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Reactive Probes for Manipulating Polyketide Synthases, and Photoreactive Probes for Strained Alkyne Click Chemistry

A Dissertation Presented

By

JON WILLIAM AMOROSO

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

February 2014

Department of Chemistry
REACTIVE PROBES FOR MANIPULATING POLYKETIDE SYNTHASES, AND PHOTOREACTIVE PROBES FOR STRAINED ALKYNE CLICK CHEMISTRY

A Dissertation Presented

By

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To my Mother Patricia for supporting me in everything I have ever tried and encouraging me to be better than I thought I could be, my Father William for telling a younger me that shopping carts pull people down the street, my sister Sara for keeping me grounded yet adventurous, and my loving and patient wife Elizabeth, my North Star. Without you none of this would have been possible.
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I would also like to thank my group members who volunteered their support on these projects. Especially Gitanjali Prasad and Adam Gann for all of the hard work and wisdom they contributed.
ABSTRACT

REACTIVE PROBES FOR MANIPULATING POLYKETIDE SYNTHASES, AND PHOTOREACTIVE PROBES FOR STRAINED ALKYNE CLICK CHEMISTRY

February, 2014

JON WILLIAM AMOROSO, Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by Dr. Nathan Schnarr

Polyketides are a broad class of natural products that have received attention from the scientific community because they are a rich mine of bioactive structures. The common thread that binds the class together is the method by which they are synthesized, by large enzymatic complexes called polyketide synthases (PKSs) which display assembly line like organization. A great deal of effort has been put into studying PKSs, but their mechanistic steps are still not perfectly understood. In order to further the study of PKSs and their components, we have developed a series of reactive small molecules that covalently modify specific sites of PKS components. We have shown that β-lactones are able to selectively load holo-acyl carrier proteins (ACPs) with polyketide-like functionality while leaving similarly functionalized components such as the ketosynthase domain virtually untouched. We have also shown that β-lactams are able discriminate between apo- and holo-ACPs and have used this difference in reactivity to develop a method of purifying holo-ACPs. Another β-lactam based probe has been shown to directly modify the active portion of holo-ACPs with malonate-like functionality, and may be starting point for introducing novel functionality into difficult to access sites of polyketides.

During the course of these investigations the copper catalyzed azide-alkyne cycloaddition has been used heavily for conjugation reactions involving small molecules and proteins. The presence of copper has been a serious problem, often leading to lost and damaged proteins. Copper-free azide-alkyne conjugation methods exist, but suffer from drawbacks of their own such as slow reaction rates and difficult targetability. In order to maintain the positive aspects of copper free click yet overcome the drawbacks, we have been developing photosensitive probes which upon irradiation rapidly react with azides in a click like reaction. Although this reaction was initially developed with bioconjugation in mind, further experiments have indicated that this may not be the best use. However, experiments are currently being carried out to optimize their performance and explore their usefulness in other chemical conjugation applications.
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CHAPTER I

INTRODUCTION AND BACKGROUND OF POLYKETIDES, POLYKETIDES SYNTHASES, AND METHODS OF INVESTIGATING AND MANIPULATING POLYKETIDE SYNTHASES

A. Introduction: Polyketides as Natural Products

Polyketides are a broad and diverse class of natural products that have received a great deal of attention from the scientific community. They are mainly produced by soil bacteria, but are also known to be produced by fungi, plants, and marine organisms. The attention they have received is due to the high degree of pharmacologically relevant bioactivity displayed by this class, having provided numerous antibiotics, anticancer compounds, antirejection compounds, antifungals, antiparasitics, toxins, antilipodemic agents, and many others. They have been of particular interest as a source of novel antibiotics due to the ever present threat of drug-resistant organisms. Although their structures are as varied as their functions (Figure 1.1), the common thread that binds polyketides together as a class is the way they are synthesized: by repeated condensations of acyl-derived units in a manner similar to fatty acid synthases.

Polyketides can be further divided according to the method by which their synthases work. Type I polyketides, also called modular polyketides, are synthesized by multimodal megasynthases with numerous active sites and tend to produce macrolides with highly varied structures. Type II polyketides function iteratively to produce a polyketone chain that is further processed into highly aromatized or oxidized products. Type III polyketides tend to be small aromatic molecules. The work in this thesis is mainly concerned with components from type I polyketide synthases (PKSs).
Figure 1.1: Polyketide structures with functions and source organism labeled.

B. Modular Polyketide Biosynthesis

The defining feature of modular PKSs is their assembly line-like organization. These linearly organized megasynthases are divided into units called modules, each of which is responsible for fabricating a particular chemical feature in the finished polyketide product. Each module is further subdivided into domains with defined structures and functions that are responsible for particular aspects of the chemical features produced by their parent module.\(^2\) Returning to the assembly line metaphor, each module is a workstation on an assembly line and each domain is a worker at that workstation. They perform defined tasks in a defined order to contribute toward the completion of a product. Once all the domains have performed their task, the partially complete piece is passed to the next station for further work and a new piece is accepted to begin the cycle anew.
Figure 1.2: Schematic representation of 6-Deoxyerythronolide-B synthase.

Peptides and modules are denoted by black lines. Individual domains are denoted by colored regions. Structures of polyketide intermediates produced by a module are shown.

1. 6-Deoxyerythronolide-B Synthase

The best studied modular PKS is 6-deoxyerythronolide B synthase (DEBS) (Figure 1.2) produced by the soil bacterium Saccharopolyspora erythraea which synthesizes 6-deoxyerythronolide B (6-DEB) the macrolide precursor of the antibiotic erythromycin$^{3-5}$ and has been used as the archetypal model of modular polyketide biosynthesis. The genes encoding DEBS have been cloned and expressed in heterologous hosts, greatly simplifying investigations.$^6$ The organization of DEBS was determined through limited proteolysis studies that revealed well defined boundaries between modules. Upon further proteolytic cleavage sequenceable domains are released, which give rise to the standalone functional units that can then be further studied. Through this approach it was determined that DEBS is composed of ketosynthase-
acyltransferase (KS-AT), dehydratase (DH), enoyl reductase (ER), ketoreductase (KR), acyl carrier (ACP), and thioesterase (TE) domains.

DEBS spans 3 peptides and is composed of six modules. DEBS1 includes modules 1 and 2, DEBS2 includes modules 3 and 4, and DEBS3 includes modules 5 and 6. Modules 1, 2, 3, 5, and 6 contain KS, AT, and ACP domains as well as KR domains, but in the case of module 3 the KR is inactive. Additionally, module 1 includes a loading didomain (LDD) composed of an AT and ACP domain, and module 6 contains a TE domain. Module 4 contains KR, AT, ACP, and KR domains as well as a DH and an ER domain. Most other modular PKSs have the same assembly line organization, but differ in the number of modules, as well as the domains present within a given module.

Figure 1.3: Simplified PKS module showing the steps of chain elongation.

2. Polyketide Biosynthesis

Polyketide biosynthesis by DEBS and other PKSs is initiated by the loading didomain. The AT, accepts onto its active site serine the starter unit which arrives as a coenzyme A (CoA) bound...
acyl group. In the case of DEBS, this is a propionyl group bound to CoA as a thioester. AT \_L then passes the acyl unit to ACP \_L which accepts it as a thioester bound to the terminal thiol of its phosphopantetheine prosthetic group. ACP \_L then passes the acyl unit downstream to the KS of the first module. The first task performed by any module is chain elongation which requires a KS, AT, and ACP domain (Figure 1.3). These domains are found in all PKS modules. The AT of this module selects the extender unit which arrives as a CoA-bound malonate thioester. In the case of DEBS, all extender units are methylmalonyl CoA. AT then passes the malonate to that module’s ACP. Once that module’s KS and ACP are loaded, KS initiates chain elongation by decarboxylating the ACP-bound malonate to produce a nucleophilic species which intercepts the KS-bound acyl unit in a Claisen condensation elongating the polyketide chain by 2 carbons and produces an ACP-bound β-keto thioester with substitution at the α-carbon if the malonate starter unit was α-substituted.\(^2,^3\)

![Diagram of β-Carbon processing](image)

**Figure 1.4:** β-Carbon processing.

Next, the oxidation of the newly fashioned segment may be adjusted depending on which tailoring domains are present within that module (Figure 1.4). If KR is present, the β-keto thioester is reduced to a β-hydroxy thioester. If DH is present, the β-hydroxy thioester is dehydrated to form an α,β-unsaturated thioester, and if ER is present, the α,β-unsaturated thioester is reduced to a product fully saturated between the α and β carbons. Each of these
tailoring domains is responsible for the stereochemistry of its product. After the completion of tailoring, the partially completed polyketide is passed from the ACP to the downstream KS, and the cycle of elongation and tailoring continues until the terminal module. There, after a final round of elongation and processing the ACP then passes the polyketide chain to the TE which frees the completed polyketide from the PKS by mediating macrocyclization in the case of macrolides, or through hydrolysis or other transfer events.\textsuperscript{2,3}

\textbf{C. Traditional Tools for Engineered Biosynthesis of Polyketides}

An interesting implication of the assembly line organization of DEBS and other PKSs is that simpler polyketide structures require greater enzymatic complexity to produce. For instance, to produce a polyketide subunit that is fully saturated would require a module to contain KS, AT, ACP, KR, DH, and ER domains, whereas production of a polyketide subunit containing a ketone only requires a module to contain KS, AT, and ACP domains. This fact has been exploited for biosynthetic engineering purposes to produce novel “unnatural” polyketides by inactivating or “knocking out” the active sites of the β-tailoring domains.\textsuperscript{9-13} Due to the array of biochemical functions that polyketides exhibit, it is desirable to obtain novel polyketides to discover and explore potential uses. Biosynthetic engineering represents an attractive approach to producing novel polyketides because the complicated structures of polyketides makes total synthesis a challenging prospect with lengthy and laborious synthetic pathways which require fine reagents, extensively use protecting groups and chiral auxiliaries, and result in overall low yields.\textsuperscript{14} In contrast, PKSs produce polyketides under mild conditions in aqueous reaction media with high yield and ee and do not require synthetic protecting groups or chiral auxiliaries.

When a module’s ER active site is inactivated or “knocked out”, it is unable to reduce the α,β-unsaturated product to the fully saturated product and the olefin-containing product is
passed to the downstream module and retained in the polyketide product. Similarly, inactivation of DH results in retention of the β-hydroxy functionality, and inactivation of KR results in retention of the β-keto functionality (Figure 1.5).

![Diagram of polyketide biosynthesis](image)

**Figure 1.5:** Products produced when various tailoring domains are knocked out.

However, there is no guarantee that downstream modules will accept the altered product, and this can come at the cost of kinetic efficiency. The knockout approach is also limited by the domains naturally present within a module. No amount of active site alteration would create a module that produces a β-hydroxy, α,β-unsaturated, or fully saturated product from a module that includes only the domains to produce a β-keto product and lacks the domains required for further elaboration. Processing domains also exhibit a natural preference for the stereochemistry of the products they produce which may not be the desired configuration of the novel product. Furthermore, the skeletal features present at the α-site of extender units are incorporated into the finished polyketide, so to alter those features new
extender units must be substituted for the natural substrates. AT, which is responsible for the selection of extender unit, with few exceptions tends to be very selective for its natural substrates and attempts to mutate the selectivity of AT have not been able to completely alter its selectivity.\textsuperscript{19}

To overcome these deficiencies, techniques to insert heterologous domains into modules to impart activity which the parent module lacked, or to swap existing domains for heterologous domains which select different starter units or produce different stereochemistry have been explored with some success.\textsuperscript{20} However, the heterologous domains may introduce instability into the engineered module or have incompatible boundaries which make operation kinetically disfavored. In order to take full advantage of the promises of polyketide biosynthetic engineering, we require tools to study the functions of PKSs and their components.

\section*{D. Traditional Tools for Investigating PKS Function}

The manner in which intermediates flow through and are modified by PKSs has been the subject of much interest. In order to better exploit the activities of PKSs, we must first understand how they function, and numerous approaches have been developed to interrogate various aspects of this biosynthetic machinery both \textit{in vitro} and \textit{in vivo}.

\subsection*{1. Isotopic Incorporation}

Incorporation of stable isotopes into PKS substrates was the first tool used to elucidate PKS functions. Cultures of \textit{S. erythrea} were fed \((2-\mathrm{H_2}, 2-\mathrm{^{13}C})\) propionate and \((2-\mathrm{S})-(2-\mathrm{H_2}, 2-\mathrm{^{13}C})\) methylmalonyl-CoA, and the polyketide product was isolated and analyzed by NMR. This provided valuable insight into the mechanisms of initiation and extension as well as stereochemical effects and supplied evidence that DEBS was behaving in a fashion similar to
fatty acid synthases. Such experiments using intact DEBS in vivo can be used to correlate starting material structures to features in the finished product, but these studies are unable to answer questions regarding protein-protein interactions, substrate recognition, and the actual mechanistic steps in elongation, processing, and intermodule transfer. Furthermore, they are hindered by the inability to isolate intermediates or directly alter reaction conditions, and the high cost of isotopically labeled substrates.

The limited proteolysis studies of DEBS makes it possible to clone standalone modules and functional domains that can be expressed in the heterologous host E. coli for examination under more easily controlled conditions in vitro. Truncated modules were produced by translocation of the TE domain from DEBS3 to the C-terminus of DEBS1 to produce DEBS1+TE or to DEBS2 to produce DEBS2+TE. These truncated modules could perform 2 discrete rounds of elongation and processing before the TE mediate release a polyketide truncated at the triketide stage (Figure 1.6). These truncated PKSs were incubated with radiolabeled substrates to execute chain elongation followed by release of cyclized products. These radiolabeled products were extracted and liquid scintillation counting was used to determine critical rate constants involved in substrate loading and product turnover. TLC coupled with autoradiography or phosphorimaging can also be used to locate and isolate products and key intermediates to identify their structures.

Figure 1.6: Truncated modules to produce triketides.
Standalone PKS components coupled with isotopically labeled substrates have allowed extensive analysis of kinetics, substrate specificities, and stereochemical preferences, but these techniques have their drawbacks. The vanishingly small quantities of products produced \textit{in vitro} makes isolation and analysis difficult and impractical for the large scale production of polyketides. Furthermore, the high cost of stable isotope incorporating substrates, and the inherent handling difficulties of radioactive substrates have limited broad implementation of these techniques for the exploration of polyketide biosynthesis to all but the most well equipped labs.

\section*{2. Mass Spectrometry}

Mass spectrometry (MS) has emerged as a powerful technique for analyzing biomacromolecules, and has been employed to study PKSs and polyketide biosynthesis. As polyketide intermediates flow through PKSs, they are covalently attached to different domains at different times, and their formula is altered as elongation and processing occurs. Each of these changes is concurrent with a change in molecular mass. MS is used to monitor these mass changes to study their attendant processes giving insight into substrate tolerances, the timing of elongation and tailoring reactions, and the timing of transfers between domains and modules.

MS coupled with limited proteolysis can identify domains present within a given module, and determine which substrates are tethered to which sites at a given time. This approach has been used to study the substrate specificities for individual AT, ACP, and KS domains within DEBS. A construct of module 1 of DEBS containing the $N$-terminal LDD and a $C$-terminal TE was incubated with propionyl-CoA, followed by limited trypsinolysis to separate components for MS analysis. This showed that propionyl-CoA was a suitable substrate for the AT, which was able to accept propionate as an initiating unit, but unsuitable for the KS, AT, or ACP of
module 1 and was therefore incapable of acting as an extender unit in 6-DEB biosynthesis. Similar experiments were also carried out using butyryl-CoA which, which has a similar mass, and MS was able to distinguish domains loaded with propionate from those loaded with butyrate.

MS coupled with complete proteolysis can also identify the specific site of attachment of a PKS substrate or intermediate within a domain, as well as to gain insight into the chemical structure of an intermediate. Standalone KS-AT and ACP domains from DEBS module 3 were expressed in *E. coli* and were incubated in various di- and triketide substrates, followed by proteolysis. MS was used to evaluate at various time points the proportion of native peptides to peptides covalently modified with substrates. This allowed rates of substrate uptake to be measured and substrate preferences to be determined. Furthermore, when methylmalonyl-ACP was included, the ketide-loaded KS was able to catalyze chain elongation. Proteolysis followed by MS was used to determine the chemical structures intermediates in the process, and comparison of the intensities of peptides and peptide-substrate complexes revealed the comparative rates of substrate uptake and polyketide elongation.

MS has several advantages over isotopic incorporation as a means of studying polyketide biosynthesis. Since the masses of peptides change predictably when substrates are covalently appended they do not require the use of exotic or radioactive isotopes to indicate incorporation. MS can also provide information about specific sites of attachment by assessment of which peptides change mass, and information about the chemical structure of the species affixed to that peptide because elongation and reduction change the product mass in predictable ways. However, MS does have its drawbacks. Due to the times required for proteolysis and LC-MS analysis, MS is not a particularly high throughput method. The reason MS
is coupled with proteolysis or used for the analysis of standalone domains is because full DEBS modules weigh approximately 300 kDa and mass spectrometers are unable to resolve the change in caused by incorporation of relatively small substrates. Incorporation of highly hydrophobic substrates can cause proteolytic products to precipitate, rendering them unsuitable for MS analysis. MS analysis is also highly dependent on the ionizability of the species of interest, and not all proteolytic fragments are amenable to this process. Furthermore, the process of chemical ionization can cause chemical changes in peptides or their attached substrates which can confound results.

**E. Traditional Tools for Loading and Manipulating PKS Components**

![Diagram of KS active site acylation](image)

*Figure 1.7*: Propionyl CoA vs. propionyl SNAc for acylation of KS active site.

In order to study the flow of intermediates through PKSs, we require tools for injecting substrates into their active sites. KS active site cysteines naturally accept acyl group from the PPant arm of ACPs. Early attempts to load KSs emulated this process by using CoA-bound thioesters as a source of simple acyl groups. However, a limited array of acyl-CoAs are available commercially and they are very expensive, whereas synthesizing acylated CoA is a laborious process. A revolution in PKS loading was brought about by use of simpler thioesters based on $N$-acetylcysteamine (SNAc) which resembles the thiol-containing terminus of CoA and can be recognized by KS which will accept acyl cargo from it (Figure 1.7), but to realize convenient reaction times, large excesses of SNAc thioesters are required. Thioesters based on
thiophenol have been shown to be more reactive, but the instability and difficult handling characteristics of S-aryl thioesters have ensured that SNAc thioesters remain the state of the art for the acylation of KS active sites.

