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Synthesis of Peptide Alkylthioesters Using the Intramolecular *N*,*S*-Acyl Shift Properties of Bis(2-sulfanylethyl)amido Peptides

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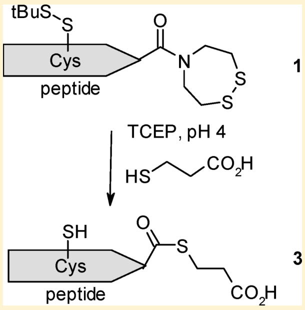
Supporting Information

ABSTRACT: The design of novel methods giving access to peptide alkylthioesters, the key building blocks for protein synthesis using Native Chemical Ligation, is an important area of research. Bis(2-sulfanylethyl)amido peptides (SEA peptides) **1** equilibrate in aqueous solution with *S*-2-(2-mercaptoethylamino)ethyl thioester peptides **2** through an *N*,*S*-acyl shift mechanism. HPLC was used to study the rate of equilibration for different C-terminal amino acids and the position of equilibrium as a function of pH. We show also that thioester form **2** can participate efficiently in a thiol−thioester exchange reaction with 5% aqueous 3-mercaptopropionic acid. The highest reaction rate was obtained at pH 4. These experimental conditions are significantly less acidic than those reported in the past for related systems. The method was validated with the synthesis of a 24-mer peptide thioester. Consequently, SEA peptides **1** constitute a powerful platform for access to native chemical ligation methodologies.



INTRODUCTION

Ligation chemistries are powerful synthetic tools for the convergent and controlled assembly of complex molecular scaffolds. In particular, native peptide ligation methods allow the formation of native peptide bonds between unprotected peptides and thus the total or hemi synthesis of proteins.[1−4] Native chemical ligation (NCL)[1,5] and related extended methodologies[6−8] are certainly the most frequently used techniques for protein total synthesis. NCL is based on the reaction of peptide thioesters with cysteinyl peptides. The reaction proceeds through a series of thiol−thioester exchanges first with an exogenous aromatic thiol used in excess and then with the side-chain thiols of cysteine residues. N-terminal cysteine leads to a key thioester-linked intermediate which rearranges spontaneously by acyl migration from sulfur to nitrogen, resulting in the formation of a native peptide bond.

Peptide alkylthioesters are frequently used for protein synthesis, using NCL or extended methodologies. In this case, NCL is carried out in the presence of an exogenous aromatic thiol such as 4-mercaptophenyl acetic acid (MPAA). MPAA converts the alkylthioester into a more reactive aryl thioester by thiol−thioester exchange.[9] MPAA maintains also cysteine thiols in a reduced form and permits the reversal of nonproductive thioester intermediates formation with internal cysteine residues.

The crucial importance of peptide alkylthioesters in NCL-based synthetic strategies has stimulated the development of various methods allowing their synthesis. The field has been reviewed very recently.[10] Historically, peptide alkylthioesters were first synthesized by using Boc/benzyl solid phase peptide synthesis (SPPS) methods.[11] This past decade, various Fmoc-SPPS methods have been reported that give also access to these key peptide derivatives. Fmoc-SPPS is by far the most popular technique for peptide synthesis.[12] However, the synthesis of peptide thioesters with Fmoc-SPPS is highly challenging due to the instability of the thioester group in the presence of bases such as piperidine used for Fmoc removal.

To circumvent this problem, additives such as hydroxybenzotriazole (HOBt) were used in combination with piperidine or other bases to remove the Fmoc group on thioester-linked peptidyl resin.[13,14] However, the most frequently used strategy is to introduce the thioester functionality after the peptide elongation step. This can be done either on the solid phase,[15−19] during the cleavage from the solid support,[20−28] in solution or in situ during NCL.[22,29−39] Among the various methods described, the intramolecular *O*,*S*-acyl shift[31,34] and later on the *N*,*S*-acyl shift-based strategies[23,29,30,32,33,35−39] have gained increasing importance in this field during the past few years. In particular, *N*,*S*-acyl shift-based methods were used either for peptide thioester synthesis[17,23,29,30,38,39] or for designing novel native ligation methods relying on the in situ generation of peptide thioesters.[32,33,35−37] In particular, peptides featuring a C-terminal *N*-alkylcysteine rearrange in acidic media into a transient peptide thioester that can be exchanged with an external thiol.[29,38,39] An application of this chemistry has been reported recently by Brik et al.[40] In this work, *N*-methylcysteine-functionalized resin was prepared by methylation of a supported *o*-nitrobenzenesulfonylcysteine residue in the presence of DBU and methyl *p*-nitrobenzenesulfonate. Removal of the *o*-nitrobenzenesulfonyl group in the presence of DBU and mercaptoethanol was followed by the remaining assembly of the target peptide. Conversion of the *N*-methylcysteine-terminated peptide into the corresponding 3-mercaptopropionic acid (MPA)−thioester was carried out at pH 1 in 20% aqueous 3-mercaptopropionic acid during 12 h.

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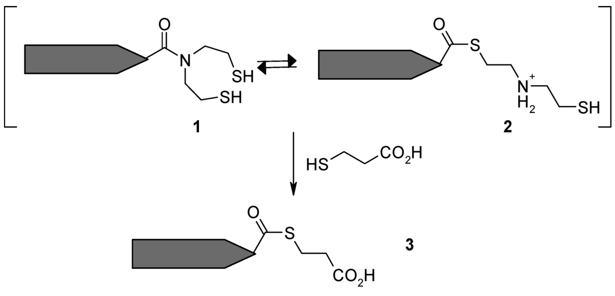
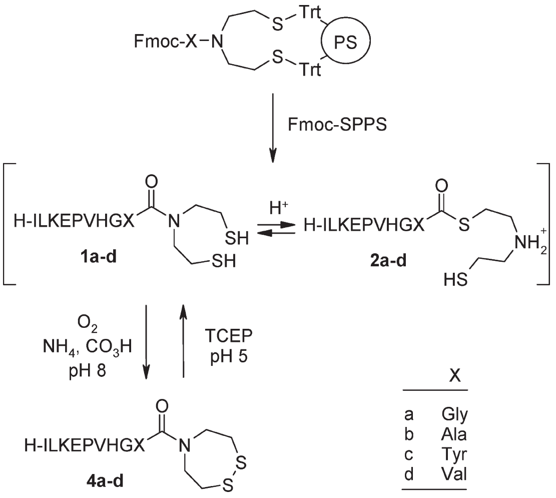
Scheme 1. Conversion of SEA Peptides 1,2 into MPA−Thioesters 3



Scheme 2. Synthesis of SEA Peptides 4a−d[a]



[a] The open form **1a**−**d** was cyclized by air oxidation. Reduction of cyclic form **4a**−**d** into open form **1a**−**d** was carried out at pH 5 in the presence of 0.1 M TCEP.

