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Diaryl Oxazoles as Cleavable Linkers for Drug Discovery Platforms

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By

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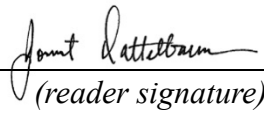

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Abstract

Within the field of medicine and pharmacology, discovering small molecule or biologic-based molecules with therapeutic potential is a difficult task. Current methods involve individually screening hundreds of compounds on a potential target biomolecule, and recent technologies have explored peptide encoded libraries (PELs) as a means of making this screening process more high-throughput. These libraries produce a large number of small molecule drug candidates each conjugated to a unique peptide fragment, functioning as a barcode. Analysis of PELs requires the capture of hit small molecules and the subsequent release of their peptide tags; however, current approaches are limited in their ability to efficiently release these peptides. My work explores the use of a diaryl oxazole as a cleavable linker that is released in mild oxidative conditions to gently liberate peptide labels from captured hits. Oxazoles are an encouraging class of cleavable linkers for peptide-based research due to their ability to be modified with functional groups necessary for solid-phase peptide synthesis, as well as their stability to the harsh conditions associated with this process. The developed cleavable linker also demonstrates compatibility with a variety of natural and abiotic amino acids. Peptide-embedded diaryl oxazoles are quantitatively cleaved in 15 minutes through single-electron oxidation with cerium (IV) ammonium nitrate under mild conditions at ambient temperature. Our reported diaryl oxazole-based linker shows promise as a novel mechanism for the mild release of peptide labels in PELs. Oxazoles as cleavable linkers provide further opportunity for screening methods in the drug discovery pathway.

Introduction

Small molecule drug discovery

Complex diseases are often manifestations of issues in the biochemical and molecular mechanisms underlying biological processes. Because of this, biomolecules such as proteins and DNA are often the culprit of disease. Although there are various forms of therapeutics to target these biomolecules, small molecule drugs continue to dominate the pharmaceutical industry (Makurvet 2021). Compared to biologic-based medicines, small molecule drugs are much lower in cost, making them more accessible to patients. This is in part due to their chemical manufacturing process and high stability (Makurvet 2021). Small molecule drugs are essential therapeutics for the treatment of various issues relating to human health.

Biologically, these drugs are small organic compounds, which bind to membrane receptors or penetrate the cell membrane to interact with intracellular proteins. The binding of small molecules to protein targets occurs in a very specific orientation to the active or allosteric sites. Therefore, only small molecules with specific functionalities and motifs are capable of binding to the desired target. This makes finding a small molecule that binds to a target biomolecule a difficult task within the drug discovery pathway. Traditionally, a chemical library of small molecules is synthesized, and each compound is individually screened against the target biomolecule (Hughes *et al*., 2011). This is a time-consuming and costly process, often requiring millions of dollars to create and screen a full chemical library (Makurvet 2021). However, recent technologies have created high-throughput screening methods that allow this initial compound screening to become more efficient.

DNA-encoded chemical libraries

One innovation in the development of high-throughput methods is the creation of DNA-encoded chemical libraries. As seen through genomes, DNA is able to store information and encode a large number of genes by creating unique sequences of the four bases. This principle has been employed in encoded libraries by conjugating a specific sequence of DNA to a small molecule candidate. Essentially, the sequence of DNA acts as a barcode that can later be used to identify small molecules that interact favorably to the target biomolecule (Neri *et al*., 2018). As opposed to individually synthesizing small molecule compounds, DNA-encoded libraries quickly build a high number of small molecule chemical motifs through split-and-pool synthesis. Chemical motifs are generalized functional groups within a structure, such as aromatic or hydrocarbon groups. In pharmacology and medicinal chemistry, chemical motifs, termed pharmacophores, are responsible for the intermolecular interactions with the biological target (Seidel *et al*., 2020). During this split-and-pool synthesis, these small molecule motifs are conjugated to a corresponding piece of DNA with known sequence. After this initial step, the pieces of DNA are split into different wells, and an additional motif is added with a corresponding DNA tag. All sequences are then continuously pooled together and split to build additional motifs (figure 1). Within hours, hundreds of chemical motifs can be created with their identities encoded within the DNA tag. The various pharmacophores comprising the library can be used to decipher the features of small molecule candidates necessary for binding.

DNA-encoded small molecule libraries have already found success within the pharmaceutical industry since they were first described in 1992. The biotechnology company Nuevolution, now owned by Amgen, was able to create a DNA-encoded library with over a trillion compounds (Makurvet 2021). A traditional library of this scale would simply not be

feasible due to the financial burden of small molecule synthesis and screening. It is estimated that the creation of 1 million compounds costs approximately 500 million dollars, but the creation of 800 million compounds by split and pool synthesis costs only $150,000 (Makurvet 2021). In addition to its practicable synthesis, these libraries have proved capable of discovering small molecules with therapeutic potential. In 2017, a DNA-encoded library was used to identify a small molecule modulator of β_2-adrenergic receptor, a G protein-coupled receptor (Ahn *et al*., 2017). DNA-encoded libraries have made great strides in the drug discovery process by creating a high throughput screening methodology of small molecules.

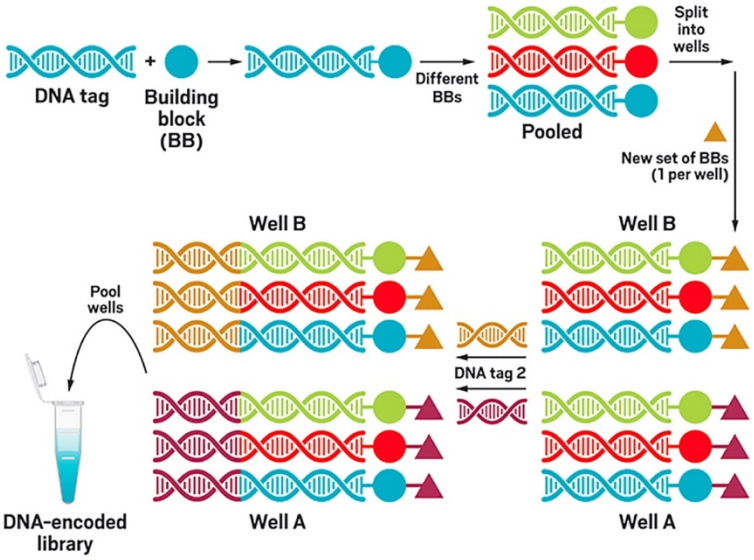

Figure 1. Creation of DNA-encoded library through split-and-pool synthesis. (Makurvet 2021).

Although DNA-encoded libraries have been a major breakthrough in the high throughput screening of small molecules, there are still limitations to this technique (Malone *et al*., 2016). Many organic reactions used during pharmaceutical synthesis require non-physiological conditions, such as the use of metals and acids. One of the most common reagents used in medicinal chemistry is palladium, as it catalyzes the formation of carbon-carbon and carbon-

nitrogen bonds. These harsh conditions, although necessary for the addition of small molecule motifs during split-and-pool synthesis, are not compatible with the DNA tag. DNA is highly degraded and unstable when exposed to common chemical reagents (Rössler *et al*., 2023). This aspect of DNA instability limits the chemical reactions compatible with synthesis of DNA-encoded libraries.

