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A Physical Organic Approach to Reagent Design for Bioconjugation: Development of
Acyl Silane and Oxaziridine Probes for Chemoproteomic Profiling

By

Annika Caroline Skaar Page

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Chemistry

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor F. Dean Toste, Chair

Professor Thomas Maimone

Professor Gerard Marriott

Summer 2024

A Physical Organic Approach to Reagent Design for Bioconjugation: Development of
Acyl Silane and Oxaziridine Probes for Chemoproteomic Profiling

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Annika Caroline Skaar Page

Abstract

A Physical Organic Approach to Reagent Design for Bioconjugation: Development of Acyl Silane and Oxaziridine Platforms for Chemoproteomic Profiling

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Annika Caroline Skaar Page

Doctor of Philosophy in Chemistry

University of California, Berkeley

Professor F. Dean Toste, Chair

Identifying potent and selective small molecule binders for specific protein targets is a central challenge in drug discovery. Most bioactive compounds do not covalently bind their protein target, and instead have a transient, reversible interaction based on affinity to the binding site of the protein. To support non-covalent drug discovery efforts, chemists and chemical biologists have developed strategies using covalent small molecule probe compounds to covalently capture these small molecule-protein interactions. This dissertation focuses on two of the most commonly used current strategies for live-cell target identification: residue-agnostic Photoaffinity Labeling (PAL) and residue-specific Activity-Based Protein Profiling (ABPP).

I first discuss work to develop a novel class of PAL probes based on the UV-triggered 1,2-photo-Brook rearrangement of acyl silanes. These new probes, particularly the *i*Pr-substituted acyl silanes, demonstrated efficient photolabeling capabilities with minimal background, showcasing their potential for use in photoaffinity labeling campaigns. Building on these advancements in PAL, we later extended this technique to live cell applications using the same acyl silane-based probes. Our work highlights the effectiveness of acyl silane photoaffinity probes in live cell photoaffinity workflows and their complementarity to existing diazirine-based probes. We also developed a modular synthetic route to generate acyl silane scaffolds with improved labeling properties, expanding the toolkit available for *in vivo* studies of small molecule-protein interactions.

In another area, we explored residue-specific protein bioconjugation by applying methionine-selective redox-activated chemical tagging (ReACT) strategies to ABPP workflows. This enabled the identification of new ligandable sites on cyclin-dependent kinase 4 (CDK4) and unveiled a potential role for the liganded methionine site as an allosteric regulator of protein phosphorylation. Lastly, we explored the role of chirality in protein regulation by developing enantiomeric oxaziridine reagents to investigate pro-chiral methionine oxidation sites. This approach revealed that chiral regulation of methionine oxidation can significantly affect protein function, as demonstrated in oxidative stress models where selective (*R*)-methionine sulfoxide formation influenced downstream protein modifications. Our work introduces a platform for studying asymmetric methionine modifications and their functional implications, contributing to a deeper understanding of chiral regulation in proteins.

DEDICATION

Dedicated to my mom, who encouraged my passion for learning.

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LIST OF ABBREVIATIONS

231 MFP	Mammary fat pad xenograft of MDA-MB-231 cells
ABPP	Activity-based protein profiling
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BD1	Bromodomain 1
BET	Bromo- and extra-terminal
BME	Beta-mercaptoethanol
BP	Benzophenone
BPHL	Biphenyl hydrolase-like protein
BRD3	Bromodomain-containing protein 3
BRD4	Bromodomain-containing protein 4
BSA	Bovine serum albumin
CAK	Cyclin-activating kinase
CCK-8	Cell counting kit-8
CCND1	Cyclin-D1
CDCl ₃	Deuterated chloroform
CDK1	Cyclin-dependent kinase 1
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
ChURRO	Chiral Unveiling by Redox-based Reactivity with Oxaziridines
CuAAC	Copper-catalyzed azide-alkyne cycloaddition
DA	Diazirine
DBCO	Dibenzocyclooctyne
DCM	Dichloromethane
DFT	Density functional theory
DMF	Dimethylformamide
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DTB-N3	Desthiobiotin azide
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
EI	Electron impact
ESI	Electrospray ionization
Et ₂ O	Diethyl ether
Et ₃ N	Triethylamine
EtOAc	Ethyl acetate
FA	Formic acid
FBDD	Fragment-based drug discovery
FBS	Fetal bovine serum
FDR	False discovery rate
FKBP12	FK506 Binding Protein
FLAG tag	DYKDDDDK polypeptide protein tag
GO	Gene ontology
HCTL	Homocysteine thiolactone

HC	Homocysteine
HEK	Human embryonic kidney
Hex	Hexanes
HFD	High fat diet
HPLC	High performance liquid chromatography
HRMS	High-resolution mass spectrometry
HRP	Horseradish peroxidase
IA	Iodoacetamide
IB	Immunoblot
IP	Immunoprecipitation
IPA	Isopropanol
IR	Infrared
isoTOP-ABPP	Isotopic tandem orthogonal proteolysis-enabled activity-based protein profiling
KD	Knockdown
KMnO ₄	Potassium permanganate
LC-MS	Liquid chromatography-mass spectrometry
LFD	Low fat diet
MCF-7	Michigan Cancer Foundation 7
mCPBA	<i>meta</i> -chloro peroxybenzoic acid
MD	Molecular dynamics
MeCN	Acetonitrile
MeOD	Deuterated methanol
MeOH	Methanol
MetO	Methionine sulfoxide
MetO	Methionine
MQ	Milli-Q
MS/MS	Tandem mass spectrometry
MS1	First MS for detection of initial ionization in MS/MS
MS2	Second MS for detection of fragment ionization in MS/MS
Msr	Methionine sulfoxide reductase
MsrA	Methionine sulfoxide reductase A
MsrB	Methionine sulfoxide reductase B
mTOR	Mammalian target of rapamycin
MudPIT	Multidimensional protein identification technology
NAFLD	Non-alcoholic fatty liver disease
NAM	<i>N</i> -acetyl methionine
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PAL	Photoaffinity labeling
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PIFA	Phenyliodine bis(trifluoroacetate)
PMBO	<i>para</i> -methoxybenzyl
pPSE	Prediction-aware part-sphere exposure

PTM	Post-translational modification
PVDF	Polyvinylidene difluoride
Rb	Retinoblastoma
ReACT	Redox-activated chemical tagging
ReDiMe	Reductive dimethylation
Rh-N ₃	Rhodamine azide
ROS	Reactive oxygen species
RT	Room temperature
SAR	Structure-activity relationship
SDS	Sodium dodecyl sulfate
SFC	Supercritical fluid chromatography
SILAC	Stable isotope labeling by amino acids in cell culture
TBS	<i>tert</i> -Butyldimethylsilyl
TBST	Tris-buffered saline with Tween 20
TEAB	Triethylammonium bicarbonate
TGX	Tris-glycine extended
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TMT	Tandem mass tag
UV	Ultraviolet
VCD	Vibrational circular dichroism

CHAPTER 1

Advances in Residue-Specific and Residue-Agnostic Covalent Protein Bioconjugation

1.1 Introduction

Identifying potent and selective small molecule binders for specific protein targets is a central challenge in drug discovery (1). This challenge is twofold: it involves both identifying small molecule binders of individual proteins of interest as well as profiling the reactivity of these molecules with all proteins in the proteome. Each of these challenges can be used to inform the design of new small molecule binders for specific proteins, and each of these challenges requires a different chemical strategy, some of which will be highlighted in this introductory chapter. Initial efforts in a drug discovery campaign often partition into two main approaches, *target-focused* and *outcome-focused* (2–4). Target-focused approaches aim to discover small molecule binders for specific protein targets and often utilize fragment-based drug discovery (FBDD). In contrast, outcome-focused approaches seek small molecules that produce a desired phenotype in a model system, frequently using phenotypic screening with large compound libraries.

Most small molecule drugs are designed to target or inhibit a specific protein, and interactions with off-target proteins can cause unintended side effects (5) (Figure 1.1). This underlines a need to be able to identify the interactions of a small molecule with high- and low- affinity protein targets. Identifying these interactions in the proteome has been described as finding a “needle in a giant haystack” due to the size and complexity of the proteome and the fleeting nature of most small molecule-protein interactions (6). Most bioactive compounds do not covalently bind their protein target, and instead have a transient, reversible interaction based on affinity to the binding site of the protein. This temporary interaction complicates identification of on- and off-targets of small molecules, especially in live cells, due to the rigorous washing steps employed by most non-covalent identification assays (5–7).

To support these non-covalent drug discovery efforts, chemists and chemical biologists have developed strategies using covalent small molecule probe compounds to uncover the interactions underlying these target- or outcome-focused approaches. These probe strategies mirror the approach undertaken in genetics and have been referred to as “reverse chemical genetics” and “forward chemical genetics”, respectively (8, 9). This chapter will explore probe design strategies for bioconjugation and their practical applications.

1.2 Strategies for covalent protein modification

Bioconjugation is a chemical strategy that enables a covalent modification on a protein or other biomolecule to attach chemical cargo by using a small molecule probe compound. Chemical probe requirements vary based on their intended application, though a few requirements apply to nearly all probe classes (10, 11). Firstly, a small molecule probe compound should contain some reactive group that engages a functionality found on a biomolecule. For protein-based strategies, these functionalities are the bonds found in amino acid residues. Common methods used to identify protein targets of bioactive molecules partition into residue-specific and residue-agnostic reagents, which will be discussed below. In addition to bearing a reactive moiety, chemical probes for bioconjugation should minimally perturb the biological system in which they are being deployed.

1.2.1 Residue-specific protein modification: Activity-based protein profiling

Covalent bioconjugation using reagents tailored for individual amino acids are a hallmark of chemical proteomics endeavors. In reverse chemical genetics approaches, a protein target with predicted or known pathological importance is selected and screened for small molecule binders. The selection and discovery of these physiologically-relevant proteins has been aided by the development of covalent bioconjugation methods to identify reactive amino acid residues in these proteins. While chemical modification of proteins has been known for decades (12), the development of residue-selective chemical probes with handles for further visualization is more recent (13, 14). The use of these small molecule probes to investigate reactivity of amino acid residues in proteins is termed “Activity-Based Protein Profiling (ABPP) and involves adding a reactive moiety to a small molecule, which in the proteome is captured by a reactive residue to form a covalent bond. The interrogation of specific amino acid residues enables site-specificity and investigation of reactive amino acid residues that may be targeted using small molecule drugs.

Commonly targeted amino acid residues for ABPP include cysteine and lysine, though there is a growing push to expand residue-specific probe technology beyond these classical methods (15). Cysteine contains a highly reactive thiol which can be easily targeted with mildly electrophilic reagents, and has thus seen extensive profiling (16, 17) for use in therapeutic discovery as well as protein conjugation applications. Cysteine-targeting electrophiles include maleimides (18), acrylamides (19), and iodoacetamides (20), as well as other broadly electrophilic compounds (15) (Figure 1.2). While cysteine is a commonly targeted amino acid for chemoproteomics owing to its high nucleophilicity, its relative scarcity hinders its use in target-based approaches as many therapeutically-relevant proteins do not have reactive cysteines available for targeting.

Lysine is a much more common amino acid in proteins and its reactivity is often dictated by the protein microenvironment in which it resides. Acylation of lysine is a widely-utilized strategy to covalently modify protein-bound lysine residues, and sulfonyl fluoride (21–23) and NHS ester (24) probes are generally used to target these amines, though they do exhibit reactivity with other amino acid residues as well (Figure 1.3). Global analyses of reactive lysine residues and lysine-reactive reagents have been reported (25, 26).

Recently, we (27–29) and others (30–32) have investigated chemistry to selectively label methionine residues in proteins. Methionine is a redox-susceptible protein commonly found in hydrophobic pockets and is one of the least-expressed amino acids. While this scarcity hampers proteome-wide target identification approaches, it is an attractive property for enabling selective protein modification while minimizing potential off-target interactions. Historically, methods to alkylate methionine for ABPP applications are less established than those for cysteine and lysine, but recent work in our group has established reactive oxaziridine reagents for selective methionine conjugation under biological conditions. This method, called Redox-Activated Chemical Tagging (ReACT) (27), has led to applications in antibody-drug conjugation (33) and peptide stapling (28) (Figure 1.4). Recently, we have focused on the expansion of this technology to enable proteome-wide ABPP of reactive methionine residues (29) as well as the investigation of functionally relevant, pro-chiral redox-susceptible methionines in proteins and proteomes, both of which will be discussed at length in subsequent chapters of this dissertation.

1.2.2 Residue-agnostic protein modification: Photoaffinity labeling

Photoaffinity labeling (PAL) is a bioconjugation method that has emerged as a powerful method to identify the targets of a bioactive compound. PAL is a technique used to covalently capture non-covalent ligand-protein interactions. In this method, a photoreactive moiety is covalently attached to the ligand, which, after ligand binding and upon irradiation, generates a reactive species that inserts into a nearby residue in the protein to covalently capture the protein-ligand interaction (Figure 1.5). PAL is compatible with live cells and is commonly used in target identification campaigns for compounds identified through phenotypic screening— with a bioactive compound already identified, one can attach a photoaffinity probe on the compound to investigate its protein targets (5, 34–36). Residue-specific covalent probes can also be used in phenotypic screens, but as they target specific residues in proteins, their use is limited to identifying proteins that contain that reactive residue in the binding pocket. Crosslinkers for photoaffinity probes are generally designed to have “residue agnostic” reactivity and can insert into a wider selection of amino acid residues (37). This property aids in the broad applicability of photoaffinity probes in phenotypic screens.

Since its introduction in the 1960’s by Knowles (38, 39) and Westheimer (34, 40) PAL has evolved into a useful strategy that can be employed to map the binding sites of a protein, identify the proteins with which a compound of interest interacts, and determine off-target interactions of the compound (37). To act as a suitable photoaffinity probe, a crosslinker must meet several criteria: 1) It should have a long wavelength absorption because many proteins are damaged or degraded with light at wavelengths below 300 nm (34, 41); 2) The probe should have a short irradiation time, on the order of minutes, to further maintain the integrity of the protein (41); 3) The photoactivated species should be sufficiently reactive to rapidly insert into a variety of residues once generated; 4) The probe should exhibit low background reactivity with biologically relevant moieties in the absence of light; 5) The photoadduct should be stable under biological conditions, so that the interactions can be identified after pulldown procedures; 6) The photoaffinity probe should be cell penetrant and not impede the binding of the ligand to the protein (40). Three probe scaffolds have enjoyed widespread use, although they do not necessarily fulfill each criteria (41).

Benzophenones, aryl azides, and diazirines have emerged as three useful, albeit imperfect, probes for photoaffinity labelling (Figure 1.6). Upon irradiation, these three probes generate a ketyl radical, a nitrene, and a carbene, respectively (Figure 1.6). Aryl azides, and the nitrenes formed from irradiation, were one of the earliest photoaffinity probes developed and were widely used due to their straightforward synthesis and thermal stability (42, 43). While aryl azides are easily synthesized, they require relatively short wavelengths for activation, which can damage biological molecules such as proteins (34). Additionally, the nitrenes generated rearrange to form a crosslinking species that is less reactive than the carbene, leading to longer reaction times and lower yields of crosslinked products (34, 43) (Figure 1.7).

Benzophenones were first used as photoaffinity probes in 1974 and are attractive due to their facile preparation and long wavelength activation (44, 45). However, benzophenones require long irradiation times, which can lead to side reactions and non-specific labelling, and are especially sensitive to methionine and can crosslink with those residues at much greater distance than most amino acids (34, 46, 47). Additionally, it can

be challenging to attach a small molecule of interest to the benzophenone scaffold as the benzophenone is difficult to derivatize (36).

A third scaffold for photoaffinity labelling is alkyl- or aryl-diazirines. These thermally stable molecules were first used for photoaffinity labelling by Knowles in 1973, and have enjoyed widespread use (39, 40). One of the main benefits of diazirines, particularly alkyl diazirines such as the “minimalist tag”, is their small size, which is beneficial as it helps maintain the affinity of the ligand for the protein and improves the cell penetrance of the probe (7, 40, 48). The carbene generated is extremely reactive, enabling fast crosslinking to proteins and minimizing non-specific labelling as unbound probes are quickly quenched with solvent and water (34). However, the excited diazirine can also rearrange to form an isomeric diazo species, and this pathway is common for alkyl diazirine probes. While photochemically active, this species generates a carbene at a slower rate and suffers from potential thermal background reactivity (Figure 1.7) (34). Due to these challenges, developing new photoaffinity warheads is desirable as it expands the toolbox of chemical biology techniques and is especially beneficial because multiple probes can be used in combination to validate protein targets. Recently (49), we have investigated the 1,2-photo-Brook rearrangement of acyl silanes to α -siloxy carbene intermediates as an alternative strategy for PAL, which will also be discussed at length in subsequent chapters of this dissertation.

1.3 Conclusion

Understanding small molecule-protein interactions remains critically important for drug medicinal chemistry and drug discovery. The challenges inherent to these endeavors are compounded by the non-covalent nature of most small molecule-protein interactions, which complicates their identification, especially in native cellular environments. To address these challenges, chemists and chemical biologists have developed a toolbox of covalent chemistries to enable residue-specific conjugation to individual amino acids in proteins as well as residue-agnostic techniques to enable unbiased proteome-wide mapping of small molecule-protein interactions. Activity-based protein profiling and photoaffinity labeling are cornerstones of modern drug discovery efforts, and will be the core focus of subsequent chapters of this dissertation.

1.4 Figures and Schemes

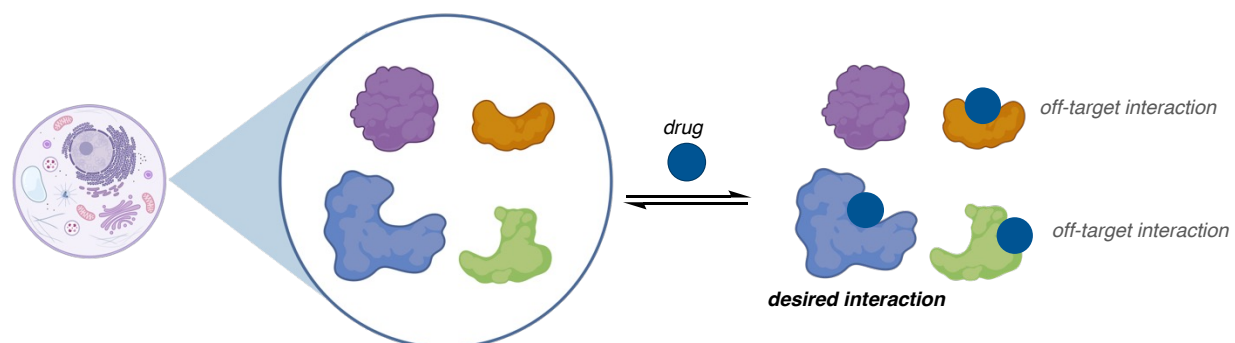


Figure 1.1. Identifying small molecule-protein interactions.

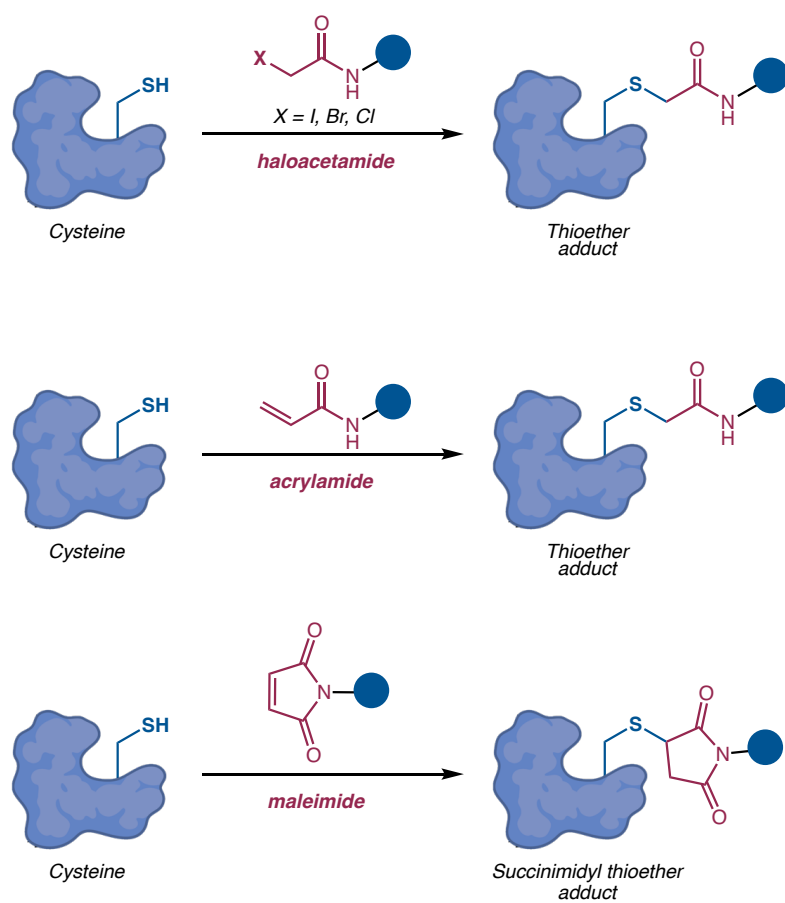


Figure 1.2. Covalent strategies for cysteine bioconjugation.

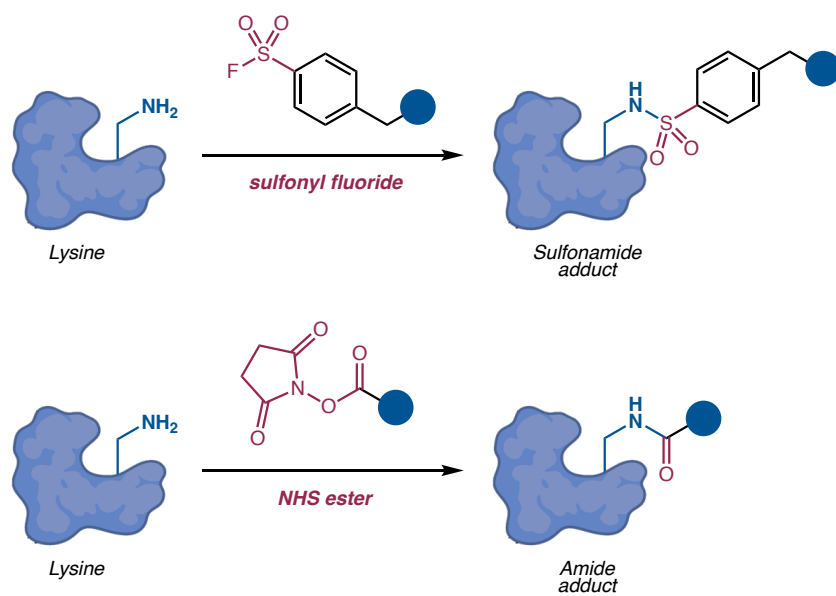


Figure 1.3. Covalent strategies for lysine bioconjugation.

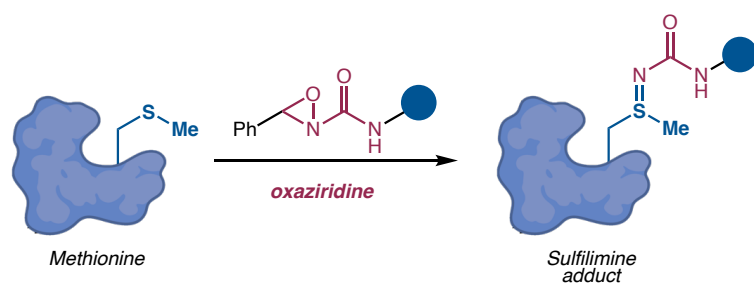


Figure 1.4. Redox-activated chemical tagging (ReACT) strategy for covalent methionine bioconjugation.

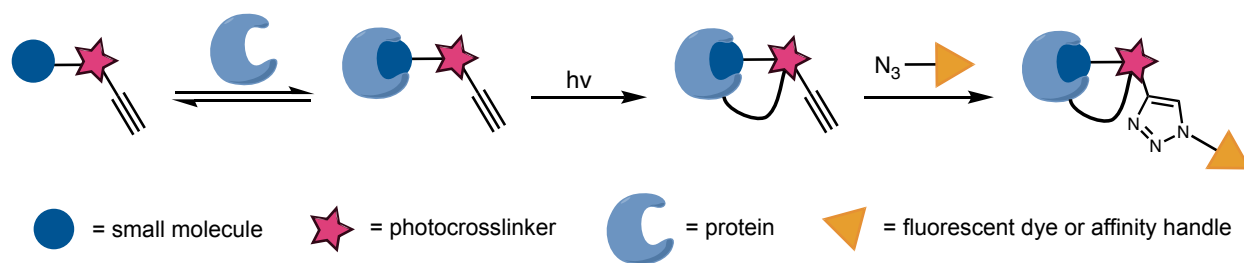


Figure 1.5. Schematic representation of photoaffinity labeling to identify small molecule-protein interactions.

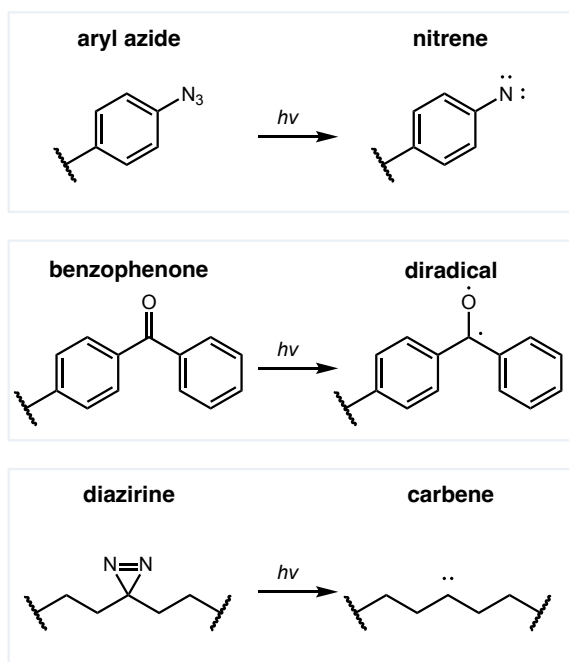


Figure 1.6. Traditional crosslinkers for photoaffinity labeling.

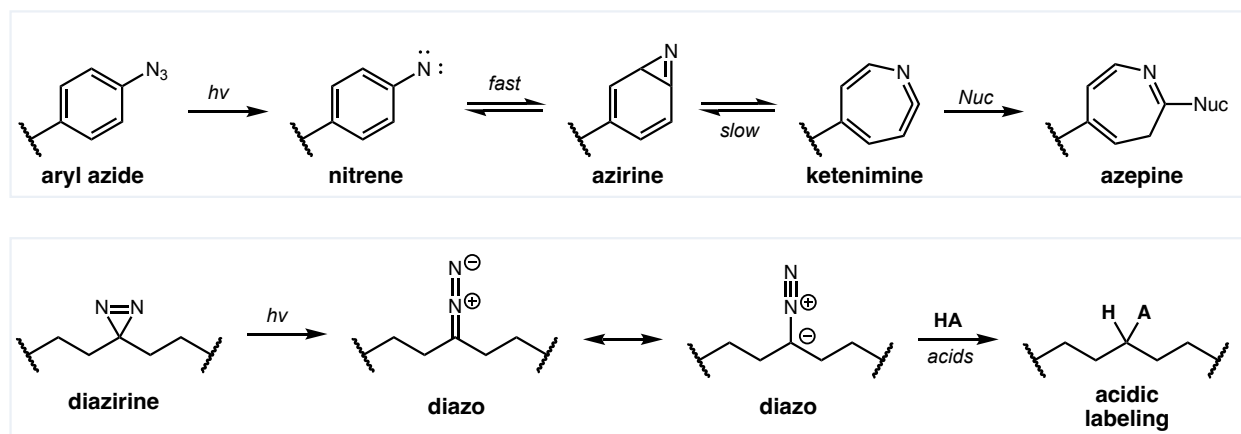


Figure 1.7. Non-specific labeling pathways of traditional crosslinkers for photoaffinity labeling.

CHAPTER 2

Photo-Brook Rearrangement of Acyl Silanes as a Strategy for Photoaffinity Probe Design

This chapter is partially based on the *Chemical Science* publication “Photo-Brook Rearrangement of Acyl Silanes as a Strategy for Photoaffinity Probe Design” (49) and has been adapted with permission from all co-authors.

2.1 Abstract

Photoaffinity labeling (PAL) is a powerful tool for the identification of non-covalent small molecule–protein interactions that are critical to drug discovery and medicinal chemistry, but this approach is limited to only a small subset of robust photocrosslinkers. The identification of new photoreactive motifs capable of covalent target capture is therefore highly desirable. Herein, we report the design, synthesis, and evaluation of a new class of PAL warheads based on the UV-triggered 1,2-photo-Brook rearrangement of acyl silanes, which hitherto have not been explored for PAL workflows. Irradiation of a series of probes in cell lysate revealed an *i*Pr-substituted acyl silane with superior photolabeling and minimal thermal background labeling compared to other substituted acyl silanes. Further, small molecule (+)-JQ1- and rapamycin-derived *i*Pr acyl silanes were shown to selectively label recombinant BRD4-BD1 and FKBP12, respectively, with minimal background. Together, these data highlight the untapped potential of acyl silanes as a novel, tunable scaffold for photoaffinity labeling.

2.2 Introduction

Identifying drivers of on- and off-target phenotypic responses to bioactive small molecules remains a significant challenge in the field of drug discovery, particularly for low-affinity or low-abundance targets (5–7, 50). One of the most powerful approaches to target deconvolution in a native cellular environment is photoaffinity labeling (PAL), which allows for the capture of transient, non-covalent small molecule-protein interactions in a proteome-wide fashion (34, 41, 43, 51, 52). In photoaffinity labeling, a molecule of interest is conjugated to a light-reactive functional group that generates a high-energy intermediate capable of covalently capturing associated proteins when exposed to UV light. Conventional photoaffinity probes employ diazirines (39), aryl azides (38), and diaryl ketones (45), which give rise to carbene, nitrene, and ketyl radical intermediates, respectively (Figure 2.1A, 1–3). All three have shown tremendous value as photoaffinity warheads in a variety of PAL applications, often in a synthetically modular fashion as exemplified by the suite of “minimalist” photocrosslinking diazirines developed by the Yao group (48, 53). However, not every PAL campaign that utilizes these probes is successful, reflecting the requirements for correct positioning and distance of the photoreactive moiety as well as overall low crosslinking efficiencies typically observed. In addition, the warhead-specific labeling of non-target proteins often results in false positive hits, potentially obscuring true cellular targets of the pharmacophore (35, 54). Taken together, the dearth of photocrosslinking functionalities is an inherent limitation for applications of PAL-based workflows—a more varied set of photoreactive motifs would greatly aid target elucidation, both by providing additional tools for capture of protein targets as well as alternative synthetic approaches for a given probe of interest. However, the development of a novel PAL warhead is contingent upon fulfilling a number of rigorous requirements. Ideally, it should possess a long-wavelength UV absorbance profile to minimize cellular and protein damage during irradiation, rapidly generate a short-lived reactive intermediate capable of residue-agnostic crosslinking, and minimally perturb the native substrate-protein interactions. As a result, only a small number of motifs have been recently disclosed meeting these criteria, including aryl tetrazole (44) and thienyl ketoamide (55) probes. Acyl silanes are an intriguing functionality with ideal properties for use as a photoaffinity warhead. Upon irradiation, acyl silanes undergo a 1,2-photo Brook

rearrangement to reveal a stabilized α -siloxy carbene, which has since been shown to insert into a variety of acidic, polar, and non-polarized bonds to yield X–H insertion products in high yields (56–61) (Figure 2.1B, **5**). The rearrangement occurs with high quantum yields to the α -siloxy carbene (**62**) at long UV wavelengths (350–420 nm) ideal for photocrosslinking. We became interested in utilizing the 1,2-photo Brook rearrangement as an orthogonal mechanism for carbene generation that could bypass deleterious competing pathways common to other carbene precursors, such as diazo formation from diazirines (**63**, **64**), and provide complementary reactivity to known warheads (Figure 2.1C). Moreover, acyl silanes allow for modular substitution on the photoactive moiety itself, offering the potential for direct tuning of desired steric and electronic properties of the probe. Herein, we report the design, synthesis, and evaluation of several acyl silane photoaffinity probes. Upon investigation, an *i*Pr-substituted acyl silane was identified that yielded superior labeling in cellular lysate relative to Me and Ph probe derivatives and avoided unwanted thermal background labeling. Subsequently, we synthesized (+)-JQ1- and rapamycin-derived *i*Pr acyl silanes and confirmed their labeling efficiency and selectivity on pure recombinant proteins. Furthermore, our *i*Pr-substituted acyl silane probe demonstrated similar levels of labeling to a “minimalist” diazirine probe in cellular lysate, suggesting the overall promise of acyl silanes as a novel scaffold for PAL.

2.3 Results and Discussion

2.3.1 Synthesis and evaluation of promiscuous acyl silane probes

We began by synthesizing a probe that contained an alcohol as a modular site of attachment for conjugation to a small molecule of interest and an alkyne handle, which could be used for either pull-down experiments or visualization after further reaction with a suitable azide dye *via* Cu-catalyzed azide–alkyne click chemistry (**14**). Additionally, a series of warheads with varying silane substitution were evaluated to probe the role of sterics on photolabeling. Starting from a TBS-protected allyl alcohol **9**, Me- and Ph-substituted silyl dithianes **11a** and **b** were obtained in 80 and 71% yields, respectively, following a Pt-catalyzed hydrosilylation/lithiation sequence. Subsequent alkylation of the lithiated dithianes and deprotection of both the alkyne and TBS moieties yielded alcohols **13a** and **b** (Scheme 2.1A). A similar sequence was followed in the preparation of *i*Pr-substituted silane **13c** from *p*-methoxybenzyl-protected allyl alcohol (Scheme 2.1B). Following elaboration of the alcohol, oxidative cleavage of the dithiane with PIFA provided the desired acyl silane probes in high yields (**65**) (Scheme 2.1C). Following deprotection, we confirmed that the acyl silanes **15a–c** each possessed a long-wavelength UV λ_{max} between 364–370 nm, which was consistent with reported values and is ideally suited for utilization as a photoaffinity warhead. The reactivity of acyl silanes **15a–c** in MeOH was also evaluated. To avoid possible damage to the protein or proteins of interest, photoaffinity probes should have short irradiation times; consistent with this, all three acyl silanes showed 80% or greater conversion over a 30-minute period upon irradiation with a simple 6 W handheld UV lamp (Figure 2.2). Analysis of the irradiation reaction in MeOH with acyl silane **15b** confirms the formation of an acetal consistent with α -siloxy carbene insertion into the O–H bond of MeOH, and in accordance with the adducts reported by Brook (**56**) (Figure S2.3).

After validating the photoactivity of the acyl silane probes with long-wavelength UV irradiation, their labeling performance in cell lysate was evaluated. Following a 30-minute incubation period, lysates treated with probes **15a–c** were irradiated for 30 minutes on ice, followed by a Cu-catalyzed click reaction with rhodamine azide. Labeled proteins were subsequently visualized by in-gel fluorescence. All probes displayed light-dependent labeling of proteins as well as an increase in labeling over DMSO control (Figure 2.3A). However, samples treated with *i*Pr-substituted probe **15c** showed significantly greater fluorescence over **15a** and **15b**. While the increased labeling may be the result of increased hydrophobicity of probe **15c**, we hypothesized the difference in labeling may be due to the inherent instability of the probes **15a** and **15b** in cell lysate prior to irradiation. Acyl silanes are known to undergo a slow, thermal 1,2-Brook rearrangement in the presence of primary amines (i.e., lysine), which could not only result in probe decomposition before crosslinking but could also result in uncontrolled background thermal labeling processes (66). A more sterically hindered probe (i.e., **15c**) could minimize probe decomposition by slowing the rate of background, thermal 1,2-Brook rearrangement. Thus, to evaluate their stability, probes **15a–c** were incubated for either 0, 0.5, 2, or 24 hours in cell lysate prior to 30 minute irradiation, with labeled proteins visualized in a similar fashion as prior (Figure 2.3B). Consistent with the previous results, *i*Pr-substituted acyl silane **15c** showed no increase in background signal over the course of the experiment and exhibited minimal loss of fluorescence. In contrast **15b**, and to a greater extent **15a**, showed increased thermal labeling upon extended incubation. Interestingly, all probes showed a decrease of light-dependent labeling over extended incubation, possibly due to instability of the proteome over extended time periods at ambient temperatures.

2.3.2 Synthesis and evaluation of fully-functionalized acyl silane probes

With these data in hand, we sought to further evaluate whether bulkier acyl silane warheads such as *i*Pr derivative **15c** could be utilized to capture targeted and specific small molecule-protein interactions; thus, we prepared two small molecule acyl silane probes utilizing the *i*Pr-substitution. The first was based on (+)-JQ1, a known inhibitor of the BET class of bromodomain proteins (67) that has seen extensive profiling (68) and has come to serve as a key benchmarking tool in the development of novel photoaffinity ligands (48, 53) (Figure 2.4, **16** and **17**). The second probe was based on rapamycin, a potent inhibitor of FKBP12, which, upon binding, forms a ternary complex with mTOR (69, 70) (Figure 4, **18** and **19**). Recently, Woo and coworkers elegantly demonstrated the ability to probe the formation of this ternary complex utilizing a diazirine-based rapamycin photoaffinity probe (71). Key to both (+)-JQ1- and rapamycin-based probe designs was identification of known points of modification, such that the acyl silane would have minimal impact on protein binding. Thus, pure recombinant BRD4-BD1 and FKBP12 protein were treated with probes **16/17** and **18/19**, respectively, followed by irradiation with a 6 W handheld UV lamp at 365 nm (Figure 2.4). Covalently labeled protein adducts were subsequently visualized by in-gel fluorescence after Cu-catalyzed click reaction with rhodamine azide. Photochemical labeling was observed for (+)-JQ1 probes **16** and **17** with no thermal background labeling. While more intense fluorescence was observed for the probe with the shorter linker (**16**), both probes could be inhibited by pretreatment of BRD4-BD1 with excess (+)-JQ1. These data are consistent with labeling being driven by

probe-protein binding, not an artifact caused by unspecific association of the acyl silane moiety with the protein target. Interestingly, the linker length for the rapamycin probes **18** and **19** had a more profound effect, as superior labeling of FKBP12 was observed with the longer linker probe **19**. Again, minimal background labeling was observed, and for both probes, labeling was inhibited by pretreatment of FKBP12 with excess rapamycin. To evaluate the relative labeling output of acyl silane probes against traditional photoaffinity warheads, we synthesized a “minimalist” diazirine probe modified with (+)-JQ1, **16-DA** (Figure 2.5). Similar diazirine probes have been used to capture specific interactions in proteins and whole cells (72). Acyl silane (+)-JQ1 probe **16**, the analogous dimethyl-substituted probe **16-Me**, and diazirine probe **16-DA** were incubated in cell lysate and irradiated for 30 minutes on ice. Following click reaction, visualization by in-gel fluorescence shows intense fluorescence for irradiated samples treated with probes **16** and **16-DA**, with minimal thermal labeling observed (Figure 2.5). Consistent with previous findings, the less bulky probe **16-Me** had both lower labeling after irradiation as well as some thermal background labeling. These results support the promise of acyl silanes as novel probes for photoaffinity labeling, as the bulkier isopropyl derivative labels cell lysate on par with current photoaffinity scaffolds. From these encouraging results, we next sought to confirm that the acyl silane probes were capable of capturing targeted and specific interactions in the proteome. Thus, (+)-JQ1-acyl silane probe **16** was incubated in 231MFP cell lysate, into which exogenous BRD4-BD1 protein was added (73). Following irradiation and click reaction of rhodamine azide, labeled proteins were visualized by in-gel fluorescence. Pretreatment of lysate with excess (+)-JQ1 led to inhibition of BRD4-BD1 labeling, indicating that the (+)-JQ1 acyl silane probe is able to capture the BRD4-BD1 protein in cell lysates (Figure 2.6). Initial attempts to identify protein adducts by mass spectrometry approaches were unsuccessful, indicating that further optimization of either the experimental workflow or the acyl silane moiety would be required for their use in target identification and chemoproteomic experiments, a major focus of our ongoing work.

2.4 Conclusions

In summary, we have identified a novel class of photoaffinity warheads with short irradiation times capable of generating α -siloxy carbene intermediates suitable for photoaffinity labeling applications at long UV wavelengths in both cell lysate and with pure protein. Further studies are underway to better understand residue chemoselectivity, as well as an in-depth evaluation of probe structure to allow for the broader use of acyl silanes in proteome profiling and site-of-modification studies.

2.5 Acknowledgements

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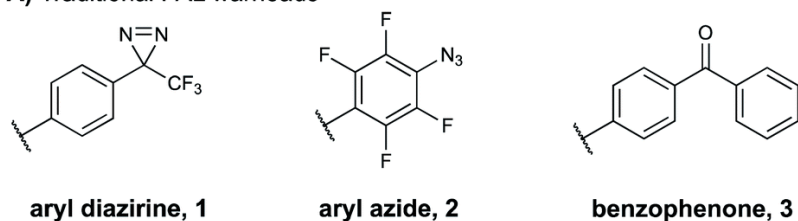
thank Dr. Alec Christian, Dr. Patti Zhang, and Dr. Carl Ward for useful discussions and advice.

2.6 Co-author contributions

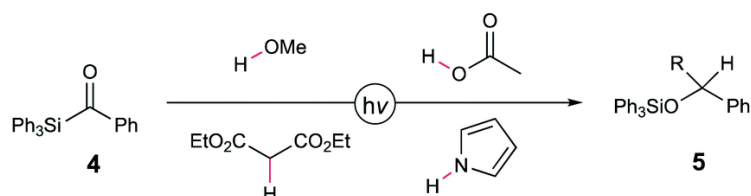
F.D.T. and S.O.S. were responsible for initial acyl silane probe design and experimental validation. A.C.S.P. and S.O.S. synthesized all compounds. K.N.K., J.N.S., and B.P.B. prepared material for experiments in cell lysate. A.C.S.P., S.O.S., K.N.K., J.N.S., and B.P.B. performed experiments in cell lysate and performed gel electrophoresis. Analysis of gels was performed by D.K.N., K.N.K., J.N.S., S.O.S., A.C.S.P., and B.P.B. The initial manuscript was prepared by S.O.S. and A.C.S.P. S.M.B., J.A.T., J.M.M., M.S., D.K.N., and F.D.T. contributed to overall experimental design and manuscript preparation. All authors contributed to manuscript edits.

2.7 Figures and Schemes

A) Traditional PAL warheads



B) Photoreactivity of acyl silanes (Brook and coworkers)



C) This Work: Utilization of acyl silanes for photoaffinity labeling

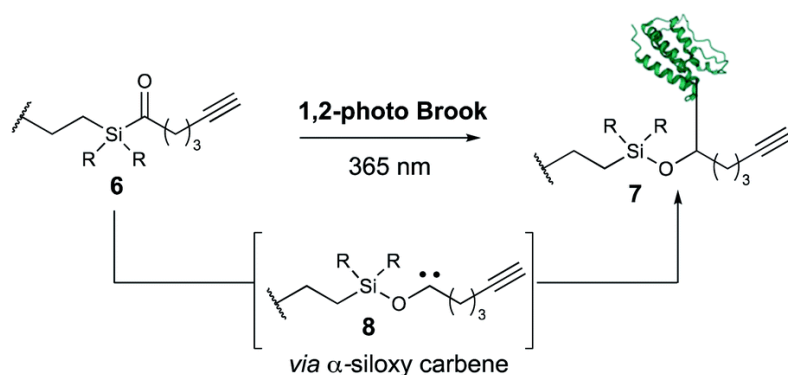
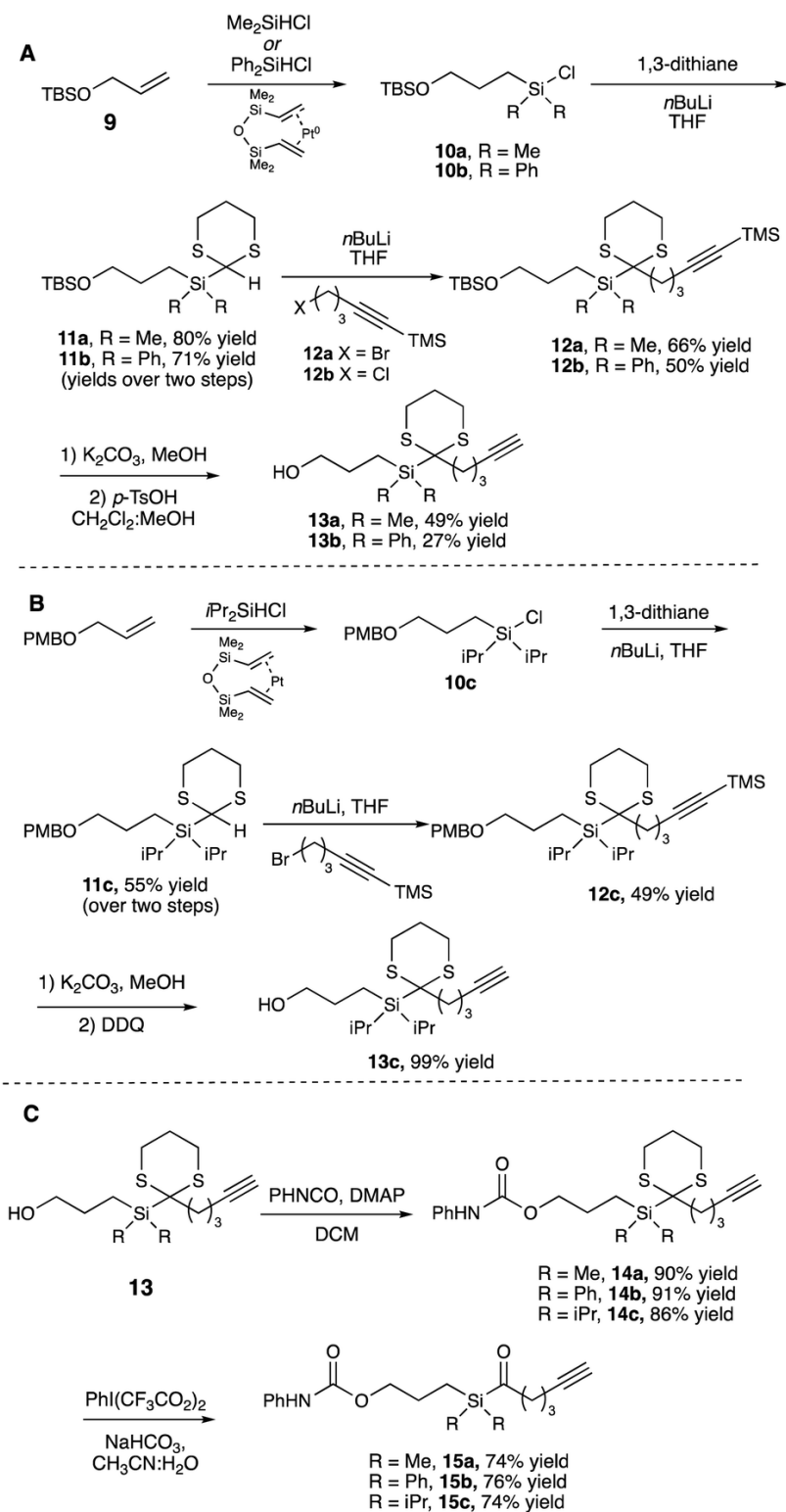


Figure 2.1. Investigation of 1,2-photo-Brook rearrangement of acyl silanes for photoaffinity labeling.



Scheme 2.1. Synthesis of acyl silanes **15a–c**.

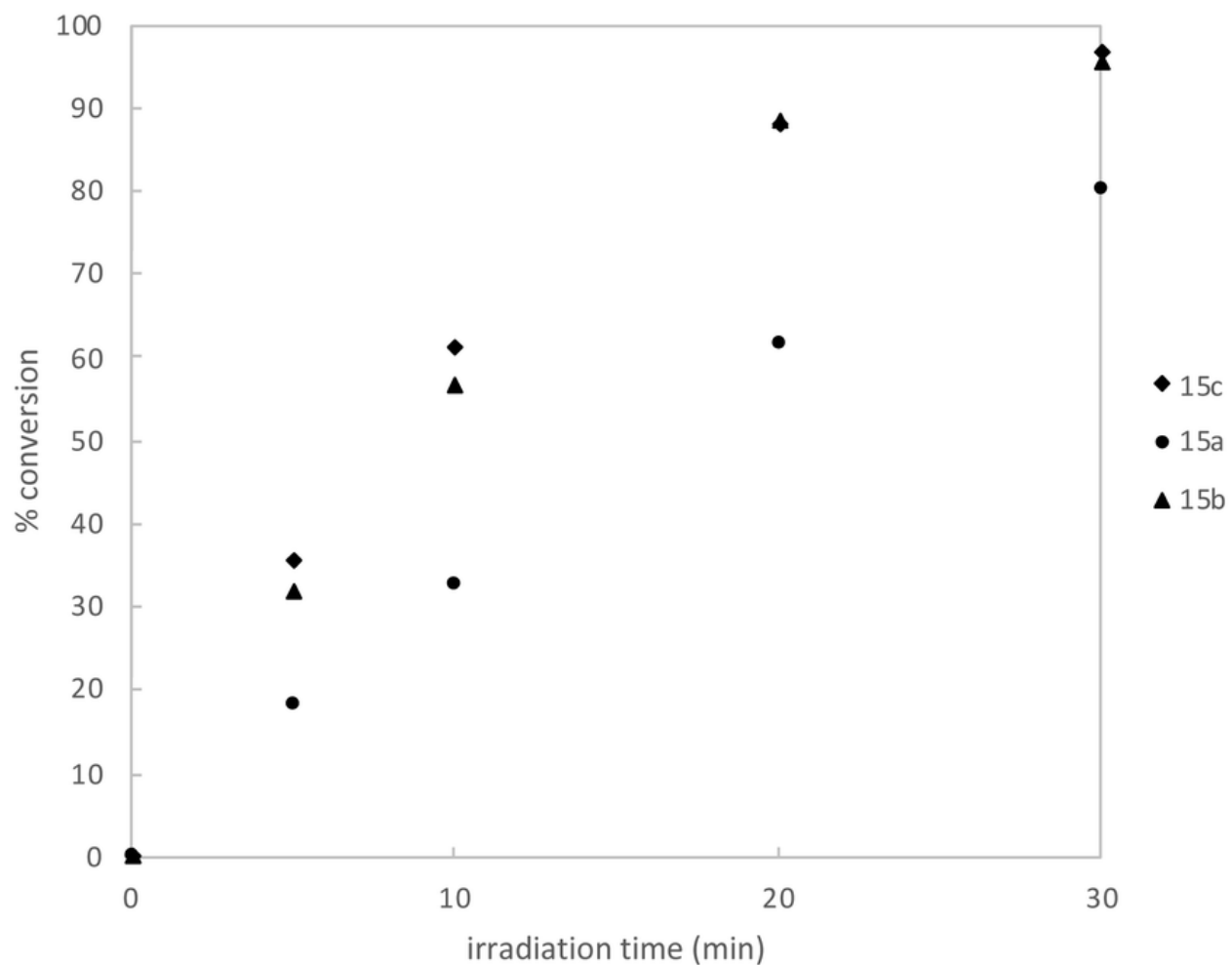


Figure 2.2. Kinetic profile of irradiation of acyl silane probes in MeOH-d₄. (n = 3, average) using a 6 W handheld UV lamp at 365 nm. Conversion was determined by ¹H NMR using an internal standard. Full details available in supplemental information.

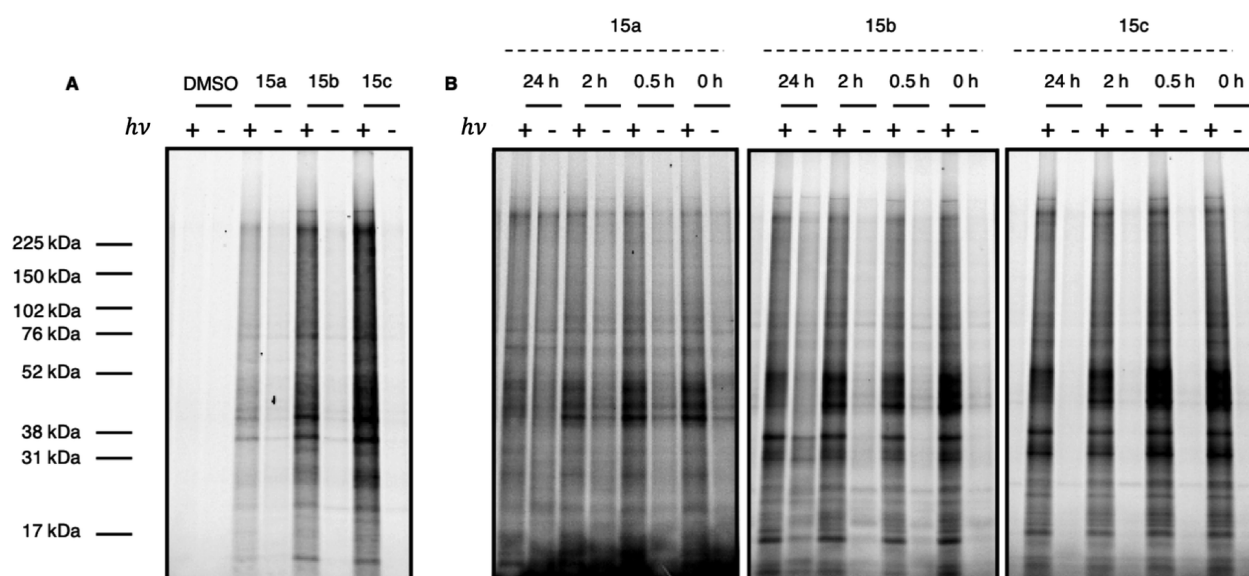


Figure 2.3. Full gel image of UV-dependent labeling of PAL probes **15a–c** in cell lysate. A. In brief, 231MFP cell lysate in pH 7.4 PBS was treated with either DMSO or 10 μ M probe **15a**, **15b**, or **15c** and incubated at ambient temperature for 30 minutes. Samples were then irradiated for 30 minutes at 4 $^{\circ}$ C with a 6 W handheld UV lamp at 365 nm. Labeled protein was visualized following Cu click reaction with Rh- N_3 . Gel quantitation available in supplemental information. B. Evaluation of probe labeling with variable incubation time. In brief, 231MFP cell lysate in pH 7.4 PBS was treated with 10 μ M of probe **15a**, **15b**, or **15c** and incubated at ambient temperature for 0, 0.5, 2, or 24 hours. Samples were then irradiated for 30 minutes at 4 $^{\circ}$ C with a 6 W handheld UV lamp at 365 nm. Labeled protein was visualized following Cu click reaction with Rh- N_3 . Gel quantitation available in supplemental information.

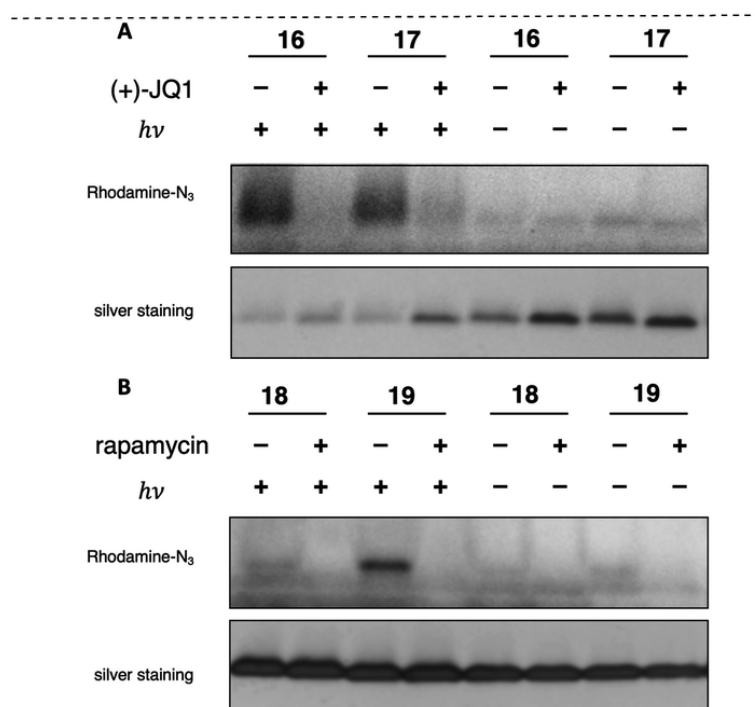
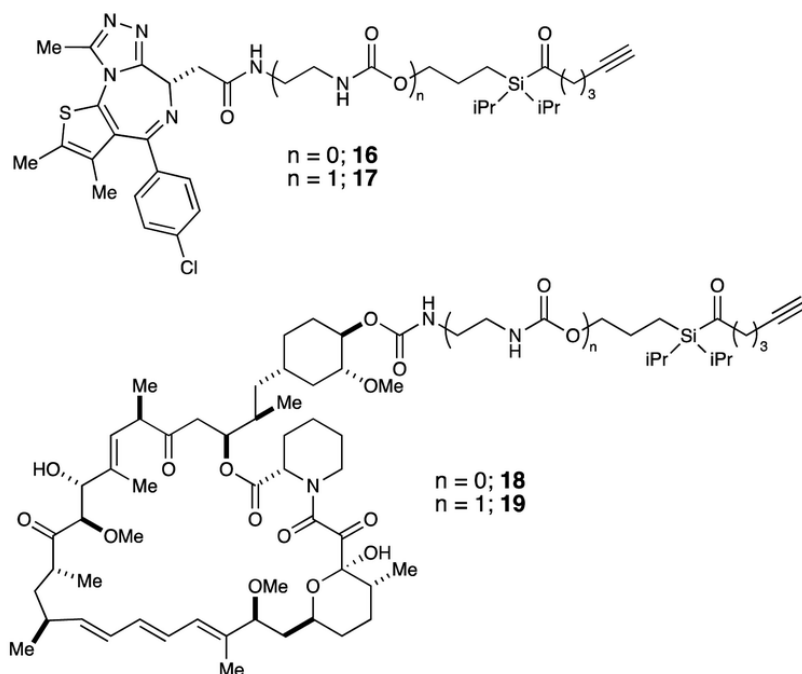


Figure 2.4. Evaluation of labeling by (+)-JQ1 (**16** and **17**) and rapamycin photoaffinity probes (**18** and **19**) with pure recombinant BRD4-BD1 and FKBP12.

(top image) by in-gel fluorescence following Cu click reaction with Rh- N_3 after irradiation at 365 nm with a 6 W handheld UV lamp. Protein loading was confirmed following Ag stain (bottom image) (a) reactions were performed using 0.6 μ g BRD4-BD1 (appx. 0.7 μ M) in 50 μ L PBS, with 10 μ M **16** or **17**, and 100 μ M (+)-JQ1 for competition. (b) Reactions were performed with 1.0 μ g FKBP12 (appx. 1.5 μ M) in 50 μ L PBS (0.1% Triton X-100) with 10 μ M **18** or **19** and 100 μ M rapamycin for competition.

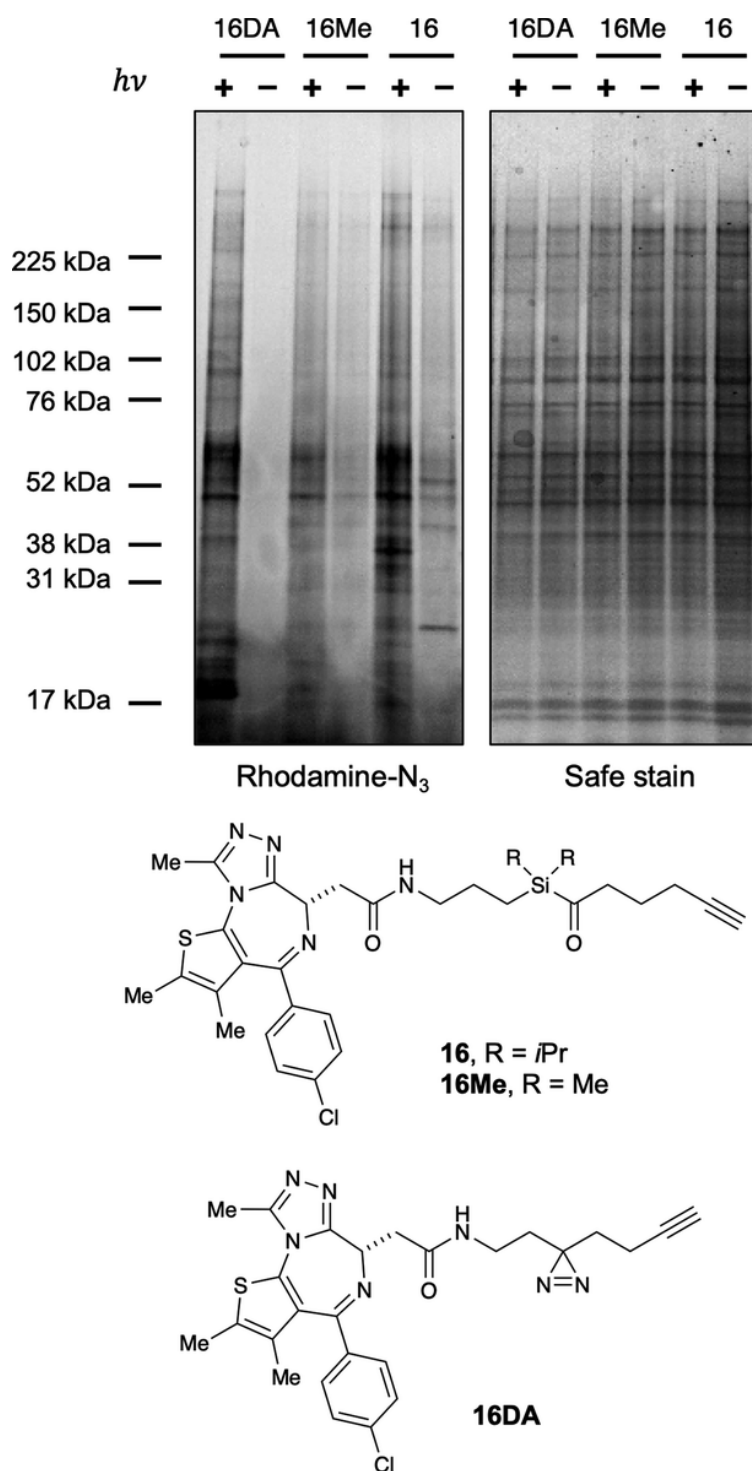


Figure 2.5. Evaluation of labeling by (+)-JQ1 photoaffinity probes (**16**, **16Me**, **16DA**) in 231MFP cell lysate.

(left image) by in-gel fluorescence following Cu click reaction with Rh-N₃ after irradiation at 365 nm with a 6 W handheld UV lamp or incubation in the dark. Reactions were performed using 50 μ L 231MFP cell lysate (normalized to 1 μ g/ μ L) in PBS, with 10 μ M probe. Protein loading was confirmed following SafeStain stain (right image) (see Supplementary information for full gel images).

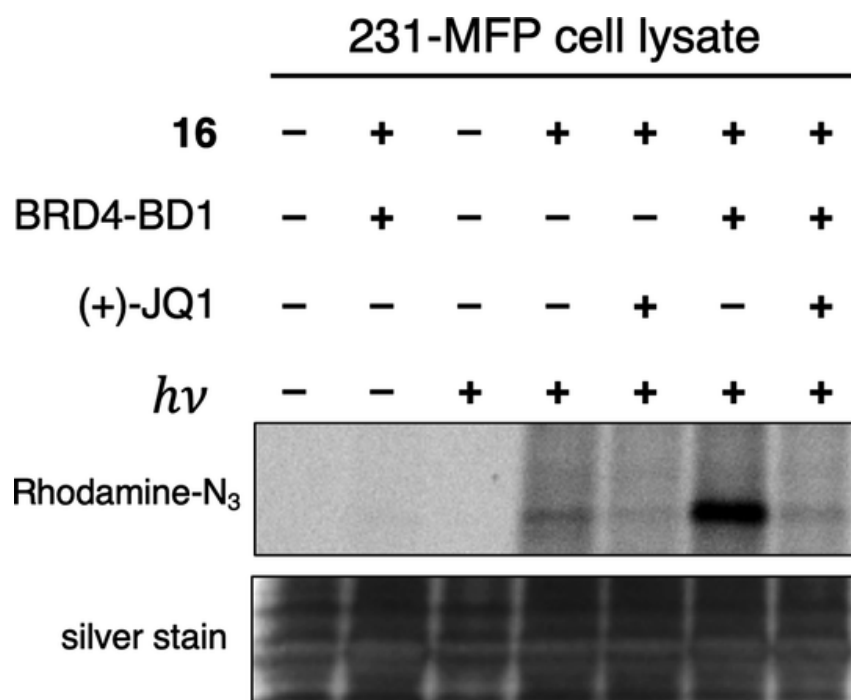


Figure 2.6. Evaluation of labeling by (+)-JQ1 photoaffinity probe **16** in 231MFP cell lysate (top image) by in-gel fluorescence following Cu click reaction with Rh–N₃ after irradiation at 365 nm with a 6 W handheld UV lamp or incubation in the dark. Reactions were performed using 50 μ L 231MFP cell lysate (normalized to 1 μ g/ μ L) spiked with 0.2 μ g BRD4-BD1 in PBS with 5 μ M **16** and 500 μ M (+)-JQ1 for competition. Protein loading was confirmed following silver stain (bottom image). See Supplementary information for full gel images.

2.8 Supplementary information for “Photo-Brook Rearrangement of Acyl Silanes as a Strategy for Photoaffinity Probe Design”

2.8.1 Supplementary figures

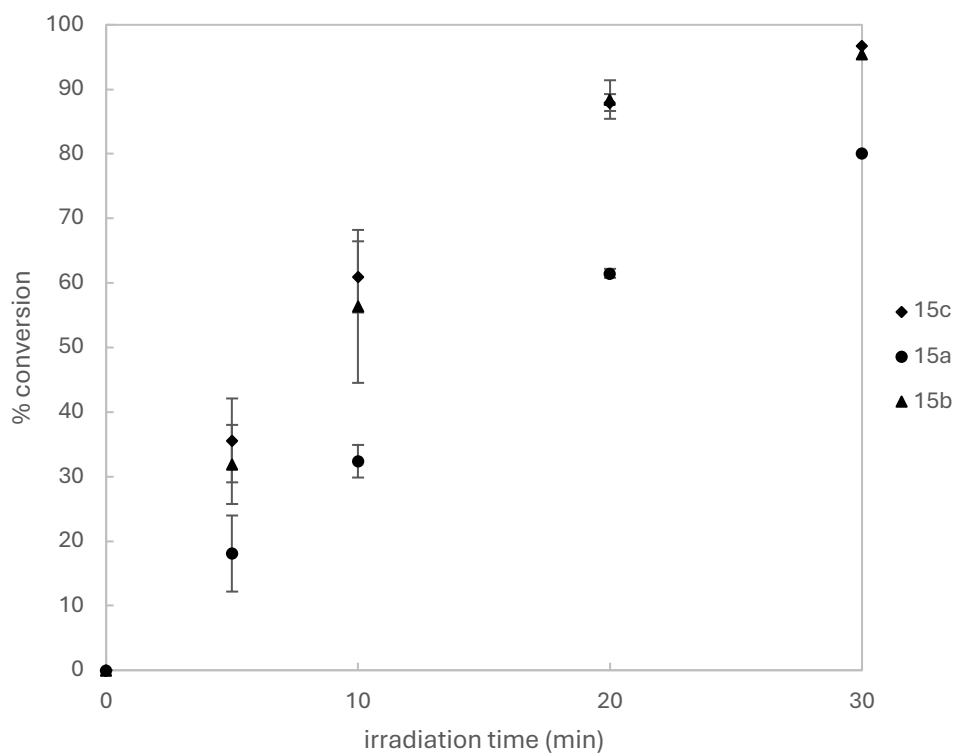


Figure S2.1. Irradiation time course of acyl silanes **15a–c** in CD₃OD.

Acyl silane prepared as a stock solution in CD₃OD was added to an NMR tube with dimethylsulfone as an internal standard for a total volume of 50 μ L or 0.024 M. Reactions were irradiated at 365 nm using a 6 W hand-held UV lamp at 25 $^{\circ}$ C. Reactions were run in triplicate and analyzed by 1 H NMR (d_1 = 10 s).

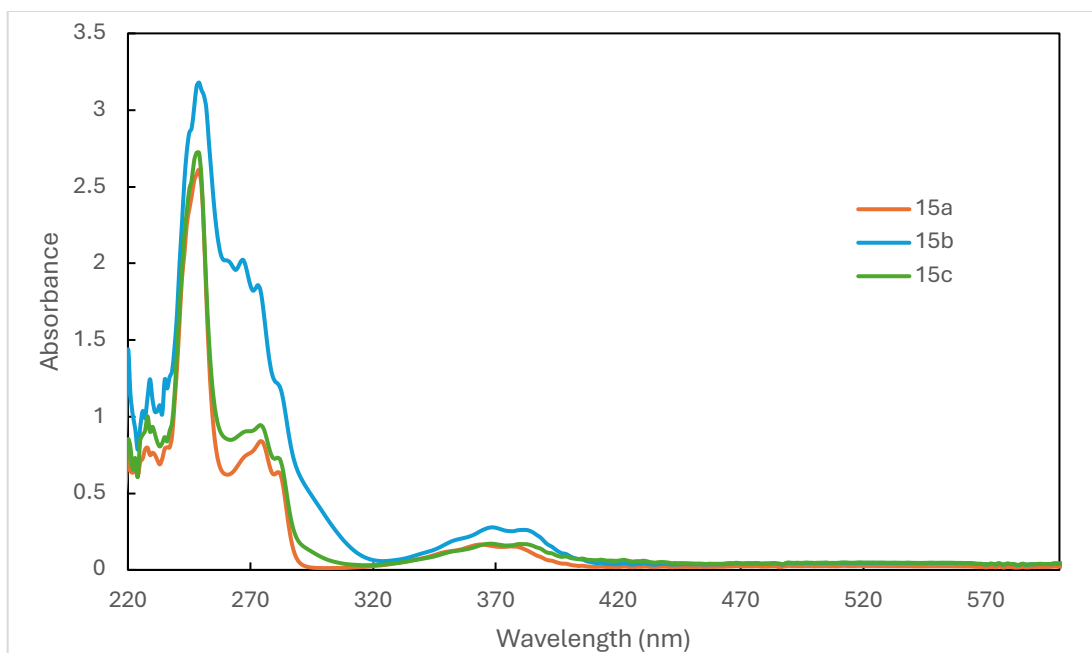
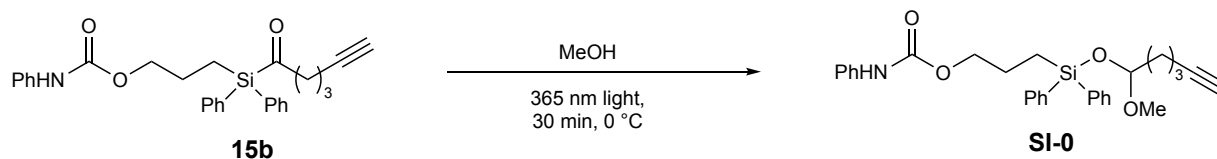


Figure S2.2. UV-Visible spectra of acyl silanes **15a–c** in MeOH.

Acyl silanes **15a**, **15b**, and **15c** show a λ_{max} = 364 nm, 370 nm, and 368 nm, respectively, and an ϵ = 115.7 M⁻¹cm⁻¹, 236.5 M⁻¹cm⁻¹, 111.0 M⁻¹cm⁻¹, respectively, at 5 mM concentration.



Irradiation of probe 15b in MeOH: Product was characterized from the crude reaction mixture. ¹H NMR (600.1 MHz, DMSO-*d*₆): *Aromatic region contains multiple overlapped species.* 9.54 (br. s), 7.60 – 7.54 (m), 7.50 – 7.40 (m), 7.29-7.24 (m), 7.00 – 6.95 (m), 4.77 (dd, *J* = 4.5, 5.6 Hz, 1H, CHOCH₃), 4.06 (t, *J* = 6.9 Hz, 2H), 3.12 (s, 3H, OCH₃), 2.72 (t, *J* = 2.6 Hz, 1H, spC–H), 2.08 (dt, *J* = 7.1, 2.7 Hz, 2H), 1.70 – 1.60 (m, 2 or 4 H), overlap with 1.63 (dt, *J* = 4.5, 7.6 Hz, 2H) 1.45 (dt, *J* = 14.7, 7.3 Hz, 2H), 1.29 – 1.26 (m, 2H).

HRMS (ESI⁺): calculated for [C₂₉H₃₃NO₄Si+Na]⁺ required *m/z* 510.2071, found *m/z* 510.2065.

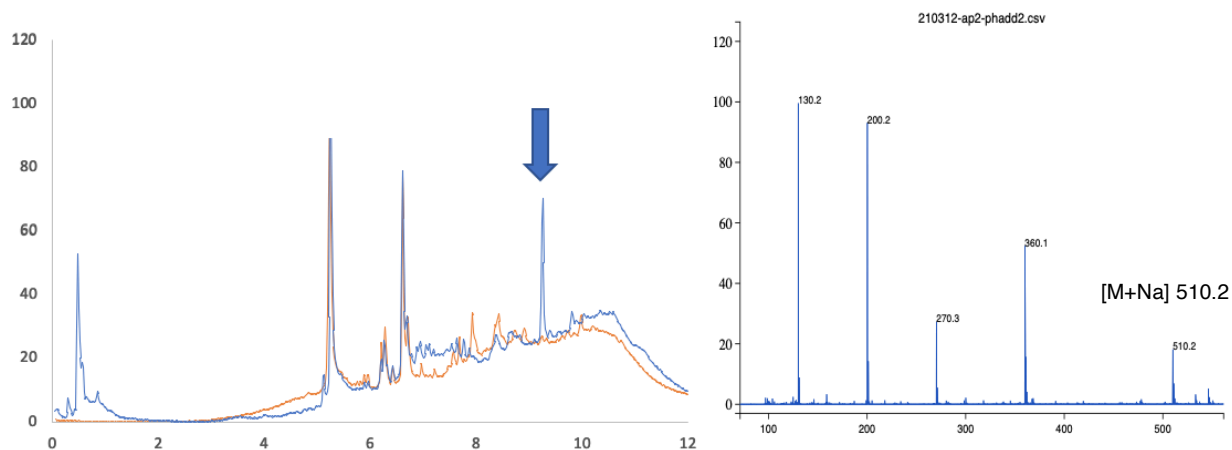
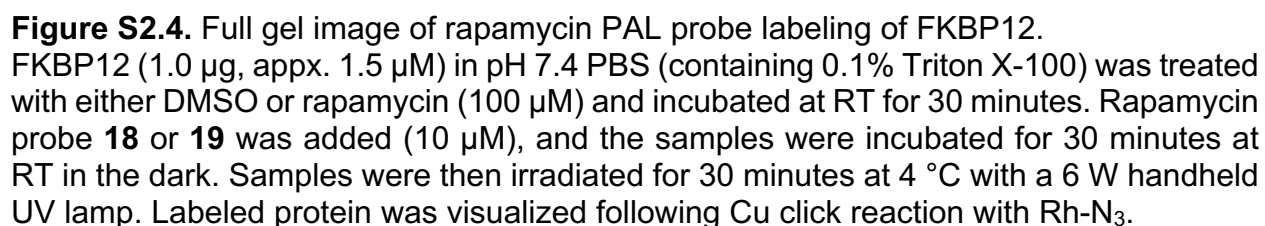


Figure S2.3. Irradiation of probe **15b** in MeOH

Left. LCMS trace (MeCN:H₂O 5-95% with 0.1% formic acid over 12 minutes) of MeOH insertion adduct of **15b**. Background profile after injection of blank sample is shown in orange. Trace following irradiation of **15b** in MeOH for 30 minutes at 0 °C shown in blue, with acetal adduct indicated by arrow. Right: Expansion of mass spectrum of acetal. LCMS (ESI⁺) calculated for [C₂₉H₃₃NNaO₄Si]⁺ requires m/z 510.2, found m/z 510.2.



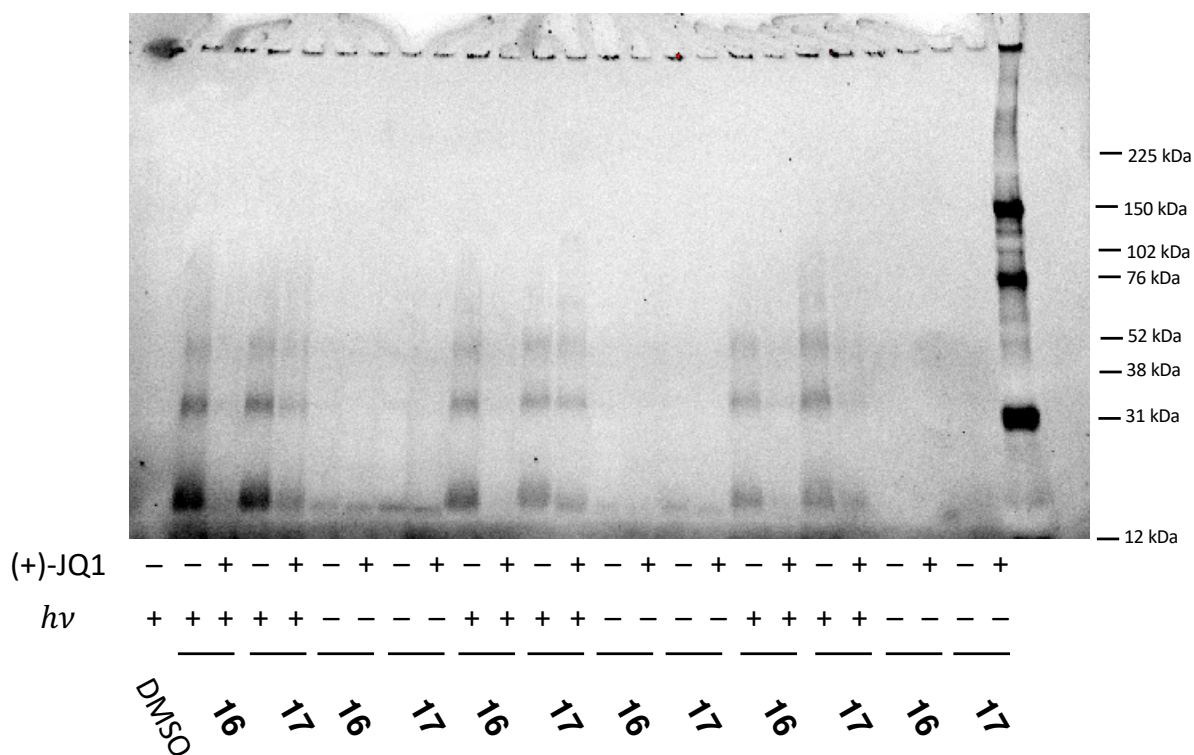


Figure S2.5. Full gel image of (+)-JQ1 PAL probe labeling of BRD4-BD1. BRD4-BD1 (0.6 μ g, appx. 0.7 μ M) in pH 7.4 PBS was treated with either DMSO or (+)-JQ1 (100 μ M) and incubated at RT for 30 minutes. (+)-JQ1 probe **16** or **17** was added (10 μ M), and the samples were incubated for 30 minutes at RT in the dark. Samples were then irradiated for 30 minutes at 4 $^{\circ}$ C with a 6 W handheld UV lamp. Labeled protein was visualized following Cu click reaction with Rh- N_3 .

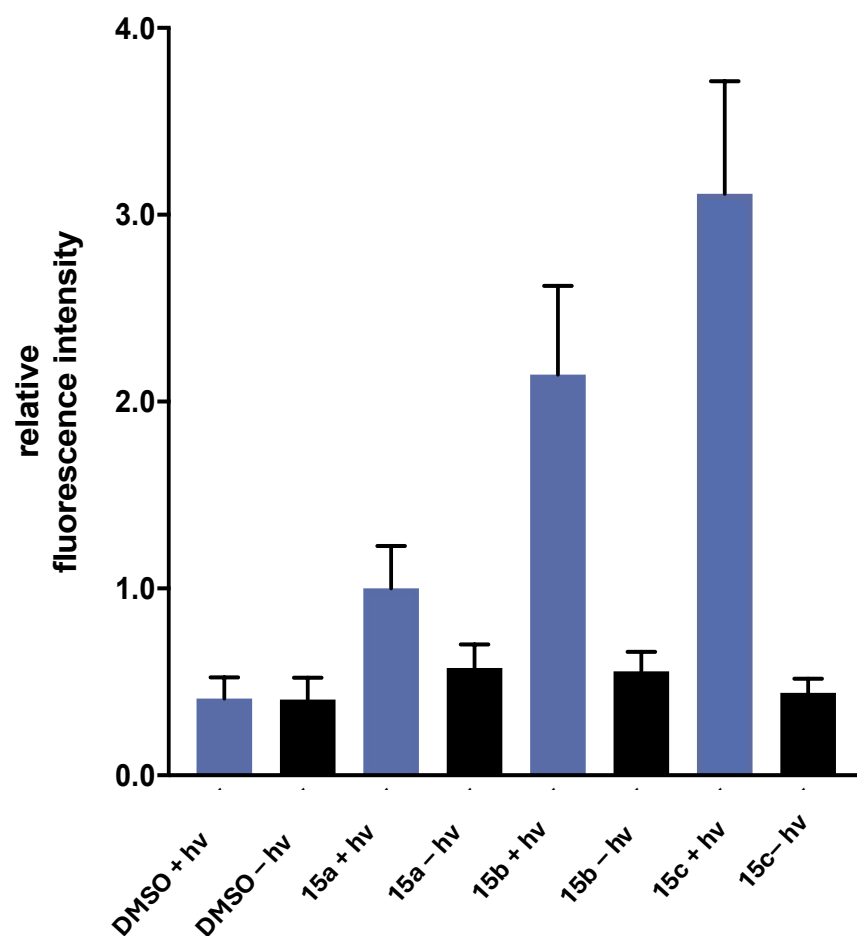


Figure S2.6. Quantitation of in-gel fluorescence for Figure 2.3A normalized to protein levels.

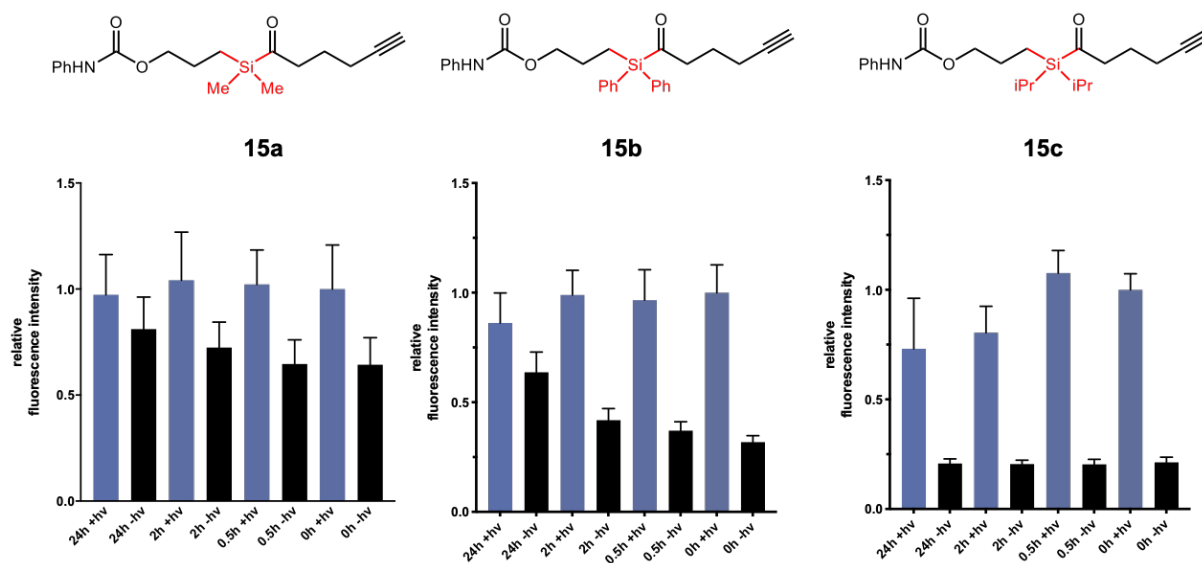


Figure S2.7. Quantitation of in-gel fluorescence for Figure 2.3B for probes **15a–c** normalized to protein levels.

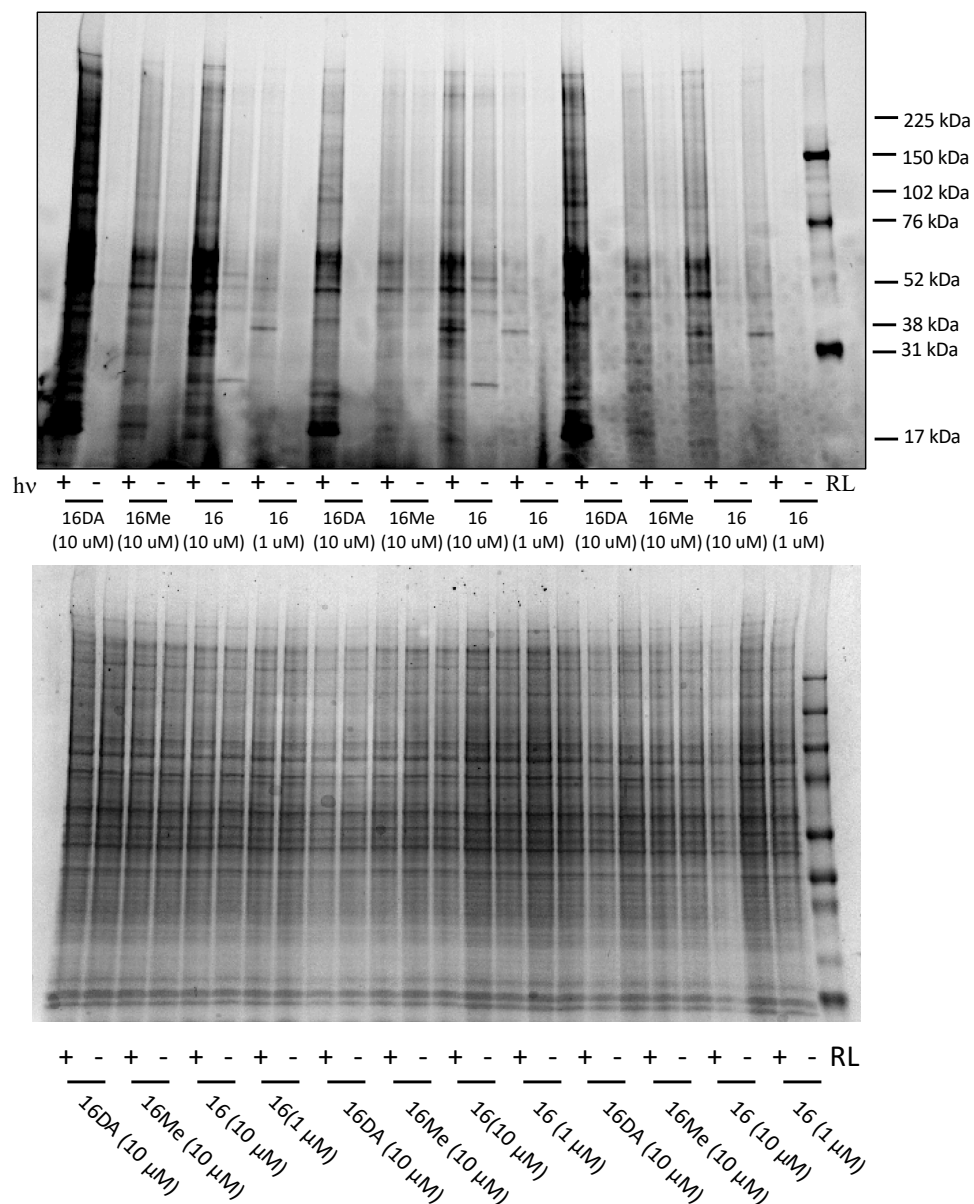
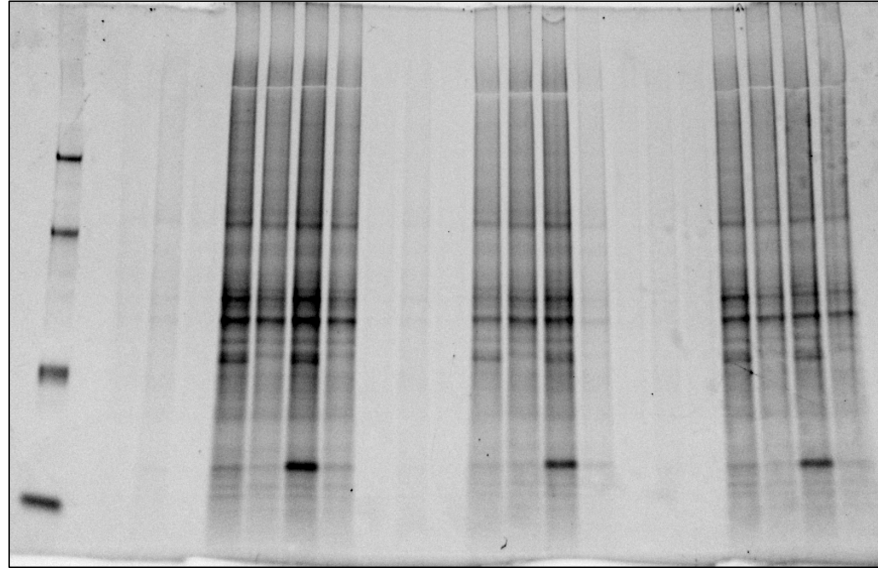


Figure S2.8. Full gel image of (+)-JQ1 probes **16**, **16DA**, **16Me** labeling 231MFP cell lysate.
(top image) by in-gel fluorescence. SafeStain gel image confirming protein labeling (bottom image)



16 (5 uM)	-	+	-	+	+	+	+	-	+	-	+	+	+	+	-	+	-	+	+	+	+
BRD4-BD1	-	+	-	-	-	+	+	-	+	-	-	-	+	+	-	+	-	-	-	+	+
(+)-JQ1	-	-	-	-	+	-	+	-	-	-	-	+	-	+	-	-	-	-	+	-	+
hv	-	-	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-	+	+	+	+

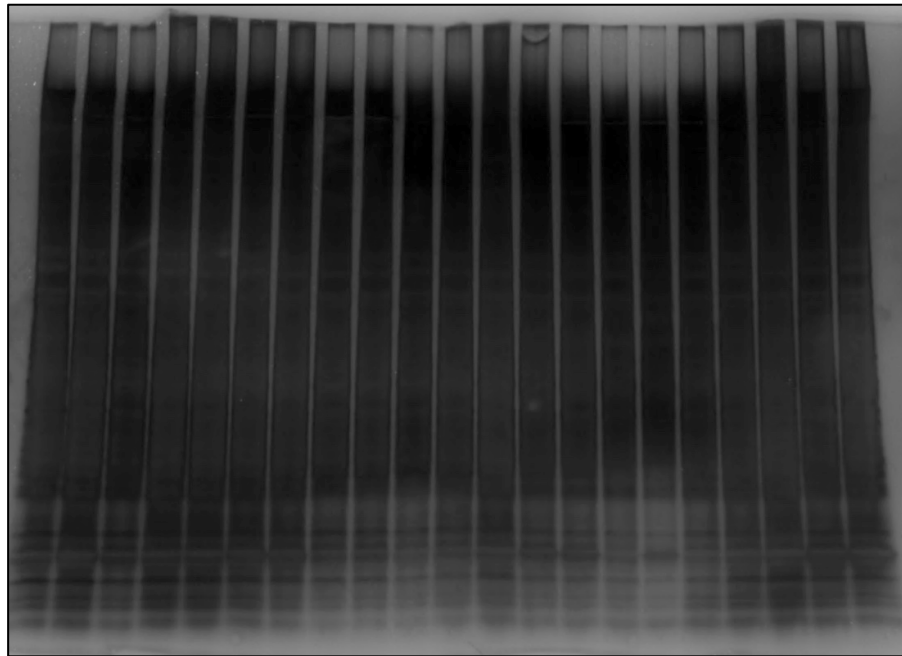
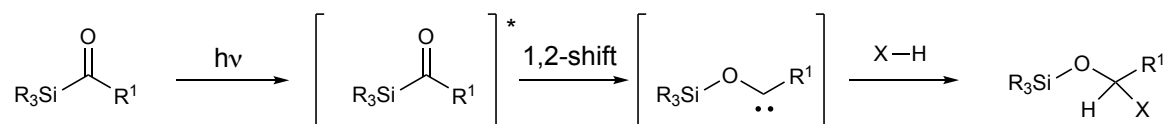
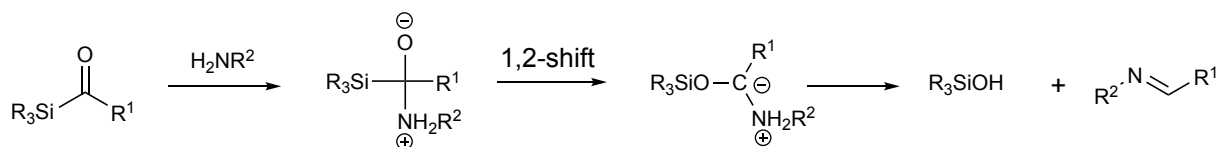


Figure S2.9. Full gel image of (+)-JQ1 PAL probe 16 labeling of 231MFP lysate spiked with BRD4-BD1. (top image) visualized by TAMRA-N₃. Full gel image of Ag stain (bottom image)

Photochemical 1,2-Brook Rearrangement:



Thermal 1,2-Brook Rearrangement:



Scheme S2.1. Mechanism of photochemical and thermal 1,2-Brook rearrangements (58, 66).

2.8.2 General information

Unless otherwise noted, all reagents were purchased from commercial suppliers and used without further purification. Tetrahydrofuran, dichloromethane, diethyl ether, toluene, trimethylamine, and N,N-dimethylformamide were purified by passage through an activated alumina column under argon. Thin-layer chromatography (TLC) analysis of reaction mixtures was performed using Merck silica gel 60 F254 TLC plates and visualized under UV or by staining with KMnO₄ or *p*-anisaldehyde. Column chromatography was performed on Merck Silica Gel 60 Å, 230 X 400 mesh. Nuclear magnetic resonance (NMR) spectra were recorded using Bruker AV-600, AV-500, DRX-500, Neo-500, AVB-400, AVQ-400, and AV-300 spectrometers. These CoC-NMR instruments at UC Berkeley are funded in part by the NIH (S10OD024998). ¹H and ¹³C chemical shifts are reported in ppm downfield of tetramethylsilane and referenced to residual solvent peak (CHCl₃; δH = 7.268 ppm and δC = 77.16ppm). Multiplicities are reported using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, hept = heptet/septet, m = multiplet, br = broad, dt = doublet of triplet resonance. Mass spectral data for EMM were obtained from the QB3 mass spectral facility at the University of California, Berkeley on a Thermo LTQ-FTICR (7T, ESI) and in the Center of Catalysis at the University of California, Berkeley. Solvent abbreviations are reported as follows: EtOAc = ethyl acetate, hex = hexanes, DCM = dichloromethane, Et₂O = diethyl ether, MeOH = methanol, THF = tetrahydrofuran, DMSO = dimethylsulfoxide, Et₃N = trimethylamine, MeCN = acetonitrile.

2.8.3 Chemicals

Rapamycin (53123-88-9) and (+)-JQ1 (1268524-70-4) were purchased from MedChemExpress (HY-10219 and HY-13030, respectively). TAMRA-PEG4-Azide was purchased from Click Chemistry Tools (AZ109). (+)-JQ1-OH was prepared from commercial (+)-JQ1 according to literature precedent without modification (74). N-boc-2-nitrobenzenesulfonamide (**SI-1**) was prepared according to literature precedent without modification (75). 2-(3-(but-3-yn-1-yl)-3*H*-diazirin-3-yl)ethan-1-amine (**SI-12**) was prepared according to literature precedent (48).

2.8.4 Biological materials

231MFP cells were obtained from Benjamin Cravatt and were derived from MDA-MB-231 cells. Pure recombinant BRD4-BD1 and FKBP12 proteins were purchased from Cayman Chemicals and Origene, respectively.

2.8.5 Analysis of small molecule probes by in-gel fluorescence

Proof-of-concept probes in cell lysate: 231MFP cell lysate (1.0 µg/µL) in pH 7.4 PBS (50 µL) was treated with a prepared stock solution of DMSO or 10 µM probe **15a**, **15b**, or **15c** in DMSO and incubated in the dark for 30 minutes at RT. Samples were irradiated for 30 minutes on ice using a 6 W handheld UV lamp at 365 nm. Labeled protein was visualized following Cu-catalyzed click reaction, performed by sequential addition of CuSO₄, (1 mM), tris(2-carboxyethyl)phosphine (1 mM), tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (34 µM) and TAMRA azide (25 µM). Following click reaction, proteins were denatured by the addition of 4X Laemmli SDS loading buffer (30 µL) and run on a

16 cm Protean II xi 10% resolving SDS-PAGE gel system (Bio-Rad) and scanned using a ChemiDoc MP (Bio-Rad).

Timecourse of proof-of-concept probes in cell lysate: 231MFP cell lysate (1.0 µg/µL) in pH 7.4 PBS (50 µL) was treated with a prepared stock solution of DMSO or 10 µM probe **15a**, **15b**, or **15c** in DMSO and incubated at ambient temperature for 0, 0.5, 2, or 24 hours. Samples were then irradiated for 30 minutes at 4 °C with a 6 W handheld UV lamp at 365 nm. Labeled protein was visualized following Cu-catalyzed click reaction, performed by sequential addition of CuSO₄, (1 mM), tris(2-carboxyethyl)phosphine (1 mM), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (34 µM) and TAMRA azide (25 µM). Following click reaction, proteins were denatured by the addition of 4X Laemmli SDS loading buffer (30 µL) and run on a 16 cm Protean II xi 10% resolving SDS-PAGE gel system (Bio-Rad) and scanned using a ChemiDoc MP (Bio-Rad).

(+)-JQ1 probes: BRD4-BD1 (0.6 µg, appx. 0.7 µM) in pH 7.4 PBS (50 µL) was treated with a prepared stock solution of (+)-JQ1 acyl silane probe in DMSO and incubated in the dark for 30 minutes at RT. During competition, BRD4-BD1 was treated with a prepared stock solution of (+)-JQ1 (10x final concentration of acyl silane) prior to acyl silane addition and incubated at RT for 30 minutes. Samples were irradiated for 30 minutes on ice using a 6 W handheld UV lamp at 365 nm. Labeled protein was visualized following Cu-catalyzed click reaction, performed by sequential addition of CuSO₄, (1 mM), tris(2-carboxyethyl)phosphine (1 mM), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (34 µM) and TAMRA azide (25 µM) (16). Following click reaction, proteins were denatured by the addition of 4X Laemmli SDS loading buffer (30 µL) and run on a 16 cm Protean II xi 10% resolving SDS-PAGE gel system (Bio-Rad) and scanned using a ChemiDoc MP (Bio-Rad).

Rapamycin probes: FKBP12 (1.0 µg, appx. 1.5 µM) in pH 7.4 PBS (0.1% Triton X-100) (50 µL) was treated with a prepared stock solution of rapamycin acyl silane probe in DMSO and incubated in the dark for 30 minutes at RT. During competition, FKBP12 was treated with a prepared stock solution of rapamycin (10x final concentration of acyl silane) prior to acyl silane addition and incubated at RT for 30 minutes. Samples were irradiated for 30 minutes on ice using a 6 W handheld UV lamp at 365 nm. Labeled protein was visualized following Cu-catalyzed click reaction, performed by sequential addition of CuSO₄, (1 mM), tris(2-carboxyethyl)phosphine (1 mM), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (34 µM) and TAMRA azide (25 µM). Following click reaction, proteins were denatured by the addition of 4X Laemmli SDS loading buffer (30 µL) and run on a 16 cm Protean II xi 10% resolving SDS-PAGE gel system (Bio-Rad) and scanned using a ChemiDoc MP (Bio-Rad).

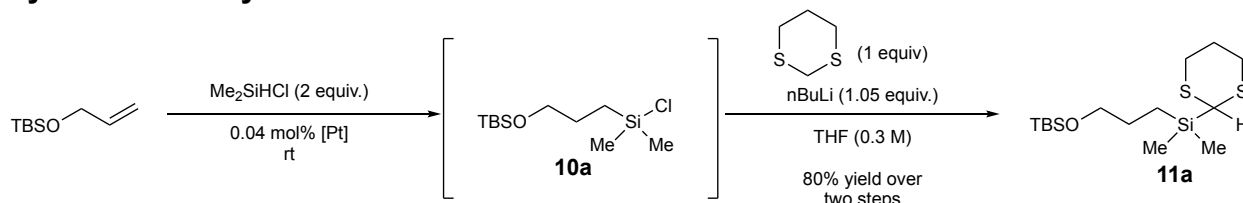
(+)-JQ1 probes in cellular lysate: 231MFP cell lysate (1.0 µg/µL) in pH 7.4 PBS (50 µL) was treated with a prepared stock solution of (+)-JQ1 probe in DMSO and incubated in the dark for 30 minutes at RT. Samples were irradiated for 30 minutes on ice using a 6 W handheld UV lamp at 365 nm. Labeled protein was visualized following Cu-catalyzed click reaction, performed by sequential addition of CuSO₄, (1 mM), tris(2-carboxyethyl)phosphine (1 mM), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (34

μM) and TAMRA azide ($25\ \mu\text{M}$). Following click reaction, proteins were denatured by the addition of 4X Laemmli SDS loading buffer ($30\ \mu\text{L}$) and run on a 16 cm Protean II xi 10% resolving SDS-PAGE gel system (Bio-Rad) and scanned using a ChemiDoc MP (Bio-Rad).

(+)-JQ1 probes in spiked cellular lysate: 231MFP cell lysate ($1.0\ \mu\text{g}/\mu\text{L}$) in pH 7.4 PBS ($50\ \mu\text{L}$) was treated with $0.2\ \mu\text{g}$ of BRD4-BD1 or DMSO control. A prepared stock solution of (+)-JQ1 probe in DMSO was added and incubated in the dark for 30 minutes at RT. For competition experiments, 231MFP lysate was treated with a prepared stock solution of (+)-JQ1 (100x final concentration of acyl silane) prior to acyl silane addition and incubated at RT for 30 minutes. Samples were irradiated for 30 minutes on ice using a 6 W handheld UV lamp at 365 nm. Labeled protein was visualized following Cu-catalyzed click reaction, performed by sequential addition of CuSO_4 (1 mM), tris(2-carboxyethyl)phosphine (1 mM), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine ($34\ \mu\text{M}$) and TAMRA azide ($25\ \mu\text{M}$). Following click reaction, proteins were denatured by the addition of 4X Laemmli SDS loading buffer ($30\ \mu\text{L}$) and run on a 16 cm Protean II xi 10% resolving SDS-PAGE gel system (Bio-Rad) and scanned using a ChemiDoc MP (Bio-Rad).

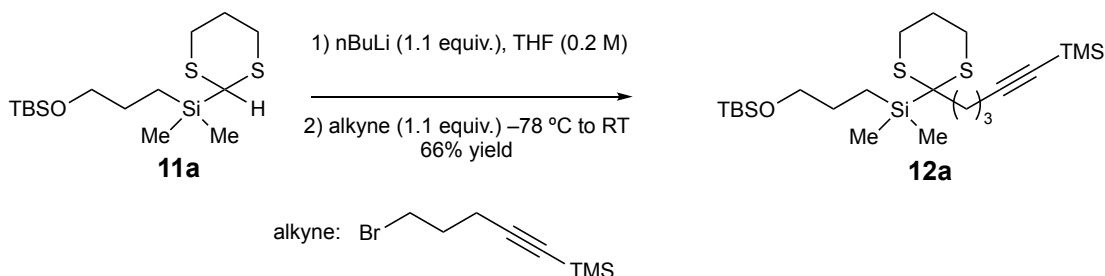
2.8.6 Synthesis of compounds

Synthesis of acyl silane 15a



(3-((1,3-dithian-2-yl)dimethylsilyl)propoxy)(*tert*-butyl)dimethylsilane (11a): To an oven-dried Schlenk flask fitted with a rubber septum and cooled under N₂ was added platinum(0)-1,3-divinyl 1,1,1,3,3-tetramethyldisiloxane complex solution (Karstedt's catalyst) [0.1 M in vinyl terminated poly(dimethylsiloxane), ca $19\ \mu\text{L}$, 0.04 mol%] and Me₂SiHCl (9.3 mmol, 1.03 mL, 2.0 equiv.) by syringe dropwise. After stirring at room temperature for 10 minutes, (allyloxy)(*tert*-butyl)dimethylsilane (4.65 mmol, 800 mg, 1.0 equiv.) was added dropwise over 15 minutes. After an additional 15 minutes stirring, the solution was cooled to 0 °C and evacuated under vacuum for 15 minutes to remove excess ClSiMe₂H. After purging with N₂ for an additional 15 minutes, silyl chloride **10a** was obtained (4.14 mmol, 1.09 g, 89% yield) and used immediately without further purification. ¹H NMR (400.0 MHz, CDCl₃): δ 3.60 (t, *J* = 6.6 Hz, 2H), 1.67 – 1.58 (m, 2H), 0.90 (s, 9H), 0.86 – 0.79 (m, 2H), 0.42 (s, 6H), 0.06 (s, 6H). A flame dried 50 mL round bottom flask cooled under vacuum was charged with 1,3-dithiane (0.411 g, 3.42 mmol, 1.0 equiv.) and THF (9 mL, 0.3 M). The reaction vessel was cooled to –30 °C and a solution of *n*BuLi (2.2 M in hexanes, 1.64 mL, 3.59 mmol, 1.05 equiv.) was added dropwise. The solution was allowed to warm to room temperature and stir for 1 hour before cooling to –78 °C and adding **10a** (1.2 equiv.) dropwise. The reaction mixture was allowed to warm to room temperature overnight and the resulting orange solution was quenched by addition of sat. aqueous NH₄Cl solution and extracted with Et₂O three times.

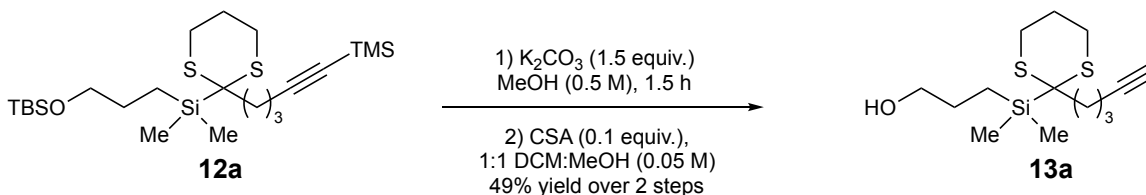
The combined organic layers were washed with saturated brine, dried over MgSO_4 , and concentrated *in vacuo*. The residual 1,3-dithiane was removed by Kugelrohr distillation under full vacuum at 70 – 100 °C to give **11a** (1.04 g, 3.08 mmol, 90% yield) as a pale oil. **^1H NMR** (400.0 MHz, CDCl_3): δ 3.73 (s, 1H), 3.56 (t, J = 6.9 Hz, 2H), 2.91 – 2.83 (m, 2H), 2.74 – 2.68 (m, 2H), 2.15 – 2.08 (m, 1H), 2.05 – 1.95 (m, 1H), 1.61 – 1.52 (m, 2H), 0.89 (s, 9H) (overlap with m, 2H), 0.67 – 0.61 (m, 2H), 0.14 (s, 6H), 0.05 (s, 6H). **^{13}C NMR** (125 MHz, CDCl_3): δ 68.1, 66.1, 33.7, 31.3, 27.1, 26.4, 26.2, 18.5, 9.3, –4.8, –5.1. **HRMS** (ESI): calculated for $[\text{C}_{15}\text{H}_{34}\text{OS}_2\text{Si}_2+\text{H}]^+$ required m/z 351.1662, found m/z 351.1664.



tert-butyl(3-(dimethyl(2-(5-(trimethylsilyl)pent-4-yn-1-yl)-1,3-dithian-2-yl)silyl)propoxy)dimethylsilane (12a):

A flame dried 50 mL round bottom flask cooled under vacuum was charged with **11a** (840 mg, 2.5 mmol, 1.0 equiv.) and THF (5 mL, 0.2 M) and cooled to –30 °C, followed by the dropwise addition of *n*BuLi (2.2 M, 1.2 mL, 1.1 equiv.). After stirring for 45 minutes at –30 °C, a solution of (5-bromopent-1-yn-1-yl)trimethylsilane (539 mg, 2.63 mmol, 1.1 equiv.) in THF (5 mL) was added dropwise. The reaction was then allowed to warm to room temperature and stir overnight. After 16 hours, the reaction was quenched by the addition of sat. aqueous NH_4Cl and extracted with a 1:1 mixture of Et_2O /hexanes. The combined organics were washed with saturated brine, dried over MgSO_4 , and concentrated to an oil. Purification of the crude material by flash column chromatography on silica gel in 95:5 hex/ EtOAc yielded the desired product **12a** as a clear, colorless oil (817 mg, 66% yield).

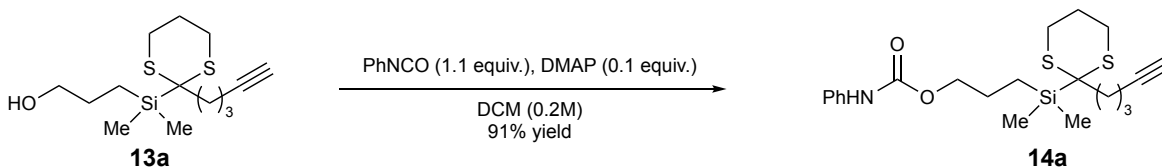
^1H NMR (500.0 MHz, CDCl_3): δ 3.57 (t, J = 7.0 Hz, 2H), 3.12 – 3.05 (m, 2H), 3.42 (dt, J = 14.3, 4.1 Hz, 2H), 2.39 – 2.33 (m, 2H), 2.30 (t, J = 6.7 Hz, 2H), 2.09–2.01 (m, 1H), 1.96 – 1.85 (m, 1H), 1.74 – 1.65 (m, 2H), 1.61 – 1.52 (m, 2H), 0.89 (s, 9H), 0.74 – 0.68 (m, 2H), 0.18 (s, 6H), 0.15 (s, 9H), 0.05 (s, 6H). **^{13}C NMR** (125.7 MHz, CDCl_3): δ 107.3, 85.2, 66.2, 38.9, 36.1, 27.5, 26.7, 26.1, 25.3, 23.3, 20.2, 18.5, 14.2, 9.3, 0.3, –4.4, –5.1. **HRMS** (ESI⁺): calculated for $[\text{C}_{12}\text{H}_{48}\text{OS}_2\text{Si}_3+\text{H}]^+$ required m/z 489.2527, found m/z 489.2524.



3-(dimethyl(2-(pent-4-yn-1-yl)-1,3-dithian-2-yl)silyl)propyl benzoate (13a): To a solution of **12a** (331 mg, 0.697 mmol, 1.0 equiv.) in MeOH (1.5 mL, 0.5 M) was added potassium carbonate (140 mg, 1.02 mmol, 1.5 equiv.). The reaction was monitored by TLC and after 4 hours quenched by addition of sat. aqueous NH_4Cl , diluted with sat. aqueous brine, and extracted with Et_2O three times. The combined organic layers were

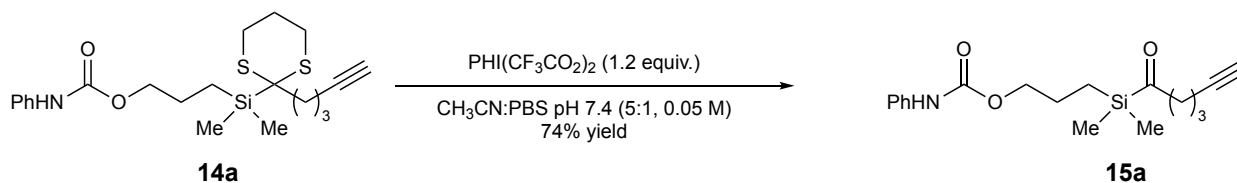
dried over MgSO_4 , filtered, and concentrated *in vacuo* to give a residue that was subsequently added to a solution of camphorsulfonic acid (15 mg, 0.068 mmol, 0.1 equiv.) in a 1:1 mixture of DCM:MeOH (0.05 M) and stirred at room temperature. After 3 hours, the solution was diluted with 1:1 hex:EtOAc, washed with sat. aqueous NaHCO_3 and sat. aqueous brine, dried, filtered, and concentrated to an oil. Purification of the crude material by gradient elution of 10–30% EtOAc in hex by flash column chromatography gave **13a** (130 mg, 49% over two steps) as a clear oil.

^1H NMR (600.1 MHz, CDCl_3): δ 3.56 (t, J = 6.7 Hz, 2H), 3.05 – 2.97 (m, 2H), 2.38 (dt, J = 14.4, 3.7 Hz, 2H), 2.32 – 2.28 (m, 2H), 2.21 (td, J = 6.8, 2.7 Hz, 2H), 2.04 – 1.93 (m, 3H), 1.84 (ddq, J = 3.2, 3.2, 12.6 Hz, 1H), 1.70 – 1.63 (m, 2H), 1.61 – 1.54 (m, 2H), 0.75 – 0.68 (m, 2H), 0.14 (s, 6H). **^{13}C NMR** (150.9 MHz, CDCl_3): δ 84.13, 68.80, 65.42, 38.50, 36.20, 27.16, 26.47, 25.00, 23.21, 18.69, 9.25, –4.47. **HRMS** (ESI^+): calculated for $[\text{C}_{14}\text{H}_{26}\text{OS}_2\text{Si}+\text{Na}]^+$ required m/z 325.1087, found m/z 325.1089.



3-(dimethyl(2-(pent-4-yn-1-yl)-1,3-dithian-2-yl)silyl)propyl benzoate (14a): A flame-dried screw-capped culture tube cooled under vacuum was charged with **13a** (161 mg, 0.532 mmol, 1.0 equiv.) and DCM (2.66 mL, 0.2 M). Phenylisocyanate (64 μL , 0.585 mmol, 1.1 equiv.) and DMAP (6.5 mg, 0.053 mmol, 0.1 equiv.) were quickly added and the resulting mixture was stirred under N_2 at room temperature overnight. After 16 hours, the resulting cloudy mixture was diluted with DCM, washed sequentially with sat. aqueous NH_4Cl , H_2O , and brine, dried over MgSO_4 , filtered, and concentrated *in vacuo* to give **14a** (204 mg, 0.483 mmol, 91% yield) as a pale yellow oil which was used in the next step without further purification.

