Rapid Additive-Free Selenocystine–Selenoester Peptide Ligation

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# Rapid Additive-Free Selenocystine–Selenoester Peptide Ligation

Nicholas J. Mitchell,[‡] Lara R. Malins,[‡] Xuyu Liu, Robert E. Thompson, Bun Chan, Leo Radom, Richard J. Payne*

School of Chemistry, Building F11, The University of Sydney, NSW 2006, AUSTRALIA

Supporting Information Placeholder

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 chemoselective deselenization chemistry. A novel pathway for the transformation is proposed, and probed through a combination of experimental and computational studies. Our new reaction manifold is showcased in the total synthesis of two proteins from *Mycobacterium tuberculosis*; a catalytically active form of the enzyme chorismate mutase and the secreted T-cell antigenic protein ESAT-6.
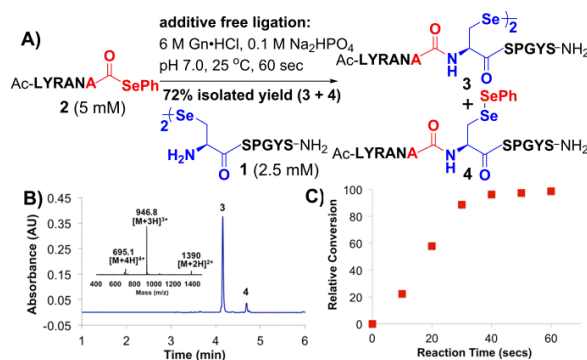
The construction of amide bonds is undoubtedly one of the most important synthetic transformations. While numerous reagents and methods have been developed and refined for amide synthesis within small molecules, large polypeptides and proteins are most commonly accessed *via* native chemical ligation methodology.[1,2] This reaction utilizes a peptide bearing an N-terminal cysteine (Cys) residue and a peptide functionalized as a C-terminal thioester (Scheme 1) and, mechanistically, proceeds through an initial transthioesterification (facilitated by the nucleophilic Cys thiol) to covalently link the two fragments, 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.

Scheme 1. Ligation–desulfurization/deselenization.
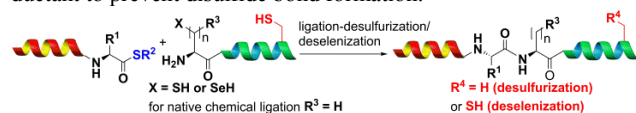
To expand the repertoire of this technology to amino acid residues other than Cys, recent efforts have focussed on thiol-derived amino acids[5] for the assembly of peptides and proteins *via* ligation–desulfurization chemistry (Scheme 1).[6-10] While native chemical ligation and the related ligation–desulfurization 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, consequently, significant thioester hydrolysis, and 2) desulfurization reactions are incompatible with the presence of Cys residues elsewhere in the sequence, as these are concomitantly desulfurized to Ala (Scheme 1).[13]

To address these limitations, ligations between selenocysteine (Sec)[14-16] or selenol-derived amino acids[17,18] and thioesters through a native chemical ligation pathway have been explored (Scheme 1).[19] Owing to the low redox potential of Sec (–381 mV),[20] 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] Aryl thiol catalysts are generally employed for reduction of the diselenide to the corresponding selenol.[15,16,21] Despite the enhanced nucleo-

philicity 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.[15,18] 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.[19,21] Rates of native chemical ligation at Cys can be enhanced by altering the acyl donor, specifically through the use of alkyl selenoesters[22] in place of thioesters. We therefore reasoned that if the increased nucleophilicity of Sec could be effectively harnessed and combined with the enhanced electrophilicity of a selenoester acyl donor, the rate of ligation should be dramatically increased. Somewhat surprisingly this reaction has not been explored to date. To avoid the undesired phosphine-mediated deselenization pathway, we sought to investigate alternative chemical and electrochemical methods for the reduction of the diselenide to 'unlock' its latent reactivity. During the course of these investigations, we were fascinated to observe 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 at pH 7.0 afforded the corresponding diselenide **3** as the major product (together with 10% of unsymmetrical diselenide **4**, Scheme 2). To our knowledge, this transformation represents unprecedented reactivity and, remarkably, proceeds at room temperature in less than 60 seconds without thiol or reductive additives (see Scheme 2B–2C). The reaction also proceeded at concentrations as low as 250 μM of **1** (reaching completion in 60 min, see Supporting Information).

Scheme 2. A) Additive-free (Sec)$_2$-selenoester peptide ligation between peptide **1** and phenylselenoester **2**; **B)** crude UPLC trace of reaction of **1** and **2** at t = 60 seconds; **C)** conversion of **1** and **2** to generate **3** and **4** over 60 seconds.

The unprecedented reactivity between phenylselenoesters and diselenides 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, see Scheme S1, Supporting Information). 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 unproductive thioester formation *via* rapid acylation of the product.

