Supplementary Information

Evaluation of Site-Diversified, Fully Functionalized Diazirine Probes for Chemical Proteomic Applications

Yan Tan,[ab] Songsen Fu,*[ab] Tao Yang,[ab] Yuxin Xie,[b] Guyi Shen,[b] Jie Yan,[b] Yufen Zhao,[ab] Feng Ni*[abc]

[a] *Institute of Drug Discovery Technology, Ningbo University, Ningbo 315211, China.*
[b] *Qian Xuesen Collaborative Research Center of Astrochemistry and Space Life Sciences, Ningbo University, Ningbo 315211, China.*
[c] *LeadArt Technologies Ltd., Ningbo, 315211, China.*

** Corresponding author: S. F. Email: fusongsen@nbu.edu.cn; F. N. Email: nifeng@nbu.edu.cn;*

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Figure S1

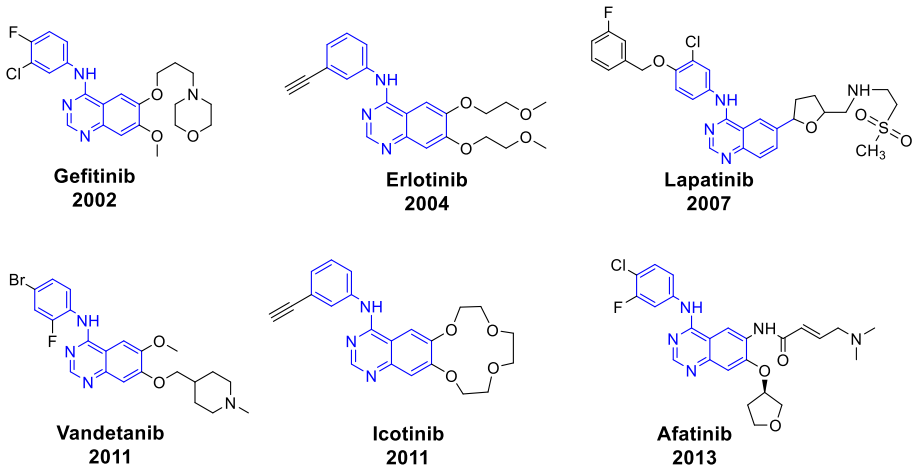


Figure S1. structures of marketed EGFR inhibitors containing 4-anilinoquinazoline scaffold (blue).

Figure S2

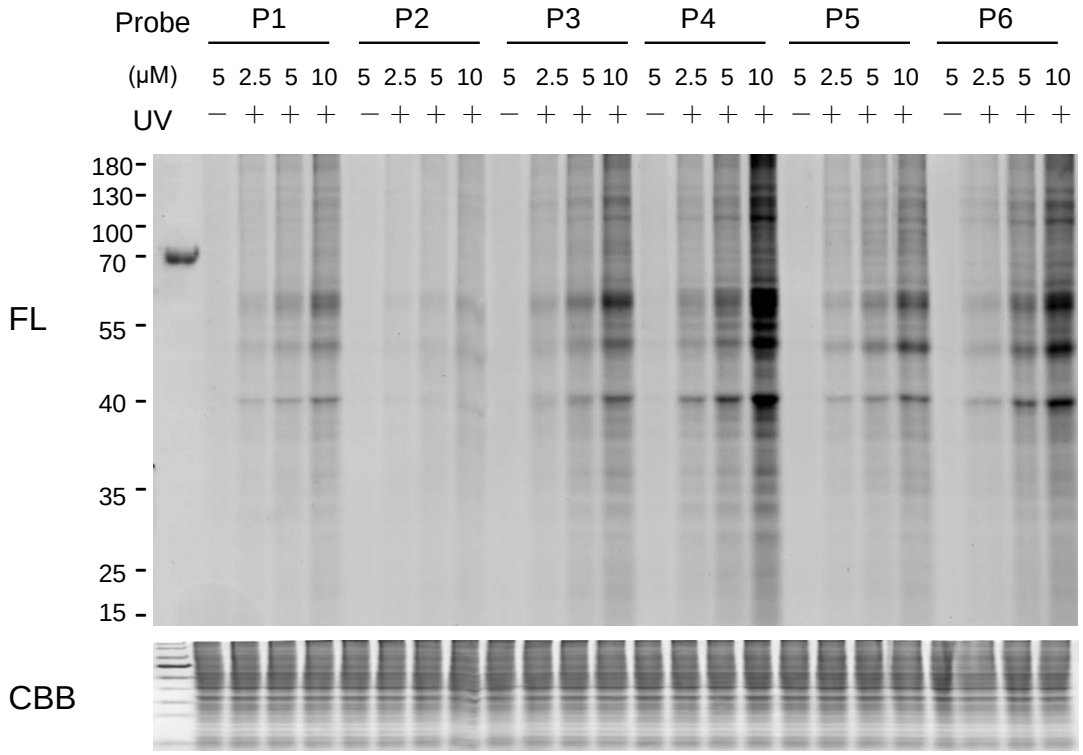


Figure S2. Hela cell lysates were treated with probes at a range of concentrations, irradiated with UV light (365 nm) for 10 min, analyzed by in-gel fluorescence. CBB-staining indicated the same amount of protein in each lane.

Figure S3

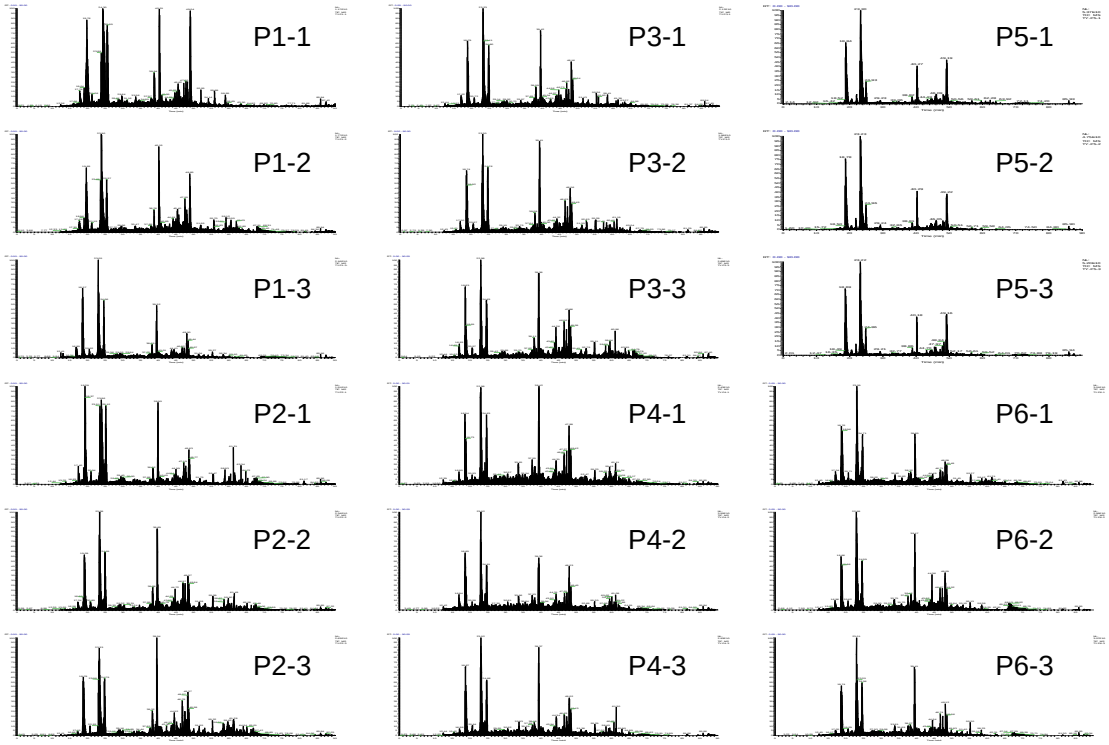


Figure S3. Total ion current chromatography of peptides of SILAC proteins enriched by site-diversified probes (light) together with negative probe (heavy) respectively in three biological replicates.

Figure S4

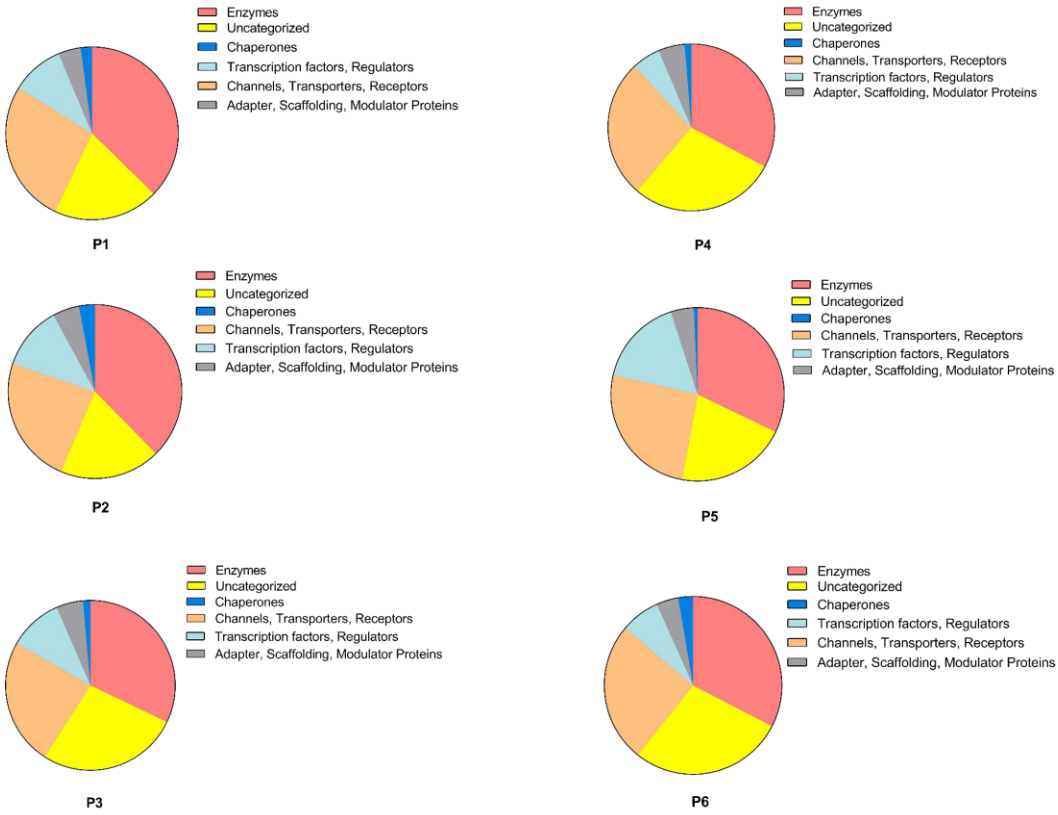


Figure S4. P1-P6 probe enriched protein classification. The classification is based on the collection of functional keywords related to the protein level from Uniprot database, and further classification into different protein families

Figure S5

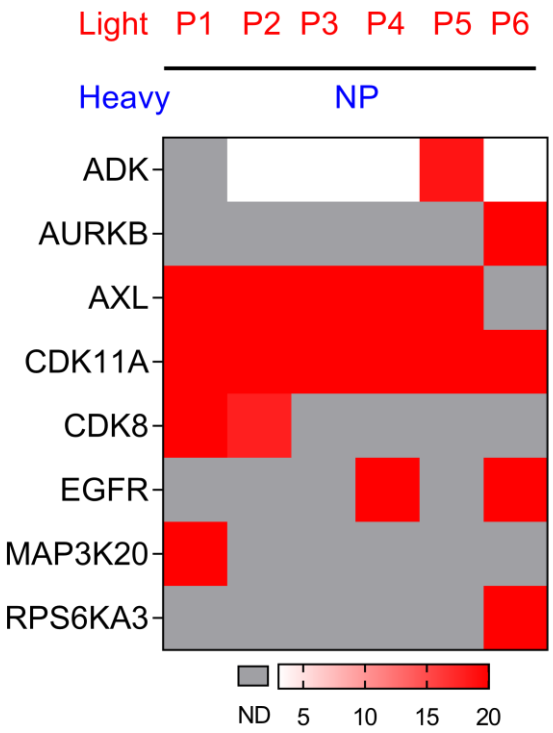


Figure S5. Heatmap showing relative kinase protein enrichment ratios of P1-P6 probes (5 μM) versus NP (5 μM) in living Hela cells.

Figure S6

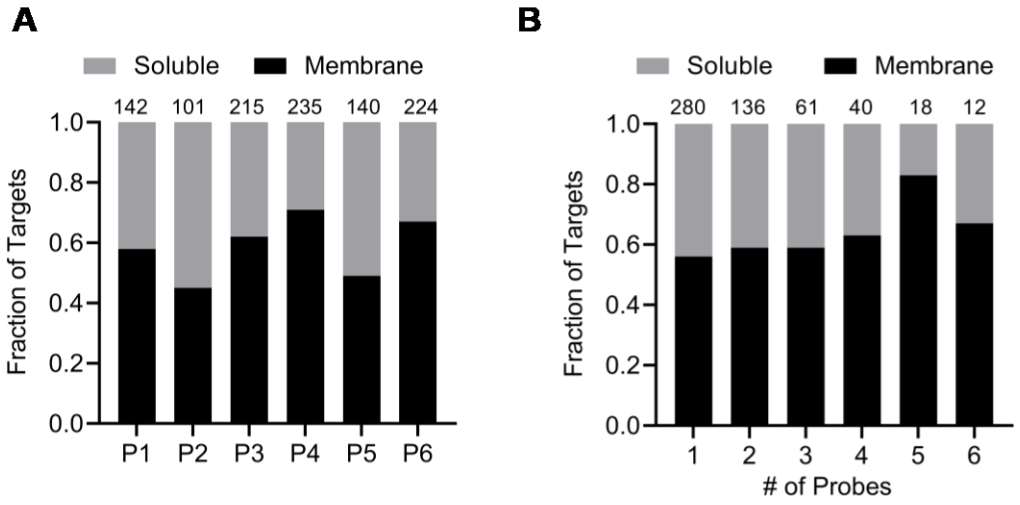


Figure S6. (A) The proportion of enriched membrane and non-membrane proteins for each probe. (B) The number and ratio of membrane and non-membrane protein targets significantly enriched by different number of probes.

Figure S7

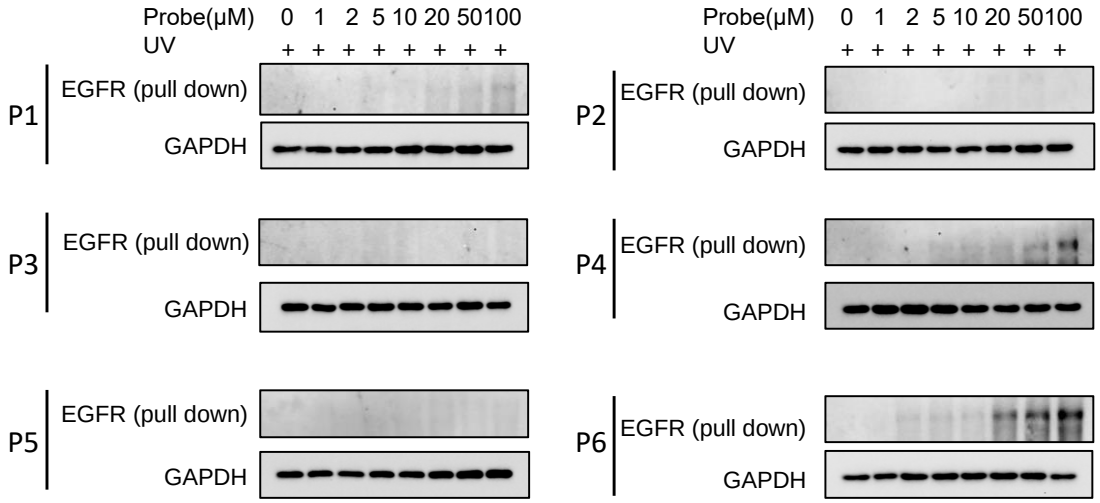


Figure S7. Experimental validation of EGFR labeling by immunoblotting. A431 cells were treated with different concentrations of probe for 1 h. The labeled proteins were reacted with Az-biotin and enriched with streptavidin beads ("EGFR (pull down)"). Equal loading is validated by immunoblotting of input samples before enrichment ("GAPDH").

Figure S8

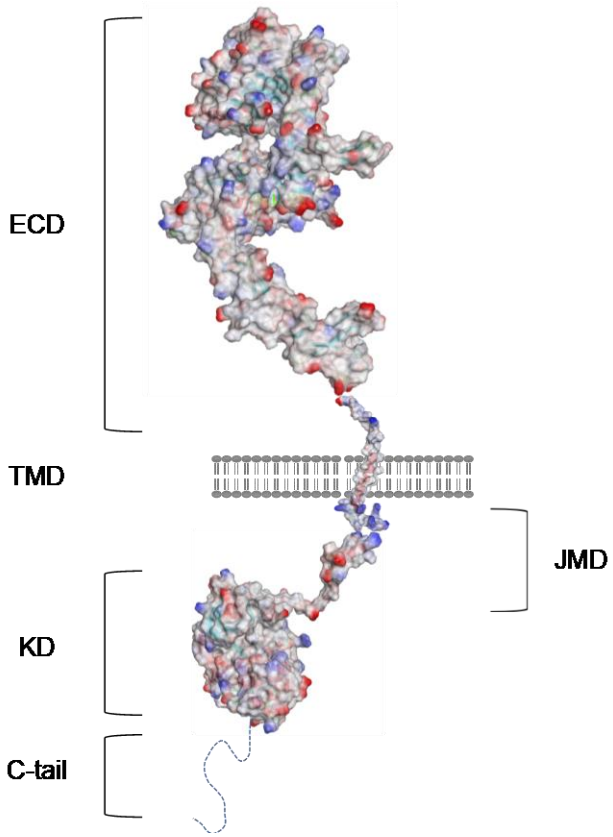


Figure S8. A schematic diagram of the structure Full-length EGFR. Published crystal structures of EGFR were used to prepare the schematic: extracellular domain (ECD) (PDB ID: 3NJP); transmembrane domain (TMD) (PDB ID:2KS1), Kinase domain (KD) with juxtamembrane domain (JMD) (PDB ID: 3GOP). The disordered C-tail has no resolved structures.

Figure S9

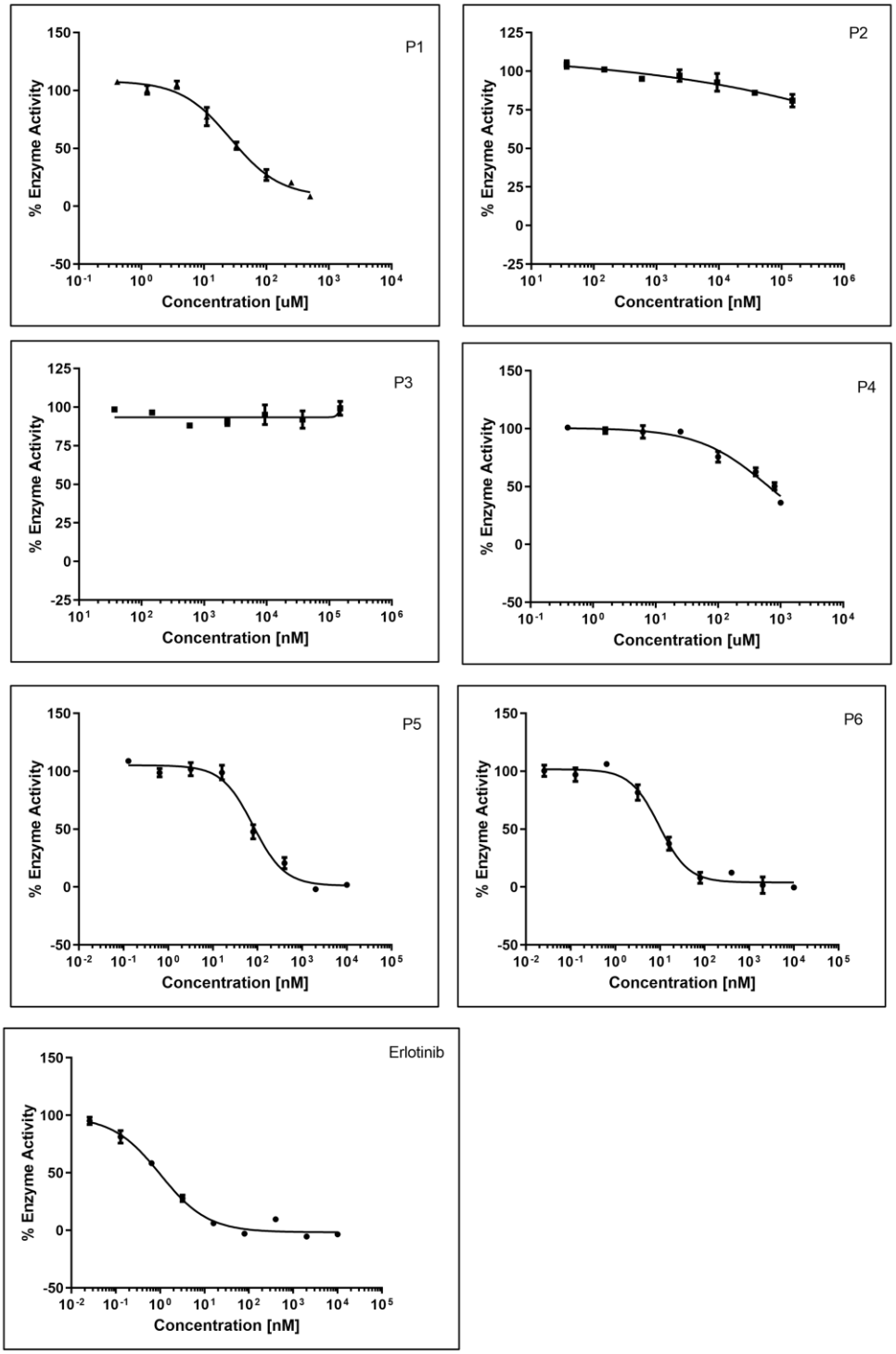
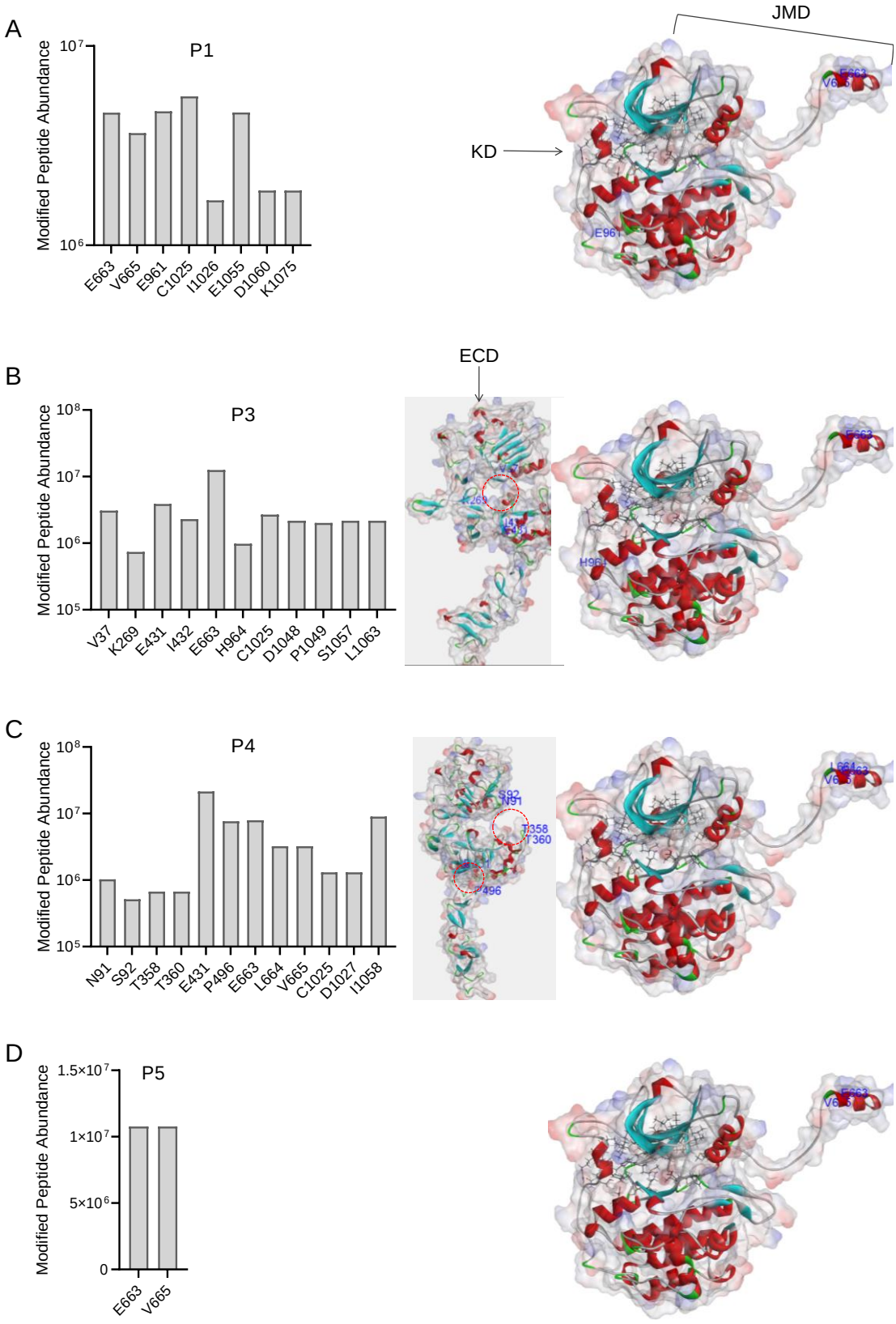


