

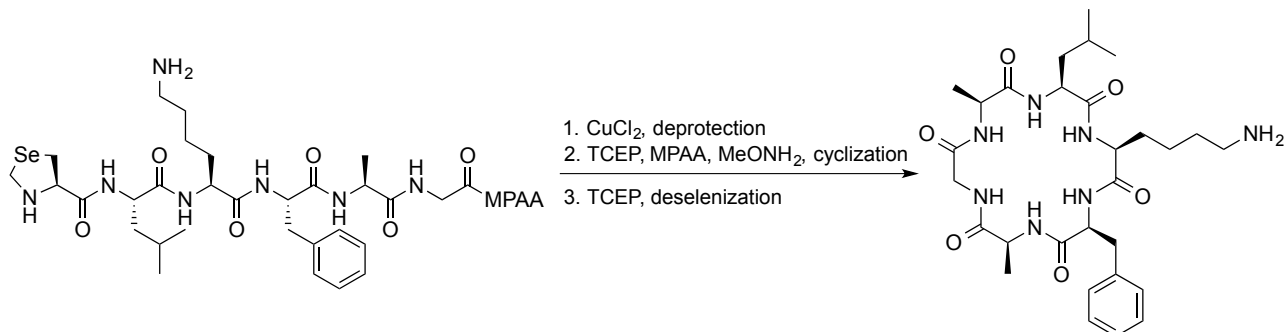
Utilizing copper-mediated deprotection of selenazolidine for cyclic peptides synthesis

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Abstract

Selenazolidine (Sez) was originally developed as a masking form of selenocysteine (Sec) for the chemical synthesis of challenging proteins. Here we utilize Sez and our recent reported copper(II)-mediated deprotection for the synthesis of cyclic peptides. This approach allows deprotection, cyclization and deselenization in one-pot, providing several different cyclic peptides in good yields. In addition, the Sec can also be retained, which enhance the oxidative folding of disulfide-rich cyclic proteins, such as the case of Kalata S.



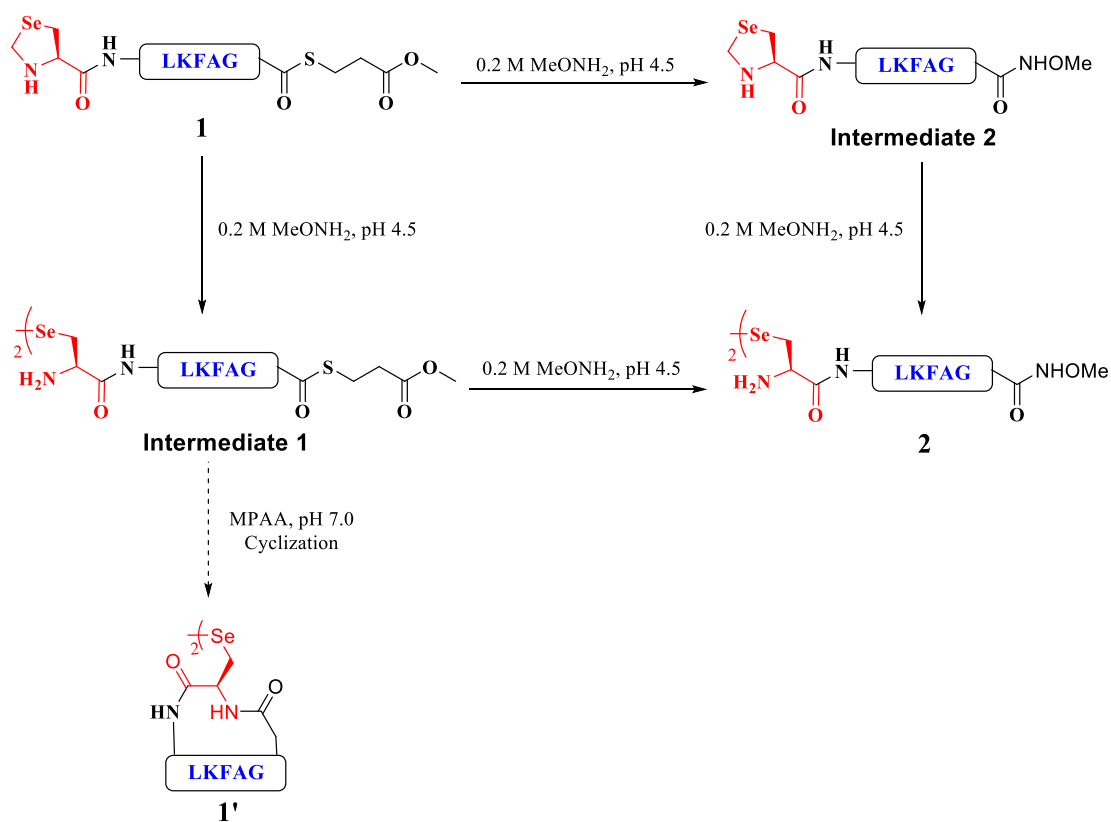
Cyclic peptides and proteins provide an attractive scaffold for developing complex, biologically active molecules due to their constrained conformation and unique structures.¹⁻⁷ In addition to their extensive distribution in nature,^{2, 8-11} cyclic peptides are promising drug candidates for a wide range of diseases.¹²⁻¹⁴ Compared to their linear counterparts, cyclic peptides often exhibit enhanced stability, increased efficiency and bioavailability.^{15, 16} For the above reasons, cyclic peptides are becoming attractive synthetic targets for chemists and chemical biologists.

