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Development of a Diaryl Oxazole-Based Cleavable Linker for Peptides

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by

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Abstract

The development of new cleavable linkers increases the diversity of compatible conditions for peptide discovery platforms. Potential applications for these linkers include high-throughput pharmaceutical candidate screening when utilized in Peptide Encoded Libraries (PELs). This thesis describes the development of a bifunctional diaryl oxazole-based cleavable linker that may be incorporated into compounds through Solid-Phase Peptide Synthesis (SPPS). This oxazole-based linker may be rapidly cleaved by cerium ammonium nitrate in aqueous conditions and is compatible with most natural amino acids and a variety of unnatural amino acids. This linker represents the first single-electron oxidant labile linker described to our knowledge and it demonstrates orthogonal cleavage to traditional oxidatively cleaved linkers. A greater diversity of cleavable linkers increases the versatility of various peptide discovery platforms and oxazole-based linkers demonstrate novel mechanisms for the mild oxidative release of attached compounds.

Introduction

Small molecule drug development

Small molecule drugs represent some of the most diverse and widely used pharmaceuticals in medicine. These drugs are desirable due to their relative stability and high bioavailability when absorbed through mucous membranes (Zurdo, 2013). The development of small molecule drugs traditionally takes an average of 13.5 years and costs over $1 billion USD per drug released to market (Hughes *et al.*, 2011). The most cost and time intensive portion of the drug development process is the pre-clinical identification of compounds which interact with a desired biological target (Zurdo, 2013). A contributing factor to the resource intensity of this process is the need for chemists to synthesize each candidate molecule and screen them individually against a biological target. Reducing the time and resources necessary to screen drug candidates would decrease the cost of pharmaceuticals and increase the rate at which new bioactive compounds are identified (Makurvet, 2021).

DNA-encoded small molecule libraries

One method to increase the rate of drug candidate identification is using high-throughput biological screening techniques to validate compounds' interactions with their targets rapidly and efficiently in one step. DNA-encoded small molecule libraries are one such technique already used in some drug development applications (Kleiner *et al.*, 2011). This method for screening potential drug candidates begins with a short segment of single-stranded DNA functionalized on its 5' end (Kleiner *et al.*, 2011). Onto this functional group, small molecules can be added through split and pool synthesis.

Split and pool synthesis involves splitting a reaction between multiple vials to react with unique components. These separate compounds are then recombined and mixed to randomly disperse the compounds together. Upon subsequent allocation to new vials and with repetition,

this process can generate hundreds of thousands of unique compounds while maintaining control of the compounds' identities at each position.

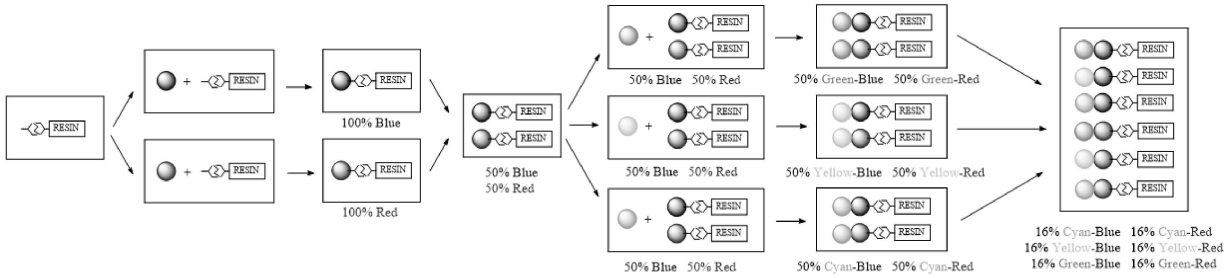


Figure 1. Example scheme of a split and pool library synthesis workflow. Unique DNA primers are represented by colors.

In DNA encoded small molecule libraries, the split and pool synthesis alternates between organic modification to a growing drug candidate and enzymatic nucleotide addition to the DNA tag encoding information about the identity of the small molecule (Kleiner *et al.*, 2011). A DNA encoded small molecule library may contain thousands of small molecules each encoded with a unique DNA tag. The library is then washed across immobilized biological targets allowing compounds that do not interact to flow off and isolating compounds which bind to the biological target (Kleiner *et al.*, 2011). Once the potentially bioactive compounds are isolated, the DNA tags are used to characterize hit compounds. Polymerase chain reaction amplification of the tags increases their concentrations to detectable levels by a DNA sequencer (Kleiner *et al.*, 2011). These tags may be thought of as barcodes, allowing the identification of compounds without having to characterize each structure individually. The decoded sequence information shows which small molecule compounds interacted with the target, facilitating the identification of hundreds to thousands of potential candidates in one screening step (Kleine *et al.*r, 2011). While the use of DNA-encoded small molecule libraries has been utilized in some drug development applications, a major limitation to their use is the relative instability of DNA to many of the reaction conditions necessary to modify organic molecules (Rössler *et al.*, 2023).

Harsher reagents, such as acids, bases, and cross-coupling reagents, can degrade the DNA tag (Rössler *et al.*, 2023). For this technique to be applicable to a greater diversity of drug types, a more stable molecule would need to be used for the encoding tag.

