Supporting information for

An Activity-Based Oxaziridine Platform for Identifying and Developing Covalent Ligands for Functional Allosteric Methionine Sites: Redox-Dependent Inhibition of Cyclin-Dependent Kinase 4

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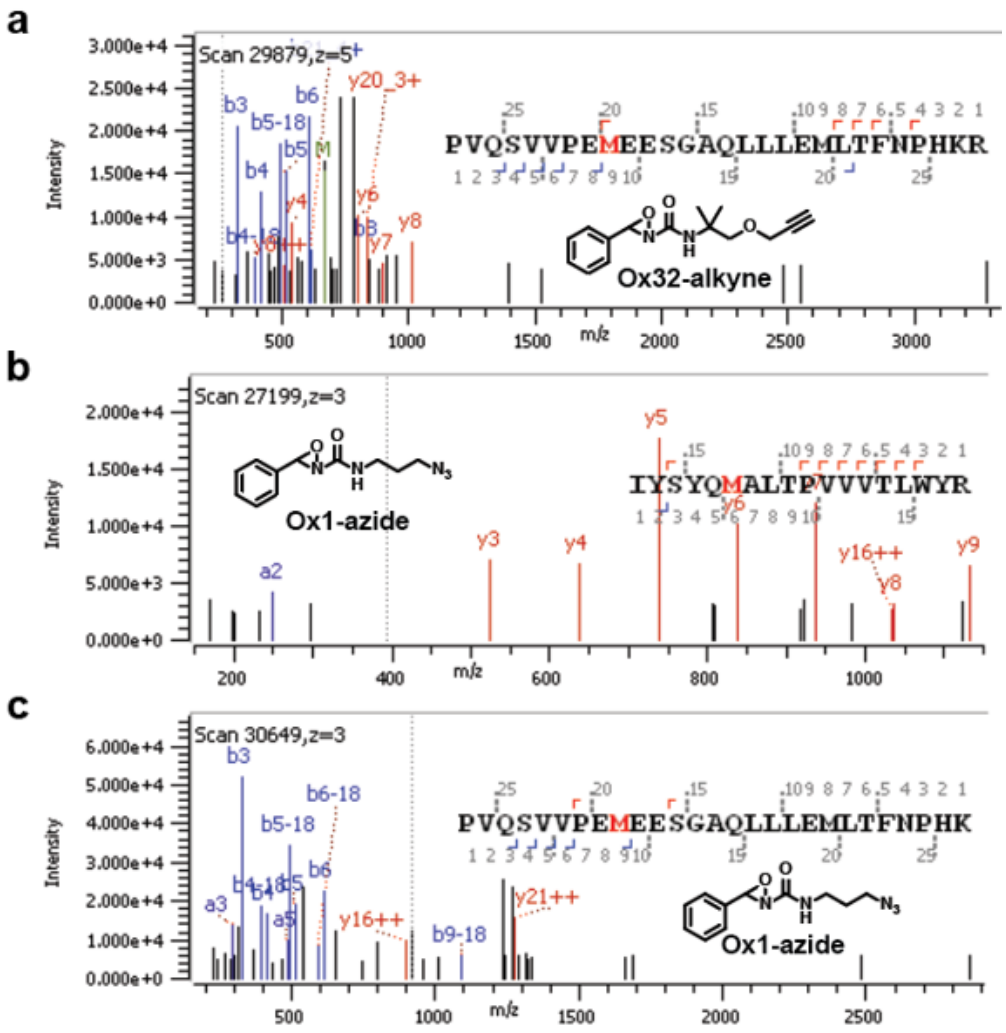
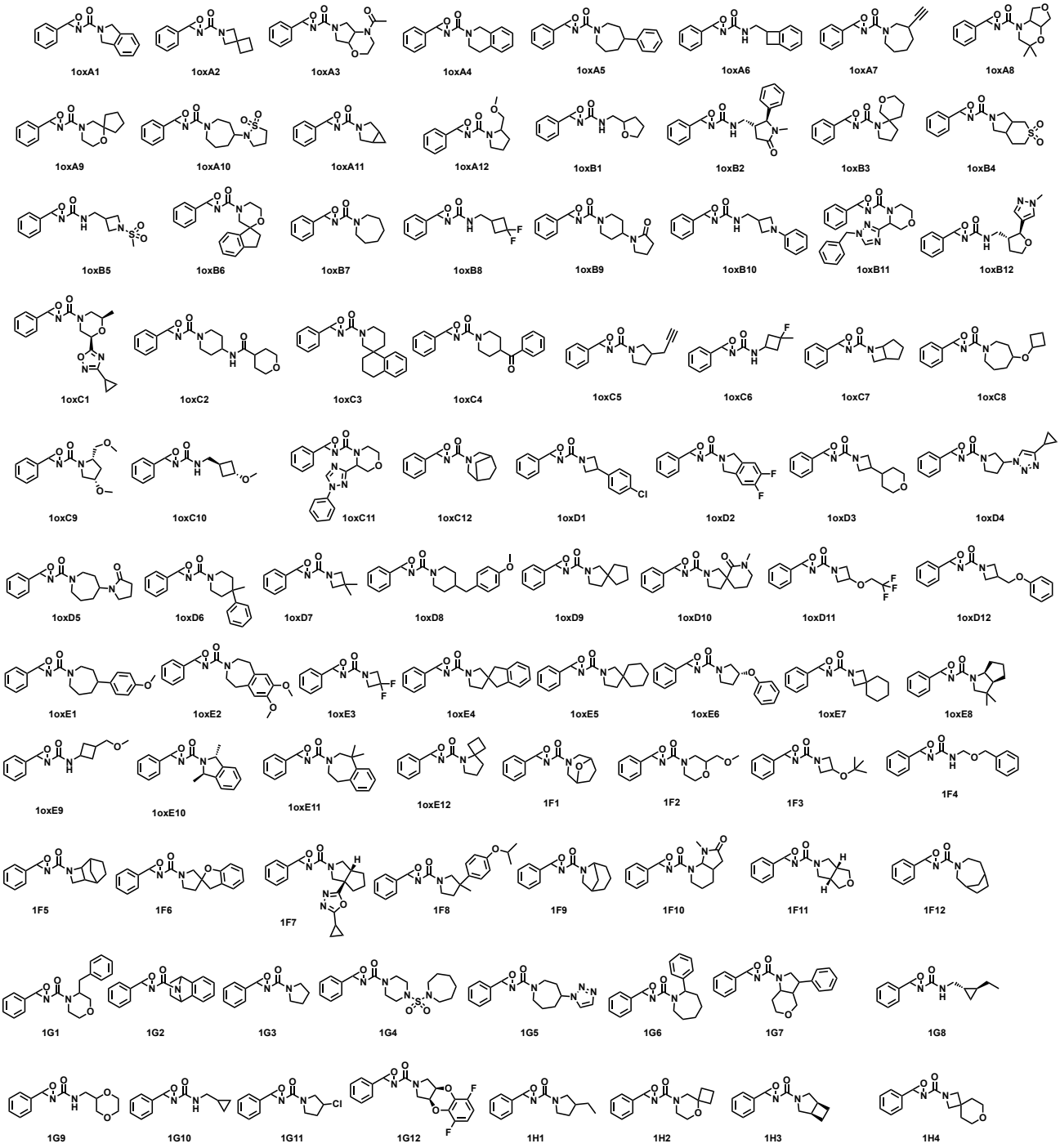


Figure S1. MS/MS of Ox1-azide and Ox32-alkyne modification on CDK4. All samples represent 50 μM probe treatment in 10 μg CDK4/CCND1 spiked into 90 μg mouse liver lysate. (a) Ox32-alkyne modification at Met264 of CDK4. (b) Ox1-azide modification at Met169 and (c) Met264 of CDK4.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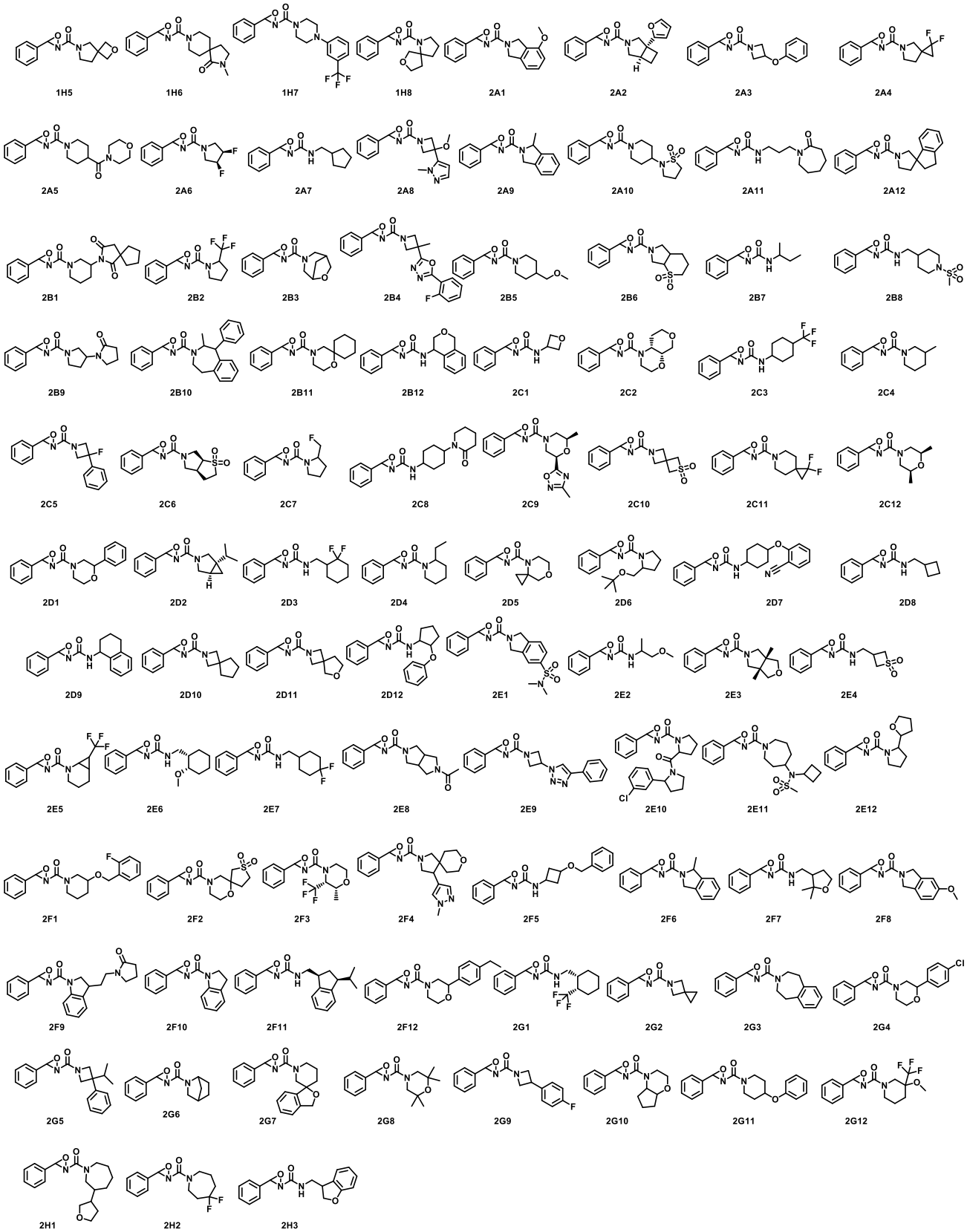


Figure S2. Oxaziridine fragment library structures**.**

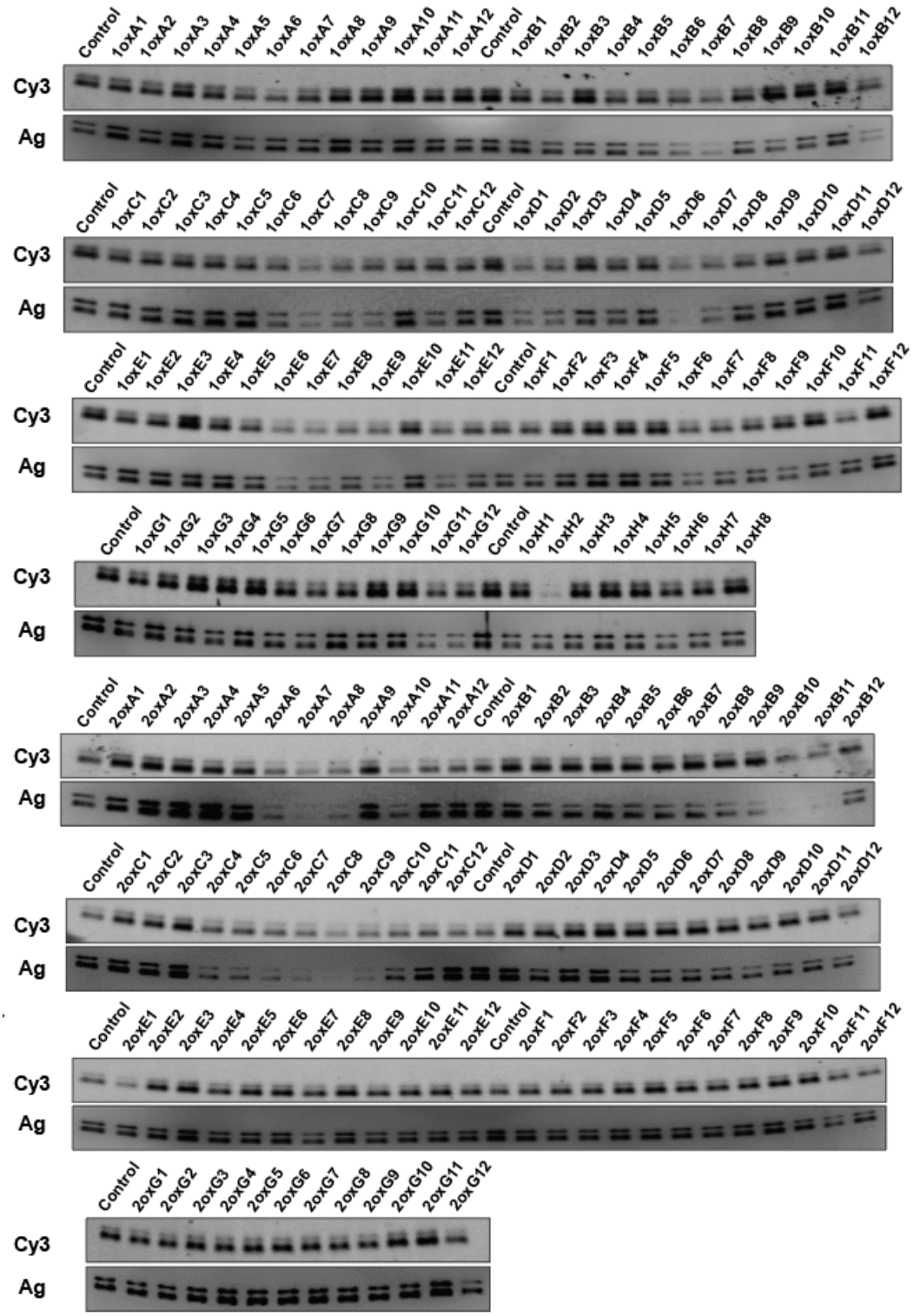


Figure S3. Oxaziridine library screen against CDK4-CCND1. Samples were treated according to gel-ABPP method outlined at 50 nM compound and 50 nM CDK4-CCND1.

