Studies on development of amide cleavage systems applicable to protein manipulation

Thesis by
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List of Abbreviations

AA	amino acid
Ac	acetyl
Alloc	allyloxycarbonyl
Boc	*tert*-butoxycarbonyl
Bu	butyl
CBB	Coomassie Brilliant Blue
CNP	C type natriuretic peptide
diMe	dimethyl
DIPCI	*N,N'*-diisopropylcarbodiimide
DIPEA	*N,N*-diisopropylethylamine
DMB	2,4-dimethoxybenzyl
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
ESI	electrospray ionization
Et	ethyl
Et_2O	diethyl ether
EDC·HCl	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
Fmoc	9-fluorenylmethoxycarbonyl
Gn	guanidine
HATU	*O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
HRMS	high resolution mass spectroscopy
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
IR	infrared spectroscopy
Me	methyl
MeCN	acetonitrile
MeOH	methanol
MESNa	sodium 2-mercaptoethanesulfonate
MPAA	4-mercaptophenylacetic acid
MS	mass spectrometry
*o*NBnoc	*ortho*-nitrobenzyloxycarbonyl
NCL	native chemical ligation

NMP	*N*-methylpyrrolidone
NMR	nuclear magnetic resonance
PhFl	9-phenyl-9-fluorenyl
$Pd(PPh_3)_4$	tetrakis(triphenylphosphine)palladium(0)
SPPS	solid-phase peptide synthesis
Su	succinimide
TCEP	tris(2-carboxyethyl)phosphine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TOF	time of flight
Trt	triphenylmethyl
UV	ultraviolet

Table of amino acids and their abbreviations (3-letters and 1-letters)

alanine	Ala	A	methionine	Met	M
cysteine	Cys	C	asparagine	Asn	N
aspartic acid	Asp	D	proline	Pro	P
glutamic acid	Glu	E	glutamine	Gln	Q
phenylalanine	Phe	F	arginine	Arg	R
glycine	Gly	G	serine	Ser	S
histidine	His	H	threonine	Thr	T
isoleucine	Ile	I	valine	Val	V
lysine	Lys	K	tryptophan	Trp	W
leucine	Leu	L	tyrosine	Tyr	Y

Preface

Amide bonds, which control the physicochemical and biochemical characters of peptides/proteins have poor electrophilicity due to their double bond nature. Consequently, chemical cleavage of amide bonds usually needs harsh conditions. In living organisms, however, amide bonds can be readily cleaved under physiological conditions through various enzymatic and non-enzymatic processes. Such amide bond cleavage processes govern many cellular regulatory functions in biological systems.

Peptides/proteins are indispensable molecules in a wide variety of vital phenomena, and elucidation of their functions is essential for understanding of the biological significance of the proteins in physiological events. Such an understanding can lead to development of new drugs with a new mechanism of action. In this context, innovative methodologies for protein manipulation including the synthesis of both chemicals which regulate protein function and functionalized proteins are highly demanded.

In this thesis, I report on the development of amide bond cleavage system for control of the functions of peptides/proteins, and also describe synthetic methodology for producing chemically modified proteins. Inspirations of these studies came from amide bond cleavage reactions in biological system.

In Chapter 1, development of a UV-responsive amide bond cleavage system is described. This chemically mimics the environment of the amide bond cleavage reaction at the carbonyl side of the critical asparagine residue found in protein splicing mediated by intein. The effects of side chain structure on the reaction rate of amide bond cleavage are also described.

In Chapter 2, development of an innovative methodology for preparation of protein thioesters from a naturally occurring peptide sequence is discussed. Thioesters are highly potent synthetic intermediates used in the preparation of chemically modified proteins. The developed methodology features the use of carboxypeptidase Y (CPY), which hydrolyzes the C-terminal end of a peptide or protein. This methodology can be applied to the synthesis of chemically modified proteins.

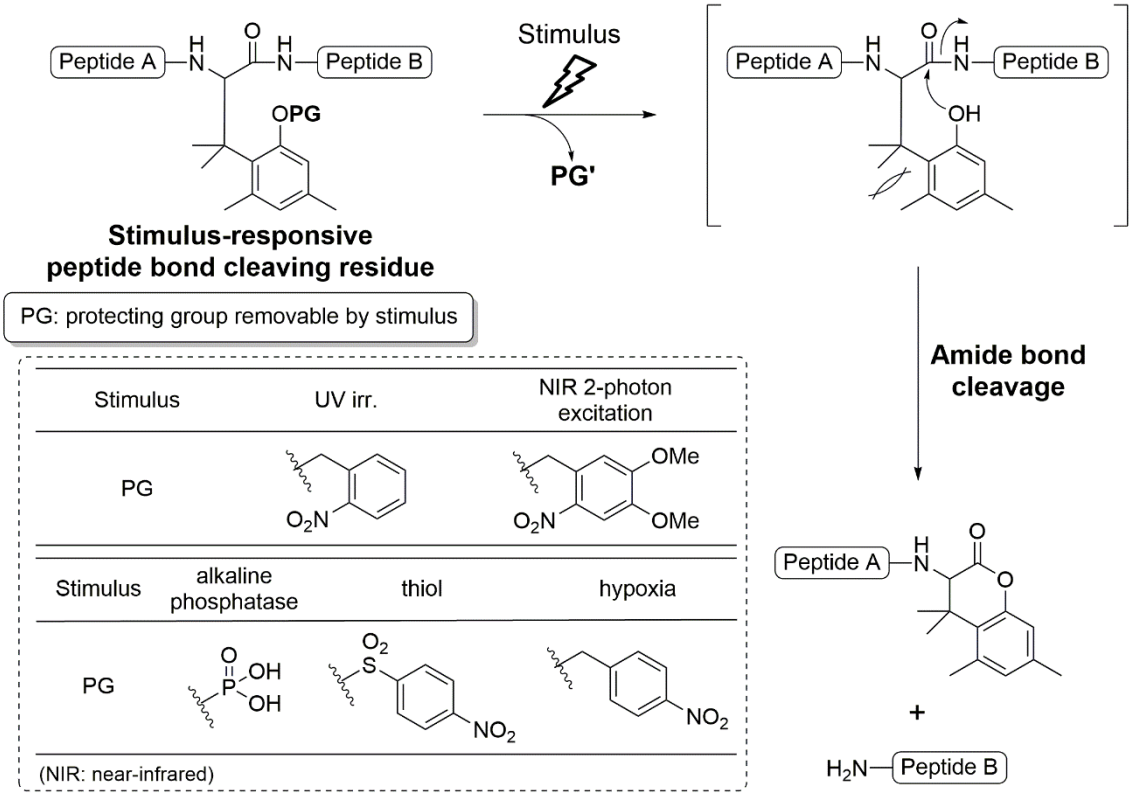
Chapter 1

An Intein-inspired stimulus responsive amide bond processing device

1.1 Introduction

Development of a methodology to control peptide/protein functions in a spatiotemporal manner is indispensable in various research fields such as chemical biology and drug delivery. Photo-induced main chain cleavage[1] or conformational changes[2] of peptides or proteins have been successfully applied to control of such functions. Recently, we developed stimulus-responsive peptide-bond-cleaving residue (Spr)[3] based on trimethyl-lock system[4] (Figure 1.1). Spr induces amide bond cleavage after stimulus-induced removal of a phenolic protective group (PG) from Spr, followed by lactonization. The stimuli available for amide cleavage vary according to the phenolic PGs of choice. Spr has shown its great utility in chemical biology fields.[3] In conjunction with our studies on Spr, we started to develop an alternative new scaffold.

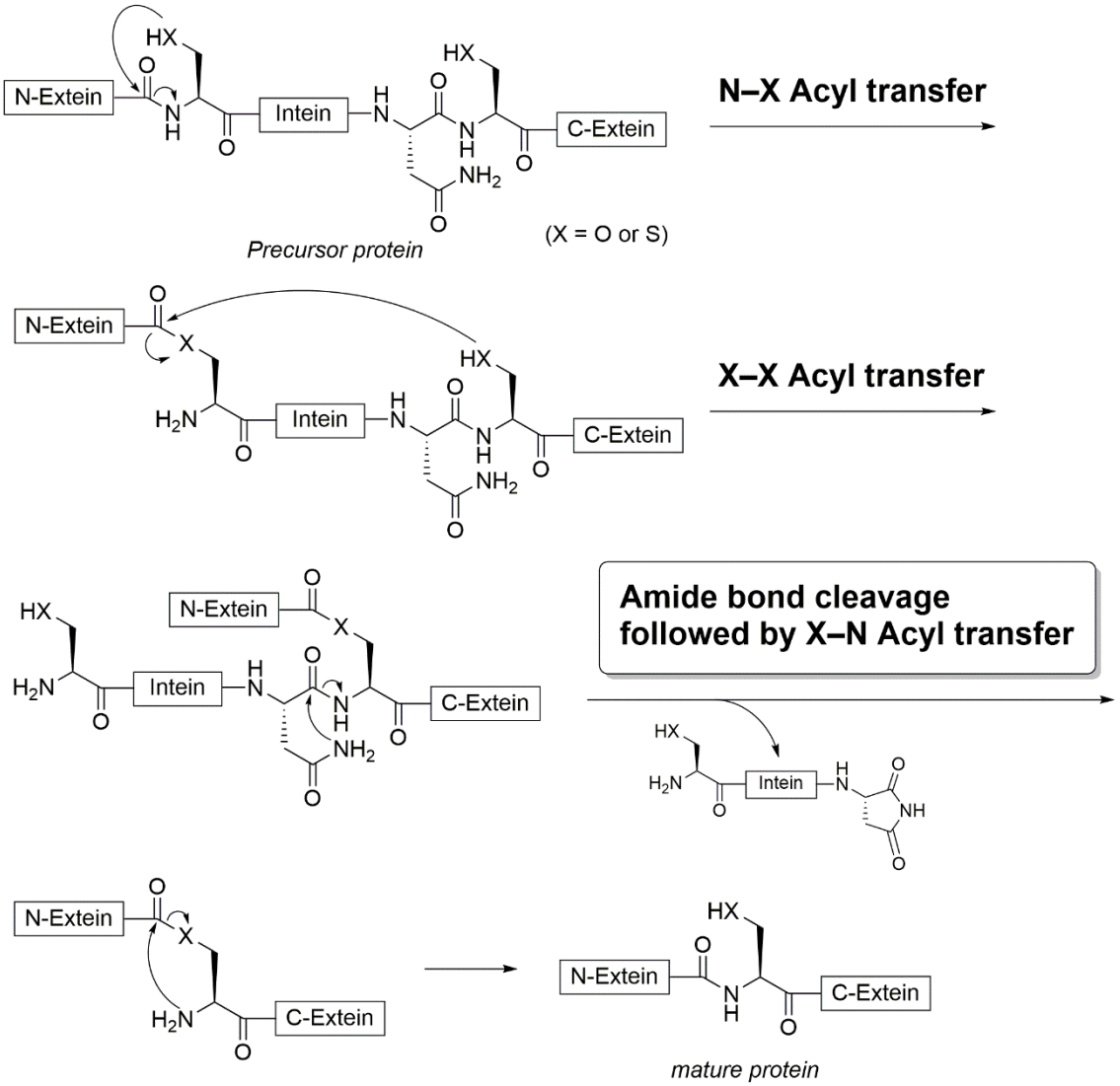
Figure 1.1. Stimulus-responsive peptide-bond cleaving residue (Spr).



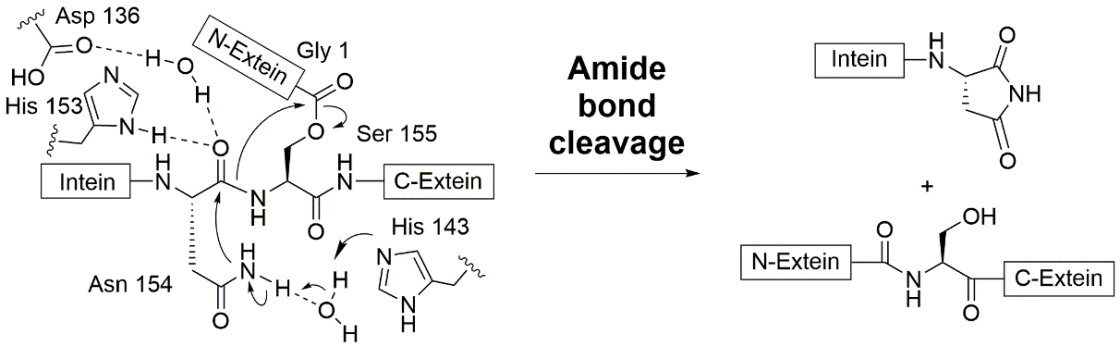
1.2 Design and synthesis of UV-responsive peptide bond processing device

Intein proteins[5,6], which are found in a wide variety of unicellular organisms[6], mediate the self-splicing of proteins containing inteins to produce intein-removed splicing proteins through sequential N–X (X = S or O), X–X, and X–N acyl transfers. (Scheme 1.1).[7] The third step, X–N acyl transfer starts from the imide cyclization-mediated cleavage of an asparagine (Asn)–cysteine (Cys) (or serine (Ser)) bond at the intein–C-extein junction followed by transfer of the *X*-peptidyl unit to the liberated Cys or Ser amino group. Such a sequence of reactions has several requirements including enhancement of nucleophilicity of the amide side chain of Asn, activation of the peptide bond and appropriate arrangement of the functional groups involved in the reactions. The structural basis for this reaction obtained from X-ray crystallographic analysis indicates that appropriately arranged functional units, including water molecules assist in the cleavage of the peptide bond by means of an acid-base-catalyzed mechanism (Scheme 1.2).[8]

Scheme 1.1. Intein-mediated protein splicing.



Scheme 1.2. Proposed mechanism of amide bond cleavage during protein splicing.



This cleavage mechanism inspired the design of a new amide bond cleavage system with a modified Asn structure (Figure 1.2). We envisioned the modifications shown in Figure 1.2 partly met the requirements responsible for the amide cleavage of the intein−C-extein junction. Incorporation of a pendant secondary amine would provide an intramolecular base[9], which could enhance the nucleophilicity of the amide nitrogen via a five-membered ring structure. Furthermore, incorporation of a geminal dimethyl group would lead to a Thorpe-Ingold effect[10], which would assist the modified Asn side chain to form the succinimide ring responsible for the amide cleavage. Lastly, masking the basic character of the secondary amine with *N*-protecting group, such as an *o*-nitorobenzyloxycarbonyl (*o*NBnoc) unit[11], which can be removed photolytically, could provide a simple platform for the development of stimulus-responsive amide bond cleavage structure.

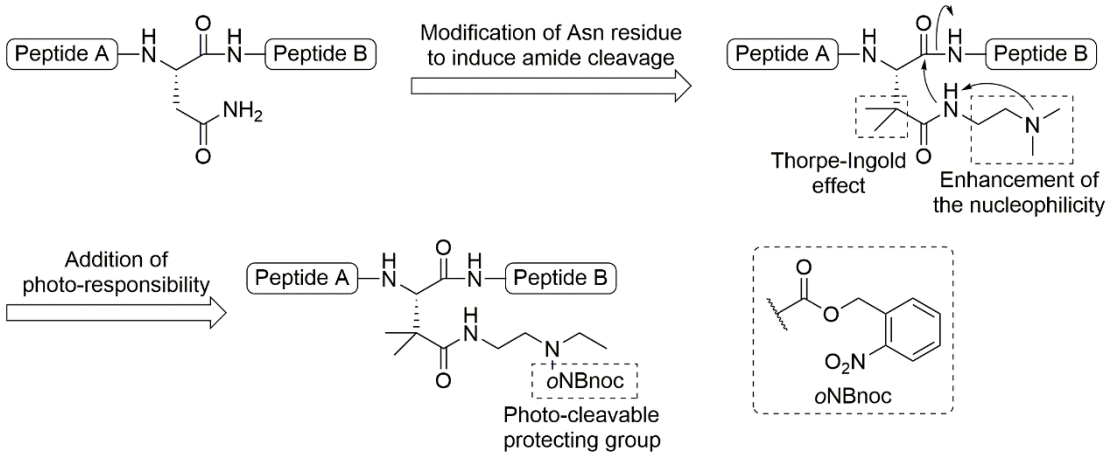
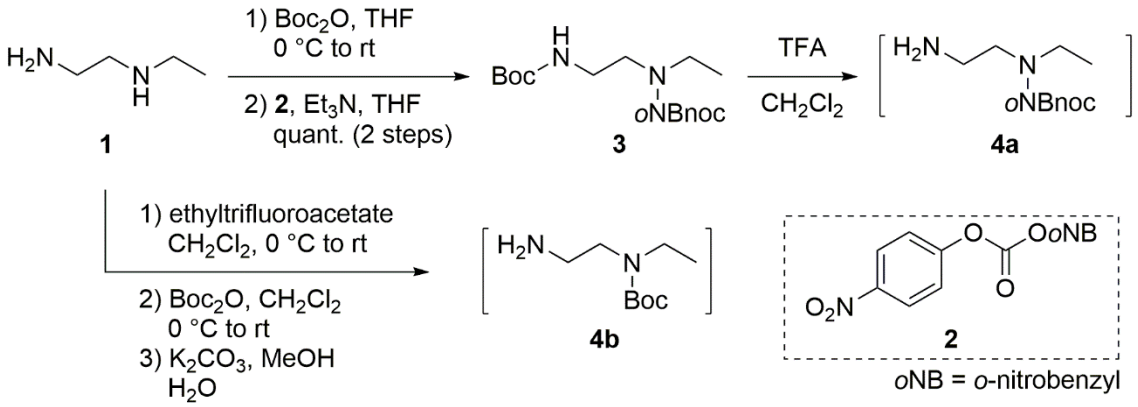


Figure 1.2. Design of intein-mediated UV-responsive amide bond cleavage device.

1.3 Synthesis of an amide bond processing device and its incorporation into peptides

Preparation of pendant secondary amine capable of responding to UV irradiation began with *N*-ethylethylenediamine **1** (Scheme 1.3). Selective protection of the primary amine in **1** with a *t*-butyloxycarbonyl (Boc) group was performed using Boc_2O in THF. Subsequent introduction of an *o*-nitrobenzyloxycarbonyl group onto the secondary amine with *p*-nitrophenylformate **2**[12] in THF in the presence of triethylamine (Et_3N) afforded the requisite compound **3** in quantitative yield (over two steps). Removal of the Boc group from **3** with TFA gave the pendant amine unit **4a**. Synthesis of the Boc-protected pendant unit **4b** was initiated by the trifluoroacetylation of the primary amine in **1** followed by introduction of the Boc group and subsequent hydrolysis of the trifluoroacetyl group.

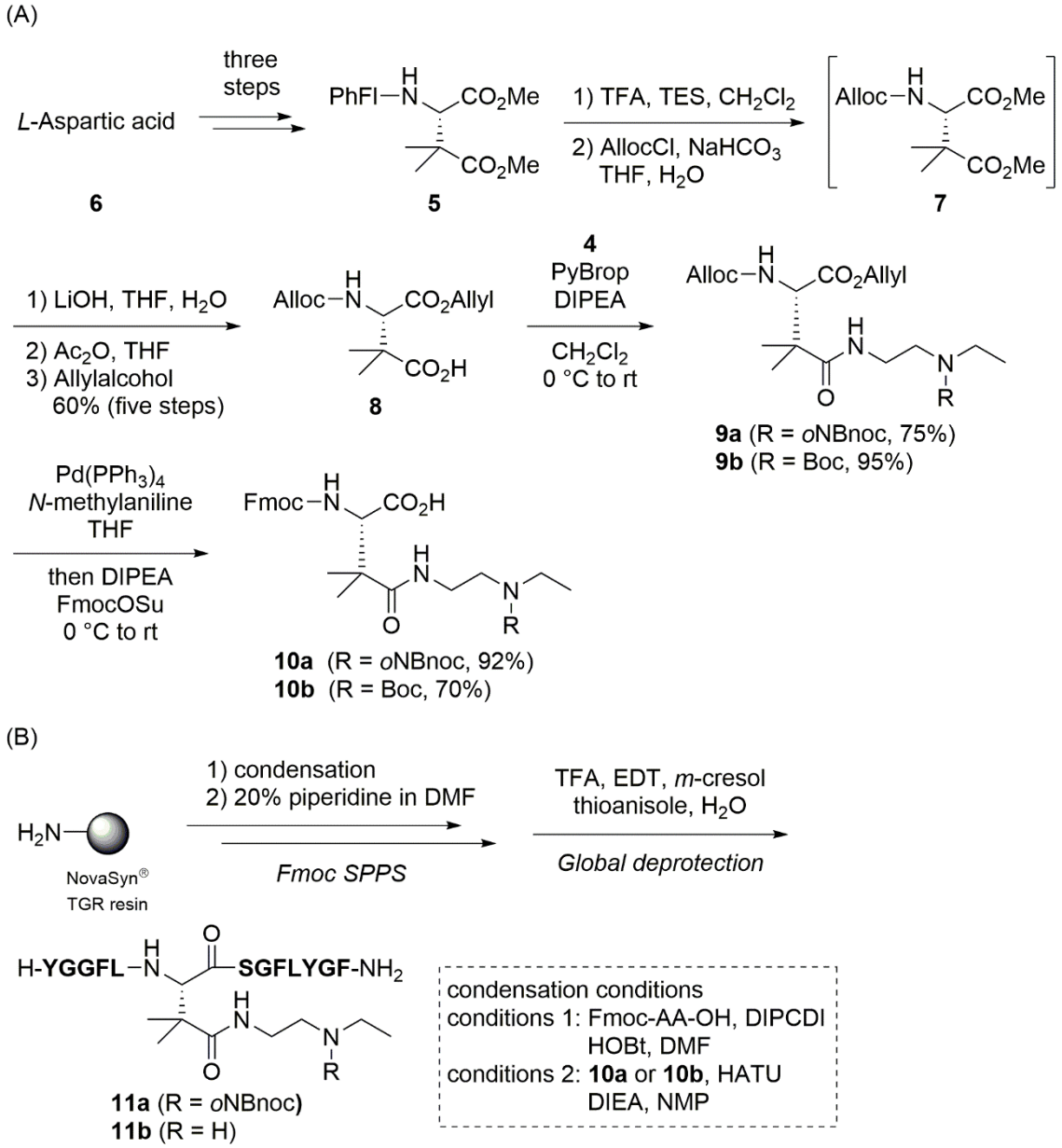
Scheme 1.3. Synthesis of pendant secondary amine **3**.



