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Investigation of peptide thioester formation via *N*→*Se* acyl transfer

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Native chemical ligation is widely used for the convergent synthesis of proteins. The peptide thioesters required for this process can be challenging to produce, particularly when using Fmoc-based solid-phase peptide synthesis. We have previously reported a route to peptide thioesters, following Fmoc solid-phase peptide synthesis, via an *N*→*S* acyl shift that is initiated by the presence of a C-terminal cysteine residue, under mildly acidic conditions. Under typical reaction conditions, we occasionally observed significant thioester hydrolysis as a consequence of long reaction times (~48 h) and sought to accelerate the reaction. Here, we present a faster route to peptide thioesters, by replacing the C-terminal cysteine residue with selenocysteine and initiating thioester formation via an *N*→*Se* acyl shift. This modification allows thioester formation to take place at lower temperatures and on shorter time scales. We also demonstrate how application of this strategy also accelerates peptide cyclization, when a linear precursor is furnished with an N-terminal cysteine and C-terminal selenocysteine.

Keywords: native chemical ligation; selenocysteine; acyl transfer; thioester

Introduction

The synthesis and semi-synthesis of proteins often rely on techniques by which peptides and proteins can be ligated under mild conditions. The advent of native chemical ligation (NCL) saw the formation of a native peptide bond, in aqueous solution and at near neutral pH, between two components adorned with an N-terminal cysteine and a C-terminal thioester, respectively [1,2]. The generation of the peptide or protein C-terminal thioester is frequently the most significant obstacle to this ligation strategy. A thioester is generally unstable to Fmoc-based chemistry, which is a popular method of peptide synthesis due to the common perception that Fmoc-based chemistry is more compatible with chemically fragile side-chain appendages. Consequently, strategies for the construction of peptide thioesters, particularly those employing Fmoc-based chemistry, have been sought and developed [3–7].

Native peptides and proteins harboring a C-terminal cysteine residue can spontaneously fragment to a C-terminal thioester and cysteine in a reverse NCL-type process upon exposure to sodium 2-mercaptoethanesulfonic acid (MESNa) at mildly acidic pH [8–11]. Maximum conversion to thioester is generally observed by heating the precursor peptide to between 50 °C and 60 °C for 24–48 h. In many cases, these conditions are tolerated, although thioester hydrolysis and peptide hydrolysis at aspartate residues (at lower pH) have been observed as significant side reactions. Additionally, while the reaction can differentiate between Xaa–Cys junctions, exhibiting some preference for forming His, Cys, and Gly thioesters, it is unlikely that we could distinguish efficiently between two such motifs within a single peptide sequence.

Although we have found this reaction operationally simple and effective in many instances, we have been keen to accelerate thioester formation to minimize hydrolysis, decrease heating and heating time, and additionally to improve the selectivity of the reaction, reducing the need for protecting groups on cysteines at sites other than the target Xaa–Cys junction. Consequently, we investigated selenocysteine as an acyl transfer facilitator.

The use of selenocysteine (Sec) as a means to improve known reactions involving cysteine is widespread. For example, its use in NCL was reported by two groups in 2001 [12,13], and substitution of the N-terminal cysteine for selenocysteine gave a more rapid ligation to a peptide fragment comprising a C-terminal thioester. This approach has since been extended to selenocysteine-mediated cyclization of peptides via NCL [14] as well as the semi-synthesis of proteins using expressed protein ligation [15,16]. The selenocysteine incorporated into the peptide or protein at the ligation site can be reduced, eliminated, or alkylated at the selenol to yield alanine, dehydroalanine, or an unnatural amino acid, respectively [14,17,18].

Selenocysteine can readily be incorporated into synthetic peptides during Fmoc solid-phase peptide synthesis (SPPS) as the Se-*p*-methoxybenzyl (PMB) protected derivative, with PMB deprotection and resin cleavage occurring simultaneously [19]. Compared with cysteine, selenocysteine has a similar electronegativity, but a selenolate anion (RSe^-) is a better nucleophile than a thiolate (RS^-) at lower pH, a property partly attributable to the lower pKa of the selenol [20] (pK_A Sec–SeH = 5.24, Cys–SH = ~8 [21]). Consequently, we hypothesized that, by introducing selenocysteine at the C-terminus of a peptide, we could generate thioesters more rapidly [22], proceeding via an initial *N*→*Se* acyl shift (Scheme 1). The product thioester would then be used in NCL as normal. Although selenocysteine is known to be prone to diselenide and selenosulfide formation, as well as deselenation [14,18], it is hoped that the lower pH (pH < 6) might minimize these undesirable reactions.

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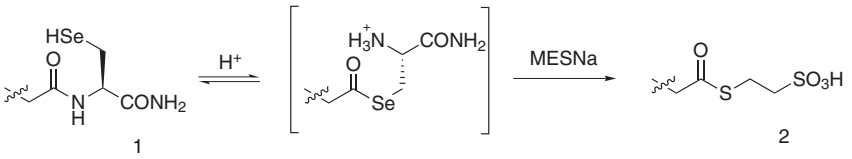

Scheme 1. Proposed reverse NCL employing C-terminal selenocysteine peptide **1** as the nucleophile and leaving group, resulting in thioester **2**.

In this study, we prepared model thioester precursors, including ^{13}C-labeled Sec-containing peptides and examined thioester formation under a range of conditions. The advantages and disadvantages of using Sec in place of Cys became apparent in acyl transfer processes, and an optimized procedure was employed to effect the cyclization of an antimicrobial peptide derived from a β-defensin.

Materials and Methods

Materials

All reagents and solvents (excluding HPLC solvents) were obtained from Sigma (Sigma-Aldrich Ltd. Dorset, UK) as standard laboratory grade and used as supplied unless otherwise stated. All standard resins and Fmoc amino acids for peptide synthesis were purchased from Novabiochem (Merck KGaA, Darmstadt, Germany).

Instruments

^{1}H NMR spectra were recorded at 500 MHz or 400 MHz and ^{13}C NMR spectra at 125 MHz or 100 MHz on Bruker (Bruker, Coventry, UK) 500 MHz and 400 MHz instruments, respectively. Chemical shifts (δ) were reported in ppm and coupling constants (J) in Hz, signals were sharp unless stated as broad (br), s, singlet, d, doublet, t, triplet, q, quartet, and m: multiplet. Mass spectra were obtained on Waters (Waters MS Technology Centre, Manchester, UK) uPLC/SQD-LC series electrospray mass spectrometer. LC-MS was performed using a gradient of 1–95% acetonitrile containing 0.1% formic acid over 10 min (flow rate of 0.6 ml/min). Semi-preparative HPLC was performed using a Phenomenex (Phenomenex, Cheshire, UK) LUNA C18 column and a gradient of 5–60% acetonitrile containing 0.1% TFA over 45 min (flow rate of 4.0 ml/min).

