Development of novel methodologies for the preparation of peptide/protein thioesters applicable to naturally occurring sequences

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Abbreviations

AA: amino acid
Ac: acetyl
ANP: atrial natriuretic peptide
CNP: C type natriuretic peptide
DIPCDI: *N,N'*-diisopropylcarbodiimide
DMAP: 4-dimethylaminopyridine
DMF: *N*,*N*-dimethylformamide
DMSO: dimethyl sulfoxide
DTDE: dithiodiethanol
EDTA: ethylenediaminetetraacetic acid
ESI: electrospray ionization
Et_2O: diethyl ether
Fmoc: 9-fluorenylmethoxycarbonyl
Gn: guanidine
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HexB: β-hexosaminidase B
HPLC: high performance liquid chromatography
HRP: horseradish peroxidase
^{i}PrOH: isopropanol
MeCN: acetonitrile
MeOH: methanol
MESNa: sodium 2-mercaptoethanesulfonate
MPAA: 4-mercaptophenylacetic acid
MS: mass spectrometry
NCL: native chemical ligation
TCEP: tris(2-carboxyethyl)phosphine
TFA: trifluoroacetic acid
TFE: trifluoroethanol
TFMSA: trifluoromethanesulfonic acid
TOF: time of flight
Tris: *tris*(hydroxymethyl)aminomethane
Trt: triphenylmethyl

SEAlide: *N*-sulfanylethylanilide

SPPS: solid-phase peptide synthesis

Abbreviation of amino acids

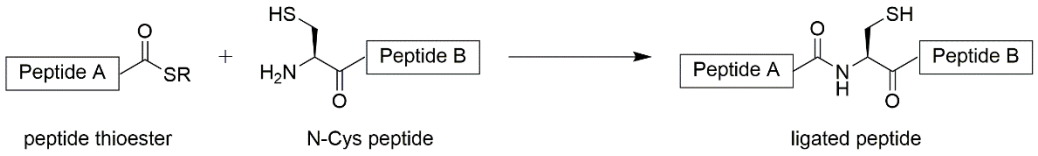
A (Ala) Alanine
C (Cys) Cysteine
D (Asp) Aspartic acid
E (Glu) Glutamic acid
F (Phe) Phenylalanine
G (Gly) Glycine
H (His) Histidine
I (Ile) Isoleucine
K (Lys) Lysine
L (Leu) Leucine
M (Met) Methionine
N (Asn) Aspargine
P (Pro) Proline
Q (Gln) Glutamine
R (Arg) Arginine
S (Ser) Serine
T (Thr) Threonine
V (Val) Valine
W (Trp) Tryptophan
Y (Tyr) Tyrosine

Contents

Preface

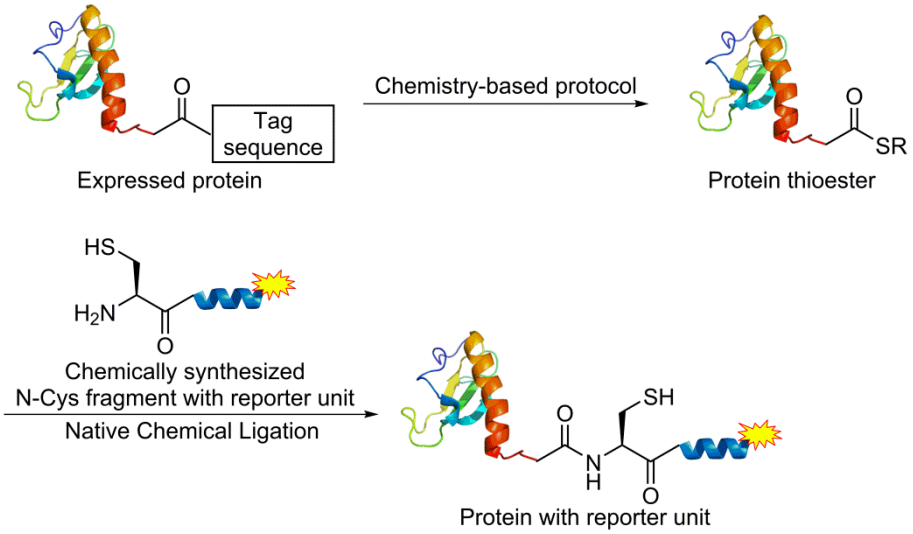
Proteins are indispensable factors in a wide variety of vital phenomena. For elucidation of protein function, chemically synthesized proteins that include artificial units such as fluorescent dye are often utilized. Chemical protein synthesis consists of two steps: 1) preparation of peptide thioester and N-terminal cysteinyl peptide by solid phase peptide synthesis (SPPS); 2) condensation of resulting fragments in solution by use of native chemical ligation (NCL) (Scheme 1). However, chemical synthesis of proteins consisting of over 100 residues has involved complicated procedures including multiple condensations followed by purifications on HPLC, resulting in low chemical yield.

Scheme 1. Native chemical ligation.



Protein semisynthesis is one way to overcome these problems. In the semisynthesis of proteins, NCL between protein thioester prepared from expressed protein and chemically synthesized N-terminal cysteinyl peptide gives chemically functionalized protein in one step. There are only a few protocols for preparation of protein thioesters from expressed proteins, such as intein- or sortase-mediated methods. However, these biochemical protocols do not always satisfy the increasing demand for thioester preparation. In this context, we began to develop a chemistry-based methodology for preparation of thioesters applicable to naturally occurring sequences utilizing a short peptide sequence that mimics the function of inteins (Scheme 2).

Scheme 2. Envisioned strategy for semisynthesis of proteins.



In Chapter 1, development of Ni(II)-triggered thioesterification reaction via DTDE esterification is described. A system utilizing sequential quadruple acyl transfer (SQAT) for preparation of peptide/protein thioesters applicable to naturally occurring sequences is presented in Chapter 2.

Chapter 1

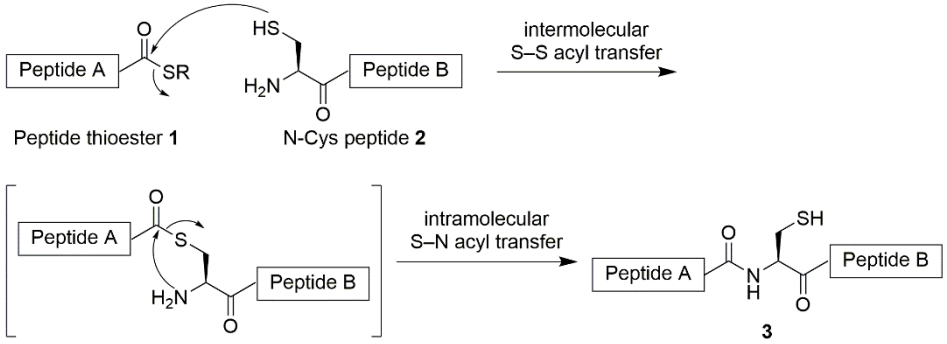
Development of Ni(II)-triggered thioesterification reaction via DTDE esterification

1.1 Introduction

Chemical protein synthesis

In general, chemical synthesis of proteins consists of preparation of peptide fragments by solid phase peptide synthesis (SPPS) and condensation of the resulting fragments in solution. Native chemical ligation (NCL) as a reaction for condensation of peptide fragments was reported by Kent and co-workers in 1994.[1] In the NCL technique, chemoselective intermolecular S–S acyl transfer between peptide thioester **1** and N-terminal cysteinyl peptide **2** followed by intramolecular S–N acyl transfer affords ligated peptide **3** (Scheme 1.1). These fragments are selectively ligated without protection of functional groups on the peptides. However, in the case of chemical synthesis of proteins consisting of over 100 residues, multiple condensations followed by purifications of the resulting products on HPLC are required, because of a limitation in the peptide chain length that can be prepared by SPPS.

Scheme 1.1. Native chemical ligation for chemical protein synthesis.

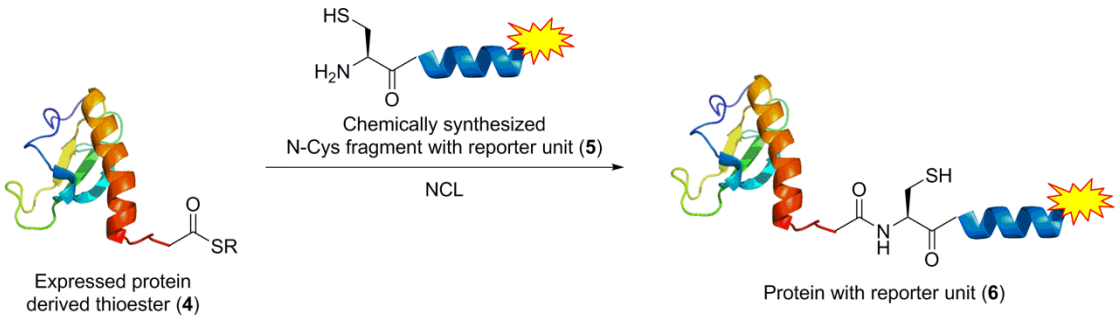


Protein semisynthesis

Protein semisynthesis can overcome the problem mentioned above. In general, protein semisynthesis is defined as synthesis of proteins using NCL between protein thioester **4** prepared from expressed protein and chemically synthesized N-terminal

cysteinyl peptide **5** (Scheme 1.2). This methodology allows for facile production of chemically functionalized long chain proteins in a short step and in high yield.

Scheme 1.2. Protein semisynthesis.



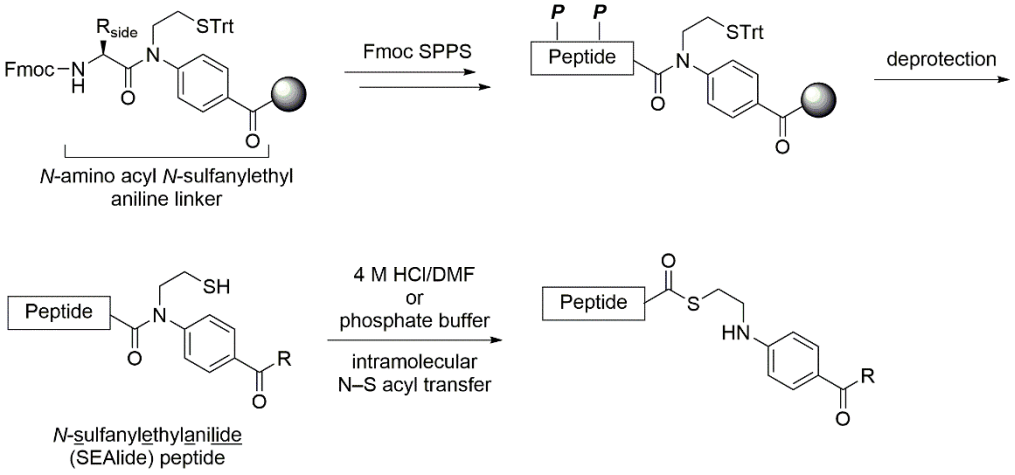
Recent development of methodologies for preparation of thioesters

Several synthetic methods of peptide thioesters have been developed because thioesters are indispensable synthetic intermediates for NCL.[2] However, the methodologies applicable to expressed proteins are limited to only a few biochemical protocols.

・Chemical protocols

Chemical protocols for producing thioesters have been in continual development based on wide variety of strategies. In this context, a methodology for preparation of peptide thioesters using *N*-sulfanylethylanilide (SEAlide) peptides as precursor of thioester was reported by our group (Scheme 1.3).[3] SEAlide peptides have *N*-amino acyl *N*-sulfanylethylaniline linker which is introduced in SPPS. SEAlide peptides are converted to the corresponding thioesters via intramolecular N–S acyl transfer under acidic conditions or in the presence of phosphate salts under neutral conditions. Several chemical protocols utilizing N–S acyl transfer for preparation of peptide thioesters have been reported. However, these protocols cannot be applied to thioesterification of expressed proteins because of necessity to introduce chemical units such as *N*-amino acyl *N*-sulfanylethylaniline linker in SPPS.

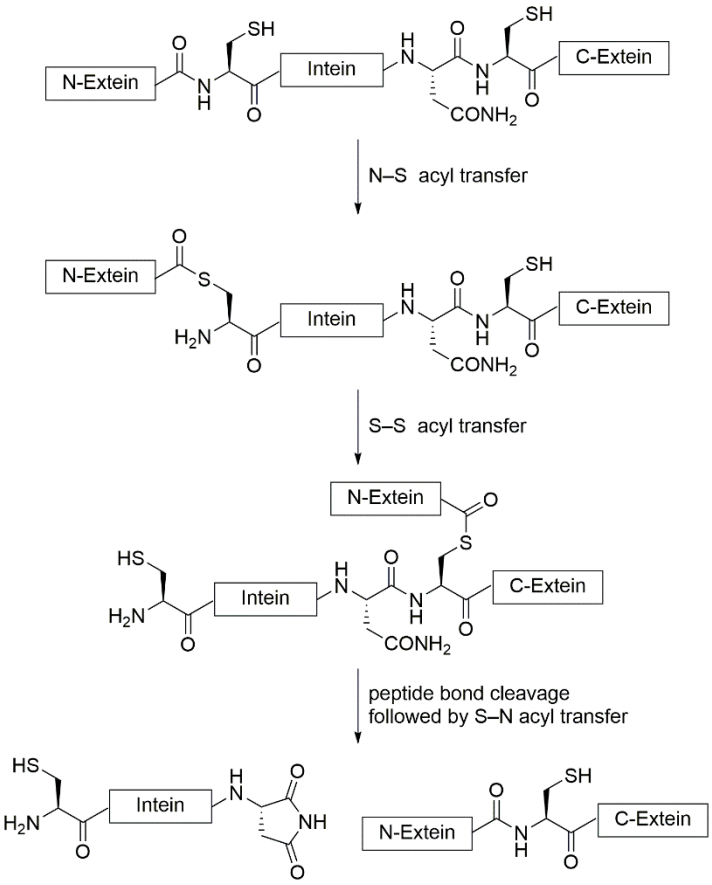
Scheme 1.3. Synthesis of peptide thioester using SEAlide peptide.



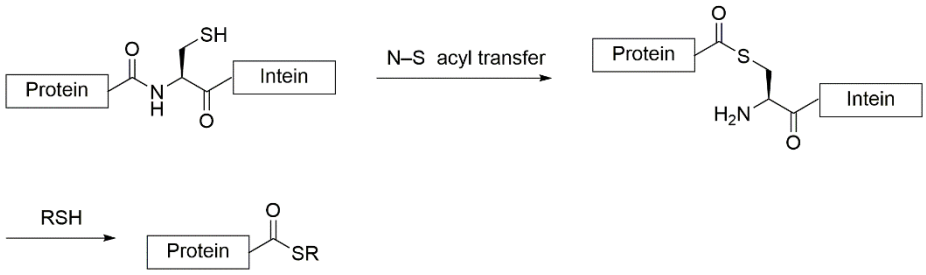
・Biochemical protocols

In 1998, a protocol for preparation of protein thioesters from expressed proteins using inteins, which are associated with protein splicing was reported by Muir and co-workers.[4] Protein splicing is one of the post-translational modifications. In protein splicing, the intervening protein domain (intein) is removed from protein precursors, while flanking regions (exteins) are ligated. Reaction mechanism of intein-mediated protein splicing consists of three crucial steps: 1) autocatalytic N–S acyl transfer of proteins, including intein sequence; 2) intramolecular S–S acyl transfer; 3) cleavage of main chain followed by intramolecular S–N acyl transfer. Consequently, extein sequences are ligated with release of intein sequence (Scheme 1.4).[5] Muir and co-workers developed the protocol for preparation of protein thioesters using intein-fused proteins (Scheme 1.5).

Scheme 1.4. Reaction mechanism of protein splicing.