^1H NMR (500.0 MHz, CDCl_3): δ 7.41 – 7.36 (m, 2H), 7.3 (t, J = 7.5 Hz, 2H), 7.05 (tt, J = 1.2, 7.5 Hz, 1H), 4.14 (t, J = 6.8 Hz, 2H), 3.07 (ddd, J = 2.7, 12.5, 14.7 Hz, 2H), 2.44 (ddd, J = 2.7, 2.7, 14.3 Hz, 2H), 2.38 – 2.34 (m, J = 3.7, 5.0, 7.9 Hz, 2H), 2.27 (ddd, J = 2.7, 6.7, 6.7 Hz, 2H), 2.09 – 2.02 (m, 1H), 2.00 (t, J = 2.5 Hz, 1H), 1.90 (ddq, J = 3.3, 3.3, 12.7 Hz, 1H), 1.80 – 1.69 (m, 4H), 0.85 – 0.80 (m, 2H), 0.21 (s, 6H).

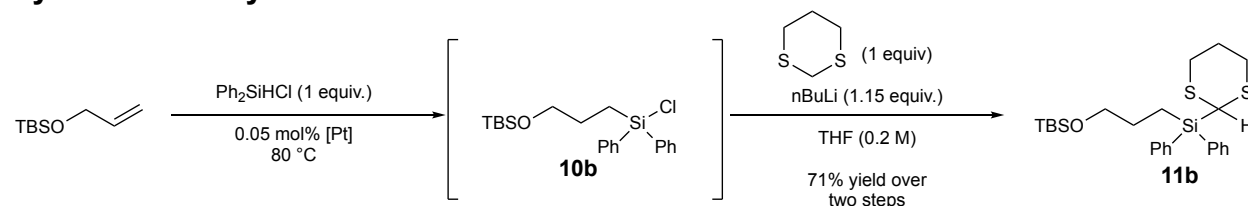


3-(hex-5-ynoyldimethylsilyl)propyl phenylcarbamate (15a) A flame-dried scintillation vial was charged with **14a** (138 mg, 0.328 mmol 1.0 equiv.) and a 5:1 solution of MeCN:PBS 7.4 buffer (0.05 M, 6 mL). To the rapidly stirred mixture was added PIFA (169 mg, 0.393 mmol, 1.2 equiv.), and the resulting solution was allowed to stir at room temperature. After 2 hours, the mixture was diluted with Et_2O , washed sequentially with sat. aqueous NH_4Cl , H_2O , and brine, dried over MgSO_4 , filtered, and concentrated *in*

vacuo. The crude material was purified using flash column chromatography (hex:EtOAc 90:10) to give acyl silane **15a** (80 mg, 74% yield) as a colorless clear oil.

¹H NMR (600.1 MHz, CDCl₃): δ 7.48 – 7.36 (m, 2H), 7.30 (t, *J* = 7.6 Hz, 2H), 7.06 (t, *J* = 7.2 Hz, 1H), 6.68 (br. s, 1H), 4.12 (t, *J* = 6.6 Hz, 2H), 2.77 (t, *J* = 7.1 Hz, 2H), 2.20 (dt, *J* = 2.6, 6.8 Hz, 2H), 1.93 (t, *J* = 2.6 Hz, 1H), 1.75 (ddd, *J* = 7.0, 7.0, 13.9 Hz, 2H), 1.73 – 1.68 (m, 2H), 0.82 – 0.77 (m, 2H), 0.23 (s, 6H). **¹³C NMR** (150.9 MHz, CDCl₃): δ 247.3, 153.6, 138.1, 129.2, 123.5, 118.8, 83.9, 69.1, 67.3, 47.2, 23.4, 20.8, 17.9, 10.0, –4.6. **HRMS** (ESI⁺): calculated for [C₁₈H₂₅NO₃Si+Na]⁺ required *m/z* 354.1496, found *m/z* 354.1499.

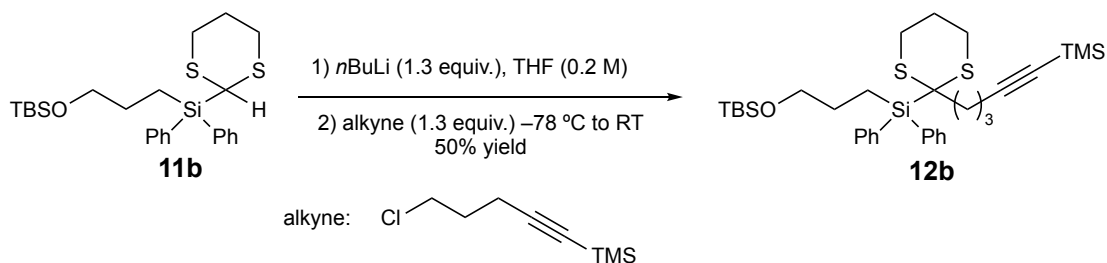
Synthesis of acyl silane **15b**



(3-((1,3-dithian-2-yl)diphenylsilyl)propoxy)(*tert*-butyl)dimethylsilane (11b**):** To an oven-dried Schlenk flask fitted with a rubber septum and cooled under N₂ was added platinum(0)-1,3-divinyl 1,1,1,3,3-tetramethyldisiloxane complex solution (Karstedt's catalyst) [0.1 M in vinyl terminated poly(dimethylsiloxane), ca 15 μL, 0.05 mol%] and Ph₂SiHCl (0.6 mL, 3.09 mmol, 1 equiv.) by syringe drop-wise. The resulting solution was stirred for 10 minutes at room temperature, then (allyloxy)(*tert*-butyl)dimethylsilane (0.531 g, 3.09 mmol, 1 equiv.) was added as a neat liquid by syringe and the solution was heated in an 80 °C oil bath for 25 minutes. After cooling to room temperature, the reaction mixture (**10b**) was used immediately in the next step without further purification. **¹H NMR** (400 MHz, CDCl₃): δ 7.66 – 7.60 (m, 4H), 7.47 – 7.39 (m, 6H), 3.61 (t, *J* = 6.4 Hz, 2H), 1.76 – 1.66 (m, 2H), 1.42 – 1.35 (m, 2H), 0.89 (s, 9H), 0.03 (s, 6H).

A flame dried 50 mL round bottom flask cooled under vacuum was charged with 1,3-dithiane (0.312 g, 2.6 mmol, 1.0 equiv.) and THF (13 mL, 0.2 M). The reaction vessel was cooled to –30 °C and a solution of *n*BuLi (2.99 mmol, 1.15 equiv.) was added dropwise. The solution was allowed to warm to room temperature and stir for 1 hour before cooling to –78 °C and adding **10b** (1.2 equiv.) dropwise. The reaction mixture was allowed to warm to room temperature overnight and the resulting orange solution was quenched by addition of sat. aqueous NH₄Cl solution and extracted with EtOAc three times. The combined organic layers were washed with saturated brine, dried over MgSO₄, and concentrated *in vacuo*. The resulting oil was purified using flash column chromatography (hex:EtOAc = 95:5) to give **11b** (0.894 g, 1.84 mmol, 71% over two steps) as an oil.

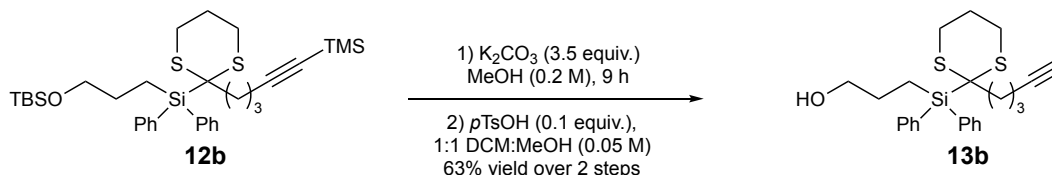
¹H NMR (300.1 MHz, CDCl₃): δ 7.70 – 7.63 (m, 4H), 7.46 – 7.34 (m, 6H), 4.25 (s, 1H), 3.60 (t, *J* = 6.6 Hz, 2H), 2.92 (ddd, *J* = 3.3, 11.6, 14.3 Hz, 2H), 2.71 (dt, *J* = 14.1, 3.7 Hz, 2H), 2.16 – 1.99 (m, 2H), 1.74 – 1.61 (m, 2H), 1.32 – 1.23 (m, 2H), 0.89 (s, 9H), 0.03 (s, 6H). **¹³C NMR** (125.7 MHz, CDCl₃): δ 135.8, 132.5, 130.0, 127.9, 65.8, 32.6, 31.8, 27.1, 26.15, 26.10, 22.15, 18.5, 7.5, –5.1 **HRMS** (ESI⁺): calculated for [C₂₅H₃₈OS₂Si₂+Na]⁺ required *m/z* 497.1795, found *m/z* 497.1792.



tert-butyl(3-(diphenyl(2-(5-(trimethylsilyl)pent-4-yn-1-yl)-1,3-dithian-2-yl)silyl)propoxy)dimethylsilane (12b):

A flame dried 50 mL round bottomed flask cooled under vacuum was charged with **11b** (0.500 g, 1.05 mmol, 1.0 equiv.) and THF (5.27 mL, 0.2 M) and cooled to $-78\text{ }^{\circ}\text{C}$, followed by the dropwise addition of *n*BuLi (2.4 M, 0.57 mL, 1.3 equiv.). After stirring for 5 minutes at $-78\text{ }^{\circ}\text{C}$, the reaction was allowed to warm to room temperature over an hour, then returned to $-78\text{ }^{\circ}\text{C}$ prior to the dropwise addition of (5-chloropent-1-yn-1-yl) trimethylsilane (0.239 g, 1.37 mmol, 1.3 equiv.). The reaction was then allowed to warm to room temperature and stir overnight. After 16 hours, the reaction was quenched by the addition of sat. aqueous NH_4Cl and extracted with a 1:1 mixture of Et_2O /hexanes. The combined organics were washed with saturated brine, dried over MgSO_4 , and concentrated to an oil. Purification of the crude material by flash column chromatography on silica gel in 95:5 hex/EtOAc yielded the desired product **12b** as a clear, colorless oil (0.333 g, 50% yield).

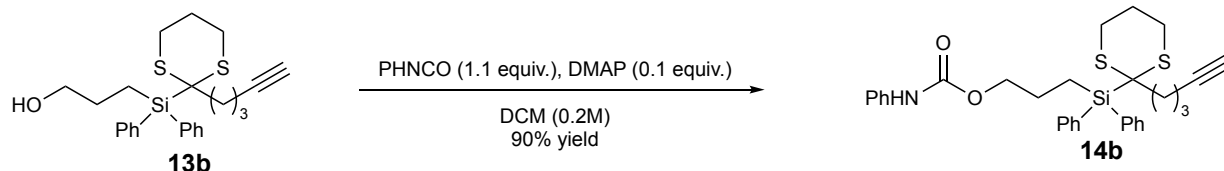
^1H NMR (500.0 MHz, CDCl_3): δ 7.81 (br.d $J = 7\text{ Hz}$, 4H), 7.46 – 7.31 (m, 6H), 3.53 (t, $J = 6.0\text{ Hz}$, 2H), 3.14 – 3.05 (m, 2H), 2.47 – 2.40 (m, 2H), 2.30 – 2.25 (m, 2H), 2.09 (t, $J = 6.6\text{ Hz}$, 2H), 1.60 – 1.53 (m, 2H), 1.48 – 1.42 (m, 2H), 1.41 – 1.35 (m, 2H), 1.34 – 1.24 (m, 2H), 0.87 (s, 9H), 0.12 (s, 9H), 0.0 (s, 6H). **^{13}C NMR** (125.7 MHz, CDCl_3): δ 136.57, 132.52, 129.81, 127.68, 107.27, 84.89, 65.71, 39.69, 36.44, 27.57, 26.42, 26.09, 25.38, 24.98, 23.93, 18.44, 7.66, 0.27, -5.14 . **HRMS** (ESI^+): calculated for $[\text{C}_{33}\text{H}_{52}\text{OS}_2\text{Si}_3+\text{H}]^+$ required m/z 613.2840, found m/z 613.2838.



3-((2-(pent-4-yn-1-yl)-1,3-dithian-2-yl)diphenylsilyl)propan-1-ol (13b): A flame dried scintillation vial was charged with **12b** (0.436 g, 0.711 mmol, 1 equiv.), K_2CO_3 (146 mg, 1.06 mmol, 1.5 equiv.) and MeOH (1.5 mL, 0.5 M) and stirred rapidly. Partial conversion was observed by TLC after workup following 6 hours of stirring, at which point the material was charged with an additional batch of K_2CO_3 (278 mg, 2.0 equiv) and MeOH (3mL), until full consumption of **12b** was observed by TLC, approximately 3 hours. The reaction was transferred to a separatory funnel with Et_2O and hexanes, washed with water, dried over MgSO_4 , and concentrated. The crude material was then transferred to a scintillation vial charged with *p*-toluenesulfonic acid (13 mg, 0.075 mmol, 0.1 equiv.) and a 1:1 solution of DCM:MeOH (0.05 M, 14 mL). After stirring for 4 hours, the solution was diluted with EtOAc, washed sequentially with sat. aqueous NH_4Cl , H_2O , and brine, dried over MgSO_4 ,

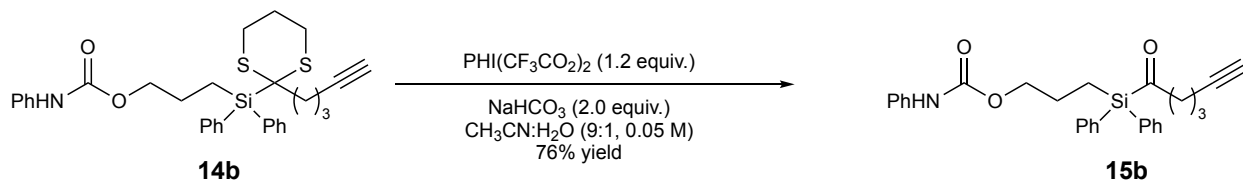
filtered, and concentrated *in vacuo* to give **13b** (191 mg, 0.448 mmol, 63% yield over two steps) as a viscous oil.

¹H NMR (500.0 MHz, CDCl₃): δ 7.80 (app d, *J* = 7.5 Hz, 4H), 7.45 – 7.35 (m, 6H), 3.57 (t, *J* = 6.4 Hz, 2H), 3.07 (ddd, *J* = 3.1, 12.0, 14.7 Hz, 2H), 2.45 (dt, *J* = 14.4, 3.8 Hz, 2H), 2.30 – 2.26 (m, 2H), 2.08 – 2.01 (m, 2H), 2.07 – 1.93 (m, 2H), 1.88 (t, *J* = 2.5 Hz, 1H), 1.64 – 1.56 (m, 2H), 1.55 – 1.47 (m, 2H), 1.46 – 1.40 (m, 2H). **¹³C NMR** (125.7 MHz, CDCl₃): δ 136.48, 132.22, 129.84, 127.68, 84.12, 68.63, 65.51, 39.49, 36.64, 27.56, 26.42, 24.80, 23.94, 18.56, 7.72. **HRMS** (ESI⁺): calculated for [C₂₄H₃₀OS₂Si+Na]⁺ required *m/z* 449.1400, found *m/z* 449.1396.



3-((2-(pent-4-yn-1-yl)-1,3-dithian-2-yl)diphenylsilyl)propyl benzoate (14b): A flame-dried screw-capped culture tube cooled under vacuum was charged with **13b** (156.4 mg, 0.36 mmol, 1.0 equiv.) and DCM (0.91 mL, 0.2 M). Phenylisocyanate (43 uL, 0.40 mmol, 1.1 equiv.) and DMAP (4.4 mg, 0.036 mmol, 0.1 equiv.) were quickly added and the resulting mixture was stirred under N₂ at room temperature overnight. After 16 hours, the resulting cloudy mixture was diluted with DCM, washed sequentially with sat. aqueous NH₄Cl, H₂O, and brine, dried over MgSO₄, filtered, and concentrated *in vacuo* to give **14b** (178.6 mg, 0.327 mmol, 90% yield) as a crude white solid which was used in the next step without further purification.

¹H NMR (400.1 MHz, CDCl₃): δ 7.80 (d, *J* = 7.0 Hz, 4H), 7.48 – 7.28 (m, 10H), 7.09 – 7.02 (m, 1H), 6.55 (br. s, 1H), 4.10 (t, *J* = 6.5 Hz, 2H), 3.14 – 3.02 (m, 2H), 2.48 (dt, *J* = 14.1, 2.6 Hz, 2H), 2.34 – 2.26 (m, 2H), 2.08 – 2.03 (m, 2H), 1.88 (t, *J* = 2.7 Hz, 1H), 1.70 – 1.54 (m, 6H), 1.50 – 1.44 (m, 2H).

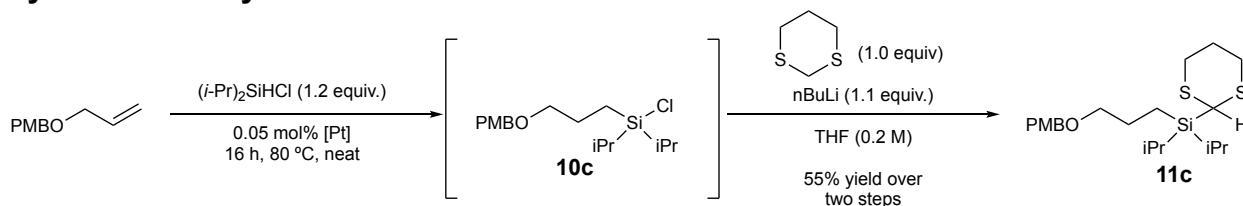


3-(hex-5-ynoyldiphenylsilyl)propyl phenylcarbamate (15b): A flame-dried scintillation vial was charged with **14b** (17 mg, 0.037 mmol 1.0 equiv.), NaHCO₃ (6.2 mg, 0.075 mmol, 2.0 equiv.), and 9:1 solution of MeCN:H₂O (700:75 uL, 0.05 M). To the rapidly stirred mixture was added PIFA (19 mg, 0.045 mmol, 1.2 equiv.), and the resulting orange solution was allowed to stir at room temperature. After 2 hours, the mixture was diluted with Et₂O, washed sequentially with sat. aqueous NH₄Cl, H₂O, and brine, dried over MgSO₄, filtered, and concentrated *in vacuo*. The crude material was purified using flash column chromatography (hex:EtOAc 95:5) to give **15b** (13 mg, 76% yield) as a pale yellow oil.

¹H NMR (600.1 MHz, CDCl₃): δ 7.58 (br. d, *J* = 7.0 Hz, 4H), 7.46 (tt, *J* = 6.4, 1.4 Hz, 2H), 7.40 (app. t, *J* = 7.5 Hz, 4H), 7.35 (br. d, *J* = 7.5 Hz, 2H), 7.30 (tt, *J* = 7.3, 2.0 Hz, 2H), 7.05 (t, *J* = 7.2 Hz, 1H), 6.54 (br. s, 1H), 4.14 (dd, *J* = 6.5 Hz, 2H), 2.78 (t, *J* = 7.0 Hz, 2H),

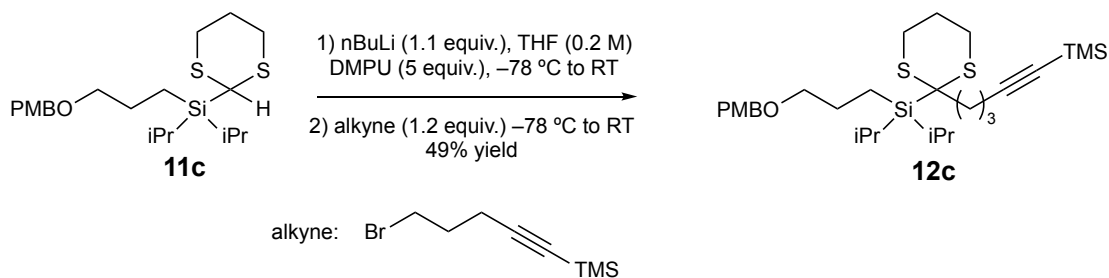
2.12 (td, $J = 7.0, 2.6$ Hz, 2H), 1.87 (t, $J = 2.6$ Hz, 1H), 1.82 – 1.76 (m, 2H), 1.71 (ddd, $J = 13.9, 7.0, 7.0$ Hz, 2H), and 1.38 – 1.33 (m, 2H). ^{13}C NMR (150.9 MHz, CDCl_3): δ 243.76, 187.88, 137.58, 135.38, 131.35, 130.34, 129.04, 128.37, 123.38, 83.67, 68.94, 48.23, 29.72, 23.12, 20.81, 17.70, and 7.83. HRMS (ESI^+): calculated for $[\text{C}_{28}\text{H}_{29}\text{NO}_3\text{Si}+\text{Na}]^+$ required m/z 478.1809, found m/z 478.1819.

Synthesis of acyl silane 15c



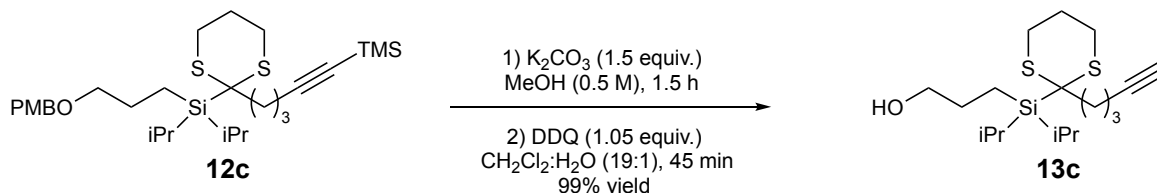
(1,3-dithian-2-yl)diisopropyl(3-((4-methoxybenzyl)oxy)propyl)silane (11c): To an oven-dried Schlenk flask fitted with a rubber septum and cooled under N_2 was added platinum(0)-1,3-divinyl 1,1,1,3,3-tetramethyldisiloxane complex solution (Karstedt's catalyst) [0.1 M in vinyl terminated poly(dimethylsiloxane), ca 150 μL , 0.05 mol%] and $i\text{Pr}_2\text{SiHCl}$ (4.5 g, 30 mmol, 1.0 equiv.) by syringe dropwise. The resulting yellow solution was stirred for 10 minutes at room temperature, then 1-((allyloxy)methyl)-4-methoxybenzene (**9c**, 4.92 g, 30 mmol, 1.0 equiv.) was added as a neat liquid by syringe. The vessel was heated to 80 °C in an oil bath for 16 hours. After cooling to room temperature, the reaction mixture (**10c**) was used immediately in the next step without further purification. ^1H NMR: (300.1 MHz, CDCl_3) δ 7.27 (d, 2H, $J = 8.7$ Hz), 6.90 (d, 2H, $J = 8.7$ Hz), 4.45 (s, 2H), 3.82 (s, 3H), 3.44 (t, 2H, $J = 6.7$ Hz), 1.79–1.69 (m, 2H), 1.11 – 1.06 (m, 14H), 0.89 – 0.83 (m, 2H). A flame dried 250 mL round bottom flask cooled under vacuum was charged with 1,3-dithiane (2.57 g, 21.4 mmol, 1.0 equiv.) and THF (70 mL, 0.3 M). The reaction vessel was cooled to –30 °C and a solution of $n\text{BuLi}$ (2.4 M in hexanes, 9.83 mL, 23.6 mmol, 1.1 equiv.) was added dropwise resulting in a persistent, but faint yellow color. The solution was maintained at –30 °C for 1 hour before cooling to –78 °C and adding **10c** (1.2 equiv.) as a solution in THF (10 mL) dropwise. The reaction mixture was allowed to warm to room temperature overnight and then quenched by addition of 200 mL sat. aqueous NH_4Cl solution and extracted with a 1:1 mixture of Et_2O /hex (3x, 100 mL). The combined organic layers were washed with saturated brine, dried over MgSO_4 , and concentrated *in vacuo*. The resulting residue was purified by flash column chromatography on silica gel in 95:5 hex:EtOAc to yield a clear, colorless oil (4.84 g, 55% yield).

^1H NMR: (500.0 MHz, CDCl_3) δ 7.29 (d, $J = 8.5$ Hz, 2H), 6.89 (d, $J = 8.6$ Hz, 2H), 4.47 (s, 2H), 3.91 (s, 1H), 3.81 (s, 3H), 3.44 (t, $J = 7.0$ Hz, 2H), 2.90 (ddd, $J = 14.7, 12.2, 2.8$ Hz), 2.71 (dt, $J = 13.8, 3.9$ Hz, 2H), 2.16 – 2.00 (m, 2H), 1.83 – 1.76 (m, 2H), 1.26 – 1.17 (m, 2H), 1.14 (d, $J = 7.4$ Hz, 6H), 1.13 (d, $J = 7.0$ Hz, 6H), 0.77 – 0.72 (m, 2H). ^{13}C NMR (125.7 MHz) δ 159.1, 130.8, 129.3, 113.8, 73.3, 72.5, 55.3, 32.1, 31.9, 26.6, 24.2, 18.59, 18.56, 11.0, 5.1. HRMS (ESI^+): calculated for $[\text{C}_{21}\text{H}_{36}\text{O}_2\text{S}_2\text{Si}+\text{Na}]^+$ required m/z 435.1823, found m/z 435.1829.



(5-(2-(diisopropyl(3-((4-methoxybenzyl)oxy)propyl)silyl)-1,3-dithian-2-yl)pent-1-yn-1-yl)trimethylsilane (12c): A flame dried 100 mL round bottom flask cooled under vacuum was charged with **11c** (1.95 g, 4.73 mmol, 1.0 equiv.) and THF (24 mL, 0.2 M) and cooled to $-78\text{ }^{\circ}\text{C}$, followed by the dropwise addition of *n*BuLi (2.4 M, 2.27 mL, 1.1 equiv.). After stirring for 5 minutes at $-78\text{ }^{\circ}\text{C}$, the reaction was allowed to warm to room temperature over an hour, resulting in an orange solution that was then returned to $-78\text{ }^{\circ}\text{C}$ prior to the addition of anhydrous DMPU (2.7 mL, 23.7 mmol, 5 equiv.) and (5-bromopent-1-yn-1-yl) trimethylsilane (1.24 g, 5.66 mmol, 1.2 equiv.). The reaction was then allowed to warm to room temperature and stir overnight. After 16 hours, the reaction was quenched by the addition of 100 mL sat. NH_4Cl and extracted with a 1:1 mixture of Et_2O /hexanes (3x50 mL). The combined organics were washed with saturated brine, dried over MgSO_4 , filtered, and concentrated to an oil. Purification of the crude material by flash column chromatography on silica gel in 95:5 hex:EtOAc yielded the desired product as a clear, colorless oil (1.29 g, 49% yield).

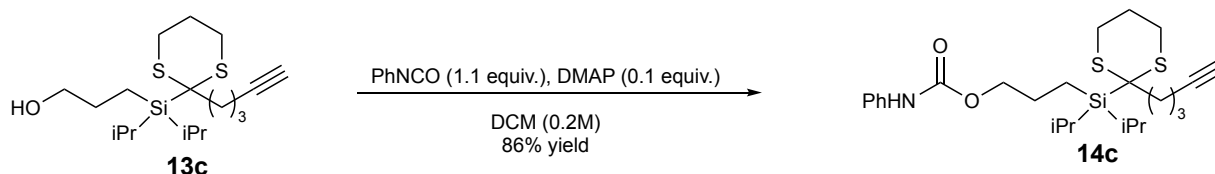
^1H NMR (500.0 MHz, CDCl_3) δ 7.28 (d, $J = 9.2\text{ Hz}$, 1H), 6.88 (d, $J = 8.8\text{ Hz}$, 1H), 4.45 (s, 2H), 3.81 (s, 3H), 3.42 (t, $J = 6.9\text{ Hz}$, 2H), 3.14 (ddd, $J = 14.3, 11.8, 2.7\text{ Hz}$, 2H), 2.49 – 2.44 (m, 2H), 2.38 (dt, $J = 14.1, 3.5\text{ Hz}$, 2H), 2.33 (t, $J = 6.5\text{ Hz}$, 2H), 2.07 – 2.00 (m, 1H), 1.99 – 1.89 (m, 1H), 1.87 – 1.80 (m, 1H), 1.80 – 1.73 (m, 2H), 1.37 (hept, $J = 7.5\text{ Hz}$, 2H), 1.19 (d, $J = 7.2, 6\text{ Hz}$), 1.18 (d, $J = 7.5, 6\text{ Hz}$), 0.16 (s, 9H). **^{13}C NMR** (125.7 MHz, CDCl_3) δ 159.2, 131.0, 129.4, 113.9, 107.3, 85.2, 73.5, 72.5, 55.4, 40.5, 36.9, 26.9, 25.2, 25.0, 23.8, 20.2, 19.8, 19.8, 12.3, 6.4, 0.3. **HRMS** (ESI^+) calculated for $[\text{C}_{29}\text{H}_{50}\text{O}_2\text{S}_2\text{Si}_2+\text{Na}]^+$ required m/z 573.2683, found m/z 573.2686.



3-(diisopropyl(2-(pent-4-yn-1-yl)-1,3-dithian-2-yl)silyl)propan-1-ol (13c): A flame dried 25 mL round bottom flask was charged with **5** (1.29 g, 2.24 mmol, 1 equiv.), K_2CO_3 (464 mg, 3.36 mmol, 1.5 equiv.) and MeOH (5 mL, 0.5 M) and stirred rapidly until full consumption of **5c** was observed by TLC, approximately 1.5 hours. The reaction was transferred to a separatory funnel with Et_2O , washed with water, dried over MgSO_4 , and concentrated. The crude product was transferred to a 25 mL round bottom flask and dissolved in a mixture of CH_2Cl_2 : H_2O (19:1, 12 mL). To the stirred solution was added DDQ (497 mg, 2.19 mmol, 1.05 equiv.) resulting in a dark purple color. After stirring for 45 min, the reaction was quenched by the addition of sat. NaHCO_3 sol. and filtered through a short pad of Celite washing with CH_2Cl_2 . The filtrate was washed with water,

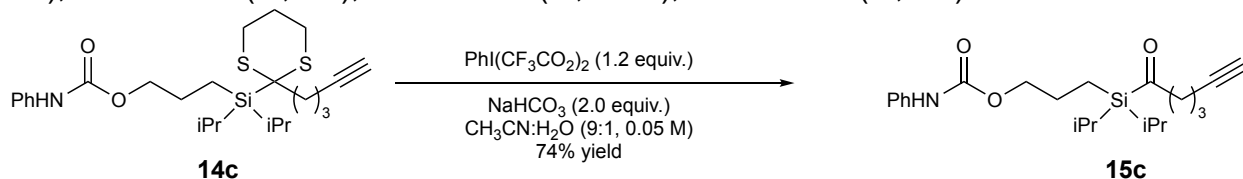
brine, dried over MgSO_4 , and concentrated to a colored oil. The crude material was purified by a gradient column (9:1 to 8:2 hex:EtOAc) to yield the desired product as a clear, colorless oil (735 mg, 99% yield).

^1H NMR (300.1 MHz, CDCl_3): δ 3.60 (t, J = 6.7 Hz, 2H), 3.09 (ddd, 14.9, 12.7, 3.0 Hz, 2H), 2.45 – 2.42 (m, 2H), 2.38 (dt, J = 14.3, 3.9 Hz, 2H), 2.28 (td, J = 6.74, 2.70, 2H), 2.05 – 2.02 (m, 1H), 1.99 (t, J = 2.63 Hz, 1H), 1.96 – 1.88 (m, 1H), 1.82–1.75 (m, 4H), 1.67 (br. s, 1H), 1.39 – 1.33 (m, 2H), 1.18 – 1.16 (m, 12H), 0.85 – 0.82 (m, 2H). **^{13}C NMR** (125.7 MHz, CDCl_3): δ 84.3, 69.0, 66.1, 40.4, 37.2, 28.0, 27.0, 25.1, 23.9, 19.8, 19.7, 18.8, 12.3, 6.1. **HRMS** (ESI^+) calculated for $[\text{C}_{18}\text{H}_{34}\text{OS}_2\text{Si}+\text{Na}]^+$ required m/z 381.1740, found m/z 381.1712.



3-(diisopropyl(2-(pent-4-yn-1-yl)-1,3-dithian-2-yl)silyl)propyl phenylcarbamate (14c): A flame-dried culture tube cooled under vacuum was charged with **13c** (65 mg, 0.18 mmol, 1.0 equiv.) and DCM (0.91 mL, 0.2 M). Phenylisocyanate (22 μL , 0.20 mmol, 1.1 equiv.) and DMAP (2.2 mg, 0.018 mmol, 0.1 equiv.) were quickly added and the resulting mixture was stirred under N_2 at room temperature overnight. After 16 hours, the resulting cloudy mixture was diluted with DCM, washed sequentially with sat. aqueous NH_4Cl , H_2O , and brine, dried over MgSO_4 , filtered, and concentrated *in vacuo* to give **14c** (74.2 mg, 0.155 mmol, 86% yield) as a crude white solid which was used in the next step without further purification.

^1H NMR (400.0 MHz, CDCl_3): δ 7.41 – 7.36 (m, 2H), 7.33 – 7.28 (m, 2H), 7.09 – 7.03 (m, 1H), 6.61 (br. s, 1H), 4.14 (t, J = 6.7 Hz, 2H), 3.15 – 3.05 (m, 2H), 2.49 – 2.35 (m, 4H), 2.29 (dt, J = 2.7, 6.7 Hz, 2H), 2.08 – 2.00 (m, 2H), 1.97 – 1.88 (m, 2H), 1.84 – 1.75 (m, 2H), 1.43 – 1.33 (m, 2H), 1.21 – 1.16 (m, 12 H), 0.92 – 0.85 (m, 2H).

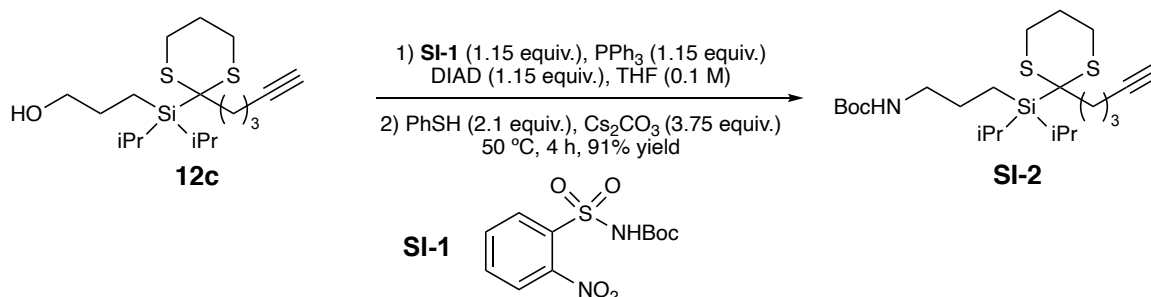


3-(hex-5-ynoyldiisopropylsilyl)propyl phenylcarbamate (15c): A flame-dried scintillation vial was charged with **14c** (9 mg, 0.023 mmol, 1.0 equiv.), NaHCO_3 (3.9 mg, 0.047 mmol, 2.0 equiv.), and a solution of $\text{MeCN}:\text{H}_2\text{O}$ (9:1, 0.05 M, 0.5 mL). To the rapidly stirred mixture was added PIFA (12 mg, 0.028 mmol, 1.2 equiv.), and the resulting yellow solution was allowed to stir at room temperature. After 30 min, the mixture was diluted with Et_2O , washed sequentially with sat. aqueous NH_4Cl , H_2O , and brine, dried over MgSO_4 , filtered, and concentrated *in vacuo*. The crude material was purified using flash column chromatography (hex:EtOAc 95:5) to give acyl silane **15c** (6.7 mg, 0.017 mmol, 74% yield) as a clear solid.

^1H NMR (500.0 MHz, CDCl_3): δ 7.42 – 7.34 (m, 2H), 7.33–7.28 (m, 2H), 7.06 (t, J = 7.3 Hz, 1H), 6.62 (br. s, 1H), 4.14 (t, J = 6.7 Hz, 2H), 2.73 (t, J = 7.0 Hz, 2H), 2.20 (dt, J = 6.9, 2.7 Hz, 2H), 1.95 (t, J = 2.7 Hz, 1H), 1.80 – 1.72 (m, 4H), 1.28 – 1.17 (m, 2H (overlap with EtOAc)), 1.09 – 1.04 (m, 12H), 0.88 – 0.83 (m, 2H). **^{13}C NMR** (125.7 MHz, CDCl_3): δ

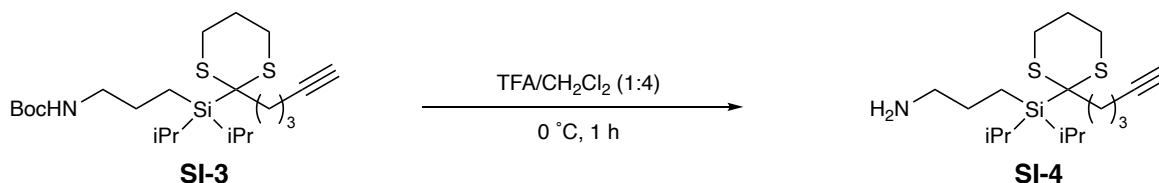
137.9, 135.4, 129.1, 128.4, 123.4, 83.7, 69.0, 67.6, 49.3, 23.5, 20.4, 18.2, 18.1, 17.8, 10.6, 4.7. **HRMS** (ESI⁺): calculated for [C₂₂H₃₃NO₃Si+Na]⁺ required *m/z* 410.2122, found *m/z* 410.2126.

Synthesis of acyl silane (+)-JQ1 probes

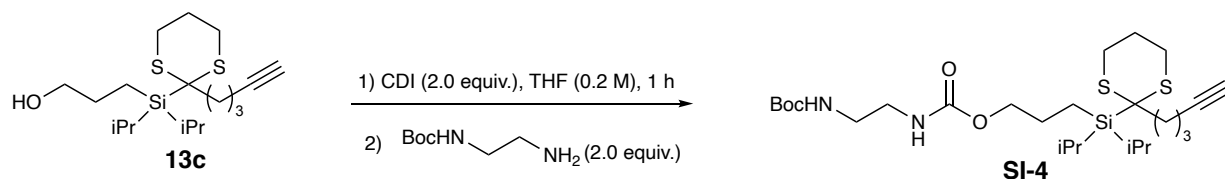


tert-butyl (3-(diisopropyl(2-(pent-4-yn-1-yl)-1,3-dithian-2-yl)silyl)propyl)carbamate (SI-2): A 10 mL flame dried round bottom flask cooled under vacuum was charged with **13c** (100 mg, 0.280 mmol, 1 equiv.), N-boc-2-nitrobenzenesulfonamide (**SI-1**, 100 mg, 0.32 mmol, 1.15 equiv., prepared according to (75)), triphenyl phosphine (84 mg, 0.32 mmol, 1.15 equiv.), and DIAD (63 μ L, 0.32 mmol, 1.15 equiv.) in THF (0.1 M). The mixture was stirred for 16 hours before the addition of thiophenol (60 μ L, 0.588 mmol, 2.1 equiv.) and Cs₂CO₃ (340 mg, 1.05 mmol, 3.75 equiv.). The reaction was heated to 50 °C and vigorously stirred for 4 hours before cooling to room temperature. The reaction was quenched by the addition of 0.5 M NaOH (5 mL) and extracted with Et₂O (3 x 10 mL). The combined organic layers were washed with brine, dried over MgSO₄ before concentrating to an oil. Purification of the crude product by flash chromatography by gradient elution of 9:1 to 8:2 hex:EtOAc yield the N-Boc protected amine (115 mg, 91% yield). The product appears faintly on TLC under UV, but presents as a significant dark band upon heating in *p*-anisaldehyde stain.

¹H NMR: (500.0 MHz, CDCl₃) δ 4.64 (bs, 1H), 3.10 (apparent t, *J* = 13.2 Hz, 2H) 2.45 – 2.41 (m, 2H), 2.38 (dt, *J* = 14.3, 3.74 Hz, 2H), 2.28 (td, *J* = 6.7, 2.6 Hz, 2H), 2.06 – 2.02 (m, 1H), 2.34 (t, *J* = 2.34 Hz, 1H), 1.96–1.88 (m, 1H), 1.81 – 1.75 (m, 2H), 1.74 – 1.67 (m, 2H), 1.44 (s, 9H), 1.34 (hept, *J* = 7.4 Hz, 1H) 1.17 (d, *J* = 7.4 Hz, 6H), 1.16 (d, *J* = 7.6 Hz, 6H), 0.83 – 0.79 (m, 2H). **¹³C NMR** (125.7 MHz, CDCl₃) δ 156.1, 84.3, 79.0, 69.1, 44.1, 40.3, 37.3, 28.6, 27.0, 25.1, 23.9, 19.8, 19.7, 18.9, 12.3, 7.5. **HRMS** (ESI⁺) calculated for [C₂₃H₄₃NO₂S₂Si+H]⁺ required *m/z* 458.2577, found *m/z* 458.2609.

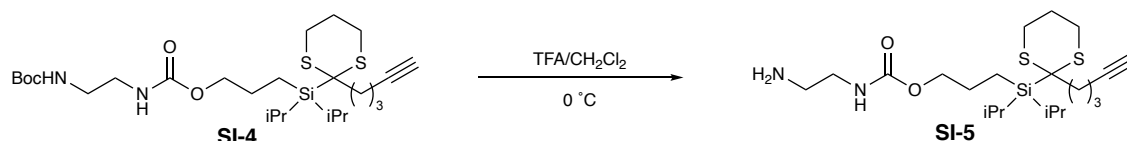


Amine linker SI-4: N-Boc protected amine **SI-3** (91 mg, 0.200 mmol, 1 equiv.) was dissolved in DCM (1 mL, 0.2 M) and cooled to 4 °C before adding TFA (0.25 mL). After 1 hour, the reaction was complete by TLC and concentrated, diluted with Et₂O and washed with 1 N NaOH. The organic layer was concentrated to a yellow oil and immediately used in the next reaction without further purification (crude mass 70 mg, crude yield 98%).

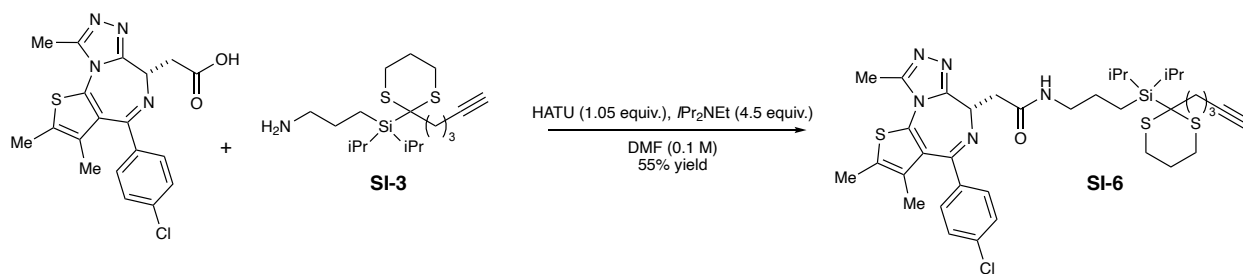


N-Boc protected amine linker SI-4: A one-dram vial was charged with **13c** (50 mg, 0.140 mmol, 1.0 equiv.), THF (0.7 mL, 0.2 M), and CDI (45 mg, 0.280 mmol, 2.0 equiv) and stirred at ambient temperature for 1 hour. Subsequently, *N*-Boc-ethylenediamine (67 mg, 0.420 mmol, 3.0 equiv.) and DMAP (51 mg, 0.420 mmol, 3.0 equiv.) were added. The reaction was stirred overnight before diluting with water and extracting the mixture with EtOAc. The organic extract was washed with brine, dried over MgSO₄, filtered, and concentrated *in vacuo*. The crude residue was purified by flash chromatography of EtOAc in hexanes to yield **SI-4** (67 mg, 88% yield).

¹H NMR (500.0 MHz, CDCl₃) δ 5.11 (s, 1H), 4.93 (s, 1H), 4.00 (t, *J* = 6.1 Hz, 2H) 3.26 – 3.24 (m, 4H), 3.09 (td, *J* = 14.9, 12.8, 2.7 Hz, 2H), 2.45 – 2.40 (m, 2H) 2.38 (dt, *J* = 14.2, 3.7 Hz, 2H), 2.28 (td, *J* = 6.7, 2.7 Hz, 2H), 2.05 – 2.00 (m, 2H), 1.95–1.76 (m, 5H), 1.42 (s, 9H), 1.35 (hept, *J* = 7.4 Hz, 2H), 1.17 (d, *J* = 7.5 Hz, 6H), 1.16 (d, *J* = 7.6 Hz, 6H), 0.88 – 0.77 (m, 2H). **¹³C NMR** (125.7 MHz, CDCl₃) δ 157.2, 156.5, 84.3, 79.6, 69.1, 67.9, 41.4, 40.8, 40.3, 37.3, 28.5, 27.0, 25.0, 24.4, 23.9, 19.8, 19.7, 18.9, 12.3, 6.3. **HRMS** (ESI⁺) calculated for [C₂₆H₄₈O₄S₂Si+Na]⁺ required *m/z* 567.2717, found *m/z* 567.2718.

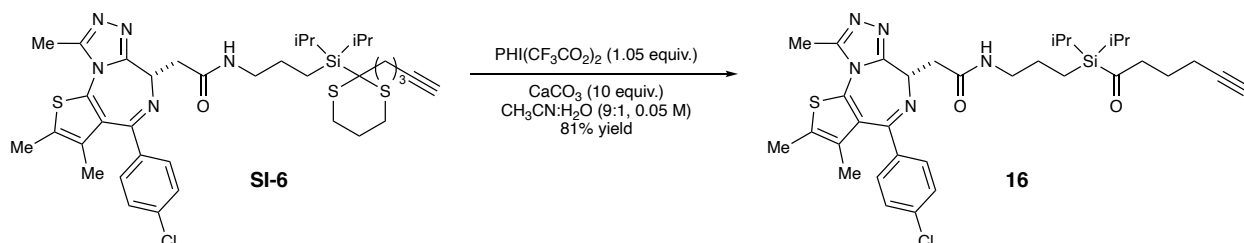


Amine linker SI-5: A one-dram vial was charged with **SI-4** (67 mg, 0.123 mmol) and DCM (1.4 mL, 0.09 M) and cooled in an ice bath. Subsequently, TFA (0.11 mL, 1.4 mmol, 11.3 equiv.) was added and stirred until complete by TLC. The reaction was concentrated, diluted with Et₂O and washed with 1 N NaOH. The organic layer was concentrated to 84 mg of a yellow oil and immediately used in the next reaction without further purification.



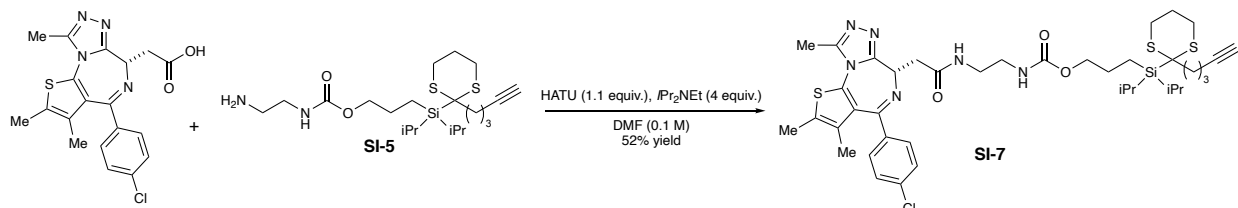
Acyl silane dithiane precursor to 16 (SI-6): To a solution of (+)-JQ1-OH (50 mg, 0.125 mmol, 1.0 equiv.) in a two-dram vial was added DMF (1.25 mL, 0.1 M), HATU (50 mg, 0.131 mmol, 1.05 equiv.), and *i*-PrNEt₂ (65 μL, 0.575 mmol, 4.5 equiv.). After 10 min, freshly prepared **SI-3** was added (46 mg, 0.138 mmol, 1.05 equiv.) and the reaction was allowed to stir for 16 hours before removing the DMF *in vacuo* and purifying by silica gel chromatography using a gradient elution of 99.5:0.05 – 96:4 DCM:MeOH. The product was isolated as a white solid (50 mg, 55% yield).

¹H NMR (500.0 MHz, CDCl₃) δ 7.39 (d, *J* = 8.5 Hz, 2H), 7.31 (d, *J* = 8.7 Hz, 2H), 6.70 (t, *J* = 5.7 Hz, 1H), 4.61 (t, *J* = 6.8 Hz, 1H), 3.71 (hept, *J* = 6.7 Hz, 1H), 3.51 (dd, *J* = 14.4, 6.9 Hz, 1H), 3.37 – 3.29 (m, 2H), 3.25 – 3.14 (m, 2H), 3.08 (ddd, *J* = 15.2, 12.8, 2.7 Hz, 2H), 2.65 (s, 3H), 2.45 – 2.40 (m, 2H), 2.38 (s, 4H), 2.35 (dt, *J* = 14.0, 3.8 Hz, 2H), 2.27 (td, *J* = 6.7, 2.7 Hz, 2H), 2.03 – 1.99 (m, 1H), 2.01 (s, *J* = 2.6 Hz, 1H), 1.98 – 1.86 (m, 1H), 1.82 – 1.72 (m, 4H), 1.36 – 1.29 (m, 1H), 1.18 – 1.10 (m, 12H), 0.84 – 0.78 (m, 2H). **¹³C NMR** (125.7 MHz, CDCl₃) δ 170.4, 164.0, 155.8, 150.0, 136.8, 136.7, 132.2, 131.0, 130.5, 129.9, 128.8, 84.3, 69.1, 54.5, 43.7, 43.3, 40.3, 39.4, 37.2, 26.9, 25.0, 24.8, 23.9, 19.8, 19.8, 19.7, 18.8, 14.4, 13.2, 12.22, 12.21, 11.9, 7.7. **HRMS** (ESI⁺) calculated for [C₃₇H₅₁N₅OCIS₃Si+H]⁺ required *m/z* 740.2714, found *m/z* 740.2708.



Acyl silane 16: To a solution of **SI-6** (48 mg, 0.0649 mmol, 1 equiv.) and CaCO₃ (64.9 mg, 0.649 mmol, 10 equiv.) in CH₃CN:H₂O (9:1, 0.05 M, 1.4 mL) cooled in an ice bath to 4 °C was added PIFA (29.3 mg, 0.0681 mmol, 1.05 equiv.) dropwise in CH₃CN (0.1 mL). The solution was stirred for 30 minutes in the ice bath before dilution with EtOAc. The resulting mixture was washed with sat. aqueous NaHCO₃, brine, and dried over MgSO₄ before concentrating *in vacuo*. The crude material was purified by flash column chromatography using a gradient elution of 99.5:0.05 – 96:4 DCM:MeOH to give **16** as a white solid (34 mg, 81% yield).

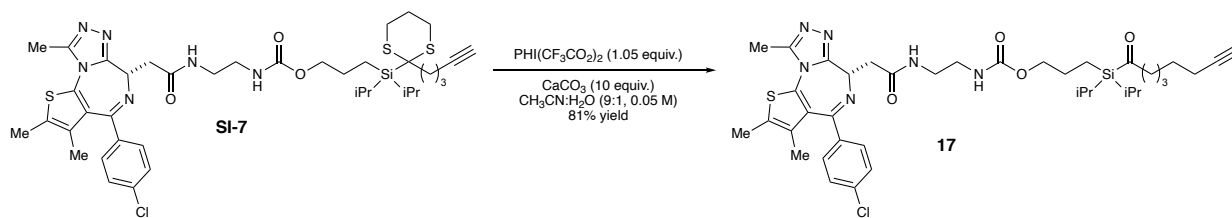
¹H NMR (500.0 MHz, CDCl₃) δ 7.34 (d, *J* = 8.3 Hz, 2H), 7.26 (d, *J* = 8.6 Hz, 2H), 6.76 (t, *J* = 6.0 Hz, 1H), 4.57 (t, *J* = 6.9 Hz, 1H), 3.50 (dd, *J* = 14.3, 7.5 Hz, 1H), 3.29 (dd, *J* = 14.4, 6.3 Hz, 1H), 3.24 (p, *J* = 6.6 Hz, 1H), 3.19 (p, *J* = 6.6 Hz, 1H), 2.64 (t, *J* = 7.0 Hz, 2H), 2.60 (s, 3H), 2.33 (s, 3H), 2.12 (td, *J* = 6.9, 2.6 Hz, 2H), 1.90 (t, *J* = 2.6 Hz, 1H), 1.67 (p, *J* = 6.9 Hz, 2H), 1.60 (s, 3H), 1.59–1.49 (m, 2H), 1.11 (hept, *J* = 7.3 Hz, 2H), 1.00 – 0.92 (m, 12H), 0.74 – 0.68 (m, 2H). **¹³C NMR** (125.7 MHz, CDCl₃) δ 246.6, 170.2, 164.7, 155.4, 150.2, 137.5, 135.9, 132.2, 131.7, 131.3, 130.5, 130.2, 128.9, 84.0, 69.1, 54.3, 49.5, 43.0, 38.8, 24.1, 20.6, 18.3, 18.2, 17.9, 14.5, 13.3, 11.8, 10.7, 5.9. **HRMS** (ESI⁺) calculated for [C₃₄H₄₅N₅O₂ClSSi+H]⁺ required *m/z* 650.2752, found *m/z* 650.2744.



Acyl silane dithiane precursor to 17 SI-7: To a solution of (+)-JQ1-OH (30 mg, 0.075 mmol, 1.0 equiv.) in a two-dram vial was added DMF (0.75 mL, 0.1 M), HATU (31 mg, 0.0825 mmol, 1.1 equiv.), and *i*-Pr₂NEt₂ (52 μL, 0.30 mmol, 4.0 equiv.). After 10 min, freshly prepared **SI-5** was added (36 mg, 0.0825 mmol, 1.1 equiv.) and the reaction was

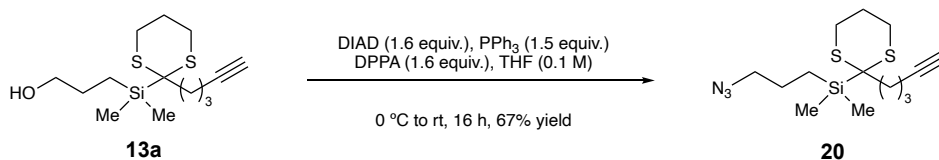
allowed to stir for 16 hours before removing the DMF *in vacuo* and purifying by silica gel chromatography using a gradient elution of 99.5:0.05 – 96:4 DCM:MeOH. The product was isolated as a white solid (32 mg, 52% yield).

¹H NMR (500 MHz, CDCl₃) δ 7.72 (br. s, 1H), 7.38 (d, *J* = 8.4 Hz, 2H), 7.30 (d, *J* = 8.6 Hz, 2H), 6.03 (m, 1H), 4.69 (t, *J* = 7.0 Hz, 1H), 4.00 – 3.93 (m, 1H), 3.58 (dd, *J* = 14.7, 7.7 Hz, 1H), 3.54 – 3.46 (m, 1H), 3.43 (dd, *J* = 14.6, 5.9 Hz, 1H), 3.40–3.25 (m, 3H), 3.08 (d, *J* = 13.1 Hz, 2H), 2.69 (s, 3H), 2.44 – 2.36 (m, 2H), 2.40 (s, 3H), 2.30 – 2.22 (m, 2H), 2.06 – 1.99 (m, 2H), 1.97 – 1.89 (m, 1H), 1.88 – 1.73 (m, 4H), 1.67 (s, 3H), 1.35 (hept, *J* = 7.3 Hz, 2H), 1.20 – 1.14 (m, 12H), 0.85 – 0.78 (m, 2H). **¹³C NMR** (125.7 MHz, CDCl₃) δ 171.2, 164.1, 157.2, 155.9, 150.1, 136.9, 136.6, 132.1, 131.1, 131.0, 130.7, 130.0, 128.9, 84.3, 69.1, 67.9, 54.4, 53.6, 41.2, 40.3, 39.9, 39.0, 37.3, 26.9, 25.1, 24.5, 23.9, 19.8, 19.7, 18.9, 14.5, 13.2, 12.3, 11.9, 6.2. **HRMS** (ESI⁺) calculated for [C₄₀H₄₄N₆O₃ClSi₃H]⁺ required *m/z* 827.3034, found *m/z* 827.3034.

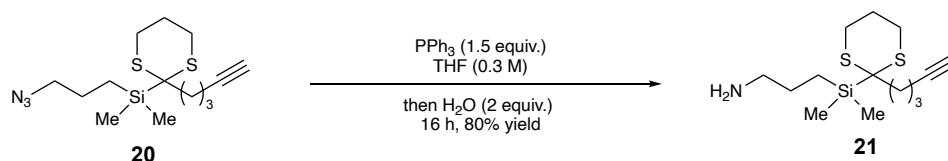


Acyl silane 17: To a solution of **SI-7** (32 mg, 0.0387 mmol, 1 equiv.) and CaCO₃ (38.7 mg, 0.387 mmol, 10 equiv.) in CH₃CN:H₂O (9:1, 0.05 M, 0.8 mL) cooled in an ice bath to 4 °C was added PIFA (17.5 mg, 0.0407 mmol, 1.05 equiv.) dropwise in CH₃CN (0.1 mL). The solution was stirred for 30 minutes in the ice bath before dilution with EtOAc. The resulting mixture was washed with sat. aqueous NaHCO₃, brine, and dried over MgSO₄ before concentrating *in vacuo*. The crude material was purified by flash column chromatography using a gradient elution of 99.5:0.05 – 96:4 DCM:MeOH to give **17** as a white solid (23 mg, 81% yield).

¹H NMR (500.0 MHz, CDCl₃) δ 7.59 (t, *J* = 5.6 Hz, 1H), 7.39 (d, *J* = 8.3 Hz, 2H), 7.31 (d, *J* = 8.4 Hz, 2H), 5.91 (t, *J* = 5.5 Hz, 1H), 4.70 (t, *J* = 7.1 Hz, 1H), 4.03 – 3.97 (m, 2H), 3.59 (dd, *J* = 14.7, 7.7 Hz, 1H), 3.52 – 3.46 (m, 1H), 3.43 (dd, *J* = 14.7, 6.5 Hz, 1H), 3.40 – 3.25 (m, 3H), 2.71 (t, *J* = 7.0 Hz, 2H), 2.69 (s, 3H), 2.41 (s, 3H), 2.19 (td, *J* = 6.9, 2.6 Hz, 2H), 1.96 (t, *J* = 2.6 Hz, 1H), 1.73 (p, *J* = 6.9 Hz, 2H), 1.67 (s, 3H), 1.20 (m, 1H), 1.05 (d, *J* = 4.8 Hz, 7H), 1.03 (d, *J* = 4.9 Hz, 6H), 0.82 – 0.75 (m, 2H). **¹³C NMR** (125.7 MHz, CDCl₃) δ 246.6, 170.8, 165.1, 157.1, 155.3, 150.3, 137.7, 135.5, 132.5, 131.8, 130.4, 128.9, 84.0, 69.1, 67.4, 54.1, 49.5, 41.1, 40.0, 38.4, 23.7, 20.6, 18.3, 18.2, 17.9, 14.6, 13.3, 11.9, 10.7, 4.6. **HRMS** (ESI⁺) calculated for [C₃₇H₄₉N₆O₄ClSSi₃H]⁺ required *m/z* 737.3072, found *m/z* 737.3075.

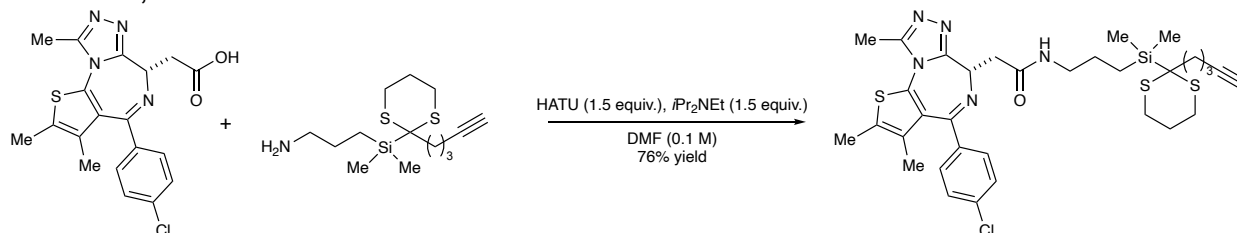


(3-azidopropyl)dimethyl(2-(pent-4-yn-1-yl)-1,3-dithian-2-yl)silane (20): To a solution of triphenylphosphine (0.393 g, 1.5 mmol, 1.5 equiv.) in THF (6 mL) at 0 °C was added DIAD (0.316 mL, 1.6 mmol, 1.6 equiv.). After stirring for 10 minutes, alcohol **13a** (0.302 g, 1.00 mmol, 1.0 equiv.) was added to the cloudy white suspension and allowed to stir at 0 °C for 30 minutes, at which point diphenylphosphoryl azide (0.346 mL, 1.6 mmol, 1.6 equiv.) was added dropwise and the reaction was allowed to stir overnight. After 16h, the reaction was quenched by addition of H₂O, extracted thrice with EtOAc, and concentrated to an oil. The crude residue was purified by flash column chromatography (hex:EtOAc = 93:7) to give **20** (220 mg, 67% yield) as a clear oil which contained 92% product by mass. **¹H NMR** (500 MHz, CDCl₃) δ 3.26 (td, *J* = 7.0, 1.4 Hz, 2H), 3.11 – 3.01 (m, 2H), 2.48 – 2.39 (m, 2H), 2.38 – 2.29 (m, 2H), 2.05 (dddt, *J* = 12.9, 4.5, 3.0, 1.5 Hz, 1H), 1.99 (td, *J* = 2.6, 1.1 Hz, 1H), 1.96 – 1.83 (m, 1H), 1.76 – 1.64 (m, 4H), 0.85 – 0.76 (m, 2H), 0.19 (d, *J* = 1.4 Hz, 6H). **¹³C NMR** (125.7 MHz, CDCl₃): δ 84.17, 68.85, 54.43, 38.46, 36.38, 26.59, 25.06, 23.89, 23.35, 18.79, 11.07, -4.37. **HRMS** (EI⁺) calculated for [C₁₄H₂₅N₃S₂Si]⁺ required *m/z* 327.1259, found *m/z* 327.1259.



3-(dimethyl(2-(pent-4-yn-1-yl)-1,3-dithian-2-yl)silyl)propan-1-amine (21) To a solution of **20** (110 mg, 0.336 mmol, 1.0 equiv.) in THF (1 mL) cooled to 0 °C was added triphenylphosphine (132 mg, 0.505 mmol, 1.5 equiv.) and the mixture was allowed to stir for 1 hour at room temperature before adding H₂O (10 uL, 0.672 mmol, 2.0 equiv.) and the reaction was allowed to stir for 16 hours. The mixture was concentrated and purified by flash column chromatography using a gradient elution of 98:2 – 8:2 DCM:MeOH to give **21** (88 mg, 80% yield) as a clear oil.

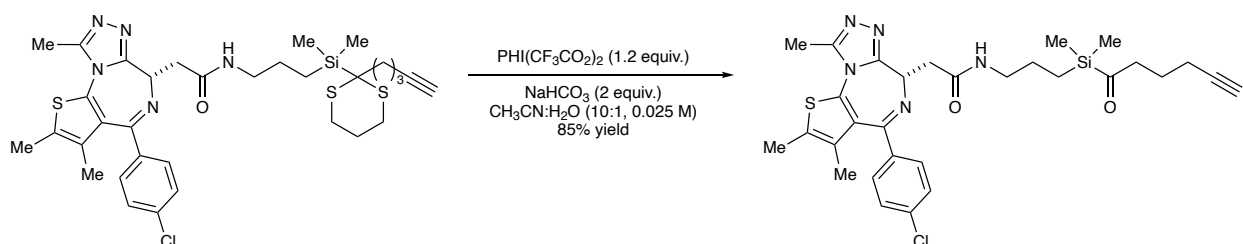
¹H NMR (500 MHz, CDCl₃) δ 3.06 (ddd, *J* = 14.8, 12.6, 2.7 Hz, 2H), 2.76 – 2.70 (m, 2H), 2.43 (dt, *J* = 14.2, 3.8 Hz, 2H), 2.38 – 2.32 (m, 2H), 2.26 (td, *J* = 6.7, 2.7 Hz, 5H), 2.10 – 2.01 (m, 1H), 1.99 (t, *J* = 2.6 Hz, 1H), 1.90 (qt, *J* = 13.0, 3.4 Hz, 1H), 1.71 (dq, *J* = 11.8, 6.9 Hz, 2H), 1.54 (ddd, *J* = 16.6, 11.1, 5.9 Hz, 2H), 0.80 – 0.73 (m, 2H), 0.18 (s, 6H). **¹³C NMR** (125.7 MHz, CDCl₃): δ 84.24, 68.83, 45.09, 38.60, 36.34, 27.49, 26.59, 25.10, 23.33, 18.80, 10.68, -4.37. **HRMS** (ESI⁺) calculated for [C₁₄H₂₇NS₂Si+H]⁺ required *m/z* 302.1247, found *m/z* 302.1243.



Dithiane precursor to acyl silane 16Me SI-8. To a solution of (+)-JQ1-OH (26 mg, 0.0625 mmol, 1.0 equiv.) in a two-dram vial was added DMF (0.625 mL, 0.1 M), HATU (35.6 mg, 0.0938 mmol, 1.5 equiv.), and *i*-Pr₂NEt₂ (16 μL, 0.0938 mmol, 1.5 equiv.). After 10 min, **21** was added (19.7 mg, 0.0656 mmol, 1.05 equiv.) and the reaction was allowed

to stir for 16 hours before removing the DMF *in vacuo* and purifying by silica gel chromatography using a gradient elution of 99:1 – 96:4 DCM:MeOH. The product was isolated as a white solid (33 mg, 76% yield).

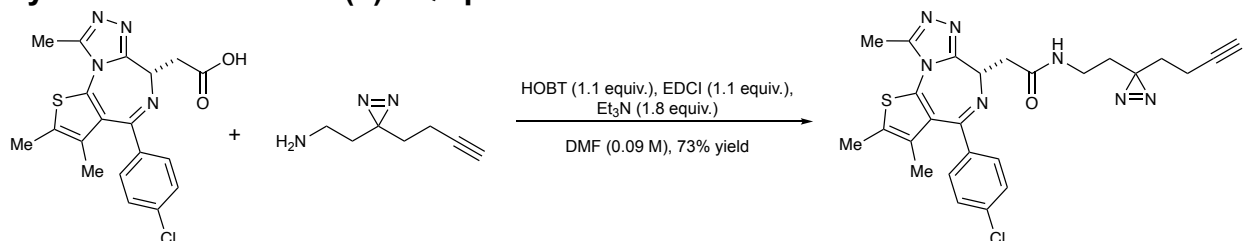
¹H NMR (600 MHz, CDCl₃) δ 7.40 (dd, *J* = 8.7, 3.6 Hz, 2H), 7.32 (d, *J* = 8.6 Hz, 2H), 6.57 (t, *J* = 5.8 Hz, 1H), 4.62 (t, *J* = 6.9 Hz, 1H), 3.71 (pd, *J* = 6.7, 4.1 Hz, 1H), 3.56 – 3.49 (m, 1H), 3.33 (dt, *J* = 13.1, 6.5 Hz, 2H), 3.22 (dt, *J* = 12.7, 6.3 Hz, 1H), 3.17 (qd, *J* = 7.4, 4.2 Hz, 1H), 3.09 – 3.01 (m, 2H), 2.66 (s, 3H), 2.45 – 2.40 (m, 2H), 2.39 (s, 3H), 2.37 – 2.30 (m, 2H), 2.26 (td, *J* = 6.7, 2.7 Hz, 2H), 2.08 – 2.04 (m, 1H), 2.01 (t, *J* = 2.6 Hz, 1H), 1.90 (qt, *J* = 12.7, 3.3 Hz, 1H), 1.74 – 1.67 (m, 2H), 1.66 (s, 3H), 1.65 – 1.57 (m, 2H), 1.44 (d, *J* = 6.6 Hz, 2H), 1.41 (d, *J* = 6.6 Hz, 2H), 0.80 – 0.72 (m, 2H), 0.17 (d, *J* = 1.6 Hz, 6H). **¹³C NMR** (151 MHz, CDCl₃) δ 170.48, 164.06, 155.80, 150.02, 136.94, 136.73, 132.25, 131.07, 131.01, 130.61, 129.96, 128.87, 84.35, 69.02, 55.63, 54.60, 43.62, 42.91, 39.52, 38.70, 36.47, 28.29, 26.70, 25.20, 24.44, 23.46, 18.90, 18.72, 17.33, 14.50, 13.21, 12.63, 11.94, 11.16, -4.21 (2 extraneous peaks). **HRMS** (ESI⁺): calculated for [C₃₃H₄₂ClN₅OS₃Si+H]⁺ required *m/z* 684.2082, found *m/z* 684.2094.



Acyl silane 16Me. To a solution of **SI-8** (5 mg, 0.00714 mmol, 1.0 equiv.) and NaHCO₃ (1.2 mg, 0.0413 mmol, 2.0 equiv.) in a solution of MeCN:H₂O (0.025 M, 270 uL:30 uL) was added PIFA (3.7 mg, 0.00857 mmol, 1.2 equiv.) and the resulting yellow solution was allowed to stir for 30 minutes, at which point the solvent was removed *in vacuo* and the residue was purified by flash column chromatography using a gradient elution of 99:1 – 97:3 DCM:MeOH to give **16Me** (3.7 mg, 85% yield) as a white powder.

¹H NMR (600 MHz, CDCl₃) δ 7.40 (d, *J* = 8.5 Hz, 2H), 7.33 (d, *J* = 8.7 Hz, 2H), 6.62 (t, *J* = 6.0 Hz, 1H), 4.60 (dd, *J* = 7.9, 6.1 Hz, 1H), 3.57 (dd, *J* = 14.1, 8.0 Hz, 1H), 3.36 – 3.19 (m, 3H), 2.74 (t, *J* = 7.1 Hz, 2H), 2.67 (s, 3H), 2.40 (s, 3H), 2.19 (td, *J* = 6.9, 2.7 Hz, 2H), 1.97 (t, *J* = 2.6 Hz, 1H), 1.74 (p, *J* = 7.0 Hz, 2H), 1.57 – 1.48 (m, 2H), 0.72 – 0.65 (m, 2H), 0.19 (d, *J* = 4.6 Hz, 6H). **¹³C NMR** (151 MHz, CDCl₃) δ 247.08, 170.54, 164.04, 155.75, 150.02, 136.94, 136.71, 132.23, 131.03, 130.97, 130.61, 129.92, 128.86, 83.90, 69.13, 54.68, 47.27, 42.50, 39.66, 29.80, 23.84, 22.79(?), 20.75, 17.89, 14.47, 13.19, 11.91, 10.71, -4.74. **HRMS** (ESI⁺) calculated for [C₃₀H₃₆ClN₅O₂SSi+H]⁺ required *m/z* 594.2121, found *m/z* 594.2127.

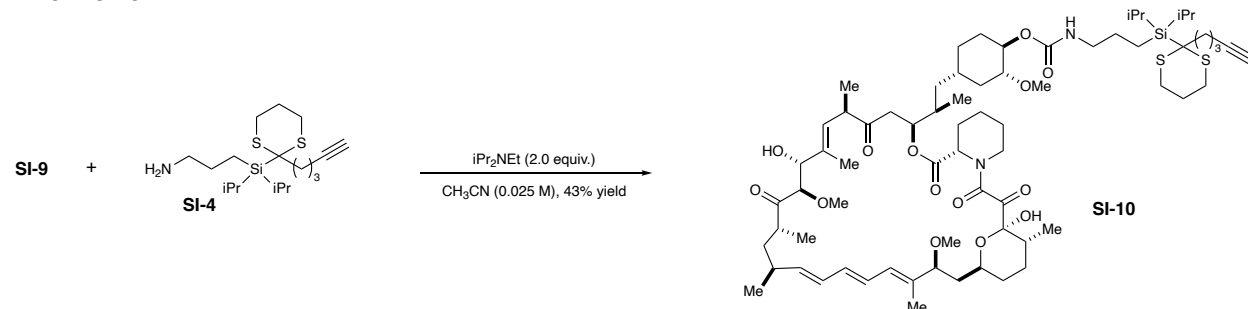
Synthesis of diazirine (+)-JQ1 probe



¹H NMR (500 MHz, CDCl₃) δ 7.41 (d, *J* = 8.2 Hz, 2H), 7.34 (d, *J* = 8.4 Hz, 2H), 6.89 (t, *J* = 6.0 Hz, 1H), 4.63 (dd, *J* = 7.8, 6.0 Hz, 1H), 3.61 (dd, *J* = 14.3, 7.8 Hz, 1H), 3.37 (dd, *J* = 14.3, 6.1 Hz, 1H), 3.17 (ddt, *J* = 16.8, 13.9, 7.0 Hz, 2H), 2.68 (s, 3H), 2.41 (s, 3H), 2.05 – 1.95 (m, 3H), 1.68 (s, 3H), 1.72 – 1.58 (m, 4H). **¹³C NMR** (126 MHz, CDCl₃) δ 170.62, 164.15, 137.05, 136.55, 131.11, 131.09, 130.62, 130.00, 128.87, 82.86, 69.51, 54.58, 39.43, 34.57, 32.77, 32.14, 31.71, 26.89, 22.78, 14.52, 14.25, 13.36, 13.23, 11.93, 1.14. **HRMS** (ESI⁺): calculated for [C₂₆H₂₆ClN₇OS+Na]⁺: required *m/z* 542.1500, found *m/z* 542.1502.

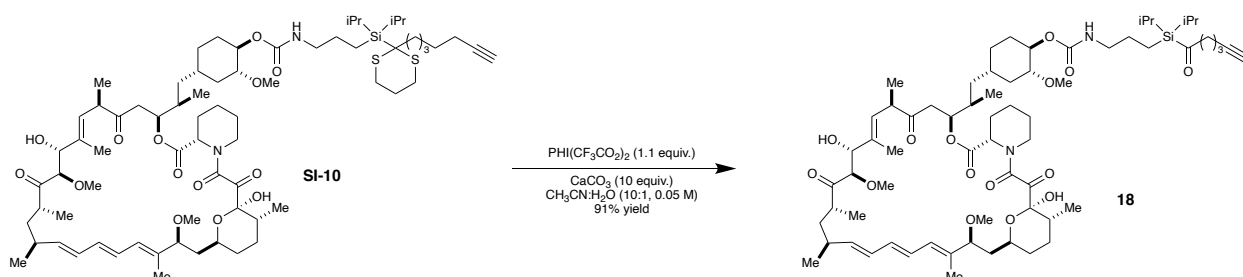
Chemical reaction scheme showing the synthesis of SI-9. The starting material is a complex macrocyclic compound with multiple stereocenters, including a piperidine ring and a cyclohexanol derivative. It reacts with 4-nitrobenzoyl chloride (4.0 equiv.) in the presence of pyridine (10 equiv.) in CH_2Cl_2 (0.04 M). The product, SI-9, is a macrocyclic compound where the cyclohexanol derivative has been converted to a 4-nitrobenzoate ester.

HRMS (ESI⁺) calculated for [C₅₈H₈₂N₂O₁₇+Na]⁺ required *m/z* 1101.5511, found *m/z* 1101.5491.



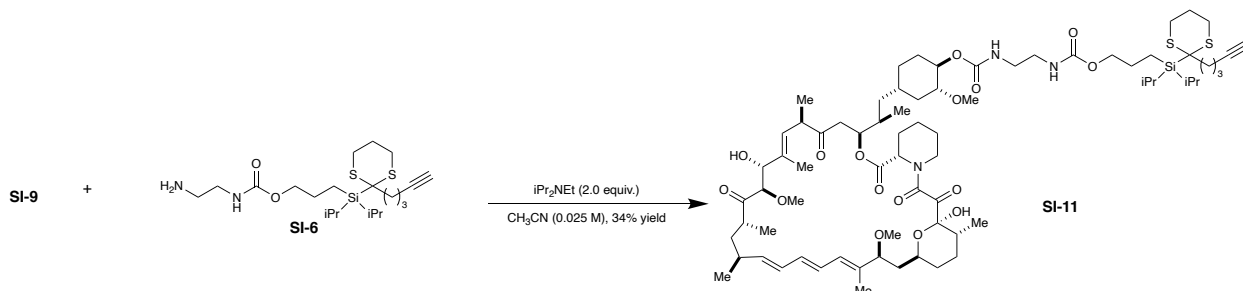
Dithiane rapamycin precursor (SI-10): A two-dram vial was charged with **SI-9** (32 mg, 0.0300 mmol, 1 equiv.), freshly prepared **SI-4** (14 mg, 0.0306 mmol, 1.3 equiv.), *i*-Pr₂NEt (10.4 μ L, 0.0600 mmol, 2.0 equiv.) and CH₃CN (0.6 mL, 0.05 M). The reaction was stirred for 16 hours before diluted with EtOAc. The solution was washed with 0.5 M HCl cooled in an ice bath and brine, before drying over NaSO₄ and concentrating *in vacuo*. The crude material was purified by flash column chromatography using a gradient of 99:1 – 96:4 CH₂Cl₂:MeOH. The product was isolated as a white solid (20 mg, 53% yield). For NMR data see Table **S2.1**.

HRMS (ESI⁺) calculated for [C₇₀H₁₁₂N₂O₁₄S₂Si+Na]⁺ required *m/z* 1319.7222, found *m/z* 1319.7217.



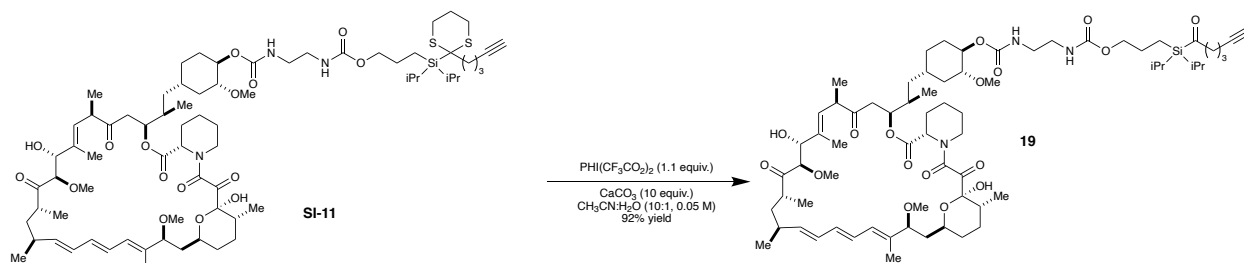
Rapamycin acyl silane (18): To a solution of **SI-10** (20 mg, 0.0154 mmol, 1 equiv.) and CaCO₃ (16 mg, 0.154 mmol, 10 equiv.) in CH₃CN:H₂O (9:1, 0.025 M, 0.62 mL) cooled in an ice bath to 4 °C was added PIFA (7.0 mg, 0.0163 mmol, 1.06 equiv.) dropwise in CH₃CN (0.1 mL). The solution was stirred for 30 minutes in the ice bath before dilution with EtOAc. The resulting mixture was washed with sat. aqueous NaHCO₃, brine, and dried over MgSO₄ before concentrating *in vacuo*. The crude material was purified by flash column chromatography using a gradient elution of 99:1 – 96:4 DCM:MeOH to give **18** as a white solid (17 mg, 91% yield). For NMR data see Table **S2.1**.

HRMS (ESI⁺) calculated for [C₆₇H₁₀₆N₂O₁₅Si+Na]⁺ required *m/z* 1229.7260, found *m/z* 1229.7241.



Dithiane rapamycin precursor (SI-11): A two-dram vial was charged with **SI-9** (50 mg, 0.0463 mmol, 1 equiv.), freshly prepared **SI-6** (22.2 mg, 0.0510 mmol, 1.1 equiv.), *i*-Pr₂NEt (16.2 μ L, 0.0930 mmol, 2.0 equiv.) and CH₃CN (1.85 mL, 0.025 M). The reaction was stirred for 16 hours before diluted with EtOAc. The solution was washed with 0.5 M HCl cooled in an ice bath and brine, before drying over NaSO₄ and concentrating *in vacuo*. The crude material was purified by flash column chromatography using a gradient of 99:1 – 96:4 DCM:MeOH. The product was isolated as a white solid (22 mg, 34% yield). For NMR data see Table **S2.1**.

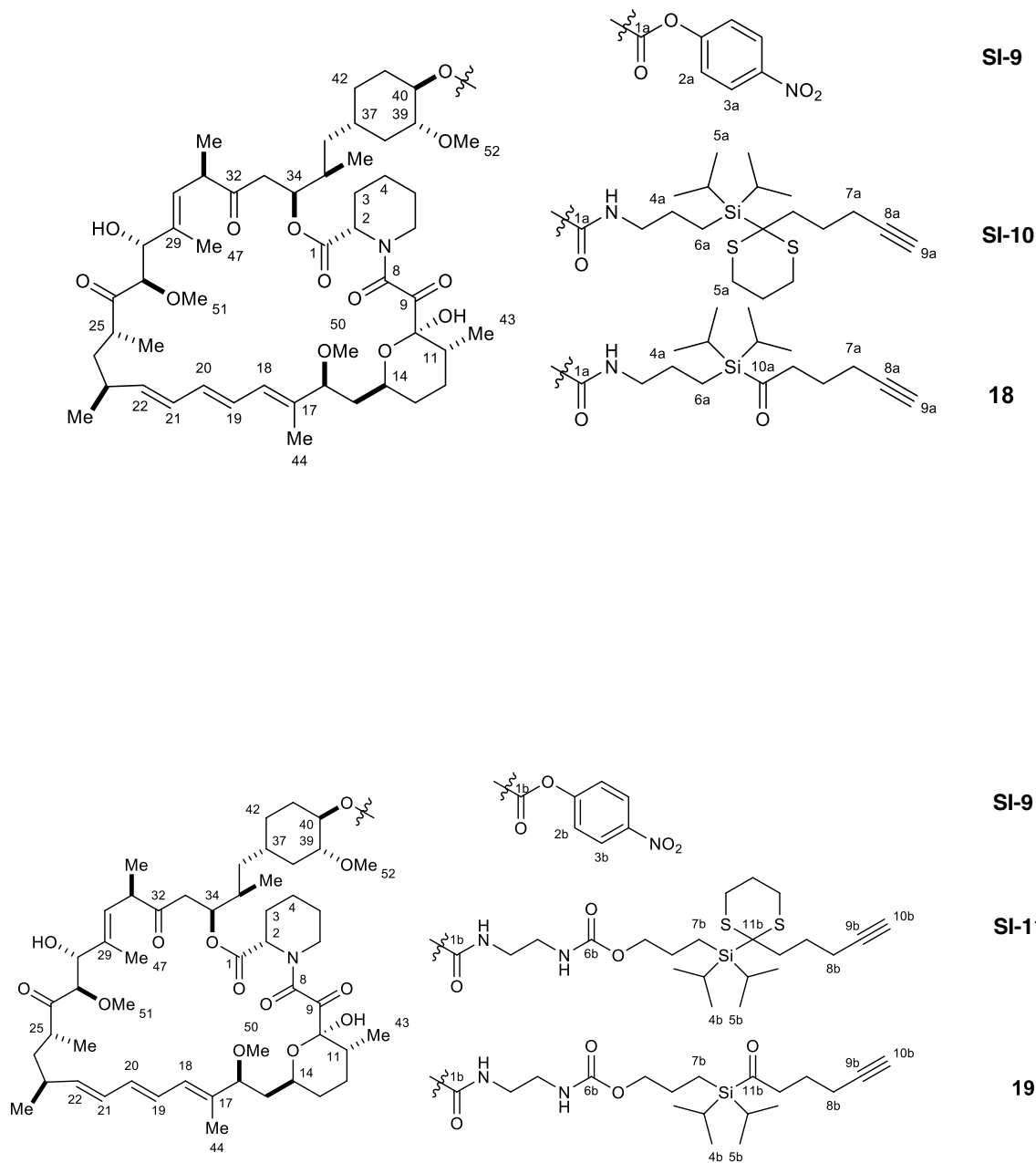
HRMS (ESI⁺) calculated for [C₇₃H₁₁₇N₃O₁₆S₂Si+Na]⁺ required *m/z* 1406.7542, found *m/z* 1406.7537.



Rapamycin acyl silane (19): To a solution of **SI-11** (22 mg, 0.0152 mmol, 1 equiv.) and CaCO₃ (15.2 mg, 0.152 mmol, 10 equiv.) in CH₃CN:H₂O (9:1, 0.05 M, 0.3 mL) cooled in an ice bath to 4 °C was added PIFA (6.8 mg, 0.0159 mmol, 1.05 equiv.) dropwise in CH₃CN (0.1 mL). The solution was stirred for 30 minutes in the ice bath before dilution with EtOAc. The resulting mixture was washed with sat. aqueous NaHCO₃, brine, and dried over MgSO₄ before concentrating *in vacuo*. The crude material was purified by flash column chromatography using a gradient elution of 99:1 – 96:4 DCM:MeOH to give **19** as a white solid (17 mg, 92% yield). For NMR data see Table **S2.1**.

HRMS (ESI⁺) calculated for [C₇₁H₁₁₄N₃O₁₇Si+Na]⁺ required *m/z* 1316.7580, found *m/z* 1316.7569.

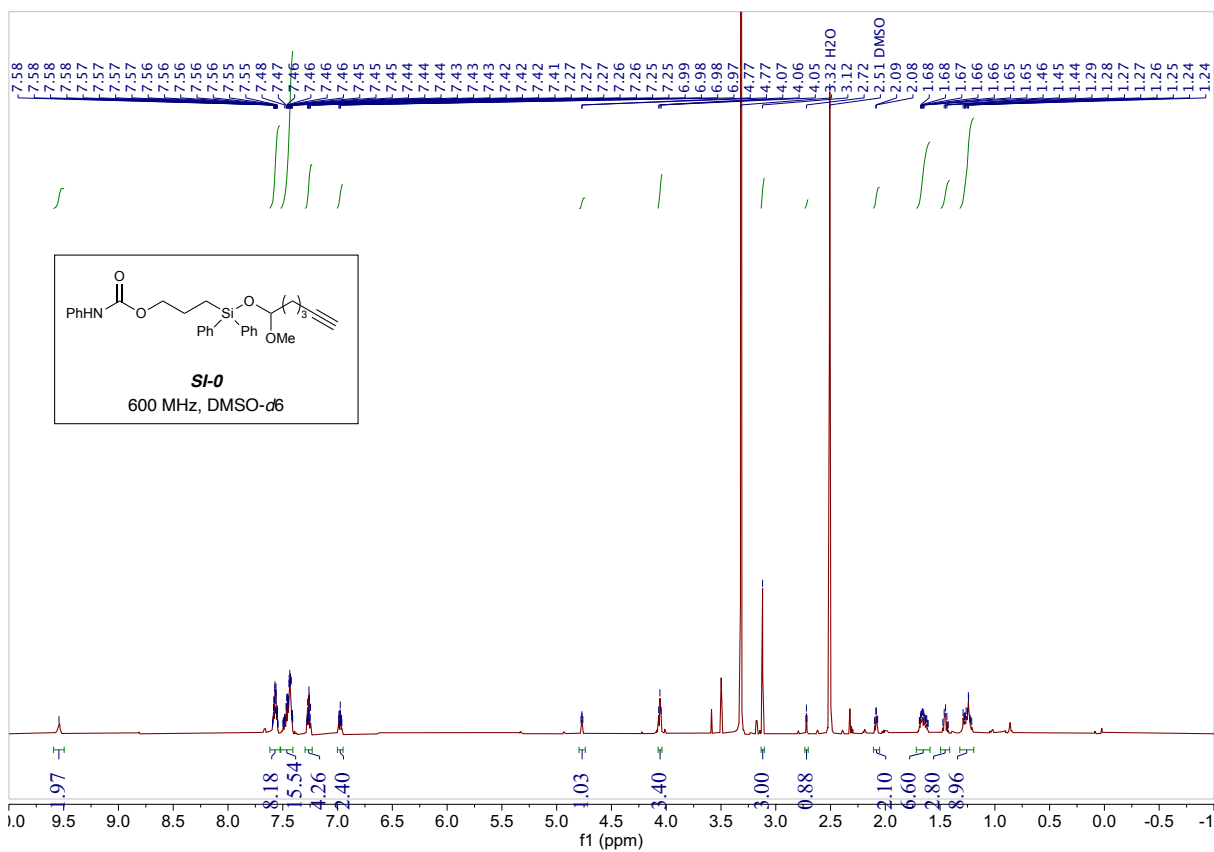
Table S2.1: Structural assignment for rapamycin probes:



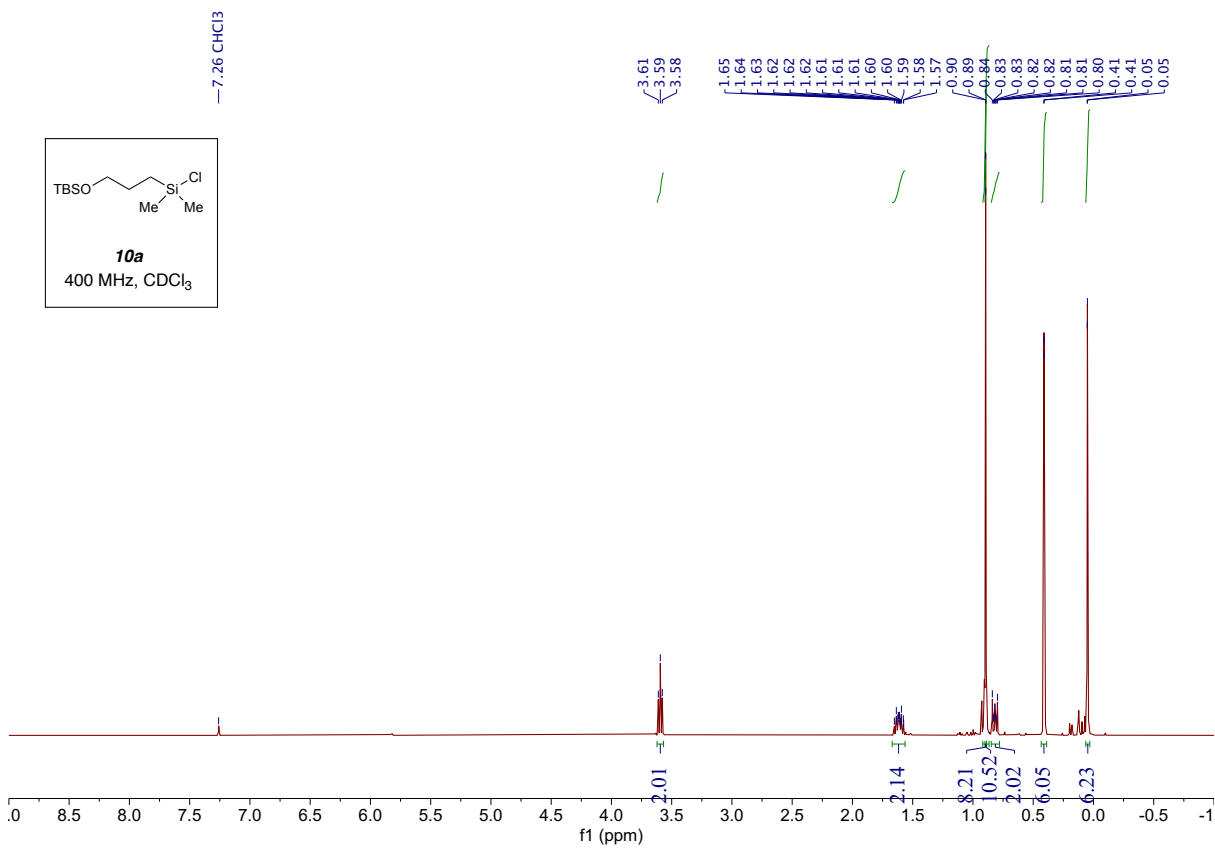
	SI-9		Dithiane precursor SI-10		Acyl silane 18	
Carbon #	Major Carbon (δ)	Major Proton (δ)	Major Carbon (δ)	Major Proton (δ)	Major Carbon (δ)	Major Proton (δ)
1	169.3		169.3		169.4	
2	51.3	5.26	51.4	5.27	51.4	5.28
3	27.0	2.32	27.2	2.32	27.2	2.35
4	20.7		20.8		20.8	
6		3.63, 3.54		3.65, 3.56		3.66, 3.57
8	166.8		166.9		166.9	
9	192.8		192.7		192.6	
10	98.5		98.6		98.6	
11	??	1.95				
14	67.2	3.86	67.3	3.86	67.3	3.86
15		1.85		1.85		1.85
16	84.3	3.65	84.3	3.68	84.5	3.68
17	135.9		135.7		135.7	
18	129.4	5.94	129.7	5.96	129.7	5.97
19		6.36		6.38		6.39
20	133.5	6.27	133.7	6.30	133.8	6.31
21	130.3	6.11	130.3	6.13	130.3	6.14
22	140.0	5.50	140.2	5.54	140.3	5.55
26	215.1		215.4		215.6	
27	84.7	3.74	84.9	3.74	84.9	3.74
28		4.17		4.18		4.18
29	136.1		136.1		136.2	
30		5.38		5.41		5.42
31	46.6		46.7		46.8	
32	208.2		208.4		208.4	
33						
34	75.7	5.17	75.6	5.16	75.6	5.17
40		3.27				
44		1.62		1.65		1.65
47		1.74		1.75		1.75
50	55.9	3.10	56.0	3.13	56.0	3.14
51	59.2	3.30	59.4	3.32	59.5	3.33
52		3.41		3.37		3.38
1a	155.7		156.3		156.3	
2a	125.3	7.34				
3a	121.8	8.26				
4a			19.8	1.16	18.3	1.06
5a			19.7	1.15	18.2	1.04
6a			7.5		5.7	0.78
7a				2.28		2.20
8a			69.1		69.1	
9a			84.4	2.02	84.0	1.96
10a					246.6	

	SI-9		Dithiane precursor SI-11		Acyl silane 19	
Carbon #	Major Carbon (δ)	Major Proton (δ)	Major Carbon (δ)	Major Proton (δ)	Major Carbon (δ)	Major Proton (δ)
1	169.3		169.4		169.4	
2	51.3	5.26	51.4	5.27	51.4	5.28
3	27.0	2.32	27.2	2.32	27.2	2.35
4	20.7		20.8		20.8	
6		3.63, 3.54		3.66, 3.57		3.66, 3.57
8	166.8		166.9		166.9	
9	192.8		192.6		192.7	
10	98.5		98.6		98.6	
11		1.95				
14	67.2	3.86	67.3	3.86	67.3	3.86
15		1.83		1.85		1.85
16	84.3	3.65	84.5	3.68	84.5	3.68
17	135.9		135.7		135.7	
18	129.4	5.94	129.7	5.96	129.7	5.97
19		6.36		6.38		6.38
20	133.5	6.27	133.7	6.31	133.8	6.31
21	130.3	6.11	130.3	6.14	130.3	6.14
22	140.0	5.50	140.3	5.55	140.3	5.55
26	215.1		215.5		215.5	
27	84.7	3.74	84.9	3.73	84.9	3.73
28		4.17		4.18		4.19
29	136.1		136.2		136.2	
30		5.38		5.41		5.41
31	46.6		46.8		46.8	
32	208.2		208.4		208.4	
33						
34	75.4	5.17	75.6	5.16	75.6	5.17
40		3.27				
44		1.62		1.65		1.65
47		1.73		1.75		1.75
50	55.9	3.10	56.0	3.14	56.0	3.14
51	59.2	3.30	59.5	3.33	59.5	3.33
52		3.41		3.37		3.38
1b	155.7		156.7		156.7	
2b	125.3	7.34				
3b	121.8	8.26				
4b			19.8	1.18	18.3	1.05
5b			19.7	1.17	18.2	1.04
6b			157.3		157.2	
7b			6.4		4.7	0.78
8b				2.29		2.20
9b			69.1		69.1	
10b			84.3	2.02	84.0	1.96
11b					246.5	

2.8.7 NMR spectra

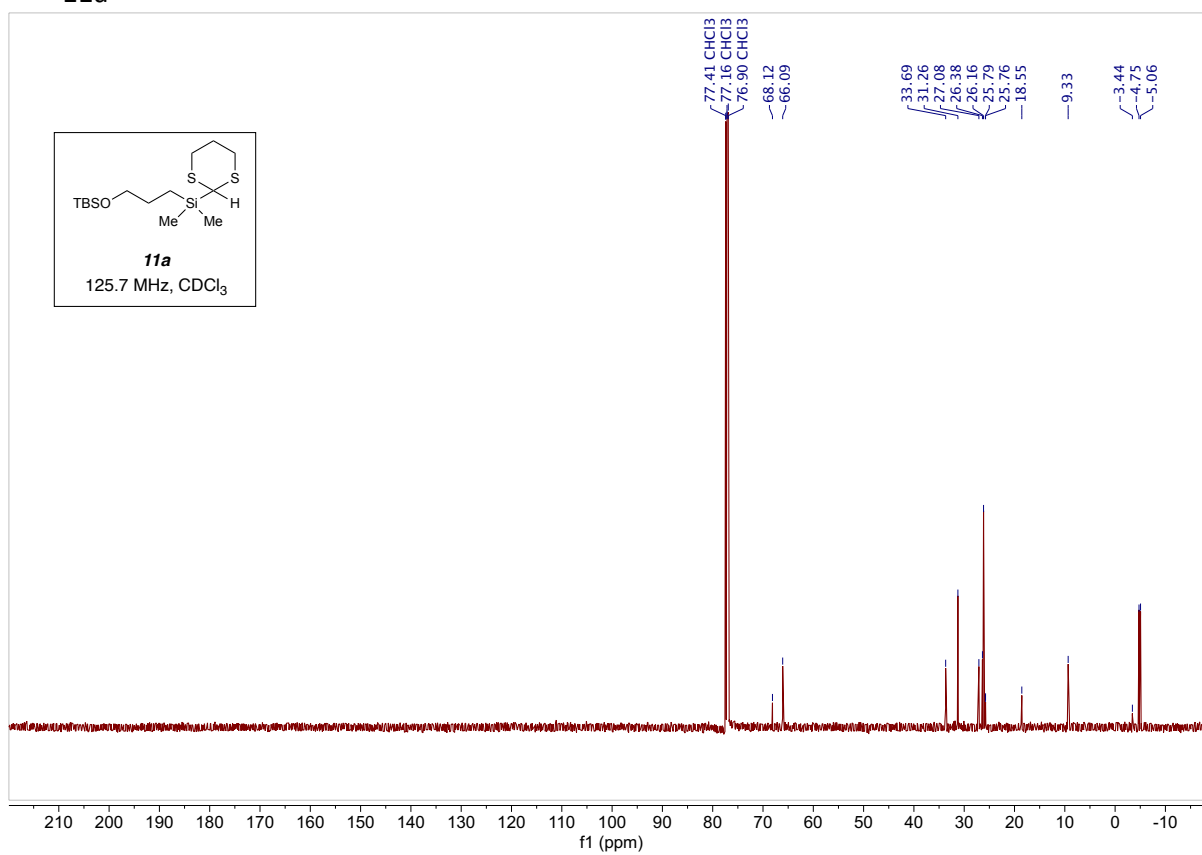


10a



[illegible]

11a



Chemical structure of **12a** is shown in the inset. The structure is a 1,3-dithiane derivative with a TMS group at position 2, a TBSO group at position 4, and a propyl chain at position 5. The spectrum is recorded in CDCl_3 at 500 MHz.

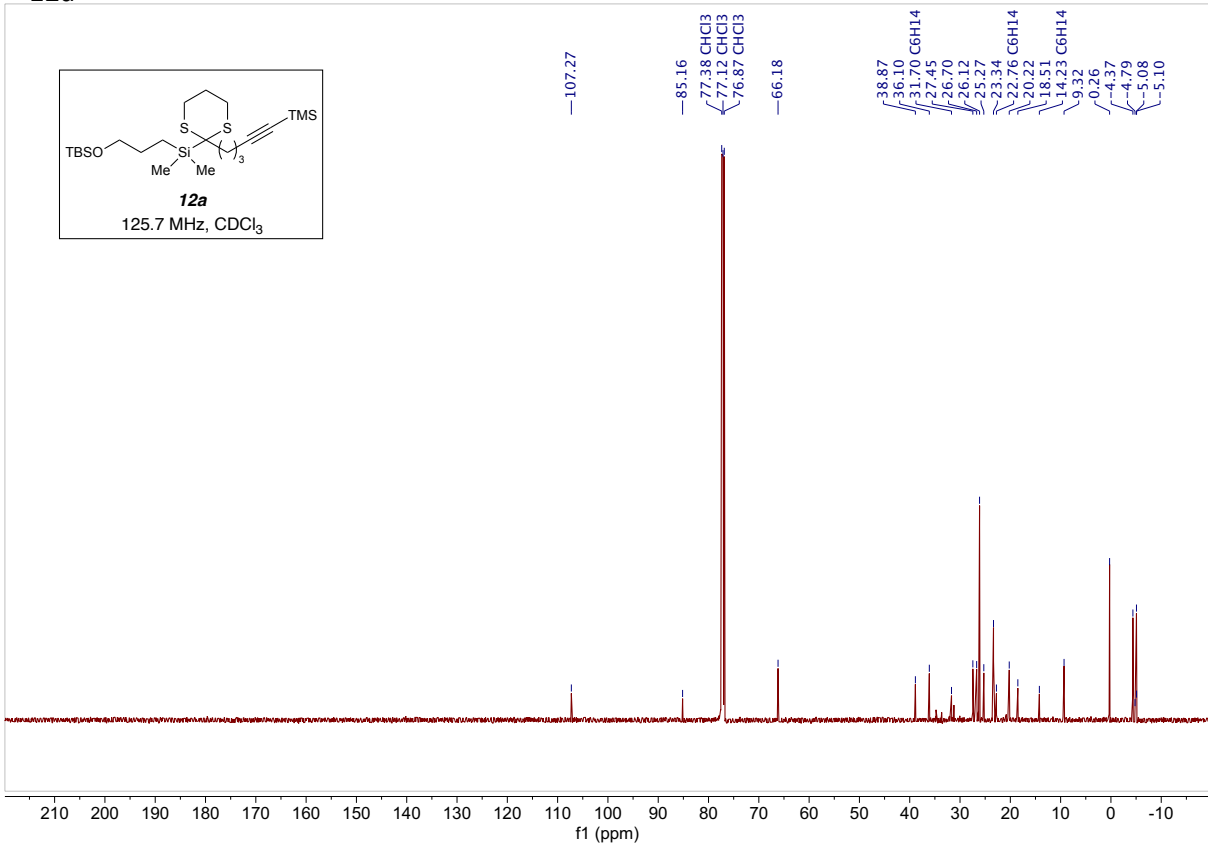
The ^1H NMR spectrum shows the following peaks (ppm) and integrations:

- 7.26 (s, 1H, integration 2.00)
- 3.59 (s, 1H, integration 1.80)
- 3.58 (s, 1H, integration 1.97)
- 3.57 (s, 1H, integration 1.79)
- 3.56 (s, 1H, integration 1.79)
- 3.55 (s, 1H, integration 1.08)
- 3.54 (s, 1H, integration 1.08)
- 3.53 (s, 1H, integration 2.16)
- 3.52 (s, 1H, integration 2.72)
- 3.51 (s, 1H, integration 2.59)
- 3.50 (s, 1H, integration 9.88)
- 3.49 (s, 1H, integration 1.78)
- 3.48 (s, 1H, integration 5.62)
- 3.47 (s, 1H, integration 8.63)
- 3.46 (s, 1H, integration 5.92)

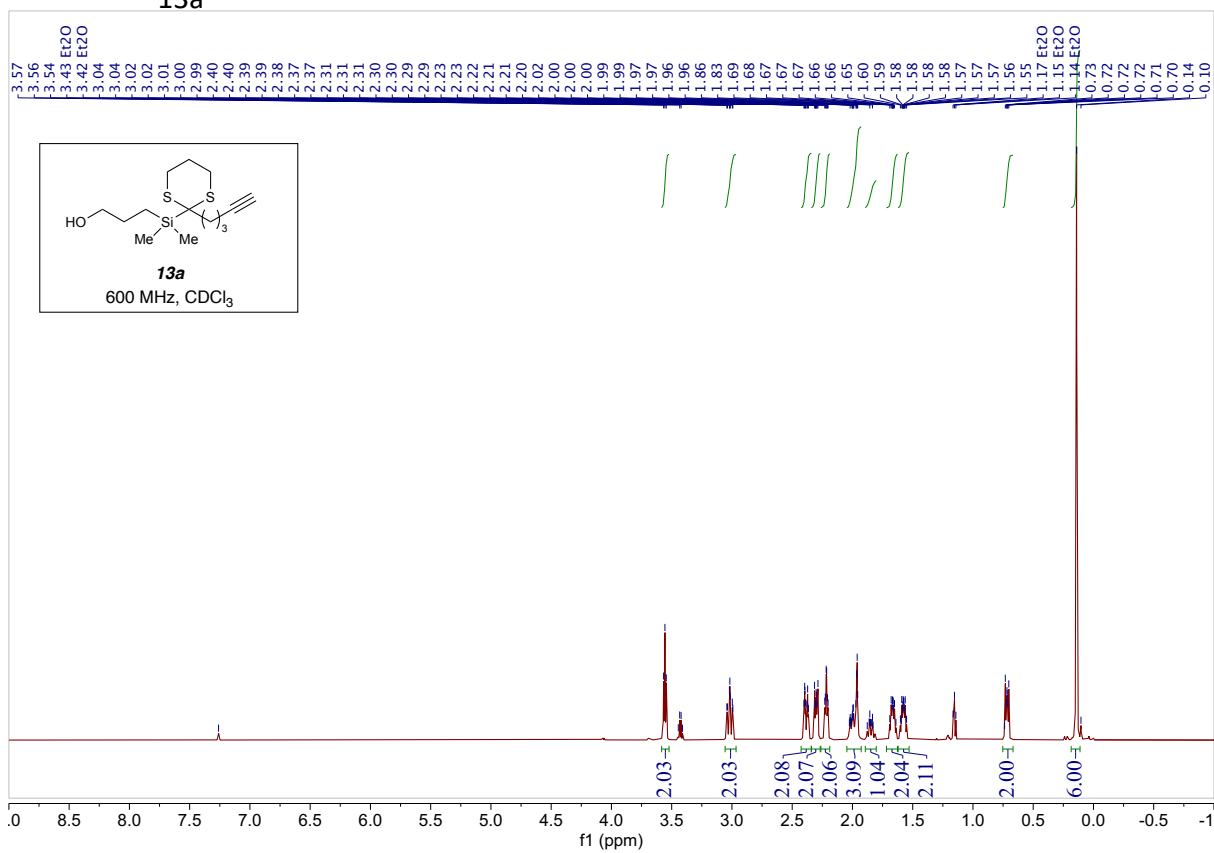
The chemical shifts (ppm) are listed on the right side of the spectrum:

- 7.26
- 3.59
- 3.58
- 3.57
- 3.56
- 3.55
- 3.54
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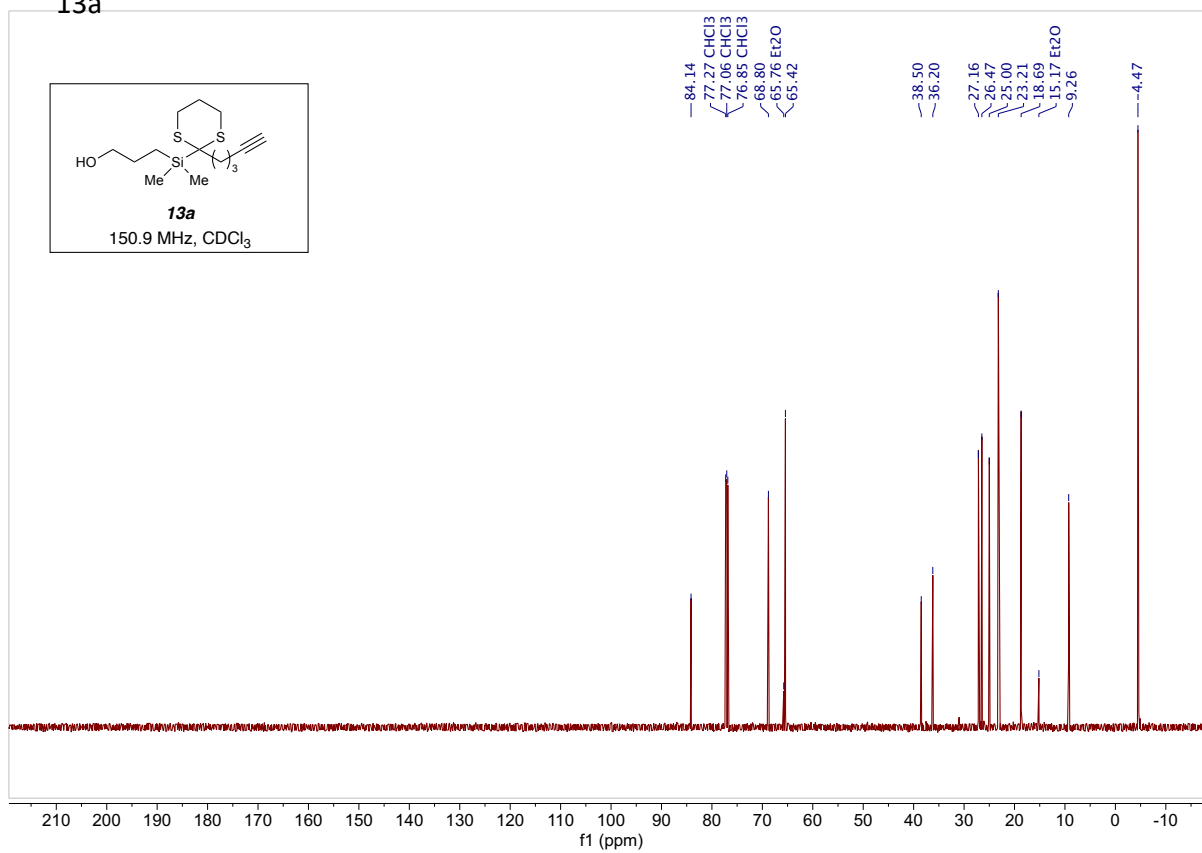
12a

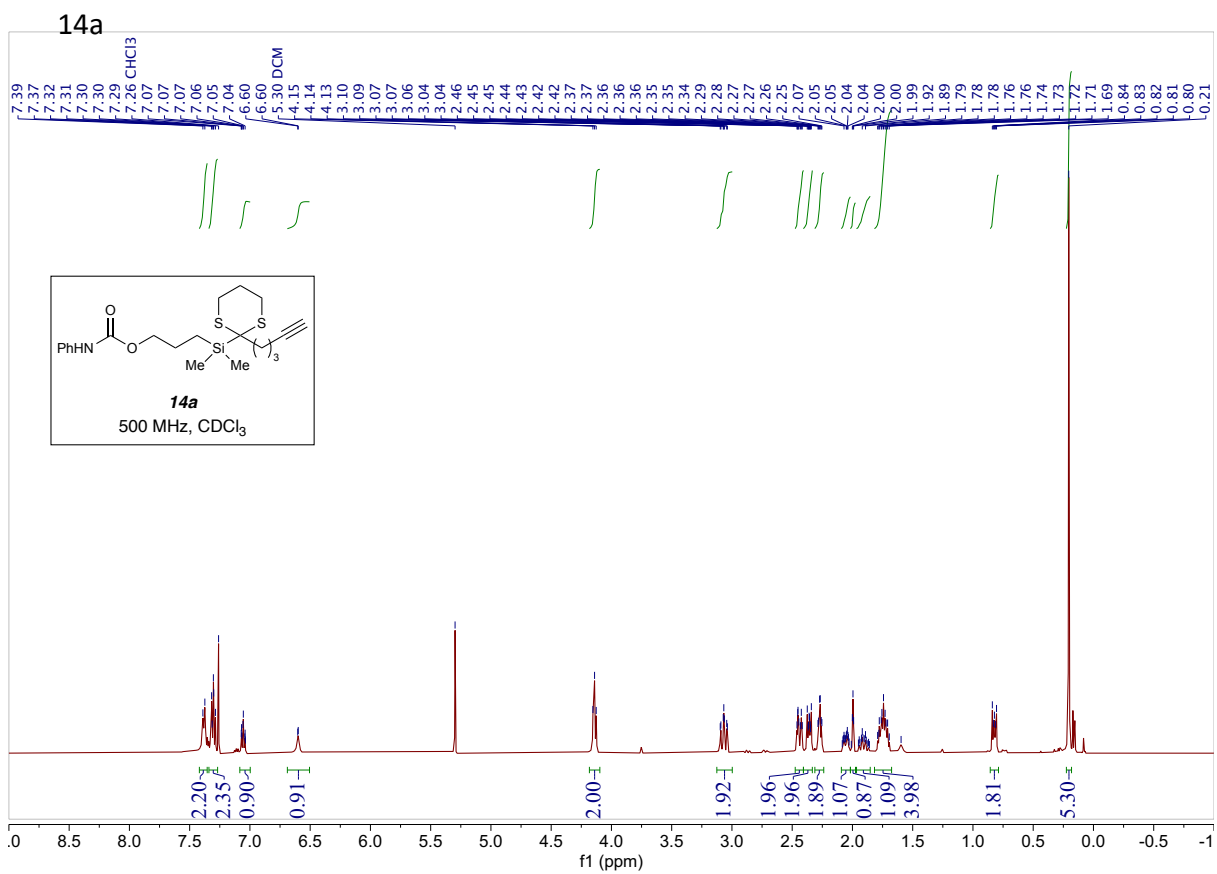


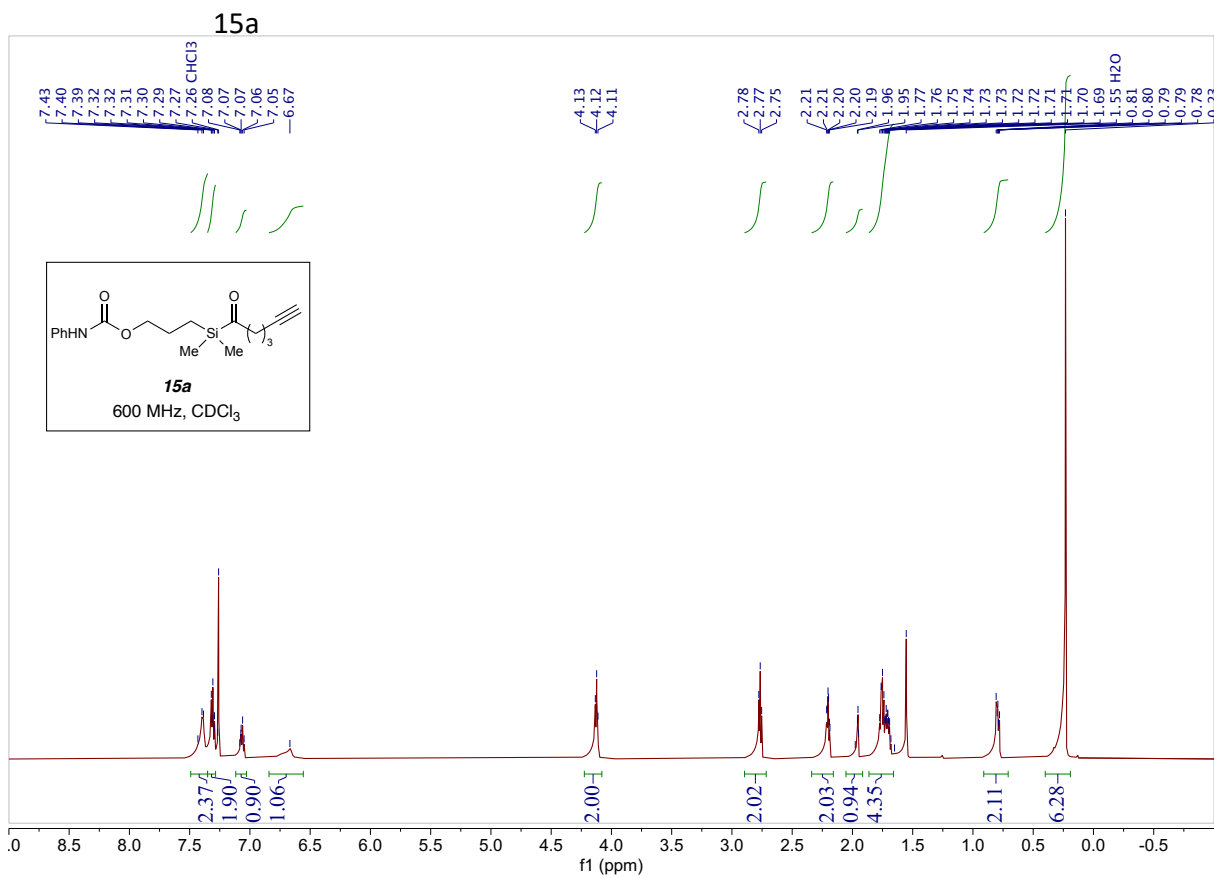
13a



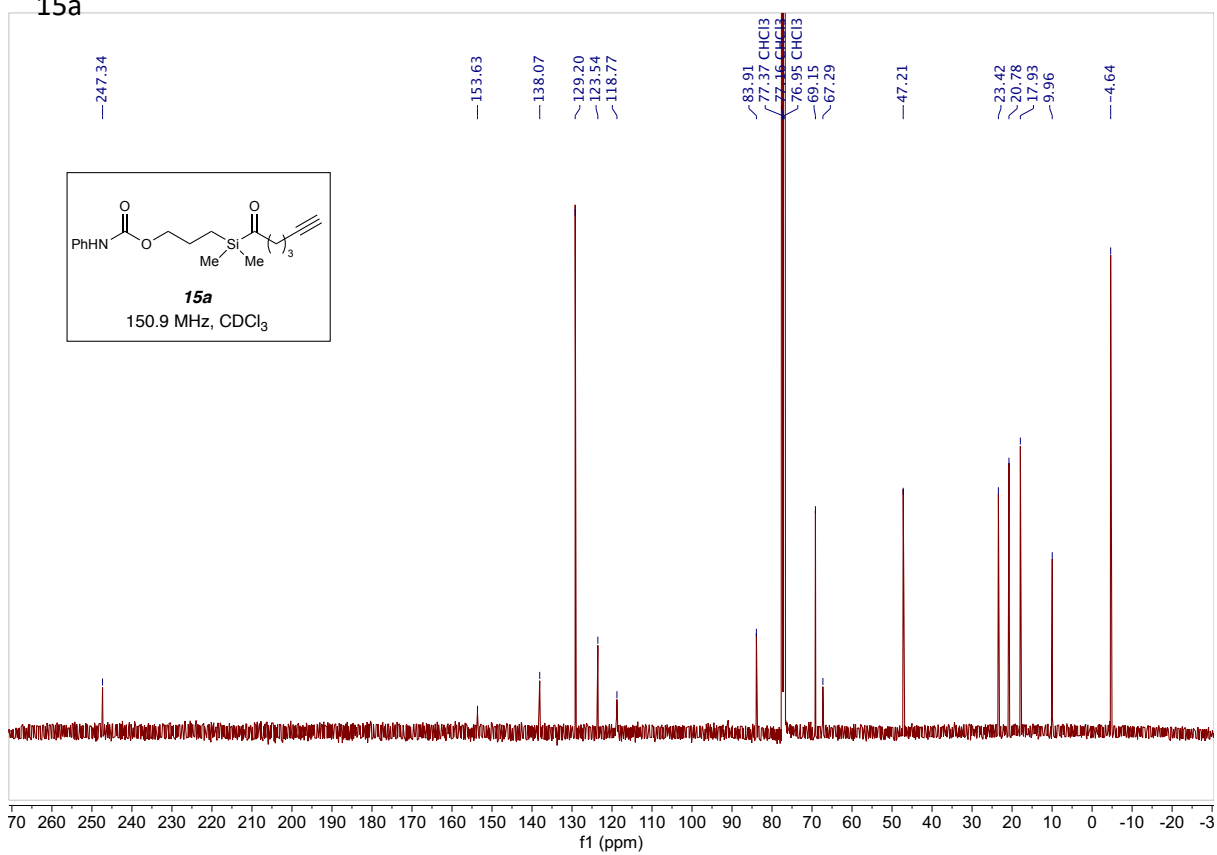
13a



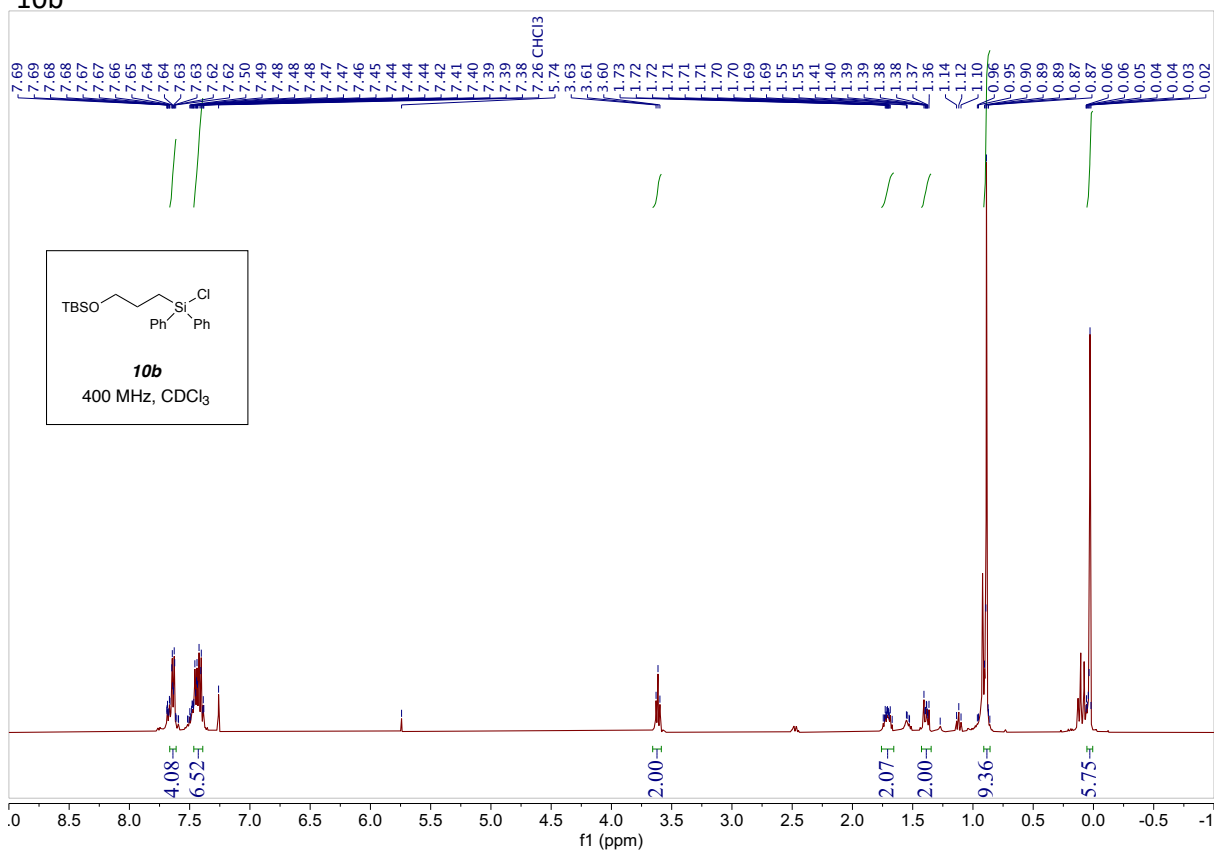




15a

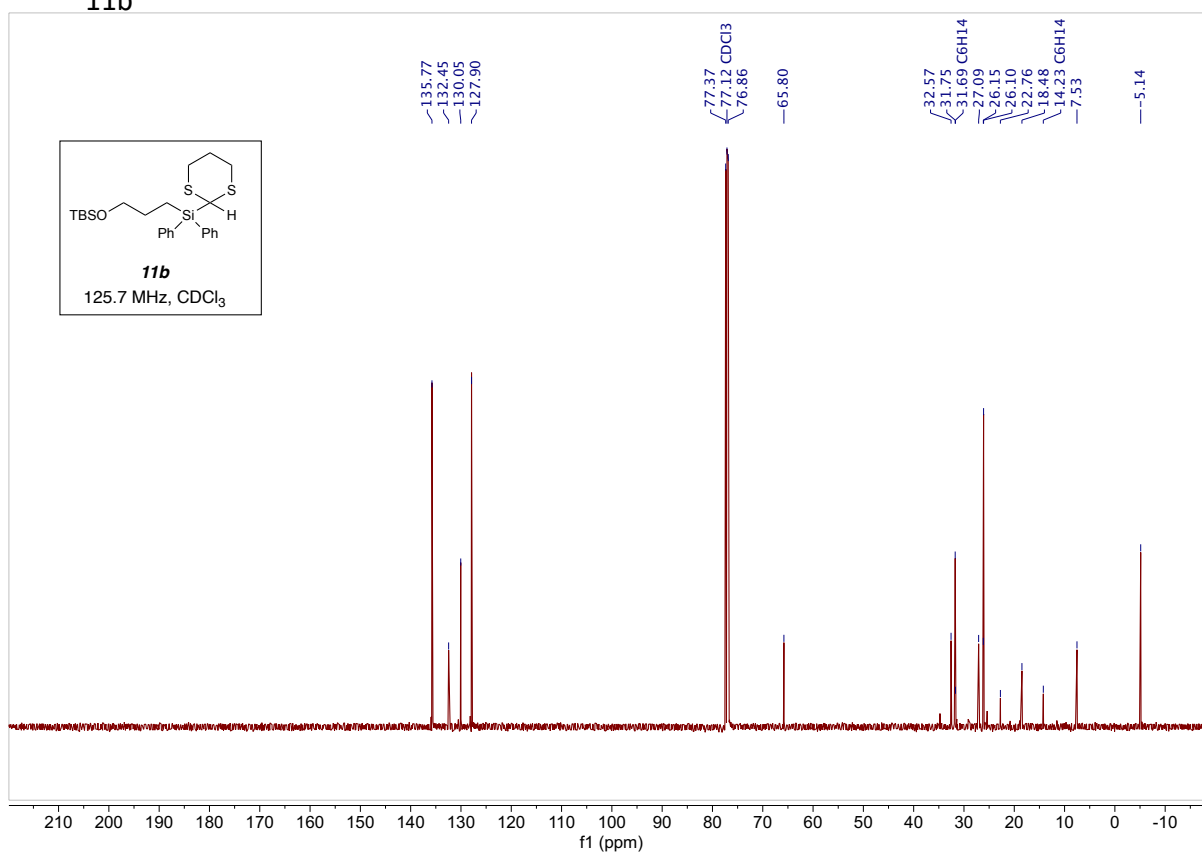


10b



[illegible]

11b



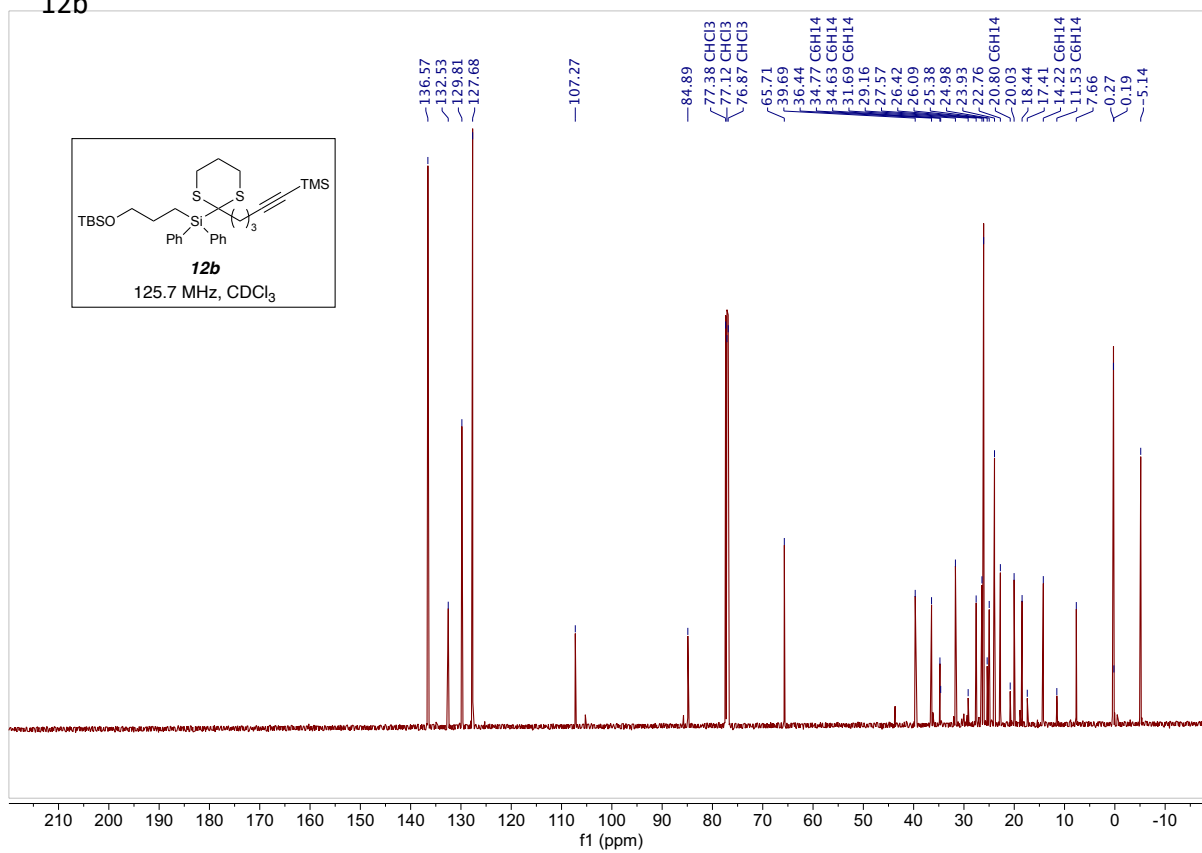
12b
500 MHz, CDCl₃

Chemical structure of **12b** is shown in the top left. It is a cyclohexane derivative with a TBSO group, a phenyl group, and a trimethylsilyl (TMS) group.

