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Solid phase synthesis of peptide-selenoesters

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ABSTRACT

The synthesis of proteins by native chemical ligation greatly enhances the application of chemistry to complex molecules such as proteins. The essential building blocks for this approach traditionally have been peptide-thioester segments that are linked chemoselectively in consecutive reactions. By using peptide selenoesters instead of thioesters, the ligation rate can be significantly accelerated permitting couplings at difficult sites and potentially enabling new ligation strategies. To facilitate the routine synthesis of selenoester peptides, a general and straightforward procedure has been developed that generates a suitably functionalized resin from which the desired selenoester peptide can be readily synthesized. This simple approach utilizes readily available and cheap chemical agents and enables production of peptide selenoesters of excellent quality in short time and with high recovery. In addition, the stability of peptide selenoesters was examined under different native chemical ligation conditions and compared to thioesters. Selenoesters are slightly more reactive and more susceptible to hydrolysis and aminolysis than thioesters but sufficiently stable under mildly acidic conditions (pH 6.5). Under these conditions, rapid selenoester-mediated ligation is kinetically favoured.

1. Introduction

Proteins are the essential ingredients of life, being involved in virtually all known biological processes. Chemists were the first to realize their potential and early on sought of ways to manipulate and study these molecules with the ultimate aim to chemically synthesize these highly complex biopolymers. Today, in the post-genome era, the discovery of novel bioactive peptides and proteins has assumed a breathtaking pace. Many of these peptides and medium-sized proteins have therapeutic or diagnostic potential, which necessitates reliable techniques to facilitate the validation of their structure, detailed structure–function studies and their production on large scale in a timely fashion. Many of these requirements can only satisfactorily be achieved through chemical means and our need for robust technologies that permit total chemical access to large peptides and proteins is higher than ever before. Two major developments in the last 50 years have significantly contributed bringing us closer to these goals: the invention of solid phase peptide synthesis by Merrifield in 1963[1] and the conceptual development of so called orthogonal chemical ligation techniques in the 80s and 90s of the last century which culminated in the discovery of native chemical ligation (NCL) by Dawson and Kent in 1994.[2] The former allows linking of amino acid building blocks in rapid and precise fashion yielding peptides of up to 50 amino acids in length. The latter discovery provides a practical means to chemoselectively join these unprotected peptides to form much longer polypeptides (>200 amino acids) thereby extending chemical access from relatively small polypeptides to the realm of full-fledged proteins and enzymes.

Of the many chemical ligation approaches developed, NCL is by far the most widely used due to its robustness, versatility and ability to yield a native peptide bond at the site of ligation.[3,4] NCL is the reaction of an unprotected peptide carrying a C-terminal α-thioester with another unprotected peptide equipped with an N-terminal cysteine residue (Scheme 1). The nucleophilic sulfhydryl group of the cysteine residue attacks the mildly activated carbonyl carbon of the thioester moiety and replaces the thiol leaving group effectively 'capturing' both peptide segments (transesterification). The new thioester is short-lived and rapidly re-arranges through an S→N acyl shift presumably involving a favorable five-membered transition state to form a native amide bond. In mechanistic terms it is important to point out that the key to the exquisite chemoselectivity lies in the reversibility of the first transesterification step and the (near)[5] irreversibility of the second acyl transfer step. The former ensures that unproductive thioesters (e.g., thioesters involving non-ligation site cysteines) are short-lived and in conjunction with the latter guarantees near quantitative (typically >95%) reactions in short time (typically 2 h). NCL is normally carried out in aqueous guanidine HCl under mild conditions (neutral pH and room temperature) using thiol catalysts

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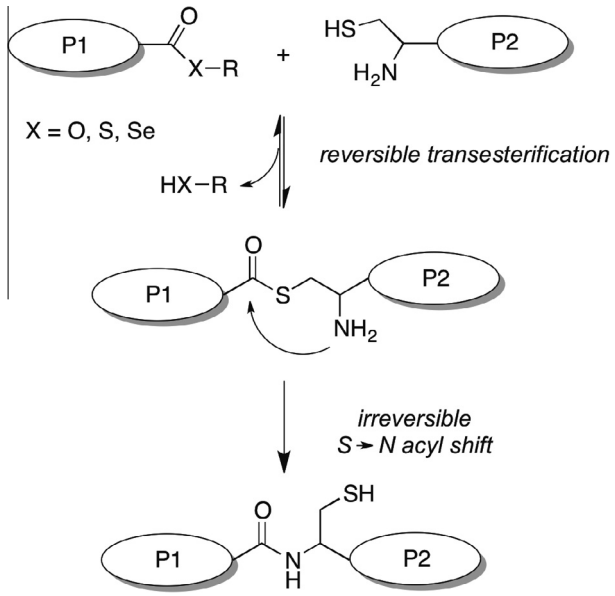


Scheme 1. Mechanism of native chemical ligation. P1 and P2 designate unprotected polypeptide segments.