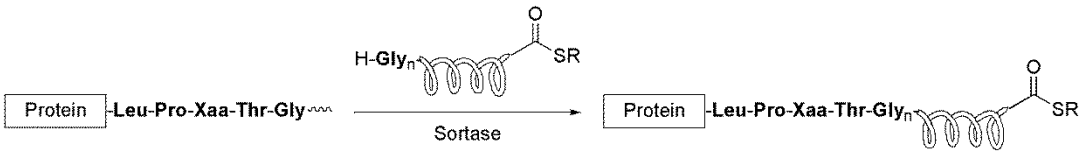


Scheme 1.5. Intein-mediated protocol for preparation of protein thioester.



In 2012, on the other hand, Pentelute and co-workers developed a sortase-mediated thioeserification reaction applicable to expressed proteins.[6] Sortase enables ligation between cell wall peptidoglycans and Leu-Pro-Xaa-Thr-Gly (Xaa: any amino acid) sequence-containing cell surface proteins. Thioesterification using sortase affords protein thioesters through the amide cleavage of Thr-Gly bond in Leu-Pro-Xaa-Thr-Gly sequence along with amide formation of olygoglycine thioester peptides (Scheme 1.6).

Scheme 1.6. Sortase-mediated protocol for preparation of protein thioester.



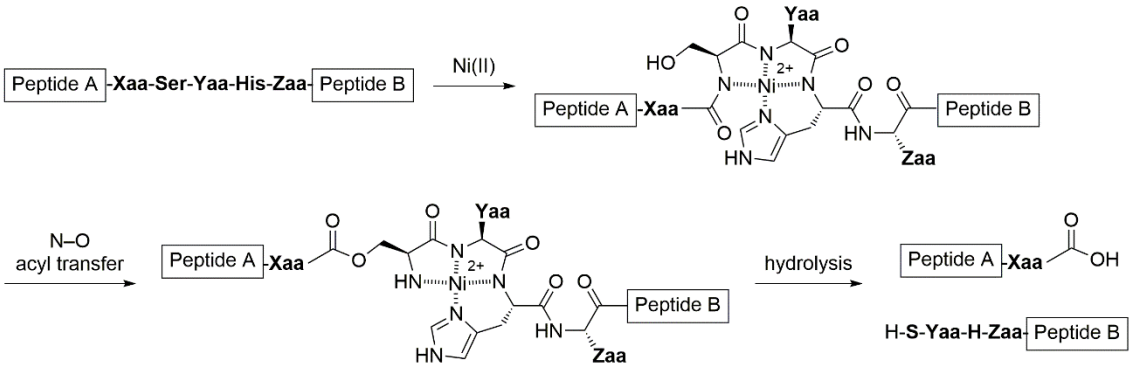
As mentioned here, methodology applicable to thioester preparation from expressed proteins is, at present, limited to the use of an intein or sortase classified as biochemical protocol. However, these biochemical protocols do not always satisfy the increasing demand for thioester preparation. From this point of view, we attempted to develop a novel chemical protocol for thioester production applicable to expressed proteins.

1.2 Strategy for preparation of thioesters from naturally occurring peptides

Although only a few biochemical protocols for preparation of thioesters from expressed proteins have been reported, these protocols do not always give satisfactory results. In this context, development of chemical protocols for preparation of thioesters applicable to naturally occurring sequences has been required. To develop a novel chemical methodology for sequence-specific thioesterification of proteins, we attempted to use a short peptide sequence that mimics the function of inteins which consist of more than 100 amino acids.

Initially, we focused on Ni(II)-triggered sequence-specific hydrolysis of peptide bond as reported by Bal and co-workers (Scheme 1.7).[7] In this reaction, coordination of Xaa-Ser-Yaa-His-Zaa (Xaa, Yaa, and Zaa are any amino acids) sequence in the peptide to Ni(II) activates amide bond between Xaa and Ser. Intramolecular N–O acyl transfer at the peptide bond then results in the formation of *O*-acyl isopeptide. Finally, the resulting *O*-acyl isopeptide is hydrolyzed. The fact that such intramolecular N–O acyl transfer as seen in the cleavage of Xaa-Ser amide is also found in the first step of intein-mediated protein splicing encouraged us to evaluate the applicability of the Ni(II)-assisted peptide hydrolysis to conversion of the Xaa-Ser-Yaa-His-Zaa-containing peptide sequence to the corresponding Xaa-thioester.

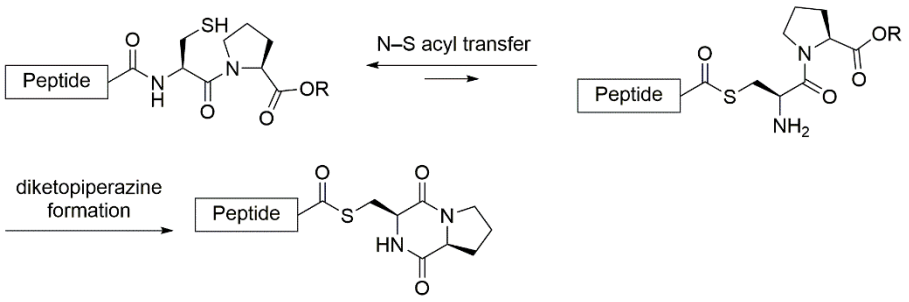
Scheme 1.7. Sequence-specific Ni(II) dependent peptide bond hydrolysis.



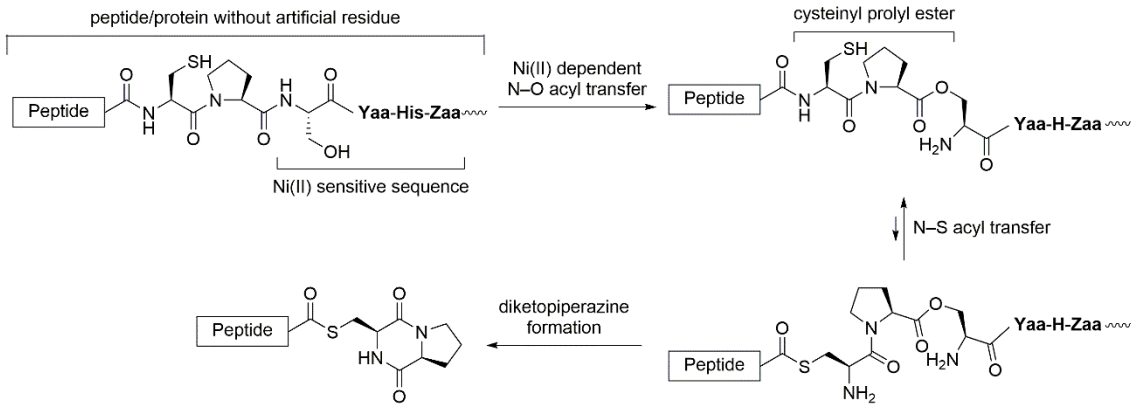
1.3 Thioesterification reaction via DTDE esterification

Kawakami and Aimoto reported that cysteinyl prolyl ester (CPE) peptides function as precursors of peptide thioesters (Scheme 1.8).[8] Under neutral conditions, CPE peptides are converted to thioesters via N–S acyl transfer followed by diketopiperazine formation. We speculated that combination of CPE chemistry and Ni(II)-assisted peptide bond hydrolysis might enable transformation of naturally occurring peptides/proteins into thioesters. Treatment of Cys-Pro-Ser-Yaa-His-Zaa sequence-containing peptide with Ni(II) would afford CPE peptide through N–O acyl transfer at Pro-Ser. Next, the resulting CPE peptide would be converted to thioester according to CPE chemistry (Scheme 1.9).

Scheme 1.8. Cysteinyl prolyl ester peptide.



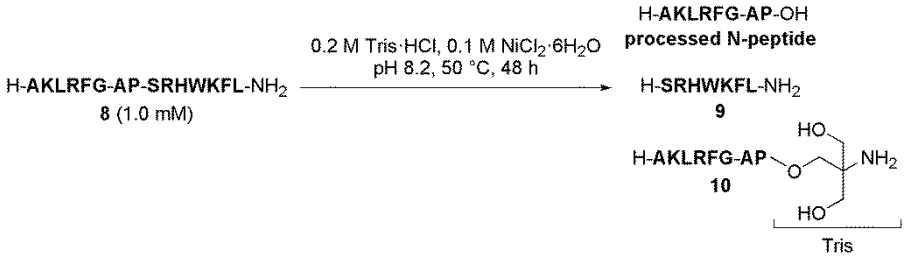
Scheme 1.9. Our strategy for thioesterification.



Initially, we used a model peptide **7** (H-**AKLRFG-CP-SRHWKFL**-NH_2) for evaluation of thioesterification reaction as mentioned above. **SRHW** sequence was chosen as Ni(II)-sensitive sequence for coordination to Ni(II), because this sequence showed the best result in the evaluation of efficacy of Ni(II)-mediated hydrolysis in the report by Bal and group.[7] Contrary to our expectation, the desired peptide thioester

(H-**AKLRFG**-SR) was not obtained. We assumed the cause of the failure was the presence of cysteine near the Ni(II)-sensitive sequence. Next, to examine the influence of cysteine on the reaction, we evaluated Ni(II)-mediated hydrolysis reaction using peptide **8** (H-**AKLRFG-AP-SRHWKFL**-NH_2), with substitution of alanine for cysteine (Scheme 1.10). The Ni(II)-mediated hydrolysis of **8** was conducted in the presence of 0.1 M $NiCl_2 \cdot 6H_2O$ in 0.2 M tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl) buffer at pH 8.2. After reaction at 50 °C for 48 h, Tris-adducted peptide **10** (H-**AKLRFGAP**-Tris) was slightly obtained with hydrolyzed peptide of **8** (Figure 1.1). We speculated that Tris-adducted peptide **10** was produced by nucleophilic attack of Tris to ester moiety of the *O*-acyl isopeptide formed via Ni(II)-mediated N–O acyl transfer. Therefore, we envisioned that *O*-acyl isopeptide could be converted to ester by the action of alcohol as nucleophile and the resulting ester could be transformed into thioester.

Scheme 1.10. Ni(II)-mediated hydrolysis resulted in formation of Tris-adducted peptide.



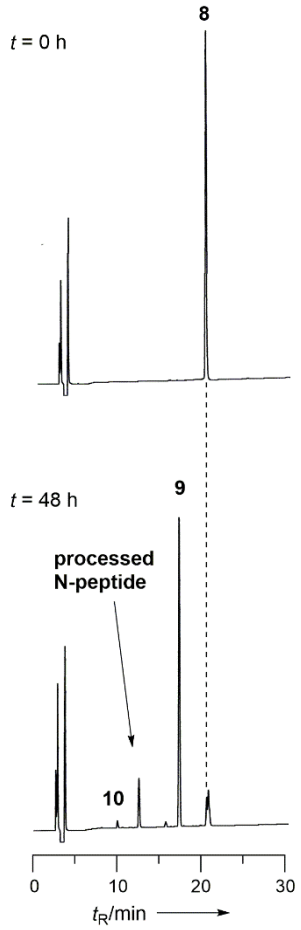
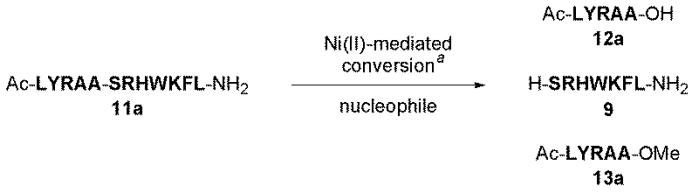


Figure 1.1. HPLC monitoring of Ni(II)-mediated hydrolysis reaction of peptide **8**. Analytical HPLC conditions: Cosmosil 5C_{18} AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (15:85–35:65 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

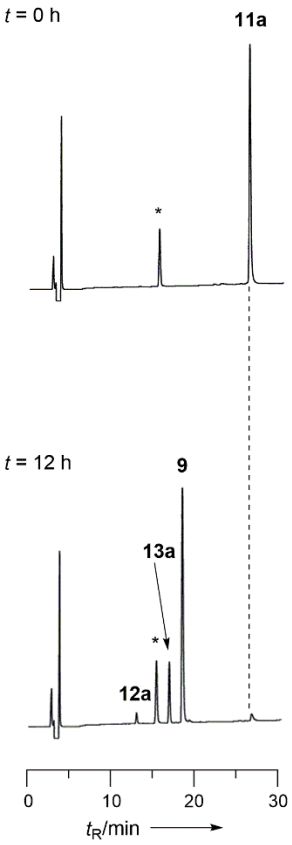
Next, we evaluated the Ni(II)-mediated alcoholysis of model peptide **11a** with Ala-Ser cleavage junction, which was supposed to be more susceptible to the action of Ni(II). The alcoholysis reaction of the peptide **11a** was conducted in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing Ni(II) and a variety of alcohols (Table 1.1). When we used trifluoroethanol (TFE) or isopropanol (^{i}PrOH) as alcohol, the corresponding peptide esters could not be formed (entries 1 and 2). On the other hand, the use of methanol in this reaction successfully yielded peptide methyl ester **13a** (entry 3) (Figure 1.2).

Table 1.1. Examination of Ni(II)-mediated alcoholysis.



Entry	Nucleophile	Fraction converted[b]
1	50% (v/v) TFE	-[c]
2	50% (v/v) ^{i}PrOH	-[c]
3	50% (v/v) MeOH	0.72

[a]Reactions were performed in 0.2 M HEPES buffer at 37 °C for 12 h in the presence of 1 mM of **11a**. [b]The fraction converted was determined by HPLC separation and integration of **13a** (integ. **13a**) as a fraction of the sum of the unreacted **11a** (integ. **11a**) + hydrolyzed **12a** (integ. **12a**) + integ. **13a**. [c]Oxyesters were not obtained.



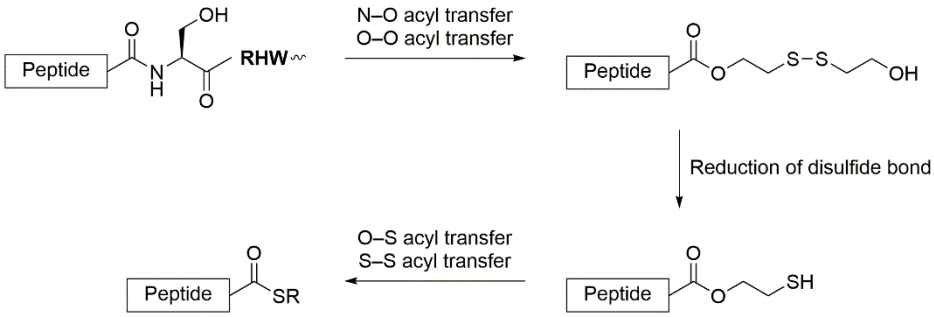
*Internal standard (*p*-toluenesulfonamide)

Figure 1.2. HPLC monitoring of methylesterification of peptide **11a**. Analytical HPLC conditions: Cosmosil 5C_{18} AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (15:85–35:65 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

As mentioned above, treatment of the model peptide containing Ni(II)-sensitive sequence with methanol gave the corresponding methylester. Next, we evaluated the conversion of oxyester formed via Ni(II)-triggered esterification into thioester. To date, several methodologies for preparation of peptide thioesters from peptide oxyesters have been reported.[9]

We envisioned thioesterfication reaction via mercaptoethyl ester (Scheme 1.11). Firstly, treatment of Ni(II)-sensitive sequence-containing peptide with Ni(II) and dithiodiethanol (DTDE) should yield DTDE ester peptide **14**. Secondly, reduction of disulfide bond of DTDE ester should give mercaptoethyl ester. Thirdly, resulting mercaptoethyl ester should be converted to thioester via intramolecular O–S acyl transfer and intermolecular S–S acyl transfer in the presence of thiol additive.