One strategy for preparing cyclic peptides is the lactamization of protected linear peptides in organic solvents after cleavage from solid support.¹⁷⁻²⁰ A major limitation of this strategy is the use of coupling reagents to activate the acylating moiety, which in turn are known to cause epimerization at the peptide C-terminal residue.²¹ Further, the cyclization reaction must be conducted at high dilution to minimize polymerization side-reactions. Furthermore, side-chain protection is necessary, which decreases peptide solubility as its size increases. To overcome these challenges, a variety of approaches to synthesize cyclic peptides from linear unprotected peptide precursors have been developed.^{20, 22-30} Native chemical ligation (NCL),³¹ which allows chemoselective intramolecular cyclization of C-terminal thioester and N-terminal cysteine to form unprotected cyclic peptide^{23, 25, 32} is perhaps the most used and effective approach. This method exhibits several advantages, including limited polymerization side-reactions, virtually no epimerization, and efficient reactions on unprotected peptides in aqueous media.

Possessing properties similar to Cys, selenocysteine (Sec, U)-mediated NCL (Sec-NCL) was developed for the synthesis of seleno-proteins.³³⁻⁴¹ Later, its use was expanded to many wild-type proteins without Sec upon the discovery that deselenization can be performed chemoselectively, without affecting unprotected Cys residues in the protein sequence.³⁷⁻⁴⁷ The synthesis of cyclic peptides by Sec-NCL has been developed using Fmoc-Sec(Mob)-OH.^{36, 48} Yet, Mob deprotection can be challenging under standard cleavage conditions,⁴⁹ and requires the addition of 2,2'-dithiobis(5-nitropyridine) (DTNP),⁵⁰ in which a large excess of thiols (e.g. DTT) must be added to remove the 5-nitropyridine-2-thiol (5-Npys) group from Sec.⁵¹ Although using ascorbate to reduce the selenosulfide was reported,^{51, 52} the ascorbate will inhibit the deselenization reaction,^{39, 44} which make one-pot chemical synthesis of cyclic peptides challenging. Selenazolidine (Sez)^{39, 53, 54} was developed as a protected form of Sec, which has been particularly useful for the synthesis of middle segment peptides in multi-NCL reactions for proteins with non-strategically positioned Cys

residues.^{39-41, 45, 55}

Similar to thiazolidine (Thz),⁵⁶ Sez deprotection is usually implemented by MeONH₂.³⁹ Yet, we sought on utilizing Sez in the synthesis of cyclic peptides using our newly developed Cu(II)-mediated Sez-deprotection approach, followed by intramolecular NCL cyclization and final deselenization step. Thus, peptide **1**, Sez-LKFAG-COSR, was prepared as a model peptide containing N-terminal Sez and a C-terminal thioester. MeONH₂ was tested for Sez deprotection in **1** followed by NCL/cyclization. However, MeONH₂ reacted with thioester to form the methoxyamide bond (**Intermediate 2**) simultaneously, which stopped the further cyclization of **1** (Scheme 1), and instead of cyclic peptide **1'**, peptide **2** was the major product (Scheme 1, Scheme S1 and Figure S1 in SI). This result is consistent with previous work by other groups.⁵⁶⁻⁵⁹ To overcome this limitation, we decided to use our recently reported Cu(II)-mediated deprotection of Sez for the synthesis of cyclic peptides and proteins⁵⁵ in one-pot deprotection and cyclization followed by deselenization without additional HPLC purifications.



Scheme 1. MeONH₂ deprotects Sez and simultaneously reacts with the thioester to give **2**.

To develop an efficient one-pot deprotection, cyclization and deselenization method, we explored the performance of a Sez-peptide-arylthioester (Sez-peptide-MPAA)⁶⁰ (Figure 1a). We reasoned that the cyclization step should proceed efficiently with the highly activated arylthioester, with no other MPAA additives in solution.

To test this approach, peptide **3a** (Sez-LKFAG-MPAA) was synthesized using an acylurea thioester precursor.^{61, 62} After deprotection of **3a** with CuCl₂ (0.2 M PB, 6 M Gn·HCl, 1.5 equiv CuCl₂, pH 6, 25 °C)⁵⁵, TCEP and ascorbate were added to the reaction, and pH was adjusted to 6.5-7. In total, the deprotection and cyclization were completed in 1 h (Figure 1b). Yet, the subsequent deselenization was greatly inhibited due to the presence of MPAA³⁷ and ascorbate.^{37, 39, 44} We tested if a PD-10 desalting column could be used to remove those additives.⁶³ However, deselenization reaction was still slower than anticipated (Figure 1b), indicating that PD-10 desalting column failed to completely remove MPAA/ascorbate. Fortunately, we found that the cyclization reaction could be completed within 30 min using only 1 equiv of MPAA without ascorbate.³⁷ Simple ether extraction successfully removed MPAA, and a subsequent deselenization reaction could be completed within 3 h (Figure 1c).