Peptide-encoded chemical libraries

Peptide-encoded libraries seek to improve upon established encoded library techniques by replacing DNA tags with peptides (Rössler *et al*., 2023). Amino acids, the building blocks of peptides, are able to encode information similarly to the base pairs in DNA. Using the same principle as DNA-encoded libraries, the unique amino acid sequence within peptides can be used to store information on the small molecule conjugate. These amino acids are easily incorporated onto small molecules motifs through solid phase peptide synthesis (SPPS). A large peptide library can be generated using the high number of natural and unnatural amino acids with unique molecular weights. These peptide sequences can then be decoded through liquid chromatography-tandem mass spectrometry (LC-MS/MS). This technique determines the peptide sequence based on the mass-to-charge ratios and peak intensities of peptide fragments within the mass spectrum (Rössler *et al*., 2023).

Although similar in principle, peptide-encoded libraries diverge from DNA-encoded libraries in their compatibility with common chemical synthesis methods. Peptides are inherently stable due the strength of the peptide bonds within the backbone. Tautomerization between the carbonyl and adjacent amine within peptides causes the nitrogen-carbon bond to have double bond character (Kamiya *et al*., 2006). The high stability of peptides can be leveraged within the

synthesis of peptide-encoded libraries. In addition to their high stability, the reactive side chains of amino acids are protected in PELs, limiting the potential of off-target reactivity. These characteristics make amino acids much more compatible with common reagents used in chemical synthesis, such as oxidants, acids, bases, and metals like palladium compared to DNA (Rössler *et al*., 2023). This widens the substrate scope and methodology compatible with this method, making it possible to synthesize various chemical motifs. Peptide-encoded libraries are an innovative technology that allow for the synthesis of many chemical motifs using established synthesis techniques.

In order to analyze a peptide-encoded library, hit compounds must first be captured and their peptide tags subsequently released for sequencing. Many purification techniques, such as the affinity of biotin for streptavidin, can facilitate the capture of these hits from the library mixture. However, the release of the peptide tag is a more difficult process given the high affinity of biotin for streptavidin. Cleavable linkers are an avenue for the efficient separation of the small molecule from the peptide tag for sequencing, an essential component for the successful application of peptide-encoded libraries.

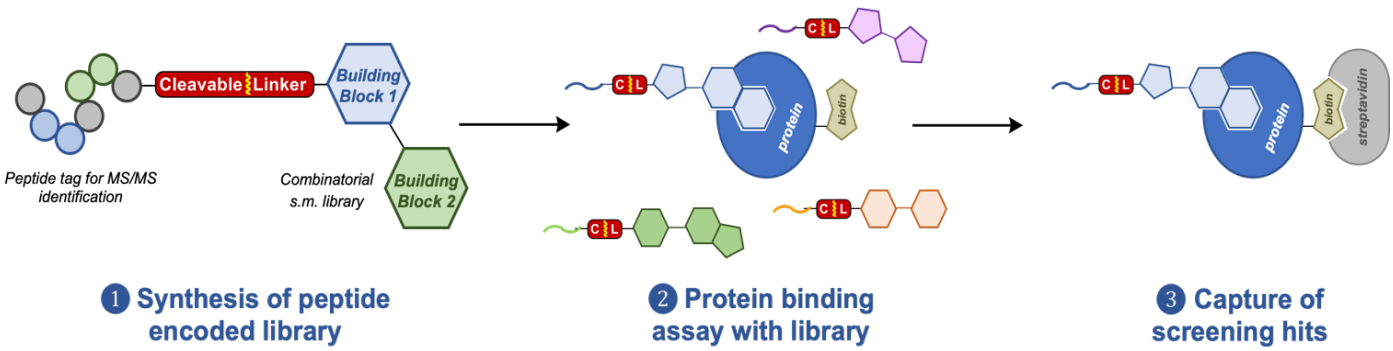

Figure 2. Peptide-encoded libraries allow the high throughput screening of small molecules to a biomolecule, such as proteins.

Oxazoles as cleavable linkers

In general, cleavable linkers connect two biological or chemical species and are cleaved under specific conditions. Many cleavable linkers have been developed within the fields of organic chemistry and chemical biology, which has led to their prominence in biopharmaceuticals. Cleavable linkers are a major component of antibody drug conjugates (ADCs), which are a class of cancer treatments that utilizes an antibody to selectively deliver a cytotoxic payload to cancerous cells. In these systems, the linker facilitates the release of the payload from the antibody (Bargh *et al*., 2019). The specific linker and associated cleavage conditions are dependent on the application. Cleavable linkers, such as those within ADCs, with biological applications are typically cleaved by enzymes or nucleophilic thiols within the body (Bargh *et al*., 2019). In addition to enzymes and nucleophiles, various cleavable linkers have been developed to be cleaved by reducing agents, photo-irradiation, electrophiles, metals, and oxidants (Leriche *et al*., 2012). However, many of these developed cleavable linkers are not stable to the harsh conditions of solid phase peptide synthesis, making them unsuitable in peptide-encoded libraries.

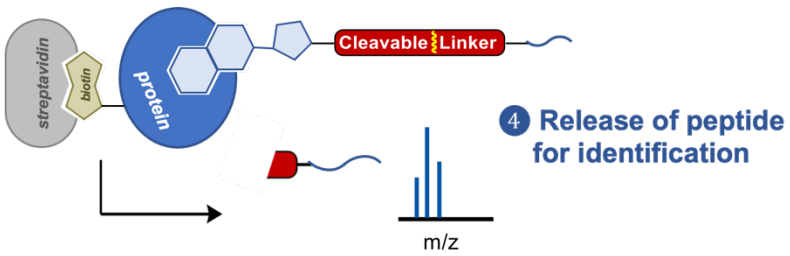

Figure 3. Cleavable linkers facilitate the release of peptide tags for MS/MS sequencing

We aim to investigate oxazoles as potential cleavable linkers for use in peptide-encoded libraries. Oxazoles are 5-membered heterocycles that contain an oxygen and nitrogen separated

by a single carbon. The aromaticity of these rings provides stability to most chemical conditions, which may make them compatible with the harsh acidic conditions of solid-phase peptide synthesis (SPPS). Although stable, aryl oxazoles can be selectively cleaved through single-electron oxidations by cerium ammonium nitrate (Evans *et al.,* 2006). The oxidation of the oxazole causes the ring to open and split into two distinct fragments (figure 4).

