Heterocycles as Peptide-Based Cleavable Linkers

John Blobe

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Heterocycles as Peptide-Based Cleavable Linkers

By

John Blobe

Honors Thesis

Submitted to:

Biochemistry & Molecular Biology Program
University of Richmond
Richmond, VA

April 29, 2024

Advisor: Dr. Christopher Shugrue
This thesis has been accepted as part of the honors requirements
In the Program in Biochemistry and Molecular Biology

Christopher R. Shigve
(advisor signature)
5/1/2024
(date)

Jonathan Dattelbaum
(reader signature)
4/29/2024
(date)
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Abstract

As cancer cases continue to rise, the need for advancing the treatment options for cancer is ever increasing. Current cancer therapeutics, while effective at treating localized cancers and a fraction of advanced cancers, generally lack the specificity needed to target more advanced cancers. Recent advancements in cancer treatments have leveraged antibodies to target certain cancers. A class of drugs that utilize antibodies to deliver anti-cancer therapy preferentially to cancer cells, termed antibody-drug conjugates (ADCs), have been quite effective in treating certain advanced forms of certain cancers. These ADCs could be more effective if they could allow for the facile release of the anti-cancer therapy by engineering in a cleavable linker. Here we explore the use of heterocycles as peptide-based cleavable linkers, which could be used in the development of novel ADCs. Two primary heterocycles were explored for their utility as peptide-based cleavable linkers, oxazoles and benzothiazoles. Oxazoles can function as cleavable linkers through the 4+2 cycloadditions with alkynes. Benzothiazoles can function as cleavable linkers through nucleophilic aromatic substitution ($S_N$Ar). We demonstrate that benzothiazoles have promise as peptide-based cleavable linkers due to their ability to be added to peptides through the $S_N$Ar with cysteine’s nucleophilic thiol. Peptide-bound benzothiazoles are quantitatively cleaved from peptides within 3 hours at room temperature. Further exploration of benzothiazoles as cleavable linkers could assist in the development of new therapeutics for cancer as well as other diseases.
Introduction

Antibodies and peptides

Antibodies are proteins naturally produced in the body by immune cells to mediate the adaptive immune response against foreign antigens (Aziz et al., 2023). An antibody is made up of four polypeptide chains, including two identical heavy and two identical light chains. The specificity of antibodies for antigens comes from the fragment antigen-binding (Fab) region of antibodies. The ability for antibodies to be directed towards any target has made the use of antibodies valuable in clinical settings. Antibodies are used in the treatment of bacterial infections, to provide passive immunity to infected patients. Additionally, antibodies can be used to treat autoimmune diseases by binding and inhibiting inflammatory mediators. Furthermore, antibodies have also been found to be effective in treating cancer (Aziz et al., 2023).

While antibodies show promise in various situations, their large size and physical properties limits their use in clinical and research settings (Trier et al., 2019). Peptides, like antibodies are also naturally produced in the body and are made up of amino acids. However, unlike antibodies, which are made up of four polypeptide chains, peptides are typically smaller molecules made up of a chain of 2 or more amino acids, making them a more practical biomolecule to work with.

Cancer and cancer therapeutics

Cancer is a significant global health problem and is the second leading cause of death behind cardiovascular disease (Siegel et al., 2024). In 2024, there are projected to be just over 2 million new cancer cases and approximately 611,000 deaths related to cancer in the United States. Progress in decreasing cancer-related mortality has been slow in the past decades despite
advances in earlier detection of cancer and improved treatment options for many cancers (Siegel et al., 2024).

A significant limitation in the advancement of cancer treatment is related to the drug discovery process and finding drugs that specifically target cancer cells (Anand et al., 2022). In contrast to antimicrobials, which can target microbes with high specificity due to the significant physiological differences between microbial and host cells, the similarity of cancer cells to host cells makes the development of drugs that are cytotoxic to cancer cells but not to non-cancer cells very difficult. The lack of specificity of chemotherapeutics increases the cytotoxicity towards non-cancer cells, leading to more side effects and less effective treatment of the cancer. Limiting the toxicity to non-target cells reduces side effects of the chemotherapy, which allows for higher doses to be utilized. Additionally, lower specificity associated with chemotherapeutics increases the likelihood of a recurrence of the cancer (Anand et al., 2022).

