Letter

Peptide Backbone Cleavage and Transamidation via Thioester-to-Imide Acyl Transfer

Bengt H. Gless,* Sabrina H. Schmied, and Christian A. Olsen*

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ABSTRACT: Cysteine thioesters are involved in a myriad of central biological transformations due to their unique reactivity. Despite their well-studied properties, we discovered an unexpected transamidation reaction of cysteine thioesters that leads to peptide backbone cleavage. S-Acylcysteine-containing peptides were found to spontaneously fragment by cleavage of the amide bond in the i-1 position to the acylated cysteine residue at pH 8−10. We present compelling evidence of a mechanism involving a central reversible thioester-to-imide acyl transfer step. The discovered trans-amidation reaction was found to be highly sequence dependent and to occur in peptides containing post-translational modifications (PTMs) such as cysteine S-acetylation and S-palmitoylation as well as in peptide−peptide branched thioesters, mimicking class I intein splicing. Thus, the inherent reactivity of peptide backbones containing S-acylcysteine residues should represent a starting point for investigation of endogenous protein behavior and may serve as a foundation for the discovery of mild new peptide and protein transformations.

KEYWORDS: thioesters, acyl transfer, amide bond cleavage, post-translational modification, cysteine acylation

Cysteine thioesters play an essential role in various important biological processes (Figure 1A). Their hydrolytic stability and selective reactivity at physiological pH[1,2] place thioesters as one of Nature's most important acyl transfer intermediates, involved in the complement system,[3−5] protein degradation,[6−8] non-ribosomal peptide synthesis,[9−11] intein-mediated protein splicing[12,13] and enzymatic peptide bond formation.[14,15] Furthermore, the chemical properties of thioesters alongside their synthetic accessibility[16,17] are the foundation of many widely used chemoselective ligation methods for peptides and proteins.[18−21] Post-translational modifications (PTMs) of cysteine residues in form of S-palmitoylation for membrane anchoring are widely present in the proteome and represent stable biological thioesters.[22−24] In addition, cysteine S-acetylation was recently reported as a PTM involved in metabolic regulation.[25]

We recently developed a Förster resonance energy transfer (FRET) system for the rapid investigation of reactivities of diverse thioesters.[26] Our investigations included the N-acetylcysteine-containing substrate **1** (Figure 1B), which we found unable to reach full conversion during hydrolysis experiments at pH 10. By further scrutiny of this reaction,
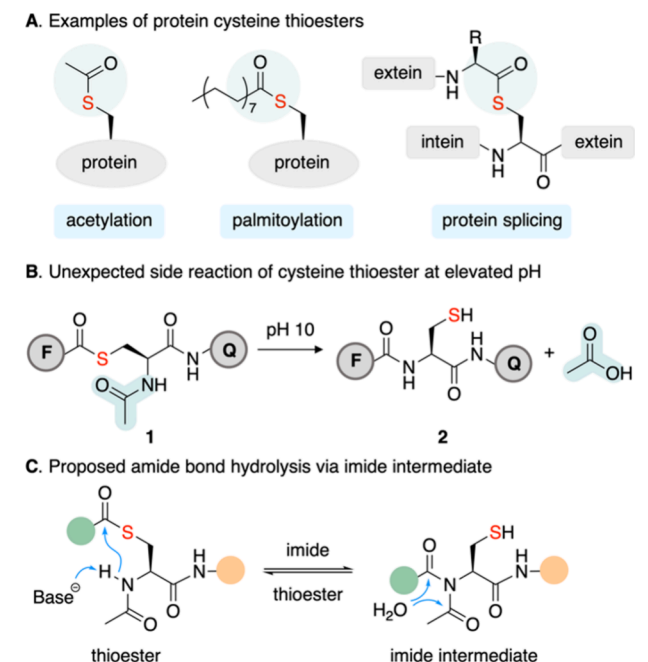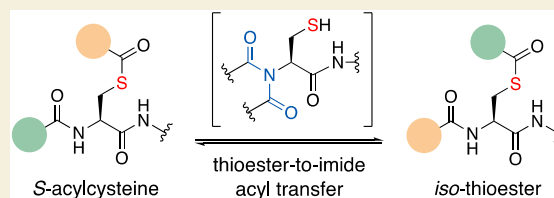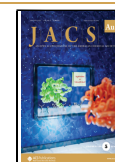

