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Isopeptide bond formation mediated by δ -selenolysine for chemical ubiquitination

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Protein ubiquitination is involved in nearly all biological processes in Eukaryotes. To gain precise insights into the function of ubiquitination in these processes, researchers frequently employ ubiquitinated protein probes with well-defined structures. While chemical protein synthesis has afforded a variety of ubiquitinated protein probes, there remains a demand for efficient synthesis methods for complex probes, such as ubiquitinated glycoproteins and ubiquitinated cysteine-containing proteins. In this study, we introduce a new method to obtain ubiquitinated proteins through isopeptide bond formation mediated by δ -selenolysine residues. We synthesized δ -selenolysine derivatives in both L- and D-forms starting from DL- δ -hydroxy-DL-lysine, accomplished by substituting the δ -mesylate with KSeCN and by enzymatic optical resolution with L- and D-aminoacylase. We synthesized ubiquitin (46–76)- α -hydrazide with a δ -seleno-L-lysine residue at position 48, as well as ubiquitin (46–76)- α -thioester, using solid-phase peptide synthesis. Subsequently, the δ -selenolysine-mediated ligation of these peptides, followed by one-pot deselenization, provided the desired isopeptide-linked ubiquitin peptide. The new δ -selenolysine-mediated isopeptide bond formation offers an alternative method to obtain complex ubiquitin- and ubiquitin-like probes with multiple post-translational modifications. These probes hold promise for advancing our understanding of ubiquitin biology.

KEYWORDS

ubiquitin, δ -selenolysine, enzymatic optical resolution, isopeptide bond, peptide ligation, deselenization

1 Introduction

Protein ubiquitination is a major post-translational modification in Eukaryotes and is involved in nearly all biological processes, i.e., protein degradation, DNA repair, and cell signaling. Hence, unraveling its precise role in health and disease is important (Chaugule and Walden, 2016; Swatek and Komander, 2016). Ubiquitin (Ub) is a 76-mer protein found in almost all Eukaryotes. In protein ubiquitination, the C-terminal carboxylate of Ub is attached to the ϵ -amino group of the lysine residue in the acceptor protein through an isopeptide bond. Ub can form homopolymers, heteropolymers, and branched polymers through its seven Lys residues and one N-terminal Met. This structural diversity allows the formation of the “Ub code” (Komander and Rape, 2012; Swatek and Komander, 2016).

To gain precise insights into the complex Ub codes formed by diverse polyUb chains, researchers frequently employ chemical probes with polyUb chains having a defined linkage

attached to a defined position of the acceptor protein (Mulder et al., 2020). Certain polyUb chains with defined linkage and length can be prepared using specific E2 or E3 enzymes. However, this method has some limitations, e.g., polyUb chains attach to the acceptor protein (Faggiano et al., 2016). Chemical protein synthesis has been extensively developed to overcome these limitations (Mali et al., 2017; Gui et al., 2021; Huppelschoten and van der Heden van Noort, 2022). Three strategies have been developed for the chemical synthesis of native isopeptide bonds: Auxiliary mediated ligation, first reported by Muir (Chatterjee et al., 2007); δ -mercaptolysine- (Kumar et al., 2009) or γ -mercaptolysine- (Yang et al., 2009; Merkx et al., 2013) mediated ligation; and isoUb ligation (Tang et al., 2017).

δ -Mercaptolysine-mediated ligation has been extensively used for the synthesis of diUb chains (Kumar et al., 2010), tetra-ubiquitinated peptides (Bavikar et al., 2012), polyubiquitinated α -synuclein (Haj-Yahya et al., 2013), and ubiquitinated and glycosylated H2B (Seenaiah et al., 2015). We also used δ -mercaptolysine-mediated ligation for the synthesis of ubiquitinated glycoprotein CCL1 in different folding states (Izumi et al., 2020). These reports demonstrate the usefulness of δ -mercaptolysine-mediated ligation for the synthesis of complex ubiquitinated protein probes. However, this approach requires subsequent desulfurization after isopeptide bond formation, necessitating the orthogonal protection of cysteine side-chain thiols to prevent undesired desulfurization. Unfortunately, the efficiency and overall yield of this process are compromised by additional deprotection and HPLC purification steps.

Metanis reported that deselenization with tris(2-carboxyethyl) phosphine (TCEP) proceeds selectively in the presence of free cysteine thiol without concomitant desulfurization (Metanis et al., 2010; Reddy et al., 2016). To avoid the risk of desulfurizing native Cys residues, γ -selenolysine-mediated ligation was developed (Dardashti et al., 2020). In the same study, they also attempted the synthesis of a δ -selenolysine derivative from L-glutamic acid. However, they could not obtain the desired product with good yields and purity. Here, we report the synthesis of δ -selenolysine derivative in both L- and D-forms and its use in the formation of traceless isopeptide bonds. We sought to prepare δ -selenolysine in both L- and D-forms because doing so will allow access to the ubiquitinated protein in both its L- and D-forms (Lander et al., 2023), which is required for racemic protein crystallography (Yeates and Kent, 2012; Yan et al., 2017). Although the quasi-racemic and monomer/oligomer quasi-racemic X-ray structures of diUb and triUb were reported (Pan et al., 2016; Wang et al., 2016), true racemic protein crystallography will be more useful for elucidating the X-ray structures of ubiquitinated proteins.

2 Results and discussion

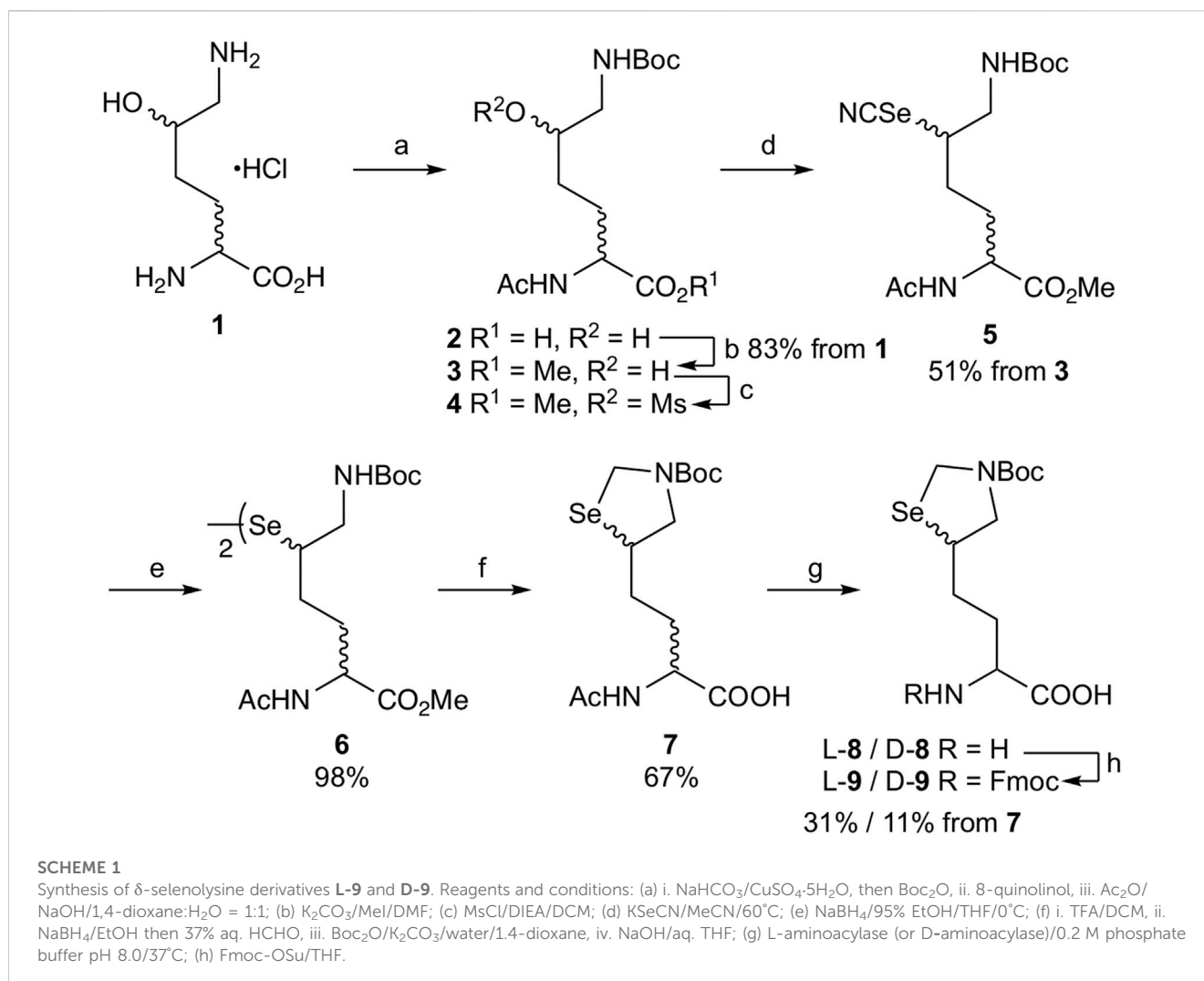
2.1 Synthesis of δ -selenolysine derivatives L-9 and D-9

To synthesize the δ -seleno-L-lysine derivative suitably protected for solid-phase peptide synthesis, we turned our attention to the synthesis route of δ -mercapto-L-lysine derivatives. Kumar et al. reported the synthesis starting from L-glutamic acid (Kumar et al., 2009; Haj-Yahya et al., 2010), while Virdee et al. used δ -hydroxy-L-lysine as a starting

material (Virdee et al., 2011). We decided to examine whether commercially available racemic DL- δ -hydroxy-DL-lysine can be employed to synthesize the δ -selenolysine derivative in both its L- and D-form after optical resolution.