We next moved to explore the scope of the technology for a range of phenylselenoester coupling partners bearing a variety of C-terminal residues. Synthesis of **2** and selenoesters **6–14** was achieved by Fmoc-strategy solid-phase peptide synthesis (SPPS) and the stereochemical integrity confirmed by NMR spectroscopy (see Scheme S2, Supporting Information and Table 1). A number of model additive-free ligations were performed. Peptide dimer **1** was reacted with selenoesters **2** and **6–13** in 6 M Gn•HCl, 0.1 M phosphate buffer at a final reaction pH of 6.2 and a concentration of 2.5 mM with respect to dimer **1** (Table 1). With the exception of the reactions with Ile and Val selenoesters (**12** and **13**), a yellow precipitate of diphenyldiselenide (DPDS) formed within 60 seconds after addition of the selenoester, indicating completion of the reactions (see Scheme S3, Supporting Information). Indeed, HPLC-MS analysis after this time revealed that reactions proceeded cleanly to afford symmetric diselenides (**15a–f**) as the major products, together with the unsymmetrical diselenide products (**16a–f**) and no detectable selenoester hydrolysis (see Supporting Information). Following reverse-phase HPLC purification, the ligation products were isolated in excellent yields (72–87%, see Table 1). Notable examples include reaction at C-terminal Leu and Thr residues, which usually proceed slowly under standard native chemical ligation conditions but were complete within 60 seconds here.[12] For reactions involving sterically hindered selenoesters, such as C-terminal Ile **12** and Val **13**, comparatively longer reaction times of 10 and 5 min, respectively, were required for complete conversion. For these two examples, a slight excess of the selenoester (1.25 equiv) was also necessary. Nonetheless, the desired products (**15g/16g** and **15h/16h**) for these sterically hindered examples were isolated in good yields (Table 1). Finally, the reaction was also performed on peptides containing both an N-terminal (Sec)2 and an internal Cys residue. These ligations were also complete within 60 seconds (see Supporting Information).

We next investigated the potential extension of the selenium-mediated ligation to a one-pot ligation–deselenization protocol[17,18,21] to afford native Ala at the ligation junction. To this end, **1** was reacted with selenoesters **2** and **6–13** and, upon completion, the insoluble DPDS was extracted prior to *in situ* treatment with TCEP and DTT to effect deselenization. It is important to note that extraction of DPDS from the reaction mixture is necessary so as to prevent quenching of the deselenization reaction. Gratifyingly, this one-pot ligation–deselenization methodology afforded native peptide products **17a–17h** in good yields following reverse-phase HPLC purification (Table 1).

As an initial foray into unravelling the mechanism of our new transformation, we sought to probe the effect of pH, known to have a dramatic effect on the rate of native chemical ligation.[14] To this end, peptide **1** was reacted with peptide selenoester **8** bearing a C-terminal Leu selenoester in ligation buffer ranging from pH 1.6–8.3. At a pH range of 5.0–7.7, reactions proceeded cleanly with similar endpoints (1–3 minutes, see Supporting Information). Reactions at or above pH 8.0 do not proceed efficiently due to rapid selenoester decomposition at basic pH, while reactions at highly acidic pH (1.6) returned only starting material. Remarkably, 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,[22] as the corresponding Pro thioesters are known to be poor acyl donors in native chemical ligation.[12,23] To this end, peptide **1** was reacted with peptide selenoester **14** under the additive free conditions. However, after 12 h no ligation product had formed (Table 1). Interestingly, upon addition of TCEP (50 mM) to reduce the diselenide in **1**, ligation with **14** proceeded rapidly (under a native chemical ligation mechanism) to afford 80% of the ligation prod-

uct together with 15% of deselenized **1** (see Supporting Information).

Table 1. Reaction times and yields for (Sec)2–selenoester ligation and one-pot ligation–deselenization.
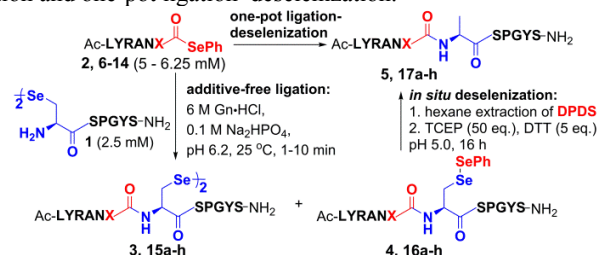

Selenoester (**X =**)	Reaction time (seconds)	Isolated yield ligation[c]	Isolated yield ligation–deselenization
Ala (**2**)	60[a]	72% (**3+4**)	60% (**5**)
Ser (**6**)	60[a]	73% (**15a+16a**)	57% (**17a**)
Thr (**7**)	60[a]	84% (**15b+16b**)	71% (**17b**)
Leu (**8**)	60[a]	87% (**15c+16c**)	66% (**17c**)
Phe (**9**)	60[a]	83% (**15d+16d**)	58% (**17d**)
Met (**10**)	60[a]	72% (**15e+16e**)	56% (**17e**)
Lys (**11**)	60[a]	79% (**15f+16f**)	97% (**17f**)
Ile (**12**)	600[b]	63% (**15g+16g**)	63% (**17g**)
Val (**13**)	300[b]	67% (**15h+16h**)	79% (**17h**)
Pro (**14**)	NR	-	-

