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Sequential native chemical ligation utilizing peptide thioacids derived from newly developed Fmoc-based synthetic method

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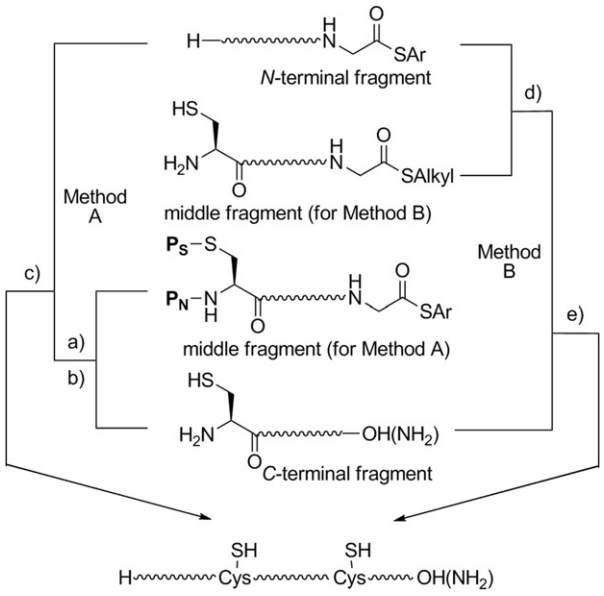
ABSTRACT

The first facile Fmoc-based synthetic procedure for peptide thioacids was developed. Successful application of the resulting thioacids to sequential native chemical ligation (NCL) in the N to C direction was achieved. Conversion of the peptide thioacids to the corresponding thioesters with Ellman's reagent followed by NCL in the presence of tris(2-carboxyethyl)phosphine (TCEP) and thiophenol was accomplished in a one-pot manner.

1. Introduction

Native chemical ligation (NCL) has shown great utility in the preparation of proteins in a synthetic or semi-synthetic manner.[1] Intermolecular chemoselective reaction of the thiol group on N-terminal cysteine (Cys) residue with thioesters followed by *S-N* acyl shift allows the two peptide fragments without protecting groups to be condensed.

That is not the case for repetitive NCL with more than one thioester fragment, whereby the N and/or S functions on the N-terminal Cys residue in the middle thioester fragment require protection to avoid intramolecular NCL, thus leading to the formation of cyclic peptides (Scheme 1, Method A).[2] To this end, various types of protecting groups are introduced onto the N-terminal cysteine to mask intramolecular NCL-reactivity of thioesters. An elegant alternative developed by Kent et al. is the use of intermolecular reaction of the free N-terminal Cys residue in peptide alkylthioesters with peptide arylthioesters, where the sulfhydryl group reacts intermolecularly to the arylthioester in a kinetically controlled manner (Scheme 1, Method B).[3] Masking the reactivity of thioesters is another potential option to establish facile sequential NCL protocols, but masked thioesters requires easy conversion to active thioesters through simple manipulations. With this in mind, we re-evaluated the synthetic applicability of peptidyl thioacids as thioester precursors to sequential NCL in solution phase synthesis.



Scheme 1. Sequential native chemical ligation for preparation of peptides/proteins. Method A: $\mathbf{P_N}$ and $\mathbf{P_S}$=protections for amino and sulfanyl groups, respectively; (a) first native chemical ligation (first NCL); (b) removal of $\mathbf{P_N}$ and/or $\mathbf{P_S}$; (c) second NCL (second NCL). Method B: (d) first NCL (kinetically controlled conditions); (e) second NCL.

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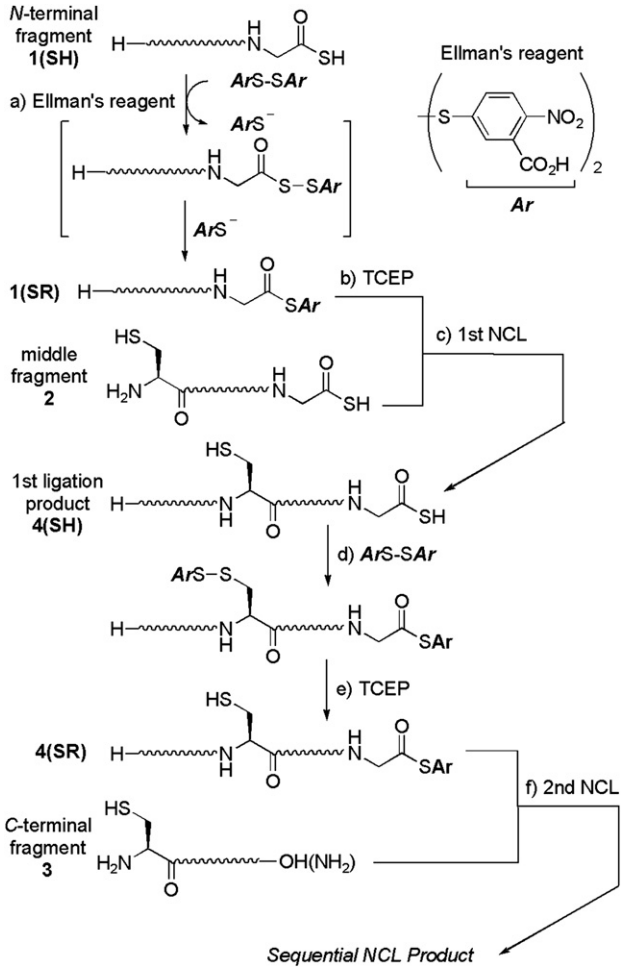
Peptidyl thioacids were first introduced into peptide chemistry by Li et al.[4] They applied the conversion of thioacids to the corresponding active acyl components by treatment with silver nitrate or diaryl disulfides in the synthesis of α-inhibin-92. The formation of peptidyl thioesters from the reaction of thioacids with Ellman's reagent with applications to inter-[5a–c] and intramolecular[5d] NCL was later reported. Among reports concerning peptide thioacids, it is worth noting that reversed-type N to C elongation was achieved using peptide thioacids.[6,7] In this research, condensation of N-terminal Cys thioacid fragments on resin-bound thioester followed by activation of the thioacids to the corresponding thioesters with alkylating agents, such as bromoacetic acid, is involved. The resulting thioester could be subjected to further NCL reaction after washing to remove bromoacetic acid.

