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Article

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Catalysis of thiol-thioester exchange by water soluble alkyldiselenols applied to the synthesis of peptide thioesters and SEA-mediated ligation

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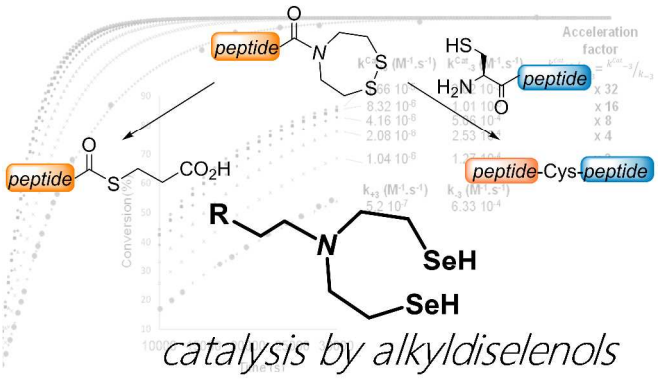


Abstract

***N*-Alkyl-*bis*(2-selenoethyl)amines catalyze the synthesis of peptide thioesters or peptide ligation from *bis*(2-sulfanylethyl)amido (SEA) peptides. These catalysts are generated *in situ* by reduction of the corresponding cyclic diselenides by *tris*(2-carboxyethyl)phosphine (TCEP). They are particularly efficient at pH 4.0 by accelerating the thiol-thioester exchange processes, which are otherwise rate-limiting at this pH. By promoting SEA-mediated reactions at mildly acidic pH, they facilitate the synthesis of complex peptides such as cyclic *O*-acyl isopeptides that are otherwise hardly accessible.**

Introduction

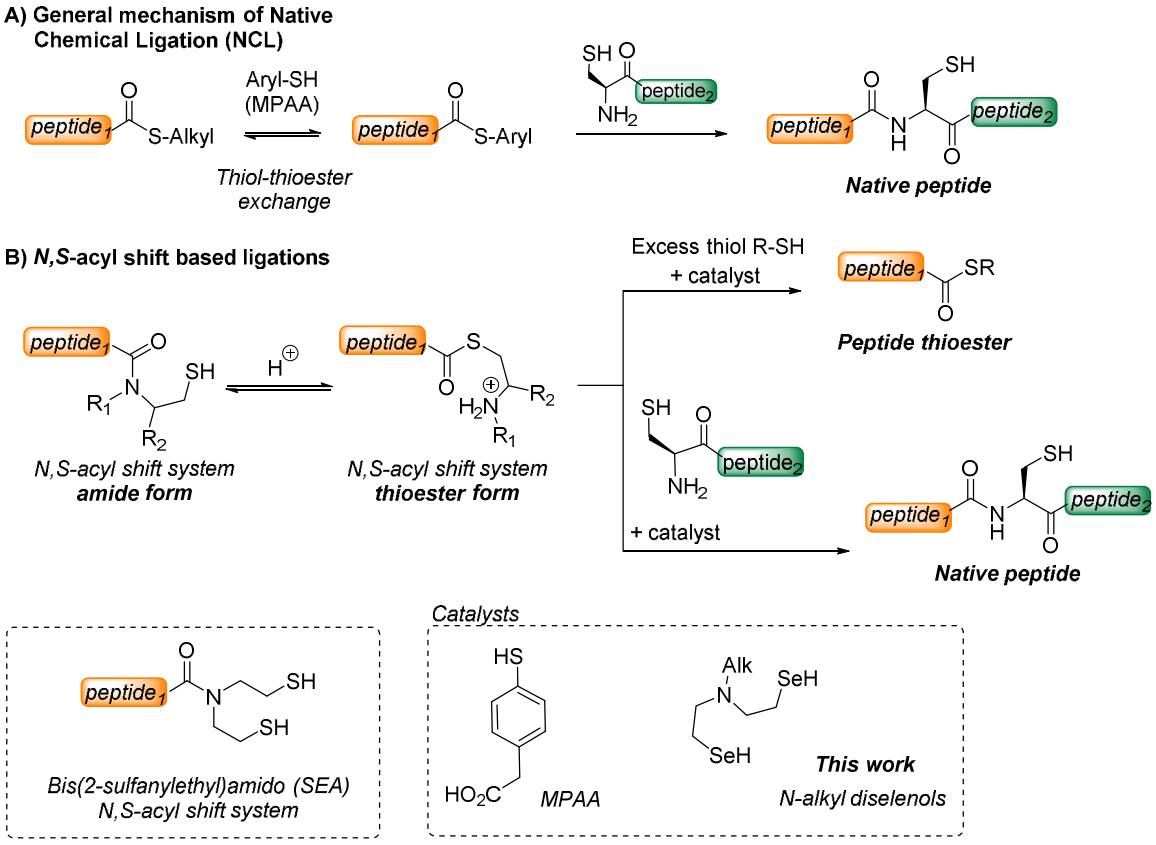
The native chemical ligation (NCL[1-2]) is a powerful chemoselective peptide bond forming reaction that is intensively used for protein chemical synthesis (Scheme 1A).[3] NCL consists in reacting a peptide thioester with a cysteinyl peptide to produce a native peptide bond to cysteine in water at neutral pH. Several thiol catalysts of the NCL reaction with peptide alkylthioesters were developed among which 4-mercaptophenylacetic acid (MPAA) is the most popular.[4-8] This thiol additive acts through a thiol-thioester exchange process that is the rate-limiting step of NCL. Since the arylthiol MPAA is more acidic and therefore a better leaving group than the departing alkylthiol, the newly formed peptide arylthioester is a better acyl donor than the starting peptide alkylthioester.[9] Preformed peptide thio[10-12] or seleno[13-15] aryl esters can be used as well in NCL and are particularly powerful acyl donors.

The use of *N,S*-acyl shift systems based on the 2-mercaptoethylamide scaffold are also popular in the field (Scheme 1B).[16-26] They can be used either as precursors of peptide thioesters by exchange with thiol additives or as thioester surrogates in NCL due to their capacity to rearrange *in situ* into transient thioesters. Considering the increased use of *N,S*-acyl shift systems alone or in combination with NCL for protein chemical synthesis, the search for novel catalysts or conditions adapted to these chemical systems is of great importance. However, novel developments in this area are complicated by the lack of mechanistic insight for these reactions.

We are particularly interested in developing new conditions and catalysts for the *bis*(2-sulfanylethyl)amido (SEA)[19] thioester surrogate whose utility for protein total synthesis has been demonstrated.[27-29] A motivation for this work is to accelerate SEA-based reactions but also to exploit SEA thioester surrogates under experimental conditions that cannot be attained by NCL for extending the range of polypeptides amenable to chemical synthesis.

Toward this end, we performed detailed kinetic investigations of the SEA *N,S*-acyl shift process and of the SEA amide-thiol exchange reaction under mildly acidic conditions. The data enabled to extract the apparent kinetic constants for the individual steps involved in these chemical processes and reveal that thiol-thioester exchanges are rate limiting in the working conditions. With this knowledge, we further designed *N*-alkyl diselenols which are able to catalyze SEA-mediated reactions by accelerating thiol-thioester exchanges (Scheme 1B). The catalysts proved particularly useful for promoting SEA-mediated reactions at difficult amino acids such as Val, Ile or Thr and also for preparing peptides that are otherwise hardly accessible such as cyclic *O*-acyl isopeptides. Therefore, the novel SEA peptide/5-alkyl-1,2,5-diselenazepane catalyst combination represents a useful addition to existing ligation techniques.

Scheme 1. A) Principle of NCL. B) *N,S*-acyl shift systems based on the 2-sulfanylethylamide scaffold enable the synthesis of peptide thioesters or can act as thioester surrogates in NCL. Structure of SEA peptides and of the *N*-alkyl diselenol catalysts developed in this work.



Results and discussion

Kinetic study and mechanistic model

We first investigated the kinetics of the uncatalyzed SEA group – alkylthiol exchange reaction in the presence of 3-mercaptopropionic acid (MPA) in large excess (Scheme 2A), a useful reaction that enables the preparation of peptidyl MPA-thioesters. As this reaction was shown to proceed optimally at pH 4.0 in previous studies,[30] we carried out a detailed kinetic study at this pH to determine the rate constants for the individual steps involved in this transformation and identify the rate-limiting step of the process.

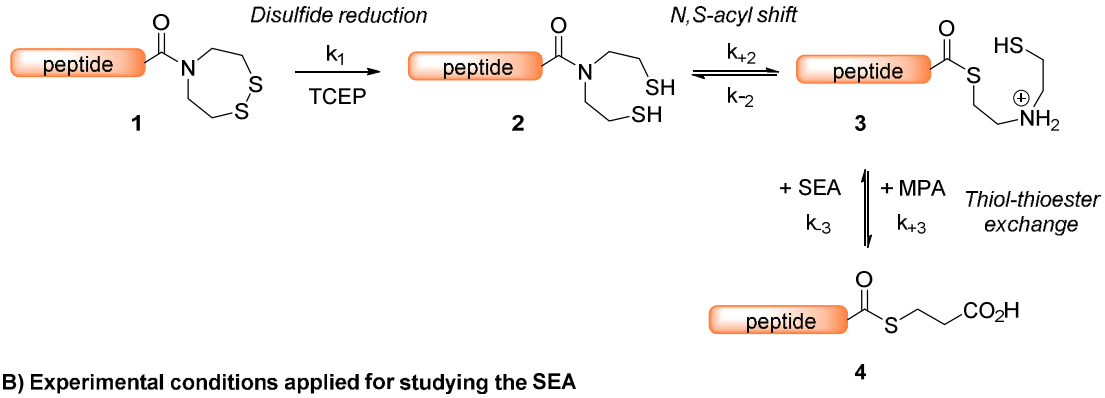
SEA peptides are produced by Fmoc SPPS in the form of a cyclic disulfide SEA^{off}. Therefore, the conversion of SEA peptides into peptide MPA thioesters in the presence of TCEP and MPA involves three different chemical steps (Scheme 2A): i) the reduction of the SEA^{off} disulfide into an active SEA dithiol (SEA^{on}), i.e. **1** → **2**, ii) the rearrangement of the SEA^{on} amide into the SEA thioester by intramolecular *N,S*-acyl migration, i.e. **2** → **3**, iii) and finally the thiol-

thioester exchange enabling the exchange of the SEA thioester by MPA, i.e. **3** → **4**. Throughout this study, a model SEA peptide **1a** featuring a C-terminal alanine residue was used as the acyl donor component (Scheme 2B,C).

