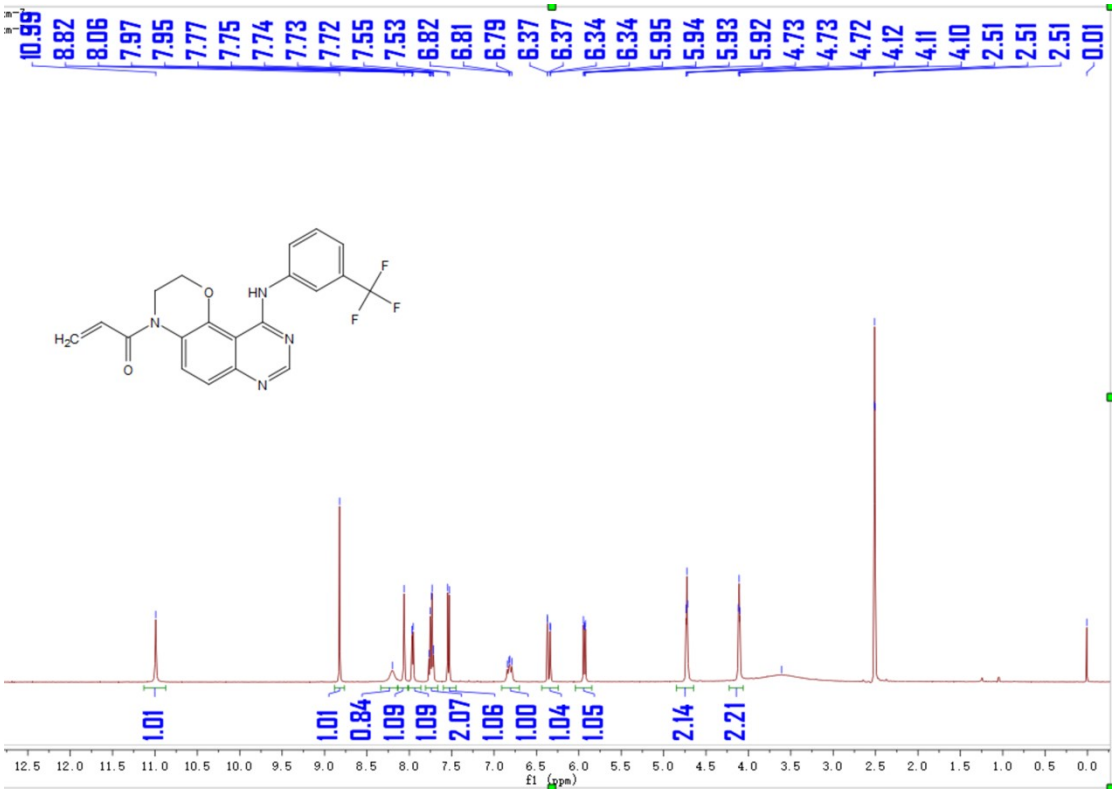


Fig. S7. ^{1}H NMR spectrum of compound **4c**

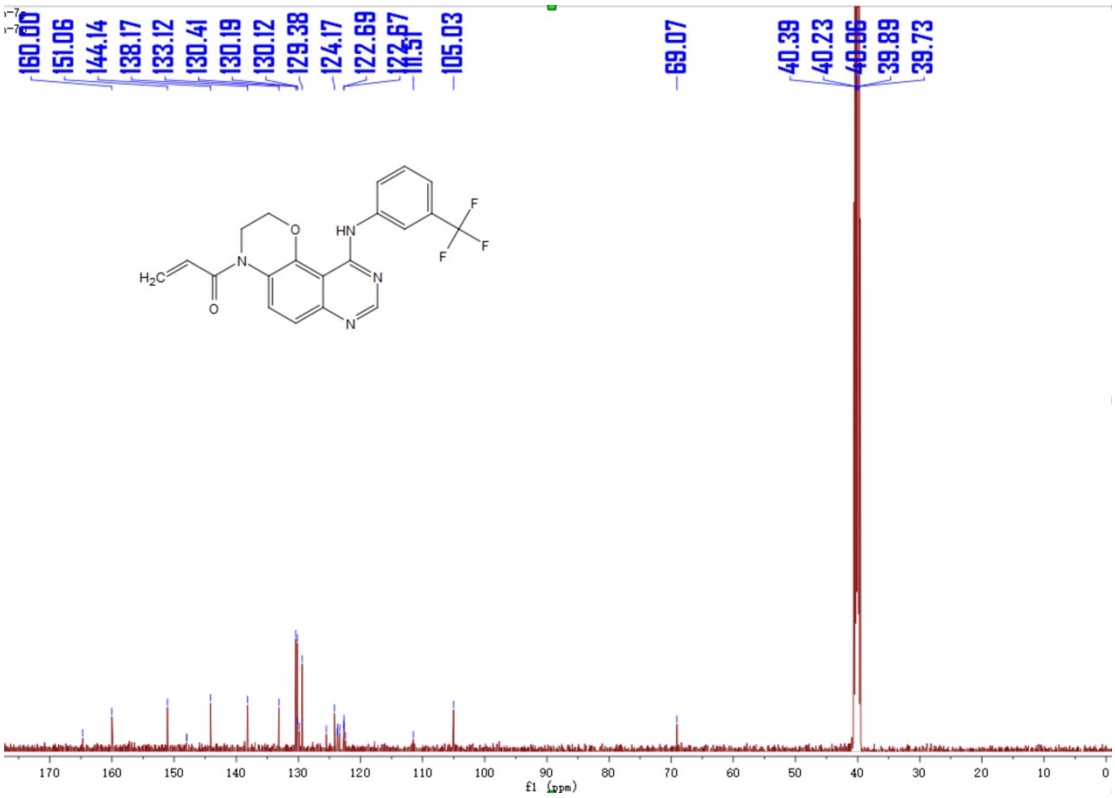


Fig. S8 ^{13}C NMR spectrum of compound **4c**

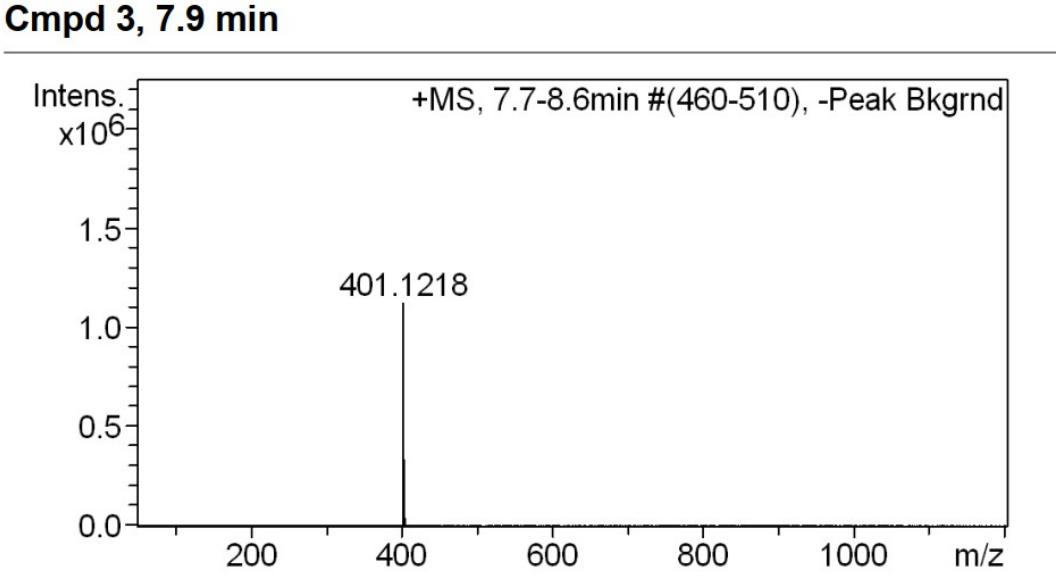


Fig. S9 HRMS spectrum of compound **4c**

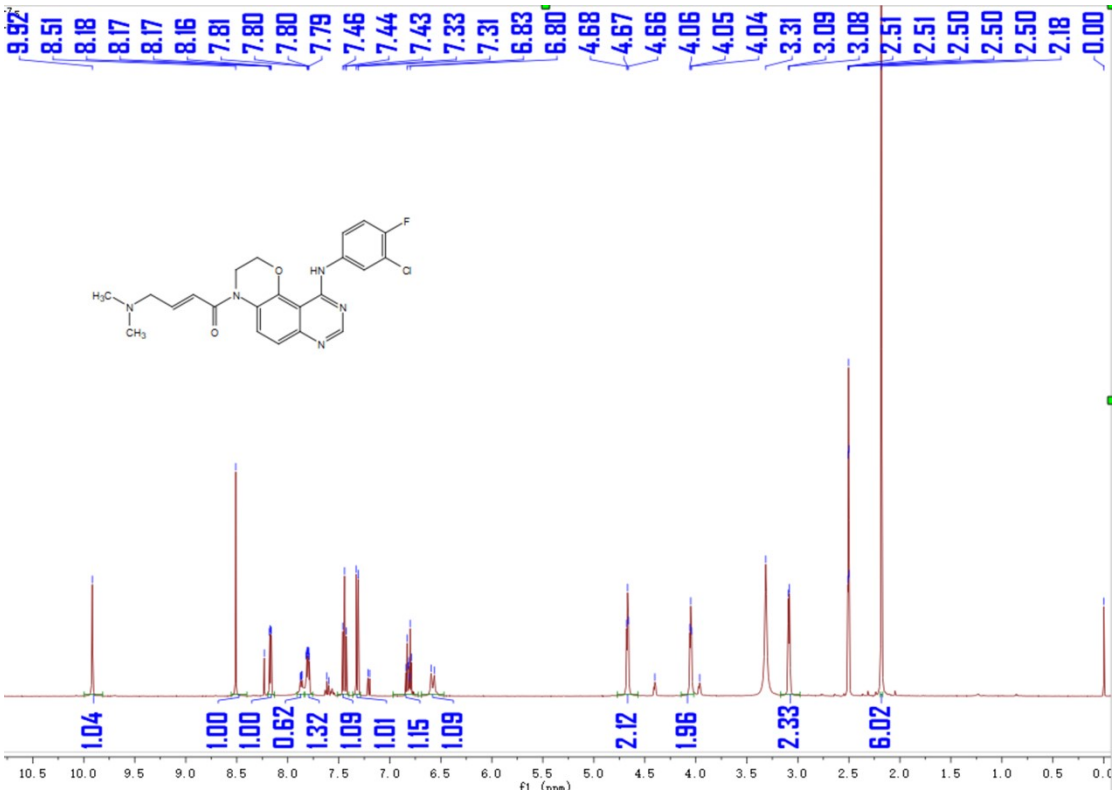


