Supplementary Information for

Title:

A covalent fragment-based strategy targeting a novel cysteine to inhibit activity of mutant EGFR kinase

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Experimental Procedures

Protein expression and production

DNAs encoding the kinase domain of human EGFR (Uniprot ID: P00533) were ordered from GeneArt (Thermo Fisher Scientific) codon optimised for *Spodoptera frugiperda*. Wild type EGFR (EGFR_wt), residues 694-1022 were encoded with an N-terminal twin Strep-tag followed by a TEV cleavage site leaving a Glycine-Serine scar. The mutants EGFR_wtX, EGFR_SM, EGFR_DM, EGFR_DMX, EGFR_TM and EGFR_TMX (see below) were encoded with either an N-terminal 6His or a twin 6His-tag followed by TEV cleavage site leaving a Glycine scar. The mutant EGFR constructs used for crystallography (EGFR_wtX, EGFR_DMX, EGFR_TMX) also contained a C-terminal Asparagine-Serine scar. The sequences were cloned into pFastBac1 and baculovirus prepared using standard techniques. Proteins were expressed in Sf9 cells at 27°C with shaking for 72 hours, harvested by centrifugation at 6,000xg for 30 mins and the cell pellet frozen at -80°C until used.

All steps were performed at 4°C. Cell pellets were thawed and resuspended in lysis buffer (buffer A supplemented with DNaseI and Protease Inhibitor cocktail) and lysed by Frech pressure cell press homogenisation. The lysate was centrifuged at 40,000xg for 1 hour and the supernatant applied to affinity chromatography columns connected to an AKTA Pure pre-equilibrated in buffer A. For constructs containing a twin Strep-tag a 5 mL Streptactin XT Superflow column was used (IBA Lifesciences). Sample was loaded onto the column at 1 mL/min and all remaining steps were performed at 5 mL/min. After sample loading the column was washed to UV baseline with buffer A (100 mM HEPES[pH 8.0], 500 mM NaCl, 10% glycerol, 2 mM TCEP) followed by a step elution to 100% buffer B (buffer A + 50 mM biotin). For constructs containing a His-tag a 5 ml HiTrap TALON crude column (Cytiva) was used. Sample was loaded onto the column at 1 mL/min and all remaining steps were performed at 5 mL/min. After sample loading the column was washed to UV baseline with buffer A (50 mM HEPES [pH 8.0], 300 mM NaCl, 10% glycerol, 1 mM TCEP) before a series of step elutions, 5%, 10% and 100% buffer B (buffer A + 400 mM imidazole). Affinity purified proteins were TEV cleaved overnight during dialysis, followed by removal of protease by negative IMAC. Proteins were subsequently purified by size exclusion chromatography using a HiLoad 26/600 Superdex 200 column. Final buffer for EGFR_wt was 25 mM HEPES [pH 8.0], 150 mM NaCl, 2 mM TCEP. Final buffer for EGFR mutants was 25 mM Tris [pH 8.0], 100 mM NaCl, 2 mM TCEP. The fractions containing the target protein were pooled, frozen in liquid nitrogen and stored at -80°C until used.

To obtain EGFR_SM2 and EGFR_DM2 (see below) for LC-MS analysis, N-terminal His-GST tagged EGFR (694-1022, L858R) and His-GST tagged EGFR (694-1022, L858R, C775S) were expressed in Sf9 insect cells using pFB vector. They were purified by Ni affinity chromatography followed by Size exclusion chromatography (SEC) and were concentrated to 0.8 mg/mL in buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM TCEP, and 5%(v/v) glycerol.

Name	Mutations	Name	Mutations	Name	Mutations
EGFR_wt	None	EGFR_wtX	E865A, E866A, K867A		
EGFR_SM	T790M	EGFR_SM2	L858R		
EGFR_DM	T790M, L858R	EGFR_DMX	T790M, L858R, E865A, E866A, K867A	EGFR_DM2	L858R, C775S
EGFR_TM	T790M, L858R, C797S	EGFR_TMX	T790M, L858R, C797S, E865A,		

			E866A, K867A		

Fragment screen using the DiscoverX kinomescan platform

In brief, the kinomescan assay detects displacement of DNA tagged kinase from binding to a bead-immobilised ligand, measured by a quantitative qPCR method.[1] The 1350-member Vernalis fragment library was screened for binding to EGFR_wt and EGFR_DMX at 200 (81 hits) and 20 (71 hits) µM concentration where a hit is defined as a signal at 1.5 standard deviation from the plate mean after B-score normalization.[2,3] 20 hits bound at both concentrations, of which 6 also bound to EGFR_wt. In addition, 25 additional compounds were chosen by visual inspection of the assay data, giving a total of 157 overall fragment hits. 56 of the fragment hits (classified into 9 chemotypes) inhibited EGFR_TM in the Lance assay with a ligand efficiency of greater 0.39; crystal structures were determined of most of the 56 fragment hits, however, only the fragment **1** which formed a covalent bond to C775 was judged suitable for optimisation to give the desired selectivity for EGFR_TM.

Functional assay

EGFR kinase activity is measured using a LANCE Ultra kinase activity assay available from Perkin Elmer. The assay is a homogeneous time resolved-fluorescence resonance energy transfer (TR-FRET) assay that measures phosphorylation of a Ulight-labelled JAK1 substrate peptide (Peptide sequence: CAGAGAIETDKEYYTVKD), the product of EGFR kinase activity. The phosphorylated peptide is recognized by a generic Eu-W1024-labelled anti-phosphotyrosine PT66 antibody and, subsequently, the phosphorylated peptide can be quantified by the extent of TR-FRET between the europium donor and Ulight acceptor.

The EGFR wild-type kinase used in this assay was obtained from Carna Biosciences and comprises residue 669 to 1210 of the full length human wild-type EGFR, expressed as a N-terminal GST-fusion protein. EGFR_DM and EGFR_TM were prepared as described above.

The kinase reactions are performed in a 10 µL volume in 384-well plates (Corning #4513). The kinase reaction was initiated by the addition of EGFR at final concentration of 0.5 nM EGFR_wt , 0.5 nM EGFR_DM, or 0.25 nM EGFR_TM. Final assay conditions were 1 µM ATP (EGFR_wt and EGFR _TM) or 10 µM ATP (EGFR_DM), 50 nM Ulight-JAK1 peptide, 50 mM Hepes pH 7.5, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 0.015 % Brij35, 5 % DMSO. The reaction mixture was incubated for 1.5 hours at 23°C, after which the reaction was terminated by addition of 25 mM EDTA in 1x LANCE buffer (Perkin Elmer). Product was then detected after the addition of 1 nM terbium-labelled anti-phosphotyrosine PT66 antibody in 1x LANCE buffer (final volume 15 µl). The mixture was further incubated for 1 hour at 23°C.

TR-FRET measurements were performed on a Biotek Synergy Neo plate reader. TR-FRET was measured by excitation of the Europium-donor at 330 nm and subsequent (delay time 100 µs) measurement of europium and Ulight emission at 620 nm and 665 nm, respectively, over a collection time of 200 µs. The TR-FRET signal was calculated as the emission-ratio at 665 nm over 620 nm.

The TR-FRET ratio readout for test compounds was normalized against 0% inhibition controls wells and 100% inhibition (no protein) control wells. Test compound potency (IC_{50}) was estimated by nonlinear regression using the sigmoidal dose-response (variable slope) using Xlfit 4 (IDBS, Guildford, Surrey, UK, model 205). Were the IC_{50} could not be determined the % inhibition at the highest tested concentration is given.

y = (A+((B-A)/(1+((C/x)^D))))

where y is the normalized TR-TRET ratio measurement for a given concentration of test compound, x is the concentration of test compound, A is the estimated efficacy (% inhibition) at infinite compound dilution, and B is the maximal efficacy (% inhibition). C is the IC_{50} value and D is the Hill slope coefficient.

Pre-incubation assay

DMSO solutions of tested compounds were pre-incubated with 250 pg/μL EGFR_TM (residues 694-1022) in reaction buffer (20 mM HEPES (pH7.4), 5 mM $MgCl_2$, 1 mM $MnCl_2$, 0.003% Brij-35, 0.004% Tween-20, 2 mM DTT) for specific pre-incubation times (0, 20, 60 minutes, respectively). After pre-incubation, Srctide (PEPTIDE-INST) solution was added at the final concentration of 1.5 μM. Simultaneously, ATP (SIGMA-ALDRICH) solution was added at the specific final concentrations (1 mM for "ATP 1 mM" condition, or 5 μM for "ATP Km" condition). After incubation at 28°C for 45 minutes (ATP 1 mM condition) or 90 minutes (ATP Km condition), reaction was stopped with stop solution (100 mM HEPES (pH7.4), 0.015% Brij-35, 40 mM EDTA, 0.1% Coating Reagent). EGFR phosphorylation was measured with LabChip EZ Reader II (PerkinElmer, Inc.) at 488 nm excitation and 530 nm emission.

Kinase panel

Kinase selectivity was evaluated by an accustomed panel of pre-incubation assay. Briefly, DMSO solutions of tested compounds (1 μM in final assay reagent) were pre-incubated with the specific concentration of each kinase in reaction buffer (as described in pre-incubation assay section for mutant EGFR proteins, and 100 mM HEPES (pH7.4), 0.003% Brij-35, 0.004% Tween-20, 1 mM DTT for other kinases) for 15 minutes. Substrate solution containing ATP and Substrate Peptide (at the final concentration of 1 mM and 1.5 μM, respectively) was added with specific additives for some kinases (in detail, see Table S3 and S4). After incubation for 45 minutes, reaction was stopped and substrate phosphorylation was measured as described in pre-incubation assay assay section.

Computational methods

Software used for modelling was from Schrödinger, LLC, New York, NY, 2021; (versions 2018-U1, 2018-U4, 2019-U2), using default settings unless otherwise specified.

The Protein Preparation Wizard protocol was used to prepare the X-ray crystallography structure of **1** bound to EGFR_DMX for covalent docking.[4] Sidechains and hydrogens were added, and protonation states and water networks optimized using default settings. Only the hydrogens were energy minimized to RMSD of 0.3 Å. Waters and ligand **1** were then removed, and the hydrogen added to Cys775 and the structure further minimized. Ligands were transformed from 2D SD files to 3D with Ligprep using default settings, except the ionization pH range was changed to 7±0. The 'Covalent Docking' protocol was used for docking the ligands to Cys775.[5,6] Depending on the ligands, the appropriate reaction chemistry was chosen. Some reactions required 'custom files' of the type .cdock which for version 2018-U1 could be obtained from https://www.schrodinger.com/kb/1848 Most of the work involved Michael Additions with acrylamides. The center for the grid was chosen such that the center of similar sized molecules would have to be near the kinase hinge and was kept the same for all covalent docking. No constraints/restraints were specified. The Pose Prediction 'Thorough' algorithm used.

Crystallisation and structure determination

EGFR_wtX was concentrated to ~15 mg/ml and set up for crystallisation in vapour diffusion sitting drops, against commercial crystallisation screens. Initial crystals were obtained overnight in several conditions, of which best appeared in 0.1M Tris buffer pH 7.0 and 0.8 M Sodium citrate at 292K. Initial crystals diffracted only to 3.8-4.0 Å. Subsequently crystallisation conditions were optimised with the final crystallisation conditions were 0.1 M Tris pH 8.5, 0.15 M sodium citrate 25% Peg400.

Crystals of EGFR_DMX and EGFR_TMX were obtained in the same conditions as crystals of EGFR_wtX. The conditions used for compound soaking and cryoprotection have been the same across all EGFR variants.

For compound soaking, the best-looking single crystals of colourless trapezoid appearance were transferred to solution containing crystallisation solution with 2 mM compound solution in DMSO. Crystals were soaked for 48 hrs, except **1** and **14**, which were soaked overnight. Crystals were subsequently harvested, streaked through cryoprotection solution (crystallisation solution with added 20% glycerol) and flash frozen in a liquid nitrogen. Such prepared crystals were used for data collection at in-house equipment (Bruker D8 Venture TXS Generator coupled with Bruker Photon 100 CMOS detector) or Rigaku MicroMax-007 with HyPix6000 detector) or at the Diamond (beamlines i04-1 and i24) and Soleil (beamlines PX1 and PX2) synchrotrons – details in Table S1.

Diffraction data were indexed and integrated using XDS or the Bruker software SAINT and SADABS (Version 8.37A (2015), Bruker AXS Inc., Madison, Wisconsin, USA), scaled and truncated using SCALA or XSCALE and the CCP4 suite of programs.[7,8] The structures were solved by molecular replacement with MolRep with 5EDQ structure used as a starting model.[9] All structures were refined using Refmac and model building was done with Coot.[10,11] Topology files for the compounds were created by AceDrg.[12] Refinement statistics are reported Table S6.

MS and LC-MS methods

Compound **14** (0.3 µM, 1 µM, 3 µM) and EGFR_SM2 or EGFR_DM2 (0.5 mg/ml) were incubated in buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM TCEP, and 5% (v/v) glycerol for 1 hour at room temperature followed by an addition of SDS (final 1%) and boiling at 95^{o}C for 10 min. Before the LC-MS analysis, SDS was removed using SDS-eliminant (ATTO #AE-1390). LC-MS analysis was performed on an Ultimate 3000 UHPLC system (Thermo Fisher Scientific) coupled with a Q-Exactive mass spectrometer (Thermo Fisher Scientific). EGFR proteins were separated on a PLRP-S 1000A column (8 µm, 50 x 2.1 mm, Agilent) at a flow rate of 0.6 mL/min. The mobile phase consisted of solution A (H_2O (0.02% TFA, 0.1% HCO_2H)) and solution B (CH_3CN). The UHPLC separation was used a linear gradient program of 20–70% B for 7 min and 70% B for 7.5 min. The column temperature was 60^{o}C. The Q-Exactive MS was operated in the positive ion electrospray mode.

