Synthesis of 3-Trifluoromethyl-3-Pyrimidinyl Diazirines as Photoreactive Amino Acid Analogs

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Dedication

To my parents, for supporting me unconditionally for my entire life.
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Photoaffinity labeling is a biochemical technique used for determining binding behavior of biomolecules. A labeled ligand and a target of interest are covalently crosslinked in response to a specific wavelength of light. These irreversibly bound complexes can then be analyzed by Western blot, mass spectrometry, or any method that can distinguish bound and unbound states. The most common photoactivatable groups used in photoaffinity labeling are aryl azides, benzophenones, and diazirines. Unlike diazirines, benzophenones are large and can disrupt binding interactions, while aryl azides require a short activation wavelength that can degrade proteins. Diazirines possess neither of these disadvantages, however some common diazirine-based photoaffinity labels, the 3-phenyl-3-trifluoromethyl diazirines, are limited in their utility in biologically relevant environments due to their poor stability in ambient light and poor solubility in aqueous media. In response to this limitation, 3-pyridyl- and 3-pyrimidinyl-3-trifluoromethyl diazirine photoaffinity labels that improve on these qualities have been previously characterized.

Photoreactive amino acid analogs are used in photoaffinity labeling of targets that bind to amino acids. These compounds allow a photoactivatable group to be attached directly to a peptide of interest without further chemical conjugation. Photoreactive aromatic amino acid analogs are less common than their aliphatic counterparts, and those that are used, such as trifluoromethyldiazirinyl phenylalanine, lack adequate stability in ambient light (as most diazirines do). Therefore, expansion of the biochemical toolbox to include a photoreactive aromatic amino acid analog with improved ambient light stability is appealing. This work describes efforts towards the synthesis of a 3-pyrimidinyl-3-trifluoromethyl diazirine containing amino acid analog for use in photoaffinity labeling. Negishi coupling is used to form a carbon-carbon bond between an aryl halide and a β-iodo amino acid to form a novel amino acid analog. Since trifluoromethyl diazirines can be
synthesized from trifluoromethyl ketones, significant efforts were made towards trifluoroacetylation of 5-halopyrimidines. However, these halides are not adequately reactive towards metal-halogen exchange. Finally, different experimentally explored and proposed synthetic routes towards these photoreactive amino acid analogs and potentially useful advanced intermediates are explored.
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Table 1. Reaction Conditions for Trifluoroacetylation of 2-Chloro-5-Iodopyrimidine
List of Abbreviations and Symbols

Boc$_2$O = di-tert-butyl dicarbonate
Cat. = catalyst
CDCl$_3$ = deuterated chloroform
Con A = concanavalin A
DCM = dichloromethane
DIBAL-H = di-isobutyl aluminum hydride
DIEA = N,N-diisopropylethylamine
DMAP = 4-dimethylaminopyridine
DMSO = dimethyl sulfoxide
DMF = dimethyl formamide
HTS = high throughput screen
iPr = isopropyl
LC/MS = liquid chromatography-mass spectrometry
Me = methyl
nBuLi = n-butyllithium
NMR = nuclear magnetic resonance
RT = room temperature
SDS-PAGE = sodium dodecyl sulfate poly(acrylamide) gel electrophoresis
$S_N$$_2$ = bimolecular nucleophilic substitution
TBAF = tetrabutyl ammonium fluoride
TBDPS = tert-butyl diphenyl silyl
TBS = tert-butyl dimethyl silyl
THF = tetrahydrofuran
TMEDA = N,N,N',N'-tetramethylethylenediamine
TMS = trimethyl silyl
Ts = para-toluene sulfonoyl
UV = ultraviolet
Chapter 1. Background

1.1 Photoaffinity Labeling

Photoaffinity labeling is a biochemical technique used to elucidate binding interactions. Typical photoaffinity labeling experiments investigate affinity interactions by forming a covalent bond between a labeled probe and the receptor of interest in response to light. Generally, ligand binding is reversible, so this covalent bond forms an irreversibly bound covalent complex which can be analyzed to provide information on solution-phase molecular interactions that might otherwise be difficult to probe. Different families of photolabels exist, however the typical experimental procedure is common between them: a probe labeled with a photoactivatable group is incubated with a protein of interest after which a specific wavelength of light causes the probe to reach an excited state and to form a reactive species. The excited probe then forms a covalent bond to whatever molecule is nearest in space, usually either a bound biomolecule or a molecule of solvent. This covalent complex can then be identified by any suitable method such as Western blot or mass spectrometry.

1.2 Common Photoaffinity Probes

The most common photoactivatable groups are aryl azides, benzophenones, and diazirines. Each of these forms a unique reactive intermediate which bonds the labeled probe to the biomolecule under study\(^1\) (Figure 1).
Figure 1. Mechanism of Activation of Common Photoaffinity Labels\(^1\).

### 1.2.1 Aryl Azides

Aryl azides lose a mole of nitrogen gas when exposed to UV light with a wavelength of 254-400 nm to form nitrenes. These nitrenes react indiscriminately with protein functional groups\(^2\) (Figure 2), but also can rearrange via ring expansion to form a less reactive intermediate, 1,2-dehydrodiazepine, limiting their photochemical yield and thus their utility for photoaffinity labeling\(^2\). The resulting 1,2-dehydrodiazepine reacts less readily with biologically relevant functional groups; for example, it cannot alkylate phenol or ribose\(^3\). Furthermore, the short wavelength for activation can also hinder its efficacy in biological environments, as shorter wavelengths are closer to the absorption wavelengths of proteins (e.g. 280 nm for aromatic residues and 200 nm for peptide bonds), and can potentially degrade and damage proteins of interest\(^4\).
1.2.2 Benzophenones

Benzophenones, in contrast to aryl azides, require a longer wavelength of activation, meaning wavelengths that excite benzophenones are less likely to damage proteins and other biomolecules. The reactive excited state of benzophenones is a triplet diradical, which alkylates proteins via an abstraction-recombination mechanism\(^2\) (Figure 2). The benzophenone oxygen radical first abstracts a hydrogen from the protein, leaving behind a carbon radical (in the case of a C-H bond), a nitrogen radical (in the case of an N-H bond), or an oxygen radical (O-H). This resulting radical then recombines with the benzophenone carbon radical to form a covalent bond between the photoprobe and the analyte of interest (Figure 2). One inherent drawback of benzophenones is their large size relative to other photoactivatable groups. Incorporation of larger functional groups near the binding site increases the likelihood that the photolabel will be too large to fit into the binding site.

