

RESEARCH ARTICLE

Sequence sensitivity and pH dependence of maleimide conjugated N-terminal cysteine peptides to thiazine rearrangement

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Thiazine formation during the conjugation of N-terminal cysteine peptides to maleimides is an underreported side reaction in the peptide literature. When the conjugation was performed at neutral and basic pH, we observed the thiazine isomer as a significant by-product. Nuclear magnetic resonance (NMR) spectroscopy confirmed the structure of the six-membered thiazine and ultra-high performance liquid chromatography (UHPLC) combined with tandem mass spectrometry (MS/MS) allowed for facile, unambiguous detection due to a unique thiazine mass fragment. Furthermore, substitution of various amino acids adjacent to the N-terminal cysteine in a tripeptide model system resulted in different rates of thiazine formation, albeit within the same order of magnitude. We also determined that varying the N-substitution of the maleimide affects the thiazine conversion rate. Altogether, our findings suggest that thiazine rearrangement for N-terminal cysteine-maleimide adducts is a general side reaction that is applicable to other peptide or protein systems. Performing the conjugation reaction under acidic conditions or avoiding the use of an N-terminal cysteine with a free amino group prevents the formation of the thiazine impurity.

1 | INTRODUCTION

The thiol-maleimide reaction is one of the most widely used strategies for the covalent modification of peptides and proteins.^{1–3} The reaction has been used to install a variety of chemical labels onto biomolecules via thiol conjugation including fluorescent dyes,^{4–6} PEG moieties,⁷ radiolabels,⁸ and small molecules.⁹ A key aspect of thiol-maleimide chemistry's success is the chemoselectivity and rapid kinetics of the reaction between maleimides and thiols at or slightly below

neutral pH.^{10,11} Despite its advantages and widespread use, thiol-maleimide conjugation is prone to various side reactions including hydrolysis of the thiosuccinimide linkage, retro-Michael reaction and subsequent cross-reactivity with other thiols, and thiazine formation when conjugation takes place using an N-terminal cysteine.^{12–14} In fact, the instability of maleimido-cysteine conjugates has prompted the development of other strategies for thiol-selective bioconjugation.¹⁵ Although the hydrolysis side reaction has been shown to be advantageous in improving the pharmacokinetics and pharmacodynamics of antibody drug conjugates,¹⁶ the aforementioned side reactions are typically detrimental to the production and efficacy of maleimido bioconjugates. Interestingly, the rearrangement of succinimidyl thioether linkages to a thiazine structure during the conjugation of N-terminal cysteine containing peptides appears to be

Abbreviations: MHH, 2,5-dihydro-N-(6-hydroxyhexyl)-2,5-dioxo-1H-pyrrole-1-hexanamide or maleimido-N-6-hydroxyhexyl-1-hexanamide; MPA, 3-maleimidopropionic acid; PEGMA10K, methoxy PEG maleimide MW: 10 kDa.

Isaiah N. Gober and Alexander J. Riemen contributed equally to this work.

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an underreported side reaction in the peptide literature. Formation of the thiazine impurity can complicate the purification, characterization, and storage of peptide conjugates and may lead to significant product loss if the succinimidyl thioether linkage is intended in the final product. Furthermore, because the succinimidyl thioether and thiazine isomers have identical molecular weights, it is not trivial to distinguish the two by routine liquid chromatography–mass spectrometry (LC–MS).

We investigated both the pH dependence and sequence dependence of the succinimidyl thioether-to-thiazine side reaction using a tripeptide H-Cys-Xxx-Phe-OH (CXF) model system (where X can be substituted for different amino acids). We observed a general occurrence of thiazine rearrangement in maleimide conjugated N-terminal cysteine peptides when conjugation was performed at or above physiological pH. Consistent with previous literature,¹⁴ we found that the conversion of succinimidyl thioethers to thiazine occurs more rapidly at elevated pH. Although nuclear magnetic resonance (NMR) characterization was necessary for structure determination, we demonstrated that UHPLC–MS/MS analysis allows for rapid detection and differentiation of the succinimidyl thioether from the thiazine isomer. Additionally, we demonstrated that the rate of thiazine formation was influenced by the identity of the neighboring amino acid, although measured rates of rearrangement were within the same order of magnitude for the various peptides studied. Finally, we examined the effect of different maleimide linkers on thiazine formation and observed similar propensities for the side reaction (Figure 1).

2 | RESULTS AND DISCUSSION

2.1 | Rearrangement of succinimidyl thioether to thiazine via transcyclization

Peptides containing cysteine react readily with maleimides to form succinimidyl thioether conjugates (Scheme 1). For peptides that are conjugated to maleimides through an N-terminal cysteine, the resulting succinimide is susceptible to nucleophilic attack from the

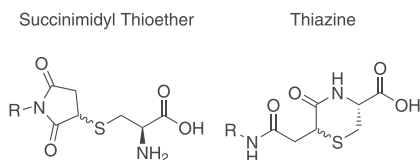
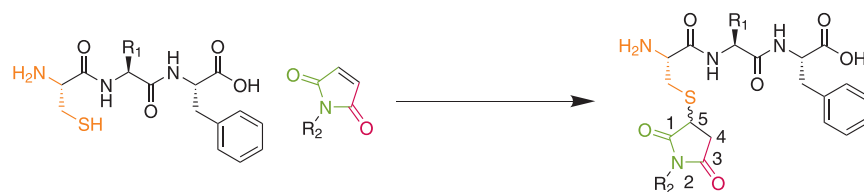


FIGURE 1 Chemical structure of the succinimidyl thioether versus the thiazine isomer



SCHEME 1 Reaction of maleimide with N-terminal Cys containing peptide via Michael addition

N-terminal amine of the cysteine. This nucleophilic attack can occur at the carbonyl at Position 1 or the carbonyl at Position 3 (Scheme 2). Transcyclization occurs through a fused bicyclic tetrahedral intermediate, which allows for the formation of a six-membered thiazine product. Although nucleophilic attack at Position 3 in Scheme 2 to form the seven-membered ring product is possible, this isomer was not detected during this study.

