Development of synthetic procedures using novel thioester derivatives for chemical synthesis of proteins

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Abbreviations

AAA	amino acid analysis
Ac	acetyl
Acm	acetamidomethyl
Ar	aryl
Boc	*tert*-butoxycarbonyl
Bom	benzyloxymethyl
BrZ	2-bromobenzyloxycarbonyl
*t*Bu	*tert*-butyl
Bzl	benzyl
CD	circular dichroism
ClZ	2-chlorobenzyloxycarbonyl
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DEAD	diethyl azodicarboxylate
DIC	*N*,*N'*-diisopropylcarbodiimide
DIEA	*N*,*N*-diisopropylethylamine
DMF	*N*,*N*-dimethylformamide
DTT	dithiothreitol
EDT	1,2-ethanedithiol
ESI	electrospray ionization
ETFE	ethyl trifluoroacetate
EtOAc	ethyl acetate
Fmoc	9-fluorenylmethyloxycarbonyl
For	formyl
Gn·HCl	guanidine hydrochloride
GSH	reduced glutathione
GSSG	oxidized glutathione
HATU	1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium 3-oxide hexafluorophosphate
HBTU	1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium-3-oxide hexafluorophosphate
HCTU	1-[bis(dimethylamino)methylene]-5-chloro-1*H*-benzotriazolium-3-oxide hexafluorophosphate

*c*Hex	cyclohexyl
HOBt	1-hydroxybenzotriazole
Hoc	cyclohexyloxycarbonyl
HPLC	high-performance liquid chromatography
HRMS	high resolution mass spectrometry
KCL	kinetically controlled ligation
MBHA	4-methylbenzhydrylamine hydrochloride
MeBzl	4-methylbenzyl
$MeONH_2 \cdot HCl$	methoxyamine hydrochloride
MPA	3-mercaptopropionic acid
MPAA	4-mercaptophenylacetic acid
Ms	methanesulfonyl
MS	mass spectrometry
NCL	native chemical ligation
NH_4OAc	ammonium acetate
NMA	*N*-methylaniline
NMP	*N*-methylpyrrolidone
NMR	nuclear magnetic resonance
Ns	*o*-nitrobenzenesulfonyl
PAM	4-(hydroxymethyl)phenylacetamidomethyl
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
Ph_3P	triphenylphosphine
PhSH	thiophenol
ProTx-I	protoxin-I
PTH	phenylthiohydantoin
PyBOP	1-benzotriazolyloxy-tris-pyrrolidinophosphonium hexafluorophosphate
SPPS	solid-phase peptide synthesis
SEAlide	*N*-sulfanylethylanilide
TCEP	tris(2-carboxyethyl) phosphine
TES	triethylsilane
Tfa	trifluoroacetyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIS	triisopropylsilane
TOF	time of flight

Tos	toluenesulfonyl
Trt	trityl
UV	ultraviolet
VA-044	2,2′-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride
Xan	xanthenyl
6-Cl-HOBt	1-hydroxy-6-chloro-benzotriazole

The commonly used one- and three- abbreviations for amino acids:

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

Contents

Experimental sections

Preface

The chemical synthesis of peptides and proteins is well recognized as a crucial technology for the study of protein-related biological phenomena. Furthermore, synthetic methodology provides access to large quantities of peptides, as well as peptides possessing post-translational modifications and unnatural amino acids. Solid-phase peptide synthesis (SPPS) is a vital methodology in peptide synthesis. However, preparation of homogeneous long-chain peptides is difficult to achieve with conventional stepwise SPPS because of the formation of impurities such as deleted, truncated, or terminated materials. These problems have been overcome by the development and continuous improvement of native chemical ligation (NCL), which is a reliable synthetic methodology for long-chain peptides featuring chemoselective condensation of high-performance liquid chromatography (HPLC)-purified unprotected peptide fragments. The condensation uses the chemoselective reaction of peptide thioesters with N-terminal Cys-peptides in neutral aqueous buffers to afford ligated peptides. One key requirement for NCL is the successful synthesis of peptide thioesters.

In this study, I investigate the effect of thiol groups present in thioester fragments on the promotion of NCL and the development of a novel peptide thioester and thioester equivalent that applicable to Boc- and Fmoc-SPPS, respectively. In Chapter 1, I describe a novel NCL-mediated methodology for Cys-rich peptides that does not require thiol additives, which were previously thought to be essential for the acceleration of NCL. This newly developed protocol facilitates NCL even when bulky thioesters such as Ile and Thr are used and enables a one-pot synthesis comprising NCL and subsequent oxidative folding to produce Cys-rich peptides. In Chapter 2, on the basis of the results reported in Chapter 1, I present a highly active peptide thioester prepared by Boc-SPPS. The novel thioester (peptide-TfaC) can be readily prepared using conventional Boc-SPPS without any side reactions. Furthermore, the considerable difference in the reactivities of peptide-TfaC and conventional alkylthioesters allows the novel thioester to be used in a kinetically controlled ligation in the same manner as an arylthioester. In Chapter 3, the preparation of the peptide *N*-sulfanylethylanilide (SEAlide) as a highly versatile crypto-thioester using Fmoc-SPPS is described.

I believe that these methods can be widely used in the field of NCL-mediated synthesis of many types of peptides and proteins.

Chapter 1

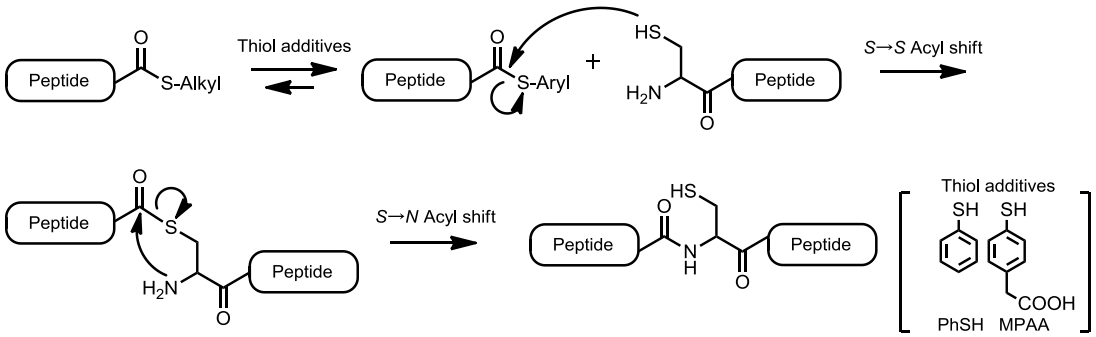
Thiol-additive-free native chemical ligation enables facile synthesis of cysteine-rich peptides

1.1 Importance of the synthesis of peptides containing disulfide bonds

Various venom peptides isolated from natural sources, such as scorpions, snakes, and spiders, have several disulfide bonds that are indispensable for exhibiting their biological activity as specific binders to receptors or ion channels.[1] Chemical synthesis of such Cys-rich peptides serves as an indispensable research means for elucidating of their biological significance and action. Furthermore, chemical synthesis enables access to the large quantities of peptide material necessary for investigation of the biological events related to specific binding proteins, the refinement of proposed primary structures, and 3D-structural analysis. It also allows access to highly active or selective peptides, facilitating the development of novel and useful peptide ligands.[2]

1.2 The role of thiol additives in native chemical ligation

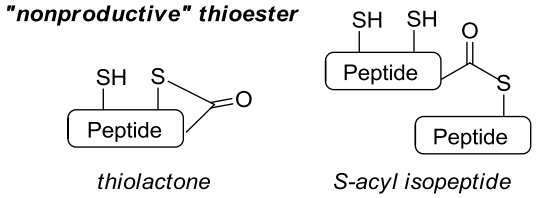
Native chemical ligation (NCL), which exploits the chemoselective reaction of peptide thioesters with N-terminal Cys-peptides to afford ligated peptides, is the most reliable synthetic strategy for Cys-rich peptides.[3] In general, peptide alkylthioesters with moderate reactivity are synthesized using Boc solid-phase peptide synthesis (Boc-SPPS)[4] and subsequently converted into their corresponding highly reactive arylthioesters in the presence of thiol additives prior to being subjected to NCL with N-terminal Cys-peptides (Scheme 1.1). Arylthiols such as thiophenol (PhSH)[5] and 4-mercaptophenylacetic acid (MPAA)[6] have been frequently used as thiol additives; however, the toxicity and smell of PhSH has led to odorless MPAA becoming adopted as the gold standard thiol additive used in NCL. One drawback with the use of MPAA is its co-elution with the ligated peptide during HPLC product purification, resulting in a low isolated yield of the desired peptide.[7] In the case of Cys-rich peptides, contamination with MPAA sometimes influences their oxidative folding reaction, causing the formation of incorrectly disulfide-bonded peptides. This situation has necessitated the study of thiol-additive-free NCL for the synthesis of Cys-rich peptides.



Scheme 1.1. Mechanism of native chemical ligation.

1.3 Effects of thiol additives on the NCL of Cys-rich peptides

In the case of NCL-mediated synthesis of Cys-rich peptides, the presence of arylthiols is thought to be essential not only for the promotion of NCL but also to prevent the intra- or intermolecular formation of "nonproductive" thioester species.[8] For example, intramolecular thiol exchange of Cys-containing thioesters affords the corresponding thiolactone species, and *S*-acyl isopeptide[9] species are formed through intermolecular thiol exchange reactions (Scheme 1.2). As a model Cys-rich peptide, I selected protoxin-I (ProTx-I, 33 amino acids, 6 Cys residues, 3 disulfide bonds) isolated from the venom of the tarantula *Thrixopelma pruriens*.[10] To examine the effect of thiol additives on the preparation of ProTx-I using NCL, the entire sequence of ProTx-I was divided into N- and C-fragments corresponding to ProTx-I (1-14) and (15-33), respectively. The N-fragments required for NCL were synthesized by Boc-SPPS as conventional alkylthioesters (thiol unit: 3-mercaptopropionyl-Leu-NH_2 (MPA-Leu-NH_2)),[11] both with *S*-acetamidomethyl (Acm) protection and without *S*-protection. The C-fragments were also synthesized with and without the *S*-protection. The fragments synthesized for the experiment are listed in Table 1.1. Here, the use of *S*-Acm protected fragments **1** and **2** in the NCL does not afford the nonproductive thioester species.



Scheme 1.2. Structures of "nonproductive" thioester species (thiolactone and *S*-acyl isopeptide)

Table 1.1. Fragments of ProTx-I synthesized.

1: H-**EC**(Acm)**RYWLGGC**(Acm)**SAGQT**-SCH_2CH_2CO-**L**-NH_2
2: H-**CC**(Acm)**KHLVC**(Acm)**SRRHGWC**(Acm)**VWDGTFS**-OH
3: H-**ECRYWLGGCSAGQT**-SCH_2CH_2CO-**L**-NH_2
4: H-**CCKHLVCSRRHGWCVWDGTFS**-OH

In NCL, tris(2-carboxyethyl)phosphine (TCEP) in the ligation buffer functions as a reducing agent to maintain the N-terminal Cys residue in its active reduced form.[12] Initially, I checked whether TCEP influences the progress of NCL. NCL of *S*-Acm protected N-fragment **1** with *S*-Acm protected C-fragment **2** was conducted in a ligation buffer (6 M guanidine hydrochloride (Gn·HCl), 100 mM Na_2HPO_4, 100 mM sodium ascorbate, pH 7.8) in the presence of different concentrations of TCEP under thiol-additive-free conditions. Sodium ascorbate was used to prevent the desulfurization of Cys residues.[13] As shown in Figure 1.1, NCL was accelerated by increasing the TCEP concentration, although the reactions could not reach completion. This result indicates that TCEP works not only as a reducing agent but also as an activator for NCL.[14] Thus, I performed all further NCL reactions using a TCEP concentration of 50 mM.

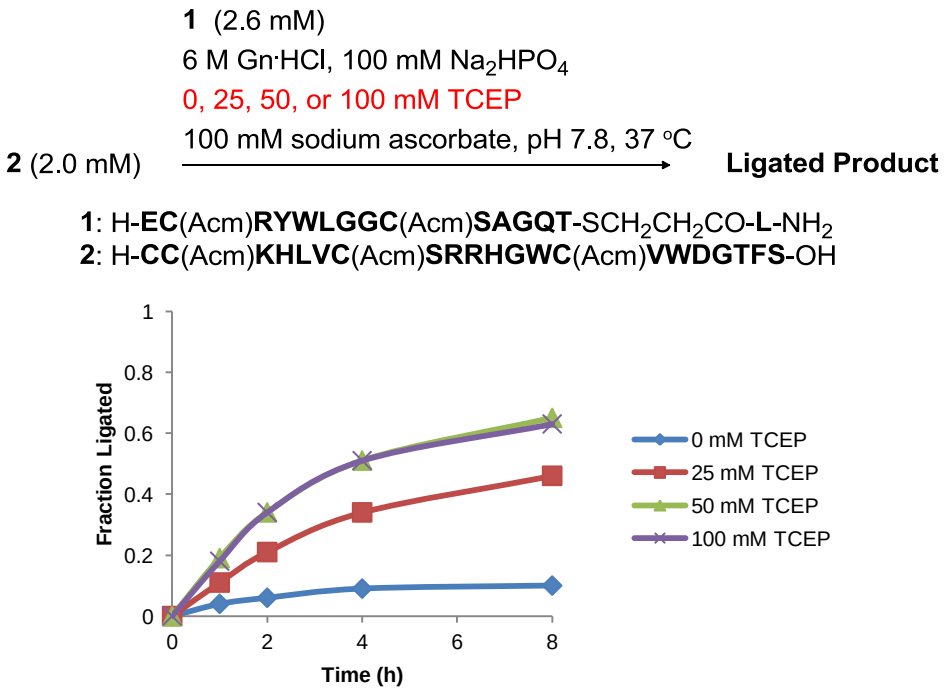


Figure 1.1. Progress of NCL reactions between *S*-Acm protected N-fragment **1** and *S*-Acm protected C-fragment **2** in the presence of different concentrations of TCEP. The progress of the NCL reaction was quantified by integration of the ligated product as a fraction of the sum of the unreacted C-fragment **2** and the ligated product, as obtained from HPLC (220 nm).

I next examined the effect of MPAA or PhSH (100 mM) on the progress of NCL between the alkylthioester **1** and the Cys peptide **2**. The data in Figure 1.2 indicates that MPAA is a NCL promoter superior to PhSH, and the addition of arylthiols is crucial for completion of NCL at bulky Thr-Cys sites.[15]

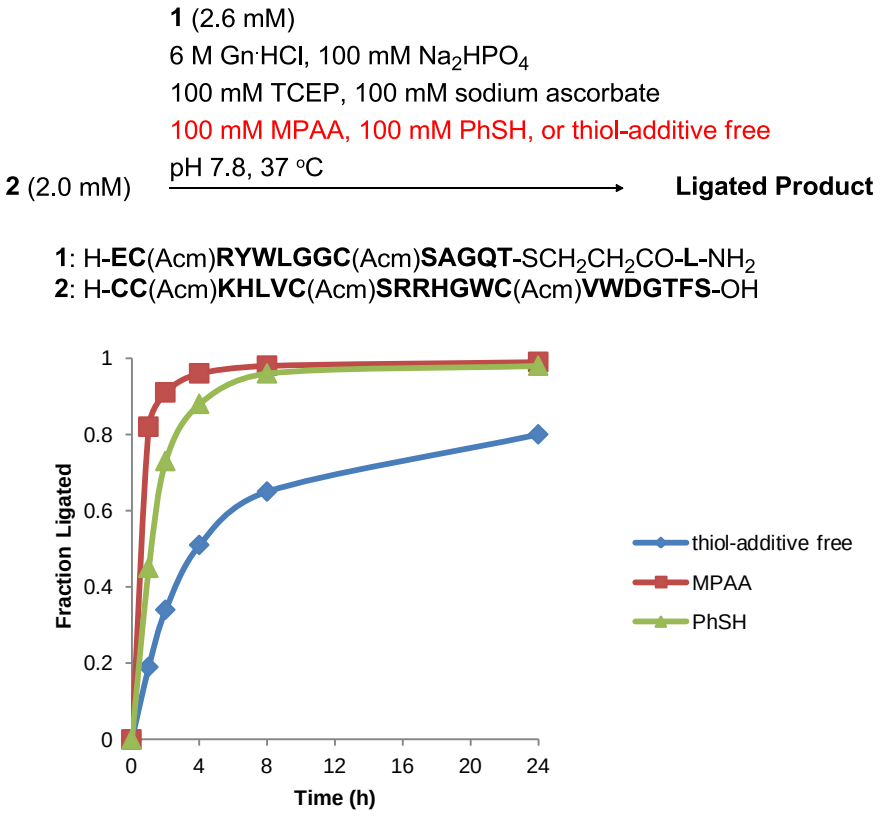


Figure 1.2. Effect of thiol additives on NCL reactions between N-fragment **1** and C-fragment **2**. The extent of NCL was quantified by integration of the ligated product as a fraction of the sum of the unreacted C-fragment **2** and the ligated product, as obtained from HPLC (220 nm).

Next, I attempted NCL between N- and C-fragments **3** and **4** without *S*-Acm protection to evaluate the effect of MPAA and PhSH (Figure 1.3). To our great surprise, the reaction attempted under thiol-additive-free conditions proceeded with efficiency comparable with that observed when MPAA was used and reached virtual completion within 8 h. This unexpected observation is contradictory to the widely held belief that an excess quantity of a thiol additive is needed to prevent the formation of "nonproductive" thioesters.[8]

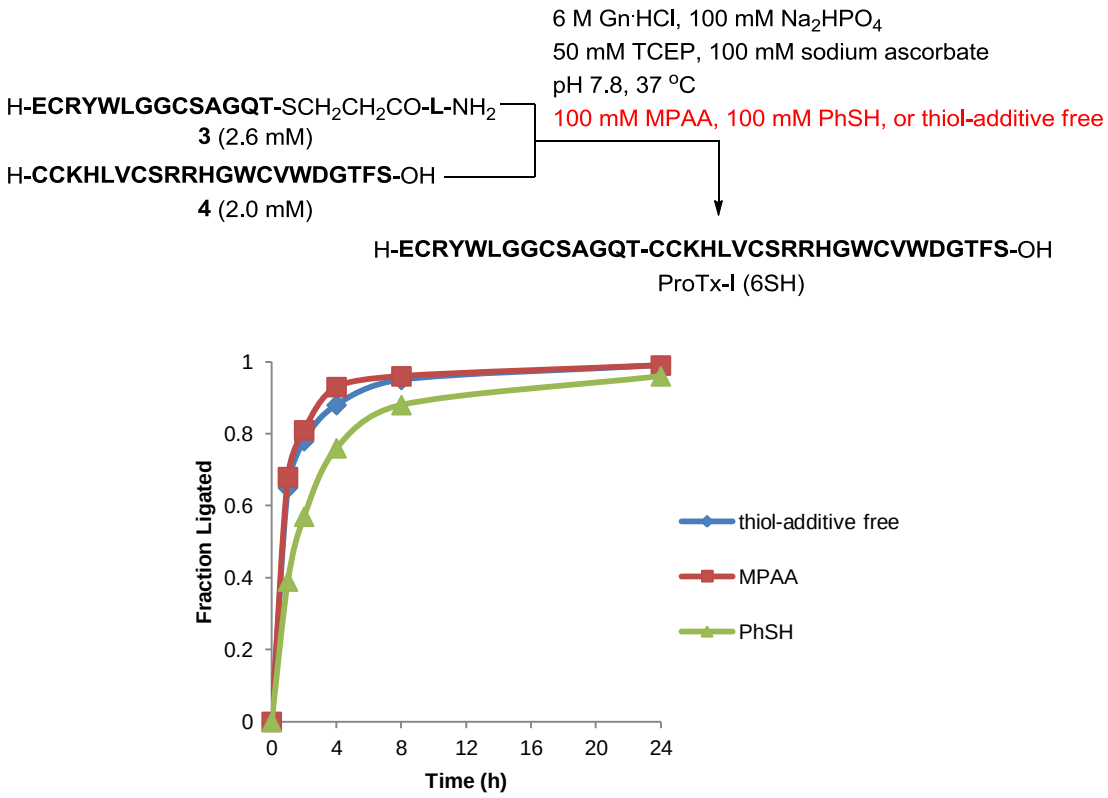


Figure 1.3. Effect of thiol additives on NCL reactions between N-fragment **3** and C-fragment **4**. The extent of NCL was quantified by integration of the ligated product as a fraction of the sum of the unreacted C-fragment **4** and the ligated product, as obtained from HPLC (220 nm).