PhFl-*β*,*β*-diMe-Asp(OMe)-OMe **5**[13] was synthesized in three steps from *L*-aspartic acid **6** following Goodman's procedure (Scheme 1.4). Deprotection of the 9-phenylfluorenyl (PhFl) group of **5** with trifluoroacetic acid (TFA) and triethylsilane (TES) in CH_2Cl_2 followed by reprotection with allylchloroformate (AllocCl) gave Alloc-*β*,*β*-diMe-Asp(OMe)-OMe **7**. Hydrolysis of both the methyl esters of **7** was achieved using LiOH in THF-H_2O at 0 °C to room temperature. Reaction of the obtained carboxylic acid with Ac_2O in THF at reflux temperature followed by alcoholysis with allyl alcohol gave the *α*-allylester **8** in 60% isolated yield (over five steps). Incorporation of the UV-responsive pendant amine unit **4a** to the sterically-crowded *β*-carboxylic group was accomplished by a reaction using bromotripyrrolidinophosphonium hexafluorophosphate (PyBrop) and diisopropylethyl amine (DIPEA) in CH_2Cl_2 to yield the fully protected Asn derivative **9a** in 75% isolated yield. Conversion of **9a** to the 9-fluorenylmethyloxycarbonyl (Fmoc) derivative suitable for Fmoc solid-phase peptide synthesis (SPPS) was performed by deprotection of the allyl and allyloxycarbonyl (Alloc) groups by the action of $Pd(PPh)_4$ and *N*-methylaniline followed by reprotection of the regenerated amine with an Fmoc group, and the desired *o*NBnoc-type Fmoc-protected Asn derivative **10a** was obtained in 92% isolated yield. Similarly, Boc-type Fmoc-protected Asn derivative **10b** was obtained in 70% isolated yield from **9b**.

Having requisite Asn derivatives in hand, we next synthesized a model peptide **11** (H–**YGGFL**–**X**–**SGFLYGF**–NH_2 (**X** = Asn or Asn derivatives)) to examine the self-processing of peptides. Fmoc amino acids were condensed on NovaSyn® TGR resin using diidopropylcarbodiimide (DIPCDI) and 1-hydroxybenztriazole (HOBt) in dimethylformamide (DMF) with the exception of the condensation of **10a**. For incorporation of **10a**, 1-[bis-(dimethylamino)methylene]1*H*-1,2,3-triazolo[4,5-β]pyridine-3-oxide hexafluorophosphate (HATU) and DIEA in *N*-methylpyrrolidone (NMP), which has superior reactivity to DIPCDI/HOBt, were used. Exposure of the resulting completed peptide resin to TFA-ethanedithiol (EDT)-*m*-cresol-thioanisole-H_2O at room temperature for 2 h afforded a mixture of two peptide materials whose mass value are identical to that of the desired material (Figure 1.3 (A)).

Scheme 1.4. (A) Synthesis of intein-inspired amide cleavage structures **10a** and **10b**. (B) Preparation of model peptides **11a** and **11b**.



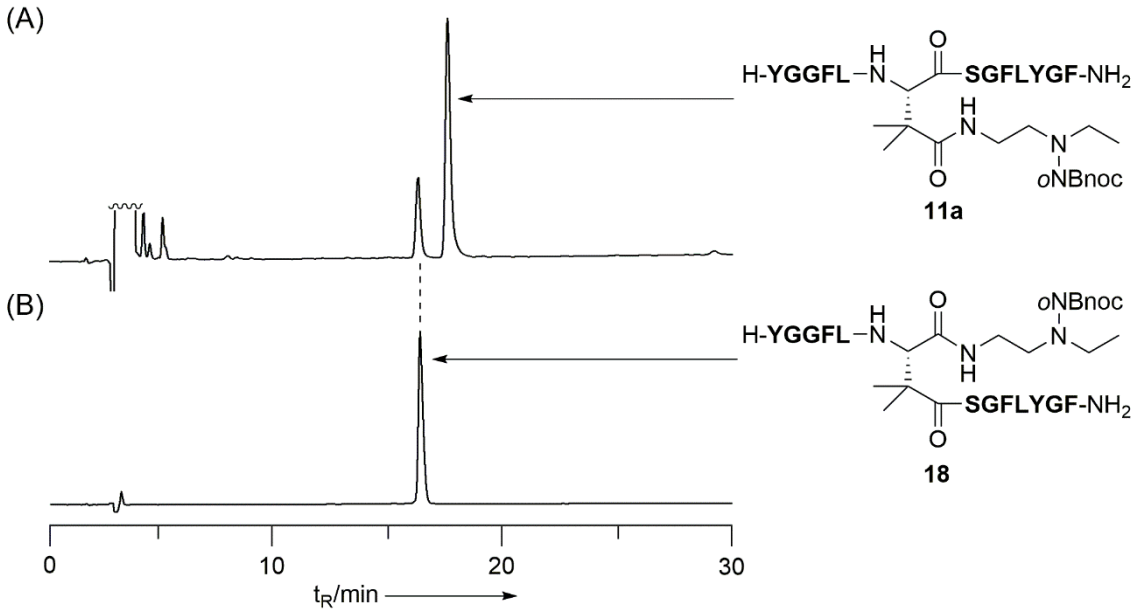
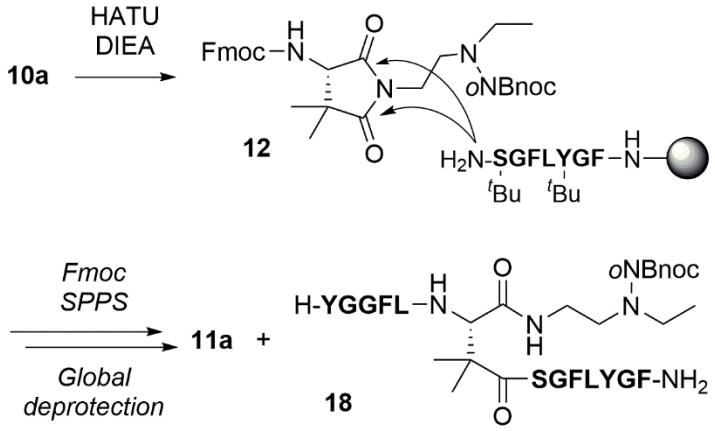


Figure 1.3. HPLC profiles of (A) a crude peptide prepared using **10a** (**11a** and **18** were obtained in 3:1 ratio) and (B) a purified peptide **18** prepared using **16** (as shown in Scheme 1.6). HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column (4.6 × 250 mm; detection 220 nm) with a linear gradient of 0.1% TFA in MeCN (38–50% over 30 min) in 0.1% TFA aq. at flow rate 1.0 mL/min.

The production of two peptides was thought to be attributable to the formation of a succinimide species **12** by activation of **10a** followed by aminolysis involving the amino group of the growing peptide chain (Scheme 1.5). Since the *β*-carboxyl group of the two possible electrophilic sites in the succinimide species is sterically crowded by the neighboring geminal dimethyl group, the major product was thought to be the desired *α*-peptide **11a**. Although preventing the formation of such a succinimide should be possible by incorporation of benzyl-type protection on the amide nitrogen,[14] an attempt to synthesize the dimethoxybenzyl (DMB)-protected Asn derivative **13** failed (Scheme 1.6). Although condensation of the *N*-dimethoxybenzyl diamine derivative **14a** with **8** gave the desired compound **15** in 50% isolated yield, subsequent removal of the protecting groups induced the release of the pendant amine molecule **14a**, possibly through nucleophilic attack of the *α*-carboxylate on the substituted amide. The *α*-amide protected derivative **16**, however, could be synthesized from the *β*-allyl ester **17** which was obtained as an isomer of Alloc-*β*,*β*-diMe-Asp(OH)-OAllyl **8**.

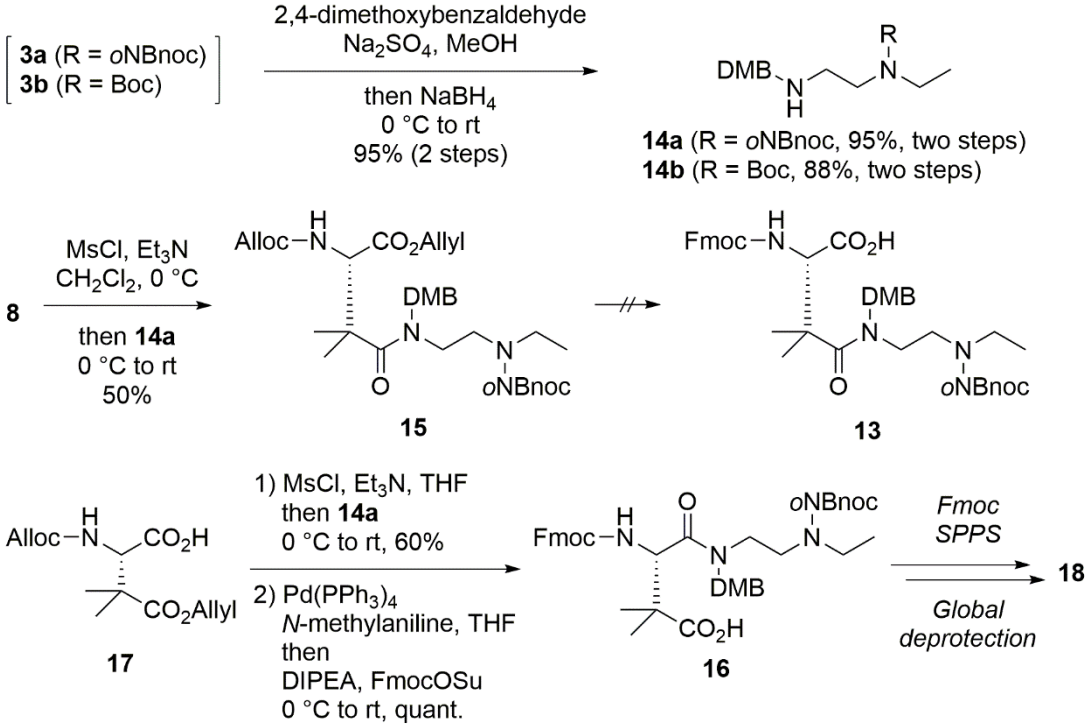
Scheme 1.5. Possible mechanism for the generation of byproduct **18**.



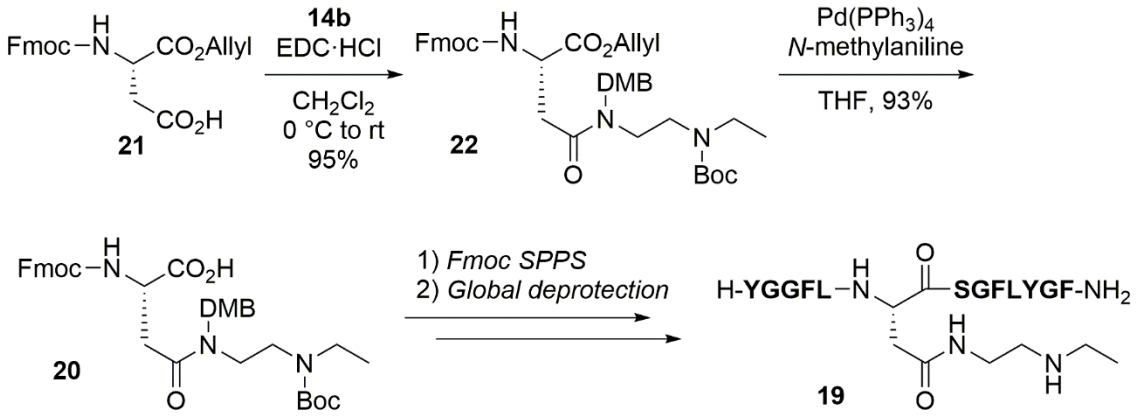
The resulting *β*-carboxylic derivative **16** which can tolerate imidation was also incorporated into a peptide resin in a manner similar to that employed for **11a**. Deprotection of the resin afforded the *β*-peptide **18** (Figure 1.3 (B)). This result clearly indicates that major product **11a** and minor product **18** (Figure 1.3 (A)) are *α*- and *β*-peptides, respectively. The peptide without oNBoc group **11b** was also prepared as stable TFA salts using the Boc-protected secondary amine **10b** in a manner similar to that employed for **11a**. As observed in the case of incorporation of **10a**, deprotection of the protected resin afforded a mixture of *α* and *β*-peptides.

The peptide **19** was also prepared to determine the effects of the geminal dimethyl group and secondary amine on the outcome of the amide bond cleavage (Scheme 1.7). A protected Asn derivative lacking the geminal dimethyl group **20** was prepared by coupling of the *β*-carboxylic acid of the Asp derivative Fmoc-Asp(OH)-OAllyl **21**[15] with *N*-DMB-*N'*-Boc-*N'*-ethylethylenediamine **14b** followed by removal of allyl ester of **22**. Fmoc-based incorporation of the resulting amino acid in the resin followed by acidic deprotection afforded the desired peptide **19** possessing an Asn residue modified by the pendant secondary amine but lacking protection of the secondary amine. During both the acidic deprotection and the HPLC purification, no significant side reactions such as peptide bond hydrolysis were observed.

Scheme 1.6. Synthetic approach for **13** and synthesis of an isomeric peptide **18**.



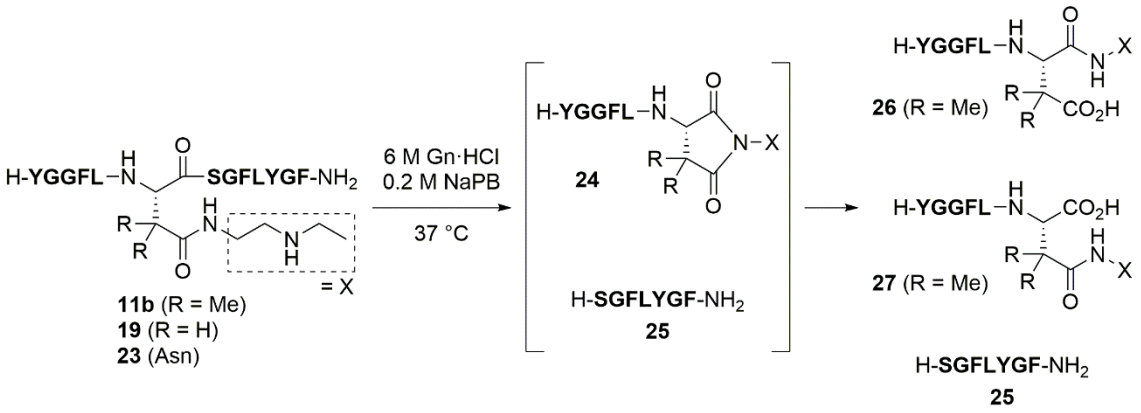
Scheme 1.7. Synthesis of model peptide **19**.



1.4 Evaluation of the effect of side chain structure on the amide bond cleavage

The Asn-containing peptide **23** was synthesized by standard Fmoc protocols and self-processing of synthetic peptides was examined (Scheme 1.8). Peptide samples were dissolved in buffer solution (6 M guanidine hydrochloride (Gn·HCl)-0.2 M Na phosphate) and amide bond cleavage was monitored by HPLC analysis of the reactions. As expected, the presence of both the secondary amine as an intramolecular base and the geminal dimethyl group as an inducer of cyclization greatly facilitated the cleavage of the amide bond. The cleavage reaction at pH 7.4, 37 °C in 24 h went to near completion to afford a mixture of split peptides consisting of the N-half imide peptide **24**, the C-half peptide **25** and succinimide-opening peptides **26** and **27** (Figure 1.4 (A)). We conducted comparison experiments using reference peptides **19** (without dimethyl group) and **23** (without dimethyl group and amine unit) and time course of disappearance of each substrate is shown in Figure 1.4 (B). These results indicate that modifications which mimic environments involved in the intein-induced amide cleavage are responsible for the envisioned artificial amide bond cleavage.

Scheme 1.8. Self-processing reaction of model peptides **11b**, **19** and **23**.



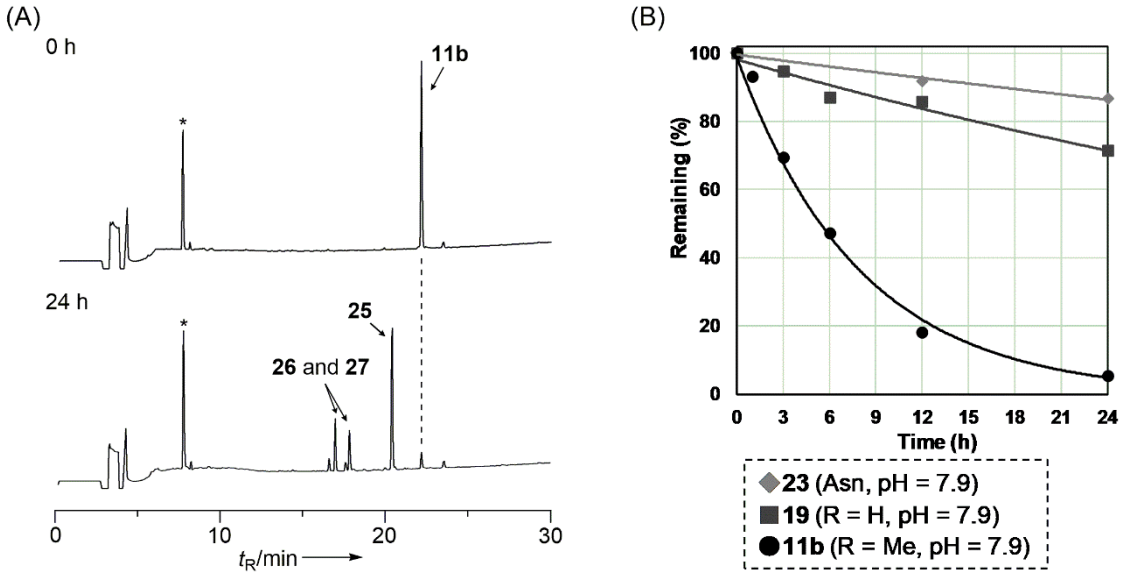
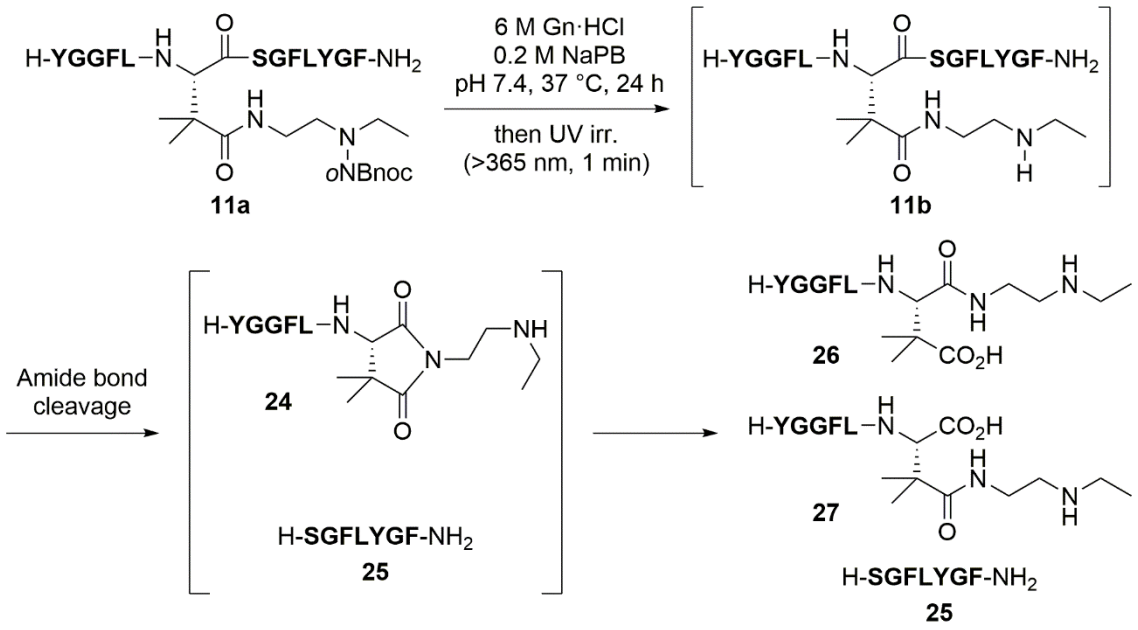


Figure 1.4. (A) HPLC monitoring of the amide bond cleavage reaction of peptide **11b**. *Internal standard. HPLC conditions: Cosmosil $5C_{18}$-AR-II analytical column (4.6 × 250 mm; detection 220 nm) with a linear gradient of 0.1% TFA-MeCN (1–60% over 30 min) in 0.1% TFA aq. at flow rate 1.0 mL/min. (B) Time course of remaining substrate **11b**, **19** or **23**. The percentage of substrate was estimated based on HPLC peak area.

1.5 UV-responsive amide bond cleavage

Encouraged by potential utility of the modified Asn **10a** (see Scheme 1.4) as a stimulus-responsive processing structure, we examined the photo-responsive peptide bond cleavage of the synthetic peptide **11a** (Scheme 1.9). The *o*NBnoc-protected peptide **11a** incubated in 6 M Gn·HCl-0.2 M Na phosphate, pH 7.4 at 37 °C for 24 h with no irradiation, remained almost intact (Figure 1.5). Irradiation of the reaction mixture induced removal of *o*NBnoc group from the secondary amine unit to produce peptide **11b**, which was degraded to the processing peptides. These results clearly indicate that the modified Asn **11a** could serve as a stimulus-responsive processing structure alternative to Spr designed using the concept of trimethyl lock system.

Scheme 1.9. UV-responsive amide bond cleavage reaction.



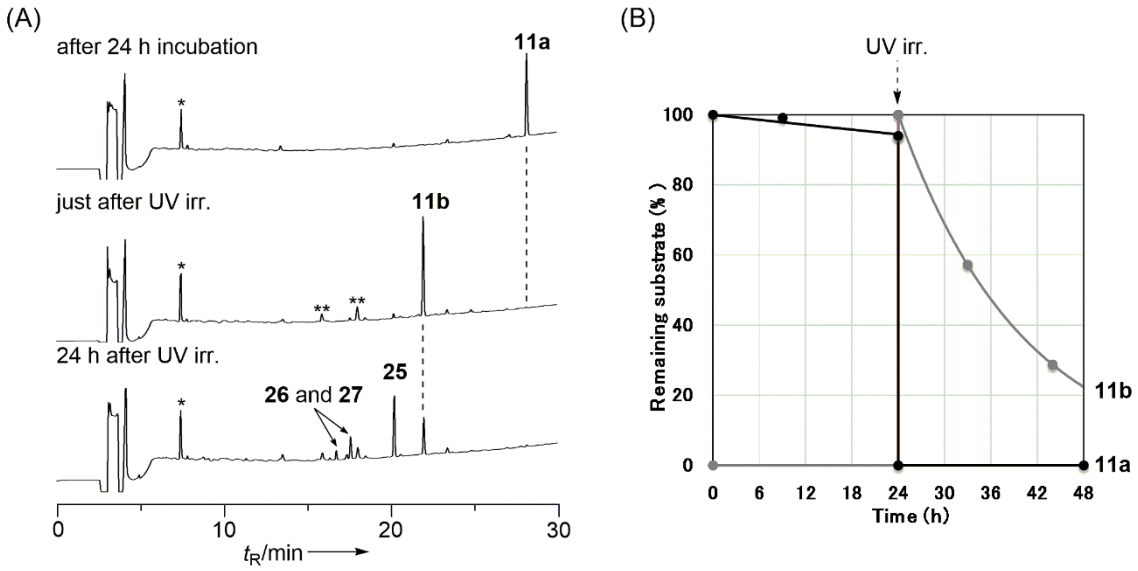


Figure 1.5. (A) HPLC monitoring of the UV-responding amide bond cleavage reaction of peptide **18**. *Internal standard. **Not peptidyl compounds, probably derived from deprotected *o*NBnoc group with UV irradiation. HPLC conditions: Cosmosil 5C_{18}-AR-II analytical column (4.6 × 250 mm; detection 220 nm) with a linear gradient of 0.1% TFA-MeCN (1–60% over 30 min) in 0.1% TFA aq. at flow rate 1.0 mL/min. (B) Time course of remaining substrate **11a** and **11b**. The percentage of substrate was estimated from the HPLC peak area.