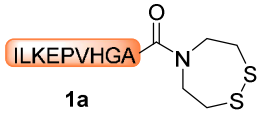
To simplify the determination of the kinetic constants, we first built a kinetic model for the conversion of SEA^{off} disulfide into SEA thioester (**1** → **3** in Scheme 2A) which could be studied separately by replacing the thiol MPA by propionic acid (Scheme 2B). This reaction was studied in the pH range 3.0-4.4 with a step of 0.2 pH units. The proportion of SEA^{off} peptide **1a**, SEA^{on} peptide **2a** and SEA thioester **3a** were quantified by LC-MS. The data presented in Fig. 1A correspond to the experiment carried out at pH 4.0. This figure clearly shows the reduction of SEA^{off} peptide **1a** into SEA^{on} peptide **2a** in the initial phase of the reaction, and then the conversion of SEA^{on} peptide **2a** into SEA thioester **3a** until the establishment of an equilibrium. The apparent kinetic constants k_1, k_2 and k_{-2} were extracted by fitting the model to the data using KinTek® chemical kinetics software.[31] The relationship between k_2 or k_{-2} and pH can be seen in Fig. 1B. The logarithm of the equilibrium constant for the SEA^{on} peptide **2a**/SEA thioester **3a** equilibrium determined experimentally and calculated from rate constants is shown in Fig. 1C. The good correspondence between the two series of values for K shows the quality of the fit and the pertinence of the kinetic model.

Scheme 2. Kinetic model for the SEA-MPA exchange reaction and experimental conditions.

A) Kinetic model for the SEA-MPA exchange reaction



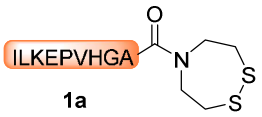
B) Experimental conditions applied for studying the SEA amide - SEA thioester equilibrium 1a ⟶ 2a ⇌ 3a



3 mM peptide **1a**, 100 mM TCEP, 574 mM propionic acid,
Phosphate buffer 0.1 M, 6 M Gn-HCl, 37° C

pH = 3.0-4.4

C) Experimental conditions applied for studying the SEA amide - MPA thioester equilibrium 1a ⟶ 2a ⇌ 3a ⇌ 4a



3 mM peptide **1a**, 100 mM TCEP
574 mM (5% by vol) 3-mercaptopropionic acid (MPA)
Phosphate buffer 0.1 M, 6 M Gn-HCl, 37° C

pH = 4.0

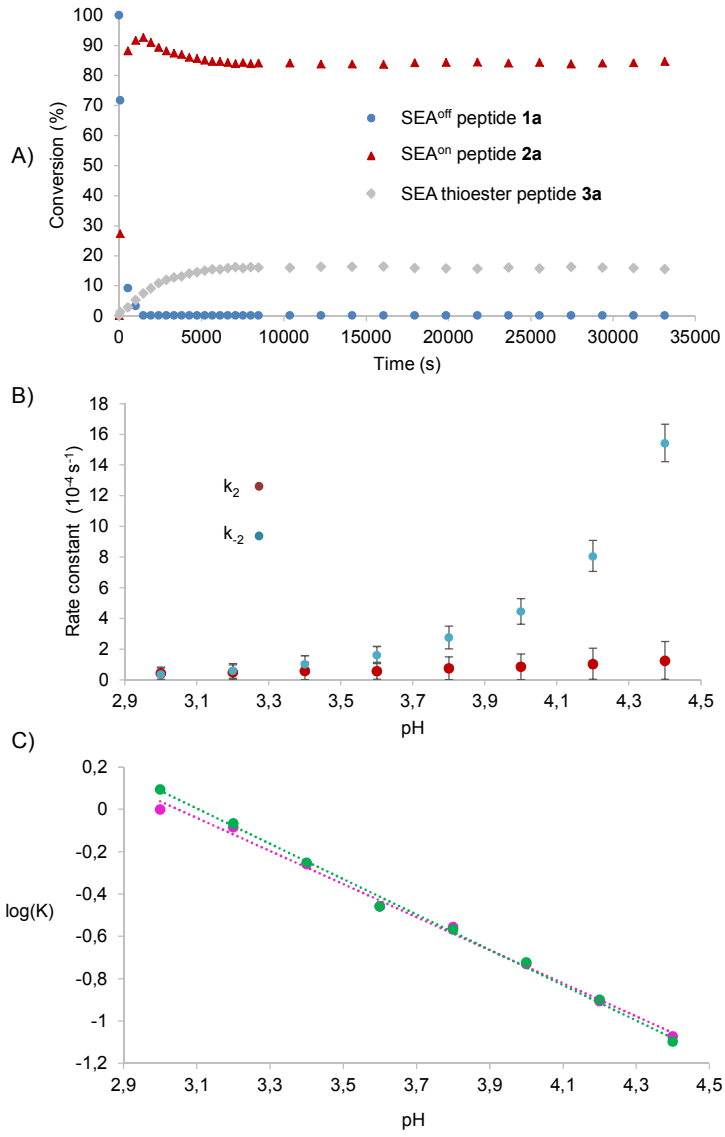


Figure 1. Kinetic study of the reduction of SEA^{off} peptide **1a** into SEA^{on} peptide **2a** and of the equilibration of SEA^{on} peptide **2a** into SEA thioester **3a**. A) The proportion of each species in the mixture at pH 4.0 was determined by HPLC using UV detection at 215 nm. $k_1 = 0.049 \pm 1.2\ 10^{-3}\ M^{-1}.s^{-1}$, $k_2 = 8.38\ 10^{-5} \pm 1.5\ 10^{-6}\ s^{-1}$, $k_{-2} = 4.44\ 10^{-4} \pm 5.9\ 10^{-5}\ s^{-1}$. B) Rate constants k_2 (red circles) and k_{-2} (blue circles) as a function of pH. The error bars correspond to the standard deviation. C) Logarithm of the equilibrium constant as a function of pH. Green circles: experimental K values calculated as $\log(\frac{[\mathbf{3a}]_{eq}}{[\mathbf{2a}]_{eq}})$ were $[\mathbf{2a}]_{eq}$ and $[\mathbf{3a}]_{eq}$ are the concentrations of **2a** and **3a** at equilibrium; magenta circles: values of K calculated using rate constants k_2 and k_{-2} determined by data-fitting, $\log(\frac{k_{+2}}{k_{-2}})$.

With these data in hand we next performed additional experiments by replacing propionic acid by MPA with the aim of building a kinetic model for the uncatalyzed conversion of SEA^{off} peptide **1a** into MPA peptide thioester **4a** (Scheme 2A,C; Fig. 2). This novel dataset was combined with the rate constants k_2 and k_{-2} determined above for extracting the apparent rate constants k_3 and k_{-3} for the exchange of the SEA peptide thioester **3a** by MPA ($k_3 = 5.21\ 10^{-7} \pm$ nd $M^{-1}.s^{-1}$, $k_{-3} = 6.33\ 10^{-4} \pm 1.0\ 10^{-4}\ M^{-1}.s^{-1}$).

The kinetic model tells us that the conversion of SEA peptide thioester **3a** into peptide thioester **4a** is rate limiting at the working pH of 4.0. The fact that the thiol-thioester exchange step of the process shown in Scheme 1A is rate limiting is consistent with the accumulation of the SEA peptide thioester **3a** at the beginning of the reaction (Fig. 2A). Interestingly, the kinetic model enables to predict the impact of adding a catalyst of the thiol-thioester process on the time-course of the reaction (Fig. 2B). The catalyst will increase the apparent rate constants k_{+3} and k_{-3} without changing the equilibrium constant k_{+3}/k_{-3}. An acceleration factor of 2 relative to the uncatalyzed reaction is predicted to have already a real impact on the global rate, while the fastest rates can be achieved for acceleration factors greater than 15. This prompted us to search for additives that could restore a fast thiol-thioester regime, thereby allowing the SEA-based reactions to fully benefit from the fast *N,S*-acyl shift that is observed at mildly acidic pH. Such catalysts would be especially useful in the case of reactions at difficult amino acids such as Thr or Val.