1.2.3. Trifluoromethyl Diazirines

Trifluoromethyl diazirines, like benzophenones, have a wavelength of activation above the region that proteins absorb (around 350 nm). However, unlike benzophenones, they are smaller and thus more easily incorporated without interfering with binding interactions. Upon irradiation with light, diazirines irreversibly release nitrogen gas to form a carbene, which reacts rapidly to form the photocrosslinked covalent complex between the analyte of interest and the labeled substrate\(^4\). The rapid insertion kinetics of diazirines also reduce pseudolabeling, or dissociation of an excited photoprobe from the binding site. Pseudolabeling results in off-site covalent modifications of proteins and decreases the accuracy of a photoaffinity labeling experiment. Additionally, diazirines can undergo a rearrangement to form the linear diazo intermediate (Figure 1) which is relatively stable and spectroscopically detectable. Upon further irradiation, the diazo
isomer too forms the carbene, the same reactive intermediate which then undergoes photocrosslinking\(^2\).

**Figure 2.** Insertion Mechanisms for Reactive Intermediates of Common Photoaffinity Labels\(^2\).

### 1.3 Photoaffinity Labeling Experiments

Photoaffinity experiments rely on probes bearing three characteristics: an affinity element, a reporter tag, and a photoactivatable group. The affinity element is the portion of the molecule that binds to the target of interest, determining the binding interaction to be investigated in a specific experiment. The reporter tag allows the labeled proteins to be distinguished from unlabeled proteins. For example, biotinylated photoaffinity probes can be photocrosslinked to a protein of interest. These labeled proteins can then be visualized using anti-biotin horseradish peroxidase antibodies following SDS-PAGE. Finally, the photoactivatable group allows for the irreversible
formation of the covalent bond between the probe and the receptor of interest. In this manner, transient binding interactions that would otherwise be difficult to characterize can be investigated. One possible application of photoaffinity labeling is target identification. For therapeutic compounds whose targets are unknown, photoactivatable analogs can be synthesized and the target can be identified based on what proteins are crosslinked to the labeled probe. For example, one class of histone deacetylase inhibitors, pimelic diphenylamides, was shown by photoaffinity labeling to specifically target histone deacetylase 3 over other histone deacetylases\(^5\).

Another common use of photoaffinity labeling is to determine protein-protein interactions. For example, benzophenones were used to crosslink transcriptional activators, a family of proteins whose protein-protein interactions are poorly characterized due to their highly transient nature\(^6\). This was accomplished by genetically encoding \(p\)-benzoyl-L-phenylalanine, an unnatural amino acid, into proteins of interest, crosslinking with UV light, and analyzing the results via mass spectrometry.

### 1.4 Photoreactive Amino Acid Analogs

An unnatural amino acid that contains a photoactivatable group is called a photoreactive amino acid analog. Diazirine-containing isosteres of leucine and methionine have been synthesized\(^7\).

![Photo-reactive amino acids](image)

**Figure 3.** Commercially Available Photoreactive Amino Acid Analogs and Their Natural Counterparts.
These analogs can be recognized by endogenous protein synthesis machinery and can thus be incorporated into proteins by culturing the organism that expresses the protein of interest in a medium with a lower concentration of the corresponding amino acid. Photoreactive amino acid analogs can also be incorporated into synthetic peptides by solid-phase peptide synthesis, which expands the utility of the class of compounds beyond those which can be incorporated biochemically. For example, a derivative of phenylalanine with a trifluoromethyldiazirine was synthesized in 1984, and subsequently incorporated into a peptide via solid-phase peptide synthesis to probe binding of the calmodulin-binding domain of the plasma membrane Ca\textsuperscript{2+} channel to another region of the same protein. Photoreactive amino acid analogs can also be incorporated into proteins by genetic engineering via stop codon suppression. For example, the previously mentioned phenylalanine derivative was encoded to the amber UAG codon in the adaptor protein growth factor receptor-bound protein 2 and used to crosslink that protein and the epidermal growth factor receptor protein in human embryonic kidney cells. In these three ways, photoreactive amino acid analogs can be incorporated into proteins and peptides for \textit{in vitro} and \textit{in vivo} photoaffinity labeling.

\textbf{1.5. Chemical Synthesis of Amino Acids}

Amino acids and their derivatives are a highly sought-after class of compounds that can be synthesized in several different ways. Since \(\alpha\)-amino acids are chiral, stereoselective methods are preferred as they provide only the desired stereoisomer, whereas reactions with no stereochemical control have a maximum theoretical yield of 50%.
1.5.1 Strecker Reaction

The Strecker amino acid synthesis is used to prepare an α-aminonitrile from an aldehyde. The resulting nitrile can then be hydrolyzed to the carboxylic acid, finally forming the α-amino acid. Asymmetric hydrocyanation, the key step of the Strecker synthesis (Figure 4), of imines has been accomplished with high yield and stereoselectivity using urea-containing Schiff bases as a catalyst\textsuperscript{11}. Metal complexes, such as Al(III)-salen, have also been used to accomplish the same transformation\textsuperscript{12}.

\textbf{Figure 4.} Mechanism of the Strecker Reaction.

1.5.2 Petasis Reaction

The Petasis reaction, also called the boronic acid Mannich reaction, is a multicomponent reaction that can be used to synthesize amino acids by condensation of an amine, an α-ketocarboxylic acid, and a boronic acid (Figure 5)\textsuperscript{13}. Asymmetric variants of the Petasis reaction have also been documented, such as the use of chiral biphenol catalysts, achieving up to 98:2 enantiomeric ratio\textsuperscript{14}.