2.2 | Initial conjugation reaction time course

A linear model peptide, H-Cys-Xxx-Phe-OH (CXF), was chosen to investigate the thiazine side reaction that occurs during the conjugation of a maleimide with an unprotected N-terminal cysteine peptide. This model system was chosen to ensure facile peptide synthesis and analysis and to allow for investigation of the effect of neighboring amino acids by changing the amino acid derivative at the X position. Initial experiments were conducted using H-Cys-Gly-Phe-OH (CGF) and 3-maleimidopropionic acid (MPA) in 0.1-M potassium phosphate buffer (with 10% acetonitrile) at pH 7.3. Because the aqueous solutions contain organic cosolvent, it is important to note that the pH values reported were the apparent measured pH based on readings from a benchtop pH meter. Additionally, all peptides used were not corrected for trifluoroacetic acid (TFA) and water content prior to weighing. The formation of the thiazine impurity was studied at various time points (0, 0.5, 1, 2, and 24 h). The percent conversion to the thiazine structure was determined by integrating the UHPLC peak area with respect to the total conjugate-related peak area (the response factor for the absorbance at 220 nm of the succinimidyl thioether and thiazine isomers was assumed to be the same). To collect each time point, the reactions were quenched with 1% TFA in water. For the 0-h time point, peptide dissolved in phosphate buffer solution was premixed with 1% TFA in water before adding the MPA solution to prevent the formation of the thiazine impurity.

Conjugation of the H-CGF-OH peptide to MPA was rapid, as the starting material was almost completely consumed even under the acidic conditions of the “time 0 h” measurement (Figure 2, black trace). Two peaks of roughly equal abundance were formed, corresponding to the two diastereomers of the succinimidyl thioether conjugation product. As the reaction was allowed to proceed to longer time points, a third peak became visible in the UHPLC chromatograph and increased in abundance while the two succinimidyl thioether peaks decreased in abundance.

UHPLC–MS analysis confirmed that all three peaks were CGF-MPA conjugate isomers with the same molecular weight. To

SCHEME 2 Thiazine side reaction formed after nucleophilic attack of N-terminal amine at the carbonyl of the succinimide and trancyclization to give a six-membered ring product

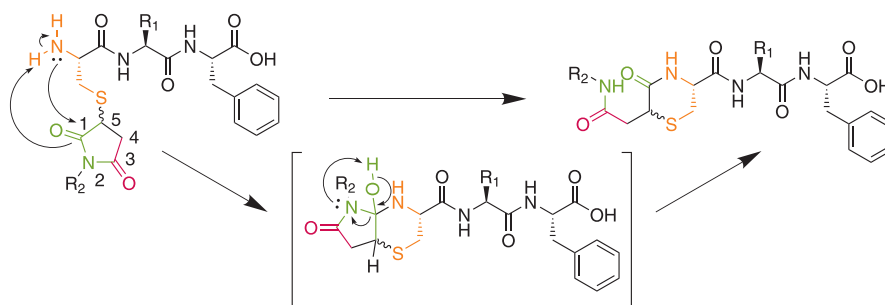
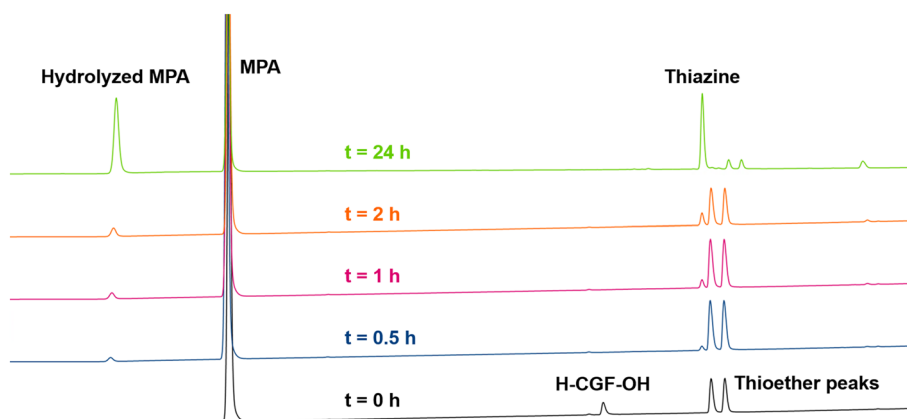
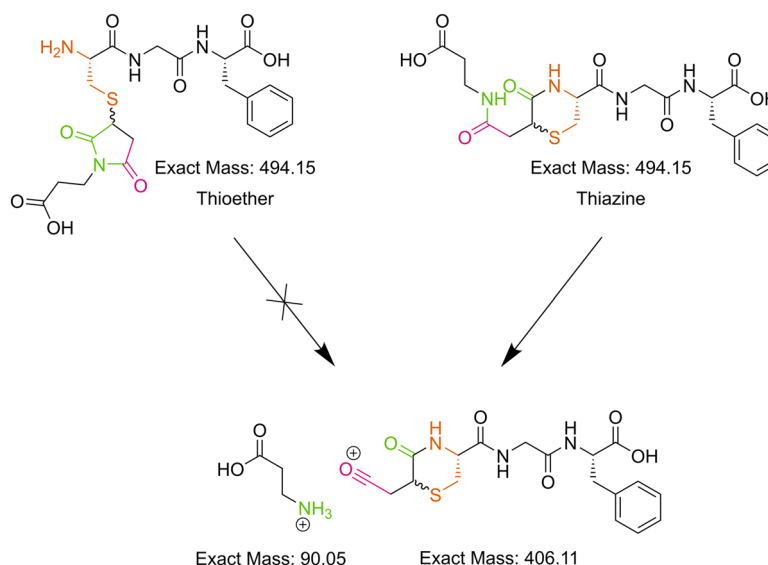


FIGURE 2 Ultra-high performance liquid chromatography (UHPLC) analysis of CGF conjugation to 3-maleimidopropionic acid (MPA) at pH 7.3. The reaction mixture was analyzed at 0 h (black), 0.5 h (blue), 1 h (cyan), 2 h (orange), and 24 h (green)



SCHEME 3 Differentiation of the thiazine isomer from the succinimidyl thioether isomer based on the unique tandem mass spectrometry (MS/MS) fragmentation of the thiazine isomer



distinguish between the succinimidyl thioether and thiazine isomers, tandem mass spectrometry (MS/MS) was used. Differentiation of the isomers was possible due to the unique fragmentation pattern of the thiazine isomer (Scheme 3). Fragmentation of the amide bond at the aminopropionic acid moiety of the thiazine isomer should yield a fragment with a mass of 90.05 Da and another fragment with a mass of 406.11 Da. In the MS/MS spectrum, a species with a mass of 406.11 Da was detected for the peak corresponding to the supposed thiazine isomer (supporting information). In the case of the succinimidyl thioether isomer, cleavage of either amide bond of the aminopropionic acid moiety should not produce two fragments

due to the cyclic nature of the succinimide. Consequently, for the two peaks corresponding to the supposed succinimidyl thioether diastereomers, a species with a mass of 406.11 Da was not detected during MS/MS analysis (supporting information).

