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THE THIOL-MALEIMIDE REACTION DOWNSIDE: SECRETS OF AN IMPORTANT BY-PRODUCT REVEALED

SEQUENCE SENSITIVITY AND PH DEPENDENCE OF MALEIMIDE CONJUGATED N-TERMINAL CYSTEINE PEPTIDES TO THIAZINE REARRANGEMENT

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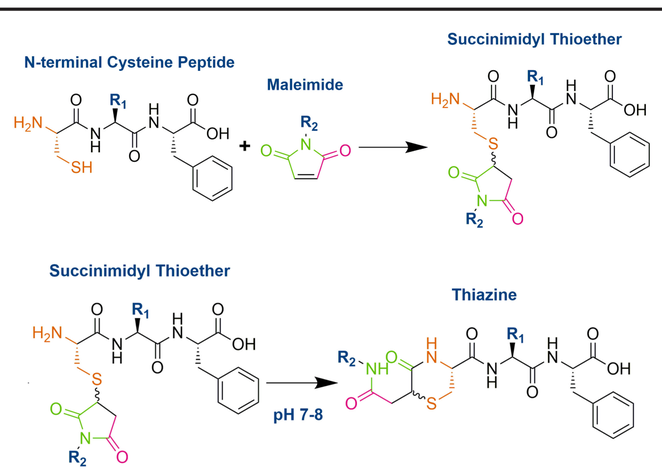
Introduction

At Bachem, technological leadership and innovative strength have been the cornerstones of our success since the very beginning of our company. We strive for technology and highest quality of our products. Investing in research in order to constantly improve our manufacturing process is one of our driving forces. In this context, we have studied and provided further optimized tools for one of the most widely used strategies for the covalent modification of peptides and proteins, the thiol-maleimide reaction. In this chemical modification reaction, however, side-reactions occur and are challenging to address. Thiazine formation during the conjugation of N-terminal cysteine peptides to maleimides is one of them and has been revealed to be underreported in peptide literature. This research effort resulted in a recently published article in Journal of Peptide Science [1].

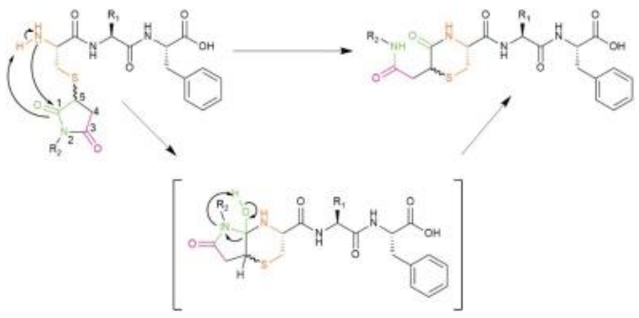


Summary and outlooks:

- *Thiazine is a by-product formed during the thiol-maleimide coupling when occurring on a N-terminal Cysteine of a peptide.*
- *Thiazine rearrangement is a general side-reaction regardless of the sequence or the maleimide linker.*
- *Thiazine by-product is rapidly formed at neutral and basic pH, whereas at pH 5 the rate of formation decreases significantly.*
- *Conjugation to an unprotected N-terminal cysteine should be avoided.*
- *If possible, 3-mercaptopropionic acid can be used to replace the N-terminal Cysteine, or the N-terminus can be acetylated.*

1. Rearrangement of succinimidyl thioether to thiazine via transcyclization

For peptides that are conjugated to maleimides through an N-terminal cysteine, the resulting succinimide is susceptible to nucleophilic attack from the N-terminal amine of the cysteine. This nucleophilic attack can occur at the carbonyl at Position 1 or the carbonyl at Position 3 (Scheme 1). Transcyclization occurs through a fused bicyclic tetrahedral intermediate, which allows for the formation of a six-membered thiazine product.



Scheme 1 - Mechanism of the Thiazine formation: Thiazine side reaction is formed after nucleophilic attack of N-terminal amine at the carbonyl of the succinimide leading to the intermediate product. Then a trancyclization occurs to give a six-membered ring product.