1.6 Conclusion

In conclusion, mimicking the chemical environment involved in the intein-mediated protein splicing step has led to development of a stimulus-responsive amide bond cleavage structure. Masking the secondary amine unit with a photo-cleavable protection endows photo-responsibility on the designed amino acid residue.

Chapter 2

Protease-mediated protocol for preparation of protein thioesters

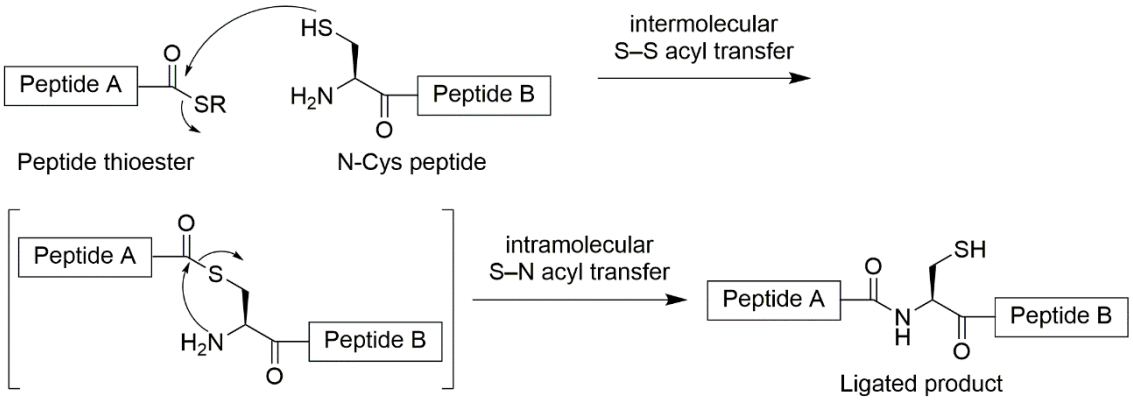
2.1 Introduction

Chemically or post-translationally modified proteins have served as powerful tools for understanding the biological involvement of such proteins in physiologically important events. Chemical synthesis[16] and semi-synthesis[17] of proteins are strategies commonly used to introduce a wide variety of structural modifications into proteins. At present, both approaches mainly rely on native chemical ligation (NCL)[18], which is a chemoselective reaction forming an amide (Scheme 2.1). This reaction features chemoselective intermolecular S–S acyl transfer between a peptide thioester and a peptide with an N-terminal cysteine followed by intramolecular S–N acyl transfer to afford a ligated peptide without side chain protection.

Chemical synthesis of proteins using an NCL protocol consists of the following

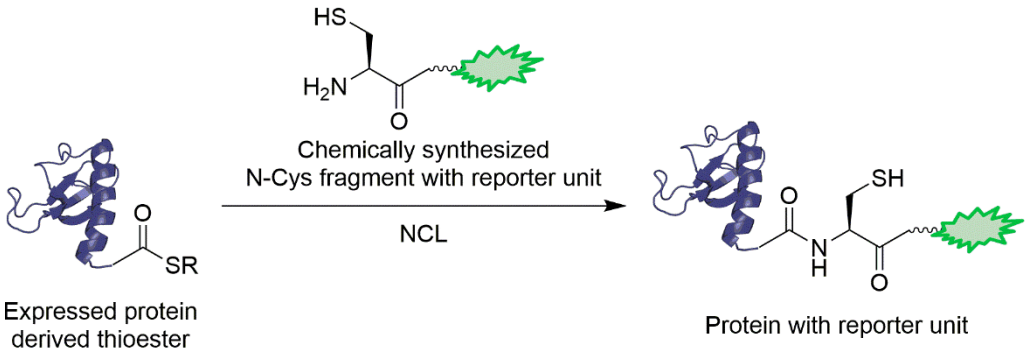
two steps: (1) preparation of peptide thioesters and N-terminal cysteinyl peptides by solid phase peptide synthesis (SPPS) and (2) condensation of the two peptide fragments in solution by NCL.[19] The NCL-mediated chemical protocol enables the synthesis of proteins consisting of ~100 residues with comparative ease, but that is not always the case with larger proteins with >100 residues, due to size limitations of the peptide chains amenable to the SPPS protocol. In such cases, complicated procedures including multiple condensations followed by HPLC purifications of resulting intermediary products are necessary.

Scheme2.1. Native chemical ligation (NCL).



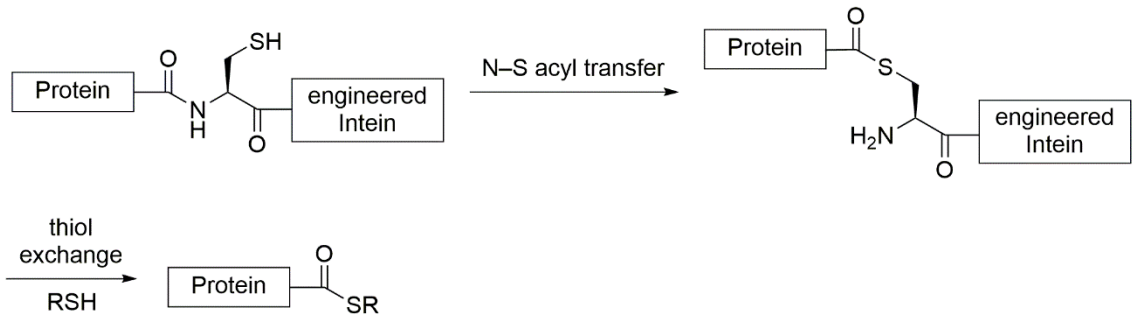
Protein semi-synthesis using NCL has the potential to solve the problems of chemical synthesis to give a chemically functionalized large protein in a few steps (Scheme 2.2).[17] This semi-synthetic approach is based on the NCL of a synthetic cysteinyl peptide with a protein thioester derived from a recombinant protein.

Scheme 2.2. Protein semi-synthesis using NCL.



Facile and efficient preparation of protein thioesters from expressed proteins is an indispensable means that can enjoy success in protein semi-synthesis using NCL protocol. In this way, challenges for preparation of protein thioesters from naturally occurring peptide sequences have been extensively investigated. One of the most used protocol for production of thioesters is the intein-mediated protocol (Scheme 2.3).[17b,20] Inteins are self-splicing elements that can be engineered to generate a protein thioester upon self-catalyzed N–S acyl transfer followed by thiol exchange with an external thiol. However, the use of an intein protocol does not always produce the desired thioester with satisfactory efficiency.[21]

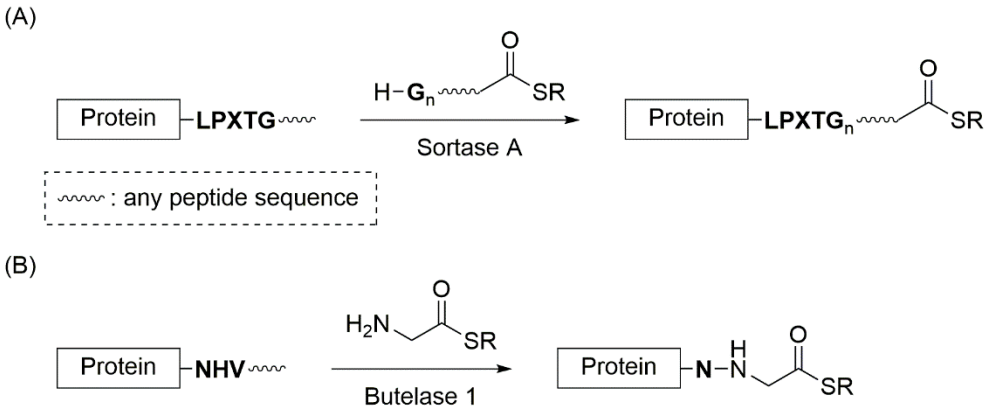
Scheme 2.3. Intein-mediated protocol for preparation of protein thioester.



Other protocols using enzymes such as sortase[22] or butelase[23] have also been reported (Scheme 2.4). Sortase A, which is a transpeptidase, recognizes the –**LPXTG**– (**X**: any amino acid) sequence and cleaves the threonine–glycine bond to form new amide bond with oligoglycine thioester (Scheme 2.4 (A)). Butelase 1 recognizes the –**NHV**– sequence and cleaves the asparagine–histidine bond to produce C-terminal glycyl thioesters through a transpeptidyl reaction with glycine thioester (Scheme 2.4 (B)). These

enzyme-mediated protocols are applicable to expressed proteins, but these protocols give only thioesters with the recognition sequence involved in the enzymatic reaction.

Scheme 2.4. (A) Sortase- or (B) butelase-mediated protocol for preparation of protein thioester.



Recently, we developed two chemistry-based protocols applicable to naturally occurring peptide sequences (Figure 2.1). One is a sequence-dependent thioesterification protocol using a sequential quadruple acyl transfer (SQAT) system (Figure 2.1 (A)).[24] This protocol includes four acyl transfers: (1) Ni(II)-mediated acyl migration of the peptide bond preceding a –**SRHW**– sequence to its serine side chain (N–O acyl transfer); (2) methanolysis of the resulting isopeptide (O–O acyl transfer); (3) conversion of the methyl ester to a hydrazide (O–N acyl transfer); and (4) conversion of the resulting hydrazide to a thioester (N–S acyl transfer) using the protocol described by Liu.[25] Although thiolysis or hydrazinolysis of the Ser-isopeptide is tempting for affording desired materials, these attempts resulted in failure. Another is a regioselective S-cyanylation/hydrazinolysis protocol (Figure 2.1(B)).[26] In this protocol, a zinc-finger sequence fused to C-terminal end of the peptide of interest, in which cysteine and histidine form a complex with zinc(II) ions, is employed for achievement of the regioselective cyanylation. Cysteine residues in the fused peptide are protected by photoremoval *O*-nitroveratryl group in the presence of zinc ions, and this results in regioselective protection of the cysteine in the peptide. Removal of the zinc protection on the zinc finger sequence followed by S-cyanylation of the regenerated sulfanyl group gives an *S*-cyanopeptide, which can be converted to a peptide hydrazide by hydrazinolysis. The resulting hydrazide can be converted to the corresponding peptide thioester.

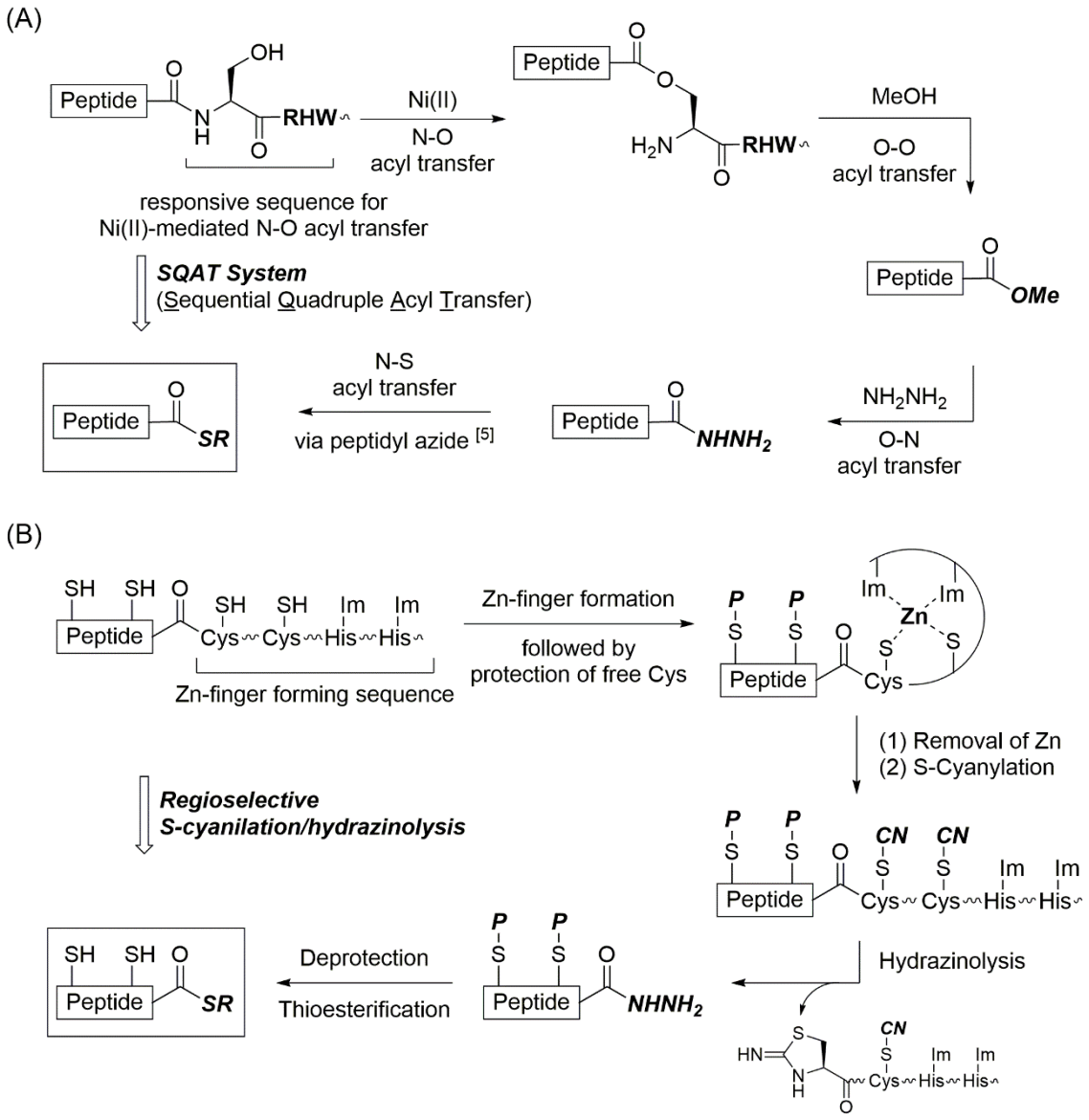


Figure 2.1. Chemistry-based protocols based on (A) sequential quadruple acyl transfer (SQAT) system and (B) regioselective S-cyanilation/hydrazinolysis.

However there remains much room for improvement; the use of MeOH in SQAT system sometimes induces precipitation of proteins and the S-cyanylation-mediated approach, which requires a multi-step conversion, generally results in low isolated yields of thioesters. In this context, we attempted to develop a new method for the preparation of protein thioesters.

2.2 Strategy and initial attempts at CPY-mediated thioester synthesis

On the basis of the biochemical aspects of enzymatic hydrolyses, we focused on carboxypeptidase Y (CPY) (EC 3.4.16.5)[27], which is easily available from commercial sources or baker's yeast.[28] This enzyme is an exopeptidase which hydrolyzes the peptide bond at the C-terminal end of a peptide or protein. In this hydrolysis process, an *O*-acyl enzyme intermediate should be involved because CPY is a serine protease (Figure 2.2(A)). Hydrolysis of this intermediate affords a peptide/protein acid.

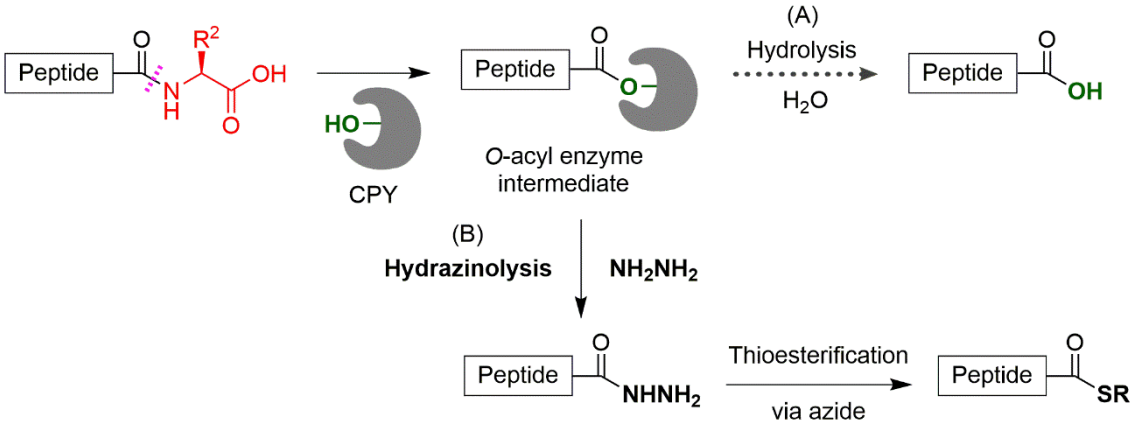


Figure 2.2. (A) CPY-mediated hydrolysis of a peptide bond and (B) strategy of CPY-mediated thioester synthesis.

Inspired by this mechanism, we envisioned that hydrazinolysis of the *O*-acyl enzyme intermediate should lead to the formation of a hydrazide, which can in turn be converted to the corresponding thioester via a peptide azide (Figure 2.2(B)). This hypothesis prompted us to attempt at the hydrazinolysis of a model peptide **28** (Ac–**ALYGAA**–OH) in the presence of CPY in hydrazine-containing aqueous solution (Figure 2.3).

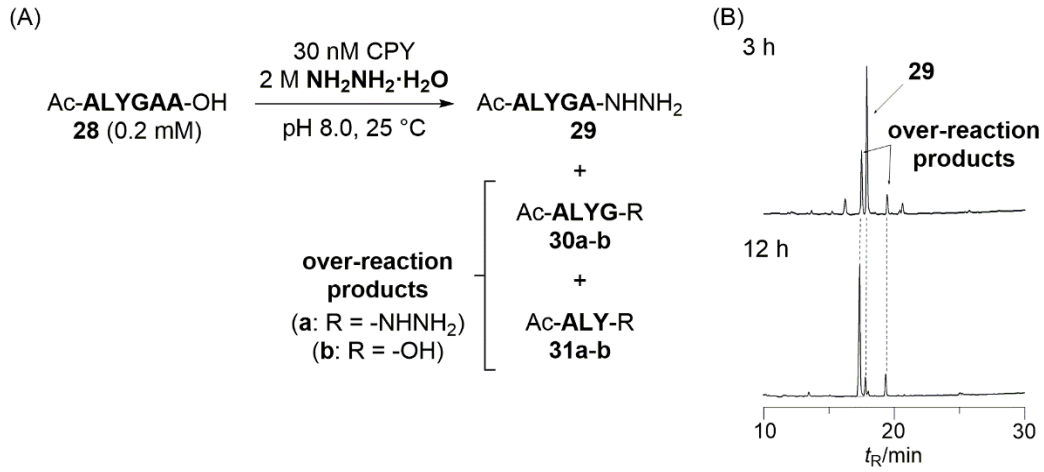


Figure 2.3. (A) CPY-mediated digestion of model peptide **28** in the presence of hydrazine. (B) HPLC monitoring of CPY-mediated hydrazinolysis of peptide **28**. HPLC conditions: a linear gradient of 0.1% TFA-MeCN (1–40% over 30 min) in 0.1% TFA aq.

Although the desired peptide hydrazide **29** was generated, products of over-reaction (**30**, **31**) were also observed and the proportion of these products increased as the reaction time increased. This result is consistent with a previous report that CPY-mediated aminolysis affords C-terminal amide peptides but these amides undergo further degradation.[29] We examined some other nucleophiles including thiol instead of hydrazine, but these attempted reactions resulted in failure. Theorizing that the amidase activity of CPY[30] is related to the degradation of the resulting peptide hydrazide **29**, we attempted to suppress this over-reaction.

2.3 Suppression of CPY-mediated over-reaction

First, we attempted to protect the desired hydrazide **29** from over-reaction by achieving hydrazone formation with a carbonyl compound. In initial screening of carbonyl compounds as additives, cyclohexanone was proven to suppress the CPY-induced over-reaction and the yield of **29** approached 70% (Figure 2.4). However, further significant improvement of the reaction was not observed by tuning the reaction conditions, including addition of aniline as a catalyst for hydrazone formation.[31]

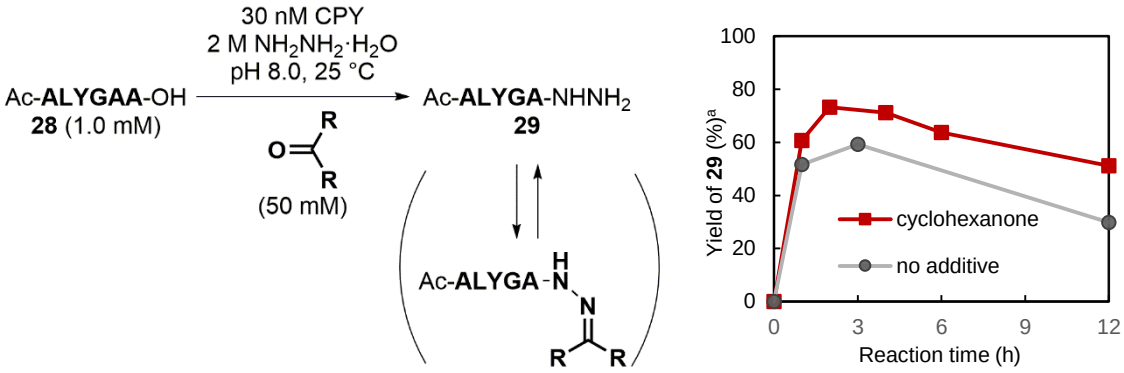


Figure 2.4. Partial suppression of over-reaction by use of cyclohexanone to form a hydrazone. [a]Yield (%) was determined by HPLC separation and integration of product **29** (integ. **29**) as a fraction of the sum of the unreacted **28** (integ. **28**) + products derived from **28** (integ. **others**) + integ. **29**.