Figure 1. Discovery of peptide bond cleavage. A) Examples of protein cysteine thioester PTMs and intermediates. B) Amide bond hydrolysis as a side reaction of FRET thioester **1** (F, fluorophore; Q, quencher). C) Proposed amide bond hydrolysis mechanism.
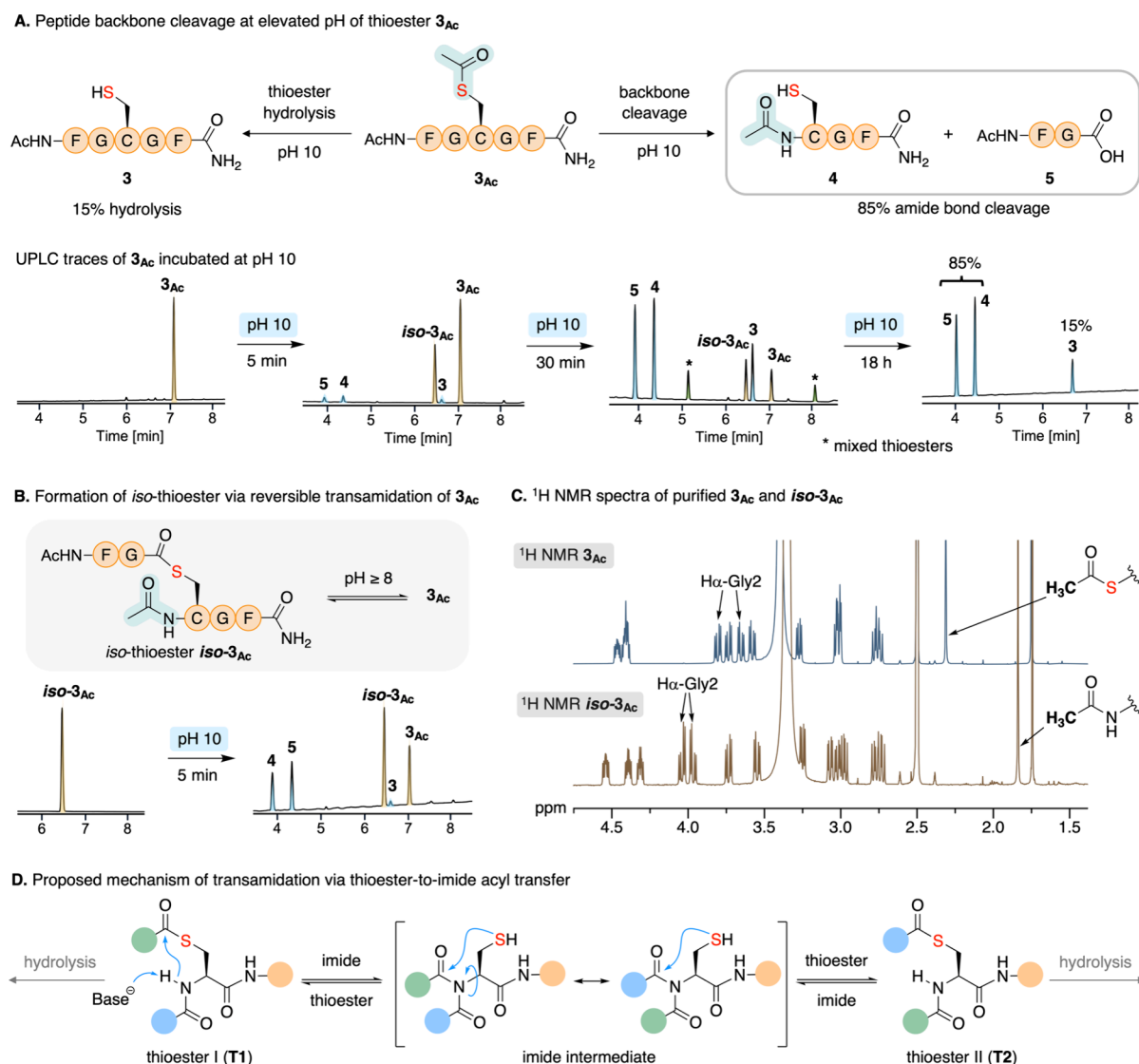
Figure 2. Peptide bond cleavage via transamidation of branched thioesters. A) Peptide bond cleavage of 3_{Ac} at pH 10 followed by UPLC. *Mixed thioesters were formed via transthioesterification of $3 + 3_{Ac}$ and $4 + iso\text{-}3_{Ac}$. B) Reversible formation of iso-thioester $iso\text{-}3_{Ac}$ via transamidation of 3_{Ac}. C) ^1H NMR spectra of purified 3_{Ac} and $iso\text{-}3_{Ac}$. D) Proposed mechanism of transamidation.