Synthesis of Fmoc-Sec(PMB)-OH [19]

p-Methoxybenzyl chloride

p-Methoxybenzyl alcohol (3.90 g, 28.5 mmol) was dissolved in dry chloroform (20 ml) and cooled to 5 °C. Thionyl chloride (3.1 ml, 42.8 mmol) was added with care to keep the temperature below 10 °C. The reaction mixture was allowed to warm to room temperature and stirred for 2 h. The reaction was then basified to pH 8.0 by slow and careful addition of saturated sodium bicarbonate solution. The product was extracted using chloroform (3 × 30 ml), and the combined organic layers were washed with distilled water (3 × 20 ml) then dried over magnesium sulfate. The solution was filtered under gravity and the solvent removed under vacuum to afford the crude product (4.03 g, 25.7 mmol, 90%) as an oil that was used without further purification. TLC: R_f 0.73 (30% ethyl acetate in *n*-hexane). *m/z* (ESI-TOF-MS) calculated $[MH]^+$ 156.03364, found $[MH]^+$ 156.03348. ^{1}H NMR: δ_H (500 MHz, $CDCl_3$) 7.33 (2H, d, J = 8.6, Ar*H*), 6.89 (2H, d, J = 8.6, Ar*H*), 4.58 (2H, s, CH_2), 3.82 (3H, s, O–CH_3). ^{13}C NMR: δ_C (125 MHz, $CDCl_3$) 130.18 (*C*H), 114.23 (*C*H), 55.40 (O–CH_3), 46.42 (CH_2).

p-Methoxybenzyl selenocysteine

Sodium borohydride (0.45 g, 11.84 mmol) was added portion-wise to an ice-cooled solution of selenocystine (0.50 g, 0.50 mmol) in 0.5-M sodium hydroxide (1.3 ml). The mixture was kept in an ice bath and stirred until the yellow color had disappeared. To the mixture was added 2-M sodium hydroxide (3.9 ml), followed by dropwise addition of *p*-methoxybenzyl chloride (1.22 g, 7.77 mmol). The reaction mixture was stirred vigorously at 4 °C for 4 h. The mixture was acidified with concentrated hydrochloric acid to form a precipitate of the crude product that was collected by filtration and washed thoroughly with diethyl ether and water to afford a white solid (0.52 g, 1.8 mmol, 60%), which was used without further purification. TLC: *Rf* 0.04 (chloroform : methanol : acetone, 8.5 : 1 : 0.5).

Fmoc-p-methoxybenzyl selenocysteine

Fmoc succinimide (0.42 g, 1.23 mmol) was dissolved in acetonitrile (2.22 ml) and added to an ice-cooled suspension of *p*-methoxybenzyl selenocysteine (0.37 g, 1.28 mmol) in a mixture of distilled water (2.22 ml) and triethylamine (178 µl, 1.28 mmol). Further, triethylamine was added (178 µl 1.28 mmol) to maintain a pH above pH 8, and the reaction was stirred at room temperature for 1 h. The mixture was then acidified with 1-M hydrochloric acid and the crude product extracted using ethyl acetate. The combined organic layers were washed with 1-M hydrochloric acid followed by saturated sodium chloride solution. The organic phase was then dried over magnesium sulfate and filtered under gravity, and the solvent removed under vacuum. The resulting oil was washed in *n*-hexane (5 ml), which was decanted, and the remaining solvent removed by vacuum to afford the product (0.47 g, 0.91 mmol, 71%) as a white foam. TLC: R_f 0.70 (chloroform : methanol : acetone, 8.5 : 1 : 0.5). NMR ^{1}H: δ_H (500 MHz, $CDCl_3$) 7.79–6.21 (12H, m, Ar*H*), 4.58 (1H, m, *CH*α), 3.79–3.69 (5H, Fmoc *CH*, Fmoc CH_2, Se–CH_2–Ar), 3.65 (3H, s, OCH_3), 2.93 (1H, m, CH_2), 2.82 (1H, m, CH_2). *m/z* (ESI-TOF-MS) calculated $[M + Na]^+$ 534.0770, found 534.0796.

Fmoc-glycine (^{13}C-1)

As reported in Kang *et al*. 2009 [23]. Glycine (^{13}C-1) (0.25 g, 3.25 mmol) was dissolved in distilled water (3.13 ml), and triethylamine (453 µl, 3.25 mmol) was added while the mixture was continuously stirred. 9-fluorenylmethyl succinimidyl carbonate (1.06 g, 3.155 mmol) was then dissolved with heating in acetonitrile (3.13 ml) and added in one portion to the amino acid solution. Further, triethylamine (453 µl, 3.25 mmol) was added to maintain a pH between pH 8.5 and pH 9.0. The reaction mixture was stirred at room temperature for 0.5 h and then concentrated *in vacuo*. The concentrate was poured into 1.5-M hydrochloric acid (14 ml). The solution was then filtered under gravity and the product extracted with DCM (3 × 6.25 ml). The combined organic extracts were washed with 1.5-M hydrochloric acid (6.25 ml), distilled water (6.25 ml), and a solution of saturated aqueous sodium chloride (6.25 ml). The organic phase was dried

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over anhydrous magnesium sulfate and the solvent removed *in vacuo*. The addition of petroleum ether (2.5 ml) afforded a white precipitate that was filtered and dried *under vacuo* to a white solid (0.81 g, 2.7 mmol, 83%). ^{1}H NMR: (DMSO, 400 MHz) δ_H/ppm: 7.90 (2H, d, J = 8.0, FmocAr–H), 7.73 (2H, d, J = 8.0, Fmoc Ar–H), 7.62 (1H, t, J = 6.0, NH), 7.42 (2H, t, J = 8.0, Fmoc Ar–H), 7.34 (2H, t, J = 8.0, Fmoc Ar–H), 4.30 (2H, d, J = 4.0 Fmoc CH_2–O), 4.23 (1H, m, Fmoc C*H*–CH_2), 3.67 (2H, t, J = 6.0, Gly–αCH_2); ^{13}C NMR: (DMSO, 100 MHz) δ_C/ppm: 172.6 (CO_2H). *m/z* (ESI-TOF-MS) calculated $[M + Na]^+$ 321.0933, found 321.0928.

General Synthesis Procedures for Sec-containing Peptides (Manual Procedures)

Fmoc-Sec(PMB)-Rink amide MBHA resin

Rink amide MBHA resin, loading = 0.56 mmol/g, (89 mg, 0.05 mmol) was treated with 20% piperidine (v/v) in DMF (2 ml, 15 min). The solution was then filtered and the resin exhaustively washed with DMF and DCM. The resin was then coupled to Fmoc-Sec(PMB)-OH (127.6 mg, 0.25 mmol) with 0.45-M HBTU/HOBt (0.55 ml) and DIPEA (75 μl) in dry DMF (0.55 ml), on a horizontal shaker (300 rpm) at room temperature for 4 h. The resin was then filtered off and washed exhaustively with DMF and DCM. Following Fmoc analysis to determine coupling efficiency, the washed resin was then transferred to a reaction vessel for automated SPPS for elongation to the desired peptide sequence.