The KS active site is not the only site which must be acylated for polyketide biosynthesis to proceed. The PPant thiol of ACP must also be loaded with either an acyl- or malonylthioester for elongation and further processing to occur, and the nature of the group loaded onto the ACP thiol directly influences the substitution at the α-site of the polyketide product. Traditionally, ACP acylation has been accomplished by using the natural activity of AT domains which have evolved to acylated ACPs, but ATs have also evolved to be very selective for their natural substrates. While the traditional picture of AT as an inflexible enzyme is being revised, loading ACPs with novel cargo remains a nontrivial process. An important advance in loading ACPs with novel cargo has come about by using the phosphopantetheinyl transferase Sfp from B. subtilis to load prefunctionalized PPant groups directly onto ACPs, but the functionality tolerated by this approach is mainly limited to thioethers which cannot participate in the natural functions of PKSs.

In order for the study of PKSs to advance, it would be convenient to have access to new tools for modifying the active portions of both KS and ACP domains, in standalone modules and intact PKS modules with novel acyl groups. These techniques should also interface well with the traditional techniques which have won many advances in our understanding of PKS structure and function, but circumvent some of their shortcomings. To this end, we have synthesized a series of reactive probes to covalently modify specific sites of PKSs with flexible functionality and explored their ability to react with and manipulate PKS components as a means of augmenting traditional methods of studying PKSs.
F. References


CHAPTER II

DIRECT AND SELECTIVE ACYLATION OF HOLO-ACYL CARRIER PROTEINS WITH β-LACTONES

A. Introduction

ACP s are a key domain in PKS modules which along with KS and AT are required for polyketide chain extension. They are small peptides, only about 10 kDa and possess no known catalytic activity, but are critical because they serve as the primary site of attachment of polyketide intermediates to the PKS during elongation and processing. To do so, they must be capable of presenting intermediates to up to five domains within their own module (Figure 2.1), as well as to the downstream KS or TE domain for further processing. This requires a great deal of flexibility.

![Figure 2.1: Representation of the domains which an ACP may interact with. KS₀, AT, KR, DH, and ER are found within the ACP’s host module. KSₙ₊₁ is found within the downstream module.](image)

The flexibility of ACPs stems in part from their phosphopantetheine prosthetic group which is approximately 20Å long and contains a terminal thiol which holds polyketide intermediates via thioester bonds. Standalone ACPs or modules incorporating ACPs are initially
synthesized by the ribosome in their apo-form which lacks the PPant group and is inactive in PKS biosynthesis. They are converted to their active holo-form by phosphopantetheinyl transferases which transfer PPant from CoA to a serine found in a specific peptide sequence.\(^1\) Apo to holo conversion is normally performed *in vivo* by the PKS’s host organism but since these host organisms tend to be inconvenient to work with most ACPs for research are expressed in *E. coli* which lacks the necessary enzymes to convert non-native ACPs.

![Figure 2.2: apo-ACPs are converted to holo-ACPs by the enzyme Sfp which transfers a PPant group from CoA to a specific site on the ACP’s surface.](image)

The study of PKSs was greatly advanced by the discovery of the promiscuous PPant transferase *Sfp* from *B. subtilis* which is capable of converting a wide variety of ACPs\(^2\) (Figure 2.2). *Sfp* can function both *in vitro* and *in vivo*, and a recombinant strain of *E. coli* called BAP-1 has been developed which naturally expresses this enzyme. Therefore, ACPs or PKS modules expressed in BAP-1 are isolated in their holo form, and those expressed in standard organisms such as BL-21 are isolated in their apo forms. *Sfp* will tolerate functionality on the terminal thiol of the PPant moiety which it transfers to ACPs. This has been used to create modified phosphopantetheinylated peptides which have found numerous uses in biochemistry and chemical biology.\(^3,4,5\) However, the nature of the attachment point to PPant in these experiments has been limited largely to thioethers, and requires laborious chemical synthesis of the modified CoA substrate.\(^6\)
**B. Hypothesis**

ACPs acylated with polyketide-like functionality could be useful tools to study PKS function, and at the start of this work there existed no way to load a *holo*-ACP in such a way without the use of AT domains which are selective in the substrates which they will accept. We hypothesized that it may be possible to use a reactive small molecule probe to directly load the thiol of *holo*-ACPs with polyketide-like functionality which may include varied and novel structures, and which may be further processed by PKSs.

![Obafluorin](image1.png)

*Obafluorin*
*Antibiotic*
*Pseudomonas fluorescens*

![Ebelactone A](image2.png)

*Ebelactone A*
*Lipase inhibitor*
*Streptomyces aburaviensis*

![Orlistat](image3.png)

*Orlistat*
*Lipase inhibitor*
*FDA approved*

**Figure 2.3:** β-Lactone Containing Natural Products and Drugs.

The probes chosen to investigate this process are based on the oxetan-2-one (β-lactone) skeleton. Several aspects of β-lactones indicate that they would be amenable to directly loading *holo*-ACPs with polyketide-like functionality. Strain relieved by opening of their 4-membered ring renders β-lactones good electrophilic partners toward nucleophilic species, but they possess biocompatible reactivity as demonstrated by their presence in natural products and drugs (Figure 2.3). β-Lactones may react with nucleophiles through acylation or alkylation.
pathways (Figure 2.4) but β-lactone containing structures are known to react with nucleophilic protein residues through the acylation pathway. Furthermore, the exposed nature of PPant thiols indicates that they may be more reactive than other thiols found within PKSs which are located at more restricted sites (Figure 2.5). Therefore, if β-lactones can react with the PPant thiol of ACPs via the acylation pathway, they could directly load them with β-hydroxy thioesters (Figure 2.6), a motif commonly found within polyketide intermediates.

![Figure 2.4: Reaction of a β-lactone with sulfur-nucleophiles.](image)

A) Acylation of a thiol nucleophile by a β-lactone. B) Alkylation of a thiol nucleophile by a β-lactone

![Figure 2.5: Representative scheme showing exposed holo-ACP thiol and shielded KS thiol.](image)

Figure 2.4: Reaction of a β-lactone with sulfur-nucleophiles.

Figure 2.5: Representative scheme showing exposed holo-ACP thiol and shielded KS thiol.
Figure 2.6: Acylation of holo-ACP thiol by a β-lactone-based reagent forming a polyketide-like product.

C. Results and Discussion

Figure 2.7: β-lactone panel (1 – 5) and Sulforhodamine-azide 6.

The primary question is whether or not β-lactones can acylate the PPant thiol of holo-ACPs. However, β-lactones have also been shown to be reactive toward KS active site cysteine thiols.¹⁰ The goal of these experiments is to find conditions under which β-lactones selectively acylate the PPant thiol of holo ACPS in vitro, but leave competing nucleophilic sites in other PKS components unmodified. To ascertain how β-lactones react with PKS components, a small panel of β-lactone containing reagents was prepared (Figure 2.7). These reagents were then incubated with apo- and holo-ACPs from DEBS modules 2 and 3 expressed in BL-21 and BAP-1
respectively, and with the KS-AT didomain from DEBS module 6. After incubation, the products are subjected to tryptic digestion and analysis by LC-MS. By comparing the intensities of β-lactone modified peptides to unmodified peptides, the site and extent of modification can be determined\(^1\) (Figure 2.8).

**Figure 2.8:** Representative scheme for reacting proteins with β-lactones, followed by trypsinolysis and LC-MS analysis to determine the site and extent of loading.

**Figure 2.9:** Saturation of ACPs and KSAT with lactone-based reagents.

Saturation curves produced from the reaction of *holo*-ACP 2 (blue) and 3 (yellow) and from and KSAT6 (red) of DEBS with compound 1, followed by trypsinolysis and LC-MS analysis. Lines added for clarity, each point is the average of 3 experiments, standard deviation shown as error bars.
First a saturation curve was prepared by incubating ACPs and KS-AT with increasing concentrations 1 at 5, 10, 25, 50, and 75 equivalents of β-lactone with respect to protein, followed by trypsinolysis and LC-MS analysis. The goal of this experiment was to determine the relative rates of loading of sites of interest within these three species. The site examined in ACPs is the phosphopantetheinylatable DSL containing peptide, and in KS-AT was the peptide derived from the KS active site. These experiments indicated that 1 is unreactive toward apo-ACPs (no modification was observed). Because the only difference between apo- and holo-ACP is the presence of the PPant group, this provides evidence that the PPant thiol is the site of modification. This curve also shows that holo-ACPs are more reactive toward β-lactones than the active site thiol of KS. At 10 equivalents of 1, holo-ACPs are approximately 50% modified but at the same proportion of β-lactone, the KS active site peptide is only 7% loaded, indicating that β-lactones are in fact selective reagents for modifying the PPant thiol of holo-ACPs with respect to the active site thiol of KS.\textsuperscript{11} The rest of the panel was tested at 10 and 50 equivalents of β-lactone against holo-ACPs and KS-AT, and interesting trends emerged (Table 2.1).

<table>
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<tr>
<th>Compound</th>
<th>Lactone equivalents</th>
<th>ACP2</th>
<th>ACP3</th>
<th>KSAT6</th>
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\textbf{Table 2.1:} Compounds and equivalents vs. loading of the PPant-containing peptide of ACPs and the active site peptide of KS-AT
The lactones 1 - 3 substituted only at the β-position were quite reactive toward both ACPs, whereas the disubstituted lactone 5 shows little reactivity toward ACPs, even at 50 equivalents. 2 is the most reactive of all lactones tested, and as the length of the β-substituent is increased, reactivity decreases, possibly due to steric or hydrophobic effects. Furthermore, ACP3 tends to be more reactive than ACP2, which seems to indicate that ACPs from later modules in DEBS are more nucleophilic which may favor with the forward progression of intermediates through the PKS machinery. ACPs are more reactive toward non α-substituted 1-3 than α-substituted 5, but the situation is reversed in KS. This indicates that the substitution pattern of the substrates plays a subtle yet important role in reactivity. Finally, nitrophenyl substituted 4 underperformed all other lactones as an electrophilic partner with both ACPs and KS-AT, indicating that not all functionality is well tolerated by PKS components, and that further experiments should be carried out to shed light upon PKS’s tolerance for highly unnatural substrates which previously could not be investigated.

![Figure 2.10](image)

**Figure 2.10:** Global reporter scheme.

A) When thiol-containing peptides react with 1, an alkyne handle is presented which can be appended with a chromophore producing a fluorescent peptide. B) If the peptide fails to react with 1, the chromophore cannot be appended, resulting in a nonfluorescent peptide.

LC-MS is a valuable tool for measuring the extent of loading of specific sites of ACPs and KSs, but under the LC-MS conditions employed not all tryptic products are detectable. It is possible that there could be alternate sites within PKSs which react with β-lactones that this
method does not reveal. In order to rule out off-target reactivity, a method is required which reports if any site at all in these proteins reacts with a β-lactone. The method chosen for global reporting depends on the alkyne-substituted β-lactone \( \text{1} \). Upon reaction with any proteinaceous nucleophile, an alkynyl handle would be covalently attached to the protein which could be appended with a fluorescent reporter via the copper catalyzed (3+2) azide-alkyne “click” reaction.\(^{12}\)

![Figure 2.11: PAGE analysis of global reporter experiments.](image)

Reaction with compound \( \text{1} \) and subsequent click reaction with \( \text{6} \). Lanes are marked above with the corresponding protein component. Markers to the left indicate expected locations of the indicated species. The right two lanes depict transfer of the acylation product from preloaded ACP to KSAT. ACP2 contains a C-terminal linker region accounting for its larger mass.

This global reporter is employed by incubating \( \text{apo} \)- and \( \text{holo} \)-ACPs as well as KS-AT with \( \text{1} \), followed by addition of azide-functionalized \( \text{6} \) in the presence of a copper(I) catalyst. Those reaction products are separated by SDS-PAGE, and analyzed for fluorescence (Figure 2.10). Proteins which react with \( \text{1} \) at any site are rendered fluorescent and detectable by this method.
The results of the global reporter assay (Figure 2.11) support the results of the LC-MS analysis. If there is a second site of attachment in ACPs, both apo- and holo-ACPs would produce luminous bands, but only holo ACPs produce luminous bands. The only difference between apo- and holo-ACPs is the presence of the PPant group which indicates that the PPant thiol is the sole point of attachment and that no second reactive site is present. Also mirroring the LC-MS saturation results is the fact that holo-ACPs produce strongly luminous bands at 10 equivalents of 1 indicating extensive modification, whereas KS-AT is dark at 10 equivalents, indicating relatively little modification. However, KS-AT does produce a strongly luminous band at 50 equivalents of 1, indicating that much higher proportions of β-lactone are required for loading and that β-lactone are in fact selective reagents for loading holo ACPs\textsuperscript{11}.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure212.png}
\caption{Schematic representation of acyl product transfer from ACP PPant to KS active site.}
\end{figure}

The chemical nature of the reaction product of β-lactones and holo-ACPs was not clear at the onset of experiments. β-lactones are capable of reacting with nucleophiles via acylation pathways which in the case of sulfur nucleophiles would produce polyketide-like β-hydroxy thioesters, or via alkylation which would produce unpolyketide-like thioethers. ACPs are known to naturally transfer acyl units from their PPant thiols to active site cysteines of KS domains and this process would be unlikely to occur with thioethers. When holo-ACPs reacted with 1 and
fluorescently tagged with 6 are incubated with KS-AT and subjected to SDS-PAGE, it is apparent that the fluorescent tag is transferred to the KS-AT, indicating that the reaction product is in fact a polyketide-like thioester (Figures 2.11, 2.12).

**Figure 2.13**: Holo- and apo-SpnB subjected to the global reporter scheme and analyzed by PAGE.

The global reporter method was also applied to an intact PKS module to determine the overall selectivity of β-lactones for holo-ACPs. SpnB is a standalone PKS module from spinosyn synthase. It contains the full complement of PKS elongation and processing domains, weighs approximately 200 kDa, and includes numerous nucleophilic sites in addition to the KS active site. Because it contains an ACP domain, it can be expressed in BAP-1 to produce holo-SpnB, or in BL-21 to produce apo-SpnB. When apo- and holo-SpnB were incubated with 1 and tagged with 6, then subjected to SDS-PAGE, it is clear that only holo-SpnB produces luminous bands and therefore β-lactones are selective for the PPant thiol of holo-ACP domains over all other competing sites within a PKS module (Figure 2.13). Considering the results of the saturation curve, fluorescence transfer experiments, and SpnB labeling together, it can be stated that β-
lactones are able to directly and selectively acylate the phosphopantetheine thiol of holo-ACPs\textsuperscript{11}.

The selective acylation of holo-ACPs by $\beta$-lactones could be used as a tool to study PKS functions. Acylated ACPs are able to transfer acyl units to the active sites of KS domains, but the inherent limits on what ACPs can naturally be loaded with has limited the study of this process. $\beta$-Lactones with novel and unnatural functionality can be used to directly load holo-ACPs to study the substrate tolerances of this transfer and shed light on the scope of the functional flexibility of the transfer of acyl units between PKS components to determine how alterations in substrate electronics, steric, hydrophobics, and stereochemistry affect this process. Furthermore, the ACP within a PKS module can be selectively modified with novel and unnatural acyl units to study the tolerance and functional flexibility of DH and ER domains. Because $\beta$-lactones are selective for the ACP, they could be added in excess to a PKS construct containing a TE domain to promote multiple turnovers, amplifying the amount of product produced making structural analysis a more feasible process (Figure 2.14). The knowledge of how PKSs handle novel substrates which $\beta$-lactones can provide could inform future efforts to utilize PKSs for engineered biosynthesis by revealing which processes are inefficient or inflexible and would require additional work to optimize. With such knowledge in hand, the engineered biosynthesis of polyketides could be rendered more facile and provide unprecedented access to libraries of novel polyketide-like structures to be tested for pharmacological activity without the need to rely on complicated chemical syntheses.

If a substrate is tolerated by the $\beta$-processing domains, it will produce a processed product. If it is not tolerated by the $\beta$-processing domains, it will simply produce a ring-opened
product. TE allows for multiple turnovers by freeing the ACP thiol for additional loadings with β-lactones, amplifying the amount of products produced.

\[
\text{(R,S)} \quad \text{(S,R)}
\]

**Figure 2.14**: Using β-lactone based reagents to study substrate tolerance of a putative PKS module.

**D. Experimental Details**

Details of critical experiments are included below.

**1. Chemical Syntheses**

Synthetic schemes and details for critical compounds are included below.

**a. 5-Hexynal**

\[
\text{HO} \quad \text{Oxallyl chloride, DMSO} \quad \text{DCM, -78 °C}
\]

**Fig. 2.15**: Synthesis of 5-hexynal.

In a flamed dried RBF with PTFE-coated stir bar, septum, and an argon inlet, oxalyl chloride (1.10 mL, 13.0 mmol) in 20 mL of anhydrous dichloromethane (DCM) is cooled to -78°C in a dry-ice/acetone bath. DMSO (0.92 mL, 13.0 mmol) with 10% v/v DCM is added dropwise via
syringe. After gas evolution ceases, the mixture is stirred for five minutes, then 5-hexyn-1-ol (0.57 mL, 5.20 mmol) is added via syringe and stirred for a further five minutes. Triethylamine (3.63 mL, 26.01 mmol) is added via syringe and the mixture stirred for an additional 15 minutes then warmed to RT. The product is washed with 0.5 M HCl and ½ saturated NaHCO₃, then dried over MgSO₄. The product is purified by flash chromatography using silica gel as the stationary phase with 9:1 Hexanes:EtOAc as the mobile phase.

**5-hexynal:** 0.4200 g (4.37 mmol, 84%), colorless oil.

$^1$H NMR (400 MHz, CDCl₃) δ: 9.811 (s, 1H), 2.619 (t, J = 7.05 Hz, 2H), 2.278 (dt, J = 6.70 Hz, 2.45 Hz, 2H), 1.999 (t, J = 2.38 Hz, 1H), 1.858 (quint J = 7.00 Hz, 2H)

$^{13}$C NMR (100 MHz, CDCl₃) δ: 201.719, 83.188, 69.386, 42.530, 20.809, 17.772

**b. Synthesis of β-Lactones from Aldehydes**

Procedure 1: aluminum-catalyzed synthesis of β-lactones from aldehydes and acid chlorides:¹⁴ In a flamed dried RBF with PTFE-coated stir bar, septum, and an argon inlet, a suspension of anhydrous AlCl₃ (0.1 eq) and AgSbF₆ (0.3 eq) in DCM is cooled to -60°C in an ethanol/ethylene glycol-dry ice bath. Next, in order, added by syringe: N,N'-diisopropylethylamine (DIEA) (1.5 eq), acid chloride (1.5 eq) and aldehyde (1 eq). The mixture is stirred for five hours at -60°C then warmed to RT and filtered through a plug of silica gel. Then product is purified by flash chromatography using silica gel as the stationary phase with hexanes:ethyl acetate mobile phase.

