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Construction of Challenging Proline–Proline Junctions via Diselenide–Selenoester Ligation Chemistry

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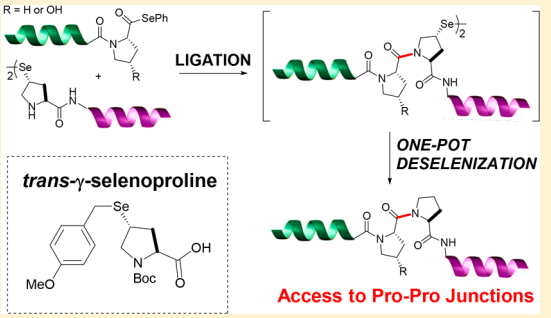
Supporting Information

ABSTRACT: Polyproline sequences are highly abundant in prokaryotic and eukaryotic proteins, where they serve as key components of secondary structure. To date, construction of the proline–proline motif has not been possible owing to steric congestion at the ligation junction, together with an n → π* electronic interaction that reduces the reactivity of acylated proline residues at the C-terminus of peptides. Here, we harness the enhanced reactivity of prolyl selenoesters and a *trans*-γ-selenoproline moiety to access the elusive proline–proline junction for the first time through a diselenide–selenoester ligation–deselenization manifold. The efficient nature of this chemistry is highlighted in the high-yielding one-pot assembly of two proline-rich polypeptide targets, submaxillary gland androgen regulated protein 3B and lumbricin-1. This method provides access to the most challenging of ligation junctions, thus enabling the construction of previously intractable peptide and protein targets of increasing structural complexity.



INTRODUCTION

Peptide ligation chemistry has revolutionized protein science by providing a means to access large polypeptide and protein targets,[1−3] including those that cannot be generated by recombinant expression technologies. This includes the ability to site-specifically incorporate nonproteinogenic amino acids, post-translational modifications, and/or isotopic or fluorescent labels.[2] The most widely adopted method for the convergent assembly of peptide fragments to afford large polypeptides and proteins is undoubtedly native chemical ligation (NCL). This technology, first reported by Kent and co-workers,[4] involves the chemoselective reaction of a peptide possessing a C-terminal thioester functionality with a second fragment bearing an N-terminal cysteine (Cys) residue. While this technology has enabled access to numerous protein targets to date,[1−3] the conventional reaction is limited by the requirement of a Cys residue at the ligation junction. This restriction has, however, been recently addressed through the development of NCL at thiol-derived amino acids,[5] selenocysteine,[6−8] and a number of synthetic selenol-derived amino acids[9] that can serve as cysteine surrogates in reactions with peptide thioesters. Importantly, following the ligation reaction, these thiol and selenol auxiliaries can be removed through desulfurization or deselenization chemistry, respectively, to afford native polypeptides and proteins.[10] One further limitation of the NCL manifold is that reactions typically proceed slowly at sterically encumbered thioesters, particularly those containing a C-terminal β-branched residue, e.g., valine (Val), threonine (Thr), or isoleucine (Ile).[11] Furthermore, thioesters bearing a C-terminal proline (Pro) residue have proven to be unreactive acyl donors in traditional NCL reactions, including at Cys, selenocysteine (Sec), and thiol/selenol amino acids.[12,13] This lack of reactivity has been attributed to a reduction in electrophilicity of the thioester due to a stabilizing n → π* electron donation from the proximal amide carbonyl to the carbonyl of the thioester moiety.[14,15] This stabilizing interaction is enabled by the rigid *trans*-configured peptide bond induced by the cyclic Pro residue.[14,15] Attempts to overcome the challenge of performing ligation chemistry at Pro thioesters have focused on enhancing the reactivity of the acyl donor component. One approach has involved substituting the prolyl thioester with a more reactive selenoester functionality (Scheme 1A).[16] These selenoester acyl donors have been reported to react with N-terminal Cys-containing peptides, albeit in the presence of a selenol catalyst and a large molar excess of the acyl donor fragment. More recently, Dong et al. have designed a prolyl thioester whereby the γ-position of the Pro ring is functionalized with a thiol moiety (Scheme 1B).[17] This modified Pro thioester reacts via a bicyclic thiolactone intermediate, which leads to activation of the carbonyl through the generation of a highly strained cyclic thioester. While this is a very elegant strategy, the γ-thiol

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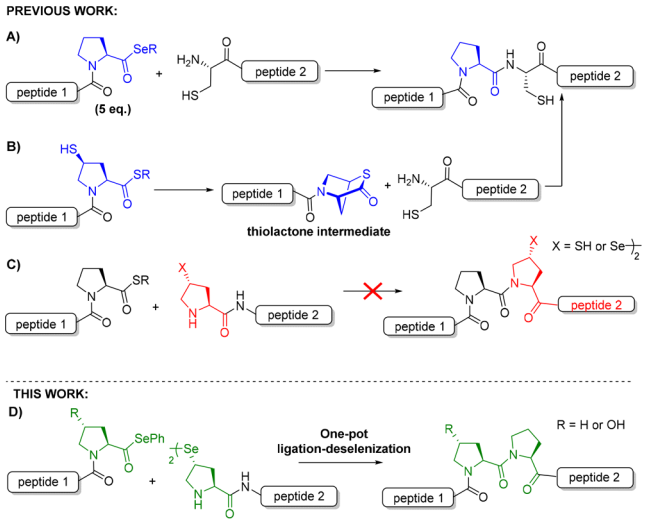
Scheme 1. Ligation Reactions between (A) a Prolyl Selenoester and Cysteinyl Peptide; (B) a Thiolated Prolyl Thioester and Cysteinyl Peptide That Proceeds via a Thiolactone Intermediate; (C) Thioesters and Thio- or Selenoproline Peptides; (D) a Prolyl Selenoester and Selenoproline Peptides via Diselenide–Selenoester Ligation (DSL)