\textbf{Figure 5.} Synthesis of Alkenyl Amino Acids via the Petasis Reaction\textsuperscript{13}.
1.5.3. O’Donnell Amino Acid Synthesis

The O’Donnell amino acid synthesis (Figure 6) can be used to synthesize amino acids by alkylation of the alpha position of the benzophenone imine of glycine esters\textsuperscript{15}. These O’Donnell Schiff bases are readily deprotonated under basic conditions and can be used to alkylate various electrophiles to provide diverse amino acid derivatives following imine hydrolysis. The reaction typically occurs in biphasic conditions using phase transfer catalysis. By using a chiral phase transfer catalyst, asymmetric alkylation can be achieved\textsuperscript{16}. This procedure is distinct from the Strecker and Petasis methods because the amino acid backbone is already in place and only the side chain is modified during the reaction.

![Figure 6. Asymmetric Variant of O’Donnell’s Amino Acid Synthesis\textsuperscript{16}.](image)

1.5.4 Negishi Coupling

Negishi coupling can also be used to prepare diverse aromatic amino acid derivatives using a starting material that already contains the amino acid backbone (Figure 7). Under metal-catalyzed conditions (typically palladium or nickel catalysts) a zinc iodide prepared from serine is cross-coupled to an aryl halide, forming a new carbon-carbon bond between an amino acid and an aromatic side chain. In this application, the coupling takes place away from the alpha carbon of
the amino acid, preventing racemization and allowing chiral starting materials (e.g. L-serine) to retain their absolute stereochemistry.

![Chemical structure](image)

**Figure 7.** Negishi Coupling of Iodoserine and Aryl Halides.

### 1.6 Pyridine and Pyrimidine Photoaffinity Labels

One common class of photoaffinity labels, 3-phenyl-3-trifluoromethyl diazirines, demonstrate poor solubility in water and poor stability in ambient light, characteristics that limit the utility of these compounds in biochemical experiments. Inspired by this limitation, the Manetsch group synthesized heteroaromatic derivatives (2 and 3) of these labels that improve on these characteristics (Figures 8 and 9).

![Structures](image)

**Figure 8.** Brunner’s Diazirine (1) and Manetsch Group Second-Generation Photolabels (2 and 3).
Figure 9. Synthesis of 3-Aryl-3-Trifluoromethyl Photoaffinity Labels$^{17}$.

Stability of these compounds toward ambient light was studied using $^{19}$F NMR, which showed that the nitrogens in the heteroaromatic substituent significantly stabilize the photoactivatable group. Interestingly, the CF$_3$ group of the linear diazo isomer of 1 is spectroscopically detectable and can be seen at around -60 ppm.
Figure 10. Photoactivation and Carbene Insertion of 1-3 into Methanol-$d_4$. 

![Chemical Diagram](image-url)
To study the labeling capabilities of these compounds, biotin-labeled photoactivatable derivatives of mannose (7-9) were synthesized. Concanavalin A, a lectin that binds mannose, was labeled using the synthetic photolabels. SDS-PAGE followed by anti-biotin peroxidase antibody Western blot demonstrated that photolabeling of Con A occurred successfully only upon photoactivation of the probes. To demonstrate that the binding of the labeled probe was specific to the binding site of mannose, mannose was added to inhibit the binding of the photoaffinity label. Photocrosslinking was significantly suppressed based on concentration of mannose (Figure 12).

**Figure 11.** $^{19}$F NMR Study of Photolabel Stability in Ambient Light$^{17}$. Diazirines (1-3), Methanol Adducts (4-6), and Diazo Isomer of 1 (-60 ppm) are All Detectable.
Chapter 2. Pyrimidine-Containing Photoreactive Amino Acid Analogs

2.1 Purpose

To expand the utility of the Manetsch group photoaffinity label 3, this photoactivatable group was proposed as the side chain for a novel photoreactive aromatic amino acid analog. Incorporation of photoreactive amino acid analogs into the primary structure of proteins and peptides, as opposed to conjugation of a photolabel to a side chain, allows for labeled species to retain a higher degree of structural similarity to their wild-type counterparts. The most commonly used photoreactive amino acid analogs, photo-leucine and photo-methionine, are aliphatic amino acids, so a binding
site of interest that does not contain these aliphatic residues cannot be studied using these labels. Furthermore, these compounds lack the stabilizing trifluoromethyl substituent, which is a major source of the stability of trifluoromethyl diazirine photolabels in the dark. In the case of aromatic amino acid analogs, a trifluoromethyl-diazarinyl analog of phenylalanine was first synthesized in 1984\(^8\), and has been used in photoaffinity labeling experiments, such as aforementioned labeling of the calmodulin-binding domain of the plasma membrane Ca\(^{2+}\) pump\(^9\). However, this compound demonstrates a problem common to 3-phenyl-3-trifluoromethyl-diazirines: it is not stable in ambient light. Based on the results of the \(^19\)F NMR experiments on previous Manetsch group photoaffinity labels, substitution of a pyrimidine in place of the phenyl group in this trifluoromethyl-diazarinyl phenylalanine should improve the stability of the compound toward ambient light. In summary, incorporation of the trifluoromethylpyrimidinyldiazirine as an amino acid side chain expands the utility of photoreactive aromatic amino acid analogs for photoaffinity labeling of peptides and proteins by conferring the ambient light stability improvements of the heterocyclic derivatives over the previous-generation phenylalanine analogues.
2.2 Design of the Proposed Probe

The pyrimidine containing probe (Figure 13) is designed to be structurally similar to aromatic amino acids, particularly phenylalanine and tyrosine, and previous-generation Manetsch group photoaffinity labels. To match these structures, the trifluoromethyl diazirine is retained at the 5-position while the 2-position substituent leads to the amino acid backbone.

