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Rapid Additive-Free Selenocystine–Selenoester Peptide Ligation

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Supporting Information

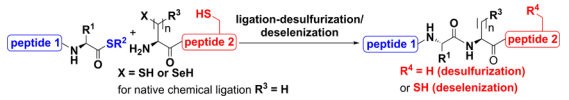
ABSTRACT: We describe an unprecedented reaction between peptide selenoesters and peptide dimers bearing N-terminal selenocystine that proceeds in aqueous buffer to afford native amide bonds without the use of additives. The selenocystine-selenoester ligations are complete in minutes, even at sterically hindered junctions, and can be used in concert with one-pot deselenization chemistry. Various pathways for the transformation are proposed and probed through a combination of experimental and computational studies. Our new reaction manifold is also showcased in the total synthesis of two proteins.

The construction of amide bonds is undoubtedly one of the most important synthetic transformations. While numerous reagents and methods have been developed and refined for amide synthesis within small molecules, large polypeptides and proteins are most commonly accessed via native chemical ligation methodology.[1,2] This reaction utilizes a peptide bearing an N-terminal Cys residue and a peptide functionalized as a C-terminal thioester (Scheme 1) and, mechanistically, proceeds through an initial transthioesterification, followed by a rapid intramolecular S→N acyl transfer to generate the native peptide bond. Usually a large excess of a thiol additive is required to generate a reactive thioester from less reactive alkyl thioester precursors,[3,4] and reactions are normally supplemented with an additional reductant to prevent disulfide bond formation.

To expand the repertoire of this technology to amino acid residues other than Cys, recent efforts have focused on thiol-derived amino acids[5] for the assembly of peptides and proteins via ligation-desulfurization chemistry (Scheme 1).[6−10] While ligation-desulfurization technologies have revolutionized synthetic protein chemistry,[11] the methods suffer from two shortcomings: (1) ligation rates at sterically hindered C-terminal thioesters are very slow, leading to prolonged reaction times (>48 h)[12] and, thus, significant thioester hydrolysis, and (2) desulfurization reactions are incompatible with Cys residues elsewhere in the sequence, as these are concomitantly desulfurized to Ala (Scheme 1).[13]

To address these limitations, ligations between selenocysteine (Sec)[14−17] or selenol-derived amino acids[18,19] and thioesters through a native chemical ligation pathway have been explored (Scheme 1).[20] Owing to the low redox potential of Sec (−381 mV),[21] selenopeptides exist as the corresponding diselenide dimers under standard conditions and do not participate in ligation chemistry in the absence of an external reductant[16], such as an aryl thiol catalyst, which serves to generate the reactive selenol.[15,16,22] Despite the enhanced nucleophilicity of selenols relative to thiols, the weak reductive power of aryl thiols leads to a low steady-state concentration of selenol which often slows the rate of Sec ligations compared with Cys.[15,19] Unfortunately, the use of stronger reducing agents, such as phosphines, promotes homolysis of the weak C-Se bond of Sec, a transformation that has been exploited for the chemoselective deselenization of Sec to Ala in the presence of free Cys.[20,22] Rates of native chemical ligation at Cys can be enhanced by altering the acyl donor, specifically through the use of alkyl selenoesters[23] in place of thioesters. We thus reasoned that if the increased nucleophilicity of Sec could be effectively harnessed and combined with the enhanced electrophilicity of a selenoester acyl donor, the ligation rate should dramatically increase. To avoid the undesired phosphine-mediated deselenization pathway, we investigated alternative chemical and electrochemical methods for the reduction of the diselenide to "unlock" its latent reactivity. We were fascinated to observed that a control experiment involving peptide dimer **1** bearing an N-terminal selenocystine [$(Sec)_2$] moiety (2.5 mM) and peptide **2** containing a C-terminal Ala phenylselenoester (5 mM) in denaturing buffer, pH 7.0, afforded the corresponding diselenide **3** as the major product (together with 10% of unsymmetrical diselenide **4**, Scheme 2A,B). To our knowledge, this transformation represents unprecedented reactivity and, remarkably, proceeds at room temperature in less than 60 s without thiol or reductive additives (Scheme 2B,C). The reaction also proceeded at concentrations as low as 250 μM of **1** (reaching completion in 60 min, SI).