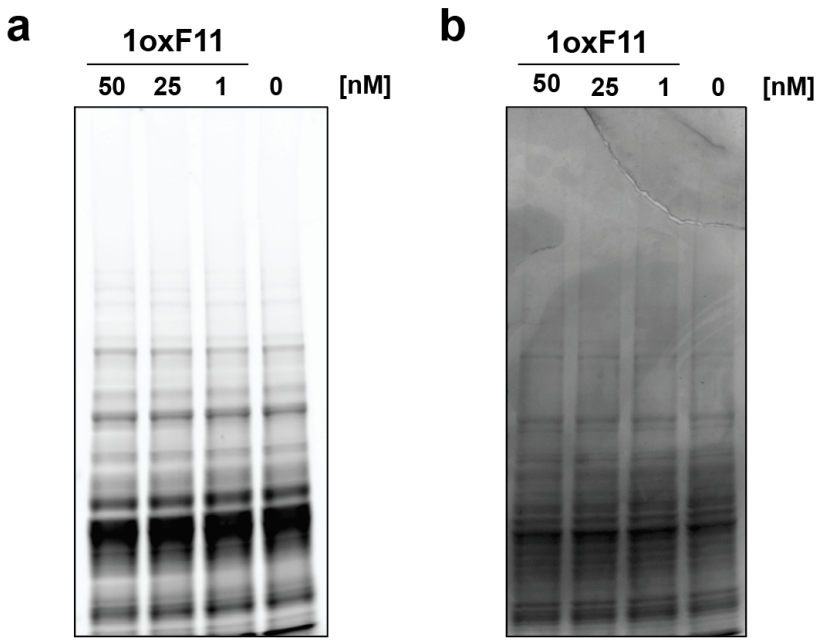


Figure S4. Aggregation test with 1oxF11. Lysates were treated with varying doses of 1oxF11 according to gel-ABPP method outlined. (a) Cy3 channel image, (b) silver stain image.

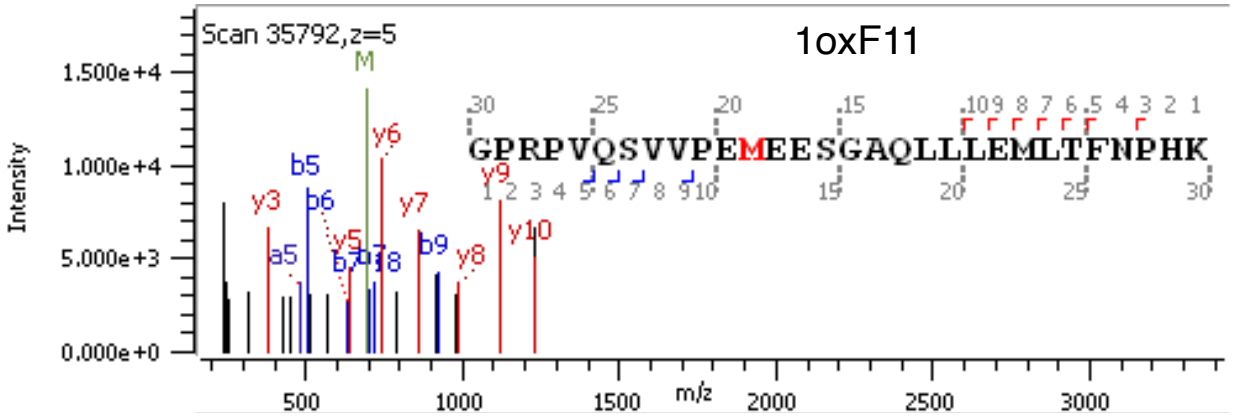


Figure S5. Additional MS/MS spectrum. MS/MS of CDK4, modified with 1oxF11 at M264. Isolated CDK4-CCND1 spiked in mouse liver lysate was treated with 1oxF11 as shotgun proteomics method outlined.

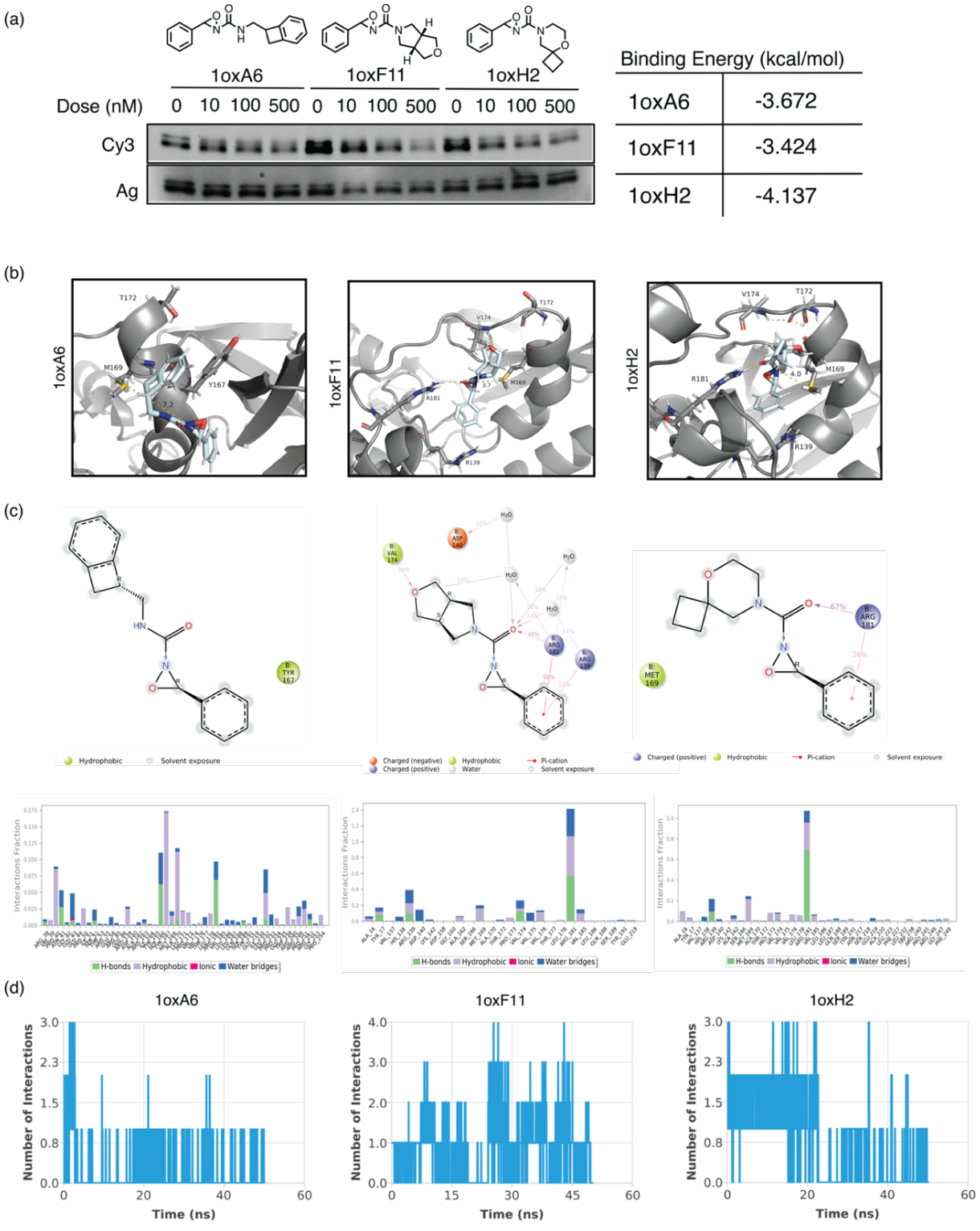


Figure S6. Fragment screening structure-activity relationship data. (a) Left: Gel-ABPP data of CDK4 binders. Right: Computed docking energies of CDK4 binders. (b) Non-covalent docking images of CDK4 binders (c) Top: Structure interaction diagrams of CDK4 binders with 10%

interaction time across a 50 ns MD simulation. Bottom: Non-covalent interactions by residue. (d) Hydrogen-bonding interaction map of CDK4 binders across a 50 ns MD simulation.

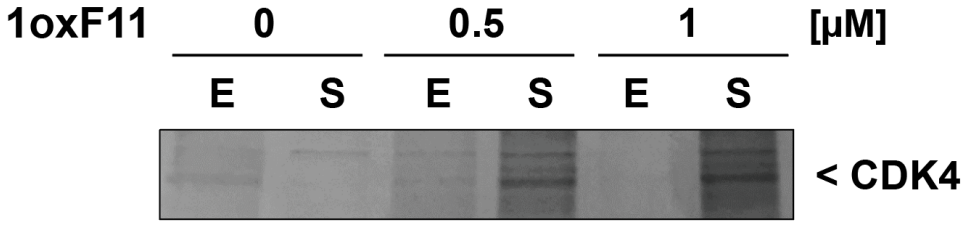


Figure S7. Competition of 1oxF11 and 1oxF11yne by gel. Lysate was collected from MCF-7 cells overexpressed with CDK4 and treated with DMSO or 1oxF11 as indicated. Then, all samples were treated with 500 µM 1oxF11yne, followed by a click step to DTB-N_3 and subsequent pulldown onto high-capacity streptavidin agarose beads overnight at 4 °C. Supernatant was saved and run as “S” lanes, proteins bound to resin were eluted and run as “E” lanes. Samples were separated by SDS-PAGE and bands visualized by Coomassie staining. CDK4 appears at 36 kDa.

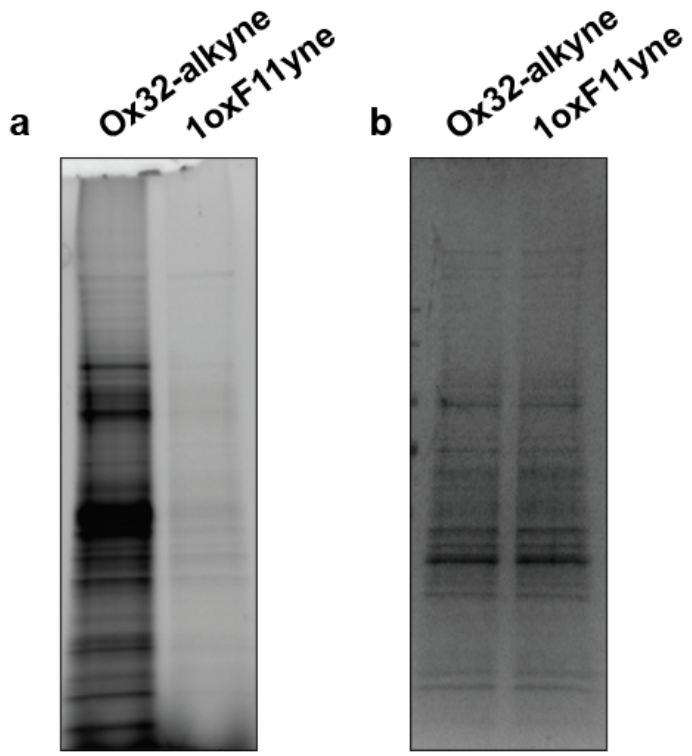


Figure S8. Reactivity comparison of Ox32-alkyne and 1oxF11yne. Lysate was collected from MCF-7 cells overexpressed with CDK4 and treated with either 10 μM Ox32alkyne or 10 μM 1oxF11yne. Then, Alexa488-azide was appended to tagged proteins via CuAAC. Samples were boiled, separated by SDS-PAGE, and visualized. (a) Alexa488-azide channel image. (b) Silver stain image.

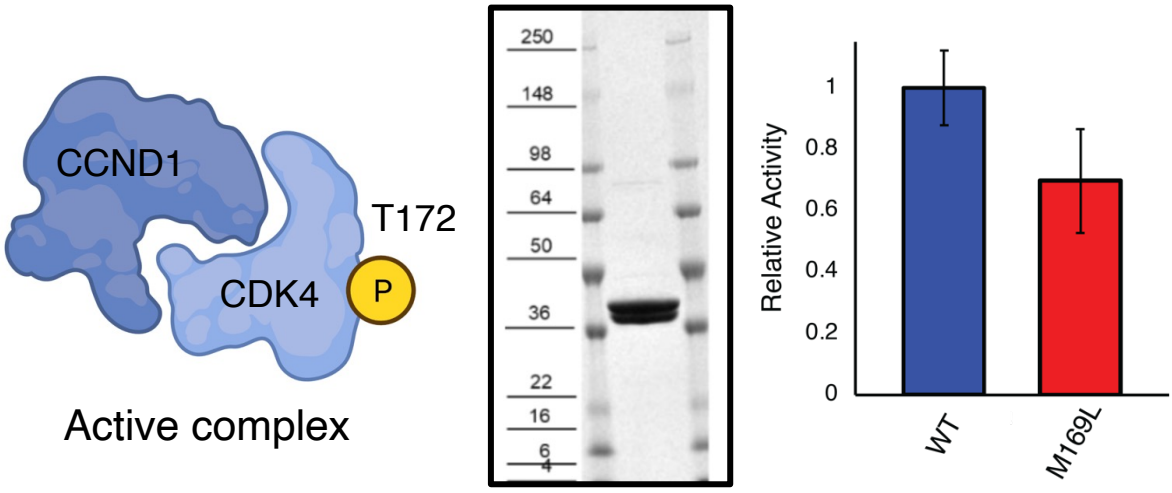
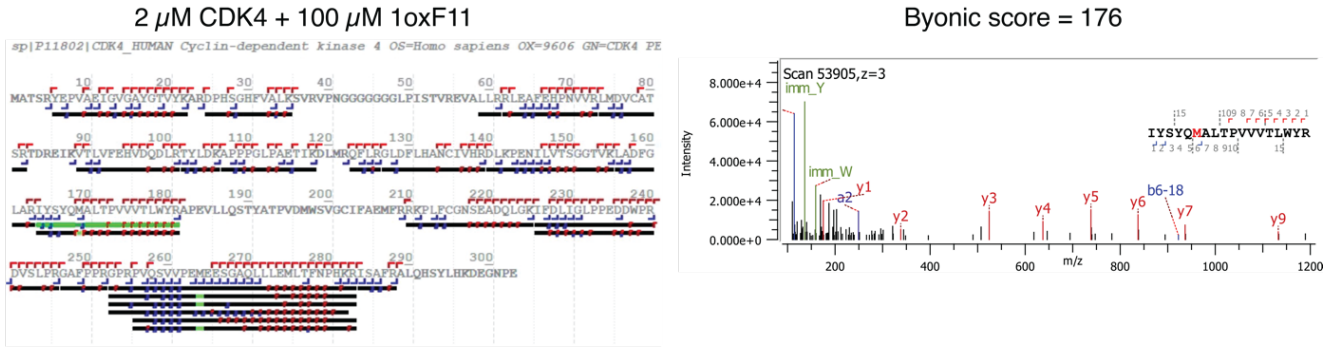


Figure S9. Relative activities of WT and M169L CDK4. Representative gel of purified CDK4/CCND1 in its active form are shown to the left, and relative activity profiles of WT and M169L variant CDK4 are shown to the right.

