CHEMISTRY
A European Journal

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ChemPubSoc Europe

Accepted Article

Title: Selenolysine: a new tool for traceless isopeptide bond formation

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To be cited as: *Chem. Eur. J.* 10.1002/chem.202000310

Link to VoR: http://dx.doi.org/10.1002/chem.202000310

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Selenolysine: a new tool for traceless isopeptide bond formation

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Abstract: Despite their biological importance, post-translationally modified proteins are notoriously difficult to produce in a homogeneous fashion using conventional expression systems. Chemical protein synthesis or semi-synthesis offers a solution to this problem; however, traditional strategies often rely on sulfur-based chemistry that is incompatible with the presence of any cysteine residues in the target protein. To overcome these limitations, we present the design and synthesis of γ-selenolysine, a selenol-containing form of the commonly modified proteinogenic amino acid, lysine. The utility of γ-selenolysine is demonstrated with the traceless ligation of the small ubiquitin-like modifier protein, SUMO-1, to a peptide segment of human glucokinase. The resulting polypeptide is poised for native chemical ligation and chemoselective deselenization in the presence of unprotected cysteine residues. Selenolysine's straightforward synthesis and incorporation into synthetic peptides marks it as a universal handle for conjugating any ubiquitin-like modifying protein to its target.

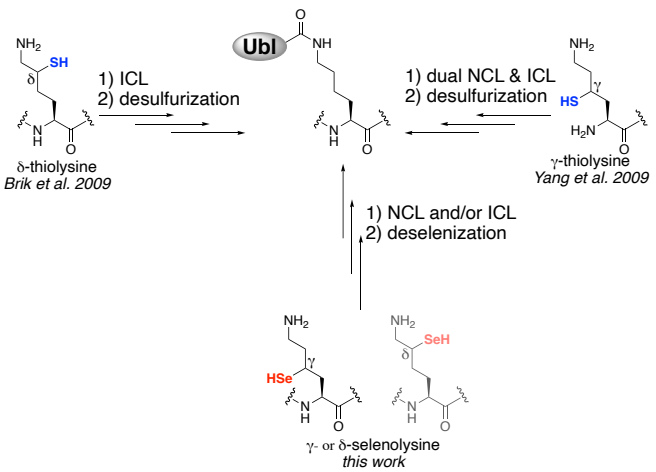
Following ribosomal translation and folding, expressed proteins can undergo a great number of post-translational modifications (PTMs) before fulfilling their biological functions.[1] These PTMs instruct the cell to carry out a variety of regulatory activities including transportation and degradation, to name a few.[2] Additionally, Lys, which is one of the more reactive amino acids under biological conditions, can be modified with post-translationally modifying proteins such as ubiquitin and other ubiquitin-like modifiers (Ubl).[3]

Techniques for controlling PTMs in cellular expression are not yet sufficiently well-developed to allow recombinant expression of homogenously modified proteins.[4] Alternatively, some methods for semi-selective modification of expressed proteins have been developed, most employing Cys chemistry. For example, dehydroalanine (Dha), a suitable electrophile for Michael addition reactions, can be generated from Cys,[5] Ser,[6] thioethers,[7] and several selenium-containing unnatural amino acids.[8] Michael addition to the resulting Dha has been used to introduce many small-molecule PTMs and entire PTM proteins, as in the case of ubiquitylated α-globin.[9] However, a stereoselective method for addition to Dha has yet to be developed,[9] a drawback in the chiral world of biology.

Chemical protein synthesis (CPS) and semi-synthesis, on the other hand, offers a direct route to selectively modified proteins.[10] Based on solid-phase peptide synthesis (SPPS)[11] and native chemical ligation (NCL)[12], this approach allows the preparation of modified proteins, including proteins with multiple sites of different PTMs.[4]