Fortunately, some forms of cancer provide an opportunity for more targeted therapies due to their differential expression of either unique or overexpressed proteins, especially proteins found on the cell surface. The most common methods for targeted drug delivery involve the use of antibodies (Anand et al., 2022). Antibodies, specifically humanized monoclonal antibodies (mAbs), can be used in the treatment of cancer by selecting them to bind antigens present or overexpressed on the cell surface of cancer cells (Zahavi & Weiner, 2020). These mAbs can cause cancer cell death through a variety of mechanisms. The primary way that mAbs kill cancerous cells is by blocking the growth factor signaling that fuels the uncontrolled growth of cancer cells. This is accomplished by either preventing ligand binding or receptor complex formation, which decreases the activation of growth factor signaling. Another way that mAbs lead to tumor cell death is through the activation of the complement system, which results in
phagocytosis of the cancer cells by macrophages or cytotoxic granule release primarily by natural killer cells (Zahavi & Weiner, 2020).

**Antibody-drug conjugates**

While mAbs have been shown to be effective in the treatment of cancer, due to their limited direct anticancer effects, combining mAbs with payloads has been explored to improve their efficacy (Marei et al., 2022). This combination of a mAb with a payload has led to the development of ADCs. ADCs have three primary structural units, including an antibody, a payload, and a linker that connects the antibody to the payload (Figure 1).

![Figure 1. Structure of antibody-drug conjugates (Joubert et al., 2020).](image)

The selectivity of mAbs and the potent cytotoxicity of chemotherapeutics brought together by the linker in ADCs allows for the effective targeting and killing of cancer cells. The efficacy of ADCs in treating their target depends greatly on the three components of ADCs. The antibodies used must have very high specificity for their target antigen. Additionally, while there are many types of antibodies, IgG antibodies are the most commonly used antibody in ADCs as they have
their own anticancer effects by activating the complement system and leading to cancer cell lysis. However, utilizing IgG antibodies over antibody fragments has its limitations due to their large size. IgG antibodies account for approximately 95% of an ADC’s size, hindering optimal penetration especially into solid tumors. Based on findings that only a small proportion of mAbs targeting tumors can penetrate the tumor, the payload bound to the mAb in ADCs must be highly cytotoxic at low quantities, ideally in sub-nanomolar concentrations. Furthermore, to increase the quantity of payload delivered per antibody, the drug-antibody ratio can be manipulated and must be optimized to provide sufficient cytotoxicity to cancer cells while limiting toxicity to surrounding tissues. Also, to limit system toxicity, the linker combining the drug and payload must be stable enough to reach its target, but also labile enough to release and deliver the payload in the tumor microenvironment. These linkers may be cleavable or non-cleavable. Cleavable linkers may be cleaved in acidic or reducing conditions or by proteolytic enzymes (for peptide-based linkers). To modulate the stability of the cleavable linker, changing the linker length or steric hindrance have been shown to improve the stability of the linker. Lastly, manipulating the chemical properties of linkers in ADCs, specifically the polarity of the linkers, has been effective in limiting the resistance of cancer cells to ADCs (Marei et al., 2022).

**Heterocycles as cleavable linkers**

While cleavable linkers are already utilized in ADCs, the development of novel cleavable linkers has the potential to expand the effectiveness and increase the specificity of ADCs as well as other pharmaceuticals. For example, almost all cleavable linkers used in ADCs work in non-specific conditions, such as acidic or reducing conditions or in the presence of enzymes within cells, which could allow for non-selective release of the payload. To improve the selectivity of
ADCs, the development of reagent-controlled cleavable linkers would be advantageous over those that cleave in non-specific conditions.

Aromatic heterocycles represent a promising class of molecules that could function as reagent-controlled cleavable linkers. The two main heterocycles we investigated for their functionality as cleavable linkers are oxazoles and benzothiazoles.

**Oxazoles as cleavable linkers**

Oxazoles are aromatic 5-membered heterocyclic compounds that contain an oxygen and a nitrogen separated by one carbon. While oxazoles are normally stable heterocycles due to their aromaticity, they can engage in 4+2 cycloadditions with alkynes (Levin & Laakso, 2003). The 4+2 cycloaddition of oxazoles with alkynes splits the oxazole, resulting in a furan and a nitrile product (Figure 2).