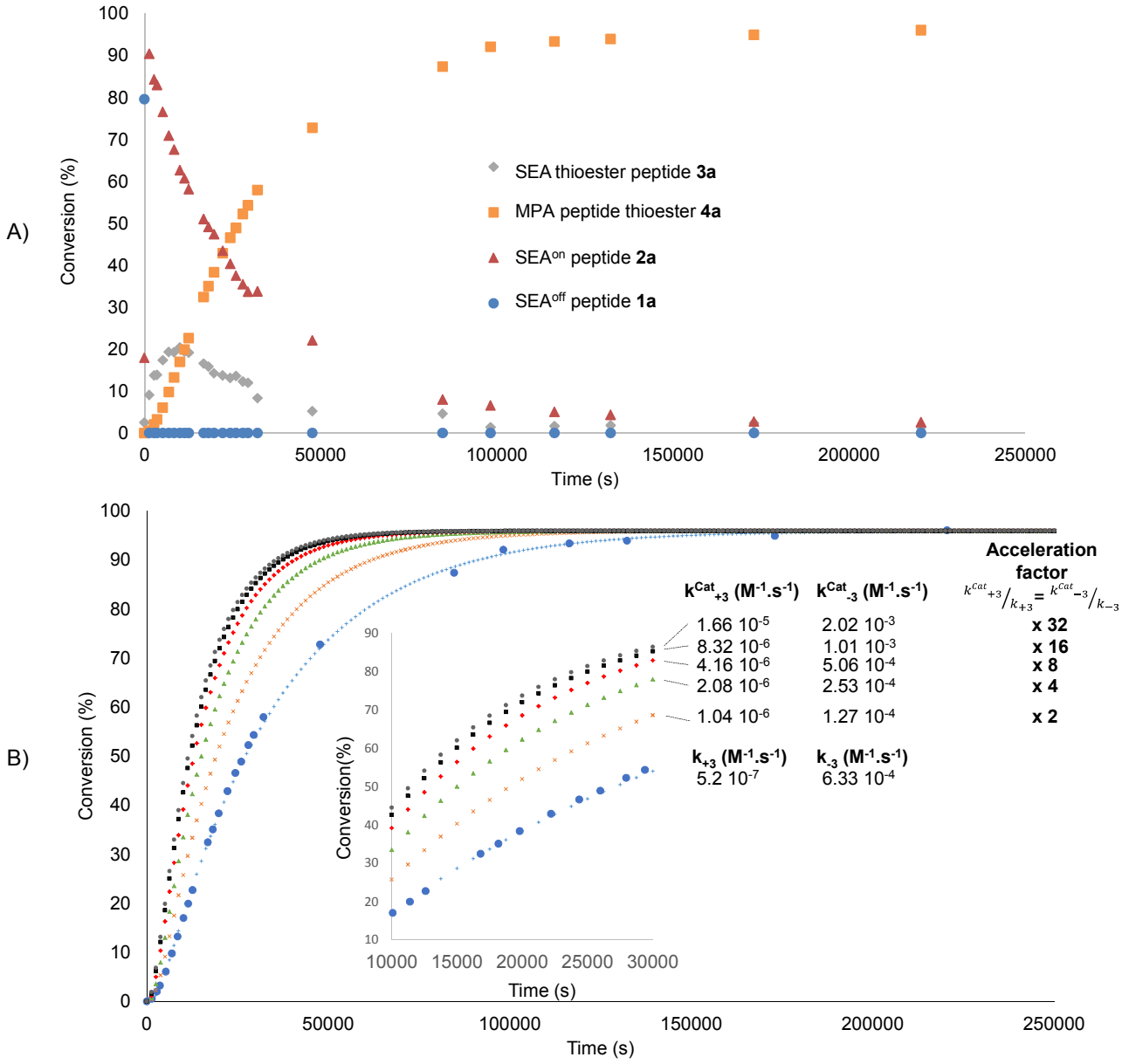


Figure 2. Kinetic study of the conversion of SEA^{off} peptide **1a** into MPA peptide thioester **4a** at pH 4.0. A) Time-course of the reaction. The proportion of each species in the mixture was determined by HPLC using UV detection at 215 nm. B) The kinetic model enables to predict the favorable impact of adding a catalyst of the thiol-thioester exchange process on the rate. Blue circles: experimental points for the uncatalyzed reaction. Blue crosses: kinetic model fitted to experimental data. The other traces were calculated using the kinetic model and the k^{Cat}_{+3} and k^{Cat}_{-3} values corresponding to the different acceleration factors indicated on the right.

Catalysis by classical arylthiol or selenol additives

We first examined the capacity of classical thiol or selenol additives, i.e. MPAA or phenylselenide PhSeH, to promote the formation of peptide thioester **4a**. For this purpose, SEA peptide **1a** was treated with the thiol MPA and TCEP in 6 M Gn·HCl at pH 4.0 in presence or absence of the additive (Fig. 3).[30] The kinetic profiles were determined by HPLC and fitted to a pseudo-first order kinetic law from which half-reaction times (t½) were extracted (Table 1).

In the absence of catalyst, formation of peptide thioester **4a** proceeded with a t½ of 7.28 h (Fig. 3, entry 1, Table 1). MPAA accelerated the SEA-thiol exchange reaction (Fig. 3), albeit with a limited effect (4.86 h, entry 3, Table 1). The fitting of the kinetic data to the model depicted in Scheme 2A shows that MPAA accelerated the thiol-thioester exchange step by a factor of about 2.5.

Replacing MPAA by PhSeH did not improve the reaction rate (Fig. 3, entry 4, Table 1) although the concentration of nucleophilic chalcogenate species at pH 4.0 is significantly larger for PhSeH vs MPAA due to the lower pK_a of the former.[5,32] Since selenophenyl esters are more powerful acylating species than thioesters derived from MPAA,[33] the poor catalytic properties of selenophenol is suggestive of an unfavourable selenophenol-alkylthioester exchange process. This lack of reactivity arises probably from the larger thermodynamic stability of alkylthioesters relative to selenophenylesters which disfavour the formation of the later in the reaction mixture.[13]

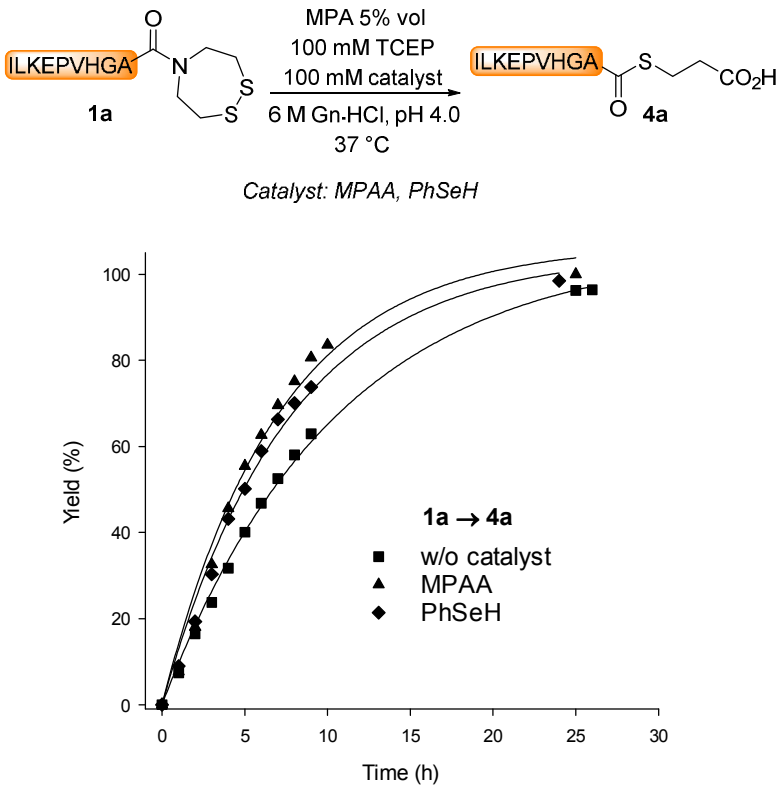


Figure 3. Exchange of the SEA group by MPA catalyzed by MPAA or selenophenol (generated by *in situ* reduction of diphenyldiselenide with TCEP). The continuous curves correspond to the fitting to a pseudo first order kinetic law from which t½ were extracted (see Table 1).

Table 1. Catalysis of SEA-thiol exchange.[a]

entry	SEA peptide	catalyst	catalyst conc. (mM)	$t_{1/2}$ (h) [b]
1	**1a**	ø	-	7.28
2	**1a**	MPAA	200	4.08
3	**1a**	MPAA	100	4.86
4	**1a**	PhSeH [c]	100	5.25
5	**1a**	**5a**	100	2.04
6	**1a**	**5a**	50	2.03
7	**1a**	**5c**	25	1.95
8	**1a**	**5c**	12.5	2.35
9	**1a**	**5c**	6.25	3.00
10	**1a**	**5c**	3.13	3.35
11	**1b**	ø	-	67.96
12	**1b**	MPAA	100	51.73
13	**1b**	**5a**	100	20.03
14	**1b**	**5b**	100	24.32
15	**1b**	**5c**	100	19.36
16	**1c**	ø	-	8.86
17	**1c**	**5b**	100	3.12
18	**1d**	ø	-	50.0 [d]
19	**1d**	**5b**	100	31.6

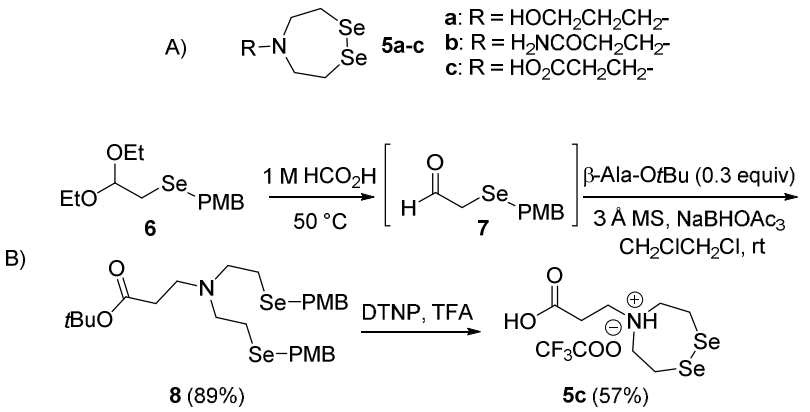
[a] Conditions: [**1a**] = [**1b**] = [**1c**] = 1 mM, 37 °C under inert atmosphere, 6 M Gn.HCl, MPA 5% v/v, 100 mM TCEP.HCl, pH 4.0. [b] Determined by HPLC. The value corresponds to the time needed to reach 50% conversion. [c] Selenophenol was formed *in situ* from PhSeSePh and was soluble in our conditions. [d] The data could not be fitted to a pseudo-first order kinetic law.