(a)



(b)

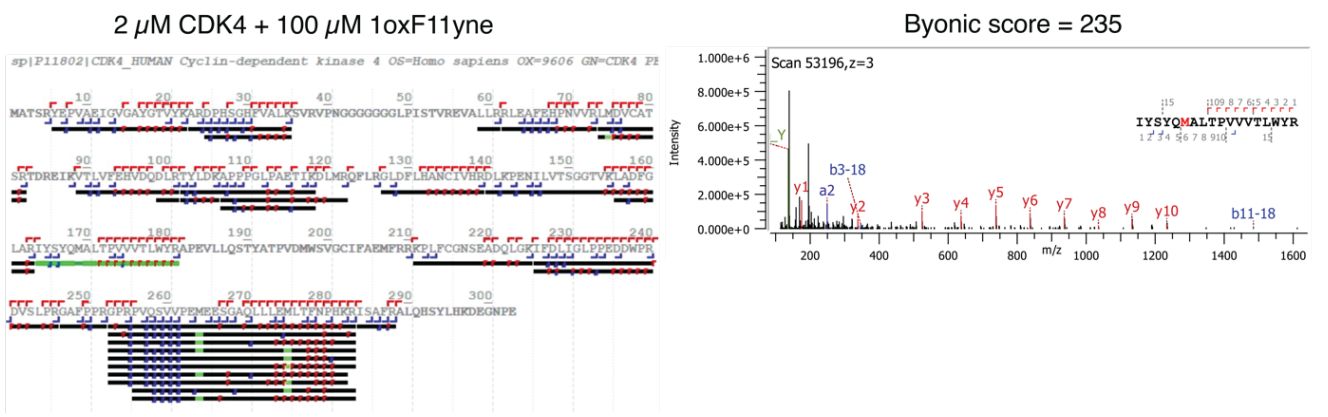


Figure S10. Protein coverage map of CDK4 treated with (a) 1oxF11 and (b) 1oxF11yne

1. General methods

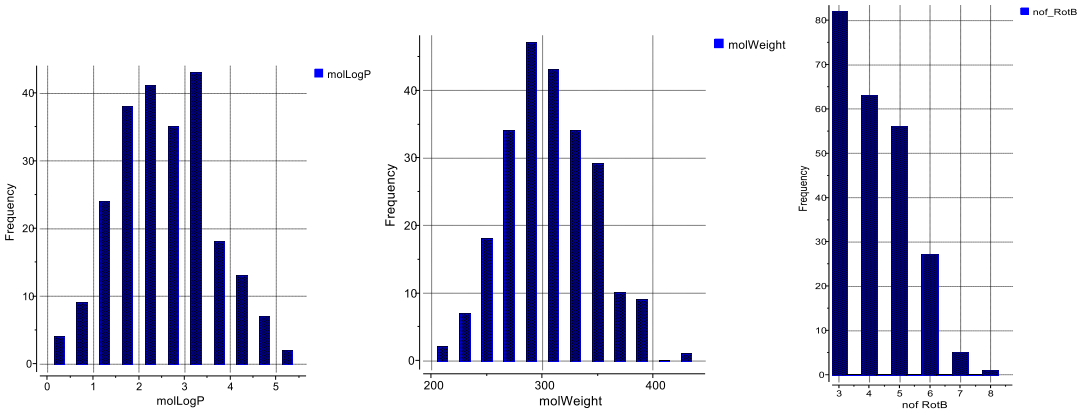
Reactions using moisture- or air-sensitive reagents were carried out in flame-dried glassware under an inert atmosphere of N_2. Solvent was passed over activated alumina and stored over activated 3 Å molecular sieves before use when dry solvent was required. All other commercially purchased chemicals were used as received (without further purification). SiliCycle 60 F254 silica gel pre-coated sheets (0.25 mm thick) were used for analytical thin layer chromatography and visualized by fluorescence quenching under UV light. Silica gel P60 (SiliCycle) was used for column chromatography. ^{1}H and ^{13}C NMR NMR spectra were collected at 298 K in $CDCl_3$ or CD_3OD (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C using Bruker AV300, AVQ-400, AVB-400, AV-500, or AV-600 instruments at the College of Chemistry NMR Facility at the University of California, Berkeley. All chemical shifts are reported in the standard notation of *δ* parts per million relative to the residual solvent peak at 7.26 ($CDCl_3$) or 3.31 (CD_3OD) for ^{1}H and 77.16 ($CDCl_3$) or 49.00 (CD_3OD) for ^{13}C as an internal reference. Splitting patterns are indicated as follows: br, broad; s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublets. Low-resolution electrospray mass spectral analyses were carried out using LC-MS (Agilent Technology 6130, Quadrupole LC/MS and Advion Expression-L Compact Mass Spectrometer). High-resolution mass spectral analyses (ESI-MS) were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley. Ox1-alkyne, Ox32-alkyne, and Ox1-azide were synthesized using published procedures[1]. All aqueous solutions were prepared using Milli-Q water, and all *in vitro* experiments were carried out in PBS, pH 7.4, unless otherwise noted. All biological experiments were prepared using freshly prepared aliquots.

Design methodology for diversity selection of oxaziridine library. The design of the oxaziridine library was initiated through selection of the diversity element, namely the corresponding amines required to synthesize the desired oxaziridine products. There were 43,950 available amines for selection (Enamine); utilizing ICM Chemist Pro (Molsoft LLC) an initial sub-structure selection was made: azetidine (286), pyrrolidine (2711), piperidine (3889), cycloheptylamines (328), iso-propylamines (4452), iso-butylamines (627), morpholines (409), ethylamine (6057), cyclo-heptylamines (328), 2,3,4,5-tetrahydro-1H-benzo[d]azepine (19) and iso-indolene (37). These choices were driven by learnings from our initial study to identify oxaziridine reagents that produced more stable methionine adducts.[2]

Chemical sub-structure	Number of derivatives	Number of initial clusters	Tanimoto coefficient	Compounds per cluster	Selected
Azetidine	**286**	**80**	**0.37**	**1-41**	**28**
Pyrrolidine	**2711**	**524**	**0.3**	**1-61**	**64**
Piperidine	**3889**	**595**	**0.3**	**1-138**	**19**
Ethyl amine	**6057**	**1199**	**0.3**	**1-142**	**-**
Iso-butyl amine	**627**	**142**	**0.3**	**1-45**	**28**
Iso-propyl amine	**4452**	**672**	**0.3**	**1-175**	**17**
morpholine	**409**	**75**	**0.3**	**1-61**	**36**

Cycloheptyl amine	**328**	**96**	**0.3**	**1-45**	**29**
Iso-indoline	**37**	**16**	**0.20**	**1-7**	**9**
2,3,4,5-tetrahydro-1H-benzo[d]azepine	**19**	**9**	**0.25**	**1-6**	**4**

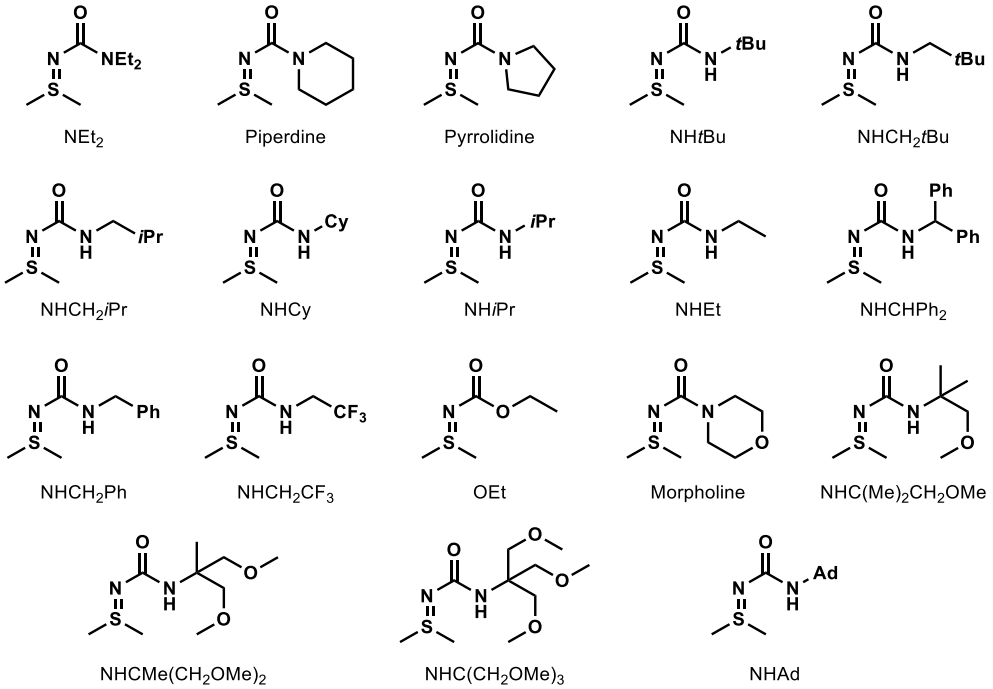
Clustering analysis for each selected amine set was conducted using *ICM Chemist Pro* (Molsoft LLC) using (Tanimoto <0.4 as noted) generating the detailed clusters for each sub-type. Thereafter, 2 rounds of selections based on diversity and selecting the square root of the population of each cluster were conducted followed by a visual inspection to remove compounds containing foreseen chemoselectivity issues based on reactivity with *m*CPBA. The resulting composite set was stripped of duplicate selections from within the sub-sets and the resultant 234 amines were then derivatized to the corresponding oxaziridine (from benzaldehyde), with the logP and MW distributions shown below for the products; it should be noted that the library also was analyzed showing all compounds with HBD ≤1, the number of rotatable bonds was ≤8 with the majority being 3-5, and tPSA distribution was 27-78Å.[2] These properties were not constraints for the library as the initial selections were made for structural diversity. All compounds were all selected for conformational analysis.



Each of the base 234 structure coded as smiles was converted to 3D using Corina with the following flags "-d wh,stergen,axchir,preserve,msc=2".[3] Symmetry equivalents were removed, then conformers were constructed using omega2[omega2] on default settings.[4] The conformers were optimized and IR frequencies computed using Gaussian09[G09] with the keywords "opt(maxcycle=250) freq pop=nbo def2tzvp m062x".[5] For each conformer, the most intense frequency F between 1600.0 and 1740.0 cm-1 for the sulfimines, and 1700.0 and 1950.0 cm-1 for ureas, and the sum of the electronic and thermal Free Energies were collected. The final figures are derived from a Boltzmann weighted average of all the conformers of a structure.

Automated Parameter Extraction: The automated computational workflow[6] was validated by obtaining the stretching frequencies from the set of compounds used to generate the reported hydrolysis kinetic model,[2] and compared to the frequencies obtained manually (**Table S1**). It was found that the automatically calculated stretching frequencies correlated very strongly with those obtained manually (**Scheme S2**), with a slope and R^2 = 0.98. As a further point of validation, the published rates of hydrolysis were plotted against the automatically calculated stretching

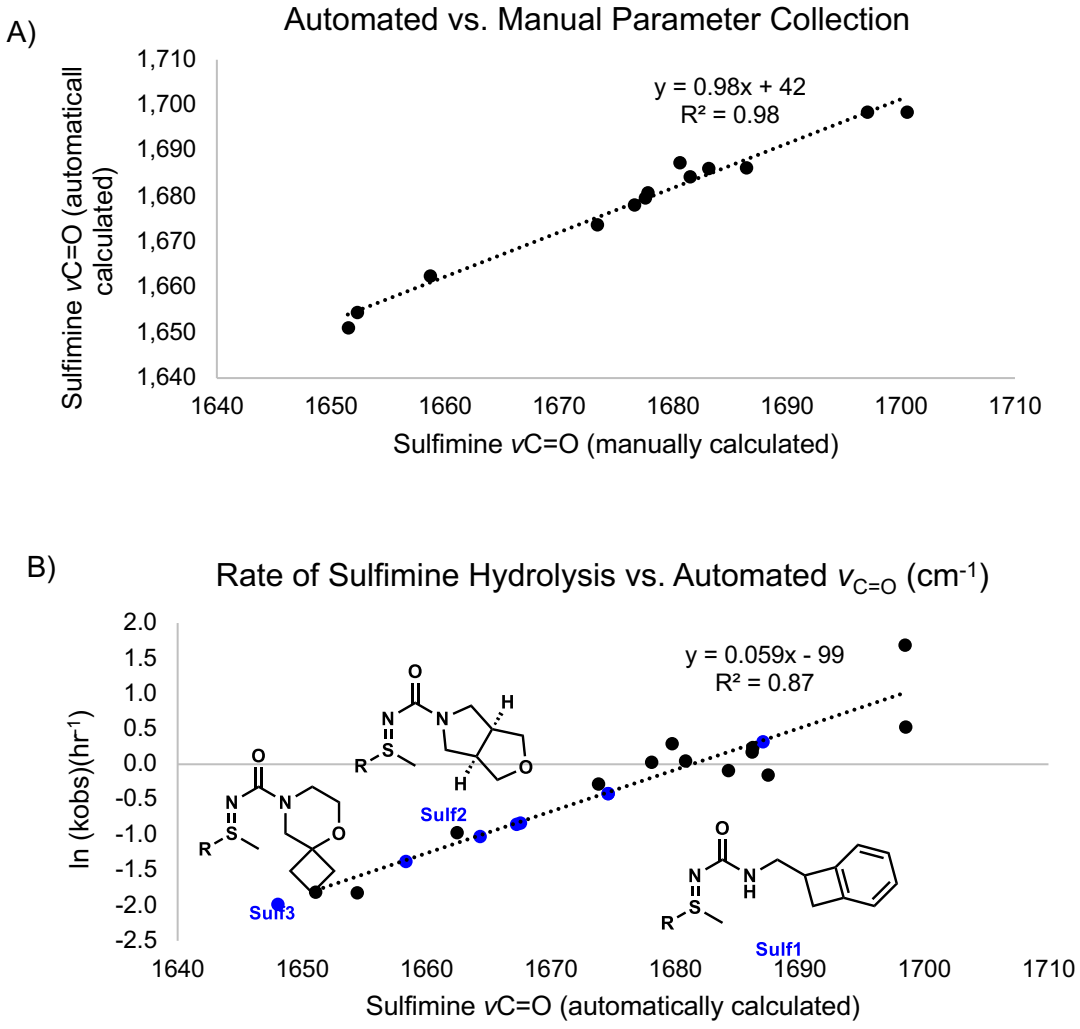
frequency, which produced a similar model to the one obtained from manual calculated stretching frequencies. With this validation in hand, the stretching frequencies of a novel library of untested oxaziridines were extracted, to predict their respective sulfimine adduct stabilities towards hydrolysis. The predicted K_{obs} are computed as 0.0595*(vC=O)-112.8 for sulfimines, and 0.0498*(vC=O)-102.8 for ureas.[2] Equivalent structures in the two series are paired up in the output. Of the 234 input pairs, 1 sulfimine and 1 urea failed to converge, even after restarting, possibly due to unresolved steric crowding. The full list of sulfimine and urea stretching frequencies, as well as predicted stabilities can be seen in the attached Excel file (CDK4_SI_OxaziridineLibraryFrequenciesAndStabilities)



Sulfimine	Man. Collected	Auto. Collected	Rate of Hydrolysis ($ln(k_{obs})$) (hr^{-1})
NEt_2	1652.31	1654.42	-1.82
Piperidine	1651.49	1651.09	-1.81
Pyrrolidine	1658.72	1662.44	-0.96
NH*t*Bu	1673.34	1673.81	-0.28
$NHCH_2$*t*Bu	1680.60	1687.47	-0.15
$NHCH_2$*i*Pr	1681.51	1684.25	-0.085
NHCy	1676.60	1678.08	0.032
NH*i*Pr	1677.78	1680.79	0.044
NHEt	1683.11	1686.17	0.17
$NHCHPh_2$	1686.45	1686.23	0.24
NHBn	1677.57	1679.74	0.29

$NHCH_2CF_3$	1697.01	1698.51	0.53
OEt	1700.52	1698.47	1.69
Morpholine	1659.09	1664.30	-1.02*
$NHCMe(CH_2OMe)_2$	1666.60	1667.23	-0.85*
$NHC(CH_2OMe)3$	1667.09	1667.55	-0.83*
NHAd	1671.38	1674.58	-0.41*
$NHCMe_2CH_2OMe$	1675.08	1674.60	-0.41*

Table S1: Sulfimines used for validating the automated workflow for obtaining C=O stretching frequencies. Manually calculated parameters and rate of hydrolysis obtained from previous report[2] (*denotes predicted hydrolysis rates)



Scheme S2. A) Comparison of *v*C=O obtained from automated vs. manual workflows and B) recreated kinetic model using the automatically calculated *v*C=O with predicted hydrolysis rates of select sulfimines (data points in blue)

Gel-based ABPP. CDK4 was diluted in PBS to 50 nM, then 50 µL was added to each well of a 96-well PCR plate (Thermo Fisher Scientific). Ligands were dissolved fresh in DMSO to 5 µM, and 1 µL was added so each well contained a unique ligand at the indicated concentration. Wells were mixed and allowed to incubate 1 h at 23 °C. Ligands were then chased with fresh Ox1-alkyne with 1 µL of 5 µM added to each well (100 nM final), wells mixed and allowed to incubate 1 h at 23 °C. Excess oxaziridine was quenched with N-acetyl methionine (Sigma; 01310) via addition of 1 µL of 10 mM DMSO stock (200 µM final), wells mixed and allowed to incubate 1 h at 23 °C. A stock was prepared 1:5 of 12.5 µM DBCO-Cy3 (Click Chemistry Tools; A140) in DMSO : 1.2% SDS/PBS. To each well was added 6 µL of this stock, the wells mixed and allowed to incubate overnight protected from light at 23 °C. The next morning 30 µL of 4X Laemmli's buffer (Bio-Rad Laboratories, Inc.; 1610747) containing 10% BME was added to each well. The plate was sealed and brought to 95 °C for 6 min. Samples were loaded and separated on precast 4–20% TGX gels (Bio-Rad Laboratories, Inc.) and scanned by ChemiDoc MP (Bio-Rad Laboratories, Inc) for measuring in-gel fluorescence. After that, the total protein level on the gel was assayed by silver staining (Thermo Fisher Scientific; 24612) according to the manufacturer's protocol and scanned by ChemiDoc MP.