Fmoc-Sec(PMB)-NovaSyn TGA resin

NovaSyn TGA resin, loading = 0.22 mmol/g, (128 mg, 0.05 mmol) was allowed to swell in DCM (15 min). The resin was then filtered off and then *double* coupled to Fmoc-Sec(PMB)-OH as earlier.

Fmoc-G(^{13}C-1)U(PMB)-Rink amide MBHA resin and Fmoc-G(^{13}C-1)U (PMB)-NovaSyn TGA resin

The washed and dried Fmoc-Sec(PMB)-Rink amide MBHA resin or Fmoc-Sec(PMB)-NovaSyn TGA resin was treated with 20% piperidine (v/v) in DMF (15 min), the solution filtered off, and the resin then washed exhaustively with DMF and DCM. The resin was then coupled to Fmoc-Gly(^{13}C-1)-OH (75 mg, 0.25 mmol) with 0.45-M HBTU/HOBt (0.55 ml) and DIPEA (75 μl) in dry DMF (0.55 ml), on a horizontal shaker at room temperature for 4 h. The resin was then filtered off and washed exhaustively with DMF and DCM. Following ninhydrin test analysis, the washed resin was then transferred to a 433A reaction for elongation to the desired peptide sequence.

Automated Peptide Synthesis

Automated solid-phase synthesis was carried out on 0.05-mmol scale, on an Applied Biosystems ABI 433A peptide synthesizer (Applied Biosystems – Life Technologies Ltd, Paisley, UK) according to the manufacturers' instructions. The Fast-moc™ protocol for SPPS was employed, using 10 equivalents of each standard Fmoc-protected amino acid in the sequence, and each coupling reaction was carried out using HBTU/HOBt in the presence of DIPEA (see Table 1 for assembled sequences).

Post-Synthesis Procedures

Following chain assembly, the resin was then removed from the reaction vessel, washed exhaustively with DMF and DCM, and dried. The dry resin was treated with a cleavage cocktail (4.0 ml) comprised of TFA (97.5% v/v) and thioanisole (2.5% v/v) with 2,2′-dithiobis(5-nitropyridine) (DTNP) (see Table 1 for quantities of DTNP added) for 5 h at room temperature with gentle stirring.

Table 1. Target peptide sequences[a] and quantities of DTNP used during cleavage

Sequence	DTNP (eq)
H-MEELYKSHU- Rink Amide resin	1.3
H-MEELYKSG(^{13}C-1)U-Rink Amide resin	1.3
H-MEELYKSG(^{13}C-1)U- NovaSyn TGA resin	1.3
H-DTHFPICIFCCGU- Rink Amide resin	10
H-DTHFPIC(Acm)IFC(Acm)C(Acm)GU- Rink Amide resin	1[b]
H-CRKFFARIRGGRGU- Rink Amide resin	5

[a]Unless otherwise stated, the resin-bound peptides carry the standard side-chain protecting groups for Fmoc-SPPS.
[b]In this case, thioanisole was omitted from the cleavage cocktail.

After exposure to the cleavage cocktail for 5 h, the resin was filtered off and the filtrate added to cold diethyl ether (10× volume) to induce precipitation of the peptide. The precipitate was collected by centrifugation (1500 rpm, 4 °C, 15 min), the supernatant removed, and the precipitated peptide resuspended in diethyl ether and centrifuged once more. The white precipitate was dissolved in water and then purified by semi-preparative reverse phase HPLC (gradient: 5–60% acetonitrile/45 min). Fractions containing the product were identified by LC-MS and lyophilized to afford pure peptides as white fluffy solids (Table 2).

General procedure for thioester formation

Sec(5-Npys)-terminated peptide **4** (1 mg) was dissolved in 0.1-M sodium phosphate buffer; pH 5.8 (0.9 ml), containing MESNa (100 mg) and *tris*-carboxyethylphosphine (TCEP) (5 mg) to a total volume of approximately 1 ml, and shaken on a Eppendorf thermomixer (Eppendorf, Stevenage, UK) at temperatures ranging from 40–60 °C. 20-μl aliquots of reaction mixture were removed for analysis by analytical HPLC over a period of 48 h. After 48 h, a 3-μl aliquot of each reaction mixture was subjected to LC-MS analysis and up to three species derived from **4** were generally observed with masses: 1161.4 Da $[MH]^+$ corresponding to the thioester **7**, 1325.4 Da $[MH]^+$ corresponding to the seleno-sulfide adduct **6**, and 1037.4 Da $[MH]^+$ derived from hydrolysis of **7**.

*General procedure for thioester formation in ^{13}C-labeled precursors **8**,**10**, **11**, and **12***

Reactions monitored by ^{13}C NMR were prepared in a similar fashion to that described earlier for model peptide **4**. Briefly, ^{13}C-labeled peptides were dissolved to final concentrations of 1 mg/ml in 0.1-M sodium phosphate buffer; pH 5.8 (prepared in D_2O) containing MESNa (10% w/v) and TCEP (0.5% w/v). 0.6-ml aliquots were dispensed into six separate 1.5-ml Eppendorf tubes, and the reactions were shaken in an Eppendorf thermomixer at 60 °C for 48 h. The contents of each Eppendorf tube, once transferred to an NMR tube, were used to obtain an independent estimation of reaction progress at 0, 1, 3, 6, 24, and 48 h by ^{13}C NMR spectroscopy.

*General procedure for thioester formation in hepcidin-derived peptides **13** and **19***

0.1-M sodium phosphate buffer; pH 5.8 in guanidinium hydrochloride was prepared by a 10-fold dilution of 1-M sodium phosphate buffer; pH 5.8 into 6-M guanidinium hydrochloride. Peptide species

Table 2. HPLC retention times (t_R), mass spectrometry characterization, and isolated yields for Sec-containing thioester precursors