2. Results and discussion

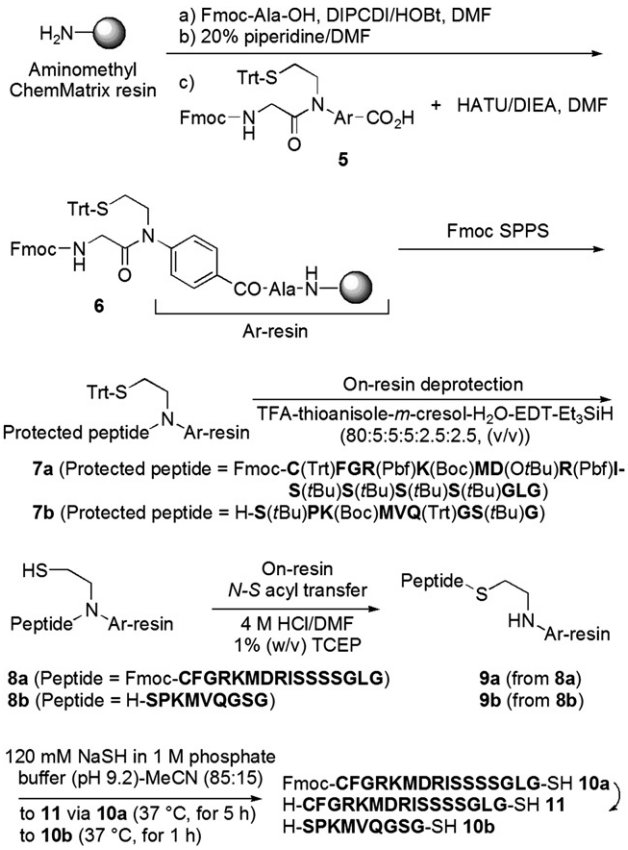
As shown in Scheme 2, we speculated that thioesterification of peptide thioacid **1**(**SH**) by aryl disulfides, such as Ellman's reagent, followed by the first NCL with middle thioacid fragment **2** in the presence of tris(2-carboxyethyl)phosphine (TCEP) for reduction of excess Ellman's reagent should result in formation of ligated peptide thioacid **4**(**SH**) in a one-pot manner. After purification of **4**(**SH**), the resulting thioacids could be brought to the second NCL with C-terminal fragment **3** according to the procedure described for the first NCL to give sequential NCL product. In this envisioned scheme, ligation of peptide thioacid (**1**(**SH**) or **4**(**SH**)) via conversion to the corresponding peptide thioester (**1**(**SR**) or **4**(**SR**), respectively) with N-terminal Cys fragments, such as **2** or **3** can be conducted in a one-pot manner in the presence of TCEP without requiring purification of the thioesters. Notably, the use of N-terminal Cys thioacid fragments, such as **2**, in the NCL step allows the coupling to be performed in a repetitive manner in the N to C direction.[8]



Scheme 2. Envisioned sequential native chemical ligation utilizing peptide thioacids as N-terminal and middle fragments. TCEP=tris(2-carboxyethyl)phosphine.

Nevertheless, widespread use of peptide thioacids in peptide chemistry, including ligation chemistry, has not been seen. This seems to be partly dependent on the fact that while Fmoc SPPS continues to increase in utility, it could not be used for preparation of peptide thioacids. Thus, development of Fmoc-based protocols for synthesizing peptide thioacids has been required in reinforcing the general applicability of sequential NCL protocols using peptide thioacids. Here, we present sequential NCL using peptide thioacids, including a new preparative protocol for peptide thioacids by Fmoc SPPS. We selected one-disulfide bond-containing 32-residue human natriuretic peptide (hBNP) as the model target peptide.

First, Fmoc-based synthesis of peptide thioacids was established for preparation of N-terminal hBNP (1–9)- and middle hBNP (10–25)-fragments (**10b** and **11**) as shown in Scheme 3.[9] Successive



Scheme 3. Fmoc-based preparation of peptide thioacids using (*N*-Fmoc-glycyl-*N*-sulfanylethyl)aminobenzoic acid linker **5**.[10a]

condensation of Fmoc-Ala-OH and 4-[Fmoc-glycyl-(2-tritylsulfanylethyl)amino]benzoic acid **5**[10a] on aminomethyl ChemMatrix resin afforded the linker-incorporated resin **6**. Standard Fmoc SPPS on the resulting resin **6** followed by deprotection with TFA-based reagent cocktail gave deprotected peptide amide resins **8**, which was then treated with 4 M HCl/DMF in the presence of 1% (w/v)