Recently also, microwave-assisted tandem *N*,*S*-acyl migration and MPA−thioester exchange permitted the preparation of a glycosylated peptide thioester.[23] Microwave irradiation was also found to induce an *N*,*S*-acyl shift within Cys peptides and the preparation of peptide thioesters by MPA−thioester exchange.[41] Even more recently, peptide anilides featuring a thiol group in the β position relative to the anilide were rearranged in 4 M HCl/DMF for 8 h into peptide thioesters.[30]

Clearly, *N*,*S*-acyl shift strategies constitute a promising access to peptide thioester building blocks. A significant improvement would be to design chemical systems able to rearrange with use of mild experimental conditions, rather than in strong acid media as reported before. We have described very recently the synthesis of bis(2-sulfanylethyl)amido peptides using Fmoc-SPPS.[33,42] These peptide derivatives, called SEA peptides, ligate chemoselectively and regioselectively with cysteinyl or homocysteinyl peptides at pH 7 to give a native peptide bond at the ligation site. Later on, Liu et al. described a similar system.[43] Interestingly, this group reported the synthesis of a pentapeptide thioester by transthioesterification of a bis(2-sulfanylethyl)amido peptide in 20% MPA at pH 2. It described also the direct use of SEA peptides in NCL at pH 4−6, suggesting that conversion of SEA peptides into alkylthioesters might be carried out with milder experimental conditions. We independently discovered the ease of transthioesterification of bis(2-sulfanylethyl)amido peptides by MPA and wish to disclose our findings in this field. We first describe hereinafter a study of the *N*,*S*-acyl shift equilibrium (rate of equilibration, effect of pH) using model nonapeptides featuring Gly, Ala, Tyr, or Val C-terminal residues. Next, we show that SEA peptides are cleanly converted into the corresponding MPA−thioesters in 5% aqueous MPA. The effect of pH on the rate of MPA−thioester formation was examined. The highest rate was obtained at pH 4. These experimental conditions are significantly less acidic than those required for the *N*,*S*-acyl shift chemical systems reported to date. Importantly, lowering the pH to 3.2 increased the proportion of thioester form in equilibrium with bis(2-sulfanylethyl)amido peptide but reduced the rate of thioester formation. The method permitted the synthesis of a 24 amino acid long MPA−thioester derived from HGF K1 domain, showing the interest of SEA peptides for the synthesis of large peptide thioesters.

■ RESULTS AND DISCUSSION

The conversion of SEA peptides into MPA−thioesters is illustrated in Scheme 1. SEA peptide amide form **1** is in equilibrium with thioester form **2**. The proportion of thioester form **2** increases by decreasing the pH of the solution. We reasoned that conversion of peptide amide **1** into MPA−thioester **3** could proceed through transthioesterification of the transient thioester form **2** by MPA.

We first characterized the equilibrium between peptide amide **1** and thioester **2**. Indeed, the rate of equilibration as well as the fraction of thioester form **2** at equilibrium is expected to influence the rate of MPA−thioester formation. We used for this purpose model SEA peptides **4a**−**d** (Scheme 2). Their synthesis was already described elsewhere.[33] In brief, we used a supported bis(2-sulfanylethyl)amino reagent linked to a trityl polystyrene resin through both sulfur atoms. This solid support is fully compatible with standard automated Fmoc-SPPS. Deprotection and cleavage of the peptide from the resin furnished peptide amides **1a**−**d**. To avoid any interference of peptide amide −-peptide thioester **2** equilibrium during RP-HPLC purification, **1a**−**d** were converted into the cyclic disulfides **4a**−**d** by air oxidation in basic medium. The isolated yields were in the range 23−47%.

The cyclic disulfides **4a**−**d** were reduced into peptides **1a**−**d** in the presence of 0.1 M TCEP at pH 5. At this pH, the amide form **1** is the unique species detected by RP-HPLC. Thioester forms **2a**−**d** were observed below pH 4.5 and eluted earlier by RP-HPLC than the corresponding amide forms **1a**−**d**, probably due to the protonated thiol handle present within thioester form **2**. LC-MS analysis of the **1b**/**2b** mixture showed the same molecular ion for the two peaks (see the Supporting Information), in accord with the proposed structures. Additional evidence for structure **2** is provided by the capacity of the β-amino thiol moiety within thioesters **2a**−**d** to form a thiazolidine by reacting with glyoxylic acid at pH 1.[44]

To study the rate of equilibration between peptide amide form **1** and thioester form **2**, the pH was rapidly adjusted to 1−2 after the reduction step with TCEP. The time course of the equilibration between forms **1a**−**d** and **2a**−**d** was monitored by RP-HPLC using a linear water−acetonitrile gradient at pH 2 (Figure 1). We have verified that equilibration between forms **1** and **2** during RP-HPLC analysis was not significant. Thus, the fraction of forms **1** and **2** determined by using this analytical

