

Development of Novel Chemical Probes for the Elucidation of Bacterial and Viral Pathogenesis and Mechanisms of Infection

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Abstract

Bioactive small molecules have been a major source of therapeutics for the treatment of human disease. Advances in synthetic and semi-synthetic methods have greatly expanded the repertoire of available small molecules for use in the study of the functional characteristics of biological systems. Chemical proteomic methods, which in some cases harness bioactive small molecules to enhance proteomic techniques, have been developed to study protein targets in physiological conditions. Such approaches allow for maintenance of post-translational modifications, protein-protein interactions, and interactions with endogenous regulators. Functional proteomics, using tandem-labeling strategies that harness bioorthogonal chemistry, have permitted the study of the molecular targets of bioactive small molecules. To this end, herein bio-orthogonal methods and functional proteomics to study the molecular targets of many bioactive small molecules have been developed. In **chapter 2**, three previously reported bioactive small molecules were screened for activity against HCV replication and 6-hydroxydopamine (6-OHDA) was identified as a potential inhibitor. By generating a novel chemical probe based on 6-OHDA, we determined that 6-OHDA was able to covalently modify a large range of biological targets and initiate cellular oxidative stress, both of which contribute to the antiviral activity of 6-OHDA. In **chapter 3**, an affinity probe (AfBP) based on the methyltransferase inhibitor sinefungin was developed and used to profile eukaryotic enzyme targets. Using in-gel fluorescence scanning and mass spectrometry analysis, we identified several proteins including methyltransferases and other candidate proteins that may be associated with epigenetic mechanisms. In **chapter 4**, the characterization of the bacterial and eukaryotic targets of a novel antibacterial small molecule armeniaspirole A is reported. Using a medicinal chemistry strategy, the Cl-ARM-A-yne affinity-based probe was developed and was capable of covalently capturing proteogenic molecular targets.

Following mass spectrometry analysis, several lead molecular targets were identified that may be implicated in the antimicrobial effects of armeniaspirole A. Herein, the targets of a novel chemical probe allowed the characterization and elucidation of the previously unknown molecular targets of armeniaspirole A. Finally, related probes were explored as novel photoaffinity ligands of cytosporone B, a small molecule agonist for the nuclear orphan receptor 77, which could be highly effective at capturing and analysing the Nur77 associated co-activators or co-repressors towards exploring the biology of this mysterious receptor.

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List of Abbreviations

Symbol	Definition
2DE	Two-dimensional gel electrophoresis
3'-UTR	3'-untranslated region
5'-UTR	5'-untranslated region
6OHDA	6-Hydroxydopamine
ABP	Activity-based probe
ABPP	Activity-based protein profiling
AF	Activation domain
AfBP	Affinity-based protein profiling
Ago	Argonaute protein
AMR	Antimicrobial resistance
ARM	Armeniaspirole
ATA	Aurintricarboxylic acid
BpyneSF	Benzophenone sinefingn
Bp	Benzophenone
CBI	Call wall biosynthesis inhibitor
CE/MS	capillary electrophoresis mass spectrometry
CYP450	Cytochrome P450
DBD	DNA-binding domain
DCF	2',7'-dichlorofluorescein
DCFDA	2',7'-dichlorofluorescein diacetate
DGCR8	diGeorge syndrome chromosomal region 8
DiME	Dimethyl labeling
ER	Estrogen receptor
ERG	ETS-related gene
EV	Ebola Virus
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GR	Glucocorticoid receptor
GSH	Glutathione
H ₂ O ₂	Hydrogen peroxide
HCP	Health care practitioners
HCV	Hepatitis C virus
HDAC	Histone deacetylase
Hek293	Human embryonic kidney 293 cells
HIV	Human immunodeficiency virus
HRE	Hormonal response element
HSP	Heat shock protein

HTS	High-throughput screen
Huh7	Hepatocellular carcinoma cell line
IC	Inhibitory concentration
iTRAQ	Isobaric Tags for Relative and Absolute Quantification
kDa	Kilodalton
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LBD	Ligand binding domain
LTQ	Linear ion trap
MDR	Multi-drug resistant
mg	Milligram
MIC	Minimum inhibitory concentration
mmol	Millimole
miRNA	MicroRNA
MRSA	Methicillin-resistant streptococcus aureus
MS	Mass spectrometry
MT	Methyltransferase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MudPIT	Multidimensional tandem mass spectrometry
NP	Natural product
NR	Nuclear receptor
Nur77	Nuclear receptor 77
PAL	Photoaffinity labels
PBS	Phosphate buffered saline
PEG	Polyethelyne glycol linker
PPAR	Peroxisome proliferator-activated receptor
PPI	Protein-protein interactions
Pre-miRNA	precursor microRNA
PRG	Photoreactive group
Pri-miRNA	Primary microRNA
PRSP	Penicillin-resistant Streptococcus pneumonia
PTM	Post-translational modification
RISC	RNA induced silencing complex
RPM	Rotations per minute
ROS	Reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SAR	Structure-activity relationship
SC	Subcutaneous
SERM	Selective estrogen receptor modulator
SF	Sinefungin
SILAC	Stable Isotope Labeling by Amino Acids in Cell Culture
SM	Small molecule

SNP	Single nucleotide polymorphisms
SPAAC	Strain-promoted azide-alkyne cycloadditions
SUR	Suramin
TBHP	Tert-butyl hydrogen peroxide
TMT	Tandem mass tags
VRE	Vancomycin-resistant <i>Enterobacterium faecium</i>

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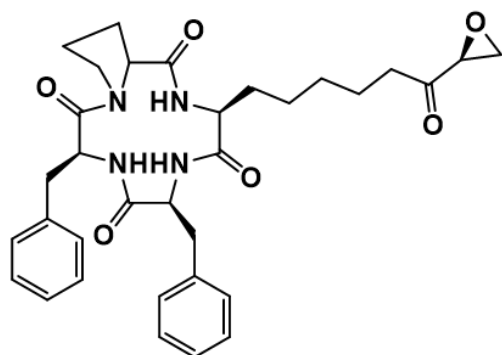
“We aspire to attain our highest potential”

-Métis Nation of Ontario

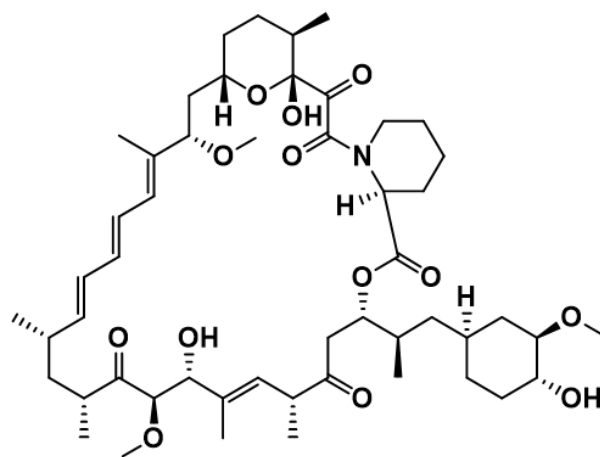
Chapter 1. Introduction to bioactive small molecules and chemical biology

1.1 Bioactive Small Molecules as Chemical Probes

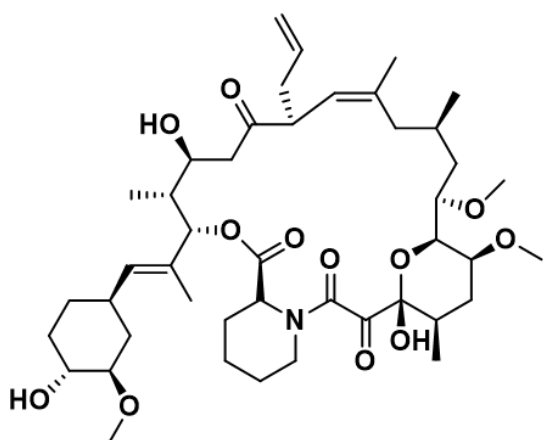
Historically, natural products (NPs) extracted from organisms found in the natural environment have been the principle source of lead compounds for development of therapeutics for human health[1]. Advances in synthetic organic chemistry and methodology have expanded the toolkit of available synthetic and semi-synthetic methods to derive analogues of NPs[2-4]. To-date, approximately 40% of available therapeutic agents are either natural products or natural product-derived molecules[5, 6]. Given that NPs are presumed to have evolved to selectively and specifically bind and fit biological macromolecules like proteins, they are privileged structures that can be harnessed not only for therapeutic drug development but also to develop bioactive small molecules as chemical probes to explore the functioning of biological systems [7-11]. There are several advantages to using NP-derived bioactive small molecules in studying the function of biological systems, given that they can penetrate the cell membrane, bind their cognate receptors, and induce a change in the cell or organisms under study[12]. For example, bioactive small molecules may afford spatial and temporal control over protein function without any concurrent changes in protein expression and the consequences of treatment often occur rapidly[13]. Additionally, small-molecule treatments can be tailored to allow for a desired phenotypic response through the control of probe concentration and duration of probe treatment [14, 15]. For example, Morgan and colleagues were able to elucidate the temporal requirements of protein tyrosine phosphorylation in sperm following treatment with the chemical probe 1NM-PP1[14]. The final advantage of NP-derived chemical probes is that probe treatment is often reversible, following either metabolic degradation or experimental wash-out and treatment is conditional on a specific experimental method[16, 17].



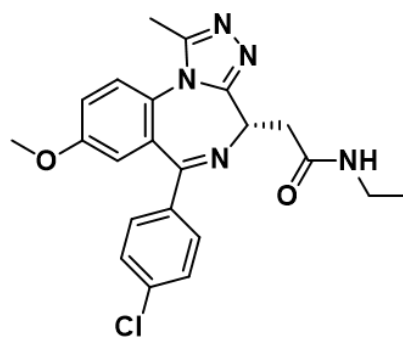
Trapoxin



Rapamycin



FK₅₀₆



I-BET

Figure 1.1. Examples of bioactive small molecules used as chemical probes.

One of the first reported studies to use a natural product based chemical probe was undertaken by Schreiber and co-workers in 1996 [18, 19]. In this work, Schreiber and colleagues developed a resin, taking advantage of the electrophilic moiety found on trapoxin, to enrich and identify the protein targets of the bioactive small molecule trapoxin (**figure 1.1**). Trapoxin was initially isolated from the fungi *Helicoma ambiens* and was previously shown to covalently modify histone deacetylases through nucleophilic attack by active-site amino acid residues[20, 21]. Using this method, they identified a previously unknown histone deacetylase that was 60% identical to Rpd3, a known transcriptional repressor[19]. Not only can bioactive small molecules be harnessed to identify new biological functionalities, but they can also be used to identify distinct biological pathways. For example, rapamycin and FK506 have been used to expand our understanding of the diverse functions of biological pathways (**figure 1.1**). Previous work has shown that the biological function of rapamycin, which has been implicated in cell growth, proliferation, cell survival, protein synthesis and transcription, is governed by its interaction with peptidyl-prolyl *cis/trans* isomerase or FK-binding protein (FKBP) [22, 23]. Initially identified as an antibiotic, rapamycin was eventually used as a potent immunosuppressive and antiproliferative agent[24]. Though similar in structure to rapamycin, immunosuppressive drug FK506 is a known target of FKBP and functions through the T-cell signal transduction and interleukin-2 (IL-2) production, whereas rapamycin-FKBP complex targets mammalian target of rapamycin (mTOR), which inhibits response to IL-2[25-27]. Finally, Nicodeme and co-workers identified the synthetic compound I-BET through a cell-based screen for modulators of apolipoprotein AI production, which ultimately led to the discovery of a previously unknown regulatory bromodomain[28]. These compounds highlight how chemical probes can be harnessed to understand the function of biological systems.

1.2 Target-centric Strategies

1.2.1 *Reverse genetics*

Following the sequencing of the human genome, genetic information was harnessed to study the function of proteins on metabolic processes through the manipulation of genetic sequences. In reverse genetic methods, a candidate gene is manipulated through genetic engineering, mutation, deletion, or functional ablation to ascertain the phenotypic responses[29-33]. However, a major limitation to reverse genetic methods is that pleiotropic effects may result following genetic manipulation. For example, previous work has shown that gene deletion mutations can simultaneously cause multiple mutant phenotypes[34-36]. The major challenge following gene deletion mutation is identifying whether a specific mutant phenotype results from the loss of function from a single gene or of multiple functions encoded by a single gene. Complicating reverse genetic analysis is the complexity of human diseases, of which the majority are often caused by subtle genetic changes, like point mutations, insertions, deletions, large deletions, and chromosomal re-arrangements, that impact the structure and function of proteins[37]. Reverse genetic methods are ill-suited to produce subtle genetic changes given the requirement to incorporate positive selection markers and recombinase recognition sites, which may affect the phenotype, and given that human diseases may not result from a single gene but may be caused by multiple mutations, which would require multiple genetic mutations to induce the disease phenotype[37].

1.2.2 *Reverse chemical genetics*

Driven by advances and developments in molecular biology, reverse chemical genetics has been the pre-dominant method used to develop first-in-class medicines[38-42]. Referred to as a

hypothesis-based method, the identification of a known protein allows the researcher to develop a hypothesis that links the role of the protein of interest or gene to an observable phenotype (**figure 1.2**). Screening can identify different types of interactions and thus new biology. In this method, a protein of interest or clinically-validated molecular target, which responds dose-dependently to a small molecule treatment and mediates a known disease process, undergoes high-throughput screening (HTS) against a library of bioactive small molecules. HTS permits the identification of bioactive small molecules that target the protein of interest and permits the study of the mode of action of the SM[43] (**figure 1.3**). This method has been used to identify inhibitors and activators of enzymes, receptors, and permitted the study of protein-protein interactions[41, 42, 44]. For

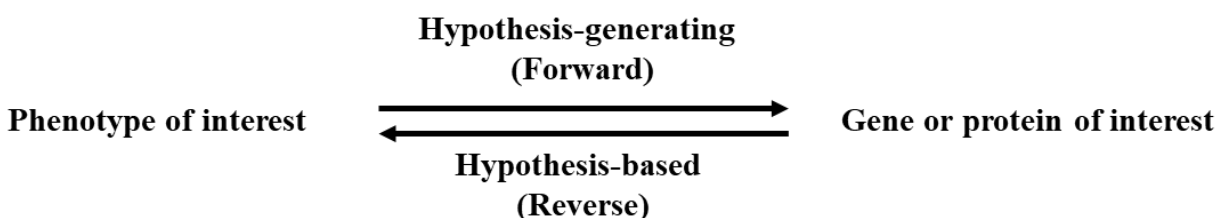


Figure 1.2. Target-based vs. chemocentric-based strategies for target deconvolution.

example, HTS screening against peptidase domain (PEP), which is important in quorum-sensing in bacterial organisms, resulted in the identification of a small molecule that could allosterically inhibit PEP and biofilm formation[45].

Reverse chemical genetics is advantageous compared with genetic methods because no direct perturbation of genetic sequences is required, reducing or eliminating compensatory changes in an organism. It further permits the study of the mode of action of a bioactive small molecule, given the reliability of enzyme assays and bioactive small molecule design based on structural biology[46]. Despite the success of reverse chemical genetics, several challenges remain.

Validation of the target protein's role in a biochemical or metabolic pathway, in either disease or healthy state, is complicated given that proteins are involved in multi-protein pathways[47-50]. As a result, establishing a link between small molecule targeting of a protein and induced phenotypes is complicated given the polypharmacological nature of bioactive small molecules[51]. Finally, biochemical assays often use recombinant protein or protein fragments to test bioactive small molecules instead of full-length endogenous proteins. These recombinant proteins do not necessarily reflect the conformation or activity of proteins in a physiological environment due to the lack of interacting regulatory domains, endogenous activators and inactivators, incorrect protein folding, or post-translational modifications[52]. As a result, the efficacy of a bioactive small molecule may not be reproduced in the cell or animal model[12].

1.3 Phenotype-based Strategies

1.3.1 Forward chemical genetics

In forward genetics, a phenotype of interest is studied under experimental selection pressure and the gene or genes responsible for a phenotype are identified that link the phenotype change to the genetic etiology[29, 53]. This method permits the generation of hypotheses that link the change in phenotype to a given protein of interest or gene (**figure 1.2**). Although forward genetic methods permit the study of a link between a genetic mutation and a disease or non-disease mutation, collecting genetic information on a cellular population can be laborious. In recent years, the contribution of forward chemical genetic strategies to the discovery of first-in-class medicines have exceeded the contributions of reverse chemical genetic strategies[54]. These first-in-class medicines include the blood cholesterol regulator ezetimibe and the histone deacetylase inhibitor and anti-tumor agent vorinostat[55, 56]. Phenotype-based methods screen bioactive small

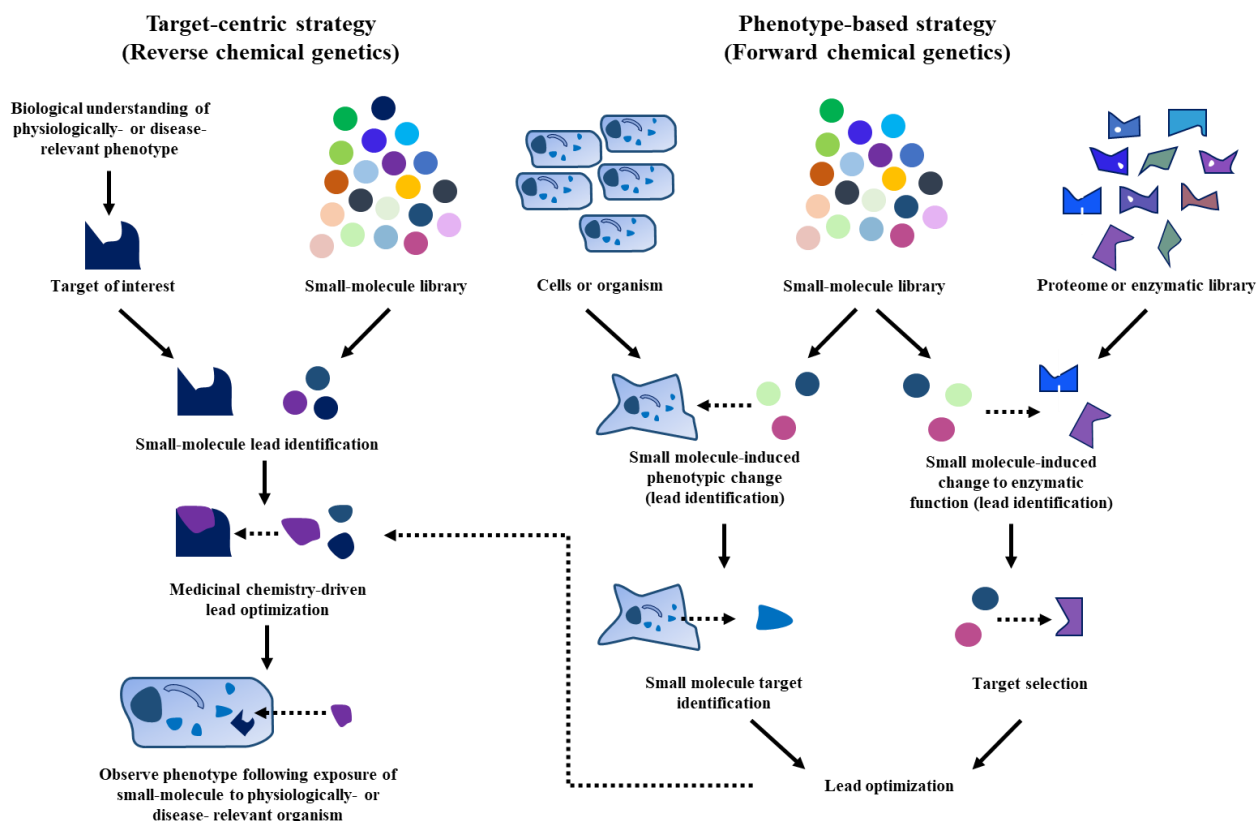


Figure 1.3. Target- and phenotype-based strategy for bioactive small molecule discovery. In a target-centric strategy, a protein of interest or clinically-validated molecular target, which responds dose-dependently to a small molecule treatment and mediates a known disease process, undergoes high-throughput screening (HTS) against a library of bioactive small molecules. This strategy permits the identification of bioactive small molecule targets for proteins of interest and allows the study of the mode of action of protein targets. Following lead identification, medicinal chemistry-driven optimization is undertaken followed by exposure to a physiologically- or disease- relevant organism to observe a phenotypic change. In a phenotype-based strategy, bioactive small molecules are screened against a physiologically relevant cell model or a proteome or enzymatic library to ascertain its impact on a biological process or phenotype.

molecules against a physiologically relevant cell model to ascertain its impact on a biological process or phenotype[57-59] (**figure 1.3**). In contrast with reverse chemical genetics, forward chemical genetics permit the evaluation of the cytotoxicity and the cell permeability of the small molecule simultaneously. This method does not require an *a priori* knowledge of the molecular target, which is advantageous given that the phenotypic response following treatment reflects the functional impact on a protein of interest in a biological pathway[12]. Several limitations of forward chemical genetics exist, including laborious nature of target validation and the low number of bioactive SMs that produce a desired phenotype[60, 61]. Kwok *et. al.* screened 14,100 small molecules against *Caenorhabditis elegans* (*C. elegans*) in search of a calcium channel antagonist and only 3.5% of screened drugs displayed the desired phenotype[62].

Since bioactive small molecules are polypharmacological, phenotypic responses may result from “off-target” interactions with non-cognate proteins or with more than one cognate protein[63]. For example, Yu and colleagues identified a nucleoside analog IB-MECA that could agonise both nuclear receptors (NR) homologs peroxisome proliferator-active receptors (PPARs) delta (Δ) and gamma (γ)[64]. Additionally, minor phenotypic changes may reflect the sum effect of multiple protein targets, thus requiring characterization of the biologically important target and off-targets of bioactive small molecules prior to development as therapeutic agents[65]. Following target identification, validation often requires biochemical assays which do not reflect the myriad of protein folding, protein-protein interactions, or endogenous interactions with regulator proteins or substrate[52]. Despite the challenges listed above, forward chemical genetics can be harnessed to identify small molecule chemical probes that induce phenotypic changes in biological systems[66].

1.4 Chemical Proteomics Methods

There exist approximately 20,000 protein coding genes in the human genome, of which a small fraction of these genes are targeted by therapeutic small molecules[67]. Most of the approximately 200 proteins that have been validated as therapeutic targets are cell surface proteins (G-protein coupled receptors (GPCR), ion channels, protein transporter) and intracellular proteins (kinases, nuclear receptors, and metabolic enzymes)[67]. Previous methods for identification of bioactive small molecules relied on the use of protein-based assays not only to identify SM inhibitors for uncharacterized enzymes but also for those SM that have been synthetically inaccessible to probe development[68-71]. The main disadvantage of these protein-based methods is that the scope of the study is limited to available protein assays.

In response to the limitations of protein-based assays, chemical methods using proteomics were developed to study the proteome in its native cellular environment. Chemoproteomic methods permit the study of proteins in their native, physiological environment like cell extracts, cell fractions, cell-based assays or animal-based models[52]. Chemoproteomic methods are advantageous given that experimental conditions can be carefully controlled to maintain protein folding and integrity, post-translations modifications (PTM), protein-protein interactions (PPI), and interactions with endogenous regulators[72, 73]. Furthermore, target deconvolution and identification can be undertaken without the need for extensive biochemical assays, thereby increasing the efficiency of target deconvolution and identification. Chemical proteomic methods have been used extensively to identify small molecule molecular targets and their mode of action and impact on protein function, which has greatly expanded our understanding of the molecular consequences of SM treatment[13, 46, 74].

1.4.1 *Proteomics*

Since the completion of the Human Genome Project, whole-genome sequencing has revealed that the human genome comprises of approximately 20,000 protein-coding genes and approximately 10,000 proteins are expressed in a cell[67, 75]. Given that proteins are responsible for executing the effects of genes through catalysis, signalling, and physical interactions with other biomolecules, the study of the whole proteome, defined as proteomics, is fundamental in understanding the role of proteins in cellular metabolic and regulatory systems. The goal of proteomics is to assign functional characteristics to protein(s) in a cellular network(s) and to understand the structural and functional features of proteins that are dysregulated in disease[76, 77]. The effort to study proteins is complicated by the dynamic, multi-dimensional nature of the proteome. Unlike the genome, which does not vary structurally from cell to cell, the proteome consists of a myriad array of functionally diverse proteins that are engaged in metabolic and regulatory mechanisms. Protein properties, like abundance levels, post-translational modifications (PTMs), protein-protein interactions, sub-cellular localization, protein synthesis and degradations rates, and allosteric and intra-steric regulation are highly dynamic and vary through the course of a biological process[78, 79]. For example, post-translational modifications (PTM), like phosphorylation and ubiquitylation, are responsible for modifying 30,000 distinct sites on proteins and 19,000 distinct sites on 5,000 proteins, respectively[80, 81]. Furthermore, the generation of different proteoforms through splice isoforms and single nucleotide polymorphisms (SNP) further complicates the study of the proteome[82, 83]. Thus, methodologies that can report on the myriad of post-translational modifications and proteoforms can greatly expand our understanding of the dynamic nature of protein form and function.

Prior to the inception of mass spectrometry (MS) as a method for whole proteome analysis, other techniques like two-dimensional gel electrophoresis (2DE), yeast two-hybrid analysis, and protein microarrays were used to study proteins. However, these techniques lacked the depth, sensitivity, and scope of MS-based proteome analysis[77]. MS analysis of proteins has permitted the interrogation of a diverse level of biological complexity, from protein complexes to human patient populations[84, 85]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has enabled the study of a wide range of biological systems and has permitted the analysis of global proteomic changes across different biological replicates[86]. For example, in shotgun proteomics (bottom-up proteomics), proteins are isolated from a biological system of interest, purified, and digested enzymatically to obtain peptide fragments. Then, peptides are separated by chromatography, their mass-to-charge ratio is detected using tandem MS, and the individual peptide ions are selected individually to obtain sequence information[77, 85, 87]. Bottom-up methods have been used to study a diverse range of biological phenomena, from identify post-translational modifications like phosphorylation and ubiquitination to analysing global proteomic changes that result from bacterial infections[88, 89]. Shotgun proteomics is advantageous given its capacity to analyze a complex mixture of peptides but is limited by dynamic range, which biases the technique towards more abundant proteins. Further, the peptides derived from shotgun proteomics may come from more than one isoform or from the same functional pool, complicating characterization efforts[78, 90]. Thus, developing complementary methods that can reduce the abundance bias and expand the dynamic range of proteomics would contribute positively to identifying relevant biological changes in protein expression following SM treatment.

1.4.2 Label-free Liquid Chromatography-Mass Spectrometry

Prior to the use of proteomic methods for the analysis of proteins, gel-based methods for protein analysis, like radioligand labeling and immunoblotting, were the primary method for protein analysis. These techniques were limited by not only the sensitivity but also the type of proteins that could be visualized, complicating the visualization of membrane or highly basic proteins. Furthermore, these methods were limited to the availability of recombinant antibodies. Liquid chromatography mass spectrometry (LC/MS)-based proteomics has become an important approach to analyzing biological samples[86]. In this method, proteins are extracted, denatured, degraded enzymatically and separated prior to mass spectrometry (MS). General proteomic approaches are useful tools for examining protein expression levels during bioactive SM treatment. Label-free protein expression profiling involves resolving the proteome by 2D gel electrophoresis, followed by protein abundance assessment by LC/MS to compare two biological conditions. A major advantage of label-free methods is that they do not require additional experimental steps, like label-based methods, and comparative quantification can be undertaken between several samples simultaneously[91]. Additionally, previous work has shown that the reproducibility of label-free methods is comparable to label-based methods like SILAC[92-94]. Thus, label-free methods can be harnessed to analyze the expression of proteins in a biological system.

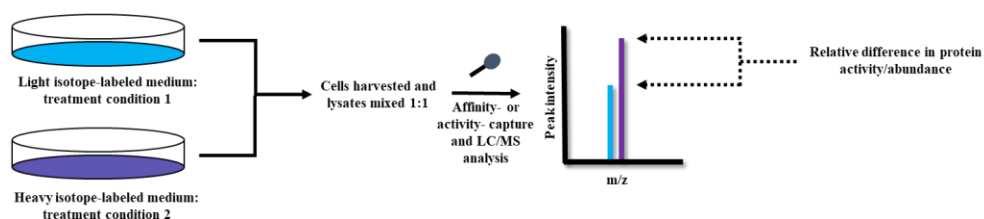
1.5 Label-based chemoproteomic techniques

Though label-free proteomic analysis depends on qualitative methods and provide only a qualitative assessment of protein expression, isotope-coded affinity tags (ICAT) specifically labels sulfhydryl groups found in cellular proteins. This method permits the covalent capture and analysis of sulfhydryl containing proteins within biological systems, decreasing the complexity of peptide

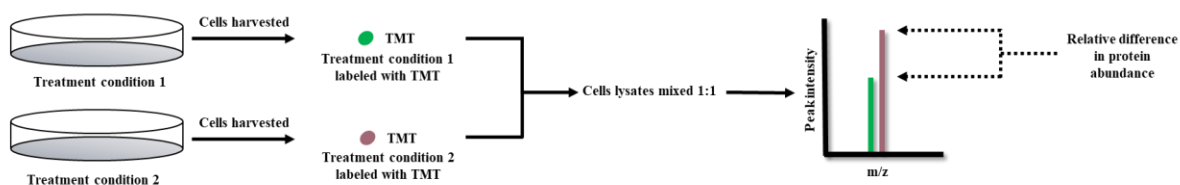
mixtures[95, 96]. In an ICAT experiment, two protein mixtures are labeled with light and heavy ICAT isotope reagents, which covalently modify cysteine residues in proteins. The two protein mixtures are combined, proteolyzed with trypsin, and analyzed using LC-MS/MS[96]. ICAT can be used to analyze up to 2,000 proteins in a single experiment, but this is a major limitation given that there are 10,000 distinct proteins expressed in a mammalian cell[97, 98]. In addition to ICAT, stable isotope labeling by amino acids in cell culture (SILAC) relies on metabolic isotope labeling of cellular proteins (**figure 1.4A**)[99]. This method permits the direct quantitative comparison of the difference in protein abundance between two or more protein samples. SILAC has been harnessed to identify the molecular targets of numerous small molecules, such as kinase inhibitor K252a and piperlongumine[100, 101]. Beneficially, the method reduces variability between samples that result from experimental error or sample handling. The peptides derived from heavy and light media can be distinguished by their mass offset, permitting a relative comparison of protein abundance under two experimental conditions via relative peak intensity.

In addition to SILAC, recent developments in the study of protein abundance have included tag-based approaches that do not require metabolic incorporation of isotopes but required post-lysis labeling using isobaric tags referred to as tandem mass tags (TMTs) that attach to the N-terminus of peptides following digestion with trypsin (**figure 1.4B**) [102]. Desouza and colleagues identified 9 previously unknown biomarkers for endometrial cancer using TMTs[103]. This technique has also been used to identify previously unknown biomarkers for Alzheimer's and Parkinson's disease, and prostate cancer[104-106]. To-date, up to 12 different isobaric tags for iTRAQ are available commercially, allowing the simultaneous study of 12 different biological conditions. In contrast to SILAC and iTRAQ, stable dimethyl (DiMe) labeling is advantageous as it can be incorporated into any system under study (**figure 1.4C**) [107]. Furthermore, it is

A. Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC)



B. Isobaric Tag for Relative and Absolute Quantification (iTRAQ)



C. Dimethyl-labeling (DiME)

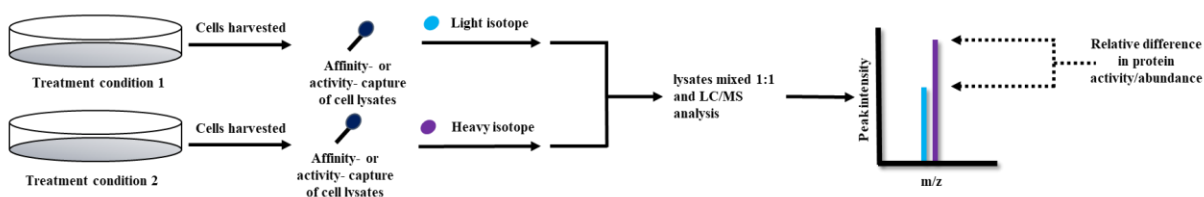


Figure 1.4. Label-based chemoproteomic methods. (A) In SILAC, isotope labels are metabolically incorporated into proteins through supplementation of the cell media with amino acids containing heavy isotopes. Proteins from two different cell populations, either treated or untreated, are grown in media containing either heavy or light isotopes, the cells are harvested, mixed, undergo proteolytic digestion, and then are analyzed by single mass spectrometry (MS) run. (B) isobaric tag for relative and absolute quantification (iTRAQ), a unique TMT label is added to a biological condition of interest following cell lysis, and treated samples are combined and analyzed by LC/MS where proteins from each biological conditions and TMT treatment are observed on the basis of the TMT-induced mass shift in sets of peaks of intensity directly reflecting the protein abundance in each sample (C) differently treated samples by an ABP-assisted pull-down, after which trypsin-digested enzymes are labeled by heavy (red) and light (green) formaldehyde, mixed in a 1:1 ratio and analyzed by LC/MS.

inexpensive, rapid, specific, and does not perturb cell function as labeling occurs post-lysis[108]. DiMe has been used extensively to characterize the global expression profiles of proteins and post-translational modifications[109-111]. In contrast with SILAC, which is limited to cell culture-based systems, DiMe is advantageous given mixing samples limits uneven sample loss due to labeling[107].

1.6 Functional Proteomics

As outlined above, expression proteomics is disadvantaged by the bias towards highly abundant proteins – particularly those involved in cellular stress response and cell homeostasis. Though traditional proteomics have greatly expanded our understanding of the global proteomic shifts that occur in a biological system following manipulation, the abundance of a protein does not correlate to its activity in a metabolic or regulatory pathway[52]. Functional proteomics aims to provide more information than traditional expression proteomics by considering the manifold layers of protein regulation, with the goal of identifying the biological mechanisms of proteins and understanding cellular mechanisms at a molecular level[112].