2.3 | Structure elucidation of purified CGF-MPA isomer

To isolate the succinimidyl thioether and thiazine isomers for analysis by NMR, the conjugation was performed on a larger scale using

500 mg of peptide. To prepare the succinimidyl thioether isomers, the conjugation of CGF to MPA was performed in unbuffered water with 10% acetonitrile. Dissolving the peptide and maleimide in unbuffered aqueous solution allowed the reaction conditions to remain acidic because MPA is a weak acid and because the peptide was used as a TFA salt. This prevented the thiazine side reaction from occurring during the conjugation. The reaction was monitored by analytical high performance liquid chromatography (HPLC), and after reaching completion, the two succinimidyl thioether peaks were purified by reversed-phase HPLC (supporting information). The earlier eluting peak was obtained in 95.9% purity (with 1.8% of the second peak), and the later eluting peak was obtained in 97.4% purity (with 2.6% of the first peak present in the sample).

For preparation of the thiazine isomer, the larger scale conjugation reaction was base stressed by performing the reaction at pH 8.5 (0.1-M potassium phosphate solution with 10% acetonitrile). The reaction was monitored by UHPLC, and after 48 h, the conjugated species were isolated by reversed-phase HPLC. The earlier eluting peak corresponding to the supposed thiazine isomer was obtained in 94.8% purity and contained 2.5% of the putative succinimidyl thioether isomer. Analysis of the purified thiazine isomer by MS/MS also produced a species with a mass of 406.11 Da, indicative of the unique fragmentation of the thiazine isomer.

The purified isomers were then characterized by NMR spectroscopy. All the ^1H NMR chemical shifts were assigned using a combination of 1-D and 2-D NMR experiments (supporting information). For protons with overlapping chemical shift signals, DQF-COSY experiments were used to make the assignments. Position assignments are indicated in Figure 3. Correlations in the ^1H - ^{13}C Heteronuclear Single Quantum Coherence (HSQC) NMR spectrum were used for numbering the protons in the thiazine isomer of CGF-MPA.

The thiazine structure could be elucidated based on correlations between H^9 and C^{18} , H^{1a} and C^{20} , H^{1a} and C^5 , H^5 and C^1 , H^5 and C^{18} , H^5 and C^{19} , and H^9 and C^{20} in the ^1H - ^{13}C Heteronuclear Multiple Bond Correlation (HMBC) NMR spectrum (Figure 3). The key correlation for differentiating the succinimidyl thioether and thiazine isomers was the H^9 and C^{18} correlation. For the six-membered thiazine structure, C^{18} is separated from H^9 by three bonds, which allowed for detection by ^1H - ^{13}C HMBC NMR.¹⁷ Because the H^9 - C^{18} correlation was observed, the succinimidyl thioether structure (where H^9 and C^{18} are five bonds away) could be excluded.

2.4 | Investigation of the influence of pH on thiazine rearrangement

To investigate the role of pH on the formation of the thiazine impurity, the conjugation reaction was monitored under acidic conditions at pH 5.0, near neutral conditions at pH 7.3, and under basic conditions at pH 8.4 (Figure 4). The reactions were all carried out in aqueous 0.1-M potassium phosphate solution to ensure that any differences observed in rate and extent of thiazine formation were not due to differences in buffer. The purified H-CGF-OH peptide was

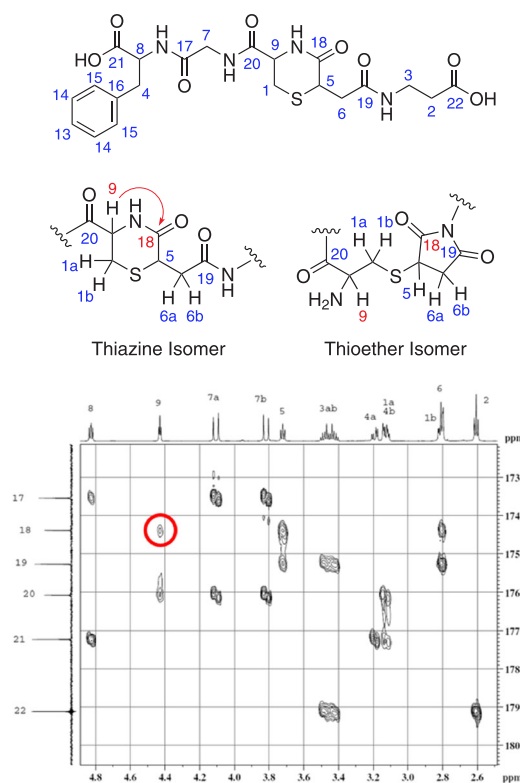


FIGURE 3 Top: Labeling of ^1H and ^{13}C peaks for CGF-3-maleimidopropionic acid (MPA) in the nuclear magnetic resonance (NMR) spectra (^{13}C signals are in order of increasing chemical shift). Bottom: Confirmation of the formation of the thiazine isomer using ^1H - ^{13}C Heteronuclear Multiple Bond Correlation (HMBC) NMR. The key correlation for excluding the possibility of the succinimidyl thioether structure (H^9 to C^{18}) is indicated in red in the 2-D NMR spectrum

incubated with MPA in potassium phosphate solution for 24 h before analyzing by UHPLC. Reactions were quenched using a solution of 1% TFA in water.

At pH 5.0, the conjugation of MPA to CGF proceeded rapidly, producing two peaks that corresponded to the succinimidyl thioether diastereomers of the CGF-MPA conjugate. However, at 24 h, only 0.1% of the thiazine isomer was observed, and hydrolysis of MPA and the succinimidyl thioether isomers was negligible. This result is consistent with the supposed mechanism of thiazine rearrangement, because protonation of the N-terminal amino group under acidic conditions should prevent intramolecular nucleophilic attack of the succinimide carbonyl and preserve the succinimidyl thioether structure.