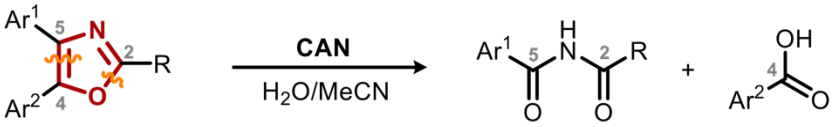

Figure 4. Oxidation of oxazoles promoted by cerium ammonium nitrate (CAN)

The addition of aryl oxazoles within peptide fragments and the subsequent cleavage by cerium ammonium nitrate would create oxazoles functional cleavable linkers. Throughout the oxidation process, it is important to maintain the integrity of the peptide fragment. Previous research has shown that cerium ammonium nitrate does not oxidize or degrade amino acids, making it compatible with our method (Seim *et al*., 2011). Here we report the development of oxazoles as a new class of peptide-based cleavable linkers through single-electron oxidation.

Experimental Methods

The described experimentation was done in collaboration with Dr. Christopher Shugrue, Evan Wolff, and Pamira Yanar.

Abbreviations

Alloc	*N*-Allyloxycarbonyl
DCM	Methylene chloride
DMF	*N*,*N*-Dimethylformamide
DIPEA	*N*,*N*-Diisopropylethylamine
EDT	1,2-ethanedithiol

Fmoc	Fluorenylmethyloxycarbonyl
HATU	Hexafluorophosphate azabenzotriazole tetramethyl uronium
MeCN	Acetonitrile
$NaHCO_3$	Sodium bicarbonate
NMR	Nuclear magnetic resonance spectroscopy
SPPS	Solid-phase peptide synthesis
TIPS	Triisopropylsilane
TFA	Trifluoroacetic acid

Small molecule oxazole oxidations

Single-electron oxidations using cerium ammonium nitrate (CAN) were conducted on various oxazoles following the procedure of Evans *et al*. This reaction was performed on 2,4-diphenyloxazole; methyl 2-methyloxazole-4-carboxylate; 5-ethoxy-4-methyloxazole; and 2-(5-Methyl-2-phenyl-1,3-oxazol-4-yl)ethan-1-ol. All oxidations were performed using 50 milligrams of oxazole substrate. The oxazole was first dissolved in acetonitrile (15 mL/mmol) and water (1.64 mL/mmol). Cerium ammonium nitrate was then added (3.8 equivalents) and the reaction was stirred at room temperature overnight. A liquid-liquid extraction was performed using ethyl acetate and water. The resulting organic layers were washed with $NaHCO_3$ and brine, then dried over sodium sulfate. The solvent was removed by rotary evaporation to give the crude product. The small molecule oxazole oxidation was analyzed by ^1H NMR.

Bifunctional oxazole synthesis

Dr. Christopher Shugrue synthesized a bifunctional diaryl oxazole according to the synthesis scheme in figure 5. This oxazole contains both a carboxylic acid and alloc-protected amine, making it compatible with SPPS. The amine allows for the additional coupling of amino acids or small molecules during solid-phase peptide synthesis. The synthesis of the bifunctional oxazole required a total of eight steps with a total yield of 43%.

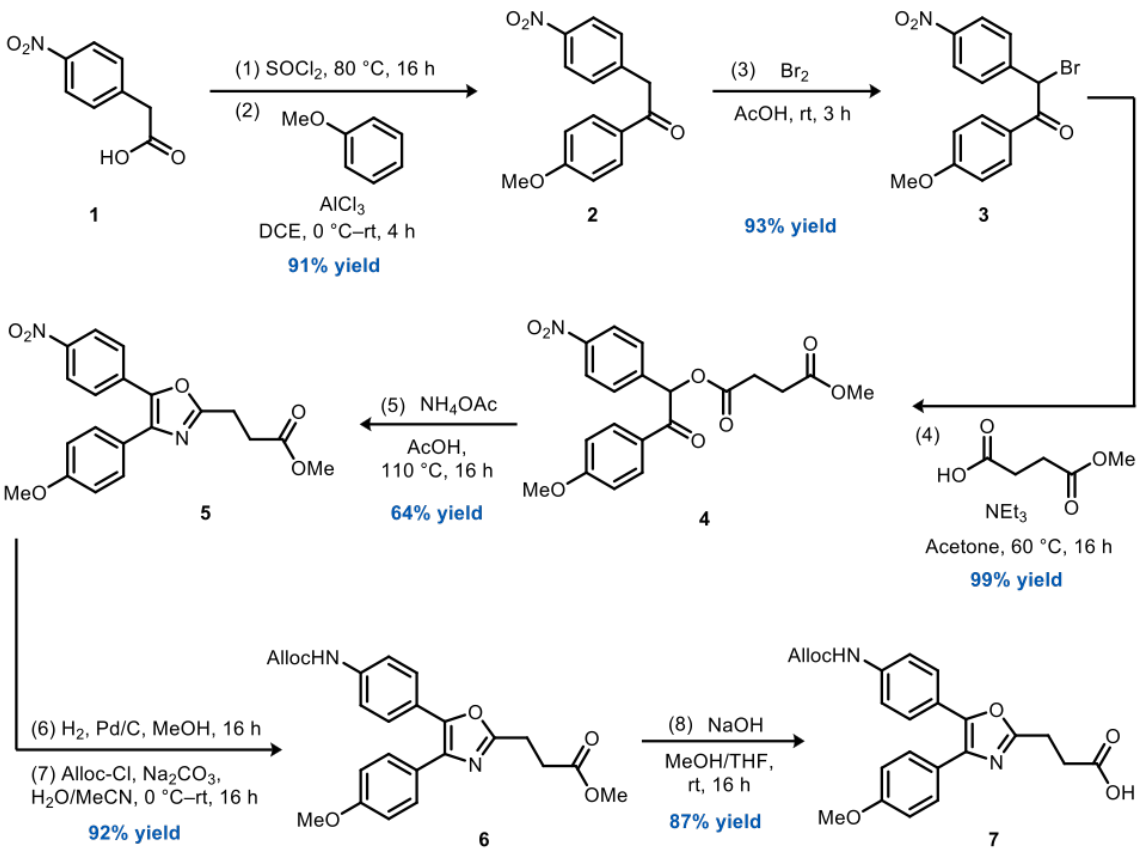

Figure 5. Synthesis scheme of a bifunctional diaryl oxazole (7) containing a carboxylic acid and alloc-protected amine. This synthesis had a total yield of 43%. Conducted by Dr. Christopher Shugrue.

Incorporating oxazoles into peptides

All peptides were synthesized through solid-phase peptide synthesis. The peptide sequences are built on 400 mg of ChemMatrix rink-amide resin (0.2 mmol) from the C to N terminus. Each Fmoc protected amino acid (2 mmol, 10 equivalents) was dissolved in 0.39 M HATU coupling reagent (5 mL) and DIPEA (6 mmol) was added just prior to coupling. The amino acid was coupled for 10 minutes, and the resin was washed with DMF. The amino acid was then deprotected under basic conditions with 20% piperidine for 5 minutes to create a free amine for subsequent coupling. These steps were then repeated for each additional amino acid in the direction of the N-terminus (figure 6).