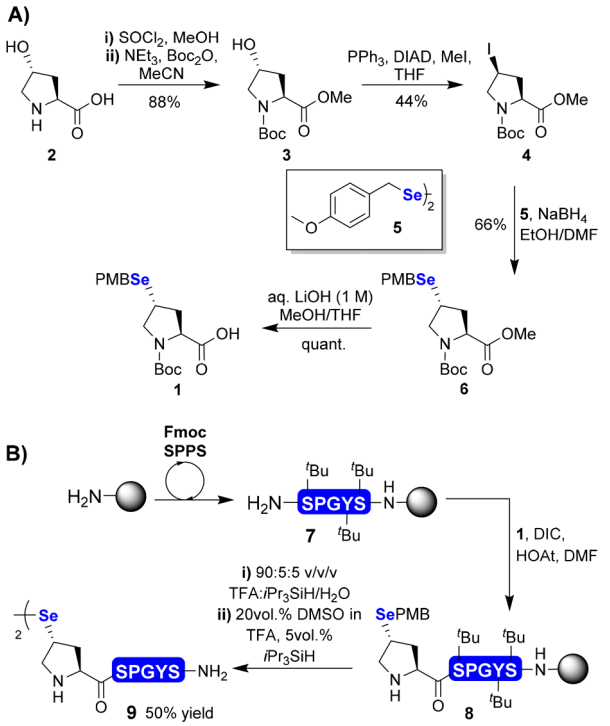
auxiliary must be removed via a postligation desulfurization protocol and therefore lacks chemoselectivity in the presence of native Cys residues found elsewhere in the sequence. Danishefsky and co-workers have reported the use of both thioproline and selenoproline residues in NCL reactions with peptide thioesters (Scheme 1C).[13,18] These ligation reactions worked well at unhindered sites, but were inefficient at more sterically encumbered C-terminal thioesters, e.g., Val, even with the enhanced nucleophilicity of the selenoproline moiety. Importantly, when peptides bearing N-terminal thioproline or selenoproline residues were reacted with peptide thioesters bearing a C-terminal Pro, no reaction was observed. This inability to forge Pro–Pro was attributed to the deactivation of the Pro thioester component through the n → π^* interaction from the adjacent carbonyl (*vide supra*).

We have recently reported the development of an additive-free peptide ligation reaction between peptides bearing a C-terminal selenoester and peptides containing an N-terminal selenocystine residue (the oxidized form of Sec).[12] Importantly, these represent the fastest ligation reactions known, proceeding efficiently in aqueous buffer without the addition of external catalysts or reductants to afford selenopeptide products in minutes (including at selenoesters bearing C-terminal β-branched amino acids). Upon reaction completion, and without purification, the selenocystine at the ligation junction can be cleanly converted to alanine or serine through reductive[19,20] or oxidative deselenization,[21,22] respectively. This diselenide–selenoester ligation (DSL) manifold has been extended to alternative selenol-derived amino acids including β-selenoaspartate, β-selenoleucine, and γ-selenoglutamate and has been successfully employed for the rapid and high-yielding synthesis of a number of protein and selenoprotein targets.[23,24] Following the establishment of the DSL technology, we were interested in probing whether the method could be used for the construction of peptides at more challenging junctions. Herein, we describe our efforts to extend the application of DSL–deselenization to amino acid junctions containing Pro; we outline the first example of the construction of challenging and hitherto inaccessible Pro–Pro junctions through ligation chemistry (Scheme 1D), as well as the application of this method in the synthesis of two large Pro-rich polypeptide targets.

RESULTS AND DISCUSSION

Design and Synthesis of a γ-Selenoproline Building Block. Before embarking on the development of the DSL methodology at Pro, we first needed to prepare a suitably protected selenol-derived Pro residue that would be compatible with solid-phase synthesis protocols and the ensuing ligation chemistry. Danishefsky and co-workers have previously reported the preparation of a γ-selenoproline diselenide amino acid.[13] We initially replicated this synthetic route (see Supporting Information), but as reported for other diselenide building blocks,[12] we observed that only one-half of the diselenide amino acid was able to couple to a resin-bound peptide chain (i.e., the other selenoproline unit within the diselenide dimer was unable to couple to another peptide N-terminus). This is particularly problematic in larger immobilized targets or when peptides are more highly loaded on resin supports (>0.5 mmol/g). In order to mitigate these issues, we sought to synthesize a monomeric selenoproline building block **1**, whereby the γ-selenol moiety was orthogonally protected with a *p*-methoxy benzyl group (PMB). The synthesis began with the C-terminal protection of commercially available *trans*-γ-hydroxyproline **2** as methyl ester **3** (Scheme 2A). Conversion to *cis*-γ-iodoproline **4** was then performed under Mitsunobu conditions with inversion of stereochemistry at the γ-position. Direct treatment of iodide **4** with 4-methoxybenzyldiselenide **5** in the presence of $NaBH_4$ then provided *trans*-γ-selenoproline methyl ester **6**. Finally,

Scheme 2. (A) Synthetic Route to *trans*-γ-Selenoproline 1; (B) Incorporation of *trans*-γ-Selenoproline 1 Building Block into Model Peptide 7 *via* Fmoc-SPPS



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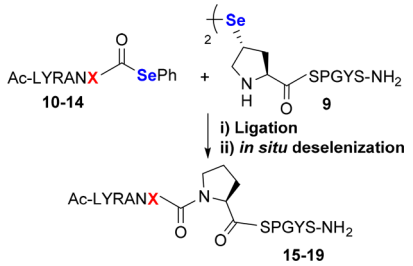
saponification of the methyl ester afforded the target *trans*-γ-selenoproline amino acid **1** in good yield over the four steps.