![Diagram of the pyrimidinyl diazirine photoreactive amino acid analog](image)

**Figure 13.** Design of the Pyrimidinyl Diazirine Photoreactive Amino Acid Analog.
2.3 Synthetic Overview

Synthesis of the amino acid probe was designed in four main stages:

1. *Preparing the amino acid coupling partner*

Iodoserine, an amino acid with a beta-iodide, has been used in metal-catalyzed cross-couplings with diverse aryl halides\(^\text{18,19}\). Typically, the iodide used in cross coupling has the amino acid N-terminus and C-terminus protected, most commonly with a tert-butylcarbamate and a methyl ester respectively. The iodide can be synthesized from the corresponding optically pure protected amino acid in two or fewer steps.

2. *Cross-coupling of the heteroarene to the amino acid backbone*

Under anhydrous conditions, the L-serine derived iodide is converted to the zinc iodide using zinc powder. This organozinc compound can then undergo a palladium- or nickel-catalyzed cross-coupling reaction with an aryl halide.

3. *Trifluoroacetylation of the aryl halide*

An aryl halide can be made nucleophilic via metalation, e.g. by n-butyllithium, in order to alkylate a trifluoromethyl electrophile, such as methyl trifluoroacetate or N-methoxy-N-methyltrifluoroacetamide, to provide the trifluoromethyl ketone, a common intermediate in synthesis of trifluoromethyl diazirines. Unfortunately for this project, 5-trifluoroacetylations of pyrimidines are rare in the literature, in fact only two procedures are published that trifluoroacetylate a 5-halopyrimidine. A 2018 paper shows an alternative procedure using “turbo Grignard”, or iPrMgCl\(\cdot\)LiCl, to form the nucleophilic carbon\(^\text{20}\).

4. *Modification of the trifluoromethyl ketone to form the diazirine*

The next steps after formation of the trifluoromethyl ketone are formation of the O-tosyloxime, cyclization in liquid ammonia, and oxidation of the resulting diaziridine to the diazirine.
2.4 First-generation Synthetic Route

The first-generation synthetic route (Figure 14) followed the above outline as written. The amino acid coupling partner was prepared from L-serine in 40% overall yield over three steps. This beta-iodo amino acid was cross-coupled to 2-iodo-5-bromopyrimidine. Following trifluoroacetylation, the diazirine could be synthesized from this material based on literature procedures.  

![Chemical Structures Diagram]

**Figure 14.** First Proposed Synthetic Route for the Pyrimidinyl Photoreactive Amino Acid Analog 10.
2.4.1. Preparing the Amino Acid Coupling Partner

Starting from L-serine methyl ester, the amine was protected using di-tertbutyl dicarbonate according to the procedure published by Chen et al.\textsuperscript{21}, forming the N-tert-butyl carbamate protected amino acid methyl ester 11 (figure 15). The primary alcohol was then activated as a leaving group using para-toluene sulfonyl chloride to form 12 in 66\% yield according to a literature procedure by Tabanella et al.\textsuperscript{22}. This tosylate was then displaced using sodium iodide in acetone, an S\textsubscript{N}2 Finkelstein-like procedure from the same publication\textsuperscript{22} to form iodide 13. The classical Finkelstein reaction uses sodium iodide to displace a bromide or chloride, after which the resulting salt (either sodium chloride or sodium bromide) precipitates out due to its poor solubility in acetone, driving the equilibrium towards the desired product. Similarly, sodium tosylate is not soluble in acetone, and can be separated from the reaction mixture via filtration to afford the desired product in 69\% yield. The product, L-iodoserine (13) is then ready for Negishi cross-coupling.

Figure 15. Preparation of Iodoserine 13.

2.4.2. Negishi Coupling

Reaction conditions for Negishi coupling were adapted from a literature procedure\textsuperscript{18}. The coupling partners in this route were protected iodoserine 13 and 2-iodo-5-bromopyrimidine (Figure 16). Addition of the aryl iodide to the zinc iodide (formed in situ) prevents zinc insertion into the aryl halides.
Initially, residual zinc powder made extractions and washes practically difficult, however implementation of a filtration step following dilution of the completed crude reaction mixture solved this issue with no negative effect on yield of 5-bromopyrimidinyl amino acid 14.

**2.4.3 Trifluoroacetylation of the Aryl Halide**

With the 5-bromopyrimidinyl amino acid 14 in hand, the next step of the synthesis is the formation of the trifluoromethyl ketone. The previous-generation pyrimidinyl photoaffinity label was synthesized by metalating the bromide with n-butyllithium followed by nucleophilic addition to methyl trifluoroacetate by the resulting aryllithium species, so this was the first method used to attempt that reaction. Consumption of the starting material was low and the desired product was not detected via LC/MS. This observation prompted the screening of reaction conditions to see if any conditions improve the conversion and yield.

**Figure 16.** Negishi Coupling of Protected Iodoserine 13 and 2-Iodo-5-Bromopyrimidine.
The desired product was not isolated from any of the screened reactions, and residual starting material was detectable across different reaction conditions, suggesting that the bromide is not very reactive. Under conditions where one equivalent of n-butyl lithium was used, low conversion (<10%) was observed via LC/MS, perhaps due to the quenching of the n-butyl lithium by either the methyl ester alpha proton or the carbamate proton. Increasing the amount of nBuLi to two equivalents increased the conversion to about 35%, however the desired product is not formed even after addition of more electrophile. Use of higher amounts of nBuLi, such as five equivalents, resulted in complete consumption of starting material, however neither the quenched intermediate nor the desired product were detected by LC/MS. Magnesium, on the other hand, did not participate at all in the metal-halogen exchange, resulting in no consumption of the starting material. Similarly, LC/MS monitoring of reactions with iPrMgCl•LiCl did not detect the desired product. These observations prompted a reorganization of the synthetic route to minimize the impact of this poor reactivity.
2.5 Reorganized Synthetic Route

The poor reactivity of the 5-bromide in the synthetic amino acid suggested that 5-halopyrimidines may not be readily metalated under typical conditions. In order to counteract this poor reactivity, the steps of the synthetic route were rearranged such that the problematic step occurs earlier in the synthesis, preventing late-stage modifications with low yield from severely depleting the quantity of advanced material available (Figure 18). Trifluoroacetylating the pyrimidine as the first step in the synthesis of that fragment would allow later-stage modifications to not be affected by the potentially poor yield of that transformation. Furthermore, the synthesis was redesigned to include an iodide in place of a bromide, predicting that the higher reactivity of the iodide in comparison to the bromide would enable the desired trifluoroacetylation.