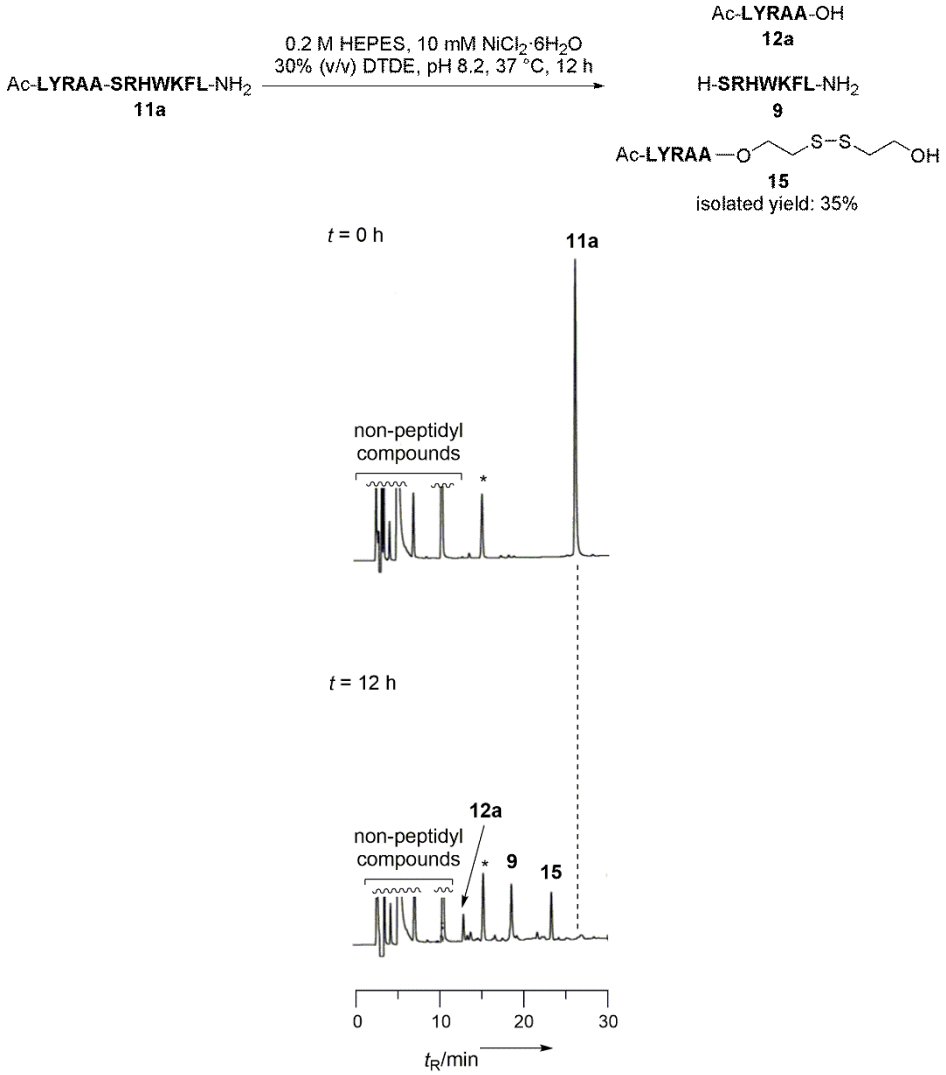
Scheme 1.11. Strategy for DTDE esterification-mediated thioesterification.



Treatment of the model peptide **11a** with Ni(II) and DTDE successfully yielded DTDE ester peptide **15** in 35% isolated yield (Scheme 1.12, Figure 1.3). To convert the resulting DTDE ester peptide **15** to the corresponding thioester, reduction of the peptide **15** under neutral conditions was attempted; however, only hydrolyzed peptide of **15** was obtained. This was caused by formation of ethylene sulfide via intramolecular cyclization of mercaptoethyl ester peptide, which resulted from reduction of DTDE ester peptide.[10] According to a report by Tam and group, on the other hand, mercaptoethyl ester **16** was successfully converted to 4-methylphenyl thioester **17** by treatment of *p*-thiocresol as thiol under strongly acidic conditions via tandem thiol switch (Scheme 1.13).[11] We focused on this reaction and attempted to convert DTDE ester **15** to 4-methylphenyl thioester **18** through reduction of disulfide followed by tandem thiol switch. 4-Methylphenyl thioester **18** was successfully obtained by treatment of DTDE ester **15** with 0.1% (v/v) trifluoromethanesulfonic acid (TFMSA) in TFA in the presence of 5% (v/v) *p*-thiocresol (Scheme 1.14, Figure 1.4). Resulting 4-methylphenyl thioester **18** was eluted as a single peak on HPLC analysis. However, we were concerned that strongly acidic conditions would induce epimerization of

C-terminal amino acid in O–S acyl transfer step (Scheme 1.15). Initially, nucleophilic attack of sulfhydryl group of mercaptoethyl ester peptide to carbonyl carbon would result in the formation of 5-membered ring intermediate. Elimination of water molecule followed by deprotonation of α position proton would then induce epimerization of C-terminal amino acid. To confirm whether 4-methylphenyl thioester was epimerized or not, we attempted to evaluate this reaction using an alternative peptide.

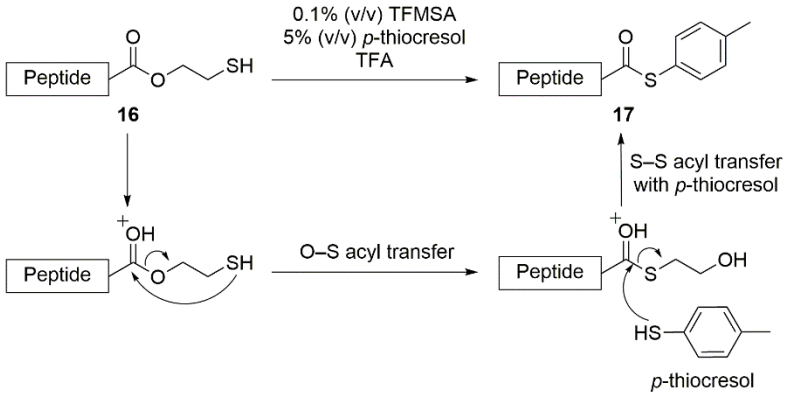
Scheme 1.12. Ni(II)-mediated DTDE esterification.



*Internal standard (*p*-toluenesulfonamide)

Figure 1.3. HPLC monitoring of DTDE esterification reaction of peptide **12a**. Analytical HPLC conditions: Cosmosil 5C_{18} AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (15:85–35:65 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

Scheme 1.13. Tandem thiol switch.



Scheme 1.14. Conversion of DTDE ester to thioester.

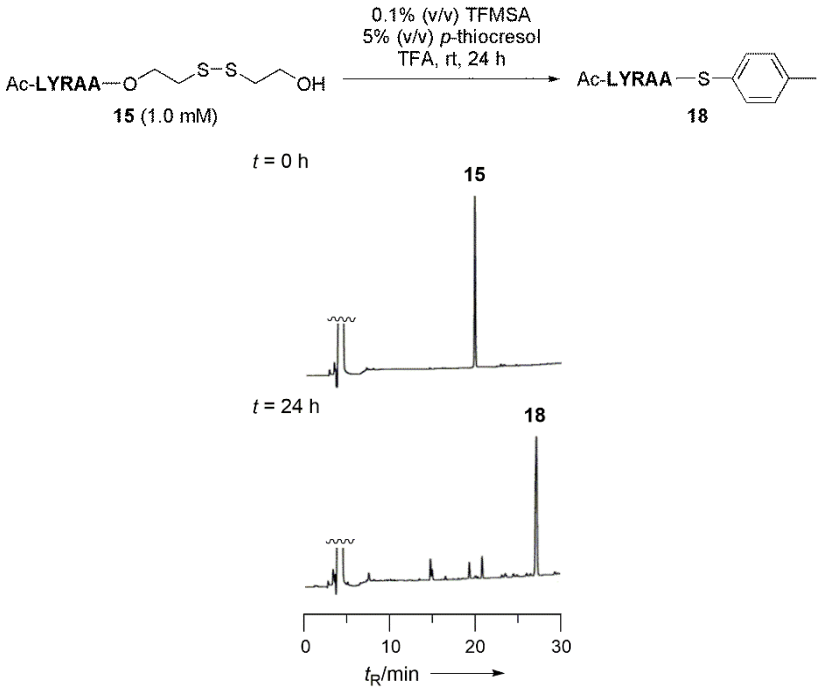
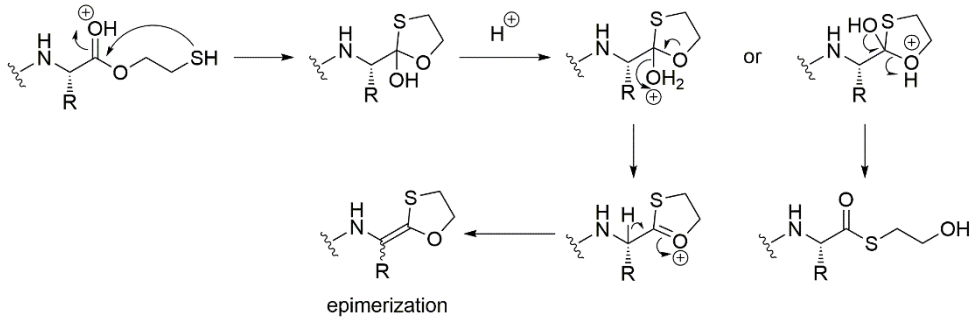


Figure 1.4. HPLC monitoring of conversion of DTDE ester to thioester. Analytical HPLC conditions: Cosmosil $5C_{18}$ AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (10:90–50:50 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

Scheme 1.15. Putative mechanism for epimerization of C-terminal amino acid.



Initially, to check the epimerization in DTDE esterification step, a model peptide **19** was subjected to the Ni(II)-mediated esterification reaction in HEPES buffer-DTDE (1:1) and the reaction was analyzed on HPLC with co-injection of standard DTDE ester peptides **20** (**L-Ala**) or **21** (**D-Ala**). HPLC analysis clearly indicated that no epimerization occurred in the initial DTDE esterification step (Scheme 1.16, Figure 1.5).

Scheme 1.16. Evaluation of epimerization at DTDE esterification step.

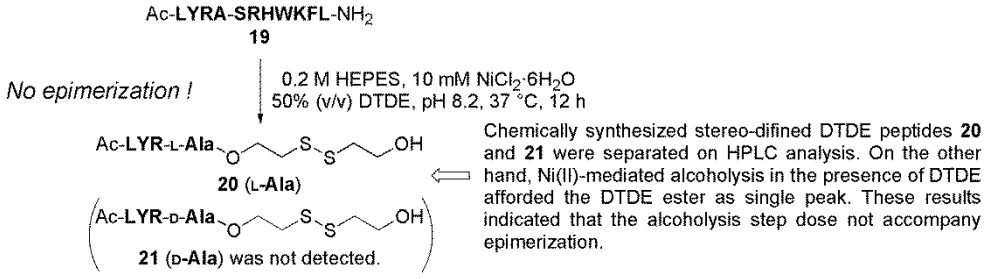
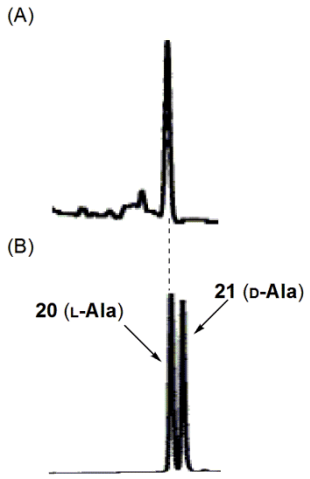


Figure 1.5. A) HPLC chart after 12 h of DTDE esterification of **19**. B) HPLC chart of synthesized **L-Ala** (**20**) and **D-Ala** (**21**) containing DTDE ester peptide. Analytical HPLC conditions: Cosmosil 5C_{18} AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (1:99–50:50 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.



Next, we checked the epimerization in O–S acyl transfer step of the resulting DTDE ester. Diastereomerically pure DTDE ester peptide was treated with 0.1% (v/v) TFMSA in TFA in the presence of 5% (v/v) *p*-thiocresol for the conversion of the DTDE oxyester to the corresponding thioester via tandem thiol switch. HPLC analysis of the reaction showed the appearance of two peaks with identical molecular mass (Scheme 1.17, Figure 1.6). To identify the configuration of C-terminal amino acid of the resulting thioesters, NCLs of each isolated thioester with N-terminal cysteinyl peptide **26** were attempted. The reactions were analyzed by HPLC with co-injection of stereo-defined peptides **24** (**L-Ala**) and **25** (**D-Ala**) as authentic samples. As a result, the peaks were identified as peptide **22** (**L-Ala**) and **23** (**D-Ala**) (Figure 1.7). This result indicated that epimerization of C-terminal amino acid proceeded in O–S acyl transfer step under strongly acidic conditions.

Scheme 1.17. Evaluation of epimerization at thioesterification step.

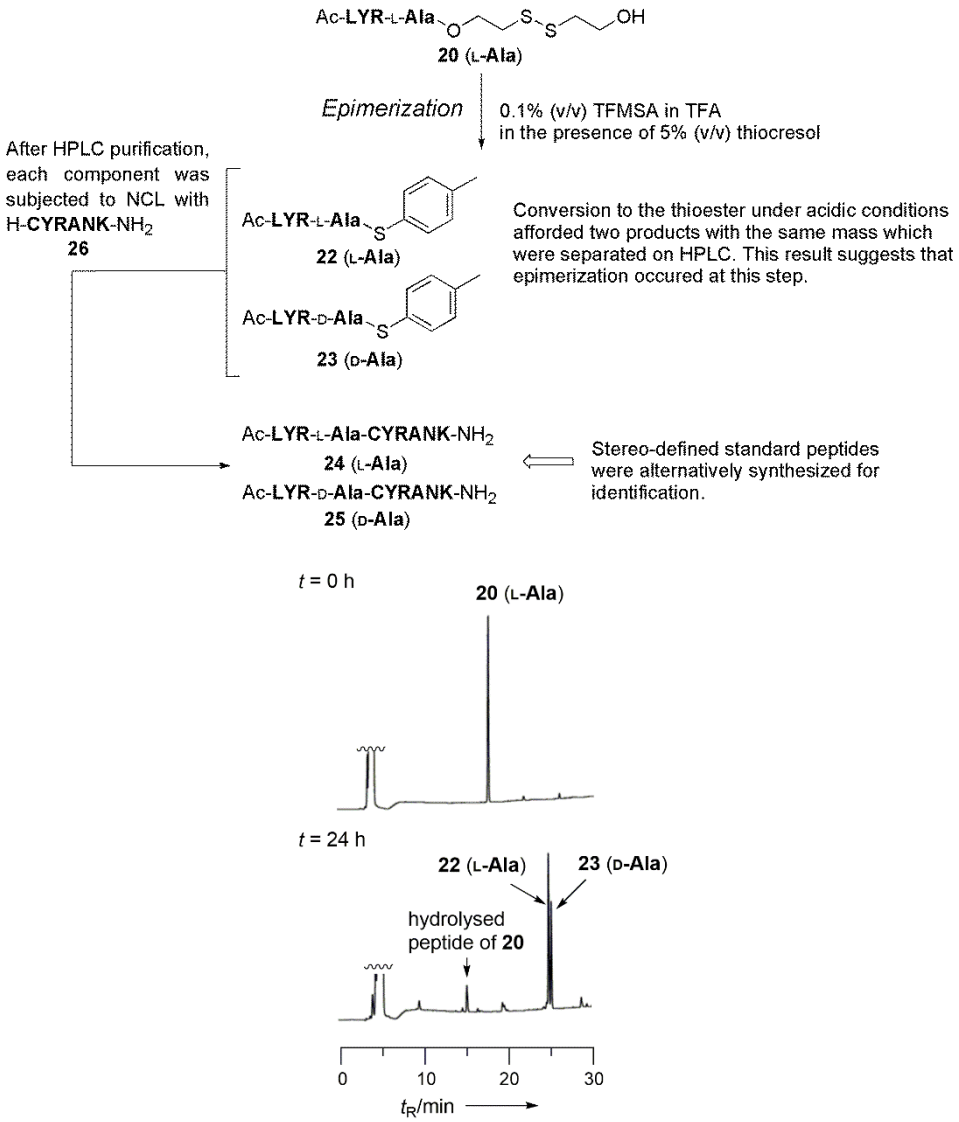


Figure 1.6. HPLC monitoring of thioesterification of **20** (**L-Ala**). Analytical HPLC conditions: Cosmosil 5C_{18} AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (20:80–60:40 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

