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Facile synthesis of C-terminal peptide thioacids under mild conditions from *N*-sulfanylethylanilide peptides

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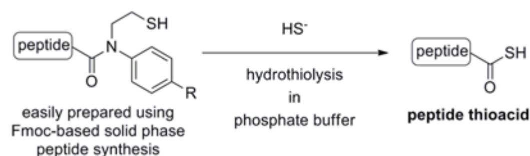

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ABSTRACT

A facile procedure has been developed for the synthesis of C-terminal peptide thioacids under mild conditions. A series of *N*-sulfanylethylanilide peptides prepared using Fmoc-based solid-phase peptide synthesis were successfully converted to the corresponding thioacids via a hydrothiolysis reaction in a phosphate buffer with only trace epimerization of the C-terminal amino acid.

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1. Introduction

Native chemical ligation (NCL) has shown great utility in chemical protein synthesis.[1,2] This methodology allows for the chemoselective condensation of a peptide thioester with an N-terminal cysteinyl peptide via sequential *S-S* and *S-N* acyl transfer reactions to afford the corresponding ligated product under mild aqueous conditions. Therefore, auxiliary-based peptide thioester precursors that can be readily converted to the corresponding thioesters via *N-S* acyl transfer, as necessary, have recently been developed for the chemical synthesis of proteins.[3,4] Several research groups, including our own, have reported that C-terminal peptide thioacids can be also used for protein synthesis.[5,6] However, only a few synthetic methods have been developed to date for the preparation of C-terminal peptide thioacids. Furthermore, most of these methods involve the use of Boc-based solid phase peptide synthesis (Boc SPPS),[6a-c,7] which requires the treatment with highly toxic hydrogen fluoride or some other harsh acid for the global deprotection of the peptide and its cleavage from the resin. Crich *et al.* recently reported a new method for the preparation of peptide thioacids using a 9-fluorenylmethyl thioester linker, which was compatible with Boc SPPS.[8] Notably, this method did not require the use of hydrogen fluoride or any other harsh acid, and allowed for the release of the resulting peptide thioacid from the resin by piperidine treatment. Although this method provided an improved process for the global deprotection of the peptide product, it required several laborious steps for the preparation of the amino acid building blocks bearing specialized 9-fluorenylmethyl type side-chain protecting groups. In contrast, Fmoc-based solid-phase peptide synthesis (Fmoc SPPS) is used much more often for the synthesis of peptides because it is technically less challenging and requires milder reagents compared with Boc SPPS. Several research groups, including our own,[5,7e,9] have therefore developed Fmoc SPPS-compatible strategies for the preparation of peptide thioacids. In our previous study, we developed an *N*-sulfanylethylanilide (SEAlide) peptide as a peptide thioester precursor using standard Fmoc SPPS.[4i] This material was subsequently converted to a peptide thioacid via a hydrogen chloride-induced *N-S* acyl transfer reaction in the presence of 1% (w/v) tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) followed by the hydrothiolysis of the resulting thioester with sodium hydrogen sulfide (NaSH) (Scheme 1).[5a] However, we observed the epimerization of the C-terminal amino thioacid residue during this reaction, which was attributed to the use of the strong acid hydrogen chloride. The development of an epimerization-free SEAlide-based method is therefore highly desired for the practical application of this method. We recently discovered that the SEAlide peptide **1** shown in Scheme 2 could be equilibrated with the corresponding thioester under neutral conditions in the presence of phosphate. Furthermore, peptide **1** could be used directly in an NCL process without any epimerization at the ligation site.[10,11] These results prompted us to use this equilibrium process to generate the peptide thioacid **2** from the SEAlide peptide **1** under mild conditions without the use of hydrogen chloride. Herein, we describe the development of an Fmoc SPPS-compatible method for the preparation of peptide thioacids using the SEAlide peptides.

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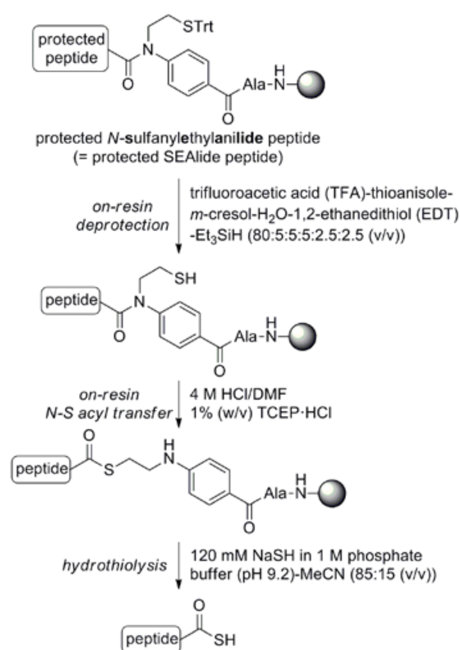
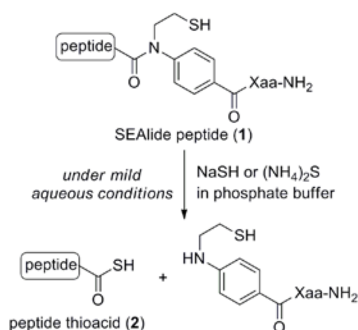