1.6.1 Activity-based Chemical Proteomic Methods

Complementary to global proteomic analyses, activity-based protein profiling (ABPP) is a functional proteomic method that permits the study of enzyme activity in native biological environments and accounts for the myriad layers of enzymatic regulation, like allosteric and intrasteric regulation, post-translational modifications, and endogenous substrates and interactions[113]. ABPP has been used to study and identify dysregulated enzymes in diseases, for example to a) identify inhibitors for enzymes, b) characterize enzyme active sites, c) elucidate the biological targets of bioactive small molecules, d) identify the agonists and antagonists of enzymes

A. *In situ* labeling and proteomics

B. *In vitro* labeling and proteomics

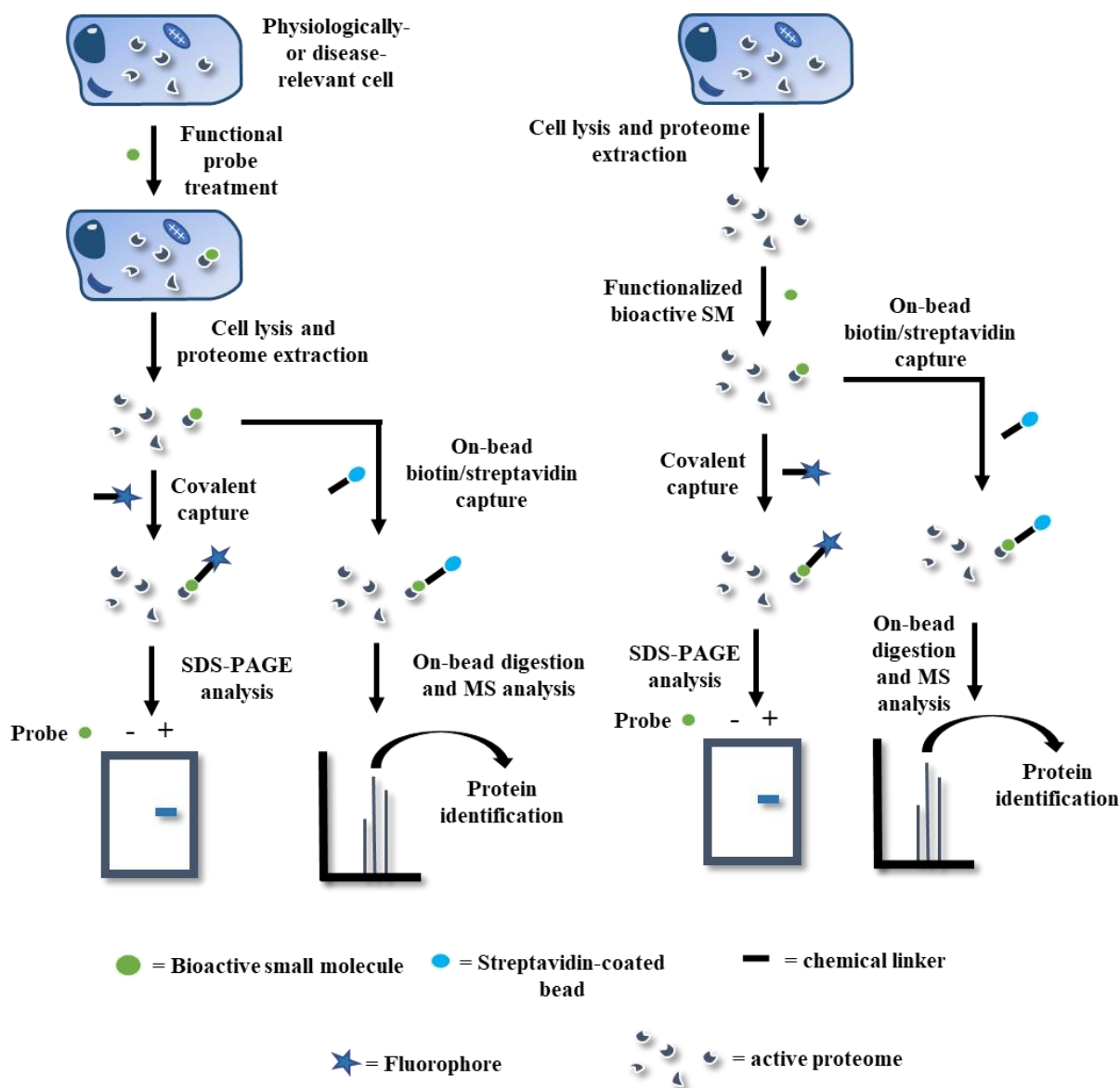


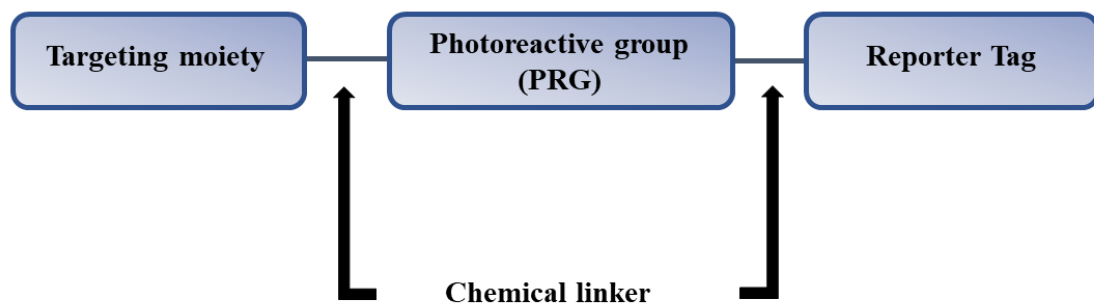
Figure 1.5. General methodology for *in vitro* or *in situ* labeling and proteomic analysis. (A) For *in situ* labeling, physiologically- or disease-relevant cell is exposed to the functionalized bioactive small molecule chemical probe, followed by cell lysis and proteome extraction. The functionalized probe can then be covalently captured and visualized using fluorescence on an SDS-PAGE gel. If the proteome is to be analyzed using MS, the functionally-labelled proteome is captured using biotin-streptavidin, digested with trypsin, and analyzed using MS. (B) For *in vitro* labeling, cells are harvested first prior to treatment with the functionalized bioactive SM.

in conjunction with HTS, and e) visualize the localization of enzymes in a cell[113, 114]. With ABPP, a physiologically relevant cell line can be treated with the functionalized ABP, which is harnessed to analyze the direct molecular targets of the bioactive SM (**figure 1.5**). Several different enzyme classes have been studied, including but not limited to proteases, phosphatases, hydrolases, and kinases[114]. ABPP requires the use of a modified bioactive small molecule (activity-based probe; ABP), which can target the catalytic site of an enzyme and report on the functional impact of binding[114] (**figure 1.6**). ABP probes are often designed based on a mechanistic or structural feature of the enzyme's active site and probes often contain an electrophilic warhead that can participate in the catalytic mechanism. Broad-based ABPs, which are broad with respect to the targeting of protein families, can be used to profile normal and disease states. The main disadvantage of these broad-based ABPP methods is that they lack selectivity and can miss changes in low abundance or unreactive molecular targets. Tailored-ABP can be used to study and detect low abundance proteins in a biological system that may be masked by high-abundance or more reactive proteins[52].

1.6.2 Affinity-based Chemical Proteomic Methods

ABP employ the use of active-site directed, electrophilic probes that participate directly in the catalytic mechanism of action of the enzyme of interest. However, some electrophilic small molecules target non-catalytic residues in the enzyme binding site. Affinity-based protein profiling (AfBPP), a subclass of ABPP, aims to characterize the selectivity and specificity of a given bioactive small molecule[115]. Binding of the AfBP is principally driven by the affinity of the small molecule, which determines the selectivity and the utility of the probe[116]. AfBP has been used to profile disease relevant enzymes including histone deacetylases, metalloproteases, and

A.



B.

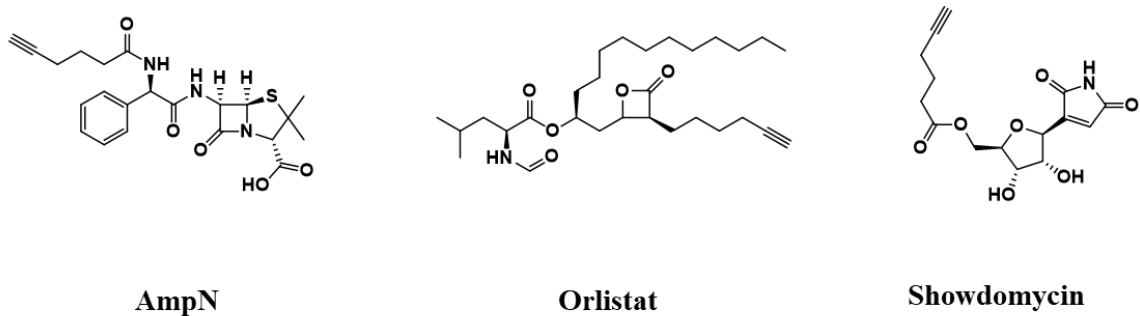


Figure 1.6. General anatomy of an affinity- or activity- based chemical probe. (A) The natural product is used as a targeting moiety in an ABPP experiment. The photoreactive group (PRG) can be either a benzophenone, diazirine, or a phenyl azide. The reporter tag can be either an alkyne, azide, biotin, or a fluorophore. (B) Functionalized bioactive small molecules.

methionine aminopeptidase[117, 118]. Further, AfBPs have been used to profile carbohydrate binding proteins, kinases, phosphatases, and ligand-gated ion channels[116].

1.7 General Design of Activity-based Probes

1.7.1 Targeting moiety

The targeting moiety of an ABP can be any bioactive small molecule that has an affinity for the active site of a given protein and is either selective for a class of proteins with shared structural or mechanistic characteristics or induces a desirable phenotypic change, which will require further validation (**figure 1.6A**). Generally, a targeting moiety should have an in vitro potency of less than 100 nM and have greater than 30-fold selectivity against other homologous proteins[119]. Common targeting moieties tend to be electrophilic traps like epoxides, α -halo carbonyl, isothiocyanates, β -lactams, and β -lactones[120] (**figure 1.6B**). To reduce the risk that appending a linker will disrupt binding of the molecule to the protein, the target moiety should originate from studies that have undertaken a structure-activity relationship (SAR) study of the SM binding to the target protein. If the bioactive small molecule does not originate from a SAR study, structural biology information can be used to design the ABP[121].

1.7.2 Chemical linkers

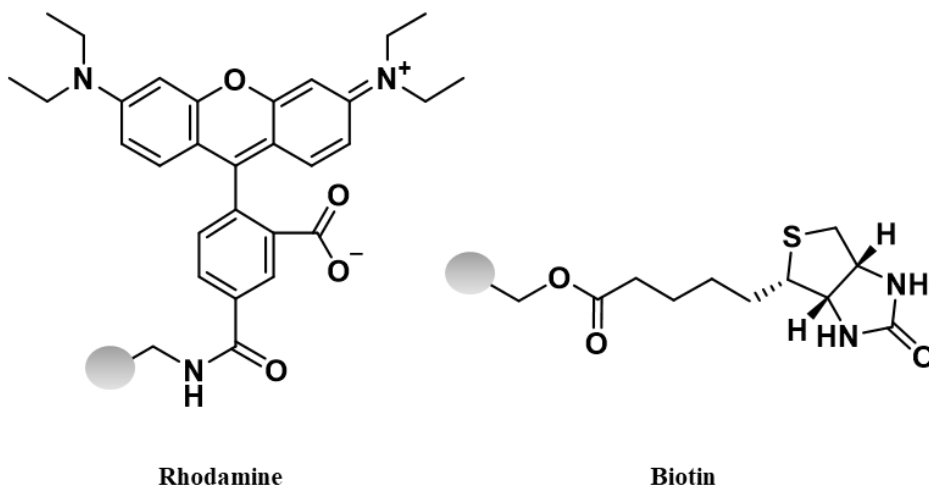
To prevent disruption of small molecule binding to the target of interest, chemical linkers are often used to extend the distance between the targeting moiety and the reporter group; the length of the linker group is important in determining whether effective covalent capture of proteins will occur[122]. If the linker length is too short, self photo-cross linking is possible, reducing labeling efficiency. If the linker length is too long, photo-cross linking may not occur with the protein. Furthermore, common linkers employ the use of polyethylene glycol (PEGs) linkers, which can

increase cellular penetration of the chemical probes[123]. Additionally, PEGs are ideal functional groups to use in affinity proteomics given their ease of installation and stability towards cellular environments. Another consideration must be given to the length and nature of the linker. Balancing between the hydrophobicity and hydrophilicity is crucial when considering non-specific binding of proteins, which could mask low abundance, high affinity protein binding to the targeting group. Previous work has shown that the chemical nature of the linker can have a significant impact on the capture of proteins[124, 125].

1.7.3 Photoreactive Groups

In addition to a targeting group, ABP may require a photo-reactive group (PRG) that can cross-link with proteins using ultraviolet radiation, which is generally required if the targeting group binds non-covalently with the protein target[126] (**figure 1.7B**). Photo-affinity labels (PALs), which are ABP that incorporate a PRG, have been around since the 1960s when Frank Westheimer introduced an aliphatic diazo group into the enzyme chymotrypsin[127]. Since then, PALs have been used extensively in medicinal chemistry[115, 116]. For PALs to be effective chemical probes they must be stable at a range of pH values, have a high degree of similarity to the parent bioactive small molecule with comparable activity and affinity levels, have little steric disruption of protein binding, be activated at a wavelength that does no or minimal damage to cellular biomolecules, and the newly formed bond following photo-irradiation must be stable enough to travel through the experimental workflow[126]. Additionally, photo-probes generally have a half-life that is shorter than the rate of dissociation of the ligand-protein complex, which ensures that covalent capture of the protein occurs. Finally, the wavelength required to activate PRG should be greater than the absorption wavelength of proteins to avoid photo-degradation of proteins. Excess absorption of UV light by proteins can result in the formation of electronically excited state

A. Reporter tags



B. Photoaffinity groups

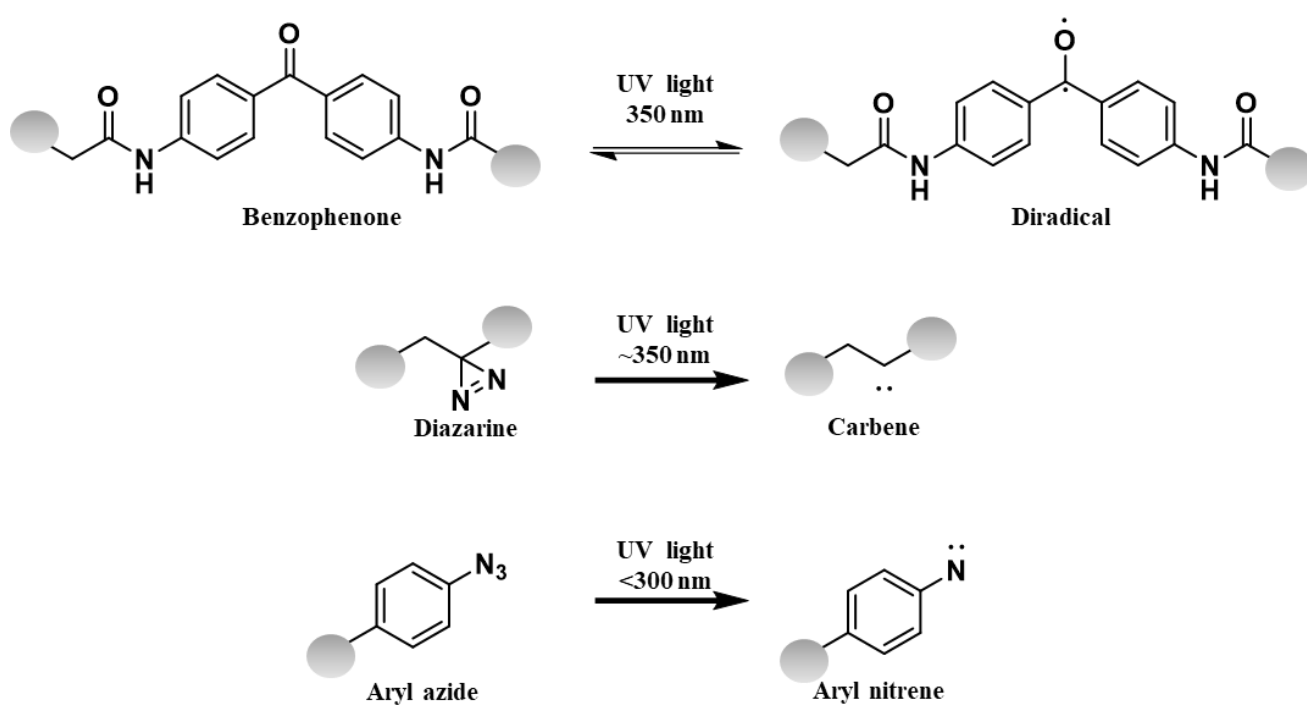


Figure 1.7. General design of an activity-based probe. (A) Structure of common reporter tags. (B) Chemical structure of the three most common photoreactive groups (PRGs). Following UV irradiation, benzophenone generates a reversible diradical. In the presence of UV light, diazirine and aryl azide irreversibly generate a carbene and an aryl nitrene, respectively.

biomolecules, which can go through degradative chemical transformations that could cause profound change to the cell[126, 128, 129]. There are three distinct PRGs that exist for use in photo-probes: aromatic azides, aliphatic and aromatic diazirines, and benzophenones. Phenyl azides are most commonly used in photo-affinity labeling due to their commercial availability and ease of synthesis[130]. The major disadvantage of phenyl azides is that the photo-irradiation wavelength is less than or equal to 300 nm, which can cause substantial damage to cellular biomolecules. Additionally, phenyl azides can be reduced to amines by cellular reducing agents, further decreasing photo-labeling efficiency[131]. Finally, photo-irradiation of phenyl azides to nitrenes can result in un-desirable formation of benzairines and dehydroazepines/ketenimines, which can be limited by addition of trifluoro groups[132]. Phenyl azides do have an advantage that they are less sterically encumbered than benzophenones moieties, which can limit steric disruption of probe binding to proteins.

In comparison with phenyl azides, aliphatic diazirines form reactive carbene intermediates following irradiation between 350 and 380 nm and greatly decreases the damage to biomolecules. Aliphatic and phenyl diazirines are stable in reducing and oxidizing environments and strongly basic and acidic solutions, expanding the synthetic utility of this PRG[133, 134]. They react rapidly and have a short half-life, which increases cross-linking with biomolecules. However, the reactivity of the diazirine's carbene means that they are quickly quenched by water to form an alcohol[135]. This is counter-balanced with rapid reactivity that reduces potential background labeling of the proteome. An additional disadvantage of diazirines is their susceptibility to rearrangement to non-reactive intermediates like linear diazo isomer, which limit the efficiency of labeling but also reduces background labeling[123, 136]. Benzophenone (Bz) is excited at a wavelength of 350-360 nm and generate a diradical species, which limits the damage to cellular

systems. Unlike the previous examples, Bz moieties can undergo reversible photochemical transformations. A major advantage of Bz is the commercial availability of multiple building blocks and the synthetic ease in expanding the available repertoire of Bz probes. The disadvantage of Bz is that they are large and bulky functional groups, which may impair their ability to transit across the cell membrane and may have a negative impact on binding to the target of interest if the linker length has not been optimized. Further, Bz requires longer irradiation times since it is less reactive, which may increase labeling of non-specific proteins[137]. Finally, they are susceptible to covalent attachment to methionines[138].

1.7.4 Reporter tags

To facilitate the isolation and visualization of probe labeled proteins, reporter tags are incorporated directly or indirectly in the photoprobe (**figure 1.7A**). Radio-isotopes (radiolabels) are advantageous because their small size limits the perturbation of probe-protein binding. They are also able to be chemically installed with relative ease and cause minimal change to the parent compound's activity and affinity and permits the detection of proteins with high sensitivity and rapid detection[139]. However, several limitations exist with radiolabels: a) there is no direct means to isolate and identify the labeling protein following capture, b) fast degradation of radio-labels places a temporal limit on the experimental workflow, and c) they require more care in the laboratory.

In addition to radioligands, affinity tags are often used as reporter groups. The most commonly used reporter tag is biotin, which has a high affinity for streptavidin. A major disadvantage of biotin is its low cell permeability, which limits its use to *in vitro* applications. Additionally, given biotin's high affinity for avidin and that there are several biotinylated proteins that can also be purified by avidin resins, increase background labeling may result if biotin is used

as a reporter group. Peptide sequences that are recognized by specific antibodies have also been used as reporter tags, but their large size restricts incorporation into a bioactive small molecule because they have limited cell permeability[126].

Fluorophores are another common group of reporter tag that is used to report on the degree of small molecule binding to a protein target. Fluorophores are generally hydrophobic and can thus penetrate the cell membrane, which is advantageous because this enhances the permeability of the labeled probe. Since the first reported use of fluorophores in ABP, fluorophores are now commonly used as reporter tags[117, 140]. Some fluorophores are susceptible to photo-bleaching, like fluorescein and rhodamine, but most fluorophores are ideal reporters because they have high absorptions coefficients, narrow absorptions peaks, high quantum yields, and large stokes shifts.

1.8 Tandem Labeling Strategies

Bioorthogonal labeling describes any chemical reaction that can occur inside a cell without disrupting cellular activity. Since the development of bioorthogonal reactions, many biomolecules have been studied, like lipids, carbohydrates, proteins, and post-translational modifications of biomolecules[141]. Unlike traditional genetic reporters, which require genetic incorporation of fluorescent tags like green fluorescent protein (GFP) that can perturb the interaction between the targeting moiety and the biological target, bioorthogonal reporters are not native to a biological system and thus are biocompatible, they do not perturb cellular activity, and can be modified to provide diverse chemical readouts [142-144]. In tandem photoaffinity-bioorthogonal labeling, a surrogate tag is chemically introduced into the ABP and the biological system is treated[46]. Following treatment, the ABP is photo-irradiated to covalently link the probe to the biomolecule of interest. Then the reporter tag is introduced exogenously and reacts selectively with the surrogate tag. This method is advantageous given that there is limited perturbation of targeting

group interaction with the host biomolecular target[145]. There are several important criteria when evaluating the bioorthogonality of a reporter system: a) the surrogate and reporter tag must react exclusively with the reporter tag, b) the reaction must be high yielding, c) the reaction must be bio-compatible and proceed under physiological conditions, d) the reaction must proceed with fast kinetics on the time scale of biological processes to prevent diminishing labeling efficiency, e) the tags must be non-toxic and stable, and f) the tags must not disrupt the structure or function of the biomolecule under study[145].

To overcome the disadvantages of the reporter tags outlined above, tandem photo-affinity labeling-bioorthogonal conjugation has been harnessed to minimize target disruptions and enhance protein target labeling. The first tandem labeling strategy was developed by Bertozzi and colleagues, where carbohydrates were tag-modified using the Staudinger ligation[146]. A second tandem labeling strategy, originally developed by Sharpless and coworkers, but expanded on by Cravatt and co-workers, uses the Huisgen [3+2] cycloaddition of alkyne and azides facilitated by a copper catalyst, commonly referred to as “click” chemistry [147, 148] (**figure 1.8**). Recent efforts have focused on the development of copper free methods for tandem labeling, given copper toxicity in cellular systems. Strain promoted azide-alkyne cycloadditions (SPAAC) do not require a metal for catalysis and covalent capture of probe-labelled proteins[149].

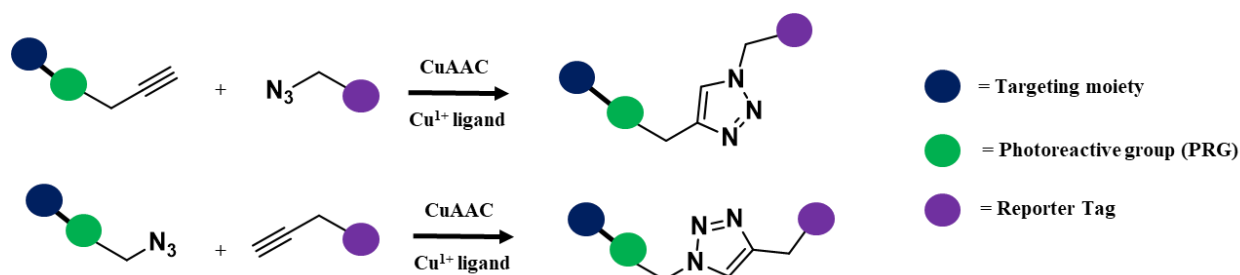


Figure 1.8. Copper-catalyzed [3+2] cycloaddition

1.9 Competitive Strategies using Activity-based Probes

A major challenge using a tandem photo-affinity labeling-bioorthogonal conjugation strategy with a bioactive small molecule is the risk of capturing proteins that are not directly targeted by the targeting moiety (a false positive) or is not physiologically relevant to the study. Since each protein that is captured must be independently validated, a false-positive capture can be laborious and wasteful[52]. One must be able to separate the physiologically relevant but low abundance high affinity proteins from the low affinity, high abundance non-binding proteins. Further, hydrophobic alkyl linkers may favor non-specific bonding of proteins[13]. To reduce the degree of non-specific protein binding, competitive ABP can be used. In this method, increasing concentrations of the unmodified parent molecule are used along with the modified ABP, which will compete for binding with the modified ABP and report on the specificity of the modified ABP through changes in the relative fluorescence signal or abundance of the target protein(s). For example, if the modified ABP probe targets the protein(s) specifically, a decrease in the signal intensity would result following competitive labeling. If the modified ABP binds non-specifically to the target protein(s), then there would be no change in the relative signal intensity following competitive labelling. Furthermore, to identify non-specific binding proteins the linker alone or an inactive analog can be used. In the latter step, this may significantly increase the amount of time to synthesize an alternative probe[52].

1.10 Conclusion

The effectiveness of chemical probes for identifying and characterizing the role of proteins in biological processes is well-proven. Chemical probes have been used extensively to identify molecular targets of bioactive small molecules and to characterize the role of the molecular

target(s) in a biological or metabolic pathways[46]. Furthermore, chemical probes can be used to ascertain the degree of target engagement and identify potential off-target interactions[72].

Chapter 2: Inhibition of Hepatitis C Virus by 6-Hydroxydopamine

The entirety of this work was published in:

Lafreniere, M.A.; Powdrill, M.H.; Singaravelu, R.; Pezacki, J.P. 6-Hydroxydopamine Inhibits the Hepatitis C Virus through Alkylation of Host and Viral Proteins and the Induction of Oxidative Stress. *ACS Infect. Dis.*, **2016**, 2 (11), pp 863–871.

Abstract

Many viruses, including the hepatitis C virus (HCV), are dependent on the host RNA silencing pathway for replication. In this study, we screened previously reported small molecule probes 6-hydroxydopamine (6-OHDA), suramin (SUR), and aurintricarboxylic acid (ATA), against an HCV cell model to examine their effects on viral replication. We found that 6-OHDA inhibited HCV replication; however, 6-OHDA was a less potent inhibitor of RISC than either SUR or ATA. By generating a novel chemical probe (6-OHDA-yne), we determined that 6-OHDA covalently modifies host and virus proteins. Moreover, 6-OHDA was shown to be an alkylating agent that is capable of generating adducts with a number of enzymes involved in the oxidative stress response. Furthermore, modification of viral enzymes with 6-OHDA and 6-OHDA-yne was found to inhibit their enzymatic activity. Our findings suggest that 6-OHDA is a probe for oxidative stress and protein alkylation, and these properties together contribute to the antiviral effects of this compound.

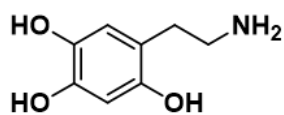
Statement of Research Contribution

As the principal author, I contributed significantly to the intellectual and experimental development of the research herein. J.P. Pezacki, Ragunath Singaravelu and I conceived of the research idea and experimental plan. I performed all the synthesis, *in vitro* labeling, mass spectrometry analysis, and oxidative stress experiments. Dr. Megan Powdrill assisted with labeling of viral proteins and the helicase assay. I wrote the first draft of this manuscript and editing was performed principally by Megan H. Powdrill and J.P. Pezacki. I would like to thank Mr. Don Leek of the National Research Council of Canada for this NMR support, Dr. Gleb Mironov for the CE-MS analysis, and Dr. Zhibin Ning for his help with mass spectrometry analysis of the biological targets of 6-OHDA-yne. I would like to further thank Dr. Megan Powdrill for her work in the DNA unwinding assay and Dr. Ragunath Singaravelu for this critical reading of the manuscript.

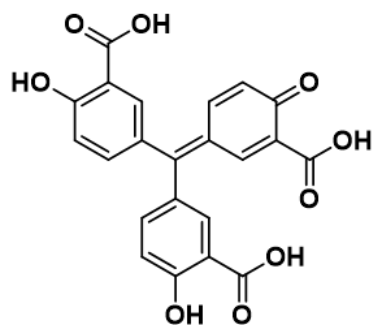
2.1 Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that are estimated to regulate in excess of 60% of protein-coding genes[150]. MicroRNA transcripts (pri-miRNAs) are processed in the nucleus by the Microprocessor complex, which is composed of Drosha and DGCR8 proteins, to form pre-miRNAs of approximately 70 nucleotides. Following processing, the pre-miRNA is transported to the cytoplasm by exportin 5 and processed by the Dicer complex to form mature miRNAs of approximately 22 nucleotides[151]. Mature miRNAs bind to Argonaute (Ago) proteins to form an RNA-Induced Silencing Complex (RISC), and partial base pairing between miRNAs and mRNA targets modulates gene expression through either translational repression or RNA destabilization[152].

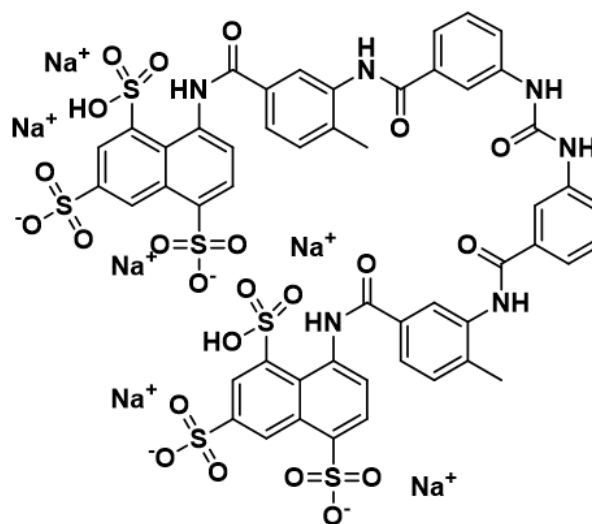
Like many other host cellular components, miRNA activities are targeted by viruses to create an environment conducive to viral replication. The importance of these small RNAs during viral infections results from the fact that many viruses, including Ebola virus (EV), influenza A virus, vaccinia virus, and the human immunodeficiency virus (HIV) have been shown to encode viral proteins and biomolecules that are capable of suppressing RNA silencing in host organisms, promoting viral replication and proliferation[153-155]. Hepatitis C virus (HCV) has been shown to alter host miRNA levels to modulate the expression of genes involved in pathways crucial for efficient replication[156]. Beyond the manipulation of miRNA levels, the 5'-untranslated region (5'-UTR) of HCV has been shown to form direct interactions with miR-122, a liver-specific miRNA that constitutes 70% of the total miRNA pool in hepatocytes[157-159]. Interactions between viral RNA and the host miRNA have been proposed to protect the HCV genome from degradation, support replication, and aid in the translation of viral proteins[160-163]. Sequestration of miR-122 significantly reduces viral RNA levels, emphasizing the importance of



2.1
6-hydroxydopamine



2.2
Aurintricarboxylic acid



2.3
Suramin

Figure 2.1. Chemical structures of small molecule inhibitors of RISC loading.

this host miRNA in HCV replication[157]. The dependence of HCV on miR-122 has been previously exploited to disrupt viral replication by antisense oligonucleotides, which are currently in development and have proven to be highly effective at inhibiting viral replication[164]. It is possible that inhibitors targeting other RISC components could also produce antiviral effects.

A recent study by Tan and colleagues identified three small molecules that disrupt RISC loading (**figure 2.1**)[165]. Aurintricarboxylic acid (ATA), suramin (SUR), and 6-hydroxydopamine (2,4,5-trihydroxyphenylethylamine; 6-OHDA) were all shown to inhibit binding of Ago2 to miRNA with low micromolar potency, based on results from a fluorescence polarization assay. SUR is an anti-trypanosomal drug with anti-tumor activity that has been demonstrated to inhibit cullin-RING E2 ubiquitin ligases through disrupting the recruitment of E2 Cdc34[166]. ATA is an inhibitor of nucleases and nucleic acid binding enzymes that has been shown to inhibit loading of miRNA onto Ago2[165]. 6-OHDA is a neurotoxin widely used to induce dopaminergic neurodegeneration in animals as a model for Parkinson's disease. Cytotoxicity of 6-OHDA is linked to the generation of reactive oxygen species (ROS), autooxidation of 6-OHDA, and the production of hydrogen peroxide (H₂O₂)[167].

In this chapter, we examined whether the use of small molecule inhibitors targeting RISC loading, an important step in miRNA biogenesis, would likewise interfere with viral replication. Both inhibition of RISC loading and additional mechanisms of action appeared to contribute to the antiviral effects. Using 6-OHDA-derived probe, we determined that 6-OHDA is an alkylating agent that forms adducts with cellular and viral proteins, including several components of the oxidative stress response pathway. Modification of viral proteins with 6-OHDA had an inhibitory effect on HCV helicase enzyme activity. This may contribute to its antiviral properties, while

additional effects were likely mediated through the generation of ROS. Our study highlights the utility of chemical probes for identification of biological targets of small molecule inhibitors.