Additionally, Lys-mediated chemical ligation to form an isopeptide bond, typically referred to as isopeptide chemical ligation (ICL), has been used as a tool to conjugate post-translationally modifying proteins to their targets. Previously, Muir and coworkers reported ubiquitylated protein synthesis using a modified Lys with an auxiliary that was removed post-ligation;[13] more recently, the Liu group employed an auxiliary to produce di- and tri-ubiquitin chains.[14] Other studies utilized orthogonal protecting groups to incorporate isopeptide bonds on-resin,[15] or installed dipeptides at desired modification sites to allow for downstream ligation.[16] Brik and coworkers introduced δ-thiolysine in the conjugation of ubiquitin and synthesis of ubiquitylated proteins or ubiquitin chains.[17] Another Lys derivative, γ-thiolysine, has been used as a handle in dual NCL reactions,[18] in which the γ-thiolysine plays a role in both main chain and isopeptide ligation. Global desulfurization of the final products gives a native Lys at the ligation site (Scheme 1).

We sought to develop selenium-containing analogs of modified Lys residues in order to facilitate traceless isopeptide bond formation through ICL. A subsequent chemoselective deselenization reaction in the presence of unprotected Cys residues (Scheme 1) would give the native Lys.

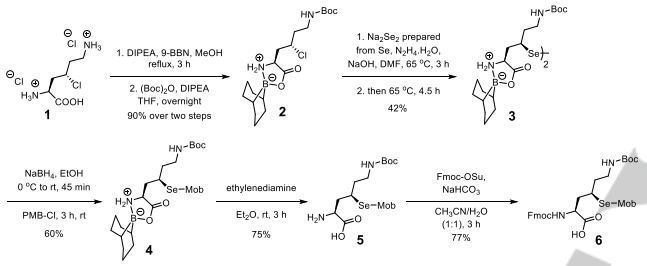


Scheme 1. Previously developed δ-thiolysine, by Brik,[16, 17] and γ-thiolysine, by Yang,[19, 20] used for NCL and ICL followed by desulfurization for the conjugation of Ubl proteins to Lys residues. Our designed γ/δ-selenolysines for NCL and/or ICL followed by chemoselective deselenization for traceless conjugation of Ubl proteins to Lys residues.

The aforementioned γ/δ-thiolysines used in ICL/desulfurization approaches are not compatible in the presence of proteins with natural Cys residues in their sequences, unless orthogonal Cys protecting groups are installed. This presents a true challenge in expressed protein ligation (EPL),[15] where installation of protected Cys residues in Ubl proteins would require specialized expression methods. In contrast, thanks to selenium's unique chemical properties, γ/δ-selenolysine (Se-Lys, Sek) could be used in an ICL/deselenization approach in the target protein, without the risk of desulfurizing natural Cys residues.[19]

Here we report the synthesis and use of γ-Se-Lys as a new tool for traceless isopeptide bond formation. The design and synthesis of δ-Se-Lys, which could not be completed due to spontaneous Se-elimination, is described in the SI (Scheme S1).

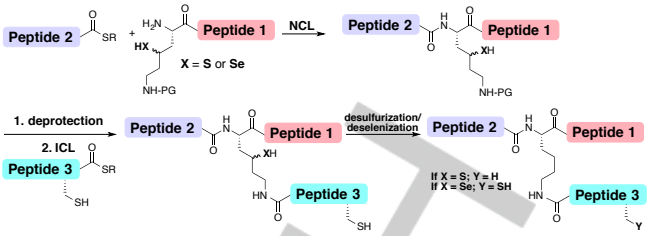
To obtain a protected form of γ-Se-Lys suitable for SPPS, we turned to work by Merkx *et al.*, where an Fmoc-Lys[S^{γ}(*S*t**Bu**)-N^{ε}(Boc)]-OH was successfully synthesized from γ-chlorolysine.[20] We opted to follow the previous protocol until selenium incorporation, protecting the α-amino and carboxylate groups of γ-chlorolysine with 9-BBN and then the ε-NH_2 group with Boc to give **2** (Scheme 2).



Scheme 2. Synthesis of Fmoc-Lys[Se^{γ}(Mob)-N^{ε}(Boc)]-OH

Nucleophilic substitution of Cl to Se in DMF was performed using Na_2Se_2, generated *in situ* from Se powder, hydrazine monohydrate, and NaOH.[21] The reaction gave the γ-diselenide product **3** in 42% yield. A reduction with $NaBH_4$ and protection with *p*-methoxybenzyl chloride (Mob-Cl or PMB-Cl) gave **4** in 60% yield. Finally, removal of 9-BBN and subsequent Fmoc-protection gave the desired Fmoc-Lys[Se^{γ}(Mob)-N^{ε}(Boc)]-OH, **6**, in 77% yield (Scheme 2).