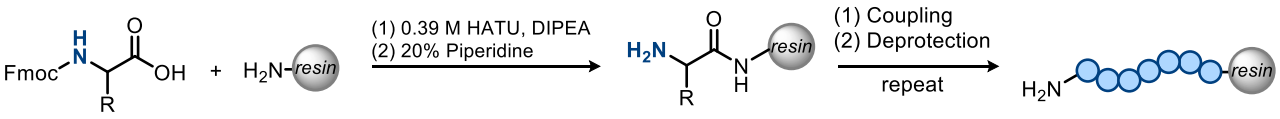

Figure 6. Schematic of solid-phase peptide synthesis (SPPS). The repeated coupling and deprotection of amino acids to the primary amine creates the desired peptide chain.

Both the unnatural amino acid oxaprozin and a bifunctional oxazole were incorporated at the N-terminus of peptides using HATU coupling reagents. Oxaprozin was incorporated into peptides using the same procedure as standard Fmoc-protected amino acids. The bifunctional linker (4 equivalents) was coupled overnight. The bifunctional oxazole (0.05 mmol) was then subsequently deprotected to allow further coupling of amino acids. The alloc protecting group of the bifunctional oxazole was deprotected using $Pd(PPH_3)_4$ (0.005 mmol) in 1 mL of DCM. Phenylsilane (2.5 mmol) was then added to the resin and the reaction proceeded for 1 hour. This was then repeated once. An additional amino acid, either phenylalanine or tyrosine, was coupled to the bifunctional oxazole using HATU reagents before cleavage from resin.

Following the incorporation of oxazoles into peptides, the peptide is cleaved from the rink-amide resin under acidic conditions. For 400 mg of resin, a total of 10 mL of cleavage cocktail is prepared (94% TFA, 2.5% water, 2.5% EDT, and 1% TIPS). The cocktail is added to the resin and mixed at room temperature for 2 hours. The TFA is then removed by N_2 gas. The resulting resin is suspended in cold diethyl ether and pelleted by centrifugation. The supernatant is poured off and the process is repeated to remove any excess TFA. The pellet is then dissolved in 95:5 acetonitrile: water and the remaining resin is removed using a fritted syringe. The peptide was purified using a C_{18} reverse phase column by the Biotage Selekt Flash Purification System with a gradient of water (0.1% TFA) and acetonitrile (0.1% TFA) as the mobile phase. Following

purification, the peptide is lyophilized to provide the peptide as a solid white powder. The identity and purity of the peptide was determined using MALDI-TOF.

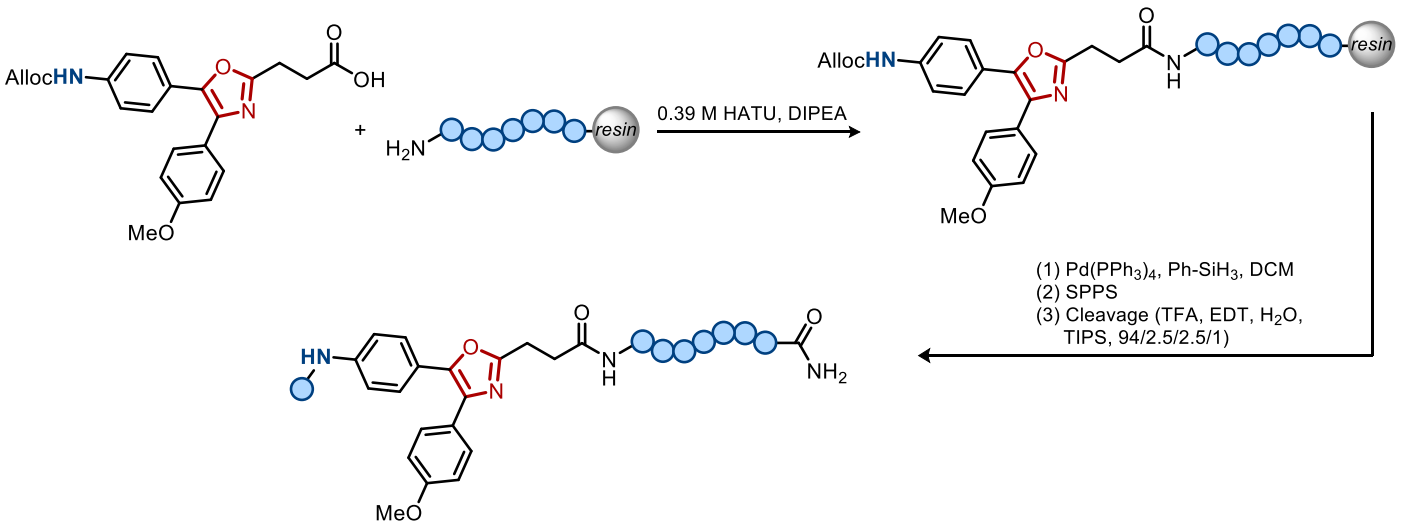

Figure 7. Incorporation of bifunctional oxazole into peptides. After the alloc protecting group is removed by palladium, an additional amino acid is coupled via SPPS.

Peptide library synthesis

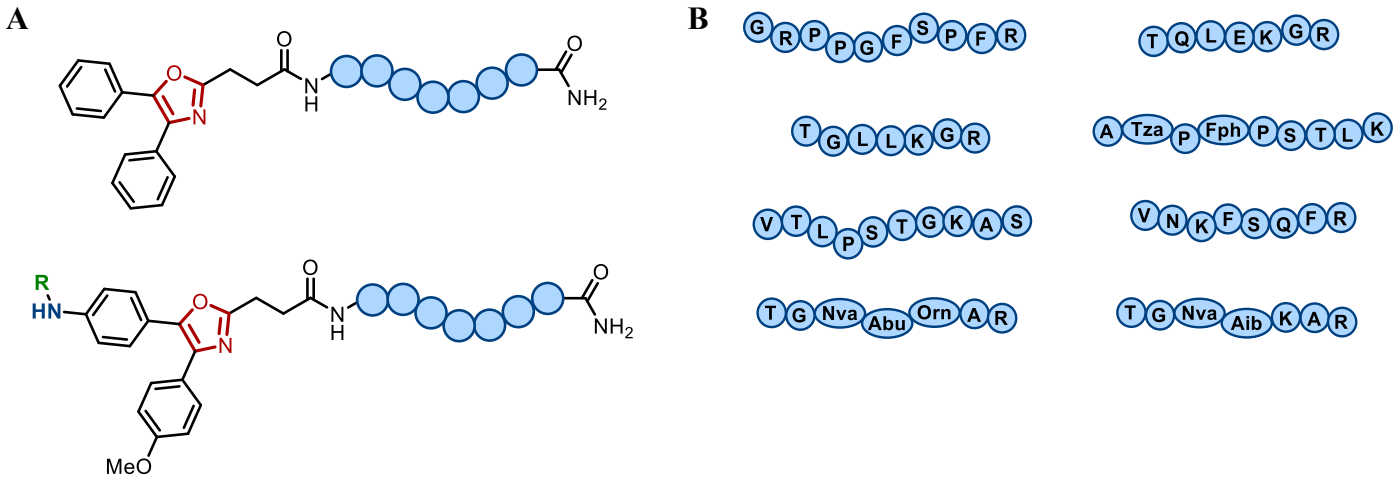

Figure 8. Synthesis of peptide library. (A) Both oxaprozin and the bifunctional linker was coupled to each peptide sequence. R=Tyr or Phe. (B) Eight unique peptide sequences containing a variety of natural and unnatural amino acids

A peptide library comprising 8 unique amino acids sequences was synthesized according to the previously described solid-phase peptide synthesis procedure. These sequences encompassed a high number of natural and unnatural amino acids. Both oxaprozin and the bifunctional oxazole were coupled to each sequence, creating a total of 16 peptides (figure 8). This library was synthesized primarily by Evan Wolff and Pamira Yanar.