Consequently, we next turned our attention to substrate preference of CPY.[32] This enzyme has strong preference concerning C-terminal amino acids of the substrate; C-terminal hydrophobic residues such as Leu, Phe and Val are favored amino acids and proteins are cleaved very rapidly at these residues, whereas hydrophilic or cyclic residues including Arg, Lys and Pro are disfavored amino acid residues where cleavage is quite slow. Therefore, the C-terminal "–(disfavored amino acid)–(favored amino acid)–OH" sequence would be a combination of amino acids that could yield a single amino acid-deleted hydrazide (Figure 2.5). To test this hypothesis, Ac–**ALYGPL**–OH **32** (1 mM) was subjected to hydrazinolysis protocol (2.4 μM CPY, 2 M $NH_2NH_2·H_2O$, 50 mM cyclohexanone, pH 8.0, 25 °C). After a 12 h reaction of **32**, the desired Ac–**ALYGP**–$NHNH_2$ **33** was formed as the major product along with trace amounts of products from over-reactions. Ac-**ALYGXL**-OH (X = R, K) was also subjected to the reaction mentioned above, but the over-reactions cannot be efficiently suppressed.

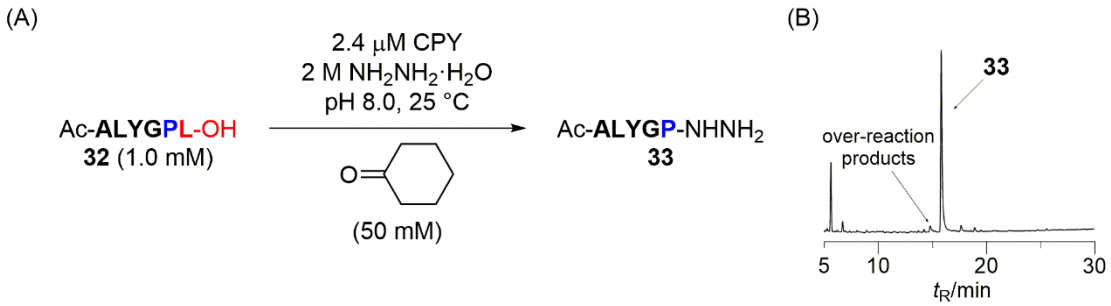


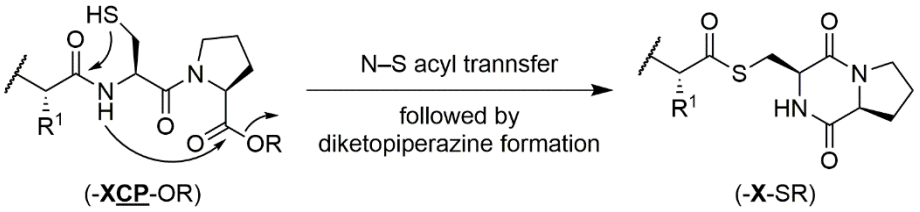
Figure 2.5. (A) Suppression of over-reaction by use of substrate preference of CPY in the presence of cyclohexanone. (B) HPLC monitoring of CPY-mediated hydrazinolysis of peptide **32**. HPLC conditions: a linear gradient of 0.1% TFA-MeCN (5−40% over 30 min) in 0.1% TFA aq.

The resulting hydrazide **33** could be successfully converted to the corresponding thioester (Ac–**ALYGP**–SR) via a peptide azide (Ac–**ALYGP**–N_3) according to Liu's protocol; however, the thioesters that were obtained were limited to only the prolyl thioester (Ac–**ALYGP**–SR). Furthermore, this prolyl thioester is not a versatile thioester in NCL reactions because of its low reactivity as generally reported.[19a] Consequently, we next examined the step-wise conversion to various thioesters via prolyl thioesters.

2.4 Synthesis of versatile thioester by using CPE-like thioesterification

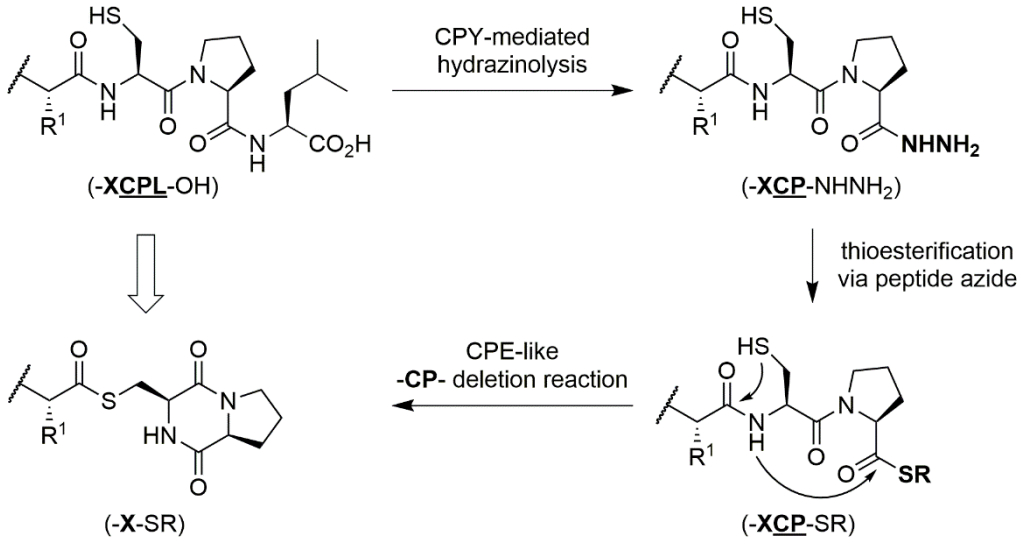
We paid our attention to the cysteinyl prolyl ester (CPE) system, which was developed by Kawakami and Aimoto[33] for preparation of peptide thioesters (Scheme 2.5). Under weakly basic conditions (pH > 7.8), CPE peptides (–**XCP**–OR) are converted to thioesters (–**X**–SR) in which two residues have been deleted via an N–S acyl transfer followed by diketopiperazine formation.

Scheme 2.5. Cysteinyl prolyl ester (CPE) system.



Inspired by this system, we envisaged a novel strategy for the synthesis of versatile thioesters using a combination of CPY-mediated hydrazinolysis and CPE-like two-residue deletion (Scheme 2.6). In this strategy, a C-terminal –**CPL**–OH peptide, (–**XCPL**–OH) is converted to the prolyl hydrazide (–**XCP**–$NHNH_2$) by CPY-mediated hydrazinolysis. The obtained hydrazide is converted to the corresponding thioester (–**XCP**–SR) via a peptide azide, followed by CPE-like two-residue deletion reaction. This sequential reaction was thought to allow for the synthesis of thioesters other than prolyl thioesters (–**X**–SR).

Scheme 2.6. Envisioned strategy for synthesis of thioester other than prolyl thioester.



A model peptide **34** (H–**LYRAACP**–$NHNH_2$, 1 mM) was treated with $NaNO_2$ in 6 M guanidine·HCl–50 mM Na phosphate (pH 3.0) at -10 °C for 30 min followed by the addition of mercaptophenyl acetic acid (MPAA) and the N-terminal cysteinyl peptide

35 (pH 6.5) at room temperature (Figure 2.6). After 3 min post-addition, thioesters **36** (H–**LYRAACP**–SAr (Ar = $–C_6H_4CH_2CO_2H$)) and **37** (H–**LYRAA**–S–DKP (DKP = diketopiperazine)) and **38** (H–**LYRAA**–SAr), concomitantly formed from the release of Cys–Pro diketopiperazine, were observed. Without isolating these thioesters, **35** underwent NCL with **38** but not with **36**, to afford the desired ligated peptide **39** almost quantitatively. The reaction of Pro-SR to afford thioesters proceeded in mild acidic condition, while that of Pro-OR requires weakly basic condition (pH > 7.8) which induce partial hydrolysis of desired thioesters.[33]

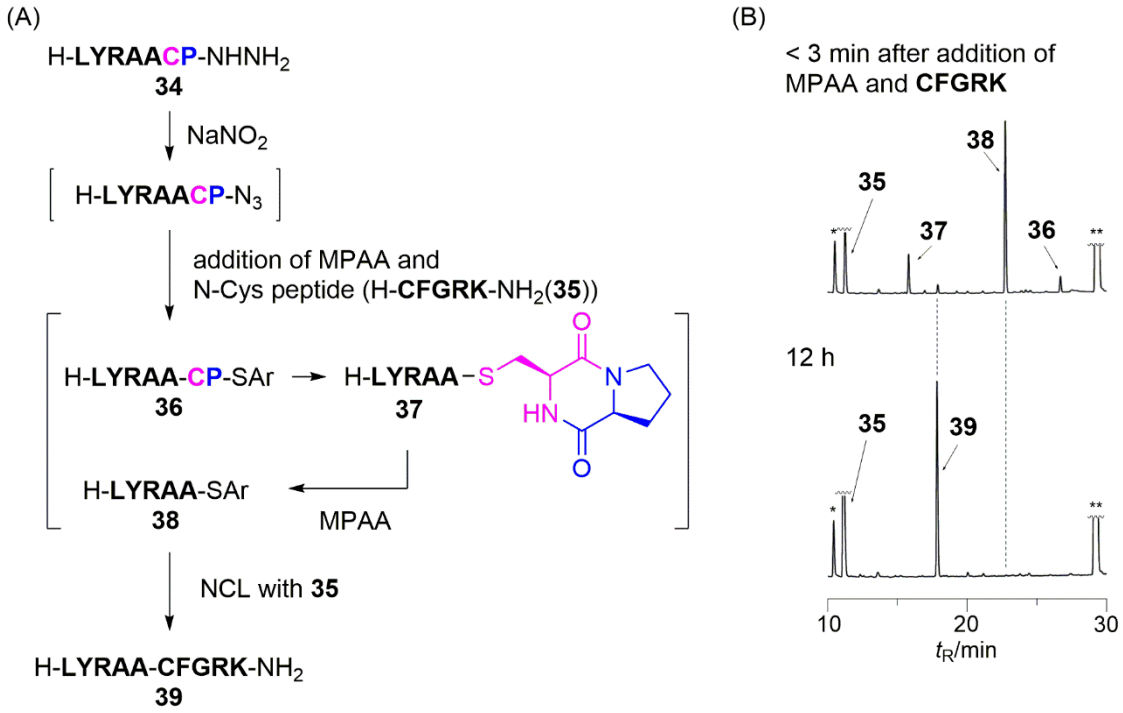


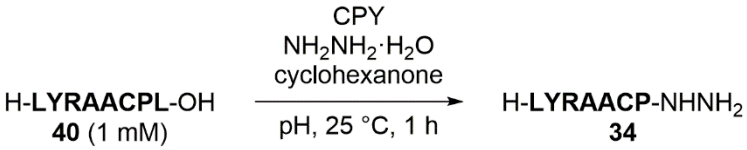
Figure 2.6. (A) NCL of model hydrazide **34**. (B) HPLC monitoring of NCL of peptide **34**. HPLC conditions: a linear gradient of 0.1% TFA-MeCN (5−35% over 30 min) in 0.1% TFA aq. *Non-peptidic impurity. **MPAA.

2.5 Optimization of reaction conditions for CPY-mediated hydrazinolysis

For efficient production of the C-terminal –**XCP**–$NHNH_2$ peptide, we optimized the reaction conditions of the CPY-mediated hydrazinolysis step (Table 2.2). The C-

terminal **–CPL**–OH peptide **40** (H–**LYRAA<u>CPL</u>**–OH) was also efficiently converted to the hydrazide **34** by CPY-mediated hydrazinolysis (entry 1). Concentrations of hydrazine and cyclohexanone in the range of 0.2–1.0 M (entries 1−3) and 60–90 mM (entries 1, 5 and 6), respectively, had no effect on the conversion efficiency. The optimal pH was found to be in the range 5.4–7.4 (entries 1 and 7–9) and the effective concentration of CPY is 1.2 μM (entries 3 and 10). On the basis of these experiments, we concluded that the conditions of entry 3 (1.2 μM CPY in the presence of 0.2 M $NH_2NH_2{\cdot}H_2O$, 60 mM cyclohexanone, pH 6.4, at 25 °C) are optimum for the efficient conversion of the C-terminal –**CPL**–OH peptide to –**CP**–$NHNH_2$.

Table 2.2. Optimization of CPY-mediated hydrazinolysis.

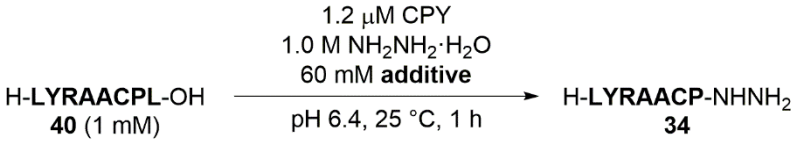


Entry	CPY (μM)	$NH_2NH_2{\cdot}H_2O$ (M)	cyclohexanone (mM)	pH	Substrate **40** (%)	Yield of **34** (%)[a]
1	1.2	1.0	60	6.4	0	>97
2	1.2	0.5	60	6.4	0	>97
3	1.2	0.2	60	6.4	0	>97 (76)[b]
4	1.2	0.05	60	6.4	0	89
5	1.2	0.2	90	6.4	0	>97
6	1.2	0.2	30	6.4	0	93
7	1.2	0.2	60	7.4	0	>97
8	1.2	0.2	60	5.4	0	>97
9	1.2	0.2	60	4.4	0	83
10	0.1	0.2	60	6.4	39	60

[a]Yield (%) was determined by HPLC separation and integration of product **34** (integ. **34**) as a fraction of the sum of the unreacted **40** (integ. **40**) + other products derived from **40** (integ. **others**) + integ. **34**. [b]Isolated yield.

Carbonyl compounds were re-evaluated as additives for CPY-mediated hydrazinolysis and the results are summarized in Table 2.3. Aryl aldehydes (entries 1 and 2) and aliphatic aldehydes (entries 3–5) were inefficient, whereas higher aliphatic ketones with larger number of carbon atoms gave better results (entries 6–9). Heterocyclic ketones (entries 10 and 11) and a dicarbonyl compound (entry 12) produced moderate yields. From these results, it was concluded that cyclohexanone is the most effective and most economical additive for CPY-mediated hydrazinolysis.

Table 2-3. Re-evaluation of additives for CPY-mediated hydrazinolysis.

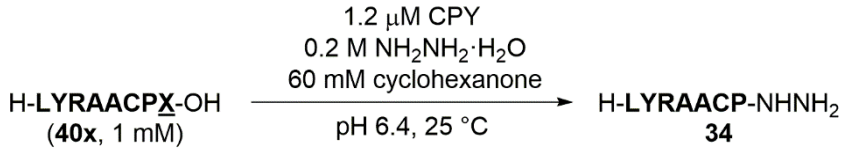


Entry	Additive	Substrate **40** (%)	Yield of **34** (%)	Over reaction products (%)
	non-additive	0	47	58
1	2-formylpyridine	0	25	75
2	3-formylpyridine	0	54	46
3	acetone	0	27	73
4	isobutylaldehyde	0	64	36
5	pivalaldehyde	0	27	73
6	3-pentanone	0	87	13
7	cyclopentanone	0	94	6
8	cyclohexanone	0	97	3
9	cycloheptanone	0	97	3
10	4-piperidone·HCl	0	86	14
11	2,2,6,6-tetramethyl-4-piperidone·HCl	0	85	15
12	1,4-cyclohexanedione	0	76	24

[a]Yield (%) was determined by HPLC separation and integration of product **40** (integ. **40**) as a fraction of the sum of the unreacted **34** (integ. **34**) + other products derived from **40** (integ. **others**) + integ. **34**.

We also evaluated the influence of C-terminal amino acid of substrate **40x** on the reaction efficiency (Table 2.4). C-Terminal hydrophobic amino acids (entries 1–3), including Ala (entry 4) could be converted to the desired hydrazide **34** very efficiently, whereas the reaction of C-terminal aromatic amino acids resulted in only a moderate yield of hydrazide **34** (entries 5 and 6). The reaction rate with C-terminal Trp, Gly and hydrophilic amino acids was quite slow (entries 7–11). From these results, it was concluded that Leu, Ile and Ala are good as the C-terminal amino acid of a substrate in CPY-mediated hydrazinolysis.

Table 2.4. Evaluation of C-terminal amino acid of substrate for CPY-mediated hydrazinolysis.



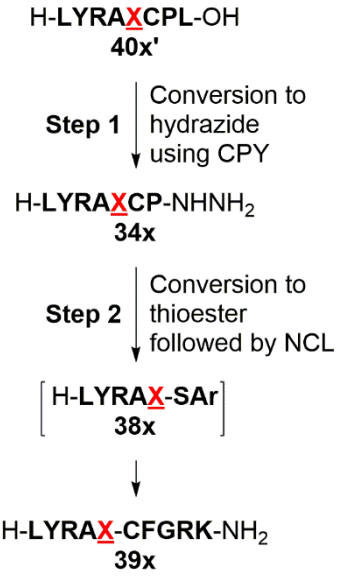
Entry	X	Substrate **40x** (%)	Hydrazide **34** (%)	Over reaction products (%)
1	Met (**40a**)	0	>97	3
2	Ile (**40b**)	0	>97	3
3	Val (**40c**)	0	95	5
4	Ala (**40d**)	0	>97	3
5	Phe (**40e**)	0	89	11
6	Tyr (**40f**)	9	91	5
7	Trp (**40g**)	73	16	11
8	Gly (**40h**)	90	9	-
9	Lys (**40i**)	71	29	-
10	Asp (**40j**)	88	12	-
11	Asn (**40k**)	77	23	-

[a]Yield (%) was determined by HPLC separation and integration of product **29** (integ. **29**) as a fraction of the sum of the unreacted **28** (integ. **28**) + products derived from **28** (integ. **others**) + integ. **29**.

2.6 Preparation of peptide thioesters using CPY-mediated protocol and chemical synthesis of natural peptides

Since the sequential reaction of CPY-mediated hydrazinolysis and the CPE-like two-residue deletion reaction yielded peptide thioesters other than the prolyl thioester, we used model peptides **40x'** (H–**LYRAXCPL**–OH, **X** = various amino acids) to assess the applicability of the protocol to various amino acids adjacent to the C-terminal –**CPL**–OH sequence. As shown in Table 2.5, all examined peptides **40x'** were smoothly converted to the corresponding hydrazides **34x** (H–**LYRAXCP**–$NHNH_2$) with >90% conversion (entries 1–8, 10–18). Although the reaction of peptides possessing hydrophobic amino acids accompanied the formation of over-reaction products, employment of a reduced quantity of CPY suppressed the over-reaction (entries 14–18). It is known that a thioester of C-terminal aspartic acid cannot be used for NCL because of the formation of a *β*-ligated byproduct under conditions of NCL, and thus we did not examine the application to Asp (entry 9).[34] The obtained hydrazides **34x** were shown to function as precursors of thioesters **38x** (H–**LYRAX**–SR) yielding the corresponding ligated products **39x** via NCL with the N-terminal cysteinyl peptide **35** (H–**CFGRK**–NH_2).

Table 2.5. Application of developed protocol to various C-terminal amino acid thioesters.



Entry	X	Step 1		Step 2	
		Time (h)	Yield of **34x** (%)[a]	Time (h)	Yield of **39x** (%)[a]
1	Gly (**40a'**)	1	>97	1	>97
2	Arg (**40b'**)	3	>97	3	>97
3	Lys (**40c'**)	6	>97	3	91
4	His (**40d'**)	3	>97	3	>97
5	Ser (**40e'**)	3	>97	3	91
6	Thr (**40f'**)	1	92	12	94
7	Cys (**40g'**)	1	94	6	83
8	Asn (**40h'**)	1	>97	3	>97
9	Asp	-	-	-	-
10	Gln (**40i'**)	1	>97	6	94
11	Glu (**40j'**)	3	>97	3	80
12	Met (**40k'**)	1	94	3	94
13	Trp (**40l'**)	1	95	6	>97
14[b]	Tyr (**40m'**)	1	94	3	96
15[b]	Phe (**40n'**)	1	>97	1	>97
16[c]	Leu (**40o'**)	1	94	12	96[d]
17[c]	Ile (**40p'**)	1	90	24	81[d]
18[c]	Val (**40q'**)	1	93	24	92[d]

Step 1: peptide **40x'** (1 mM) was treated with aqueous solution containing 1.2 μM CPY, 0.2 M $NH_2NH_2 \cdot H_2O$, 60 mM cyclohexanone, pH 6.4 at 25 ˚C for 1 h. Step 2: Peptide **34x** (1.5 mM) was treated with 50 mM Na phosphate buffer containing 6 M Gn· HCl, 7.5 mM $NaNO_2$, pH 3.0 at -10 ˚C for 30 min. Then 50 mM Na phosphate buffer containing 6 M Gn· HCl, 50 mM MPAA and 2.0 mM H-**CFGRK**-NH_2 was added (**34x**: 1.0 mM) to the reaction mixture and the pH was adjusted to pH 6.5. the reaction mixture was incubated at rt. [a]Yield (%) was determined by HPLC separation and integration of product (integ. product) as a fraction of the sum of the unreacted substrate (integ. substrate) + other products derived from substrate (integ. others) + integ. product. [b]Step 1 was conducted with 0.3 μM CPY. [c]Step 1 was conducted with 0.1 μM CPY. [d]The NCL was conducted at 37 ˚C.

To test the feasibility of producing thioesters using this developed protocol, an attempt was made to synthesize the reduced form of the C-type natriuretic peptide (CNP 53)[35] (**41**), consisting of 53 amino acids (Figure 2.7). Initially, the peptide thioester **42**, corresponding to CNP (1-36) was prepared. The 39-residue peptide **43** with an additional –**CPL**–OH sequence at the C-terminus, prepared by Fmoc-SPPS, was converted to 38-residue peptide hydrazide **44** by CPY-mediated hydrazinolysis in 70% isolated yield (Figure 2.7 (A, B)). The resulting hydrazide **44** was converted to the desired 36-residue thioester **42** followed by NCL with the N-terminal cysteinyl peptide **45** to afford the reduced form of CNP 53 in 88% isolated yield (Figure 2.7 (A, C)).