Procedure 2: Nucleophile-catalyzed production of β-lactones from aldehydes and acid Chlorides:¹⁵ For some reactions 1,4-diazabicyclo[2.2.2]octane (DABCO) is substituted for the quinine-based catalyst.¹¹
In a flame-dried RBF with PTFE-coated stir bar, septum, and an argon inlet, a solution of anhydrous LiClO₄ (1.0 eq) and trimethylsilyl quinine¹⁶ (TMSq) (0.1 eq) or DABCO (0.2 eq) is prepared in 3:1 v:v DCM:Et₂O, and cooled to -78°C in a dry ice-acetone bath. DIEA (3.0 eq), and aldehyde (1.0 eq) are added by syringe, then acid chloride (1 - 2 eq diluted in DCM) is added dropwise over ca. 3 hours. The mixture is stirred for 8 hrs at -78°C then diluted with an equal volume of ether and allowed to warm to room temperature. The mixture is filtered through a silica gel plug then purified by flash chromatography using silica gel as the stationary phase with hexanes:ethyl acetate mobile phase.

i. 4-(Pent-4-yn-1-yl)oxetan-2-one (1)

![Chemical structure of 4-(Pent-4-yn-1-yl)oxetan-2-one (1)](image)

**Fig. 2.16:** Synthesis of 1.

*Procedure 1:* AlCl₃ (0.0057 g, 0.0427 mmol), AgSbF₆ (0.0440 g, 0.1281 mmol), acetyl chloride (0.0469 g, 0.5976 mmol), DIEA (0.11 mL 0.67 mmol), 5-hexynal (0.0413 g, 0.4300 mmol).

1: 0.0330 g (0.2389 mmol, 56%), pale yellow oil.

¹H NMR (400 MHz, CDCl₃) δ: 4.567 – 4.539 (m, 1H), 3.529 (dd, J = 16.76 Hz, 1.76 Hz, 1H), 3.129 (dd, J = 16.29 Hz, 1.67 Hz, 1H), 2.286 (dt, J = 6.61 Hz, 1.46 Hz, 2H), 2.001 (t, J = 2.4, 1H), 1.720 – 1.644 (m, 4H).

¹³C NMR (100 MHz, CDCl₃) δ: 168.101, 83.236, 70.785, 69.355, 43.029, 33.590, 23.867, 17.982.

Hi-res FAB MS calculated m/z for C₈H₁₁O₂ [m+H]⁺ = 139.075356. Observed m/z = 139.0766.

ii. 4-Propyloxetan-2-one (3)
Fig. 2.17: Synthesis of 3.

Procedure 1: AlCl₃ (0.1320 g, 0.99 mmol), AgSbF₆ (0.9965 g, 2.9 mmol), acetyl chloride (0.78 mL, 11 mmol), DIEA (1.89 mL, 11), butanal (0.88 mL, 9.8 mmol).

3: 0.9490 g (8.134 mmol, 83%), colorless oil.

¹H NMR (400 MHz, CDCl₃) δ: 4.530 (dt, J = 2.96 Hz, 5.76 Hz, 1H), 3.522 (dd, J = 16.31 Hz, 5.78 Hz, 1H), 3.067 (dd, J = 16.29 Hz, 4.28 Hz), 1.1.871 – 1.838 (m, 1H), 1.833 – 1.745 (m, 1H), 1.56 – 1.36 (m, 1H), 0.986 (t, J = 7.40 Hz)

¹³C NMR (100 MHz, CDCl₃) δ: 166.477, 69.238, 41.036, 34.826, 16.429, 11.802

iii. 4-(4-Nitrophenyl)oxetan-2-one (4)

Fig. 2.18: Synthesis of 4.

Procedure 2: DABCO (0.0673 g, 0.6 mmol), DIEA (1.55 mL, 9 mmol), LiClO₄ (0.3192 g, 3 mmol), p-nitrobenzaldehyde (0.4534 g, 3.0 mmol), acetyl chloride (0.21 mL, 3.0 mmol).

4: 0.2608 g (1.35 mmol, 45%), pale yellow oil.

¹H NMR (400 MHz, CDCl₃) δ: 8.30 (d, J = 8.88, 2H), 7.60 (d, J = 8.46 Hz, 2H), 5.63 (dd, J = 6.3 Hz, J = 4.5 Hz, 1H), 4.04 (dd, J = 16.4, J= 6.3, 1H), 3.44 (dd, J = 16.5, J = 4.5, 1H)

¹³C NMR (100 MHz, CDCl₃) δ: 166.51, 148.42, 144.40, 126.50, 124.36, 69.51, 46.90

iv. 3-Methyl-4-propyloxetan-2-one (5)
Fig 2.19: Synthesis of 5.

Procedure 2: TMSq (.1189 g, 0.3 mmol), DIEA (1.29 mL, 7.5 mmol), LiClO₄ (0.3192 g, 3.0 mmol), butyraldehyde (0.27 mL, 3.0 mmol), propionyl chloride (0.26 mL, 3.0 mmol).

6: 0.2153 g (1.68 mmol, 56%), colorless oil.

¹H NMR (400 MHz, CDCl₃) δ: 4.60 – 4.55 (m, 1H), 3.79 – 3.17 (m, 1H), 1.80 – 1.69 (m, 1H), 1.68 – 1.61 (m, 1H), 1.59 – 1.49 (m, 1H), 1.47 – 1.38 (m, 1H), 1.28 (d, J = 7.8 Hz, 1H), 1.00 (t, J = 7.4 Hz, 1H)

¹³C NMR (100 MHz, CDCl₃) δ: 172.86, 75.56, 47.22, 32.00, 18.79, 13.79, 8.06

c. Sulforhodamine-B Azide (6)

Fig. 2.20: Synthesis of 6.

In an RBF with PTFE-coated stir bar, 3-azido-1-aminopropane¹⁷ (0.220 g, 0.22 mmol) and triethylamine (0.070 mL, 0.5 mmol) are dissolved in 1 mL of 5:1 v:v DCM:DMF and cooled to 0°C in an ice-water bath. Sulforhodamine B sulfonyl chloride (mixture of ortho- and para- sulfonyl chloride isomers) (0.1154 g, 0.20 mmol) is added portion-wise over ca. 30 min and stirred overnight at room temperature. The product is purified by flash chromatography using silica gel as the stationary phase with 90:5:5 DCM : acetonitrile : methanol mobile phase.
The ortho isomer is distinguished by its reversible decolorization in pH 9.0 buffer. Ortho-6 was found to perform best in the click reaction and was utilized for labeling experiments.

Para-6: 0.0218 g (0.0340 mmol, 17%), deep purple-red solid with metallic green luster.

Ortho-6: 0.0179 g (0.0280 mmol, 14%), deep purple-red solid with metallic green luster.

NMR spectra are of poor quality due to long relaxation times. Product is pure by LC-MS.

Hi-res FAB MS calculated m/z for C_{30}H_{37}N_{6}O_{6}S_{2} [M+H]^+ = 641.221049. Observed m/z = 641.2216.

2. Plasmid Construction

pCAD01 is prepared from an *E. coli* optimized synthetic construct (DNA2.0, Menlo Park, CA). The synthetic gene is excised from the shipping vector via flanking NdeI and NotI restriction sites and ligated into pET21b.

3. Protein Expression and Isolation

*SpnB* is expressed from pCAD01. KSAT6 is expressed from pAYC11. ACP2 is expressed from pNW6, ACP3 is expressed from pVYA05. Apo-*SpnB* and ACPs are expressed in *E. coli* BL-21 cells and holo- *SpnB* and ACPs are expressed in *E. coli* BAP-1. pCAD01, pAYC02, and pAYC11 contain ampicillin resistance vectors; pNW6 and pVYA05 contain kanamycin resistance vectors.

General Procedure for Protein Expression and Isolation: Cells are grown in 1 L shake cultures of LB-antibiotic media at 37°C in a New Brunswick Scientific Excella E24 Incubator Shaker until the OD600 is between 0.6 and 0.8 AU. Overexpression is induced with 200 μL of 1 M IPTG (per liter of culture) and carried out at 18°C for 18 hours, after this point all work is carried out at 4°C. Cells are pelleted by spinning at 3000 RPM for 10 minutes in a Sorvall RC6 Plus with a FiberLite F21S-8x50 rotor and resuspended in 50 mL of lysis buffer (20 mM Tris-HCl,
150 mM NaCl, 1 mM Na$_2$EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium Na$_2$VO$_4$, 1 μg/mL leupeptin, pH 7.5). Cells are lysed using a Misonics ultrasonic converter without microtip, amplitude 30 for five 30 second intervals with a 60 second cool down period between each cycle. Cell debris are pelleted at 10,000 rpm and with a FiberLite F1055 6x500 Y rotor for 60 minutes. The lysate is equilibrated with 3 mL of PerfectPro Ni-NTA bead slurry for 60 minutes by stirring with a PTFE-coated stir bar at 60 RPM for 60 minutes. The lysate is then poured into a 15 mL column and the supernatant eluted. The column is then washed with two 15 mL portions of wash buffer (50 mM phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0), and eluted with 3 mL of elution buffer (50 mM phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0). The purified protein is loaded into an Amicon Ultra centrifugal concentrator and diluted to 15 mL with storage buffer (100 mM Tris, 1 mM EDTA, 1 mM dithioerythritol, 10% glycerol pH 8) and spun at 3000 rpm in Eppendorf Centrifuge 5810 R with swinging-bucket rotor. Dilution and filtration is repeated a total of three times. Protein concentration is determined by Bradford assay, average concentration is approximately 500 μM. Proteins re flash frozen in liquid nitrogen and stored at -80°C until use.

4. Loading Proteins with β-Lactones

Loading reactions are performed in Microfuge tubes. Reactions for gel analysis are performed at 20 μL total volume; reactions for LC-MS analysis are performed at 50 μL total volume. Final concentrations are reported in specific procedures. DMSO concentration is maintained below 20%.

Loading ACPs with β-lactones: Phosphate buffer (100 mM, pH 7), tris(2-carboxyethyl)phosphine (2.5 mM), and holo- or apo-ACPs 2 and 3 (0.025 mM) are reacted at ambient temperature for 15 min to reduce thiols. Then a β-Lactone (solution in DMSO) is added.
to the appropriate concentration (1, 5, 10, 20, 50, or 75x with respect to protein for saturation experiments, 10 or 50x with respect to protein for loading experiments) and the mixture is reacted at ambient temperature for 60 min.

Loading KSATs with β-lactones: Phosphate buffer (100 mM, pH 7), tris(2-carboxyethyl)phosphine (2.5 mM), and KSAT 6 (0.025 mM) are reacted at ambient temperature for 15 min to reduce thiols, then β-Lactone (solution in DMSO) is added (1, 5, 10, 20, 50, or 75x with respect to protein for saturation experiments, 10 or 50x with respect to protein for loading experiments) and the mixture is reacted at ambient temperature for 60 min.

Loading SpnB with β-lactones: Phosphate buffer (100 mM, pH 7), tris(2-carboxyethyl)phosphine (2.5 mM), and SpnB (0.015 mM) are reacted at ambient temperature for 15 min to reduce thiols, then β-Lactone (solution in DMSO) is added (10, 50, or 75x with respect to protein) and the mixture incubated at ambient temperature for 60 min.

5. Proteolysis

Promega Sequencing Grade Modified Trypsin is added to prepared protein samples so that the final trypsin:protein ratio is 1:50 (w/w). The mixture is incubated at 30°C for 18 hours. Digestion is quenched by addition of an equal volume of 10% formic acid. Digests are flash frozen in liquid nitrogen and stored at -20°C until analysis.

6. LC-MS

Separation is performed with a Waters 1525 system. The mobile phase gradient employed is A = water + 0.1% formic acid, B = acetonitrile + 0.1% formic acid, 5-95% B over 60 min the column is a Vydac 218TP C18 5u column (4.6 x 150 mm). Mass spectra are acquired with a Waters Micromass ZQ mass detector in El+ mode: Capillary voltage = 3.50 kV, cone
voltage = 30 V, extractor = 3 V, RF lens = 0.0 V, source temp = 100°C, desolvation temp = 200°C, desolvation gas = 300 L/hr, desolvation gas = 0.0 L/hr The system is operated and spectra are processed using the Waters Empower software suite.

7. Chromophore Attachment

The reaction is carried out at 25 μL total volume, final concentrations reported. 6, (2x alkyne concentration), sodium ascorbate (1 mM), and copper(II) sulfate (1 mM) are added to samples which are labeled with 1. The reaction is performed at ambient temperature for 60 minutes.

8. Acyl Transfer

Phosphate buffer (100 mM, pH 7), tris(2-carboxyethyl)phosphine (2.5 mM), and holo-ACP 2 or 3 (0.025 mM) are reacted at ambient temperature for 15 min to reduce thiols. Then 10 equivalents of β-Lactone (solution in DMSO) is added and the mixture reacted at ambient temperature for 60 min.

Phosphate buffer (100 mM, pH 7), tris(2-carboxyethyl)phosphine (2.5 mM), and KSAT 6 (0.025 mM) are reacted at ambient temperature for 15 min to reduce thiols.

The two samples are combined and allowed to react at room temperature for 60 min.

9. In Gel Fluorescence Assay

Labeled samples are diluted to 35 μL with gel-loading buffer. ACPs and KSATs are separated by 12.5% SDS-PAGE with 5% stacking gel (100 V, 50 mA, 135 min). SpnB is separated by 4 – 20% gradient HEPES-PAGE (100 V, 50 mA, 90 min). Gels are developed in 10% acetic acid
to visualize 6 and imaged on a BioDoc-It Imaging System with UV transilluminator. Total protein is stained using GelCode Blue stain.

E. References


CHAPTER III

β-LACTAMS FOR SELECTIVE LOADING AND PURIFICATION OF HOLO ACPS, AND DIRECT LOADING OF ACPS WITH MALONATE

A. Introduction

β-Lactones are competent reagents for the selective acylation of the PPant thiols holo-ACPs, but a large excess of reagent is required to achieve a high degree of saturation,\(^1\) and it would be convenient to have procedures which feature shorter reaction times or use smaller quantities of these precious electrophiles which in most cases must be specially prepared. Given the inherent difficulties in altering the electronics of β-lactones, we sought alternate probes which might provide increased activity, yet still acylate protein thiols with minimal off-target reactions. Azetadín-2-ones (β-Lactams), like β-lactones, are strained electrophiles and are a known structural feature of drugs and natural products which acylate thiol nucleophiles with a high degree of activity.\(^2\) Unlike the heterocyclic oxygen of β-lactones, the heterocyclic nitrogen β-lactams can be modified with a variety of functional groups which modulate their reactivity. Electron-donating substituents decrease the electrophilic character of β-lactones, and electron-withdrawing substituents increase it (Figure 3.1). To investigate the reactions of PKSs components, a panel of N-substituted β-lactones was prepared by using common protecting groups which provide a cross section of electronics, sterics, and hydrophobics. The substitution of β-lactam based reagents can also be further modified to produce reagents which directly load holo-ACPs with substrates which differ in the oxidation of their β-sites.
A concern was that the increased reactivity of β-lactams might come at the cost of reduced selectivity for the PPant thiol over other thiol nucleophiles present within PKSs such as the KS active site. To avoid this competition altogether, standalone domains can be used in loading experiments because the ACPs from DEBS contain no thiols other than the PPant thiol of holo-ACPs. Since standalone domains tend to retain their functions, ACPs modified by β-lactams could then be combined with other PKS components to study their behavior.

B. Results and Discussion

Figure 3.2 Lactone panel (7 – 12) and azide-functionalized biotin 13.
Saturation curves produced from the reaction of holo-ACPs 2 (A) and 3 (B) of DEBS with compounds 8 - 12, followed by trypsinolysis and LC-MS analysis. Lines added for clarity, each point is the average of 3 experiments, standard deviation shown as error bars.

To determine the effects of electronics, sterics, and hydrophobics on the relative reactivity of β-lactam-based reagents, the β-lactam panel (Figure 3.2) was used to create saturation curves by reacting increasing equivalents of reagent with holo-ACPs 2 and 3 from DEBS. The reaction product was subjected to tryptic digestion followed by LC-MS analysis and the ratio of modified to unmodified phosphopanteheinylated peptide was used to determine the extent of loading (Figure 3.3). The results indicate that in general β-lactams show excellent acylation efficiencies, with most compounds producing 50% saturation levels of ACPs at a mere 1 to 2 equivalents. The exception is Boc-functionalized 6, but the lowered loading efficiency may be an artifact due to degradation of the Boc group under the ionization conditions used. The curves also indicate that the reactivity of the β-lactams is largely insensitive toward the functionality on the heterocyclic nitrogen, so long as it is electron-withdrawing in nature.
Unfortunately, the activated $\beta$-lactams were also highly active toward the KS active site (Figure 3.4), and selectivity between KS and holo-ACPs was not practical with these reagents.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3_4.png}
\caption{Saturation of KSAT with lactam-based reagents.}
Saturation curves produced from the reaction of KSAT6 of DEBS with compounds 8 - 12, followed by trypsinolysis and LC-MS analysis. Lines added for clarity.
\end{figure}

Though $\beta$-lactam-based reagents are not selective for holo-ACPs with respect to the KS active site, they do exhibit excellent selectivity for holo-ACPs over apo-ACPs as a modification of the global reporter strategy shows (Figure 3.5). When apo- and holo- ACPs are incubated with alkyne-functionalized $\beta$-lactam 12, then reacted with 6 in the presence of a Cu(I) catalyst, and separated by SDS-PAGE, fluorescent bands are observed with holo-ACPs at a mere 5 equivalents of lactone, but apo-ACPs remain dark at all concentrations of lactam tested. This can be attributed to the fact that the apo-ACPs of DEBS lack cysteine residues, but upon conversion to their holo form, they acquire a thiol nucleophile as part of the PPant group.$^3$
Figure 3.5: Global reporter results from ACPs 2 and 3.

The identity of the ACP and the equivalents of 12 are indicated.