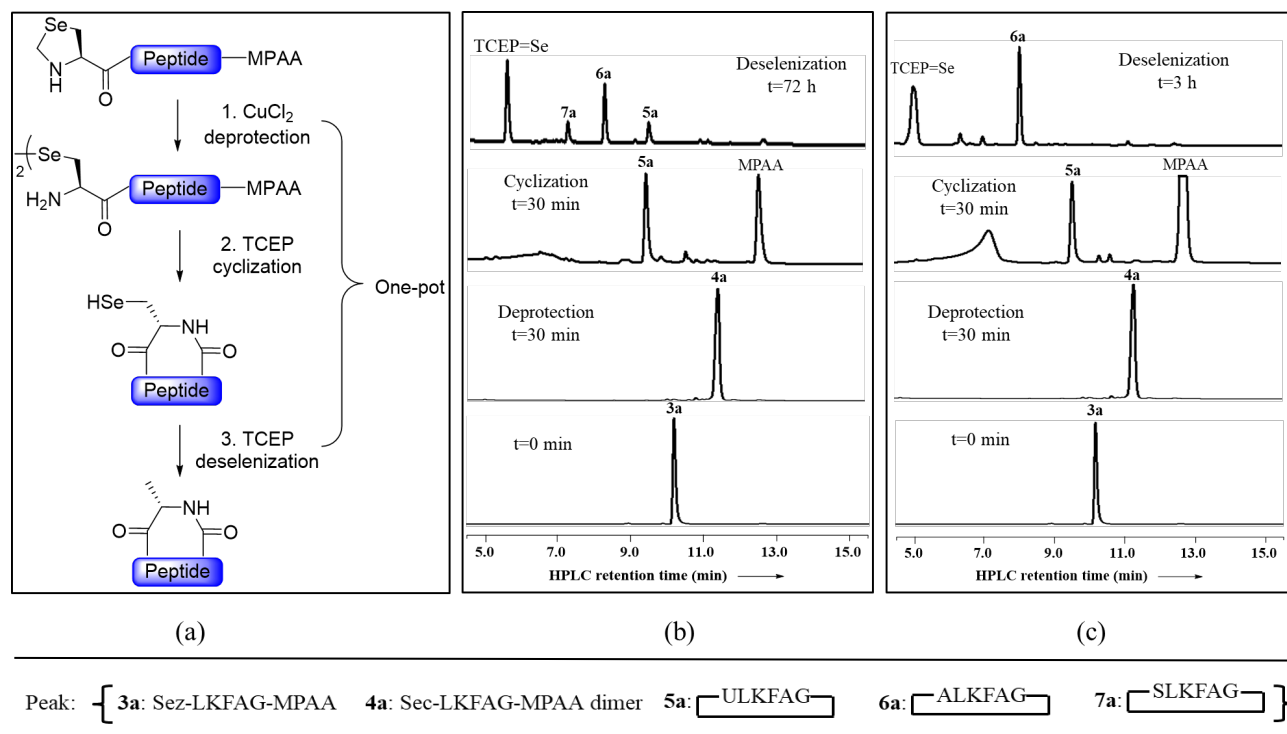


Figure 1. (a) The synthesis of cyclic peptide from Sez-peptide-MPAA in one-pot. Deprotection: 1.5 equiv CuCl₂, pH 6 took 30 min; (b) Approach I: cyclization: TCEP (25 equiv), ascorbate (50 equiv), pH 7 took 30 min; deselenization: after PD-10 column, TCEP (200 equiv) at pH 5 was not over even after 72 h; (c)

Approach II: cyclization: TCEP (5 equiv), MPAA (1 equiv), pH 7 took 30 min; deselenization: after ether extraction, TCEP (200 equiv), pH 5 took 3 h.

Encouraged by these results, peptide **3b** (Sez-LKFAT-MPAA) was also tested using this approach.⁶⁴ Under the same reaction conditions, however, only minimal cyclization product was observed and peptide **3b** (with N-terminal Sez) was regenerated due to the slow cyclization reaction at β -branched C-terminal thioesters, in this case Thr (Figure S3).⁶⁴ We note that the released formaldehyde (1 equiv) in the deprotection reaction, reacts again with the free N-terminal Sec to regenerate Sez, which inhibited the cyclization reaction. When MeONH₂ (5 equiv) was added to capture the released formaldehyde, no regeneration of **3b** was observed, and the cyclization reaction was completed within 30 min (Figure S3). Following ether extraction to remove MPAA, the deselenization reaction was completed (3 h) and cyclic product **6b** was isolated in 47 % yield over the three steps (entry 2, Table 1). Further tests on four other representative peptides (**3c-3f**), in which Ala, Phe, Lys and Val were installed at the C-terminal, the target cyclic peptides (**6c-6f**) could be prepared in good isolated yields (39%-68%) under our optimized conditions (Table 1).

Table 1. One-pot deprotection, cyclization, and deselenization for six different representative peptides^a

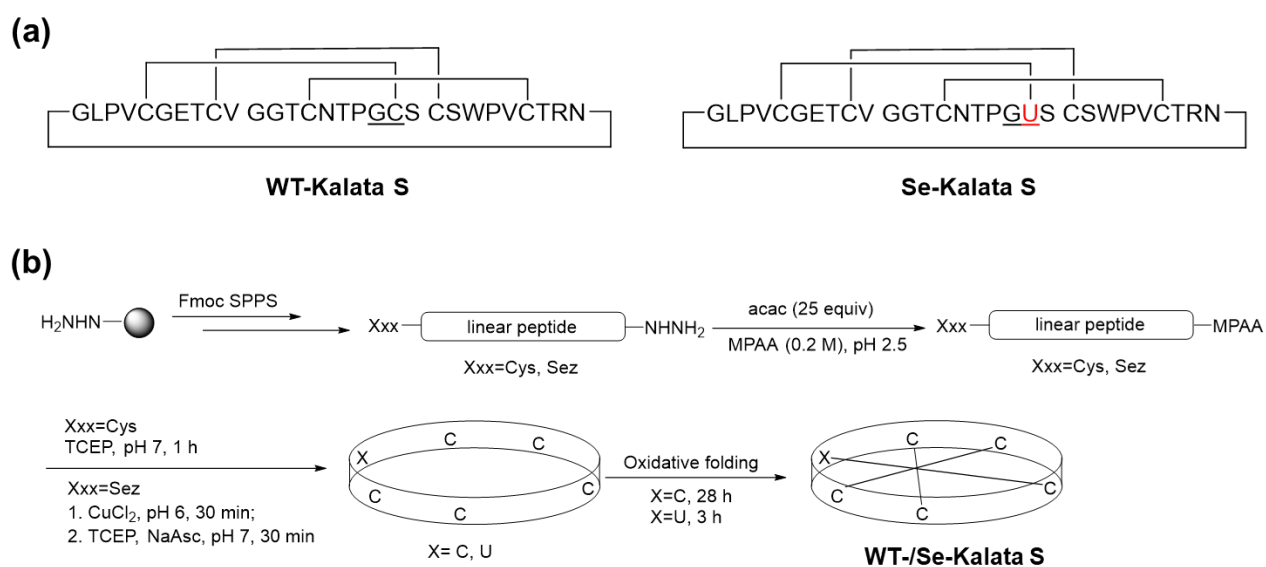
Entry	Peptides	Products	Isolated yield (%)	Average yield per step (%)
1	3a ; X=G	6a	68	88
2	3b ; X=T	6b	47	78
3	3c ; X=A	6c	67	88
4	3d ; X=F	6d	59	84
5 ^b	3e ; X=K	6e	39	73
6	3f ; X=V	6f	55	82