2.2 Results and discussion

2.2.1 Small Molecule RISC Inhibitors Interfere with Replication of HCV

Many viruses are dependent on the RISC pathway for processing both viral and host miRNAs to modulate expression of genes essential for viral pathogenesis[168]. Manipulation of this pathway by small molecule inhibitors can provide insight into the mechanisms through which viruses promote pathogenesis. Here, we used three small chemical inhibitors previously shown to interfere with RISC loading (i.e., ATA, SUR, and 6-OHDA) to monitor the effects of inhibiting the silencing complex on replication of HCV. We chose HCV as a model virus since it requires the

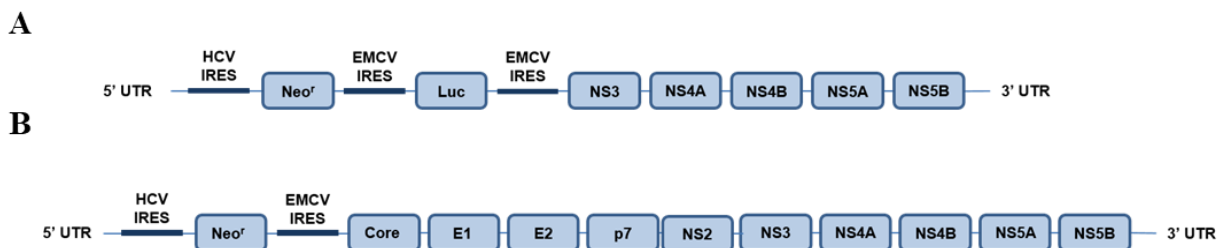


Figure 2.2. Hepatitis C virus (HCV) replicon models. (A) The HCV sub-genomic replicon (HCV-SGR) encoding the non-structural proteins NS3-NS5B and a luciferase reporter. (B) The HCV full genomic replicon (FGR) encodes both the HCV structural and non-structural proteins.

multicomponent RNA-induced silencing complex (RISC) due to HCV's dependence on host miR-122[169]. HCV sub-genomic replicons (HCV-SGR), which encode both a luciferase reporter and the HCV non-structural proteins NS3 through NS5B, can be used to monitor HCV replication (**figure 2.2A**). A full genomic replication (HCV-FGR), encoding both the HCV structural and non-structural proteins, that does not produce infectious virus was used for studies not requiring a

fluorescent reporter (**figure 2.2B**). Treatment of hepatocellular carcinoma cell line Huh7 expressing the HCV-SGR with SUR did not show dose-dependent effects on viral replication. In contrast, ATA treatment was associated with significant cytotoxicity (**figure 2.3C**). Treatment with **2.1** for 24 h resulted in dose-dependent inhibition of HCV replication with an EC₅₀ of 8.2 μ M and no significant cytotoxicity up to concentrations of 500 μ M (**figure 2.4B**). These results suggest that **2.1** can impair viral replication at concentrations well below those at which cytotoxic effects are observed. The toxicity associated with **2.2** precludes further consideration as an antiviral; however, small molecules targeting the RNA silencing pathway offer an alternative approach to antiviral therapy, thus **2.1** still has potential as a probe[165, 170]. Despite the success of recently approved direct acting antivirals (DAA) targeting HCV, new approaches that may be effective against multiple viruses with similar dependencies on host cell pathways are still desired. Therefore, there is an interest in identifying additional approaches to disrupt the viral lifecycle. Host factors are particularly interesting due to their high barrier to the development of resistance. The dependence of viruses on host miRNAs for various aspects of their lifecycle makes targeting the RNA silencing pathway a promising approach. Previously, HCV has been targeted by an oligonucleotide that sequesters miR-122, reducing HCV levels in infected chimpanzees[171]. A phase 2a clinical study of this inhibitor, miravirsin, a locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide, showed dose-dependent decreases in HCV levels without the selection of resistance[164]. Further development of HCV anti-infective drugs is ongoing, indicating the clinical relevance of this approach for targeting viral infection[172].

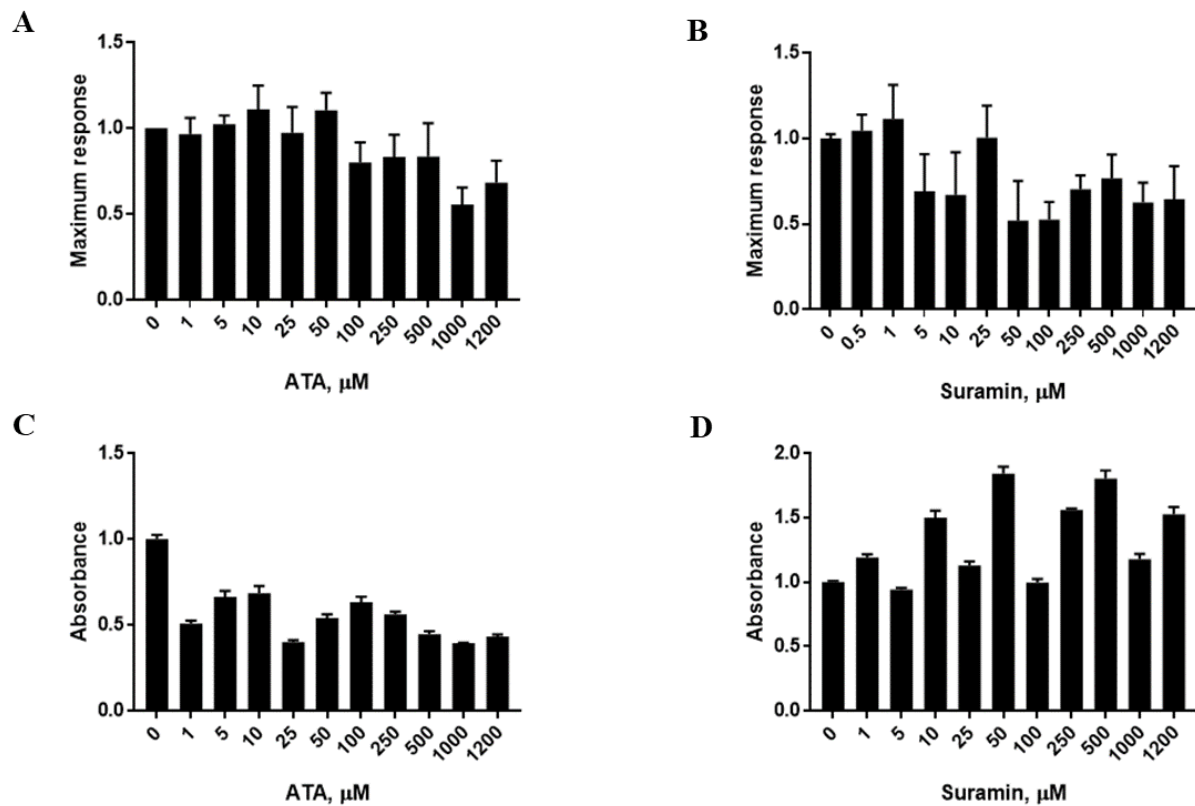


Figure 2.3. Inhibition of HCV replication by ATA and SUR. Huh7 cells expressing the HCV-SGR were treated with designated concentrations of either ATA (A) or SUR (B). In parallel, an MTT assay was performed for cells treated with ATA (C) or SUR (D) to monitor cytotoxicity.

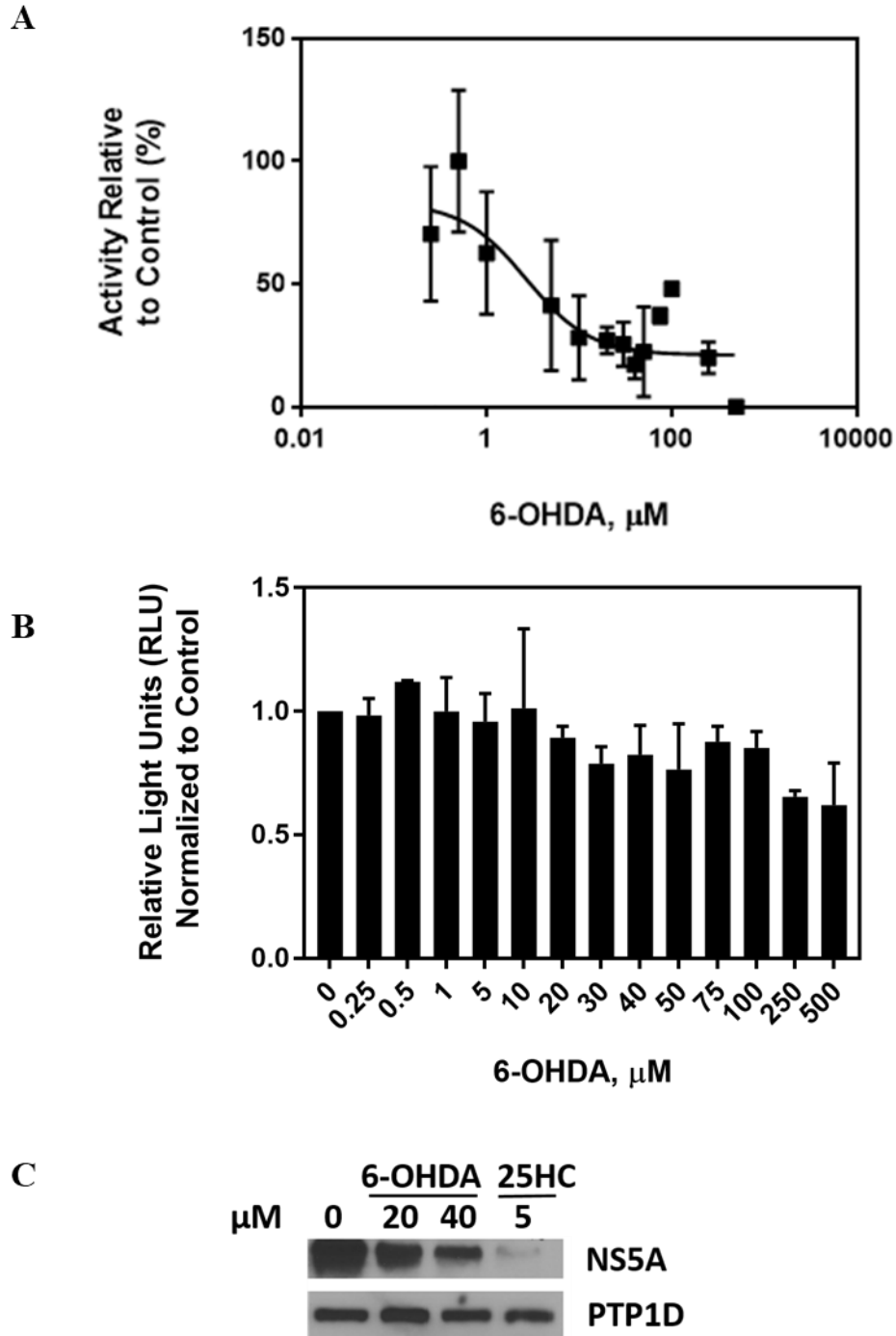


Figure 2.4. 6-OHDA is an inhibitor of HCV replication. (A) EC_{50} curve showing concentration-dependent inhibition of HCV replication by 6-OHDA. Huh7 cells expressing the HCV-SGR were treated with designated concentrations of 6-OHDA for 24 h, and HCV replication levels measured using a bioluminescence reporter. Values represent the mean $\pm$ SD of three replicates. (B) Cytotoxicity of 6-OHDA at 24 h as determined by MTT assay. (C) Immunoblot showing that the levels of the HCV NS5A protein in HCV-FGR are decreased following treatment with 6-OHDA. As a control, cells were treated with 5 μM 25-hydroxycholesterol, a compound known to inhibit HCV: PTP1D was used as a loading control.

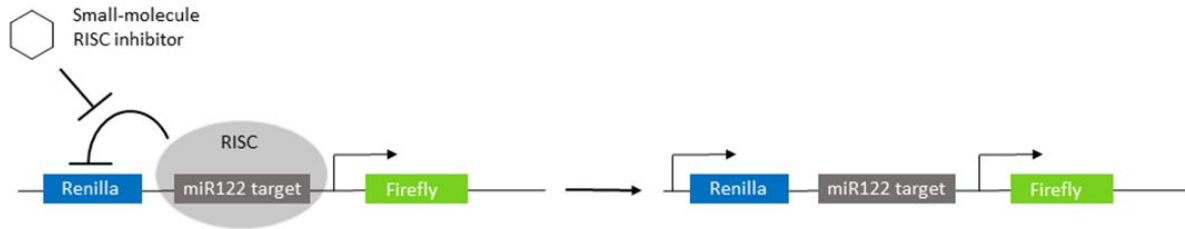
2.2.2 6-OHDA Treatment Does Not Significantly Affect miR-122 Levels

Inhibition of RISC should decrease miRNA activity in the cell, including that of miR-122. Since miR-122 is essential for HCV replication, this could be the mechanism through which 6-OHDA exerts its antiviral effects. To explore this possibility, we used a psiCHECK-2 reporter plasmid (Promega, Madison, WI) containing both a Renilla luciferase gene and a firefly luciferase gene; the firefly gene is independently transcribed and is used to normalize for variations in transfection efficiency. Two miR-122 target sites were inserted into the 3' UTR of the Renilla luciferase reporter gene, and following miR-122 binding, reduced Renilla luciferase expression can be detected by luminescence (**figure 2.5A**). We treated Huh7.5 cells expressing the miR-122 reporter construct with 6-OHDA, ATA, and SUR and measured miR-122 activity using a luciferase reporter assay. As shown in **figure 2.5B** treatment with ATA or SUR alleviated Renilla luciferase reporter repression, increasing fluorescence. This suggests that these small molecules can inhibit binding of miR-122 to the reporter's 3'-UTR. 6-OHDA treatment resulted in an approximately 2-fold increase in luciferase expression, demonstrating a modest inhibition of RISC. These data suggest that the observed antiviral effects of 6-OHDA against HCV could be mediated through additional mechanisms rather than exclusively through interference with the RNA silencing pathway.

2.2.3 Treatment with 6-OHDA Generates ROS Which May Contribute to Reduced Viral Replication

The neurotoxicity associated with 6-OHDA has been attributed in part to the production of ROS. Autoxidation of 6-OHDA can generate H₂O₂, which is reduced in the cell leading to the generation of hydroxyl radicals[167]. Oxidative stress occurs when the equilibrium between reactive oxygen species and anti-oxidative countermeasures is disrupted. Oxidative stress is widely reported during

A



B

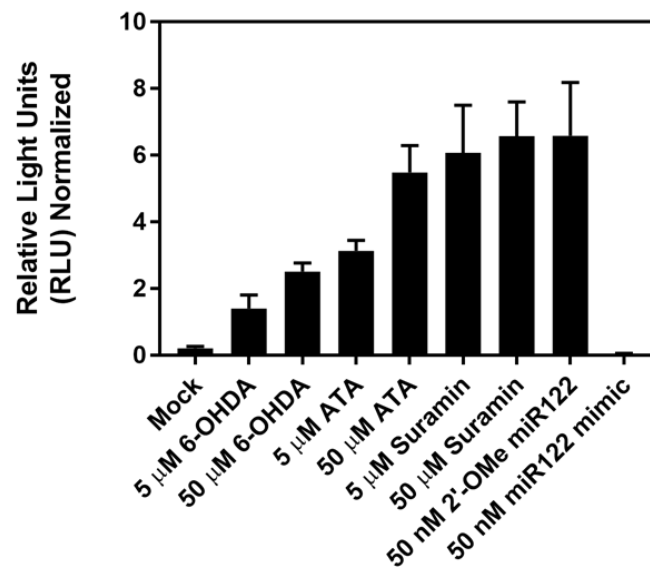


Figure 2.5. miR-122 activity is not significantly reduced following treatment with 6-OHDA. (A) miR-122 reporter construct. Inhibition of RISC diminishes miR-122 activity, permitting expression of Renilla luciferase. (B) Huh7.5 cells were transfected with a miR-122 dual reporter plasmid. At 24 h post-transfection, cells were treated with 6-OHDA, ATA or SUR for 24 h then relative miR-122 activity measured by a luciferase reporter assay. 2'-O-methylated antisense miR-122 targets miR-122 and restores luciferase expression, while the miR-122 mimic represses expression.

infection with HCV[173]. The HCV core protein enhances calcium-induced mitochondrial superoxide production, causing cellular oxidative injury through the generation of ROS[174]. Other viral proteins have been reported to cause dysregulation of mitochondrial calcium homeostasis, which results in glutathione oxidation in the mitochondria and increased levels of ROS[175]. We examined whether treatment with 6-OHDA resulted in increased production of ROS. Using an assay based on a fluorogenic dye that measures hydroxyl and peroxy species, we observed the production of ROS following treatment with 40 μ M 6-OHDA (**figure 2.6**). These findings are consistent with previous reports demonstrating that **2.1** treatment generates ROS[167]. In the context of HCV infection, previous work has shown that ROS interferes with HCV replication mainly through disruption of replication complexes[173, 176]. Therefore, ROS generation is likely a contributing mechanism to the antiviral effects of 6-OHDA.

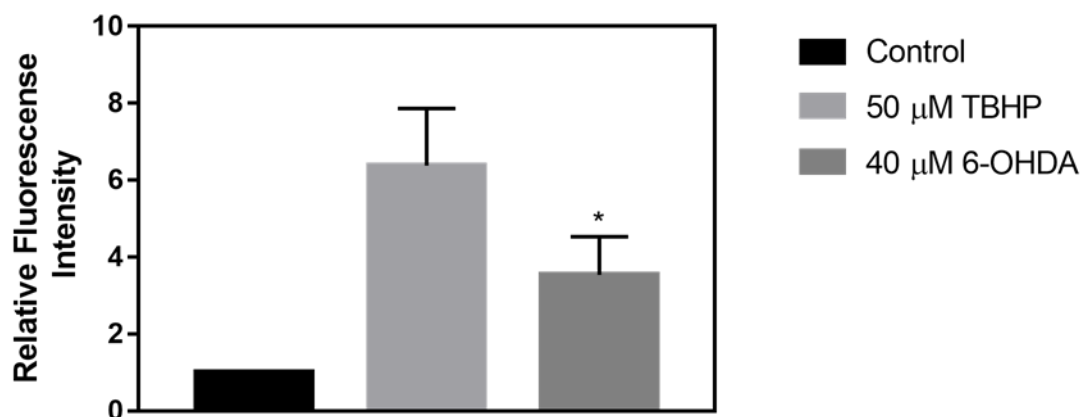


Figure 2.6. 6-OHDA treatment generates reactive oxygen species. Huh7.5 cells were stained with 20 μ M 2',7'-dichlorofluorescein diacetate (DCFDA). DCFDA is deacetylated to a non-fluorescent compound by cellular esterases. Cells were then treated for 3 h with either 50 μ M of the control, tert-butyl hydrogen peroxide (TBHP), or 40 μ M 6-OHDA. ROS oxidize DCFDA into 2',7'-dichlorofluorescein (DCF) which is detected using a fluorescent plate reader. The values represent the mean $\pm$ SD of three biological replicates.

2.2.4 6-OHDA is an Alkylating Agent

To gain further insights into the molecular mechanisms involved in the impairment of HCV replication, we studied the chemical characteristics of 6-OHDA. Previous research has suggested that 6-OHDA can generate ROS through autoxidation[177, 178]. The proposed mechanism suggests that triplet oxygen abstracts two hydrogens from the 2,4,5-trihydroxyphenyl ring of 6-OHDA and through a radical cascade forms an α,β -unsaturated carbonyl. The autoxidation of 6-OHDA to α,β -unsaturated carbonyl is prone to Michael addition by nucleophiles, which could result in covalent attachment to cellular or viral proteins. Previous work using dopamine, an analogue of 6-OHDA, has shown that molecular oxygen generates the formation of o-quinone, a Michael acceptor[179]. As indicated in **chapter 1**, electrophilic moieties are used as AfBP or ABP for the covalent capture of proteins. Glutathione (GSH) is a cellular antioxidant that exists mainly in a reduced state that can be oxidized to GSSG during oxidative stress and then reverted to GSH by GSH reductase[180]. In its reduced state, GSH acts as a radical scavenger[178, 181]. In our study, we attempted to establish whether 6-OHDA adducts could be generated through nucleophilic addition by GSH. To examine this possibility, GSH was incubated with 6-OHDA. After 24 h, the product was examined by capillary electrophoresis coupled mass spectrometry (CE/MS) (**figure 2.7**). A product with a mass-to-charge ratio of 475.09 was observed, indicating the formation of a 6-OHDA-GSH covalent adduct.

2.2.5 A Wide Range of Cellular and Viral Proteins are Covalently Modified by 6-OHDA

Once we established that 6-OHDA could potentially modify biologically relevant molecules, we sought to develop a chemical probe and identify cellular targets. To this end, we designed and

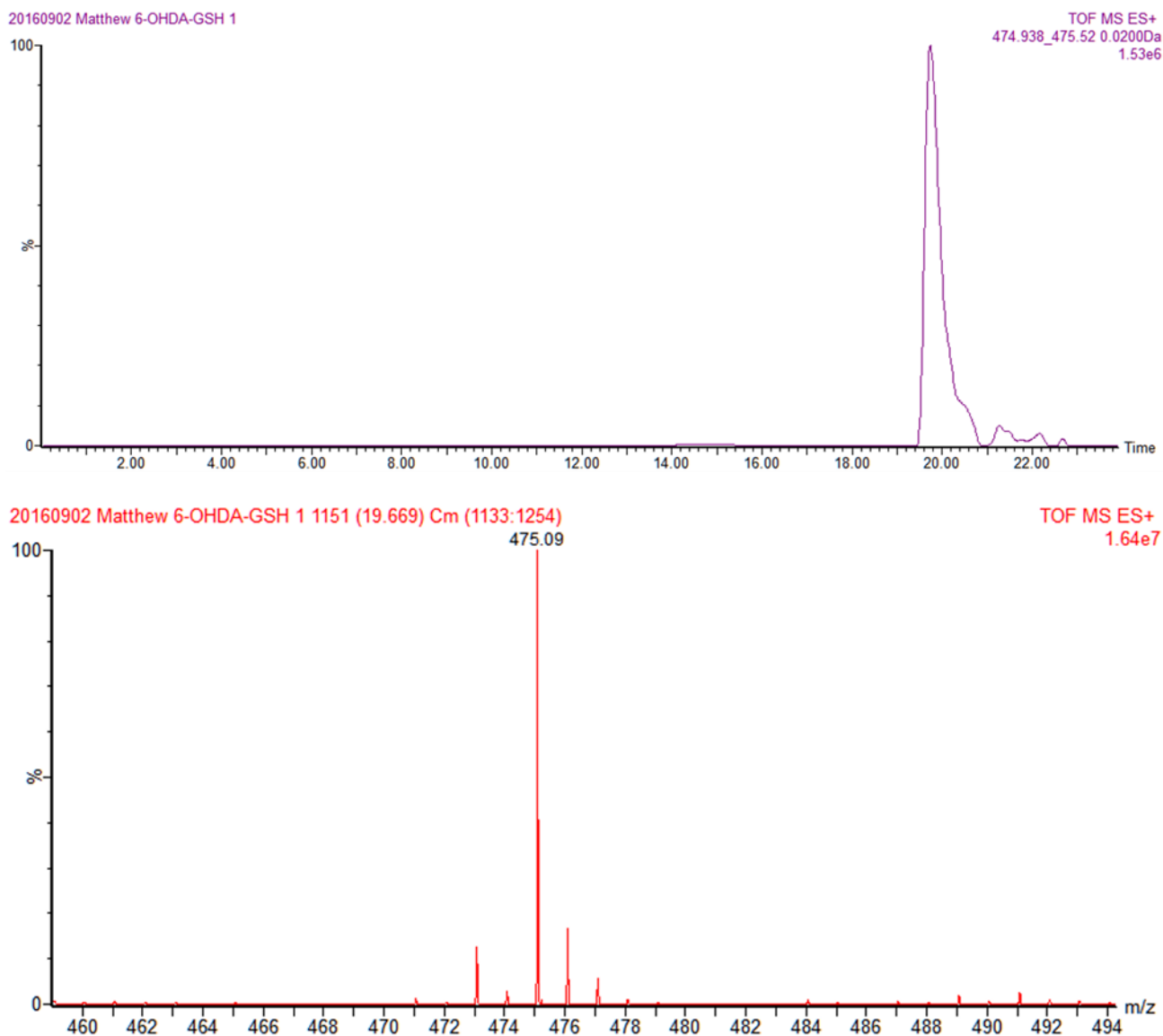
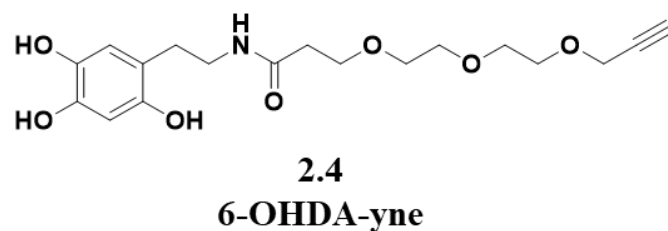


Figure 2.7. Capillary electrophoresis and mass spectrometry (CE/MS) trace of 6-OHDA-GSH adduct. 6-OHDA and GSH were reacted for 24 h at 37 C under ambient conditions, followed by capillary electrophoresis mass spectrometry analysis. A product with a retention time of 19.05 seconds, with a corresponding mass of 6-OHDA-GSH [M + 1] of 475.09 was identified.

synthesized a chemical probe to identify proteins alkylated by 6-OHDA. Chemical probes are ubiquitously used in chemical biology to identify target proteins and/or to characterize enzyme function in native biological environments[182]. We synthesized 6-OHDA-yne, a probe with an appended alkyne, for biorthogonal conjugation to a desired reporter (**figure 2.8A**). We performed *in vitro* labeling of freshly prepared HCV-FGR lysates and labeled with various concentrations of the 6-OHDA-yne probe (**figure 2.8B**). After incubation for 30 min at room temperature, 6-OHDA-yne was tagged to rhodamine-azide using copper(I)-catalyzed azide-alkyne cycloaddition[183]. The observed in-gel fluorescence scan revealed dose-dependent labeling of a wide range of proteins with 6-OHDA-yne. To confirm the extent of labeling, we undertook a competitive labeling experiment with 6-OHDA and 6-OHDA-yne (**figure 2.9**). Decreased labeling was observed as increasing concentrations of unmodified 6-OHDA were added, suggesting that 6-OHDA labeling is selective. Maleimide was used as a control to confirm that 6-OHDA and 6-OHDA-yne labeled cysteine residues (**figure 2.9**). To identify the labeled proteins, we treated HCV-SGR lysates with 6-OHDA-yne as described before but tagged the probe with biotin-azide. Proteins were subsequently isolated with streptavidin-coated beads and digested with trypsin for mass spectrometry analysis. A DMSO control was done to identify non-specific binding of proteins to the streptavidin-coated beads. We identified a set of labeled proteins and noted an over-representation of proteins involved in cellular stress and redox regulation, such as glutathione-S-transferase and peroxiredoxin. Induction of the cellular stress response during acute viral infection has been associated with up-regulation of proteins involved in the oxidative stress response, including both peroxiredoxin-1 and glutathione S-transferase[182]. Previous studies have shown that viral replication can be inhibited by blocking protein folding through alkylation and that viral genomic RNA can be susceptible to damage by free radical-mediated RNA damage[184].

A



B

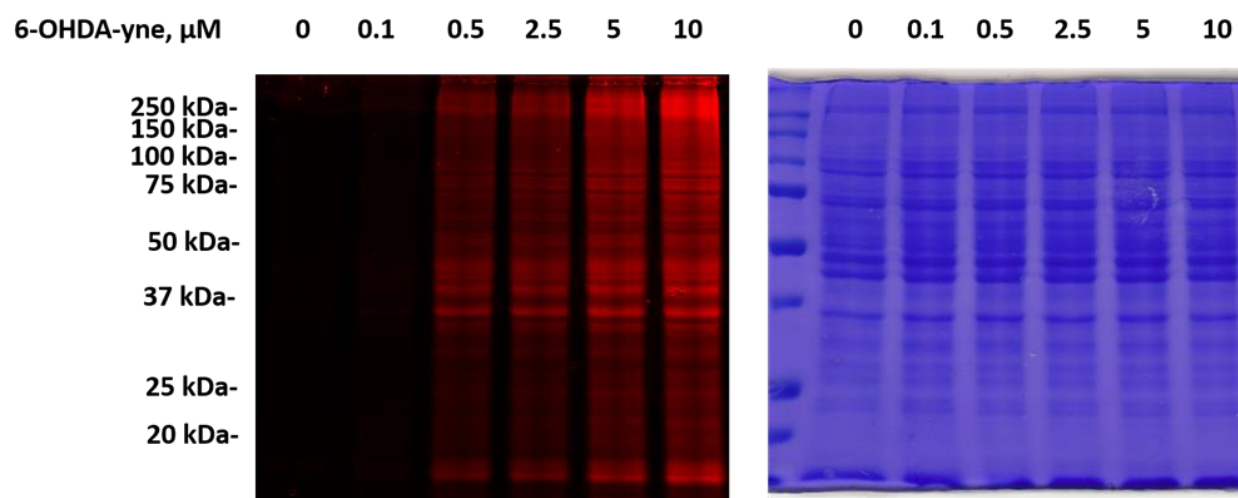


Figure 2.8. Proteome labeling by 6-OHDA. (A) 6-OHDA-yne is formed from the reaction between 6-OHDA and propargyl-PEG3-acid. (B) Labeling of Huh7.5-FGR cell lysates using the 6-OHDA-yne. Increasing concentrations of 6-OHDA-yne were incubated with Huh7.5-FGR. Labeled proteins were visualized by in-gel fluorescence. A Coomassie stain of the gel was performed to ensure equal loading.

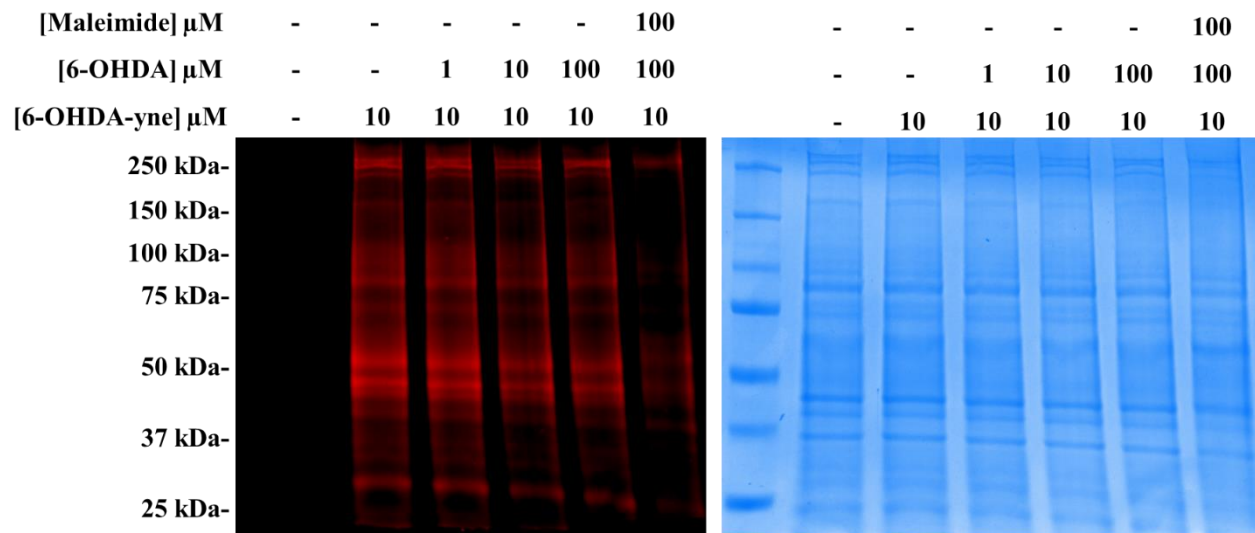
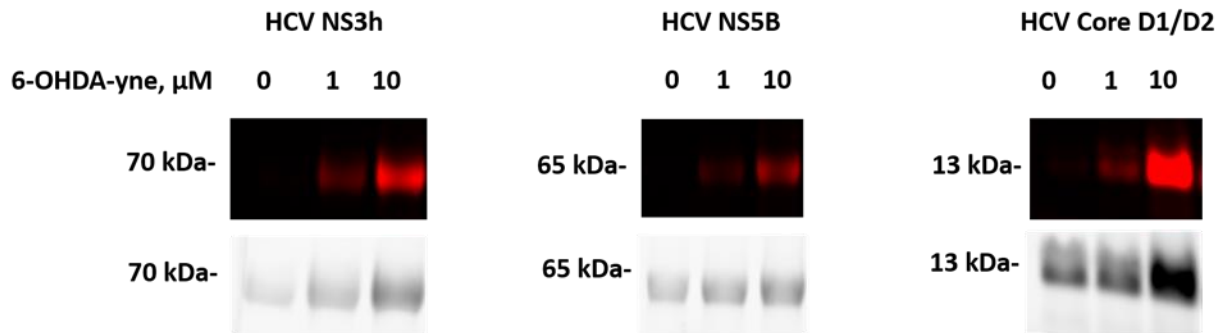


Figure 2.9. Competitive labeling of HCV-FGR with unmodified 6-OHDA and 6-OHDA-yne. (A) Unmodified 6-OHDA was pre-incubated with the cell lysates for 30 minutes, followed by the addition of 6-OHDA-yne. Labeled proteins were visualized by in-gel fluorescence. (B) a Coomassie stain of the gel was performed to ensure equal loading.

A



B

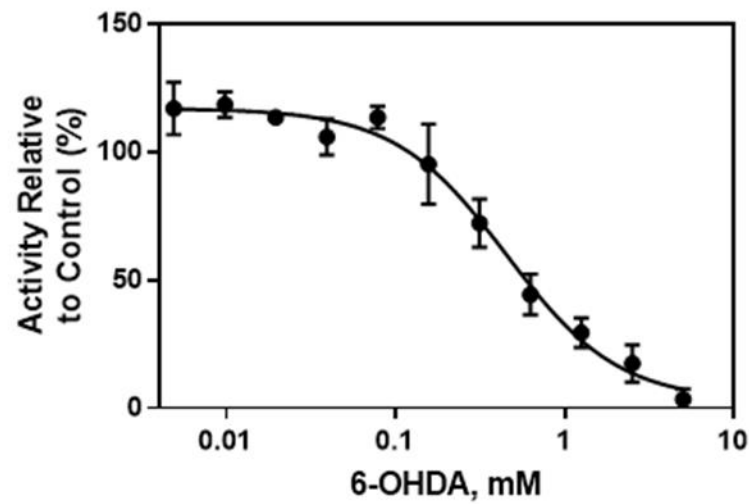


Figure 2.10. In-gel labeling of viral proteins with 6-OHDA-yne. (A) Labeling of NS3h HCV helicase, HCV NS5B polymerase, and domains 1/2 of the core protein with various concentration of 6-OHDA-yne. (B) Dose-response curve showing decreased helicase activity following incubation with 6-OHDA.

Alkylation of cellular proteins involved in the oxidative stress response could have further negative effects on viral replication, as indicated by the effects of 6-OHDA. In addition, we speculated that **2.1** might also react directly with the viral proteome. To test this interaction, we used the **2.4** probe with rhodamine-azide as described above and labeled the recombinantly expressed HCV core (domain 1 and 2), the helicase domain of HCV NS2 (NS3h), and the NS5B polymerase (**figure 2.10A**). These three recombinant proteins were selected not only given their role in viral replication and propagation but also the importance of cysteine residues in their function[185-187]. Chemical probe **2.4** labels each of the viral proteins displayed in **figure 2.10A**. To examine the functional outcome of viral protein alkylation, we pre-incubated NS3h with **2.4** and performed a DNA unwinding assay (**figure 2.10B**). Alkylation of the viral helicase resulted in functional impairment, demonstrated by decreased unwinding activity in the presence of 6-OHDA although full inhibition was only achieved at a high concentration of 5 mM. To confirm that functionalization of **2.1** did not impair its inhibitory effect on the unwinding activity of NS3h, we examined the effects of **2.1** and **2.4** on helicase unwinding and did not observe a functional difference, with both compounds inhibiting approximately 40% at a concentration 750 μ M. In our assay, modification of the helicase can affect unwinding. In the context of the cell, effects could extend to disruption of protein-protein interactions. It is possible that the effects of **2.1** are amplified in a cellular setting. These results support our observations that protein modification by 6-OHDA is a contributing mechanism to antiviral activity of the compound.