TCEP to give peptide thioester resins **9** via *N*-*S* acyl transfer on resin.[10] Treatment of the resulting thioesters **9** with NaSH in 1 M phosphate buffer (pH 9.2)/MeCN (85:15) yielded N^{α}-Fmoc protected peptide thioacid **10a** and peptide thioacid **10b** within 15 min.[11] To avoid the intramolecular NCL-mediated release of middle peptide fragment from the resin, protection on the α-amino group was required. To this end, we selected the Fmoc group among the potential candidates that are stable under TFA-mediated deprotection conditions. Here, we were pleased to find that the continuous NaSH-treatment in the presence of peptide-detached resin (linker) resulted in the formation of Fmoc-deprotected peptide thioacid **11**, whereas reaction without the resin gave little N^{α}-amine peptide (Fig. 1).[12] The N^{α}-Fmoc protection exerted its utility in the Fmoc-based preparation of peptide thioacids, such as **11**, because conversion of the thioester to thioacid proceeded more rapidly than deprotection of the

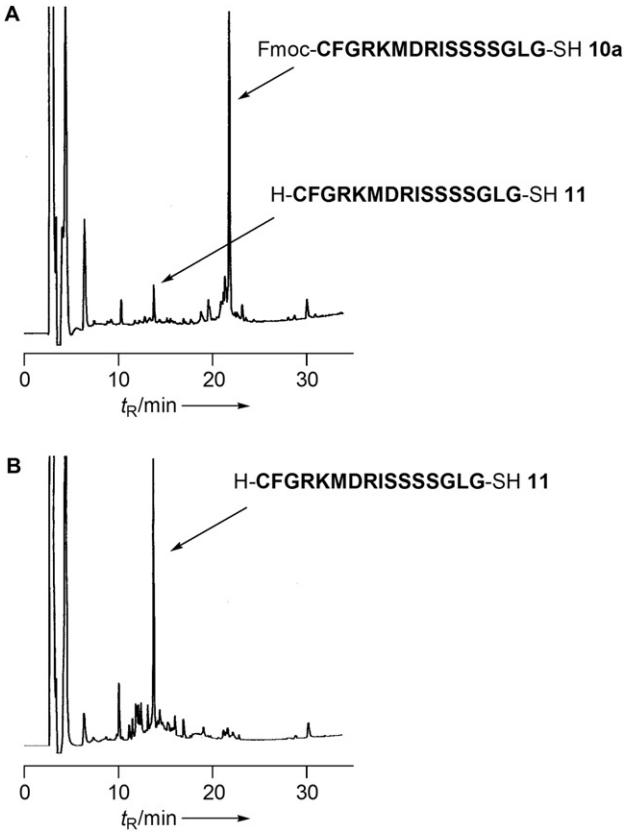


Figure 1. HPLC monitoring of release of peptide thioacids from resin by treatment with NaSH in 1 M phosphate buffer (pH 9.2)/MeCN (85:15). (A) After 1 h incubation of resin **9a**, the filtrate was analyzed. (B) After 8 h incubation of the resin. HPLC conditions: Cosmosil 5C_{18}-AR-II column (4.6×250 mm) with a linear gradient of 0.1% TFA/MeCN/0.1% TFA aq (5:95–65:35 over 30 min) at a flow rate of 1.0 mL min^{-1}, detection at 220 nm.

Fmoc group. The C-terminal fragment (H-**CKVLRRH**-OH **12**: hBNP (26–32)) was prepared by standard Fmoc SPPS. Generality of the treatment with NaSH for preparation of N-terminal Cys peptide thioacids was confirmed by successful application to the synthesis of H-CYAVTGRGDSPAASSG-SH. Alternatively, addition of piperidine (to 5% (v/v)) into the reaction mixture containing NaSH also afforded the desired peptide thioacid (Fig. 2).

Having three requisite fragments for construction of the hBNP backbone, we attempted repetitive NCL in the N to C directive manner as shown in Scheme 4.

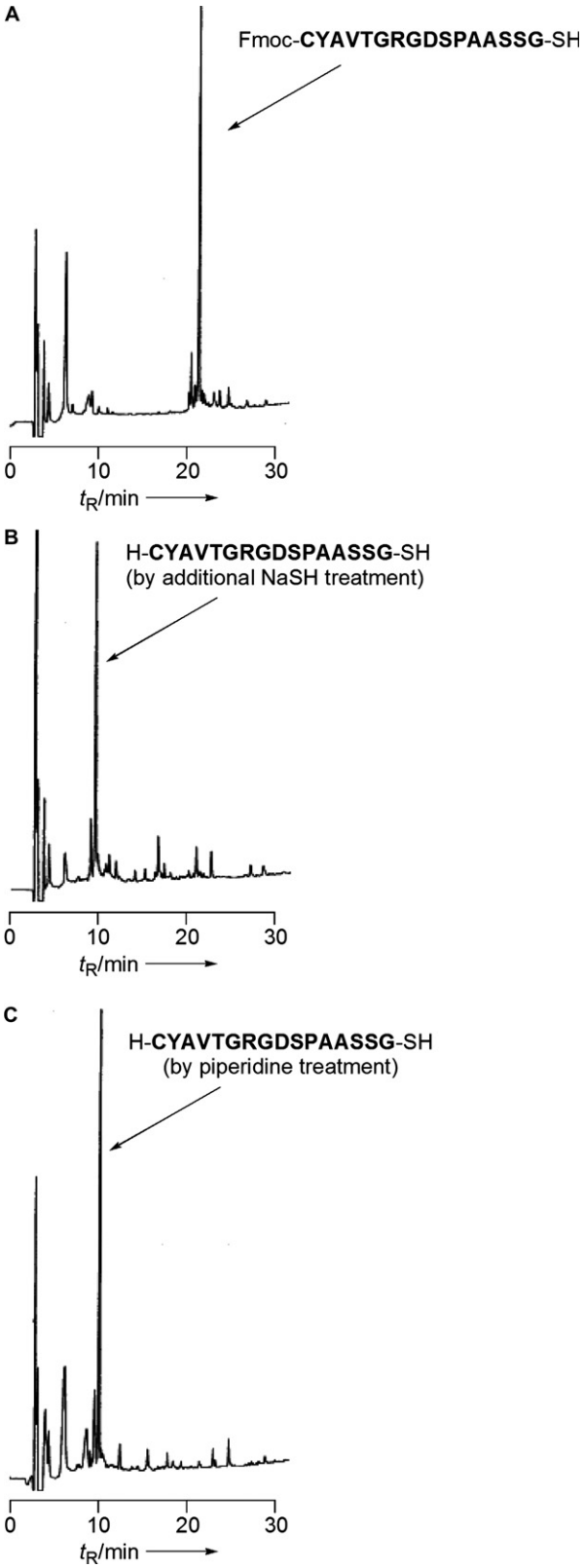
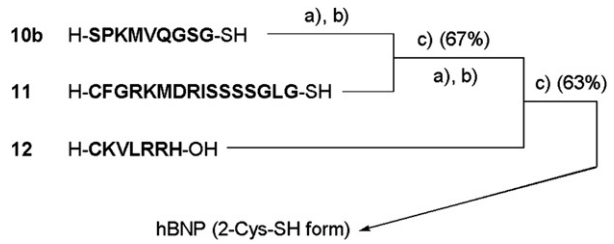


