Development of Lysine-Targeted Probes for Protein Kinases

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Abstract

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Lysine is one of the most common amino acids in the proteome, but relatively few probes have been designed to label specific lysine. Properties of lysine such as its low intrinsic nucleophilicity and its ubiquity has made it a challenge to develop probes that label active site lysine in protein kinases. In this thesis, I describe the design, synthesis, and testing of three different lysine-targeted type I kinases probes based on different scaffolds. Two of these probes are fluorosulfates (Probe 1 and Probe 2) displayed from either a quinazoline or pyridine-pyrimidine scaffold. The third (Probe 3) is a sulfonyl fluoride probe with a trans-cyclooctyne (TCO) click handle, which is based on the previously developed probe XO44 probe. Based on lysate labeling experiments and sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, neither alkyne-containing nor TCO-containing versions of Probe 1 and Probe 2 are able to specifically label protein kinases. Lysate labeling

experiments followed by liquid chromatography tandem mass spectrometry (LC/MS/MS) demonstrate that Probe 3 can specifically label at least 7 protein kinases. Together, these results represent a comprehensive analysis of lysine-targeted type I kinases probes.

The supplementary spreadsheets include the whole proteomic data for Probe 3 LC/MS/MS pull-down experiment (‘Proteomic data for Probe 3’); the protein kinases with non-significant label free intensities difference between the ‘Probe 3’ group and the ‘Probe 3 + Competitor 2’ group (‘Non-significant difference PKs’); the protein kinases only contain one label free intensity in the ‘Probe 3’ group (‘Non-representative PKs’); the protein kinases labeled by Probe 3 (‘Labeled PKs’).

Introduction:

Protein kinases are the largest family of enzymes encoded by the human genome[1], which participate in a variety of biological activities, including cell cycle progression, signaling, proliferation and death of cells, protein transport, and immunological response. Additionally, they play a role in a variety of human disorders.[2] Due to the frequent dysregulation of kinase-dependent signaling pathways in illnesses such as inflammation, degenerative diseases, infectious diseases, and cancer, kinases have become the primary therapeutic targets.[3] Cell information processing relies on kinase-dependent pathways to produce highly dynamic, complex and interconnected signal networks.[4]

DNA or RNA content analysis alone is insufficient for understanding cell life and disease. Additionally, the human genome has an estimated 30,000–40000 protein-coding genes, and 10–100 times this number of distinct proteins can be created by post-transcriptional and post-translational processing and modification.[5] Therefore, research on protein kinases is becoming even more important. Sensitivity to small molecules and the role of dysregulated kinases in many diseases make them very attractive drug targets.[3] Since 2001, when the FDA approved the first small molecule kinase inhibitor, Imatinib, for the treatment of cancer and other diseases, an increasing number of small molecule kinase medicines have been approved.[6] Determining the structure–activity relationship and the capacity to recognize cell targets in lysate and tissues is critical for the development of kinase inhibitor medicines.[3] Researchers can focus on compounds with high specificity and minimal side effects in the body, to cope with disease.[7]

Almost all methodologies make use of conserved ATP binding sites to establish a common platform for investigation.[8]

The overall structure of the protein kinase catalytic domain is highly conserved. It consists of 250–300 residues.[1] The catalytic domain of kinase is composed of β-chain rich N-lobes and α-helix C-lobes, and the active or ATP binding site is located between the two. When the kinase is activated, the αC helix in the N lobe contains a highly conserved Glu that forms a salt bridge with the catalytic lysine on the β3 chain. Adjacent to the active site are three highly conserved residues, Asp, Phe, and Gly, collectively referred to as the DFG motif, which initiate the activation loop. The DFG motif can take on two distinct conformations: one in which the Asp side chain points toward the ATP binding site and positions a magnesium that coordinates ATP, which is referred to as DFG-in, and another in which the Asp side chain points away from the ATP binding site, which is referred to as DFG-out depending on the binding mode and kinase conformation. The majority of currently developed kinase inhibitors are type I, which bind to the ATP binding site in the DFG-in conformation.[6] Among them, lysine is rich in content, widely distributed in and around a druggable site,[6] and it may play an active role in the phosphate transfer mechanism[9]. Moreover, functional lysine cannot undergo point mutations.[6]

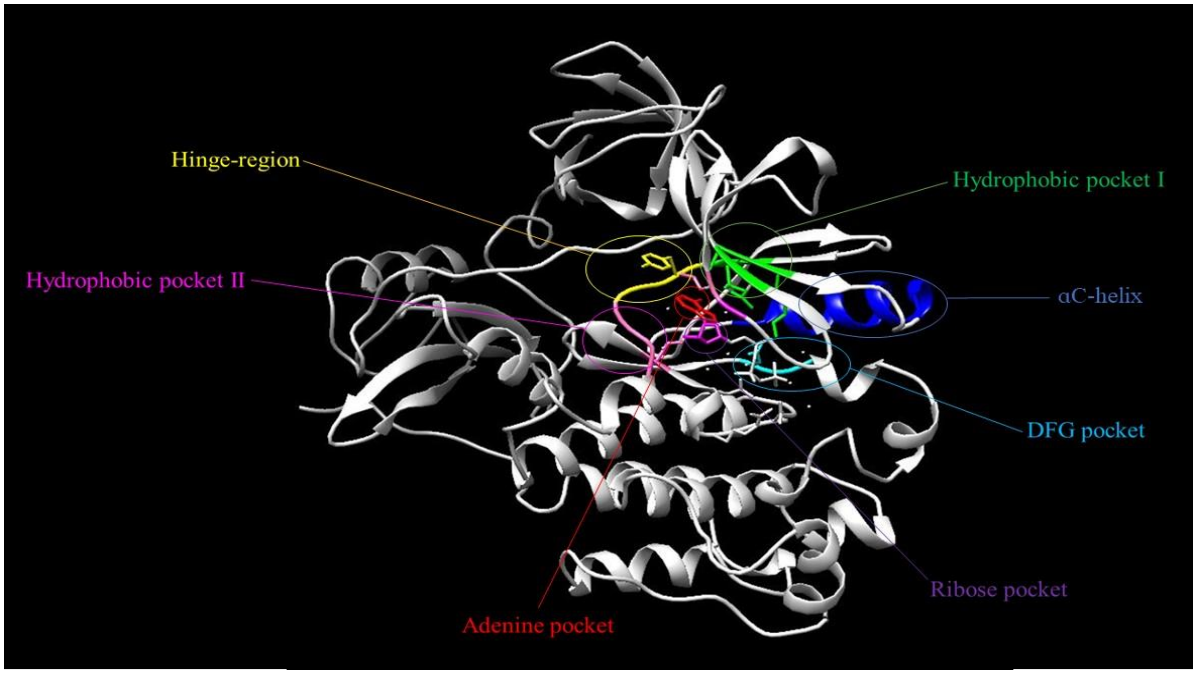
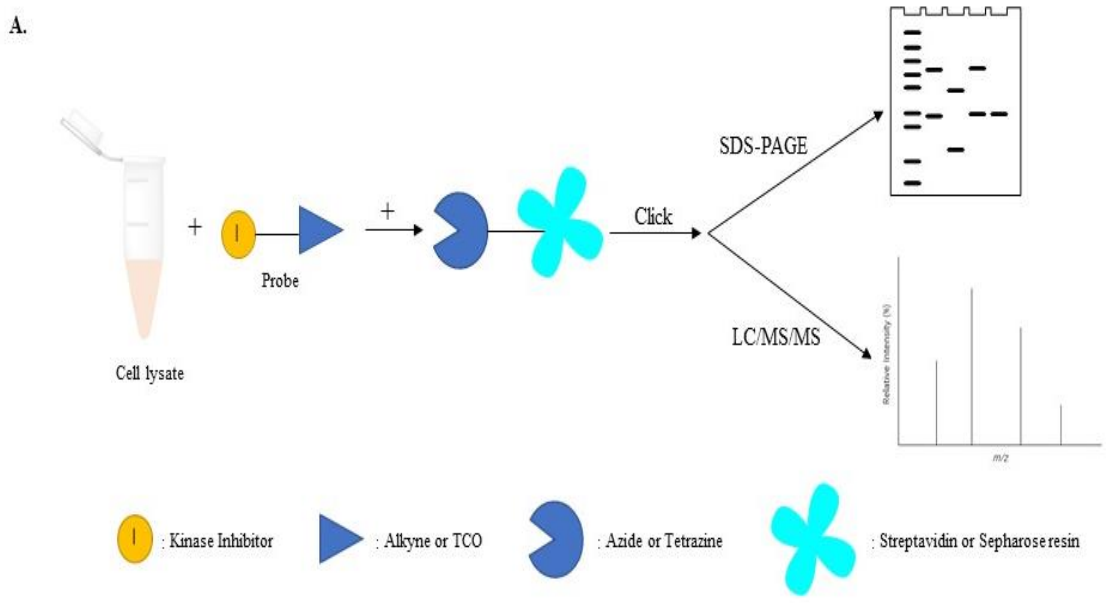


Figure.1: Crystal structure of c-SRC kinase. (PDB: 2src).

Probes can be designed to modify specific active site residues in a way that requires the activity of the target enzyme. Such probes are called activity-based protein profiling (ABPP) probes to reflect their demand for active enzymes, thereby facilitating covalent modification.[5] ABPPs are composed of at least two elements: a reactive group for binding and covalently labeling the active site; and one or more reporter tags (biotin and/or fluorescent group), used for rapid detection and separation of probe-labeled enzymes.[10]

There are two strategies for analyzing ABPP experiments: gel (such as 1D gel SDS-PAGE) and gel-free (LC/MS/MS). The former is more suitable for rapid comparison of a large number of proteomes, which has the advantage of higher throughput; the latter is more suitable for in-depth analysis of limited samples, and it will show higher resolution when analyzing probe-labeled proteomes.[11]

In this experiment, two new fluorosulfate type I kinases inhibitors (Probe 1 and Probe 2) with alkyne or TCO click handles were designed to target lysine in the ATP binding site of protein kinases. And a sulfonyl fluoride type I kinases inhibitor, XO44, was redesigned with a TCO click handle (Probe 3) for the experiment. The difference between these three probes and traditional ABPP probes is that these three probes target non-activated nucleophilic lysine. The chemoproteomics results were analyzed using SDS-PAGE and LC/MS/MS.



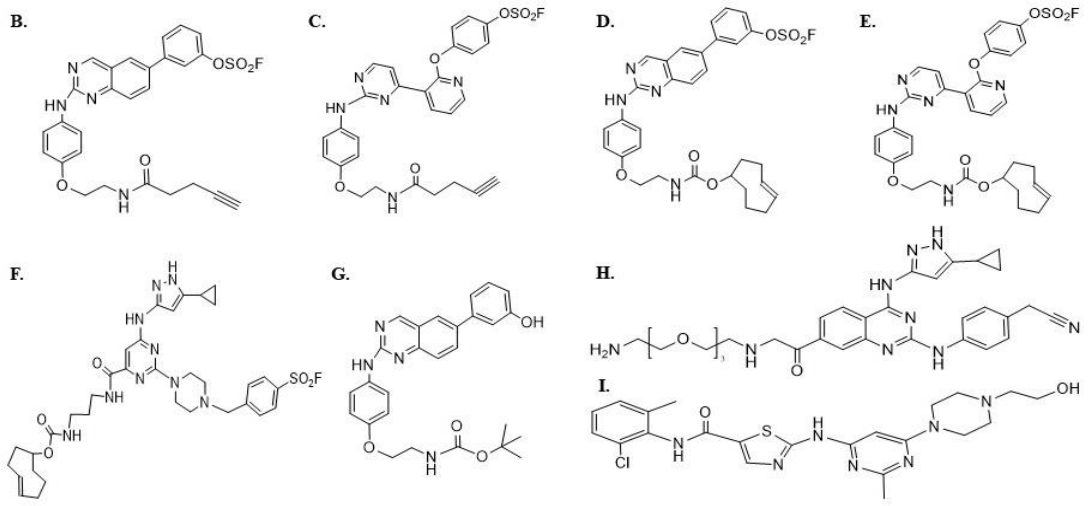
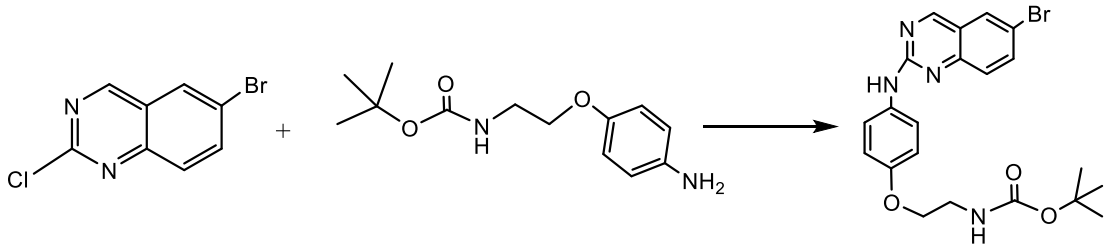


Figure.2: (A). Schematic for click chemistry in this experiment and analysis of probe-bound protein complexes. (B). Structure for Probe 1. (C). Structure for Probe 2. (D). Structure for Probe 1-TCO. (E). Structure for Probe 2-TCO. (F). Structure for Probe 3. (G). Structure for Competitor 1. (H). Structure for Competitor 2. (I). Structure for Competitor 3.

Method:

Probes' synthesis:



6-Bromo-2-chloroquinazoline

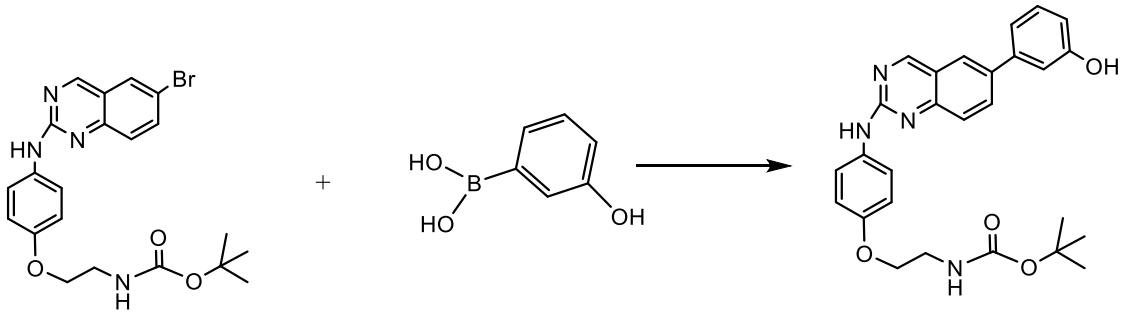
tert-Butyl 2-(4-aminophenoxy)ethylcarbamate

Compound 1

Scheme.1: Synthesis of Compound 1.

589.0 mg (2.4190 mmol) 6-Bromo-2-chloroquinazoline and 533.6 mg (2.1149 mmol) tert-Butyl 2-(4-aminophenoxy)ethylcarbamate were dissolved in 13.5 mL isopropanol with 262 µL (3.4215 mmol) trifluoroacetic acid (TFA). The tube was sealed; the color of the suspension was yellow. The suspension was stirred overnight at 70°C. 1.5 mL (10.7619 mmol) triethylamine (TEA) was added to neutralize the suspension, and the color of this mixture was yellow green. It was concentrated *in vacuo*. The Compound 1 was purified by silica flash chromatography (0%-100% ethyl acetate/hexane). The yellow product was 421.2 mg (0.9170 mmol). The yield was 43%. ^{1}H NMR (300 MHz, chloroform-d) δ 9.00 (s, 1H), 7.88 (d, J = 2.2 Hz, 1H), 7.80 (dd,

J = 9.0, 2.2 Hz, 1H), 7.68 (d, *J* = 8.7 Hz, 2H), 7.60 (d, *J* = 8.9 Hz, 2H), 6.98 – 6.90 (m, 2H), 5.04 (br s, 1H), 4.07 (t, *J* = 4.53Hz, 2H), 3.59-3.53 (m, 2H), 1.48 (s, 9H). MS *m/z* ($C_{21}H_{23}BrN_4O_3$) calc'd = 459.3, observed M+Na = 483.0.

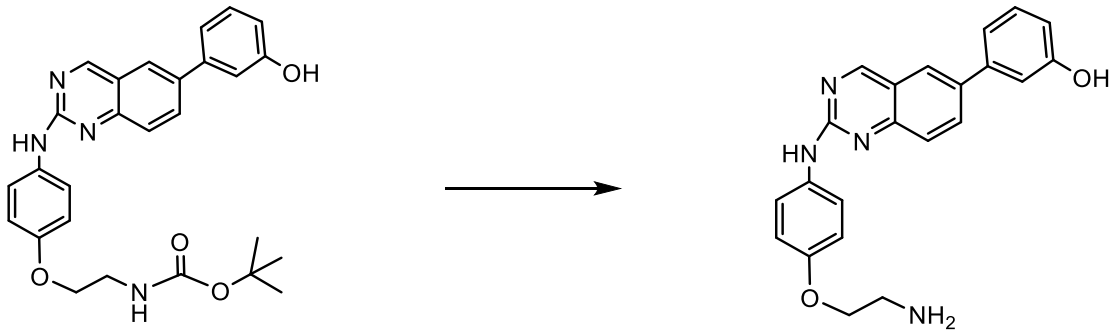


Compound 1 + 3-Hydroxybenzeneboronic acid → Compound 2

Scheme.2: Synthesis of Compound 2 (Competitor 1).

299.2 mg (0.6514 mmol) Compound 1 and 112.4 mg (0.8149 mmol) 3-Hydroxybenzeneboronic acid were dissolved in a 3:1 mixture of dimethoxyethane (DEM)/distilled water (6 mL) with 23.9 mg (0.2068 mmol) tetrakis(triphenylphosphine)palladium and 154.4 mg (1.4567 mmol) sodium carbonate. The color of the solution was black. The mixture was stirred overnight at 85°C. After the mixture was cooled to room temperature, it was diluted by 30 mL ethyl acetate. The solution was extracted with distilled water (20 mL * 3). The organic extracts were washed with 30 mL brine, dried by sodium sulfate and concentrated *in vacuo*. The Compound 2 was purified by silica flash chromatography (0%-100% ethyl acetate/hexane). The yellow product was 110.1 mg (0.2330 mmol). The yield was 36%. ^{1}H NMR (300 MHz, chloroform-d) δ 9.15 (s, 1H), 8.03 (dd, *J* = 8.8, 2.1 Hz, 1H), 7.93 (d, *J* = 2.0 Hz, 1H), 7.80 (d, *J* = 8.7 Hz, 2H), 7.69 (d, *J* = 8.9 Hz, 2H), 7.37 (t, *J* = 7.9 Hz, 1H), 7.24 (d, *J* = 7.7 Hz, 1H), 7.16 (s, 1H), 6.98-6.94 (m, 2H), 6.89

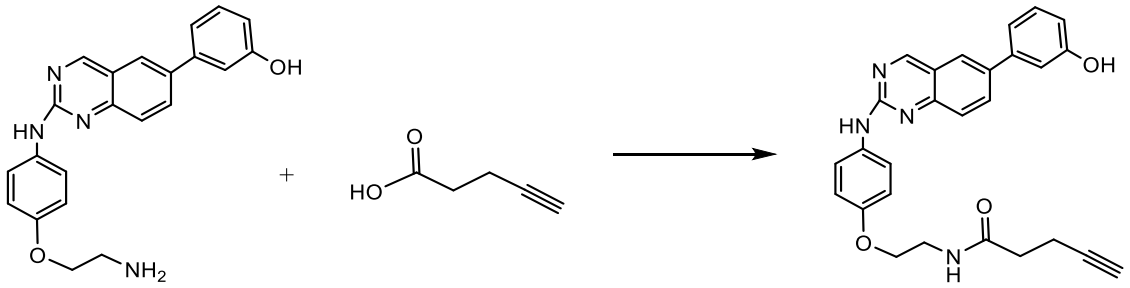
(dd, *J* = 8.1, 2.5 Hz, 1H), 5.21 (br s, 1H), 5.03 (br s, 1H), 4.09 (t, *J* = 5.30 Hz, 2H), 3.61-3.53 (m, 2H), 1.49 (s, 9H). MS *m/z* ($C_{27}H_{28}N_4O_4$) calc'd = 472.5, observed M+1 = 473.2.



Compound 2 Compound 3

Scheme.3: Synthesis of Compound 3.

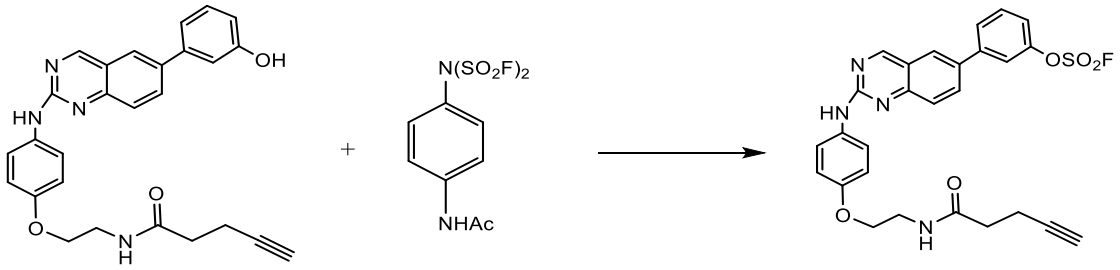
110.1 mg (0.2330 mmol) Compound 2 was added to 3.84 mL dichloromethane (DCM) with 1.68 mL (0.0219 mol) TFA. The solution was stirred at room temperature for 3h. The liquid's color was dark yellow. After 3h reaction, the solution was concentrated *in vacuo*. The mass of the orange product Compound 3 was 85.9 mg (0.2307 mmol). The yield was 99%. ^{1}H NMR (300 MHz, methanol-d4) δ 9.50 (s, 1H), 8.31 – 8.19 (m, 2H), 7.86 – 7.59 (m, 4H), 7.46 – 7.01 (m, 2H), 6.96 – 6.71 (m, 3H), 5.82 (br s, 1H), 4.36 – 4.27 (m, 3H), 3.43 (t, *J* = 5.0 Hz, 2H), 1.58 (s, 2H). MS *m/z* ($C_{22}H_{20}N_4O_2$) calc'd = 372.4, observed M+1 = 373.1.



Compound 3 4-Pentynoic acid Compound 4

Scheme.4: Synthesis of Compound 4.

32.0 mg (0.0859 mmol) Compound 3 and 12.0 mg (0.1223 mmol) 4-Pentynoic acid were added to 255.4 µL DCM with 17.4 mg (0.1121 mmol) 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI); 15.6 mg (0.1155 mmol) hydroxybenzotriazole (HOBT); and 44.9 µL (0.2578 mmol) *N,N*-Diisopropylethylamine. The solution color was yellow. The solution was stirred at room temperature overnight. The mixture was concentrated *in vacuo* and purified by reverse phase chromatography (HPLC). The mass of the orange product Compound 4 was 15.9 mg (0.0352 mmol). The yield was 41%. ^{1}H NMR (300 MHz, methanol-d4) δ 9.13 (s, 1H), 8.04 – 7.93 (m, 2H), 7.67 – 7.53 (m, 4H), 7.48 – 7.34 (m, 1H), 7.33 – 7.15 (m, 1H), 7.08 – 6.98 (m, 2H), 6.88 (dd, *J* = 7.83, 2.31 Hz, 1H), 6.13 (br s, 1H), 5.71 (br s, 1H), 4.07 – 3.92 (m, 2H), 3.49 (t, *J* = 5.2 Hz, 2H), 2.81 – 2.69 (m, 1H), 2.58 – 2.46 (m, 1H), 2.42 – 2.22 (m, 1H). MS *m/z* ($C_{27}H_{24}N_4O_3$) calc'd = 452.2, observed M+1 = 453.2.



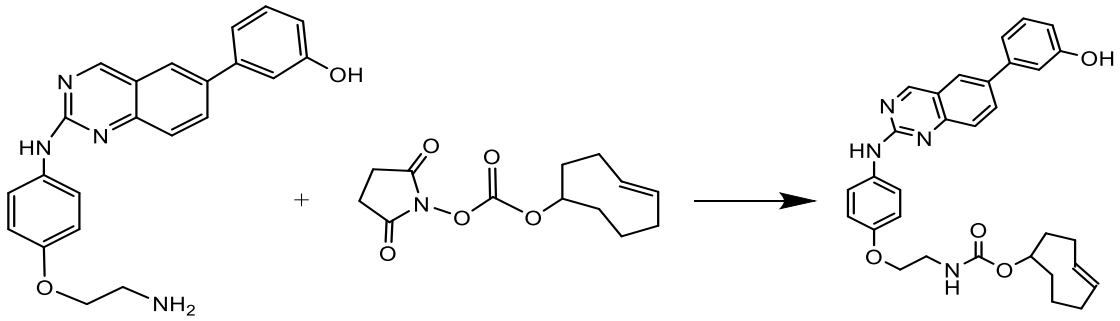
Compound 4

4-(Acetylamino)phenyl] imidodisulfuryl difluoride

Compound 5

Scheme.5: Synthesis of Compound 5 (Probe 1).

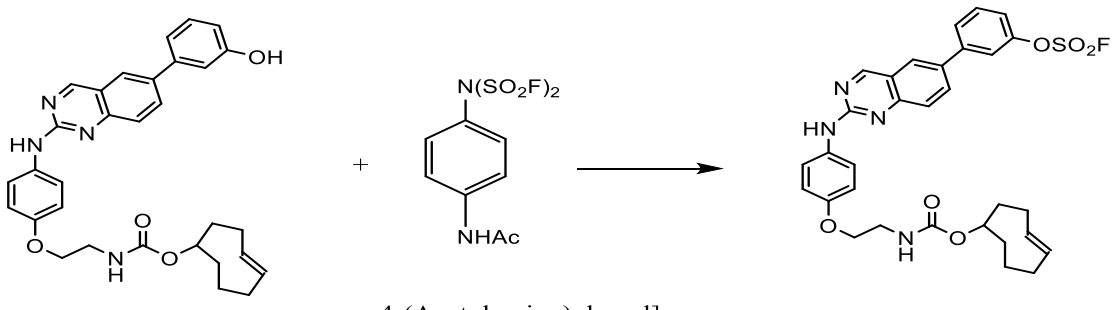
15.9 mg (0.0352 mmol) Compound 4 and 14.1 mg (0.0449 mmol) 4-(Acetylamino)phenyl]imidodisulfuryl difluoride (AISF) were added to 1 mL tetrahydrofuran (THF) followed by 36 µL (0.2412 mmol) 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) over a period of 30 seconds. The solution color was yellow. The solution was stirred at room temperature for 10 minutes. The mixture was diluted with 15 mL ethyl acetate. The solution was extracted with 0.5N HCl (15 mL * 2). The organic extracts were washed with 15 mL brine, dried by sodium sulfate and concentrated *in vacuo*. and purified by HPLC. The mass of the yellow product Compound 5 was 4.7 mg (0.0088 mmol). The yield was 25%. ^{1}H NMR (300 MHz, methanol-d4) δ 9.29 (s, 1H), 8.19 – 8.08 (m, 2H), 7.82 – 7.62 (m, 4H), 7.60 – 7.46 (m, 1H), 7.45 – 7.31 (m, 1H), 7.36 – 7.27 (m, 1H), 7.21 – 7.10 (m, 1H), 7.02 (dd, J = 9.1, 2.9 Hz, 1H), 5.83 (br s, 1H), 4.12 (t, J = 5.6 Hz, 2H), 3.66 – 3.57 (m, 2H), 2.93 – 2.81 (m, 2H), 2.70 – 2.57 (m, 2H), 2.54 – 2.34 (m, 1H). MS *m/z* ($C_{27}H_{23}FN_4O_5S$) calc'd = 534.6, observed M+1=533.2.