Fig. S10. ^{1}H NMR spectrum of compound **6a**

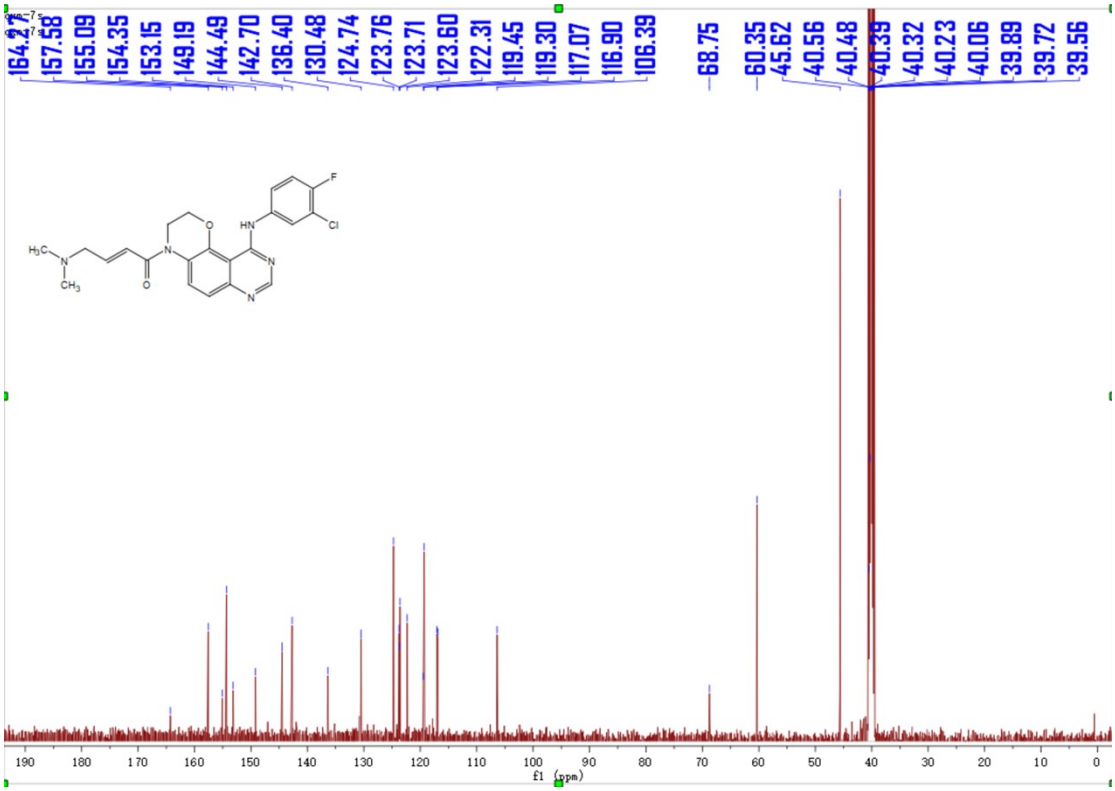


Fig. S11 ^{13}C NMR spectrum of compound **6a**

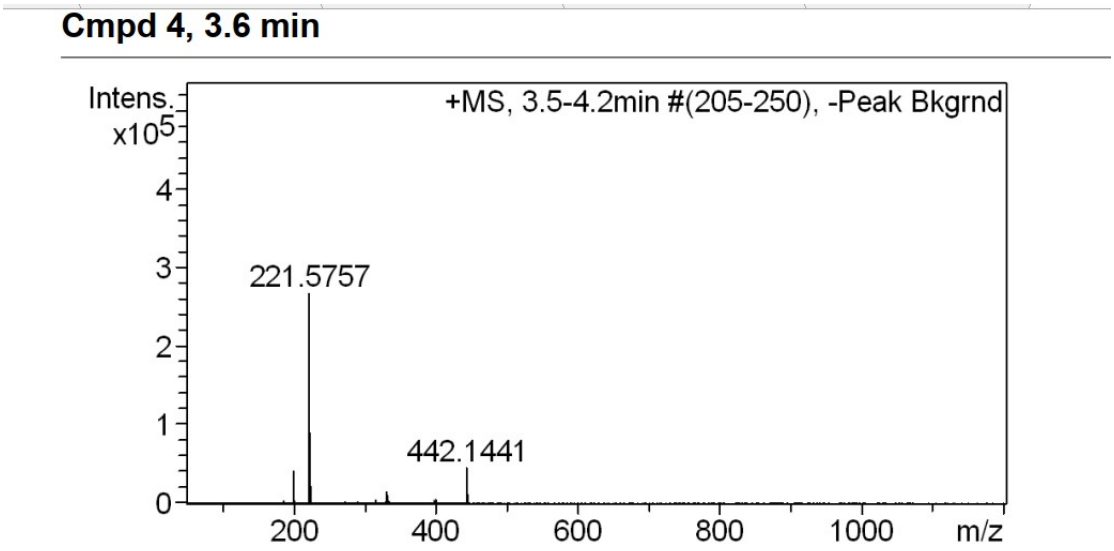


Fig. S12 HRMS spectrum of compound **6a**

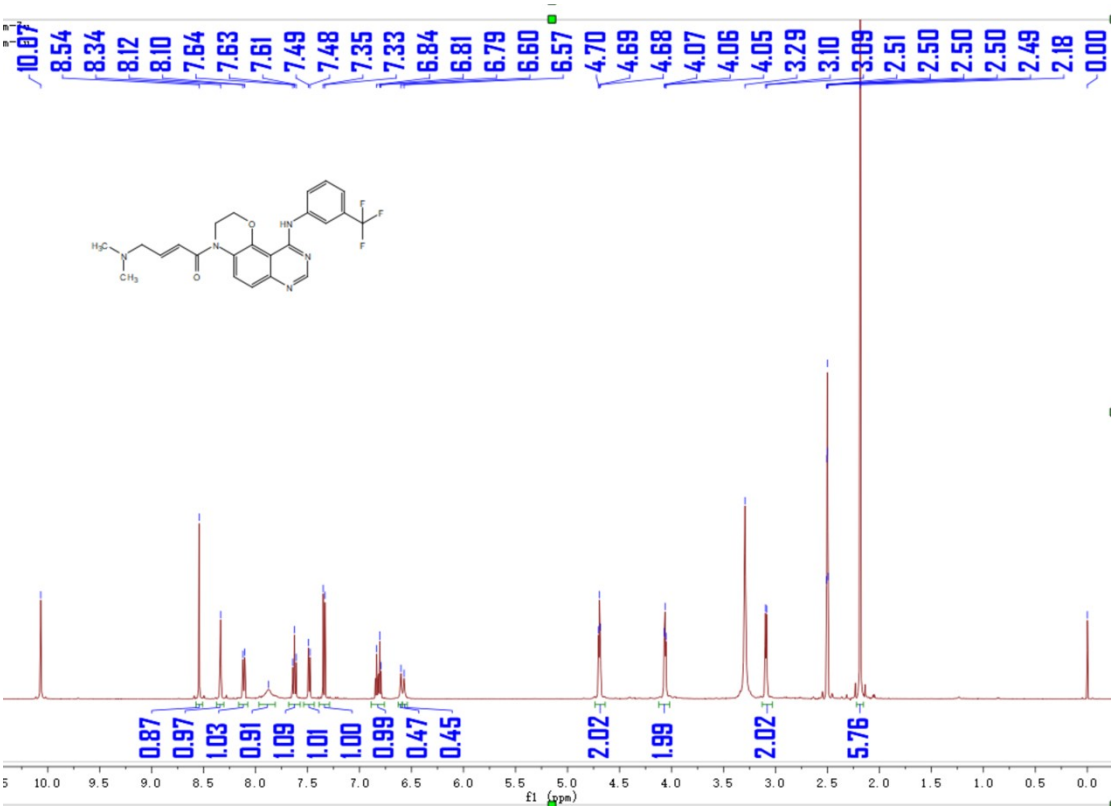


Fig. S13. ^{1}H NMR spectrum of compound **6b**

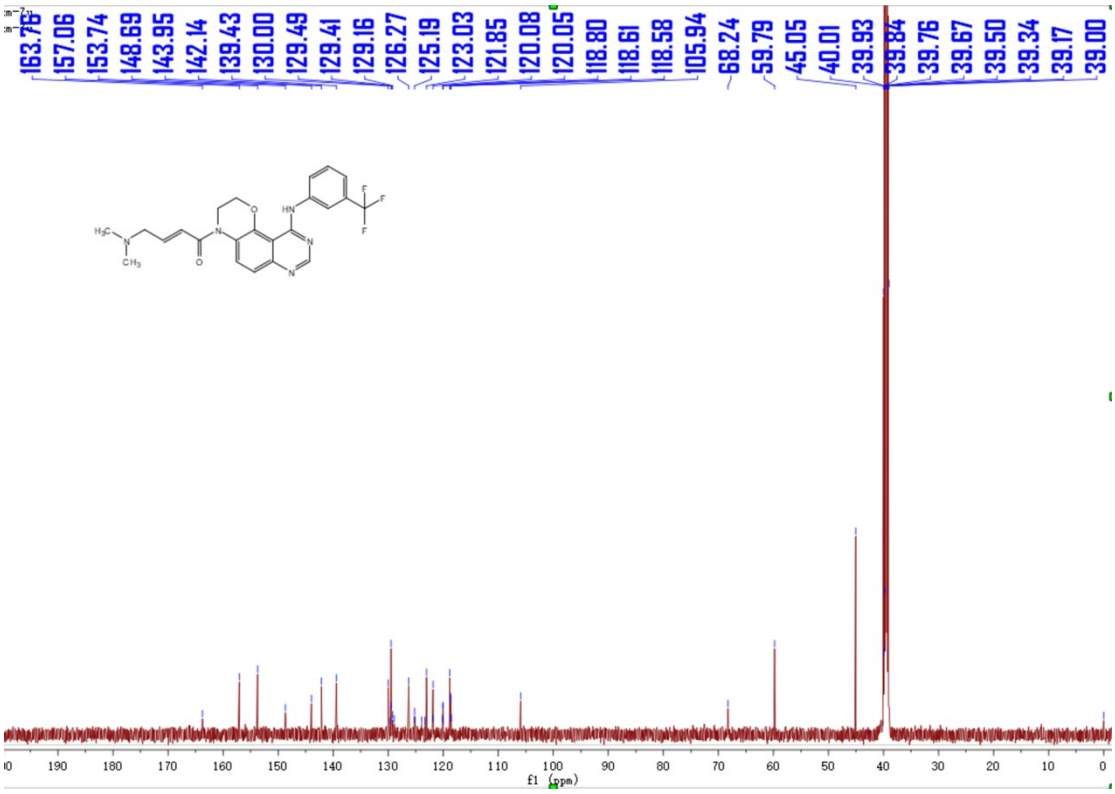