Figure 2. HPLC monitoring of release of peptide thioacid (Fmoc-**CYAVTGRGDSPAASSG**-SH) and deprotection of the Fmoc group by additional NaSH treatment or piperidine treatment. (A) After 15 min treatment of resin (Fmoc-**CYAVTGRGDSPAASSG**-*S*-Ar-resin) with 120 mM NaSH in 6 M guanidine-0.1 M sodium phosphate buffer (pH 9.2) at 37 °C. (B) After additional treatment with NaSH aq for 24 h in the presence of the peptide-detached resin. (C) Fifteen min hydrothiolitic release of the peptide followed by addition of piperidine (5%, (v/v)) with additional 15 min treatment. HPLC conditions identical to that of Figure 1 were used.



Scheme 4. Sequential NCL for the preparation of hBNP. (a) Ellman's reagent, $KHCO_3$, H_2O/DMF (8:2); (b) TCEP, thiophenol; (c) NCL.

First, N-terminal thioacid fragment **10b** (1 mM) was converted to the corresponding Ar thioester **10b**′ by reaction with Ellman's reagent (3 mM) in the presence of $KHCO_3$ (3 mM) in H_2O/DMF (80:20) at room temperature (Fig. 3, A and B).[13,14] After reduction of adjustment of the reaction pH to around 7.4 at 4 °C.[15] The first NCL completed in 2 h to yield ligated peptide thioacid **13** corresponding to hBNP (1–25) (Fig. 3, C and D). After HPLC purification of the crude material, purified thioacid **13** was subjected to the second NCL step in the presence of thiophenol after conversion to the corresponding Ar thioester **13**′ by treatment with Ellman's reagent followed by TCEP reduction[16] as mentioned in the transformation of **10b** (Fig. 3, E and F). Attempted second NCL with C-terminal fragment **12** also efficiently proceeded to yield 2-Cys-SH hBNP (Fig. 3, G and H). Then, HPLC-purified Cys-SH peptide was oxidized to hBNP by the action of DMSO.

In order to evaluate epimerization of chiral C-terminal amino acids, model thioacid fragments were synthesized for application to NCL. Using 4-[Fmoc-L- (or D-)alanyl-(2-tritylsulfanylethyl)-amino]-benzoic acid, protected peptide resins corresponding to H-HRFA(L- or D-)A-SH were constructed on aminomethyl ChemMatrix resin by standard Fmoc SPPS. After on-resin deprotection of the completed