Scheme 1. Ligation-Desulfurization/Deselenization



This unprecedented reactivity prompted exploration of similar ligations using alternative acyl donors and/or N-terminal functionalities. Reactions of peptide dimers bearing N-terminal $(Sec)_2$ do not proceed with alkyl or arylthioesters, consistent with prior ligation studies without a reductant,[16] while reactions at alkylselenoesters do proceed, albeit sluggishly (<10% over 2.5 h, Scheme S1). Interestingly, peptide dimers with N-terminal cystine in place of $(Sec)_2$ only react with arylselenoesters (of the acyl donors studied) but are slower and stall at ca. 50% due to thioester formation via acylation of the product.

Next, we explored the scope of the technology for a range of phenylselenoester coupling partners bearing a variety of C-

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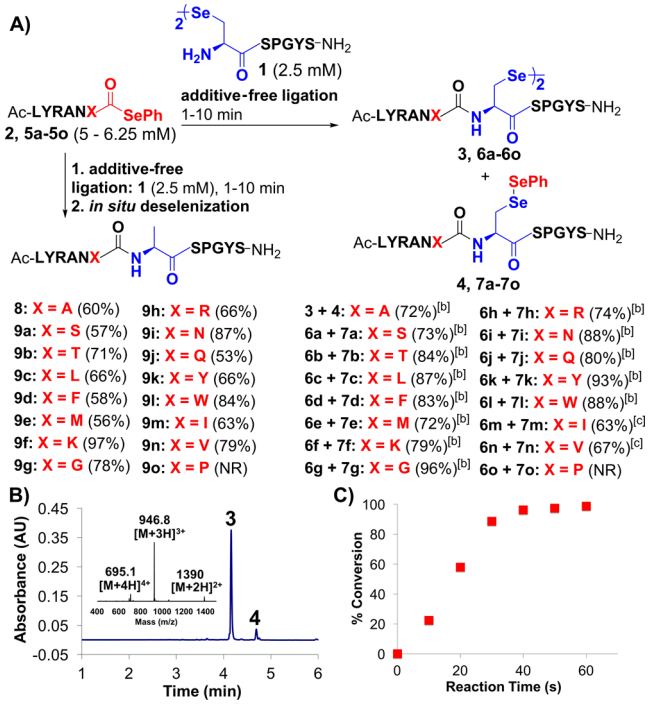
Scheme 2. (A) Additive-Free $(Sec)_2$-Selenoester Peptide Ligation and One-Pot Ligation-Deselenization between Peptide 1 and Phenylselenoesters 2 and 5a−5o; (B) Crude UPLC Trace of Reaction of 1 and 2 at t = 60 s; (C) Conversion of 1 and 2 To Generate 3 and 4 over 60 s[a]



[a]Conditions: additive-free ligation; 6 M Gdn·HCl, 0.1 M Na_2HPO_4, pH 6.2, 25 °C; in situ deselenization; (i) hexane extraction, (ii) TCEP (50 equiv), DTT (5 equiv), pH 5.0, 16 h. [b]0.5 equiv dimer **1** (1.0 equiv of H-USPGYS-NH_2) to 1.0 equiv selenoester, reaction time = 60 s. [c]0.5 equiv dimer **1** to 1.25 equiv of selenoester, reaction time = 600 s (X = I) or 300 s (X = V). Yield calculated from combined diselenide products (**6** + **7**). NR = no reaction.

terminal residues. Synthesis of **2** and selenoesters **5a-5o** was achieved by Fmoc-strategy solid-phase peptide synthesis (SPPS) and the stereochemical integrity confirmed by NMR spectroscopy (Scheme S2). Several model additive-free ligations were performed. Peptide dimer **1** was reacted with selenoesters **5a-5n** in 6 M Gdn·HCl, 0.1 M phosphate buffer at a final reaction pH of 6.2 and concentration of 2.5 mM with respect to dimer **1** and a stoichiometry of 1:1 of selenoester/monomer of **1** (Scheme 2A). With the exception of the reactions with Ile and Val selenoesters (**5m** and **5n**), a yellow precipitate of diphenyldiselenide (DPDS) formed within 60 s after addition of the selenoester, indicating completion of the reactions (Scheme S3), as confirmed by HPLC-MS analysis. After reverse-phase HPLC purification, the ligation products were isolated as the symmetric diselenides (**6a-6l**) as the major products, together with the unsymmetrical diselenide products (**7a-7l**) in excellent combined yields (72-96%, Scheme 2A). Notable examples include reaction at C-terminal Leu and Thr residues, which were complete within 60 s.[12] For reactions involving sterically hindered selenoesters, such as C-terminal Ile **5m** and Val **5n**, comparatively longer reaction times of 10 and 5 min, respectively, were required for complete conversion. For these, a slight excess of the selenoester (1.25 equiv) was also necessary. Nonetheless, the desired products (**6m**/**7m** and **6n**/**7n**) were isolated in good yields (Scheme 2A). Finally, the reaction was also performed on a peptide containing an N-terminal $(Sec)_2$ and an internal Cys residue, as well as with **1** in the presence of a competing peptide containing an internal Cys (SI). These ligations were also complete within 60 s and suggest that the manifold can be compatible with unprotected Cys.