and strong reducing agents such as tris(2-carboxyethyl)phosphine (TCEP) in order to prevent formation of unproductive thioesters and disulfides, respectively. Many of the original limitations of NCL, such as the requirement for a cysteine at the site of ligation or chemical access to the essential peptide thioester segments by more user-friendly and milder Fmoc SPPS protocols (rather than the traditional Boc SPPS strategies)[6] have been resolved in the past and are covered by recent excellent reviews.[7,8]

However, of the few remaining challenges, the issue relating to the dependence of the NCL reaction rate on the identity of the amino acid adjacent to the thioester moiety has been poorly addressed (Xaa in a peptidyl-Xaa-[COSR] + Cys-peptide ligation). Hackeng et al. observed early, that β-branched amino acids such as Thr, Val and Ile in this position react extremely slowly under standard NCL reaction conditions (>48 h).[6] Long NCL reaction times are generally discouraged, due to potential side-reactions (thioester hydrolysis, desulfurization of cysteine and methionine oxidation)[9] under the conditions employed, hence, ligations at these sites traditionally have been avoided. Furthermore, proline in this position has been found to react even more sluggishly with, at best, conversions of around 20% after 48 h.[6,10,11] As demonstrated by Danishefsky and coworkers, the problem can be partially solved by employing *p*-nitrophenyl oxo-esters (50% conversion after 15 h), however, hydrolysis of the highly activated esters even at slightly acidic pH values largely prevents quantitative conversions.[12]

By extending this line of thought of replacing sulfur with its chalcogen cousins, we recently investigated peptidyl-selenoesters as acyl donors in NCL reactions.[13] We reasoned that a selenoate would be a superior leaving group than the corresponding thiolate and alkoxide due to the better polarizibility of selenium versus sulfur and oxygen.[14] Detailed kinetic studies confirmed that selenoesters are indeed superior acyl donors in NCL reactions with observed rate enhancements of up to 350 times for ligations at proline. Stereochemistry and chemoselectivity were maintained under these conditions. Hence, this poorly studied compound class appears to have great potential not only as a practical way to enable NCL at difficult sites, but also in the optimization of existing chemical ligation strategies or the development of new ligation chemistries.

Here we outline two approaches for the solid phase chemical synthesis of peptide selenoester building blocks and discuss their advantages and limitations. We also compare the stability of seleno- and thioesters towards hydrolysis and aminolysis allowing a critical evaluation of these compounds under typical NCL reaction conditions.

2. Results and discussion

2.1. Synthesis of peptide selenoesters by solution phase approaches

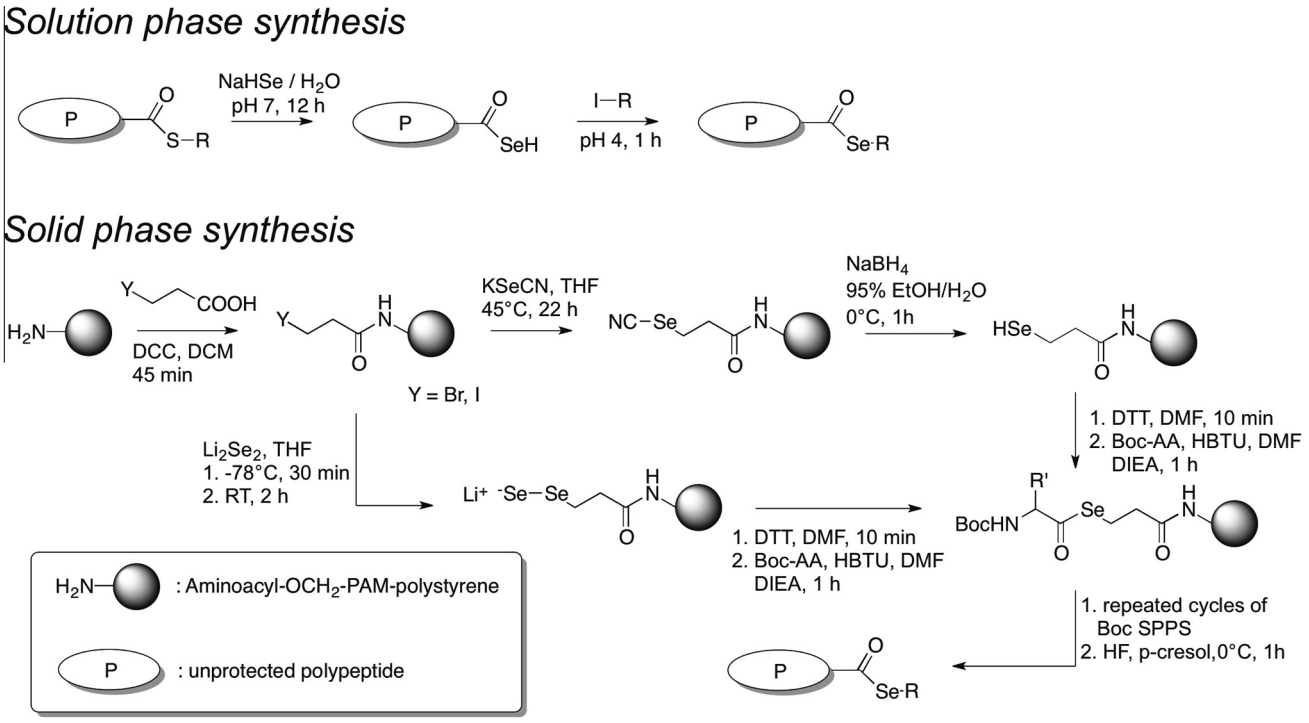
There are surprisingly few chemical approaches available for the incorporation and intermediate protection of selenium into organic molecules, when compared to its chalcogen peers sulfur and oxygen.[15] Most approaches have focused on selenocysteine (Sec, the '21st amino acid'), which plays an important role in enzymatic redox pathways[16] and also has some utility in Sec-mediated NCL.[9,17–19] Selenols have significantly lower pK_a values than thiols of similar structure, which makes them considerably more nucleophilic at neutral and acidic pH.[20] In addition, their reduction potentials are substantially lower (more negative) and are more rapidly oxidized to diselenides than thiols to disulfides.[21]