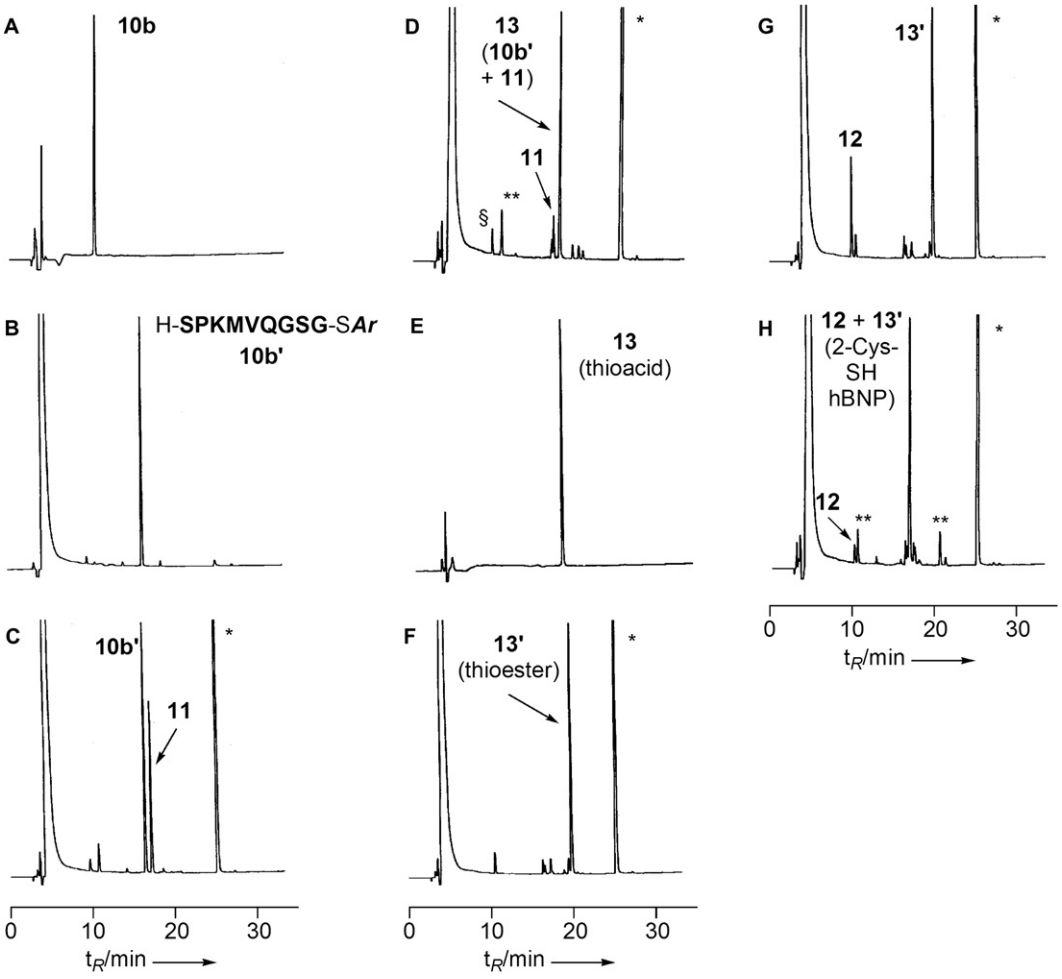


Figure 3. HPLC monitoring of sequential NCL. (A) HPLC analysis of N-terminal thioacid fragment **10b**. (B) Conversion of **10b** to the corresponding Ar thioester **10b**′ by the reaction of **10b** with Ellman's reagent in the presence of $KHCO_3$ in H_2O/DMF (2:8) at room temperature for 1.5 h. *Ar*=–$C_6H_4(NO_2)(CO_2H)$. (C) The first NCL (before adjustment of pH): To the crude reaction mixture of **10b** and Ellman's reagent were successively added TCEP, thioacid fragment **11** and thiophenol followed by the adjustment of pH to 7.5. (D) The first NCL (t=2 h) at 37 °C, H-**SPKMVQGSGCFGRKMDRISSSSGLG**-SH (**13**). §Hydrolyzed product of **10b**′. (*E*) HPLC-purified **13**. (F) Conversion of **13** to the corresponding Ar thioester **13**′ (H-**SPKMVQGSGCFGRKMDRISSSSGLG**-S*Ar*) by the reaction of **13** with Ellman's reagent in the presence of $KHCO_3$ in H_2O/DMF (2:8) at room temperature for 1.5 h followed by addition of TCEP. (G) The second NCL (before adjustment of pH): To the crude reaction mixture containing **13**′ were added C-terminal fragment **12** and thiophenol followed by the adjustment of pH to 7.5. (H) The second NCL (t=2 h) at 37 °C. HPLC conditions: Cosmosil 5C_{18}-AR-II column (4.6×250 mm) with a linear gradient of 0.1% TFA/MeCN/0.1% TFA aq (5:95–55:45 over 30 min) at a flow rate of 1.0 mL min^{-1}, detection at 220 nm. *5-mercapto-2-nitrobenzoic acid, **Non-peptidic impurities.

excess Ellman's reagent with TCEP, the first NCL was initiated by successive addition of equimolar amount of middle thioacid fragment **11** and thiophenol[5d] to the reaction mixture followed by resins, *N*-*S* acyl transfer on the resin was achieved by treatment of the amide-type resins with TFA at ambient temperature for 20 h in the presence of TCEP (1% (w/v)).[17] Treatment of the resulting

thioester resin with NaSH in 1 M phosphate buffer (pH 9.2)/MeCN (85:15) afforded H-HRFA(L- or D-)A-SH peptide. Each peptide thioacid resulting from L- or D-Ala-linked resin was converted to the corresponding thioester by reaction with Ellman's reagent followed by NCL with peptide **12** in the presence of TCEP and thiophenol to give the desired ligated peptide while accompanied by no epimerized material (Fig. 4).