Molecular Cloning and Site-Directed Mutagenesis. A FLAG-TEV-CDK4 gene was inserted into a pcDNA3.1(+) vector for transient mammalian expression using restriction digestion cloning methods. Briefly, the gene was amplified by PCR with primers containing HindIII and XbaI restriction sites. Both plasmid and PCR product were digested with the indicated restriction enzymes at 37°C for 4 H, followed by purification using standard commercial kits (Qiagen). Insert and plasmid were mixed in 0:1, 3:1, and 5:1 insert:vector ratios and ligated using a T7 DNA ligase (New England Biolabs) overnight at 23°C. Plasmids were then transformed into XL1-blue *E. Coli* strain via heat-shock, grown overnight, and purified plasmids were sequence verified. Site-directed mutagenesis was then performed on the verified plasmid via QuikChange strategy using the indicated M169L primers. The PCR product was directly transformed, grown, purified, and sequence verified. Prior to mammalian transfection, plasmids were grown for endotoxin-free preparation using commercial EndoFree kits (Qiagen).

Primers:

HindIII CDK4 forward: 5' – AACGACAAGCTTGCCACCATGGGAGACTATAAG – 3'

XbaI CDK4 reverse: 5' – AAAGATCTAGACTCGAGTCATTCTGGATTACCCTCATCTTTG – 3'

M169L forward: 5' – GAATCTATAGCTACCAGCTGGCACTGACTCCTGTGG – 3'

M169L reverse: 5' – CCACAGGAGTCAGTGCCAGCTGGTAGCTATAGATTC – 3'

Spiked shotgun proteomics. To more accurately assess residue reactivity of oxaziridines on CDK4, 10 µg of CDK4 was diluted with 90 µg whole cell extract derived from mouse liver to a total volume of 100 µL in PBS. Protein mixture was treated with 50 µM oxaziridine (DMSO) and allowed to incubate at 23 °C for 30 min. Labeled protein was precipitated via addition of 900 µL MeOH at -80 °C overnight. The next day, sample was spun at max speed at 4 °C for 10 min. The pellet was gently washed 3 times with a solution of ice cold MeOH. The supernatant was then removed, and

the pellet resuspended in 30 µL freshly prepared 8 M urea/PBS. A 5X stock of ProteaseMAX (Promega; V2071) was prepared by dissolved the pellet in 100 µL ammonium bicarbonate. To the protein mixture was added 30 µL 1X ProteaseMAX, 40 µL ammonium bicarbonate, and 10 µL of 110 mM freshly prepared TCEP (Pierce; 20490). The sample was then incubated at 60 °C for 30 min. To the sample was then added 2.5 µL of 500 mM freshly prepared iodoacetamide (Sigma Aldrich; I1149), and the sample was incubated protected from light at 23 °C for 30 min. 120 µL of PBS was then added, followed by 1.2 µL 5X ProteaseMAX. The sample was vortexed thoroughly. Sequencing grade Trypsin/Lys-C mix (Promega; V5071) was reconstituted in 40 µL trypsin buffer, and 4 µL was added to the sample. The sample was allowed to digest at 37 °C overnight. The next day the sample was acidified with 12 µL formic acid and spun at max speed for 30 min. The supernatant was taken to a low-adhesion tube and stored at -80 °C until MS analysis.

Shotgun proteomics M169L and WT-CDK4

To more accurately assess residue reactivity of oxaziridines on CDK4, 20 µg of CDK4 (WT or M169L variant) was diluted to a total volume of 100 µL in PBS. Protein mixture was treated with 50 µM oxaziridine (DMSO) and allowed to incubate at 23 °C for 30 min. Labeled protein was precipitated via addition of 900 µL MeOH at -80 °C overnight. The next day, sample was spun at max speed at 4 °C for 10 min. The pellet was gently washed 3 times with a solution of ice cold MeOH. The supernatant was then removed, and the pellet resuspended in 100 µL freshly prepared 6M urea/PBS and incubated at 60 °C for 1 hour. Samples were then treated with 5 µL of freshly prepared 110 µM TCEP (Pierce; 20490). The samples were then incubated at 60 °C for 20 min. To the samples was then added 4 µL of 400 mM freshly prepared iodoacetamide (Sigma Aldrich; I1149), and the sample was incubated protected from light at 37 °C for 30 min. The reaction was quenched by addition of 550 µL of 50 mM ammonium bicarbonate. Sequencing grade Trypsin/Lys-C mix (Promega; V5071) was reconstituted in 40 µL trypsin buffer, and 8 µL was added to the samples. The sample was allowed to digest at 37 °C overnight. The next day samples were dried down in a SpeedVac (60 °C, 1 hour). The samples were then reconstituted in 100 µL ultrapure water + 0.1% TFA and desalted using Pierce C18 stage tips.

Mass spec analysis. Peptides from all experiments were analyzed on a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific); pure proteins were analyzed on a C18 only column, while complex proteomes were analyzed via a five step Multidimensional Protein Identification Technology (MudPIT).[7] In both cases, Inlines (IDEX; M-520) were fitted with ~20 cm of 250 µm tubing and prepacked with 4 cm of Aqua C18 reverse-phase resin (Phenomenex; 04A-4299). Columns were made from 100 µm tubing pulled with a P-2000 laser (Sutter Instruments Co.) and packed with either 13 cm of Aqua C18 reverse-phase resin (pure proteins) or packed first with 10 cm of Aqua C18 reverse-phase resin followed by 3 cm of strong-cation exchange resin (Phenomenex; 04A-4398; MudPIT). Both inlines and columns were equilibrated after packing prior to use on an Agilent 1260 HiP AIS coupled to an Agilent 1260 Quat Pump using the following gradient: 100% buffer A to 100% buffer B over 10 min, hold at buffer B for 5 min, finish with a wash with 100% buffer A over 15 min (where buffer A is 95:5 H_2O:MeCN/0.1% formic acid, and buffer B is 80:20 H_2O:MeCN/0.1% formic acid). For best performance, columns were equilibrated the day of sample run.

Peptides were pressure-loaded onto an equilibrated inline until dry. The tubing was cut to ~2 cm above the resin bed and an appropriate equilibrated column was attached to the opposite end of the inline. The sample was attached to the LC-MS/MS via a MicroTee PEEK 360µm fitting (Thermo Fisher Scientific; p-888) and the column tip aligned with the MS source opening. Data

was collected in positive-ion mode using data-dependent acquisition mode with dynamic exclusion enabled (60 s) between 400 and 1800 m/z and a mass resolution of 70,000, with one MS1 scan followed by 15 MS2 scans of the nth most abundant ions. Nanospray voltage was set at 2.75 kV and heated capillary temperature at 200 °C. The MudPIT program utilized for all samples consists of five separate programs run sequentially, where each begins with either 0, 25, 50, 80, or 100% salt bump (buffer C, 500 mM ammonium acetate/H_2O) followed by a gradient of 5-55% buffer B in buffer A. Pure proteins were run on only the first program (0% salt bump) from the MudPIT program. The flow was kept at 0.1 mL/min throughout.

Data were analyzed with Byologic (Protein Metrics Inc.). Raw files were searched directly against the Uniprot human or mouse database using the Byos HCP workflow, with decoys and common contaminants added. Peptides were assumed fully tryptic. All searches included the following modifications: Acetyl (+42.010565; Protein N-term; variable - rare1), carbamidomethyl (+57.021464; C; Fixed), and oxidation (+15.994915; M; variable - common1). Other modifications to methionine depended on the probe added to the sample and were all treated as variable - common1: 1oxF11yne (+235.09569), 1oxF11 (+154.07423), 1oxH2 (+168.08988). Peptides with b and y ions flanking the site of modification were assumed modified.

ReDiMe Peptide Prep. MCF-7 cells were expressed with FLAG-TEV-CDK4, lysed, and normalized to 4 mg/mL as described below. 4 mg of protein was treated with 500 µM 1oxF11yne or DMSO control for 1 H at 23 °C. Labeled proteins were then clicked with 1.2 eq of DTB-N_3 compound with TBTA, TCEP, and $Cu_2(SO_4)$, and the CuAAC was allowed to proceed for 1 H at 23°C. The samples were added to 9 mL of MeOH:$CHCl_3$ (4:1.5) and precipitated by vortexing. Samples were then spun at max speed for 10 min at 4 °C. Pellet was resuspended in cold MeOH and samples spun again. Pellet was washed once more before resuspension in 500 µL 1.2% SDS/PBS via probe-tip sonication (20% A, 5 s on, 20 s off, 6-8 pulses). High-capacity streptavidin beads (350 µL per sample) (Thermo Fisher Scientific; 20357) were washed 2X in PBS and 2X in MQ. The supernatant of the samples was added to the washed beads and allowed to incubate at 4 °C overnight. The next morning the samples were allowed to rock at 23°C to resolubilize the SDS. The beads were washed thoroughly with 5X PBS and 5X MQ in micro bio-spin columns (Bio-Rad; 7326204). The beads were then resuspended in 500 µL 6 M urea, transferred to a 2.0 mL tube, and incubated with 25 µL TCEP (110 mM) at 65°C for 20 mins. Then, 25 µL 400 mM Iodoacetamide was added and incubated at 37°C for 30 mins in the dark. The reaction was quenched by adding 950 µL PBS and spinning at 1,400 xgs for 3 mins to remove the supernatant. A premixed solution of 200 µL 2 M urea/PBS, 2 µL $CaCl_2$, and 16 µL Trypsin/LysC solution. (20 µg lyophilized Trypsin/LysC reconstituted in 40 µL Trypsin/LysC buffer) was added and incubated at 37°C overnight. The next day, the beads were washed 3X with PBS and 3X with MQ. Peptides were eluted from beads via addition of 2 X 200 µL 0.1% FA (50% ACN/MQ) and spun at 3000 x g for 3 min. The eluent was dried via SpeedVac concentration. Peptides were reconstituted in 100 µL of MQ + 0.1% FA and quantified using a commercial colorimetric kit (Pierce; 23275).

ReDiMe Labeling. Samples were reconstituted in 100 µL of 100 mM TEAB. To the DMSO-treated control sample was added 4 µL of 4% (v/v) formaldehyde for isotopically light labeling. To the 1oxF11yne-treated sample was added 4 µL of 4% (v/v) CD_2O for isotopically heavy labeling. Then, 4 µL of 0.6M $NaBH_3CN$ was added to each sample and incubated in a fumehood for 30 minutes at room temperature while mixing. The reaction was quenched by addition of 16 µL of 1% (v/v) ammonia solution. Formic acid (16 µL) was added to further quench the reaction and acidify the samples for LC-MS analysis. 500 ng of each of the differentially labeled samples were

then combined and aliquoted into low-retention tubes for 1 µg samples in triplicate. Differentially labeled samples were then dried down (SpeedVac) and analyzed by LC-MS/MS.

ReDiMe Mass spec analysis. An n = 3 of 1 µg multiplexed samples were reconstituted in 20 µL of MQ H_2O + 0.1% formic acid by bath sonication for 30 mins. Peptides were analyzed on a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). The samples were separated by liquid chromatography using a VanquishNeo UHPLC (Thermo Fisher Scientific) in Trap-and-Elute configuration using a PepMap Neo (5 µM x 300 µM x 5 mm) trap (Thermo Fisher Scientific) and an Ultimate 25 cm C18 analytical column (IonOpticks) operating at a 350 nL/min flow rate at a column temperature of 50°C. Peptides were eluted over a 120 min gradient from 0-80% H_2O/ACN and data were collected in positive-ion mode using data-dependent acquisition mode with dynamic exclusion enabled (30 s) between 400 and 1800 m/z and a mass resolution of 70,000, with one MS1 scan followed by 15 MS2 scans of the nth most abundant ions. Nanospray voltage was set at 2.50 kV and heated capillary temperature at 200 °C.

Data were analyzed using Fragpipe (v18.0). Raw files were searched against the Uniprot human database with 50% decoys and common contaminants added. Searches were performed using a custom ReDiMe workflow derived from the 'SILAC3' workflow with the following modifications:

MS Fragger: lysc added under protein digestion. Variable modifications n^K (Δ m = 28.0313) and n^K (Δ m = 32.05641) were added for light and heavy dimethyl labels, respectively with max occurrences = 2.

Validation: Run MSBooster checked, generate peptide-level, and protein-level summary checked.

Quant: IonQuant enabled. Light label assigned as K28.0313, heavy label as K32.05641 with Re-quantify checked. Normalize intensity across runs enabled with unique+razor peptides examined.

Only validated peptides with spectral counts identified across 2 independent experiments were included in the final dataset. Proteomic data were filtered and cleaned to extract unique Uniprot IDs using an in-house python script. The data were then run through UniprotKB Retrieve/ID mapping tool to append subcellular localization identifiers to each unique ID. These data were then analyzed against a second python script to search for subcellular localization based on user-defined identifiers (e.g. Nucleus = 'Nucle[a-z]*', Cytoplasm = 'Cytoplasm[a-z]*'). Percent localization was determined by dividing the number of proteins localized to a specific organelle over the total number of proteins. Molecular activity was determined via PANTHER GO analysis using the extracted Uniprot IDs and plotted using the following identifiers: Binding (GO: 0005488), Catalytic activity (GO: 0003824), Structural molecule activity (GO: 0005198), Translation regulator protein (GO: 0003743), ATP-dependent activity (GO:0140657), Transporter activity (GO:0005215), Molecular adaptor activity (GO:0060090).