Compound 3 TCO-NHS ester Compound 11

Scheme.6: Synthesis of Compound 11.

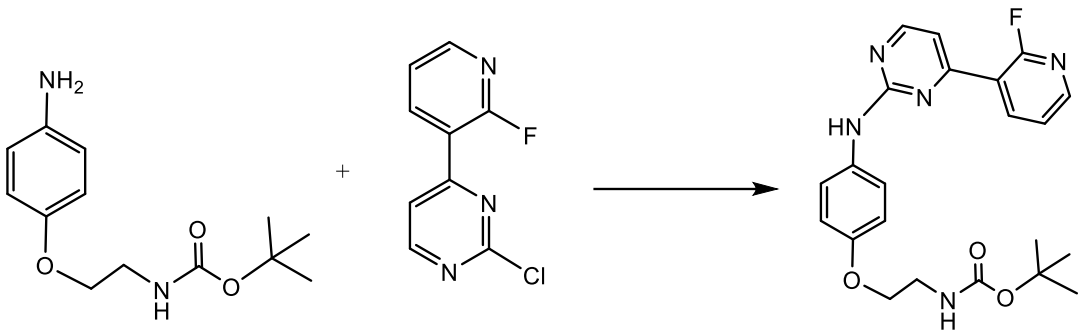
30.1 mg (0.0808 mmol) Compound 3 and 89.9 mg (0.3364 mmol) TCO-NHS ester were added to 4 mL DCM with 200 µL TEA (1.4339 mmol). The solution color was yellow. The solution was stirred at room temperature for 3h. It was concentrated *in vacuo*. The Compound 11 was purified by silica flash chromatography (0%-100% ethyl acetate/hexane). The yellow product was 30.6 mg (0.0583 mmol). The yield was 72%. ^{1}H NMR (300 MHz, methanol-d4) δ 9.16 (s, 1H), 8.20 (br s, 1H), 8.07 – 7.96 (m, 2H), 7.80 – 7.53 (m, 2H), 7.61 – 7.56 (m, 1H), 7.30 (t, *J* = 7.9 Hz, 1H), 7.20 – 7.11 (m, 1H), 7.09 – 6.87 (m, 1H), 6.81 (dd, *J* = 11.5, 4.7 Hz, 1H), 5.72 – 5.41 (m, 2H), 4.36 (br s, 1H), 4.15 – 4.06 (m, 1H), 4.02 (t, *J* = 5.5 Hz, 2H), 3.69 (s, 1H), 3.53 – 3.33 (m, 2H), 2.44 – 2.28 (m, 4H), 2.01 – 1.55 (m, 4H), 1.51 – 1.34 (m, 2H), 1.32 – 1.18 (m, 1H). MS *m/z* ($C_{31}H_{32}N_4O_4$) calc'd = 524.6, observed M+1 =525.2.



Compound 11 4-(Acetylamino)phenyl] imidodisulfuryl difluoride Compound 12

Scheme.7: Synthesis of Compound 12 (Probe 1-TCO).

30.6 mg (0.0583 mmol) Compound 11 and 25.3 mg (0.0805 mmol) AISF were added to 2 mL THF followed by 132 μL (0.8844 mmol) DBU over a period of 30 seconds. The solution color was yellow. The solution was stirred at room temperature for 10 minutes. The mixture was diluted with 15 mL ethyl acetate. The solution was extracted with 0.5N HCl (15 mL * 2). The organic extracts were washed with 15 mL brine, dried by sodium sulfate and concentrated *in vacuo*, and purified by HPLC. The mass of the orange product Compound 12 was 7.6 mg (0.0125 mmol). The yield was 21%. ^{1}H NMR (300 MHz, methanol-d4) δ 9.22 (s, 1H), 8.17 – 8.03 (m, 2H), 7.88 – 7.55 (m, 3H), 7.47 – 7.38 (m, 1H), 7.33 – 7.24 (m, 1H), 7.11 – 7.02 (m, 1H), 6.93 (dd, *J* = 9.0, 3.1 Hz, 1H), 5.78 (s, 1H), 5.59 – 5.42 (m, 2H), 4.30 (br s, 1H), 4.08 – 3.94 (m, 2H), 3.47 – 3.38 (m, 2H), 3.05 – 3.00 (m, 1H), 2.35 – 2.26 (m, 4H), 2.02 – 1.80 (m, 4H), 1.77 – 1.49 (m, 2H), 1.35 – 1.20 (m, 1H). MS *m/z* ($C_{31}H_{31}FN_4O_6S$) calc'd = 606.7, observed M+1 = 607.1.



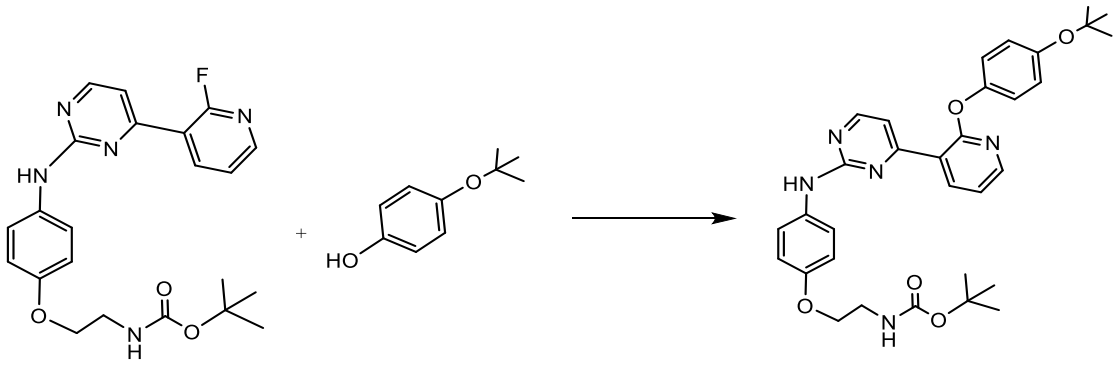
tert-Butyl 2-(4-aminophenoxy) ethylcarbamate

2-Chloro-4-(2-fluoro-3-pyridinyl)pyrimidine

Compound 6

Scheme.8: Synthesis of Compound 6.

563.5 mg (2.2334 mmol) tert-Butyl 2-(4-aminophenoxy)ethylcarbamate and 663.0 mg (3.1630 mmol) 2-Chloro-4-(2-fluoro-3-pyridinyl)pyrimidine were added to 50 mL dimethyl sulfoxide (DMSO), 790.2 mg (5.2021 mmol) cesium fluoride, and 2 mL (0.0115 mol) *N,N*-Diisopropylethylamine. The color of the solution was brown. The solution was stirred at 90℃ for 2h. The reaction mixture was diluted with 30 mL distilled water. The solution was extracted with ethyl acetate (40 mL * 3). The organic extracts were washed by 30 mL brine, dried by sodium sulfate and concentrated *in vacuo*. The Compound 6 was purified by silica flash chromatography (10%-100% ethyl acetate/hexane). The yellow product was 433.2 mg (1.0182 mmol). The yield was 46%. ^{1}H NMR (300 MHz, DMSO-d6) δ 9.62 (s, 1H), 8.60-8.53 (m, 2H), 8.40 (d, *J* = 4.82 Hz, 1H), 7.58 (t, *J* = 8.25 Hz, 1H), 6.90 (d, *J* = 8.8 Hz, 1H), 6.64 (d, *J* = 8.4 Hz, 2H), 6.49 (d, *J* = 8.3 Hz, 2H), 4.60 (s, 1H), 3.95-3.91 (t, *J* = 5.9 Hz, 2H) 3.81-3.77 (t, *J* = 6.1 Hz, 2H), 1.38 (s, 9H). MS *m/z* ($C_{22}H_{24}FN_5O_3$) calc'd = 425.5, observed M+1 = 426.1.

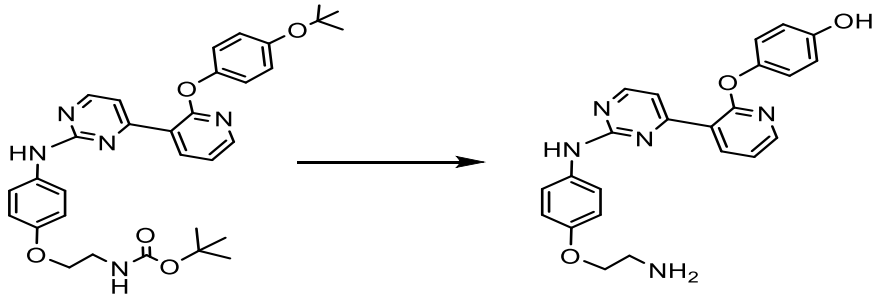


Compound 6 4-tert-Butoxyphenol Compound 7

Scheme.9: Synthesis of Compound 7.

134.4 mg (0.3159 mmol) Compound 6 and 77.7 mg (0.4675 mmol) 4-tert-Butoxyphenol were added with 5 mL DMSO and 301.8 mg (0.9263 mmol) cesium carbonate. The color of the

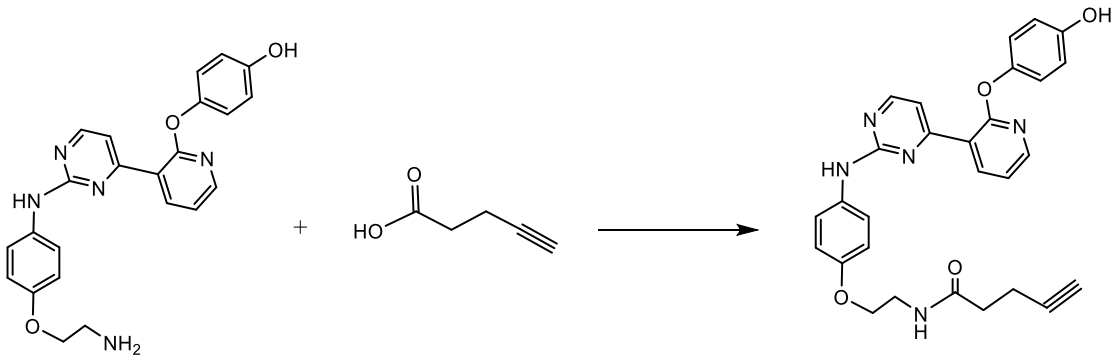
solution was black. The solution was stirred at 120℃ for 2h. The reaction mixture was diluted by 30 mL ethyl acetate after the solution cooled to room temperature. The solution was extracted with distilled water (30 mL * 2). The organic extracts were washed with brine (20 mL * 2), dried by sodium sulfate and concentrated *in vacuo*. The Compound 7 was purified by silica flash chromatography (0%-100% dichloromethane/methanol). The red brown product was 46.7 mg (0.0817 mmol). The yield was 26%. ^{1}H NMR (300 MHz, chloroform-d) δ 8.51 (dd, *J* = 7.6, 2.0 Hz, 1H), 8.44 (d, *J* = 5.3 Hz, 1H), 8.25 (dd, *J* = 4.8, 2.0 Hz, 1H), 7.57 (t, *J* = 8.4 Hz, 1H), 7.49 – 7.43 (m, 1H), 7.22 – 6.99 (m, 4H), 6.93 (d, *J* = 8.6 Hz, 4H), 5.13 (s, 1H), 4.07 – 4.01 (m, 2H), 3.48 – 3.40 (m, 2H), 1.38 (s, 18H). MS *m/z* ($C_{32}H_{37}N_5O_5$) calc'd = 571.7, observed M-BOC group = 472.1.



Scheme.10: Synthesis of Compound 8.

46.7 mg (0.0817 mmol) Compound 7 was added to 3.2 mL DCM with 1.4 mL (18.2950 mmol) TFA. The solution was stirred at room temperature for 3h. The liquid color was dark red. After 3h reaction, the solution was concentrated *in vacuo*. The dark brown product Compound 8 was 33.6 mg (0.0809 mmol). The yield was 99%. ^{1}H NMR (300 MHz, methanol-d4) δ 8.53 (dd, *J* = 7.7, 2.0 Hz, 1H), 8.40 (d, *J* = 6.1 Hz, 1H), 8.22 (dd, *J* = 4.9, 1.9 Hz, 1H), 7.76 (d, *J* = 6.0 Hz,

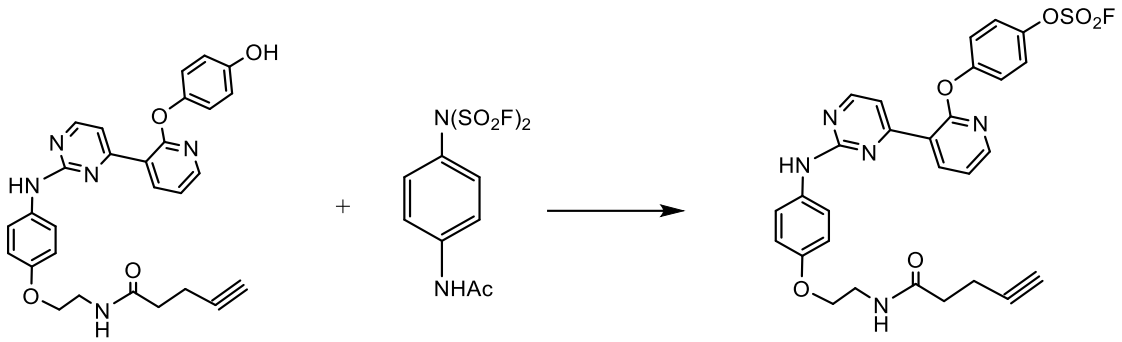
1H), 7.65 – 7.54 (m, 2H), 7.24 (dd, *J* = 7.7, 4.8 Hz, 1H), 7.13 – 7.01 (m, 2H), 7.05 – 6.94 (m, 2H), 6.91 – 6.79 (m, 2H), 5.13 (br s, 1H), 4.10 (br s, 1H), 4.26 (t, *J* = 5.1 Hz, 2H), 3.40 (t, *J* = 5.0 Hz, 2H), 1.58 (s, 2H). MS *m/z* ($C_{23}H_{21}N_5O_3$) calc'd = 415.5, observed M+1 = 416.0.



Compound 8 4-Pentynoic acid Compound 9

Scheme.11: Synthesis of Compound 9.

33.6 mg (0.0809 mmol) Compound 8 and 20.7 mg (0.2110 mmol) 4-Pentynoic acid were added to 1.618 mL DCM with 37.7 mg (0.2428 mmol) EDCI; 34.8 mg (0.2575 mmol) HOBT; and 110 µL (0.6315 mmol) *N,N*-Diisopropylethylamine. The solution color was orange. The solution was stirred at room temperature overnight. The mixture was concentrated *in vacuo* and purified by HPLC. The mass of the yellow product Compound 9 was 31.1 mg (0.0628 mmol). The yield was 78%. ^{1}H NMR (300 MHz, methanol-d4) δ 8.54 (dd, *J* = 7.6, 2.0 Hz, 1H), 8.44 (d, *J* = 5.2 Hz, 1H), 8.31 (br s, 1H), 8.20 (dd, *J* = 4.9, 2.0 Hz, 1H), 7.65 – 7.54 (m, 2H), 7.48 (d, *J* = 5.2 Hz, 1H), 7.29 (dd, *J* = 7.6, 4.9 Hz, 1H), 7.20 (s, 4H), 7.01 – 6.81 (m, 2H), 4.75 (br s, 1H), 4.06 (t, *J* = 5.5 Hz, 2H), 3.60 (t, *J* = 5.5 Hz, 2H), 2.84 (t, *J* = 7.1 Hz, 2H), 2.62 (t, *J* = 7.0 Hz, 2H), 2.51 – 2.42 (m, 1H). MS *m/z* ($C_{28}H_{25}N_5O_4$) calc'd = 495.5, observed M+1 = 496.2.



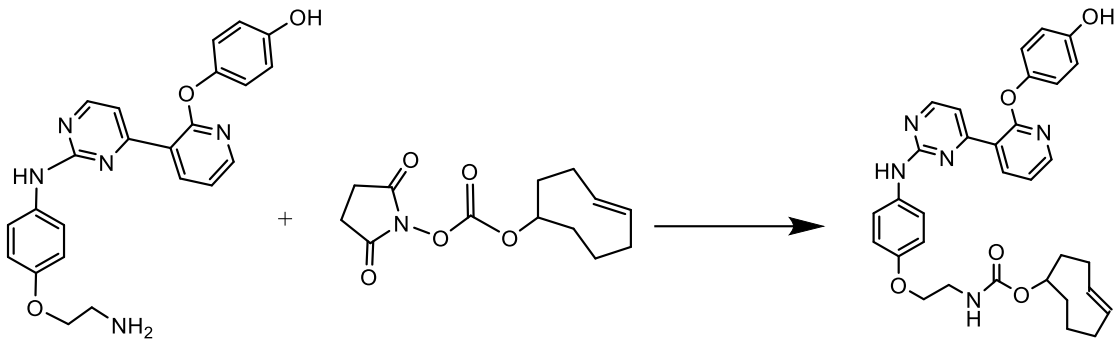
Compound 9

4-(Acetylamino)phenyl] imidodisulfuryl difluoride

Compound 10

Scheme.12: Synthesis of Compound 10 (Probe 2).

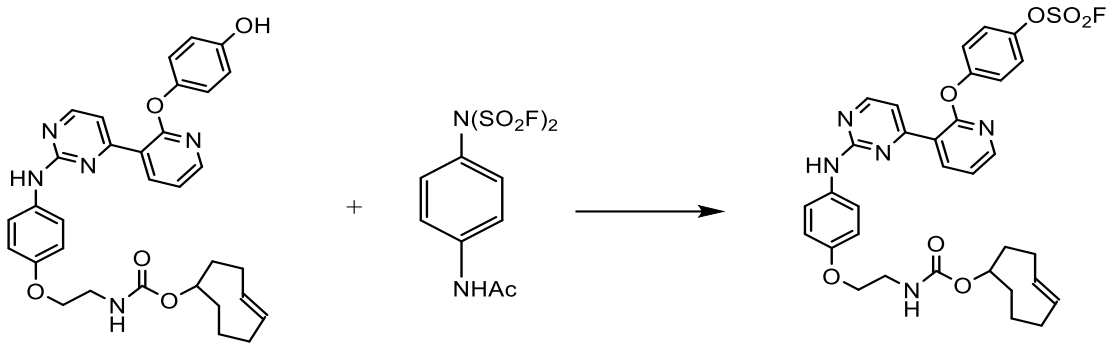
31.1 mg (0.06228 mmol) Compound 9 and 24.6 mg (0.0783 mmol) AISF were added to 1.5 mL THF followed by 60 µL (0.4020 mmol) DBU over a period of 30 seconds. The solution color was dark yellow. The solution was stirred at room temperature for 10 minutes. The mixture was diluted by 15mL ethyl acetate. The solution was extracted with 0.5N HCl (15 mL * 2). The organic extracts were washed with 15 mL brine, dried by sodium sulfate and concentrated *in vacuo*, and purified by HPLC. The mass of the yellow product Compound 10 was 7.9 mg (0.0137 mmol). The yield was 22%. ^{1}H NMR (300 MHz, methanol-d4) δ 8.56 (dd, *J* = 7.6, 2.0 Hz, 1H), 8.40 (d, *J* = 5.7 Hz, 1H), 8.25 (dd, *J* = 4.9, 2.0 Hz, 1H), 7.62 (d, *J* = 5.7 Hz, 1H), 7.60 – 7.50 (m, 2H), 7.42 – 7.25 (m, 1H), 7.21 (s, 4H), 7.02 – 6.93 (m, 2H), 4.64 (br s, 1H), 4.08 (t, *J* = 5.5 Hz, 2H), 3.60 (t, *J* = 5.5 Hz, 2H), 2.85 (t, *J* = 7.0 Hz, 2H), 2.68 – 2.56 (m, 2H), 2.56 – 2.33 (m, 1H). MS *m/z* ($C_{28}H_{24}FN_5O_6S$) calc'd = 577.6, observed M+1 = 576.3.



Compound 8 TCO-NHS ester Compound 13

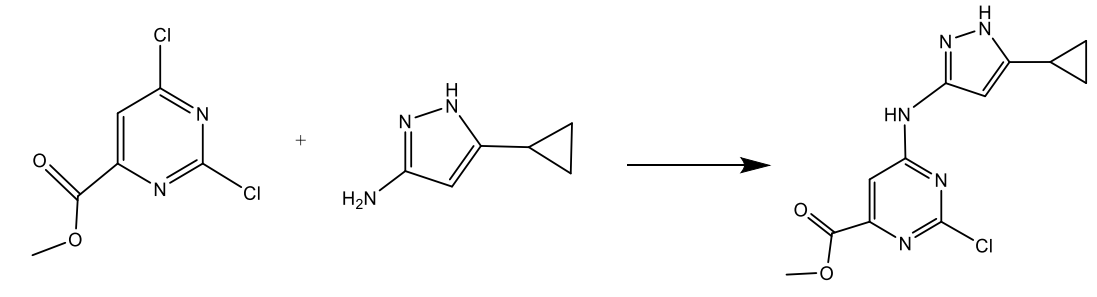
Scheme.13: Synthesis of Compound 13.

21.9 mg (0.0527 mmol) Compound 8 and 59.9 mg (0.2241 mmol) TCO-NHS ester were added to 3 mL DCM with 200 µL TEA (0.7170 mmol). The solution color was dark yellow. The solution was stirred at room temperature for 3h. It was concentrated *in vacuo*. Compound 13 was purified by silica flash chromatography (0%-100% ethyl acetate/hexane). The yellow product was 22.7 mg (0.0400 mmol). The yield was 76%. ^{1}H NMR (300 MHz, methanol-d4) δ 8.52 (dd, *J* = 7.6, 2.0 Hz, 1H), 8.42 (d, *J* = 5.2 Hz, 1H), 8.14 (dd, *J* = 4.9, 2.0 Hz, 1H), 7.65 – 7.54 (m, 2H), 7.50 (d, *J* = 5.3 Hz, 1H), 7.22 (dd, *J* = 7.6, 4.9 Hz, 1H), 7.04 – 6.94 (m, 2H), 6.94 – 6.79 (m, 4H), 6.63 (br s, 1H), 5.69 – 5.38 (m, 2H), 4.35 (s, 1H), 4.18 – 4.06 (m, 1H), 4.01 (t, *J* = 5.6 Hz, 2H), 3.46 (t, *J* = 5.6 Hz, 2H), 3.33 (s, 1H), 2.42 – 2.27 (m, 4H), 2.04 – 1.87 (m, 4H), 1.83 – 1.54 (m, 2H), 1.33 – 1.21 (m, 1H). MS *m/z* ($C_{32}H_{33}N_5O_5$) calc'd = 567.7, observed M+1 = 568.3.



Scheme.14: Synthesis of Compound 14 (Probe 2-TCO).

22.7 mg (0.0400 mmol) Compound 13 and 17.9 mg (0.0570 mmol) AISF were added to 2 mL THF followed by 132 µL (0.8844 mmol) DBU over a period of 30 seconds. The solution color was yellow. The solution was stirred at room temperature for 10 minutes. The mixture was diluted with 15 mL ethyl acetate. The solution was extracted with 0.5N HCl (15 mL * 2). The organic extracts were washed with 15 mL brine, dried by sodium sulfate and concentrated *in vacuo*, and purified by HPLC. The mass of the orange product Compound 14 was 7.1 mg (0.0109 mmol). The yield was 27%. ^{1}H NMR (300 MHz, methanol-d4) δ 8.50 (dd, *J* = 7.6, 2.0 Hz, 1H), 8.36 (d, *J* = 5.5 Hz, 1H), 8.19 (dd, *J* = 4.9, 2.0 Hz, 1H), 7.55 – 7.42 (m, 3H), 7.39 – 7.23 (m, 3H), 6.92 – 6.83 (m, 4H), 5.58 – 5.40 (m, 2H), 4.94 – 4.88 (m, 1H), 4.29 (br s, 1H), 3.96 (t, *J* = 5.6 Hz, 2H), 3.46 – 3.32 (m, 2H), 2.34 – 2.21 (m, 4H), 2.00 – 1.82 (m, 4H), 1.78 – 1.49 (m, 2H), 1.28 – 1.22 (m, 1H). MS *m/z* ($C_{32}H_{32}FN_5O_7S$) calc'd = 649.7, observed M+1 = 650.2.



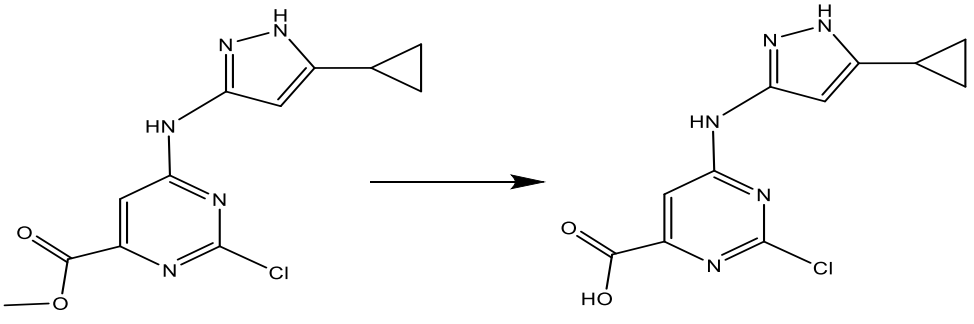
methyl 2,6-dichloropyrimidine -4-carboxylate

5-cyclopropyl-1*H*-pyrazol-3-amine

Compound 15

Scheme.15: Synthesis of Compound 15.

2.5031 g (0.0121 mol) methyl 2,6-dichloropyrimidine-4-carboxylate was dissolved in 16.6 mL THF at 0℃. Then, 1.1 mL (0.0122 mol) 5-cyclopropyl-1H-pyrazol-3-amine and 2.5 mL (0.0144 mol) *N,N*-Diisopropylethylamine were added to this solution. The reaction was stirred at room temperature for 2h. The mixture color was brown. The solvent was evaporated *in vacuo*; and 16.6 mL methanol was added at 80℃ for 1h. The mixture was a yellow suspension. After the mixture cooled to room temperature, the precipitate was filtered and rinsed by cooled methanol. The yellow product (Compound 15) was 2.0031 g (0.0068 mol). The yield was 56%. ^{1}H NMR (300 MHz, DMSO-d6) δ 12.30 (br s, 1H), 10.78 (d, J = 41.0 Hz, 1H), 8.29 (s, 0.5H), 7.35 (s, 0.5H), 6.38 (s, 0.5H), 5.68 (s, 0.5H), 3.87 (s, 3H), 1.91 (br s, 1H), 1.00 – 0.86 (m, 2H), 0.75 – 0.64 (m, 2H). MS *m/z* ($C_{12}H_{12}ClN_5O_2$) calc'd = 293.7, observed M+1 = 295.2.