![Figure 2](image-url)

**Figure 2.** How oxazoles can function as cleavable linkers through 4+2 cycloaddition of oxazoles and alkynes.

The presence of multiple substituents (R) on oxazoles allows for the conjugation of biomolecules and the selective cleavage of oxazoles in the presence of alkynes would provide a novel reagent-controlled cleavable linker.
Benzothiazoles as cleavable linkers

Benzothiazoles include a thiazole ring, which is an aromatic 5-membered heterocyclic compound that contains a sulfur and a nitrogen separated by one carbon and a benzyl ring attached to carbon 4 and 5 of the thiazole ring. Although benzothiazoles are stable molecules due to their aromatic bicyclic structure, they are susceptible to $S_N$Ar. Specifically, methylsulfonyl benzothiazoles can engage in $S_N$Ar reactions with nucleophiles, like cysteine (Zhang et al., 2012). The $S_N$Ar reactions of alkyl sulfanyl benzothiazoles lead to the substitution of the alkyl sulfonyl group for the nucleophile, resulting in a sulfinic acid and a benzothiazole-nucleophile product (Figure 3).

![Figure 3. $S_N$Ar reaction of methylsulfonyl benzothiazole and nucleophile.](image)

The reactivity of methylsulfonyl benzothiazoles with nucleophiles, cysteine in particular, as well as the abundance of sites available for the conjugation of biomolecules suggests that benzothiazoles hold potential as effective reagent-controlled cleavable linkers (Motiwala et al., 2020).
Experimental Methods

The following experiments were performed in collaboration with Dr. Christopher Shugrue and Abigail Dalton.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MeCN</td>
<td>Acetonitrile</td>
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<tr>
<td>$^1$H NMR</td>
<td>Proton nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<tr>
<td>Et$_3$N</td>
<td>Triethylamine</td>
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<td>THF</td>
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<tr>
<td>mCPBA</td>
<td>meta-Chloroperoxybenzoic acid</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>βME</td>
<td>Beta-mercaptoethanol</td>
</tr>
<tr>
<td>HATU</td>
<td>Hexafluorophosphate azabenzotriazole tetramethyl uronium</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>EDT</td>
<td>Ethane-1,2-dithiol</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenylmethyloxycarbonyl protecting group</td>
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Cycloadditions of oxazoles

The 4+2 cycloadditions of oxazoles with alkynes were conducted on various oxazoles and alkynes. These reactions were conducted with the following oxazoles: 2,4-diphenyloxazole; 5-ethoxy-4-methyloxazole; 5-methoxy-2,4-diphenyloxazole; ethyl oxazole-4-carboxylate; and 5-dimethylamine-2-methyl-4-phenyloxazole. Additionally, these reactions were conducted with the following alkynes: diethyl acetylenedicarboxylate; and bicyclonon-4-yn-9-ylmethanol. The reactions were completed in the following solvents: toluene; acetonitrile (MeCN); 1:1 H$_2$O:toluene; H$_2$O; H$_2$O+5M ammonium chloride; methanol; and chloroform. The oxazole (1 mmol) and alkyne (1 mmol) were dissolved in solvent (1 mL/mmol). The reaction was stirred vigorously at room temperature for at least 24 hours. The product was extracted with ethyl
acetate and dried over sodium sulfate if the reaction was done in the presence of any proportion of H$_2$O. The solvent was removed by rotary evaporation and analyzed by proton nuclear magnetic resonance spectroscopy ($^1$H NMR).