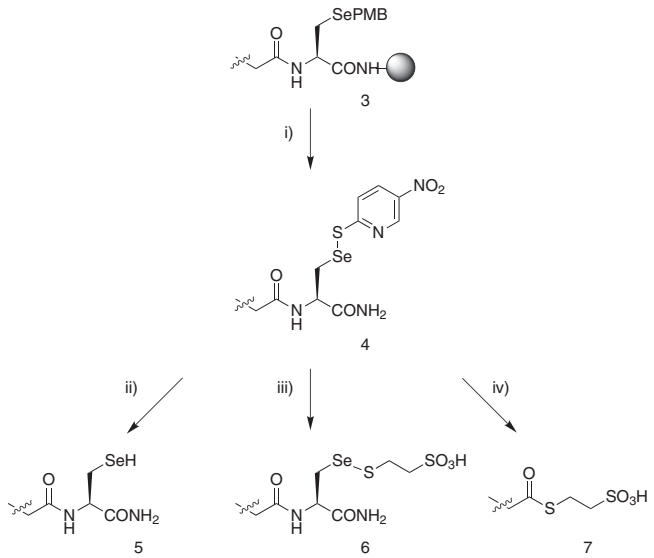
Peptide	t_R/min	Calculated mass/Da (no. of 5-Npys)	Observed mass/Da (ESI-MS) $[MH]^+$	Isolated yield (%)[a]
4	24.8	1339.4 (1)	1340.8	31
8	26.0	1260.2 (1)	1261.2	5
10	25.9	1261.2 (1)	1262.6	7
13A	41.0,	2121.2 (4)	1810.6 (2 × 5-Npys)	1
13B	42.4,		1810.4 (2 × 5-Npys)	2
13C	48.7		2120.8	3
19	33.4	1872.0 (1)	3434.2 (Se–Se dimer)	17
20	27.3	1981.2 (2)	1981.1	30

[a]Isolated yields are based on initial resin loading.

isolated by semi-preparative HPLC and assigned, by LC-MS, as Sec (5-Npys) or Cys(5-Npys) capped derivatives of **13** or **19** were then dissolved to a final concentration of 1 mg/ml in the prepared phosphate buffer solution containing MESNa (10% w/v) and TCEP (0.5% w/v). The reaction was then shaken on an Eppendorf thermomixer at 60 °C for 24 h. 3-μl aliquots of the reaction mixture were subjected to LC-MS analysis, after 6 and 24 h.

Peptide cyclization via N→Se acyl shift

Peptide **20** was dissolved in 0.1-M sodium phosphate buffer (pH 5.8), with MESNa (10% w/v) and sodium ascorbate (5% w/v). The final peptide concentration was 1 mg/ml. The reaction mixture was degassed by freezing the sample in liquid nitrogen and placing the frozen reaction mixture on a freeze drier for 10 min. TCEP-HCl (0.5% w/v) was then added to the thawed sample under a blanket of nitrogen, and the reaction was shaken on an Eppendorf thermomixer at 60 °C and purified after 24 h by semi-preparative reverse-phase HPLC. Fractions containing the cyclic peptide were lyophilized to yield **21** as a white fluffy solid (4.3 mg, 61%), t_R 24.9 min, calculated mass: 1505.8 Da, observed mass (ESI-MS) $[MH_2]^{2+}$ + 753.5 Da, which deconvolutes to 1505.1 Da.



Scheme 2. Synthesis of a peptide thioester **7** from Sec-terminated **4**. Reagents and conditions: (i) 95% v/v TFA, 1.3 equivalent 2,2′-dithiobis (5-nitropyridine). (ii) 50-mM DTT, Na phosphate buffer; pH 7.5. (iii) 10% w/v MESNa, 0.1-M Na phosphate buffer pH 5.8, 60 °C. (iv) 10% w/v MESNa, 0.1-M Na phosphate buffer pH 5.8, 0.5% w/v TCEP-HCl, 60 °C, 6 h.

Results and Discussion

For model experiments, selenocysteine was introduced to synthetic peptides using the Fmoc-Sec(PMB)-OH building block. Although not especially ideal, and highlighting the dearth of available orthogonal protecting groups for Sec, we envisaged that the transformation of Sec(PMB) to the 5-nitropyridyl-2-selenosulfide [Sec(5-Npys)] derivative **4** would enable our proposed study (Scheme 2). Therefore, model peptide **4** (sequence: H-MEELYK-SHU(5-Npys)-NH_2), derived from the C-terminal residues of green fluorescent protein, was obtained in 31% yield following cleavage from Rink amide resin in the presence of DTNP [24]. We first attempted the conversion of the 5-Npys-protected peptide to the free selenol **5**. However, upon exposure to 50-mM dithiothreitol (DTT) at pH 7.5, **5** could only be observed in small quantities and the reaction mixture was comprised mainly of the diselenide after 4 h. Longer exposure to DTT resulted in decomposition to several species after 16 h, suggesting that it may be more beneficial to use the selenosulfide **4** directly as the thioester precursor. Unfortunately, exposure of **4** to typical reaction conditions (10% w/v MESNa, 0.1-M Na phosphate buffer; pH 5.8, 60 °C) did not result in thioester formation, but we instead observed quantitative conversion to selenosulfide **6**.

Upon introduction of TCEP-HCl to the reaction mixture, the desired product **7** could, in fact, be observed and gratifyingly the reaction appeared, by HPLC analysis, to be almost complete within 6 h at 60 °C. The corresponding cysteinyl peptide underwent less than 20% conversion to **7** in the same time.

Furthermore, good conversion from **4** to **7** was observed at lower temperatures (40–50 °C) and was extremely encouraging (Figure 1). At 40 °C, thioester formation, while slow, proceeded smoothly resulting in 73% conversion to **7** within 24 h. The reaction was, however, incomplete, and a small amount (6%) of **4**, and intermediates derived from **4**, remained. After 6 h, less than 11% of **7** had been hydrolyzed, and by 24 h, the amount of hydrolyzed thioester had not increased significantly. At 50 °C, after 6 h, the rate of thioester formation appeared similar to that at 40 °C, reaching 75% conversion to **7** in 24 h; however, at this temperature, all of **4** was consumed. **7** was more prone to hydrolysis at this higher temperature, and at 24 h, 14% of the peptide had been hydrolyzed. At both temperatures, the formation of **7** was fastest within the first 6 h. In contrast, the hydrolysis of **7** occurred at a much slower rate throughout the reactions. At 60 °C, maximum conversion to **7** was reached in 6 h (77%). A