Chemical synthesis

General

Proton nuclear magnetic resonance spectra (^{1}H-NMR) were recorded on a JEOL JNM-ECS400 spectrometer or an AVANCE III HD 400 (400 MHz for 1H, BRUKER) in the indicated solvent. Chemical shifts (δ) are reported in parts per million relative to the internal standard tetramethylsilane. Abbreviations of multiplicity are as follows: s: singlet, d: doublet, dd: double doublet, t: triplet, q: quartet, m: multiplet, and br: broad. Data are presented as follows: chemical shift (multiplicity, integration, coupling constant). APCI/ESI mass spectra were recorded on Agilent Technologies Agilent 1100 or 1200 series LC/MS. HRMS was carried out by using a liquid chromatography-mass spectrometry (LC-MS) system composed of a Waters Xevo Quadrupole Time-of-Flight Mass Spectrometer and an Acquity UHPLC system. Compound purity was confirmed to exceed 95% by the DAD signal area (%), which was calculated with an Agilent Infinity 1260 LC-MS system. The conditions used were: column, Develosil Combi-RP-5 2.0 mmID × 50 mmL; gradient elution, 0.1% HCO_2H-H_2O/0.1% HCO_2H-MeCN = 98/2 to 0/100 (v/v); flow rate, 1.2 mL/min; UV detection, 254 nm; column temperature, 40°C; ionization, APCI/ESI. Purity ≥95% was determined by elemental analysis for each of the tested compounds. Flash column chromatography was performed using Purif-Pack® SI 30 μm and Purif-Pack® NH 30 μm supplied by Shoko Scientific or Merck silica gel 60 (230–400 mesh).

All compounds used in the experiments (whether purchased or synthesised) were >95% purity

N-(2-methoxy-3-pyridyl)prop-2-enamide (**S-1**)

To a solution of 3-amino-2-methoxypyridine (376 mg, 3.03 mmol, 1.0 eq) and triethylamine (0.42 mL, 3.03 mmol, 1.0 eq) in CH_2Cl_2 (10 mL) was added acryloyl chloride (0.26 mL, 3.03 mmol, 1.0 eq) at 0°C and the mixture was stirred for 3h under a nitrogen atmosphere. The resulting mixture was concentrated *in vacuo* and purified by silica gel column chromatography (SiO_2, *n*-hexane/ethyl acetate (2% CH_2Cl_2); 98:2 to 50:50) to afford compound **S-1** (455 mg, 84%) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 4.03 (3H, s), 5.80 (1H, dd, *J* = 10.1, 1.2 Hz), 6.29 (1H, dd, *J* = 17.0, 10.1 Hz), 6.44 (1H, dd, *J* = 17.0, 1.2 Hz), 6.92 (1H, dd, *J* = 8.0, 4.9 Hz), 7.77 (1H, br s), 7.87 (1H, dd, *J* = 4.9, 1.8 Hz), 8.69 (1H, dd, *J* = 7.7, 1.8 Hz). ESI-LRMS: calcd for $C_9H_{11}N_2O_2$ [$(M+H)^+$], 179.08; found, 179.2.

N-(2-oxo-1**H**-pyridin-3-yl)prop-2-enamide (**2**)

To a solution of compound **S-1** (219 mg, 1.23 mmol, 1.0 eq) in 1,4-dioxane (3 mL) was added conc. HCl (1 mL, 32.0mmol 26 eq) and water (2 mL) and the mixture was stirred at 60°C for 5h. The resulting mixture was concentrated *in vacuo* and purified by silica gel column chromatography (SiO_2, *n*-hexane/ethyl acetate; 80:20 to 0:100 to ethyl acetate/methanol; 100:0 to 90:10). The residue was suspended in *n*-hexane/ethyl acetate (10:1) and filtered. The solid was washed with *n*-hexane to afford compound **2** (43 mg, 21%)

as a purple solid. ^{1}H-NMR (DMSO-d_6) δ: 5.70 (1H, dd, J = 10.4, 1.8 Hz), 6.18-6.27 (2H, m), 6.82 (1H, dd, J = 16.9, 10.1 Hz), 7.12 (1H, dd, J = 6.7, 1.8 Hz), 8.35 (1H, dd, J = 6.7, 1.8 Hz), 9.53 (1H, s), 11.99 (1H, br s). ESI-HRMS: calcd for $C_8H_9N_2O_2$ [$(M+H)^+$], 165.0658; found, 165.0661.

N-(3-acetylphenyl)prop-2-enamide (**3**)

To a solution of 1-(3-aminophenyl)ethanone (126 mg, 0.93 mmol, 1.0 eq) and triethylamine (0.130 mL, 0.93 mmol, 1.0 eq) in CH_2Cl_2 (3 mL) was added acryloyl chloride (0.080 mL, 0.93 mmol, 1.0 eq) at 0°C and the mixture was stirred for 0.5h under a nitrogen atmosphere. The resulting mixture was concentrated *in vacuo* and purified by silica gel column chromatography (SiO_2, *n*-hexane/ethyl acetate; 95:5 to 20:80). The residue was suspended in *n*-hexane/ethyl acetate (5:1) and filtered. The solid was washed with *n*-hexane to afford compound **3** (125 mg, 71%) as a white solid. ^{1}H-NMR ($CDCl_3$) δ: 2.62 (3H, s), 5.82 (1H, dd, J = 10.4, 1.2 Hz), 6.30 (1H, dd, J = 17.0, 10.4 Hz), 6.48 (1H, dd, J = 17.0, 1.2 Hz), 7.43-7.48 (1H, m), 7.67-7.75 (2H, m), 7.99 (1H, d, J = 8.0 Hz), 8.11 (1H, s). ESI-HRMS: calcd for $C_{11}H_{12}NO_2$ [$(M+H)^+$], 190.0862; found, 190.0877.

N-(6-acetamido-2-pyridyl)prop-2-enamide (**5**)

To a solution of **N**-(6-amino-2-pyridyl)acetamide (120 mg, 0.79 mmol, 1.0 eq) and triethylamine (0.120 mL, 0.79 mmol, 1.0 eq) in CH_2Cl_2 (3 mL) was added acryloyl chloride (0.069 mL, 0.79 mmol, 1.0 eq) at 0°C and the mixture was stirred for 1h under a nitrogen atmosphere. The resulting mixture was concentrated *in vacuo* and purified by silica gel column chromatography (SiO_2, *n*-hexane/ethyl acetate; 80:20 to 0:100 to ethyl acetate/methanol; 100:0 to 90:10). The residue was suspended in *n*-hexane/ethyl acetate (5:1) and filtered. The solid was washed with *n*-hexane to afford compound **5** (68.4 mg, 42%) as a white solid. ^{1}H-NMR (DMSO-d_6) δ: 2.11 (3H, s), 5.78 (1H, dd, J = 9.8, 1.8 Hz), 6.30 (1H, dd, J = 16.9, 2.1 Hz), 6.66 (1H, dd, J = 16.9, 10.1 Hz), 7.72-7.78 (2H, m), 7.80-7.86 (1H, m), 10.09 (1H, s), 10.30 (1H, s). ESI-HRMS: calcd for $C_{10}H_{12}N_3O_2$ [$(M+H)^+$], 206.0924; found, 206.0934.

N-(1-methylbenzimidazol-4-yl)prop-2-enamide (**7**)

To a solution of 4-amino-1-methylbenzimidazole dihydrochloride (144 mg, 0.65 mmol, 1.0 eq) and triethylamine (0.270 mL, 1.96 mmol, 3.0 eq) in CH_2Cl_2 (3 mL) was added acryloyl chloride (0.059 mL, 0.69 mmol, 1.05 eq) at 0°C and the mixture was stirred for 1h under a nitrogen atmosphere. The resulting mixture was concentrated *in vacuo* and purified by silica gel column chromatography (SiO_2, ethyl acetate/methanol (2% CH_2Cl_2); 100:0 to 90:10). The residue was suspended in *n*-hexane/ethyl acetate (5:1) and filtered. The solid was washed with *n*-hexane to afford compound **7** (70.9 mg, 54%) as a white solid. ^{1}H-NMR ($CDCl_3$) δ: 3.86 (3H, s), 5.80 (1H, dd, J = 9.8, 1.8 Hz), 6.41 (1H, dd, J = 16.6, 9.8 Hz), 6.50 (1H, dd, J =

16.6, 1.8 Hz), 7.14 (1H, d, *J* = 7.4 Hz), 7.30-7.36 (1H, m), 7.80 (1H, s), 8.36 (1H, d, *J* = 8.0 Hz), 8.63 (1H, br s). ESI-HRMS: calcd for $C_{11}H_{12}N_3O$ [$(M+H)^+$], 202.0975; found, 202.0975.

N-(7-quinolyl)prop-2-enamide (**8**)

To a solution of 7-aminoquinoline (123 mg, 0.85 mmol, 1.0 eq) and triethylamine (0.119 mL, 0.85 mmol, 1.0 eq) in CH_2Cl_2 (3 mL) was added acryloyl chloride (0.073 mL, 0.85 mmol, 1.0 eq) at 0°C and the mixture was stirred for 1h under a nitrogen atmosphere. The resulting mixture was concentrated *in vacuo* and purified by silica gel column chromatography (SiO_2, *n*-hexane/ethyl acetate; 95:5 to 20:80). The residue was suspended in *n*-hexane/ethyl acetate (5:1) and filtered. The solid was washed with *n*-hexane to afford compound **8** (110 mg, 65%) as a white solid. ^{1}H-NMR ($CDCl_3$) δ: 5.79 (1H, dd, *J* = 10.2, 1.2 Hz), 6.33 (1H, dd, *J* = 17.0, 10.2 Hz), 6.49 (1H, dd, *J* = 17.0, 1.2 Hz), 7.33 (1H, dd, *J* = 8.0, 4.3 Hz), 7.78 (1H, d, *J* = 9.2 Hz), 8.04-8.14 (3H, m), 8.25 (1H, br s), 8.86 (1H, dd, *J* = 4.3, 1.8 Hz). ESI-HRMS: calcd for $C_{12}H_{11}N_2O$ [$(M+H)^+$], 199.0866; found, 199.0855.

N-[7-(dimethylamino)-2-methyl-pyrazolo[1,5-a]pyrimidin-5-yl]prop-2-enamide (**9**)

To a solution of **N**7,**N**7,2-trimethylpyrazolo[1,5-a]pyrimidine-5,7-diamine (61 mg, 0.30 mmol, 1.0 eq) and triethylamine (0.43 mL, 0.30 mmol, 1.0 eq) in CH_2Cl_2 (3 mL) was added acryloyl chloride (0.027 mL, 0.30 mmol, 1.0 eq) at 0°C and the mixture was stirred for 1h under a nitrogen atmosphere. The resulting mixture was concentrated *in vacuo* and purified by silica gel column chromatography (SiO_2, *n*-hexane/ethyl acetate; 80:20 to 0:100). The residue was suspended in *n*-hexane/ethyl acetate (5:1) and filtered. The solid was washed with *n*-hexane to afford compound **9** (12.1 mg, 16%) as a white solid. ^{1}H-NMR ($CDCl_3$) δ: 2.45 (3H, s), 3.36 (6H, s), 5.84 (1H, d, *J* = 10.4 Hz), 6.05 (1H, s), 6.23 (1H, dd, *J* = 16.5, 10.4 Hz), 6.47 (1H, d, *J* = 16.5 Hz), 7.24 (1H, s), 8.04 (1H, br s). ESI-HRMS: calcd for $C_{12}H_{16}N_5O$ [$(M+H)^+$], 246.1349; found, 246.1351.

N-pyrazolo[1,5-a]pyridin-2-ylprop-2-enamide (**10**)

To a solution of pyrazolo[1,5-a]pyridin-2-amine (120 mg, 0.86 mmol, 1.0 eq) and triethylamine (0.12 mL, 0.86 mmol, 1.0 eq) in CH_2Cl_2 (3 mL) was added acryloyl chloride (0.074 mL, 0.86 mmol, 1.0 eq) at 0°C and the mixture was stirred for 2h under a nitrogen atmosphere. The resulting mixture was concentrated *in vacuo* and purified by silica gel column chromatography (SiO_2, *n*-hexane/ethyl acetate; 90:10 to 0:100). The residue was suspended in *n*-hexane/ethyl acetate (5:1) and filtered. The solid was washed with *n*-hexane to afford compound **10** (107 mg, 67%) as a white solid. ^{1}H-NMR ($CDCl_3$) δ: 5.80 (1H, dd, *J* = 10.2, 1.2 Hz), 6.27 (1H, dd, *J* = 17.0, 10.2 Hz), 6.48 (1H, dd, *J* = 17.0, 1.2 Hz), 6.70-6.75 (1H, m), 7.08 (1H, s), 7.10-7.15 (1H, m), 7.45-7.49 (1H, m), 8.26 (1H, d, *J* = 8.0 Hz), 8.68 (1H, br s). ESI-HRMS: calcd for $C_{10}H_{10}N_3O$ [$(M+H)^+$], 188.0818; found, 188.0820.

N-(2,3-dimethylindazol-6-yl)prop-2-enamide (**11**)

To a solution of 2,3-dimethyl-2**H**-indazol-6-amine (127 mg, 0.76 mmol, 1.0 eq) and triethylamine (0.106 mL, 0.76 mmol, 1.0 eq) in CH_2Cl_2 (3 mL) was added acryloyl chloride (0.066 mL, 0.76 mmol, 1.0 eq) at 0°C and the mixture was stirred for 1h under a nitrogen atmosphere. The resulting mixture was concentrated *in vacuo* and purified by silica gel column chromatography (SiO_2, CH_2Cl_2/methanol; 100:0 to 88:12). The residue was suspended in *n*-hexane/ethyl acetate (5:1) and filtered. The solid was washed with *n*-hexane to afford compound **11** (84.9 mg, 52%) as a light-yellow solid. ^{1}H-NMR (DMSO-d_6) δ: 2.56 (3H, s), 4.00 (3H, s), 5.75 (1H, dd, *J* = 10.1, 2.1 Hz), 6.26 (1H, dd, *J* = 16.9, 2.1 Hz), 6.46 (1H, dd, *J* = 16.9, 10.1 Hz), 7.04 (1H, dd, *J* = 9.0, 1.5 Hz), 7.59 (1H, d, *J* = 9.0 Hz), 8.08 (1H, s), 10.10 (1H, s). ESI-HRMS: calcd for $C_{12}H_{14}N_3O$ [$(M+H)^+$], 216.1131; found, 216.1119.