To employ L- or D-aminoacylase for enzymatic optical resolution (Morishita et al., 2015), the α -amino group should be protected with an acetyl group (Scheme 1). Selective protection of the ϵ -amino group of DL- δ -hydroxy-DL-lysine hydrochloride **1** was achieved by forming a copper (II) complex and then reacting it with Boc₂O (Virdee et al., 2011). Acetylation of the α -amino group with acetic anhydride and sodium hydroxide yielded acetamide **2**, which was subsequently treated with iodomethane for 3.5 h under basic conditions to obtain methyl ester **3** in 83% yield from **1**. Prolonging this reaction (5 h) afforded δ -lactone as a byproduct along with methyl ester **3**. In such a case, δ -lactone can be easily converted back to **3** by treating the resulting mixture with sodium methoxide. Next, δ -alcohol **3** was treated with MsCl to produce δ -mesylate **4** for the substitution reaction. Reacting **4** with potassium selenocyanate in acetonitrile at 60°C for 42 h yielded δ -selenocyanate **5** in 51% yield in two steps (Malins and Payne, 2012). Selenocyanate **5** was converted to diselenide **6** (98% yield) by treatment with NaBH₄. Then, diselenide **6** was converted to carboxylate **7** by the following reaction sequence: the Boc group was removed by treatment with TFA. The diselenide bond was reduced by NaBH₄, and the resulting 1,2-aminoselenol group was protected as selenazolidine by treatment with 37% HCHO under acidic pH, and the ϵ -imino group was protected again with the Boc group to obtain methyl ester. However, undesired partial hydrolysis of methyl ester was observed during this reaction sequence, and the isolation of methyl ester reduced the overall yield. Thus, the crude product was treated with aqueous NaOH, and carboxylate **7** was obtained in 67% yield from **6** after purification via silica gel column chromatography.

Enzymatic optical resolution using L-aminoacylase was examined using racemic DL- δ -seleno-DL-lysine **7**. Racemic **7** was treated with L-aminoacylase in phosphate buffer at pH 8, and the reaction progress was monitored by LC/MS. As shown in Figure 1A, a new peak corresponding to the desired deacetylated compound L-**8** was observed. However, the peak of **7** did not disappear after 21 h. Fmoc-OSu was added to the reaction mixture to produce Fmoc-protected L-**9** in 31% isolated yield from racemic **7**. We also examined the enzymatic optical resolution of racemic **7** by D-aminoacylase. This reaction also resulted in two peaks: one corresponding to the deacetylated product D-**8** and the other to remaining **7** (Figure 1B). Treatment with Fmoc-OSu produced D-**9** in 11% isolated yield from racemic **7**. We speculate that the small-scale reaction resulted in a lower yield of D-**9** than that of L-**9**, because the LC/MS analysis results suggested that the enzymatic deacetylation of **7** by L-aminoacylase and D-aminoacylase proceeded to a similar degree. The **7** remaining after hydrolysis with D-aminoacylase was isolated and treated with L-aminoacylase. The HPLC peak corresponding to **7** weakened, while the L-**8** peak appeared. However, the peak corresponding to **7** did not disappear even after 18 h (Supplementary Figure S1A). The results suggest that enzymatic hydrolysis did not proceed to completion. Compound **7** was also not completely hydrolyzed by 24-h treatment with L-aminoacylase followed by additional 24-h treatment with D-aminoacylase (Supplementary Figure S1B). These results suggest that the remaining **7** after each enzymatic hydrolysis is

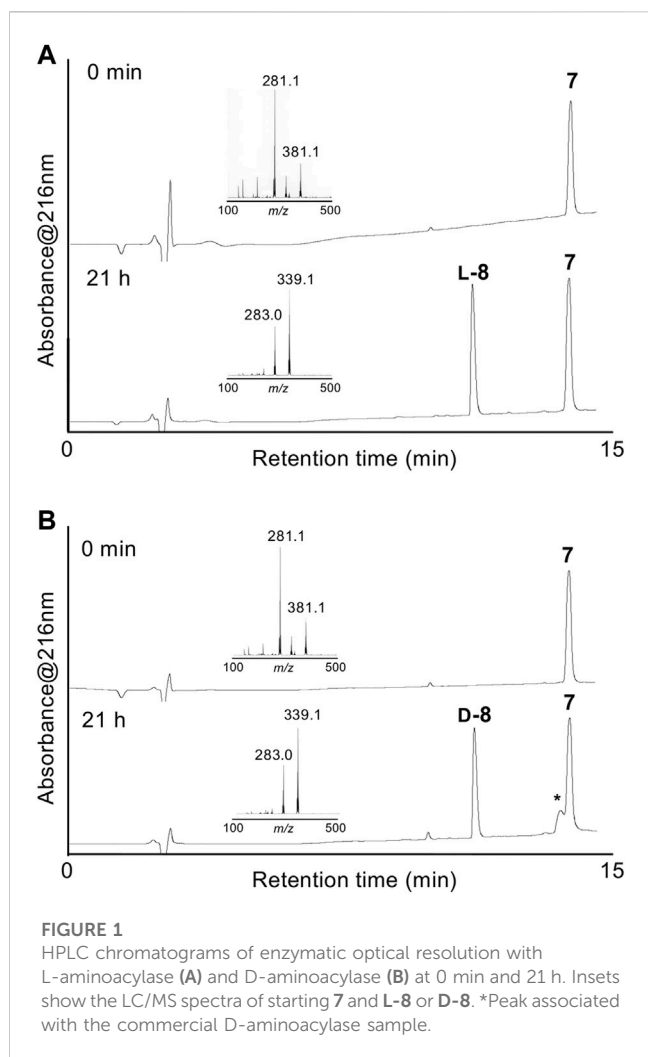


not optically pure. Thus, an efficient approach for obtaining both **L-9** and **D-9** could involve recovering the unreacted **7** from the L-aminoacylase reaction mixture and utilizing it as a substrate for D-aminoacylase.

To determine the absolute configuration at the α -position of synthetic **L-9** and **D-9**, pentapeptide FKPSD was synthesized by Fmoc-solid-phase peptide synthesis (SPPS) using both **L-9** and **D-9** (Supplementary Scheme S1) (Kumar et al., 2009). Pentapeptides containing a δ -selenolysine residue **L-10** and **D-10** were treated with $\text{MeONH}_2 \cdot \text{HCl}$ to convert them to diselenides **L-11** and **D-11**, which were then deselenized by treatment with TCEP to yield pentapeptides **L-12** and **D-12**, respectively. We also synthesized pentapeptides F(L-K)PSD and F(D-K)PSD using commercial Fmoc-L-Lys(Boc) and Fmoc-D-Lys(Boc), respectively, to compare them with **L-12** and **D-12**. The retention time of each pentapeptide on reversed-phase (RP) HPLC confirmed that **L-12** is F(L-K)PSD and **D-12** is F(D-K)PSD (Supplementary Figure S2). HPLC analyses did not show any F(D-K)PSD or F(L-K)PSD peak from the pentapeptide sample synthesized using **L-9** or **D-9**, respectively. Hence, it can be suggested that the optical purity of the α -position of **L-9** and **D-9** is high enough to be used in the SPPS (Supplementary Figure S2).