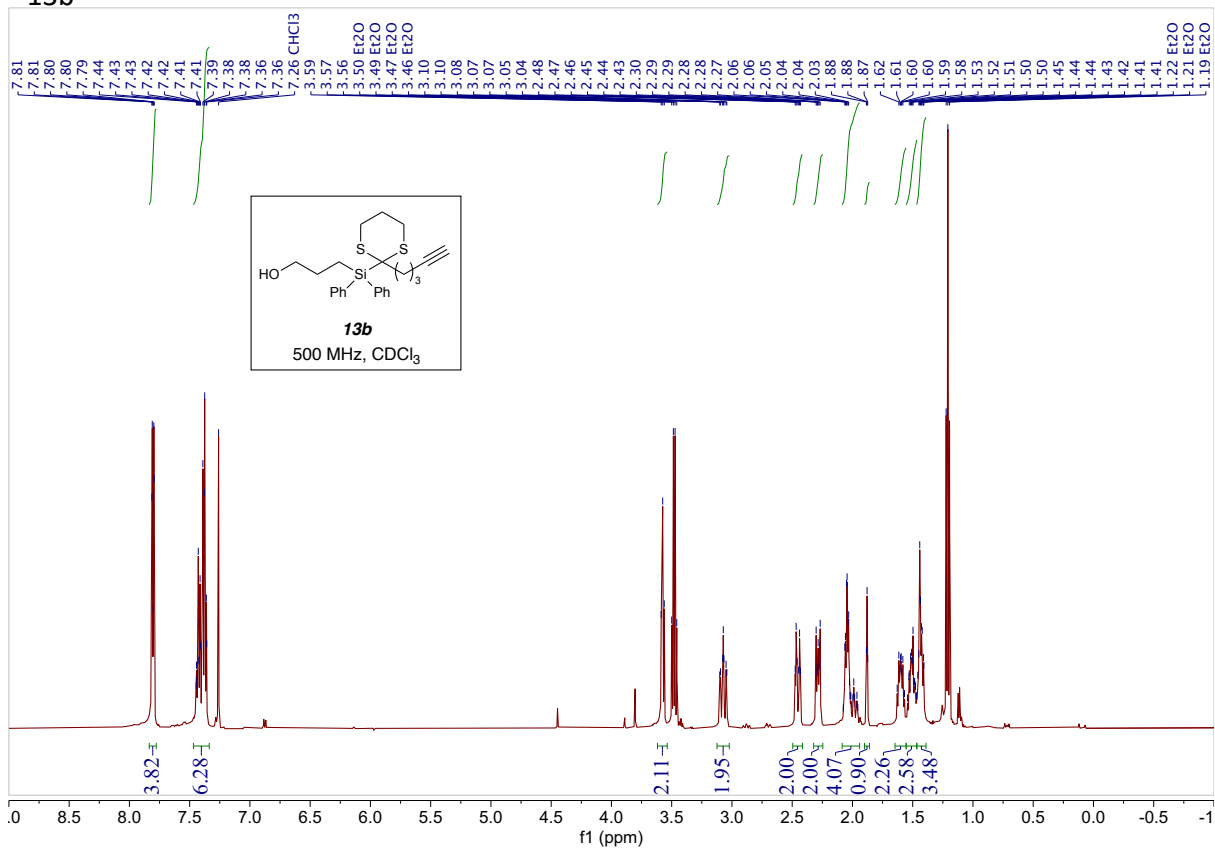
The ¹H NMR spectrum (500 MHz, CDCl₃) shows the following peaks (ppm) and integrations:

Chemical Shift (ppm)	Integration
7.83, 7.82, 7.81, 7.80, 7.43, 7.42, 7.41, 7.40, 7.38, 7.37, 7.36, 7.35, 7.26 (CHCl ₃)	4.00, 6.15
3.56, 3.55, 3.54, 3.53, 3.11, 3.10, 2.47, 2.46, 2.44, 2.43, 2.42, 2.31, 2.30, 2.29, 2.28, 2.27, 2.22, 2.11, 2.10, 2.08, 2.03, 2.02, 1.60, 1.59, 1.58, 1.57, 1.56, 1.55, 1.47, 1.46, 1.45, 1.44, 1.42, 1.41, 1.31, 1.30, 1.27, 0.98, 0.97, 0.90, 0.89, 0.88, 0.13, 0.01	1.99, 2.07, 2.29, 2.08, 2.18, 2.38, 2.36, 1.97, 4.72, 2.31, 1.66, 1.20, 0.81, 5.91

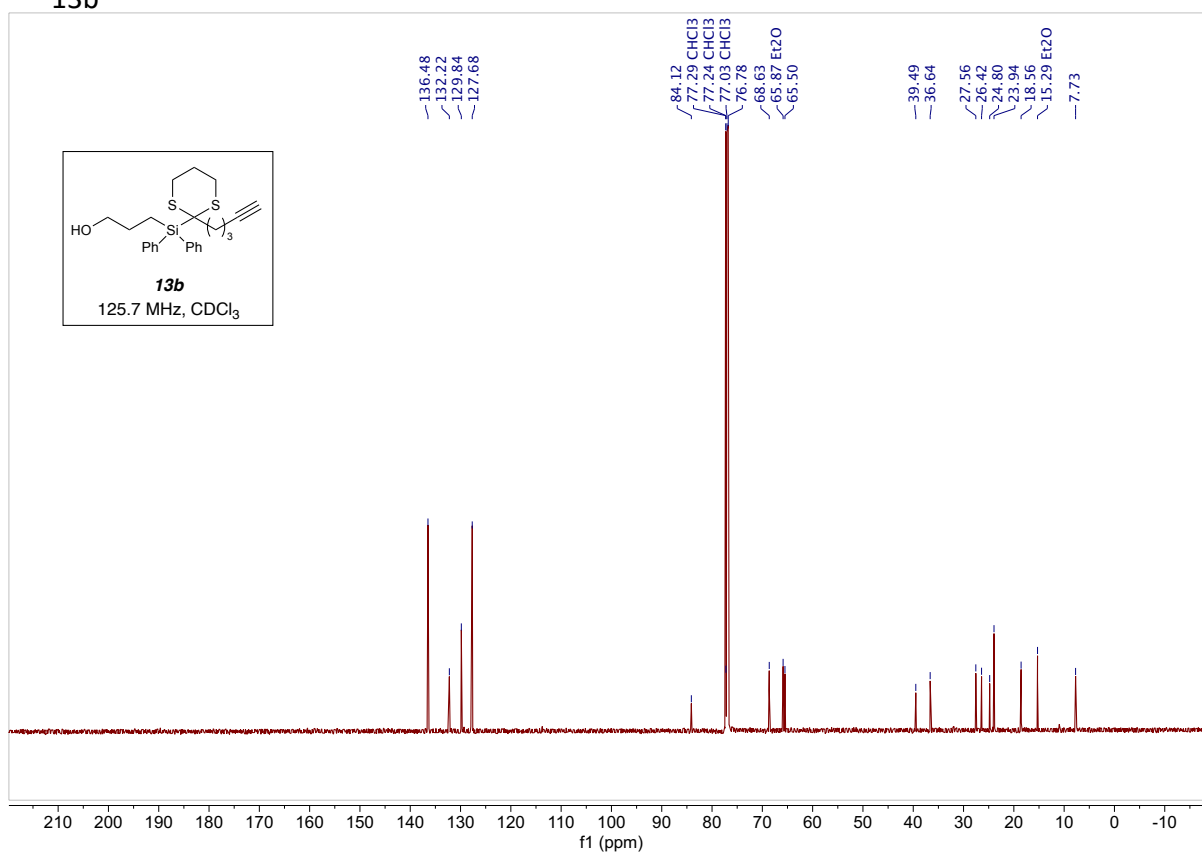
12b



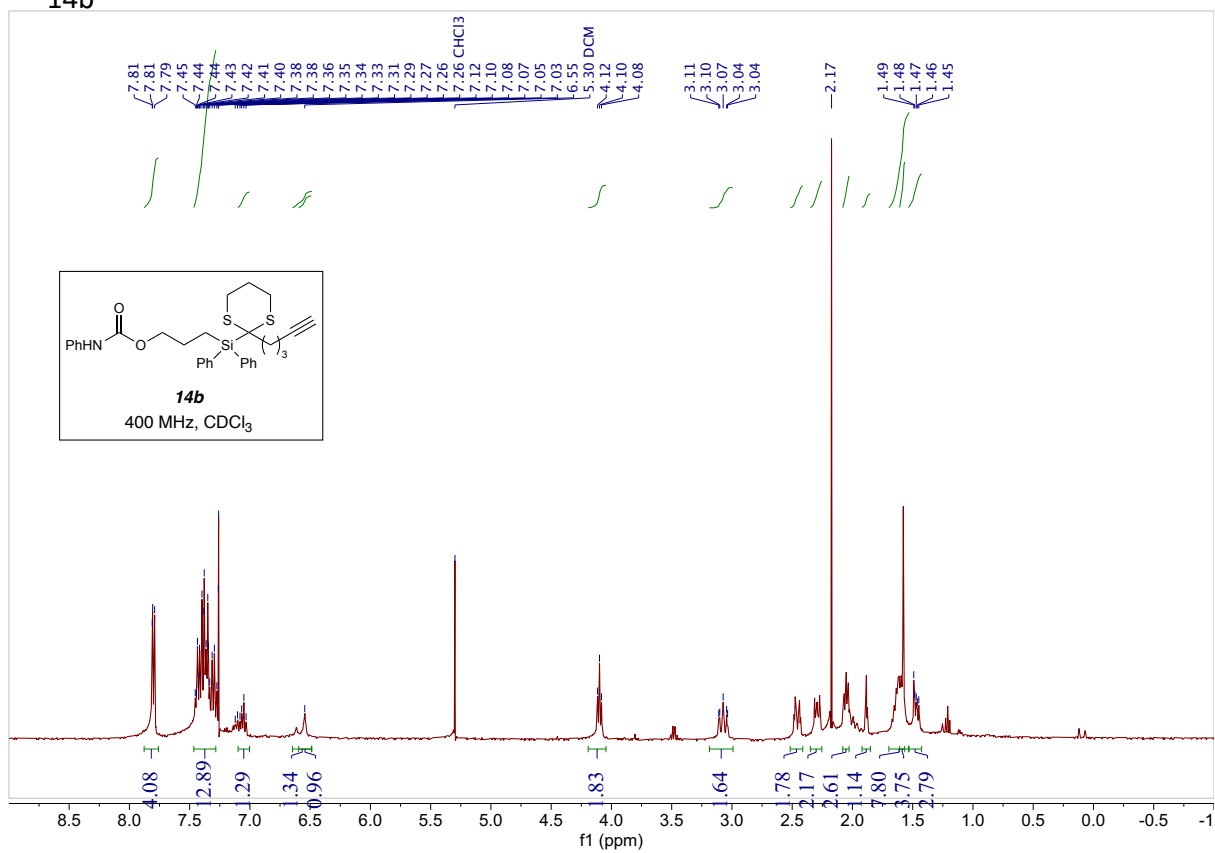
13b

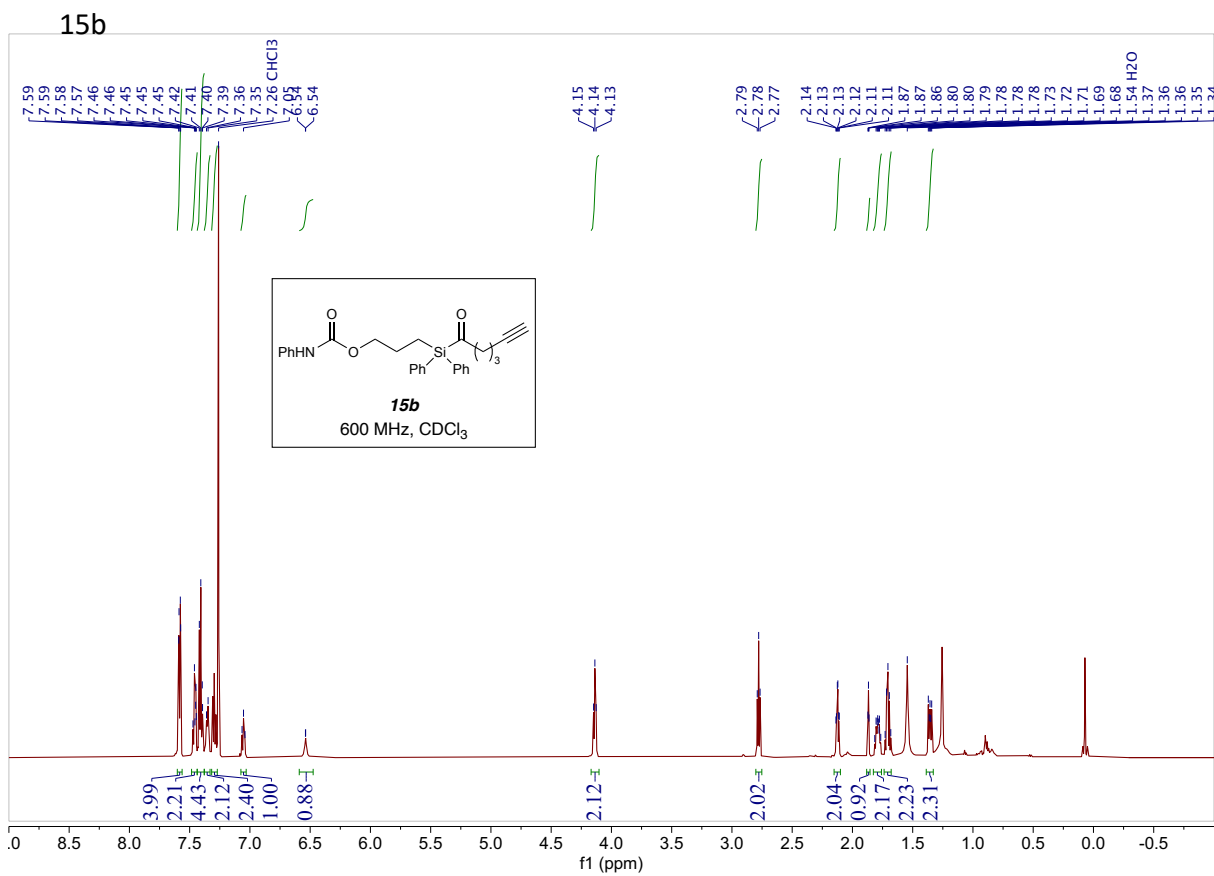


13b

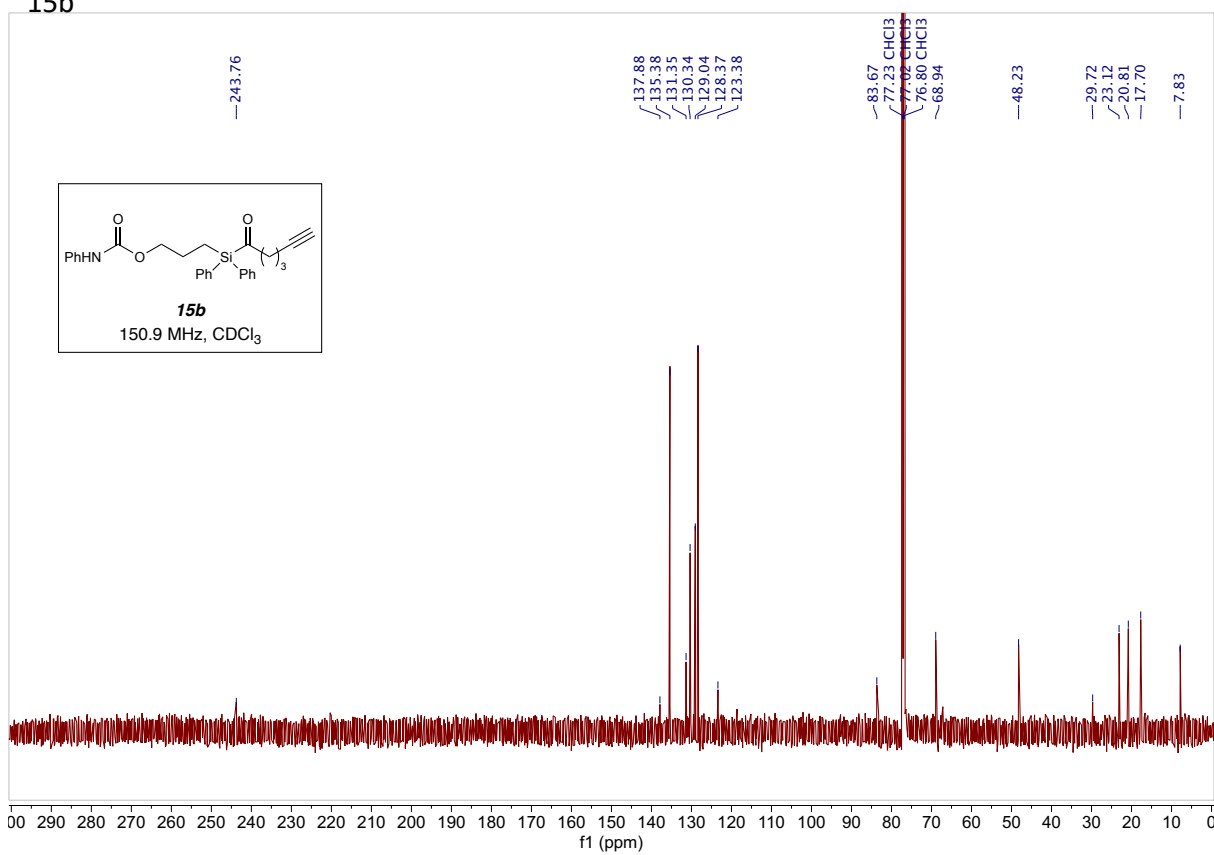


14b

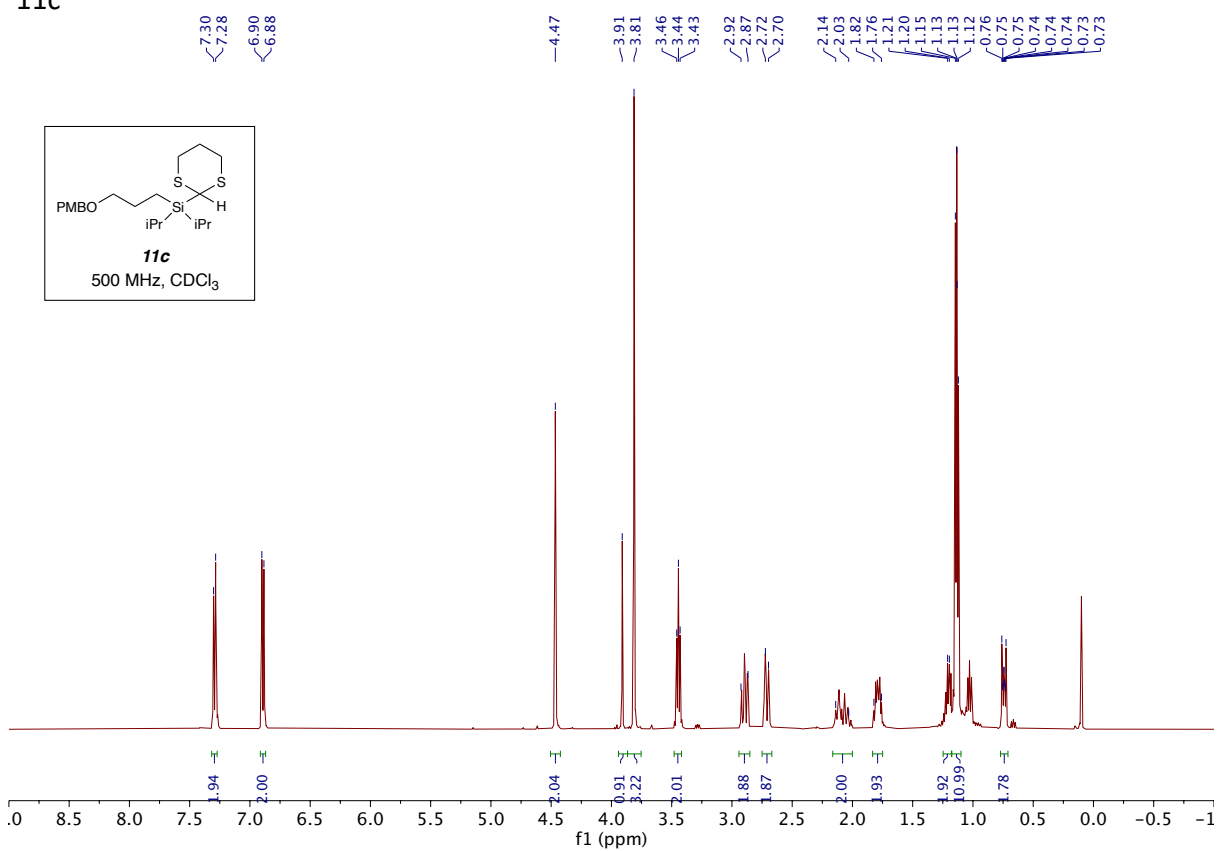




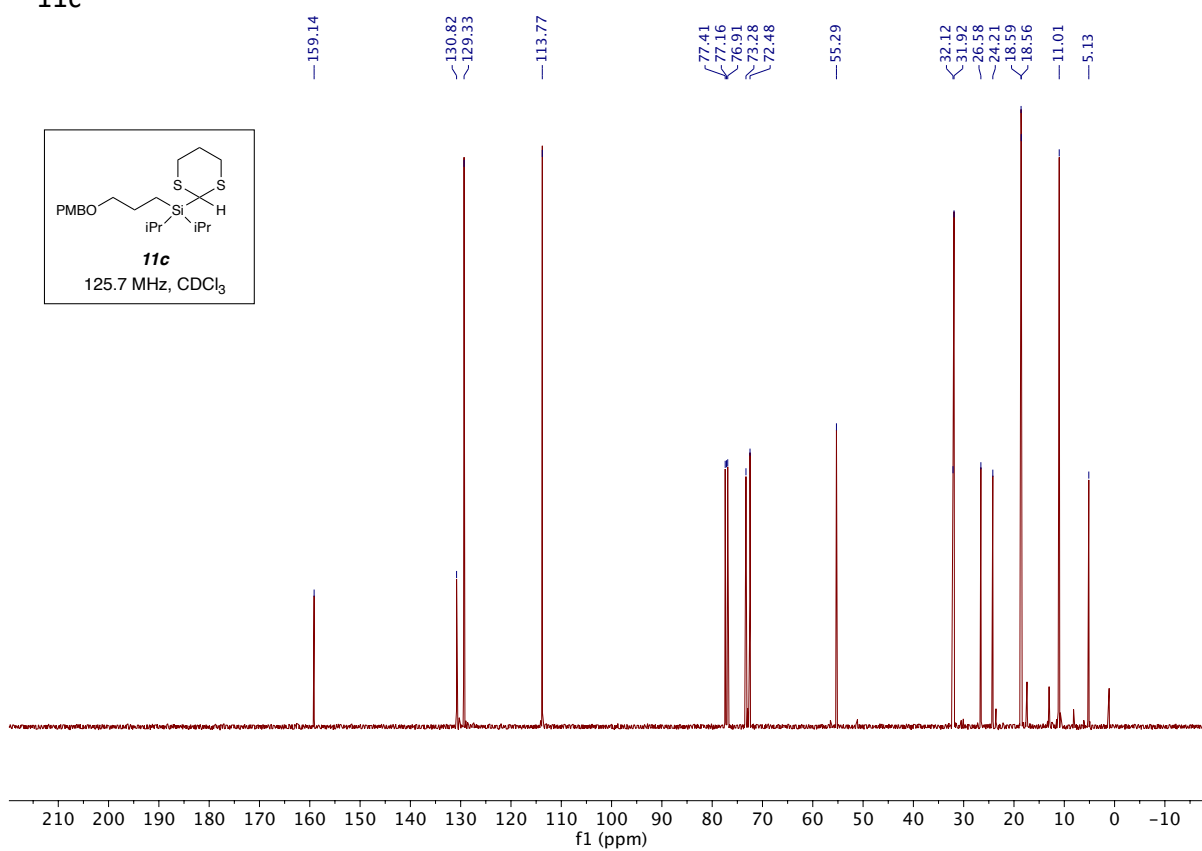
15b



11c



11c

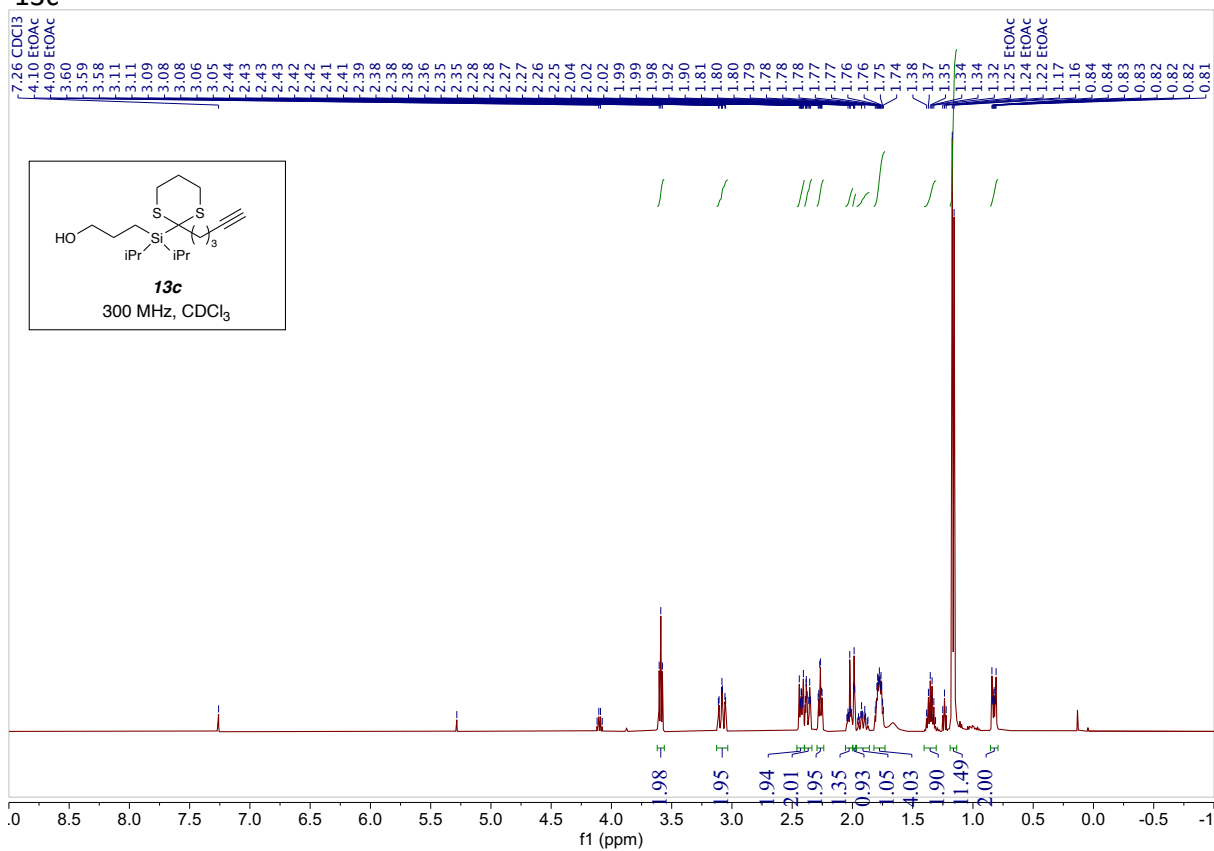


[illegible]

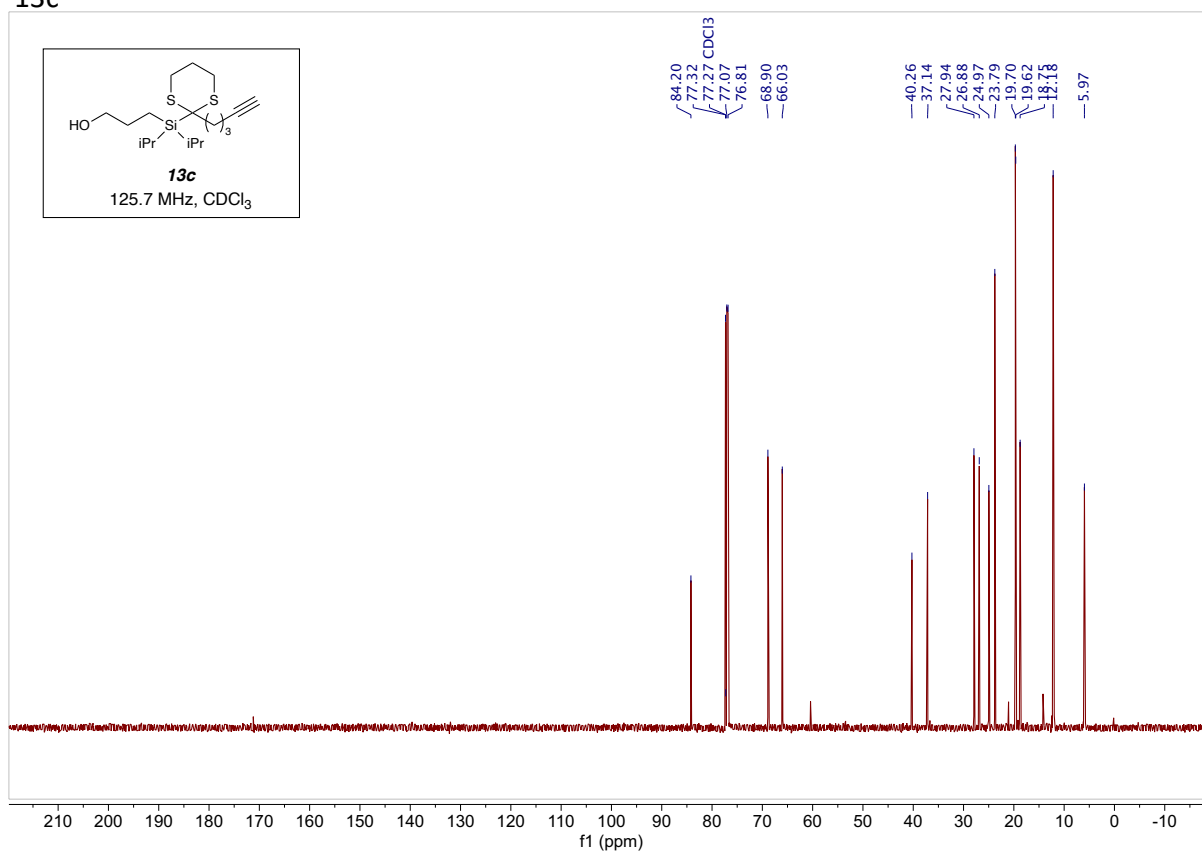

12c
125.7 MHz, CDCl₃

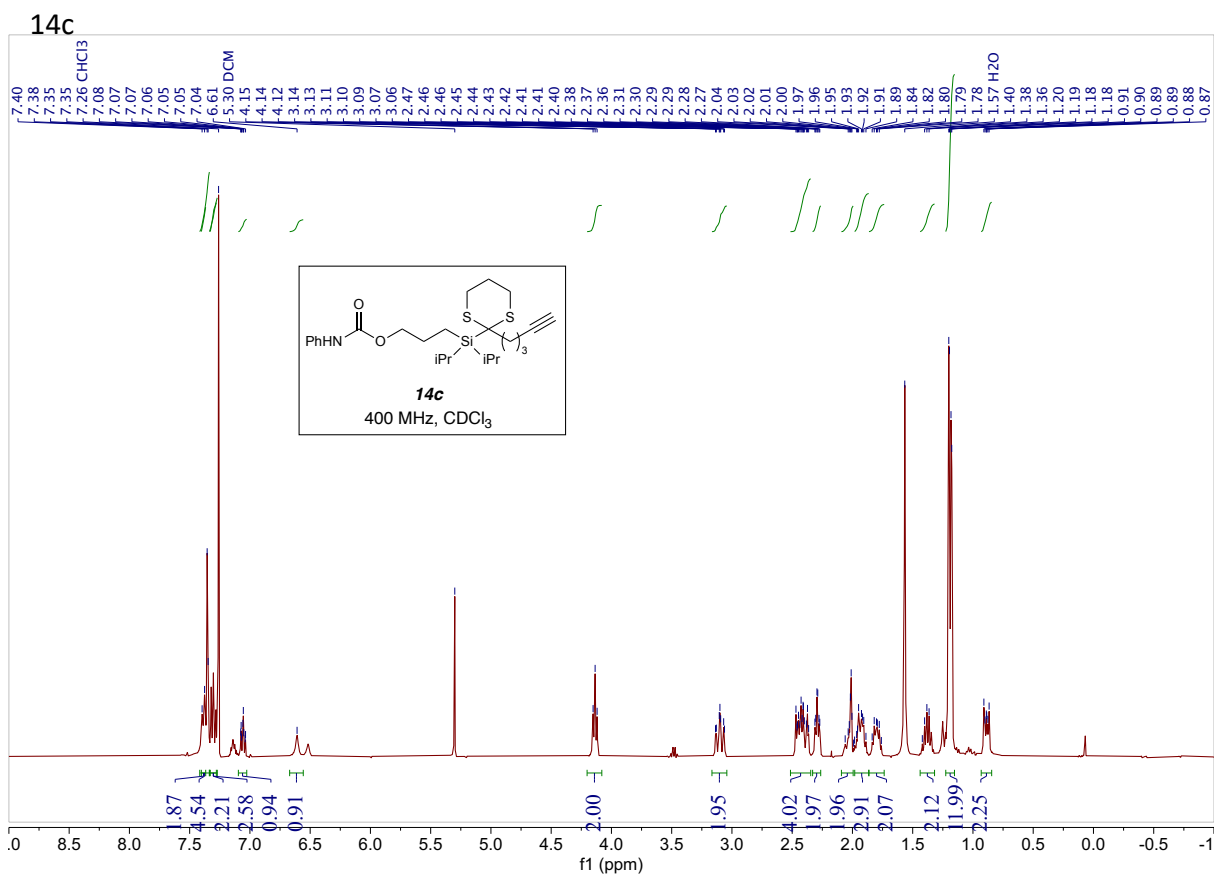


13c



13c





15c
500 MHz, CDCl₃

Chemical structure of **15c**: CC(C)(C)C(=O)Si(C(C)C)CCOC(=O)c1ccccc1

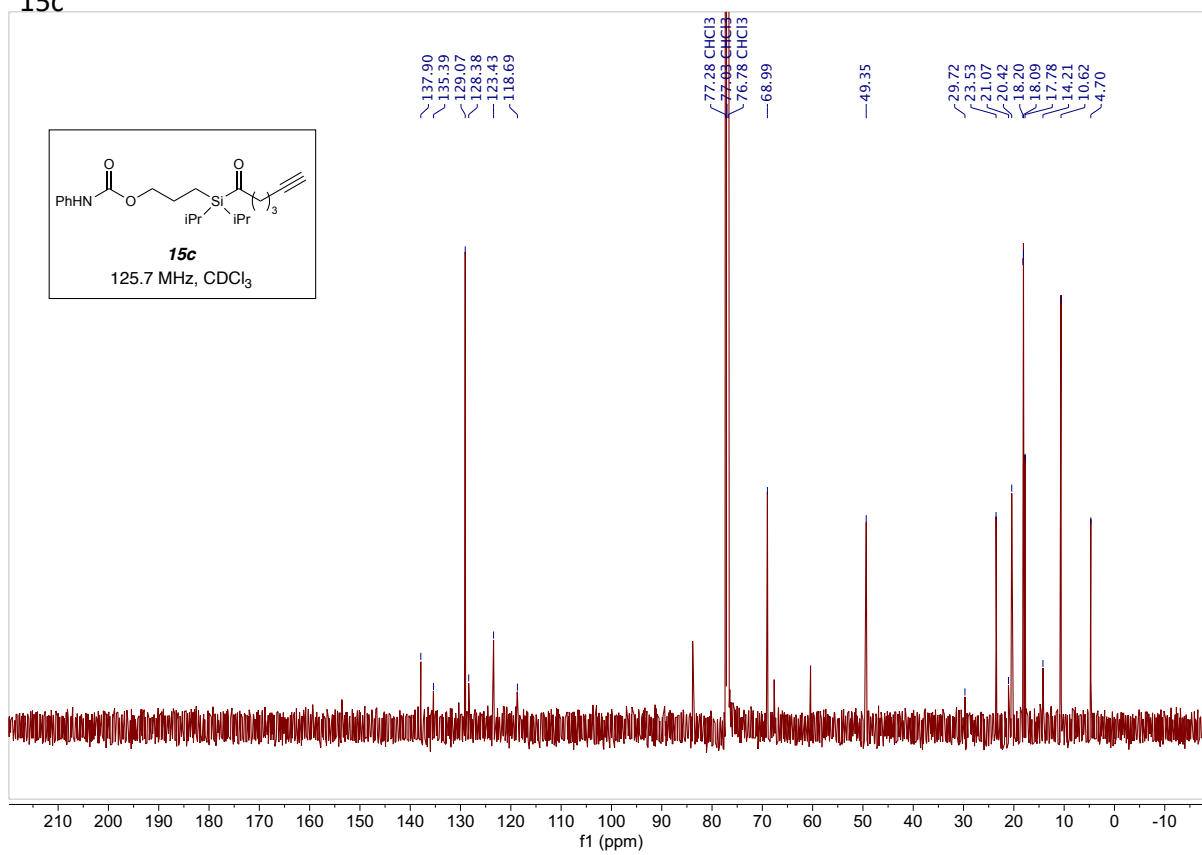
¹H NMR spectrum (500 MHz, CDCl₃) of compound **15c**. The spectrum shows peaks corresponding to the structure, with integration values indicated below the peaks.

Chemical structure of **15c** is shown in the inset.

Integration values (from left to right): 2.35, 2.30, 1.00, 1.00, 2.43, 1.95, 1.91, 0.88, 4.19, 2.17, 4.62, 12.18, 2.29.

Peak assignments (from left to right): Aromatic protons (7.39, 7.38, 7.37, 7.36, 7.35, 7.34, 7.33, 7.32, 7.31, 7.30, 7.26, 7.08, 7.07, 7.06, 7.06, 7.05, 7.04, 6.63), NH (7.05), CH₂ (4.15, 4.14, 4.13, 4.11, 4.10, 4.09, 4.08, 4.07, 4.06, 4.05, 4.04, 4.03, 4.02, 4.01, 4.00, 3.99, 3.98, 3.97, 3.96, 3.95, 3.94, 3.93, 3.92, 3.91, 3.90, 3.89, 3.88, 3.87, 3.86, 3.85, 3.84, 3.83, 3.82, 3.81, 3.80, 3.79, 3.78, 3.77, 3.76, 3.75, 3.74, 3.73, 3.72, 3.71, 3.70, 3.69, 3.68, 3.67, 3.66, 3.65, 3.64, 3.63, 3.62, 3.61, 3.60, 3.59, 3.58, 3.57, 3.56, 3.55, 3.54, 3.53, 3.52, 3.51, 3.50, 3.49, 3.48, 3.47, 3.46, 3.45, 3.44, 3.43, 3.42, 3.41, 3.40, 3.39, 3.38, 3.37, 3.36, 3.35, 3.34, 3.33, 3.32, 3.31, 3.30, 3.29, 3.28, 3.27, 3.26, 3.25, 3.24, 3.23, 3.22, 3.21, 3.20, 3.19, 3.18, 3.17, 3.16, 3.15, 3.14, 3.13, 3.12, 3.11, 3.10, 3.09, 3.08, 3.07, 3.06, 3.05, 3.04, 3.03, 3.02, 3.01, 3.00, 2.99, 2.98, 2.97, 2.96, 2.95, 2.94, 2.93, 2.92, 2.91, 2.90, 2.89, 2.88, 2.87, 2.86, 2.85, 2.84, 2.83, 2.82, 2.81, 2.80, 2.79, 2.78, 2.77, 2.76, 2.75, 2.74, 2.73, 2.72, 2.71, 2.70, 2.69, 2.68, 2.67, 2.66, 2.65, 2.64, 2.63, 2.62, 2.61, 2.60, 2.59, 2.58, 2.57, 2.56, 2.55, 2.54, 2.53, 2.52, 2.51, 2.50, 2.49, 2.48, 2.47, 2.46, 2.45, 2.44, 2.43, 2.42, 2.41, 2.40, 2.39, 2.38, 2.37, 2.36, 2.35, 2.34, 2.33, 2.32, 2.31, 2.30, 2.29, 2.28, 2.27, 2.26, 2.25, 2.24, 2.23, 2.22, 2.21, 2.20, 2.19, 2.18, 2.17, 2.16, 2.15, 2.14, 2.13, 2.12, 2.11, 2.10, 2.09, 2.08, 2.07, 2.06, 2.05, 2.04, 2.03, 2.02, 2.01, 2.00, 1.99, 1.98, 1.97, 1.96, 1.95, 1.94, 1.93, 1.92, 1.91, 1.90, 1.89, 1.88, 1.87, 1.86, 1.85, 1.84, 1.83, 1.82, 1.81, 1.80, 1.79, 1.78, 1.77, 1.76, 1.75, 1.74, 1.73, 1.72, 1.71, 1.70, 1.69, 1.68, 1.67, 1.66, 1.65, 1.64, 1.63, 1.62, 1.61, 1.60, 1.59, 1.58, 1.57, 1.56, 1.55, 1.54, 1.53, 1.52, 1.51, 1.50, 1.49, 1.48, 1.47, 1.46, 1.45, 1.44, 1.43, 1.42, 1.41, 1.40, 1.39, 1.38, 1.37, 1.36, 1.35, 1.34, 1.33, 1.32, 1.31, 1.30, 1.29, 1.28, 1.27, 1.26, 1.25, 1.24, 1.23, 1.22, 1.21, 1.20, 1.19, 1.18, 1.17, 1.16, 1.15, 1.14, 1.13, 1.12, 1.11, 1.10, 1.09, 1.08, 1.07, 1.06, 1.05, 1.04, 1.03, 1.02, 1.01, 1.00, 0.99, 0.98, 0.97, 0.96, 0.95, 0.94, 0.93, 0.92, 0.91, 0.90, 0.89, 0.88, 0.87, 0.86, 0.85, 0.84, 0.83, 0.82, 0.81, 0.80, 0.79, 0.78, 0.77, 0.76, 0.75, 0.74, 0.73, 0.72, 0.71, 0.70, 0.69, 0.68, 0.67, 0.66, 0.65, 0.64, 0.63, 0.62, 0.61, 0.60, 0.59, 0.58, 0.57, 0.56, 0.55, 0.54, 0.53, 0.52, 0.51, 0.50, 0.49, 0.48, 0.47, 0.46, 0.45, 0.44, 0.43, 0.42, 0.41, 0.40, 0.39, 0.38, 0.37, 0.36, 0.35, 0.34, 0.33, 0.32, 0.31, 0.30, 0.29, 0.28, 0.27, 0.26, 0.25, 0.24, 0.23, 0.22, 0.21, 0.20, 0.19, 0.18, 0.17, 0.16, 0.15, 0.14, 0.13, 0.12, 0.11, 0.10, 0.09, 0.08, 0.07, 0.06, 0.05, 0.04, 0.03, 0.02, 0.01, 0.00).

15c

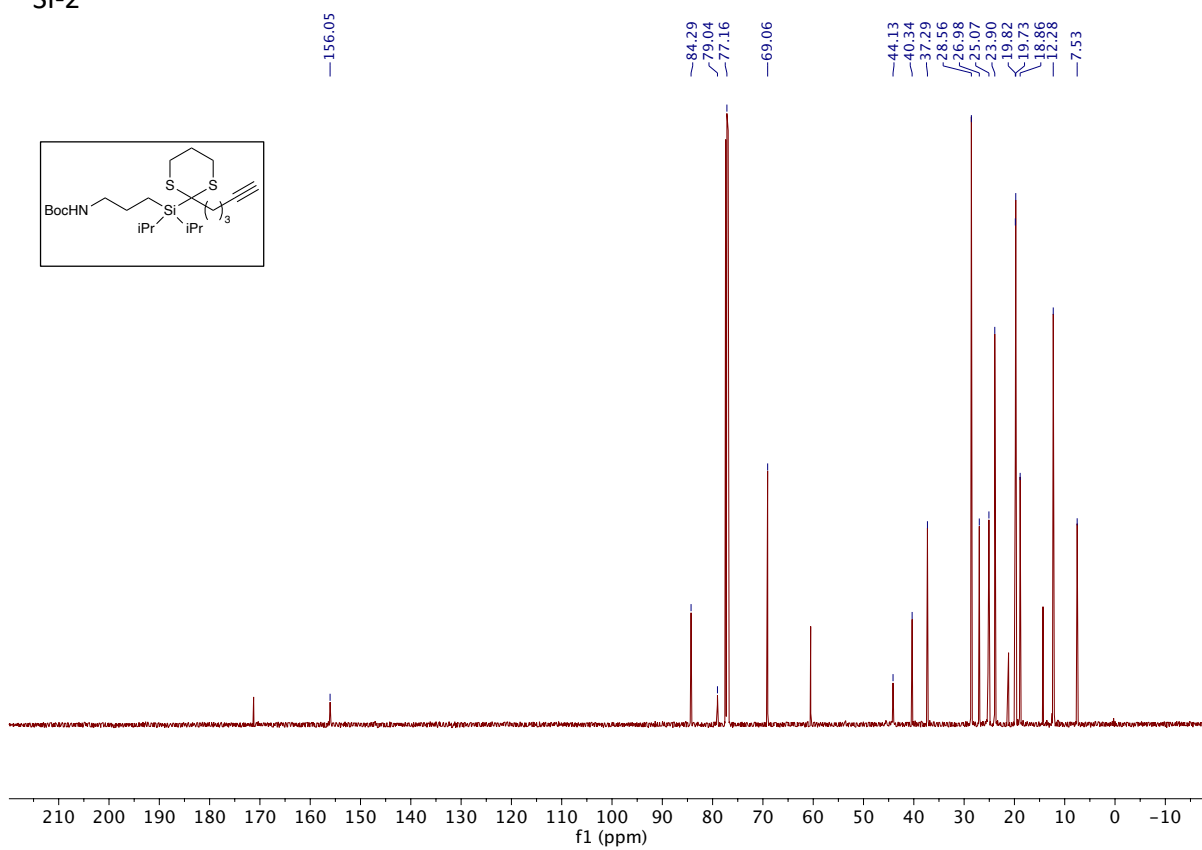




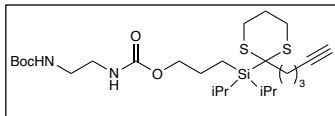
CC(C)(C)SC1(C(C)(C)C)SC(C1)CCCCOC(C)(C)C



SI-2



7.27 CDCI3
5.29 DCM



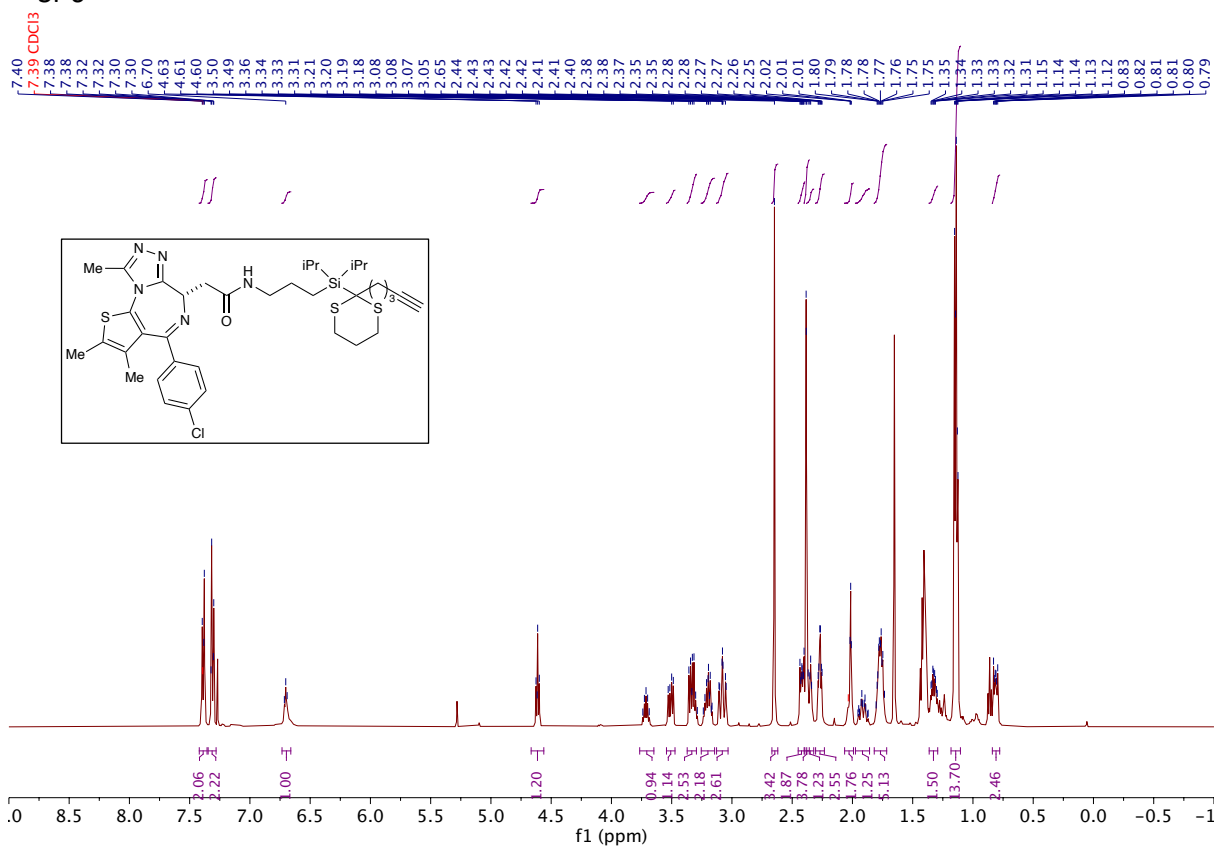
Chemical structure of the compound is shown in the inset:

CC(C)(C)Si(C(C)(C)C)CSCCSCC(C#C)C(=O)NCCNC(=O)OCC(C)(C)C

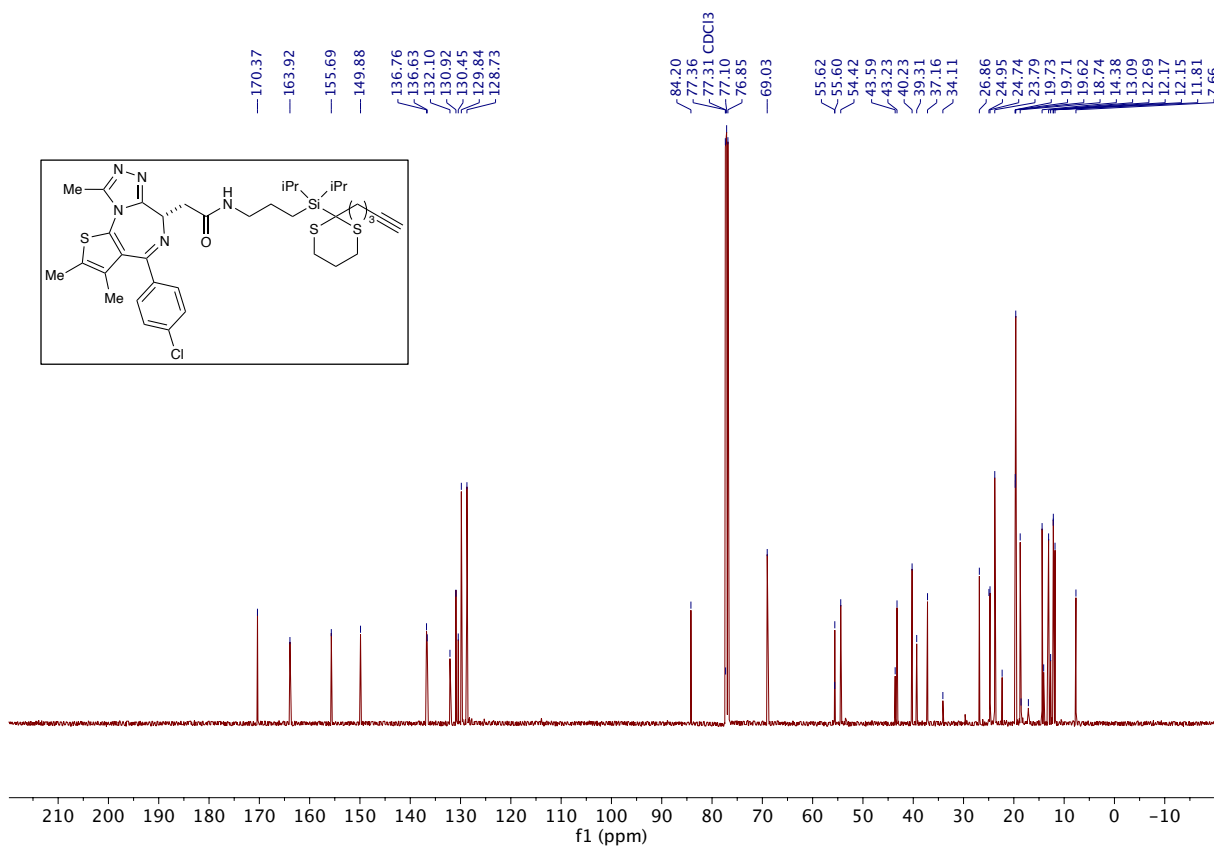
¹³C NMR spectrum (f1 (ppm)) showing peaks at:

- 157.25, 156.48 (Carbonyl carbons)
- 84.30, 79.61, 77.16 (Solvent and alkyne carbons)
- 69.06, 67.92 (Alkyne carbons)
- 41.40, 40.81, 40.27, 37.27, 28.48, 26.95, 25.04, 24.41, 23.88 (Aliphatic carbons)
- 19.79, 19.71, 18.95 (Aliphatic carbons)
- 6.31 (Aliphatic carbon)

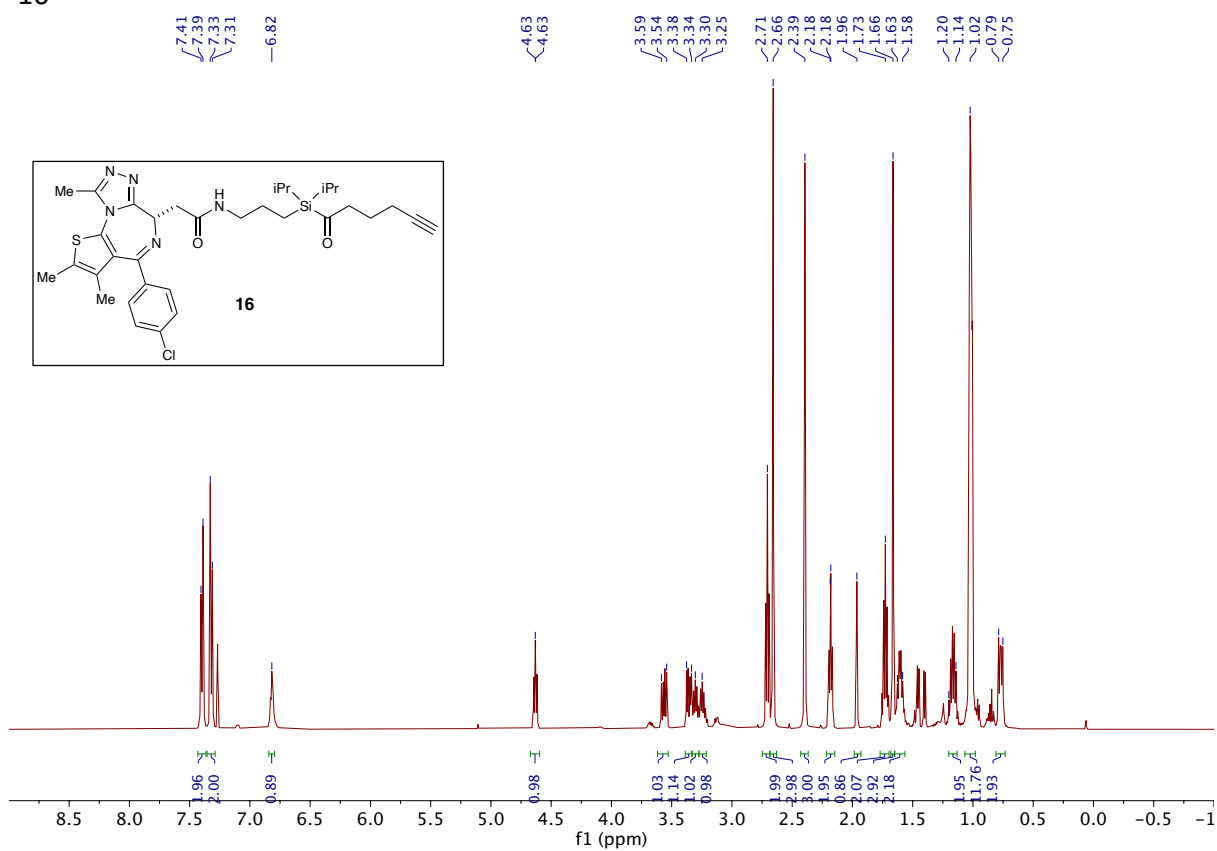
SI-6



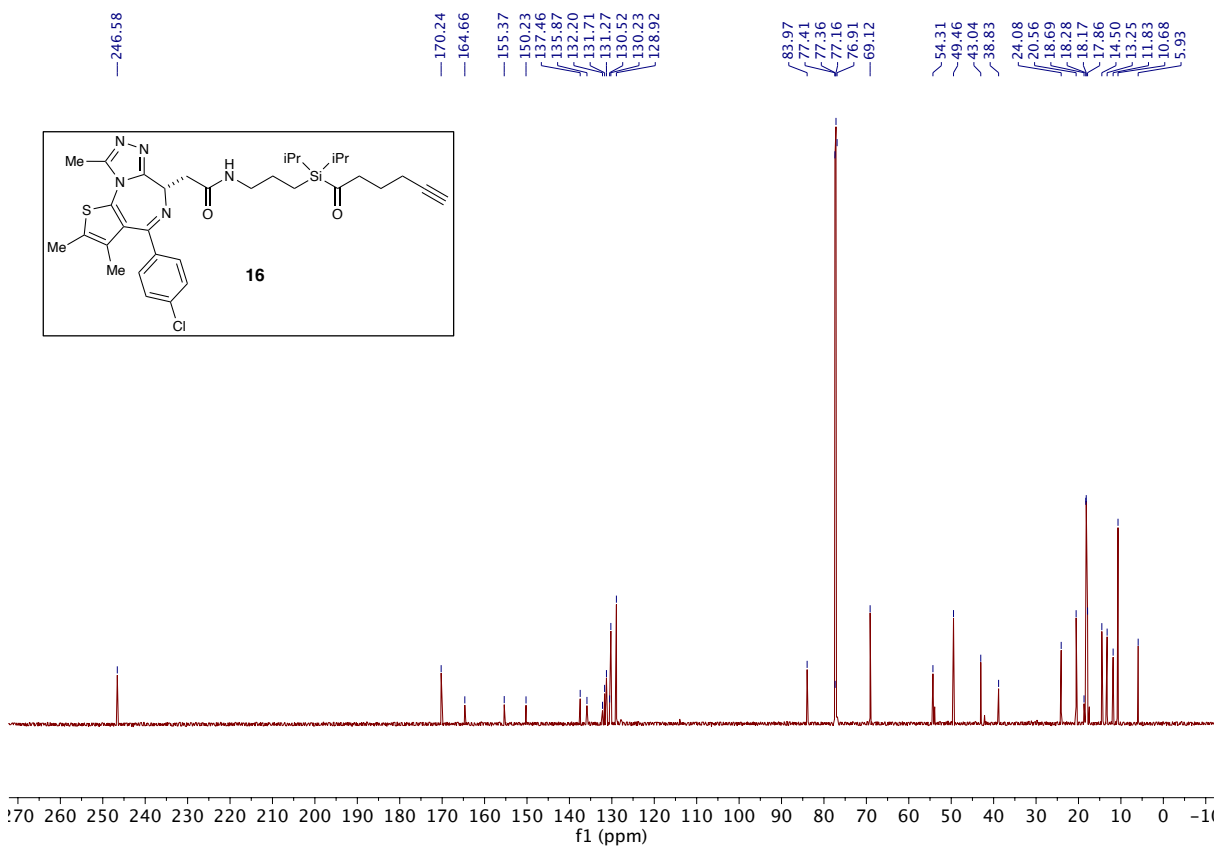
SI-6



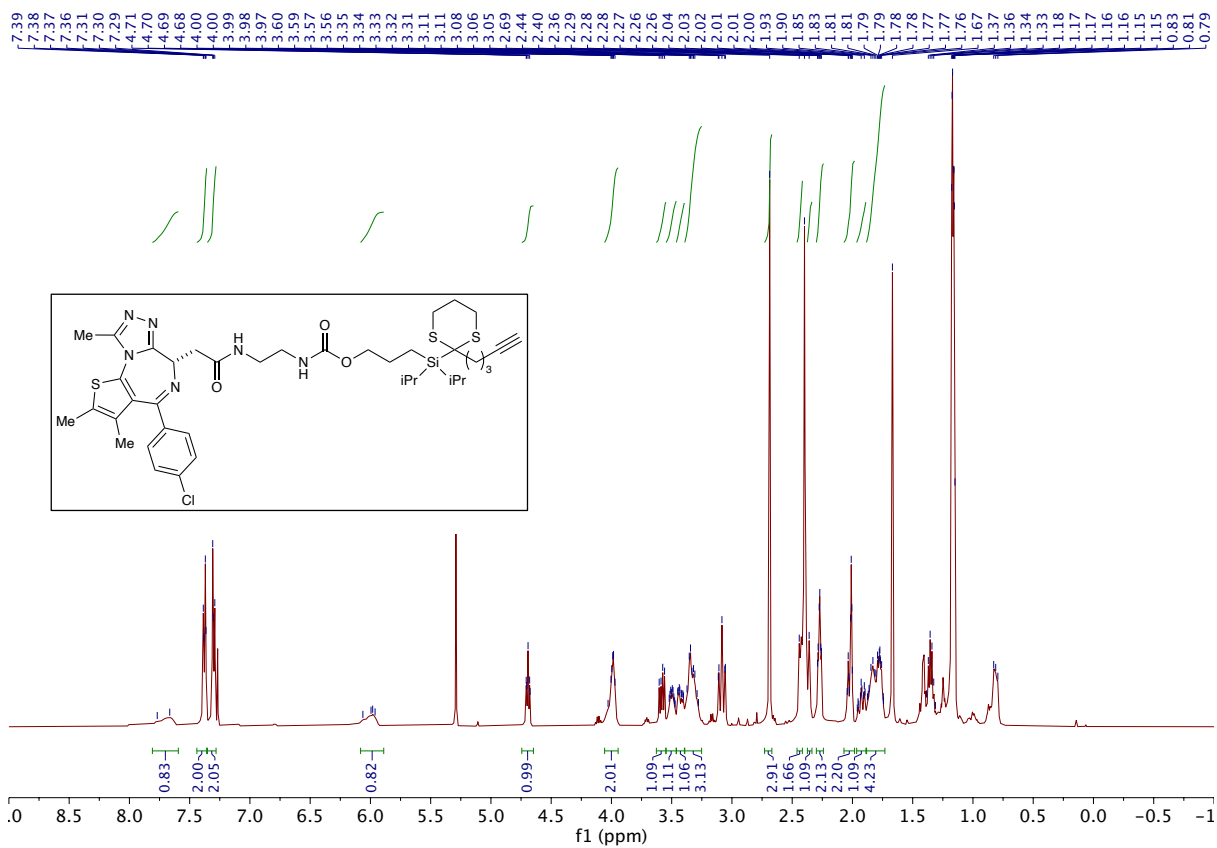
16



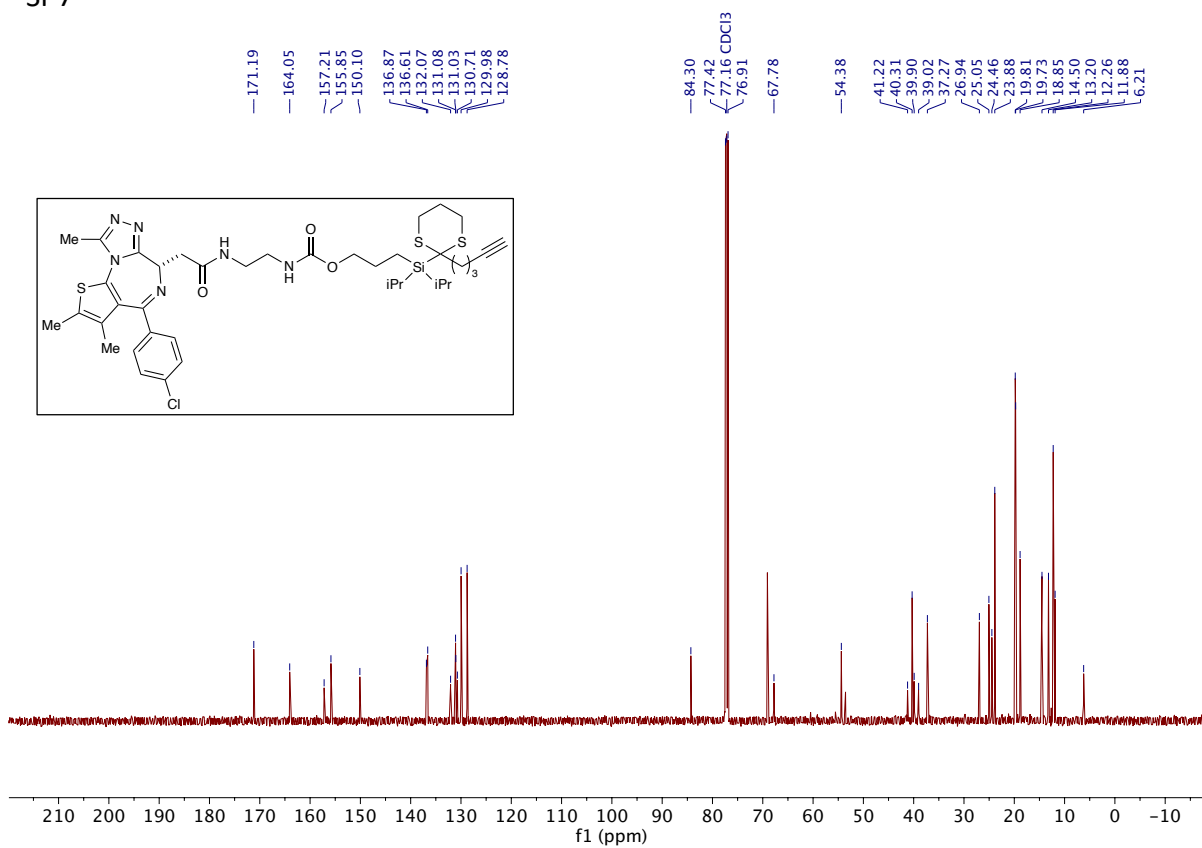
16



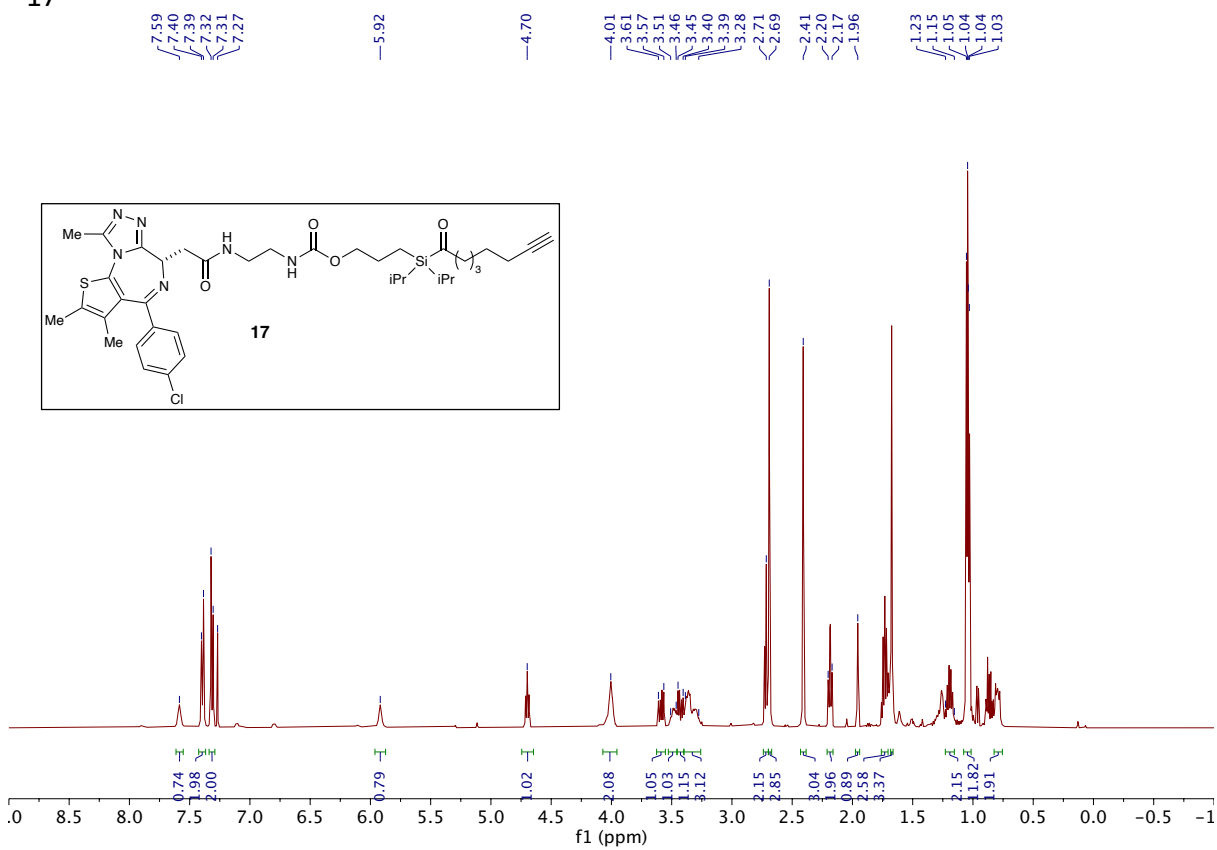
SI-7



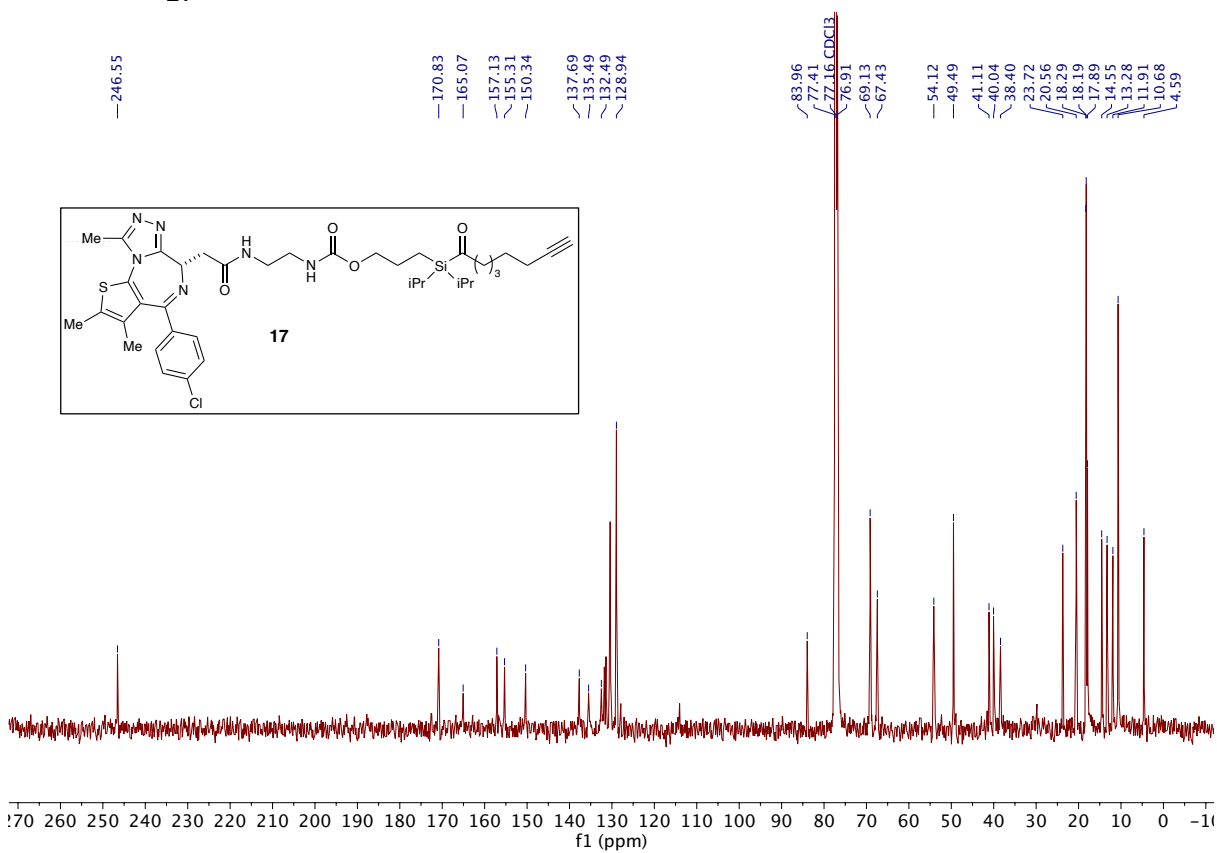
SI-7



17



17



20

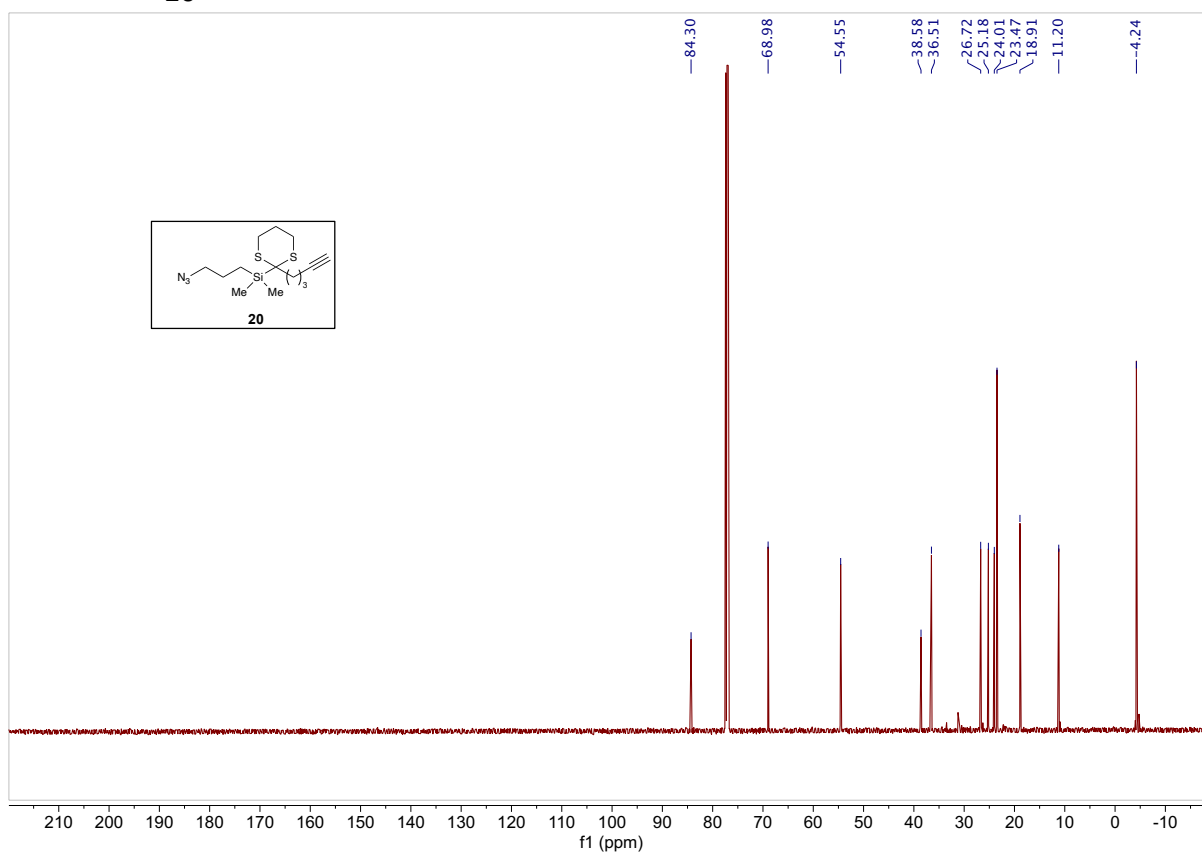
CN=[N+]#NCCCC1(C)SCC(C#CC)S1

7.46
3.48
3.48
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3.46
3.425
3.425
3.099
3.099
3.077
3.066
3.066
3.04
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0.81
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0.19
0.18

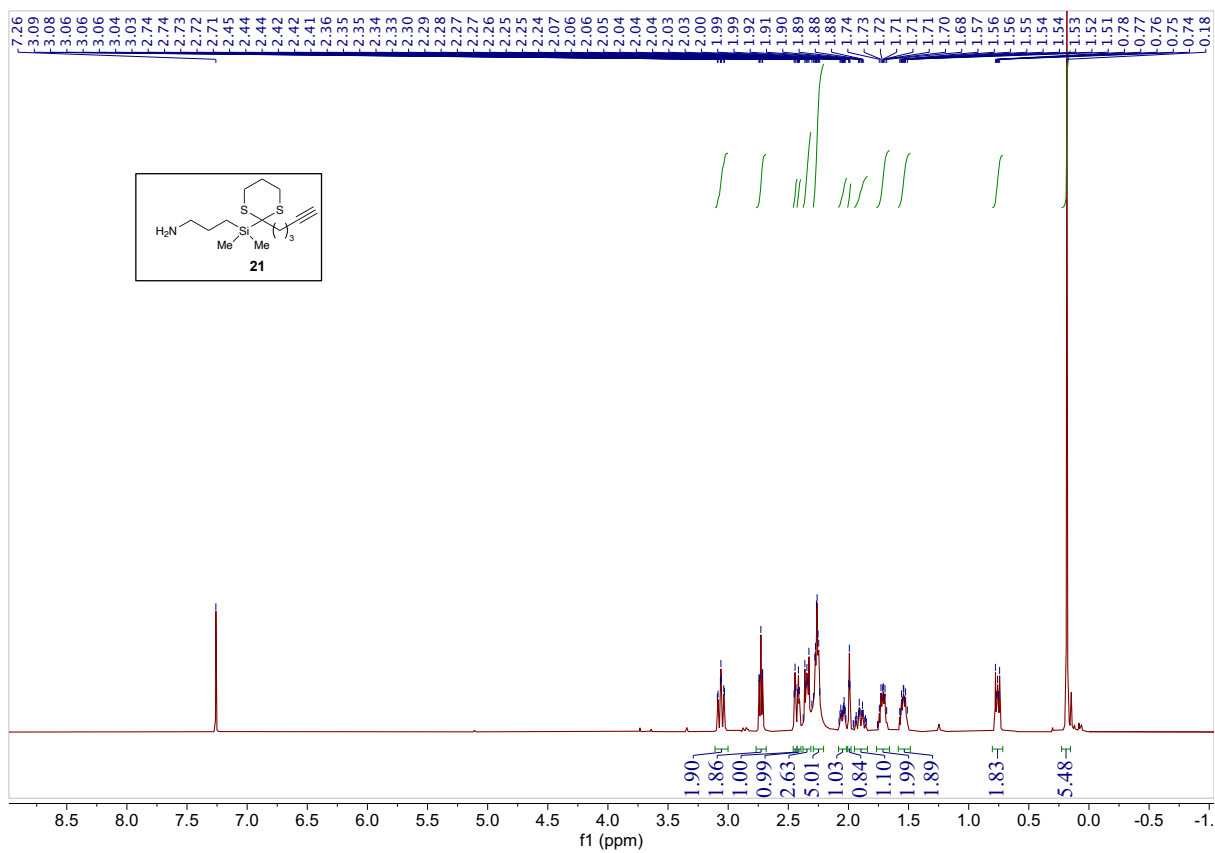
2.13
1.95
1.00
0.96
1.94
2.00
1.05
0.93
1.17
4.10
1.96
5.70

f1 (ppm)

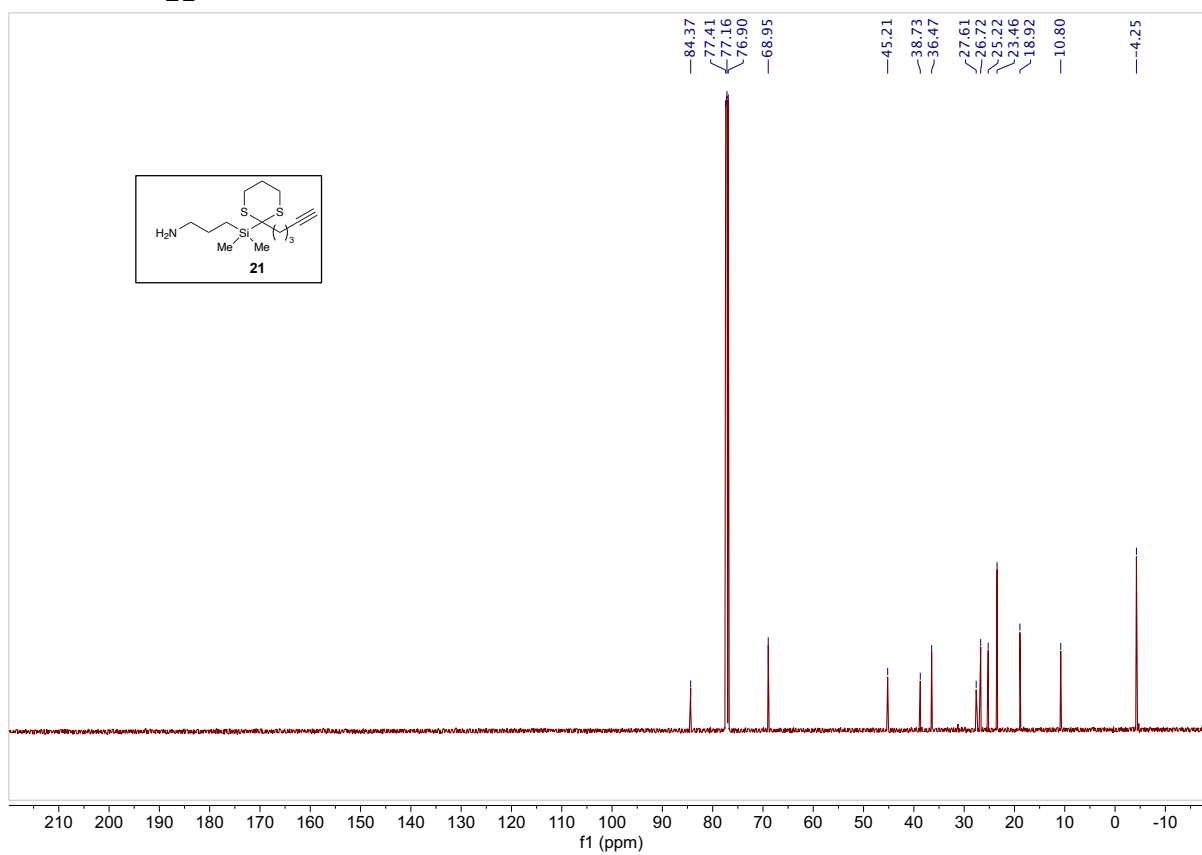
20



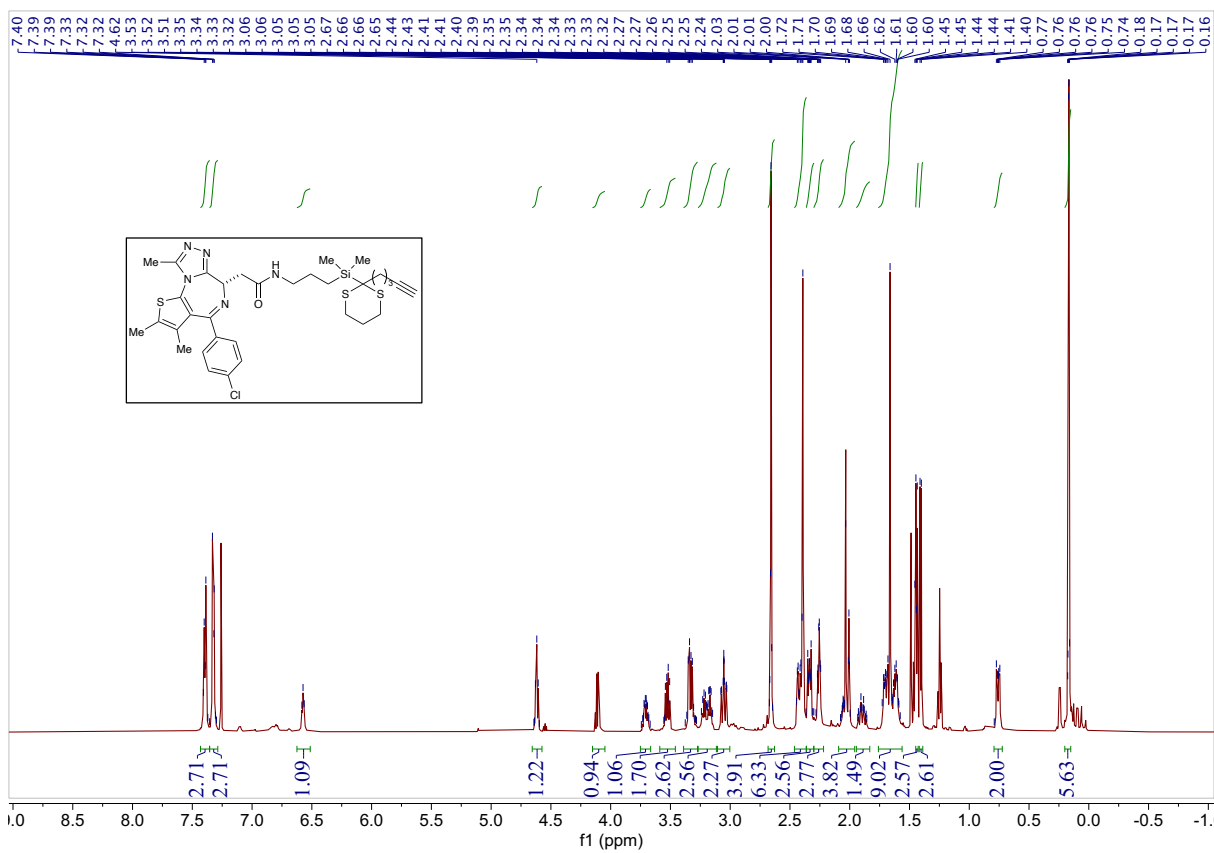
21



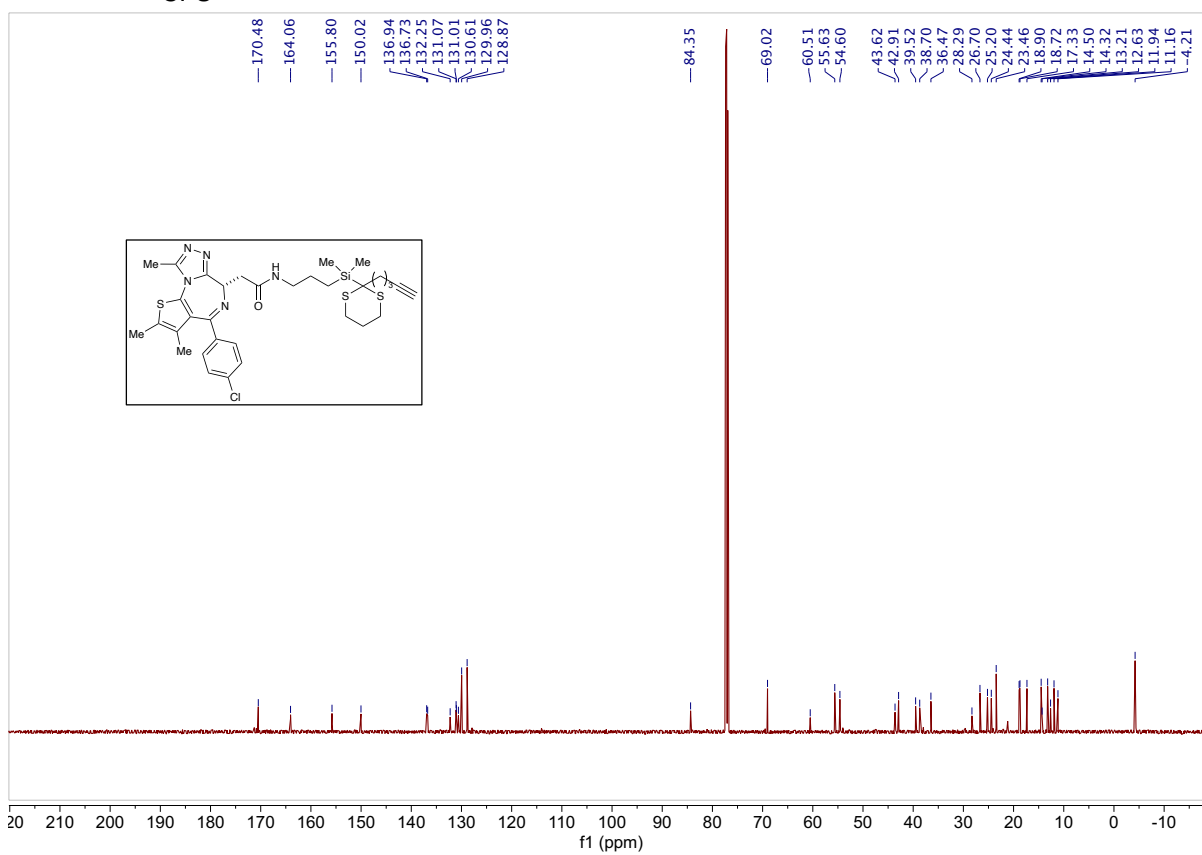
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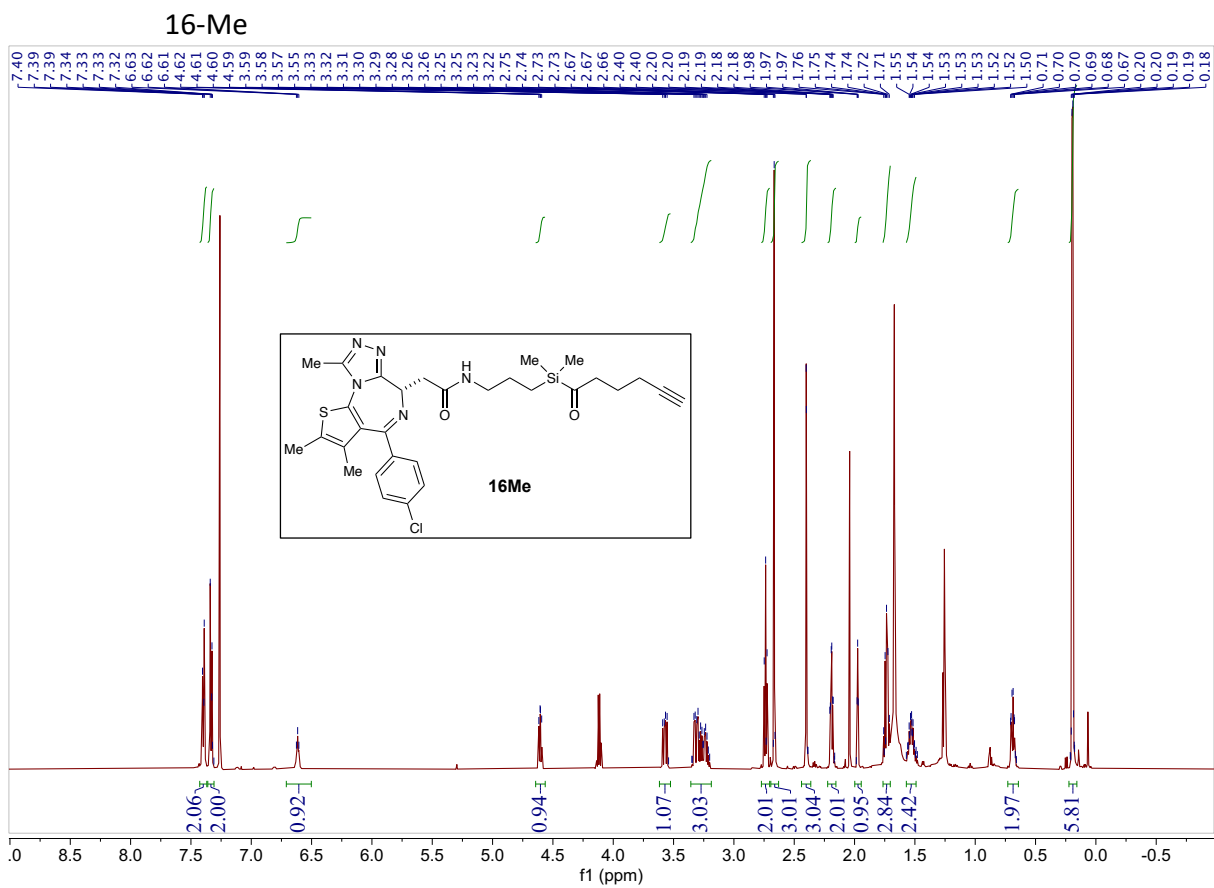


SI-8

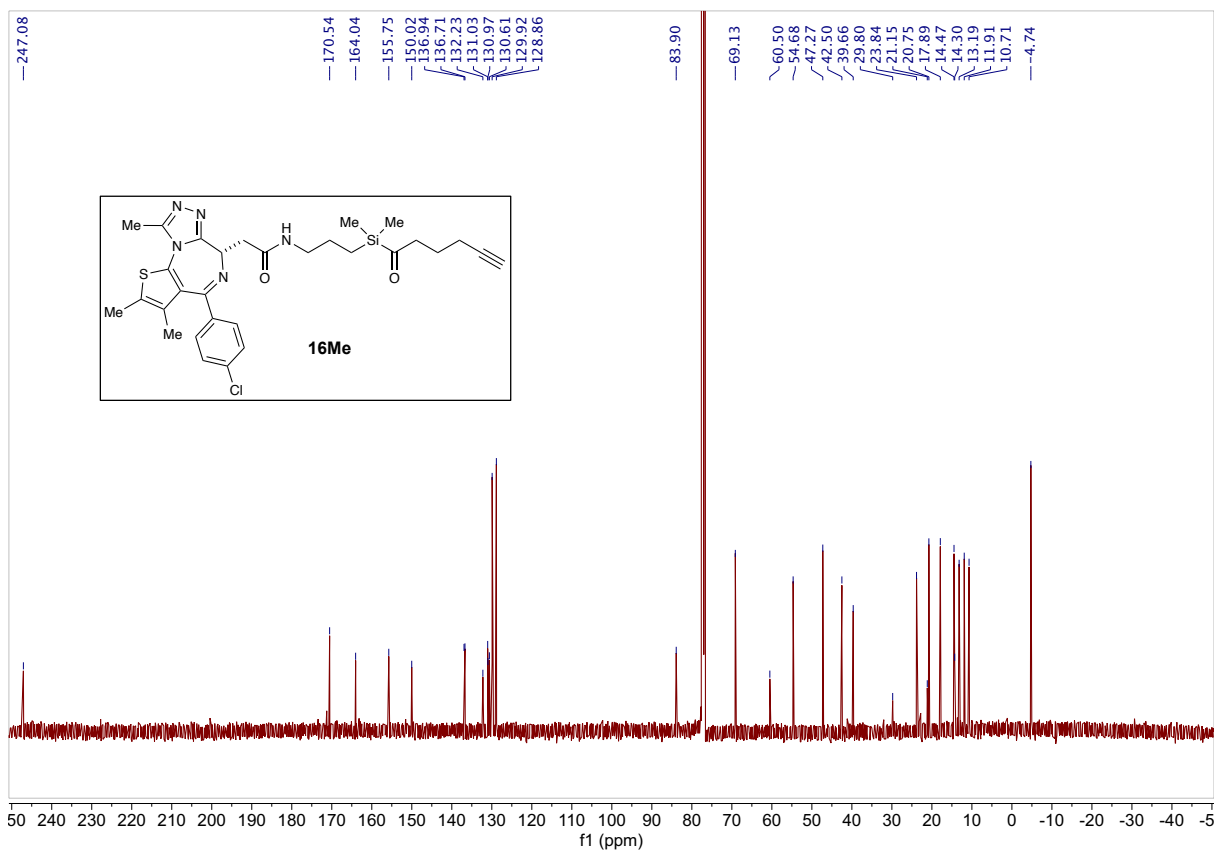


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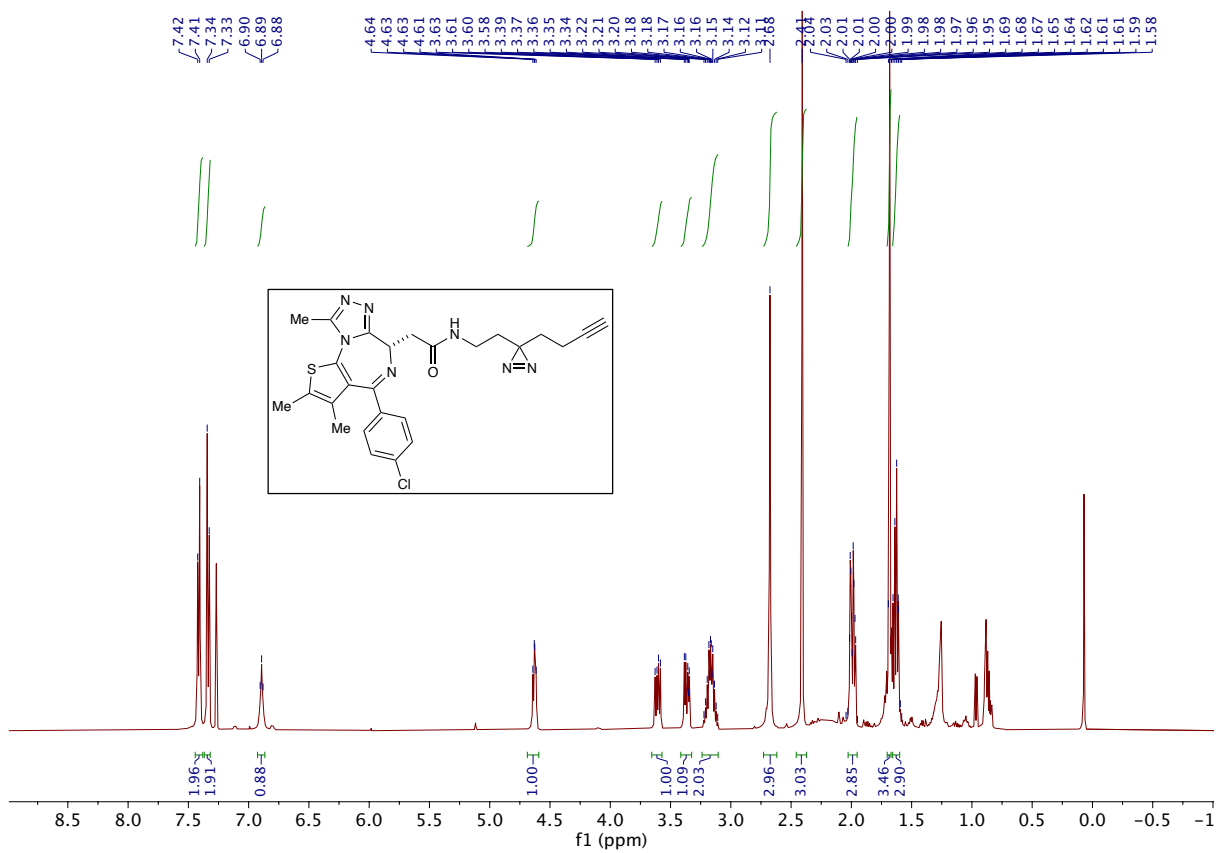