Synthesis of Peptides Bearing γ-Selenoproline. With monomeric selenoproline building block **1** in hand, we next investigated the efficiency of incorporation into the N-terminus of Rink amide resin immobilized pentapeptide **7** loaded at 0.7 mmol/g (Scheme 2B). We were pleased to observe complete coupling to **7** using only a slight excess of **1** (1.2 equiv) under standard coupling conditions [hydroxyazabenzotriazole (1.2 equiv), (dimethylamino)isopropyl chloride hydrochloride (1.2 equiv), in dimethylformamide] to generate resin-bound selenopeptide **8**. Acidolytic side-chain deprotection with concomitant cleavage from the resin followed by deprotection of the PMB group (using 20% dimethylsulfoxide, 5% iPr$_3$SiH in trifluoroacetic acid) and purification via reverse-phase HPLC provided the model diselenide dimer peptide **9** in 50% yield (based on the resin loading of the first amino acid).

One-Pot DSL−Deselenization at γ-Selenoproline. With model peptide **9** in hand, the ability of the diselenoproline motif to participate in additive-free peptide ligation chemistry with a range of model selenoesters (Ac-LYRANX-SePh) was investigated (Table 1). We first assessed the reaction of **9** with

Table 1. Model Ligation−Deselenization Reactions between Diselenide Dimer 9 and Selenoesters 10−14[a]



entry	Ac-LYRAN**X**-SePh **10−14** (X =)	ligation time (min)	yield for one-pot ligation−deselenization
1	Ala (**10**)	5	56% (**15**)
2	Met (**11**)	10	52% (**16**)
3	Tyr (**12**)	10	60% (**17**)
4	Leu (**13**)	10	51% (**18**)
5	Val (**14**)	45	54% (**19**)

[a]Ligation: **9** and **10−14** in ligation buffer (6 M Gdn·HCl, 0.1 M Na_2HPO_4), rt, pH 6.2, [**9**] = 2.5 mM, [**10−14**] = 6.5 mM, 5−45 min. *In situ* deselenization: Hexane extraction followed by addition of an equivalent volume of TCEP (250 mM) and DTT (20 mM) in buffer (6 M Gdn·HCl, 0.1 M Na_2HPO_4, pH 4.5−5.5).

peptide selenoesters **10−14** bearing C-terminal Ala (A), Met (M), Tyr (Y), Leu (L), and Val (V) residues as a representative selection of the proteinogenic amino acids (see Supporting Information for details on peptide selenoester synthesis). Specifically, diselenide dimer **9** and peptide selenoesters **10−14** (1.3 equiv with respect to monomeric **9**) were simply dissolved in 6 M guanidinium chloride (Gdn·HCl) and 0.1 M phosphate buffer at pH 6.2 without the addition of any other exogenous additives. Equal volumes of the solutions were combined to a final concentration of 2.5 mM diselenide dimer **9** and 6.5 mM selenoester **10−14**. In all cases the ligation reached completion within 5−45 min (as judged by UPLC-MS analysis) to afford the ligation product as a mixture of the symmetrical diselenide and the product additionally acylated at the γ-selenol moiety via a trans-selenoesterification reaction with excess selenoester starting material (also observed with the parent reaction at Sec;[12] see Supporting Information). The mixture of ligation products formed is ultimately inconsequential, as all products converge to the desired native peptide following *in situ* deselenization.

It should be noted that a fine precipitate of diphenyl diselenide (DPDS) is produced during the ligation reaction, which serves as a visual indication of reaction completion.[12] Upon completion of the ligation reaction, the DPDS precipitate was removed via hexane extraction and the deselenization step was effected by treatment with tris(2-carboxyethyl)phosphine (TCEP, 250 mM) and dithiothreitol (DTT, 20 mM) in 6 M Gdn·HCl/0.1 M phosphate buffer at pH 5.0. *In situ* deselenization of the γ-selenoproline moiety proceeded to completion within 16 h to afford the desired native Pro-containing peptides. Purification via reverse-phase HPLC provided **15−19** in excellent yields (51−60%) over the two steps. Having established that the γ-selenoproline moiety is competent in additive-free DSL−deselenization chemistry, we next investigated whether the method could be expanded to include C-terminal Pro selenoesters in an attempt to forge previously intractable Pro−Pro junctions. Importantly, polyproline sequences are highly abundant in peptides and proteins across all taxa; more than 33% of *E. coli* proteins[25] and ca. 25% of human proteins (see Supplementary Table) possess one or more polyproline motifs. A method to construct such junctions would therefore be highly valuable. As an initial assessment of the ligation efficiency, Ac-LYRANP-SePh **20** (2.0 equiv) was reacted with diselenide dimer peptide **9** (1.0 equiv with respect to the monomer) in 6 M Gdn·HCl/0.1 M phosphate buffer at pH 6.2. While the reaction proceeded more slowly than with selenoesters **10−14**, we were delighted to observe clean and complete conversion within 16 h (Scheme 3A). Extraction of the DPDS precipitate and *in situ* deselenization then provided the desired Pro−Pro-containing peptide **21** in 54% yield over two steps. To our knowledge, this represents the first successful example of a peptide ligation at the sterically encumbered and electronically disarmed Pro−Pro junction, thus providing a unique advantage of the DSL reaction manifold. Encouraged by the successful Pro−Pro ligation under the DSL manifold, we next explored the scope of the chemistry at another Pro-derived residue, namely, γ-hydroxyproline (Hyp), a common post-translationally modified residue found in proteins, e.g., collagens. Studies began with the synthesis of model peptide selenoester **22**, possessing a C-terminal Hyp residue (Ac-LYRANP(OH)-SePh, see Supporting Information for details). Pleasingly, the additive-free DSL reaction between **22** and **9** also proceeded to completion within 16 h, and, following *in situ* deselenization and purification by HPLC, peptide product **23** was isolated in 55% yield (Scheme 3A). In order to show that DSL reactions at prolyl selenoesters were also competent with other selenoamino acids, we next performed ligation between peptide selenoester **20** and peptides bearing N-terminal selenocystine and β-selenoaspartate residues as the corresponding diselenide dimers (see Supporting Information). These reactions proceeded cleanly within 16 h, and subsequent deselenization provided products containing Ala and Asp at the ligation junction in good yield.