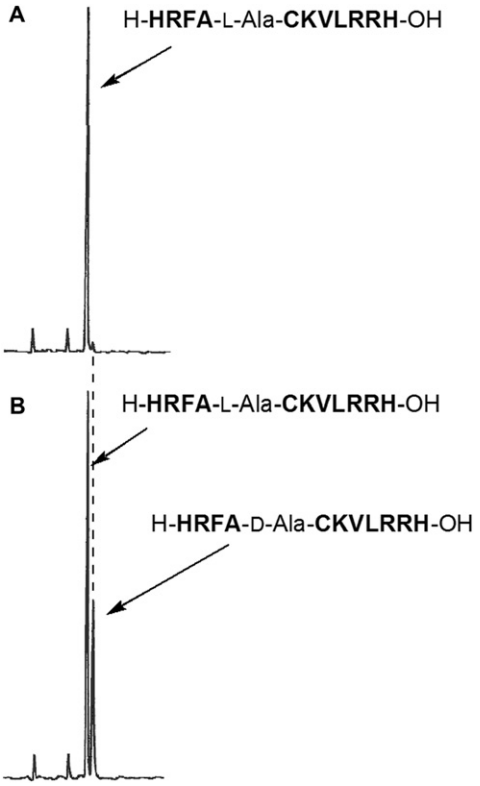


Figure 4. HPLC examination of epimerization of C-terminal chiral amino acid. (A) HPLC analysis of crude material after NCL completion (H-**HRFA**-L-Ala-SH+H-**CKVLRRH**-OH). (B) Co-injection of the crude L-Ala peptide and purified D-Ala peptide. HPLC conditions: Cosmosil $5C_{18}$AR-II column (4.6×250 mm) with a linear gradient of 0.1% TFA/MeCN/0.1% TFA aq (5:95–30:70 over 30 min) at a flow rate of 1.0 mL/min, detection at 220 nm. Only a critical retention time region of the HPLC charts was enlarged.

3. Conclusion

We established the first facile Fmoc-based synthetic protocols for peptide thioacids using the *N-S* acyl transfer methodology. Resulting peptide thioacids have been successfully applied to sequential NCL procedures. The thioacid moiety serves as a masked thioester with its ready conversion to the corresponding active thioester via reaction with aryl disulfide and participates in the NCL step in the presence of TCEP in a one-pot manner. Recent growing interest of thioacids in peptide/protein chemistry[18] allows our presented protocols to gain unequivocal utility in protein synthesis. Extension of our methods for preparation of proteins, including modification of proteins, is currently under investigation.

4. Experimental section

4.1. General methods

Exact mass spectra were recorded on Waters MICROMASS LCT PREMIER. For HPLC separations, a Cosmosil $5C_{18}$-AR-II analytical column (Nacalai Tesque, 4.6×250 mm, flow rate 1 mL/min) or a $5C_{18}$-AR-II semi-preparative column (Nacalai Tesque, 10×250 mm, flow rate 3.0 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% TFA aq solution (v/v, solvent A) and 0.1% TFA in MeCN (v/v, solvent B) was used for HPLC elution. All Chemicals were purchased from either Kanto Chemicals, Sigma Aldrich Japan, Tokyo Chemical Industry, Wako Pure Chemical Industries or Watanabe Chemical Industry.

4.2. Preparation of resin bound peptide thioesters 9a and 9b

On aminomethyl ChemMatrix® resin (1.0 mmol amine/g, 40 mg, 0.040 mmol) was coupled Fmoc-Ala-OH (40 mg, 0.12 mmol) as an internal standard amino acid in the presence of DIPCDI (19 μL, 0.12 mmol) and HOBt·H_2O (21 mg, 0.13 mmol) in DMF at room temperature for 2 h followed by Fmoc removal by 20% piperidine/DMF to afford internal standard-incorporated resin. The resulting resin was treated with a preactivated acid **5** (59 mg, 0.080 mmol) with HATU (30 mg, 0.075 mmol) and DIPEA (14 μL, 0.075 mmol) to yield aniline-linked resin **6**. On the resulting resin **6**, standard Fmoc SPPS (Fmoc amino acid (3.0 equiv), DIPCDI (3.0 equiv) and HOBt·H_2O (3.3 equiv) in DMF for acylation and 20% piperidine/DMF (10 min) for Fmoc removal) was performed for the chain elongation to give protected peptide resin **7a** or **7b**. The resulting completed resin **7a** or **7b** was treated with TFA-thioanisole-*m*-cresol-H_2O-EDT-Et_3SiH (80:5:5:5:2.5:2.5, (v/v)) at room temperature for 1.5 h to give deprotected peptide resin **8a** or **8b**, respectively. After being washed with CH_2Cl_2, each amide-type resin **8a** or **8b** was subjected to *N-S* acyl transfer reaction in 4 M HCl/DMF in the presence of TCEP (1% (w/v)) at room temperature for 20 h followed by washing with CH_2Cl_2 to yield the corresponding on-resin thioester peptide **9a** or **9b**, respectively.

4.3. Hydrothiolytic release of peptide *N*-Cys thioacid 11

On-resin peptide thioester **9a** was incubated with 120 mM NaSH in 1 M sodium phosphate buffer (pH 9.2)/MeCN (85:15) at 37 °C. Release of N^{α}-Fmoc protected peptide thioacid **10a** was confirmed by HPLC analysis within 15 min of incubation. After additional incubation for 8 h, the Fmoc group on peptide **10a** was completely removed to give crude materials, which were then subjected to semi-preparative HPLC purification to afford the desired Fmoc-deprotected peptide thioacid **11**. The residual resin was hydrolyzed with 6 M HCl aq in the presence of phenol (0.2%, (v/v)). The resulting hydrolysate was subjected to amino acid analysis (AAA) and the release yield was estimated to be 62% based on the internal Ala.

Compound **10a**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 65% over 30 min, retention time=21.7 min. MS (ESI-TOF) *m/z* calcd for ($[M+2H]^{2+}$) 969.9, found 970.0.

Compound **11**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 65% over 30 min, retention time=13.7 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 5 to 15% over 30 min. MS (ESI-TOF) *m/z* calcd for ($[M+2H]^{2+}$) 858.8, found 858.8.