[a] 0.5 eq. H-USPGYS-NH2 dimer to 1.0 eq. selenoester. [b] 0.5 eq. H-USPGYS-NH2 dimer to 1.25 eq. of selenoester [c] Yield calculated from combined diselenide products (**15 + 16**). NR = no reaction

Mechanistically, the intricacies of the additive-free (Sec)2–selenoester ligation methodology cannot be explained by a native chemical ligation pathway alone. While it is likely that the reaction involves linking of the two peptide fragments through an intermediate selenoester followed by an Se→N acyl shift as the amide bond-forming step, in the absence of an external reductant, the reaction cannot *initiate* through nucleophilic attack of a selenol onto the carbonyl carbon of the selenoester. Generation of the putative selenoester intermediate must therefore proceed through a mechanistically distinct process. We initially probed the feasibility of initiation *via* selenol electron-relay catalysis[24] facilitated by selenoester hydrolysis and concomitant generation of catalytic amounts of aryl selenolate. However, the lack of observable selenoester hydrolysis in productive ligations and the viability of the reaction even at acidic pH (where the likelihood of hydrolysis is further reduced) suggest that selenol catalysis as an *initiation* process is unlikely. We also probed a possible radical mechanism, fuelled by reports of phenyl selenoesters serving as acyl radical precursors[25-27] and recent evidence that light-mediated dynamic diselenide exchange occurs through a radical process.[28] However, we saw no evidence of 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 (see Supporting Information). Interestingly, after synthesis of model peptides bearing a range of electron-rich and electron-poor arylselenoesters, evaluation of the effect of aryl substitution on ligation rate revealed that electron-rich selenoesters react more rapidly than their electron-poor counterparts [e.g. $t_{1/2}$ (*p*-methoxyphenyl selenoester = 24 seconds; $t_{1/2}$ (*p*-nitrophenyl selenoester) = 72 seconds, see Supporting Information]. Given that electron-rich aryl substituents attenuate the electrophilicity of

the selenoester carbonyl carbon, these results contradict a native chemical ligation mechanism and suggest that, in the initiation phase, the arylselenoester may not be the electrophilic species.

We therefore proceeded to explore an intriguing mechanistic hypothesis in which the initiation step involves nucleophilic attack by the Se atom of the aryl selenoester onto the diselenide (Scheme 3) generating one equivalent of peptide selenolate **I** and a charged intermediate **II** that is stabilized by electron-donating substituents on the aromatic ring of the arylselenoester. The charged intermediate may then undergo a direct Se→N acyl shift to generate a new amide bond (through a six-membered ring intermediate, path a in Scheme 3A), or rapid trans-selenoesterification with selenolate **I** (path b, Scheme 3A) to generate an intermediate selenoester identical to that produced in a native chemical ligation-type pathway (e.g. **III**, Scheme 3B). Notably, a control reaction with a peptide dimer bearing an N-acetylated (Sec)₂ residue resulted in rapid formation of a selenoester intermediate (see Supporting Information for details) suggesting that the feasibility of the pathway is not dependent on the Se→N acyl-transfer, and that direct selenolate attack onto the charged intermediate may be a viable pathway.
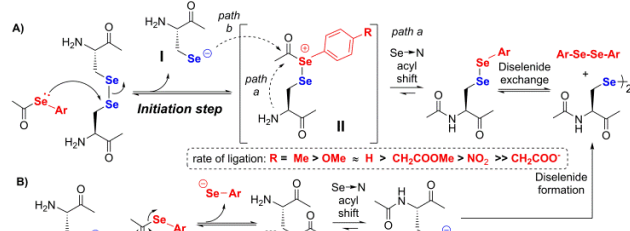
Scheme 3. A) Proposed *initiation* step of the (Sec)₂–selenoester ligation; B) native chemical ligation-type pathway.

In order to further probe the proposed initiation mechanism, we carried out computational quantum chemistry calculations using density functional theory and the Gaussian 09 program using the species depicted in Scheme 3 as models.[29] Gas-phase energies were obtained at the M06-2X/6-311+G(3df,2p)//M05-2X/6-31G(d) level, with the effect of solvation incorporated through the SMD continuum model at the M05-2X/6-31G(d) level for both geometry optimization as well as single-point energy calculations. The calculated relative energies of the putative charged intermediates are consistent with the experimentally observed trends (see Supporting Information). In particular, they provide support for the specificity of the reaction for arylselenoesters, for which the energies are markedly lower than those for the corresponding intermediates derived from aryl thioesters or aryl oxo-esters. In addition, lower calculated energies were obtained for charged intermediates derived from electron-rich aryl selenoesters (ΔG (*p*-OMe) = 137.0 kJ/mol) than electron-poor aryl selenoesters (ΔG (*p*-NO₂) = 139.1 kJ/mol), corroborating the experimentally observed rate enhancement afforded by electron-donating aryl substituents (see Supporting Information). It is also worth noting that peptide selenolate **I** may facilitate propagation of the reaction *via* a native chemical ligation pathway (Scheme 3B) and/or selenol catalysis, which likely proceed in tandem with the proposed initiation pathway. Interestingly, the rate of reaction showed further dependence on the solubility of the aryl diselenide derived from the starting aryl selenoester, with insoluble diselenides (e.g. DPDS) serving as a positive driving force for the reaction, while selenoesters that generated water-soluble diselenides (e.g. 4-selenophenylacetic acid diselenide) led to a dramatic reduction in ligation rate. The reaction rate for this example could be enhanced using a less soluble seleneoester analogue (e.g., the methyl ester of selenophenylacetic acid). We note that, while we have substantial evidence consistent with the pathways described above, several