Peptide-encoded small molecule libraries

Just as DNA may encode information within its sequence of nucleotides, peptides can similarly encode information within their sequence of amino acids. To encode this information, amino acids may be added to peptides during Solid Phase Peptide Synthesis (SPPS). Unlike DNA, amino acids demonstrate a greater stability to a variety of organic reaction conditions when protected on solid-support. For this reason peptides are an attractive alternative to the DNA tags used in DNA-encoded small-molecule libraries. Peptide-Encoded Libraries (PELs) have been reported to be an effective technique to encode complex compound identity while surviving the diverse array of reaction conditions necessary to construct some drug candidates (Rössler, 2023).

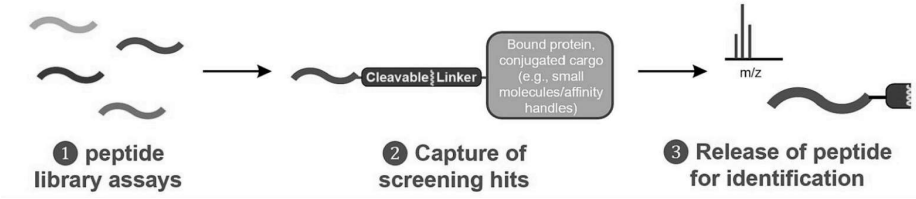


Figure 2. PEL screening and peptide tag decoding.

The exploration of PEL's has only recently been possible due to significant advancements in the sensitivity of nano Liquid Chromatography Tandem Mass Spectrometry (nLC-MS/MS) technology (Rössler *et al.*, 2023). Since peptide tags cannot be amplified like DNA, the resulting pool of peptide tags after target interaction screening exist in concentrations close to one nanomolar per peptide. Such a dilute amount of peptide is close to the detection limit for our current nLC-MS/MS technology (Rössler *et al.*, 2023). The gentle separation of

peptide tags from small molecule drugs is vital for two reasons: degradation peptide tags would push their final concentration below the nLC-MS/MS detection limit and separating the small molecule from the peptide before MS/MS fragmentation provides higher accuracy in computational reconstruction of the peptide sequence. To achieve the soft release of these peptide tags, cleavable peptide linkers have been used (Rössler *et al.*, 2023). These linkers can release the peptide tag from an attached small molecule under specific conditions, mild enough to maintain the integrity of the tag's structure. Development of PEL's have great potential to decrease the cost of drug development and increase the speed at which new potential bioactive compounds are identified.

Cleavable linkers

A cleavable linker describes any compound which may attach molecules together and facilitate their separation under specific conditions. A wide variety of linker types exist whose cleavage can be activated by a diverse array of stimuli including: electromagnetic radiation, enzymes, metals, acids, nucleophiles, electrophiles, reductants, and oxidants (Leriche, *et al.*, 2012). The application of these linkers are similarly diverse, ranging from targeted drug release to pharmaceutical development. In each of these applications, it is vital that the structure of the connected molecules is not affected by the linker's cleavage conditions. While many types of cleavable linker have already been described, there is great value in increasing the diversity of potential linkers, as linkers with new characteristics may be applied to previously incompatible conditions.

The identification of potential cleavable linker candidates often begins with a review of already described reactions involving controlled molecule decomposition under specific conditions. Oxazoles have previously been utilized to introduce carboxyl groups onto sensitive compounds via a reaction with cerium ammonium nitrate in which the oxazole ring decomposes

into a carboxyl group and an imide (Evans *et al.*, 2006). This reaction effectively separates an R group and an aryl group on either side of the oxazole ring.

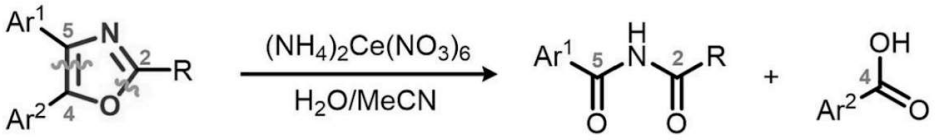


Figure 3. Cerium ammonium nitrate mediated oxazole cleavage.

If the R group and aryl group were able to be functionalized, compounds could be added to either side of the ring and the oxazole would serve as an oxidative cleavable linker. To our knowledge, a cleavable linker which is labile to single-electron oxidants such as cerium ammonium nitrate has not been described in literature. Prior research has found the aryl group in the 5 position of the oxazole ring to be important for cleavage (Evans *et al.*, 2006). Herein, we report the development of a 4,5-diaryloxazole-based cleavable linker.