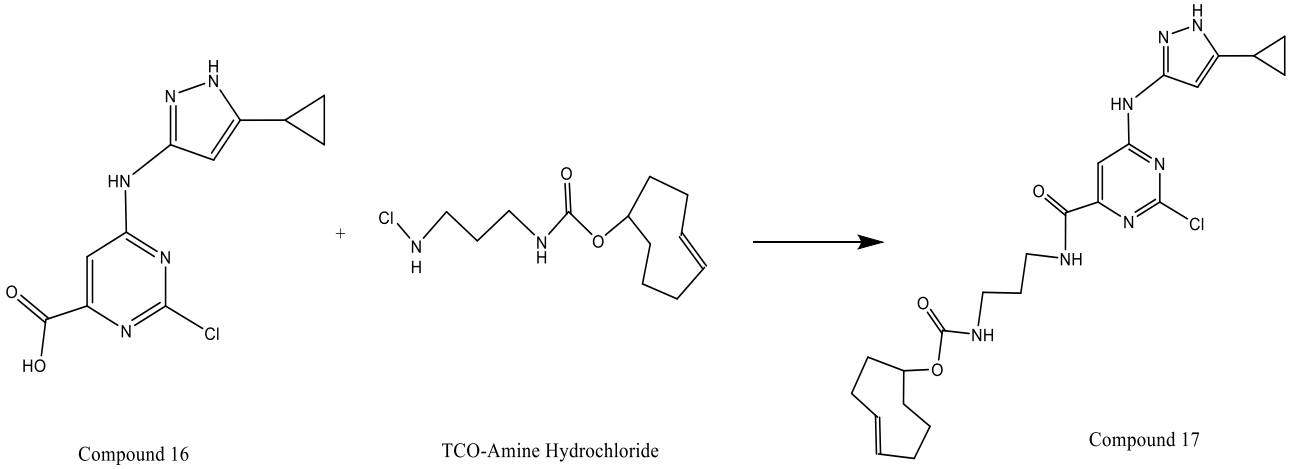


Compound 15

Compound 16

Scheme.16: Synthesis of Compound 16.

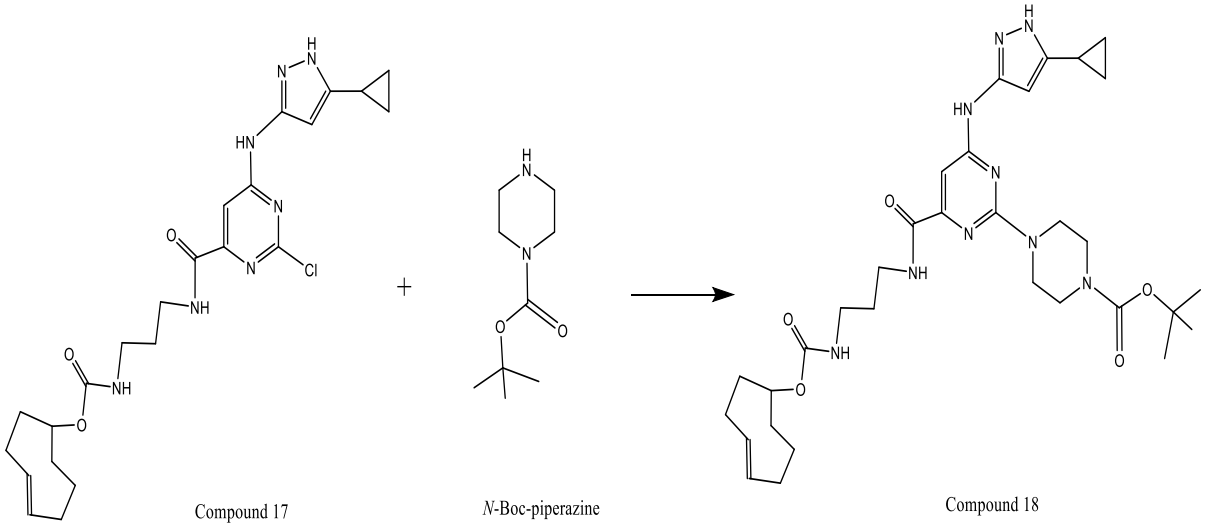
1.8433 g (0.0063 mol) Compound 15 was dissolved in 35 mL dioxane. 35 mL of 1M NaOH was added over 10 minutes. The reaction was stirred at room temperature for 20 minutes. The mixture was an orange liquid. Then, 1M HCl was added to the solution to acidify to pH=4, when a white precipitate was formed. The precipitate was filtered and rinsed with cooled distilled water. The white product (Compound 16) was 1.6177 g (0.0058 mol). The yield was 92%. ^{1}H NMR (300 MHz, DMSO-d6) δ 12.33 (br s, 1H), 10.71 (d, *J* = 40 Hz, 1H), 8.24 (s, 0.5H), 7.33 (s, 0.5H), 6.37 (s, 0.5H), 5.68 (s, 0.5H), 1.91 (tt, *J* = 8.5, 5.0 Hz, 1H), 1.00 – 0.86 (m, 2H), 0.75 – 0.63 (m, 2H). MS *m/z* ($C_{11}H_{10}ClN_5O_2$) calc'd = 279.7, observed M+1 = 280.0.



Scheme.17: Synthesis of Compound 17.

96 mg (0.3432 mmol) Compound 16 and 85.1 mg (0.3238 mmol) TCO-Amine Hydrochloride were added in 7.5 mL DMF with 1.25 mL (0.0072 mol) *N,N*-Diisopropylethylamine. 208.4 mg (0.5481 mmol) HATU was added over 30 minutes. The reaction was stirred at room

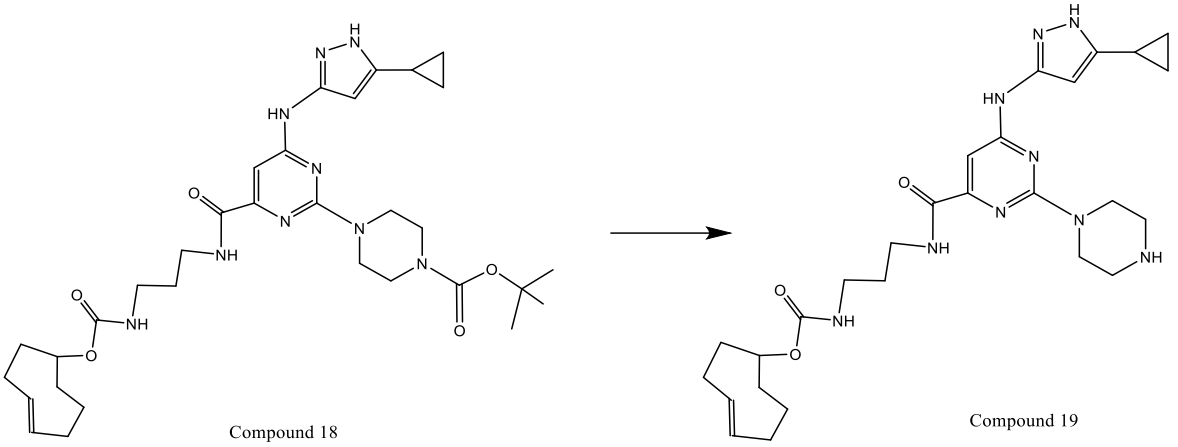
temperature under nitrogen, overnight. The mixture was an orange liquid. The reaction mixture was diluted with 30 mL ethyl acetate. The solution was extracted with distilled water (30 mL * 2), dried by sodium sulfate and concentrated *in vacuo*. The Compound 17 was purified by silica flash chromatography (10%-100% ethyl acetate/hexane). The yellow product was 121.7 mg (0.2494 mmol). The yield was 77%. ^{1}H NMR (300 MHz, methanol-d4) δ 8.80 (d, *J* = 11.7 Hz, 1H), 8.10 (s, 0.5H), 8.00 (s, 1H), 7.57 (s, 0.5H), 6.74 (s, 0.5H), 6.17 (s, 0.5H), 5.70 – 5.39 (m, 2H), 4.97 (br s, 1H), 4.11 (q, *J* = 7.2 Hz, 2H), 4.30 (br s, 1H), 3.43 (t, *J* = 4.2 Hz, 2H), 3.16 (t, *J* = 6.5 Hz, 2H), 2.40 – 2.21 (m, 4H), 2.03 (m, 1H), 2.01 – 1.84 (m, 4H), 1.84 – 1.66 (m, 2H), 1.33 – 1.14 (m, 1H), 1.07 – 0.87 (m, 2H), 0.82 – 0.69 (m, 2H). MS *m/z* ($C_{23}H_{30}ClN_7O_3$) calc'd = 488.0, observed M+1 = 488.1.



Scheme.18: Synthesis of Compound 18.

121.7 mg (0.2494 mmol) Compound 17 and 102.2 mg (0.5487 mmol) *N*-Boc-piperazine were dissolved in 10 mL DMF. The mixture was stirred at 100℃ for 2h. The mixture was a yellow color. The solution was concentrated *in vacuo* and mixed with 30 mL ethyl acetate. The organic

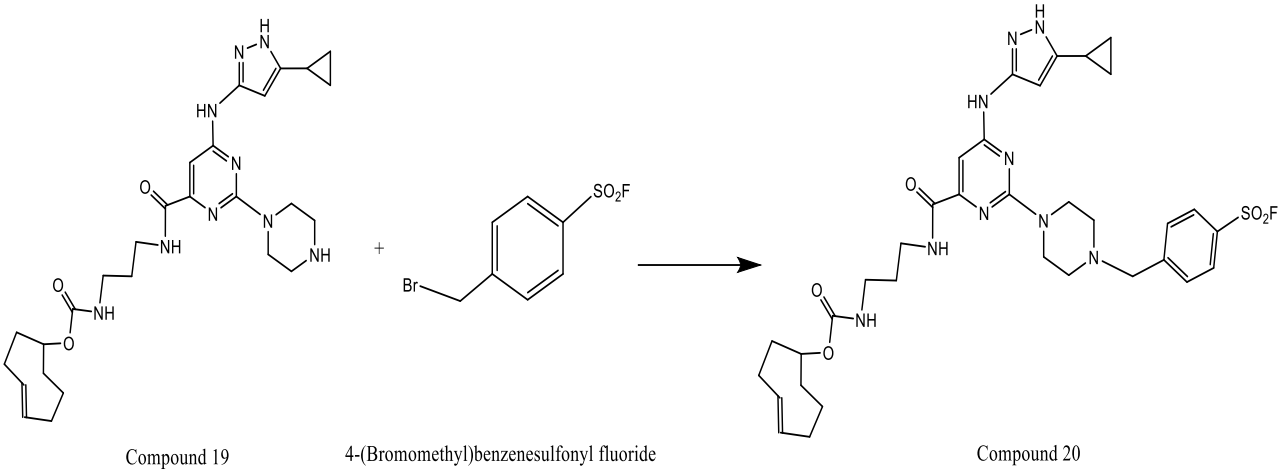
extracts were washed with 30 mL saturated ammonium chloride, 30 mL saturated sodium bicarbonate, and 30 mL brine. The organic extracts were dried by sodium sulfate and concentrated *in vacuo*. The Compound 18 was purified by silica flash chromatography (0%-100% methanol/dichloromethane). The yellow product was 114.0 mg (0.1787 mmol). The yield was 72%. ^{1}H NMR (300 MHz, methanol-d4) δ 8.08 (s, 0.5H), 7.99 (s, 1H), 6.84 (s, 0.5H), 6.72 (s, 0.5H), 6.10 (s, 0.5H), 5.67 – 5.38 (m, 2H), 5.13 (br s, 1H), 4.31 (br s, 1H), 4.15 (q, *J* = 7.6 Hz, 2H), 3.92 – 3.83 (m, 4H), 3.61 – 3.47 (m, 4H), 3.41 (t, *J* = 6.7 Hz, 2H), 3.14 (d, *J* = 6.5 Hz, 2H), 2.41 – 2.25 (m, 4H), 2.03 (m, 1H), 2.00 – 1.85 (m, 4H), 1.81 – 1.58 (m, 2H), 1.51 (s, 9H), 1.29-1.20 (m, 1H), 1.07 – 0.86 (m, 2H), 0.82 – 0.69 (m, 2H). MS *m/z* ($C_{32}H_{47}N_9O_5$) calc'd = 637.79, observed M+Na = 660.4.



Scheme.19: Synthesis of Compound 19.

114.0 mg (0.1787 mmol) Compound 18 was added to 7mL DCM with 3 mL (0.0382 mol) TFA. The solution was stirred at room temperature for 3h. The liquid was dark yellow in color. After 3h reaction, the solution was concentrated *in vacuo*. The dark brown product Compound 19 was 96.0 mg (0.1785 mmol). The yield was 99%. ^{1}H NMR (300 MHz, methanol-d4) δ 8.11 (s,

0.5H), 7.99 (s, 1H), 7.53 (d, *J* = 7.3 Hz, 1H), 6.92 (s, 0.5H), 6.89 (s, 0.5H), 6.16 (s, 0.5H), 5.65 (m, 2H), 4.67 (br s, 1H), 4.18 (br s, 1H), 4.09 (q, *J* = 7.1 Hz, 2H), 3.81 – 3.71 (m, 4H), 3.56 – 3.48 (m, 4H), 3.44 (t, *J* = 6.7 Hz, 1H), 3.23 – 3.13 (m, 2H), 2.82 (br s, 1H), 2.09 – 1.93 (m, 4H), 1.83 – 1.70 (m, 4H), 1.58 (s, 1H), 1.39 – 1.22 (m, 2H), 1.20 – 1.09 (m, 1H), 0.95 – 0.89 (m, 2H), 0.89 – 0.82 (m, 2H). MS *m/z* ($C_{27}H_{39}N_9O_3$) calc'd = 537.7, observed M+1 = 538.3.



Scheme.20: Synthesis of Compound 20 (Probe 3).

29.8 mg (0.0554 mmol) Compound 19 and 19.9 mg (0.0786 mmol) 4-(Bromomethyl)benzenesulfonyl fluoride were added to 5 mL DMF with 300 µL (1.7222 mmol) *N,N*-Diisopropylethylamine. The mixture was stirred at room temperature for 20 minutes. The mixture was yellow in color. The solution was concentrated in *vacuo* and mixed with 30 mL ethyl acetate. The organic extracts were washed with 30 mL saturated sodium bicarbonate, and 30 mL brine. The organic extracts were dried by sodium sulfate and concentrated *in vacuo*. The mixture was purified by HPLC. The mass of the white product Compound 20 was 5.7 mg (0.0080 mmol). The yield was 14%. ^{1}H NMR (300 MHz, methanol-d4) δ 8.98 (br s, 1H), 8.25

(d, *J* = 8.3 Hz, 1H), 8.13 – 7.99 (m, 1H), 7.93 (d, *J* = 8.2 Hz, 1H), 7.85 – 7.76 (m, 1H), 6.96 (s, 1H), 6.06 (s, 1H), 5.33 – 5.21 (m, 2H), 4.81 (br s, 1H), 4.64 (br s, 1H), 4.57 (br s, 1H), 3.44 – 3.38 (m, 2H), 3.19 (t, *J* = 5.2 Hz, 2H), 2.84 – 2.74 (m, 4H), 2.42 – 2.31 (m, 4H), 2.42 – 2.36 (m, 2H), 2.15 (s, 2H), 2.00 – 1.88 (m, 4H), 1.95 (s, 1H), 1.81 – 1.69 (m, 4H), 1.62 – 1.50 (m, 2H), 1.27 – 1.19 (m, 1H), 1.08 – 0.95 (m, 2H), 0.82 – 0.70 (m, 2H). MS *m/z* ($C_{34}H_{44}FN_9O_5S$) calc'd = 709.8, observed M+1 = 710.4.

Cells incubation:

Cells (Hela SRC Wild Type (WT) cells or HCT116 cells) were grown in McCoy's medium supplemented with 10% FBS. Cells were grown at 37°C in a humidified incubator with 5% CO_2.

Cells were plated on 15 cm plates to 90% confluency. Media was aspirated and cells were washed once with ice cold 1X PBS. 1 mL Ice cold lysis buffer 1 (20 mM Tris-HCl, 137 mM NaCl (pH = 7.5), 1 mM EDTA, 1% IGEPAL CA-630, phosphatase inhibitor cocktails 2 + 3 (Sigma-Aldrich), protease inhibitor tablet (ThermoFisher)) was added to each plate. Cells were then scraped and transferred into 1.5 mL Eppendorf tubes. Then, the 1.5mL Eppendorf tubes were sonicated on the water bath sonicator. 10 x 10-sec sonications with 10 sec breaks were carried out on ice. Finally, the soincated lysate was rotated for 10 minutes at 4°C. Samples were then centrifuged at 17xG for 10 minutes at 4°C. The supernatant was then collected and placed in separate 1.5 mL Eppendorf tubes.

Concentration of cell lysate detection:

2.5 mg Bovine serum albumin (BSA) was dissolved in lysis buffer (PBS + 1% IGEPAL) to prepare 2 mg/mL solution under ice-cold condition. The BSA solution was serial diluted to 1 mM, 0.5 mM, 0.25 mM, 0.125 mM, 0.0625 mM, and 0.03125 mM. 5 µL of serial diluted BSA solution and cell lysate were added in the microplate; each sample was added twice in two different wells. 150 µL Protein assay reagent was added for each well. The mixture was incubated for 5 minutes. Accounting for the absorbance by serial diluted BSA solution and the calibration line, the concentration of cell lysate would be detected. The cell lysate would be diluted with cell lysate buffer to desired concentration.

Probe 1 and Probe 2 click chemistry preparation for SDS-PAGE:

10 mM DMSO mixture for Competitor 1; and 4 mM DMSO mixture for Probe 1 and for Probe 2 were prepared. 50 µL of 1 mg/mL Hela SRC WT cell lysate was separately added to 14 of the 1.5 mL Eppendorf tubes. Two tubes were labeled as ‘Competitor 1’, and ‘Positive control’. The remaining 12 tubes were separated into 3 groups; 4 tubes in each group were labeled as ‘Probe 1’; ‘Probe 2’; ‘Competitor + Probe1’; and ‘Competitor 1 + Probe 2’. ‘Competitor 1’, ‘Competitor 1 + Probe 1’, and ‘Competitor 1 + Probe 2’. 0.5 µL of 10mM Competitor 1 was separately added to ‘Competitor 1’, ‘Competitor 1 + Probe 1’, and ‘Competitor 1 + Probe 2’, and 0.5 µL DMSO was separately added to the other tubes. The mixture was incubated with rotation at 4°C for 30 minutes. 0.5 µL of 4 mM Probe 1 was separately added to ‘Probe 1’ and

‘Competitor 1 + Probe 1’; 0.5 µL of 4 mM Probe 2 was separately added to ‘Probe 2’ and ‘Competitor + Probe 2’. 0.5 µL of DMSO was added to ‘Competitor’. The mixture was incubated with rotation at 4℃ for 1h (first group); 2h (second group); 4h (third group). Only 0.5 µL of 10 mM iodoacetamide (IAA)-alkyne was added to the ‘Positive control’ for 4h incubation. The concentration of cell lysate for positive control, Competitor 1, Probe 1, and Probe 2 in each group was 100 µM, 100 µM, 40 µM, and 40 µM, respectively. Then 90 µL of click chemistry solution was prepared with 15 µL of 50 mM $CuSO_4$ (dissolve in MilliQ water); 15 µL of Biotin-N_3 solution (1.875 µL of 10 mM Biotin-N_3 dissolved in 13.125 µL DMSO); 15 µL of 50 mM tris(2-carboxyethyl)phosphine (dissolved in MilliQ water); and 45 µL of 1.7 mM tris(benzyltriazolylmethyl)amine (dissolved in 1:4 DMSO/t-BuOH), and 6 µL of the click chemistry solution was separately added to each tube. The mixture was incubated under rotation at room temperature for 1h.

Probe 1-TCO and Probe 2-TCO click chemistry preparation for SDS-PAGE:

10 mM DMSO mixture for Competitor 1; and 4 mM DMSO mixture for Probe 1-TCO and for Probe 2-TCO were prepared. 50 µL of 1 mg/mL Hela SRC WT cell lysate was separately added to 6 of the 1.5 mL Eppendorf tubes. Two tubes were labeled as ‘Competitor 1’ and ‘Positive control’. The other 4 tubes in each group were labeled as ‘Probe 1-TCO’; ‘Probe 2-TCO’; ‘Competitor 1 + Probe1-TCO’; and ‘Competitor 1 + Probe 2-TCO’. 0.5 µL of 10 mM Competitor 1 was separately added to ‘Competitor 1’, ‘Competitor 1 + Probe 1-TCO’, and ‘Competitor 1 + Probe 2-TCO’, and 0.5 µL DMSO was separately added to the other two tubes.

The mixture was incubated with rotation at 4℃ for 30 minutes, and 0.5 μL of 4 mM Probe 1-TCO was separately added to 'Probe 1-TCO' and 'Competitor 1 + Probe 1-TCO'; 0.5 μL of 4 mM Probe 2-TCO was separately added to 'Probe 2-TCO' and 'Competitor 1 + Probe 2-TCO'. 0.5 μL of DMSO was added to 'Competitor'. Only 0.5 μL of 10 mM IAA-TCO was added to the 'Positive control'. The mixture was incubated with rotation at 4℃ for 4h. The concentration for positive control, Competitor 1, Probe 1-TCO, and Probe 2-TCO in each group was 100 μM, 100 μM, 40 μM, and 40 μM, respectively. 2.5 μL of 2 mM tetrazine (Tz)-PEG5-biotin was separately added to each tube. The mixture was incubated under rotation for 1h at room temperature.

Concentration variables experiment of Probe 1-TCO click chemistry preparation for SDS-PAGE:

10 mM DMSO mixture for Competitor 1; and 2 mM, 1 mM, and 0.5 mM DMSO mixture for Probe 1-TCO were prepared. 50 μL of 1 mg/mL Hela SRC WT cell lysate was separately added to 8 of the 1.5 mL Eppendorf tubes. Two tubes were labeled as 'Competitor 1' and 'Positive control', and the 6 other tubes were separated into 3 groups for the different concentrations, with 2 tubes in each group being labeled as 'Probe 1-TCO' and 'Competitor 1 + Probe 1-TCO'. 0.5 μL of 10 mM Competitor 1 was separately added to 'Competitor 1' and 'Competitor 1 + Probe 1-TCO', and 0.5 μL DMSO was separately added to the other tubes. The mixture was incubated with rotation at 4℃ for 30 minutes. 0.5 μL of 2 mM Probe 1-TCO was separately added to the first group of 'Probe 1-TCO' and 'Competitor 1 + Probe 1-TCO'; 0.5 μL of 1 mM

Probe 1-TCO was added to the second group; and 0.5 μL of 0.5 mM Probe 1-TCO was added to the third group. 0.5 μL of DMSO was added to 'Competitor 1'. Only 0.5 μL of 10 mM IAA-TCO was added to the 'Positive control'. The mixture was incubated with rotation at 4℃ for 4h. The concentration for positive control and Competitor 1 was 100 μM, and the concentrations for Probe 1-TCO in these three groups were 20 μM, 10 μM, and 5 uM. Then, 2.5 μL of 2 mM Tz-PEG5-biotin was separately added to each tube. The mixture was incubated under rotation for 1h at room temperature.

Dasatinib-beads pull-down experiment of Probe 1, Probe 2, Probe 1-TCO, and Probe 2-TCO preparation for SDS-PAGE:

4 mM of DMSO mixture for Probe 1, Probe 2, Probe 1-TCO, and Probe 2-TCO were prepared. 50 μL of 1 mg/mL Hela SRC WT cell lysate was separately added to 5 of the 1.5 mL Eppendorf tubes. These tubes were labeled as 'Control'; 'Probe 1'; 'Probe 2'; 'Probe 1-TCO'; and 'Probe 2-TCO'. 0.5 μL DMSO was added to the 'Control'. 0.5 μL of probes were added to the remaining groups corresponding to their labeled names. The concentrations for these probes were 20 μM. The mixtures were incubated for 4h at 4℃. Dasatinib-beads (in a 50% slurry dissolved in 20% ethanol) were washed with ice-cold cell lysis buffer 1 (3 X 500 μL per each time). 20 μL of dasatinib-beads were separately added to each incubated mixture, followed by 1h incubation at 4℃. (Only the 'Control' remained as a 20 μL mixture without beads, as 'No-beads incubation'.) After incubation, the samples were spun in a centrifuge, and 50 μL

supernatant was collected for each sample. Then, the samples with beads were 3X washed by ice-cold cell lysate buffer 1 (500 µL each time).

Probe 3 click chemistry preparation for SDS-PAGE:

2 mM DMSO mixture for Probe 3, 2 M DMSO mixture for cyclopropyl-aminopyrazole-quinazoline (Competitor 2). and for dasatinib (Competitor 3) were prepared. 50 µL 1 mg/mL of Hela SRC WT cell lysate was separately added to 4 of the 1.5 mL Eppendorf tubes; and 50 µL 1 mg/mL of HCT116 cell lysate was separately added to the 4 other 1.5 mL Eppendorf tubes. Two tubes in each cell lysate group were labeled as 'Competitor (2 or 3)' and 'Positive control'. The other two tubes in each group were labeled as 'Probe 3' and 'Competitor (2 or 3) + Probe 3'. For Hela SRC WT group, 0.5 µL of 2 mM Competitor 3 was separately added to 'Competitor 3' and 'Competitor 3 + Probe 3', and for HCT116 group, 0.5 µL of 2 mM Competitor 2 was separately added to 'Competitor 2' and 'Competitor 2 + Probe 3', while 0.5 µL DMSO was separately added to the other tubes. The mixture was incubated with rotation at 4°C for 30 minutes. 0.5 µL of 2 mM Probe 3 was separately added to 'Probe 3' and 'Competitor (2 or 3) + Probe 3' in each group. 0.5 µL of DMSO was added to 'Competitor (2 or 3)' in each group. 0.5 µL of 10 mM IAA-TCO was only added to the 'Positive control'. The concentration for positive control was 100 µM. The samples were incubated with rotation at 4°C for 1h. The concentration for competitors was 20 µM, and the concentration for Probe 3 was 20 µM. Then, 2.5 µL of 2 mM Tz-PEG5-biotin was separately added to each tube. The mixture was incubated under rotation for 1h at room temperature.

Preparation of tetrazine labeling conditions matrix experiment for SDS-PAGE:

The 24 of the 1.5 mL Eppendorf tubes were equally divided into two groups, and 50 μL of 1 mg/mL HCT116 cell lysate was added to each tube. 0.5 μL DMSO was separately added to one group of cell lysate; 0.5 μL IAA-TCO was separately added to the other group. The mixture was incubated with rotation at 4℃ for 1h. The two groups were subjected to the same Tz-PEG5-biotin matrix experiment. After adding Tz-PEG5-biotin, the incubation time variables at room temperature were 5 min, 15 min, 30 min, and 1h. The concentration variables of the final concentration for Tz-PEG5-biotin in the cell lysate were 1 μM, 10 μM, and 100 μM.

SDS-PAGE process; and proteins-membranes electrophoretic transfer process, and image:

After preparation, 16 μL of 4X SDS labeling dye was added to each tube. **(For dasatinib-beads pull-down experiment:** 16.7 μL of 4X SDS labeling dye was added to each tube with supernatant, and 16.7 μL of 1X SDS labeling dye was added to each tube containing beads, while 6.7 μL of 4X SDS labeling dye was added to the 'No-beads incubation'.**)** The samples with SDS labeling dye were boiled at 95℃ for 5 min. 5 μL of PageRuler™ Plus Prestained Protein Ladder and 15 μL of each sample was separately added to lanes of the SDS-page gel. The process of protein separation took 45 min at 150 V. The protein on SDS-page gel was transferred to PVDF membrane by Trans-Blot Turbo Transfer System. The membrane was soaked by blocking solution (5% non-fat dry milk in 1X TBST) with rotation for 1h at room temperature. **(For concentration variables experiment of Probe 1-TCO:** After blocking, the

membrane was soaked in Mouse monoclonal IgG (dissolved in blocking buffer to 1:4000 dilution) for 1h incubation with rotation at room temperature.**) (For dasatinib-beads pull-down experiment:** After blocking, the membrane was soaked in SRC Rabbit mAb (dissolved in blocking buffer to 1:1000 dilution) for overnight incubation with rotation at 4°C.**)** Then, the membrane was soaked in streptavidin solution (0.8 mg/mL streptavidin was dissolved in blocking buffer to make 1:1000 dilution.) with rotation for 1h at room temperature. **(For concentration variables experiment of Probe 1-TCO:** After adding streptavidin, Goat anti-mouse (dissolved in blocking buffer to 1:10000 dilution) was added for 1h incubation with rotation at room temperature.**) (For dasatinib-beads pull-down experiment:** No streptavidin was needed.**)** The membrane was washed with 1XTBS + 0.1%Tween 3 times (5 min each time). **(For dasatinib-beads pull-down experiment:** After washing, the membrane was soaked in Goat anti-Rabbit (dissolved in blocking buffer to 1:10000 dilution) for 1h with rotation at room temperature, followed by 1XTBS + 0.1%Tween 3 times (5 min each time).**)** The image of the membrane was captured using LI-COR Odyssey IR imager.