Novel catalyst design and synthesis

In search for alternative catalysts that could perform better than MPAA, we were interested by the findings of Makriyannis *et al*. who showed that an alkylthioester can be exchanged by an alkyselenol at mildly acidic pH.[34] Inspired by this work, which shows the formation of an alkylselenoester from an alkylthioester in the typical pH range for the SEA-mediated reactions, we undertook the synthesis of *N*-alkyldiselenides **5a-c** (Scheme 3A). These compounds are highly water-soluble due to their protonation at acidic pH. Moreover, the presence of an ammonium group closeby the selenol contributes to lower its pK_a (estimated pK_a 4.5) and thus the formation of selenoate species at the working pH of 4.0, i.e. the active species involved in the catalysis of the thiol-thioester exchange process. Moreover and as will be shown later, the *N*-alkyl group has no effect on catalysis. Therefore, it can be varied depending on the final application. For example, the carboxylic acid group of catalyst **5c** enables potentially its attachment to a solid support.

N-alkylated diselenides **5a-c** were initially prepared starting from 1,2,5-diselenazepane by alkylation with 3-bromo-1-propanol (for **5a)** or by silica gel-catalyzed aza-Michael addition[35] to acrylamide or acrylic acid (for **5b** or **5c** respectively, see Experimental Section). We also developed the third method described in Scheme 3 that facilitates the production of catalyst **5c** on a larger scale. In this case, the tertiary amine scaffold was produced by double reductive alkylation of β-alanine *tert*-butyl ester with aldehyde **7** (PMB: 4-methoxybenzyl). The precursor for aldehyde **7**, i.e. acetal **6**, was easily produced by adapting the method of Abbas *et al*.[36] Advantageously, cyclic diselenide formation and *t*Bu protecting group removal were performed simultaneously in TFA in the presence of 2,2′-dithiobis(5-nitropyridine) (DTNP).[37] This practical route provided diselenide **5c** with an overall yield of 51% and can potentially be applied to the synthesis of other *N*-alkylated perhydro-1,2,5-diselenazepane derivatives.

Scheme 3. Structure of catalysts 5a-c (A) and synthesis of diselenide 5c (B).



Catalysis of SEA thioester-thiol exchange reaction by N-alkyl-diselenides

Diselenide **5a** which is *in situ* reduced into the active alkyldiselenol significantly accelerated the formation of peptide thioester **4a** (Fig. 4B) and consequently reduced the t½ of the reaction by a factor 3.6 compared to the uncatalyzed reaction (entries 1 and 5, Table 1). The fitting of the kinetic data to the model described in Scheme 2A indicates that the exchange of the SEA thioester by MPA is accelerated more than 15 times compared to the uncatalyzed reaction.

Catalyst **5a** was then compared to its analogs **5b** and **5c** which differ by the functionality present on the exocyclic alkyl side chain. For this purpose, peptide **1b** which features a sterically demanding C-terminal Val residue was used as starting material (Fig. 4C). The three catalysts converted **1b** into the thioester peptide **4b** with almost equal efficiency (entries 13-15, Table 1), while MPAA had a minor effect compared to the control experiment without catalyst (compare entries 11 & 12, Table 1). Note also that catalyst **5b** promoted the SEA-thiol exchange reaction in the cases of SEA peptides equipped with a C-terminal Thr (**1c**, entries 16 and 17, Table 1) or Ile (**1d**, entries 18 and 19, Table 1) residues.

As mentioned above, diselenide **5a** was significantly more efficient than MPAA in catalyzing the SEA-thiol exchange reaction (compare entries 2 & 5, Table 1). Since the bivalency of catalyst **5a** might introduce a bias in the comparisons, we compared the catalytic potency of diselenide **5a** at 100 or 50 mM (entries 5,6 in Table 1) with those of MPAA used at 200 or 100 mM respectively (entries 2,3 in Table 1). In each case the rate of SEA-thiol exchange was significantly faster with diselenide **5a** than with MPAA, excluding the possibility that the differ-

ence observed at 100 mM for both catalysts might be due to a doubling of selenol concentration for **5a**. We also decreased the diselenide catalyst **5c** concentration to see if the SEA-MPA exchange is still accelerated at low catalyst concentration (see entries 7-10 in Table 1). Interestingly, diselenide catalyst **5c** had a significant effect on the rate even at 3 mM, and was more powerful at this concentration than MPAA at 200 mM.

We further examined the stability of our catalysts in the presence TCEP. While the metal-free desulfurization of Cys thiols requires a phosphine such as TCEP and a radical initiator to proceed at an appreciable rate,[38] the radical initiator is not necessary for the deselenization of alkylselenols by TCEP.[39] The deselenization of the catalysts could dramatically alter their catalytic potency over long reaction times. We therefore determined the extent of catalyst deselenization by quantifying the amount of TCEP-derived selenophosphine produced in the reaction mixture. This study indicated that less than 5% of the catalyst was deselenized after 24 h even for the lowest catalyst concentrations tested (see Supporting Information).

The usefulness of catalyst **5c** was then confirmed on preparative scale by the synthesis of peptide **4b** with 63% yield (Scheme 4). Chiral GC analysis of peptide **4b** after acid hydrolysis indicated a D-Val content below detectable levels (< 0.1%), and thus that no epimerization occurred during the exchange process. In another example, the 46 amino acids SEA peptide **1e** which is equipped with a C-terminal Thr residue was successfully converted into thioester **4e** using the same protocol (36%, Scheme 4).

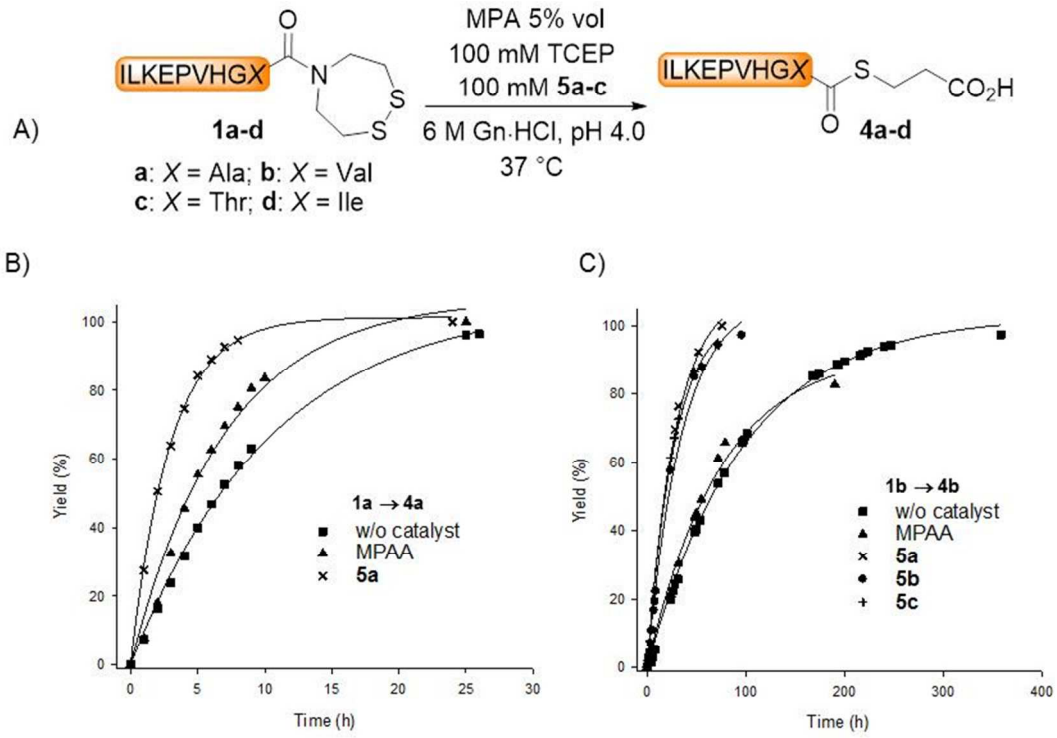
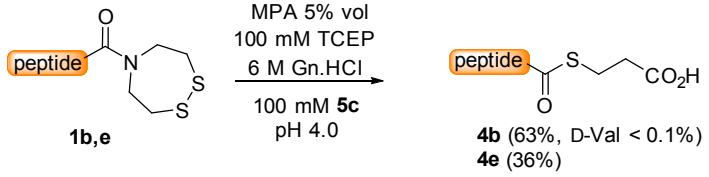


Figure 4. Exchange of the SEA group by MPA catalyzed by MPAA, selenophenol or diselenides **5a-c**. A) Model reactions. B) Conversion of peptide **1a** into **4a** catalyzed by MPAA or diselenide **5a**. C) Conversion of peptide **1b** into **4b** uncatalyzed, catalyzed by MPAA or diselenides **5a-c**. The continuous curves correspond to the fitting to a pseudo first order kinetic law from which t½ were extracted (see Table 1).