Oxidation of peptide-embedded oxazoles

Solid peptides were dissolved in water to a final concentration of 5 mM. Oxidation conditions were optimized using peptide sequences GRPPGFSPFR and TGLLKGR containing oxaprozin at a final concentration of 1 mM. The components of the oxidation reaction were oxazole-containing peptide, cerium ammonium nitrate, buffer, water (95%), and acetonitrile (5%) to a final volume of 20 µL. Citrate (pH 3 and 5), TRIS (pH 7), phosphate (pH 7), and HEPES (pH 7) buffers were screened at a final concentration of 10, 50, and 100 mM. The concentration of cerium ammonium nitrate was also varied at 5, 50, and 100 mM. Reactions proceeded at room temperature for 1 hour and were then quenched with 50 mM sodium thiosulfate (20 µL).

The optimized reaction conditions were performed on a 250 µM scale, according to table 1. Peptides containing oxaprozin or the bifunctional oxazole were oxidized at room temperature with cerium ammonium nitrate for 1 hour or 15 minutes respectively. Reactions were quenched with 20 µL of 50 mM sodium thiosulfate before analysis by MALDI-TOF and HPLC. Oxidations of each peptide were performed in triplicate.

Table 1. Optimized oxazole oxidation conditions

Compound	Stock concentration	Volume (µL)	Final concentration
peptide	5 mM	1	250 µM
Cerium ammonium nitrate	100 mM	2	10 mM
HEPES buffer (pH 7)	100 mM	2	10 mM
water	neat	14	95%
acetontrile	neat	1	5%

Analysis of oxazole oxidation on peptides

Matrix Assisted Laser Desorption/ Ionization- time of flight (MALDI-TOF) was used to identify peptides based on molecular mass. MALDI-TOF is a form of soft mass spectrometry that utilizes a matrix to ionize biomolecules without fragmentation, revealing the molecular ion with high accuracy. The plate was first spotted with 1 µL of 5 mg/mL sinapinic acid in acetone. After this first matrix spot is dry, 1 µL of the quenched reaction mixture is spotted with 1 µL of 5 mg/mL sinapinic acid in 75:25 acetonitrile: water (0.1% TFA). The peptide Angiotensin was used to calibrate the MALDI instrument. The mass of peptides was determined using Shimadzu AXIMA Confidence on reflectron mode.

High performance liquid chromatography (HPLC) was used to quantify the conversion of oxidation reactions with cerium ammonium nitrate. HPLC is a type of liquid chromatography that utilizes high pressures to separate compounds based on polarity. A reverse phase C_{18} column was used as the stationary phase. A gradient of water (0.1% TFA) and acetonitrile (0.08% TFA) was the mobile phase at a flow rate of 0.5 mL/min. The concentration of acetonitrile was linearly increased from 5% to 95% over the course of the one-hour run. The quenched reaction mixture was centrifuged and filtered to remove excess salt or oxidant. Peptides were detected at the wavelength of 254 nm.

Results and Discussion

Aryl substituents are required for oxazole oxidation

The oxidation of oxazoles promoted by cerium ammonium nitrate is dependent on the functionality of the oxazole. When exposed to cerium ammonium nitrate, the oxazoles methyl 2-methyloxazole-4-carboxylate (figure 9A) and 5-ethoxy-4-methyloxazole (figure 9B) had no conversion to product. These reactions reveal that cerium ammonium nitrate oxidation is not compatible with all oxazoles, such as those containing ester and ethoxy groups.

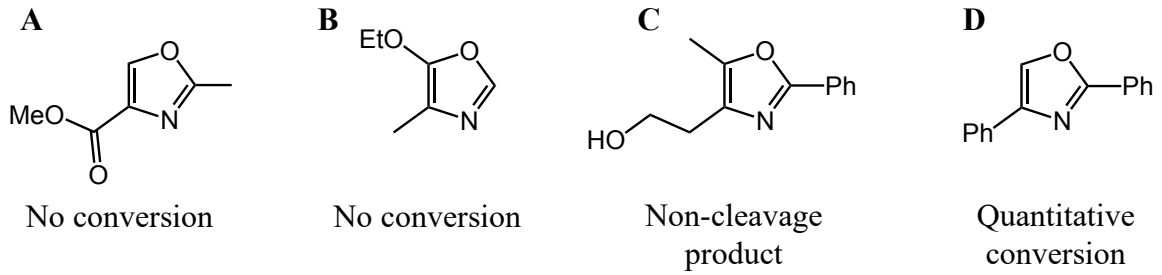

Figure 9. Reactivity of oxazoles containing various substituents. Oxazoles lacking phenyl rings are not oxidatively cleaved.

The reaction of 2,4-diphenyloxazole (figure 9D) with cerium ammonium nitrate resulted in the complete conversion to the expected product (figure 10A). The oxidation product was revealed by NMR, due to the creation of an imide which has a broad signal and high chemical shift at 9.29 ppm. The two adjacent carbonyls and attached phenyl rings explain the high chemical shift compared to a typical secondary amine.

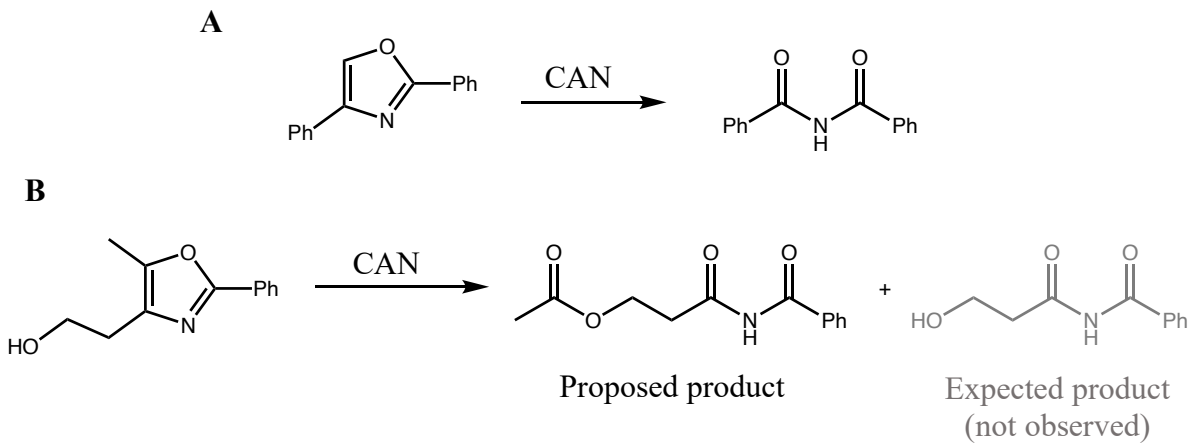

Figure 10. Oxidation of oxazoles containing aryl substituents by cerium ammonium nitrate (CAN). (A) The oxidation of a diphenyl oxazole creates the expected imide product. (B) Observation of an unexpected cleavage product after oxidation by CAN.