LC/MS/MS pull-down experiment for Probe 3:

3 mL of HCT116 cell lysate (1.5 mg/mL) was prepared. The experiment was divided into two groups, 'Competitor 2 + Probe 3' and 'Probe 3'; three replicate samples were run for each group. 30 µL of Sepharose-beads coupled with ethanolamine was separately added to 6 of the 1.5 mL Eppendorf tubes. The Sepharose-beads were washed three times (1st time: 500µL of cell lysate buffer 2 (100 mM HEPES pH = 7.5, 150 mM NaCl, 0.1% NP-40, 1X complete

EDTA-free protease inhibitor cocktail (Roche)); 2nd and 3rd time: ice-cold washing buffer (100 mM HEPES pH 7.5, 150 mM NaCl, 0.1% NP-40), 500 µL for each wash). Then, the cell lysate was separated equally into 6 of the 1.5 mL Eppendorf tubes with incubation for 1h at 4˚C for reducing background contaminants. 5 µL of 2 mM Competitor 2 was added to the 'Competitor 2 + Probe 3' group; and 5 µL of DMSO was added to the 'Probe 3' group, followed by 30 min rotated incubation at 4˚C. Then, 5 µL of 0.2 mM Probe 3 was added to these two groups with rotated incubation for 1h at 4˚C. The concentration for Probe 3 was 2 µM; for the Competitor 2 it was 20 µM. The cell lysate supernatant was transferred into 6 of the new 1.5 mL Eppendorf tubes, with each tube containing 30 µL Tz-Beads (Tz-Beads were prewashed by 3 X 500 µL ice-cold washing buffer). The mixture was incubated at 4˚C for 30 min under rotation. After the incubation, the samples were spun in a centrifuge, and the supernatant was quickly removed by aspiration. The beads were washed with 6 X 500 µL ice-cold washing buffer. 50 µL of Denaturing buffer was added to each tube (Tris base = 50 mM, pH = 8.5; Guanidinium Chloride = 6 M; TCEP = 5 mM; 2-Chloroacetamide = 10 mM), and boiled at 95 ˚C for 5 min. 50 µL of 100 M tetraethylammonium bromide (TEAB) was added to each tube, after cooling down. 1N NaOH was added to adjust the pH value to 8-9. 1 µg Lys-c Lysyl Endopeptidase was added to each tube with 2h incubation in a thermal mixer agitating at 1400 rpm at 37 ˚C. Then, 100 mM TEAB and 1 µg MS Grade Trypsin were added with overnight incubation in a thermal mixer agitating at 1400 rpm at 37 ˚C (the pH value for the mixtures was kept at 8-9). After the incubation, 200 µL of Buffer A (5% acetonitrile, 0.1% TFA, MilliQ water) was added, and the pH value was adjusted by formic acid to 2-3. C18 StageTip was used to desalt the supernatant, followed by washing once with 50 µL Buffer A. (The C18 StageTip was prewashed with 50

μL Buffer B (80% acetonitrile, 0.1% TFA, MilliQ water) and 50 μL Buffer A). The C18 StageTip bound with peptide was used for LC/MS/MS analysis. The samples were processed by Thermo-Dionex RSLCNano UHPLC instrument (Sunnyvale, CA), FTMS, and ITMS MS/MS.

Statistical Analyses for LC/MS/MS results:

The original data was processed by MaxQuant to obtain label-free quantification (LFQ) intensity and sequence coverage (%). Log2 (Fold change) and P-value in Student's *T*-test was obtained by Excel. *Fold change = (Average LFQ intensity for three replicates in 'Probe 3' group) – (Average LFQ intensity for three replicates in 'Probe 3 + Competitor 2' group)* P-value was less than 0.05, which meant that there was a significant difference of LFQ intensities between these two groups.

Results and Discussion:

The kinases bind to probes via hydrogen bond interactions with the kinases' peptide backbone, as well as hydrophobic and electrostatic interactions between the kinases' side chain and the well-defined pocket in the active site.[2] Type I kinases inhibitors are ATP competitors that mimic the adenine portion of ATP's purine ring. They interact with the catalytic sites of kinases that are involved in conformational phosphorylation. These kinases inhibitors interact with active conformation sites and alter the structural conformation of the kinase.[12] Probe 1 and Probe 2

designed in this experiment are typical type I kinases inhibitors. (Figure.3A & 3B) Both of them have a heterocyclic ring system, which can occupy the adenine portion as the purine ring. The heterocyclic ring can supply two hydrogen bonds to the Hinge region, stabilizing it in the structure of kinases, and the side rings occupy the hydrophobic pocket II and hydrophobic pocket I. These structures are conducive to the close combination of probes and kinase, so as to achieve the experimental purpose. In addition, at functional protein sites it is easy for lysine to have nucleophilic reaction with fluorosulfate, based on sulfate being more electrophilic.[10] (Figure.3D)

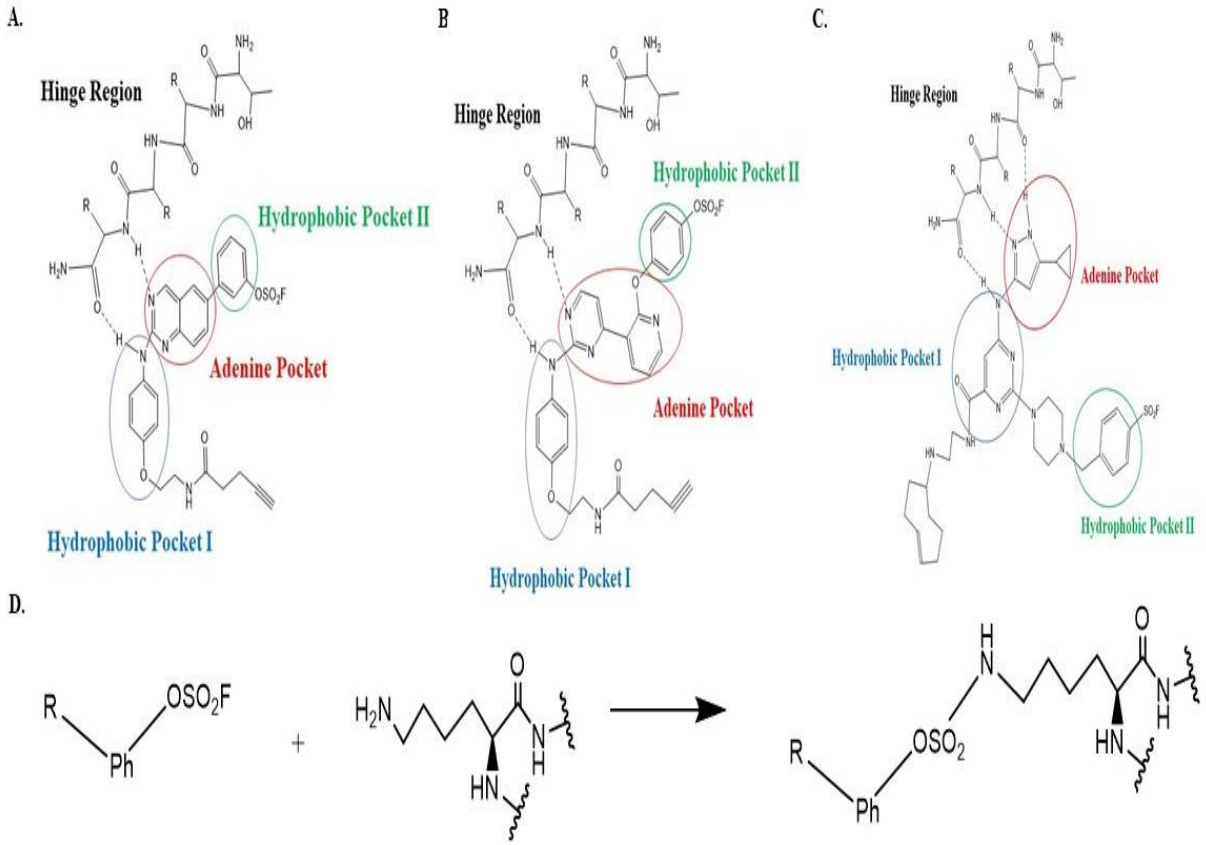


Figure.3: (A). Schema for Probe 1 conjugate with active site of kinase. (B). Schema for Probe 2 conjugate with active site of kinase. (C). Schema for Probe 3 conjugate with active site of kinase. (D). Schema for the fluorosulfate attach to target lysine.

For these two kinds of probes' synthesis, a series of chemical synthesis methods were used. For Probe 1, the process started with 6-Bromo-2-chloroquinazoline and tert-Butyl 2-(4-aminophenoxy)ethylcarbamate by nucleophilic aromatic substitution. Then, the Suzuki coupling reaction was used to add the phenol. Palladium was the catalyst in this reaction, and sodium carbonate was used in the reaction to increase the reactivity of boric acid to palladium-halide by converting boric acid to the corresponding organic borate.[13] TFA was used to remove the tert-butyloxycarbonyl protecting group (BOC group). After that, there were two versions for this probe which could be considered, alkyne version and TCO version. For the alkyne version synthesis, EDCI was used as a carboxyl activator and HOBT was used to generate active esters that can effectively acylate amino groups,[14] and the *N,N*-Diisopropylethylamine was used as the hindered base.[15] Both of these materials can efficiently produce the primary amide coupling experiment. For the TCO version synthesis, TEA was used as the base for the primary amide coupling reaction. The final steps for these two versions are sulfurylation by AISF with DBU (as catalyst and non-nucleophilic base). This reaction is not affected by the electron-withdrawing or electron-donating substituents present on the phenol, and is resistant to heterocycles and sterically hindered substrates.[16] Compared with the traditional sulfuryl fluoride gas method, this reaction condition is mild and safe. To synthesize Probe 2, almost all steps are similar to Probe 1, the first step and second step were nucleophilic aromatic substitution. Cesium fluoride is a useful base, which also can provide a source of the fluoride anion to prevent emergence of the byproduct.[17] And cesium carbonate was used, which is a mild base, and this unique feature is essential for selective deprotonation and it is suitable for base-catalyzed reactions.[18] Moreover, the selection of the starting martial in this step is

incredibly important. If hydroquinone is used instead of 4-tert-Butoxyphenol, it will easily produce dimer byproduct (4,4'-(((oxybis(4,1-phenylene))bis(oxy))bis(pyridine-2,3-diyl))bis(N-(4-(2-aminoethoxy)phenyl)pyrimidin-2-amine)), and the desired products are very difficult to obtain. Furthermore, the yields for sulfurylation in these experiments (~25%) were much lower than the yield mentioned in the cited article[16] (97%). The reason is hard to figure out; it may be due to the experiment of the cited article using simple starting materials, 4-Benzoylphenol; however, these probes have a more complex structure. Therefore, under the same reaction time and conditions, these probes are harder to sulfurylate.

Chemical proteomics research has extensively utilized the cycloaddition reaction of alkynes-azide labels. These bio-orthogonal conjugation reactions enable the use of small molecule probes with chemical handles that have a negligible effect on their solubility and binding properties.[19] It has been possible to combine two molecular building blocks under mild biocompatible conditions with almost no by-products. The reaction is a Cu-catalyzed azide/alkyne cycloaddition reaction (CuAAC), based on Huisgen's research on 1,3-dipolar cycloaddition reaction that was discovered in the mid-20th century. [20] CuAAC is not affected by various functional groups and can be realized by $CuSO_4$ catalysts and solvents (including aqueous solutions) from various sources. Moreover, the reaction's triazole bridge is deemed biologically stable.[20] However, at low biomolecule concentrations, alkyne-azide cycloadditions are insufficient due to moderate kinetics, and it may damage the viability of the system.[21] TCO-Tz is an attractive alternative. This linkage chemistry is based on the reverse demand Diels-Alder cycloaddition reaction between TCO and TZ pairs to form dihydropyridazine bonds. The

TCO-Tz connection pair features ultrafast kinetics, selectivity, and water stability.[21] And the TCO-Tz connection also can enhance fluorescence for image.[21] Streptavidin is a very useful detective reagent, since it is fast, specific, and not easily reversible non-covalent interaction with biotin. Biotin is locked to the active site of streptavidin by 8 hydrogen bonds and van der Waals interactions between non-polar groups.[22] (Figure.4)

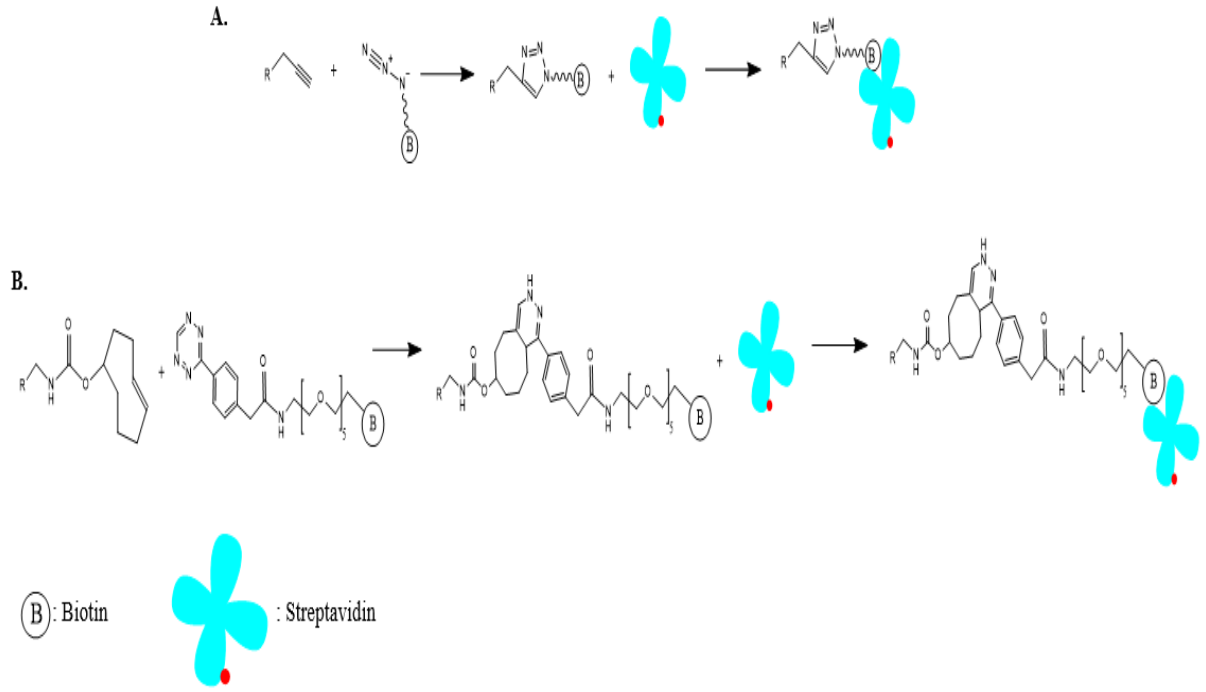


Figure.4: (A). Schema for CuAAC and biotin-streptavidin conjugation. (B). Schema for TCO-Tz Diels-Alder cycloaddition and biotin-streptavidin conjugation.

To resolve the issue of whether the designed Probe 1 and Probe 2 can label the target lysine on protein kinases' active sites, the SDS-PAGE was used. For the alkyne version probes, the SDS-PAGE results showed that the positive control IAA-alkyne can successfully react with biotin-N_3, and the results of positive control evidence that the click chemistry was effective. The

specific competitor has an almost identical structure to these probes, which can confirm the specificity of the bands created by protein labeling with the specific probes. If the bands in the gel only appear on the probes' lanes, and not on the lanes of probes with the competitor, it can be considered that these bands are the specific labeling with the probes, due to the competitor already binding with the kinases' active sites. It can be determined that the probes are bound with kinases' active sites and conjugated with target amino acids, instead of binding with some side chain amino acids. In Figure.5, the results cannot prove that these new probes specifically label kinases. The signal toward the top of the membrane seen in all the lanes ('white rectangle') is slightly odd, it could be the streptavidin binding with impurities. However, it is harder to figure out what this is. Besides, the 'yellow circles' in Figure.5 are also elusive. This band only disappeared in the lanes for 'Probe 1 + Competitor 1 (1h)', 'Probe 2 + Competitor 1 (1h)', 'Probe 1 (2h)', 'Probe 2 (2h)', and 'Probe 1 + Competitor 1 (2h)'. The SDS-PAGE results for alkyne version probes had no value for this experiment.

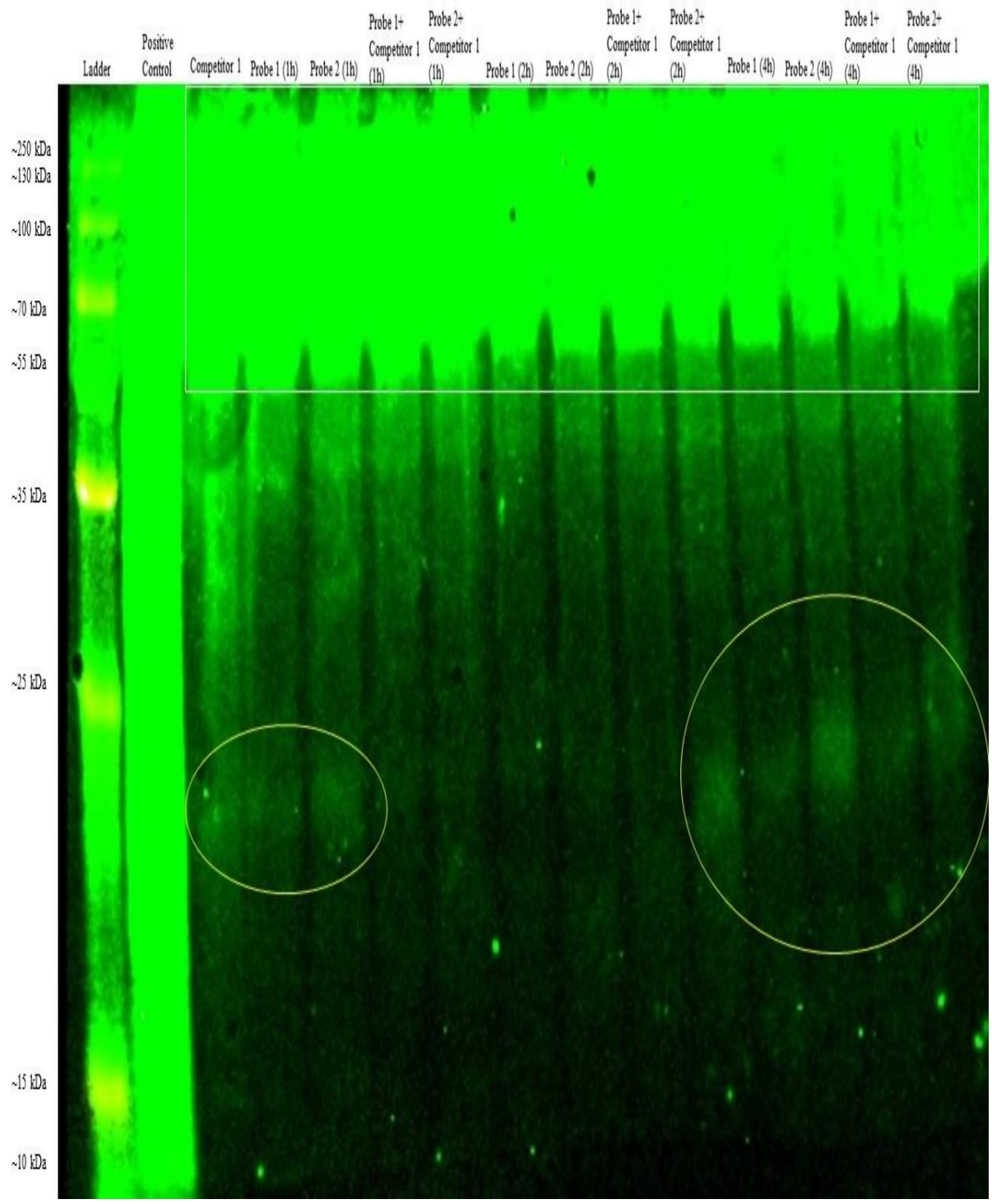


Figure.5: SDS-PAGE results for Probe 1 and Probe 2 click chemistry, and probes' incubation time (1h, 2h, 4h) test. The cell lysate type is Hela SRC WT. The concentration in cell lysate for Probe 1 and Probe 2 is 40 μM. The Competitor 1 concentration is 100 μM with 30 min incubation. The positive control IAA-alkyne (100 μM, 4h) can successfully bind with the biotin-N_3 (25 μM, 1h). 'White rectangle' is the signal in all the lanes. The 'yellow circles' signal

is only in the lanes for ‘Competitor 1’, ‘Probe 1 (1h)’, ‘Probe 2 (1h)’, ‘Probe 2 + Competitor 1 (2h)’, ‘Probe 1 (4h)’, ‘Probe 2 (4h)’, ‘Probe 1 + Competitor 1 (4h)’, and ‘Probe 2 + Competitor 1 (4h)’; however, it disappeared in the lanes of ‘Probe 1 + Competitor 1 (1h)’, ‘Probe 2 + Competitor 1 (1h)’, ‘Probe 1 (2h)’, ‘Probe 2 (2h)’, and ‘Probe 1 + Competitor 1 (2h)’.

For further detection, the Probe 1-TCO and Probe 2-TCO were used. Even though the SDS-PAGE results for alkyne version probes are not representative, it can be considered that the 4 hours incubation could have the obvious bands for all the tests. In Figure.6, the lanes of ‘Competitor 1’, ‘Probe 2-TCO’, and ‘Probe 2-TCO + Competitor 1’ have the identical shade and positive of the bands. From these results, it can be considered that Probe 2-TCO may occupy kinases with its scaffold, without the target lysine binding. Compared to other lanes, the lane for ‘Probe 1-TCO’ has more and clearer bands in 55kDa-15kDa, and the Probe 1-TCO + Competitor 1’ is the same as for the ‘Competitor 1’. Going by preliminary estimates, fluorosulfate on Probe 1-TCO could bind with the target lysine on kinases, occupying the kinases’ active sites.

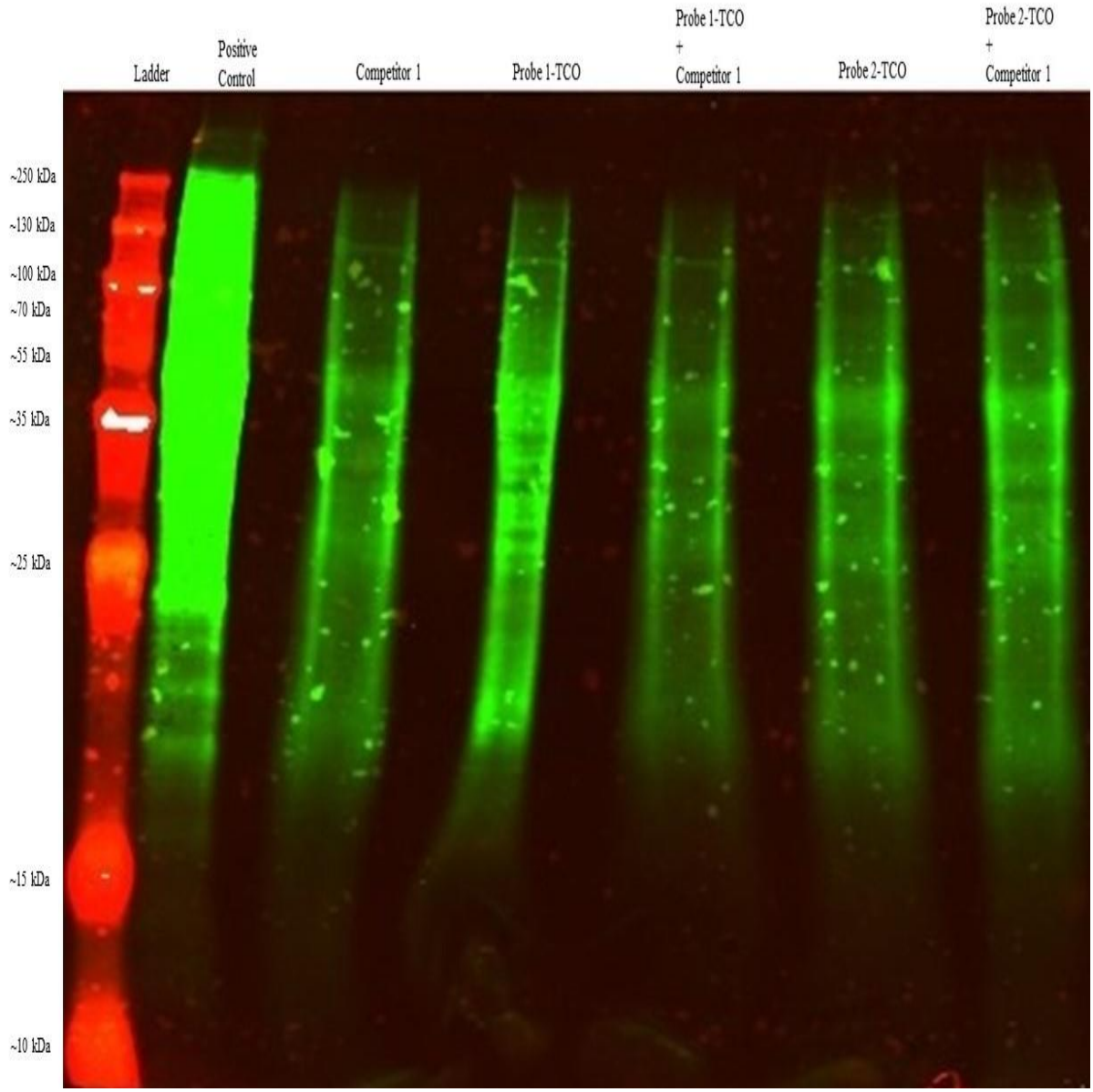


Figure.6: SDS-PAGE results for Probe 1-TCO and Probe 2-TCO click chemistry. The cell lysate type is Hela SRC WT. The concentration in cell lysate for Probe 1-TCO and Probe 2-TCO is 40 μM with 4h incubation. Competitor 1 is 100 μM with 30 min incubation. The positive control IAA-TCO (100 μM, 4h) can successfully bind with the Tz-PEGs-biotin (100 μM, 1h). Lane for 'Probe 1-TCO' contains more and clearer bands than the lanes for 'Competitor 1' and 'Probe 1-TCO + Competitor 1'. Lanes for Competitor 1', 'Probe 2-TCO', and 'Probe 2-TCO + Competitor 1' are identical.