Solvent Accessibility. Residue solvent accessibility calculations of methionines on CDK4 protein were computed using the Discovery Studio 2021 platform from Dassault Systemes BIOVIATM. The 2W9Z pdb file for CDK4 was utilized and submitted to a "Solvent Accessibility" calculation. The software was set up with grid points per atom at 240 and probe radius at 1.4 Å.

***In vitro* activity assay.** Effects of 1oxF11 on *in vitro* activity of CDK4 were determined via commercially available ADP-Glo assay (Promega; V6930). Isolated WT-CDK4 and M169L variant CDK4 were provided by Novartis. Retinoblastoma protein (aa 773-928) was obtained from commercial sources (Millipore Sigma; 12-439). Briefly, 500 nM WT-CDK4/CCND1 or M169L-

CCND1 and 5 μM retinoblastoma protein were premixed in PBS buffer containing 5 mM $MgCl_2$, 50 μM Na-orthovanadate, and 0.1% (v/v) Triton X-1000 then added to a white 384-well plate in quadruplicate. Then, 1oxF11 was titrated into each well at the desired concentrations and incubated for 1 H at 23°C. The reaction was quenched by incubating with 1 μL of 25 μM N-acetyl methionine for 30 minutes at room temperature Then, 50 μM ATP was added to commence the kinase reaction and incubated for 2 H at 37°C. Activity was measured following the manufacturer's protocols.

Cell culture. Cells were maintained by the UC Berkeley Tissue Culture Facility. All cells were maintained as a monolayer in exponential growth at 37 °C in a 5% CO2 atmosphere. MCF-7 and HepG2 were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Seradigm). HT-29 and SW-48 were maintained in RPMI 1640 Medium (Gibco) supplemented with 10% FBS.

Cell synchronization. Cells were synchronized to G_0/G_1 via serum starvation. Cells were plated at 50% confluency and allowed to adhere overnight in serum-containing media. The next morning, the cells were gently washed twice with HBSS, and serum-free media was added. Cells were allowed to incubate an additional 24 h, for a total of 48 h without serum. Final cell confluency should remain below 75% for optimal results.

Cell treatment. All cells dosed with oxaziridine were treated using a 2% DMSO/media solution. Oxaziridines were dissolved in DMSO and used the same day as treatment. Media was removed from cells and replaced with fresh media containing oxaziridine in DMSO for a final concentration of 2% DMSO. Control wells were treated with 2% DMSO/media. Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

Cell viability assay. Commercially available Cell Counting Kit-8 Assay (Dojindo) was used to investigate cell viability after treatment with oxaziridines. Cells were plated in 96-well plates (black/clear bottom; Thermo Fisher) and grown to 75% confluency. Media was removed and replaced with media containing 2% DMSO and indicated compound concentration. Cells were allowed to incubate 24 h. To assess viability, media was removed and replaced with 100 μL media containing 10% CCK-8 assay solution. Plates were incubated at 37 °C in a 5% CO_2 atmosphere for 1-4 h until an orange color was visible. Viability was quantified via plate reader (monofilter) with absorption at 450 nm.

Western blot analysis. Cells were seeded in full media at 8e5 cells/well in a 6-well plate. They were then serum-synchronized and treated with compound as described previously. Cells were monitored by eye for cell death and harvested once high-dose cells were round but not detached. Cells were transferred to ice and washed twice gently with 1 mL ice-cold PBS. 10 mL of lysis buffer was prepared by dissolved a protease-inhibitor table (Pierce; A32953) and a phosphatase-inhibitor tablet (Roche; 4906845001) in 10 mL PBS (1% Triton X-100). Cells were scraped to harvest, transferred to 1.5 mL Eppendorf tubes, and incubated at 4 °C for 30 min on a rotator. Samples were spun at 5000 x g for 10 min to clarify lysate. Supernatant was transferred to a new tube and protein concentration quantified via BCA assay (Pierce; 23225). Samples were normalized to the lowest concentration using chilled lysis buffer. Samples were diluted with 4X Laemmli's buffer (10% BME) (Bio-Rad; 1610747) and loaded at 25 μg per lane on a 4-20% Tris-Gly SDS-PAGE gel. The gel was run at 160 V for 80 min and semi-dry electrotransferred to a PVDF membrane at 25 V, 2.5 Å, for 10 min. Blots were blocked with 5% BSA/TBST for 1 h, then washed 2 X TBST for 5 min, and cut for incubation with separate antibodies. Antibodies used

were rabbit anti-pRb Ser807/811 (CST; 9308), rabbit anti-pRb Ser780 (CST; 3590), rabbit anti-β actin (CST; 4970), mouse phospho-T172 CDK4 (NB8-AD9),[8] and rabbit anti-CDK4 (CST; 12790). All antibodies were diluted at 1:1000 in 5% BSA/TBST, with the exception of AD9 diluted at 1:500, at 4 °C overnight. The next morning the blots were washed 3 X with TBST prior to incubation with anti-rabbit IgG HRP conjugated secondary (CST; 7074) (1:3000 TBST) or anti-mouse IgG HRP conjugated secondary (CST; 7076S) (1:3000 TBST) for 2 h at room temp. Blots were quickly washed 3 X with TBST prior to incubation with ECL western blotting substrates (Promega; W1001) for 1 min and imaging with ChemiDoc MP.

2D gel electrophoresis. Serum-synchronized MCF-7 cells were treated with compound according to method outlined. Cells were monitored by eye for cell death and harvested once high-dose cells were round but not detached, about 2 hours. Media was removed and cells washed with HBSS. Trypsin was added to detach cells. Cells were transferred to a falcon tube containing 7 mL of complete DMEM and centrifuged at 1200 rpm for 2 min. Supernatant discarded and pellet gently resuspended in 1 mL PBS and transferred to an Eppendorf tube. Sample was spun once more, after which the pellet was quickly rinsed with ice cold MQ and spun a final time. Pellet was aspirated, flash frozen with LN2, and stored at -80 °C.

Samples were shipped on dry ice to K. Coulonval who proceeded with analysis. Cell pellets were solubilized in cold 30 mM Tris buffer pH 8.5 containing 7 M urea, 2 M thiourea and 4% CHAPS with continuous vortexing until unfrozen and then kept agitated for 20 min. After centrifugation at 15,700 g for 10 min at 4°C, proteins were quantified. An equal volume of 2-D-sample buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.4% 3–10 Pharmalytes, and 0.4% DTT) was added to samples normalized to 150 µg proteins. Proteins were separated by isoelectrofocusing on immobilized linear gradient (pH 5 to 8 [11 cm], Bio-Rad) strips, separated by SDS-PAGE and immunoblotted with antibodies against CDK4 (D9G3E, rabbit monoclonal) or phospho-T172 CDK4 (NB8-AD9, mouse).[8] Secondary antibodies were coupled to horseradish peroxidase (Cell Signaling Technology). The proteins were detected using Western Lightning Plus ECL (Perkin Elmer) and viewed in Fusion FX gel documentation system using the Solo7S camera (Vilber Lourmat, France).

Expression of CDK4 in MCF-7 cells. MCF-7 cells were grown to 40% confluency in 3 mL DMEM media (Gibco) containing 10% (v/v) FBS in a 6-well chamber at 37°C, 5% CO_2. Transfection was then performed as per Lipofectamine 2000 protocol (Invitrogen). Briefly, 0 or 2.5 µg of pcDNA3.1(+)-FLAG-TEV-CDK4 expression construct was introduced at 0:0, 2:1 and 3:1 transfection reagent:DNA. The lipid-DNA complex was incubated for 30 mins at 23°C in Opti-MEM media (Gibco). Then, 250 µL complex was added to 2.75 mL DMEM containing no FBS. 3 mL DMEM + DNA was added to each well, while control wells received DMEM only. Cells were incubated for 6 H at 37°C, 5 % CO_2. The media was then aspirated, and cells were given 3 mL DMEM + 10% FBS and left to incubate for an additional 30 H at 37°C, 5 % CO_2. Cells were washed 3X in 500 µL ice-cold PBS then harvested by scraping in 200 µL ice-cold PBS + 1% Triton X-100 containing EDTA-free protease inhibitors (Roche). Cells were lysed at 4°C for 30 mins while rocking. Lysate was clarified by centrifugation at 10,000 x g at 4°C for 15 mins. The supernatant was then transferred to a fresh prechilled 1.5 mL microcentrifuge tube. Protein concentration was normalized to 2.0 mg/mL via BCA assay (Pierce; 23225). Samples were denatured in 4X Laemmli's buffer + 10% BME (Bio-Rad; 1610747) and boiled at 95°C for 8 mins. 30 µg protein was loaded onto a 4-20% Tris-Gly SDS-PAGE gel run at 160 V for 70 mins. Proteins were then electro-transferred to a PVDF membrane (25 V/2.5 A for 10 mins). Membranes were

then blocked in a solution of TBST + 5% BSA (w/v) and rocked at 23°C for 1 H. Membranes were washed 3X in TBST for 5 mins each while rocking and cut using a razor blade along the protein ladder for separate antibody incubation. Membranes were then blotted with primary rabbit anti-CDK4 (CST; 12790) or rabbit anti-β actin (CST; 4970) (both as 1:1000 TBST + 5% BSA suspensions) at 4°C overnight. The following morning membranes were washed 3X in TBST for 5 mins then incubated with anti-rabbit IgG HRP conjugated secondary (1:3000 TBST) (CST; 7076S) for 2 H at 23°C. Blots were quickly washed 3 X with TBST prior to incubation with ECL western blotting substrates (Promega; W1001) for 1 min and imaging with ChemiDoc MP. CDK4 expression was quantified by densitometry (ImageJ) and normalized to the actin loading control.

Constitutive KD+OE of Variant CDK4 and Cytotoxicity Assay with 1oxF11. MCF-7 cells were seeded to 70% confluency in DMEM + 10% FBS in a 6-well chamber. Each well was transfected with either 50 pmol of siCDK4 (Sigma #AM16708) or 2.5 µg of pcDNA3.1(+) vector bearing FLAG-tagged CDK4 using Lipofectamine 2000 (Invitrogen) for 48 H according to the manufacturer's protocols. Constitutive KD + OE were performed by co-transfecting siCDK4 and the pcDNA vector followed by tryptic harvesting at the indicated time points. Cells were lysed in PBS + 1% Triton X-100 containing EDTA-free protease inhibitors (Roche) and quantified by BCA assay (ThermoScientific). 20 µg protein was run on a 4-20% Tris-Gly SDS-PAGE gel followed by transfer to a PVDF membrane. Immunoblotting was performed by blocking the membrane in TBST + 5% BSA for 1 H at 23°C while rocking. The membrane was washed 3 x 10 mins in TBST then incubated with mouse a-FLAG (1:500 TBST + 3% milk, Sigma #F1804), rabbit a-CDK4 (1:1000 TBST + 5% BSA; CST #12790), or rabbit a-β-actin (1:1000 TBST + 5% BSA; CST #4970) overnight at 4°C while rocking. Primary antibodies of the same molecular weight were blotted by stripping, re-blocking, and blotting with the commensurate antibody. The membrane was then washed 3 x 10 mins in TBST and incubated with secondary mouse-HRP conjugate (1:3000 TBST, CST #7076) or secondary rabbit-HRP conjugate (1:3000 TBST, CST #7074) for 2 H at 23°C while rocking. Blots were imaged by incubating with ECL substrates. Cell viability was assessed by seeding MCF-7 cells to 70% confluency in DMEM + 10% FBS in a 96-well chamber. Cells were constitutively KD+OE with either WT or M169L CDK4 by scaling down by a multiplication factor of 0.17 and incubated for 24 H. The media was then aspirated, and cells were treated with the indicated concentrations of 1oxF11 in DMEM + 10% FBS containing 1% DMSO for 24 H. Cell viability was assessed by CCK-8 assay (Dojindo) according to the manufacturer's protocols.

Competition and pulldown of 1oxF11yne/1oxF11. MCF-7 cells were expressed with FLAG-TEV-CDK4 and normalized to 2 mg/mL as described. To 50 µL of this lysate was added 1 µL of a stock of 1oxF11 in DMSO for a final concentration of 500 µM, 250 µM, or 0 µM 1oxF11. A sample was set aside as "just lysate," which was not treated with any compounds. After incubation with 1oxF11 for 1 hour at 22 °C, 1 µL of 25 mM 1oxF11yne was added to each sample as indicated for a final concentration of 500 µM 1oxF11yne. Samples were incubated again for 1 hour at 22 °C. DTB-N_3 was added to each compound with TBTA, TCEP, and $Cu_2(SO_4)$, and CuAAC was allowed to proceed for 1 h at 22 °C. 450 µL of MeOH was added to each sample and proteins were precipitated at -80 °C for 12 h. Samples were then spun at max speed for 10 min at 4 °C. Pellet was resuspended in cold MeOH and samples spun again. Pellet was washed once more before resuspension in 150 µL 0.2% SDS/PBS. Samples were then boiled for 5 min and spun at 6500 x g for 5 min. Meanwhile, high-capacity streptavidin beads (10 µL per sample) (Thermo Fisher Scientific; 20357) were washed 2X in PBS and 2X in MQ. The supernatant of the samples was added to 10 µL of the washed beads and allowed to incubate at 4 °C overnight. The next morning the samples were allowed to rock at 22 °C to resolubilize the SDS. The supernatant was

collected and set aside for analysis. The beads were washed thoroughly with 2X PBS and 2X MQ and transferred to micro bio-spin columns (Bio-Rad; 7326204). Peptides were eluted from beads via addition of 2X 75 µL 0.1% FA (50% MeCN/MQ). Eluent was collected and beads washed once more with 20 µL of elution buffer. Columns were spun at 3000 x g for 3 min. Eluent was lyophilized to remove MeCN. After samples were dry, they were reconstituted in 75 µL PBS. Protein was diluted with 4X Laemmli's buffer (Bio-Rad Laboratories, Inc.; 1610747) containing 10% BME and brought to 95 °C for 6 min. Samples were loaded and separated on precast 4–20% TGX gels (Bio-Rad Laboratories, Inc.) The gel was run at 160 V for 80 min and semi-dry electrotransferred to a PVDF membrane at 25 V, 2.5 Å, for 10 min. A separate gel was run and stained for total protein via Coomassie. Blot was blocked with 5% BSA/TBST for 1 h, then washed 2 X TBST for 5 min. Rabbit anti-CDK4 (CST; 12790) diluted at 1:1000 in 5% BSA/TBST was used to blot for CDK4 signal at 4 °C overnight. The next morning the blot was washed 3 X with TBST prior to incubation with anti-rabbit IgG HRP conjugated secondary (CST; 7074) (1:3000 TBST) for 2 h at 22 °C. Blots was quickly washed 3 X with TBST prior to incubation with ECL western blotting substrates (Promega; W1001) for 1 min and imaging with ChemiDoc MP.