Interestingly, the oxidation of 2-(5-Methyl-2-phenyl-1,3-oxazol-4-yl)ethan-1-ol (figure 9C) did not progress as expected. During the expected reaction, the methyl substituent of the oxazole is cleaved from the product, however it was still present in the NMR. To further investigate the identity of the product, infrared spectroscopy (IR) was conducted on the product. Although a hydroxyl group is in the expected product, a corresponding broad signal in the range of 3300 cm^{-1} was not present in the IR spectrum. Based on the NMR and IR spectra, we propose the formation of the following non-cleavage product (figure 10B). The hydroxyl group likely participated in the reaction mechanism to create the observed product. Although this reaction did not follow the expected mechanism, it does support that this oxazole is oxidatively cleaved. Based on the four oxazoles investigated, an aryl substituent is required for the oxidation by cerium ammonium nitrate. The mechanism of oxazole oxidation remains unknown, but the proposed mechanism predicts the formation of a radical or cation (Seim *et al*., 2011). These aryl substituents likely function to stabilize these species during a single-electron oxidation. Upon

oxidation, aryl substituents are able to delocalize the radical or cation intermediate through resonance.

Oxazoles are compatible with solid-phase peptide synthesis

An essential requirement for our cleavable linker is its incorporation into peptides through solid-phase peptide synthesis. During SPPS, coupling of an amino acid is facilitated by its carboxylic acid functional group reacting with the primary amine of the growing peptide chain. Oxaprozin is a commercially available oxazole containing a carboxylic acid, making it capable of coupling to peptides as an unnatural amino acid during SPPS. The successful synthesis of the peptide GRPPGFSPFR containing oxaprozin was confirmed by the molecular ion in MALDI-TOF (figure 11A). Oxaprozin was successfully incorporated into peptides using standard HATU coupling reagents. In addition, oxaprozin was not degraded by the harsh cleavage conditions from resin, which involves 94% trifluoroacetic acid (TFA). Oxaprozin is not only able to integrate into peptides through standard coupling techniques but is stable to the cleavage conditions from resin.

The incorporation of oxaprozin into peptides displays the compatibility of oxazoles with SPPS. However, oxaprozin is limited in its ability to function as a cleavable linker because it lacks an amine to facilitate the additional coupling of amino acids or small molecule cargo. A bifunctional oxazole was therefore synthesized in the Shugrue lab to overcome this limitation. This oxazole contains both a carboxylic acid for coupling to peptides as well as a protected amine for the subsequent addition of molecules. The amine is initially protected by an Alloc group to prevent off target reactions during the initial coupling step. Following the coupling of the bifunctional linker to the peptide GRPPGFSPFR, an additional amino acid, tyrosine, was

then successfully coupled to the amine of the oxazole (figure 11B). This synthetic scheme using the bifunctional linker demonstrates that oxazoles can be coupled to peptides and allow the additional incorporation of other molecules, such as amino acids.

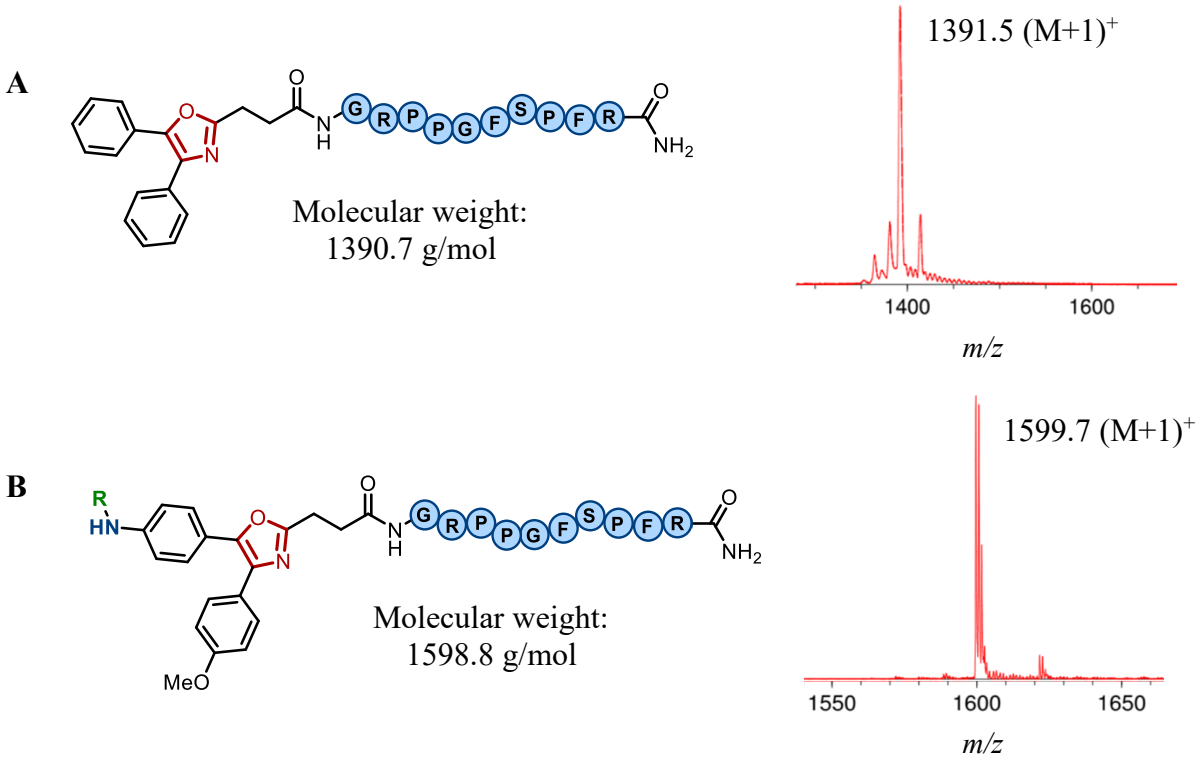

Figure 11. Successful synthesis of oxazole-containing peptides confirmed by MALDI-TOF. The molecular weight of peptide GRPPGFSPFR incorporating oxaprozin (A) and the bifunctional oxazole (B) is the major peak in MALDI-TOF. R=Tyr.