^aAll reactions were carried out in PB buffer (0.2 M NaH₂PO₄, 6 M Gn•HCl)

^bCyclization conditions: MeONH₂ (10 equiv) to inhibit the regeneration of **3e**.

The unique chemistry of Sec not only allows its use in NCL and its chemoselective deselenization to Ala or Ser,^{37, 39, 41, 43, 44} but also can be used to enhance the oxidative folding of Cys-rich proteins without any major structural or biological function perturbation.⁴¹ To demonstrate the applicability of our protocol for the synthesis of cyclic proteins, we chose the Cys-rich cyclic protein Kalata S as target (Scheme 2a). Kalata S is a plant cyclotide, which is a unique family of cyclic cystine knotted proteins with a head-to-tail cyclized backbone.^{9, 65} Kalata S, also called Varv A, was isolated from *V. arvensis* Murray twenty years ago,⁶⁶ although its exact function is still unknown, it differs from Kalata B1 (which shows various biological activities)^{10, 67, 68} by a single substitution of threonine to serine.⁶⁹

Like many members of the plant cyclotides, Kalata S contains six Cys residues in its sequence, which forms three disulfide bonds in its folded native state (Scheme 2). Four of these Cys residues are adjacent to β -branched residues (Thr and Val) that are not preferred for intramolecular NCL cyclization.⁶⁴ We chose the Gly-Cys site for NCL/cyclization (underlined in the sequence in Scheme 2a). We synthesized both wild-type (WT) Kalata S and its seleno-analogue Se-Kalata S, using our one-pot deprotection and cyclization strategy, followed by efficient oxidative folding (Scheme 2b).



Scheme 2. Synthesis of WT-Kalata S and its seleno-analogue, Se-Kalata S. **(a)** The sequence of WT-Kalata S and Se-Kalata S. **(b)** The synthetic approach for the two cyclic proteins.

The linear peptide hydrazides of WT- and Se-Kalata S analogue (Scheme 2b) were readily

synthesized on hydrazine-functionalized 2-chlorotrityl-resin by standard Fmoc-SPPS.⁷⁰ Using the Knorr pyrazole method⁷¹ for thioester formation, and subsequent NCL/cyclization, the WT-Kalata S was synthesized in 37% isolated yield (Scheme 2 and Figure 3a). For oxidative folding of WT-Kalata S, we used the established method for Kalata B1 folding,⁷² and the final folded state was obtained after 28 h in 57% isolated yield (Figure 3a, and Figure S6).

For Se-Kalata S, the Sez-linear peptide-MPAA was also synthesized by using Knorr pyrazole method.⁷¹ The deprotection of Sez by CuCl_2 at pH 6 took 30 min, after which TCEP and ascorbate were added to the reaction mixture, and pH was adjusted to 7 to allow for the cyclization reaction to proceed, which was completed in 30 min, giving Se-Kalata S in 40% isolated yield for cyclization. Under the same reaction condition the oxidative folding of Se-Kalata S was roughly 10 times faster (3 h) than WT-Kalata S, with a similar 62% isolated yield of folded Se-Kalata S (Figure 3b, and Figure S9).