2.2 δ -Selenolysine-mediated isopeptide bond formation

We next examined the formation of isopeptide bonds using Ub (46–76) peptide fragment and ligation position at Lys48. We selected Lys48 of Ub as a linkage position because protein degradation through the ubiquitin-proteasome system mediated by Lys48-linked polyUb is well-studied (Hershko and Ciechanover, 1998). In addition, the Ub (49–76) peptide resin can be used as a common intermediate for the synthesis of both proximal and distal Ub (46–76) fragments, Ub (46C^{AcM}–48SeK–76) and Ub (46C^{AcM}–76), respectively (Scheme 2). The use of the Ub (46–76) peptide fragment with the mutation of Ala46 to Cys allows the fabrication of a full-length Ub polypeptide by native chemical ligation–desulfurization with Ub (1–45)- α -thioester (Erlich et al., 2010). Side-chain thiol of the N-terminal Cys was protected with an acetamidomethyl (AcM) group. Although SPPS of a full-length Ub chain is already reported (El Oualid et al., 2010; Bavikar et al., 2012), synthesizing such chains containing δ -selenolysine in their middle does not seem practical. We synthesized both the distal and proximal Ub (46–76) peptide fragments as peptide hydrazide, because, after isopeptide bond formation, the C-terminal hydrazide of the isopeptide can be

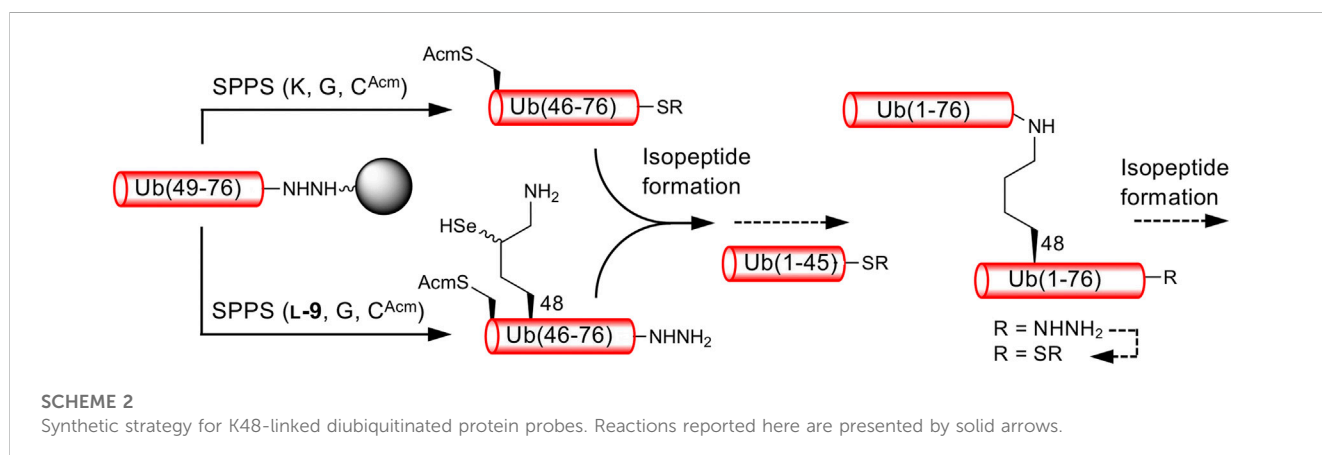


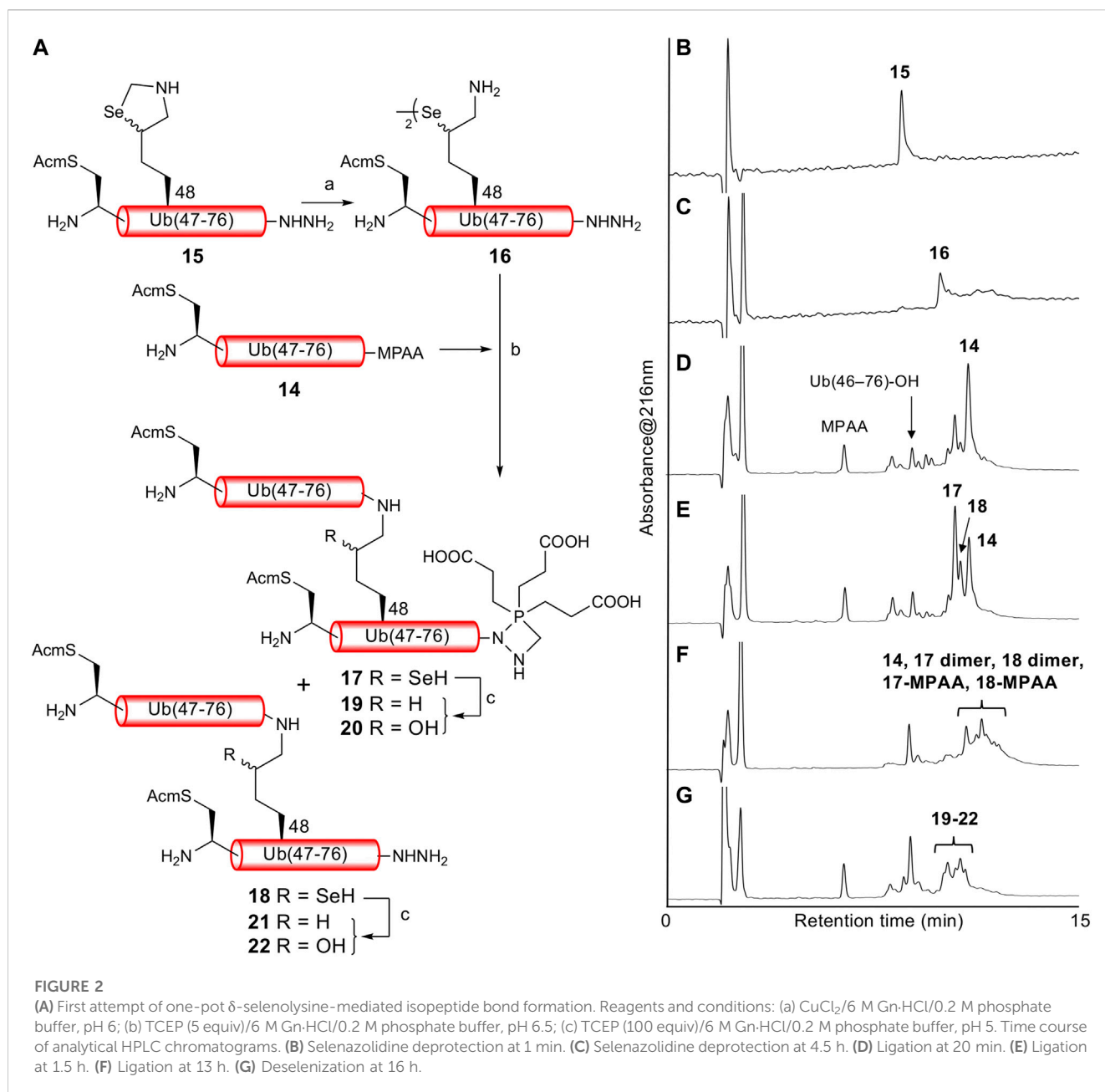
converted to thioester and further used for the chemical polyubiquitination of the acceptor protein (Scheme 2).

Ub (46C^{Acm}-76)- α -hydrazide was synthesized using Fmoc-SPPS on the Fmoc-hydrazine 2-chlorotrityl resin (Huang et al., 2014). To synthesize the 31-mer peptide on the polystyrene-based 2-chlorotrityl resin, the loading of the first amino acid was adjusted to 0.4–0.5 mmol/g and the coupling of the amino acid

was carried out using Fmoc-AA (10 equiv) with DIC/OxymaPure in NMP at 60°C for 20 min (Bacsa et al., 2008). Dipeptides Fmoc-Leu-Ser(ψMe, Meprō) and Fmoc-Asp(OtBu)-(Dmb)Gly were used at positions 56–57 and 52–53, respectively, according to the assembly of a full-length Ub chain. Boc-Cys(Acm) was coupled at the N-terminus. Global deprotection and cleavage by treatment with TFA/TIPS/water (95/2.5/2.5) yielded the desired Ub (46C^{Acm}-76)- α -hydrazide 13 in 17% yield after RP-HPLC purification using a C4 column (Supplementary Figure S3). Then, the peptide-hydrazide 13 was converted to thioester 14 in 70% yield by treatment with acetylacetone and 4-mercaptophenylacetic acid (MPAA) at pH 3 (Supplementary Figure S4) (Flood et al., 2018). The Ub (46–76) peptide fragment containing a δ -seleno-L-lysine residue at position 48 was synthesized by a similar procedure. Coupling of L-9 (2 equiv) with the Ub (49–76)-peptide resin was performed with DIC/OxymaPure at 60°C for 20 min. The coupling was performed twice to ensure the incorporation of L-9 into the resin-bound peptide. After the completion of peptide elongation, global deprotection and cleavage were performed using TFA/TIPS/water/MeONH₂-HCl (94/2.5/2.5/0.2) (Dardashti et al., 2020). The desired Ub (46C^{Acm}-48K^{Sez}-76)- α -hydrazide 15 was obtained in 10% yield after RP-HPLC purification (Supplementary Figure S5). We also attempted to synthesize Ub (46C-48K^{Sez}-76)- α -hydrazide with an N-terminal free cysteine residue, which can be first ligated with Ub (1–45)- α -thioester and then used for δ -selenolysine-mediated isopeptide bond formation. However, a selenosulfide bond was formed between Cys46 and δ -selenoLys48 residues, suggesting that selenazolidine is unstable at neutral pH when a free thiol group is present in the same peptide. Protecting selenols in δ -selenolysine with stable protecting groups such as methoxybenzyl might be helpful in avoiding undesired deprotection.