Peptide-embedded oxazoles are cleaved by CAN

The cleavage of oxazoles by cerium ammonium nitrate is demonstrated when incorporated onto peptides. However, the specific reaction conditions impact both the rate of reaction and the total conversion. Preliminary oxidations on 1 mM peptide with various concentrations of buffer and cerium ammonium nitrate revealed the optimal conditions involve buffer and cerium ammonium nitrate at a concentration of 50 mM. Under these conditions, there

was complete oxidation of peptide after one hour, as the starting material was no longer detected by MALDI-TOF. However, in addition to the expected imide product, a side product was also formed. The oxidation of oxaprozin caused the formation of a benzil byproduct, which was also observed by Evans *et al.* Interestingly, the formation of this side product could be controlled by the buffer. The use of phosphate and TRIS buffers resulted in a high proportion of benzil byproduct observed (figure 12B). However, the use of HEPES buffer minimized the formation of this side product, with the expected product making up the majority of the mixture (figure 12C).

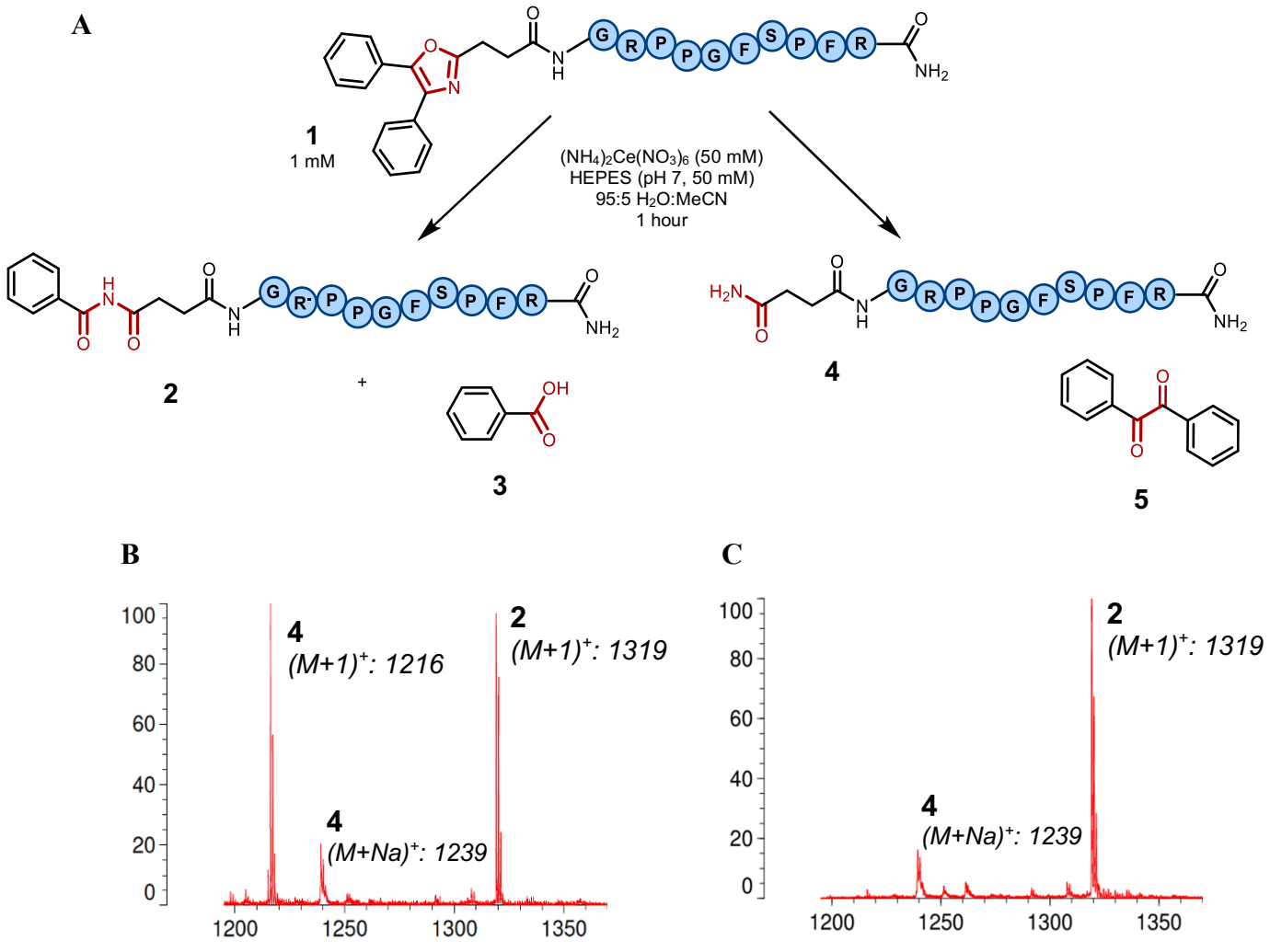

Figure 12. Oxaprozin cleavage by cerium ammonium nitrate causes formation of two distinct products. (A) Oxidation of oxaprozin creates both the expected imide product (2) as well as a side product 4. This byproduct is formed by the release of a benzil molecule (5). (B) MALDI-TOF of reaction with TRIS buffer results in the formation of both products at approximately equal amounts. (B) MALDI-TOF of reaction with HEPES buffer favors the expected imide product. Both buffers were at pH 7 and 50 mM concentrations.

MALDI-TOF provides information on the identity of the peptide based on its molecular weight. However, this alone does not give quantitative information on the conversion and purity of the reaction. High-performance liquid chromatography (HPLC) was used to assess these aspects of the reaction. The change in retention time supports the change in polarity between the peptide before and after oxidation. In addition, the number of peaks reveals the purity of the reaction product. Oxaprozin within peptide TQLEKGR was completely cleaved at a concentration of 250 μM. The formation of the expected product was confirmed by MALDI-TOF, and the reaction was highly pure, as shown by HPLC (figure 13). Diaryl oxazoles are able to be quantitatively cleaved at mild pH and low concentration by cerium ammonium nitrate.

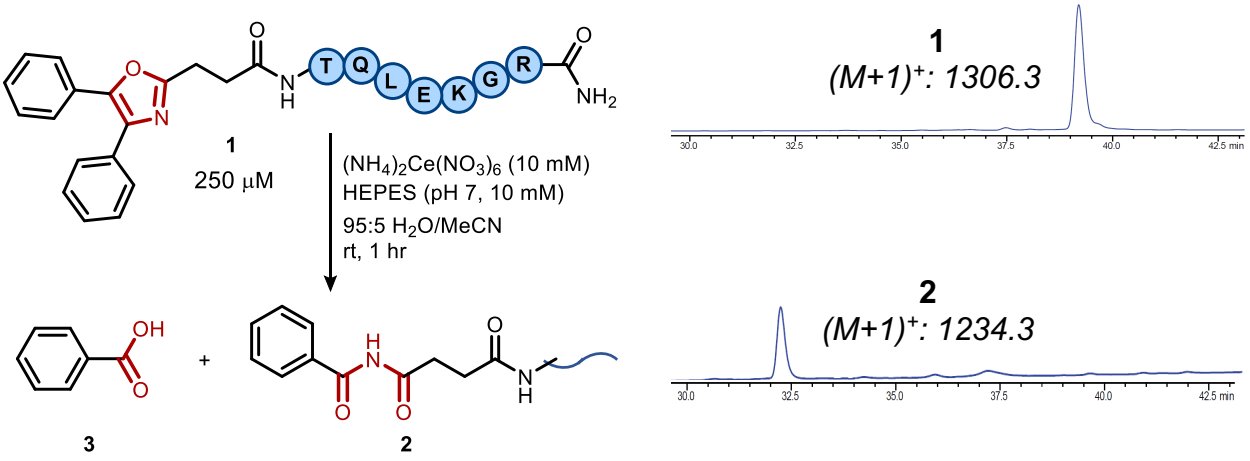

Figure 13. Oxaprozin cleavage is highly pure according to HPLC. The oxaprozin containing peptide (1) and its cleavage product (2) have retention times of approximately 39.25 and 32.25 minutes respectively. HPLC runs of both peptides display high purity.