Molecular Dynamics. All calculations were performed in Maestro 2022-2. The oxaziridine ligands and protein were prepared separately. Oxaziridine fragments were first energetically minimized using 'Minimization' in MacroModel with the OPLS4 force field and water as the solvent. Energetically favorable conformers were elucidated by monte-carlo sampling using 'Conformational search' in MacroModel with Torsional Sampling (MCMM) as the method. CDK4 (PDB: 2W9Z) was prepared by only modeling the CDK4 chain of the heterodimer with the 'Preparation Workflow' in Prime with 'Preprocess', 'Optimize H-bond assignments', and 'Minimize and Delete Waters' selected. A docking grid was prepared with a 12 $Å^3$ grid centered on the M169 residue of CDK4 with 'Receptor Grid Generation' in Glide. Ligands were then docked using 'Ligand Docking' in Glide using the optimized conformers, prepared protein structure, and receptor grid. MD simulations were then performed by first generating a model system with 'System Builder' in Desmond using the SPC solvent model, minimized volume, and 150 mM NaCl as the salt concentration. A 50 ns simulation was then performed for each fragment independently with 'Molecular Dynamics' in Desmond with a 25 ps interval, T = 300 K, P = 1.01325 bar, and 'Relax model system before simulation' implemented. Structure relationships were deduced by performing 'Simulation Interaction Diagram' in Desmond and trajectories were exported to PyMOL to generate movies.

2. Synthesis of oxaziridine probes

General methods

All non-aqueous reactions were performed under an inert atmosphere of dry nitrogen in flame dried glassware sealed with a rubber septum unless stated otherwise. Nitrogen was supplied through a glass manifold. Reactions were stirred magnetically and monitored by thin layer chromatography (TLC). Analytical thin layer chromatography was performed using MERCK Silica Gel 60 F254 TLC glass plates and visualized by ultraviolet light (UV). Additionally, TLC plates were stained with aqueous potassium permanganate (KMnO4) [1.5 g KMnO4, 200 mL H2O, 10 g K2CO3, 1.25 mL 10% NaOH]. Concentration under reduced pressure was performed by rotator evaporation at 40 °C at the appropriate pressure. Chromatographic purification was performed as flash chromatography on MERCK silica gel 60 Å (230 x 400 mesh) at 0.2–0.5 bar overpressure. Purified compounds were dried further under high vacuum (0.01 0.1 mbar). Yields refer to the purified compound.

Chemicals

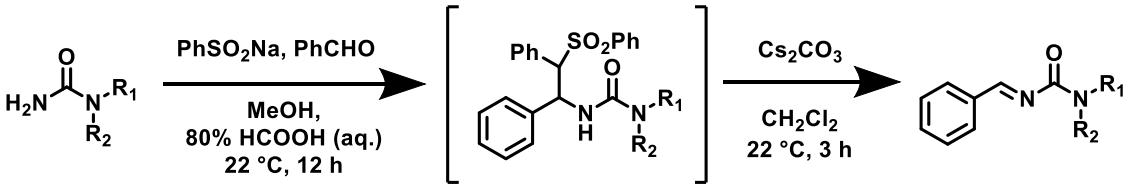
All chemicals and solvents were used as received from the commercial supplier without further purification unless mentioned otherwise.

Analytics

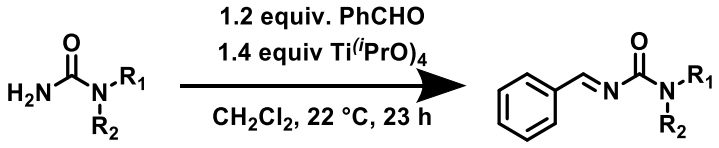
Nuclear Magnetic Resonance (NMR) spectra were recorded on BRUKER AV (600 MHz and 300 MHz), AVB (400 MHz), AVQ (400 MHz) and NEO (500 MHz) spectrometers. Measurements were carried out at ambient temperature. Chemical shifts (δ) are reported in ppm with the residual solvent signal as internal standard (chloroform at 7.26 and 77.00 ppm for ^{1}H NMR and ^{13}C NMR spectroscopy, respectively). The data is reported as (s = singlet, d = doublet, t = triplet, q = quartet, p = quintet, m = multiplet or unresolved, br = broad signal, coupling constant(s) in Hz, integration). 13C NMR spectra were recorded with broadband 1H decoupling.

Mass spectrometry (MS) analyses were obtained at the Catalysis Center at the College of Chemistry, University of California, Berkeley.

Experimental procedures and characterization

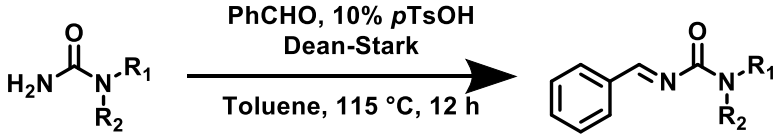


General imine synthesis 1. Scheme is based off work by Lillo et. al.[9] Desired urea (10 mmol) and $PhSO_2Na$ (2 eq) were dissolved in 20 mL of water. Then PhCHO (2 eq) in MeOH (8 mL) was added thereto followed by aqueous 80% HCOOH (0.8 mL). The reaction was stirred at room temperature overnight. If a precipitate was formed it was filtered, washed with water, hexanes, and dried. If no precipitate was observed, HCl was added to until the reaction mixture reached pH 3. In our cases, after overnight stirring the precipitate was formed and collected after filtration, washing with water and hexanes, and dried.

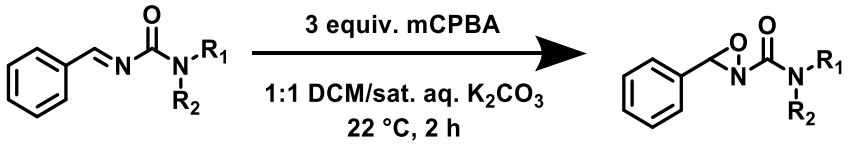


General imine synthesis 2. To a solution of corresponding urea (115 mmol) and benzaldehyde (127 mmol) in dry CH_2Cl_2 (20 mL) was added Ti(*i*PrO)$_4$ (140 mmol) dropwise at room temperature. The mixture was stirred at room temperature 23 h. The solvent was removed *in vacuo* to afford

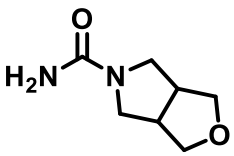
crude imine which was used immediately in the next step. The reaction was controlled by 1H NMR via appearance of the imine proton signal intensity.



General imine synthesis 3. To a flamed dried 3-neck round bottom equipped with a Dean-Stark trap and reflux condenser was added urea (10 mmol, 1 equiv.) and toluene (200 mL). To this mixture was added benzaldehyde (25 mmol, 2.5 equiv.) and pTsOH•H_2O (2 mmol, 0.2 equiv.). The mixture was refluxed for 12 hours. The mixture was then concentrated under reduced pressure and was used in the next step without further purification.

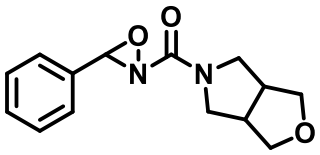


General oxaziridine synthesis. In a 25 mL round-bottom flask, mCPBA (75%, 538 mmol) was pre-stirred in 1:1 CH_2Cl_2/sat. aq. K_2CO_3 (140 mL) at room temperature for 10 minutes. A suspension formed. A solution of imine was added dropwise, but rapidly, using CH_2Cl_2. Intense stirring at this step was observed to be essential. Additionally, if a partial dissolution of solids was observed after imine addition, sat. K_2CO_3 was added to reaction mixture immediately. After 2 h, the reaction was diluted with water (150 mL) and extracted with CH_2Cl_2 (3 x 100 mL). The organic layer was separated, washed with diluted aq. K_2CO_3 (3 times) and water (2 times), dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was purified by column chromatography.

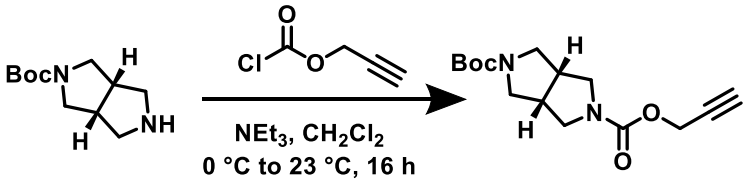


1oxF11 urea characterization. Compound provided by Enamine. **^{1}H NMR** (400 MHz, DMSO-d6) δ 5.72 (br s, 2H), 3.75 (dd, J = 8.8, 6.7 Hz, 2H), 3.45 – 3.31 (m, 4H), 3.10 (dd, J = 10.8, 3.3 Hz, 2H), 2.82 (dq, J = 7.2, 3.8 Hz, 2H). **^{1}H NMR** (600 MHz, $CDCl_3$) δ 4.54 (br s, 2H), 3.93 (dd, *J*

= 9.0, 6.6 Hz, 2H), 3.72 – 3.59 (m, 4H), 3.32 (dd, *J* = 10.5, 3.3 Hz, 2H), 3.00 (tp, *J* = 8.3, 4.3 Hz, 2H). **^{13}C NMR** (101 MHz, DMSO-d6) δ 157.90, 73.48, 50.73, 43.42. **HRMS (ESI + TOF)** calcd for $C_7H_{13}N_2O_2$ [MH+]: 157.0977. Found 157.0793.

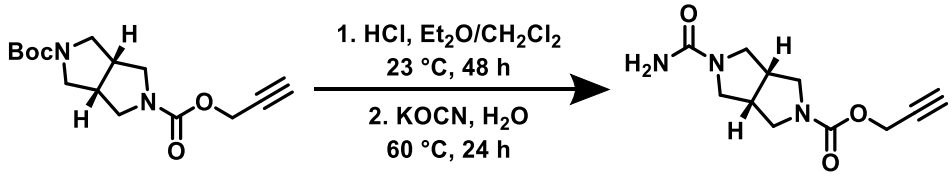


1oxF11 characterization. Compound provided by Enamine. **^{1}H NMR** (600 MHz, $CDCl_3$) δ 7.48 (m, 2 H), 7.45 (m, 1H), 7.41 (m, 2H), 5.22 (s, 1 H), 3.90 (m, 2 H), 3.77 (m, 2 H), 3.66 (m, 2 H), 3.50 (m, 2 H), 2.99 (m, 2 H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 160.14, 160.05, 133.08, 133.03, 130.91, 128.79, 128.05, 78.02, 77.96, 73.75, 73.59, 73.46, 73.29, 51.71, 51.44, 51.11, 50.83, 44.29, 44.25, 42.51. **HRMS (ESI)** calcd for $C_{14}H_{16}N_2O_3$ [MH+]: 261.1234. Found 261.1504.



A solution of amine (3.0 g, 14.1 mmol, 1.0 equiv.) in CH_2Cl_2 (31 mL) was cooled to 0 °C. Triethylamine (2.9 mL, 21 mmol, 1.5 equiv.) was added at 0 °C, followed by the dropwise addition of propargylchloroformate (1.5 mL, 15.5 mmol, 1.1 equiv.). The solution was warmed to room temperature overnight. After 16 h, the reaction mixture was diluted with CH_2Cl_2 (50 mL), washed with 1M aq. HCl (20 mL), sat. aq. $NaHCO_3$ (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo* to afford the desired product as a pale yellow oil which solidified upon standing to a pale yellow solid (4.2 g, >95%) containing traces of CH_2Cl_2.

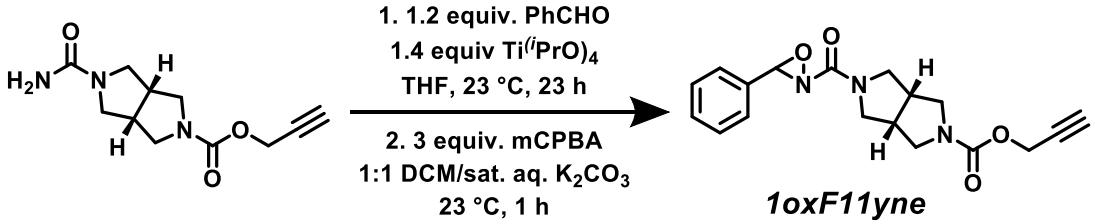
^{1}H NMR (500 MHz, $CDCl_3$) δ 4.66 (t, *J* = 2.6 Hz, 2H), 3.58 (m, 4H), 3.36 – 3.13 (m, 4H), 2.83 (s, 2H), 2.44 (t, *J* = 2.4 Hz, 1H), 1.42 (s, 9H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 154.5, 153.9, 79.7, 78.6, 74.6, 52.8, 50.2, 49.7, 49.5, 42.5, 41.6, 40.7, 28.5. **HRMS (ESI):** calcd for $C_{15}H_{22}N_2O_4Na$ [M+Na]+: 317.1472. Found 317.1479.



To a solution of Boc-protected amine (2.0 g, 6.8 mmol, 1.0 equiv.) in CH_2Cl_2 (13 mL) was added HCl (2 M in Et_2O, 12 mL, 24 mmol, 3.5 equiv.) at room temperature. After 48 h, the solvent was removed in vacuo to afford the desired product containing ~10% remaining starting material. This material was resubjected to the same conditions using HCl (2 M in Et_2O, 6 mL, 12 mmol, 1.8 equiv.). After 16 h, the solvent was removed *in vacuo* to afford the corresponding amine hydrochloride salt (1.5 g, 96%) as a white solid which was directly used in the next step.

To a solution of the amine hydrochloride salt (1.45 g, 6.3 mmol, 1.0 equiv.) in water (6.3 mL) was added KOCN (1.5 g, 19 mmol, 3 equiv.) at room temperature. The reaction was sealed and stirred at 60 °C. After 24 h, the reaction was cooled to room temperature and extracted with EtOAc (5 x 100 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo* to afford the desired urea product (1.3 g, 88%) as a white solid.

^{1}H NMR (500 MHz, DMSO) δ 5.74 (s, 2H), 4.65 (t, *J* = 2.9 Hz, 2H), 3.53 (m, 2H), 3.48 (t, *J* = 2.4 Hz, 1H), 3.43 (m, 2H), 3.10 (m, 4H), 2.92 – 2.74 (m, 2H). **^{13}C NMR** (126 MHz, DMSO) δ 157.4, 153.2, 79.3, 77.3, 52.2, 50.2, 49.6, 49.4, 41.6, 40.6. **HRMS (ESI):** calcd for $C_{11}H_{16}N_3O_3$ [M+H]+: 238.1186. Found 238.1199.