With both Ub (46C^{Acm}-76)- α -thioester 14 and Ub (46C^{Acm}-48K^{Sez}-76)- α -hydrazide 15 in hand, we examined the δ -selenolysine-mediated isopeptide bond formation. We investigated the one-pot copper-mediated selenazolidine deprotection and native chemical ligation conditions (Zhao and Metanis, 2019). In our first attempt (Figure 2; Supplementary Figure S6), we initially treated selenazolidine 15 with CuCl₂ at pH 6 to produce diselenide 16 and then added thioester 14 and TCEP to the solution. The desired ligated product 18 was observed after 1.5 h, along with 17 (+262 Da) as a major byproduct. We subsequently added TCEP and adjusted the pH to 5 for deselenization. The LC/MS analysis results showed

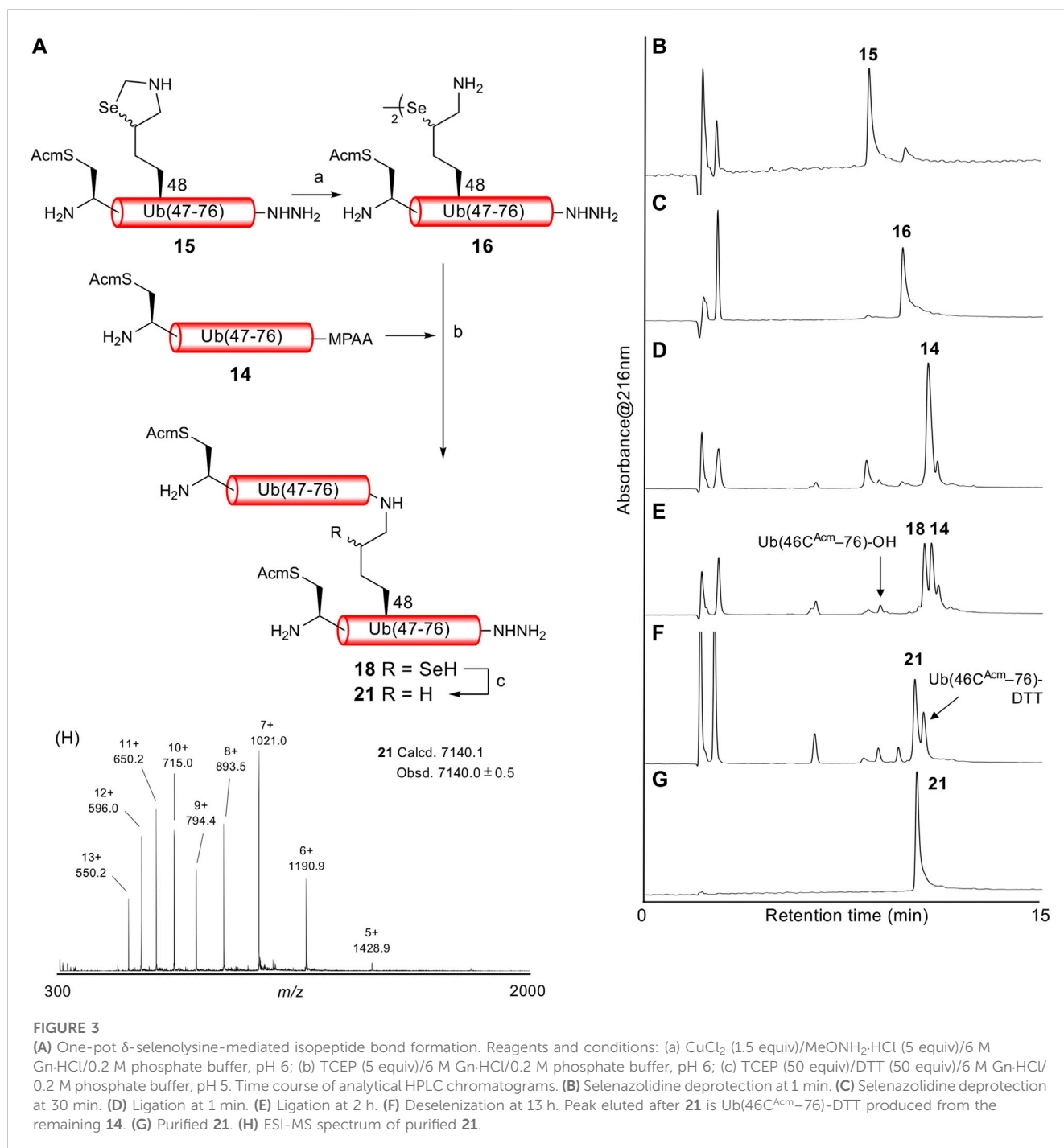




the formation of the desired deselenized product **21**; however, deselenized **19** (+262 Da) was still a major byproduct. Oxidized byproducts **20** and **22** were also formed because of insufficient degassing of the reaction buffer. We speculated that the major byproduct with a molecular weight of +262 Da was formed by a reaction with formaldehyde released from selenazolidine and TCEP with a C-terminal hydrazide. To confirm this speculation, selenazolidine **15** was deprotected by treatment with CuCl_2 and the liberated formaldehyde was removed by solid-phase extraction. Then, diselenide **16** was ligated with thioester **14**. This stepwise synthesis did not yield any byproduct of molecular weight +262 Da, suggesting that formaldehyde was involved in the formation of this byproduct (data not shown). Finally, we performed one-pot selenazolidine deprotection– δ -selenolysine-mediated ligation–deselenization (Figure 3; Supplementary Figure

S7). First, selenazolidine **15** was treated with CuCl_2 for 30 min in the presence of $\text{MeONH}_2\text{-HCl}$ as a scavenger of formaldehyde to yield diselenide **16**. Then, thioester **14** and 5 equiv of TCEP were added to perform ligation for 2 h. Deselenization was performed by adding 50 equiv of both TCEP and DTT at pH 5 for 13 h (Wang et al., 2020). The desired isopeptide Ub [46C^{Acm}–48K{Ub (46C^{Acm}–76)}–76]– α -hydrazide **21** was obtained in 53% yield and in good purity after RP-HPLC purification. The structure of **21** was confirmed by trypsin digestion (Supplementary Figure S8). The LC/MS analysis results confirmed the formation of the desired isopeptide fragment. Hence, it can be suggested that the formation of isopeptide bonds involves a δ -selenolysine residue at position 48.

The reaction time of δ -selenolysine-mediated ligation (2 h) was much shorter than that of the γ -selenolysine-mediated ligation (22 h) (Dardashti et al., 2020). However, these two reactions cannot be



compared directly because the sequence of peptides containing γ/δ -selenolysine residues and peptide thioesters used are different, although both have Gly-Gly at the C-terminus. Additionally, we used an excess amount of highly reactive MPAA-thioester to push the reaction to completion in a short period of time because, in the selenium-mediated ligation, a longer reaction time results in the formation of inactive diselenide, which slows down the reaction. We assume that the faster reaction of δ -selenolysine-mediated ligation is not because of the five-membered ring transition rather than the six-membered ring transition because γ - and δ -mercaptolysine-mediated ligation proceeds at a similar rate (Merkx et al., 2013).

3 Materials and methods

For general experimental and instrumental methods, and full compound characterization, see the [Supplementary Material S1](#).

3.1 *N*- α -acetyl-*N*- ϵ -(*tert*-butoxycarbonyl)- δ -DL-hydroxy-DL-lysine methyl ester (**3**)

Crude *N*- ϵ -(*tert*-butoxycarbonyl)- δ -DL-hydroxy-DL-lysine (2.64 g), prepared from δ -DL-hydroxy-DL-lysine hydrochloride

1 (2.00 g, 10.1 mmol) by Chin's procedure (Virdee et al., 2011), was dissolved in a mixture of water and 1,4-dioxane (1:1, v/v, 13.5 mL). Ac₂O (1.9 mL, 20.1 mmol) was divided into three portions and added to the solution. The pH of the resulting solution was adjusted each time to 11 with 5 M NaOH. After adding all Ac₂O, the solution was carefully neutralized with 1 M HCl and concentrated *in vacuo*. The residue was desalted by chromatography on C18 silica gel (washed with water and then eluted with 15% aqueous MeCN) to yield *N*-α-acetyl-*N*-ε-(*tert*-butoxycarbonyl)-δ-DL-hydroxy-DL-lysine **2** (3.52 g) as a colorless syrup.; HR-ESI-FTMS *m/z* calcd for C₁₃H₂₄N₂O₆ [M + Na]⁺ 327.1527, found: 327.1527; ¹H NMR (400 MHz, CD₃OD) δ 4.30–4.21 (m, 1H), 3.67–3.53 (m, 1H), 3.17–3.07 (m, 1H), 3.07–2.94 (m, 1H), 2.00 (s, 3H), 1.96–1.50 (m, 4H), 1.45 (s, 9H); ¹³C NMR (100 MHz, CD₃OD) δ 177.8, 177.7, 171.12, 171.08, 157.2, 78.6, 70.3, 69.8, 54.9, 54.5, 46.0, 30.6, 30.4, 28.8, 28.7, 27.4, 21.4.