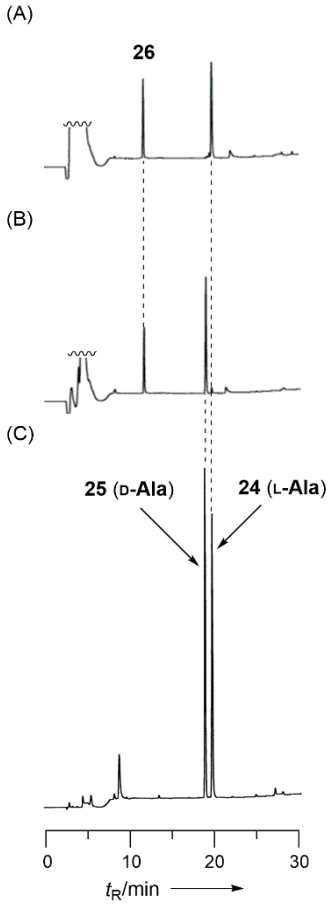


Figure 1.7. A) HPLC chart after 1 h of NCL reaction of **22** (**L-Ala**) with **26**. B) HPLC chart after 1 h of NCL reaction of **23** (**D-Ala**) with **26**. C) Analytical HPLC chart of mixture of synthesized lagated product **24** (**L-Ala**) and **25** (**D-Ala**). Analytical HPLC conditions: Cosmosil $5C_{18}$ AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (20:80–60:40 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

1.4 Conclusion

A novel reaction enabling thioesterification of naturally occurring peptides was developed. This reaction consists of two crucial steps: 1) Ni(II)-mediated intramolecular N–O acyl transfer followed by intermolecular O–O acyl transfer with DTDE; 2) reduction of disulfide bond of the resulting DTDE ester followed by intramolecular O–S acyl transfer based on tandem thiol switch mechanism. However, epimerization of C-terminal amino acid occurred because of strongly acidic conditions in O–S acyl transfer step. Therefore, we next attempted to develop an alternative methodology for thioesterification, without epimerization.

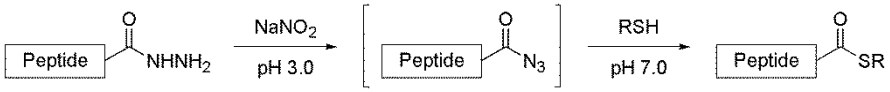
Chapter 2

Development of SQAT system applicable to naturally occurring sequences

2.1 Introduction

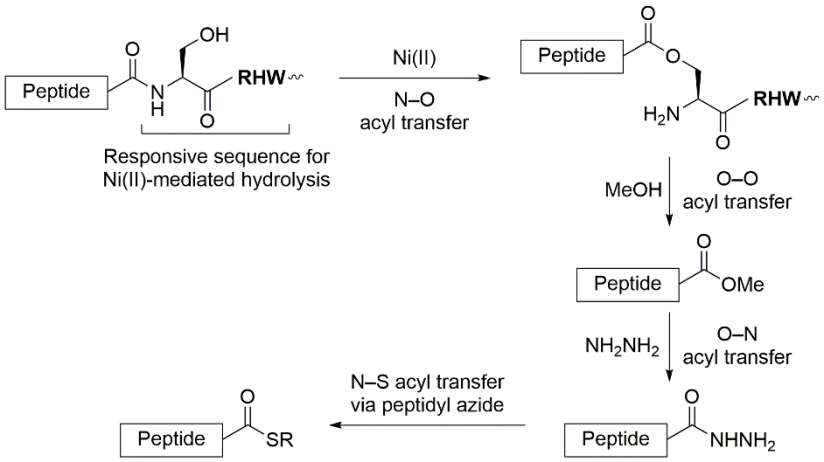
As mentioned in Chapter 1, we developed a reaction for preparation of thioesters via DTDE oxyesters although epimerization of C-terminal amino acid occurred because of strongly acidic conditions for the conversion of mercaptoethyl ester to thioester through O–S acyl transfer. To overcome this drawback, we began to develop an alternative methodology to the O–S acyl transfer step. We focused on peptide hydrazides as precursor of peptide thioesters. In 2011, Liu and co-workers developed a reaction for conversion of peptide hydrazides into thioesters (Scheme 2.1, hereinafter referred to as "Liu's protocol").[12] In this reaction, peptide hydrazides are converted to peptide azides with the action of sodium nitrite under acidic conditions. The resulting peptidyl azides are transformed to peptide thioesters by addition of thiol into the reaction mixture under neutral conditions without epimerization.

Scheme 2.1. Conversion of peptide hydrazide to peptide thioester via peptidyl azide.



We envisioned that combination of Ni(II)-mediated esterification and Liu's protocol would result in conversion of naturally occurring peptides/proteins to thioesters without epimerization (Scheme 2.2).[12] Firstly, Ni(II)-triggered N–O acyl transfer at Ser in Ser-Arg-His-Trp sequence-containing peptide should afford *O*-acyl isopeptide. Secondly, the *O*-acyl isopeptide should be transformed into the corresponding methyl ester by O–O acyl transfer with methanol. Thirdly, addition of hydrazine into the reaction mixture should afford peptide hydrazide via O–N acyl transfer. In the last conversion, peptide hydrazide should be converted to thioester via peptidyl azide by N–S acyl transfer based on Liu's protocol.

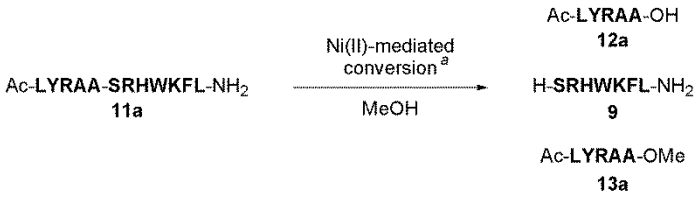
Scheme 2.2. Envisioned strategy for thioesterification of naturally occurring sequences.



2.2 Optimization of reaction conditions for Ni(II)-mediated methanolysis

As mentioned in the previous chapter, the Ni(II)-mediated methanolysis proceeded successfully. To optimize the reaction conditions, the Ni(II)-mediated methanolysis of the model peptide **11a** was attempted under various conditions (Table 2.1). Conversion efficiency of this reaction increased with the increase of concentration of MeOH (entries 1–3). On the other hand, concentration of Ni(II) within the range of 1 to 20 mM did not affect the conversion efficiency (entries 2, 4 and 5). At pH 8.2, the Ni(II)-assisted methanolysis proceeded with the highest conversion efficiency (entries 2, 6 and 7). In consideration of application of this reaction to expressed proteins, Ni(II)-mediated methanolysis in the presence of guanidine hydrochloride (Gn·HCl) as denaturating reagent was attempted. The reaction endured the presence of Gn·HCl, although the reaction efficiency decreased (entry 8). These results indicated that the conditions of entry 3 (10 mM Ni(II), pH 8.2, 50% (v/v) MeOH) are optimum for Ni(II)-mediated methanolysis.

Table 2.1. Optimization of Ni(II)-mediated methylesterification.



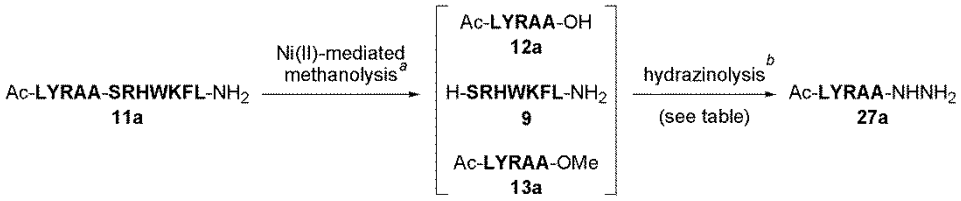
Entry	$NiCl_2 \cdot 6H_2O$ (mM)	pH	% (v/v) of MeOH	Fraction converted[b]
1	10	8.2	10	0.44
2	10	8.2	30	0.70
3	10	8.2	50	0.72
4	1	8.2	30	0.69
5	20	8.2	30	0.68
6	10	7.8	30	0.61
7	10	8.6	30	0.53
8[c]	10	8.2	30	0.43

[a]Reactions were performed in 0.2 M HEPES buffer at 37 °C for 12 h in the presence of 1 mM of **11a**. [b]The fraction converted was determined by HPLC separation and integration of **13a** (integ. **13a**) as a fraction of the sum of the unreacted **11a** (integ. **11a**) + hydrolyzed **12a** (integ. **12a**) + integ. **13a**. [c]In the presence of 6 M guanidine hydrochloride (Gn·HCl).

2.3 Evaluation of hydrazinolysis through methanolysis

We next investigated the reaction conditions of hydrazinolysis via methanolysis. Treatment of a model peptide **11a** under the optimum condition of methanolysis (0.2 M HEPES, 10 mM $NiCl_2 \cdot 6H_2O$, 50% (v/v) MeOH, pH 8.2, 37 ºC, 12 h) as described in the previous section, followed by addition of hydrazine into the reaction mixture under various conditions, were conducted (Table 2.2). The use of 5% (v/v) hydrazine at 25 ºC afforded peptide hydrazide **27a** with the best conversion efficiency (entry 3, Figure 2.1).

Table 2.2. Hydrazinolysis via Ni(II)-mediated methanolysis.



Entry	% (v/v) of NH_2NH_2	Reaction temperature	Fraction converted[c]
1	5	37 ℃	0.56
2	2	37 ℃	0.27
3	5	25 ℃	0.70

[a]Reactions were performed in 0.2 M HEPES buffer (10 mM $NiCl_2 \cdot 6H_2O$, 50% (v/v) MeOH, pH 8.2) at 37 °C for 12 h in the presence of 1 mM of **11a**.[b]Reaction mixture was incubated for 3 h. [c]The fraction converted was determined by HPLC separation and integration of **27a** (integ. **27a**) as a fraction of the sum of the unreacted **11a** (integ. **11a**) + hydrolyzed **12a** (integ. **12a**) + integ. **27a**.

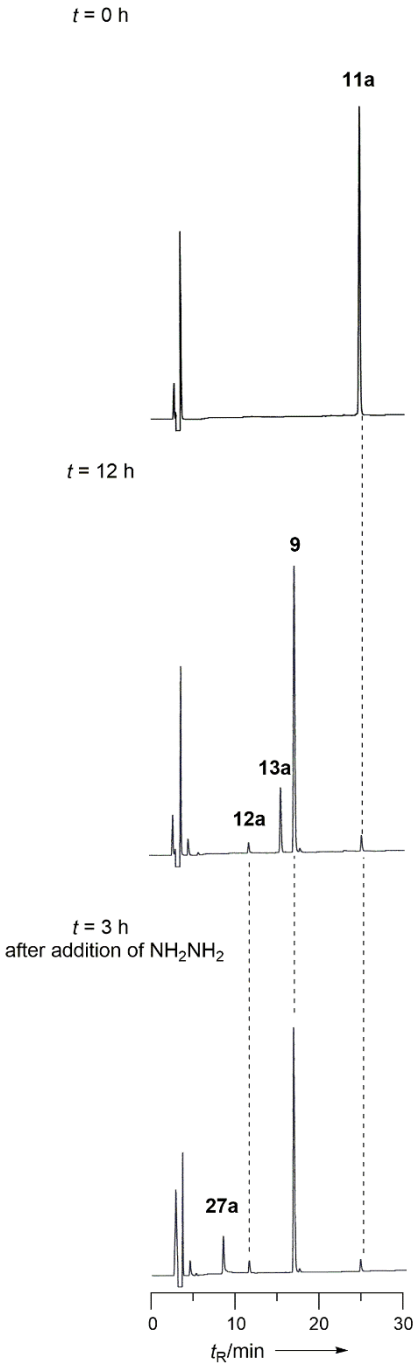


Figure 2.1. HPLC monitoring of hydrazinolysis via Ni(II)-mediated methanolysis of peptide **11a** (entry 3). Analytical HPLC conditions: Cosmosil 5C_{18} AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (15:85–35:65 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

Next, conversion of the resulting peptide hydrazide to peptide thioester was attempted. Under the optimum reaction conditions of methanolysis and hydrazinolysis as described above, peptide hydrazide **27a** was obtained in 80% isolated yield. The

resulting peptide hydrazide **27a** was smoothly transformed into peptide thioester **29a** (**L-Ala**) via peptidyl azide **28a** following Liu's protocol (Scheme 2.3, Figure 2.2). Furthermore, epimerization of C-terminal amino acid was not observed on HPLC analysis with co-injection of peptide thioester **29a** (**L-Ala**) and alternatively synthesized **29u** (**D-Ala**) (Figure 2.3). We named this sequential system for preparation of thioesters SQAT, because the thioesters were prepared by sequential quadruple acyl transfer (N–O, O–O, O–N, and N–S acyl transfers), as shown in Scheme 2.2.

Scheme 2.3. Thioesterification of peptide hydrazide via peptidyl azide.