Figure S9. Kinase inhibitory plots for P1-P6 and erlotinib against *in vitro* EGFR. IC_{50} values of P1-P6 and erlotinib were calculated. Data shown are average ± SEM, n=2 biological replicates per group.

Figure S10


A
P1
Modified Peptide Abundance
10^7
10^6
E663
V665
E961
C1025
I1026
E1055
D1060
K1075
JMD
KD
B
P3
Modified Peptide Abundance
10^8
10^7
10^6
10^5
V37
K269
E431
I432
E663
H964
C1025
D1048
P1049
S1057
L1063
ECD
C
P4
Modified Peptide Abundance
10^8
10^7
10^6
10^5
N91
S92
T358
T360
E431
P496
E663
L664
V665
C1025
D1027
I1058
D
P5
Modified Peptide Abundance
1.5×10^7
1×10^7
5×10^6
0
E663
V665

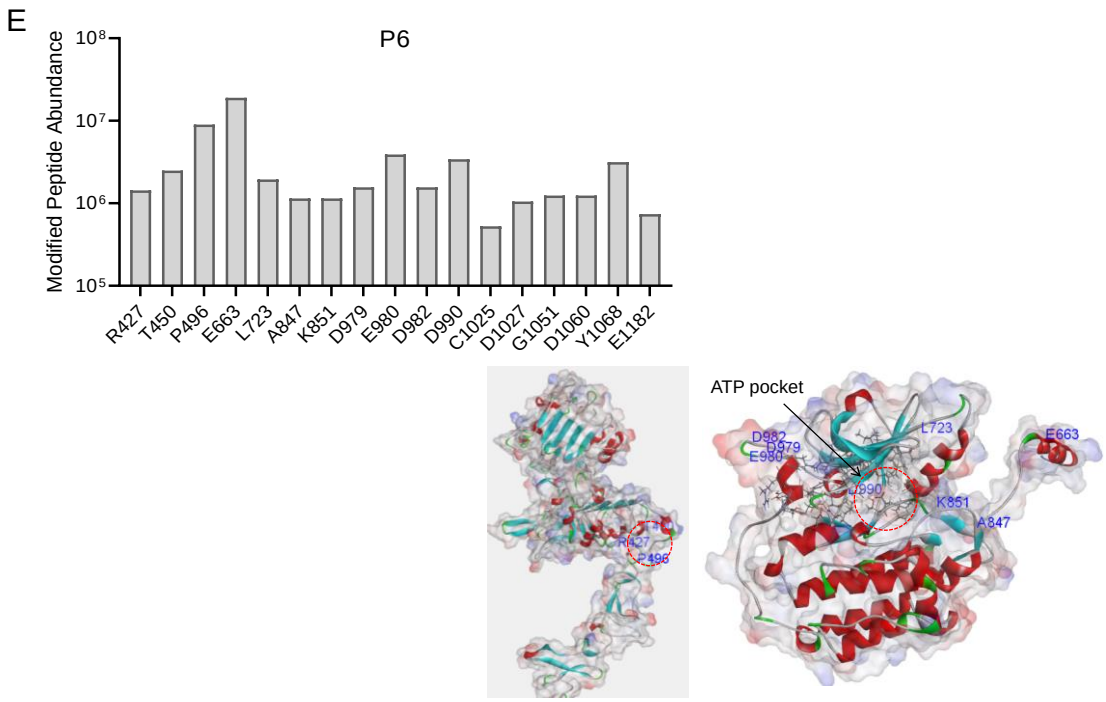


Figure S10. Bar charts show (A) **P1**-, (B) **P3**-, (C) **P4**-, D) **P5**-, (E) **P6**-modified peptide abundances, and the amino acid sites are labeled on 3D structures of extracellular domain ECD (PDB ID: 3NJP), Kinase domain (KD) and juxtamembrane domain (JMD) (PDB ID: 3GOP). Note that the numberings of amino acids are offset by 24 from that in Uniprot.

Figure S11

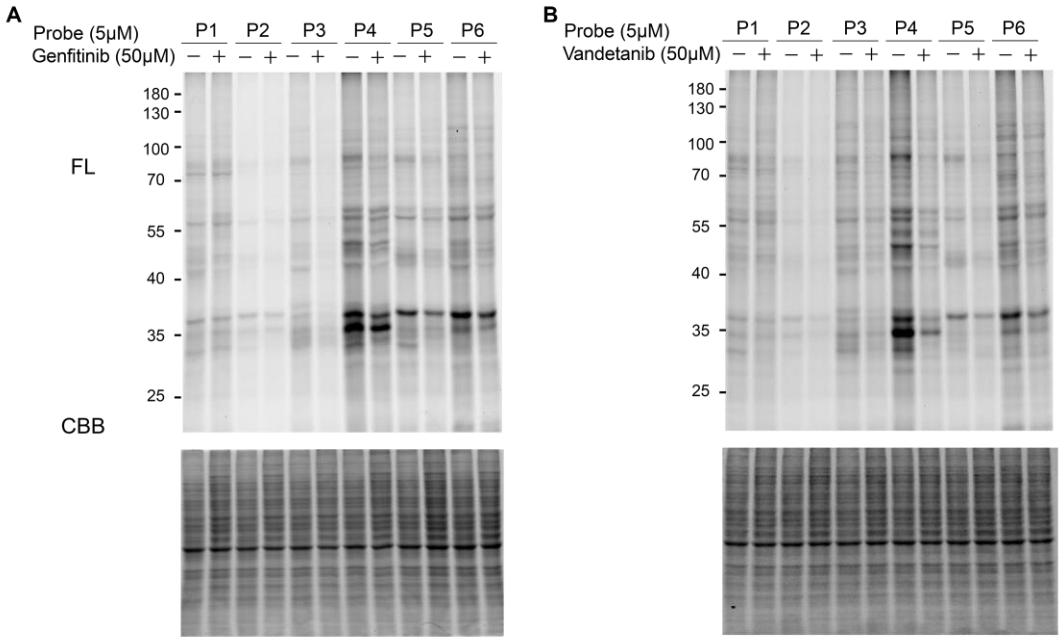


Figure S11. Gel-based competitive labeling of potential proteins by P1-P6 probes with 10× (A) Genfitinib or (B) Vandetinib. CBB-staining indicted the same amount of protein in each lane.

Figure S12

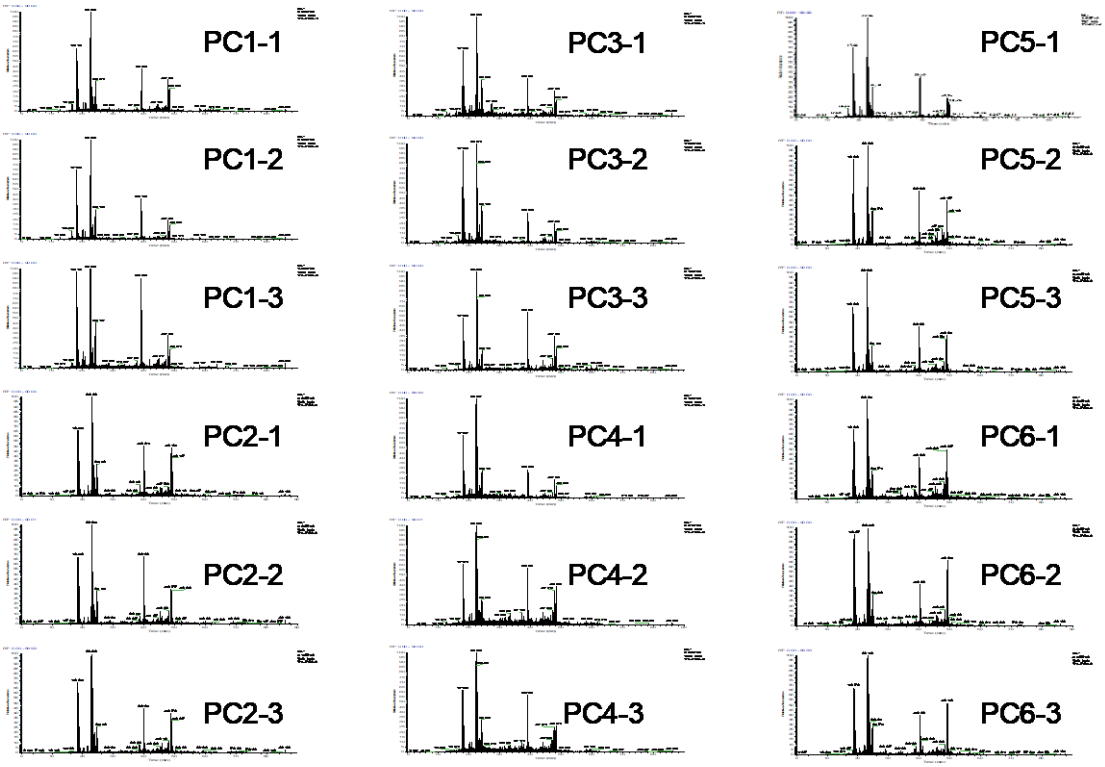


Figure S12. Total ion current chromatography of peptides of SILAC proteins enriched by site-diversified probes and either DMSO (light) or 10× erlotinib (heavy) respectively in three biological replicates.

Figure S13

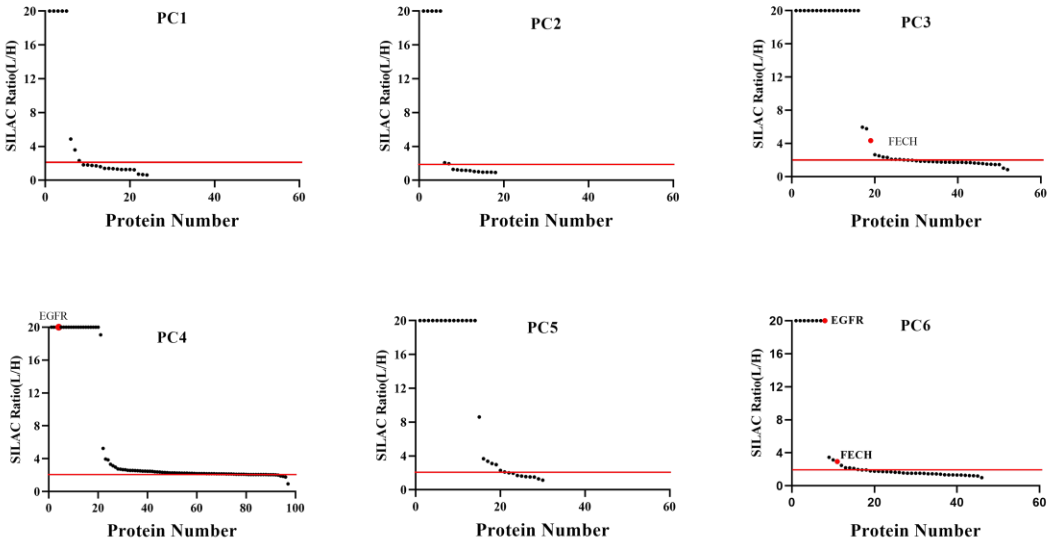
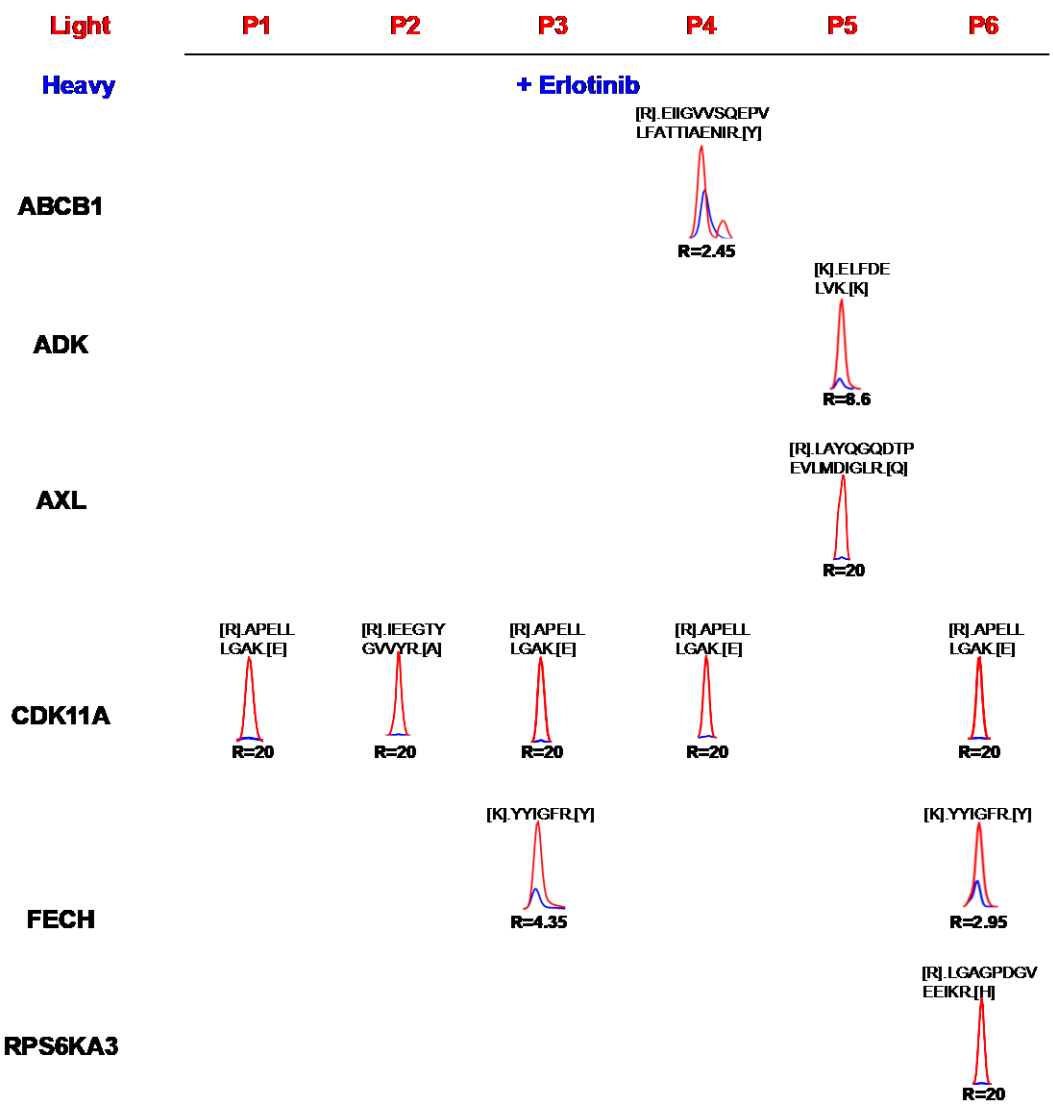


Figure S13. SILAC plots for total proteins identified in experiments comparing Hela cells treated with P1-P6 probe (5μM) and either DMSO (light), or 10× erlotinib (50 μM, heavy). Red dashed line marks a light/heavy ratio of 2; protein ratio at or higher than this line indicates substantial competition.

Figure S14

A

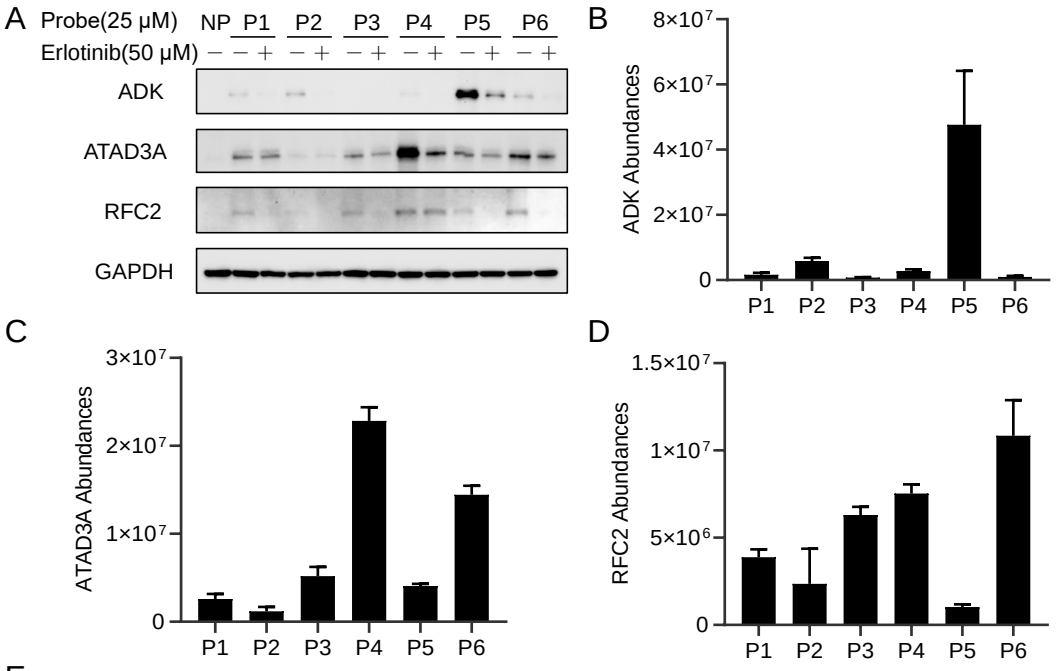


B

target	Activity	Assay	PubMed ID
ABCB1	IC50 = 26μM	Inhibition of human MDR1	22541068
ADK	Kd > 30μM	Kinobead profiling	29191878
AXL	Kd > 10μM	Binding constant determination	18183025
CDK11A	Kd > 10μM	Binding constant determination	18183025
FECH	Kd = 6.905μM	Kinobead profiling	29191878
RPS6KA3	Kd > 10μM	Binding constant determination	18183025

Figure S14. (A) Representative MS1 peptide traces for off-targets of erlotinib that have been validated by in vitro assays. (B) The activity data of these proteins quoted from PubChem database.

Figure S15



E

Accession	Description	$R_{(P1/P1+E)}$	$R_{(P2/P2+E)}$	$R_{(P3/P3+E)}$	$R_{(P4/P4+E)}$	$R_{(P5/P5+E)}$	$R_{(P6/P6+E)}$
P55263	Adenosine kinase OS=Homo sapiens (Human) OX=9606 GN=ADK PE=1 SV=2	-	-	-	-	8.60	-
Q9NVI7	ATPase family AAA domain-containing protein 3A OS=Homo sapiens (Human) OX=9606 GN=ATAD3A PE=1 SV=2	-	-	1.42	2.15	-	1.51
P35250	Replication factor C subunit 2 OS=Homo sapiens (Human) OX=9606 GN=RFC2 PE=1 SV=3	-	-	-	1.09	20	14.09

Figure S15. (A) Validation of representative off-targets by western blotting. Equal loading is validated by immunoblotting of input samples before enrichment. (B-D) MS abundances of ADK, ATAD3A and RFC2, respectively. (E) Quantitative ratios of competitive displacement by erlotinib in each probe group. $R_{(P1/P1+E)}$ represents the competitive quantitative ratio in P1 group. R: Ratio; E: Erlotinib.