Fig. S14 ^{13}C NMR spectrum of compound **6b**

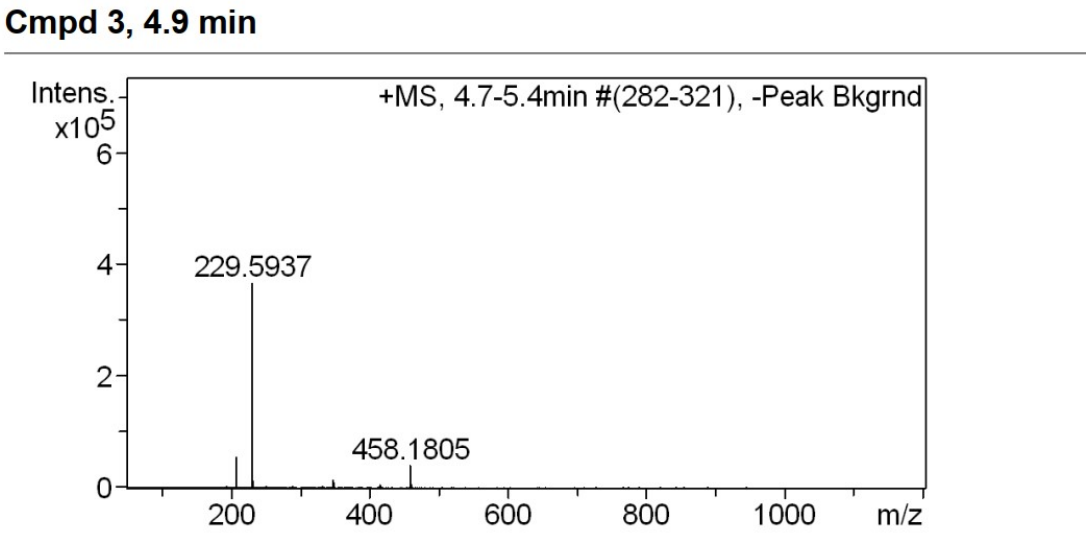


Fig. S15 HRMS spectrum of compound **6b**

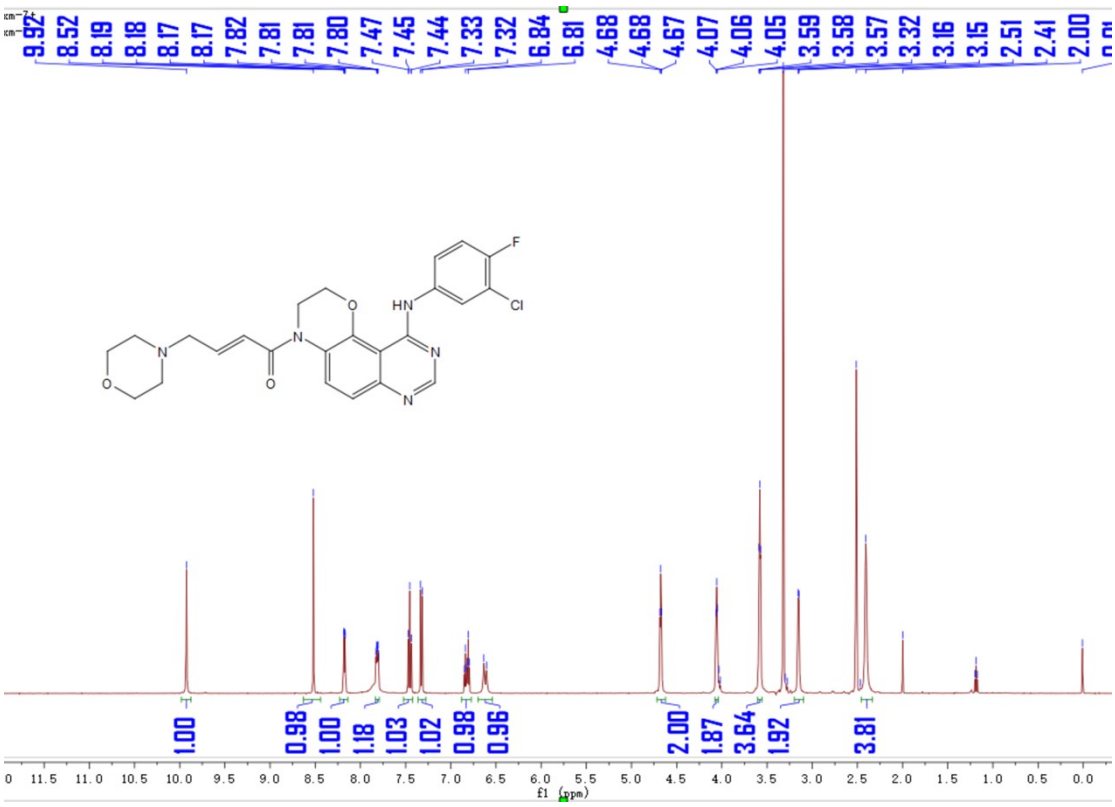


Fig. S16. ^{1}H NMR spectrum of compound **6c**

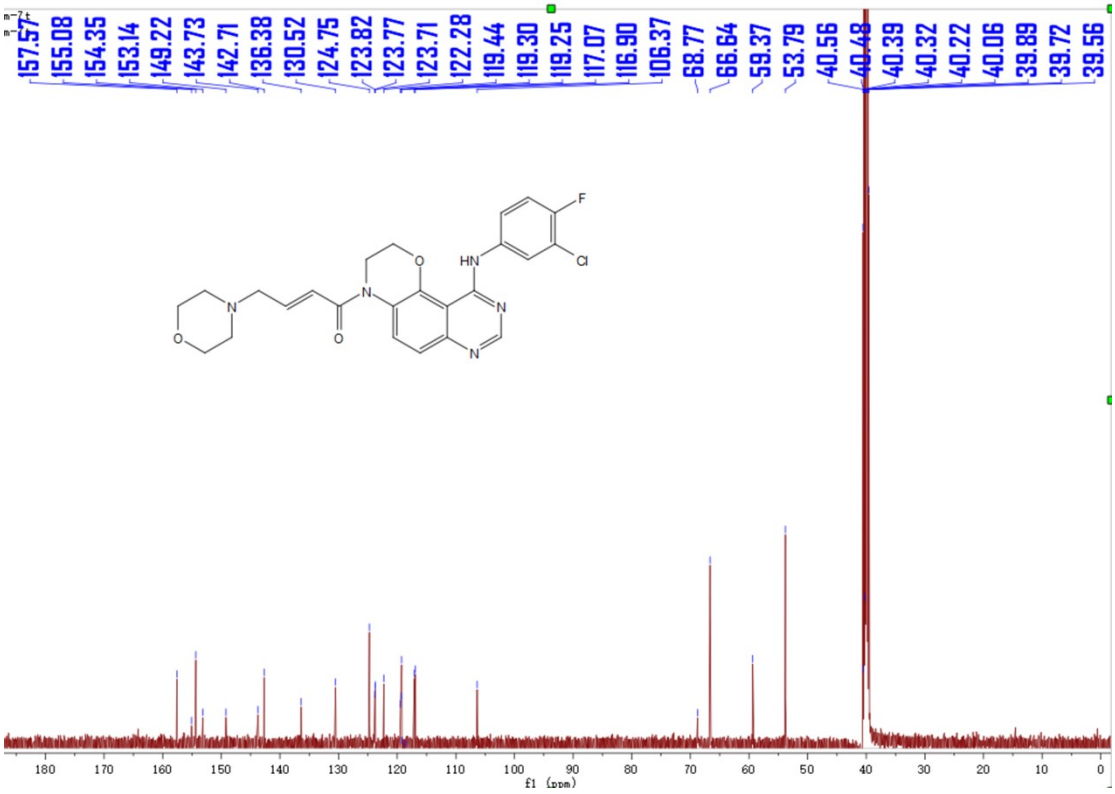


Fig. S17 ^{13}C NMR spectrum of compound **6c**