we have isolated compound **2** as the side product (Figure 1B and Supporting Figure S1), which we envision could be explained by a mechanism involving the formation of an imide intermediate (Figure 1C). To illuminate this mechanism and probe the scope of the reaction, we first prepared the peptide 3_{Ac} (Supporting Scheme S1), containing an S-acetylcysteine (C_{ac}) residue, and investigated its degradation products resulting from elevated pH treatment over time.

Incubation of peptide 3_{Ac} at pH 10 led to only 15% thioester hydrolysis in the form of peptide **3**, and 85% of 3_{Ac} underwent formal peptide backbone cleavage to give fragments **4** and **5** (Figure 2A). However, when investigating the progress of the reaction over time, by neutralizing the reaction mixture and isolating the individual intermediates, we identified the formation of the iso-thioester $iso\text{-}3_{Ac}$ (Figures 2B,C and Supporting Figures S2−S4). The transamidation of 3_{Ac} to give $iso\text{-}3_{Ac}$ occurred within minutes, even before significant hydrolysis was observed. This enabled transthioesterification between $iso\text{-}3_{Ac}$ and **3**, as well as 3_{Ac} and **4**, to furnish mixed thioesters that further complicated the chromatogram recorded

after 30 min (Figure 2A and Supporting Figure S3). Interestingly, incubation of isolated $iso\text{-}3_{Ac}$ led to rapid formation of 3_{Ac}, providing evidence for the reversibility of the discovered transamidation reaction (Figure 2B). Based on the identified intermediates and final products, we propose a thioester-to-imide acyl transfer as a key step in the observed transamidation reaction (Figure 2D).

The first step of our proposed mechanism is a base-assisted attack of the amide nitrogen on the carbonyl of thioester I (**T1**), leading to an acyl group transfer to form an imide intermediate. Rotation around the $N−C_\alpha$ bond of the formed imide species then enables acyl transfer back to the cysteine thiol to give thioester II (**T2**). Because hydrolysis can occur at both the thioester and imide stages and the resulting hydrolysis products can cross react with **T1** and **T2**, leading to mixed thioesters, all observed intermediates can be accounted for (see Supporting Figure S2 for the full mechanism).