To a solution of **2** (3.52 g) in DMF (100 mL), K₂CO₃ (3.22 g, 23.3 mmol) was added, and the mixture was stirred for 10 min at room temperature. Iodomethane (1.1 mL, 17.5 mmol) was added to the suspension, and the resulting suspension was stirred for 3.5 h at room temperature. The solid was filtered off, and the filtrate was concentrated *in vacuo*. The residue was concentrated again from toluene. The residue was dissolved in EtOAc and washed with brine. The organic layer was dried over MgSO₄, filtered, and concentrated. The residue was purified by silica gel chromatography (EtOAc) to yield **3** (2.65 g, 83% from **1**) as a colorless syrup.; HR-ESI-FTMS *m/z* calcd for C₁₄H₂₆N₂O₆ [M + Na]⁺ 341.1683, found: 341.1684; ¹H NMR (400 MHz, CDCl₃) δ 6.43, 6.36 (br, 1H), 5.00 (br, 1H), 4.66–4.58 (m, 1H), 3.75 (s, 3H), 3.71 (br, 1H), 3.31–3.21 (br, 1H), 3.09–2.97 (m, 1H), 2.03 (s, 3H), 2.00–1.75 (m, 2H), 1.57–1.43 (m, 1H), 1.45 (s, 9H); ¹³C NMR (100 MHz) δ 172.93, 172.89, 170.3, 170.1, 157.1, 156.9, 79.9, 79.7, 71.2, 71.0, 52.50, 52.45, 52.0, 51.8, 46.7, 46.6, 30.1, 30.0, 29.3, 28.6, 28.4, 23.16, 23.13.

3.2 *N*-α-acetyl-*N*-ε-(*tert*-butoxycarbonyl)-δ-DL-cyanoseleno-DL-lysine methyl ester (**5**)

A solution of **3** (2.23 g, 7.00 mmol) in DCM (30 mL) and DIEA (2.4 mL, 14 mmol) was cooled in an ice bath, and MsCl (0.651 mL, 8.4 mmol) was added dropwise. The solution was gradually warmed to room temperature and stirred for 2 h. The solution was diluted with DCM and washed with saturated aqueous NH₄Cl and brine, sequentially. The organic layer was dried over MgSO₄, filtered, and concentrated *in vacuo* to yield *N*-α-acetyl-*N*-ε-(*tert*-butoxycarbonyl)-δ-DL-methanesulfonyloxy-DL-lysine methyl ester **4** (2.90 g) as a syrup.; ESI MS calcd. for C₁₅H₂₈N₂O₈S [M + Na]⁺ 419.1, found 419.1; ¹H NMR (400 MHz, CDCl₃) δ 6.21 (br, 1H), 4.96 (br, 1H), 4.78–4.72 (m, 1H), 4.64–4.56 (m, 1H), 3.76 (s, 3H), 3.47–3.43 (m, 1H), 3.32–3.26 (m, 1H), 3.06 (s, 3H), 2.04 (s, 3H), 2.01–1.97 (m, 1H), 1.87–1.67 (m, 3H), 1.45 (s, 9H); ¹³C NMR (100 MHz) δ 172.4, 170.2, 156.1, 80.9, 80.1, 52.6, 51.8, 51.6, 43.8, 43.6, 38.5, 28.3, 28.22, 28.18, 28.0, 27.7, 23.1.

To a solution of **4** (2.90 g) in MeCN (40 mL), KSeCN (2.11 g, 14.6 mmol) was added, and the mixture was stirred for 42 h at 60°C. The solution was concentrated, and the residue was dissolved in DCM (15 mL) and poured onto water (30 mL). The aqueous layer was extracted three times with DCM. The combined organic layers were dried over MgSO₄, filtered, and concentrated *in vacuo*. The residue

was purified by silica gel chromatography (EtOAc:hexane = 2:1 then EtOAc) to yield **5** (1.45 g, 51%) as a syrup. HR-ESI-TOFMS *m/z* calcd for C₁₅H₂₅N₃O₅Se [M + Na]⁺ 430.0852, found 430.0868; ¹H NMR (400 MHz, CDCl₃) δ 6.65 (s, 1H), 5.54 (s, 1H), 4.70–4.56 (m, 1H), 3.77 (s, 3H), 3.65–3.48 (m, 2H), 3.43–3.30 (m, 1H), 2.18–2.05 (m, 1H), 2.04 (s, 3H), 2.00–1.68 (m, 3H), 1.45 (s, 9H); ¹³C NMR (100 MHz, CDCl₃) δ 172.4 (Ac), 170.3 (COOH), 156.1, 156.0 (Boc-CO), 100.8, 100.6 (SeCN), 80.2 (tBu-C), 52.7, 52.6 (OMe), 51.6, 51.4 (α), 49.9, 49.8 (δ), 45.6 45.1 (ε), 30.3, 29.5, 28.3 (β,γ), 23.0 (tBu).

3.3 *N*-α-acetyl-*N*-ε-(*tert*-butoxycarbonyl)-δ-DL-seleno-DL-lysine methyl ester dimer (**6**)

A solution of NaBH₄ (18 mg, 0.47 mmol) in 95% aqueous EtOH (0.5 mL) was added dropwise to a solution of **5** (94.4 mg, 0.23 mmol) in THF (3.5 mL) at 0°C. The solution was stirred for 15 min at 0°C, diluted with EtOAc (10 mL), and poured onto 1 M aqueous HCl (15 mL). The organic layer was separated, and the aqueous layer was extracted with EtOAc (3 × 10 mL). Combined organic layers were dried over MgSO₄, filtered, and concentrated. The residue was purified by silica gel chromatography (EtOAc) to yield **6** (86.6 mg, 98%) as a yellow oil.; ESI MS calcd. for C₂₈H₅₀N₄O₁₀Se₂ [M + Na]⁺ 785.2, found: 785.2; ¹H NMR (400 MHz, CDCl₃) δ 6.77–6.59 (m, 1H), 5.45–5.10 (m, 1H), 4.61–4.48 (m, 1H), 3.72 (s, 3H), 3.38–3.22 (m, 2H), 3.09–2.96 (m, 1H), 2.12–2.05 (m, 1H), 2.03 (s, 3H), 1.97–1.52 (m, 3H), 1.45 (s, 9H); ¹³C NMR (100 MHz) δ 172.73, 172.67, 170.4, 170.2, 156.2, 79.6, 52.5, 51.82, 51.78, 45.8, 45.7, 45.2, 45.0, 44.8, 30.4, 29.7, 29.1, 29.0, 28.4, 23.0.

3.4 *N*-α-acetyl-*N*-ε-(*tert*-butoxycarbonyl)-δ,ε-Se,*N*-methylene-δ-DL-seleno-DL-lysine (**7**)

To a solution of **6** (0.226 g, 0.297 mmol) in DCM (4 mL), 10% v/v TFA/DCM (0.45 mL, 5.9 mmol) was added, and the mixture was stirred for 3 h at room temperature. The solution was concentrated *in vacuo*, and the residue was concentrated twice from toluene to yield crude *N*-α-acetyl-δ-DL-seleno-DL-lysine methyl ester dimer (0.307 g) as a syrup. The syrup was dissolved in 0.05 M aqueous NaOH (5.9 mL) and EtOH (1.6 mL). The solution was bubbled with Ar for 5 min, and NaBH₄ (39.3 mg, 1.01 mmol) was added in three portions to this solution over 10 min under an Ar atmosphere. When the solution was stirred for 30 min, it became colorless. It was then cooled to 0°C, and the pH was adjusted to 4–5 by adding 5 M HCl. To this solution, 37% aqueous formaldehyde (222 μL, 3.0 mmol) was added over 25 min. The resulting solution was stirred first for 30 min at 0°C and then for 20 h at room temperature. The solution was concentrated *in vacuo* at 30°C, and the resulting aqueous solution was lyophilized to yield crude *N*-α-acetyl-δ,ε-Se,*N*-methylene-δ-DL-seleno-DL-lysine methyl ester (0.460 g) as a white solid. The solid was dissolved in water (3.1 mL), and K₂CO₃ (94.2 mg, 0.888 mmol) was added to it at 0°C. The resulting solution was stirred for 10 min at 0°C, and then a solution of Boc₂O (0.163 mL, 0.71 mmol) in 1,4-dioxane (3.1 mL) was added dropwise. The obtained solution was first stirred for 5 min at 0°C, and then for an additional 14 h at room temperature. The solution was concentrated to yield crude *N*-α-acetyl-*N*-ε-(*tert*-butoxycarbonyl)-δ,ε-Se,*N*-

methylene- δ -DL-seleno-DL-lysine methyl ester (0.602 g); ESI MS $C_{15}H_{26}N_2O_5Se$ $[M + H]^+$ calcd: 394.1, found: 394.4; 1H NMR (400 MHz, $CDCl_3$) δ 6.29–6.02 (m, 1H), 4.85–4.72 (m, 1H), 4.68–4.51 (m, 2H), 3.76 (s, 3H), 3.75–3.68 (m, 2H), 3.66–3.55 (m, 1H), 2.03 (s, 3H), 1.99–1.50 (m, 4H), 1.48 (s, 9H); ^{13}C NMR (100 MHz) δ 172.73, 172.67 (Ac), 170.4, 170.2 (COOH), 156.2 (Boc-CO), 79.6 (tBu-C), 52.5 (OMe), 51.82, 51.78 (α), 45.8, 45.7, 45.2, 45.0, 44.8 (δ, ϵ), 30.4, 29.7, 29.1, 29.0 (β, γ), 28.4 (tBu), 23.0 (Ac).