![Figure 18. Reorganized Synthetic Route towards Photoreactive Amino Acid Analogs.](image)

2.5.1 Improved Synthesis of Iodoserine

The previous strategy of preparing iodoserine from the N-(tert-butyl carbamate) protected serine methyl ester by a subsequent tosylation and Finkelstein-like displacement with sodium iodide worked acceptably and provided the desired iodoserine 13 in approximately 40% yield over three steps. However, one issue with that synthetic strategy is that it is time-consuming: each of the three
reactions takes place overnight, meaning the quickest possible timeframe to isolate iodoserine is four days. To overcome this issue, an improved experimental procedure\textsuperscript{23} based on the Appel reaction was employed to transform the side-chain alcohol to an iodide. Triphenylphosphine, imidazole, and iodine are used in an Appel-like reaction to generate an iodide from an alcohol. Upon activation by triphenylphosphine, the OH-derived leaving group is displaced by the iodide generated \textit{in situ}. In the classical Appel reaction, an alcohol is displaced by a halide formed from the corresponding tetrahalomethane. The electrophilic halide source in this modified procedure is molecular iodine. The reactive intermediate, [PPh\textsubscript{3}I]\textsuperscript{+}, is common to both variants. This method eliminates the need for toslyating the alcohol and takes considerably less time than the previous-generation of overnight reactions. The yield of the transformation was also increased to 80%.

![Figure 19. Mechanism of the Classical Appel Reaction.](image)

2.5.2 Trifluoroacetylation of 2,5-Dihalopyrimidines

To ameliorate the poor reactivity of the first-generation 5-bromopyrimidine, 5-iodopyrimidines were selected as a starting material for the second-generation synthesis based on the principle that reactivity increases going down the halogens (i.e. iodides are more reactive than bromides). However, preliminary experiments showed that even 5-iodopyrimidines proved not to be very reactive either under conditions used in the literature to trifluoroacetylate 5-halopyrimidines (Figure 20 and Table 1).
Figure 20. Trifluoroacetylation of 2-Chloro-5-Iodopyrimidine.

Table 1. Reaction Conditions for Trifluoroacetylation of 2-Chloro-5-Iodopyrimidine.

<table>
<thead>
<tr>
<th>Reaction Number</th>
<th>Conditions</th>
<th>Yield*</th>
</tr>
</thead>
<tbody>
<tr>
<td>85</td>
<td>iPrMgCl•LiCl, 0 °C</td>
<td>8%</td>
</tr>
<tr>
<td>87</td>
<td>iPrMgCl•LiCl, 0 °C</td>
<td>n.d.</td>
</tr>
<tr>
<td>89</td>
<td>nBuLi, TMEDA, -78 °C</td>
<td>7%</td>
</tr>
</tbody>
</table>

n.d. = not detected

* Reactions 85 and 89 were chromatographed and concentrated, after which the $^1$H NMR spectrum suggested the presence of significant impurities. To determine the percent yield, the percent purity (based on $^1$H integrals) was multiplied by the measured mass and this value was compared to the theoretical yield.

One potential problem with this approach is regiochemistry. Halides at the 2-position of pyrimidine are more activated than halides at the 5-position. Therefore, if this activated 2-chloride is similar in reactivity to the 5-iodide, then regiochemical selectivity issues should be expected.

Selection of a chloride for the 2-position was meant to minimize this issue in comparison to more reactive 2-halopyrimidines. (i.e. 2-bromo- or 2-iodopyrimidines).

2.5.3 Negishi Coupling of 5-Trifluoroacetyl Pyrimidines

The purpose of the 2-chloride in 17 is to permit cross-coupling following trifluoroacetylation. Negishi coupling of deactivated aryl chlorides to iodoserine has been shown. Therefore the 2-
chloro-5-trifluoroacetyl pyrimidine 17 should undergo Negishi coupling with L-serine zinc iodide under similar conditions to the literature$^{24}$.

![Diagram of Negishi coupling reaction]

**Figure 21.** Proposed Negishi Coupling of 2-Halo-5-Trifluoroacetyl Pyrimidines.

In the case that the 2-chloride is not suitably reactive for cross-coupling, one possible solution is to exchange the halide for a more reactive species by nucleophilic aromatic substitution. Reports of pyrimidine 2-chlorides being exchanged for bromides$^{25}$ and iodides$^{26}$ exist, and both of these would be more reactive Negishi coupling partners. However, with this substrate, another electrophile is present. Since the molecule already has a trifluoromethyl ketone, it is possible that the selectivity of the nucleophile for the 2-chloride over the trifluoromethyl ketone may not be acceptable.