In our initial attempt to generate peptide selenoesters,[13] we developed a solution phase approach in which unprotected peptide thioesters were converted to selenoacids by treatment with NaHSe at pH 7 followed by alkylation with alkyl halides at pH 4 to yield the corresponding selenoesters (Scheme 2). This sequence has to be carried out in one-pot under inert conditions as the intermediate selenoacids are highly unstable when exposed to air.[22] While this approach is compatible with a variety of functionalized amino acids and long polypeptides,[13] our continuing studies suggest that a few amino acids (most likely Trp) and certain side chain protecting groups (e.g., Cys(Fm)) can undergo substantial side reactions. These side reactions primarily occur during the first reaction step in the presence of the highly nucleophilic and reducing hydrogenselenide agent rather than during the alkylation step. While these major side products were not fully characterized, it is evident that novel approaches for the synthesis of selenoesters are needed.

We initially resorted to two well-established strategies that have proven useful for the Fmoc SPPS of peptide thioesters: Dawson's *N*-acyl-benzimidazolinone (Nbz) approach and Liu's hydrazide method.[23,24] Both approaches rely on relatively unreactive precursors during chain assembly (*o*-aminoanilides and hydrazides, respectively) that can be activated under mild conditions (to give Nbz and azides, respectively) and thiolysed with mercaptans yielding the corresponding peptide thioesters. We anticipated that these precursors might be sufficiently activated towards displacement with selenols. LYRAF-Nbz and LYRAF-azide were prepared as described and treated with 30 mM diphenyldiselenide, 100 mM TCEP in 6 M GdmHCl, 0.1 M sodium phosphate buffer, pH 7.0 under argon atmosphere. However, analysis of the reaction mixture after 24 h indicated that only trace amounts of the expected LYRAF-[COSe]-phenyl ester were formed and the majority of peptide was still in the precursor form. This suggests that selenophenol is a comparatively poor nucleophile under these conditions, in agreement with our earlier observations.[13] This problem is further compounded by the poor solubility of organic diselenides in aqueous solution and the strong tendency of selenols to oxidize rapidly and quantitatively, which make it difficult to achieve and maintain high concentrations of free selenol nucleophile. Hence, we next turned our attention to solid phase strategies to avoid most of the limitations of the solution phase methods.

2.2. Synthesis of peptide selenoesters by solid phase approaches

To enable the synthesis of selenoester peptides, a general selenoester-generating resin linker for Boc SPPS was developed



Scheme 2. Synthetic strategies for the solution and solid-phase synthesis of peptide selenoesters.

(Scheme 2). This linker (HSe-CH_2-CH_2-CONH-CH(R)-COO-CH_2-PAM-Polystyrene) is a selenol variant of the thioester-generating resin described by Hackeng et al.[6] and employs the HF-labile PAM linker.[25] Starting from pre-loaded Ile-OCH_2-PAM-resin, 3-bromo- or 3-iodopropionic acid was coupled by using symmetric anhydride conditions and dicyclohexylcarbodiimide (DCC). Introduction of the selenol functionality was achieved by two alternative procedures as shown in Scheme 2.

In our initial attempt lithium diselenide (Li_2Se_2) was generated from the reduction of stoichiometric amounts of elemental selenium with lithium triethylborohydride.[26] The resulting mixture was used to selenate the halogenated resin under inert conditions. Diselenides were reduced with 1,4-dithiothreitol (DTT, in DMF) and the selenol acylated with the first Boc-protected amino acid building block using standard SPPS protocols with an extended coupling time (1 h).[27] Manual peptide synthesis by standard protocols was then continued and the completed peptide selenoester was cleaved from the resin with concomitant side chain deprotection using HF/*p*-cresol (9:1 (v/v)) at −5 °C. The crude peptide (LYRAF-[COSe-CH_2-CH_2-CO]-Ile) was recovered in good yield (70% based on resin loading) and purity (Fig. 1a). The described approach facilitates generation of peptide selenoesters though it does require the handling of toxic and malodorous selenide solution under inert conditions and demands safety precautions for the potentially hazardous hydrogen. In addition, the Li_2Se_2 solution has to be freshly prepared every time, which may limit the attractiveness of this approach.