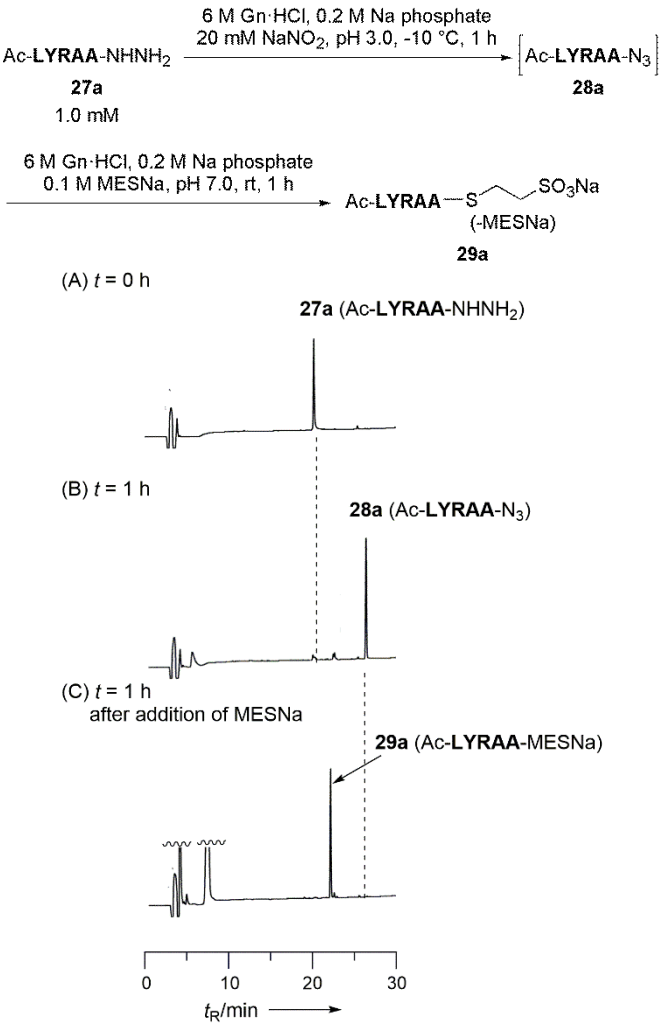


Figure 2.2. HPLC monitoring of thioesterification of peptide hydrazide **27a**. A) HPLC chart after 0 h of azidation of peptide **27a** to peptide azide **28a**. B) HPLC chart after 1 h of azidation of peptide **27a** to peptide azide **28a**. C) HPLC chart after 1 h of thioesterification of peptide **28a** to peptide thioester **29a**. Analytical HPLC conditions: Cosmosil $5C_{18}$ AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (1:99–35:65 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

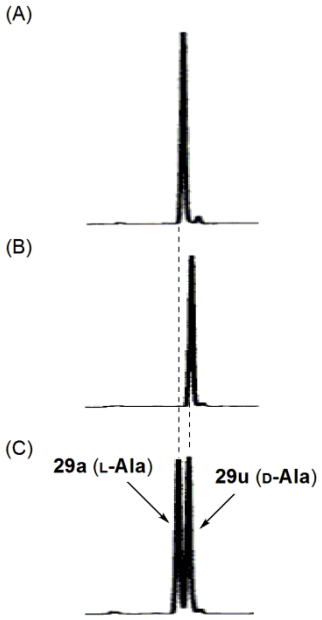
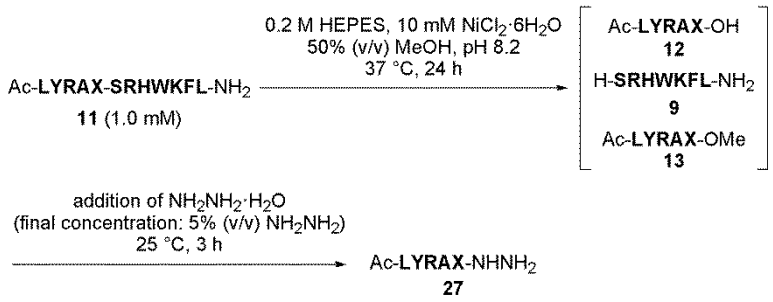


Figure 2.3. A) Analytical HPLC chart of crude **29a** (**L-Ala**). B) Analytical HPLC chart of crude **29u** (**D-Ala**). C) Analytical HPLC chart of mixture of crude **29a** (**L-Ala**) and **29u** (**D-Ala**). Analytical HPLC conditions: Cosmosil 5C_{18} AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (10:90–50:50 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

We speculated that the amino acids adjacent to the Ni(II)-sensitive sequence (**SRHW** sequence) might affect the reaction efficiency in SQAT system. To examine the substrate versatility of SQAT system, Ni(II)-mediated methanolysis followed by hydrazinolysis was attempted using model peptides including **X-SRHW** (X: 20 naturally occurring amino acids) sequence (Table 2.3). In the cases of **11e** (**X** = Asn) and **11f** (**X** = Gln), the desired methyl esters were not obtained, because of the formation of peptidyl imide in Ni(II)-mediated methanolysis step. Although Ni(II)-mediated methanolysis of **11i** (X = Cys) was attempted, N-processed peptides **12i** and **13i** were not observed on HPLC analysis. We speculated that coordination of side chain of Cys to Ni(II) would induce several side reactions, although detailed examinations of the side reaction were not carried out. N–O Acyl transfer and methanolysis of **11n** (Ile) did not proceed. We assumed that steric hindrance at β position would impede the reaction progress of methanolysis, because similar phenomenon was observed in methanolysis of **11k** (Val).

Table 2.3. Substrate versatility of SQAT system.



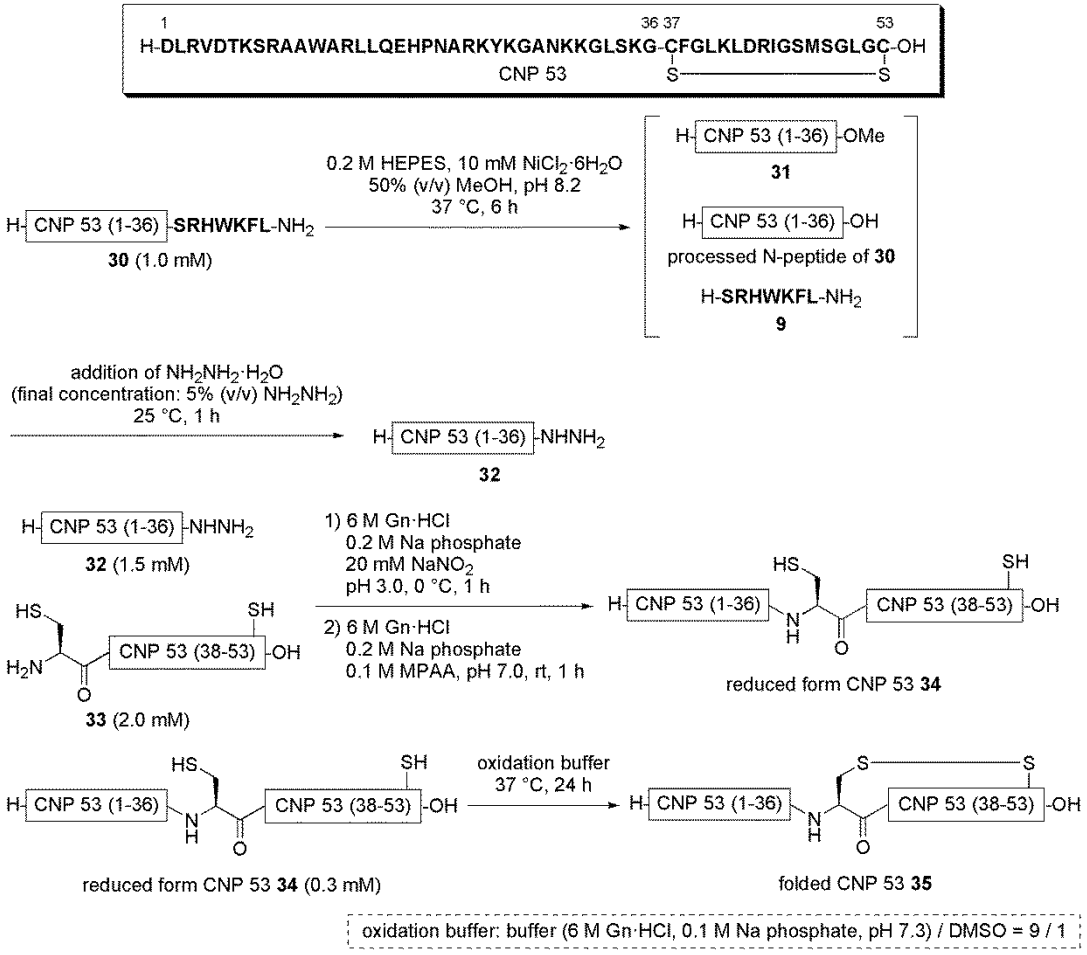
X	Fraction converted[a]	X	Fraction converted[a]
Ala (**11a**)	0.61	Val (**11k**)	0.12
Gly (**11b**)	0.66	Met (**11l**)	0.58
Asp (**11c**)	0.40	Leu[e] (**11m**)	0.66
Glu (**11d**)	0.69	Ile (**11n**)	-[f]
Asn (**11e**)	-[c]	Tyr (**11o**)	0.40
Gln (**11f**)	-[c]	Phe (**11p**)	0.41
Ser (**11g**)	0.42	His (**11q**)	0.57
Thr (**11h**)	0.48	Lys (**11r**)	0.65
Cys (**11i**)	-[d]	Arg (**11s**)	0.59
Pro (**11j**)	0.09	Trp (**11t**)	0.62

[a]The fraction converted was determined by HPLC separation and integration of **27** (integ. **27**) as a fraction of the sum of the unreacted **11** (integ. **11**) + hydrolyzed **12** (integ. **12**) + integ. **27**. [b]Under optimized conditions, **11a** was obtained in 80% isolated yield. [c]Two peptides possessing desired molecular weight were obtained provably because of the formation of cyclic imide. [d]No N-processed peptides (**12i**, **13i**, and **27i**) were observed. [e]For the satisfactory HPLC purification, N-terminally extended peptide Ac-**KLYRALSRHWKFL**-NH_2 (**11m**) was used. [f]Although the initial *N*-*O* acyl shift proceeded, subsequent reactions went to incompletion.

2.4 Preparation of peptide thioesters using SQAT system and chemical synthesis of natural peptides

To confirm the feasibility of preparation of thioesters using SQAT system, chemical synthesis of C-type natriuretic peptide (CNP 53)[13], consisting of 53 amino acids, was attempted (Scheme 2.4). First, 43-residue Ni(II)-sensitive sequence-fused peptide **30** was prepared by SPPS. Peptide **30** did not have Cys which would be able to coordinate to Ni(II). Ni(II)-mediated methanolysis of peptide **30** yielded peptide methyl ester **31** as main product. Then, addition of hydrazine into the reaction mixture gave peptide hydrazide **32** in 69% isolated yield (Figure 2.4). After conversion of peptide hydrazide **32** to peptide thioester via peptidyl azide in accordance with Liu's protocol, NCL between the resulting thioester and N-terminal cysteinyl peptide **33** yielded reduced form CNP 53 **34** in 47% isolated yield (Figure 2.5). Finally, folding of the reduced peptide **34** gave oxidized form CNP 53 **35** in 66% isolated yield (Figure 2.6).[14]

Scheme 2.4. Chemical synthesis of CNP 53 using SQAT system.



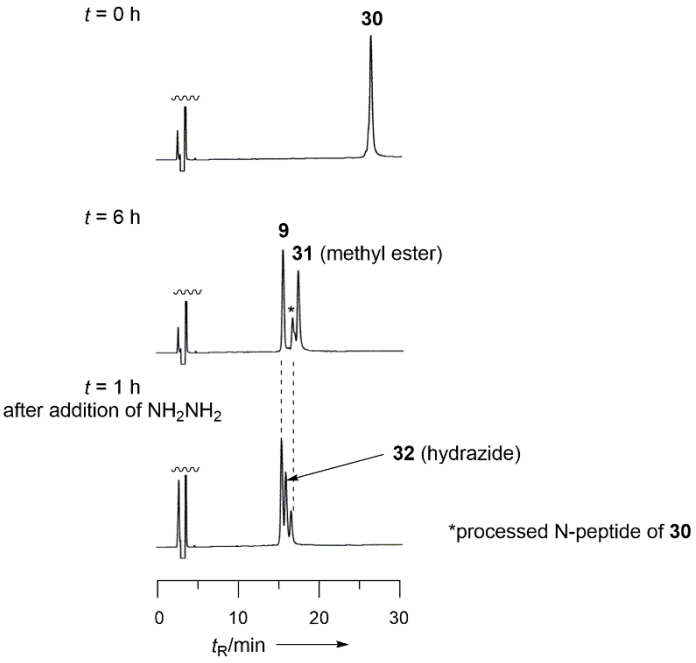


Figure 2.4. HPLC monitoring of hydrazinolysis via Ni(II)-mediated methanolysis of peptide **30**. Analytical HPLC conditions: Cosmosil 5C_{18} AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (18:82–26:74 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

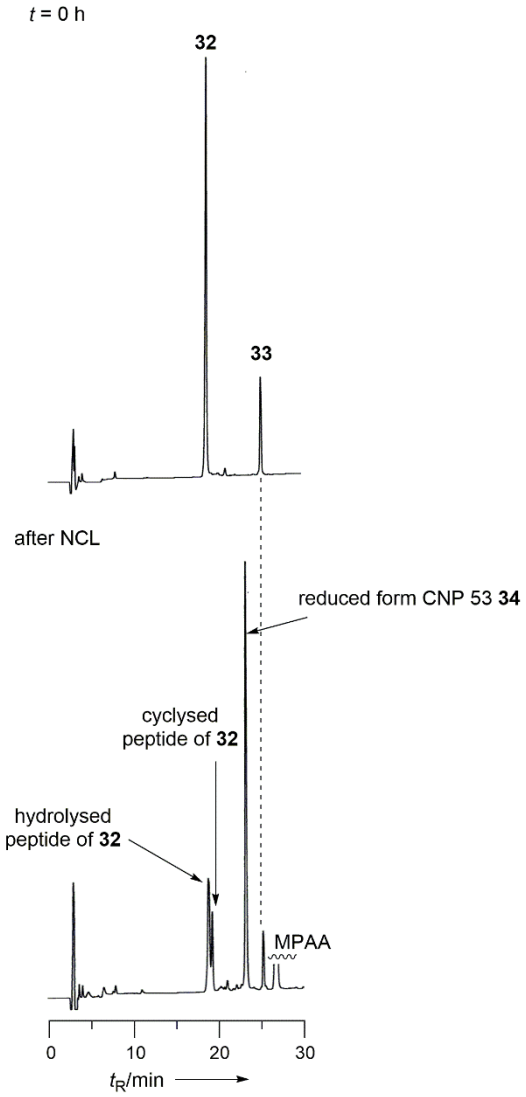


Figure 2.5. HPLC monitoring of NCL of peptide hydrazide **32** with N-terminal cysteinyl CNP fragment **33**. Analytical HPLC conditions: Cosmosil $5C_{18}$ AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (10:90–40:60 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