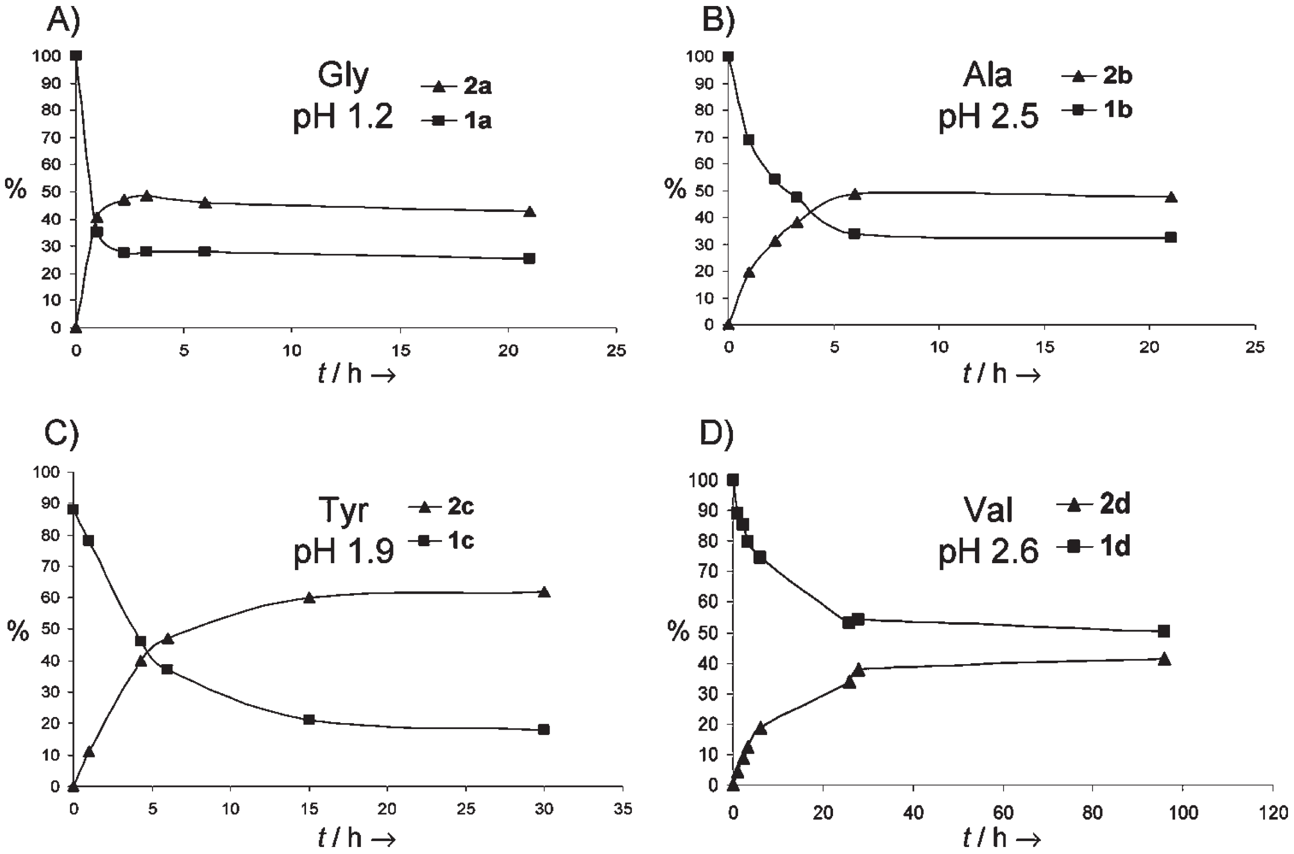


Figure 1. The rate of equilibration of peptide amide **1** into peptide thioester **2** was monitored by RP-HPLC (detection at 215 nm). The fraction of each form **1** and **2** in the mixture is plotted as a function of time (h). Equilibration of (A) Gly analogue **1a**, (B) Ala analogue **1b**, (C) Tyr analogue **1c**, and (D) Val analogue **1d**.

method (Figure 1) corresponds to the fraction of **1** and **2** in the reaction mixture.

Gly analogue **1a** equilibrated rapidly in about 2 h (Figure 1A). Ala and Tyr analogues **1b** and **1c** equilibrated in about 5 h (Figure 1B and C), whereas Val analogue required up to 30 h to reach equilibrium (Figure 1D). Clearly, the time required to reach equilibrium is in the order Gly > Ala, Tyr > Val and is correlated with the bulkiness of the amino acid residue.

Next, the fraction of peptide amide **1** and peptide thioester **2** at equilibrium for different pH values was determined by RP-HPLC (Figure 2). The relationship between the fraction of peptides **1**,**2** and pH is very similar for Ala **1b**, Tyr **1c**, and Val **1d** analogues. Typically, the proportion of peptides **1**,**2b**−**d** is equal at pH ~3, whereas peptide thioesters **2b**−**d** are almost the unique specie detected at pH ~1. Gly analogue behaves slightly differently as both forms **1a** and **2a** are in equal proportion at pH ~2, whereas peptide amide **1a** is still present in significant amounts at pH below 1. Another interesting point to mention is that the fraction of peptide thioester **2** at equilibrium at pH 4 is in the range 4−10% for the four analogues studied. This result suggests that the conversion of peptide **1** into MPA−thioester **3** may take place at pH 4.

To test this hypothesis, peptide **4b** was reacted with MPA (5% by volume) at pH 3.2, 4.0, or 5.0. The reaction was carried out in the presence of 0.1 M TCEP to maintain the thiol groups in reduced form. The time course of MPA−thioester **3b** formation was followed by RP-HPLC (Figure 3).

This experiment revealed the rapid conversion of peptide **4b** into MPA−thioester **3b** for the three pH values tested. Formation of MPA−thioester **3b** appeared to be optimal at pH 4.0. The time required to reach 50% conversion ($t_{1/2}$) was 120 min. Interestingly, the rate of MPA−thioester **3b** formation was only slightly diminished at pH 5. Consequently, the conversion of amide form **1** into thioester **3** takes place efficiently even if thioester form **2** is barely detectable by RP-HPLC. The fraction of thioester form **2b** is significantly higher at pH 3.2 (~20%) than at pH 4.0 (8%) as shown in Figure 2. However, lowering the pH from 4.0 to 3.2 decreased significantly the rate of thioester **3b** formation ($t_{1/2}$ = 180 min). The effect of pH on the rate of MPA−thioester formation suggests that the secondary amine in the thiol handle within **2b** might facilitate the attack of the external thiol by an intramolecular general-base catalysis mechanism. Thus a balance between the fraction of thioester form **2b** in the mixture and the fraction of free secondary amine might govern the rate of MPA−thioester **3b** formation. In the rest of this study, conversion of peptides **4** into MPA−thioesters **3** was carried out at pH 4.0.

Next, the optimal experimental conditions determined above were used for the synthesis of MPA−thioesters **3a**−**d** (Table 1). Isolated yields were in the range 62−87%, showing the efficiency of the method. The lowest yield (62%, Table 1, entry 4) as well as the longest reaction time was obtained for Val C-terminal residue. This result is in accord with the time required for Val derivative **1d** to equilibrate compared to Gly, Ala, and Tyr derivatives **1a**−**c** (Figure 1). Typical RP-HPLC traces for the conversion of Gly analogue **1a** into MPA−thioester **3a** are shown in Figure 4. MPA−thioesters **3b**−**d** were also analyzed