During our studies we came across potassium selenocyanate as a suitable alternative to Li_2Se_2/NaHSe for incorporation of the selenol functionality.[28,29] KSeCN is a stable salt, less odorous than selenides, easily handled, cheap and the cyano group effectively functions as an intermediate protecting group to prevent undesired oxidation or substitution reactions.[15,28,29] Substitution of the halogenated resin was achieved by a modest excess of KSeCN (5 equiv) in under 24 h at ambient temperature (45 °C) and the cyano group removed by treatment with sodium borohydride at 0 °C for 1 h. Acylation of the free selenol with the first Boc-protected amino acid was then carried out as described above and included a DTT reduction step in order to reverse any potential

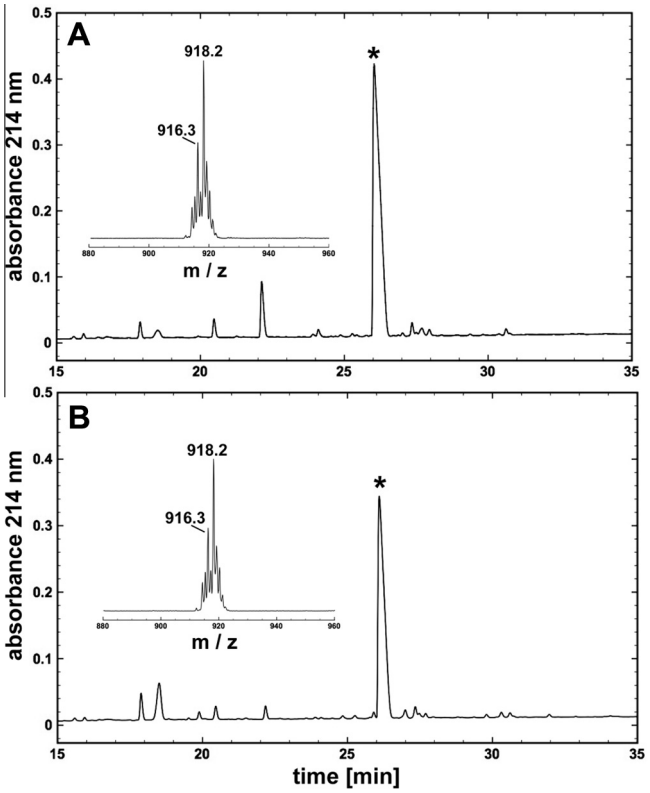


Fig. 1. HPLC and MS analysis of crude peptide selenoester LYRAF-[COSe-CH_2-CH_2-CO]-Ile obtained by the Li_2Se_2 (A) or KSeCN (B) SPPS approaches. The insert shows an ESI-MS of the crude peptide (calculated monoisotopic mass (most abundant isotope composition): 918.4 Da) and the desired product is indicated by an asterisk. HPLC analysis was done using an Agilent Zorbax 300SB-C18, 4.6 × 250 mm Column and a gradient of 10–50% buffer B in buffer A over 35 min.

diselenide formation during the washing steps. Test peptide LYRAF-[COSe-CH_2-CH_2-CO]-Ile was obtained in similar yields and slightly higher purity when compared to the same peptide prepared by the Li_2Se_2 approach (Fig. 1b).

To demonstrate the applicability of this approach to the synthesis of longer peptides, two medium-sized peptide selenoesters corresponding to segments 1–13 and 14–30 of the spider toxin PnTx2-6 from *Phoneutria nigriventer* were synthesized.[30] This 48 amino acid toxin (also known as 'spider-viagra') contains ten cysteine residues, which should facilitate its synthesis by a number of ligation strategies. However, the most practicable ligation sites are exclusively 'slow ligation sites' (i.e., either T/I/V/P-C, six in total) making it an ideal target for selenoester-mediated NCL. Both peptides were recovered in high yield (1–13: 83%; 14–30: 71% of the theoretical yield based on resin loading) and purity following HF cleavage (see Fig. 2). Thus, the described procedure is straightforward, simple, utilizes readily available and cheap chemical agents and facilitates production of any desired peptide selenoester in short time, high recovery and excellent quality.

PnTx2-6[14-30]-selenoester was ligated to peptides CFRANK and PnTx2-6[31-48] (CRQGYFWIAWYKLANC(Acm)KK) using conditions described previously.[13] More than 80% conversion was achieved within 2 h, thus demonstrating the utility of selenoester-mediated NCL for the synthesis of longer polypeptides at difficult ligation sites as well as the genuine structure of the synthetic selenoesters obtained by the described direct SPPS approach.