Pro−Pro Ligation in the Presence of Additives. Together with the additive-free DSL−deselenization chemistry, we have previously shown that diselenide peptides (with the exception of those bearing N-terminal β-selenoaspartate or γ-selenoglutamate residues[22]) can be successfully ligated with

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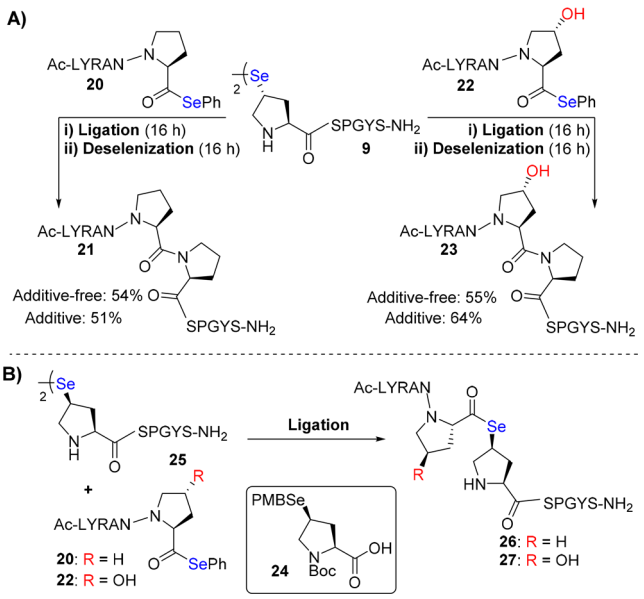
Scheme 3. (A) Ligation−Deselenization between Diselenide Dimer 9 Bearing an N-Terminal *trans*-γ-Selenoproline and Selenoesters 20 and 22 under Additive-Free and Additive Conditions;[a] (B) Ligation between *cis*-γ-Selenoproline Diselenide Dimer 25 and Selenoesters 20 and 22 Leading to Unrearranged Selenoester Intermediates 26 and 27



[a]1 equiv of **20** or **22** used in the additive reaction with 50 mM TCEP and 20 mM DPDS.

peptide selenoesters in the presence of additives, specifically the phosphine reductant TCEP. Since TCEP is able to facilitate deselenization reactions, a radical-trapping agent such as ascorbic acid[26] or DPDS must also be used to avoid this deleterious side reaction (until the ligation has reached completion). We were interested in exploring whether the inclusion of additives would accelerate the ligation reaction at the challenging Pro−Pro junctions. We also envisaged that the reductant would be capable of regenerating phenylselenolate from DPDS, which would perform selenolysis of the product selenoesters that are generated during the reaction, thus enabling the reduction of the molar excess of the selenoester component required (2.0 equiv under additive-free conditions, *vide supra*). We chose to use TCEP as the reductant and DPDS as the radical trap, which can be easily extracted prior to *in situ* deselenization. Ligations between peptide diselenide dimer **9** and selenoesters **20** and **22** were repeated in the presence of TCEP (50 mM) and DPDS (20 mM) using 1 equiv of the selenoester component (Scheme 3A). These ligations proceeded to completion with only ca. 3−5% deselenization of the starting diselenide dimer **9** over a period of 16 h but, interestingly, did lead to substantial deselenization of the ligation product. Nonetheless, upon extraction of the excess DPDS with hexane and treatment with TCEP and DTT, a single deselenized product could be obtained. Purification via reverse-phase HPLC afforded the desired peptide products **21** and **23** bearing Pro−Pro and Hyp−Pro motifs in 51% and 64% yield, respectively. Importantly, these experiments demonstrate that DSL reactions at γ-selenoproline can be performed using reductants in the presence of a radical quenching agent (in this case DPDS); however, there is no benefit to the rate of the ligation reaction. Finally, we envisaged that the use of additives in forging Pro−Pro junctions via DSL chemistry would be beneficial for substrates possessing unprotected Cys residues. Specifically, the inclusion of TCEP and DPDS would enable any unproductive thioesters that may form to be selenolyzed with phenylselenolate. Toward this end, reactions were performed with and without additives (TCEP, DPDS) on a model peptide containing an unprotected internal Cys residue (see Supporting Information). Gratifyingly, the reactions proceeded smoothly and at similar rates under both additive- and additive-free manifolds. Following chemoselective deselenization[19] of γ-Se-Pro, native peptides bearing an intact internal Cys residue were generated in good yields (see Supporting Information).

Effects of γ-Selenoproline Stereochemistry on Ligation Efficiency. Having shown that the reaction was tolerant of a number of selenoester reactants, we were next interested in investigating the effect of the stereochemistry at the γ-position of the selenoproline unit on the productivity of the ligation. As such, we prepared *cis*-γ-selenoproline building block **24** using a similar route to that developed for **1** (Scheme 3B). Stereochemical inversion at the γ-position was effected by treatment of a tosylated variant of γ-hydroxyproline with diselenide **5** under reducing conditions (see Supporting Information for full synthetic details). The *cis*-γ-selenoproline **24** was subsequently coupled to resin-bound peptide **7** and subjected to acidolytic cleavage, side-chain deprotection, and PMB removal. The final HPLC-purified diselenide dimer peptide **25** bearing *cis*-γ-selenoproline was obtained in 66% yield (based on the loading of the first amino acid). Interestingly, ligation of **25** to both Ac-LYRANP-SePh **20** and Ac-LYRANP(OH)-SePh **22**, under both additive-free and additive conditions, failed to afford the desired ligation product over 16 h. Instead, formation of unrearranged selenoesters of **25** (i.e., branched selenoesters **26** and **27**) were observed as the major products (Scheme 3B). This was confirmed upon treatment of **26** and **27** with aqueous hydrazine to effect cleavage of the side-chain selenoester to reafford **25** and form the acyl hydrazide of **20** and **22** (see Supporting Information). Importantly, the results of this study suggest that the normally rapid Se → N acyl shift is unable to proceed when the γ-seleno moiety is in the *S*-configuration, i.e., *cis*-configured with respect to the α-amine moiety.