N-(4-phenylpyridin-2-yl)prop-2-enamide (**12**)

To 2-Amino-4-phenylpyridine (100 mg, 0.59 mmol, 1 eq) in DCM (6 mL) was added triethylamine (0.04 mL, 0.29 mmol, 0.5 eq) and stirred at 0°C for 10min. Acryloyl chloride (0.04 mL, 1.12 g/mL, 0.53 mmol, 0.9 eq) was added drop wise and the reaction stirred for 1h over an ice bath. The reaction was quenched with saturated $NaHCO_3$ (aq), extracted with DCM and the organics filtered through a phase separator and evaporated to a crude residue. The pure compound was isolated by preparative HPLC automated flash chromatography (Teledyne ISCO ACCQ, Prep HPLC Column: Gemini 5 μm; pH=neutral Dimensions: 21 mm x 150 mm; elution of A/B (95/5) to A/B (4/96), (A: water; B: MeCN) and selected fractions were combined and freeze dried to afford the title compound (5.1 mg, 0.02 mmol, 4.3%) as a cream solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 5.74 (dd, J = 10.1, 1.9 Hz, 1H), 6.27 (dd, J = 17.0, 1.9 Hz, 1H), 6.59 (dd, J = 17.0, 10.1 Hz, 1H), 7.38 (dd, J = 5.2, 1.7 Hz, 1H), 7.38 - 7.47 (m, 1H), 7.43 - 7.53 (m, 2H), 7.62 - 7.71 (m, 2H), 8.34 (dd, J = 5.3, 0.8 Hz, 1H), 8.47 (dd, J = 1.8, 0.8 Hz, 1H), 10.78 (s, 1H). ESI-HRMS: calcd for $C_{14}H_{13}N_2O$ [$(M+H)^+$], 225.1022; found, 225.1023.

4-(1-methylindol-3-yl)pyridin-2-amine (**S-2**)

To a suspension of 2-amino-4-chloropyridine (100 mg, 0.78 mmol, 1.0 eq), 1-methyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1**H**-indole (300 mg, 1.17 mmol, 1.5 eq) and K_3PO_4 (495 mg, 2.33 mmol, 3.0 eq) in 1,4-dioxane (3 mL), ethanol (1 mL) and water (0.8 mL) was added XPhos Pd G2 (61.2 mg, 0.078 mmol, 0.1 eq) and the mixture was stirred at 120°C with microwave irradiation for 3h under a nitrogen atmosphere, and then cooled to room temperature. The reaction mixture was purified by silica gel column chromatography (SiO_2, CH_2Cl_2/methanol; 97:3 to 89:11) to afford compound **S-2** (122 mg, 70%) as a pale-yellow solid. ^{1}H-NMR ($CDCl_3$) δ: 3.85 (3H, s), 4.45 (2H, s), 6.81-6.83 (1H, m), 6.96 (1H, dd,

J = 5.5, 1.6 Hz), 7.20-7.25 (1H, m), 7.28-7.33 (1H, m), 7.36-7.40 (2H, m), 7.94-7.98 (1H, m), 8.06-8.09 (1H, m). ESI-LRMS: calcd for $C_{14}H_{14}N_3$ [$(M+H)^+$], 224.12; found, 224.2.

N-[4-(1-methylindol-3-yl)-2-pyridyl]prop-2-enamide (**13**)

To a solution of compound **S-2** (60.0 mg, 0.27 mmol, 1.0 eq) and triethylamine (0.112 mL, 0.81 mmol, 3.0 eq) in CH_2Cl_2 (5 mL) was added acryloyl chloride (0.063 mL, 0.78 mmol, 2.9 eq) at -78°C and the mixture was stirred for10 minutes under a nitrogen atmosphere. Saturated aqueous $NaHCO_3$ and water were added and the mixture was warmed up to room temperature with vigorous stirring. The resulting mixture was extracted with CH_2Cl_2 and the combined organic layers were washed with water, saturated aqueous $NaHCO_3$ and brine, then dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (SiO_2, CH_2Cl_2/methanol; 100:0 to 94:6) and purified again by silica gel column chromatography (SiO_2, *n*-hexane/ethyl acetate; 61:39 to 40:60) to afford compound **13** (35 mg, 47%) as a white solid. ^{1}H-NMR ($CDCl_3$) δ: 3.86 (3H, s), 5.83 (1H, dd, *J* = 10.3, 1.2 Hz), 6.33 (1H, dd, *J* = 17.0, 10.3 Hz), 6.51 (1H, dd, *J* = 17.0, 1.2 Hz), 7.27-7.34 (2H, m), 7.38-7.42 (2H, m), 7.55 (1H, s), 8.06-8.11 (1H, m), 8.26-8.28 (1H, m), 8.61 (1H, br s), 8.68-8.69 (1H, m). ESI-HRMS: calcd for $C_{17}H_{16}N_3O$ [$(M+H)^+$], 278.1288; found, 278.1276.

6-(1-methylindol-3-yl)pyrimidin-4-amine (**S-3**)

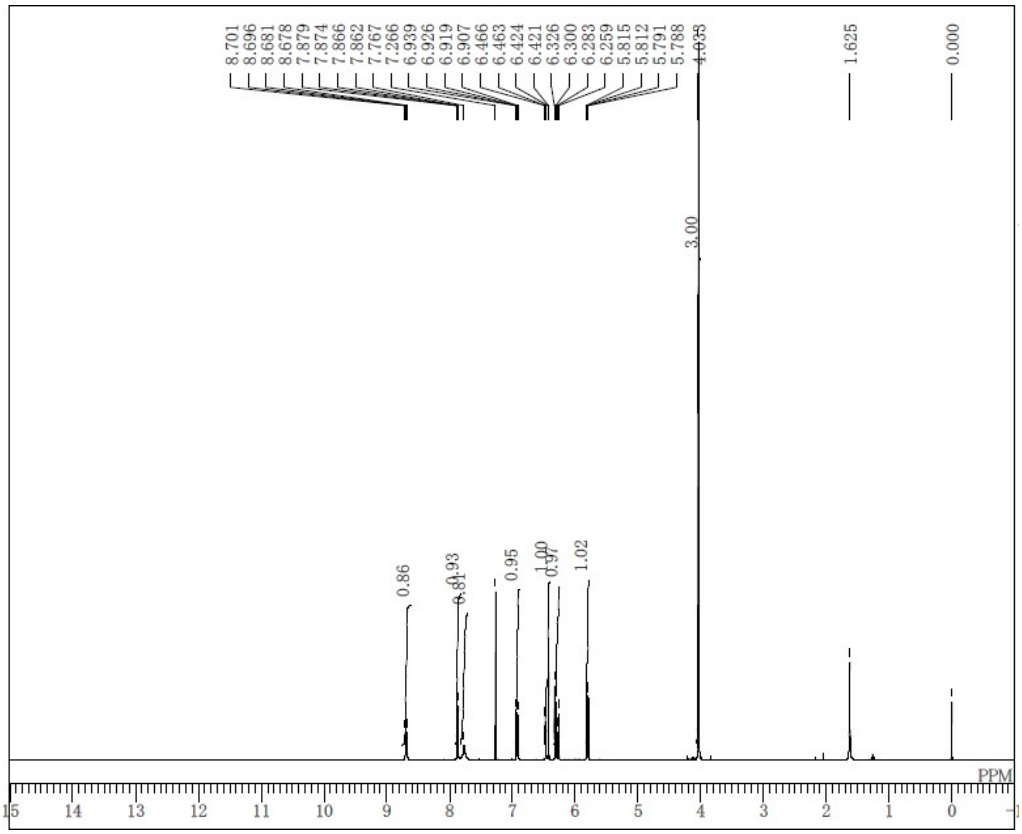
To a suspension of 4-amino-6-chloropyrimidine (147 mg, 1.13 mmol, 1.0 eq), 1-methyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1**H**-indole (326 mg, 1.27 mmol, 1.12 eq) and K_3PO_4 (725 mg, 3.42 mmol, 3.01 eq) in 1,4-dioxane (3 mL) and water (0.6 mL) was added $Pd(dppf)_2Cl_2$ (144 mg, 0.176 mmol, 0.155 eq) and the mixture was stirred at 110°C with microwave irradiation for 2h under a nitrogen atmosphere, and then cooled to room temperature. The reaction mixture was filtered through a Celite pad, washed with 1,4-dioxane and CH_2Cl_2 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (SiO_2, *n*-hexane/ethyl acetate (2% CH_2Cl_2); 90:10 to 0:100 to ethyl acetate/methanol (2% CH_2Cl_2); 100:0 to 90:10) to afford compound **S-3** (57 mg, 22%) as a brown solid. ^{1}H-NMR (DMSO-d_6) δ: 3.86 (3H, s), 6.67 (2H, br s), 6.83 (1H, d, *J* = 1.2 Hz), 7.16-7.21 (1H, m), 7.22-7.27 (1H, m), 7.52 (1H, d, *J* = 8.0 Hz), 8.07 (1H, s), 8.22 (1H, d, *J* = 8.0 Hz), 8.35 (1H, d, *J* = 1.2 Hz). ESI-LRMS: calcd for $C_{13}H_{13}N_4$ [$(M+H)^+$], 225.11; found, 225.1.

N-[6-(1-methylindol-3-yl)pyrimidin-4-yl]prop-2-enamide (**14**)

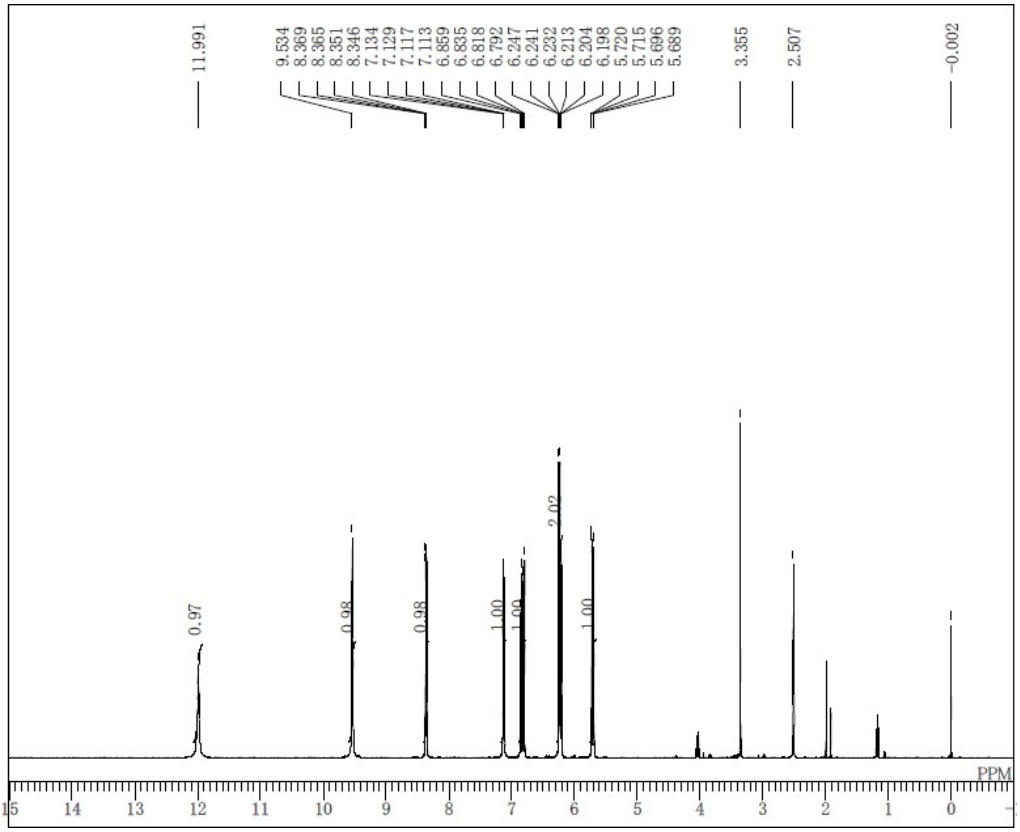
To a solution of compound **S-3** (57.0 mg, 0.254 mmol, 1.0 eq) and triethylamine (0.11 mL, 0.79 mmol, 3.1 eq) in CH_2Cl_2 (1.2 mL) and DMF (0.6 mL) was added acryloyl chloride (0.06 mL, 0.742 mmol, 2.92 eq) at -78°C and the mixture was stirred for 1.5h under a nitrogen

atmosphere. The reaction was quenched with saturated aqueous $NaHCO_3$ and water. The mixture was warmed up to room temperature and vigorously stirred for 2.5h. The resulting mixture was extracted with ethyl acetate and the combined organic layers were washed with water and brine, then dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (SiO_2, *n*-hexane/ethyl acetate (2% CH_2Cl_2); 90:10 to 0:100 to ethyl acetate/methanol (2% CH_2Cl_2); 100:0 to 95:5) to afford compound **14** (30.6 mg, 43%) as a white solid. ^{1}H-NMR (DMSO-d_6) δ: 3.91 (3H, s), 5.89 (1H, dd, J = 10.0, 1.8 Hz), 6.41 (1H, dd, J = 17.2, 1.8 Hz), 6.66 (1H, dd, J = 17.2, 10.0 Hz), 7.23-7.32 (2H, m), 7.54-7.58 (1H, m), 8.30-8.36 (2H, m), 8.60 (1H, d, J = 1.2 Hz), 8.83 (1H, d, J = 1.2 Hz), 11.02 (1H, br s). ESI-HRMS: calcd for $C_{16}H_{15}N_4O$ [$(M+H)^+$], 279.1240; found, 279.1270.