Supplementary Table

Table S1

Table S1. Full proteomic data sets for quantitative MS (SILAC) studies. Data sets are provided as tabs within the accompanying Excel file.

Tab legend.

Tab (1): Compiled target list for site-diversified probes P1–P6, with corresponding averaged SILAC ratios derived from site-diversified probe versus NP enrichment experiments conducted at 5 μM in Hela cells. Protein family, DrugBank, and membrane annotations as well as associated reported Hela iBAQ values[1] are also included.

Tab (2): Supplementary information for tab (1): the median SILAC ratios for all quantified tryptic peptides per target and calculated *t*-test p values from three biological replicates.

Tab (3): Supplementary information for tab (1): the median abundances for protein target from three biological replicates. Abundance for a protein was calculated by summing the precursor mass abundances of all peptides identified of the protein.

Tab (4): Compiled target list for erlotinib identified by site-diversified probes P1–P6, with corresponding averaged SILAC ratios derived from site-diversified probe versus probe with excess erlotinib enrichment experiments in Hela cells. Protein family, DrugBank, and membrane annotations are also included.

Tab (5): Supplementary information for tab (4): the median SILAC ratios for all quantified tryptic peptides per target and calculated *t*-test p values from three biological replicates.

Tab (6): The peptide spectrum matches (PSMs)regard to probe-labeled peptides of EGFR. All the PSMs have been manually validated with the characteristic ion corresponding to probe fragment generated during HCD fragmentation.

Table S2

Table S2. The protein targets enriched by at least 5 probes of P1-P6. The target genes in green font were potential targets of probes, while in red were well-known non-specific background proteins.

Gene	SILAC Ratio (L/H)						Protein Classification	Membrane	iBAQ	# of Probe
	P1	P2	P3	P4	P5	P6				
ATAD3B	20	-	20	20	20	20	Enzymes	TRUE	5.38	5
ATP6AP2	20	20	20	20	-	20	Channels, Transporters, Receptors	TRUE	6.42	5
AXL	20	20	20	20	20	-	Enzymes	TRUE	4.62	5
CDK11A	20	20	20	20	20	20	Enzymes	FALSE	-	6
CTSD	15.47	19.60	20	20	20	17.92	Enzymes	FALSE	6.88	6
ECH1	2.65	9.68	7.19	20	7.28	8.44	Enzymes	FALSE	7.07	5
VDAC1	19.11	20	20	20	20	20	Channels, Transporters, Receptors	TRUE	8.11	6
VDAC2	9.13	8.32	20	20	20	19.23	Channels, Transporters, Receptors	TRUE	4.86	6
VIM	8.23	6.57	5.85	9.19	5.69	6.70	Uncategorized	FALSE	8.74	6
COX7A2	20	20	20	20	20	20	Enzymes	TRUE	7.16	6
EPHX1	12.68	11.49	20	20	4.00	20	Enzymes	TRUE	7.00	5
GOLGB1	-	20	20	20	20	20	Transcription factors, Regulators	TRUE	6.03	5
LPCAT3	5.02	20	6.34	5.96	20	-	Enzymes	TRUE	6.66	5
MARCHF5	20	20	20	20	20	20	Enzymes	TRUE	6.51	6
MTCH2	6.02	3.25	5.31	7.20	8.71	9.50	Channels, Transporters, Receptors	TRUE	7.42	5
NIPA2	-	20	20	20	20	20	Channels, Transporters, Receptors	TRUE	5.26	5
NQO1	17.18	6.42	6.23	8.81	7.79	11.16	Enzymes	FALSE	-	6
OCIAD2	20	-	20	20	20	20	Uncategorized	FALSE	5.52	5
PEX16	20	-	20	20	20	20	Uncategorized	TRUE	5.68	5
PSAP	7.95	5.25	17.68	15.02	12.32	-	Adapter, Scaffolding, Modulator Proteins	FALSE	7.06	5
SCARB1	20	20	20	20	-	13.43	Channels, Transporters, Receptors	TRUE	6.62	5
SLC16A3	6.82	8.87	6.24	10.71	3.54	8.95	Channels, Transporters, Receptors	TRUE	7.21	5
SLC18B1	20	20	20	20	20	20	Channels, Transporters, Receptors	TRUE	-	6
SLC25A10	11.68	11.07	14.09	-	16.09	18.15	Channels, Transporters, Receptors	TRUE	6.08	5
SLC25A15	20	20	-	20	20	20	Channels, Transporters, Receptors	TRUE	5.90	5
SLC25A20	16.96	16.96	20	19.63	19.62	20	Channels, Transporters, Receptors	TRUE	5.60	6
SPNS1	20	16.43	17.36	17.88	6.24	20	Channels, Transporters, Receptors	TRUE	4.98	6
SSR1	9.59	5.32	7.97	19.56	13.11	18.06	Uncategorized	TRUE	7.11	6
TMEM259	20	-	20	20	20	20	Uncategorized	TRUE	5.69	5
TOMM22	17.97	-	20	20	20	20	Channels, Transporters, Receptors	TRUE	7.66	5

Biological Methods

a. Cell culture

Hela cells were cultured in Dulbecco's modified Eagle medium (DMEM, HyClone) supplemented with 10% (v/v) fetal bovine serum (FBS, Biological Industries), and maintained in a humidified 37 ℃incubator with 5% CO_2. For SILAC experiments, SILAC DMEM (Thermo) deficient in L-lysine and L-arginine supplement with 10% dialyzed FBS (Thermo) and either [$^{13}C_6$, $^{15}N_2$]-L-lysine and [$^{13}C_6$, $^{15}N_2$]- L-arginine (100 μg/mL each) or L-lysine· HCl and L-arginine· HCl (100 μg/mL each) (Cambridge Isotope Laboratories). Heavy and light Hela cells were maintained in parallel and cell aliquots were frozen after six passages in SILAC DMEM and stored in liquid N_2 until needed.

b. In *vitro* EGFR kinase activity assay

Compounds were evaluated using an ADP-Glo kinase assay kit.[2] The compounds were dissolved and diluted in DMSO. 2 μL (2 ng) of EGFR-WT recombinant proteins were co-incubated with 1 μL of compound or 5% DMSO, 2 μL of substrate/ATP mix (5 μM ATP, 0.2 μg/μL PolyE4Y1) for 60 min at 25℃. After the kinase reaction, 5 μL of ADP-Glo™ Reagent was added to the reaction and incubated at 25℃for another 40 min. Then, the kinase detection reagent was added and incubated at 25℃for 30 min. Finally, the luminescence was measured with a luminometer. IC_{50} values were calculated by GraphPad Prism 8.0.

c. *In situ* labeling of Hela cells with P1-P6 probes

Experiments were performed similarly as previously reported.[3] For gel-based experiments, Hela cells were seeded in 6-well plates and grown to 90% confluence at the time of experiment. After suction of the medium and PBS washing, DMEM containing indicated probe with a final DMSO concentration of 0.5% was added and incubated for 1 h at 37℃/5% CO_2. The mediums were discarded and the plates were washed twice with pre-cooled 2 mL of PBS. Then

1 mL of PBS was added and the cells were irradiated by UV light (365 nm) for 10 min at 4℃ For MS-based experiments, the operation of cell labeling is similar to that described above. The 'light' and 'heavy' SILAC Hela cells were grown in 10 cm plates. In probe-versus-**NP** experiments, light cells were treated with 5 μM each of **P1**-**P6** probes, while the heavy cells were treated with 5 μM **NP**. In competition experiments, 5 μM each of **P1**-**P6** probes was added to the light cells, and 5 μM each of **P1**-**P6** probes and 50 μM erlotinib were added to the heavy cells. After treatments and UV irradiation, cells were harvested with a cell scraper, then were centrifuged at 1000 × g for 5 min at 4℃ and the supernatant was removed to yield cell pellets. The pellets were resuspended in 100 μL of 0.1% Triton X-100 (in PBS) and lysed by sonication. After centrifuged (15000 × *g*, 10 min, 4℃), the supernatant was collected as cell lysates. The protein concentration of cell lysates was determined by BCA protein assay and adjusted to 2 mg/mL.

d. Labeling of Hela cell lysates with P1-P6 probes

The collected hela cells were resuspended in PBS containing 1 × protease inhibitor cocktail and lysed by sonication and centrifuged (15000 × *g*, 10 min, 4℃). After centrifuged (15000 × *g*, 10 min, 4℃), the supernatant was collected as cell lysates. The protein concentration of cell lysates was determined by BCA protein assay and adjusted to 2 mg/mL. cell lysates (100 μL) were combined with probes in 48-well plates and incubated for 0.5 h at 25℃, then were irradiated by UV light (365 nm) for 10 min at 4℃

e. Gel-based analysis of crosslinked proteins

To each sample (100 μL), 3 μL of a freshly prepared 'click' reagent mixture containing 0.05 mM TBTA (3.4 mM in DMSO), 1mM $CuSO_4$ (200 mM in H_2O), 1mM TCEP (200 mM in H_2O), and 50 μM TAMRA-azide (20 mM in DMSO) was added. Each solution was immediately mixed by vortex and gently rotated at room temperature for 2 h before quenching the reactions with 25 μL of 5× SDS loading buffer. Finally, 15 μg of protein (per gel lane) was resolved by SDS-PAGE (10% acrylamide), then visualized by

in-gel fluorescence scanning (Bio-Rad ChemiDoc ™ MP) and Coomassie Brilliant Blue (CBB) staining.

f. Preparation of labeled proteome for MS-based analysis

To the combined mixture of heavy and light soluble proteomes (1 mg), a mixture of TBTA (7.5 µL, 3.4 mM in DMSO), $CuSO_4$ (2.5 µL, 200 mM in H_2O), TCEP (2.5 µL, 100 mM in H_2O) and Biotin-N_3 (1 µL, 50 mM in DMSO) was added and each sample was rotated at room temperature. After 2 h, the mixture was transferred to a 15 mL tube, then a 4-fold volume of cold acetone was added. Precipitated proteins were collected by centrifuged (10000 × *g*, 5 min, 4℃) and washed twice with cold MeOH. Subsequently, the pellet was resuspended in 1.5 mL of PBS with 0.2% SDS. 78.9 µL of TCEP (200 mM in PBS) was added to the resuspended solution and incubated for 20 min at 65℃. Reduced thiols were alkylated by adding 54.5 µL of iodoacetamide (600 mM in PBS) for 40 min at room temperature away from light. Then the solution was centrifuged (14000 × *g*, 10 min, 25℃) and the supernatant was transferred to a new 15 mL tube. To each solution, 0.5 mL of 1.2% SDS (in PBS) and 0.5 mL of PBS were added and incubated with 3 mL of 0.2% SDS including streptavidin beads for 2 h at room temperature on a rotator. The beads were collected by centrifugation (1400 × *g*, 2 min), and sequentially washed with 0.2% SDS in PBS (1 × 5 mL), 6 M urea in PBS (1 × 5 mL), 2 M urea in PBS (1 × 5 mL), PBS (1 × 5 mL) and ddH_2O (1 × 5 mL). The beads were transferred to a Protein LoBind tube (Eppendorf), resuspended with 200 µL of 100 mM NH_4HCO_3 and incubated with trypsin (1 µg, Promega) and $CaCl_2$ (1 mM) at 37℃ overnight. The supernatant was transferred to a fresh Protein LoBind tube. The beads were washed twice with 50 µL of 100 mM NH_4HCO_3 and washings were combined with previously separated supernatant. Each solution was acidified with formic acid (FA)and desalted with C_{18} tips according to the product instruction, then was dried and stored at -80℃ until analyzed.

g. Preparation of probe-labeled peptides for MS-based analysis

A431 cells were grown in 10-cm plates with DMEM medium supplemented with 10 % FBS to ~90 % confluence at the time of treatment. The medium was aspirated, and the cells were incubated with 2 mL of serum-free media containing each probe of

P1-**P6** for 60 min at 37 °C. The plates were then directly exposed to 365 nm UV light for 20 min. cells were harvested with a cell scraper, washed (×2) with cold PBS, then were centrifuged at 1000 × g for 5 min at 4℃and the supernatant was removed to yield cell pellets. Cell pellets were resuspended in 500 µL PBS and lysed by sonication. Protein concentrations were normalized (2 mg/mL). To each sample (1 mg of proteins) was added solutions of TBTA (7.5 µL, 3.4 mM in DMSO), $CuSO_4$ (2.5 µL, 200 mM in H_2O), TCEP (2.5 µL, 100 mM in H_2O) and DADPS (1 µL, 50 mM in DMSO) and shaken at room temperature for 2 h. The mixture was transferred to a 15 mL tube, then a 4-fold volume of cold acetone was added. Precipitated proteins were collected by centrifuged (10000 × *g*, 5 min, 4℃) and washed twice with cold MeOH. Subsequently, the pellet was resuspended in 1.5 mL of PBS with 0.2% SDS. 78.9 µL of TCEP (200 mM in PBS) was added to the resuspended solution and incubated for 20 min at 65℃. Reduced thiols were alkylated by adding 54.5 µL of iodoacetamide (600 mM in PBS) for 40 min at room temperature away from light. Then the solution was centrifuged (14000 × *g*, 10 min, 25℃) and the supernatant was transferred to a new 15 mL tube. To each solution, 0.5 mL of 1.2% SDS (in PBS) and 0.5 mL of PBS were added and incubated with 3 mL of 0.2% SDS including streptavidin beads for 2 h at room temperature on a rotator. The beads were collected by centrifugation (1400 × *g*, 2 min), and sequentially washed with 0.2% SDS in PBS (1 × 5 mL), 6 M urea in PBS (1 × 5 mL), 2 M urea in PBS (1 × 5 mL), PBS (1 × 5 mL) and ddH_2O (1 × 5 mL). The beads were transferred to a Protein LoBind tube (Eppendorf), resuspended with 200 µL of 100 mM NH_4HCO_3 and incubated with trypsin (1 µg, Promega)) and $CaCl_2$ (1 mM) at 37℃ overnight. The beads were washed twice with 50 µL of 100 mM NH_4HCO_3. The beads were transferred to LoBind microcentrifuge tubes with water, and the water was removed by centrifugation (500 x g, 10 min). The beads were incubated with a solution of formic acid/water (200 µL, 10 % v/v) for 0.5 h with gentle shaking. The supernatants were combined. The beads were then washed with acetonitrile/water (50 % v/v with 1 % formic acid, 400 µL). The washing was combined with the supernatants, and solvents were removed with vacuum centrifugation. The residue was redissolved in FA solution (0.1 % in water v/v) and was stored at -80 °C until ready for mass spectrometric analysis.

h. Liquid chromatography-mass spectrometry (LC-MS) analysis

Peptides were resuspended in 20 µL of 0.1% FA and analysed using Orbitrap Fusion Lumos mass spectrometer. The peptides were loaded onto an Easy-Spray column heated at 50°C (C_{18}, 3 µm particle size, 50 × 150 mm; Thermo Scientific) and eluted using a 90 min LC gradient (0–32% B, 0–78 min; 32–90% B, 78–80 min; 90% B, 80–88 min; 90–2% B, 88-90 min; followed by pre-equilibration, where mobile phase A consisted of 0.1% formic acid in H_2O and mobile phase B consisted of 0.1% formic acid in acetonitrile) at a flow rate of 300 nL/min. The voltage applied to the nano-LC electrospray ionization source was 2.5 kV. Survey scans were acquired over 400–1,800 m/z at a resolution of 120,000 in data-dependent acquisition (DDA) approach in positive mode. The precursor automatic gain control (AGC) target was set to 3×10^6, and the maximum injection time was set to 50 ms. The most abundant ions (with a charge stage of 2–7) were selected for fragmentation by HCD (NCE of 30%) with a 30 sec dynamic exclusion in a top-speed mode. The resolution of MS2 was 30,000. The fragment automatic gain control (AGC) target was set to 1×10^5, and the maximum injection time was set to 100 ms.

For analysis of probe-labeled peptides, peptides were eluted using a 120 min LC gradient (0 – 1 min, 0 – 5%; 1 – 91 min, 5 – 27%; 91 – 115 min, 27 – 98%; 115 – 120 min, 98% – 0%; followed by pre-equilibration, where mobile phase A consisted of 0.1% formic acid in H_2O and mobile phase B consisted of 0.1% formic acid in acetonitrile) at a flow rate of 350 nL/min. Tandem MS was performed on the most abundant precursors exhibiting a charge state. HCD fragmentation was applied with 35% collision energy and resulting fragments detected using the normal scan rate in the ion trap.

i. Protein identification and quantification

Raw files were analyzed in Proteome Discoverer 2.3 (Thermo Fisher Scientific) and were searched using the SEQUEST HT algorithm against homo sapiens (TaxID=9606, Uniprot release – 01/28/2021) peptide sequence. Precursor and fragment mass tolerance was set to 10 ppm and 0.02 Da respectively, with up to two missed cleavages allowed. Cysteine carbamidomethylation (+57.021 Da) was set as a static modification, acetylation of protein N-terminus (+42.011 Da),

methionine oxidation (+15.995 Da) and light- and heavy-isotope (+8.014 Da for K, +10.008 Da for R) of SILAC were set as variable modifications. The false discovery rates (FDR) were less than 1% at the peptide and protein level.

j. Proteomic analysis

For all SILAC experiments, proteins were filtered to those detected with high "Protein FDR Confidence", and each protein ratio was resulted from at least two unique peptides in all of the triplicate analyses. Swiss contaminants, keratin and trypsin proteins were manually removed from protein lists. Identification of probe targets in probe versus control probe and probe competition experiments represent averaged values from two or more biological replicate experiments.

For probe versus control experiments, in addition to the above criteria, to be classified as protein targets, proteins must be following additional criteria: (1) SILAC ratio derived from at least 2 biological replicate experiments, and derived from two or more quantified peptides, (2) average SILAC ratio ≥5 were required and (3) a p-value ≤0.05 calculated by a two-sided one sample Student's test over 2 or 3 replicates. For competition experiments, proteins that (1) were designated probe targets for the probe being used, as described above, (2) with SILAC ratios ≥2 derived from at least 2 biological replicate experiments, and (3) with p-values ≤0.05 calculated by a two-sided one sample Student's test over 2 or 3 replicates were designated as targets of the competitor.

Protein targets were queried against the DrugBank database (https://go.drugbank.com/contact) and fractionated into DrugBank and non-DrugBank proteins. Protein targets were queried against the KEGG and UniProtKB/Swiss-Prot Protein Knowledge database for protein classification. Membrane proteins defined as proteins possessing known or predicated transmembrane domains (Uniprot analysis), and the remaining targets were considered soluble.

k. Identification of probe-labeling sites

Photoaffinity probe binding site analysis was performed with the processing software Proteome Discoverer 2.3 (Thermo Fisher Scientific). Peptide sequences were identified by matching proteome databases with experimental fragmentation patterns via the SEQUEST HT algorithm. Fragment tolerances were set to 0.6 Da, and precursor mass tolerances set to 10 ppm with two missed cleavage sites allowed. Carbamidomethyl (C, +57.02146) and oxidation (M, +15.994915) were defined as variable modifications. Photoaffinity probe binding sites were assigned as variable modifications on any amino acid (P1~P6: +499.2696). Spectra were searched against the Homo sapiens proteome database using a false discovery rate of 1 % (Percolator). The peptide spectrum matches (PSMs) with probe-modification were considered as high confidence if the characteristic ion (+1, 520.28) corresponding to probe fragment could be found in the MS/MS spectra.

l. Protein enrichment by pull down experiment

Cells were plated out on 10 cm dishes and grown to 90 % confluence prior to the labeling experiment. At the time of experiment, the mediums were replaced with 5 mL of fresh medium. For control group, the cells were incubated with **NP** for 1 h. For probe group, the cells were incubated with each probe of **P1**-**P6** for 1 h. For competition group, the cells were incubated with erlotinib (10 x) combined with each probe of **P1**-**P6** for 1 h. The mediums were discarded and the dishes were washed twice with pre-cooled PBS (2 x 3 mL). Then 3 mL PBS was added and the cells were irradiated with 365 nm UV light at 4℃ for 10 min. The cells were harvested with a cell scraper, then were centrifuged at 1000 × g for 5 min at 4℃ and the supernatant was removed to yield cell pellets. The pellets were resuspended in 100 µL of 0.1% Triton X-100 (in PBS) and lysed by sonication. After centrifuged (15000 × *g*, 10 min, 4℃), the supernatant was collected as cell lysates. The protein concentration of cell lysates was determined by BCA protein assay and adjusted to 2 mg/mL. To the combined mixture of heavy and light soluble proteomes (1 mg), a mixture of TBTA (7.5 µL, 3.4 mM in DMSO), $CuSO_4$ (2.5 µL, 200 mM in H_2O), TCEP (2.5 µL, 100 mM in H_2O) and Biotin-N_3 (1 µL, 50 mM in DMSO) was added and each sample was rotated at room temperature. After 2 h, the mixture

was transferred to a 15 mL tube, then a 4-fold volume of cold acetone was added. Precipitated proteins were collected by centrifuged (10000 × *g*, 5 min, 4℃) and washed twice with cold MeOH. Subsequently, the pellet was resuspended in 1.5 mL of PBS with 0.2% SDS. 78.9 µL of TCEP (200 mM in PBS) was added to the resuspended solution and incubated for 20 min at 65℃. Reduced thiols were alkylated by adding 54.5 µL of iodoacetamide (600 mM in PBS) for 40 min at room temperature away from light. Then the solution was centrifuged (14000 × *g*, 10 min, 25℃) and the supernatant was transferred to a new 15 mL tube. To each solution, 0.5 mL of 1.2% SDS (in PBS) and 0.5 mL of PBS were added and incubated with 3 mL of 0.2% SDS including streptavidin beads for 2 h at room temperature on a rotator. The beads were collected by centrifugation (1400 × *g*, 2 min), and sequentially washed with 0.2% SDS in PBS (1 × 5 mL), 6 M urea in PBS (1 × 5 mL), 2 M urea in PBS (1 × 5 mL), PBS (1 × 5 mL) and ddH_2O (1 × 5 mL). The beads were transferred to a Protein LoBind tube (Eppendorf). The enriched proteins were eluted by 1 × loading buffer at 95 °C for 10 min and separated by SDS-PAGE (10%).

m. Western Blotting

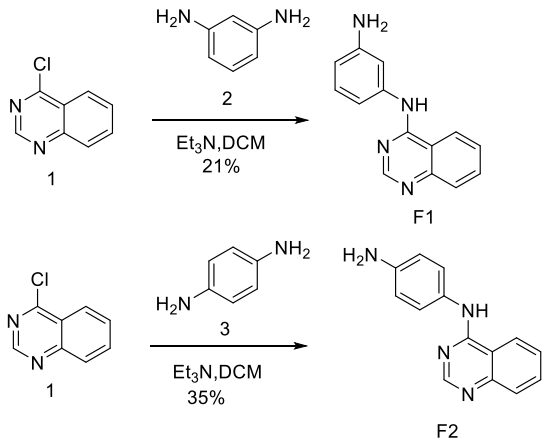
For Western blotting experiments, samples were resolved by SDS−polyacrylamide gels and transferred to poly membranes. Membranes were then blocked with 5% milk in TBST (0.1% Tween in Tris-buffered saline) for 1 h at room temperature. After blocking, membranes were incubated with the corresponding primary antibody (purchased from proteintech. GAPDH: 60004-1-IG; EGFR: 51071-2-AP; ADK: 66929-1-IG; ATAD3A: 16610-1-AP; RFC2: 10410-1-AP) overnight at 4 °C. After incubation, membranes were washed with TBST (4 ×10 min) and then incubated with an appropriate secondary antibody. Finally, blots were washed again with TBST before incubated with ECL chemiluminescence substrate reagent, and visualized by chemiluminescence scanning (Bio-Rad ChemiDoc ™ MP).