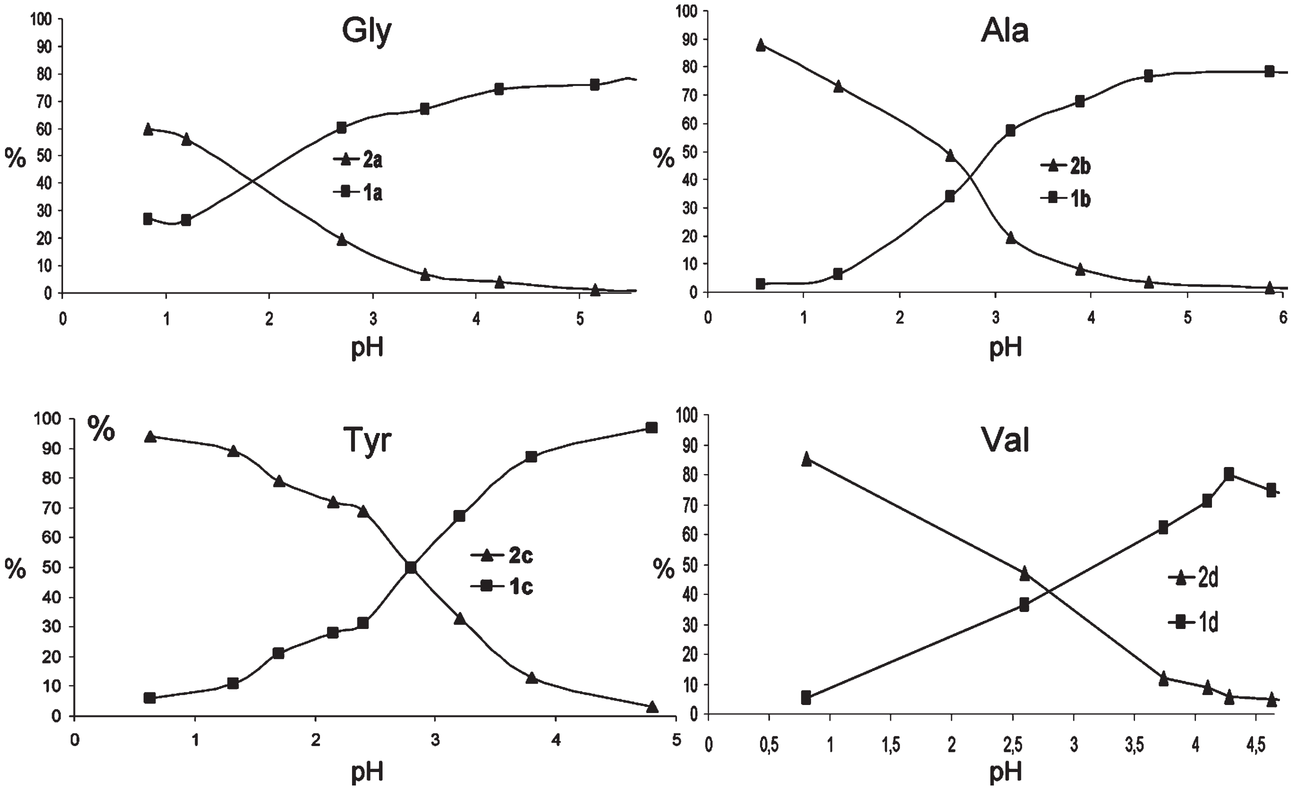


Figure 2. The fraction of peptide amide **1** and peptide thioester **2** at equilibrium at different pH was determined by RP-HPLC (detection at 215 nm). The fraction of each form **1** and **2** in the mixture is plotted as a function of pH. Data for (A) Gly analogue **1a**, (B) Ala analogue **1b**, (C) Tyr analogue **1c**, and (D) Val analogue **1d**.

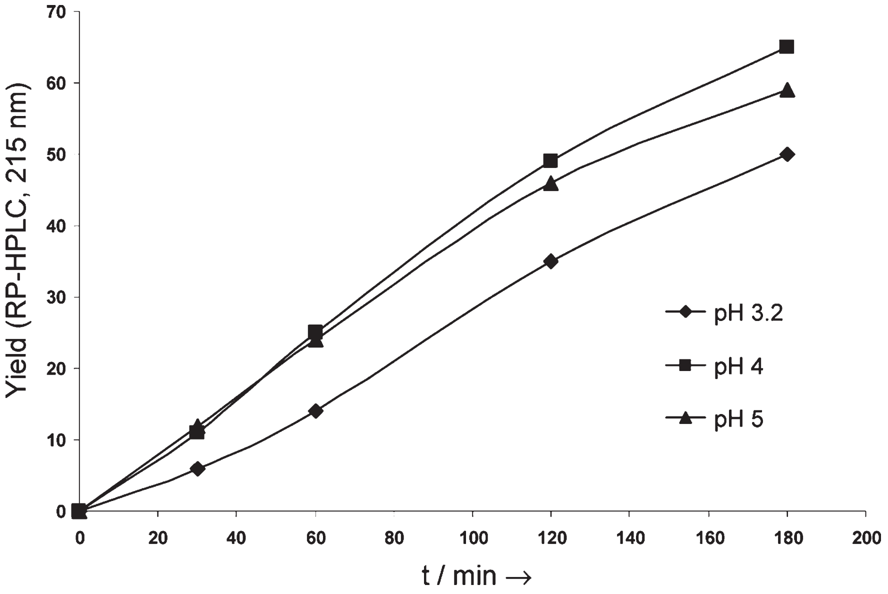


Figure 3. Rate of conversion of peptide **4b** into MPA−thioester **3b** for different pH values: pH 3.2 (◆), pH 4.0 (■), or pH 5.0 (▲). Peptide **4b** (1 mM final concentration) was solubilized in 0.2 M pH 7.0 phosphate buffer at 37 °C containing 0.1 M TCEP and 5% MPA by volume. The pH was adjusted with NaOH 5 N and 1 N.

by chiral GC-MS analysis after acid hydrolysis (Table 1).[45] No racemization was observed for C-terminal Ala, Tyr, or Val residues.

Next, we have verified that MPA−thioesters synthesized in this study were efficient partners in NCL. Typical examples are provided in Scheme 3. NCL of MPA−thioesters **3c,d** with cysteinyl peptide **5** in the presence of MPAA[9] furnished successfully the corresponding native peptides **6c,d** as expected.

Finally, to illustrate the utility of the method for the synthesis of large peptide thioesters we undertook the synthesis of

Table 1. Conversion of Peptides 4a–d into MPA–Thioesters 3a–d

entry	MPA–thioester 3	C-terminal residue	isolated yield (%)[a]	reaction time (h)	D-enantiomer[b] (%)
1	**a**	Gly	70	16	
2	**b**	Ala	71	16	0.37
3	**c**	Tyr	87	16	0.6
4	**d**	Val	62	94	0.1

[a] Peptides were purified by RP-HPLC. [b] Determined by chiral GC-MS analysis after acid hydrolysis.

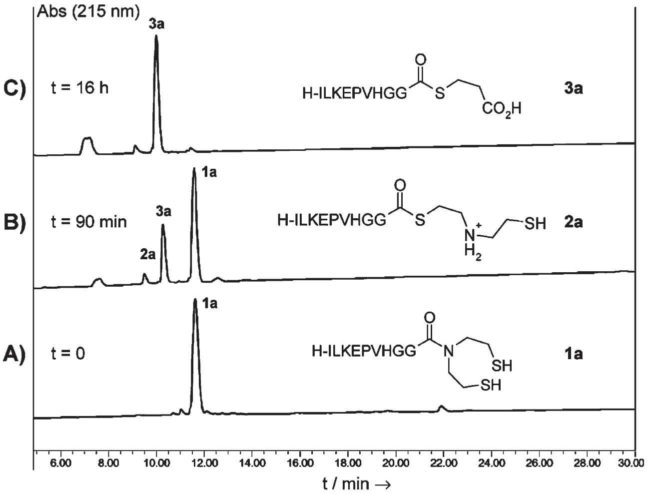
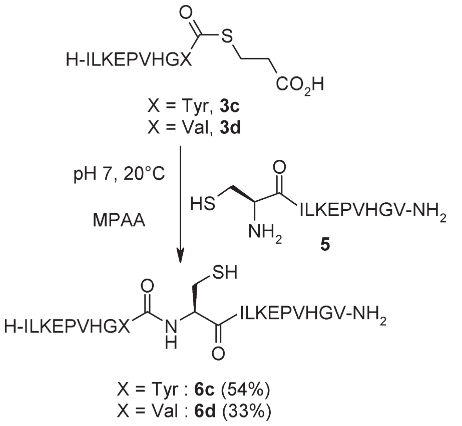
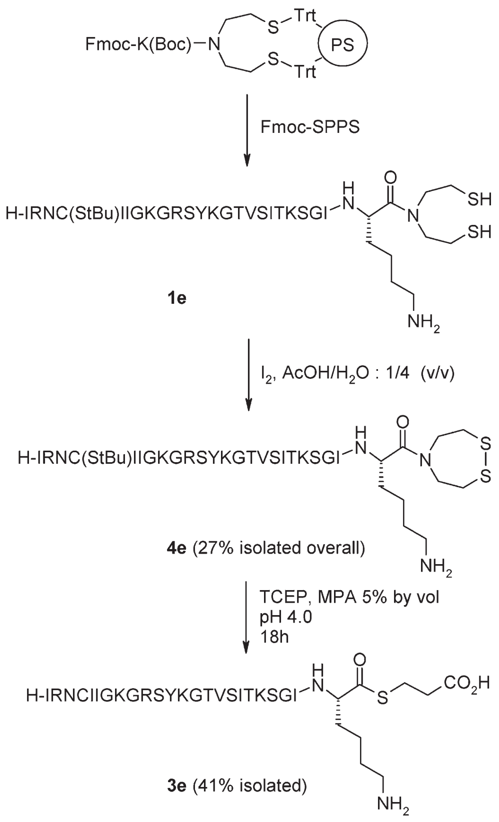


Figure 4. RP-HPLC monitoring of the conversion of Gly analogue **1a** into MPA–thioester **3a** (**4a** 1 mM, TCEP 0.1 M, MPA 5% by volume, pH 4.0, 37 °C under inert atmosphere).