S-1_^{1}HNMR



2_^{1}HNMR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6.232
6.213
6.204
6.198
5.720
5.715
5.696
5.689
3.355
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0.98
0.98
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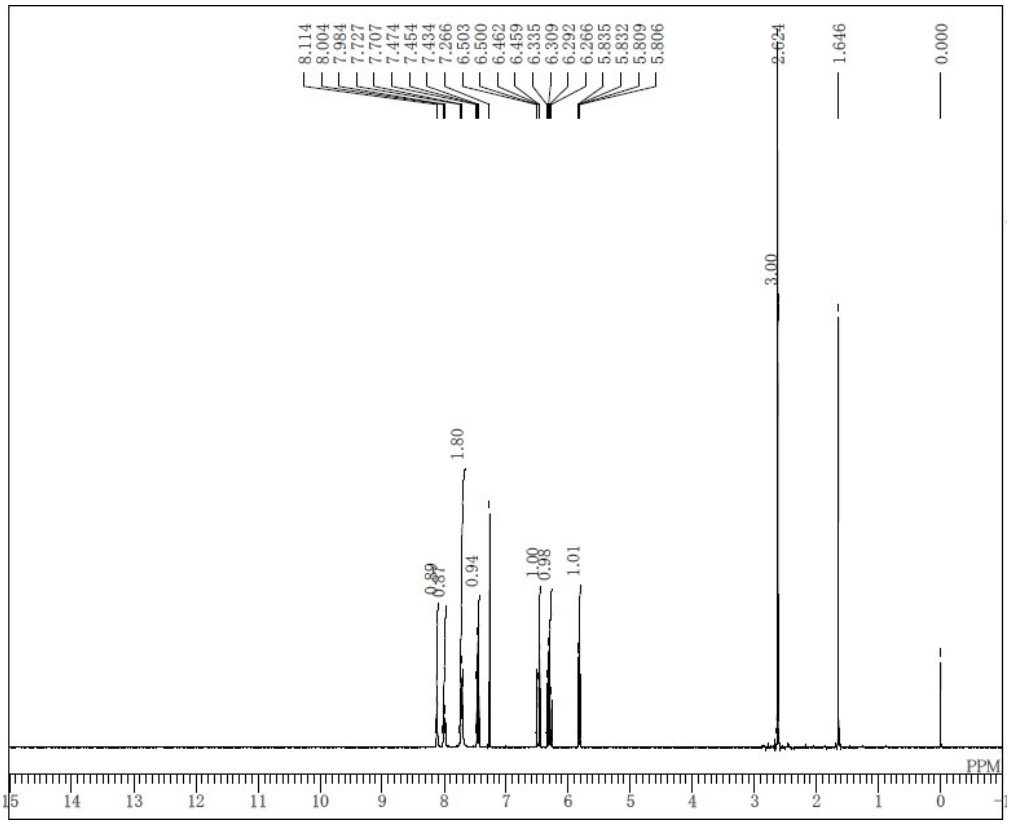
1.09
2.02
1.00
PPM

3_^{1}HNMR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6.309
6.292
6.266
5.835
5.832
5.809
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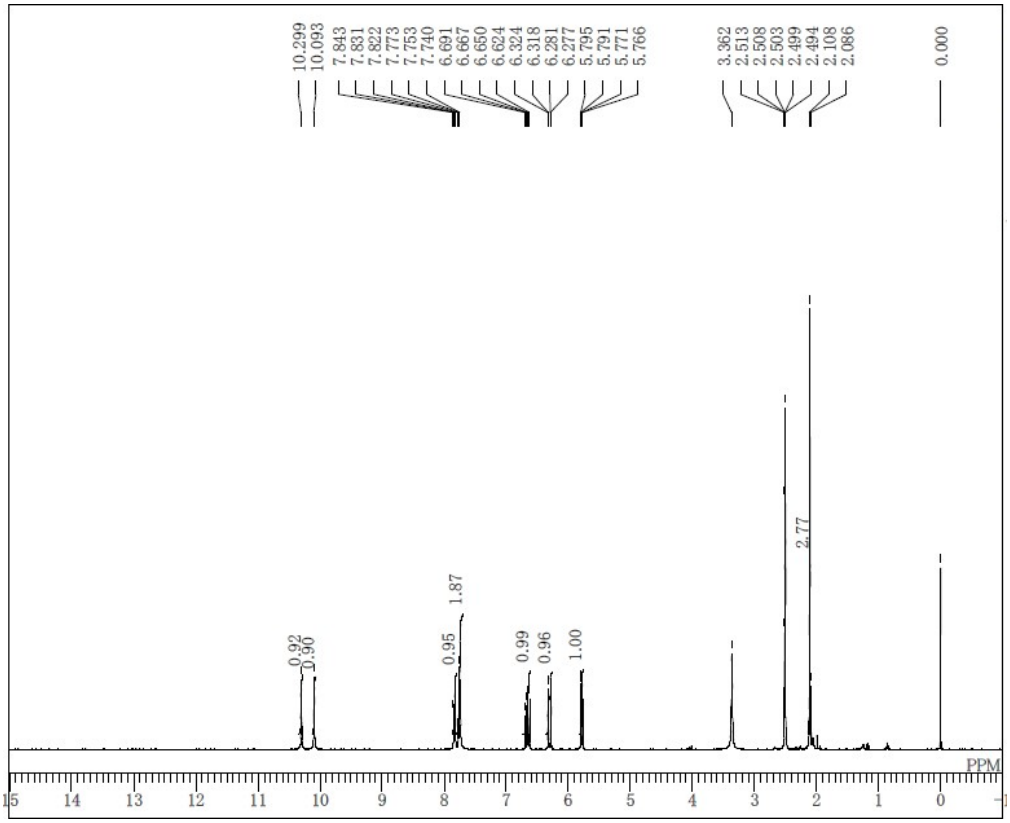
3.00
1.80
0.94
1.00
0.98
1.01
PPM

5_^{1}HNMR



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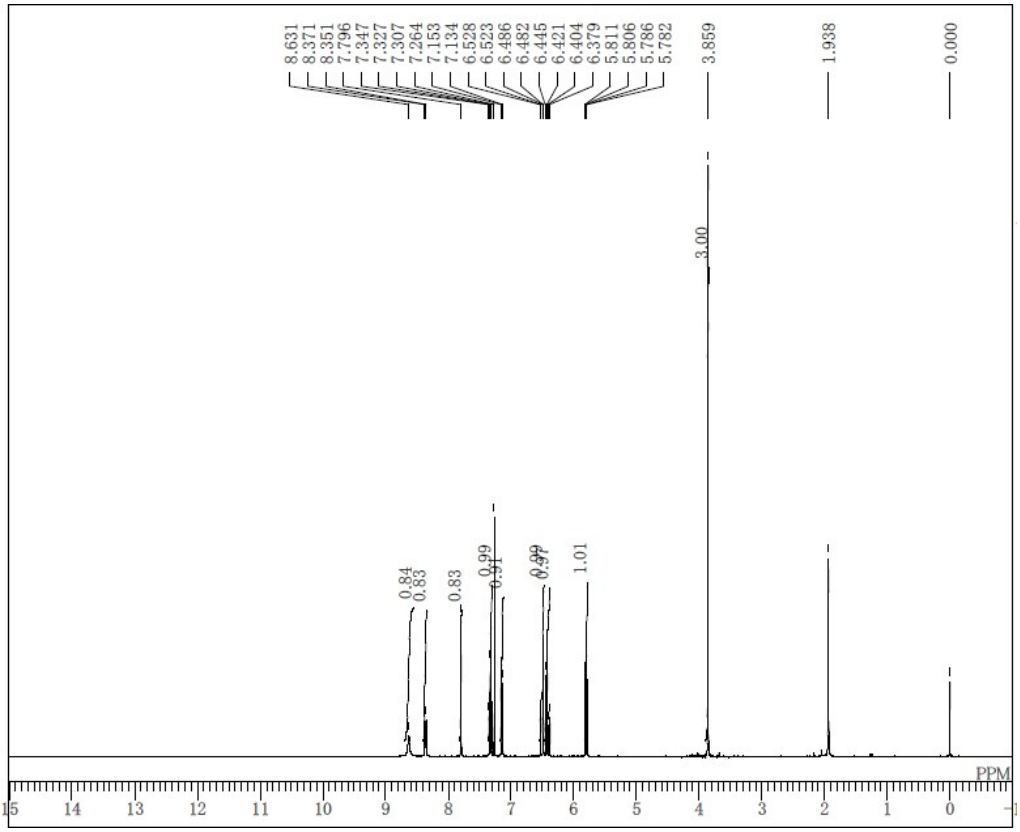

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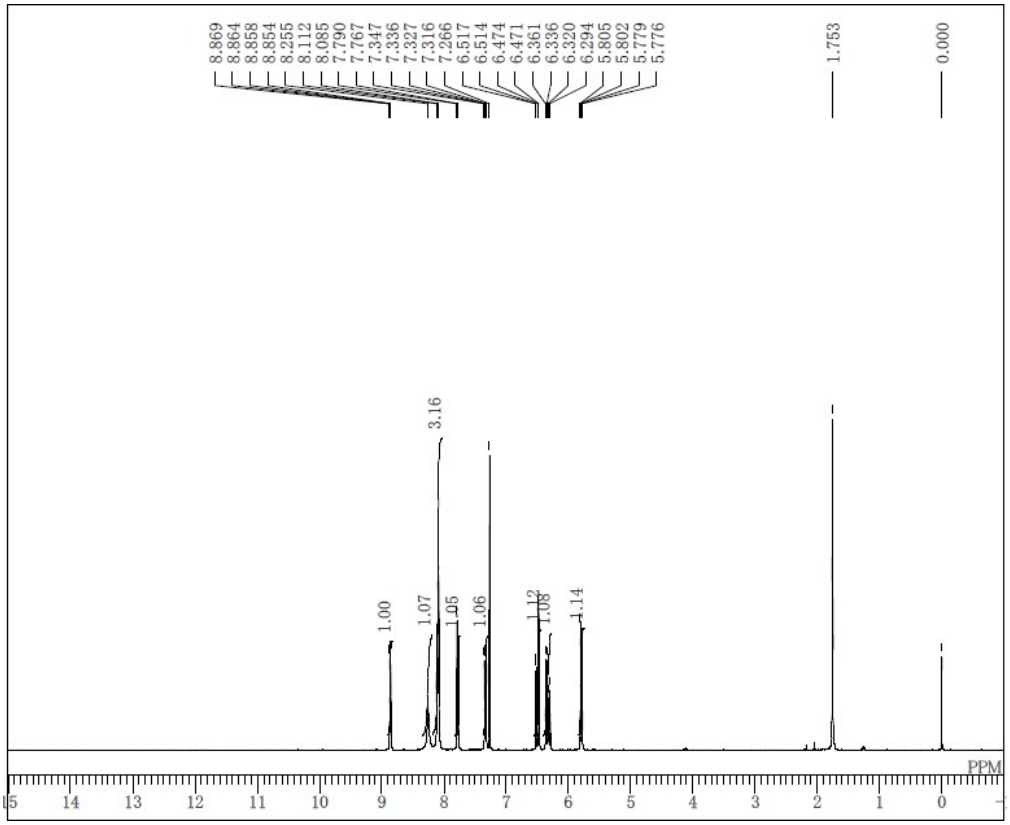