We next investigated the potential extension of the selenium-mediated ligation to a one-pot ligation-deselenization protocol[18,19,22] to afford native Ala at the ligation junction. For this, **1** was reacted with selenoesters **2** and **5a-5n**, and upon completion, the insoluble DPDS was extracted prior to *in situ* treatment with TCEP and DTT to effect deselenization. Note that extraction of DPDS from the reaction mixture is necessary to prevent quenching of the deselenization reaction. This one-pot ligation-deselenization methodology afforded native peptide products **8** and **9a-9n** in good yields following reverse-phase HPLC purification (Scheme 2A).

We next probed the effect of pH, known to have a dramatic effect on the rate of native chemical ligation, on the additive-free ligation.[14] Reactions between C-terminal Leu selenoester **5c** and peptide **1** were examined in pH 1.6-8.3. At a pH of 5.0-7.7, reactions proceeded cleanly with similar end points (1-3 min, SI). Reactions at or above pH 8.0 led to rapid selenoester decomposition, while reactions at highly acidic pH (1.6) returned only starting material. Ligation reactions still proceed cleanly at pH 2.3, albeit with a longer reaction time of 5 h. In addition, we performed a ligation with a peptide selenoester bearing a C-terminal Pro residue,[23] as the corresponding Pro thioesters are known to be poor acyl donors in native chemical ligation.[12,24] Peptide **1** was reacted with Pro selenoester **5o** under the additive-free conditions. However, after 12 h no ligation product had formed (Scheme 2A). Interestingly, upon addition of TCEP (50 mM) to reduce the diselenide in **1**, ligation with **5o** proceeded rapidly (under a native chemical ligation mechanism)[23] to afford 80% of the ligation product and 15% of deselenized **1** (SI).

As the precipitation of DPDS in the aqueous reaction media provided a strong visual indication of the success of our model ligations (Scheme S3), we next investigated the potential role of this event as an accelerating force for the ligation manifold. We prepared a peptide arylselenoester derived from the water-soluble 4-selenophenylacetic acid (SePAA) diselenide (SI), a selenium analogue of the highly efficient thiol native chemical ligation additive 4-mercaptophenylacetic acid (MPAA).[4] This SePAA-derived selenoester reacted substantially slower ($t_{1/2}$ > 1 h) than the corresponding phenylselenoester ($t_{1/2}$ = 24 s). Methyl ester derivatization of the carboxylate moiety of SePAA, however, resulted in a dramatic ligation rate enhancement ($t_{1/2}$ = 48 s) relative to the more water-soluble counterpart.

These data preliminarily suggest that the reaction is an equilibrium process, whereby the forward reaction is promoted by removal of diaryldiselenide from solution by precipitation. Based on this observation and the precedent for native chemical ligation-type reactions at Sec residues[14−16] or with alkyl selenoesters,[23] we hypothesize that the additive-free $(Sec)_2$-selenoester ligation might propagate through a ligation redox cycle (Scheme 3A), a reversible manifold that is ultimately driven by oxidation of displaced arylselenolate to form an insoluble diaryldiselenide. In this native chemical ligation-type mechanism, we postulate that selenolate $\mathbf{I}^-$, derived from the starting peptide diselenide **I**, reacts in an initial trans-selenoesterification reaction with a peptide selenoester, displacing aryl selenolate $\mathbf{II}^-$ and forming the selenoester intermediate **III**. An Se→N acyl shift[25] of intermediate **III** then generates the selenolate ligation product $\mathbf{IV}^-$. A coupled redox process, whereby electrons are relayed[26] from selenolate product $\mathbf{IV}^-$ to peptide starting material **I**, would

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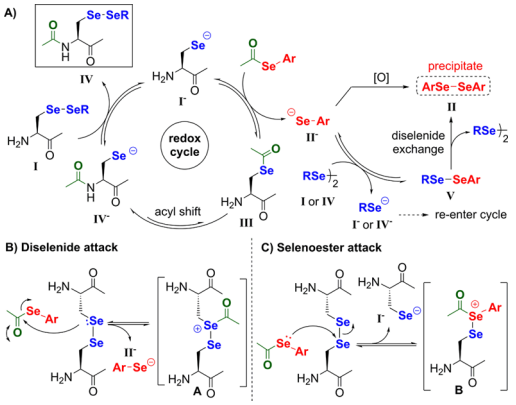
Scheme 3. (A) Proposed Ligation Cycle; Initiation via (B) Diselenide Attack and (C) Selenoester Attack