Scheme 3. NCL of Peptides 3c,d with Cysteinyl Peptide 5 and Synthesis of Peptides 6c,d

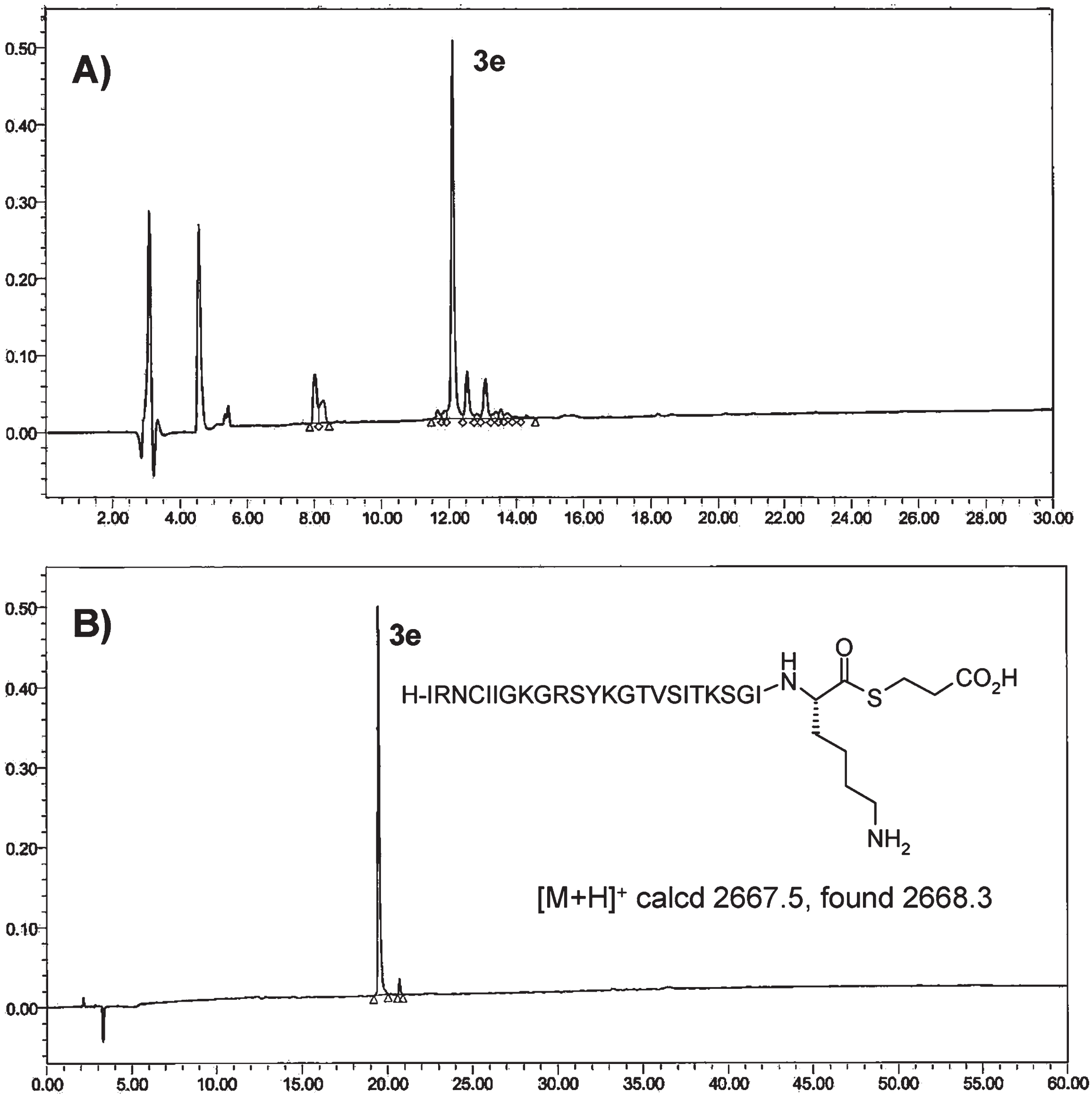


Scheme 4. Synthesis of MPA–Thioester 3e



MPA–thioester **3e** (Scheme 4). The sequence of peptide **3e** corresponds to the first 24 amino acid residues of K1 domain of human hepatocyte growth factor.[46] For this, Fmoc-L-Lys-(Boc)-OH was coupled to bis(2-sulfanylethyl)amino trityl polystyrene resin, using PyBrop/DIEA activation as described above. Then, the peptide was assembled by using standard Fmoc-SPPS. Deprotection and cleavage furnished amide form **1e**, which was directly oxidized into the cyclic derivative **4e** to facilitate RP-HPLC purification. Oxidation was carried out with iodine in aqueous acetic acid. Cys residue in the fourth position was introduced as Cys(S*t*Bu) during SPPS to avoid the formation of unwanted disulfide bonds during this oxidation step. Next, peptide amide **4e** was reacted with MPA/TCEP, using the experimental conditions described above (pH 4.0). Again, we observed the clean conversion of **4e** into MPA–thioester **3e**. The RP-HPLC trace of the crude reaction mixture is shown in Figure 5A. The isolated yield for peptide **3e** after RP-HPLC purification was 41% overall. Finally, chiral GC-MS analysis of peptide **3e** after acid hydrolysis showed the absence of racemization for C-terminal lysine residue.[45]

In conclusion, bis(2-sulfanylethyl)amido peptides (SEA peptides) constitute a powerful platform for native chemical ligation methodologies. By themselves, SEA peptides ligate with Cys peptides to give the corresponding native peptides. Alternately, SEA peptides can be converted efficiently into MPA–thioesters that are commonly used in NCL or extended methodologies. The important finding reported here is that conversion of SEA peptides into MPA–thioesters takes place efficiently at pH 4. These experimental conditions are much milder than those reported before for related systems.



$[M+H]^+$ calcd 2667.5, found 2668.3

Figure 5. RP-HPLC traces of peptide **3e**: (A) crude reaction mixture after 18 h and (B) C18 RP-HPLC purified.

Importantly, SEA peptides are easily synthesized by using Fmoc-SPPS. Consequently, the method described here should facilitate the synthesis of these key building blocks and especially of acid-sensitive MPA−thioesters, and extends the usefulness of *N*,*S*-acyl shift methodologies.