alternative pathways can be considered. These mechanistic possibilities are presented in the Supporting Information, together with additional experiments and computational studies.
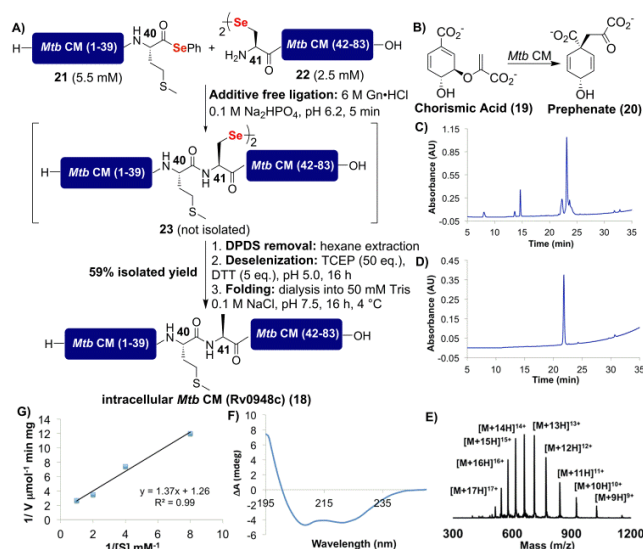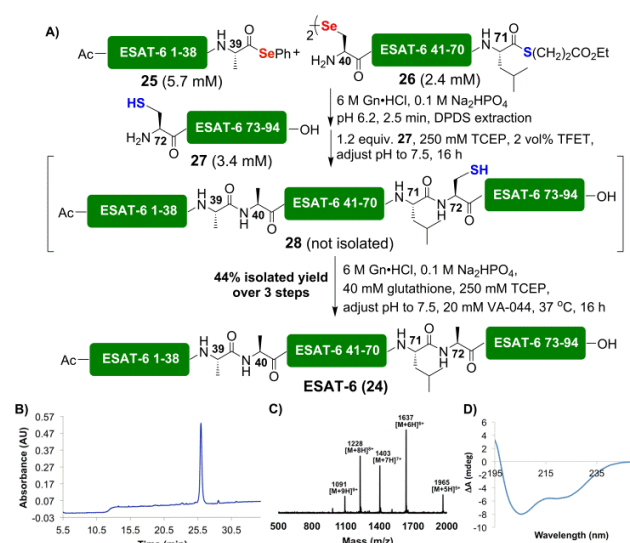

Scheme 4. A) Synthesis of *Mtb* CM **18** *via* additive-free (Sec)₂–seleneoester ligation–deselenization; **B)** Claisen rearrangement catalyzed by chorismate mutase; **C)** crude analytical HPLC after ligation–deselenization; **D)** analytical HPLC after purification and folding; **E)** ESI mass spectrum of purified and folded *Mtb* CM; **F)** CD spectrum of folded *Mtb* CM in 50 mM Tris, 0.1 M NaCl, pH 7.5; **G)** Lineweaver-Burk plot for the kinetics of chorismate to prephenate conversion by synthetic *Mtb* CM **18** (Kₘ = 1.1 mM and Vₘₐₓ = 0.79 μmol min⁻¹ mg⁻¹).[30,31]

Having thoroughly explored the scope of the additive-free ligation on a number of model systems and interrogated the mechanism of the reaction, we next moved to probe 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*) **18**,[30,31] the etiological agent of TB (Scheme 4A). This 83 residue enzyme is responsible for the conversion of chorismate **19** to prephenate **20** (see Scheme 4B) through a Claisen rearrangement, and is a crucial enzyme *en route* to aromatic amino acid synthesis in *Mtb*. The enzyme was disconnected to reveal two targets: *Mtb* CM 1–40 as a C-terminal methionine phenylselenoester (**21**) and *Mtb* CM 41–83 bearing an N-terminal (Sec)₂ moiety (**22**). Both fragments were synthesized *via* Fmoc-strategy SPPS (see Supporting Information). These two fragments were dissolved in 6 M Gn•HCl, 0.1 M phosphate buffer to give a final pH of 6.2 and after 5 min the additive-free ligation had proceeded to completion to afford exclusively the symmetrical diselenide **23** together with precipitated DPDS. Without purification, DPDS was extracted before treating the reaction mixture with DTT and TCEP to effect *in situ* deselenization and afford full length *Mtb* CM with excellent crude purity (Scheme 4C). Following reverse-phase HPLC, the protein was folded by dialysis into 50 mM Tris and 0.1 M NaCl to provide *Mtb* CM **18** in an excellent 59% overall yield (Scheme 4D and 4E). Importantly, our synthetic folded enzyme had similar structure and activity to that reported for the recombinant protein[30,31] as determined by circular dichroism (CD) spectroscopy (Scheme 4F) and by a kinetic assay with chorismate (Scheme 4G), respectively.