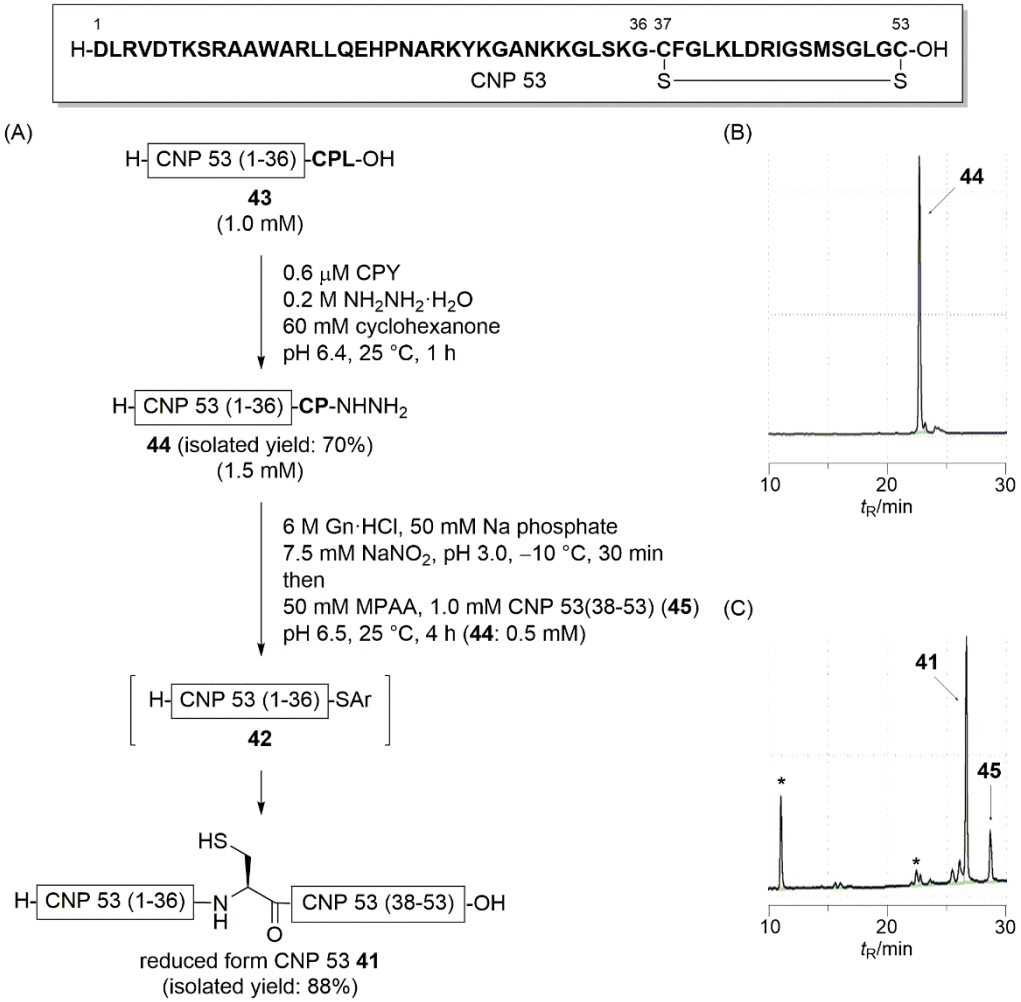


Figure 2.7. Preparation of the thioester fragment using CPY-mediated protocol applied to the synthesis of the reduced form CNP 53 (**41**). (A) Synthetic scheme of the reduced form CNP 53 (**41**). (B, C) HPLC monitoring of reactions. (B) After 1 h of hydrazinolysis of peptide **43**. (C) After 4 h of NCL. HPLC conditions: a linear gradient of 0.1% TFA-MeCN (5−35% over 30 min) in 0.1% TFA aq. *Non-peptidic impurity.

2.7 Application to expressed protein

The thioesterification of chemically synthesized peptides described in the previous section was successful. We next evaluated the applicability of developed CPY-mediated protocol to an expressed protein (Figure 2.8). As a model of an expressed protein, we used the 225-residue DsRED protein.[36] CPY-mediated hydrazinolysis of DsRED protein **46** with a C-terminal –**CPL**–OH sequence, afforded the protein hydrazide **47**. After conversion of **47** to the corresponding thioester, the biotinylated peptide **48** was ligated by NCL to yield a biotinylated product **49** (Figure 2.8 (A, C)). After these reactions, the new band corresponding to ligated product **49** was observed and a biotinylated signal was detected from the same band by SDS-PAGE (Figure 2.8 (B), lane 3). From Coomassie Brilliant Blue (CBB) stained gel analysis, the conversion yield was estimated to be ~70%. These results indicate that CPY-mediated protocol is applicable to expressed proteins.

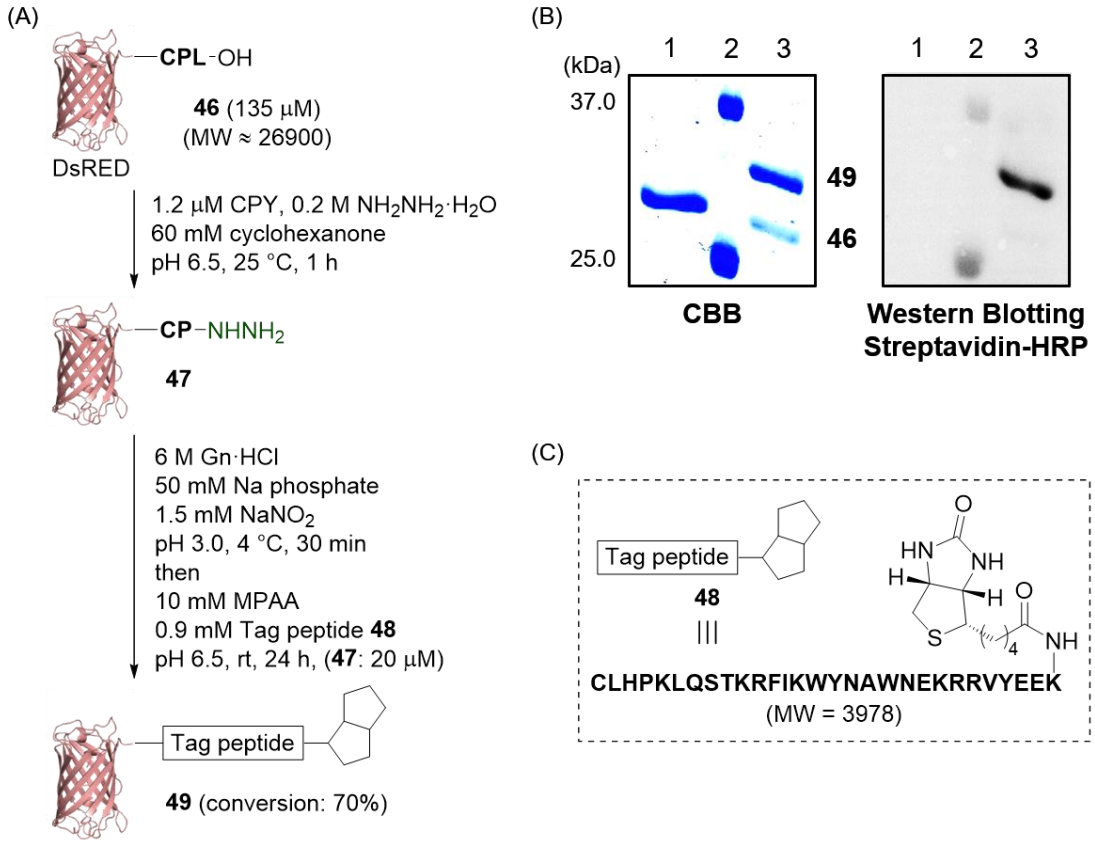


Figure 2.8. (A) Application of CPY-mediated protocol to DsRED protein. Conversion was calculated from the ratio of the band intensity of each protein band **49** and **46**. (B) SDS-page analysis of modification of DSRED **46**. Lane 1, intact DsRED **46**; Lane 2, standard; Lane 3 crude mixture after thioesterification using CPY-mediated protocol followed by NCL with biotinylated peptide **48**. (C) Structure of biotinylated peptide **48**.

2.8 Conclusion

We developed a CPY-mediated protocol for producing thioesters from naturally occurring peptide sequences. This protocol enables conversion of C-terminal –**CPL**–OH peptides to thioesters in a traceless manner and is applicable to the synthesis of a wide variety of thioesters containing various C-terminal amino acids. This CPY-mediated protocol was successfully applied to modification of DsRED as expressed proteins.

Chapter 3

Conclusion

1. An intein-inspired UV-responsive molecular architecture which cleaves amide bonds has been developed. In principle, the amide bond cleaving structure should respond to stimuli simply by replacing the oNBnoc group with other stimulus-removable protecting groups.

2. A novel methodology for production of protein thioesters from naturally occurring peptide sequences was developed. This protocol features CPY-mediated hydrazinolysis followed by CPE-like thioesterification, and has enabled synthesis of a wide variety of thioesters containing various C-terminal amino acids in a traceless manner.

We developed chemical modifications and synthetic methodology intended to understand the function of proteins. The CPY-mediated protocol could be a practical method for synthesis of large, chemically modified proteins possessing functional moieties, such as a UV-responsive peptide bond cleaving unit. Several studies of additional applications to expressed proteins are currently underway in our laboratory.

Experimental section — General Methods

General experimental

All reactions were carried out under an atmosphere of argon. All commercial reagents were used without further purification. Colum chromatography was performed using silica gel (spherical, 63−210 μm; KANTO CHEMICAL Co, Inc.). Preparative thin layer chromatography (PTLC) was carried out on silica gel precoated plates (TLC Silica gel 60G F_{254}; <Merck Millipore). Mass spectra were recorded on Waters MICROMASS® LCT PREMIER™ by electrospray ionization time-of-flight (ESI-TOF) reflection experiments. Each peptide structure is assigned by MS analysis. NMR spectra were measured using a Bruker AV400N at 400 MHz frequency for ^{1}H, and on JEOL GSX300 at 75 MHz frequency for ^{13}C. Chemical shifts were calibrated to the solvent signal. The following abbreviations were used to explain NMR peak muntiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. IR spectra and optical rotations were measured using a JASCO FT-IR 6200 and a JASCO P-2200 polarimeter (concentration in g/100 mL), respectively. For LC-MS analysis (Shimadzu, Japan, Prominence-I LC-2030, LCMS-2020), a Cosmosil 5C18-AR-II analytical column (Nacalai Tesque, Japan, 4.6×250 mm, flow rate 1 mL min^{-1}) was employed, and eluting products were detected by UV at 250 nm and MS. For HPLC separation, Cosmosil $5C_{18}$-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), Cosmosil $5C_{18}$-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL/min), or Cosmosil $5C_{18}$-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10.0 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. Photolysis was performed using Moritex MUV-202U with the filtered output (>365 nm) of a 3000 mW/cm^2 HG-Xe lamp.

General procedure for peptide synthesis

Unless otherwise description, peptides used in this work were synthesized by Fmoc solid-phase peptide synthesis (Fmoc SPPS) on NovaSyn® TGR resin (Rink amide type: 0.25 mmol amine/g), Rink Amide AM resin (0.62 mmol amine/g), Wang resin (0.80 mmol alcohol/g) or HMPB-ChemMatrix resin (0.5 mmol alcohol/g). Fmoc SPPS was performed according to the following protocol.

1. Removal of Fmoc groups was carried out using 20% (v/v) piperidine in DMF for 10 min at room temperature.
2. The resin was washed with DMF (10 times)
3. A standard Fmoc-protected amino acid (4 equiv.) was coupled with the aid of *N*,*N*-diisopropylcarbodiimide (DIPCI) (4 equiv.) and 1-hydroxybenzotriazole monohydrate (HOBt·H_2O) (4 equiv.) or *N*,*N*-diisopropyletthylamine (DIPEA) (4.0 equiv.) and *N*,*N*,*N'*,*N'*-tetramethyl-O-(benzotriazole-1-yl)uronium hexafluorophosphate (HBTU, 3.9 equiv.) in DMF for 1.5 h. Coupling of asparagine derivatives **10a**, **10b**, **16**, **20** or **s3** (2 equiv.) was performed using HATU (1.95 equiv.) and DIPEA (4.0 equiv.) in NMP for 2 h. Completion of the coupling reaction was checked by the Kaiser ninhydrin test. The coupling reaction was repeated until the Kaiser test became negative.
4. The resin was washed with DMF (5 times).

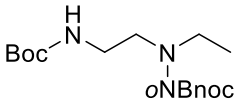
A cycle of steps 1 to 4 was repeated.

Deprotection of acid-lable protecting groups with concomitant release of peptides from a resin was achieved using a cocktail of 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. The resin was filtered off and the filtrate was concentrated by N_2 stream. Then cooled diethyl ether (Et_2O) was added to the concentrate and the formed precipitate was collected by centrifugation. The obtained precipitate was thoroughly washed with cooled Et_2O, and purified by preparative HPLC.

Experimental section − Chapter 1

S1.1 Synthesis of intein-inspired amide bond processing derivatives and its incorporation in to peptides

2-Nitrobenzyl {2-[(*tert*-butoxycarbonyl)amino]ethyl}ethyl carbamate (3)

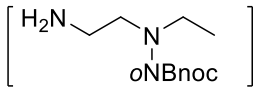


3

To a solution of *N*-ethylethylenediamine (**1**) (5.37 mL, 50.0 mmol) in THF (100 mL) was added a solution of Boc_2O (3.27 g, 15.0 mmol) in THF (30 mL) dropwise at 0 °C. The reaction mixture was stirred at room temperature for 5 h, and then concentrated in vacuo. The obtained residue was subsequently diluted with EtOAc and sat. $NaHCO_3$ aq. The obtained mixture was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. The obtained crude carbamate (2.82 g, 15.0 mmol, quant., a pale yellow powder) was used for the next step without further purification.

The obtained carbamate (2.82 g) in THF (30 mL) was treated sequentially with Et_3N (1.62 mL, 11.6 mmol) and 2-nitrobenzyl 4-nitrophenyl carbonate (**2**)[12] (3.69 g, 11.6 mmol). The reaction mixture was stirred at room temperature for 4 h and then concentrated in vacuo and diluted with EtOAc and 5% (w/v) $KHSO_4$ aq. The obtained mixture was extracted three times with EtOAc. The combined organic layer was washed with sat. $NaHCO_3$ aq. and brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. The crude material was purified by column chromatography (*n*-hexane/EtOAc = 8/1 then 1/1 (v/v)) to afford *o*NBnoc-diamine **3** (4.26 g, 11.6 mmol, quant.) as a yellow oil: IR ($CHCl_3$) ν_{max}, cm^{-1} 1364, 1477, 1529, 1701, 2875, 2977, 3358; ^1H NMR (DMSO-d_6, 100 °C, 300 MHz) δ = 1.09 (3H, t, J = 7.0 Hz), 1.38 (9H, s), 3.11 (2H, dt, J = 6.6, 6.6 Hz), 3.22–3.35 (4H, m), 5.39 (2H, s), 6.35–6.47 (1H, br m), 7.60 (1H, dd, J = 8.1, 7.5 Hz), 7.67 (1H, d, J = 7.3 Hz), 7.76 (1H, dd, J = 7.3, 7.5 Hz), 8.05 (1H, d, J = 8.1 Hz); ^{13}C NMR (DMSO-d_6, 60 °C, 75 MHz) δ = 13.1, 28.0, 41.9, 62.7, 77.5, 124.3, 128.7, 128.8, 132.1, 133.6, 147.2, 154.5, 155.3; HRMS (ESI-TOF) *m/z* calcd for $C_{17}H_{25}N_3NaO_6$ ($[M + Na]^+$) 390.1641, found 390.1643.

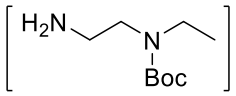
2-Nitrobenzyl (2-aminoethyl) ethylcarbamate (4a)



4a

Carbamate **3** (2.00 g, 2.72 mmol) in CH_2Cl_2 (1.36 mL) was treated with TFA (1.36 mL). The reaction mixture was stirred at room temperature for 45 min and concentrated in vacuo. After dilution of the resulting residue with EtOAc and sat. $NaHCO_3$ aq., the solution was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. Crude *o*NBnoc amine **4a** (1.45 g) was obtained as a yellow powder. The obtained crude **4a** was used for preparation of **9a** and **14a** without further purification.

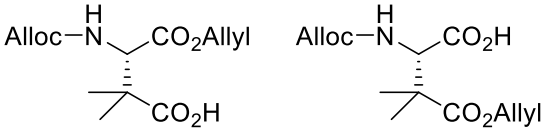
***tert*-Butyl (2-aminoethyl)(ethyl)carbamate (4b)**



4b

To a stirred mixture of *N*-ethylethylenediamine (**3**) (2.39 mL, 22.7 mmol) in CH_2Cl_2 (50 mL) was added ethyl trifluoroacetate (3.46 mL, 22.7 mmol) in CH_2Cl_2 (50 mL) dropwise over 40 min at 0 °C. The reaction mixture was stirred at room temperature for 1 h and then concentrated in vacuo. After dilution of the resulting residue with CH_2Cl_2 (100 mL), Boc_2O (4.95 g, 22.7 mmol) in CH_2Cl_2 (5.0 mL) was added to the solution at 0 °C. The reaction mixture was stirred at room temperature for 1.5 h and then diluted with EtOAc and sat. $NaHCO_3$ aq. The solution was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. The obtained crude material in MeOH (90 mL) and H_2O (10 mL) was treated with K_2CO_3 (2.00 g). The reaction mixture was refluxed for 2 h and then concentrated in vacuo. The mixture was extracted three times with EtOAc. The combined organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. Crude Boc amine **4b** (4.27 g) was obtained as a pale yellow oil. The obtained crude **4b** was used for preparation of **9b** and **14b** without further purification.

(*S*)-3-{[(Allyloxy)carbonyl]amino}-2,2-dimethylsuccinic acid 4-(allyl)ester (8) and (*S*)-4-(allyloxy)-2-{[(allyloxy)carbonyl]amino}-3,3-dimethyl-4-oxobutanoic acid (17)



8 **17**

To a solution of PhFl-*β*,*β*-diMe-Asp(OMe)-OMe (**5**)[13] (1.83 g, 4.05 mmol) in CH_2Cl_2 (10.2 mL) were added Et_3SiH (1.43 mL, 14.2 mmol) and TFA (10.2 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1 h and concentrated in vacuo. The obtained mixture was diluted with 1 M HCl aq. The precipitate was filtrated and washed with MeOH. The filtrate was concentrated in vacuo and the resulting crude amine was used for the next step without further purification.

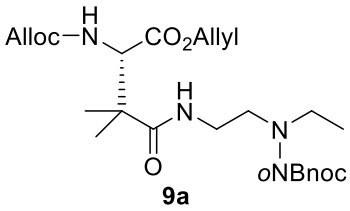
The crude amine in THF (12.5 mL) and H_2O (8.96 mL) was treated sequentially with $NaHCO_3$ (2.51 g, 29.9 mmol) and allyl chloroformate (63.6 μL, 5.98 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 9 h and diluted with H_2O and EtOAc. The mixture was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. The obtained crude Alloc-*β*,*β*-diMe-Asp(OMe)-OMe (**7**) was used for the next step without further purification.

The crude **9** in THF (10.9 mL) and H_2O (30 mL) was treated with 1 M LiOH aq. (17.4 mL, 17.4 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 10 h and diluted with CH_2Cl_2. The aqueous layer was washed three times with CH_2Cl_2 and then acidified (pH ~ 3) with 3 M HCl aq. To the aqueous layer were added EtOAc and NaCl. The mixture was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. Crude carboxylic acid (1.22 g) was obtained as a colorless oil. A 600 mg portion was used for the next step without further purification.

The stirred mixture of obtained crude carboxylic acid (600 mg) in THF (2.43 mL) was treated with Ac_2O (616 μL, 6.56 mmol). The reaction mixture was refluxed for 18 h and then concentrated in vacuo. To the obtained crude anhydride was added allyl alcohol (7.5 mL). The reaction mixture was stirred at room temperature for 23 h and concentrated in vacuo. The obtained crude material was purified by column chromatography (chloroform/MeOH = 400/1 then 50/1 (v/v)) to afford **8** (449 mg, 1.47 mmol, 61% over five steps from **5**) as a colorless oil and **17** (131 mg, 0.459 mmol, 19% over five steps from **5**) as a colorless oil. **8**: $[\alpha]^{28}_D$ −12.3 (*c* 1.56, $CHCl_3$); IR ($CHCl_3$) ν_{max}, cm^{-1} 1330, 1519, 1713, 2886, 2942, 3084, 3349; ^1H NMR ($CDCl_3$, 400 MHz) δ = 1.26 (3H, s), 1.37

(3H, s), 4.58–4.67 (5H, m), 5.21–5.27 (2H, m), 5.29–5.36 (2H, m), 5.66 (br d, J = 9.6 Hz), 5.85–5.98 (2H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 22.2, 23.3, 45.7, 59.8, 66.0, 66.4, 118.2, 118.7, 131.9, 132.5, 156.5, 175.1, 175.3; HRMS (ESI-TOF) *m/z* calcd for $C_{13}H_{19}N_1NaO_6$ ([M + Na]$^+$) 308.1110, found 308.1115. **17**: $[\alpha]^{28}_D$ −11.8 (*c* 2.40, $CHCl_3$), IR ($CHCl_3$) ν_{max}, cm^{-1} 932, 1251, 1525, 1724, 2886, 2944, 2984, 3088, 3350; ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.23 (3H, s), 1.34 (3H, s), 4.57–4.67 (5H, m), 5.20–5.26 (2H, m), 5.28–5.36 (2H, m), 5.68 (1H, br d, J = 10.4), 5.81–5.98 (2H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 22.1, 23.3, 45.6, 59.9, 66.3, 66.4, 118.2, 119.3, 131.3, 132.6, 156.4, 170.2, 181.5; HRMS (ESI-TOF) *m/z* calcd for $C_{25}H_{19}N_1NaO_6$ ([M + Na]$^+$) 308.1110, found 308.1121.

Allyl (*S*)-2-{[(allyloxy)carbonyl]amino}-4-{[2-ethyl(2-nitrobenzyloxycarbonyl) aminoethyl]amino}-3,3-dimethyl-4-oxobutanoate (9a)

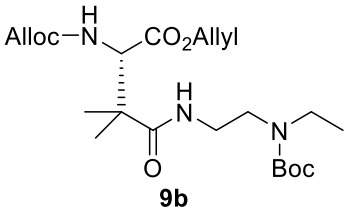


9a

To a solution of **8** (34.5 mg, 0.121 mmol) in CH_2Cl_2 (605 μL) were added crude **4a** (93.8 mg), bromotripyrrolidinophosphonium hexafluorophosphate (PyBrop) (152 mg, 0.454 mmol) and *N*,*N*-diisopropylethylamine (DIPEA) (77.2 μL, 0.454 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 34 h and then diluted with EtOAc and 5% (w/v) $KHSO_4$ aq. The solution was extracted three times with EtOAc. The combined organic layer was washed with sat. $NaHCO_3$ aq., dried over Na_2SO_4, filtrated and concentrated in vacuo. The obtained crude material was purified with column chromatography (*n*-hexane/EtOAc = 1/1 then 1/2 (v/v)) to afford amide **9a** (48.0 mg, 0.904 mmol, 75%) as a colorless oil: $[\alpha]^{28}_D$ −2.9 (*c* 1.25, $CHCl_3$); IR ($CHCl_3$) ν_{max}, cm^{-1} 1268, 1342, 1427, 1526, 1650, 1703, 2875, 2939, 2973, 3079, 3361; ^{1}H NMR (DMSO-d_6, 70 °C, 300 MHz) δ = 1.08 (3H, t, J = 7.1 Hz), 1.13 (3H, s), 1.14 (3H, s), 3.19–3.35 (6H, m), 4.46–4.59 (5H, m), 5.13–5.24 (2H, m), 5.24–5.36 (2H, m), 5.4 (2H, s), 5.81–5.98 (2H, m), 7.16 (1H, br d, J = 2.9 Hz), 7.46–7.55 (1H, br m), 7.60 (1H, dd, J = 7.1, 8.0 Hz), 7.69 (1H, br d, J = 7.2 Hz), 7.78 (1H, dd, J = 7.1, 7.2 Hz), 8.07 (1H, d, J = 8.0 Hz); ^{13}C NMR (DMSO-d_6, 70 °C, 75 MHz) δ =13.0, 21.1, 22.6, 37.7, 41.8, 44.1, 45.3, 59.7, 62.7, 64.4, 64.5, 116.6, 117.4, 124.2, 128.7, 128.9, 131.9, 132.0, 133.1, 147.3, 154.5,

155.7, 169.7, 174.6; HRMS (ESI-TOF) *m/z* calcd for $C_{25}H_{34}N_4NaO_9$ ([M + Na]$^+$) 557.2223, found 557.2244.