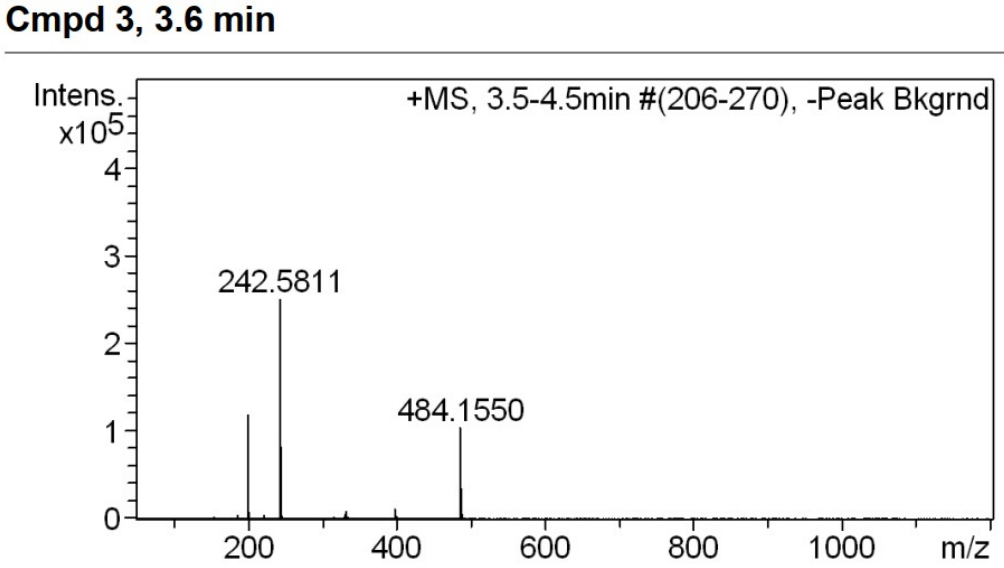


Fig. S18 HRMS spectrum of compound **6c**

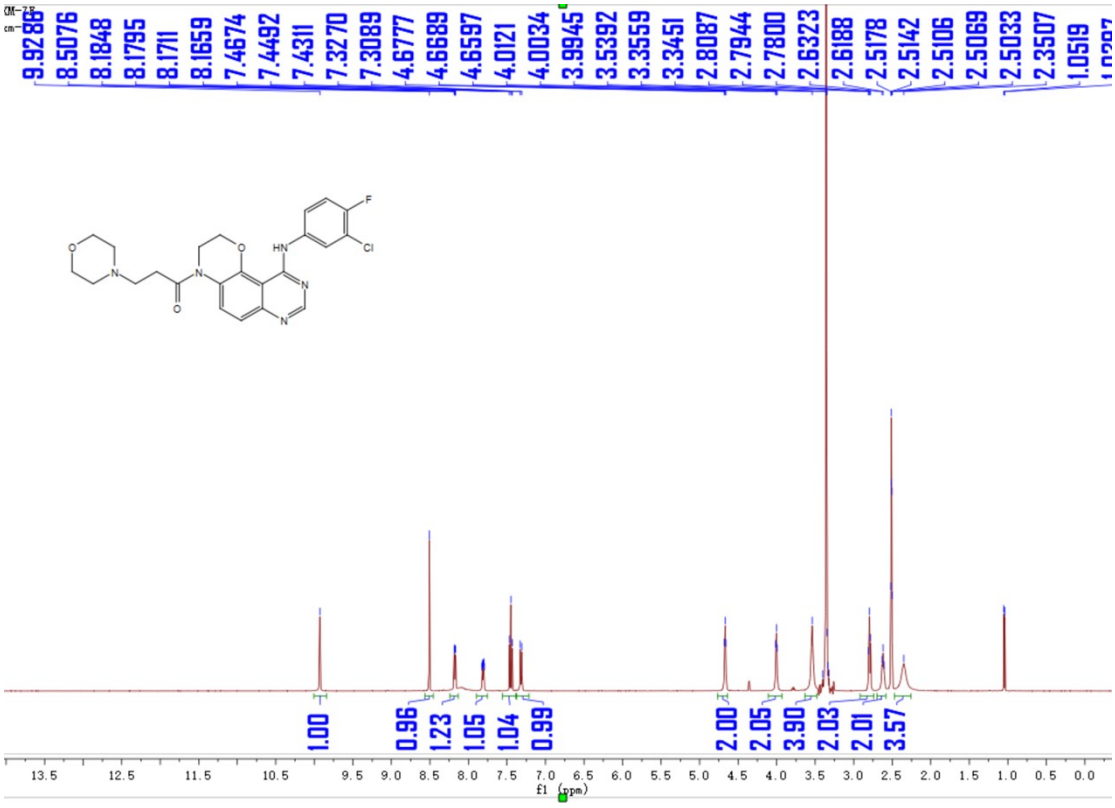


Fig. S19. ^{1}H NMR spectrum of compound **8a**

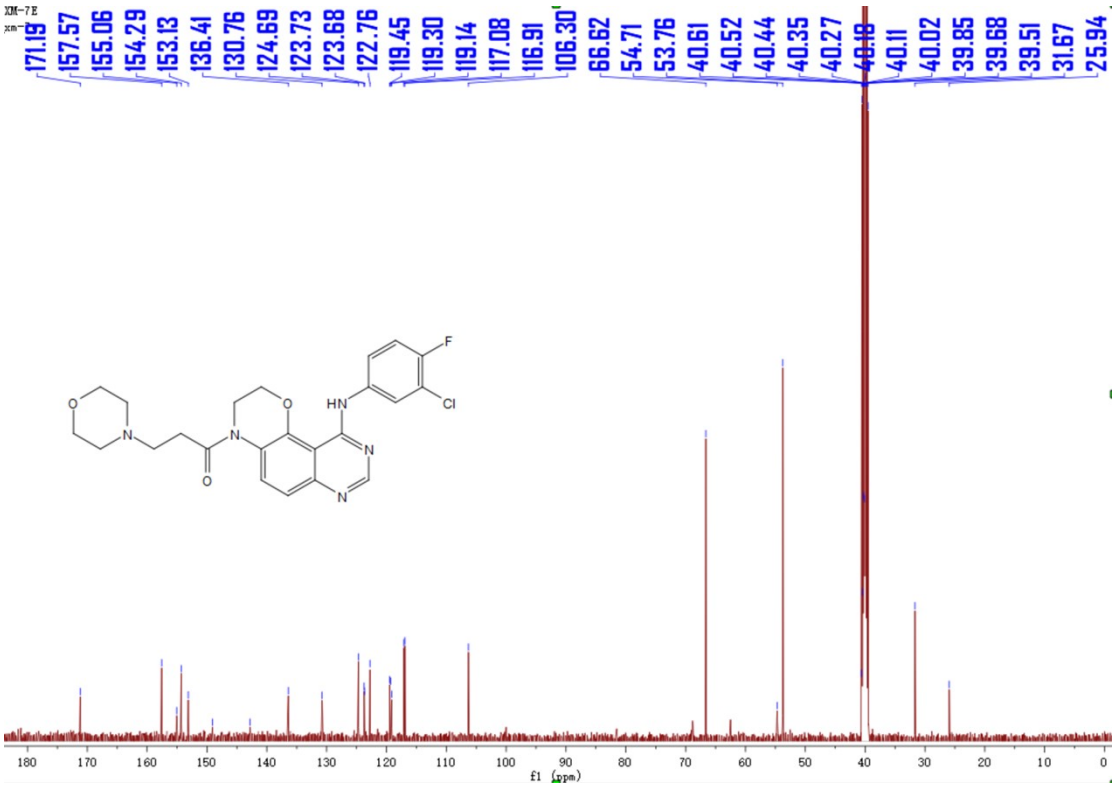


Fig. S20 ^{13}C NMR spectrum of compound **8a**

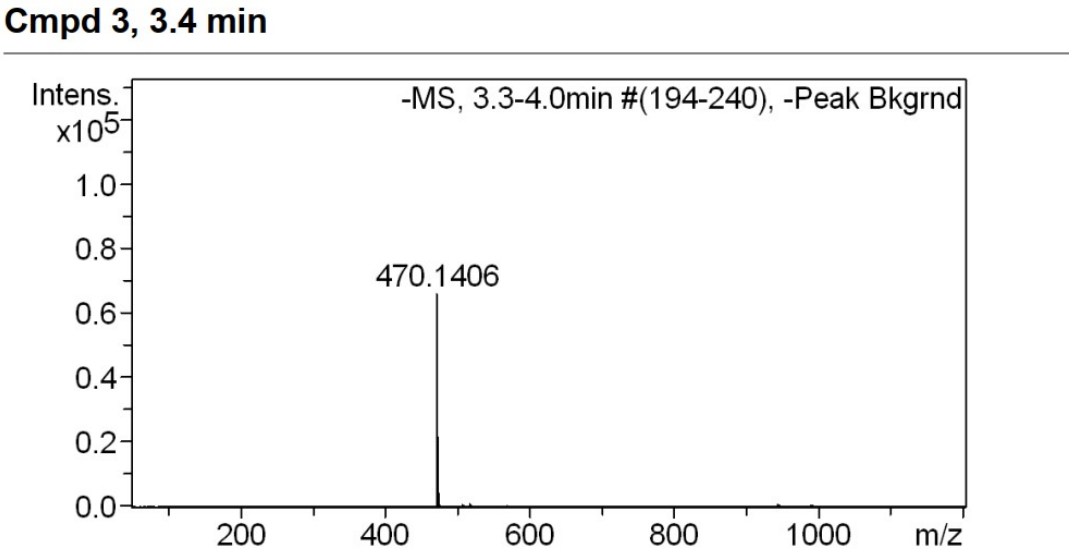