Scheme 4. Preparative synthesis of peptide thioesters using diselenide 5c.



b: 50 h, 37 °C. peptide = ILKEPVHGV-
e: 19 h, 30 °C. peptide = RLSPEYYDLARAHLRDEEKSC(S*t*Bu)PC(S*t*Bu)LAQEGPQGDLLTKT QELGRDYRT- (S*t*Bu groups are absent in **4e**)

Catalysis of SEA-mediated ligation by N-alkyl-diselenides

Having demonstrated the usefulness of diselenides **5** for catalyzing the SEA-thiol exchange reaction, we next examined if they could accelerate the mechanistically related SEA-mediated ligation as well. The optimal pH for such ligation catalyzed by MPAA is 5.5 and its rate decreases as the pH is lowered from 5.5 to 4.0 (Fig. 5). As for the SEA-MPA exchange reaction, diselenide **5b** was more powerful in promoting the SEA-based ligation of peptide **1b** at pH 4.0 than MPAA at pH 4.0 or 5.5. To illustrate the synthetic usefulness of diselenide **5c**, peptide **10** was synthesized on preparative scale (59%). Here again, no significant epimerization (0.19%) was observed for the valine involved in the formed junction. In another example, the 38 amino acids SEA peptide **1f** which is equipped with a C-terminal Val residue was successfully cyclized into cyclic peptide **11** in the presence of diselenide **5c** (Scheme 5). In this case *N*-octyl glucoside was used as a non-ionic detergent to avoid the gelification of the ligation mixture that had been observed when ligation was attempted in 6 M Gn·HCl. Note also that the high hydrophilicity of catalyst **5c** enabled the direct purification of the crude ligation mixtures by HPLC. This is a great advantage over MPAA which must be removed from the ligation mixture prior to the isolation step.[7,29,40]

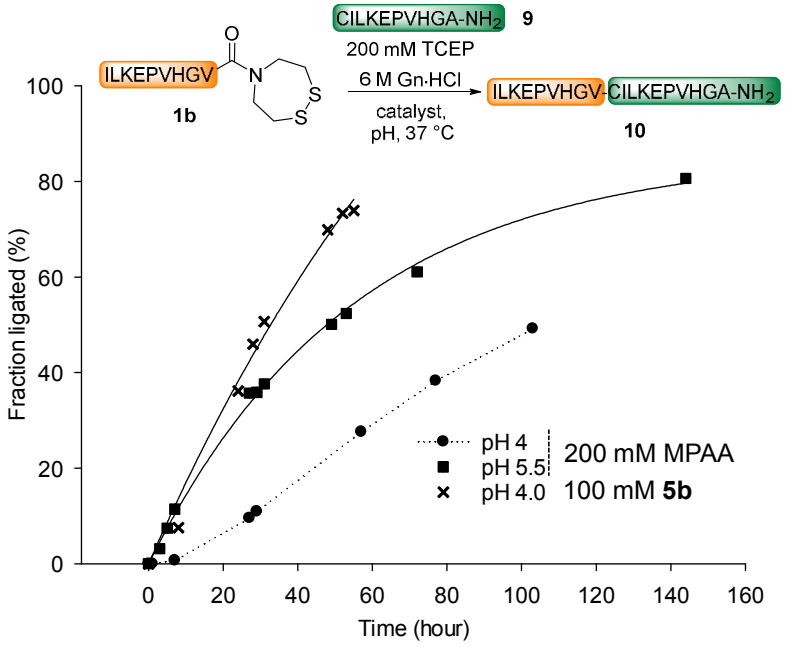
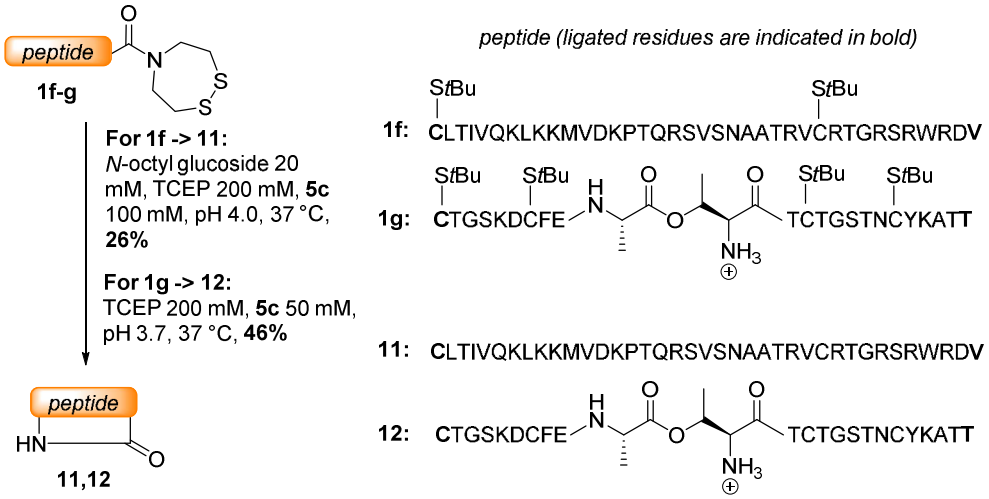


Figure 5. Catalysis of the SEA-mediated ligation by diselenide **5b** or MPAA. Peptide concentration was 7 mM.

The possibility to promote SEA-mediated ligations at pH 4.0 broadens the scope of the peptides amenable to chemical synthesis. We undertook the synthesis of cyclic peptide **12** (Scheme 5) whose linear precursor **1g** features a C-terminal Thr residue and an internal *O*-acyl isopeptide unit.[41-43] The later rearranges spontaneously by *O,N*-acyl shift above pH 5 to produce a native peptide bond.[41-43] Therefore, the classical conditions for NCL (MPAA, pH 7), SEA ligation (MPAA, pH 5.5) or for any other *N,S*-acyl shift mediated ligation cannot cyclize peptide **1g** without inducing the rearrangement of the *O*-acyl isopeptide unit. In contrast, the combination of a SEA peptide precursor and diselenide **5c** successfully yielded cyclic peptide **12** in about 10 h. Here again as for the other examples discussed above, the crude product could be directly purified by HPLC. The presence of the *O*-acyl isopeptide unit in the final product and the cyclic structure of peptide **12** was demonstrated by rearranging peptide **12** at neutral pH and by analyzing the resulting cyclic peptide *c*[CTGSKDCFEA TTCTGSTNCYKATT] **13** after alkylation and trypsin digestion (see Supporting Information).

Scheme 5. Cyclative SEA-mediated ligation catalyzed by diselenide 5c.



Conclusion

In conclusion, we describe a novel family of water-soluble alkyldiselenol catalysts for the *bis*(2-sulfanylethyl)amido (SEA)-thiol exchange reaction or the SEA-mediated ligation which are particularly useful for promoting the reactions at difficult amino acids. The performance of the novel catalysts at pH 4.0 is superior to those of MPAA at any pH. They act by accelerating thiol-thioester exchanges which are rate-limiting at pH 4.0. The catalysts have an excellent water-solubility due to the presence of a protonated tertiary amine within their structure and enable the direct purification of the reaction mixtures by HPLC. Besides accelerating the SEA-mediated reactions, working at pH 4.0 is potentially an advantage when the peptide segments are not soluble at higher pH. Ligation at pH 4.0 also extends the scope of peptides amenable to chemical synthesis such as cyclic *O*-acyl isopeptides which cannot be produced at neutral or slightly acidic pH. The novel catalysts should find various applications in the synthesis of complex peptides or challenging proteins.

EXPERIMENTAL SECTION

General Methods

Reagents and solvents

2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium fluorophosphate (HBTU) and *N*-Fmoc protected amino acids were obtained from Iris Biotech GmbH. Side-chain protecting groups used for the amino acids were Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Phe-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH, Fmoc-Cys(S*t*Bu)-OH or Fmoc-Cys(Trt)-OH. Synthesis of *bis*(2-sulfanylethyl)aminotrityl polystyrene (SEA PS) resin was carried out as described elsewhere.[19]

4-mercaptophenylacetic acid (MPAA), 3-mercaptopropionic acid (MPA), *tris*(2-carboxyethyl)phosphine hydrochloride (TCEP), guanidine hydrochloride (Gn·HCl) were purchased from Sigma-Aldrich. All other reagents were purchased from Acros Organics or Merck and were of the purest grade available.

Peptide synthesis grade *N,N*-dimethylformamide (DMF), dichloromethane (CH_2Cl_2), diethylether (Et_2O), acetonitrile (CH_3CN), heptane, LC–MS-grade acetonitrile (CH_3CN, 0.1% TFA), LC–MS-grade water (H_2O, 0.1% TFA), *N,N*-diisopropylethylamine (DIEA), acetic anhydride (Ac_2O) were purchased from Biosolve and Fisher-Chemical. Trifluoroacetic acid (TFA) was obtained from Biosolve. Water was purified with a Milli-Q Ultra-Pure Water Purification System.

Analyses

The reactions were monitored by analytical LC–MS (Waters 2695 LC/ZQ 2000 quadripole) on a reverse phase column XBridge BEH300 C18 (3.5 µm, 300 Å, 4.6 × 150 mm) unless otherwise stated. The elutions were carried out at 30 °C using a linear gradient of 0-100% of eluent B in eluent A over 30 min at a flow rate of 1 mL/min (eluent A = 0.1% TFA in H_2O; eluent B = 0.1% TFA in CH_3CN/H_2O: 4/1 v/v). The column eluate was monitored by UV at 215 nm and by evaporative light scattering (ELS, Waters 2424 detector). The peptide masses were measured by on-line LC–MS: Ionization mode: ES+, m/z range 350–2040, capillary voltage 3 kV, cone voltage 30 V, extractor voltage 3 V, RF lens 0.2 V, source temperature 120 °C, dessolvation temperature 350 °C.

MALDI-TOF mass spectra were recorded with a BrukerAutoflex Speed using alpha-cyano-4-hydroxycinnaminic acid, sinapinic acid or 2,5-dihydroxybenzoic acid (DHB) as matrix. The observed m/z corresponded to the monoisotopic ions, unless otherwise stated.

High resolution mass spectra (HRMS) were recorded with an Orbitrap mass spectrometer (ThermoFischer). ESI spectra (positive mode, 100-600 uma) were obtained by direct injection of the compounds.