Following our successful synthesis of a protected Se-Lys building block for SPPS, we set out to design an analog with an orthogonal protecting group at the ε-NH_2 site to allow for dual chemical ligation. In the proposed application, Se-Lys with an orthogonal protection at ε-NH_2 would first be used in NCL along the main peptide chain. After NCL, the protecting group of ε-NH_2 would be removed to allow for a second ligation, forming an isopeptide bond with the desired PTM protein-thioester. Final deselenization would give native Lys at the PTM site (Scheme 3). Disappointingly, using γ-chlorolysine, we were unsuccessful in incorporating an orthogonal protecting group on the ε-NH_2. Instead, an alternative route was pursued from known aldehyde **10**, which can be obtained from *L*-aspartic acid precursor in 3 steps (Scheme 4).[22] In this synthesis, the aldehyde **10** was treated with methyl (triphenylphosphoranylidene)acetate to afford the α,β-unsaturated ester **11** (85% for 2 steps).[23] Michael addition on **11** with Mob-selenolate (generated *in situ* from (Mob- Se-)$_2$ and $NaBH_4$)[24] gave compound **12** in 90% yield.

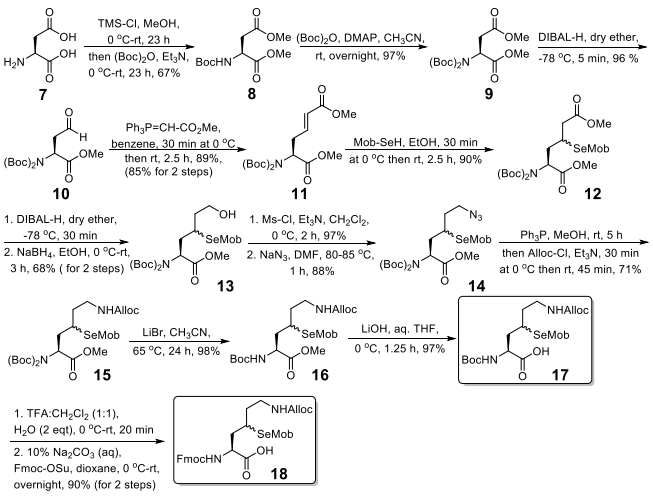


Scheme 3. γ-thiolysine[18] and γ-selenolysine as tools for dual NCL and ICL at Lys. Selenolysine has the advantage of allowing chemoselective deselenization in the presence of unprotected Cys residues.

The less sterically hindered ester in **12** was selectively converted to an aldehyde with DIBAL-H in dry ether at -78 °C, which was treated with $NaBH_4$ in EtOH to give alcohol **13** (68% for 2 steps). The hydroxyl group in **13** was converted to mesylate using MsCl and Et_3N in CH_2Cl_2 followed by treatment with NaN_3 in DMF at 80 °C, which afforded the azide compound **14** in 88% yield. Reduction of azide **14** with Ph_3P in MeOH, followed by treatment with Alloc-Cl and Et_3N, gave an Alloc-protected amine **15** (71%). Conversion of *di*-Boc (**15**) to *mono*-Boc (**16**) with LiBr[25] was carried out in CH_3CN at 65 °C for 24 h in 98% yield. Finally, hydrolysis of methyl ester with LiOH[17a] in aqueous THF gave the carboxylic acid **17** in 97% yield (**17** can be used in Boc-SPPS). Compound **17** was treated with TFA in CH_2Cl_2 to give an amine-salt, which was finally converted to Fmoc-protected amino acid **18** using Fmoc-OSu and aqueous Na_2CO_3 in 90% yield. The final product, Fmoc-Lys[Se^{γ}(Mob)-N^{ε}(Alloc)]-OH, was achieved in 17% overall yield for 13 steps of synthesis (Scheme 4).