In order to determine whether the result of 40 μM Probe 1-TCO contained off-target binding, serial dilutions of Probe 1-TCO were used. Antibodies were used to exhibit that there was the same amount of protein to control the experimental background in this test. Given this experimental result (Figure.7), it cannot be considered that the Probe 1-TCO combines with target lysine. Compared with the previous 40 μM, there is a significant likelihood that the 40 μM is off target. More importantly, in all SDS-PAGE results, the lanes for probes only and competitor only are almost the same, which makes it even more difficult to know whether the probes can occupy the active sites of kinases. Therefore, in order to prove more effectively and pertinently whether the two probes (both alkyne and TCO version) can occupy the active sites of kinases, pull-down assay protocol was introduced. Pull-down analysis is an in vitro technique used to detect physical interactions between two or more proteins. It is a valuable tool for confirming predicted protein–protein interactions or identifying new interaction partners.[23] The two versions of these probes would combine with the target kinase, and are 'filtered' by kinobeads. Kinobeads are a set of promiscuous kinase inhibitors immobilized on sepharose beads that are used to comprehensively enrich endogenously expressed protein kinases from cell lines and tissues.[24] All kinases that do not bind to the probe will bind to the kinobeads; and these only contain kinases occupied by the probes in the supernatant. SRC antibodies were used to determine the amount of SRC kinase here. The 'No-beads incubation' exhibited that the cell lysate contains the band for SRC. The supernatant for all the probes had the same lighter bands for SRC; and the lanes of the beads for probes also had these brighter bands. Therefore, the probes may have lower affinity to SRC. However, there is a phenomenon that breaks this hypothesis. The bands for 'Control (supernatant)' have the same brightness as

the bands for all the probes' supernatants. Based on the fact that the 'Control (supernatant)' did not contain any probes, the only reason is that there were not enough beads to enrich all the SRC. A small part of SRC kinase remained in the supernatant because it did not bind to dasatinib-beads. Therefore, it can be inferred that the probes themselves did not bind to SRC (Figure.8) The reason why these two probes cannot occupy kinases' active sites is harder to figure out. Based on the similar structure in the experiments of the reference articles[1, 19], they used quinazoline scaffold and pyrimidine-pyridine scaffold to make type II inhibitor, and their structure can occupy DFG-out pocket to stabilize in kinases. In addition, type I inhibitor is more sensitive to the serine/threonine kinases; and type II inhibitor can always potently inhibit SRC, ABL, LCK, PYK2, HCK, CSK, c-KIT, and p38.[1] And, whether the fluorosulfate in these two probes attaches to the target lysine cannot be known. If the fluorosulfate cannot attach to the target lysine, the reason may be that the fluorosulfate is not in the proper orientation to react with active site lysines.[25] In addition, the nucleophilic property of lysine is low. The pKa of typical lysine is about 10.4.[6] Therefore, lysine is quite sensitive to pH value. For example, when the physiological pH value is 7.4, lysine is protonated and positively charged, so it cannot be used as a nucleophilic reagent.[6]

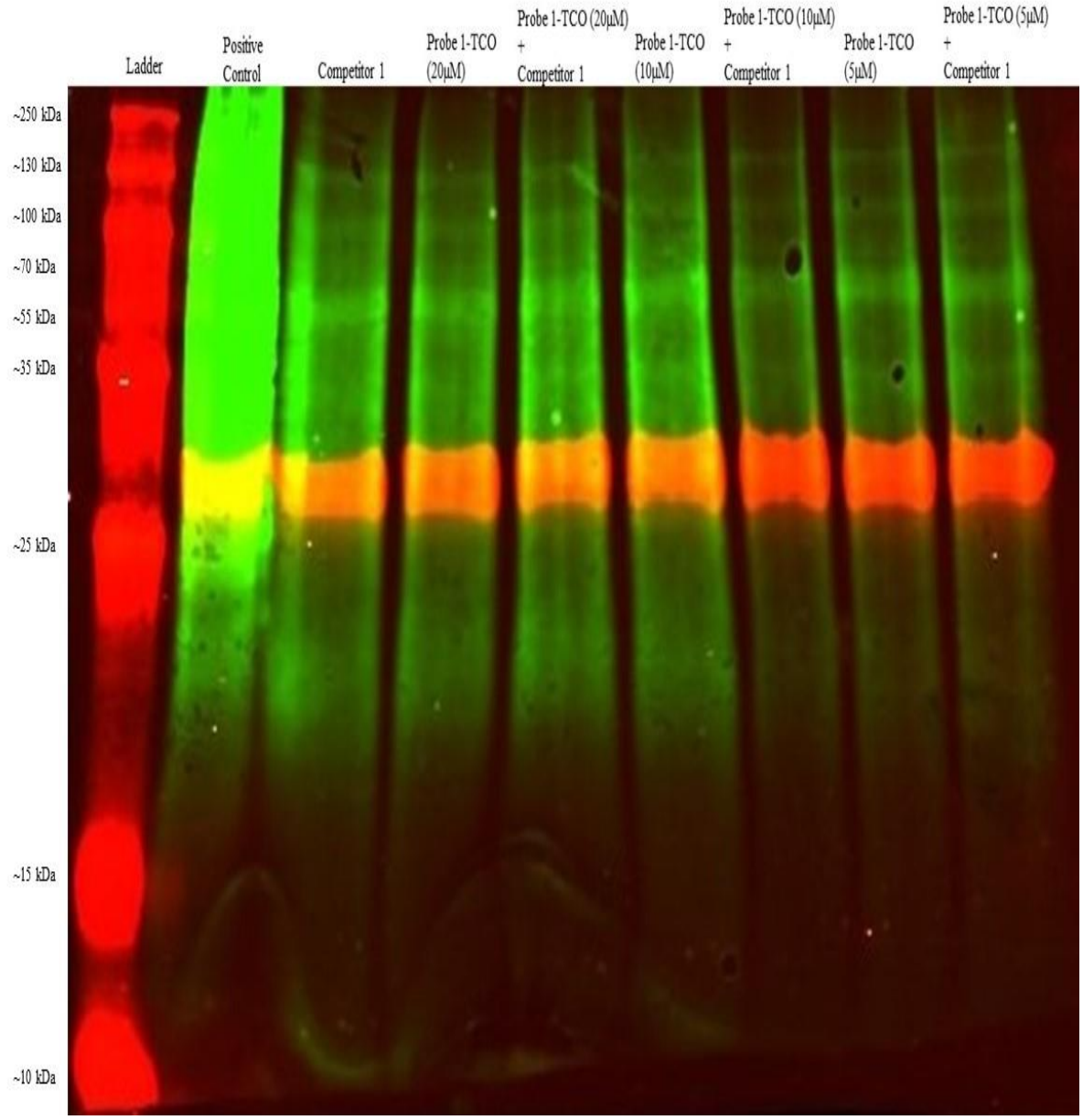


Figure.7: SDS-PAGE results for different concentrations (20 μM, 10 μM, 5 μM) of Probe 1-TCO in cell lysate. The incubation time for probes is 4h. The cell lysate is Hela SRC WT. Competitor 1 is 100 μM with 30 min incubation. The positive control IAA-TCO (100 μM, 4h) can successfully bind with the Tz-PEGs-biotin (100 μM, 1h). The bands (orange color bands) labeled by the antibodies (1^{st}: Mouse monoclonal IgG, 2^{nd}: Goat anti-mouse) exhibited that there is the same amount of protein. The shades of the bands in each samples' lanes are the same.

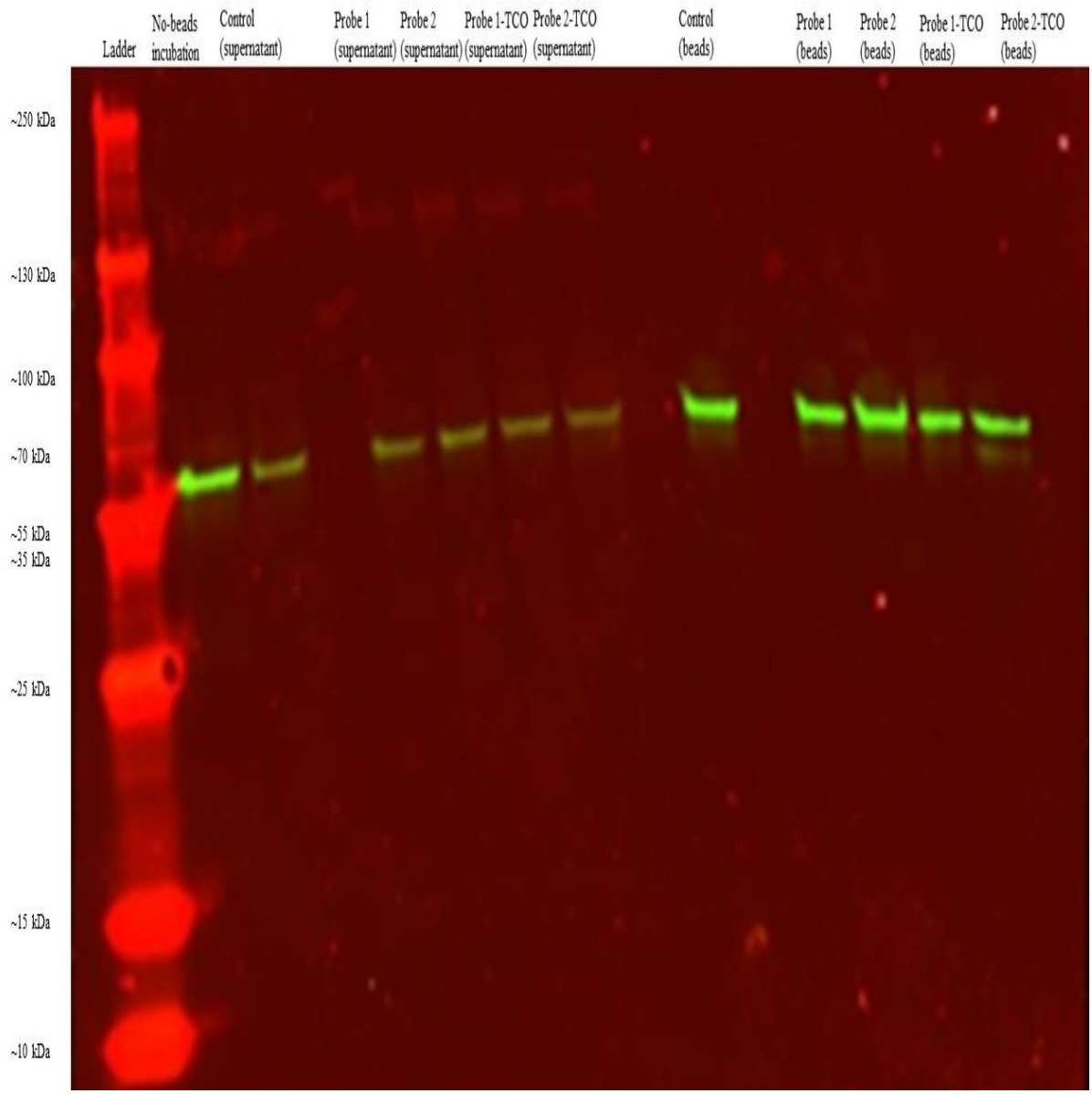


Figure.8: SDS-PAGE results for dasatinib-beads pull-down experiment of Probe 1, Probe 2, Probe 1-TCO, and Probe 2-TCO. The concentration in cell lysate for all the probes is 20 μM with 4h incubation. The cell lysate type is Hela SRC WT. The antibodies are SRC Rabbit mAb (1^{st}) and Goat anti-Rabbit (2^{nd}) to quantify the amount of SRC. 'No-beads incubation' (the cell lysate was without beads and probes.) showed the cell lysate contained SRC kinase. The lanes of 'beads' for the probes have brighter bands. The lanes of supernatant for the probes contain lighter bands for SRC. The 'Control (supernatant)' has the same bands as the lanes for the supernatant of all the probes. ('Control' was only incubated with beads, without probes.)

The Probe 3 is designed based on the 133 endogenous kinases in Jurkat T cells labeled by XO44[25]. According to XO44's structure, the Probe 3 is redesigned with TCO click handle, to optimize and improve the performance of the probe. The typical type I inhibitor, Probe 3, can form 3 hydrogen bonds with Hinge region, and the scaffold can occupy an adenine pocket; hydrophobic pocket I and hydrophobic pocket II. (Figure.3C) The ingenious aspect of the design of this probe is that the short linker (3–5Å) attached to the pyrimidine at C2 is best for positioning the sulfonyl fluoride close to the target lysine.[25] In addition, the sulfonyl fluoride makes the sulfate more electrophilic than the fluorosulfate, due to phenol is more electron-withdrawing.[26, 27] The synthesis for Probe 3 starts from methyl 2,6-dichloropyrimidine-4-carboxylate and 5-cyclopropyl-1*H*-pyrazol-3-amine. The reaction types are similar to Probe 1 and Probe 2, without sulfurylation. The second step is a hydrolysis reaction. And in the third step, HATU is used as a reagent for generating active esters from carboxylic acids.[28] In addition, it should be noted that the final step is adding 4-(Bromomethyl)benzenesulfonyl fluoride, since too many additional products will lead to low yield.

From the results of SDS-PAGE for Probe 3 (Figure. 9), it is harder to consider that the probe can bind with the targeted lysine on the active sites of kinases. For the Hela SRC WT cell lysate, even though 'Probe 3 + Competitor 3' has the lighter bands, the lane for 'Competitor 3' is the same as that for 'Probe 3'. The lighter bands for 'Probe 3 + Competitor 3' may be due to a slight difference in loading. In addition, the drop in signal did not seem all that drastic either, and there have been the same results in HCT116 cell lysate. It is also worth noting that positive control has strong background labeling. It may be due to the higher concentration and longer

incubation time for tetrazine, which can lead to a strong uncertainty of the experiment. In Figure.10, it can be determined that the 1h incubation of 100 μM of Tz-PEGs-biotin without IAA-TCO also can induce the clear bands in SDS-PAGE. The proteomics experiment was introduced according to the results of XO44[25], to prove the function of Probe 3.

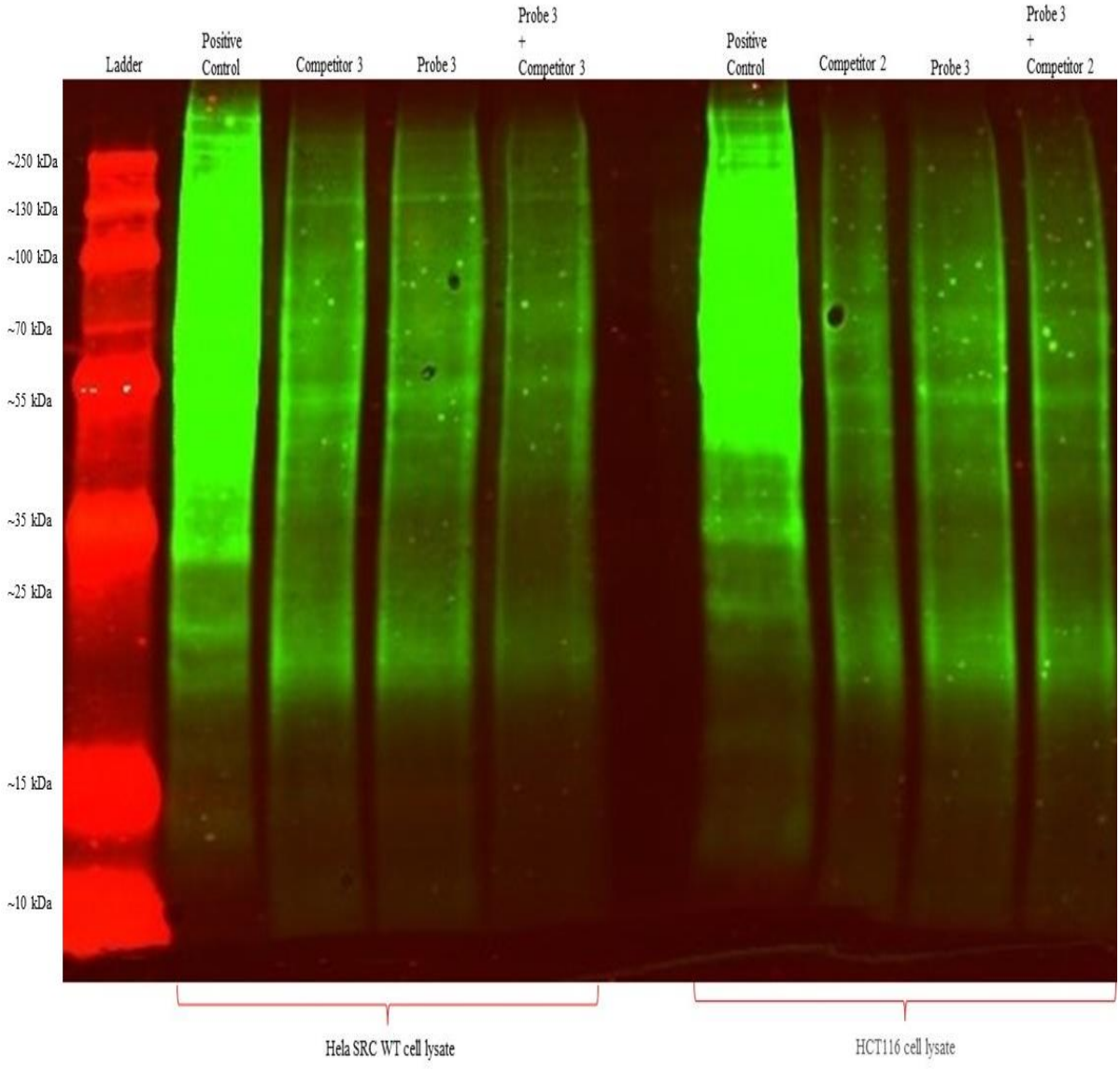


Figure.9: SDS-PAGE results for Probe 3 in Hela SRC WT cell lysate and HCT116 cell lysate. The positive control IAA-TCO (100 μM, 1h) can successfully react with the Tz-PEGs-biotin (100 μM, 1h). The incubation time for Probe 3 is 1h with 20 μM concentration. Competitor 2 and Competitor 3 are 20 μM with 30 min incubation. The bands in the lanes of 'Competitor 2

+ Probe 3', 'Competitor 2', and 'Probe 3' in HCT116 cell lysate are the same. The bands for 'Probe 3 + Competitor 3' in Hela SRC WT cell lysate are lighter than for 'Competitor 3' and 'Probe 3'. The bands in the lanes of 'Competitor 3' are identical to those in the lanes of 'Probe 3' in Hela SRC WT cell lysate.

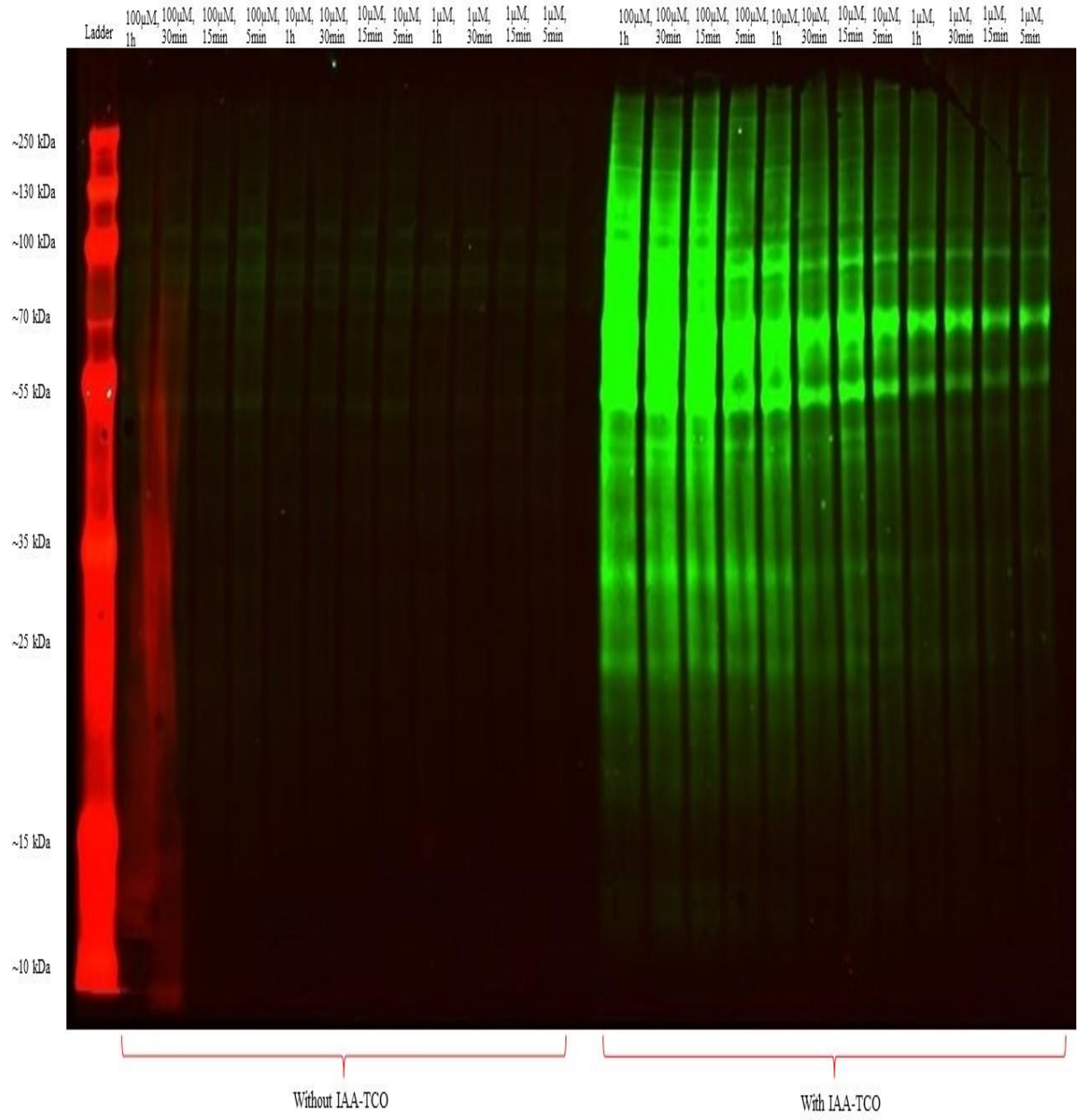


Figure.10: SDS-PAGE results for tetrazine labeling conditions in HCT116 cell lysate. There are two groups—one is with IAA-TCO (100 μM, 1h), the other is not. The matrix condition test is based on different incubation times (1h, 30 min, 15 min, 5 min) and different Tz-PEGs-

biotin concentrations (100 μM, 10 μM, 1 μM). The 100 μM group has higher background labeling, and the 1 μM group does not contain obvious labeling. 10 μM with 15 min or 5 min incubation can induce clear labeling with IAA-TCO and relatively unobtrusive labeling without IAA-TCO.

Based on the LC/MS/MS pull-down experiment data analysis, P-value of less than 0.05 means Probe 3 had significant labeling. In Figure.11, there are 4 protein kinases (ZAK, DDR1, RIPK2, PLK1) that have P-value of less than 0.05, and three of them (ZAK, DDR1, RIPK2) that have log2 differences >1. The protein kinases with LFQ intensities that were only contained in the 'Probe 3' group also exhibited that Probe 3 worked (LYN, LIMK2, and MAP3K1). Therefore, Probe 3 appears to specifically label at least 7 protein kinases' active sites. Compared with the results for XO44 probe[25], Probe 3 labeled a smaller number of protein kinases. Moreover, the protein kinases with non-significant LFQ intensities difference (P-value larger than 0.05) are possible to be labeled by Probe 3. In these 21 protein kinases with non-significant difference (ARAF, ATR, BUB1, CDC2/CDK1, CDK2, CDK7, CSK, EPHA2, GAK, IRAK1, MAP2K3, MAP2K4, MARK2, MLKL, NEK9, PBK, RIPK1, SRPK1, TNIK, TTK, VRK3), there have 4 protein kinases (CDC2/CDK1, CDK2, CSK, RIPK1) with higher sequence coverage (over 30%) and the sequence coverages in the 'Probe 3' group were almost same as the sequence coverages in the 'Probe 3 + Competitor 2' group. After the pull-down experiment, the protein kinases combined with the Competitor 2 would be removed, the protein kinases combined with Probe 3 were the major component in the two groups. In addition, the scaffold of Competitor 2 and Probe 3 are not exactly the same. Therefore, these protein kinases with similarly high sequence coverages can be the potential targets for Probe 3. And these 4 protein kinases could be labeled

by XO44 probe.[25] However, it need to need to be validated with further experimentation. From the data obtained, 11 protein kinases (BAZ1B, BRAF, CAMK4, CDK5, MARK3/MARK1, MELK, MET, RIOK3, SRC, SRPK2, VRK2) only contained one LFQ intensity in the 'Probe 3' group. These kinases represent lower confidence hit. The reason for this result may be the inappropriate concentration of the probe, or the inadequate incubation time between the probe and cell lysate, or the deficient incubation time for Tz-beads. In addition, according to the results of the purity assessment for Probe 3 by liquid chromatography–mass spectrometry, it can be found that the probe contained 29% impurity with 902.4 *m/z*. If the impurity had more affinity to some protein kinases, there can be some biases in the experiment. And the research for XO44 used living cell to detect the labeled protein kinases, the actual probe concentration in living cells cannot be compared with the actual probe concentration in cell lysate.

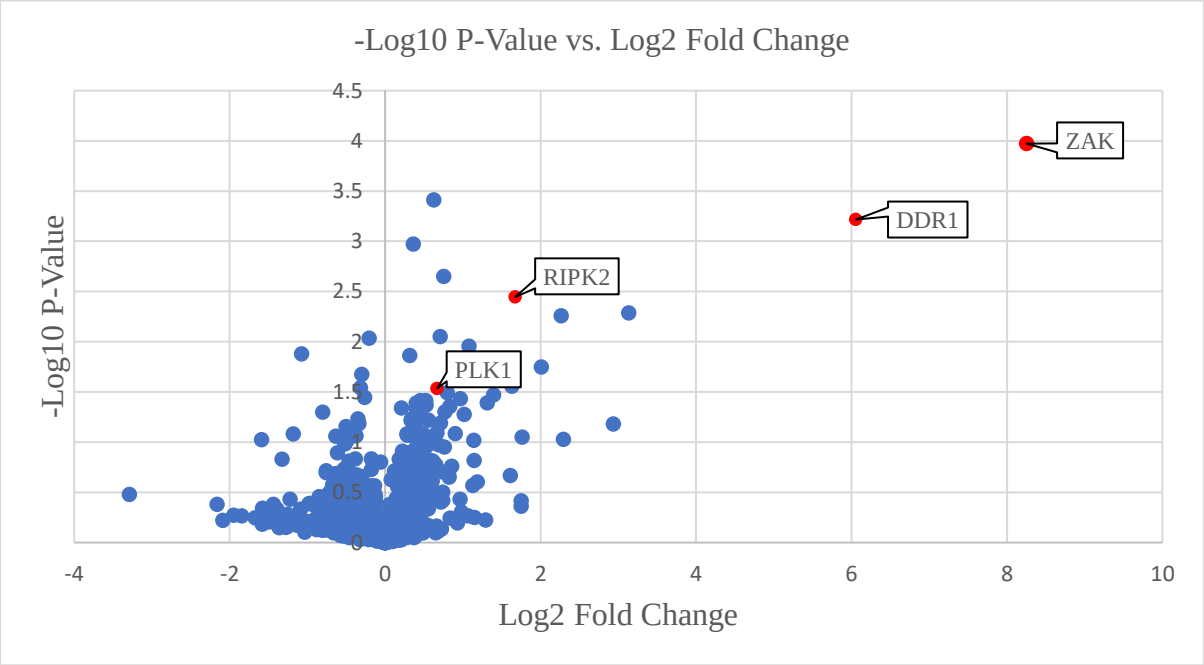


Figure.11: Volcano plot for -Log10 (P-Value) against Log2 (Fold change). The plot and data were processed by Excel. Fold change is the difference between the average LFQ intensity for

the three replicates in the 'Probe 3' group and average LFQ intensity for the three replicates in the 'Probe 3 + Competitor 2' group. The P-value in the Student's *T*-test was less than 0.05, which means the probe can have a significant binding. Only ZAK, DDR1, RIPK2, and PLK1 were the protein kinases with significant labeling. The data without P-value has not been included in this plot.