Analysis of CGF and MPA conjugation at pH 7.3 revealed the thiazine isomer as the major product at the 24 h mark in 70% abundance. Conversely, the abundance of the succinimidyl thioether isomers decreased, which was indicative of the rearrangement. Expectedly, the increase in the rate of thiazine formation at pH 7.3 compared to pH 5.0 was substantial. Significant hydrolysis of excess MPA was also observed, but minimal hydrolysis products of the CGF-MPA conjugate were detected.

FIGURE 4 Ultra-high performance liquid chromatography (UHPLC) analysis of CGF conjugation to 3-maleimidopropionic acid (MPA) after 24 h at pH 5.0 (black), pH 7.3 (green), and pH 8.4 (orange)

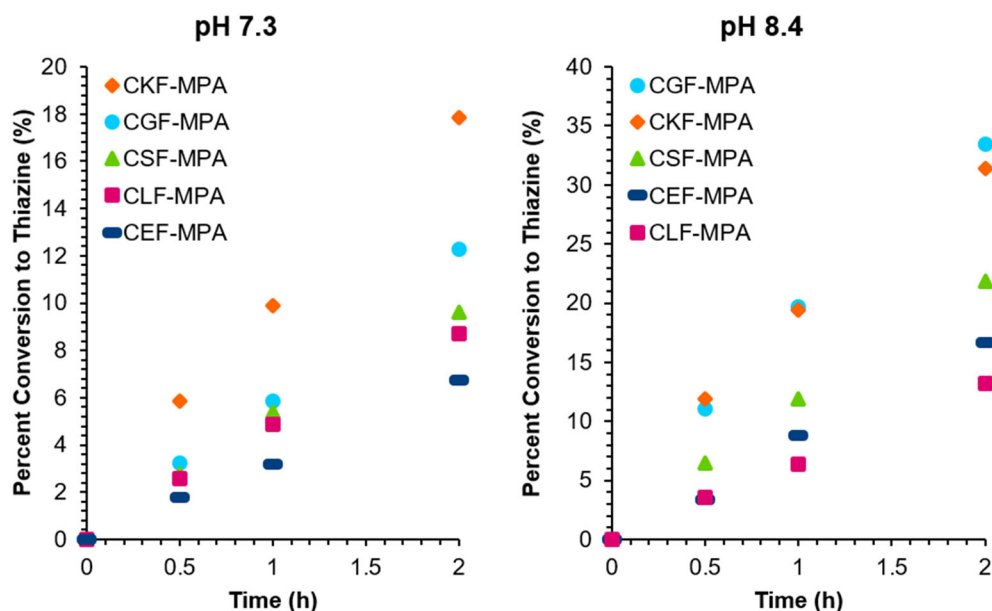
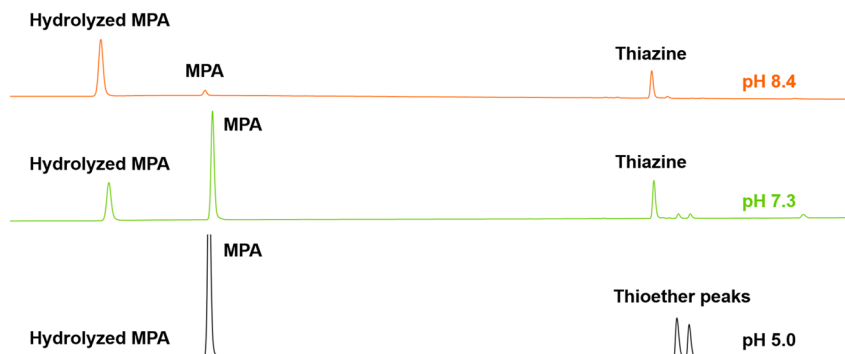


FIGURE 5 Left: Increase in thiazine formation over time for different neighboring amino acids of CXF-3-maleimidopropionic acid (MPA) at pH 7.3 in 0.1-M potassium phosphate buffer with 10% acetonitrile. For CLF, the conjugation reaction was carried out in 0.1-M potassium phosphate buffer with 26% acetonitrile added to maintain solubility. Right: Increase in thiazine formation over time for different neighboring amino acids of CXF-MPA at pH 8.4 in aqueous 0.1-M potassium phosphate solution with 10% acetonitrile. For CLF, the conjugation reaction was carried out in 0.1-M potassium phosphate solution with 26% acetonitrile added to maintain solubility

Thiazine formation was rapid at pH 8.4, with nearly 90% of the succinimidyl thioether isomers converted to the thiazine isomer after 24 h. The considerable increase in the rate of thiazine formation at higher pH again supports a base-dependent mechanism involving nucleophilic attack of the succinimide by the N-terminal amine. Due to the higher pH, most of the excess MPA was hydrolyzed over this period of time. On the other hand, the thiazine isomer appeared to be stable under the base stressed conditions.

2.5 | Investigation of sequence dependence on thiazine rearrangement kinetics

To assess the influence of neighboring amino acids (relative to N-terminal cysteine) on the thiazine side reaction, a selection of five peptides was prepared: H-CGF-OH, H-CEF-OH, H-CKF-OH,

H-CSF-OH, and H-CLF-OH. Side chains were selected to provide a sampling of functional groups and potential stereoelectronic interactions. Because it is the smallest amino acid, glycine was selected with the expectation of it having the least conformational restriction due to steric hindrance. Glutamic acid and lysine were chosen to investigate the effect of acidic and basic ionizable side chains, respectively, while serine was chosen to test the role of a neighboring hydroxyl functional group. Finally, leucine was chosen for a representative hydrophobic sequence.

Conjugation reactions with MPA were carried out at pH 5.0, pH 7.3, and pH 8.4 for various time points. The percent conversion of succinimidyl thioether to thiazine was measured at 0-, 0.5-, 1-, and 2-h time points by UHPLC (Figure 5). At pH 5.0, thiazine rearrangement was suppressed for all the CXF peptides. Even after 336 h, only 1.1% conversion occurred in the fastest instance, which made it impractical to calculate a rate of thiazine conversion at pH 5.0

(Table 1). In the case of the pH 7.3 experiments, all five peptides showed extensive conversion to the thiazine isomer. CKF and CGF gave the fastest rates of conversion with 8.7% and 6.1% thiazine formed per hour, respectively (Table 2). For CLF-MPA, the rate of thiazine conversion was the second slowest with a rate of 4.3% thiazine formed per hour. The slowest rate of rearrangement corresponded to the CEF peptide, which was over 2 times slower than the fastest result.