Experimental Methods

Abbreviations

Alloc	N-Allyloxycarbonyl
Boc	tert-butyloxycarbonyl
DCM	Methylene chloride
DMF	N,N-Dimethylformamide
DIPEA	N,N-Diisopropylethylamine
EDT	1,2-ethanedithiol
Fmoc	Fluorenylmethyloxycarbonyl
HATU	Hexafluorophosphate azabenzotriazole tetramethyl uronium
MeCN	Acetonitrile
NaHCO3	Sodium bicarbonate
NMR	Nuclear magnetic resonance spectroscopy
SPPS	Solid-phase peptide synthesis
TIPS	Triisopropylsilane
TFA	Trifluoroacetic acid
Trityl	Triphenylmethyl

Synthesis of bifunctional oxazole

A bifunctional liker was synthesized containing a diaryl oxazole analogous to oxaprozin with its phenol groups substituted for an alloc-protected 4-aminobenzyl group and a 4-methoxybenzyl group. The presence of a carboxylic acid and a protected amine in the reported linker allows for its incorporation onto peptides during SPPS and the addition of further compounds through HATU mediated amide coupling. While multiple derivatives of oxaprozin have been studied as potential pharmaceuticals, this linker is a novel analogue to our knowledge. The linker required an eight step synthesis with a total yield of 46% (Fig. 4). In addition to the standard alloc-protected amine, an additional bifunctional linker was synthesized with a Boc-protected phenylalanine residue already coupled to the linker.

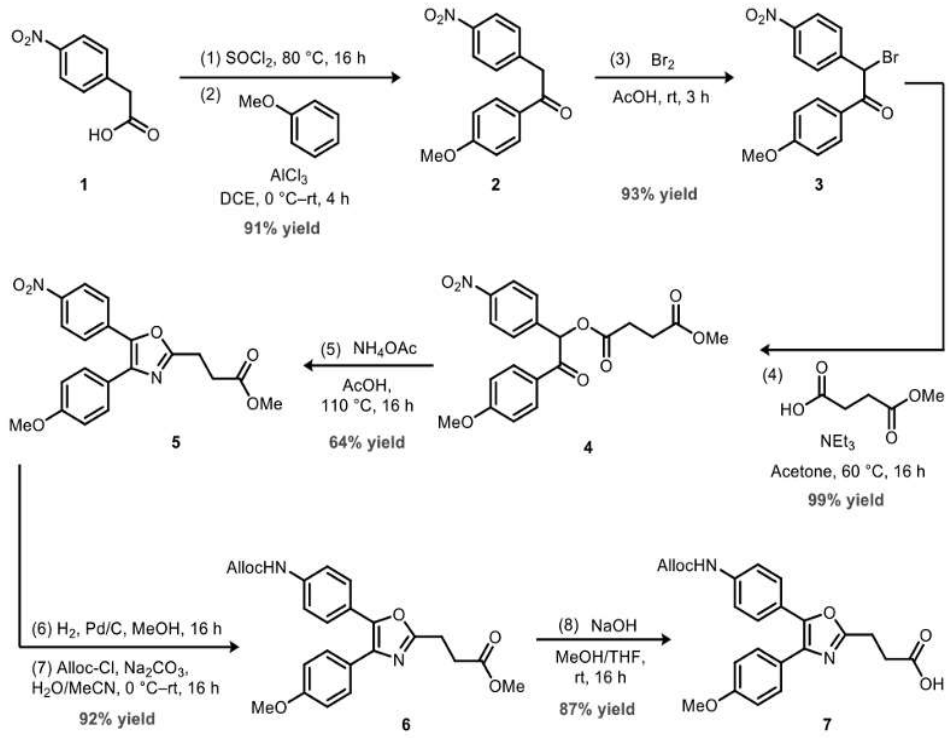


Figure 4. Reaction scheme for bifunctional oxazole-based linker synthesis.

General Peptide Synthesis

All peptides were synthesized through SPPS on rink-amide resin. In a fritted syringe, rink-amide resin (0.2mmol) was swelled in DMF for 30 minutes with occasional stirring. Fmoc-protected amino acids (10 equivalents) were dissolved in a 0.39M solution of HATU (5mL). Before amino acid addition, DIPEA (6 mmol) was added to the solution and mixed for 15 seconds. After swelling, the resin was drained under vacuum. For each amino acid coupling, the resin was incubated with an amino acid solution for 30 minutes with occasional stirring, drained, washed with DMF, deprotected with a 20% piperidine solution for 15 minutes, then washed again with DMF. This procedure was repeated for each amino acid addition from the C-terminal end of the growing peptide to the N-terminus. Additional compounds could be added to the N-terminus of peptide through this SPPS procedure including unnatural amino acids, small

molecules, and the bifunctional oxazole. Coupling of the bifunctional oxazole was performed overnight with 4 equivalents of the linker.

To remove the peptide from its solid support, the resin was washed with DCM and dried under vacuum. The peptide-coupled resin was added to a centrifuge tube along with 5 mL of cleavage solution (94% TFA, 2.5% H_2O, 2.5% EDT, and 1% TIPS). The resin and cocktail were mixed for 2 hours or 4 hours if the C-terminal amino acid was Arginine. The TFA in solution was evaporated by bubbling N_2 through the solution until the resin appeared dry. -80℃ diethyl ether was added to the resin, mixed, then centrifuged at 4,500 RPM for 3 minutes. The resulting solution was decanted from the centrifuged pellet and this process was repeated one more time. After the final decanting, 5 ml of 95:5 H_2O:MeCN was added to the pellet to dissolve free peptide and filtered to remove remaining resin (Fig. 5). The peptide solution was purified on a 12g C_{18} Column on a Biotage Selekt Flash Purification System with a gradient of polar (0.1% TFA H_2O) and nonpolar (0.1%TFA MeCN) solvents. Column fractions containing peptide were combined and concentrated under vacuum until their volume was approximately 5mL. This solution was cooled in a -80℃ freezer and lyophilized, resulting in a solid white powder. The peptide's identity and purity were confirmed using MALDI-TOF spectroscopy.

