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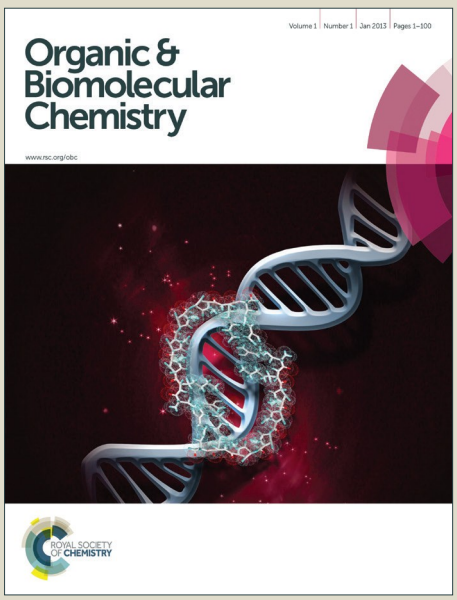

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Insight into the SEA amide thioester equilibrium. Application to the synthesis of thioesters at neutral pH

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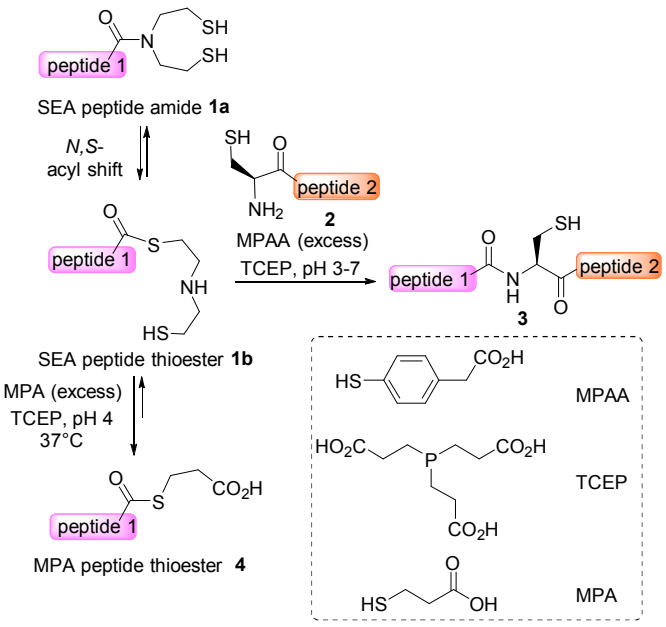
The *bis*(2-sulfanylethyl)amide (SEA) *N,S*-acyl shift thioester surrogate has found a variety of useful applications in the field of protein total synthesis. Here we present novel insights into the SEA amide/thioester equilibrium in water which is an essential step in any reaction involving the thioester surrogate properties of the SEA group. We show also that the SEA amide thioester equilibrium can be efficiently displaced at neutral pH for accessing peptide alkylthioesters, i.e. the key components of the native chemical ligation (NCL) reaction.

Introduction

Modern protein total synthesis relies on the coupling of unprotected peptide segments in water using chemoselective amide bond forming reactions.[1-6] Peptide thioester chemistry plays a central role in the field since the discovery of the native chemical ligation (NCL) by Dawson and coworkers.[1,4] This reaction is based on the chemoselective reaction of a C-terminal peptide thioester with an N-terminal cysteinyl (Cys) peptide in water at neutral pH. The reaction produces a native peptide bond to cysteine and since its introduction in 1994 it has been extended to the formation of nearly all types of peptide junctions using desulfurization,[7-9] deselenization[10,11] or thiol auxiliary approaches.[12-14] During the last decade, several *N,S*-acyl shift systems have been designed for acting as thioester surrogates[3,15-23] (for recent reviews see[24,25]). A few of them have the capacity to act as thioester surrogates at neutral pH, and thus to react spontaneously and chemoselectively with Cys peptides in a manner analogous to the NCL reaction.[3,22,23,26] Although these *N,S*-acyl shift systems are becoming more and more popular in the field of protein total synthesis, their mechanism of action is far from being fully understood.[27]