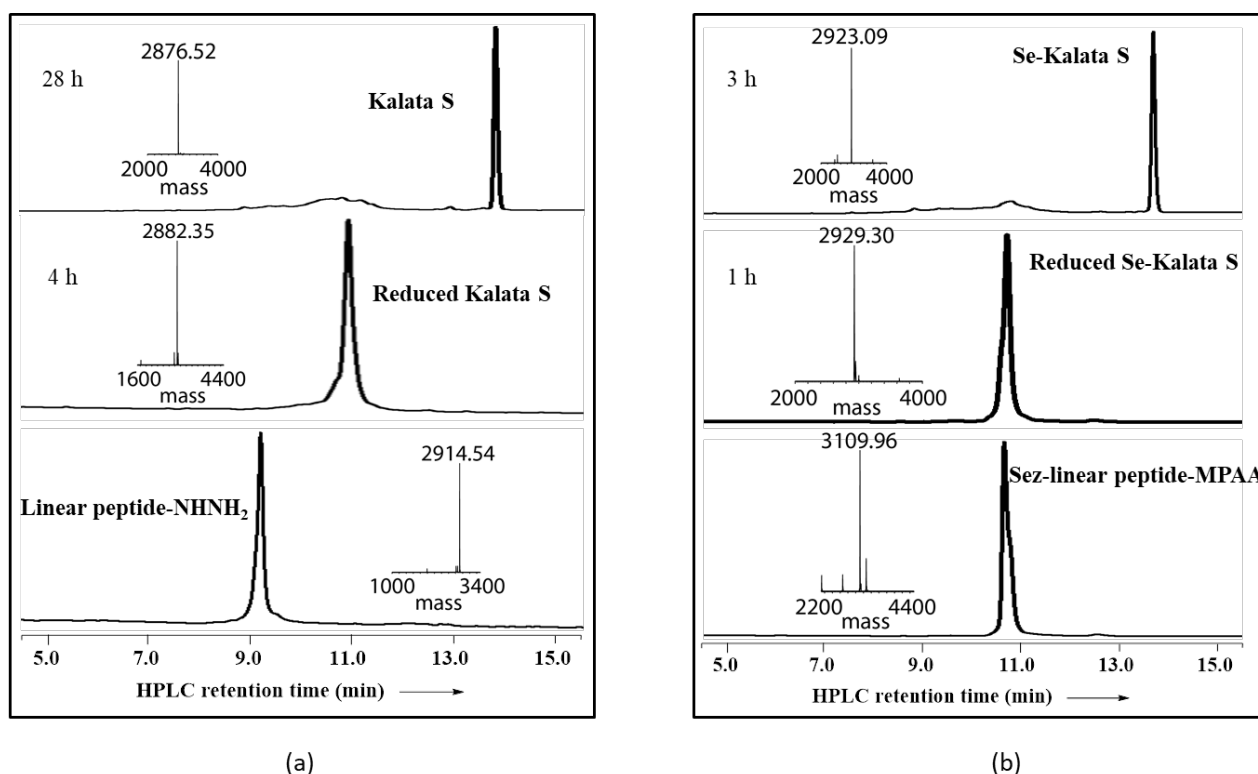


Figure 3. Synthesis of WT-Kalata S and its Se-analogue. Analytical HPLC and ESI-MS for the product in every step of (a) linear WT-Kalata S-NHNH₂ (obs. 2914.54 Da, calc. 2914.21 Da), reduced cyclic WT-Kalata S (obs. 2882.35 Da, calc. 2882.17 Da), folded WT-Kalata S (obs. 2876.52 Da, calc. 2876.13 Da); and (b) Sez-linear peptide-MPAA (obs. 3109.96 Da, calc. 3110.14 Da), reduced cyclic Se-Kalata S (obs. 2929.30 Da, calc. 2930.12 Da), folded Se-Kalata S (obs. 2923.09 Da, calc. 2924.07 Da).

In summary, we used our recently developed copper-mediated deprotection of Sez for the preparation of cyclic peptides and proteins. After deprotection, the intramolecular cyclization reaction was smoothly completed in a one-pot fashion. We could then implement deselenization of cyclic selenopeptides without intermediate HPLC-purification. Using this approach, a variety of cyclic peptides were prepared in good isolated yields. Furthermore, the good performance of both deprotection and cyclization steps were highlighted in one-pot synthesis of Se-Kalata S, in which the presence of Sec in its sequence accelerated oxidative folding compared with wild-type Kalata S. These advances provide new tools for chemical synthesis of cyclic peptides and proteins.

EXPERIMENTAL SECTION

General Methods and Materials. *Solvents and reagents.* Buffers were prepared using MilliQ water (Millipore, Merck). Disodium hydrogen phosphate dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$), 4-mercaptophenylacetic acid (MPAA), *N,N'*-Diisopropylcarbodiimide (DIC), triisopropylsilane (TIPS), acetylacetone (acac), DL-dithiothreitol (DTT), tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl), sodium ascorbate, concentrated HNO_3 were purchased from Sigma-Aldrich. Ultrapure guanidinium chloride ($\text{Gn} \cdot \text{HCl}$, MP Biomedicals, LLC, France) was used in all cyclization reactions. All Fmoc-amino acids were obtained from Matrix Innovation (Quebec City, Canada), with the following side chain protecting groups: Arg(Pbf), Glu(OtBu), Ser(*t*Bu), Thr(*t*Bu), Cys(Trt), Lys(Boc), Trp(Boc), Asn(Trt). TentaGel® R RAM resin (loading 0.18 mmol/g), Rink amide resin (loading 0.3~0.6 mmol/g), Wang resin (loading 0.3~0.8 mmol/g) and Chlorotriyl resin (loading 1.8 mmol/g) were purchased from Rapp Polymer GmbH (Germany), GL Biochemical (China) or Chem-impex (USA). *N,N,N',N'*-Tetramethyl-*O*-(6-chloro-1H-benzotriazol-1-yl) uronium hexafluorophosphate (HCTU) and Ethyl cyano(hydroxyimino)acetate (OxymaPure) were purchased from Luxembourg Biotechnologies Ltd. (Rehovot, Israel). All solvents: *N,N*-dimethylformamide (DMF), Diethyl ether (Et_2O), hydrochloric acid (HCl), ethyl acetate, dichloromethane (DCM), acetonitrile (MeCN), *N,N*-diisopropylethyl amine (DIEA), Trifluoroacetic acid (TFA) and piperidine (Pip) were purchased from Bio-Lab (Jerusalem, Israel) and were peptide synthesis, HPLC or ULC-grade. Boc-Sez-OH synthesis was reported previously.³⁹

Analyses and methods. **High Performance Liquid Chromatography (HPLC).** Analytical reversed-phase (RP) HPLC analyses were performed on a Waters Alliance HPLC with UV detection (220 nm and 280 nm) using a XSelect C18 column (3.5 μm , 130 Å, 4.6 × 150 mm). Preparative and semi-preparative RP-HPLC were performed on a Waters 150 Q LC system using a XSelect C18 column (5 μm , 30 × 250 mm) and XSelect C18 column (5 μm , 10 × 150 mm). Linear gradients of MeCN (with 0.1 % TFA, buffer B) in water (with 0.1 % TFA, buffer A) were used for all systems to elute bound peptides. The flow rates were 1 mL/min (analytical, column heated at 30 °C), 3.4 mL/min (semi-preparative), and 20 mL/min (C18 preparative).