**Synthesis of oxazoles with an electron-donating group**

The synthesis of oxazoles with an electron-donating group (EDG) was a two-step process. First, phenylalanine methyl ester (3.53 mmol, 1 eq.) was dissolved in H$_2$O (15 mL/mmol) with sodium carbonate (68.06 mmol, 19.28 eq.). A solution of benzoyl chloride (3.53 mmol, 1 eq.) in dioxane (15 mL/mmol) was then added to the reaction mixture and the reaction was stirred for 1 hour. A liquid-liquid extraction was performed with ethyl acetate. The organic layers were washed sequentially with saturated sodium bicarbonate, 10% hydrochloric acid (HCl), and saturated brine, then dried over sodium sulfate. The solvent was removed by rotary evaporation resulting in a solid product. Second, triethylamine (Et$_3$N) (4 mmol, 4 eq.) was added dropwise to a mixture of triphenylphosphine (2 mmol, 2 eq.) and iodine (2 mmol, 2 eq.) dissolved in ½ of the dichloromethane (DCM) (2 mL/mmol). The reaction mixture was stirred for 10 minutes. The solid product from the first step of the synthesis was dissolved in ½ of the DCM and added to the reaction mixture. The reaction was stirred overnight. The solvent was removed by rotary evaporation and the compound was purified by column chromatography.

**Small-molecule reactions of benzothiazoles**

As a proof of concept, the reaction scheme involving the addition of benzothiazoles to cysteine through SNAr, oxidation, and cleavage from cysteine through an additional SNAr
reaction was completed. The three-step process was completed according to the scheme shown in Figure 4.

**Figure 4.** Reaction scheme of the addition, oxidation, and cleavage of benzothiazole 1 and cysteine methyl ester.

**Addition of benzothiazoles to peptides**

Peptides were synthesized using solid-phase peptide synthesis (SPPS). SPSS involves building a peptide on resin. Peptides were synthesized on 200 mg of resin (0.1 mmol) from C- to N-terminus. Resin was first deprotected under basic conditions with 20% piperidine for 15 minutes to allow for coupling, then washed with dimethylformamide (DMF). Each Fmoc-protected amino acid (1 mmol) was dissolved in a 0.39 M hexafluorophosphate azabenzotriazole tetramethyluronium (HATU) solution in DMF (2.5 mL). N,N-diisopropylethylamine (500 µL) was added to the solution before coupling. The amino acid was coupled for 30 minutes, then washed with DMF. The amino acid was deprotected with 20% piperidine for 15 minutes to allow
for subsequent coupling, then washed with DMF. These steps were repeated for each amino acid.

After completing the synthesis of the entire peptide, the peptide was cleaved from resin by mixing with a cleavage cocktail consisting of 94% Trifluoroacetic acid (TFA), 2.5% Ethane-1,2-dithiol (EDT), 2.5% H$_2$O, and 1% Triisopropylsilane for two hours.

Solid peptides were dissolved in H$_2$O to make a stock concentration of 10 mM. The addition of benzothiazoles to peptides was done on the cysteine-containing peptide sequence FCPFGLLKGR, at a final concentration of 1 mM. The components of the reaction mixture included the cysteine-containing peptide, benzothiazole 1, Tris(2-carboxyethyl)-phosphine (TCEP), phosphate buffer, H$_2$O (95%), and DMF (5%) to a final volume of 100 µL. The optimized reaction conditions were determined to include the cysteine-containing peptide (1 mM), benzothiazole (2.5 mM), TCEP (2.5 mM), phosphate buffer (200 mM), H$_2$O (95%), and DMF (5%) to a final volume of 100 µL. Reactions were done at room temperature overnight. Analysis of the reaction was done by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

Oxidation of benzothiazoles on peptides

After the successful addition of benzothiazoles to peptides was confirmed, oxidizing conditions were assessed. To the existing reaction mixture from the addition of benzothiazoles to peptides, an oxidant was added. The following oxidants were assessed: meta-chloroperoxy-benzoic acid (mCPBA), hydrogen peroxide, and sodium periodate at a final concentration of 2.5, 5, 10, 25, 50 mM. Oxidizing conditions were not able to be optimized for peptide reactions in solution.
Synthesis of benzothiazoles with an electron-withdrawing group

A benzothiazole with an electron-withdrawing group (EWG) was synthesized according to the synthesis scheme shown in Figure 5. The synthesis of the benzothiazole was a two-step process.