1.4 Consideration of why thiol additives are not required in the NCL of Cys-rich peptides

The fact that NCL using *S*-unprotected fragments **3** and **4** proceeded efficiently under thiol-additive-free conditions, whereas thiol additives had a considerable effect on NCL between *S*-Acm protected fragments **1** and **2**, indicated the indispensable involvement of thiol groups of the internal Cys residues on progress of NCL.

To evaluate the involvement of the free internal Cys residues, I attempted NCL under thiol-additive-free conditions with the combinations of N- and C-fragments as follows: (A) **1** and **2**; (B) **3** and **2**; (C) **1** and **4**; and (D) **3** and **4**. The results were summarized in Figure 1.4. The NCL did complete within 24 h to afford the corresponding ligated products for all combinations except for (A) using **1** and **2**. These results led us to hypothesize that the internal Cys residues were not involved in the formation of "nonproductive" thioesters such as thiolactones and *S*-acyl isopeptides but

were involved in the formation of "productive" thioesters, thereby promoting NCL. In other words, thiolactones and *S*-acyl isopeptides, which have previously been believed to be "nonproductive" thioesters, seem to be "productive" thioesters.

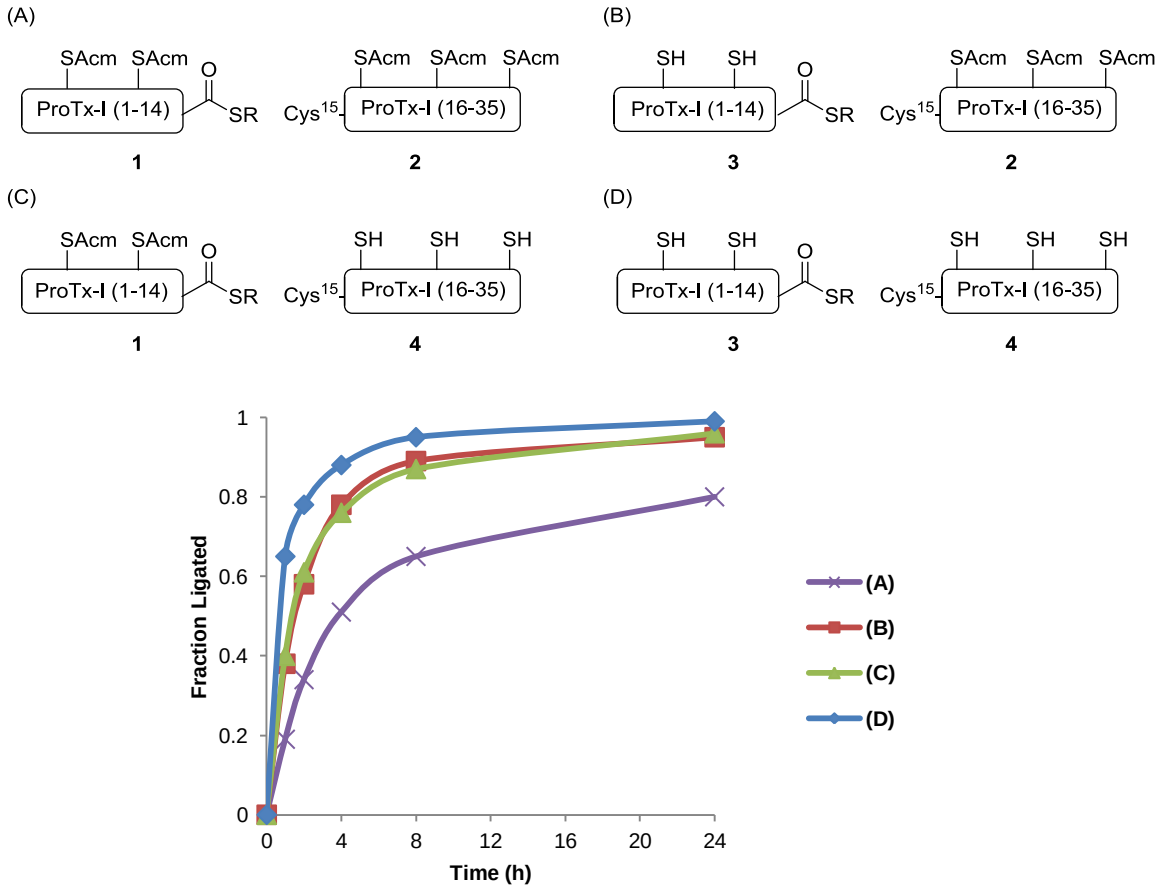


Figure 1.4. Progress of thiol-additive-free NCL reactions between N- and C-fragments: (A) **1** + **2**, (B) **3** + **2**, (C) **1** + **4**, and (D) **3** + **4**.

To confirm this hypothesis, I performed model reactions using thiolactone **5**, *S*-acyl isopeptide **6**, the conventional alkylthioester **7**, and arylthioester **8** with the Cys peptide **9** (Figure 1.5). As expected, thiolactone **5** and *S*-acyl isopeptide **6** showed a higher reactivity than the conventional MPA-Leu-NH_2 thioester **7**, although they exhibited slightly lower reactivity than MPAA thioester **8**. One possible explanation for this result is that the acidity of the thiols as leaving groups contributes to their reactivity in NCL. The reaction rates of the model NCLs shown in Figure 1.5 correlated well with the pK_a values of the thiols employed in the reaction (MPAA: pK_a 6.6; Cys residue thiol: pK_a 8.7 (estimated from reduced glutathione); mercaptopropionic acid: pK_a >

10).[6,16] A possible reason for the acceleration of NCL in the preparation of ProTx-I (Figure 1.3) under thiol-additive-free conditions is that the pK_a values of the thiol in ProTx-I are lowered by their surrounding chemical environments. A similar environment-dependent effect is seen for the active-site Cys residue in the protein disulfide isomerase.[17]

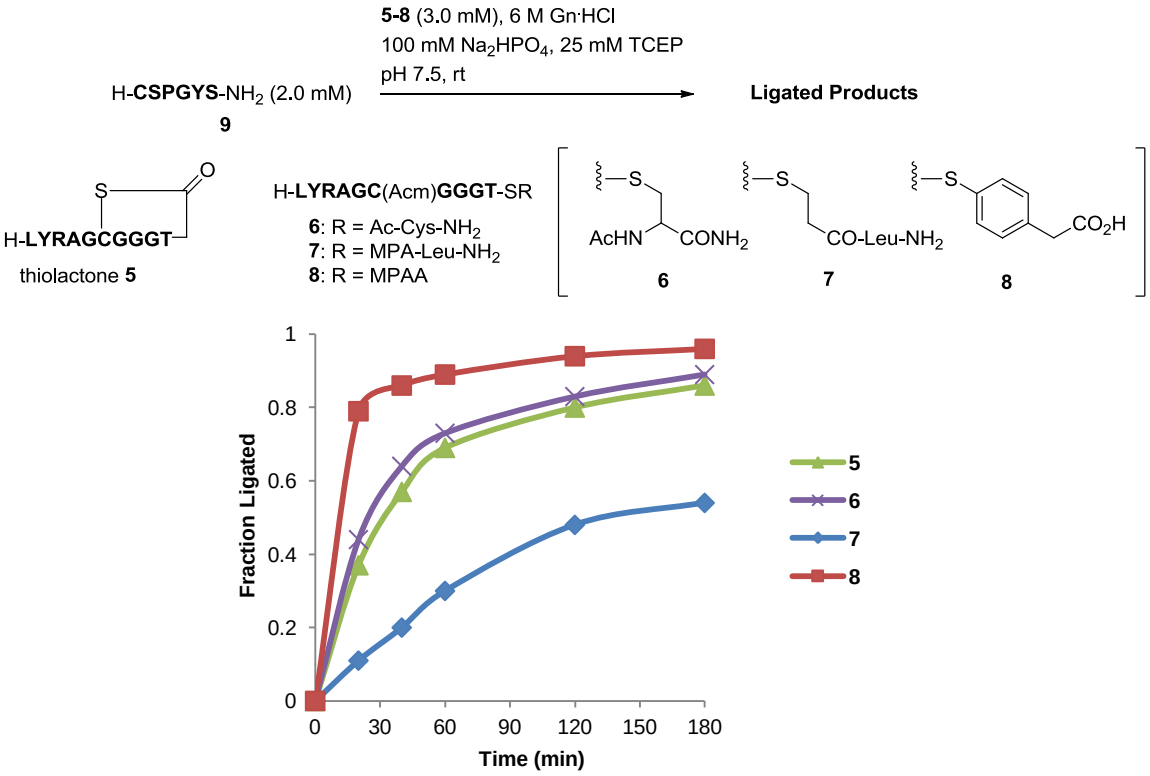


Figure 1.5. Progress of model NCL reactions between N-fragments **5**–**8** and C-fragment **9**. The extent of NCL was quantified by integration of the ligated product as a fraction of the sum of the unreacted C-fragment **9** and the ligated product, as obtained from HPLC (220 nm).

1.5 Synthesis of ProTx-I, [Ile^{14}]-ProTx-I, kurtoxin, and orexin-A using thiol-additive-free NCL

Owing to the absence of thiol additives in this system, there is no need to prevent contamination of the product or unfolded intermediate by thiol additives. Thus, I synthesized oxidative ProTx-I using thiol-additive-free NCL and the subsequent oxidative folding reaction in a one-pot manner. NCL in a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 100 mM sodium ascorbate, pH 7.8) between **3** and **4** reached completion within 20 h, and the subsequent one-pot oxidative folding reaction diluted

with a buffer (1 M NH_4OAc, pH 7.8) in the presence of reduced and oxidized glutathione (GSH/GSSG) for 24 h afforded the product **11** in 56% isolated yield after HPLC purification (Figure 1.6). In addition, the disulfide bond patterns of **11** were consistent with the native disulfide bond patterns detected by chemical method (see Experimental section, Table E1.1 and Table E1.2). This result confirms that it is possible to apply thiol-additive-free NCL and the subsequent oxidative folding without contamination from thiol additives.

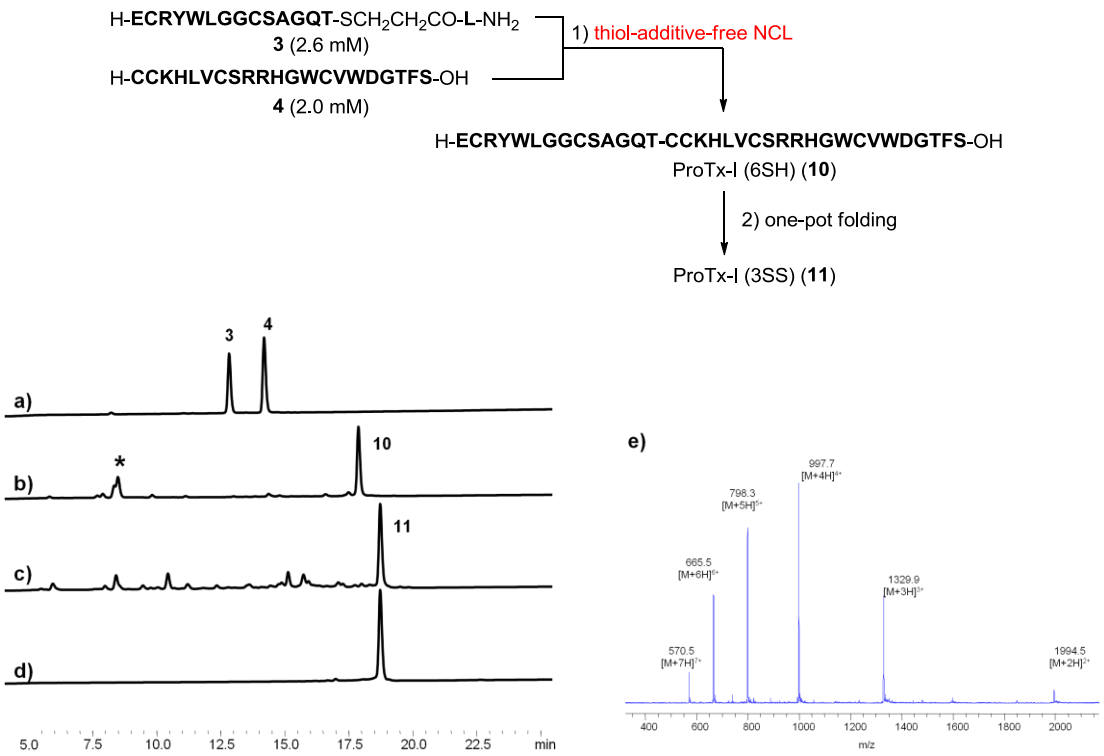


Figure 1.6. HPLC profiles of one-pot synthesis of ProTx-I (3SS) (**11**). a) NCL (t = 0 h), b) NCL (t = 20 h), c) folding reaction (t = 24 h), d) purified product **11**, e) ESI-MS of **11**. *Thiolactone and hydrolysate of N-fragment **3**. HPLC conditions: column, DAISO-PAK SP-120-5-ODS-BIO (4.6 × 150 mm); elution, 20–40% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL/min; detection, 220 nm.

In a similar way, the mutated [Ile^{14}]-ProTx-I **13** having the most bulky Ile^{14}-Cys^{15} site could be achieved in 45% isolated yield (Figure 1.7). Furthermore, I could also synthesize kurtoxin **16** (63 amino acids)[18] at the Leu^{26}-Cys^{27} site in 55% isolated yield (Figure 1.8), and orexin-A **19** (33 amino acids)[19] at the Thr^{11}-Cys^{12} site in 61% isolated yield (Figure 1.9), respectively. These results confirm that thiol-additive-free NCL can be used for the synthesis of Cys-rich peptides.

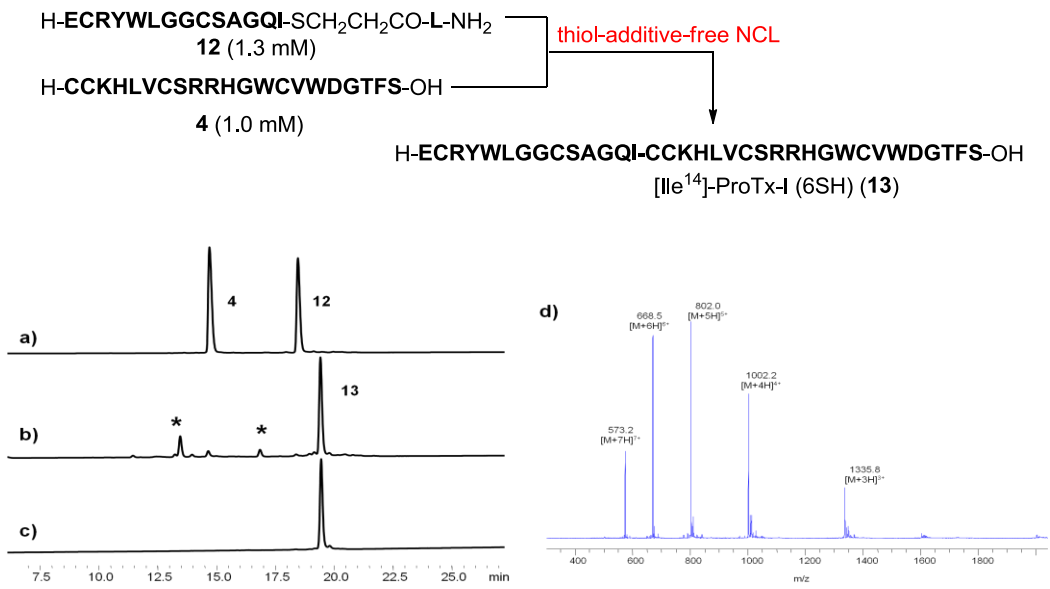


Figure 1.7. HPLC profiles for the synthesis of [Ile^{14}]-ProTx-I (6SH) (**13**). a) NCL (t = 0 h), b) NCL (t = 32 h), c) purified product **13**, d) ESI-MS of **13**. *Thiolactone of the N-fragment **12**. HPLC conditions: column, DAISO-PAK SP-120-5-ODS-BIO (4.6 × 150 mm); elution, 20–40% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL/min; detection, 220 nm.