Following successful main-chain NCL, the Alloc protecting group can be removed off-resin with a ruthenium catalyst[26] and the resulting peptide can be subjected to isopeptide ligation. Studies showing the utility of Fmoc-Lys[N^{ε}(Alloc)-Se^{γ}(Mob)]-OH in dual chemical ligation are currently underway.

To assess the utility of γ-Se-Lys in protein semi-synthesis, we incorporated our first-generation Se-Lys **6** (Scheme 2) into post-translationally modified human glucokinase (GCK). Prior studies demonstrated that the pancreatic isoform of this key glucose



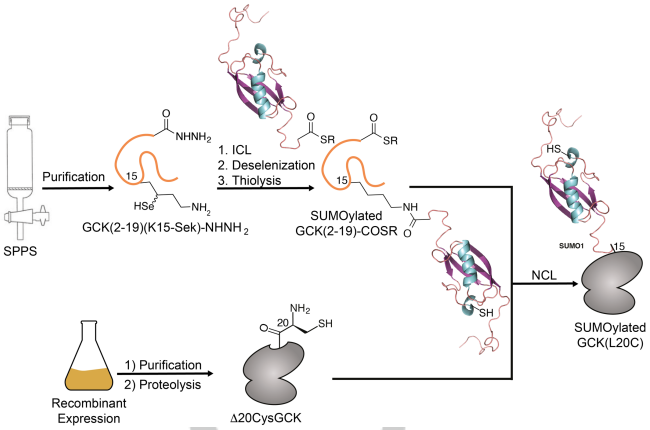
Scheme 4. Synthesis of Fmoc-Lys[Se^{γ}(Mob)-N^{ε}(Alloc)]-OH.

homeostatic enzyme is modified by the small ubiquitin-like modifier protein, SUMO-1, at one or more lysine residues near the N-terminus.[27] Unfortunately, the structural and functional impact of site-specific GCK SUMOylation has been difficult to assess due to an inability to produce homogenously SUMOylated GCK. We viewed the SUMO-1-GCK conjugate as an attractive target, since both proteins contain unprotected cysteine residues, and thus require the unique chemical properties of γ-Se-Lys.

We devised a three-segment strategy to obtain semisynthetic SUMOylated human GCK (Scheme 5). First, SPPS was used to incorporate our Fmoc-protected γ-Se-Lys **6** at residue 15*,[28] within a peptide consisting of the first 18 residues of GCK, GCK(2-19)-$NHNH_2$ (Scheme 5, Figure S1). This peptide also contained a C-terminal masked thioester for downstream use in NCL.[29] Next, SUMO-1 was produced as a C-terminal fusion with the MxeGyrA C-terminal intein (Figures S2 and S3),[30] which was subsequently converted to its 2-mercaptoethane sulfonic acid thioester (SUMO-1-Mes) for ICL (Figure S4, details and method in SI). Following ICL, the product underwent a one-pot deselenization/thioesterification (Figure S5), giving an overall yield of 12.3% of the GCK(2-19) segment linked to SUMO-1 via an isopeptide bond at Lys15 (Scheme 5, Figure 1).

We expected the ligation step to proceed efficiently, as the size of the amino acid adjacent to the thioester typically dictates the rate of the ligation reaction.[31] SUMO-1 contains a desirable Gly-Gly sequence adjacent to the thioester. However, the ligation step was surprisingly slow. SUMO-1-Mes was converted to the more reactive SUMO-1-MPAA thioester within 1 hour. Yet, ligation of this adduct with the small GCK peptide took up to 22 hours to complete despite a large excess of the peptide and the presence of TCEP and sodium ascorbate, which are known to accelerate the selenium-mediated NCL reaction.[19b, 32] It is unlikely that the γ-position of the selenol contributes to a slower rate of ligation, based on a previous report indicating that this has no impact on ligation rate.[33] Instead, a direct comparison of isopeptide ligation in the synthesis of diubiquitin with γ- and δ-thiolysine also showed a slower-than-expected rate of ligation.[20] In fact, the pK_a of the peptide's amino groups is likely to blame, as the N-terminal α-amino group of a peptide has an average pK_a of 7.7[34], while the ε-amino group of Lys has a pK_a of 10.5[35], making it significantly less reactive at pH 7, the optimized pH for NCL. Together, these observations suggest that the inefficiency lies in the nature of isopeptide bond formation, as the first, typically rate-determining nucleophilic attack in ICL is performed by the less reactive ε-amine.