**Chapter 3. Conclusions and Future Directions**

**3.1 Summary**

3-trifluoromethyl-3-aryl diazirines are one of the most prevalent photoactivatable groups due to their high wavelength of activation and small size, allowing for activation without photodegradation of proteins and incorporation near the binding site without disrupting binding interactions. However, traditional 3-trifluoromethyl-3-aryl diazirines are not stable in ambient light, a property that has been improved upon by use of a pyridine or pyrimidine analogue$^{17}$. These 3-heteroaryl-3-trifluoromethyl diazirines are also more soluble in water than their previous-
generation phenyl analogues, a property which expands their utility in biologically relevant environments. Incorporation of the improved pyrimidinyl photoactivatable groups as an amino acid side chain affords a novel photoreactive aromatic amino acid analog with improved light stability and solubility. Photoreactive amino acid analogs are used for photoaffinity labeling of peptides and proteins. However, the most common photoreactive amino acid analogs, photo-leucine and photo-methionine, have limited stability due to their lack of the stabilizing 3-trifluoromethyl substituent present across 3-trifluoromethyl-3-aryl diazirines. Furthermore, these photolabels are not useful in proteins that do not have methionine or leucine residues in their binding site. Therefore, expansion of the biochemical toolbox to include aromatic photoreactive amino acid analogs is desirable, and in fact trifluoromethyl diazirine-containing analogs of phenylalanine have been used⁹. However, similarly to non-amino acid 3-trifluoromethyl-3-phenyl diazirines, these are only stable in the dark. Inclusion of the 3-pyrimidinyl-3-trifluoromethyl diazirine moiety as an amino acid side chain would allow for photoaffinity labeling of sites containing aromatic residues, such as phenylalanine or tyrosine, while avoiding the issues of poor solubility and stability observed in traditional 3-trifluoromethyl-3-aryl diazirine photoaffinity labels.

To this end, a synthetic route towards a 3-trifluoromethyl-3-pyrimidyldiazirine-containing amino acid photolabel is proposed. The synthetic route is centered around Negishi coupling of an aryl iodide to an amino acid side chain, which is appealing due to its ability to retain the absolute stereochemistry of the amino acid alpha carbon. Another key step is the trifluoroacetylation of the 5-position of pyrimidine, which proved difficult due to the poor reactivity of halides at the 5-position.
3.2 Other Synthetic Possibilities

Several other approaches towards the 5-trifluoroacetyl 17 intermediate have promise, and if a scalable synthesis of this compound is achieved then the diazirine can be synthesized in only five more steps.

3.2.1 Pyrimidine Synthesis

Primary syntheses of pyrimidines are rare, but one method used to synthesize pyrimidines is called the Traube synthesis, in which an amidine, urea, or guanidine condenses with a 1,3-dicarbonyl to form the corresponding pyrimidine. Incorporation of relevant substituents on the starting materials for a Traube-like synthesis could shorten the overall synthetic route towards the pyrimidinyl amino acid photolabels. For example, 2-aminopyrimidine-5-ethyl ester 18 was synthesized\textsuperscript{27} from guanidine and ethyl 2-formyl-3-oxopropanoate (Figure 22). This compound could also be potentially used to access the trifluoromethyl Negishi coupling partner 17 in the previously proposed synthetic route (Figure 23).

![Chemical Structure of 2-Aminopyrimidine-5-Ethyl Ester 18](image)

**Figure 22.** Primary Synthesis of 2-Aminopyrimidine-5-Ethyl Ester 18.
3.2.2 Other intermediates via 5-halopyrimidines

Considering the difficulty of trifluoroacetylation of 5-halopyrimidines, perhaps the desired trifluoromethyl ketone 17 could be prepared from a different substituent at the 5-position (such as an aldehyde, ester, or other carboxylic derivative) followed by substitution of the carboxyl derivative with a trifluoromethyl nucleophile (Figure 24). For example, 2-amino-5-pyridine methyl ester 20 has been synthesized via palladium-catalyzed formylation using CO gas in methanol\textsuperscript{28}. Reduction of the ester to an aldehyde affords 2-amino-5-pyrimidine-5-carbaldehyde\textsuperscript{29} 19. Pyrimidine 5-carbaldehydes have been alkylated using trimethyl(trifluoromethyl)silane and cesium fluoride to afford a secondary alcohol which can then be oxidized to the ketone using either MnO\textsubscript{2} or Dess-Martin periodinane\textsuperscript{30}. The 2-amine can also be converted to a chloride via the Sandmeyer reaction\textsuperscript{31}. This strategy, although it has more steps, could afford the trifluoromethyl ketone without need for direct nucleophilic addition to a trifluoromethyl nucleophile.

**Figure 23.** Retrosynthesis of Pyrimidinyl Photoreactive Amino Acid Analogs from 2-Aminopyrimidine-5-Ethyl Ester 18.
Figure 24. Alternative Synthesis of 5-Trifluoroacetyl Pyrimidines.

3.2.3 Other Approaches to Amino Acid Derivatives

Another promising approach to synthesizing advanced intermediates toward the pyrimidinyl photoreactive amino acid analogs is using different synthetic methods to assemble the pyrimidinyl side chain and the amino acid core (Figure 25). For example, 2-(2-pyrimidinyl)-acetaldehyde 21 could be used in the Strecker reaction to form an alpha-amino nitrile, which could then be further functionalized and hydrolyzed to the alpha-amino carboxylic acid (+/-)-10.

Figure 25. Strecker Synthesis of (2-Pyrimidinyl) Amino Acids.

This 2-(2-pyrimidinyl)-acetaldehyde 21 can in principle be accessed from ethyl 2-(2-pyrimidinyl)-acetate, which has been synthesized\textsuperscript{32} in two steps from 2-chloropyridine via alkylation of the 2 position and decarboxylation (Figure 26).
Figure 26. Proposed Synthesis of 2-(2-Pyrimidinyl)-Acetaldehyde 21 from Literature

Synthesis\textsuperscript{32} of Analogous Esters (e.g., R=H)

3.3 Photochemistry

Once the pyrimidinyl photoreactive amino acid analog 10 is synthesized, the next steps will be to demonstrate its viability as a photoaffinity label. These experiments should be similar to those mentioned previously (section 1.6), including thermal and ambient light stability, photoactivation to form the carbene, and photolabeling of an example ligand-receptor pair.

3.3.1 Thermal Stability

Triplicate NMR samples of the photoaffinity probe should be prepared in $d_4$-methanol and stored in the dark at room temperature. The $^{19}$F NMR spectrum should be measured periodically starting at day zero. Additionally, samples of 3-trifluoromethyl diazirinyl phenylalanine should be prepared and analyzed for comparison.