EXPERIMENTAL SECTION

Synthesis of Peptide 3a. Peptide **4a** was prepared as described elsewhere.[33] Reaction buffer was prepared by dissolving 500 mg of tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl, 2 mmol) and 1 mL of 3-mercaptopropionic acid (MPA) (5% by volume, 11.5 mmol) in 20 mL of degassed sodium phosphate buffer (pH 7.0, 0.2 M). The pH of the solution was then adjusted at 4.0 by adding 5 M aqueous NaOH. Peptide **4a** (20 mg, 14.2 μmol) was dissolved in the above solution and placed under nitrogen atmosphere at 37 °C under magnetic stirring. The reaction was monitored by RP-HPLC. Prior to the analysis, the excess of MPA was removed from 50 μL aliquots by adding 50 μL of 10% aqueous TFA and then extracting with diethyl ether (3 × 1 mL). Analytical HPLC was performed by using a Waters Alliance instrument (column: Waters XBridge BEH300 C18 Å, 5 μm, 4.6 × 250 mm) and solvents A (0.05% TFA in H_2O) and B (80% acetonitrile, 20% H_2O, 0.05% TFA) in a linear gradient (0−100% B in 30 or 60 min at 30 °C) with a flow rate of 1 mL/min. Detection was achieved with a UV−vis detector at wavelength λ = 215 nm.

The crude peptide was purified by semipreparative RP-HPLC on a C18 nucleosil 120 Å, 5 μm column (230 nm, 6 mL/min, rt, buffer A water containing 0.05% TFA, buffer B CH_3CN/water 4/1 containing 0.05% TFA, linear gradient of 0−30% B in 40 min) to give 13.7 mg of peptide **3a** (70% yield).

MALDI-TOF matrix: α-cyano-4-hydroxycinnaminic acid. Calcd for $C_{46}H_{76}N_{12}O_{13}S$ $[M + H]^+$ 1037.5, found 1037.6.

^1H and ^{13}C NMR for peptide **3a**: ^1H NMR (300 MHz, H_2O/D_2O: 9/1 by volume, 20 °C) δ 8.68 (d, *J* = 7.6 Hz, 1H), 8.61 (s, 1H), 8.59 − 8.45 (m, 4H), 8.34 (d, *J* = 6.9 Hz, 1H), 8.25 (d, *J* = 6.8 Hz, 1H), 7.32 (s, 1H), 4.44 (m, 2H), 4.31 (q, *J* = 7.0 Hz, 1H), 4.16 (d, *J* = 6.0 Hz, 2H), 4.01−3.85 (m, 3H), 3.78−3.64 (m, 3H), 3.35−3.18 (m, 2H), 3.13 (t, *J* = 6.8 Hz, 2H), 2.98 (s, 2H), 2.65 (t, *J* = 6.8 Hz, 2H), 2.47 (q, *J* = 6.5 Hz, 2H), 2.41−2.18 (m, 2H), 2.14−1.57 (m, 18H), 1.52−1.39 (m, 4H), 1.28−1.13 (m, 2H), 0.98−0.83 (m, 16H); ^{13}C NMR (75 MHz, H_2O/D_2O: 9/1 by volume, 20 °C) δ 203.2, 180.4, 179.8, 176.8, 176.6, 176.3, 176, 175.4, 174.7, 174.6, 174.1, 172, 136.4, 131.2, 120.2, 103.8, 60.41, 6.12, 55.3, 54.5, 53.6, 51.8, 45.5, 45.2, 44, 42.5, 42.1, 39.2, 36.9, 32.8, 32.2, 32.2, 29.1, 29.1, 28.7, 27.4, 27, 26.7, 26.5, 24.8, 24.6, 23.9, 21, 20.6, 16.8, 13.2.

Moreover, COSY, HSQC, and ROESY NMR experiments were in accord with the proposed structure (see the Supporting Information).

Synthesis of Peptide 3b. The synthesis of peptide **3b** was performed similarly on a 13.9 μmol scale. Purification was done by RP-HPLC on a C18 nucleosil 120 Å, 5 μm column (230 nm, 6 mL/min, rt, buffer A water containing 0.05% TFA, buffer B CH_3CN/water 4/1 by volume containing 0.05% TFA, linear gradient of 0−30% B in 40 min) to give 13.8 mg of peptide **3b** (71% yield).

Chiral GC-MS analysis: 0.37% of D-Ala.

MALDI-TOF matrix: 2,5-dihydroxybenzoic acid. Calcd for $C_{47}H_{78}N_{12}O_{13}S$ $[M + H]^+$1051.6, found 1051.5; $[M + Na]^+$ 1073.5, found 1073.5.

^{1}H and ^{13}C NMR for peptide **3b** (selected NMR data): ^{1}H NMR (600 MHz, $H_2O + D_2O$, 20 °C) δ 8.66 (d, *J* = 7.4 Hz, 1H), 8.61 (s, 1H), 8.58 (m, 2H), 8.51 (d, *J* = 6.1 Hz, 1H), 8.36 (d, *J* = 7.0 Hz, 1H), 8.27 (d, *J* = 7.1 Hz, 1H), 7.32 (s, 1H), 4.52 (m, 1H), 4.46−4.39 (m, 2H), 4.31 (q, *J* = 7.2 Hz, 1H), 4.01 (m, 1H), 3.97 (t, *J* = 5.9 Hz, 2H), 3.86−3.79 (m, 2H), 3.73−3.67 (m, 1H), 3.24 (ddd, *J* = 23.7, 15.5, 7.2 Hz, 2H), 3.11 (t, *J* = 6.8 Hz, 2H), 3.02−2.95 (m, 2H), 2.76−2.69 (m, 1H), 2.68 (t, *J* = 6.8 Hz, 2H), 2.56−2.43 (m, 2H), 2.41−2.22 (m, 2H), 2.13−1.54 (m, 18H), 1.52−1.31 (m, 8H), 1.21 (m, 1H), 0.98−0.85 (m, 23H); ^{13}C NMR (151 MHz, $H_2O + D_2O$, 20 °C) δ 179.9, 179.2, 176.8, 176.6, 176.3, 176, 174.8, 174.7, 174.1, 173.9, 173.8, 172.1, 165.8, 165.6, 137, 136.9, 136, 135.9, 121.9, 121.4, 120.5, 120, 118.1, 116.1, 103.8, 63, 62.9, 58.6, 58.6, 58.6, 58.5, 56.2, 56.1, 56.1, 55.3, 55.2, 55.2, 53.5, 45.3, 45.1, 42.1, 39.5, 39.2, 39, 37.9, 36.9, 36.8, 36.5, 36.1, 35.3, 33.1, 33.1, 32.3 32.2, 32.2, 31.9, 29.9, 29.4, 29.1, 28.8, 27.9, 27.4, 27.2, 26.8, 26.7, 26.3, 26, 25.3, 24.8, 19.8, 19.3, 13.5, 12.9.

Moreover, COSY, HSQC, and ROESY NMR experiments were in accord with the proposed structure (see the Supporting Information).

Synthesis of Peptide 3c. Synthesis was done on a 8.5 μmol scale. Purification was done by RP-HPLC on a C18 nucleosil 120 Å, 5 μm column (215 nm, 6 mL/min, rt, buffer A water containing 0.05% TFA, buffer B CH_3CN/water 4/1 by volume containing 0.05% TFA, linear gradient of 0−30% B in 40 min) to give 10.9 mg of peptide **3c** (87% yield).