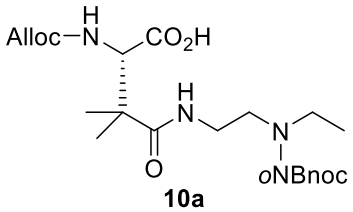
Allyl (*S*)-2-{[(allyloxy)carbonyl]amino}-4-{[2-(*tert*-butoxy carbonyl)(ethyl)amino ethyl]amino}-3,3-dimethyl-4-oxobutanoate (9b)



9b

Amide **9b** was prepared from carboxylic acid **8** (72.0 mg, 0.252 mmol) and crude **4b** (96 mg) in a manner similar to that described for the synthesis of **9a**. Compound **9b** (109 mg, 0.239 mmol, 95%) was obtained as a colorless oil: $[\alpha]^{28}_D$ −6.7 (*c* 2.18, $CHCl_3$); IR ($CHCl_3$) ν_{max}, cm^{-1} 1341, 1431, 1525, 1709, 2880, 2934, 2975, 3317; ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.10 (3H, t, *J* = 7.0 Hz), 1.25 (3H, s), 1.35 (3H, s), 1.46 (3H, s), 3.21 (2H, q, *J* = 7.0 Hz), 3.28–3.50 (4H, br m), 4.32 (2H, d, *J* = 9.2 Hz), 4.57 (2H, ddd, *J* = 1.6, 1.6, 5.6 Hz), 4.60 (2H, ddd, *J* = 1.6, 1.6, 5.6 Hz), 5.16–5.25 (2H, m), 5.26–5.35 (2H, m), 5.82–5.97 (2H, m), 6.38 (2H, br d, *J* = 9.2 Hz), 7.05–7.20 (1H, br m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ =13.9, 23.5, 24.6, 28.6, 41.1, 43.0, 44.5, 45.5, 61.3, 65.9, 80.3, 117.6, 118.6, 131.8, 132.9, 156.5, 157.6, 170.6, 176.3; HRMS (ESI-TOF) *m/z* calcd for $C_{22}H_{37}N_3NaO_7$ ([M + Na]$^+$) 478.2529, found 478.2539.

(*S*)-2-{[(9H-Fluoren-9-yl)methoxycarbonyl]amino}-4-{[2-ethyl(2-nitrobenzyloxy carbonyl)amino ethyl]amino}-3,3-dimethyl-4-oxobutanoic acid (10a)

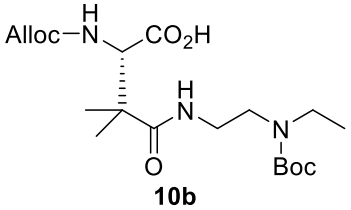


10a

To a stirred mixture of **9a** (20.2 mg, 37.4 μmol) in THF (374 μL) were added $Pd(PPh_3)_4$ (6.49 mg, 5.61 μmol) and *N*-methylaniline (40.8 μL, 0.374 mmol). The reaction mixture was stirred at room temperature for 6 h. To the reaction mixture were added DIPEA (15.3 μL, 89.8 μmol) and FmocOSu (15.1 mg, 44.9 μmol) at 0 °C. The reaction mixture was stirred at room temperature for 10 h and diluted with EtOAc and 5% (w/v) $KHSO_4$ aq.

The solution was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. The obtained crude material was purified with column chromatography ($CHCl_3$/MeOH = 150/1 then 30/1 (v/v)) to afford carboxylic acid **10a** (21.7 mg, 34.3 μmol, 92%) as a pale yellow oil: $[\alpha]^{28}_D$ −1.1 (*c* 1.30, $CHCl_3$); IR ($CHCl_3$) ν_{max}, cm^{-1} 930, 1249, 1367, 1530, 1672, 1726, 2875, 2934, 2975, 3079, 3350; ^{1}H NMR ($CDCl_3$, 50 °C, 300 MHz) δ = 1.03–1.20 (6H, m), 1.25 (3H, s), 3.30 (2H, q, *J* = 7.1 Hz), 3.34–3.57 (4H, m), 4.18 (1H, t, *J* = 6.8 Hz), 4.37 (2H, d, *J* = 6.8 Hz), 4.42–4.58 (1H, m), 5.47 (2H, s), 6.03–6.13 (1H, m), 6.79–6.94 (1H, m), 7.24–7.61 (9H, m), 7.71 (2H, d, *J* = 7.5 Hz), 7.98 (1H, d, *J* = 7.9 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ =13.9, 23.2, 23.6, 40.7, 43.0, 45.5, 45.9, 47.3, 60.0, 64.7, 67.5, 120.1, 125.2, 125.3, 127.2, 127.9, 128.9, 129.1, 129.3, 132.3, 133.8, 141.4, 143.8, 143.9, 147.9, 149.0, 156.9, 157.6, 172.3; HRMS (ESI-TOF) *m/z* calcd for $C_{33}H_{37}N_4O_9$ ($[M + H]^+$) 633.2561, found 633.2549.

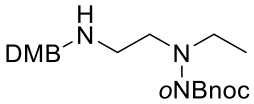
(*S*)-2-{[(9H-Fluoren-9-yl)methoxycarbonyl]amino}-4-{[2-(*tert*-butoxycarbonyl) (ethyl)aminoethyl] amino}-3,3-dimethyl-4-oxobutanoic acid (10b)



10b

Carboxylic acid **10b** was prepared from amide **9b** (88.8 mg, 0.195 mmol) in a manner similar to that described for the synthesis of **10a**. Compound **10b** (72.2 mg, 0.136 mmol, 70%) was obtained as a pale yellow oil: $[\alpha]^{28}_D$ −1.0 (*c* 2.16, $CHCl_3$); IR ($CHCl_3$) ν_{max}, cm^{-1} 1366, 1450, 1479, 1531, 1709, 2875, 2934, 2975, 3329; ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.11 (3H, t, *J* =7.2 Hz), 1.24 (3H, s), 1.36 (3H, s), 1.46 (9H, s), 3.22 (2H, q, *J* = 7.2 Hz), 3.3–3.47 (4H, m), 4.22 (1H, t, *J* = 7.2 Hz), 4.30 (2H, d, *J* = 7.2 Hz), 4.56 (1H, br d, *J* = 8.0 Hz), 6.11–6.26 (1H, br m), 7.31 (2H, dd, *J* = 7.6, 7.6 Hz), 7.39 (2H, dd, *J* = 9.2, 7.6 Hz), 7.57–7.65 (2H, m), 7.75 (2H, d, *J* = 9.2 Hz), 7.77–7.85 (1H, br m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ =13.8, 23.4, 23.7, 28.5, 41.5, 43.2, 45.3, 45.5, 47.3, 60.1, 67.4, 80.8, 120.1, 125.3, 127.2, 127.8, 141.4, 143.8, 144.0, 156.8, 157.8, 172.1, 178.9; HRMS (ESI-TOF) *m/z* calcd for $C_{30}H_{39}N_3NaO_7$ ($[M + Na]^+$) 576.2686, found 576.2672.

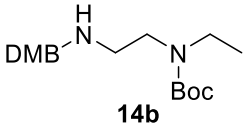
2-Nitrobenzyl {2-[(2,4-dimethoxybenzyl)amino]ethyl}(ethyl)carbamate (14a)



14a

Crude amine **4a** (1.24 g) in MeOH (8.9 mL) was treated with 2,4-dimethoxybenzaldehyde (1.23 g, 7.41 mmol), AcOH (278 μL, 4.86 mmol) and Na_2SO_4 (3.29 g, 46.3 mmol). The reaction mixture was stirred at room temperature for 2 h. To the reaction mixture was added $NaBH_4$ (700 mg, 18.5 mmol) at 0 °C. The reaction mixture was additionally stirred at room temperature for 1 h and then diluted with sat. $NaHCO_3$ aq. The mixture was extracted three times with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. The obtained crude material was purified with column chromatography (*n*-hexane/EtOAc = 2/1 then EtOAc/MeOH 3/1 (v/v)) to afford DMB-*o*NBnoc amine **14a** (1.84 g, 4.41 mmol, 95% over two steps) as a light brown oil: IR ($CHCl_3$) ν_{max}, cm^{-1} 1343, 1423, 1465, 1529, 1613, 1701, 2836, 2935, 3340; ^{1}H NMR (DMSO-d_6, 80 °C, 300 MHz) δ =1.09 (3H, t, J = 7.0 Hz), 1.38 (9H, s), 3.11(2H, dt, J = 6.6, 6.6 Hz), 3.21-3.37 (4H, m), 5.39 (2H, s), 6.33-6.48 (1H, br m), 7.60 (1H, dd, J = 8.1, 7.6 Hz), 7.68 (1H, d, J = 7.3 Hz), 7.76 (1H, dd, J = 7.3, 7.5 Hz), 8.05 (1H, d, J = 8.1 Hz); ^{13}C NMR (DMSO-d_6, 60 °C, 75 MHz) δ =13.1, 28.0, 38.5, 41.9, 46.1, 62.7, 77.5, 124.3, 128.7, 128.8, 132.1, 133.6, 147.2, 154.5, 155.3; HRMS (ESI-TOF) *m/z* calcd for $C_{21}H_{28}N_3O_6$ ($[M + Na]^+$) 418.1978, found 418.1988.

***tert*-Butyl {2-[(2,4-dimethoxybenzyl)amino]ethyl}(ethyl)carbamate (14b)**

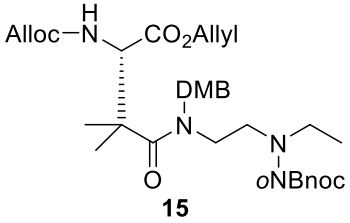


14b

DMB-Boc amine **14b** was prepared from crude amine **4b** (500 mg) and 2,4-dimethoxybenzaldehyde (221 mg, 1.33 mmol) in a manner similar to that described for the synthesis of **14a**. Compound **14b** (396 mg, 1.17 mmol, 88%) was obtained as a yellow oil: IR ($CHCl_3$) ν_{max}, cm^{-1} 1156, 1366, 1463, 1507, 1613, 1690, 2837, 2933, 2973, 3342; ^{1}H NMR (DMSO-d_6, 60 °C, 300 MHz) δ = 1.02 (3H, t, J = 7.0 Hz), 1.38 (9H, s), 2.69 (2H, t, J = 6.8 Hz), 3.17 (2H, q, J = 7.0 Hz), 3.25 (2H, t, J = 6.8 Hz), 3.70 (2H, s), 3.76 (3H, s), 3.78 (3H, s), 6.59 (1H, br s), 6.48 (1H, dd, J = 8.3, 2.2 Hz), 6.55 (1H, d, J = 2.2 Hz), 7.18 (1H, d, J = 8.3 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 13.6, 28.5, 42.5, 46.6, 47.3,

48.7, 55.4, 55.5, 70.6, 77.2, 79.4, 98.6, 103.8, 120.2, 130.6, 158.7, 160.3; HRMS (ESI-TOF) *m/z* calcd for $C_{18}H_{31}N_2O_4$ ([M + H]$^+$) 339.2284, found 339.2281.

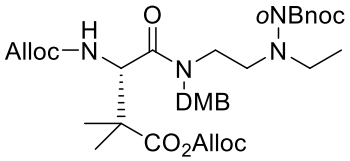
Allyl (*S*)-2-[(allyloxycarbonyl)amino]-4-[(2,4-dimethoxybenzyl)(2-ethyl-2-nitro benzyloxycarbonyl aminoethyl)amino]-3,3-dimethyl-4-oxobutanoate (15)



15

A solution of carboxylic acid **8** (118 mg, 0.413 mmol) in CH_2Cl_2 (1.5 mL) was treated sequentially with Et_3N (173 μL, 1.24 mmol). After the addition of MsCl (38.4 μL, 0.496 mmol) in CH_2Cl_2 (100 μL) at 0 °C, the reaction mixture was stirred at the same temperature for 2 h. To the mixture was added **14a** (199 mg, 0.476 mmol) in CH_2Cl_2 (1.5 mL) at 0 °C and the resulting mixture was stirred at room temperature for additional 17 h and then diluted with EtOAc and 5% (w/v) $KHSO_4$ aq. The solution was extracted three times with EtOAc. The combined organic layer was washed with sat. $NaHCO_3$ aq. and brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. The obtained crude material was purified with column chromatography (*n*-hexane/EtOAc = 13/7 then 3/2 (v/v)) to afford amide **15** (140 mg, 0.204 mmol, 50%) as a colorless oil: $[\alpha]^{29}_D$ −0.4 (*c* 0.80, $CHCl_3$); IR ($CHCl_3$) ν_{max}, cm^{-1} 1208, 1423, 1477, 1528, 1614, 1707, 2838, 2939, 2972, 3084, 3314, 3443; ^{1}H NMR (DMSO-*d*$_6$, 100 °C, 300 MHz) δ = 1.04 (3H, t, *J* = 7.0 Hz), 1.27 (3H, s), 1.33 (3H, s), 3.21(3H, q, *J* = 7.0 Hz), 3.26–3.44 (4H, m), 3.76 (3H, s), 3.78 (3H, s), 4.50–4.64 (7H, m), 5.12–5.24 (2H, m), 5.26–5.38 (4H, m), 5.77–6.11 (2H, m), 6.49 (1H, dd, *J* = 8.4, 2.0 Hz), 6.56 (1H, d, *J* = 2.0 Hz), 6.85 (1H, br d, *J* = 9.3 Hz), 6.99 (1H, d, *J* = 8.4 Hz), 7.54–7.63 (2H, m), 7.71 (1H, t, *J* = 7.5 Hz), 8.03 (1H, d, *J* = 8.1 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz, rotamer) δ = 13.2, 13.9, 24.2, 25.3, 25.5, 29.6, 42.4, 43.1, 43.6, 43.7, 44.1, 44.3, 45.5, 46.4, 47.6, 55.1, 55.3, 63.2, 63.5, 63.7, 65.7, 98.4, 104.0, 116.6, 117.0, 117.4, 117.5, 117.9, 118.1, 124.8, 128.2, 128.3, 128.4, 128.5, 128.7, 131.8, 131.9, 132.7, 133.1, 133.2, 133.5, 133.6, 155.0, 155.4, 156.8, 157.9, 160.2, 160.3, 170.7, 176.2, 176.3, 176.4; HRMS (ESI-TOF) *m/z* calcd for $C_{34}H_{44}N_4NaO_{11}$ ([M + Na]$^+$) 707.2904, found 707.2924.

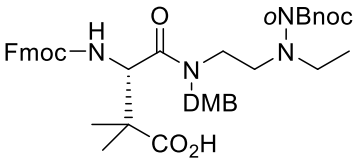
Allyl (*S*)-3-(allyloxycarbonyl)amino-4-{[2,4-dimethoxybenzyl][2-(ethyl-2-nitro benzyloxycarbonyl amino)ethyl]amino}-2,2-dimethyl-4-oxobutanoate (S1)



S1

Carboxylic acid **17** (90.6 mg, 0.318 mmol) in THF (3.1 mL) was treated with Et_3N (133 μL, 0.953 mmol). After the addition of MsCl (29.5 μL, 0.381 mmol) at 0 °C, the reaction mixture was stirred at the same temperature for 30 min. To the mixture was added **14a** (199 mg, 0.476 mmol) at 0 °C and the reaction mixture was stirred at room temperature for an additional 20 h. After that the mixture was diluted with EtOAc and 5% (w/v) $KHSO_4$ aq. The solution was extracted three times with EtOAc. The combined organic layer was washed with sat. $NaHCO_3$ aq. and brine, dried over Na_2SO_4, filtrated and concentrated in vacuo. The obtained crude material was purified with column chromatography (*n*-hexane/EtOAc = 2/1 then 1/1 (v/v)) to afford amide **S1** (130 mg, 0.190 mmol, 60%) as a colorless oil: $[\alpha]^{28}_D$ −16.8 (*c* 1.03, $CHCl_3$); IR ($CHCl_3$) ν_{max}, cm^{-1} 1209, 1343, 1426, 1509, 1529, 1645, 1709, 2939, 2976, 3196; ^1H NMR (DMSO-d_6, 100 °C, 300 MHz) δ = 1.04 (3H, br t, *J* = 6.1 Hz), 1.15 (3H, s), 1.24 (3H, s), 3.13–3.62 (6H, br m), 3.76 (6H, s), 4.33–4.64 (6H, m), 4.74–4.97 (1H, br m), 5.08–5.23 (2H, m), 5.23–5.34 (2H, m), 5.37 (2H, s), 5.78–6.02 (2H, m), 6.45 (1H, d, *J* = 8.2 Hz), 6.55 (1H, s), 6.85 (1H, br d, *J* = 9.0 Hz), 7.03 (1H, d, *J* = 8.2 Hz), 7.52–7.67 (2H, m), 7.73 (1H, dd, *J* = 7.4, 7.4 Hz), 8.04 (1H, dd, *J* = 8.1 Hz); ^{13}C NMR (DMSO-d_6, 100 °C, 75 MHz) δ = 12.7, 21.0, 22.5, 41.6, 43.3, 43.4, 44.9, 45.5, 54.8, 55.1, 55.8, 62.4, 64.1, 64.4, 98.4, 104.7, 116.5, 116.8, 123.9, 128.4, 128.7, 131.6, 132.2, 132.8, 133.1, 147.3, 154.2, 155.1, 157.9, 159.8, 169.3, 174.6; HRMS (ESI-TOF) *m/z* calcd for $C_{34}H_{44}N_4NaO_{11}$ ($[M + Na]^+$) 707.2904, found 707.2933.

(*S*)-3-{[(9H-Fluoren-9-yl)methoxycarbonyl]amino}-4-{[2,4-dimethoxybenzyl][2-ethyl(2-nitrobenzyl oxycarbonyl)aminoethyl]amino}-2,2-dimethyl-4-oxobutanoic acid (16)

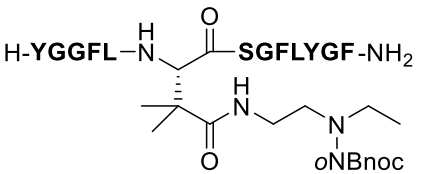


16

Carboxylic acid **16** was prepared from amide **S1** (55.5 mg, 81.1 μmol) in a manner similar to that described for the synthesis of **10a**. Compound **16** (63.0 mg, 80.5 μmol, quant.) was obtained as a pale yellow amorphous solid: $[\alpha]^{28}_{D}$ −8.2 (*c* 1.49, $CHCl_3$); IR ($CHCl_3$) ν_{max}, cm^{-1} 1342, 1452, 1508, 1526, 1613, 1645, 1708, 2853, 2931, 2961, 3068, 3421; ^1H NMR (DMSO-d_6, 100 °C, 300 MHz) δ = 0.97 (3H, t, *J* = 7.0 Hz), 1.04 (3H, s), 1.14 (3H, s), 3.12–3.43 (6H, br m), 3.66 (3H, s), 3.68 (3H, s), 4.01–4.57 (5H, br m), 4.75 (1H, br d, *J* = 1.8 Hz), 5.31 (2H, s), 6.34 (1H, dd, *J* = 8.3, 2.0 Hz), 6.47 (1H, d, *J* = 2.0 Hz), 6.84–7.07 (2H, m), 7.16–7.29 (2H, m), 7.34 (2H, dd, *J* = 7.5, 7.1 Hz), 7.46–7.72 (5H, m), 7.79 (2H, d, *J* = 7.5 Hz), 7.97 (1H, 7.9 Hz); ^{13}C NMR (DMSO-d_6, 75 MHz, rotamer) δ = 13.1, 13.7, 20.8, 21.0, 24.3, 42.2, 42.3, 43.1, 44.9, 45.8, 45.9, 46.7, 55.0, 55.4, 55.6, 55.9, 63.2, 65.9, 98.1, 98.3, 104.3, 104.5, 116.4, 117.0, 120.2, 124.7, 125.4, 127.0, 127.7, 128.7, 129.0, 132.3, 132.6, 134.1, 134.2, 140.7, 143.5, 143.7, 143.8, 154.3, 154.7, 156.0, 156.3, 158.0, 158.2, 159.7, 160.2, 169.9, 177.5, 177.7; HRMS (ESI-TOF) *m/z* calcd for $C_{42}H_{46}N_4NaO_{11}$ ($[M + Na]^+$) 805.3061, found 805.3063.

Preparation of model peptides 11a and 18

Peptides were synthesized on NovaSyn® TGR resin (Rink amide type: 0.25 mmol amine/g) by Fmoc SPPS described in *"Experimental section — general methods; general procedure for Fmoc SPPS"*.



11a

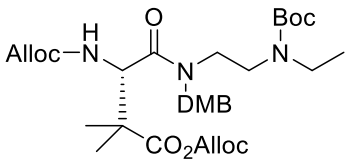
18

*Fmoc SPPS using **10a***. Peptide **11a** (major peak): a white lyophilized powder (2.08 mg, 1.19 μmol, 6.2%); retention time = 16.2 min (analytical HPLC conditions: linear gradient

of solvent B in solvent A, 38–50% over 30 min); retention time = 24.2 (semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 38–50% over 30 min); LRMS (ESI-TOF) *m/z* calcd for ([M + 2H]$^{2+}$) 859.9, found 859.7. Peptide **18** (minor peak): a white lyophilized powder (1.30 mg, 0.699 μmol, 3.9%); retention time = 17.6 min (analytical HPLC conditions: linear gradient of solvent B in solvent A, 38–50% over 30 min); retention time = 22.9 (semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 38–50% over 30 min); LRMS (ESI-TOF) *m/z* calcd ([M + 2H]$^{2+}$) 859.9, found 859.8.

*Fmoc SPPS using **16**.* Peptide **18**: retention time = 16.3 min (analytical HPLC conditions: linear gradient of solvent B in solvent A, 38–50% over 30 min); LRMS (ESI-TOF) *m/z* calcd for ([M + 2H]$^{2+}$) 859.9, found 859.8.