The formation of an imide intermediate represents a form of amide bond activation[27,28] and has been observed in the spontaneous amide bond hydrolysis in O-glycosylated
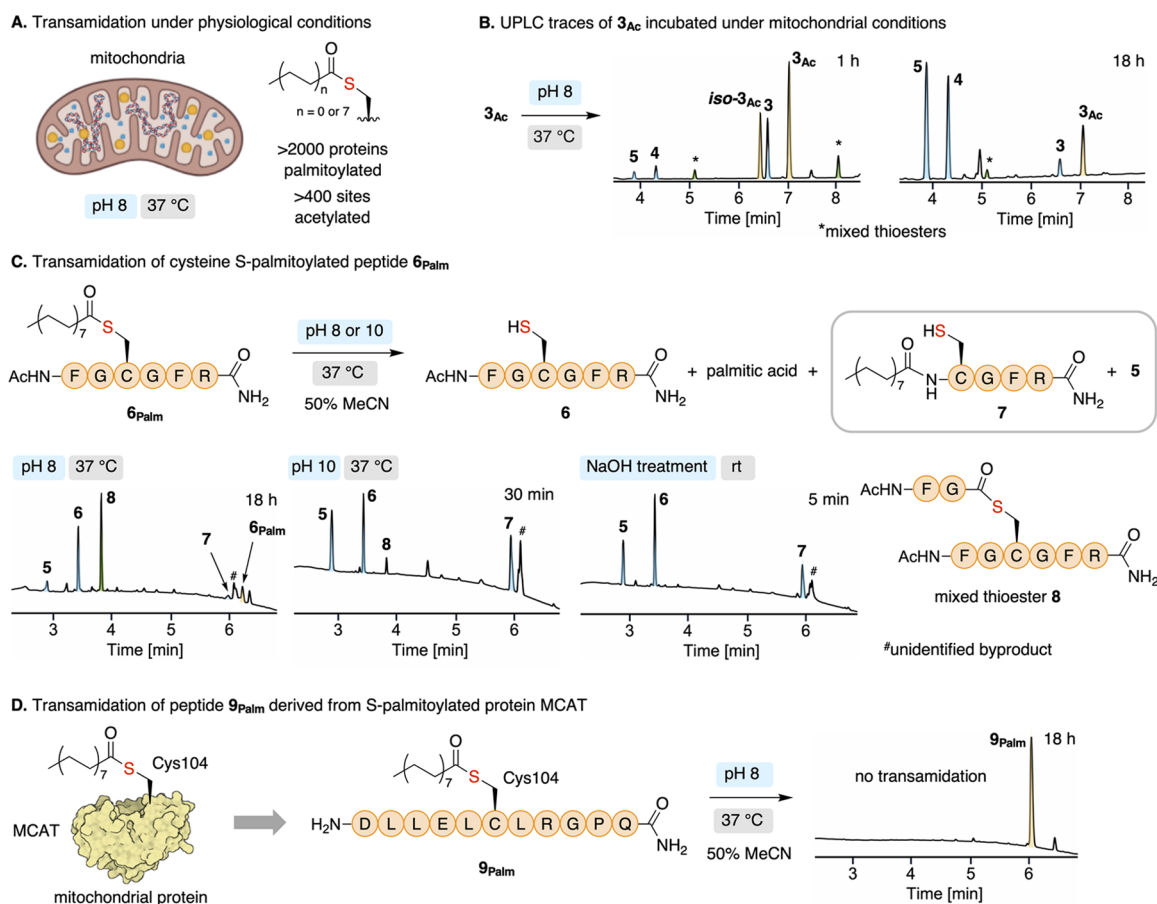
Figure 3. Cysteine thioester transamidation under physiological conditions. A) Mitochondria and cysteine palmitoylation. B) Transamidation of peptide 3_{Ac} at mitochondrial pH monitored by UPLC. *Mixed thioesters formed via *trans*-thioesterification of $3 + 3_{Ac}$ and $4 + iso\text{-}3_{Ac}$. C) Transamidation of peptide 6_{Palm} at pH 8, pH 10, or in 2 M NaOH. D) Transamidation of peptide 9_{Palm} derived from the S-palmitoylated mitochondrial protein malonyl-CoA acyl carrier protein transacylase (MCAT).

proteins[29,30] as well as in a previously reported thioester-to-imide acyl transfer reaction for constrained thiolactone-containing peptides.[31] Further, imide-based chemistries have been developed to activate amide bonds in selective peptide backbone cleavage reactions.[32−34] These studies establish support for the formation of a backbone imide intermediate, as outlined in our proposed mechanism.