**Figure 5.** Synthesis of benzothiazole 8.

Synthesis of oxidized benzothiazoles on resin-bound peptides

The synthesis of oxidized benzothiazoles on resin-bound peptides was done according to the synthesis scheme in Figure 6. To selectively deprotect cysteine, a Fmoc-cysteine with a tert-butyl-disulfide protecting group on cysteine’s thiol R-group was used. After the synthesis was completed, the peptide was cleaved from resin, purified and analyzed through MALDI-TOF MS.
Cleavage of oxidized benzothiazoles on peptides

The cleavage of benzothiazoles from peptides was assessed with a variety of nucleophiles. The components of the cleavage reaction included a peptide, a nucleophile, buffer, \( \text{H}_2\text{O} \) (95%), and acetonitrile (5%) with a final volume of 20 \( \mu \text{L} \). The concentration of peptides 12a and 12b were tested at 0.5 mM and 1 mM. Nucleophiles, including Beta-mercaptoethanol (\( \beta \text{ME} \)), 4-methoxybenzenethiol, Sodium borohydride, and TCEP were assessed at various final concentrations including 5, 10, 25, 50, 100, and 400 mM. Phosphate (pH 7 and 8) and tris(hydroxymethyl)aminomethane (pH 7) buffers were screened at a final concentration of 25, 50, 200 mM. Reactions occurred at room temperature from 3 hours to overnight.
The optimized reaction conditions were performed with 0.5 mM of peptide 12b, 10 mM of TCEP, 25 mM of pH 8 phosphate buffer, 95:5 H2O:MeCN at room temperature for three hours, after which the reactions were analyzed through MALDI-TOF MS (Figure 7).

**Figure 7.** Optimized cleavage conditions of benzothiazoles from peptides.

**Synthesis of benzothiazoles “clicked” onto peptides**

To attach the benzothiazole to the alkyne-containing EWG to a peptide, a “click” reaction, a Cu-Catalyzed Azide-Alkyne Cycloaddition (CuAAC) was done (Figure 8). A peptide with the unnatural amino acid, azidolysine, which contains an azide group instead of the typical amine group of lysine was synthesized using SPPS. The peptide (4.5 µmol) was dissolved in a mixture of 2:1 H2O:butanol (1.11 mL/µmol). The alkyne-containing benzothiazole (6.75 µmol) was added to the solution. Sodium ascorbate (30.0 µmol) and copper (II) sulfate pentahydrate (20.0 µmol) were added to the reaction mixture, which was then stirred overnight. The solvent was removed through rotary evaporation and purified through reverse-phase column chromatography. The resulting product was analyzed through MALDI-TOF MS.
Figure 8. “Click” reaction between the azide on azidolysine and the alkyne on benzothiazole 8.

Analysis of reactions on peptides

MALDI-TOF MS was utilized to determine the mass of peptides. MALDI-TOF MS is an ionization technique that utilizes a matrix that absorbs the energy from a laser to allow for more gentle vaporization of the target compound without fragmentation. The resulting MALDI-TOF MS shows the molecular ion of the compound. The plate used to spot samples for analysis by MALDI-TOF MS was first spotted with 0.75 µL of 5 mg/mL α-Cyano-4-hydroxycinnamic acid (4-HCCA) in acetone. Then 0.75 µL of the sample is spotted, followed immediately by 0.75 µL of 4-HCCA in 75:25 MeCN:H₂O with 0.1% TFA.
Results and Discussion

Electron-donating groups increase reactivity of oxazoles

The 4+2 cycloadditions of oxazoles with alkynes of unfunctionalized oxazoles are slow in a variety of solvent conditions. The addition of functional groups in oxazoles’ R-groups significantly impacts their reactivity in 4+2 cycloadditions. Under the same reaction conditions, 2,4-diphenyloxazole (Figure 9A) only had 33% conversion to the furan product after 96 hours and ethyl oxazole-4-carboxylate (Figure 9B) had no conversion to the furan product after 16 hours. Conversion was calculated by comparing the integrations of $^1$H NMR peaks that correspond to the starting material to that of the product.

Figure 9. Reaction conversion to furan product with various oxazoles.
These reactions indicate that unfunctionalized oxazoles as well as oxazoles with an EWG do not engage in 4+2 cycloadditions with alkynes rapidly. Meanwhile the reaction of an oxazole with an EDG, 5-ethoxy-4-methyloxazole, under the same reaction conditions resulted in quantitative conversion to the expected furan product (Figure 9C) after 16 hours. The conversion to furan product was detected by $^1$H NMR by the disappearance of the peak at 2.03 ppm, corresponding to the methyl peak present on the oxazole starting material.