Fig. S21 HRMS spectrum of compound **8a**

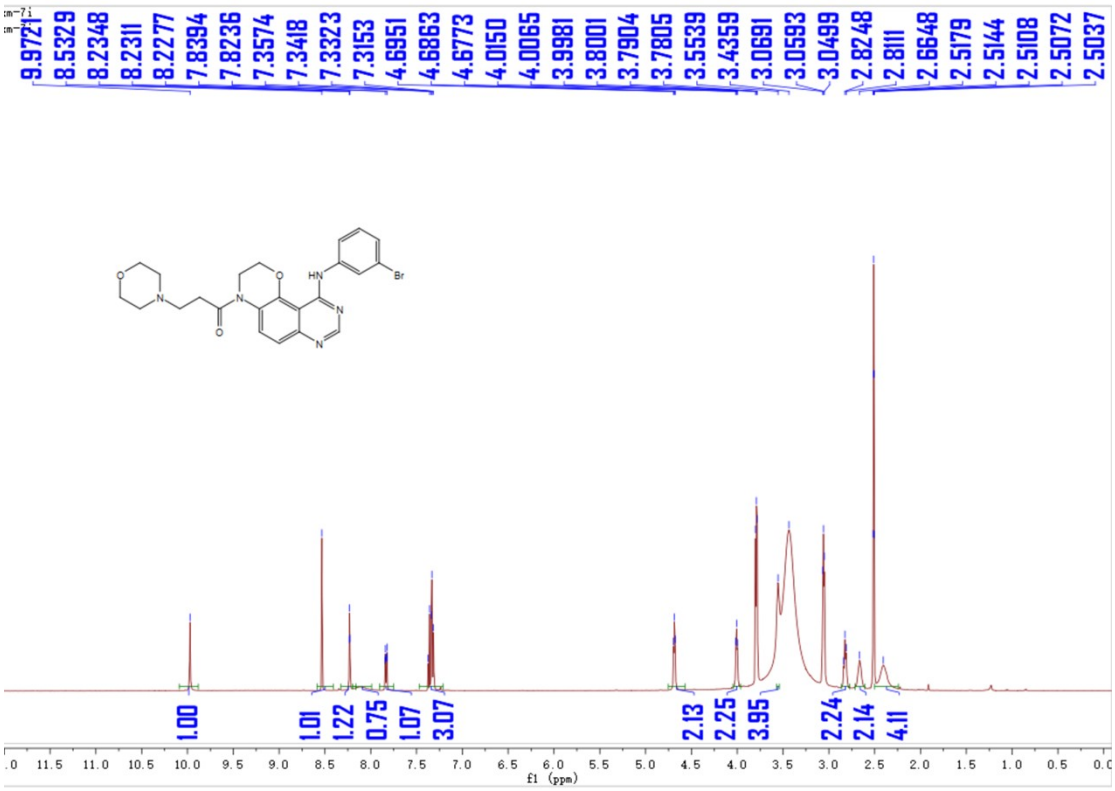


Fig. S22. ^{1}H NMR spectrum of compound **8b**

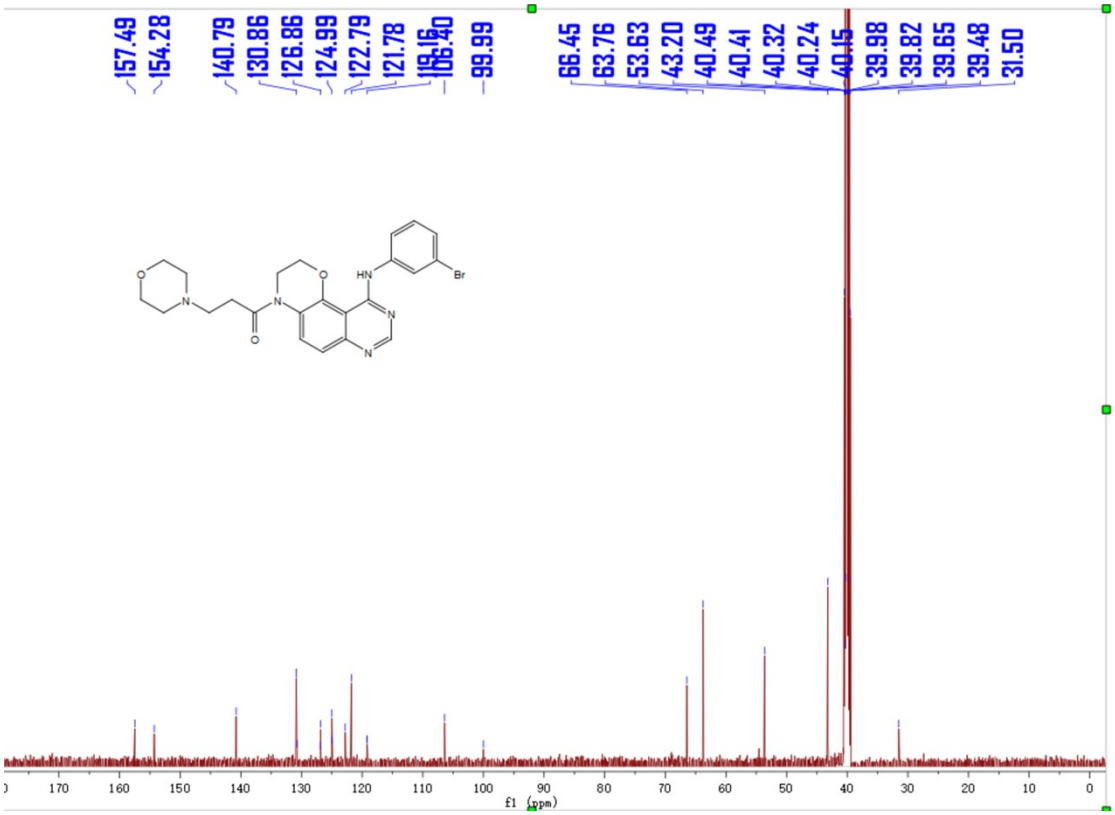


Fig. S23 ^{13}C NMR spectrum of compound **8b**

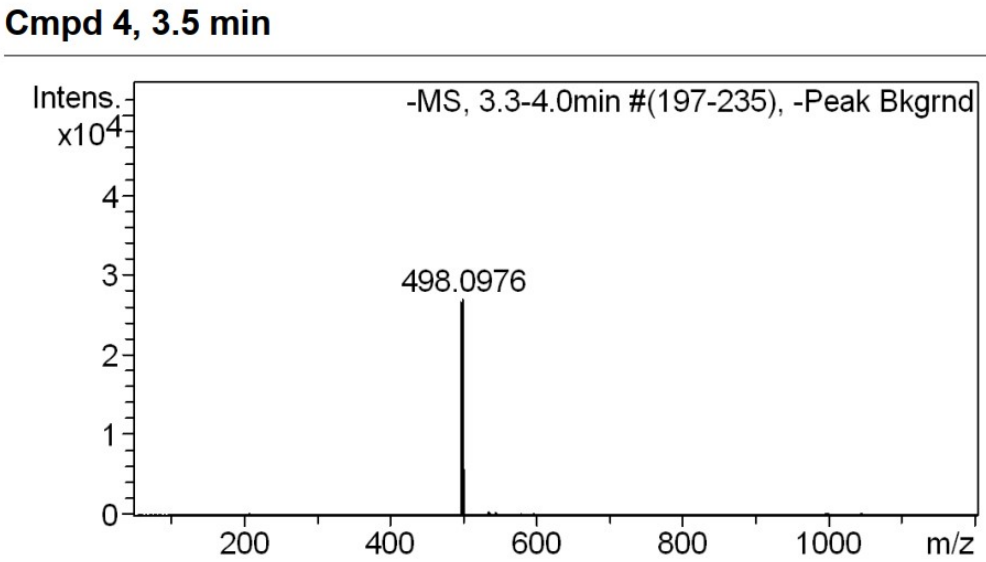


Fig. S24 HRMS spectrum of compound **8b**

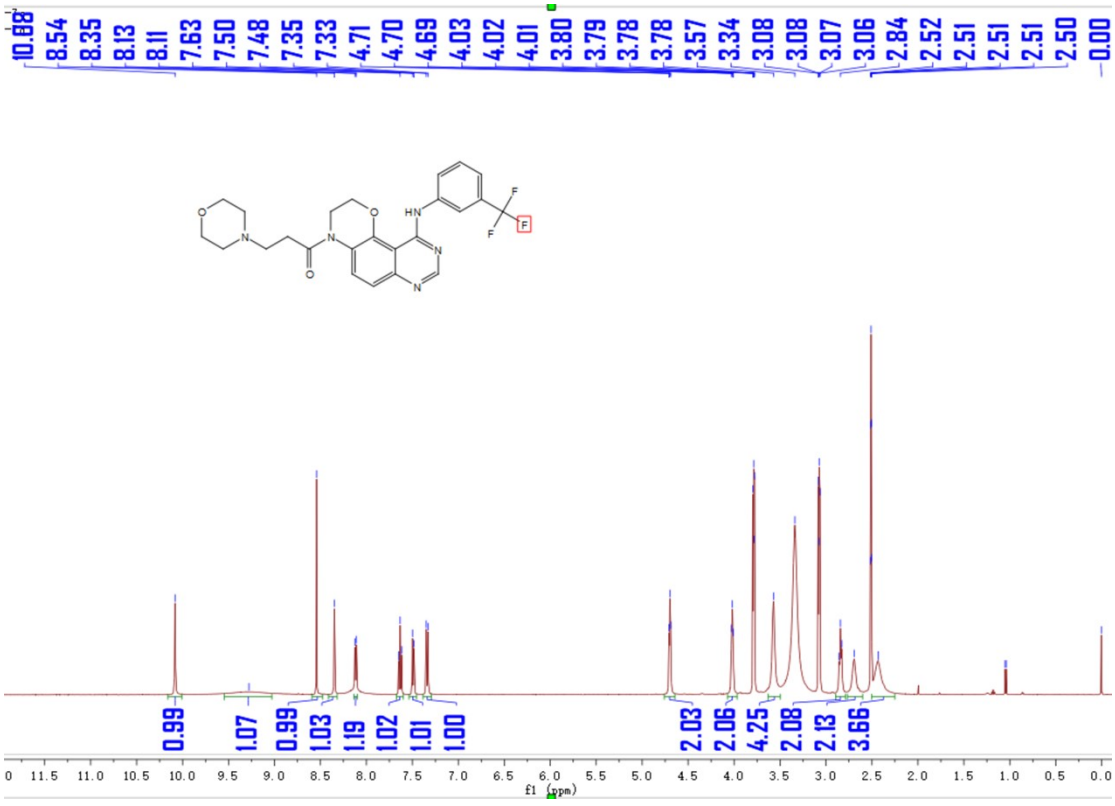