Crude methyl ester (0.602 g) was dissolved in THF (3 mL), and 1 M NaOH (3 mL) was added to the solution. The solution was stirred for 1.5 h at room temperature, and neutralized with Dowex 50W-X8 (H^+ form). The resin was filtered off, and the filtrate was concentrated. The residue was purified by silica gel chromatography (EtOAc then EtOAc:MeOH = 4:1) to yield **7** (0.150 g, 67% from **6**) as a colorless syrup; HR-ESI-TOFMS m/z calcd. for $C_{14}H_{24}N_2O_5Se$ $[M + Na]^+$ 403.0743, found: 403.0754; 1H NMR (400 MHz, $CDCl_3$) δ 6.97–6.60 (br, 1H), 6.55–6.02 (br, 1H), 4.83–4.72 (m, 1H), 4.63–4.51 (m, 2H), 3.84–3.68 (m, 2H), 3.68–3.57 (m, 1H), 2.07 (s, 3H), 2.01–1.90 (m, 1H), 1.90–1.50 (m, 3H), 1.48 (s, 9H).

3.5 *N*- α -(9-fluorenylmethoxycarbonyl)-*N*- ϵ -(*tert*-butoxycarbonyl)- δ, ϵ -Se,*N*-methylene- δ -DL-seleno-L-lysine (**L-9**)

L-Aminoacylase (68 mg) was added to a solution of compound **7** (0.136 g, 0.36 mmol) in 0.2 M phosphate buffer (pH 8.0, 3.6 mL) and the resulting suspension was incubated for 21 h at 37°C. The LC/MS analysis [CAPCELLPAK C18 (2.0 mm \times 150 mm), solvent A:solvent B = 80:20 to 5:95 over 10 min at a flow rate of 0.2 mL/min] results suggested that approximately half of **7** was converted to **L-8**; ESI-MS m/z calcd. for $C_{12}H_{22}N_2O_4Se$ $[M + H]^+$ 339.1, found: 339.1; The suspension was centrifuged, and the supernatant was separated. The precipitate was washed twice with 0.2 M phosphate buffer. The combined supernatant and washings (total 5.6 mL) were added to a solution of Fmoc-OSu (0.0733 g, 0.217 mmol) in THF (5.6 mL). The solution was stirred overnight at room temperature. It was then concentrated *in vacuo* and the residue was purified by silica gel chromatography (EtOAc:hexane = 1:3, then 1:3 containing 1% AcOH) to yield **L-9** (0.0629 g, 31%) as a colorless syrup; HR-ESI-FTMS m/z calcd. for $C_{27}H_{32}N_2O_6Se$ $[M + Na]^+$ 583.1318, found: 583.1317; 1H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, J 7.5 Hz, 2H), 7.63–7.50 (m, 2H), 7.39 (t, J 7.4 Hz, 2H), 7.30 (t, J 7.4 Hz, 2H), 5.66–5.34 (br, 1H), 4.86–4.65 (m, 1H), 4.63–4.49 (m, 1H), 4.48–4.34 (m, 2H), 4.21 (t, J 6.7 Hz, 1H), 3.90–3.57 (m, 3H), 2.08–1.93 (m, 1H), 1.93–1.53 (m, 3H), 1.48 (s, 9H); ^{13}C NMR (100 MHz) δ 175.6 (COOH), 156.2, 154.3 (Fmoc-CO, Boc-CO), 143.8, 143.7, 141.3 (Fmoc-ArC), 127.8, 127.1, 125.1, 120.0 (Fmoc-ArCH), 81.2 (tBu-C), 67.2 (Fmoc-CH₂O), 55.2 (br, ϵ), 53.4 (α), 47.1 (Fmoc-CH), 43.7 (br, δ), 39.7 (br, SeCH₂N), 32.2, 31.6 (β, γ), 28.3 (tBu-Me).

3.6 *N*- α -(9-fluorenylmethoxycarbonyl)-*N*- ϵ -(*tert*-butoxycarbonyl)- δ, ϵ -Se,*N*-methylene- δ -DL-seleno-D-lysine (**D-9**)

D-Aminoacylase (7.0 mg) was added to a solution of compound **7** (14 mg, 37 μ mol) in 0.2 M phosphate buffer (pH 8.0, 0.34 mL) and

the resulting suspension was incubated for 21 h at 37°C. The LC/MS analysis results suggested that approximately half of **7** was converted to **D-8**; ESI-MS m/z calcd. for $C_{12}H_{22}N_2O_4Se$ $[M + H]^+$ 339.1, found: 339.1; A solution of Fmoc-OSu (7.6 mg, 23 μ mol) in THF (0.59 mL) was added to this suspension. The resulting suspension was stirred overnight at room temperature and then concentrated *in vacuo*. The residue was purified by silica gel chromatography (EtOAc:hexane = 1:3, then 1:3 containing 1% AcOH) to yield **D-9** (2.3 mg, 11%) as a colorless syrup; ESI-MS m/z calcd. for $C_{27}H_{32}N_2O_6Se$ $[M + Na]^+$ 583.1, found: 583.1; 1H NMR (500 MHz, $CDCl_3$) δ 7.75 (d, J 7.5 Hz, 2H), 7.63–7.50 (m, 2H), 7.39 (t, J 7.4 Hz, 2H), 7.34 (t, J 7.4 Hz, 2H), 5.66–5.34 (br, 1H), 4.86–4.65 (m, 1H), 4.63–4.49 (m, 1H), 4.48–4.34 (m, 2H), 4.21 (t, J 6.7 Hz, 1H), 3.90–3.57 (m, 3H), 2.08–1.93 (m, 1H), 1.93–1.53 (m, 3H), 1.48 (s, 9H).

3.7 Determination of the absolute configuration of the α -position of **L-9** and **D-9**

Aminomethyl-ChemMatrix resin was treated with HMPA (2.5 equiv), TBTU (2.5 equiv), and 4-ethylmorpholine (2.5 equiv) in DMF for 3.5 h at room temperature to yield the HMPA-ChemMatrix rein. The first amino acid Fmoc-Asp(OtBu) (5 equiv) was coupled with MSNT (5 equiv) and 1-methylimidazole (3.75 equiv) in DCM for 1 h. The Fmoc group was deprotected by treatment with 20% piperidine in DMF for 15 min. Each amino acid (4 equiv) was coupled using DIC (4 equiv) and HOBt (4 equiv) in DMF for 1 h. For the coupling of **L-9** and **D-9**, 1.3 equiv of amino acid, DIC, and HOBt were used. The peptides were cleaved off from the resin by treatment with TFA/H₂O/TIPS (95/2.5/2.5) for 2 h. The resin was filtered off and the filtrate was concentrated *in vacuo* to yield crude pentapeptides containing a δ -selenolysine residue. The crude peptides (3 μ mol) were treated with 0.2 M MeONH₂-HCl, 6 M Gn-HCl, 0.2 M phosphate buffer at pH 4.5 (550 μ L). Diselenides **L-11/D-11** were purified by RP-HPLC [CAPCELLPAK C18 (10 mm \times 250 mm), solvent A:solvent B = 95:5 to 75:25 over 30 min at a flow rate of 2 mL/min) and lyophilized. Purified diselenides were dissolved in 25 mM DTT, 6 M Gn-HCl, 0.2 M phosphate buffer at pH 6.5 (582 μ L), purged with Ar for 3 min, and stood for 10 min. Then, a solution of 200 mM TCEP, 6 M Gn-HCl, 0.2 M phosphate buffer at pH 5.4 (632 μ L) was added to the solution and stood overnight. RP-HPLC purification [CAPCELLPAK C18 (10 mm \times 250 mm), solvent A:solvent B = 95:5 to 70:30 over 45 min at a flow rate of 2 mL/min) and lyophilization yielded the pentapeptide **L-12/D-12**, which were analyzed by RP-HPLC [CAPCELLPAK C18 (4.6 mm \times 150 mm), solvent A:solvent B = 98:2 to 80:20 over 15 min at a flow rate of 1 mL/min] by co-injection with pentapeptide samples synthesized separately using Fmoc-L-Lys(Boc) or Fmoc-D-Lys(Boc).

3.8 Preparation of hydrazine resin

2-Chlorotriptyl chloride resin (76.9 mg, 100 μ mol, 1.30 mmol/g loading) was washed with DMF \times 2, DCM \times 5, and DMF \times 3, and was swollen in DCM (1.385 mL) for 15 min at 0°C. A solution of

9-fluorenylmethyl carbazate (25.7 mg, 100 μmol) in DMF (1.77 mL) and DCM (0.385 mL) was added to the suspension, followed by DIEA (44.3 μL , 250 μmol , 2.5 equiv). The suspension was first shaken for 5 min at 0°C and then for an additional 2.5 h at room temperature. MeOH (81 μL , 2.0 mmol, 20 equiv) and DIEA (340 μL , 2.0 mmol, 20 equiv) were added to the suspension and shaken for 1 h. The resin was filtered off and washed with DCM/MeOH/DIEA = 17/2/1 (2 mL \times 3), DCM \times 5, DMF \times 5, water \times 5, DMF \times 5, DCM \times 5, DMF \times 5, MeOH \times 3, and Et₂O \times 3 and dried in a vacuum desiccator overnight. The dried resin was swollen in DMF/DCM = 1/1 for 10 min, washed with DCM \times 5, and DMF \times 5, and treated with 20% piperidine in DMF (4 mL) for 15 min. Fmoc quantification suggested that the amount of loaded hydrazine was 42 μmol (0.52 mmol/g loading).