Synthetic Methods

Chemistry materials

Solvents and chemicals were purchased from commercial vendors and used without further purification. Starting materials of amine substituted 4-aniline quinazolines were obtained from customized synthesis. All reactions were monitored by analytical thin layer chromatography (TLC) on pre-coated silica plates and spots were visualized by UV (254 nm or 280 nm), iodine, or heating with $KMnO_4$. The products were purified by high performance liquid chromatography (HPLC). NMR spectra (^{1}H-NMR, ^{13}C-NMR) were recorded at room temperature on a Bruker Ascend 500 MHz NMR spectrometer. Chemical shifts were recorded in ppm using tetramethylsilane (TMS) or residual solvent peaks (TMS=0.00 ppm; DMSO-d6=2.50 ppm for ^{1}H NMR; DMSO-d6=39.53 ppm for ^{13}C NMR). The following abbreviations were used in reporting spectra, br s (broad singlet), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets). High-resolution mass spectra (HRMS) were obtained on a Q Exactive Plus mass spectrometer.

a. Synthesis of Intermediate Compound F1 and F2



Scheme S1. Synthetic route of intermediate compound F1 and F2

N1-(quinazolin-4-yl) benzene-1,3-diamine (F1)

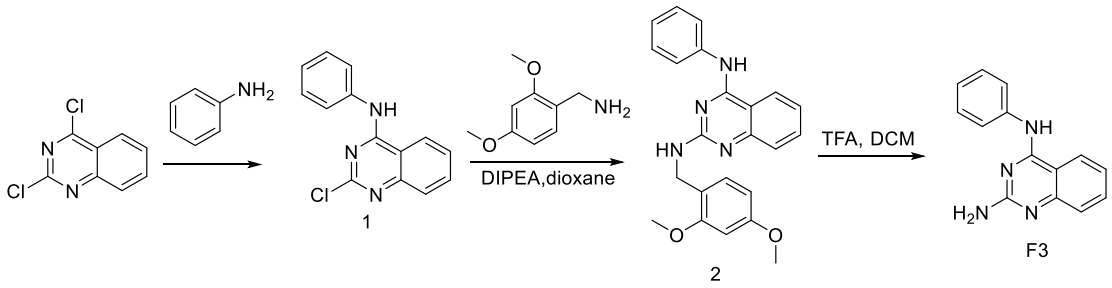
To a solution of compound 1 (2 g, 12.1 mmol) in DCM (10 mL) was added compound

2 (1.96 g, 18.2 mmol) and stirred for sixteen hours at room temperature. The precipitant was collected by vacuum filtration and washed with DCM (25 mL) to give F1 (0.6 g, 21%) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d6) δ ppm 9.55 (s, 1H), 8.54 (s, 1H), 8.51 (s, 1H), 7.84 - 7.75 (m, 3H), 7.10 - 6.93 (m, 3H), 6.38 (d, J = 7.6 Hz, 1H), 5.07 (bs, 2H).

N1-(quinazolin-4-yl) benzene-1,4-diamine (F2)

To a solution of 4-chloroquinazoline (1 g, 6.07 mmol) in DCM (10 mL) was added benzene-1,4-diamine (984 mg, 9.1 mmol) and stirred for sixteen hours at room temperature. The precipitant was collected by vacuum filtration and washed with DCM (25 mL) to give F2 (0.5 g, 35%) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d6) δ ppm 9.54 (s, 1H), 8.47 (s, 1H), 8.45 (s, 1H), 7.82 - 7.57 (m, 3H), 7.36 (d, J = 8.4Hz, 2H), 6.61 (d, J = 8.4 Hz, 2H), 4.99 (bs, 2H).

b. Synthesis of Intermediate Compound F3



Scheme S2. Synthetic route of intermediate compound F3

2-chloro-N-phenylquinazolin-4-amine (compound 1)

A mixture of 2,4-dichloroquinazoline (5 g, 25.12 mmol), aniline (2.57 g, 27.6 mmol) and sodium acetate (2.06 g, 25.12 mmol) in THF (37.5 mL) and water (12.5 mL), was stirred at room temperature for 2 h. When the reaction was observed to be finished by TLC, the solution was diluted with ethyl acetate (30 mL). The layers were separated and was washed three times with brine (10 mL) and dried over Na_2SO_4. The crude was then purified by recrystallized with ethanol and water, filtered, and rinsed with cold ethanol to yield the title compound (7.3 g, crude) as a brown solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85-7.66 (m, 5H), 7.64-7.54 (m, 1H), 7.49 (ddd, J = 8.3,

6.8, 1.4 Hz, 1H), 7.39-7.33 (m, 2H), 7.16-7.11 (m, 1H).

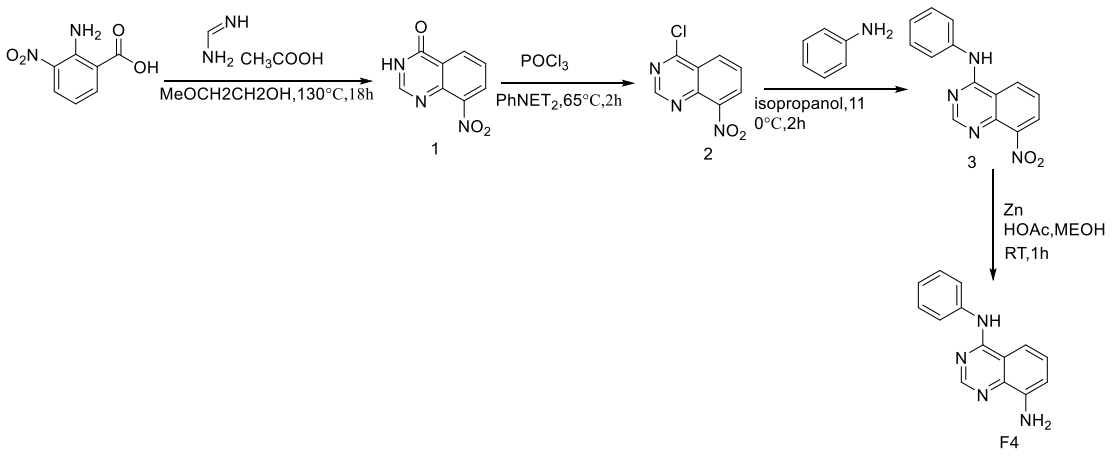
N2-(2,4-dimethoxybenzyl)-N4-phenylquinazoline-2,4-diamine (compound 2)

A mixture of compound 1 (4.5 g, 17.6 mmol), (2,4-dimethoxyphenyl) methylamine (4.4 g, 26.4 mmol) and DIPEA (4.5 g, 35.2 mmol) in dioxane (60 mL) was stirred at 90℃ for 16 h. After completion by LC/MS, the reaction mixture was concentrated and diluted with ethyl acetate (100 mL). The layers were separated and was washed three times with brine (50 mL), dried over Na_2SO_4 and concentrated to give the crude product (2.5 g, crude), which was no purification and directly to next step.

N4-phenylquinazoline-2,4-diamine (F3)

To a solution of compound 2 (2.5 g, crude) in DCM (20 mL) was added TFA (10 mL). The reaction mixture was then stirred at rt for 12 h. LCMS indicated completion. The reaction mixture was concentrated and diluted with EA (50 mL) and washed with $NaHCO_3$ aq. (50 mL) and brine (50 mL), dried over sodium sulfate and concentrated to give the crude product (1.2 g). Another batch (2.89 g, crude) to give the crude product (1.5 g). The combined crude product (2.7 g) was purified with Flash (C18 column, 5%-90% ACN in water, 40 min) to afford F3 (526 mg, 9% for three steps) as a light yellow solid. ^{1}H NMR (400 MHz, DMSO-d6): δ 9.34 (s, 1H), 8.29 (d, J = 8.1 Hz, 1H), 7.95 (d, J = 7.9 Hz, 2H), 7.56 (t, J = 7.4 Hz, 1H), 7.35 (t, J = 7.9 Hz, 2H), 7.28 (d, J = 8.3 Hz, 1H), 7.10 (dt, J = 23.4, 7.5 Hz, 2H), 6.28 (s, 2H) ppm.

c. Synthesis of Intermediate Compound F4



Scheme S3. Synthetic route of intermediate compound F4

8-nitro-3l4,4l5-quinazolin-4-one (compound 1)

To a solution of 2-amino-3-nitrobenzoic acid (10 g, 55 mmol) in $MeOCH_2CH_2OH$ (200 mL), formimidamide acetate (50 g, 481 mmol) was added. The solution was stirred at 130℃for 18 h. LCMS indicated the reaction was completed, the reaction mixture was cooled to room temperature and concentrated under reduced pressure. The residue was diluted with EtOAc (100 mL) and saturated $NaHCO_3$ aq. (100 mL). phases were separated and the organic layer was dried over anhydrous Na_2SO_4. Filtered and concentrated to give a crude residue, which was purified by silica gel column chromatography (PE: EtOAc = 7: 3) to give compound 2 (3.3 g, 31% yield) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d6): δ 12.73 (s, 1H), 8.35 (dd, J = 8.0, 1.2 Hz, 1H), 8.29 (d, J = 7.8 Hz, 1H), 8.24 (s, 1H), 7.67 (t, J = 7.9 Hz, 1H) ppm.

4-chloro-8-nitroquinazoline (compound 2)

Compound 1 (3 g, 15.7 mmol) was dissolved in $POCl_3$ (24 g, 157 mmol). The mixture reaction was stirred at room temperature for 10 mins. Then $PhNEt_2$ (3.5 g, 23.5 mmol) was added dropwise. The reaction mixture was stirred at 65℃for 2 hours. When the reaction was completed by TLC, the reaction mixture was concentrated to give a crude residue (6 g, 100%), which was used directly to the next step without further purification.

8-nitro-N-phenylquinazolin-4-amine (compound 3)

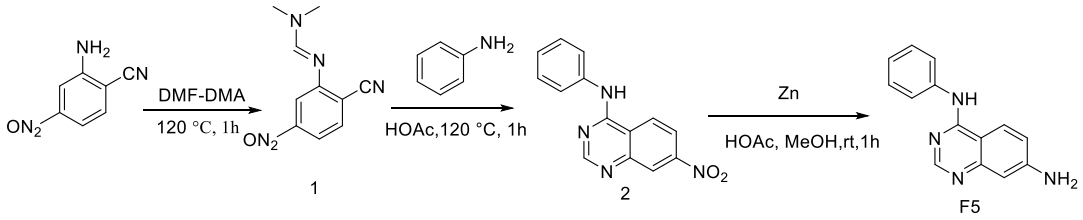
A solution of compound 3 (6 g, crude) in isopropanol (20 mL) was stirred at room temperature for 10 mins. Then aniline (1.5 g, 15.7 mmol) was added dropwise. After the addition, the reaction mixture was stirred at 110℃for 2 h. The reaction mixture was cooled to room temperature and filtered to give compound 4 (3 g, 72 %) as a yellow oil.^{1}H NMR (400 MHz, DMSO-d6): δ 10.95 (s, 1H), 9.06 (d, J = 8.4 Hz, 1H), 8.70 (s, 1H), 8.51 (d, J = 7.2 Hz, 1H), 7.87-7.79 (m, J = 7.2 Hz 3H), 7.51-7.41 (m, 2H), 7.26 (t, J = 7.2 Hz, 1H) ppm.

N4-phenylquinazoline-4,8-diamine (F4)

To a solution of compound 3 (1.7 g, 6.4 mmol) in MeOH (17 mL) and AcOH (17 mL), was added Zn powder (2 g, 31.9 mmol) in portions. The solution was stirred at room temperature for 1 h. LC/MS indicated the reaction was completed. The reaction mixture was filtered through a pad of celite and the filtrate was evaporated. The

residue was diluted with EtOAc (100 mL) and washed with saturated $NaHCO_3$ aqueous solution (100 mL) and extracted by EtOAc (2 x 100 mL). The combined organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated to give a crude residue, which was purified by silica gel column chromatography (PE: EtOAc = 1:1) to give the title compound (500 mg, 33% yield) as a yellow solid.^{1}H NMR (400 MHz, DMSO-d6): δ 9.49 (s, 1H), 8.49 (s, 1H), 7.85 (d, J = 7.6 Hz, 2H), 7.61 (d, J = 8.4Hz, 1H), 7.38 (t, J = 7.6 Hz, 2H), 7.30 (t, J = 8.0 Hz, 1H), 7.11 (t, J = 7.6 Hz, 1H), 6.96 (d, J = 7.6 Hz, 1H), 5.84 (s, 2H) ppm.

d. Synthesis of Intermediate Compound F5



Scheme S4. Synthetic route of intermediate compound F5

(E)-N'-(2-cyano-5-nitrophenyl)-N, N-dimethylformimidamide (compound 1)

A solution of 2-amino-4-nitrobenzonitrile (5 g, 30.7 mmol) in DMF-DMA (10 mL) was heated to 120℃and stirred for 1 h. LC/MS indicated the reaction was completed, the reaction mixture was cooled to room temperature and filtered to afford compound 2 (6 g, yield 90%) as yellow solid. ^{1}H NMR (400 MHz, DMSO-d6): δ 8.24 (s, 1H), 8.04 (d, J = 2.1 Hz, 1H), 7.89 (d, J = 8.5 Hz, 1H), 7.73 (dd, J = 8.5, 2.1 Hz, 1H), 3.14 (s, 3H), 3.05 (s, 3H) ppm.

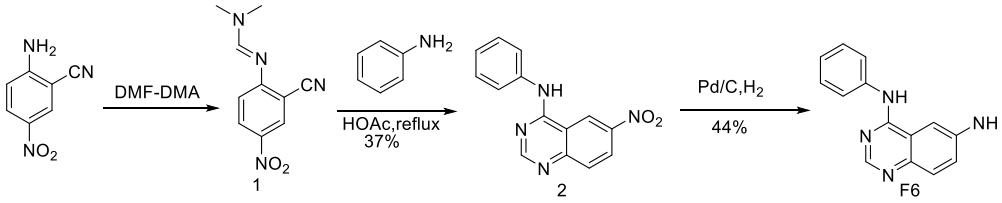
7-nitro-N-phenylquinazolin-4-amine (compound 2)

To a solution of compound 1 (3 g, 13.6 mmol) in HOAc (15 mL) was added aniline (3 g, 32.3 mmol) dropwise. The mixture was stirred at 120℃for 1 h. LC/MS indicated the reaction was completed, the reaction mixture was cooled to room temperature and filtered to afford the compound 3 (3 g, 81% yield) as a brown solid. ^{1}H NMR (400 MHz, DMSO-d6): δ 10.19 (s, 1H), 8.83 (d, J = 9.0 Hz, 1H), 8.71 (s, 1H), 8.50 (s, 1H), 8.37 (d, J = 8.5 Hz, 1H), 7.85 (d, J = 7.6 Hz, 2H), 7.44 (t, J = 7.3 Hz, 2H), 7.20 (t, J = 7.0 Hz, 1H) ppm.

N4-phenylquinazoline-4,7-diamine (F5)

To a solution of compound 2 (3 g, 11.3 mmol) in MeOH (30 mL) and AcOH (30 mL) was added Zn (3.6 g, 56.4mmol) in portions. The solution was stirred at room temperature for 1 h. LC/MS indicated the reaction was completed. The reaction mixture was filtered through a pad of Celite and the filtrate was evaporated. The residue was diluted with EtOAc (100 mL) and washed with saturated $NaHCO_3$ aqueous solution (100 mL) and extracted by EtOAc (100 mL) twice more. The combined organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated to give a crude residue, which was purified by silica gel column chromatography (PE: EtOAc = 1:1) to give the title compound (500 mg, 19% yield) as a yellow solid.^{1}H NMR (400 MHz, DMSO-d6): δ 9.57-9.07 (m, 1H), 8.56-8.30 (m, 1H), 8.30-7.94 (m, 1H), 7.94-7.59 (m, 2H), 7.59-7.16 (m, 2H), 7.16-6.78 (m, 2H), 6.78-6.49 (m, 1H), 6.27-5.78 (m, 2H) ppm.

e. Synthesis of Intermediate Compound F6



Scheme S5. Synthetic route of intermediate compound F6

(E)-N'-(2-cyano-4-nitrophenyl)-N, N-dimethylformimidamide (compound 1)

To a solution of 2-amino-5-nitrobenzonitrile (5.0 g, 30.6 mmol) in dimethylformamide dimethyl acetal (5 mL, 33.7 mmol). The mixture was heated up to reflux for 1.5 h. The resulting mixture was cooled to room temperature and refrigerated overnight. The yellow precipitated was filtered, washed with ethyl ether to give compound 1 (4.0 g) which was used to next step without further purification.

6-nitro-N-phenylquinazolin-4-amine (compound 2)

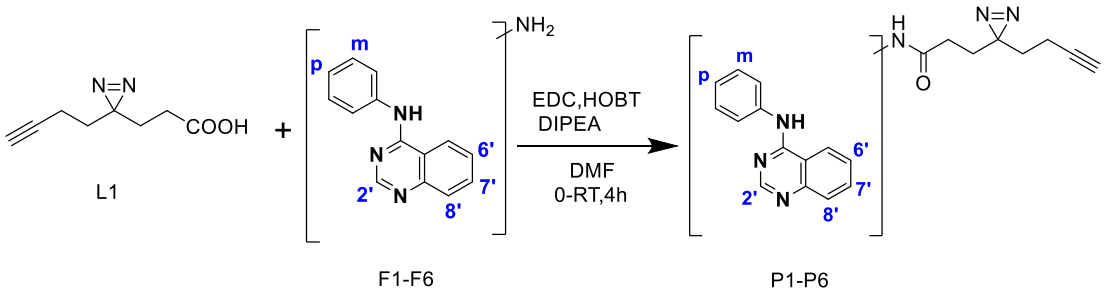
To a solution of compound 1 (4.0 g, 18.3 mmol) in acetic acid (50 mL) was added aniline (1.7 g, 18.3 mmol). The mixture was heated under reflux for 3 h. After completion of the reaction, the resulting mixture was cooled to room temperature. The solid separated was filtered and washed with ether to give compound 2(1.8 g,

37%). ^{1}H NMR (400 MHz, DMSO-d6) δ ppm 10.45(s, 1H), 9.67(s, 1H), 8.71(s, 1H), 8.56 (d, J=9.2 Hz, 1H), 7.94 (d, J=9.2 Hz, 1H), 7.84 (d, J=7.6 Hz, 2H), 7.44 (t, J=8.0 Hz, 2H), 7.21 (t, J=8.0 Hz, 1H).

6-(l2-azanyl)-N-phenylquinazolin-4-amine (F6)

To a solution of compound 2 (1.8 g, 6.8 mmol) in methanol (30 mL) was added palladium on carbon (10%, 0.2 g). The reaction was stirred under 1 bar hydrogen atmosphere for 18 hours. The resulting mixture was filtered through Celite and the filtrate concentrated in vacuum to provide the crude product. Purification of the residue by column chromatography, eluting with a gradient of 5 % of MeOH in DCM, provided F6 (0.7 g, 44%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d6) δ ppm 9.29(s, 1H), 8.28(s, 1H), 7.81(d, J=10.4 Hz, 2H), 7.50 (d, J=12.0 Hz, 1H), 7.35-7.29 (m, 3H), 7.21 (d, J=11.6 Hz, 1H), 7.04 (t, J=9.6 Hz, 1H), 5.54 (s, 2H).

f. Synthesis of Probe P1-P6

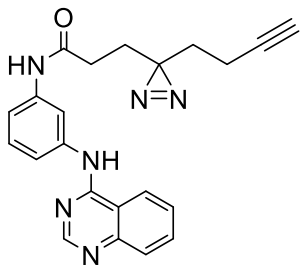


Scheme S6. Synthetic route of photoaffinity probes P1-P6[4].