Conducting the studies at pH 8.4 resulted in a considerable increase in the rate of thiazine formation for each of the peptides (Figure 5 and Table 2). However, a different order of reactivity was observed at elevated pH, with CGF giving the most rapid rate of conversion at 17.6% thiazine formed per hour while the CLF peptide was the slowest at 6.6% thiazine formed per hour. The trend of the CLF and CEF peptides being considerably slower than the other peptides was also observed for the pH 8.4 experiments.

Although the thiazine rearrangement kinetics data appear suggestive of side-chain specific interactions that may influence the rearrangement reaction, a more nuanced analysis via modeling of the stereochemical constraints and probabilities of side-chain dihedral angles is necessary in order to fully elucidate the observed differences in rates of rearrangement for the CXF peptides.^{18,19} However, we postulated that the rate of thiazine conversion for CGF-MPA adducts was among the most rapid due to the small size of glycine and the presumed freedom of rotation around the Cys-Gly bond. In the case of CKF, the rationale for the faster rate of rearrangement was less clear. Although intramolecular base catalysis has been reported for

thiosuccinimide hydrolysis when using beta amino maleimides,²⁰ the amine of Lys does not appear to be well suited for base catalysis as it has a pKa of 10.5 and would therefore be protonated at pH 7.3 and pH 8.4.²¹

In addition, because the conjugation and subsequent rearrangement was performed in water, it was not certain whether the Lys amine, the Glu carboxylate, or the Ser hydroxyl in CKF, CEF, and CSF (respectively) would participate in electrostatic or hydrogen bonding interactions that directly affected thiazine formation via interaction with the N-terminal amine. In the absence of a hydrophobic binding pocket, these types of noncovalent interactions are often negligible in water due to solvation and hydrogen bonding between water and the polar functional groups of amino acid side chains.^{22–24} Furthermore, the presence of salt (0.1-M potassium phosphate) was expected to weaken electrostatic or hydrogen-bonding interactions that may have existed between side chain functional groups and the amine of the N-terminus.²⁵

It is also important to note that for the CLF peptide, a higher percentage by volume of acetonitrile (26%) was present in the conjugation solution compared to the other experiments. To evaluate the effect of the greater organic solvent content, the conjugation studies were also performed using the CGF peptide and MPA in pH 7.4 potassium phosphate buffer solution containing 26% acetonitrile (supporting information). In comparison with the CGF-MPA studies with 10% acetonitrile cosolvent, the rate of thiazine rearrangement was 1.3 times slower. These results support the supposition that the slower rate of thiazine conversion for the CLF peptide was also due to the physiochemical properties of leucine rather than just the presence of more acetonitrile in the conjugation solution.

TABLE 1 Thiazine conversion for CXF-MPA adducts at pH 5.0 after 336 h

Thiazine conversion after 336 h	
Peptide conjugate	pH 5.0 (%)
CGF-MPA	1.1
CEF-MPA	1.0
CKF-MPA	0.5
CSF-MPA	0.9
CLF-MPA	0.3

Abbreviation: MPA, 3-maleimidopropionic acid.

TABLE 2 Rate of thiazine formation for CXF-MPA adducts at pH 7.3 and pH 8.4

Thiazine conversion after 1 h		
Peptide conjugate	pH 7.3 (%)	pH 8.4 (%)
CGF-MPA	6.1	17.6
CEF-MPA	3.4	8.4
CKF-MPA	8.7	16.8
CSF-MPA	4.8	11.2
CLF-MPA	4.3	6.6

Abbreviation: MPA, 3-maleimidopropionic acid.

2.6 | Investigation of maleimide N-substitution on thiazine rearrangement

Previous reports have shown that maleimides with more electron-withdrawing substituents at the nitrogen of the maleimide have greater electrophilic character.^{16,20,26} This leads to more rapid hydrolysis of both the maleimide and the cysteine-conjugated thioether adduct. Two linkers, maleimido (hydroxyhexyl)hexanamide (MHH) and a methoxy PEG maleimide with an average molecular weight of 10,000 Da (PEGMA10K) were chosen to compare with MPA (Figure 6). Because its longer alkyl chain is less electron withdrawing than the propionic acid linker of MPA, we anticipated that the MHH linker may provide higher stability toward conversion of peptide conjugates to the thiazine isomer. Additionally, PEGMA10K was selected in order to assess the analytical challenge of detecting the thiazine isomer in the context of a polydisperse, large molecular weight conjugate. PEGMA10K was suitable for comparison to MPA because it maintains a similar linker length (maleimido propionic amide vs. maleimido propionic acid) but has a significantly higher overall molecular weight.

The conjugation of CXF peptides to MHH was performed at pH 7.3 in 0.1-M potassium phosphate buffer with 26% acetonitrile added to ensure solubility during the course of the reaction (for conjugation of CLF to MHH, 28% acetonitrile was present). The thiazine concentration was measured at 0, 0.5, 1, and 2 h by UHPLC. As seen in Figure 7, CGF reacted rapidly with MHH to give complete conjugation within 30 min. The emergence of a new peak corresponding to the thiazine isomer was observed over time. After 24 h, about 33% of the succinimidyl thioether adduct was converted to the thiazine isomer. When compared to the generation of the CGF-MPA thiazine adduct at pH 7.3 with 26% acetonitrile cosolvent, the rate of thiazine rearrangement for the CGF-MHH adduct was over two times slower. In addition, the extent of hydrolysis of MHH over time was significantly lower compared to MPA as the majority of the maleimide remained intact after 24 h. Overall, these results demonstrated that linker chemistry has a notable effect on the degree of thiazine side reaction encountered for maleimide conjugated N-terminal cysteine containing peptides.

Conjugation of CKF, CEF, CSF, and CLF peptides to MHH produced succinimidyl thioether adducts that also suffered from thiazine rearrangement over time but at slower rates. Compared to the MPA studies, a similar order of reactivity was also observed with CGF and CKF giving the fastest rates of rearrangement and CLF and CEF producing the slowest rates (Figure 8 and Table S6.1). Interestingly, all MHH conjugates were between 3 and 5 times slower toward thiazine

conversion than the MPA counterparts were. The greater stability of the MHH linker was likely due to its longer hexanamide alkyl chain that is inductively electron donating with respect to the maleimido nitrogen. Despite the greater stability of the MHH linker, these data support the conclusion that the susceptibility of succinimidyl thioethers to thiazine rearrangement is a general property of all maleimido linkers.