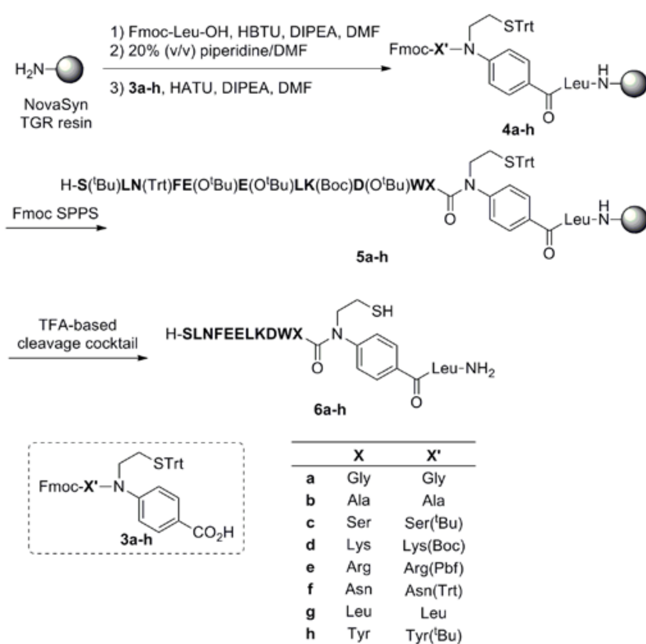
Scheme 1. Previously reported Fmoc-based preparation of peptide thioacids using the SEAlide peptide.[5a]



Scheme 2. Conversion of the SEAlide peptide to the corresponding thioacids under the mild conditions used in this study.

2. Results and discussion

The SEAlide peptides **6** were prepared using Fmoc SPPS according to the previously reported method (Scheme 3).[12] N-Terminal human RFamide-related peptide-1 (hRFRP-1) (1-11) was selected as a model peptide for this study.[13] The conversion of SEAlide peptide **6a** to thioacid **7a** was initially attempted with 120 mM NaSH in phosphate buffer containing TCEP·HCl as an inhibitor of disulfide formation and sodium ascorbate as an alternative to thiol additive[14] in the presence of 10% (v/v) *N*-methylpyrrolidone (NMP) (Table 1). We initially evaluated the effect of the phosphate salt for confirmation of our hypothesis that the presence of phosphate is essential for the hydrothiolysis reaction. Results in Table 1 (entries 1–4) clearly showed that the yield of peptide thioacid **7a** varied with the concentration of phosphate salt, indicating that the presence of phosphate was essential for the hydrothiolysis reaction. In the absence of NMP, we observed a subtle decrease in the product yield, which was attributed to the instability of the intermediate thioester in the absence of an organic solvent (Table 1, entries 4 and 5).[15]

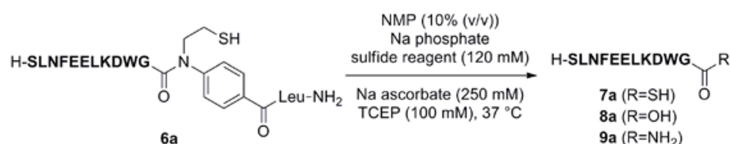


	X	X'
a	Gly	Gly
b	Ala	Ala
c	Ser	Ser(tBu)
d	Lys	Lys(Boc)
e	Arg	Arg(Pbf)
f	Asn	Asn(Trt)
g	Leu	Leu
h	Tyr	Tyr(tBu)

Scheme 3. Synthesis of SEAlide peptide **6a**-**h** using Fmoc SPPS.

We then proceeded to investigate the effect of the peptide concentration on the reaction (Table 1, entries 4, 6 and 7). The results of these experiments revealed that the increase in the concentration of peptide **6a,** decreasing the equivalent of the hydrosulfide ion source, led to a decrease in the yield of the product. The pH also had a significant impact on the yield of the desired product, especially under acidic conditions (Table 1, entries 4, 8 and 9). We subsequently examined the effect of a hydrosulfide anion source to the reaction. The replacement of NaSH with $(NH_4)_2S$ led to a significant increase in the yield of the desired thioacid **7a**, although the aminolysis byproduct **9a** was detected (Table 1, entries 4 and 10, and Fig 1).[16] These results therefore suggested that $(NH_4)_2S$ was more suitable for the hydrothiolysis of the SEAlide peptide. The phosphate salt was also critical for the hydrothiolysis reaction using $(NH_4)_2S$ as the case with the use of NaSH (Table 1, entries 10 and 11). This result repeatedly indicated that the existence of phosphate was essential for the hydrothiolysis reaction. Melnyk *et al.*[9b] recently reported the synthesis of peptide thioacids at neutral pH using bis(2-sulfanylethyl)amido (SEA) peptides. According to their report, the reaction of the model SEA peptide with an excess of NaSH at pH 7 in the presence of TCEP and 4-mercaptophenylacetic acid did not provide direct access to any of the peptide thiocarboxylate. The conversion of the SEA peptide to the corresponding peptide thioacid therefore required the use of triisopropylsilylthiol in the presence of an amino acyl thioester, which was used as a scavenger for the resulting SEA moiety. In contrast, we successfully obtained the peptide thioacid **7a** via the hydrothiolysis reaction of SEAlide peptide **6a** with $(NH_4)_2S$ under neutral conditions.