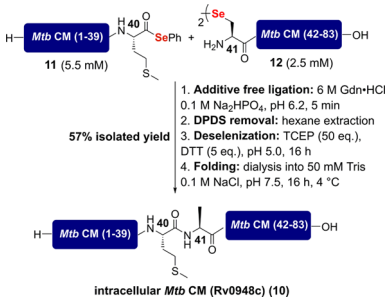
then turn over the cycle through regeneration of selenolate $\mathbf{I}^-$ and concomitant oxidation of $\mathbf{IV}^-$ to afford diselenide ligation product **IV**. Given the reversibility of the trans-esterification step and the recent disclosure that Se→N acyl shifts are also reversible processes,[27,28] this reaction manifold could in principle proceed in either direction. However, the oxidation of arylselenolate $\mathbf{II}^-$ (displaced in the initial trans-esterification step) to the insoluble diaryldiselenide **II** is a powerful promoter of the forward reaction. The oxidation of $\mathbf{II}^-$ might also be coupled to reduction of starting diselenide **I** generating selenolate $\mathbf{I}^-$ to propagate the forward cycle. This electron-relay process might proceed through asymmetric diselenide **V**, which we have shown through crossover experiments can undergo facile diselenide exchange leading to the precipitation of **II** in ligation buffer (see SI). This exchange affords DPDS from asymmetric diselenide precursors, but is substantially slower for the SePAA analogue.

In the absence of additives or external reductants, the propagation cycle outlined in Scheme 3A requires initiation through an internal reduction event (to effect selenolate formation). Generation of any of the intermediates in the cycle would, in principle, initiate the reaction. We therefore investigated a number of possible initiation pathways using both experimental and computational methods. We first evaluated the feasibility of initiation through selenoester hydrolysis and direct generation of catalytic amounts of aryl selenolate anion $\mathbf{II}^-$. However, the slow rate of ligation with the SePAA-derived selenoester relative to the phenylselenoester (which on the basis of electrophilicity should hydrolyze at comparable rates) and the viability of the reaction even at acidic pH, where the likelihood of hydrolysis is significantly reduced, suggest that selenoester hydrolysis as an initiation event is unlikely. We next probed a possible radical mechanism for the direct generation of intermediate **III**, fueled by reports of phenylselenoesters serving as acyl radical precursors[29−31] and recent evidence that light-mediated dynamic diselenide exchange occurs through a radical process.[32] However, we saw no evidence of acyl radical species in the presence of radical spin traps, e.g., *N*-*t*-butyl-α-phenylnitrone (PBN) and 2-methyl-2-nitrosopropane (MNP), or upon examination of the reaction using EPR spectroscopy. Given the potent electrophilicity of the carbonyl carbon of aryl selenoesters, we also investigated initiation via direct diselenide attack onto the selenoester acyl donor (Scheme 3B). This would result in formation of arylselenolate $\mathbf{II}^-$, which might facilitate turnover of the redox ligation cycle through oxidation to the insoluble aryl diselenide, coupled with reduction of a peptide diselenide (e.g., **I**). The process of diselenide attack would also generate the high-energy charged intermediate **A**. An alternative pathway involves attack of the selenoester onto the peptide diselenide, which would generate the productive selenolate $\mathbf{I}^-$ along with charged intermediate **B** (Scheme 3C). Notably, amide bond formation might also be possible through a direct Se→N acyl shift of either intermediate **A** or **B**.

To differentiate between the hypothetical pathways outlined in Scheme 3B,C, we probed the stability of **A** and **B** with computational quantum chemistry calculations using the species depicted in the Scheme as models (SI). Relative to the neutral peptide starting materials (with Ar = Ph), similar calculated energies were observed for intermediates **A** (111.8 kJ/mol) and **B** (118.3 kJ/mol). On a simplified model system, calculated energies for **A** decreased when arylselenoesters with electron-withdrawing substituents were employed (Table S5); the opposite trend was observed for **B** (Table S6). Based on these discrete computational predictions, we sought to probe the effect of electron density in the aryl ring of the selenoester on the rate of peptide ligation. Several model peptide selenoesters bearing a range of electron-withdrawing and electron-donating substituents on the aryl ring were prepared (Table S7), but no clear trend was observed between the reaction rate and relative electron-density in the aryl ring. Indeed, solubility of the aryldiselenide, rather than electronics, remained the most prominent predictor of selenoester reactivity. As such, despite a number of additional experimental and computational studies, we are currently unable to distinguish between the two pathways and, additionally, cannot definitively rule out the possibility of an alternative, associative mechanism, in which neither **A** nor **B** is involved (SI). Further mechanistic exploration of the additive-free ligation platform will therefore be the subject of future work in our laboratory.