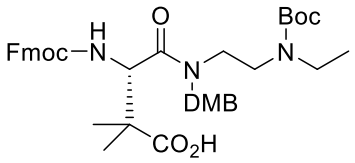
Allyl (*S*)-3-[(allyloxycarbonyl)amino]-4-{[2-ethyl(*tert*-butoxycarbonyl)aminoethyl] [2,4-dimethoxybenzyl]amino}-2,2-dimethyl-4-oxobutanoate (S2)



S2

Amide **S2** was prepared from **17** (63.5 mg, 0.223 mmol) and **14b** (113 mg) in a manner similar to the described for the synthesis of **S1**. Compound **S2** (74.6 mg, 0.122 mmol, 55%) was obtained as a colorless oil: $[\alpha]^{28}_{D}$ −1.8 (*c* 1.07, $CHCl_3$); IR ($CHCl_3$) ν_{max}, cm^{-1} 1366, 1507, 1646, 1693, 1723, 2832, 2875, 2934, 2975; ^{1}H NMR (DMSO-d_6, 100 °C, 300 MHz) δ = 0.88–1.11 (3H, m,), 1.16 (3H, s), 1.13 (3H, s), 1.38 (9H), 3.03–3.60 (6H, br m), 3.77(3H, s), 3.79 (3H, s), 4.38–4.65 (6H, m), 4.84 (1H, br d, *J* = 9.0 Hz), 5.11–5.24 (2H, m), 5.24–5.41 (2H, m), 5.75–6.06 (2H, m), 6.48 (1H, br d, *J* = 7.7 Hz), 6.57 (1H, s), 6.83 (1H, br d, *J* = 9.0 Hz), 7.03 (1H, br d, *J* = 7.7 Hz); ^{13}C NMR (DMSO-d_6, 100 °C, 75 MHz) δ = 12.8, 21.1, 22.6, 27.6, 41.3, 43.5, 44.8, 45.5, 54.8, 55.0, 55.8, 64.1, 64.4, 78.1, 98.3, 104.7, 116.4, 116.7, 128.7, 132.2, 132.8, 153.9, 155.0, 157.8, 159.8, 169.2, 174.6; HRMS (ESI-TOF) *m/z* calcd for $C_{31}H_{47}N_3NaO_9$ ([M + Na]$^+$): 628.3210, found: 628.3234.

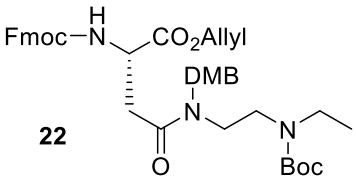
(*S*)-3-{[(9H-Fluoren-9-yl)methoxycarbonyl]amino}-4-{[2-ethyl(*tert*-butoxy carbonyl)aminoethyl] [2,4-dimethoxybenzyl]amino}-2,2-dimethyl-4-oxobutanoic acid (S3)



S3

Carboxylic acid **S3** was prepared from amide **S2** (55.9 mg, 92.3 μmol) in a manner similar to that described for the synthesis of **10a**. Compound **S3** (64.7 mg, 92.0 μmol, quant.) was obtained as pale a yellow amorphous solid: $[\alpha]^{25}_{D}$ −1.5 (*c* 0.80, $CHCl_3$); IR ($CHCl_3$) ν_{max}, cm^{-1} 1160, 1210, 1455, 1508, 1616, 1643, 1692, 1718, 2928, 2973, 3277; ^{1}H NMR (DMSO-d_6, 100 °C, 300 MHz) δ = 0.10 (3H, t, *J* = 7.1 Hz), 1.12 (3H, s), 1.22 (1H, s), 1.38 (9H, s), 3.10–3.44 (6H, br m), 3.72 (3H, s), 3.77 (3H, s), 4.07–4.25 (1H, br m), 4.25–4.41 (2H, br m), 4.41–4.61 (2H, br m) 4.81 (1H, br d, *J* = 6.2 Hz), 6.42 (1H, dd, *J* = 8.3, 2.0 Hz), 6.55 (1H, d, *J* = 2.0 Hz), 7.25–7.36 (2H, m), 7.40 (2H, dd, *J* = 7.3, 7.5 Hz), 7.67 (2H, d, *J* = 7.0 Hz), 7.84 (2H, d, *J* = 7.3 Hz); ^{13}C NMR (DMSO-d_6, 75 MHz, rotamer)[19] δ =13.5, 20.8, 20.9, 24.1, 24.3, 28.0, 42.8, 43.6, 44.8, 45.8, 46.6, 46.7, 55.0, 55.2, 55.3, 55.7, 65.9, 66.0, 78.5, 78.7, 98.2, 98.3, 104.3, 104.4, 116.4, 116.9, 120.1, 125.3, 125.4, 127.0, 127.3, 127.7, 128.9, 140.7, 143.5, 143.6, 143.8, 156.0, 156.3, 157.8, 158.0, 159.6, 159.9, 169.9, 177.6, 177.7; HRMS (ESI-TOF) *m/z* calcd for $C_{39}H_{49}N_3NaO_9$ ($[M + Na]^+$) 726.3367, found 726.3365.

Allyl *N*^{2}-{[(9H-Fluoren-9-yl)methoxy]carbonyl}-*N*^{4}-{2-[(*tert*-butoxycarbonyl)(ethyl) amino]ethyl}-*N*^{4} -(2,4-dimethoxybenzyl)-L-asparaginate (22)

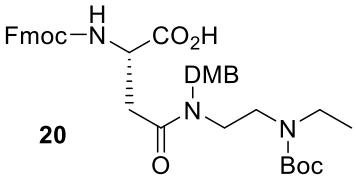


22

To a stirred mixture of Fmoc-L-Asp(OH)-OAllyl (**21**)[15] (350 mg, 0.886 mmol) and **14b** (250 mg, 0.739 mmol) in CH_2Cl_2 (10 mL) was added EDC· HCl (170 mg, 0.886 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 1 h and diluted with EtOAc and 5% (w/v) $KHSO_4$ aq. The solution was extracted three times with EtOAc. The combined organic layer was washed with brine, sat. $NaHCO_3$ aq. and brine, dried over $MgSO_4$, filtrated and concentrated in vacuo. The obtained crude material was purified

with column chromatography (*n*-hexane/EtOAc = 1/1 (v/v)) to afford amide **22** (500 mg, 0.698 mmol, 95%) as a pale yellow oil: $[\alpha]^{28}_{D}$ 22.8 (*c* 2.13, $CHCl_3$); IR ($CHCl_3$) ν_{max}, cm^{-1} 1289, 1454, 1506, 1642, 1690, 1725, 2838, 2934, 2972, 3438; ^{1}H NMR (DMSO-d_6, 100 °C, rotamer, 300 MHz) δ =1.01 (3H, t, *J* = 7.0 Hz), 1.39 (9H, s), 2.89–2.95 (2H, m), 3.09–3.23 (4H, m), 3.33 (2H, br t, *J* = 6.3 Hz), 3.75 (3H, s), 3.79 (3H, s), 4.20–4.28 (1H, m), 4.30–4.36 (2H, m), 4.38–4.51 (2H, m), 4.56–4.63 (3H, m), 5.18 (1H, dd, *J* = 10.4, 1.5 Hz), 5.31 (1H, dd, *J* = 17.2, 1.5 Hz), 5.89 (1H, ddt, *J* = 17.2, 10.4, 5.3 Hz), 6.47 (1H, br d, *J* = 8.2 Hz), 6.58 (1H, s), 7.02 (1H, d, *J* =, 8.2 Hz), 7.05–7.21 (1H, m), 7.31 (2H, dd, *J* = 7.5, 7.1 Hz), 7.41 (2H, dd, *J* = 7.5, 7.1 Hz), 7.67 (2H, d, *J* = 7.3 Hz), 7.85 (2H, d, *J* = 7.5 Hz); ^{13}C NMR (DMSO-d_6, 75 MHz, rotamer) δ =13.3, 13.8, 28.0, 34.0, 34.7, 41.5, 42.1, 42.7, 43.6, 44.7, 44.9, 46.3, 46.6, 50.5, 50.9, 55.1, 55.2, 55.3, 55.4, 64.9, 65.0, 65.8, 78.4, 78.9, 98.2, 98.5, 104.3, 104.5, 116.3, 117.4, 117.6, 120.1, 125.2, 127.1, 127.6, 128.2, 128.4, 128.9, 132.3, 132.4, 140.7, 143.7, 155.7, 155.8, 157.9, 159.7, 160.2, 169.1, 169.6, 171.1, 171.3; HRMS (ESI-TOF) *m/z* calcd for $C_{40}H_{49}N_3NaO_9$ ($[M + Na]^+$) 738.3367, found 738.3389.

N^2-{[(9H-Fluoren-9-yl)methoxy]carbonyl}-N^4-{2-[(*tert*-butoxycarbonyl)(ethyl) amino]ethyl}-N^4-(2,4-dimethoxybenzyl)-L-asparagine (20)



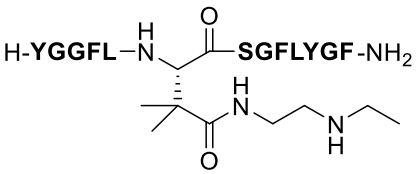
20

To a solution of amide **20** (450 mg, 0.629 mmol) in THF (6.0 mL) were added $Pd(PPh_3)_4$ (72.7 mg, 62.9 μmol) and *N*-methylaniline (685 μL, 6.29 mmol). The reaction mixture was stirred at room temperature for 1 h and concentrated in vacuo. The obtained crude material was purified with column chromatography (*n*-hexane/EtOAc = 1/1 then EtOAc/MeOH = 10/1 (v/v)) to afford carboxylic acid **20** (394 mg, 0.583 mmol, 93%) as a pale yellow amorphous solid: $[\alpha]^{28}_{D}$ 39.5 (*c* 1.26, $CHCl_3$); IR ($CHCl_3$) ν_{max}, cm^{-1} 1289, 1506, 1610, 1643, 1690, 1718, 2843, 2972, 3314, 3427; ^{1}H NMR (DMSO-d_6, 120 °C, rotamer, 300 MHz) δ =1.02 (3H, t, *J* = 7.0 Hz), 1.40 (9H, s), 2.86–2.96 (2H, m), 3.15 (2H, q, *J* = 7.0 Hz), 3.19–3.27 (2H, br m), 3.30–3.42 (2H, br m), 3.75 (3H, s), 3.80 (3H, s), 4.20–4.29 (1H, m), 4.29–4.35 (2H, m), 4.42–4.58 (3H, m), 6.47 (1H, dd, *J* = 8.4, 2.2 Hz), 6.58 (1H, d, *J* = 2.2 Hz), 6.86 (1H, br d, *J* = 7.5 Hz), 7.04 (1H, d, *J* = 8.4 Hz), 7.31 (2H, dd, *J* = 7.5, 7.0 Hz), 7.41 (2H, dd, *J* = 7.5, 7.0 Hz), 7.68 (2H, d, *J* = 7.5 Hz), 7.84 (2H, d, *J* = 7.5 Hz); ^{13}C NMR (DMSO-d_6, 75 MHz, rotamer) δ =13.2, 13.8, 28.0, 33.9, 34.6, 41.5,

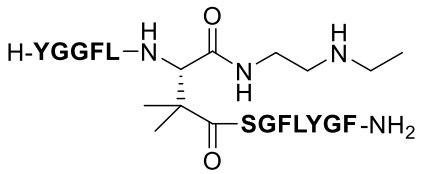
42.2, 42.8, 43.6, 44.0, 44.4, 44.9, 45.3, 46.4, 46.6, 50.5, 50.8, 55.1, 55.2, 55.3, 65.8, 66.3, 78.4, 78.7, 78.9, 98.1, 98.5, 104.3, 104.5, 116.4, 117.1, 120.1, 125.2, 127.1, 127.6, 128.2, 128.4, 128.7, 140.7, 143.8, 154.1, 154.6, 155.7, 155.8, 157.9, 159.7, 160.1, 169.4, 169.8, 172.9, 173.2; HRMS (ESI-TOF) *m/z* calcd for $C_{37}H_{45}N_3NaO_9$ ($[M + Na]^+$) 698.3054, found 698.3051.

Preparation of model peptides 11b, S4, 19 and 23

Peptides were synthesized according to the section "general procedure for Fmoc SPPS"



11b



S4

*Fmoc SPPS using **10b***. Peptide **11b** (major peak): a white lyophilized powder (0.75 mg, 0.411 μmol, 8.2%); retention time = 18.9 min (analytical HPLC conditions: linear gradient of solvent B in A, 25–40% over 30 min); retention time = 21.4 (semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 27–42% over 30 min); LRMS (ESI-TOF) *m/z* calcd for $C_{78}H_{108}N_{16}O_{17}$ ($[M + 2H]^{2+}$) 770.4, found 770.2. Peptide **S4** (minor peak): a white lyophilized powder (0.39 mg, 0.214 μmol, 4.3%); retention time = 20.3 min (analytical HPLC conditions: linear gradient of solvent B in solvent A, 25–40% over 30 min); retention time = 23.0 (semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 27–42% over 30 min); LRMS (ESI-TOF) *m/z* calcd ($[M + 2H]^{2+}$) 770.4, found 770.3.

*Fmoc SPPS using **S3***. Peptide **S4**: retention time = 20.3 min (analytical HPLC conditions: linear gradient of solvent B in solvent A, 25–40% over 30 min); LRMS (ESI-TOF) *m/z* calcd ($[M + 2H]^{2+}$) 770.4, found 770.2.

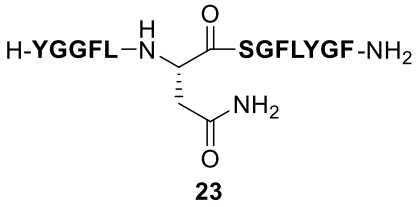
*Fmoc SPPS using **20***.

19

Peptide **19**: a white lyophilized powder (12.0 mg, 6.70 μmol, 50%); retention time = 20.4 min (analytical HPLC conditions: linear gradient of solvent B in solvent A, 30–40% over

30 min); retention time = 24.3 (semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 27–41% over 30 min); LRMS (ESI-TOF) *m/z* calcd for ([M + 2H]$^{2+}$) 756.4, found 756.3.

Fmoc SPPS using Fmoc-Asn(Trt)-OH

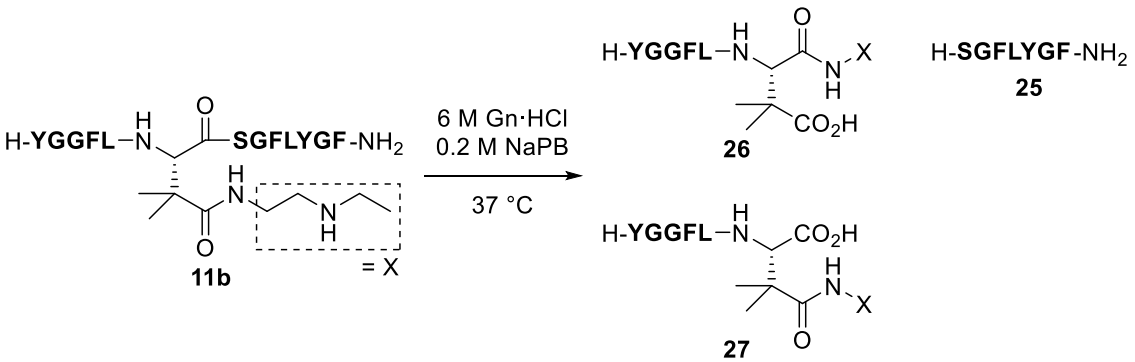


23

Peptide **23**: a white lyophilized powder (8.32 mg, 5.26 µmol, 53%); retention time = 23.5 min (analytical HPLC conditions: linear gradient of solvent B in solvent A, 5–60% over 30 min); retention time = 28.0 (semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 28–42% over 30 min); LRMS (ESI-TOF) *m/z* calcd for ([M + H]$^{+}$) 1440.7, found 1441.0.

S1.2 Self-processing of peptides 11b, 19 and 23

Self-processing of peptide 11b



A solution of model peptide **11b** (45.0 µg, 25.9 nmol) and benzenesulfonic acid sodium salt (internal standard, 20.7 ng, 0.115 nmol) in 0.2 M Na phosphate (pH 7.4 and 7.9, 550 µL) containing 6 M guanidine hydrochloride (Gn·HCl) was incubated at 37 °C and the reaction was monitored by analytical HPLC. Analytical HPLC conditions: a linear gradient of solvent B in solvent A, 1–60% over 30 min.

The remaining substrate was calculated on the basis of peak areas (= *A*) of HPLC as follow. $A^{t=0}$ indicates peak areas at the beginning of the reaction (t = 0).

$$\text{remaining substrate (\%)} = \frac{A_{\text{substrate}} \,/\, A_{\text{internal standard}}}{A^{t=0}_{\text{substrate}} \,/\, A^{t=0}_{\text{internal standard}}} \times 100$$

11b: retention time = 21.9 min.

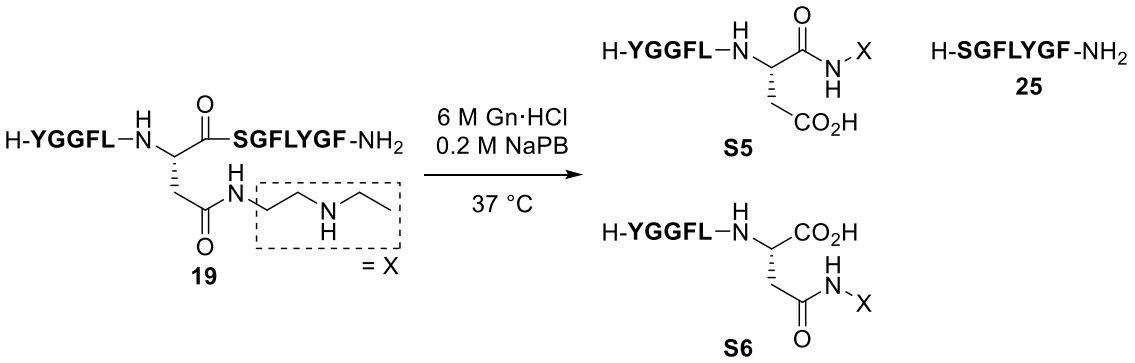
26 or **27**: retention time = 16.4 min; LRMS (ESI-TOF) *m/z* calcd for ([M + H]$^+$) 769.4, found 769.3.

26 or **27**: retention time = 17.4 min; LRMS (ESI-TOF) *m/z* calcd for ([M + H]$^+$) 769.4, found 769.3.

25: retention time = 20.1 min; LRMS (ESI-TOF) *m/z* calcd for ([M + H]$^+$) 789.4, found 789.2.

Benzenesulfonic acid sodium salt (internal standard): retention time = 7.5 min.

Self-processing of peptide 19



The procedure for self-processing of **19** was conducted in a similar to that described for **11b** (pH = 7.9).

19: retention time = 21.9 min.

S5 or **S6**: retention time = 15.6 min; LRMS (ESI-TOF) *m/z* calcd for ([M + H]$^+$) 741.4, found 741.3.

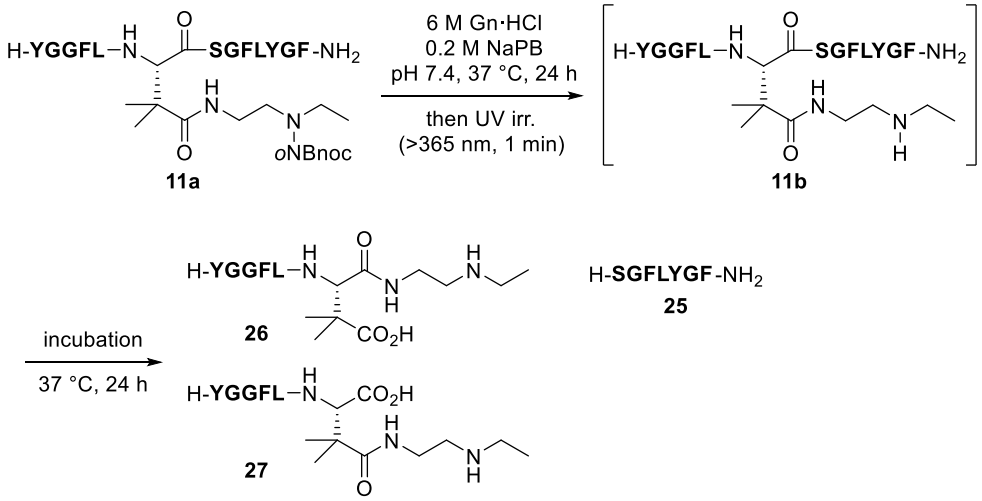
S5 or **S6**: retention time = 16.3 min; LRMS (ESI-TOF) *m/z* calcd for ([M + H]$^+$) 741.4, found 741.3.

Self-processing of peptide 23

The procedure for self-processing of **23** was conducted in a manner similar to that described for **11b** (pH = 7.9). Almost no split peptide was observed within 24 h.

23: retention time = 23.5 min.

S1.3 Photoresponsible amide bond cleavage of peptide 11a



A solution of photoresponsible peptide **11a** (45.0 μg, 24.2 nmol) and benzenesulfonic acid sodium salt (internal standard, 3.00 ng, 16.7 pmol) in 0.2 M Na phosphate (pH 7.4, 515 μL) containing 6 M Gn·HCl was incubated at 37 °C for 24 h, and the reaction mixture was then irradiated by UV (>365 nm) for 1 min. The resulting solution was incubated at 37 °C. The reaction was monitored by analytical HPLC. Analytical HPLC conditions: a linear gradient of solvent B in solvent A, 1–60% over 30 min.

11a: retention time = 28.2 min.

Experimental section — Chapter 2

S2.1 Initial attempt for CPY-mediated hydrazinolysis

Preparation of the peptides 28 and 32

Peptides were synthesized on Fmoc-Xaa-O-Wang resin (see below) by Fmoc SPPS (see general methods).

Preparation of Fmoc-Xaa-O-Wang resin: On Wang resin (0.80 mmol/g), Fmoc-Xaa-OH (10 equiv.) was coupled with the aid of HBTU (9.9 equiv.), DIPEA (10 equiv.) and DMAP (0.05 equiv.) in DMF at room temperature for 3 h. The resulting resin was treated with Ac_2O (10 equiv.) and pyridine (10 equiv.) in DMF at room temperature for 30 min to cap unreacted hydroxyl groups with Ac group. After capping, the loading of Xaa was checked by quantification of the Fmoc group.

Characterization data of peptides are shown in Table S2.1.

Table S2.1. Characterization data of synthesized peptides.

Peptide	Analytical HPLC[a]		Preparative HPLC[b]	*m/z*		Yield
	Retention time (min)	Gradient (%)	Gradient (%)	Calcd	Found	(%)
28	20.4	1–40	13–23	607.3$[M + H]^+$	607.2	45
32	25.7	5–40	22–32	675.4$[M + H]^+$	675.3	56

[a]Cosmosil $5C_{18}$-AR-II analytical column and [b]Cosmosil $5C_{18}$-AR-II preparative column were employed with a linear gradient of solvent B in solvent A over 30 min.

CPY-mediated hydrazinolysis of the peptide 28.