Fig. S25. ^{1}H NMR spectrum of compound **8c**

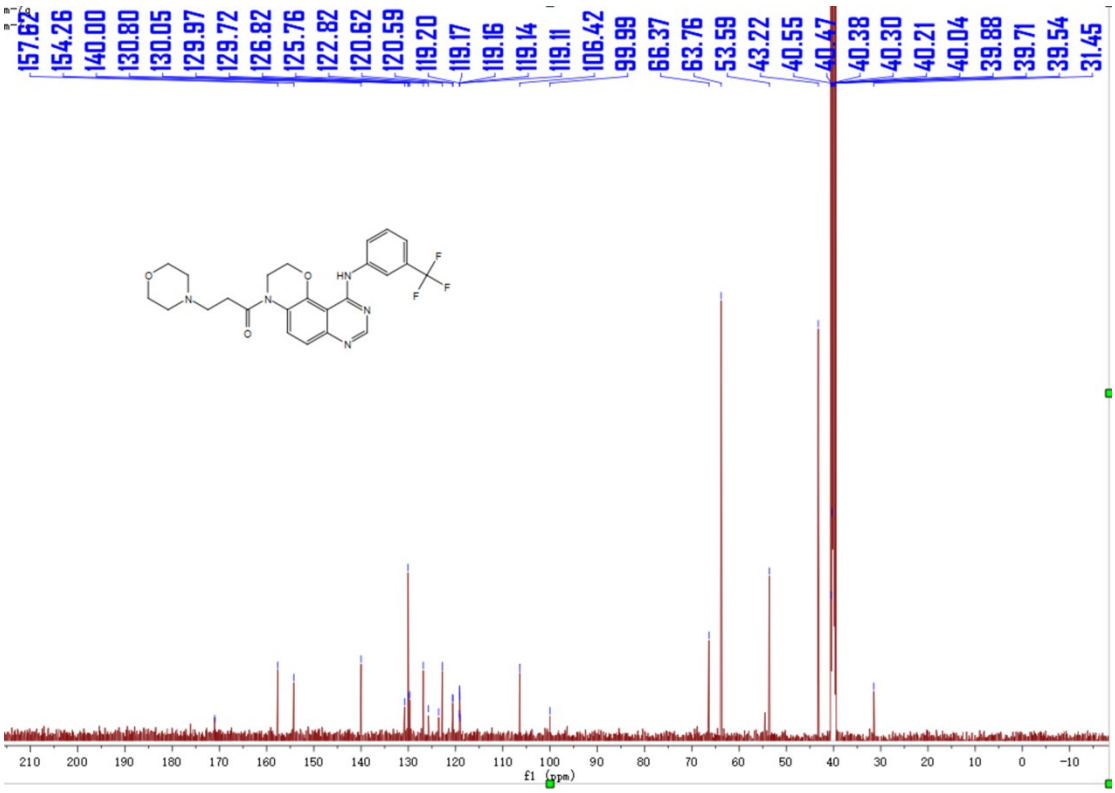


Fig. S26 ^{13}C NMR spectrum of compound **8c**

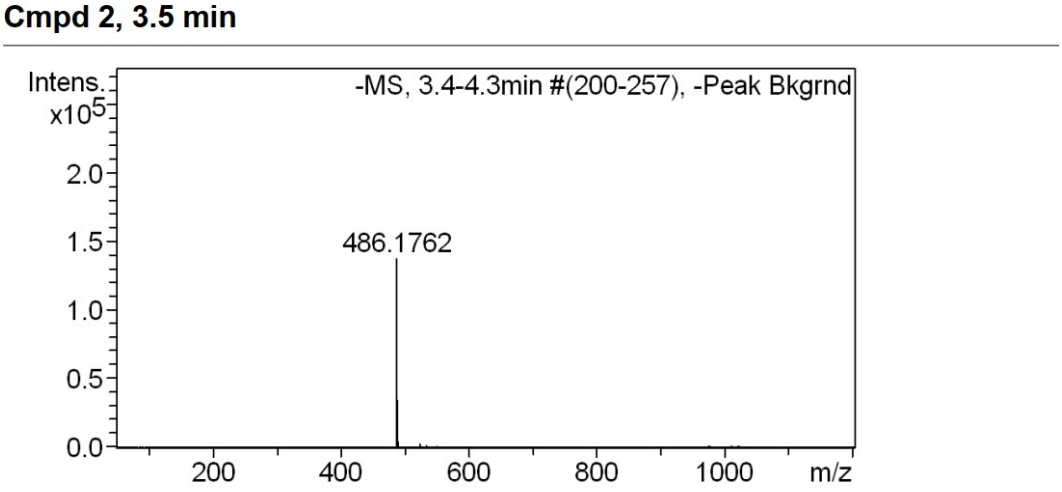
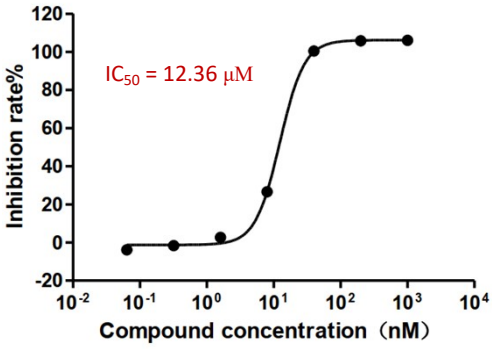


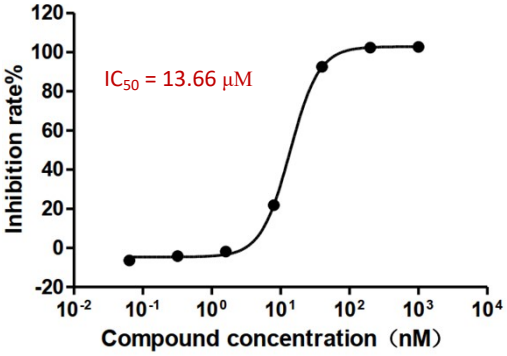
Fig. S27 HRMS spectrum of compound **8c**