Computational Studies. In order to help rationalize the strict stereochemical requirement for the Se → N acyl shift of the selenoester intermediate, and therefore productive ligation at Pro−Pro, we turned to computational studies. Specifically, using model systems for the selenoester intermediate generated with *trans*- and *cis*-γ-selenoproline diastereoisomers (Figure 1A), we extensively examined the possible conformational space for both to determine the likelihood of the acyl shift proceeding. Toward this end, we assessed a large number of initial conformers using a Monte Carlo algorithm together with molecular mechanics. These structures were then optimized with density functional theory (DFT) using the PBE/6-31G(d) method; improved single-point energies were subsequently obtained using the higher-level M06-2X/6-311G(2df,p) method (see Supporting Information). Using this method we found that the low-energy structures for the *trans*-isomer (e.g., **I** in Figure 1B) have the carbonyl carbon of the selenoester moiety in close proximity to the Pro α-NH and at a suitable geometry to facilitate the intramolecular acyl shift, a pathway that is observed experimentally. Logically, one can envisage that inversion of the stereochemistry at the γ-selenoproline unit would place the Pro α-NH away from this more optimal location. Indeed, despite sampling hundreds of conformations (e.g., **II**) for the *cis*-isomer, none place the carbonyl carbon of the selenoester in a position

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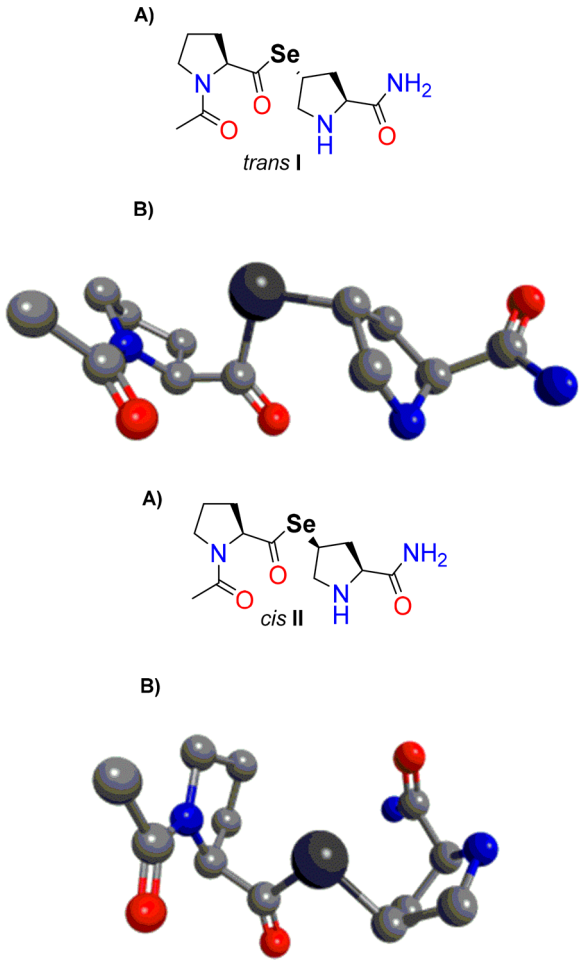

Figure 1. Proposed selenoester intermediates for *trans*- (**I**, not observed experimentally) and *cis*- (**II**, observed experimentally) γ-selenoproline isomers: (A) ChemDraw representations; (B) low-energy structures. Hydrogen atoms are omitted for clarity.

that would lead to a favorable Se → N shift with the Pro α-NH. It is interesting to note that our calculations suggest that the Se → N acyl shift would be further favored for the *trans*-isomer from an acyl-selenonium species if generated under the reaction conditions (see Supporting Information for data).[12] In summary, our DFT results explain the experimental observation that the selenoesters, **26** and **27**, are formed but do not rearrange.

Synthesis of Proline-Rich Polypeptides. Having successfully developed a robust method for the one-pot DSL−deselenization at γ-selenoproline and, more importantly, demonstrated the first successful ligation at Pro−Pro, we next turned our attention to the application of this technology for the preparation of larger polypeptides. For this purpose we first chose to target the synthesis of the Pro-rich polypeptide SMR3B (submaxillary gland androgen regulated protein 3B, **28**) using the additive-free manifold.[27] The 57-amino-acid sequence of SMR3B is Cys- and Ala-free and contains a total of 30 Pro residues (53%), seven of which form a centrally located polyproline stretch. We envisaged a disconnection between Pro27 and Pro28 within this motif that would facilitate a simple two-fragment assembly via the Pro−Pro DSL−deselenization method. Fragment **29** (SMR3B 1−27) bearing a C-terminal proline selenoester and **30** (SMR3B 28−57) containing an N-terminal *trans*-γ-selenoproline diselenide dimer were first synthesized via Fmoc-SPPS on 2-chlorotrityl chloride resin (see Supporting Information). The resulting fragments **29** (10 mM final conc) and **30** (5 mM final conc with respect to the monomer) were then subjected to ligation in 6 M Gdn·HCl/0.1 M phosphate buffer at a final pH of 6.4. The reaction was monitored by UPLC-MS and reached completion within 16 h. Extraction of the precipitated DPDS with hexane, followed by *in situ* deselenization with TCEP and DTT, successfully generated the native polypeptide. Purification via reverse-phase HPLC then provided SMR3B **28** in 52% yield.