We next moved to assess the efficiency of the reaction for the chemical synthesis of proteins. Our first target was an enzyme, namely the intracellular chorismate mutase (CM) from *Mycobacterium tuberculosis* (*Mtb*) which converts chorismate to prephenate through a Claisen rearrangement (Scheme 4 and

Scheme 4. One-Pot Synthesis of *Mtb* CM 10



SI).[33] Reaction of *Mtb* CM 1-40 phenylselenoester (**11**) and *Mtb* CM 41-83 bearing an N-terminal $(Sec)_2$ moiety (**12**) under the additive-free ligation conditions proceeded to completion in 5 min to exclusively afford the symmetrical diselenide, which following *in situ* deselenization, provided full length *Mtb* CM with excellent crude purity (SI). Purification by reverse-phase HPLC and folding provided *Mtb* CM **10** in an excellent 57% yield. Importantly, the synthetic enzyme had similar structure and activity to that reported for the recombinant protein[33] (SI).

We also focused on the synthesis of the *N*-acetylated Cys-free 94 residue early secretory antigenic protein-6 (ESAT-6) **13**, a virulence factor and potent T cell antigen also from *Mtb*.[34] The

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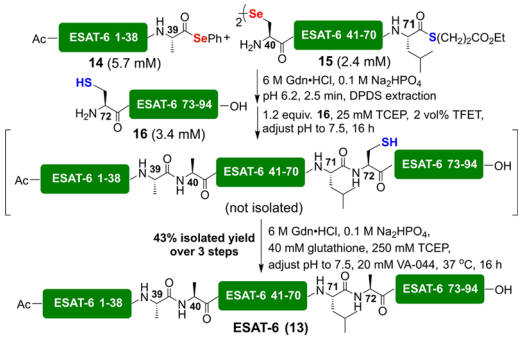
Scheme 5. Synthesis of *Mtb* ESAT-6 13 via a Three-Component One-Pot Ligation Strategy



protein was disconnected into three fragments, ESAT-6 1-39 **14** as a C-terminal phenylselenoester, ESAT-6 40-71 dimer **15** with an N-terminal $(Sec)_2$ moiety and C-terminal alkyl thioester, and ESAT-6 72-94 **16**, which we aimed to unify via a one-pot, three-component ligation reaction using both native chemical ligation and the $(Sec)_2$-selenoester ligation (Scheme 5). Selenoester **14** and bifunctional peptide dimer **15** were first reacted in 6 M Gdn·HCl, 0.1 M phosphate buffer at pH 6.2 and, within 2.5 min, a precipitate formed and the reaction was judged to have reached completion by LC-MS analysis. At this point, C-terminal fragment **16** was added, together with TCEP and the thiol additive TFET,[35] before adjusting the pH to 7.5. After 16 h, the native chemical ligation reaction was complete with concomitant phosphine-mediated deselenization of Sec-40 to Ala.[22] This product was not isolated, but the crude reaction was dosed with glutathione, further TCEP and the pH adjusted to 7.5 before the addition of the radical initiator VA-044[7] to effect desulfurization of Cys-72 to Ala. HPLC purification then provided ESAT-6 (**13**) in 43% yield over the multiple-step, one-pot process.

In summary, we describe a novel, additive-free peptide ligation reaction between peptide selenoesters and peptide diselenide dimers bearing an N-terminal $(Sec)_2$ that enables rapid ligation within minutes, even at sterically hindered junctions. We have proposed several putative reaction pathways for the transformation, which we have investigated through a combination of experimental and computational studies. Importantly, we have demonstrated that the methodology has wide scope, is applicable to protein synthesis, and can be used in conjunction with other peptide ligation technologies. Future work in our laboratory will involve more detailed investigations into the mechanism of this unique ligation reaction and use of the chemistry for the synthesis of modified proteins.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b07237.

Additional methods and data (PDF)
Movie of ligation reactions (MPG)
Movie of ligation reactions (MPG)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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Scheme 2 footnote notations were corrected November 11, 2015.

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