^1H and ^{13}C NMR spectra were recorded on a Bruker Advance-300 spectrometer operating at 300 MHz and 75 MHz respectively. The spectra are reported as parts per million (ppm) down field shift using tetramethylsilane or dimethylselenide as internal references. The data are reported as chemical shift (δ), multiplicity, relative integral, coupling constant (J Hz) and assignment where possible.

The determination of optical purity for the C-terminal amino acid was done by chiral GC-MS following total acid hydrolysis in deuterated aqueous acid (C.A.T. GmbH & Co. Chromatographie und Analysen-

technik KG, Heerweg 10, D-72070 Tübingen, Germany).[44]

HPLC purification

Preparative reverse phase HPLC of crude peptides were performed with a preparative HPLC Waters system using a reverse phase column XBridge BEH300 Prep C18 (5 µm, 300 Å, 10 × 250 mm) and appropriate linear gradient of increasing concentration of eluent B in eluent A (flow rate of 6 mL/min). Selected fractions were then combined and lyophilized.

Preparative reverse phase HPLCs of crude selenium compounds were performed with an Autopurification prep HPLC–MS Waters system using a reverse phase column XBridge ODB prep C-18 (5 µm, 300 Å, 19 × 100 mm) and appropriate gradient of increasing concentration of eluent B in eluent A (flow rate of 25 mL/min). The fractions containing the purified target compound were identified on-line using MS (ZQ 2000 quadripole). Selected fractions were combined and lyophilized.

Kinetic studies

Kinetic model (see Scheme 2)

For the kinetic study of the MPA – SEA thioester exchange process (see Scheme 2), all reactions were set up from a single batch of lyophilized SEA^{off} peptide **1a**. Also, freshly prepared buffers and newly opened reagents were used for the whole series of experiments.

In order to determine the rate constants involved in the different steps of the SEA thioester – MPA exchange process, the system was decomposed to simpler reactions and the protocol was gradually complexified. Data were fitted and rate constants were determined using KinteK Explorer Software^{TM} (Version 7.2.180216., Kintek Corporation - https://kintekcorp.com/software/)

Step 1. Study of the SEA amide / SEA thioester equilibrium (see Figure 1)

The reduction and the rearrangement of the SEA peptide were investigated in the absence of MPA. In order to maintain the concentration of carboxylic acids to the same level in all reactions, MPA was replaced by propionic acid (574 mM).

The reaction mixture was prepared as follows: TCEP (28.65 mg, 100 mM) was dissolved in a 6 M Gn.HCl solution in phosphate buffer 0.1 M (1 mL). 958 µL of this solution was added to propionic acid (42 µL, 574 mM) and the pH was adjusted to the desired value by adding a 6 M NaOH solution. Lyophilized SEA^{off} peptide **1a** (~ 850 µg, ~0.6 µmol) was weighed directly in an HPLC vial and dissolved in the above described buffer (~200 µL, 3 mM).

The reaction mixture was left at 37°C in a temperature-controlled well plate autosampler (Dionex Ultimate 3000 RS) and 1 µL was injected for analysis at regular time intervals (eluent A 0.1% TFA in water, eluent B 0.1% TFA in CH_3CN, Column Acquity UPLC® Peptide BEH C18 300 Å, 1.7 µm, 2.1 mm x 100 mm, gradient 10-35% B in 3 min, 0.7 mL/min, 37° C). The chromatograms were processed on the basis of the absorbance signal at 215 nm to deduce the conversion ratio of the different species (no absorbance correction was applied as SEA^{off}, SEA^{on} amide and SEA thioester were considered to have the same molecular extinction coefficient).

All datasets were fitted with a four-phase exponential analytical function to obtain an estimate of the standard deviation. A subsequent global fit allowed determining rate constants k_1, k_{+2}, k_{-2} as well standard deviations at each studied pH (see Supporting Information).

Step 2. Kinetic study of the MPA - SEA thioester exchange (see Figure 2)

TCEP (28.65 mg, 100 mM) was dissolved in a 6 M Gdn•HCl solution in phosphate buffer 0.1 M (1 mL). 950 µL of this solution was added to mercaptopropionic acid (50 µL, 574 mM) and the pH was adjusted to 4.0 by adding a 6 M NaOH aqueous solution.

SEA^{off} peptide **1a** (~ 850 µg, ~0.6 µmol) was weighed in an eppendorf tube and dissolved in the above described buffer (~200 µL, 3 mM). The reaction mixture was left at 37°C under nitrogen atmosphere. 3 µL of the reaction mixture were collected every ~20 minutes and added to 100 µL of a 20% acetic acid aqueous solution. The sample was extracted four times by Et_2O and the aqueous layer was analyzed by

HPLC (eluent A 0.1% TFA in water, eluent B 0.1% TFA in CH_3CN/water: 4/1 by vol. C18 Xbridge BEH 300 Å 5 µm (4.6 × 250 mm) column, gradient 0-50% B in 15 min, 1 mL/min, 30° C, detection at 215 nm)

The chromatograms were processed on the basis of the absorbance signal at 215 nm to deduce the conversion ratio of the different species (no absorbance correction was applied as SEA^{off}, SEA^{on} amide and SEA thioester were considered to have the same molecular extinction coefficient). The data were processed as described above for Step 1.

SEA thioester-MPA exchange (see Figure 3 and 4)

General procedure illustrated with peptide **1a** and MPAA as catalyst

To a solution of TCEP·HCl (28.67 mg, 100 µmol, 100 mM final concentration) in 6 M Gn·HCl, 0.2 M, pH 7.2 sodium phosphate buffer (1 mL) was added MPAA (16.82 mg, 0.1 mmol, 100 mM final concentration) dissolved in 20 µL 5 M NaOH. Note that the presolubilization step in NaOH was not required for diselenide catalysts.

The SEA peptide **1a** (0.994 mg, 0.6 µmol) was dissolved in the previous solution (final peptide concentration 1 mM). To the resulting mixture was added MPA (5% v/v). The pH was adjusted to 4.0 by addition of 5 M NaOH and the reaction mixture was then stirred at 37 °C under nitrogen atmosphere.

The progress of the reaction was monitored by HPLC. For each point, a 2 µL aliquot was taken from the reaction mixture and quenched by adding 50 µL of 0.1% aqueous TFA. The sample was then extracted with Et_2O to remove MPAA prior to HPLC analysis. Note that this extraction step was not required for diselenide catalysts.

SEA-mediated ligation (see Figure 5)

General procedure illustrated with peptide **1b**, Cys peptide **8** and MPAA as catalyst

To a solution of TCEP·HCl (11.47 mg, 40 μmol, 200 mM final concentration) in 6 M Gn·HCl, pH 7.2, 0.1 M sodium phosphate buffer (200 μL) was added MPAA (6.72 mg, 40 μmol, 200 mM final concentration) dissolved in 20 μL 5 M NaOH. The pH was then adjusted to 4-5.5 by addition of 5 M NaOH. Note that the presolubilization step in NaOH was not required for diselenide catalysts.

The SEA peptide **1b** (1.498 mg, 1 μmol, 7 mM final concentration) and Cys peptide **8** (2.22 mg, 1.5 μmol, 10.5 mM, 1.5 equiv) were dissolved in the previous solution. The reaction mixture was stirred at 37 °C under nitrogen atmosphere.

The progress of the reaction was monitored by HPLC. For each point, a 2 μL aliquot was taken from the reaction mixture and quenched by adding 50 μL of 0.1% aqueous TFA. The sample was then extracted with Et_2O to remove MPAA prior to HPLC analysis. Note that this extraction step was not required for diselenide catalysts.

Peptide synthesis

General protocol for automated peptide synthesis

Peptide elongation was performed using standard Fmoc/*tert*-butyl chemistry on an automated peptide synthesizer (0.2 mmol scale). Couplings were performed using 5-fold molar excess of each Fmoc-L-amino acid, 4.5-fold molar excess of HBTU, and 10-fold molar excess of DIEA. A capping step was performed after each coupling with Ac_2O/DIEA in DMF. At the end of the synthesis, the resin was washed with CH_2Cl_2, diethylether (3 × 2 min) and dried *in vacuo*.

Synthesis of SEA^{off} peptide segments

Peptide elongation was performed on SEA PS resin[19] (0.2 mmol, 0.16 mmol/g). Typical procedures for the synthesis of SEA^{off} peptide segments were described in previous papers.[19,29,45]

For the synthesis of SEA^{off} peptide **1g**, Boc-Thr(Fmoc-Ala)-OH and the following amino acid were coupled manually using HBTU/DIEA activation in DMF.

Peptides **1a-d** were already described elsewhere.[19]

RLSPEYYDLARAHLRDEEKSC(S*t*Bu)PC(S*t*Bu)LAQEGPQGDLLTKTQELGRDYRT-SEA^{off} (peptide **1e**): 26 mg (8% yield, 0.05 mmol scale). MALDI-TOF m/z calcd for $[M+H]^+$ (average): 5618.48, found: 5617.2.

C(S*t*Bu)LTIVQKLKKMVDKPTQRSVSNAATRVC(S*t*Bu)RTGRSRWRDV-SEA^{off} (peptide **1f**): 11.5 mg (4% yield, 0.05 mmol scale). MALDI-TOF m/z calcd for $[M+H]^+$ (average): 4683.8, found: 4683.3.