In addition to SMR3B, we also chose to prepare the 62-amino-acid antimicrobial peptide lumbricin-1 via one-pot DSL−deselenization chemistry at γ-selenoproline. Lumbricin-1 is a Pro-rich peptide (15% Pro content) isolated from the earthworm *Lumbricius rubellus*.[28] It has been shown to exhibit antimicrobial activity *in vitro* against a broad spectrum of microorganisms including Gram-positive (e.g., *Staphylococcus aureus*) and Gram-negative bacteria (e.g., *E. coli*) as well as some fungi (e.g., *Candida albicans*). Given the absence of Cys or suitably positioned Ala residues in the sequence, together with the abundance of Pro, lumbricin-1 was considered a second ideal target to showcase the DSL−deselenization method at Pro−Pro. s4

We chose to disconnect lumbricin-1 (**31**) centrally between Pro33 and Pro34 for assembly through additive-free DSL−deselenization in one pot. This led to two peptide targets for synthesis, the 33-amino-acid peptide **32**, bearing a C-terminal Pro selenoester, and peptide dimer **33**, bearing an N-terminal *trans*-γ-selenoproline. Both fragments were synthesized on 2-chlorotrityl chloride resin via Fmoc-SPPS (see Supporting Information for details). Fragments **32** (10 mM final conc) and **33** (5 mM final conc with respect to the monomer) were then ligated by dissolving in 6 M Gdn·HCl/0.1 M phosphate buffer at a final pH of 6.2. The reaction was monitored by UPLC-MS and reached completion within 16 h. On this occasion extraction of the DPDS, followed by *in situ* deselenization with TCEP and DTT, did not proceed to completion, attributed to the inability of DTT to thiolyze the product selenoester generated in the reaction. Therefore, the ligation was repeated and, upon completion, the crude reaction mixture was treated with 2 vol % hydrazine for 10 min, which led to complete hydrazinolysis of the product selenoester, generating diselenide ligation product **34** exclusively. Hexane extraction of residual DPDS, *in situ* deselenization with TCEP and DTT, and purification via reverse-phase HPLC then afforded lumbricin-1 (**31**) in 52% yield over two steps. The synthetic lumbricin-1 was subsequently assessed for activity against *Staphylococcus aureus* (SH1000 strain) and exhibited activity consistent with that reported for the material isolated from the earthworm (IC_{50} = 80 μM).[28] s5

CONCLUSIONS

In summary, an efficient one-pot DSL−deselenization strategy has been developed at γ-selenoproline that has enabled access to Pro−Pro ligation sites for the first time. The reaction was shown to be equally efficient with or without the inclusion of reductive additives. However, the stereochemistry of the γ-selenol moiety was shown to be crucial for peptide bond formation. The power of this technology was demonstrated via a one-pot synthesis of submaxillary gland androgen regulated protein 3B and the antimicrobial peptide lumbricin-1 in excellent overall yields. The simplicity and efficiency of the ligation−deselenization chemistry developed here should see this technology applied to the synthesis of numerous Pro-rich peptide and protein targets in

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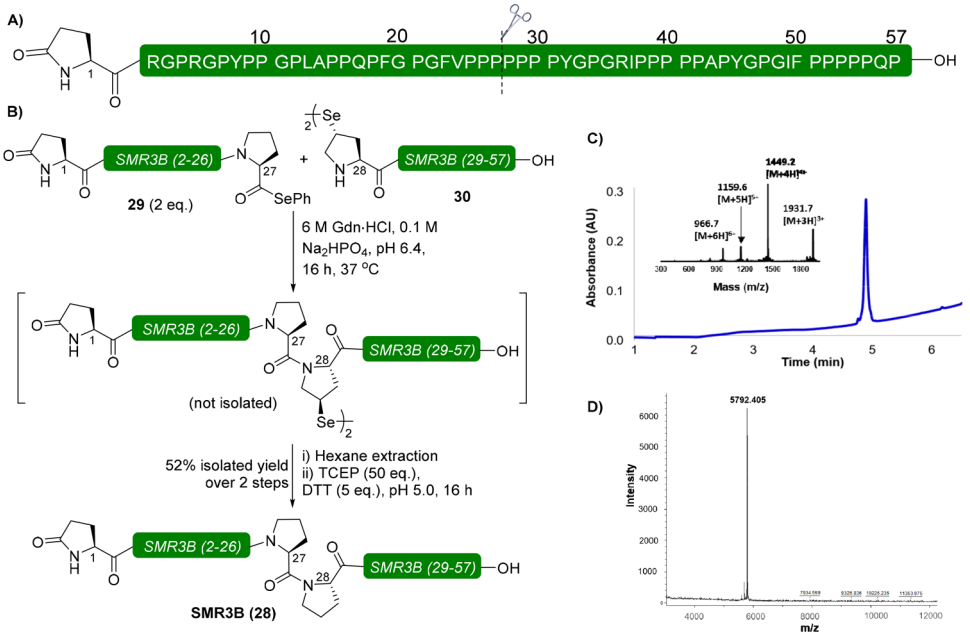
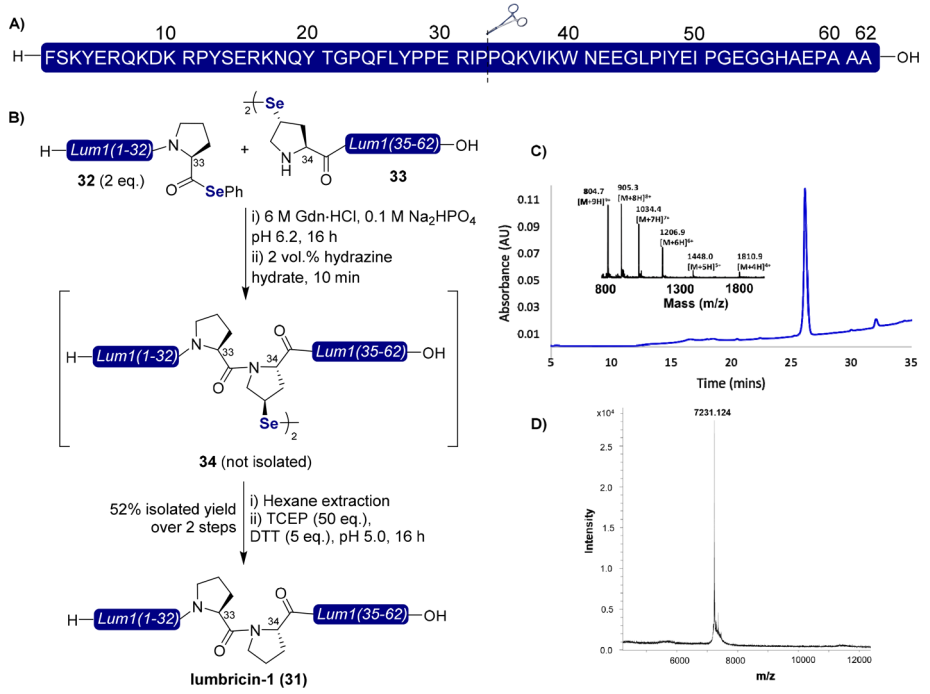
Scheme 4. (A) Primary Structure of SMR3B; (B) Synthesis of SMR3B (28) *via* a One-Pot Additive-Free Ligation−Deselenization of Fragments 29 and 30; (C) Analytical HPLC and ESI Mass Spectrum (Inset) of Purified Synthetic SMR3B; (D) MALDI-TOF Mass Spectrum of Purified Synthetic SMR3B