1. Purification of Holo-ACPs using a β-Lactam-Based Reagent

Although β-lactam-based reagents are unable to discriminate well between holo-ACPs and the KS active site, they show excellent discrimination between apo- and holo-ACPs, and this can still be a useful tool for the manipulation of PKS components. The majority of ACPs produced for research are converted from their apo- to holo-form using Sfp.5 While this transferase is able to convert a broad range of ACPs, its activity is not perfect. To achieve full conversion in vitro, a large excess of expensive CoA may be required in addition to the extra expression and purification steps needed to produce Sfp. It would seem that the more convenient method of acquiring holo-ACPs would be to express them in BAP-1 which naturally
produces holo-ACPs, but as expression levels rise BAP-1’s endogenous Sfp struggles to match ACP expression levels, and the isolated ACP is often a mixture of apo- and holo-material. However, reagents which are able to discriminate between apo- and holo-ACPs can be used as the basis of a method to separate these two forms of ACP (Figure 3.6).

Figure 3.6: Schematic representation of lactam-based purification scheme.

A mixture of apo and holo-ACPs are exposed to 12, then appended with 13. Streptavidin-coated beads selectively immobilize biotinylated proteins, and hydrazine selectively cleaves thioester bonds.

Alkyne substituted 12 can be used in conjunction with affinity reagents to separate apo- and holo-ACPs. When a mixture of apo- and holo-ACP from DEBS is incubated with 12, it selectively acylates the PPant thiol and in the process presents a terminal alkyne handle. This covalently attached alkyne can be appended with azide-functionalized biotin via the copper-catalyzed (3+2) click reaction, then the reaction product is incubated with streptavidin-coated beads. The selectively biotinylated holo-ACPs are immobilized, and the nonbinding apo-material can be simply washed away with buffer. To elute the purified holo-material, the unique reactivity of thioesters can be taken advantage of. Thioesters are hydrolytically stable, but labile toward nitrogen nucleophiles. The acyl side of the thioester is tethered to the beads and the
thiol side is connected to the ACP, so hydrazinolysis frees the purified holo-material which can be washed away from the beads with buffer. Because there is only 1 thiol in holo-ACPs and none in apo-ACPs, off target attack by 12 would form a linkage which is resistant to hydrazinolysis.

**Figure 3.7:** Intact LC-MS of material before purification procedure (A), and after (B).

Apo- and holo-ACPs are labeled, as is observed m/z. No traces of apo-ACPs remain after purification.

When the purified material is analyzed by LC-MS (*Figure 3.7*), there are no traces of apo-material remaining. Furthermore, the recovered holo-ACPs retain their activity as shown by their ability to accept methylmalonate from methylmalonyl-CoA when incubated in the presence of a KS-AT didomain (*Figure 3.8*).

The overall recovery of this process is approximately 30%. When the concentration of free protein is analyzed at various points in the procedure (*Figure 3.9*), it is apparent that 50% of the material is lost before it is applied to the strep beads while 65% of material applied to the strep beads is recovered. The major loss can be attributed to damage caused by the Cu(I) catalyst, and losses to centrifugal concentrators used to remove excess reagents. For saturation to occur in a timely manner 12 must be used in excess, and for efficient click labeling to occur unreacted 12 must be removed as the click reaction is favored with unbound alkynes and azides.
Furthermore, strep beads preferentially bind free biotin, so unreacted 13 must also be removed before protein is applied to them. It is expected that scale up of the purification procedure would reduce process losses to centrifugal concentrators, but losses due to copper damage may be unavoidable without resorting to Cu-free methods.

**Figure 3.8:** LC-MS analysis purified ACP loaded with methyl malonate followed by tryptic digestion.

**Figure 3.9:** Concentration of free protein during purification procedure as determined by Bradford assay. Concentrations reported in µg/mL.
2. Fluorescence Transfer

The ability to rapidly acylate holo-ACPs can also be used to study the function of PKS components due to the ability of ACPs to transfer acyl units to the active site of KS. In the natural course of polyketide biosynthesis, the KS of a particular PKS module accepts an acyl unit from the upstream ACP. In polyketide biosynthetic engineering, the KS active site may be loaded with novel or unnatural substrates, but the ability of the active site to accept those substrates is not immediately apparent, so experimental methods are required to assess its substrate tolerance. This can be investigated using proteolysis coupled with LC-MS to determine the extent of loading of the KS active site peptide, but LC-MS methods tend to be low throughput. Radiolabeled substrates coupled with radiography or dosimetry can also be used to determine the extent of loading but require a laboratory equipped to handle radioactive isotopes. It would be convenient to have a method for studying the substrate tolerance of KSs which functions in a high throughput manner and which does not rely upon radioactive reagents. β-Lactam-based reagents can be used to bring about these requirements.

![Fluorescence transfer scheme.](image)

**Figure 3.10:** Fluorescence transfer scheme.

When KSAT is preincubated with cerulenin or propionyl-SNAC (14), the KS active site is covalently blocked and cannot receive the fluorescently-functionalized acyl group from the loaded ACP. When the active site is available, it can receive the fluorescently-functionalized group.
The nucleophilic portion of the KS active site is the thiol of a cysteine residue. Sulfur atoms in the -2 oxidation state can only effectively participate in 2 bonds at a time, and one of these bonds is required to link it to the KS backbone. Consequently, if the active site is occupied or ‘blocked’ by an acyl substrate, it would be unable to accept an acyl group from an ACP (Figure 3.10). Therefore, if a portion of a population of KS is loaded with an experimental substrate, the remainder which is unblocked can be loaded with a fluorescently labeled reporter substrate and the degree of fluorescence of the KS can be used to determine the extent of loading of the first substrate; i.e. if the KS is very tolerant of the experimental substrate, it will be blocked to a great extent and will only accept a small amount of fluorescently labeled reporter substrate producing a weakly fluorescent band in a gel, and if it is intolerant of an experimental substrate it will be largely unblocked and accept a large amount of fluorescently labeled reporter substrate producing a strongly fluorescent band in a gel.

Figure 3.11: PAGE analysis of fluorescence transfer from ACP 2 to KSAT 6.

Lanes marked “1” are preincubated with blocking agents 14 or cerulenin for 60 min prior to incubation with fluorescently loaded ACP. Lanes marked “2” are mixed with fluorescently loaded ACP and blocking agent simultaneously. Lane marked “3” is incubated with 12 and 6 pre-reacted in the presence of Cu(I). Bright KSAT bands indicate a great degree of fluorescence transfer due to a low degree of blocking. Dim KSAT bands indicate a low degree of fluorescence transfer due to a great degree of blocking.
To test this hypothesis, KS-AT from DEBS module 6 was preincubated with 14 or cerulenin, compounds known to modify its active site thiol through covalent modification.\textsuperscript{10} Next, the loaded KS-AT was incubated with ACP2 from DEBS which was preloaded with a fluorescent sulforhodamine reporter by first acylating with 12 followed by the click reaction with 6. Control experiments were conducted by omitting propionyl-SNAc/cerulenin, or by preincubating KS-AT with fluorescently-loaded ACP. When analyzed by SDS-PAGE, it is apparent that KS-AT is able to accept the fluorescently linked substrate from ACP, but when blocked with cerulenin or propionyl-SNAc it is unable to accept the fluorescently linked substrate. Addition of propionyl-SNAc/cerulenin after preincubation with the fluorescent reporter did not perturb the fluorescence (Figure 3.11). These experiments demonstrate that when the KS active site is preloaded it is unable to accept a fluorescent reporter from ACP which was installed using a β-lactam-based reagent and that the degree of fluorescence indicates the extent of blocking of the KS active site.

This approach was expanded to determine the substrate tolerance of the KS from DEBS module 6 for a variety of SNAc-thioesters with regard to its preference for acyl group length and sterics.\textsuperscript{11} KS-AT is incubated with SNAc-thioesters of increasing alkyl chain length (Figure 3.12), followed by incubation with fluorescently-linked ACP2 and SDS-PAGE analysis (Figure 3.13). As the length of the alkyl chain of the SNAc thioesters increases, the fluorescence intensity of the bands they produce in the SDS-PAGE gel decreases. This indicates that SNAc-thioesters with longer acyl chains are favored substrates for this domain. This observation can be rationalized because module 6 is the terminal module of DEBS and would bear longer polyketide intermediates. Similar investigations were carried out to investigate the effects of sterics and electronics on the loading of KS 6 with SNAc thioesters. The same information regarding substrate tolerance can be obtained from proteolysis/LC-MS analysis, or through the use of
radiolabeled SNAC-thioesters, but the blocking+fluorescence transfer protocol rapidly provides this information in a high throughput manner without the need of radioactive reagents.

![SNAC thioesters](image)

**Figure 3.12:** SNAC thioesters used to investigate KS active site chain length preference.

![PAGE analysis](image)

**Figure 3.13:** PAGE analysis of blocking+fluorescence transfer protocol with SNAC thioesters 14 – 19.

Lane marked “control” is incubated with fluorescently loaded ACP only. Bright bands indicate a low degree of blocking by experimental thioester; dim bands indicate a large degree of blocking.

3. Direct Loading of *holo*-ACPs with Malonate-like Functionality Using a β-Lactam-Based Reagent

Thus far the β-lactam-based reagents discussed react with *holo*-ACPs to produce β-amino thioesters, but this product structure is not an inherent limitation. The β-site of the lactams can be functionalized with leaving groups in which case the lactam ring acts like a safety catch; while it is intact the nitrogen’s lone pair is heavily involved in resonance with the two electron-withdrawing groups which decrease its ability to displace the leaving group. When the
ring is opened the influence of one of the withdrawing groups is removed which renders the nitrogen more nucleophilic. If it is active enough to displace the leaving group, the product would become a \( \beta \)-imino thioester. The resultant imine could hydrolyze to form a \( \beta \)-carbonyl thioester, a motif commonly seen in polyketide intermediates. Furthermore, if the newly formed carbonyl is an aldehyde, it could be oxidized to form malonate (Figure 3.14).

**Figure 3.14:** Reaction of an ACP with a lactam-based reagent to form malonate.

X indicates a leaving group, R indicates an electron withdrawing group, curved arrows indicate flow of electrons. The structure of 20 is depicted.

**Figure 3.15** LC-MS of DEBS ACP2 reacted with 20.
This safety catch hypothesis was tested by synthesizing 20, a β-lactam which bears an S-pyridyl leaving group at the β-position and a propargyloxy carbamoyl activating group on the heterocyclic nitrogen for use in the global reporter strategy. *Holo*-ACP2 incubated with 20 and analyzed by LC-MS shows two populations. One possesses a mass consistent with ACP-2 loaded with malonate, the other is consistent with ACP-2 loaded with 20 which had not converted from the thioaminal to the imine stage (Figure 3.15). Genuine malonate-loaded ACP2 cannot be easily produced for comparison due to the inherent limitations of the ATs of DEBS which only transfers methylmalonate.

![A and B](image)

**Figure 3.16:** Possible behavior of β-lactones and –lactams bearing multiple leaving groups at the 4-position.

A) Proposed reaction of a *holo*-ACP with a doubly-substituted lactam-based reagent to produce a malonamide. B) Proposed reaction of a *holo*-ACP with a doubly-substituted lactone-based reagent to produce malonate.

This work was unfortunately discontinued due to deficiencies in the chemistry of the reactive probes required to directly load ACPs with malonate. Conversion from thioaminal stage
to the carbonyl stage did not go to completion in a timely manner, and oxidation of the aldehyde stage is an indirect pathway to malonate. While it would initially seem that a better strategy might be to include two leaving groups at the $\beta$-position to directly yield a malonate, this would likely result in formation of a malonamide (Figure 3.16 A) which is not seen in the standard picture of polyketide biosynthesis. Substituting a $\beta$-lactone with two leaving groups at the $\beta$-position would also seem like a good strategy (Figure 3.16 B) but the chemistry to reliably produce such lactones did not exist at the time this work was undertaken.

\[ \text{Figure 3.17: Chemical acyl transferase scheme.} \]

Inactivation of a module’s native AT ($\text{AT}^0$) would render it unable to accept its native substrate. Instead, ACP is directly loaded with novel malonate-like functionality using a substrate similar to 20. This malonate then may be able to participate in polyketide chain elongation, incorporating novel functionality at the $\alpha$-site.

Nevertheless, the notion of directly loading holo-ACPs with malonate remains tantalizing given the inherent selectivity of AT domains. Acting as the ‘gate keeper domain’, ATs select the functionality at the $\alpha$-site of a polyketide intermediate and no good method currently exists for rationally altering their selectivity.\(^{12}\) This selectivity could be circumvented with appropriately functionalized probes that could be used as a ‘small molecule acyltransterase’ to directly load
holo-ACPs with novelly-substituted malonate (Figure 3.17) unlocking the previously off limits \( \alpha \)-site and finally providing a means to fully explore the substrate tolerance of PKSs. The small molecule acyltransferase could also be coupled with traditional knockout methods to produce a multitude of novel polyketide-like products varying in the substitution at \( \alpha \)- and \( \beta \)-sites.

C. Experimental Details

Below are details of critical experiments.

1. Chemical Syntheses

Below are details of the syntheses of critical compounds.

a. 1-[(4-Methylphenyl)sulfonyl]azetidin-2-one\(^13\) (8)

![Reaction equation]

**Fig. 3.18: Synthesis of 8.**

In a flame-dried RBF with PTFE-coated stir bar, septum, and an argon inlet, cooled to \(-78^\circ\text{C}\) in a dry ice-acetone bath, 2-Azetidinone (0.0720 g, 1 mmol) is dissolved in THF (5 mL). NaHMDS (0.3670 g, 2 mmol) is dissolved in THF (1 mL) and added dropwise. 4-Toluenesulfonyl chloride (0.7630 mg, 4 mmol) dissolved in THF (5 mL) is added to the reaction mixture dropwise over 15 min. The reaction mixture is stirred until all the starting material is consumed (monitored by TLC), \( \text{ca.} \ 8 \text{ h} \). The reaction is washed with sodium bicarbonate (10 mL \( \times \)3) and extracted with DCM (25 mL \( \times \)3). The combined organic layers are dried over Na\(_2\)SO\(_4\), and the solvent is removed by rotary evaporation. The crude material is purified by flash
chromatography using silica gel as the stationary phase with 1:1 hexanes:ethyl acetate as the mobile phase.

**Yield of 8**: 0.1780 g (0.79 mmol, 79%) colorless solid.

$^1$H NMR (400 MHz, CDCl$_3$)$\delta$ (ppm): 7.89 (d, $J = 8.29$, 2H), 7.34 (d, $J = 8.48$, 2H), 3.65 (t, $J = 5.2$, 2H), 3.03 (t, $J = 5.1$, 2H), 2.46 (s, 3H)

$^{13}$C NMR (100 MHz, CDCl$_3$)$\delta$ (ppm): 161.9, 143.4, 128.3, 125.7, 38.0, 34.9, 19.9.

IR : $\bar{v} = 2973, 1776, 1362, 1154, 682, 98$ cm$^{-1}$,

LC-MS (ESI$^+$) $[M + H]^+$ = 227

**b. Benzyl 2-oxazetidine-1-carboxylate$^{14}$ (9)**

![Synthesis of 9](image)

Fig. 3.19: Synthesis of 9.

In flame dried RBF A with PTFE-coated stir bar, septum, and an argon inlet, cooled to -78°C in a dry ice-acetone bath, HMDS (0.2500 g, 1.5 mmol) is dissolved in THF (8 mL). nBuLi (0.0960 g, 1.5 mmol) is added dropwise and stirred for 30 min. In RBF B cooled to -78°C, 2-azetidinone (0.1066 mg, 1.5 mmol) is dissolved in THF (5 mL). The contents of A are transferred via cannula to B and stirred for 1 h. Benzylchloroformate (0.2560 g, 1.5 mmol) is added in portions and stirred for 2 h at −78 °C. The reaction mixture is allowed to warm to ambient temperature and stirred for an additional 4 h. The reaction mixture is diluted with water and extracted with DCM (50 mL × 3). Combined organic phases are washed with brine and dried.
over Na₂SO₄ and solvent is removed by rotary evaporation. The material is purified by flash chromatography using silica gel as the stationary phase with 5:1 hexanes:ethyl acetate as the mobile phase.

**Yield of 9**: 0.2850 g (1.39 mmol, 93%) oil.

^1^H NMR (400 MHz, CDCl₃) δ (ppm): 7.28–7.16 (m, 5H), 5.1 (s, 2H), 3.42 (t, J = 5.3, 2H), 2.83 (t, J = 5.3, 2H).

^1^C NMR (100 MHz, CDCl₃) δ (ppm): 167.8, 155.0, 147.1, 126.8, 126.1, 65.9, 36.1, 34.7, 23.7

IR: $\tilde{\nu} = 3040, 2985, 1811, 1724, 1388, 1330, 1214, 1177, 1120, 1044, 763, 699$ cm⁻¹,

LC-MS (ESI⁺) [M + H]^⁺ = 206

c. (9H-Fluoren-9-yl)methyl 2-oxoazetidin-1-carboxylate (10)

![Chemical Structure](image)

**Fig. 3.20**: Synthesis of 10.

In a flame-dried RBF with PTFE-coated stir bar, septum, and an argon inlet, cooled to -78°C in a dry ice-acetone bath, 2-Azetidinone (0.0720 g, 1 mmol) is dissolved in THF (5 mL), and LiHMDS (0.1670 g, 1 mmol) dissolved in THF (2 mL) is added dropwise over a 15 min and stirred for an additional 30 min. 9-Fluorenymethyl chlorof orm ate (0.2590 g, 1 mmol) dissolved in THF (2 mL) is added dropwise. The resulting mixture is stirred for 1 h at -78 °C, then allowed to warm to R.T and stirred for 1 h further at which point the starting material had been completely
consumed (monitored by TLC). The reaction mixture is poured into water and extracted with DCM (3 × 25 mL). The combined extracts are washed with brine and dried over Na$_2$SO$_4$, and solvent is removed by rotary evaporation. The material is purified by flash chromatography using silica gel as the stationary phase with DCM:ethyl acetate, 95:5 as the mobile phase.

**Yield of 10**: 0.0620 g (0.62 mmol, 86%), off-white solid.

$^1$H NMR (400 MHz, CDCl$_3$)δ (ppm): 7.73–7.80 (m, 4H), 7.29 – 7.46 (m, 4H), 4.47 (d, 2H), 4.32 (t, 1H), 3.70 (t, 2H), 3.13 (t, 2H)

$^{13}$C NMR (100 MHz, CDCl$_3$)δ (ppm): 162.1, 148.3, 141.3, 139.4, 126.4, 125.5, 123.5, 118.1, 66.8, 44.3, 34.8, 36.2

IR: $\bar{\nu}$ = 3015, 2922,1770, 1722, 1449, 1388, 1313,1119, 1044, 964, 738 cm$^{-1}$.