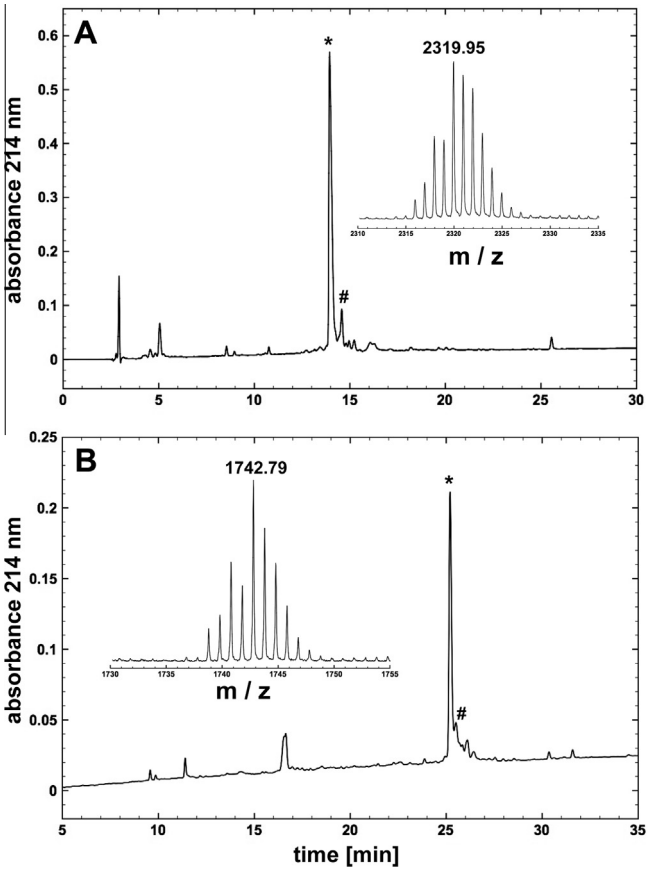


Fig. 2. HPLC and MALDI-MS analysis of crude peptide selenoesters obtained using the KSeCN approach. (A) crude *Thz*-DC(Acm)C(Acm)GERGEC(Acm)VC(Acm)GGPC (Acm)I-[COSe-CH_2-CH_2-CO]-Ile after HF cleavage. The asterisk indicates the desired product and the insert depicts a high-resolution MALDI-MS (calculated monoisotopic mass (most abundant isotope composition): 2319.78 Da). *(B)* ATC(Acm)AGQDQPC(Acm)KET-[COSe-CH_2-CH_2-CO]-Ile after HF cleavage. The asterisk indicates the desired product and the insert depicts a high-resolution MALDI-MS (calculated monoisotopic mass (most abundant isotope composition): 1742.66 Da). HPLC analysis was done using an Agilent Zorbax 300SB-C18, 4.6x250 mm column and a gradient of 10-50% buffer B in buffer A over 40 min. The hash symbol indicates a side product corresponding to the loss of one Acm group.

2.3. Evaluation of the stability of selenoesters versus thioesters under NCL conditions

Selenoesters have a higher reactivity than comparable thioesters towards thiol nucleophiles in the first transesterification step of NCL.[13] One could reasonably argue that this higher reactivity of selenoesters also extends towards other nucleophiles, for example, amines and hydroxyl ions. This would have direct consequences for their applicability in NCL, because such (undesired) reactivity would affect chemoselectivity and/or stability towards alkaline hydrolysis at even slightly elevated pH. To compare the stability of selenoesters and thioesters under typical NCL conditions, peptides LYRAF-[COS-CH_2-CH_2-CO]-Gly and LYRAF-[COSe-CH_2-CH_2-CO]-Ile were incubated at a concentration of 1 mg/mL (approximately 1.1 mM) in 6 M guanidine HCl, 200 mM Na_2HPO_4 which was adjusted to different pH values. A thiol or selenol catalyst was not included, as this would give rise to transesterification reactions that would complicate data analysis. Hence, the data should be interpreted with caution as NCL is usually carried out in the presence of aryl thiol catalysts that activate the poorly reactive alkyl thioester. The resulting aryl thioester is typically short-lived but also likely more prone to hydrolysis than the alkyl thioesters studied here. The mixtures were analyzed by HPLC and MS and the fraction of remaining ester expressed as a function of time (Fig. 3). Our data indicate that selenoesters at pH 7.0 and 7.5 decompose substantially with half-lives of 12 h and 7 h, respectively. The hydrolyzed ester was the dominant product in both cases, however small amounts of cyclic LYRAF and a guanidino adduct were also detected, suggesting that direct aminolysis of the selenoester may also play a role.[31] In contrast, thioesters are reasonably stable at pH 7.5. The minor hydrolysis observed, however, could be of significance if ligations are carried out over longer periods (>24 h), suggesting ligations may best be carried out at lower pH values. At a pH of 6.5 the selenoester was essentially as stable as a thioester of similar structure at pH 8.0 ($t_{1/2}$ in both cases approximately 20 h).