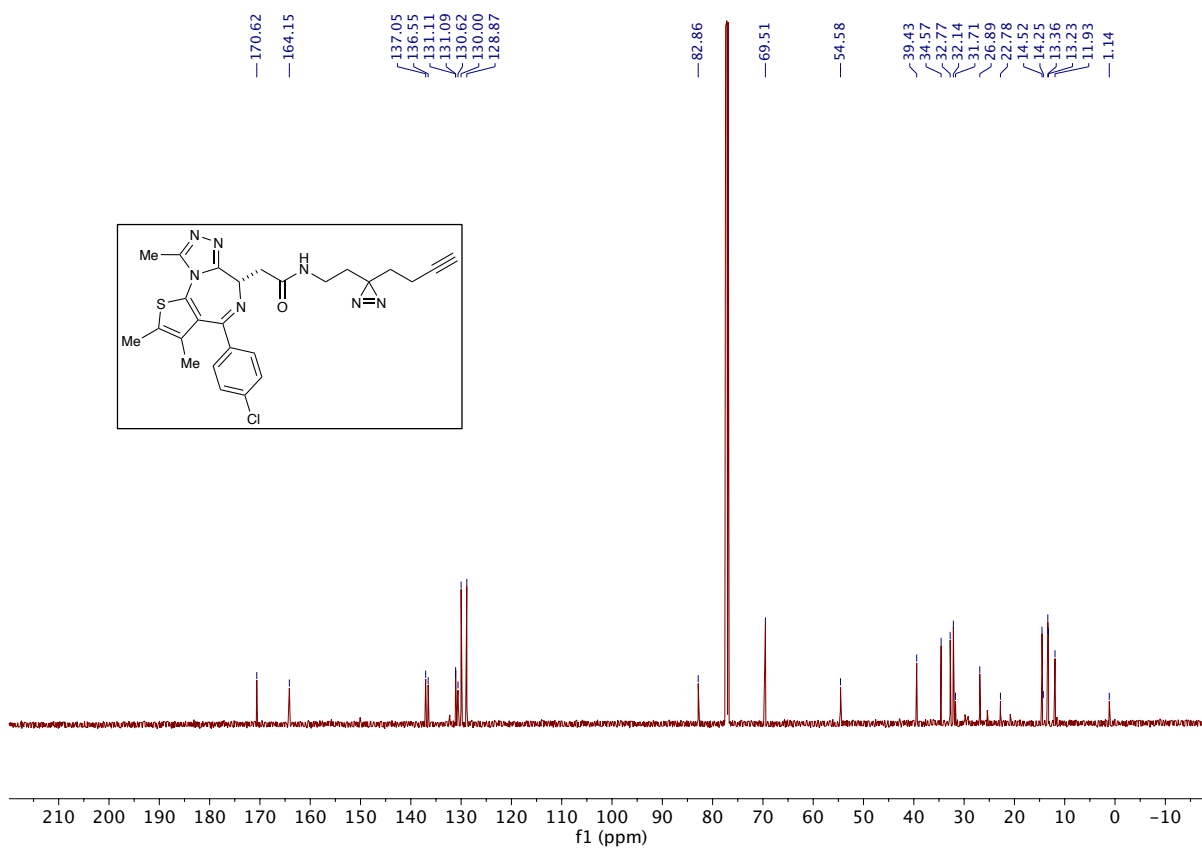
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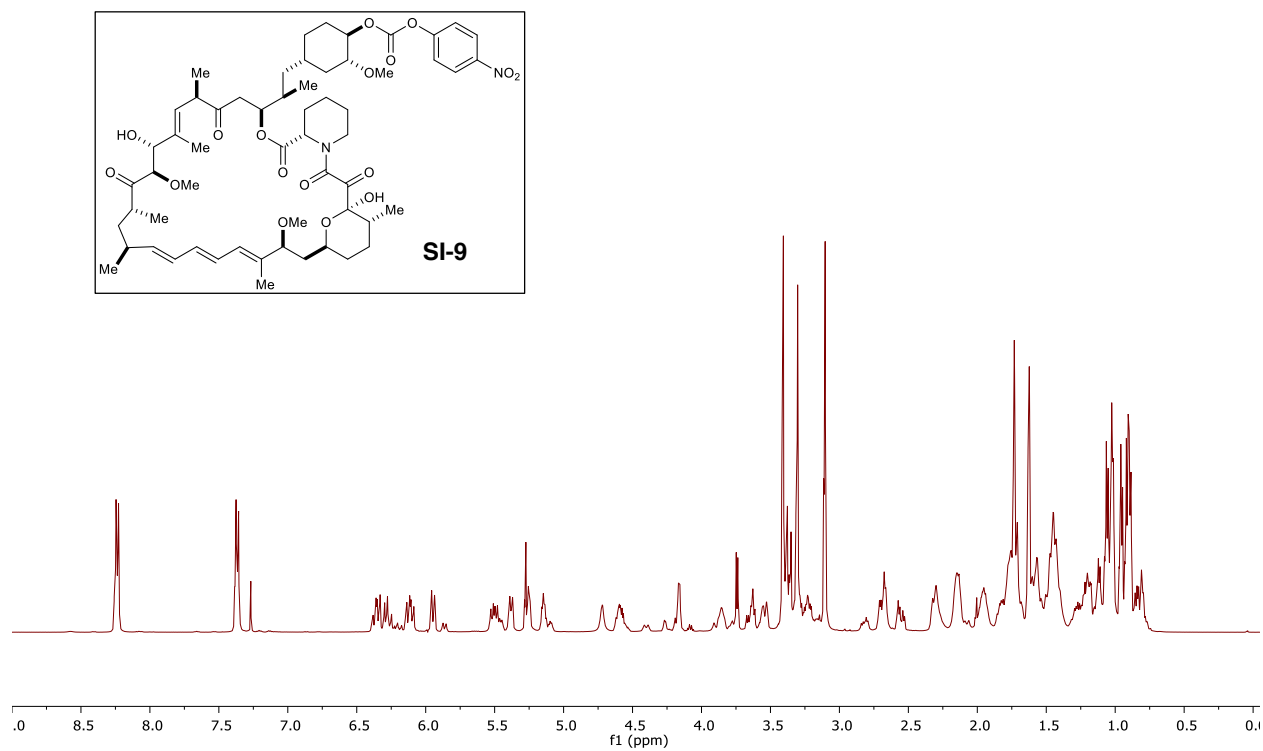
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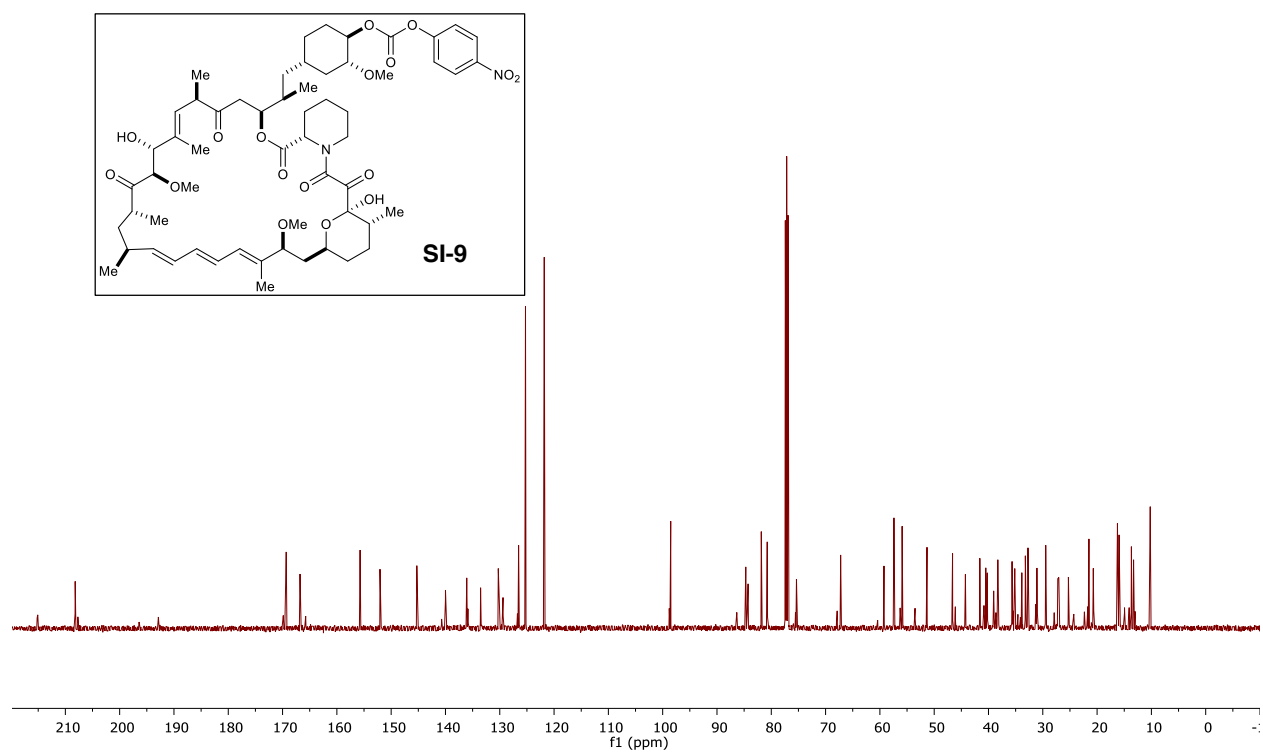


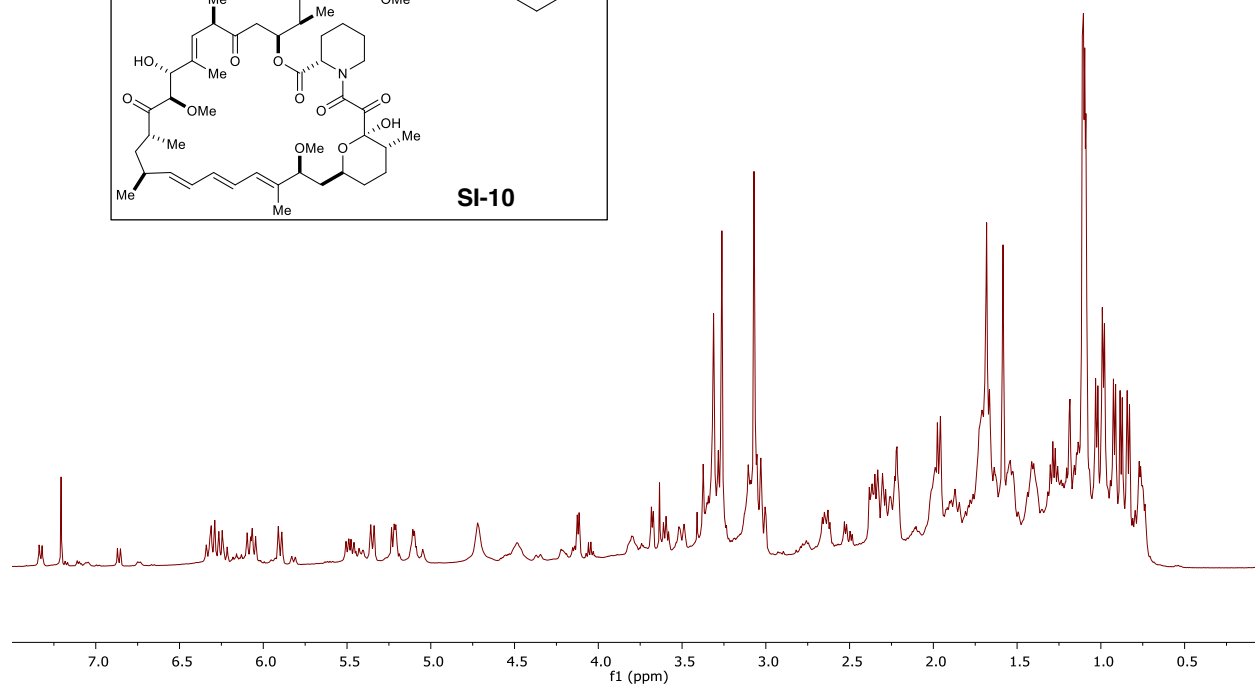
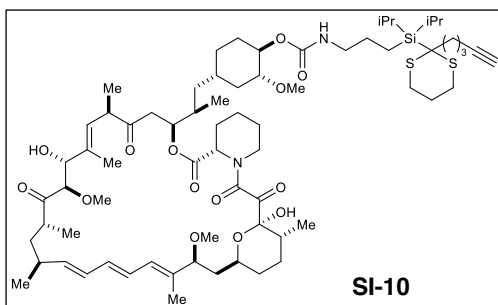
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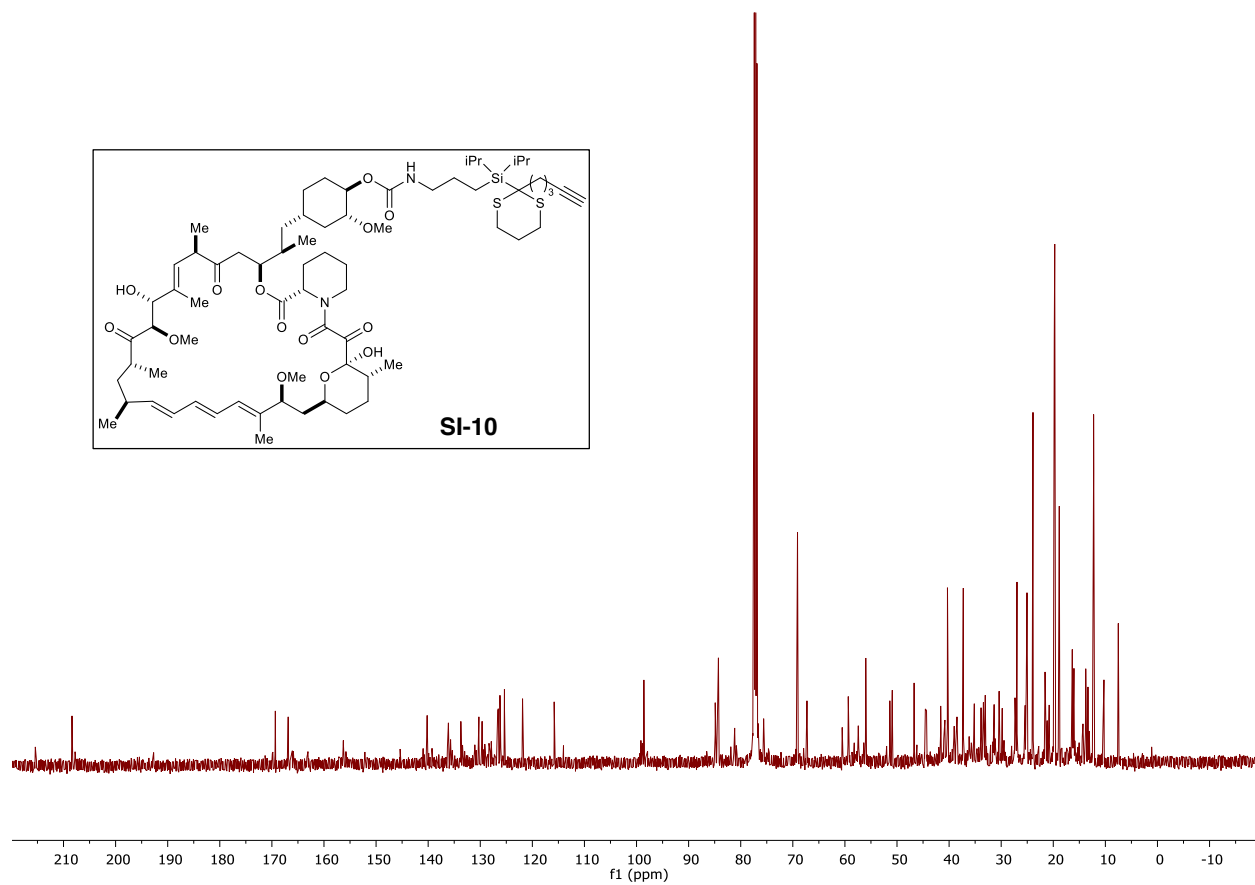


SI-9

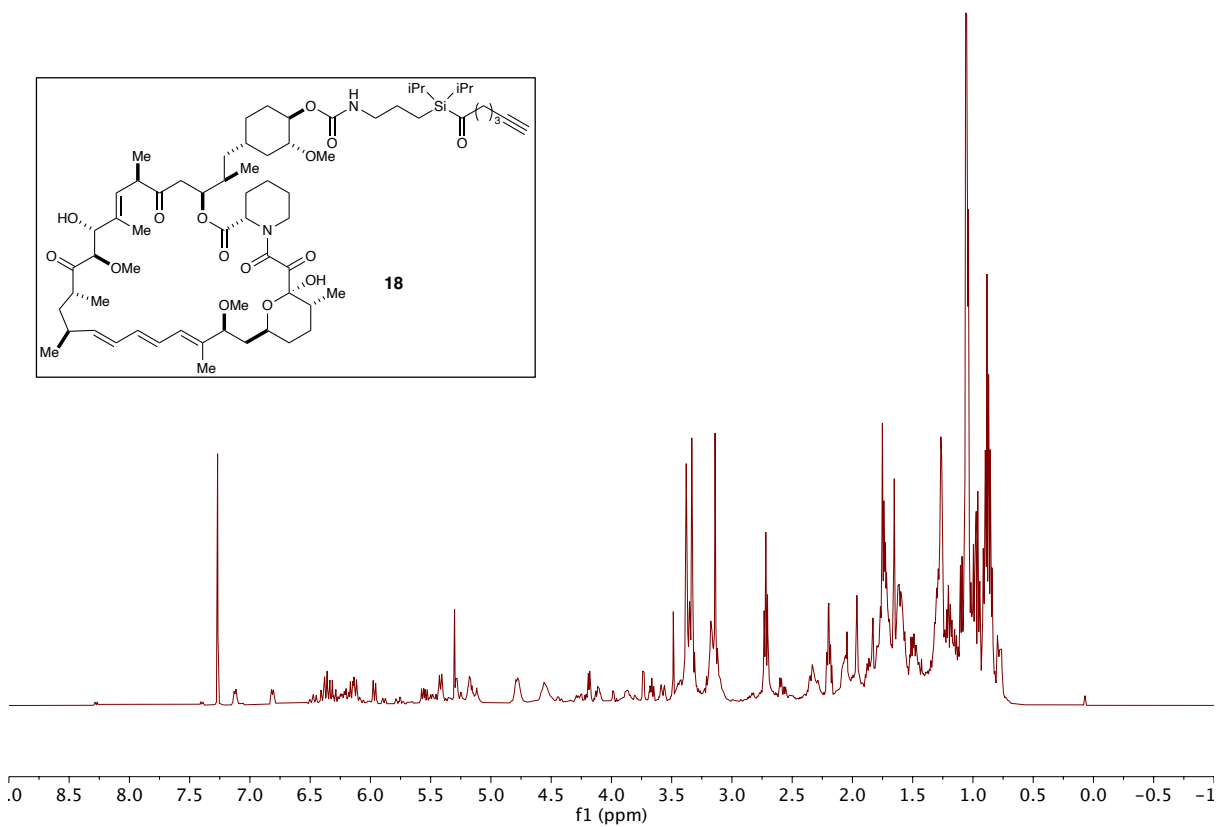




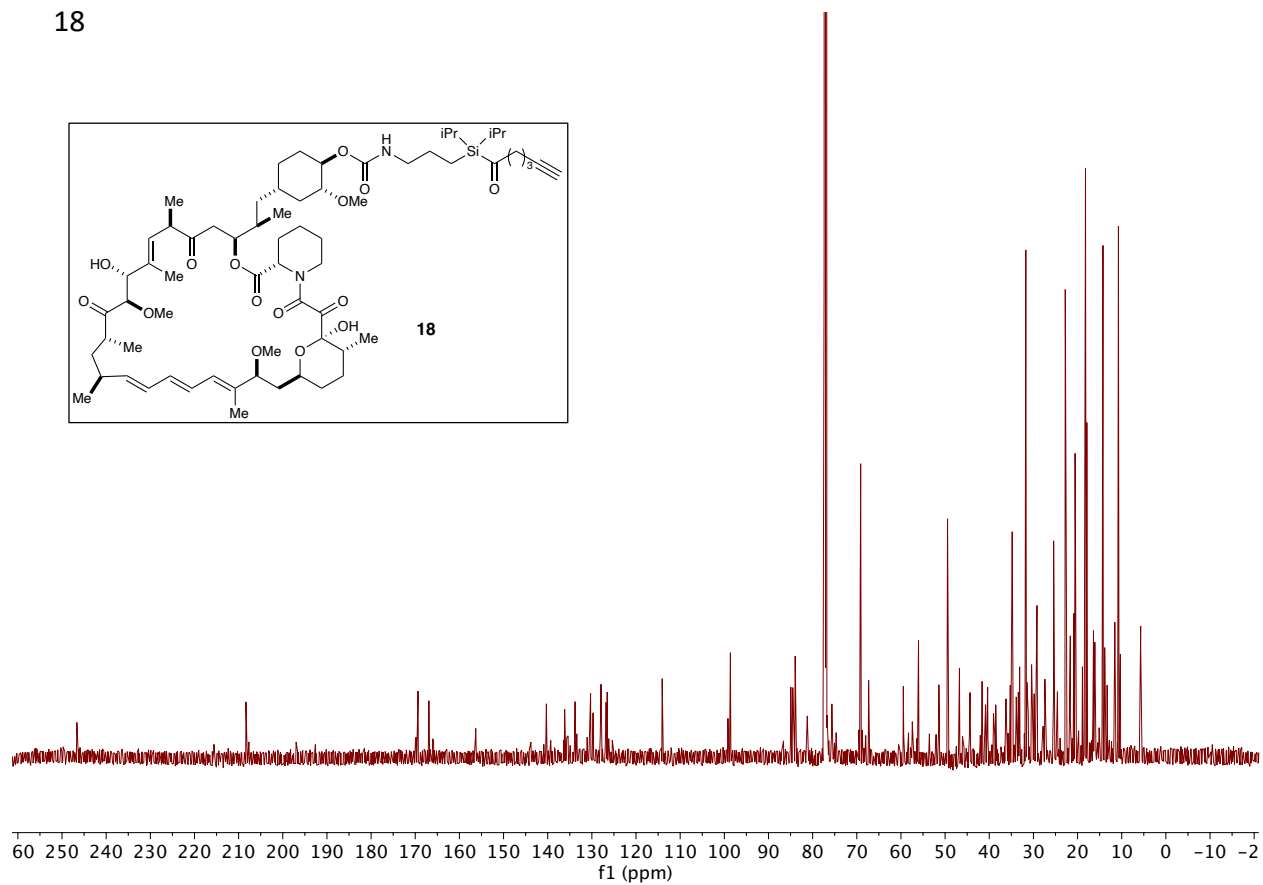




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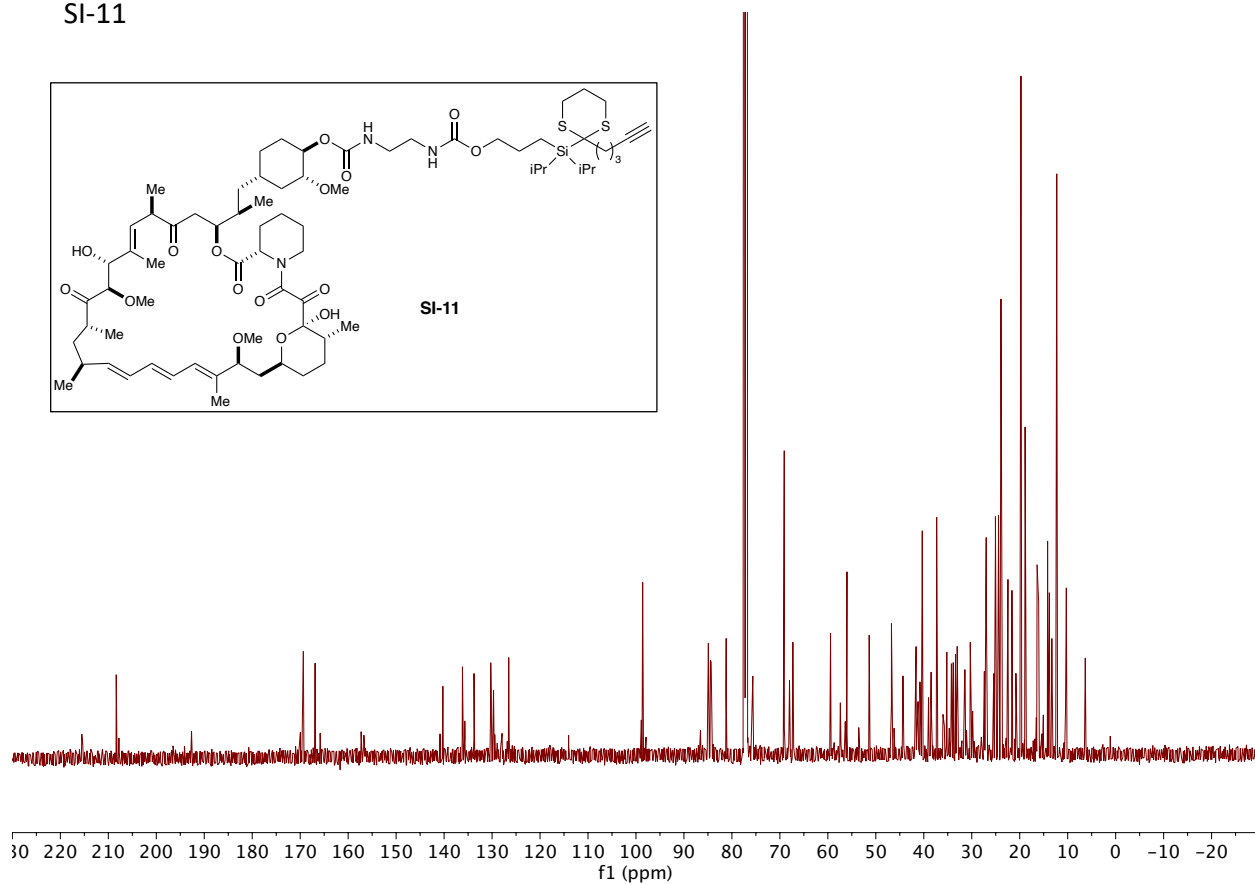
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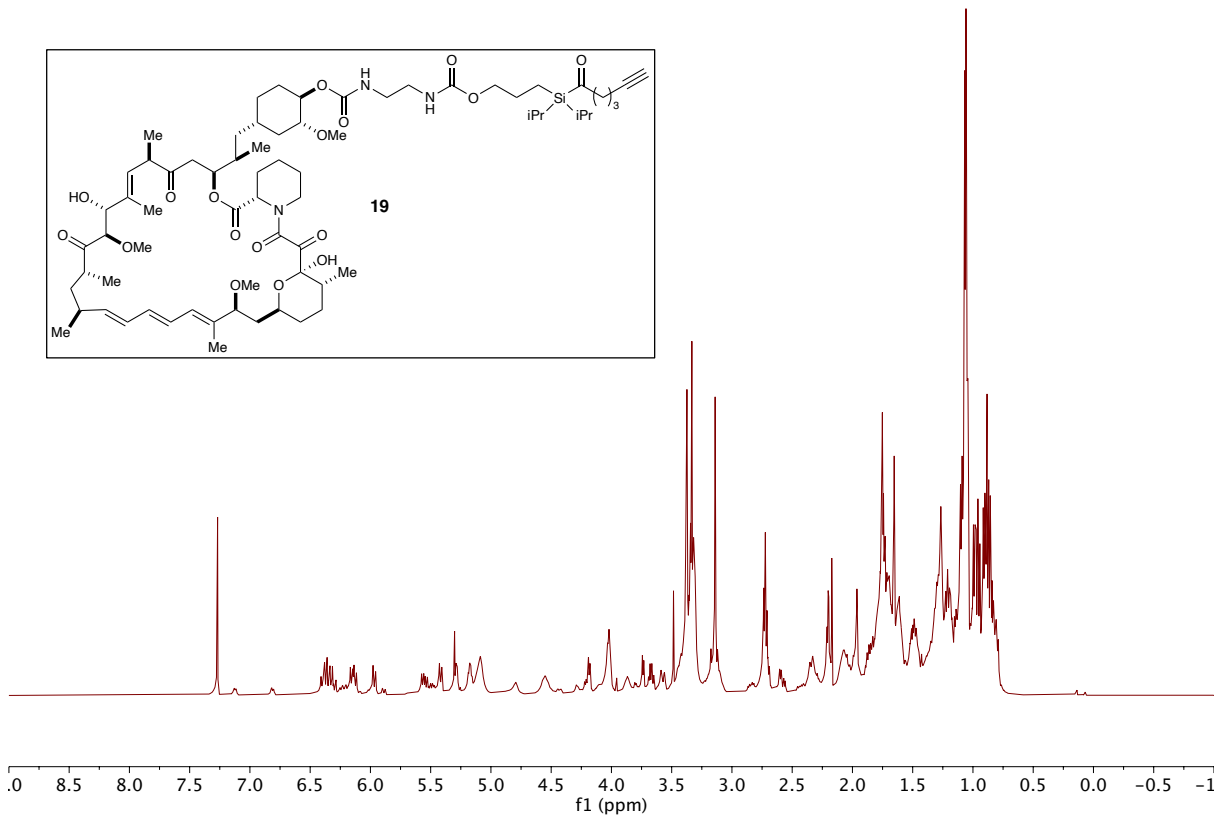
Chemical structure of **SI-11** is shown in the inset. It is a complex molecule featuring a central cyclohexane ring substituted with a methoxycarbonyl group and a side chain containing a piperidine ring, a carboxylic acid, and a long alkene chain. The molecule also includes a trimethylsilyl group and a long alkyne chain.

The ¹H NMR spectrum (CDCl₃) is displayed below the structure. The x-axis represents the chemical shift in ppm, ranging from 0 to 10. The spectrum shows several sharp peaks in the aromatic region (6.5-7.5 ppm), a broad peak around 5.5 ppm, and a large, complex multiplet in the aliphatic region (0.5-4.5 ppm), characteristic of the complex structure of SI-11.

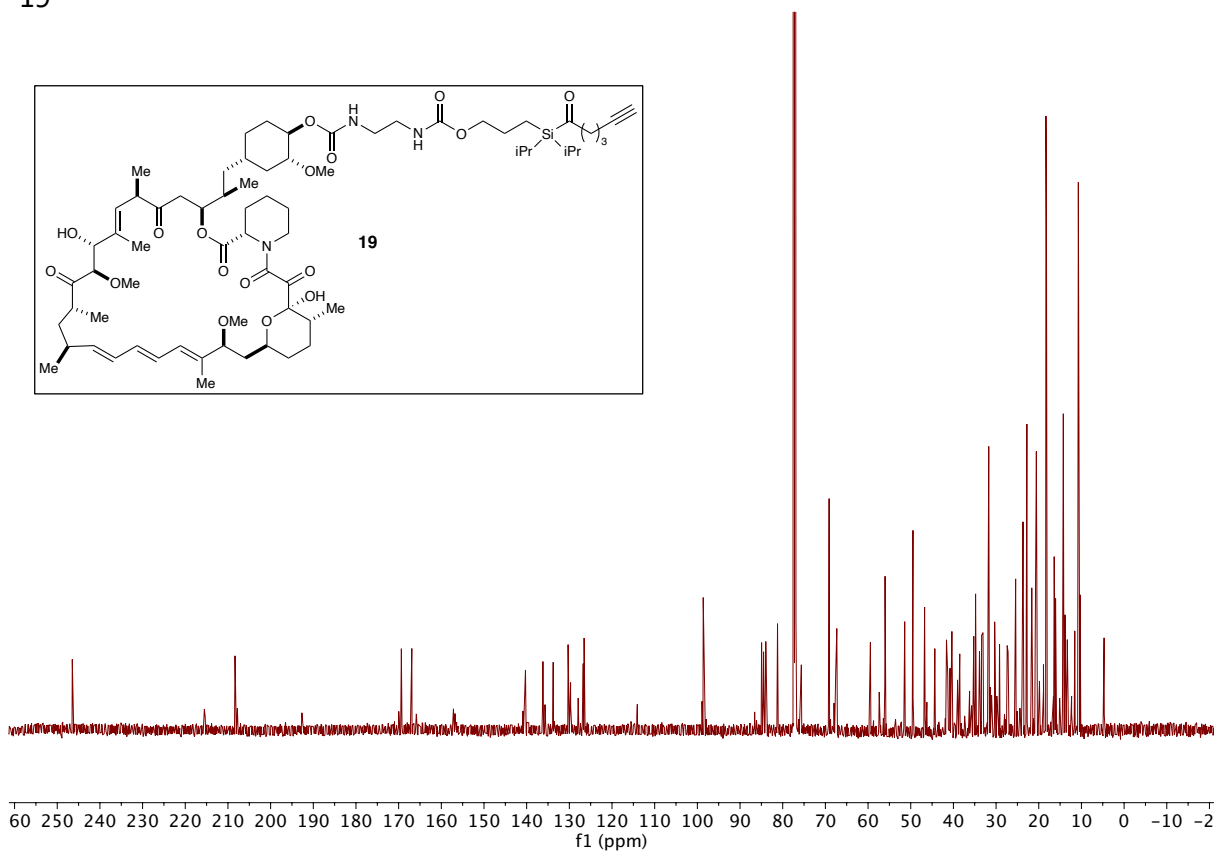
SI-11



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CHAPTER 3

An Activity-Based Oxaziridine Platform for Identifying and Developing Covalent Ligands for Functional Allosteric Methionine Sites

This chapter is partially based on the *Journal of the American Chemical Society* publication “An Activity-Based Oxaziridine Platform for Identifying and Developing Covalent Ligands for Functional Allosteric Methionine Sites: Redox-Dependent Inhibition of Cyclin-Dependent Kinase 4” (29) and has been adapted with permission from all co-authors.

3.1 Abstract

Activity-based protein profiling (ABPP) is a versatile strategy for identifying and characterizing functional protein sites and compounds for therapeutic development. However, the vast majority of ABPP methods for covalent drug discovery target highly nucleophilic amino acids such as cysteine or lysine. Here, we report a methionine-directed ABPP platform using Redox-Activated Chemical Tagging (ReACT), which leverages a biomimetic oxidative ligation strategy for selective methionine modification. Application of ReACT to oncoprotein cyclin-dependent kinase 4 (CDK4) as a representative high-value drug target identified three new ligandable methionine sites. We then synthesized a methionine-targeting covalent ligand library bearing a diverse array of heterocyclic, heteroatom, and stereochemically rich substituents. ABPP screening of this focused library identified 1oxF11 as a covalent modifier of CDK4 at an allosteric M169 site. This compound inhibited kinase activity in a dose-dependent manner on purified protein and in breast cancer cells. Further investigation of 1oxF11 found prominent cation- π and H-bonding interactions stabilizing the binding of this fragment at the M169 site. Quantitative mass-spectrometry studies validated 1oxF11 ligation of CDK4 in breast cancer cell lysates. Further biochemical analyses revealed cross-talk between M169 oxidation and T172 phosphorylation, where M169 oxidation prevented phosphorylation of the activating T172 site on CDK4 and blocked cell cycle progression. By identifying a new mechanism for allosteric methionine redox regulation on CDK4 and developing a unique modality for its therapeutic intervention, this work showcases a generalizable platform that provides a starting point for engaging in broader chemoproteomics and protein ligand discovery efforts to find and target previously undruggable methionine sites.

3.2 Introduction

Covalent small molecules that target specific amino acid residues are powerful chemical tools that can reveal fundamental new protein function and identify lead candidates for accelerating drug discovery (76–83). Indeed, led by advances across broad fields encompassing organic chemistry, chemical biology, cell biology, and bioinformatics, covalent therapeutics now constitute approximately 30% of enzyme-targeting FDA-approved drugs (84). In this context, activity-based protein profiling (ABPP), where chemical probes measure protein function rather than protein abundance (85, 86), has enabled new modalities for fragment-based drug discovery by applying small-molecule screening efforts in conjunction with chemoproteomics for target and site identification and characterization (13, 22, 24, 25, 87–106). These technologies rely on residue-specific covalent warheads that can be used from proteins to proteomes (15, 80, 107–112), yet the majority of reactive probe development to tackle this vast undruggable space has targeted cysteine (16, 113) or lysine (24–26, 95), with relatively limited expansion of this chemical toolbox to other nucleophilic residues like tyrosine (114) and glutamate/aspartate (115, 116).

Motivated to contribute to this area as part of a larger program in our laboratories to create and use activity-based chemical probes for biological applications (117–120), we and others have developed bioconjugation methods for methionine (27, 28, 30, 32, 33, 121), one of two privileged sulfur-containing amino acids, along with cysteine. Methionine is distinguished by its characteristic thioether moiety, which endows this

hydrophobic amino acid with high redox activity and low nucleophilicity relative to its highly redox-active and nucleophilic cysteine congener. The methionine sulfur atom not only enables greater rotational freedom through lower-strain gauche interactions but also provides the opportunity for unique single-atom post-translational modifications (PTM) through a reversible two-electron oxidation to generate both (R)- and (S)-methionine sulfoxide products (122). Akin to kinase writer and phosphatase eraser pairs for installing and removing phosphate PTMs, respectively, stereospecific reduction of methionine sulfoxide sites catalyzed by methionine sulfoxide reductase (Msr) eraser proteins can remove these single-oxygen PTMs. This reversible methionine thioether/sulfoxide cycle plays an integral part in the redox regulation of cell signaling events, antioxidant function, and other forms of protein regulation (123–125).

In contrast to cysteine and other protein nucleophiles, the reactivity of protein-bound methionine is dictated by its redox potential rather than by its pKa. As such, we developed Redox-Activated Chemical Tagging (ReACT), a versatile bioconjugation method that targets methionine through the use of oxaziridine reagents that promote selective nitrene fragment transfer reactivity that is isoelectronic to native methionine oxidation by oxygen atom transfer (27). Methionine functionalization with ReACT proceeds selectively and rapidly at physiological pH and generates stable, mass-spectrometry-compatible sulfimine adducts, enabling further chemoproteomic characterization of putative protein targets and sites of modification. Indeed, ReACT has found utility in the context of the synthesis of stapled cyclic peptides (28), production of antibody–drug conjugates (MetMAb) (33), proximity-activated imaging reporters for protein function (PAIR) (121), ¹⁸F radioimaging tracers, and probes for protein and nucleic acid biotinylation (BioReACT) (126). Here, we present the development of ReACT as a versatile methionine-targeting ABPP platform for covalent ligand discovery. We highlight its application to the study of the cancer-driver protein cyclin-dependent kinase 4 (CDK4), a serine/threonine kinase that serves as a master regulator of mitogenic signaling responsible for G1–S phase progression of the cell cycle (127–129). CDK4 is a high-value therapeutic target that is commonly misregulated in a variety of cancers and is one of many CDKs targeted in cancer drug therapy efforts (130–132). ReACT ABPP identified three new hyperreactive, ligandable methionine residues on CDK4, including an allosteric M169 site that is proximal to an activating T172 phosphorylation site. We then designed and synthesized a focused 179-member oxaziridine fragment library featuring a diverse array of heterocyclic, heteroatom, and stereochemically rich substituents. Gel-based ABPP screening and chemoproteomic characterization identified 1oxF11 as a covalent modifier of the CDK4/Cyclin-D1 (CDK4/CCND1) heterodimer at the M169 site. This compound inhibited the kinase activity of CDK4 in a dose-dependent manner on purified protein and in live cells. Structure–activity relationship (SAR) studies revealed that 1oxF11 is a privileged scaffold for CDK4 binding at the M169 site through prominent cation- π and H-bonding interactions. Quantitative chemoproteomic studies conducted with an alkynyl derivative of 1oxF11 revealed ligation of CDK4 in complex proteomes. Interestingly, further biochemical studies using 1D- and 2D-immunoblot analyses with a phospho-specific pT172 CDK4 antibody revealed reciprocal cross-talk between M169 oxidation and T172 phosphorylation, where M169 oxidation by 1oxF11 prevented phosphorylation of the activating T172 site, thus blocking cell cycle progression at the S-phase checkpoint. The collective data identify a new mechanism for allosteric methionine

regulation on CDK4 and a unique redox vulnerability for therapeutic intervention. Owing to the generality of the ReACT ABPP workflow, this platform provides a starting point for expanding the repertoire of chemical tools for identifying and characterizing new functional methionine sites and developing methionine-targeting covalent ligands for accelerating drug discovery.

3.3 Results and Discussion

3.3.1 ReACT identifies new ligandable methionine sites on the cancer-driver protein CDK4

As a starting point to develop a methionine-targeting ABPP platform for covalent ligand discovery, we chose to study CDK4 as a representative kinase and high-value drug target. Kinases present a unique challenge in the design of selective therapeutics as they share a highly conserved ATP-binding active site among other common domains (133, 134). Indeed, off-target binding of promiscuous kinase inhibitors, many of which are substrate analogues of ATP, has led to the failure of numerous drug candidates in clinical trials due to undesirable side effects (135). As such, identification of allosteric sites distal from the ATP-binding active site pocket provides a promising alternative approach for tuning kinase selectivity.

Against this backdrop and combining the synergistic approaches of fragment-based drug discovery (136–138) and covalent ligand development (77, 80), we sought to expand the scope of proteins that can be targeted by small molecules. This bottom-up approach involves the screening of covalent ligand fragment libraries against broadly reactive, but amino acid-specific, bioconjugation warheads to discover novel binding pockets for covalent modification (139). SARs can then be interrogated to further optimize selectivity and potency after identification of promising fragment leads. To identify potential new ligandable methionine sites in CDK4, we applied a set of three unique ReACT oxaziridine probes to this target: Ox1-alkyne, Ox1-azide, and Ox32-alkyne (Figure 3.1a). Interestingly, each ABPP probe showed a different pattern of covalent methionine labeling on our protein of interest, presaging that these sites can be preferentially targeted. Ox1-azide modifies three reactive sites: M169, M264, and M275 (Figure 3.1b and Figure S3.1). In contrast, Ox1-alkyne labeled only M169, whereas Ox32-alkyne engaged only M264. Sites of modification were determined via shotgun proteomics (Figure 3.1c). The frequency of modifications observed on each methionine site correlated with the solvent accessibility of that residue. M169 displayed the highest reactivity, followed by M264, with M275 being the least solvent-accessible and least reactive. Notably, the newly identified hyperreactive M169 site is proximal to a known T172 phosphorylation site on CDK4 that activates protein function (140, 141).

3.3.2 Design and synthesis of a methionine-targeting covalent ligand library

After identifying hyperreactive methionine residues as potentially new ligandable sites for CDK4, we next sought to develop a methionine-directed covalent ligand platform to engage this protein target through the design and synthesis of a focused oxaziridine fragment library. To this end, we first optimized a suite of synthetic routes to such products, which included three methods for imine synthesis followed by oxidation via mCPBA (Scheme 3.1). As oxaziridine synthesis limited available functional groups to those stable to the final oxidation of the imine intermediate and the oxaziridine itself can

show sensitivity to acid, base, reductants, metals, and thiols, we sought to prepare a focused library to achieve sufficient structural diversity under these constraints.

The design of the oxaziridine library was initiated through a triaging selection of the key diversity element, namely, the corresponding amine component. A total of 43,950 amines were available for selection (Enamine); utilizing ICM Chemist Pro (Molsoft LLC), sub-structure selections were made driven by learnings from our initial study (28). Clustering analysis for each selected amine set was conducted using ICM Chemist Pro (Molsoft LLC), generating the detailed clusters for each sub-type (SI). Thereafter, rounds of selections based on diversity and removal of compounds containing foreseen chemoselectivity issues resulted in a preferred set of 234 amines. These amines were derivatized to the corresponding sulfinimine or urea in silico, and a 3D conformational search allowed C=O stretching frequencies to be determined. In order to ensure the formation of N-transfer sulfinimine over O-transfer sulfoxide products upon reaction with methionine, we employed principal component analysis (PCA) methods to optimize this parameter and found that rates of sulfinimine hydrolysis correlated with the calculated sulfinimine adduct $\nu_{\text{C=O}}$ values (Figure 2a). The finalized library of 179 unique oxaziridine fragments featured a diverse array of functional groups, including spirocycles, halogens, azoles, ethers, and amides (Figure 3.2c and Figure S3.2).

3.3.3 Methionine-directed ABPP screen against CDK4 identifies lead compound fragment 1oxF11

With this oxaziridine fragment library in hand, we proceeded to screen for methionine-directed modifiers of CDK4 via a gel-based ABPP platform (Figure 3.2b). We chose the Ox1-azide ReACT probe as it was the most promiscuous and could engage three hyperreactive methionine sites in competitive binding assays with potential covalent oxaziridine ligands. Briefly, isolated CDK4 was treated either with DMSO (vehicle) or a covalent ligand from the oxaziridine library (ligand-treated). Samples were then treated with Ox1-azide probe followed by a quench step with N-acetyl methionine (NAM) to remove any excess oxaziridine. DBCO-Cy3 was then introduced by strain-promoted click chemistry to provide a fluorescence readout. The samples were subsequently separated via SDS-PAGE, and fluorescence signals were normalized using a silver stain to triage any covalent ligands that induced general protein aggregation, which would generate a false positive Cy3 signal (Figure 3.2b).

Using ReACT, ABPP screening of the focused oxaziridine fragment library on CDK4 revealed 1oxF11 as a candidate for further study (Figure 3.2d and Figure S3.3). This fragment showed competition with the Ox1-azide probe for CDK4 binding in a dose-dependent manner. We then tested the isoform specificity with two closely related congeners, CDK1 and CDK6. CDK1 is ubiquitously expressed and essential for healthy cell function, and CDK6 shows the highest structural similarity to CDK4 (142). Indeed, all CDK4 inhibitors currently in therapeutic use also display activity against CDK6. Along these lines, we were pleased to observe that 1oxF11 displayed no dose-dependent labeling against either CDK1 or CDK6 by gel analysis (Figure 3.2e). Additionally, we confirmed that this compound did not induce general aggregation in cell lysates (Figure S3.4).

3.3.4 1oxF11 is a covalent modifier of CDK4 at the M169 site and inhibits activity on purified protein

We next characterized the sites of modification and subsequent effect on protein activity on CDK4 by 1oxF11 treatment in vitro. To start, we performed shotgun proteomics with 1oxF11 on the purified and active CDK4/CCND1 heterodimer. We identified that the primary site of modification on CDK4 by 1oxF11 was M169, with minor labeling at M264 (Figure 3.3a and Figure S3.5). We then mutated the M169 site to leucine in order to further investigate the effects of M169 ligation. When this site is mutated, the primary site of modification was identified as M264 (Figure 3.3b). Next, we demonstrated that 1oxF11 inhibits activity of the CDK4/CCND1 complex in a dose-dependent manner using a luciferase-based activity assay as a proxy for kinase activity. Here, we observed an IC50 value of 1.89 μ M for the WT form and 3.00 μ M for the M169L variant (Figure 3.3c). We posit that the primary negative modulator for kinase activity is M169 ligation, while M264 ligation may also affect protein activity through another allosteric mechanism.

3.3.5 1oxF11 is a privileged scaffold for binding the M169 site of CDK4

To better understand the propensity for 1oxF11 to bind the M169 site, we performed molecular dynamics studies on 1oxF11 (Figure 3.4a and Movie S3.2). These studies revealed that 1oxF11 binds the M169 site through prolonged cation- π interactions with R139/R181 of CDK4 at the phenyl moiety. Critically, the R181 residue has a strong, pronounced hydrogen-bonding interaction with the carbonyl present on the urea moiety of 1oxF11, which is further stabilized by transient H-bonding interactions with V174 (Figure 3.4b). We determined the strength of these interactions by calculating the percent interaction time with each residue of CDK4 across the simulation (Figure 3.4c). Furthermore, we observed that the tetrahydrofuranyl moiety of 1oxF11 is conformationally locked in a position that enables a hydrogen bonding interaction with the amide backbone of V174. Together, these data inform the binding mode of 1oxF11 in the M169 pocket, positioning the oxaziridine for proximal M169 ligation (Figure 3.4d). We also performed molecular dynamics simulations on two other CDK4 binders, 1oxA6 and 1oxH2, and found that their noncovalent interactions are much less pronounced, leading to reduced occupancy time (Figure S3.6 and Movies S3.1 and S3.3).

3.3.6 1oxF11 decreases cell viability and inhibits cellular CDK4 activity

Turning our attention to cellular studies, we next sought to identify a model cell line that displayed heightened sensitivity to 1oxF11. We screened 1oxF11 across a small panel of cancer cell lines with sensitivity to ribociclib, a clinically approved CDK4/6 inhibitor (Figure 3.5a). We selected the human breast adenocarcinoma line MCF-7 as a model for further study due to its pronounced sensitivity to 1oxF11 treatment (Figure 3.5a). Additionally, the contributions of CDK4 to uncontrolled cell division in the MCF-7 line have been well documented (143, 144). Indeed, we observed a dose-dependent decrease in the cell viability of MCF-7 cells in response to 1oxF11, with an EC50 of 329 μ M (Figure 3.5b). This lower EC50 observed in cells compared to in vitro is likely the result of several factors including cell permeability, non-productive consumption by thiols, and/or off-target effects. We also investigated the effects of 1oxF11 treatment specific to the M169 site in cellulo by generating constructs for the transient mammalian transfection of both FLAG-tagged WT and M169L variants of CDK4. We began by constitutively

knocking down endogenous CDK4 expression, followed by transient expression of both variants to control for background inhibition of endogenously expressed CDK4. We observed EC50 values of 189 and 296 μ M for transfected WT and M169L variants, respectively (Figure 3.5b). This complements our in vitro results, suggesting that modification of M169 leads to decreased MCF-7 cell viability. We attribute the decreased EC50 values for transfected cells compared to the untransfected cells to the increased sensitivity of mammalian cells following treatment with harsh transfection reagents.

To further probe how 1oxF11 contributes to the observed cellular phenotype, we synthesized a 1oxF11 analogue, 1oxF11yne, containing an alkyne handle for detection and enrichment using click chemistry (Figure 3.5c). Shotgun proteomics experiments revealed that the 1oxF11yne probe modifies CDK4 selectively at the same M169 site as the parent 1oxF11 fragment (Figure 3.5d). We then sought to test the effects of both probes on CDK4 activity within a cellular context. To achieve this goal, we turned to immunoblot analysis, monitoring the phosphorylation status of retinoblastoma protein (Rb), the main cellular substrate of CDK4. When active, CDK4 in complex with its cognate cyclin partner phosphorylates Rb at one of 14 sites (144) (Figure 3.5e). We used phospho-responsive antibodies specific for three of these sites—S780, S807, and S811—as a method to assess CDK4 activity in cells. To control for increased background levels of pRb due to cells being in different stages of the cell cycle, we serum-synchronized MCF-7 cells to the G0/G1 phase. Indeed, incubation of serum-synchronized MCF-7 cells with either 1oxF11 or 1oxF11yne at a dose of 500 μ M resulted in a marked decrease in signal from the pRb antibodies, suggesting a decrease in cellular CDK4 activity under these conditions (Figure 3.5f). Finally, we confirmed CDK4 target engagement in cells using a competition binding assay between 1oxF11 and 1oxF11yne (Figure 3.5g). To overcome detection challenges with the low endogenous expression levels of CDK4 even in cells known to upregulate proteins such as MCF-7, we transfected cells with WT CDK4 to achieve transient expression in these models. Treatment of cells with a titration of 1oxF11 prior to incubation with 500 μ M 1oxF11yne probe showed a dose-dependent decrease in signal in eluted proteins and a corresponding increase in signal in the respective supernatant, indicating competition between the two compounds and engagement of CDK4 in a cellular context (Figure S3.7). Additionally, 1oxF11yne displayed lower promiscuity in lysate compared to Ox32-alkyne, further suggesting its heightened selectivity in a complex milieu of proteins (Figure S3.8).

3.3.7 ReDiMe-ABPP identifies CDK4 as a target of 1oxF11yne in MCF-7 lysates

Following our promising immunoblotting and substrate promiscuity results, we next sought to apply quantitative chemoproteomics to identify targets of 1oxF11yne in a proteome-wide context. With the 1oxF11yne probe in hand, we enriched the protein targets of 1oxF11yne by first treating MCF-7 lysates expressing CDK4 with 500 μ M of the probe followed by CuAAC to a desthiobiotin azide, enabling streptavidin pulldown of the labeled targets. In order to exclude proteins that non-specifically bind to the streptavidin interface, we also treated MCF-7 lysates with a vehicle control followed by CuAAC and enrichment. We incorporated reductive dimethylation (ReDiMe) labeling to our ABPP platform, enabling sample multiplexing of 1oxF11yne targets against non-specific binders and providing an isotopic signature to distinguish between the two protein groups (Figure 3.6a). Through these studies, we identified 59 protein targets that are engaged by

1oxF11yne including CDK4 (Figure 3.6b) with no overlap to the DMSO control. A full list of these protein targets was also generated (SI). We further analyzed the subcellular distribution of these targets and found that the majority of these proteins are cytosolically or nuclearly localized, providing further evidence of probe quenching in the cytosolic environment through off-target effects (Figure 3.6c). We also assessed the function of these protein targets through PANTHER GO analyses, demonstrating a clear enrichment in proteins involved in protein–protein/protein–small molecule binding as well as catalysis (Figure 3.6d). Together, these data provide a promising avenue for future structure optimization to increase the selectivity of 1oxF11 for CDK4 and thereby increase its potency in a proteome-wide context.

3.3.8 Biochemical studies of CDK4 inhibition by 1oxF11 reveal reciprocal crosstalk between M169 oxidation and T172 phosphorylation

With data establishing 1oxF11 as a covalent modifier of CDK4 at a newly identified allosteric M169 site along with its ability to inhibit CDK4 activity on purified protein and in cells and decrease cell viability in a dose-dependent manner combined with chemoproteomic verification of target engagement in cellulo, we next sought to further interrogate its potential mechanism of action at the biochemical level. In this context, CDK4 plays a key role in the cell cycle in clearing the cell for division, only allowing for passage through the S-phase checkpoint when properly activated. In particular, this signaling pathway relies on proper binding of CDK4 to its respective cyclin as well as phosphorylation at T172 by cyclin-dependent activating kinase (CAK) to activate the protein (145, 146). Owing to the proximity of M169 to this activating T172 phosphorylation site, we hypothesized that M169 may act as an allosteric redox regulatory switch at this S-phase checkpoint.

Oxidation can transform the normally hydrophobic methionine residue into a hyperpolarized and sterically demanding methionine sulfoxide congener, which vastly modulates the electrostatic and steric environment of this residue. We hypothesized that oxidation could block access of CAK to T172 and prevent its phosphorylation, thus causing the cell to fail the S-phase checkpoint (Figure 3.7a). This type of cross-talk between methionine oxidation and adjacent phosphorylation sites has been reported for other systems (147, 148). Indeed, M169 and T172 lie in a flexible region of CDK4 that can come within 7 angstroms of each other, a distance observed to undergo this phenomenon previously (149) (Figure 3.7b). We performed 2D-immunoblot analyses utilizing a pCDK4-Thr172-specific antibody to investigate the effects of 1oxF11 treatment on CDK4 activation. Through 2D-analysis, we were able to separate distinct proteoforms of pCDK4, with spot 3 corresponding to CDK4 in its T172 phosphorylated state and spot 1 corresponding to unphosphorylated CDK4. We observed that treatment with 1oxF11 was indeed able to diminish T172 phosphorylation status on CDK4 in MCF-7 cells in a dose-dependent manner (Figure 3.7c). To control for differences in protein expression, we normalized pCDK4-T172 levels to unphosphorylated CDK4 (spot 3 normalized to spot 1). These data, along with supporting evidence showing engagement of CDK4 through chemoproteomics and immunoblotting as well as inhibition of its activity both in vitro and in these same MCF-7 cell models, support a model in which M169 oxidation/T172 phosphorylation cross-talk offers a potential new redox vulnerability where oxidative

modification of CDK4 at an allosteric M169 by 1oxF11 inhibits CDK4 activity by hindering phosphorylation at its activating T172 site.

3.4 Conclusions

We have presented a methionine-directed ABPP platform for identifying and developing covalent ligands for new functional methionine sites. We highlighted the potential value of this approach using CDK4 as a representative high-value target to showcase the application of ReACT probes to reveal fundamental new chemical functions on proteins and accelerate drug discovery efforts by expanding covalent ligand development beyond the more common cysteine and lysine protein nucleophiles. Indeed, we applied methionine-directed ReACT probes with broad reactivity to identify novel hyperreactive, ligandable methionine sites on CDK4. We then optimized the synthesis of oxaziridine probes and used these methods to design and synthesize a focused covalent ligand library of ca. 180 oxaziridine fragments bearing chemically diverse functional groups, including spirocycles, halogens, azoles, ethers, and amides. Synthesis of the fragment library was guided by computational design to ensure efficient N-transfer rates and sulfimine stability of the subsequent products with methionine. We moved on to establish that this ReACT ABPP platform was useful for fragment-based screening efforts against the representative oncoprotein CDK4. Chemoproteomic experiments revealed that fragment 1oxF11 was a covalent modifier of CDK4 that selectively labeled its allosteric M169 site with isoform specificity over CDK1 and CDK6. We derived structure–activity relationships from molecular dynamics studies that suggest that the tetrahydrofuranyl moiety on 1oxF11 uniquely positions it toward a hydrogen bonding interaction with the amide backbone of V174 in addition to cation- π interactions with the phenyl moiety alpha to the oxaziridine. Biochemical and cell-based assays showed that 1oxF11 can inhibit CDK4 activity on purified protein and in cells and decrease cell viability in a dose-dependent manner, with detection of target engagement in cells enabled by the synthesis of a 1oxF11yne probe bearing an alkyne handle for detection and enrichment. Quantitative chemoproteomics studies demonstrated that 1oxF11yne engages CDK4 in a proteome context with 59 other unique protein targets identified. Additionally, biochemical studies uncovered a novel redox regulatory mechanism for kinase inactivation through reciprocal oxidation/phosphorylation cross-talk between proximal M169 and T172 residues in CDK4, where M169 oxidation hinders phosphorylation at the protein-activating T172 site. Indeed, use of a phospho-specific pT172-CDK4 antibody in 1D- and 2D-immunoblot analyses established that treatment with the M169-modifying 1oxF11 covalent ligand diminished T172 phosphorylation and CDK4 activity. The resulting loss of CDK4 function prevented downstream Rb phosphorylation at S780 and S807/811, leading to cell cycle arrest by failure at the S-phase checkpoint. Our findings thus support a role for M169 as a redox sensor site at the S-phase checkpoint, preventing cell division under highly oxidative conditions by sterically preventing phosphorylation at T172. This newly discovered redox vulnerability in CDK4 provides an alternative modality for therapeutic intervention. Current and future efforts are geared to increase the size and diversity of methionine-directed activity-based probes and covalent ligand platforms to improve potency and selectivity, pursue other high-value targets and therapeutic modalities, and expand these approaches to other native amino acids in the proteome beyond cysteine, lysine, and methionine.

3.5 Acknowledgements

We thank Novartis Institutes for BioMedical Research and the Novartis-Berkeley Center for Proteomics and Chemistry Technologies (NB-CPACT) for supporting this work. We also thank the NIH (R01 GM139245 and ES28096 to C.J.C. and R35 GM118190 to F.D.T.) for funding. A.G.R., A.G.-V., and A.H.C. thank the NSF Graduate Fellowship Program for financial support. A.G.R. and A.G.-V. were partially supported by a Chemical Biology Training Grant from the NIH (T32GM066698). P.J.M. thanks the Natural Sciences and Engineering Research Council of Canada (NSERC) for a postdoctoral fellowship. S.W.M.C. is supported by the AGBT-Elaine R. Mardis Fellowship in Cancer Genomics from the Damon Runyon Cancer Research Foundation and The Genome Partnership, Inc. (DRG-2395-20). X.X. and D.H. are Tang Distinguished Scholars of the University of California, Berkeley. P.Z.M. thanks the American Cancer Society for a postdoctoral fellowship (PF-18-132-01-CDD). C.J.C. is a CIFAR Fellow. P.P.R. and K.C. thank WALInnov (CICLIBT-EST 1710166) and the FRS-FNRS (J.0141.19) for funding. P.P.R. is a Senior Research Associate of the FRS-FNRS. We thank Alison Killilea (UC Berkeley Tissue Culture Facility) and Prof. Daniel Nomura for technical assistance, Dr. Hasan Celik for assistance with NMR spectroscopy (NIHS10OD024998), and Drs. Kathleen Durkin and David Small for computational support (NIH S10OD023532).

3.6 Figures and Schemes

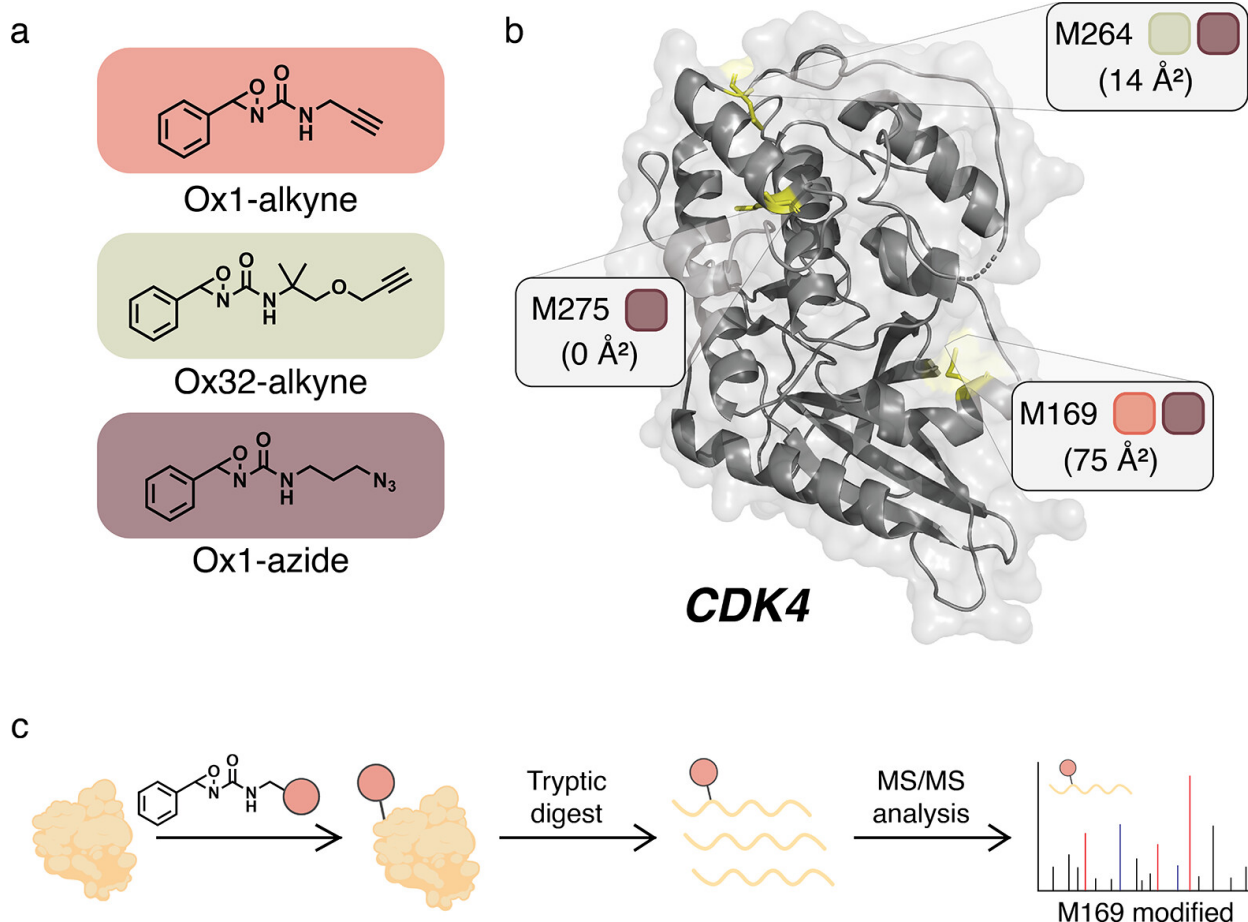
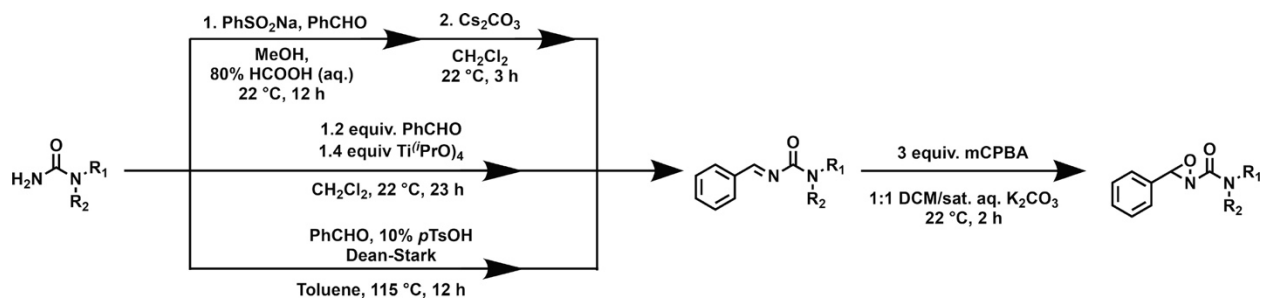


Figure 3.1. Activity-based protein profiling (ABPP) using oxaziridine probes for Redox-Activated Chemical Tagging (ReACT) identifies new hyperreactive, ligandable methionine sites on CDK4.

(a) Structures of Ox1-alkyne, Ox32-alkyne, and Ox1-azide ABPP probes. (b) Ribbon diagram of CDK4 (PDB: [2W9Z](#)). Methionine residues modified by oxaziridines are highlighted, with colored squares representing the corresponding ReACT reagents found to modify each site. Each residue is additionally labeled with its calculated solvent accessibility. (c) Shotgun proteomics general workflow. Isolated protein is first incubated with compound. After excess compound is neutralized, protein is tryptic-digested and analyzed via MS/MS to reveal compound site(s) of modification.



Scheme 3.1. General synthetic routes to oxaziridine compounds for creating a focused covalent ligand fragment library.

All syntheses began with primary or secondary amine synthons. Three routes were utilized to generate imine intermediates depending on the starting amine. All imines were converted to oxaziridines using the same convergent method outlined.

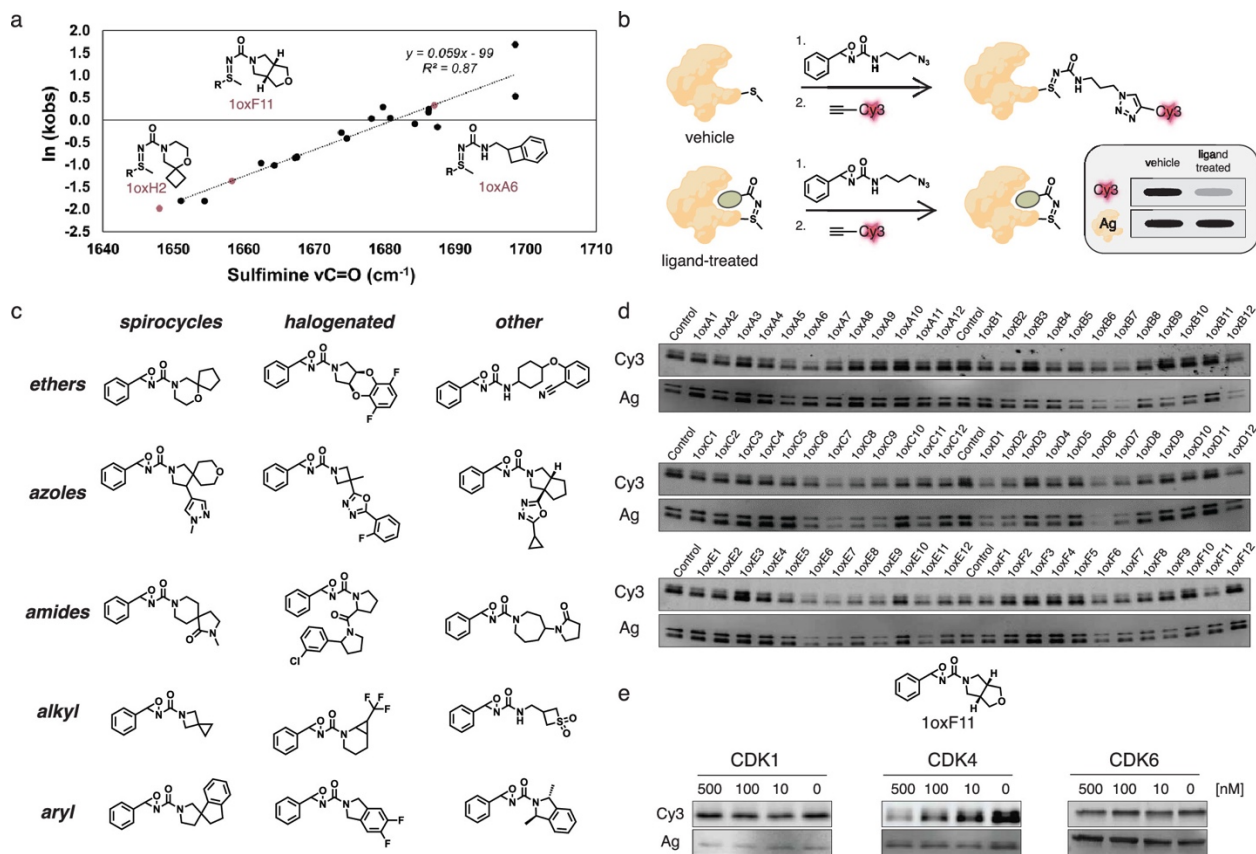


Figure 3.2. Design, synthesis, and evaluation of an oxaziridine-based covalent ligand library for targeting methionine sites and identification of 1oxF11 as a covalent modifier of CDK4 via gel-based ABPP screening.

(a) Design of oxaziridine fragments that favor the formation of *N*-transfer sulfimine over *O*-transfer sulfoxide products upon reaction with methionine using principal component analysis (PCA). Rates of sulfimine hydrolysis correlate with calculated $\nu\text{C}=\text{O}$ values. (b) Schematic of gel-based ABPP screening workflow. The protein target is preincubated with a covalent ligand followed by treatment with an Ox1-azide probe. Excess oxaziridine is then quenched with *N*-acetyl methionine (NAM), and the sample was treated with DBCO-Cy3 overnight for fluorescence detection. Samples are finally separated by SDS-PAGE. Loss of fluorescent signal suggests competitive ligand binding to the protein. Silver stain is used to identify and exclude samples where signal loss corresponds to overall protein loss, likely due to aggregation induced by the ligand. (c) Representative structure types within the oxaziridine fragment library, organized by common functional groups. (d) Representative data from gel-based ABPP screen. CDK4 and ligand incubated at equimolar doses. (e) Structure of 1oxF11 fragment identified in gel-based ABPP screens as a competitive ligand for CDK4. Dose-dependent treatment of 1oxF11 against CDK4, CDK1, and CDK6 shows selective loss of fluorescent signal only with CDK4, suggesting isoform-specific engagement of this target.

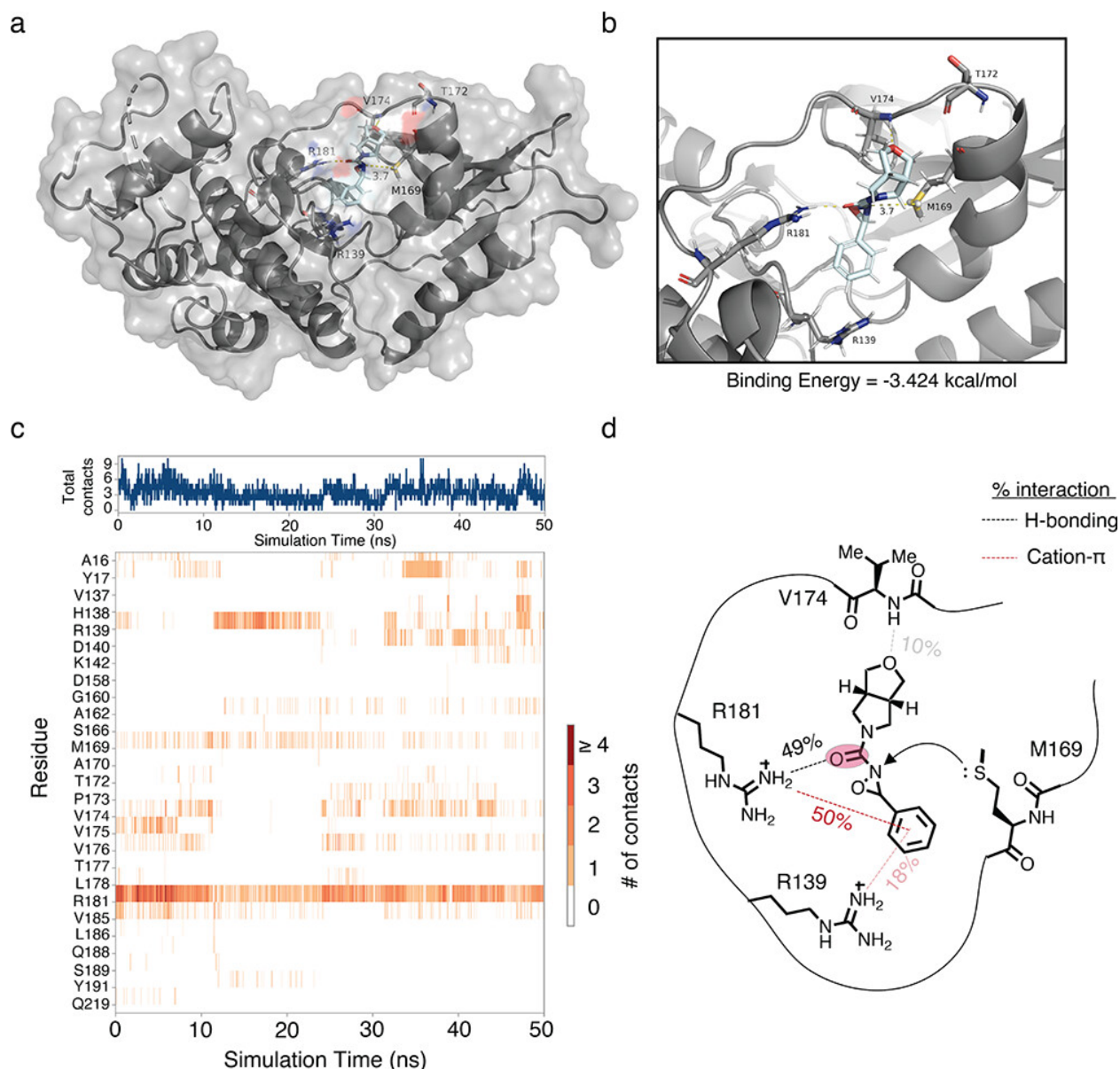


Figure 3.4. Molecular dynamics of 1oxF11 bound to CDK4.

(a) Full structure of CDK4 with 1oxF11 noncovalently bound. (b) Expansion of M169 binding pocket. (c) Ligand-residue interaction diagram over time. (d) Schematic of 1oxF11 binding.

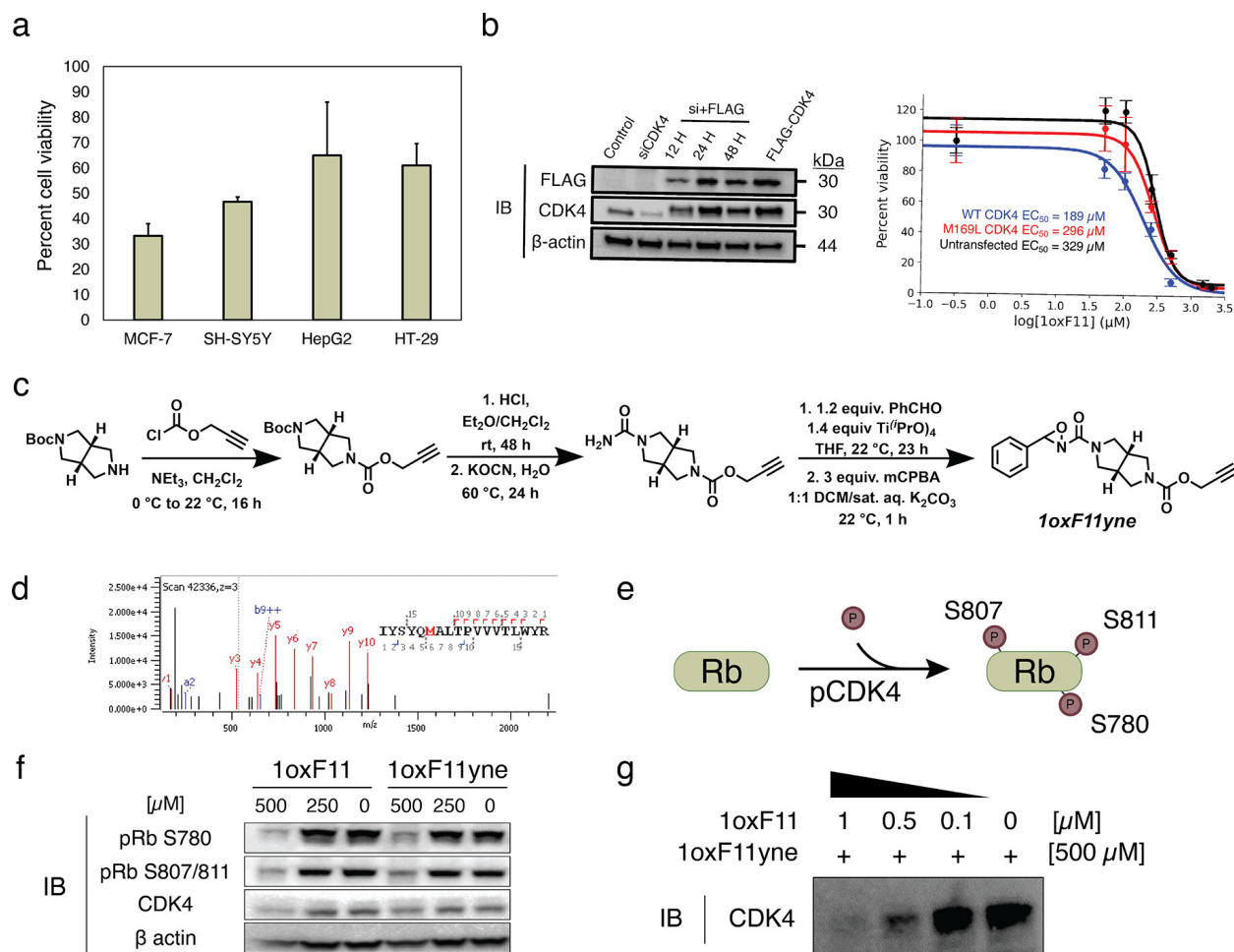


Figure 3.5. Decrease in cell viability and inhibition of cellular CDK4 activity by 1oxF11 and its 1oxF11yne analogue in various cancer cell models.

(a) Screening of ribociclib-sensitive cell lines by treatment with 500 μM 1oxF11 showing differential cell viability profiles across cancer types. The MCF-7 cell line displays the highest sensitivity to 1oxF11. Error bars represent standard deviation of $n = 3$ biological replicates. (b) Left: Immunoblot timecourse of constitutive knockdown and overexpression of transiently expressed CDK4. Right: Dose-response curves of untransfected and transfected MCF-7 cells transiently expressing CDK4 treated with 1oxF11. Error bars represent standard deviation of $n = 3$ biological replicates. (c) Synthetic route of 1oxF11yne bearing an alkyne handle for bio-orthogonal detection and enrichment purposes. (d) MS^2 data showing that 1oxF11yne is a covalent modifier of CDK4 at the same M169 site as the parent 1oxF11 fragment. (e) Simplified scheme of cellular CDK4 activity, where Rb represents the native substrate of CDK4. Immunoblot analyses measuring the extent of Rb phosphorylation provides a method to detect intracellular CDK4 activity. (f) Immunoblot analysis of MCF-7 cells treated with a titration of 1oxF11 and 1oxF11yne to assess changes in cellular CDK4 activity. (g) Competition binding assay between 1oxF11 and 1oxF11yne, providing evidence for target engagement in MCF-7 cells.

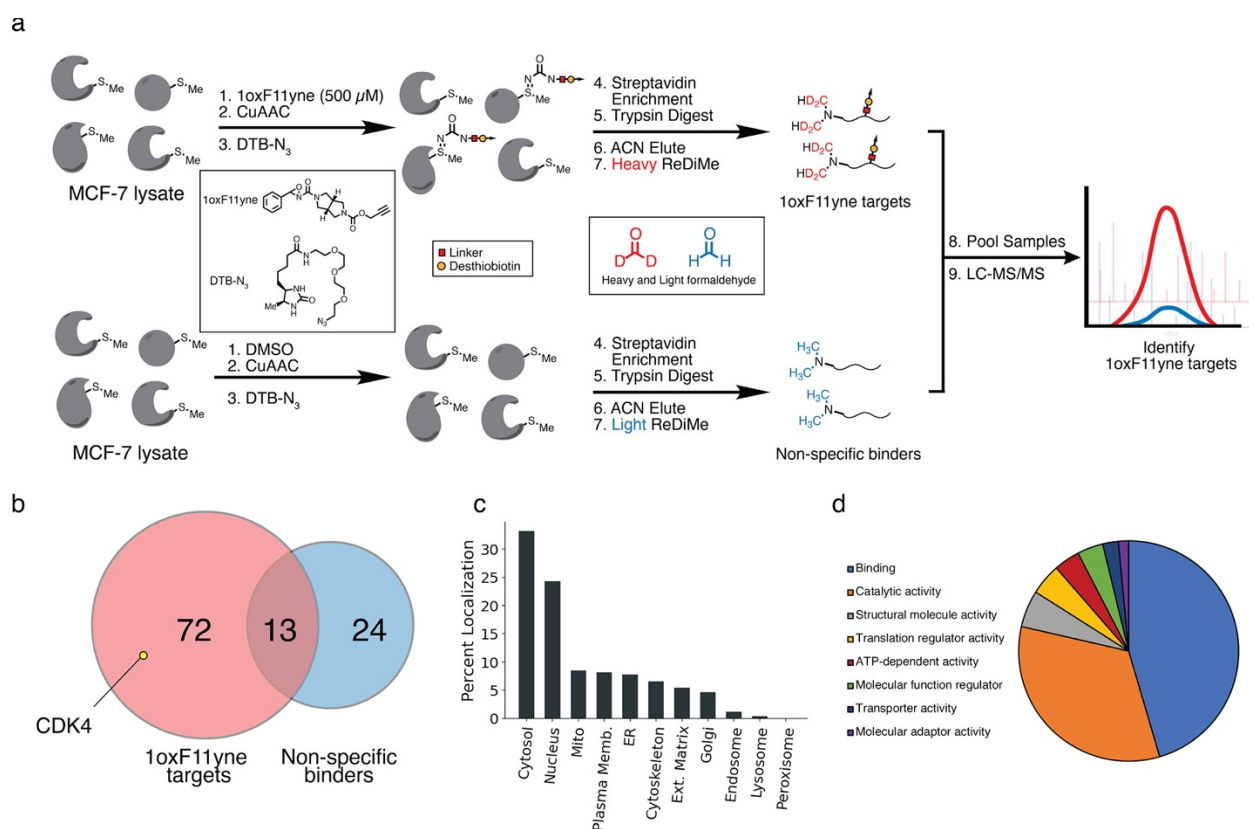


Figure 3.6. ReDiMe-ABPP platform to identify targets of 1oxF11yne in MCF-7 lysates. (a) Schematic of the chemoproteomic platform to identify protein targets of 1oxF11yne. MCF-7 lysates transiently expressing CDK4 were treated with either 500 μ M 1oxF11yne or vehicle followed by CuAAC to a DTB- N_3 . Samples were then separately enriched with streptavidin–agarose beads and tryptically digested. Following peptide elution, the samples were differentially labeled with light or heavy isotopes at their N termini through reductive dimethylation to enable identification of 1oxF11yne targets compared to proteins that non-specifically bind to the streptavidin–agarose interface. Samples were multiplexed and analyzed across $n = 3$ biological replicates. (b) Unique protein targets identified by ReDiMe-ABPP. A total of 72 unique protein targets with $n = 2$ statistical representation were identified as 1oxF11yne targets including CDK4, with some overlap to non-specific binders. (c) Subcellular distribution of identified 1oxF11yne targets. (d) PANTHER GO protein function of identified 1oxF11yne targets.

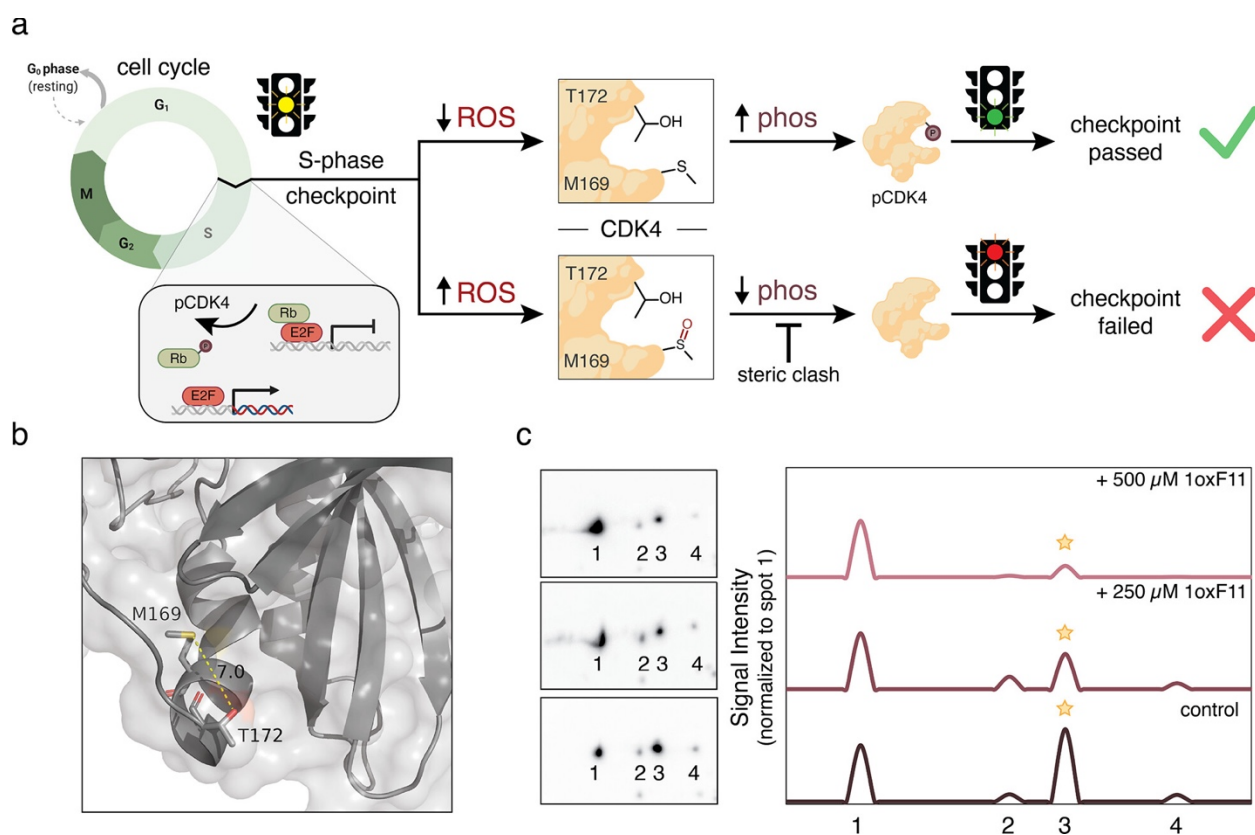


Figure 3.7. The ReACT covalent ligand probe platform enables discovery of a reciprocal oxidation/phosphorylation crosstalk pathway in CDK4 through proximal allosteric M169 and T172 sites, where M169-targeted oxidation can inhibit CDK4 activity by preventing phosphorylation at T172.

(a) Schematic outlining oxidation/phosphorylation cross-talk between CDK4 M169 and T172. Under low oxidative conditions, T172 of CDK4 is phosphorylated by cyclin-dependent activating kinase (CAK) as part of a critical activating step, leading to cell division to pass the S-phase checkpoint. High oxidative conditions can lead to oxidation at M169, which blocks the T172 phosphorylation site, thus preventing cell division via S-phase checkpoint failure. This cross-talk identifies a methionine redox-dependent vulnerability for potential therapeutic intervention. (b) Ribbon structure (PDB: [2W9Z](#)) highlighting proximity of M169 and T172. (c) Monitoring CDK4 phosphorylation status using 2D-immunoblot analyses with phospho-specific CDK4 antibodies. Phosphorylation at T172 decreases with increasing concentrations of added 1oxF11 in MCF-7 cells. Spots 1, 2, 3, and 4 represent different phosphorylation states of CDK4, with spot 1 being unphosphorylated and spot 3 being monophosphorylated at T172. Peaks represent changes in intensity of spots normalized to peak 1 intensity. A clear decrease in spot 3 is observed upon treatment with increasing doses of 1oxF11, consistent with a model where this covalent ligand inhibits CDK4 activity by promoting M169 oxidation to block T172 phosphorylation.

3.7 Supplementary information for “An Activity-Based Oxaziridine Platform for Identifying and Developing Covalent Ligands for Functional Allosteric Methionine Sites”

3.7.1 Supplementary figures

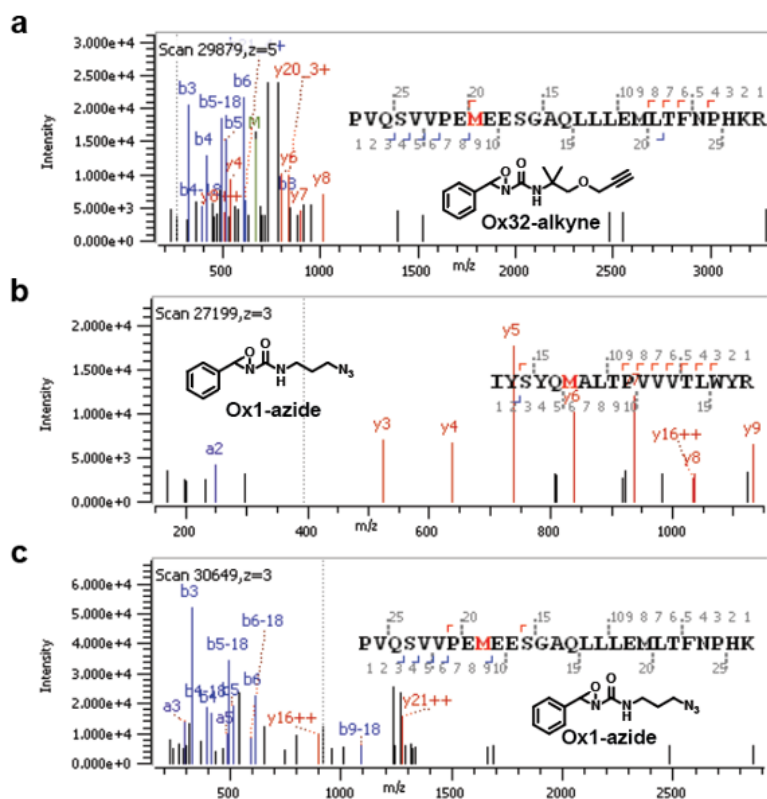
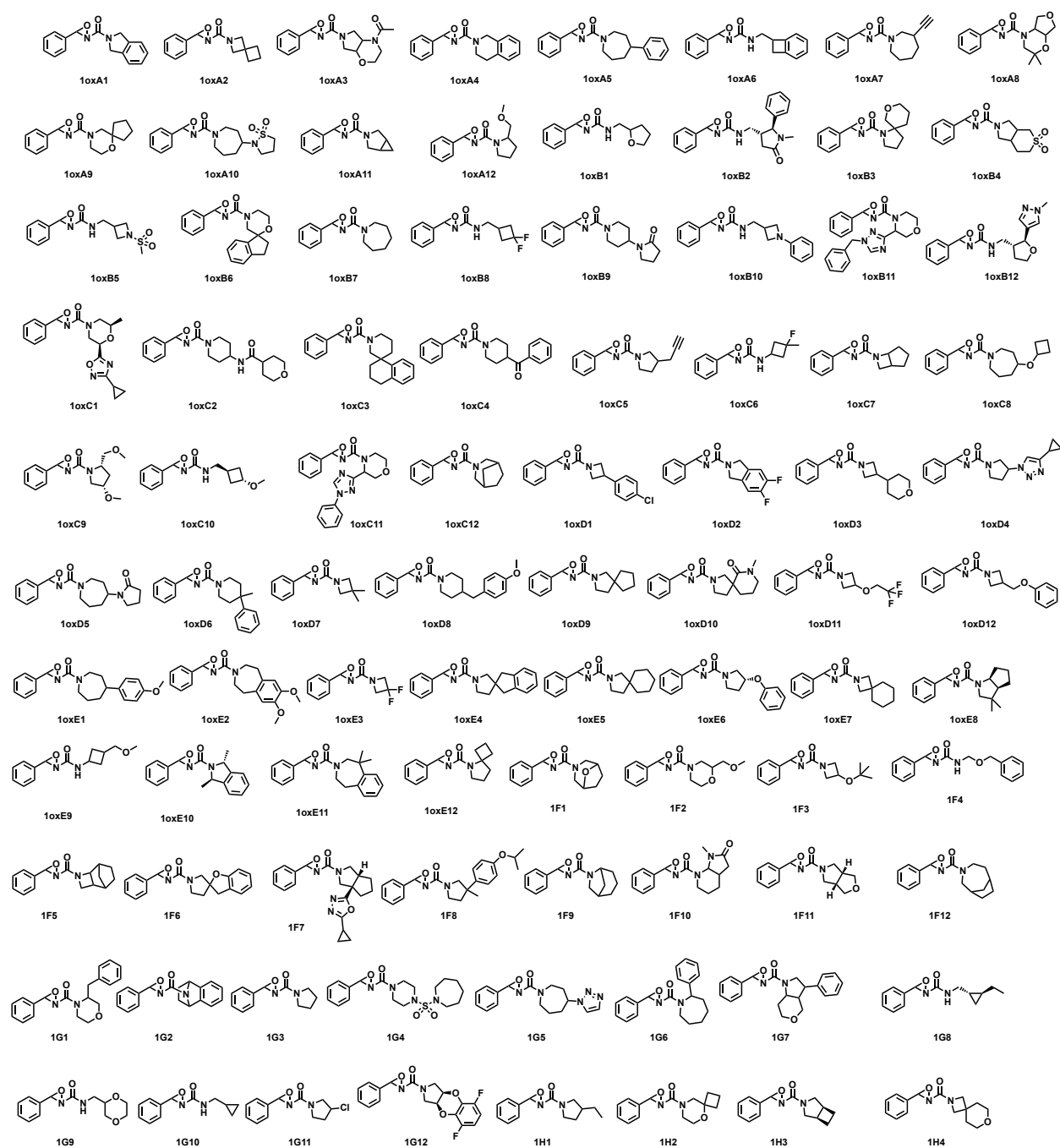


Figure S3.1. MS/MS of Ox1-azide and Ox32-alkyne modification on CDK4.

All samples represent 50 μ M probe treatment in 10 μ g CDK4/CCND1 spiked into 90 μ g mouse liver lysate. (a) Ox32-alkyne modification at Met264 of CDK4. (b) Ox1-azide modification at Met169 and (c) Met264 of CDK4.



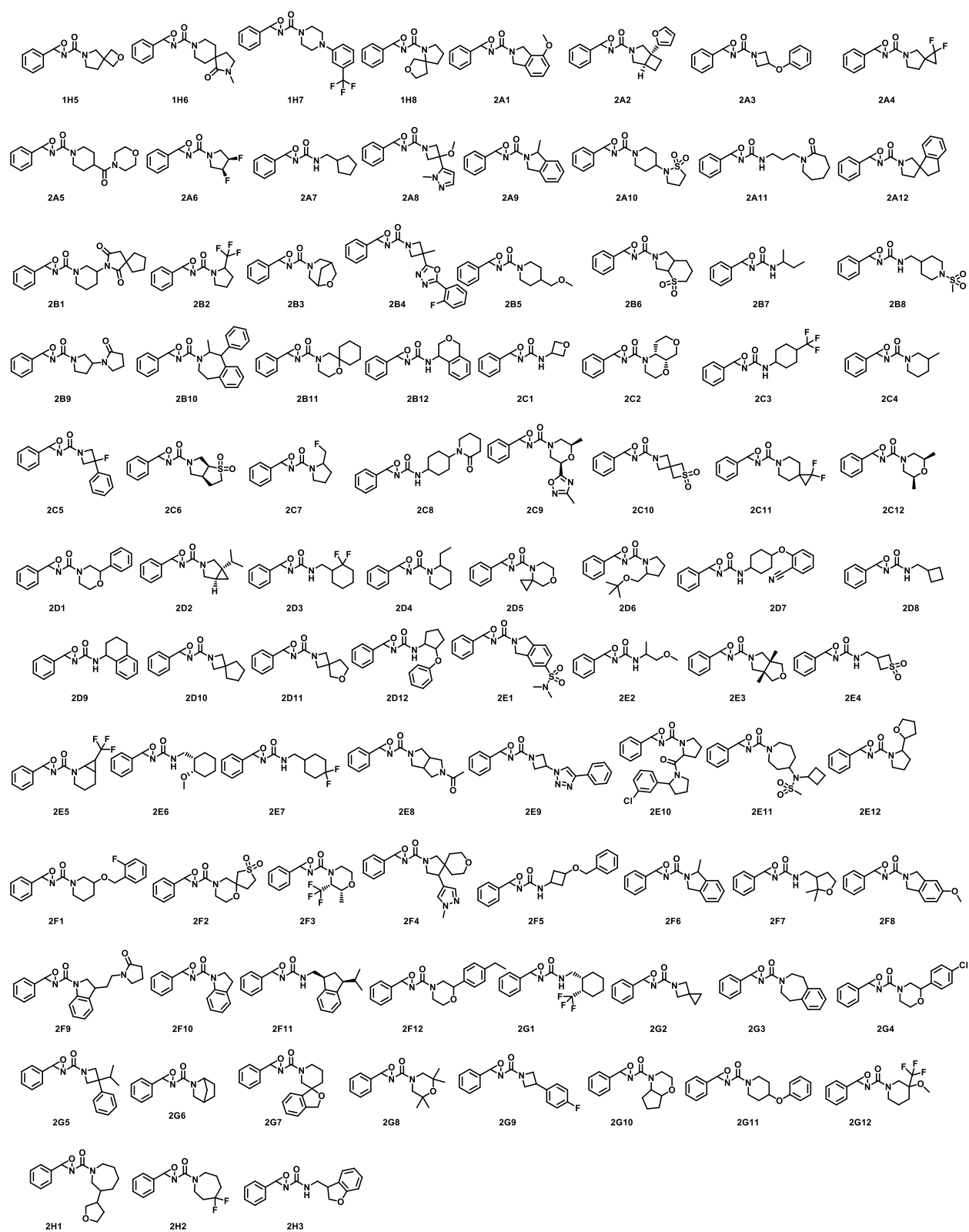


Figure S3.2. Oxaziridine fragment library structures.

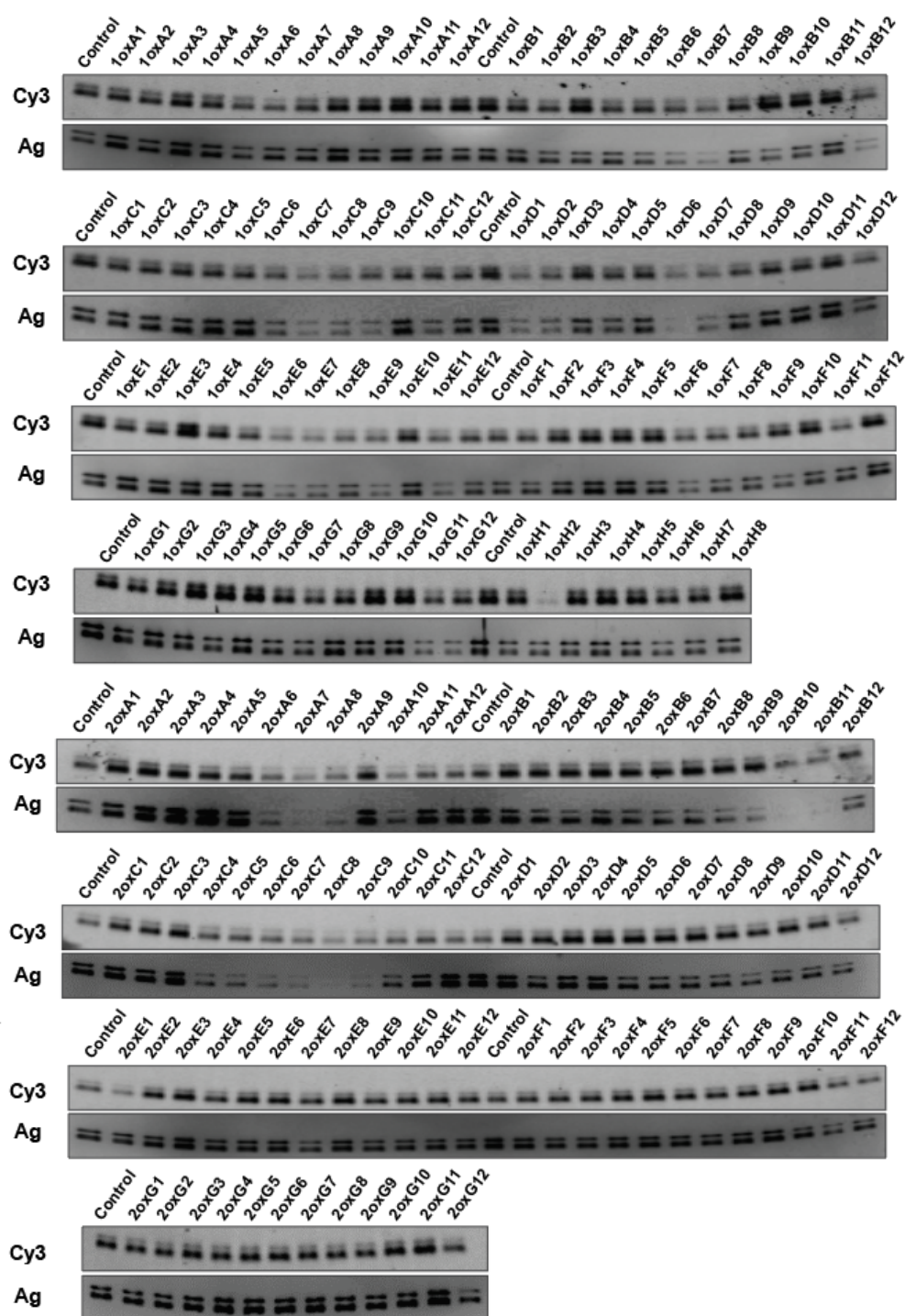


Figure S3.3. Oxaziridine library screen against CDK4-CCND1.

Samples were treated according to gel-ABPP method outlined at 50 nM compound and 50 nM CDK4-CCND1.

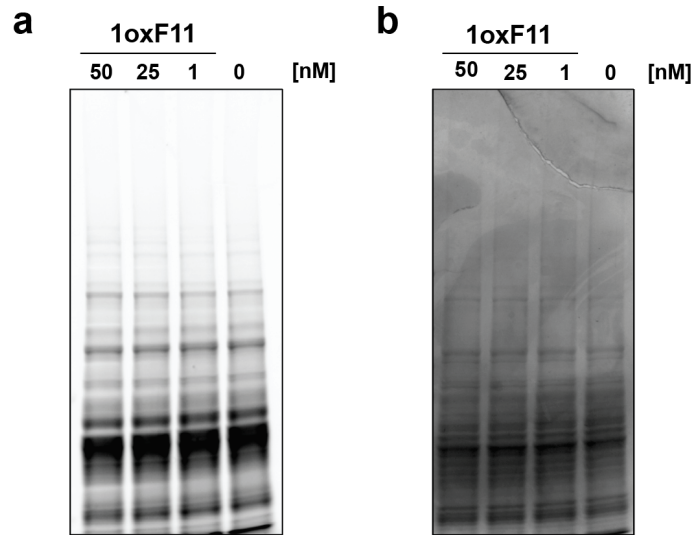


Figure S3.4. Aggregation test with 1oxF11. Lysates were treated with varying doses of 1oxF11 according to gel-ABPP method outlined. (a) Cy3 channel image, (b) silver stain image.

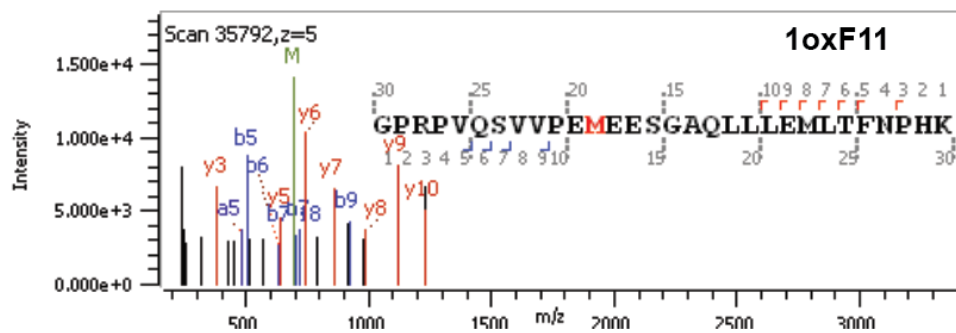


Figure S3.5. Additional MS/MS spectrum of CDK4, modified with 1oxF11 at M264. Isolated CDK4-CCND1 spiked in mouse liver lysate was treated with 1oxF11 as shotgun proteomics method outlined.

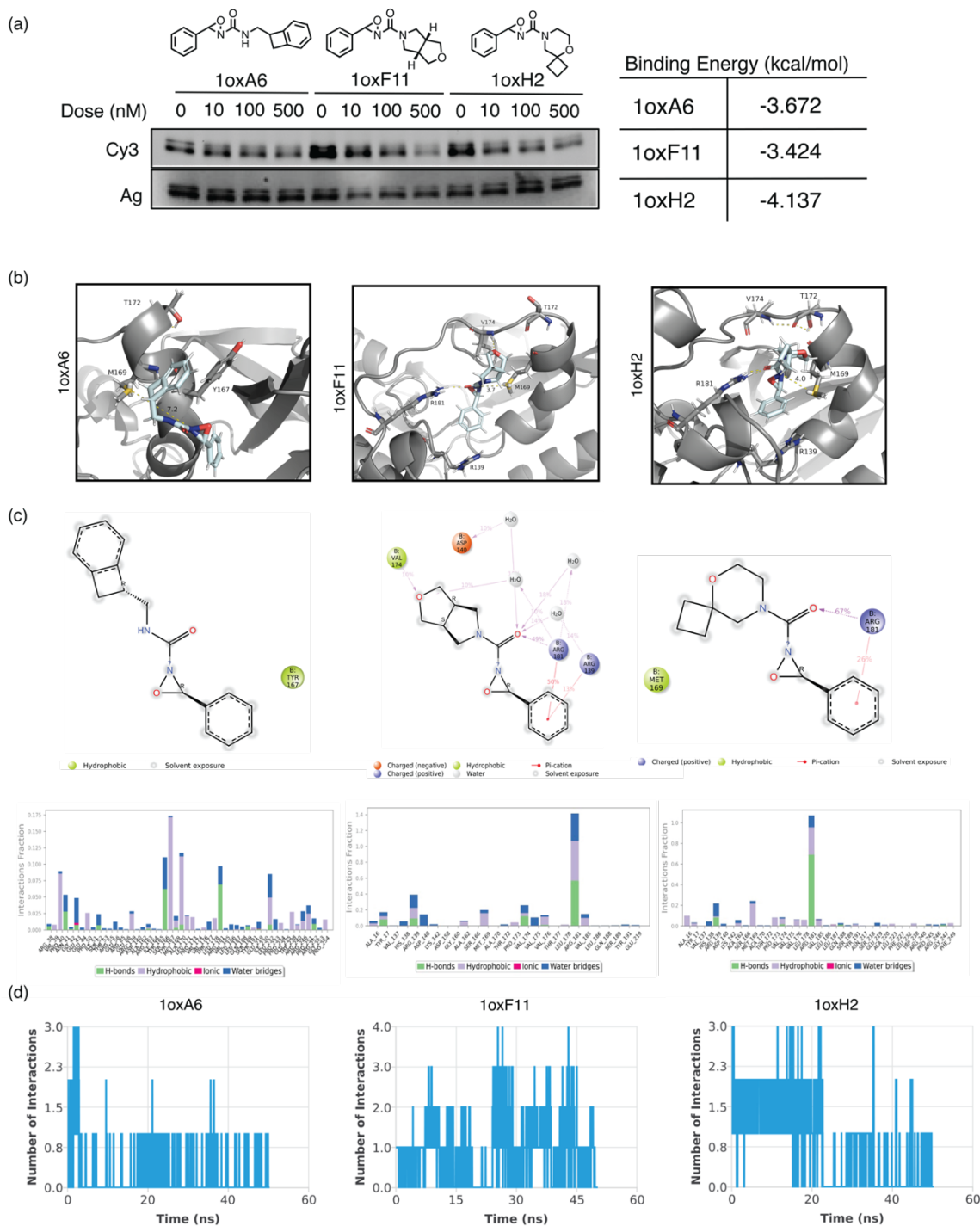


Figure S3.6. Fragment screening structure-activity relationship data.

(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.

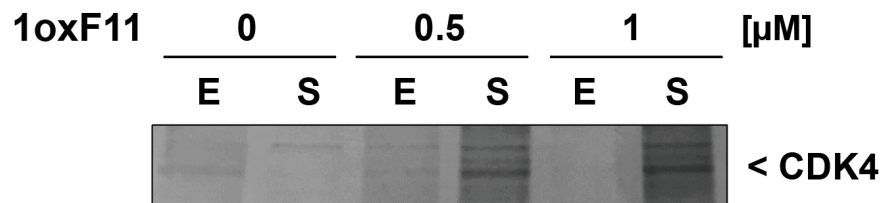


Figure S3.7. Competition of 1oxF11 and 1oxF11yne by gel.

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 μ M 1oxF11yne, followed by a click step to DTB-N₃ 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.

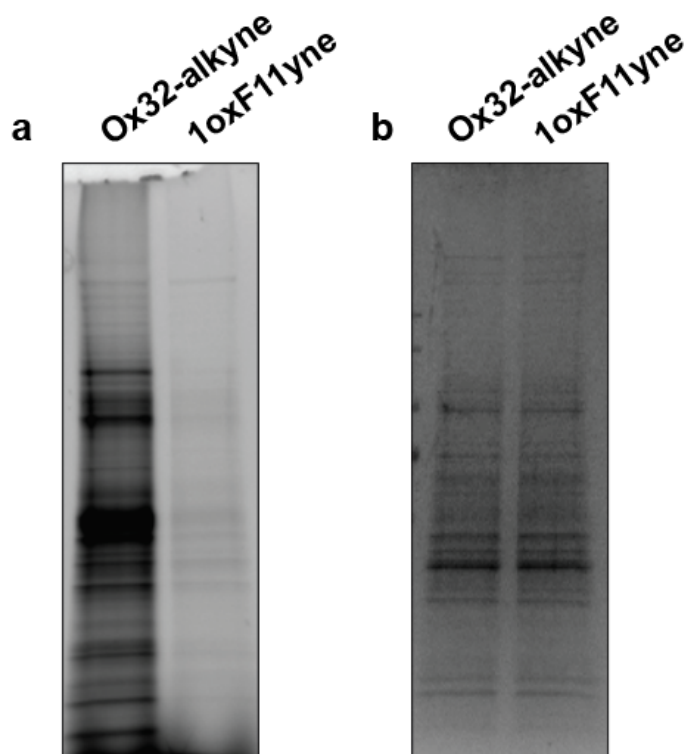


Figure S3.8. Reactivity comparison of Ox32-alkyne and 1oxF11yne.

Lysate was collected from MCF-7 cells overexpressed with CDK4 and treated with either 10 μ M Ox32alkyne or 10 μ M 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.

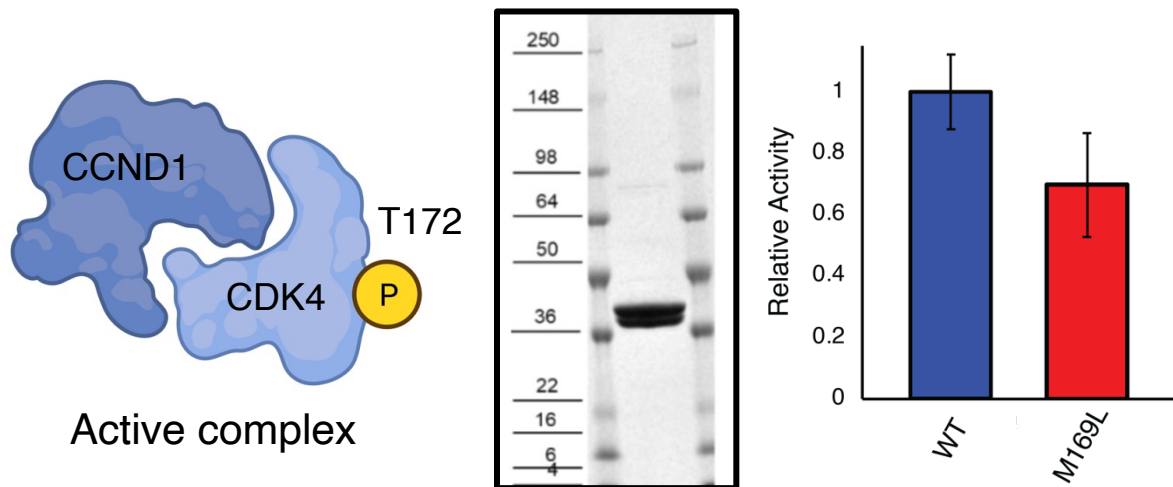


Figure S3.9. Relative activities of WT and M169L CDK4. Representative gel of purified CDK4/CCND1 in its active form are shown to the left, and relative activity profiles of WT and M169L variant CDK4 are shown to the right.