The *bis*(2-sulfanylethyl)amide (SEA[3]) group belongs to this growing family of useful thioester surrogates which can substitute classical peptide thioesters or be combined with them for designing efficient protein synthetic strategies (Scheme 1).[5,28-31] SEA peptide amide **1a** rearranges spontaneously in water into the SEA thioester form **1b**. Apparently, this rearrangement takes place in a wide range of pH since SEA ligation with Cys peptides **2** to produce peptide **3** has been shown to proceed from pH 3 to 7.[3,32] The SEA thioester form **1b** can also be trapped *in situ* by simple alkylthiols such as 3-mercaptopropionic acid (MPA) to produce MPA peptide thioesters of type **4** which can be isolated (Scheme 1).[33] This transformation must be carried out at an acidic pH of 4 to be of synthetic value.

This study provides novel insights into the SEA amide/thioester equilibrium, which is the starting point of all the reactions involving the SEA thioester surrogate. We characterized also the SEA/MPA exchange reaction at different pH. Since the level of SEA/MPA exchange was modest at neutral pH, we sought to displace the equilibrium toward the MPA peptide thioester for providing an access to these valuable peptide derivatives using very mild conditions.



Scheme 1. Principle of SEA *N,S*-acyl shift system and of SEA native peptide ligation.

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Results and discussion

A. SEA amide/thioester equilibrium

The SEA amide/thioester equilibrium was studied using SEA peptides **5a-d** (Fig 1A) which were produced by Fmoc SPPS as described elsewhere.[3] The SEA cyclic disulfide was reduced into the active dithiol form in the presence of TCEP at pH 6.5 and then diluted in the appropriate solution to fix the pH (HCl, citrate phosphate buffer, citrate buffer or phosphate buffer depending on the final pH, see Supporting Information). The peptide solutions were equilibrated at 37 °C and then analyzed by HPLC using UV detection at 215 nm to quantify the relative amount of SEA amide and thioester forms. At pH ~2, peptide **5a** equilibrated in about 2 h, peptides **5b,c** in about 5 h while peptide **5d** required up to 30 h to reach the equilibrium. The logarithm of the ratio [SEA thioester **7**/SEA amide **6**] was plotted as a function of the pH (Fig. 1B). Fig. 1B shows that Log(SEA thioester **7**/SEA amide **6**) is linearly correlated with the pH for all the tested peptides. The lines corresponding to the linear fits are nearly identical for peptides **5b-d** featuring a C-terminal Ala, Tyr or Val residue respectively. These lines differ slightly from the one obtained for peptide **5a** equipped with a C-terminal Gly. No useful data could be obtained above pH 5 by HPLC using UV detection due to the low proportion of the SEA thioester form **7** in the mixture at equilibrium. A higher signal-to-noise ratio could be achieved by analyzing the mixtures by LC-MS and using the total ion current (TIC) for the detection. Using this detection technique we could estimate the value of Log(SEA thioester **7b**/amide **6b**) at pH 5.4 (-2.1456) and pH 7.1 (-2.6566). These data are indicated by a red arrow in Fig. 1B. Fig. 1C shows the HPLC trace for the **6b**/**7b** mixture equilibrated at pH 7.1. The extrapolation of the linear fit for Log(SEA thioester **7b**/amide **6b**) (continuous red line in Fig. 1B) gives values of -2.06 at pH 5.4 and -3.23 at pH 7.1. These calculated values are close to those determined experimentally. Note that the experimental value for Log(SEA thioester **7b**/amide **6b**) at pH 7.1 is probably overestimated due to the partial rearrangement of the SEA amide peptide **6b** during the elution step, which is carried out at pH ~ 1.8). We thus consider that at pH 7 a proportion of 0.1% for the SEA thioester form **7** at equilibrium is a good estimate, meaning a concentration of the order of a few µM if the SEA peptide amide is solubilized at a mM concentration as is done usually.