To examine whether the transamidation reaction might have endogenous relevance in cells, we mimicked the conditions found in the mitochondria (pH ∼ 8, 37 °C) where high acyl-CoA concentrations are believed to lead to increased cysteine acylation, in particular S-acetylation[35,36] (Figure 3A). Intriguingly, incubation of peptide 3_{Ac} under these conditions led to fast transamidation followed by peptide bond cleavage to yield **4** and **5** upon extended reaction time (Figure 3B and Supporting Figure S4). The S-palmitoylcysteine (C_{palm}) is well established as a post-translationally modified residue, and transamidation at C_{palm} sites could therefore present a naturally occurring transformation. Thus, we prepared S-palmitoylated peptide 6_{Palm} (Supporting Scheme S1) and incubated it at pH 8 and 10 (Figure 3C and Supporting Figure S5). In both cases, we were able to identify the transamidation products **5** and **7**, demonstrating that this reaction can also take place at mitochondrial pH.

We also found that treating 6_{Palm} with 2 M NaOH led to transamidation and backbone cleavage, which warrants caution for alkaline hydrolysis procedures when handling C_{palm}

thioesters (Figure 3C). Next, we selected a mitochondrial protein with an identified C_{palm} site, malonyl-CoA acyl carrier protein transacylase (MCAT),[37] and synthesized an undeca-peptide (9_{Palm}), containing the C_{palm} residue flanked by amino acids based on the sequence of the protein (Figure 3D). When the peptide 9_{Palm} was incubated at pH 8, no thioester hydrolysis or transamidation was detected for up to 18 h, showing that thioester hydrolysis and transamidation of C_{palm}-containing proteins is sequence dependent under mitochondrial conditions. Incubation of peptide 9_{Palm} under more forcing conditions (pH 10 or with 2 M NaOH) led to amide bond cleavage products although to a lower extent than for peptide 6_{Palm} (Supporting Figure S6).