4.4. Hydrothiolytic release of peptide thioacid 10b

On-resin peptide thioester **9b** was incubated with 120 mM NaSH in 1 M sodium phosphate buffer (pH 9.2)/MeCN (85:15) at 37 °C for 15 min to yield hydrothiolytically released peptide thioacid **10b**. The crude material was purified by semi-preparative HPLC. The residual resin was hydrolyzed with 6 M HCl aq in the presence of phenol (0.2%, (v/v)). The resulting hydrolysate was

subjected to AAA and the release yield was estimated to be 69% based on internal Ala.

Compound **10b**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time=10.3 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 5 to 10% over 30 min. MS (ESI-TOF) *m*/*z* calcd for ($[M+H]^+$) 906.4, found 906.5.

4.5. Confirmation of generality of the preparative method for N-terminal Cys peptide thioacid

On-resin peptide Fmoc-Cys(Trt)-Tyr(^{t}Bu)-Ala-Val-Thr(^{t}Bu)-Gly-Arg(Pbf)-Gly-Asp(^{t}Bu)-Ser(^{t}Bu)-Pro-Ala-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-Gly-*N*-Ar-resin was prepared on Leu-aminomethyl ChemMatrix® resin as mentioned above using Fmoc protocol. Deprotection of the completed resin with TFA-thioanisole-*m*-cresol-H_2O-EDT-Et_3SiH (80:5:5:5:2.5:2.5, (v/v)) at room temperature for 1.5 h followed by the treatment with 4 M HCl/DMF in the presence of TCEP (1% (w/v)) at room temperature for 20 h gave deprotected on-resin peptide thioester Fmoc-Cys-Tyr-Ala-Val-Thr-Gly-Arg-Gly-Asp-Ser-Pro-Ala-Ala-Ser-Ser-Gly-*S*-Ar-resin. The on-resin peptide thioester was incubated with 120 mM NaSH in 6 M guanidine-0.1 M sodium phosphate buffer (pH 9.2) at 37 °C for 15 min to yield the hydrothiolytically released peptide thioacid Fmoc-Cys-Tyr-Ala-Val-Thr-Gly-Arg-Gly-Asp-Ser-Pro-Ala-Ala-Ser-Ser-Gly-SH (Fig. 2-A). After additional incubation for 24 h, complete removal of the Fmoc group was confirmed by HPLC analysis (Fig. 2-B). When piperidine was added to the crude mixture of the N^{α}-Fmoc protected peptide thioacid obtained after hydrothiolysis, removal of the Fmoc group was completed within 15 min of incubation (Fig. 2-C).

Fmoc-Cys-Tyr-Ala-Val-Thr-Gly-Arg-Gly-Asp-Ser-Pro-Ala-Ala-Ser-Ser-Gly-SH: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 65% over 30 min, retention time=21.4 min. MS (ESI-TOF) *m*/*z* calcd for ($[M+H]^+$) 1735.7, found 1735.8.

H-Cys-Tyr-Ala-Val-Thr-Gly-Arg-Gly-Asp-Ser-Pro-Ala-Ala-Ser-Ser-Gly-SH: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 65% over 30 min, retention time=10.0 min. MS (ESI-TOF) *m*/*z* calcd for ($[M+H]^+$) 1513.6, found 1513.6.

4.6. Synthesis of hBNP-32 by sequential native chemical ligation utilizing peptide thioacids

First cycle. Peptide thioacid **10b** (630 μg, 0.69 μmol) was dissolved at a final concentration of 1.0 mM in DMF/H_2O (690 μL, 2:8 (v/v)) containing 3.0 mM Ellman's reagent and 3.0 mM $KHCO_3$. The resulting mixture was shaken at room temperature for 1.5 h and conversion of thioacid **10b** to thioester **10b′** was confirmed by HPLC analysis. After reduction of an excess Ellman's reagent with 1% (w/v) TCEP (6.9 mg), *N*-Cys thioacid fragment **11** (1.2 mg, 0.69 μmol) and thiophenol (6.9 μL) were added to the mixture. Then 10% K_2CO_3 aq solution (50 μL) was added to the reaction mixture at 4 °C to adjust the pH around 7.3. After incubation of the mixture at 37 °C for 2 h, the starting materials were disappeared and the crude material was purified by semi-preparative HPLC to give ligated peptide thioacid **13** (1.2 mg, 67% yield).

Second cycle. The purified peptide thioacid **13** (1.2 mg, 0.46 μmol) was dissolved at a final concentration of 1.0 mM in DMF/H_2O (460 μL, 2:8(v/v)) containing 3.0 mM Ellman's reagent and 3.0 mM $KHCO_3$. The resulting mixture was shaken at room temperature for 1.5 h. Then 1% (w/v) TCEP (4.6 mg) was added to the reaction mixture to reduce an excess Ellman's reagent and the hetero disulfide generated from the cysteinyl sulfhydryl group and 5-mercapto-2-nitrobenzoic acid. The conversion of thioacid **13** to thioester **13′** was confirmed by HPLC analysis. *N*-Cys fragment **12** (500 μg, 0.55 μmol) and thiophenol (4.6 μL) were added to the reaction mixture and then the pH of the reaction was adjusted around 7.5 by addition of 10% K_2CO_3 solution (31 μL) at 4 °C. After incubation of the mixture at 37 °C for 2 h, the starting materials were disappeared and the crude material was purified by semi-preparative HPLC to give ligated peptide 2-Cys-SH hBNP (1.0 mg, 63%). Oxidation of the purified 2-Cys-SH hBNP (0.3 mM) was performed in 0.1 M sodium phosphate buffer containing 6 M guanidine hydrochloride (pH 7.3)/DMSO (9:1). After incubation of the mixture at 37 °C for 12 h, hBNP was isolated by semi-preparative HPLC.