Conclusion:

In this experiment, three different scaffold type I inhibitor probes were tested. Based on the results, neither alkyne version nor TCO version for Probe 1 and Probe 2 can label the active sites of kinases. Even though it was found in subsequent experiments that the incubation time and concentration of Tz can bring experimental errors, this result can be confirmed again through dasatinib-beads pull-down experiment. Therefore, it is not known whether the fluorosulfate in these two probes can label the target lysine or not. Based on the cited articles[1, 19], these two scaffold probes are useful as type II inhibitors. To design the lysine targeted type I inhibitor, some successful type I inhibitor scaffolds can be used, such as thiadiazole triazines scaffold[29] or 2,7-naphthyridinone scaffold[30]. Based on the XO44 designed, a longer bond length linker (such as piperazine) can be used for sulfonyl fluoride to attach the target lysine.[25]

Probe 3 can specifically label at least 7 protein kinases in a HCT116 cell lysate. Compared with the alkyne version of Probe 3, this is a smaller subset of kinases. In future experiments, optimal incubation time and concentration for Probe 3 or Tz-beads can be tested. Also, future experiments should be conducted with a probe free of impurities. For example, a longer

gradient for HPLC purification can be used to remove impurities. In addition, a cell lysate mixture (such as HEK293 cell lysate with HCT116 cell lysate) could be used, to increase the number of protein kinases.

In brief, probes for protein kinases are capable of labeling several target kinases in a single experiment. Thus, an adequate probe may be used in the future for illness diagnosis and therapy; for example, by examining the functions of enzymes in cancer to obtain insight into the metabolic and signaling networks that contribute to cancer pathogenesis.[31]

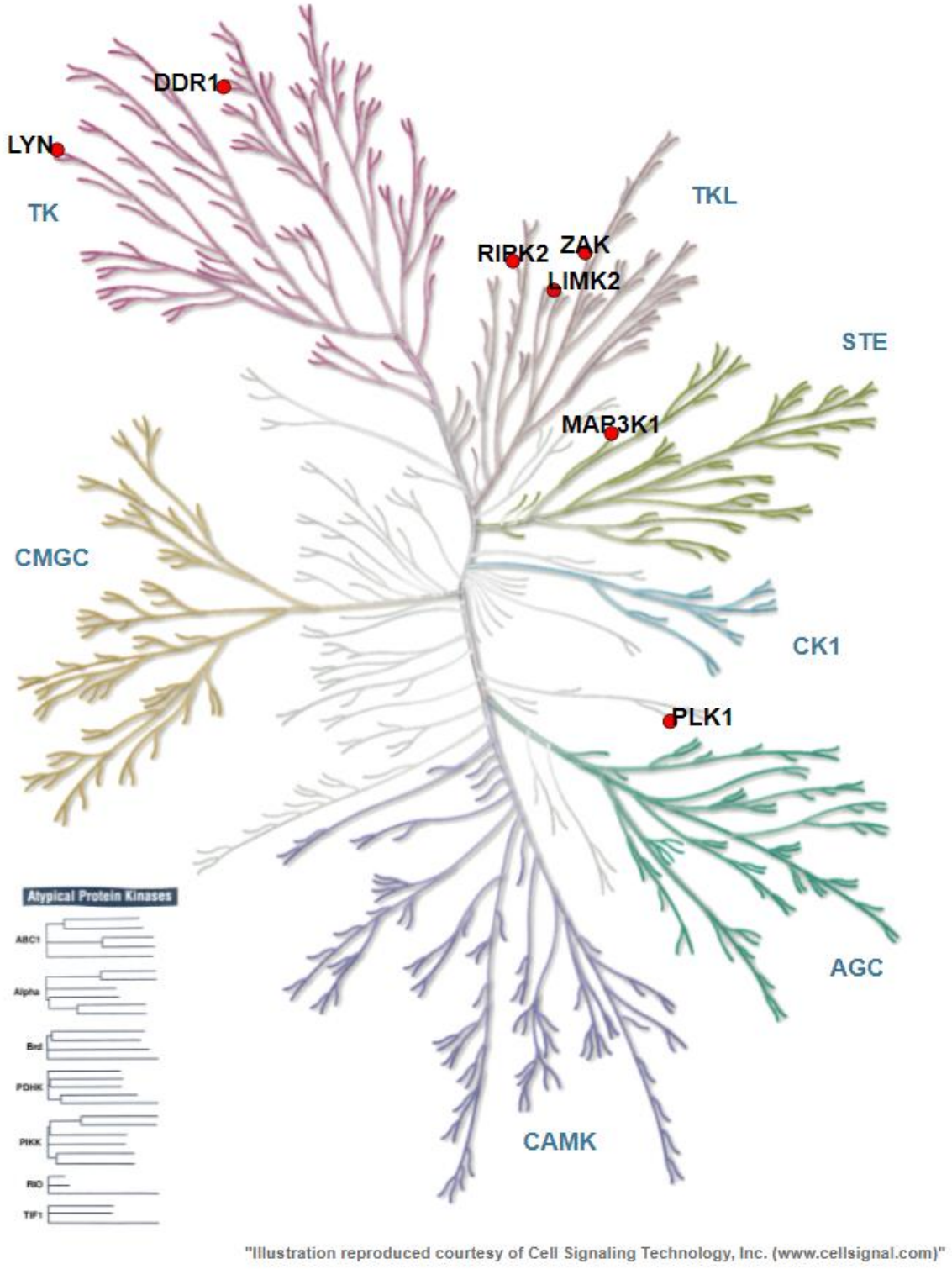


Figure.12: Protein kinases coverage of Probe 3 based on chemoproteomic experiments in HCT116 cell lysate. Only LYN, DDR1, RIPK2, ZAK, LIMK2, MAP3K1, and PLK1 were covered.

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Required For Anchoring ATP. *Proceedings of the National Academy of Sciences*. **1993**, *90* (2), 442-446. DOI: 10.1073/pnas.90.2.442
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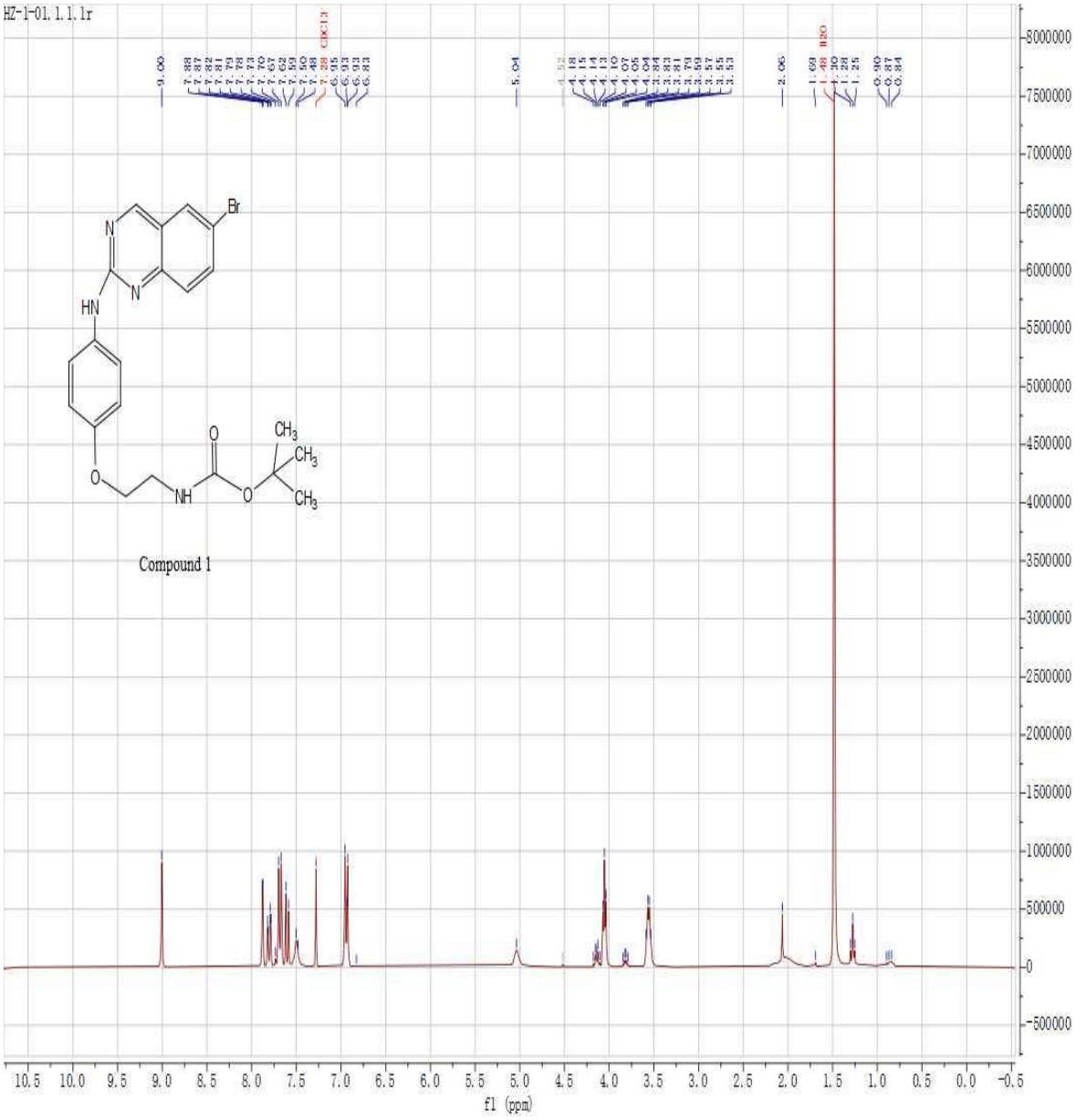
Appendix:

NMR spectra:

Compound 1:



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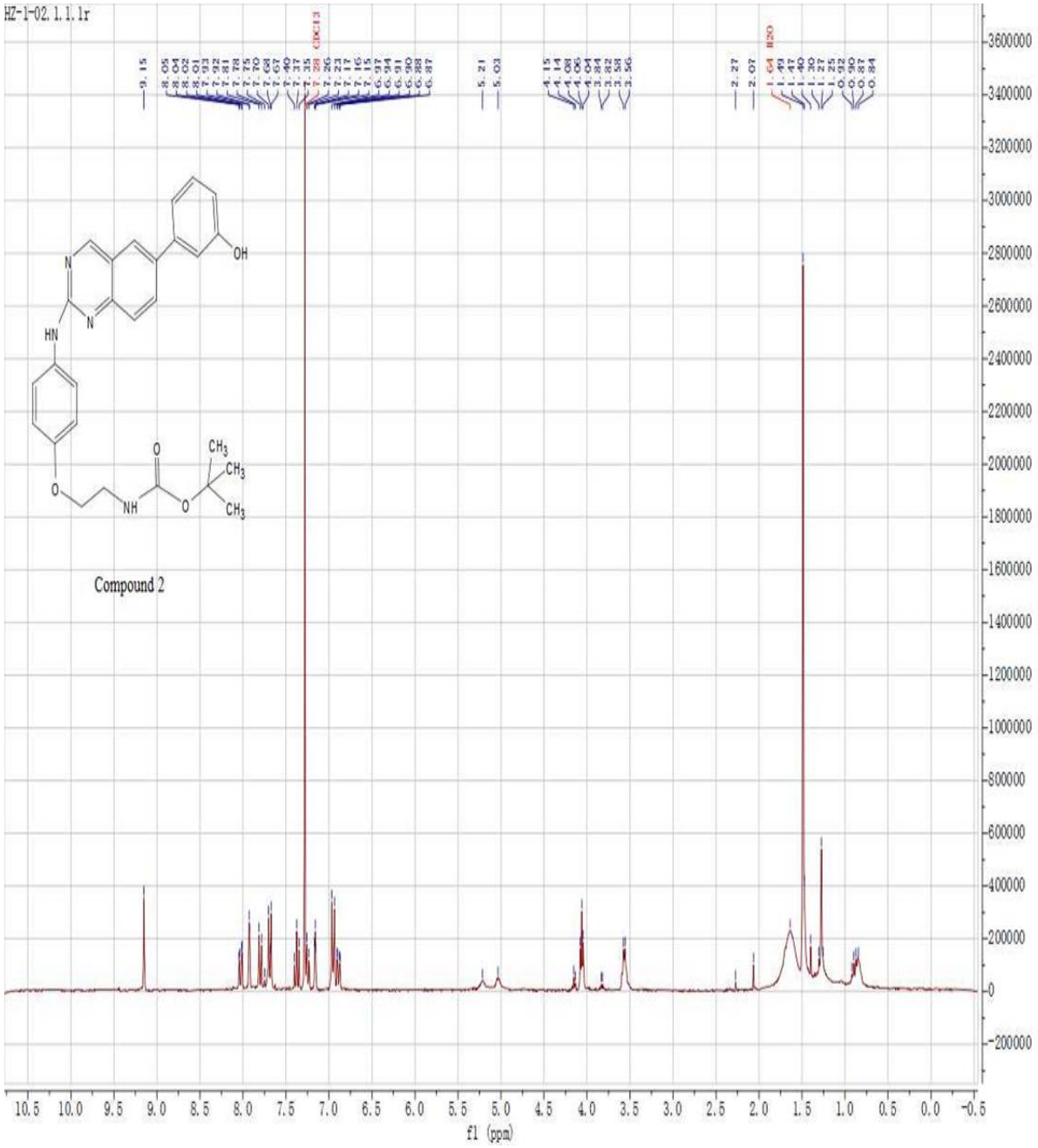

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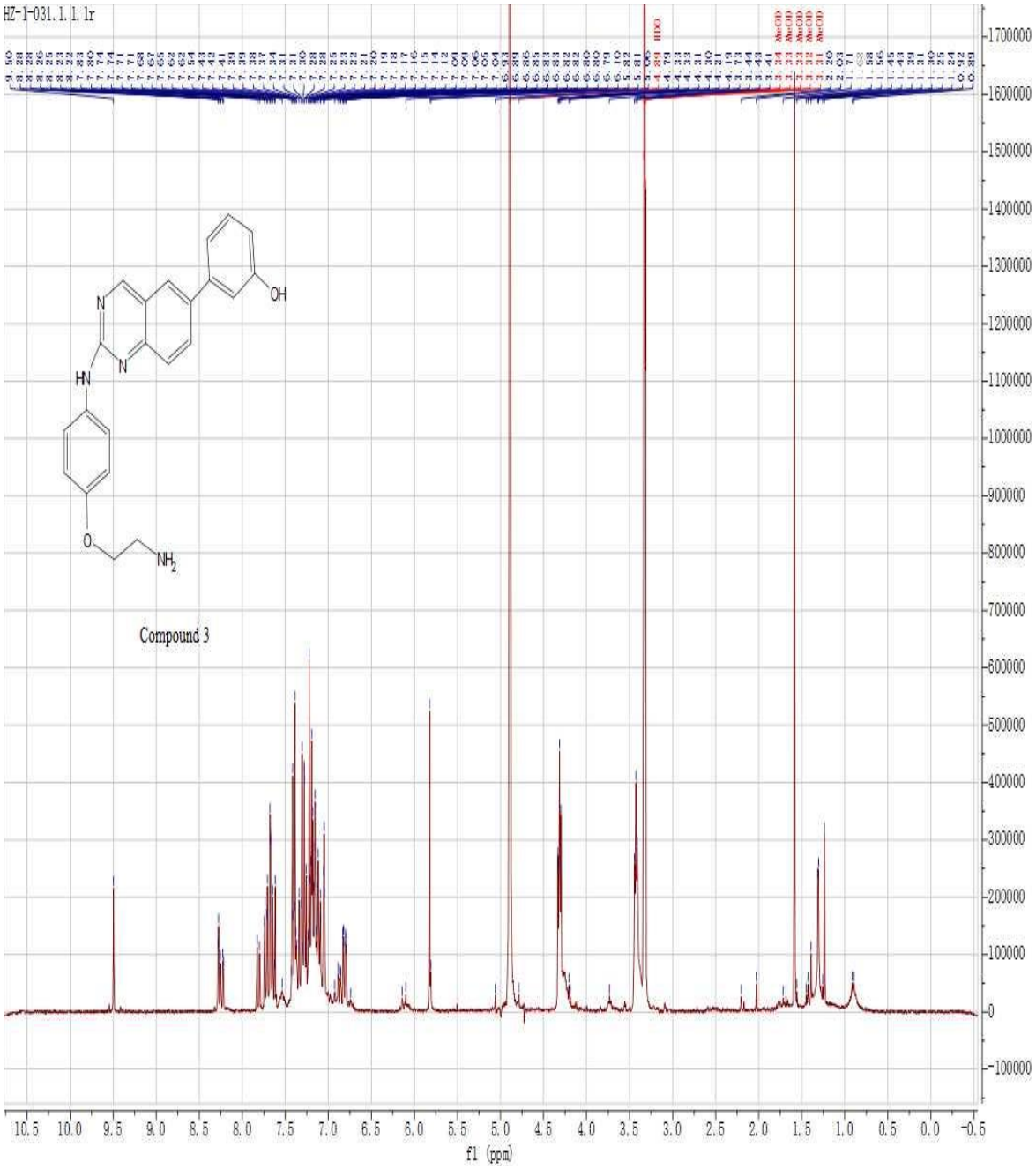
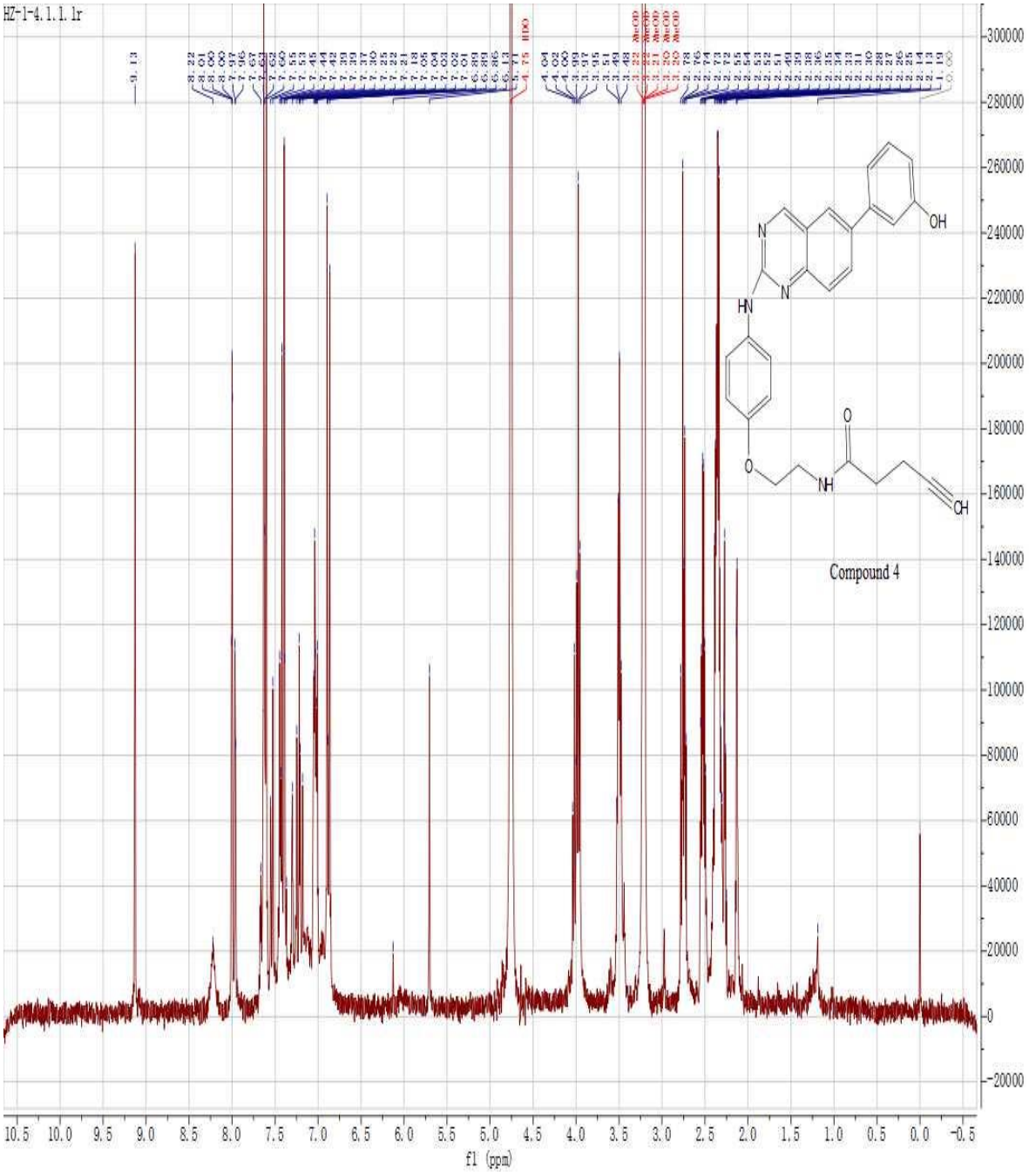
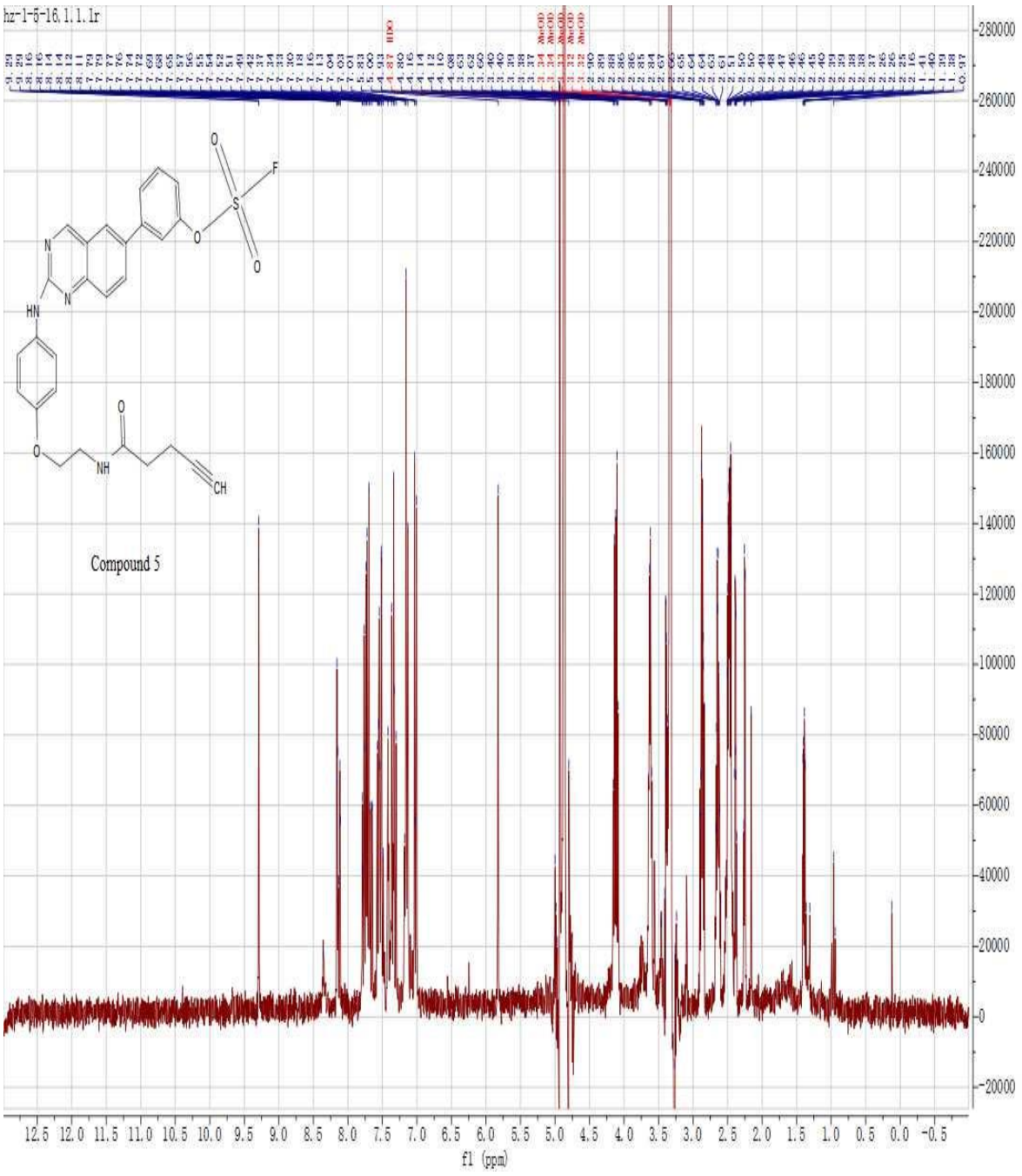


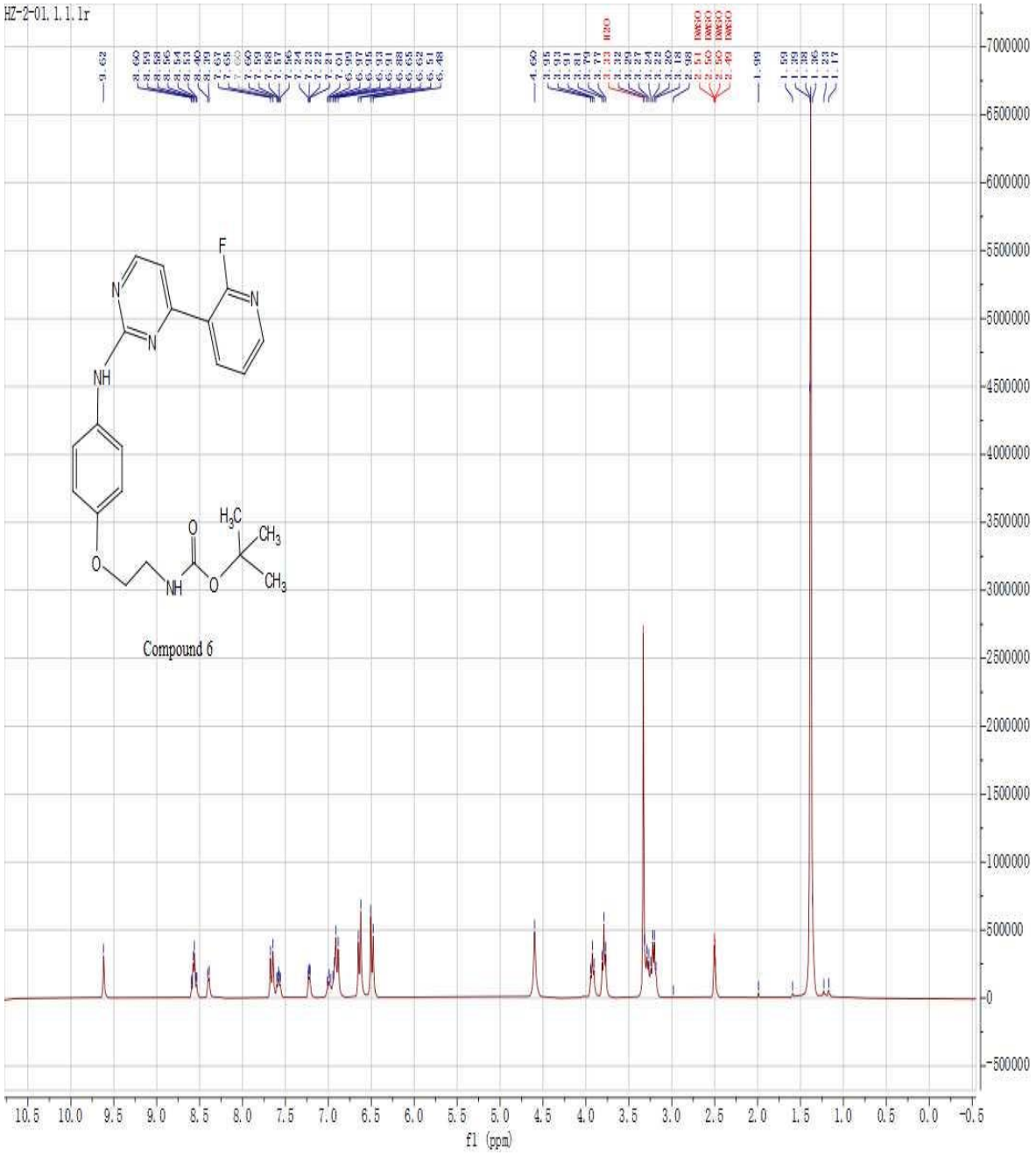
Compound 4:



Compound 5:



Compound 6:



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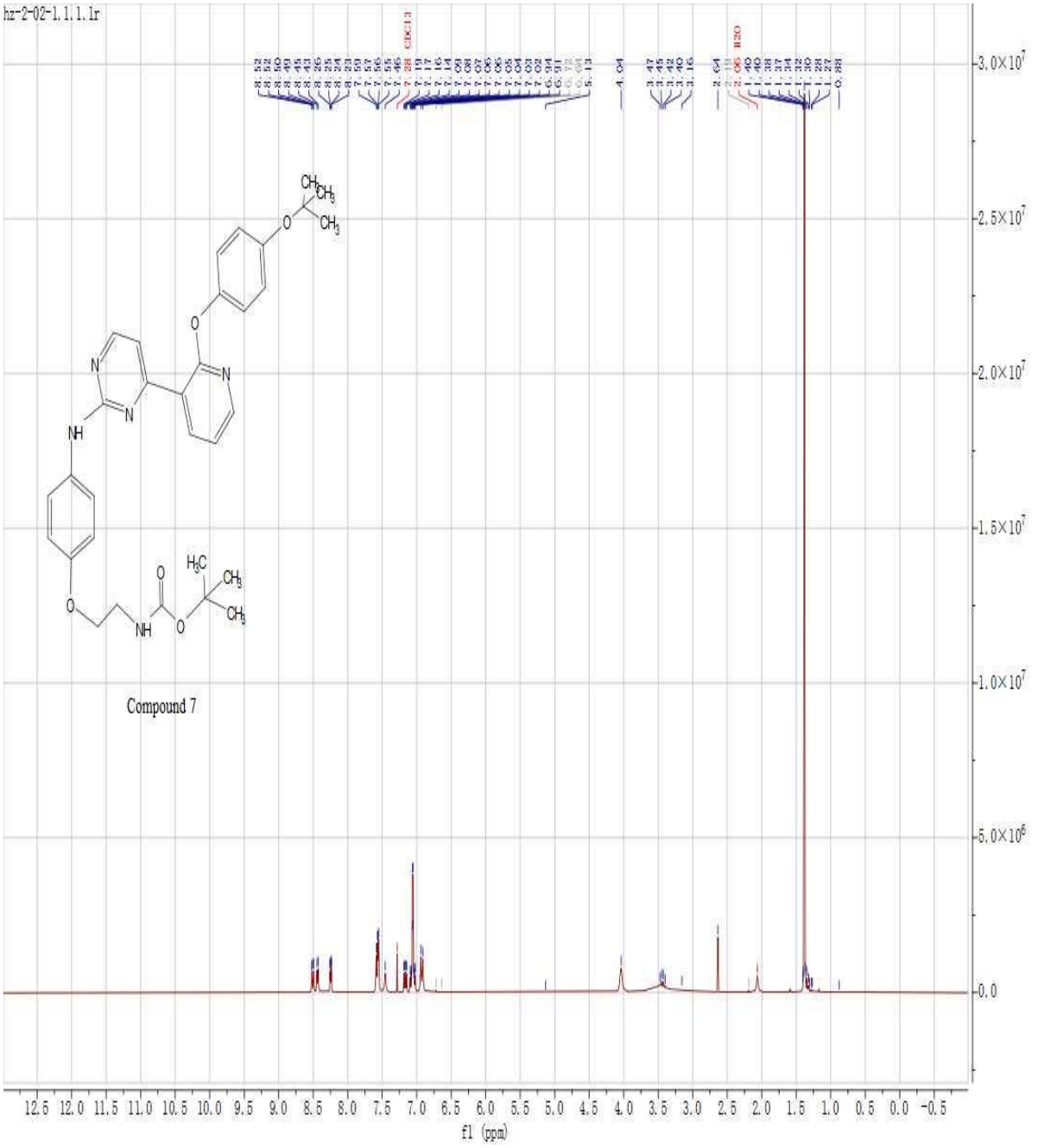

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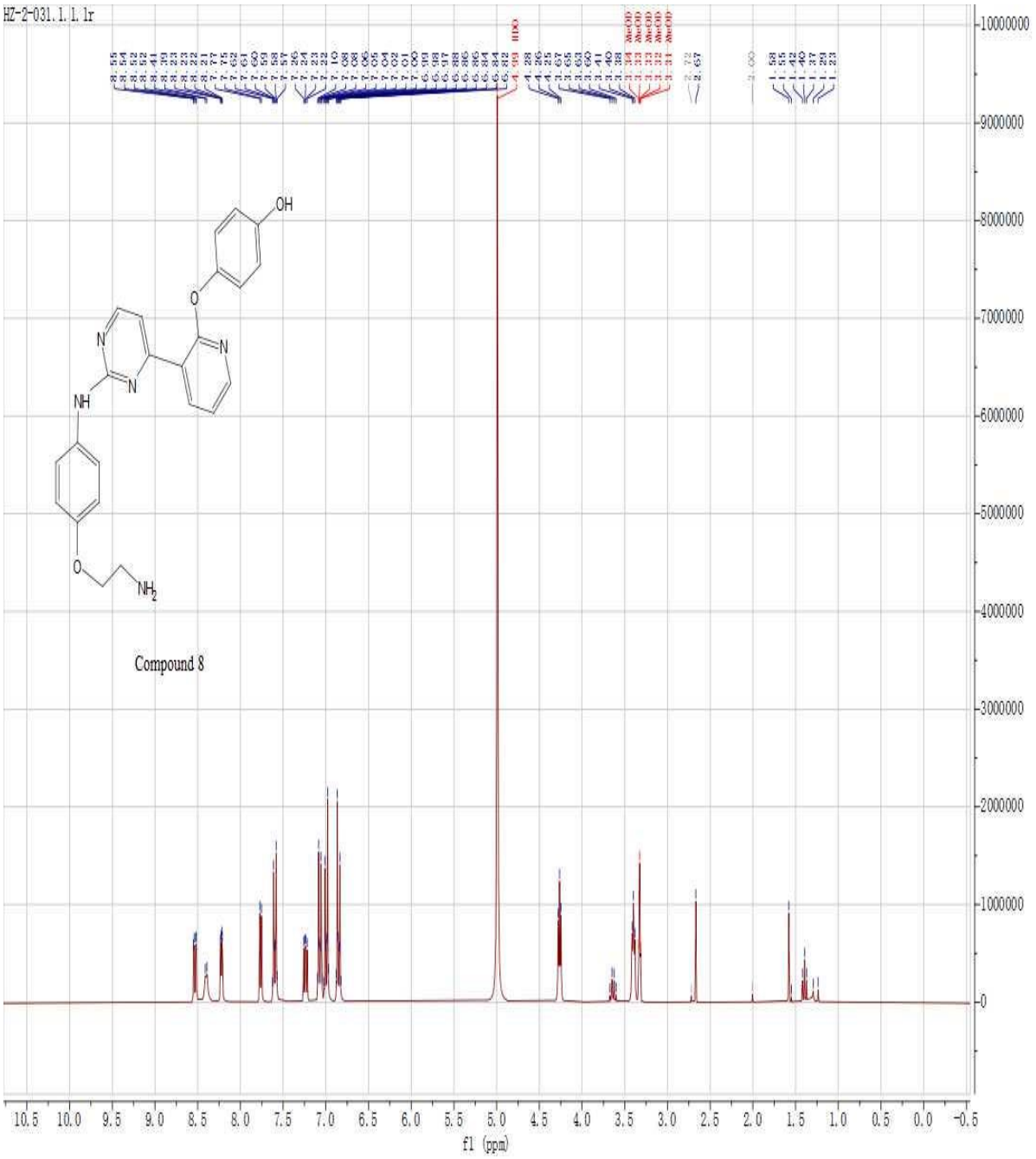

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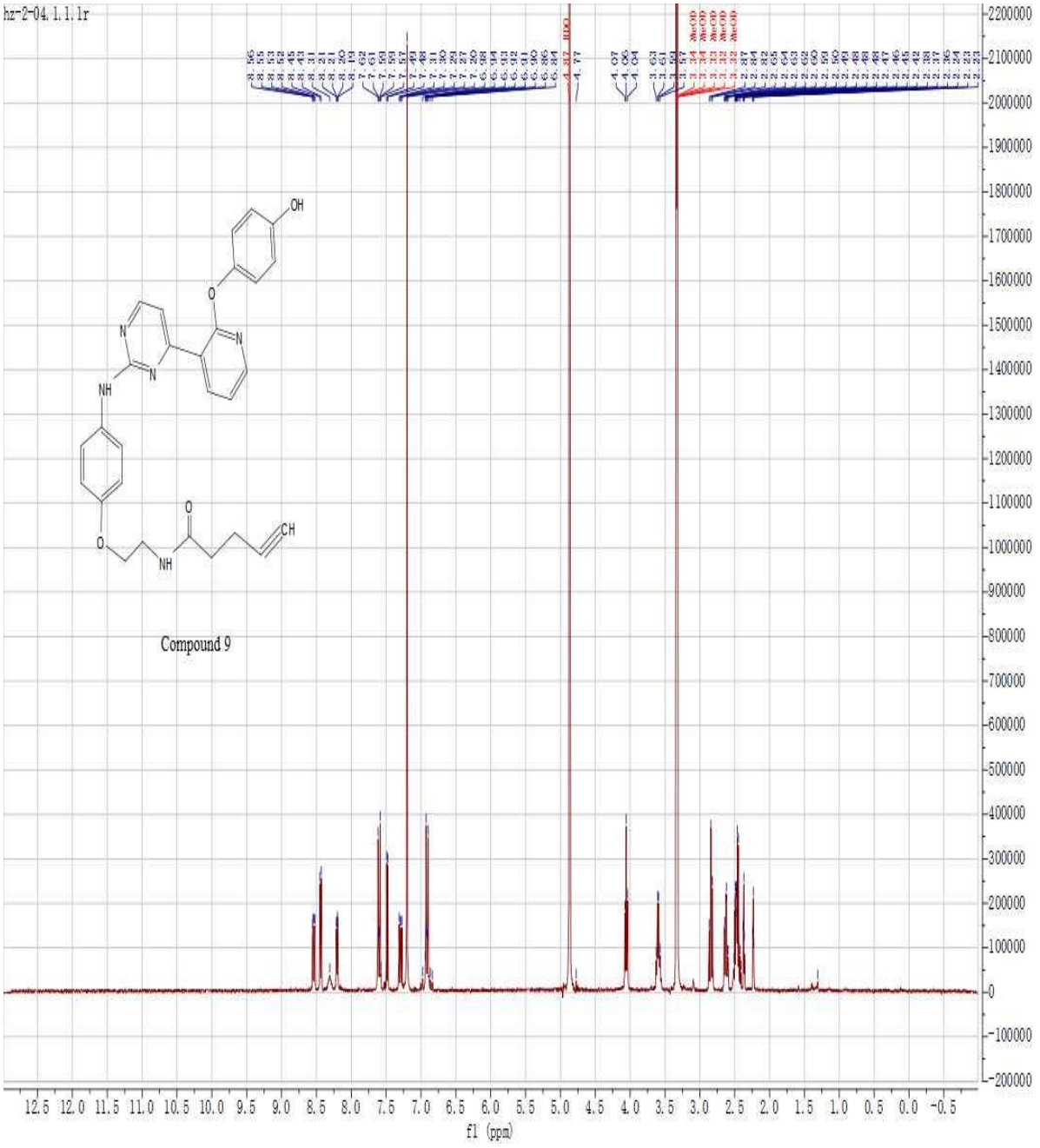

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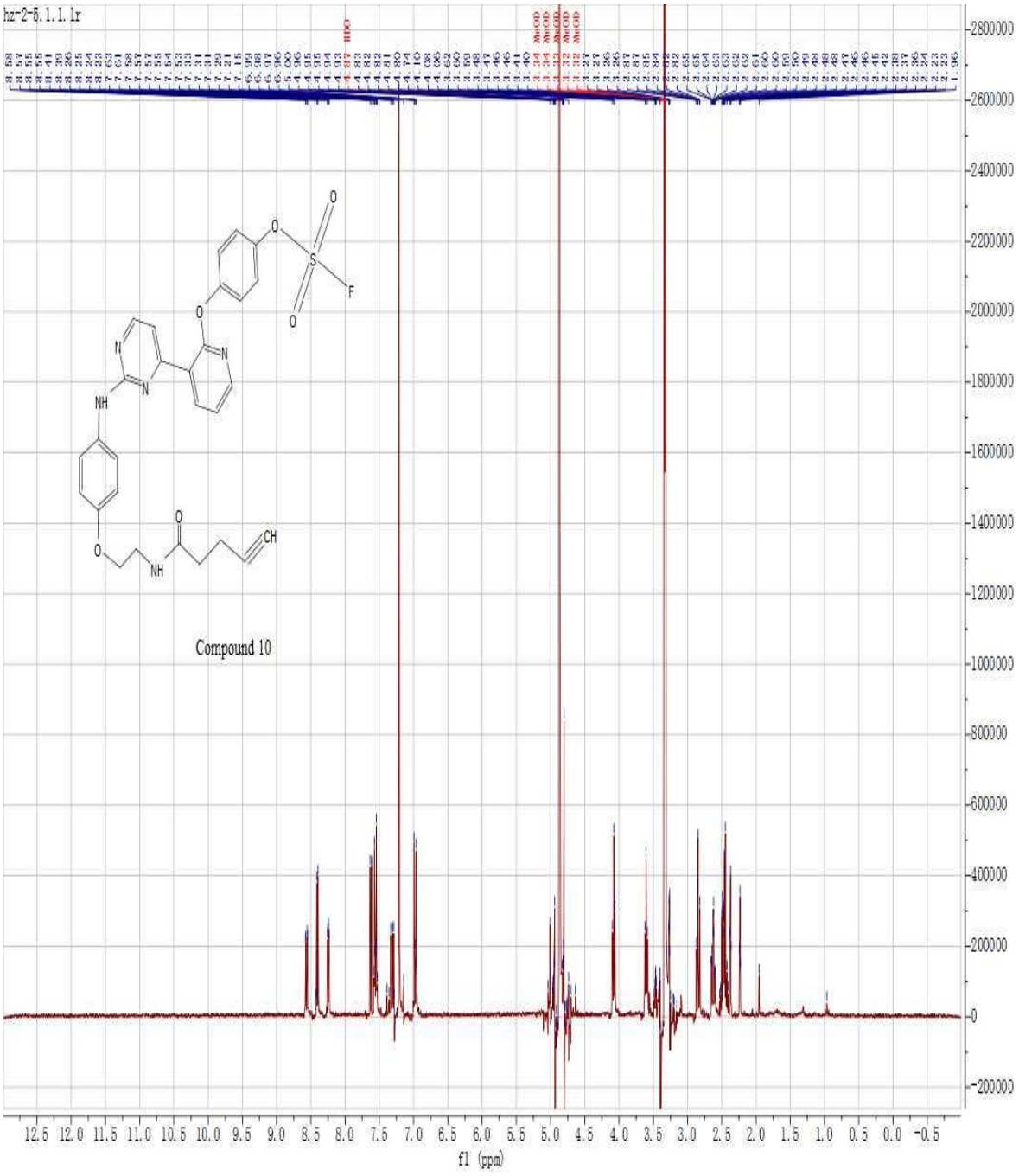

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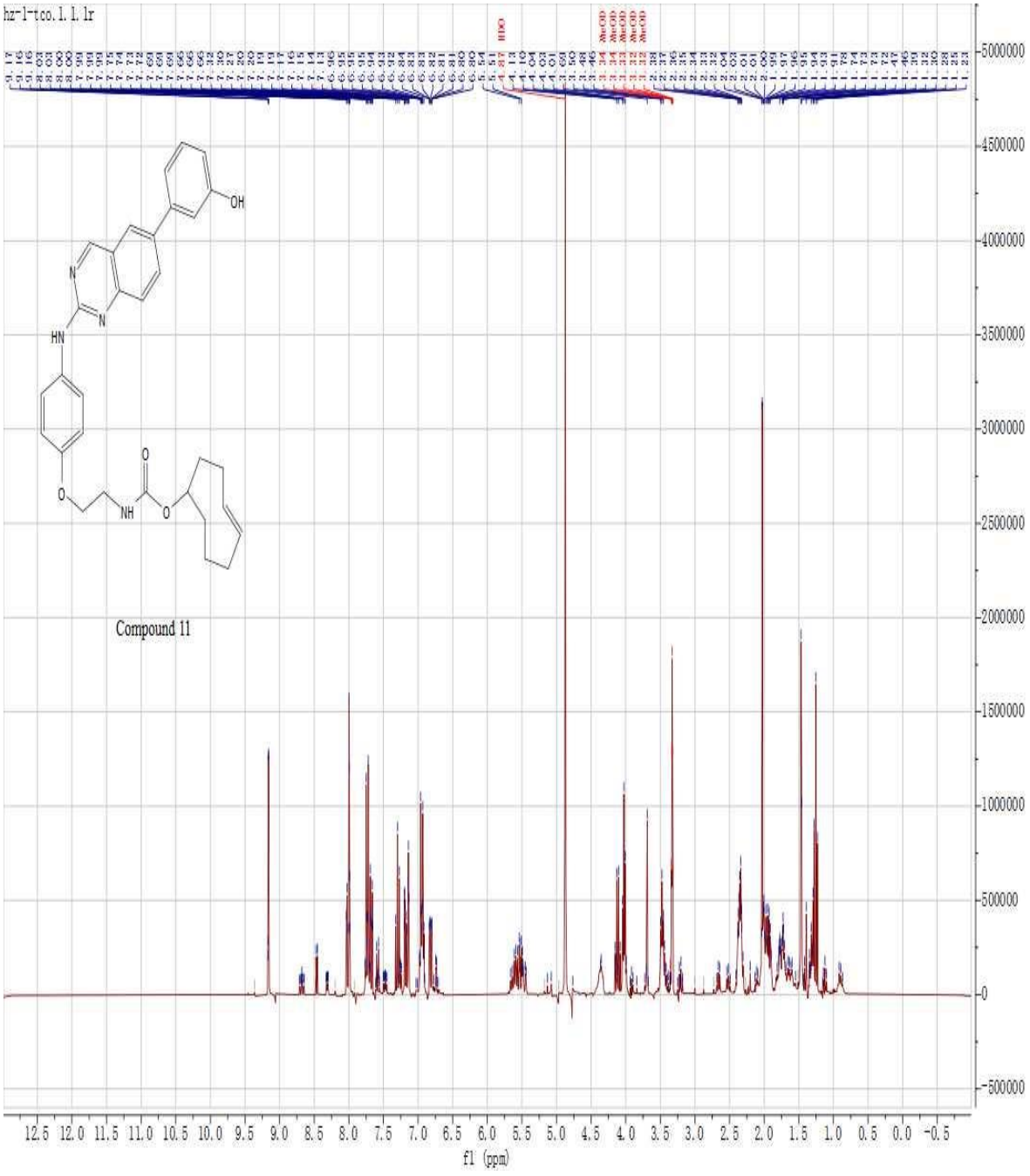

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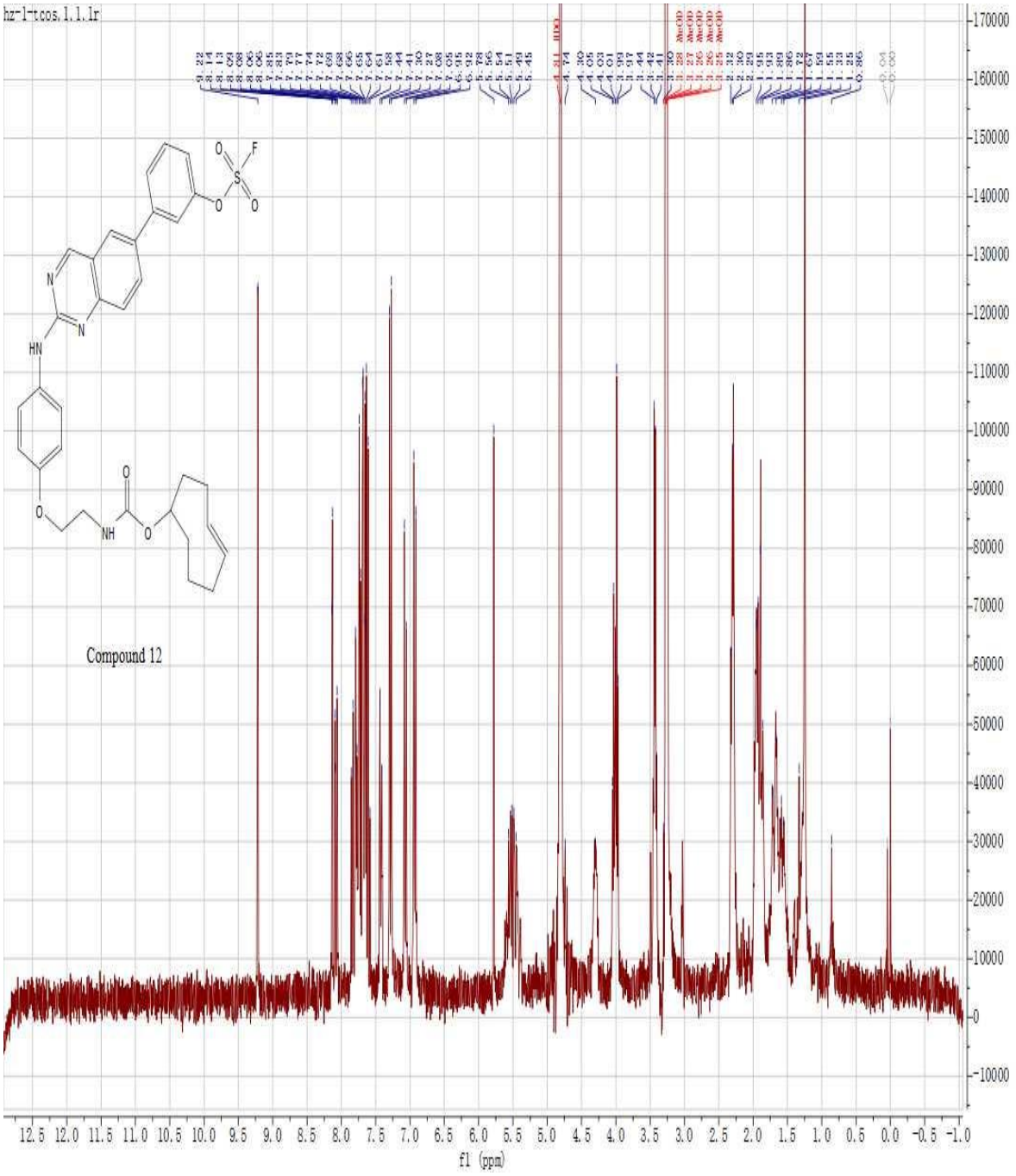

