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# Synthesis and Utility of β-Selenol-Phenylalanine for Native Chemical Ligation–Deselenization Chemistry

**Lara R. Malins and Richard J. Payne***

*School of Chemistry, The University of Sydney, NSW 2006, Australia*

*richard.payne@sydney.edu.au*

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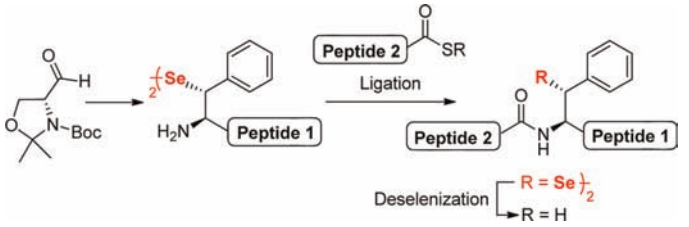
## ABSTRACT




**An efficient synthetic route to a suitably protected β-selenol-phenylalanine derivative from commercially available Garner's aldehyde is described. The incorporation of this building block into peptides and its application in native chemical ligation reactions with peptide thioesters are demonstrated. Ligation products were chemoselectively deselenized (including in the presence of unprotected cysteine residues) to provide native peptides.**

Native chemical ligation is the most widely used method for the chemoselective assembly of unprotected peptide fragments to afford polypeptides and proteins.[1] The method relies on the reaction of a peptide bearing an N-terminal cysteine (Cys) residue with a peptide possessing a C-terminal thioester (or more recently a selenoester[2]) moiety. The reaction involves a reversible thioesterification step followed by a rapid intramolecular S to N acyl shift to generate the native peptide bond in high yield and under mild reaction conditions. The power of this methodology is exemplified by its application in the synthesis of hundreds of proteins to date.[1b,3]

The requirement for a Cys residue at the N-terminus of a peptide fragment has prompted significant research effort directed toward extending the repertoire of native chemical ligation-based transformations to other amino acids. This concept was fuelled by the work of Dawson and co-workers who demonstrated that peptides and proteins produced *via* native chemical ligation could be desulfurized to provide an alanine (Ala) residue at the ligation junction.[4] In the same report, the authors proposed the concept of further expanding native chemical ligation to any thiol-derivatized proteinogenic amino acid at the N-terminus of peptide fragments through the use of ligation–desulfurization chemistry.[4,5]

This concept has now been expanded through the use of β-, γ-, or δ-thiol amino acids.[6−12] Although much progress has been made to maximize the scope of native chemical

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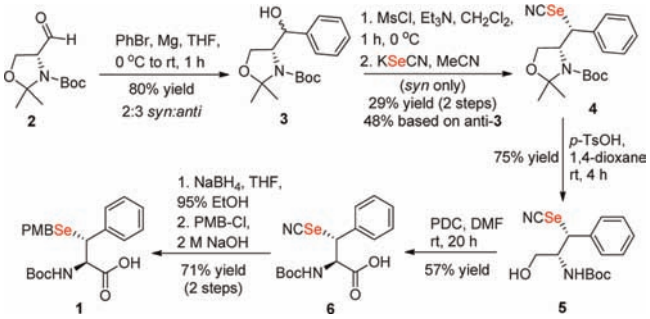
ligation–desulfurization chemistry, the various conditions (reductive or radical) used to effect desulfurization are not selective in the presence of other Cys residues in the ligation product which are concomitantly converted to Ala. This unwanted side reaction can be prevented by global protection of Cys side chains in the sequence;[13] however, this prevents the use of biologically expressed peptide and protein fragments and the use of expressed protein ligation (EPL) methodologies.[14] A recent report from Dawson and co-workers elegantly circumvents this issue by demonstrating that peptides obtainable from selenocysteine (Sec)-mediated native chemical ligation reactions[15] can be chemoselectively deselenized with tris(2-carboxyethyl)phosphine (TCEP) and dithiothreitol (DTT) to afford an Ala residue without affecting unprotected Cys residues.[16]

**Scheme 1.** $\beta$-Selenol-Mediated Ligation–Deselenization


Given the potential utility of this chemoselective deselenization methodology, we envisaged the use of unnatural, $\beta$-selenol amino acid derivatives (in a similar manner to $\beta$-thiol amino acids) so as to expand the number of available ligation sites by taking advantage of a ligation-deselenization theme (Scheme 1). Recently, this type of methodology was shown to be achievable at 4-selenoproline residues at the N-terminus of peptide fragments.[11b] Herein, we report the preparation of the first $\beta$-selenol-derived amino acid, namely the suitably protected $\beta$-selenol-Phe building block **1**. In addition, we demonstrate the utility of this building block in selenol-mediated native chemical ligation–deselenization reactions. Particular emphasis is placed on the compatibility of this methodology with unprotected Cys residues.