Due to the success of the functionalization of oxazoles, specifically with the addition of an EDG to position 5 on the oxazole, additional oxazoles with an EDG present on position 5 were synthesized, making electron-rich oxazoles. The following two electron-rich oxazoles were synthesized, 5-methoxy-2,4-diphenyloxazole (oxazole 15) and 5-dimethylamine-2-methyl-4-phenyloxazole (oxazole 16) (Figure 10).

![Figure 10. Oxazoles with electron-donating groups.](image)

The reaction of oxazole 15 was attempted in deuterated chloroform with diethyl acetylenedicarboxylate overnight at room temperature; however, the expected furan product was not observed. Interestingly, the $^1$H NMR indicated the presence of an aldehyde in the reaction mixture by the presence of a singlet peak at ~10 ppm. To determine if oxazole 15 was decomposing and responsible for this peak, a $^1$H NMR of the purified oxazole was collected. The $^1$H-NMR showed that oxazole 15 likely decomposed to produce an aldehyde. Additionally, oxazole 16 showed a similar pattern of an aldehyde peak in a $^1$H NMR.
Based on these $^1$H NMR results of the electron-rich oxazoles, it was determined that while electron-rich oxazoles do rapidly engage in 4+2 cycloadditions with alkynes, they are too unstable to function as cleavable linkers in peptide workflows. This instability could be due to their sensitivity to acidic conditions. In the purification of the electron-rich oxazoles, normal-phase chromatography was conducted using a silica-based medium. Silica is mildly acidic, which could have led to acid-mediated decomposition of the electron-rich oxazoles. Meanwhile, the unfunctionalized oxazoles were quite stable, but did not quickly react with alkynes.

Because of the limited ability of oxazoles to function as reagent-controlled cleavable linkers through 4+2 cycloadditions, reactions of a related heterocycle, benzothiazoles, were explored. Specifically, the $S_{\rm N}Ar$ of benzothiazoles was tested to evaluate their potential as a reagent-controlled cleavable linker.

**Benzothiazoles function as cleavable linkers on cysteine**

Benzothiazoles have already been shown to selectively react with the nucleophilic thiol group on cysteine over other nucleophilic amino acids through $S_{\rm N}Ar$, however, their ability to function as a cleavable linker on cysteine has not been explored. To assess benzothiazoles’ ability to function as cleavable linkers on cysteine, the reaction scheme in Figure 4 was conducted. The ability of benzothiazoles to be readily attached to and removed from cysteine through $S_{\rm N}Ar$ reactions demonstrate their potential to function as cleavable linkers on cysteine-containing peptides. Therefore, a similar reaction scheme as shown in Figure 4 was tested on cysteine-containing peptide with benzothiazoles.
**Benzothiazoles are compatible with solid-phase peptide synthesis.**

The oxidation of benzothiazoles on cysteine-containing peptides led to the decomposition of peptides while in solution. To improve the stability of peptides in oxidizing conditions, benzothiazoles were incorporated onto peptides on-resin (see Figure 6 for scheme). After synthesizing peptide 9 through SPPS the cysteine residue was selectively deprotected with dithiothreitol (DTT) and the benzothiazole was added onto the peptide by a SₘAr reaction with the free cysteine residue on peptide 10. The successful addition of benzothiazole to peptide 10 to produce peptide 11a was confirmed through MALDI-TOF MS with a test cleavage of a small proportion of the resin (Figure 11A). After confirming that benzothiazoles can be added onto peptides while on resin, the oxidation of benzothiazoles’ sulfide to a sulfone was performed. The successful oxidation of peptide 11a to peptide 12a was confirmed by MALDI-TOF MS (Figure 11B). MALDI-TOF MS analysis indicated that an extra oxygen was added onto the peptide; however, the obtained masses are 2 m/z below what would be expected for the M+H peaks of the peptides with an additional oxygen. We are still exploring this finding. The extra oxygen was likely added to the sulfide present on the thiazole ring as shown on peptide 12a. Additionally, the oxidized benzothiazole on peptide 12a was stable in the harsh cleavage conditions from resin, including >90% TFA. One modification made to the cleavage conditions was the removal of EDT due to its potential to engage in SₘAr with benzothiazoles.
Successful addition and oxidation of benzothiazoles to peptides confirmed by MALDI-TOF MS. The major peaks present in the MALDI-TOF MS spectra correspond to the molecular weight of peptides 11a and 12a.