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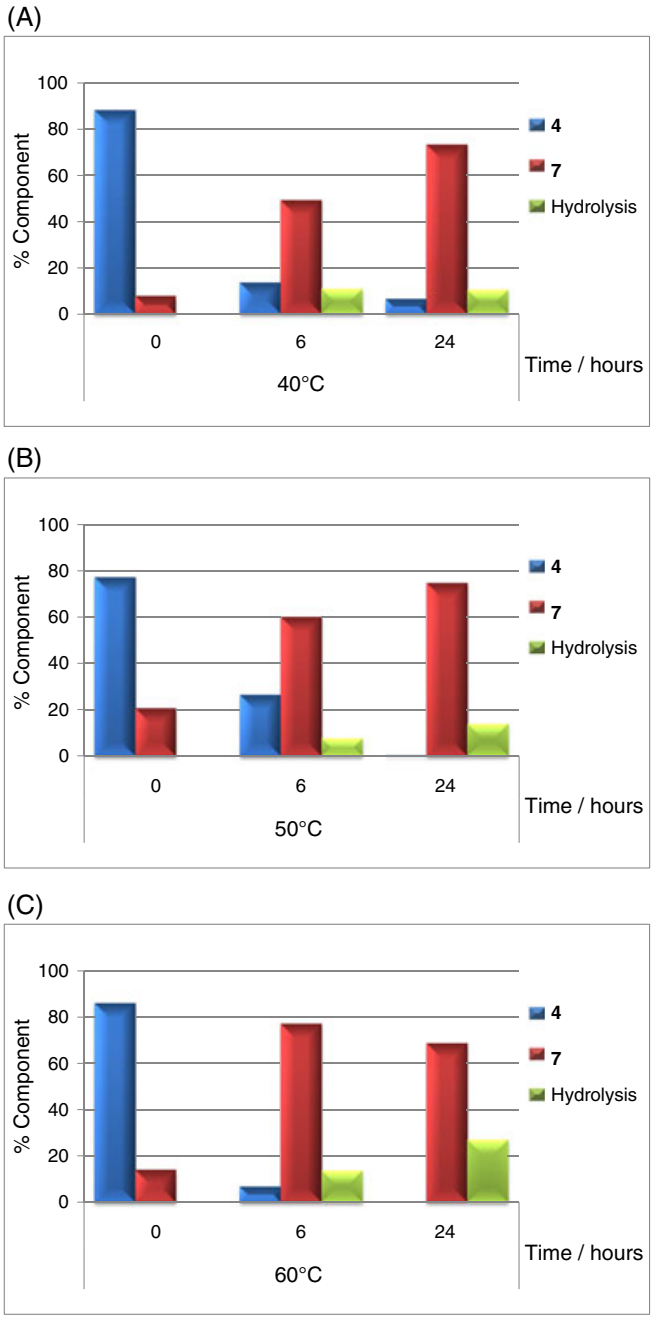

Figure 1. HPLC analysis of the formation of thioester **7** from Sec-terminated **4**. Reagents: 10% w/v MESNa, 0.1-M Na phosphate buffer pH 5.8, 0.5% w/v TCEP-HCl, at (A) 40, (B) 50, and (C) 60 °C. Upon reduction with TCEP, at $t = 0$ h, **4** is transformed to a mixture comprising selenol **5**, selenosulfide **6**, and diselenide, which are consumed as the reaction progresses. The % **4** given above corresponds to the summed total integration for these species, where present.

small quantity of **4** remained but was fully consumed after 24 h. However, at this temperature, **7** was being hydrolyzed more rapidly such that the total amount of **7** was reduced after 24 h, and 27% of the hydrolysis product had accumulated. Although hydrolysis was less prevalent over extended reaction times at lower temperatures, the thioester was formed fastest at 60 °C. The amount of hydrolysis seen at this temperature after 6 h was also comparable with that seen after 24 h at the lower temperatures.

The significant decrease in reaction time by using selenocysteine rather than cysteine was verified by ^{13}C NMR spectroscopy employing a ^{13}C-1-labeled glycine analogue of **4**; **8** (sequence: H-MEELYKS**G**(^{13}**C-1**)U-NH_2, Figure 2). Conversion to the corresponding MESNa thioester **9** (sequence: H-MEELYKS**G**(^{13}**C-1**)-$SCH_2CH_2SO_3H$) could be easily confirmed through appearance of the characteristic thioester signal at ~200 ppm (Figure 2(A)). Notably there was no obvious trace of an *Se*-peptide intermediate, but a small signal at 190 ppm, observed in some spectra, may be indicative of its presence.

^{13}C NMR analysis of the reaction was generally in agreement with the HPLC data, in that the conversion of Sec(5-Npys) precursor to thioester was nearly complete within 6 h and had reached completion within 24 h. When ^{13}C-labeling data for **8** and the corresponding Cys-terminated peptide **12** (H-MEELYKS**G**C-NH_2) were compared, the rate enhancement attributable to the presence of the Sec residue was clear (Figure 2(B)). Interestingly, and as seen previously [9], thioester formation was initially faster with carboxyl-terminated peptide **10** (sequence: H-MEELYKS**G**U-OH) than with **8** affording 60% conversion to thioester after only 3 h. However, this reaction showed little further conversion to thioester after this time, and the ^{13}C NMR spectrum remained largely unchanged after 24 h. In this case, LC-MS indicated that a significant side reaction had occurred, resulting in a product that lacked selenium. The molecular mass of this product coincided exactly with that of the Ala analogue (sequence: H-MEELYKS**G**A-OH) indicating the increased sensitivity of Sec to deselenation, because no analogous side reaction is observed in the corresponding Cys-terminated peptides under identical reaction conditions. Although the simplified isotopic distribution of the molecular ion in the mass spectrum was sufficient to indicate loss of selenium, further analysis of the ^{1}H NMR spectrum of this purified side product supported alanine formation by the presence of a characteristic doublet for the side-chain methyl group at 1.40 ppm. We repeated the reaction of **10** in the presence of 5% w/v sodium ascorbate [25] and did not observe any deselenation. However, once formed, **9** appeared much more susceptible to hydrolysis in the presence of sodium ascorbate. Here, the elimination of one problematic side reaction only exacerbated another, and so, the presence of sodium ascorbate was considered poorly compatible with this process.

Interestingly, thioester hydrolysis appeared generally more prevalent during HPLC analysis of reaction mixtures containing the His-Sec-terminated model peptide when compared with ^{13}C NMR analyses of Gly–Sec-terminated peptide where it is a minor component, if observed at all. This indicated that thioester hydrolysis was either more prevalent in His rather than Gly thioesters or that significant hydrolysis occurred during HPLC sampling of reaction mixtures. Thioester observed at $t = 0$ h in HPLC monitored experiments was also not evident in ^{13}C NMR spectra of Gly–Sec-terminated peptides. Thioester formation across the ^{13}C-labeled Gly–Sec-terminated peptide was then also subjected to HPLC analysis and was in full agreement with the ^{13}C NMR data, supporting the increased reactivity of the His–Sec motif over the Gly–Sec motif.

Having confirmed that thioester formation across these Xaa–Sec motifs can proceed to near completion in approximately 6 h, we were keen to explore whether the reaction had the potential to occur selectively at a single site, even in the presence of additional unprotected Xaa–Cys motifs.