Scheme 5. (A) Primary Structure of Lumbricin-1; (B) Synthesis of Lumbricin-1 (31) *via* a One-Pot Additive-Free Ligation−Deselenization of Fragments 32 and 33; (C) Analytical HPLC and ESI Mass Spectrum (Inset) of Purified Synthetic Lumbricin-1; (D) MALDI-TOF Mass Spectrum of Purified Synthetic Lumbricin-1



the future, particularly those that can only be accessed via Pro-Pro/Hyp-Pro ligations, e.g., antimicrobial peptides and collagens.

EXPERIMENTAL SECTION

One-Pot Additive-Free DSL−Deselenization at Proline−Proline. Peptide prolyl selenoesters (2.0 equiv) and N-terminal selenoproline diselenide dimer peptides (1.0 equiv with respect to the monomer) were dissolved separately in ligation buffer (6 M Gdn·HCl/100 mM Na_2HPO_4, pH 7.2). The solutions were combined to give an overall concentration of 10 mM with respect to the peptide selenoester and 5 mM with respect to monomeric selenoproline peptide, and the pH was adjusted to 6.2−6.5 with aqueous 1 M NaOH. The ligation progress was monitored by UPLC-MS until the selenoproline peptide was completely consumed. The precipitated DPDS was extracted from the crude ligation solution with hexane (×3). The solution was then thoroughly degassed with Ar(g) sparging. To effect deselenization, an equal volume of degassed buffer containing TCEP (0.25 M) and DTT

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(20 mM) at pH 4.5−5.5 was added. Following the completion of the reaction (16 h), the solution was purified via semipreparative reverse-phase HPLC (see Supporting Information for column and gradient information). All peptides were isolated as white solids following lyophilization.

One-Pot Additive DSL−Deselenization at Proline−Proline. The N-terminal selenoproline diselenide dimer (1.0 equiv with respect to the monomer) was dissolved in a solution of TCEP (100 mM) and DPDS (40 mM) in ligation buffer (6 M Gdn·HCl/100 mM Na_2HPO_4, pH 6−7). The proline selenoester peptide (1.0 equiv) was dissolved separately in ligation buffer to a concentration of 10 mM. The two solutions were combined to give an overall concentration of 5 mM with respect to both peptide fragments (final additive concentrations: 50 mM TCEP, 20 mM DPDS), and the pH was adjusted to 6.2−6.5. The ligation reaction was allowed to proceed to completion (as judged by analytical UPLC and UPLC-MS analysis). The precipitated DPDS was extracted from the crude ligation solution with hexane (×3). The solution was then thoroughly degassed with Ar(g) sparging. To effect deselenization, an equal volume of degassed buffer containing TCEP (0.25 M) and DTT (20 mM) at pH 4.5−5.5 was added. Following the completion of the reaction (16 h), the solution was purified via semipreparative reverse-phase HPLC (see Supporting Information for column and gradient information), and the desired ligation products were isolated as white solids following lyophilization.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b07877.

Experimental, reaction, characterization, and computational data (PDF)

Table of human proteins possessing polyproline sequences (XLSX)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Kent, S. B. H. Total chemical synthesis of proteins. *Chem. Soc. Rev.* **2009**, *38* (2), 338−351.

(2) Kulkarni, S. S.; Sayers, J.; Premdjee, B.; Payne, R. J. Rapid and efficient protein synthesis through expansion of the native chemical ligation concept. *Nat. Rev. Chem.* **2018**, *2*, 0122.

(3) Bondalapati, S.; Jbara, M.; Brik, A. Expanding the chemical toolbox for the synthesis of large and uniquely modified proteins. *Nat. Chem.* **2016**, *8*, 407−418.

(4) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. Synthesis of proteins by native chemical ligation. *Science* **1994**, *266* (5186), 776−779.

(5) Malins, L. R.; Payne, R. J. Synthetic amino acids for applications in peptide ligation−desulfurization chemistry. *Aust. J. Chem.* **2015**, *68*, 521−537.