HRMS (EI$^+$) Calculated for C$_{18}$H$_{15}$NO$_3$, 293.1052; Found, 293.1046

d. tert-Butyl 2-oxoazetidine-1-carboxylate$^{15}$ (11)

![Fig. 3.21: Synthesis of 11.](image)

In a flame-dried RBF with PTFE-coated stir bar, septum, and an argon inlet, cooled to 0°C in an ice-water bath, 2-Azetidinone (0.0720 g, 1 mmol) and DMAP (0.0122 g, 0.1 mmol) are dissolved in acetonitrile (5 mL) at 0 °C. Di-tert-butyl dicarbonate (0.2180 mg, 1.1 mmol) is added to the reaction mixture in portions. The reaction mixture is stirred at 0 °C for 2 h then warmed to ambient temperature and stirred overnight. The reaction mixture is diluted with ethyl
acetate and washed with 1 N HCl then brine. The combined aqueous layers are extracted with ethyl acetate (25 mL ×3). Combined organic layers are dried over Na₂SO₄ and solvent is removed by rotary evaporation. The material is purified by flash chromatography using silica gel as the stationary phase with 2:1, hexanes:ethyl acetate as the stationary phase.

**Yield of 11**: 0.1540 g (0.9 mmol, 90%), oil.

$^1$H NMR (400 MHz, CDCl₃)δ (ppm): 3.54 (t, J = 5.1, 2H), 2.96 (t, J = 5.2, 2H), 1.45 (s, 9H)

$^{13}$C NMR (100 MHz, CDCl₃)δ (ppm): 205.4, 162.1, 146.5, 81.2, 36.8, 35.0, 29.8, 26.2

IR : $\tilde{\nu}$ = 2984, 1795, 1719, 1331, 1157, 1043 cm⁻¹

LC-MS (ESI⁺) [M + H]$^+$ = 172

**e. Prop-2-yn-1-yl 2-oxoazetidine-1-carboxylate (12)**

Fig. 3.22: Synthesis of 12.

In a flamed dried RBF with PTFE-coated stir bar, septum, and an argon inlet, cooled to -78°C in a dry ice-acetone bath, 2-Azetidinone (0.1070 g, 1.5 mmol) is dissolved in THF (7 mL). LiHMDS (0.3300 g, 2 mmol) dissolved in THF (2 mL) is added dropwise over 10 min and the reaction mixture is stirred for 30 min. Propargyl chloroformate (0.1810 g, 1.5 mmol) dissolved in THF (2 mL) is added dropwise over 10 min and the reaction mixture is stirred at −78 °C for 2 h then warmed to ambient temperature. The reaction mixture is diluted with water and extracted with DCM (50 mL ×3). The combined organic layers are washed with brine and dried over
\[ \text{Na}_2\text{SO}_4. \text{ Solvent is removed by rotary evaporation. The material is purified by flash chromatography using silica gel as the stationary phase with 95:5 DCM:methanol as the mobile phase.} \]

**Yield of 12**: 0.1060 g (0.69 mmol, 46%), pale yellow solid.

\[ ^1\text{H NMR (400 MHz, CDCl}_3\text{)} \delta (\text{ppm}): 4.80 (d, J = 2.3, 1H), 3.66 (t, J = 5.3, 2H), 3.08 (t, J = 5.3, 2H), 2.54 (t, J = 2.4, 1H). \]

\[ ^{13}\text{C NMR (100 MHz, CDCl}_3\text{)} \delta (\text{ppm}): 162.2, 146.3, 74.0, 51.8, 36.1, 34.9, \]

IR : \( \bar{\nu} = 3251, 2925, 2134, 1782, 1722, 1313, 1187, 1114, 1045, 615 \text{ cm}^{-1}. \)

HRMS (EI+) Calculated for C\(_7\)H\(_7\)NO\(_3\), 153.0426; Found, 153.0402

**f. Biotin Azide (13)**

![Chemical structure](image)

**Fig. 3.23**: Esterification of biotin.

In an RBF with PTFE-coated stir bar, D-(+)-biotin (0.0730 g, 0.300 mmol) is dissolved in 3 mL of DMF by briefly heating the mixture with a heat gun. The mixture is cooled to 0 °C in an ice-water bath and 6-chloro-1-hexanol (0.1200 g, 0.900 mmol), and N,N-dimethylamino pyridine (0.0730 g, 0.600 mmol) are added. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (0.1150 g, 0.600 mmol) is added in portions over \( \text{ca.} 60 \text{ min} \) and the reaction mixture is warmed to room temperature. Stirring is continued for 24 h. When biotin had been consumed (monitored by TLC ), the reaction mixture is diluted with ethyl acetate (100 mL). The
organic phase is washed with concentrated NaHCO$_3$ (3 × 50 mL), and pH 2 saturated Na$_2$SO$_4$ (5 × 50 mL portions). Solvent is removed by rotary evaporation and the material is used without further purification.

![Chemical structure](image)

Fig. 3.24: Synthesis of 13.

In an RBF with PTFE-coated stir bar, the material from the ester coupling is dissolved in 5 mL of DMF and NaN$_3$ (0.0400 g, 0.600 mmol) and a catalytic amount of KI are added. A reflux condenser is attached and the reaction mixture is heated to 80 °C for 16 h. The material is diluted with 100 mL of ethyl acetate and washed with brine and the solvent is removed by rotary evaporation. The product is purified by flash chromatography using silica gel as the stationary phase with 5% MeOH in DCM as the mobile phase.

**Yield of 13:** 0.0670 g (0.182 mmol, 61%), waxy pale yellow solid.

$^1$H NMR (400 MHz, CDCl$_3$) δ (ppm): 6.03 (s, 1 H), 5.67 (s, 1 H), 4.51 (dd, J = 7.71, 5.05, 1 H), 4.31 (ddd, J = 7.67, 4.71, 1.14, 1 H), 4.06 (t, J = 6.63, 2 H), 3.28 (t, J = 6.88, 2 H), 3.16 (ddd, J = 8.34, 6.38, 4.74, 1 H), 2.91 (dd, J = 12.82, 4.99, 1 H), 2.74 (d, J = 12.76, 1 H), 2.33 (t, J = 7.45, 2 H), 1.56–1.80 (m, 8 H), 1.32–1.52 (m, 6 H)

$^{13}$C NMR (100 MHz, CDCl$_3$) δ (ppm): 173.7, 163.7, 65.6, 61.2, 60.8, 56.1, 51.3, 40.5, 33.9, 29.9, 28.7, 28.4, 27.2, 26.3, 25.5, 24.8

IR: $\tilde{\nu}$ = 3230, 2934, 2101, 1733, 1700, 1264, 1175 cm$^{-1}$
HRMS (EI+) Calculated for C_{16}H_{27}N_{5}O_{3}S, 369.1848; Found, 369.1814

\begin{align*}
\text{O} \quad \text{OH} \\
\text{O} \quad \text{H} \\
\text{N} \quad \text{S} \\
\text{H} \\
\text{O} \\
\text{S} \\
\text{N} \\
\text{H} \\
\text{O} \\
\text{n} = 1 - 6
\end{align*}

\[ \text{EDC-HCl, HOBT-H_2O} \]

\[ \text{TEA, DCM} \]

**Fig. 3.25:** SNAc thioester formation.

General method for preparation of all N-acetylcysteamine (SNAc) thioester derivatives:

In a flamed dried RBF with PTFE-coated stir bar, septum, and an argon inlet, to a solution of triethylamine (2.80 mmol) in dichloromethane (10 mL) is added the appropriate acid (1.4 mmol), (3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.40 mmol), 1-hydroxybenzotriazole hydrate (1.40 mmol) and N-acetylcysteamine (1.35 mmol) under argon. The reaction mixture is stirred overnight. The organic layer is washed with saturated NaHCO$_3$, 0.1 N HCl and brine. It is then dried over anhydrous sodium sulfate, solvent is removed by rotary evaporation, and purified by flash column to provide the final product in pure form.

g. **S-Propionyl N-acetylcysteamine (14)**

**Yield of 14:** 140 mg, 74%) as a pale yellow oil.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm): 0.86 (t, $J = 7.58$ Hz, 3H) 1.69 (s, 3H) 2.28 (q, $J = 7.4$ Hz, 2H) 2.72 (t, $J = 6.8$ Hz, 2H) 3.07 (q, $J = 6.6$ Hz, 2H) 7.39 (br s, 1H)

$^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ (ppm): 199.4, 170.6, 39.0, 36.8, 28.0, 22.6, 9.4

IR : $\tilde{\nu} = 3282, 2979, 1690, 1650, 1546, 1373, 1288, 1090, 935$ cm$^{-1}$

LC-MS (ESI$^+$) [M + H]$^+$ = 176
**h. S-Butyryl N-acetylcysteamine (15)**

**Yield of 15**: 0.1814 g (0.96 mmol, 71%), Pale yellow solid

$^1$H NMR (400 MHz, CDCl$_3$) δ (ppm): 6.12 (s, 1H), 3.41 (q, 2H), 3.00 (t, 2H), 2.54 (t,2H), 1.95 (s, 3H), 1.67-1.69 (m,2H), 0.94 (t, J=, 3H)

$^{13}$C NMR (100 MHz, CDCl$_3$) δ (ppm): 197.97, 168.64, 44.12, 37.64, 26.55, 21.27, 17.17, 11.54

FT-IR: ν 3291.13, 2967.08, 1651.12, 1547.47, 1436.23, 1370.50, 1284.55, 1112.64, 988.76, 905.33, 723.31 cm$^{-1}$

LC-MS (ESI$^+$) m/z Calculated for C$_8$H$_{15}$SNO$_2$ [M+H]$^+$ = 190.089, found 190.0

**i. S-Pentanoyl N-acetylcysteamine (16)**

**Yield of 16**: 0.2251 g, (1.11 mmol, 82%), Pale yellow solid

$^1$H NMR (400 MHz, CDCl$_3$) δ (ppm): 6.22 (s, 1H), 3.38 (q, J=6.5 Hz, 2H), 2.96 (t, J=6.6 Hz, 2H), 2.55 (t, J=6.6 Hz, 2H), 1.95 (s, 3H), 1-58-1.66 (m, J=6.6 Hz, 2H), 1.3-1.36 (m, J=6.6 Hz, 2H), 0.89 (t, J=6.6 Hz, 3H)

$^{13}$C NMR (100 MHz, CDCl$_3$) δ (ppm): 197.92, 168.64, 41.83, 37.58, 26.38, 25.69, 21.10, 20.08, 11.71

FT-IR: ν = 3288.16, 3077.00, 2959.74, 2933.28, 2873.68, 1651.25, 1546.86, 1287.53, 1016.73, 731.82 cm$^{-1}$

LC-MS (ESI$^+$) m/z calculated for C$_9$H$_{17}$SNO$_2$ [M+H]$^+$ = 204.105, found 204.1

**j. S-Hexanoyl N-acetylcysteamine (17)**
Yield of 17: 0.2142 g (0.99 mmol, 73%), Colorless solid.

$^1$H NMR (400 MHz, CDCl$_3$) δ (ppm): 6.33 (s, 1H), 3.38 (q, J=6.3 Hz, 2H), 2.99 (t, J=6.6 Hz, 2H), 2.53 (t, J=6.6 Hz, 2H), 1.93 (s, 3H), 1.58-1.66 (m, J=6.3 Hz, 2H), 1.25-1.31 (m, J=6.3 Hz, 4H), 0.86 (t, J=6.3 Hz, 3H)

$^{13}$C NMR (100 MHz, CDCl$_3$) δ (ppm): 198.24, 168.45, 42.16, 37.77, 29.13, 26.48, 23.41, 21.25, 20.35, 11.93

FT-IR: $\tilde{\nu}$ =3293.04, 3093.17, 2952.97, 2930.26, 2870.06, 1641.09, 1551.33, 1408.42, 1358.88, 1292.89, 970.52, 745.99 cm$^{-1}$

LC-MS (ESI$^+$) m/z Calculated for C$_{10}$H$_{19}$SNO$_2$ [M+H]$^+$ 218.120, found 218.0

k. S-Heptanoyl N-acetylcysteamine (18)

Yield of 18: 0.1874 g (0.81 mmol, 60%), Pale yellow solid.

$^1$H NMR (400 MHz, CDCl$_3$) δ (ppm): 6.28 (s, 1H), 3.52 (q, J=6.6 Hz, 2H), 2.99 (t, J=6.4 Hz, 2H), 2.53 (t, J=6.6 Hz, 2H), 1.93 (s, 3H), 1.58-1.66 (m, J=7.5 Hz, 2H), 1.23-1.33 (m, J=6.3 Hz, 6H), 0.85 (t, J=6.3 Hz, 3H),

$^{13}$C NMR (100 MHz, CDCl$_3$) δ (ppm): 197.94, 168.59, 42.12, 37.61, 29.41, 26.59, 26.38, 23.61, 21.10, 20.44, 12.02

FT-IR: $\tilde{\nu}$ = 3290.00, 3103.58, 2954.74, 2929.89, 2871.55, 1681.0, 1635.24, 1557.27, 1442.49, 1370.78, 1293.34, 1045.19, 971.98, 760.76 cm$^{-1}$

LC-MS (ESI$^+$) m/z Calculated for C$_{11}$H$_{21}$SNO$_2$ [M+H]$^+$, 232.136, found 232.1

l.S-Octanoyl N-acetylcysteamine (19)
Yield of 19: 0.2783 g (1.13 mmol, 84%), Colorless solid

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm): 6.27 (s, 1H), 3.40 (q, J=6.3 Hz, 2H), 2.99 (t, J=6.6 Hz, 2H), 2.54 (t, J=6.6 Hz, 2H), 1.94 (s, 3H), 1.58-1.66 (m, J=7.4 Hz, 2H), 1.20-1.30 (m, J=6.3 Hz, 9H), 0.86 (t, J=6.3 Hz, 3H)

$^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ (ppm): 198.21, 168.47, 42.19, 37.76, 29.66, 26.94, 26.46, 23.72, 21.23, 20.64, 12.12

FT-IR: $\tilde{\nu}$ = 3291.33, 3101.19, 2922.78, 2848.61, 1682.4, 1637.18, 1555.34, 1294.29, 1178.47, 1045.81, 967.51, 728.99 cm$^{-1}$

LC-MS (ESI$^+$) m/z Calculated for C$_{12}$H$_{23}$SNO$_2$ [M+H]$^+$246.1522, found 246.1

**m. 4-(Pyridin-2-ylsulfanyl)azetidin-2-one**

![Chemical structure image]

**Fig. 3.26**: Synthesis of 4-(pyridin-2-ylsulfanyl)azetidin-2-one.

In a 20 mL vial with PTFE-coated stir bar cooled in an ice-water bath, 2-mercapto pyridine (0.223 g, 2.00 mmol) is dissolved in 2.0 mL of methanol and 2.0 mL of 1N KOH. 4-acetoxy-2-azetidinone (0.2582 g, 2.00 mmol) dissolved in 2 mL of water is added, and the mixture is sired for 60 min. The crude material is dissolved in ether and washed with water, then dried over MgSO$_4$. Material is pure enough for use without further purification.

4-(pyridin-2-ylsulfanyl)azetidin-2-one: 0.3685 g (1.87 mmol, 93.4%), colorless solid.
$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm): 8.41 (d, $J = 4.4$ Hz, 1 H), 7.54 (dt, $J = 1.8$, 7.7 Hz, 1 H), 7.20 (d, $J = 8.1$ Hz, 1 H), 7.05 (dd, $J = 5.2$, 6.9 Hz, 1 H), 6.86 (br. s., 1 H), 5.49 (dd, $J = 2.5$, 5.1 Hz, 1 H), 3.58 - 3.34 (m, 1 H), 2.99 (d, $J = 15.2$ Hz, 1 H)

**n. Prop-2-yn-1-yl 2-oxo-4-(pyridin-2-ylsulfanyl)azetidine-1-carboxylate (20)**

![Chemical Structure](image)

**Fig. 3.27: Synthesis of 20.**

In a 20 mL vial with PTFE-coated stir bar, septum, and Ar-inlet cooled in an ice-water bath, 4-(pyridin-2-ylsulfanyl)azetidin-2-one (0.0756 g, 0.70 mmol) is dissolved in 5 mL of DCM. Propargyl chloroformate (0.20 mL, 1.4 mmol) is added via syringe, followed by triethylamine (0.19 mL, 2.1 mmol) and the mixture is stirred for 60 min. The mixture is diluted with 100 mL of ethyl acetate and washed with saturated NaHCO$_3$, 0.1 N HCl, and brine. It is then dried over anhydrous sodium sulfate, solvent is removed by rotary evaporation. The material is purified by flash chromatography using silica gel as the stationary phase and a gradient of 70 – 30% hexanes:ethyl acetate with a 10% step between.

**Yield of 20:** 0.0267 g (0.11 mmol, 15.2%), pale yellow solid.

$^1$H NMR (400MHz, d6-DMSO) $\delta$: 10.85 (br. s., 1 H), 8.59 (br. s., 1 H), 7.89 (br. s., 1 H), 7.68 (d, $J = 8.7$ Hz, 2 H), 7.44 (br. s., 1 H), 5.88 (d, $J = 13.6$ Hz, 1 H), 4.83 (br. s., 2 H), 3.65 (br. s., 1 H), 1.34 - 1.05 (m, 2 H)
LC-MS calculated for $\text{C}_{12}\text{H}_{10}\text{SN}_{2}\text{OS}$ [m+H]$^+$ = 263.048489, found 263.29

2. Protein Expression and Isolation

ACP2 is expressed from pNW06, ACP3 is expressed from pVYA05. KS6AT6 is expressed from pAYC11; pNW6 and pVYA05 contain kanamycin resistant vectors. Apo-ACPs are harvested from *E. coli* BL-21 and holo-ACPs are harvested from *E. coli* BAP-1.