T[C(S*t*Bu)TGSKDC(S*t*Bu)FEA]TC(S*t*Bu)TGSTNC(S*t*Bu)YKATT-SEA^{off} (peptide **1g**): 0.1 mmol scale, 36.1 mg (11%). MALDI-TOF m/z calcd for $[M+H]^+$ (monoisotopic): 2962.1, found: 2962.2

Synthesis of Cys peptides

The synthesis of peptide **9** has been described elsewhere.[19]

*Synthesis of diselenide catalysts **5a-c***

Synthesis of diselenide **5a** (alkylation of 1,2,5-diselenazepane with 3-bromopropanol/CsOH)

Cesium hydroxide monohydrate (509 mg, 3.00 mmol, 2 equiv) was added to activated powdered 4 Å molecular sieve (400 mg) in anhydrous *N,N*-dimethylformamide (1 mL). The white suspension was vigorously stirred for 10 min at rt. Then, the 1,2,5-diselenazepane trifluoroacetate salt[46-47] (350 mg, 1.5 mmol) was dissolved in DMF (2.5 mL) and added to the suspension of molecular sieve. The mixture was stirred for 30 min before adding 3-bromopropanol (402 µL, 4.55 mmol, 3 equiv). The reaction mixture was stirred for 20 h at rt, diluted with water containing 0.1% TFA (20 mL) and centrifuged. The supernatant was collected, filtered and purified by RP-HPLC (XBridge C18 OBD column, eluent A: water containing 0.1% TFA by vol, eluent B: water/acetonitrile: 1/4 by vol containing 0.1% TFA by vol, detection at 215 nm, 25 mL/min, 0-15% eluent B in 10 min). The purified fractions were collected, frozen and lyophilized to give 19.53 mg (4.5%) of **5a** as the trifluoroacetate salt.
The synthesis of **5a** was unsatisfatory and was discontinued.
Catalyst **5a**: HRMS calcd. for $[M+H]^+$ $C_7H_{16}ONSe_2$ 289.9557, found 289.9552 (-1.67 ppm).

^{1}H NMR (300 MHz) spectrum for compound **5a** (DMF-d7, 300 K). δ 3.97 (m, 4H), 3.69 – 3.65 (m, 3H), 3.46 – 3.40 (m, 6H), 2.00 (m, 2H) ppm.

^{13}C JMOD NMR (75 MHz) spectrum for compound **5a** (DMF-d7, 300 K). δ 59.2 (CH_2), 54.6 (4 × CH_2), 22.30 (CH_2) ppm. One ^{13}C signal is not seen due to overlap with the residual signal from the solvent.

Synthesis of diselenide catalyst **5b** (reaction of 1,2,5-diselenazepane with acrylamide)

The 1,2,5-diselenazepane trifluoroacetate salt[47] (43.2 mg, 0.125 mmol, 1 equiv) and acrylamide (44.4 mg, 0.625 mmol, 5 equiv) were dissolved separately in acetonitrile (1 mL for each reactant). The two solutions were combined and silica gel (50 % w/w relative to the diselenide) and triethylamine (17 µL, 0.125 mmol, 1 equiv) were then successively added to the mixture. Note that the 1,2,5-diselenazepane trifluoroacetate salt precipitates partially upon Et_3N addition but after a few time the precipitate disappears probably due to its adsorption on silica gel.

The suspension was stirred at 60 °C and the progress of the reaction was monitored by LC-MS. After 24 h, more acrylamide (5 equiv) was added to the reaction mixture and the resulting suspension was stirred at 60 °C for two further days. When the reaction was completed, the supernatant was collected and 2 mL of water-TFA 0.1% were added to the residue of silica gel. The mixture was stirred for 30 min and the supernatant was collected. This washing procedure was repeated twice. All supernatants were combined and concentrated *in vacuo* to remove the acetonitrile. This operation must be carefully controlled to avoid evaporation to dryness.

The solution was filtered and purified by RP-HPLC (XBridge C18 OBD column, eluent A : water containing 0.1% TFA by vol, eluent B: water/acetonitrile : 1/4 by vol containing 0.1% TFA by vol, detection at 215 nm, 25 mL/min, 0-15% eluent B in 20 min). The purified fractions were collected, frozen and lyophilized to give 30.9 mg (40%) of **5b** as the trifluoroacetate salt.

Catalyst **5b**: HRMS calcd. for $[M+H]^+$ $C_7H_{15}ON_2Se_2$ 302.9509, found 302.9503 (-1.98 ppm).

^{1}H NMR (300 MHz) spectrum for compound **5b** (DMF-d7, 333 K). δ 7.59 (s, 1H), 6.84 (s, 1H), 3.77 – 3.74 (m, 4H), 3.42 – 3.36 (t, J = 6.9 Hz, 2H), 3.33 – 3.29 (m, 4H), 2.73 – 2.64 (t, J = 6.9 Hz, 2H) ppm.

^{13}C JMOD NMR (75 MHz) spectrum for compound **5b** (DMF-d7, 291 K). δ 172.49 (C), 55.93 (2 × CH_2), 53.90 (CH_2), 23.33 (2 × CH_2) ppm. One ^{13}C signal is not seen due to overlap with the residual signal from the solvent.

Synthesis of catalyst **5c** (Scheme 3)

Synthesis of (2,2-diethoxyethyl)(4-methoxybenzyl)selane **6**

To a solution of 1,2-*bis*(2,2-diethoxyethyl)diselane[36] (1.94 g, 4.94 mmol) in ethanol (34 mL) was added portionwise (30 min) at 0 °C $NaBH_4$ (500 mg, 13.2 mmol, 2.7 equiv). Once the emission of H_2 stopped, the reaction mixture was warmed to 25 °C for 30 min. Then the reaction mixture was cooled to 0 °C and 4-methoxybenzyl chloride (1.24 mL, 9.19 mmol, 1.9 equiv) was added. The reaction mixture was stirred at room temperature for 2 h. Water (150 mL) was added afterwards and the obtained aqueous layer was extracted with Et_2O (2 × 100 mL). The combined organic layers were dried over $MgSO_4$ and the solvent was evaporated under reduced pressure. Purification of the crude by column chromatography (cyclohexane/ethyl acetate 95:5) provided the expected selenide **6** (2.75 g, 94%) as an oil.

Compound **6**: ESI-MS $[M+Na]^+$ calcd. 341.06, found 341.09.

^{1}H NMR (300 MHz) spectrum for compound **6** ($CDCl_3$, 293 K). δ 7.23 (d, J = 8.6 Hz, 2H), 6.82 (d, J = 8.6 Hz, 2H), 4.59 (t, J = 5.6 Hz, 1H), 3.82 (s, 2H), 3.78 (s, 3H), 3.59-3.71 (m, 2H), 3.45-3.57 (m, 2H), 2.63 (d, J = 5.6 Hz, 2H), 1.21 (d, J = 7.0 Hz, 6H) ppm.

^{13}C JMOD NMR (75 MHz) spectrum for compound **6** ($CDCl_3$, 293 K). δ 158.4 (C), 131.3 (C), 130.0 (2 × CH), 113.9 (2 × CH), 103.7 (CH), 62.0 (2 × CH_2), 55.2 (CH_3), 27.1 (CH_2), 26.3 (CH_2), 15.3 (2 × CH_3) ppm.

Synthesis of *tert*-butyl 3-(bis(2-((4-methoxybenzyl)selanyl)ethyl)amino)propanoate **8**

The selenide **6** (1.24 g, 3.90 mmol) was suspended in formic acid (1 M, 16 mL). The reaction mixture was stirred at 50 °C overnight. Water (50 mL) was added and the obtained aqueous layer was extracted with Et_2O (2 × 50 mL). The combined organic layers were dried over $MgSO_4$ and the solvent evapo-

rated under reduced pressure. The aldehyde **7** contained in the residue was dissolved under argon atmosphere in anhydrous 1,2-dichloroethane (8 mL). Activated powdered 3 Å molecular sieves (1 g), sodium triacetoxyborohydride (700 mg, 4.3 mmol, 3.3 equiv) and a solution of β-alanine *t*-butyl ester hydrochloride (236 mg, 1.3 mmol) in DCE (5 mL) were successively added to the solution of aldehyde **7**. After 24 h stirring at RT, the reaction mixture was filtrated on Büchner funnel and the filtered solid was washed with additional portion of DCM. The filtrate was evaporated under reduced pressure. Then, 10% K_2CO_3 (25 mL) and brine (30 mL) were added to residue and the obtained aqueous layer was extracted with DCM (3 × 50 mL). The combined organic layers were dried over $MgSO_4$ and the solvent was evaporated under reduced pressure. Purification of the crude by column chromatography (cyclohexane/ethyl acetate 95:5) provided the amine **8** (695 mg, 89% from the β-amino acid).

Compound **8**: ESI-MS $[M+H]^+$ calcd. 602.1, found 602.2.

^{1}H NMR (300 MHz) spectrum for compound **8** ($CDCl_3$, 305 K). δ 7.20 (d, J = 8.7 Hz, 4H), 6.82 (d, J = 8.7 Hz, 4H), 3.76 (s, 6H), 3.76 (s, 4H), 2.69 (t, J = 7.1 Hz, 2H), 2.56-2.62 (m, 4H), 2.46-2.51 (m, 4H), 2.28 (t, J = 7.1 Hz, 2H), 1.44 (s, 9H) ppm.

^{13}C JMOD NMR (75 MHz) spectrum for compound **8** ($CDCl_3$, 293 K). δ 171.6 (C), 158.2 (2 × C), 131.1 (2 × C), 129.7 (4 × CH), 113.7 (4 × CH), 80.1 (C), 55.0 (2 × CH_3), 54.1 (2 × CH_2), 49.0 (CH_2), 33.8 (CH_2), 28.0 (3 × CH_3), 26.3 (2 × CH_2), 21.4 (2 × CH_2) ppm.

Synthesis of catalyst **5c**

To the amine **8** (50 mg, 0.08 mmol) dissolved in TFA (0.5 mL) was added dropwise a solution of DTNP (51.36 mg, 0.16 mmol, 2 equiv) in TFA (0.5 mL). The reaction mixture was stirred during 1 h at r.t. Then, cold Et_2O was added (40 mL) and the mixture was evaporated under reduced pressure. Water (10 mL) was added and the solution was purified by RP-HPLC (XBridge C18 OBD column, eluent A: water containing 0.1% TFA by vol, eluent B: water/acetonitrile: 1/4 by vol containing 0.1% TFA by vol, detection at 215 nm, 25 mL/min, 0-15% eluent B in 15 min). The purified fractions were collected, frozen and lyophilized to give 19.8 mg (57%) of **5c** as the trifluoroacetate salt.