We then decided to focus on implications of the discovered reactivity with regard to protein splicing, which is a widely applied technique to manipulate recombinant proteins.[38] Branched cysteine thioesters play a key role in intein-mediated protein splicing, a spontaneous intramolecular process in which the internal protein segment (intein) is excised, thereby fusing the N-terminal and C-terminal (N/C-extein) segments of the protein (Figure 4A).[12] The discovered transamidation reaction resembles the final steps in class 1 cysteine intein splicing, where the N-extein is linked via a cysteine thioester to the C-extein. The asparagine residue in the *i*-1 position to the cysteine cyclizes to release a C-terminal succinimide fragment, leading to an S−N acyl shift within the remaining segment to join the two exteins[39] in an acyl transfer step similar to the
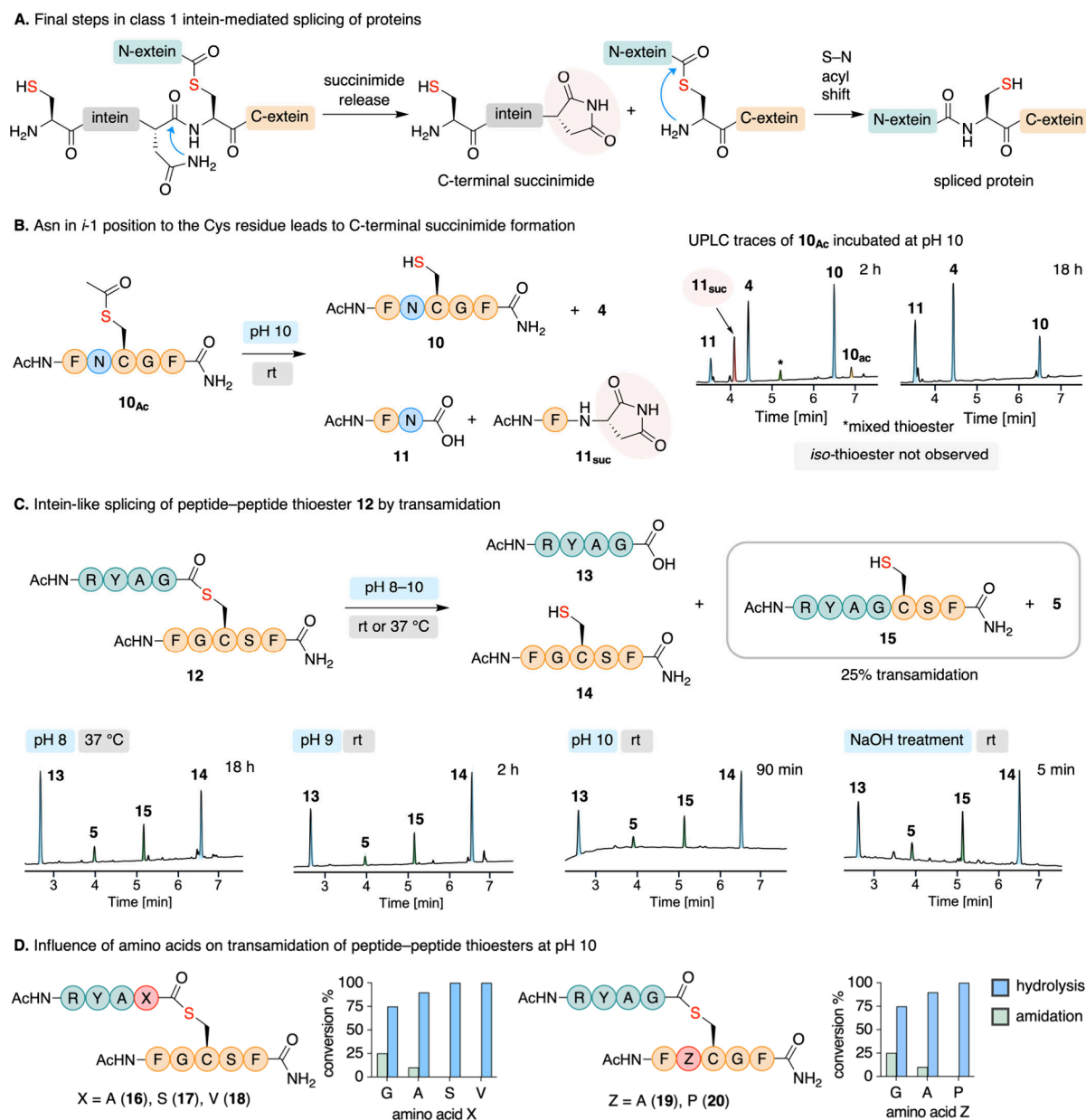
Figure 4. Intein-like splicing of peptide−peptide thioesters by transamidation. A) Final steps in class 1 intein splicing. B) Transamidation of peptide 10_{Ac} at pH 10 led to succinimide formation. *Mixed thioester formed via transthioesterification between **4** and 10_{Ac}. C) Intein-like splicing of peptide−peptide thioester **12** via transamidation at pH 8, pH 9, pH 10, or in 2 M NaOH. D) Effect of the C-terminal thioesters and amino acids in the *i*-1 position on transamidation of peptide−peptide thioesters **16−20**.

native chemical ligation reaction (Figure 4A).[40,41] First, we investigated whether the observed transamidation reaction would lead to the formation of C-terminal succinimide for sequences containing Asn-Cys. To address this question, we synthesized peptide 10_{Ac}, containing an asparagine residue in the *i*-1 position to the acylated cysteine as present in the intein splicing step (Figure 4B). When we incubated 10_{Ac} at pH 10, we were able to identify the succinimide 11_{suc} after 2 h, which was fully hydrolyzed to **11** after 18 h, providing a similar final product distribution as observed for 3_{Ac} above (Figure 4B and Supporting Figure S7). Further, no *iso*-thioester of 10_{Ac} could be detected, indicating that formation of succinimide happens more rapidly than that of the backbone imide intermediate. A peptide containing aspartic acid in the *i*-1 position gave rise to the same distribution of intermediates and products (Support-

ing Figure S8). Notably, succinimide formation has gained recent attention because C-terminal imides have been identified as markers for protein degradation.[42] Similar to our findings, a recent study reported base-induced succinimide formation and splicing of a branched Asn-Ser-containing depsipeptide.[43]