3.3.2 Ambient Light Stability

Triplicate NMR samples of the pyrimidyl- and phenyl-diazirinyl photoreactive amino acid analogs should be prepared in $d_4$-methanol and stored in ambient light (linear fluorescent lamps). As above, the $^{19}$F spectrum should be measured periodically to monitor photo-degradation of the probes.
3.3.3 Photoactivation in UV Light

To determine the kinetics of the formation of the carbene, NMR samples should be prepared as above in $d_4$-methanol and exposed to UV light (350 nm) for two minutes, after which the $^{19}$F spectrum should be taken. This procedure should be repeated until only the methanol insertion products are observed.

3.4 Photoaffinity Labeling

Photoaffinity labeling using the proposed photoreactive aromatic amino acid analogs is limited only by the presence of an aromatic amino acid residue at or near a binding site of interest.

3.4.1 Labeled Peptides and Proteins

Photolabeled peptides can be synthesized using solid-phase or solution-phase peptide synthesis. These labeled peptides can be used to investigate biologically relevant peptides and regions of proteins containing aromatic residues. For compatibility with solid-phase peptide synthesis, the N-fluorenylmethyloxyl carbonyl (Fmoc) protected amino acid should be prepared.

Photolabeled proteins can also be made via genetic incorporation of the unnatural amino acid analogs. The most common method to genetically encode these amino acids is stop codon suppression, wherein an engineered mutant tyrosine tRNA synthetase (TyrRS) is used to acylate a tRNA that corresponds to the stop codon (UAG) with the photolabeled amino acid. Then, culturing the organism in a medium enriched with the photoreactive amino acid analog results in the expression of photolabeled proteins. These proteins can then be used to determine other proteins and small molecules that bind the protein of interest via high-throughput screening.
Chapter 4. Experimental Procedures and Supplementary Data

4.1 Experimental Procedures

All reagents and solvents were purchased from commercial sources and used without further purification. Reactions were carried out under inert atmosphere (Ar) as needed. Flash chromatography was performed on 60 Å silica gel (230 x 400 mesh). $^1$H and $^{13}$C NMR spectra were obtained on a 500 MHz Varian spectrometer at room temperature in CDCl$_3$. Reported chemical shifts (δ) are given in parts per million (ppm) and are referenced to chloroform-d (δ=7.26) or tetramethylsilane (δ=0.00). NMR spectra was processed using MestReNova. Mass spectra were collected on an Agilent 6120 single quadrupole mass spectrometer.

$N$-(tert-butoxycarbonyl)-L-serine methyl ester (11)

To a suspension of methyl-L-serinate hydrochloride (11.9g, 76.4 mmol) in THF (150 mL) was added triethylamine (15.5 g, 153 mmol). The solution was cooled to 0 °C, Boc$_2$O (16.7 g, 76.4 mmol) was added, and the reaction was allowed to stir overnight. The reaction mixture was concentrated under reduced pressure and partitioned between Et$_2$O (100 mL) and H$_2$O (100 mL). The aqueous layer was extracted with Et$_2$O (2 x 60 mL) and the combined organic phase was washed with 1N HCl (60 mL), saturated NaHCO$_3$ (60 mL), and saturated NaCl (100 mL), then dried over anhydrous Na$_2$SO$_4$. The solvent was removed under reduced pressure to afford $N$-(tert-butoxycarbonyl)-L-serine methyl ester 11 (12.8g, 80%) as a colorless oil which was used without further purification. Analytical data are in agreement with literature values$^{21}$. $^1$H NMR (500 MHz, CDCl$_3$) δ 5.43 (s, 1H), 4.40 (s, 1H), 4.02 – 3.94 (m, 1H), 3.87-3.94 (m, 1H), 3.79 (s, 3H), 1.46 (s, 9H). ESI-MS m/z: 120.0 [M-Boc+H$^+$]. TLC (silica, 1% MeOH in DCM) R$_f$ = 0.1.
**N-(tert-butoxycarbonyl)-O-tosyl L-serine methyl ester (12)**

N-(tert-butoxycarbonyl)-L-serine methyl ester 11 (12.8 g, 39.0 mmol) was dissolved in pyridine (125 mL) and para-toluene sulfonyl chloride (14.9 g, 77.9 mmol) was added. The reaction was allowed to stir overnight, diluted with ice water (200 mL), and extracted with ethyl acetate (2 x 100 mL). The organic phase was washed successively with 1M citric acid (3 x 50 mL), saturated NaHCO$_3$ (2 x 50 mL), and saturated NaCl (50 mL), then dried over anhydrous Na$_2$SO$_4$ and concentrated under reduced pressure. The residue was purified via normal phase flash chromatography (silica gel, ethyl acetate:hexanes 1:10-1:2) to afford N-(tert-butoxycarbonyl)-O-tosyl L-serine methyl ester 12 (14.5 g, 66%) as a white solid. Analytical data are in agreement with literature values$^{33}$. $^1$H NMR (500 MHz, CDCl$_3$) δ 7.76 (d, J = 8.3 Hz, 2H), 7.35 (d, J = 8.3 Hz, 2H), 5.29 (d, J = 7.3 Hz, 1H), 4.55 – 4.45 (m, 1H), 4.39 (dd, J = 10.1, 3.0 Hz, 1H), 4.29 (dd, J = 10.1, 3.0 Hz, 1H), 3.70 (s, 3H), 2.45 (s, 3H), 1.42 (s, 9H). ESI-MS m/z: 274.1 [M-Boc+H$^+$]. TLC (silica, ethyl acetate:hexanes 1:2) $R_f$ = 0.4.

**Methyl (R)-2-((tert-butoxycarbonyl)amino)-3-iodopropanoate (13)**

Iodoserine 13 can be prepared via two different methods. Procedure A involves sodium iodide-mediated $S_{N}2$ displacement of the tosyl alcohol of 12 while Procedure B uses a modified Appel reaction to produce iodide 13 directly from primary alcohol 11.