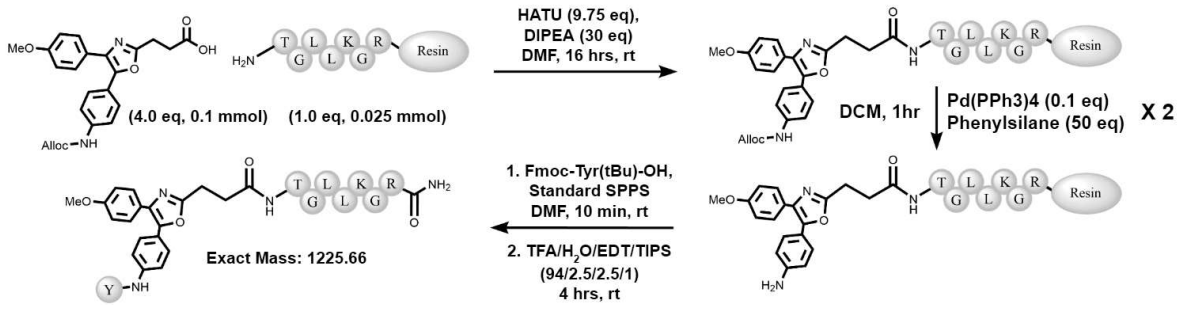


Figure 5. Workflow diagram for coupling of the bifunctional linker onto peptides during SPPS.

Alloc Deprotection

For peptides containing the bifunctional oxazole, the alloc-protected amine required deprotection before the next compound could be coupled. The alloc-containing peptide on resin was swollen in DCM for 30 minutes. After draining, $Pd(PPH_3)_4$ (0.005 mmol) was added to the resin in 1mL of DCM along with phenylsilane (2.5 mmol). The reaction mixture incubated for 1 hour with occasional stirring. This process was repeated one more time to ensure complete deprotection of the peptide. To remove any additional $Pd(PPH_3)_4$ stuck to the peptide or resin, the resin was washed with DCM (X3), DMF (X3), 0.5% sodium diethyldithiocarbamate trihydrate in DMF (2X5min), and DMF (X3).

Cleavage of peptide-embedded oxazoles

After cleavage from resin, solid oxazole-containing peptides were dissolved in water to a concentration of 5mM. Reaction conditions were optimized across various reaction temperatures, peptide concentrations, reaction time, and buffer identities. The final oxidation conditions varied depending on characteristics of the peptide. The standard cleavage condition for peptides continuing the bifunctional linker included 250 μM of peptide, 10 mM cerium ammonium nitrate, 10 mM pH 7 HEPES, water (95%), and acetonitrile (5%) in a final volume of 20 μL. This reaction was performed at room temperature for 15 minutes and quenched with 20 μL of 50 mM sodium thiosulfate (Fig. 6).

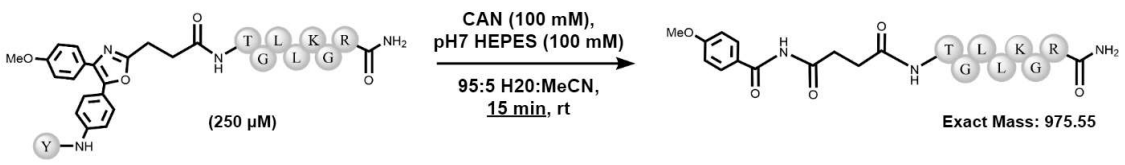


Figure 6. Reaction scheme for bifunctional linker cleavage

Peptides containing tyrosine and tryptophan residues are more sensitive to oxidative conditions and were cleaved under conditions with 250 μM of peptide, 10 mM cerium ammonium nitrate, 25 mM pH 7 TRIS, water (95%), and acetonitrile (5%) in a final volume of 20 μL. This reaction was performed at room temperature for 1 minute and quenched with 20 μL of 50 mM sodium thiosulfate.

While the cleavage of most peptides resulted in the formation of a terminal imide, some sequences were partially hydrolyzed to a terminal carboxylic acid. For these sequences, the standard cleavage condition was used and the reaction was quenched with a solution of 50 mM sodium thiosulfate and 50 mM NaOH (20 μL).

Cleavages for peptides containing the bifunctional linker analog oxaprozin were performed under the standard cleavage conditions at room temperature, but required 60 minutes to reach full conversion.