To examine if intein-like peptide splicing could occur via the discovered transamidation reaction, we prepared branched peptide−peptide thioesters linked via an internal cysteine residue (Figure 4C,D). Based on the previous results, we anticipated that the C-terminal amino acid of the thioester and the residue in the *i*-1 position to the cysteine would determine the reactivity. We therefore synthesized a peptide−peptide branched thioester with glycine in both positions (**12**) as the starting point (Supporting Schemes S2,3). Incubation of **12** at

pH 8 for 18 h gave 25% transamidation products **5/15**, where **15** resembles a splicing product formed via an intein splicing (Figure 4C and Supporting Figure S9). Interestingly, incubation of **12** under different alkaline conditions (pH 8−10 or NaOH treatment) led to nearly the same ratio of transamidation products **5/15** to hydrolysis products **13/14**; albeit, at different reaction times (Supporting Figure S9). We then assessed the effect of the C-terminal thioester position on the reaction at pH 10 while keeping the *i*-1 position as a glycine residue **16−18** (Figure 4D). With increasing steric bulk of the amino acid side chains (Ala−Ser−Val) **16−18**, the ratio of transamidation to hydrolysis changed significantly (Figure 4D). For alanine (**16**) only 8% compared to 25% for glycine (**12**) of the spliced peptide was detected, while for serine (**17**) only traces could be found (Supporting Figures S10,11). The valine thioester **18** did not lead to any detectable transamidation product. Interestingly, however, the hydrolysis of the valine thioester resulted in complete racemization at the C-terminus, which we could validate by also following the hydrolysis of a MESNa thioester of the corresponding valine peptide (Supporting Figures S12,13).

Finally, we examined the *i*-1 position by testing peptide−peptide thioesters with alanine (**19**) and proline (**20**) in *i*-1, while keeping the C-terminal thioester as a glycine residue (Figure 4D). The alanine analogue **19** led to 7% of the spliced peptide compared to hydrolysis, which is comparable to construct **16**, and the proline analogue (**20**) did not result in any detectable transamidation as expected, supporting the proposed thioester-to-imide acyl transfer mechanism (Supporting Figures S14,15).

In summary, we discovered an unexpected transamidation reaction of branched cysteine thioester-containing peptides that leads to the cleavage of the peptide backbone. We provide evidence for a mechanism involving a reversible intramolecular thioester-to-imide acyl transfer reaction, which competes with thioester hydrolysis. Upon completion of the reaction, the ratio of final products is dependent on the structure of the thioester and the amino acid in the *i*-1 position to the cysteine. The inherent reactivity driving this transformation at known PTM sites such as C_{palm} and C_{ac} under mitochondrial conditions (pH 8, 37 °C) may suggest that this peptide/protein backbone cleavage could represent a previously unknown biologically relevant reaction. We expect that our present work will provide a foundation for exploring biological implications of the discovered reaction as well as for the development of novel ligation and intein-mimicking chemistries.[44,45]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.4c01143.

> Detailed characterization of transamidation intermediates; schemes depicting the synthesis of substrates and compounds used; experimental methods; chemical synthesis and compound characterization data; as well as copies of HPLC traces and ^{1}H and ^{13}C NMR spectra (PDF)

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Author Contributions

CRediT: **Bengt H. Gless** conceptualization, formal analysis, investigation, methodology, supervision, visualization, writing-original draft, writing-review and editing; **Sabrina H. Schmied** investigation, methodology, writing-review and editing; **Christian A. Olsen** conceptualization, resources, funding acquisition, project administration, supervision, visualization, writing-original draft, writing-review and editing.

Notes

The authors declare no competing financial interest.

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