2.3 Conclusion

In this project, we have identified **2.1** as an inhibitor of HCV replication and a probe for the role of oxidative stress. Although subtle changes in the level of RISC inhibition were observed following the treatment with 6-OHDA, it is likely that additional mechanism contribute to the

antiviral effects of **2.1**. Our data suggests that both oxidative stress and protein alkylation contribute to 6-OHDA's antiviral effect. The bioactive SM **2.1** was shown to generate ROS and is highly reactive with viral and host cellular proteins, many of which are involved in stress response. Alkylation of viral proteins impaired function, and this is likely the case with host proteins. Taken together, these results point to 6-hydroxydopamine as a useful probe for oxidative stress in the context of virus-host interactions and other cellular models of disease.

2.4 Experimental section

General information

All reagents and solvents were purchased from Sigma-Aldrich, unless otherwise noted, and used without further purification. Alkyne-PEG3-acid was purchased from BroadPharm. 6-OHDA HCl was purchased from Sigma Aldrich. Deuterated solvents were purchased from Cambridge Isotope Laboratories. Thin layer chromatography was performed using Analtech Uniplate® silica gel plates (60A F254, layer thickness 250 μm). Flash column chromatography was performed by silica gel (60A, particle size 40 to 63 μm). ^1H NMR and ^{13}C NMR spectra were recorded using a Bruker-DRX-400 spectrometer at a frequency of 400.13 MHz for ^1H and 100.61 MHz for ^{13}C and processed with Bruker TOPSPIN 2.1 software. Chemical shifts are reported in parts per million (δ) using residual solvent resonance as an internal reference. The following abbreviations were used to designate chemical shift multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet or unresolved, br = broad single and J = coupling constant in Hz.

Tissue culture

Huh7 or Huh7.5 cells expressing the HCV-SGR and HCV-FGR, respectively, were grown in Dulbecco's Modified Eagle Medium (GIBCO-BRL, Burlington, Canada) supplemented with 10% fetal bovine serum (FBS), 100 nM non-essential amino acids (ThermoFisher), 100 U/mL penicillin-streptomycin, and 250 µg/mL Geneticin (GIBCO-BRL, Burlington, Ontario).

Luciferase reporter assay

HCV-SGR expressing cells were seeded in a 12-well plate at a density of 1.5×10^4 cells/well and maintained for 24 h in culture media until a confluency of 60-70% was reached. Cells were then treated with 6-OHDA (0 to 500 µM). At 24 h post-treatment, the cells were washed twice with phosphate buffered saline (PBS), and lysed with cell culture lysis buffer (Promega, Madison, WI). The culture plate was incubated at room temperature for 10 min on a shaker and then lysates stored at -20 C overnight. Prior to usage, samples were equilibrated to room temperature. Luciferase assay substrates (25 mM glycylglycine, 5 mM K_xPO_4 , pH 8, 4 mM EGTA, 2 mM ATP, 1 mM DTT, 15 mM $MgSO_4$, 0.1 mM Coenzyme A, and 75 µL luciferin) were added and expression of luciferase determined by bioluminescence measurement on an Lmax luminometer (Molecular Devices Corporation, Sunnyvale, CA). Total protein concentration was measured by Bio-Rad DC Protein Assay (Bio-Rad, Mississauga, Canada) according to manufacturer's protocol. EC_{50} values were determined by plotting the relative enzymatic activity against the log-transformed concentration values and fit using a four-parameter equation: $Y = min + \left(\frac{(max-min)}{1+10^{((X-logEC_{50}) \times Hill\ slope)}} \right)$, where X is the inhibitor concentration, min and max are the minimum and maximum observed responses, respectively, and the Hill slope is the slope factor.

MTT assay

Cells expressing HCV-SGR were treated as described above. After 24 h, cells were washed twice with PBS followed by the addition of 200 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Alfa-Aesar, Ward Hill, MA) at 2.5 mg/mL in PBS and incubated for 3 h. Excess media was removed and precipitated crystals were solubilized in 200 μ L of DMSO. The absorbance was read at 562 nm using SpectarMax M2 (Molecular Devices Corporation, Sunnyvale, CA) and the data was recorded using Softmax Pro 4.7 software.

miR-122 reporter assay

DNA sequences containing miR-122 binding sites were as follows: reverse, GGCCGCTGGAGTGTGACAATGGTGTGTTGGTGATTGGAGTGTGACAATGGTGTGTTGC; forward,

TCGAGCAAACACCATTGTCACACTCCAAATCACCAAACACCATTGTCACACTCCAGC

. These sequences were annealed and digested with XhoI/NotI for cloning into a psiCHECK-2 vector. Huh7.5 cells were seeded in a 24-well plate at a density of 6.0×10^4 cells/well and maintained in antibiotic free media for 24 h. The psiCHECK-2 plasmid (200 ng) containing the miR-122 sites was transfected into cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocols. Twenty-four hours following the transfection of psiCHECK-2, cells were treated with the inhibitors at 5 or 50 μ M for 24 h. Alternatively, cells were transfected with 50 nM of the miRNA-122 mimic or 2'O-methyl miR-122 (Dharmacon, Lafayette USA). After 24 h, cells were washed with PBS and lysed using passive cell lysis buffer (100 μ L; Promega) and a dual luciferase assay performed to assess miR-122 activity.

Cellular reactive oxygen species (ROS) detection assay

Cellular ROS were analyzed with Abcam's cellular reactive oxygen species detection assay kit (Abcam Inc., Toronto, ON, Canada). The assay uses the cell permeable reagent 2',7'-dichlorofluorescein diacetate (DCFDA) that measures the level of hydroxyl, peroxy, and other reactive oxygen species (ROS) within a cell. Huh7.5s were seeded in a 96-well plate at a density of 2.5×10^4 cells/well and maintained for 24 h in cell culture media. Media was removed after 24 h and cells washed with 100 μ L of 1X buffer according to the manufacturer's protocol. The cells were incubated with DCFDA solution for 45 min at 37 C. After 45 min, the solution was removed and 1X buffer was added followed by the immediate measurement of fluorescence intensity. After measuring the initial fluorescence intensity using a SpectraMax I3 plate reader (Molecular Devices, Sunnyvale, CA, USA) with the excitation and emission spectra of 485 nm and 535 nm, respectively, the 1X buffer was removed and the cells were treated with either the control reagent (Tert-butyl hydroperoxide, TBHP) or 6-OHDA for 24 h. The results were expressed as a fold increase in fluorescence intensity with respect to the untreated control.

Capillary electrophoresis mass spectrometry

All reaction solutions were filtered through nylon membrane filter with a 0.22 μ m pore size (Millipore, Nepean, ON, Canada). The bare-silica capillary was purchased from Polymicro (Phoenix, USA). The sample storage was maintained at 4 ± 0.5 C and capillary temperature was maintained at 15 ± 0.5 C. The electric field in CE separation was 330 V/cm with a positive electrode at the injection end. For all samples, the capillary was 90 cm long with an inner and outer diameter of 50 μ m and 360 μ m, respectively. Each sample was injected into the capillary from the inlet end by a $10 \text{ s} \times 5 \text{ psi}$ pressure pulse. Prior to each experimental sample, the capillary

was rinsed at 75 psi pressure with 0.1 M NH_4OH for 2 min, followed by ddH₂O for 2 min, and finally with 5% formic acid for 2 min. A Synapt G2 high definition mass spectrometer with T-Wave ion mobility from Waters (UK) was coupled with a PA800plus Pharmaceutical Analysis CE system through a CE-ESI sprayer from Micromass (UK). The electrospray ionization conditions were as follows: capillary voltage 3.2 kV, positive mode, sampling cone voltage 25 V, extraction cone voltage 5 V, source temperature 120°C, cone gas 50 L/h, nano flow gas 0.15 Bar, purge gas 3 L/h. Sheath liquid (50:50 methanol:1% acetic acid) was delivered with flow rate of 1.5 $\mu\text{L}/\text{min}$. MS data was acquired from $m/z=100$ to $m/z=500$.

Preparation of cell lysates for profiling and mass spectrometry

Cells expressing HCV-FGR were seeded at 1×10^5 cells in a 100 mm dish. After 24 h, the subconfluent cells were washed twice with PBS and 1 mL of 0.01% Triton X-100 in PBS was added. The lysates were sonicated then centrifuged for 5 min at 14 000 rpm, 4 C to precipitate cell debris. The protein concentration of the supernatant was quantified via DC protein assay (Bio-Rad).

Protein labeling in vitro

For *in vitro* protein labeling, various concentrations of 6-OHDA-yne were added to fresh cell lysates in 100 μL PBS and 0.05% Triton X-100 buffer. Samples were incubated with the probe for 30 min (**figure 2.10**). Then, 100 μL of freshly prepared click chemistry mix in PBS consisting of rhodamine-azide (100 μM), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA, 200 μM), CuSO_4 (2 mM), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 2 mM), were added and incubated for 2 h at room temperature with gentle mixing. The reactions were terminated by

addition of 1 mL of acetone and stored at -80 C for at least 30 min. The samples were then centrifuged for 15 min at 4 C, 14 000 rpm to precipitate protein. The protein pellets were air dried for 10 min, resuspended in 35 Laemmli buffer containing beta-mercaptoethanol, shaken for 15 min and heated for 5 min at 95 C. The samples were then resolved by 10% SDS-PAGE and then visualized by in-gel fluorescence scanning using a FMBIO fluorescence scanner (Hitachi). Visualization of loading was undertaken using Coomassie Brilliant Blue.

Competitive labeling of HCV-FGR cell lysates in vitro

For *in vitro* protein labeling, fresh HCV-SGR lysates (0.7 mg/mL) in 100 μ L PBS and 0.05% Triton X-100 buffer were incubated with increasing concentrations of 6-OHDA for 1 h, followed by the addition of 6-OHDA-yne. Samples were incubated with the probe for 30 min. Then, 100 μ L of freshly prepared click chemistry mix in PBS consisting of rhodamine-azide (100 μ M), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA, 200 μ M), CuSO₄ (2 mM), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 2 mM), were added and incubated for 2 h at room temperature with gentle mixing. The reactions were terminated by addition of 1 mL of acetone and stored at -80 C for at least 30 min. The samples were then centrifuged for 15 min at 4 C, 14 000 rpm to precipitate protein. The protein pellets were air dried for 10 min, resuspended in 35 μ L Laemmli buffer containing beta-mercaptoethanol, shaken for 15 min and heated for 5 min at 95 C. The samples were then resolved by 10% SDS-PAGE and then visualized by in-gel fluorescence scanning using a FMBIO fluorescence scanner (Hitachi). Visualization of loading was undertaken using Coomassie Brilliant Blue.

6-OHDA-yne pull-down

Biotin-azide click chemistry, streptavidin-enrichment, and trypsin digest of probe-labelled proteomes was performed as previously described[183]. However, an acetone precipitation step was included following the incubation with the click chemistry mix. Following protein enrichment, agarose-streptavidin beads (Pierce) were washed three times with 100 μ L PBS in a Biospin column. Using 200 μ L of PBS, beads were transferred to probe-labeling cell lysates and incubated for 90 min. For the on-bead digestion, the beads were washed five times with 50 mM ammonium bicarbonate, transferred to an Eppendorf tube and heated for 15 min at 60 C in 500 μ L of 10 mM DTT in 50 mM ammonium bicarbonate (ABC). After 15 minutes, 25 μ L of 500 mM iodoacetamide was added and lysates were incubated in the dark for 30 min. Samples were centrifuged for 2 min at 1400 rpm and 100 μ L of ABC was added followed by 2 μ L of 0.5 mg/mL of trypsin. Samples were rotated at 37 C overnight, followed by bead pelleting and the transfer of the supernatant for MS analysis.

Mass spectrometry

HPLC-ESI-MS/MS was used for MS analysis. The system consisted of an Agilent 1100 micro-HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled with an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA) equipped with a nano-electrospray interface operated in positive ion mode. Pre-column was packed in-house with reverse phase Magic C18AQ resins (5 μ m; 120 Å pore size; Dr. Maisch GmbH, Ammerbuch, Germany), and analytical column of 75 μ m $\times$ 100 mm was packed with reverse phase Magic C18AQ resins (1.9 μ m; 120 Å pore size; Dr. Maisch GmbH, Ammerbuch, Germany). The mobile phases consisted of 0.1% (v/v) FA in water as buffer A and 0.1% (v/v) FA in acetonitrile as buffer B. The 4 μ L sample was loaded

onto the pre-column using a buffer containing 9% A at a flow rate of 4 $\mu\text{L}/\text{min}$ for 5 min. Subsequently, a gradient from 5% to 35% buffer B was performed, at a flow rate of $\sim 300\text{ nL}/\text{min}$ obtained from splitting a 20 $\mu\text{L}/\text{min}$ through a restrictor, in 60 min. The MS method consisted of one full MS scan from 350 to 1700 m/z followed by data-dependent MS/MS scan of the 5 most intense ions, with dynamic exclusion repeat count of 2, and repeat duration of 90 s. As well, for the experiments on the Orbitrap MS the full MS was performed in the Orbitrap analyzer with $R = 60,000$ defined at m/z 400, while the MS/MS analysis were performed in the LTQ. To improve the mass accuracy, all the measurements in Orbitrap mass analyzer were performed with internal recalibration (“Lock Mass”). On the Orbitrap, the charge state rejection function was enabled, with single and “unassigned” charge states rejected.

Analysis of mass spectrometry data

Raw files were generated using LTQ-Orbitrap and processed and analyzed using MaxQuant, Version 1.3.0.5 using the Uniprot fasta protein database (2013, July version) in addition to the HCV con1 sequence including the commonly observed contaminants. When accessing the protein databases, the following parameters were used: enzyme specificity was set to trypsin; protein N-terminal acetylation, methionine oxidation, and cysteine carbamidomethylation was selected as fixed modification as previously described[188-192]. A maximum of two missing trypsin cleavages were permitted. Precursor ion mass tolerances were set to 7 ppm, and the fragment ion mass tolerance was 0.8 Da for the MS/MS spectra. If the identified peptide sequences of one protein were contained within or equal to another protein’s peptide set, the proteins were grouped together and reported as a single protein group. The false discovery rate (FDR) for peptide and

protein was set to 1 % and a minimum length of six amino acids was used for peptide identification. Normalized LFQ intensity was used for protein quantification.

Labeling of purified viral proteins

Purified HCV core (D1/D2) was provided by Dr. Matthew Goodmurphy (University of Ottawa). The HCV NS5B polymerase and the HCV NS3 helicase were a kind gift from Dr. Matthias Götter at the University of Alberta. For labeling of viral proteins *in vitro*, various concentration of 6-OHDA-*yne* were added to purified viral proteins in 100 μ L PBS and 0.05% Triton X-100 buffer. Samples were incubated with the probe for 30 min. After 30 min, 100 μ L of freshly prepared click chemistry mix in PBS consisting of rhodamine-azide (100 μ M), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA, 200 μ M), CuSO₄ (2 mM), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 2 mM), were added and incubated for 2 h at room temperature with gentle mixing. The reactions were terminated with the addition of 1 mL of acetone and stored at -80 C for at least 30 min. The samples were then centrifuged for 15 min at 4 C, 14 000 rpm to precipitate the protein. The protein pellets were air dried for 10 min, resuspended in Laemmli buffer containing beta-mercaptoethanol, shaken for 15 min and heated for 5 minutes at 95 C. The samples were then resolved by 12% SDS-PAGE and then visualized by in-gel fluorescence scanning using the ChemiDoc MP system (BioRad). Visualization of protein loading was undertaken using stain-free technology (BioRad).

Helicase unwinding IC₅₀ assay

The HCV NS3h helicase (75 nM) was incubated for 2 h with a range of 6-OHDA concentrations up to 10 mM in a buffer containing 25 mM MOPS pH 6, 10 mM NaCl, 3 mM MgCl₂, and 0.1%

Tween-20. Following the 2 h incubation, 5 nM of a partially single-stranded DNA duplex (top strand, 5'-Cy5/GCTCCCCAATCGATGAACGGGGAGC/IBQ/-3' and bottom strand 5'-GCTCCCCGTTTCATCGATTGGGGAGC(T)₂₀) was added[192]. The unwinding reaction was initiated by the addition of 0.5 mM ATP and upon excitation at 635 nm, the fluorescence emission at 670 nm was monitored on a SpectraMaxi3 (Molecular Devices) at 1 min intervals. Unwinding of the duplex results in the top strand forming a hairpin structure and quenching of the Cy5 signal by IBQ. After 25 min the unwinding reaction was complete, and the amount of unwinding at each concentration of 6-OHDA was determined as a percentage of unwinding in the absence of any inhibitor (control). Unwinding activity was plotted against the log concentration of inhibitor and fit to the equation: $Y = min + \left(\frac{(max-min)}{1+10^{((X-logEC50) \times Hill\ slope)}} \right)$, where X is the inhibitor concentration, min and max are the minimum and maximum observed responses, respectively, and the Hill slope is the slope factor.

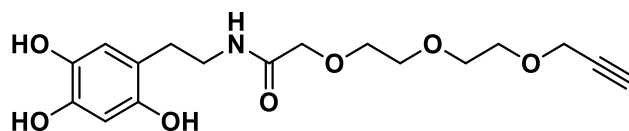
Statistical analysis

Statistical analysis of HCV replication and ROS generation was performed using a paired Student's t-test in GraphPad Prism 4.01 (GraphPad Software Inc., La Jolla, CA). A p-value of < 0.05 was considered as significant.

2.5 Synthetic methods and characterization

All reagents and solvents were purchased from Sigma-Aldrich, unless otherwise noted, and used without further purification. Deuterated solvents were purchased from Cambridge Isotope Laboratories and thin layer chromatography was done using Analtech Uniplate® silica gel plates (60A F254, layer thickness 250 µm). Flash column chromatography was performed by silica gel

(60A, particle size 40 to 63 μm). ^1H NMR and ^{13}C NMR spectra were recorded using a Bruker-DRX-400 spectrometer at a frequency of 400.13 MHz for ^1H and 100.61 MHz for ^{13}C and processed with Bruker TOPSPIN 2.1 software. Chemical shifts are reported in parts per million (δ) using residual solvent resonance as an internal reference. The following abbreviations were used to designate chemical shift multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet or unresolved, br = broad single and J = coupling constant in Hz.



6-OHDA-yne: Twenty milliliters of DMF was added to an oven-dried flask. The mixture was degassed for 30 min using argon and then alkyne-PEG3-OH (263 mg, 1.02 mmol) and HATU (208 mg, 0.111 mmol) were added. The reaction mixture was stirred for 30 min under argon and then 6-hydroxydopamine hydrochloride (208 mg, 1.01 mmol) was added. The reaction was stirred for additional 15 min at 60 C under argon and then diisopropylamine (340 μL , 2.03 mmol) was added. The reaction was stirred at 60 C under argon overnight. Upon completion of the reaction, the mixture was concentration *in vacuo*. The residue was purified using HPLC eluting 10 to 60% acetonitrile/water with 0.1% formic acid. ^1H NMR (400 MHz, MeOD) δ 6.24 (s, 1H), 6.05 (s, 1H), 3.88 (d, J = 2.40, 2H), 3.43-3.30 (m, 10H), 3.08 (t, J = 7.14, 2H), 2.54 (t, J = 2.38, 1H), 2.37 (t, J = 7.12, 2H), 2.15 (t, J = 6.10, 2H). ^{13}C NMR (100 MHz, MeOD) δ 174.14 (C), 149.41(C), 145.31(C), 138.88(C), 118.40 (C), 117.25(CH), 104.57(CH), 80.57 (C), 75.94(C), 71.37(CH₂), 71.29(CH₂), 71.25(CH₂), 70.08(CH₂), 68.21(CH₂), 59.01(CH₂), 41.25(CH₂), 37.63(CH₂), 30.42(CH₂). **LRMS** (ESI⁺): *m/z* calculated for C₃₁H₃₁N₃O₇ [M+H]⁺ = 368.16. Found 368.

6-OHDA/GSH reaction

Glutathione (100 mg, 0.325 mmol) was added to a round bottom flask containing milliQ water at pH 7.8. This was followed by the addition of 6-OHDA (66.7 mg, 0.325 mmol). The reaction was stirred overnight at 37 C under ambient conditions. Compound identified using CE/MS. LRMS (ESI⁺): *m/z* calculated for C₁₈H₂₆NO₉S [M+H⁺] = 474.14. Found [M/2 + 1] = 474.9

Chapter 3: A Sinefungin-based Affinity Probe for Methyltransferase Enzymes

The entirety of this work was published in:

Lafreniere, M.A.; Desrochers, G.F.; Mekbib, K.; Pezacki, J.P. An affinity-based probe for methyltransferase enzymes based on sinefungin. *Can. J. Chem.*, **2017**, 95(10): 1059-1063.

Abstract

Epigenetic modifications of cellular biomolecules controls numerous cellular processes, such as gene transcription, signal transduction, and protein stabilization. An understanding of epigenetic mechanisms can lead to the development of therapeutic agents for various diseases. Herein, we report the design and synthesis of a sinefungin-based affinity probe (BpyneSF) that targets methyltransferase enzymes and other proteins involved in recognition of methylation. This probe contains a bioorthogonal alkyne residue for conjugation using the copper- catalyzed azide-alkyne cycloaddition and a photoactivatable crosslinker group for covalent attachment of the probe to its proteomic targets. We investigate the efficiency and selectivity of the probe to inhibit and label methyltransferase enzymes and we demonstrate through in-gel fluorescence, on-bead digestion, and tandem mass spectrometry, that BpyneSF can label methyltransferase SETD2 and reader proteins *in vitro*. These results establish the utility of BpyneSF as a tool for activity-based protein profiling in complex biological environments.

Statement of Research Contributions

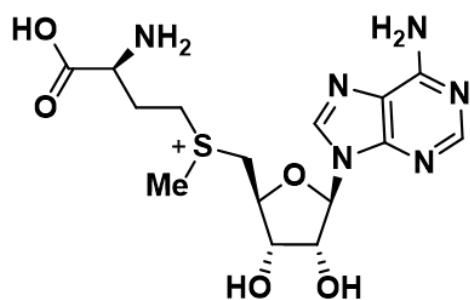
This chapter includes data previously published in the *Canadian Journal of Chemistry* titled: An affinity-based probe for methyltransferase enzymes based on sinefungin. This publication was authored by M.A. Lafrenière, Kedous Mehkib, Geneviève Desrochers, and J.P. Pezacki. As the principal author, I contributed significantly to the intellectual and experimental development of the research herein. J.P. Pezacki and I conceived of the research idea and experimental plan. I performed all the synthesis required and *in vitro* labeling. Geneviève Desrochers assisted with *in vitro* methyltransferase labeling and Kedous Mehkib assisted with *in vitro* labeling of eukaryotic cell lysates. I wrote the first draft of the manuscript and editing was performed by all authors.

3.1 Introduction

Epigenetics refers to changes in heritable phenotypic traits caused by manipulating gene expression rather than caused by alterations in the genetic code. Control of phenotype expression involves the diverse interaction between regulator RNAs, DNA methylation, and histone modification[193]. Disruption of one of these essential epigenetic regulators can result in epigenetic diseases, such as congenital disorders, cancers, and neurodegenerative diseases[194, 195]. Importantly, methylation of DNA and proteins regulate numerous biological processes, such as gene transcription, signal transduction, and protein stabilization[195, 196]. Given the importance of methylation to normal physiological function and its contribution to human disease, DNA and protein methyltransferases have emerged as an important group of enzymes targeted for development of novel pharmacological agents[197-199]. In reactions catalyzed by methyltransferases, cofactor S-(5'-Adenosyl)-L-methionine (SAM) (**figure 3.1**) is used to transfer a methyl group to biological substrates, such as CpG islands in DNA or histone tails in nucleosomes[194, 200, 201]. SAM and S-(5'-Adenosyl)-L-homocysteine (SAH), a selective feedback inhibitor that results from methyl transfer, and its analogues have been developed as versatile tools to study SAM-dependent methyltransferases[202].

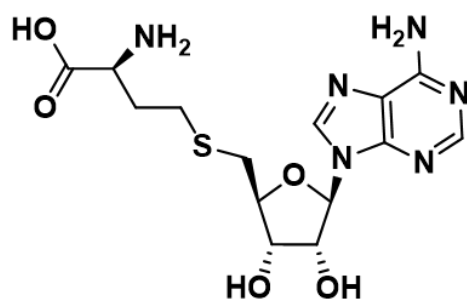
3.1.1 Sinefungin as an inhibitor of methyltransferases

Sinefungin (**figure 3.1**) was originally isolated from *Streptomyces* sp. K05-0178 and tested as a novel antibiotic for *Trypanosoma brucei*, the parasitic source for human African trypanosomiasis or sleeping sickness[203]. The bioactive SM **3.3** is a nucleoside analogue of SAH and has been used as a scaffold to design selective inhibitors for protein lysine methyltransferases[204-206]. Previous reports have used **3.3** to investigate the mechanism by which SET domain-containing methyltransferases catalyze the transfer of methyl groups to protein biomolecules[205]. A major



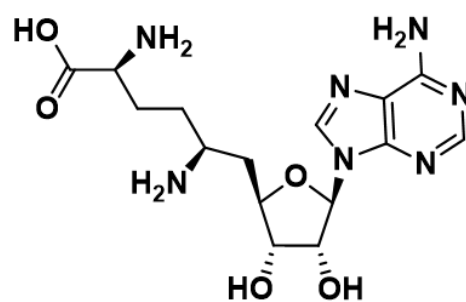
3.1

S-adenosylmethionine (SAM)



3.2

S-adenosylhomocysteine (SAH)



3.3

Sinefungin (SF)

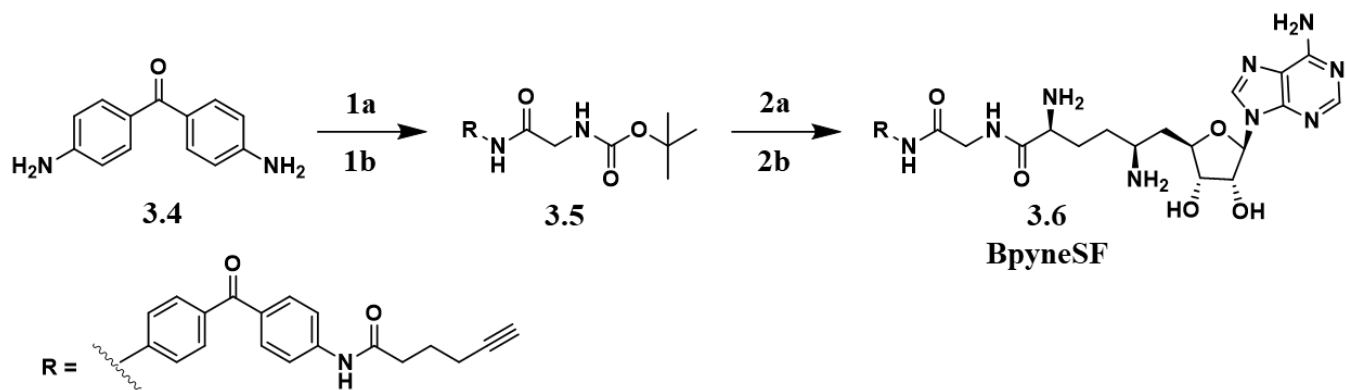
Figure 3.1. Structure of bioactive small molecules important in SAM-dependent epigenetic regulation.

drawback of sinefungin is that it is a reversible, non-selective inhibitor of methyltransferases with an IC_{50} range of between 0.1 μ m and 20 μ m[197, 207, 208]. However, the lack of SF selectivity is advantageous as it permits the study of a broad variety of methyltransferases in parallel. In this study, we develop a photoactive chemical probe based on the reversible inhibitor sinefungin (**scheme 3.1**) and undertook a preliminary investigation into its effectiveness as a methyltransferase activity probe.

3.2 Results and discussion

3.2.1 Development of sinefungin affinity-based probe

To prepare our photoactive chemical probe **3.6**, we introduced the photoactive moiety benzophenone (Bp) to the core probe structure (**scheme 3.1**). The inclusion of Bp enables the covalent modification of target methyltransferases and associated binding proteins, as has been previously demonstrated with other Bp-containing activity probes and as outlined in **chapter 1**[209, 210]. In addition to the Bp moiety, a biologically inert alkyne was appended to the probe to facilitate the incorporation of a reporter tag through copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC)[147, 211]. Initially, **3.3** (**figure 3.1**) was protected with di-tert-butyl dicarbonate in water to facilitate amide coupling to the Bpyne. As shown in **scheme 3.1**, 4,4'-diaminobenzophenone was first coupled to 5-hexynoic acid, followed by coupling to *N*-(tert-Butoxycarbonyl)glycine by HATU-mediated coupling[212]. Trifluoroacetic acid was used to deprotect Bpyne-glycine-BOC and Bpyne-glycine-NH₂ was subsequently coupled to (Boc)₂-sinefungin. Finally, (Boc)₂-sinefungin was deprotected in acid to yield BpyneSF. Following the



Scheme 3.1. Design and synthesis of BpyneSF. 1a) 5-Hexynoic acid, HATU, trimethylamine, DMF, Argon, 80°C (13% yield); 1b) *N*-(*tert*-Butoxycarbonyl)glycine, HATU, trimethylamine, DMF, Argon, 65°C (35% yield); 2a) (Boc)₂-Sinefungin, HATU, Diisopropylamine, anhydrous DMF, Argon, 60°C. 2b) Trifluoroacetic acid, RT (48% yield).

synthesis, UV-induced covalent attachment of BpyneSF to target enzymes was visualized through in-gel fluorescent (**figure 3.2B**).

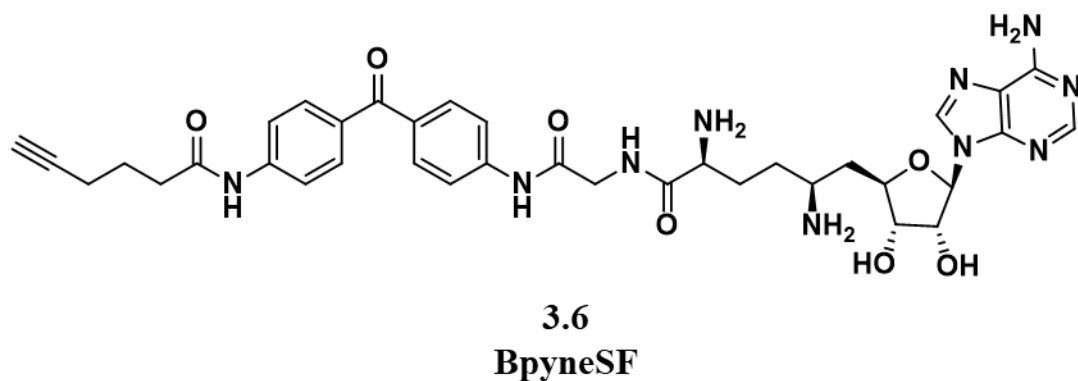
3.2.2 Targeting eukaryotic proteins with BpyneSF

To validate the effectiveness of **3.6** as an affinity probe, we evaluated *in vitro* labeling of human embryonic kidney cells (Hek293). Hek293 cell line and its derivatives are commonly used in a wide variety of experiments ranging from signal transduction to protein interaction studies and represent an ideal cell line for study[213]. Optimal labeling concentration of BpyneSF was determined by treatment of isolated Hek293 proteome with various probe concentrations (**figure 3.2B**) followed by covalent attachment of rhodamine-azide via CuAAC. Levels of background labeling by BpyneSF were assessed with a no ultraviolet light exposed sample (**figure 3.2B**). Total protein content was also assessed to ensure equal protein loading (**figure 3.4A**). Further optimization was undertaken to determine the ideal probe incubation time (**figure 3.3A**) and rhodamine-azide incubation time (**figure 3.3B**). To limit background intensity, the labeling concentration and incubation time were determined to be 10 μ M and 5 minutes, respectively. Following incubation, the sample was UV irradiated for 15 minutes at room temperature. The intense bands detected in the region around 100, 50, and 40 kDa by in-gel fluorescent detection suggests that the BpyneSF probe has a greater selectivity for these proteins. Overall, these results indicate that BpyneSF can selectively target enzymes in Hek293 proteome.

3.2.3 Target validation of BpyneSF

To elucidate the targeted subclasses of methyltransferase enzymes by BpyneSF, we undertook competitive labeling of Hek293 proteome with 1-Benzyl-3,5-*bis*-(3-bromo-4-

A



B

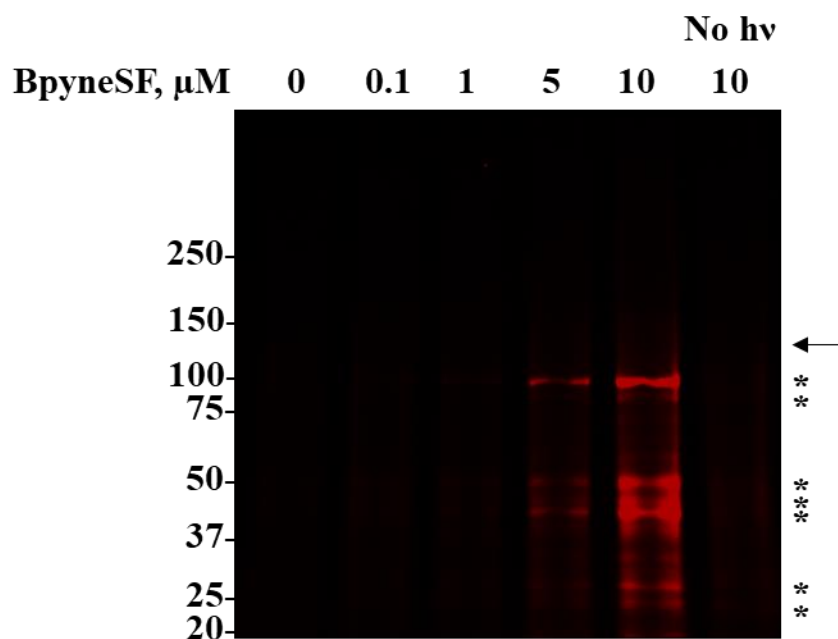


Figure 3.2. *In vitro* activity labeling of Hek293 proteome. (A) Structure of BpyneSF activity probe. (B) BpyneSF concentration-dependent labeling of Hek293 proteomes (0.5 mg/ml). Asterisks indicates concentration-dependent increases in band intensity. Arrow indicates location of SETD2.

hydroxylbenzylidene)piperidin-4-one (**3.7**), a small molecule inhibitor of coactivator-associated arginine methyltransferase (CARM1)[214]. We treated freshly isolated Hek293 with 10 μ M and 100 μ M inhibitor or DMSO control, followed by incubation with 10 μ M BpyneSF, irradiation with 365 nm wavelength, and CuAAC-mediated tagging of rhodamine-azide. The reactions were then quenched with cold acetone, separated by SDS-PAGE, and rhodamine-tagged BpyneSF was detected by in-gel fluorescence scanning (**figure 3.3D**). Treatment with 100 μ M of **3.7** resulted in a concentration-dependent decrease in fluorescence signal intensity compared with the no inhibitor control and the 10 μ M CARM1 inhibitor (**figure 3.3D**). Total protein content was assessed to ensure equal loading (**figure 3.4B**). CARM1 inhibitor is a cell-permeable (*bis-benzylidene*)piperidinone derivative that is reported to display selective inhibition of CARM1/Protein arginine methyltransferase 4 (PRMT4), with low or no activity against a diverse panel of arginine and histone lysine methyltransferase enzymes[214]. As previously reported, CARM1 can inhibit protein arginine N-methyltransferase 1 (PRMT1) and CARM1/PRMT4 with IC_{50} values of 63 μ M and 7.1 μ M, respectively[214]. We observed a decrease in signal intensity across the gradient of protein masses in the 100 μ M CARM1 inhibitor lane, suggesting that at higher concentrations the CARM1 inhibitor can inhibit PRMT1 and other off-target proteins. Previous research has shown that SF can target PRMT1, which methylates arginine on histone H4 and has a molecular weight of approximately 40 kDa[215]. As observed in **figure 3.3D**, the decrease in signal intensity at 40 kDa suggests that this protein band is PRMT1 but further validation of this observation using recombinant enzyme assay is required. Overall, these results suggest that BpyneSF can covalently modify subclasses of methyltransferase enzymes.