Due to the slow rate of ligation, a significant portion of SUMO-1-MPAA underwent hydrolysis to inactive SUMO-1-OH rather than to the desired ligation. As a result, the maximum yield we observed was 22% for the ligation step, which severely limited the expected yield of the overall strategy. Notably, for the subsequent deselenization reaction, the unprotected, native Cys52 of SUMO-1 was unaffected by this procedure[16a] and unmasking of the



Scheme 5. Proposed synthesis of the semisynthetic SUMOylated GCK.

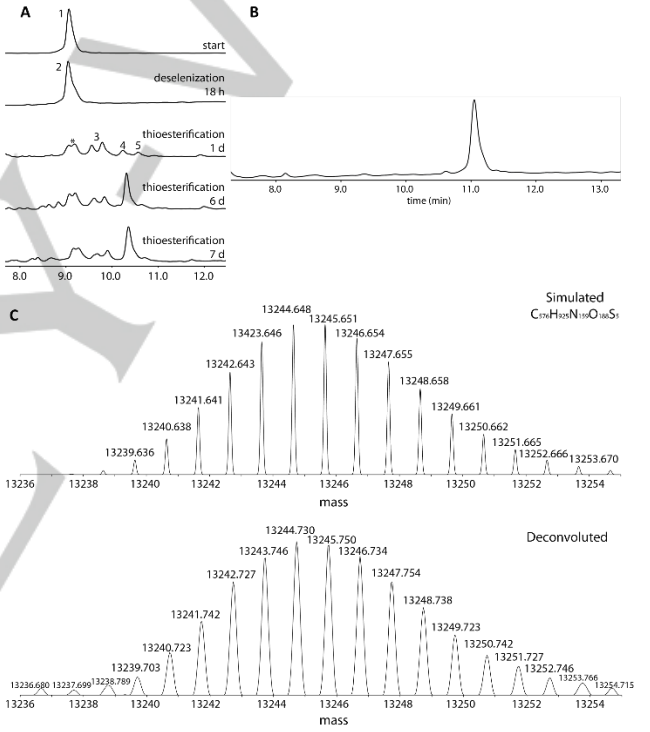


Figure 1. A. HPLC traces of one-pot SUMO-1-GCK deselenization and thioesterification over time. **1** is SUMO-1-GCK(2-19)(K15Sek)-$NHNH_2$, **2** is SUMO-1-GCK(2-19)-$NHNH_2$, **3** shows two peaks, both with the mass of the first intermediate in thioesterification (Schiff base),[29b] **4** is the final SUMO-GCK(2-19)-MPAA, and **5** is the second intermediate in thioesterification (Knorr pyrazole).[29b] * shows a mass that most likely is truncated protein from expression of the intein, which coeluted with all intermediate products. The slow rate of thioesterification is attributed to a C-terminal Ile[31]; **B**. HPLC trace for the

* Using online analysis programs such as SUMOplot™ or SUMOsp 2.0, which predict possible SUMOylation sites, K12 and K15 are predicted as likely SUMOylation sites in the N-terminus of pancreatic GCK.[27b, 28a] The first site, K12, is part of SUMO consensus motif ψ-K-x-D/E, where ψ is a large hydrophobic amino acid, K is the lysine at which the SUMO is linked, x is any amino acid and D/E is an acidic amino acid (Asp or Glu, respectively).[28c] The second SUMOylation site is K15, which is not part of a typical SUMO consensus motif. Both analysis programs did not predict K13 as a possible SUMOylation site. In early crystal structures of GCK (PDB entries 1V4S, 1V4T)[28b] the N-terminus was truncated to facilitate crystallization. In these structures the N-terminal region of GCK, which begins with Lys12, adopts an α-helix.[28d] Although K12, K13 and K15 are adjacent residues, each residue would face a different environment as part of the α-helix, with some inaccessible to the SUMOylation system. Indeed, based on a more recent crystal structure (PDB entry 3IDH),[28d] only K15 is solvent exposed, while K12 and K13 are on the opposite face of the helix and make electrostatic and hydrogen bonding interactions with the rest of the protein. Based on these observations, we initially focused on Lys15 as the SUMOylation site in pancreatic GCK; however, our method should be applicable to Lys12 or any Lys residue in a protein sequence.