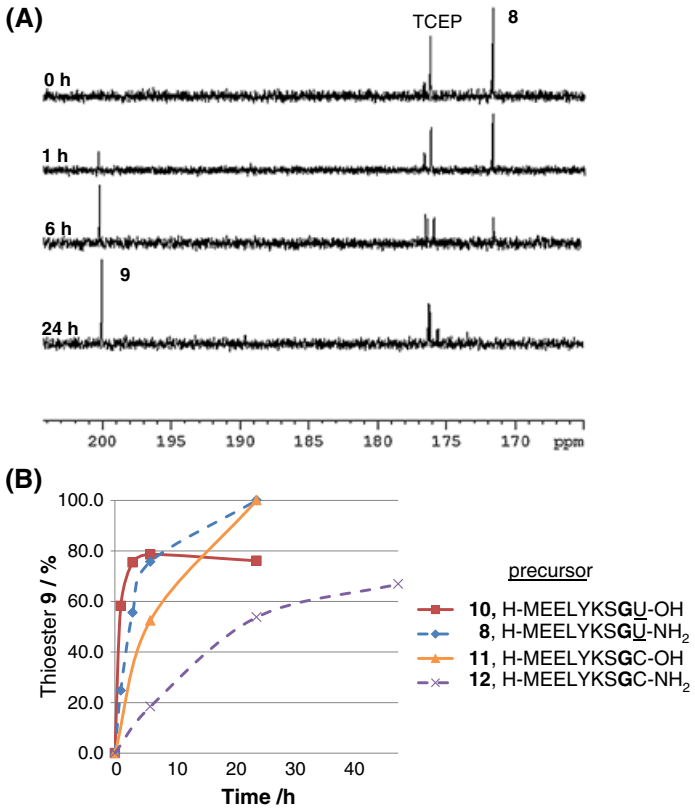

Figure 2. (A) Thioester formation from precursor peptide **8** monitored by ^{13}C NMR after 0, 1, 6, and 24 h. Reagents and conditions: 1 mg/ml peptide, 10% w/v MESNa, 0.1-M Na phosphate buffer pH 5.8 (in D_2O), 0.5% w/v TCEP. (B) Comparison of thioester formation in Sec-terminated and Cys-terminated peptides.

A peptide based on a fragment of human hepcidin comprising residues 1–12 and furnished with a C-terminal Sec(PMB) carboxamide, **13**, was synthesized (Figure 3). Thioesterification of the C-terminal Sec carboxamide was examined because the synthesis was more straightforward, and the conversion to thioester had been observed to take place more rapidly when compared with the C-terminal cysteinyl carboxylate. Additionally, thioester formation, when conducted on peptide **8**, had also been free from reduction of Sec to Ala. This fragment of hepcidin contained three unprotected cysteine residues in addition to the terminal selenocysteine. Furthermore, it possessed a Cys–Cys motif, known to be favorable to form thioesters in the presence of MESNa at pH 5.8. By subjecting this peptide to thioester formation, it is hoped to establish whether the faster thioester formation at Gly–Sec would lead to selectivity being observed for this site, over the internal Xaa–Cys sites.

Upon cleavage of this peptide from the resin, initially employing stoichiometric quantities of DTNP and then with an excess of DTNP, a mixture of peptides protected to varying degrees with the 5-Npys group were obtained. The occurrence of this heterogeneous mixture of 5-Npys-protected starting materials lowered the isolated yield of **13** significantly. In cases where incomplete 5-Npys modification was observed, we were unable to ascertain whether the thiols on the cysteines or the selenol group had been protected, but we suspected that the 5-Npys group had facilitated the formation of an intramolecular selenosulfide bond between a cysteine residue and selenocysteine [24]. We next subjected this mixture of peptides to the reaction conditions, anticipating that the presence of TCEP-HCl would eradicate the variation in the starting material(s) by reducing any disulfide and selenosulfide bonds, as well as cleaving the 5-Npys groups. The reaction was carried out in guanidine hydrochloride to aid the solubility of **13**, and, prior to the addition of TCEP-HCl under nitrogen, the reaction mixture was degassed with the hope of lowering the risk of deselenation.

At $t = 0$ h, and upon addition of all reagents, LC-MS indicated that the starting peptide was indeed mostly reduced peptide, **14**. After 6 h, some of the desired thioester (**15**) had already formed via reaction at the Gly–Sec site on the C-terminus; however, a considerable quantity of unreacted **14** remained, which was in stark contrast to the model studies. After 24 h, the reaction contained a mixture of thioesters formed at the C-terminal Gly–Sec site, as well as the internal Cys–Cys (**16**) site and remarkably at the internal Phe–Cys site (**17**). A small amount of unreacted starting peptide also remained. Unfortunately, also observed to a significant extent was deselenated peptide, **18**, where the C-terminal Sec had been converted to Ala. Notably, after 24 h, the observed mass (1503.9 Da, MH^+) for the *reduced* starting material **14** was low (calculated $m/z = 1505.5$ Da, MH^+) and the sluggish progress of the desired reaction was possibly attributable to the formation of a persistent intramolecular selenosulfide bond, which would also compromise the selectivity as the reaction progressed.

From this investigation, we concluded that hepcidin-derived **13** was too ambitious for an initial substrate on which to demonstrate the benefits of the C-terminal Sec-mediated thioester formation or observe selectivity for thioester formation at Sec over Cys, due to its cysteine-rich nature. Whereas thioester formation across the Ile–Cys junction was not observed, as reported previously, a species corresponding to the Phe-thioester **17** was particularly well represented in the combined total ion chromatogram (TIC). This raises the possibility that direct thiolysis of this Phe–Cys motif or, intriguingly, its production form thioester-terminated **16** is also favorable.