Table 1. Hydrothiolysis of SEAlide peptide **6a** under various reaction conditions.



Entry	SEAlide peptide [mM]	Na phosphate [M] (salt)	Sulfide reagent	pH	Reaction time [h]	Yield of **7a**[a] [%]	Yields of the byproducts[b] [%]	Recovery of **6a**[c] [%]
1	1.0	0.0[d]	NaSH	6.9	24	12	9	79
2	1.0	0.2	NaSH	6.9	24	35	18	48
3	1.0	0.5	NaSH	6.9	24	39	23	38
4	1.0	1.0	NaSH	6.9	24	49	20	31
5[e]	1.0	1.0	NaSH	6.9	24	43	24	33
6	2.0	1.0	NaSH	6.9	24	28	27	45
7	5.0	1.0	NaSH	6.9	24	8	27	65
8	1.0	1.0	NaSH	5.9	24	6	16	78
9	1.0	1.0	NaSH	8.3	24	31	49	21
10	1.0	1.0	$(NH_4)_2S$	7.1	24	74	12	14
11	1.0	0.0[d]	$(NH_4)_2S$	7.0	24	18	10	72

Yield of **7a**, yields of the byproducts and recovery of **6a** were determined by HPLC separation (detection at 220 nm) using the following calculations (integ. = peak area).

[a]Yield of **7a** = integ. **7a**/(integ. **6a** + integ. **7a** + integ. **8a** + integ. **9a**)×100.

[b]Yields of the byproducts = (integ. **8a** + integ. **9a**)/(integ. **6a** + integ. **7a** + integ. **8a** + integ. **9a**)×100.

[c]Recovery of **6a** = integ. **6a**/(integ. **6a** + integ. **7a** + integ. **8a** + integ. **9a**)×100.

[d]HEPES buffer (0.2 M) was employed instead of phosphate buffer.

[e]This reaction was conducted without the addition of NMP.

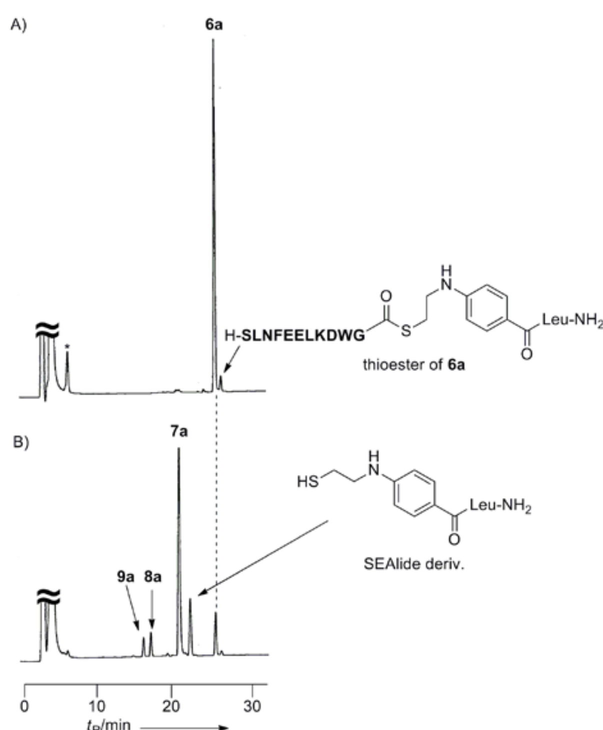


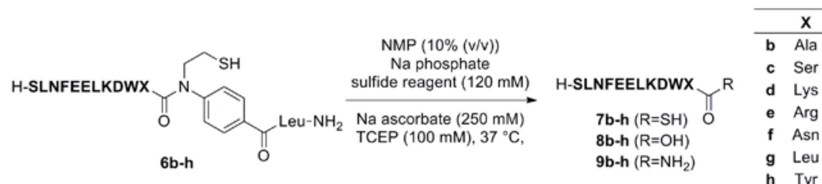
Figure 1. HPLC monitoring of the hydrothiolysis reaction of SEAlide **6a** with $(NH_4)_2S$ (Table 1, entry 10) (A) Reaction time = 0 min. (B) Reaction time = 24 h. The HPLC conditions are described in the experimental section. The asterisked peak indicates a non-peptidyl compound that was derived from reaction media.

We then proceeded to investigate the epimerization of the C-terminal amino acid. Our previous method, which involved a hydrogen chloride-induced *N-S* acyl transfer reaction, was accompanied by 20% of the epimerization at the C-terminal amino acid residue.[4i] It was envisaged that the milder reaction conditions of our new method would suppress the epimerization of the C-terminal residue. Peptide **6b** bearing a C-terminal alanine residue was converted to the corresponding thioacid **7b** under the conditions described above in entry 10 of Table 1. These conditions resulted in only 2% epimerization, which was significantly lower than that observed using hydrogen chloride (Fig 2).