For PEGMA10K, conjugation was performed using CGF and CKF, the two peptides to give the fastest rates of thiazine rearrangement in the MPA and MHH studies. Reactions were run in two steps to facilitate monitoring the progress of the reaction.

First, the CXF peptide and PEGMA10K were dissolved in unbuffered water with 10% acetonitrile at a ratio of 2:1 peptide to maleimide. After 1 h, a second equivalent of PEGMA10K was added, and the conjugation reaction was allowed to proceed for another hour. The pH of the reaction was adjusted to pH 7.7 using 0.1-M potassium phosphate solution with 10% acetonitrile, and the reaction was quenched with dilute TFA in water after 48 h. As shown in Figure 9, CGF reacted completely with PEGMA10K after the addition of the second portion of the maleimide. After increasing the pH, a new peak with a slightly shifted retention time was observed, and the peak corresponding to the CGF-PEGMA10K succinimidyl thioether was absent. When CKF was used during the conjugation with PEGMA10K, conversion of the CKF-PEGMA10K adduct to the thiazine isomer could be detected even in unbuffered water with 10% acetonitrile upon the addition of the second equivalent of PEGMA10K as evidenced by the emergence of a second peak in the UHPLC chromatogram (Figure 10, green trace). After base stressing the conjugation reaction mixture at pH 7.7 for 48 h, nearly complete conversion to the thiazine isomer was observed (Figure 10, orange trace).

Direct infusion MS/MS analysis was used to confirm the presence of the thiazine isomer for both the CGF and CKF conjugation reactions after incubation with PEGMA10K at pH 7.7 for 48 h (supporting information). Fragmentation of the CGF-PEGMA10K thiazine isomer produced a unique mass fragment with a mass of 406.11 Da, which was not possible for the succinimidyl thioether isomer. Similarly, fragmentation of the CKF-PEGMA10K adduct gave a 477.18-Da mass fragment, which was unique for the thiazine isomer. These results demonstrated the power of our MS/MS strategy for unambiguous

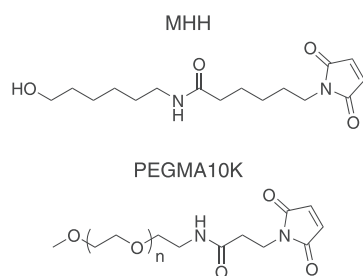
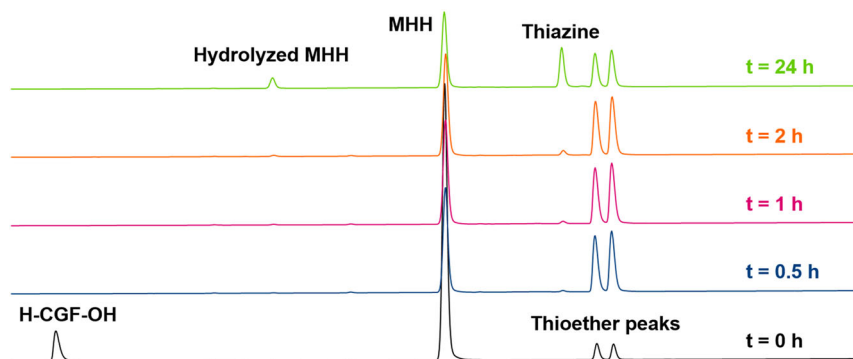


FIGURE 6 Chemical structures of the maleimido (hydroxyhexyl) hexanamide (MHH) and methoxy PEG maleimide MW: 10 kDa (PEGMA10K) maleimide reagents

FIGURE 7 Ultra-high performance liquid chromatography (UHPLC) analysis of H-CGF-OH conjugation to maleimido (hydroxyhexyl) hexanamide (MHH) at pH 7.3. The reaction mixture was analyzed at 0 h (black), 0.5 h (blue), 1 h (cyan), 2 h (orange), and 24 h (green)



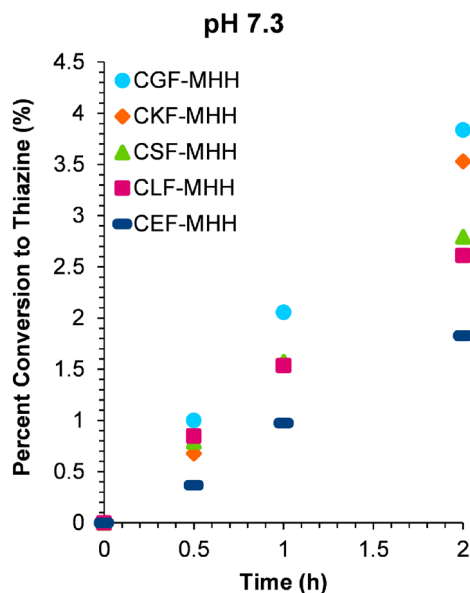


FIGURE 8 Increase in thiazine formation over time for different neighboring amino acids of CXF-maleimido (hydroxyhexyl) hexanamide (MHH) adducts at pH 7.3 in 0.1-M potassium phosphate buffer with 26% acetonitrile. For CLF, the conjugation reaction was carried out in 0.1-M potassium phosphate buffer with 28% acetonitrile added to maintain solubility

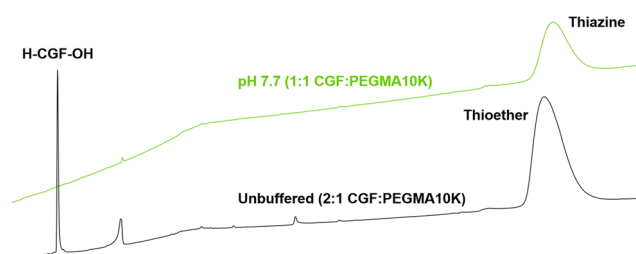


FIGURE 9 Ultra-high performance liquid chromatography (UHPLC) analysis of H-CGF-OH conjugation to methoxy PEG maleimide MW: 10 kDa (PEGMA10K). Black trace: conjugation of CGF to PEGMA10K (2:1 ratio of peptide to maleimide) in unbuffered water with 10% acetonitrile. Green trace: thiazine rearrangement of CGF-PEGMA10K conjugate after 48 h (1:1 ratio of peptide to maleimide) at pH 7.7 in 0.1-M potassium phosphate solution with 10% acetonitrile

detection of the thiazine isomer. Because of the nonuniformity of pegylated maleimide reagents, analysis of thiazine rearrangement of succinimidyl thioethers by routine LC-MS presents an analytical challenge. Typically, confirmation of the rearrangement would require purification and isolation of the desired isomer followed by advanced 2-D NMR experiments. However, an MS/MS detection approach greatly simplified the detection of the thiazine isomer and even allowed confirmation of the rearrangement without preparative chromatographic separation or specialized sample preparation prior to MS/MS analysis.