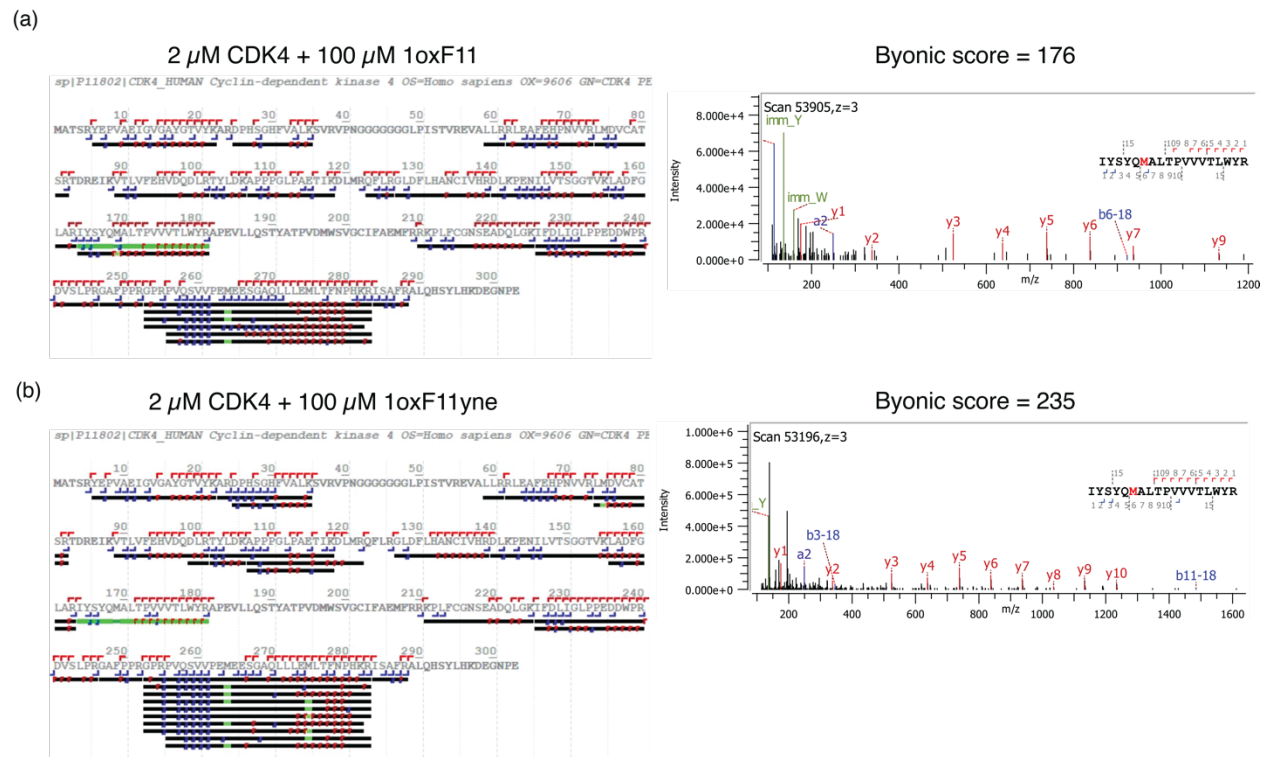


Figure S3.10. Protein coverage map of CDK4 treated with (a) 1oxF11 and (b) 1oxF11yne.

3.7.2 General methods

Reactions using moisture- or air-sensitive reagents were carried out in flame-dried glassware under an inert atmosphere of N₂. 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. ¹H and ¹³C NMR spectra were collected at 298 K in CDCl₃ or CD₃OD (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 (CDCl₃) or 3.31 (CD₃OD) for ¹H and 77.16 (CDCl₃) or 49.00 (CD₃OD) for ¹³C 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 (27). 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.

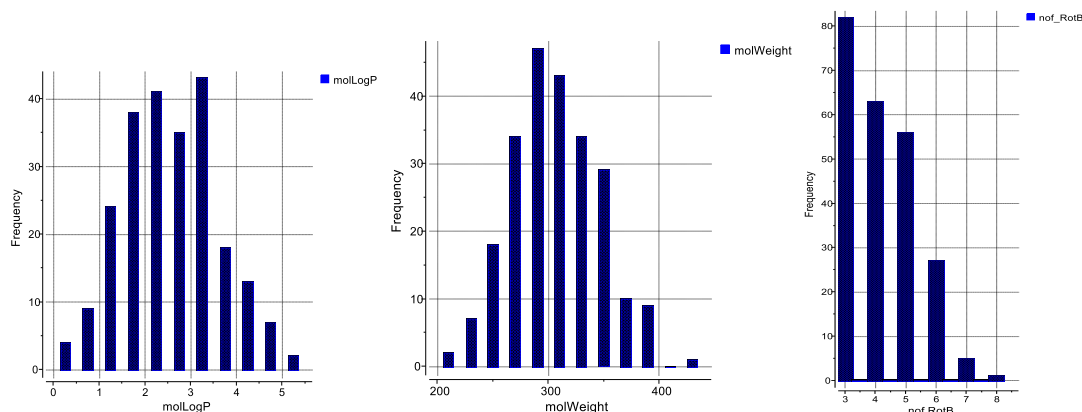
3.7.3 Design methodology for diversity selection of oxaziridine library.

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), 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 (28).

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

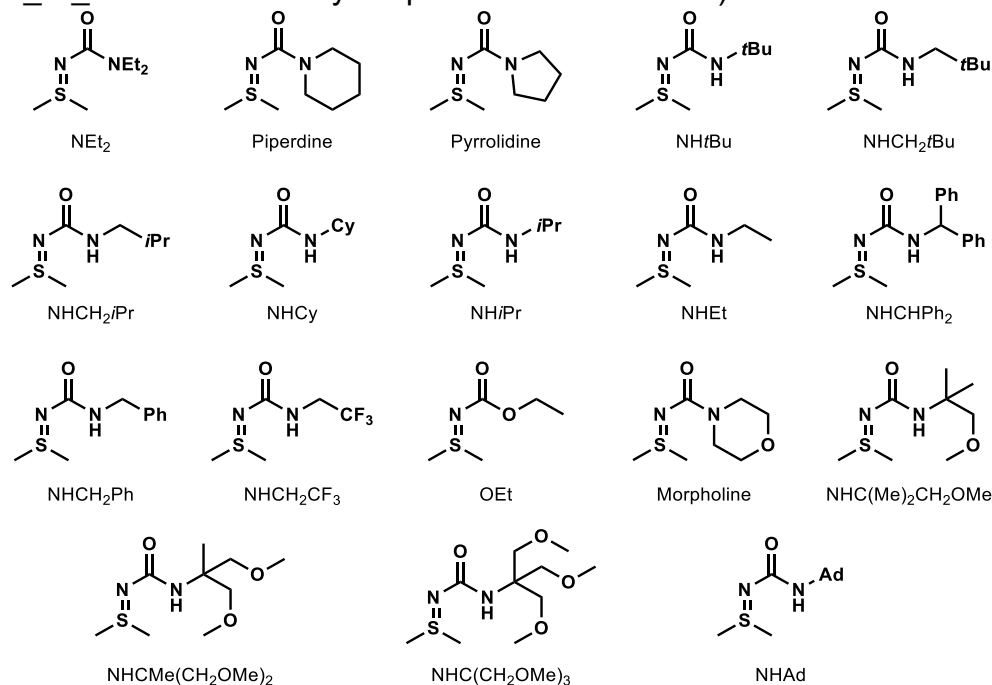
Clustering analysis for each selected amine set was conducted using *ICM Chemist Pro* (Molsoft LLC) using (Tanimoto <0.4 as noted) generating the detailed clusters for each sub-type. Thereafter, 2 rounds of selections based on diversity and selecting the square root of the population of each cluster 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 (28). 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” (150). Symmetry equivalents were removed, then conformers were constructed using omega2[omega2] on default settings (151). The conformers were optimized and IR frequencies computed using Gaussian09[G09] with the keywords “opt(maxcycle=250) freq pop=nbo def2tzvp m062x” (152). 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 (153) was validated by obtaining the stretching frequencies from the set of compounds used to generate the reported hydrolysis kinetic model (28), and compared to the frequencies obtained manually (**Table S3.1**). It was found that the automatically calculated stretching frequencies correlated very strongly with those obtained manually (**Scheme S3.2**), with

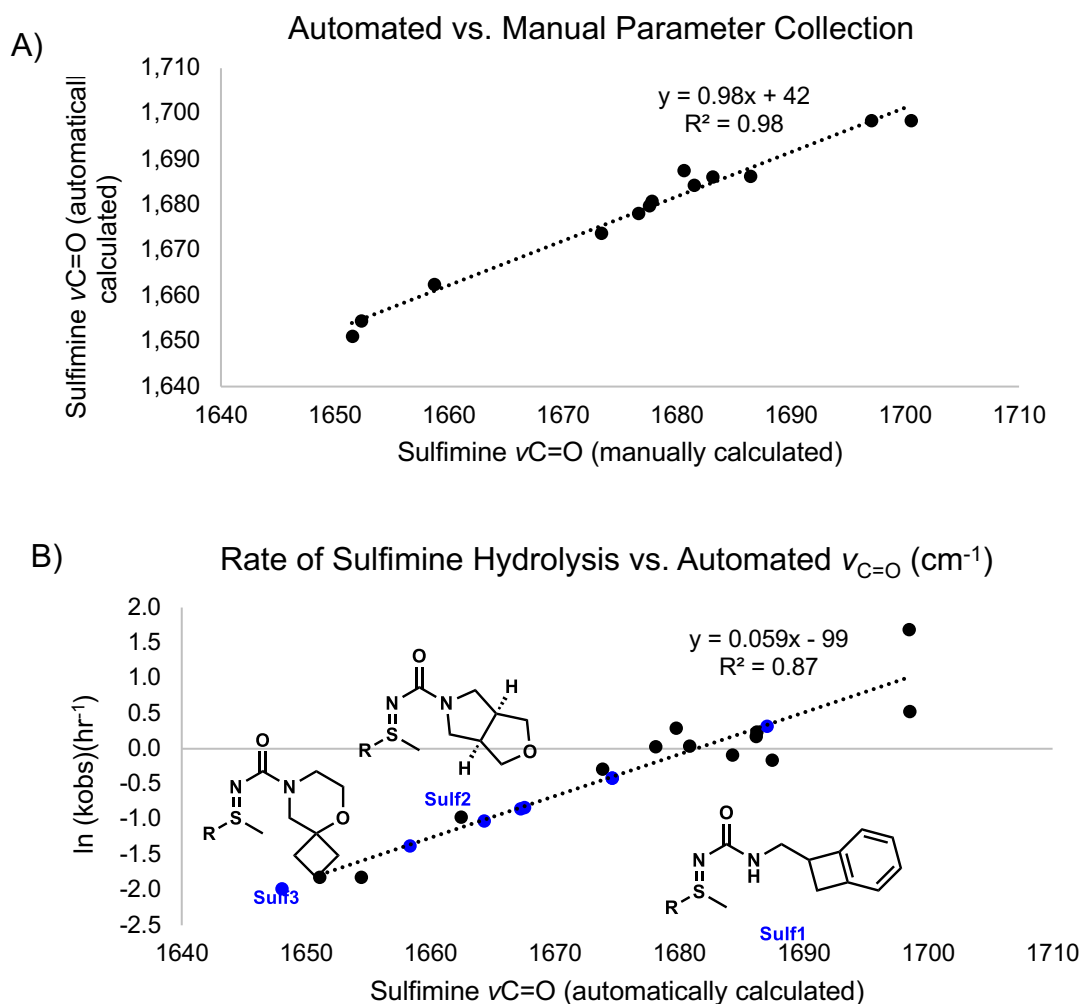
a slope and $R^2 = 0.98$. As a further point of validation, the published rates of hydrolysis were plotted against the automatically calculated stretching frequency, which produced a similar model to the one obtained from manual calculated stretching frequencies. With this validation in hand, the stretching frequencies of a novel library of untested oxaziridines were extracted, to predict their respective sulfinimine adduct stabilities towards hydrolysis. The predicted K_{obs} are computed as $0.0595 \cdot (\nu_{\text{C=O}}) - 112.8$ for sulfinimines, and $0.0498 \cdot (\nu_{\text{C=O}}) - 102.8$ for ureas (28). Equivalent structures in the two series are paired up in the output. Of the 234 input pairs, 1 sulfinimine and 1 urea failed to converge, even after restarting, possibly due to unresolved steric crowding. The full list of sulfinimine and urea stretching frequencies, as well as predicted stabilities can be seen in the attached Excel file (CDK4_SI_OxaziridineLibraryFrequenciesAndStabilities)



Sulfinimine	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
NHtBu	1673.34	1673.81	-0.28
NHCH ₂ tBu	1680.60	1687.47	-0.15
NHCH ₂ iPr	1681.51	1684.25	-0.085
NHCy	1676.60	1678.08	0.032
NH/iPr	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 ₂ CF ₃	1697.01	1698.51	0.53
OEt	1700.52	1698.47	1.69
Morpholine	1659.09	1664.30	-1.02*
NHCMe(CH ₂ OMe) ₂	1666.60	1667.23	-0.85*
NHC(CH ₂ OMe) ₃	1667.09	1667.55	-0.83*
NHAd	1671.38	1674.58	-0.41*
NHCMe ₂ CH ₂ OMe	1675.08	1674.60	-0.41*

Table S3.1: Sulfinimes used for validating the automated workflow for obtaining C=O stretching frequencies. Manually calculated parameters and rate of hydrolysis obtained from previous report (28) (*denotes predicted hydrolysis rates).



Scheme S3.2. A) Comparison of $\nu_{C=O}$ obtained from automated vs. manual workflows and B) recreated kinetic model using the automatically calculated $\nu_{C=O}$ with predicted hydrolysis rates of select sulfinimes (data points in blue)

Gel-based ABPP. CDK4 was diluted in PBS to 50 nM, then 50 μ L was added to each well of a 96-well PCR plate (Thermo Fisher Scientific). Ligands were dissolved fresh in DMSO to 5 μ M, and 1 μ L was added so each well contained a unique ligand at the indicated concentration. Wells were mixed and allowed to incubate 1 hour at 23 $^{\circ}$ C. Ligands were then chased with fresh Ox1-alkyne with 1 μ L of 5 μ M added to each well (100 nM final), wells mixed and allowed to incubate 1 hour at 23 $^{\circ}$ C. Excess oxaziridine was quenched with N-acetyl methionine (Sigma; 01310) via addition of 1 μ L of 10 mM DMSO stock (200 μ M final), wells mixed and allowed to incubate 1 hour at 23 $^{\circ}$ C. A stock was prepared 1:5 of 12.5 μ M DBCO-Cy3 (Click Chemistry Tools; A140) in DMSO : 1.2% SDS/PBS. To each well was added 6 μ L of this stock, the wells mixed and allowed to incubate overnight protected from light at 23 $^{\circ}$ C. The next morning 30 μ L 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 $^{\circ}$ C for 6 minutes. 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.

Primers:

HindIII CDK4 forward: 5' – AACGACAAGCTTGCCACCATGGGAGACTATAAG – 3'
 XbaI CDK4 reverse: 5' – AAAGATCTAGACTCGAGTCATTCTGGATTACCCCTCATCTTTG – 3'
 M169L forward: 5' – GAATCTATAGCTACCAGCTGGCACTGACTCCTGTGG – 3'
 M169L reverse: 5' – CCACAGGAGTCAGTGCCAGCTGGTAGCTATAGATTC – 3'

150

resuspended in 30 μ L freshly prepared 8 M urea/PBS. A 5X stock of ProteaseMAX (Promega; V2071) was prepared by dissolving the pellet in 100 μ L ammonium bicarbonate. To the protein mixture was added 30 μ L 1X ProteaseMAX, 40 μ L ammonium bicarbonate, and 10 μ L of 110 mM freshly prepared TCEP (Pierce; 20490). The sample was then incubated at 60 °C for 30 minutes. To the sample was then added 2.5 μ L of 500 mM freshly prepared iodoacetamide (Sigma Aldrich; I1149), and the sample was incubated protected from light at 23 °C for 30 minutes. 120 μ L of PBS was then added, followed by 1.2 μ L 5X ProteaseMAX. The sample was vortexed thoroughly. Sequencing grade Trypsin/Lys-C mix (Promega; V5071) was reconstituted in 40 μ L trypsin buffer, and 4 μ L was added to the sample. The sample was allowed to digest at 37 °C overnight. The next day the sample was acidified with 12 μ L formic acid and spun at max speed for 30 minutes. 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 μ g of CDK4 (WT or M169L variant) was diluted to a total volume of 100 μ L in PBS. Protein mixture was treated with 50 μ M oxaziridine (DMSO) and allowed to incubate at 23 °C for 30 minutes. Labeled protein was precipitated via addition of 900 μ L MeOH at -80 °C overnight. The next day, sample was spun at max speed at 4 °C for 10 minutes. 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 μ L freshly prepared 6M urea/PBS and incubated at 60 °C for 1 hour. Samples were then treated with 5 μ L of freshly prepared 110 μ M TCEP (Pierce; 20490). The samples were then incubated at 60 °C for 20 minutes. To the samples was then added 4 μ L of 400 mM freshly prepared iodoacetamide (Sigma Aldrich; I1149), and the sample was incubated protected from light at 37 °C for 30 minutes. The reaction was quenched by addition of 550 μ L of 50 mM ammonium bicarbonate. Sequencing grade Trypsin/Lys-C mix (Promega; V5071) was reconstituted in 40 μ L trypsin buffer, and 8 μ L 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 μ L 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) (154). In both cases, inlines (IDEX; M-520) were fitted with ~20 cm of 250 μ m tubing and prepacked with 4 cm of Aqua C18 reverse-phase resin (Phenomenex; 04A-4299). Columns were made from 100 μ m 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 minutes (where buffer A is 95:5 H₂O:MeCN/0.1% formic acid,

and buffer B is 80:20 H₂O: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 *n*th 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/H₂O) 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 hour at 23 °C. Labeled proteins were then clicked with 1.2 eq of DTB-N₃ compound with TBTA, TCEP, and Cu₂(SO₄), and the CuAAC was allowed to proceed for 1 hour at 23°C. The samples were added to 9 mL of MeOH:CHCl₃ (4:1.5) and precipitated by vortexing. Samples were then spun at max speed for 10 minutes 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 minutes. Then, 25 µL 400 mM Iodoacetamide was added and incubated at 37°C for 30 minutes in the dark. The reaction was quenched by adding 950 µL PBS and spinning at 1,400 xgs for 3 minutes to remove the supernatant. A premixed solution of 200 µL 2 M urea/PBS, 2 µL CaCl₂, 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 μ L 0.1% FA (50% ACN/MQ) and spun at 3000 x g for 3 minutes. The eluent was dried via SpeedVac concentration. Peptides were reconstituted in 100 μ L of MQ + 0.1% FA and quantified using a commercial colorimetric kit (Pierce; 23275).

ReDiMe labeling. Samples were reconstituted in 100 μ L of 100 mM TEAB. To the DMSO-treated control sample was added 4 μ L of 4% (v/v) formaldehyde for isotopically light labeling. To the 1oxF11yne-treated sample was added 4 μ L of 4% (v/v) CD₂O for isotopically heavy labeling. Then, 4 μ L of 0.6M NaBH₃CN 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 μ L of 1% (v/v) ammonia solution. Formic acid (16 μ L) 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 μ g 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 μ g multiplexed samples were reconstituted in 20 μ L of MQ H₂O + 0.1% formic acid by bath sonication for 30 minutes. 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 μ m x 300 μ m 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 minutes gradient from 0-80% H₂O/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^AK (Δ m = 28.0313) and n^AK (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 BIOVIA. 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 MgCl₂, 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 hour 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 hours 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 G₀/G₁ 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 hours, for a total of 48 hours 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% CO₂ 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 hours. To assess viability, media was removed and replaced with 100 μ L media containing 10% CCK-8 assay solution. Plates were incubated at 37 °C in a 5% CO₂ atmosphere for 1-4 hours 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 tablet (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 minutes on a rotator. Samples were spun at 5000 x g for 10 minutes 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 μ g per lane on a 4-20% Tris-Gly SDS-PAGE gel. The gel was run at 160 V for 80 minutes and semi-dry electrotransferred to a PVDF membrane at 25 V, 2.5 Å, for 10 minutes. Blots were blocked with 5% BSA/TBST for 1 hour, 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) (155), 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 hours at room temp. Blots were quickly washed 3 X with TBST prior to incubation with ECL western blotting substrates (Promega; W1001) for 1 minutes 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 minutes. 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 minutes. After centrifugation at 15,700 g for 10 minutes 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% CO₂. 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 minutes 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 hours at 37°C, 5 % CO₂. The media was then aspirated, and cells were given 3 mL DMEM + 10% FBS and left to incubate for an additional 30 hours at 37°C, 5 % CO₂. 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 minutes while rocking. Lysate was clarified by centrifugation at 10,000 x g at 4°C for 15 minutes. 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 minutes. 30 µg protein was loaded onto a 4-20% Tris-Gly SDS-PAGE gel run at 160 V for 70 minutes. Proteins were then electro-transferred to a PVDF membrane (25 V/2.5 A for 10 minutes). Membranes were then blocked in a solution of TBST + 5% BSA (w/v) and rocked at 23°C for 1 hour. Membranes were washed 3X in TBST for 5 minutes 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 minutes then incubated with anti-rabbit IgG HRP conjugated secondary (1:3000 TBST) (CST; 7076S) for 2 hours at 23°C. Blots were quickly washed 3 X with TBST prior to incubation with ECL western blotting substrates (Promega; W1001) for 1 minutes 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 hours 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 μ g 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 hour at 23°C while rocking. The membrane was washed 3 x 10 minutes in TBST then incubated with mouse α -FLAG (1:500 TBST + 3% milk, Sigma #F1804), rabbit α -CDK4 (1:1000 TBST + 5% BSA; CST #12790), or rabbit α - 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 minutes 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 hours 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 hours. The media was then aspirated, and cells were treated with the indicated concentrations of 1oxF11 in DMEM + 10% FBS containing 1% DMSO for 24 hours. 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 μ L of this lysate was added 1 μ L of a stock of 1oxF11 in DMSO for a final concentration of 500 μ M, 250 μ M, or 0 μ M 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 μ L of 25 mM 1oxF11yne was added to each sample as indicated for a final concentration of 500 μ M 1oxF11yne. Samples were incubated again for 1 hour at 22 °C. DTB-N₃ was added to each compound with TBTA, TCEP, and Cu₂(SO₄), and CuAAC was allowed to proceed for 1 hour at 22 °C. 450 μ L of MeOH was added to each sample and proteins were precipitated at -80 °C for 12 hours. Samples were then spun at max speed for 10 minutes at 4 °C. Pellet was resuspended in cold MeOH and samples spun again. Pellet was washed once more before resuspension in 150 μ L 0.2% SDS/PBS. Samples were then boiled for 5 minutes and spun at 6500 x g for 5 minutes. Meanwhile, high-capacity streptavidin beads (10 μ L per sample) (Thermo Fisher Scientific; 20357) were washed 2X in PBS and 2X in MQ. The supernatant of the samples was added to 10 μ L 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 μ L 0.1% FA (50% MeCN/MQ). Eluent was collected and beads washed once more with 20 μ L of elution buffer. Columns were spun at 3000 x g for 3 minutes. Eluent was lyophilized to remove MeCN. After samples were dry, they were reconstituted in 75 μ L PBS. Protein was diluted with 4X Laemmli's buffer (Bio-Rad Laboratories, Inc.; 1610747) containing 10% BME and brought to 95 °C for 6 minutes. Samples were loaded and separated on precast 4–20% TGX gels (Bio-Rad Laboratories, Inc.) The gel was run at 160 V for 80 minutes and semi-dry electrotransferred to a PVDF membrane at 25 V, 2.5 Å, for 10 minutes. A separate gel was run and stained for total protein via Coomassie.

Blot was blocked with 5% BSA/TBST for 1 hour, then washed 2 X TBST for 5 minutes. 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 hours at 22 °C. Blots was quickly washed 3 X with TBST prior to incubation with ECL western blotting substrates (Promega; W1001) for 1 minutes 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 Å³ 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.

3.7.5 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

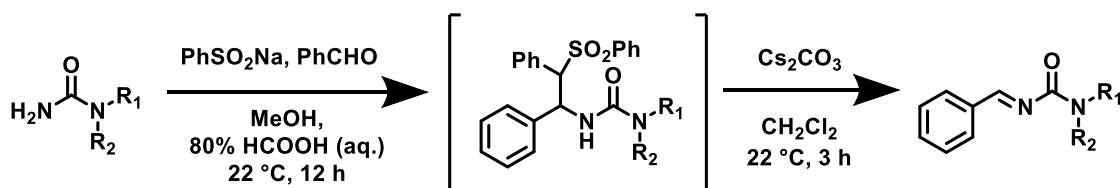
All chemicals and solvents were used as received from the commercial supplier without further purification unless mentioned otherwise.

Analytatics

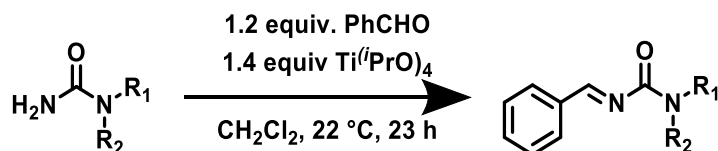
Nuclear Magnetic Resonance (NMR) spectra were recorded on BRUKER AV (600 MHz and 300 MHz), AVB (400 MHz), AVQ (400 MHz) and NEO (500 MHz) spectrometers. Measurements were carried out at ambient temperature. Chemical shifts (δ) are reported in ppm with the residual solvent signal as internal standard (chloroform at 7.26 and 77.00 ppm for ^1H 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). ^{13}C 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.

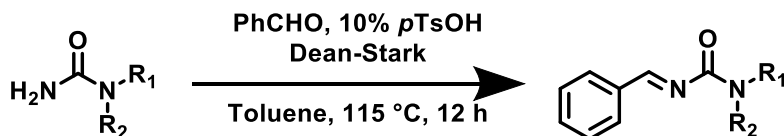
Experimental procedures and characterization



General imine synthesis 1. Scheme is based off work by Lillo et al (156). 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.

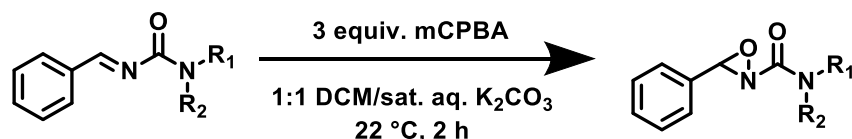


General imine synthesis 2. To a solution of corresponding urea (115 mmol) and benzaldehyde (127 mmol) in dry CH_2Cl_2 (20 mL) was added $\text{Ti}(\text{iPrO})_4$ (140 mmol) dropwise at room temperature. The mixture was stirred at room temperature for 23 hours. The solvent was removed *in vacuo* to afford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

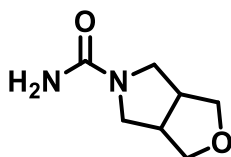


General imine synthesis 3. To a flamed dried 3-neck round bottom equipped with a Dean-Stark trap and reflux condenser was added urea (10 mmol, 1 equiv.) and toluene (200 mL). To this mixture was added benzaldehyde (25 mmol, 2.5 equiv.) and

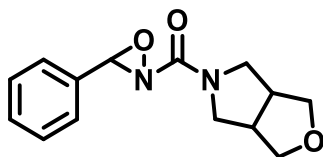
pTsOH•H₂O (2 mmol, 0.2 equiv.). The mixture was refluxed for 12 hours. The mixture was then concentrated under reduced pressure and was used in the next step without further purification.



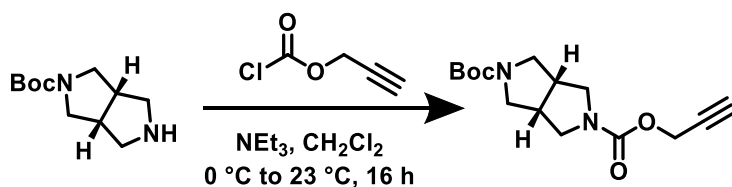
General oxaziridine synthesis. In a 25 mL round-bottom flask, mCPBA (75%, 538 mmol) was pre-stirred in 1:1 CH₂Cl₂/sat. aq. K₂CO₃ (140 mL) at room temperature for 10 minutes. A suspension formed. A solution of imine was added dropwise, but rapidly, using CH₂Cl₂. Intense stirring at this step was observed to be essential. Additionally, if a partial dissolution of solids was observed after imine addition, sat. K₂CO₃ was added to reaction mixture immediately. After 2 hours, the reaction was diluted with water (150 mL) and extracted with CH₂Cl₂ (3 x 100 mL). The organic layer was separated, washed with diluted aq. K₂CO₃ (3 times) and water (2 times), dried over Na₂SO₄, filtered, and concentrated *in vacuo*. The residue was purified by column chromatography.



1oxF11 urea characterization. Compound provided by Enamine. ¹H 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). ¹H NMR (600 MHz, CDCl₃) 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). ¹³C NMR (101 MHz, DMSO-d₆) δ 157.90, 73.48, 50.73, 43.42. HRMS (ESI + TOF) calcd for C₇H₁₃N₂O₂ [M+H]⁺: 157.0977. Found 157.0793.

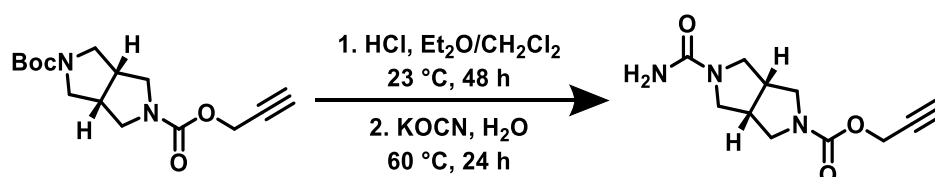


1oxF11 characterization. Compound provided by Enamine. ¹H NMR (600 MHz, CDCl₃) δ 7.48 (m, 2 H), 7.45 (m, 1H), 7.41 (m, 2H), 5.22 (s, 1 H), 3.90 (m, 2 H), 3.77 (m, 2 H), 3.66 (m, 2 H), 3.50 (m, 2 H), 2.99 (m, 2 H). ¹³C NMR (126 MHz, CDCl₃) δ 160.14, 160.05, 133.08, 133.03, 130.91, 128.79, 128.05, 78.02, 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 C₁₄H₁₆N₂O₃ [M+H]⁺: 261.1234. Found 261.1504.



A solution of amine (3.0 g, 14.1 mmol, 1.0 equiv.) in CH₂Cl₂ (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 hours, the reaction mixture was diluted with CH₂Cl₂ (50 mL), washed with 1M aq. HCl (20 mL), sat. aq. NaHCO₃ (20 mL), and brine (20 mL). The organic layer was dried over Na₂SO₄, 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 CH₂Cl₂.

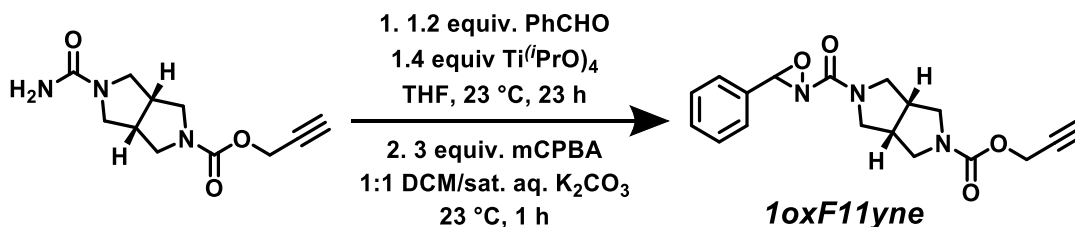
¹H NMR (500 MHz, CDCl₃) δ 4.66 (t, *J* = 2.6 Hz, 2H), 3.58 (m, 4H), 3.36 – 3.13 (m, 4H), 2.83 (s, 2H), 2.44 (t, *J* = 2.4 Hz, 1H), 1.42 (s, 9H). **¹³C NMR** (126 MHz, CDCl₃) δ 154.5, 153.9, 79.7, 78.6, 74.6, 52.8, 50.2, 49.7, 49.5, 42.5, 41.6, 40.7, 28.5. **HRMS (ESI)**: calcd for C₁₅H₂₂N₂O₄Na [M+Na]⁺: 317.1472. Found 317.1479.



To a solution of Boc-protected amine (2.0 g, 6.8 mmol, 1.0 equiv.) in CH₂Cl₂ (13 mL) was added HCl (2 M in Et₂O, 12 mL, 24 mmol, 3.5 equiv.) at room temperature. After 48 hours, 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 Et₂O, 6 mL, 12 mmol, 1.8 equiv.). After 16 hours, 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 hours, the reaction was cooled to room temperature and extracted with EtOAc (5 x 100 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo* to afford the desired urea product (1.3 g, 88%) as a white solid.

¹H 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). **¹³C 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 C₁₁H₁₆N₃O₃ [M+H]⁺: 238.1186. Found 238.1199.



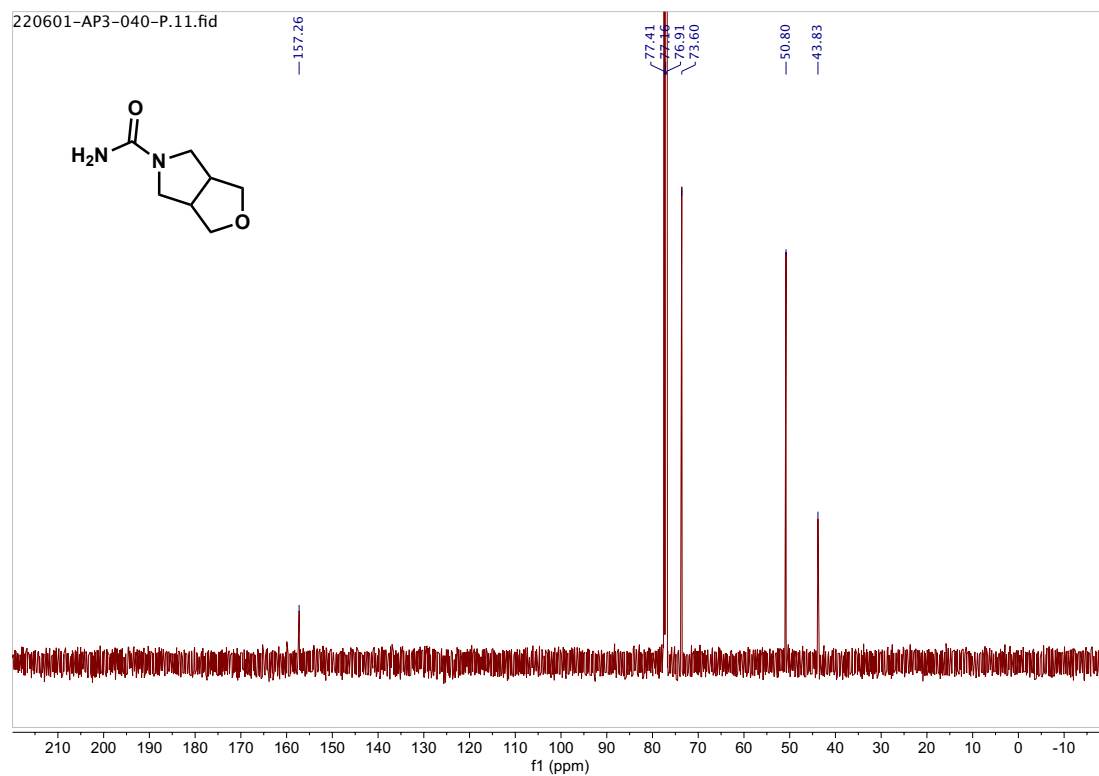
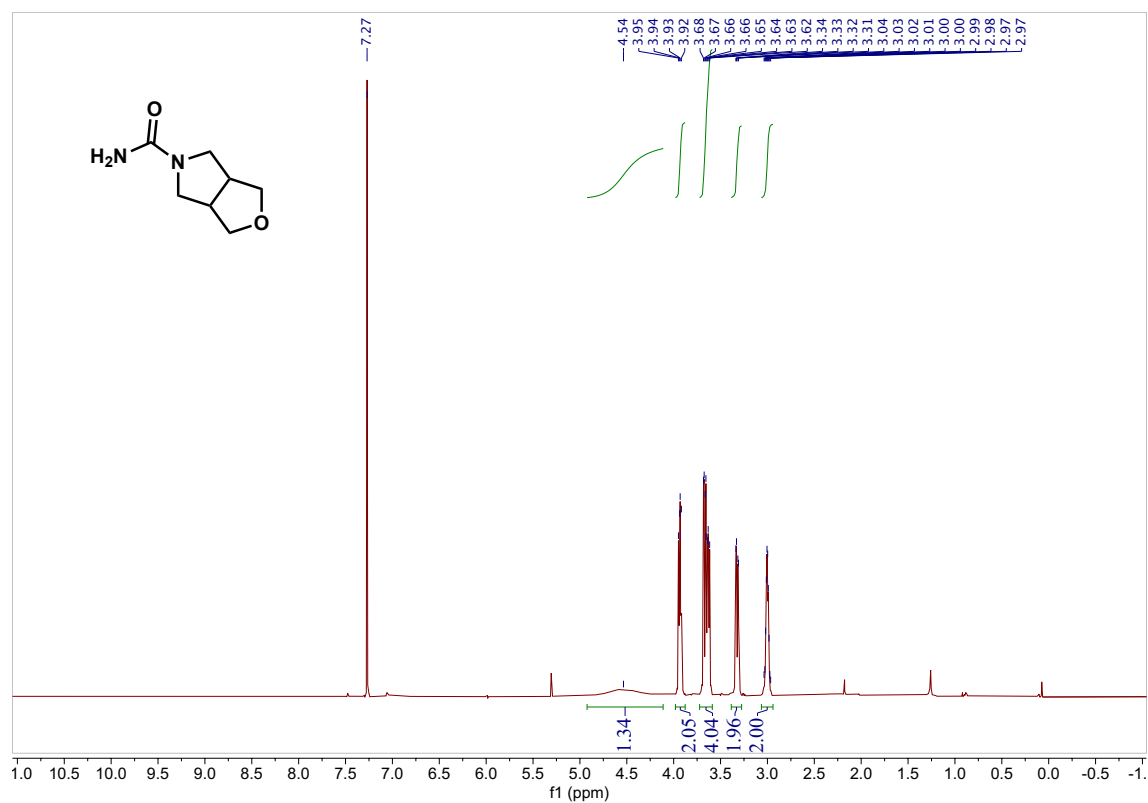
To a solution of corresponding urea (237 mg, 1.0 mmol, 1.0 equiv.) and benzaldehyde (0.12 mL, 1.2 mmol, 1.2 equiv.) in dry THF (3 mL) was added Ti(*i*PrO)₄ (0.42 mL, 1.4 mmol, 1.4 equiv.) dropwise at room temperature. The mixture was stirred

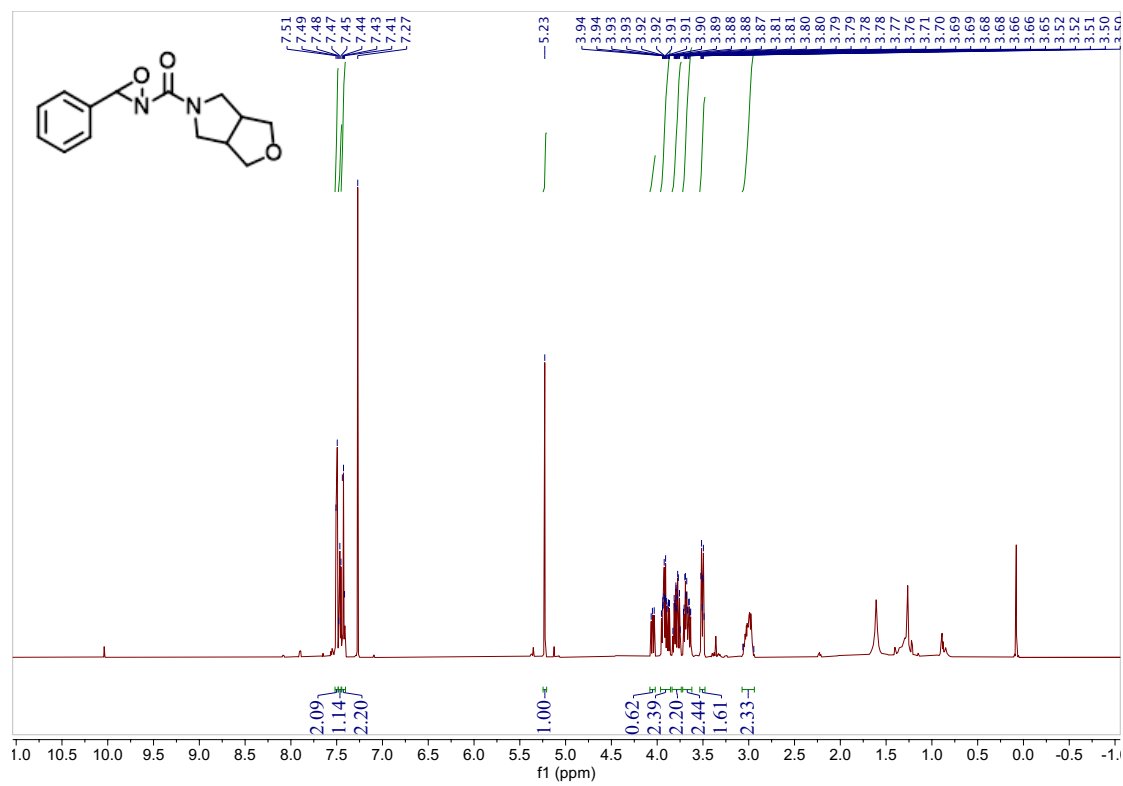
at room temperature for 23 hours. The solvent was removed *in vacuo* to afford crude imine which was used immediately in the next step.

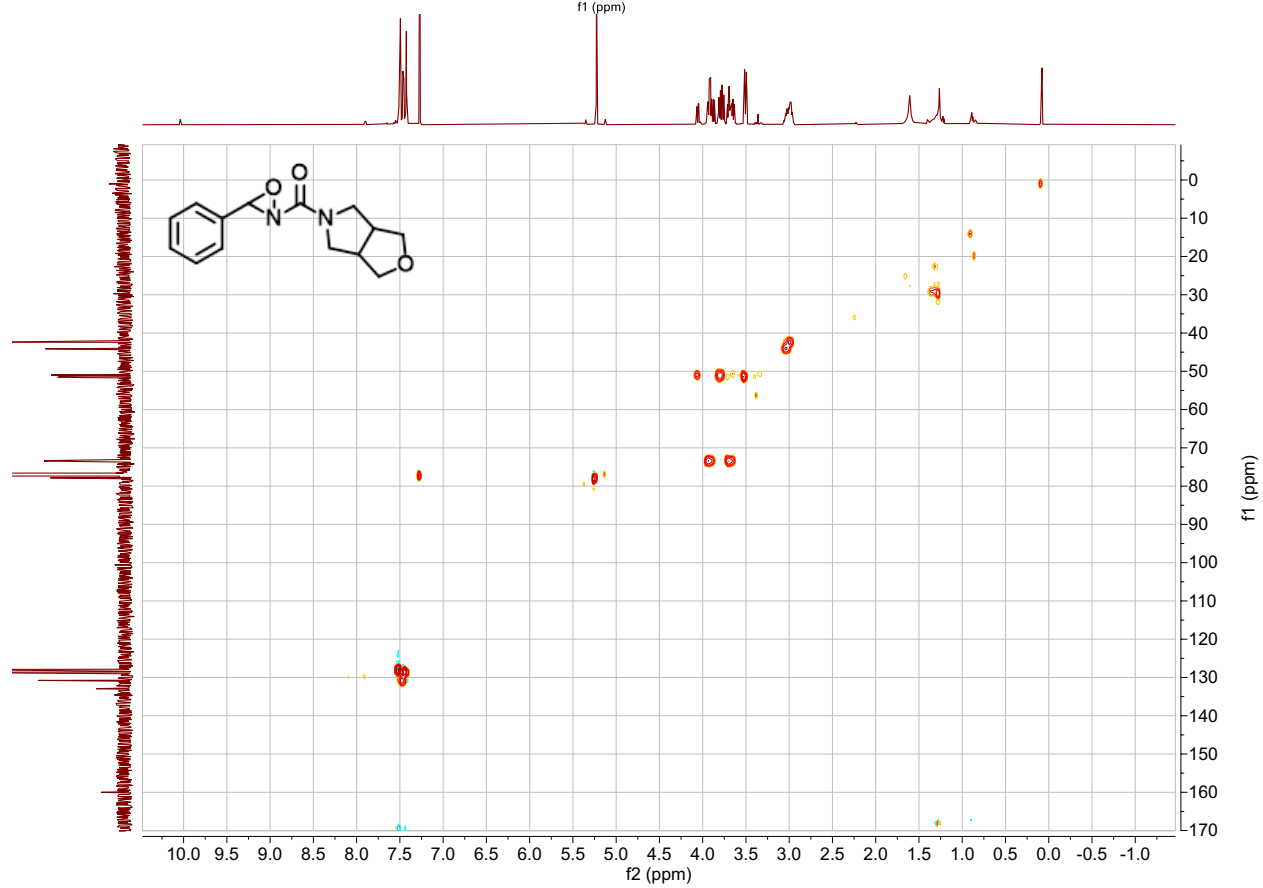
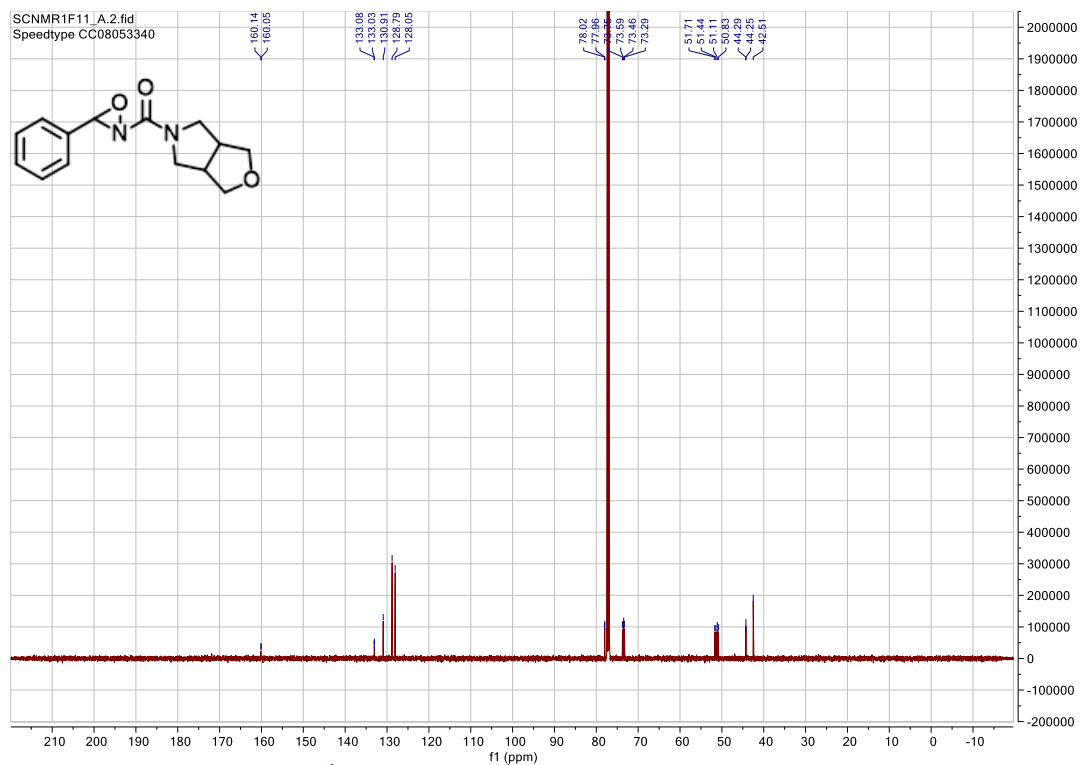
In a 25 mL round-bottom flask, mCPBA (75%, 692 mg, 3.0 mmol, 3 equiv.) was pre-stirred in 1:1 CH₂Cl₂/sat. aq. K₂CO₃ (8 mL) at room temperature for 10 minutes. A solution of crude imine from the previous step (in 1 mL CH₂Cl₂) was added dropwise, using additional CH₂Cl₂ (2 x 1 mL) rinses for a quantitative transfer. After 1 hour, the reaction was diluted with water (30 mL) and extracted with CH₂Cl₂ (3 x 30 mL). The combined organic layers were washed with brine (30 mL), dried over Na₂SO₄, 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.

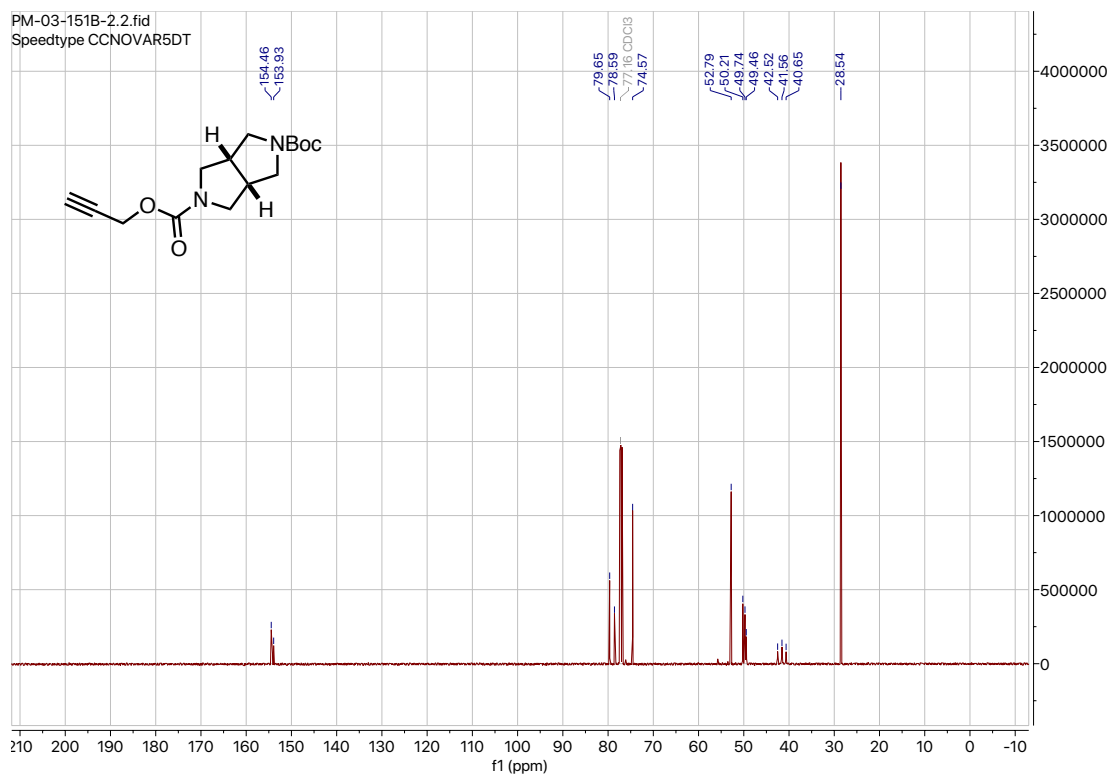
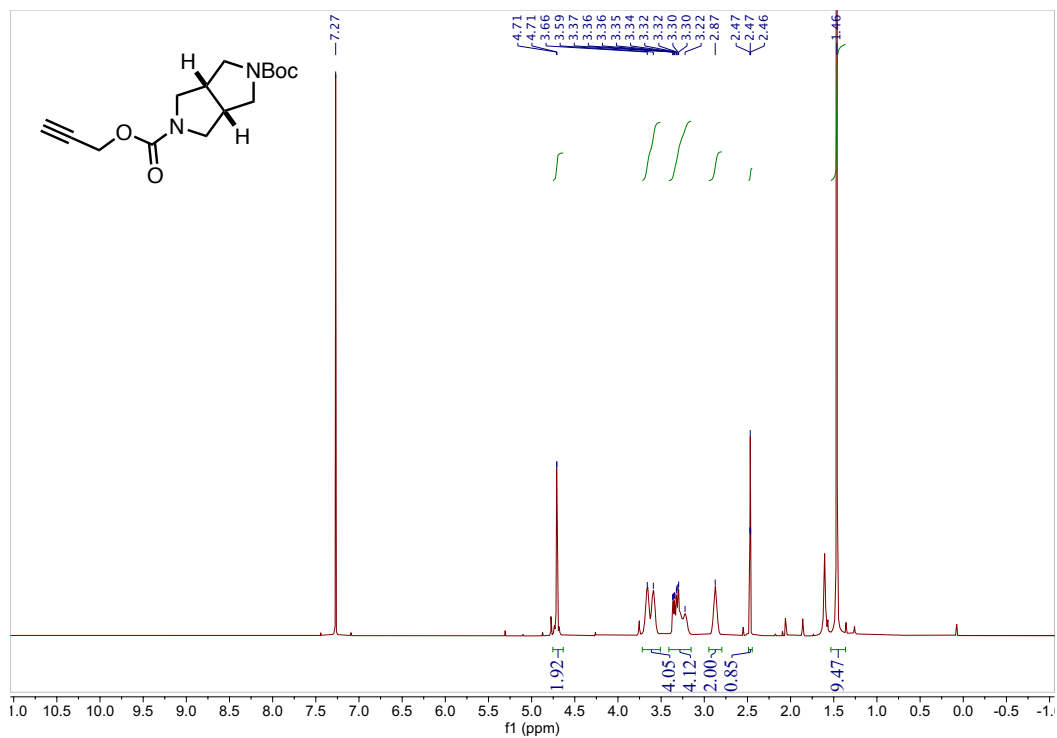
¹H NMR (500 MHz, CDCl₃) δ 7.44 (m, 5H), 5.22 (s, 1H), 4.78 – 4.60 (m, 2H), 4.05 (m, 0.4H, *minor rotamer*), 3.84 – 3.64 (m, 4H), 3.50 – 3.29 (m, 3.6H), 3.01 – 2.87 (m, 2H), 2.46 (m, 1H). **¹³C NMR** (126 MHz, CDCl₃) δ 160.4, 153.9, 130.9, 128.8, 128.03, 128.00, 78.5, 78.0, 74.7, 53.0, 50.7, 50.6, 50.1, 50.0, 41.1, 40.1. **HRMS (ESI)**: calcd for C₁₈H₂₀N₃O₄ [M+H]⁺: 342.1449. Found 342.1442.

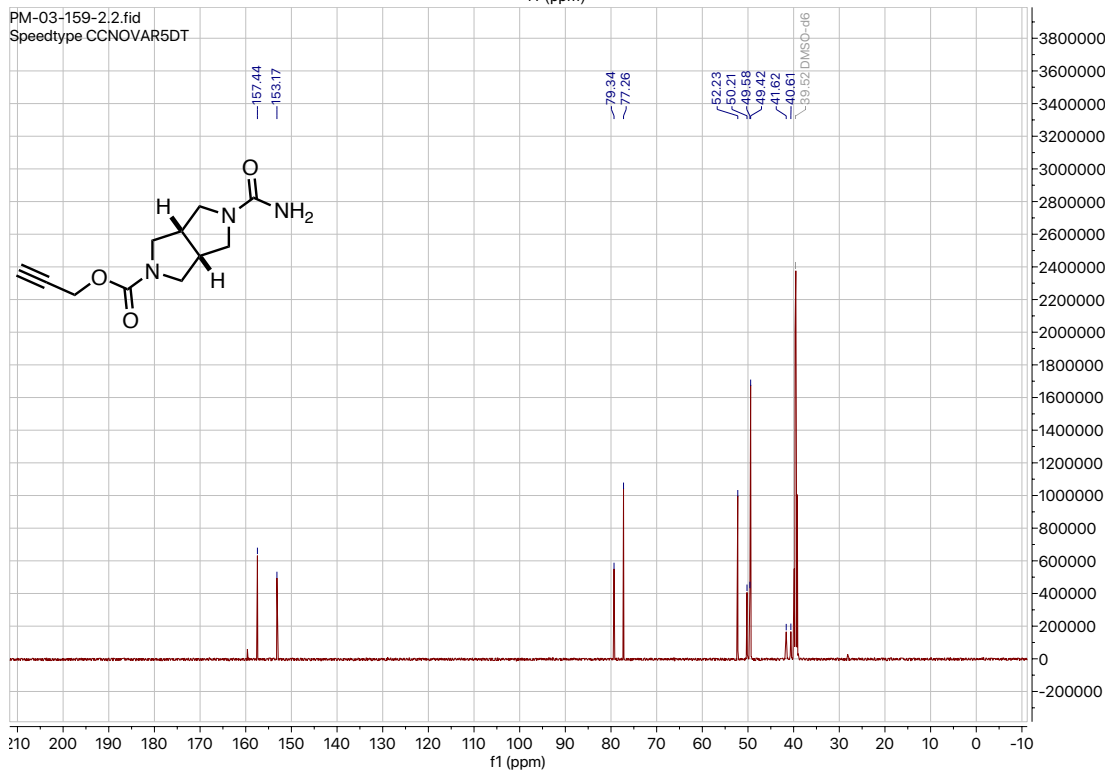
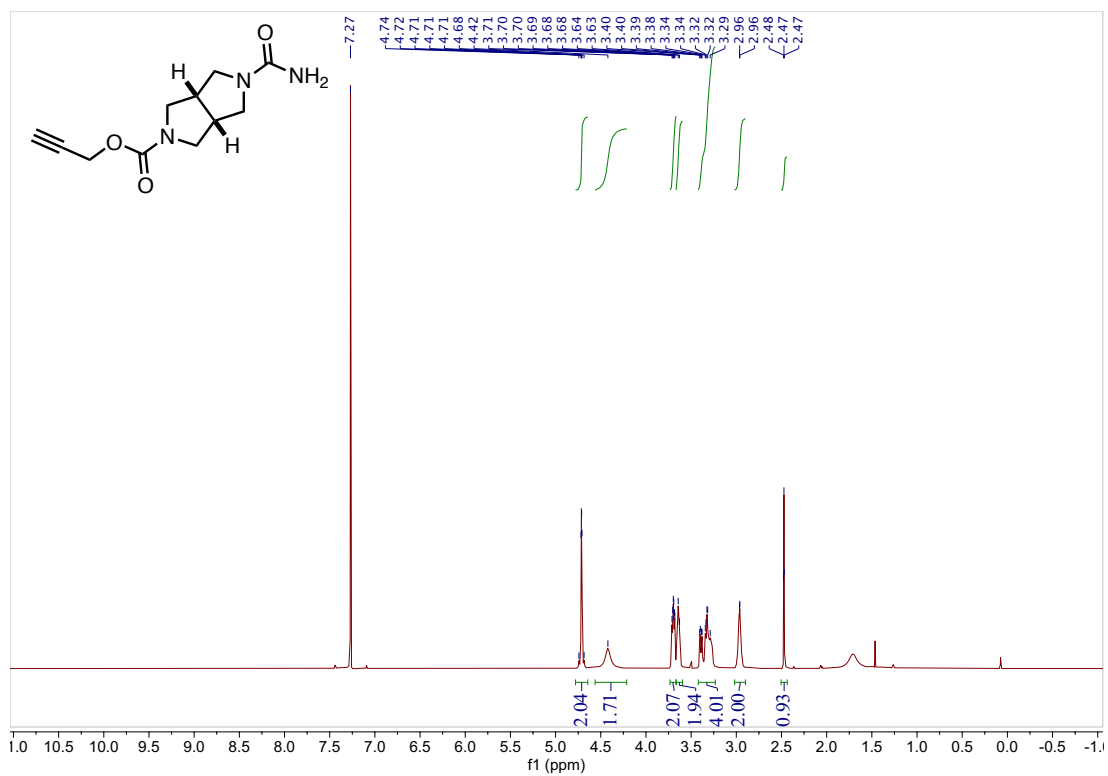
3.7.6 NMR spectra

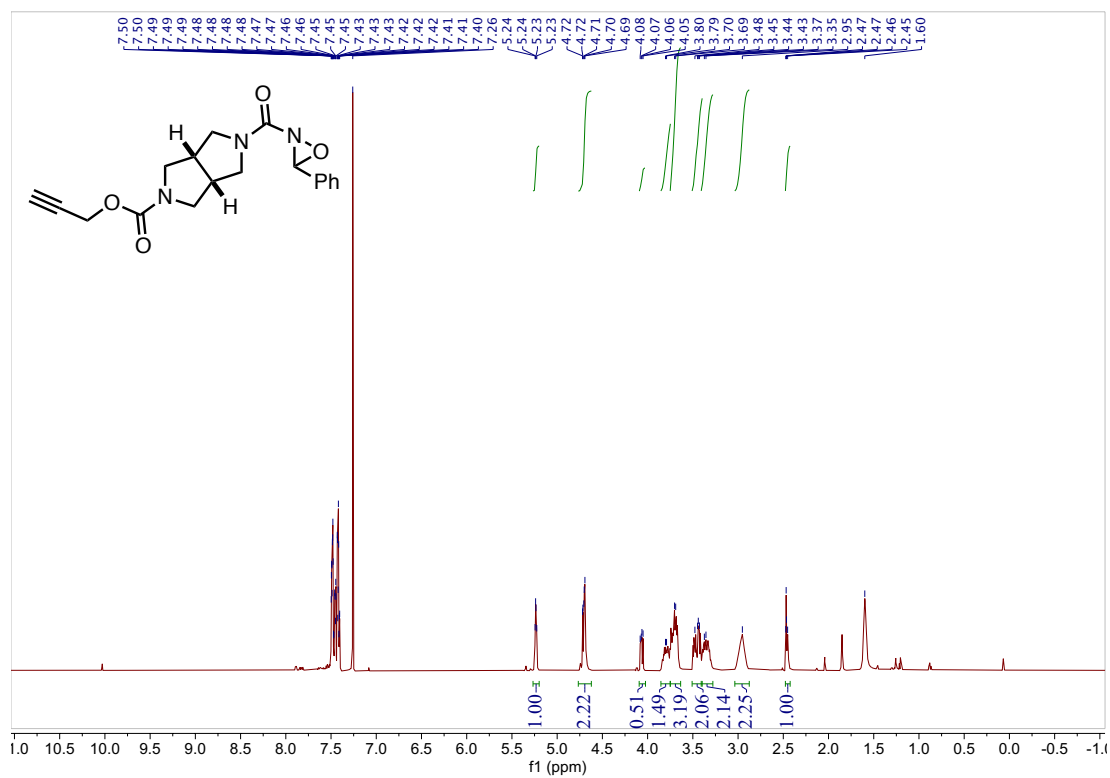












CHAPTER 4

Chiral Methionine Oxidation Reagents Reveal Stereospecific Proteome Modifications

This chapter is partially based on work submitted to *Science* entitled “Chiral methionine oxidation reagents reveal stereospecific proteome modifications” and has been adapted with permission from all co-authors.

4.1 Abstract

Life is predicated on chirality, a molecular asymmetry akin to the left and right versions of human hands. Here we show that privileged protein residues are predisposed for chiral regulation. We developed enantiomeric oxaziridine reagents that systematically identify pro-(*S*) and pro-(*R*) methionine oxidation sites across proteomes that can be erased by stereospecific methionine sulfoxide reductase enzymes A and B, respectively. These probes reveal that chiral regulation of methionine oxidation-reduction processes can allosterically regulate protein function, as shown in cell and mouse models of oxidative stress where selective (*R*)-methionine sulfoxide formation on M69 of biphenyl hydrolase-like protein leads to amplification of *N*-homocysteinylation modifications. This work introduces a platform for characterizing sites of asymmetric methionine oxidation and the functional consequences concomitant with an individual chiral single-atom modification.

4.2 Introduction

Chirality, or the asymmetric orientation of atoms in space, is an intrinsic property of life (157, 158). Nature has evolved preferential handedness as a progenitor for many fundamental aspects of biology. Indeed, the diverse stereochemical ornamentation of natural products and the enantiopurity present in biomacromolecules such as left-handed amino acids or right-handed nucleic acids provide molecular surfaces for selective recognition and regulation events (159, 160) (Figure 4.1A). Within proteins, this homochiral structuring is further stratified through the introduction of post-translational modifications (PTMs). In this context, methionine (Met), one of only two sulfur-containing amino acids, is highly susceptible to post-translational modification. Upon reaction with monooxygenases or reactive oxygen species (ROS), Met converts into methionine sulfoxide (MetO) (161). The oxidation of the thioether moiety introduces a chiral center at sulfur that partitions into two distinct diastereomers: (*S*)- and (*R*)-MetO (122, 162, 163). These (*S*) and (*R*) single-atom oxygen marks on proteins can be removed through stereospecific reduction of the sulfoxide using methionine sulfoxide reductases (Msr) A and B, respectively (164, 165) (Figure 4.1A). Whereas there is direct evidence for the stereospecific reduction of MetO epimers on proteins, the extent of stereospecific oxidation of Met on proteins has not yet been fully realized (166–168) (Figure 4.1A). Critically, Met-based redox events are associated with negative consequences such as the development and progression of neurodegenerative (169) and cardiovascular diseases (170), but also positive regulation of life span (171), protein function (123, 172, 173), and signal transduction (174).

Systematically identifying chiral single-atom modification sites on proteins has remained an elusive endeavor, with most examples performed on a protein-by-protein basis using Msr enzymes (123, 175, 176). With growing evidence of stereospecific MetO redox regulation impacting physiology (123, 173, 177), along with incongruencies in the subcellular distribution of both MsrA and MsrB enzymes to fully resolve racemic MetO epimer formation in eukaryotes (178), we sought to decipher the extent of stereospecific Met oxidation at the proteome level. Inspired by work by Cravatt that identified enantioenriched druggable hotspots in proteomes (23, 101), along with work by our laboratories and others on synthetic methods for selective protein Met bioconjugation (27, 30–32), we developed a pair of enantiopure oxaziridine oxidant probes to systematically identify and interrogate protein chiral microenvironments in which diastereoselective Met

oxidation may occur. This method, termed **ChURRO** (**Chiral Unveiling by Redox-based Reactivity with Oxaziridines**) (Fig. 1A) provides a chemical framework for the deconvolution of prochiral Met oxidation sites in purified proteins, cell lysates, and tissues, allowing for the rapid identification of putative regulatory Met sites that are privileged for stereospecific post-translational modifications.

4.3 Results and Discussion

4.3.1 Development of ChURRO for identifying prochiral methionine oxidation sites in proteins and proteomes

Drawing inspiration from reports of enhanced diastereoselectivity for oxidation of sterically constrained thioethers as well as certain Met oxidation sites on proteins with ROS (179, 180), we reasoned that the extent of diastereoselectivity in Met oxidation could be predicated by the protein microenvironment in which a given Met residue resides. The asymmetric oxidation of Met using ROS would then operate by accessibility of either the pro-(*S*) or pro-(*R*) lone pair on the sulfur atom. We hypothesized that a pair of enantiomeric probes could discriminate between asymmetric Met environments, and we sought to generate enantiopure versions of a previously reported scout ligand, Ox32yne, which exhibits broad Met reactivity to form sulfilimine adducts as isoelectronic proxies for MetO while allowing for downstream functionalization and enrichment of ligated proteins (29) (Figure 4.1A). In this design, the chiral *C*-phenyl moiety of Ox32yne could serve as a hydrophobic directing group for the pan-reactive Met warhead to access putative prochiral Met oxidation sites by differential protein binding of the enantiomers (Figure 4.1A). To meet this goal, (*S*)- and (*R*)-Ox32yne were isolated at preparative scale with >99% ee through chiral SFC separation of the racemic material (Figure S4.1), and the absolute configuration of both enantiomers was determined through experimental and computational vibrational circular dichroism measurements (Figure S4.2). Initial reactivity studies of (*S*)- and (*R*)-Ox32yne with a model substrate, *N*-acetyl-L-methionine methyl ester, revealed that reaction with either enantiomer proceeds to quantitatively form the (*S*)-sulfilimine diastereomer (Figure 4.1B, Figure S4.3, Figure S4.4). Density functional theory computations indicate that the increased steric constraints in the transition state imposed by coordination of Ox32yne lead to differential reactivity of the sulfur lone pairs in the reactive conformation, suggesting that these reagents may be used to probe Met oxidation sites in which diastereoselectivity is controlled by the environment encompassing the Met site (Figure S4.5).

We next sought to apply ChURRO in model proteins and proteomes. We screened a modest set of proteins with Met oxidation sites whose selective reduction by MsrA or MsrB enzymes has been previously reported (123, 179, 181, 182). In-gel fluorescence, as well as LC tandem MS (LC-MS/MS) measurements, revealed the ability for ChURRO to differentially ligate and identify these Met oxidation sites in proteins (Figure S4.6 and Table S4.1). With this proof-of-concept in hand, we envisioned validating pro-(*S*) or pro-(*R*) Met oxidation site detection across proteomes by performing quantitative proteomic studies in a model of (*S*)- or (*R*)-Met oxidation in HEK-293T cells. We generated these mirrored models through genetic knock-down of the stereospecific reductases present in mitochondria, MsrA or MsrB2, followed by oxidative stress through mitochondrial uncoupling to exacerbate (*S*)- or (*R*)-MetO formation, respectively. We anticipated that we would observe stereochemically-biased protein labeling when a prochiral Met

oxidation site is blocked (Figure 4.1C). Across the two experiments, we identified 213 pro-(S) and 157 pro-(R) unique Met oxidation sites that were enriched following accumulation of (S)- or (R)-MetO, respectively, establishing the ability of the ChURRO platform to systematically detect prochiral Met oxidation sites in proteomes using the respective enantioprobe (Figure 4.1D, Table S4.2, Table S4.3).

4.3.2 ChURRO identifies prochiral methionine oxidation sites in the mitochondria

After showing that ChURRO can discriminate prochiral Met oxidation sites in a high-throughput manner, we applied this method to profile stereospecific Met oxidation in isolated mitochondria derived from liver tissue. The liver is an essential organ for maintaining metabolic homeostasis and is highly enriched in mitochondria, a subcellular hotspot for redox-mediated processes. As such, we reasoned that this model would reveal a potential wealth of functional prochiral Met oxidation sites. We employed a combination of sucrose and Percoll gradients to isolate enriched mitochondria via density ultracentrifugation from wild-type mouse livers (Figure 4.2A). The enrichment of mitochondria was confirmed by immunoblot analyses of validated subcellular protein markers (Figure 4.2B). Additionally, we attempted traditional sucrose gradient- or immunoprecipitation-based techniques for isolation of mitochondria from tissues and cells, respectively, but found that a combinatorial gradient approach afforded the highest purity mitochondria (Figure S4.7). With these purified mitochondria in hand, we envisioned a quantitative chemoproteomic approach to identify prochiral Met oxidation sites in the mitochondria by equimolar treatment of both enantioprobes to allow for direct comparison of unperturbed pro-(S) and pro-(R) Met oxidation sites (Figure 4.2C). A rapid assessment of putative pro-(S) and pro-(R) Met oxidation sites by protein gel assays displayed sufficient resolution to distinguish prochiral sites (Figure 4.2D). Encouraged by these qualitative results, we then employed our quantitative chemoproteomics approach and identified 138 significant Met oxidation sites, of which 60 were highly enriched with 39 pro-(S) and 21 pro-(R) sites described (Figure 4.2E and Table S4.4). We also identified hyper-reactive Met sites in these mitochondria by treating with low and high doses of racemic Ox32yne and determined that the previously identified prochiral Met oxidation sites were not simply hyper-reactive Met sites (Figure S4.8 and Table S4.5), showcasing a stereochemical distinction in prochiral Met oxidation that supersedes hyper-reactivity.

4.3.3 Structural and functional characteristics of pro-(S) and pro-(R) methionine oxidation sites

With this wealth of newly revealed prochiral Met oxidation sites across both human and mouse proteomes, we next sought to identify key biochemical parameters that govern structural environments that promote asymmetric Met oxidation and assess their impact on protein function. In this context, a protein's fold is described by its amino acid torsion angles, phi and psi. Plotted together, these angles provide a quantitative stereochemical assessment of local fold. Using the AlphaFold database (183), we extracted the phi and psi angles of each uniquely identified pro-(S) and pro-(R) Met oxidation site determined in the MsrA and MsrB2 knock-down experiments in HEK-293T cells to generate (S)- and (R)-Ramachandran plots (Figure 4.3A, Table S4.6). We determined the distribution of prochiral Met oxidation sites in the phi and psi regions for common secondary protein structures. Both pro-(S) and pro-(R) Met oxidation sites have strong right-handed alpha

helical character, with fractions of 0.63 and 0.66, respectively. Interestingly, the pro-(S) sites displayed slightly enhanced beta sheet and left-handed alpha helical character when compared to the pro-(R) sites, where the latter instead displayed more disorder. Protein Met sites that were shared across the two datasets displayed more equal helical and beta sheet distributions, which mirror the Ramachandran plot of an average Met residue (184) (Figure S4.9 and Table S4.6). Taken together, these results suggest that right-handed alpha helical structures may serve as privileged scaffolds for asymmetric Met oxidation.

The list of putative functional prochiral Met oxidation sites identified from the mitochondrial dataset enabled a more selective proteome analysis by comparing to annotated mitochondrial proteins (185). We determined the subcellular distribution of our identified sites and found a clear enrichment of mitochondrially-localized proteins, validating our purification and immunoblotting results (Figure 4.3B and Table S4.7). The steric environment of all 138 identified Met sites was compared to all 4848 possible mitochondrial methionines by prediction-aware part-sphere exposure (pPSE) analysis, where larger pPSE values denote a more crowded environment (186). From these data, we determined a bimodal distribution of crowdedness for the average mitochondrial Met, giving rise to two populations of either more buried or more accessible Met sites (Figure 4.3C and Table S4.8). In contrast, the same analysis on our identified prochiral Met oxidation sites revealed a unimodal distribution in only the more buried environments, suggesting a role for steric bias in asymmetric Met oxidation. Moreover, predicted mutation pathogenicity scoring through AlphaMissense analysis of the 138 prochiral Met oxidation sites revealed that 72.4% of all possible 2626 missense mutations are predicted to be pathogenic, and that 97.1% of the 138 sites have at least one predicted pathogenic mutation, highlighting the biological significance of these privileged residues that are predisposed to stereospecific oxidation (187) (Figure 4.3D and Table S4.9). Along these lines, gene ontology (GO) analysis revealed a strong enrichment in proteins involved with amino acid biosynthesis, particularly proteinogenic and non-proteinogenic sulfur amino acids, suggesting potential regulatory roles in amino acid metabolism for certain prochiral Met oxidation sites (Figure 4.3E and Table S4.10).

4.3.4 Human BPHL contains a regulatory allosteric prochiral methionine oxidation site at M69

To showcase the consequences of asymmetric Met oxidation on protein function, we chose to characterize a highly enriched and significant pro-(R) M69 site present on mouse biphenyl hydrolase-like protein (BPHL) as a representative example of prochiral Met regulation. BPHL is a mitochondrially-localized serine hydrolase that is most commonly associated with prodrug activation but is also suggested to play a significant role in homocysteine thiolactone (HCTL) detoxification (188, 189). HCTL levels reciprocally correlate with those of homocysteine (HC), a non-proteinogenic amino acid involved in cysteine and Met biosynthesis, where HC is converted to HCTL through an error-editing mechanism of methionyl-tRNA synthetase (190, 191). Aberrant overproduction of HCTL from HC introduces a highly electrophilic species that reacts with protein-bound amines via *N*-homocysteinylation (*N*-Hcylation). *N*-Hcylation adducts are known to induce protein damage, aggregation, immunogenicity, and cellular toxicity (192) (Figure 4.4A). Analysis of available crystallographic data for human BPHL (hBPHL) revealed that the identified M69 site lies in an allosteric hydrophobic pocket proximal to the catalytic triad (193)

(Figure 4.4B), and a multiple-sequence alignment revealed sequence conservation of this Met site in humans, mice, and many orders of animal phyla, suggesting this site may have an evolved functional role (Figure S4.10). To further understand the functional consequences of this M69 site, we generated recombinant wild-type and M69 to leucine (M69L) variants of hBPHL and measured their activities using the native substrate HCTL following oxidation with hydrogen peroxide (H_2O_2) or a chiral camphor-derived Davis oxaziridine (OT-Ox) previously used as an asymmetric oxidation reagent to form (*R*)-sulfoxides (194) (Figure S4.11). Increasing doses of either oxidant led to catalytic inactivation of wild-type hBPHL, which was attenuated by the M69L mutation, suggesting HCTLase activity is affected by allosteric M69 redox regulation (Figure 4.4C and Table S4.11). Moreover, in-gel fluorescence and dose-response studies on purified wild-type hBPHL establish that the (*R*)-Ox32yne probe is the favored enantiomer to ligate hBPHL and inhibits the protein with an IC_{50} of 336 nM (Figure 4.4D, Figure S4.12, Table S4.12).

4.3.5 Protein *N*-homocysteinylolation is regulated by a BPHL/MsrB2 axis in cell and tissue models of oxidative stress

Following *in vitro* characterization of BPHL's prochiral regulatory M69 site on purified protein models, we explored how this stereospecific single-atom modification impacts downstream protein *N*-Hcylation at the proteome level. To this end, we devised a cellular *N*-Hcylation assay that utilizes mitochondrial uncoupling reagents for *in situ* ROS generation, followed by dosing of HCTL, to examine the extent of protein *N*-Hcylation by leveraging a chemoselective native chemical ligation strategy reported by Wang (195) (Figure 4.5A). Screening a panel of hepatic and renal cell lines, we found that HepG2, HuH-7, and HEK-293T cells all showed high relative expression levels of BPHL compared to other models (Figure S4.14 and Table S4.13). We chose HEK-293T cells for *in cellulo* characterization of protein *N*-Hcylation levels owing to their ease of transfection for subsequent genetic perturbation studies. Expression of an epitope-tagged BPHL confirmed selective ligation of (*R*)-Ox32yne in these cells by immunoprecipitation studies (Figure S4.15). To further validate (*R*)-MetO formation and regulation, we measured MsrA or MsrB2 engagement of BPHL *in cellulo*, revealing an increase of MsrB2 co-precipitating with BPHL under conditions of oxidative stress, suggesting that MsrB2 is an eraser of BPHL (*R*)-MetO (Figure 4.5B). We next measured protein *N*-Hcylation levels following knock-down of either MsrA or MsrB2 and treatment with paraquat to induce oxidative stress. Global protein *N*-Hcylation levels were comparable for control and MsrA knock-down treatments but displayed a significant 2-fold increase upon knock-down of MsrB2 (Figure 4.5C, Figure S4.16, Table S4.14). These cellular data support the *in vitro* studies and suggest BPHL activity is stereospecifically regulated by Met redox status, which in turn amplifies downstream protein *N*-Hcylation across proteomes.

The crosstalk between MetO, a chiral single-atom modification, and global protein *N*-Hcylation prompted us to study the biochemical relationship between these two PTMs in a physiologically relevant model of oxidative stress. Specifically, non-alcoholic fatty liver disease (NAFLD) is an increasingly prevalent chronic liver ailment worldwide, with an estimated 1 in 4 individuals affected by this disease (196). The progression of NAFLD leads to the onset of liver fibrosis, cirrhosis, carcinoma, and death (197). While the molecular mechanisms of NAFLD pathogenesis are not fully elucidated, there is evidence for disruptions in homocysteine metabolism and increased oxidative stress associated

with NAFLD pathophysiology and severity (198–200). We therefore sought to investigate a potential connection between protein *N*-Hcylation and chiral Met oxidation status proteome-wide. We elected for a diet-induced murine liver model of NAFLD, with low-fat diet (LFD) mice serving as controls and high-fat diet (HFD) mice serving as disease models (Figure S4.17). Following tissue extraction, we performed immunoblot analyses of BPHL, MsrA, and MsrB2 enzymes and found no significant change in BPHL or MsrA, but a significant reduction in MsrB2 levels in HFD liver tissue (Figure S4.18 and Table S4.15). These findings mirror our *in cellulo* *N*-Hcylation studies, which revealed that deficiencies in MsrB2 led to increased proteome *N*-Hcylation under conditions of oxidative stress. Therefore, we envisioned a pulse-chase experiment to systematically profile the pro-(*R*) Met oxidation landscape in these tissues enabled by (*R*)-Ox32yne to identify sites that may influence *N*-Hcylation (Figure 4.5D). To achieve this goal, we performed two-dimensional proteomic profiling of both LFD and HFD tissues with one dimension normalizing for protein abundance and the other dimension characterizing changes in pro-(*R*) Met oxidation status under vehicle and H₂O₂ treatment (Figure 4.5E, Table S4.16, Table S4.17). To our delight, nearly all pro-(*R*) Met oxidation sites that significantly changed in oxidation status following addition of H₂O₂ were significantly enriched in LFD tissue. These data indicate a loss of (*R*)-Ox32yne labeling in HFD tissue, suggesting that these sites are predisposed to (*R*)-MetO formation in models of NAFLD. Indeed, BPHL M69 is one such protein site that is oxidized in HFD tissue (Figure S4.19, Figure S4.20, Table S4.18, and Table S4.19). Moreover, many of the other pro-(*R*) Met oxidation sites that are redox susceptible in HFD tissue are involved in lipid regulatory processes. Included is PLIN2, which displays an inverse relationship following oxidative stress, suggesting a preference for its M265 site to be in the reduced state in HFD tissues. Assessment of protein *N*-Hcylation in these tissues found a significant dose-dependent increase in protein *N*-Hcylation in the HFD tissues upon titration of HCTL (Figure 4.5F, Figure S4.21, Table S4.20). Taken together, these data support a model where a chiral single-atom modification on M69 of BPHL, a biocatalyst for HCTL degradation, inactivates its activity leading to higher levels of HCTL and amplification of downstream protein *N*-Hcylation across the proteome.

4.4 Conclusions

Reversible, regulated oxidation-reduction cycles on protein Met residues give rise to chiral single-atom modifications across the proteome. The stereochemistry that engenders these events has been partially elucidated through characterization of MetO reduction events catalyzed by stereospecific Msr enzymes. Here, we show an inherent stereospecificity in PTM oxidation events that occur at privileged prochiral Met oxidation sites driven by the structural asymmetry present in protein pockets. We challenge the dogma of stereochemically non-specific MetO formation in proteins through development of a biomimetic chiral oxidation reagent platform to detect these privileged protein sites. The ChURRO method enables systematic identification of prochiral Met oxidation sites on purified proteins and in whole proteomes. Using this approach, we identified a diverse array of new prochiral Met oxidation sites in human whole-cell proteomes, as well as in mouse liver-derived mitochondria, a subcellular locus of redox homeostasis and regulatory processes. In-depth analysis of the local microenvironments of these prochiral Met oxidation sites revealed an enrichment of sterically occluded, right-handed alpha

helices around these residues, which we posit favor asymmetric Met oxidation. Together, these data elucidate foundational local environment preferences as well as protein functions that stratify and distinguish prochiral Met oxidation sites from other Met residues within the proteome.

To establish the functional consequences for regulation of prochiral Met oxidation sites, we performed extensive biochemical studies of a representative identified pro-(*R*) Met oxidation site, M69, on the mitochondrial serine hydrolase BPHL. We show that this prochiral redox site plays an important role in HC metabolism through the hydrolysis of the reactive intermediate HCTL. Through site-directed mutagenesis studies, we revealed that HCTLase activity is dependent upon M69 redox status and its oxidation to (*R*)-MetO can function as a negative allosteric regulator of protein activity, resulting in increased HCTL levels and protein *N*-Hcylation in cells. We also found that BPHL is a selective substrate for the (*R*)-MetO reductase MsrB2, but not the corresponding (*S*)-MetO reductase MsrA, when subjected to elevated ROS levels, suggesting stereochemically privileged (*R*)-MetO formation and reduction on this protein. We further characterized pro-(*R*) Met oxidation sites in murine models of NAFLD, where loss of MsrB2 induced by a high-fat diet led to increased levels of M69 oxidation on BPHL and subsequent amplification of proteome-wide protein *N*-Hcylation. Indeed, these findings support a newly identified PTM crosstalk pathway between Met oxidation and protein *N*-Hcylation, where chiral regulation of oxidation-reduction events at a specific M69 site on BPHL leads to formation of (*R*)-MetO and allosteric inhibition of BPHL activity, resulting in elevated HCTL levels and downstream protein *N*-Hcylation. This work presages that chiral protein Met modifications can serve as privileged allosteric signaling moieties that reveal new native physiology and disease vulnerabilities at the single-atom level for potential detection and intervention.

4.5 Acknowledgements

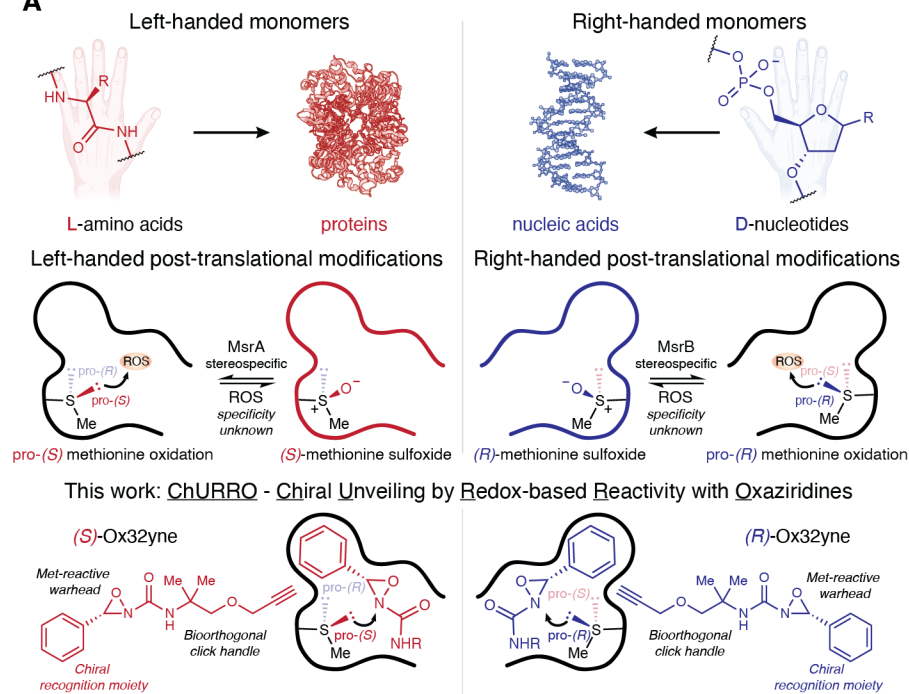
We thank Dr. A. Killilea and her staff at the UC Berkeley Cell Culture Facility for cell culture support (RRID: SCR_017924). We thank J. Nafie of BioTools, Inc. for VCD analyses. We thank Dr. A. Jain and Prof. R. Zoncu for providing cells for mito-IP comparison and suggestions for mitochondrial purification. We thank Dr. T. Xiao for providing mouse liver tissue. We thank Dr. H. Celik for assistance with NMR spectroscopy (NIH S10OD024998). We thank Drs. V. Roytman, K. Durkin, and D. Small for computational support (NIH S10OD034382).

4.6 Co-author contributions

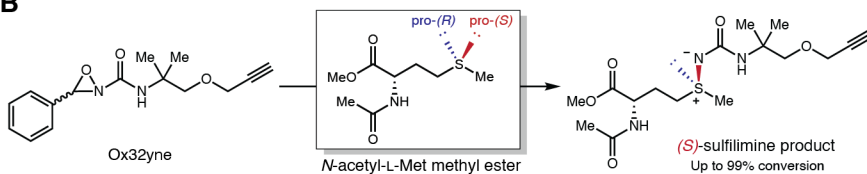
A.G.-V., A.C.S.P., A.G.R., F.D.T., and C.J.C. conceived of this study. A.C.S.P., A.G.-V., J.M.B., and T.G.C. synthesized the chemical probes used in this study. S.C.V. performed the SFC chiral separation. A.C.S.P. performed DFT computations. A.G.-V. and C.J.C. designed the proteomic experiments and A.G.-V. and A.C.S.P. performed the proteomic experiments. A.G.-V. and A.C.S.P. purified mitochondria. A.G.-V. performed biochemical experiments on purified proteins. A.L.G. and A.S. raised and fed mice and harvested tissue. X.X., R.R.S., A.A.A., and N.D. performed bioinformatic analyses for this study based on discussions with A.G.-V., J.M.B., and D.H. The initial manuscript was written by A.G.-V. and A.C.S.P. All authors contributed to the editing of this manuscript.

4.7 Figures and Schemes

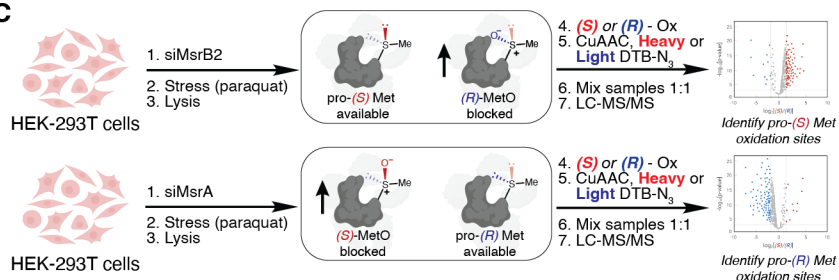
A



B



C



D

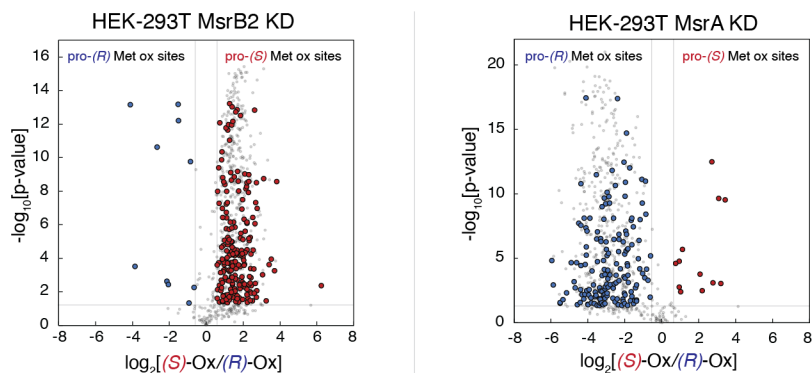


Figure 4.1. ChURRO for detection of prochiral methionine oxidation sites.

(A) (Top) Homochirality paradigm present within the biomacromolecules of life. (Middle) Chiral post-translational modification of Met and native Met redox regulation. (Bottom) ChURRO strategy for the global detection of prochiral Met oxidation sites. **(B)** Model studies of (*R*)- or (*S*)-Ox32yne (50 mM) incubated with *N*-acetyl-L-methionine methyl ester (55 mM) in cosolvent (1:1 MeOD:D₂O) at room temperature for 30 minutes. Product was confirmed by ¹H NMR and VCD measurement. **(C)** ChURRO-ABPP strategy to identify prochiral Met oxidation sites. HEK-293T cells were incubated with siRNA (50 nM) to knock-down endogenous MsrB2 or MsrA followed by addition of paraquat (100 μM) to bias (*R*)- or (*S*)-MetO formation on proteins. Following lysis, lysates were treated with equimolar (200 μM) doses of (*S*)- or (*R*)-Ox32yne and labeled with isotopically-encoded desthiobiotin tags through Cu-mediated click chemistry. Proteomes were then combined, enriched using streptavidin, tryptically digested, and analyzed by LC-MS/MS. **(D)** Volcano plot of ChURRO-ABPP identifying prochiral Met oxidation sites in oxidatively-stressed HEK-293T lysates lacking either (*R*)- or (*S*)-MetO rescue mechanisms (n = 12 technical MS replicates).

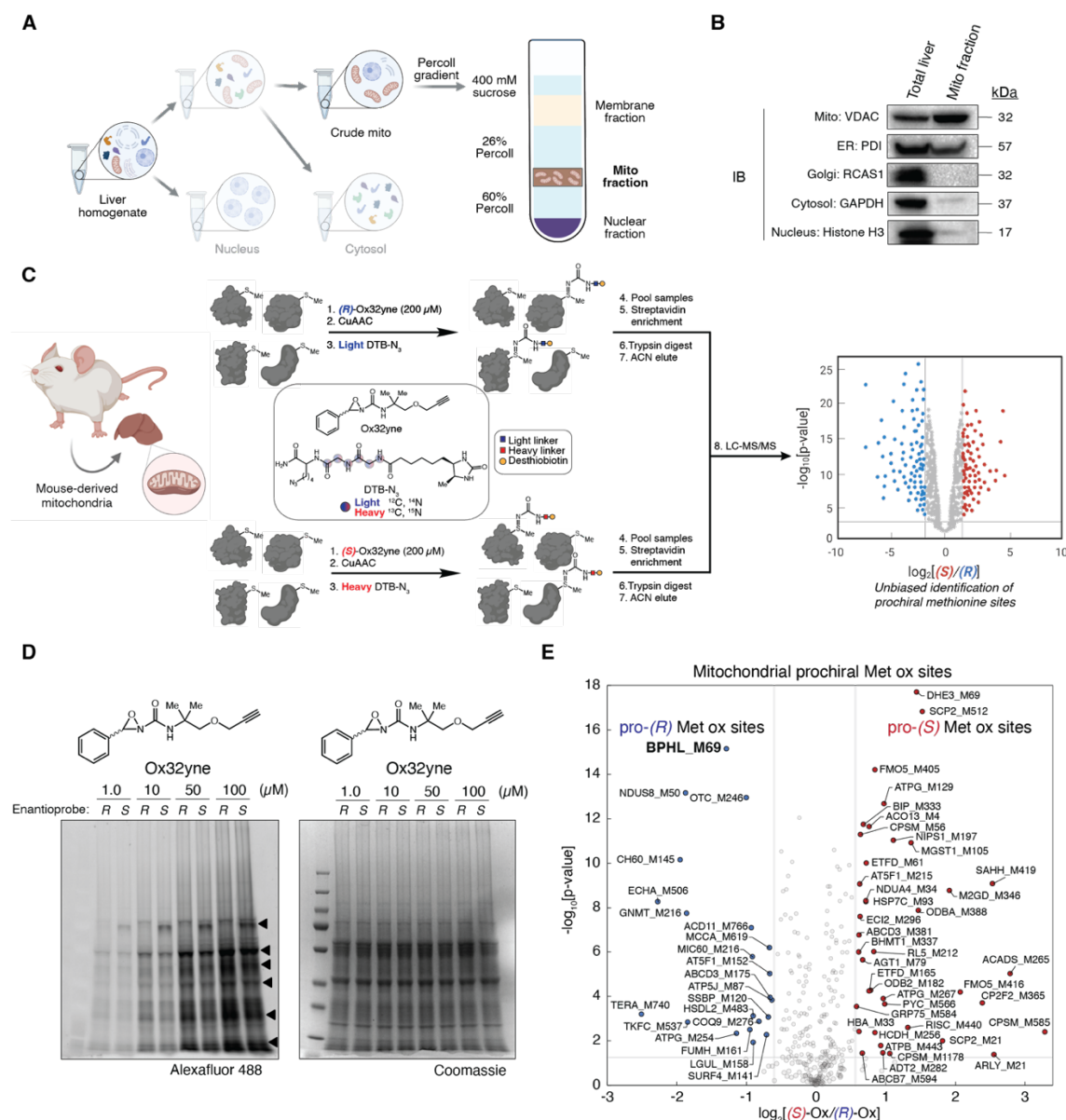


Figure 4.2. Chemoproteomic detection of prochiral methionine oxidation sites in the mitochondria.

(A) Density centrifugation and Percoll gradient scheme for isolation of highly pure mitochondria from mouse liver tissue. (B) Immunoblot analysis of mitochondrial purity using various subcellular protein markers. (C) ChURRO proteomic strategy to identify prochiral Met oxidation sites. Mitochondria were incubated with equimolar doses of (R)- or (S)-Ox32yne and labeled using isotopically-encoded desthiobiotin tags through Cu-mediated click chemistry. Proteomes were then combined, enriched using streptavidin, tryptically digested, and analyzed by LC-MS/MS. (D) (R)- and (S)-Ox32yne prochiral Met profile in purified mitochondria with increasing doses of reagent visualized by in-gel fluorescence. (E) Volcano plot analysis of statistically significant and highly enriched pro-(R) and pro-(S) Met oxidation sites in the mitochondria (n = 12 technical MS replicates)

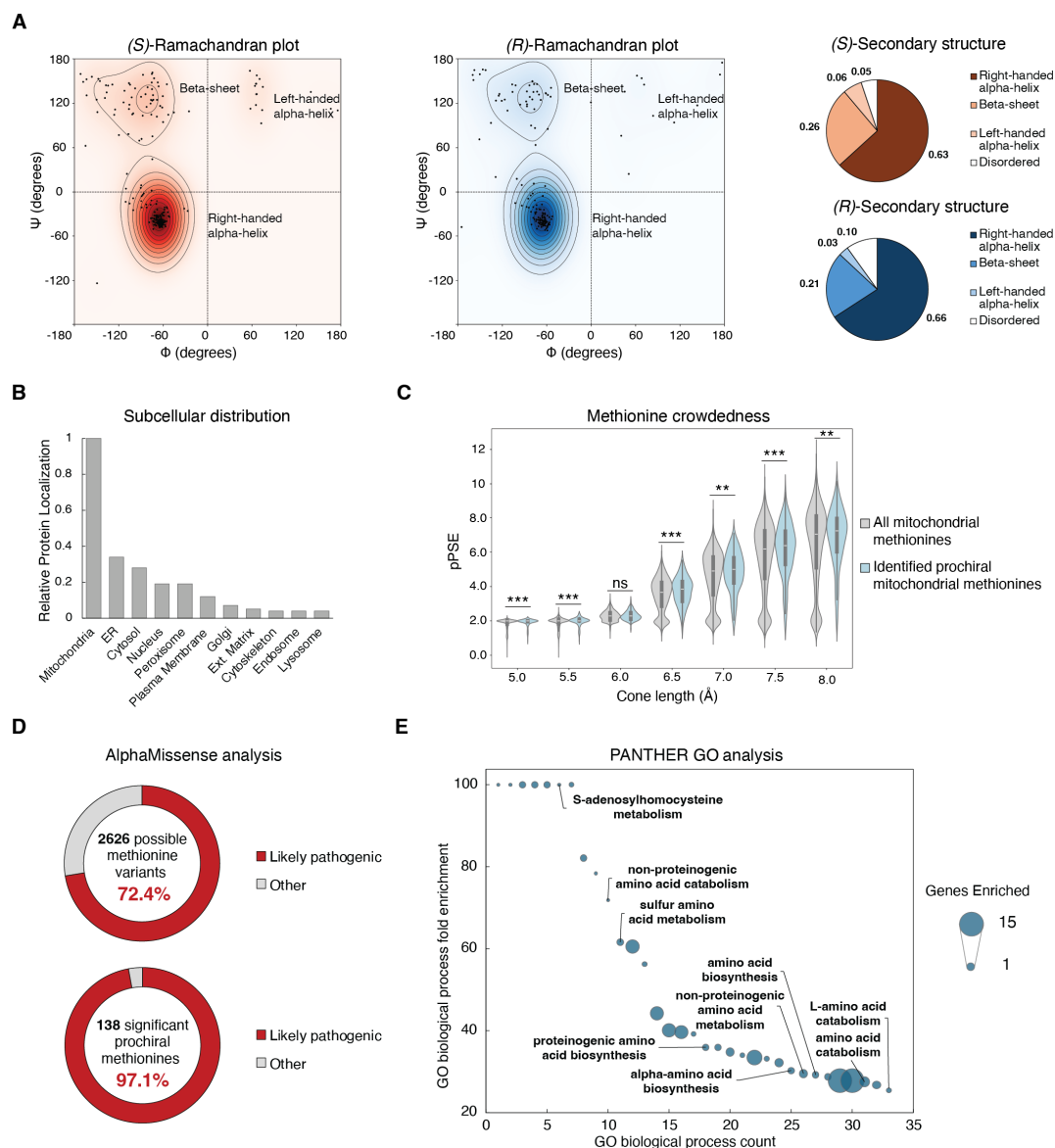


Figure 4.3. Mapping the microenvironment and functional landscape of prochiral methionine oxidation sites in proteins and proteomes.

(A) Ramachandran plots of enriched pro-(S) and pro-(R) Met oxidation sites determined from ChURRO experiments in Msr-deficient HEK-293T cells. Phi and psi angles of identified prochiral Met oxidation sites were extracted from the AlphaFold database to visualize and quantify local environment. (B) Normalized subcellular distribution of proteins identified from mitochondrial prochiral Met oxidation site dataset. (C) Met crowdedness of the entire mitochondrial methionome compared to the identified mitochondrial prochiral Met oxidation sites. Crowdedness was determined by counting the number of residues within a projected cone of varying sizes using part-sphere exposure (pPSE) analysis for each individual Met site ($n = 4848$ total mito Met sites, $n = 138$ identified prochiral Met oxidation sites). (D) AlphaMissense prediction of 2626 missense variants of identified mitochondrial prochiral Met oxidation sites. (E) Gene ontology analysis of mitochondrial prochiral Met oxidation sites.

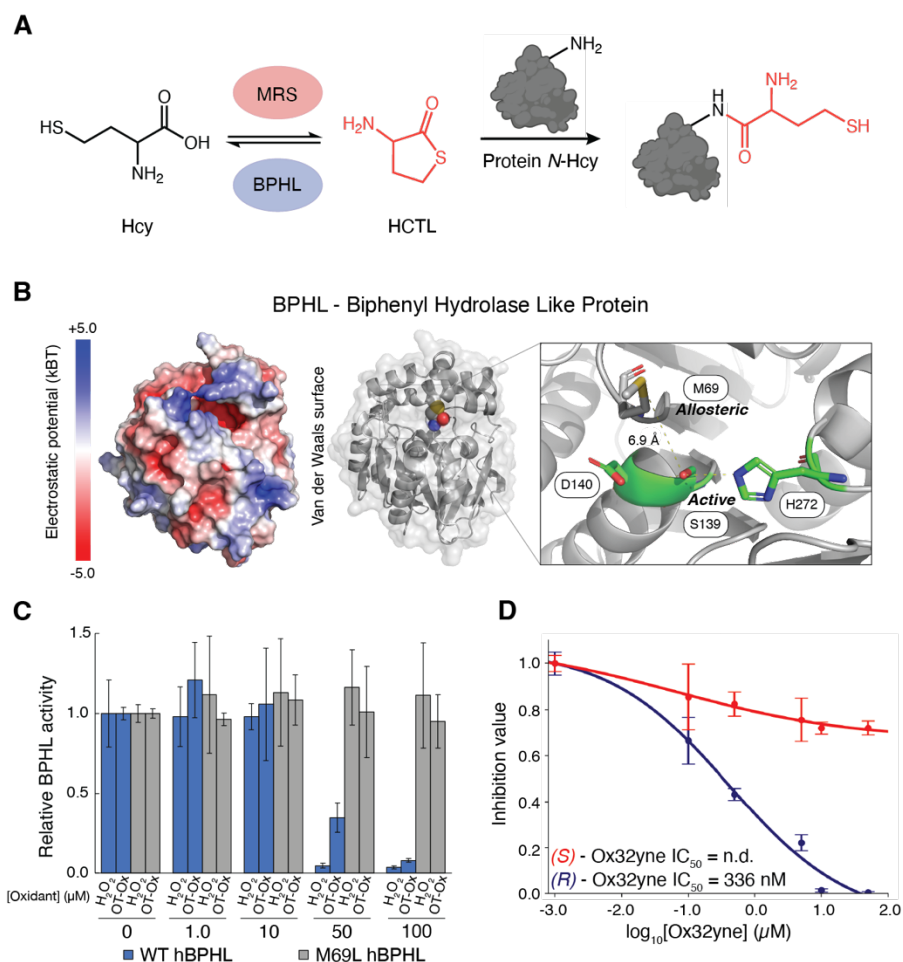


Figure 4.4. Human BPHL contains an allosteric regulatory M69 site.

(A) Homocysteine thiolactone (HCTL) regulation by BPHL and methionyl-tRNA synthetase (MRS) and subsequent protein *N*-homocysteinylation with HCTL. (B) Crystal structure of human BPHL (PDB 2OCG) with catalytic triad. (C) Activity assay of WT and M69L variant of BPHL under increasing doses of various oxidants, showing that allosteric M69 oxidation inhibits BPHL activity ($n = 3$ technical replicates, error bars represent standard deviation). (D) Dose-response curve of WT BPHL treated with (*S*)- and (*R*)-Ox32yne with calculated IC_{50} values, showing selectivity for the (*R*) enantiomer ($n = 3$ technical replicates, error bars represent standard deviation).

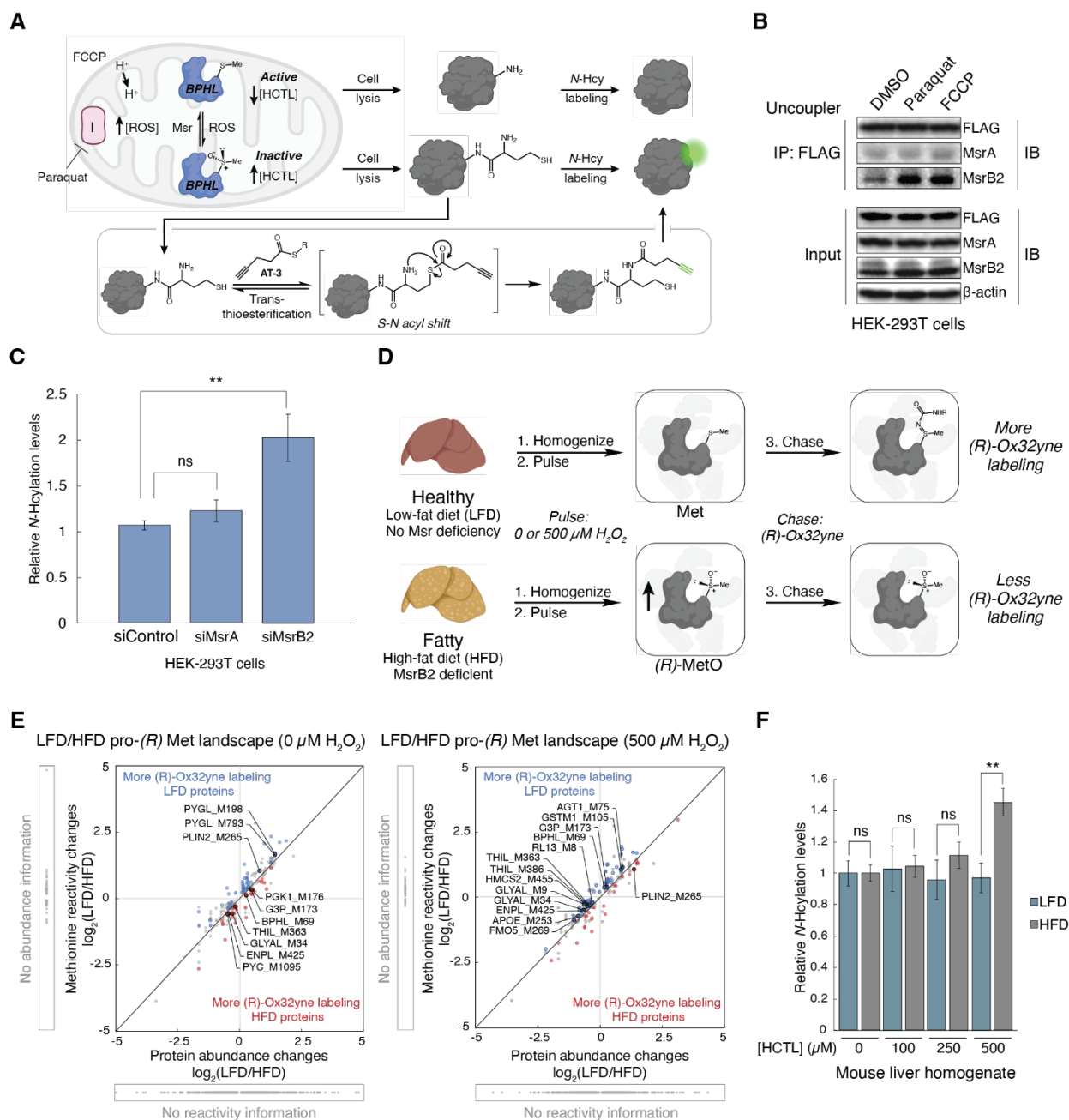


Figure 4.5. MsrB2 regulates protein *N*-homocysteinylation through stereospecific methionine redox regulation of BPHL in cells and in mouse models of non-alcoholic fatty liver disease.

(A) Mitochondrial uncoupler assay to assess protein *N*-homocysteinylation status as a function of BPHL Met redox status using thioester probe AT-3 to label sites of protein *N*-homocysteinylation. (B) BPHL and Msr co-immunoprecipitation analysis following mitochondrial uncoupling in HEK-293T cells. (C) Relative protein *N*-homocysteinylation levels following loss of mitochondrial (S)- or (R)-MetO rescue mechanisms ($n = 3$ cell technical replicates, error bars represent standard deviation). (D) Pulse-chase experimental design for assessing changes in the pro-(R) Met landscape in healthy and MsrB2 deficient fatty mouse liver tissue. (E) 2D proteomic analysis of the pro-(R) Met

landscape in healthy and fatty mouse liver tissue. Changes in pro-(*R*) Met reactivity (n = 3 bioreplicates, n = 3 technical MS replicates) are plotted on the Y-axis while changes in protein abundance (n = 3 bioreplicates, n = 8 technical MS replicates) are plotted on the X-axis. Values above or below the y = x line are sites that shift due to changes in pro-(*R*) Met reactivity. Pro-(*R*) Met sites that significantly change in reactivity across both experiments are labeled. **(F)** Relative *N*-homocysteinylation levels in healthy and fatty mouse liver following oxidative stress (n = 3 bioreplicates, error bars represent standard deviation).

4.8 Supplementary information for “Chiral Methionine Oxidation Reagents Reveal Stereospecific Proteome Modifications”

4.8.1 Supplementary figures

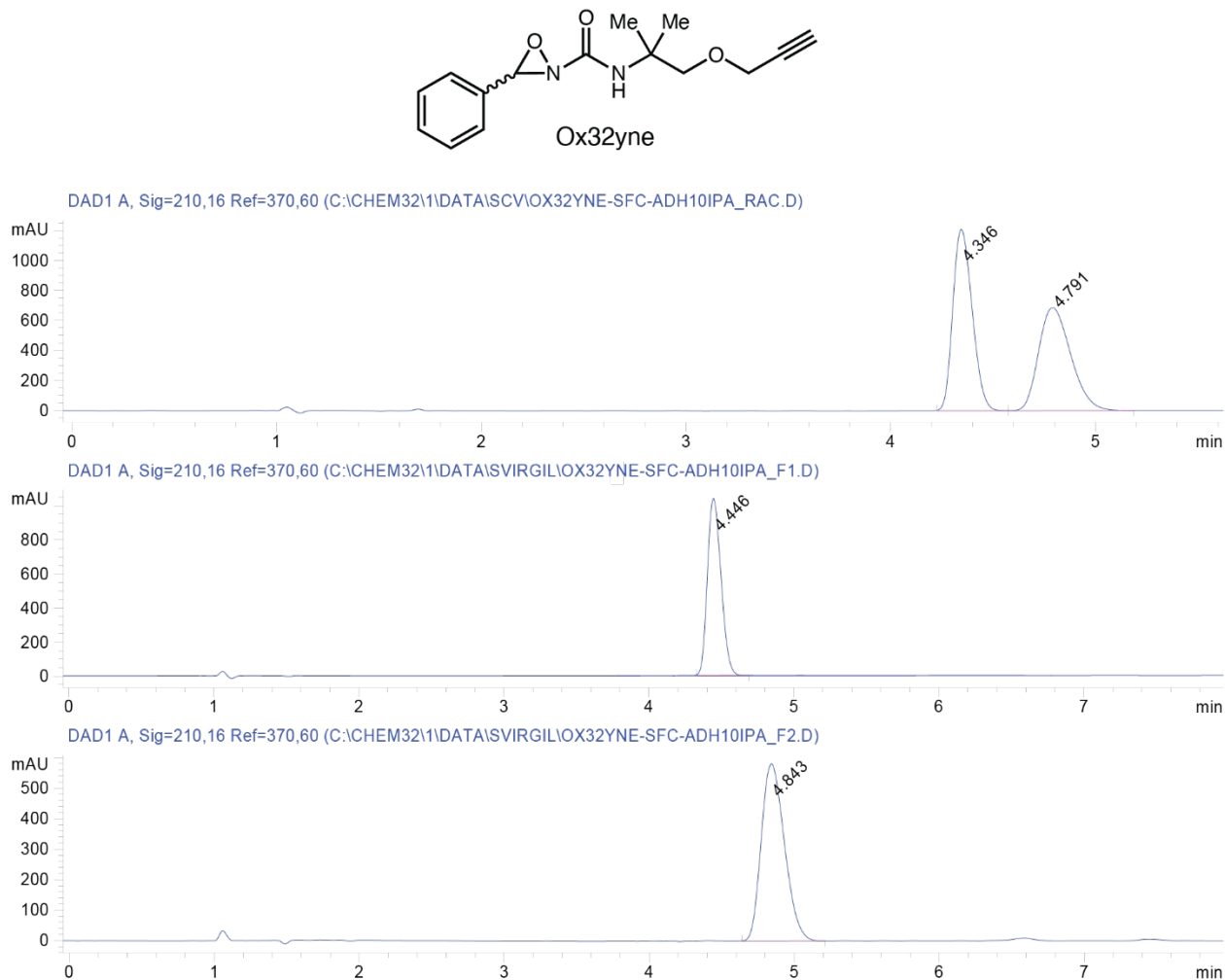


Figure S4.1. scCO₂-based separation of the two Ox32yne enantiomers.

The enantiomeric mixture of Ox32yne (200 mg) was purified on a JASCO System 2000 preparative SFC system with 25 mL/min of 25% IPA/CO₂ on a 20 x 250 mm Chiral Technologies OZ-H column to afford 92 mg of each enantiomer. The fractions were analyzed on a Thar SFC system with 2.5 mL/min of 10% IPA/CO₂ on a 4.6 x 250 mm Chiral Technologies AD-H column. (Top) SFC chromatogram (AD-H, 10% IPA/CO₂) of racemic Ox32yne (51.5:48.5 e.r). (Middle) SFC chromatogram (AD-H, 10% IPA/CO₂) of Fraction 1 of Ox32yne at a purity exceeding 99%, assigned as (*R*)-Ox32yne by VCD. (Bottom) SFC chromatogram (AD-H, 10% IPA/CO₂) of Fraction 2 of Ox32yne at a purity exceeding 99%, assigned as (*S*)-Ox32yne by VCD.