It has long been noted that oxoesters and analogous thioesters have very similar reactivities towards hydroxide ion nucleophiles

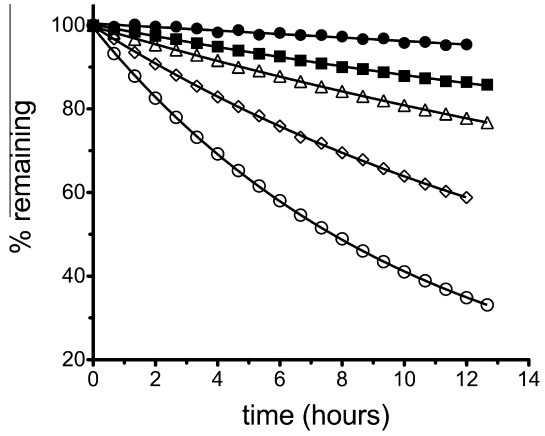


Fig. 3. Stability of seleno- and thioester-peptides under NCL conditions. Peptides LYRAF-[COS-CH_2-CH_2-CO]-Gly (thioester) or LYRAF-[COSe-CH_2-CH_2-CO]-Ile (selenoester) were incubated in 6 M guanidine HCl, 200 mM Na_2HPO_4, adjusted to different pH values and the mixtures analyzed by HPLC at the indicated times. Data were fitted to a single exponential equation. Legend and calculated half-lifes ($t_{1/2}$): (●) thioester, pH 7.5, $t_{1/2} \sim 20{,}000$ h; (■) thioester, pH 8.0, $t_{1/2} = 19$ h; (△) selenoester, pH 6.5, $t_{1/2} = 22$ h; (◇) selenoester, pH 7.0, $t_{1/2}$=12 h; (○) selenoester, pH 7.5, $t_{1/2}$=7 h.

during alkaline hydrolysis.[32] Our results indicate that selenoesters are more susceptible than thioesters towards base-mediated hydrolysis under NCL conditions. This is in agreement with results from Chu and Mautner, who found that selenoesters are hydrolyzed approximately 3–10 times faster than corresponding thioesters across a wide pH range.[33] Interestingly, the same report found that the rate of hydrolysis is significantly accelerated by phosphate ions, suggesting that under NCL conditions, selenoesters may be stabilized by substituting the widely used phosphate buffer. More importantly, however, these changes are relatively small on the grand kinetic scale and similar to rate changes observed when comparing thioesters to oxoesters.

In contrast, thioesters are much more reactive than oxoesters of similar structure towards 'softer' nucleophiles such as amines and thiols.[32,34] This trend appears to continue in the chalcogen series with selenoesters being substantially more reactive towards thiol[13] and amine nucleophiles[33] than thioesters. For example it was observed that aminolysis of selenoesters is about 100 times faster than the same reaction of thioesters under identical conditions (oxoesters did not react).[33] Hence, the greater susceptibility of selenoesters to hydrolysis when compared to thioesters does not diminish their value in NCL as long as a safe optimum pH, where the rate of thiolysis far exceeds the rate of hydrolysis, is maintained.[35] Depending on the exact reaction conditions, selenoester-mediated NCLs are typically quantitative within minutes to a few hours, even at slightly acidic pH values and in the worst-case scenario (Pro-Cys ligations).[13] Thus, under most conditions selenoester-mediated NCL will be kinetically favoured.

3. Conclusions

In summary, herein we describe two high-yield strategies for the efficient Boc-based synthesis of peptidyl selenoesters directly on the solid phase. The use of KSeCN and Li_2Se_2 nucleophiles for on-resin selenoester synthesis provides an alternative means to access medium-length peptides through NCL reactions at difficult sites that are otherwise impractical via traditional thioester ligation chemistry. The solid phase approaches described overcome several obstacles encountered in our solution-state synthesis, such as considerable side reactions with protecting groups and Trp amino acids. In addition, these protocols are quick, user friendly, and result in excellent yields with high purity. Though they are more prone to hydrolysis and aminolysis than thioesters, selenoesters may be used for NCL in slightly acidic buffers for relatively short ligation periods with minimal losses. The utility of selenoesters in peptide ligation chemistry is a subfield in its infancy and widespread utility awaits further development such as the development of Fmoc-compatible techniques.

4. Materials and methods

4.1. Peptide synthesis

All peptides were manually synthesized by step-wise Boc SPPS using highly optimized in-situ neutralization protocols.[27] Peptide thioesters were synthesized on Trt-S-CH_2-CH_2-CONH-Xaa-O-CH_2-PAM resins as described previously.[6] Peptide selenoesters were synthesized on HSe-CH_2-CH_2-CONH-Xaa-O-CH_2-PAM resins as described below. Coupling efficiencies were measured using the quantitative ninhydrin test.[36] Boc-protected amino acids were used with the following side chain protecting groups: D(OcHx); E(OcHX); H(DNP); K(Cl-Z); N(Xan); Q(Xan); R(Tos); S(Bzl); T(Bzl); Y(BrZ). All cysteine side-chains were protected with acetamidomethyl (Acm).