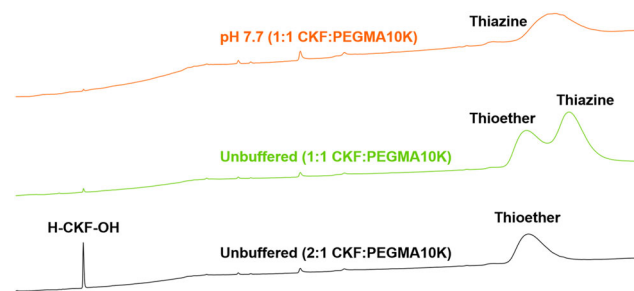


FIGURE 10 Ultra-high performance liquid chromatography (UHPLC) analysis of H-CKF-OH conjugation to methoxy PEG maleimide MW: 10 kDa (PEGMA10K). Black trace: conjugation of CKF to PEGMA10K (2:1 ratio of peptide to maleimide) in unbuffered water with 10% acetonitrile. Green trace: conjugation of CKF to PEGMA10K (1:1 ratio of peptide to maleimide) in unbuffered water with 10% acetonitrile. Orange trace: thiazine rearrangement of CKF-PEGMA10K conjugate after 48 h (1:1 ratio of peptide to maleimide) at pH 7.7 in 0.1-M potassium phosphate solution with 10% acetonitrile

3 | CONCLUSION

In this work, we explored the pH and sequence dependence of the rearrangement of succinimidyl thioethers to thiazine isomers in the context of maleimide conjugation to N-terminal cysteine containing peptides. A prominent increase in the rate of thiazine formation was observed at basic pH, which was consistent with previous work indicating that the rearrangement is base promoted. When the amino acid adjacent to the N-terminal cysteine was substituted for various amino acids, we observed generation of the thiazine impurity in all instances, though the rates of thiazine formation differed for the CXF peptides that were studied. Using a maleimide linker that we anticipated to be more stable also gave substantial thiazine formation, which provides additional support that the side reaction is general.

NMR analysis conclusively confirmed the presence of the thiazine impurity, which we observed as a significant side reaction during the conjugation of CXF peptides to MPA, MHH, and PEGMA10K maleimides. We also developed a powerful MS/MS strategy for identifying the thiazine isomer that has general applicability and is independent of the N-substitution of the maleimide. This provides excellent analytical utility because MS/MS capabilities are common to many research labs, while instrumentation for advanced 2-D NMR experiments and the requisite expertise to perform such experiments are less common.

Due to the general nature of the thiazine side reaction, it is advisable to avoid the use of N-terminal cysteine in peptide conjugates where the succinimidyl thioether linkage is desired. Although performing the conjugation under acidic conditions near pH 5 prevents thiazine formation, the subsequent purification, storage, and application of the peptide conjugates must also be carried out under acidic conditions to avoid loss of the succinimidyl thioether. Alternatively, acetylation of the N-terminal cysteine can be performed to prevent formation of the thiazine impurity (supporting information).^{12,13}

4 | MATERIALS AND METHODS

4.1 | Materials

MHH was purchased from AldLab (Woburn, MA), and PEGMA10K was purchased from JenKem Technology USA (Plano, TX). Dimethylformamide was purchased from Univar Solutions Inc. (Downers Grove, IL). N,N-diisopropylethylamine was purchased from Spectrum Laboratory Products Inc. (Gardena, CA). Piperidine was purchased from Tedia Company Inc. (Fairfield, OH). Isopropyl alcohol was purchased from Brenntag Pacific Inc. (Santa Fe Springs, CA). Diisopropyl ether was purchased from Neuchem (Sparks, NV). N,N'-diisopropylcarbodiimide, triisopropylsilane, 1,2-ethanedithiol, potassium phosphate monobasic, and potassium phosphate dibasic were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade trifluoroacetic acid was purchased from Alfa Aesar (Haverhill, MA). HPLC grade water and acetonitrile were obtained from MilliporeSigma (Burlington, MA). All other amino acid derivatives and reagents were obtained from Bachem AG, Bubendorf, Switzerland.

Preparative HPLC purification was performed using a Waters Prep LC system. HPLC and UHPLC analyses were performed on a Thermo Scientific UltiMate 3000 UHPLC system using an Atlantis T3 100 Å, 3 µm, 4.6 mm × 150-mm column and a Waters Acquity HSS T3 100 Å, 1.8 µm, 2.1 × 150 mm column, respectively. A Thermo Scientific Vanquish UHPLC system with a Waters Acquity CSH C18 130 Å, 1.7 µm, 2.1-mm × 150-mm column was used for UHPLC-MS separations. MS analysis was performed in positive ion mode on a Thermo Scientific Q Exactive Focus Hybrid Quadrupole-Orbitrap™ mass spectrometer. For MS/MS analyses, high-energy C-trap dissociation (HCD) was used for fragmentation.

The apparent pH of the solutions used during the CXF and maleimide conjugations was measured using a Mettler Toledo™ SevenCompact™ S220-Basic pH/ion benchtop meter equipped with a Mettler Toledo™ InLab Solids Go-ISM® electrode (pH 1–11; 0–80°C). The electrode was calibrated before each series of measurements using standard buffer solutions purchased from Millipore Sigma (Burlington, MA). All measurements were taken at ambient temperature.