MALDI-TOF

Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) is a form of soft-ionization mass spectroscopy which maintains the structure of compounds through the use of matrix assisted laser ionization. This technique allows the validation of compound identity through molecular weight analysis. A Shimadzu AXIMA Confidence was used to analyze peptide samples. To prepare compounds for analysis, a metal spotting plate was first coated with 1 μL of 5 mg/mL sinapinic acid in acetone and allowed to fully dry. The peptide sample (1 μL) was then added to the plate in quick succession to 1 μL of 5 mg/mL alpha-cyano-4-hydroxycinnamic acid in 75:25 MeCN:H_2O (0.1% TFA) and allowed to fully dry. By incorporating the sample into the sinapinic acid, the peptide is protected from harsh ionization by the excitatory laser and the photosensitive matrix acts to aerosolize the peptide sample and transfer charge to the sample in lower energy excitations. Samples were fired in reflectron mode and analyzed from a mass range of 500-4,000 m/z.

HPLC

Due to the non-quantitative nature of MALDI-TOF purity analysis, High-Performance Liquid Chromatography (HPLC) was used for purity analysis of peptide cleavage samples. This technique separates compounds by charge through high-pressure column chromatography and quantifies their concentrations through UV/Vis absorption. A Shimadzu HPLC was used with a reverse phase C_{18} column. Samples were de-salted using a preparatory filter before being added to the column. Chromatography was performed with a gradient of H_2O (0.1% TFA) and MeCN (0.08% TFA) with a flow rate of 0.5 mL/min. This gradient decreased in polarity over the course of the run as MeCN concentration was linearly increased from 5% to 95%. Absorbance of elutants were detected at 254 nm and 280 nm by a diode array detector. Quantitative cleavage of compounds was verified by the presence of only two peaks outside the buffer elution region of the HPLC, matching the elution times of the carboxylated peptide tag and attached small molecule respectively.

Results and Discussion

Importance of aryl substituted and EDGs

Since oxazoles have three positions available for modification, the electronic environment of the oxazole ring can be modified through addition of various R groups. During initial solvent-phase experimentation exploring the oxidation of substituted oxazoles, a variety of oxazoles with different R groups were exposed to cerium ammonium nitrate and their reactions were analyzed for formation of desirable products. This experimentation revealed that diaryl oxazoles were cleaved by cerium ammonium nitrate with the best speed and with least byproduct formation. While the mechanism for oxazole cleavage is not fully understood, it was theorized that the formation of a radical inside the oxazole ring is an important intermediate step during single-electron oxidation with cerium ammonium nitrate. Diaryl substitution of the oxazole ring may stabilize the radical intermediate leading to the more rapid reaction times observed.

The identification of diaryl oxazole as a promising linker candidate led to the selection of oxaprozin, a common anti-inflammatory drug, as a model linker. Oxaprozin contains a diaryl oxazole and a free carboxylic acid which could be reacted with the terminal amine on growing peptide sequence through HATU mediated SPPS. Oxaprozin was successfully added to a peptide on solid support and survived the rink amide cleavage conditions needed to separate the peptide from resin (Fig. 7).

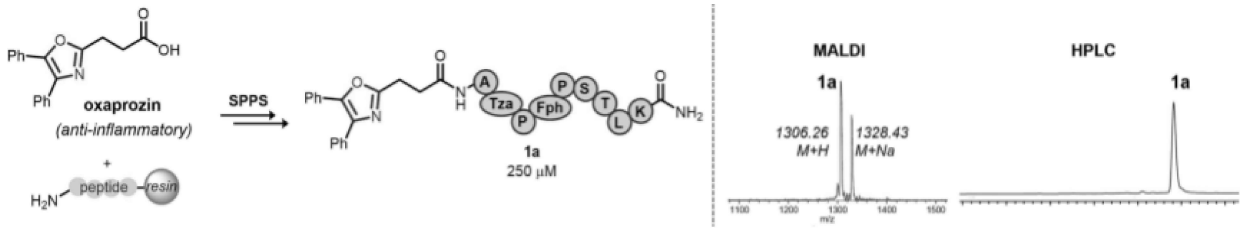


Figure 7. Coupling of oxaprozin onto a peptide during SPPS with verification of product identity via MALDI-TOF and HPLC.

Due to the widespread use of ring-amide for peptide synthesis, the stability of potential linkers to the highly acidic conditions of resin cleavage was an important consideration in our linker's development. While oxaprozin served as a strong model for our eventual linker, it lacks a secondary functional group on the distal end of the molecule to which further components could be added.

In order to create a bifunctional oxazole-based linker, an eight step total synthesis was necessary and resulted in the diaryl oxazole containing an alloc-protected 4-aminobenzyl group and an 4-methoxybenzyl group. These amino and methoxy electron donating groups make the oxazole electron rich. When performing cleavages on the bifunctional oxazole, the reaction proceeded to completion in one-fourth of the time required for quantitative cleavage of oxaprozin. We hypothesize that the presence of the electron donating groups on the aryl substituents of the oxazole further increased the speed of the reaction by stabilizing radical formation (Fig. 8).

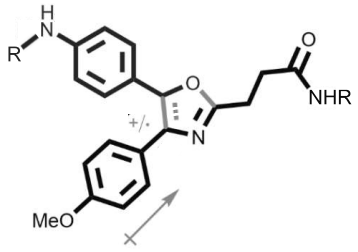


Figure 8. Hypothesized stabilization of radial intermediates by electron rich aryl substituent.