Catalyst **5c**: HRMS calcd. for $[M+H]^+$ $C_7H_{14}O_2NSe_2$ 303.9350, found 303.9348 (-0.13 ppm).

^{1}H NMR (300 MHz) spectrum for compound **5c** (DMF-d7, 310 K). δ 3.78 – 3.75 (t, *J* = 5.3 Hz, 4H), 3.41 – 3.36 (t, *J* = 7.2 Hz, 2H), 3.31 – 3.27 (t, *J* = 5.4 Hz, 4H), 2.81 – 2.76 (t, *J* = 7.2 Hz, 2H) ppm

^{13}C JMOD NMR (75 MHz) spectrum for compound **5c** (DMF-d7, 310 K). δ 172.50 (C), 55.66 (2 × CH_2), 52.16 (CH_2), 24.61 (2 × CH_2) ppm. One ^{13}C signal is not seen due to overlap with the residual signal from the solvent.

Preparative synthesis of peptide thioesters by SEA-thiol exchange

Synthesis of the MPA thioester **4b**

TCEP·HCl (14.33 mg, 50 μmol, 100 mM final concentration) was dissolved in 6 M Gn·HCl, pH 7.2 0.2 M sodium phosphate buffer (0.5 mL). To the resulting solution (448 μL) were added diselenide **5c** (18.64 mg, 50 μmol, 100 mM final concentration), peptide **1b** (4.55 mg, 3.1 μmol, final peptide concentration 7 mM) and MPA (22 μL, 5% v/v). The pH was adjusted to 4.0 using 5 M NaOH and the reaction mixture was stirred at 37 °C under nitrogen atmosphere. After completion of the reaction, the mixture was diluted with water-TFA 0.1% (5 mL) and purified by reversed-phase HPLC using a linear water-acetonitrile gradient containing 0.1% TFA to give the peptide **4b** (2.8 mg, 63% yield).

The determination of the optical purity for the C-terminal Val residue was performed by chiral GC-MS analysis after acid hydrolysis in deuterated acid. The analysis was done by C.A.T. GmbH & Co. company (Chromatographie und Analysen technik KG, Heerweg 10, D-72070 Tübingen, Germany). The analysis indicated a D-Val content < 0.1%.

Peptide **4b**: ESI-MS m/z = 1079.8 ($[M+H]^+$), 540.6 ($[M+2H]^{2+}$); calcd. for M (average): 1079.3, found: 1079.0. MALDI-TOF matrix = α-cyano-4-hydroxycinnamic acid, positive detection mode, m/z calcd for $[M+H]^+$ (monoisotopic): 1079.6, found: 1079.3.

Synthesis of the MPA thioester **4e**

TCEP·HCl (8.6 mg, 30 μmol, 100 mM final concentration) was dissolved in 6 M Gn·HCl, 0.2 M pH 7.2 sodium phosphate buffer (300 μL). To the solution were added diselenide **5c** (12.48 mg, 30 μmol,

100 mM final concentration) and MPA (15 µL, 5% v/v). The pH was then adjusted to 4.0 using 6 M NaOH.

Peptide **1e** (13.32 mg, 2 µmol) was dissolved in the previous solution (287 µL, final peptide concentration 7 mM) and the reaction mixture was stirred at 37 °C under nitrogen atmosphere. The progress of the reaction was monitored by HPLC.

After completion of the reaction, the mixture was diluted with water-TFA 0.1% (5 mL). The crude peptide was purified by RP-HPLC (eluent A = water containing 0.1% TFA, eluent B = acetonitrile in water 4/1 containing 0.1% TFA, 60 °C, detection at 215 nm, 6 mL/min, 0-25% eluent B in 10 min then 25-35% eluent B in 60 min, C3 Zorbax column) to give 4.65 mg of peptide **4e** (36%).
Peptide **4e**: ESI-MS m/z = 1354.2 ($[M+4H]^{4+}$), 1083.8 ($[M+5H]^{5+}$), 903.1 ($[M+6H]^{6+}$), 774.2 ($[M+7H]^{7+}$), 677.5 ($[M+8H]^{8+}$), 602.3 ($[M+9H]^{9+}$); calcd. for M (average) 5412.04, found: 5412.50. MALDI-TOF matrix = sinapinic acid, positive detection mode, m/z calcd for $[M+H]^{+}$ (average): 5413.0, found: 5413.0.

Preparative SEA-mediated ligations

Synthesis of peptide **10** by reaction of the SEA^{off} peptide **1b** with Cys peptide **9**

TCEP·HCl (28.66 mg, 100 µmol, 200 mM final concentration) was dissolved in 6 M Gn·HCl, 0.1 M pH 7.2 sodium phosphate buffer (0.5 mL). To the resulting solution was added diselenide **5c** (20.76 mg, 50 µmol, 100 mM). The pH was then adjusted to 4.0 by addition of 6 M NaOH.
The SEA peptide **1b** (5.06 mg, 3.5 µmol, 7 mM) and Cys peptide **9** (7.52 mg, 10.5 mM, 1.5 equiv) were dissolved in the previous solution (499 µL) and the reaction mixture was stirred at 37 °C under nitrogen atmosphere. After completion of the ligation, the mixture was diluted with water-TFA 0.1% (5 mL) and purified by reversed-phase HPLC using a linear water-acetonitrile gradient containing 0.1% TFA to give the purified ligation product **10** (4.69 mg, 51% yield)**.**

The determination of the optical purity for the C-terminal amino acid residue within peptide **10** was performed by chiral GC-MS analysis after acid hydrolysis in deuterated acid. The analysis was done by C.A.T. GmbH & Co. company (Chromatographie und Analysen technik KG, Heerweg 10, D-72070

Tübingen, Germany). The analysis indicated a D-Val content of 0.19%.
Peptide **10**: ESI-MS m/z = 1034.2 ($[M+2H]^{2+}$), 689.7 ($[M+3H]^{3+}$), 517.5 ($[M+4H]^{4+}$); calcd. for M (average): 2066.5, found 2066.1. MALDI-TOF matrix = α-cyano-4-hydroxycinnamic acid, positive detection mode, m/z calcd for $[M+H]^{+}$ (monoisotopic): 2066.2, found: 2066.5.

Synthesis of cyclic peptide **11** by cyclization of peptide **1f**

N-octyl glucoside (1.17 mg, 4 μmol, 20 mM) was dissolved in 0.1 M pH 7.2 sodium phosphate buffer (200 μL). To the solution were added TCEP·HCl (11.47 mg, 40 μmol, 200 mM) and diselenide **5c** (6.45 mg, 20 μmol, 100 mM). The pH was then adjusted to 4.0 by addition of 6 M NaOH.
Peptide **1f** (2.75 mg, 0.5 μmol, 3 mM) was dissolved in the previous solution (154 μL) and the reaction mixture was stirred at 37 °C under nitrogen atmosphere. The progress of the reaction was monitored by HPLC.

After completion of the cyclisation, the mixture was diluted with 6 M Gn·HCl in water to achieve 500 μL final volume. The crude peptide was purified by RP-HPLC (eluent A = water containing 0.1% TFA, eluent B = acetonitrile in water 4/1 containing 0.1% TFA, 60 °C, UV-detection at 215 nm, 6 mL/min, 0-25% eluent B in 10 min then 25-45% eluent B in 60 min, C3 Zorbax column) to give 0.67 mg of cyclic peptide **11** (26%).
Peptide **11**: ESI-MS m/z = 875.3 ($[M+5H]^{5+}$), 729.5 ($[M+6H]^{6+}$), 625.4 ($[M+7H]^{7+}$), 547.3 ($[M+8H]^{8+}$); calcd. for M (average): 4371.2, found 4370.9. MALDI-TOF matrix = sinapinic acid, positive detection mode, m/z calcd for $[M+H]^{+}$ (average): 4372.2, found: 4372.7.

Synthesis of cyclic peptide **12** by cyclization of peptide **1g**

TCEP·HCl (37.05 mg, 129 μmol, 200 mM) and diselenide **5c** (13.37 mg, 32 μmol, 50 mM) were added to a 6 M Gn.HCl phosphate buffer 0.1 M (650 μL). The pH was adjusted to 3.64 by addition of 6 M NaOH. Peptide **1g** (4.78 mg, 1.45 μmol, 2.25 mM) was dissolved in the previous solution and the reaction mixture was stirred at 37 °C under nitrogen atmosphere. The progress of the reaction was monitored by HPLC.

After completion of the cyclisation (i.e. ~10 h), the mixture was diluted in a 0.1% aqueous TFA solution (4.5 mL final volume). The crude peptide was immediately purified by RP-HPLC (eluent A = water containing 0.1% TFA, eluent B = acetonitrile in water 4/1 containing 0.1% TFA, 50 °C, UV-detection at 215 nm, 6 mL/min, 0-10% eluent B in 5 min then 10-30% eluent B in 35 min, C18 Zorbax column) to give 1.81 mg of cyclic peptide **12** still containing the *O*-acyl isopeptide unit (46 %).
Peptide **12**: ESI-MS m/z 1238.6 ($[M+2H]^{2+}$), 826.2 ($[M+3H]^{3+}$), calcd. for M 2475.7 (average), found 2475.3. MALDI-TOF matrix sinapinic acid, positive detection mode, $[M+H]^{+}$ calcd (monoisotopic) 2474.99, observed mass: 2474.98 (monoisotopic).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Supporting Information describes in more detail the kinetic experiments and model, and the characterization of all compounds (PDF).

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