Electron-withdrawing groups improve the $S_N$Ar of benzothiazoles

After obtaining the oxidized benzothiazole incorporated on a peptide, cleavage conditions were assessed with a variety of nucleophiles. Unfunctionalized benzothiazoles appeared to be resistant to engaging in $S_N$Ar with a variety of different nucleophiles leading the reactions to proceed slowly. To improve the reactivity of benzothiazoles, an EWG group was added to a benzothiazole based on literature precedent that EWGs accelerate the rate at which benzothiazoles engage in $S_N$Ar (Motiwala et al., 2020). The EWG may improve the reactivity of $S_N$Ar reactions of benzothiazoles by stabilizing the Meisenheimer complex formed as an intermediate in the $S_N$Ar of benzothiazoles.
The cleavage of benzothiazoles with an EWG from peptides by a nucleophile was shown after obtaining the oxidized benzothiazole incorporated onto peptides. Various nucleophiles and cleavage conditions were assessed to determine the optimal conditions. In the testing of various thiol-based nucleophiles, a reducing agent was used to prevent the formation of disulfide bonds by the oxidation of the thiols. The reducing agent, TCEP, was found to be a better nucleophile than the thiol-based nucleophiles tested. The optimal conditions for cleavage were determined to be TCEP at a concentration of 10 mM, pH 8 phosphate buffer at 25 mM, and peptide at 0.5 mM. With these conditions full cleavage of the benzothiazole from the peptide was found to occur after 3 hours, leaving the sulfonic acid product. Disappearance of the starting material was followed using MALDI-TOF MS (Figure 12).

**Figure 12.** Successful cleavage of the benzothiazole present on peptide **12b** to peptide **13** was confirmed by MALDI-TOF MS.
While the difference in the masses in the MALDI-TOF MS spectra are consistent with the difference in the masses of peptides 12b and 13, the masses from the MALDI-TOF MS spectra are 2 m/z below what would be expected for the M+H peaks of the peptides. This is consistent with what is found for peptide 12a. Further studies on additional peptide sequences and MS/MS analysis of the peptides are essential to determine the exact structures corresponding to the masses present in the MALDI-TOF MS spectra.

**Benzothiazoles are compatible with “click” reaction**

To work around the unexplained mass differences in the MALDI-TOF MS spectra of peptides 12a, 12b, and 13, the compatibility of benzothiazoles with the CuAAC “click” reaction was assessed. A peptide containing azidolysine was synthesized to bind to the alkyne present on benzothiazole 8, which does not contain an additional oxygen. The successful CuACC reaction between the azidolysine-containing peptide and alkyne-containing benzothiazole was detected by MALDI-TOF MS (Figure 13).

![Diagram](image.png)

**Figure 13.** Successful synthesis of peptide 14 was confirmed by MALDI-TOF MS. The molecular weight of peptide 14 with sodium was the major peak in MALDI-TOF MS.
Preliminary results indicate that the same cleavage conditions of 10 mM TCEP, pH 8 phosphate buffer at 25 mM, and peptide at 0.5 mM work to cleave benzothiazoles “clicked” onto peptides like peptide 14.

**Conclusions**

Heterocycles show promise as cleavable linkers due to their relative stability in most conditions and their susceptibility to react in specific conditions. Benzothiazoles in particular are promising due to their ability to cleave in the presence of a variety of nucleophiles, which will continue to be explored. Additionally, their compatibility with peptides through the addition to peptides by S$_N$Ar with cysteine’s nucleophilic thiol group and by CuAAC of the alkyne present on benzothiazoles with azidolysine make them useful for peptide-based research. One application of benzothiazoles is their utility as cleavable linkers in ADCs. ADCs have made significant contributions to the treatment of various forms of cancer and show promise in the treatment of many diseases. To improve the scope of cancers and other diseases that ADCs can treat, it is essential to continue the development of cleavable linkers.
References