Ac-**ALYGAA**-OH (**28**) (0.050 μmol) was incubated in 250 μL of aqueous solution containing 30 nM CPY and 2 M $NH_2NH_2{\cdot}H_2O$ (pH 8.0) at 25 ˚C for 12 h. The reaction was monitored and analyzed by HPLC. Before analysis, the reaction solution was diluted twice with quenching buffer[a] and incubated at 37 ˚C for 10 min. [a]The quenching buffer: 50 mM Na phosphate, 100 mM TCEP, pH 6.8.

Table S2.2. Characterization data of peptides.

Peptide	Analytical HPLC[a]		*m/z*	
	Retention time (min)	Gradient (%)	Calcd	Found
28	20.5	1–40	607.3$[M + H]^+$	607.4
29	17.8	1–40	550.3$[M + H]^+$	550.3
30a	17.5	1–40	479.3$[M + H]^+$	479.4
30b	19.4	1–40	536.3$[M + H]^+$	536.3
31a	17.5	1–40	422.2$[M + H]^+$	422.2

[a]Cosmosil $5C_{18}$-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min.

For the attempt at direct conversion to the thioester, **28** (0.050 μmol) was incubated in 250 μL of 50 mM HEPES buffer containing 100 nM CPY and 1 M sodium 2-mercaptoethanesulfonate (pH 6.0 and 7.0 and 8.0) at 25 ˚C for 24 h. the reaction was monitored and analyzed by HPLC. Before analysis, the reaction solution was diluted twice with quenching buffer[a] and incubated at 37 ˚C for 10 min. [a]The quenching buffer: 50 mM Na phosphate, 100 mM TCEP, pH 6.8.

To suppress CPY-mediated over-reaction, **28** (0.050 μmol) was incubated in 250 μL of aqueous solution containing 30 nM CPY, 2 M $NH_2NH_2{\cdot}H_2O$ and 50 mM cyclohexanone (pH 8.0) at 25 ˚C for 12 h. The reaction was monitored and analyzed by HPLC. Before analysis, the reaction solution was diluted twice with quenching buffer[a] and incubated at 37 ˚C for 10 min. [a]The quenching buffer: 50 mM Na phosphate, 200 mM $MeONH_2{\cdot}HCl$, 100 mM TCEP, pH 6.8. The HPLC yield was calculated as described in Figure 2.4.

CPY-mediated hydrazinolysis of the peptide 32

Ac-**ALYGPL**-OH (**32**) (0.050 μmol) was incubated in 50 μL of aqueous solution containing 2.4 μM CPY and 2 M $NH_2NH_2{\cdot}H_2O$ with 50 mM cyclohexanone (pH 8.0) at 25 ˚C for 12 h. The reaction was monitored and analyzed by LC-MS. Before analysis, the reaction solution was diluted twice with quenching buffer[a] and incubated at 37 ˚C for 10 min. [a]The quenching buffer: 50 mM Na phosphate, 200 mM $MeONH_2{\cdot}HCl$, 100 mM TCEP, pH 6.8.

S2.2 CPE-like thioesterification followed by NCL

Preparation of the hydrazide 34 and N-terminal Cys peptide 35

H-**LYRAACP**-$NHNH_2$ (**34**) was synthesized on hydrazine 2-Cl Trt resin by Fmoc SPPS (see general methods). The hydrazine 2-Cl Trt resin was prepared according to the protocol described by Liu.[25b] LC-MS conditions: A linear gradient of solvent B in solvent A, 5–35% over 30 min, retention time = 14.8 min. Semi-preparative HPLC condtions: A linear gradient of solvent B in solvent A, 5–18% over 30 min. MS (ESI-TOF) *m/z* calcd ($[M+H]^+$) 807.4, found 807.4.

H-**CFGRK**-NH_2 (**35**) was synthesized on NovaSyn® TGR resin by Fmoc SPPS according to the protocol described by Otaka.[37]

CPE-like thioesterification followed by NCL using hydrazide 34

The hydrazide **34** (0.100 μmol) was dissolved in 64.5 μL of 50 mM Na phosphate buffer containing 6 M Gn·HCl (pH 3.0) and the reaction mixture was stored at -10 ˚C. Then, 2.5 μL of 0.2 M $NaNO_2$ aq. was added to the solution and the reaction mixture was stored at -10 ˚C for 30 min. After that, 33 μL of 50 mM Na phosphate containing 6 M Gn·HCl and 150 mM MPAA was added to the mixture, and the pH of the mixed solution was adjusted to pH 6.5 with 1.0 M NaOH aq.. To the mixture was added N-terminal cysteinyl peptide **35** (0.200 μmol) and the mixed solution was incubated at room temperature. The reaction was monitored and analyzed by LC-MS. Before analysis, the reaction solution was diluted twice with quenching buffer[a] and incubated at 37 ˚C for 10 min. [a]The quenching buffer: 6 M Gn·HCl, 50 mM Na phosphate, 100 mM TCEP, pH 6.8. Characterization data of observed peptides are shown in Table S2.4.

Table S2.4. Characterization data of observed peptides.

Peptide	Analytical HPLC[a]		*m/z*	
	Retention time (min)	Gradient (%)	Calcd	Found
34	14.8	5–35	807.4$[M + H]^+$	807.4
35	11.0	5–35	606.3$[M + H]^+$	606.4
36	26.8	5–35	943.4$[M + H]^+$	943.4
37	15.7	5–35	775.4$[M + H]^+$	775.4
38	22.8	5–35	743.4$[M + H]^+$	743.4
39	17.7	5–35	592.4$[M + 2H]^{2+}$	592.3

[a]Cosmosil $5C_{18}$-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min.

S2.3 Optimization of reaction conditions for CPY-mediated hydrazinolysis

Preparation of the C-terminal –CPX–OH peptides 40 and 40a–k

All peptides were prepared according to the protocol described in *"Experimental section — Chapter 2; S2.1 — Synthesis of peptide **28** and **32**"*.

Characterization data of peptides are shown in Table S2.5.

Table S2.5. Characterization data of synthesized peptides.

Peptide	Analytical HPLC[a]		Preparative HPLC[b]	*m/z*		Yield (%)
	Retention time (min)	Gradient (%)	Gradient (%)	Calcd	Found	
40	25.9	5–30	17–24	905.5$[M + H]^+$	905.4	52
40a	22.8	5–30	13–23	923.4$[M + H]^+$	923.2	27
40b	25.5	5–30	15–25	905.5$[M + H]^+$	905.6	49
40c	22.6	5–30	13–23	939.5$[M + H]^+$	939.7	57
40d	18.1	5–30	10–20	863.4$[M + H]^+$	863.6	52
40e	28.2	5–30	16–26	939.5$[M + H]^+$	939.7	52
40f	22.5	5–30	13–23	955.5$[M + H]^+$	955.7	61
40g	28.8	5–30	15–26	978.5$[M + H]^+$	978.7	20
40h	17.2	5–30	8–18	849.4$[M + H]^+$	849.6	13
40i	15.6	5–30	8–18	920.5$[M + H]^+$	920.6	36
40j	17.2	5–30	9–19	907.4$[M + H]^+$	907.7	48
40k	15.3	5–30	8–13	906.4$[M + H]^+$	906.6	17

[a]Cosmosil 5C_{18}-AR-II analytical column and [b]Cosmosil 5C_{18}-AR-II preparative column were employed with a linear gradient of solvent B in solvent A over 30 min.

CPY-mediated hydrazinolysis of C-terminal -CPX-OH peptide

The standard procedure (Table 2.2, entry 3): H-**LYRAACPL**-OH (**40**) (0.050 μmol) was incubated in 50 μL (1 mM peptide) of aqueous solution of 1.2 μM CPY, 0.2 M $NH_2NH_2 \cdot H_2O$, 60 mM cyclohexanone, pH 6.4 at 25 ˚C. The reaction was monitored and analyzed by LC-MS. Before analysis, the reaction solution was diluted twice with quenching buffer[a] and incubated at 37 ˚C for 10 min. [a]The quenching buffer: 50 mM Na phosphate, 200 mM $MeONH_2 \cdot HCl$, 200 mM TCEP, pH 6.8.

To optimize the reaction conditions, the concentrations of $NH_2NH_2{\cdot}H_2O$, cyclohexanone and CPY and the pH were separately changed from the standard procedure. HPLC yield was calculated as described in Table 2.2.

To re-evaluate carbonyl compounds as additives for this reaction, each carbonyl compound was used instead of cyclohexanone. HPLC yield was calculated as described in Table 2.3.

To evaluate the influence of C-terminal amino acid of substrate, each peptide **40a–k** was used instead of peptide **40**. HPLC yield was calculated as described in Table 2.4.

To isolate hydrazide **34**, peptide substrate **40** (2.00 μmol) was dissolved in 0.4 mL (5 mM peptide) of hydrazinolysis solution [0.3 μM CPY, 0.2 M $NH_2NH_2 \cdot H_2O$, 60 mM cyclohexanone, pH 6.4] and incubated at 25 ˚C for 1 h. After completion of the reaction, the reaction solution was diluted twice with the quenching buffer. The crude material was analyzed by LC-MS and purified by semi-preparative HPLC to give the hydrazide **34** (1.2 mg, 1.50 μmol, 76%). Semi-preparative HPLC condition was same as described in the preparation of **34**.

S2.4 Preparation of peptide thioesters using CPY-mediated protocol

Preparation of the C-terminal –XCPL–OH peptides 40a'–q'

All peptides were prepared according to the protocol described in *"Experimental section — Chapter 2; S2.1 — Synthesis of peptide **28** and **32**"*.

Characterization data of peptides are shown in Table S2.6.

Table S2.6. Characterization data of synthesized peptides.

Peptide	Analytical HPLC[a]		Preparative HPLC[b]	*m/z*		Yield (%)
	Retention time (min)	Gradient (%)	Gradient (%)	Calcd	Found	
40a'	25.0	5–30	14–24	892.5$[M + H]^+$	892.6	39
40b'	24.2	5–30	16–26	991.5$[M + H]^+$	991.5	44
40c'	23.5	5–30	12–22	963.5$[M + H]^+$	963.6	35
40d'	23.8	5–30	13–23	972.5$[M + H]^+$	971.6	26
40e'	25.0	5–30	16–26	922.5$[M + H]^+$	922.6	43
40f'	25.8	5–30	16–26	936.5$[M + H]^+$	936.5	41
40g'	27.9	5–30	16–36	938.5$[M + H]^+$	938.6	35
40h'	24.4	5–30	14–24	949.5$[M + H]^+$	949.6	49
40i'	25.2	5–30	15–25	963.5$[M + H]^+$	963.5	50
40j'	26.0	5–30	14–24	964.5$[M + H]^+$	964.5	44
40k'	26.0	5–35	18–28	967.2$[M + H]^+$	9675	54
40l'	22.4	5–40	22–32	1021.5$[M + H]^+$	1021.4	6
40m'	25.2	5–35	16–26	998.5$[M + H]^+$	998.5	31
40n'	25.5	5–40	22–32	982.5$[M + H]^+$	982.6	49
40o'	27.5	5–35	18–28	948.5$[M + H]^+$	948.6	52
40p'	18.6	5–55	20–30	948.5$[M + H]^+$	948.3	42
40q'	29.2	5–35	18–28	934.5$[M + H]^+$	934.7	28

[a]Cosmosil $5C_{18}$-AR-II analytical column [b]Cosmosil $5C_{18}$-AR-II preparative column were employed with a linear gradient of solvent B in solvent A over 30 min.

CPY-mediated hydrazinolysis of C-terminal -XCPL-OH peptides 40a'–q'

To examine the compatibility and efficiency of the CPY-mediated hydrazinolysis, CPY-mediated hydrazinolysis of peptides **40a'–q'** was conducted according to the protocol described in *"Experimental section — Chapter 2; S2.3 — CPY-mediated hydrazinolysis of C-terminal –CPX–OH peptide — The standard procedure"*. The HPLC yield was calculated as described in Table 2.5.

Preparation of the hydrazide 34a–q

Except for **34l**, hydrazides **34a–q** were prepared by CPY-mediated hydrazinolysis in a

manner described below.

Peptide (1.00 μmol) was incubated in 0.2 mL (5 mM peptide) of aqueous solution containing 0.3 μM CPY, 0.2 M $NH_2NH_2{\cdot}H_2O$ and 60 mM cyclohexanone (pH 6.4) and incubated at 25 ˚C for 1 h. After completion of the reaction, the solution was diluted twice with the quenching buffer. The crude material was analyzed by LC-MS and purified by semi-preparative HPLC to give the corresponding hydrazide.

34l was prepared by Fmoc SPPS according to the protocol for preparation of **34**. Characterization data of peptides are shown in Table S2.7.

Table S2.7. Characterization data of peptides.

Peptide	Analytical HPLC[a]		Semi-preparative HPLC[b]	*m/z*		Yield (%)
	Retention time (min)	Gradient (%)	Gradient (%)	Calcd	Found	
34a	14.0	5–30	6–16	793.4$[M + H]^+$	793.5	64
34b	14.2	5–30	7–17	892.5$[M + H]^+$	892.6	66
34c	14.0	5–30	7–17	864.5$[M + H]^+$	864.5	72
34d	14.2	5–30	6–16	873.5$[M + H]^+$	873.6	95
34e	14.1	5–30	7–17	823.4$[M + H]^+$	823.4	76
34f	14.8	5–30	7–17	837.4$[M + H]^+$	837.5	39
34g	16.5	5–30	9–19	839.4$[M + H]^+$	839.4	26
34h	13.6	5–30	6–16	850.4$[M + H]^+$	850.6	62
34i	14.6	5–30	8–18	864.4$[M + H]^+$	864.4	66
34j	15.2	5–30	5–15	865.4$[M + H]^+$	865.4	56
34k	16.5	5–35	10–20	867.4$[M + H]^+$	867.4	59
34l	20.5	5–35	8–18	922.5$[M + H]^+$	922.7	2
34m	18.2	5–30	10–20	899.5$[M + H]^+$	899.5	86
34n	19.7	5–35	13–23	883.5$[M + H]^+$	883.6	66
34o	18.5	5–35	14–24	849.5$[M + H]^+$	849.6	37
34p	17.4	5–35	10–20	849.5$[M + H]^+$	849.5	67
34q	15.8	5–35	10–20	835.5$[M + H]^+$	835.5	71

[a]Cosmosil $5C_{18}$-AR-II analytical column [b]Cosmosil $5C_{18}$-AR-II semi-preparative column were employed with a linear gradient of solvent B in solvent A over 30 min.

CPE-like Thioesterification followed by NCL using peptide 34a–q

Using **34a–q**, we examined the compatibility and efficiency of the CPE-like ligation according to the protocol described in *"Experimental section — Chepter 2; S2.3 — CPE-like thioesterification followed by NCL using hydrazide **34**"*. The HPLC yield was calculated as described in Table 2.5.

S2.5 Chemical synthesis of reduced form CNP 53 (41)

Preparation of peptide fragments 43 and 45

Both peptides were prepared according to the protocol described in *"Experimental section — Chapter 2; S2.1 — Synthesis of peptides **28** and **32**"*.

Characterization data of peptides are shown in Table S2.8.

Table S2.8. Characterization data of synthesized peptides.

Peptide	Analytical HPLC[a]		Preparative HPLC[b]	*m/z*		Yield (%)
	Retention time (min)	Gradient (%)	Gradient (%)	Calcd	Found	
43	22.1	5–35	14–20	547.9$[M + 8H]^{2+}$	547.8	7
45	23.9	5–30	22–32	878.9$[M + 2H]^{2+}$	878.7	16

[a]Cosmosil $5C_{18}$-AR-II analytical column and [b]Cosmosil $5C_{18}$-AR-II preparative column were employed with a linear gradient of solvent B in solvent A over 30 min.

CPY-mediated hydrazinolysis of peptide 43.

Peptide **43** (0.854 μmol) was incubated in 1.0 mL (0.854 mM peptide) of aqueous solution containing 0.6 μM CPY, 0.2 M $NH_2NH_2{\cdot}H_2O$ and 60 mM cyclohexanone (pH 6.4) and incubated at 25 ˚C for 1 h. After completion of the reaction, the solution was diluted twice with the quenching buffer[a]. The crude material was analyzed by HPLC and purified by semi-preparative HPLC to give hydrazide **44** (2.6 mg, 0.595 μmol, 70%). [a]The quenching buffer: 50 mM Na phosphate, 200 mM $MeONH_2{\cdot}HCl$, 200 mM TCEP, pH 6.8.

CPE-like Thioesterification followed by NCL using peptide 44 and 45

Hydrazide **34** (0.595 μmol) was dissolved in 387 μL of 50 mM Na phosphate buffer containing 6 M Gn·HCl (pH 3.0) and the reaction mixture was stored at -10 ˚C. Then, 15 μL of 0.2 M $NaNO_2$ aq. was added to the solution, and the reaction mixture was stored at -10 ˚C for 1 h. After that, 198 μL of 50 mM Na phosphate containing 6 M Gn·HCl and 150 mM MPAA was added to the solution, and the pH of the mixed solution was adjusted to pH 6.5 with 1.0 M NaOH aq.. To the mixture was added N-terminal Cys peptide **45** (1.2 μmol) in 600 μL of 50 mM Na phosphate containing 6 M Gn·HCl and 50 mM MPAA (pH6.5) and the reaction mixture was incubated at room temperature. The reaction was monitored and analyzed by HPLC. Before analysis, the reaction solution was diluted twice with quenching buffer[a] and incubated at 37 ˚C for 10 min. [a]The quenching buffer: 6 M Gn·HCl, 50 mM Na phosphate, 100 mM TCEP, pH 6.8. After completion of the reaction, the solution was diluted twice with the quenching buffer. The crude material was purified by semi-preparative HPLC to give CNP 53 **41** (3.0 mg, 0.522 μmol, 88%).

Table S2.9. Characterization data of synthesized peptides.

Peptide	Analytical HPLC[a]		Semi-preparative HPLC[b]	*m/z*		Yield (%)
	Retention time (min)	Gradient (%)	Gradient (%)	Calcd	Found	
44	22.8	5–35	13-23	1070.1[M+4H]	1070.2	70
41	23.9	5–35	14-24	1161.0[M+5H]	1161.1	88

[a]Cosmosil 5C_{18}-AR-II analytical column and [b]Cosmosil 5C_{18}-AR-II preparative column were employed with a linear gradient of solvent B in solvent A over 30 min.

S2.6 Application to expressed protein

DsRED protein **46** (14.5 nmol) was incubated in 266 μL (55 μM) of aqueous solution containing 1.2 μM CPY, 0.2 M $NH_2NH_2{\cdot}H_2O$, 60 mM cyclohexanone (pH 6.4) at 25 ˚C for 1 h. The reaction mixture was quenched with 266 μL of 50 mM Na phosphate containing 200 mM DTT, 200 mM $MeONH_2{\cdot}HCl$. The mixture was incubated at 25 ˚C for 10 min and then buffer-exchanged, by use of a centrifugal filter equipped with a 10 kDa molecular weight cut off, into 50 mM Na phosphate (pH 6.8, 150 μL) by repeated dilution/concentration about 10 times. A part of the protein mixture (3.0 nmol, 53 μL) was mixed with 50 μL of 50 mM Na phosphate containing 6 M Gn·HCl and the pH was

adjusted to 3.0 with 1 N HCl aq.. To the mixture, 10 μL of 1.48 mM $NaNO_2$ aq. was added at 4 ˚C and the reaction mixture was stored at 4 ˚C for 30 min. Then biotinylated N-terminal Cys peptide **48** (96.3 nmol) in 51 μL of 50 mM Na phosphate containing 6 M Gn·HCl and 30 mM MPAA was added to the reaction mixture and the mixture was incubated at room temperature. After that the crude protein was obtained by trichloroacetic acid (TCA) precipitation[38] to remove small molecules and analyzed by SDS-PAGE (CBB stain and Western blot analysis using streptavidin-HRP). The conversion yield was estimated by quantitation of the ligated protein from CBB-stained gel image using imageJ software.

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List of publications

Publications regarding this thesis

Chapter 1

Development of an intein-inspired amide cleavage chemical device

Chiaki Komiya[†], Keisuke Aihara[†], Ko Morishita, Hao Ding, Tsubasa Inokuma, Akira Shigenaga, and Akira Otaka[*] ([†]equal contribution)

J. Org. Chem. **2016**, *81*, 699–707.

Chapter 2

Preparation of protein thioesters enabled by carboxypeptidase

Chiaki Komiya, Jun Tsukimoto, Ueda Masahiro, Takuya Morisaki, Tsubasa Inokuma, Akira Shigenaga, Kohji Itoh, and Akira Otaka[*]

Manuscript in preparation

Other publications

1. Design and synthesis of a hydrogen peroxide-responsive amino acid that induces peptide bond cleavage after exposure to hydrogen peroxide

   Miku Kita, Jun Yamamoto, Takuya Morisaki, **Chiaki Komiya**, Tsubasa Inokuma, Licht Miyamoto, Koichiro Tsuchiya, Akira Shigenaga*, and Akira Otaka*

   Tetrahedron Letters **2015**, *56*, 4228–4231.

2. Synthesis of lactam-bridged cyclic peptides by using olefin metathesis and diimide reduction

   Keisuke Aihara, Tsubasa Inokuma, **Chiaki Komiya**, Akira Shigenaga, and Akira Otaka*

   Tetrahedron **2015**, *71*, 4183–4191.

3. A photoinduced growth system of peptide nanofibers addressed by DNA hybridization

   Masahiro Furutani, Akihito Uemura, Akira Shigenaga, **Chiaki Komiya**, Akira Otaka, and Kazunori Matsuura*

   Chemical Communications **2015**, *51*, 8020–8022.

4. Liquid-phase synthesis of bridged peptides using olefin metathesis of a protected peptide with a long aliphatic chain anchor

   Keisuke Aihara, **Chiaki Komiya**, Akira Shigenaga, Tsubasa Inokuma, Daisuke Takahashi, and Akira Otaka*

   Organic Letters **2015**, *17*, 696–699.

5. Development of a fluoride-responsive amide bond cleavage device that is potentially applicable to a traceable linker

Jun Yamamoto[†], Nami Maeda[†], **Chiaki Komiya**, Tomohiro Tanaka, Masaya Denda, Koji Ebisuno, Wataru Nomura, Hirokazu Tamamura, Youichi Sato, Aiko Yamauchi, Akira Shigenaga[*], and Akira Otaka[*] ([†]equal contribution)

Tetrahedron **2014**, *70*, 5122–5127.

6. Development of a traceable linker containing a thiol-responsive amino acid for the enrichment and selective labelling of target proteins

Jun Yamamoto, Masaya Denda, Nami Maeda, Miku Kita, **Chiaki Komiya**, Tomohiro Tanaka, Wataru Nomura, Hirokazu Tamamura, Youichi Sato, Aiko Yamauchi, Akira Shigenaga[*], and Akira Otaka[*]

Organic & Biomolecular Chemistry **2014**, *12*, 3821–3826.