5. *E*nzymatic inhibitory activity of target compounds against EGFR

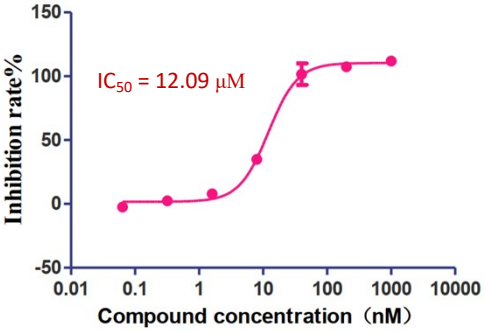
Compound **4a**



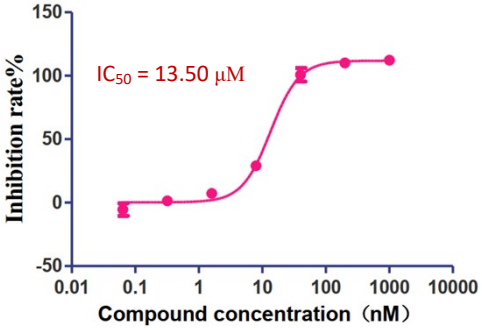
Compound **4b**



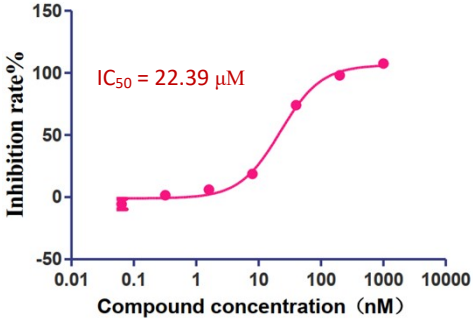
Compound **4c**



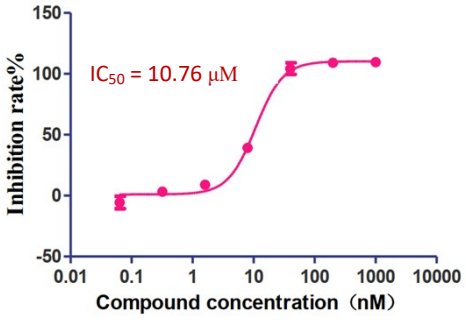
Compound **6a**



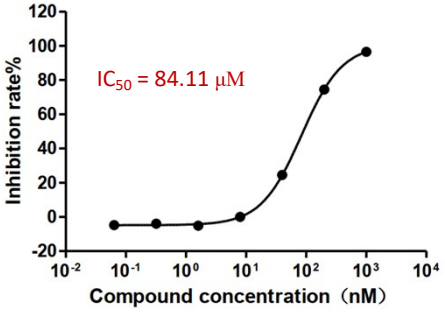
Compound **6b**



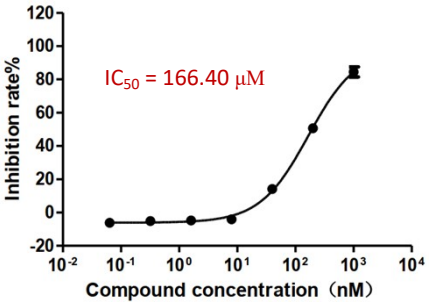
Compound **6c**

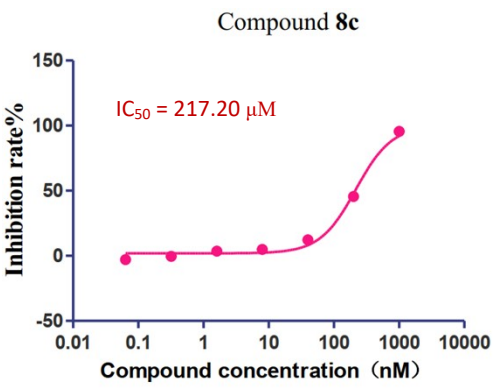


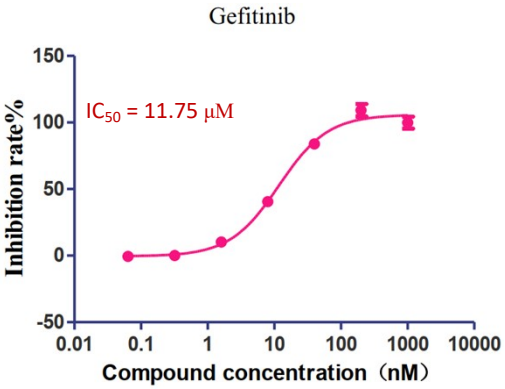
Compound **8a**



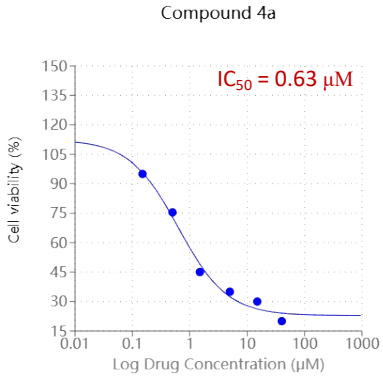
Compound **8b**

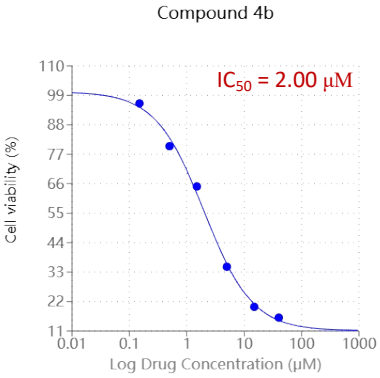


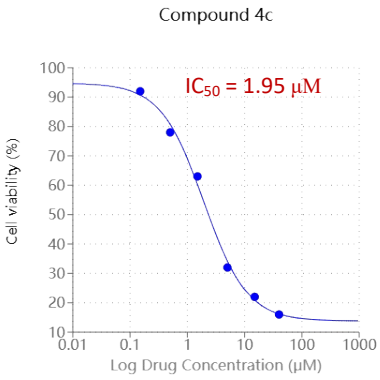


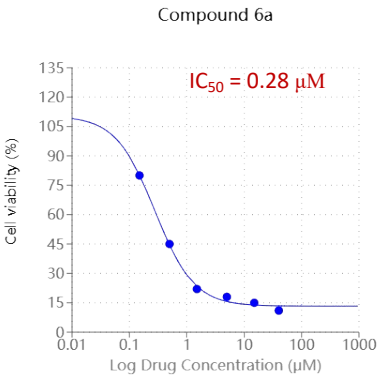


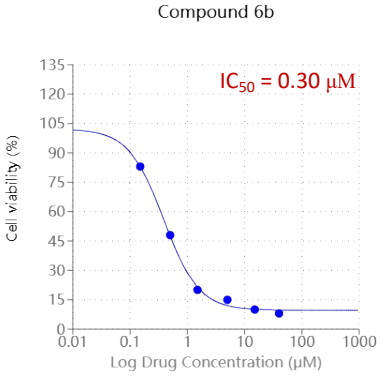
6. Anti-H1975 cells proliferation assay

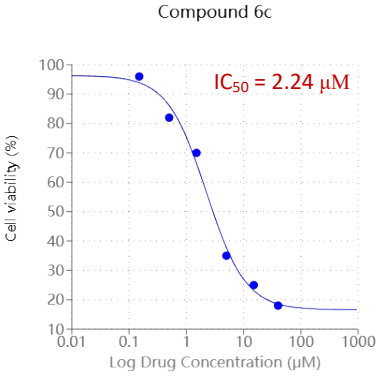