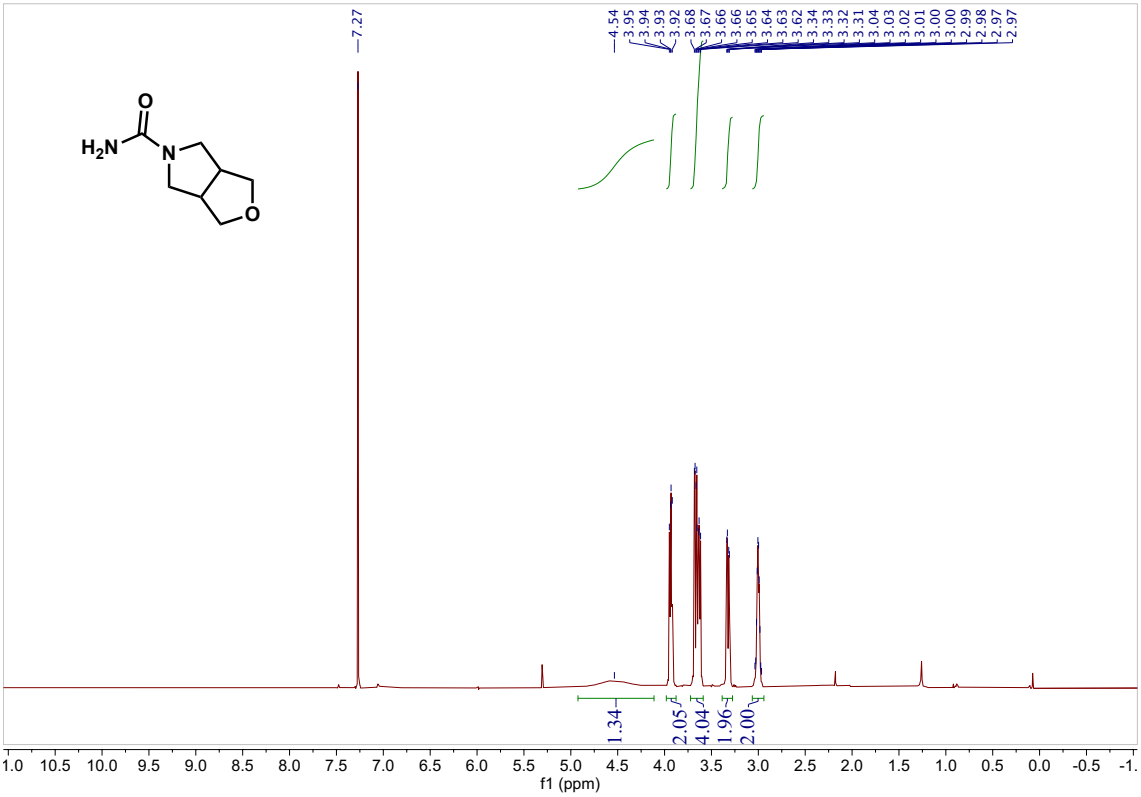


To a solution of corresponding urea (237 mg, 1.0 mmol, 1.0 equiv.) and benzaldehyde (0.12 mL, 1.2 mmol, 1.2 equiv.) in dry THF (3 mL) was added Ti(*i*PrO)$_4$ (0.42 mL, 1.4 mmol, 1.4 equiv.) dropwise at room temperature. The mixture was stirred at room temperature 23 h. The solvent was removed *in vacuo* to afford crude imine which was used immediately in the next step.

In a 25 mL round-bottom flask, mCPBA (75%, 692 mg, 3.0 mmol, 3 equiv.) was prestirred in 1:1 CH_2Cl_2/sat. aq. K_2CO_3 (8 mL) at room temperature for 10 minutes. A solution of crude imine

from the previous step (in 1 mL CH_2Cl_2) was added dropwise, using additional CH_2Cl_2 (2 x 1 mL) rinses for a quantitative transfer. After 1 h, the reaction was diluted with water (30 mL), and extracted with CH_2Cl_2 (3 x 30 mL). The combined organic layers were washed with brine (30 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (75% to 85% EtOAc/Hexane) afforded the corresponding oxaziridine (194 mg, 57%) as a white foam.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44 (m, 5H), 5.22 (s, 1H), 4.78 – 4.60 (m, 2H), 4.05 (m, 0.4H, *minor rotamer*), 3.84 – 3.64 (m, 4H), 3.50 – 3.29 (m, 3.6H), 3.01 – 2.87 (m, 2H), 2.46 (m, 1H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 160.4, 153.9, 130.9, 128.8, 128.03, 128.00, 78.5, 78.0, 74.7, 53.0, 50.7, 50.6, 50.1, 50.0, 41.1, 40.1. **HRMS (ESI):** calcd for $C_{18}H_{20}N_3O_4$ [M+H]+: 342.1449. Found 342.1442.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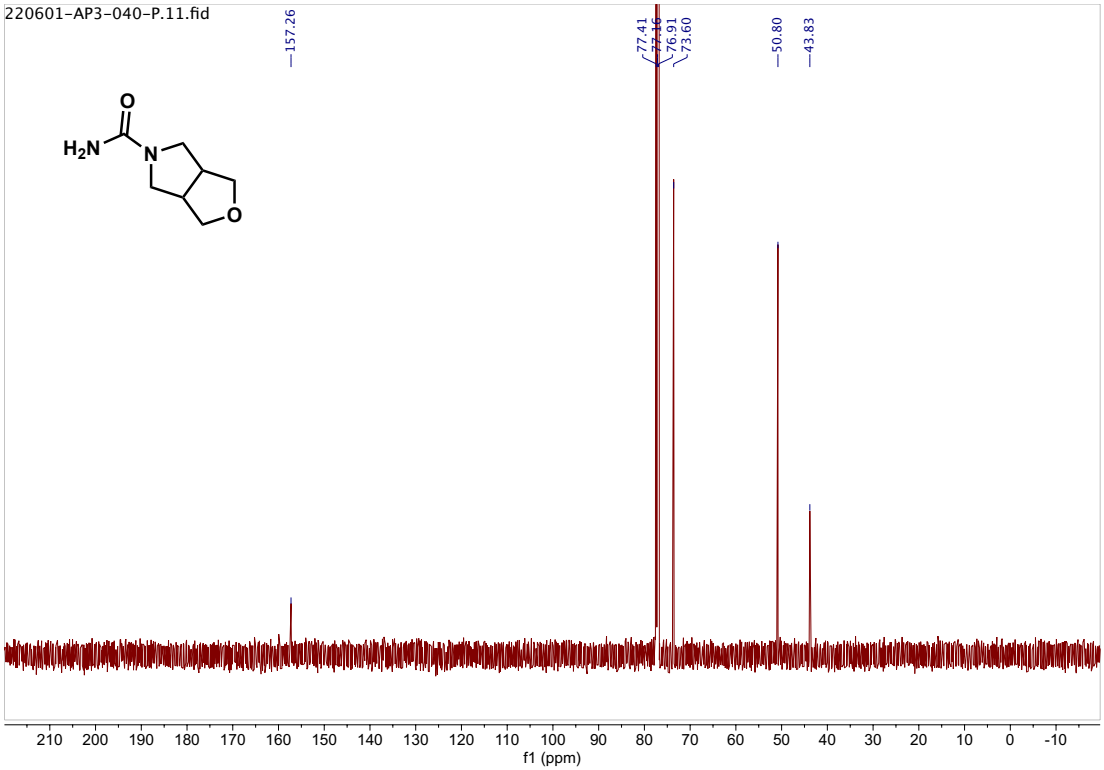
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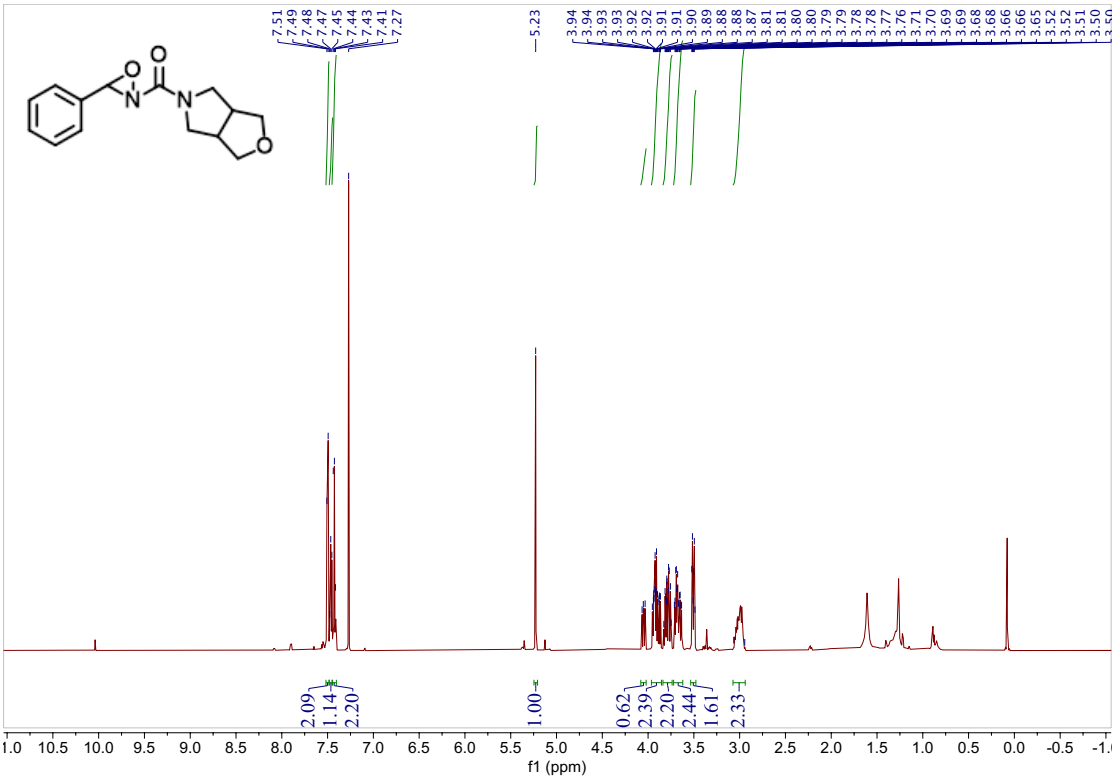
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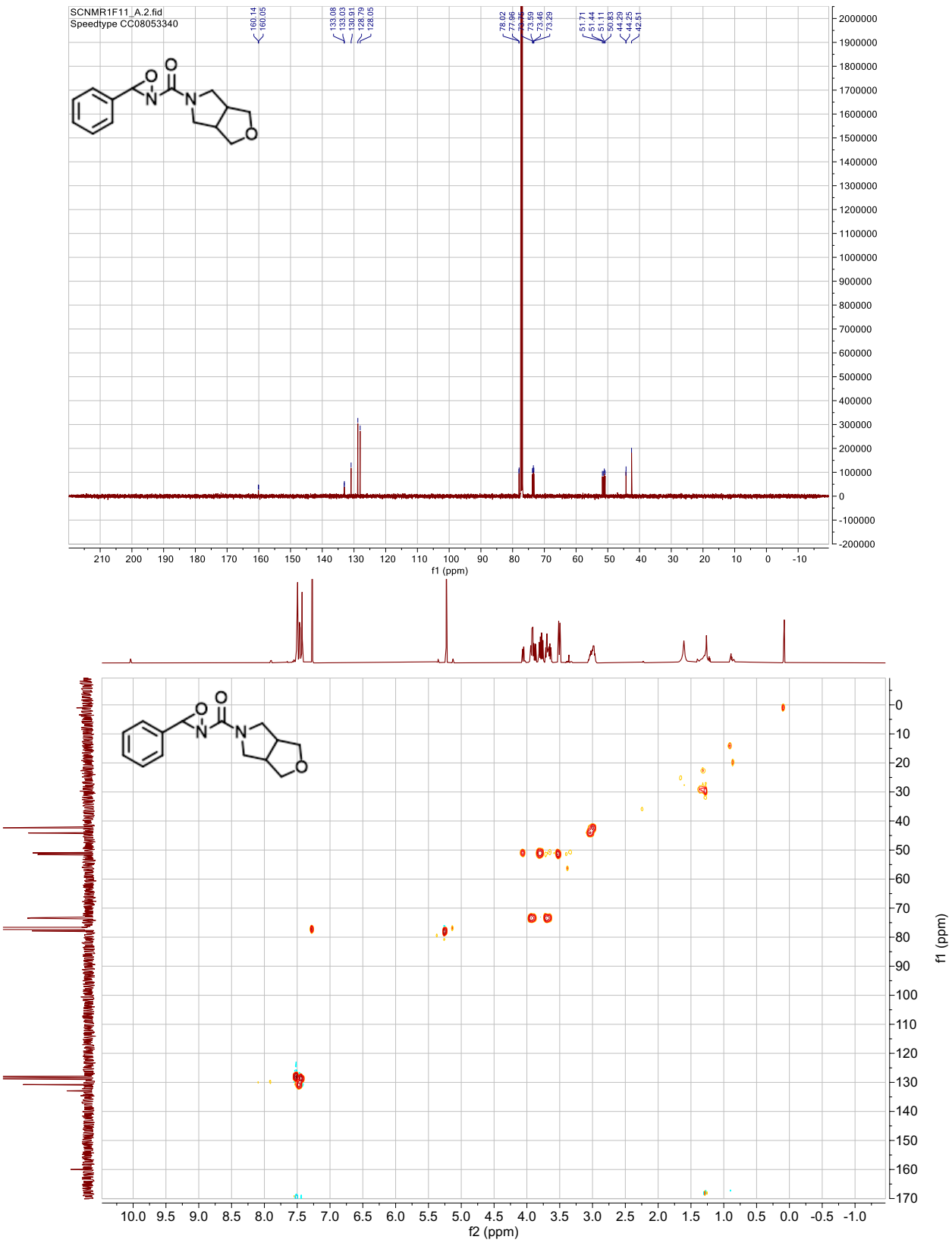
f1 (ppm)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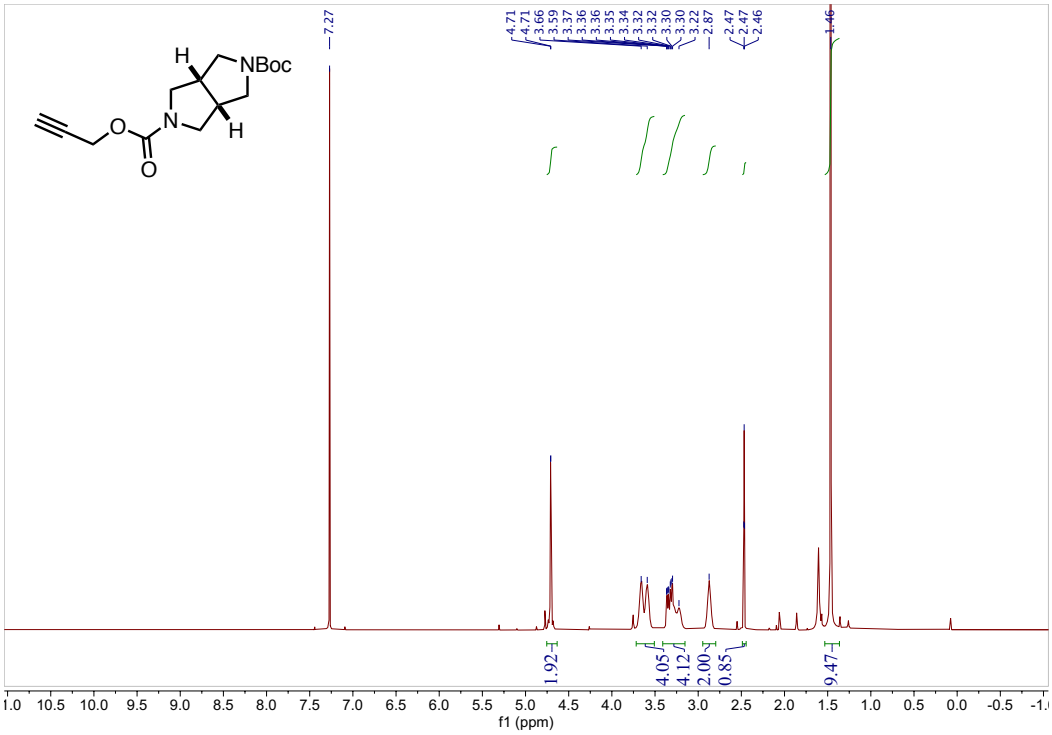
f2 (ppm)
f1 (ppm)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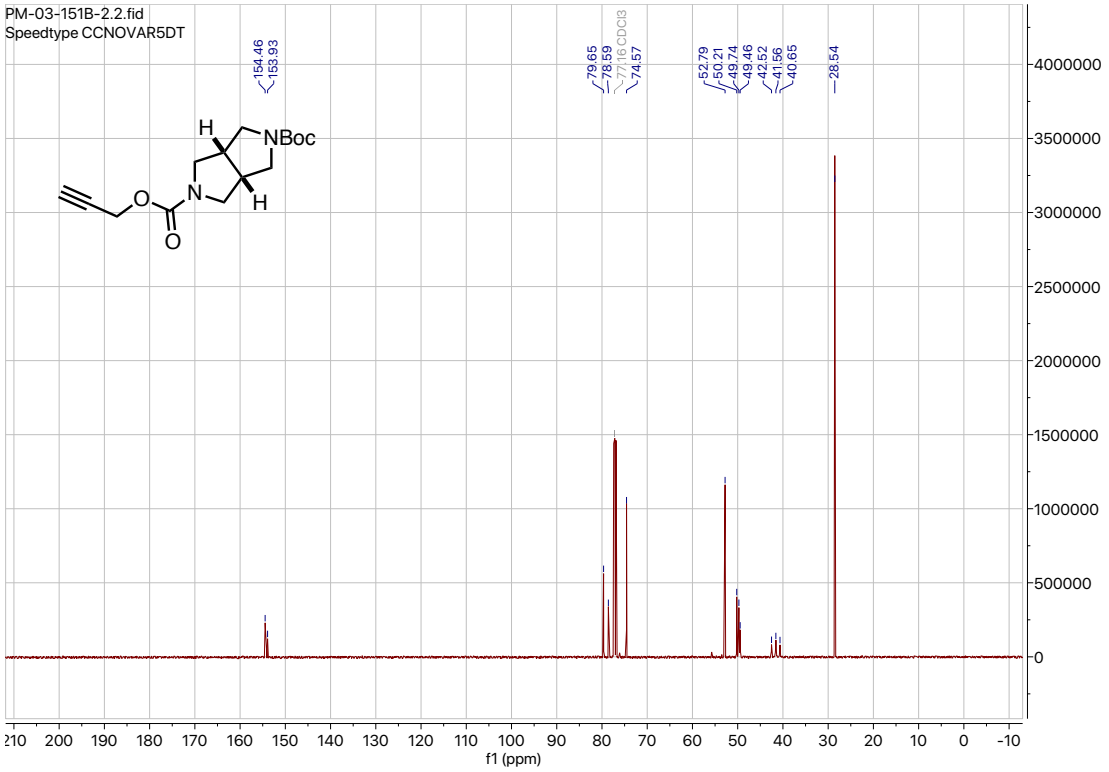
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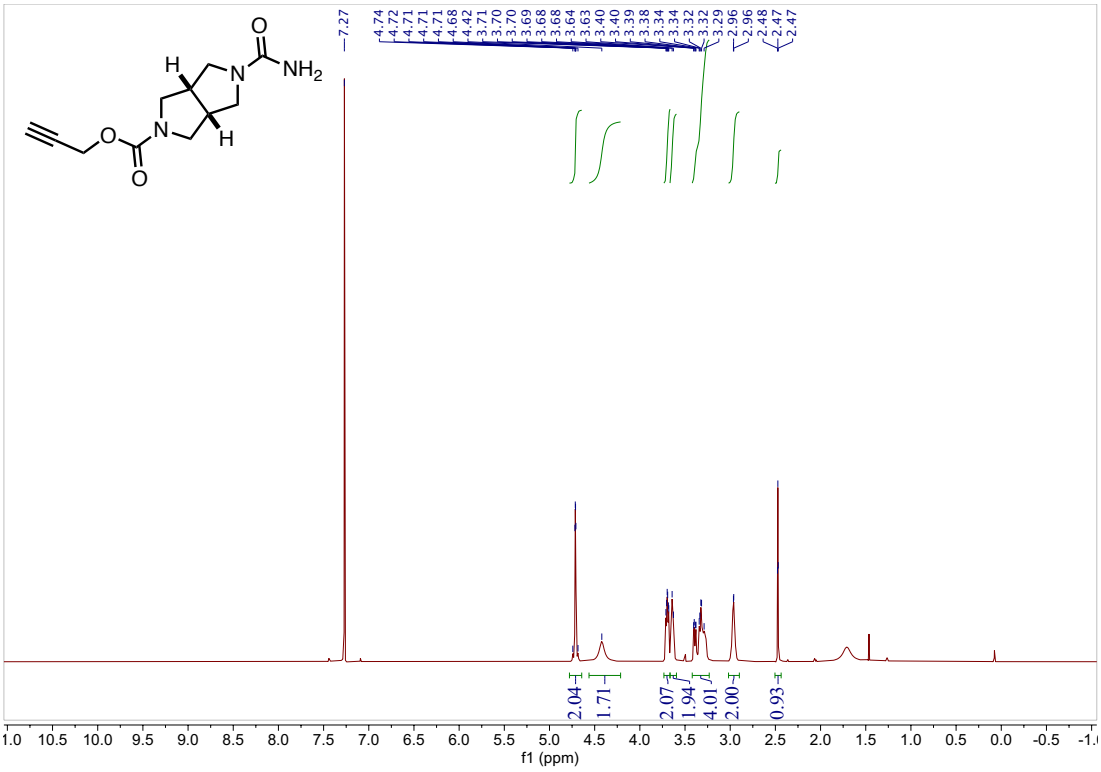
f1 (ppm)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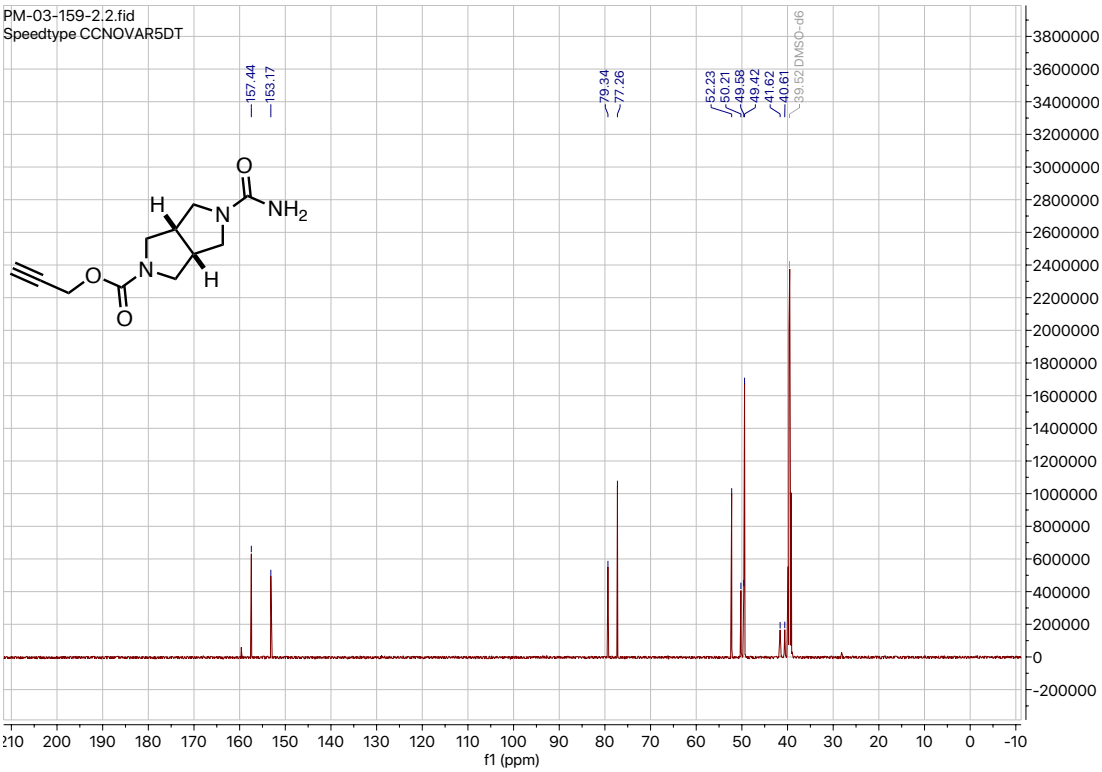
f1 (ppm)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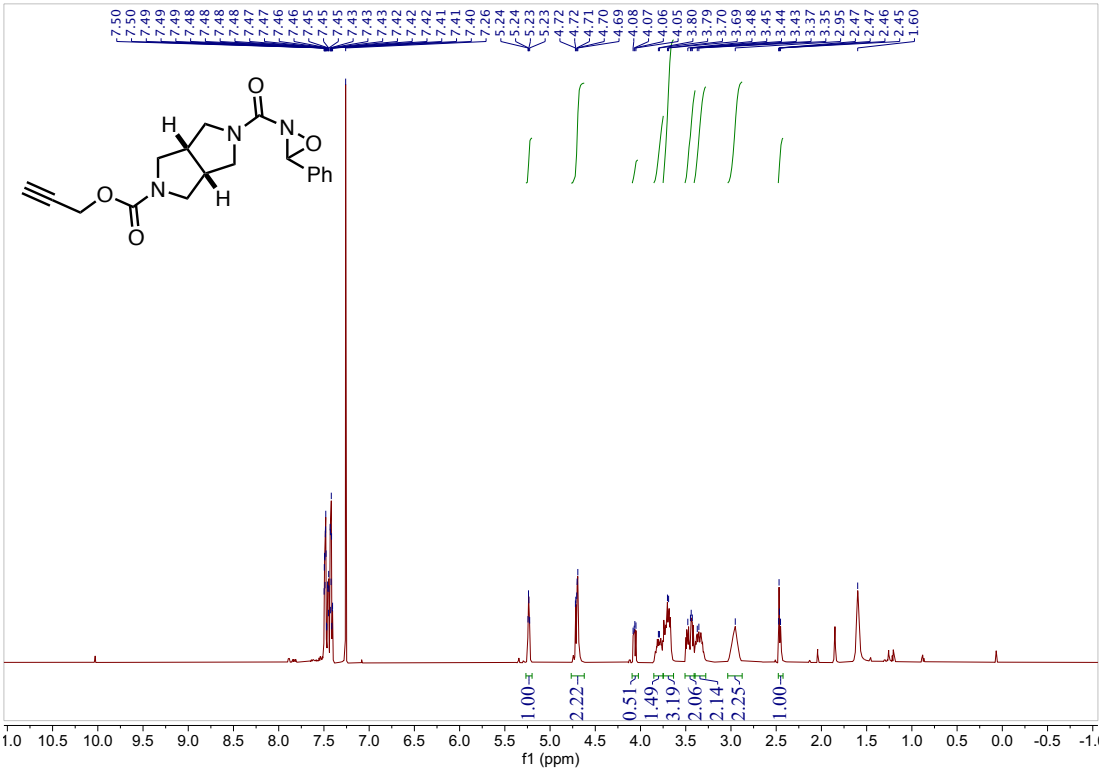
f1 (ppm)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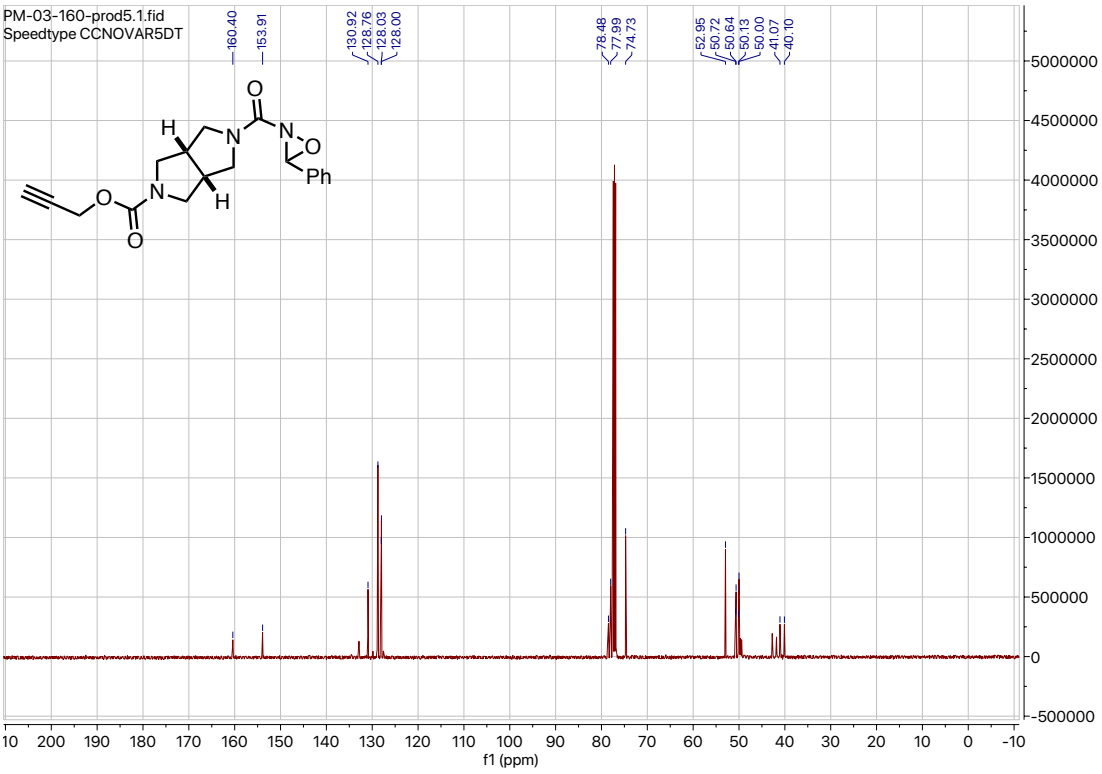
f1 (ppm)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f1 (ppm)