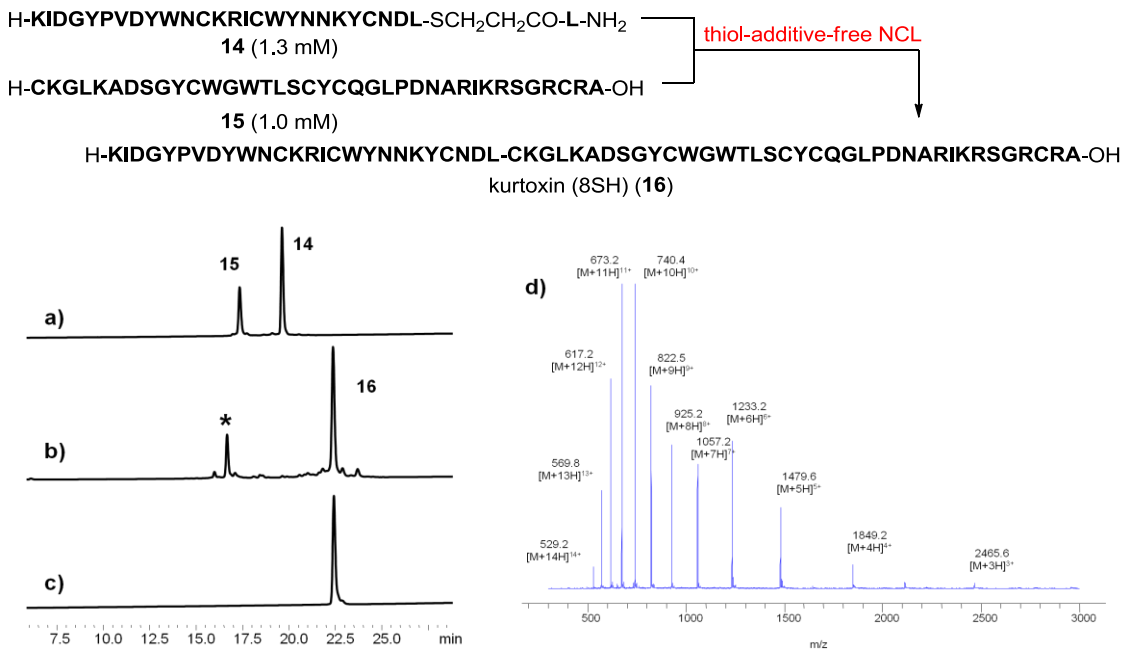


Figure 1.8. HPLC profiles for the synthesis of kurtoxin (8SH) (**16**). a) NCL (t = 0 h), b) NCL (t = 18 h), c) purified product **16**, d) ESI-MS of **16**. *Thiolactone and hydrolysate of N-fragment **14**. HPLC conditions: column, DAISO-PAK SP-120-5-ODS-BIO (4.6 × 150 mm); elution, 20–40% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL/min; detection, 220 nm.

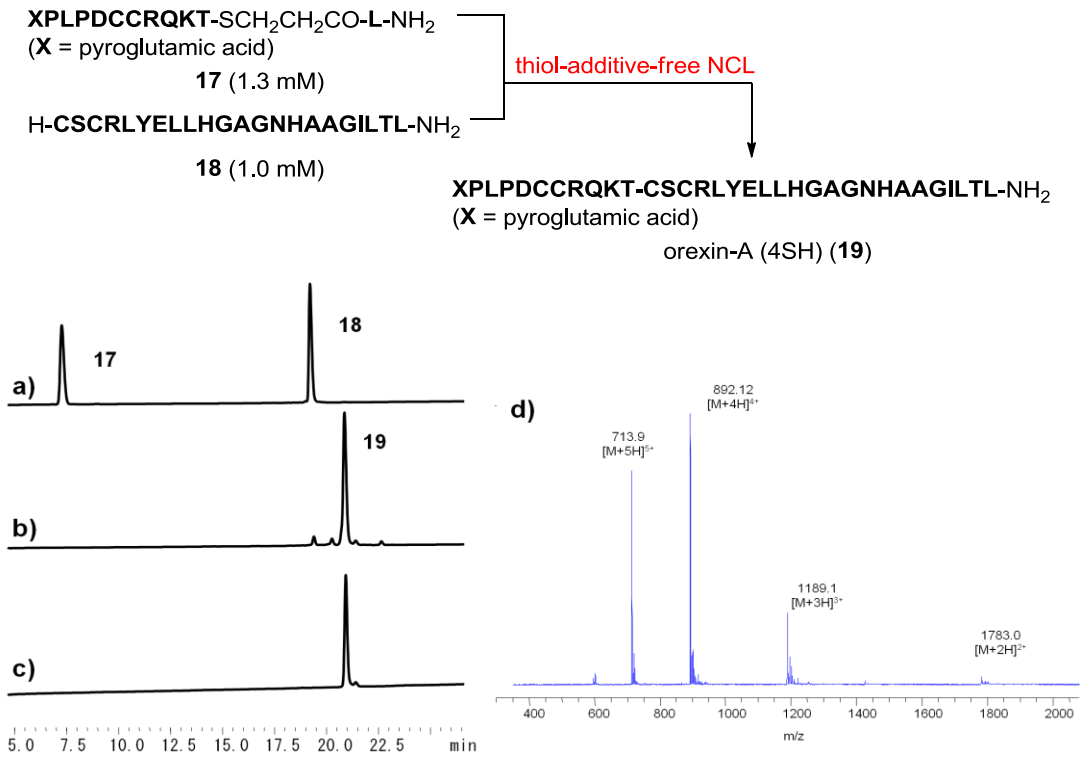


Figure 1.9. HPLC profiles for the synthesis of orexin-A (4SH) (**19**). a) NCL (t = 0 h), b) NCL (t = 24 h), c) purified product **19**, d) ESI-MS of **19**. HPLC conditions: column, DAISO-PAK SP-120-5-ODS-BIO (4.6 × 150 mm); elution, 10–60% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL/min; detection, 220 nm.

1.6 Conclusion

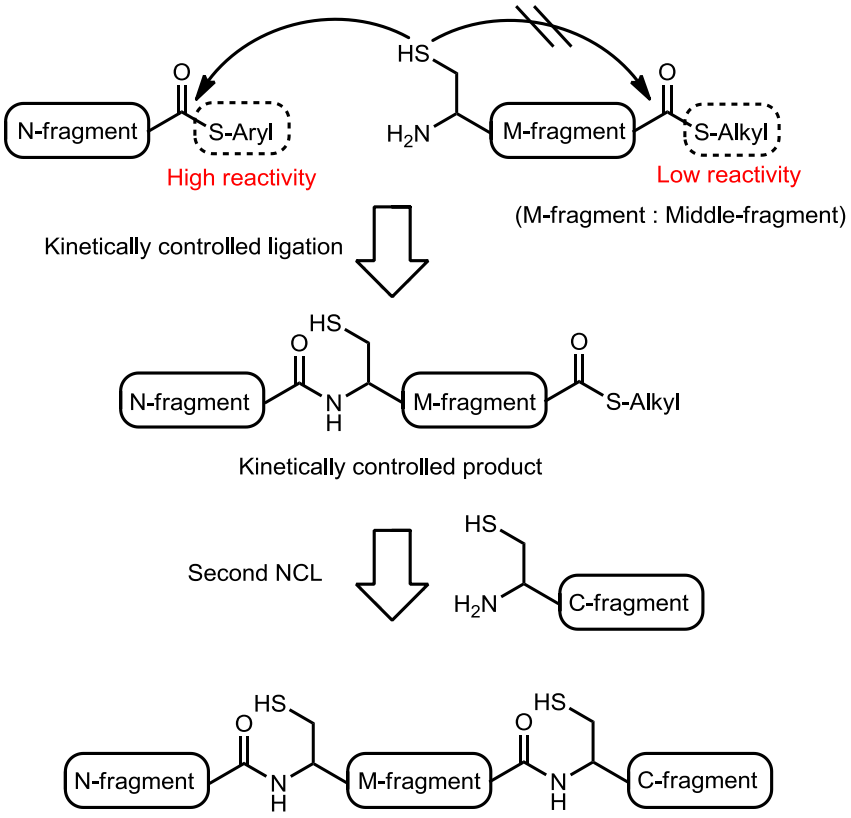
I performed the synthesis of Cys-rich peptides such as ProTx-I, [Ile^{14}]-ProTx-I, kurtoxin, and orexin-A using thiol-additive-free NCL. This procedure, which mitigates problems associated with contamination by thiol additives, facilitated the HPLC purification of the ligated product and the subsequent oxidative folding reaction, leading to an improvement in the synthetic yield of the Cys-rich peptides. The scope of the reaction and its mechanism, which is thought to involve the lower pK_a of Cys residues owing to the influence of their surrounding environments, are currently under investigation. In the next chapter, I discuss the application of highly reactive alkylthioesters in this reaction.

Chapter 2

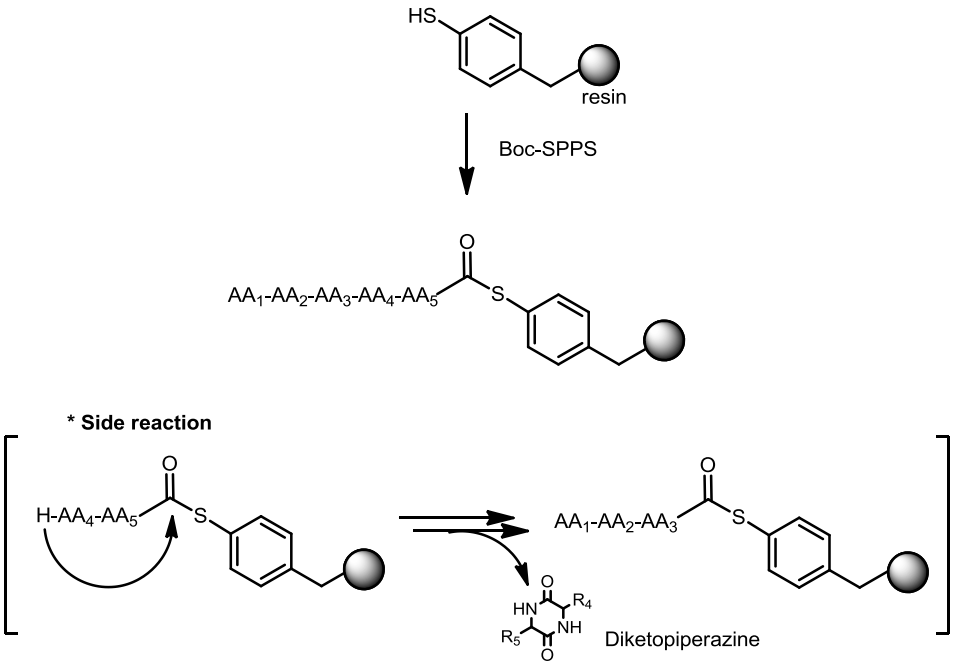
On-resin synthesis of peptide-TfaC for kinetically controlled ligation

2.1 Arylthioesters applicable to kinetically controlled ligation: a methodology for total synthesis of long peptides

Stepwise SPPS can only afford peptide fragments comprising a maximum of 30–40 amino acids. To overcome the size limitation of SPPS, conventional NCL has been developed for chemical synthesis of peptides. However, alternative NCL protocols are required for preparation of long peptides containing over 100 amino acids. These protocols involve that the division of target peptides into multiple peptide fragments having 30–40 amino acids each, followed by sequential ligations. As one potential solution, kinetically controlled ligation (KCL) has been reported by Kent.[20] Under thiol-additive-free conditions, KCL works based on the principle that a highly reactive arylthioester reacts faster with an N-terminal Cys moiety in a kinetically controlled manner than a less reactive alkylthioester. In other words, the N-terminal Cys residue of a middle-fragment-containing alkylthioester does not react intramolecularly with its alkylthioester unit but rather reacts intermolecularly with the arylthioester of an N-fragment to yield the ligated alkylthioester as the kinetically controlled product (Scheme 2.1). The resulting alkylthioester can then be brought to the second NCL step in a one-pot manner. As mentioned above, arylthioesters have shown great utility in KCL with N-terminal cysteinyl alkylthioesters; however, their preparation by Boc-SPPS is problematic owing to the highly electrophilic nature of the arylthioester moiety, with diketopiperazine formation sometimes occurring (Scheme 2.2).[21] Therefore, less reactive alkylthioesters easily obtainable by Boc-SPPS, such as MPA thioesters, have been converted to the corresponding arylthioester in aqueous buffers, which requires laborious multiple synthesis and purification steps.[22] Consequently, I envision that development of a Boc-SPPS-based synthetic protocol for a novel thioester possessing reactivity comparable with that of arylthioesters will facilitate the practical and efficient chemical synthesis of long peptides.



Scheme 2.1. Mechanism of kinetically controlled ligation.



Scheme 2.2. Side reaction occurring during the preparation of arylthioesters.

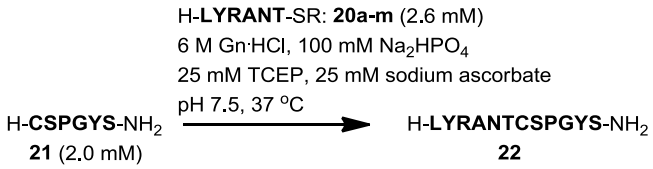
Based on the fact that the peptidyl-*S*-Cys derivatives as peptide alkylthioesters exhibited much higher reactivity than conventional MPA thioesters in NCL (as reported in Chapter 1), I hypothesized that optimization of the Cys scaffold in the peptidyl-*S*-Cys species should result in the development of a highly reactive alkylthioester easily accessible by Boc-SPPS. In this chapter, I examined the reactivity of peptidyl-*S*-Cys derivatives with different Cys-unit structures and their applicability to KCL.

2.2 Investigation of the reactivities of alkylthioesters possessing different Cys units

In addition to MPA-Leu-NH_2 alkylthioester **20a** and MPAA arylthioester **20b** as reference peptide thioesters, alkylthioesters possessing different Cys units (**20c**-**m**) were subjected to model NCL reactions. The results of NCL reactions of peptide thioesters **20a**-**m** (2.6 mM) with N-terminal Cys-peptide **21** (2.0 mM) under thiol-additive-free conditions were summarized in Table 2.1 and Figure 2.1. The thioesters **20b-k** (except for **20c**) were prepared by thioester exchange of **20a** with large excess amounts of different Cys derivatives. The MPA-Cys-NH_2 thioester **20c** was prepared by conventional Boc-SPPS,[4] and peptidyl-*S*-Cys thioesters **20l** and **20m** were synthesized by Boc-SPPS, as discussed later.

As expected, **20a** and **20b** exhibited the lowest and highest reactivities in model NCL, respectively (entries 1 and 2). Unlike the previous literature,[23] the MPA-Cys-NH_2 thioester **20c** did not react faster than the MPAA thioester **20b** under our conditions. Steric hindrance owing to the side chains of the amino acids at the N-terminal side of the Cys residue affected the efficiency of NCL (entries 5-8). Conversely, the efficiency of NCL was hardly affected by the nature of the amino acids at the C-terminal side (entries 4, 5, 9, and 10). As shown in entries 5, 11, and 12, peptidyl-*S*-Cys thioesters **20k** and **20l**, which possessed a strongly electron withdrawing trifluoroacetyl (Tfa) and methanesulfonyl (Ms) group, respectively, exhibited slightly higher reactivity than acetyl counterpart **20e**. I speculated that a decrease in the pK_a value by the electron withdrawing group resulted in improved NCL. Although the toluenesulfonyl (Tos) group is also strongly electron withdrawing, the steric hindrance of the Tos-containing thioester **20m** most likely suppresses the promotion of NCL. Thus, the peptidyl-*S*-Cys alkylthioesters bearing Tfa (peptide-TfaC) and Ms (peptide-MsC) groups were chosen for further investigation, wherein accessibility by Boc-SPPS and applicability to KCL were examined.

Table 2.1. NCL reactions of thioesters **20a-m** with Cys peptide **21** under thiol-additive-free conditions.



SR =

	Entry	Thioesters	Fraction ligated[a] (60 min)	Fraction ligated (180 min)
20a : *MPA*-Leu-NH_2	1	**20a**	0.30	0.54
20b : *MPAA*	2	**20b**	0.93	0.97
20c : *MPA*-Cys-NH_2	3	**20c**	0.52	0.79
20d : Ac-*Cys*-NH_2	4	**20d**	0.71	0.90
20e : Ac-*Cys*-Leu-NH_2	5	**20e**	0.67	0.87
20f : Ac-Gly-*Cys*-Leu-NH_2	6	**20f**	0.68	0.87
20g : Ac-His-*Cys*-Leu-NH_2	7	**20g**	0.47	0.68
20h : Ac-Glu-*Cys*-Leu-NH_2	8	**20h**	0.44	0.65
20i : Ac-*Cys*-His-NH_2	9	**20i**	0.64	0.83
20j : Ac-*Cys*-Glu-NH_2	10	**20j**	0.64	0.85
20k : Tfa-*Cys*-Leu-NH_2	11	**20k**	0.78	0.91
20l : Ms-*Cys*-Leu-NH_2	12	**20l**	0.80	0.92
20m : Tos-*Cys*-Leu-NH_2	13	**20m**	0.68	0.84

[a] The fraction ligated was quantified by integration of the ligated product **22** as a fraction of the sum of the unreacted C-fragment peptide **21** and the ligated product **22**, as obtained from HPLC (220 nm). Structures written in italic fonts (**20a-m**) are involved in thioesters as the thiol counterpart.

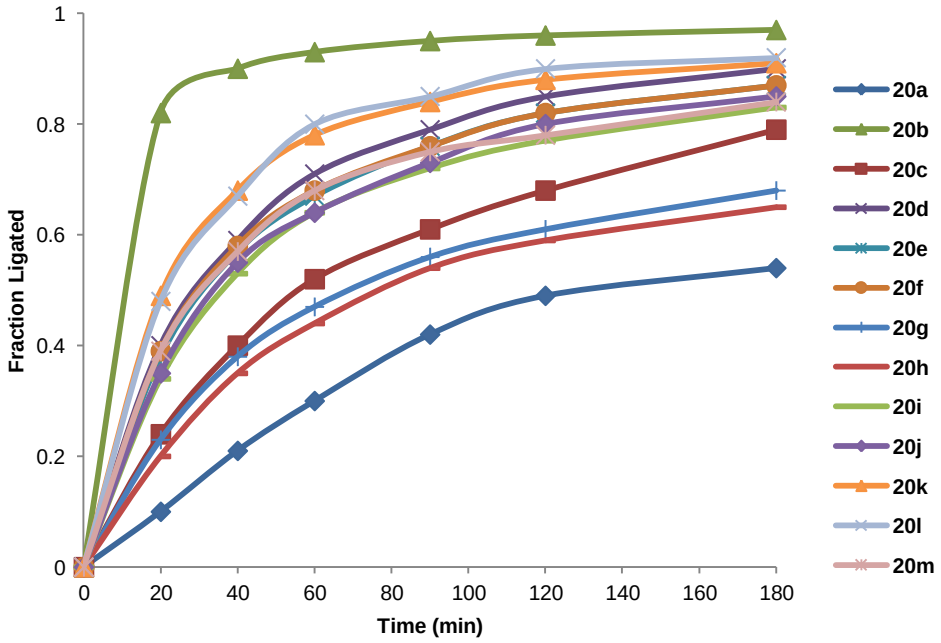
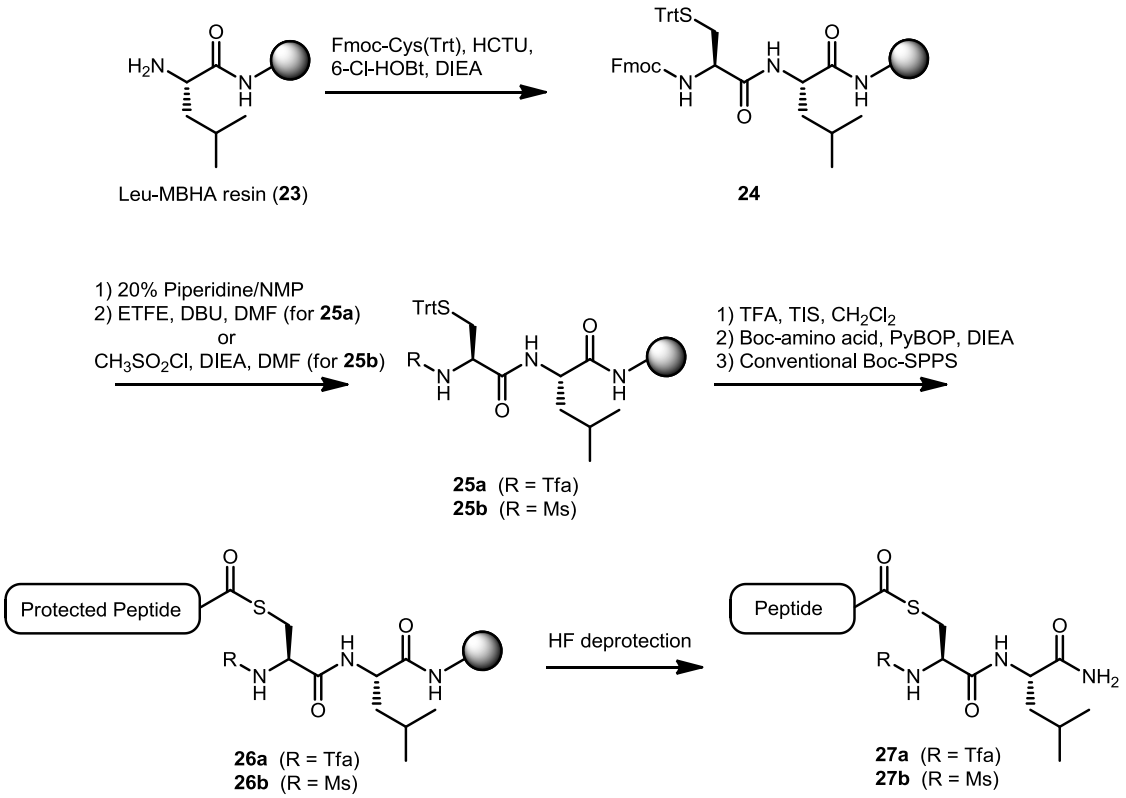


Figure 2.1. Progress of NCL reactions between thioesters **20a-m** and Cys peptide **21** under thiol-additive-free conditions.