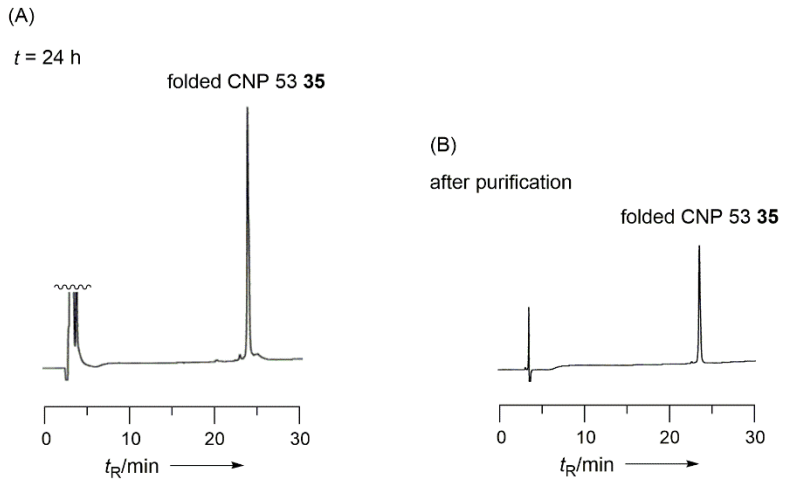
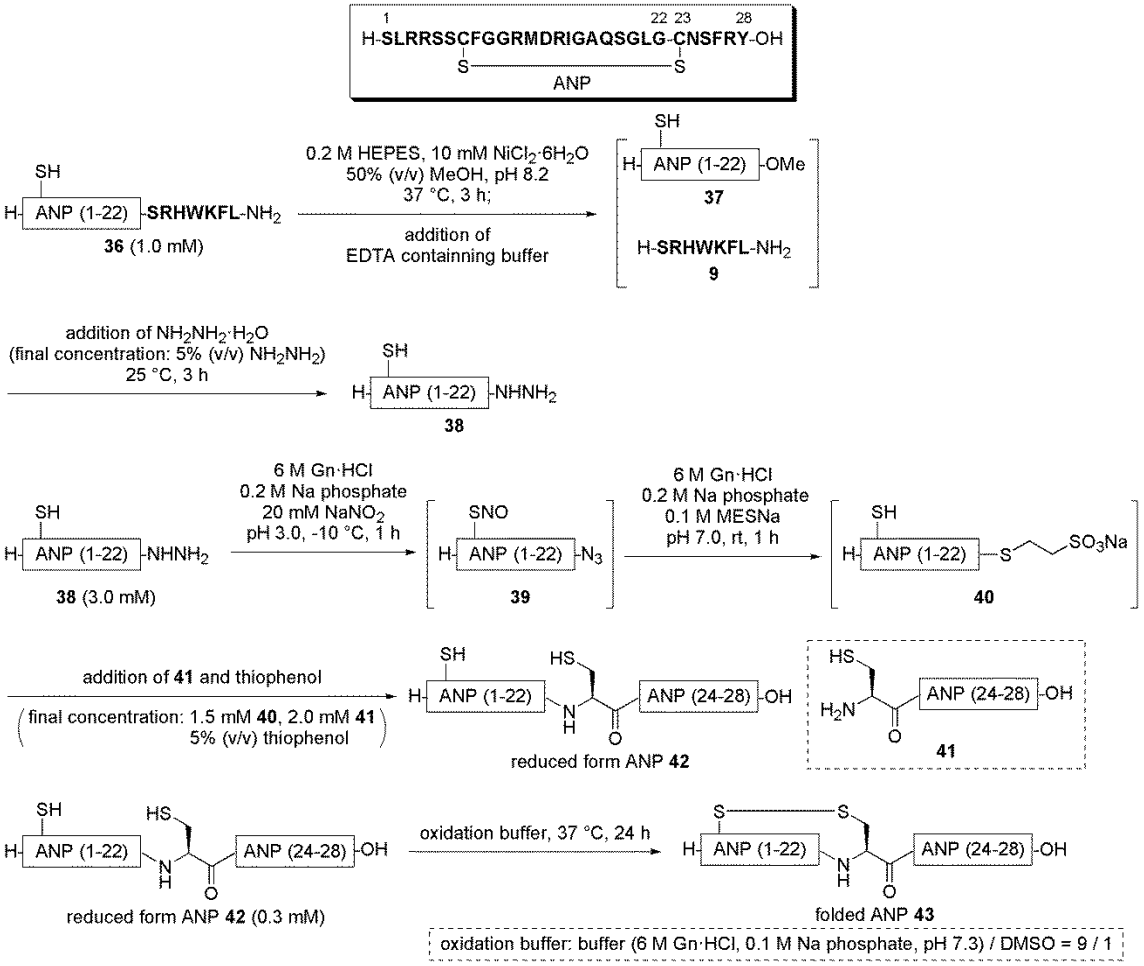


Figure 2.6. A) HPLC monitoring of the folding of reduced form CNP 53 **34** (t = 24 h). B) HPLC chart of folded CNP 53 **35** after purification. Analytical HPLC conditions: Cosmosil 5C_{18} AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (15:85–35:65 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

Next, evaluation of hydrazinolysis via methanolysis of Cys-containing peptide was attempted. Atrial natriuretic peptide (ANP)[15] was chosen as the synthetic target (Scheme 2.5). Initially, we synthesized Cys-containing peptide **36**, which is Ni(II)-sensitive sequence-fused N-terminal fragment of ANP by SPPS. Treatment of peptide **36** with Ni(II) and methanol yielded peptide methyl ester **37**. We next attempted to convert the resulting peptide methyl ester **37** to peptide hydrazide **38** by addition of hydrazine into the reaction mixture. However, the desired peptide hydrazide **38** was not observed on HPLC analysis. This was probably caused by adsorption of the Cys-containing peptide on insoluble Ni species formed by reduction of Ni(II) with hydrazine.[16] Addition of ethylenediaminetetraacetic acid (EDTA) as a chelating reagent followed by hydrazinolysis gave peptide hydrazide **38** in 71% isolated yield (Figure 2.7). The resulting peptide hydrazide **38** was then converted to peptide thioester **40** via peptidyl azide **39** by Liu's protocol (Figure 2.8). Although *S*-nitroso peptide was observed in the azidation step, addition of excess thiols induced de-nitrosation of the peptide in the thioesterification step. NCL of the resulting peptide thioester **40** with N-terminal cysteinyl peptide **41** afforded reduced form ANP **42** in 70% isolated yield. Finally, treatment of reduced form ANP **42** with folding conditions gave oxidized form ANP **43** in 86% isolated yield (Figure 2.9).

Scheme 2.5. Chemical synthesis of ANP using SQAT system.



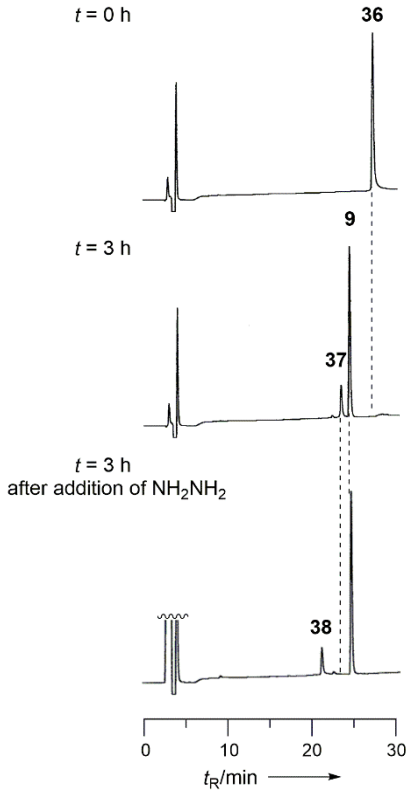


Figure 2.7. HPLC monitoring of hydrazinolysis via Ni(II)-mediated methanolysis of peptide **36**. Analytical HPLC conditions: Cosmosil 5C_{18} AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (10:90–35:65 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

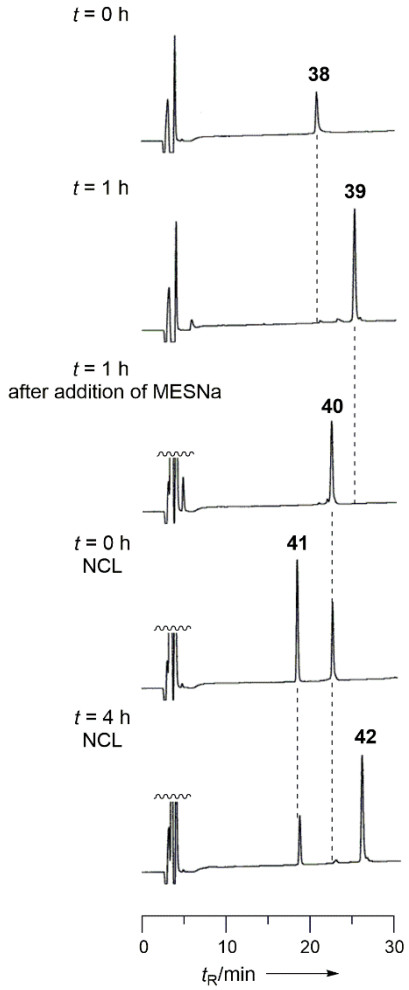


Figure 2.8. HPLC monitoring of NCL of peptide hydrazide **38** with N-terminal cysteinyl ANP fragment **41**. Analytical HPLC conditions: Cosmosil $5C_{18}$ AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (10:90–35:65 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

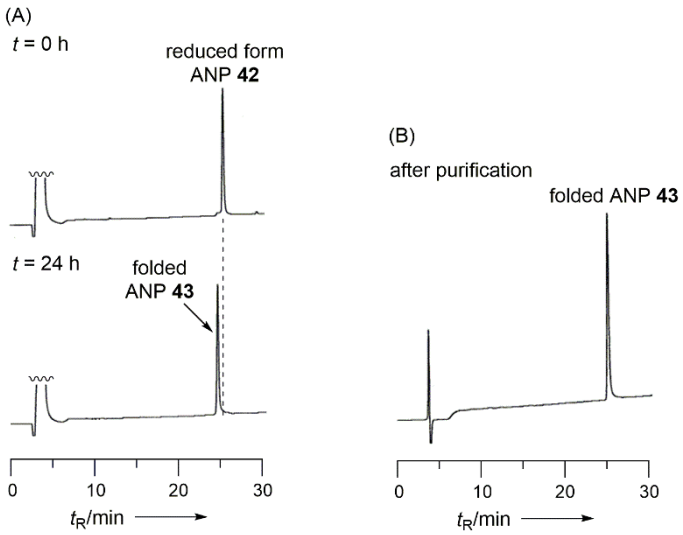
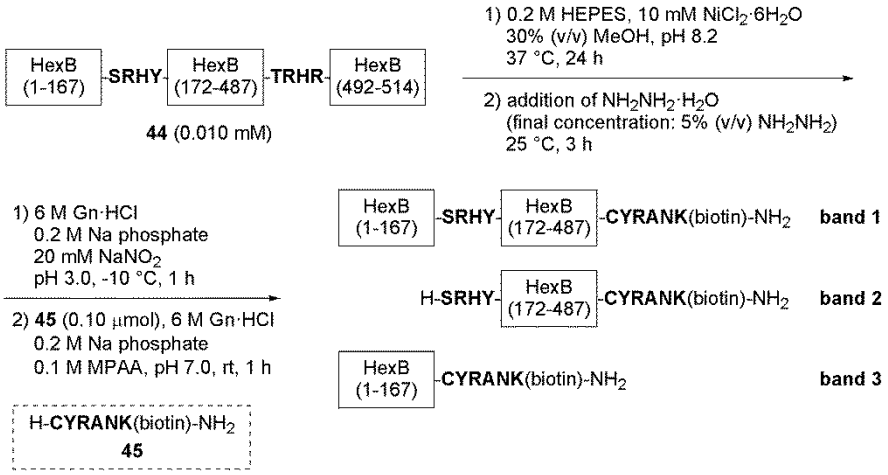


Figure 2.9. A) HPLC monitoring of the folding of reduced form ANP **42**. B) HPLC chart of folded ANP **43** after purification. Analytical HPLC conditions: Cosmosil 5C_{18} AR-II column (4.6 × 250 mm) with a linear gradient of MeCN (10:90–35:65 over 30 min) in 0.1% TFA aq. at a flow rate 1.0 mL/min, detection at 220 nm.

2.5 Application of SQAT system to expressed proteins

We succeeded in the thioesterification of chemically synthesized peptides as described in the previous section. Finally, evaluation of application of SQAT system to expressed proteins was attempted. Beta-hexosaminidase B (HexB) **44**, a 514-residue glycoprotein, was chosen as a model of expressed protein.[17] HexB has originally two Ni-sensitive sequences (**SRHY** and **TRHR**). Thioesterification of HexB **44** using SQAT system followed by NCL of the resulting thioesters with biotinylated peptide **45** was attempted (Scheme 2.6). As a result, three reaction products were observed by western blotting (Figure 2.10). These products were not identified by mass spectrometry because of heterogeneity of sugar chains. We assumed that these products have their origin in the reaction at **SRHY** or **TRHR** or both sites. This result indicated that SQAT system can be applied to expressed proteins.

Scheme 2.6. Application of SQAT system to HexB protein.



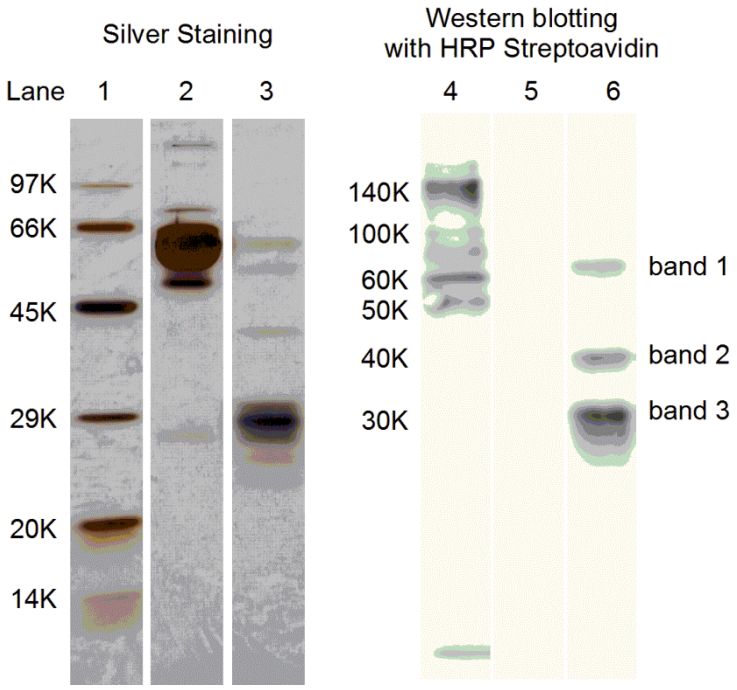


Figure 2.10. SDS-page analysis of SQAT-mediated editing of HexB. Lane 1, standard; Lanes 2 and 5, intact HexB; Lanes 3 and 6, crude mix after thioesterification using SQAT system followed by NCL with biotinylated peptide **45**; Lane 4, biotinylated standard.

2.6 Conclusion

We developed SQAT system enabling preparation of thioesters from naturally occurring peptide sequences without epimerization of C-terminal amino acid. SQAT system consists of sequential quadruple acyl transfers: 1) Ni(II)-triggered N–O acyl transfer; 2) O–O Acyl transfer with methanol; 3) O–N Acyl transfer with hydrazine; 4) Conversion of peptide hydrazide to peptidyl azide followed by N–S acyl transfer with thiol. SQAT system was successfully applied to thioesterification of chemically synthesized peptides and HexB as expressed protein.

Chapter 3

Conclusions

1. A novel methodology for preparation of thioesters applicable to naturally occurring sequences was developed. This method features the use of Ni(II)-triggered N–O acyl transfer followed by O–O acyl transfer with DTDE. The resulting DTDE ester peptide was converted to the corresponding thioester by reduction of disulfide followed by O–S acyl transfer based on tandem thiol switch. However, this protocol induced epimerization of C-terminal amino acid.

2. SQAT system, enabling formation of thioesters from naturally occurring peptide sequences without epimerization of C-terminal amino acid, was developed. SQAT system consists of sequential quadruple acyl transfer (N–O, O–O, O–N, and N–S acyl transfers). Chemical synthesis of CNP 53 and ANP using thioesters prepared by SQAT system was achieved. Furthermore, SQAT system was successfully applied to thioesterification of HexB protein.

Experimental section

General Methods

Mass spectra were recorded on a Waters MICROMASS® LCT PREMIER™. For HPLC separations, a Cosmosil 5C_{18}-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), a Cosmosil 5C_{18}-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL/min) or a Cosmosil 5C_{18}-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% TFA aqueous solution (v/v, solvent A) and 0.1% TFA in MeCN (v/v, solvent B) was used for HPLC elution.

Chapter 1

Preparation of Peptides **7** and **8**

H-**AKLRFGCPSRHWKFL**-NH_2 **7**

H-**AKLRFGAPSRHWKFL**-NH_2 **8**

General procedure: Protected peptide resin corresponding to peptide **7** or **8** was prepared by Fmoc SPPS on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.23 g, 0.05 mmol). The resulting completed resin was treated with TFA-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin) at room temperature for 2 h, and then the resin was filtrated off. To the filtrate was added cooled Et_2O to give precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide **7** or **8**. The crude peptide was purified by preparative HPLC to give the purified peptide **7** or **8**.

7: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 22.5 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 19% to 29% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 3H]^{3+}$) 615.7, found 615.7.

8: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 22.2 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 19% to 29% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 907.0, found 906.7.

Ni(II)-mediated Hydrolysis of Peptide **8**

Peptide **8** (0.25 mg, 0.1 μmol) was dissolved in 0.2 M Tris·HCl buffer containing 0.1 M $NiCl_2 \cdot 6H_2O$ (pH 8.2, 0.1 mL, 1.0 mM peptide). The reaction mixture was incubated at 50 °C and the reaction progress was monitored by analytical HPLC.