General Procedure 1: synthesis of photoaffinity probes

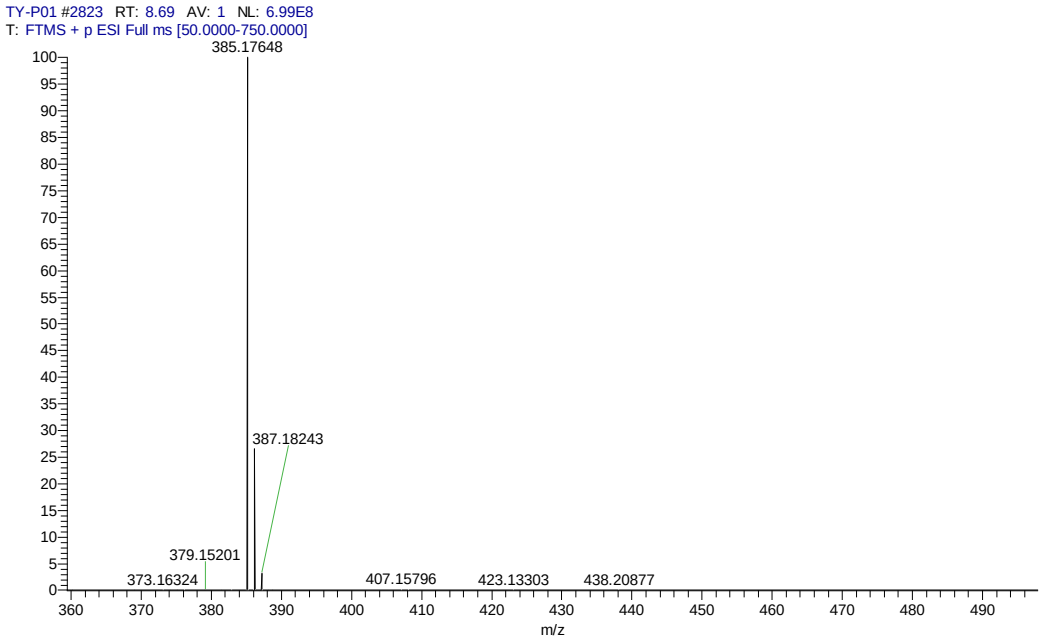
To a 5 mL vial containing 3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl) propanoic acid (L1, 25.2 mg, 0.15 mmol,1 eq) in DMF, HATU (85.74 mg, 0.225 mmol, 1.5 eq), DIPEA (77.54 mg, 0.6 mmol, 0.4 eq) and amine (20.9mg, 0.225 mmol, 1.5 eq). Reaction mixture was stirred at room temperature for 4 h to overnight until TLC indicated the reaction was completed, then 2 mL of H_2O was added to quench the reaction and extracted by ethyl acetate (3 x 5 mL). The combined organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated to give a crude residue, which was purified by SiO_2 flash column chromatography.

3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(3-(quinazolin-4-ylamino) phenyl) propenamide (P1)

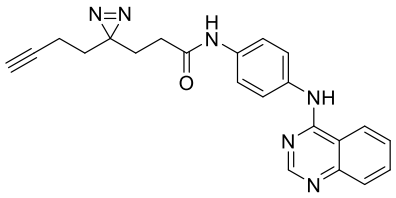


P1

General Procedure 1. Purified by SiO_2 flash chromatography (Hexane/EtOAc, 3:1→1:1) to afford **P1** as a white solid (20 mg, 55%); ^{1}H NMR (500 MHz, DMSO-d6) δ 9.99 (s, 1H), 9.82 (s, 1H), 8.58 (d, J = 7.5 Hz, 2H), 8.14 (d, J = 2.0 Hz, 1H), 7.90 – 7.83 (m, 1H), 7.79 (d, J = 7.5 Hz, 1H), 7.64 (t, J = 7.0 Hz, 1H), 7.54 (d, J = 8.5 Hz, 1H), 7.36 (d, J = 8.5 Hz, 1H), 7.30 (t, J = 8.0 Hz, 1H), 2.86 – 2.81 (m, 1H), 2.21 – 2.10 (m, 2H), 2.07 – 1.97 (m, 2H), 1.78 (t, J = 8.0 Hz, 2H), 1.62 (t, J = 7.5 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d6) δ 170.21, 158.33, 154.94, 150.18, 139.87, 139.76, 133.50, 129.02, 128.26, 126.72, 123.57, 118.09, 115.66, 115.26, 113.93, 83.68, 72.27, 32.02, 30.97, 28.76, 28.27, 13.17. ESI-HRMS (m/z): calcd. for $C_{22}H_{20}N_6O$ $[M+H]^+$ 385.17768; found 385.17648. Purity 96.7% (HPLC).

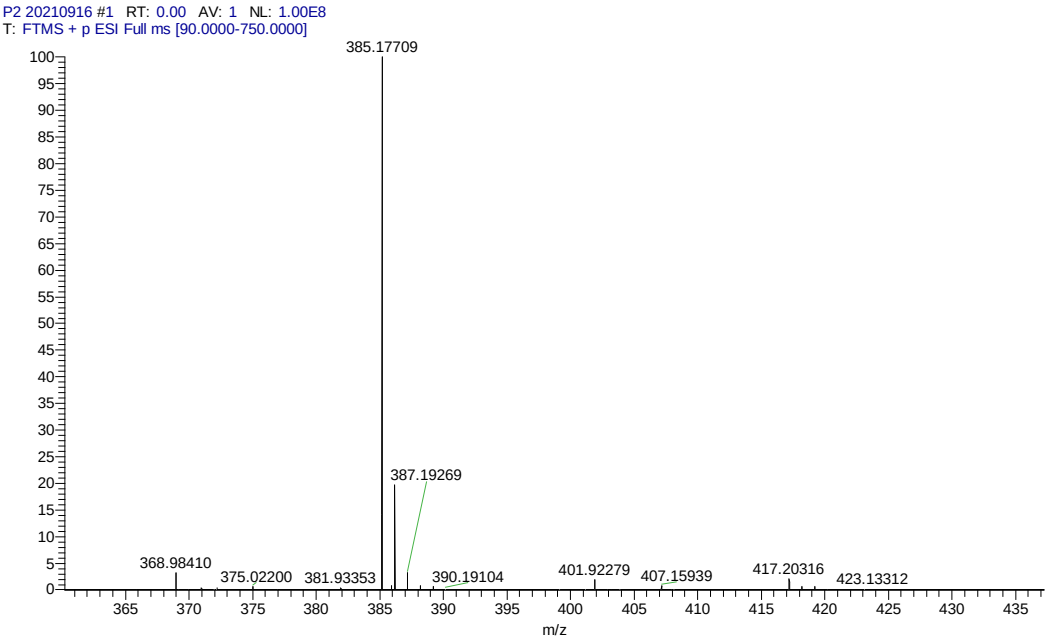


3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(4-(quinazolin-4-ylamino) phenyl) propenamide (P2)

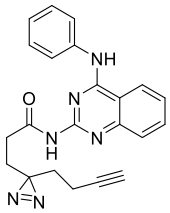


P2

General Procedure 1. Purified by SiO_2 flash chromatography (Hexane/EtOAc, 3:1→1:1) to afford **P2** as a white solid (30 mg, 63%). ^{1}H NMR (500 MHz, DMSO-d6) δ 9.95 (s, 1H), 9.76 (s, 1H), 8.54 (d, J = 7.0 Hz, 2H), 7.88 – 7.81 (m, 1H), 7.80 – 7.73 (m, 3H), 7.66 – 7.57 (m, 3H), 2.85 (t, J = 3.0 Hz, 1H), 2.19 – 2.11 (m, 2H), 2.04 (td, J = 7.5, 3.0 Hz, 2H), 1.81 – 1.74 (m, 2H), 1.63 (t, J = 7.5 Hz, 2H). ^{13}C NMR (125 MHz, DMSO-d6) δ 155.1, 150.1, 128.2, 126.6, 123.5, 123.4, 119.7, 83.7, 72.3, 32.0, 30.9, 28.8, 28.3, 13.2. ESI-HRMS (m/z): calcd. for $C_{22}H_{20}N_6O$ $[M+H]^+$ 385.17768; found 385.17709. Purity 94.5% (HPLC).

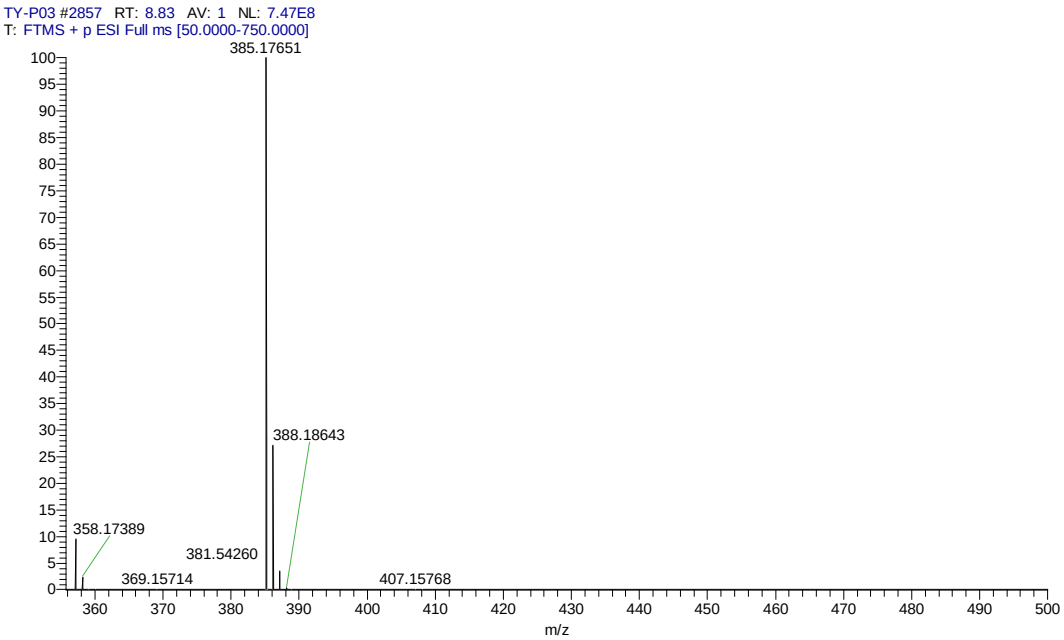


3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(4-(phenylamino) quinazolin-2-yl) propenamide (P3)

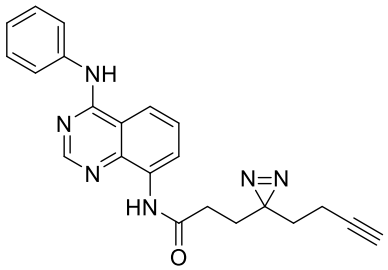


P3

General Procedure 1. Purified by SiO_2 flash chromatography (Hexane/EtOAc, 5:1→3:1) to afford **P3** as a light-yellow solid (15 mg, 58%). ^{1}H NMR (500 MHz, DMSO-d6) δ 10.23 (s, 1H), 9.73 (s, 1H), 8.53 (d, J = 8.0 Hz, 1H), 8.16 (d, J = 7.5 Hz, 2H), 7.81 – 7.74 (m, 1H), 7.60 (d, J = 7.5 Hz, 1H), 7.50 – 7.44 (m, 1H), 7.38 (t, J = 7.5 Hz, 2H), 7.11 (t, J = 7.5 Hz, 1H), 2.83 (t, J = 3.0 Hz, 1H), 2.42 (t, J = 7.5 Hz, 2H), 2.03 (td, J = 7.5, 2.5 Hz, 2H), 1.75 (t, J = 7.5 Hz, 2H), 1.62 (t, J = 7.0 Hz, 2H). ^{13}C NMR (125 MHz, DMSO-d6) δ 158.9, 154.3, 151.2, 139.8, 133.7, 128.9, 127.0, 124.6, 123.9, 123.6, 122.2, 113.2, 83.7, 72.2, 32.1, 31.2, 28.8, 28.1, 13.2. ESI-HRMS (m/z): calcd. for $C_{22}H_{20}N_6O$ $[M+H]^+$ 385.17768; found 385.17651. Purity 95.9% (HPLC).

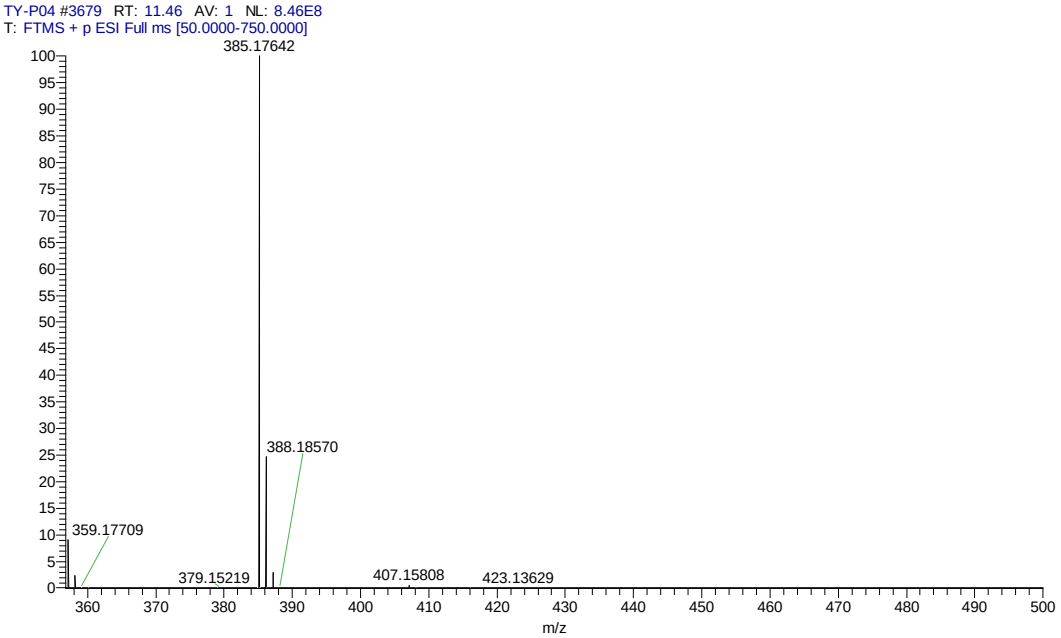


3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(4-(phenylamino) quinazolin-8-yl) propenamide(P4)

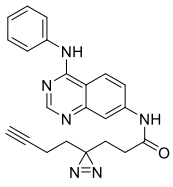


P4

General Procedure 1. Purified by SiO_2 flash chromatography (Hexane/EtOAc, 5:1→2:1) to afford **P4** as a brown solid (30 mg, 65%). ^{1}H NMR (500 MHz, DMSO-d6) δ 9.97 (s, 1H), 9.85 (s, 1H), 8.71 – 8.62 (m, 2H), 8.22 (d, J = 8.5 Hz, 1H), 7.86 (d, J = 8.0 Hz, 2H), 7.58 (t, J = 8.5 Hz, 1H), 7.41 (t, J = 7.0 Hz, 2H), 7.16 (t, J = 7.5 Hz, 1H), 2.86 – 2.80 (m, 1H), 2.46 (t, J = 7.5 Hz, 2H), 2.04 (td, J = 7.5, 2.5 Hz, 2H), 1.78 (t, J = 7.5 Hz, 2H), 1.65 (t, J = 7.5 Hz, 2H). ^{13}C NMR (125 MHz, DMSO-d6) δ 170.9, 158.3, 153.8, 140.6, 139.4, 134.5, 129.0, 126.6, 124.5, 123.2, 120.6, 117.0, 115.2, 83.7, 72.23, 60.2, 31.9, 31.2, 28.7, 28.5, 14.6, 13.2. ESI-HRMS (m/z): calcd. for $C_{22}H_{20}N_6O$ $[M+H]^+$ 385.17768; found 385.17642. Purity 99.1% (HPLC).

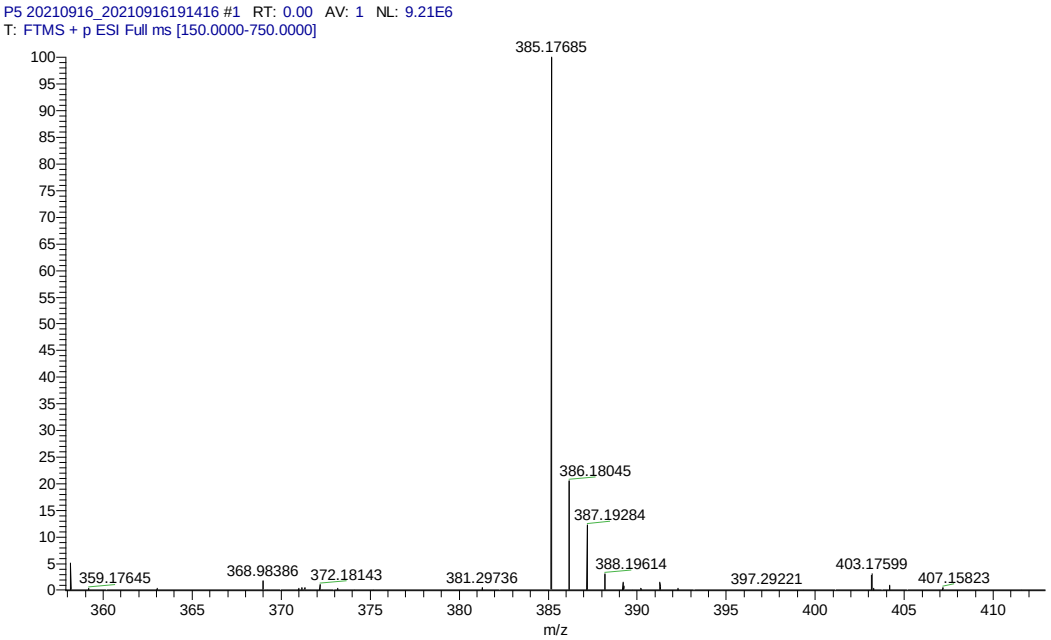


3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(4-(phenylamino) quinazolin-7-yl) propenamide (P5)

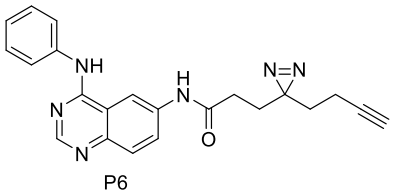


P5

General Procedure 1. Purified by SiO_2 flash chromatography (Hexane/EtOAc, 2:1→1:2) to afford **P5** as a white solid (26 mg, 63%). ^{1}H NMR (500 MHz, DMSO-d6) δ 10.36 (s, 1H), 9.67 (s, 1H), 8.58 – 8.37 (m, 2H), 8.10 (d, J = 2.2 Hz, 1H), 7.84 (d, J = 8.0 Hz, 2H), 7.70 (dd, J = 9.1, 2.2 Hz, 1H), 7.39 (t, J = 7.9 Hz, 2H), 7.12 (t, J = 7.4 Hz, 1H), 2.85 (t, J = 2.7 Hz, 1H), 2.24 (t, J = 7.6 Hz, 2H), 2.04 (td, J = 7.4, 2.7 Hz, 2H), 1.81 (t, J = 7.6 Hz, 2H), 1.63 (t, J = 7.4 Hz, 2H). ^{13}C NMR (125 MHz, DMSO-d6) δ 171.06, 157.79, 155.50, 143.40, 139.71, 128.94, 124.29, 124.07, 122.77, 119.14, 115.15, 111.41, 83.68, 72.28, 32.00, 31.13, 28.71, 28.05, 13.16. ESI-HRMS (m/z): calcd. for $C_{22}H_{20}N_6O$ $[M+H]^+$ 385.17768; found 385.17685. Purity 98.1% (HPLC).

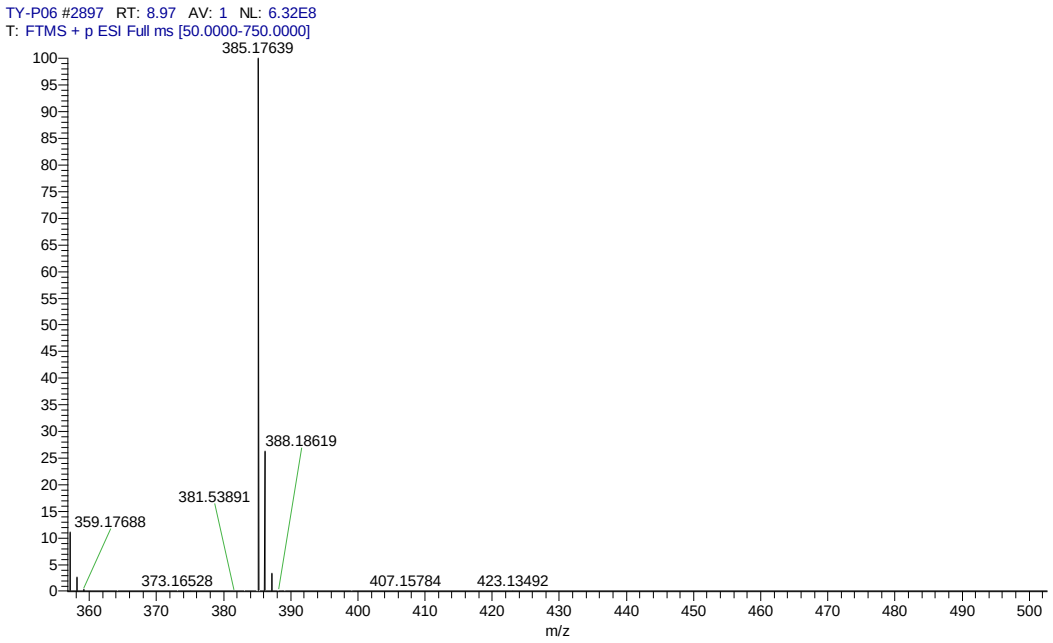


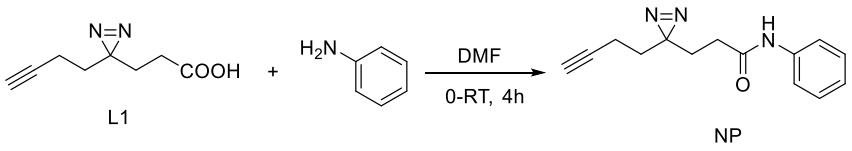
3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-(4-(phenylamino) quinazolin-6-yl) propenamide (P6)



P6

General Procedure 1. Purified by SiO_2 flash chromatography (Hexane/EtOAc, 2:1→1:2) to afford **P6** as a yellow solid (27mg, 65%). ^{1}H NMR (500 MHz, DMSO-d6) δ 10.26 (s, 1H), 9.80 (s, 1H), 8.65 (d, J = 2.5 Hz, 1H), 8.51 (s, 1H), 7.92 – 7.69 (m, 4H), 7.38 (t, J = 7.5 Hz, 2H), 7.12 (t, J = 7.5 Hz, 1H), 2.85 (t, J = 2.5 Hz, 1H), 2.24 (t, J = 7.5 Hz, 2H), 2.04 (td, J = 7.5, 2.5 Hz, 2H), 1.83 (t, J = 8.0 Hz, 2H), 1.65 (t, J = 7.0 Hz, 2H).^{13}C NMR (125 MHz, DMSO-d6) δ 170.4, 158.0, 153.7, 147.0, 139.9, 137.2, 128.9, 128.8, 127.4, 124.0, 123.0, 116.0, 112.4, 83.7, 72.3, 32.0, 30.8, 29.5, 28.8, 28.2, 13.2. ESI-HRMS (m/z): calcd. for $C_{22}H_{20}N_6O$ $[M+H]^+$ 385.17768; found 385.17639. Purity 94.4% (HPLC).