2.3 Evaluation of synthetic procedures using peptide-TfaC and -MsC

Having confirmed the potential utility of peptide-TfaC and -MsC for NCL in model experiments, I next attempted the preparation of these peptides by Boc-SPPS according to Scheme 2.3 in order to check for side reactions during the syntheses. Coupling of Fmoc-Cys(Trt)-OH on Leu-MBHA resin **23** (where MBHA resin = 4-methylbenzhydrylamine hydrochloride resin) in the presence of 1-[bis(dimethylamino)methylene]-5-chloro-1*H*-benzotriazolium 3-oxide hexafluorophosphate (HCTU), 1-hydroxy-6-chloro-benzotriazole (6-Cl-HOBt), and *N*,*N*-diisopropylethylamine (DIEA) in *N*,*N*-dimethylformamide (DMF) afforded the Fmoc-Cys(Trt)-incorporated resin **24**. Removal of the Fmoc group with 20% piperidine/DMF followed by acylation or sulfonylation with ethyl trifluoroacetate (ETFE)[24] or methanesulfonyl chloride afforded the corresponding trifluoroacetylated product **25a** or methanesulfonylated product **25b**, respectively. Treatment of the resin with TFA for removal of the Trt group regenerated the thiol group. Condensation of Boc-Ala-OH with the thiol group in the presence of 1-benzotriazolyloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) and DIEA followed by sequential coupling/deprotection with the appropriate Boc-amino acids using conventional Boc-SPPS methodology yielded the completed resin **26**. Treatment of the resulting resin with HF/*p*-cresol for 1 h afforded peptidyl-*S*-Cys thioesters **27** (i.e., Ile^{1}-Leu-Gln-Arg-Gly-Ser^{6}-Gly^{7}-Thr^{8}-Ala^{9}-TfaC-Leu-NH_2 (**27a**), or Ile^{1}-Leu-Gln-Arg-Gly-Ser^{6}-Gly^{7}-Thr^{8}-Ala^{9}-MsC-Leu-NH_2 (**27b**)). In the case of **27a**, the two-residue deleted byproduct (Ile^{1}-Leu-Gln-Arg-Gly-Ser^{6}-Gly^{7}-TfaC-Leu-NH_2, **27a'**) derived from diketopiperazine formation was detected at a level of only < 1%, and the purity of the crude products was estimated to be ca. 88% by HPLC analysis (Figure 2.2). However, 5–10% formation of the two-residue deleted byproduct (Ile^{1}-Leu-Gln-Arg-Gly-Ser^{6}-Gly^{7}-MsC-Leu-NH_2, **27b'**) accompanied the preparation of **27b**. Although the reason for such difference between peptide-TfaC and -MsC is still not disclosed, peptide-TfaC was brought to further evaluation of side reactions.



Scheme 2.3. Synthetic scheme for the preparation of peptide-TfaC and peptide-MsC.

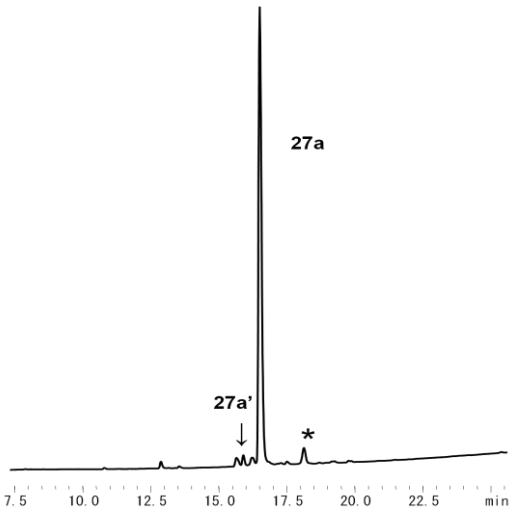


Figure 2.2. HPLC profile of the crude deprotected peptide-TfaC **27a** following treatment with HF. The purity obtained from HPLC was ca. 88%. The peptide **27a'** is the two-residue deleted byproduct derived from diketopiperazine formation (H-**ILQRGSG**-TfaC-**L**-NH_2). **p*-cresol. HPLC conditions: column, DAISO-PAK SP-120-5-ODS-BIO (4.6 × 150 mm); elution, 1–60% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL/min; detection, 220 nm.

Next, I evaluated whether epimerization occurred during synthesis of peptide-TfaC (Leu-Tyr-Arg-Ala-Asn-L-Phe-TfaC-Leu-NH_2, **28a**). The corresponding epimeric peptide (Leu-Tyr-Arg-Ala-Asn-D-Phe-TfaC-Leu-NH_2, **28b**) was also synthesized using Boc-SPPS, and both peptides were subjected to HPLC analysis for evaluation of epimerization during the synthetic process. However, sufficient separation of the epimers on HPLC analysis was not achieved. Therefore, NCL of each unpurified epimer with N-terminal Cys-peptide **21** under standard NCL conditions (6 M Gn·HCl, 100 mM Na_2HPO_4, 1% PhSH, 37 °C) followed by HPLC analysis of the diastereomer products (**28a'**, **28b'**) was performed. The HPLC analysis of both products (**28a'**, **28b'**) clearly indicated that negligible epimerization occurred during the synthesis of peptide-TfaC (Figure 2.3). The facile side-reaction-free synthetic access to peptide-TfaC by Boc-SPPS made it an excellent candidate for a highly reactive thioester to be used in KCL.

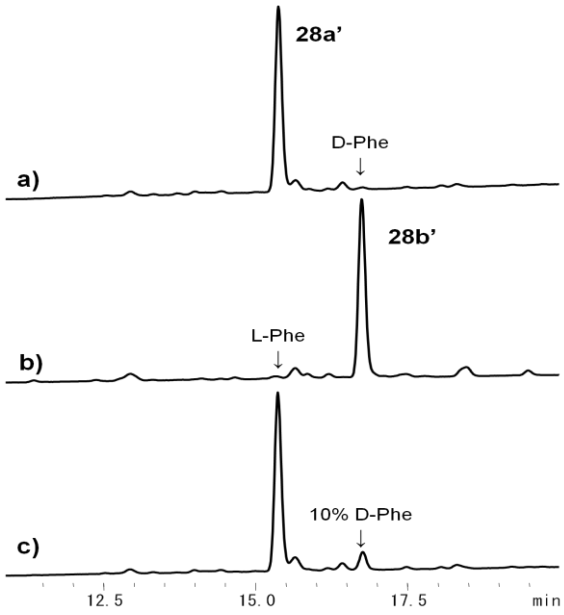
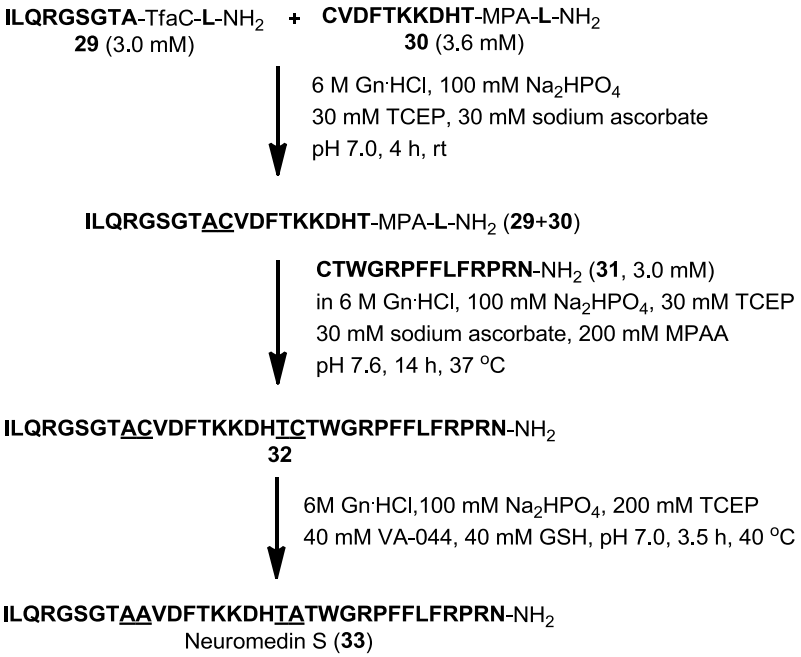


Figure 2.3. HPLC monitoring of epimerization during the synthesis of peptide-TfaC. (a) HPLC profile of NCL reaction of crude peptide **28a** (H-**LYRANF**-TfaC-**L**-NH_2) with Cys peptide **21**. The diastereomeric D-Phe byproduct content was 0.7%. (b) HPLC profile of NCL reaction of crude **28b** (H-**LYRANf**-TfaC-**L**-NH_2) with Cys peptide **21**. The diastereomeric L-Phe byproduct content was 1.1%. (c) co-injection of (a) and (b) (v/v, 9/1). HPLC conditions: column, DAISO-PAK SP-120-5-ODS-BIO (4.6 × 150 mm); elution, 10–30% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL/min; detection, 220 nm.

2.4 Synthesis of neuromedin S by kinetically controlled ligation using peptide-TfaC

The synthetic utility of peptide-TfaC was evaluated through preparation of neuromedin S (33 amino acids)[25] as shown in Scheme 2.4 and Figure 2.4. A one-pot KCL protocol followed by desulfurization[26,27] was employed. The first NCL of peptide-TfaC (Ile-Leu-Gln-Arg-Gly-Ser-Gly-Thr-Ala-TfaC-Leu-NH_2, **29**) with N-terminal cysteinyl MPA-Leu-NH_2 thioester (Cys-Val-Asp-Phe-Thr-Lys-Lys-Asp-His-Thr-MPA-Leu-NH_2, **30**) under thiol-additive-free conditions (6 M Gn·HCl, 100 mM Na_2HPO_4, 30 mM TCEP, 30 mM sodium ascorbate, pH 7.0) was sufficiently completed in 4 h to afford a reaction mixture containing the desired ligated product without a significant amount of side products. Then, addition of Cys peptide **31** in a 200 mM MPAA-containing buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 30 mM TCEP, 30 mM sodium ascorbate, pH 7.6) to the reaction mixture at 37 °C initiated the second NCL to yield the desired fully ligated peptide **32** after 14 h in 38% isolated yield. Finally, desulfurization of HPLC-purified peptide **32** with 40 mM VA-044[27] in a buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 400 mM TCEP, 40 mM GSH, pH 7.0) successfully proceeded to give neuromedin S **33** in 73% HPLC-isolated yield based on **32**.



Scheme 2.4. Synthetic scheme of neuromedin S (**33**) for the one-pot KCL and subsequent desulfurization.

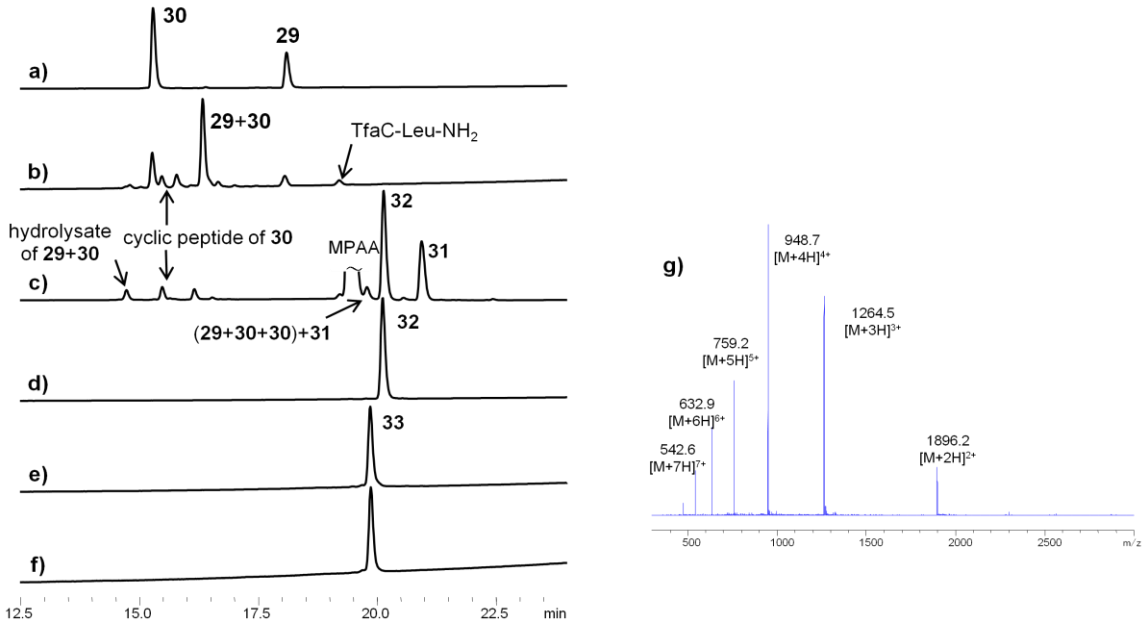


Figure 2.4. HPLC profiles of the synthesis of neuromedin S (**33**) by one-pot KCL and subsequent desulfurization; (a) the first NCL between **29** and **30** (t = 0 h), (b) the first NCL (t = 4 h), (c) the second NCL (t = 14 h), (d) purified product **32**, (e) desulfurization of **32** (t = 3.5 h), (f) purified product **33**, (g) ESI-MS of **33**. HPLC conditions: column, DAISO-PAK SP-120-5-ODS-BIO (4.6 × 150 mm); elution, 1–60% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL/min; detection, 220 nm.

2.5 Conclusion

In Chapter 2, the reactivities of different peptidyl-*S*-Cys derivatives were investigated. In conclusion, I report that "peptide-TfaC" is a novel, highly reactive peptidyl-*S*-Cys alkylthioester and is applicable to KCL protocols. Furthermore, the facile preparation of peptide-TfaC by Boc-SPPS proceeds without the occurrence of side reactions, including diketopiperazine formation and epimerization byproducts, enabling us to adopt peptide-TfaC as a substitute for arylthioesters. I am currently studying further applications of peptide-TfaC, which will assist the practical and efficient chemical synthesis of long peptides.

Chapter 3

Synthesis of *N*-sulfanylethylanilide (SEAlide) peptides via *N*→*S* acyl shift

3.1 Peptide thioester synthesis by Fmoc-SPPS

Synthesis of peptide thioesters using conventional Fmoc-SPPS protocols is a challenging task because of decomposition and/or epimerization of the C-terminal thioester unit by base treatment (Figure 3.1).[28] Therefore, Boc-SPPS has served as a reliable methodology for preparation of peptide thioesters. However, highly acidic HF treatment required for Boc chemistry is not suitable for synthesis of post-translationally modified peptides such as phosphorylated or glycosylated peptides owing to their instability in the presence of HF. These problems have prompted peptide chemists to prepare peptide thioesters using Fmoc-SPPS.[29-31]

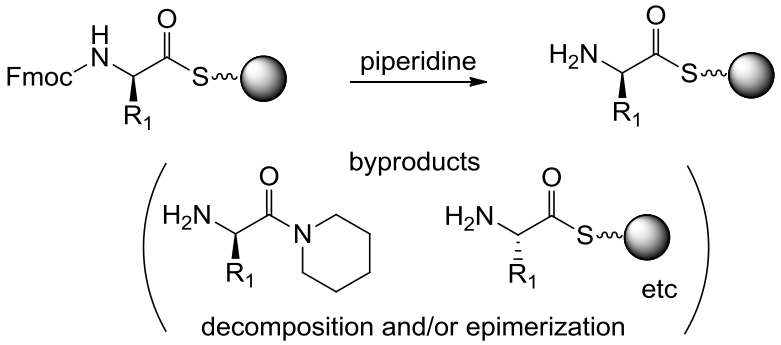


Figure 3.1. Side reactions occurring during the synthesis of peptide thioesters using conventional Fmoc-SPPS.

3.2 Previous reports of peptide thioesters synthesized by Fmoc-SPPS

Many groups have reported Fmoc-based methodologies for the synthesis of peptide thioesters with the aid of species such as Kenner's safety catch linker,[31a] orthothioester,[31b] *N*-acyl urea linker,[31g,31i] or hydrazide linker.[31h] Alternatively, Aimoto et al. developed *N*→*S* acyl shift protocols based on the side reaction they observed in the acidic treatment of a ligation auxiliary.[30a] Independently, our group also reported an *N*→*S* acyl shift protocol using an *N*-acyl oxazolidinone system.[30b] In this system, *N*-acyl oxazolidinone **34**[30b] in which inhibition of delocalization of the amide nitrogen lone pair

to the exo-carbonyl π* orbital by the ring carbonyl weakens the peptide-oxazolidinone linkage **35** and thereby leads to the formation of peptide thioester **36** through nucleophilic involvement of the thiol group neighboring to the activated amide (Figure 3.2). However, a new scaffold alternative to the oxazolidinone system has been required because the peptide-oxazolidinone linkage was partially decomposed, and epimerization of the C-terminal residue was observed during treatment with 20% piperidine/DMF. Therefore, I selected the *N*-sulfanylethylanilide (SEAlide) peptide **37** as a candidate of crypto-thioesters fully comparable to Fmoc-SPPS for the following reasons that (1) *N*-acyl anilide derivatives such as the SEAlide unit are sufficiently resistant to 20% piperidine/DMF, as reported previously,[32] and (2) destabilization of the amide bond adjacent to consecutive sp^2-atoms in **37** promote *N*→*S* acyl shift for preparation of the thioester.