Electrospray Ionization Mass Spectrometry (ESI-MS) and High-Resolution Mass

Spectrometry (HR-MS). ESI-MS was performed on LCQ Fleet Ion Trap mass spectrometer (Thermo Scientific) in the positive mode. Peptide masses were calculated from the experimental mass to charge (m/z) ratios from the observed multiply charged species of a peptide. The HR-MS were recorded on a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific) with a ESI source with 140'000 FWHM, a method that the AGC target was set to 1e6, and scan range was 400-2800 m/z . The raw data was deconvoluted by the MagTran v1.03.

Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Trace copper analyses were performed on an Agilent 7500cx Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Sample preparation and chemical analysis were performed in the clean laboratory of the Institute of Earth Sciences at the Hebrew University of Jerusalem. The peptides and proteins were digested in concentrated HNO_3 for 36 h, and diluted to 1% HNO_3 solution with distilled water. Before the analysis, the ICP-MS was calibrated with a series of multielement standard solutions (Merck; ME VI). The contribution of metals by the reagents used in the procedures was determined by measuring procedural blanks. The precision and accuracy of the ICP-MS were $\pm 5\%$.

The determination of protein concentration. The solutions of WT-Kalata S and Se-Kalata S served as stock and their concentrations were determined by UV-Vis spectrophotometer (using theoretical molar extinction coefficient of $\epsilon_{280\text{ nm}} = 5690\text{ M}^{-1}\text{ cm}^{-1}$ for the two proteins).

Circular Dichroism (CD). The secondary structure content of WT-Kalata S and Se-Kalata S were compared using UV CD spectroscopy (200 to 320 nm). CD spectra of these variants (30 μM for WT-Kalata S and Se-Kalata S) were recorded at 25 °C in NH_4HCO_3 buffer solution (50 mM NH_4HCO_3 ; 10 mM GSH, pH 7.5 containing 50% *i*PrOH) in a quartz cuvette with a path length of 0.1 cm using a J-810 spectropolarimeter (Jasco). Spectra were obtained by averaging 5 wavelength scans from 200 to 320nm in 1 nm steps, with a signal averaging time of 2 s and a bandwidth of 1 nm. As shown in Figure S26, the UV CD spectra of folded WT-Kalata S and Se-Kalata S are similar.

General procedure for Fmoc solid-phase peptide synthesis. Peptides were synthesized manually by Fmoc-SPPS typically on a 0.25 mmol scale. Fmoc-deprotection was carried out with 20% piperidine in DMF (10 min \times 2). Fmoc-amino acids (1 mmol in 5 mL DMF, 4 equiv) activated with HATU (1 mmol in 5 mL DMF, 4 equiv) and DIEA (2 mmol in 5 mL DMF, 8 equiv) for 5 min and allowed to couple for 30 min, with constant shaking. Sez coupling was performed by DIC/OxymaPure procedure using 2 equiv of Boc-Sez-OH.¹ The resulting resins were washed with DMF (x3) and DCM (x3) and methanol (x3) and dried.

General procedure for synthesis of peptide thioesters. The peptide thioesters were synthesized first on Fmoc-(Me)Dbz-resin (0.25 mmol scale). Fmoc-(Me)Dbz-OH (3 equiv)^{61, 62} activated with HCTU (3 equiv)/DIEA (6 equiv) in DMF was doubly coupled to the free amine of TentaGel[®] R RAM resin (0.18 mmol/g, 0.25 mmol scale) for 1 h. Sez was manually doubly coupled for 2 h (3.0 equiv Boc-Sez-OH activated on ice for 5 min using 3.0 equiv OxymaPure and 2.9 equiv DIC).

*On resin (Me)Nbz formation*⁶²: After synthesis completion, the resin was washed well with DMF and DCM and dried well under vacuum. A solution of *p*-nitrophenyl chloroformate (5 equiv) in

DCM (10 mL/0.125 mmol resin) was then added, shaken for 2 h at room temperature and washed with DCM (3 × 5 mL) and DMF (3 × 5 mL). This step was repeated one more time. Next, 5 mL of 0.5 M DIEA in DMF was added and shaken for additional 1 h to complete Nbz formation (repeated twice), and washed well with DMF (3 × 5 mL), DCM (3 × 5 mL) and dried under vacuum.

Deprotection and cleavage: The peptide was cleaved off resin using TFA:triisopropylsilane (TIPS):H₂O (95:2.5:2.5) cocktail for 2 h at room temperature. The cleavage mixture was filtered and the resin was washed with TFA. The combined solutions were concentrated by N₂ bubbling, to which 8-fold volume of cold ether was added dropwise. The precipitated crude peptides were centrifuged, ether was removed and the crude peptides dissolved in MeCN-water (1:1) containing 0.1% TFA and was further diluted to ~25% MeCN with water and lyophilized.