Incorporation of bifunctional oxazole into peptides during SPPS

The bifunctional oxazole was developed to be compatible with SPPS. Similarly to oxaprozin, the reported linker contains a free carboxylic acid which can be attached to terminal amines of a peptide. The bifunctional linker is stable to HATU mediated coupling conditions. After addition of the bifunctional liner, the alloc-protected amine can be deprotected using a palladium based catalyst, yielding a free amine ready for further coupling reactions. Addition of the bifunctional linker to peptides was performed at a lower equivalence than standard amino acid couplings and allowed to react overnight to ensure efficient use of the synthesized linker. Addition of the linker to both polar, non-polar and aromatic amino acids were demonstrated. The linker showed stability to rink-amide cleave conditions, surviving a solution of 94% TFA for 4 hours. The bifunctional linker could also be incorporated onto unprotected lysine residues.

Cleavage of oxazole embedded peptides

The cleavage of oxazole embedded peptides occurs through oxidation by cerium ammonium nitrate. Both buffer identity and concentrations had an effect on product formation and reaction rate. During condition optimization, a side reaction was identified in which the oxazole cleaved to form an amide and a benzil fragment. The desired cleavage products were an imide fragment and a carboxylic acid fragment. Using the buffer HEPES at a pH of 7 minimized this side reaction. Various concentrations of the buffer were also tested and it was

found that when buffer concentrations were higher than cerium ammonium nitrate concentrations, the reaction rate was slowed. The optimized conditions reported are on a 250 μM scale of peptide with 10 mM of cerium ammonium nitrate and 10 mM of pH 7 HEPES (95:5 H_2O:MeCN, 15 minutes, rt). Cleavage yielded the desired imide and carboxylic acid fragments confirmed by MALDI-TOF. Quenching the reaction with 50 mM sodium thiosulfate neutralized the remaining oxidant and prevented additional oxidations to the peptide tag from occurring.

Compatibility of amino acid residues to cleavage conditions

For oxazole-based cleavable linkers to be used in PELs their cleavage conditions must show compatibility with a variety of natural and unnatural amino acid sequences. Due to the low concentrations of peptide recovered after PEL screening, any degradation of amino acid side chains by cleavage conditions might reduce peptide concentrations below the detectable limit by ns-MS/MS. In order to test the compatibility of our cleavage conditions with various amino acid residues, a library of 10 peptides containing the oxazole-based linker was synthesized. Each peptide was first tested with the incorporation and cleavage of oxaprozin to ensure that the sequence could be dissolved in 95:5 H_2O:MeCN and purified via column chromatography. In addition to natural amino acids, various unnatural amino acids were Incorporated into the peptide library. Unnatural amino acids are diverse in structure and molecular weight making them a beneficial edition to PELs which depend on the determination of linker identity in a pool of hundreds of thousands of peptides.The standard cleavage conditions were found to be compatible with all natural amino acids except for tyrosine, tryptophan, and cysteine as well as for a variety of unnatural amino acids (Fig. 9). Peptides containing histidine and methionine residues experienced secondary oxidation at those residues, but the secondary oxidations were predictable and did not affect the stability of the peptide. Unfavorable interactions with cysteine were hypothesized before testing due to the strong nucleophilic nature of cysteine's sulfur-containing R group. Oxazole-based linker’s incompatibility with cysteine is consistent with previously reported oxidation labile linkers. Tryptophan and tyrosine residues are reactive to

oxidative conditions and it was hypothesized that their oxidation products degraded the peptide label under the standard cleavage condition.

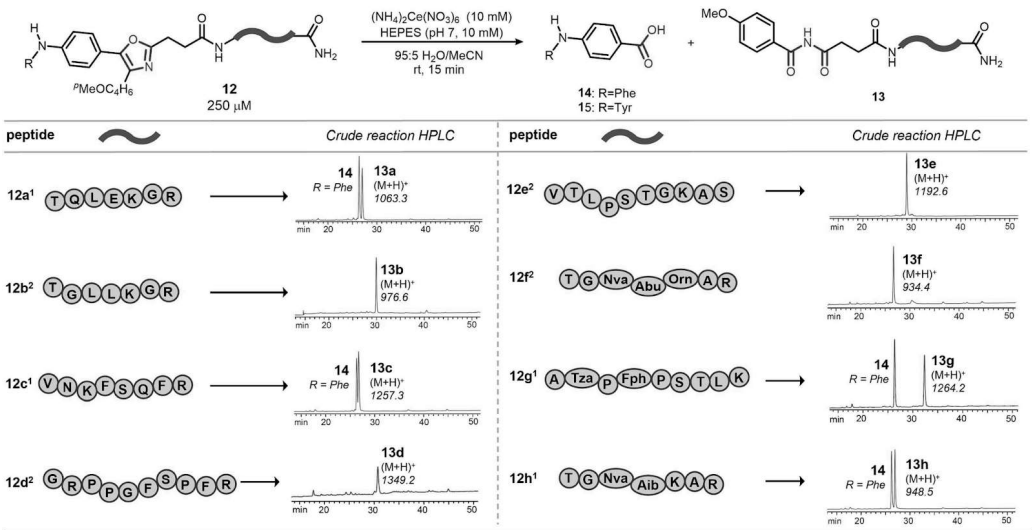


Figure 9. Cleavage results of bifunctional linker across an eight member peptide library with MALDI-TOF analysis of products