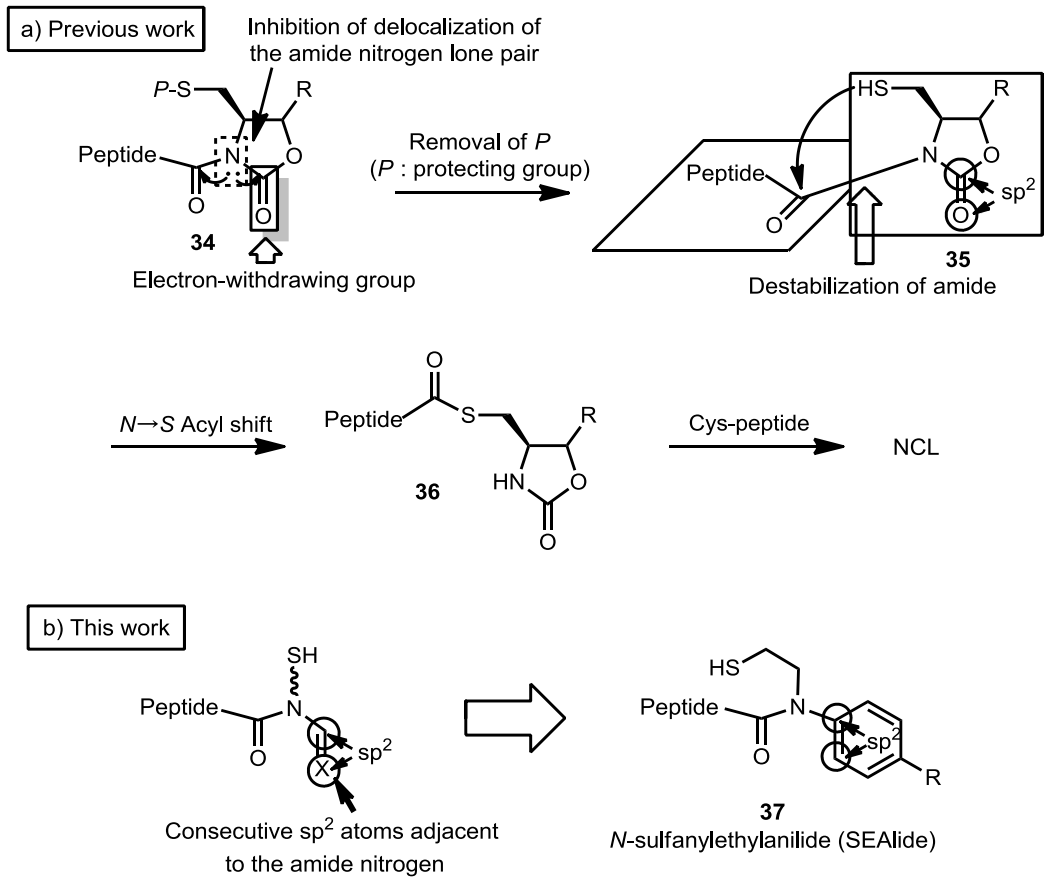
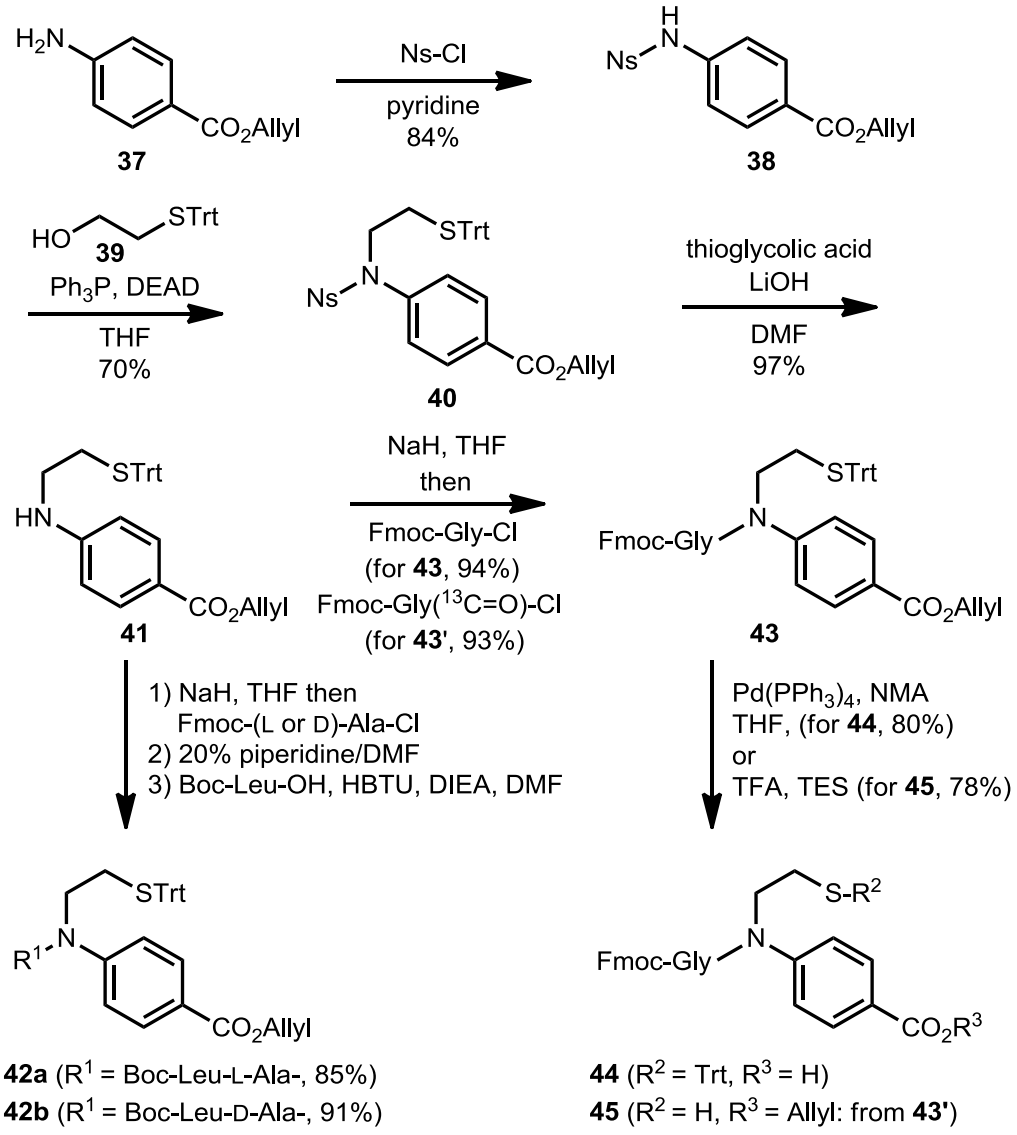


Figure 3.2. General concept for the synthesis of peptide thioesters using *N*→*S* acyl shift protocols. (a) previous synthesis of thioesters using the *N*-acyl oxazolidinone system. (b) new synthesis of thioesters using the SEAlide system.

3.3 Synthesis of SEAlide units

The synthetic strategy for SEAlide derivatives was exhibited in Scheme 3.1. Starting from the known compound **37**,[33] the amino group of **37** was protected with an *o*-nitrobenzenesulfonyl (Ns) group to form Ns-protected allyl ester **38**. The following Mitsunobu reaction using Trt-protected thiol unit **39**[34] gave *N*-sulfanylethyl compound **40**. The Ns group of **40** was removed using thioglycolic acid under the basic condition to form the *N*-ethylaniline intermediate **41**. After acylation of **41** with Fmoc-L- or -D-Ala-Cl in the presence of NaH, removal of the Fmoc group and subsequent coupling



Scheme 3.1. Synthesis of SEAlide derivatives.

with Boc-Leu-OH using 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate (HBTU) afforded the diastereomer **42a** or **42b** for use in base-stability tests. Moreover, reaction of **41** with Fmoc-Gly-Cl or Fmoc-Gly(^{13}C=O)-Cl gave the glycine-incorporated material **43** or **43'** for investigation of *N*→*S* acyl shift. Deprotection of the allyl group in **43** with Pd(PPh_3)$_4$/*N*-methylaniline (NMA) afforded carboxylic acid **44** for attachment of the SEAlide unit to a resin. Removal of the Trt group with TFA/TES yielded the thiol compound **45** for evaluation of *N*→*S* acyl shift by HPLC and NMR analyses.

3.4 Stability of the SEAlide unit during 20% piperidine treatment

Treatment of **42a** with 20% piperidine/DMF at room temperature to evaluate the stability of the anilide linkage and epimerization of the C-terminal Ala clearly indicated that neither significant decomposition nor epimerization was observed by HPLC analysis (Table 3.1 and Figure 3.3). From these results, I concluded that the SEAlide unit is fully compatible with Fmoc chemistry in terms of peptide-chain elongation.

Table 3.1. Examination of stability of **42a** in 20% piperidine/DMF.[a], [b]

	Reaction (h)				
	0	2	6	12	24
42a/pyrene	1.00	1.01	0.98	0.95	0.98

[a] Mixture of **42a** and pyrene (internal standard) was treated with 20% piperidine/DMF at room temperature. Reaction aliquots were analyzed using HPLC after 2, 6, 12, and 24 h. The ratio of **42a** to pyrene was calculated. [b] Ratio of **42a** to pyrene at 0 h is defined as 1.00.

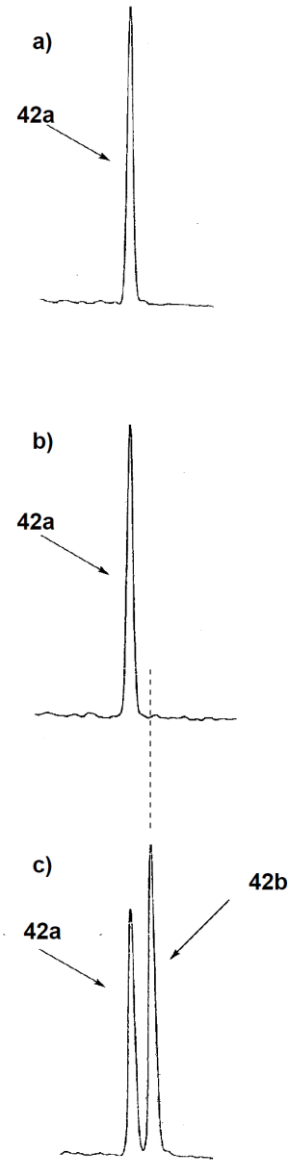


Figure 3.3. HPLC examination of the C-terminal L-Ala epimerization of **42a** during 20% piperidine/DMF treatment. (a) t = 0 h, (b) t = 24 h, (c) co-injection of **42a** and **42b**. HPLC conditions: column, Cosmosil $5C_{18}$ARII column (4.6 × 250 mm; tandem); elution, 65–80% CH_3CN in 0.1% TFA (120 min) at room temperature; flow rate, 1.0 mL/min; detection, 215 nm.

3.5 N→S Acyl shift in the SEAlide unit as analyzed by HPLC and ^{13}C NMR

Inspired by *N→S* acyl shift in acidic solution presented by Aimoto et al., I examined the conversion of the SEAlide compound **45** into the corresponding thioester

45' under acidic conditions.[30a] Treatment of **45** with 4 M HCl/dioxane in the presence of 1% (w/v) TCEP for 2 h afforded the corresponding thioester **45'**, as revealed by HPLC analysis (Figure 3.4, left). Moreover, ^{13}C NMR study also supported the conversion by *N*→*S* acyl shift of **45** into the corresponding thioester **45'**, because the signal at 167.8 ppm (amide carbonyl) shifted to 197.3 and 199.6 ppm (thioester carbonyl) (Figure 3.4, right). I surmised that these two signals were derived from the rotamers of the thioesters, as mentioned previously,[30d] although the details are currently under study.

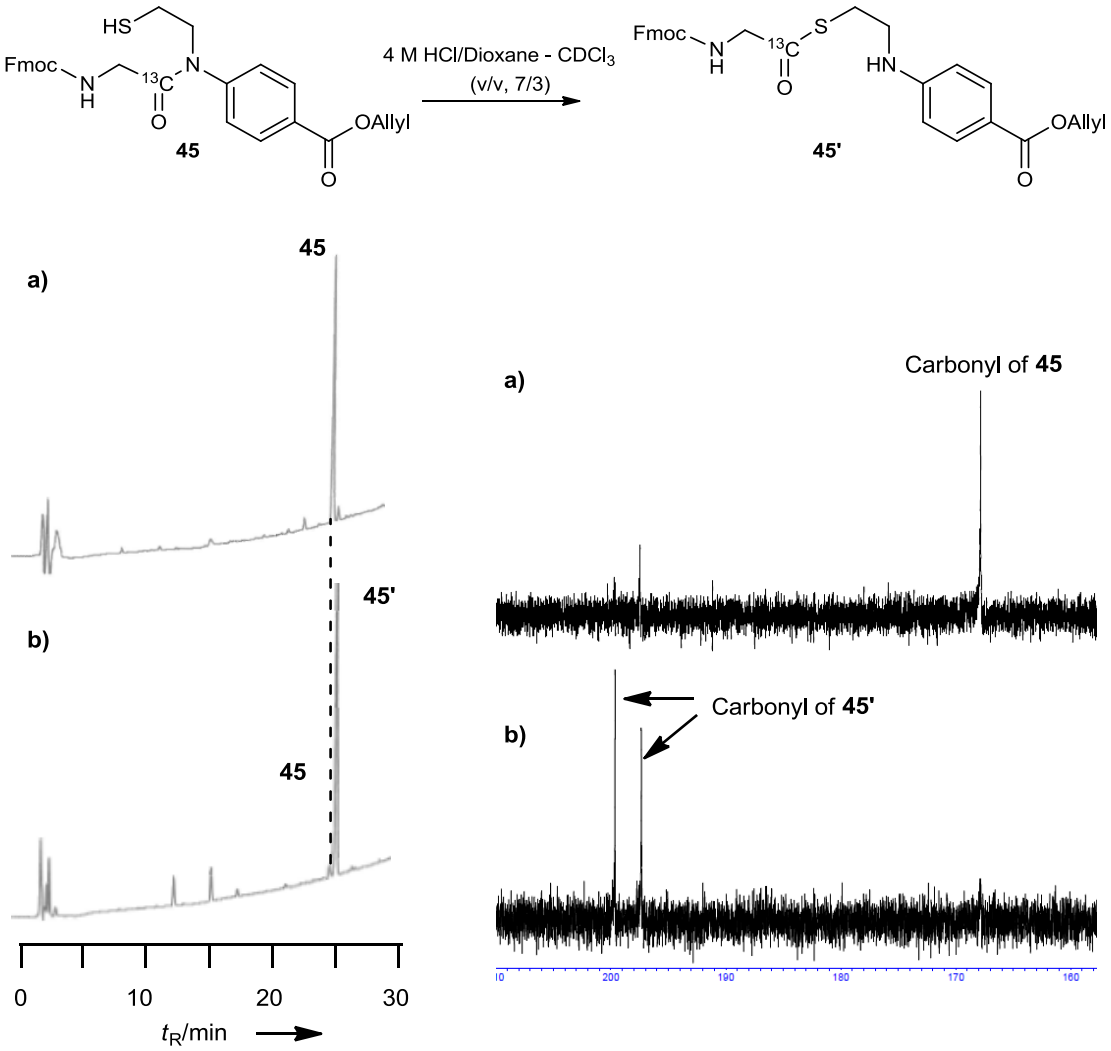
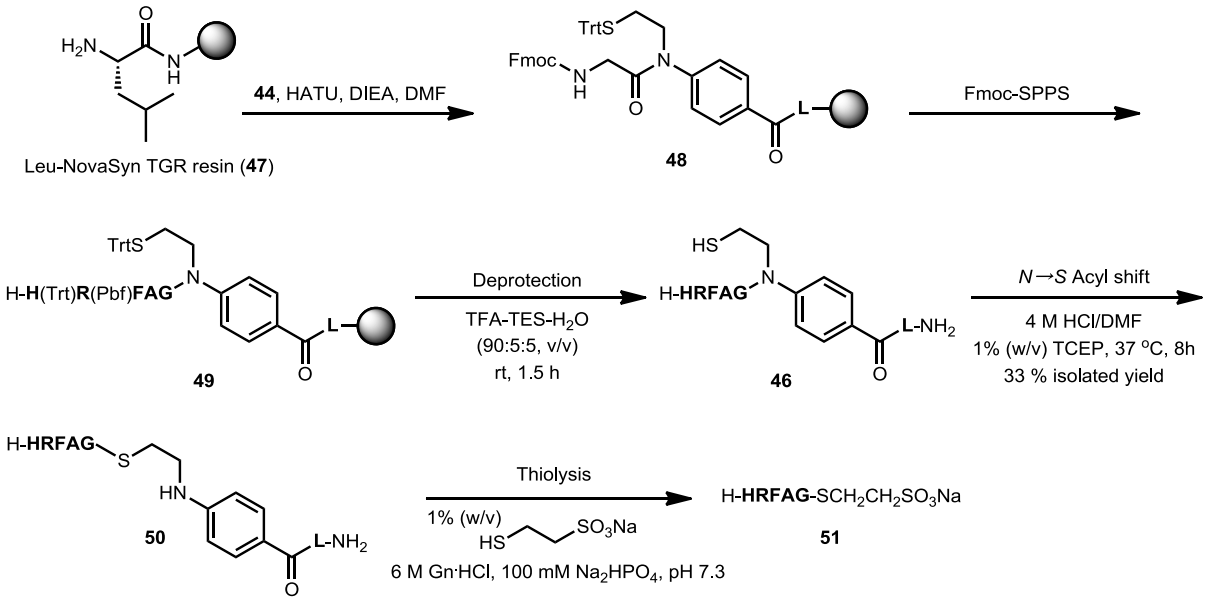


Figure 3.4. HPLC monitoring (left) and ^{13}C NMR analyses (right) of *N*→*S* acyl shift by treatment with HCl/dioxane-$CDCl_3$ (v/v, 7:3) (a) *N*→*S* acyl shift (t < 3 min), (b) *N*→*S* acyl shift (t = 60 min). HPLC conditions: column, Cosmosil $5C_{18}$ARII column (4.6 × 250 mm); elution, 30–90% CH_3CN in 0.1% TFA (30 min) at room temperature; flow rate, 1.0 mL/min; detection, 220 nm.