3. References

1. Lin, S.; Yang, X.; Jia, S.; Weeks, A. M.; Hornsby, M.; Lee, P. S.; Nichiporuk, R. V.; Iavarone, A. T.; Wells, J. A.; Toste, F. D.; Chang, C. J. Redox-Based Reagents for Chemoselective Methionine Bioconjugation. *Science* **2017**, *355* (6325), 597–602.
2. Christian, A. H.; Jia, S.; Cao, W.; Zhang, P.; Meza, A. T.; Sigman, M. S.; Chang, C. J.; Toste, F. D. A Physical Organic Approach to Tuning Reagents for Selective and Stable Methionine Bioconjugation. *J. Am. Chem. Soc.* **2019**, *141* (32), 12657–12662.
3. Corina version 4.2.0, Molecular Networks GmbH, www.mn-am.com.
4. Hawkins, P.C.D.; Skillman, A.G.; Warren, G.L.; Ellingson, B.A.; Stahl, M.T. Conformer Generation with OMEGA: Algorithm and Validation Using High Quality Structures from the Protein Databank and the Cambridge Structural Database. J. Chem. Inf. Model. 2010, 50, 572-584.
5. Gaussian 09, Revision E.01, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, T. Keith, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, O. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski, and D. J. Fox, Gaussian, Inc., Wallingford CT, 2013.
6. https://github.com/EdMiller0886/Thesis-Chapter-5/
7. Wolters, D. A.; Washburn, M. P.; Yates, J. R. An Automated Multidimensional Protein Identification Technology for Shotgun Proteomics. *Anal. Chem.* **2001**, *73* (23), 5683–5690.
8. Coulonval, K.; Vercruysse, V.; Paternot, S.; Pita, J. M.; Corman, R.; Raspé, E.; Roger, P. P. Monoclonal Antibodies to Activated CDK4: Use to Investigate Normal and Cancerous Cell Cycle Regulation and Involvement of Phosphorylations of P21 and P27. *Cell Cycle* **2021**, 1–21.
9. Lillo, V. J.; Mansilla, J.; Saá, J. M. The Role of Proton Shuttling Mechanisms in Solvent-Free and Catalyst-Free Acetalization Reactions of Imines. *Org. Biomol. Chem.* **2018**, *16* (24), 4527–4536.