General procedure for protein expression and isolation: Cells are grown in 1 L shake cultures of LB-antibiotic media at 37°C in a New Brunswick Scientific Excella E24 Incubator Shaker until the OD600 is between 0.6 and 0.8 AU. Overexpression is induced with 200 μL of 1 M IPTG (per liter of culture) and carried out at 18°C for 18 hours, after this point all work is carried out at 4°C. Cells are pelleted by spinning at 3000 RPM for 10 minutes in a Sorvall RC6 Plus with a FiberLite F21S-8x50 rotor and resuspended in 50 mL of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium Na3VO4, 1 μg/mL leupeptin, pH 7.5). Cells are lysed using a Misonics ultrasonic converter without microtip, amplitude 30 for five 30 second intervals with a 60 second cool down period between each cycle. Cell debris are pelleted at 10,000 rpm and with a FiberLite F105S 6x500 Y rotor for 60 minutes. The lysate is equilibrated with 3 mL of PerfectPro Ni-NTA bead slurry for 60 minutes by stirring with a PTFE-coated stir bar at 60 RPM for 60 minutes. The lysate is then poured into a 15 mL column and the supernatant eluted. The column is then washed with two 15 mL portions of wash buffer (50 mM phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0), and eluted with 3 mL of elution buffer (50 mM phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0). The purified protein is loaded into an Amicon Ultra centrifugal concentrator and diluted to 15 mL with storage buffer (100 mM Tris, 1 mM EDTA, 1 mM dithioerythritol, 10% glycerol pH 8) and spun at 3000 rpm in Eppendorf Centrifuge 5810 R
with swinging-bucket rotor. Dilution and filtration is repeated a total of three times. Protein concentration is determined by Bradford assay, average concentration is approximately 500 μM. Proteins reflash frozen in liquid nitrogen and stored at -80°C until use.

3. Acylation of Proteins with β-Lactams

Labeling reactions for gel analysis are performed at 20 μL total volume; reactions for LC-MS analysis are performed at 50 μL total volume. Phosphate buffer (100 mM, pH 7), tris(2-carboxyethyl)phosphine (TCEP) (2.5 mM), and protein (0.025 mM) are reacted at ambient temperature for 15 min. β-Lactam (stock solution in DMSO so that final DMSO concentration is less than 20%) is added to achieve the appropriate final concentration. The mixture is equilibrated at ambient temperature for 60 min.

4. Proteolysis

Promega Sequencing Grade Modified Trypsin is added to prepared protein samples so that the final trypsin:protein ratio is 1:50 (w/w). The mixture is incubated at 30°C for 18 hours. Digestion is quenched by addition of an equal volume of 10% formic acid. Digests are flash frozen in liquid nitrogen and stored at -20°C until analysis.

5. LC-MS

Separation of digests is performed with a Waters 1525 system. The mobile phase gradient employed is A = water + 0.1% formic acid, B = acetonitrile + 0.1% formic acid, 5-95% B over 60 min the column is a Vydac 218TP C18 5μ column (4.6 x 150 mm).

Intact proteins are analyzed by direct infusion without a column.
Mass spectra are acquired with a Waters Micromass ZQ mass detector in EI+ mode: Capillary voltage = 3.50 kV, cone voltage = 30 V, extractor = 3 V, RF lens = 0.0 V, source temp = 100°C, desolvation temp = 200°C, desolvation gas = 300 L/hr, cone gas = 0.0 L/hr. The system is operated and spectra are processed using the Waters Empower software suite.

6. Chromophore Attachment

Reactions are carried out at 25 μL total volume; final concentrations reported. 6, (2× with respect to 12), sodium ascorbate (1 mM), and copper(II) sulfate (1 mM) are added to samples which had been labeled with 12. The mixture is allowed to react for 60 min at ambient temperature.

7. ACP to KS Fluorescence Transfer

General Procedure for Fluorescence Transfer: Holo-ACP (25 μM) is modified with 10 equivalents of 12 followed by copper-catalyzed click reaction with 6 as described above. For transfer of the fluorescent product, KS6AT6 (25 μM) is introduced to fluorescently-modified-ACP (25 μM) and incubated for 1 h.

Blocking KS with Cerulenin or SNAC Thioesters: KS6AT6 is pretreated with cerulenin (5 mM) or the appropriate SNAC thioester (10 mM) for 1 h before introduction to lactam-modified-ACP2.

8. Gel Assay

Fluorescently labeled samples are diluted to 35 μL with gel-loading buffer. ACP 2 is separated by 12% SDS-PAGE gels with 5% stacking gel, and ACP 3 with 15% SDS-PAGE gels with 5% stacking gel, run at 100 V, 50 mA, for 135 min. Gels are developed in 10% acetic acid to visualize 6 and imaged on a BioDoc-It Imaging System with UV transilluminator. Total protein is
stained using GelCode Blue stain. For the ACP to KS transfer experiments, proteins were separated using a 4–20% gradient HEPES-PAGE gel (100V, 50mA, 90 min), then developed and imaged using the same method as single protein experiments.

9. Affinity Purification of Holo-ACPs

Lactam modification of ACPs: Unless otherwise stated, phosphate buffer refers to 100 mM, pH 7.0 phosphate. 1 mL of an 80:20 mixture of holo:apo-ACP2 (25 μM total protein) is equilibrated at ambient temperature for 15 min in phosphate buffer containing 2.5 mM TCEP. 12 (25× with respect to total ACP concentration) is added and the mixture is equilibrated at ambient temperature for 1 h. To remove excess 12, the mixture is loaded into a 3kDa NMW concentrator and the volume reduced to 100 μL by spinning in a centrifuge cooled to 4 °C. The mixture is diluted to 500 μL with phosphate buffer, and then concentrated to 100 μL again. This process is repeated a total of 3 times. Protein is removed from the concentrator by inverting it and spinning. The concentrator is then washed several times with phosphate buffer which is added to the protein sample so that the filtered protein is reconstituted to 900 μL.

Click Attachment of Biotin: In a separate microfuge tube, DMSO (100 μL), 13 (50× with respect to protein), THPTA21 (1.1 mM), sodium ascorbate (10 mM), and CuSO4 (1 mM) are combined. The combined click reagents are added to the protein from the previous step and allowed to react at ambient temperature for 6 h. Excess biotin reagent is removed using the same centrifugal concentration procedure for the removal of excess lactam. The modified protein is reconstituted to 1000 μL in phosphate buffer.

Immobilization of modified ACPs: Streptavidin Sepharose High Performance beads (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, United Kingdom) (10× with respect to protein) are washed 3× with 1000 μL of phosphate buffer and the supernatant is removed. The
modified protein from the previous step is added to the beads, and the mixture equilibrated at ambient temperature for 60 min with agitation on an orbit shaker. The supernatant is removed and the beads are washed 5× with 1000 μL of phosphate buffer, 5× with 1000 μL of TRIS buffer (100 mM, pH 9.0), and once with 1000 μL of 100 mM TRIS, +10 mM N2H4, pH 9.0.

Elution of immobilized ACPs: The washed beads from the previous step are resuspended in 1000 μL of 100 mM ammonium formate containing 100 mM N2H4 (100 mM pH 9.0) and equilibrated overnight at 4° C with agitation on an orbit shaker. The supernatant is removed and concentrated in a 3kDa NMW concentrator and subjected to buffer exchange for further experiments, or combined with TCEP for direct infusion LC-MS experiments.

10. Loading Holo-ACP with Malonate using KSAT

To a buffered solution of KSAT619,20,22,23 is added methylmalonyl-CoA and holo-ACP2 eluted from the purification procedure above. After 30 min of reaction time, the mixture is subjected to trypsinolysis and the resulting peptide fragments are separated and analyzed via LC-MS under conditions for examining intact proteins.

11. Direct loading of Holo-ACP with 20

Labeling reactions are performed at 50 μL total volume. Phosphate buffer (100 mM, pH 7), tris(2-carboxyethyl)phosphine (TCEP) (0.050 mM), and protein (0.025 mM) are reacted at ambient temperature for 15 min. 20 (1.25 mM) is added and the reaction mixture is incubated in a 37°C water bath for 18 hours. Samples are frozen in liquid nitrogen prior to LC-MS analysis.

D. References


CHAPTER IV

DEVELOPMENT AND PROSPECTIVE APPLICATIONS OF THE BENZYNE LIGHT INDUCED CYCLOADDITION (BLINC) REACTION AS A TARGETABLE COPPER-FREE CLICK REACTION

A. Introduction

The work discussed in chapters 2 and 3 has heavily relied on the copper catalyzed azide-alkyne click CuAAC reaction to append chromophores or affinity groups to alkynes which are covalently attached to proteins via reactive probes, and several of these experiments have been hindered by the harmful effects of copper. For instance, SpnB is particularly sensitive to copper, and the correct amount of copper which would provide convenient reaction rates was difficult to balance against harmful effects to the protein. Furthermore, in the β-lactam-based separation of apo- and holo-ACPs, the presences of copper contributed to the low overall recovery. These difficulties are not unique, and various solutions have been enacted.

The CuAAC reaction is undoubtedly a workhorse reaction in modern chemistry. Its mild reaction conditions and broad substrate tolerance have made it useful in a numerous conjugation applications including small molecules, polymers, surfaces, and even bioconjugation. However, it is not a truly bioorthogonal conjugation method. The problem is the copper catalyst: it is toxic to effectively all cells and damages proteins. Ligands have been developed which mitigate some of these harmful effects, but so long as the copper remains, so does the possibility of detrimental outcomes.

1. Strained Alkyne Click

The most popular method for removing copper from chemical conjugations relies on the (2+3) cycloaddition of cyclooctyne-based dipolarophiles with azides (Figure 4.1). Due to the sp
hybridization of the carbon atoms involved, alkynes prefer to align their bonds at 180°, as exemplified by acetylene or 2-butyne, but the alkyne functional group can also be incorporated into a ring which restricts available bonding angles. As ring size grows smaller, the bonding angle of the alkyne becomes distorted from the ideal alignment which induces a great deal of ring strain.\textsuperscript{6} This increases the reactivity of the alkyne to a degree where it spontaneously adds to azides under biologically relevant conditions. The driving force for this reaction is the ring strain relieved when the alkyne adds to an azide to form a 1,2,3-triazole.

![Figure 4.1: Copper free click reaction between cyclooctyne and an azide to form a 1,2,3-triazole.](image)

Strain-promoted alkyne-azide cycloaddition provides convenient click reactivity without the need for harmful copper and has seen uses ranging from proteins \textit{in vitro} to \textit{in vivo} applications within cell cultures and even multicellular organisms.\textsuperscript{7} However, reliance on cyclooctyne as the dipolarophile does have its drawbacks. Firstly, the reaction rate between simple cyclooctynes and azides is disappointingly slow. Methods have been developed to increase the reactivity of cyclooctyne-based reagents, the most successful of which relies on installing \textit{benzo}-groups flanking the alkyne to impart a greater deal of strain. Unfortunately, the increased rate from these \textit{benzo}-groups comes at the cost of increased hydrophobicity and steric demand (Figure 4.2).
Figure 4.2: Click reaction with dibenzo cyclooctyne dipolarophile.

Another drawback of cyclooctyne-based click reactions is difficulties in precise targetability. For instance, if a protein of interest in a cell culture is uniformly modified with an azide partner, it would be difficult to confine cycloalkyne-labeling to a specific region of interest such as an individual cell, because the cyclooctyne partner can diffuse through the media and will eventually react with whichever azide it encounters, regardless of location.

2. Benzyne Click

Due to their innocuous nature and pervasiveness in labs which have already adapted to azide-alkyne chemistry, it makes sense to maintain the azide partner in conjugation reactions. However, it would be convenient to have a more active and targetable dipolarophile partner. A structure which seems to be able to keep the advantages of copper-free click while providing enhanced reactivity and targetability is benzyne. A well established reactive intermediate in organic chemistry,\(^8\)\(^{-10}\) benzyne contains the same aromatic sextet as benzene but also contains a formal triple bond formed from the overlap of two \(sp^2\) orbitals (Figure 4.3). The result of this unusual arrangement is a highly strained and reactive bond. Benzyne cannot be isolated and must be generated and consume \textit{in situ}.
Benzyne contains the same formal aromatic sextet as benzene and an additional bond created by the overlap of $sp^2$ orbitals.

A popular method for generating benzyne is via the reaction of a trimethylsilyl (TMS) substituted arene bearing a leaving group ortho to the TMS; formation of the Si-F bond promotes elimination of the leaving group and formation of the formal triple bond\textsuperscript{11} (Figure 4.4). The popularity of this method stems from its mild reaction conditions, occurring at room temperature in acetonitrile (ACN) without the need for strong bases. Benzyne generated through this method has also been shown to act as a dipolarophile in (3+2) cycloadditions with azides to form benzotriazoles.\textsuperscript{11-16} Interestingly, these experiments indicate that benzyne shows selectivity for azides over other functional groups it is known to react with, even when those groups are present within the same molecule. Benzotriazoles have been synthesized through this method from azide-substituted alkenes, alkynes, esters, amides, alcohols, and more.
interestingly carbohydrates and in one case a nucleoside.\textsuperscript{12} Benzyne’s selectivity for azide suggests that it may be a competent partner in bioconjugation reactions.

To explore its utility in such applications, a hurdle which must first be cleared is the matter of the solvent these reactions occur in. The fluoride-promoted elimination reaction is universally carried out in ACN, which is incompatible with biological systems. Preliminary attempts to react a TMS-based reagent with various fluoride sources in water did not produce any product, presumably due to the strong hydration of fluoride. There may be ways to increase fluoride’s activity in water, but this not an attractive prospect. The intent of strain-promoted click is to remove toxic copper from the equation, and substituting it for toxic fluoride is a poor trade. Fortunately benzyne generation is not limited to chemical methods.

3. Hypothesis: Benzyne Light Induced Cycloaddition (BLInC) Reaction

Numerous compounds are known to generate benzyne upon irradiation,\textsuperscript{17-21} many of these precursors are stable in water under biologically relevant conditions. We propose that a benzyne precursor can be irradiated to produce benzyne in the presence of azide-functionalized partners which then participate in a (3+2) cycloaddition to form benzotriazoles in a process that has been dubbed the “benzyne light-induced cycloaddition” (BLInC) reaction (Figure 4.5). If this reaction proceeds in buffered aqueous solutions, then it could be adapted to bioconjugation applications.

![Figure 4.5: Basic conception of the BLInC reaction.](image-url)
Toxic co-reagents are not required for the generation or cycloaddition of benzyne, and its highly reactive nature means that the cycloaddition proceeds very rapidly. The BLInC reaction could also be easily targeted spatiotemporally. The reactive species is only generated where and when light is applied, and the fleeting lifetime of benzyne\textsuperscript{22} prevents it from migrating far outside the target area, reducing the possibility of off-target conjugation.

![Figure 4.6: Triazeny1benzoic acid synthesis and photoreaction.](image)

(A) Synthesis of TBAs via diazoamination. (B) TBA participation in the BLInC reaction: excitation results in loss of CO\textsubscript{2}, N\textsubscript{2}, and an amine to form benzyne which undergoes a (2+3) cycloaddition with an azide.

In order to test this hypothesis, a benzyne-precursor is required. Of the known photoactivatable benzyne-precursors, one that stands out as an attractive prospect for several reasons is based on the ortho-triazenylbenzoic acid (TBA) core. TBAs are simple to synthesize through diazoamination, reacting diazotized anthranilic acid with a secondary amine\textsuperscript{23} (Figure 4.6), a reaction which can be done using water as the only solvent. Triazenes are also chemically stable: they are not degraded by water or bases, they can tolerate mild heat and acid, and can be stored for extended periods of time. Another interesting trait of triazenes is their inherent
photocleavability,\textsuperscript{20,21} a feature which could be exploited to shed one functionality while simultaneously gaining another via cycloaddition (Figure 4.6).

### B. Results and Discussion

In order to explore the feasibility of the BLInC reaction, benzyne-precursors were synthesized and subjected to irradiation in the presence of various organic azides to determine if benzotriazole formation takes place. When possible, results are compared to traditional chemical methods.

#### 1. First Generation Probe

![Figure 4.7: First generation probe 21.](image)

The first generation probe 21 synthesized to explore the BLInC reaction was an $N,N$-dialkyl TBA, which used morpholine as the secondary amine because it can act as a solublizing group (Figure 4.7). 21 was prepared by diazotizing anthranilic acid with NaNO\textsubscript{2}/HCl in water at 0°, then making the solution basic and reacting the diazonium with morpholine. After the reaction was complete, acidification of the solution caused 21 to precipitate.

The mechanism of photochemical benzyne generation from triazeneobenzoic acids is not established in the literature. Triazenes are known to undergo photoinitiated decomposition \textit{via} a radical pathway,\textsuperscript{24-26} but to form benzyne the carboxyl group would also have to exit \textit{via} a
radical pathway which seems unlikely in the absence of any groups to activate the carboxyl in such a way. There is one suggestion that triazene-based reagents can form diazonium ions via photolysis, but the diazonium species was not directly observed and was inferred from the way in which a triazene-based reagent interacted with plasmid DNA. However, a benzyne generation pathway which includes a diazonium ortho-carboxylate (DOC) intermediate makes sense because DOCs are well established as photochemical benzyne precursors.

![Diagram of DOC formation](image)

**Figure 4.8**: Phenol trapping of DOC to form azobenzene 22.

DOC is produced by diazotization (A) or by photolysis of 21 (B).

The presence of a DOC intermediate in the photolysis of TBAs was established through two methods. Upon irradiation 21, a species with the calculated mass of the DOC can be directly detected by LC-MS. This species has the same mass as the DOC produced by diazotization of anthranilic acid. The second method used to establish the presence of the DOC intermediate is chemical trapping. Diazonium ions are known to react with electron rich arenes to form brightly colored azobenzenes. When 21 is co-irradiated with basic phenol, the reaction mixture becomes yellow, and produces a compound which is indistinguishable by LC-MS from the
azobenzene produced via the reaction of diazotized anthranilic acid and phenol (Figures 4.8, 4.9). This color change is also a useful chemical test during the synthesis of triazenes because it indicates when all diazonium has been consumed during the diazoamination procedure. Furthermore, TLC plates can be treated with basic phenol and irradiated with a spot lamp causing triazene-containing spots to turn yellow, aiding in the isolation of these compounds.

Figure 4.9: LC-MS of 22 TIC extracted at m/z = 243.1.

(A) 22 produced from diazotized anthranilic acid. (B) 22 produced by coirradiation of 21 with phenol.
2. Reaction of Photochemical vs. Chemically Generated Benzyne

To establish if TBAs are reacting with azides in a benzyne-like manner, a chemical standard is required. 23 reacts with CsF in ACN to produce benzyne, and when performed in the presence of 13 produces the benzotriazole product 24 (Figure 4.10) which was analyzed by LC-MS. When 21 is reacted with 13 via the BLInC reaction in phosphate buffer and the product analyzed by LC-MS, it produces a peak with the expected mass and retention time of 24. When the chemical standard is combined with the experimental photochemical product and analyzed by LC-MS, the products have the same retention time and the same mass (Figure 4.11), indicating that 21 is reacting like benzyne upon irradiation. Furthermore, the reaction of 21 and 13 requires irradiation to occur, and 21 coirradiated with unfunctionalized biotin did not produce any detectable products indicating that the reaction is selective for the azide functional group, even in an aqueous environment.