3.6 Preparation of different peptide thioesters using the SEAlide unit

I next checked whether a model peptide **46** (H-His-Arg-Phe-Ala-Gly-SEAlide-Leu-NH_2) could be converted to the corresponding thioester by *N*→*S* acyl shift. The synthetic scheme for the SEAlide peptide was shown in Scheme 3.2. The Leu-incorporated resin **47** was coupled with the SEAlide unit **44** with the aid of 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU) and DIEA in DMF to afford the resin **48**, which was subjected to the peptide-chain elongation step by Fmoc-SPPS to give the protected resin **49**. Deprotection of the completed resin **49** with TFA and concomitant cleavage of peptides from resin gave crude anilide-type SEAlide peptides **46**. Subsequent peptide-chain elongation was performed with conventional Fmoc-SPPS. The side-chain protecting groups of the resin **49** were removed with TFA to form the crude SEAlide peptide **46**. Treatment of **46** with 4 M HCl/DMF produced the corresponding peptide thioester **50** in 33% isolated yield after HPLC purification. I chose 4 M HCl/DMF instead of 4 M HCl/dioxane because peptide **46** was not soluble in dioxane, but soluble in DMF. The following thiol-thiol exchange with a large amount of sodium 2-mercaptoethanesulfonate in a phosphate buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, pH 7.3) afforded peptide alkylthioester **51** (Figure 3.5). These results clearly indicated that the SEAlide peptide served as novel thioester precursor compatible with Fmoc chemistry.



Scheme 3.2. Synthesis of peptide alkylthioester **51** using SEAlide unit.

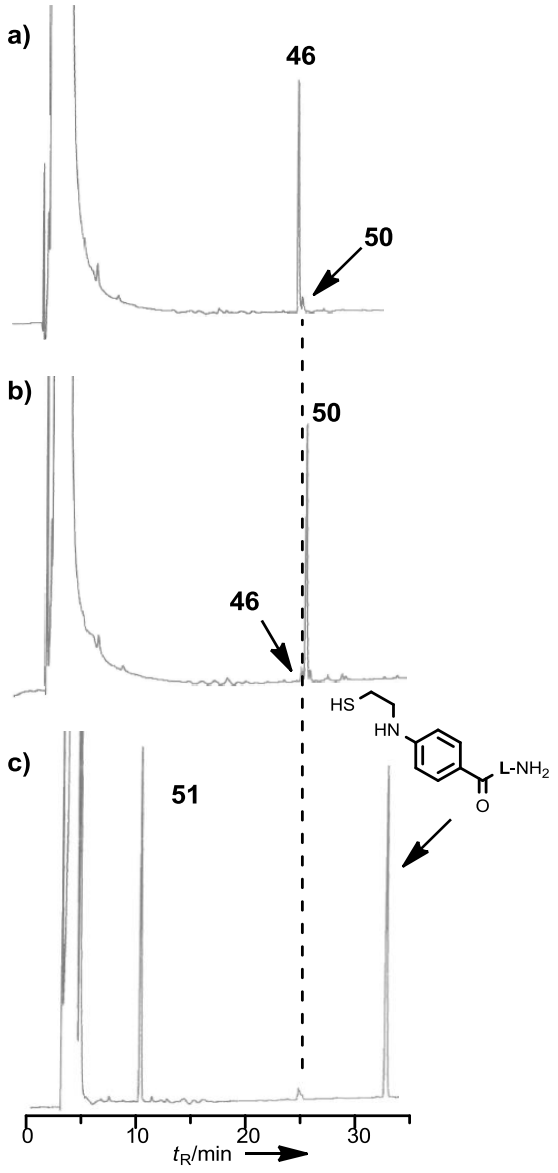
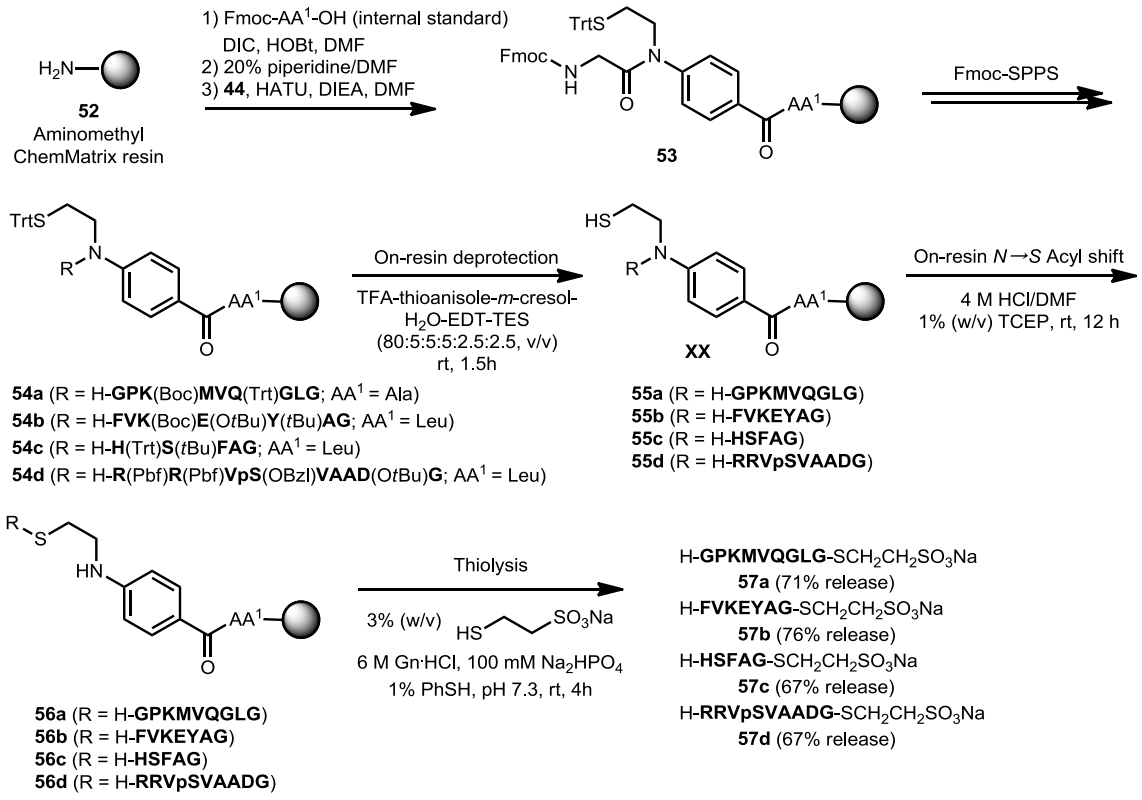


Figure 3.5. HPLC monitoring of *N*→*S* acyl shift in the SEAlide peptide **49** by treatment with 4 M HCl/DMF. (a) *N*→*S* acyl shift of **46** (t < 5 min), (b) *N*→*S* acyl shift (t = 8 h), (c) thiol-thiol exchange reaction with sodium 2-mercaptoethanesulfonate (t = 1 h). HPLC conditions: column, Cosmosil $5C_{18}$ARII column (4.6 × 250 mm); elution, 5–30% CH_3CN in 0.1% TFA (30 min) at room temperature; flow rate, 1.0 mL/min; detection, 220 nm.

To further expand the utility of SEAlide peptides, I investigated an on-resin *N*→*S* acyl shift followed by the direct thiolytic release of peptide thioesters with an aqueous buffer. As shown in Scheme 3.3, I synthesized four model peptides **57a-d** including a phosphopeptide **57d**.[36] The internal standard amino acid (Ala or Leu) for

amino acid analysis (AAA) and SEAlide unit **44** were successively coupled on aminomethyl ChemMatrix resin **52**[37] to produce resin **53**. Fmoc-SPPS was then performed to form the fully protected peptide resins **54a-d**. Side-chain protecting groups on each resin were removed using TFA to afford the side-chain-deprotected resins **55a-d**. After conversion of **55a-d** with 4 M HCl/DMF into the corresponding peptide thioester resins **56a-d**, the thiolytic release in the presence of 3% (w/v) sodium 2-mercaptoethanesulfonate and 1% (v/v) PhSH in a phosphate buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, pH 7.3) afforded the standard peptide alkylthioesters **57a-d** with high purity in approximately 70% releasing yields, as calculated by AAA analysis (**57a**, 71%; **57b**, 76%; **57c**, 67%; **57d**, 67%) (Figure 3.6).



Scheme 3.3. Synthesis of peptide thioesters **57a-d** using an on-resin *N*→*S* acyl shift followed by direct thiolytic release with an aqueous buffer.

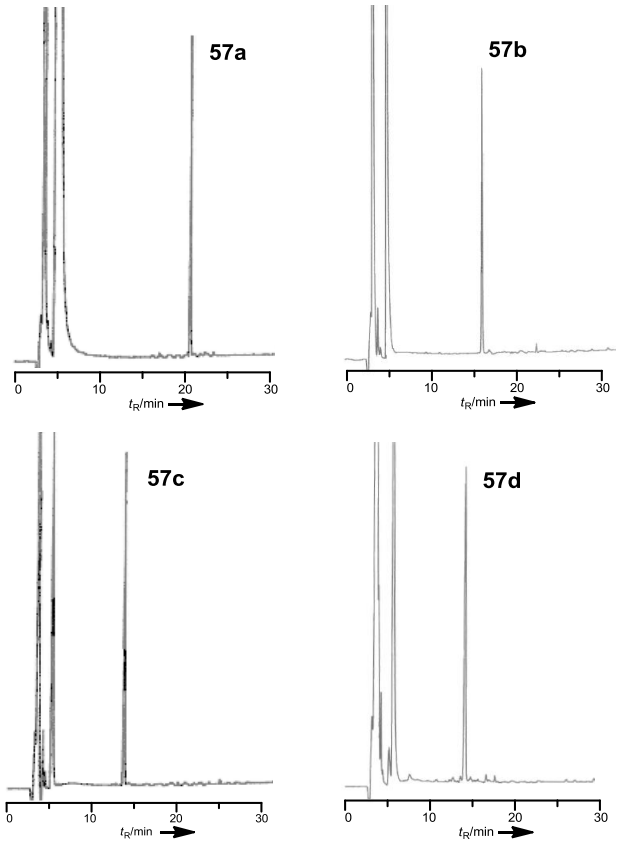


Figure 3.6. HPLC profiles of crude peptide thioesters **57a-d** using an on-resin *N*→*S* acyl shift followed by direct thiolytic release with an aqueous buffer. (a) H-**GPKMVQGLG**-$SCH_2CH_2SO_3Na$ (**57a**) (b) H-**FVKEYAG**-$SCH_2CH_2SO_3Na$ (**57b**) (c) H-**HSFAG**-$SCH_2CH_2SO_3Na$ (**57c**) (d) H-**RRVpSVAADG**-$SCH_2CH_2SO_3Na$ (**57d**) HPLC conditions: column, Cosmosil $5C_{18}$ARII column (4.6 × 250 mm); elution, 2–25% (for **57a-c**) or 2–30% (for **57d**) CH_3CN in 0.1% TFA (30 min) at room temperature; flow rate, 1.0 mL/min; detection, 220 nm.

3.7 Evaluation of epimerization during N→S acyl shift in the SEAlide unit

Occurrence of epimerization of the C-terminus during *N*→*S* acyl shift under acidic conditions was checked through the use of model epimers **58** (i.e. H-Phe-Ala-L-Ala-*N*-Ar (**58a**) and H-Phe-Ala-D-Ala-*N*-Ar (**58b**)) (Figure 3.7). Partial epimerization in the corresponding thioesters **59** was detected by treatment of **58a** or **58b** with 4 M HCl/DMF. Conversely, no epimerization of **59** was detected by treatment of **58a** or **58b** with TFA, although the *N*→*S* acyl shift with TFA was slower than with 4 M HCl/DMF.

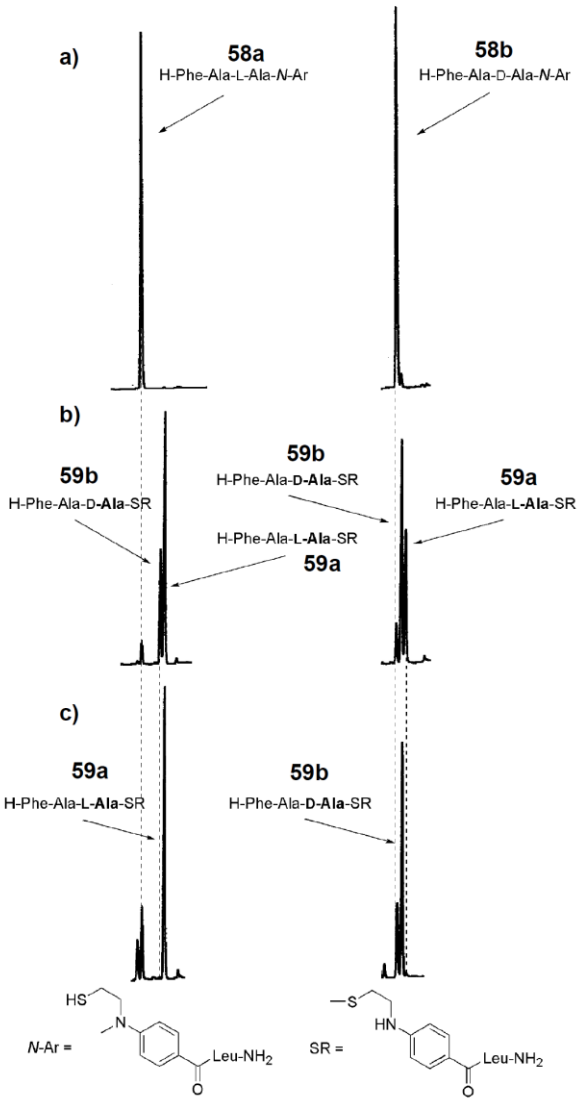


Figure 3.7. HPLC monitoring of the epimerization occurring during *N*→*S* acyl shift in model epimers **58a** or **58b** by treatment with 4 M HCl/DMF (b) or TFA (c). (a) **58a** or **58b** in DMF, (b) 4 M HCl/DMF treatment (t = 8h) (c) TFA treatment (t = 6 h). HPLC conditions: column, Cosmosil $5C_{18}$ARII column (4.6 × 250 mm); elution, 2–40% CH_3CN in 0.1% TFA (30 min) at room temperature; flow rate, 1.0 mL/min; detection, 220 nm.

One possible explanation for this result is that the difference in the acidity of HCl and TFA influences the protonation of the five-membered intermediate **60** shown in Figure 3.8. While TFA ($pK_a = 0$) can only protonate the N atom of intermediate **60** to afford the desired product **61**, HCl ($pK_a = -7$) can protonate both the N atom and the OH group of intermediate **60** to yield the product **61** via formation of undesirable

intermediate **62**, which is susceptible to epimerization.[38] Fortunately, further investigation indicated that SEAlide peptides are applicable to NCL in neutral phosphate buffers without epimerization.[39]

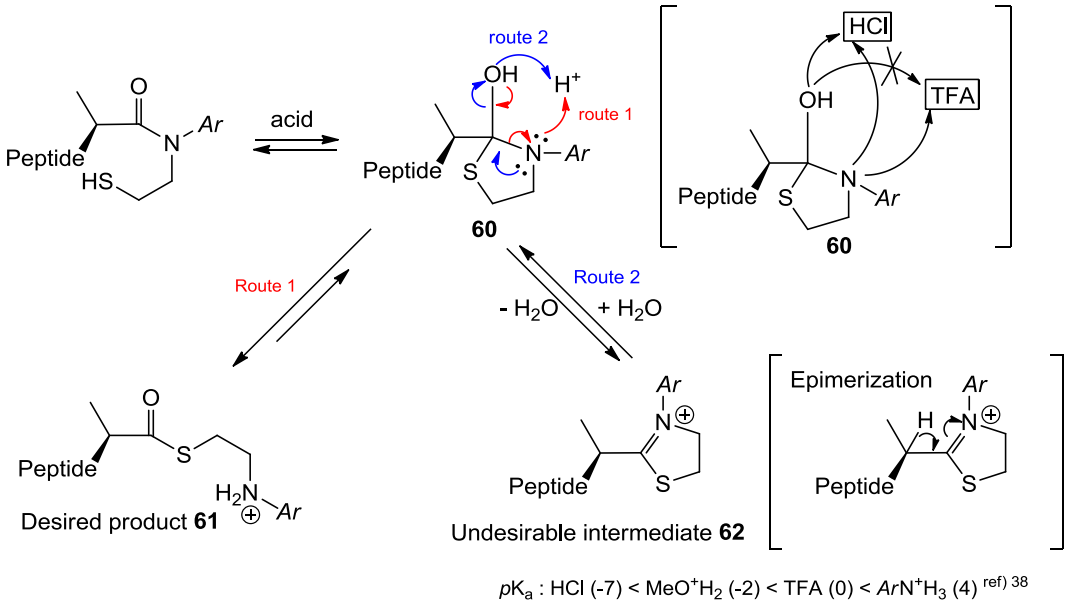


Figure 3.8. Putative mechanism for epimerization during *N*⟶*S* acyl shift by HCl/DMF or TFA treatment.

3.8 Conclusion

I have demonstrated the applicability of a SEAlide peptide as a novel surrogate for peptide thioester. This crypto-thioester is sufficiently compatible with conventional Fmoc-SPPS without decomposition and epimerization. I synthesized various model peptides, including a phosphopeptide using this methodology. SEAlide was first reported in 2009 in Organic Letters as our original thioester. Thanks to the subsequent efforts of our co-workers,[40] I believe that this Fmoc-based unit could be one of the most practical units for chemical synthesis of proteins, such as post-translationally modified proteins, in the world.

Chapter 4

Conclusions

1. The synthesis of Cys-rich peptides using thiol-additive-free NCL was reported. This procedure, which avoids the problems associated with contamination of thiol additives, facilitated HPLC purification and the subsequent oxidative folding reaction, leading to improvement of the synthetic yields of Cys-rich peptides. The scope of the reaction and its mechanism are currently under investigation. I believe that this methodology will help research into the roles of bioactive peptides having disulfide bonds.

2. The reactivities of different peptidyl-*S*-Cys derivatives were investigated. I observed that "peptide-TfaC" is a novel, highly reactive peptidyl-*S*-Cys alkylthioester that is applicable for KCL protocols. Furthermore, the facile preparation of peptide-TfaC by Boc-SPPS proceeds without the occurrence of side reactions, including diketopiperazine formation and epimerization byproducts, enabling us to adopt peptide-TfaC as a substitute for arylthioesters. I am currently assessing further applications of peptide-TfaC, which will assist the practical and efficient chemical synthesis of long peptides.