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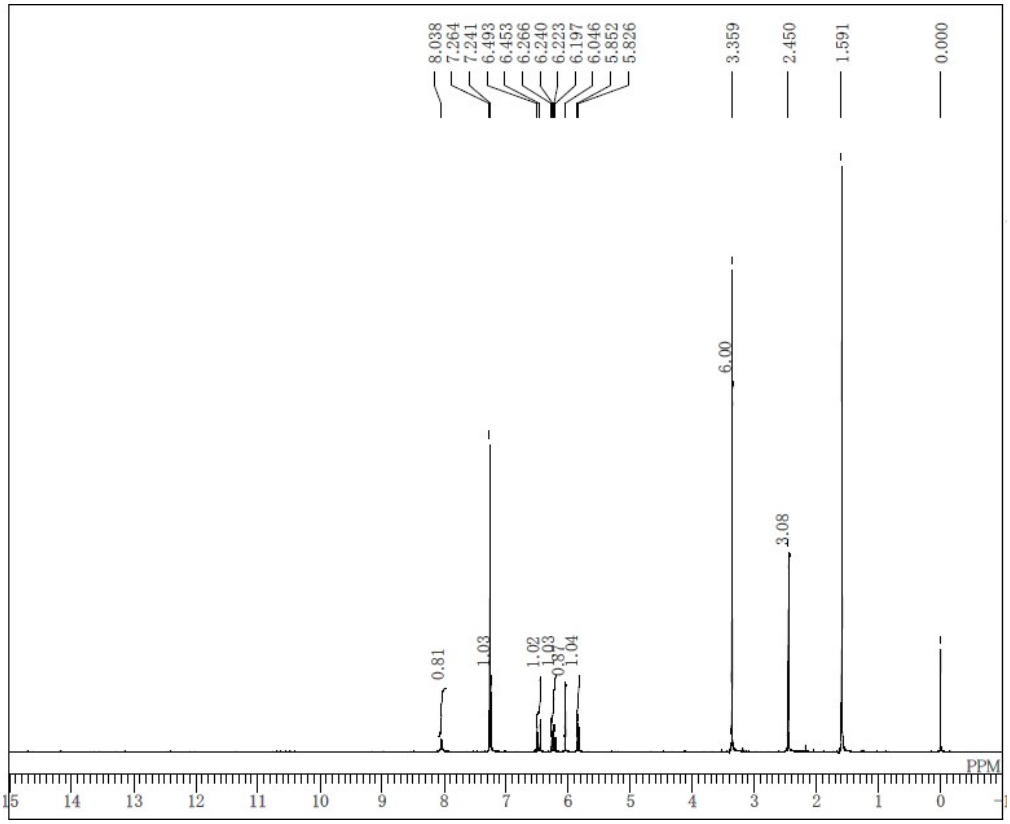
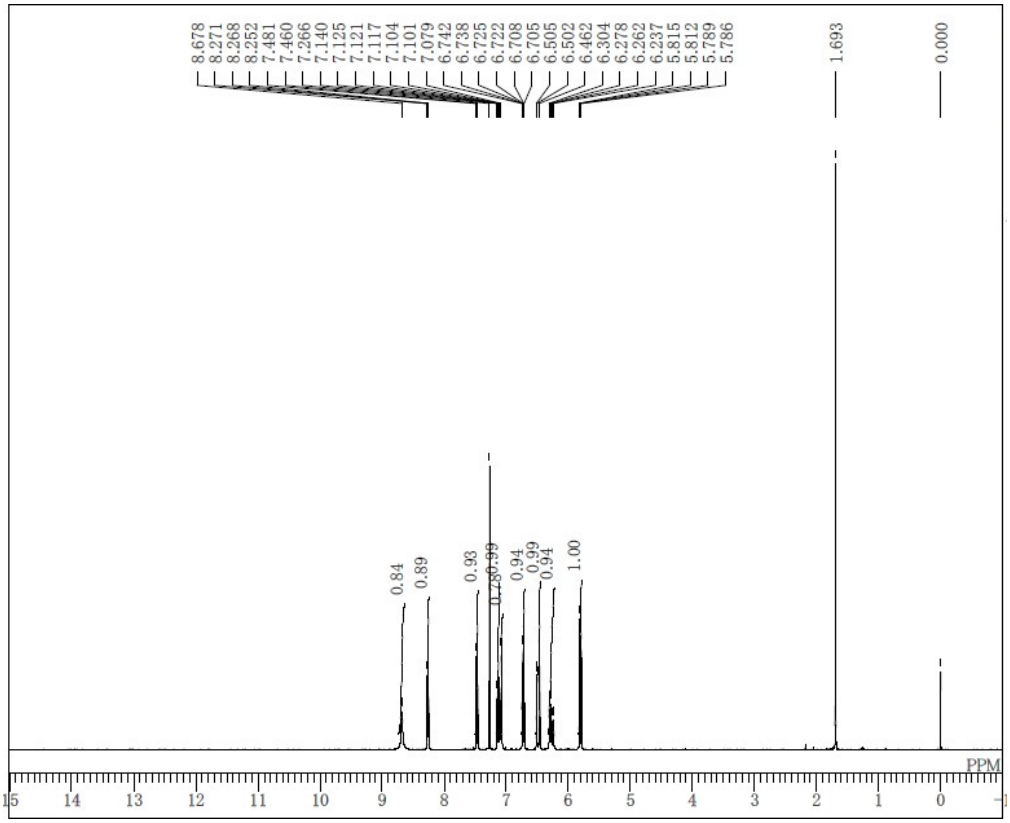
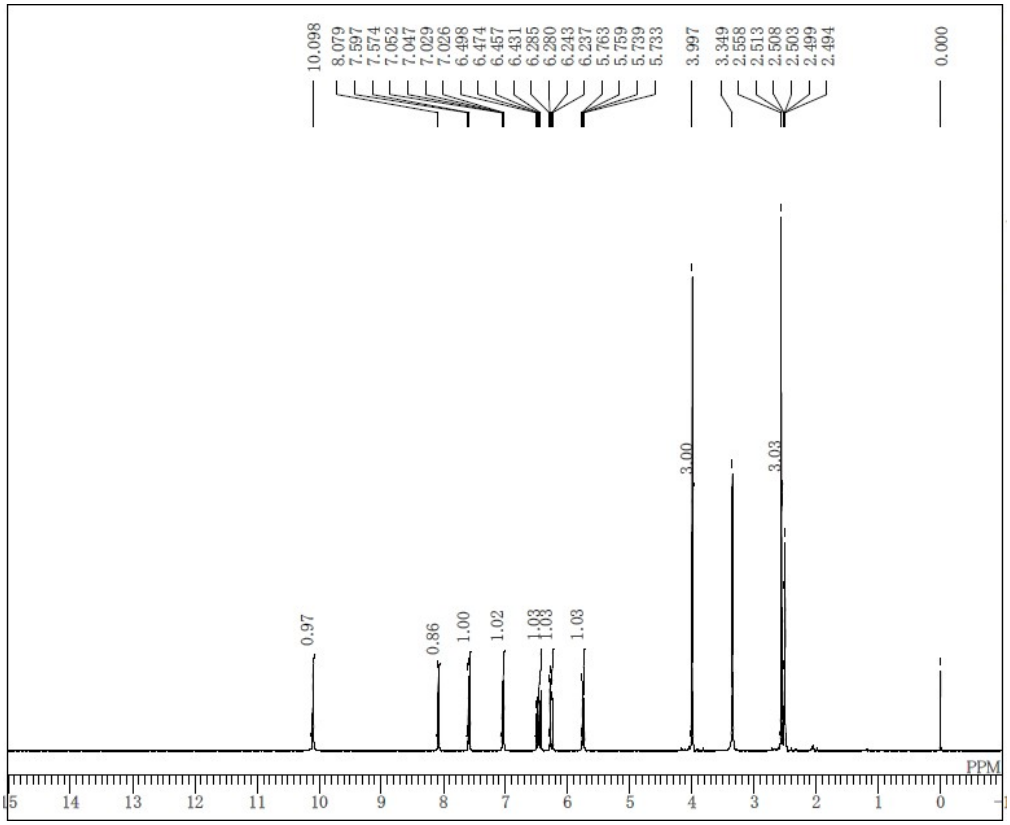