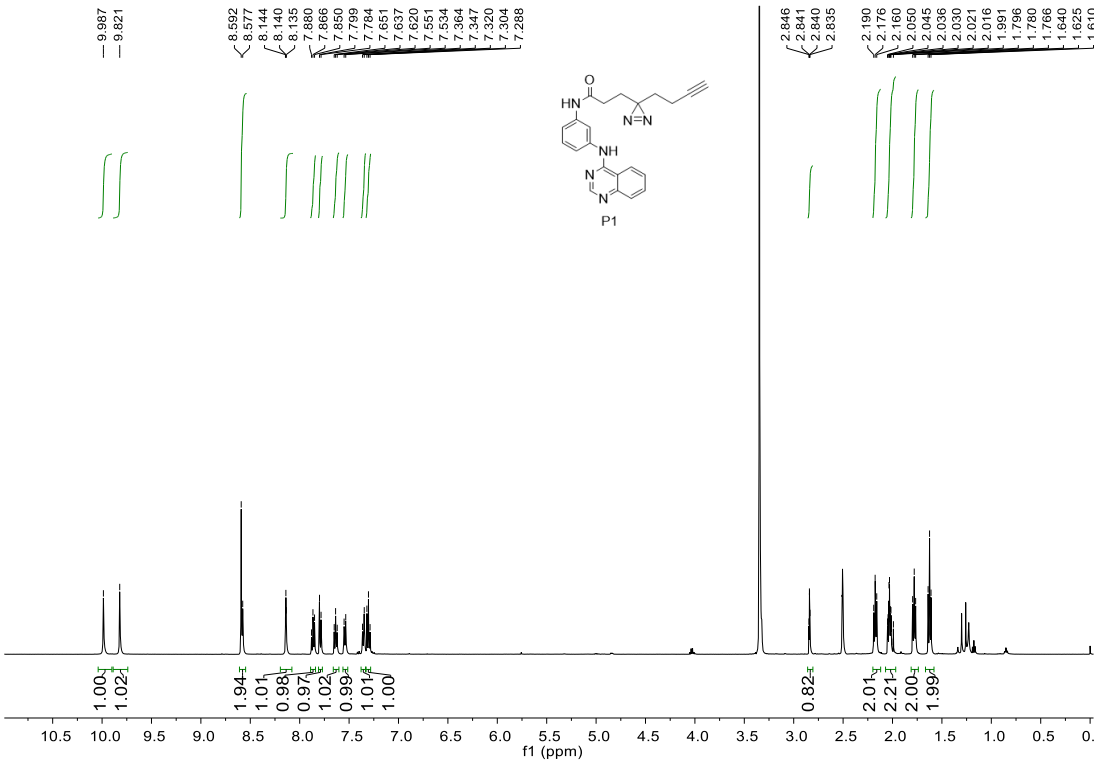
Scheme S7. Synthetic route of negative probe NP.

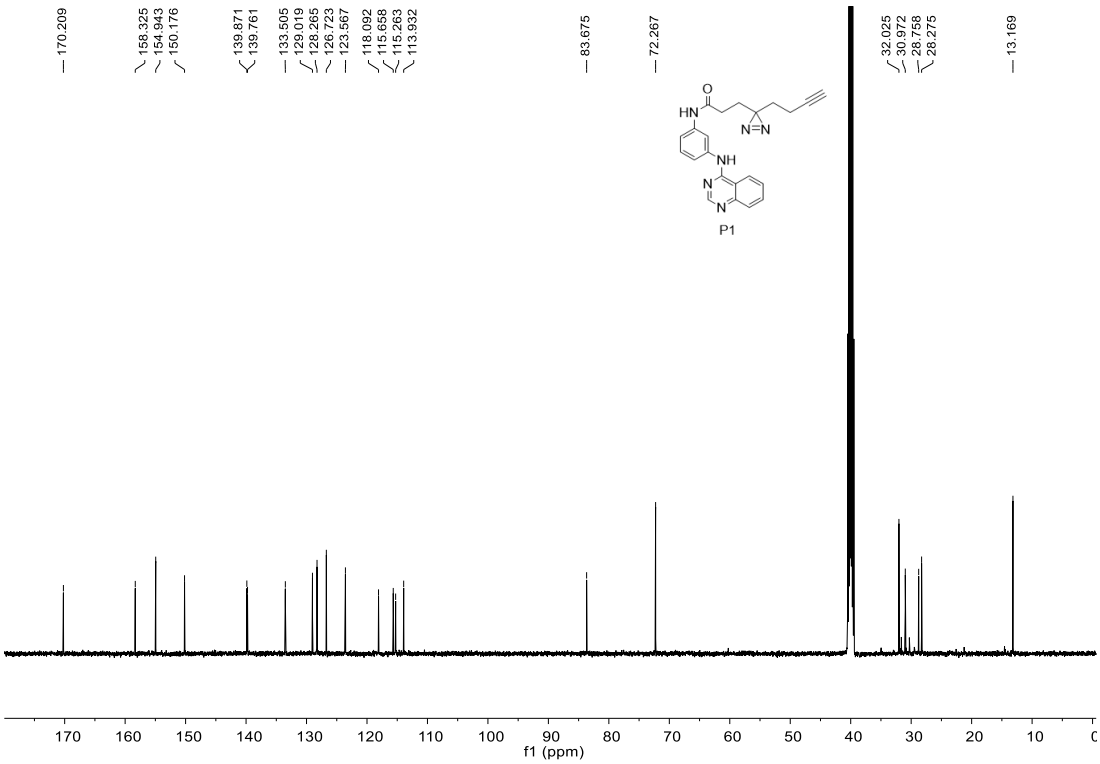
3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)-N-phenylpropanamide (NP)

General Procedure 1. Purified by SiO_2 flash chromatography (Hexane/EtOAc, 5:1→3:1) to afford **NP** as a colorless oil (12 mg, 48%). ^{1}H NMR (500 MHz, DMSO-d6) δ 9.90 (s, 1H), 7.56 (d, J = 8.0 Hz, 2H), 7.29 (t, J = 7.5 Hz, 2H), 7.03 (t, J = 7.5 Hz, 1H), 2.83 (t, J = 2.5 Hz, 1H), 2.16 – 2.11 (m, 2H), 2.02 (td, J = 7.0, 2.6 Hz, 2H), 1.79 – 1.73 (m, 2H), 1.61 (t, J = 7.5 Hz, 2H). 13C NMR (125 MHz, DMSO-d6) δ 170.1, 139.6, 129.2, 123.56, 119.5, 83.7, 72.3, 32.0, 31.0, 28.7, 28.3, 13.2. ESI-HRMS (m/z): calcd. for $C_{14}H_{15}N_3O$ $[M+H]^+$, 242.1293; found 242.1257. Purity 99.2% (HPLC).

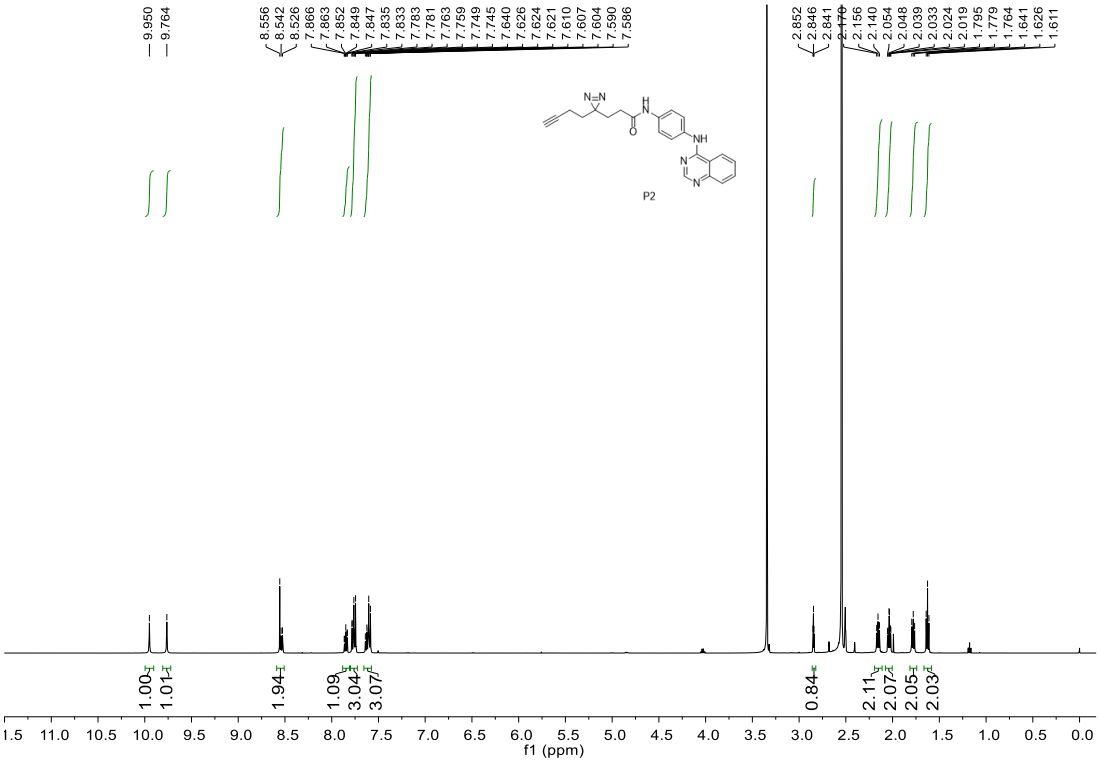
NMR Spectra

P1





P2


9.950
9.764
8.556
8.542
8.526
7.866
7.863
7.852
7.849
7.847
7.835
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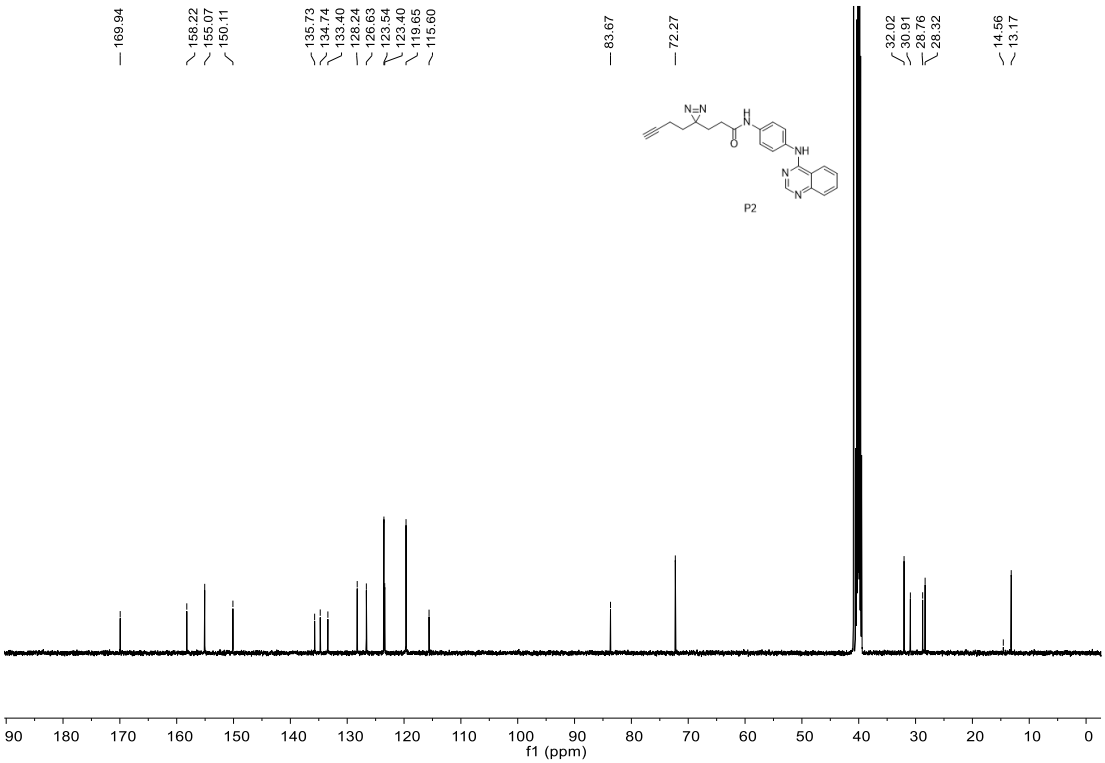
7.783
7.781
7.763
7.759
7.749
7.745
7.640
7.626
7.624
7.621
7.610
7.607
7.604
7.590
7.586
2.852
2.846
2.841
2.156
2.140
2.054
2.048
2.039
2.033
2.024
2.019
1.795
1.779
1.764
1.641
1.626
1.611
P2
1.00
1.01
1.94
1.09
3.04
3.07
0.84
2.11
2.07
2.05
2.03
f1 (ppm)


169.94
158.22
155.07
150.11
135.73
134.74
133.40
128.24
126.63
123.54
123.40
119.65
115.60
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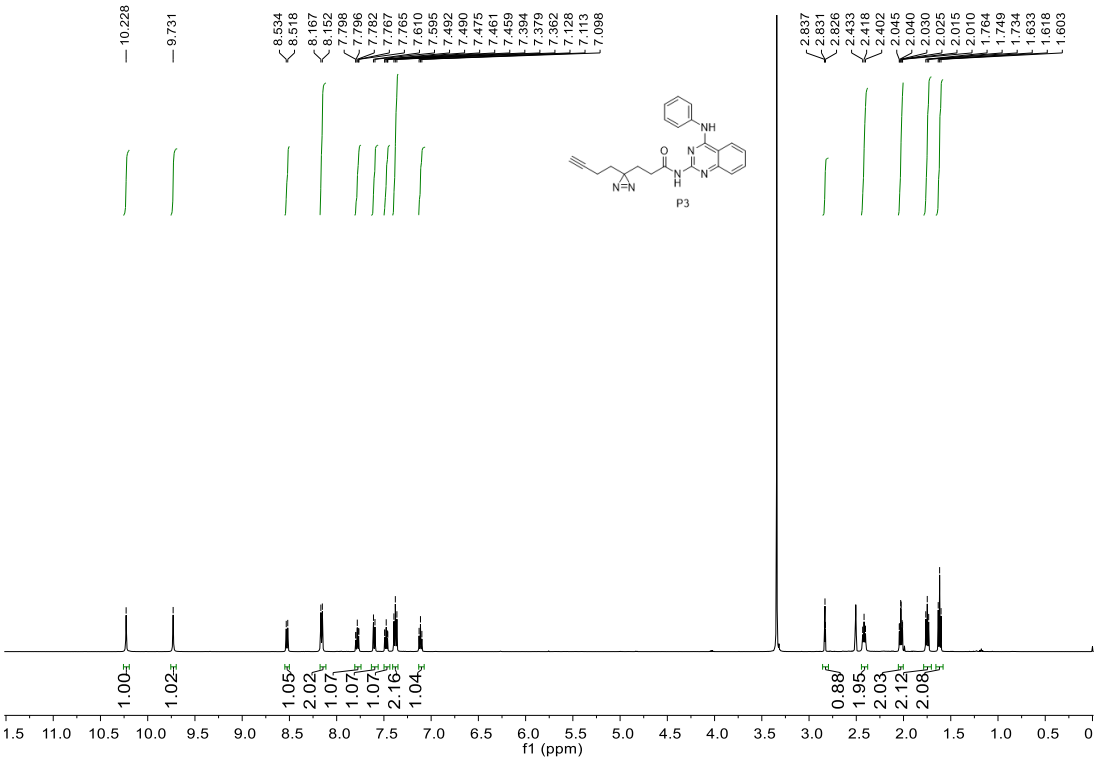
72.27
32.02
30.91
28.76
28.32
14.56
13.17
P2
f1 (ppm)

P3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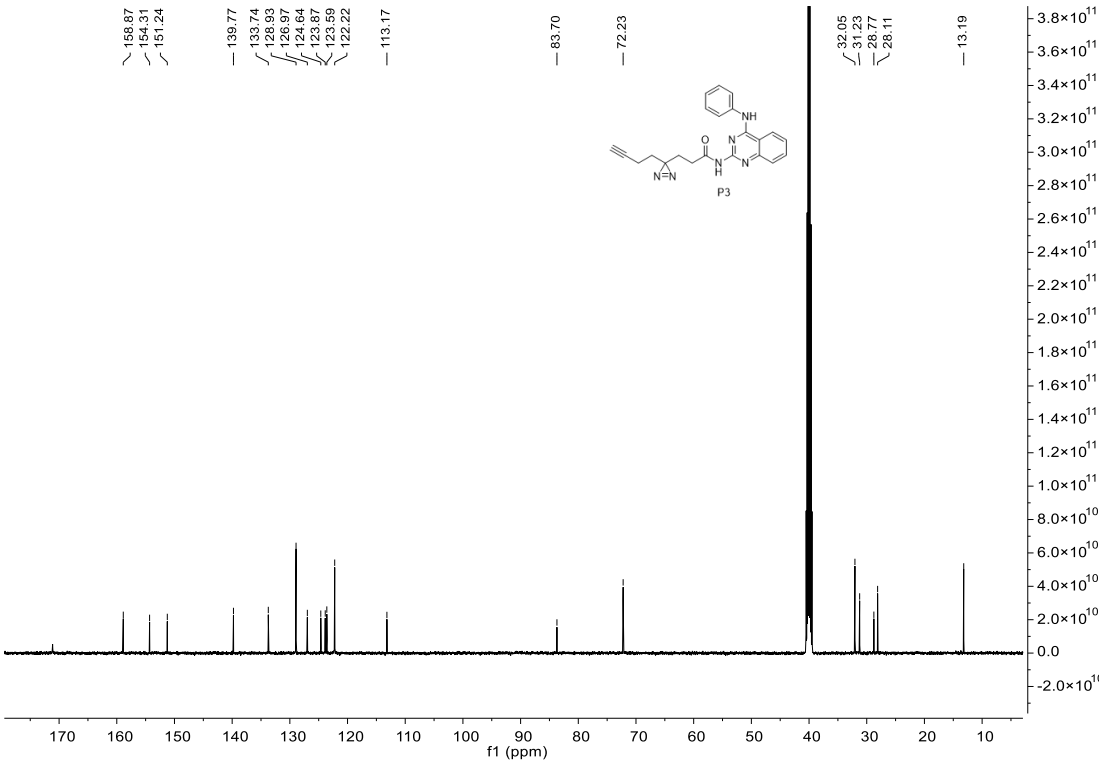
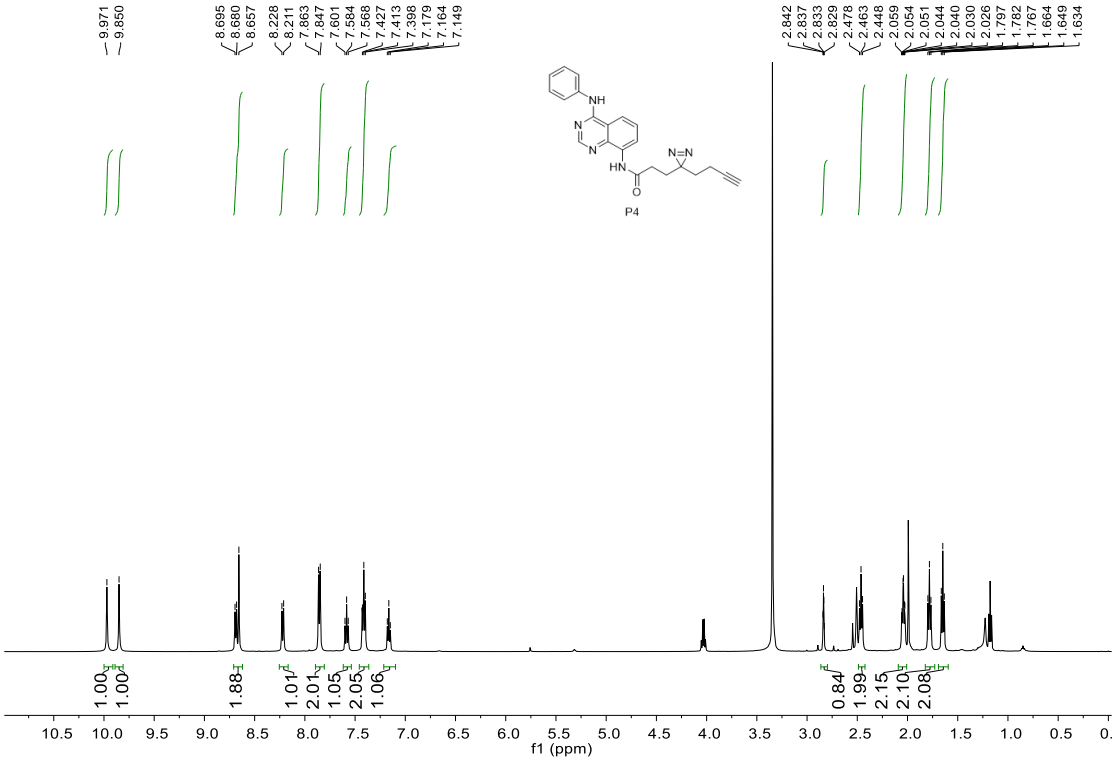
8.534
8.518
8.167
8.152
7.798
7.796
7.782
7.767
7.765
7.610
7.595
7.492
7.490
7.475
7.461
7.459
7.394
7.379
7.362
7.128
7.113
7.098
2.837
2.831
2.826
2.433
2.418
2.402
2.045
2.040
2.030
2.025
2.015
2.010
1.764
1.749
1.734
1.633
1.618
1.603
NH
N
N
O
N
H
N=N
P3
1.00
1.02
1.05
2.02
1.07
1.07
1.07
2.16
1.04
0.88
1.95
2.03
2.12
2.08
f1 (ppm)