Processed N-peptide: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 12.4 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 859.5, found 859.3.

9: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 17.0 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 972.6, found 972.2.

10: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30

min, retention time = 9.8 min. MS (ESI-TOF) *m*/*z* calcd ([M + 2H]$^{2+}$) 481.8, found 481.9.

Preparation of Peptides **11a** for Their Ni(II)-mediated Conversion

Ac-**LYRAASRHWKFL**-NH_2

11a

Protected peptide resins corresponding to the title peptides were constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.05 g, 0.01 mmol) using standard Fmoc SPPS. TFA cleavage (TFA-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide.

11a: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 25.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + 3H]$^{3+}$) 530.3, found 530.6.

Evaluation of Ni(II)-mediated Conversion of Peptide **11a** to Oxyester

Peptide **11a** (0.20 mg, 0.10 μmol) was treated with 10 mM $NiCl_2 \cdot 6H_2O$ in 0.2 M HEPES-alcohol (TFE, ^{i}PrOH, MeOH) buffer (96 μL, pH 8.2) in the presence of 0.05% (w/v) *p*-toluenesulfonamide in H_2O (4 μL) aq. as an internal standard, the reaction progress was monitored by analytical HPLC (a linear gradient of solvent B in solvent A, 15% to 35% over 30 min). Fraction converted was determined by HPLC separation and integration of **13a** (integ. **13a**) as a fraction of the sum of the unreacted **11a** (integ. **11a**) + hydrolyzed **12a** (integ. **12a**) + integ. **13a**.

12a: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 12.6 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^{+}$) 635.3, found 635.3.

13a: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 16.4 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^{+}$) 649.4, found 649.2.

Conversion of Peptide **11a** to DTDE Ester Peptide **15**

Peptide **11a** (3.07 mg, 1.5 μmol) was dissolved in 0.2 M HEPES buffer containing 10 mM $NiCl_2 \cdot 6H_2O$ and 30% (v/v) DTDE (pH 8.2, 1.5 mL, 1.0 mM peptide). The reaction mixture was incubated at 37 °C for 12 h. After confirmation of the completion of the reaction by HPLC

analysis, the solution was diluted with 0.1% TFA aq (1.5 mL). The crude material was purified by semi-preparative HPLC to give the purified DTDE ester **15** (0.46 mg, 0.52 μmol, 35%).

15: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 23.0 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 26% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 771.3, found 771.2.

Conversion of DTDE Ester Peptide **15** to 4-Methylphenyl Thioester Peptide **18**

DTDE ester peptide **15** (0.09 mg, 0.1 μmol) was dissolved in TFA containing 0.1% (v/v) TFMSA, 5% (v/v) *p*-thiocresol (100 μL, 1.0 mM peptide). The reaction mixture was incubated at room temperature for 24 h. The reaction progress was monitored by analytical HPLC (a linear gradient of solvent B in solvent A, 10% to 50% over 30 min).

4-Methylphenyl thioester 18: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 50% over 30 min, retention time = 27.0 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 771.3, found 771.2.

Because the resulting thioester **18** was eluted as a single peak on HPLC analysis, risk of epimerization during the converting step could not be verified by the use of peptide **11a** and alternative peptide therefore was synthesized.

Examination of Epimerization During the Conversion of a SRHW-tagged Parent Peptide to the Corresponding DTDE Ester and 4-Methylphenyl Thioester

As mentioned above, the parent peptide **11a** was unsuitable for the validation of epimerization, alternative parent peptide, Ac-**LYRASRHWKFL**-NH_2 **19**, was synthesized in a manner similar to those employed for **11a**.

1. Preparation of Peptide **19**

Ac-**LYRASRHWKFL**-NH_2

19

Protected peptide resin corresponding to **19** was constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.05 g, 0.01 mmol) using standard Fmoc SPPS. TFA cleavage (TFA-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin), at room

temperature for 2 h) followed by HPLC purification afforded the desired peptide **19**.

19: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 25.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 3H]^{3+}$) 759.4, found 759.3.

2. Conversion of Peptide **19** to DTDE Ester Peptide **20** (L-**Ala**)

Peptide **19** (3.95 mg, 2.0 μmol) was dissolved in 0.2 M HEPES buffer containing 10 mM $NiCl_2 \cdot 6H_2O$ and 50% (v/v) DTDE (pH 8.2, 2.0 mL, 1.0 mM peptide). The reaction mixture was incubated at 37 °C for 12 h. After confirmation of the completion of the reaction by HPLC analysis, the solution was diluted with 0.1% TFA aq (2.0 mL). HPLC analysis of the crude material clearly indicated that product corresponding to the DTDE ester appeared as single peak on HPLC chart (Figure 5 (A)). As mentioned later, obtained DTDE ester had L-**Ala** configuration. The crude material was purified by semi-preparative HPLC to give the purified DTDE ester **20** (L-**Ala**) (0.73 mg, 0.90 μmol, 45%).

20 (L-**Ala**): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 22.9 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^{+}$) 700.3, found 700.3.

3. Preparation of Stereo-defined DTDE Ester Peptides **20** (L-**Ala**) and **21** (D-**Ala**)

Ac-**LYR**-L-**Ala**-DTDE　　Ac-**LYR**-D-**Ala**-DTDE

20 (L-**Ala**)　　**21** (D-**Ala**)

2-Chlorotrityl resin 50 mg (1.57 mmol/g) was swollen in DMF for 30 min. To the resin were added dithiodiethanol (0.1 mL, 10 equiv. for resin) and pyridine (0.07 mL, 10 equiv. for resin) in DMF. Then, incorporation of the C-terminal AA was performed using Fmoc-L-**Ala**-OH (or Fmoc-D-**Ala**-OH) and DIPCDI (10 equiv. each for resin) in the presence of DMAP (0.1 equiv. for resin). Standard elongation steps by Fmoc protocol followed by deprotection and subsequent HPLC purification afforded the desired reference peptide **20** (L-**Ala**) or **21** (D-**Ala**). Peptides **20** and **21** were well resolved each other on HPLC analysis (Figure 5 (B)). Based on the HPLC analysis in Figure 5, we concluded that no epimerization occurred during oxyesterification step.

20 (L-**Ala**): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 23.5 min. Preparative HPLC conditions: A linear gradient of

solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 700.3, found 700.2.

21 (**D-Ala**): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 24.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 700.3, found 700.1.

4. Conversion of Stereo-defined DTDE Ester Peptide **20** (**L-Ala**) to 4-Methylphenyl Thioester Peptides **22** (**L-Ala**) and **23** (**D-Ala**)

Stereo-defined DTDE ester peptide **20** (**L-Ala**) (2.4 mg, 3.0 μmol) was converted to the methylthiophenyl ester by the action of TFA containing 0.1% (v/v) TFMSA, 5% (v/v) thiocresol (3 mL, 1.0 mM peptide) for 24 h at room temperature. HPLC analysis of the crude material indicated that two components corresponding to the 4-methylphenyl thioesters appeared as separable peaks (Figure 6). Although the procedure for characterization of the configuration of the thioester part was described later, the resulting two components were homogeneously purified to give **L-Ala**-containing 4-methylphenyl thioester **22** (**L-Ala**) (0.50 mg, 0.64 μmol, 21%) and **D-Ala**-containing 4-methylphenyl thioester **23** (**D-Ala**) (0.15 mg, 0.19 μmol, 6%).

L-Ala-containing 4-methylphenyl thioester 22 (**L-Ala**): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 60% over 30 min, retention time = 23.4 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 60% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 670.3, found 670.2.

D-Ala-containing 4-methylphenyl thioester 23 (**D-Ala**): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 60% over 30 min, retention time = 23.8 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 60% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 670.3, found 670.2.

Hydrolysed peptide of 20: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 60% over 30 min, retention time = 15.2 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 564.3, found 564.3.

5. Preparation of Stereo-defined Ligated Products **24** (L-**Ala**) and **25** (D-**Ala**)

In order to determine the configuration of alanine of **22** and **23**, resulting methylthiophenyl ester **22** and **23** were subjected to NCL with N-terminal cysteinyl peptide, H-**CYRANK**-NH_2 **26**, and then, resulting ligated peptides were analyzed by HPLC using stereo-defined Ac-**LYR**-L-**Ala**-**CYRANK**-NH_2 **24** and Ac-**LYR**-D-**Ala**-**CYRANK**-NH_2 **25** as authentic samples.

Ac-**LYR**-L-**Ala**-**CYRANK**-NH_2 **24** (L-**Ala**)

Ac-**LYR**-D-**Ala**-**CYRANK**-NH_2 **25** (D-**Ala**)

Protected peptide resins corresponding to the title peptides were constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.05 g, 0.01 mmol) using standard Fmoc SPPS. TFA cleavage (TFA-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide.

L-Ala-containing ligated product 24 (L-**Ala**): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 19.4 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 14% to 24% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 649.8, found 649.8.

D-Ala-containing ligated product 25 (D-**Ala**): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 18.7 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 14% to 24% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 649.8, found 649.8.

6. Preparation of N-Terminal Cysteinyl Peptide **26**

H-**CYRANK**-NH_2 **26**

Protected peptide resin corresponding to **26** was constructed NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.05 g, 0.01 mmol) using standard Fmoc SPPS. TFA cleavage (TFA-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide.

N-Terminal cysteinyl peptide 26: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 11.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 13% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M

+ H]$^+$) 753.4, found 753.2.

7. NCL of 4-Methylphenyl Thioester Peptides **22** (L-**Ala**), **23** (D-**Ala**) with N-Terminal Cysteinyl Peptide **26**

4-Methylphenyl thioester peptide **22** (L-**Ala**) or **23** (D-**Ala**) (0.08 mg, 0.1 μmol) and N-terminal cysteinyl peptide **26** (0.14 mg, 0.13 μmol) were dissolved in 6 M Gn·HCl-0.2 M Na phosphate buffer containing 20 mM TCEP and 50 mM sodium ascorbate (pH 7.0, 0.1 mL) to perform NCL reaction. One hour reaction at 37 °C followed by HPLC purifications gave ligated peptides. Analysis of each obtained peptide by HPLC using authentic stereo-defined samples **24** (L-**Ala**) and **25** (D-**Ala**) showed that thioester peptides **22** and **23** had C-terminal L-**Ala** and D-**Ala** residues, respectively.

Chapter 2

Evaluation of Ni(II)-mediated Methanolysis of Peptide **11a**

Peptide **11a** (0.20 mg, 0.10 μmol) was treated with 1-20 mM $NiCl_2 \cdot 6H_2O$ in 0.2 M HEPES-alcohol (10-50% MeOH) buffer (96 μL, pH 7.8-8.2) in the presence of 0.05% (w/v) *p*-toluenesulfonamide in H_2O (4 μL) aq. as an internal standard, the reaction progress was monitored by analytical HPLC (a linear gradient of solvent B in solvent A, 15% to 35% over 30 min). Fraction converted was determined by HPLC separation and integration of **13a** (integ. **13a**) as a fraction of the sum of the unreacted **11a** (integ. **11a**) + hydrolyzed **12a** (integ. **12a**) + integ. **13a**.

Preparation of Peptides **11b-l**, **n-t**, and **u** for Their Ni(II)-mediated Conversion

Ac-**LYRAXSRHWKFL**-NH_2

11b-l, **n-u**

Protected peptide resins corresponding to the title peptides were constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.05 g, 0.01 mmol) using standard Fmoc SPPS. TFA cleavage (TFA-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide.

11b (X = Gly): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 16.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 787.9, found 787.8.

11c (X = Asp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 17.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 21% to 31% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 816.9, found 816.8.

11d (X = Glu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 17.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 21% to 31% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 823.9, found 823.8.

11e (X = Asn): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 16.8 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 21% to 31% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 816.4, found 816.3.

11f (X = Gln): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 16.8 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 823.5, found 823.3.

11g (X = Ser): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 16.7 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 802.9, found 802.8.

11h (X = Thr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 17.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 21% to 31% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 809.9, found 809.8.

11i (X = Cys): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 18.3 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 810.9, found 810.8.

11j (X = Pro): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 16.4 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 807.9, found 807.8.

11k (X = Val): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 21.8 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 25% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 809.0, found 808.8.

11l (X = Met): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 19.8 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 28% to 36% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 824.9, found 824.8.

11n (X = Ile): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 45% over 30 min, retention time = 23.4 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 27% to 37% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 816.0, found 815.8.

11o (X = Tyr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 18.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 841.0, found 840.8.

11p (X = Phe): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 21.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 27% to 37% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 833.0, found 832.8.

11q (X = His): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 15.7 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 28% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 828.0, found 827.8.

11r (X = Lys): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 15.6 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 21% to 29% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 823.5, found 823.3.

11s (X = Arg): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 15.8 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 21% to 29% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 837.5, found 837.3.

11t (X = Trp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 21.0 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 27% to 37% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 852.5, found 852.2.

11u (X = D-Ala): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 24.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 2H]^{2+}$) 794.9, found 794.8.

Preparation of Peptide **11m**

Ac-**KLYRALSRHWKFL**-NH_2

11m

Protected peptide resin corresponding to **11m** was constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.05 g, 0.01 mmol) using standard Fmoc SPPS. TFA cleavage (TFA-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide.

11m: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 50% over 30 min, retention time = 19.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 25% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 3H]^{3+}$) 880.0, found 879.8.

Conversion of Peptides **11** to Peptide Hydrazides **27**

General procedure: Peptide **11** (0.20 mg, 0.1 μmol) was dissolved in 0.2 M HEPES buffer containing 10 mM $NiCl_2 \cdot 6H_2O$ and 50% (v/v) MeOH (pH 8.2, 0.1 mL, 1.0 mM peptide). The reaction mixture was incubated at 37 °C for 24 h, followed by addition of $NH_2NH_2 \cdot H_2O$ (4.9 μL) into the reaction mixture (final concentration; 5% (v/v) NH_2NH_2). And then, additional reaction at 25 °C for 3 h gave peptide hydrazide **27**. The reaction progress was monitored by analytical HPLC. Fraction converted was determined by HPLC separation and integration of **27** (integ. **27**) as a fraction of the sum of the unreacted **11** (integ. **11**) + hydrolyzed **12** (integ. **12**) + integ. **13** + integ. **27**.

27a (X = Ala): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 8.5 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 649.4, found 649.3.

12b (X = Gly): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 10.2 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 621.4, found 621.3.

13b (X = Gly): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 13.1 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 635.3, found 635.3.

27b (X = Gly): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 7.7 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 635.4, found 635.4.

12c (X = Asp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 10.1 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 679.3, found 679.3.

13c (X = Asp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 12.9 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 693.3, found 693.3.

27c (X = Asp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 8.1 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 693.4, found 693.3.

12d (X = Glu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 10.8 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 693.3, found 693.3.

13d (X = Glu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 14.0 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 707.4, found 707.2.

27d (X = Glu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 8.4 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 707.4, found 707.3.

12g (X = Ser): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 9.6 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 651.3, found 651.3.

13g (X = Ser): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 12.0 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 665.4, found 665.3.

27g (X = Ser): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 7.7 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 665.4, found 665.3.

12h (X = Thr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 10.5 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 665.4, found 665.3.

13h (X = Thr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 13.3 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 679.4, found 679.3.

27h (X = Thr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 8.4 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 679.4, found 679.3.

27j (X = Pro): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 10.5 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 675.4, found 675.4.

13k (X = Val): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 19.4 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 677.4, found 677.4.

27k (X = Val): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 9.3 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 677.4, found 677.4.

12l (X = Met): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 18.2 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 695.3, found 695.3.

13l (X = Met): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 22.9 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 709.4, found 709.2.

27l (X = Met): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 14.0 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 709.4, found 709.3.