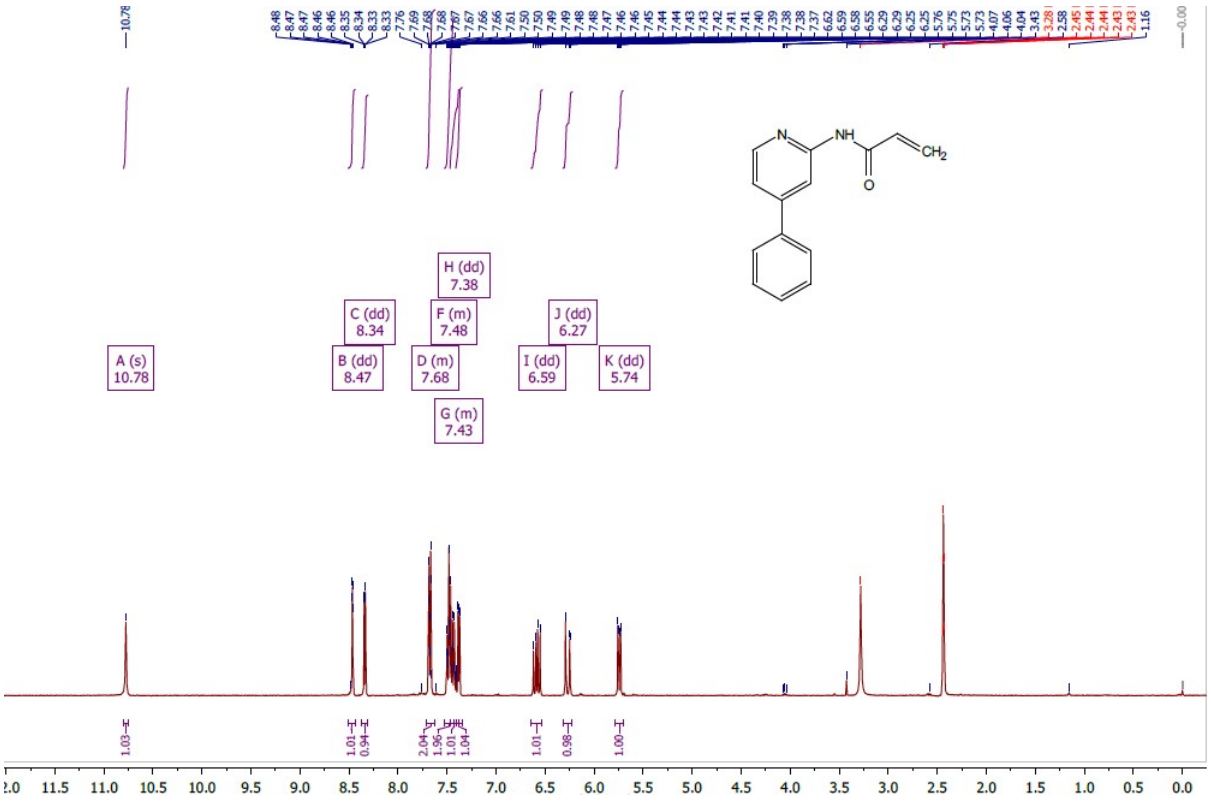
10_^{1}HNMR



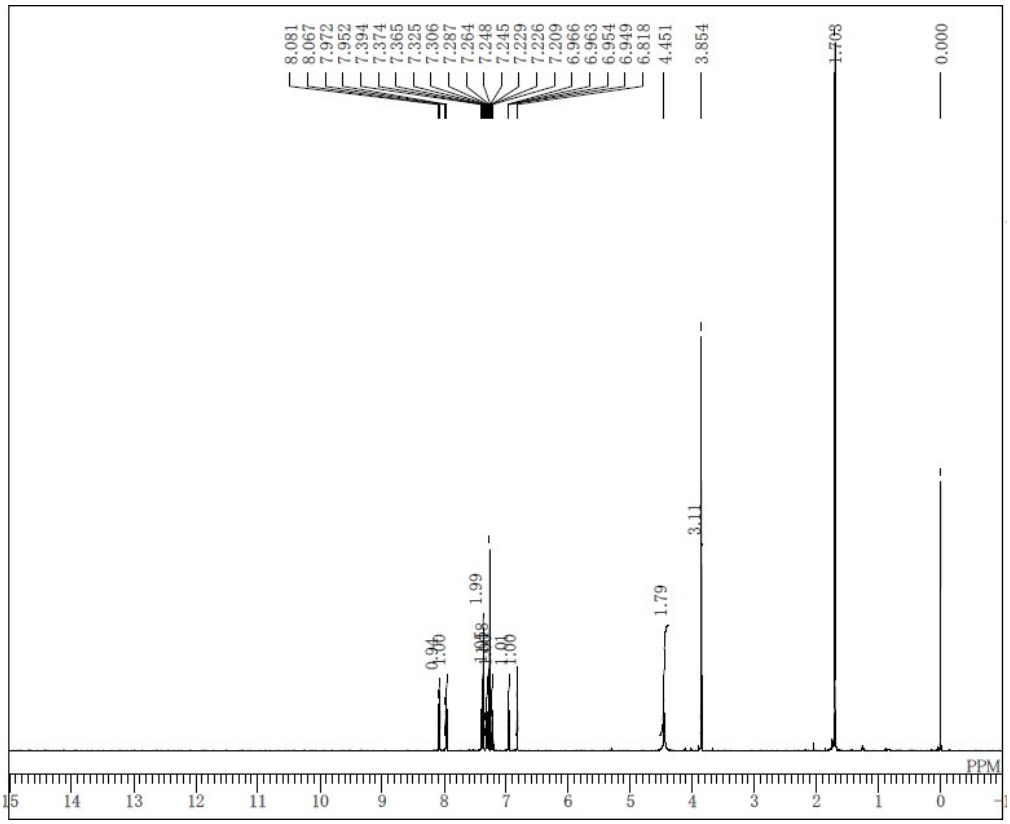
11_^{1}HNMR



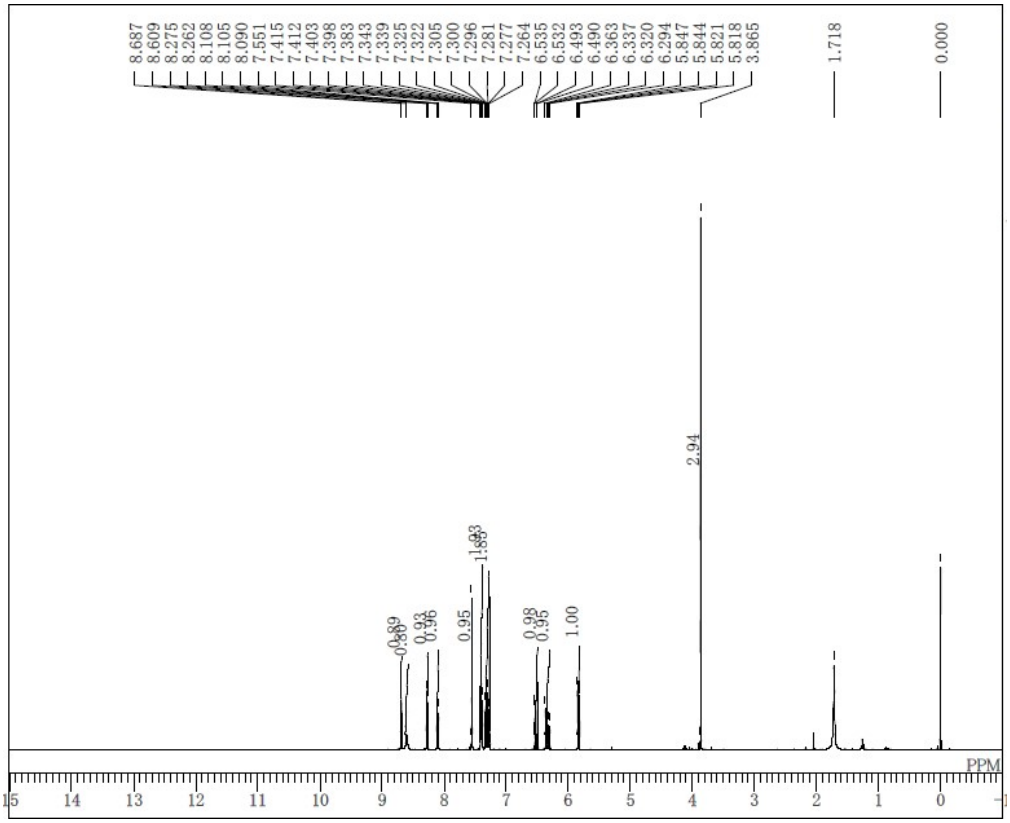
12_^{1}HNMR



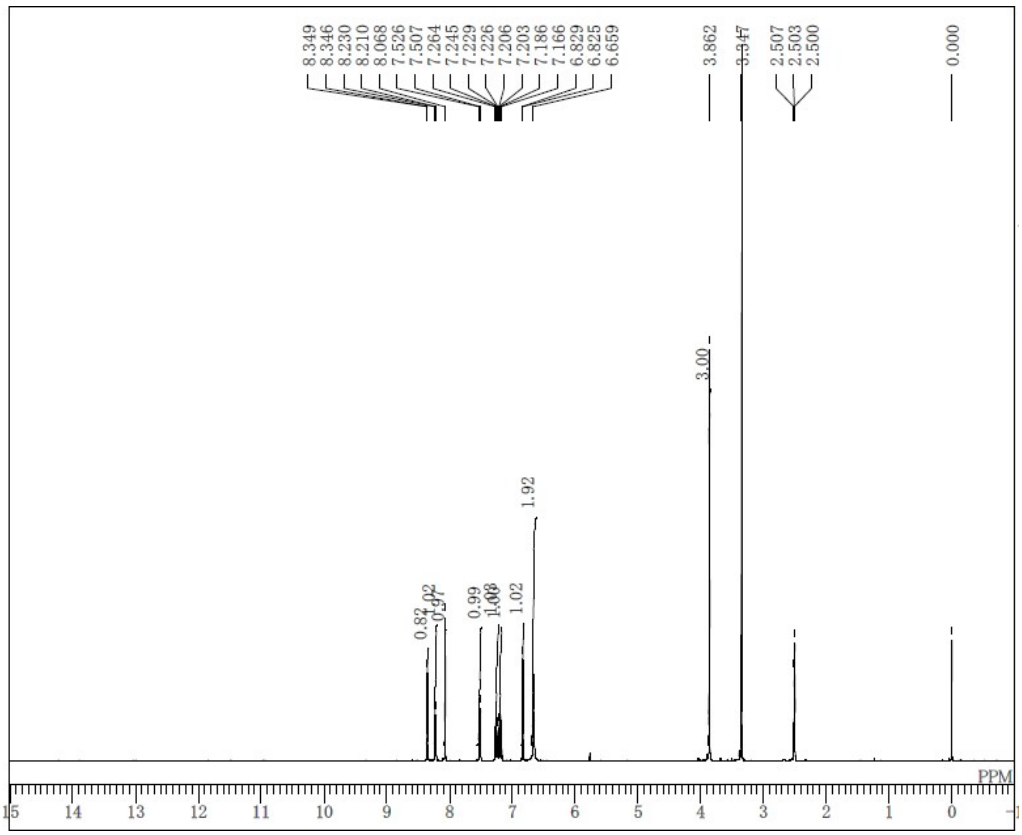
S-2_^{1}HNMR



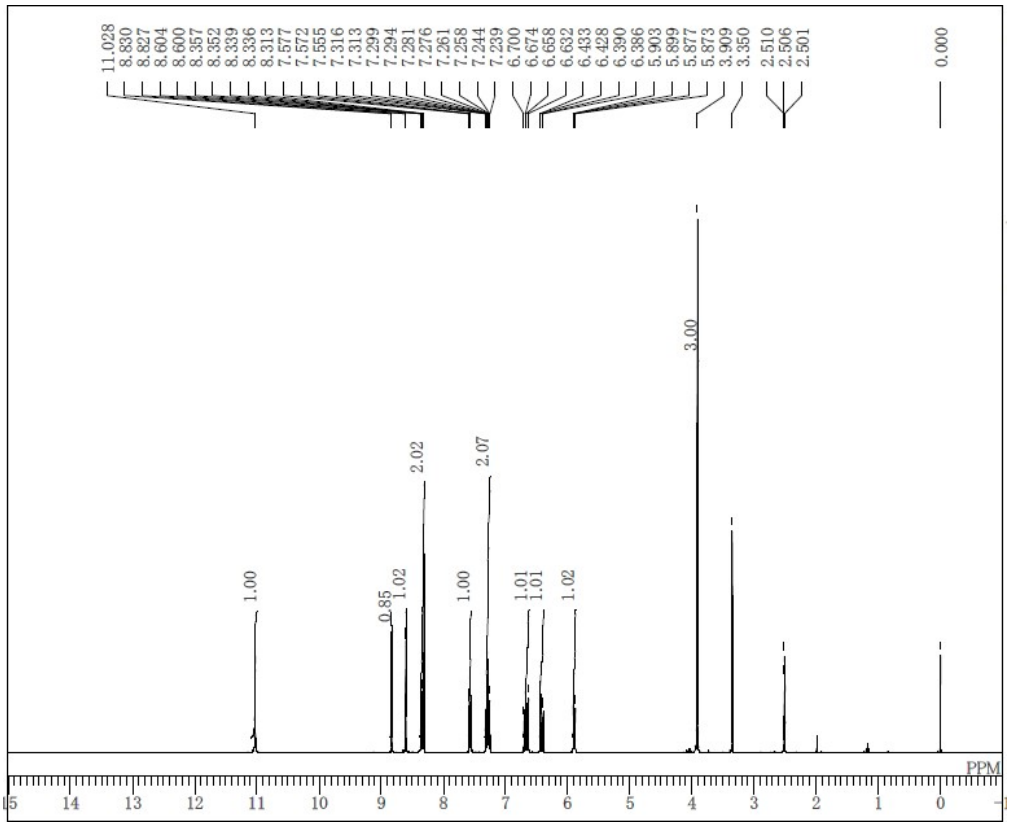
13_^{1}HNMR



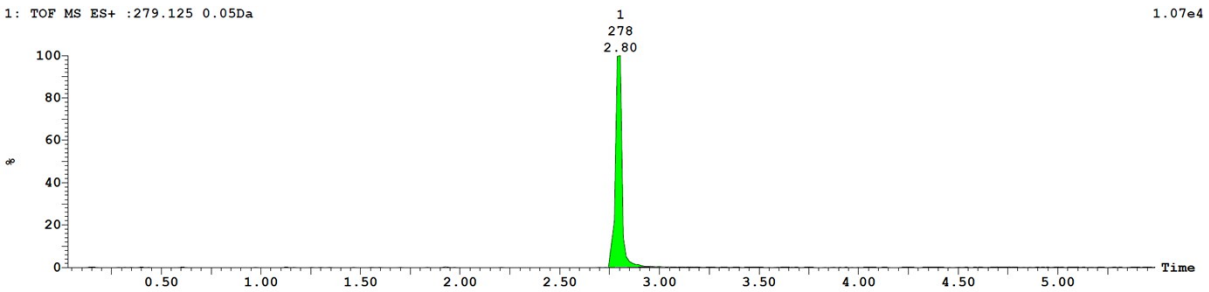
S-3_^{1}HNMR



14_^{1}HNMR

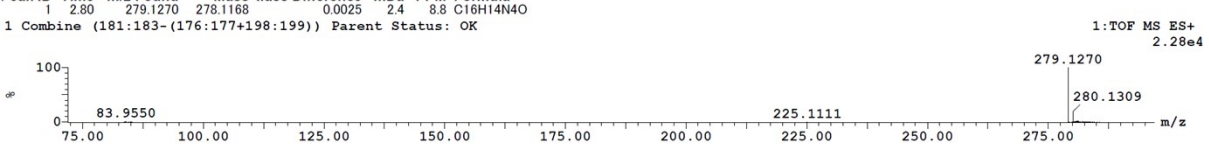


14_ESI-HRMS



Peak ID	Time	m/z Found	Mass	Mass Difference	mDa	PPM	Formula
1	2.80	279.1270	278.1168	0.0025	2.4	8.8	C16H14N4O

1 Combine (181:183-(176:177+198:199)) Parent Status: OK



Supplementary Figures

Figure S1: overlay of ATP binding pocket in the structures of EGFR, TTBK1 and MELK kinases

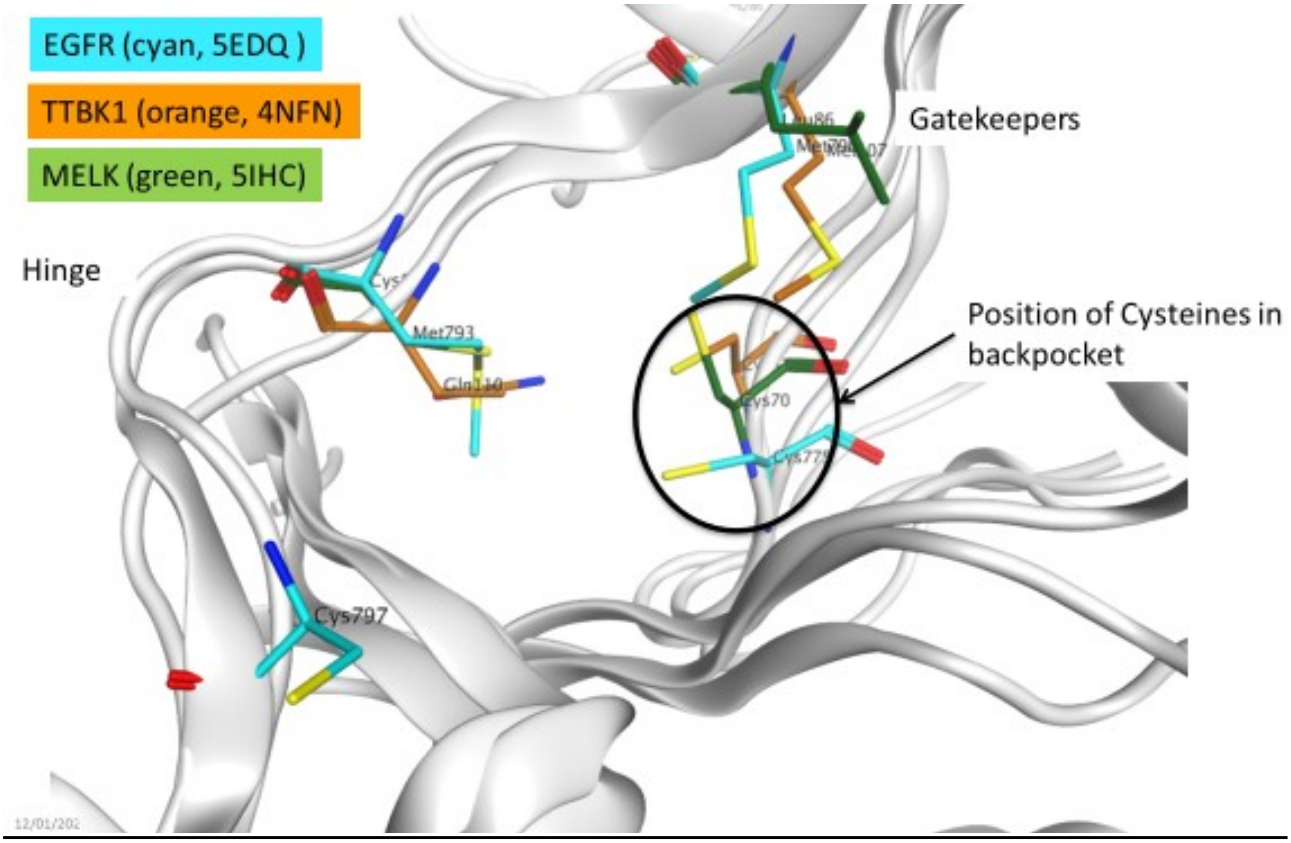
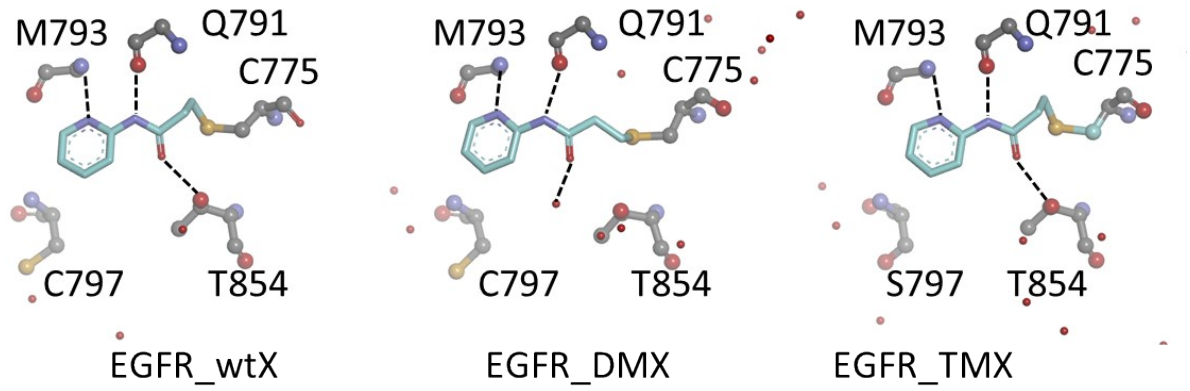
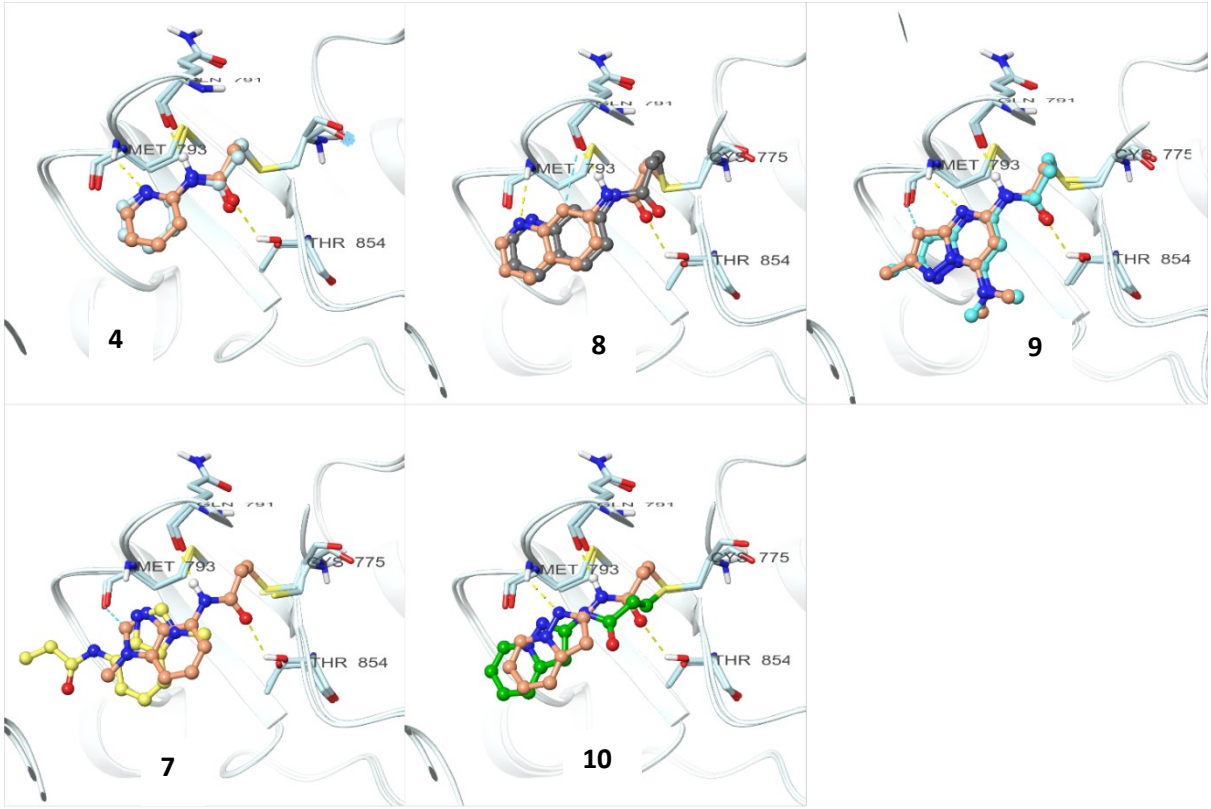