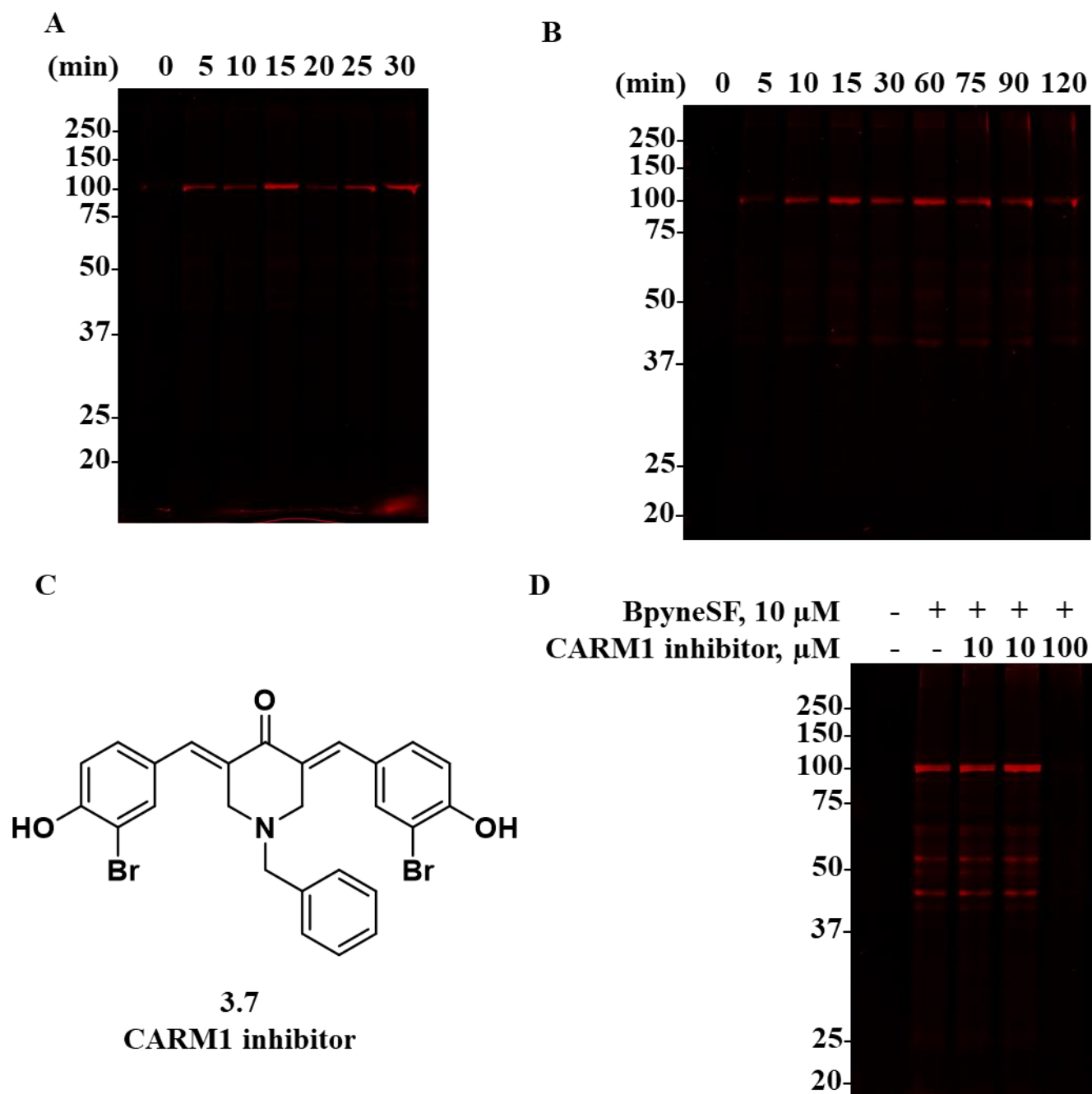


Figure 3.3. *In vitro* activity labeling of Hek293 proteome. (A) Optimization of BpyneSF incubation time for labeling Hek293 proteome with 10 μ M of BpyneSF. (B) Optimization of BpyneSF incubation with rhodamine azide for labeling Hek293 proteome with 10 μ M of BpyneSF. (C) Structure of CARM1 inhibitor. (D) Competitive inhibition of BpyneSF labeling. Hek293 proteome was first treated with CARM1 inhibitor, 1-Benzyl-3,5-*bis*-(3-bromo-4-hydroxylbenzylidene)piperidin-4-one, for 30 min at RT, followed by incubation for 5 min with BpyneSF (10 μ M).

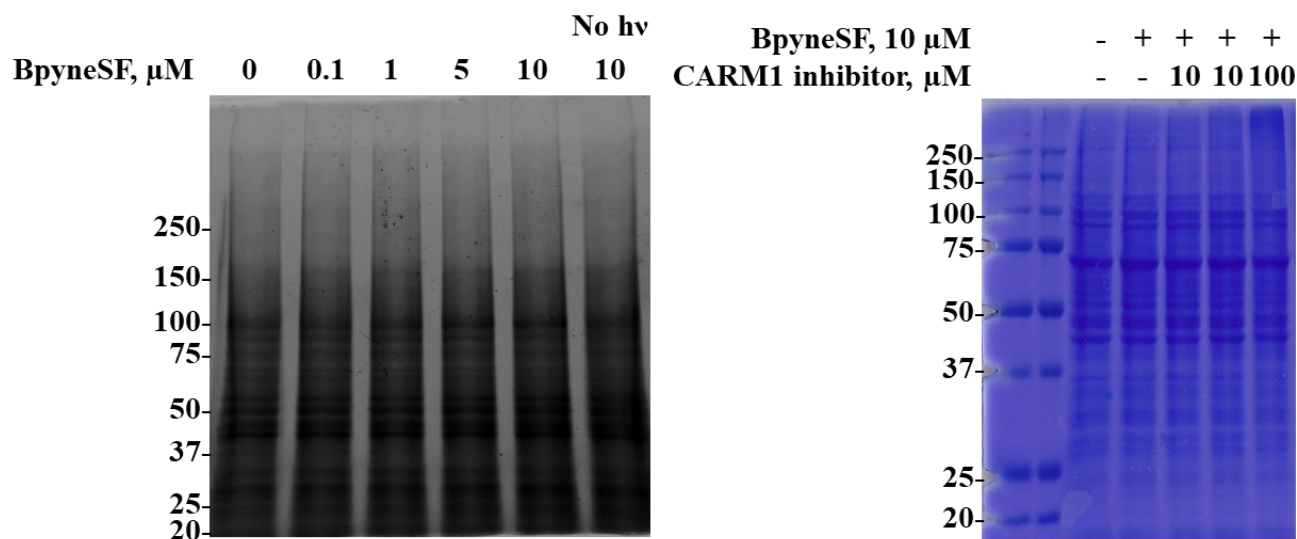


Figure 3.4. Imaging total protein content following in-gel fluorescence labeling with BpyneSF using the gel-free imaging protocol on the ChemiDoc MP imager (Bio-Rad) or coomassie stain.

3.2.4 Molecular targets of sinefungin affinity-based probe

To identify methyltransferase enzymes and the binding proteins that recognize methylated biological targets, we treated Hek293 lysates with 10 μ M BpyneSF as previously described and tagged the probe with biotin-azide. In this method, labeled proteins can be isolated with streptavidin-coated beads and digested with trypsin for mass spectrometry analysis. A DMSO control was done to identify non-specific binding of proteins to the streptavidin-coated beads. Following mass spectrometry analysis, we identified histone-lysine N-methyltransferase (SETD2) as a target of BpyneSF in addition to several proteins involved in recognition of methylated biomolecules (**table 3.1**). SETD2 tri-methylates lysine position 36 (Lys26) on histone H3 (H3K36me3), which results in the recruitment of protein complexes that carry out transcription elongation, RNA processing, and DNA repair[216, 217]. Previous research has demonstrated that sinefungin can specifically target SETD2 enzyme, but SETD2 was not visualized fluorescently in our experiment [202, 204]. The lack of fluorescent visualization of SETD2 enzyme may be due to low SETD2 abundance in Hek293 proteome or due to high foreground fluorescent intensity that may limit band visualization. To validate our LC–MS/MS results and confirm the selectivity of **3.6** for methyltransferase enzymes, recombinant SETD2, PRMT1, and CARM1 were labelled *in vitro* as previously described (**figure 3.5**). We observed fluorescent bands at the molecular weight of each recombinant methyltransferase in the presence of **3.6** and 365 nm radiation and no fluorescence in the absence of BpyneSF. These results suggest that BpyneSF labels methyltransferase enzymes *in vitro*. In addition to targeting SETD2, BpyneSF also captured methyl methanesulfonate-sensitivity protein 22 (MMS22L) and tudor domain-containing protein 6 (TDRD6). MMS22L is a histone chaperone and in complex with TONSL has been shown to bind to methylated lysine on histones H3 and H4 and regulates the replicative state of

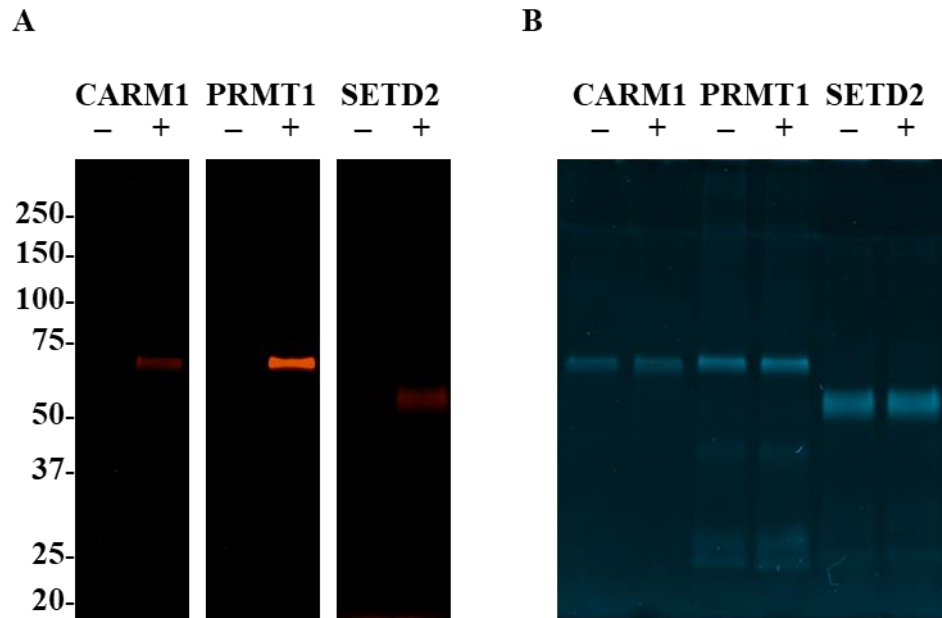


Figure 3.5. *In vitro* activity labeling of purified methyltransferases CARM1, PRMT1, and SETD2. 1 μ g of purified protein was incubated for 30 min with BpyneSF (10 μ M) and UV irradiated for 45 minutes, followed by click-attachment to rhodamine azide. CARM1 was visualized at 66 kDa, PRMT1 at 68 kDa, and SETD2 at 60 kDa.

Table 3.1. Proteins labelled *in vitro* by BpyneSF and identified by on-bead digestion followed by tandem MS

Accession	Description
H7BZ93	Histone-lysine N-methyltransferase SETD2
A0A087WWT3	Serum albumin OS=Homo sapiens
Q96L17	FMN2 protein (Fragment) OS=Homo sapiens
Q8NDY3	[Protein ADP-ribosylarginine] hydrolase-like protein 1 OS=Homo sapiens
E2QRD4	Protein MMS22-like OS=Homo sapiens
O75132	Zinc finger BED domain-containing protein 4 OS=Homo sapiens
H7BZ93	Histone-lysine N-methyltransferase SETD2 (Fragment) OS=Homo sapiens
A2IB45	Tudor domain containing 6 OS=Homo sapiens
E9PS68	Pyruvate carboxylase, mitochondrial OS=Homo sapiens
H7C1I9	Microtubule-associated serine/threonine-protein kinase 4 (Fragment) OS=Homo sapiens
B4DZA1	cDNA FLJ60986, moderately similar to Homo sapiens golgi autoantigen, golgin subfamily a, 8A (GOLGA8A), transcript variant 3, mRNA OS=Homo sapiens
Q4G1H2	Abnormal spindle-like microcephaly associated splice variant 3 OS=Homo sapiens

DNA[218, 219]. Tudor domain proteins are epigenetic “readers” that directly interact with other proteins through methylated arginine or lysine residues[220, 221]. Both MMS22L and TDRD6 are associated with methylated lysine and arginine residues and were likely non-specifically captured by our probe. Further validation of a wider range of proteomes is required to confirm that BpyneSF can non-specifically bind proteins involved in the methyl group recognition. Despite the limitations outline above, these results suggest that BpyneSF is capable of labeling methyltransferase and binding proteins that recognize methylated biological targets.

3.3 Conclusion

In this chapter, we report the design and synthesis of a SF probe that can covalently modify methyltransferase enzymes using a photo-crosslinking strategy, labeling several proteins simultaneously in an affinity-dependent manner. Our work demonstrates that the reversible binding of SF to methyltransferase enzymes can be employed to permanently modify target enzymes, permitting identification and characterization of enzyme targets. We further demonstrated that in conjunction with small molecule inhibitors, BpyneSF can be used to deconvolute the potential targets of chemical probes and associated binding proteins. Future work will involve the further refinement of the probe design, as well as the identification and characterization of target methyltransferases and interacting proteins involved in recognition of biological methylation in disease models.

3.4 Experimental Section

Tissue culture and preparation of cell lysates

Hek293 cells were grown and maintained in MEM medium (GIBCO-BRL, Burlington, Ontario) and supplemented with 50 ml of 10% fetal bovine serum (FBS, PAA Laboratories), 5 ml of a 50

U/ml mixture of penicillin/streptomycin, and 5 ml of Sodium Pyruvate (GIBCO). For active proteome extraction, subconfluent Hek293 cells were washed twice with phosphate buffered saline (PBS), followed by the addition of 1 ml of 0.05% Triton-X in PB. The cell lysates were scrapped, pooled, and kept at 0 C. Lysates were sonicated (15 pulses/sample, 50 % duty cycle; Sonifier 250, Branson Ultrasonic, Danbury, CT) and centrifuged for 15 min (14,000 rpm, 4 C). The supernatant was transferred to a sterile Eppendorf tube and quantified via DC protein assay (BioRad).

In vitro protein labeling

For *in vitro* protein labeling, various concentrations of BpyneSF were added to fresh cell lysates in 100 μ L 0.05% Triton-X in PBS. Lysate samples were incubated with the probe for 5 minutes, followed by UV irradiation at 365 nm for 5 minutes on ice. After the UV irradiation, 100 μ L of freshly prepared click chemistry mix in PBS consisting of Rhodamine-azide (100 μ M, 100 mM stock solution in DMSO), Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA, 200 μ M, 50 mM stock solution in 4:1 DMSO:tert-butanol), copper sulfate (2 mM, 50 mM freshly prepared stock solution in deionized water), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 2 mM, 50 mM freshly prepared stock in deionized water) were added and incubated for 15 minutes at room temperature with gentle mixing. The addition of 1000 μ L of acetone terminated the reaction and the samples were stored in a -80 C for at least 30 minutes. After at least 30 minutes, protein precipitation was done by centrifugation for 15 min at 4 C and 14 000 rpm. Acetone was removed and the protein pellets were allowed to dry for 5 minutes followed by the addition of 35 μ L of 2x Laemmli buffer containing beta-mercaptoethanol. Samples were shaken for 15 minutes and heated for 5 minutes at 95 C. The samples were separated by 10% SDS-PAGE (Bio-Rad, TGX Stain-Free™ FastCast™ Acylamide Kit, 10%) and then visualized by in-gel fluorescent scanning using ChemiDoc MP (Bio-Rad) imager. Gel visualization was undertaken using both Coomassie

blue staining and stain-free imaging, as previously described[222].

Enrichment and mass spectrometry of labelled proteins

Biotin-azide click chemistry, streptavidin-enrichment, and trypsin digest of probe-labelled proteomes was performed as previously described[183]. However, acetone precipitation step was included following the incubation with click mix. Following protein enrichment, agarose-streptavidin beads (Pierce) were washed three times with 100 μ L PBS in a Biospin column. Using 200 μ L of PBS, beads were transferred to probe-labelling cell lysates and incubated for 90 min. For the on-bead digestion, the beads were washed five times with 50 mM ammonium bicarbonate, transferred to an Eppendorf tube and were heated for 15 min at 65 C in 500 μ L of 10 mM DTT in 50 mM ammonium bicarbonate (ABC). After 15 minutes, 25 μ L of 500 mM iodoacetamide was added and lysates were rotated in the dark for 30 min. Samples were centrifuged for 2 min at 1400 rpm and 100 μ L of ABC was added followed by 2 μ L of 0.5 mg/mL of Trypsin was added. Samples were rotated at 37 C overnight, followed by bead pelleting and the transfer of the supernatant for MS analysis. HPLC-ESI-MS/MS was used for all MS analyses. The system consisted of an Agilent 1100 micro-HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled with an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA) equipped with a nano-electrospray interface operated in positive ion mode. Pre-column was packed in-house with reverse phase Magic C18AQ resins (5 μ m; 120 Å pore size; Dr. Maisch GmbH, Ammerbuch, Germany), and analytical column of 75 μ m $\times$ 100 mm was packed with reverse phase Magic C18AQ resins (1.9 μ m; 120 Å pore size; Dr. Maisch GmbH, Ammerbuch, Germany). The mobile phases consisted of 0.1 % (v/v) FA in water as buffer A and 0.1% (v/v) FA in acetonitrile as buffer B. The 4 μ L sample was loaded onto the pre-column using a buffer containing 98% A at a flow rate of 4 μ L/min for 5 min. Subsequently, a gradient from 5% to 35% buffer B was performed, at a

flow rate of ~300 nL/min obtained from splitting a 20 μ L/min through a restrictor, in 60 min. The MS method consisted of one full MS scan from 350 to 1700 m/z followed by data-dependent MS/MS scan of the 5 most intense ions, with dynamic exclusion repeat count of 2, and repeat duration of 90 s. As well, for the experiments on the Orbitrap MS the full MS was performed in the Orbitrap analyzer with $R = 60,000$ defined at m/z 400, while the MS/MS analysis were performed in the LTQ. To improve the mass accuracy, all the measurements in Orbitrap mass analyzer were performed with internal recalibration (“Lock Mass”). On the Orbitrap, the charge state rejection function was enabled, with single and “unassigned” charge states rejected.

Analysis of mass spectrometry data

Raw files were generated using LTQ-Orbitrap and processed and analyzed using MaxQuant, Version 1.3.0.5 using the Uniprot fasta protein database (2013, July version). A maximum of two missing trypsin cleavages were permitted. Precursor ion mass tolerances were set to 7 ppm, and the fragment ion mass tolerance was 0.8 Da for the MS/MS spectra. If the identified peptide sequences of one protein were contained within or equal to another protein’s peptide set, the proteins were grouped together and reported as a single protein group. The false discovery rate (FDR) for peptide and protein was set to 1% and a minimum length of six amino acids was used for peptide identification. Normalized LFQ intensity was used for protein quantification.

In vitro recombinant protein labeling

1 μ g of CARM1 (Sigma-Aldrich), SETD2 (Sigma-Aldrich), and PRMT1 (Sigma-Aldrich) were dissolved in 100 μ L 0.05% Triton-X in PBS. Recombinant proteins were incubated with the probe for 30 minutes, followed by UV irradiation at 365 nm for 45 minutes on ice. After the UV irradiation, 100 μ L of freshly prepared click chemistry mix in PBS consisting of Rhodamine-azide

(100 μ M, 100 mM stock solution in DMSO), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA, 200 μ M, 50 mM stock solution in DMSO), copper sulfate (2 mM, 50 mM freshly prepared stock solution in deionized water), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 2 mM, 50 mM freshly prepared stock in deionized water) were added and incubated for 45 minutes at room temperature with gentle mixing. The addition of 1000 μ L of acetone terminated the reaction and the samples were stored in a -80 C for at least 30 minutes. After at least 30 minutes, protein were pelleted by centrifugation for 15 min at 4 C and 14 000 rpm. Acetone was removed and the protein pellets were allowed to dry for 5 minutes then solubilised in SDS-PAGE loading buffer (0.1 M Tris, pH 6.8, 10% glycerol, 4% SDS, 0.02% bromophenol blue, 30 mM DTT). Samples were vortexed and heated for 5 minutes at 95 C. The samples were separated by 10% SDS-PAGE (Bio-Rad, TGX Stain-Free™ FastCast™ Acylamide Kit, 10%) and then visualized by in-gel fluorescent scanning using ChemiDoc MP (Bio-Rad) imager. Total protein visualization was undertaken using both Coomassie blue staining and stain-free imaging as previously described[222].

High-Performance Liquid Chromatography Mass Spectrometry

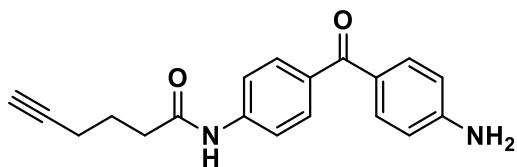
Thermo Easy nLC II coupled with an Orbitrap Q Exactive Plus mass spectrometer (Thermo Scientific, San Jose, Ca) was used for HPLC-MS analysis of small molecules, using an Acclaim PepMap RSLC 75 μ m ID x 150 mm length separation column (Thermo Scientific, San Jose, Ca). 18 μ L of the BpyneSF were injected and separated by the following gradient (A – 0.1% formic acid in H₂O, B – 80% acetonitrile, 0.1% formic acid in H₂O) with the flow of 200 nl/min: 0.0-80.0 min 0-40% B, 80.0-80.1 min 40-80% B, 80.1-90.0 min 80% B, 90.0-90.1 min 80-2% B, 90.1-115.0 min 2% B. The following parameters were used: Nano-ESI conditions: spray voltage in

positive mode – 2000 V; ion transfer tube temperature – 275 C; S-lens RF level – 60. Initial scans of small molecule precursors from 300 to 1500 m/z were performed at 60K resolution (at 200 m/z) with a 2×10^5 ion count target and maximum injection time of 50 ms. Tandem MS was performed by isolation at 0.7 Th with the quadrupole, HCD fragmentation with collision energy of 30% with 5% step, and normal scan MS analysis in the ion trap. The MS² ion count target was set to 10^4 and the max injection time was 35 ms. Precursors with charge state 2–6 were sampled for MS². The dynamic exclusion duration was set to 60 s with a 10 ppm tolerance around the selected precursor and its isotopes. The instrument was run in top speed mode with 4 seconds per cycles.

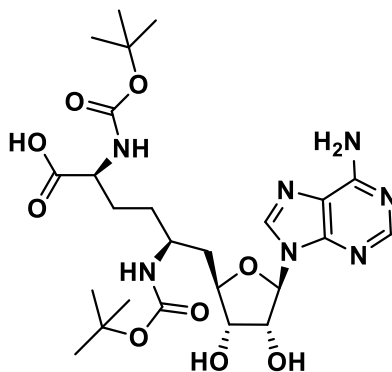
3.5 Synthetic methods and characterization

General information

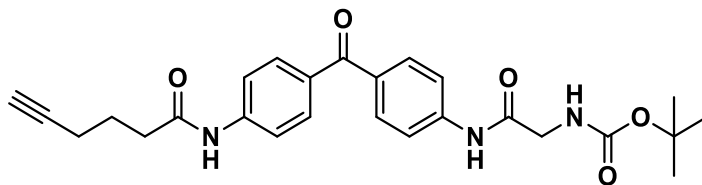
All reagents and solvents were purchased from Sigma-Aldrich, unless otherwise noted, and used without further purification. Deuterated solvents were purchased from Cambridge Isotope Laboratories and thin layer chromatography was done using Analtech Uniplat[®] silica gel plates (60A F254, layer thickness 250 μm). Flash column chromatography was performed by silica gel (60A, particle size 40 to 63 μm). ¹H NMR and ¹³C NMR spectra were recorded using a Bruker-DRX-400 spectrometer at a frequency of 400.13 MHz for ¹H and 100.61 MHz for ¹³C and processed with Bruker TOPSPIN 2.1 software. Chemical shifts are reported in parts per million (δ) using residual solvent resonance as an internal reference. The following abbreviations were used to designate chemical shift multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet or unresolved, br = broad single and J = coupling constant in Hz.



N-(4-(4-aminobenzoyl)phenyl)hex-5-ynamide was synthesized per previously published reports[210].

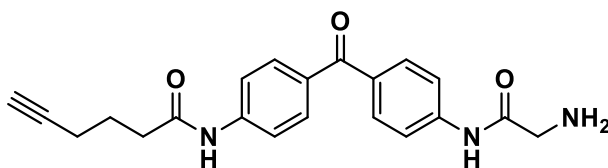


(BOC)₂Sinefungin was synthesized per previously published reports[223]

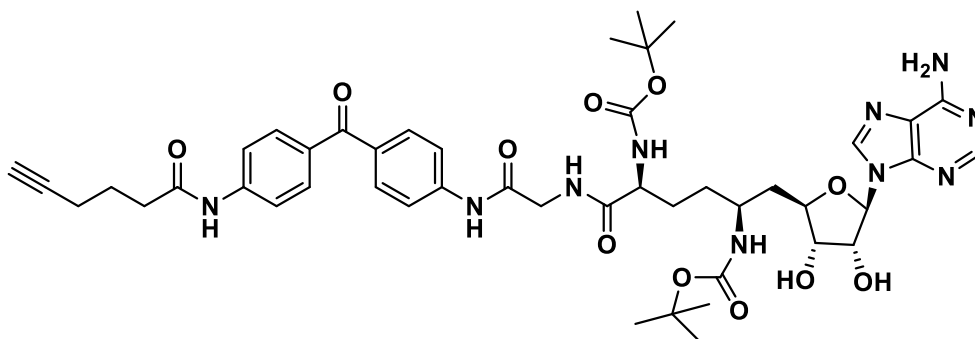


Bpyne-gly-BOC. In a 100 mL flask was added *N*-(*tert*-Butoxycarbonyl)glycine (Boc-gly-OH) (743 mg, 4.31 mmol), 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU) (1.70 g, 4.47 mmol), triethylamine (1.15 mL, 8.07 mmol) and DMF (40 mL) The mixture was stirred for 5 minutes and then Bpyne (866.3 mg, 2.83 mmol) was added in one portion, and the reaction was stirred at 80°C under argon overnight. After stirring overnight, the mixture was concentrated *in vacuo* and the residue was purified over silica (60% ethyl acetate in hexane) to yield a yellow, crystalline solid (138.2 mg, 11%). ¹H NMR (400 MHz, CDCl₃) δ 9.07 (s, 1H), 8.41 (s, 1H), 7.64 (m, 8H), 5.75 (s, 1H), 4.00 (d, *J* = 5.8 Hz, 2H), 2.55 (t, *J* = 7.3 Hz, 2H), 2.27 (td, *J* = 6.7, 2.5 Hz, 2H), 1.97 (t, *J* = 2.5 Hz, 2H), 1.93 (q, *J* = 7.1 Hz, 1H),

1.44 (s, 9H). ^{13}C NMR (100 MHz, CDCl_3) δ 194.95, 194.87, 171.61, 168.76, 149.95, 142.13, 141.62, 133.59, 141.62, 133.59, 133.32, 133.00, 131.46, 131.43, 120.80, 119.20, 119.12, 83.47, 69.65, 36.11, 28.42, 23.97, 17.95. LRMS m/z calcd for $\text{C}_{26}\text{H}_{29}\text{N}_3\text{O}_5$ (M+H): 464.21. Found: 464.2.

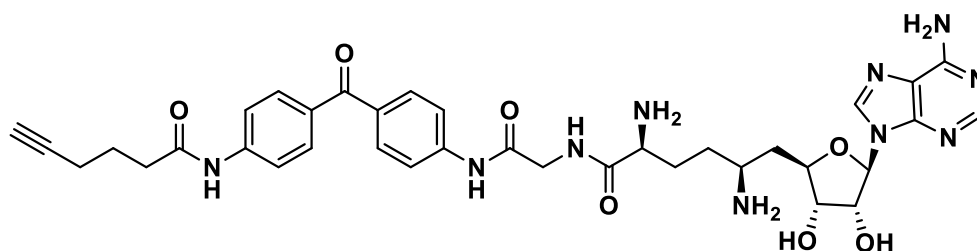


Bpyne-gly-NH₂. In a 5 mL flask was added Bpyne-gly-BOC (50 mg, 0.107 mmol) in CHCl_3 (2 mL), followed by the addition of trifluoroacetic acid (TFA) (200 μL , 2.612 mmol). The reaction was stirred for 3 h and then concentration *in vacuo* to yield a yellow, crystalline solid (31.5 mg, 80%). ^1H NMR (400 MHz, CDCl_3) δ 7.50 (m, 8H), 3.56 (s, 2H), 2.32 (t, J = 7.4 Hz, 2H), 2.06 (td, J = 6.8, 2.6 Hz, 2H), 1.84 (t, J = 2.5 Hz, 2H), 1.69 (q, J = 7.1 Hz, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 196.07, 173.14, 143.33, 142.19, 133.81, 132.81, 132.93, 131.77, 131.74, 119.39, 83.64, 78.19, 77.87, 77.55, 69.60, 49.86, 36.07, 24.54, 18.21. LRMS m/z calcd for $\text{C}_{21}\text{H}_{21}\text{N}_3\text{O}_3$ (M+H): 364.158. Found: 364.2.



BpyneSF(BOC)₂. In a 5 mL flask was added (BOC)₂Sinefungin (29.9 mg, 0.051 mmol), (HATU) (22.2 mg, 0.058 mmol), and DMF (1 mL). Then, diisopropylethylamine (DIPEA) (10 μL , 0.058) was added and the reaction was stirred for 20 minutes. Then, Bpyne-gly-NH₂ (17.7 mg, 0.048 mmol) was added to the reaction mixture and the reaction was stirred overnight at 60°C under

argon. After 24 h, product synthesis was confirmed through LC/MS (eluting 10 to 60 % acetonitrile/water with 0.1 % formic acid) and solution was concentration *in vacuo*. Residue was purified on HPLC eluting 10 to 60 % acetonitrile/water with 0.1 % formic acid and yielded a light yellow, crystalline solid (15.2 mg, 33.7%). ^1H NMR (400 MHz, CDCl_3) δ 8.23 (s, 1H), 8.20 (s, 1H), 7.73 (m, 8H), 5.95 (d, $J = 4.9$ Hz, 1H), 4.83-4.76 (m, 2H), 4.16 (s, 2H), 4.05 (s, 2H), 3.70 (m, 1H), 3.35 (s, 1H) 2.56 (t, $J = 7.4$ Hz, 2H), 2.28 (m, 3H), 2.02 (m, 1H), 1.90 (m, 4H), 1.70 (m, 1H), 1.59 (m, 2H), 1.42 (s, 9H), 1.40 (s, 9H). LRMS m/z calcd for $\text{C}_{46}\text{H}_{58}\text{N}_{10}\text{O}_{11}$ ($\text{M}+\text{H}$): 927.43. Found: 927.8.



BpyneSF. In a 5 mL flask was added BpyneSF(BOC) $_2$ (17.7 mg, 0.0019 mmol) and TFA (1 mL). The solution was stirred for 3 h and then the reaction mixture was concentrated *in vacuo* to quantitatively yield a yellow, crystalline solid (13.8 mg). Compound was identified using HPLC/MS. HRMS m/z calcd for $\text{C}_{36}\text{H}_{42}\text{N}_{10}\text{O}_7$ ($\text{M}+\text{H}$): 727.32. Found: 727.33269.

Chapter 4: Other probes for elucidation of prokaryotic and eukaryotic targets

Abstract

The development of chemical probes can be harnessed to understand how proteins function in biological systems through elucidating the molecular targets of bioactive small molecules. Previous work has isolated and characterized a series of chlorinated streptopyrroles, called armeniaspiroles, that can inhibit bacterial propagation through an unknown mechanism. Given the rise in antimicrobial resistance, the isolation and development of novel antibacterial small molecules is of critical importance. Herein, we investigate the molecular targets of armeniaspirole through the synthesis and development of a novel affinity-based probe (Cl-ARM-A-yne). We demonstrate that Cl-ARM-A-yne is capable of labeling prokaryotic and eukaryotic proteins *in vitro* and using functional proteomics, we identify several proteins that may be important in protein homeostasis in bacterial organisms. These initial results provide a preliminary understanding of the molecular targets and function of armeniaspiroles in prokaryotic and eukaryotic organisms. In addition to the development of an armeniaspirole probe, we highlight our work towards the development of an affinity probe for nuclear receptors. Nuclear receptors (NRs) are a class of signaling receptors responsible for cellular development, reproduction, and metabolism and only half of the known nuclear receptors have endogenous ligands. Given the importance of nuclear receptors in cellular function, the identification and development of chemical probes for orphan nuclear receptors is an important step towards revealing their cellular function and identifying associated binding proteins. Herein, we highlight the development of a cytosporone chemical probe (CSN-B-yne) that will be able to report on the function of nuclear orphan receptor 77 and associated binding protein co-activators and repressors.