Synthesis of **1** began with a Grignard addition of bromobenzene to readily available Garner's aldehyde **2** which provided **3** as an inseparable mixture of diastereoisomers (2:3 *syn*/*anti*) in 80% yield (Scheme 2). Anticipating the potential for significantly different relative reactivities of the *anti*- and *syn*-diastereoisomers, we chose to progress this mixture without further attempts to optimize the selectivity of the Grignard addition. As such, activation of alcohol **3** as the corresponding mesylate, followed by introduction of the crucial selenium moiety *via* $S_N2$ displacement with potassium selenocyanate, afforded compound **4** in 29% yield over the two steps. Importantly, this inversion reaction enabled resolution of the two diastereoisomers, as only the *anti*-mesylate proved to be competent in the $S_N2$ process. This exclusively provided the desired *syn*-selenocyanate, which, based on prior work on $\beta$-thiol leucine ligations,[9b] was predicted to facilitate more facile peptide ligations than the corresponding $\beta$-epimer. It is worth noting, however, that an oxidation/reduction protocol could be used to obtain *anti*-enriched **3** (1:8 *syn*/*anti*), thereby improving the overall yield of the inversion to provide *syn*-diastereoisomer **4** in 55% yield over two steps (see Supporting Information (SI) for details). Acidic cleavage of the hemiaminal protecting group followed by oxidation with pyridinium dichromate (PDC), yielded carboxylic acid **6** in 57% yield. Importantly, under these reaction conditions, no selenium oxidation byproduct was observed. Reduction of the selenocyanate functionality with sodium borohydride followed by protection afforded the corresponding *p*-methoxybenzyl (PMB) protected $\beta$-selenol building block **1**, ready for incorporation into peptides.

**Scheme 2.** Synthesis of $\beta$-Selenol-Phe Building Block **1** from Garner's Aldehyde **2**




Compound **1** was subsequently incorporated at the N-terminus of model hexapeptides using Fmoc-strategy solid-phase peptide synthesis (SPPS) starting from Rink amide resin (Scheme 3). Following Fmoc-SPPS, cleavage from the resin and purification, peptide **7** was isolated in 52% yield (see SI for synthetic details). The protected seleno-peptide was then treated with 2,2′-dithiobis(5-nitropyridine) (DTNP)[17] in trifluoroacetic acid (TFA) to afford the symmetrical diselenide **8** in 88% yield. A variety of C-terminal peptide thioesters (Ac-LYRANX-S$(CH_2)_2$-$CO_2$Et, X = Gly, Ala, Met, Phe, Val) were also prepared, as previously described,[18] thus enabling the scope of the proposed ligation reaction to be investigated.

With $\beta$-selenol peptide **8** and an array of peptide thioesters now in hand, we next turned our attention to ligation reactions which we investigated under native chemical ligation conditions. After screening a variety of conditions and thiol additives, optimal results were obtained using ligation buffer [6 M Gn·HCl, 100 mM $Na_2HPO_4$, 5 mM

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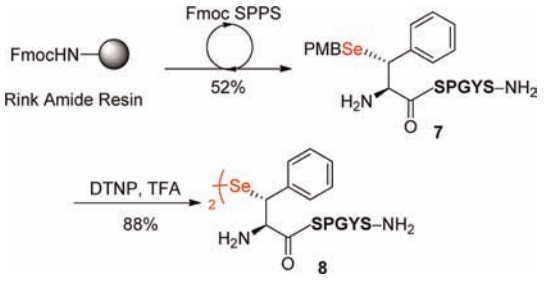
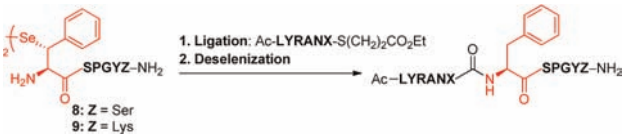
**Scheme 3.** Synthesis of Model Diselenide Peptide **8**