This result encouraged us to examine the effect of different C-terminal amino acid residues on the hydrothiolysis reaction (Table 2). The C-terminal amino acid was replaced with a series of typical amino acids (e.g., Ala, Ser, Lys, Arg, Asn, Leu or Tyr), and the resulting peptides were treated with NaSH or $(NH_4)_2S$ as a hydrosulfide source. For peptides **6b–e** and **6h** (Ala, Ser, Lys, Arg or Tyr derivative), the corresponding thioacids **7b–e** and **7h** were successfully formed. Higher yields of the thioacid were observed when $(NH_4)_2S$ was used instead of NaSH (Table 2, entries 1–8). Whereas two peaks their mass was identical to the **7c** and its epimer was observed in entry 4 (X = Ser, the major peak/the minor peak = 95/5 based on HPLC peak area), the minor peak that would be an epimer of **7** was not detected in the case of **6b**, **6d**, **6e** and **6h**.[17] Unfortunately, no peptide thioacid was obtained when X was Asn (Table 2, entries 9 and 10). In this reaction, the formation of the aspartimide byproduct **10**[18] was confirmed by HPLC separation followed by MS analysis (Fig 3). When the C-terminal amino acid was Leu, the reaction

was very slow and gave a complex mixture. In addition, the HPLC analysis of this reaction mixture revealed the formation of multiple peaks derived from byproducts other than **8g** and **9g** (data not shown). Given that this phenomenon was not observed when the C-terminal amino acid was Lys or Arg, it was assumed that the presence of a methyl group at the γ-position of Leu was having an adverse impact on the rate of the hydrothiolysis through steric hindrance, and that the side reactions therefore proceeded at a relatively higher rate to generate a complex mixture of products.

Table 2. Hydrothiolysis reactions of the SEAlide peptides containing various amino acids at their C-terminal position



Entry	SEAlide peptide [mM]	X	Na Phosphate [M] (salt)	Sulfide reagent	pH	Reaction time [h]	Yield of **7**[a] [%]	Yields of the byproducts[b] [%]	Recovery of **6**[c] [%]
1	1.0 (**6b**)	Ala	1.0	NaSH	6.9	24	12	39	50
2	1.0 (**6b**)	Ala	1.0	$(NH_4)_2S$	7.0	24	79	3	19
3	1.0 (**6c**)	Ser	1.0	NaSH	6.9	24	23	20	57
4	1.0 (**6c**)	Ser	1.0	$(NH_4)_2S$	7.2	24	44	8	48
5	1.0 (**6d**)	Lys	1.0	NaSH	6.9	24	18	5	77
6	1.0 (**6d**)	Lys	1.0	$(NH_4)_2S$	7.1	24	60	<1	40
7	1.0 (**6e**)	Arg	1.0	NaSH	6.9	24	5	34	62
8	1.0 (**6e**)	Arg	1.0	$(NH_4)_2S$	7.1	24	58	6	36
9	1.0 (**6f**)	Asn	1.0	NaSH	6.9	24	-[d]		
10	1.0 (**6f**)	Asn	1.0	$(NH_4)_2S$	7.1	24	-[d]		
11	1.0 (**6g**)	Leu	1.0	$(NH_4)_2S$	7.0	76		complex mixture	
12	1.0 (**6h**)	Tyr	1.0	$(NH_4)_2S$	6.9	24	33	2	65

The yield of **7**, yields of the byproducts and recovery of **6** were determined by HPLC separation (detection at 220 nm) using the following calculations (integ. = peak area).

[a]Yield of **7** = integ. **7**/(integ. **6** + integ. **7** + integ. **8** + integ. **9**)×100.

[b]Yields of the byproducts = (integ. **8** + integ. **9**)/(integ. **6** + integ. **7** + integ. **8** + integ. **9**)×100.

[c]Recovery of **6** = integ. **6**/(integ. **6** + integ. **7** + integ. **8** + integ. **9**)×100.

[d]Not detected.

The isolated yields of the peptide thioacids **7a–e** were estimated and the results are summarized in Table 3. The trend in the isolated yields was identical to that of the HPLC-based yields for **7a–e** shown in Tables 1 and 2. The isolated yields of the SEAlide peptides with Ala (**7b**) and Lys (**7d**) at the C-terminus were higher than those of the other peptides prepared in this study. These results therefore suggested that our newly developed method is practical when the C-terminal amino acid was appropriately chosen.

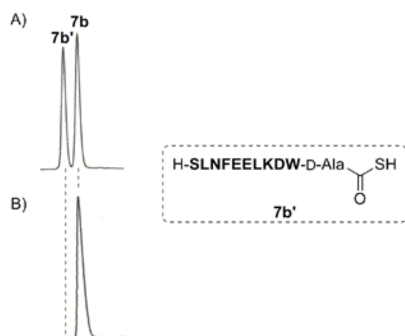


Figure 2. HPLC estimation of the epimerization of **7b** during the hydrothiolysis. (A) Mixture of **7b** and its epimer at the C-terminal Ala residue (**7b'**). (B) Crude reaction mixture after the hydrothiolysis of **6b**. The reaction conditions are shown in entry 2 of Table 2.