final product SUMO-GCK(2-19)-MPAA, and **C**. Overlay of the simulated HR-MS (above) and deconvoluted HR-MS (below).

peptide's cryptic thioester provided a segment poised for NCL. As a final step in the preparation of SUMOylated human GCK, we recombinantly produced a human GCK variant containing a Factor Xa recognition sequence directly upstream of a Cys at position 20 (Scheme 5, and Figure S6). Following purification and Factor Xa digestion, this procedure yielded a truncated, N-terminal Cys containing GCK variant, Δ20CysGCK, that displayed wild-type kinetic characteristics (Figure S7). Unfortunately, efforts to perform NCL between Δ20CysGCK and the SUMO-1-GCK(1-19)-COSR segment proved unsuccessful. We observed no ligation after prolonged incubation of the reaction components in the absence of denaturants, whereas inclusion of guanidium hydrochloride in the ligation buffer caused extensive precipitation of the protein, as has been reported earlier.[36] Nonetheless, we expect that the methods presented here will provide a route towards Cys-rich Ubl-protein conjugates. Further exploration of this topic could include an investigation of bacterial expression using the orthogonal tRNA synthetase/tRNA pairs, as has been done for thiol-containing lysine analogs.[37]

In summary, lysine, often a site of ubiquitylation, SUMOylation, and other Ubl protein PTM conjugations, was designed with an artificial γ-selenol moiety. The resulting amino acid was successfully used for traceless isopeptide ligation of SUMO-1 on a peptide of its natural target protein, human glucokinase. Additionally, a selenolysine analog, possessing orthogonal protecting groups to be used in traceless dual chemical ligation, was designed and synthesized. Selenolysine presents an attractive building block in the synthesis of post-translationally modified proteins.

Acknowledgements

We thank the Dr. Tsafi Danieli, Dr. Mario Lebendiker, and Dr. Yael Keren for their assistance in SUMO-1-COSR expression and purification in the Wolfson Centre for Applied Structural Biology at HUJI. Thanks to Dr. Bill Breuer of the Mass Spectrometry Unit at the Institute of Life Sciences, HUJI for assistance with HR-MS. We wish to thank Prof. Dr. Huib Ovaa (Leiden University Medical Center, The Netherlands) for the generous gift of chlorolysine. RND was supported by the Kaete Klausner Ph.D. scholarship, SK was supported by PBC fellowship for postdocs, NM acknowledges the support of ISF (783/18) and the US-Israel Binational Science Foundation (BSF) (2014167), and BGM acknowledges the support of the NIH (GM115388).

Keywords: Chemical protein synthesis • post-translational modification • deselenization • expressed protein ligation • SUMOylation

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10.1002/chem.202000310

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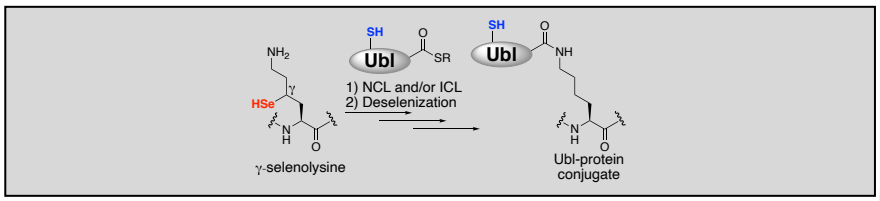

Insert text for Table of Contents here. The design and synthesis of γ-selenolysine, a selenol-containing form of the commonly modified proteinogenic amino acid, lysine is reported. The utility of γ-selenolysine is demonstrated with the traceless ligation of the small ubiquitin-like modifier protein, SUMO-1, to a peptide segment of human glucokinase.