(6) Hondal, R. J.; Nilsson, B. L.; Raines, R. T. Selenocysteine in native chemical ligation and expressed protein ligation. *J. Am. Chem. Soc.* **2001**, *123* (21), 5140−5141.

(7) Quaderer, R.; Sewing, A.; Hilvert, D. Selenocysteine-mediated native chemical ligation. *Helv. Chim. Acta* **2001**, *84*, 1197−1206.

(8) Gieselman, M. D.; Xie, L.; van der Donk, W. A. Synthesis of a selenocysteine-containing peptide by native chemical ligation. *Org. Lett.* **2001**, *3* (9), 1331−1334.

(9) Malins, L. R.; Mitchell, N. J.; Payne, R. J. Peptide ligation chemistry at selenol amino acids. *J. Pept. Sci.* **2014**, *20* (2), 64−77.

(10) Mousa, R.; Dardashti, R. N.; Metanis, N. Selenium and selenocysteine in protein chemistry. *Angew. Chem., Int. Ed.* **2017**, *56* (50), 15818−15827.

(11) Hackeng, T. M.; Griffin, J. H.; Dawson, P. E. Protein synthesis by native chemical ligation: Expanded scope by using straightforward methodology. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96* (18), 10068−10073.

(12) Mitchell, N. J.; Malins, L. R.; Liu, X.; Thompson, R. E.; Chan, B.; Radom, L.; Payne, R. J. Rapid additive-free selenocystine−selenoester peptide ligation. *J. Am. Chem. Soc.* **2015**, *137* (44), 14011−14014.

(13) Townsend, S. D.; Tan, Z.; Dong, S.; Shang, S.; Brailsford, J. A.; Danishefsky, S. J. Advances in proline ligation. *J. Am. Chem. Soc.* **2012**, *134* (8), 3912−3916.

(14) Pollock, S. B.; Kent, S. B. An investigation into the origin of the dramatically reduced reactivity of peptide-prolyl-thioesters in native chemical ligation. *Chem. Commun.* **2011**, *47* (8), 2342−2344.

(15) Raibaut, L.; Seeberger, P.; Melnyk, O. Bis(2-sulfanylethyl)amido peptides enable native chemical ligation at proline and minimize deletion side-product formation. *Org. Lett.* **2013**, *15*, 5516−5519.

(16) Durek, T.; Alewood, P. F. Preformed selenoesters enable rapid native chemical ligation at intractable sites. *Angew. Chem., Int. Ed.* **2011**, *50* (50), 12042−12045.

(17) Gui, Y.; Qiu, L.; Li, Y.; Li, H.; Dong, S. Internal activation of peptidyl prolyl thioesters in native chemical ligation. *J. Am. Chem. Soc.* **2016**, *138* (14), 4890−4899.

(18) Shang, S.; Tan, Z.; Dong, S.; Danishefsky, S. J. An advance in proline ligation. *J. Am. Chem. Soc.* **2011**, *133* (28), 10784−10786.

(19) Metanis, N.; Keinan, E.; Dawson, P. E. Traceless ligation of cysteine peptides using selective deselenization. *Angew. Chem., Int. Ed.* **2010**, *49* (39), 7049−7053.

(20) Dery, S.; Reddy, P. S.; Dery, L.; Mousa, R.; Dardashti, R. N.; Metanis, N. Insights into the deselenization of selenocysteine into alanine and serine. *Chem. Sci.* **2015**, *6* (11), 6207−6212.

(21) Malins, L. R.; Mitchell, N. J.; McGowan, S.; Payne, R. J. Oxidative deselenization of selenocysteine: applications for programmed ligation at serine. *Angew. Chem.* **2015**, *127* (43), 12907−12912.

(22) Mitchell, N. J.; Kulkarni, S. S.; Malins, L. R.; Wang, S.; Payne, R. J. One-pot ligation−oxidative deselenization at selenocysteine and selenocystine. *Chem. - Eur. J.* **2017**, *23* (4), 946−952.

(23) Wang, X.; Sanchez, J.; Stone, M.; Payne, R. J. Sulfation of human cytomegalovirus protein UL22A enhances binding to the chemokine RANTES. *Angew. Chem., Int. Ed.* **2017**, *56* (29), 8490−8494.

(24) Mitchell, N. J.; Sayers, J.; Kulkarni, S. S.; Clayton, D.; Goldys, A. M.; Ripoll-Rozada, J.; Pereira, P. J. B.; Chan, B.; Radom, L.; Payne, R. J. Accelerated protein synthesis via one-pot ligation-deselenization chemistry. *Chem.* **2017**, *2* (5), 703−715.

(25) Qi, F.; Motz, M.; Jung, K.; Lassak, J.; Frishman, D. Evolutionary analysis of polyproline motifs in *Escherichia coli* reveals their regulatory role in translation. *PLoS Comput. Biol.* **2018**, *14* (2), e1005987.

(26) Reddy, P. S.; Dery, S.; Metanis, N. Chemical synthesis of proteins with non-strategically placed cysteines using selenazolidine and selective deselenization. *Angew. Chem., Int. Ed.* **2016**, *55* (3), 992−995.

DOI: 10.1021/jacs.8b07877
J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

(27) Isemura, S.; Saitoh, E.; Sanada, K. Isolation and amino acid sequences of proline-rich peptides of human whole saliva. *J. Biochem.* **1979**, *86*, 79−86.
(28) Cho, J. H.; Park, C. B.; Yoon, Y. G.; Kim, S. C. Lumbricin I, a novel proline-rich antimicrobial peptide from the earthworm: purification, cDNA cloning and molecular characterization. *Biochim. Biophys. Acta, Mol. Basis Dis.* **1998**, *1408*, 67−76.

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J. Am. Chem. Soc. XXXX, XXX, XXX−XXX