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. 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. This model system was chosen to ensure easy 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. 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 (Figure 1).

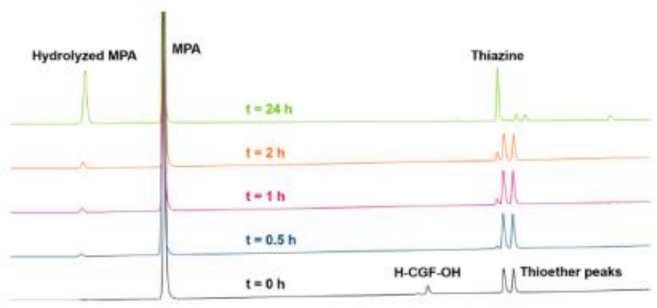
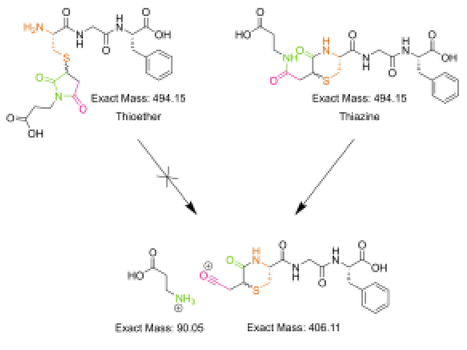


Figure 1 - Ultra-high performance liquid chromatography (UHPLC) analysis of CGF conjugation to 3-maleimidopropionic acid (MPA) at pH 7.3. The reaction mixture was analyzed at 0 h (black), 0.5 h (blue), 1 h (cyan), 2 h (orange), and 24 h (green).

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 1, 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 corresponding to the thiazine byproduct 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. MS/MS analysis enabled to distinguish the isomers due to their unique fragmentation pattern (Scheme 2).

Scheme 2 - Differentiation of the thiazine isomer from the succinimidyl thioether isomer based on the unique tandem mass spectrometry (MS/MS) fragmentation of the thiazine isomer.



3. Structure elucidation of purified CGF-MPA isomers

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**. 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 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.

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} shown in Figure 2.

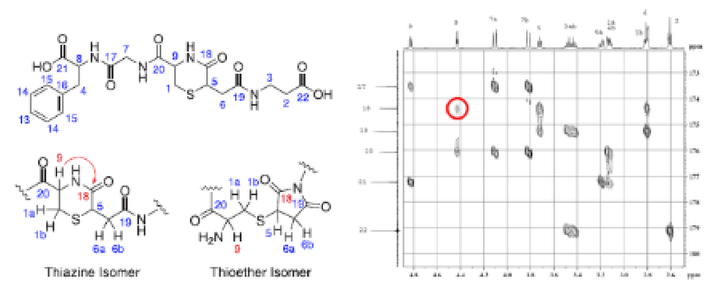


Figure 2 - Left: Labeling of ^1H and ^{13}C peaks for CGF-3-maleimidopropionic acid (MPA) in the nuclear magnetic resonance (NMR) spectra (^{13}C signals are in order of increasing chemical shift). Left: Confirmation of the formation of the thiazine isomer using ^1H–^{13}C HMBC NMR. The key correlation for excluding the possibility of the succinimidyl thioether structure (H_9 to C_{18}) is indicated in red in the 2-D NMR spectrum.

4. Influence of pH on thiazine rearrangement

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. The peptide H-CGF-OH was used as model for the experiments. H-CGF-OH was 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.

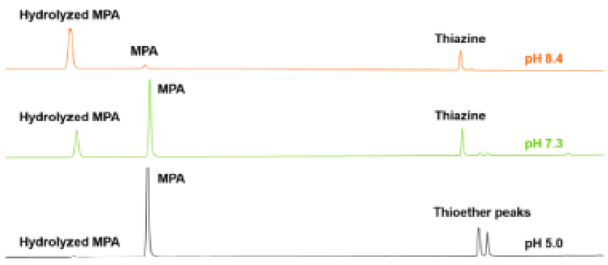


Figure 3 - UHPLC analysis of CGF conjugation to 3-maleimidopropionic acid (MPA) after 24h at pH 5.0 (black), pH 7.3 (green), and pH 8.4 (orange).