Statement of Research Contributions

Work towards the development of an affinity-based probe was done exclusively by Dr. Mark H. Dornan. I undertook the *in vitro* labeling experiments in both prokaryotic and eukaryotic cell lines and prepared the cell lysates for mass spectrometry analysis. I would like to thank Dr. Gleb Mironov for his work with the MS analysis of the proteomic samples.

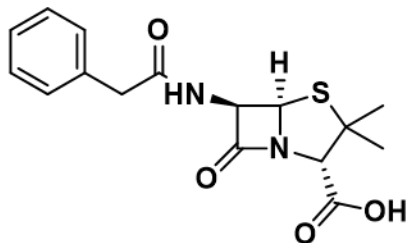
4.1 Probing the Molecular Targets of Armeniaspirole A

4.1.1 Antibiotic resistance

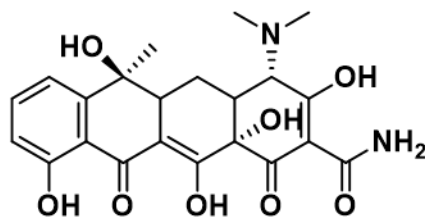
The discovery and application of antibiotic molecules in the treatment of infection and disease was a major turning point in twentieth century medicine[224]. Prior to the antibiotic era, one of the leading cause of morbidity and mortality was principally due to infections[225]. Since the beginning of the antibiotic era, antibiotics have been applied to decrease the risk of infection following surgical procedures, deployed in treatment of chronic diseases like diabetes and arthritis, and provided to patients undergoing immune compromising chemotherapy[226-228]. As a result, the broad use of antibiotics has had a positive impact on the morbidity and mortality of patients undergoing medical procedures and in management of chronic disease. Unfortunately, there has been an increase in multi-drug resistant (MDR) bacteria resulting from the overuse of antibiotics by health care practitioners (HCP) and industry organizations, which has resulted in approximately 700,000 deaths worldwide due to antimicrobial resistance (AMR) and is anticipated to increase to 10 million by 2050[229]. The increase in MDR bacteria has been concurrently impacted by the decrease in pharmaceutical investment in research and development of antibiotics[227, 230]. The inherited ability of microorganisms to grow and resist high concentrations of antibiotic is driven by intrinsic mechanisms like preventing access to drug targets, induced changes in the drug target's structure, and direct modification or inactivation of the antibiotic[231, 232]. Given the current challenges with increasing bacterial resistance and the simultaneous decreasing in antibiotic effectiveness, increased research and development into new microbial agents is needed to hasten the replenishment of the antibiotic toolbox.

4.1.2 *Molecular targets of antibiotic molecules*

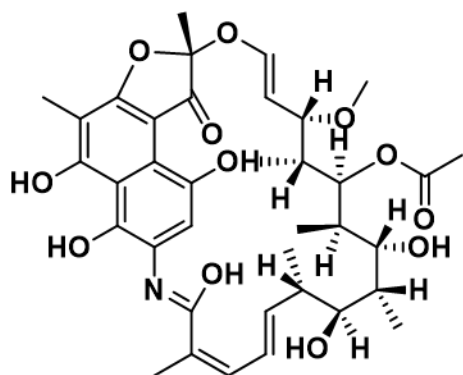
To develop progressively better antibiotics, it is necessary to understand the mechanism of action of antibiotics currently available. Following the discovery of penicillin in 1942, several bacterial mechanisms have been targeted by a range of structurally diverse antibiotics[233] (**figure 4.1**). For example, the peptidoglycan cell wall of bacteria is important in maintaining bacterial viability and, ultimately, its pathogenicity[234, 235]. Cell wall biosynthesis inhibitors (CBI) are the principle means by which antibiotics like penicillin and vancomycin can inhibit bacterial growth[236]. Tetracyclines and other macrolides target the translational machinery of bacteria, specifically the 50S ribosomal subunit of bacterial ribosomes, and prevent the exit of the polypeptide from the ribosome[237]. Fluoroquinolones, like levofloxacin, and related quinolones impair bacterial propagation by targeting DNA gyrase and topoisomerase, two enzymes responsible for cellular replication and packaging of DNA[233]. Antibiotics like rifamycin target the β -subunit of DNA-dependent RNA polymerase, which results in blocking RNA elongation[238]. Finally, isoniazid and sulfonamide antibiotics, like sulfadoxine, target mycolic acid synthesis and folic acid synthesis, respectively. Despite the range of structurally diverse antibiotics and range of molecular targets, AMR has emerged for all those antibiotic molecules described above[224, 227]. Given that AMR is a critical challenge in healthcare, increased research towards the discovery and development of novel antibiotic molecules is required to prevent a post-antibiotic era where the prevalence of resistant bacteria will have detrimental health effects on humans[239].



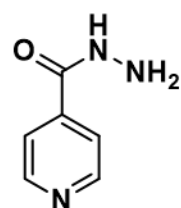
Penicillin



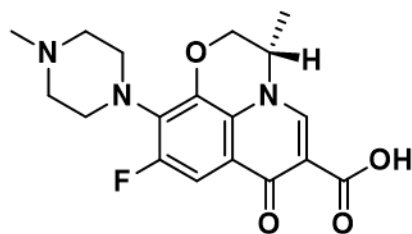
Tetracycline



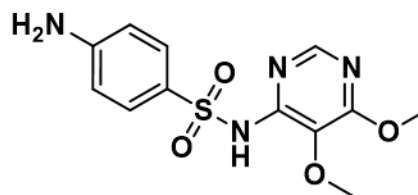
Rifamycin



Isoniazid



Levofloxacin



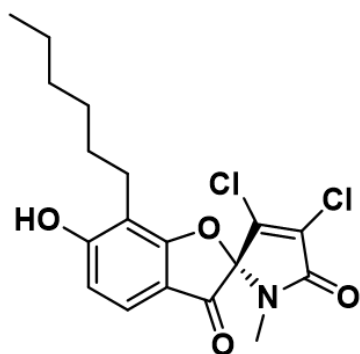
Sulfadoxine

Figure 4.1. Examples of different classes of antimicrobial agents

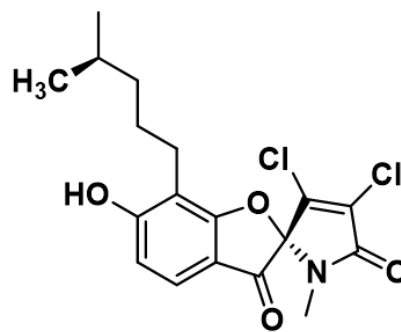
4.1.3 Armeniaspiroles as novel antibiotic molecules

To discover bioactive small molecules with antimicrobial properties, bacterial organisms are investigated since they are the source of most clinically available antibiotics to-date[1, 5, 240]. To this end, Dufour and colleagues investigated gram-positive *Streptomyces armeniacus* strain DSM19369 and isolated three natural products following cultivation on a malt-containing medium, extraction with methanol, and isolation through preparative high-performance liquid chromatograph (HPLC) [241]. Following preparative HPLC isolation, three independent methods were used to characterize the compounds: chemical degradation followed by NMR spectroscopy, computer-assisted structure prediction, and X-ray crystallography[241]. Compounds **4.1** through **4.3**, which were not previously known in the literature, contained a novel chlorinated spiro[4.4]non-8-ene which differed by whether the alkyl change on the 6-position of aromatic ring is linear or branched (**figure 4.2**).

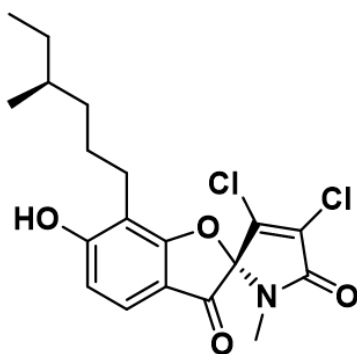
Following the chemical characterization of the natural products, Dufour *et al.* investigated the antimicrobial properties of armeniaspirole (ARM) A-C. As shown in **table 4.1**, compound **4.1**, **4.2**, and **4.3** were shown to have a moderate to high *in vitro* activity in comparison to ciprofloxacin, which has a minimal inhibitory concentration of less than 0.5 $\mu\text{g mL}^{-1}$ against all the bacteria outlined in **table 4.1**. Remarkably, the armeniaspirole compounds were able to inhibit the growth of several antibiotic resistant strains, like gram-positive methicillin-resistant *Streptococcus aureus* (MRSA), vancomycin-resistant *Enterobacterium faecium* (VRE) and penicillin-resistant *Streptococcus pneumoniae* (*S. pneumoniae*) *in vitro*[241, 242]. They were also shown to be ineffective against gram-negative bacteria like *Escherichia coli* and *P. aeruginosa* and fungi *C. albicans*, which suggests that armeniaspiroles are not effective broad-spectrum antibiotics, which target both gram-positive and gram-negative bacteria. Importantly, **4.1**



4.1
Armeniaspirole A



4.2
Armeniaspirole B



4.3
Armeniaspirole C

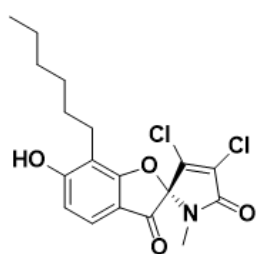
Figure 4.2. Armeniaspiroles isolated from methanolic extracts of *Streptomyces armeniacus* strain DSM19369.

through **4.3** did not display cytotoxicity against human liver cancer cell line HepG2 up to 30 μM and no bacterial resistance was observed against treatment with **4.1**, indicating this compound could be harnessed in a clinical setting[243].

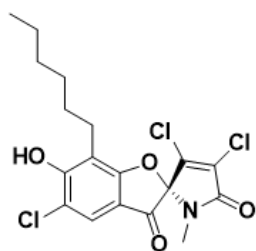
To ascertain the effectiveness of **4.1** *in vivo*, MRSA-induced septicemia model was used to investigate antimicrobial properties of **4.1** and to ascertain the impact of **4.1** on the survivability of the host organism. Survivability to treatment was dose-dependent in contrast with vancomycin (**figure 4.4A**) [241]. Following intravenous injection, **4.1** decreased blood bacteremia in a dose-dependent manner but at higher doses adverse cardiac effects were observed and infection could not be cured. Following the initial investigation of **4.1** activity *in vitro* and *in vivo*, Couturier and colleagues designed a series of semi-synthetic analogues of armeniaspirole A-C and tested their potency against a range of multi-drug resistant, gram-positive bacterial pathogens (**figure 4.3**) [243]. As outlined in **figure 4.4B**, a number of the synthetically derived armeniaspirole derivatives had good *in vitro* activities with inhibitor concentration (IC_{80}) values in the low micromolar (μM) range against MRSA, penicillin-resistant *Streptococcus pneumoniae* (PRSP), and VRE. Interestingly, chlorination of the 2-position on the aromatic ring slightly decreased antimicrobial activity of **4.4** relative to **4.1** but resulted in increased metabolic stability, increasing its effectiveness in a diverse range of uses[243]. Compound **4.1** exhibited no interactions with cytochrome CYP3A4 and CYP2D6 ($\text{IC}_{50} > 10 \mu\text{M}$) and no activity on the ERG cardiac channel (inhibition $< 35\%$ at $10 \mu\text{M}$). Further, **4.1** exhibited moderate clearance in male swiss mice (1.2 L/h/kg), a large volume of distribution ($V_{ss} = 2.4 \text{ L/kg}$), a moderate half-life in plasma ($t_{1/2} = 3\text{h}$), and a c_{max} of $27.4 \mu\text{g/ml}$. Interestingly, *in vitro* studies indicated that there was a decrease in antimicrobial activity in the presence of fetal calf serum, suggesting that **4.1** can react with the

Table 4.1. *In vitro* activities of isolated armeniaspirole compounds against pathogens

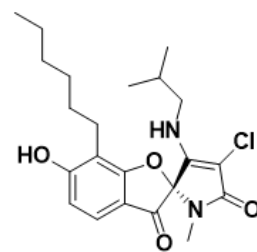
Strain	4.1		4.2		4.3	
	IC ₈₀ [µg mL ⁻¹]	MIC [µg mL ⁻¹]	IC ₈₀ [µg mL ⁻¹]	MIC [µg mL ⁻¹]	IC ₈₀ [µg mL ⁻¹]	MIC [µg mL ⁻¹]
<i>S. aureus</i> (ATCC13709)	0.3	1	0.7	1	0.5	1
<i>S. aureus</i> (ATCC29213)	0.35	1	0.85	2	0.75	2
<i>S. aureus</i> (ATCC33592)	<0.125	0.25	<0.125	0.25	<0.125	0.125
<i>S. pneumonia</i> (DSM11865)	2.30	4	1.80	4	1.80	4
<i>S. pyogenes</i> (ATCC12344)	6.20	16	5.80	32	4.30	64
<i>S. faecium</i> (DSM17050)	8.20	16	5.80	8	5.90	8
<i>E. faecalis</i> (ATCC29212)	25.20	32	17.80	32	24.70	64
<i>M. smegmatis</i> (ATCC607)	0.30	2	0.60	8	0.50	4
<i>P. aeruginosa</i> (ATCC27853)	>64	>64	>64	>64	>64	>64
<i>E. coli</i> (ATCC25922)	>64	>64	>64	>64	>64	>64
<i>C. albicans</i> (FH2173)	>64	>64	>64	>64	>64	>64



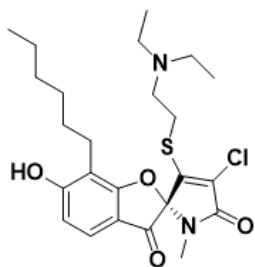
Armeniaspirole A



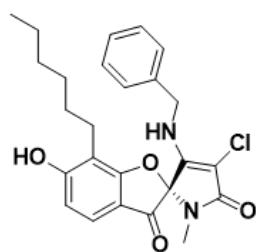
4.4



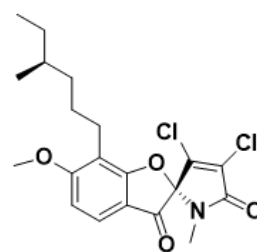
4.5



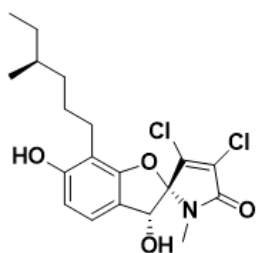
4.6



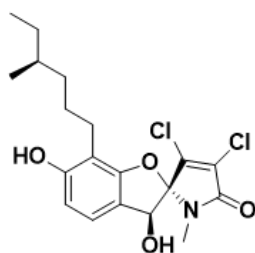
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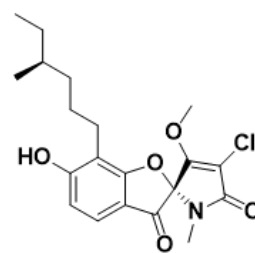
4.8



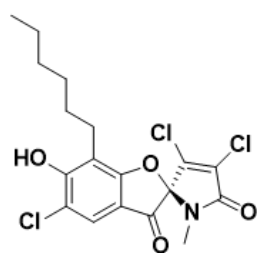
4.9



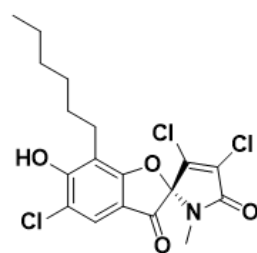
4.10



4.11



4.12



4.13

Figure 4.3. Structures of armeniaspirole analogues designed and synthesized by Couturier and colleagues.

A

	4.1			Vancomycin
Iv dose [mg kg ⁻¹ d ⁻¹]	3	10	15	20
Surviving animals [%]	0	33.3	67	100
Log [CFU reduction]	0.05	0.9	2.8	4.2

B

Activity	MRSA (ATCC33592)	PRSP (DSM11865)	VRE (DSM17050)	E. Coli-LacR (ATCC35218)	P. Aeruginosa (ATCC27853)
4.1	0.125	2.3	8.2	>30	>30
4.13	5.1	1.7	8.5	>30	>30
4.13 + 4.12	3.1	1.3	5.2	>30	>30
4.12	1.4	0.5	6.7	>30	>30
Vancomycin	0.4	0.2	>30	n/a	n/a

Figure 4.4. Summary of the biological consequences of armeniaspirole treatment. (A) Survivability of host organisms following treatment with compound 4.1 compared with vancomycin. (B) the impact of synthetically derived armeniaspiroles on the grow of several multi-drug resistant, gram-positive bacterial pathogens. IC₈₀ values are represented in table A and B.

high number thiols contained with fetal calf serum[244]. Finally, following subcutaneous (sc) injections of **4.1** *in vivo*, significant negative effects were observed on the host organism[243].

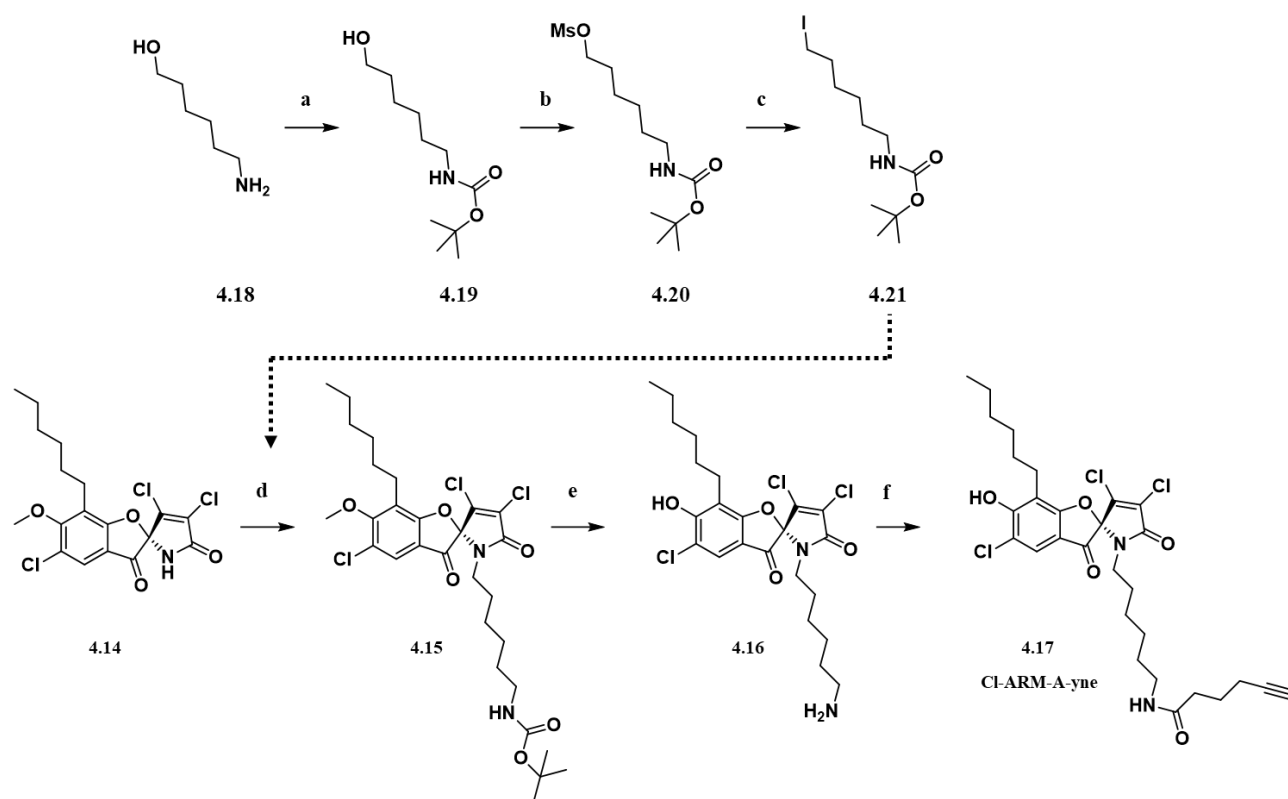
4.1.4 Molecular targets of armeniaspirole

Provided the metabolic stability of **4.13** and its *in vitro* activities in comparison with **4.1**, we investigated the bacterial and eukaryotic targets of armeniaspirole with the aim of identifying and understanding not only the antimicrobial mechanism of action but also to identify the molecular mechanisms and structures that lead to adverse survivability following treatment.

4.2 Results and discussion

4.2.1 Development of a chloro-armeniaspirole B chemical probe

To undertake a preliminary investigation of the molecular targets of **4.13**, Dr. Mark H. Dornan synthesized an alkylated derivative of chloro-armeniaspirole (Cl-ARM-A-yne) that could be used to capture and isolate target biomolecules *in vitro* and *in vivo* (**scheme 4.1**). To this end, we synthesized compound **4.14** according to previously published reports[243]. To help minimize potentially deleterious steric clash between the chemical probe and the target biomolecule(s), the alkyne linker **4.21** was synthesized. Following the synthesis of **4.21**, compound **4.14** was coupled to the alkyne linker using sodium hydride to produce intermediate **4.15**. To deprotect both the BOC and to remove the methyl group from the methoxy at 1-position of the aromatic ring, boron tribromide (BBr₃) deprotection was undertaken at room temperature for 30 minutes in 1,2-dichloroethane (DCE). Following the deprotection of **4.16**, succinimide-activated 5-hexynoic



Scheme 4.1. Synthesis of Cl-ARM-A-yne. a.) di-tert-butyl decarbonate (BOC_2O), methanol, 1.5 hrs, >99%; b.) methanesulfonyl chloride (MsCl), triethylamine, dichloromethane; c.) sodium iodide, acetonitrile, rt, 70% Synthesis of chloro-armeniaspirole A. a.) sodium hydride (NaH), DMF, 0 C to rt, 5hr, 26%; b.) BBr_3 , DCE, rt, 30 min, 87%; c.) succinimide-activated 5-hexynoic acid, Et_3N , DMF, rt, 3 hr, 70%.

acid was reacted to form the final compound **4.17**. Following the synthesis of **4.17**, the minimum inhibitor concentration (MIC) was evaluated and determined to be 8.0 µg / mL in *Bacillus subtilis*. Finally, to evaluate the cytotoxicity of compound **4.17**, an MTT assay was used to evaluate the concentration at which cytotoxicity occurs. Following a 24 h treatment of human embryonic kidney (Hek293) cells, cells were washed twice with phosphate buffered saline (PBS) and incubated with MTT for 3 hrs, followed by colorimetric analysis (**figure 4.5A**). Minimal cytotoxicity is observed for concentrations of **4.17** up to 30 µM, indicating that eukaryotic cells lines can tolerate **4.17** with minimal cytotoxicity.

4.2.2 Cl-ARM-A-yne can covalently label prokaryotic and eukaryotic proteins

Following the synthesis of the chemical probe **4.17** and the evaluation of its cytotoxicity and MIC, we evaluated the probe's ability to covalently label proteins. Given that electrophilic natural products are prevalent in the biology and convey several biological effects like antifungal, antibiotic, and antitumor activity, we determined that the Michael acceptor moiety found in **4.17** would be capable of covalently labeling proteins[245-247]. To this end, we validated the effectiveness of **4.17** as an affinity-based probe by *in vitro* labeling of the gram-positive bacterial strain *Bacillus subtilis* (*B. subtilis*), human embryonic kidney (Hek293), and Henrietta Lacks (HeLa) cells. *B. subtilis* is a diverse and stable non-pathogenic bacterial species that is used ubiquitously in antimicrobial analysis of bioactive small molecules[248, 249]. Hek293 and HeLa cells have been used extensively in experiments ranging from antimicrobial research to cancer biology, thus providing a robust cellular platform on which to study the properties of bioactive small molecules[250, 251]. *B. subtilis* cell lysates were harvested and treated for 1 h with the inhibitor Cl-ARM-A (**4.14**), followed by the addition and incubation for 1 h of **4.17**. Following incubation with **4.17**, samples were treated with rhodamine-azide to visualize labeling (**figure**

4.5B). To ensure that the fluorescence signal was not due to unequal loading of protein, the protein concentration in each gel was assessed qualitatively (**figure 4.6**). As can be seen in **figure 4.5B**, there was a decrease in the relative band intensity across the gradient of protein masses in the lanes that contained inhibitor **4.14**. The bands with diminished intensity suggest strongly that **4.17** can target these proteins selectively and specifically. A pronounced band can be visualized at a mass of approximately 55 kDa and the intensity is diminished with inhibitor treatment, indicating that this protein is a potential molecular target of **4.17**. In **figure 4.5C** and **figure 4.5D**, there was a concentration-dependent increase in fluorescent intensity in both Hek293 and HeLa labeling. Similar to *B. subtilis* labeling, the increase in fluorescence intensity in both eukaryotic cell lines suggest a greater selectivity for these proteins. There does appear to be a comparative difference in the fluorescent labeling profiles of Hek293 and HeLa using following **4.17** treatment, which suggest that there is differential activity of enzymes between two distinct cell lines. Overall, these results strongly suggest that not only does **4.17** covalently labeling proteins but also that tissue-specific targeting may be involved.

4.2.3 Molecular targets of Cl-ARM-A-yne

To identify the molecular targets of **4.17** in both prokaryotic and eukaryotic cells, we treated both cell lines as previously described and tagged the probe with biotin-azide, which permits the capture of biological targets with streptavidin-coated beads[113]. Following capture, the labeled proteins are digested with trypsin and analyzed using mass spectrometry (MS). Using MS, we identified several proteins that were targeted by **4.17** in *B. subtilis*: Adenosine triphosphate (ATP)-dependent protease ClpY, trifunctional nucleotide phosphoesterase YfkN, and hydrolase MtnU.

Proteolytic mechanisms in biological systems are an important component in preventing protein toxicity that may result from misfolding proteins and aggregation, both of which have been

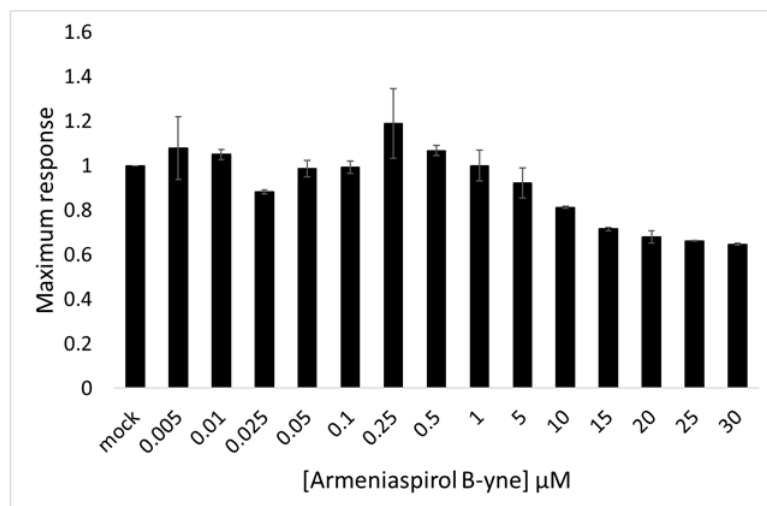
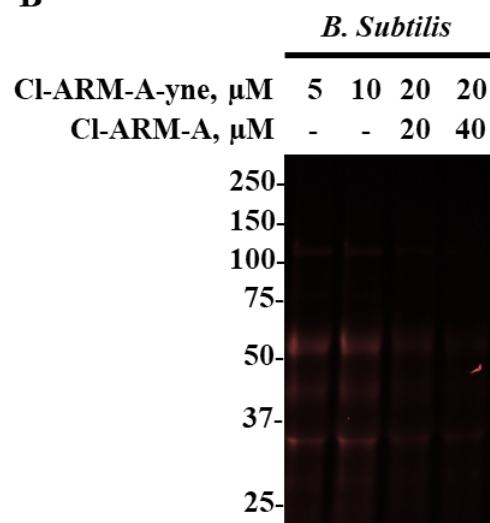
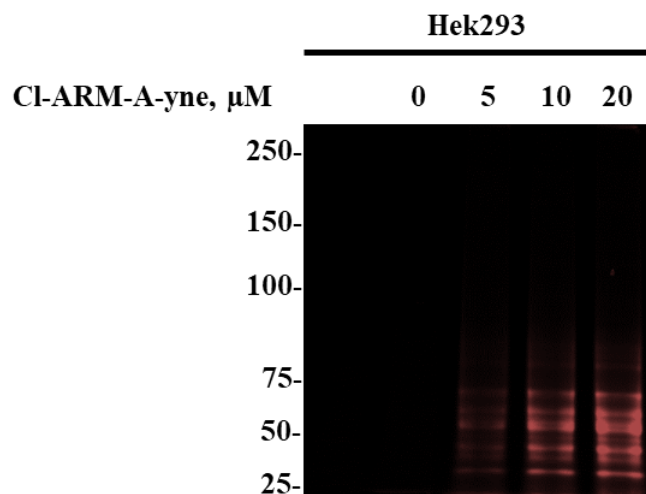
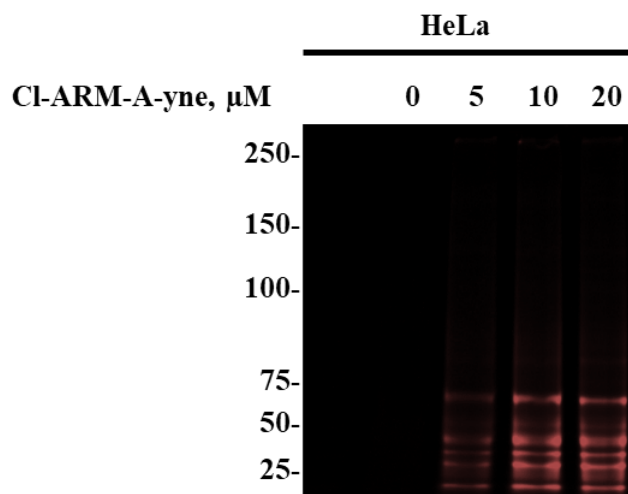
A**B****C****D**

Figure 4.5. Visualization of the molecular targets of CI-ARM-A-yne. (A) MTT analysis of cytotoxicity in Hek293 using compound **4.17**. (B) *in vitro* labeling of *B. subtilis* proteome by derivatized **4.17** and competitive inhibitor **4.14**. (C) *in vitro* labeling of human embryonic kidney (Hek293) cells. (D) *in vitro* labeling of HeLa cells.

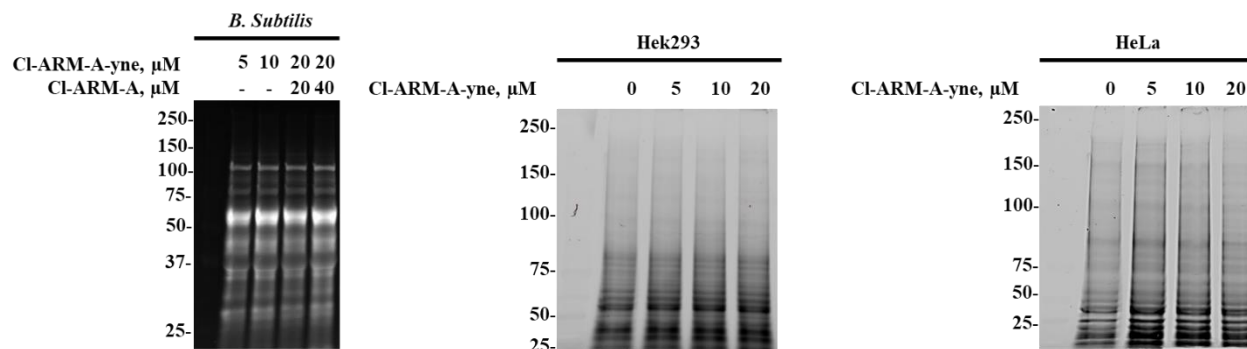


Figure 4.6. Imaging total protein content following in-gel fluorescent labeling with Cl-ARM-A-yne using gel-free method imaging protocol on the ChemiDoc MP imager (Bio-Rad).

implicated in numerous human diseases[252]. ATP-dependent proteases like ClpYQ are important in controlling the proteotoxicity of proteins in an organism. Adenosine triphosphate (ATP)-dependent protease ClpY, part of the multicomponent ClpYQ protease, selectively recognizes, unfolds, and translocates proteins to the catalytic core of serine protease ClpQ for degradation[253-255]. The ClpQY complex functions as a chaperone, which recognizes, unfolds and transfers proteins to the proteolytic core[256]. Several small molecules have been developed that can specifically target the Clp proteins and cause a decrease in proteolytic activity or cause de-oligomerization of Clp proteins, resulting in inactivation of the ClpYQ function[257, 258].

In addition to ClpY, the hydrolase MtnU was identified and has been previously implicated as a potentially important component of the methylthioribose (MTR) recycling pathway, part of the methionine salvage pathway in *B. subtilis*[259]. Previous work has shown that methylthioribose, a byproduct of spermidine and spermine metabolism, is metabolized to methionine through the methionine salvage pathway[260]. Work done by Sekowska and

colleagues has shown that in the absence of MtnW, which shares similarities to MtnU, MTR becomes toxic to the bacterial cell[259]. It is possible that **4.17** targets and inactivates the MtnU component of the MTR recycling pathway, inducing a buildup of MTR, which results in bacterial cytotoxicity.

An additional target identified by **4.17** is trifunctional nucleotide phosphoesterase YfkN, which catalyzes the release of phosphate from 2,3-cyclic nucleotides through consecutive 2,3-phosphodiesterase and 3-(or 2-) nucleotidase activities[259, 261]. Previous work has also shown YfkN's role as a sortase, which are known to be essential in *B. subtilis* and *S. aureus* and catalyze the covalent C-terminal anchoring of virulence proteins and adhesion proteins to the bacterial cell wall[262]. Small molecule inhibitors have been developed that can reduce the virulence of bacterial strains and it is possible that **4.17** inhibits the sortase activity of YfkN and thus the virulence of *B. subtilis*[263, 264].

In Hek293 cells, heat shock protein 70 (Hsp70), which is important in proper protein folding and function was targeted. Previous work has suggested that small molecule targeting of Hsp70 ATPase activity can cause apoptosis[265]. In HeLa cells, tyrosine-protein phosphatase nonreceptor 12 (PTPN12), which is a common enzyme involved in post-translational modifications (PTM) was also targeted. Previous work has shown that inhibition of phosphatase can induce apoptosis[266]. Validation of MS identified proteins is required to definitively conclude that **4.17** targets the labeled proteins. Despite this, these results suggest that not only can **4.17** covalently label both prokaryotic and eukaryotic molecular targets through a nucleophilic addition to the electrophilic moiety on chloro-armeniaspirole A but, importantly, the MS results suggest that the cytotoxicity of the probe may arise from impairing cellular machinery critical for protein homeostasis and folding.