with respect to seleno-peptide **8**] in the presence of 200 mM 4-mercaptophenylacetic acid (MPAA) at room temperature and a final pH of 7.0−7.3, a slight modification of conditions used by Dawson and co-workers for Sec ligations.[16] Gratifyingly, the first ligation of peptide **8** with a peptide thioester bearing a C-terminal Gly residue (entry 1, Table 1) proceeded to completion in less than 24 h and in 56% isolated yield, comparable to previously reported Sec-mediated ligations.[15,16] Under these reaction conditions, the ligation product was obtained as a mixture of the diselenide dimer and the selenyl-MPAA sulfide adduct which were separated using reversed-phase HPLC (see SI). Ligations with peptide thioesters bearing C-terminal Ala, Met, and Phe (entries 2−4, Table 1) also proceeded to completion within 24 h, and the desired products were isolated in 51−53% yields. Unfortunately, ligation of **8** with a peptide thioester bearing a sterically encumbered Val residue led to poor conversion to the desired ligation product after 96 h (21%, see entry 5, Table 1). The prolonged ligation times are likely a result of the low steady state concentration of free selenol present in solution,[15c] owing to the stability of the diselenide and use of a relatively mild reductant (MPAA) in the ligation reaction.[16] The chemoselectivity of the ligation in the presence of an unprotected Lys residue was also demonstrated by the ligation of a modified N-terminal peptide ($\beta$-Se-FSPGYK-$NH_2$ dimer, **9**) with two model thioesters (Entries 6 and 7, Table 1) which provided the desired ligation products with no direct aminolysis byproduct detected.[19]

After successfully demonstrating the feasibility and scope of $\beta$-selenol-Phe mediated ligations, we subjected our recombined mixture of purified ligation products to deselenization using the optimized conditions reported previously (9.4 equiv of DTT, 2.9 equiv of TCEP in 0.1 M phosphate buffer, pH = 5.1).[16] In our hands, however, these conditions afforded the desired deselenization product along with a significant quantity (~50%) of two diastereomeric hydroxylated byproducts, consistent with the conversion of the enantiopure $\beta$-selenol-Phe residue within the peptide ligation products into a mixture of the corresponding $\beta$-hydroxylated compounds (see SI). Dawson and co-workers have also reported a minor byproduct upon deselenization in the presence of excess

**Table 1.** Scope of $\beta$-Selenol-Phe Ligation−Deselenizations




| entry | thioester (**X** =) | peptide (**Z** =) | ligation yield[a] | ligation−deselenization yield[a] |
|---|---|---|---|---|
| 1 | Gly | **8:** Ser | 56%[b] | 35%[e,g] |
| 2 | Ala | **8**: Ser | 53%[b] | 38%[e,g] |
| 3 | Met | **8**: Ser | 51%[b] | 47%[f] |
| 4 | Phe | **8**: Ser | 53%[b] | 43%[f] |
| 5 | Val | **8**: Ser | 21%[c] | 21%[f] |
| 6 | Gly | **9**: Lys | 52%[b] | N.D. |
| 7 | Phe | **9**: Lys | 54%[d] | N.D. |

[a] Isolated yields; reaction conditions: ligation: buffer (6 M Gn·HCl, 100 mM $Na_2HPO_4$, 5 mM with respect to seleno-peptide fragment **8** or **9**), 200 mM MPAA, rt, pH 7.0−7.3. [b] $t$ = 24 h. [c] $t$ = 96 h. [d] $t$ = 48 h; one-pot deselenization: addition of TCEP (42 equiv) and DTT (4.9 equiv) to ligation mixture. [e] $t$ = 48 h. [f] $t$ = 24 h. [g] Additional TCEP (42 equiv) and DTT (4.9 equiv) were added after 24 h.

TCEP that is consistent with Sec to Ser conversion.[16] The relative prevalence of the hydroxylation pathway in our system is likely due to the enhanced stability of the benzylic radical formed in the course of the deselenization of $\beta$-selenol-Phe over the alanyl radical formed in the corresponding deselenization of Sec residues.[16]

Given the difficulties associated with purification of the ligation product mixture and in an attempt to suppress the unwanted hydroxylation pathway, we were interested in exploring a one-pot ligation−deselenization protocol which would avoid any intermediary purification steps and hopefully provide further insight into the intricacies of the deselenization process. To this end, ligation reactions with N-terminal peptide **8** and a range of thioesters were carried out using the conditions previously described and subjected directly (without purification) to deselenization with DTT (4 equiv) and excess TCEP (40 equiv) (Table 1). The deselenization reactions were relatively slow, requiring 24−48 h and, in some cases, additional dosing with DTT and TCEP to reach completion (see SI). As previously suggested,[16,20] the sluggish reaction rates may be attributed to the large excess (200 mM) of MPAA present in the ligation buffer, which acts as a competitive radical scavenger. Nonetheless, we were pleased to find that the one-pot ligation−deselenization protocol afforded the desired peptide products in an average of 60−70% yield per step (35−47% over two steps). Most notably, the hydroxylation pathway was almost entirely suppressed (< 5% hydroxylated byproduct observed) likely a positive effect of the excess MPAA, which may also act as a suitable hydrogen donor to rapidly trap the reactive benzylic radical and prevent unwanted side reactions.[21]