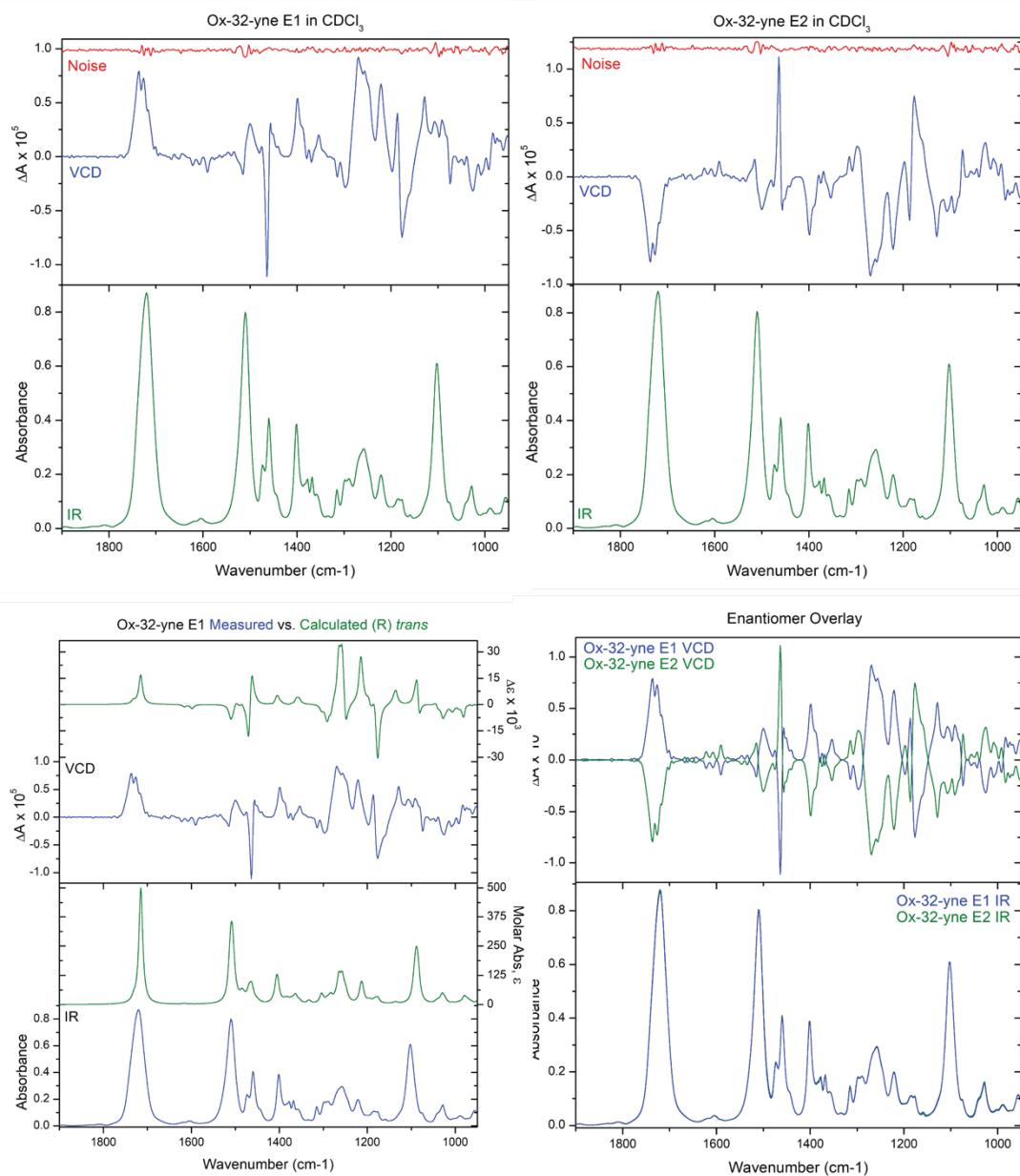
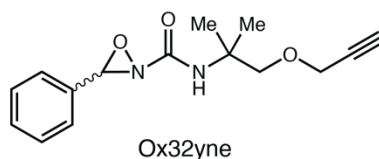


Figure S4.2. VCD analysis of Ox32yne.

(Top Left) Measured VCD and IR spectra of first eluting enantiomer of Ox32yne. (Top Right) Measured VCD and IR spectra of second eluting enantiomer of Ox32yne. (Bottom Left) Measured and calculated IR and VCD spectra of first eluting enantiomer of Ox32yne, assigned as (*R*)-Ox32yne. (Bottom Right) Overlay of measured VCD and IR spectra of enantiomers of Ox32yne, assigned as (*R*) and (*S*), respectively, in order of elution on AD-H column.

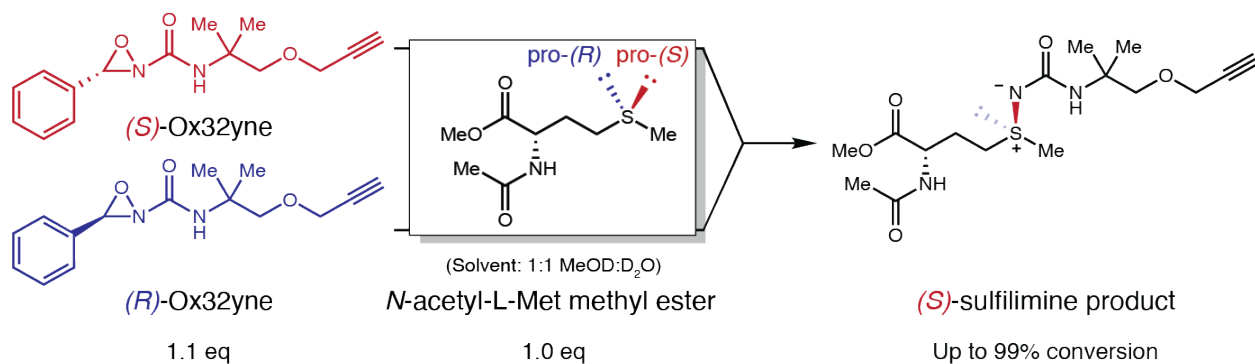


Figure S4.3. Model diastereoselectivity studies of Ox32yne.

(*R*)- or (*S*)-Ox32yne (50 mM) were incubated with *N*-acetyl-L-methionine methyl ester (55 mM) in cosolvent (1:1 MeOD:D₂O) at room temperature for 30 minutes. Product was confirmed by ¹H NMR and VCD measurement.

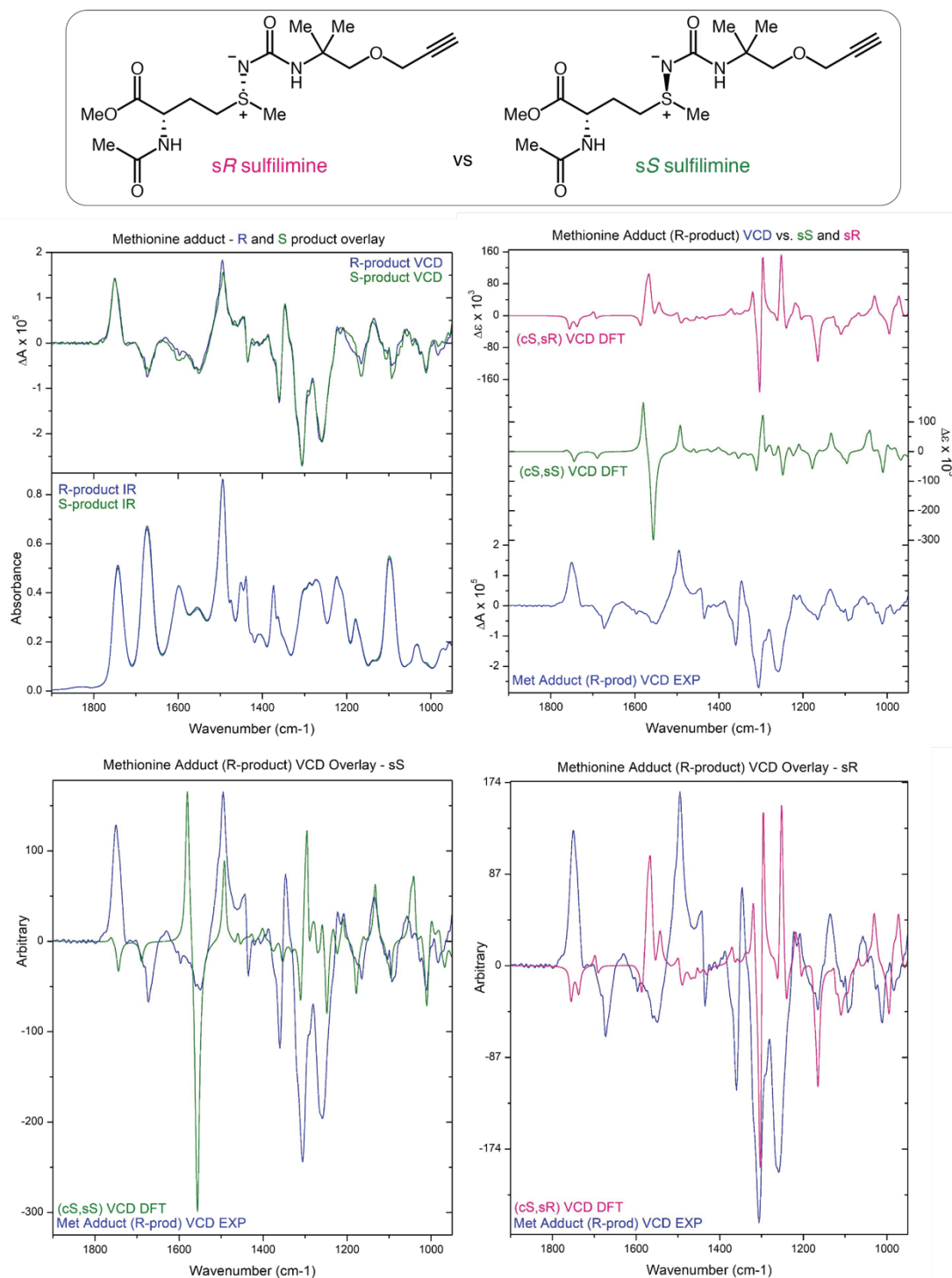


Figure S4.4. VCD analysis of sulfilimine products.

(Top Left) Overlay of measured VCD and IR spectra of products isolated from reaction of (*R*)-Ox32yne and (*S*)-Ox32yne with *N*-acetyl methionine methyl ester. Near-perfect overlap allows for assignment of sulfilimine product from both *R*-Ox32yne and *S*-Ox32yne as identical. (Top Right) Measured VCD spectrum of isolated sulfilimine product from (*R*)-Ox32yne reaction vs calculated VCD spectra of (*sR*) and (*sS*) sulfilimine diastereomers. (Bottom Left) Overlay of measured VCD spectrum of isolated sulfilimine product from (*R*)-

Ox32yne reaction with calculated VCD spectrum of (sS) sulfilimine. Excellent overlap in the 1200-950 cm^{-1} region allows for assignment of isolated sulfilimine products as (sS). (Bottom Right) Overlay of measured VCD spectrum of isolated sulfilimine product from (*R*)-Ox32yne reaction with calculated VCD spectrum of (s*R*) sulfilimine showcasing mismatches in the 1200-950 cm^{-1} region.

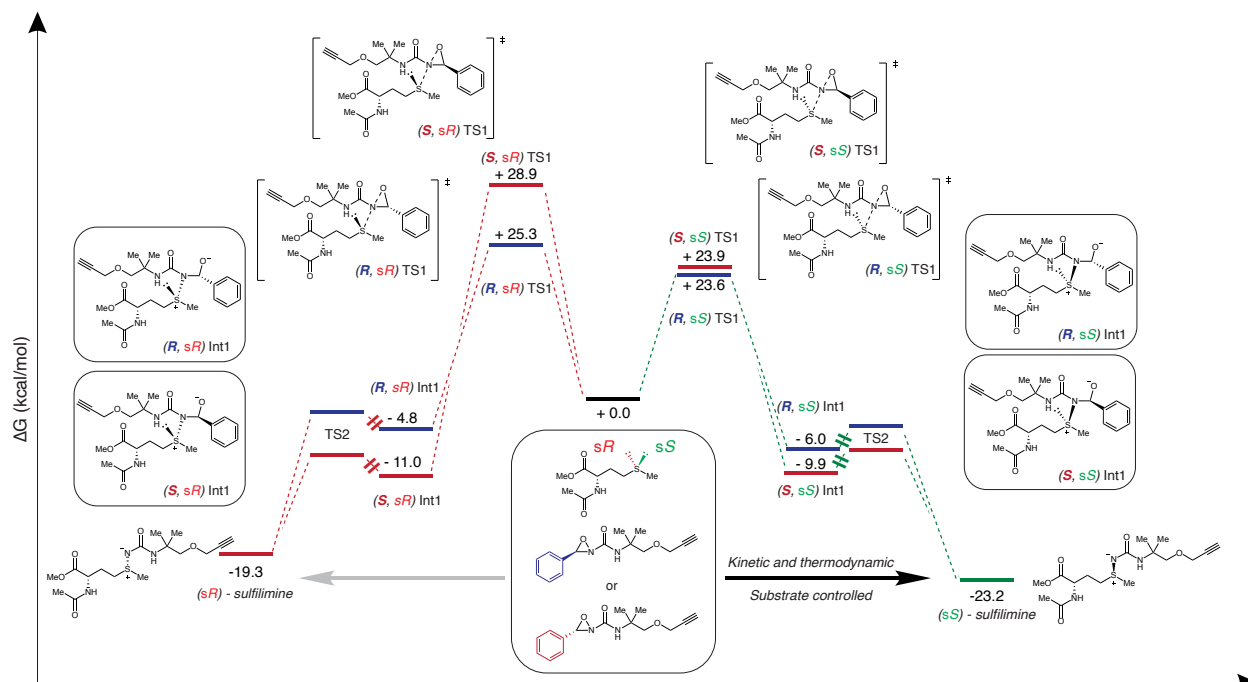


Figure S4.5. Reaction coordinate diagram of (*R*)- or (*S*)-Ox32yne reacting with a model methionine substrate.

Legend

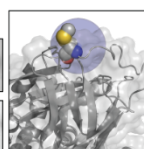
● ● Evidence for unique stereospecific MetO reduction

Alpha 1 antitrypsin

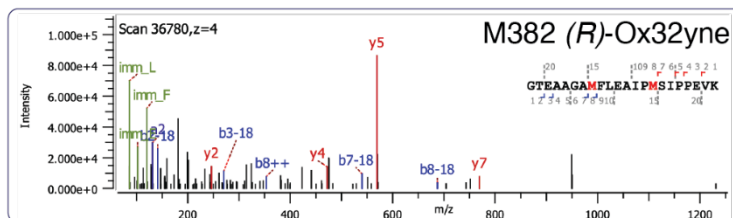
Enantioprobe: 1.0 eq *R* *S*

488

Safestain



M382

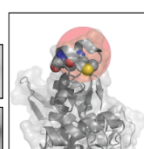


G actin

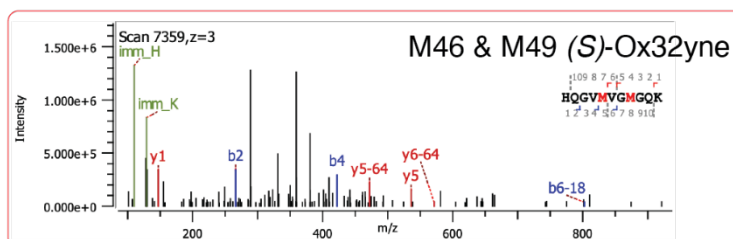
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488

Safestain



M46, M49

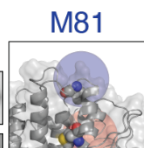


Glutathione S transferase

Enantioprobe: 1.0 eq *R* *S*

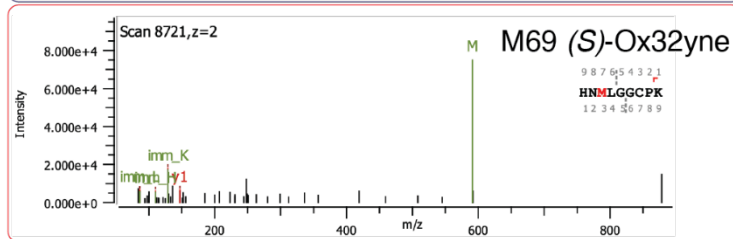
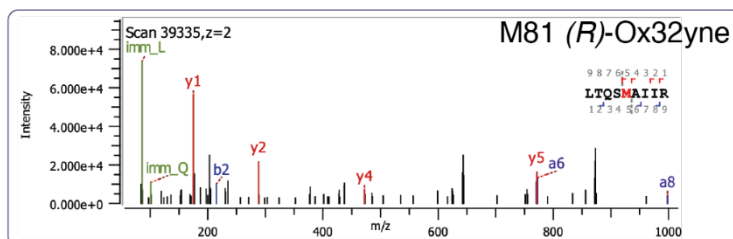
488

Safestain



M81

M69

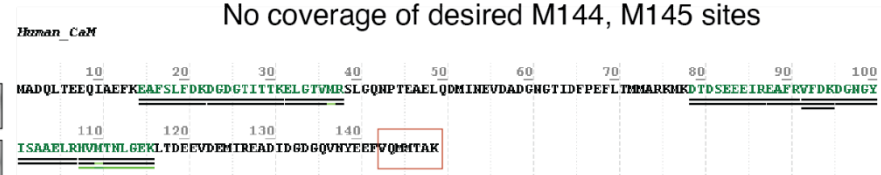


Calmodulin

Enantioprobe: 1.0 eq *R* *S*

488

Safestain



No coverage of desired M144, M145 sites

Figure S4.6. ChURRO labeling of previously characterized proteins that have evidence for stereospecific MetO reduction assessed by in-gel fluorescence and shotgun proteomics.

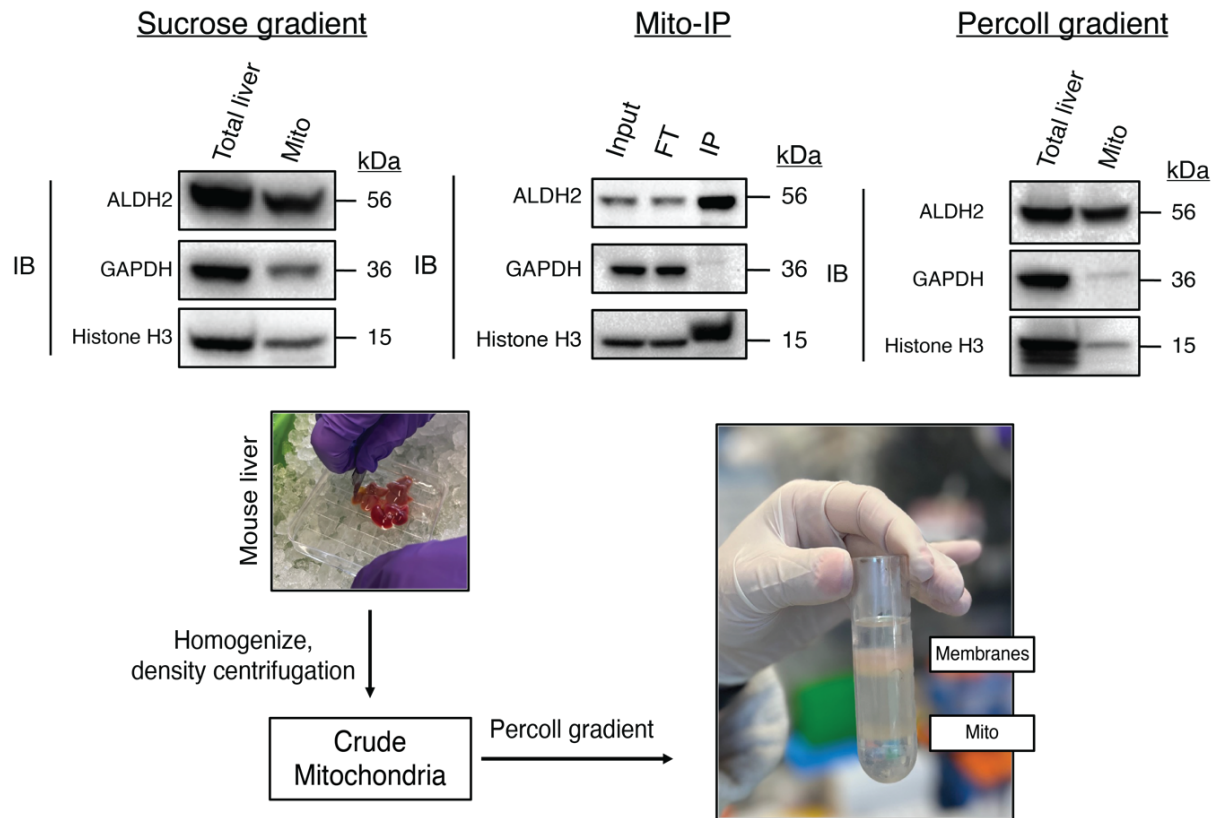


Figure S4.7. Comparison of mitochondrial enrichment methods. Comparison of mitochondrial enrichment methods assessed by immunoblotting of ALDH2 (mitochondrial), GAPDH (cytosolic), or Histone H3 (nuclear). Sucrose gradient protocol adapted from Dias et.al (201) and Mito-IP protocol adapted from Chen et. al (202).

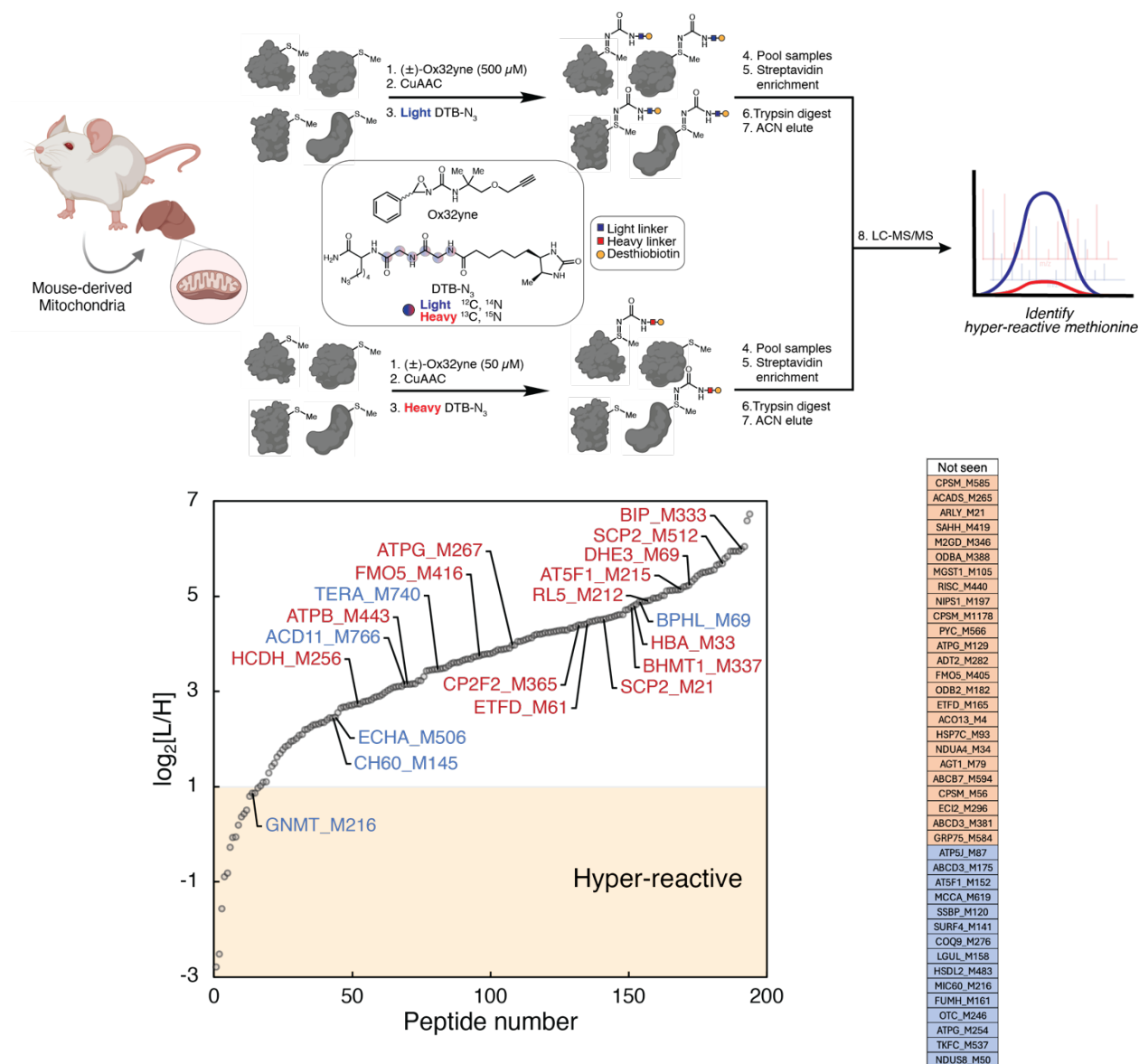


Figure S4.8. Identification of hyperreactive methionine sites in the mitochondria. (Top) isoTOP-ABPP workflow to identify hyperreactive methionine sites in the mitochondria. (Bottom) Reactivity profiles of Met sites in mitochondria with identified prochiral Met oxidation sites labeled (n = 1 technical MS replicates).

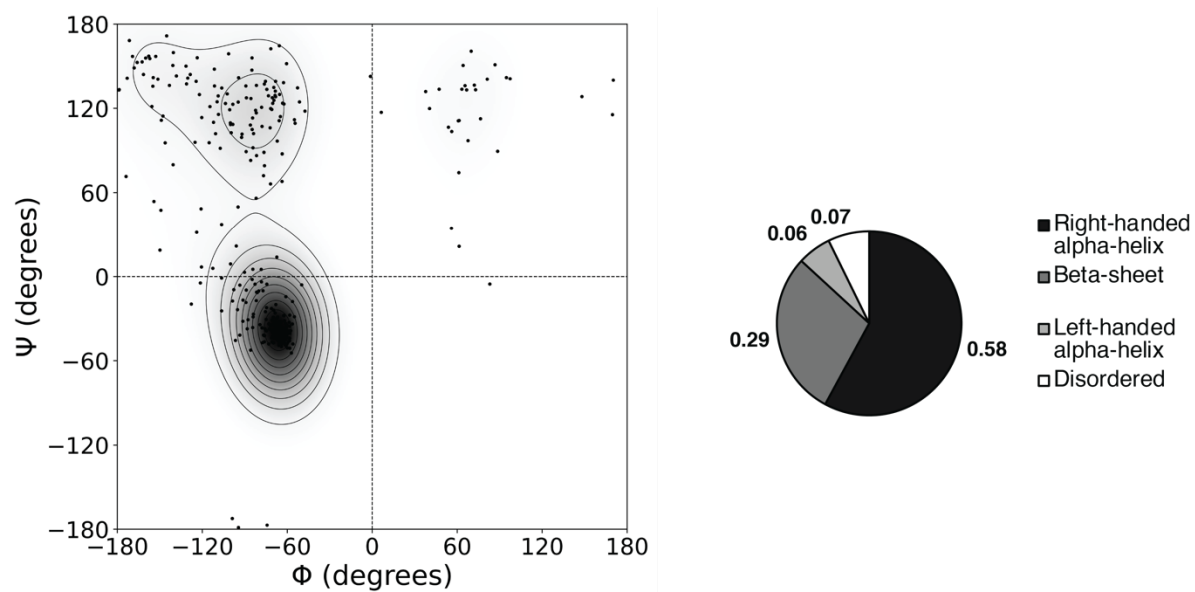


Figure S4.9. Ramachandran plot of methionine sites enriched by both (*R*)- and (*S*)-Ox32yne.

Human_BPHL	-----MVAVLGGRGVLRRLRLLSALKPGI-HVPRA-GPAAAFGTSVTSAKVAVNGVQLH	52
Chimpanzee_BPHL	-----MFRNLLYSL-----LSS-HLSPHFSTSVTSAKVAVNGVQLH	35
Bonobo_BPHL	-----MVAVLGGRGVLRRLRLLSALKPGI-HVPRA-GPAAAFGTSVTSAKVAVNGVQLH	52
Mouse_BPHL	-----MATATVRPAAQRLRLRLLSPLKSRI-CVPQA-EPVATFGTAVTSAKVAVNGVHLH	52
Rat_BPHL	-----MATAAVHPVALRLRLRLLSPLKSRI-CVPQA-EHAATFGTAVTSAKAAVNGIHLH	52
Dog_BPHL	MHTPRPMAAVLDRRASRQLRLRLLSALKAGS-RVPRA-GPAAAFGSSVTSKTVAVNGVHLH	58
Cat_BPHL	-----MAEVLGGRAVRQVRLRLLSVLKPGN-HVPYA-GPAAAFGTSVTSAKVAVNGVHLH	52
Cow_BPHL	-----MAAALGSRAARMRHLLSALKPGI-QIPRA-GPAAAFGSSVTSAKAAVNGVHLH	52
Whale_BPHL	MAAAAAAAAAAQLWGARRLRFLSTLKPGI-HVPQA-EPVAAFSSVTSAKVAVNGVHLH	58
Snake_BPHL	-----MTRLAGAALWRLRLFLSHLSRSATLAATP-RSTAAYSTAITSAKVEVNGVHLH	52
ZebraFish_BPHL	-----MATL-----M-FI-----NRILRKAATQKRSY-SVSVCKLSVNGVNLH	37
Frog_BPHL	-----MALSL-----RKSPLRSVAV-QFLRGVFLR-QGATSYCTATISERVNVNGINLH	46
M69 :		
Human_BPHL	YQQTGEGDHAVLLLPGLGSGGTDGFGPQLKNLNKKLFTVVAVNDPRGYGHSRPPDRDFFAD	112
Chimpanzee_BPHL	YQQTGEGDHAVLLLPGLGSGGTDGFGPQLKNLNKKLFTVVAVNDPRGYGHSRPPDRDFFAD	95
Bonobo_BPHL	YQQTGEGDHAVLLLPGLGSGGTDGFGPQLKNLNKKLFTVVAVNDPRGYGHSRPPDRDFFAD	112
Mouse_BPHL	YQRVGEGEHAILLLPGLGSGGTDGFGPQLQSLNKKRFTLVAVNDPRGYGSRPPDRDFFPD	112
Rat_BPHL	YQRVGEGEHAVLLLPGLGSGGTDGFGPQLQSLNKKRFTLVAVNDPRGYGESRPPDRDFFPD	112
Dog_BPHL	YQQTGEGEHAVLLLPGLGSGGTDGFGPQIKNLNKKLFTVVAVNDPRGYGHSRPPDRDFFMD	118
Cat_BPHL	YQRTGEGEHAVLLLPGLGSGGTDGFGPQIKNLNKKLFTVVAVNDPRGYGHSRPPDRDFFPD	112
Cow_BPHL	YLRTGGGEHAVLLLPGLGSGGTDGFGPQIENLNKKLFTVVAVNDPRGYGHSRPPDRDFFVD	112
Whale_BPHL	YLRTGGGEHAVLLLPGLGSGGTDGFGPQIENLNKKLFTVVAVNDPRGYGSRPPDRDFFVE	118
Snake_BPHL	YQQTGNGNHAVLLLPGLGSGGTDGFGPQLKNMNKEIFTVVAVNDPRGYGKSIPPIRDFPLD	112
ZebraFish_BPHL	YQRAGEGKHTVLLLPGLGSGGTDGFGPQLSALDSSRFTVSVNDPRGYGSRPPDRDFFPLH	97
Frog_BPHL	YQRTGWDHAVLLLPGLGSGGTDGFGPQLKSLDKEAFTIATVNDPRGYGYSVPPSRDYPFLH	106
* . *		
Human_BPHL	FFERDAKDAVDLMKALKFKKVSLLGWSGGGITALIAAAKYPYSIHKMVIWGANAYVTDED	172
Chimpanzee_BPHL	FFERDAKDAVDLMKALKFKKVSLLGWSGGGITALIAAAKYPYSIHKMVIWGANAYVTDED	155
Bonobo_BPHL	FFERDAKDAVDLMKALKFKKVSLLGWSGGGITALIAAAKYPYSIHKMVIWGANAYVTDED	172
Mouse_BPHL	FFERDAKDAVDLMKALQFKQVSLGWSGGGITALIAAAKYPYSIRKMVIWGANAYVTEED	172
Rat_BPHL	FFERDAKDAVDLMKALQFKQVSLGWSGGGITALIAAAKYPYSIRKMVIWGANAYVTEED	172
Dog_BPHL	FLERDAKDAVDLMKTLNFKKVSLLGWSGGGITALIAAAKYPYLYINKMVIWGANAYVTDED	178
Cat_BPHL	FFERDAKDAVDLMKTLNFKKVSLLGWSGGGITALIAAAKYPYSINKMVIWGANAYVTEED	172
Cow_BPHL	FFERDAKDAVDLMKTLNFKKVSLLGWSGGGITALIAAAKYPYSYINKMVIWGANAYVTEED	172
Whale_BPHL	FFERDAKDAVDLMKMLKFKKVSLLGWSGGGITALIAAAKYPYSINKMVIWGANAYVTDDQ	178
Snake_BPHL	FFERDAKDAVDLMQALKFKQKVSLLGWSGGGITALIAAAKYPELIHKMVIWGANAYVTEED	172
ZebraFish_BPHL	FFHRDAKDAVDLMQALGFRFSLGWSGGGITALIAAALNPTLVNKLIVWGANAFVSEED	157
Frog_BPHL	FFERDAKDAVDLMQALNFKKVSLLGWSGGGITALIGAGTYPYSLIRKLIVWGANASVTEED	166
* . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . *		
Human_BPHL	SMIYEGIRDVSKWSETRKPLEALYGYDYFARTCEKWVDGIRQFKHLPDGNICRHLLPRV	232
Chimpanzee_BPHL	SMIYEGIRDVSKWSETRKPLEALYGYDYFARTCEKWVDGIRQFKHLPDGNICRHLLPRV	215
Bonobo_BPHL	SMIYEGIRDVSKWSETRKPLEALYGYDYFARTCEKWVDGIRQFKHLPDGNICRHLLPRV	232
Mouse_BPHL	SRIYQGIIRDVSKWSEKARKPLEALYGYDYLAKECEDWVDGISQFKQLPEGNICRHLLPLV	232
Rat_BPHL	SRIYQGIIRDVSKWSEKARKPLEALYGHYDYFAKTEKWVDGINQFKHLPDGNICRHLLPLI	232
Dog_BPHL	EKIYQGIIRDVSKWSEKARKPLETLYGYDYFAKTEKWVDGIMQQFKHLPDGNICRHLLPLV	238
Cat_BPHL	ETIYQGIIRDVSKWSETRKPLETLYGYDYFTKTCEKWVDGIKQFKHLSGDNICRHLLPLV	232
Cow_BPHL	TEIYQGIIRDVSKWSEKARKPLEALYGYDYFARTCEKWVDGIEQFKHLPDGSICRDLLPLV	232
Whale_BPHL	EELIYRGIRDVSKWSETRKPLEALYGYDYFARTCEKWVDGIKQFKHLPDGNICRHLLPLV	238
Snake_BPHL	VKIYNGIRDVSKWSDKTRKPLEKMYGHEYFSTKCSAWVDGISQFVQKSDGDIQCHLLPHI	232
ZebraFish_BPHL	IQIYQSLRDVSLWSEMRMRPMEQMYGAQYFKQFTWERVDGISQFINKPQGSICVDLLPQI	217
Frog_BPHL	LKLYNAIKDVSNWSEKMRKPMEDLYGKEYFADTFKAWCEAMCKLASRTDGNICRHLLPLI	226
* . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . *		
Human_BPHL	QCPALIVHGEKDPVPRFHADFIHKHVGSRRLHLMPEGKHNHLRFADEFNKLAEFLQ-	291
Chimpanzee_BPHL	QCPALIVHGEKDPVPRFHADFIHEHVKGSRRLHLMPEGKHNHLRFADEFNKLAEFLQ-	274
Bonobo_BPHL	QCPALIVHGEKDPVPRFHADFIHEHVKGSRRLHLMPEGKHNHLRFADEFNKLAEFLQ-	291
Mouse_BPHL	QCPTLIVHGEKDPVPRFHADFLLEHVKGSRRLHLMPEGKHNHLRFADEFNRLVEDFLQ-	291
Rat_BPHL	QCPTLIVHGEKDPVPRFHADFLLEHVKGSRRLHLMPEGKHNHLRFADEFNRLVRQYCNL	292
Dog_BPHL	QCPTLIVHGEKDPVPRFHADFIHKHVGSRRLHLMPEGKHNHLRFADEFNKLVEDFLQ-	290
Cat_BPHL	QCPTLIVHGEKDPVPRFHADFIHKHVGSRRLHLMPEGKHNHLRFADEFNKLAEFL--	297
Cow_BPHL	QCPTLIVHGEKDPVPRFHADFLHRHVGSRRLHLMPEGKHNHLRFADEFNRLAEGFTQ-	291
Whale_BPHL	QCPTLIVHGEKDPVPSFHADFIHKHVGSRRLHLMPEGKHNHLRFADEFNKLAEFLQ-	297
Snake_BPHL	KCPTLIIHGEKDPVPRFHAIEYIHKHIQGSRLHFMPEGKHNHLRFAEEFNKMEFFLV-	291
ZebraFish_BPHL	SCPTLIIHGAQDPVVPAYHPQLQDSISGSRHLVFPKGKHNHLRYSAEFNTLVEQFLNE	277
Frog_BPHL	NCPTLIIHGEKDPMPVPSFHPQYIHQIKGSRRLHLMSEKGNHLRLRYAEFNGLVEDFLCE	286
* . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . *		
Human_BPHL	----- 291	
Chimpanzee_BPHL	----- 274	
Bonobo_BPHL	----- 291	
Mouse_BPHL	----- 291	
Rat_BPHL	ERRFTLLHQ 302	
Dog_BPHL	----- 297	
Cat_BPHL	----- 290	
Cow_BPHL	----- 291	
Whale_BPHL	----- 297	
Snake_BPHL	----- 291	
ZebraFish_BPHL	----- 277	
Frog_BPHL	K----- 287	

LEGEND (. weakly similar) (: strongly similar) (* complete conservation)

Figure S4.10. Clustal omega multiple sequence alignment of BPHL across multiple organisms.

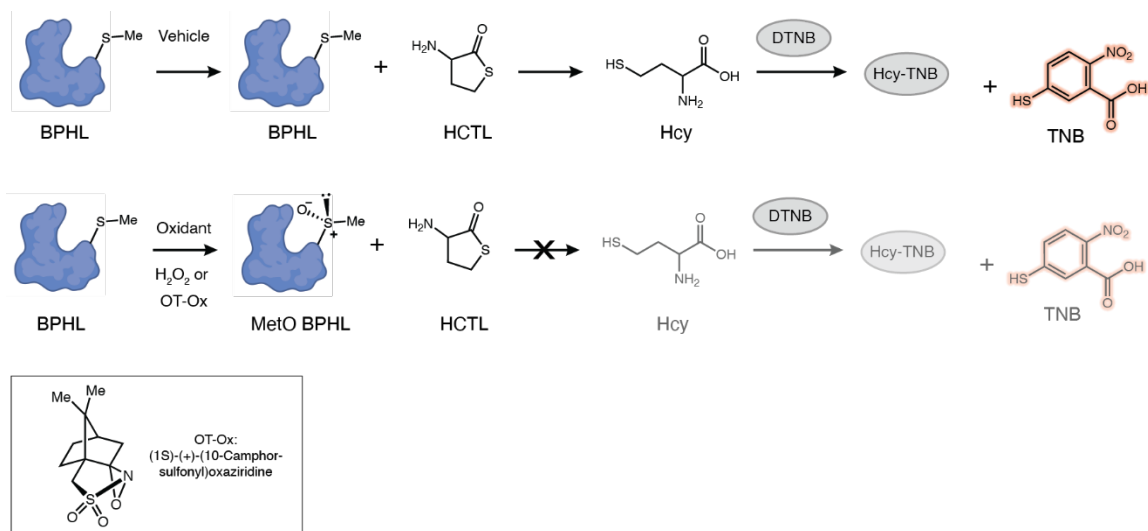


Figure S4.11. Schematic of *in vitro* BPHL activity assay using native HCTL substrate and Ellman's reagent (DTNB) to oxidize Hcy and form TNB as a colorimetric readout.

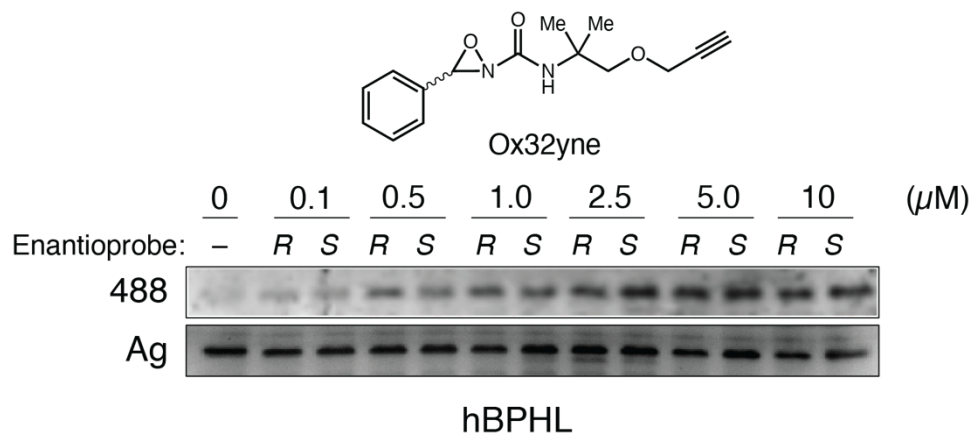


Figure S4.12. Dose-titration Gel-ABPP of recombinant human BPHL using (*R*)- and (*S*)-Ox32yne.

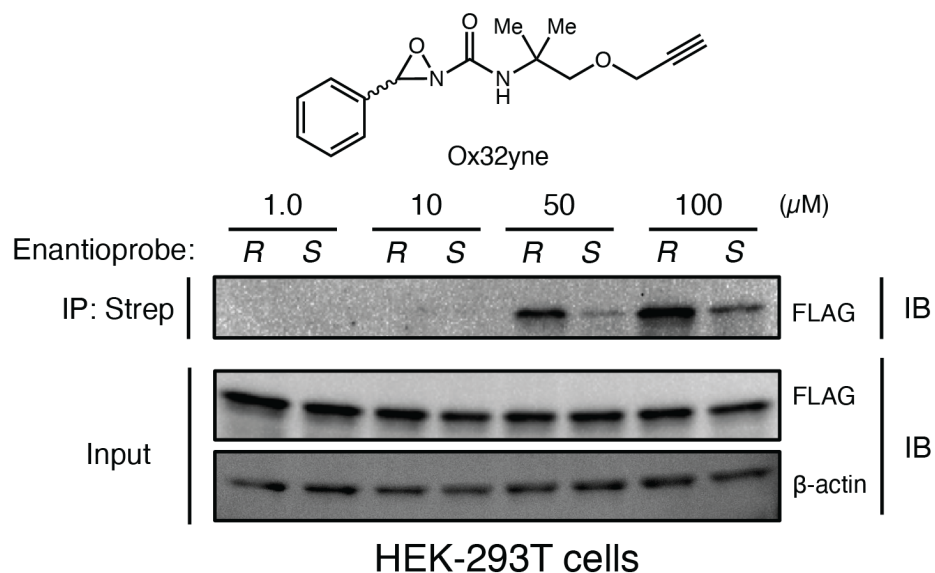


Figure S4.13. Immunoprecipitation studies of FLAG-BPHL expressed in HEK-293T cells using (*R*)- and (*S*)-Ox32yne.

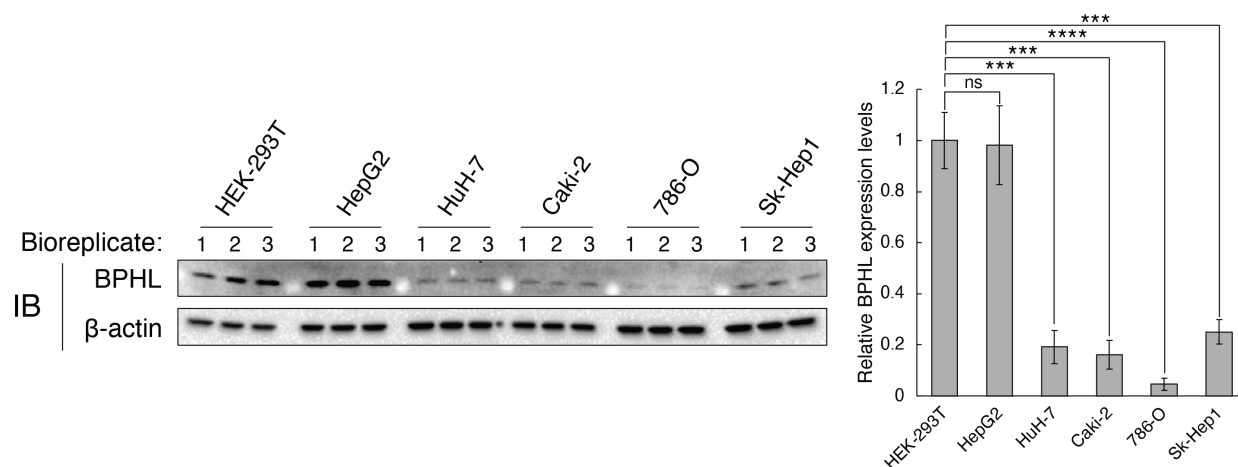
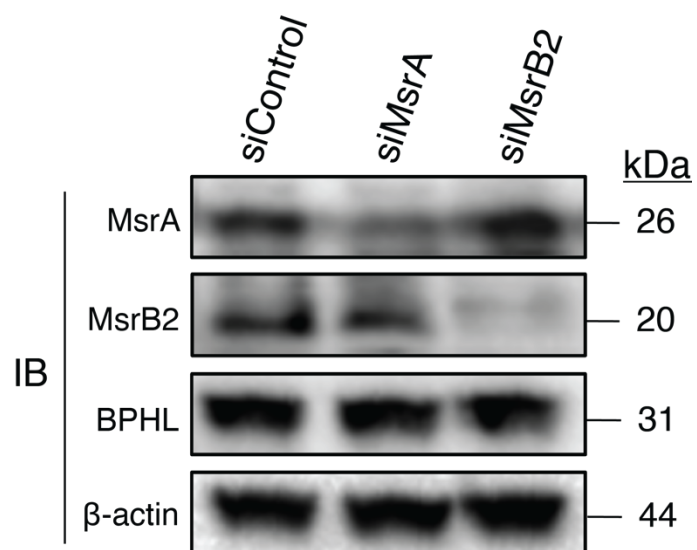


Figure S4.14. BPHL expression screen in renal and hepatic cell lines assessed by immunoblotting.
(n = 3 technical cell replicates, error bars represent standard deviation).



HEK-293T cells

Figure S4.15. Assessment of Msr knock-down efficiency in HEK-293T cells.

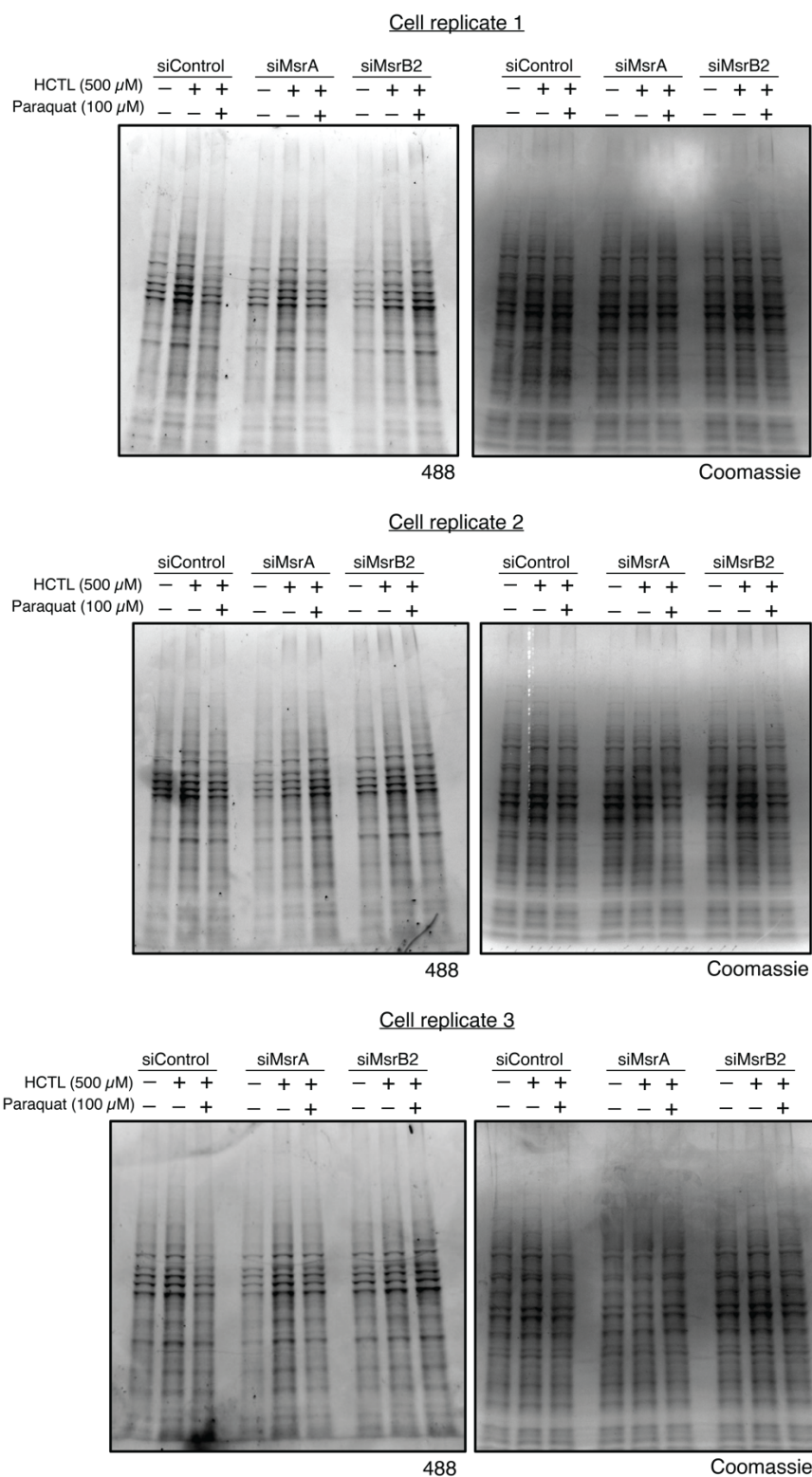


Figure S4.16. Gel data for *N*-homocysteinylation assay in HEK-293T cells with MsrA or MsrB2 knocked down.
(n = 3 technical cell replicates).

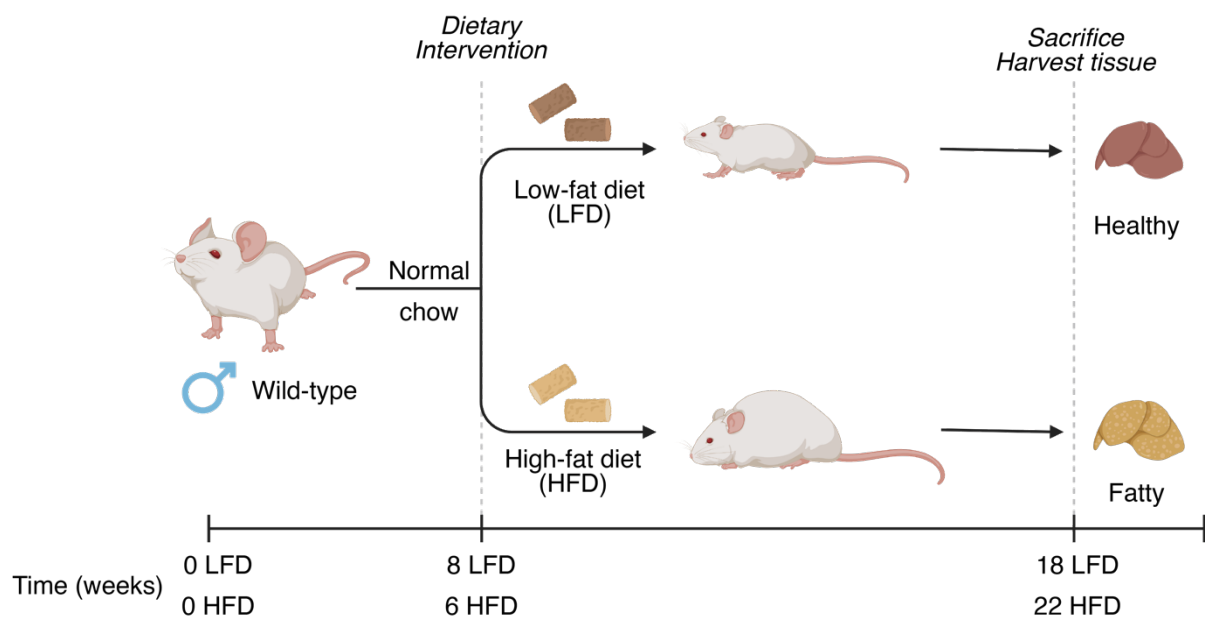


Figure S4.17. Mouse feeding schematic for diet-induced model of NAFLD.

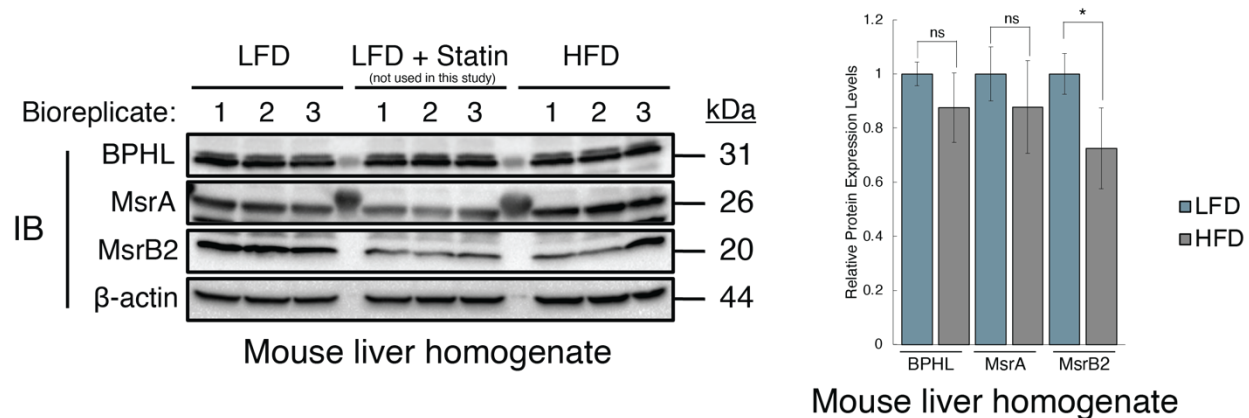


Figure S4.18. Immunoblot data of LFD and HFD mouse liver.

(Left) Immunoblot data of LFD and HFD mouse liver against BPHL, MsrA, MsrB2, and loading control. (Right) Densitometry quantification of immunoblot results (n = 3 bioreplicates, error bars represent standard deviation).

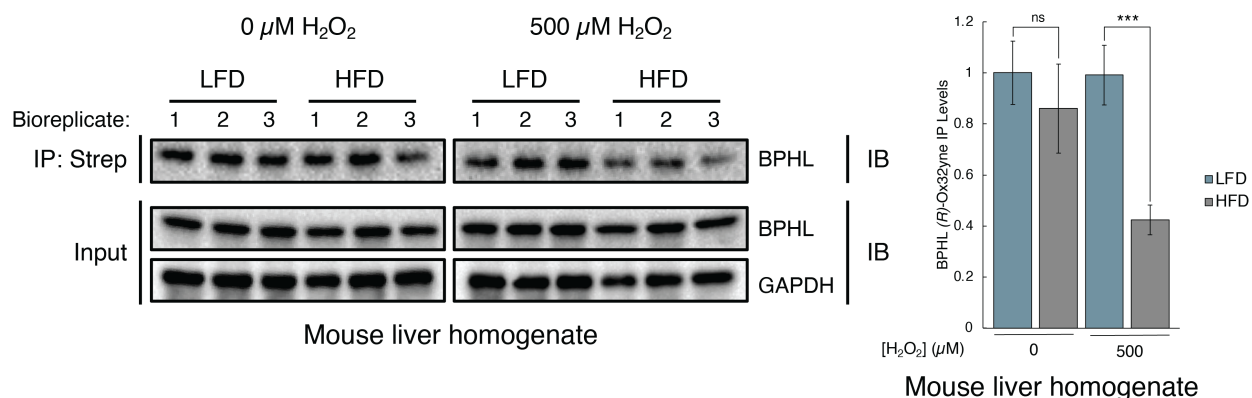


Figure S4.19. Immunoprecipitation of BPHL in LFD and HFD mouse liver. (Left) Immunoprecipitation of BPHL in LFD and HFD mouse liver using (R)-Ox32yne under normal and oxidative stress conditions. (Right) Densitometry quantification of immunoprecipitation results (n = 3 bioreplicates, error bars represent standard deviation).

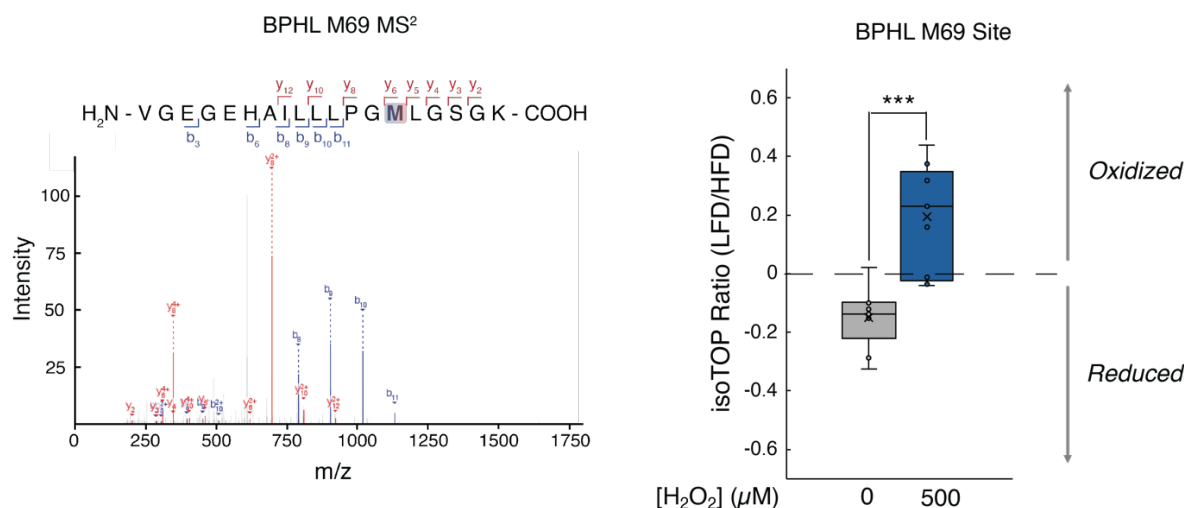
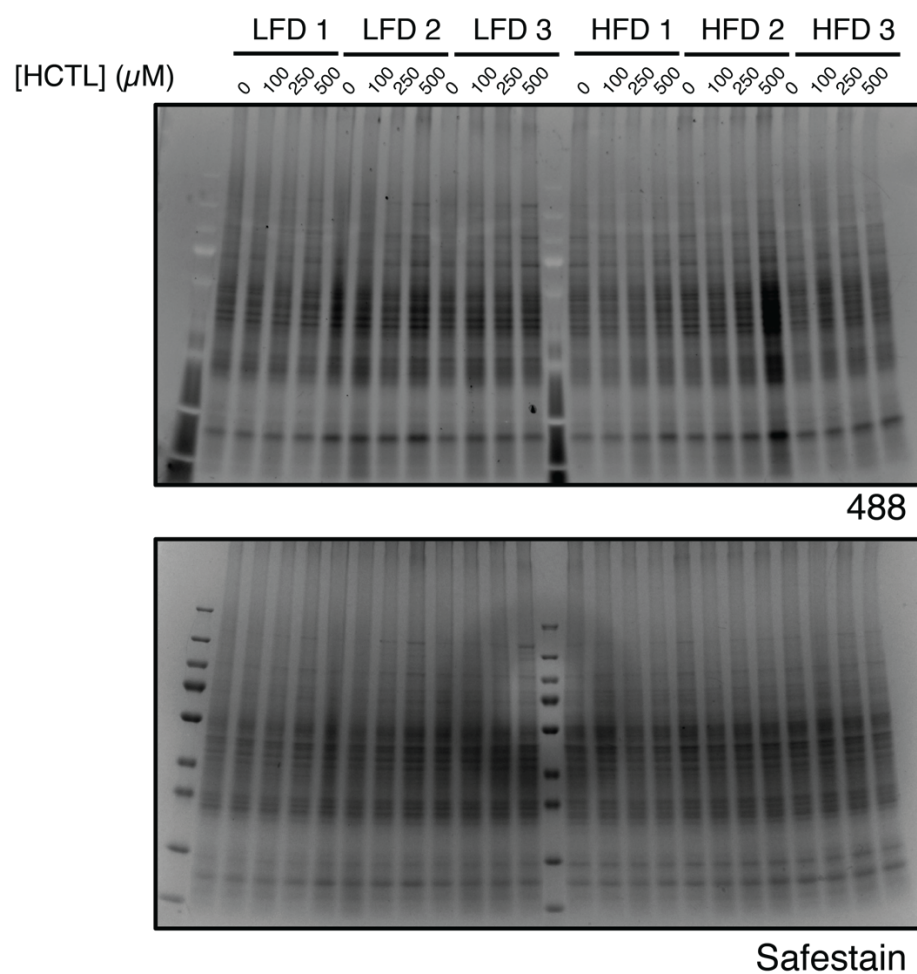


Figure S4.20. Additional quantification of BPHL M69 oxidation status. (Left) BPHL M69 peptide MS2 spectrum. (Right) Quantification of BPHL M69 reactivity towards (R)-Ox32yne in LFD and HFD mouse liver under normal and oxidative stress conditions (n = 3 bioreplicates, n = 3 technical MS replicates for isoDTB and n = 8 technical MS replicates for ReDiMe).



Mouse liver homogenate

Figure S4.21. LFD and HFD mouse liver *N*-homocysteinylation assay with increasing titration of HCTL under conditions of oxidative stress.
(*n* = 3 bioreplicates).

4.8.2 List of supplementary tables

(to access, please contact authors)

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Table S4.15.

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Table S4.16.

pro-(*R*) Met landscape in LFD and HFD mouse livers under normal conditions.

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pro-(*R*) Met landscape in LFD and HFD mouse livers under oxidative stress conditions.

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Densitometry quantification of BPHL assessed by (*R*)-Ox32yne immunoprecipitation studies in LFD and HFD mouse livers under normal and oxidative stress conditions.

Table S4.19.

Quantification of (*R*)-Ox32yne labeling on BPHL M69 peptide in LFD and HFD mouse livers under normal and oxidative stress conditions.

Table S4.20.

Densitometry quantification of protein *N*-homocysteinylation with a titration of HCTL in LFD and HFD mouse livers under oxidative stress conditions.

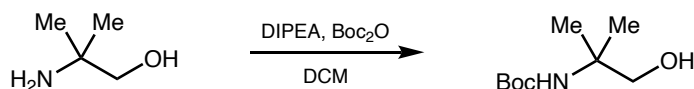
4.8.3 General methods

Reactions using moisture- or air-sensitive reagents were carried out in flame-dried glassware under an inert atmosphere of N₂. 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). Compound **AT-3** was prepared according to Chen et. al [\(195\)](#) without modification. 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. ¹H and ¹³C NMR spectra were collected at 298 K in CDCl₃ or CD₃OD (Cambridge Isotope Laboratories, Cambridge, MA) using Bruker AVQ-400, AVB-400, AV-500, AV-600, NEO-500, or NEO-501 instruments at the Pines Magnetic Resonance Center's Core 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 ^1H 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. Enantioenrichment was determined by chiral HPLC using a Daicel CHIRALPAK® ADH column (4.6 x 250 mm) located in the Toste group at the University of California, Berkeley or by chiral SFC using a Chiral Technologies AD-H column (4.6 x 250 mm) located at the California Institute of Technology. Absolute configuration of oxaziridine enantiomers and sulfilimine products was determined by VCD analysis using a DualPEM FT-VCD spectrometer located at BioTools (Jupiter, FL). 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 using an Agilent 6230 LC-TOF (ESI) located in the Toste group at the University of California, Berkeley. All aqueous solutions for biological experiments 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.

4.8.4 Chemistry methods

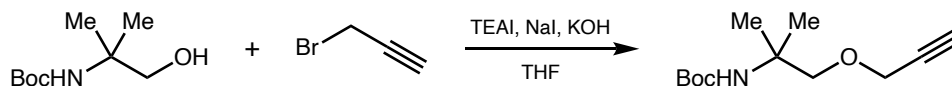
Chemical synthesis of Ox32yne probe:



tert-Butyl (1-hydroxy-2-methylpropan-2-yl)carbamate (S4.1)

To a solution of 2-amino-2-methyl propanol (9.54 mL, 100 mmol, 1.11 equiv.) in DCM (20 mL) was added DIPEA (15.68 mL, 90 mmol, 1.00 equiv.) and Boc anhydride (19.64 g, 90 mmol, 1.00 equiv.) in DCM (23.75 mL). The reaction was allowed to stir at room temperature overnight. After 16 hours, the reaction was quenched by addition of 10% aqueous citric acid, diluted with DCM, and washed successively with water and brine. The organic layers were dried over MgSO_4 , filtered, and concentrated under reduced pressure to give **S4.1** as a white powder (14.50 g, 76.6 mmol, 85% yield).

^1H NMR (600 MHz, CDCl_3) δ 4.65 (s, 1H), 4.00 (s, 1H), 3.65 – 3.50 (m, 2H), 1.43 (s, 9H), 1.25 (s, 6H). ^{13}C NMR (151 MHz, CDCl_3) δ 156.30, 79.94, 71.05, 54.48, 28.51, 24.88. Compound did not ionize on ESI.

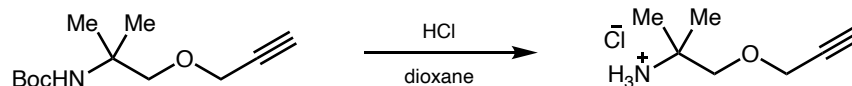


tert-Butyl (2-methyl-1-(prop-2-yn-1-yloxy)propan-2-yl)carbamate (S4.2)

A flame-dried round bottomed flask equipped with a magnetic stir bar was charged with THF (20 mL), alcohol **S4.1** (1.50 g, 7.93 mmol, 1.0 equiv.), propargyl bromide (80 wt% in toluene, 1.06 mL, 9.52 mmol, 1.2 equiv.), sodium iodide (0.204 g, 1.36 mmol, 0.17 equiv.), TEAI (0.204 g, 0.793 mmol, 0.1 equiv.), and KOH (0.890 g, 15.86 mmol, 2.0 equiv.). The resulting orange suspension was stirred vigorously overnight and quenched by addition of 20 mL H_2O and washed with EtOAc (3 x 50 mL). The combined organic layers were

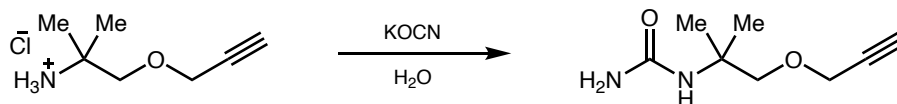
dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude yellow oil was purified using flash column chromatography (15% EtOAc/Hex) to give **S4.2** as a yellow oil (1.062 g, 4.67 mmol, 59% yield).

¹H NMR (600 MHz, CDCl₃) δ 4.70 (s, 1H), 4.16 (d, *J* = 2.4 Hz, 2H), 3.47 (s, 2H), 2.42 (t, *J* = 2.4 Hz, 1H), 1.43 (s, 9H), 1.30 (s, 6H). **¹³C NMR** (151 MHz, CDCl₃) δ 154.95, 79.81, 79.01, 76.25, 74.61, 58.67, 52.66, 28.58, 24.38. Compound did not ionize on ESI.



2-Methyl-1-(prop-2-yn-1-yloxy)propan-2-aminium chloride (**S4.3**)

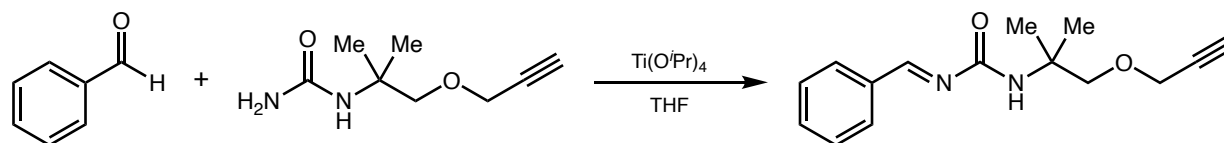
To a flame-dried round bottomed flask equipped with a magnetic stir bar was added a solution of carbamate **S4.2** (2.85 g, 12.52 mmol, 1.00 equiv.) and anhydrous HCl (27.54 mmol, 2.2 equiv., 4M in 1,4-dioxane) in anhydrous MeOH (25 mL). The reaction was allowed to stir overnight and the resulting pink solution was concentrated under reduced pressure. The crude pink solid was washed with cold Et₂O to afford the HCl salt of the amine as a white crystalline solid (1.50 g, 9.17 mmol, 73% yield) that was used immediately in the next step without further purification.



1-(2-Methyl-1-(prop-2-yn-1-yloxy)propan-2-yl)urea (**S4.4**)

To a solution of amine **S4.3** (1.50 g, 9.17 mmol, 1.00 equiv.) in H₂O (9.17 mL) was added potassium isocyanate (2.98 g, 36.68 mmol, 4.00 equiv.). The reaction was heated to 60 °C and allowed to stir overnight. After 16 hours, the reaction was cooled to room temperature and extracted with EtOAc (5 x 100 mL). The combined organic layers were dried over MgSO₄, filtered, and concentrated under reduced pressure to afford the urea **S4.4** as a white powder (1.05 g, 6.19 mmol, 68% yield).

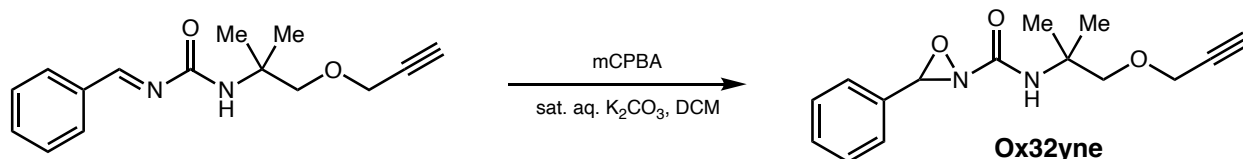
¹H NMR (500 MHz, CDCl₃) δ 4.86 (s, 1H), 4.47 (s, 2H), 4.17 (d, *J* = 2.4 Hz, 2H), 3.51 (s, 2H), 2.44 (t, *J* = 2.4 Hz, 1H), 1.34 (s, 6H). **¹³C NMR** (126 MHz, CDCl₃) δ 158.41, 79.64, 76.96, 74.80, 58.69, 52.99, 24.73. **HRMS** (ESI) calculated for [C₈H₁₄N₂O₂+H]⁺: required *m/z* 171.1128, found *m/z* 171.1124.



1-Benzylidene-3-(2-methyl-1-(prop-2-yn-1-yloxy)propan-2-yl)urea (**S4.5**)

A flame-dried round bottomed flask equipped with a magnetic stir bar was charged with urea **S4.4** (1.05 g, 6.19 mmol, 1.00 equiv.) and evacuated and backfilled with N₂ twice. The urea was dissolved in THF (18.76 mL), to which benzaldehyde (0.76 mL, 7.43 mmol, 1.20 equiv.) and Ti(O^{*i*}Pr)₄ (2.57 mL, 8.67 mmol, 1.40 equiv.) were added. The resulting

mixture was stirred vigorously overnight. After 16 hours, the reaction was concentrated under reduced pressure and used immediately in the next step without further purification.



***N*-(2-Methyl-1-(prop-2-yn-1-yloxy)propan-2-yl)-3-phenyl-1,2-oxaziridine-2-carboxamide (Ox32yne)**

To a solution of DCM (20 mL) and sat. aq. K₂CO₃ (20 mL) was added *m*-CPBA (3.16 g, 18.57 mmol, 3.00 equiv.). The resulting slurry was stirred vigorously at room temperature for 10 minutes at which point a suspension of crude imine **SI-5** in DCM (15 mL) was added. The resulting yellow solution was allowed to stir at room temperature. After 8 hours, the reaction was quenched by addition of H₂O (100 mL) and extracted with DCM (3 x 50 mL). The combined organic layers were dried over MgSO₄, filtered, and concentrated under reduced pressure. The resulting oil was purified using flash column chromatography (DCM/MeOH) to afford the racemic **Ox32yne** as a clear oil. (226.4 mg, 0.83 mmol, 13% yield over 2 steps). Enantiomeric ratio was determined via SFC (AD-H column, 10% IPA/CO₂, 2.5 mL/min, 210 nm) retention times: 4.3 minutes (51%), 4.8 minutes (49%).

¹H NMR (600 MHz, CDCl₃) δ 7.50 – 7.38 (m, 5H), 6.20 (s, 1H), 4.98 (s, 1H), 4.24 – 4.16 (m, 2H), 3.59 (d, *J* = 9.0 Hz, 1H), 3.50 (d, *J* = 9.0 Hz, 1H), 2.47 (t, *J* = 2.4 Hz, 1H), 1.41 (s, 3H), 1.39 (s, 3H). **¹³C NMR** (151 MHz, CDCl₃) δ 161.12, 132.81, 131.02, 128.73, 128.12, 79.56, 79.33, 75.43, 75.02, 58.68, 53.99, 23.98, 23.73. **HRMS** (ESI) calculated for [C₁₅H₁₈N₂O₃+H]⁺: required *m/z* 275.1390, found *m/z* 275.1383.

4.8.5 SFC separation

Preparative oxaziridine separation was conducted at the California Institute of Technology by Dr. Scott Virgil via chiral supercritical fluid chromatography (SFC). The enantiomeric mixture of Ox32yne (200 mg) was purified on a JASCO System 2000 preparative SFC system with 25 mL/min of 25% IPA/CO₂ on a 20 x 250 mm Chiral Technologies OZ-H column to afford 92 mg of each enantiomer. The fractions were analyzed on a Thar SFC system with 2.5 mL/min of 10% IPA/CO₂ on a 4.6 x 250 mm Chiral Technologies AD-H column.

(*R*)-Ox32yne is the first eluting enantiomer on AD-H column (retention time 4.4 min) and the second eluting enantiomer on OZ-H column.

(*S*)-Ox32yne is the second eluting enantiomer on AD-H column (retention time 4.8 min) and the first eluting enantiomer on OZ-H column.

(*R*)-Ox32yne: **¹H NMR** (500 MHz, CDCl₃) δ 7.50 – 7.37 (m, 5H), 6.20 (s, 1H), 4.98 (s, 1H), 4.20 (t, *J* = 2.3 Hz, 2H), 3.59 (d, *J* = 9.0 Hz, 1H), 3.49 (d, *J* = 9.0 Hz, 1H), 2.47 (t, *J* = 2.4 Hz, 1H), 1.40 (d, *J* = 5.8 Hz, 6H). **¹³C NMR** (126 MHz, CDCl₃) δ 161.12, 132.78, 131.03, 128.73, 128.12, 79.55, 79.32, 75.41, 75.03, 58.67, 53.98, 23.96, 23.72. **HRMS** (ESI) calculated for [C₁₅H₁₈N₂O₃+Na]⁺: required *m/z* 297.1210, found *m/z* 297.1211.

Chiral SFC (AD-H column, 10% IPA/CO₂, 2.5 mL/min, 210 nm) retention time: 4.4 minutes (99%).

(S)-Ox32yne: ¹H NMR (500 MHz, CDCl₃) δ 7.50 – 7.37 (m, 5H), 6.20 (s, 1H), 4.98 (s, 1H), 4.20 (t, *J* = 2.3 Hz, 2H), 3.59 (d, *J* = 9.1 Hz, 1H), 3.49 (d, *J* = 9.1 Hz, 1H), 2.47 (t, *J* = 2.4 Hz, 1H), 1.40 (d, *J* = 5.8 Hz, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 161.11, 132.78, 131.02, 128.73, 128.11, 79.55, 79.32, 75.40, 75.03, 58.66, 53.98, 23.96, 23.71. **HRMS** (ESI) calculated for [C₁₅H₁₈N₂O₃+Na]⁺: required *m/z* 297.1210, found *m/z* 297.1210. **Chiral SFC** (AD-H column, 10% IPA/CO₂, 2.5 mL/min, 210 nm) retention time: 4.8 minutes (99%).

4.8.6 VCD analysis

Vibrational circular dichroism (VCD) measurements were conducted at BioTools (Jupiter, FL) by Jordan Nafie as follows:

Ox-32-yne:

Measurements: Dissolved E1 (first eluting enantiomer of Ox32yne) or E2 (second eluting enantiomer of Ox32yne) (10 mg) in 150 µL CDCl₃ (previously run through a plug of activated basic alumina) and transferred to a 100 µm BaF₂ IR cell. Simultaneous measurement of IR and VCD were taken for ~18 hours in one-hour blocks on a BioTools (Jupiter, FL) DualPEM FT-VCD spectrometer at 4 cm⁻¹ resolution and PEM maximum frequencies both set to 1400 cm⁻¹. The first block of IR was solvent and water vapor corrected, and offset to zero at 2000 cm⁻¹. VCD was block averaged and corrected by half difference ((E1 – E2) / 2). Noise spectra were block averaged and used as is.

Calculations: The (*R*) enantiomer of Ox32yne was constructed using BioTools (Jupiter, FL) ComputeVOA software. A thorough conformational search was performed at the molecular mechanics level (GMMX) using the MMF94 force field in a 7 kcal / mol energy window. The resulting 121 conformations were optimized at the DFT (152) level (B3LYP / 6-31G(d) with CPCM - Chloroform) and the IR and VCD frequencies were also calculated at the same level. The conformations were separated into cis and trans oxaziridine isomers, and the 20 lowest energy conformers from each group (cis and trans) were re-optimized and frequencies calculated at the B3PW91 / cc-pVTZ level. The spectra from the resulting conformers were Boltzmann averaged using the electronic energy values and plotted using a line width of 5 cm⁻¹ HWHH, and an x-axis scaling factor of 0.976 for comparison with the experimental IR and VCD data. Of the geometric isomers, it was determined that the trans was a good match with the data and the cis a poor match.

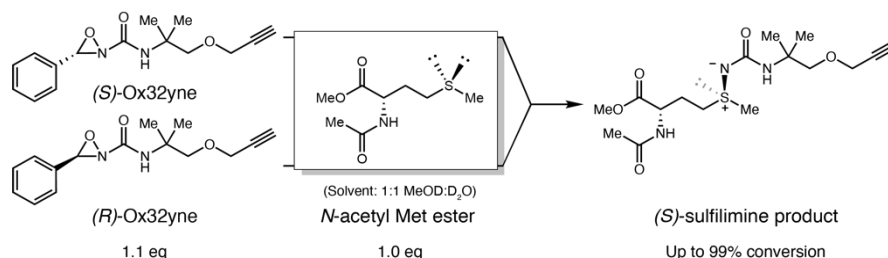
Methionine adduct:

Measurements: Two samples were received which were derived from (*R*) and (*S*) oxaziridine starting material. Each sample (~10 mg) was separately dissolved in 125 – 150 µL CDCl₃ (previously run through a plug of activated basic alumina) and transferred to a 100 µm BaF₂ IR cell. Simultaneous measurement of IR and VCD were taken for ~18 hours in one-hour blocks on a BioTools (Jupiter, FL) DualPEM FT-VCD spectrometer at 4 cm⁻¹ resolution and PEM maximum frequencies both set to 1400 cm⁻¹. The first block of IR was solvent and water vapor corrected, and offset to zero at 2000 cm⁻¹. VCD was block

averaged and corrected by subtraction of a solvent baseline which was previously acquired (18 hours averaged). Noise spectra were block averaged and used as is. The two samples had identical IR and nearly identical VCD – based on this, and the fact that the calculated (sR) and (sS) diastereomers had very different VCD spectra, the samples were determined to be identical.

Calculations: The (sR, S) and (sS, S) diastereomers were separately constructed using BioTools (Jupiter, FL) ComputeVOA software. A thorough conformational search was performed on each at the molecular mechanics level (GMMX) using the MMF94 force field in a 7 kcal / mol energy window. The resulting ~150 conformations for each diastereomer were optimized at the DFT (152) level (B3LYP / 6-31G(d) with CPCM - Chloroform) and the IR and VCD frequencies were also calculated at the same level. The 20 lowest energy conformers from each group (sR or sS) were re-optimized and frequencies calculated at the B3LYP / 6-311G(3df,2pd) level. The spectra from the resulting conformers were Boltzmann averaged using the electronic energy values and plotted using a line width of 5 cm⁻¹ HWHH, and an x-axis scaling factor of 0.982 for comparison with the experimental IR and VCD data. The (sS, S) DFT spectra were a much better match to the experimental than the (sR, S).

4.8.7 Diastereoselectivity studies



To an NMR tube was added (*R*)- or (*S*)-Ox32yne (9.1 mg, 0.033 mmol, 1.1 equiv.) and *N*-acetyl-L-methionine methyl ester (6.2 mg, 0.03 mmol, 1.0 equiv.) in 0.6 mL 1:1 MeOD:D₂O. The reaction was incubated and monitored by ¹H NMR. After 30 minutes the reaction was concentrated and purified using flash column chromatography (10% MeOH/DCM eluent) to give sulfilimine product which was analyzed by ¹H NMR, HRMS, VCD, and IR to confirm absolute configuration at sulfur.

(*R*)-Ox32yne product: 11.1 mg isolated. ¹H NMR (600 MHz, MeOD) δ 4.58 – 4.52 (m, 1H), 4.16 (d, *J* = 2.3 Hz, 2H), 3.74 (d, *J* = 1.4 Hz, 3H), 3.56 – 3.47 (m, 2H), 3.06 – 2.87 (m, 2H), 2.82 (t, *J* = 2.4 Hz, 1H), 2.63 (s, 3H), 2.30 – 2.23 (m, 1H), 2.10 – 2.02 (m, 1H), 2.01 (d, *J* = 3.0 Hz, 3H), 1.29 (d, *J* = 2.0 Hz, 6H, *overlap with grease*). **HRMS** (ESI): calculated for [C₁₆H₂₇N₃O₅S+H]⁺: required *m/z* 374.1744, found *m/z* 374.1754.

(*S*)-Ox32yne product: 8.7 mg isolated. ¹H NMR (600 MHz, MeOD) δ 4.60 – 4.51 (m, 1H *overlap with impurity*), 4.16 (d, *J* = 2.4 Hz, 2H), 3.74 (d, *J* = 1.4 Hz, 3H), 3.56 – 3.47 (m, 2H), 3.07 – 2.87 (m, 2H), 2.82 (t, *J* = 2.4 Hz, 1H), 2.63 (s, 3H), 2.30 – 2.23 (m, 1H), 2.10 – 2.02 (m, 1H), 2.01 (d, *J* = 2.9 Hz, 3H), 1.29 (d, *J* = 2.0 Hz, 6H, *overlap with grease*).

HRMS (ESI): calculated for $[C_{16}H_{27}N_3O_5S+H]^+$: required m/z 374.1744, found m/z 374.1754.

4.8.8 Biochemistry methods

Stereospecifically reduced protein Gel-ABPP: Proteins (Alpha 1 antitrypsin: Sigma, Cat. 178251; G-actin: Cytoskeleton, Cat. AKL95-B; Glutathione S transferase: abcam, Cat. Ab89494, Calmodulin: Enzo Life Sciences, Cat. BML-SE325-0001) (123, 179, 181, 182) were diluted to 2 μ M in PBS. To 50 μ L of protein was added 1 μ L of a stock of either (*R*)- or (*S*)-Ox32yne in DMSO for a final concentration of 0, 0.2, 1.0, 2.0, 4.0, 10, or 20 μ M. Samples were allowed to incubate at 23 °C for 1 hour. Excess oxaziridine was then quenched by addition of 1 μ L *N*-acetyl methionine (100 μ M) and incubation at 23 °C for 30 minutes. 1 μ L of Alexafluor488- N_3 (1.2 equiv.) was added to each sample with 3 μ L TBTA (stock in 1:4 DMSO: t BuOH, 100 μ M), 1 μ L TCEP (1 mM), and 1 μ L $CuSO_4$ (1 mM). Proteins were unfolded by addition of 5 μ L 1.2% SDS in PBS (0.1% w/v) and CuAAC was allowed to proceed at 23 °C for 1 hour with rocking. Then, 20 μ L of 4X Laemmli's buffer (Bio-Rad Laboratories, Inc.; 1610747) + 10% BME was added, and samples were boiled at 95 °C for 8 minutes. 30 μ L of sample was loaded per well and separated on precast 4–20% Novex Tris-Gly SDS-PAGE gels (Invitrogen) run at 160 V for 70 minutes. The gel was fixed in MQ water containing 10% acetic acid, 30% EtOH overnight then visualized using ChemiDoc MP (Bio-Rad) for measuring in-gel fluorescence (ex = 499 nm, em = 520 nm). The gel was rehydrated in MQ water for 1 hour then total protein level was assessed by Pierce Silver Staining (Thermo Fisher Scientific; 24612) according to the manufacturer's protocol or Safestain incubation (Invitrogen; LC6065) and visualized under brightfield.

hBPHL Gel-ABPP: hBPHL was diluted to 2 μ M in PBS. To 50 μ L of protein was added 1 μ L of a stock of either (*R*)- or (*S*)-Ox32yne in DMSO for a final concentration of 0, 0.1, 0.5, 1.0, 2.5, 5.0, or 10 μ M. Samples were allowed to incubate at 23 °C for 1 hour. Excess oxaziridine was then quenched by addition of 1 μ L *N*-acetyl methionine (100 μ M) and incubation at 23 °C for 30 minutes. 1 μ L of Alexafluor488- N_3 (1.2 equiv.) was added to each sample with 3 μ L TBTA (stock in 1:4 DMSO: t BuOH, 100 μ M), 1 μ L TCEP (1 mM), and 1 μ L $CuSO_4$ (1 mM). Proteins were unfolded by addition of 5 μ L 1.2% SDS in PBS (0.1% w/v) and CuAAC was allowed to proceed at 23 °C for 1 hour with rocking. Then, 20 μ L of 4X Laemmli's buffer (Bio-Rad; 1610747) + 10% BME was added, and samples were boiled at 95 °C for 8 minutes. 30 μ L of sample was loaded per well and separated on precast 4–20% Novex Tris-Gly SDS-PAGE gels (Invitrogen) run at 160 V for 70 minutes. The gel was fixed in MQ water containing 10% acetic acid, 30% EtOH overnight then visualized using ChemiDoc MP (Bio-Rad) for measuring in-gel fluorescence (ex = 499 nm, em = 520 nm). The gel was rehydrated in MQ water for 1 hour then total protein level was assessed by coomassie brilliant blue staining and visualized under brightfield.

Mitochondrial proteome Gel-ABPP: Mitochondrial lysate was diluted to 2.0 mg/mL in PBS. To 100 μ L of protein was added 1 μ L of a stock of either (*R*)- or (*S*)-Ox32yne in DMSO for a final concentration of 0, 1.0, 10, 50, or 100 μ M. Samples were allowed to incubate at 23 °C for 1 hour. Excess oxaziridine was then quenched by addition of 1 μ L *N*-acetyl methionine (100 μ M) and incubation at 23 °C for 30 minutes. 2 μ L of

Alexafluor488-N₃ (1.2 equiv.) was added to each sample with 6 μ L TBTA (stock in 1:4 DMSO:BuOH, 100 μ M), 2 μ L TCEP (1 mM), and 2 μ L CuSO₄ (1 mM). Proteins were unfolded by addition of 10 μ L 1.2% SDS in PBS (0.1% w/v) and CuAAC was allowed to proceed at 23 °C for 1 hour with rocking. Then, 40 μ L of 4X Laemmli's buffer (Bio-Rad; 1610747) + 10% BME was added, and samples were boiled at 95 °C for 8 minutes. 30 μ L of sample was loaded per well and separated on precast 4–20% Novex Tris-Glyc SDS-PAGE gels (Invitrogen) run at 160 V for 70 minutes. The gel was fixed in MQ water containing 10% acetic acid, 30% EtOH overnight then visualized using ChemiDoc MP (Bio-Rad) for measuring in-gel fluorescence (ex = 499 nm, em = 520 nm). The gel was rehydrated in MQ water for 1 hour then total protein level was assessed by coomassie brilliant blue staining and visualized under brightfield.

***In vitro* BPHL HCTLase activity assay:** All additions were performed using multi-channel pipettes. 100 μ L of a 100 nM protein solution containing either WT or M69L BPHL was aliquoted into a black, clear flat-bottom 96-well plate and allowed to equilibrate to 23 °C. Wells were then treated by 1 μ L addition of the appropriate concentration of (R)- or (S)-Ox32yne, H₂O₂, or OT-Ox, while controls were treated with vehicle (DMSO). Oxidation was allowed to occur for 30 minutes at 23 °C, followed by quenching with 1 μ L of *N*-acetyl methionine (1 mM) for 15 minutes at 23 °C. A solution containing both 40 mM HCTL and 4 mM DTNB was then prepared in the interim, and 5 μ L was administered to each well. Absorbance at 412 nm was immediately monitored at 37 °C for 10 minutes using a plate reader (BioTek). Linear rates for the first 120 - 180 seconds were calculated to compute relative activities.

General *N*-homocysteinylation assay: 100 μ L of desired proteome (4 mg/mL) was diluted to a concentration of 1 mg/mL using 8 M urea, 27 mM TCEP, pH 6.0 as the diluent for a final concentration of 6 M urea, 20 mM TCEP, pH 6.0 then incubated with 1 mM AT-3 thioester (48) for 30 minutes at 23 °C while rotating. Reaction was quenched by adding 20 μ L NH₂OH (50% v/v) and 50 μ L iodoacetamide (400 mM) for 1 hour at 23 °C in the dark. Proteins were then precipitated by addition of 4 volumes of MeOH:CHCl₃ (4:1.5), vortexed, and centrifuged at 12,000 xg for 15 minutes. The collected protein pellet was washed 1X with 500 μ L chilled (-20 °C) MeOH via probe-tip sonication (5 s on, 20 s off, 20% A, 4-6 pulses) and resuspended in 200 μ L 1.2% SDS/PBS via probe-tip sonication (5 s on, 20 s off, 20% A, 6-8 pulses). CuAAC was performed by addition of 4 μ L Alexafluor488-N₃ (1.2 equiv.), 12 μ L TBTA (stock in 1:4 DMSO:BuOH, 100 μ M), 4 μ L TCEP (1 mM), and 4 μ L CuSO₄ (1 mM). Then, 56 μ L of 4X Laemmli's buffer (Bio-Rad;1610747) + 10% BME was added and samples were boiled at 95 °C for 8 minutes. 20 μ L of sample was loaded per well and separated on precast 4–20% Novex Tris-Glyc SDS-PAGE gels (Invitrogen) run at 160 V for 70 minutes. The gel was fixed in MQ water containing 10% acetic acid, 30% EtOH overnight then visualized using ChemiDoc MP (Bio-Rad) for measuring in-gel fluorescence (ex = 499 nm, em = 520 nm). The gel was rehydrated in MQ water for 1 hour then total protein level was assessed by coomassie brilliant blue staining and visualized under brightfield. Band intensities were quantified via densitometry (ImageJ).

rhBPHL cloning and M69L mutagenesis:

WT Sequence (hBPHL, Uniprot ID: Q86WA6)

MVAVLGGRGVLRLRLLSALKPGIHVPRAGPAAAFGTSVTSKVAVNGVQ
LHYQQTGEDHAVLLLPGLGSGETDFGPQLKNLNKKLFTVVAWDPRGYG
HSRPPDRDFPADFFERDAKDAVDLMKALKFKKVSLLGWSDGGITALIAAA
KYPYIHKMVIWGANAYVTDEDSMIYEGIRDVSKWSERTRKPLEALYGYD
YFARTCEKWVDGIRQFKHLPDGNICRHLLPRVQCPALIVHGEKDPLVPRF
HADFIHKHVKGSRHLHLMPEGKHNLHLRFADEFNKLAEDFLQ*

Cloned Sequence (rhBPHL)

MVAVLGGRGVLRLRLLSALKPGIHVPRAGPAAAFGTSVTSKVAVNGVQ
LHYQQTGEDHAVLLLPGL**ML**GSGETDFGPQLKNLNKKLFTVVAWDPRGYG
HSRPPDRDFPADFFERDAKDAVDLMKALKFKKVSLLGWSDGGITALIAAA
KYPYIHKMVIWGANAYVTDEDSMIYEGIRDVSKWSERTRKPLEALYGYD
YFARTCEKWVDGIRQFKHLPDGNICRHLLPRVQCPALIVHGEKDPLVPRF
HADFIHKHVKGSRHLHLMPEGKHNLHLRFADEFNKLAEDFLQHHHHHH*

5' – BPHL XbaI FOR – 3'

CCCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGTAGCTGTGT
TAGGCG

5' – BPHL XhoI REV – 3'

GTGCTCGAGTTGTAAAAAGTCTTCGGCCAG

5' – BPHL M69L FOR – 3'

GTATTACTGCTGCCCGGT**C**TGTTGGGTAGCGGTGAAAC

5' – BPHL M69L REV – 3'

GTTTCACCGCTACCCAAC**A**GACCGGGCAGCAGTAATAC

Molecular cloning was performed using a pET24a vector via traditional double-restriction digest using a codon-optimized synthetic gene-block of rhBPHL for *E. coli* expression (IDT) and overhang oligonucleotide primers (IDT). Insert PCR product was amplified and purified using QIAquick PCR purification (Qiagen). Both insert and vector were then double-restriction digested using XbaI and XhoI endonucleases (NEB). Plasmid was gel-purified using QIAquick gel purification (Qiagen) and insert was PCR purified. The digested insert and vector were then mixed at 0:1, 3:1, and 5:1 insert:vector ratios and ligated using T4 DNA ligase in 1X T4 DNA ligase buffer (NEB). Ligation was incubated overnight at 23 °C then transformed into chemically-competent XL1-blue cells and plated on 50 µg/mL kanamycin agar plates. Cells were grown at 37 °C overnight and colonies were picked for culturing and Sanger sequencing (QuintaraBiosciences).

Site-directed mutagenesis was performed via Quikchange using complementary oligonucleotide primers (IDT) that change a single codon from M to L shown in bold. Forward and reverse reactions were carried out in separate PCR tubes for 5 cycles, then combined 1:1 for 13 additional cycles. Methylated DNA was digested via addition of 20 U DpnI (NEB) and incubation at 37 °C for 1 hour. 5 µL of PCR reaction was directly

transformed into chemically-competent XL1-blue cells and plated on 50 µg/mL kanamycin agar plates. Cells were grown at 37 °C overnight and colonies were picked for culturing and Sanger sequencing (QuintaraBiosciences).

FLAG-BPHL cloning:

WT Sequence (hBPHL, Uniprot ID: Q86WA6)

MVAVLGGRGVLRLRLLSALKPGIHVPRAGPAAAFGTSVTSKAVAVNGVQ
LHYQQTGEGDHAVLLLPGMLGSGETDFGPQLKNLNKKLFTVVAWDPRGYG
HSRPPDRDFPADFFERDAKDAVDLMKALKFKKVSLGWSGGITALIAAA
KYPSYIHKMVIWGANAYVTDEDSMIYEGIRDVSKWSERTRKPLEALYGYD
YFARTCEKWVDGIRQFKHLPDGNICRHLLPRVQCPALIVHGEKDPLVPRF
HADFIHKHVKGSRLLHLMPEGKHNLHLRFADFNKLAEDFLQ

Cloned Sequence (FLAG-BPHL)

MGDYKDDDDKENLYFQSGGSGGMMVAVLGGRGVLRLRLLSALKPGIHVPRAGPAAAF
GTSVTSKAVAVNGVQLHYQQTGEGDHAVLLLPGMLGSGETDFGPQLKNLNKKLFTV
AWDPRGYGHSRPPDRDFPADFFERDAKDAVDLMKALKFKKVSLGWSGGITALIAA
AKYPSYIHKMVIWGANAYVTDEDSMIYEGIRDVSKWSERTRKPLEALYGYDYFARTCE
KWVDGIRQFKHLPDGNICRHLLPRVQCPALIVHGEKDPLVPRFHADFIHKHVKGSRLLH
LMPEGKHNLHLRFADFNKLAEDFLQ*

5' – BPHL HindIII FOR– 3'

CGACAAAGCTTGCCACCGCCACCATGGGAGACTATAAG

5' – BPHL XbaI REV– 3'

AATATTCTAGACTCGAGTCACTGAAGAAAATCTTCAGCC

Molecular cloning was performed using a pcDNA3.1(+) vector via traditional double-restriction digest using a codon-optimized synthetic gene-block of rhBPHL for mammalian expression (IDT) and overhang oligonucleotide primers (IDT). Insert PCR product was amplified and purified using QIAquick PCR purification (Qiagen). Both insert and vector were then double-restriction digested using HindIII and XbaI endonucleases (NEB). Plasmid was gel-purified using QIAquick gel purification (Qiagen) and insert was PCR purified. The digested insert and vector were then mixed at 0:1, 3:1, and 5:1 insert:vector ratios and ligated using T4 DNA ligase in 1X T4 DNA ligase buffer (NEB). Ligation was incubated overnight at 23 °C then transformed into chemically-competent XL1-blue cells and plated on 100 µg/mL ampicillin agar plates. Cells were grown at 37 °C overnight and colonies were picked for culturing and Sanger sequencing (QuintaraBiosciences). Plasmids were bacterial endotoxing purified using Endofree purification kit (Qiagen) prior to mammalian transfection.

Recombinant protein expression and purification: pET24a-rhBPHL was transformed into a chemically-competent Origami2 (DE3) expression host. Following kanamycin selection on agar plates (50 µg/mL), three separate 5 mL starter cultures were grown in LB media containing kanamycin (50 µg/mL) at 37 °C overnight. These cultures were then inoculated into three 1 L cultures of LB media and grown at 37 °C to OD₆₀₀ = 0.6 then

induced with 0.5 mM IPTG. Protein was expressed for 6 hours at 37 °C then pelleted by centrifugation at 5,000 xg, 4 °C for 25 minutes. Overexpressed pellets were then flash-frozen in liquid nitrogen and stored at -80 °C. Protein pellets were thawed on ice then resuspended in 35 mL lysis buffer containing EDTA-free protease inhibitors (Roche). Cells were kept on ice and lysed via sonication (5 s on, 20 s off, 40% A, 6 pulses) then centrifuged at 15,000 xg, 4 °C for 25 minutes. Lysate was further clarified by 0.45 µm syringe filtration. Clarified lysate was then loaded onto a pre-equilibrated HisGraviTrap TALON column (Cytiva). Bound protein was washed by addition of 10 mL wash buffer, then eluted with 3 mL elution buffer. Protein was then dialyzed against 3 L SEC buffer at 4 °C for 4 hours before being loaded a HiLoad 16/600 Superdex 75 pg size-exclusion column and separated at a 0.25 mL/min flow rate. Fractions containing HCTLase activity (see *in vitro* BPHL HCTLase activity assay) were pooled, concentrated, and quantified by A280.

Buffers

Lysis – 20 mM Tris, 300 mM NaCl, 5 mM imidazole, 5% glycerol, pH 8.0

Wash – 20 mM Tris, 300 mM NaCl, 15 mM imidazole, 5% glycerol, pH 8.0

Elution – 20 mM Tris, 300 mM NaCl, 300 mM imidazole, 5% glycerol, pH 8.0

SEC – 20 mM Tris, 150 mM NaCl, 5% glycerol, pH 8.0

Treatment of FLAG-BPHL with Ox32yne enantiomers for Strep-IP: Adapted from Zanon et. al (203). Transfected HEK-293T cells bearing pcDNA3.1(+)-FLAG-BPHL were prepared as described below (see FLAG-BPHL transfection). Cells were resuspended in 200 µL ice-cold PBS + 1% Triton X-100 (v/v) containing EDTA-free protease inhibitors (Roche). Cells were lysed at 4 °C for 30 minutes while rotating. Lysate was clarified by centrifugation at 12,000 x g at 4 °C for 15 minutes. The supernatant was then transferred to a fresh prechilled 1.7 mL microcentrifuge tube. Protein concentration was normalized to 2.0 mg/mL via BCA assay (Thermo; 23225). 100 µL of this sample was then aliquoted into separate 2.0 mL tubes and allowed to equilibrate to 23 °C. Samples were treated with 1 µL of either (*R*)- or (*S*)-Ox32yne at the desired concentration in DMSO and incubated at 23 °C for 1 hour. 2 µL of DTB-N₃ (1.2 equiv.) was added to each sample with 6 µL TBTA (stock in 1:4 DMSO:BuOH, 100 µM), 2 µL TCEP (1 mM), and 2 µL CuSO₄ (1 mM). Proteins were unfolded by addition of 20 µL 1.2% SDS in PBS (0.1% w/v) and CuAAC was allowed to proceed at 23 °C for 1 hour with rocking. Proteins were then precipitated by addition of 4 volumes of MeOH:CHCl₃ (4:1.5), vortexed, and centrifuged at 12,000 xg for 15 minutes. The collected protein pellet was washed 1X with 500 µL chilled (-20 °C) MeOH via probe-tip sonication (5 s on, 20 s off, 20% A, 4-6 pulses) and resuspended in 100 µL 1.2% SDS/PBS via probe-tip sonication (5 s on, 20 s off, 20% A, 6-8 pulses).

Treatment of LFD or HFD mouse livers with (*R*)-Ox32yne for Strep-IP: LFD or HFD diet mouse liver homogenates were prepared as described below (see Tissue homogenization). Mouse liver homogenates were normalized to 2.0 mg/mL via BCA assay (Thermo; 23225). 100 µL of this sample was then aliquoted into separate 2.0 mL tubes and allowed to equilibrate to 23 °C. Samples were treated with either 1 µL of vehicle (DMSO) or (*R*)-Ox32yne (50 µM) in DMSO and incubated at 23 °C for 1 hour. 2 µL of DTB-N₃ (1.2 equiv.) was added to each sample with 6 µL TBTA (stock in 1:4

DMSO:*t*BuOH, 100 μ M), 2 μ L TCEP (1 mM), and 2 μ L CuSO₄ (1 mM). Proteins were unfolded by addition of 20 μ L 1.2% SDS in PBS (0.1% w/v) and CuAAC was allowed to proceed at 23 °C for 1 hour with rocking. Proteins were then precipitated by addition of 4 volumes of MeOH:CHCl₃ (4:1.5), vortexed, and centrifuged at 12,000 xg for 15 minutes. The collected protein pellet was washed 1X with 500 μ L chilled (-20 °C) MeOH via probe-tip sonication (5 s on, 20 s off, 20% A, 4-6 pulses) and resuspended in 100 μ L 1.2% SDS/PBS via probe sonication (5 s on, 20 s off, 20% A, 6-8 pulses).

General Strep-IP protocol: High-capacity streptavidin agarose beads (50 μ L per sample) (Thermo Fisher Scientific; 20357) were washed 3X in PBS then transferred to a fresh 2.0 mL microcentrifuge tube containing 1.1 mL PBS. The reconstituted protein samples (100 μ L) were then transferred to tubes containing streptavidin beads and allowed to incubate at 23 °C for 2 hours. The samples were centrifuged at 1,400 xg for 3 minutes and the supernatant was removed. The beads were then washed with 500 μ L PBS (5X) and 500 μ L MQ (5X) in micro bio-spin columns (Bio-Rad; 7326204) on a vacuum manifold. With the final wash, samples were transferred to a fresh 1.7 mL microcentrifuge tube, centrifuged at 1,400 xg for 3 minutes, and the supernatant was removed. Beads were resuspended in 100 μ L of a premixed 1X Laemmli's buffer (Bio-Rad; 1610747) + 10% BME diluted with PBS and boiled at 95 °C for 8 minutes. 20 μ L of bead slurry was loaded per well and separated on precast 4–20% Novex Tris-Glyc SDS-PAGE gels (Invitrogen) run at 160 V for 70 minutes. Proteins were then electrotransferred to a PVDF membrane (25 V/2.5 A for 30 minutes). Membranes were then blocked in a solution of TBST + 5% BSA (w/v) and rocked at 23 °C for 1 hour. Membranes were washed 3X in TBST for 5 minutes each while rocking and cut using scissors along the protein ladder for separate antibody incubation. Membranes were then blotted with primary mouse anti-FLAG M2 (Sigma; F1804), rabbit anti-BPHL (Sigma; HPA036752), rabbit anti- β actin (CST; 4970), or rabbit anti-GAPDH (CST; 5174) (1:1000 TBST + 5% BSA) at 4 °C overnight. The following morning membranes were washed 3X in TBST for 5 minutes then incubated with either anti-mouse IgG HRP conjugated secondary (CST; 7076) or anti-rabbit IgG HRP conjugated secondary (CST; 7074) (1:3000 TBST) for 2 hours at 23 °C. Blots were quickly washed 3X with TBST prior to incubation with ECL western blotting substrates (Sigma; 34580) for 5 minutes and imaged with ChemiDoc MP. Band intensities were quantified via densitometry when applicable (ImageJ).

Co-IP of BPHL and Msr proteins: Transfected HEK-293T cells bearing pcDNA3.1(+)-FLAG-BPHL were prepared as described below (see Co-IP transfection and uncoupler treatment). Cells were resuspended in 200 μ L ice-cold PBS + 1% Triton X-100 (v/v) containing EDTA-free protease inhibitors (Roche). Cells were lysed at 4 °C for 30 minutes while rotating. Lysate was clarified by centrifugation at 12,000 xg at 4 °C for 15 minutes. The supernatant was then transferred to a fresh prechilled 1.7 mL microcentrifuge tube. Protein concentration was normalized to 4.0 mg/mL via BCA assay (Thermo; 23225). 100 μ L of input sample was boiled with 4X Laemmli's buffer (Bio-Rad; 1610747) + 10% BME at 95 °C for 8 minutes and stored at -20 °C for downstream analysis. Anti-FLAG agarose beads (50 μ L per sample) (Sigma; A2220) were aliquoted into 1.7 mL microcentrifuge tubes and washed 3X in 500 μ L PBS by centrifugation at 1,400 xg for 3 minutes and removal of the supernatant. The resulting beads were then resuspended in 300 μ L PBS

and 100 μ L of protein sample was added for a final protein concentration of 1.0 mg/mL. The samples were incubated for 2 hours at 23 °C while rocking then washed 3X with 500 μ L PBS. Beads were resuspended in 100 μ L of a premixed 1X Laemmli's buffer (Bio-Rad; 1610747) + 10% BME diluted with PBS and boiled at 95 °C for 8 minutes. 20 μ L of bead slurry was loaded per well and separated on precast 4–20% Novex Tris-Glyc SDS-PAGE gels (Invitrogen) run at 160 V for 70 minutes. Proteins were then electro-transferred to a PVDF membrane (25 V/2.5 A for 30 minutes). Membranes were then blocked in a solution of TBST + 5% BSA (w/v) and rocked at 23 °C for 1 hour. Membranes were washed 3X in TBST for 5 minutes each while rocking and cut using scissors along the protein ladder for separate antibody incubation. Membranes were then blotted with primary mouse anti-FLAG M2 (Sigma; F1804), rabbit anti-MsrA (Invitrogen; PA5-88562), rabbit anti-MsrB2 (abcam; ab229940), or rabbit anti- β actin (CST; 4970) (1:1000 TBST + 5% BSA) at 4 °C overnight. The following morning membranes were washed 3X in TBST for 5 minutes then incubated with either anti-mouse IgG HRP conjugated secondary (CST; 7076) or anti-rabbit IgG HRP conjugated secondary (CST; 7074) (1:3000 TBST) for 2 hours at 23 °C. Blots were quickly washed 3X with TBST prior to incubation with ECL western blotting substrates (Sigma; 34580) for 5 minutes and imaged with ChemiDoc MP.

4.8.9 Cell culture methods

Cell line maintenance: 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. HEK-293T cells were maintained in Dulbecco's Modified Eagle Medium + GlutaMAX (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Seradigm), referred to below as complete media.

FLAG-BPHL transfection: HEK-293T cells were grown to 70% confluency in 10 mL complete media in a 10 cm plate at 37 °C, 5% CO₂. Transfection was then performed as per Lipofectamine 3000 protocol (Invitrogen). Briefly, 25 μ g of pcDNA3.1(+)-FLAG-BPHL expression construct was introduced at 1:1 transfection reagent:DNA ratio. The lipid-DNA complex was incubated for 30 minutes at 23 °C in 2 mL Opti-MEM media (Gibco). Then, 2 mL complex was added to 8 mL complete media. 10 mL of this solution was then added to each 10 cm plate. Cells were incubated for 24 hours at 37 °C, 5% CO₂. The media was then aspirated, and cells were harvested by trypsinization. Cells were washed 1X with 10 mL of ice-cold PBS, pelleted at 300 xg, and flash-frozen in LN₂ for future analysis.

Co-IP transfection and uncoupler treatment: HEK-293T cells were grown to 70% confluency in 10 mL complete media in a 10 cm plate at 37 °C, 5% CO₂. Transfection was then performed as per Lipofectamine 3000 protocol (Invitrogen). Briefly, 25 μ g of pcDNA3.1(+)-FLAG-BPHL expression construct was introduced at 1:1 transfection reagent:DNA ratio. The lipid-DNA complex was incubated for 30 minutes at 23 °C in 2 mL Opti-MEM media (Gibco). Then, 2 mL complex was added to 8 mL complete media. 10 mL of this solution was then added to each 10 cm plate. Cells were incubated for 24 hours at 37 °C, 5 % CO₂. The media was then aspirated, and fresh complete media containing vehicle (DMSO), 100 μ M paraquat, or 2.5 μ M FCCP was added. Cells were incubated for 18 hours at 37 °C, 5 % CO₂. The following day, cells were harvested by trypsinization.

Cells were washed 1X with 10 mL of ice-cold PBS, pelleted at 300 xg, and flash-frozen in LN₂ for future analysis.

siRNA knock-down for *N*-homocysteinylation: HEK-293T cells were grown to 70% confluency in 10 mL complete media in a 10 cm plate at 37 °C, 5% CO₂. Transfection was then performed as per Lipofectamine RNAiMax protocol. Briefly, 500 pmol of desired siRNA, siControl (Thermo; 4390843), siMsrA (Thermo; AM16708), or siMsrB2 (Thermo; AM16708), was introduced to 25 µL lipid. The lipid-siRNA complex was incubated for 30 minutes at 23 °C in 2 mL Opti-MEM media (Gibco). Then, 2 mL complex was added to 8 mL complete media. 10 mL of this solution was then added to each 10 cm plate containing a final siRNA concentration of 50 nM. Cells were incubated for 24 hours at 37 °C, 5 % CO₂. The media was then aspirated, and fresh complete media containing a combination of 500 µM HCTL and either vehicle (DMSO) or 100 µM paraquat was added. Cells were incubated for 18 hours at 37 °C, 5 % CO₂. The following day, cells were harvested by trypsinization. Cells were washed 1X with 10 mL of ice-cold PBS, pelleted at 300 xg, and flash-frozen in LN₂ for future analysis.

siRNA knock-down for immunoblotting: HEK-293T cells were grown to 70% confluency in 3 mL complete media in a 6-well plate at 37 °C, 5% CO₂. Transfection was then performed as per Lipofectamine RNAiMax protocol. Briefly, 150 pmol of desired siRNA, siControl (Thermo; 4390843), siMsrA (Thermo; AM16708), or siMsrB2 (Thermo; AM16708), was introduced to 7.5 µL lipid. The lipid-siRNA complex was incubated for 30 minutes at 23 °C in 500 µL Opti-MEM media (Gibco). Then, 500 µL complex was added to 2.5 mL complete media. 10 mL of this solution was then added to each 10 cm plate containing a final siRNA concentration of 50 nM. Cells were incubated for 24 hours at 37 °C, 5 % CO₂. The following day, cells were harvested by trypsinization. Cells were washed 1X with 1 mL of ice-cold PBS, pelleted at 300 xg, and flash-frozen in LN₂ for future analysis.

siRNA knock-down for proteomics: HEK-293T cells were grown to 70% confluency in 25 mL complete media in a 15 cm plate at 37 °C, 5% CO₂. Transfection was then performed as per Lipofectamine RNAiMax protocol. Briefly, 1250 pmol of desired siRNA, siControl (Thermo; 4390843), siMsrA (Thermo; AM16708), or siMsrB2 (Thermo; AM16708), was introduced to 62.5 µL lipid. The lipid-siRNA complex was incubated for 30 minutes at 23 °C in 5 mL Opti-MEM media (Gibco). Then, 5 mL complex was added to 20 mL complete media. 25 mL of this solution was then added to each 15 cm plate containing a final siRNA concentration of 50 nM. Cells were incubated for 24 hours at 37 °C, 5 % CO₂. The media was then aspirated, and fresh complete media containing 100 µM paraquat was added. Cells were incubated for 18 hours at 37 °C, 5 % CO₂. The following day, cells were harvested by trypsinization. Cells were washed 2X with 10 mL of ice-cold PBS, pelleted at 300 xg, and flash-frozen in LN₂ for future analysis.

Determination of Msr knock-down efficiency by immunoblotting: Transfected HEK-293T cells bearing the corresponding siRNA were prepared as described above. Cells were resuspended in 200 µL ice-cold PBS + 1% Triton X-100 (v/v) containing EDTA-free protease inhibitors (Roche). Cells were lysed at 4 °C for 30 minutes while rotating. Lysate

was clarified by centrifugation at 12,000 x g at 4 °C for 15 minutes. The supernatant was then transferred to a fresh prechilled 1.7 mL microcentrifuge tube. Protein concentration was normalized to 2.0 mg/mL via BCA assay (Thermo; 23225). 100 µL of sample was boiled with 4X Laemmli's buffer (Bio-Rad; 1610747) + 10% BME diluted at 95 °C for 8 minutes. Approximately 30 µg of protein sample was loaded per well and separated on precast 4–20% Novex Tris-Gly SDS-PAGE gels (Invitrogen) run at 160 V for 70 minutes. Proteins were then electro-transferred to a PVDF membrane (25 V/2.5 A for 30 minutes). Membranes were then blocked in a solution of TBST + 5% BSA (w/v) and rocked at 23 °C for 1 hour. Membranes were washed 3X in TBST for 5 minutes each while rocking and cut using scissors along the protein ladder for separate antibody incubation. Membranes were then blotted with primary rabbit anti-BPHL (Sigma; HPA036752), rabbit anti-MsrA (Invitrogen; PA5-88562), rabbit anti-MsrB2 (abcam; ab229940), or rabbit anti-β actin (CST; 4970) (1:1000 TBST + 5% BSA) at 4 °C overnight. The following morning membranes were washed 3X in TBST for 5 minutes then incubated with anti-rabbit IgG HRP conjugated secondary (CST; 7074) (1:3000 TBST) for 2 hours at 23 °C. Blots were quickly washed 3X with TBST prior to incubation with ECL western blotting substrates (Sigma; 34580) for 5 minutes and imaged with ChemiDoc MP.

4.8.10 Animal methods

Animal raising and feeding: All animal experiments were approved and performed under the guidelines and ethical regulations established by the UC Berkeley Animal Care and Use Committee. Male wildtype C57BL/6J mice (JAX, stock #000664) were fed a regular chow diet (LabDiet 5053) until 6-8 weeks of age under ambient temperature at 23 °C. For the control group, after 8 weeks of age mice were fed a low-fat diet (10% kcal fat; Research Diets D12450J) for 10 weeks prior to tissue collection. For the oxidative stress model we utilized diet induced obesity, which has been shown to exhibit a NAFLD phenotype in the liver (55). After 6 weeks of age mice were fed a high fat diet (60% kcal fat; Research Diets D12492) for 16 weeks prior to tissue collection. Mice were fasted for 4 – 5 hours prior to sacrificing and tissue harvesting. All animal studies were performed using age-matched male mice (18-22 weeks).