Procedure of thioesterification: The dried crude peptides were dissolved in 20 mL PB buffer (0.2 M sodium phosphate, 6 M Gn·HCl, pH 7) and treated with 5% MMP (Methyl 3-mercaptopropionate) or 200 mM MPAA (4-Mercaptophenylacetic acid) for 1 h~2 h at room temperature. The reaction was monitored by analytical HPLC (XSelect C18 column, 3.5 μm, 130 Å, 4.6 × 150 mm) at 220 nm, and confirmed by ESI-MS. The peptide thioesters were purified by preparative RP-HPLC, using a gradient of 25-45% B over 51 min.

MeONH₂ deprotects Sez and reacts with the thioester to give 2

Peptide **1** (1.6 mg, 2 μmol, final conc. 2 mM) was dissolved in 1 mL PB buffer (0.2 M NaH₂PO₄, 6 M Gn·HCl, 0.2 M MeONH₂, pH 4.5) at room temperature. After 17 h, peptide **2** was formed in 95% yield (HPLC) (Figure S1). The progress of reaction was followed by analytical HPLC using a XSelect C18 column (3.5 μm, 130 Å, 4.6 × 150 mm) at 220 nm.

One-pot Cu(II)-mediated deprotection of Sez, cyclization and deselenization for the synthesis of cyclic peptides.

Three one-pot deprotection, cyclization and deselenization strategies were investigated (Figure S3). **(I)**: 1.5 equiv CuCl₂ was added to 500 μL PB buffer (0.2 M NaH₂PO₄, 6 M Gn·HCl, pH 6), and peptide **3a** (2 μmol) was then added to the buffer. The reaction mixture was shaken every five minutes under aerobic conditions. After 30 min, 7.1 mg TCEP (0.05 M) and 9.9 mg sodium ascorbate (0.1 M) were added to the reaction mixture and retained at pH 7 (adjusted with 1 M NaOH). The cyclization reaction was completed within 30 min. **(II)**: 1.5 equiv CuCl₂ was added to 500 μL of the same PB buffer, and peptide **3a** (2 μmol) was then added to the above buffer. The reaction mixture was shaken every five minutes. After 30 min, 100 μL TCEP (5 equiv) and MPAA (1 equiv) solution was added to the reaction mixture and retained at pH 7 (adjusted with 1 M NaOH). The cyclization reaction was completed within 30 min. The reaction mixture was extracted three times by ether, TCEP (200 equiv) was added to the reaction mixture and deselenization reaction was completed within 3 h at pH 5 (adjusted with 1 M NaOH) under the argon atmosphere, giving the desired cyclic peptide c(ALKFAG) as the major product. **(III)** 1.5 equiv CuCl₂ was added to 500 μL of the same PB buffer, and **3b** (2 μmol) was then added to the above buffer. The reaction mixture was shaken every five minutes. After 30 min, 100 μL TCEP (5 equiv), MPAA (1 equiv) and MeONH₂ (5 equiv) solution was added to the reaction mixture and retained pH 7 (with 1 M NaOH). The cyclization reaction was completed within 30 min under these conditions. The reaction mixture was extracted three times by ether, TCEP (200 equiv) was added to the reaction mixture and deselenization reaction could be completed within 3 h at pH 5 (adjusted with 1 M NaOH) under argon atmosphere giving the desired cyclic peptide c(ALKFAT) as the

major product. The progress of reactions were monitored by analytical HPLC (linear gradient of 5% to 70% B over 25 min, 1 mL/min) using a XSelect C18 column (3.5 μm , 130 \AA , 4.6 \times 150 mm) at 220 nm.

General procedure for the synthesis of cyclic peptide. 1.5 equiv CuCl_2 was incubated in 500 μL PB buffer (0.2 M NaH_2PO_4 , 6 M $\text{Gn}\cdot\text{HCl}$, pH 6) for 5 min, peptide thioesters (2 μmol) were then added to the above buffer. The reaction mixture was shaken every five minutes. After 30 min, 100 μL TCEP (5 equiv), MPAA (1 equiv) and MeONH_2 (5 equiv) solution was added to the reaction mixture and retained pH 7 (adjusted with 1 M NaOH). The cyclization reaction could be completed within 30 min. The reaction mixture was extracted three times by ether, TCEP (200 equiv) was added to the reaction mixture and deselenization reaction could be completed within 3 h at pH 5 (adjusted with 1 M NaOH) under argon atmosphere. The progress of reactions was monitored by analytical HPLC (linear gradient of 5% to 70% B over 25 min, 1 mL/min) using a XSelect C18 column (3.5 μm , 130 \AA , 4.6 \times 150 mm) at 220 nm. Isolated yields were calculated based on the amount of each peptide thioester used.

The synthesis of WT-Kalata S

The synthesis of linear WT-Kalata S was carried out on hydrazide functionalized chlorotriyl resin (1.8 mmol/g, 0.25 mmol scale) on automated peptide synthesizer. Chlorotriyl resin (1.8 mmol/g, 0.25 mmol scale) was swelled in DMF for 1 h. The resin was doubly treated with freshly prepared 10% hydrazine in DMF for 30 min and drained. The resin was washed well with DMF and then treated with 10% MeOH in DMF for 30 min. Subsequent steps were completed with standard machine assisted Fmoc-SPPS. After peptide chain assembly, the resin was washed well with DMF/DCM, and dried under vacuum. The peptide was cleaved using TFA:water:TIS (95%: 2.5%: 2.5%) cocktail for 2 h. The cleavage mixture was filtered and washed by TFA. The combined solution was concentrated with N_2 bubbling, to which 8-fold volume of cold ether was added dropwise. The precipitated crude peptide was centrifuged (5000 rpm, 10 min), ether was removed and the crude peptide dissolved in MeCN-water (1:1) containing 0.1% TFA and was further diluted to ~25% MeCN with water and lyophilized. The crude peptide were dissolved in MeCN/ H_2O and purified by prep RP-HPLC (XSelect C18 column: 5 μm , 30 \times 250 mm) using a gradient of 28-48% B over 50 min at 220 nm. The isolated yield was 20%. The objective peak was determined by ESI-MS (obs. 2914.54 Da, calc. 2914.21 Da, Figure S4).