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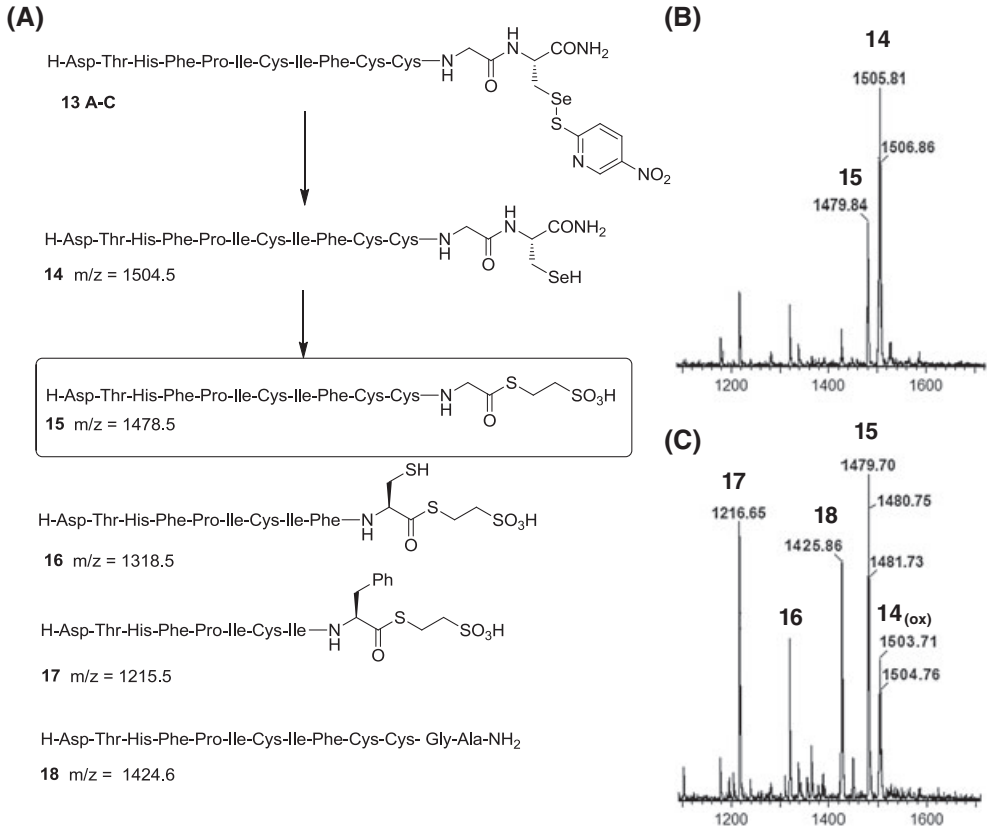
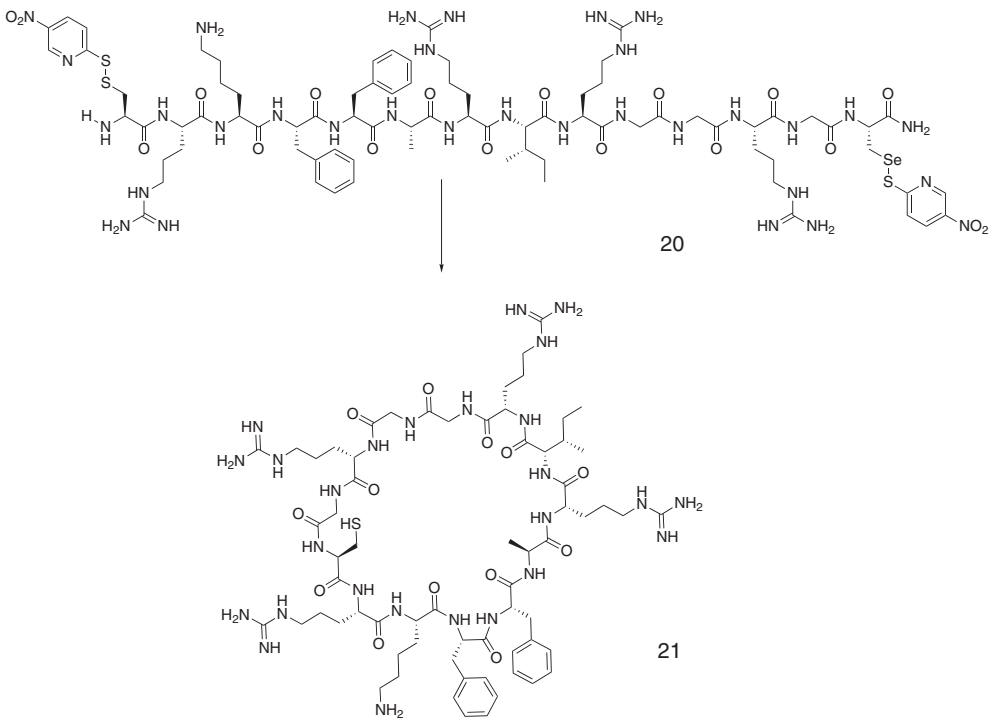


Figure 3. (A) Attempted synthesis of peptide thioester **15** from Sec-terminated **14**. Reagents and conditions: 10% w/v MESNa, 0.1-M Na phosphate buffer pH 5.8, 0.5% w/v TCEP-HCl, 60 °C, 24 h. (B) MS analysis showing $[MH]^+$ of components present in the reaction after 6 h. (C) MS analysis showing $[MH]^+$ of components present in the reaction mixture after 24 h.



Scheme 3. Conversion of linear thioester precursor **20** to cyclic **21**. Reagents and conditions: **20**, 10% w/v MESNa, 0.1-M Na phosphate buffer pH 5.8, 5% w/v Na ascorbate, 0.5% w/v TCEP.

In an attempt to make this hepcidin fragment more compatible with our chemistry, we decided to next protect the Cys residues with Acm protecting groups. However, the synthesis of the Acm-protected thioester precursor **19** was not as straightforward as it was hoped because of the labile nature of the Acm protecting groups in the presence of DTNP. To preserve the Acm groups, we used 1 equivalent of DTNP and omitted thioanisole from the cleavage cocktail [24]. The resulting peptide was isolated as a dimer, presumably joined by an intermolecular diselenide bond, and therefore did not possess any 5-Npys protecting groups. When subjected to thioester formation, we did not see reduction of this bond, which appeared inert to 0.5% w/v (17.5 mM) TCEP. After 6 h, the peptide had undergone quantitative deselenation to afford the alanine terminated product.

In light of this, we wanted to apply our reaction to a substrate that was not quite as cysteine-rich as the fragment of hepcidin. We have previously shown a fragment of Defb14 (sequence: H-CRKFFAR-IRGGRGC-OH), the mouse orthologue of the human β-definsin-3 (hBD-3), to undergo cyclization by virtue of a C-terminal and an N-terminal cysteine and subjected to thioester formation at pH 5.8. Upon conversion to the C-terminal thioester, the peptide spontaneously underwent intramolecular NCL [26].

We isolated the desired thioester precursor containing seleno-cysteine at the C-terminus (**20**, sequence: H-C(5-NPys)RKFFAR-IRGGRGU(5-Npys)-NH_2) in 30% yield and subjected it to our usual reaction conditions (10% w/v MESNa, 0.1-M Na phosphate buffer; pH 5.8, 0.5% w/v TCEP, 60 °C). After 6 h, a small amount of thioester and cyclic peptide could be observed by LC-MS; however, the reaction appeared to be mainly comprised of **20** and deselenated **20** (sequence: H-CRKFFARIRGGRGA-NH_2). After 24 h, the reaction contained mostly deselenated material.

To eliminate deselenation, likely initiated by the presence of air and TCEP, we reintroduced the antioxidant sodium ascorbate to the reaction [25], and in this case, the major product after 24 h was the cyclic peptide **21** (Scheme 3), which we were able to isolate in 61% yield. In contrast to what we observed in the case of peptide **10**, hydrolysis of the presumed intermediate thioester (which was enhanced in the presence of sodium ascorbate) was not observed. This was likely to be due to the transient nature of the thioester, which was not observed by LC-MS during the reaction.