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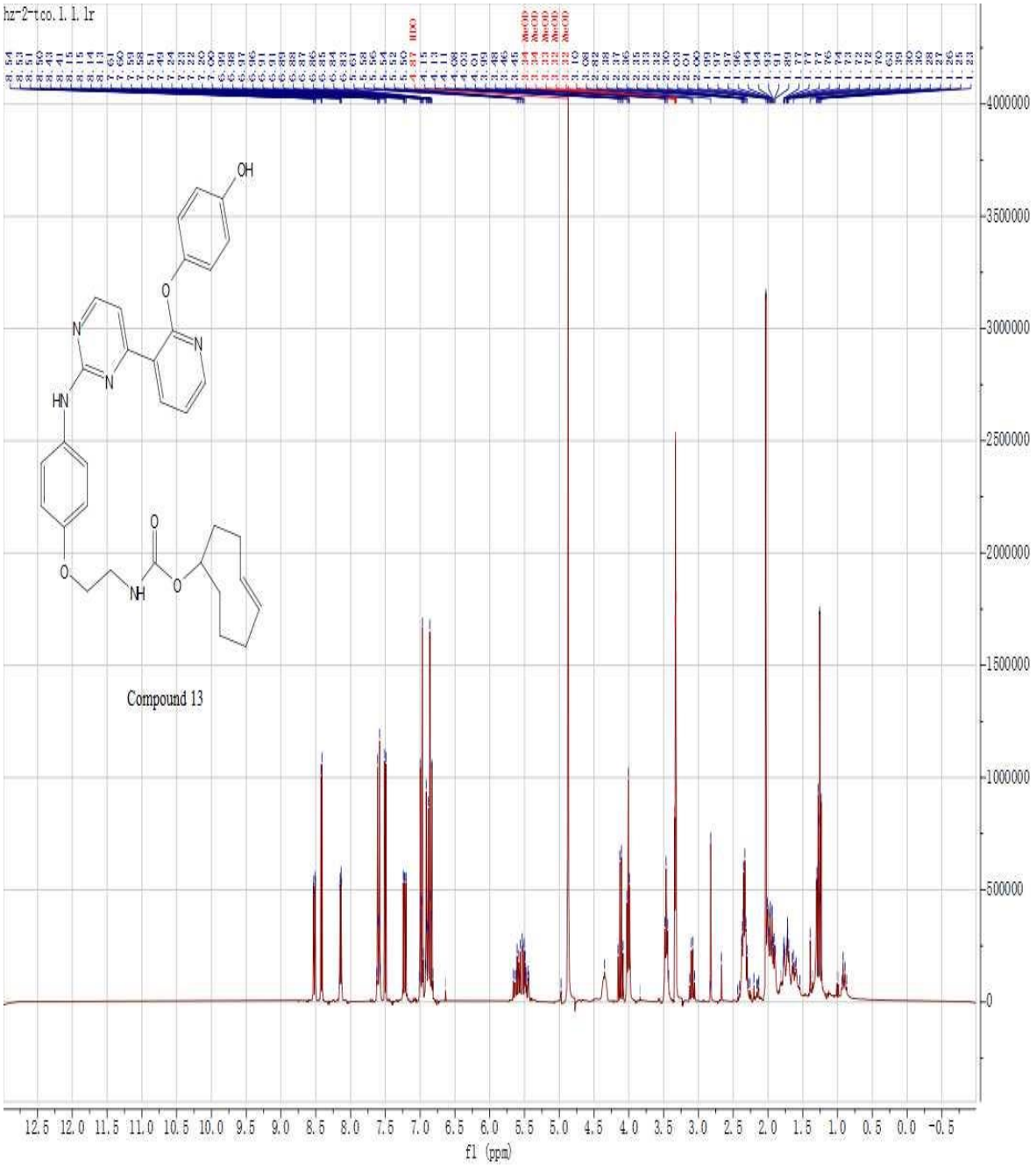
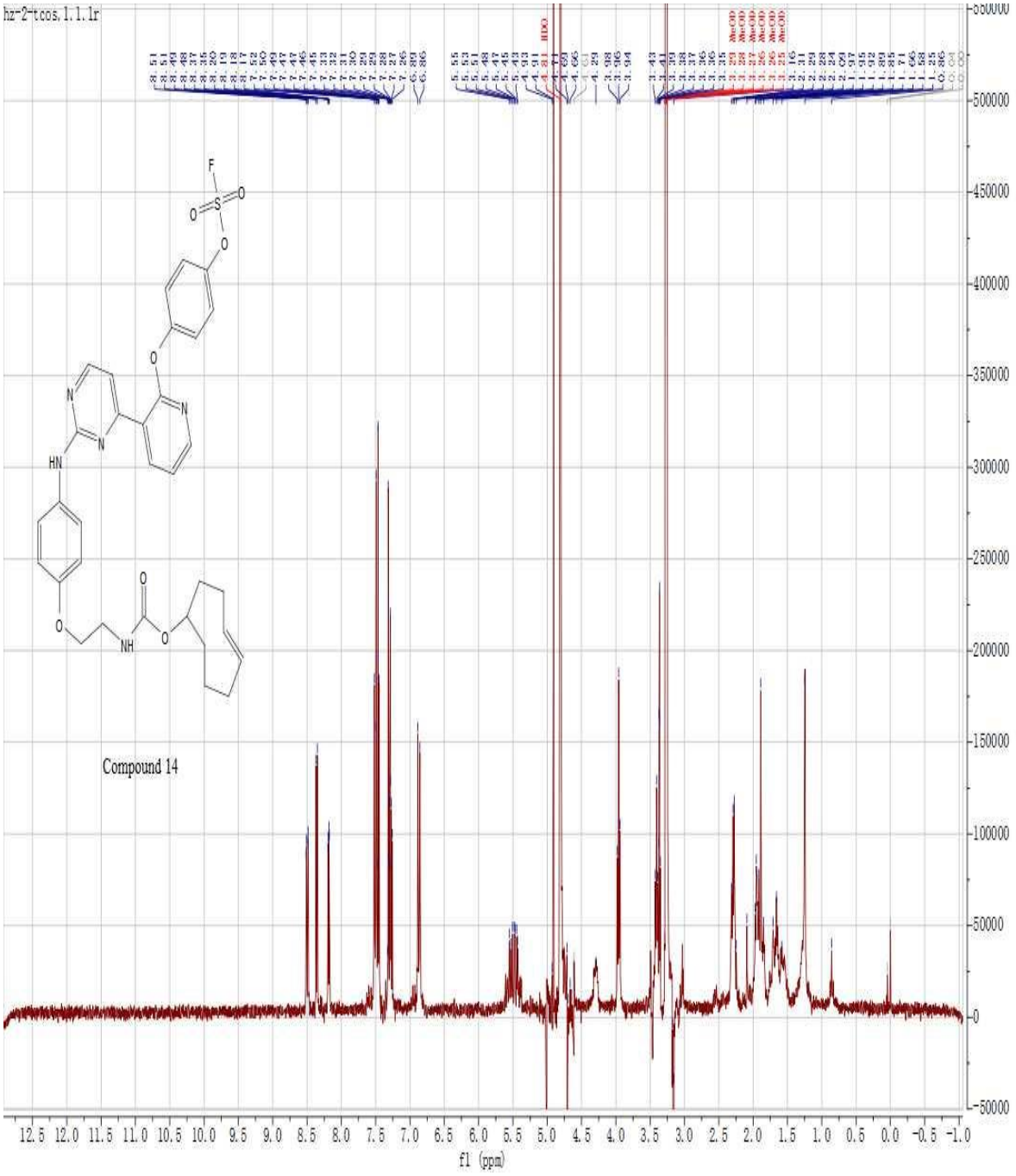
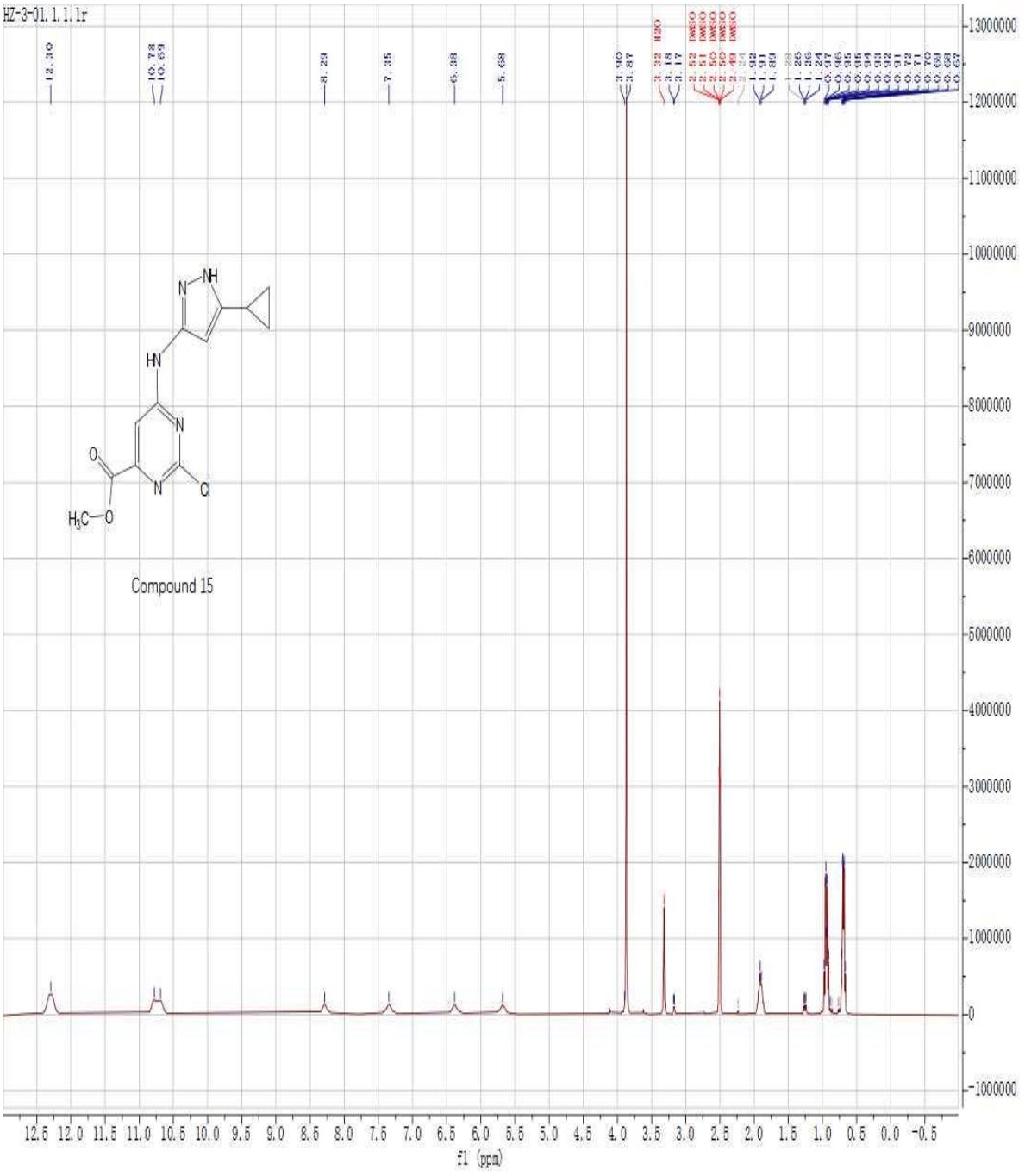

hz-2-tco.1.1.1r
OH
O
N
N
N
HN
O
O
NH
O
Compound 13
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
f1 (ppm)

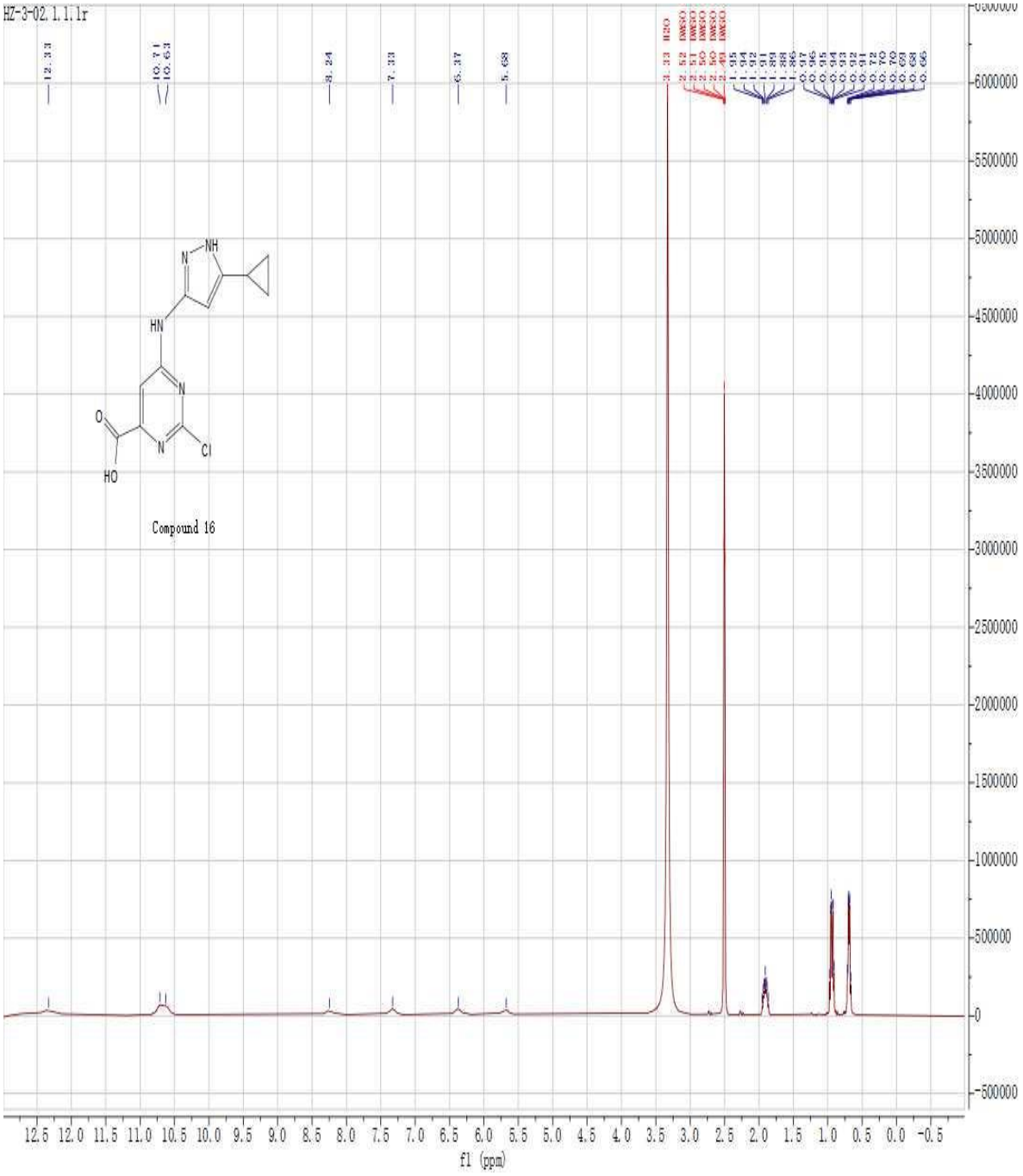
Compound 14:



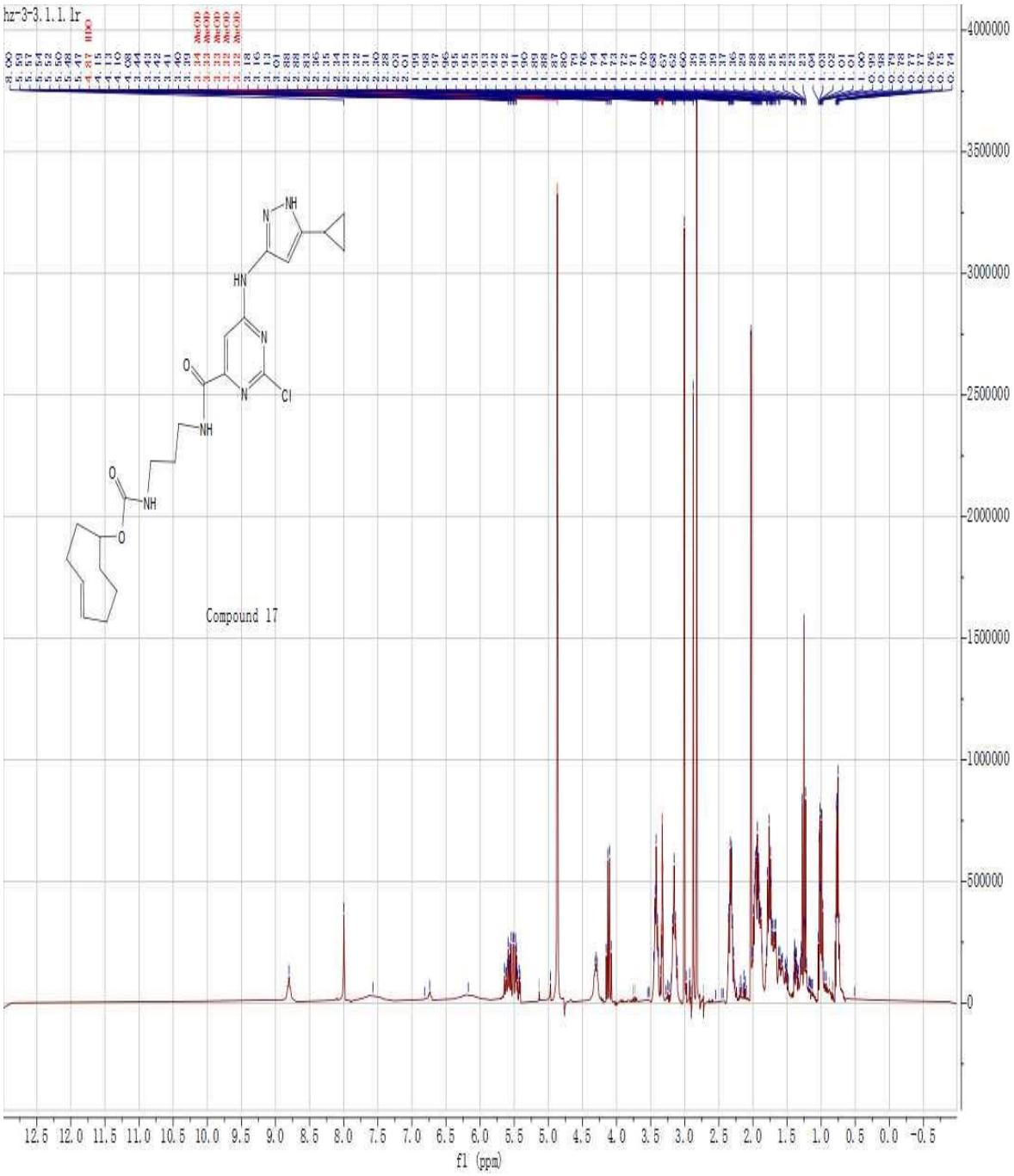
Compound 15:


HZ-3-01.1.1.1r
12.30
10.78
10.63
8.29
7.35
6.38
5.68
3.90
3.87
3.32
H2O
3.18
3.17
DMSO
2.52
2.51
2.50
2.50
2.49
1.92
1.91
1.89
1.26
1.25
1.24
0.97
0.96
0.95
0.94
0.93
0.92
0.91
0.72
0.71
0.70
0.69
0.68
0.67
13000000
12000000
11000000
10000000
9000000
8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
-1000000
12.5 12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)
NH
N
HN
N
O
N
Cl
H3C
O
Compound 15

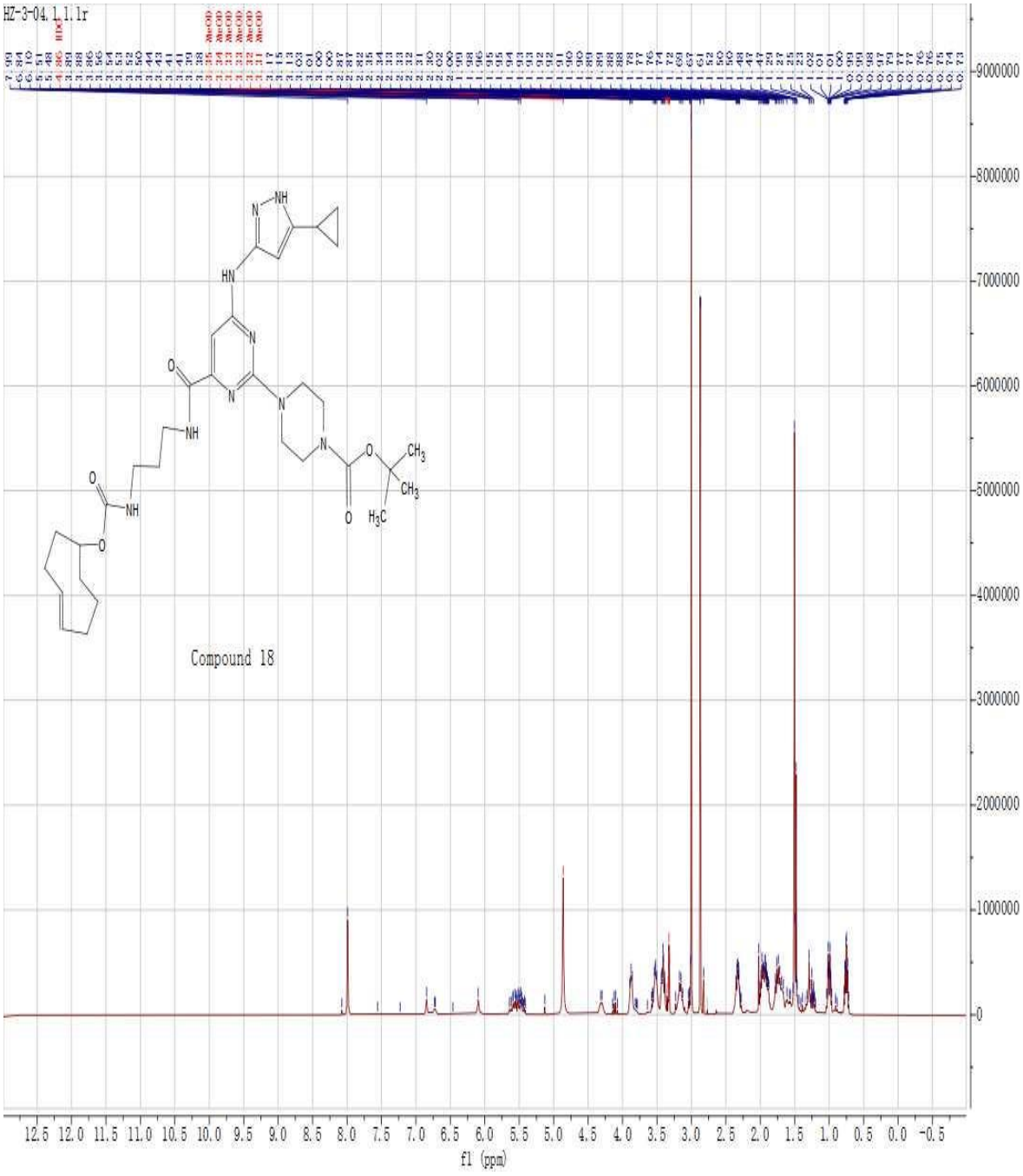
Compound 16:


HZ-3-02.1.1.1r
12.33
10.71
10.63
8.24
7.33
6.37
5.68
3.33 H2O
2.52 DMSO
2.51 DMSO
2.50 DMSO
2.49 DMSO
1.95
1.94
1.92
1.91
1.89
1.88
1.86
1.47
0.96
0.95
0.94
0.93
0.92
0.91
0.72
0.70
0.69
0.68
0.66
6000000
5500000
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
-500000
NH
N
HN
N
O
HO
N
Cl
Compound 16
12.5 12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

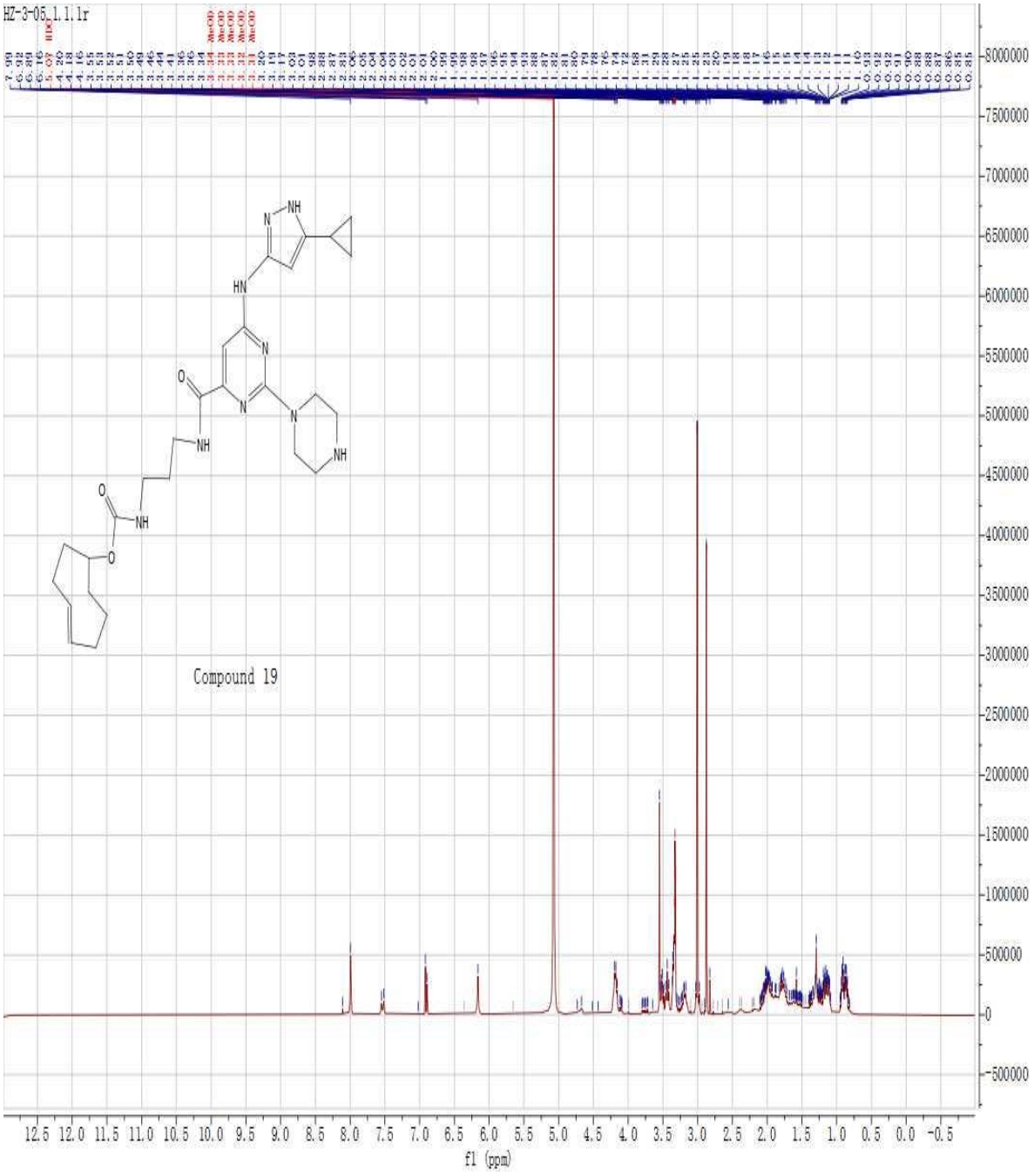
Compound 17:



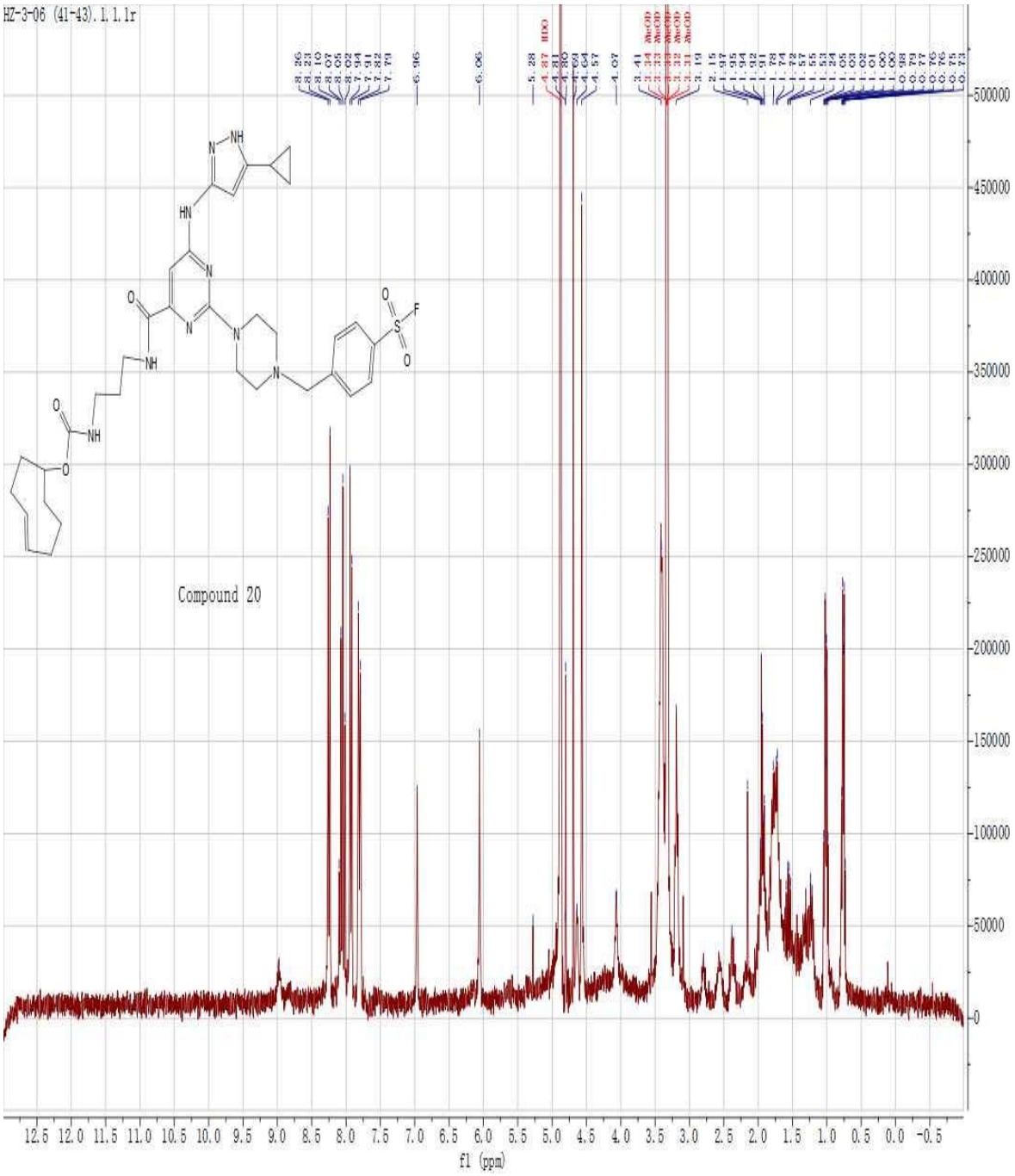
Compound 18:



Compound 19:



Compound 20:



Purity assessment for Probe 3 by liquid chromatography–mass spectrometry:

Probe 3 (71% purity)