158.87
154.31
151.24
139.77
133.74
128.93
126.97
124.64
123.87
123.59
122.22
113.17
83.70
72.23
32.05
31.23
28.77
28.11
13.19
NH
N
N
O
N
H
N=N
P3
f1 (ppm)

P4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9.850
8.695
8.680
8.657
8.228
8.211
7.863
7.847
7.601
7.584
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7.427
7.413
7.398
7.179
7.164
7.149
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2.837
2.833
2.829
2.478
2.463
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2.044
2.040
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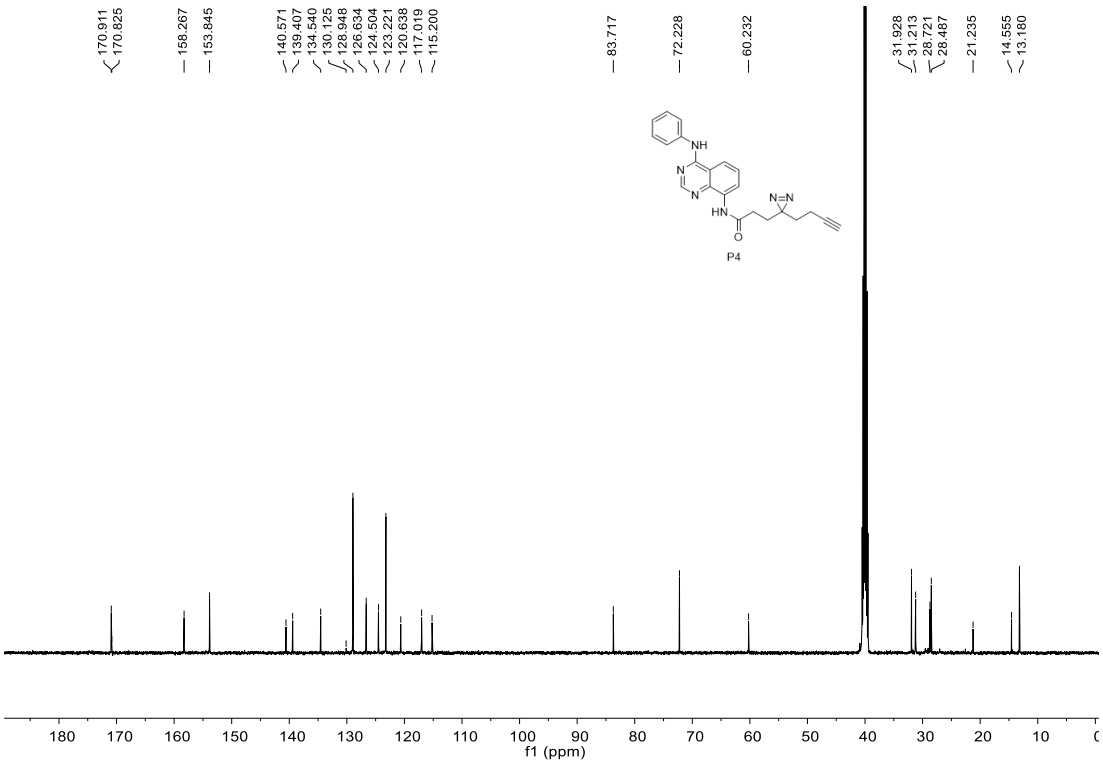
1.782
1.767
1.664
1.649
1.634
NH
N
N
HN
O
N=N
P4
1.00
1.00
1.88
1.01
2.01
1.05
2.05
1.06
0.84
1.99
2.15
2.10
2.08
f1 (ppm)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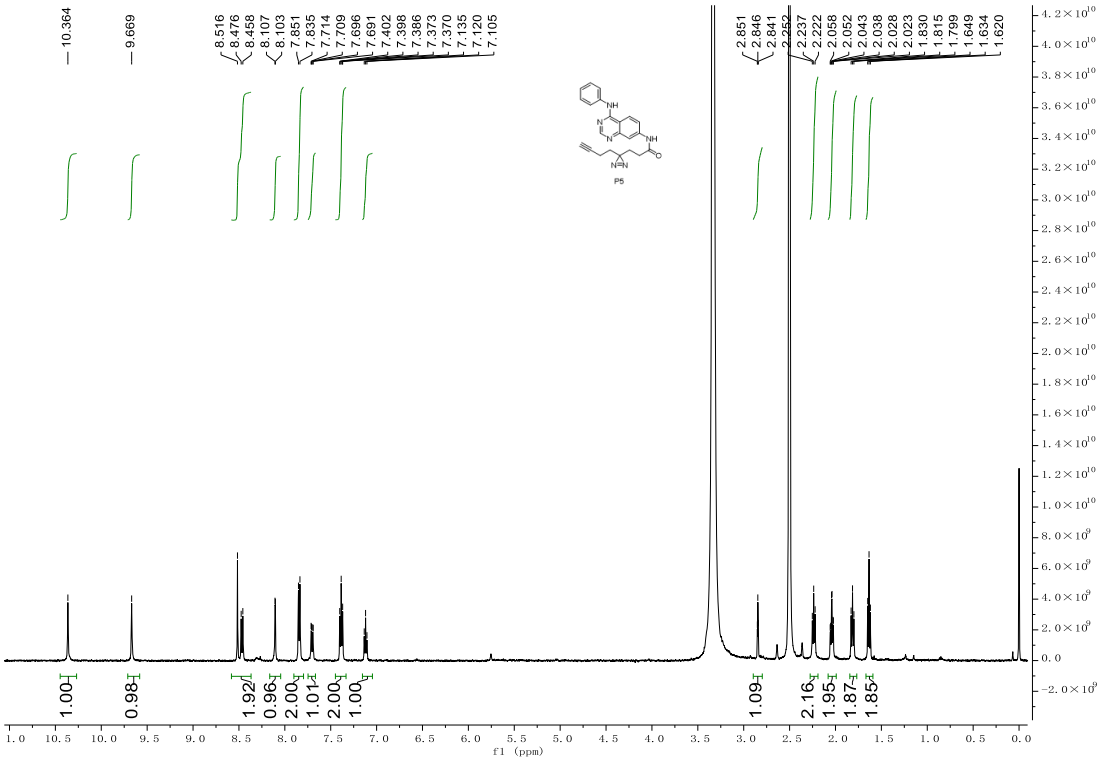
170.825
158.267
153.845
140.571
139.407
134.540
130.125
128.948
126.634
124.504
123.221
120.638
117.019
115.200
83.717
72.228
60.232
31.928
31.213
28.721
28.487
21.235
14.555
13.180
NH
N
N
HN
O
N=N
P4
f1 (ppm)

P5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9.669
8.516
8.476
8.458
8.107
8.103
7.851
7.835
7.714
7.709
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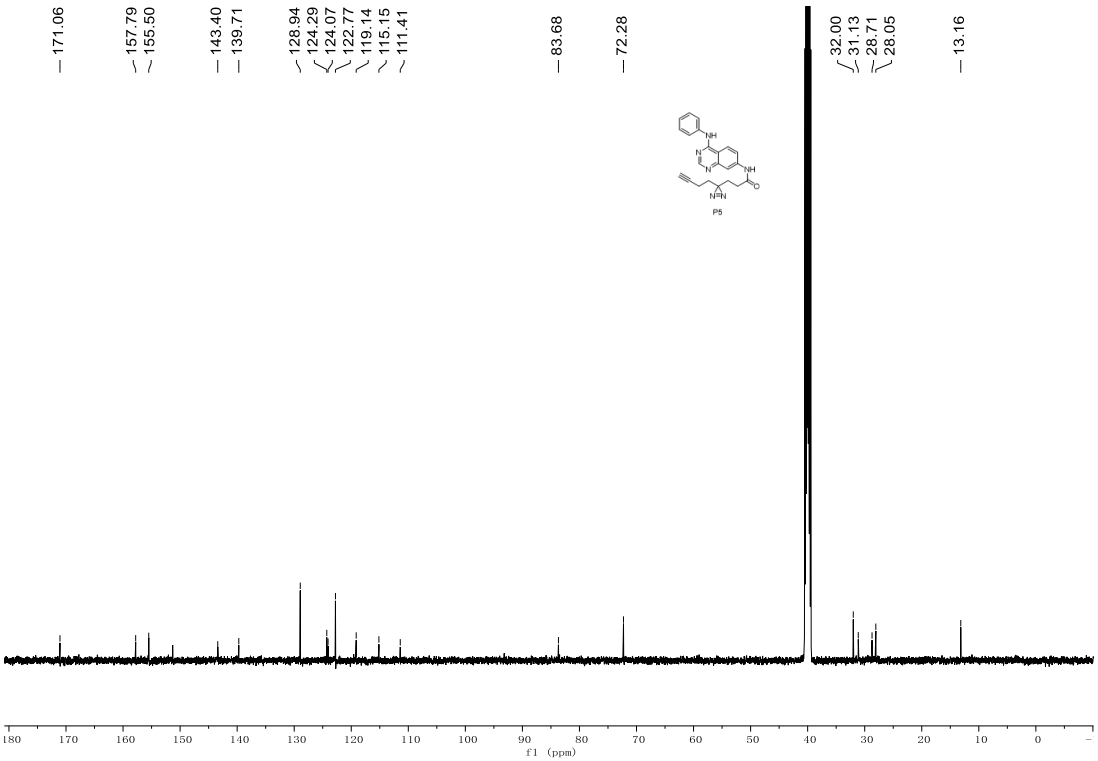
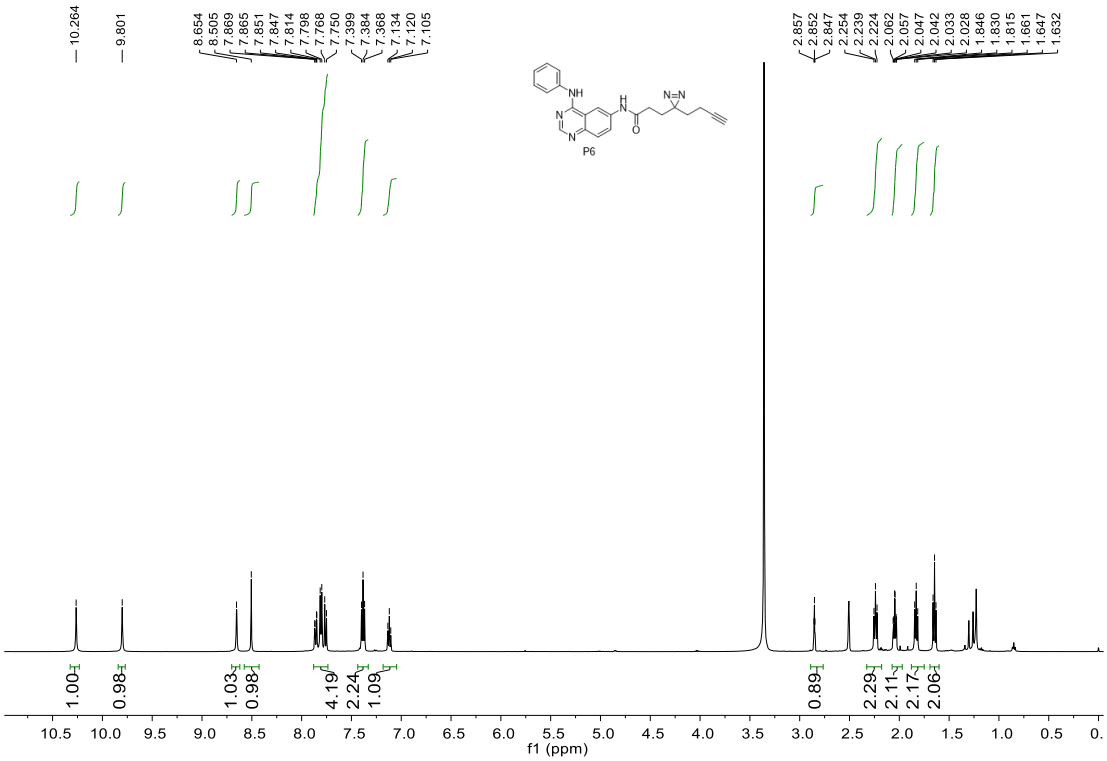
7.691
7.402
7.398
7.386
7.373
7.370
7.135
7.120
7.105
2.851
2.846
2.841
2.237
2.222
2.058
2.052
2.043
2.038
2.028
2.023
1.830
1.815
1.799
1.649
1.634
1.620
1.00
0.98
1.92
0.96
2.00
1.01
2.00
1.00
1.09
2.16
1.95
1.87
1.85
f1 (ppm)


171.06
157.79
155.50
143.40
139.71
128.94
124.29
124.07
122.77
119.14
115.15
111.41
83.68
72.28
32.00
31.13
28.71
28.05
13.16
f1 (ppm)

P6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8.505
7.869
7.865
7.851
7.847
7.814
7.798
7.768
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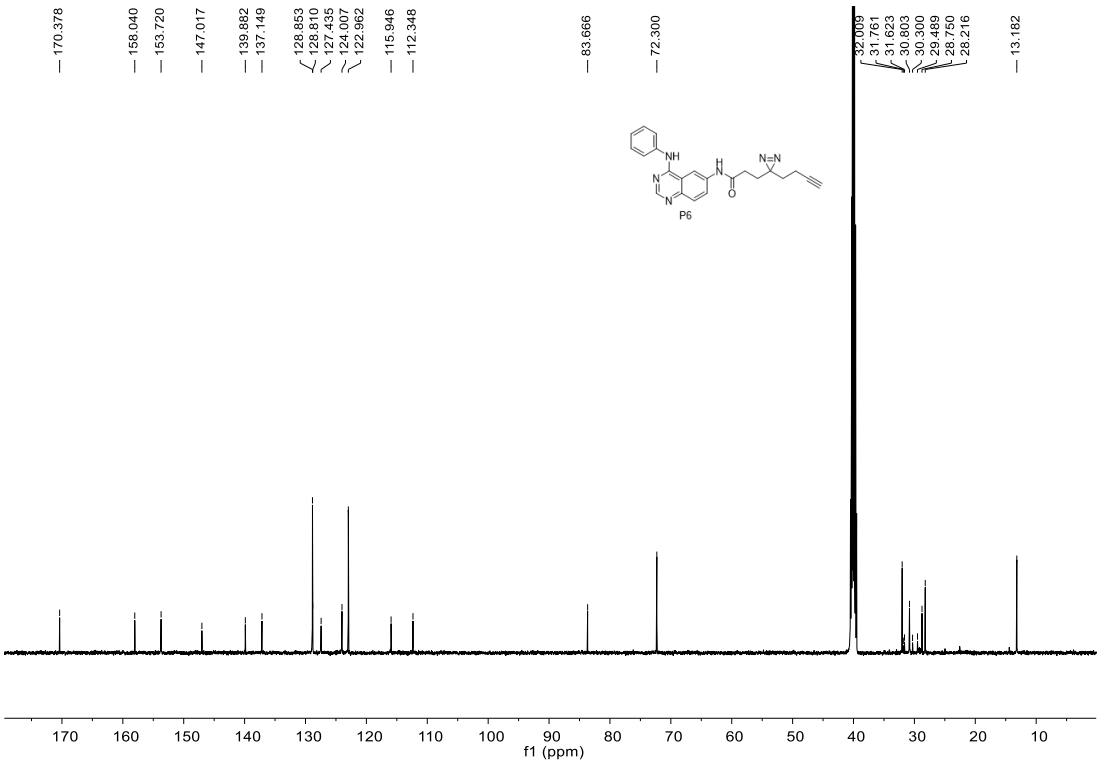
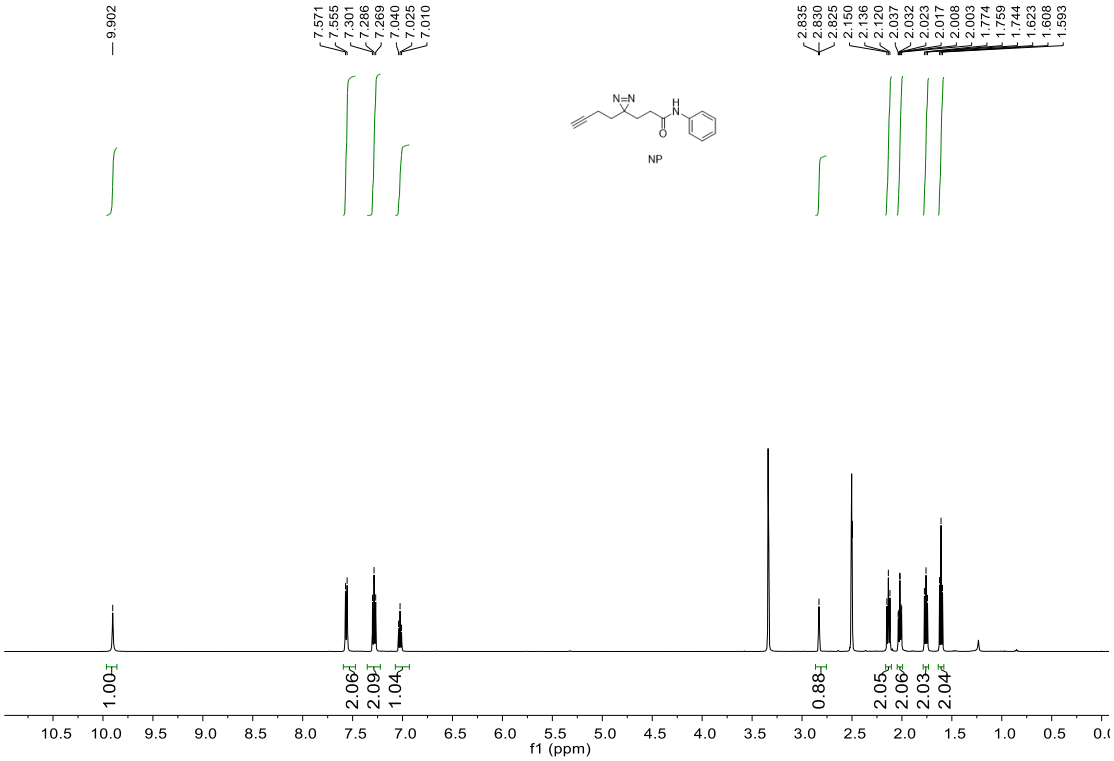
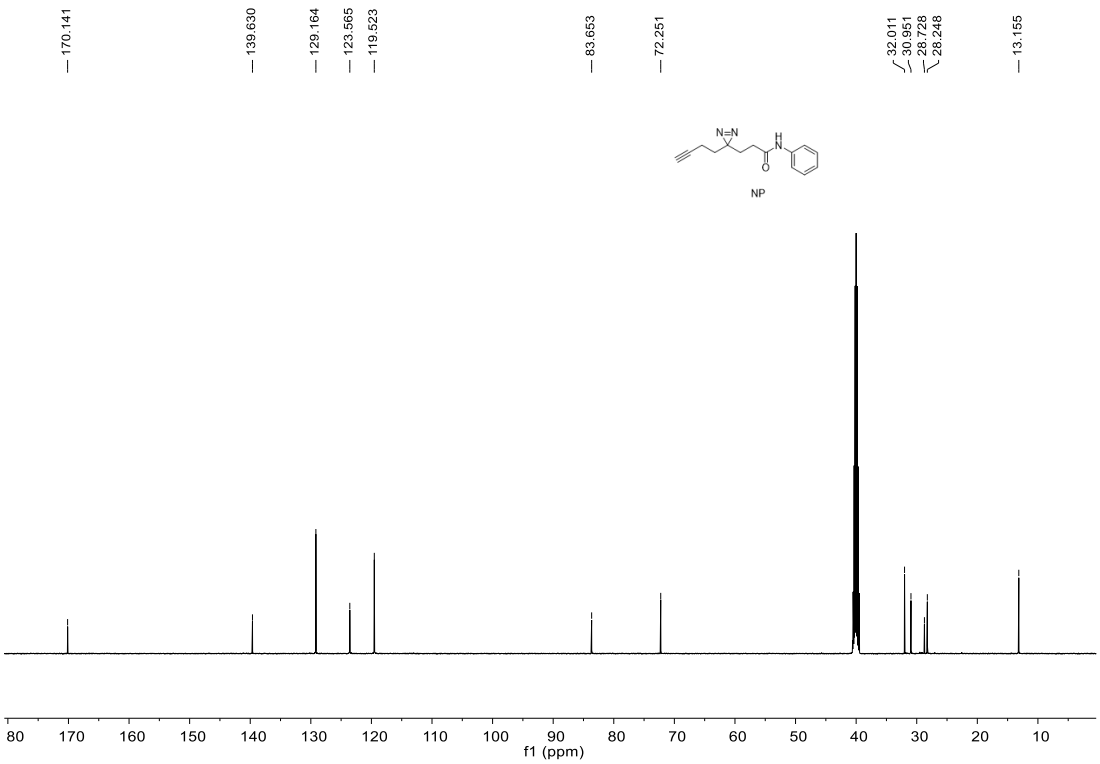
7.384
7.368
7.134
7.120
7.105
2.857
2.852
2.847
2.254
2.239
2.224
2.062
2.057
2.047
2.042
2.033
2.028
1.846
1.830
1.815
1.661
1.647
1.632
NH
N
N
H
N
O
N=N
P6
1.00
0.98
1.03
0.98
4.19
2.24
1.09
0.89
2.29
2.11
2.17
2.06
f1 (ppm)


170.378
158.040
153.720
147.017
139.882
137.149
128.853
128.810
127.435
124.007
122.962
115.946
112.348
83.666
72.300
32.009
31.761
31.623
30.803
30.300
29.489
28.750
28.216
13.182
NH
N
N
H
N
O
N=N
P6
f1 (ppm)