Analysis of CGF and MPA conjugation at pH 7.3 and 8.4 revealed the thiazine isomer as the major product at the 24h. Furthermore, the increase in the rate of thiazine formation at pH 7.3 compared to pH 5.0 was substantial.

Thiazine formation was faster at pH 8.4, with nearly 90% compared to 70% at pH 7.3 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.

5. Sequence dependence on thiazine rearrangement kinetics

Five H-CXF-OH tripeptides were prepared and conjugated with MPA at different pH as shown in Figure 3. Various neighboring amino-acids were selected to provide a sampling of different side chain functional groups and potential stereoelectronic interactions. The thiazine formation occurring during the conjugation reaction was monitored by UHPLC at different time points.

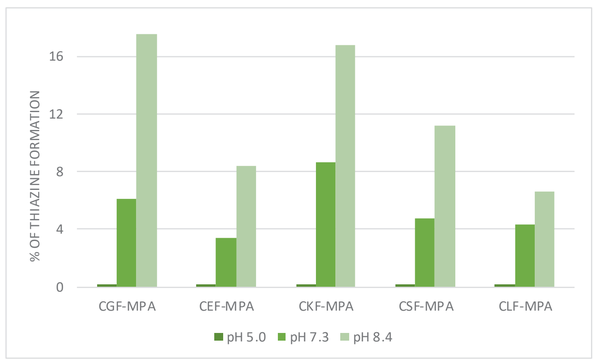


Figure 3 – Rate of thiazine formation for CXF-MPA conjugates at pH 5.0, 7.3, 8.4 after 1h of reaction. The pH 5.0 values reflect the thiazine conversion after 336 h. Any thiazine formation at pH 5.0 after 1 hour has been detected.

At pH 5.0, thiazine rearrangement was suppressed for all the CXF peptides. 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. 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. Conducting the studies at pH 8.4 resulted in a considerable increase in the rate of thiazine formation for each of the peptides.

6. Investigation of maleimide N-substitution on thiazine rearrangement

Maleimides bearing more electron-withdrawing groups at the nitrogen have been shown to have greater electrophilic character [2]. 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.

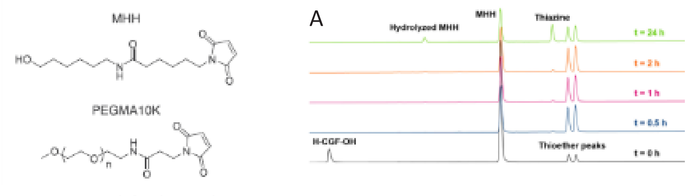


Figure 4 – Top left: chemical structure of MHH and PEGMA10K. A: UHPLC analysis of CGF conjugation to MHH at pH 7.3. B: UHPLC analysis of CGF conjugation to PEGMA10K. Black trace: 2:1 ratio of CGF:PEGMA10K in 10% acetonitrile in water. Green trace: 1:1 ratio of CGF:PEGMA10K at pH 7.7 in 0.1M potassium phosphate solution with 10% acetonitrile.

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. 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-PEGAMA10K succinimidyl thioether was absent.

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.

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.

References

[1] I. N. Gober, A. J. Riemen and M. Villain *J. Pep. Sci.* e3323 (**2021**).

[2] S. D. Fontaine, R. Reid, L. Robinson, G. W. Ashley, D. V. *Bioconjug Chem*. 26(1), 145–152 (**2015**); R.P. Lyon, J.R. Setter, T. D. Bovee, et al. *Nat Biotechnol*. 32(10), 1059–1062 (**2014**); R. J. Christie, R. Fleming, B. Bezabeh, R. Woods, S. Mao, J. Harper, A. Joseph, Q. Wang, Z. Q. Xu, H. Wu, C. Gao, N. *J Controlled Release*. 220(Pt B), 660–670 (**2015**).