4.3 The Development of a Cytosporone-based Affinity Probe for Nuclear Receptor 77

4.3.1 *The structure and function of nuclear receptors*

Nuclear receptors (NRs) are a family of ligand-dependent transcription factors that regulate the expression of proteins involved in an assortment of physiological processes like embryonic development, organ physiology, cell differentiation and homeostasis[267-271]. Dissimilar from cell surface receptors, which bind intercellular ligands to initiate communication between the cell and the extracellular environment, intercellular ligands that target NRs diffuse across the plasma membrane and bind their targets directly[272, 273] (**figure 4.6B**). Once bound to a ligand, NRs diffuse across the nuclear membrane to transcriptionally control the expression of specific genes, recruiting co-activators and binding to specific enhancer DNA sequences located far upstream from the target gene[273, 274]. In the absence of a ligand, NRs recruit co-repressors that prevent the expression of gene sequences regulated by the NRs. Though NRs have been traditionally seen to regulate nuclear functions, Wu and colleagues have shown that non-nuclear regulation of cell migration can be achieved when estrogen receptor binds the estrogen hormone, suggesting a greater physiological role for NRs outside of transcriptional regulation[275].

NRs are activated not only by steroid hormones such as estrogen and progesterone but also by various other lipid-soluble molecules like retinoic acid, oxysterols, and thyroid hormone ligands[276]. The full-length NR complementary DNAs (cDNAs) for glucocorticoid, estrogen, and thyroid receptor were isolated in between 1985 and 1987 and permitted the analysis of the structural and functional features of NRs[272, 277-279]. Nuclear receptors share common structural features, comprising of an N-terminal transactivation domain AF1, a highly conserved DNA-binding domain (DBD) containing a C₂ zinc finger, a hinge domain responsible for nuclear localization, and the carboxyl-terminal ligand-binding domain (LBD), constructed from 12 alpha-

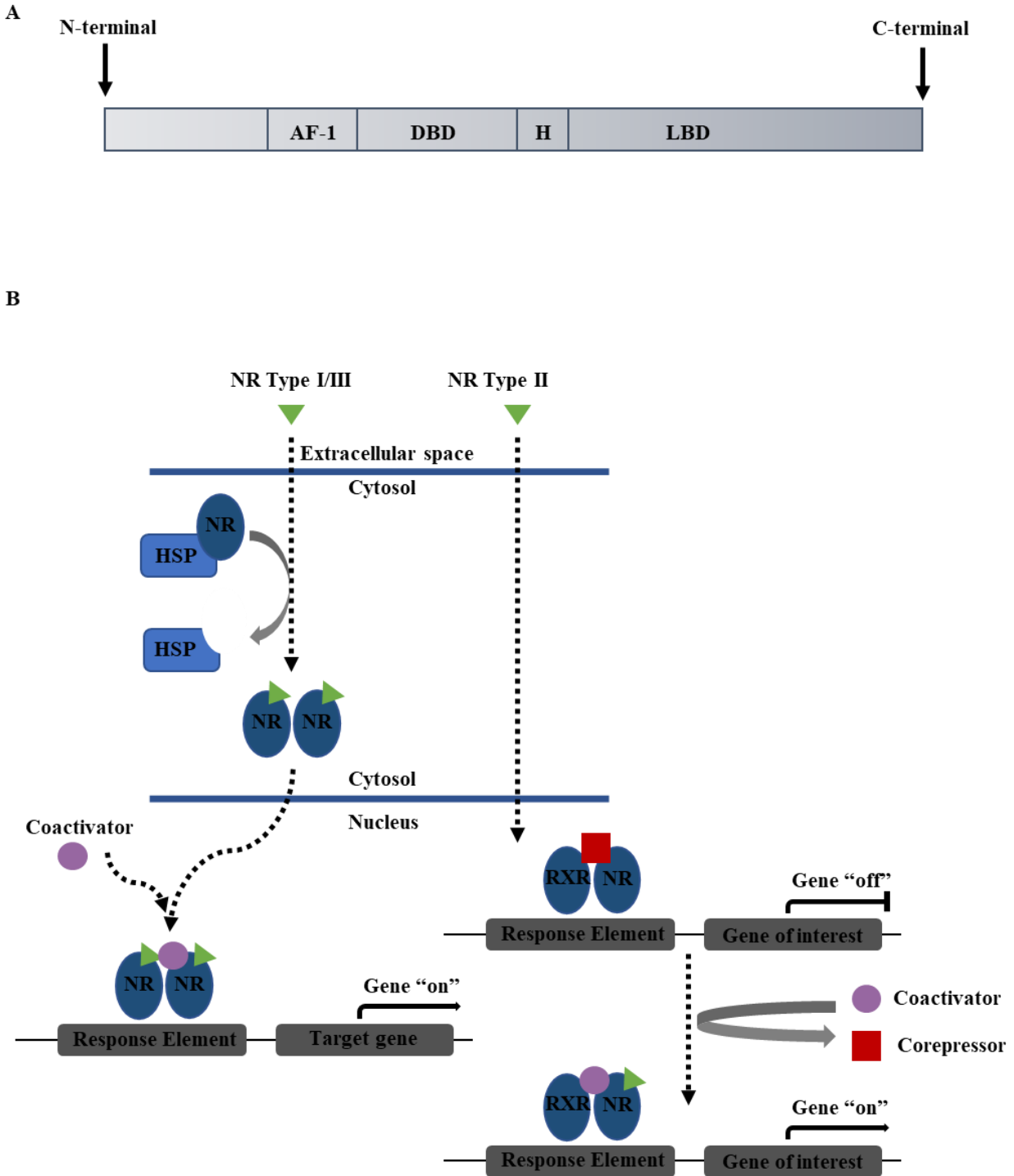


Figure 4.7. General structure and function of nuclear receptors. (A) highly variable amino-terminal domain that includes AF-1, activation domain; BDB, highly conserved DNA-binding domain that contains a C₂ zinc finger; H, a short hinge domain that is responsible for nuclear localization; LBD, a highly-conserved ligand-binding domain that not only binds the ligand but is responsible for heterodimerization or homodimerization with other nuclear receptors. (B) general function of nuclear receptor signalling. NR, nuclear receptor; RXR, retinoic acid receptor; HSP, heat-shock protein.

helical structures that form a hydrophobic pocket for ligand binding (**figure 4.6A**)[280]. Depending on the type of receptor, they can exist as monomers, homodimers, or heterodimers and recognize DNA sequences called hormonal response elements (HRE). For example, type I receptors, which include estrogen receptors and androgen receptors, are found in the cytoplasm bound to the heat shock protein (Hsp) 70 and Hsp90[281]. Following ligand binding, the receptor is freed from the Hsp and homodimerizes, exposing the hinge domains regulatory sequence, which allows translocation into the nucleus[282, 283]. Once in the nucleus, the receptor binds to coactivators and activates target genes. Type II receptors, like the thyroid and retinoic acid receptor, reside in the nucleus and are bound to specific DNA response elements in the absence of a ligand[273]. Following ligand binding to the receptor, the co-repressor dissociates and a co-activator is recruited, leading to activation of target genes[283]. Type III functions like Type I, except that it contains a direct repeat DNA sequence as opposed to an inverted DNA sequence and type IV nuclear receptors bind to half-sites in HREs.

4.3.2 Endogenous and non-endogenous ligands for nuclear receptors

Given that NRs are one of the most popular molecular targets for therapeutic small molecules and given their role in pathological processes like diabetes, cancer, and heart disease, several synthetic and semi-synthetic derivatives have been developed that control the regulation of nuclear receptors[284-287]. For example, prednisone, which is a glucocorticoid receptor (GR) ligand, is a semi-synthetic derivative of cortisone, which was originally used in the treatment of inflammatory conditions like asthma, hay fever, and allergies (**figure 4.7**) [288, 289]. Several other NRs have been targeted to control other pathological functions, like peroxisome proliferator-activated receptor gamma (PPAR γ) targeting using thiazolenediones, which improve insulin sensitivity in diabetes by controlling transcriptions of genes involved in glucose and lipid metabolism[290].

Tamoxifen, a selective estrogen receptor modulator (SERM), targets the estrogen receptor (ER) and is used a chemoprotective agent in breast cancers[276, 291]. Despite the number of clinically available small molecules that target NRs, over half of the approximately 50 genetically-encoded NRs are referred to as orphan NRs because they do not yet have a known ligand[292, 293]. Given the number of pathological functions that directly or indirectly involve NRs, the identification of ligands for orphan NRs could prove therapeutically fruitful.

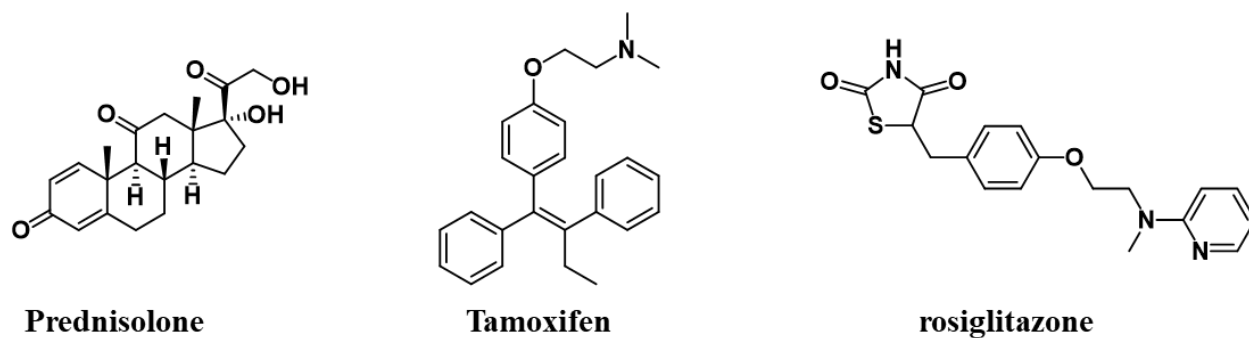


Figure 4.8. Ligands for nuclear receptors

4.3.3 Discovery and evaluation of nuclear receptor agonist cytosporone B

Given that prokaryotic and fungal organisms have been the principle source of therapeutically-relevant bioactive small molecules, Brady and colleagues originally isolated five structurally-distinct octaketides towards identifying novel biologically active secondary metabolites[294]. Using a combination of NMR and X-ray crystallography, cytosporone A-E and two cytotoxic trihydroxybenzene lactones were isolated from endophytic fungi *Cytospora* species (*Cytospora* sp.) and *Diaporthe* species (*Diaporthe* sp.), both of which were collected from *Conocarpus erecta* and *Forsteronia spicata* plants, respectively (**figure 4.8**)[294]. Antimicrobial activity was assessed

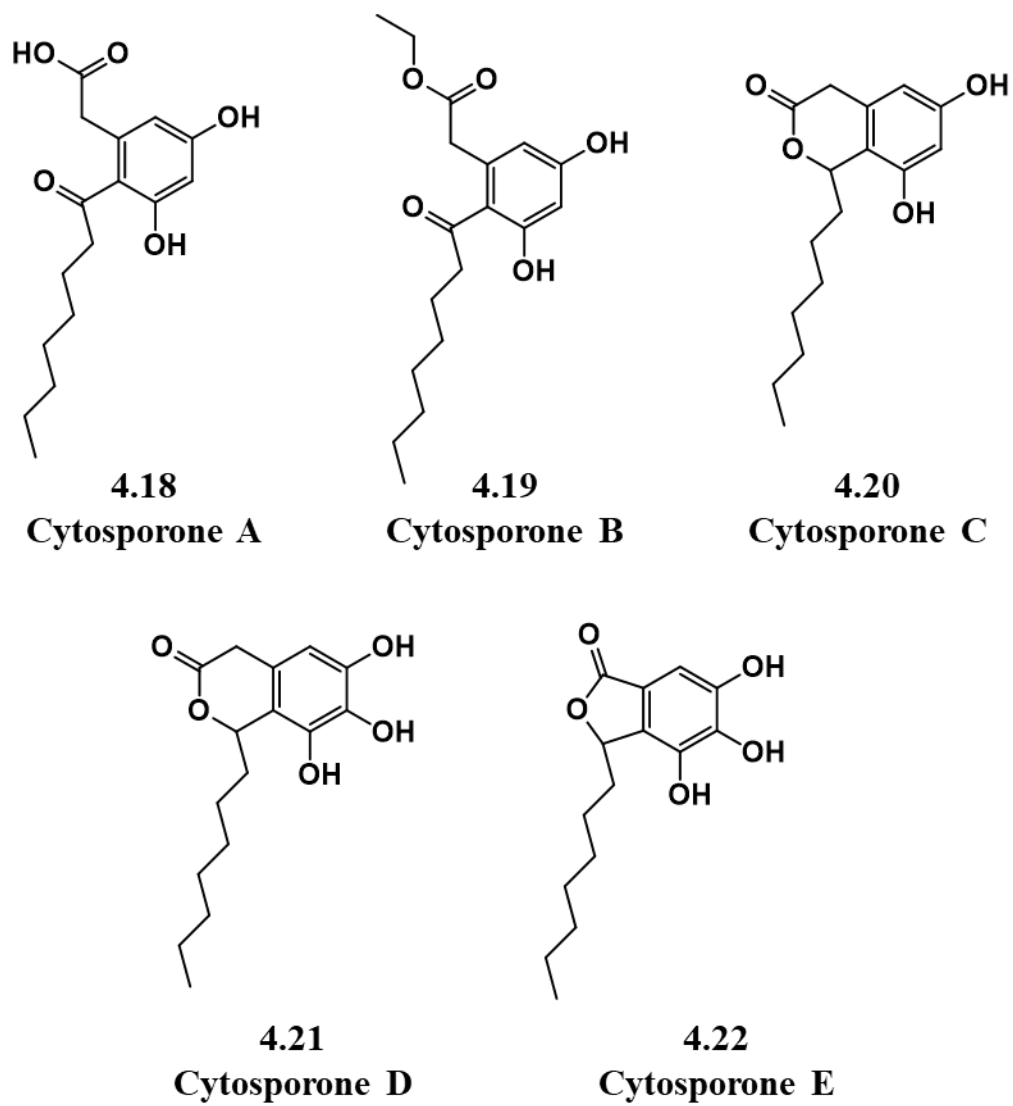


Figure 4.9. Cytosporones isolated by Brady and colleagues.

for all the isolated cytosporones and compared to the broad-spectrum antibiotic gentamicin. Only cytosporone **4.21** and **4.22** displayed antimicrobial activity against gram-positive *Staphylococcus aureus* and *Enterococcus faecalis*, gram-negative *Escherichia coli*, and fungus *Candida albicans* when compared to gentamicin[294]. Following the isolation of compounds **4.18** through **4.22**, Wu *et al.* screened a bank of endophytic natural products in search of an agonist for orphan nuclear receptor 77 (Nur77). Nur77, along with homologs Nurr1 and NOR1, belong to the nuclear receptor family 4 group A and have been shown to regulate the transcription of genes responsible for apoptosis and gluconeogenesis[295-297]. Anti-sense RNA (siRNA) knockdown of Nur77 expression or over-expression of a dominant-negative Nur77 was shown to inhibit T-cell receptor-induced apoptosis, suggesting that Nur77 is required for T-cell mediated apoptosis[298, 299]. Interestingly, following chemical stimuli that induce apoptosis, nuclear receptor Nur77 is shuttled, with assistance from a 9-retinoic acid-dependent retinoid X receptor alpha (RXR α) mechanism, to the mitochondria and induces cytochrome c release into the cytoplasm, which induces apoptosis[300-303]. The observation that NRs can have non-nuclear roles expands the scope of possible NR mechanisms in the cell, given that there is a limited amount of research on cytoplasmic roles of NRs[275]. In addition to a role in apoptosis, Nur77 has been reported to be a positive regulator of *G6pc*, *Fbp1*, *Fbp2*, and *Eno3*, which are genes involved in the stimulation of glucose production. Given that NOR1 has also been shown to be important in metabolism, through increased insulin-augmented glucose metabolism, the NR4A sub-family may be important targets in dealing with metabolic diseases[296]. Following screening of the bank of endophytic bioactive small molecules against luciferase-linked Nur77 reporter gene, Wu and colleagues identified compound **4.19** as an agonist for Nur77. Following extensive validation, **4.19** was shown to elevate blood glucose levels in fasting mice but was not observed in Nur77-null mice, indicating that **4.19**

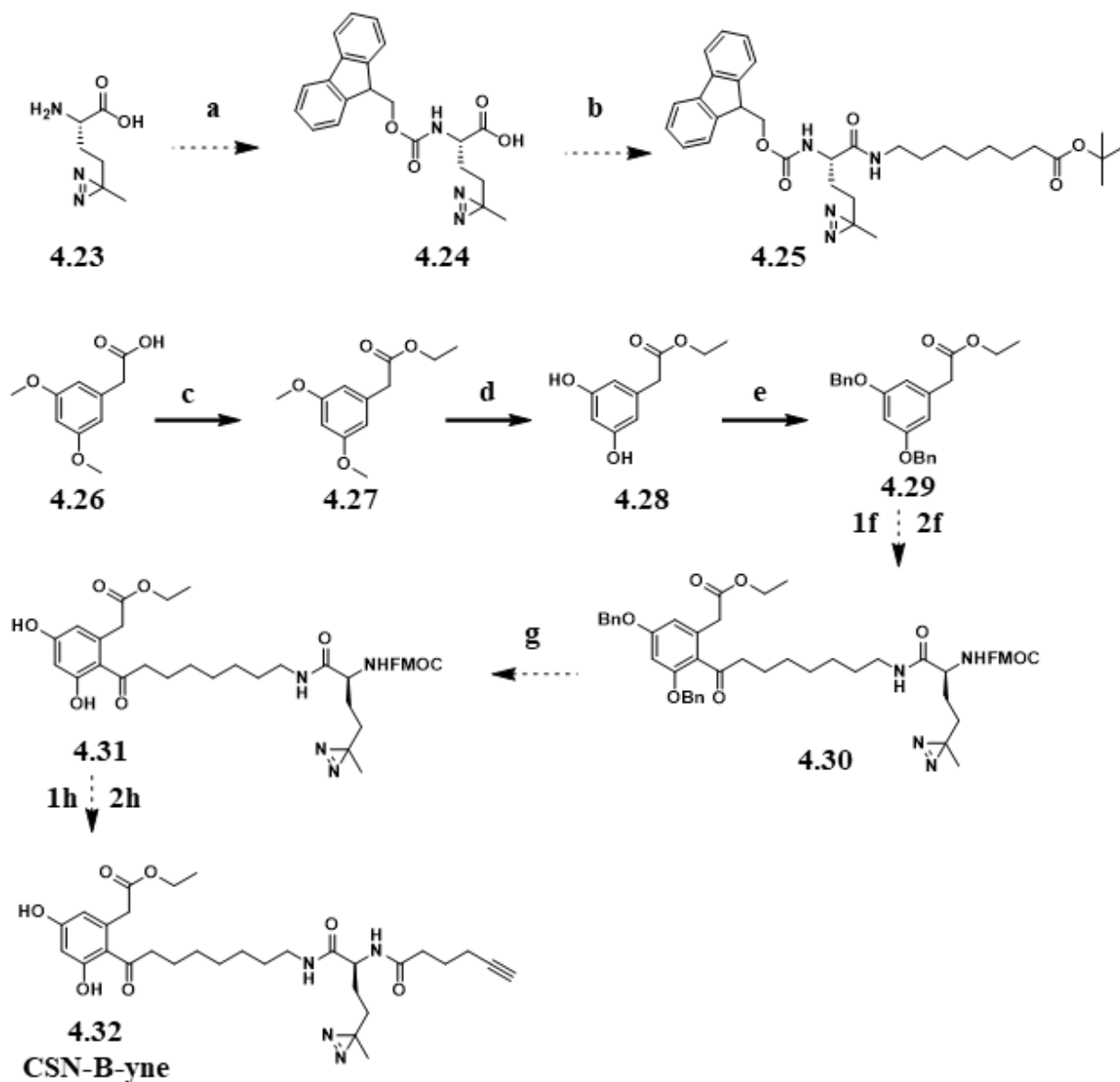
functions through direct binding to Nur77[295]. In addition to stimulating apoptosis, **4.19** was shown to retard xenograft tumor growth by inducing Nur77 expression, suggesting an anti-tumorigenesis role[295].

4.3.4 Design and synthesis of a novel cytosporone probe

Endogenous ligands for NRs, and their semi-synthetic therapeutic derivatives, often have undesirable side effects. For example, extended use of prednisone and dexamethanose can cause fat re-distribution, diabetes, cancers, and immunological disorders. Further, given the tissue-specific and promotor-specific nature of NR activation, which results from the expansive promiscuity of co-factors for NRs, a detailed understanding of co-factor binding to NRs could reveal structural and functional information relevant to NR activity[276, 304]. To this end, we endeavored to synthesize a photoaffinity ligand derived from cytosporone B to study Nur77, identify co-factors, and discover biological functions previously unknown.

4.4 Results and discussion

As outlined in the introduction, harnessing the privileged structures of bioactive small molecules has provided a robust understanding of the structure and function of biomolecules. Given the role of cytosporone B in the regulation of glucose and cancer cell viability and recognizing the diverse role of co-factors in NR regulation, we endeavored to synthesize an ABP that could provide information on the associated proteins that regulated Nur77 and similar proteins[295, 296]. To this end, we envisioned the design of an alkylated derivative of Csn-B (**scheme 4.2**). Previous work undertaken by Dawson and colleagues has provided the shortest route towards the synthesis of cytosporone derivatives[305, 306]. As outlined in **scheme 4.2**, we set out to undertake the synthesis of CSN-B-yne. To this end, we undertook the esterification of **4.26** over a period 22



Scheme 4.2. Synthesis of CSN-B-yne. a.) Fmoc-Cl, NaHCO₃, 1,4-dioxane/H₂O (2:1). b.) tert-butyl 8-aminooctanoate, HATU, DIPEA, DMF. c.) Ethanol, H₂SO₄, Benzene, Reflux, 20 h, 43.7%. d.) Boron tribromide, dichloromethane, -78 C, 0.5 h and 0 C, 4 h, 5%. e.) Benzyl bromide, K₂CO₃, acetone, 68 C, 21.5 h. f.) **4.25**, trifluoroacetic anhydride/phosphoric acid (4:1), 23 h. g.) boron tribromide, dichloromethane, -78 C, 0.5 h, and 0 C, 4 h. 1h.) 20% piperadine, DMF, 24 h. 2h.) 5-hexynoic acid, HATU, DIPEA, DMF, 24 h, rt. Reactions undertaken and completed are indicated with a solid arrow. Reactions to be completed indicated with a dash arrow.

hours using 99% ethyl alcohol and a Dean-Stark apparatus to remove water from our reaction. As previously reported, the ethyl carboxylate group is important in ligand binding to NR because it resides in a polar cleft in the LBD of the NR and hydrogen bonds with a histidine's imidazole amine [305]. Following the esterification of **4.26** to produce compound **4.27**, ether cleavage using boron tribromide in neat dichloromethane was undertaken at the 3- and 5- position on the aromatic ring. Work by Xia and colleagues has shown that not only does ether cleavage at temperatures greater than 0 C can cause mixtures of methyl ethers but also that methyl cleavage conditions may only de-methylate the 3-position[305]. We encountered difficulty with complete methyl cleavage of the 3- and 5- methyl ethers, which ultimately resulted in a significantly decreased yield compared with the literature results. Despite this, we obtained compound **4.28** and protected the phenyl alcohols with benzyl bromide using potassium carbonate as a base to generate compound **4.29**. Unfortunately, several issues were encountered with the protection of the phenyl alcohols. One of the major issues was the difficulty with complete benzyl protection of the alcohols, which was likely due to the steric difficulties with appending multiple benzyl group. Additionally, the acidification of the reaction mixture following removal of acetone under reduced pressure may have hydrolyzed the ester causing a mixture of products.

To continue the synthesis of the CSN-B-yne probe, fluorenylmethoxycarbonyl chloride (Fmoc-Cl) protection of L-photo-methionine should be undertaken, followed by HATU-mediated coupling to tert-butyl 8-aminooctanoate for form product **4.25** [306-308]. As outlined in **chapter 1**, diazirine have been demonstrated to not only limit background labeling but may decrease perturbations of protein-protein interactions *in vivo*[126, 309, 310]. Additionally, linker lengths have been demonstrated to impact covalent capture of proteins[124, 125]. Within the context of our chemical probe, the linker length is important given that the NR4A1 nuclear

receptor's ligand binding site within the ligand binding domain (LBD) is non-canonical and is located deep within the LBD, suggesting that a shorter linker would not bind with great efficiency to the LBD[92, 305, 311-313]. In addition to potentially decreasing binding efficiency, an insufficiently long linker may impact the binding of co-activators or co-repressors to Nur77. Previous work has demonstrated that following ligand binding, the AF-2 helix (Helix 12) on the LBD rotates to allow binding of co-activator or co-repressor peptide binding to an LXXLL motif on the LBD[314]. As has been demonstrated with PPAR α antagonist GW6471, disruption of H-12 rotation can impair binding of co-activator or co-repressor peptides[315-317]. As reported by Dawson and colleagues, the precursor **4.29** would then undergo an acylation with compound **4.25** to generate **4.30**. Following deprotection of the benzyl groups, compound **4.31** is formed. Once this compound is purified, we propose the Fmoc deprotection followed by HATU-mediate coupling to 5-hexynoic acid to generate the desired compound CSN-B-yne (**4.32**).

4.5 Conclusions

In this chapter, we undertake an initial analysis of the molecular targets of Cl-ARM-A, a novel antibiotic isolated from *Streptomyces armeniacus*. Using a medicinal chemistry strategy, a derivative of armeniaspirole is designed and synthesized so that covalent capture of molecular targets is feasible. Cl-ARM-A-yne was harnessed to identify the molecular targets of armeniaspirole A in prokaryotic and eukaryotic organisms. We were able to identify several lead molecular targets that have been previously shown to be important in bacterial growth. Future work will include the validation of the molecular targets of **4.17** identified via MS using recombinant proteins. In this chapter, we also demonstrate our work towards the synthesis of an AfBP based on the privileged structure of bioactive small molecule cytosporone B. Once this proposed molecule has been synthesized, the chemical probe will need to be validated in an *in*

vitro eukaryotic model to confirm that it can covalently capture nuclear receptor proteins and their associated binding proteins.

4.6 Experimental Section

General method labeling of cell lysates in vitro

For *in vitro* protein labeling of cell lysates, fresh cell lysates (0.7 mg/mL) in 100 μ L PBS and 0.05 % Triton X-100 buffer were incubated 40 μ M of Cl-ARM-B probe for 1 h, followed by the addition of the functionalized probe Cl-ARM-B-yne and incubation with the probe for 1 h. If the competitive inhibitor was not used, then cell lysates were incubated with Cl-ARM-B-yne for 1 h. Following incubation with probe **4.17**, 100 μ L of freshly prepared click chemistry mix in PBS consisting of rhodamine-azide (100 μ M), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA, 200 μ M), CuSO₄ (2 mM), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 2 mM), were added and incubated for 2 h at room temperature with gentle mixing. The reactions were terminated by addition of 1 mL of acetone and stored at -80 C for at least 30 min. The samples were then centrifuged for 15 min at 4 C, 14 000 rpm to precipitate protein. The protein pellets were air dried for 10 min, resuspended in 35 μ L Laemmli buffer containing beta-mercaptoethanol, shaken for 15 min and heated for 5 min at 95 C. The samples were then resolved by 10% SDS-PAGE and then visualized by in-gel fluorescence scanning using a FMBIO fluorescence scanner (Hitachi). Visualization of loading was undertaken using Coomassie Brilliant Blue.

General procedure for the isolation of labeling proteins

Biotin-azide click chemistry, streptavidin-enrichment, and trypsin digest of probe-labelled proteomes was performed as previously described[183]. However, an acetone precipitation step was included following the incubation with the click chemistry mix. Following protein enrichment,

agarose-streptavidin beads (Pierce) were washed three times with 100 μ L PBS in a Biospin column. Using 200 μ L of PBS, beads were transferred to probe-labeling cell lysates and incubated for 90 min. For the on-bead digestion, the beads were washed five times with 50 mM ammonium bicarbonate, transferred to an Eppendorf tube and heated for 15 min at 6 C in 500 μ L of 10 mM DTT in 50 mM ammonium bicarbonate (ABC). After 15 minutes, 25 μ L of 500 mM iodoacetamide was added and lysates were incubated in the dark for 30 min. Samples were centrifuged for 2 min at 1400 rpm and 100 μ L of ABC was added followed by 2 μ L of 0.5 mg/mL of trypsin. Samples were rotated at 37 C overnight, followed by bead pelleting and the transfer of the supernatant for MS analysis.

Mass spectrometry

HPLC-ESI-MS/MS was used for MS analysis. The system consisted of an Agilent 1100 micro-HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled with an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA) equipped with a nano-electrospray interface operated in positive ion mode. Pre-column was packed in-house with reverse phase Magic C18AQ resins (5 μ m; 120 Å pore size; Dr. Maisch GmbH, Ammerbuch, Germany), and analytical column of 75 μ m $\times$ 100 mm was packed with reverse phase Magic C18AQ resins (1.9 μ m; 120 Å pore size; Dr. Maisch GmbH, Ammerbuch, Germany). The mobile phases consisted of 0.1% (v/v) FA in water as buffer A and 0.1% (v/v) FA in acetonitrile as buffer B. The 4 μ L sample was loaded onto the pre-column using a buffer containing 98 % A at a flow rate of 4 μ L/min for 5 min. Subsequently, a gradient from 5% to 35% buffer B was performed, at a flow rate of $\sim$ 300 nL/min obtained from splitting a 20 μ L/min through a restrictor, in 60 min. The MS method consisted of one full MS scan from 350 to 1700 m/z followed by data-dependent MS/MS scan of the 5 most intense ions, with dynamic exclusion repeat count of 2, and repeat duration of 90 s. As well, for

the experiments on the Orbitrap MS the full MS was performed in the Orbitrap analyzer with $R = 60,000$ defined at m/z 400, while the MS/MS analysis were performed in the LTQ. To improve the mass accuracy, all the measurements in Orbitrap mass analyzer were performed with internal recalibration (“Lock Mass”). On the Orbitrap, the charge state rejection function was enabled, with single and “unassigned” charge states rejected.

Analysis of mass spectrometry data

Raw files were generated using LTQ-Orbitrap and processed and analyzed using MaxQuant, Version 1.3.0.5 using the Uniprot fasta protein database (2013, July version) in addition to the HCV con1 sequence including the commonly observed contaminants. When accessing the protein databases, the following parameters were used: enzyme specificity was set to trypsin; protein N-terminal acetylation, methionine oxidation, and cysteine carbamidomethylation was selected as fixed modification as previously described[183, 188].³⁵⁻²⁹ A maximum of two missing trypsin cleavages were permitted. Precursor ion mass tolerances were set to 7 ppm, and the fragment ion mass tolerance was 0.8 Da for the MS/MS spectra. If the identified peptide sequences of one protein were contained within or equal to another protein’s peptide set, the proteins were grouped together and reported as a single protein group. The false discovery rate (FDR) for peptide and protein was set to 1 % and a minimum length of six amino acids was used for peptide identification. Normalized LFQ intensity was used for protein quantification.

Cytotoxicity assay

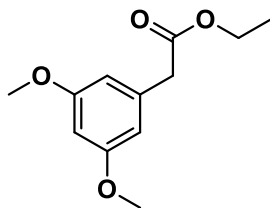
Human embryonic kidney (Hek293) cells were treated as described above. After 24 h, cells were washed twice with PBS followed by the addition of 200 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Alfa-Aesar, Ward Hill, MA) at 2.5 mg/mL in PBS and incubated

for 3 h. Excess media was removed and precipitated crystals were solubilized in 200 μ L of DMSO. The absorbance was read at 562 nm using SpectarMax M2 (Molecular Devices Corporation, Sunnyvale, CA) and the data was recorded using Softmax Pro 4.7 software.

4.7 Synthetic methods and characterization

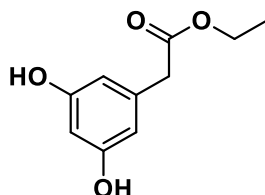
General information

All reagents and solvents were purchased from Sigma-Aldrich, unless otherwise noted, and used without further purification. Deuterated solvents were purchased from Cambridge Isotope Laboratories and thin layer chromatography was done using Analtech Uniplate $\text{\textcircled{R}}$ silica gel plates (60A F254, layer thickness 250 μ m). Flash column chromatography was performed by silica gel (60A, particle size 40 to 63 μ m). ^1H NMR and ^{13}C NMR spectra were recorded using a Bruker-DRX-400 spectrometer at a frequency of 400.13 MHz for ^1H and 100.61 MHz for ^{13}C and processed with Bruker TOPSPIN 2.1 software. Chemical shifts are reported in parts per million (δ) using residual solvent resonance as an internal reference. The following abbreviations were used to designate chemical shift multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet or unresolved, br = broad single and J = coupling constant in Hz.



Ethyl 2-(3,5-dimethoxyphenyl)acetate (4.27): To a solution containing 3,5-dimethoxyphenyl acetic acid (507 mg, 2.58 mmol) was added 99% ethyl alcohol (1 mL) and a drop of sulfuric acid. The mixture was heated at reflux for 26 hours using a Dean-Stark apparatus. After the

reflux, the solvent was cooled and mixture was evaporated prior to extraction with ethyl acetate (50 mL) and 1N sodium hydroxide (50 mL). The resulting organic mixture was then dried using magnesium sulfate, filtered, and evaporated. The product was then purified using column chromatography (40:60 Ethyl acetate: hexane) and 253 mg yellow oil was obtained (43.7%). NMR matched previously reported values[305, 306].



Ethyl 2-(3,5-dihydroxyphenyl)acetate (4.28): To a suspension of 3,5-dimethoxyphenyl acetate (430 mg, 1.9 mmol) in dry DCM was added boron tribromide (10 mL, 10.4 mmol) dropwise to the suspension at -78 C under argon. After the dropwise addition, the reaction was stirred for 30 minutes at -78 C and then raised to 0 C and stirred for 4 hours. Reaction was quenched with water and extracted with ethyl acetate. The organic layer was rinsed with brine and dried with sodium sulfate. The solvent was removed and the residue was purified by column chromatograph (46% ethyl acetate: hexane) to yield 20 mg of a white power (5%). NMR matched previously reported values[305, 306].