SEA ligation proceeds at neutral pH, suggesting that thiol/SEA thioester exchanges take place despite the very low concentration of the SEA thioester form in these conditions. SEA ligation is a transamidation process which is probably driven by the nearly irreversible formation of the peptide bond to cysteine.[25] The experiment depicted in Scheme 2 was designed to test if a SEA peptide can equilibrate at neutral pH with some *bis*(2-sulfanylethyl)amine added exogenously. To follow the rate of exchange by LC-MS we used the ^{15}N-labelled *bis*(2-sulfanylethyl)amine **10** (trifluoroacetate salt). Peptide **8** was used in this experiment since it gives only a singly charged molecular ion by LC-MS, thereby facilitating the quantitation of the exchange reaction. The data presented in Fig. 2 show that

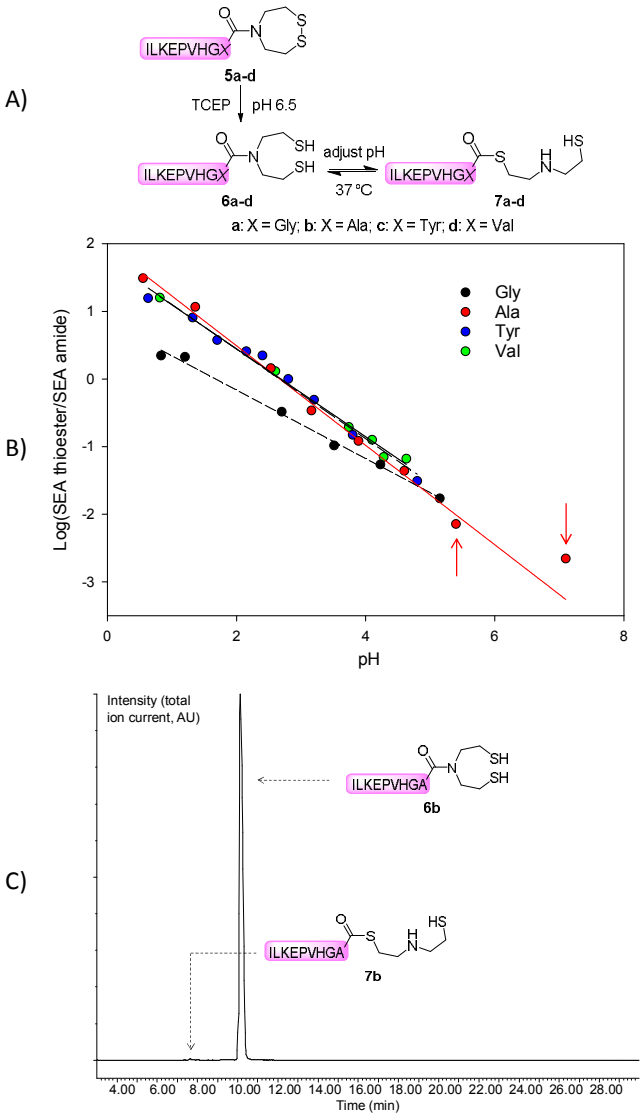
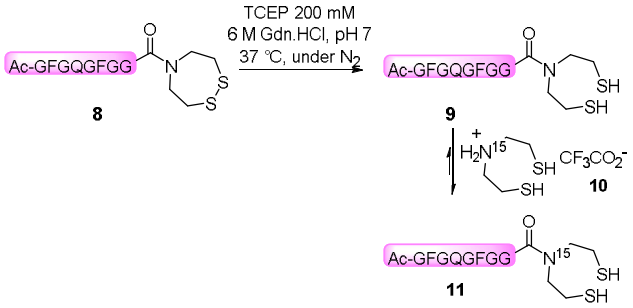