3.9 Fmoc SPPS of Ub (49–76)-hydrazide resin

A solution of Fmoc-Gly (420 μmol , 10 equiv), DIC (65 μL , 10 equiv), and OxymaPure (59.7 mg, 10 equiv) in NMP (1,050 μL) was preactivated for 3 min and added to hydrazine-2-chlorotriyl resin (42 μmol). The suspension was shaken for 20 min at 60°C. The coupling of Fmoc-Gly was performed twice. After washing the resin with DMF \times 5, it was treated with Ac₂O (0.1 mL), HOBT (4.7 mg), and DIEA (47 μL) in NMP (2.1 mL) for 10 min at room temperature. The resin was then treated with 20% piperidine in DMF for 15 min at room temperature, and the amount of Gly loading was estimated to be 26 μmol . Peptide elongation was manually carried out according to the general steps below. Coupling step: Fmoc-AA (420 μmol , 10 equiv), DIC (65 μL , 10 equiv), OxymaPure (59.7 mg, 10 equiv) in NMP (1,050 μL) was preactivated for 3 min. The solution was added to the resin and shaken for 20 min at 60°C. The coupling of Fmoc-His(Trt) was performed for 60 min at room temperature. Dipeptides Fmoc-Leu-Ser(psiMe, Mepro) and Fmoc-Asp(OtBu)-(Dmb)Gly were used for Leu56–Ser57 and Asp52–Gly53, respectively. Dipeptides were coupled via double coupling using 4 equiv followed by using 2 equiv. Deprotection step: 20% piperidine in DMF was added to the resin and sonicated with ultrasound for 30 s and 1 min.

3.10 Ub (46C^{Acm}–76)- α -hydrazide (13)

Approximately 1/4th of the aforementioned Ub (49–76)-hydrazine resin was treated with 20% piperidine in DMF (0.5 mL) for 15 min at room temperature, and the amount of the peptide was estimated to be 3.9 μmol . Peptide elongation was manually carried out according to the general steps given below. Coupling step: Fmoc-Lys(Boc) and Fmoc-Gly (50 μmol), DIC (7.75 μL , 10 equiv), and OxymaPure (7.1 mg, 10 equiv) in NMP (250 μL) were preactivated for 3 min, and the solution was added to the resin and shaken for 20 min at 60°C; Deprotection step: 20% piperidine in DMF was added to the resin and sonicated with ultrasound for 30 s and 1 min. Finally, Boc-Cys(Acm) (50 μmol), DIC (7.75 μL , 10 equiv), and OxymaPure (7.1 mg, 10 equiv) in NMP (250 μL) were preactivated for 3 min, and the solution was added to the resin and shaken for 60 min at room temperature. The resin was washed with DMF \times 5, DCM \times 5, DMF \times 5, MeOH \times 3, and Et₂O \times 3 and dried in a vacuum desiccator

overnight. The dried resin was treated with a mixture comprising TFA (950 μL), TIPS (25 μL), water (25 μL) and shaken for 2 h at room temperature. The resin was filtered off, and the filtrate was added dropwise to ice-cold Et₂O (10 mL). The precipitate was collected via centrifugation. The remaining resin was washed with TFA (1 mL), and the washing was treated in the same way. The precipitate was washed twice with ice-cold Et₂O and dried in a vacuum desiccator overnight. The dried crude peptide was purified by RP-HPLC [Proteonavi (10 mm \times 250 mm), solvent A:solvent B = 85:15 to 65:35 over 100 min at a flow rate of 2 mL/min] and lyophilized to yield Ub (46C^{Acm}–76)- α -hydrazide **13** (6.5 mg, 1.8 μmol , 17% calculated using 1/4th of the starting hydrazine-2-chlorotriyl resin) as a white fluffy solid; Analytical HPLC conditions: Proteonavi (4.6 mm \times 150 mm), solvent A:solvent B = 85:15 to 55:45 over 15 min at a flow rate of 1 mL/min; ESI-MS: calcd. for C₁₅₂H₂₅₈N₅₀O₄₈S 3586.1, obsd. 3586.1 \pm 0.2. *m/z* calcd. [M+3H]³⁺ 1196.4, [M+4H]⁴⁺ 897.5, [M+5H]⁵⁺ 718.2, [M+6H]⁶⁺ 598.7, [M+7H]⁷⁺ 513.3; obsd. [M+3H]³⁺ 1196.3, [M+4H]⁴⁺ 897.6, [M+5H]⁵⁺ 718.2, [M+6H]⁶⁺ 598.7, [M+7H]⁷⁺ 513.3.

3.11 Ub (46C^{Acm}–76)- α -MPAA (14)

Ub (46C^{Acm}–76)- α -hydrazide **13** (5.1 mg, 1.4 μmol) was dissolved in 0.2 M MPAA, 6 M Gn-HCl, 0.2 M phosphate buffer at pH 3.0 (286 μL), and 0.1 M aqueous acetylacetone (143 μL , 14.2 μmol , 10 equiv) was added. The suspension was incubated overnight at room temperature. It was then diluted with 1 M Gn-HCl, 33 mM phosphate buffer at pH 3.0 (4 mL), filtered through Millex LCR syringe filter, and purified by RP-HPLC [Proteonavi (10 mm \times 250 mm), solvent A:solvent B = 80:20 to 50:50 over 60 min at a flow rate of 2 mL/min]. Fractions containing the desired product were collected and lyophilized to yield Ub (46C^{Acm}–76)- α -MPAA **14** (3.7 mg, 0.99 μmol , 70%) as a white fluffy solid; Analytical HPLC conditions: Proteonavi (4.6 mm \times 150 mm), solvent A:solvent B = 80:20 to 50:50 over 15 min at a flow rate of 1 mL/min; ESI-MS: calcd. for C₁₆₀H₂₆₂N₄₈O₅₀S₂ 3722.2, obsd. 3722.4 \pm 0.2. *m/z* calcd. [M+3H]³⁺ 1241.7, [M+4H]⁴⁺ 931.6, [M+5H]⁵⁺ 745.5, [M+6H]⁶⁺ 621.4, [M+7H]⁷⁺ 532.8; obsd. [M+3H]³⁺ 1241.7, [M+4H]⁴⁺ 931.6, [M+5H]⁵⁺ 745.5, [M+6H]⁶⁺ 621.4, [M+7H]⁷⁺ 532.8.

3.12 Ub (46C^{Acm}–48K^{Sez}–76)- α -hydrazide (15)

Approximately 1/4th of the aforementioned Ub (49–76)-hydrazine resin was treated with 20% piperidine in DMF (0.5 mL) for 15 min at room temperature, and the amount of the peptide was estimated to be 4.4 μmol . Fmoc-Lys(Sez) **L-9** (5.6 mg, 10 μmol , 2 equiv), DIC (1.55 μL , 2 equiv), and OxymaPure (1.4 mg, 2 equiv) in NMP (100 μL) were preactivated for 3 min, and the solution was added to the resin and shaken for 20 min at 60°C. The coupling was performed twice using the same conditions. Peptide elongation using Fmoc-Gly (double coupling) and Boc-Cys(Acm) was carried out as mentioned above for the synthesis of **13**. The resin was washed with DMF \times 5, DCM \times 5, DMF \times 5, MeOH \times 3, and Et₂O \times 3 and dried in a vacuum desiccator overnight. The dried resin was treated with a mixture comprising TFA (950 μL), TIPS (25 μL), water (25 μL), MeONH₂-HCl (2.1 mg, 5 equiv) and allowed to shake for 2 h at room temperature. The crude peptide

was obtained by ice-cold Et₂O treatment, using the same method as that for **13**. The dried crude peptide was purified by RP-HPLC [Proteonavi (10 mm × 250 mm), solvent A:solvent B = 85:15 to 65:35 over 100 min at a flow rate of 2 mL/min] and lyophilized to yield Ub (46C^{Acm}-48K^{Sez}-76)-α-hydrazide **15** (3.7 mg, 1.0 μmol, 10% calculated based on the 1/4th of the starting hydrazine-2CT resin) as a white fluffy solid.; Analytical HPLC conditions: Proteonavi (4.6 mm × 150 mm), solvent A:solvent B = 85:15 to 55:45 over 15 min at a flow rate of 1 mL/min.; ESI-MS: calcd. for C₁₅₃H₂₅₈N₅₀O₄₈SSe 3677.0, obsd. 3677.3 ± 0.4. *m/z* calcd. [M+3H]³⁺ 1266.7, [M+4H]⁴⁺ 920.3, [M+5H]⁵⁺ 736.4, [M+6H]⁶⁺ 613.8, [M+7H]⁷⁺ 526.3; obsd. [M+3H]³⁺ 1226.5, [M+4H]⁴⁺ 920.4, [M+5H]⁵⁺ 736.5, [M+6H]⁶⁺ 613.9, [M+7H]⁷⁺ 526.4.