We also focussed on a second protein, the *N*-acetylated Cys-free 94 residue early secretory antigenic protein-6 (ESAT-6) **24**, to showcase the efficiency of the additive-free ligation technology (Scheme 5). ESAT-6, also from *Mtb*, is an important virulence factor and a potent T cell antigen.[32] The protein was disconnected

into three fragments, ESAT-6 1–39 **25** as a C-terminal phenylselenoester, ESAT-6 40–71 dimer **26** containing an N-terminal $(Sec)_2$ moiety and C-terminal alkyl thioester and ESAT-6 72–94 **27**, which we aimed to unify *via* a one-pot, three-component ligation reaction using both native chemical ligation and the $(Sec)_2$–selenoester ligation. Selenoester **25** and bifunctional peptide dimer **26** were first reacted in 6 M Gn•HCl, 0.1 M phosphate buffer at pH 6.2 and, after 2.5 min, a yellow DPDS precipitate formed and the reaction was judged to have reached completion by LCMS analysis. At this point, C-terminal fragment **27** was added, together with TCEP and the thiol additive TFET,[33] before adjusting the pH to 7.5. The ligation was incubated at 37 °C for 16 h, which led to completion of the native chemical ligation reaction together with concomitant phosphine-mediated deselenization of Sec-40 to Ala.[21] The reaction mixture was subsequently dosed with glutathione,[34] 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 (**24**) in 44% yield over the multiple-step, one-pot process.

Scheme 5. A) Synthesis of *Mtb* ESAT-6 **24** *via* a three-component one-pot ligation using an additive-free $(Sec)_2$–selenoester and native chemical ligation reactions; **B)** analytical HPLC of purified protein; **C)** ESI mass spectrum of purified protein; D) CD spectrum of folded synthetic ESAT-6.

In summary, we describe a novel 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. The $(Sec)_2$–selenoester ligations are performed by simple mixing of the two peptide components in aqueous buffer at neutral or acidic pH without the requirement for any reductants or additives. We have proposed a novel reaction pathway 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 the synthesis of proteins, 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

Experimental protocols, analytical ligation data, characterization of peptides and proteins, and computational data. This material is available free of charge *via* the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

Email: richard.payne@sydney.edu.au, phone: +61 2 9351 5877

Notes

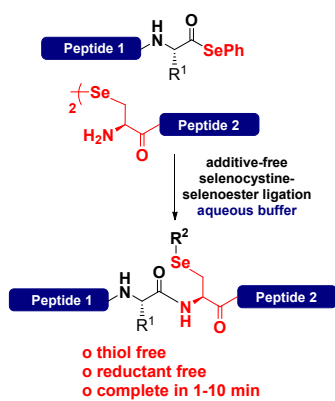The authors declare no competing financial interests.

Author Contributions
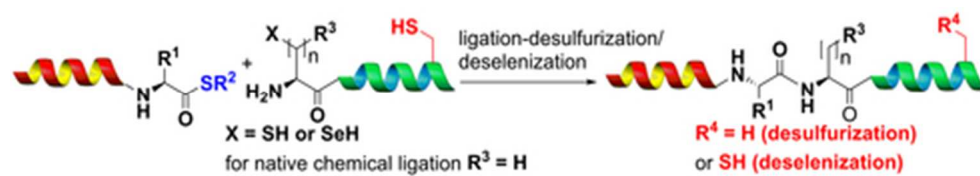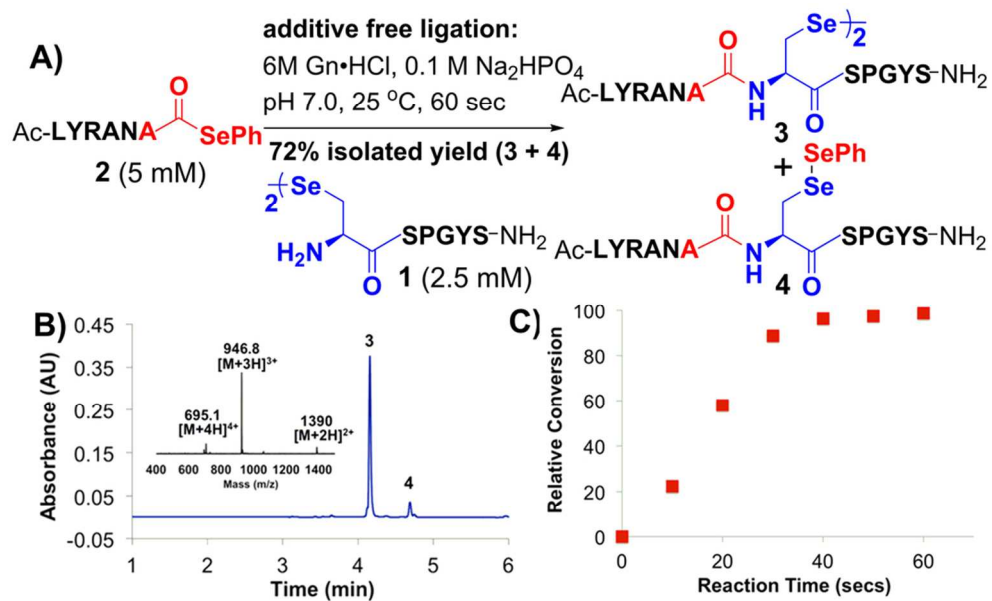
‡These authors contributed equally.