![Chemical and photochemical reaction scheme.](image)

**Figure 4.10:** Chemical and photochemical reaction scheme.

A) Photochemical reaction between 21 and 13 in phosphate-buffered saline to produce benzotriazole 24. B) Chemical reaction between 23 and 13 in ACN promoted by CsF to produce 24.
Figure 4.11: LC-MS results of photochemical and chemical conjugation reactions.

A) Photochemical reaction LC-MS TIC extracted at m/z = 455.2. B) Chemical reaction LC-MS TIC extracted at m/z = 455.2. C) Coelution of photochemical and chemical reaction LC-MS TIC extracted at m/z = 455.2.

3. First Generation Probe with Linker Attachment Point

To be useful in conjugation reactions, there must be a way to link the benzyne dipolarophile to something else, such as a surface, protein, drug, chromophore, or affinity group. Synthesis of the linker-containing 26 began from the conveniently desymmetrized amino terphthalate 25. This was coupled to a monoprotected diamine with EDC/HOBt followed by saponification to free the carboxyl which would be required for benzyne formation. Finally, diazoamination with morpholine produced 26 (Figure 4.12).
Figure 4.12: Synthetic scheme for producing 26.

The increased mass of 26 provided by the linker also allows further investigation into the photochemical intermediates produced by the irradiation of TBAs. If TBAs are producing benzyne, then it can intercept a molecule of water from the solvent and produce the corresponding phenol (Figure 4.13). When 26 is irradiated in an aqueous solution and analyzed by LC-MS, a peak is observed which has the predicted mass of the phenol product (Figure 4.14), indicating that benzyne is in fact being generated during the process.

Figure 4.13: Phenol formation by photoreaction of 26.
Unfortunately, when tested in the BLInC reaction, 26 performed disappointingly overall. While active with 13 and ethyl azidoacetate, it did not produce detectable product with benzyl azide or rhodamine-azide 6. The lack of clear structure-function relationships hampered attempts to optimize the reactivity. Furthermore, large quantities of the corresponding benzoic acid sans triazene and radical recombination products are detectable by LC-MS after irradiation, indicating this compound favored a radical generating pathway (Figures 4.15, 4.16) over benzyne formation.

Upon excitation, triazenes undergo radical decomposition to form N₂, an aminyl radical, and in the case of TBAs, a carboxyphenyl radical. The carboxyphenyl radical can be quenched to form benzoic acid. Curved arrows show the flow of electrons.
The poor click performance is likely due to electronic effects. The literature regarding cycloadditions of chemically generated benzyynes with azides indicates that electron-poor benzyne significantly underperforms its more electron-rich counterparts.\textsuperscript{11,16} Since the amide group chosen as the attachment point for the linker is electron-withdrawing in nature, this may have played a role in the reduced reactivity compared to more electron-rich 21. Another detrimental electronic effect is inherent to dialkyl TBAs in general which causes strengthening of the bonds which must be broken in the course of benzyne formation (Figure 4.17), possibly decreasing overall photoreactivity. Fortunately, there are straightforward solutions to these adverse electronic effects.
Figure 4.17: Electronic effects weaken the bonds highlighted in red and strengthen the bonds highlighted in blue.

4. Second Generation Probe Development

Since the source of the latter problem appears to originate from the lone-pair of the terminal dialkyl nitrogen, it seems that installing an electron-withdrawing group at this position may alleviate this effect. To test this hypothesis, the N-acetyl TBA 27 was synthesized and its photoactivity compared to 21. When 15 mmol samples of each are irradiated in ACN, TLC indicates that nearly all traces of 27 have disappeared within 15 min, whereas significant quantities of 21 remain even after 60 minutes.

Unfortunately, finding a practical synthetic route to N-acetyl TBAs was a difficult undertaking. Simply performing a diazoamination with anthranilic acid and a primary amine followed by acetylation is impossible as monoalkyl triazenes are decomposed by carboxylates. Masking the carboxyl as an ester initially seems like a viable strategy, but the reactive terminal nitrogen of monoalkyl triazenes is nucleophilic enough to attack the ester, forming triazinones. Attempts to adapt the Mills reaction to produce N-acetyl TBAs were also unsuccessful.

The successful route to 27 produced for these experiments is a diazoamination-acylation-oxidation pathway beginning from o-amino benzyl alcohol. This material undergoes diazoamination with methamphetamine as the amine partner to form a monoalkyl triazinyl benzyl...
alcohol which is $N$-acylated and then the benzyl site is oxidized with permanganate to yield 27 (Figure 4.18). With this much more active probe in hand, further studies were carried out.

![Synthetic scheme to produce 27](image)

Figure 4.18: Synthetic scheme to produce 27.

5. Preliminary Investigation into Bioconjugation

For the BLInC reaction to be a successful bioconjugation method, it should not only proceed in buffered aqueous solutions, but it should also perform well at high dilution without requiring large excesses of either dipolarophile or azide partner. To determine if 27 functions under these conditions, the BLInC reaction was conducted with the biologically compatible azide 28 in phosphate-buffered saline using increasing equivalents of 27 (Figure 4.19). The LC-MS results show that to achieve 50% conversion of azide to benzotriazole, over 100 equivalents of 27 are required (Figure 4.20). Due to this poor performance, work to adapt 27 as a bioconjugation method has been suspended.
BLInC Reaction of 27 and 28 in PBS was analyzed by LC-MS at varying proportions of 27:28. Product yield is calculated by comparing the intensity of click product to the intensity of unreacted 28.

### 6. Preliminary Results in Organic Solvent

To directly compare BLInC reaction to traditional benzyne-azide cycloaddition, reactions were carried out with equimolar quantities of 27 and various azides in ACN. The same azide partners were reacted with benzyne generated from 23 and CsF. The yields from the BLInC reactions are comparable to traditional conditions, in most cases falling within 10% (Figure 4.21, Table 4.1). However, the fluoride-promoted method requires reaction times of 18 - 36 hours, whereas the BLInC reactions only required 15 minutes of irradiation, a vast improvement in rate.
Furthermore, the BLInC reaction yields could likely be improved by employing an excess of 27 with respect to azide partner. Considering these results, it is safe to say that the BLInC reaction represents a method for rapidly accessing benzyne-azide click reactivity in organic solvents. Further experiments to explore substrate and solvent tolerances are currently being carried out.

![Chemical structures of compounds](image)

**Figure 4.21**: Products produced by photochemical and chemical reactions in ACN.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% yield Chemical</th>
<th>% yield Photochemical</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>63.3</td>
<td>58.5</td>
</tr>
<tr>
<td>31</td>
<td>36</td>
<td>30.9</td>
</tr>
<tr>
<td>32</td>
<td>66.4</td>
<td>68.4</td>
</tr>
<tr>
<td>33</td>
<td>45.9</td>
<td>35.9</td>
</tr>
<tr>
<td>34</td>
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<td>39.7</td>
</tr>
<tr>
<td>36</td>
<td>56.5</td>
<td>32.6</td>
</tr>
</tbody>
</table>

**Table 4.1**: Product yields produced by chemical and photochemical methods.

**C. Future Directions**

The BLInC reaction is not immediately adaptable for use as a bioconjugation reaction, but it does offer click-like reactivity that is both rapid and potentially targetable. To expand the scope of this reaction, certain modifications to the chemical structures of the TBA probes should
be explored in order to further increase its reactivity and utility. These include further optimization of TBA electronics, and an improved linker attachment point.

1. Optimization of Electronics

The BLInC reaction when, using TBAs as the benzyne precursor, goes through several discrete steps: photocleavage to produce the DOC intermediate, benzyne generation, and then the cycloaddition with azide. Electronics play in important role in each of these transformations. It has already been shown that addition of an electron-withdrawing group to the terminal nitrogen of the triazene accelerates the first step. The second and third step remain unoptimized.

![Chemical structure](image)

**Figure 4.22**: Photoreaction of 29.

A sign that the benzyne formation step may be optimized came from experiments with bromine-functionalized TBA **29 (Figure 4.22)**. When the ‘naked’ TBA **27** is irradiated at the micromole scale in PBS, 60 seconds of irradiation are required to remove all signs of starting material and DOC intermediate by LC-MS analysis. However, when 29 is irradiated under the same conditions, a large amount of the DOC is still detectable, and very little click product is produced, even with greatly increased reaction times. Electronic effects can be invoked to explain the persistence of the DOC intermediate, as well as the small amount of benzotriazole product produced.
The first is that the electron-withdrawing nature of the bromide substituent may be disfavoring conversion of DOC to benzyne. It was reasoned that if electron-withdrawing substituents disfavored this process then electron-donating substituents may favor it. To test this hypothesis, synthesis of a TBA functionalized with electron-donating substituents was attempted starting from 6-nitropiperonal. To obtain the amino benzyl alcohol substrate required for N-acetyl TBA synthesis via the diazoamination-acetylation-oxidation pathway, the nitro group was first reduced with Fe, then the aldehyde was reduced with NaBH4. The resultant amino benzyl alcohol is extraordinarily acid labile, rendering it unfit for diazotization under standard conditions (Figure 4.23).

![Figure 4.23: Attempted synthesis of electron-rich TBA.](image)

This sensitivity to acid may be due to the highly electron-rich nature of the benzyl site, favoring loss of H2O to form a benzylic carbocation which further rearranges or decomposes. One way to circumvent this behavior may be to start from an analogous o-methyl aniline, lacking the hydroxyl substituent (Figure 4.24). This could be subjected to diazoamination-acetylation to form the N-acetyl triazene moiety, followed by oxidation of the methyl group to form the carboxyl moiety. This may be accomplished either by direct oxidation with KMnO4, or through a multi-step pathway starting with a benzylic oxidation followed by further oxidation to form the carboxyl. With a more electron-rich core in hand, experiments can be conducted to explore electronic effects on the conversion of the DOC intermediate to benzyne.
Figure 4.24: proposed synthetic scheme for Electron-rich TBA.

The small amount of product produced may also be a result of the electron-deficient nature of 29. Experiments with chemically-generated benzyne show that the cycloaddition step is also susceptible to electronic effects and that electron-poor benzyynes underperform their more electron-rich counterparts. While electron-rich benzyynes do not significantly outperform electron-neutral benzyne, there are slight yield enhancements (Figure 4.25), so if electron-rich DOCs do form benzyne more efficiently, those structural alterations will likely not be a detriment to the cycloaddition step.

Figure 4.25: Reported yields of various electron-poor, -neutral, -rich benzyne click products.

2. Improved Attachment Point for Linkers

Preliminary experiments with 26 indicate that an amide or other electron-withdrawing group is unacceptable as attachment points to the TBA core, and that it would be wise to insulate the aryne from the electronics of any functionality connected to it. An insulated linker
can be installed onto a TBA core starting from 25. First the free carboxyl is converted to a primary amide followed by reduction with lithium aluminum hydride. The newly formed material presents two primary amines, both of which are susceptible to diazotization, but the benzylamine can be selectively protected with either a Cbz or trifluoroacetamine which are insensitive to conditions required for later transformations but can be removed under conditions which do not harm any of the required N-acetyl TBA functionality. The protected material can then be subjected to diazoamination-acetylation forming the corresponding N-acetyl triazenylbenzyl alcohol. From this stage a multi-step oxidation will likely be required to produce the carboxyl without oxidizing the other benzyl site, but Swern conditions or Dess-Martin reagent should selectively oxidize the benzyl alcohol to an aldehyde which can be further oxidized using Pinnick conditions to yield the TBA. De-protection of the benzylamine will unmask a primary amine connected to the core via an insulating alkyl linkage (Figure 4.26). This primary amine should act as an excellent point to attach further functionality through standard activated ester chemistry and the methylene linker should insulate the TBA core from the electronic effects of the appended substituents.

Figure 4.26: Proposed synthetic scheme for improved linker attachment point.

3. The BLInC Reaction for Surface Modification
The linker attachment point can be functionalized with an electrophile which can be used to modify thiol-coated surfaces with TBAs. The BLInC reaction can then be used to pattern that TBA-coated surface by applying dissolved azides, and irradiating selected areas. The BLInC reaction will occur only in those targeted areas, modifying the surface with whatever functionality is attached to the azide such as chromophores, affinity groups, or functionality to modify hydrophobicity. By using masks, different functionality can be targeted to distinct regions of the surface and the options for surface functionality are only limited by what can be attached to an azide.

**Figure 4.27:** Surface patterning.

A TBA-coated surface can be immersed in a solvent containing an azide, and a region masked (shaded areas), followed by irradiation. The BLInC reaction will occur in unmasked areas, shedding the acyl functionality from the TBA and gaining the functionality appended to the azide partner. This procedure can be repeated by unmasking new regions, and co-irradiating with new azides.

The inherent photocleavability of TBAs can also be taken advantage of to swap one type of surface functionality for another. For instance, if a TBA is acylated with a fatty acid, the TBA-coated surface will be hydrophobic in nature. Upon irradiation, the fatty acid will be cleaved, and the resultant benzyne can accept an azide bearing a hydrophilic functional group, creating hydrophilic regions on the surface. The unirradiated regions will remain hydrophobic. This
approach can be broadened to swap any functionality with which a TBA can be acylated with for any functionality which can be appended to an azide (Figure 4.27).

**D. Summary and Outlook for the BLInC Reaction**

The BLInC reaction offers rapid click-like reactivity that does not require copper or any other catalyst, and the reaction can be target to anywhere light can penetrate. The benzyne-azide click reaction proceeds in buffered aqueous media, but the efficiency falls off rapidly as the components become more dilute, indicating that it is not immediately of use as a bioconjugation method. However, improvements may be realized through optimization of the electronics of the TBA probes. In the short term, the more fruitful pathway seems to be utilizing ACN as a solvent. When compared to chemically generated benzyne, the photochemical reaction has similar yields, but hugely enhanced rates. Development of more efficient linker attachment points may allow TBAs and the BLInC reaction to create surfaces patterned with hugely varied functionalities through both the photoactivated cleavage of groups from TBAs, and through capturing functionalized azides.

**E. Experimental Details**

1. **Chemical Syntheses**

Below are the details of the syntheses of critical compounds.

a. **2-(Morpholin-4-yldiazenyl)benzoic acid (21)**
In a RBF with PTFE-coated stir bar cooled in an ice-water bath, anthranilic acid (0.343 g, 2.50 mmol) is dissolved in 22.5 mL of methanol and 7.5 mL of concentrated HCl. Sodium nitrite (0.173 g, 2.50 mmol) is added and the mixture is stirred for 60 min. The pH of the solution is adjusted to approximately 9 by addition of 6N KOH, and morpholine (0.66 mL, 7.5 mmol) in 10 mL of water is added and the mixture is stirred for 3 hrs. Methanol is removed by rotary evaporation and the material is purified by column chromatography using silica gel as the stationary phase and 95:5 v:v DCM:methanol as the mobile phase.

**Yield of 21**: 63% as a colorless solid.

$^1$H NMR (400MHz, CDCl$_3$) δ (ppm): 13.56 (br. s., 1 H), 7.72 (d, J = 8.3 Hz, 1 H), 7.54 (t, J = 7.5 Hz, 1 H), 7.34 (t, J = 7.7 Hz, 1 H), 4.11 - 3.73 (m, 8 H)

LC-MS Calculated for C$_{11}$H$_{13}$N$_3$O$_3$ [m+H]$^+$ = 236.102968, found 236.18

**b. 2-[(2-Hydroxyphenyl)diazenyl]benzoic acid (22)**

**Fig 4.29**: Synthesis of 22.
In a 20 mL scintillation vial with PTFE-coated stir bar cooled in an ice-water bath, anthranilic acid (0.0686 g, 0.50 mmol) is dissolved in 4.5 mL of 2:1 v:v ethanol:water containing 0.50 mL of 6M HCl. NaNO₂ (0.0335 g, 0.50 mmol) is added and the mixture is stirred for 60 min, then 3.0 mL of 1N NaOH is added, followed by phenol (0.0518 g, 0.55 mmol) and the mixture is stirred overnight at 4°C. The reaction mixture is diluted with ethyl acetate and washed with brine, the organic phase is dried over Na₂SO₄ and solvent is removed by rotary evaporation. The material is purified by flash chromatography using silica gel as the stationary phase and ethyl acetate as the mobile phase.

**Yield of 22:** 0.1223 g (0.50 mmol, >99%), yellow solid

¹H NMR (400 MHz, CDCl₃) δ: 8.08 - 8.01 (m, 1 H), 7.83 - 7.77 (m, 3 H), 7.76 - 7.72 (m, 1 H), 7.69 - 7.60 (m, 1 H), 7.59 - 7.49 (m, 1 H), 7.04 - 6.92 (m, 2 H)

LC-MS calculated for C₁₃H₁₀N₂O₃ [m+H]⁺ = 243.076419, found 243.08

c. 4-[[4-((((tert-Butoxy)carbonyl)amino)butyl)carbamoyl]-2-[2-(morpholin-4-yl)diazen-1-yl]benzoic acid (26)

![Diagram of reaction](image-url)

**Fig 4.30:** Amide coupling of 25.

Part 1: In a RBF with PTFE-coated stir bar, 3-amino-4-(methoxycarbonyl)benzoic acid (25) (1.4638 g, 7.50 mmol) is dissolved in 30 mL of DMF, then DIEA (1.65 mL, 10.0 mmol), HOBt·H₂O (1.7228 g, 11.3 mmol), and EDC·HCl (2.1566 g, 11.3 mmol) are reacted for 60 min. Then, tert-butyl (4-aminobutyl)carbamate dissolved in 10 mL of DMF are added, and the
mixture is stirred at ambient temperature for 18 hours. The reaction mixture is diluted with 250 mL of ethyl acetate and washed with 100 mL of water, 100 mL of 0.5M HCl, 100 mL of 50% saturated NaHCO₃, dried over Na₂SO₄, and solvent is removed by rotary evaporation. The material is purified by recrystallization from a minimum volume of boiling methanol.