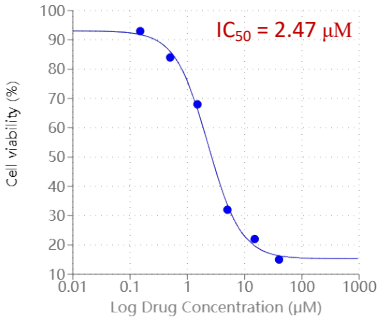




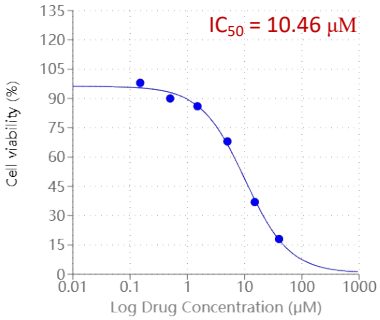




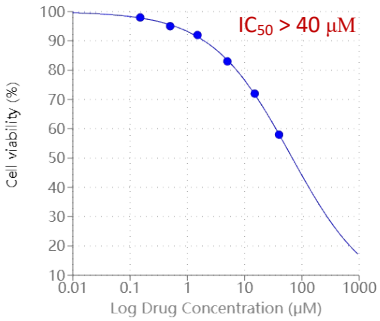
Compound 8a



Compound 8b



Compound 8c



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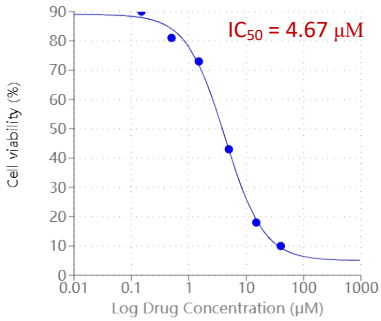
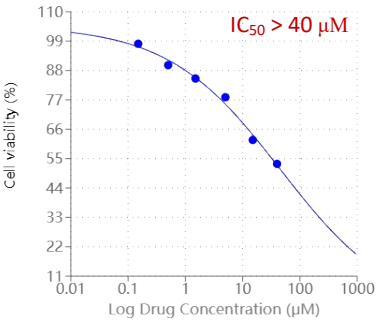
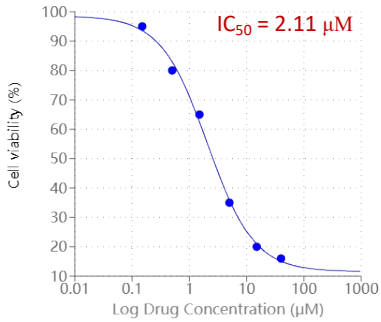


7. Anti-H1563 cells proliferation assay

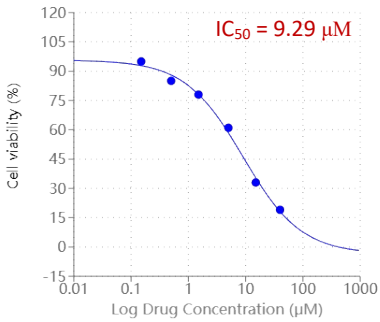
Compound 4a



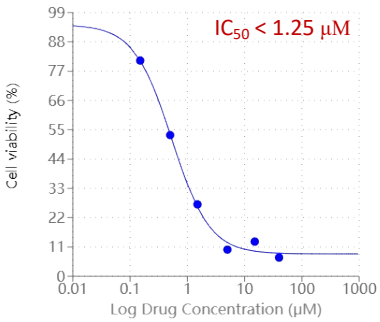
Compound 4b



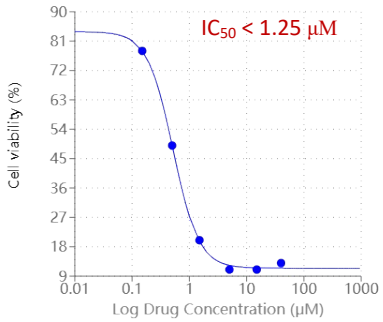
Compound 4c



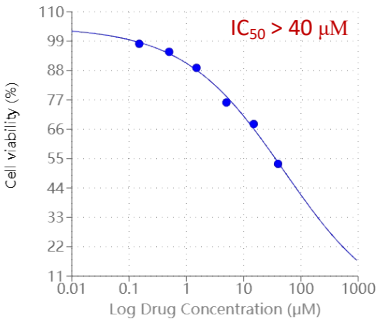
Compound 6a



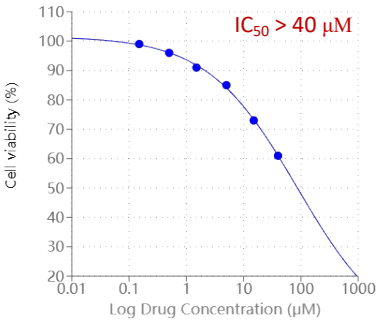
Compound 6b



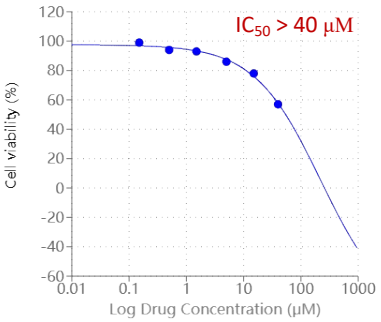
Compound 6c



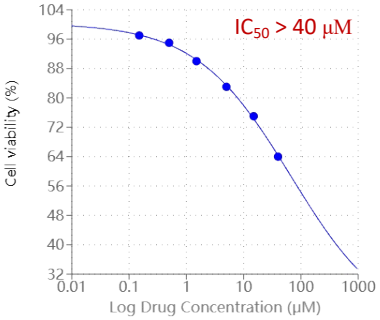
Compound 8a



Compound 8b



Compound 8c



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