3.13 One-pot selenazolidine deprotection-δ-selenolysine-mediated ligation-deselenization

Ub (46C^{Acm}-48K^{Sez}-76)-α-hydrazide **15** (0.6 mg, 0.16 μmol) was dissolved in a degassed solution of 3 mM CuCl₂ (1.5 equiv), 10 mM MeONH₂-HCl (5 equiv) in 6 M Gn-HCl, 0.2 M phosphate (81 μL, pH 6). The resulting suspension was vortexed for 30 min. The LC/MS analysis results showed the formation of diselenide **16**. The solution was degassed for 5 min with Ar bubbling, and a solution of Ub (46C^{Acm}-76)-α-MPAA **14** (0.8 mg, 0.22 μmol) in 10 mM TCEP (5 equiv), 6 M Gn-HCl, and 0.2 M phosphate (81 μL, pH 6) was added. The resulting solution was allowed to stand for 2 h. The LC/MS analysis results showed the formation of isopeptide **18**. The solution was degassed for more than 10 min with Ar bubbling. TCEP (2.4 mg, 8.2 μmol, 50 equiv) and DTT (1.2 mg, 8.2 μmol, 50 equiv) were added to the degassed solution and the pH was adjusted to 5 with 1 M NaOH. The resulting solution was again bubbled with Ar for 5 min and allowed to stand overnight. The solution was diluted with aqueous 0.04% TFA (0.8 mL) and purified by RP-HPLC [Proteonavi (10 mm × 150 mm), solvent A:solvent B = 95:5 to 87:13 over 5 min then to 63:37 over 100 min at a flow rate of 2 mL/min] and lyophilized to yield Ub [46C^{Acm}-48K[Ub (46C^{Acm}-76)]-76]-α-hydrazide **21** (0.6 mg, 53%) as a white fluffy solid.; Analytical HPLC conditions: Proteonavi (4.6 mm × 150 mm), solvent A:solvent B = 80:20 to 60:40 over 15 min at a flow rate of 1 mL/min.

Compound **16**: calcd. for C₃₀₄H₅₁₄N₁₀₀O₉₆S₂Se₂ 7328.0, obsd. 7328.5 ± 0.5. *m/z* calcd. [M+6H]⁶⁺ 1222.3, [M+7H]⁷⁺ 1047.9, [M+8H]⁸⁺ 917.0, [M+9H]⁹⁺ 815.2, [M+10H]¹⁰⁺ 733.8, [M+11H]¹¹⁺ 667.2, [M+12H]¹²⁺ 611.7, [M+13H]¹³⁺ 564.7, [M+14H]¹⁴⁺ 524.4; obsd. [M+6H]⁶⁺ 1222.4, [M+7H]⁷⁺ 1048.0, [M+8H]⁸⁺ 917.1, [M+9H]⁹⁺ 815.3, [M+10H]¹⁰⁺ 733.9, [M+11H]¹¹⁺ 667.3, [M+12H]¹²⁺ 611.7, [M+13H]¹³⁺ 564.7, [M+14H]¹⁴⁺ 524.4.

Compound **18**: calcd. for C₃₀₄H₅₁₂N₉₈O₉₆S₂Se 7219.0, obsd. 7220.0 ± 0.9. *m/z* calcd. [M+5H]⁵⁺ 1444.8, [M+6H]⁶⁺ 1204.1, [M+7H]⁷⁺ 1032.2, [M+8H]⁸⁺ 903.3, [M+9H]⁹⁺ 803.1, [M+10H]¹⁰⁺ 722.9, [M+11H]¹¹⁺ 657.3, [M+12H]¹²⁺ 602.6; obsd. [M+5H]⁵⁺ 1444.9, [M+6H]⁶⁺ 1204.3, [M+7H]⁷⁺ 1032.2, [M+8H]⁸⁺ 903.6, [M+9H]⁹⁺ 803.4, [M+10H]¹⁰⁺ 723.1, [M+11H]¹¹⁺ 657.3, [M+12H]¹²⁺ 602.6.

Compound **21**: calcd. for C₃₀₄H₅₁₂N₉₈O₉₆S₂ 7140.1, obsd. 7140.0 ± 0.5. *m/z* calcd. [M+5H]⁵⁺ 1429.0, [M+6H]⁶⁺ 1191.0, [M+7H]⁷⁺ 1021.0, [M+8H]⁸⁺ 893.5, [M+9H]⁹⁺ 794.3, [M+10H]¹⁰⁺ 715.0, [M+11H]¹¹⁺ 650.1, [M+12H]¹²⁺ 596.0, [M+13H]¹³⁺ 550.2;

obsd. [M+5H]⁵⁺ 1428.9, [M+6H]⁶⁺ 1191.0, [M+7H]⁷⁺ 1021.0, [M+8H]⁸⁺ 893.5, [M+9H]⁹⁺ 794.4, [M+10H]¹⁰⁺ 715.0, [M+11H]¹¹⁺ 650.2, [M+12H]¹²⁺ 596.0, [M+13H]¹³⁺ 550.2.

3.14 Trypsin digestion of isopeptide (21)

An aliquot of isopeptide **21** was dissolved in 50 mM ammonium bicarbonate buffer (pH 8.0, 20 μL), and a trypsin solution (20 μg/200 μL, 0.3 μL) was added. The resulting solution was incubated overnight at 37°C and analyzed by LC/MS [CAPCELLPAK C18 (Osaka soda, 5 μm, 2 × 150 mm), solution A:solution B = 98:2 to 40:60 over 10 min at a flow rate of 0.2 mL/min]. ESI-MS: (Peak a in [Supplementary Figure S8](#)) calcd. for C₂₈H₄₈N₁₀O₁₂ 716.3, obsd. 716.3. *m/z* calcd. [M + H]⁺ 717.4, [M+2H]²⁺ 359.2; obsd. [M + H]⁺ 717.5, [M+2H]²⁺ 359.2; (Peak b in [Supplementary Figure S8](#)) calcd. for C₄₆H₇₉N₁₇O₁₈S 1189.6, obsd. 1189.8 *m/z* calcd. [M+3H]³⁺ 397.5; obsd. [M+3H]³⁺ 397.6; (Peak c in [Supplementary Figure S8](#)) calcd. for C₄₇H₇₆N₁₂O₁₇ 1080.5, obsd. 1080.6. *m/z* calcd. [M + H]⁺ 1081.6, [M+2H]²⁺ 541.3; obsd. [M + H]⁺ 1081.6, [M+2H]²⁺ 541.3; (Peak d in [Supplementary Figure S8](#)) calcd. for C₄₇H₈₂N₁₄O₁₄ 1066.6, obsd. 1066.6. *m/z* calcd. [M + H]⁺ 1067.6, [M+2H]²⁺ 534.3, [M+3H]³⁺ 356.5; obsd. [M + H]⁺ 1067.7, [M+2H]²⁺ 534.3, [M+3H]³⁺ 356.6.

4 Conclusion

We have developed a δ-selenolysine-mediated pathway for the formation of isopeptide bonds. *N*-α-Fmoc-δ-selenolysine, with its side chain protected as selenazolidine, was synthesized in both L- and D-forms starting from commercially available racemic DL-δ-hydroxy-DL-lysine. Substitution of δ-mesylyate by KSeCN and enzymatic optical resolution using L- or D-aminoacylase successfully provided the desired L-**9** and D-**9**, respectively. We prepared both L- and D-forms of the δ-selenolysine derivative because the L-form is essential for the synthesis of native ubiquitinated protein probes, while the D-form is valuable for the synthesis of the mirror-image protein that is required for racemic protein crystallography. Then, we examined the isopeptide bond formation mediated by δ-selenolysine between ubiquitin (46-76)-α-thioester and ubiquitin (46-76)-α-hydrazide containing a δ-selenolysine residue at position 48. After thoroughly analyzing the byproducts, we established a one-pot protocol involving selenazolidine deprotection, δ-selenolysine-mediated isopeptide chemical ligation, and deselenization. We anticipate that the δ-selenolysine-mediated isopeptide bond formation reported here may prove helpful for the synthesis of ubiquitinated proteins with native cysteine residues, such as glycoproteins, as deselenization proceeds without desulfurization of cysteine residues. We are currently studying the synthesis of di-ubiquitins using δ-selenolysine-mediated isopeptide bond formation in our laboratory and will report the results in due course.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#), further inquiries can be directed to the corresponding author.

Author contributions

TA: Investigation, Writing–original draft. YT: Investigation, Writing–original draft. RO: Conceptualization, Writing–review and editing. YK: Conceptualization, Funding acquisition, Writing–review and editing. MI: Investigation, Conceptualization, Funding acquisition, Writing–original draft, Writing–review and editing.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fchem.2023.1307254/full#supplementary-material>

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