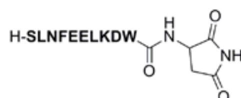


Figure 3. Structure of the aspartimide byproduct **10**.

Table 3. Isolated yields of the peptide thioacids **7a–e**[a]

Entry	Peptide thioacid	X	Isolated yield [%]
1	**7a**	Gly	30
2	**7b**	Ala	45
3	**7c**	Ser	10
4	**7d**	Lys	47
5	**7e**	Arg	26

[a]Peptides **6a–e** (1 mM), 120 mM $(NH_4)_2S$, 100 mM TCEP·HCl and 250 mM sodium ascorbate in 1.0 M sodium phosphate buffer (pH 7), 10% (v/v) NMP, 37 °C, 24 h.

3. Conclusion

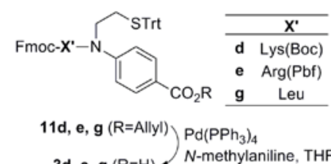
An Fmoc SPPS-compatible synthetic protocol for the synthesis of C-terminal peptide thioacids from the corresponding SEAlide peptides was developed. Characteristic chemical behavior of the SEAlide peptide in the presence of phosphate salt enabled the facile preparation of thioacids under mild conditions. After the optimization of the reaction conditions, we successfully evaluated the scope and limitations of this new process. This new method worked well for various C-terminal amino acid residues tested in this study (based on the HPLC-based yields), except for Asn and Leu (Table 2). However, the results revealed that the appropriate selection of a C-terminal amino acid was critical from a practical perspective in terms of the isolated yield (Table 3).

4. Experimental section

4.1. General methods

All of the reactions involving small molecules were carried out under a positive pressure of argon at room temperature. Purifications by column chromatography were performed over silica gel (KANTO KAGAKU N-60). Mass spectra were recorded on a Waters MICROMASS® LCT PREMIER™ (ESI-TOF) or a Bruker Esquire200T (ESI-Ion Trap). NMR spectra were measured using a Bruker AV400N at 400 MHz frequency for ^1H and a JEOL JNM-AL300 at 75 MHz frequency for ^{13}C. Purifications by HPLC were performed over a Cosmosil $5C_{18}$-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), a $5C_{18}$-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3 mL/min) or a $5C_{18}$-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10 mL/min) with UV detection at 220 nm. A solvent system consisting of 0.1% (v/v) TFA in H_2O (solvent A) and 0.1% TFA (v/v) in MeCN (solvent B) was used as the mobile phase for the HPLC purification processes. The system was eluted with a linear gradient of solvent A in solvent B over 30 min according to the description provided below unless otherwise noted. Optical rotations were measured using a JASCO P-2200 polarimeter (concentration in g/100 mL).

4.2. Typical procedure for the preparation of the 4-[(Fmoc-Xaa){2-(triphenylmethylsulfanyl)ethyl}amino]benzoic acid.



N-Methylaniline (303 µl, 2.79 mmol) and $Pd(PPh_3)_4$ (32.3 mg, 27.9 µmol) were added to a solution of allyl 4-[{Fmoc-Lys(Boc)}{2-(triphenylmethylsulfanyl)ethyl}amino]benzoate **11d**[11b] (260 mg, 279 µmol) in THF (6.2 ml), and the resulting mixture was stirred overnight. The reaction mixture was evaporated to dryness to give a residue, which was treated with monohexylamine (32 µL, 28 µmol) and ethyl acetate. The resulting mixture was stirred for 5 min and evaporated to dryness to give a solid, which was washed with Et_2O. The solid was partitioned between EtOAc and saturated aqueous citric acid, and the organic layer was collected, washed with 5% (w/v) aqueous citric acid and dried over $MgSO_4$. The solvent was subsequently evaporated under reduced pressure to afford the desired product **3d** (211 mg) in 85% isolated yield.

4-[{Fmoc-Lys(Boc)}{2-(triphenylmethylsulfanyl)ethyl}amino]-benzoic acid (**3d**)
$[\alpha]^{19}_D$ +71.0 (*c* 1.00, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz) δ = 1.01-1.30 (4H, br m), 1.31-1.52 (2H, m), 1.41 (9H, s), 2.29 (1H, m), 2.54 (1H, m), 2.95 (2H, br s), 3.31 (1H, m), 3.60 (1H, m), 4.12-4.27 (1H, m), 4.19 (1H, t, J = 7.0 Hz), 4.35 (2H, d, J = 7.0 Hz), 4.54 (1H, s), 5.68 (1H, br d, J = 8.2 Hz), 7.05-7.44 (19H, m), 7.39 (2H, t, J = 7.4 Hz), 7.59 (2H, dd, J = 5.6 and 7.7 Hz), 7.75 (2H, d, J = 7.5 Hz), 8.07 (2H, d, J = 8.3 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 22.3, 28.5, 29.3, 29.3, 32.6, 40.1, 47.2, 49.2, 51.6, 67.1, 67.2, 79.3, 120.1, 125.3, 126.8, 127.2, 127.8, 128.0, 128.4, 129.6, 129.9, 131.9, 141.4, 143.9, 143.9, 144.6, 145.0, 145.0, 156.2, 168.9, 172.3; HRMS (ESI-TOF) *m/z* calcd for $C_{54}H_{55}N_3NaO_7S$ ($[M+Na]^+$) 912.3658, found 912.3617.
4-[{Fmoc-Arg(Pbf)}{2-(triphenylmethylsulfanyl)ethyl}amino]-benzoic acid (**3e**)
Compound **11e** was prepared according to the method described in reference 11b. **3e**, 861 mg, (77%); $[\alpha]^{19}_D$ +52.4 (*c* 1.00,

$CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.27-1.35 (2H, m), 1.38 (3H, s), 1.39 (3H, s), 1.42-1.52 (2H, m), 2.03 (3H, s), 2.22-2.34 (1H, m), 2.32 (3H, s), 2.38-2.58 (2H, m), 2.52 (3H, s), 2.71-2.92 (1H, br m), 2.71-2.92 (2H, m), 3.20 (1H, ddd, J = 13.1, 9.1, 5.7 Hz), 3.73 (1H, br m), 4.16 (1H, t, J = 6.9 Hz), 4.18-4.25 (2H, m), 4.30-4.40 (2H, m), 5.96 (1H, br d, J = 8.0 Hz), 6.25 (2H, br s), 7.06 (2H, d, J = 8.0 Hz), 7.09-7.44 (20H, m), 7.54 (1H, d, J = 7.4 Hz), 7.58 (1H, d, J = 7.4 Hz), 7.75 (2H, d, J = 7.5 Hz), 8.02 (2H, d, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 12.6, 17.9, 19.4, 24.7, 28.7, 29.2, 30.8, 40.1, 43.3, 47.2, 49.1, 50.7, 67.2, 67.5, 86.5, 117.6, 120.2, 120.2, 124.8, 125.2, 126.9, 127.3, 127.9, 128.0, 128.4, 129.6, 130.5, 131.9, 132.6, 138.7, 141.4, 143.6, 143.7, 144.4, 144.5, 156.7, 156.8, 158.9, 168.5, 171.4; HRMS (ESI-TOF) *m/z* calcd for $C_{62}H_{63}N_5O_8S_2$ ([M+H]$^+$) 1070.4196, found 1070.4196.

4-[(Fmoc-Leu){2-(triphenylmethylsulfanyl)ethyl}amino]benzoic acid (**3g**)

Compound **11g** was prepared according to the method described in reference 11b. **3g**, 1.27 g, (77%); $[\alpha]^{19}_D$ +95.3 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 0.37 (3H, d, J = 6.0 Hz), 0.72 (3H, d, J = 6.1 Hz), 1.09-1.20 (1H, m), 1.34-1.51 (2H, m), 2.27-2.35 (1H, m), 2.49-2.60 (1H, m), 3.24-3.35 (1H, m), 3.57-3.68 (1H, m), 4.20 (1H, t, J = 7.1 Hz), 4.23-4.39 (3H, m), 5.50 (1H, d, J = 9.1 Hz), 7.10-7.36 (19H, m), 7.39 (2H, t, J = 7.4 Hz), 7.59 (1H, d, J = 7.2 Hz), 7.60 (1H, d, J = 7.2 Hz), 7.76 (2H, d, J = 7.5 Hz), 8.11 (2H, d, J = 8.1 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ =20.9, 23.4, 24.5, 29.4, 42.3, 47.3, 49.2, 50.5, 67.2, 120.1, 125.3, 126.8, 127.2, 127.8, 128.0, 128.6, 129.3, 129.7, 131.9, 141.5, 143.9, 144.0, 144.7, 145.5, 156.4, 169.4, 172.9; HRMS (ESI-TOF) *m/z* calcd for $C_{49}H_{46}N_2NaO_5S$ ([M+Na]$^+$) 797.3025, found 797.3036.

*4.3. General procedure for the preparation of SEAlide peptides **6***

NovaSyn TGR resin (Rink amide type: 0.22 mmol amine/g, 0.46 g, 0.10 mmol) was coupled with Fmoc-Leu-OH (106 mg, 0.30 mmol) using HBTU (110 mg, 0.29 mmol) and DIPEA (52 µL, 0.30 mmol) in DMF at room temperature for 0.5 h. The subsequent removal of the Fmoc group with 20% (v/v) piperidine in DMF gave the corresponding Leu-incorporated resin. This material was then treated with a mixture of **3** (0.20 mmol, preparation of **3a–c**, **3f** and **3h** are described in reference 4i, 11b and 19), HATU (72 mg, 0.19 mmol) and DIPEA (32 µl, 0.20 mmol) in DMF at room temperature for 2 h to yield the Fmoc-aminoacyl SEAlide-incorporated resin **4**. This resin was subjected to a standard Fmoc SPPS (coupling: Fmoc amino acid/HBTU/DIEPA (3.0/2.9/3.0 equiv), 0.5 h; Fmoc removal: 20% (v/v) piperidine in DMF, 10 min) for the elongation of the peptide chain to give the protected peptide resin **5**. The completed resin (30 or 200 mg) was exposed to a TFA-based mixture of reagents (i.e., TFA/thioanisole/*m*-cresol/EDT/H_2O – 82:5:5:3:5 (v/v), 50 µL/1 mg of the resin) at room temperature for 120–150 min. The reaction mixture was filtered and treated with cold Et_2O to afford a precipitate, which was collected by centrifugation and purified by reversed-phase preparative HPLC to give SEAlide peptide **6**.