**Yield:** 2.5269 g (6.92 mmol, 92.2 %), colorless solid

¹H NMR (400M Hz, d6-DMSO) δ: 8.41 (s, 1 H), 7.73 (d, J = 8.3 Hz, 1 H), 7.21 (d, J = 1.5 Hz, 1 H), 6.90 (dd, J = 1.7, 8.4 Hz, 1 H), 6.76 (s, 3 H), 3.32 (s, 2 H), 3.20 (d, J = 6.1 Hz, 2 H), 2.92 (d, J = 6.1 Hz, 2 H), 1.39 - 1.34 (m, 9 H)

LC-MS Calculated for C₁₈H₂₇N₃O₅ [m+H]⁺ = 366.202347, found = 366.04

![Fig 4.31: Synthesis of 26.](attachment:image.png)

Part 2: In a RBF with PTFE-coated stir bar cooled in an ice-water bath, material from step 1 (0.1020 g, 0.28 mmol) is dissolved in 15 mL of 2:1 v:v ethanol : water containing 6 drops of 6M HCl. NaNO₂ (0.024 g, 0.34 mmol) dissolved in 0.5 mL of water is added, and the mixture is stirred for 60 min. The pH of the mixture is adjusted to 9 with 1N NaOH, and morpholine (50 µL, 0.42 mmol) is added and the mixture is stirred for 3 hours. Ethanol is removed by rotary evaporation, and the mixture is acidified with HCl, then extracted with ethyl acetate. Ethyl acetate is removed by rotary evaporation, and the residue is dissolved in 4.0 mL of methanol. The mixture is heated to reflux in an oil bath, and 1.0 mL of 1N NaOH is added. The mixture is stirred for 30 min, then diluted with water and acidified with HCl to precipitate the product. The mixture is extracted
with ethyl acetate and the organic phase is dried over Na₂SO₄. Material can be used without further purification.

**Yield of 26:** 0.1073 g (0.23 mmol, 85.4%), yellow solid.

\(^1\)H NMR (400 MHz, CDCl₃) δ (ppm): 14.06 - 12.79 (Br. S, 1 H), 8.21 - 7.99 (m, 2 H), 7.59 (d, J = 7.5 Hz, 1 H), 7.40 (br. s., 1 H), 4.92 (br. s., 1 H), 4.12 - 3.72 (m, 8 H), 3.49 (d, J = 5.6 Hz, 2 H), 3.16 (d, J = 5.8 Hz, 2 H), 1.75 - 1.54 (m, 4 H), 1.42 (s, 9 H)

\(^{13}\)C NMR (100 MHz, CDCl₃) δ (ppm): 166.74, 156.31, 148.01, 139.60, 132.45, 124.09, 123.40, 115.58, 79.25, 77.33, 66.75, 64.87, 52.59, 45.11, 40.01, 39.91, 28.39, 27.73, 26.34

LC-MS Calculated for C₂₁H₃₁N₅O₆ [m+H]⁺ = 450.23471, found = 450.47

d. 2-(3-Acetyl-3-methyltriaz-1-en-1-yl)benzoic acid (27)

![Reactions](image)

**Fig. 4.32:** Diazoamination.

Part 1: In a RBF with PTFE-coated stir bar cooled in an ice-water bath, 2-aminobenzyl alcohol (1.847 g, 15.00 mmol) is dissolved in 12 mL of water and 2.75 mL of concentrated HCl. Sodium nitrite (1.076 g, 15.60 mmol) dissolved in 5 mL of water is added dropwise and the mixture is stirred for 10 min, then transferred to a dropping addition funnel and then added over ca. 10 minutes to a solution of 36 mL of 8M methyamine in ethanol (cooled to -25°C in an ethanol:glycol-dry ice bath). The reaction mixture is extracted with DCM and the combined
organics are washed with saturated NaHCO$_3$ and brine, dried over Na$_2$SO$_4$, and solvent is removed by rotary evaporation.

![Chemical reaction diagram](image)

**Fig. 4.33:** $N$-Acylation of triazene.

Part 2: The residue is dissolved in 100 mL of DCM and cooled in an ice-water bath. DIEA (10.5 mL, 60.00 mmol) is added, followed by acetyl chloride (1.07 mL, 15.00 mmol) and the mixture is stirred for 2 hours. The reaction mixture is washed with 0.1 N HCl, dried over Na$_2$SO$_4$, and solvent is removed by rotary evaporation.

![Chemical reaction diagram](image)

**Fig. 4.34:** Synthesis of 27.

Part 3: The residue is dissolved in 250 mL of acetone and KMnO$_4$ (3.34 g, 21.1 mmol) is added and the mixture is stirred for 15 hours at ambient temperature. The reaction mixture is titrated with saturated Na$_2$SO$_3$ until the purple color no longer remains. The mixture is diluted with 250 mL of ethyl acetate and carefully acidified to pH 6. The organic layer is washed with brine and dried over Na$_2$SO$_4$, then solvent is removed by rotary evaporation to yield a brown solid. The material is sonicated with 30 mL of 1:1 v:v heptane:ether to precipitate purified product.
Yield of 27: 1.3002 g (40.7%), pale brown solid.

\(^1\)H NMR (400MHz, CDCl\(_3\)) \(\delta\): 12.50 (br. s., 1 H), 8.41 (dd, \(J = 1.5, 7.8\) Hz, 1 H), 7.85 - 7.78 (m, 1 H), 7.72 - 7.64 (m, 1 H), 7.62 - 7.55 (m, 1 H), 3.56 (s, 3 H), 2.68 (s, 3 H)

\(^13\)C NMR (100MHz, CDCl\(_3\)) \(\delta\): 172.41, 165.91, 164.34, 133.87, 133.20, 130.04, 125.12, 116.60, 28.51, 22.00

LC-MS Calculated for C\(_{10}\)H\(_{11}\)N\(_3\)O\(_3\) [m+H]\(^+\) = 222.087318, found = 222.11

e. 2-(3-Acetyl-3-methyltriaz-1-en-1-yl)-5-bromobenzoic acid (29)

\[
\begin{align*}
\text{Br} & \quad \text{OH} \\
\text{O} & \quad \text{LiAlH}_4 \\
\text{NH}_2 & \quad \text{Et}_2\text{O}, 0^\circ\text{C} - \text{RT}
\end{align*}
\]

Fig. 4.35: Reduction of 5-bromoanthranilic acid.

Part 1 - Synthesis of (2-amino-5-bromophenyl)methanol: In a flame-dried 3-neck RBF with PTFE-coated stir bar, septum, glass stopper, and Ar-inlet, 50 mL of ether are cooled in an ice-water bath. LiAlH\(_4\) (0.9 g, 24 mmol) is dissolved, then 5-bromoanthranilic acid is slowly added and stirred for 16 hours at ambient temperature. The mixture was diluted with ether, and quenched with 1 mL of water, 1 mL of 15% w:v NaOH, and 3 mL of water, then filtered. the filter cake was washed with ether and the solvent is removed by rotary evaporation. The material is purified by flash chromatography using a silica gel stationary phase and 95:5 v:v DCM : methanol mobile phase.

Yield: 0.9093 g (4.50 mmol, 57.5%), colorless solid
**Fig. 4.36:** Diazoamination and N-acylation.

Part 2 - Synthesis of 1-{3-[4-bromo-2-(hydroxymethyl)phenyl]-1-methyltriaz-2-en-1-yl}ethanone: In an RBF with PTFE-coated stir bar, cooled in an ice-water bath, (2-amino-5-bromophenyl)methanol from part 1 (0.9000 g, 4.50 mmol) is dissolved in 45 mL of water with 4.5 mL of 6M HCl. NaNO₂ (0.3283 g, 4.9 mmol) is added and the mixture is stirred for 30 min. The reaction mixture is added dropwise to 8 mL of 40% methyamine in water (cooled to 0 °C). The mixture is stirred for 10 minutes and then extracted with DCM which is dried over Na₂SO₄ and removed by rotary evaporation. The residue is dissolved in 80 mL of DCM and cooled in an ice-water bath. DIEA (3.0 mL, 18.0 mmol) is added, followed by acetyl chloride (0.32 mL, 4.5 mmol). The mixture is stirred for 3 hours, then diluted with DCM and washed with 50% saturated NaHCO₃, 0.1M HCl, and brine. The organic phase is dried over Na₂SO₄, and solvent is removed by rotary evaporation. The material is purified by flash chromatography with a silica gel stationary phase and 95:5 v:v DCM : methanol mobile phase.

**Yield:** 0.6577 g (2.30 mmol, 51.6%), red solid

\(^1\)H NMR (400 MHz, CDCl₃) δ (ppm): 7.68 (d, J = 1.9 Hz, 1 H), 7.49 - 7.45 (m, 1 H), 7.43 - 7.39 (m, 1 H), 4.95 (d, J = 6.4 Hz, 2 H), 3.42 (s, 3 H), 2.57 (s, 3 H), 2.37 (s, 1 H)
Part 3 - Final Product Formation: In an RBF with PTFE-coated stir bar, material from part 2 (0.6577 g, 2.30 mmol) is dissolved in 25.0 mL of acetone. KMnO₄ (0.7265 g, 4.60 mmol) dissolved in ca. 5 mL of water is added dropwise and the mixture is stirred for 2 hours. Excess KMnO₄ is destroyed with a few drops of saturated Na₂SO₃, and the mixture is filtered through celite to remove MnO₄. The flowthrough is acidified and extracted with ethyl acetate which is dried over Na₂SO₄.

**Yield of 29**: 0.6155 g (2.05 mmol), pale yellow solid

¹H NMR (400 MHz, CDCl₃)δ (ppm): 8.48 (s, 1H), 7.75 (m, 1H), 7.65 (m, 1H), 3.25 (s, 3H), 2.65 (s, 3H)

LC-MS Calculated for C₁₀H₁₂BrN₃O₂ [m+H]+ = 286.018558, found = 286.09

**f. 2-(1H-Benzotriazol-1-yl)-1-(morpholin-4-yl)ethanone (30)**

**Chemical yield of 30**: 0.0468 g (0.19 mmol, 63.3%)

**Photochemical yield of 30**: 0.1081 g (0.44 mmol, 58.5%)
\[^{1}\text{H} \text{NMR (400 MHz, CDCl}_3\text{) } \delta: 8.08 (d, J = 7.6 \text{ Hz}, 1 \text{ H}), 7.65 - 7.59 (m, 1 \text{ H}), 7.53 (t, J = 7.7 \text{ Hz}, 1 \text{ H}), 7.45 - 7.36 (m, 1 \text{ H}), 5.51 (s, 1 \text{ H}), 3.69 - 3.57 (m, 8 \text{ H})\]

\[^{13}\text{C NMR (100 MHz, } d_6\text{-DMSO) } \delta: 164.9, 145.5, 134.4, 127.6, 124.2, 119.4, 111.5, 49.2, 45.3, 42.4\]

g. \textit{N-}[3-\text{(1H-Benzotriazol-1-yl)propyl} \text{acetamide (31)}}

\textbf{Chemical yield of 31:} 0.0236 g (0.11 mmol, 36.0%)

\textbf{Photochemical yield of 31:} 0.0505 g (0.23 mmol, 30.9%)

\[^{1}\text{H} \text{NMR (400 MHz, CDCl}_3\text{) } \delta: 8.04 (d, J = 8.3 \text{ Hz}, 1 \text{ H}), 7.58 - 7.47 (m, 2 \text{ H}), 7.39 (ddd, J = 1.3, 6.9, 8.3 \text{ Hz}, 1 \text{ H}), 6.42 (\text{br. s., 1 H}), 4.71 (t, J = 6.8 \text{ Hz}, 2 \text{ H}), 3.27 (q, J = 6.5 \text{ Hz}, 2 \text{ H}), 2.24 (\text{quin, J = 6.6 Hz}, 2 \text{ H}), 1.94 (s, 3 \text{ H})\]

\[^{13}\text{C NMR (101 MHz, CDCl}_3\text{) } \delta: 170.6, 146.0, 132.9, 127.6, 124.2, 120.0, 109.3, 45.7, 36.8, 29.0, 23.2\]

h. \textit{1-Benzyl-1H-benzotriazole (32)}

\textbf{Chemical yield of 32:} 0.0417 g (0.20 mmol, 66.4%)

\textbf{Photochemical yield of 32:} 0.1074 g (0.51 mmol, 68.4%)

\[^{1}\text{H} \text{NMR (400 MHz, CDCl}_3\text{) } \delta: 8.06 (td, J = 1.0, 8.1 \text{ Hz}, 1 \text{ H}), 7.43 - 7.26 (m, 8 \text{ H}), 5.84 (s, 2 \text{ H})\]

\[^{13}\text{C NMR (101 MHz,CDCl}_3\text{) } \delta: 146.4, 134.8, 132.8, 129.0, 128.5, 127.6, 127.4, 123.9, 120.1, 109.7, 52.3\]

i. \textit{4-tert-Butylbenzyl 1H-benzotriazol-1-ylacetate (33)}

\textbf{Chemical yield of 33:} 0.0445 g (0.14 mmol, 45.9%)
Photochemical yield of 33: 0.0870 g (0.27 mmol, 35.9%)

\[^1\text{H} \text{ NMR (400 MHz, CDCl}_3\text{)} \delta: 8.10 \text{ (dd, } J = 0.8, 8.3 \text{ Hz, 1 H}), 7.54 - 7.48 \text{ (m, 1 H), 7.45 - 7.36} \text{ (m, 4 H), 7.24 \text{ (d, } J = 8.1 \text{ Hz, 2 H), 5.45} \text{ (s, 2 H), 5.20 \text{ (s, 2 H), 1.32} \text{ (s, 9 H)}}

\[^{13}\text{C NMR (101 MHz, CDCl}_3\text{)} \delta: 166.3, 152.0, 146.1, 133.4, 131.6, 128.5, 127.9, 125.7, 124.1, 120.2, 109.3, 67.8, 49.1, 34.7, 31.3

j. 6-(1H-Benzotriazol-1-yl)hexan-1-ol (34)

Chemical yield of 34: 0.0522 g (0.24 mmol, 79.8%)

Photochemical yield of 34: 0.0757 g (0.35 mmol, 46.0%)

\[^1\text{H} \text{ NMR (400 MHz, CDCl}_3\text{)} \delta: 8.04 \text{ (d, } J = 8.3 \text{ Hz, 1 H), 7.58 - 7.51} \text{ (m, 5 H), 7.50 - 7.43} \text{ (m, 5 H), 7.36} \text{ (dt, } J = 0.9, 7.5 \text{ Hz, 1 H), 4.63} \text{ (t, } J = 7.1 \text{ Hz, 2 H), 3.61} \text{ (t, } J = 6.4 \text{ Hz, 2 H), 2.72} \text{ (br. s., 1 H), 2.00} \text{ (quin, } J = 7.3 \text{ Hz, 2 H), 1.66 - 1.48} \text{ (m, 3 H), 1.47 - 1.31} \text{ (m, 5 H)}

\[^{13}\text{C NMR (101 MHz, CDCl}_3\text{)} \delta: 145.9, 132.9, 127.2, 123.8, 119.9, 109.3, 62.4, 48.1, 32.4, 29.6, 26.4, 25.2

k. 1-(4-Methoxyphenyl)-1H-benzotriazole (35)

Chemical yield of 35: 0.0329 g (0.15 mmol, 48.7%)

Photochemical yield of 35: 0.0671 g (0.30 mmol, 39.7%)

\[^1\text{H} \text{ NMR (400MHz, CDCl}_3\text{)} \delta: 8.13 \text{ (d, } J = 8.1 \text{ Hz, 1 H), 7.72 - 7.62} \text{ (m, 3 H), 7.52} \text{ (t, } J = 7.6 \text{ Hz, 1 H), 7.46 - 7.37} \text{ (m, 1 H), 7.11} \text{ (d, } J = 8.8 \text{ Hz, 2 H), 3.90} \text{ (s, 3 H)}

\[^{13}\text{C NMR (101 MHz, CDCl}_3\text{)} \delta: 159.9, 146.3, 132.7, 130.0, 128.0, 124.6, 124.2, 120.2, 115.0, 110.2, 55.7
1. 1-Phenyl-1*H*-benzotriazole (36)

**Chemical yield of 36:** 0.0331 g (0.17 mmol, 56.5%)

**Photochemical yield of 36:** 0.0478 g (0.24 mmol, 32.6%)

$^1$H NMR (400MHz, CDCl$_3$) $\delta$: 8.15 (dd, $J = 1.0, 8.3$ Hz, 1 H), 7.82 - 7.72 (m, 3 H), 7.66 - 7.58 (m, 2 H), 7.58 - 7.47 (m, 2 H), 7.47 - 7.40 (m, 1 H)

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta =$ 146.5, 137.0, 132.3, 129.9, 128.7, 128.3, 124.4, 122.9, 120.3, 110.4

2. Benzyne-Azide Conjugation Experiments

Small-scale photoreaction of TBAs and azides: Irradiations are performed in 2 mL Microfuge tubes held in a reflective-lined chamber. Reaction volume is kept constant at 50 $\mu$L. Azide 0.25 mM (stock solution in DMSO) and TBA (stock solution in DMSO) are dissolved in PBS, keeping the concentration of DMSO below 1%. Samples are irradiated using a LedEngin(San Jose, CA) High Power 365 nm light emitting diode (200 mW radiant flux) for 60 seconds, then frozen at -80°C until analysis.

Fluoride promoted reaction of 23 and azides: In a 25 mL scintillation vial with PTFE-coated stir bar, 23 (0.35 mmol), CsF (0.60 mmol) and the appropriate azide partner (0.30 mmol) are dissolved in 0.30 mL of ACN and stirred for 18 – 36 hours. Material is purified by flash chromatography.

Large-scale photoreaction of 27 and azides: In a 25 mL scintillation vial held in a reflective-lined chamber, 0.75 $\mu$mole of 27 and 0.75 $\mu$mol of the azide partner dissolved in 5 mL of acetonitrile are irradiated using a LedEngin(San Jose, CA) High Power 365 nm light emitting
diode (200 mW radiant flux) for 15 min. Material is purified by flash chromatography. Yields are the result of 10 combined experiments.

3. LC-MS Conditions

Separation is performed with a Waters 1525 system. The mobile phase gradient employed is A = water + 0.1% formic acid, B = acetonitrile + 0.1% formic acid, 5-95% B over 60 min the column is a Waters XBridge C$_{18}$ column (4.6 x 100 mm). Mass spectra are acquired with a Waters Micromass ZQ mass detector in Ei+ mode: Capillary voltage = 3.50 kV, cone voltage = 30 V, extractor = 3 V, RF lens = 0.0 V, source temp = 100°C, desolvation temp = 200°C, desolvation gas = 300 L/hr, desolvation gas = 0.0 L/hr The system is operated and spectra are processed using the Waters Empower software suite.

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