Figure 1. A) Study of the SEA thioester/SEA amide equilibrium using SEA peptides **5a-d**. Peptides 1 mM, TCEP 50 mM, 37 °C under nitrogen atmosphere. B) Log(SEA thioester **7**/amide **6**) as a function of pH. Peak areas were determined by RP-HPLC using UV detection at 215 nm. The data indicated by a red arrow were determined by LC-MS using the total ionic current detection (peptide **5b**). C) LC-MS of the mixture **6b**/**7b** equilibrated at pH 7.1, total ion current detection. Waters XBridgeTM BEH300 C18 3.5 µm (4.6 × 150 mm). Eluent A: TFA 0.1% by vol. in water; eluent B: TFA 0.1% by vol. in CH_3CN/water 4:1 by vol. Gradient: 0-100% B in 30 min; flow rate 1 mL/min.

the level of exchange observed after 80 h at 37 °C was 90%, that is the percent of exchange that was expected at equilibrium based on the stoichiometry of the reaction (10 equiv of compound **10**).



Scheme 2. Exchange of the SEA group of peptide **8** by ^{15}N-labelled *bis*(2-sulfanylethyl)amine **10** at neutral pH.

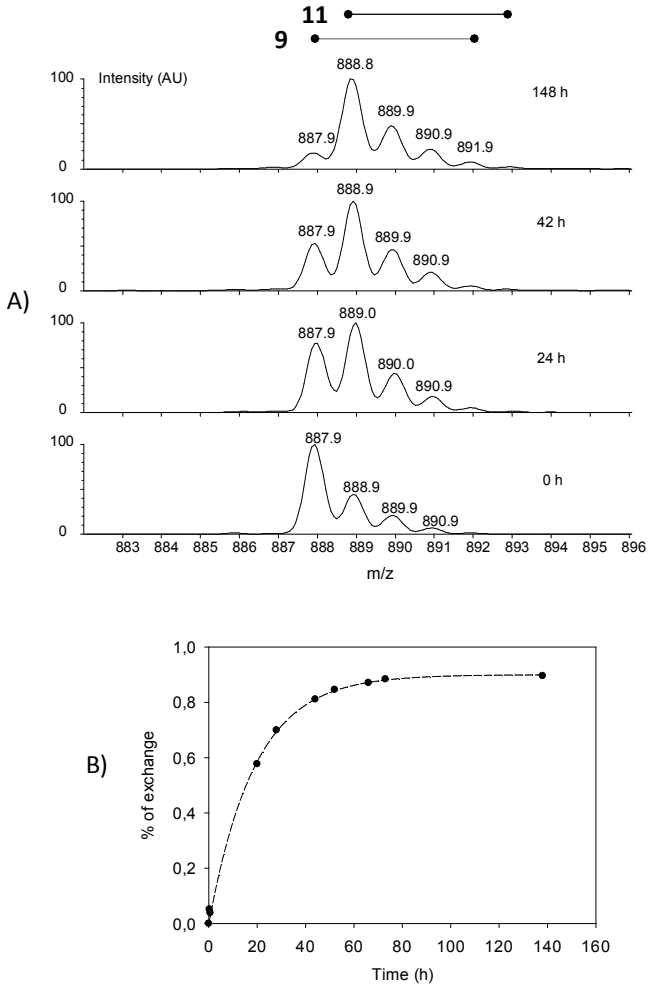


Figure 2. Exchange of the SEA group of peptide **8** by ^{15}N-labelled *bis*(2-sulfanylethyl)amine **10** at neutral pH. A) Monitoring by LC-MS (MS traces showing the molecular ion for peptides **9** and **11**). B) The time course of the exchange as monitored by LC-MS. Peptide **8** 3 mM, TCEP 200 mM, compound **10** 30 mM, 6 M Gdn.HCl 0.1 M sodium phosphate buffer pH 7, 37 °C under nitrogen atmosphere.

To conclude at this point, the SEA thioester form is present at low (µM) concentration at neutral pH. Nevertheless, it can participate in thiol/thioester exchanges and equilibrate with a thiol added exogenously.