6a, 15.6 mg (25% as **6a**·2TFA); Analytical HPLC conditions: 20-40%, retention time = 24.8 min; LRMS (ESI-Ion Trap) *m/z* calcd. for [M+H]$^+$ 1628.8, found 1628.2.

6b, 22.9 mg (36% as **6b**·2TFA); Analytical HPLC conditions: 20-40%, retention time = 25.6 min; LRMS (ESI-Ion Trap) *m/z* calcd. for [M+H]$^+$ 1642.8, found 1643.1.

6c, 21.4 mg (34% as **6c**·2TFA); Analytical HPLC conditions: 20-40%, retention time = 23.4 min; LRMS (ESI-Ion Trap) *m/z* calcd. for [M+H]$^+$ 1658.8, found 1659.0.

6d, 13.6 mg (18% as **6d**·3TFA); Analytical HPLC conditions: 20-40%, retention time = 22.7 min; LRMS (ESI-Ion Trap) *m/z* calcd. for [M+H]$^+$ 1699.8, found 1700.3.

6e, 19.4 mg (22% as **6e**·3TFA); Analytical HPLC conditions: 20-40%, retention time = 23.2 min; LRMS (ESI-TOF) *m/z* calcd. for [M+H]$^+$ 1727.8, found 1727.9.

6f, 0.5 mg (5% as **6f**·2TFA); Analytical HPLC conditions: 20-40%, retention time = 22.1 min; LRMS (ESI-Ion Trap) *m/z* calcd. for [M+H]$^+$ 1685.8, found 1686.1.

6g, 17.3 mg (33% as **6g**·2TFA); Analytical HPLC conditions: 20-50%, retention time = 23.0 min; LRMS (ESI-TOF) *m/z* calcd. for [M+2H]$^{2+}$ 842.9, found 842.7.

6h, 20.9 mg (32% as **6h**·2TFA); Analytical HPLC conditions: 20-50%, retention time = 21.4 min; LRMS (ESI-TOF) *m/z* calcd. for [M+2H]$^{2+}$ 867.9, found 867.6.

*4.4. General procedure for preparation of C-terminal peptide thioacids **7a**-**h***

To a solution of the SEAlide peptide **6** (1.0 µmol) in NMP (100 µL) were added 1.0 M Na phosphate buffer (900 µL) containing 120 mM NaSH or $(NH_4)_2S$, 100 mM TCEP·HCl and 250 mM Na ascorbate, and the resulting mixture was incubated at 37 °C. Five-microliter aliquots of the reaction mixture were collected at regular time intervals and analyzed by analytical HPLC. To determine the isolated yields, we fractionated an entire reaction mixture by semi-preparative HPLC following 24 h of the reaction and the product was obtained after lyophilization.

7a. Analytical HPLC conditions: 20-40%, retention time = 20.3 min; LRMS (ESI-TOF) *m/z* calcd. for [M+2H]$^{2+}$ 677.3, found 677.3.

8a. Analytical HPLC conditions: 20-40%, retention time = 16.7 min; LRMS (ESI-TOF) *m/z* calcd. for [M+2H]$^{2+}$ 669.3, found 669.3.

9a. Analytical HPLC conditions: 20-40%, retention time = 15.8 min; LRMS (ESI-TOF) *m/z* calcd. for [M+2H]$^{2+}$ 668.8, found 668.8.

Thioester of **6a**. Analytical HPLC conditions: 20-40%, retention time = 25.6 min; LRMS (ESI-TOF) m/z calcd. for [M+2H]$^{2+}$ 814.9, found 814.9.

SEAlide deriv. Analytical HPLC conditions: 20-40%, retention time = 21.7 min; LRMS (ESI-TOF) m/z calcd. for [M+H]$^+$ 310.2, found 310.4.

7b. Analytical HPLC conditions: 20-40%, retention time = 21.3 min; LRMS (ESI-TOF) *m/z* calcd. for [M+2H]$^{2+}$ 684.3, found 684.2.

8b. Analytical HPLC conditions: 20-40%, retention time = 16.8 min; LRMS (ESI-TOF) *m/z* calcd. for [M+2H]$^{2+}$ 676.3, found 676.2.

9b. Analytical HPLC conditions: 20-40%, retention time = 15.8 min; LRMS (ESI-TOF) *m/z* calcd. for [M+2H]$^{2+}$ 675.8, found 675.8.

7c. Analytical HPLC conditions: 20-40%, retention time = 19.0 min; LRMS (ESI-TOF) *m/z* calcd. for [M+2H]$^{2+}$ 692.3, found 692.5.