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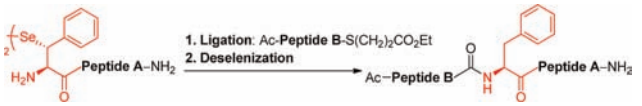
**Table 2.** One-Pot Ligation–Deselenization in the Presence of Cys




| entry | peptide A | peptide B | ligation–deselenization yield[a] |
|---|---|---|---|
| 1 | SPGY**C** (**10**) | LYRANG | 52% |
| 2 | SPGYS (**8**) | LYR**C**NG | 61% |
| 3 | SPGYS (**8**) | LYR**C**NM | 48% |

[a] Isolated yields; reaction conditions: ligation: buffer (6 M Gn·HCl, 100 mM $Na_2HPO_4$, 5 mM with respect to seleno-peptide fragments), 200 mM MPAA, rt, pH 7.0–7.3, 48 h; one-pot deselenization: addition of TCEP (42 equiv), DTT (4.9 equiv) to ligation mixture, 48 h.

Finally, to evaluate whether the modified conditions used to effect deselenization in our one-pot ligation–deselenization protocol were chemoselective in the presence of unprotected Cys residues, we synthesized an alternative peptide **10** bearing both a Cys residue and the $\beta$-selenol-Phe building block, as well as two Cys-containing thioester fragments (Ac-LYRCNX-S$(CH_2)_2$-$CO_2$Et, X = Gly, Met), and subjected these compounds to the ligation–deselenization cascade (Table 2). Ligation reactions required slightly longer reaction times to reach completion (~48 h), owing to the formation of a stable intramolecular selenyl-sulfide bond (entry 1, Table 2), and unproductive thioester formation with the unprotected Cys residues (entries 2 and 3, Table 2). Nonetheless, we were pleased to find that, in all cases, the two-step process afforded the desired peptide products bearing a native Phe residue at the ligation junction in good yields (48–61% over two steps) without affecting the internal, unprotected Cys residues (Figure 1). This represents the first study of $\beta$-selenol-mediated ligations at an amino acid other than Sec and suggests that this chemistry will be amenable to other $\beta$-selenol amino acids. The chemoselectivity of the deselenization protocol in the presence of free Cys residues has important implications for protein synthesis *via* selenol-based ligation techniques.

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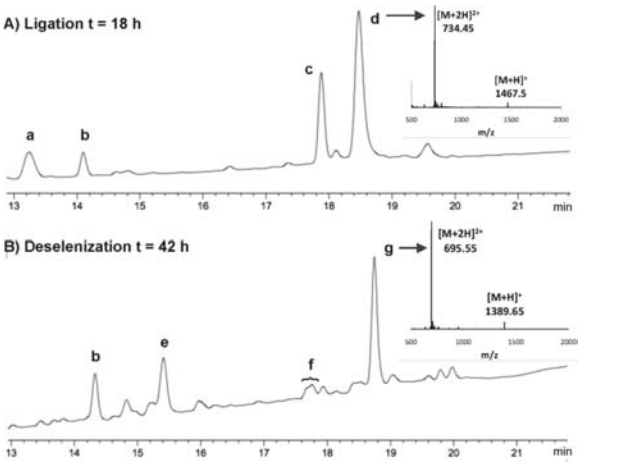


**Figure 1.** (A) Representative crude reaction mixture of ligation between peptide **10** and Ac-LYRANG-S$(CH_2)_2CO_2$Et; peak a = unreacted **10** $[M + H]^+ = 750.5$; b = hydrolyzed thioester $[M + H]^+ = 735.7$; c = MPAA-thioester $[M + H]^+ = 885.9$; d = ligation product $[M + H]^+ = 1467.5$, $[M + 2H]^{2+} = 734.45$. (B) One-pot deselenization; e = Se=P$[(CH_2)_2CO_2H]_3$ $[M + H]^+ = 331.2$ ; *f* = hydroxylated byproducts $[M + 2H]^{2+} = 703.5$; g = deselenization product $[M + H]^+ = 1389.65$, $[M + 2H]^{2+} = 695.55$.

In summary, we have developed a novel synthetic route to $\beta$-selenol-Phe **1** that can be readily modified to enable access to a range of $\beta$-selenol (and $\beta$-thiol) amino acid building blocks for use in native chemical ligation–deselenization chemistry. We have successfully utilized this building block in ligation reactions with a variety of peptide thioesters and have demonstrated a one-pot ligation–deselenization cascade that is efficient and chemoselective in the presence of unprotected Cys residues, thereby expanding the repertoire of ligation methodologies. Future work in our laboratory will focus on the synthesis of additional $\beta$-selenol amino acids and their utility in peptide ligation chemistry and protein synthesis.

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**Supporting Information Available.** Detailed experimental procedures, analytical HPLC traces and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.