B. SEA/thiol exchange

Next we were wondering if the above properties might be exploited for accessing peptide alkylthioesters at neutral pH. Accessing peptide alkylthioesters using mild conditions might facilitate the incorporation of sensitive groups into these useful building blocks. As already mentioned before, this exchange reaction proceeds efficiently at pH 4 and constitutes a useful entry toward peptide alkylthioesters.[30,33-35] Typical conditions for the exchange, i.e. at pH 4.0 using 5% by vol (0.57 M) of 3-mercaptopropionic acid (MPA), results usually in more than 95% of exchange.[33] However, we have observed that these conditions can lead to the degradation of some functional groups such as cysteine thiazolidine,[36] which is a useful protected form of cysteine for protein chemical synthesis. Cysteine thiazolidine residue is highly stable at neutral pH but not at acidic pH (pH 3-4), as already discussed by others.[37]

We re-examined the exchange of the SEA group by MPA using only 1% by vol of MPA (0.11 M) in the search for milder conditions (Fig. 3). MPAA was used as a catalyst of the thiol/thioester exchange reaction.[38,39] At pH 4, the level of exchange at equilibrium reached ~80%. Increasing the pH from 4 to pH 7 resulted in a gradual decrease of the level of exchange at equilibrium. At pH 7, the level of exchange was 20% only and thus too low for being of synthetic value.

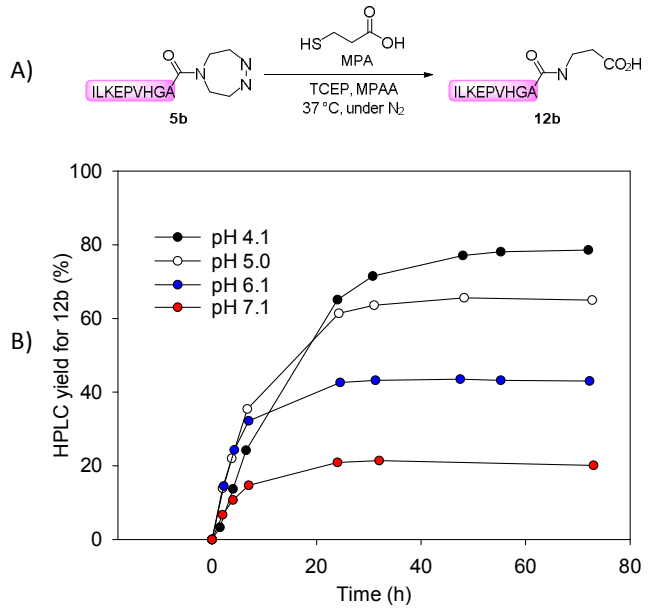


Figure 3. The influence of the pH on the SEA/thiol exchange was studied using SEA peptide **5b** and 3-mercaptopropionic acid (MPA) as the exogeneous thiol. Peptide **5b** 4.6 mM, TCEP 30 mM, MPAA 62 mM, 1% MPA by vol (0.11 M), 6 M Gdn.HCl, 37 °C under nitrogen atmosphere. The exchange was monitored by HPLC (UV detection at 215 nm).

The exchange of the SEA group by MPA favors the SEA amide form at neutral pH probably due to the greater stability of tertiary amides relative to MPA thioesters. One potential strategy for displacing the equilibrium toward the MPA peptide thioester would be to trap the *bis*(2-sulfanylethyl)amine which is released during the exchange process. Previous work showed the usefulness of simple alkylthioesters such as Gly-SCH$_2$CH$_2$CO$_2$H (Gly-MPA **14**, Fig. 4A) for