8c. Analytical HPLC conditions: 20-40%, retention time = 16.1 min; LRMS (ESI-TOF) *m/z* calcd. for[M+2H]$^{2+}$ 684.3, found 684.5.

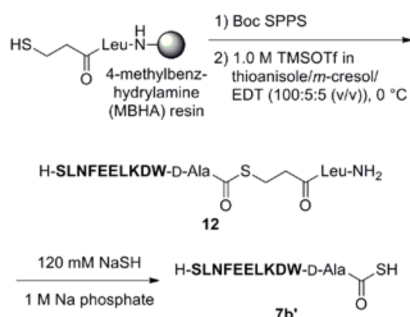
9c. Analytical HPLC conditions: 20-40%, retention time = 15.2 min; LRMS (ESI-TOF) *m/z* calcd. for [M+2H]$^{2+}$ 683.8, found 683.9.

7d. Analytical HPLC conditions: 20-40%, retention time = 16.0 min; LRMS (ESI-TOF) *m/z* calcd. for [M+2H]$^{2+}$ 712.9, found 712.8.

8d. Analytical HPLC conditions: 20-40%, retention time = 13.0 min; LRMS (ESI-TOF) m/z calcd. for $[M+2H]^{2+}$ 704.9, found 704.8
9d. Analytical HPLC conditions: 20-40%, retention time = 12.1 min; LRMS (ESI-Ion Trap) m/z calcd. for $[M+2H]^{2+}$ 704.4, found 704.6.
7e. Analytical HPLC conditions: 20-40%, retention time = 16.3 min; LRMS (ESI-TOF) m/z calcd. for $[M+2H]^{2+}$ 726.8, found 726.7.
8e. Analytical HPLC conditions: 20-40%, retention time = 13.5 min; LRMS (ESI-TOF) m/z calcd. for $[M+2H]^{2+}$ 718.9, found 718.8.
9e. Analytical HPLC conditions: 20-40%, retention time = 12.7 min; LRMS (ESI-TOF) m/z calcd. for $[M+2H]^{2+}$ 718.4, found 718.4.
7g. Analytical HPLC conditions: 15-45%, retention time = 26.6 min; LRMS (ESI-TOF) m/z calcd. for $[M+2H]^{2+}$ 705.3, found 705.2.
8g. Analytical HPLC conditions: 15-45%, retention time = 23.0 min; LRMS (ESI-TOF) m/z calcd. for $[M+2H]^{2+}$ 697.3, found 697.3.
9g. Analytical HPLC conditions: 15-45%, retention time = 22.2 min; LRMS (ESI-TOF) m/z calcd. for $[M+2H]^{2+}$ 696.9, found 696.8.
7h. Analytical HPLC conditions: 20-50%, retention time = 17.4 min; LRMS (ESI-TOF) m/z calcd. for $[M+2H]^{2+}$ 730.3, found 730.2.
8h. Analytical HPLC conditions: 20-50%, retention time = 14.6 min; LRMS (ESI-TOF) m/z calcd. for $[M+2H]^{2+}$ 722.3, found 722.3.
9h. Analytical HPLC conditions: 20-50%, retention time = 13.6 min; LRMS (ESI-TOF) m/z calcd. for $[M+2H]^{2+}$ 721.8, found 721.8.
10. Analytical HPLC conditions: 20-40%, retention time = 15.8 min; LRMS (ESI-TOF) m/z calcd. for $[M+2H]^{2+}$ 688.8, found 688.8.

4.5. Examination of the C-terminal epimerization

Analyte **7b** was prepared using the protocol described above and analyzed by HPLC using two analytical columns, which were connected in sequence. The analytical HPLC conditions were as follows: 22%–32% over 120 min, retention times = 74.3 min (**7b**) and 72.7 min (**7b'**: epimer of **7b** at the C-terminal Ala residue).



The epimer of **7b** at the C-terminal Ala residue was prepared as follows. Peptide thioester **12** was prepared via a Boc SPPS method using an *in situ* neutralization protocol[20] on $HSCH_2CH_2CO$-Leu-MBHA resin (0.70 mmol amine/g, 0.14 g, 0.10 mmol). The resulting resin was treated with a mixture of 1.0 M TMSOTf/thioanisole (50 µL/1 mg resin), *m*-cresol and EDT (100:5:5 (v/v)) at 0 °C for 2 h. The reaction mixture was filtered and treated with cold Et_2O to afford a precipitate, which was collected by centrifugation and dissolved in a mixture of 0.1% (v/v) TFA in H_2O and 0.1% TFA (v/v) in MeCN (5 mL, 1:1 (v/v)). The resulting solution was lyophilized to give crude **12**, which was dissolved in 5 mL of 1.0 M sodium phosphate containing 120 mM NaSH (pH 9.1). The resulting solution was held at room temperature for 1 h before being fractionated by preparative HPLC to give pure **7b'** after lyophilization. Analytical HPLC conditions: 20-40%, retention time = 22.7 min; LRMS (ESI-Ion Trap) m/z calcd. for $[M+H]^{+}$ 1367.6, found 1368.0.

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