3. I have demonstrated the applicability of a SEAlide peptide as a novel surrogate for peptide thioester. This crypto-thioester is sufficiently compatible with conventional Fmoc-SPPS without decomposition and epimerization. I synthesized various model peptides, including a phosphopeptide using this methodology. Thanks to the subsequent efforts of our co-workers, I believe that this SEAlide unit could be one of the most practical units for chemical synthesis of proteins, such as post-translationally modified proteins, in the world.

As mentioned above, it will be possible to develop novel synthetic procedures with the aid of internal thiol groups for the chemical synthesis of proteins. I hope to elucidate biological phenomena by the means of synthetic proteins prepared through the use of our new procedures.

Experimental sections

General Methods

All reagents and solvents were obtained from the Peptide Institute, Inc. (Osaka, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Nacalai Tesque, Inc. (Kyoto, Japan), Watanabe Chemical Industries, Ltd. (Hiroshima, Japan), and Sigma-Aldrich Co. LLC. (St. Louis, MO). All reactions except for peptide synthesis were carried out under a positive pressure of argon. For column chromatography, silica gel (KANTO KAGAKU N-60) was employed.

For Chapter 1 and 2, preparative HPLC was carried out with a DAISO-PAK SP-120-5-ODS-BIO (30 × 250 mm) and the following solvent systems: 0.1% TFA in H_2O (A) and 0.1% TFA in CH_3CN (B) at a flow rate of 20 mL min^{-1} (room temperature) with detection at 220 nm. Analytical HPLC was performed with a DAISO-PAK SP-120-5-ODS-BIO (4.6 × 150 mm) and the following solvent systems: 0.1% TFA in H_2O (A) and 0.1% TFA in CH_3CN (B) at a flow rate of 1 mL min^{-1} (40 °C) with detection at 220 nm. For Chapter 3, preparative or semi-preparative HPLC was carried out with a Cosmosil $5C_{18}$-ARII preparative column (20 × 250 mm) or a Cosmosil $5C_{18}$-ARII semi-preparative column (10 × 250 mm), respectively and the following solvent systems: 0.1% TFA in H_2O (A) and 0.1% TFA in CH_3CN (B) at flow rates of 10 mL min^{-1} or 3 mL min^{-1} (room temperature) with detection at 220 nm. Analytical HPLC was performed with a Cosmosil $5C_{18}$-ARII analytical column (4.6 × 250 mm) and the following solvent systems: 0.1% TFA in H_2O (A) and 0.1% TFA in CH_3CN (B) at a flow rate of 1 mL min^{-1} (room temperature) with detection at 220 nm. For Chapter 1 and 2, exact mass spectra of all peptide fragments were observed with an Agilent G1956B LC/MSD detector using an Agilent 1100 series HPLC system; observed masses were derived from the experimental m/z values for all observed protonation states of a molecular species, using the program ChemStation®. For Chapter 3, exact mass spectra were recorded on Waters MICROMASS® LCT PREMIERTM or Bruker Esquire200T. NMR spectra were recorded using a JEOL GSX400 spectrometer at 400 MHz frequency for ^1H and 100 MHz frequency for ^{13}C in $CDCl_3$. Chemical shifts are calibrated to the solvent signal.

Automated peptide synthesis by Boc-SPPS was performed on an ABI 433A (Forester, CA, USA) peptide synthesizer. The peptide chain was elongated using *in situ* neutralization protocols of coupling with Boc-amino acid/HCTU/6-Cl-HOBt/DIEA (4/4/4/6 equiv) in NMP (single coupling, 30 min). The acetyl capping was performed

using acetic anhydride/NMP in the presence of DIEA after each coupling step. The following side-chain-protected amino acids were employed: Arg(Tos), Asn(Xan), Asp(O*c*Hex), Cys(MeBzl), Glu(O*c*Hex), His(Bom), Lys(ClZ), Ser(Bzl), Thr(Bzl), Trp(For), Trp(Hoc), Tyr(BrZ). Manual peptide synthesis by Fmoc-SPPS was performed on a KMS-3 (Kokusan Chemical Co., Ltd, Tokyo, Japan) peptide synthesizer. The peptide chain was elongated using protocols of coupling with Fmoc-amino acid/DIC/HOBt·H_2O (3/3/3 equiv) in DMF (single coupling, 120 min). The following side-chain-protected amino acids were employed: Arg(Pbf), Asp(O*t*Bu), Cys(Trt), Glu(O*t*Bu), Gln(Trt), His(Trt), Lys(Boc), Ser(*t*Bu), Tyr(*t*Bu).

Ligation time-courses were plotted for the reaction between N-fragment thioesters and C-fragment peptides. Aliquots of 3 μL were taken from the reaction mixture at different time intervals and were quenched with 45 μL of 3% TFA in H_2O and neutral TCEP solution 5 μL (0.5 M, Sigma-Aldrich). The mixtures were analyzed by analytical HPLC at 220 nm. The extent of NCL was quantified by integration of the ligated products as a fraction of the sum of the unreacted C-fragment peptides and the ligated products by HPLC.

Chapter 1

Preparation of [Cys(Acm)$^{2, 9}$]-ProTx-I (1-14)-MPA-Leu-NH_2 (1)

The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Thr(Bzl)-SCH_2CH_2CO-Leu-MBHA resin (0.53 g, 0.25 mmol) according to the general automated Boc-SPPS. The peptide resin was treated by HF/*p*-cresol (v/v, 90/10) within the range of -5 ℃ to -2 ℃ for 1 h to give a crude product, which was purified by preparative HPLC to afford **1** (0.12 g, 25%). Analytical HPLC: Rt, 11.7 min (20–40% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1873.1, found 1872.0.

Preparation of [Cys(Acm) $^{16, 21, 28}$]-ProTx-I (15-35) (**2**)

The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Ser(Bzl)-PAM resin (0.41 g, 0.25 mmol) according to the general automated Boc-SPPS. The peptide resin was treated by HF/*p*-cresol (v/v, 90/10) in the presence of $MeONH_2$·HCl (10 equiv.) within the range of -5 ℃ to -2 ℃ for 1 h to give a crude product, which was purified by preparative HPLC to afford **2** (0.14 g, 21%). Analytical HPLC: Rt, 13.5 min (20–40% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 2694.1, found 2693.6.

Preparation of ProTx-I (1-14)-MPA-Leu-NH_2 (3)

The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Thr(Bzl)-SCH_2CH_2CO-Leu-MBHA resin (0.53 g, 0.25 mmol) according to the general automated Boc-SPPS. The peptide resin was treated by HF/*p*-cresol (v/v, 90/10) within the range of -5 ℃ to -2 ℃ for 1 h to give a crude product, which was purified by preparative HPLC to afford **3** (0.11 g, 26%). Analytical HPLC: Rt, 12.8 min (20–40% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1731.0, found 1730.4.

Preparation of ProTx-I (15-35) (4)

The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Ser(Bzl)-PAM resin (0.41 g, 0.25 mmol) according to the general automated Boc-SPPS. The peptide resin was treated by HF/*p*-cresol/1,4-butanedithiol (v/v, 80/5/15) in the presence of $MeONH_2$·HCl (10 equiv.) within the range of -5 ℃ to -2 ℃ for 1 h to give a crude product, which was purified by preparative HPLC to afford **4** (88 mg,14%). Analytical HPLC: Rt, 14.2 min (20–40% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 2480.9, found 2480.4.

Preparation of a model thiolactone (5)

The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Thr(Bzl)-SCH_2CH_2CO-Leu-MBHA resin (0.32 g, 0.15 mmol) according to the general automated Boc-SPPS. The peptide resin was treated by HF/*p*-cresol (v/v, 85/15) within the range of -5 ℃ to -2 ℃ for 1 h to give a crude product, which was purified by preparative HPLC to afford H-**LYRAGCGGGT**-SCH_2CH_2CO-**L**-NH_2. The obtained product (20 mg, 17 μmol) was dissolved in 33 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 30 mM TCEP, 30 mM sodium ascorbate, pH 7.8), and the solution was incubated at room temperature. The reaction mixture was stirred for 3 h and directly subjected to preparative HPLC to afford **5** (12 mg, 74%). Analytical HPLC: Rt, 10.4 min (1–60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 936.1, found 935.6.

Preparation of a model MPA thioloester (7)

The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Thr(Bzl)-SCH_2CH_2CO-Leu-MBHA resin (0.32 g, 0.15 mmol) according to the general automated Boc-SPPS The peptide resin was treated by HF/*p*-cresol (v/v, 85/15) within the range of -5 ℃ to -2 ℃ for 1 h to give a crude product, which was purified by preparative HPLC to afford **7** (85 mg, 46%). Analytical HPLC: Rt, 13.0 min (1–60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1225.4, found 1225.5.

Preparation of a model thioloester of Ac-Cys-NH_2 (6)

The MPA thioester **7** (20 mg, 16 μmol) and Ac-Cys-NH_2 (0.10 g, 0.62 mmol) were dissolved in 3.0 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 50 mM sodium ascorbate, pH 7.8), and the solution was incubated at room temperature. The mixture was stirred for 2 h and directly subjected to preparative HPLC to afford **6** (14 mg, 75%). Analytical HPLC: Rt, 10.5 min (1–60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 1169.3, found 1169.0.

Preparation of a model thioester of MPAA thioester (8)

The MPA thioester **7** (30 mg, 25 μmol) was dissolved in 6.0 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 50 mM MPAA, 25 mM TCEP, pH 7.8), and the solution was incubated at room temperature. The reaction mixture was stirred for 24 h and directly subjected to preparative HPLC to afford **8** (11 mg, 37%). Analytical HPLC: Rt, 13.2 min (1–60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 1175.3, found 1174.6.

Preparation of a model Cys peptide (9)

The peptide was assembled using an ABI 433A peptide synthesizer on a MBHA resin (0.42 g, 0.25 mmol) according to the general automated Boc-SPPS. The peptide resin was treated by HF/*p*-cresol (v/v, 85/15) within the range of -5 °C to -2 °C for 1 h to give a crude product, which was purified by preparative HPLC to afford **9** (0.11 g, 72%). Analytical HPLC: Rt, 8.6 min (1–60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 611.7, found 612.3.

One-pot Synthesis of ProTx-I (3SS) (11)

N-fragment **3** (5.0 mg, 2.9 μmol) and C-fragment **4** (5.5 mg, 2.2 μmol) were dissolved in 1.1 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 100 mM TCEP, 100 mM sodium ascorbate, pH 7.8), and the solution was incubated at 37 °C. The mixture was stirred for 20 h to afford **10** (not isolated) and then diluted with 1 M Gn·HCl/1 M NH_4OAc buffer (pH 7.8, 55 mL) in the presence of GSH (68 mg, 0.22 mmol) and GSSG (54 mg, 89 μmol). After stirred at 4 °C for 24 h, the reaction mixture was directly subjected to preparative HPLC to afford **11** (5.0 mg, 56%). Analytical HPLC: Rt, 18.7 min (20–40% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 3987.5, found 3987.0.

Preparation of [Ile ^{14}]-ProTx-I (1-14)-MPA-Leu-NH_2 (12)

The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Ile-SCH_2CH_2CO-Leu-MBHA resin (0.50 g, 0.25 mmol) according to the general automated Boc-SPPS. The peptide resin was treated by HF/*p*-cresol (v/v, 90/10) within the range of -5 °C to -2 °C for 1 h to give a crude product, which was purified by preparative HPLC to afford **12** (0.16 g, 38%). Analytical HPLC: Rt, 18.1 min (20–40% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1743.0, found 1742.5.

Synthesis of [Ile^{14}]-ProTx-I (6SH) (13)

N-fragment **12** (11 mg, 6.4 μmol) and C-fragment **4** (12 mg, 4.9 μmol) were dissolved in 2.5 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 100 mM TCEP, 100 mM sodium ascorbate, pH 7.8), and the solution was incubated at 37 °C. The mixture was stirred for 32 h and then treated with DTT (45 mg, 0.30 mmol). After 30 min, the pH was adjusted to < 2 by the addition of 1 M HCl aq. The reaction mixture was directly subjected to preparative HPLC to afford **13** (8.8 mg, 45%). Analytical HPLC: Rt, 19.4 min (20–40% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 4005.6, found 4005.0.

Preparation of kurtoxin (1-23)-MPA-Leu-NH_2 (14)

The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Leu-SCH_2CH_2CO-Leu-MBHA resin (0.55 g, 0.35 mmol) according to the general automated Boc-SPPS. The peptide resin was treated by HF/*p*-cresol/1,4-butanedithiol (v/v, 80/5/15) within the range of -5 ℃ to -2 ℃ for 1 h to give a crude product, which was purified by preparative HPLC to afford **14** (0.29 g, 24%). Analytical HPLC: Rt, 19.6 min (20–40% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 3488.0, found 3487.4.

Preparation of kurtoxin (24-63) (15)

The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Ala-Pam resin (0.36 g, 0.25 mmol) according to the general automated Boc-SPPS. The peptide resin was treated by HF/*p*-cresol (v/v, 80/20) within the range of -5 ℃ to -2 ℃ for 1 h to give a crude product, which was purified by preparative HPLC to afford **15** (62 mg, 6.0%). Analytical HPLC: Rt, 17.3 min (20–40% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 4124.7, found 4124.3.

Synthesis of kurtoxin (8SH) (16)

N-fragment **14** (11 mg, 3.2 μmol) and C-fragment **15** (10 mg, 2.4 μmol) were dissolved in 1.2 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 100 mM TCEP, 100 mM sodium ascorbate, pH 7.8), and the solution was incubated at 37 ℃. The mixture was stirred for 18 h and then treated with DTT (22 mg, 0.15 mmol). After 30 min, the pH was adjusted to < 2 by the addition of 1 M HCl aq. The reaction mixture was directly subjected to preparative HPLC to yield **16** (9.9 mg, 55%). Analytical HPLC: Rt, 22.4 min (20–40% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 7394.4, found 7393.7.

Preparation of Orexin A (1-11)-MPA-Leu-NH_2 (17)

The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Thr(Bzl)-SCH_2CH_2CO-Leu-MBHA resin (0.53 g, 0.25 mmol) according to the general automated Boc-SPPS. The peptide resin was treated by HF/*p*-cresol (v/v, 90/10) within the range of -5 ℃ to -2 ℃ for 1 h to give a crude product, which was purified by preparative HPLC to afford **17** (0.14 g, 38%). Analytical HPLC: Rt, 7.1 min (10–60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1471.8, found 1471.3.

Preparation of Orexin A (12-33) (18)

The peptide was assembled using an ABI 433A peptide synthesizer on a MBHA resin (0.42 g, 0.25 mmol) according to the general automated Boc-SPPS. The peptide resin was treated by HF/*p*-cresol (v/v, 90/10) in the presence of $MeONH_2 \cdot HCl$ (10 equiv) within the range of -5 °C to -2 °C for 1 h to give a crude product, which was purified by preparative HPLC to afford **18** (56 mg, 10%). Analytical HPLC: Rt, 19.1 min (10–60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 2312.7, found 2312.1.

Synthesis of orexin A (4SH) (19)

N-fragment **17** (8.3 mg, 5.6 μmol) and C-fragment **18** (10 mg, 4.3 μmol) were dissolved in 2.2 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 100 mM TCEP, 100 mM sodium ascorbate, pH 7.8), and the solution was incubated at 37 °C. The mixture was stirred for 24 h and then treated with DTT (40 mg, 0.26 mmol). After 30 min, the pH was adjusted to < 2 by the addition of 1 M HCl aq. The reaction mixture was directly subjected to preparative HPLC to afford **19** (9.4 mg, 61%). Analytical HPLC: Rt, 20.9 min (10–60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 3566.1, found 3564.6.

Ligation time-courses in the presence of TCEP in a concentration-dependent manner: NCL between 1 and 2

N-fragment **1** (1.3 equiv. 2.6 mM) and C-fragment **2** (1.0 equiv. 2.0 mM) were dissolved in ligation buffers (6 M Gn·HCl, 100 mM Na_2HPO_4, 0, 25, 50, or 100 mM TCEP, 100 mM sodium ascorbate buffer,pH 7.8) at 37 °C. The reaction aliquots were acidified with 3% TFA in H_2O to quench the reaction and then the progress of NCL was analyzed by analytical HPLC.

Ligation time-courses in the presence of thiol-additives: NCL between 1 and 2

N-fragment **1** (1.3 equiv. 2.6 mM) and C-fragment **2** (1.0 equiv. 2.0 mM) were dissolved in a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 100 mM TCEP, 100 mM sodium ascorbate buffer, pH 7.8) in the presence of different thiol additives (100 mM MPAA, 100 mM PhSH, or thiol-additive-free condition) at 37 °C. The reaction aliquots were acidified with 3% TFA in H_2O to quench the reaction and the progress of NCL was analyzed by analytical HPLC.

Ligation time-courses: NCL between 3 and 4

N-fragment **3** (1.3 equiv. 2.6 mM) and C-fragment **4** (1.0 equiv. 2.0 mM) were dissolved in a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP, 100 mM sodium ascorbate buffer, pH 7.8) in the presence of different thiol additives (100 mM MPAA, 100 mM PhSH, or thiol-additive-free condition) at 37 °C. The reaction aliquots were acidified with 3% TFA in H_2O to quench the reaction and the progress of NCL was analyzed by analytical HPLC.

Ligation time-courses: NCL of model thioesters 5-8.

N-fragments **5-8** (1.5 equiv. 3.0 mM) and C-fragment **9** (1.0 equiv. 2.0 mM) were dissolved in a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 25 mM TCEP buffer, pH 7.5) at room temperature. The reaction aliquots were acidified with 3% TFA in H_2O to quench the reaction and the progress of NCL was analyzed by analytical HPLC.

Disulfide structure determination of ProTx-I (3SS) (11)

The synthetic peptide was digested with thermolysin to obtain its Cys fragments as shown in Table E1.1. The assignment of Cys-fragment consisting of one disulfide linkage could readily reveal one of three disulfide linkages be linked between Cys^9-Cys^{21}. The Cys-fragment containing two adjacent Cys residues with two disulfide linkages, for which two possible disulfide modes could be considered, was subjected to Edman degradation to assign its disulfide structure with the guidance of the cycles detecting diPTH Cys. However, Edman degradation could not discriminate between two possible disulfide modes because diPTH Cys could be detected at the same cycles in both structures (Table E1.2). To avoid this obstacle, the acetyl group was introduced to the N-terminus and the ε-amino group at Lys^{17} of the parent molecule prior to enzymatic digestions. This provided a Cys-fragment having three peptide chains linked by two disulfide linkages, in which the peptide chain originating from the N-terminus was tagged with the acetyl group. This procedure was successfully applied to discriminating two possible structures by Edman degradation. DiPTH Cys was detected at the cycles indicated at the upper strand in Table E1.2, implying that the Cys-fragment should have the upper type of structure. From these results, the synthetic ProTx-I was confirmed to have the native disulfide pairing, *i.e.* Cys^2-Cys^{16}, Cys^9-Cys^{21} and Cys^{15}-Cys^{28}.