Since tyrosine and tryptophan are potentially important residues to include in a PEL, alternate cleavage conditions were developed to minimize unwanted byproducts. Since it was hypothesized that these residues created unfavorable by-products in oxidative conditions, a secondary cleavage condition was explored to reduce secondary oxidation. Our initial cleavage testing results had shown that increased buffer concentration and TRIS buffers slow the rate of oxidation and it was predicted that the use of these conditions may reduce the rate of unwanted secondary oxidation while still allowing oxazole cleavage to proceed to completion (Seim, 2011). Peptide labels containing tryptophan and tyrosine residues were successfully cleaved on a 250 μM scale using 10 mM cerium ammonium nitrate and 25 mM pH 7 TRIS buffer (1 minute, rt). The product of these conditions contained oxidative modifications to the tyrosine and tryptophan residues, but the integrity of the peptide label was maintained.

In summary, single-electron oxidant cleavage conditions have been developed which are compatible with 19 out of 20 natural amino acids and a variety of unnatural amino acids. This

versatility makes oxazole-based linkers strong candidates for PELs which require diverse peptide library designs.

Controlled hydrolysis of cleavage product

During amino acid sequence compatibility testing, the oxidation of two peptide sequences (H_2N-Linker-VDKFSQFR-$COONH_2$ and H_2N-Linker-GDKFSQFR-$COONH_2$) resulted in the partial hydrolysis of the imide product. Both of these sequences contain an aspartic acid at the secondary position to the oxazole-based linker. Since the partial hydrolysis could affect the detection of peptide labels, additional quenching conditions were developed to hydrolyze cleavage products to completion. The imide product of oxidation is already vulnerable to hydrolysis, so the addition of sodium hydroxide to the oxidation quenching solution would push the hydrolysis of the imide to completion (Fig. 10).

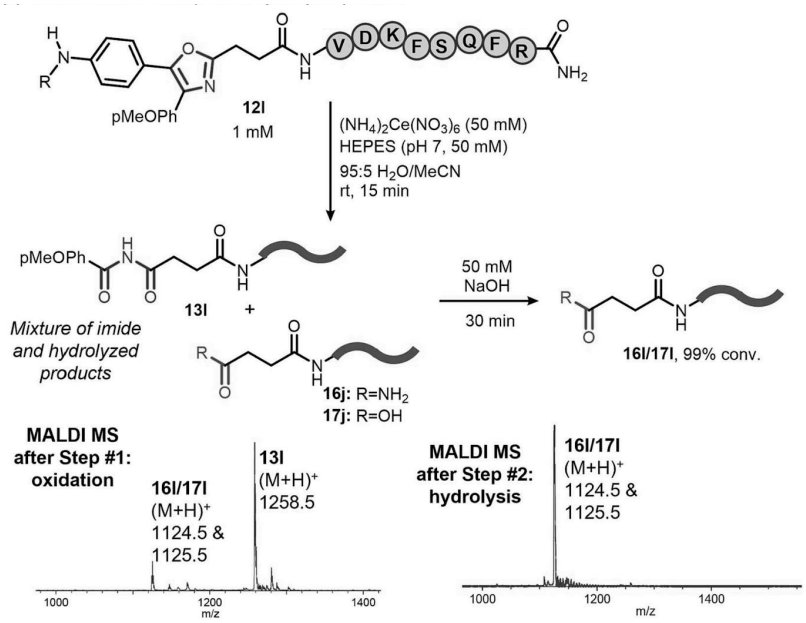
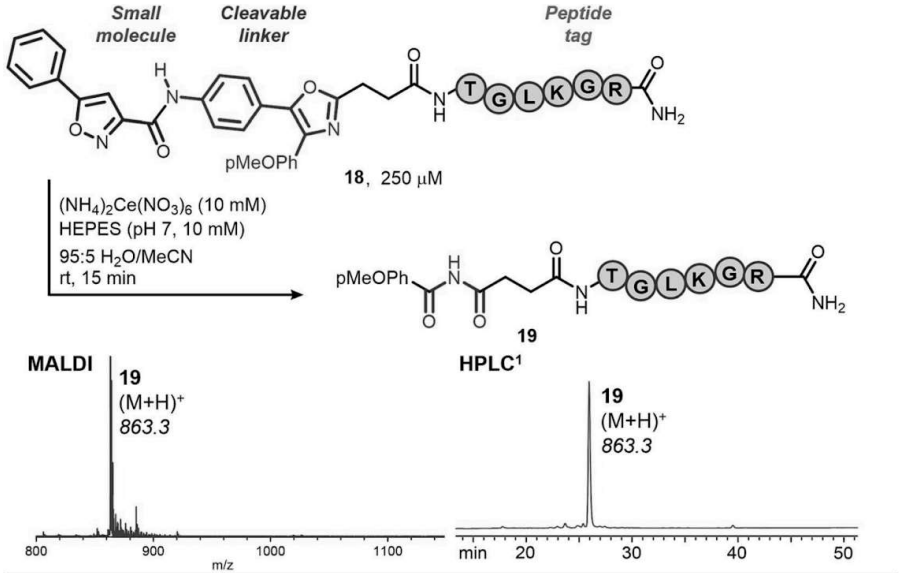


Figure 10. Controlled hydrolysis of cleavage product imide.