Compound **10b′**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time=16.3 min. MS (ESI-TOF) *m*/*z* calcd for ($[M+H]^+$) 1071.4, found 1071.5.

Compound **12**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time=10.3 min. MS (ESI-TOF) *m*/*z* calcd for ($[M+2H]^{2+}$) 456.3, found 456.2.

Compound **13**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min at a flow rate of 1.0 mL min^{-1}, retention time=17.8 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 15 to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd for ($[M+3H]^{3+}$) 863.4, found 863.4.

Compound **13′**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time=19.8 min. MS (ESI-TOF) *m*/*z* calcd ($[M+3H]^{3+}$) 918.4, found 918.5.

hBNP (2-Cys-SH form): Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time=16.5 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 15 to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd for ($[M+4H]^{4+}$) 866.9, found 866.9.

hBNP: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time=15.3 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 15 to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd for ($[M+4H]^{4+}$) 866.5, found 866.5.

4.7. Examination of epimerization during hydrothiolytic release of peptide thioacid followed by thioesterification and NCL

On-resin peptide H-His(Trt)-Arg(Pbf)-Phe-Ala-L-Ala-*N*-Ar-resin or H-His(Trt)-Arg(Pbf)-Phe-Ala-D-Ala-*N*-Ar-resin was prepared on Leu-aminomethyl ChemMatrix® resin as mentioned above using Fmoc protocol. Deprotection of the completed resin with TFA-thioanisole-*m*-cresol-H_2O-EDT-Et_3SiH (80:5:5:5:2.5:2.5, (v/v)) at room temperature for 1.5 h followed by the treatment with TFA in the presence of TCEP (1% (w/v)) at room temperature for 20 h gave deprotected on-resin peptide thioester H-His-Arg-Phe-Ala-L-Ala-*S*-Ar-resin or H-His-Arg-Phe-Ala-D-Ala-*S*-Ar-resin, respectively. The on-resin peptide thioester was incubated with 120 mM NaSH in 1 M sodium phosphate buffer (pH 9.2)/MeCN (85:15) at 37 °C for 15 min to yield the hydrothiolytically released peptide thioacid H-His-Arg-Phe-Ala-L-Ala-SH or H-His-Arg-Phe-Ala-D-Ala-SH, respectively.

Each peptide thioacid was transformed into a corresponding Ar thioester by treatment with Ellman's reagent, and it was ligated with *N*-Cys peptide **12** using sequential NCL protocol as mentioned above. After completion of NCL, the reaction mixtures were analyzed by HPLC and no epimerization was observed after these reactions (Fig. 4).

H-His-Arg-Phe-Ala-L-Ala-SH and H-His-Arg-Phe-Ala-D-Ala-SH: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 25% over 30 min, retention time=17.6 min. MS (ESI-TOF) *m*/*z* calcd for ($[M+H]^+$) 617.3, found 617.3.

H-His-Arg-Phe-Ala-L-Ala-Cys-Lys-Val-Leu-Arg-Arg-His-OH: Analytical HPLC condition: linear gradient of solvent B in solvent A,

5 to 30% over 30 min, retention time=17.7 min. MS (ESI-TOF) m/z calcd for ($[M+2H]^{2+}$) 747.4, found 747.2.

H-His-Arg-Phe-Ala-D-Ala-Cys-Lys-Val-Leu-Arg-Arg-His-OH: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 30% over 30 min, retention time=18.2 min. MS (ESI-TOF) m/z calcd for ($[M+2H]^{2+}$) 747.4, found 747.2.

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12. Various attempts at evaluating the effect of peptide-detached resin for removal of the Fmoc group were conducted; however, the reason for the facile cleavage of the Fmoc group has yet to be disclosed. For example, treatment of **10a** in the presence of (*N*-sulfanylethyl)aminobenzoic acid linked resin with NaSH in 1 M phosphate buffer (pH 9.2)/MeCN (85:15) have no effect in Fmoc removal.
13. Ellman's reagent showed the most suitable nature (reactivity to thioacids and stability of the resulting thioester) in the examined aryl disulfides including phenyl disulfide and pyridyl disulfide.
14. Treatment of peptide thioacids with Ellman's reagent under acidic conditions (around pH 4) for more than 15 min resulted in the formation of peptide thioesters via more active acyl intermediates such as acyl disulfides.
15. Since the formed peptide arylthioester is susceptible to hydrolysis under neutral conditions, addition of thiophenol and the ligation partner followed by adjustment of pH is critical.
16. Conversion of peptide thioacid **13** by reaction with Ellman's reagent (3 mM Ellman's reagent in 3 mM $KHCO_3$ in H_2O/DMF) accompanied the formation of a non-negligible amount of disulfide peptide resulting from the reaction of cysteine sulfhydryl group with the aryl disulfide. Furthermore, the remaining excess Ellman's reagent should induce hindrance of the N-terminal Cys residue in the following NCL step under neutral conditions. To prevent these undesirable reactions, the addition of TCEP as a reducing agent for disulfides is crucial for achieving the sequential NCL in a one-pot manner.
17. For the *N*-*S* acyl transfer on peptidyl aminobenzoic acid linker, treatment with 4 M HCl/DMF most efficiently induced the acyl transfer thus far in the examination; however, application of this system to chiral amino acid attached resin accompanied the partial epimerization of the C-terminal amino acid. On the other hand, the reaction with TFA encountered no epimerization although the progress of the acyl transfer is slower than that observed in the reaction with 4 M HCl/DMF. See Ref. 10a.
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