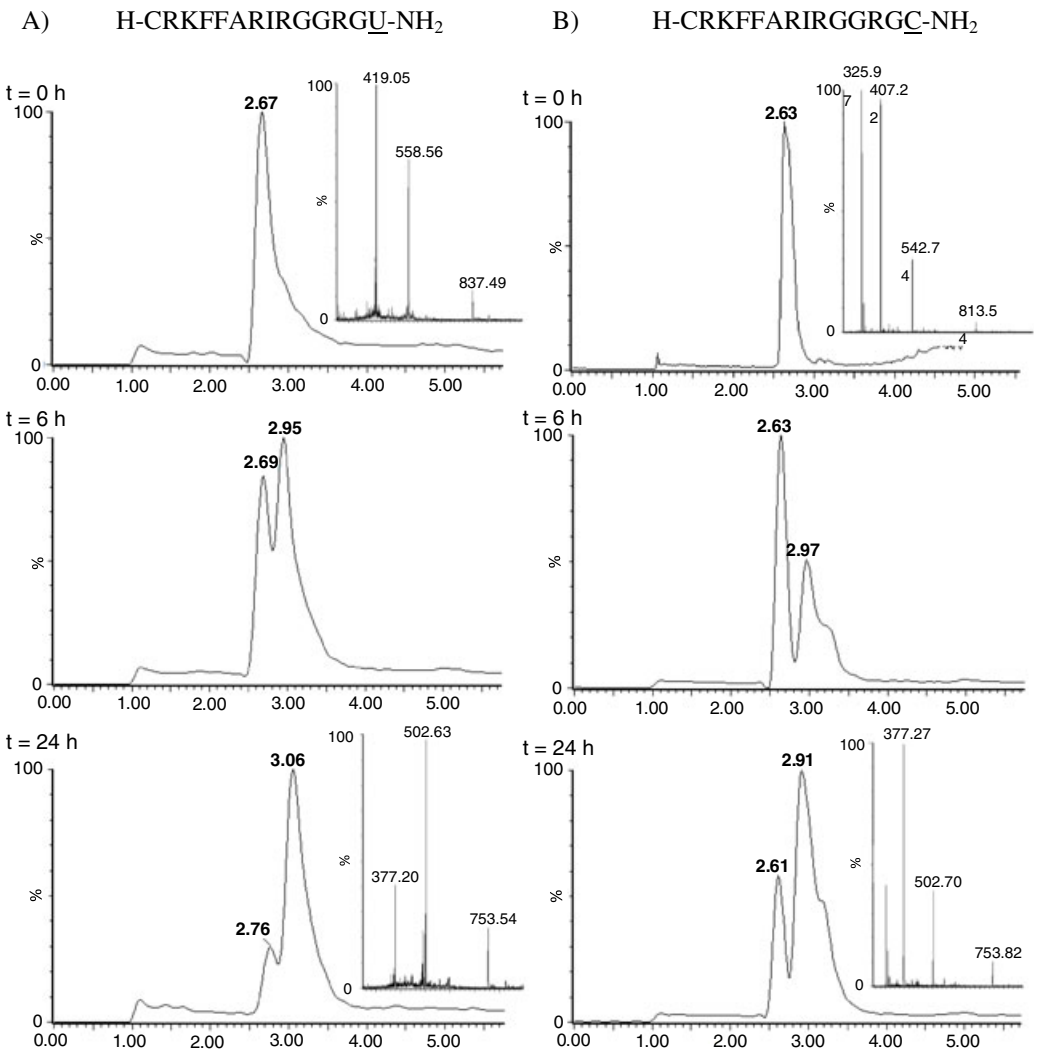


Figure 4. Comparison of Sec-terminated (panel a) and Cys-terminated (panel b) peptides as they undergo cyclization under identical reaction conditions. The figure shows TIC traces from LC-MS analysis of each reaction mixture at 0, 6, and 24 h. The mass spectra (inset) are obtained by combining the data across the full-width at half maximum peak height of the most abundant peak in the TIC. (A) H-CRKFFARIRGGRGU-NH_2 and (B) H-CRKFFARIRGGRGC-NH_2.

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This isolated yield of this cyclization (61%) certainly appeared superior to the corresponding reaction of the Cys-carboxylate-terminated peptide we had reported previously [26]. Furthermore, the reaction was essentially complete in only half of the time, 24 h rather than 48 h. For a more accurate comparison of reaction progress, cyclization of **20** was compared with that of the C-terminal Cys-carboxamide (sequence: H-CRKFFARIRGGRGC-NH_2,). Although the cyclization of the Sec-terminated precursor was not complete within 6 h, it had clearly progressed further than that of the cysteinyl peptide (Figure 4). Furthermore, the cyclic product derived from the Cys-carboxamide was only isolated in a yield of 20% yield after 24 h.

Conclusions

We have demonstrated that incorporation of a C-terminal selenocysteine residue into synthetic peptides facilitates more rapid thioester formation when compared with the corresponding cysteinyl peptide, presumably through an initial *N→Se* acyl shift. To our knowledge, this is the first time that this process has been studied or reported. Thioester formation in a model peptide was observed to proceed to approximately 80% conversion within 6 h at 60 °C, as judged by HPLC, LC-MS, and ^{13}C NMR spectroscopy.

In model studies, Sec was introduced to the reaction as the Sec (5-Npys) derivative that prevented the formation of diselenide dimers during resin cleavage yet could be reduced to the selenol upon addition of TCEP. In peptides containing multiple cysteine residues, the use of DTNP gave rise to a heterogeneous mixture of partially and fully 5-Npys-protected peptides, which compromised yields, and most likely also contained an intramolecular selenosulfide bond. Reduction of these species did allow thioester formation to proceed but at a much slower rate and with poor selectivity over internal Xaa–Cys motifs. It is possible that the use of lower temperatures (40 °C) and strictly anoxic conditions may yet improve selectivity in Sec-mediated thioester formation.

The addition of sodium ascorbate to prevent deselenation during the reaction appeared to increase hydrolysis of the product thioester. However, when the thioester is immediately consumed in ligation, there appeared little opportunity for hydrolysis to occur. The advantageous antioxidant properties of ascorbate could then be combined with accelerated thioester formation in the cyclization of peptide **20** through *in situ* thioester formation and NCL.

The use of selenocysteine clearly presents a challenge to thioester formation in the presence of additional protected or unprotected cysteine residues within the peptide. However, thioester formation through an *N→Se* acyl shift appears fast and efficient provided the tendency for Sec to interact intramolecularly or intermolecularly with additional Sec or Cys residues, or to undergo deselenation, can be controlled. If these well-documented obstacles can be ultimately overcome, then incorporation of C-terminal Sec potentially constitutes an extremely efficient Fmoc-based approach to peptide thioesters for use in NCL.

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