Figure S2: Comparison of the structure of **4** bound to EGFR_wtX, EGFR_DMX and EGFR_TMX



Labelled amino acids in ball and stick (grey carbon atoms) with main chain only for Q791 and M793, **4** in stick (light blue carbons), hydrogen bonds in dashed lines in the crystal structures of EGFR_wtX (PDB code: 8HV2), EGFR_DMX (PDB code: 8HV3) and EGFR_TMX (PDB code: 8HV4)

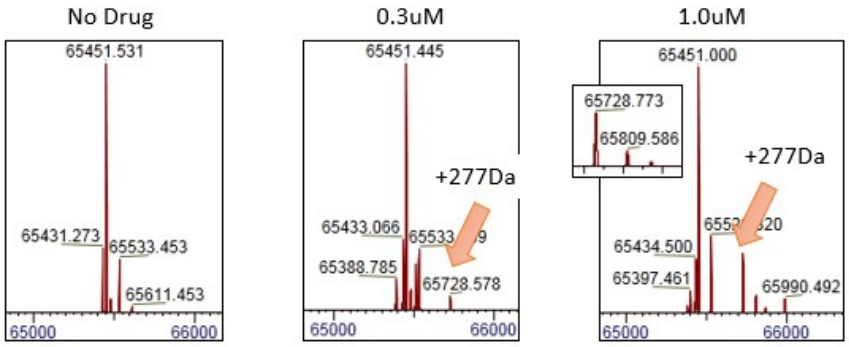
Figure S3. Comparison of the predicted binding pose of acrylamide compounds with that observed in crystal structures



Compound	**4**	**7**	**8**	**9**	**10**
PDB code	8HV4	8HV5	8HV6	8HV7	8HV8

Figure S4: MS of EGFR_SM2 and EGFR_DM2 incubated with compound **14**

EGFR_SM2



EGFR_DM2

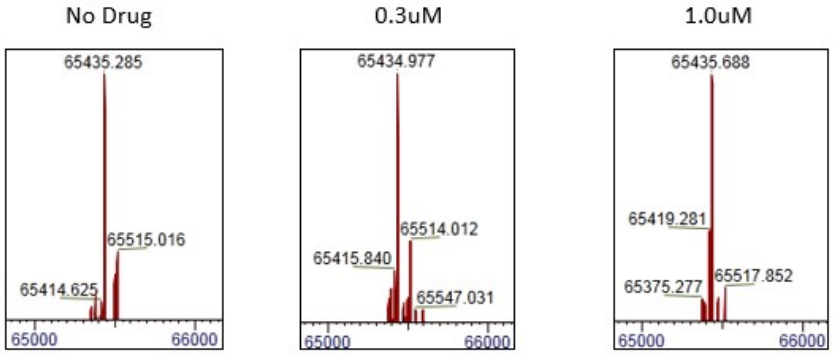
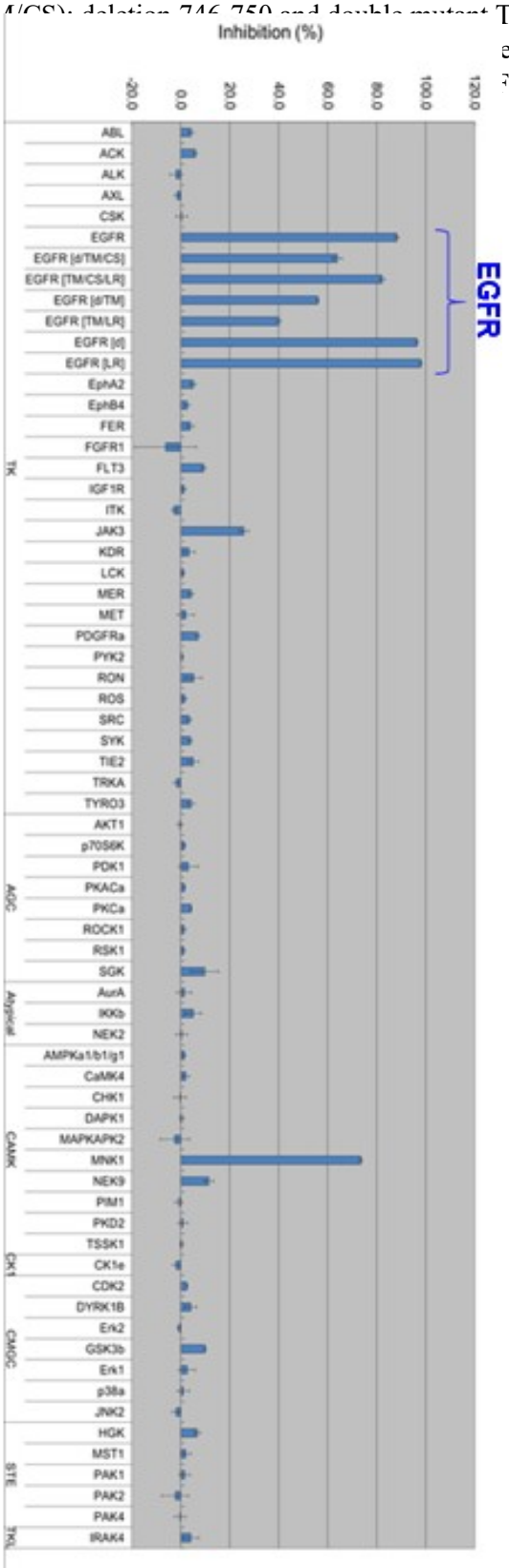


Figure S5: Inhibition of a panel of 68 kinases by compound **14**

EGFR: EGFR_wt, EGFR(*d*/TM/CS): deletion 746-750 and double mutant T790M and C797S,
EGFR(TM/CS/LR): EGFR_TM e mutant T790M,
EGFR(TM/LR): EGFR_DM, E R_SM2



Supplementary Table

Table S1 Data collection

Compound	Data collection	Space group, Unit cell dimensions	Resolution (A)[a]	Completeness (%)[a]	Unique reflections	Redundancy	Rmerge [a, b]	<I/σ(I)> [a, c]	Protein
1	Soleil PX2	I23 a=b=c=144.9	2.26 – 45.8 (2.26 – 2.32)	99.7 (96.1)	23885 (1714)	10.4 (10.0)	0.061 (1.56)	23.2 (1.4)	EGFR_DMX
4	DLS I04-1	I23 a=b=c=145.5	2.80 – 59.4 (2.80 – 2.88)	90.6 (56.2)	11590 (584)	10.8 (11.9)	0.124 (2.12)	15.4 (1.3)	EGFR_wt
4	D8Venture (Bruker)	I23 a=b=c=145.0	2.30 – 21.9 (2.30 – 2.40)	99.8 (100)	22648 (2698)	7.1 (4.6)	0.162 (0.820)	9.3 (1.4)	EGFR_DMX
4	D8Venture (Bruker)	I23 a=b=c=144.7	2.15 – 21.3 (2.15 – 2.25)	99.8 (100)	27464 (3472)	9.6 (7.7)	0.100 (0.647)	13.2 (1.8)	EGFR_TMX
7	Soleil PX1	I23 a=b=c=144.7	2.06 – 45.8 (2.06 – 2.12)	99.5 (98.3)	30952 (2257)	13.8 (13.3)	0.055 (1.39)	22.7 (1.7)	EGFR_DMX
8	D8Venture (Bruker)	I23 a=b=c=143.8	2.00 – 20.8 (2.00 – 2.10)	98.9 (93.2)	33119 (4198)	13.3 (6.5)	0.137 (0.626)	12.9 (2.4)	EGFR_TMX
9	D8Venture (Bruker)	I23 a=b=c=144.9	2.69 – 21.4 (2.69 – 2.79)	99.6 (100)	14176 (1464)	8.8 (8.0)	0.139 (0.485)	12.5 (3.4)	EGFR_TMX
10	D8Venture (Bruker)	I23 a=b=c=144.0	2.20 – 21.2 (2.20 – 2.30)	99.8 (100)	25290 (3124)	15.0 (12.1)	0.163 (0.747)	13.0 (2.0)	EGFR_TMX
12	D8Venture (Bruker)	I23 a=b=c=144.4	2.40 – 21.3 (2.40 – 2.50)	94.6 (99.7)	18682 (2249)	4.1 (3.5)	0.152 (0.684)	6.8 (1.2)	EGFR_TMX
14	MicroMax-007 (Rigaku)	I23 a=b=c=144.7	2.77 – 14.93 (2.77 – 2.92)	99.0 (99.8)	12847 (1879)	6.1 (6.8)	0.229 (2.065)	7.8 (0.6)	EGFR_TMX

[a] Values in parentheses are for highest resolution shells

[b] Rmerge = $\sum(I-\langle I\rangle) / \sum I$

[c] Signal to noise ratio of intensities.

Table S2: Procurement of tested kinases.

Kinase	Manufacturer	Catalog No.
ABL	Carna Biosciences, Inc.	08-001
ACK	Carna Biosciences, Inc.	08-196
AKT1	Carna Biosciences, Inc.	01-101
ALK	Carna Biosciences, Inc.	08-518
AMPKa1/b1/g1	Carna Biosciences, Inc.	02-113
AurA	Carna Biosciences, Inc.	05-101
AXL	Carna Biosciences, Inc.	08-107
CaMK4	Carna Biosciences, Inc.	02-108
CDK2	Carna Biosciences, Inc.	04-103
CHK1	Carna Biosciences, Inc.	02-117
CK1e	Carna Biosciences, Inc.	03-104
CSK	Carna Biosciences, Inc.	08-111
DAPK1	Carna Biosciences, Inc.	02-134
DYRK1B	Carna Biosciences, Inc.	04-131
EGFR	Carna Biosciences, Inc.	08-115
EphA2	Carna Biosciences, Inc.	08-121
EphB4	Carna Biosciences, Inc.	08-131
Erk1	Carna Biosciences, Inc.	04-142
Erk2	Carna Biosciences, Inc.	04-143
FER	Carna Biosciences, Inc.	08-139
FGFR1	Carna Biosciences, Inc.	08-133
FLT3	Carna Biosciences, Inc.	08-154
GSK3b	Carna Biosciences, Inc.	04-141
HGK	Carna Biosciences, Inc.	07-137
IGF1R	Carna Biosciences, Inc.	08-141
IKKb	Carna Biosciences, Inc.	05-084
IRAK4	Carna Biosciences, Inc.	09-145
ITK	Carna Biosciences, Inc.	08-181
JAK3	Carna Biosciences, Inc.	08-046
JNK2	Carna Biosciences, Inc.	04-164
KDR	Carna Biosciences, Inc.	08-191
LCK	Carna Biosciences, Inc.	08-170
MAPKAPK2	Carna Biosciences, Inc.	02-142
MER	Carna Biosciences, Inc.	08-108
MET	Carna Biosciences, Inc.	08-151
MNK1	Carna Biosciences, Inc.	02-145
MST1	Carna Biosciences, Inc.	07-116
NEK2	Carna Biosciences, Inc.	05-226
NEK9	Carna Biosciences, Inc.	05-133
p38a	Carna Biosciences, Inc.	04-152

p70S6K	Carna Biosciences, Inc.	01-154
PAK1	Carna Biosciences, Inc.	07-123
PAK2	Carna Biosciences, Inc.	07-124
PAK4	Carna Biosciences, Inc.	07-126
PDGFRa	Carna Biosciences, Inc.	08-157
PDK1	Invitrogen	P3001
PIM1	Carna Biosciences, Inc.	02-054
PKACa	Carna Biosciences, Inc.	01-127
PKCa	Carna Biosciences, Inc.	01-133
PKD2	Carna Biosciences, Inc.	02-158
PYK2	Carna Biosciences, Inc.	08-138
ROCK1	Carna Biosciences, Inc.	01-109
RON	Carna Biosciences, Inc.	08-152
ROS	Carna Biosciences, Inc.	08-163
RSK1	Carna Biosciences, Inc.	01-149
SGK	Carna Biosciences, Inc.	01-158
SRC	Carna Biosciences, Inc.	08-173
SYK	Carna Biosciences, Inc.	08-176
TIE2	Carna Biosciences, Inc.	08-185
TRKA	Carna Biosciences, Inc.	08-186
TSSK1	Carna Biosciences, Inc.	02-364
TYRO3	Carna Biosciences, Inc.	08-109
EGFR [d746-750/T790M/C797S]	Daiichi Sankyo RD Novare	-
EGFR [T790M/C797S/L858R]	Daiichi Sankyo RD Novare	-
EGFR [d746-750/T790M]	Carna Biosciences, Inc.	08-528
EGFR [T790M/L858R]	Carna Biosciences, Inc.	08-510
EGFR [d746-750]	Carna Biosciences, Inc.	08-527
EGFR [L858R]	Carna Biosciences, Inc.	08-502

Table S3: Sequences of Substrate Peptides.