4.2 | Manual Fmoc solid-phase peptide synthesis

The peptides H-Cys-Gly-Phe-OH (CGF), H-Cys-Leu-Phe-OH (CLF), H-Cys-Glu-Phe-OH (CEF), H-Cys-Lys-Phe-OH (CKF), and H-Cys-Ser-Phe-OH (CSF) were synthesized on a 2.5-mmol scale by first loading Fmoc-Phe-OH onto 2-chlorotrityl chloride resin (10-mmol scale, substitution = 1.3 mmol/g) and then splitting the resin into four lots for the subsequent divergent coupling reactions. Loading of the first amino acid derivative was carried out using Fmoc-Phe-OH (1.0 eq., 10 mmol) and DIPEA (1.0 eq., 10 mmol) in dimethylformamide (DMF). Coupling of the second amino acid derivative was performed using TCTU/DIPEA (1.9 eq., 4.8 mmol/2.5 eq., 6.3 mmol) in DMF. Coupling of Fmoc-Cys (Trt)-OH was performed using DIC/HOBt (6.2 eq,

15.4 mmol/2.3 eq, 5.7 mmol). All reactions were performed at room temperature. For Fmoc removal, 20% (v/v) piperidine in DMF was used. Cleavage of the peptidyl-resin to obtain crude peptides was performed using TFA/TIS/water/EDT (92.5:2.5:2.5:2.5 v/v). Crude peptides were analyzed by reversed-phase UHPLC (Waters Acquity HSS T3 100 Å, 1.8 µm, 2.1 × 150 mm) using a linear gradient of 0.1% TFA in acetonitrile. Peptides were purified by preparative reversed-phase HPLC using Luna® C18(3) 10-µm particle size media packed in an axial compression column (ModCol®, 25 × 5 iD). After purification, the peptides were lyophilized to give white powders. No correction for TFA and water content was performed prior to weighing out peptides for the maleimide conjugation experiments.

4.3 | Maleimide conjugation time course experiments

4.3.1 | MPA

A solution of MPA (2 mg/ml) and a solution of CXF peptide (2 mg/ml) were prepared separately in aqueous 0.1-M potassium phosphate solution in 10:90 acetonitrile: water (for CLF-MPA conjugation, the peptide and MPA were dissolved in 0.1-M potassium phosphate solution containing 26:74 acetonitrile: water to help solubilize the peptide). Reactions were initiated by mixing the solution of MPA with the solution of CXF. The reaction was performed in a sealed microcentrifuge tube at ambient temperature. An aliquot of the reaction solution was removed at the appropriate time point and was quenched using an equal volume of a solution of 1% TFA in water.

4.3.2 | MHH

A solution of MHH (2 mg/ml) was dissolved in 50% acetonitrile in water, and a solution of CXF peptide (2 mg/ml) was prepared in aqueous 0.1-M potassium phosphate solution in 10:90 acetonitrile: water (for CLF-MPA conjugation, the peptide and MHH were dissolved in 0.1-M potassium phosphate solution containing 28:72 acetonitrile in water). Reactions were initiated by mixing the solution of MPA with the solution of CXF. The reaction was performed in a sealed microcentrifuge tube at ambient temperature. An aliquot of the reaction solution was removed at the appropriate time point and was quenched using an equal volume of a solution of 1% TFA in water.

4.3.3 | PEGMA10K

A solution of PEGMA10K (25 mg/ml) and CXF peptide (1.55 mg/ml) was dissolved in unbuffered water with 10% acetonitrile. The solutions of peptide and maleimide were mixed and allowed to react for 1 h. After 1 h, another portion of PEGMA10K (22 mg/ml) in unbuffered water with 10% acetonitrile was added. The reaction was allowed to proceed for another hour before the pH was adjusted to

pH 7.7 using 0.1-M potassium phosphate solution in 10:90 acetonitrile:water. Reactions were performed in sealed amber vials at ambient temperature. Reaction solutions at pH 7.7 were quenched with an equal volume of a solution of 1% TFA in water.

4.4 | Preparation and purification of CGF-MPA thioether and CGF-MPA thiazine adducts

4.4.1 | Preparation and purification of CGF-MPA thioether

To a solution of 500 mg of H-CGF-OH in 25 ml of 10:90 acetonitrile:water was added a solution of 500-mg MPA in 25 ml of 10:90 acetonitrile:water. The two solutions were mixed together to initiate the conjugation reaction. The reaction progress was monitored by taking 500- μ l aliquots and adding 500 μ l of 0.2% TFA in water to the solutions, which were subsequently analyzed by analytical HPLC. Once the reaction was complete, the two isomer peaks of the CGF-MPA conjugate were purified by preparative reversed-phase HPLC using Luna $\text{\textcircled{R}}$ C18(3) 10- μ m particle size media packed in an axial compression column (ModCol $\text{\textcircled{R}}$, 25 \times 5 iD). The peptide conjugate isomers were lyophilized to give white powders.

4.4.2 | Preparation and purification of CGF-MPA thiazine

To a solution of 500 mg of H-CGF-OH in 25 ml of pH 8.5, 20-mM potassium phosphate buffer solution (10:90 acetonitrile:water) was added a solution of 500-mg MPA in 25 ml of pH 8.5, 20-mM potassium phosphate buffer solution (10:90 acetonitrile:water). The two solutions were mixed together to initiate the conjugation reaction. The reaction progress was monitored by taking 500- μ l aliquots and adding 500 μ l of 1% TFA in water to the solutions, which were subsequently analyzed by UHPLC. Once the reaction was complete, the two isomer peaks of the CGF-MPA conjugate were purified by preparative reversed-phase HPLC using Luna $\text{\textcircled{R}}$ C18(3) 10- μ m particle size media packed in an axial compression column (ModCol $\text{\textcircled{R}}$, 25 \times 5 iD). The peptide conjugate was lyophilized to give a white powder.

4.5 | NMR experiments

For characterization, 1-D ^1H and ^{13}C spectra were collected, and various 2-D NMR experiments were performed including ^1H - ^1H DQF-COSY, ^1H - ^{13}C HSQC, ^1H - ^{13}C HMBC, and ROESY. All spectra were recorded at 600 MHz on a Bruker AVNeo-600 NMR spectrometer at 277 K. Spectra were referenced to internal trimethylsilylpropanoic acid (TSP). Samples were prepared by dissolving 10 mg of the lyophilized peptide in 600 ml of D_2O with 0.1% TFA-d.

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SUPPORTING INFORMATION

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