Chiral GC-MS analysis: 0.6% of D-Tyr.

MALDI-TOF matrix: 2,5-dihydroxybenzoic acid. Calcd for $C_{53}H_{82}N_{12}O_{14}S$ $[M + H]^+$1143.6, found 1143.4; $[M + Na]^+$ 1165.6, found 1165.4.

^{1}H and ^{13}C NMR analysis of **3c** (selected NMR data): ^{1}H NMR (300 MHz, $H_2O + D_2O$) δ 8.59−8.5 (m, 3H), 8.47 (t, *J* = 7.1 Hz, 2H), 8.36 (m, 2H), 8.24 (d, *J* = 6.9 Hz, 1H), 7.26 (s, 1H), 7.13 (d, *J* = 8.5 Hz, 2H), 6.83 (d, *J* = 8.6 Hz, 2H), 4.49−4.38 (m, 2H), 4.31 (q, *J* = 6.6 Hz, 1H), 3.98 (t, *J* = 7.2 Hz, 1H), 3.93−3.82 (m, 4H), 3.82−3.73 (m, 1H), 3.72 (s, 1H), 3.63 (d, *J* = 10.2 Hz, 1H), 3.28−3.05 (m, 7H), 2.94 (m, 4H), 2.63 (t, *J* = 6.7 Hz, 2H), 2.45 (t, *J* = 7.2 Hz, 2H), 2.32−1.10 (m, 28H), 1.,02−0.85 (m, 24H); ^{13}C NMR (75 MHz, $H_2O + D_2O$) δ 205.5, 180.3, 179.7, 176.8, 176.6, 176.3, 176, 174.6, 174.1, 173.8, 172, 169.7, 157.4, 136.4, 133.4, 131.2, 130.6, 121, 120.1, 118.3, 117.1, 103.8, 63.8, 62.9, 62.7, 60.4, 56.1, 55.2, 55.1, 53.6, 50.7, 45, 42.5, 42.1, 39.2, 39, 36.8, 33.1, 32.7, 32.1, 29.1, 28.6, 27.4, 27, 26.8, 26.7, 24.8, 24.6, 23.9, 21, 20.7, 16.8, 13.2.

Moreover, COSY, HSQC, and ROESY NMR experiments were in accord with the proposed structure (see the Supporting Information).

Synthesis of Peptide 3d. Synthesis was done on a 7.4 μmol scale. Purification was done by RP-HPLC on a C18 nucleosil 120 Å, 5 μm column (230 nm, 6 mL/min, rt, buffer A water containing 0.05% TFA, buffer B CH_3CN/water 4/1 by volume containing 0.05% TFA, linear gradient of 0−35% B in 40 min) to give 6.5 mg of peptide **3d** (62% yield).

Chiral GC-MS analysis: 0.1% of D-Val.

MALDI-TOF matrix: 2,5-dihydroxybenzoic acid. Calcd for $C_{49}H_{82}N_{12}O_{13}S$ $[M + H]^+$1079.6, found 1079.7.

^{1}H and ^{13}C NMR analysis of **3d** (selected NMR data): ^{1}H NMR (300 MHz, $H_2O + D_2O$) δ 8.62 (t, *J* = 4.1 Hz, 2H), 8.57 (d, *J* = 6.7 Hz, 1H), 8.46 (m, 3H), 8.34 (d, *J* = 7.0 Hz, 1H), 8.24 (d, *J* = 7.2 Hz, 1H), 7.32 (s, 1H), 4.49−4.25 (m, 3H), 4.11−3.92 (m, 3H), 3.82 (m, 2H), 3.75−3.63 (m, 1H), 3.36−3.17 (m, 2H), 3.12 (t, *J* = 6.8 Hz, 2H), 2.98 (s, 2H), 2.65 (t, *J* = 6.8 Hz, 2H), 2.47 (q, *J* = 6.7 Hz, 2H), 2.34−2.18 (m, 2H), 2.15−1.54 (m, 14H), 1.53−1.31 (m, 3H), 1.28−1.10 (m, 1H), 1.02−0.81 (m, 23H); ^{13}C NMR (75 MHz, $H_2O + D_2O$) δ 205.9, 180.4, 179.8, 176.7, 176.6, 176.2, 176, 174.7, 174.2, 174.1, 172, 136.4, 131.2, 120.2, 68, 62.9, 62.5, 60.4, 56.1, 55.2, 53.6, 42.5, 42.1, 39.2, 37, 33, 32.8, 32.2, 29.1, 27.4, 27, 26.7, 24.8, 24.6, 23.9, 21.2, 21.1, 20.7, 19.7, 16.8, 13.2.

Moreover, COSY, HSQC, and ROESY NMR experiments were in accord with the proposed structure (see the Supporting Information).

Synthesis of Peptide 5. Peptide elongation was performed on Rink-PEG-PS resin (NovaSyn TGR, 0.25 mmol/g, 500 μmol scale) by using standard Fmoc/*tert*-butyl chemistry on an automated peptide synthesizer with HBTU/DIEA activation in DMF. A capping step was performed after each coupling with Ac_2O/DIEA. At the end of the synthesis, the Fmoc protecting group of the last amino acid was removed with 20% piperidine in DMF.

Final deprotection and cleavage from the solid support were performed with TFA/water/1,2-ethanedithiol/TIS 94.5/2.5/2.5/1 by volume for 2 h (50 mL). The crude peptide was precipitated in diethyl ether/heptane 1/1 by volume (500 mL), solubilized in 10 mL of deionized water, and lyophilized.

Purification was performed by RP-HPLC on a C18 Nucleosil 120 Å, 5 μm column (215 nm, 6 mL/min, rt, buffer A water containing 0.05% TFA, buffer B CH_3CN/water 4/1 by volume containing 0.05% TFA, 0−25% B in 30 min) to give 495 mg of peptide **5** (69%).

MALDI-TOF matrix: α-cyano-4-hydroxycinnaminic acid. Calcd for $C_{49}H_{84}N_{14}O_{12}S$ $[M + H]^+$ 1093.6, found 1093.4; $[M + Na]^+$ 1115.6, found 1115.4.

Synthesis of Peptide 6c. 4-Mercaptophenylacetic acid (MPAA, 33.63 mg, 0.2 mmol) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP.·Cl, 459 mg, 1.6 mmol) were solubilized in 0.2 M sodium phosphate buffer pH 7.3 (10 mL). NaOH (6 M) was added to adjust the pH to 7.2. Then, peptide **3c** (5.3 mg, 3.6 μmol) and peptide **5** (7.7 mg, 5.4 μmol, 1.5 equiv) were dissolved in the pH 7.3 phosphate buffer (1.8 mL). The two solutions were mixed and the reaction mixture was agitated at 20 °C. Final reaction conditions: 1 mM for peptide **3c**, 1.5 mM for peptide **5**, 10 mM MPAA, 80 mM TCEP, 0.1 M phosphate, pH 7.2. Purification was performed by RP-HPLC on a C18 Atlantis column 120 Å, 5 μm (215 nm, 25 mL/min, rt, buffer A water containing 0.05% TFA, buffer B CH_3CN/water 4/1 by volume containing 0.05% TFA, 0−35% B in 40 min) and afforded 5.2 mg of peptide **6c** (54%).