scavenging the *bis*(2-sulfanylethyl)amine during the synthesis of C-terminal peptide thioacids.[39] We therefore examined the exchange of the SEA group by MPA at pH 7 in the presence of Gly-MPA **14** (10 equiv, Fig. 4B). The use of Gly-MPA **14** as an additive resulted in a significant increase of the level of exchange (80%, red dots, Fig. 4B) compared to the experiment in the absence of Gly-MPA **14** (20% of exchange, black dots, Fig. 4B). The level of exchange in the presence of Gly-MPA **14** only (green dots, Fig. 4B) was 60%, showing the importance of combining MPA and Gly-MPA **14** for obtaining high levels of exchange. Increasing the concentration of MPA from 1 to 1.8% by vol led to no improvement that Gly-MPA **14** was present or not. Similarly, increasing the amount of Gly-MPA **14** from 10 to 17 equivalents had no significant effect (data not shown). Therefore we used 1% of MPA and 10 equivalents of Gly-MPA **14** for the exchange at pH 7 for the rest of this study. A typical HPLC profile for the exchange reaction is shown in Fig. 5 using peptide **5b**. The scavenging of the *bis*(2-sulfanylethyl)amine by Gly-MPA **14** results in the formation of Gly-SEA compound **15**, which elutes early by HPLC and does not complicate the final HPLC purification step. A small proportion of peptide thioester **12b** hydrolysis into peptide acid **13b** was also observed. Nevertheless, the extent of hydrolysis is low and does not impair the synthetic usefulness of the method.

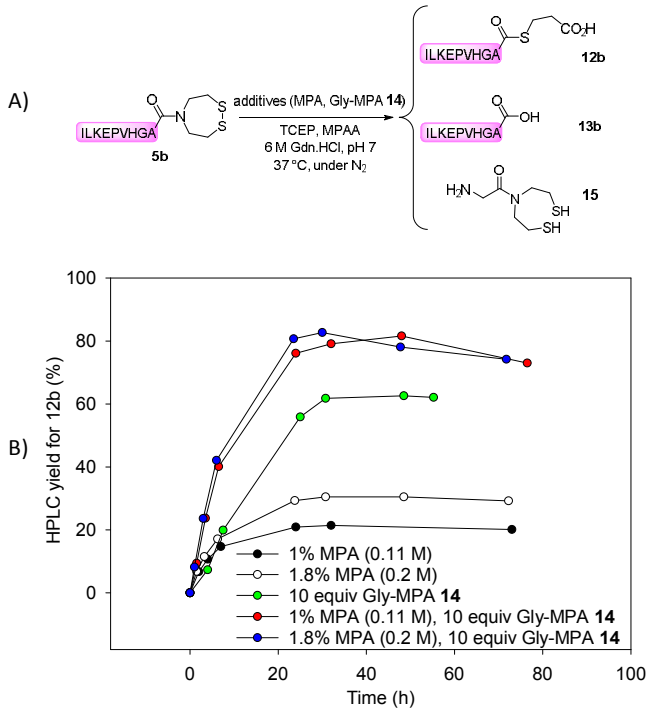


Figure 4. Displacement of the SEA/thiol exchange equilibrium using Gly-MPA thioester **14**. The reactions were monitored by HPLC (UV detection at 215 nm).

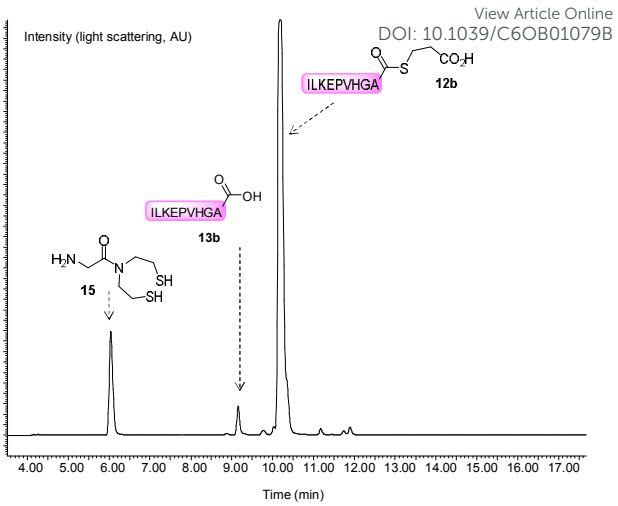


Figure. 5. LC-MS characterization of the crude exchange mixture after 72 h. Peptide **5b** 4.6 mM, TCEP 30 mM, MPAA 62 mM, 1% MPA by vol (0.11 M), 10 equiv thioester **14**, 6 M Gdn.HCl, pH 7 0.1 M sodium phosphate buffer, 37 °C under nitrogen atmosphere.