Cyclization of linear WT-Kalata S-NH NH_2 : The WT-Kalata S-NH NH_2 (0.7 μmol , 2.1 mg) was dissolved in 700 μL PB buffer (0.2 M NaH_2PO_4 , 6 M $\text{Gn}\cdot\text{HCl}$, 200 mM MPAA, pH 2.5), 10 equiv acetylacetone (acac) was added to the mixture and the reaction mixture was held at room temperature for 3 hours. The pH of the reaction mixture was adjusted to 7 with 1 M NaOH . The cyclization reaction was complete within 1 h. The reduced cyclic WT-Kalata S was obtained in 37% isolated yield (0.26 μmol , 0.75 mg) after purification by semi-preparative RP-HPLC (XSelect C18 column: 5 μm , 10 \times 150 mm) using a gradient of 5-70% B over 20 min. The product was characterized by ESI-MS analysis (obs. 2882.35 Da, calc. 2882.17 Da, Figure S5).

Oxidative folding of the reduced Kalata S⁷². The purified reduced cyclic WT-Kalata S (0.6 mg, 0.21 μmol , final concentration 0.42 mM) was oxidatively folded in the presence of reduced glutathione (10 mM; GSH) in NH_4HCO_3 buffer solution (50 mM NH_4HCO_3 ; pH 7.5 containing 50% *i*PrOH). Under these conditions, the reduced Kalata S was converted to the native structure quantitatively

(>95%) within 28 h. The product was isolated in 57% yield and characterized by ESI-MS analysis (obs. 2876.52 Da, calc. 2876.13 Da, Figure S6).

The synthesis of Se-Kalata S

The synthesis of linear Se-Kalata S was similar to the synthesis of linear WT-Kalata S, on hydrazide functionalized chlorotrityl resin (1.8 mmol/g, 0.25 mmol scale) and by automated peptide synthesizer, except that Boc-Sez-OH was coupled at the N-terminal. Sez was manually doubly coupled for 2 h (3.0 equiv Boc-Sez-OH activated on ice for 5 min using 3.0 equiv OxymaPure and 2.9 equiv DIC). After peptide chain assembly, the resin was washed well with DMF and DCM, and dried under vacuum. The peptide was cleaved using TFA:water:TIS (95%: 2.5%: 2.5%) cocktail for 2 h. The cleavage mixture was filtered and washed by TFA. The combined solution was concentrated with N₂ bubbling, to which 8-fold volume of cold ether was added dropwise. The precipitated crude peptide was centrifuged (5000 rpm, 10 min), ether was removed and the crude peptide dissolved in MeCN/H₂O (1:1) containing 0.1% TFA and was further diluted to ~25% MeCN with water and lyophilized.

Thioesterification of Sez-Kalata S-NHNH₂: The crude C-terminal hydrazide peptide was converted to the C-terminal MPAA thioester using the same procedure as for WT-Kalata S. The Sez-Kalata S-MPAA was obtained in 5% isolated yield (12.5 μmol, 39 mg) after purification by preparative RP-HPLC (XSelect C18 column: 5 μm, 30 × 250 mm, at 220 nm) using a gradient of 25-45% B over 30 min. The product was characterized by ESI-MS analysis (obs. 3109.96 Da, calc. 3110.14 Da, Figure S7).

Cyclization of Sez-Kalata S-MPAA: 1.5 equiv CuCl₂ (2.1 μmol, 0.3 mg) was added to 500 μL PB buffer (0.2 M NaH₂PO₄, 6 M Gn·HCl, pH 6) and Sez-Kalata S-MPAA (0.7 μmol, 2.2 mg) was added to the above buffer at room temperature. The reaction mixture was shaken every five minutes. The deprotection reaction was completed within 30 min. 14.3 mg TCEP (50 μmol, 71 equiv) and 19.8 mg (100 μmol, 142 equiv) sodium ascorbate were added to the reaction mixture and the pH of reaction system was adjusted to 7 (with 1 M NaOH). After 30 min, the cyclization reaction was completed, and 50 equiv DTT was added to the reaction mixture to get reduced Se-Kalata S. The reduced cyclic Se-Kalata S was obtained in 40% isolated yield (0.28 μmol, 0.8 mg) after purification by semi-preparative RP-HPLC (XSelect C18 column: 5 μm, 10 × 150 mm, at 220 nm) using a gradient of 25-45% B over 30 min. The product was characterized by ESI-MS analysis (obs. 2929.30 Da, calc. 2930.12 Da, Figure S8).

Oxidative folding of the reduced Se-Kalata S: The purified reduced cyclic Se-Kalata S (0.6 mg, 0.2 μmol, final concentration 0.4 mM) was oxidatively folded in the presence of reduced glutathione (10 mM; GSH) in NH₄HCO₃ buffer solution (50 mM; pH 7.5 containing 50% iPrOH). Under these conditions, the reduced Se-Kalata S was converted to the native structures quantitatively (>97%) within 3 h. The product was characterized by ESI-MS analysis (obs. 2923.09 Da, calc. 2924.07 Da, Figure S9).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: HPLC chromatograms, LC- MS, HRMS and CD spectra of products (PDF)

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Notes

The authors declare no competing financial interest.

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