Peptide Name	Manufacturer	Sequence	Catalog No.
FL-Peptide 1	PerkinElmer, Inc.	5FAM-AKRRRLSSLRA-COOH	760345
FL-Peptide 2	PerkinElmer, Inc.	5FAM-EAIYAAPFAKKK-CONH2	760346
FL-Peptide 4	PerkinElmer, Inc.	5FAM-EGIYGVLFKKK-CONH2	760348
FL-Peptide 6	PerkinElmer, Inc.	5FAM-GRPRTSSFAEG-CONH2	760350
FL-Peptide 7	PerkinElmer, Inc.	5FAM-HMRSAMSGLHLVKRR-COOH	760351
FL-Peptide 8	PerkinElmer, Inc.	5FAM-IPTSPITTTYFFFKKK-COOH	760352
FL-Peptide 10	PerkinElmer, Inc.	5FAM-KKKVSRSGLYRSPSMPENLNRPR-COOH	760354
FL-Peptide 11	PerkinElmer, Inc.	5FAM-KKLNRTLSVA-COOH	760355
FL-Peptide 12	PerkinElmer, Inc.	5FAM-KKLRRTLSVA-COOH	760356
FL-Peptide 14	PerkinElmer, Inc.	5FAM-KRELVEPLTPSGEAPNQALLR-CONH2	760358
FL-Peptide 15	PerkinElmer, Inc.	5FAM-KRREILSRRPpSYR-COOH	760359
FL-Peptide 16	PerkinElmer, Inc.	5FAM-KRRRALpSVASLPGL-CONH2	760360
FL-Peptide 19	PerkinElmer, Inc.	5FAM-RFARKGSLRQKNV-COOH	760363
FL-Peptide 20	PerkinElmer, Inc.	5FAM-RSRHSSYPAGT-CONH2	760364
FL-Peptide 21	PerkinElmer, Inc.	5FAM-LRRASLG-CONH2	760365
FL-Peptide 24	PerkinElmer, Inc.	5FAM-KKISGRLSPIMTEQ-CONH2	760387
FL-Peptide 25	PerkinElmer, Inc.	5FAM-VDGKEIYNTIRRK-CONH2	760388
FL-Peptide 26	PerkinElmer, Inc.	5FAM-ARKRERTYSFGHHA-COOH	760389
FL-Peptide 27	PerkinElmer, Inc.	5FAM-EFPIYDFLPAKKK-CONH2	760424
FL-Peptide 29	PerkinElmer, Inc.	5FAM-GGGPATPKKAKKL-CONH2	760429
FL-Peptide 30	PerkinElmer, Inc.	5FAM-KKKKEEIYFFF-CONH2	760430
FL-Peptide 31	PerkinElmer, Inc.	5FAM-RRRLSFAEPG-CONH2	760480
FL-Peptide 32	PerkinElmer, Inc.	5FAM-FLAKSFGSPNRAYKK-CONH2	760641
FL-T308Tide	Invitrogen	FLC-KTFCGTPEYLAPEVRR-COOH	- (custom synthesis)
Srctide	PEPTIDE INSTITUTE, INC.	FITC-EEPLYWSFPAKKK-CONH2	- (custom synthesis)
IRS1	PEPTIDE INSTITUTE, INC.	FITC-KKSRGDYMTMQIG-CONH2	- (custom synthesis)

Table S4: Assay conditions for tested kinases.

Kinase	Kinase concentration (ng/mL)	Substrate Peptide	Additives
ABL	75	FL-Peptide 2	10 mM $MgCl_2$
ACK	1500	FL-Peptide 27	10 mM $MgCl_2$
AKT1	15	FL-Peptide 6	10 mM $MgCl_2$
ALK	85	IRS1	10 mM $MgCl_2$
AMPKa1/b1/g1	600	FL-Peptide 7	10 mM $MgCl_2$
AurA	120	FL-Peptide 21	10 mM $MgCl_2$
AXL	120	FL-Peptide 30	10 mM $MgCl_2$
CaMK4	2000	FL-Peptide 11	10 mM $MgCl_2$, 0.5 mM $CaCl_2$,
			10 µg/mL Calmodulin
CDK2	20	FL-Peptide 29	10 mM $MgCl_2$
CHK1	50	FL-Peptide 10	10 mM $MgCl_2$
CK1e	890	FL-Peptide 16	10 mM $MgCl_2$
CSK	600	Srctide	10 mM $MgCl_2$, 10 mM $MnCl_2$
DAPK1	148.9	FL-Peptide 1	10 mM $MgCl_2$
DYRK1B	700	FL-Peptide 24	10 mM $MgCl_2$
EGFR	800	Srctide	10 mM $MgCl_2$, 10 mM $MnCl_2$
EphA2	600	FL-Peptide 27	10 mM $MgCl_2$
EphB4	250	FL-Peptide 27	10 mM $MgCl_2$
Erk1	150	FL-Peptide 8	10 mM $MgCl_2$
Erk2	90	FL-Peptide 8	10 mM $MgCl_2$
FER	400	Srctide	10 mM $MgCl_2$
FGFR1	300	IRS1	10 mM $MgCl_2$
FLT3	100	FL-Peptide 2	10 mM $MgCl_2$
GSK3b	50	FL-Peptide 15	10 mM $MgCl_2$
HGK	30	FL-Peptide 25	10 mM $MgCl_2$
IGF1R	60	IRS1	10 mM $MgCl_2$
IKKb	5010	FL-Peptide 1	10 mM $MgCl_2$
IRAK4	750	FL-Peptide 8	10 mM $MnCl_2$
ITK	350	FL-Peptide 27	10 mM $MgCl_2$
JAK3	50	Srctide	10 mM $MgCl_2$
JNK2	1295	FL-Peptide 14	10 mM $MgCl_2$
KDR	30	Srctide	10 mM $MgCl_2$, 0.05% CHAPSO, 0.01% BSA
LCK	50	FL-Peptide 4	10 mM $MgCl_2$
MAPKAPK2	30	FL-Peptide 12	10 mM $MgCl_2$
MER	30	FL-Peptide 27	10 mM $MgCl_2$
MET	90	FL-Peptide 30	10 mM $MgCl_2$
MNK1	440	FL-Peptide 10	10 mM $MgCl_2$
MST1	30	FL-Peptide 25	10 mM $MgCl_2$
NEK2	500	FL-Peptide 32	10 mM $MgCl_2$

NEK9	756	FL-Peptide 32	10 mM $MgCl_2$
p38a	225	FL-Peptide 8	10 mM $MgCl_2$
p70S6K	30	FL-Peptide 26	10 mM $MgCl_2$
PAK1	313	FL-Peptide 1	10 mM $MgCl_2$
PAK2	285	FL-Peptide 1	10 mM $MgCl_2$
PAK4	700	FL-Peptide 31	10 mM $MgCl_2$
PDGFRa	600	FL-Peptide 30	10 mM $MgCl_2$
PDK1	600	FL-T308Tide	10 mM $MgCl_2$, 2 μM PIFtide
PIM1	20	FL-Peptide 20	10 mM $MgCl_2$
PKACa	7	FL-Peptide 21	10 mM $MgCl_2$
PKCa	6	FL-Peptide 19	10 mM $MgCl_2$, 0.1 mM $CaCl_2$,
			10 μg/mL PS
PKD2	20	FL-Peptide 12	10 mM $MgCl_2$
PYK2	200	FL-Peptide 27	10 mM $MgCl_2$
ROCK1	225	FL-Peptide 1	10 mM $MgCl_2$
RON	280	Srctide	10 mM $MgCl_2$
ROS	20	Srctide	10 mM $MgCl_2$
RSK1	1390	FL-Peptide 11	10 mM $MgCl_2$
SGK	15	FL-Peptide 6	10 mM $MgCl_2$
SRC	74.8	FL-Peptide 4	10 mM $MgCl_2$
SYK	600	Srctide	10 mM $MgCl_2$
TIE2	150	FL-Peptide 27	10 mM $MgCl_2$
TRKA	150	FL-Peptide 27	10 mM $MgCl_2$
TSSK1	500	FL-Peptide 10	10 mM $MgCl_2$
TYRO3	84	FL-Peptide 27	10 mM $MgCl_2$
EGFR [d746-750/T790M/C797S]	350	Srctide	5 mM $MgCl_2$, 1 mM $MnCl_2$
EGFR [T790M/C797S/L858R]	200	Srctide	5 mM $MgCl_2$, 1 mM $MnCl_2$
EGFR [d746-750/T790M]	180	Srctide	5 mM $MgCl_2$, 1 mM $MnCl_2$
EGFR [T790M/L858R]	500	Srctide	5 mM $MgCl_2$, 1 mM $MnCl_2$
EGFR [d746-750]	120	Srctide	5 mM $MgCl_2$, 1 mM $MnCl_2$
EGFR [L858R]	160	Srctide	5 mM $MgCl_2$, 1 mM $MnCl_2$

Table S5 Mass spectrometry of EGFR_TMX and EGFR_DMX incubated with acrylamide fragments at pH 7.4

Compound	% product[1] EGFR_TMX	Added mass	% di-addition	% product[1] EGFR_DMX	% di-addition
2	0			0	
3	5			100	
4	55	148	25	100	no
5	0			100	
6	0			100	
7	0			100	
8	45	198	15	100	0
9	0			ND	
10	25	187	15	100	0
11	0			25	

[1] percentage of the sample which had a mass greater than that of the original protein sample alone

Table S6: Summary of refin

PDB Code	Resolution (A)[a]	No. of reflections used in refinement[a]	Completeness (%)[a]	Rwork[a,d]	Rfree[a, d, e]	Average B-factor (A^2) All atoms / Ligand	Rms deviations from ideal[f] Bonds (A) / Angles (°)	Ramachandran plot Most favoured / Allowed / Outliers (%)[g]	Protein
8HV1	2.40 – 25.0 (2.40 – 2.53)	18909 (2842)	99.9 (100)	0.189 (0.358)	0.219 (0.365)	61.5 / 83.6	0.010 / 1.58	95.7 / 4.3 / 0.0	EGFR_DMX
8HV2	2.80 – 25.0 (2.80 – 2.95)	11019 (1326)	90.6 (73.6)	0.177 (0.314)	0.216 (0.387)	80.3 / 68.9	0.007 / 1.48	95.2 / 4.8 / 0.0	EGFR_wt
8HV3	2.40 – 20.0 (2.40 – 2.53)	18888 (2841)	99.4 (100)	0.212 (0.292)	0.250 (0.305)	44.5 / 60.9	0.010 / 1.66	96.3 / 3.4 / 0.3	EGFR_DMX
8HV4	2.20 – 20.0 (2.20 – 2.32)	24015 (3637)	98.4 (99.2)	0.237 (0.352)	0.275 (0.401)	42.5 / 43.7	0.011 / 1.67	96.3 / 3.7 / 0.0	EGFR_TMX
8HV5	2.20 – 20.0 (2.20 – 2.32)	24229 (3634)	99.4 (100)	0.186 (0.244)	0.224 (0.303)	57.3 / 80.5	0.011 / 1.57	98.0 / 2.0 / 0.0	EGFR_DMX
8HV6	2.20 – 20.0 (2.20 – 2.32)	23956 (3590)	99.6 (99.8)	0.247 (0.366)	0.296 (0.400)	36.4 / 54.4	0.011 / 1.73	97.0 / 3.0 / 0.0	EGFR_TMX
8HV7	2.69 – 21.4 (2.69 – 2.76)	13193 (1019)	97.6 (99.8)	0.218 (0.284)	0.290 (0.317)	41.0 / 46.6	0.009 / 1.53	96.2 / 3.8 / 0.0	EGFR_TMX
8HV8	2.40 – 20.0 (2.40 – 2.53)	18367 (2771)	99.1 (99.8)	0.232 (0.313)	0.283 (0.383)	40.4 / 62.5	0.010 / 1.66	97.3 / 2.7 / 0.0	EGFR_TMX
8HV9	2.50 – 20.0 (2.50 – 2.63)	15629 (2472)	94.0 (100)	0.253 (0.328)	0.274 (0.290)	40.1 / 60.2	0.009 / 1.60	96.0 / 3.7 / 0.3	EGFR_TMX
8HVA	2.77 – 14.9 (2.77 – 2.92)	12206 (1845)	98.5 (99.7)	0.242 (0.681)	0.251 (0.720)	69.8 / 70.8	0.007 / 1.52	94.5 / 5.1 / 0.3	EGFR_TMX

es in parentheses are for highest resolution shells

$\sum(F_{obs}-F_{calc}) / \sum F_{obs}$

percent of reflections were randomly chosen for calculation of R_{free}.

mean square deviations from ideal geometry[13]

efined in Procheck[14]

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