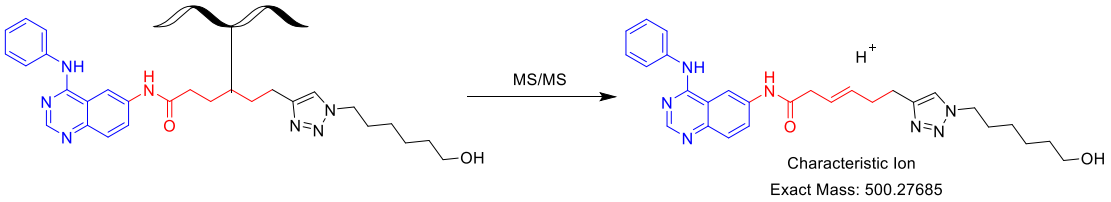
NP

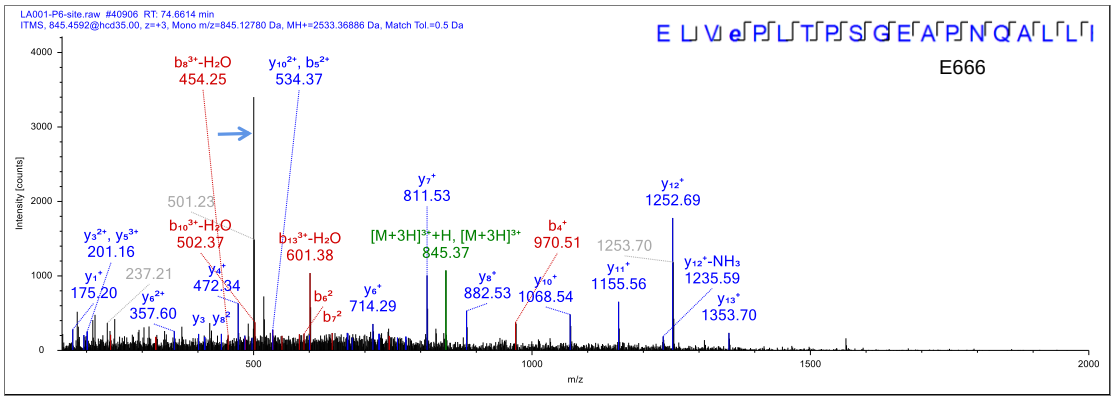

9.902
7.571
7.555
7.301
7.286
7.269
7.040
7.025
7.010
2.835
2.830
2.825
2.150
2.136
2.120
2.037
2.032
2.023
2.017
2.008
2.003
1.774
1.759
1.744
1.623
1.608
1.593
NP
1.00
2.06
2.09
1.04
0.88
2.05
2.06
2.03
2.04
f1 (ppm)

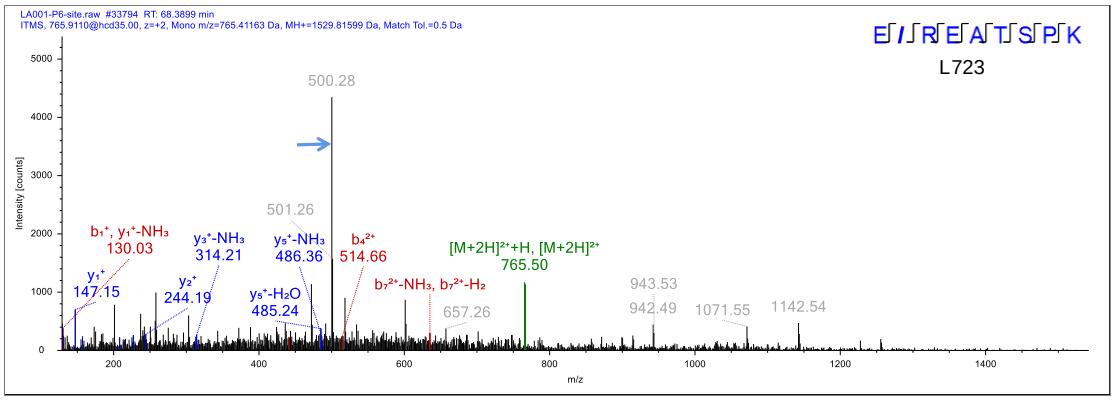

170.141
139.630
129.164
123.565
119.523
83.653
72.251
32.011
30.951
28.728
28.248
13.155
NP
f1 (ppm)

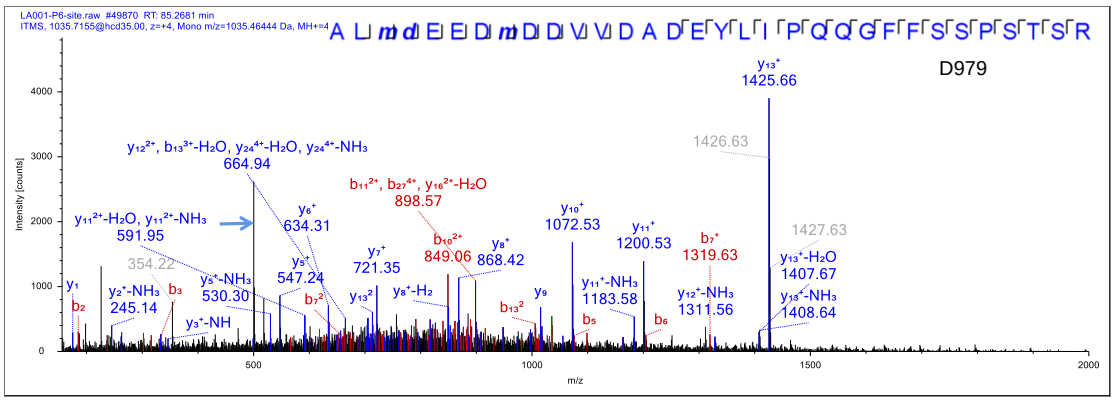
Binding site Spectra of P6-labeled peptides in EGFR

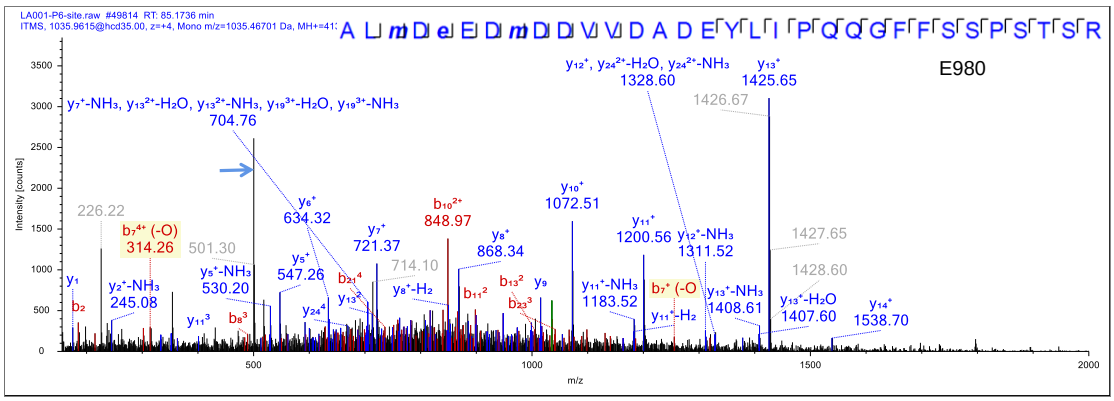
The characteristic ion corresponding to probe fragment was used to manually validate the probe-labeled peptides. The peak of characteristic ion (+1, 500.28) in each spectrum was indicated by a blue arrow.

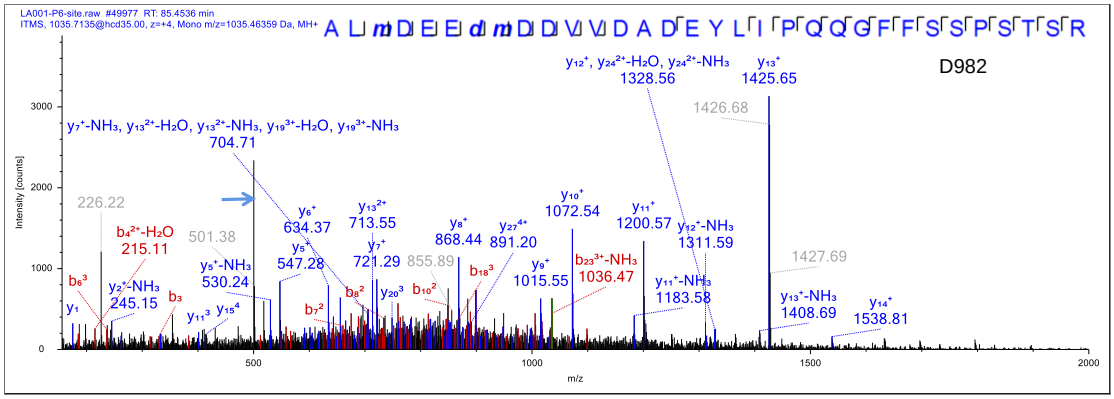


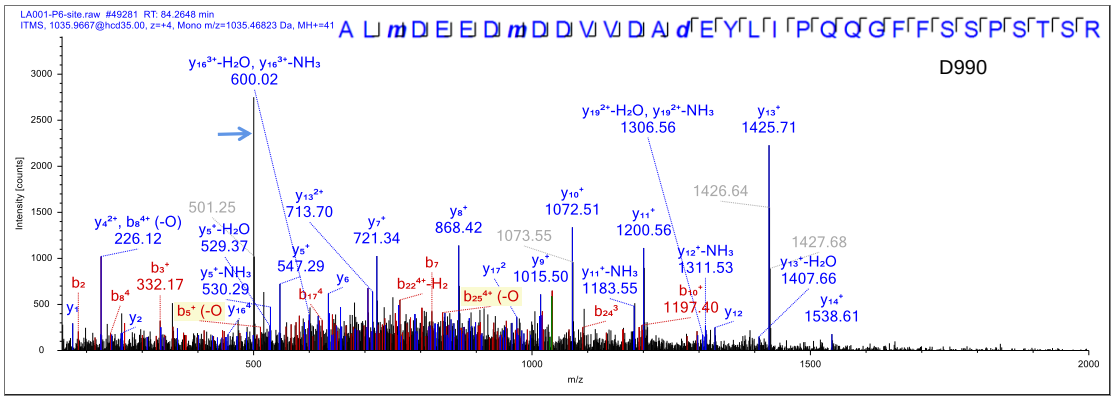


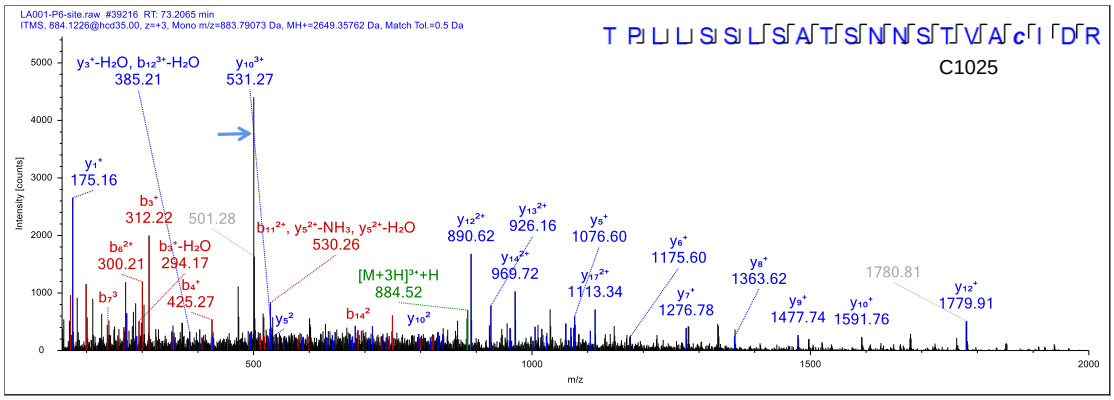


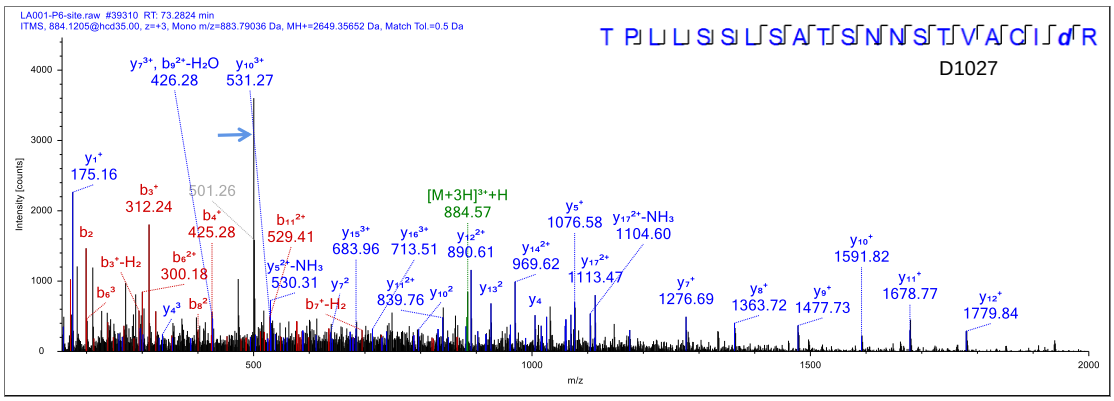


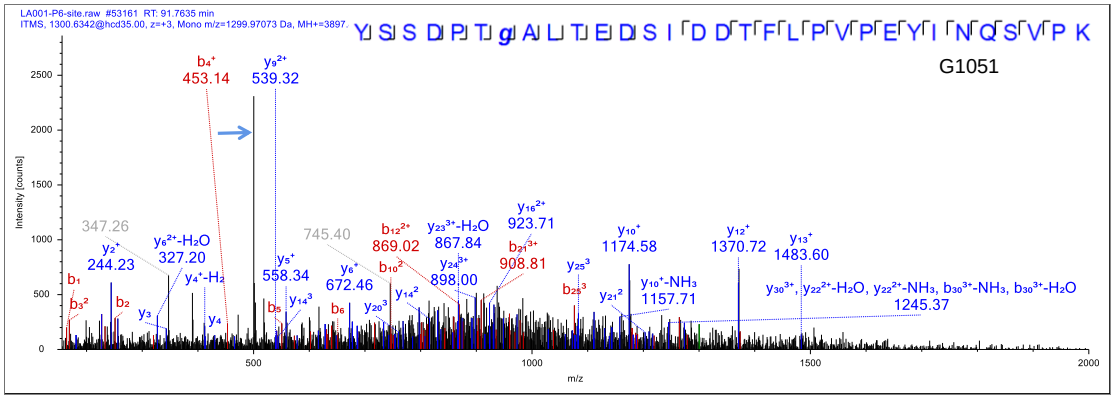

LA001-P6-site.raw #49814 RT: 85.1736 min
ITMS, 1035.9615@hcd35.00, z=+4, Mono m/z=1035.46701 Da, MH+=41
E980
y12+, y24 2+-H2O, y24 2+-NH3
1328.60
y13+
1425.65
1426.67
y7+-NH3, y13 2+-H2O, y13 2+-NH3, y19 3+-H2O, y19 3+-NH3
704.76
y10+
1072.51
y6+
634.32
b10 2+
848.97
y7+
721.37
y8+
868.34
y11+
1200.56
y12+-NH3
1311.52
1427.65
226.22
b7 4+ (-O)
314.26
501.30
y5+
547.26
714.10
y8+-H2
b21 4
b13 2
y9
y11+-NH3
1183.52
b7+ (-O
y13+-NH3
1408.61
1428.60
y13+-H2O
1407.60
y14+
1538.70
y1
y2+-NH3
245.08
b2
y11 3
b8 3
y24 4
y13 3
y5+-NH3
530.20
y11+-H2
b23 3
Intensity [counts]
m/z

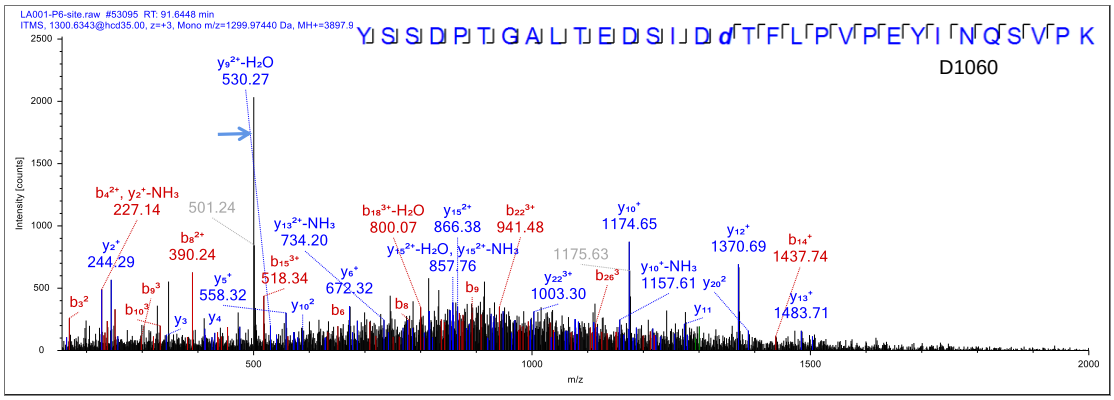

LA001-P6-site.raw #49977 RT: 85.4536 min
ITMS, 1035.7135@hcd35.00, z=+4, Mono m/z=1035.46359 Da, MH+
D982
y12+, y24 2+-H2O, y24 2+-NH3
1328.56
y13+
1425.65
1426.68
y7+-NH3, y13 2+-H2O, y13 2+-NH3, y19 3+-H2O, y19 3+-NH3
704.71
y10+
1072.54
y11+
1200.57
y12+-NH3
1311.59
y6+
634.37
y13 2+
713.55
y8+
868.44
y27 4+
891.20
226.22
b4 2+-H2O
215.11
501.38
y5+
547.28
y7+
721.29
855.89
b18 3
y9+
1015.55
b23 3+-NH3
1036.47
1427.69
y5+-NH3
530.24
b6 3
y2 2+-NH3
245.15
b3
y1
y11 3
y15 4
b7 2
b8 2
y20 3
b10 2
y11+-NH3
1183.58
y13+-NH3
1408.69
y14+
1538.81
Intensity [counts]
m/z

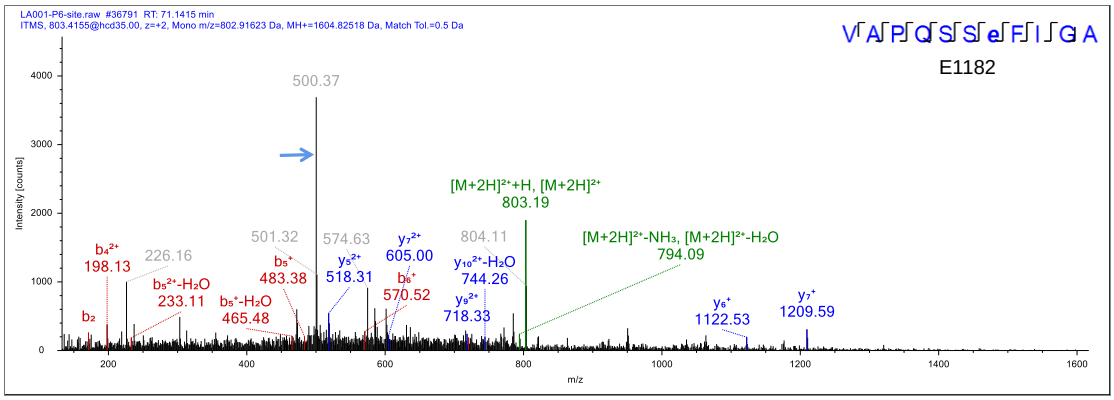

LA001-P6-site.raw #49281 RT: 84.2648 min
ITMS, 1035.9667@hcd35.00, z=+4, Mono m/z=1035.46823 Da, MH+=41
D990
y16 3+-H2O, y16 3+-NH3
600.02
y19 2+-H2O, y19 2+-NH3
1306.56
y13+
1425.71
1426.64
y13 2+
713.70
y10+
1072.51
y11+
1200.56
y4 2+, b8 4+ (-O)
226.12
501.25
y5+-H2O
529.37
y7+
721.34
y8+
868.42
1073.55
y12+-NH3
1311.53
1427.68
y13+-H2O
1407.66
y14+
1538.61
y5+
547.29
b7
y17 2
y9+
1015.50
y11+-NH3
1183.55
b3+
332.17
y5+-NH3
530.29
y6
b22 4+-H2
b25 4+ (-O
b10+
1197.40
y12
b2
b8 4
y1
y2
b5+ (-O
y16 4
b17 4
b24 3
Intensity [counts]
m/z


LA001-P6-site.raw #39216 RT: 73.2065 min
ITMS, 884.1226@hcd35.00, z=+3, Mono m/z=883.79073 Da, MH+=2649.35762 Da, Match Tol.=0.5 Da
C1025
y3+-H2O, b12 3+-H2O
385.21
y10 3+
531.27
y1+
175.16
b3+
312.22
501.28
b11 2+, y5 2+-NH3, y5 2+-H2O
530.26
y12 2+
890.62
y13 2+
926.16
y5+
1076.60
b6 2+
300.21
b3+-H2O
294.17
y14 2+
969.72
y6+
1175.60
y8+
1363.62
1780.81
[M+3H]3++H
884.52
y17 2+
1113.34
b4+
425.27
b7 3
y7+
1276.78
y9+
1477.74
y10+
1591.76
y12+
1779.91
b14 2
y5 2
y10 2
Intensity [counts]
m/z


LA001-P6-site.raw #39310 RT: 73.2824 min
ITMS, 884.1205@hcd35.00, z=+3, Mono m/z=883.79036 Da, MH+=2649.35652 Da, Match Tol.=0.5 Da
T P L L S S L S A T S N N S T V A C I d R
D1027
Intensity [counts]
m/z


LA001-P6-site.raw #53161 RT: 91.7635 min
ITMS, 1300.6342@hcd35.00, z=+3, Mono m/z=1299.97073 Da, MH+=3897.
Y S S D P T g A L T E D S I D D T F L P V P E Y I N Q S V P K
G1051
Intensity [counts]
m/z


LA001-P6-site.raw #53095 RT: 91.6448 min
ITMS, 1300.6343@hcd35.00, z=+3, Mono m/z=1299.97440 Da, MH+=3897.9
Y S S D P T G A L T E D S I J D d T F L P V P E Y I N Q S V P K
D1060
Intensity [counts]
m/z


LA001-P6-site.raw #36791 RT: 71.1415 min
ITMS, 803.4155@hcd35.00, z=+2, Mono m/z=802.91623 Da, MH+=1604.82518 Da, Match Tol.=0.5 Da
V A P Q S S e F I G A
E1182
Intensity [counts]
m/z

References

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