The bifunctional oxazole is also rapidly oxidized by cerium ammonium nitrate. Oxaprozin was completely cleaved in approximately one hour, but the bifunctional linker was cleaved in as little as 15 minutes. This rapid oxidation of the bifunctional oxazole is likely due to the presence of the methoxyphenyl substituent on the phenyl ring. This electron donating group

provides additional stability to the cation or radical created during the single-electron oxidation, as these are electron deficient species.

The oxidation of the bifunctional linker has a wide peptide substrate scope, encompassing many amino acids with various functionalities. Amino acids are largely characterized as polar, nonpolar, and charged based on their unique R-group. These functionalities may impact the stability of the peptide sequence when treated with cerium ammonium nitrate. An eight-membered peptide library was synthesized with the bifunctional linker to determine the compatibility of amino acids with this method. All eight tested peptide sequences had successful cleavage, as seen by MALDI-TOF and HPLC (figure 14). This reveals that the bifunctional cleavable linker is practical for use in large peptide libraries.

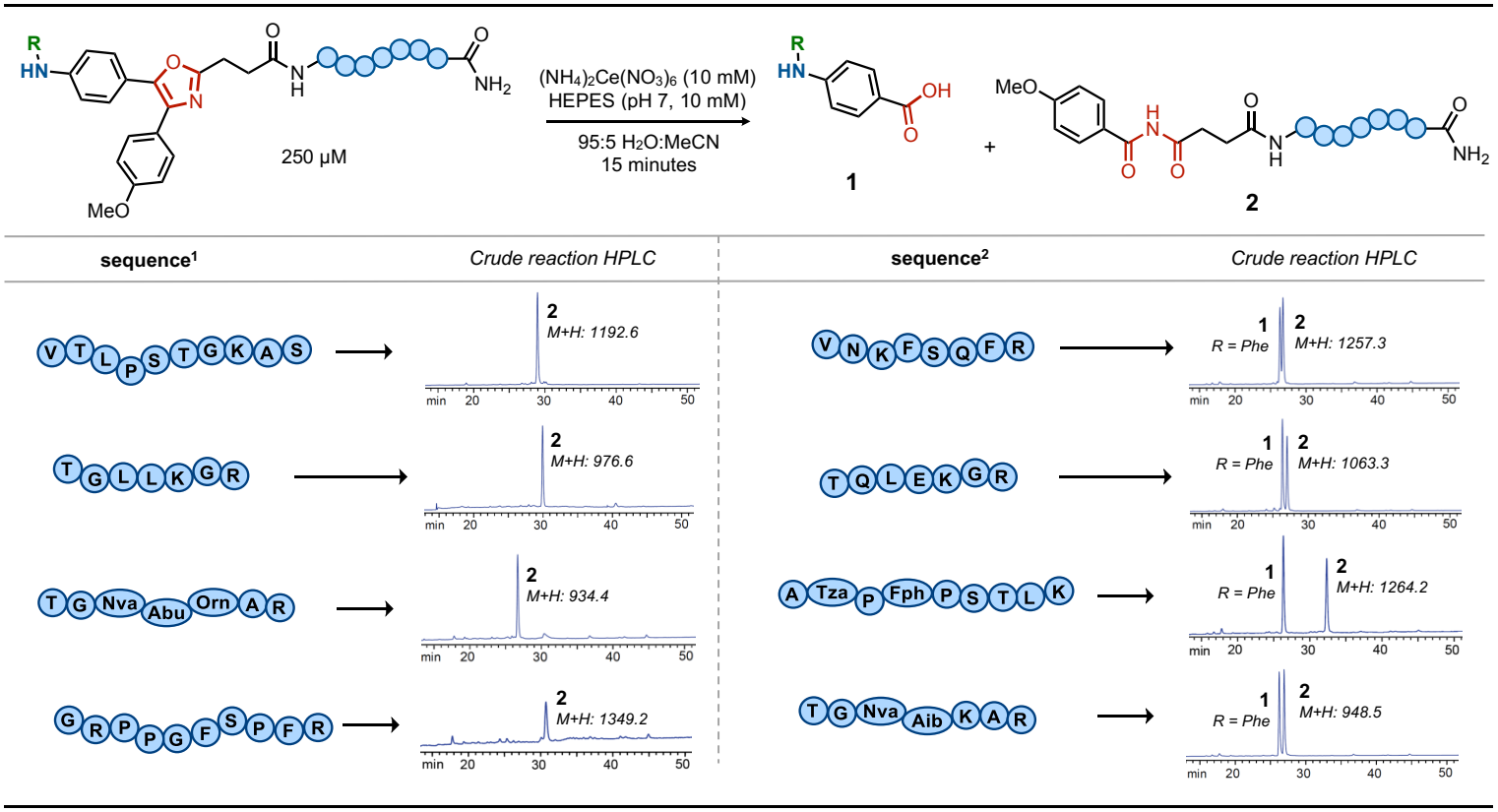

Figure 14. Peptide sequence compatibility with cerium ammonium nitrate oxidation. Most natural amino acids and many unnatural amino acids are stable to the cleavage conditions. HPLC runs reveal purity of each peptide following oxidation (2), with their masses confirmed by MALDI-TOF. Sequence[1] and sequence[2] indicate the additional R group is tyrosine and phenylalanine respectively. In peptides containing the additional phenylalanine residue, byproduct 1 was observed in the HPLC.

Conclusions

Diaryl oxazoles are promising cleavable linkers because of their inherent stability to most conditions, except cerium ammonium nitrate oxidation. During this single-electron oxidation process, diaryl oxazoles are rapidly cleaved under mild pH into two separate compounds. These oxazoles are especially useful for peptide-based research, as they are compatible with solid-phase peptide synthesis. Oxazoles can be incorporated into peptides using standard HATU coupling procedures and are stable to the acidic cleavage conditions from resin. One application of this diaryl oxazole linker is within peptide encoded libraries to release peptide-based tags. These library technologies have made significant advances in the high throughput screening of small molecule drugs, and our oxazole would continue to improve upon this technique.

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