**Procedure A:** To a solution of N-(tert-butoxycarbonyl)-O-tosyl L-serine methyl ester 12 (12.9 g, 34.6 mmol) in acetone (75 mL) was added NaI (10.4 g, 69.0 mmol) in acetone (50 mL). The reaction was allowed to stir overnight after which a white precipitate was observed. The reaction mixture was filtered, the solid was washed with acetone (2 x 50 mL), and the filtrate was
concentrated under reduced pressure. The residue was partitioned between Et₂O (100 mL) and 10% Na₂S₂O₃ (100 mL). The organic phase was washed with 10% Na₂S₂O₃ (2 x 50 mL) and saturated NaCl (2 x 50 mL), dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The residue was purified via normal phase flash chromatography (silica gel, ethyl acetate:hexanes 1:20-1:5) to afford methyl (R)-2-((tert-butoxycarbonyl)amino)-3-iodopropanoate 13 as a yellow oil that solidified upon standing (7.81 g, 69%). Analytical data are in agreement with literature values.³³ ¹H NMR (500 MHz, CDCl₃) δ 5.36 (d, J = 7.7 Hz, 1H), 4.46-4.54 (m, 1H), 3.78 (s, 3H), 3.57 (dd, J = 10.2, 3.8 Hz, 1H), 3.53 (dd, J = 10.2, 3.8 Hz, 1H), 1.44 (s, 9H). ESI-MS m/z: 229.9 [M-Boc+H⁺]. TLC (silica, ethyl acetate:hexanes 1:2) Rᵣ = 0.4.

Procedure B: PPh₃ (1.20 g, 4.56 mmol) and imidazole (311 mg, 4.56 mmol) were dissolved in DCM (40 mL) and I₂ (1.39 g, 5.47 mmol) was added in portions. The solution was allowed to stir for ten minutes. The solution was then cooled to 0°C and a solution of N-(tert-butoxycarbonyl)-L-serine methyl ester 11 (1.00 g) in DCM (10 mL) was added dropwise. The solution was stirred for an additional two hours after which the reaction mixture was washed with H₂O, 10% Na₂S₂O₃ (3 x 50 mL), saturated NaCl (3 x 50 mL), dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The residue was purified via normal phase flash chromatography (silica gel, ethyl acetate:hexanes 1:20-1:5) to afford methyl (R)-2-((tert-butoxycarbonyl)amino)-3-iodopropanoate 13 as a yellow oil that solidified upon standing (1.24 g, 83%). Analytical data are in agreement with literature values.³³ ¹H NMR (500 MHz, CDCl₃) δ 5.35 (d, J = 7.8 Hz, 1H), 4.47-4.58 (m, 1H), 3.80 (s, 3H), 3.59 (dd, J = 10.2, 3.6 Hz, 2H), 3.55 (dd, J = 10.2, 3.6 Hz, 2H), 1.46 (s, 9H). ESI-MS m/z: 229.9 [M-Boc+H⁺]. TLC (silica, ethyl acetate:hexanes 1:2) Rᵣ = 0.4.
**Methyl (S)-3-(5-bromopyrimidin-2-yl)-2-((tert-butoxycarbonyl)amino)propanoate (14)**

To a flame-dried round-bottom flask was added zinc powder (4.21 g, 64.4 mmol) and a stir bar. The flask was heated using a heat gun under vacuum to dry the zinc. Iodine (382 mg, 1.5 mmol) was added and the flask was stirred and heated with a heat gun for ten minutes. At this time, a solution of methyl (R)-2-((tert-butoxycarbonyl)amino)-3-iodopropanoate 13 (7.07 g, 21.48 mmol) in anhydrous DMF (60 mL) was added and the reaction was allowed to stir for 90 minutes under inert atmosphere. After consumption of the starting material was observed via TLC (approximately 30 minutes), Pd(PPh₃)₂Cl₂ (754 mg, 1.07 mmol) and 2-iodo-5-bromopyrimidine (8.14 g, 28.57 mmol) were added. The reaction was allowed to stir overnight at 50 °C under inert atmosphere, after which the solution was diluted with water (300 mL) and extracted with ethyl acetate (3 x 250 mL). The organic layer was filtered, washed with water (3 x 250 mL) and brine (3 x 250 mL), dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The residue was purified via normal phase flash chromatography (silica gel, hexanes - ethyl acetate:hexanes 1:5) to afford methyl (S)-3-(5-bromopyrimidin-2-yl)-2-((tert-butoxycarbonyl)amino)propanoate 14 (3.47 g, 45%).

**1H NMR (400 MHz, CDCl₃) δ 8.69 (s, 2H), 5.69 (d, J = 8.8 Hz, 1H), 4.75–4.83 (m, 1H), 3.68 (s, 3H), 3.50 (dd, J = 16.0, 5.5 Hz, 1H), 3.39 (dd, J = 16.0, 4.6 Hz, 2H), 1.41 (s, 9H).**

**13C NMR (126 MHz, CDCl₃) δ 171.97, 165.29, 157.57, 155.23, 118.24, 79.57, 52.27, 51.81, 40.15, 28.14.** ESI-MS m/z: 360.0 [M+H⁺]. TLC (silica, ethyl acetate:hexanes 1:10) Rf = 0.2. [α]²²D = +27.4 (c = 1.0, CH₂Cl₂). m.p. 79.8-81.4 °C. IR (ATR, cm⁻¹) 3368, 2975, 1747, 1715, 1542, 1500, 1164.
4.2 NMR Spectra

Figure 27. $^1$H NMR Spectrum of 11
Figure 28. $^1$H NMR Spectrum of 12
Figure 29. $^1$H NMR Spectrum of 13 (Procedure A)
Figure 30. $^1$H NMR Spectrum of 13 (Procedure B)
Figure 31. $^1$H NMR Spectrum of 14
Figure 32. $^{13}$C NMR Spectrum of 14
Chapter 5. References


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