Chapter 5: Conclusions and future directions

5.1 Preface

The research contained in this thesis focused on the development of chemical probes derived from bioactive small molecules and the identification of the molecular targets of these bioactive small molecules. Contained within each of the preceding chapters were the objectives and results of each project. This work contributes to the probe development research community, which designs and synthesizes affinity- and activity-based probes that can report on the molecular targets of bioactive small molecules. In this concluding chapter, the research completed in this thesis is summarized and evaluated based on the original research objectives. Finally, the project results will be contextualized within the greater research framework and future work will be proposed for each project.

5.2 Identifying the molecular targets of 6-hydroxydopamine

Identifying the molecular targets of bioactive small molecules permits the elucidation of the target's role in a physiologically- or disease- relevant model, expanding our understanding of biological systems and the role of specific proteins in human disease[318]. In **chapter 2**, we assessed the impact of 6-OHDA on the expression of HCV through the development of a novel affinity-based chemical probe. HCV infection carries a significant clinical burden that if left untreated can result in liver cirrhosis and hepatocellular carcinoma[319, 320]. Given the dependence of HCV replication on the expression of liver-specific miRNA-122, the development of small molecules that can target the RNA-interference machinery could provide for a novel therapeutic mechanism for the treatment of HCV[157, 321]. Based on the work undertaken by Tan and colleagues to identify small molecule regulators of RISC, we examined the effect of three bioactive small molecules on HCV replication (**figure 2.1**)[165].

Given the role of the RISC complex in HCV replication, which can regulate the expression of the HCV through binding with liver-abundance miRNA-122, we examined the biological effects of 6-OHDA, ATA, and SUR on HCV expression[157, 321]. Our initial work focused on assessing the cytotoxicity of 6-OHDA, SUR, and ATA on an HCV subgenomic replicon cell system. Following a 24 hour treatment, 6-OHDA was determined to inhibit HCV in a dose-dependent fashion without excess cytotoxicity. Given the cytotoxicity of ATA and SUR on model HCV subgenomic replicon, 6-OHDA was used to determine the impact on RISC binding to miRNA-122 reporter system. Using a reporter plasmid that contained a *Renilla* luciferase gene under the control of RISC and a firefly luciferase gene as a control for transfection efficiency, we demonstrated that 6-OHDA caused a modest two-fold increase in the expression of the *Renilla* luciferase. Interestingly, despite the previous work by Tan and colleagues, 6-OHDA did not significantly

impair RISC binding to the reporter system, which strongly suggests that 6-OHDA does not inhibit HCV replication through an exclusively RISC binding mechanism but also through an alternative mechanism.

Given the hydroxylated structure of 6-OHDA, we proposed a mechanism that generates a reactive oxygen species (ROS), which may then generate a Michael acceptor that can covalently label eukaryotic and viral proteins. For example, previous reports have indicated that oxidative stress can result through the dysregulation of HCV non-structural protein 3 and 5A[173, 175, 176]. We demonstrated that 6-OHDA could generate ROS in hepatocellular carcinoma cells (Huh7.5) and, following the synthesis of novel 6-OHDA chemical probe, could covalently label a wide range of eukaryotic and recombinant viral proteins. Previous work has shown that alkylation of viral proteins can cause inhibition of viral replication[184]. We demonstrated that 6-OHDA could cause protein un-ravelling, a potential mechanism for 6-OHDA induced HCV inhibition.

In our study, we did not assess the specific sites on recombinant viral proteins that were labeled by 6-OHDA. Previous work has shown that 6-OHDA, following oxidation, forms a stable *p*-quinone at room temperature[322, 323]. It is well-known in the literature that *p*-quinones can form site-specific thioether bonds with cysteines through an addition-elimination reaction[324]. The reactivity of *p*-quinone Michael acceptors has been harnessed in functional proteomics to evaluate the molecular targets of various quinone-containing molecules[325, 326]. Future work with 6-OHDA could focus on mapping the specific 6-OHDA labeling sites on viral proteins, which could provide insight into how 6-OHDA impairs viral proteins and ultimately HCV expression. Future work could also focus on analyzing the role of 6-OHDA in other viral models, given that oxidative stress has been implicated in several viral infections[327]. For example, Olnagier and colleagues have shown that ROS can modulate dengue virus infection in dendritic cells[328].

5.3 Validating the effectiveness of a sinefungin-based affinity probe

Despite the presence of 20,000 protein-coding genes within the human genome, post-translational modifications of mature proteins drastically increases their structural and functional diversity in eukaryotic systems. Given that post-translational modifications can cause epigenetic changes in heritable phenotypic traits, an understanding of epigenetic modifications will improve our understanding of human genetics[193]. Methyltransferases, an important class of epigenetic enzymes, have important roles in the numerous biological processes like gene transcription, signal transduction, and protein stabilization[196, 329].

In **chapter 4**, we developed a sinefungin-based PAL that can target methyltransferases enzymes. Sinefungin is an ideal targeting moiety given it is a non-selective and reversible inhibitor of methyltransferase enzymes, and thus can report on a wide-range of methyltransferases in parallel[330]. Through a number of coupling and protecting group strategies, we synthesized BpyneSF and then assessed its ability to specifically capture proteins. We demonstrated through an *in vitro* labeling strategy using hek293 that BpyneSF could label proteins with a degree of selectivity. Following this initial labeling experiment, we optimized BpyneSF incubation and irradiation time with cell lysates. Finally, to ascertain the selectivity of BpyneSF, we undertook a competitive experiment using the methyltransferase inhibitor CARM1, which demonstrated that BpyneSF could target specific methyltransferase enzymes. Using BpyneSF, we used MS to identify that BpyneSF could target methyltransferase SETD2. Furthermore, using recombinant methyltransferase proteins CARM1, PRMT1, and SETD2, we showed that BpyneSF could broadly target other methyltransferases.

In our study, we designed and synthesized a chemical probe that was chemically appended to the 5'-carboxyl end of sinefungin. Recent work by Cravatt and colleagues has shown that covalent

attach at the 6-amino end of the S-adenosyl methionine (SAM) co-factor could report on the molecular targets of SAM and profile the methyltransferase activity both *in vitro* and *in vivo*[331]. Future work should focus on chemically linking the benzophenone moiety to the 6-amino group on SF and then undertaking a comparative study of different eukaryotic cell lines. A comparative analysis of the molecular targets of SF could permit the identification of MT-associated binding proteins important in epigenetic modification. Future work should also focus on the use of extended linkers and different PRG. As indicated in **chapter 1**, variations in the linker length and branching, in addition to the differences in PRG, can have significant impacts on the molecular targets captured[124, 125]. Given the limited number of methyltransferase enzymes captured using BpyneSF, which may be the result of steric issues around probe binding, the use of different linker lengths and PRGs could expand the scope of labeled MTs in our study.

5.4 Other probes for the elucidation of prokaryotic and eukaryotic mechanisms

5.4.1 Identifying the molecular targets of Armeniaspirole B

Given the rise of antimicrobial resistance, the discovery, isolation, and development of antimicrobial small molecules with novel chemical structures may prevent the advent of a post-antibiotic era[239]. In **chapter 4**, we identified the prokaryotic and eukaryotic molecular targets of armeniaspirole A. Initially isolated by Dufour and colleagues, armeniaspiroles were shown to have antimicrobial properties in several gram-positive bacteria[241, 243]. Despite their effectiveness as antibiotics, the survivability of armeniaspirole A treatment was dose-dependent in the MRSA-induced septicemia model[243]. Towards the identification of the molecular targets of ARM-A, Cl-ARM-A-yne was synthesized by our collaborator Dr. Mark H. Dornan. Following the synthesis of the chemical probe, we undertook an initial *in vitro* labeling experiment using non-

pathogenic bacterial cell line *B. subtilis*. We demonstrated that Cl-ARM-A-yne could label *B. subtilis* in a concentration-dependent manner and was selective for prokaryotic molecular targets. Following the labeling of *B. subtilis*, we demonstrated that Cl-ARM-a-yne could also label Hek293 and HeLa cells in a concentration-dependent manner. Our initially labeling experiments in eukaryotic organisms displayed a difference in labeling profiles between the two cell lines, suggesting that ARM-A may differentially target proteins in distinct eukaryotic cell lines. Following the fluorescent labeling of proteogenic biological targets, we undertook an MS analysis of the biological targets that were visualized. In *B. subtilis*, we identified ATP-dependent protease ClpY, a component of the proteolytic complex ClpQY, as a potential target of ARM-A in addition to hydrolase MtnU and phosphoesterase YfkN. In Hek293 and HeLa, we identified several proteins involved in protein homeostasis and protein folding machinery. In our study, we did not validate the molecular targets identified by MS. Given that ClpY plays a critical role in protein homeostasis and protein folding, future work should focus on validation this as a *bona fide* target of ARM-A. Our collaborators in the Boddy Lab have undertaken a preliminary investigation of the three *B. subtilis* targets and have identified ClpY to be a reversible target of armeniaspirole A. Future work should focus on linking the inhibition of ClpY by armeniaspirole A to the phenotypic observation of inhibition of bacterial propagation. Future work can also focus validating the phosphoesterase YfkN and the hydrolase MntU as *bona fide* targets of armeniaspirole A, given their potential role in bacterial inhibition. Additionally, future work could focus on analyzing the molecular targets of armeniaspirole A within eukaryotic cells and validating Hsp70 as a possible adaptation of structure for use in human disease.

5.4.2 *Cytosporone probe for the evaluation of Nur77-binding proteins*

In **chapter 4**, we propose the development of a cytosporone-based affinity probe. Despite the number of therapeutic drugs development for proteins, nuclear receptors are considered “undruggable” because they are either activated or repressed through a series of non-covalent interactions[332]. Furthermore, protein-protein interactions are important in normal and dys-regulated disease processes[333, 334]. Future work in this project should focus on the synthesis of the proposed affinity probe, followed by the in-gel fluorescent evaluation of molecular targetin in eukaryotic cell lines. Once the probe has been evaluated, the identification of the molecular targets should be undertaken using DiME or another functional proteomic method.

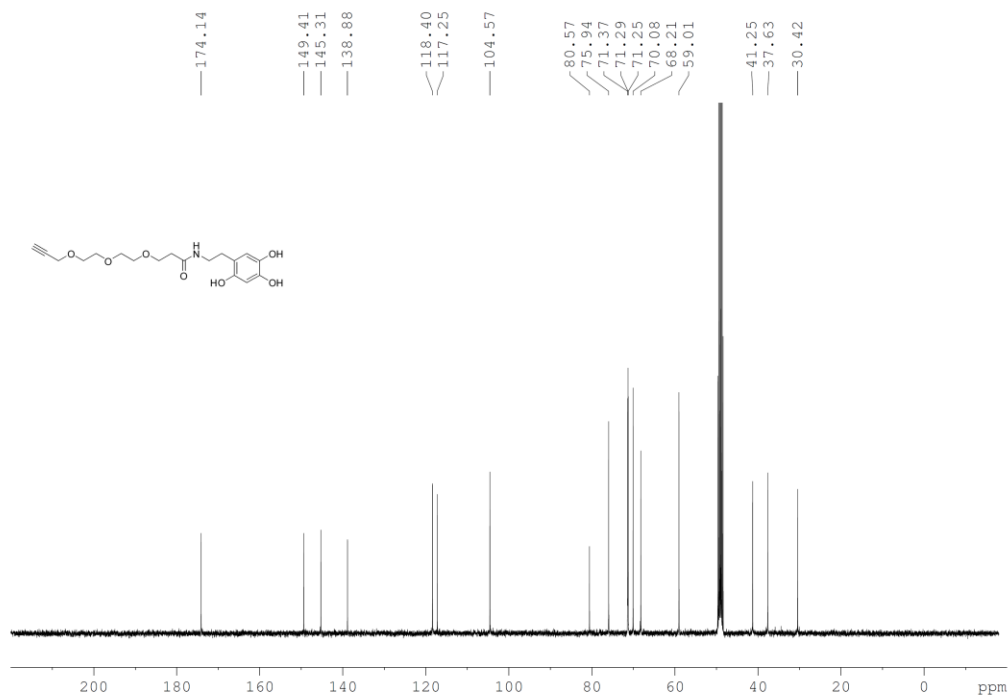
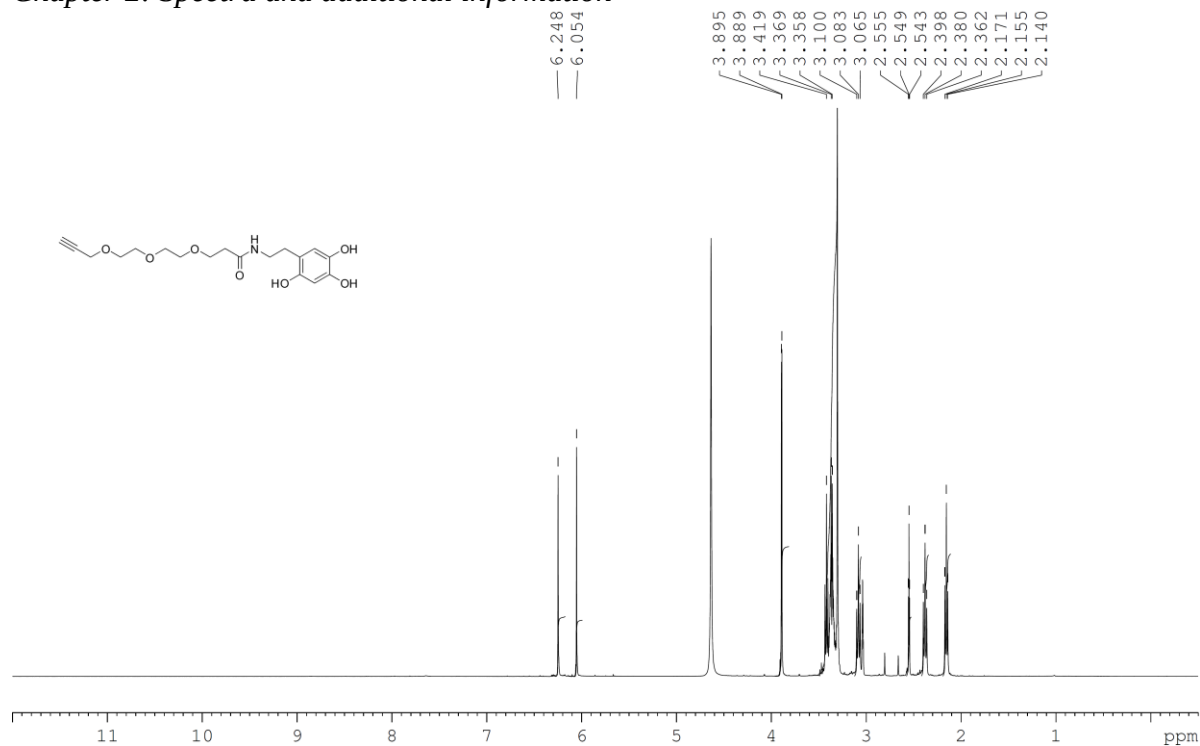
List of Publications

Lafreniere, M.A.; Powdrill, M.H.; Singaravelu, R.; Pezacki, J.P. 6-Hydroxydopamine Inhibits the Hepatitis C Virus through Alkylation of Host and Viral Proteins and the Induction of Oxidative Stress. *ACS Infect. Dis.*, **2016**, 2 (11), pp 863–871.

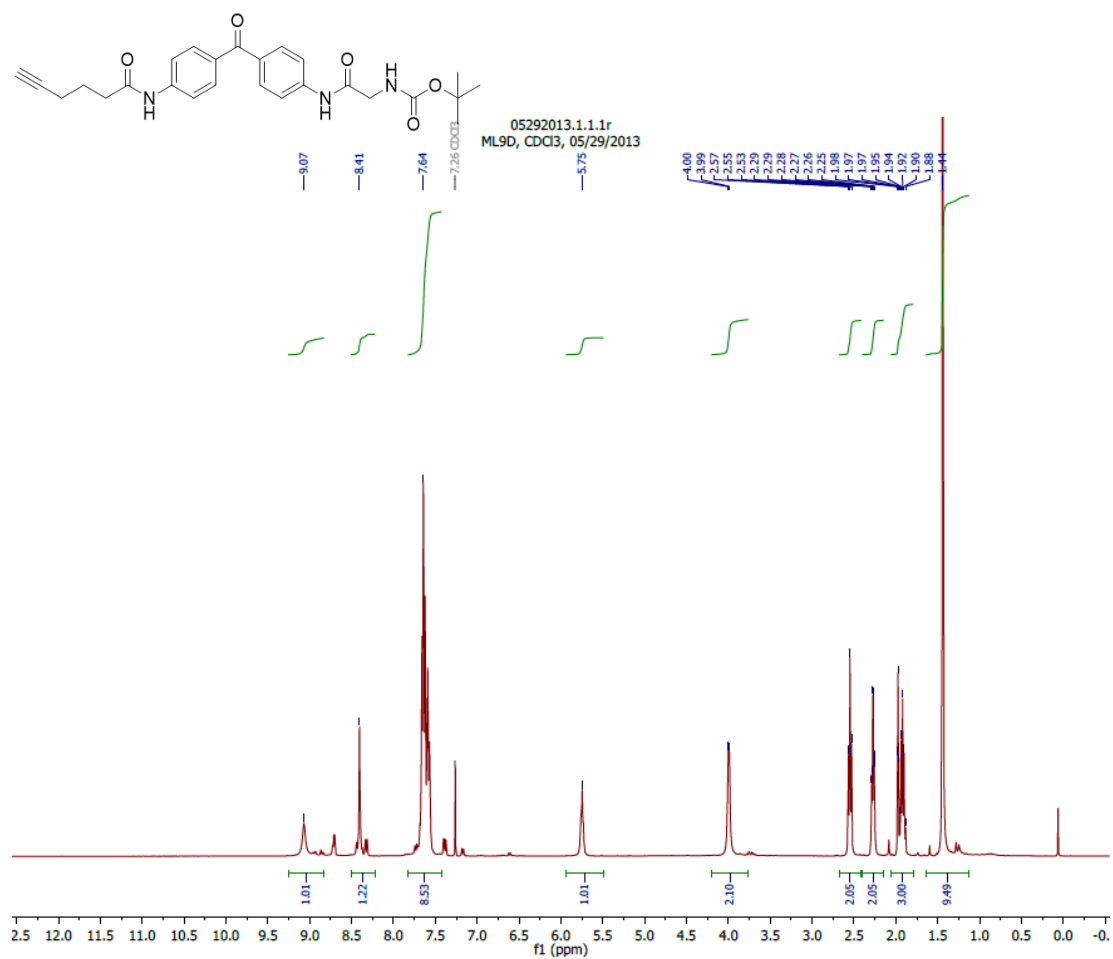
Lafreniere, M.A.; Desrochers, G.F.; Mekbib, K.; Pezacki, J.P. An affinity-based probe for methyltransferase enzymes based on sinefungin. *Can. J. Chem.*, **2017**, 95(10): 1059-1063

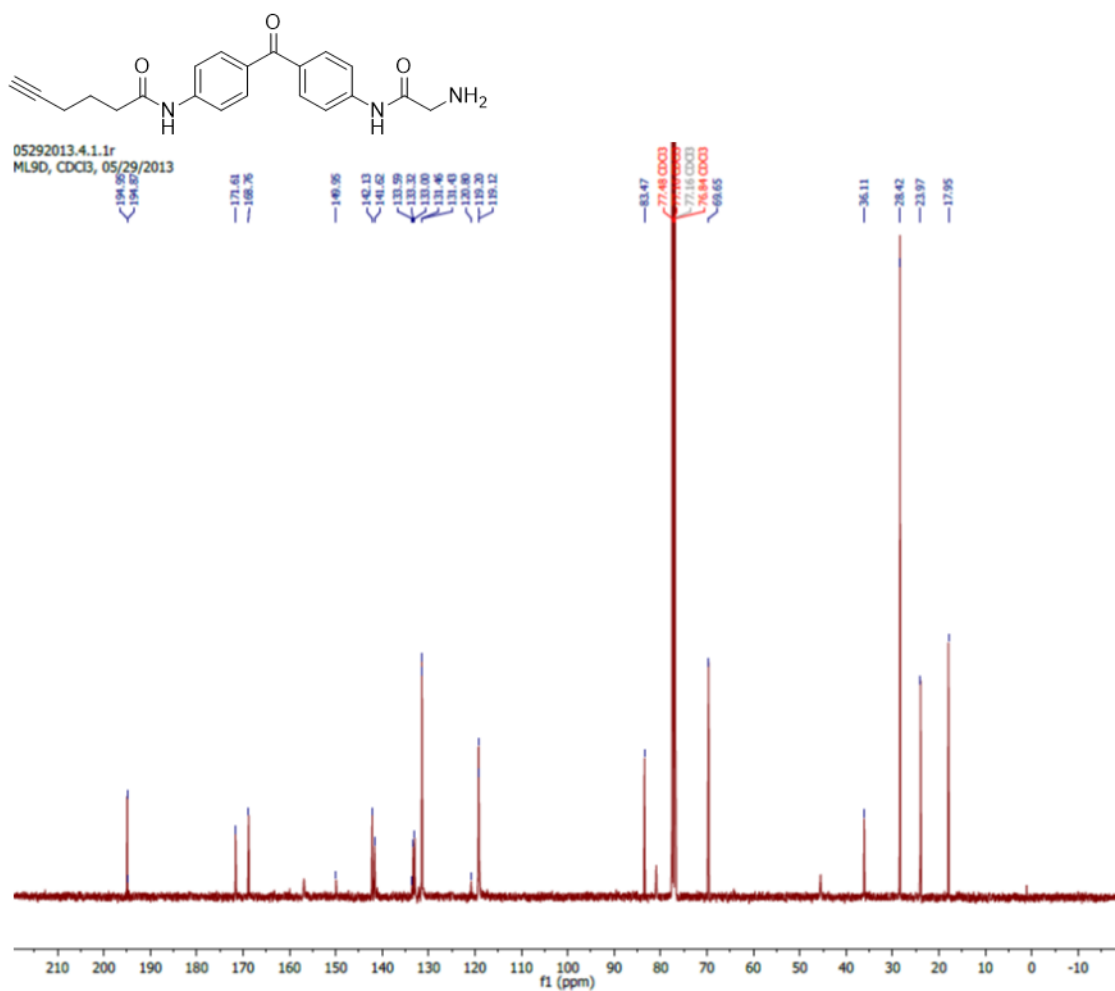
Appendix

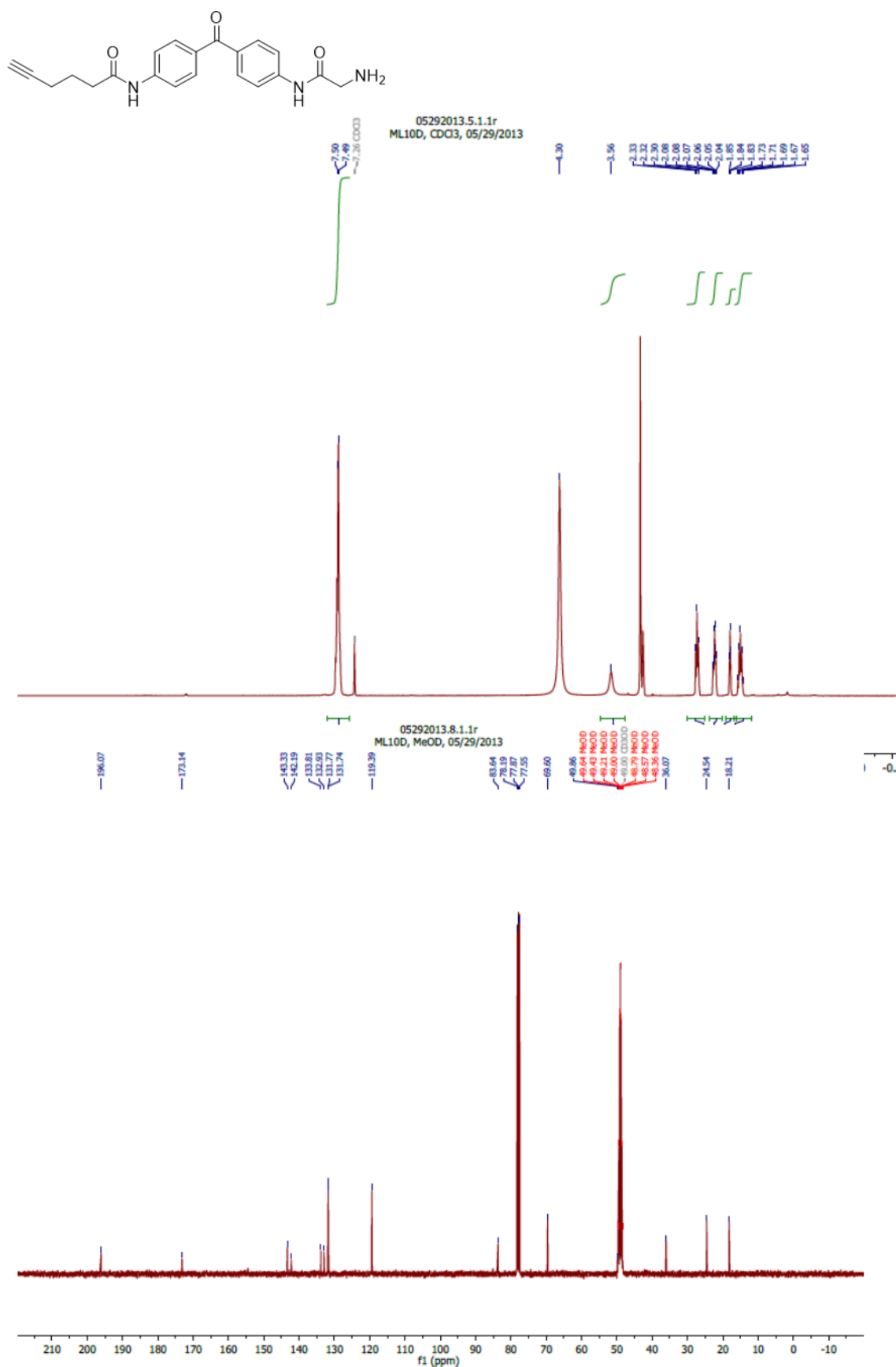
Chapter 2: Spectra and additional information

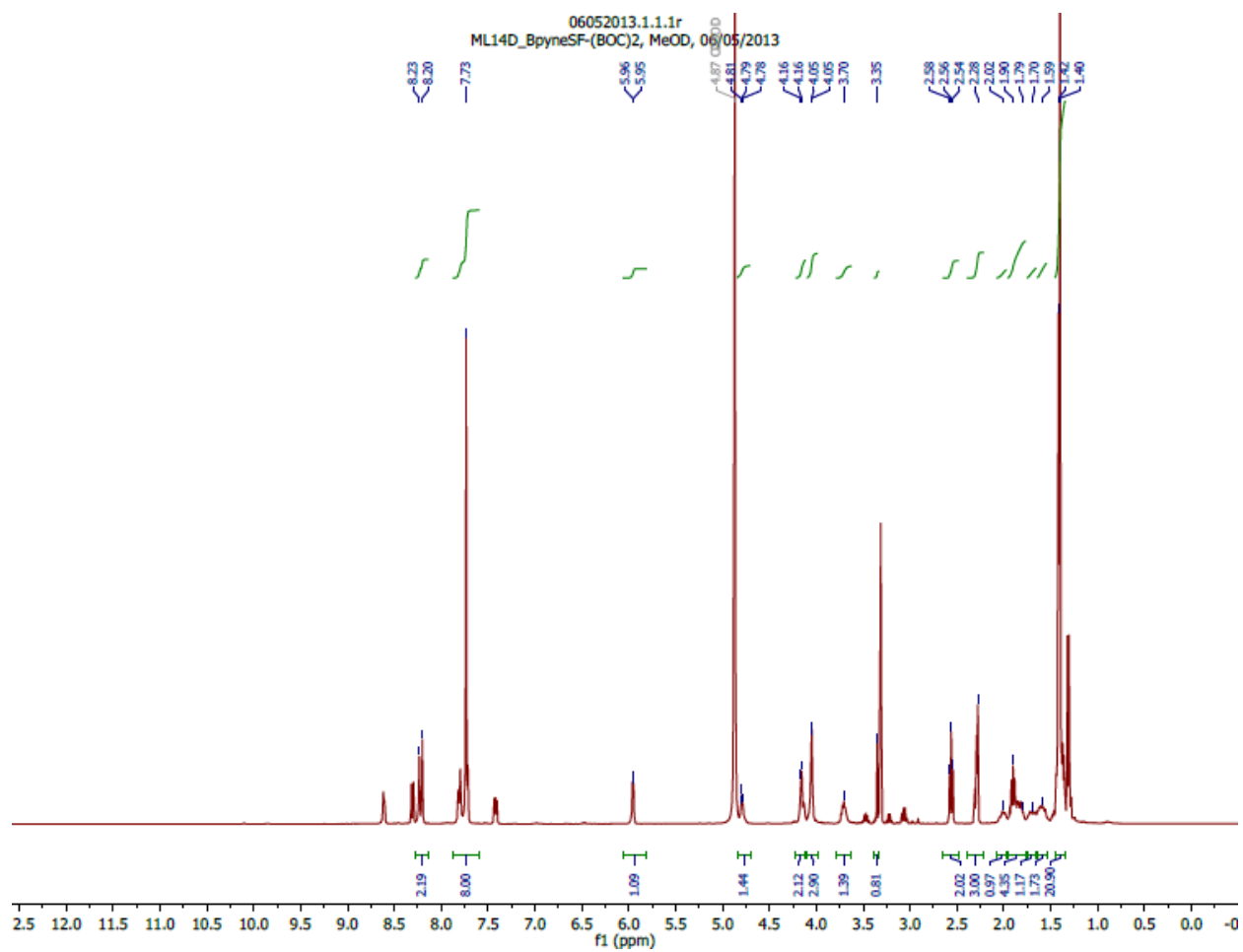
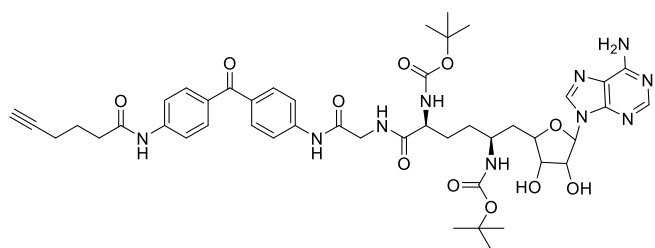


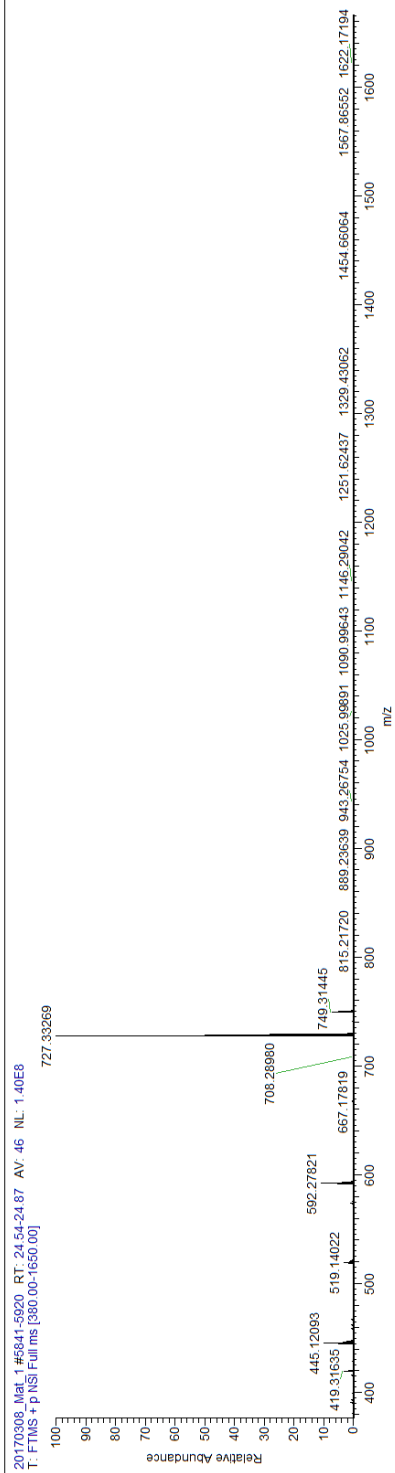
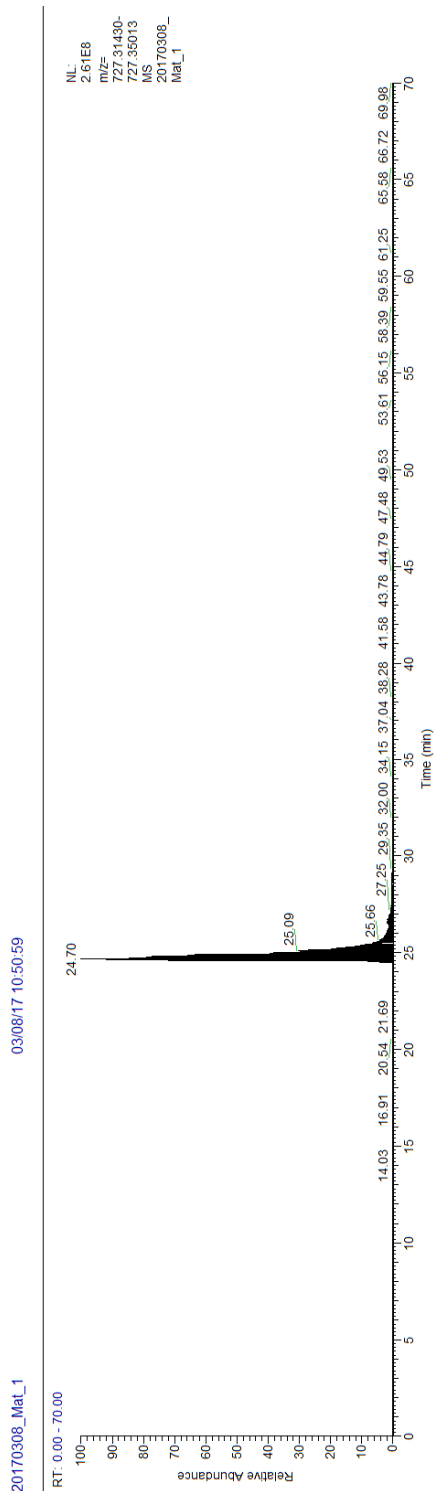
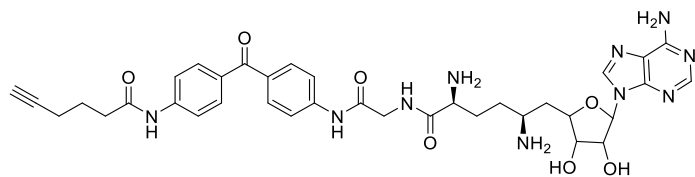
Chapter 3: Spectra and additional information











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