It was found that using a 50 mm sodium thiosulfate and 50 mm sodium hydroxide quenching solution resulted in the cleavage products of multiple peptide sequences fully hydrolyzing.

Addition of small molecule building blocks

For application in drug discovery platforms, oxazole-based linkers must be compatible with the sequential addition of small molecule components in a PEL. A proof of concept peptide encoded small molecule was synthesized using an alloc-protected lysine residue as a secondary handle connecting the bifunctional linker and small molecule portion to a modifiable peptide tag. The identity of small molecule additions could be coded onto the peptide strand using a trityl group as an orthogonal amino-protecting group to the alloc-protected amine on the N-terminal linker. Small molecules such as 2-bromothiazole-5-carboxylic acid and 5-phenylisoxazole-3-carboxylic acid were successfully incorporated onto the encoding peptide and cleavable linker using HATU mediated SPPS. Upon oxidative cleavage, the peptide tag was recovered and characterized through MALDI-TOF (Fig. 11)



.Figure 11. Cleavage and tag recovery of a peptide encoded small molecule verified via MALDI-TOF and HPLC

Orthogonality to existing cleavable linkers

Oxazole-based linkers are not the first oxidatively cleavable linker described in literature (Pomplun *et al.*, 2020). However, oxazoles are the first linkers to our knowledge that are selectively cleaved by single electron oxidants. To demonstrate the orthogonality of cleavage conditions to existing oxidatively cleavable linkers, a peptide was synthesized containing both the bifunctional oxazole and the seramox linker. Seramox is an amino alcohol based linker which shows rapid cleavage when exposed to sodium periodate cleavage conditions, a double-electron oxidant (Pomplun *et al.*, 2020). The peptide with both linkers was exposed to cerium ammonium nitrate and sodium periodate conditions independently. Reaction with cerium ammonium nitrate successfully cleaved the oxazoline-based linker while leaving the seramox linker intact, while reaction with sodium periodate conditions cleaved seramox and left the oxazole-based linker intact (Fig. 12).

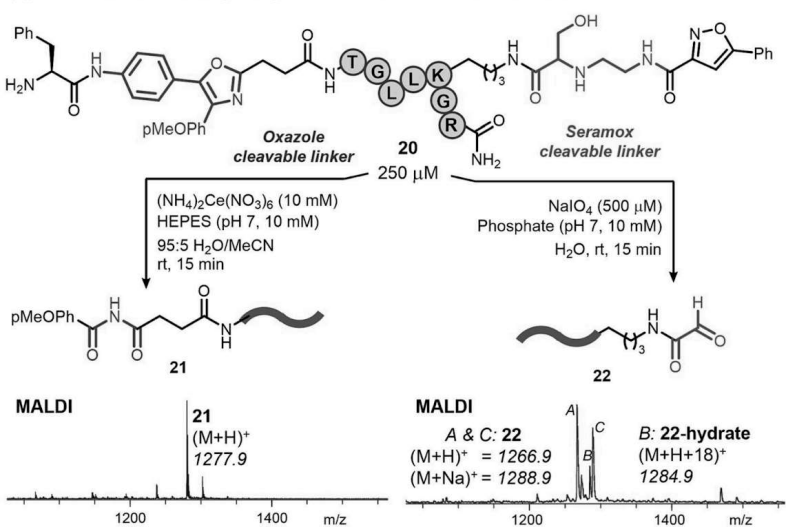


Figure 12. Orthogonal oxidative cleavage of oxazole-based linker and seramox linker.

This experiment demonstrates the orthogonality of an oxazole-based Linker to a double-electron oxidatively cleavable linker. This characteristic of oxazole-based linkers might

allow the use of traditional double-electron oxidants during small molecule PEL development, reagents which would normally be incompatible with oxidatively cleaved linkers.

Conclusions

This research describes the development of an oxazole-based cleavable peptide linker which has been optimized to undergo rapid cleavage in mild oxidative conditions with cerium ammonium nitrate. This linker was successfully incorporated onto peptides during SPPS, survived rink-amide cleavage conditions, and rapidly released a variety of attached compounds. The single-electron oxidant cleavage conditions for the oxazole-based linker was compatible with most natural amino acids and a variety of unnatural amino acids. A peptide tag was successfully retrieved after linker cleavage in a single member small molecule PEL containing the bifunctional linker. This is the first described single-electron oxidant labile linker to our knowledge and the oxidative cleavage of oxazole-based linkers is orthogonal to double-electron labile linkers. These characteristics of oxazole-based linkers expand the applicability of peptide linkers in conditions where double-electron oxidants are present. The discovery of new versatile cleavable peptide linkers continues to increase the scope and applications of PEL's and peptide discovery platforms

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