Tissue homogenization: All steps and centrifugations were carried out at 4 °C. Mouse livers were thawed on ice and rinsed 2X with 5 mL of ice-cold PBS. Livers were cut into small pieces (size of a grain of rice) using a razor blade prior to homogenization. Chopped liver pieces were transferred to a glass homogenizer on ice and homogenized with 6 – 8 strokes of the glass pestle in 10 mL/gram tissue ice-cold PBS. Tissue homogenate was then sonicated via probe-tip sonication (5 s pulse, 20 s off, 40% A, 4-6 pulses) then strained through a 100 µm cell strainer into a 50 mL conical tube and centrifuged at 12,000 xg for 20 minutes. The resulting top fatty layer was removed by pipetting and the supernatant was collected.

Mitochondrial purification: All steps and centrifugations were carried out at 4 °C. Mouse livers were thawed on ice and rinsed twice with 5 mL of ice-cold PBS. 3 mouse livers were cut into small pieces (size of a grain of rice) using a razor blade prior to homogenization. Chopped liver pieces were transferred to a glass homogenizer on ice and homogenized with 6 – 8 strokes of the glass pestle in 10 mL/gram tissue ice-cold

Buffer A. Tissue homogenate was then strained through a 100 μ M cell strainer into separate 50 mL conical tube and balanced by dilution with Buffer A. Samples were centrifuged for 30 minutes at 600 xg to remove nuclear components. The supernatant was collected and centrifuged an additional 10 minutes at 15,000 xg to yield a pellet of crude mitochondria. The crude mitochondria pellet was resuspended in 18 mL Buffer B and centrifuged for 30 minutes at 15,000 xg. The supernatant was discarded, and the pellet was resuspended in 9 mL of Buffer C. To three separate polycarbonate ultracentrifuge tubes (24 mL size) was added a 5 mL layer of Buffer D followed by careful addition (add to the side of tube and pipette extremely gently) of 9 mL Buffer E. Then, 3 mL of resuspended crude mitochondrial pellet was carefully added to the top layer of each Percoll gradient. The triphasic solutions were centrifuged with a SW 32Ti swinging-bucket rotor (Beckman; 369650) using an Optima XPN-100-IVD ultracentrifuge (Beckman; B10053) at 41,000 xg for 30 minutes. After centrifugation, enriched mitochondria were collected from the interface formed between the 26% and 60% Percoll and transferred to a 15 mL conical tube. Excess Percoll was removed from the mitochondrial fraction by resuspension in 10 mL of ice-cold PBS followed by centrifugation at 7,000 xg for 10 minutes. The resulting pellet of enriched mitochondria was then flash-frozen in LN₂ for future analysis.

Buffers

A – 225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, 1 mg/mL fatty acid-free BSA, 10 mM HEPES, pH 7.4

B – 225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, 10 mM HEPES, pH 7.4

C – 395 mM sucrose, 0.1 mM EGTA, 10 mM HEPES, pH 7.4

D – 1.28 M sucrose, 0.4 mM EGTA, 40 mM HEPES, 60% Percoll (v/v), pH 7.4

E – 1.28 M sucrose, 0.4 mM EGTA, 40 mM HEPES, 26% Percoll (v/v), pH 7.4

Determination of mitochondrial enrichment by immunoblotting: Mitochondrial lysate was normalized to 4.0 mg/mL in PBS. Samples were diluted with 4X Laemmli's buffer (Bio-Rad Laboratories, Inc.; 1610747) + 10% BME, boiled at 95 °C for 8 minutes, and loaded at 40 μ g per lane on a 4-20% Tris-Gly SDS-PAGE gel. The gel was run at 160 V for 80 minutes and semi-dry electrotransferred to a PVDF membrane at 25 V, 2.5 Å, for 10 minutes. Blots were blocked with 5% BSA/TBST for 1 hour, then washed 2X with TBST for 5 minutes, and cut for incubation with separate antibodies. Antibodies used were rabbit anti-VDAC (CST; 4661), rabbit anti-PDI (CST; 3501), rabbit anti-RCAS1 (CST; 12290), rabbit anti-GAPDH (CST; 5714), and rabbit anti-Histone H3 (CST; 4499). All antibodies were diluted at 1:1000 in 5% BSA/TBST at 4 °C overnight. The next morning the blots were washed 3X with TBST prior to incubation with anti-rabbit IgG HRP conjugated secondary (CST; 7074) (1:3000 TBST) for 2 hours at 23 °C. Blots were quickly washed 3X with TBST prior to incubation with ECL western blotting substrates (Promega; W1001) for 1 minute and imaging with ChemiDoc MP.

Determination of BPHL and Msr levels in LFD and HFD mouse livers by immunoblotting: LFD or HFD diet mouse liver homogenates were prepared as described above (see Tissue homogenization). Mouse liver homogenates were normalized to 4.0 mg/mL via BCA assay (Thermo; 23225). 100 μ L of sample was boiled

with 4X Laemmli's buffer (Bio-Rad; 1610747) + 10% BME at 95 °C for 8 minutes. Approximately 40 µg of protein sample was loaded per well and separated on precast 4–20% Novex Tris-Glyc SDS-PAGE gels (Invitrogen) run at 160 V for 70 minutes. Proteins were then electro-transferred to a PVDF membrane (25 V/2.5 A for 30 minutes). Membranes were then blocked in a solution of TBST + 5% BSA (w/v) and rocked at 23 °C for 1 hour. Membranes were washed 3X in TBST for 5 minutes each while rocking and cut using scissors along the protein ladder for separate antibody incubation. Membranes were then blotted with primary rabbit anti-BPHL (Sigma; HPA036752), rabbit anti-MsrA (Invitrogen; PA5-88562), rabbit anti-MsrB2 (abcam; ab229940), or rabbit anti-GAPDH (CST; 5174) (1:1000 TBST + 5% BSA) at 4 °C overnight. The following morning membranes were washed 3X in TBST for 5 minutes then incubated with anti-rabbit IgG HRP conjugated secondary (CST; 7074) (1:3000 TBST) for 2 hours at 23 °C. Blots were quickly washed 3X with TBST prior to incubation with ECL western blotting substrates (Sigma; 34580) for 5 minutes and imaged with ChemiDoc MP. Band intensities were quantified via densitometry (ImageJ).

Treatment of LFD and HFD mouse livers for N-homocysteinylation: LFD or HFD diet mouse liver homogenates were prepared as described above (see Tissue homogenization). Mouse liver homogenates were normalized to 4.0 mg/mL via BCA assay (Thermo; 23225). 100 µL of this sample was then aliquoted into separate 2.0 mL tubes and allowed to equilibrate to 23 °C. Samples were co-treated with 1 µL of H₂O₂ (500 µM) and 1 µL of HCTL at the desired concentration. Samples were incubated for 18 hours at 37 °C.

Treatment of LFD and HFD mouse livers for proteomics: LFD or HFD diet mouse liver homogenates were prepared as described above (see Tissue homogenization). Mouse liver homogenates were normalized to 4.0 mg/mL via BCA assay (Thermo; 23225). 500 µL of this sample was then aliquoted into separate 2.0 mL tubes and allowed to equilibrate to 23 °C. Samples were co-treated with 5 µL of vehicle (PBS) or H₂O₂ (500 µM). Samples were incubated for 18 hours at 37 °C.

4.8.11 Proteomic methods

Shotgun proteomics: Proteins (Alpha 1 antitrypsin: Sigma, Cat. 178251; G-actin: Cytoskeleton, Cat. AKL95-B; Glutathione S transferase: abcam, Cat. Ab89494, Calmodulin: Enzo Life Sciences, Cat. BML-SE325-0001) were diluted to 10 µM. To 100 µL of protein was added 1 µL of a stock of either (R)- or (S)-Ox32yne in DMSO for a final concentration of 200 µM. Samples were allowed to incubate for 1 hour at 23 °C. While samples were incubating, 10 µL each of magnetic E3 (Cytiva; 44152105050250) and E7 (Cytiva; 24152105050250) SP3 beads were mixed 1:1 and washed 3X with 1 mL MQ using a magnetic rack. The washed beads were then resuspended in 20 µL of MQ and added to each protein sample. The samples were allowed to incubate at 23 °C for 5 minutes with shaking (1000 rpm). Protein binding was induced by addition of 800 µL absolute ethanol followed by incubation at 23 °C for 5 minutes with shaking (1000 rpm). The beads were then placed on a magnetic rack and allowed to settle. The supernatant was removed, and the beads were then resuspended and washed 3X with 400 µL of 80% (v/v) ethanol. The beads were then resuspended in 200 µL of 8 M urea in PBS and incubated

with 10 μ L TCEP (10 mM) at 65 °C for 15 minutes. Then, 10 μ L of iodoacetamide (20 mM) was added and incubated at 37 °C for 30 minutes in the dark. Samples were cooled to 23 °C and incubated with 800 μ L ethanol for 5 minutes with shaking (1000 rpm). The supernatant was removed, and the beads were then resuspended and washed 3X with 400 μ L of 80% (v/v) ethanol. Then, the beads were resuspended in 200 μ L of 8 M urea in PBS and 4 μ L of reconstituted trypsin/Lys C solution (20 μ g lyophilized Trypsin/LysC reconstituted in 40 μ L Trypsin/LysC buffer) (Promega; V5073) was added and incubated at 37 °C overnight with shaking (200 rpm). The next day, the digested samples were transferred to a 15 mL conical tube and incubated with 3.8 mL MeCN at 23 °C for 10 minutes with shaking (1000 rpm). The tubes were placed on a magnetic rack and the beads were allowed to settle. The supernatant was removed, and the beads were resuspended and washed 3X with 1 mL MeCN. Then, the beads were eluted by resuspension with 100 μ L 2% (v/v) DMSO in MQ and incubated at 37 °C for 30 minutes with shaking (1000 rpm). The tubes were placed on a magnetic rack and the supernatants were collected in a microcentrifuge tube. The elution step was repeated, and the supernatants were combined. Peptides were reconstituted in 100 μ L LC-MS grade 50% ACN/H₂O + 0.1% FA (v/v) via bath sonication for 10 minutes and quantified using Pierce fluorometric assay kit (Thermo; 23290).

ChURRO-ABPP: 500 μ L of desired proteome was normalized to 4.0 mg/mL and allowed to equilibrate to 23 °C. To 500 μ L of lysate was added 5 μ L of (*R*)- or (*S*)-Ox32yne (200 μ M) and incubated at 23 °C for 1 hour. Excess oxaziridine was then quenched by addition of 5 μ L *N*-acetyl methionine (final concentration 200 μ M) and incubation at 23 °C for 30 minutes. 10 μ L of isotopically light isoDTB-N₃ (1.2 eq) (Vector labs; CCT-1565) tag was added to samples labeled with (*R*)-Ox32yne while 10 μ L isotopically heavy isoDTB-N₃ (1.2 eq) (Vector labs; CCT-1565) tag was added to samples labeled with (*S*)-Ox32yne. 30 μ L TBTA (stock in 1:4 DMSO:BuOH, 100 μ M), 10 μ L TCEP (1 mM), and 10 μ L CuSO₄ (1 mM) were added and proteins were unfolded by addition of 50 μ L 1.2% SDS in PBS (0.1% w/v). CuAAC was allowed to proceed at 23 °C for 1 hour with rocking. Light and heavy samples were combined in a 1:1 ratio and co-precipitated by addition of 4 volumes of MeOH:CHCl₃ (4:1.5), vortexed, and centrifuged at 12,000 xg for 15 minutes. The collected protein pellet was washed 1X with 1 mL chilled (-20 °C) MeOH via probe-tip sonication (5 s on, 20 s off, 20% A, 4-6 pulses) on ice and transferred to a 2 mL microcentrifuge tube and centrifuged at 4 °C for 5 minutes at 6,500 xg. The resulting pellet was resuspended in 500 μ L 1.2% SDS/PBS via probe-tip sonication (5 s on, 20 s off, 20% A, 6-8 pulses). Samples were then incubated at 90 °C for 5 minutes, cooled to 23 °C, then centrifuged for 5 minutes at 6,500 xg. High-capacity streptavidin agarose beads (200 μ L per sample) (Thermo Fisher Scientific; 20357) were washed 3X in 1 mL PBS. The washed beads were then transferred by 2X 250 μ L resuspension in PBS to a 15 mL conical tube containing 5 mL PBS (final volume 5.5 mL). The 500 μ L resuspended sample was added to the conical tube containing beads and allowed to incubate at 4 °C overnight while rotating.

The following day, samples were allowed to rock at 23 °C for 30 minutes to resolubilize the SDS. The samples were centrifuged at 1,400 xg for 3 minutes and the supernatant was removed. The beads were then washed with 5 mL 0.2% SDS/PBS (w/v), incubated

for 10 minutes on a rotator, and centrifuged for 3 minutes at 1,400 xg. The beads were then transferred to micro bio-spin columns (Bio-Rad; 7326204) by 2X 1 mL resuspension in PBS, placed on a vacuum manifold, and washed thoroughly by 5X 1 mL PBS washes and 5X 1 mL MQ washes. The beads were then transferred to low-retention 2.0 mL microcentrifuge tubes by 2X 250 μ L resuspension in 6 M urea/PBS and incubated with 25 μ L TCEP (110 mM) at 65 °C for 20 minutes, with gentle agitation every 10 minutes. Then, 25 μ L iodoacetamide (400 mM) was added and incubated at 37 °C for 30 minutes in the dark. The beads were washed 2X by addition of 1 mL PBS and centrifuged at 1,400 xg for 3 minutes. The supernatant was removed, and a premixed solution of 200 μ L 2 M urea/PBS, 2 μ L CaCl_2 (100 mM), and 8 μ L Trypsin/LysC solution (20 μ g lyophilized Trypsin/LysC reconstituted in 40 μ L Trypsin/LysC buffer) (Promega; V5073) was added and incubated at 37 °C overnight.

The following day, the beads were transferred to micro bio-spin columns (Bio-Rad; 7326204) by 2X 500 μ L resuspension in PBS, placed on a vacuum manifold, and washed thoroughly by 5X 1 mL PBS washes and 5X 1 mL MQ washes. The bio-spin columns were capped at the bottom, placed in fresh low-retention 2.0 mL microcentrifuge tubes, and peptides were eluted from beads via addition of 200 μ L LC-MS grade 50% ACN/ H_2O + 0.1% FA (v/v) and incubated for 5 minutes. The cap was removed, and samples were centrifuged at 3000 xg for 3 minutes to collect the eluent. The elution step was repeated once more, and elution fractions containing the same sample were combined. The eluent was dried via SpeedVac concentration. Peptides were reconstituted in 100 μ L LC-MS grade 50% ACN/ H_2O + 0.1% FA (v/v) via bath sonication for 10 minutes and quantified using Pierce fluorometric assay kit (Thermo; 23290).

isoTOP-ABPP: Hyper-reactivity was assessed as previously described (16). 500 μ L of desired proteome was normalized to 4.0 mg/mL and allowed to equilibrate to 23 °C. To 500 μ L of lysate was added 5 μ L of racemic Ox32yne (50 or 500 μ M final) and incubated at 23 °C for 1 hour. Excess oxaziridine was then quenched by addition of 5 μ L *N*-acetyl methionine (50 μ M or 500 μ M final) and incubation at 23 °C for 30 minutes. 10 μ L of isotopically light isoDTB- N_3 (1.2 eq) (Vector labs; CCT-1565) tag was added to samples labeled with 500 μ M racemic Ox32yne while 10 μ L isotopically heavy isoDTB- N_3 (1.2 eq) (Vector labs; CCT-1565) tag was added to samples labeled with 50 μ M racemic Ox32yne. 30 μ L TBTA (stock in 1:4 DMSO: tBuOH , 100 μ M), 10 μ L TCEP (1 mM), and 10 μ L CuSO_4 (1 mM) were added and proteins were unfolded by addition of 50 μ L 1.2% SDS in PBS (0.1% w/v). CuAAC was allowed to proceed at 23 °C for 1 hour with rocking. Light and heavy samples were combined in a 1:1 ratio and co-precipitated by addition of 4 volumes of $\text{MeOH}:\text{CHCl}_3$ (4:1.5), then vortexed and centrifuged at 12,000 xg for 15 minutes. The collected protein pellet was washed 1X with 1 mL chilled (-20 °C) MeOH via probe-tip sonication (5 s on, 20 s off, 20% A, 4-6 pulses) on ice and transferred to a 2 mL microcentrifuge tube and centrifuged at 4 °C for 5 minutes at 6,500 xg. The resulting pellet was resuspended in 500 μ L 1.2% SDS/PBS via probe sonication (5 s on, 20 s off, 20% A, 6-8 pulses). Samples were then incubated at 90 °C for 5 minutes, cooled to 23 °C, then centrifuged for 5 minutes at 6,500 xg. High-capacity streptavidin agarose beads (200 μ L per sample) (Thermo Fisher Scientific; 20357) were washed 3X in 1 mL PBS. The washed beads were then transferred by 2X 250 μ L resuspension in PBS to a 15 mL conical tube

containing 5 mL PBS (final volume 5.5 mL). The 500 μ L resuspended sample was added to the conical tube containing beads and allowed to incubate at 4 °C overnight while rotating.

The following day, samples were allowed to rock at 23 °C for 30 minutes to resolubilize the SDS. The samples were centrifuged at 1,400 xg for 3 minutes and the supernatant was removed. The beads were then washed with 5 mL 0.2% SDS/PBS (w/v), incubated for 10 minutes on a rotator, and centrifuged for 3 minutes at 1,400 xg. The beads were then transferred to micro bio-spin columns (Bio-Rad; 7326204) by 2X 1 mL resuspension in PBS, placed on a vacuum manifold, and washed thoroughly by 5X 1 mL PBS washes and 5X 1 mL MQ washes. The beads were then transferred to low-retention 2.0 mL microcentrifuge tubes by 2X 250 μ L resuspension in 6 M urea/PBS and incubated with 25 μ L TCEP (110 mM) at 65 °C for 20 minutes, with gentle agitation every 10 minutes. Then, 25 μ L iodoacetamide (400 mM) was added and incubated at 37 °C for 30 minutes in the dark. The beads were washed 2X by addition of 1 mL PBS and centrifuged at 1,400 xg for 3 minutes. The supernatant was removed, and a premixed solution of 200 μ L 2 M urea/PBS, 2 μ L CaCl_2 (100 mM), and 8 μ L Trypsin/LysC solution (20 μ g lyophilized Trypsin/LysC reconstituted in 40 μ L Trypsin/LysC buffer) (Promega; V5073) was added and incubated at 37 °C overnight while rotating.

The following day, the beads were transferred to micro bio-spin columns (Bio-Rad; 7326204) by 2X 500 μ L resuspension in PBS, placed on a vacuum manifold, and washed thoroughly by 5X 1 mL PBS washes and 5X 1 mL MQ washes. The bio-spin columns were capped at the bottom, placed in fresh low-retention 2.0 mL microcentrifuge tubes, and peptides were eluted from beads via addition of 200 μ L LC-MS grade 50% ACN/ H_2O + 0.1% FA (v/v) and incubated for 5 minutes. The cap was removed and samples were centrifuged at 3000 xg for 3 minutes to collect the eluent. The elution step was repeated once more, and elution fractions containing the same sample were combined. The eluent was dried via SpeedVac concentration. Peptides were reconstituted in 100 μ L LC-MS grade 50% ACN/ H_2O + 0.1% FA (v/v) via bath sonication for 10 minutes and quantified using Pierce fluorometric assay kit (Thermo; 23290).

ReDiMe of LFD and HFD mouse liver tissue: Adapted from Hsu et. al (205), Bak et. al (206). 50 μ L of desired proteome (with no treatment, for protein abundance normalization) was normalized to 4.0 mg/mL and allowed to equilibrate to 23 °C. Proteins were precipitated by addition of 4 volumes of MeOH: CHCl_3 (4:1.5), vortexed, and centrifuged at 12,000 xg for 15 minutes. The collected protein pellet was washed 1X with 500 μ L chilled (-20 °C) MeOH via probe-tip sonication (5 s on, 20 s off, 20% A, 4-6 pulses) on ice and transferred to a 2 mL low-retention microcentrifuge tube and centrifuged at 4 °C for 5 minutes at 6,500 xg. The resulting pellet was resuspended in 100 μ L 6 M urea/PBS via probe-tip sonication (5 s on, 20 s off, 20% A, 6-8 pulses). Samples were then incubated at 60 °C for 20 minutes, cooled to 23 °C, then centrifuged for 5 minutes at 6,500 xg. Samples were then incubated with 5 μ L TCEP (110 mM) at 65 °C for 20 minutes, with gentle agitation every 10 minutes. Then, 5 μ L iodoacetamide (400 mM) was added and incubated at 37 °C for 30 minutes in the dark. The reaction was quenched by addition of 550 μ L ammonium bicarbonate (50 mM). To this sample, 6 μ L Trypsin/LysC solution (20

µg lyophilized Trypsin/LysC reconstituted in 40 µL Trypsin/LysC buffer) (Promega; V5073) was added and incubated at 37 °C overnight while rotating. The following day, samples were dried via SpeedVac concentration. Peptides were reconstituted in 100 µL LC-MS grade 50% ACN/H₂O + 0.1% FA (v/v) via bath sonication for 10 minutes and quantified using Pierce fluorometric assay kit (Thermo; 23290). 30 µg of peptide was aliquoted in a fresh 2.0 mL low-retention microcentrifuge tube and dried via SpeedVac concentration. Samples were reconstituted in 100 µL TEAB (100 mM) via bath sonication for 15 minutes. To the LFD mouse liver sample was added 4 µL of 4% (v/v) formaldehyde for isotopically light labeling. To the HFD mouse liver sample was added 4 µL of 4% (v/v) CD₂O for isotopically heavy labeling. Then, 4 µL of NaBH₃CN (600 mM) was added to each sample and incubated in a fumehood for 30 minutes at 23 °C while rocking (1000 rpm). The reaction was quenched by addition of 16 µL of 1% (v/v) ammonia solution. 20 µL of formic acid was added to further quench the reaction and acidify the samples. The differentially labeled samples were then combined and dried via SpeedVac concentration. Samples were then reconstituted in 300 µL MQ + 0.1% FA via bath sonication for 15 minutes. Samples were then desalted and high-pH offline fractionated using Pierce RP peptide fractionation kit (Thermo; 84868). The resulting fractions were dried via SpeedVac concentration, reconstituted in LC-MS grade H₂O + 0.1% FA (v/v) via bath sonication for 15 minutes, and quantified using Pierce colorimetric assay kit (Thermo; 23275).

LC-MS/MS data collection: Peptides from all experiments were analyzed on a Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo; IQLAAEGAAPFALGMBDK). Peptides from all experiments were reconstituted in LC-MS grade H₂O + 0.1% FA (v/v) via bath sonication for 15 minutes for a final peptide concentration of 50 ng/µL. Approximately 500 ng of peptide was injected and separated via liquid chromatography using a VanquishNeo UHPLC (Thermo; VN-S10-A-01) in Trap-and-Elute configuration using a PepMap Neo (5 µM x 300 µM x 5 mm) trap (Thermo; 174500) and an Aurora Ultimate 25 cm C18 analytical column (IonOpticks; AUR3-25075C18) operating at a 350 nL/min flow rate at a column temperature of 50 °C. Peptides were eluted over a 120 minute gradient from 0-80% H₂O/ACN + 0.1% FA and data were collected in positive-ion mode using data-dependent acquisition mode with a default charge state of +2. MS1 was operated at 70,000 mass resolution, 1e6 AGC target, 30 ms maximum injection time, at a scan range of 400 – 1800 m/z. One MS1 scan was followed by 15 MS2 scans of the nth most abundant ions operated at 17,500 mass resolution, 5e4 AGC target, 50 ms maximum injection time, 27 (N)CE, with an isolation window of 1.5 m/z. A 30 s dynamic exclusion window was enabled. Nanospray voltage was set at 2.50 kV and heated capillary temperature at 200 °C.

Byos analysis: Shotgun data of purified proteins were analyzed with Byologic (Protein Metrics Inc.). Raw files were searched directly against the FASTA sequences of the respective protein pulled from Uniprot 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), oxidation (+15.994915; M; variable - common1) and Ox32yne labeling (+168.08988; M; variable - common1).

ChURRO-ABPP and isoTOP-ABPP Fraggpipe analysis: All samples were analyzed using Fraggpipe v21.1, MSFragger v4.1, IonQuant v1.10.27, Philosopher v.5.1.0, and Python v3.9.12. Raw files were directly ported into the Fraggpipe GUI under 'Workflow'. Proteomes were directly downloaded from Uniprot database (human; UP000005640, mouse; UP000005589). The default workflow was used with the following modifications.

MSFragger: Protein digestion was set to enzyme 1: trypsin and enzyme 2: lysc. Variable modifications included oxidation (M, +15.9949, max occurrences 2), *N*-terminal acetylation ([^, +42.0106, max occurrences 1), light Ox32yne adduct (M, +649.3660, max occurrences 2), and heavy Ox32yne adduct (M, +655.3735, max occurrences 2). A fixed carbamidomethyl modification (C, +57.021465) was enabled.

Validation: Run validation tools was enabled. Run MSBooster, predict RT, predict spectra was enabled. Run PSM validation, and run Percolator was enabled. Run ProteinProphet was enabled. Generate reports, remove contaminants, generate peptide-level summary, and generate protein-level summary was enabled.

Quant (MS1): Run MS1 quant was enabled. IonQuant Labeling was enabled with light modification of M649.3660 and heavy modification of M655.3735 and re-quantify enabled. Normalized intensity across runs was enabled.

After running the search, statistical significance was calculated using a two-tailed t-test of unequal variance with the first array set as the computed $\log_2(H/L)$ ratios across multiple technical replicates ($n = 12$), and the secondary array centered at $\{0,0\}$.

ReDiMe Fraggpipe analysis: All samples were analyzed using Fraggpipe v21.1, MSFragger v4.1, IonQuant v1.10.27, Philosopher v.5.1.0, and Python v3.9.12. Raw files were directly ported into the Fraggpipe GUI under 'Workflow'. Proteomes were directly downloaded from Uniprot database (mouse; UP000005589). The default workflow was used with the following modifications.

MSFragger: Protein digestion was set to enzyme 1: trypsin and enzyme 2: lysc. Variable modifications included *N*-terminal acetylation ([^, +42.0106, max occurrences 1), light dimethyl adduct (n^K, +28.0313, max occurrences 2), and heavy dimethyl adduct (n^K, +32.0564, max occurrences 2). A fixed carbamidomethyl modification (C, +57.021465) was enabled.

Validation: Run validation tools was enabled. Run MSBooster, predict RT, predict spectra was enabled. Run PSM validation, and run Percolator was enabled. Run ProteinProphet was enabled. Generate reports, remove contaminants, generate peptide-level summary, and generate protein-level summary was enabled.

Quant (MS1): Run MS1 quant was enabled. IonQuant Labeling was enabled with light modification of Kn28.0313 and heavy modification of Kn32.0564 and re-quantify enabled. Normalized intensity across runs was enabled.

After running the search, statistical significance was calculated using a two-tailed t-test of unequal variance with the first array set as the computed $\log_2(H/L)$ ratios across multiple technical replicates ($n = 8$ technical replicates), and the secondary array centered at $\{0,0\}$.

4.8.12 Computational methods

Ramachandran analysis: For each amino acid residue, the ϕ (phi) and ψ (psi) dihedral angles were extracted from their protein's predicted AlphaFold structure. The AlphaFold structures were queried from the Alpha Fold Protein Structure Database (183, 207) using the python package StructureMap (<https://github.com/MannLabs/structuremap>), adapted from Bludau et. al (208). The BioPython package (209) was then used to parse the queried CIFs and extract the psi and phi angles of the amino acids of interest. The dihedral angles of the modified methionine residues were then plotted and intensities were calculated using kernel density estimation to construct Ramachandran plots. The source code for replicating this analysis, along with detailed instructions, are available at https://github.com/ritster/ChURRO_ABPP.

Crowdedness analysis: Amino acid crowdedness (pPSE) predictions were done using StructureMap (<https://github.com/MannLabs/structuremap>), adapted from Bludau et. al (208) and White et. al (186). In short, for each amino acid residue, a spherical region is projected from the β -carbon (or the α -carbon for glycine) at various radii (4-8 Å). Within each sphere, the number of α -carbon atoms from neighboring amino acids is counted, considering the tolerance of the predicted alignment error as reported by AlphaFold. This count is referred to as the pPSE (proximal PSE) value, which reflects the crowdedness/accessibility of the local environment around each amino acid. The source code for replicating this analysis, along with detailed instructions, are available at https://github.com/ritster/ChURRO_ABPP.

Subcellular localization: An in-house python script was used to search for subcellular localization using subcellular localization data pulled from the Uniprot database and employing regex patterning with 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.

PANTHER GO: Biological activity was determined via “PANTHER GO biological process complete” analysis (210–212) using Uniprot IDs of all statistically significant protein hits and plotted based if a biological process was deemed statistically significant and > 25% enriched.

AlphaMissense: The public AlphaMissense database was used to determine predicted mutation outcomes from mitochondrial datasets. Briefly, mouse protein Met sites were aligned to their human isoforms and the human Met site information was used to map mutation outcome.

https://console.cloud.google.com/storage/browser/dm_alphamissense;tab=objects?prefix=&forceOnObjectsSortingFiltering=false&pli=1

DFT: DFT calculations were conducted at the Molecular Graphics and Computational Facility (MGCF) at the University of California, Berkeley, using the Gaussian 16 software package. All calculations, including geometry optimizations, were performed in H₂O solvent using the SMD solvent continuum model reported by Truhlar and co-workers (scrf=smd) (213). Initial geometries were constructed in GaussView16 and subjected to conformational searches using Maestro (Schrodinger). Conformers within a 5 kcal / mol window were subsequently optimized to stationary points (either minima or first-order saddle points) using the M06-2X functional and the basis set 6-31G(d,p). The nature of each stationary point was evaluated by accompanying frequency calculations (all positive eigenvalues for minima and exactly one negative eigenvalue for transition states). Several isomers were considered, but only those representing the minimum-energy paths are presented here. Transition states were connected to ground states by following the intrinsic reaction coordinate (IRC) downhill from the transition state. All other parameters were left as their defaults unless otherwise stated.

4.8.13 Energies of optimized geometries

structure	energy in kcal	Delta G (R)	Delta G (S)
methionine	-622532.5111		
(R)-Ox32yne	-575332.7136		
(S)-Ox32yne	-575332.711		
R SMs	-1197865.225	0.00	
S SMs	-1197865.222		0.00
R_sS TS1	-1197841.608	23.62	
R_sR TS1	-1197839.958	25.27	
S_sR TS1	-1197836.37		28.85
S_sS TS1	-1197841.263		23.96
R_sR int1	-1197870.053	-4.83	
S_sR int1	-1197876.253		-11.03
R_sS int1	-1197871.261	-6.04	
S_sS_int1	-1197875.102		-9.88
benzaldehyde	-216710.2069		
sR sulfilimine	-981174.3494		
sS sulfilimine	-981178.2042		
sR pdts	-1197884.556	-19.33	
sS pdts	-1197888.411		-23.19

4.8.14 XYZ coordinates of optimized geometries

N-acetyl-L-methionine methyl ester

N	0.65411900	1.28373700	-0.63063100
H	0.67394800	1.50072700	-1.62215700
C	0.20752600	-0.03821200	-0.25997900
H	-0.22647500	-0.01087900	0.74624800
C	-0.86693200	-0.52788700	-1.23471700
C	1.37210700	-1.02085700	-0.20030600
H	-0.44932300	-0.54164600	-2.24861000
H	-1.12348200	-1.55819400	-0.97942900
C	-2.12242000	0.33996300	-1.23939900
O	2.52750200	-0.74691000	-0.44520300
H	-1.90358500	1.34430500	-1.61079500
H	-2.86359700	-0.10785400	-1.90675500
S	-2.88192900	0.58957700	0.39883800
C	-3.08367700	-1.13970500	0.91043700
H	-2.11972500	-1.61318600	1.11260800
H	-3.67292400	-1.13710400	1.82885200
H	-3.62096700	-1.70526300	0.14567900
C	1.39008400	2.11387000	0.15180500
O	1.90607900	3.12942500	-0.32064700
C	1.50547500	1.76242900	1.61243800
H	0.51396600	1.67082700	2.06495300
H	2.02875800	0.81108300	1.74297300
H	2.06385000	2.55160300	2.11320800
O	0.96357100	-2.22908100	0.18730600
C	1.99157200	-3.22648400	0.30399000
H	1.48659400	-4.13253000	0.63139400
H	2.47201100	-3.38397500	-0.66284300
H	2.73335600	-2.91347100	1.04043000

(R)-Ox32yne

O	-0.75286600	-2.01503000	-1.28781200
C	-0.73823000	-1.62652100	-0.12372400
N	-1.49884200	-0.67429500	0.41967100
C	-2.54546200	0.07552400	-0.30826300
C	-3.47507900	-0.88285900	-1.05975100
H	-4.35977100	-0.33097900	-1.38772200
H	-2.99422800	-1.32019100	-1.93542800
H	-3.79658200	-1.68899300	-0.39502900
C	-3.34987100	0.84248800	0.73808100
H	-3.87264800	0.14228700	1.39580600
H	-4.09182300	1.46895400	0.23660600
H	-2.70751300	1.48497300	1.34422400

C	-1.91757400	1.03797400	-1.32715400
O	-1.33396900	2.20605600	-0.77154300
C	-0.04264300	2.04045400	-0.20766500
C	-0.08010900	1.93413100	1.26165500
C	-0.12016800	1.85755000	2.46610100
H	-0.15078100	1.78926800	3.53395200
H	0.54989300	2.92037600	-0.47967100
H	0.45425600	1.15664300	-0.62800000
H	-1.18036600	0.49685600	-1.93604000
H	-2.71414200	1.39861400	-1.98479100
H	-1.28815600	-0.39588600	1.37193500
N	0.07591300	-2.38132600	0.80051000
O	0.50089800	-1.59383500	1.95780400
C	1.43868600	-1.96146700	0.97130200
C	2.06291400	-0.85994800	0.18326000
C	2.59131100	0.23478800	0.86888600
C	3.21671400	1.25913800	0.16276500
C	3.31959000	1.18632000	-1.22581900
C	2.80694800	0.08378700	-1.90794000
C	2.18390000	-0.94560900	-1.20558300
H	1.78240400	-1.80612900	-1.73050600
H	2.89178300	0.02362100	-2.98787700
H	3.80212100	1.98756600	-1.77612200
H	3.61819100	2.11493300	0.69569800
H	2.49824600	0.28743900	1.94934100
H	2.10155500	-2.77202700	1.26786000

(S)-Ox32yne

O	0.75286000	-2.01424800	-1.28854500
C	0.73805400	-1.62625400	-0.12427900
N	1.49877100	-0.67441400	0.41970000
C	2.54560300	0.07554400	-0.30781400
C	3.34968600	0.84243500	0.73880000
H	4.09191500	1.46876500	0.23757200
H	2.70722300	1.48502000	1.34471400
H	3.87211900	0.14215800	1.39670900
C	3.47552200	-0.88278600	-1.05903200
H	2.99499600	-1.32011700	-1.93488500
H	3.79681600	-1.68892600	-0.39422100
H	4.36029700	-0.33086300	-1.38669100
C	1.91814400	1.03800900	-1.32691200
O	1.33401800	2.20602000	-0.77168000
C	0.04275500	2.04003400	-0.20777100
C	0.08027600	1.93346000	1.26151000

C	0.12037100	1.85704500	2.46595900
H	0.15097500	1.78868800	3.53381100
H	-0.45407400	1.15629400	-0.62836800
H	-0.54992600	2.91995600	-0.47948100
H	2.71505300	1.39873300	-1.98406600
H	1.18138800	0.49680500	-1.93626900
H	1.28783000	-0.39620300	1.37198700
N	-0.07620800	-2.38157800	0.79933800
O	-0.50143400	-1.59499400	1.95707200
C	-1.43905800	-1.96217900	0.97020300
C	-2.06323600	-0.86014400	0.18290100
C	-2.18462700	-0.94498100	-1.20592500
C	-2.80743500	0.08505400	-1.90755600
C	-3.31941200	1.18742600	-1.22469100
C	-3.21605500	1.25945900	0.16389800
C	-2.59093100	0.23445300	0.86927800
H	-2.49741700	0.28652700	1.94973000
H	-3.61701500	2.11513600	0.69740200
H	-3.80177200	1.98920400	-1.77436100
H	-2.89251500	0.02548200	-2.98749800
H	-1.78350700	-1.80529000	-1.73148500
H	-2.10182000	-2.77309500	1.26593900

R_sR_TS1

O	-1.70246400	-1.34129800	-1.84486900
C	-1.89360900	-1.63528100	-0.66984000
N	-3.03841200	-1.47421900	0.00094000
C	-4.27074100	-0.87188400	-0.54215000
C	-4.80240600	-1.70201400	-1.71137300
H	-5.73470600	-1.26428700	-2.07853100
H	-4.08399100	-1.73588300	-2.53254200
H	-5.00659900	-2.72252800	-1.37784200
C	-5.28185300	-0.85772300	0.60173000
H	-5.49261200	-1.87873500	0.93245800
H	-6.21497200	-0.40444200	0.25911300
H	-4.90477500	-0.28217200	1.45129000
C	-4.00501600	0.55495400	-1.02986600
O	-3.49652300	1.33206400	0.04112800
C	-3.22192700	2.66307700	-0.36039700
C	-2.50902100	3.35391500	0.71931800
C	-1.88337900	3.91326000	1.58648300
H	-1.34032300	4.42176000	2.35685600
H	-4.15358900	3.19375500	-0.59885800
H	-2.59442600	2.66755100	-1.26393200

H	-3.28836700	0.55010500	-1.86186600
H	-4.95292700	0.97863900	-1.39075700
H	-3.04373300	-1.76543300	0.97115700
N	-0.84912900	-2.19047900	0.20721400
O	-0.75097000	-3.84696000	-0.71014700
C	0.17593900	-2.84956300	-0.58988100
C	1.43062200	-3.13191100	0.19571800
C	2.60174100	-2.43606300	-0.10385900
C	3.75876800	-2.65209500	0.64220100
C	3.74837300	-3.57892600	1.68305400
C	2.58133800	-4.28638400	1.97562500
C	1.42407300	-4.06229700	1.23481000
H	0.51085300	-4.60904900	1.45033900
H	2.57604000	-5.01223700	2.78274100
H	4.64826300	-3.75401200	2.26414000
H	4.66439100	-2.09952000	0.40924200
H	2.60673500	-1.72191600	-0.92428700
H	0.41735500	-2.31268900	-1.51907900
S	-0.03728600	-0.25535500	0.96641300
C	-0.26408200	1.03672900	-0.29837000
C	-1.31197000	0.17231200	2.17178600
C	0.97750600	1.11844100	-1.17422100
H	-0.45581500	1.97976100	0.22312900
H	-1.14584900	0.78830500	-0.89270900
H	-2.24640700	0.40408800	1.65968400
H	-1.43052900	-0.69139800	2.82836400
H	-0.97717200	1.03239900	2.75569500
C	2.14189400	1.83723300	-0.46442100
H	0.73033500	1.68140400	-2.08041200
H	1.29097100	0.11712100	-1.48966700
N	1.85849400	3.24208100	-0.27942400
H	2.33344900	1.36865700	0.50492000
C	3.40289100	1.66954000	-1.29664600
H	1.77886000	3.79944700	-1.12435800
C	1.52341100	3.87926700	0.86957800
O	3.90858100	2.53871900	-1.97203900
O	3.85767900	0.42146800	-1.21971400
O	1.17939900	5.06421400	0.84712400
C	1.61171100	3.10622400	2.16168200
C	5.00192400	0.11478100	-2.03296800
H	1.36887900	3.78253100	2.97972600
H	0.90798800	2.26828100	2.17061300
H	2.61756000	2.70305900	2.30610200
H	5.19444800	-0.94545600	-1.88112400

H	4.78007900	0.31737900	-3.08177700
H	5.85774100	0.71031700	-1.71136800

R_sS_TS1

O	-0.00549200	-2.75687300	1.30268000
C	0.01748300	-1.53644600	1.22309200
N	-0.95069700	-0.69154300	1.59284200
C	-2.29850900	-1.07080500	2.06364600
C	-2.24428400	-2.14741800	3.14729700
C	-2.93477100	0.19571600	2.63215600
C	-3.11111700	-1.58501700	0.87367300
O	-3.12306500	-0.58367300	-0.13401400
C	-4.10440700	-0.78937300	-1.13352600
C	-3.92449800	-2.05059300	-1.87300100
C	-3.76742200	-3.08855200	-2.46803800
N	1.16853400	-0.81553200	0.63308500
O	2.02458200	-0.47652000	2.27484300
C	2.41040200	-1.27490200	1.22906300
C	3.62786800	-0.87590100	0.43336900
C	3.94999800	0.47094600	0.25832500
C	5.05620600	0.83305600	-0.50469100
C	5.85119000	-0.15138600	-1.09258000
C	5.53719400	-1.49701400	-0.91479700
C	4.42748900	-1.85723300	-0.15223200
S	1.08786100	-1.64285900	-1.45269400
C	0.84886000	0.03054300	-2.12625000
C	-0.52233100	-2.39791000	-1.78092200
C	-0.45756400	0.66999200	-1.64483800
C	-0.31085300	2.10623300	-1.12522100
N	0.69276400	2.18412900	-0.09023900
C	-1.67055700	2.51195300	-0.55523300
C	0.99462800	3.37492100	0.47587400
O	-1.89890100	2.70795200	0.61930400
O	-2.59903300	2.57654300	-1.50734600
O	0.54311000	4.43296200	0.02721900
C	1.92972400	3.33820000	1.65623900
C	-3.93096700	2.86969600	-1.05210000
H	-0.78818400	0.29537600	1.42077400
H	-3.24518500	-2.27075700	3.56930900
H	-1.90753100	-3.10783800	2.75776500
H	-1.56926300	-1.83457000	3.94833600
H	-3.95258800	-0.02837900	2.96051700
H	-2.36191300	0.55295500	3.49249200
H	-2.97573100	0.98767000	1.88120900

H	-2.66415900	-2.51001700	0.48210700
H	-4.13594000	-1.80338900	1.20517100
H	-5.10837800	-0.77346600	-0.68838200
H	-4.02121600	0.05472000	-1.82437500
H	-3.63227000	-4.00718900	-3.00090400
H	2.41667700	-2.35503900	1.43591900
H	3.33572100	1.23149600	0.72893000
H	5.30212900	1.88194900	-0.63732600
H	6.71564700	0.13100300	-1.68496200
H	6.15600200	-2.26539700	-1.36689100
H	4.17674300	-2.90547600	-0.00986800
H	1.73499300	0.57574300	-1.78784800
H	0.88991000	-0.04049100	-3.21483000
H	-0.70368700	-2.38468300	-2.85837900
H	-0.47510100	-3.42688600	-1.42317500
H	-1.32040200	-1.86336700	-1.26413300
H	-1.19269300	0.66905200	-2.45214700
H	-0.89473100	0.08808600	-0.83099900
H	-0.06482300	2.79559300	-1.93905400
H	1.00257200	1.31906200	0.35108700
H	1.59185400	4.07127600	2.39008200
H	1.97802300	2.34879600	2.11524700
H	2.93116100	3.62751800	1.32364200
H	-4.55725300	2.84962100	-1.94134300
H	-4.25528900	2.11185700	-0.33539900
H	-3.95725500	3.85617700	-0.58658400

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O	-1.38036100	-0.93107900	0.10181300
C	-1.79542800	0.15945600	0.48749400
N	-3.01279100	0.39437700	0.97815500
C	-4.13783900	-0.55292100	0.86457800
C	-3.93580700	-1.73413400	1.81140500
C	-5.39687100	0.22751600	1.23375800
C	-4.23896000	-1.06520000	-0.57881800
O	-4.18014400	0.04632300	-1.45868800
C	-3.92918200	-0.32058400	-2.80220800
C	-2.54380900	-0.78071100	-3.01613000
C	-1.40839900	-1.15401100	-3.18866900
N	-0.96248600	1.36751300	0.50527400
O	-1.50880700	1.87292300	-1.23957300
C	-0.27634700	1.50769100	-0.77533900
C	0.79778400	2.56483700	-0.76243800
C	2.06828500	2.28416000	-1.26410000

C	3.05692300	3.26851500	-1.26356800
C	2.77270200	4.53695600	-0.76565500
C	1.49712700	4.82489100	-0.27523700
C	0.51163500	3.84416600	-0.27895800
S	0.36641600	0.85241300	2.20526900
C	0.81353500	-0.90793900	2.44582800
C	-0.93687800	0.98745200	3.44935900
C	2.19778500	-1.21672400	1.88840500
C	2.27945300	-0.97452400	0.38114400
N	1.33972400	-1.78464100	-0.36925400
C	3.66718800	-1.13640300	-0.22781100
C	1.57762200	-3.09844000	-0.57199000
O	3.87769600	-1.04485200	-1.41984600
O	4.62121700	-1.32006500	0.67804400
O	2.66180200	-3.61340500	-0.27375500
C	0.46918500	-3.87560000	-1.23120100
C	5.95271500	-1.44886000	0.15449000
H	-3.23483400	1.36793200	1.14909700
H	-4.74474200	-2.45818300	1.67699400
H	-2.98433200	-2.23429000	1.61602500
H	-3.94996900	-1.38842700	2.84852900
H	-6.26020700	-0.44146200	1.21149500
H	-5.30564100	0.64379400	2.24109200
H	-5.56795700	1.04438900	0.52717700
H	-3.42182500	-1.76161700	-0.79470300
H	-5.19259800	-1.59829700	-0.69767700
H	-4.62638800	-1.10254800	-3.13038700
H	-4.11108300	0.57054200	-3.40818700
H	-0.40057300	-1.47967700	-3.34758500
H	0.11316500	0.56346700	-1.18673700
H	2.28461400	1.29412200	-1.66134900
H	4.04418600	3.04349400	-1.65409900
H	3.54039200	5.30422200	-0.76291000
H	1.27385500	5.81641900	0.10566900
H	-0.48650900	4.06266200	0.08902000
H	0.79307700	-1.07337100	3.52623600
H	0.03200700	-1.51842200	1.98289900
H	-0.47932700	0.98970100	4.43951600
H	-1.45764200	1.93004100	3.27731500
H	-1.63215900	0.15075500	3.35437500
H	2.94765500	-0.60353300	2.39448800
H	2.42447000	-2.26633900	2.09944000
H	2.02928100	0.07447500	0.17694000
H	0.38630100	-1.43693900	-0.45521800

H	-0.48996900	-3.35678300	-1.17744100
H	0.39173000	-4.85309000	-0.75306300
H	0.73082300	-4.03085400	-2.28181700
H	6.00711300	-2.31436600	-0.50808600
H	6.23073000	-0.54540600	-0.39033300
H	6.59683400	-1.58805100	1.01974700

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O	1.25219900	-1.84412600	-1.23755600
C	1.94150300	-1.66875100	-0.23523600
N	3.17798300	-1.16452300	-0.24505900
C	3.83689300	-0.59737500	-1.43333000
C	5.17202300	-0.04084700	-0.94190300
C	4.07186800	-1.67001000	-2.49773700
C	2.99347500	0.52816900	-2.03582900
O	2.78295900	1.53128700	-1.05458700
C	2.17333200	2.69068700	-1.58510600
C	0.75745900	2.48012900	-1.93308100
C	-0.40109100	2.31033900	-2.22744100
N	1.42620800	-2.12986900	1.06459400
O	2.87937300	-1.85947700	2.25524500
C	1.65969000	-1.25468100	2.21801800
C	1.66984200	0.25010000	2.03564300
C	2.84993200	0.95903800	2.26281500
C	2.86990100	2.34779000	2.14804300
C	1.70976500	3.03573400	1.79749900
C	0.52766700	2.33152000	1.56922700
C	0.50615300	0.94520100	1.70349600
S	-0.81439000	-2.40840000	0.79408100
C	-1.68580400	-1.87101100	-0.71624200
C	-0.51950600	-4.12414900	0.32718500
C	-2.05385100	-0.39769400	-0.67347500
C	-3.07257200	-0.06004500	0.42790300
N	-4.26910700	-0.87167700	0.36222200
C	-3.45601300	1.41845700	0.45400900
C	-5.15497200	-0.73168800	-0.64730900
O	-4.49990700	1.84945800	0.89314000
O	-2.47734600	2.19021000	-0.00753300
O	-4.99571400	0.11660000	-1.53185700
C	-6.34498600	-1.65292300	-0.63394100
C	-2.72293400	3.60467300	0.02053900
H	3.64474500	-1.10940400	0.65442500
H	5.70848300	0.41965700	-1.77515300
H	5.01607200	0.71339800	-0.16572900

H	5.78968900	-0.84585400	-0.53334200
H	4.60966800	-1.23626500	-3.34548000
H	4.67872000	-2.47796400	-2.08089000
H	3.12940100	-2.08667700	-2.85708600
H	3.54293800	0.94993800	-2.88980900
H	2.03297800	0.14275900	-2.39735900
H	2.24634200	3.46492500	-0.81678300
H	2.71618600	3.03809900	-2.47472600
H	-1.42987300	2.16813700	-2.48732600
H	0.95593000	-1.54151400	3.01463000
H	3.75042700	0.41639500	2.53347000
H	3.79142000	2.89133200	2.33130800
H	1.72355200	4.11733100	1.70558000
H	-0.38118400	2.86203100	1.30394400
H	-0.42712200	0.40620500	1.56900700
H	-1.02077600	-2.07499200	-1.55510000
H	-2.57491400	-2.50461400	-0.79805600
H	-1.47475100	-4.61406800	0.13186600
H	0.11518500	-4.14454500	-0.56060500
H	-0.01789300	-4.61401400	1.16091700
H	-1.15152800	0.20676300	-0.54182500
H	-2.48217200	-0.13088100	-1.64369800
H	-2.62796200	-0.24710100	1.41326900
H	-4.42549100	-1.57549200	1.07013400
H	-6.33748100	-2.23955600	-1.55538200
H	-6.35389100	-2.32465200	0.22475300
H	-7.25201100	-1.04454300	-0.63178600
H	-1.83086800	4.06473400	-0.40130200
H	-3.59922500	3.84206000	-0.58487300
H	-2.87908600	3.93766300	1.04809400

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O	2.06926000	0.23751300	1.64108500
C	1.97518900	-0.55538100	0.70616000
N	2.95900900	-1.25204100	0.13612100
C	4.37455200	-1.22545400	0.53389100
C	4.59918200	-2.13411400	1.74266300
H	5.63879600	-2.06827400	2.07740300
H	3.94638300	-1.83561700	2.56814800
H	4.38364600	-3.17292900	1.47965400
C	5.16501100	-1.71652500	-0.67768100
H	4.81508900	-2.70615300	-0.98683400
H	6.22498900	-1.78847000	-0.42246200

H	5.05070200	-1.02516400	-1.51761500
C	4.81855300	0.19372900	0.91106300
O	4.34697200	1.11577100	-0.05742300
C	4.31073300	2.43874200	0.44958500
C	3.64322600	3.30485200	-0.52661700
C	3.06898200	4.00747800	-1.32107200
H	2.56882200	4.63501800	-2.02974600
H	5.32560700	2.80889900	0.64834500
H	3.75182500	2.46432600	1.39567400
H	4.43145600	0.46093600	1.89860300
H	5.91674700	0.21092800	0.95199700
H	2.67179700	-1.81289000	-0.67873800
N	0.67267800	-0.79406100	0.10641100
O	1.12596800	-2.40526300	-1.61464900
C	0.45395700	-2.22519600	-0.48387000
C	-1.06542400	-2.40726300	-0.55911300
C	-1.86112300	-2.23496800	0.57862700
C	-3.23748700	-2.43625000	0.51793700
C	-3.83119100	-2.83079700	-0.68317900
C	-3.04321800	-3.00791500	-1.81802800
C	-1.66524900	-2.79322100	-1.75548000
H	-1.03820900	-2.93239300	-2.62990300
H	-3.50086400	-3.31264100	-2.75424900
H	-4.90319200	-2.99485300	-0.73228200
H	-3.84534800	-2.29084700	1.40719600
H	-1.39737900	-1.93137400	1.51437800
H	0.80541000	-2.82446200	0.38463700
S	0.23694900	0.35775100	-1.03805300
C	0.07796000	1.87614600	-0.05002200
C	1.67975900	0.75380600	-2.04037200
C	-0.96335700	1.69896900	1.04562300
H	-0.20088800	2.64291500	-0.77875100
H	1.06190800	2.10971500	0.36268100
H	2.53137300	0.99820200	-1.40241800
H	1.87363500	-0.12766600	-2.65075400
H	1.39841300	1.60702400	-2.66291000
C	-2.32809800	1.25665400	0.50614400
H	-1.06658200	2.66130800	1.55774700
H	-0.60754800	0.96814100	1.77359500
N	-2.82644600	2.20231800	-0.46212700
H	-2.22017900	0.27490900	0.02726900
C	-3.33958900	1.01371300	1.62485100
H	-2.70972000	3.18661900	-0.23997100
C	-3.79011000	1.93717800	-1.38756100

O	-4.47422200	1.43949300	1.62440700
O	-2.84085800	0.22615500	2.57282000
O	-4.32916100	2.85447100	-2.00721500
C	-4.12281200	0.48987300	-1.63822200
C	-3.75344200	-0.14399200	3.62061200
H	-4.56821800	0.04007400	-0.74572900
H	-4.83190300	0.43449600	-2.46280500
H	-3.22322600	-0.08158800	-1.88915000
H	-3.20084500	-0.82510100	4.26379300
H	-4.06209100	0.74314900	4.17571800
H	-4.62798100	-0.63928100	3.19565800

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O	0.94485200	-1.48844500	-1.60894300
C	1.32904600	-1.39063900	-0.43992800
N	2.59595000	-1.36779100	-0.01783700
C	3.76559700	-1.13362100	-0.87598000
C	4.94164600	-0.88167300	0.06528700
C	4.03787100	-2.34558000	-1.76774600
C	3.53241200	0.09023200	-1.76212100
O	3.20563900	1.20888600	-0.94998500
C	2.72405300	2.29412200	-1.72107600
C	1.34575600	2.06226000	-2.18981400
C	0.21622900	1.84897200	-2.56041900
N	0.37889000	-1.29282300	0.62143400
O	1.72287300	-1.69768700	2.58267500
C	0.85274200	-0.85319100	2.06178200
C	1.33726300	0.59590100	1.88557400
C	2.53058900	0.98748500	2.48886400
C	2.97894500	2.30412000	2.38285200
C	2.22691400	3.24559600	1.68282600
C	1.02841400	2.85987300	1.08070300
C	0.59124500	1.54195700	1.17969900
S	-0.85193700	-2.40535100	0.63460100
C	-1.90468700	-2.03411000	-0.81043600
C	-0.13160200	-3.98110900	0.15354500
C	-2.16356800	-0.54347200	-0.92830800
C	-2.88548500	0.05861400	0.28661100
N	-4.10407000	-0.63427900	0.63864400
C	-3.15904400	1.55435800	0.11815500
C	-5.19184100	-0.57502100	-0.16275500
O	-4.06022400	2.14994200	0.66552500
O	-2.24348100	2.14028200	-0.64774100
O	-5.16841900	0.04166300	-1.23232800

C	-6.42466700	-1.28817700	0.32160300
C	-2.34560300	3.56767300	-0.77304400
H	2.71644700	-1.44843700	0.99981000
H	5.85274200	-0.72092700	-0.51642400
H	4.75810600	0.00073100	0.68490000
H	5.09579300	-1.74507800	0.71957300
H	4.90559400	-2.15466500	-2.40587900
H	4.24906700	-3.22224000	-1.14976300
H	3.17609100	-2.56246700	-2.40317100
H	4.45120200	0.29382800	-2.33019600
H	2.72215000	-0.10748000	-2.47306800
H	2.74723500	3.17779700	-1.07873700
H	3.37833800	2.47992900	-2.58303200
H	-0.78502600	1.67055000	-2.89570400
H	-0.13169500	-0.80386400	2.57808400
H	3.10532200	0.24716200	3.03568200
H	3.91356400	2.59574900	2.85244100
H	2.57291500	4.27144800	1.60219200
H	0.44057600	3.58122100	0.51860700
H	-0.33020700	1.24580400	0.68872400
H	-1.38934100	-2.41534000	-1.69211400
H	-2.81820800	-2.60921200	-0.62991700
H	-0.94717900	-4.70604400	0.10724000
H	0.35665600	-3.87855300	-0.81661500
H	0.57771200	-4.25056800	0.93721600
H	-1.21138000	-0.02838600	-1.07507800
H	-2.77012100	-0.39292800	-1.82474300
H	-2.22632500	0.00353400	1.16444700
H	-4.17161300	-1.09148900	1.53727600
H	-7.21071600	-0.54561600	0.47870900
H	-6.76046700	-1.97167400	-0.46077700
H	-6.25777900	-1.84072800	1.24627700
H	-1.51084300	3.86571200	-1.40488900
H	-3.29532100	3.83350900	-1.23994800
H	-2.27093900	4.03404800	0.21071600

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O	0.37677200	-2.71268700	-0.43469100
C	0.70061100	-1.74166500	0.25063600
N	0.09065800	-1.29178100	1.35183600
C	-0.96108100	-1.99208000	2.10654000
C	-0.32274600	-3.03159700	3.03030300
H	-1.09513500	-3.59879900	3.55837300
H	0.28914200	-3.72844600	2.44967200

H	0.31269000	-2.53751900	3.77018900
C	-1.70625000	-0.93130700	2.91735200
H	-1.00512500	-0.34175100	3.51742500
H	-2.41161200	-1.42176500	3.59315900
H	-2.26249800	-0.25366500	2.26182800
C	-1.94020500	-2.71723800	1.17916100
O	-2.34685900	-1.85031600	0.13274800
C	-2.96150000	-2.56965100	-0.92327300
C	-3.21577200	-1.65946800	-2.04218400
C	-3.41371600	-0.91454700	-2.97012500
H	-3.59911300	-0.24397600	-3.78387500
H	-3.90392600	-3.02286300	-0.58687800
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H	-2.81058500	-3.02712900	1.77492400
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C	3.90396400	0.31229900	0.36176600
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C	5.99406500	-0.16947400	-0.75980000
C	6.27761100	1.19583000	-0.82329500
C	5.37741200	2.11506100	-0.28999500
C	4.19443700	1.67239400	0.30128900
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H	5.59603900	3.17755600	-0.33388000
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H	6.69526300	-0.88993400	-1.16919500
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H	2.83670700	-1.02734100	1.65307900
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H	-2.23619900	2.41309100	-2.09494400
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H	-1.94417700	2.47466400	3.60734900

S_sS_int1

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C	4.16014500	0.50324000	0.01226600
C	4.55440500	1.47874200	1.12047700
C	5.28929700	-0.49354800	-0.23923500
C	3.83435700	1.27734200	-1.26537400
O	3.47830100	0.34860300	-2.27927300
C	2.69945000	0.92588900	-3.30917900
C	1.32762500	1.23116200	-2.86454900
C	0.20357100	1.48470300	-2.50112700
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C	1.00273300	-2.08754800	-0.22937200
C	-0.39135800	-2.65854300	-0.48828700
C	-1.26918300	-2.00157100	-1.35596900
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C	-1.77637600	-1.63198900	-1.38794800
C	-2.35953600	-2.95321600	-1.89179300
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H	-2.69566800	-3.57648100	-1.05943700
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H	1.51599000	1.68023600	2.38282000
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H	2.61253800	2.19050900	0.30296000
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H	0.22707900	1.03049500	-0.90893500
C	1.78437400	1.43245200	-2.16961700
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O	2.57491900	-0.91831500	-0.18514000
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C	3.25868600	1.74754400	-2.21384900
C	3.36615000	-2.09033600	0.07272300
H	3.84805500	0.97733200	-1.70817500

H	3.56487500	1.80852600	-3.25684000
H	3.45841600	2.70392800	-1.72185500
H	3.03872800	-2.83038100	-0.65468500
H	4.42394300	-1.85968600	-0.06633200
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C	3.43851000	0.21413300	-0.32452600
C	3.99910600	-0.46290500	-1.57578400
H	4.86358900	-1.07728400	-1.31039700
H	3.24777900	-1.10360700	-2.04466000
H	4.31744600	0.29239300	-2.29987400
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H	4.16838600	1.60961100	1.18842900
H	4.65407400	1.99231100	-0.47470200
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O	2.32630900	-1.86798100	0.17323500
C	1.97023600	-2.85445200	1.12012900
C	0.94429500	-2.37753200	2.06491500
C	0.10488700	-1.97661900	2.83496900
H	-0.63599300	-1.62823400	3.52536100
H	2.85285100	-3.18864500	1.68215300
H	1.58239500	-3.71007800	0.56071400
H	4.06163100	-1.26773000	1.11837700
H	2.59222700	-0.36718700	1.57984500
H	1.76589100	0.56549400	-1.57466800
N	0.29886700	2.11201600	-0.55876700
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C	-1.45824400	4.10891200	-0.68822800
C	-1.71614100	0.46513900	1.25945800
H	-2.58509200	1.69512300	-0.35499300
H	-2.85291000	2.34093400	1.27691600
H	-2.32837300	4.55074600	-0.19920800
H	-0.71417700	4.87739800	-0.89825100
H	-1.74290700	3.58407800	-1.60133300
C	-1.76977600	-0.70883800	0.26925600
H	-2.37490500	0.23578200	2.09769900
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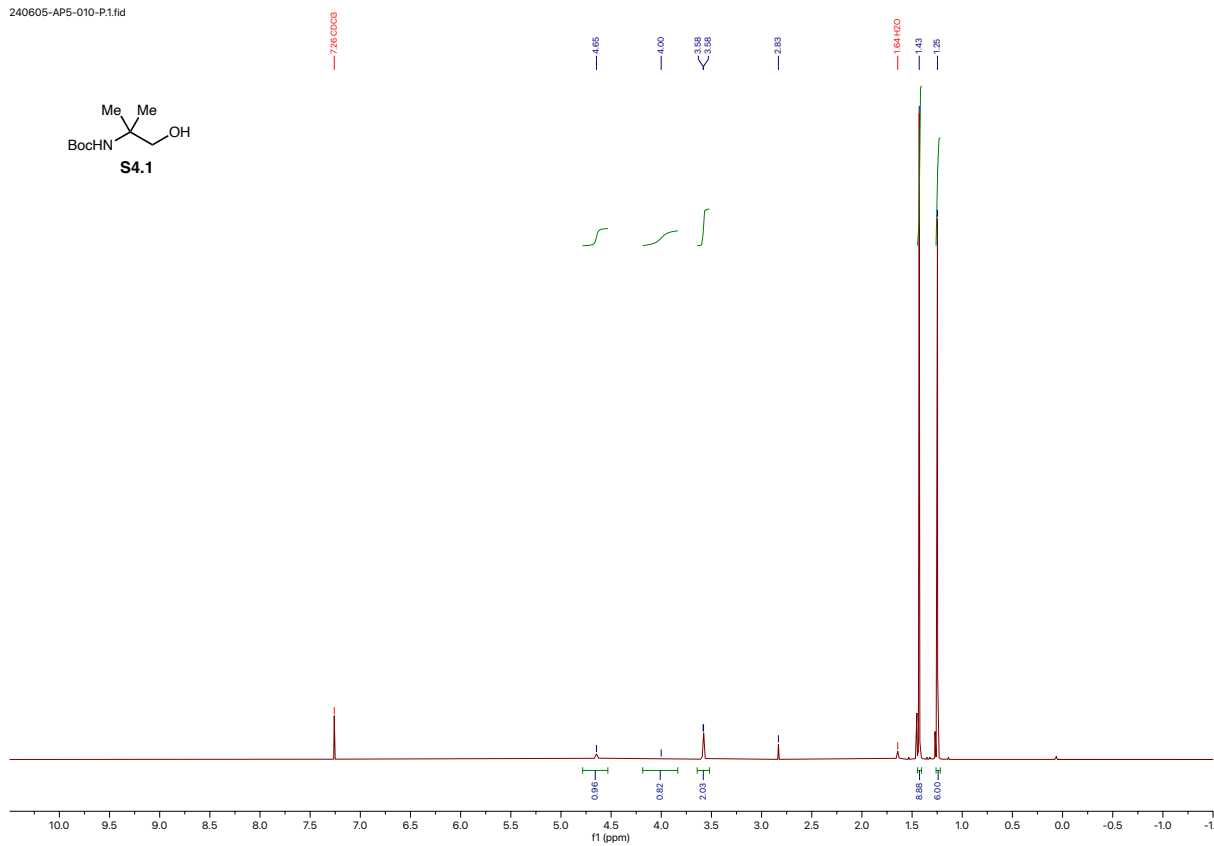
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C	-0.21789500	-1.51877800	-1.51509800
O	-3.38831600	-0.94807900	-1.53268500
O	-4.10575600	-0.87439700	0.59287500
O	0.60838000	-1.22700900	-2.39286900
C	-0.65241900	-2.94097400	-1.27586100
C	-5.45219500	-1.03260300	0.11530500
H	-0.00606600	-3.60496100	-1.84827200
H	-0.60412400	-3.20303100	-0.21546000
H	-1.68812200	-3.06898400	-1.60512400
H	-6.08094000	-1.03396500	1.00282500
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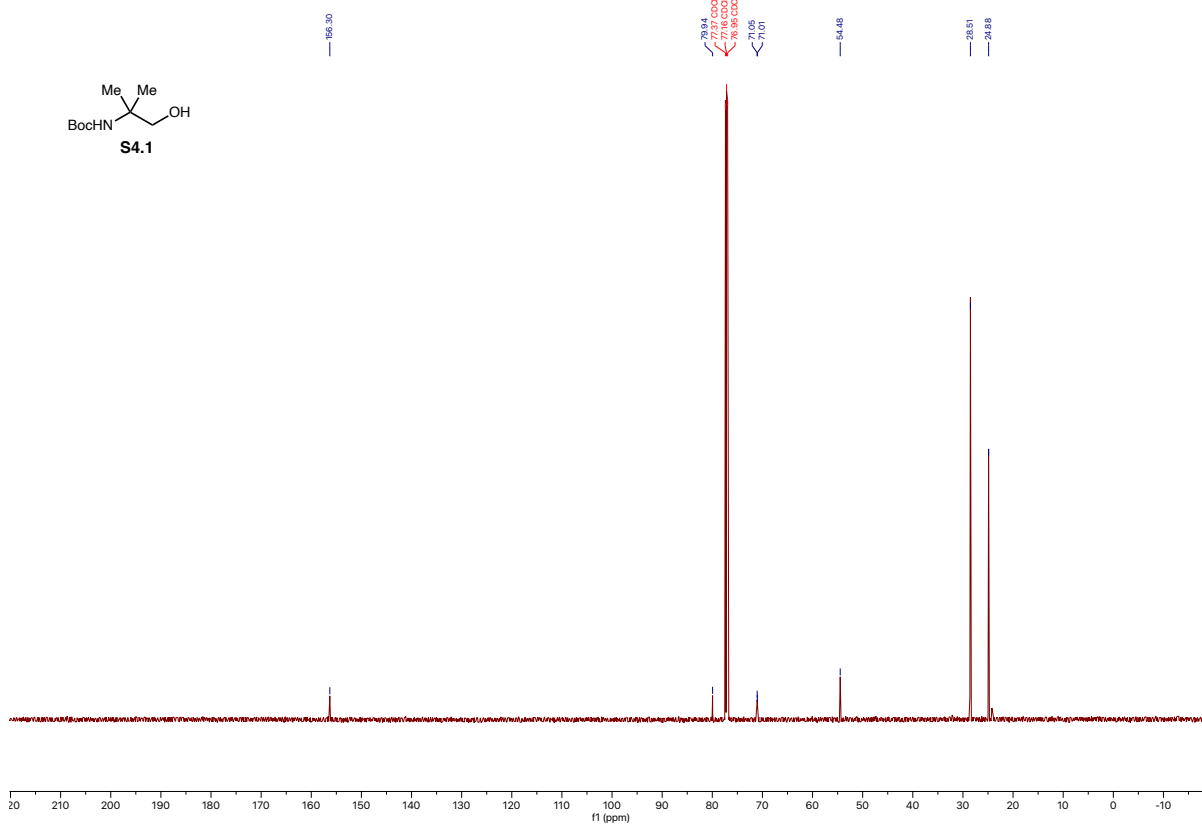
Benzaldehyde

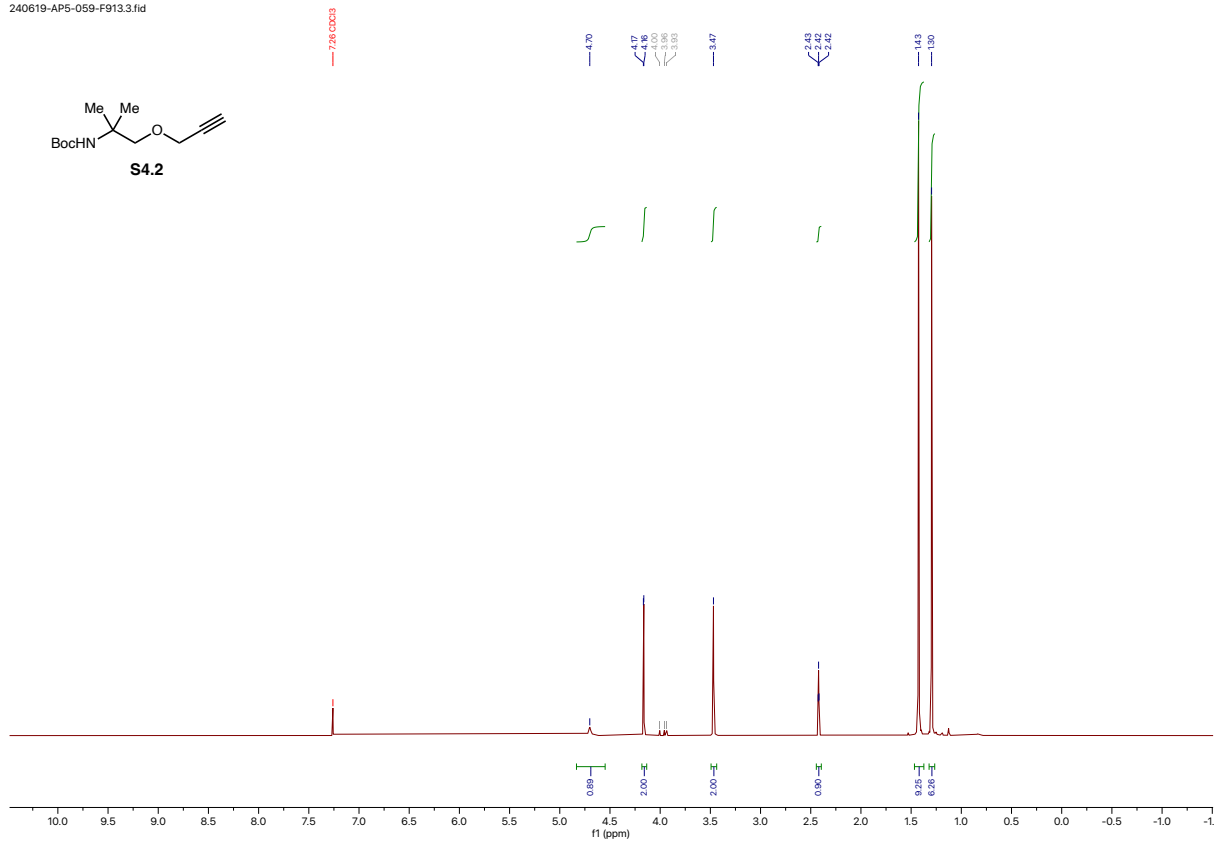
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C	-0.53047800	0.20962300	-0.00000300
C	-0.04595800	-1.10329700	-0.00000300
C	1.32366900	-1.32888600	0.00000000
H	3.27808200	-0.42668100	0.00000400
H	2.41862600	1.89699900	0.00000400
H	-0.03922800	2.30397500	-0.00000100
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4.8.14 NMR spectra

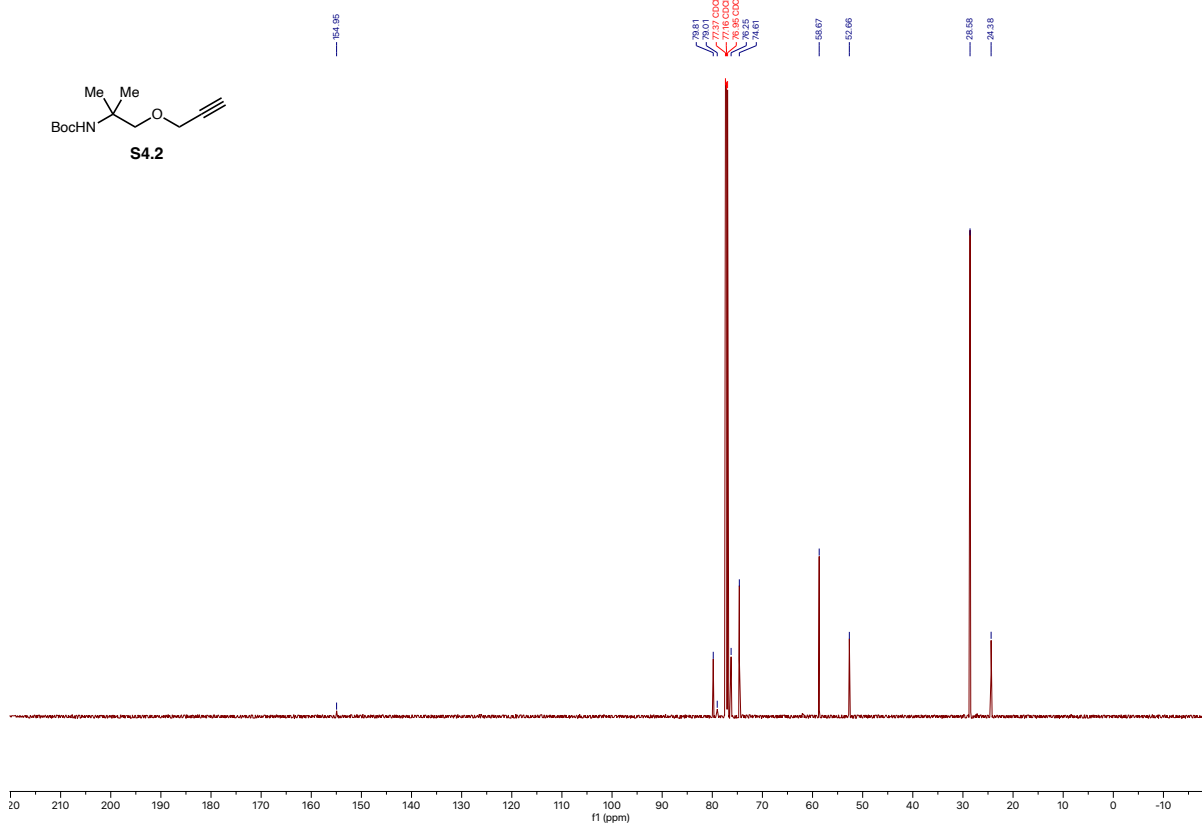
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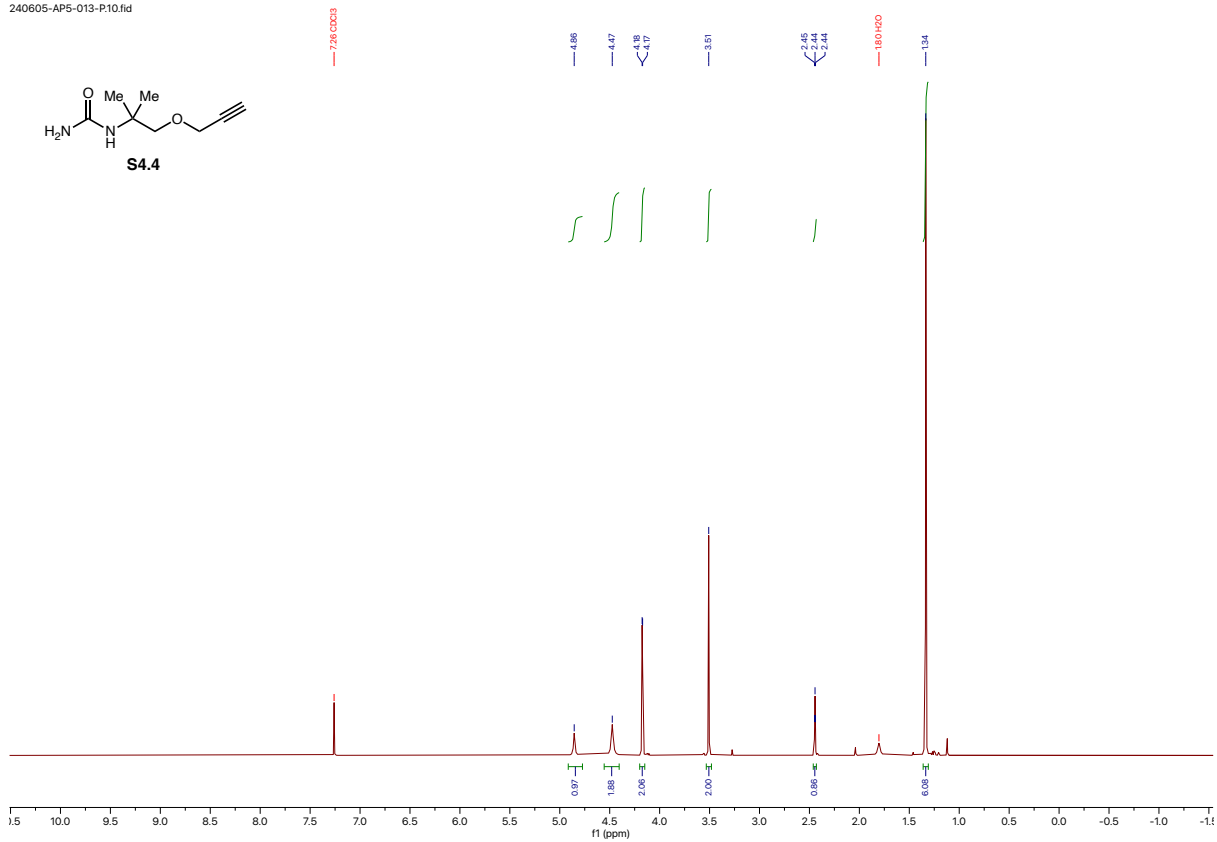
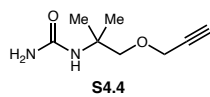




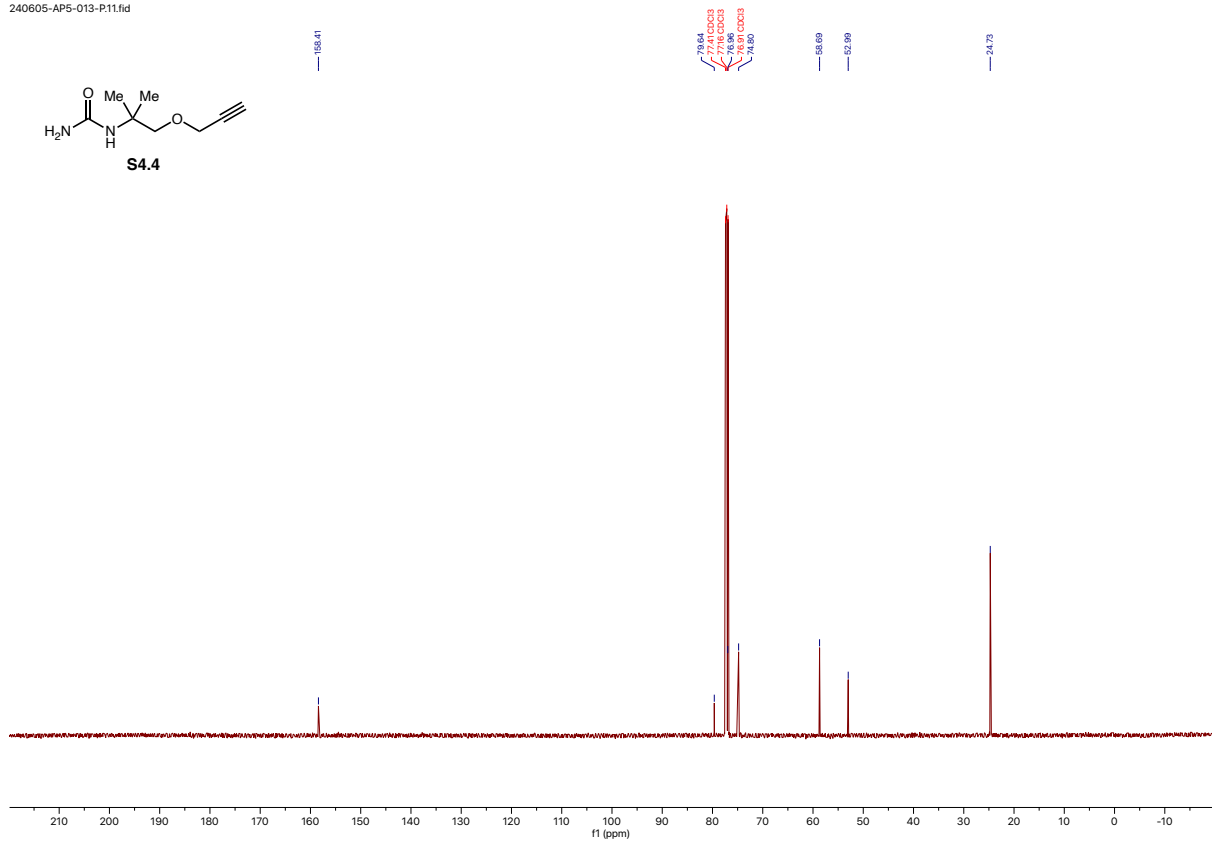


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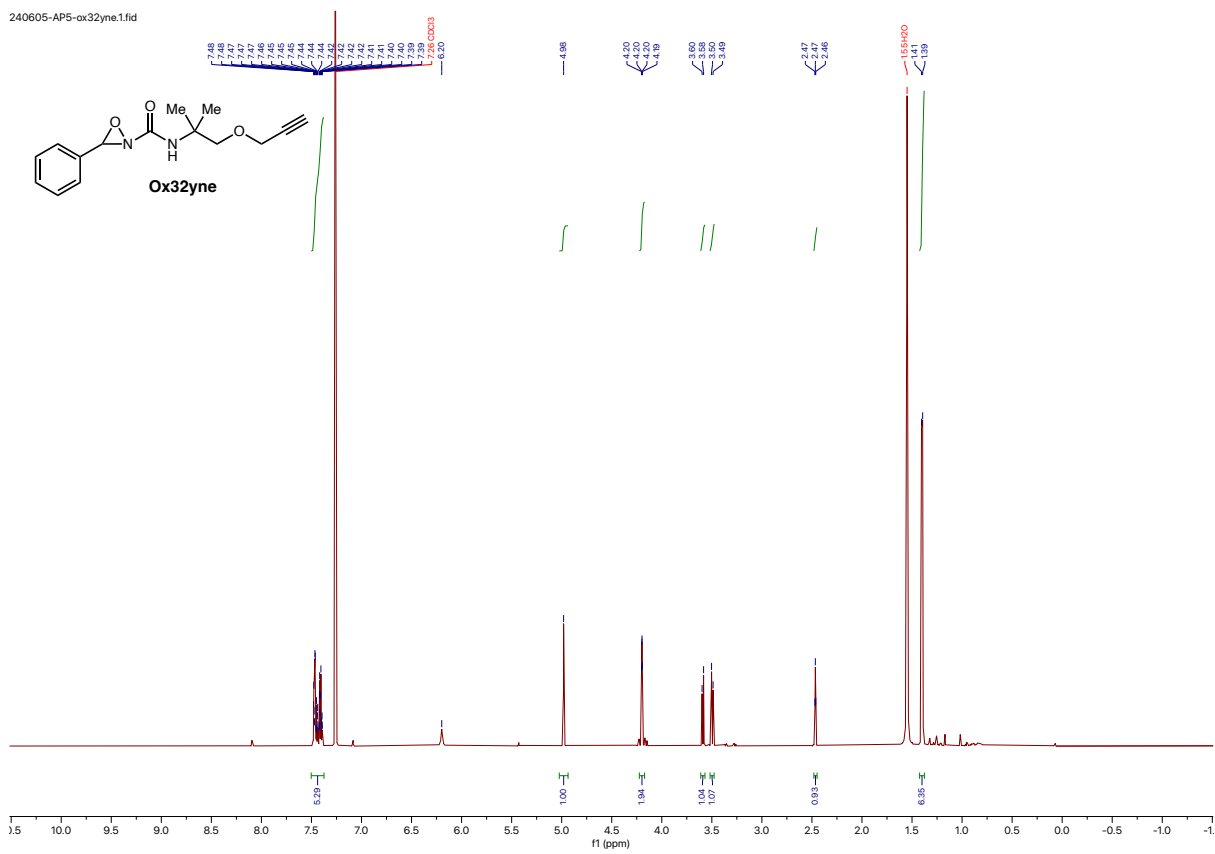




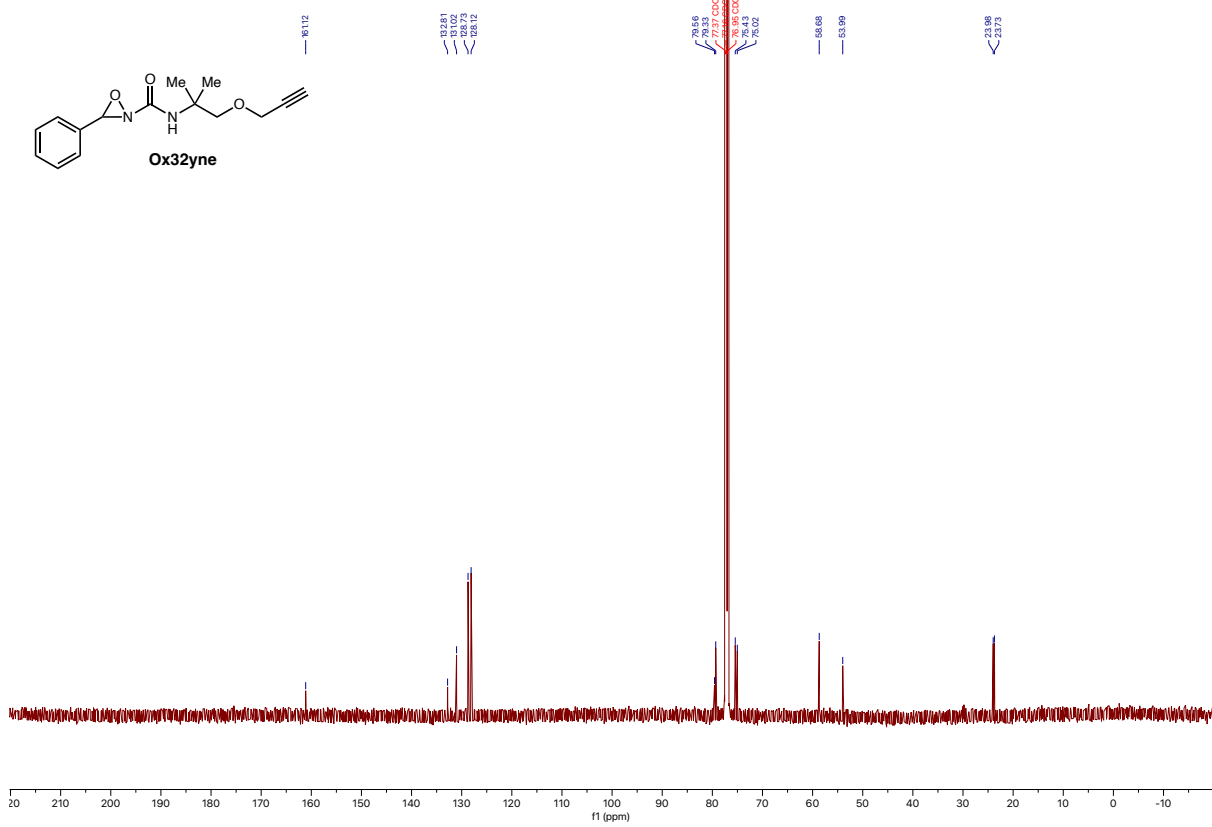
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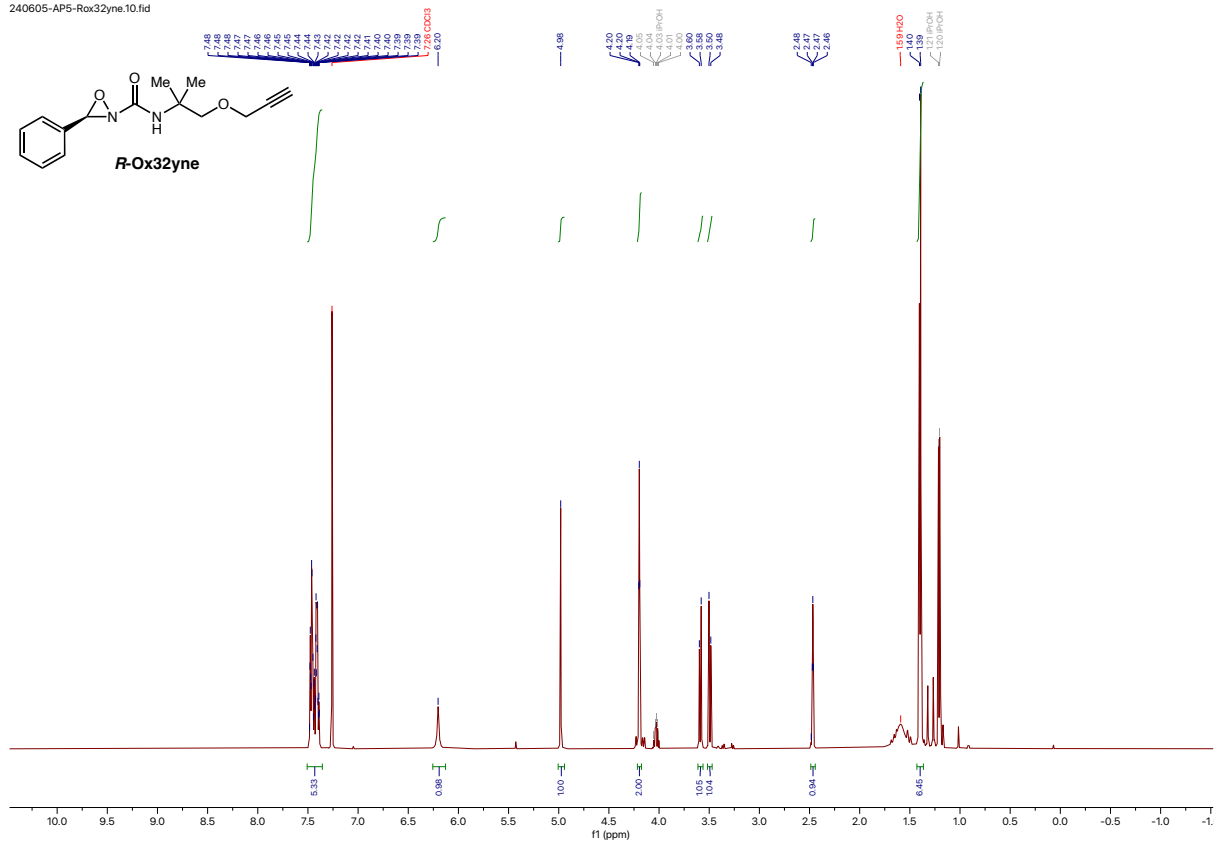
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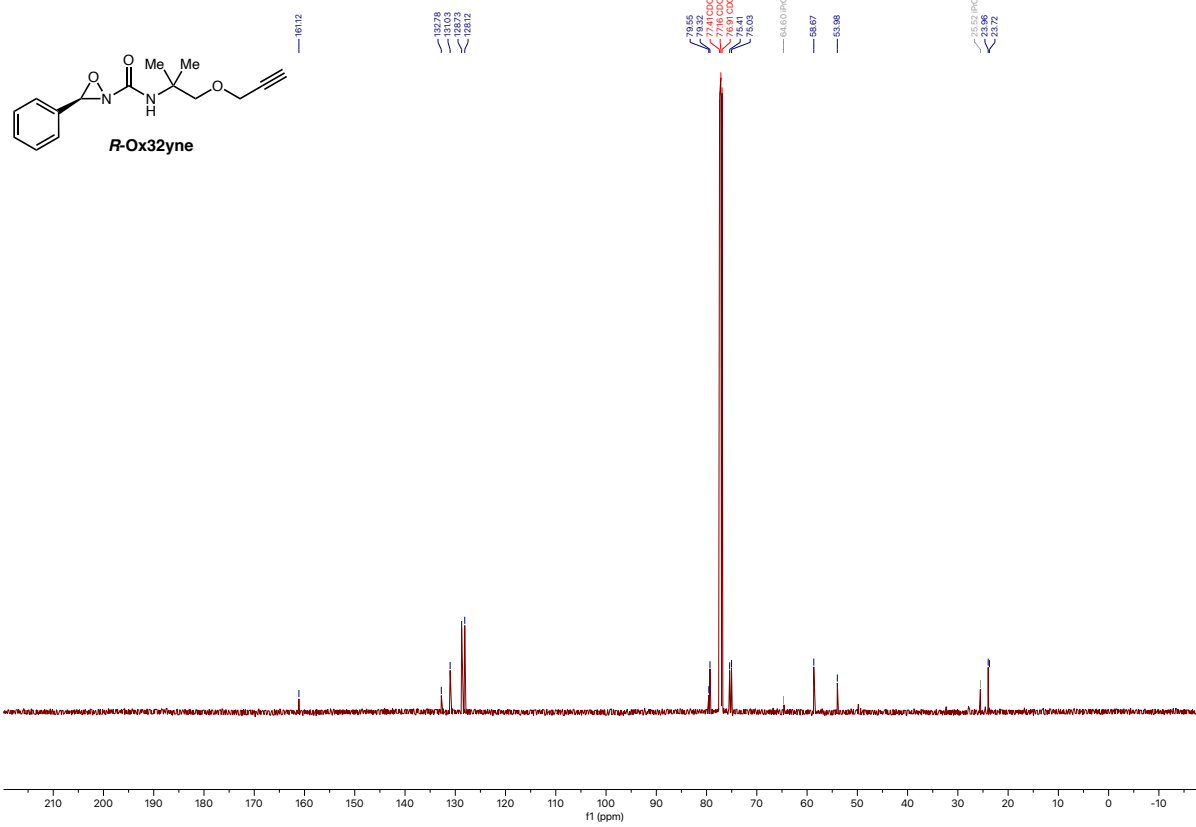
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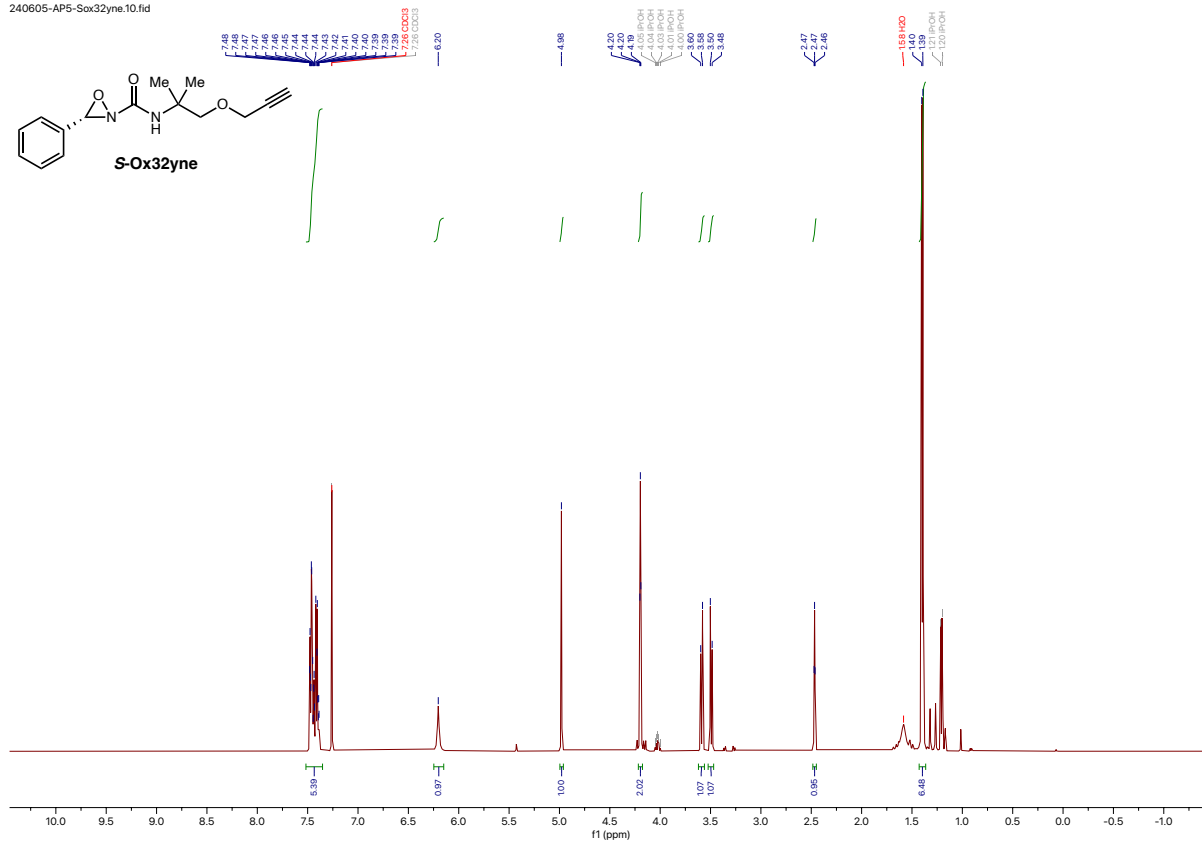
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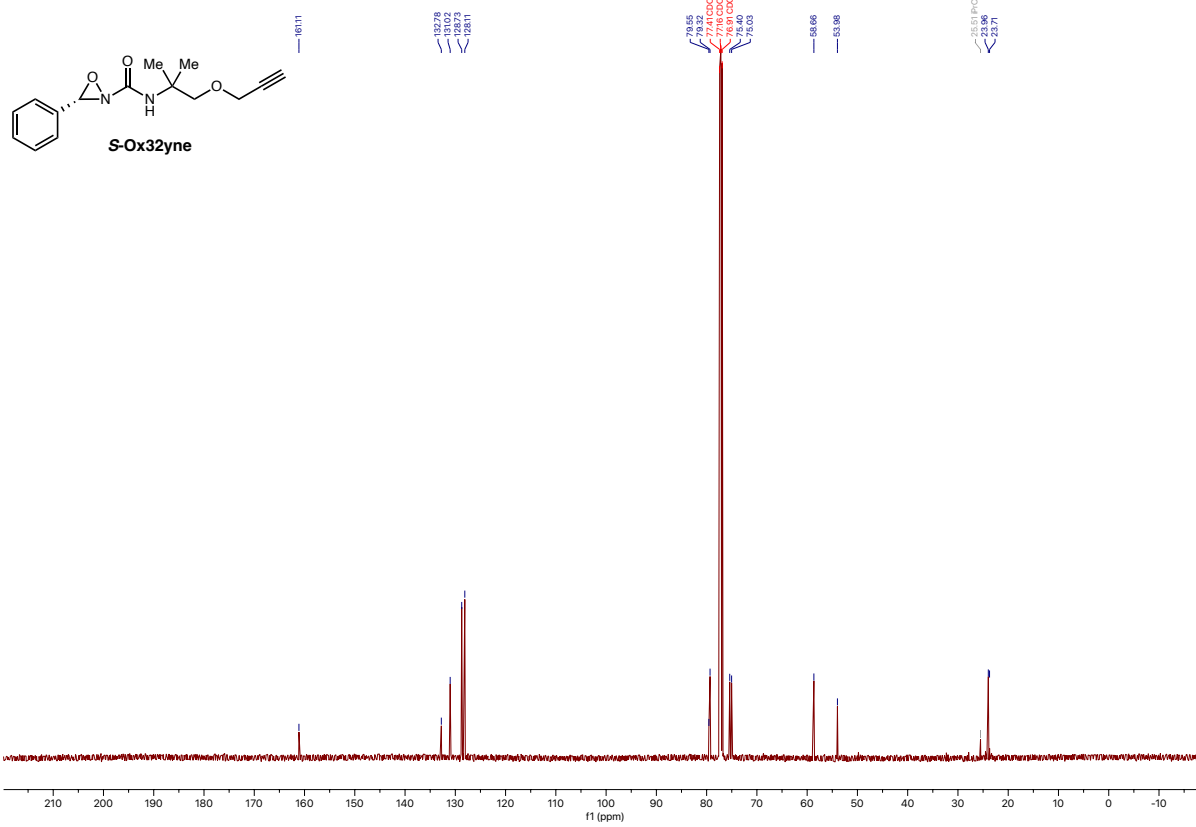
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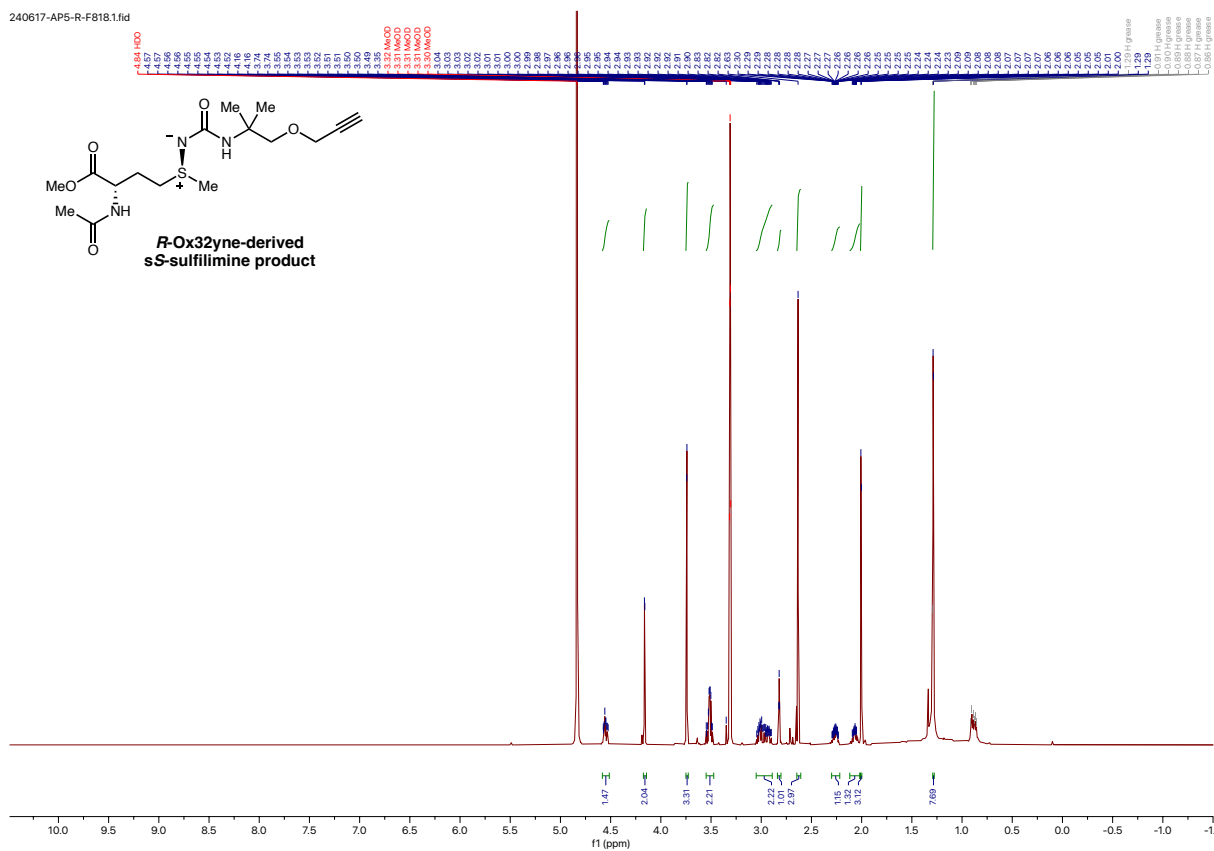


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CHAPTER 5

Development of Second-Generation Acyl Silane Photoaffinity Probes for Live Cell Chemoproteomic Profiling

Portions of this work were performed in collaboration with Bridget P. Belcher, Margot L. Meyers, Theodore G. Coffey, Lauren M. Orr, Spencer O. Scholz, Sabine Cismoski, Daniel K. Nomura, and F. Dean Toste

5.1 Abstract

Deconvolution of the protein targets of hit compounds from phenotypic screens, often conducted in live cells, is critical for understanding mechanism of action and identifying potentially hazardous off-target interactions. While photoaffinity labeling is a long-established technique for the confirmation of small molecule-protein interactions, its application into live cells is relatively new. Recently, we reported a novel chemical framework for photoaffinity labeling based on the photo-Brook rearrangement of acyl silanes and demonstrated its use in gel-based proteomics in cellular lysates. Here, we report the application of these probes to live cell photoaffinity workflows, demonstrate their complementarity to current state-of-the-art minimalist diazirine-based photoaffinity probes, and introduce a modular synthetic route to access acyl silane scaffolds with improved labeling properties.

5.2 Introduction

Understanding molecular mechanisms of action of bioactive small molecules is a critical challenge in the drug discovery process. Many small molecule drug candidates are initially identified through high-throughput phenotypic screens (3, 214), but the underlying protein targets which give rise to the desired phenotype can be challenging to ascertain. Additionally, as nearly a third of all clinical candidates fail due to undesired side-effects or toxicity, characterizing and minimizing the off-target interactions is a critical stage in the drug development process (215).

Traditional efforts to characterize protein targets of small molecule compounds are typically performed *in vitro* (5). Assessment of compound binding against panels of purified protein is a hallmark of the drug discovery process, but this method suffers severely from the challenges of obtaining large quantities of purified proteins and the inherent bias in protein selection. For analysis of compounds against a more physiologically-relevant protein sample, affinity purification has long stood as the standard for protein target identification (6). In this method, small molecule compounds are immobilized by conjugation to a solid support; then, the functionalized solid phase is treated with complex mixtures of proteins, usually cellular lysates, to induce binding events between the small molecule and its protein targets. Identification of these binding events, however, can be limited by the rigorous wash steps and buffer exchanges, which can lead to false negatives by the inclusion of only strongly bound small molecule protein interactions. While most small molecule drugs are optimized to maximize binding efficiency, even low-affinity binding events can modulate cellular function, leading to either desired phenotypic outcomes or undesired side effects. The identification and characterization of the entire interactome of a small molecule drug is therefore critical.

While many methods exist to determine small-molecule protein binding events *in vitro*, the characterization of a small molecule's interactome in the native cellular or physiological context is much more challenging. While *in vitro* assays can be implemented more quickly, identification of a compound's on- and off-targets in the native cellular environment is crucial for the success of drug programs— for example, live cell chemoproteomic efforts have aided in the identification of possible off-target interactions for suspended clinical candidates (216, 217). Two of the most commonly used current strategies for live-cell target identification include residue-specific Activity-Based Protein Profiling (ABPP) and residue-agnostic Photoaffinity Labeling (PAL). While ABPP is an

excellent method for profiling proteins that have reactive nucleophilic residues, such as kinases and other enzymes, a residue-agnostic approach is better suited to the global identification of a small molecule's protein interactions. PAL incorporates a photo-reactive crosslinker into a small molecule of interest, which forms a stable, covalent bond with an amino acid residue on its protein target upon irradiation. First proposed in the 1960's by Knowles (38, 39) and Westheimer (218, 219), PAL has become a widely-used strategy to identify protein targets of bioactive small molecules (5, 34, 37, 41, 52, 220, 221).

While PAL is widely used in industrial and academic research efforts, the photo-reactive crosslinkers utilized in this strategy have remained largely unchanged since their initial reports. Most commonly used PAL campaigns employ aryl azides (38), benzophenones (222), or diazirine (39) crosslinkers, which form reactive triplet diradicals, carbenes, and nitrenes, respectively. Since their initial reports, only a handful of new scaffolds for PAL have been reported (44, 116), and none have experienced widespread adoption to date. Presently, diazirines, particularly linear aliphatic diazirines dubbed “minimalist” probes, are usually considered the state-of-the-art crosslinker for PAL. Their small size and short irradiation times required for crosslinking offer attractive properties for use as a PAL crosslinker. Live cell diazirine applications of PAL were first reported with the development of this “minimalist” scaffold (53), which has enabled many subsequent target identification campaigns. Indeed, recent efforts by Woo et al. enabled the determination of protein targets of several common NSAID compounds by employing a linear diazirine tag ligated to the compounds of interest (37). However, it has been established that much of the labeling events observed through diazirine irradiation occurs through a diazo isomer, whose long lifetime in solution can lead to the capture of non-specific proteins after eventual formation of the carbene resulting in false positive hits (64) as well as an overrepresentation of membrane-related proteins through protonation of the diazo species and subsequent labeling by acidic residues on those proteins (63) (Figure 5.1A). Some efforts have been undertaken to reduce labeling from the diazo species (223, 224), though most PAL campaigns still use the original minimalist scaffold. Minimalist PAL probes that avoid undesired reactivity and enable live-cell target identification would thus greatly expand the available toolkit for a target identification campaign (64). With these considerations in mind, we recently reported a novel chemical framework for PAL based on the photo-Brook rearrangement of acyl silanes and demonstrated its use in gel-based proteomics in cellular lysates. In this method, photoactivation of the acyl silane species with long-wavelength UV light enables the formation of a reactive α -siloxy carbene species which can react with amino acid residues in an agnostic fashion (Figure 5.1B). We reported a small series of acyl silane probes differentiated by substitution around the photoactivatable moiety and observed improved labeling profiles as we increased steric bulk. In this initial report, we validated labeling with established small molecule protein binders in pure proteins and cell lysates and confirmed promise for proteome-wide target identification through acyl silane irradiation. Against this backdrop, we sought to develop a workflow for these probes to enable their application to live cell target identification. Here we report a method for quantitative proteomic profiling in live cells using these acyl silane reagents. We demonstrate that the avoidance of the diazo pathway leads to a reduction in non-specific membrane protein labeling, and then develop a modular synthetic route to enable access to diversified acyl silanes which can augment probe properties. Taken together, these findings establish acyl silanes as a valuable alternative

to traditional diazirine-based PAL probes and highlight the importance of modularity in a PAL probe scaffold.

5.3 Results and Discussion

5.3.1 Quantitative live cell proteomics enables comparison of acyl silane and diazirine probes

Our initial report of acyl silane photoaffinity scaffolds set out to develop a new scaffold for PAL which could avoid the deleterious side reactions involved with diazirines, while also introducing a modular handle through which photo- and physico-chemical properties could be tuned. Through a small series of probes, we determined that labeling efficiency by these probes correlates with steric bulk about the silane. Additionally, we found that the bulkier probes were more stable to thermal background reactions, likely resulting from attack by lysine followed by a thermal Brook rearrangement (66). These data led us to synthesize the bulky iPr-substituted probe **JQ1-iPr** and compare it to current state-of-the-art diazirine probes. We were gratified to find that in cellular lysate, our acyl silane probe labeled at a similar level to the diazirine probe **JQ1-DA**. With this proof-of-concept established, we thus sought to develop a workflow for quantitative live cell comparison between the acyl silane and diazirine probes. Previous reports have emphasized the benefits of utilizing multiple photoaffinity probe types in a target identification campaign to ensure broad proteome coverage. Indeed, it has been reported that the diazirine scaffold disproportionately labels membrane-bound proteins due to the presence of the undesired diazo labeling pathway (63). The consequences of this labeling preference have recently been reported, and a similar probe to **2-DA** was not able to enrich for BRD4 over DMSO vehicle (225). We sought to compare our acyl silane scaffold to the diazirine probe in a global proteomics experiment to confirm engagement of BRD4 by the acyl silane probe in whole live cells.

Thus, we performed a global pulldown chemoproteomic experiment in live K562 cells, a leukemia cell line which has commonly been used in proteomic workflows (37, 44, 225, 226). Following equimolar treatment with probes **JQ1-iPr**, **JQ1-DA**, or DMSO vehicle, K562 cells were incubated and irradiated at 4 °C for 20 minutes followed by cell lysis. Copper-catalyzed azide-alkyne cycloaddition (CuAAC) of labeled proteins with a biotin azide tag enabled pulldown and enrichment of labeled proteins from the lysate solution. Following tryptic digest, peptides were labeled with isobaric tandem-mass-tag (TMT) reagents and combined for analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS). From these data, we confirmed the non-specific labeling profile of **JQ1-DA**, which was unable to enrich for BRD4 and labeled almost entirely membrane-related proteins (Figure 5.2, Figure 5.3). Gratifyingly, analysis of acyl silane probe **JQ1-iPr** revealed significant enrichment of both BRD3 and BRD4 over DMSO vehicle (Figure 5.2). Analysis of labeled proteins from the acyl silane dataset revealed a decrease in membrane-related protein labeling compared to **JQ1-DA**, although both probes demonstrated an enrichment of membrane-related proteins compared to the expected frequency (63, 227) (Figure 5.3). Encouraged by these results, we sought to improve the labeling specificity of these acyl silane probes by attenuating their hydrophobic properties. We hypothesized that the over-enrichment of membrane-related proteins could result from probe association with hydrophobic membrane proteins. While previous efforts have investigated varying the linker of the PAL probe to improve

physicochemical properties, we aimed to take advantage of the substitution handle provided by the silane to modulate probe properties to enable better comparison to current probes while minimizing the distance between the targeting ligand and reactive species to further minimize non-specific labeling. Additionally, a small library of PAL probes which differ only by substitution on the photoactivatable warhead could enable higher-confidence target identification.

5.3.2 Synthesis of second-generation acyl silanes

As we have established the importance of steric bulk about the acyl silane for labeling efficiency, we envisioned that a diethyl- or silacyclopentyl-substituted probe could reduce hydrophilicity while preserving labeling efficiency and selected such dichlorosilanes for our initial reaction screens (Figure S5.1). We began our efforts by examining our previously reported synthetic route to acyl silane probes. In our initial studies, the silane moiety was installed through initial hydrosilylation of an alkene with a disubstituted chlorosilane (HR_2SiCl). While this route was successful for our initial series of substituents, the relative dearth of these chlorosilane reagents hampers further probe diversification. We therefore turned to the much more available dichlorosilane species for our diversification attempts. Installation of the silane was achieved by reaction of the respective dichlorosilane with a Grignard species, followed by capture of the silyl chloride intermediate by lithiated dithiane. We found that bromoethyl dioxolane was the most compatible synthon for the Grignard sequence (Figure S5.2). Installation of the alkyne moiety by deprotonation and substitution gave compounds **5.2**. The TMS functionality on the alkyne was deprotected by addition of potassium carbonate in methanol to give terminal alkynes **5.3**. Sequential acid-promoted deprotection of the acetal and reduction of the corresponding aldehyde with sodium borohydride afforded the alcohol intermediates **5.4**. Following installation of the phenyl carbamate moiety, deprotection of the dithiane by PIFA revealed substituted probes **15-Et** and **15-cyclo** (Figure 5.4).