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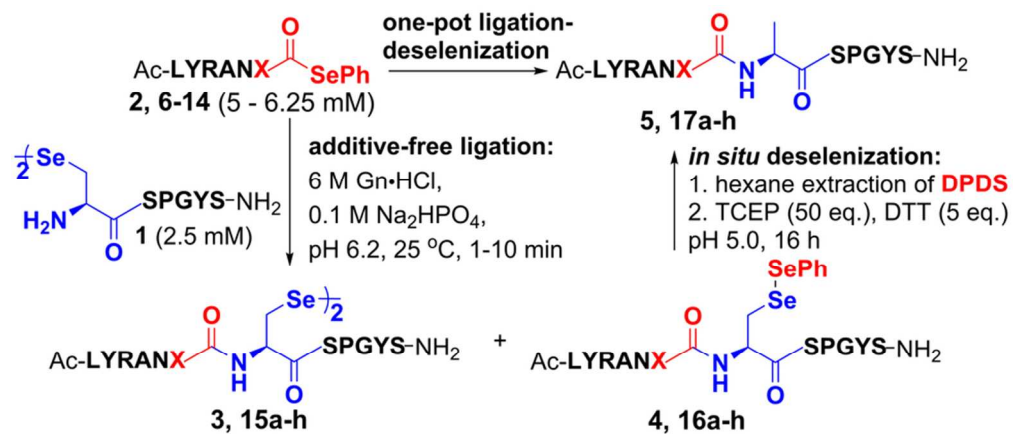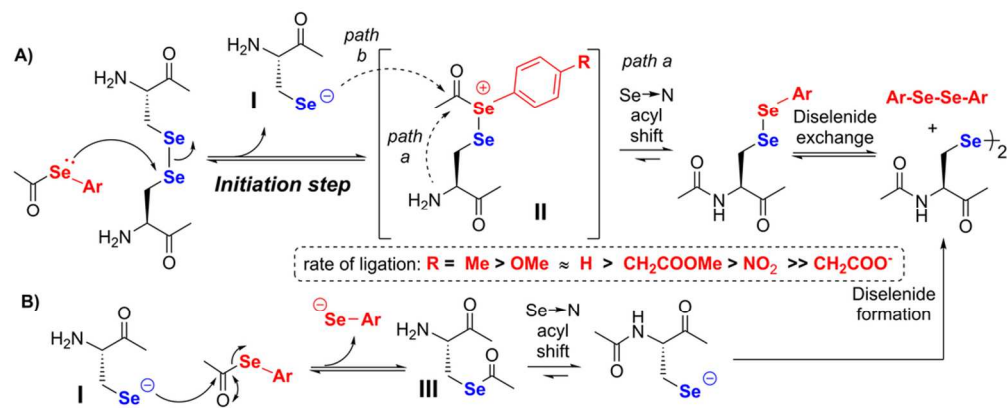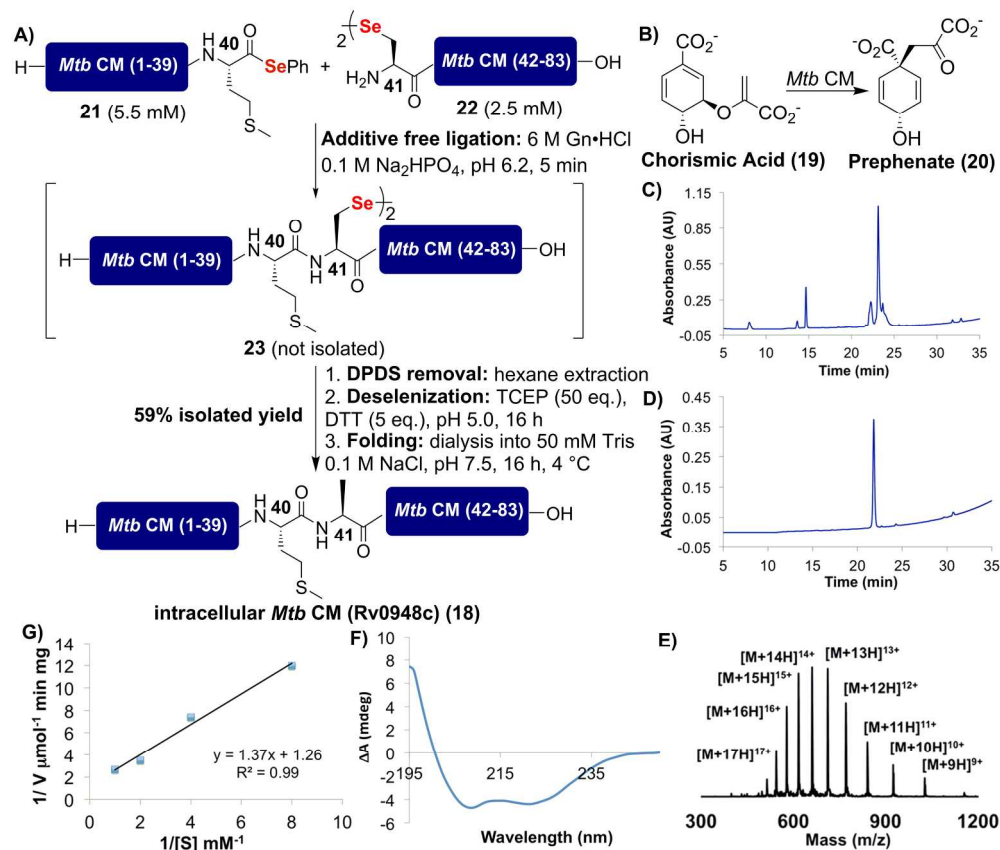o complete in 1-10 min