The time course of the exchange reaction for different C-terminal amino acid residues (Ala, Tyr, Leu, Arg) is shown in Fig. 6. The exchange proceeds in 24-30 h and is slower for peptide **5c** which features a C-terminal Tyr residue. In any case, the target peptide thioester **12** could be isolated in good yield (Table 1) with no detectable racemization of the C-terminal residue.

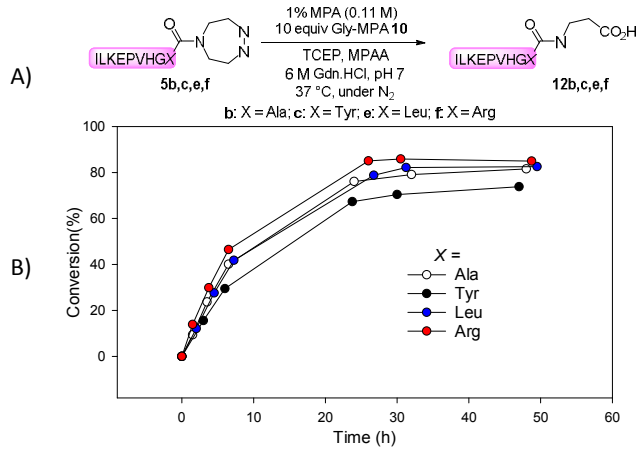
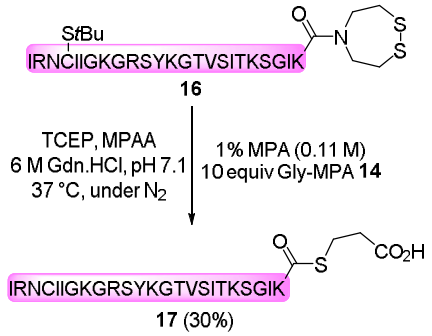


Figure 6. Time course of the SEA/thiol exchange reaction for different C-terminal amino acids. See the legend of Fig. 5 for the experimental conditions.

Finally, the method was further validated by the synthesis of the 24 amino acids peptide thioester **17**, which features an internal Cys residue (30%, Scheme 3). Note that the *tert*-butyl sulfenyl protecting group present on the internal cysteine, that is used temporarily to facilitate the synthesis of the SEA peptide, is removed during the exchange reaction.



Scheme 3. Synthesis of peptide thioester **17**.

Table 1. Synthesis of peptide thioesters by SEA/thiol exchange at neutral pH.[a]

entry	thioester	yield (%)[b]	D-AA (%)[c]
1	**12b**	54	0.96
2	**12c**	53	0.74
3	**12e**	60	0.22
4	**12f**	64	0.50
5	**17**	30	nd

[a] For the experimental conditions see the legend of Fig. 5. [b] Isolated by HPLC. [c] The percentage of racemization for the C-terminal residue was determined by chiral GC-MS after acid hydrolysis of the peptide thioesters.[40]

Conclusions

The study of the SEA amide/thioester equilibrium shows that the proportion of the SEA thioester is only ~0.1% at pH 7. Nevertheless, it can participate in thiol/thioester exchange reactions and equilibrate with a thiol added exogenously such as the *bis*(2-sulfanylethyl)amine or 3-mercaptopropionic acid (MPA). The level of exchange with MPA at pH 7 is modest (20%) but can be increased significantly up to 80% by scavenging the *bis*(2-sulfanylethyl)amine which is released during the exchange. This can be done by using Gly-MPA thioester as an additive in combination with MPA. The method was validated by the synthesis of several peptide thioesters equipped with a C-terminal Ala, Tyr, Leu, Arg or Lys residue.

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