5.3.3 Evaluation of proof-of-concept labeling by second-generation acyl silane probes

With two new substituted acyl silane probes in hand, we sought to evaluate their proof-of-concept labeling compared to the best performing acyl silane from our original report, isopropyl-substituted probe **15-iPr**. We began by evaluating their labeling performance in cell lysate. Following a 30-minute incubation period, lysates treated with probes **15-iPr**, **15-Et**, and **15-cyclo** were irradiated for 20 minutes on ice, followed by a Cu-catalyzed click reaction with rhodamine azide. Labeled proteins were subsequently visualized by in-gel fluorescence. Gratifyingly, probe **15-Et** displayed light-dependent labeling of proteins to a similar extent as probe **15-iPr** while preserving the minimal thermal background labeling as its bulkier congener (Figure 5.5). However, samples treated with silacyclopentane-substituted probe **15-cyclo** showed significantly greater fluorescence in the samples incubated in the absence of light, suggesting the susceptibility of this species towards thermal background reactivity (Figure 5.5). To better understand this thermal background pathway, we envision performing peptide mapping experiments and computational modeling by density functional theory (DFT) calculations to rationalize the increased reactivity of the silacyclopentane-substituted probe **15-cyclo**, which is the focus of continued work in our group. Irradiation of probes **15-iPr** and **15-Et**

in live K562 cells again demonstrate the promising labeling efficiency of the diethyl substitution pattern, displaying similar labeling to the diisopropyl probe while reducing the hydrophobic footprint (Figure 5.6).

5.3.4 Future directions: synthesis and evaluation of fully-functionalized second-generation acyl silane probes

To validate our hypothesis that reduced hydrophobicity could lead to improved labeling specificity by reducing the fraction of membrane-associated proteins labeled, we sought to synthesize and evaluate the (+)-JQ1-ligated probe **JQ1-Et**. Gratifyingly, the new modular route towards these second-generation probes should enable a facile reductive amination from the aldehyde intermediate after deprotection of acetal **5.3-Et** to provide the versatile amine handle **5.6-Et**. We envision that an amide coupling of the carboxylic acid derived from (+)-JQ1 to this amine will allow access to the fully-functionalized diethyl acyl silane **JQ1-Et** (Figure 5.7). With this probe in hand, we will perform quantitative proteomics to determine the impact of reduced hydrophobicity on probe specificity.

5.4 Conclusions

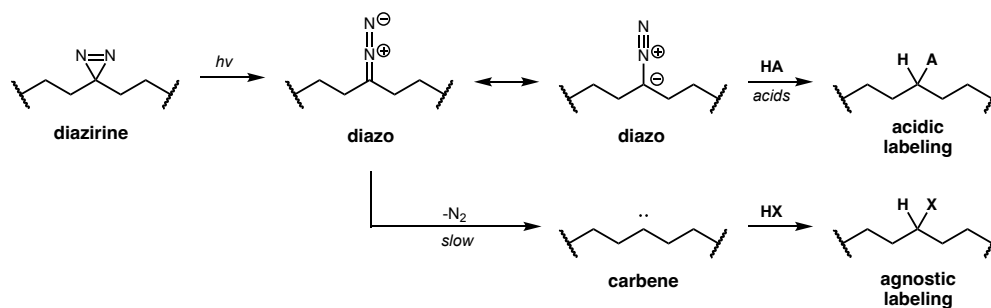
Photoaffinity labeling is a powerful tool for the deconvolution of small molecule-protein interactions, but its use in live cell applications is hampered by the non-specific labeling profiles of commonly used alkyl diazirine crosslinkers. We recently reported acyl silanes as a novel scaffold for photoaffinity labeling, based on the 1,2-photo-Brook rearrangement of acyl silanes to form an α -siloxy carbene intermediate. We show that this siloxy carbene exhibits complementary labeling to traditional diazirine probes and reduces non-specific labeling by avoiding pH-dependent labeling with acidic membrane residues. To improve probe labeling properties, we developed a modular synthetic route to enable access to acyl silanes with diverse substituents at silicon and demonstrated that reducing the hydrophobicity of the photoaffinity probe improves specificity of probe labeling. These results establish acyl silanes as a valuable tool for photoaffinity labeling whose use in target identification campaigns could enable discovery of heretofore unknown small molecule-protein interactions.

5.5 Acknowledgements

We thank Novartis Institutes for BioMedical Research, the Novartis-Berkeley Center for Proteomics and Chemistry Technologies (NB-CPACT), the Novartis-Berkeley Translational Chemical Biology Institute (NB-TCI), and the National Institutes of Health (R01CA240981, R01GM136945, P42ES004705) for supporting this work, as well as the Quantitative Biosciences Institute (Dr. Robert Maxwell) and the College of Chemistry's NMR facility supported in part by NIH S10OD024998. A.C.S.P would like to thank Deborah Zhuang for assistance with high-resolution mass spectrometry.

5.6 Figures and Schemes

A. “Minimalist” diazirine (Yao 2013)



B. “Minimalist” acyl silane (Toste 2022, *this work*)

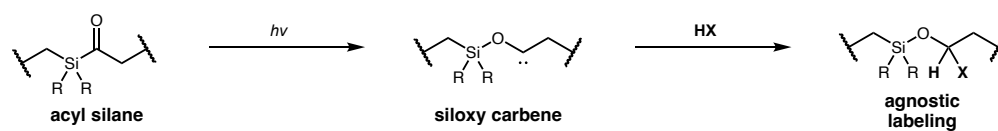


Figure 5.1. A: Pathways for photoaffinity labeling by aliphatic “minimalist” diazirines. B: Pathway for photoaffinity labeling by acyl silanes.

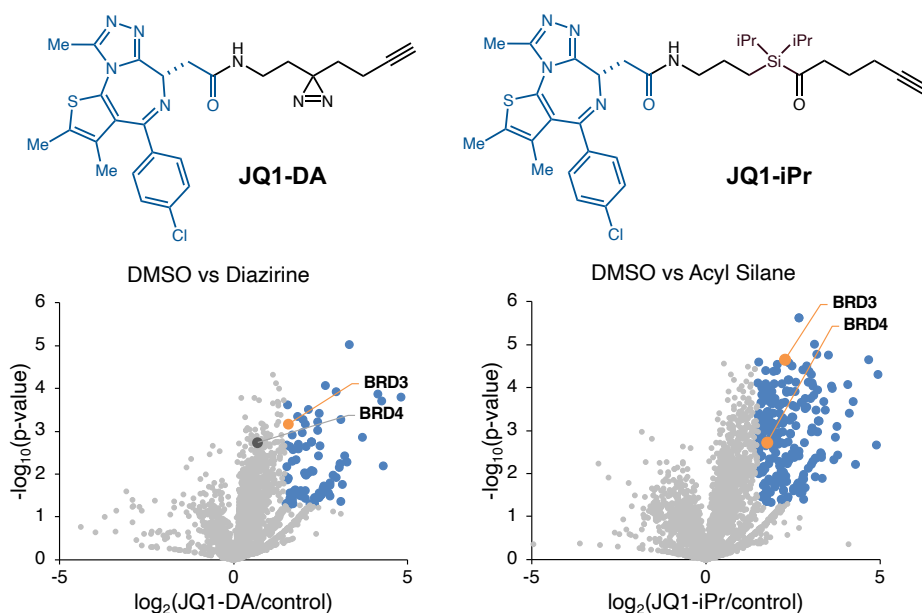


Figure 5.2. Quantitative proteomics of (+)-JQ1 photoaffinity probes in whole cells. K562 cells were treated with DMSO vehicle or PAL probe (10 μ M) for 1 hour and subjected to irradiation at 365 nm for 20 minutes. Subsequent lysates were subjected to copper-catalyzed azide-alkyne cycloaddition (CuAAC) with an azide- functionalized biotin handle, after which probe-modified proteins were avidin-enriched, eluted, and digested, and analyzed by TMT-based quantitative proteomics. Data shown are ratio of PAL probe vs. DMSO control enriched proteins and p-values from n=3 biologically independent replicates/group.

Localization of enriched proteins:

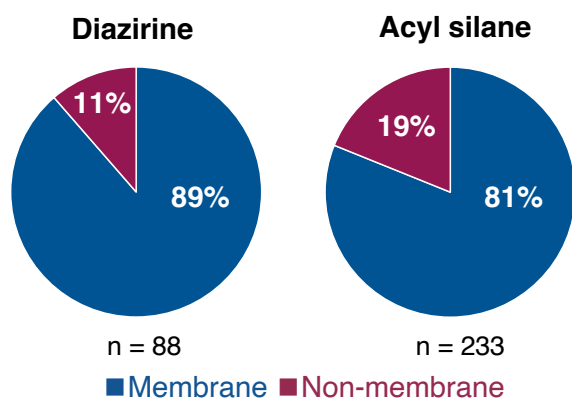


Figure 5.3. Subcellular distribution of proteins enriched by diazirine probe **JQ1-DA** (left) and acyl silane probe **JQ1-iPr** (right).

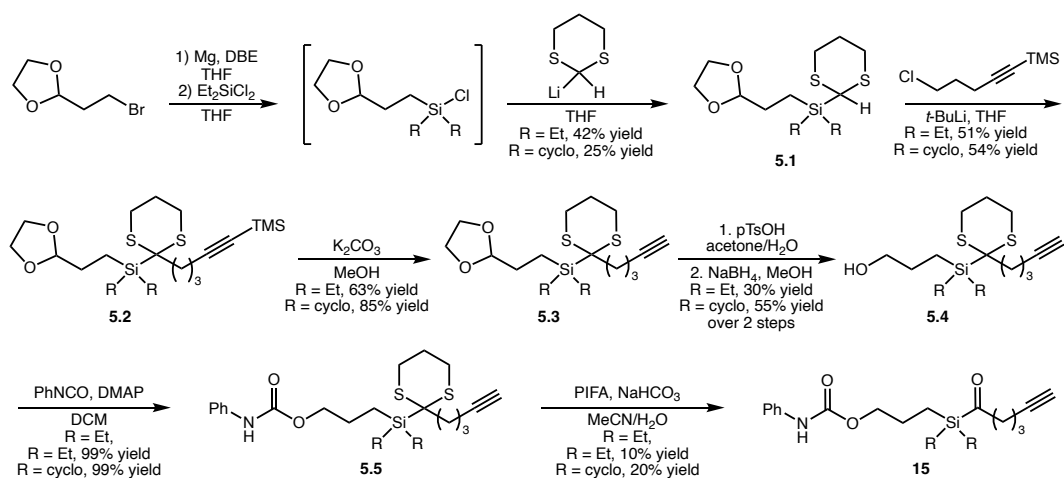


Figure 5.4. Synthesis of proof-of-concept photoaffinity probes containing diversified silane substitution.

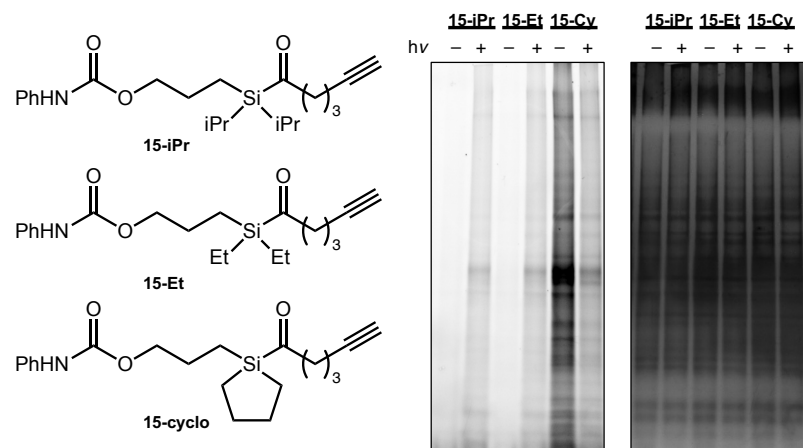


Figure 5.5. Evaluation of labeling by proof-of-concept photoaffinity probes. Evaluation of labeling by proof-of-concept photoaffinity probes (**15-iPr**, **15-Et**, and **15-cyclo**) in K562 cell lysate by in-gel fluorescence (left image) following Cu click reaction with Rh-N₃ after irradiation at 365 nm or incubation in the dark. Protein loading was confirmed following silver staining (right image) (see Supplementary information for full gel images).

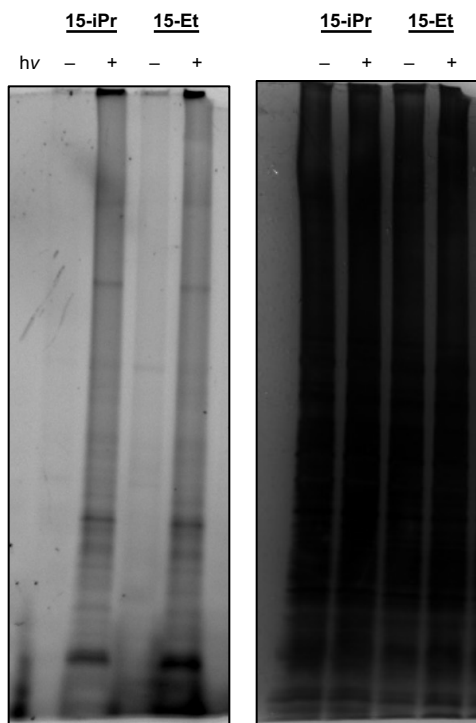


Figure 5.6. Evaluation of labeling by proof-of-concept photoaffinity probes 15-iPr and 15-Et in K562 whole cells.

(left image) Evaluation of labeling by in-gel fluorescence following Cu click reaction with Rh-N₃ after irradiation at 365 nm or incubation in the dark. Protein loading was confirmed following silver staining (right image) (see Supplementary information for full gel images).

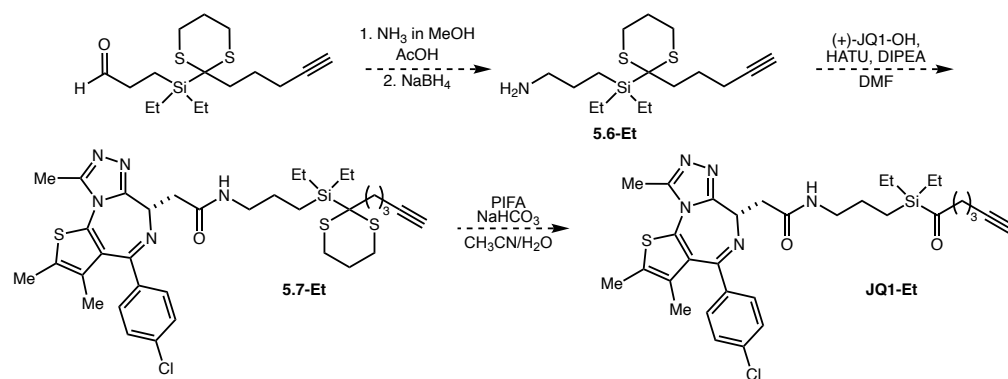


Figure 5.7. Envisioned synthetic route towards fully-functionalized probe **JQ1-Et**.

5.7 Supplementary information for “Development of Second-Generation Acyl Silane Photoaffinity Probes for Live Cell Chemoproteomic Profiling”

5.7.1 Supplementary figures

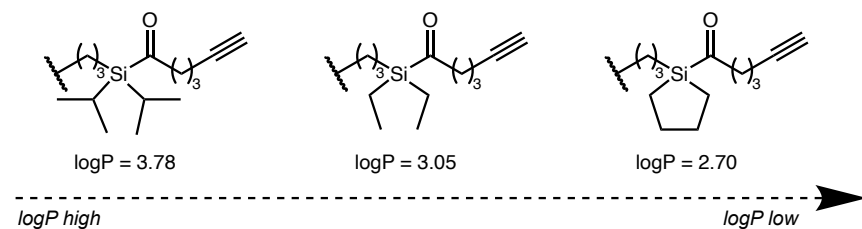
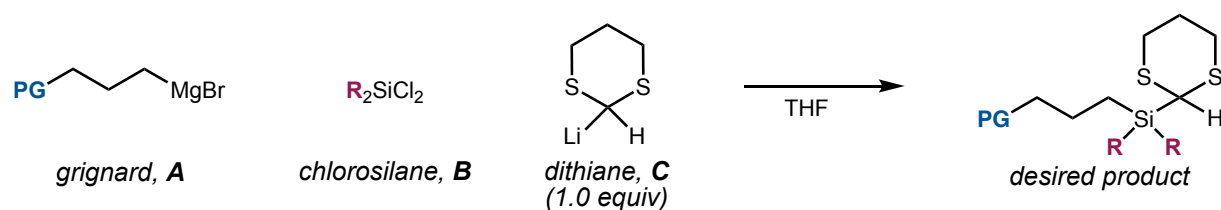


Figure S5.1. Calculated logP of acyl silane fragments.



PG (equiv A)	R (equiv B)	Order of addition	Yield
OTBS (1.2)	Et (1.1)	B to C , add to A	0%
OTHP (2.0)	Et (1.0)	C to B , add to A	13%
OTHP (1.5)	Et (1.2)	A to B , add to C	0%
OBn (1.5)	Et (1.2)	A to B , add to C	52%
dioxolane (1.5)	Et (1.2)	A to B , add C @ -78	42%
dioxolane (1.5)	silacyclopentane (1.2)	A to B , add C @ -78	25%

Figure S5.2. Condition screen for Grignard/lithiation sequence.

n/a	15-iPr		15-Et		15-Cy		n/a	15-iPr		15-Et		15-Cy		n/a	15-iPr		15-Et		15-Cy	
hv	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+

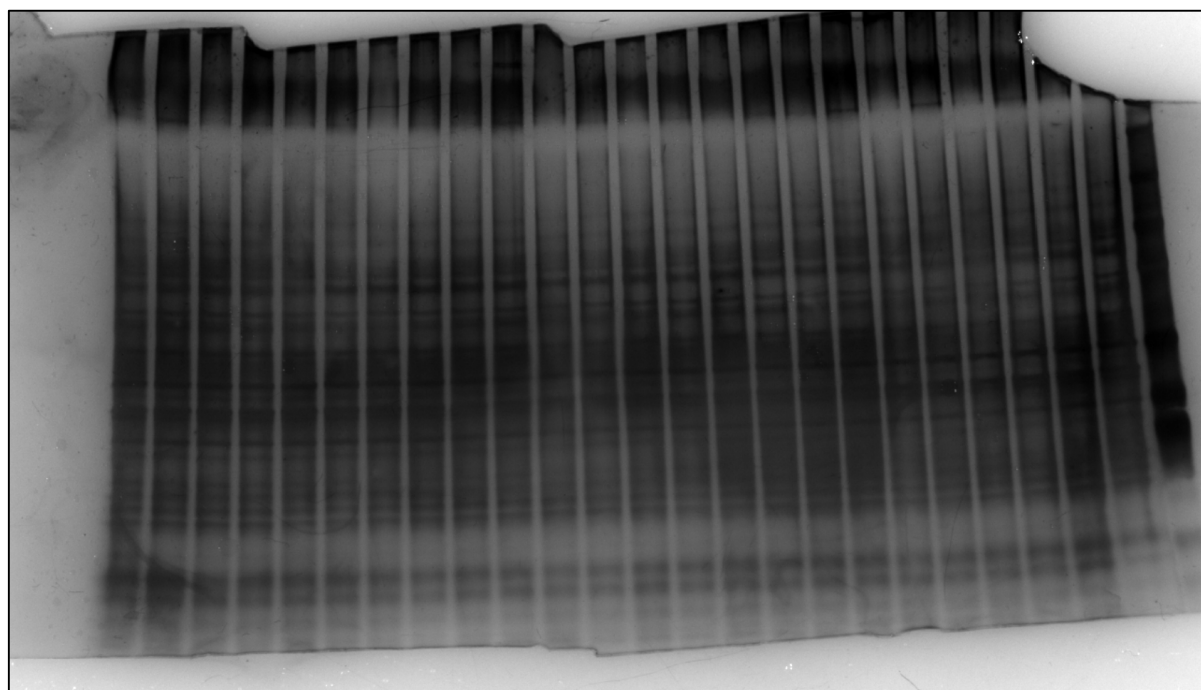
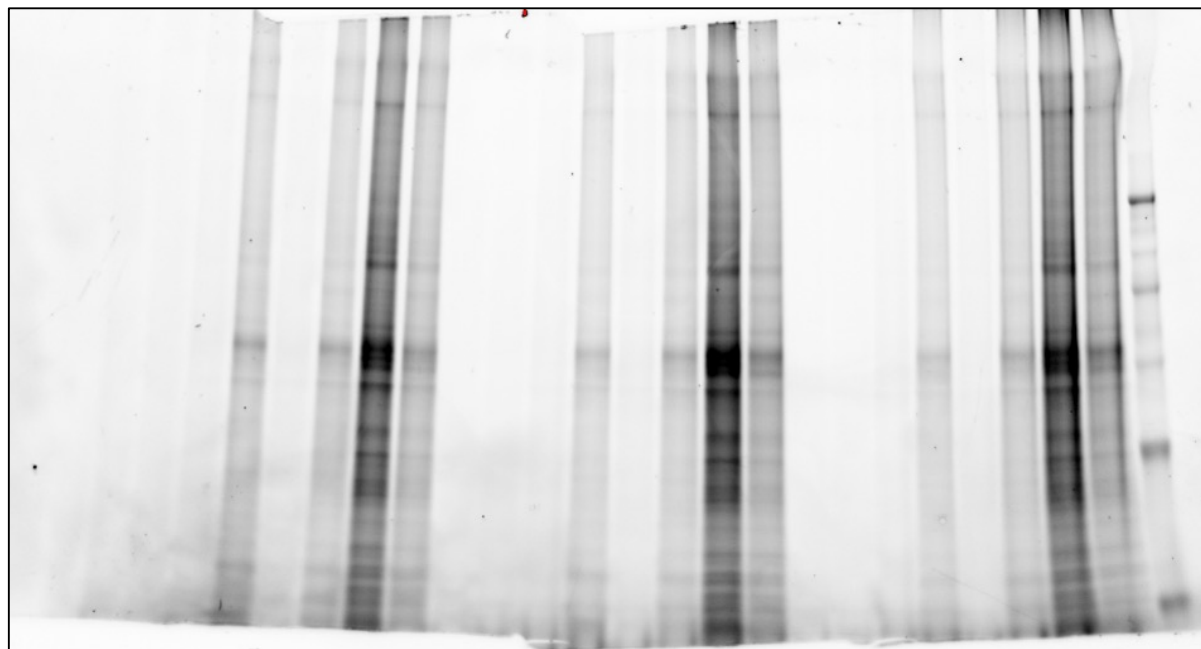


Figure S5.3. Full gel image of probes **15-iPr**, **15-Et**, and **15-cyclo** labeling K562 cell lysate (top image). Silver stain confirming protein loading (bottom image).

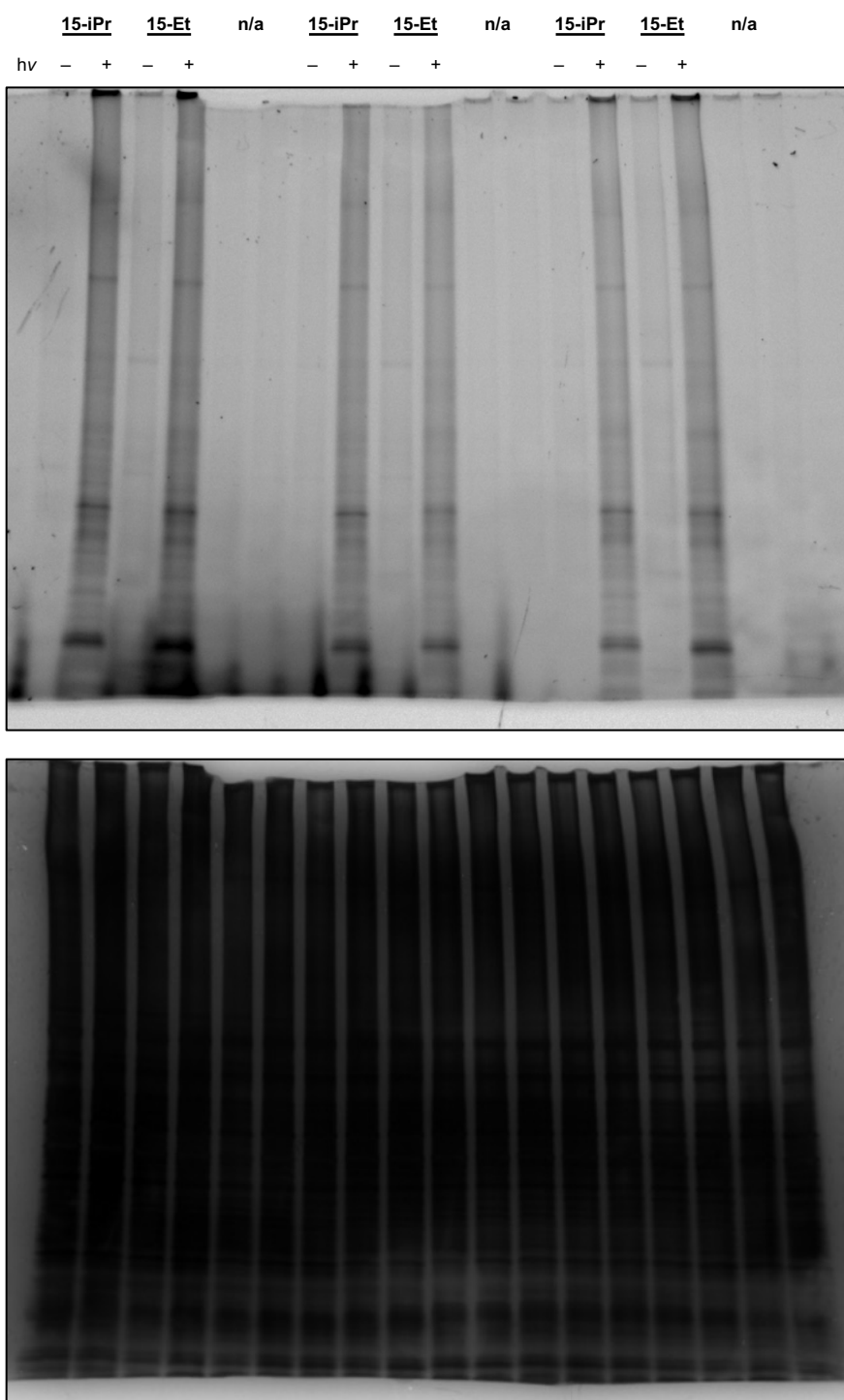


Figure S5.4. Full gel image of probes **15-iPr** and **15-Et** labeling K562 whole cells (top image). Silver stain confirming protein loading (bottom image).

5.7.2 List of supplementary tables

(to access, please contact authors)

Table S5.1.

TMT proteomics data quantifying proteins enriched by probe **JQ1-DA** vs DMSO control.

Table S5.2.

TMT proteomics data quantifying proteins enriched by probe **JQ1-iPr** vs DMSO control.

Table S5.3.

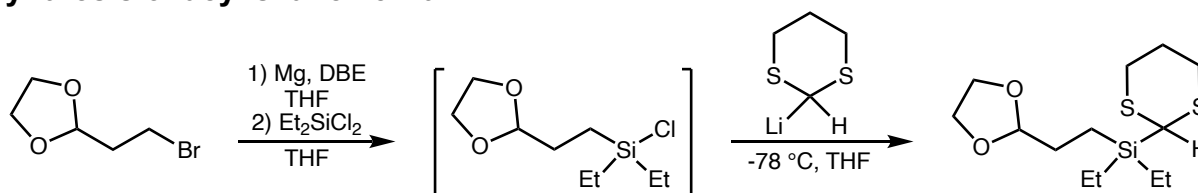
TMT proteomics data quantifying proteins enriched by probe **JQ1-iPr** vs **JQ1-DA**.

5.7.3 General methods

Unless otherwise noted, all reagents were purchased from commercial suppliers and used without further purification. Tetrahydrofuran, dichloromethane, diethyl ether, toluene, trimethylamine, and *N,N*-dimethylformamide were purified by passage through an activated alumina column under argon. Thin-layer chromatography (TLC) analysis of reaction mixtures was performed using Merck silica gel 60 F254 TLC plates and visualized under UV or by staining with KMnO_4 or *p*-anisaldehyde. Column chromatography was performed on Merck Silica Gel 60 Å, 230 X 400 mesh. Nuclear magnetic resonance (NMR) spectra were recorded using Bruker AV-600, AV-500, Neo-500, Neo-501, AVB-400, and AVQ-400 spectrometers at the Pines Magnetic Resonance Center's Core 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 ^1H and 77.16 (CDCl_3) or 49.00 (CD_3OD) for ^{13}C as an internal reference. Splitting patterns are indicated as follows: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, hept = heptet/septet, m = multiplet, br = broad, dt = doublet of triplet resonance. These NMR instruments at UC Berkeley are funded in part by the NIH (S10OD024998). High-resolution mass spectral analyses (ESI-MS) were carried out using an Agilent 1260 series LC equipped with a 6530 series Q-TOF (ESI) located in the Francis group at the University of California, Berkeley or obtained from the QB3 mass spectral facility at the University of California, Berkeley on a Thermo LTQ-FTICR (7T, ESI). Solvent abbreviations are reported as follows: EtOAc = ethyl acetate, hex = hexanes, DCM = dichloromethane, Et_2O = diethyl ether, MeOH = methanol, THF = tetrahydrofuran, DMSO = dimethylsulfoxide, Et_3N = trimethylamine, MeCN = acetonitrile.

5.7.4 Synthetic methods

Synthesis of acyl silane 15-Et

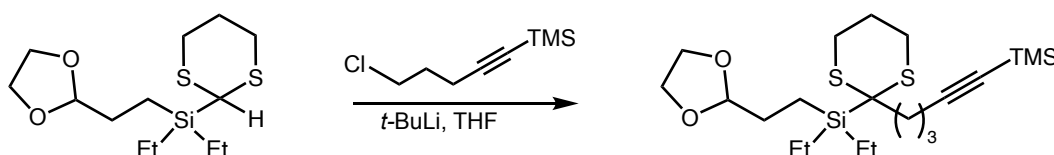


(2-(1,3-dioxolan-2-yl)ethyl)(1,3-dithian-2-yl)diethylsilane (5.1-Et)

A flame-dried three-neck flask fitted with a reflux condenser and cooled under N_2 was charged with magnesium turnings (0.6321 g, 26 mmol, 2.03 equiv.) and THF (20 mL). A small amount of 1,2-dibromoethane was added and the solution was heated with a heat gun until ethylene bubbles were observed. The flask was then placed in a water bath and dioxolane (2.35 mL, 20 mmol, 1.56 equiv.) was added slowly. The mixture was allowed to stir at 40 °C until a majority of the magnesium was consumed (ca. 2 hours). The resulting Grignard reagent was added via cannula to a separate flame-dried flask containing dichlorodiethylsilane (2.25 mL, 15.4 mmol, 1.20 equiv.) and THF (10 mL) at 0 °C. The mixture was allowed to warm to room temperature over 2 hours. Meanwhile, a separate round bottom flask cooled under vacuum was charged with 1,3-dithiane (1.54 g, 12.82 mmol, 1.0 equiv.) and THF (25 mL). The reaction vessel was cooled to -78°C and a solution of *n*BuLi (2.5 M in hexanes, 5.64 mL) was added dropwise resulting in a persistent, but faint yellow color. The flask was allowed to warm to room temperature over 30 minutes and then cooled back to -78°C and transferred via cannula to the flask

containing silyl chloride at $-78\text{ }^{\circ}\text{C}$. The solution was allowed to warm to room temperature overnight. After 16 hours, the reaction was diluted with EtOAc, quenched by addition of sat. aqueous NH_4Cl and washed with water. The combined aqueous layers were then extracted by EtOAc 2X and the combined organic layers were washed with brine, dried over MgSO_4 , filtered, and concentrated under reduced pressure. The crude oil was then purified via flash-column chromatography (gradient elution 0% – 10% EtOAc/Hex) to give **5.1-Et** as a clear oil (1.646 g, 5.369 mmol, 42% yield).

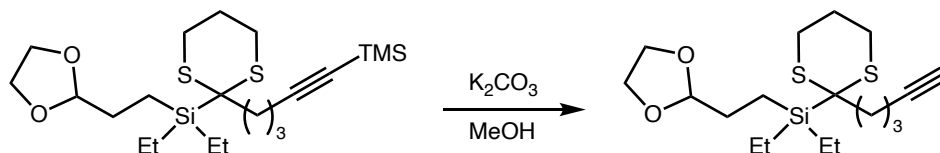
^1H NMR (600 MHz, CDCl_3): δ 4.83 (t, J = 4.6 Hz, 1H), 4.00 – 3.93 (m, 2H), 3.90 – 3.83 (m, 2H), 3.82 (s, 1H), 2.87 (ddd, J = 14.6, 12.6, 2.6 Hz, 2H), 2.70 (ddd, J = 14.0, 4.2, 2.9 Hz, 2H), 2.11 (dtt, J = 14.1, 4.7, 2.7 Hz, 1H), 2.07 – 1.97 (m, 1H), 1.75 (dt, J = 13.4, 4.5 Hz, 2H), 1.02 (t, J = 7.9 Hz, 6H), 0.83 – 0.76 (m, 2H), 0.72 (q, J = 7.9 Hz, 4H). **^{13}C NMR** (151 MHz, CDCl_3): δ 106.12, 65.10, 32.30, 31.55, 28.20, 26.51, 7.53, 4.29, 2.60. **HRMS** (ESI): calculated for $[\text{C}_{13}\text{H}_{26}\text{O}_2\text{S}_2\text{Si}+\text{H}]^+$ required m/z 307.1216, found m/z 307.1223.



(2-(1,3-dioxolan-2-yl)ethyl)diethyl(2-(5-(trimethylsilyl)pent-4-yn-1-yl)-1,3-dithian-2-yl)silane (5.2-Et)

A flame-dried round bottom flask cooled under vacuum was charged with **5.1-Et** (1.218 g, 3.97 mmol, 1.0 equiv.) and THF (20 mL, 0.2 M) and cooled to $-78\text{ }^{\circ}\text{C}$, followed by the dropwise addition of $t\text{BuLi}$ (1.6 M in pentane, 2.61 mL, 1.05 equiv.). After stirring for 30 minutes at $-78\text{ }^{\circ}\text{C}$, the solution was allowed to warm to room temperature over 30 minutes and then cooled back down to $-78\text{ }^{\circ}\text{C}$. Next, (5-chloropent-1-yn-1-yl)trimethylsilane (0.78 mL, 4.37 mmol, 1.1 equiv.) was added dropwise and the reaction was allowed to warm to room temperature overnight. After 16 hours, the reaction was quenched by the addition of sat. aqueous NH_4Cl and extracted with EtOAc. The combined organic layers were washed with saturated brine, dried over MgSO_4 , and concentrated to an oil. Purification of the crude material by flash column chromatography (5% EtOAc/hex) yielded the desired product **5.2-Et** as a clear, colorless oil (0.896 g, 2.014 mmol, 51% yield).

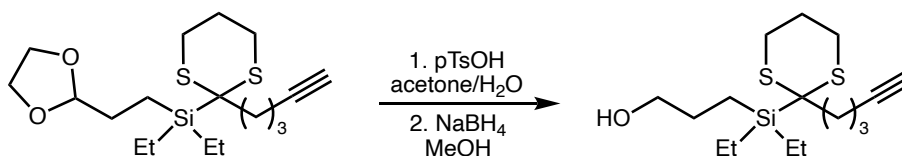
^1H NMR (600 MHz, CDCl_3): δ 4.84 (t, J = 4.6 Hz, 1H), 4.00 – 3.93 (m, 2H), 3.89 – 3.83 (m, 2H), 3.10 (ddd, J = 14.8, 12.7, 2.7 Hz, 2H), 2.39 (dt, J = 13.7, 3.8 Hz, 4H), 2.32 (t, J = 6.5 Hz, 2H), 2.07 – 1.99 (m, 1H), 1.93 (qt, J = 12.9, 3.3 Hz, 1H), 1.83 (ddd, J = 9.4, 8.2, 4.5 Hz, 2H), 1.76 – 1.67 (m, 2H), 1.06 (t, J = 7.9 Hz, 6H), 0.92 – 0.87 (m, 2H), 0.84 – 0.77 (m, 4H), 0.15 (s, 9H). **^{13}C NMR** (151 MHz, CDCl_3): δ 107.32, 106.34, 85.23, 65.08, 39.57, 36.41, 28.88, 26.86, 25.32, 23.49, 20.26, 8.27, 4.95, 3.28, 0.30, -1.04. **HRMS** (ESI) calculated for $[\text{C}_{21}\text{H}_{40}\text{O}_2\text{S}_2\text{Si}_2+\text{H}]^+$ required m/z 445.2081, found m/z 445.2112.



(2-(1,3-dioxolan-2-yl)ethyl)diethyl(2-(pent-4-yn-1-yl)-1,3-dithian-2-yl)silane (5.3-Et)

A scintillation vial was charged with **5.2-Et** (2.160 g, 4.856 mmol, 1.0 equiv.), potassium carbonate (2.013 g, 14.567 mmol, 3 equiv.), and MeOH (12 mL). The reaction was allowed to stir at room temperature for 2 hours and was then diluted with ether and washed with sat. aqueous NH_4Cl , water, and brine. The organic layers were dried over MgSO_4 , filtered, and concentrated under reduced pressure. The crude oil was purified using flash column chromatography (10% EtOAc/hex) to give alkyne **5.3-Et** (1.139 g, 3.056 mmol, 63% yield) as a clear oil.

^1H NMR (500 MHz, CDCl_3) δ 4.84 (t, J = 4.7 Hz, 1H), 4.01 – 3.92 (m, 2H), 3.91 – 3.80 (m, 2H), 3.07 (ddd, J = 15.0, 12.7, 2.7 Hz, 2H), 2.39 (ddd, J = 13.3, 6.8, 3.1 Hz, 4H), 2.27 (td, J = 6.8, 2.7 Hz, 2H), 2.08 – 2.00 (m, 1H), 1.99 (t, J = 2.6 Hz, 1H), 1.92 (qt, J = 12.9, 3.2 Hz, 1H), 1.83 (ddd, J = 9.4, 8.2, 4.5 Hz, 2H), 1.79 – 1.67 (m, 2H), 1.05 (t, J = 7.9 Hz, 6H), 0.92 – 0.85 (m, 2H), 0.81 (q, J = 8.0 Hz, 4H). **^{13}C NMR** (126 MHz, CDCl_3): δ 106.32, 84.38, 68.94, 65.08, 39.42, 36.71, 28.87, 26.86, 25.27, 23.57, 18.92, 8.26, 4.90, 3.30. **HRMS** (ESI) calculated for $[\text{C}_{18}\text{H}_{32}\text{O}_2\text{S}_2\text{Si}+\text{H}]^+$ required m/z 373.1686, found m/z 373.1699.



3-(diethyl(2-(pent-4-yn-1-yl)-1,3-dithian-2-yl)silyl)propan-1-ol (**5.4-Et**)

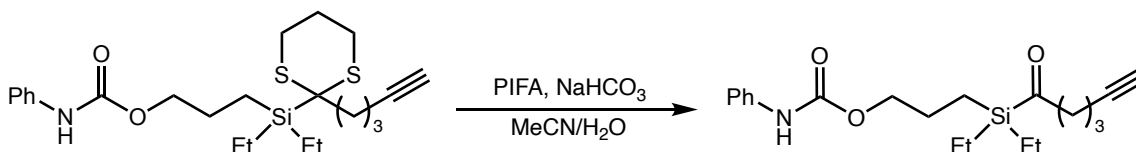
A 50 mL round-bottom flask was charged with alkyne **5.3-Et** (1.139 g, 3.056 mmol, 1.0 equiv.) and acetone (40 mL). Water (10 mL) and *p*-TsOH (3.157 g, 18.336 mmol, 6.0 equiv.) were added and the reaction was stirred at room temperature. After 16 hours, the reaction was quenched by addition of sat. aqueous NaHCO_3 and extracted with EtOAc. The combined organic layers were washed with brine, dried over MgSO_4 , filtered, and concentrated to give the aldehyde as a yellow oil which was used immediately in the next step without further purification. A scintillation vial was then charged with aldehyde and MeOH (12.25 mL). The vial was cooled to 0 °C and sodium borohydride (97 mg) was added and the reaction was allowed to stir. After one hour, an additional dose of sodium borohydride (60 mg) was added and the reaction was stirred for an additional hour. The reaction was then quenched by addition of water and extracted with EtOAc. The combined organic layers were washed with brine, dried over MgSO_4 , filtered, and concentrated under reduced pressure. The crude oil was purified using flash column chromatography (25% EtOAc/hex) to give alcohol **5.4-Et** (0.294 g, 0.889 mmol, 30% yield over two steps) as a clear oil.

^1H NMR (500 MHz, CDCl_3) δ 3.63 (t, J = 6.6 Hz, 2H), 3.08 (ddd, J = 15.2, 12.7, 2.7 Hz, 2H), 2.40 (ddd, J = 13.0, 6.7, 3.0 Hz, 4H), 2.28 (td, J = 6.8, 2.6 Hz, 2H), 2.08 – 2.02 (m, 1H), 2.00 (t, J = 2.6 Hz, 1H), 1.97 – 1.85 (m, 1H), 1.79 – 1.69 (m, 4H), 1.06 (t, J = 7.9 Hz, 6H), 0.85 – 0.78 (m, 6H). **^{13}C NMR** (126 MHz, CDCl_3) δ 68.98, 66.02, 36.66, 27.72, 26.89, 25.29, 23.55, 18.92, 14.28, 8.31, 6.85, 3.37 (*one resonance not observed*). **HRMS** (ESI) calculated for $[\text{C}_{16}\text{H}_{30}\text{OS}_2\text{Si}+\text{H}]^+$ required m/z 331.1580, found m/z 331.1613.



3-(diethyl(2-(pent-4-yn-1-yl)-1,3-dithian-2-yl)silyl)propyl phenylcarbamate (5.5-Et)

A scintillation vial was charged with alcohol **5.4-Et** (0.050 g, 0.151 mmol, 1.0 equiv.), DMAP (4 mg, 0.032 mmol, 0.2 equiv.), and DCM (0.80 mL). Phenylisocyanate (20 μ L, 0.17 mmol, 1.1 equiv.) was added and the reaction was allowed to stir at room temperature overnight. After 16 hours, the reaction was diluted with DCM, washed successively with sat. aqueous NH_4Cl , water, and brine, and the organic layer was dried over MgSO_4 , filtered, and concentrated under reduced pressure to give carbamate **5.5-Et** which was used immediately in the next step without further purification.

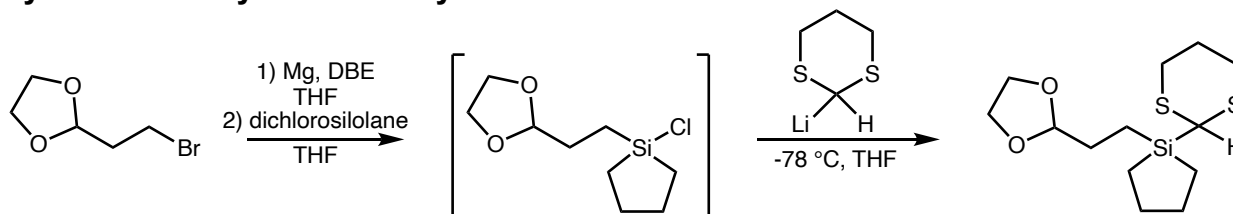


3-(diethyl(hex-5-ynoyl)silyl)propyl phenylcarbamate (15-Et)

A scintillation vial was charged with carbamate **5.5-Et** (0.151 mmol, 1.0 equiv.), NaHCO_3 (25 mg, 0.302 mmol, 2.0 equiv.), MeCN (2.72 mL), and water (0.30 mL). PIFA (78 mg, 0.313 mmol, 1.2 equiv.) was added and the reaction was stirred at room temperature. After 30 minutes, the reaction was diluted with Et_2O , washed successively with sat. aqueous NH_4Cl , water, and brine, and the organic layer was dried over MgSO_4 , filtered, and concentrated under reduced pressure. The crude material was purified using flash column chromatography (10% EtOAc/Hex) to give acyl silane **15-Et** (5.4 mg, 0.015 mmol, 10% yield) as a clear oil.

^1H NMR (600 MHz, CDCl_3) δ 7.42 – 7.35 (m, 2H), 7.34 – 7.27 (m, 2H), 7.06 (tt, J = 7.3, 1.2 Hz, 1H), 6.66 (s, 1H), 4.12 (t, J = 6.6 Hz, 2H), 2.74 (t, J = 7.0 Hz, 2H), 2.20 (td, J = 6.9, 2.7 Hz, 2H), 1.95 (t, J = 2.6 Hz, 1H), 1.80 – 1.67 (m, 4H), 0.99 (t, J = 7.9 Hz, 6H), 0.85 – 0.70 (m, 6H). **^{13}C NMR** (151 MHz, CDCl_3) δ 247.17, 138.07, 129.20, 129.17, 123.55, 120.79, 118.79, 83.93, 69.13, 48.38, 23.40, 20.64, 17.93, 7.39, 6.63, 2.57. **HRMS** (ESI): calculated for $[\text{C}_{20}\text{H}_{29}\text{NO}_3\text{Si}+\text{H}]^+$ required m/z 360.5485, found m/z 360.5421.

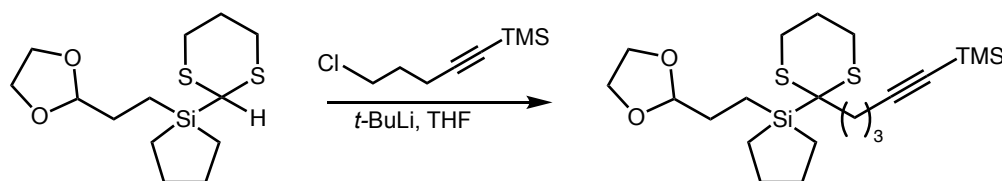
Synthesis of acyl silane 15-cyclo:



(2-(1,3-dioxolan-2-yl)ethyl)(1,3-dithian-2-yl)diethylsilane (5.1-cyclo)

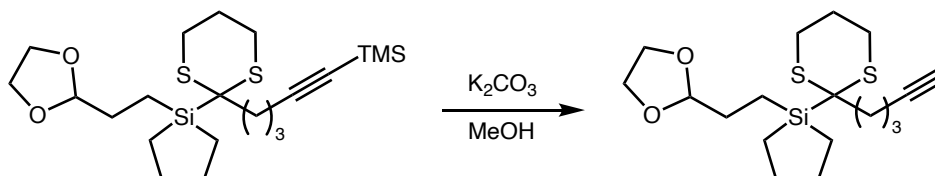
A flame-dried three-neck flask fitted with a reflux condenser and cooled under N_2 was charged with magnesium turnings (1.896 g, 78 mmol, 2.03 equiv.) and THF (60 mL). A small amount of 1,2-dibromoethane was added and the solution was heated with a heat

¹H NMR (400 MHz, CDCl₃) δ 4.85 (q, *J* = 3.5 Hz, 1H), 4.03 – 3.93 (m, 2H), 3.91 – 3.84 (m, 2H), 3.83 (d, *J* = 2.5 Hz, 1H), 2.87 (ddd, *J* = 14.7, 12.3, 2.8 Hz, 2H), 2.70 (dt, *J* = 13.9, 3.6 Hz, 2H), 2.19 – 1.92 (m, 2H), 1.74 (dt, *J* = 12.9, 4.5 Hz, 2H), 1.68 – 1.50 (m, 4H), 0.94 – 0.82 (m, 4H), 0.59 (dt, *J* = 13.0, 6.1 Hz, 2H). **¹³C NMR** (126 MHz, CDCl₃) δ 106.06, 65.09, 29.86, 28.06, 26.25, 26.17, 22.83, 12.71, 10.40. **HRMS** (ESI): calculated for [C₁₃H₂₄O₂S₂Si+Na+MeCN]⁺ required *m/z* 368.1145, found *m/z* 368.1162.



A flame-dried round bottom flask cooled under vacuum was charged with **5.1-cyclo** (2.462 g, 8.20 mmol, 1.0 equiv.) and THF (41 mL, 0.2 M) and cooled to $-78\text{ }^{\circ}\text{C}$, followed by the dropwise addition of *t*BuLi (1.7 M in pentane, 5.01 mL, 1.05 equiv.). After stirring for 30 minutes at $-78\text{ }^{\circ}\text{C}$, the solution was allowed to warm to room temperature over 30 minutes and then cooled back down to $-78\text{ }^{\circ}\text{C}$. Next, (5-chloropent-1-yn-1-yl)trimethylsilane (1.60 mL, 9.02 mmol, 1.1 equiv.) was added dropwise and the reaction was allowed to warm to room temperature overnight. After 16 hours, the reaction was quenched by the addition of sat. aqueous NH_4Cl and extracted with EtOAc. The combined organic layers were washed with saturated brine, dried over MgSO_4 , and concentrated to an oil. Purification of the crude material by flash column chromatography on silica gel in 95:5 hex/EtOAc yielded the desired product **5.2-cyclo** as a clear yellow oil (2.169 g, 4.907 mmol, 60% yield).

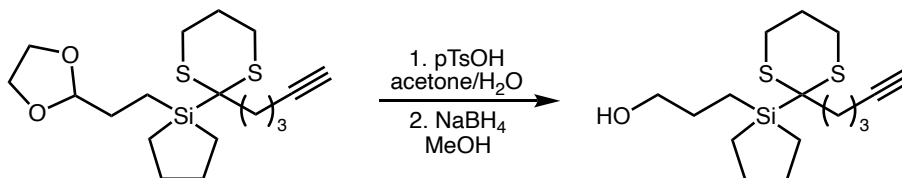
¹H NMR (500 MHz, CDCl₃) δ 4.82 (t, *J* = 4.6 Hz, 1H), 3.99 – 3.91 (m, 2H), 3.90 – 3.80 (m, 2H), 3.09 (ddd, *J* = 14.9, 12.7, 2.7 Hz, 2H), 2.46 – 2.34 (m, 4H), 2.31 (t, *J* = 6.6 Hz, 2H), 2.09 – 2.00 (m, 1H), 1.91 (qt, *J* = 13.1, 3.4 Hz, 1H), 1.73 – 1.63 (m, 6H), 1.61 – 1.50 (m, 2H), 1.00 (dt, *J* = 14.5, 7.2 Hz, 2H), 0.89 – 0.84 (m, 2H), 0.64 (dt, *J* = 14.3, 6.8 Hz, 2H), 0.16 (s, 9H). **¹³C NMR** (126 MHz, CDCl₃) δ 107.24, 105.98, 85.21, 65.09, 38.76, 36.07, 28.74, 27.64, 26.75, 25.29, 23.36, 20.20, 9.84, 5.19, 0.30. **HRMS** (ESI): calculated for [C₂₁H₃₈O₂S₂Si₂+H]⁺ required *m/z* 443.1925, found *m/z* 443.1992.



1-(2-(1,3-dioxolan-2-yl)ethyl)-1-(2-(pent-4-yn-1-yl)-1,3-dithian-2-yl)silolane (5.3-cyclo)

A scintillation vial was charged with **5.2-cyclo** (0.637 g, 1.435 mmol, 1.0 equiv.), potassium carbonate (0.595 g, 4.30 mmol, 3 equiv.), and MeOH (3.59 mL). The reaction was allowed to stir at room temperature for 90 minute and was then diluted with ether and washed with water and brine. The organic layers were dried over MgSO₄, filtered, and concentrated under reduced pressure to give alkyne **5.3-cyclo** (0.452 g, 1.215 mmol, 85% yield) as a clear oil.

¹H NMR (500 MHz, CDCl₃) δ 4.83 (t, *J* = 4.6 Hz, 1H), 4.02 – 3.91 (m, 2H), 3.91 – 3.80 (m, 2H), 3.07 (ddd, *J* = 15.0, 12.6, 2.7 Hz, 2H), 2.42 (ddd, *J* = 14.2, 4.2, 3.1 Hz, 2H), 2.39 – 2.34 (m, 2H), 2.26 (td, *J* = 6.8, 2.6 Hz, 2H), 2.09 – 2.02 (m, 1H), 1.99 (t, *J* = 2.6 Hz, 1H), 1.90 (qt, *J* = 13.0, 3.3 Hz, 1H), 1.74 – 1.63 (m, 6H), 1.57 – 1.50 (m, 3H), 1.00 (dt, *J* = 14.6, 7.4 Hz, 2H), 0.90 – 0.85 (m, 2H), 0.64 (dt, *J* = 14.3, 6.9 Hz, 2H). **¹³C NMR** (126 MHz, CDCl₃) δ 105.98, 84.33, 68.98, 65.12, 38.68, 36.27, 28.75, 27.63, 26.79, 25.28, 23.43, 18.82, 9.94, 5.16. **HRMS** (ESI): calculated for [C₁₈H₃₀O₂S₂Si+H]⁺ required *m/z* 371.1529, found *m/z* 371.1532.

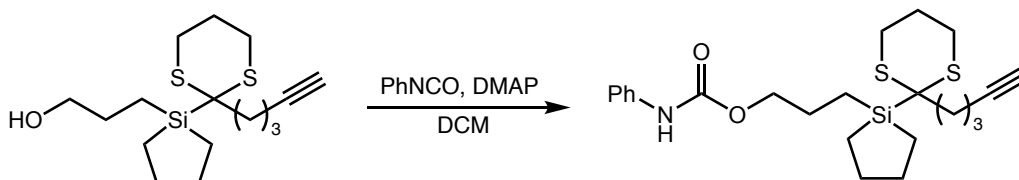


3-(1-(2-(pent-4-yn-1-yl)-1,3-dithian-2-yl)silolan-1-yl)propan-1-ol (5.4-cyclo)

A 50 mL round-bottom flask was charged with alkyne **5.3-cyclo** (0.7862 g, 2.121 mmol, 1.0 equiv.) and acetone (28.3 mL). Water (7.1 mL) and *p*-TsOH (2.191 g, 12.726 mmol, 6.0 equiv.) were added and the reaction was stirred at room temperature. After 16 hours, the reaction was quenched by addition of sat. aqueous NaHCO₃ and extracted with EtOAc. The combined organic layers were washed with brine, dried over MgSO₄, filtered, and concentrated to give the aldehyde was used immediately in the next step without further purification. A scintillation vial was then charged with aldehyde and MeOH (8.5 mL). The vial was cooled to 0 °C and sodium borohydride (67 mg) was added and the

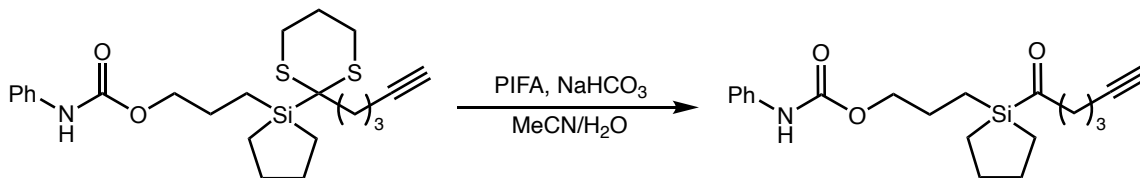
reaction was allowed to stir. After one hour, an additional dose of sodium borohydride (40 mg) was added and the reaction was stirred for an additional hour. The reaction was then quenched by addition of water and extracted with EtOAc. The combined organic layers were washed with brine, dried over MgSO_4 , filtered, and concentrated under reduced pressure. The crude oil was purified using flash column chromatography (25% EtOAc/hex) to give alcohol **5.4-cyclo** (0.380 g, 1.156 mmol, 55% yield over two steps) as a clear oil.

^1H NMR (500 MHz, CDCl_3): δ 3.62 (t, J = 6.6 Hz, 2H), 3.07 (ddd, J = 15.0, 12.7, 2.7 Hz, 2H), 2.43 (ddd, J = 14.3, 4.2, 3.1 Hz, 2H), 2.40 – 2.33 (m, 2H), 2.27 (td, J = 6.7, 2.7 Hz, 2H), 2.10 – 2.03 (m, 1H), 1.99 (t, J = 2.7 Hz, 1H), 1.90 (qt, J = 12.9, 3.3 Hz, 1H), 1.73 – 1.65 (m, 4H), 1.66 – 1.60 (m, 2H), 1.59 – 1.52 (m, 2H), 1.02 (dt, J = 14.6, 7.2 Hz, 2H), 0.83 – 0.75 (m, 2H), 0.64 (dt, J = 14.5, 6.9 Hz, 2H). **^{13}C NMR** (126 MHz, CDCl_3): δ 84.33, 68.98, 65.74, 38.71, 36.33, 27.66, 27.58, 26.78, 25.28, 23.42, 18.82, 10.03, 7.23. **HRMS** (ESI): calculated for $[\text{C}_{16}\text{H}_{28}\text{OS}_2\text{Si}+\text{H}]^+$ required m/z 329.1424, found m/z 329.1450.



3-(1-(2-(pent-4-yn-1-yl)-1,3-dithian-2-yl)silolan-1-yl)propyl phenylcarbamate (5.5-cyclo)

A scintillation vial was charged with alcohol **5.4-cyclo** (0.150 g, 0.456 mmol, 1.0 equiv.), DMAP (6 mg) and DCM (2.28 mL). Phenylisocyanate (55 μL , 0.502 mmol, 1.1 equiv.) was added and the reaction was allowed to stir at room temperature overnight. After 16 hours, the reaction was diluted with DCM, washed successively with sat. aqueous NH_4Cl , water, and brine, and the organic layer was dried over MgSO_4 , filtered, and concentrated under reduced pressure to give carbamate **5.5-cyclo** (99% yield) which was used immediately in the next step without further purification.



3-(1-(hex-5-ynoyl)silolan-1-yl)propyl phenylcarbamate (15-cyclo)

A scintillation vial was charged with carbamate **5.5-cyclo** (0.456 mmol, 1.0 equiv.), NaHCO_3 (77 mg, 0.912 mmol, 2.0 equiv.), MeCN (8.21 mL), and water (0.91 mL). PIFA (0.235 g, 0.547 mmol, 1.2 equiv.) was added and the reaction was stirred at room temperature. After 30 minutes, the reaction was diluted with Et_2O , washed successively with sat. aqueous NH_4Cl , water, and brine, and the organic layer was dried over MgSO_4 , filtered, and concentrated under reduced pressure. The crude material was purified using preparatory TLC (10% EtOAc/Hex) to give acyl silane **15-cyclo** (32 mg, 0.090 mmol, 20% yield) as a white solid.

¹H NMR (400 MHz, MeOD) δ 7.41 (d, J = 8.0 Hz, 2H), 7.31 – 7.21 (m, 2H), 7.06 – 6.96 (m, 1H), 4.09 (t, J = 6.6 Hz, 2H), 2.82 (t, J = 7.0 Hz, 2H), 2.22 (q, J = 2.8 Hz, 1H), 2.15 (td, J = 7.0, 2.7 Hz, 2H), 1.80 – 1.59 (m, 8H), 0.98 – 0.81 (m, 4H), 0.80 – 0.68 (m, 2H). **¹³C NMR** (151 MHz, MeOD) δ 248.60, 156.05, 140.17, 129.79, 124.00, 119.87, 84.40, 70.22, 67.98, 28.32, 24.72, 22.10, 18.40, 10.47, 8.52. **HRMS** (ESI): calculated for $[\text{C}_{20}\text{H}_{27}\text{NO}_3\text{Si}+\text{H}]^+$ required m/z 358.5325, found m/z 358.5286.

5.7.5 Cell culture

K562 cells were obtained from the UC Berkeley Cell Culture Facility and were cultured in RPMI medium containing 10% (v/v) fetal bovine serum (FBS) and maintained at 37°C with 5% CO₂.

5.7.6 Preparation of cell lysate

Cells were pelleted by centrifugation (1400 xg, 5 minutes, 4 °C). Pellets were resuspended in RIPA buffer containing protease inhibitor and lysed on ice, and clarified by centrifugation (12,000 xg, 10 minutes, 4 °C). Lysate was transferred to low-adhesion microcentrifuge tubes. Proteome concentrations were determined using BCA assay and lysate was diluted with PBS to appropriate working concentrations.

5.7.7 Gel-based ABPP in cell lysate

K562 cell lysate (1.0 $\mu\text{g}/\mu\text{L}$) in pH 7.4 PBS (50 μL) was treated with a prepared stock solution of DMSO or PAL probe (10 μM) in DMSO and incubated in the dark for 30 minutes at RT. Samples were irradiated for 30 minutes on ice at 365 nm. CuAAC was performed to append rhodamine-azide (1 μM final concentration) onto alkyne probe-labeled proteins. Samples were then diluted with 4x reducing Laemmli SDS sample loading buffer and heated at 95 °C for 5 minutes. The samples were separated on precast 4-20% Criterion TGX gels (Bio-Rad). Prior to analysis, gels were fixed in a solution of 10% acetic acid and 30% ethanol for 1 hour. Probe-labeled proteins were analyzed by in-gel fluorescence using a ChemiDoc MP (Bio-Rad). Protein loading was assessed by silver stain.

5.7.8 Gel-based ABPP in whole cells

K562 cells were treated with either DMSO vehicle or PAL probe (10 μM) for 1 hour in serum-free media and subsequently irradiated for 20 minutes at 365 nm. Lysate was prepared as described above. CuAAC was performed to append rhodamine-azide (1 μM final concentration) onto alkyne probe-labeled proteins. Samples were then diluted with 4x reducing Laemmli SDS sample loading buffer and heated at 95 °C for 5 minutes. The samples were separated on precast 4-20% Criterion TGX gels (Bio-Rad). Prior to analysis, gels were fixed in a solution of 10% acetic acid and 30% ethanol for 1 hour. Probe-labeled proteins were analyzed by in-gel fluorescence using a ChemiDoc MP (Bio-Rad). Protein loading was assessed by silver stain.

5.7.9 TMT proteomics

K562 cells at 80% confluency were treated with DMSO vehicle, **JQ1-iPr** (1 μM), or **JQ1-DA** (1 μM). After 1 hour of incubation, the cells were irradiated on ice for 30 minutes, harvested, and lysed via sonication. Each lysate was normalized to a concentration of 2

mg/mL, and 500 μ L of each lysate was transferred to a separate tube. To each tube containing 500 μ L of cell lysate, 10 μ L of 20 mM biotin picolyl azide (Sigma-Aldrich, 900912) in DMSO, 10 μ L of 50 mM TCEP in H₂O, 10 μ L of 50 mM CuSO₄ in H₂O, and 30 μ L of TBTA ligand (1.3 mg/mL in 1:4 DMSO/*t*-BuOH, Cayman Chemical, 18816) were added. The reaction mixture was incubated at room temperature for 60 minutes with agitation. The reaction was quenched by protein precipitation. After washing the protein pellets twice with cold MeOH (4 °C), the pellets were redissolved in 250 μ L of 1.2% SDS/PBS (w/v). The samples were heated at 95 °C for 5 minutes and then spun at 6500 xg for 5 minutes. The supernatant was transferred to new tubes containing 1.25 mL of PBS was added to the remaining sample to reduce the total SDS concentration to less than 0.2% SDS/PBS (w/v). 170 μ L of streptavidin agarose beads (ThermoFisher, 20353) were added to the lysate-containing tubes, and the samples were incubated at 4 °C on a rotator overnight. After incubation, the samples were brought to 23 °C, and the beads were spun down in a centrifuge (1300 xg, 2 minutes). The supernatant was removed, and the beads were washed three more times with 500 μ L of PBS and 500 μ L of H₂O. The beads were resuspended in 50 μ L of 0.1% SDS/PBS (w/v) and the samples were further treated with DTT (90 μ g per 1 mg protein) for 20 minutes at 65 °C. Iodoacetamide (237 μ g per 1 mg protein) was added and the samples were incubated for 1 hour at 37 °C. After incubation, 25.8 μ L of a premixed solution containing 25 μ L 100 mM triethylammonium biocarbonate (TEAB), 0.25 μ L 100 mM CaCl₂, and 0.5 μ L 20 mg/mL trypsin (Promega V5111) was added to each sample. The samples were incubated horizontally overnight at 37 °C with agitation. The collected peptide-containing samples were dried using a vacufuge and resuspended in 100 μ L of 50 mM TEAB before labeling using commercially available TMT10plex tags (ThermoFisher, PN 90110). After labeling, 30 μ g of each labeled sample was combined and dried using a vacufuge. Dried samples were redissolved with 300 μ L of 0.1% TFA in H₂O and further fractionated using high-pH reversed-phase peptide fractionation kits (ThermoFisher, PN 84868) following the manufacturer's protocol.

Mass spectrometry analysis was performed on an Orbitrap Eclipse Tribrid Mass Spectrometer with a High Field Asymmetric Waveform Ion Mobility (FAIMS Pro) Interface (Thermo Scientific) with an UltiMate 3000 Nano Flow Rapid Separation LCnano System (Thermo Scientific). Off-line fractionated samples (5 μ L aliquot of 15 μ L sample) were injected via an autosampler (Thermo Scientific) onto a 5 μ L sample loop which was subsequently eluted onto an Acclaim PepMap 100 C18 HPLC column (75 μ m x 50 cm, nanoViper). Peptides were separated at a flow rate of 0.3 μ L/min using the following gradient: 2 % buffer B (100% acetonitrile with 0.1% formic acid) in buffer A (95:5 water:acetonitrile, 0.1% formic acid) for 5 minutes, followed by a gradient from 2 – 40 % buffer B from 5 – 159 minutes, 40 – 95% buffer B from 159 to 160 minutes, holding at 95% B from 160 – 179 minutes, 95 – 2% buffer B from 179 – 180 minutes, and then 2% buffer B from 180 – 200 minutes. Voltage applied to the nano-LC electrospray ionization source was 2.1 kV. Data was acquired through an MS1 master scan (Orbitrap analysis, resolution 120,000, 400-1800 m/z, RF lens 30%, heated capillary temperature 250 °C) with dynamic exclusion enabled (repeat count 1, duration 60 s). Data-dependent data acquisition comprised a full MS1 scan followed by sequential MS2 scans based on 2 s cycle times. FAIMS compensation voltages (CV) of -35, -45, and -55 were applied. MS2

analysis consisted of: quadrupole isolation window of 0.7 m/z of precursor ion followed by higher energy collision dissociation (HCD) energy of 38% with a orbitrap resolution of 50,000.

5.7.10 GO analysis

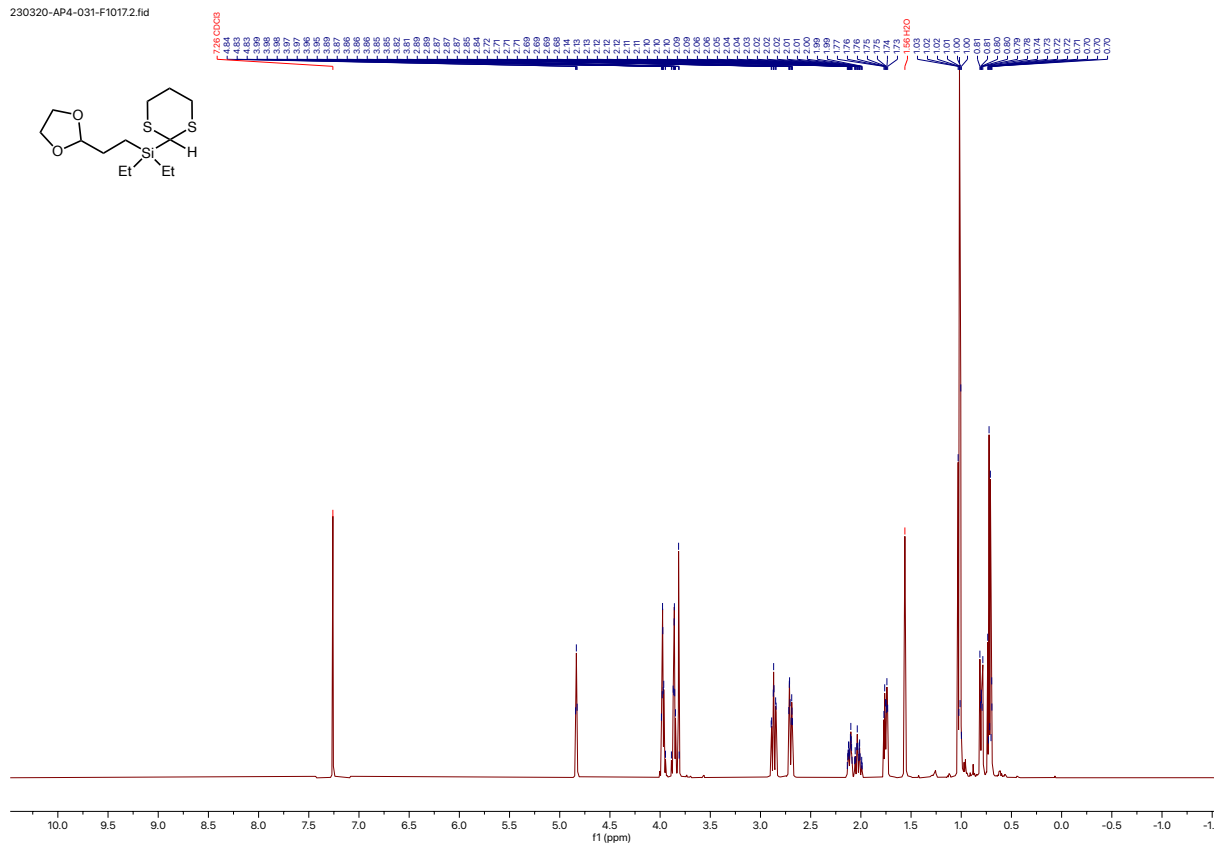
Gene ontology (GO) analysis for subcellular localization of labeled proteins was performed using the DAVID Gene Functional Classification Tool at <https://david.ncifcrf.gov/summary.jsp>.

5.7.11 LogP calculation

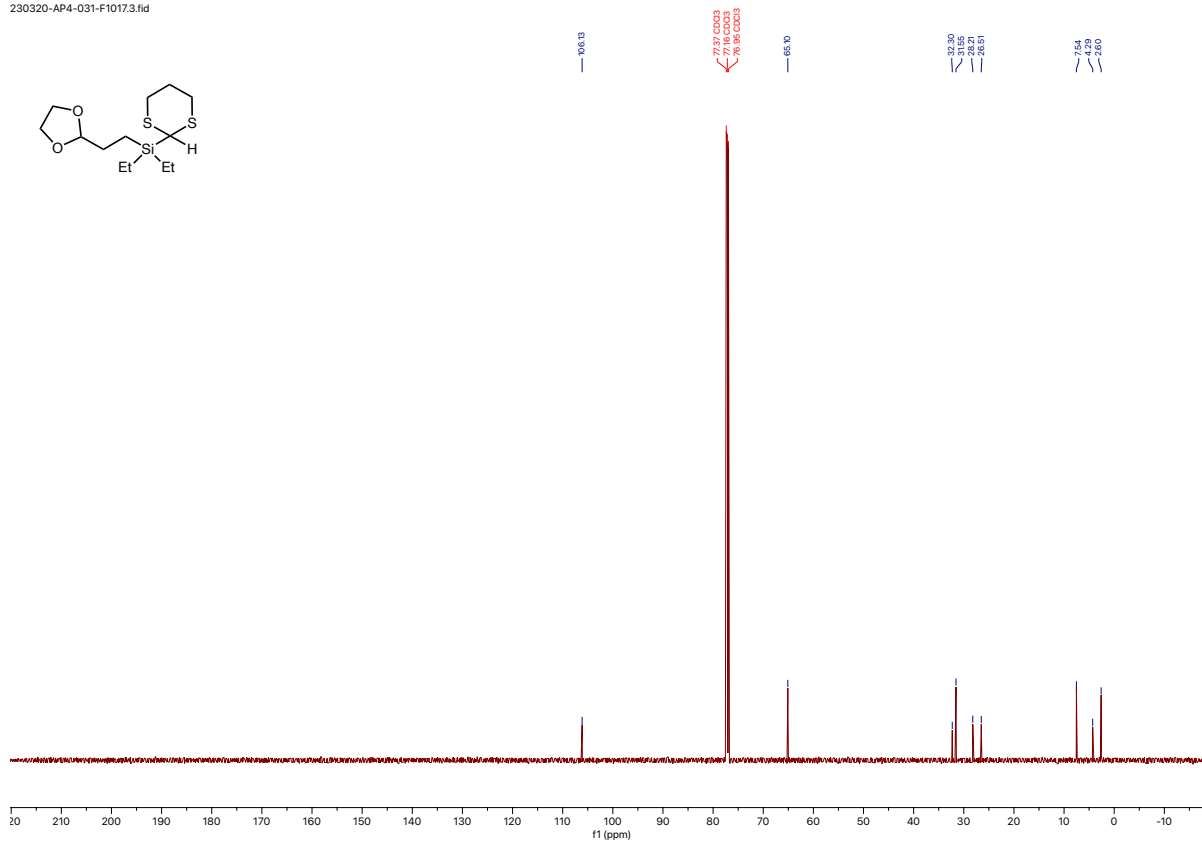
Calculation of predicted logP values were performed using the Molinspiration Cheminformatics free web services at <https://www.molinspiration.com>.

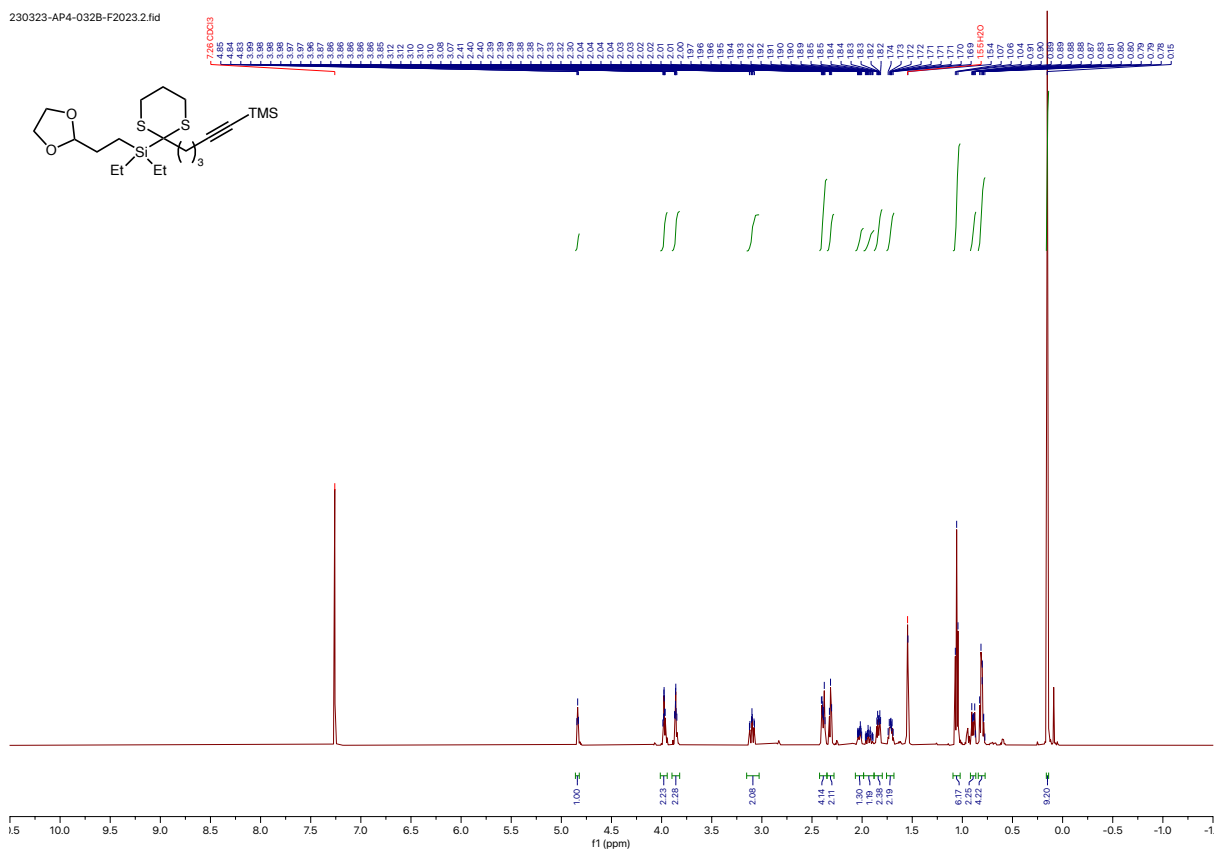
5.7.12 NMR spectra

230320-AP4-031-F1017.2.fid

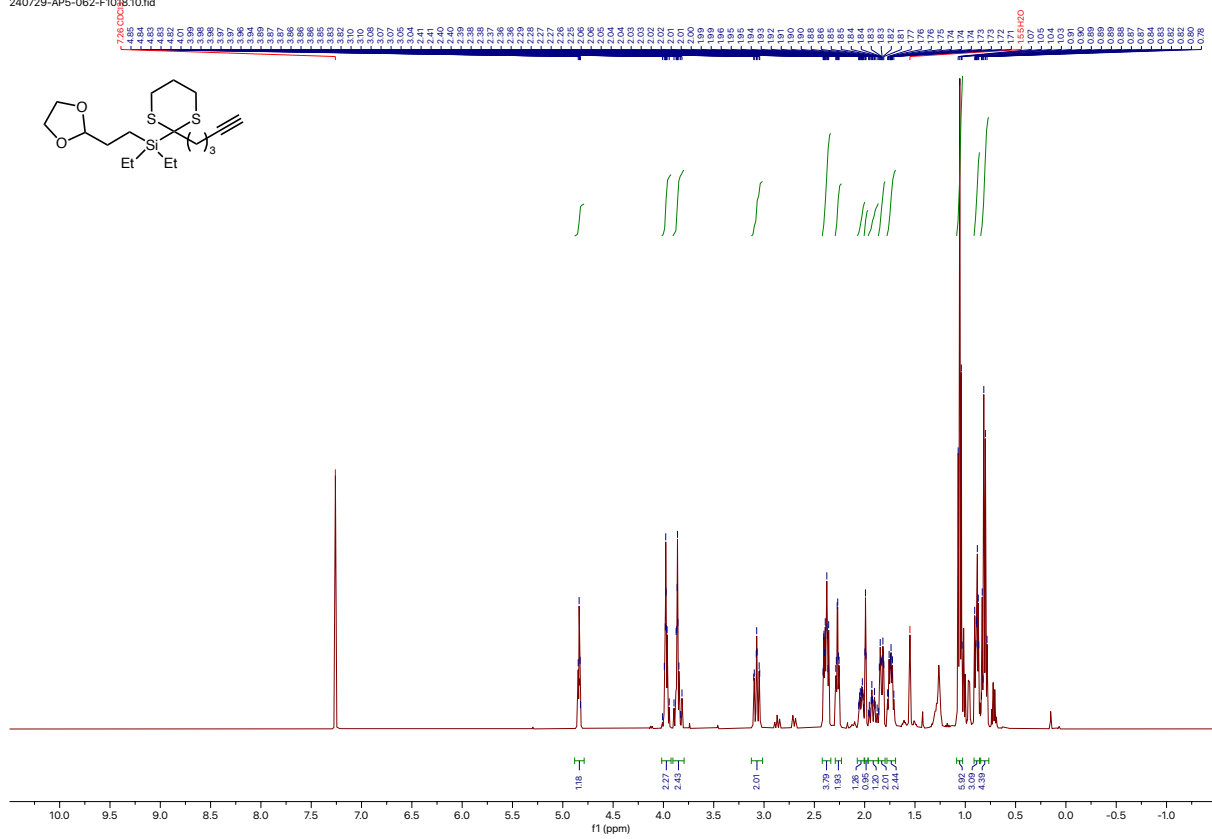


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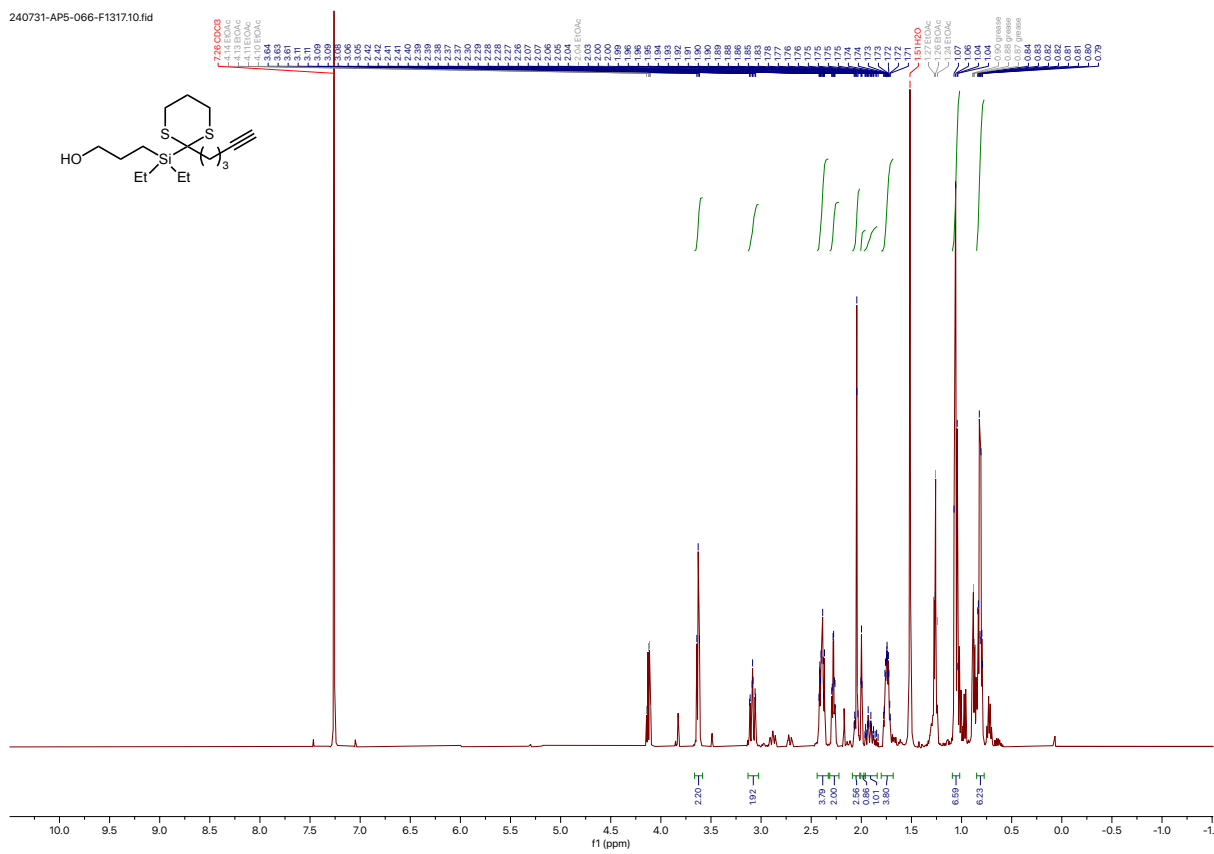


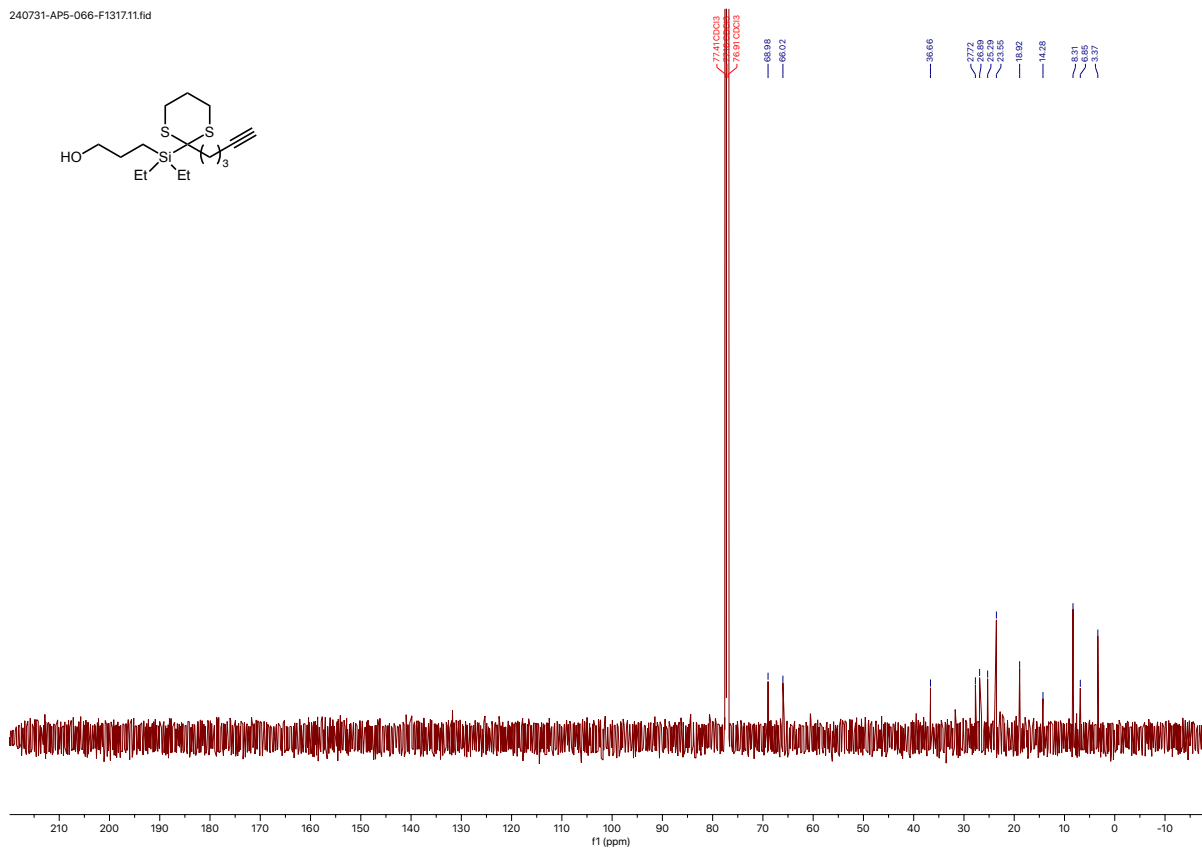




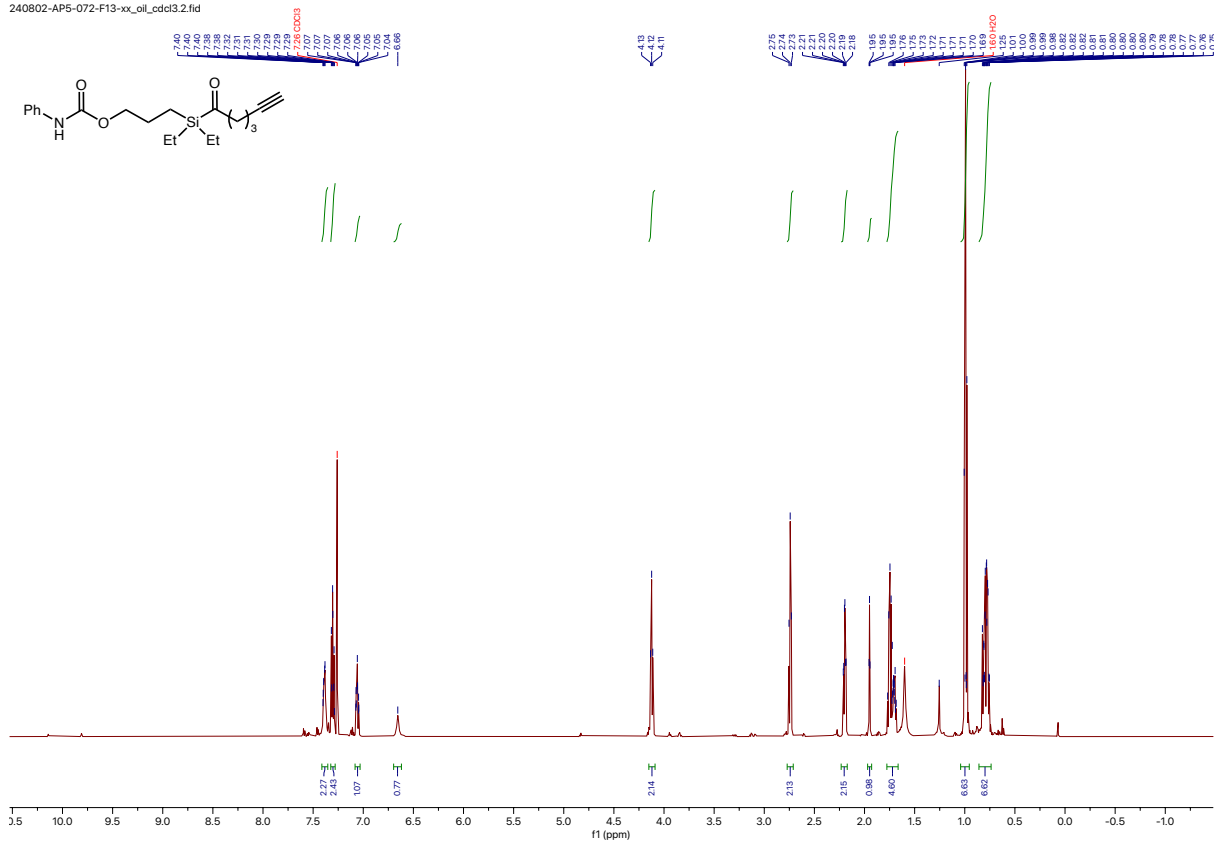


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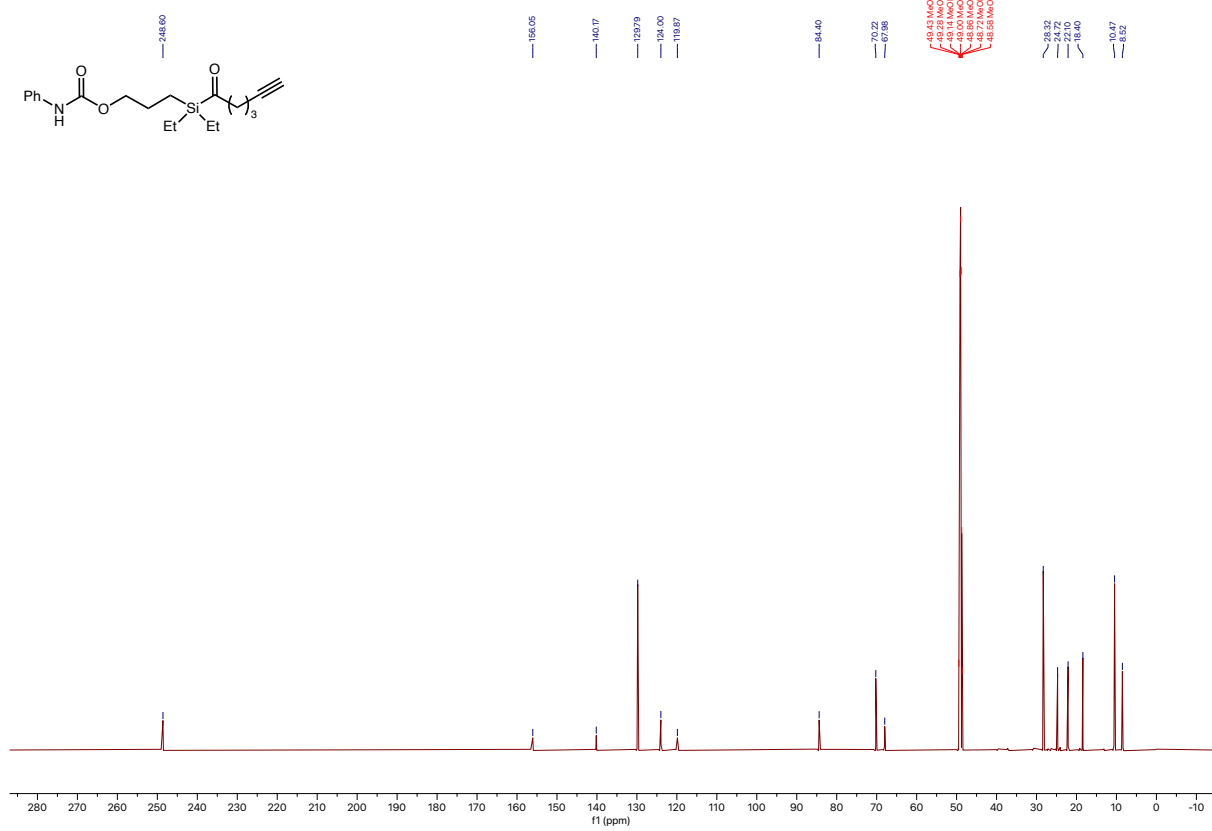


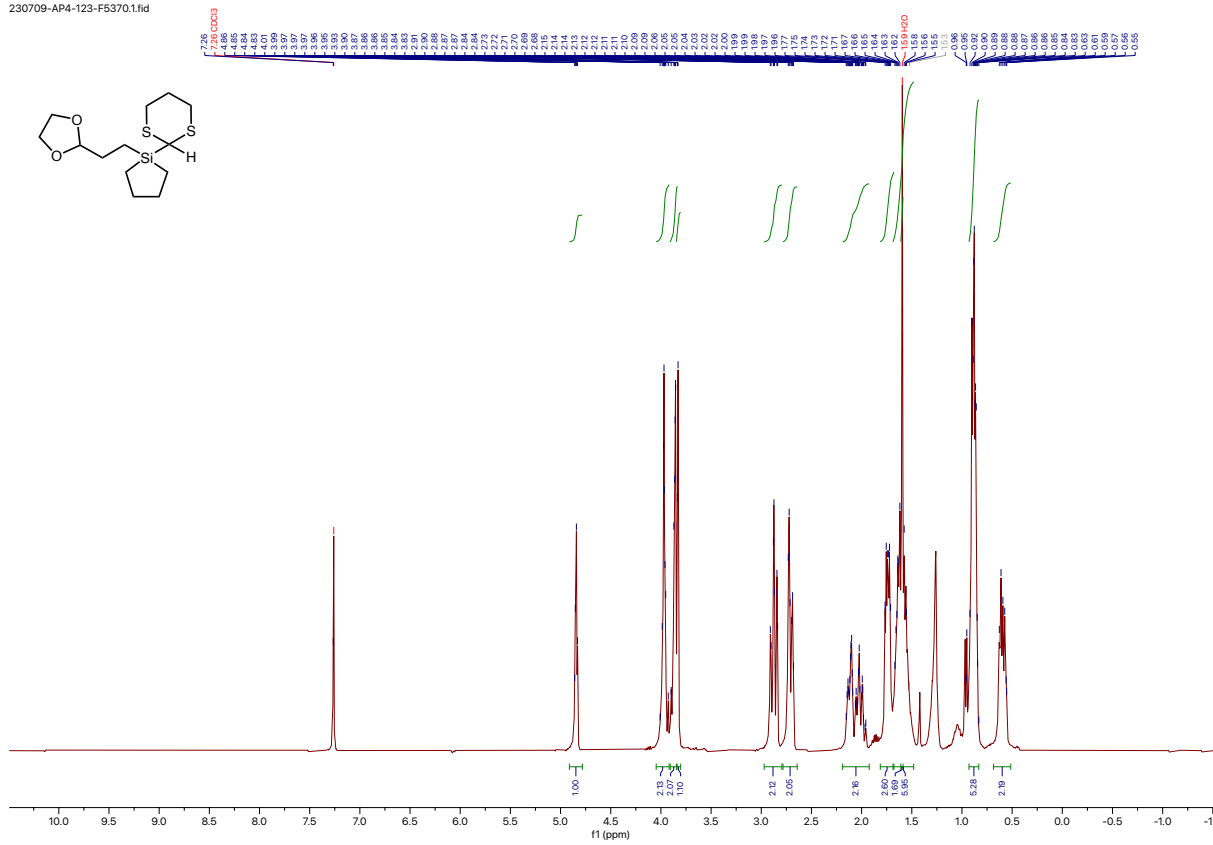


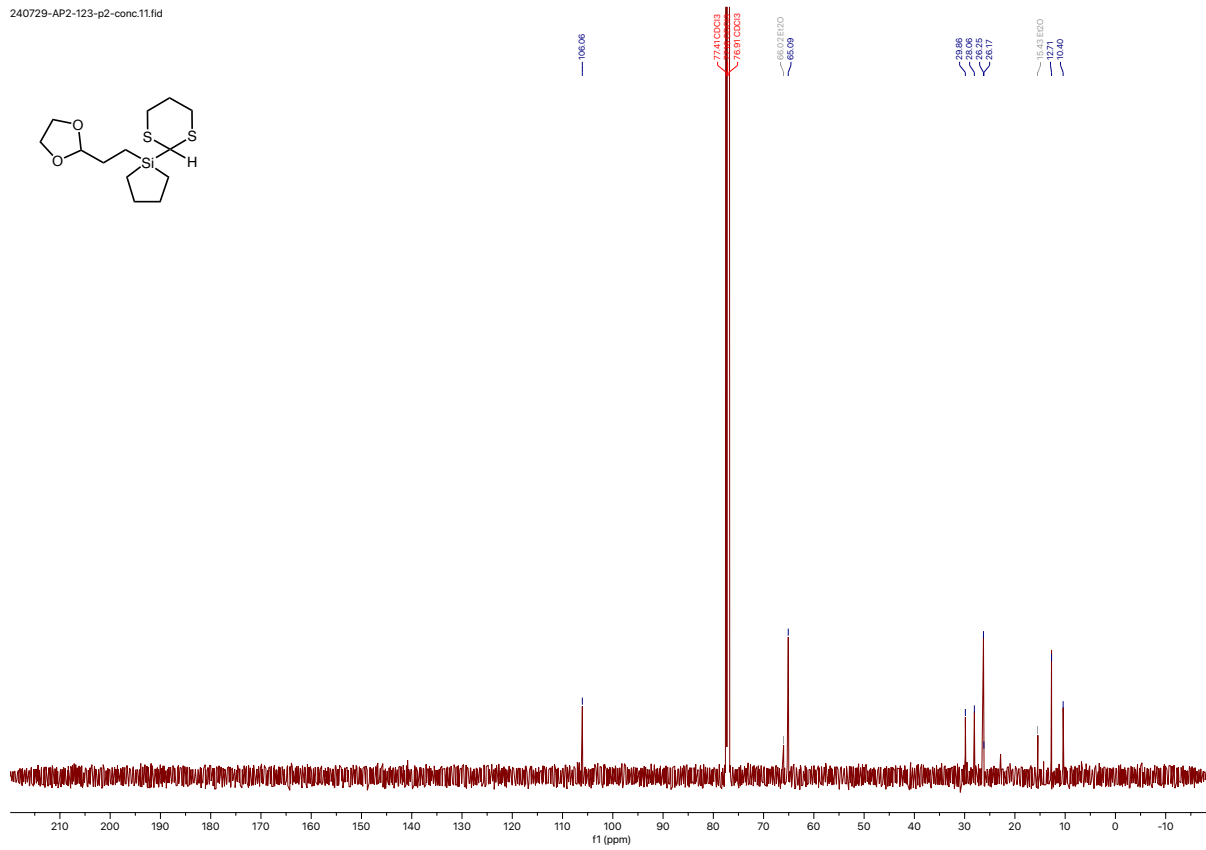
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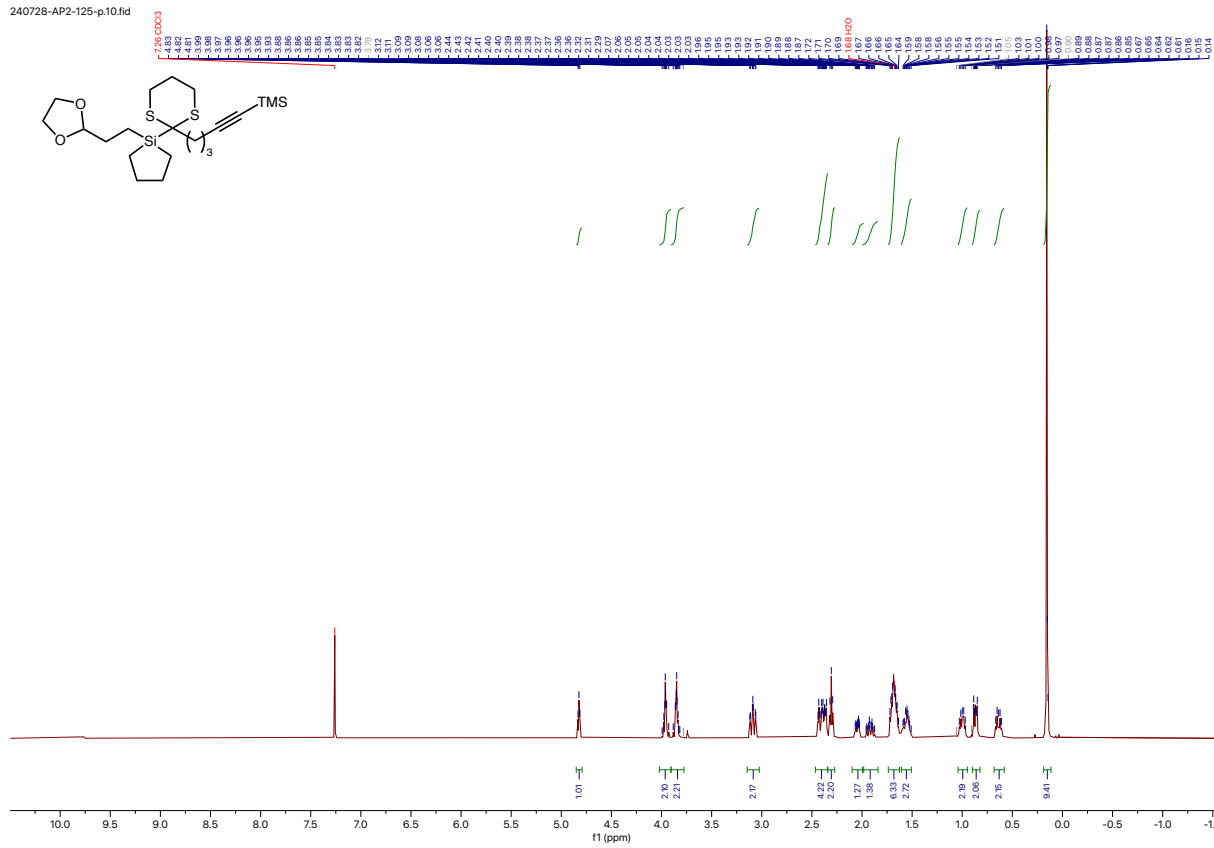


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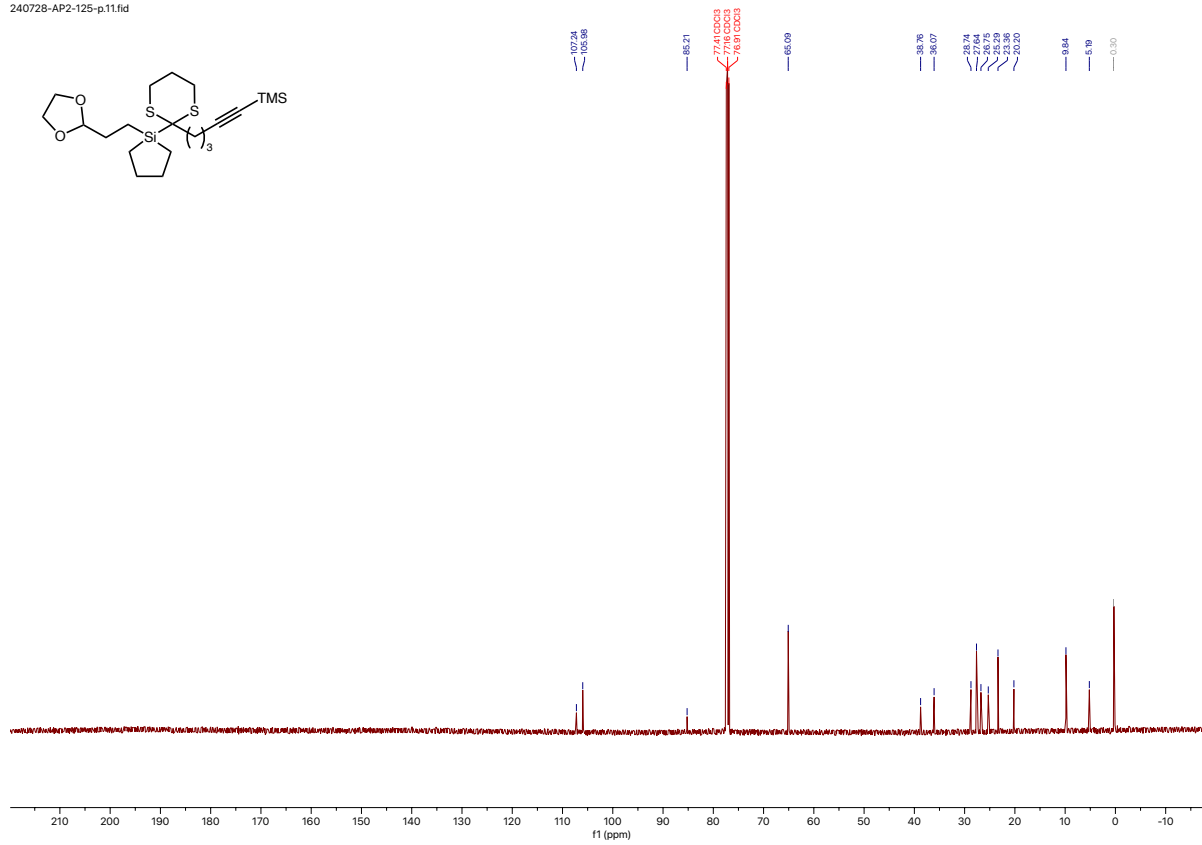




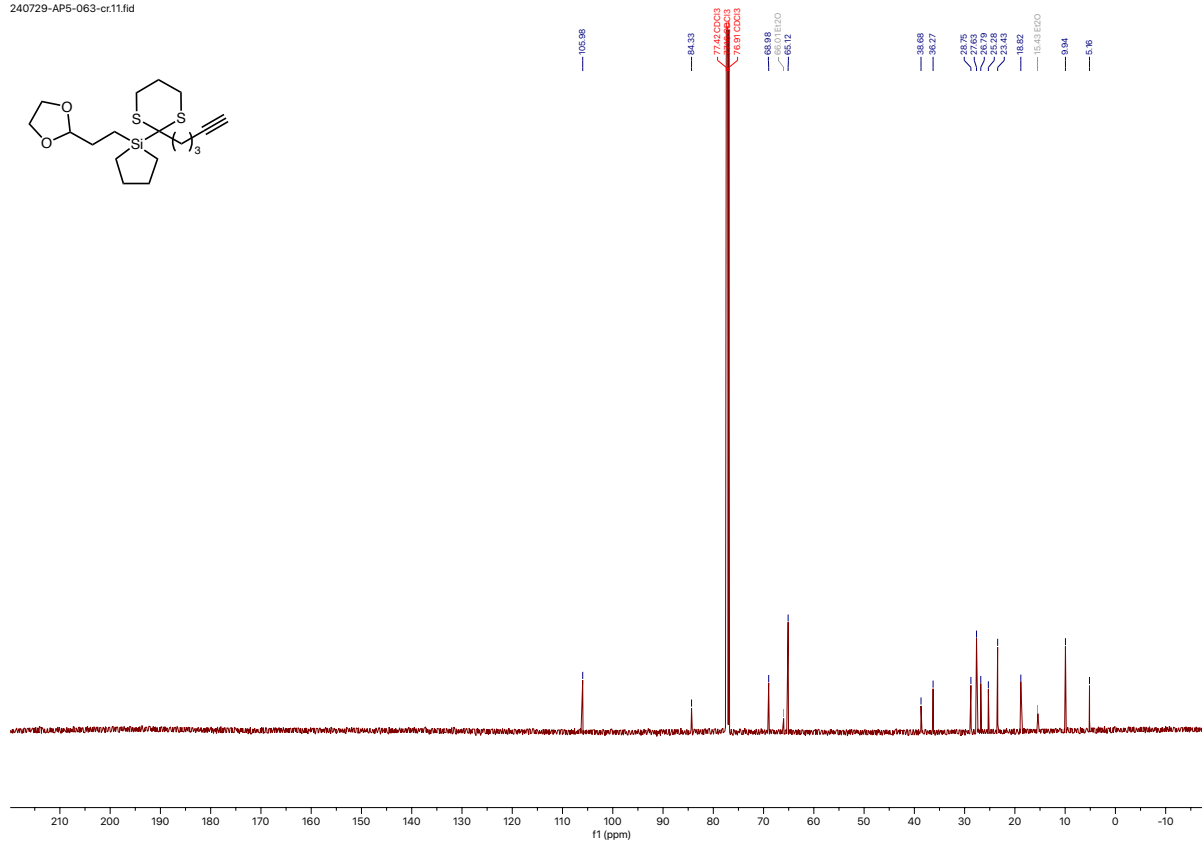




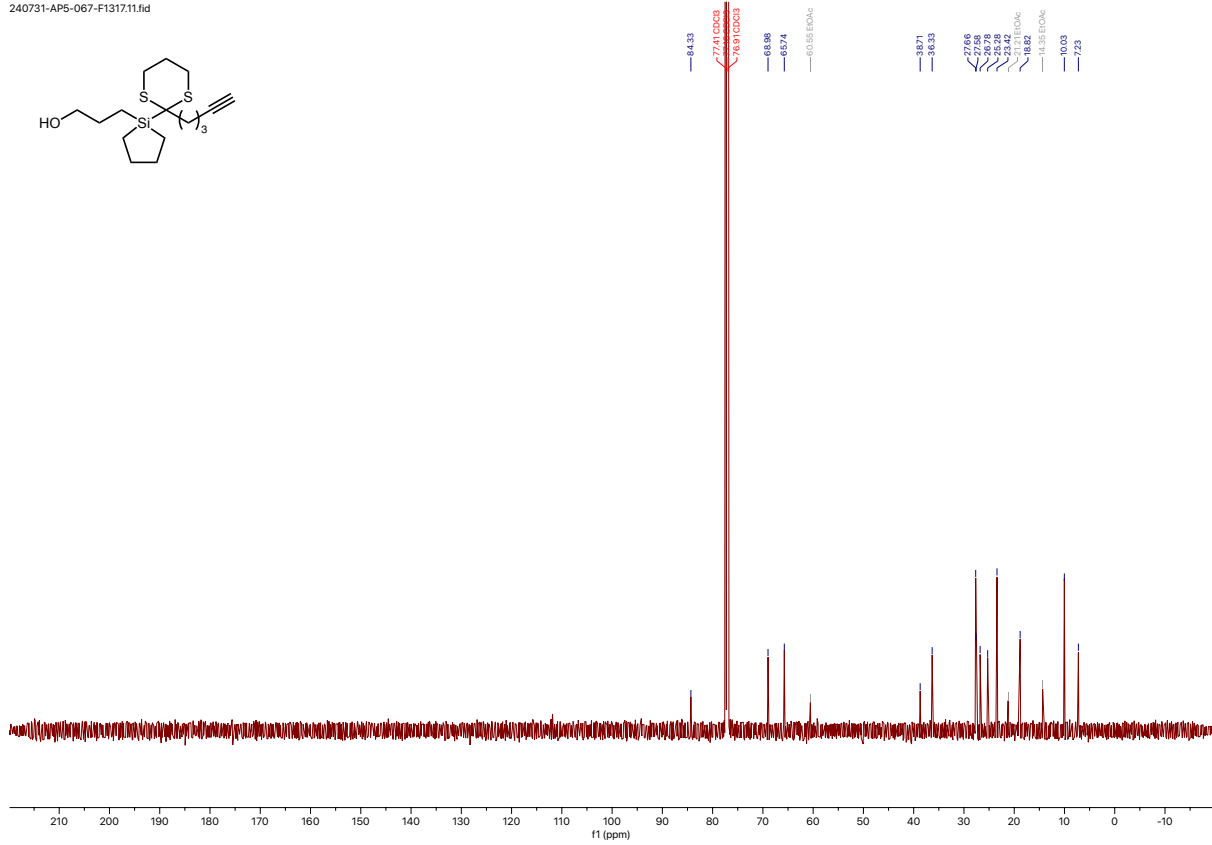
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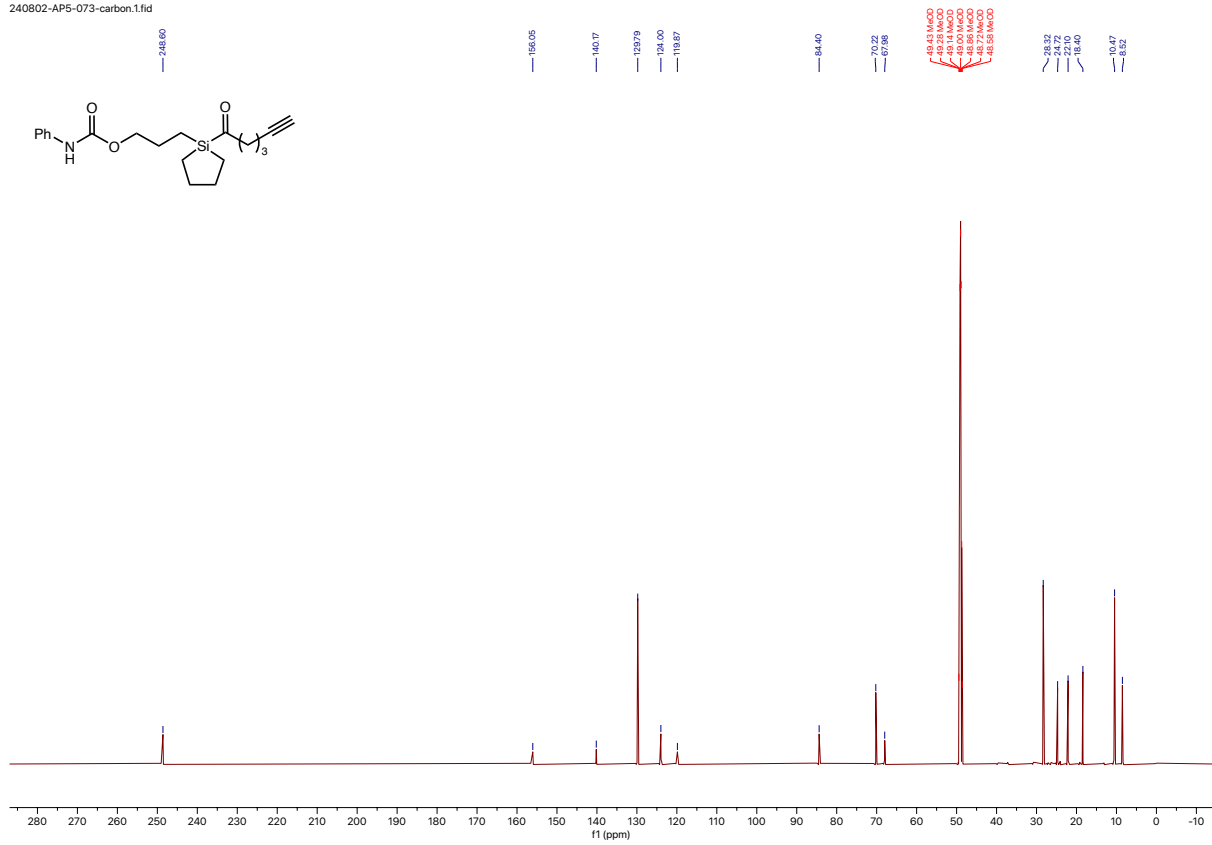
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