After chain assembly, the resin was washed with DMF and 1:1 DCM/MeOH and dried under vacuum prior to HF cleavage. Simultaneous side chain removal and cleavage of peptides from the dried resins were carried out with 9 mL of HF and 1 mL *p*-cresol as scavenger for 1.5 hour at −5 °C. Cleaved peptides were precipitated and washed twice with cold diethyl ether. The peptides were then dissolved in 50% acetonitrile, 0.1% TFA (v/v) in water and lyophilized. Peptides were purified by preparative reverse-phase (RP) HPLC and fractions containing the desired peptide were pooled, lyophilized and stored at −20 °C.

4.2. Characterization of peptides

Peptides were characterized by analytical HPLC on a Shimadzu Prominence systems using a solvent system of 0.05% TFA in water (buffer A) and 90% acetonitrile, 0.043% TFA in water (buffer B) and an Agilent Zorbax 300SB-C18, 4.6 × 250 mm column. Peptide purity and identity were assessed by ESI-MS on a API-2000 mass spectrometer (Applied Biosystems) and by MALDI-MS on a Applied Biosystems 4700 TOF/TOF.

LYRAF-[COS-CH_2-CH_2-CO]-Gly M_{found}: 814.3 Da; calculated monoisotopic mass (most abundant isotope composition): 814.4 Da

CFRANK M_{found}: 737.4 Da; calculated monoisotopic mass (most abundant isotope composition): 737.3 Da.

LYRAF-[COSe-CH_2-CH_2-CO]-Ile M_{found}: 918.3 Da; calculated monoisotopic mass (most abundant isotope composition): 918.4 Da.

ATC(Acm)AGQDQPC(Acm)KET-[COSe-CH_2-CH_2-CO]-Ile M_{found}: 1742.79 Da; calculated monoisotopic mass (most abundant isotope composition): 1742.66 Da.

Thz-DC(Acm)C(Acm)GERGEC(Acm)VC(Acm)GGP-C(Acm)I-[COSe-CH_2-CH_2-CO]-Ile M_{found}: 2319.95 Da; calculated monoisotopic mass (most abundant isotope composition): 2319.78 Da.

Thz-DC(Acm)C(Acm)GERGEC(Acm)VC(Acm)GGP-C(Acm)ICRQGYFWIAWYKLANC(Acm)KK M_{found}: 4402.5 Da; calculated mass (average isotope composition): 4402.7 Da.

Thz-DC(Acm)C(Acm)GERGEC(Acm)VC(Acm)GGP-C(Acm)ICFRANK M_{found}: 2790.6 Da; calculated mass (average isotope composition): 2790.8 Da.

4.3. Solid phase synthesis of peptide-selenoester: KSeCN protocol

1.43 g of Boc-Ile-PAM resin (0.70 mmol/g, 1 mmol) was swollen in DMF for 1 hour, after which the terminal Boc group was removed with 2 × 1 min treatment with neat TFA. The resin was flow-washed with DMF for 30 s, and neutralized with 2 × 1 min treatments with 10% DIEA in DMF (v/v). The neutralized resin was then flow washed for 30 s with DMF, then 30 s with DCM.

10 equiv of DCC (2.063 g, 10 mmol) were dissolved in 5 mL of DCM and 20 equiv of 3-iodopropionic acid (4.0 g, 20 mmol) were dissolved in ∼ 6 mL of DCM. Both solutions were cooled to 0 °C for 5 min before being combined and allowed to react at 0 °C for 30 min. The resulting solution containing 3-iodopropionic anhydride was gravity filtered to remove the dicyclohexylurea precipitate. The residue was then washed with 2 mL of DCM, filtered, and pooled with the 3-iodopropionic anhydride filtrate. The pooled filtrates were then added to the deprotected neutralized Ile-PAM resin and allowed to couple for 45 min. The resin was drained and washed with DCM for 30 s. Quantitative ninhydrin test indicated >99% coupling. The resin was then dried under vacuum. Yield: 1.49 g (new loading: 0.661 mmol/g, 0.984 mmol) yellowish resin.

550 mg (0.661 mmol/g, 0.363 mmol) of the dried acylated resin was then swollen in 10 mL of anhydrous THF for 1 h. 5 equiv of KSeCN (262 mg, 1.82 mmol) were added to the swollen resin in THF. The mixture was then incubated under argon atmosphere at 45 °C and gently agitated for 22 h (magnetic stirring should be

avoided as the stir bar will crush the resin which will later clog the SPPS synthesis vessel frits). The resin was drained and washed extensively with THF and DCM and dried under vacuum. Yield: 452 mg (new loading: 0.67 mmol/g, 0.303 mmol) of greyish-white resin.