12m (X = Leu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 15.5 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 805.5, found 805.3.

13m (X = Leu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 20.1 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 819.5, found 819.3.

27m (X = Leu): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 11.6 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 819.5, found 819.3.

12o (X = Tyr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 16.0 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 727.4, found 727.3.

13o (X = Tyr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 20.4 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 741.4, found 741.3.

27o (X = Tyr): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 11.9 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 741.4, found 741.3.

12p (X = Phe): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 20.9 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 711.4, found 711.3.

13p (X = Phe): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 25.8 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 725.4, found 725.3.

27p (X = Phe): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 16.7 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 725.4, found 725.3.

12q (X = His): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 11.2 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 701.4, found 701.3.

13q (X = His): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 13.5 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 715.4, found 715.3.

27q (X = His): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 9.8 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 715.4, found 715.3.

12r (X = Lys): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 10.5 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 692.4, found 692.3.

13r (X = Lys): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 13.0 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 706.4, found 706.3.

27r (X = Lys): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 8.7 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 706.4, found 706.3.

12s (X = Arg): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 11.3 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 720.4, found 720.3.

13s (X = Arg): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 14.0 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 734.4, found 734.3.

27s (X = Arg): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min, retention time = 9.6 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 734.4, found 734.3.

12t (X = Trp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 21.5 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 750.4, found 750.3.

13t (X = Trp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 26.6 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 764.4, found 764.3.

27t (X = Trp): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 17% to 37% over 30 min, retention time = 15.9 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 764.4, found 764.3.

12u (X = D-Ala): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 12.2 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 635.3, found 635.3.

13u (X = D-Ala): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 16.2 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 649.4, found 649.3.

27u (X = D-Ala): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min, retention time = 8.7 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 30% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 649.4, found 649.3.

Preparation of Peptide Hydrazides **27a** and **27u**

Peptide **11a** (8.18 mg, 4.0 μmol) (or peptide **11u** (8.18 mg, 4.0 μmol)) was dissolved in 0.2 M HEPES buffer containing 10 mM $NiCl_2 \cdot 6H_2O$ and 50% (v/v) MeOH (pH 8.2, 4.0 mL, 1.0 mM peptide). The reaction mixture was incubated at 37 °C for 12 h, followed by addition of $NH_2NH_2 \cdot H_2O$ (0.2 mL) into the reaction mixture (final concentration; 5% (v/v) NH_2NH_2). And then, additional reaction for 1 h at 25 °C gave peptide hydrazide **27a** (or peptide hydrazide **27u**). After confirmation of the completion of the reaction by HPLC analysis, the solution was diluted with 0.1% TFA aq. (4.0 mL). The crude material was purified by semi-preparative HPLC to give the purified peptide hydrazide **27a** (2.81 mg, 3.20 μmol, 80%) (or peptide **27u** (3.14 mg, 3.58 μmol, 90%)).

Conversion of Peptide Hydrazide **27a** (or **27u**) to MESNa Ester **29a** (or **29u**)

Peptide **27a** (0.087 mg, 0.1 μmol) (or Peptide **27u** (0.087 mg, 0.1 μmol)) was dissolved in 0.2 M Na phosphate buffer containing 6 M Gn·HCl, (pH 3.0, 0.1 mL, 3 mM peptide). The reaction mixture was stored at -10 °C. Then. 10 μL of 0.2 M $NaNO_2$ aq. was added, and the reaction mixture was stored at -10 °C for 1 h. After that, 0.2 M Na phosphate buffer containing 6 M Gn·HCl and 0.2 M MESNa (0.1 mL) was added, and pH of the mixed solution was adjusted to pH 7.0 with 2.0 M NaOH aq.. The reaction mixture was stored at room temperature for 1 h. The reaction was monitored by analytical HPLC.

28a (Ac-**LYRA-L-Ala**-N_3): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 35% over 30 min, retention time = 26.5 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 660.4, found 660.3.

29a (Ac-**LYRA-L-Ala**-MESNa): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 35% over 30 min, retention time = 22.0 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 759.3, found 759.1.

28u (Ac-**LYRA-D-Ala**-N_3): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 35% over 30 min, retention time = 27.7 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 660.4, found 660.3.

29u (Ac-**LYRA-ᴅ-Ala**-MESNa): Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 35% over 30 min, retention time = 23.1 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 759.3, found 759.1.

Preparation of 43-residue CNP Peptide **30** and N-terminal Cysteinyl CNP Fragment **33**

H-**DLRVDTKSRAAWARLLQEHPNARKYKGANKKGLSKGSRHWKFL**-NH_2

30

Protected peptide resin corresponding to **30** was constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.5g, 0.11 mmol) using standard Fmoc SPPS. TFA cleavage (TFA-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide **30**.

30: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 50% over 30 min, retention time = 16.1 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 19% to 29% over 30 min. MS (ESI-TOF) calcd (average isotopes) 5018.7, found 5018.6.

H-**CFGLKLDRIGSMSGLGC**-OH

33

Protected peptide resin corresponding to **33** were constructed on Wang resin (1.1 mmol amine/g, 0.09 g, 0.099 mmol) using standard Fmoc SPPS. TFA cleavage (TFA-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide **33**.

33: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 23.9 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 22% to 32% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + 2H]$^{2+}$) 878.9, found 878.7.

Conversion of 43-residue CNP Peptide **30** to 36-residue Peptide Hydrazide **32**

Peptide **30** (6.73 mg, 1.0 μmol) was dissolved in 0.2 M HEPES buffer containing 10 mM $NiCl_2 \cdot 6H_2O$ and 50% (v/v) MeOH (pH 8.2, 1.0 mL, 1.0 mM peptide). The reaction mixture was incubated at 37 °C for 6 h, followed by addition of $NH_2NH_2 \cdot H_2O$ (0.053 mL) into the reaction mixture (final concentration; 5% (v/v) NH_2NH_2). And then, additional reaction at 25 °C for 1 h

gave peptide hydrazide **32**. After confirmation of the completion of the reaction by HPLC analysis, the solution was diluted with 0.1% TFA aq. (4.0 mL). The crude material was purified by semi-preparative HPLC to give the purified peptide hydrazide **32** (2.80 mg, 0.69 μmol, 69%).

Processed N-peptide of 30: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 26% over 30 min, retention time = 16.6 min. MS (ESI-TOF) calcd (average isotopes) 4064.6, found 4064.5.

Methyl ester 31: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 26% over 30 min, retention time = 17.3 min. MS (ESI-TOF) calcd (average isotopes) 4078.6, found 4078.3.

Hydrazide 32: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 18% to 26% over 30 min, retention time = 15.8 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 13% to 33% over 30 min. MS (ESI-TOF) calcd (average isotopes) 4078.7, found 4078.4.

NCL for the Synthesis of Reduced Form CNP 53 **34**

Peptides **32** (3.22 mg, 0.58 μmol) and **33** (1.57 mg, 0.75 μmol) were dissolved in 0.2 M Na phosphate buffer containing 6 M Gn·HCl, (pH 3.0, 0.19 mL, 3 mM or 4 mM each peptides). The reaction mixture was stored at 0 °C. Then. 19 μL of 0.2 M $NaNO_2$ aq. was added, and the reaction mixture was stored at 0 °C for 1 h. After that, 0.2 M Na phosphate buffer containing 6 M Gn·HCl and 0.2 M MPAA (0.19 mL) was added, and pH of the mixed solution was adjusted to pH 7.0 with 2.0 M NaOH aq.. The reaction mixture was stored at room temperature for 1 h. After completion of the reaction, the solution was diluted with 30 mM TCEP aq. (pH 7.0, 0.4 mL). The crude material was purified by semi-preparative HPLC to give the reduced form CNP 53 **34** (2.00 mg, 0.27 μmol, 47%).

Reduced form CNP 53 34: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 23.1 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min. MS (ESI-TOF) calcd (average isotopes) 5803.7, found 5803.3.

Cyclysed peptide of 32: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 19.8 min. MS (ESI-TOF) calcd (average isotopes) 4046.6, found 4046.3.

Folding for Preparation of CNP 53

Reduced form CNP 53 **34** (1.94 mg, 0.26 μmol) was dissolved in 0.1 M Na phosphate buffer containing 6 M Gn·HCl, (pH 7.3, 0.79 mL) and DMSO (0.09 mL). The reaction mixture was incubated at 37 °C for 24 h. The crude material was purified by semi-preparative HPLC to give the purified folded CNP 53 **35** (1.28 mg, 0.17 μmol, 66%).

Folded CNP 53 35: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 40% over 30 min, retention time = 23.4 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 15% to 35% over 30 min. MS (ESI-TOF) calcd (average isotopes) 5801.7, found 5801.6.

Preparation of 29-residue ANP Peptide **36** and N-terminal Cysteinyl ANP Fragment **41**

H- **SLRRSSCFGGRMDRIGAQSGLGSRHWKFL**-NH_2

36

Protected peptide resin corresponding to **36** was constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.5g, 0.11 mmol) using standard Fmoc SPPS. TFA cleavage (TFA-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide **36**.

36: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 50% over 30 min, retention time = 19.7 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 20% to 27% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + 3H]^{3+}$) 1088.9, found 1088.8.

H- **CNSFRY**-OH

41

Protected peptide resin corresponding to **41** were constructed on Wang resin (1.1 mmol amine/g, 0.09 g, 0.099 mmol) using standard Fmoc SPPS. TFA cleavage (TFA-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide **41**.

41: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 50% over 30 min, retention time = 14.5 min. Preparative HPLC conditions: A linear gradient of solvent B in

solvent A, 12% to 22% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 789.3, found 789.2.

Conversion of 29-residue ANP Peptide **36** to 22-residue Peptide Hydrazide **38**

Peptide **36** (4.60 mg, 1.1 μmol) was dissolved in 0.2 M HEPES buffer containing 10 mM $NiCl_2 \cdot 6H_2O$ and 50% (v/v) MeOH (pH 8.2, 1.1 mL, 1.0 mM peptide). The reaction mixture was incubated at 37 °C for 3 h, followed by addition of 0.2 M HEPES buffer containing 0.1 M EDTA (pH 8.2, 1.1 mL). And then, $NH_2NH_2 \cdot H_2O$ (0.116 mL) was added into the reaction mixture (final concentration; 5% (v/v) NH_2NH_2). Additional reaction at 25 °C for 3 h gave peptide hydrazide **38**. After confirmation of the completion of the reaction by HPLC analysis, the solution was diluted with 0.1% TFA aq. (2.2 mL). The crude material was purified by semi-preparative HPLC to give the purified peptide hydrazide **38** (2.30 mg, 0.76 μmol, 71%).

Methylester 37: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min, retention time = 23.2 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 775.7, found 775.9.

Hydrazide 38: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min, retention time = 20.7 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + H]$^+$) 775.7, found 775.9.

NCL for the Synthesis of Reduced Form ANP **42**

Peptide **38** (0.90 mg, 0.30 μmol) was dissolved in 0.2 M Na phosphate buffer containing 6 M Gn·HCl, (pH 3.0, 0.1 mL, 3 mM peptide). The reaction mixture was stored at -10 °C. Then. 10 μL of 0.2 M $NaNO_2$ aq. was added, and the reaction mixture was stored at -10 °C for 1 h. After that, 0.2 M Na phosphate buffer containing 6 M Gn·HCl and 0.2 M MESNa (0.1 mL) was added, and pH of the mixed solution was adjusted to pH 7.0 with 2.0 M NaOH aq.. The reaction mixture was stored at room temperature for 1 h. And then, peptide **41** (0.40 mg. 0.40 μmol) and thiophenol (0.01 mL) were added into the reaction mixture (final concentration; 5% (v/v) thiophenol). After completion of the reaction, the solution was diluted with 30 mM TCEP aq. (pH7.0, 0.2 mL). The crude material was purified by semi-preparative HPLC to give the reduced form ANP **42** (0.78 mg, 0.21 μmol, 70%).

Peptidyl azide 39: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min, retention time = 24.8 min. MS (ESI-TOF) *m*/*z* calcd ([M + 3H]$^{3+}$) 789.0, found 788.9.

MESNa ester 40: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min, retention time = 22.1 min. MS (ESI-TOF) *m*/*z* calcd ([M + 3H]$^{3+}$) 812.4, found 812.2.

Reduced form ANP 42: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min, retention time = 25.3 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + 3H]$^{3+}$) 1027.8, found 1027.8.

Folding for Preparation of ANP

Reduced form ANP **42** (0.78 mg, 0.21 μmol) was dissolved in 0.1 M Na phosphate buffer containing 6 M Gn·HCl, (pH 7.3, 0.63 mL) and DMSO (0.07 mL). The reaction mixture was incubated at 37 °C for 24 h. The crude material was purified by semi-preparative HPLC to give the purified folded ANP **43** (0.67 mg, 0.18 μmol, 86%).

Folded ANP 43: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min, retention time = 24.3 min. Semi-preparative HPLC conditions: A linear gradient of solvent B in solvent A, 10% to 35% over 30 min. MS (ESI-TOF) *m*/*z* calcd ([M + 3H]$^{3+}$) 1027.1, found 1027.1.

Preparation of Peptide **45**

H-**CYRANK**(biotin)-NH_2

45

Protected peptide resin corresponding to **45** was constructed on NovaSyn® TGR resin (Rink amide type: 0.22 mmol amine/g, 0.2 g, 0.044 mmol) using standard Fmoc SPPS. TFA cleavage (TFA-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol (80:5:5:5:5 (v/v), 50 μL/1 mg resin), at room temperature for 2 h) followed by HPLC purification afforded the desired peptide.

45: Analytical HPLC conditions: A linear gradient of solvent B in solvent A, 1% to 50% over 30 min, retention time = 15.3 min. Preparative HPLC conditions: A linear gradient of solvent B in solvent A, 8% to 18% over 30 min. MS (ESI-TOF) *m*/*z* calcd ($[M + H]^+$) 979.5, found 979.1.

Application of SQAT System to Expressed Protein

HexB **44** (0.057 mg, 0.01 μmol) was dissolved in 0.2 M HEPES buffer containing 6 M Gn·HCl, 10 mM $NiCl_2 \cdot 6H_2O$ and 30% (v/v) MeOH (pH 8.2, 0.1 mL, 0.01 mM protein). The reaction mixture was incubated at 37 °C for 24 h, followed by addition of $NH_2NH_2 \cdot H_2O$ (5.3 μL) into the reaction mixture (final concentration: 5% (v/v) NH_2NH_2). And then, the reaction mixture was incubated at 25 °C for 3 h. The protein was then buffer-exchanged, by use of a centrifugal filter equipped with a 10 kDa molecular weight cut off, into 0.2 M Na phosphate buffer containing 6 M Gn·HCl, (pH 3.0, 0.4 mL) by repeated dilution/concentration and ultimately obtained in the original reaction volume of ligation buffer (0.1 mL). The reaction mixture was stored at -10 °C. Then. 10 μL of 0.2 M $NaNO_2$ aq. was added, and the reaction mixture was stored at -10 °C for 1 h. After that, 0.2 M Na phosphate buffer containing 6 M Gn·HCl and 0.2 M MPAA (0.1 mL) was added, and pH of the mixed solution was adjusted to pH 7.0 with 2.0 M NaOH aq. followed by addition of peptide **45** (0.12 mg, 0.1 μmol). The reaction mixture was stored at room temperature for 12 h, followed by addition of 30 mM TCEP aq. (pH 7.0, 0.2 mL). And then, the reaction mixture was exchanged into 0.1% TFA aqueous solution by use of a centrifugal filter equipped with a 10 kDa molecular weight cut off followed by silver staining and streptavidin-HRP blotting.

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1. Development of a chemical methodology for the preparation of peptide thioesters applicable to naturally occurring peptides using a sequential quadruple acyl transfer system

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