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91x55mm (300 x 300 DPI)

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Ac-**LYRANX**⎯C(=O)⎯SePh
2, 6-14 (5 - 6.25 mM)

one-pot ligation-deselenization →

Ac-**LYRANX**⎯C(=O)⎯N(H)⎯CH(CH₃)⎯C(=O)⎯**SPGYS**-NH₂
5, 17a-h

(½Se)⎯CH₂
H₂N⎯CH⎯C(=O)⎯**SPGYS**-NH₂
1 (2.5 mM)

additive-free ligation:
6 M Gn·HCl,
0.1 M Na₂HPO₄,
pH 6.2, 25 °C, 1-10 min

in situ **deselenization:**
1. hexane extraction of **DPDS**
2. TCEP (50 eq.), DTT (5 eq.)
pH 5.0, 16 h

Ac-**LYRANX**⎯C(=O)⎯N(H)⎯CH(CH₂-Se-½)⎯C(=O)⎯**SPGYS**-NH₂
3, 15a-h

+

Ac-**LYRANX**⎯C(=O)⎯N(H)⎯CH(CH₂-Se-SePh)⎯C(=O)⎯**SPGYS**-NH₂
4, 16a-h

82x35mm (300 x 300 DPI)

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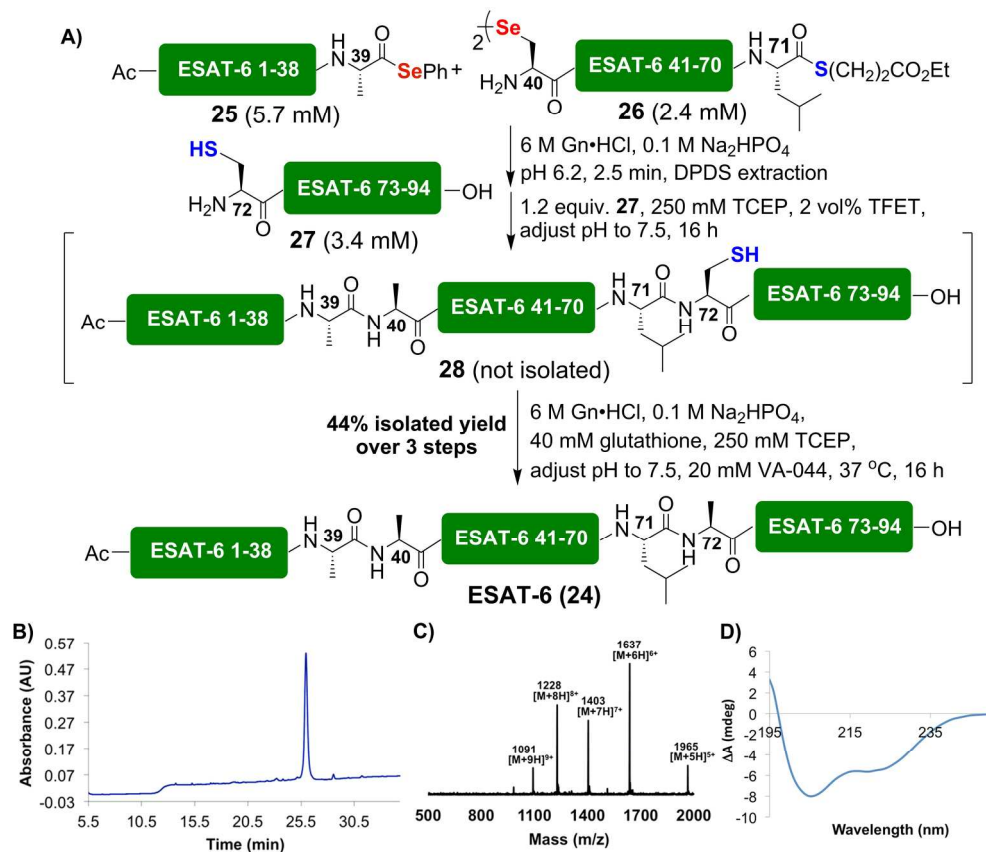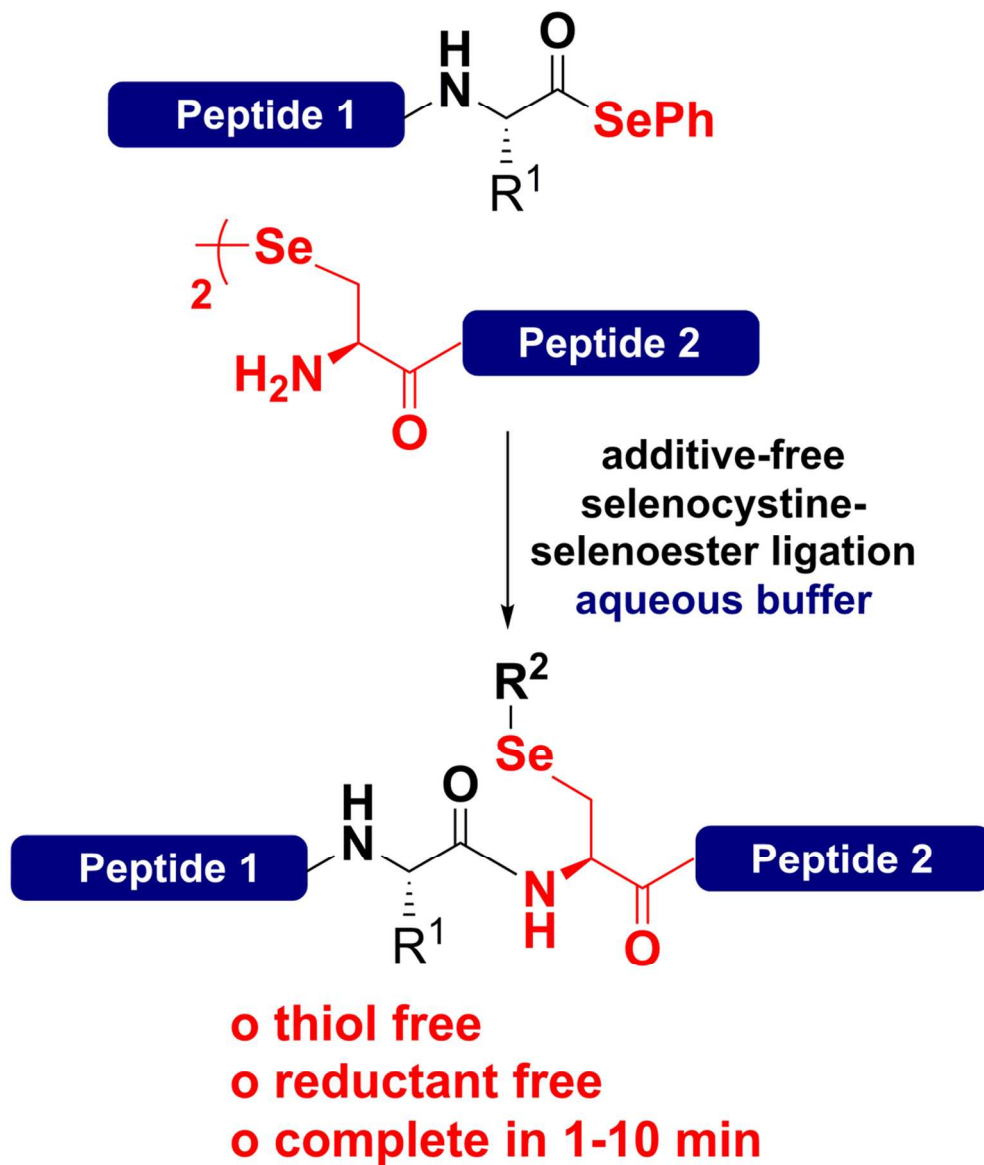

95x38mm (300 x 300 DPI)

213x181mm (300 x 300 DPI)

200x170mm (300 x 300 DPI)

101x119mm (300 x 300 DPI)