The dried resin (450 mg, 0.3 mmol) was re-swollen in 8 mL of THF for 1 h, cooled to 0 °C on an ice/water bath, and sodium borohydride (37.83 mg, 1 mmol) in 95% (v/v) EtOH/H_2O (1 mL) was added in one portion. The reaction was placed at 0 °C for 1 h with occasional agitation, drained, and washed with THF, DCM, and DMF.

Prior to coupling of the first amino acid, the resin was treated with 3 mmol 1,4-dithiothreitol (DTT) in 6 mL DMF for 10 min. The resin was then drained, and without washing, a 10 equiv pre-activated mixture of Boc-Xaa-OH (3 mmol), HATU (1140 mg, 3 mmol), and 1.038 mL DIEA (6 mmol) in 6 mL of DMF was added and allowed to couple for 1 h. The resin was then drained and washed with DMF, and the reduction and coupling steps were performed a second time. The resin was washed with DMF and stepwise synthesis was subsequently carried out using standard Boc in-situ neutralization protocols as described previously.

4.4. Solid phase synthesis of peptide-selenoester: Li_2Se_2 protocol

1.43 g of Boc-Ile-PAM resin (0.70 mmol/g, 1 mmol) was swollen in DMF for 1 h, after which Boc groups were cleaved with 2×1 min treatment with neat TFA, flow washed with DMF for 30 s, and neutralized with 2×1 min treatments with 10% DIEA in DMF (v/v). The neutralized resin was then flow washed for 30 s with DMF, then 30 s with DCM. The resin was then stored in DCM. 15 equiv of DCC (3.09 g, 15 mmol) and 30 equiv of 3-bromopropionic acid (4.59 g, 30 mmol) were each dissolved in 10 mL DCM and cooled at 0 °C for 5 min before being combined. The combined solution was then allowed to react at 0 °C for 1 h. The resulting solution containing 3-bromopropionic anhydride was then gravity filtered to remove the dicyclohexylurea precipitate. The residue was then washed with 2 mL of DCM and the filtrates pooled. The combined filtrates were added to the deprotected and neutralized Ile-PAM resin and allowed to couple for 45 min. The resin was drained and washed with DCM for 30 s. Quantitative ninhydrin test indicated more than 98% coupling efficiency and the resin was dried under vacuum.

The acylated resin was then swollen in 30 mL dry THF under argon for 1 h, then cooled to −78 °C prior to selenization with Li_2Se_2. Li_2Se_2 (6 mmol) was prepared by suspending elemental selenium powder (480 mg, 6.1 mmol) in 60 mL of dry THF under argon and slowly adding 1 equiv of $LiEt_3BH$ (1 M in dry THF, 6 mL, 6 mmol). When hydrogen evolution stopped, the solution was refluxed under argon atmosphere for 30 min. The solution was then allowed to cool to room temperature and 5 equiv (55 mL, 5 mmol) were added to the swollen resin in THF at −78 °C under argon. The mixture was allowed to stir at −78 °C for 30 min, then allowed to stir at room temperature for 2 h. The resin was then transferred to a peptide synthesis reaction vessel, drained, washed once with THF, then washed thoroughly with DMF and stored in DMF under argon.

Approximately 0.1 mmol of selenized resin was transferred to another reaction vessel in DMF and put under argon. Immediately prior to coupling of the first Boc-amino acid, the selenized resin was reduced with 5 equiv of 0.5 M DTT in DMF (1 mL, 0.5 mmol) for 10 min. The resin was then drained, and without washing, a 10 equiv pre-activated mixture of Boc-Xaa-OH (1 mmol), HATU (380 mg, 1 mmol), and 260 μL DIEA in 2 mL DMF was added and allowed to couple for 1 h. The resin was then drained and flow washed with DMF for 30 s, and reduced and coupled as described for a second time. After double coupling of the first amino acid, subsequent amino acids were coupled according to Boc in-situ neutralization protocols.

4.5. Stability assay

A 1 mg/mL solution of H-Leu-Tyr-Arg-Ala-Phe-[COS-CH_2-CH_2-CO]-Gly-OH or H-Leu-Tyr-Arg-Ala-Phe-[COSe-CH_2-CH_2-CO]-Ile-OH was prepared by dissolving 1 mg of peptide in 1 mL of ligation buffer (6 M guanidine HCl, 200 mM Na_2HPO_4, adjusted with 6 M HCl or NaOH to the indicated pH). The dissolved peptidyl esters were incubated for a total of 13.4 h at 22 °C and 10 μl aliquots were removed every 40 min and analyzed by RP-HPLC using a gradient of 20–40% of buffer B (90% acetonitrile, 0.043% TFA in water) in buffer A (0.05% TFA in water) over 40 min (Column: Agilent Zorbax 300SB-C18, 4.6×250 mm column). Peak areas were integrated using the Shimadzu Labsolutions software package and normalized to 100% at reaction time $t = 0$. Data were fitted to a single exponential decay curve.

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