LC-MS analysis of **6c**: $C_{99}H_{160}N_{26}O_{24}S$ $[M + H]^+$ calcd 2131.6, found 2132.01; $[M + 2H]^{2+}$ calcd 1066.3, found 1066.5.

Synthesis of Peptide 6d. Peptide **6d** was synthesized by using the same procedure as described above on a 2.2 μmol scale of peptide **3d** (3.1 mg). Purification by RP-HPLC on a C18 Atlantis column 120 Å, 5 μm (215 nm, 25 mL/min, rt, buffer A water containing 0.05% TFA, buffer B CH_3CN/water 4/1 by volume containing 0.05% TFA, 15−35% B in 40 min) afforded 1.9 mg of peptide **6d** (33%).

LC-MS analysis of **6d**: $C_{95}H_{160}N_{26}O_{23}S$ $[M + H]^+$ *m*/*z* calcd 2067.5, found 2068.02; $[M + 2H]^{2+}$ *m*/*z* calcd 1034.3, found 1034.6.

Synthesis of 4e

Preparation of bis(2-sulfanylethyl)amino trityl polystyrene resin: Bis({2-[(triphenylmethyl)sulfanyl]ethyl})amine[47] (933 mg, 1.5 mmol) was deprotected by treatment with TFA/TIS (97.5/2.5, v/v, 150 mL) during 30 min. The solvent was evaporated under reduced pressure and residual TFA was coevaporated with cyclohexane

(2 × 50 mL). The resulting white solid was dissolved in anhydrous DMF (36 mL) and added immediately to trityl chloride polystyrene resin cross-linked with 1% divinylbenzene (10.71 g, 1.4 mmol/g) under argon. The suspension was shaken overnight. Then, the remaining trityl chloride groups were capped with methanol (500 μL) in the presence of 2,6-lutidine (1.4 mL) during 30 min. Finally, the resin was washed with DMF (2 × 2 min), methanol (2 × 2 min), DMF (2 × 2 min), 5% (v/v) DIEA in DMF (2 × 2 min), and DMF (2 × 2 min). The loading was determined by coupling Fmoc-Gly-OH to a resin aliquot, using standard PyBrop/DIEA activation in DMF, and then by measuring the absorbance at 290 nm of the dibenzofulvene−piperidine adduct released by using 20% (v/v) piperidine in DMF.

Peptide elongation: Bis(2-sulfanylethyl)amino trityl polystyrene resin (0.5 mmol, 0.175 mmol/g) was conditioned in CH_2Cl_2. Fmoc-Lys(Boc)-OH (2.342 g, 5 mmol) was dissolved in CH_2Cl_2 (a few drops of DMF were added to favor dissolution). PyBrop (2.331 g, 5 mmol) was dissolved in the minimal volume of CH_2Cl_2 and added to the resin. DIEA (2.613 mL, 15 mmol) was added to the resin in one portion. The resin was shaken during 2 h, and then washed with CH_2Cl_2 (3 × 2 min). The resin was treated with Ac_2O/DIEA/CH_2Cl_2 10/5/85 by volume (10 mL, 2 min then 10 mL, 20 min) to cap unreacted amino groups. Finally, the resin was washed with CH_2Cl_2 (5 × 2 min).

The peptide was assembled on a 0.25 mmol scale (0.175 mmol/g) by using standard automated Fmoc-SPPS. The couplings were performed with protected amino acids (0.2 M in DMF, 4 equiv), HBTU (0.5 M in DMF, 3.6 equiv), and DIEA (2 M in DMF, 8 equiv).

Peptide deprotection and cleavage: Deprotection and cleavage were performed with TFA/TIS/DMS/thioanisole/H_2O (90/2.5/2.5/2.5/2.5 by volume, 25 mL) during 2 h 30 min. The peptide was precipitated in cold Et_2O/heptane 1/1 by volume, dissolved in deionized water, and lyophilized to give 288 mg of crude peptide **1e** (32%).

LC-MS $C_{120}H_{216}N_{36}O_{31}S_4$ $[M + H]^+$ m/z calcd (mean) 2788.5, obsd 2789.1.

Peptide **1e** was directly oxidized into **4e** before RP-HPLC purification. For this, crude **1e** (95.7 mg) was dissolved in AcOH/eau 1/4 by volume (47.9 mL). I_2 (197 mM) in DMSO (270 μL, 50 μmol, 2 equiv) was added to this solution. After 30 s of mixing, 64.8 mM dithiothréitol (DTT, 823 μL, 50 μmol, 2 equiv) was added to decompose the excess of I_2. Then, the crude peptide was immediately purified by RP-HPLC. (C18 nucleosil 120 Å, 5 μm, buffer A 100% water containing 0.05% TFA, buffer B CH_3CN/water 4/1 by volume containing 0.05% TFA, gradient: 0−20% B in 5 min, then 20−40% B in 40 min, flow rate 6 mL/min, detection at 215 nm) to give 25.8 mg of peptide **4e** (27%).

MALDI-TOF $C_{120}H_{214}N_{36}O_{31}S_4$ $[M + H]^+$ m/z calcd (monoisotopic) 2784.52, obsd 2784.6.

Synthesis of Peptide 3e. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl, 229.63 mg, 0.8 mmol) was dissolved in 0.2 M sodium phosphate buffer pH 7.3 (10 mL). 3-Mercaptopropionic acid (MPA, 0.5 mL) was added. NaOH (5 M, 340 μL) was then added to adjust the pH to 3.98.

Peptide **4e** (24.67 mg, 6.9 μmol) was dissolved in the above solution (10.3 mL). The reaction was agitated at 37 °C under argon atmosphere. The reaction was monitored by RP-HPLC on an C18 Xbridge BEH column (215 nm, 1 mL/min, 30 °C, buffer A water containing 0.05% TFA, buffer B CH3CN/water 4/1 by volume containing 0.05% TFA, 0−100% B in 30 min). For this, aliquots were acidified with 10% aqueous TFA and extracted with Et_2O to remove MPA in excess prior to analysis.

After completion of the reaction, the mixture was diluted with water (10 mL) and 10% aqueous TFA (5 mL) was added. After 3 extractions with Et_2O (3 × 15 mL) and argon bubbling during 15 min, purification by RP-HPLC on a C18 Nucleosil column 120 Å, 5 μm (215 nm, 6 mL/min, rt, buffer A water containing 0.05% TFA, buffer B CH_3CN/water 4/1 by volume containing 0.05% TFA, 0−10% B in 5 min, then 10−100% B in 150 min) afforded 9.7 mg of **3e** (41%).

MALDI-TOF matrix: sinapinic acid. $C_{115}H_{203}N_{35}O_{33}S_2$ $[M + H]^+$ m/z calcd 2667.48, found 2668.3.

■ ASSOCIATED CONTENT

Ⓢ **Supporting Information.** RP-HPLC, CZE, and MS data for all compounds, and ^1H and ^{13}C NMR spectra for peptides **3a−d**. This material is available free of charge via the Internet at http://pubs.acs.org.

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