Table E1.1. Cys-fragments obtained by enzymatic digestion of synthetic ProTx-I and Ac-ProTx-I

Assignment	Sequence	ESI MS : MW	Cycle(s) detecting $(PTH\text{-}Cys)_2$
(6-10)/(20-21)	LGGCS/VC	653.8 (653.8)[a]	4
(1-3)/(11-18)/(27-28)	ECR/AGQTCCKH/WC	1557.0 (1556.8)	5,6
Ac-(1-3)/(11-18)/(27-28)	**Ac**-ECR/AGQTCCK(**Ac**)H/WC	1640.9 (1640.8)	5

[a]( ): The values in parentheses are theoretical values.

Table E1.2 Two possible disulfide structures of Cys-fragment containing two adjacent Cys residues and ratios of $(PTH\text{-}Cys)_2$ that should appear in each cycle during Edman degradation

Peptide	Cystine segments with 2 SS* and its expected cycle(s) to detect $(PTH\text{-}Cys)_2$	cycle 4	5	6		cycle 4	5	6
ProTx-I	ECR AGQTCCKH WC	0	1	1	**Ac-**ECR AGQTCCK(Ac)H WC	0	1	0
	ECR AGQTCCKH WC	0	1	1	**Ac-**ECR AGQTCCK(Ac)H WC	0	0	1

* Two possible disulfide modes can be considered.

Chapter 2

Preparation of a model thioester of MPA-Leu-NH_2 (20a)

The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Thr(Bzl)-SCH_2CH_2CO-Leu-MBHA resin (1.1 g, 0.50 mmol) according to the general automated Boc-SPPS. The obtained peptide resin was treated with HF/*p*-cresol (v/v, 85/15) within the range of -5 ℃ to -2 ℃ for 1 h to give a crude product ,which was purified by preparative HPLC to afford **20a** (144 mg, 31%). Analytical HPLC: Rt, 14.1 min (1–60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 937.1, found 937.5.

Preparation of a model MPAA thioester (20b)

MPA thioester **20a** (25 mg, 27 μmol) was dissolved in 5.0 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 100 mM MPAA, 25 mM TCEP, pH 6.8), and the solution was incubated at room temperature. The reaction mixture was stirred for 16 h and then subjected to preparative HPLC to afford **20b** (9.8 mg, 41%). Analytical HPLC: Rt, 13.0 min (1–60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 887.0, found 887.3.

Preparation of a model thioester of MPA-Cys-NH_2 (20c)

The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Thr(Bzl)-SCH_2CH_2CO-Cys(MeBzl)-MBHA resin (0.54 g, 0.25 mmol) according to the general automated Boc-SPPS. The obtained peptide resin was treated by HF/*p*-cresol (v/v, 85/15) within the range of -5 ℃ to -2 ℃ for 1 h to give a crude product, which was purified by preparative HPLC to afford **20c** (82 mg, 35%). Analytical HPLC: Rt, 10.8 min (1–60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 927.1, found 927.3.

Preparation of a model thioester of Ac-Cys-NH_2 (20d)

The MPA thioester **20a** (16 mg, 17 μmol) and Ac-Cys-NH_2 (50 mg, 0.31 mmol) were dissolved in 1.0 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, pH 7.8), and the solution was incubated at room temperature. The reaction mixture was stirred for 2 h and then subjected to preparative HPLC to afford **20d** (14 mg, 93%). Analytical HPLC: Rt, 10.8 min (1–60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 881.0, found 881.5.

Preparation of a model thioester of Ac-Cys-Leu-NH_2 (20e)

The MPA thioester **20a** (16 mg, 17 μmol) and Ac-Cys-Leu-NH_2 (40 mg, 0.15 mmol) were dissolved in 1.0 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, pH 7.8), and the solution was incubated at room temperature. The reaction mixture was stirred for 16 h and then subjected to preparative HPLC to afford **20e** (11 mg, 65%). Analytical HPLC: Rt, 13.4 min (1–60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 994.2, found 994.4.

Preparation of a model thioester of Ac-Gly-Cys-Leu-NH_2 (20f)

The MPA thioester **20a** (16 mg, 17 μmol) and Ac-Gly-Cys-Leu-NH_2 (57 mg, 0.17 mmol) were dissolved in 1.0 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, pH 7.8), and the solution was incubated at room temperature. The reaction mixture was stirred for 1 h and then subjected to preparative HPLC to afford **20f** (10 mg, 56%). Analytical HPLC: Rt, 12.2 min (1–60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 1051.2, found 1051.4.

Preparation of a model thioester of Ac-His-Cys-Leu-NH_2 (20g)

The MPA thioester **20a** (16 mg, 17 μmol) and Ac-His-Cys-Leu-NH_2 (35 mg, 87 μmol) were dissolved in 1.0 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, pH 7.8), and the solution was incubated at room temperature. The reaction mixture was stirred for 5 h and then subjected to preparative HPLC to afford **20g** (12 mg, 63%). Analytical HPLC: Rt, 11.7 min (1–60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 1131.3, found 1131.5.

Preparation of a model thioester of Ac-Glu-Cys-Leu-NH_2 (20h)

The MPA thioester **20a** (16 mg, 17 μmol) and Ac-Glu-Cys-Leu-NH_2 (34 mg, 84 μmol) were dissolved in 1.0 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, pH 7.8), and the solution was incubated at room temperature. The reaction mixture was stirred for 4 h and then subjected to preparative HPLC to afford **20h** (14 mg, 72 %). Analytical HPLC: Rt, 13.3 min (1–60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 1123.3, found 1123.4.

Preparation of a model thioester of Ac-Cys-His-NH_2 (20i)

The MPA thioester **20a** (12 mg, 13 μmol) and Ac-Cys-His-NH_2 (28 mg, 94 μmol) were dissolved in 1.2 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, pH 7.8), and the solution was incubated at room temperature. The reaction mixture was

stirred for 2 h and then subjected to preparative HPLC to afford **20i** (4.8 mg, 36 %). Analytical HPLC: Rt, 9.7 min (1–60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 1018.2, found 1018.5.

Preparation of a model thioester of Ac-Cys-Glu-NH_2 (20j)

The MPA thioester **20a** (12 mg, 13 μmol) and Ac-Cys-Glu-NH_2 (29 mg, 0.10 mmol) were dissolved in 1.2 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, pH 7.8), and the solution was incubated at room temperature. The reaction mixture was stirred for 2 h and then subjected to preparative HPLC to afford **20j** (5.9 mg, 46 %). Analytical HPLC: Rt, 10.1 min (1–60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 1010.1, found 1010.4.

Preparation of a model TfaC thioester (20k)

The MPA thioester **20a** (8.0 mg, 8.5 μmol) and Tfa-Cys-Leu-NH_2 (14 mg, 41 μmol) were dissolved in DMF (1.0 mL) in the presence of DIEA (pH~9) and the solution was incubated at room temperature. The reaction mixture was stirred for 2 h and then subjected to preparative HPLC to afford **20k** (9.0 mg, 50 %). Analytical HPLC: Rt, 9.7 min (10–95% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 1048.1, found 1048.4.

Preparation of a model MsC thioester (20l)

H-Cys(Trt)-Leu-MBHA resin (0.53 g, 0.25 mmol) was treated with methanesulfonyl chloride (0.12 mL, 1.5 mmol) in pyridine/CH_2Cl_2 (v/v, 50/50) for 1.5 h. The Trt group was removed by TFA/TIS/CH_2Cl_2 (v/v, 10/0.50/10) for 15 min, followed by coupling of Boc-Thr(Bzl)-OH (0.31 g, 1.0 mmol) using PyBOP (0.52 g, 1.0 mmol) and DIEA (0.22 mL 1.5 mmol) in NMP for 2 h. The peptide-chain elongation was performed using an ABI 433A peptide synthesizer according to the general automated Boc-SPPS. The obtained peptide resin was then treated with HF/*p*-cresol (v/v, 85/15) within the range of -5 °C to -2 °C for 1 h to give a crude product, which was purified by preparative HPLC to afford **20l** (36 mg, 14%). Analytical HPLC: Rt, 12.8 min (1–60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1030.2, found 1030.4.

Preparation of a model thioester of Tos-Cys-Leu-NH_2 (20m)

H-Cys(Trt)-Leu-MBHA resin (0.53 g, 0.25 mmol) was treated with *p*-toluenesulfonyl chloride (0.29 g, 1.5 mmol) (0.12 mL, 1.5 mmol) in pyridine/CH_2Cl_2 (v/v, 50/50) for 1.5 h. The Trt group was removed by TFA/TIS/CH_2Cl_2 (v/v, 10/0.50/10)

for 15 min, followed by coupling of Boc-Thr(Bzl)-OH (0.31 g, 1.0 mmol) using PyBOP (0.52 g, 1.0 mmol) and DIEA (0.22 mL 1.5 mmol) in NMP for 2 h. The peptide-chain elongation was performed using an ABI 433A peptide synthesizer according to the general automated Boc-SPPS. The obtained peptide resin was treated with HF/*p*-cresol (v/v, 85/15) within the range of -5 ℃ to -2 ℃ for 1 h to give a crude product which was purified by preparative HPLC to afford **20m** (33 mg, 12%). Analytical HPLC: Rt, 16.8 min (1–60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1106.3, found 1106.4.

Preparation of a model Cys peptide (21)

The peptide was assembled using an ABI 433A peptide synthesizer on a MBHA resin (0.42 g, 0.25 mmol) according to the general automated Boc-SPPS. The peptide resin was treated with HF/*p*-cresol (v/v, 85/15) within the range of -5 ℃ to -2 ℃ for 1 h to give a crude product, which was purified by preparative HPLC to afford **21** (0.11 g, 72%). Analytical HPLC: Rt, 8.6 min (1–60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 611.7, found 612.3.

Preparation of a model peptide-TfaC (27)

The peptide was assembled using an ABI 433A peptide synthesizer on a MBHA resin (5.0 g, 3.0 mmol) according to the general automated Boc-SPPS. The obtained resin **24** was treated with 20% piperidine/DMF (20 mL) for 20 min, and the resulting H-Cys(Trt)-Leu-MBHA resin (2.1 g, 1.0 mmol) was reacted with ETFE (1.2 mL, 10 mmol) and DBU (1.5 mL, 12 mmol) in DMF (20 mL) for 1.5 h. The Trt group of Tfa-Cys(Trt)-Leu-MBHA resin **25** (0.55 g, 0.25 mmol) was removed by TFA/TIS/CH_2Cl_2 (v/v, 10/0.50/10) for 15 min, followed by coupling of Boc-Ala-OH (0.19 g, 1.0 mmol) using PyBOP (0.52 g, 1.0 mmol) and DIEA (0.22 mL 1.5 mmol) in NMP for 1.5 h. The peptide-chain elongation was performed using an ABI 433A peptide synthesizer according to the general automated Boc-SPPS. The obtained resin **26** was treated with HF/*p*-cresol (v/v, 85/15) within the range of -5 ℃ to -2 ℃ for 1 h to afford a crude product **27**. Analytical HPLC: Rt, 18.2 min (1–60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1213.3, found 1213.6.

Preparation of peptide-TfaC having L-Phe for evaluating epimerization (28a)

The Trt group of Tfa-Cys(Trt)-Leu-MBHA resin **25** (0.55 g, 0.25 mmol) was removed by TFA/TIS/CH_2Cl_2 (v/v, 10/0.50/10) for 15 min, followed by coupling of Boc-Phe-OH (0.27 g, 1.0 mmol) using PyBOP (0.52 g, 1.0 mmol) and DIEA (0.22 mL

1.5 mmol) in NMP for 1.5 h. The peptide-chain elongation was performed using an ABI 433A peptide synthesizer according to the general automated Boc-SPPS. The obtained resin was treated with HF/*p*-cresol (v/v, 85/15) within the range of -5 °C to -2 °C for 1 h to afford a crude product **28a** (155 mg, 57%). Analytical HPLC: Rt, 18.2 min (1–30% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1094.2, found 1094.4.

Preparation of peptide-TfaC having D-Phe for evaluating epimerization (28b)

The Trt group of Tfa-Cys(Trt)-Leu-MBHA resin (0.55 g, 0.25 mmol) was removed by TFA/TIS/CH_2Cl_2 (v/v, 10/0.50/10) for 15 min, followed by coupling of Boc-D-Phe-OH (0.27 g, 1.0 mmol) using PyBOP (0.52 g, 1.0 mmol) and DIEA (0.22 mL 1.5 mmol) in NMP for 1.5 h. The peptide-chain elongation was performed using an ABI 433A peptide synthesizer according to the general automated Boc-SPPS. The obtained resin was treated with HF/*p*-cresol (v/v, 85/15) within the range of -5 °C to -2 °C for 1 h to afford a crude product **28b** (147 mg, 54%). Analytical HPLC: Rt, 18.2 min (1–30% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1094.2, found 1094.4.

Preparation of neuromedin-S (1-9)-TfaC (29)

The crude peptide **27** was purified by preparative HPLC to afford **29** (162 mg, 54% based on 0.25 mmol **25** resin). Analytical HPLC: Rt, 18.2 min (1–60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1213.3, found 1213.6.

Preparation of [Cys ^{10}]-neuromedin-S (10-19)-MPA-Leu-NH_2 (30)

The peptide was assembled using an ABI 433A peptide synthesizer on a Boc-Thr(Bzl)-SCH_2CH_2CO-Leu-MBHA resin (0.54 g, 0.25 mmol) according to the general automated Boc-SPPS. The peptide resin was treated with HF/*p*-cresol (v/v, 85/15) in the presence of $MeONH_2$·HCl (10 equiv.) within the range of -5 °C to -2 °C for 1 h to give a crude product, which was purified by preparative HPLC to afford **30** (0.22 g, 62%). Analytical HPLC: Rt, 15.3 min (1–60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1393.6, found 1393.6.

Preparation of [Cys ^{20}] neuromedin-S (20-33)-NH_2 (31)

The peptide was assembled using an ABI 433A peptide synthesizer on a MBHA resin (0.33 g, 0.20 mmol) according to the general automated SPPS procedure. The peptide resin was treated with HF/*p*-cresol (v/v, 85/15) within the range of -5 °C to -2 °C for 1 h to give a crude product, which was purified by preparative HPLC to afford

31 (0.18 g, 49%). Analytical HPLC: Rt, 21.0 min (1–60% CH_3CN/0.1% TFA for 25 min); ESI MS calcd (average isotopes) 1796.1, found 1796.9.

Synthesis of [Cys $^{10, 20}$]neuromedin S (32)

Peptide-TfaC **29** (16 mg, 13 μmol) and MPA thioester **30** (22 mg, 16 μmol) were dissolved in 4.4 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 30 mM TCEP, 30 mM sodium ascorbate, pH 7.0), and the solution was incubated at room temperature. After stirred for 4 h, C-fragment **31** (24 mg, 13 μmol) in 4.4 mL of a buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 200 mM MPAA, 30 mM TCEP, 30 mM sodium ascorbate, pH 7.6) was then added to the reaction mixture at 37 °C for 14 h, followed by the treatment with DTT (40 mg, 0.32 mmol) for 30 min. After adjustment of the pH < 2 by 1 M HCl aq, the reaction mixture was directly subjected to preparative HPLC to afford **32** (12 mg, 38%). Analytical HPLC: Rt, 20.2 min (1–60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 3855.4, found 3854.8.

Synthesis of neuromedin S (33)

VA-044 (20 mg, 62 μmol) and GSH (19 mg, 62 μmol) was added to a solution of **32** (12 mg, 3.1 μmol) in 1.6 mL of a buffer (6 M Gn·HCl, 100 mM, 200 mM TCEP). The mixture was stirred at 40 ºC for 3.5 h and then directly subjected to preparative HPLC to afford **33** (8.6 mg, 73%). Analytical HPLC: Rt, 19.8 min (1–60% CH_3CN/0.1% TFA for 25min); ESI MS calcd (average isotopes) 3791.3, found 3791.0.

Ligation time-courses: NCL between 20 and 21

N-fragment **20a-m** (1.3 eq. 2.6 mM) and C-fragment **21** (1.0 eq. 2.0 mM) were dissolved in a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 25 mM TCEP, 25 mM sodium ascorbate buffer, pH 7.5) at 37 °C. The reaction aliquots were acidified with 3% TFA in H_2O to quench the reaction and the progress of NCL was analyzed by analytical HPLC.

Examination of epimerization during NCL between 20 + 28

Crude N-fragments (Leu-Tyr-Arg-Ala-Asn-L-Phe-TfaC-Leu-NH_2, **28a** and Leu-Tyr-Arg-Ala-Asn-D-Phe-TfaC-Leu-NH_2, **28b**, 1.1 mg, 1.0 μmol, respectively) and C-fragment peptide **20** (1.2 mg, 2.0 μmol) were dissolved in 0.10 mL of a ligation buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 1% PhSH, pH 7.8), and the solutions were incubated at 37 °C 3 h. The reaction mixtures were analyzed by analytical HPLC (10–30% CH_3CN/0.1% TFA for 25min).

Chapter 3

Synthesis of allyl 4-(2-nitrobenzensulfonylamino)benzoate (38)

o-Nitrobenzenesulfonyl chloride (7.2 g, 33 mmol) was added to a solution of compound **37** (5.8 g, 33 mmol) in pyridine (30 mL). The reaction mixture was stirred at room temperature for 3 h and then concentrated. The crude was quenched with 1 M HCl, and the aqueous layer was extracted with $CHCl_3$. The organic layer was washed with brine, dried over $MgSO_4$ and evaporated. The residue was purified by silica gel column chromatography (2:1 = *n*-hexane/EtOAc) followed by recrystallization from DMF/*n*-hexane/EtOAc to afford compound **38** (10 g, 84 %) as a light yellow plate: mp 137-139 °C; ^{1}H NMR ($CDCl_3$) δ 4.79 (dt, J = 5.6 and 1.5 Hz, 2H), 5.28 (dq, J = 10.5 and 1.5 Hz, 1H), 5.38 (dq, J = 17.1 and 1.5 Hz, 1H), 6.00 (ddt, J = 17.1, 10.5 and 5.6 Hz, 1H), 7.29 (d, J = 8.8 Hz, 2H), 7.45 (br, 1H), 7.61 (td, J = 7.8 and 1.5 Hz, 1H), 7.71 (td, J = 7.8 and 1.5 Hz, 1H), 7.86 (dd, J = 7.8 and 1.5 Hz, 1H), 7.92 (dd, J = 7.8 and 1.5 Hz, 1H), 7.98 (d, J = 8.8 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 65.7, 118.4, 121.1, 125.5, 127.6, 131.1, 131.7, 131.9, 132.0, 132.7, 134.3, 139.9, 148.1, 165.3. HRMS (ESI-TOF) *m*/*z* calcd for $C_{16}H_{14}N_2NaO_6S$ ([M + Na]$^+$) 385.0470, found 385.0454. anal. calcd for $C_{16}H_{14}N_2NaO_6S$: C, 53.03; H, 3.89; N, 7.73. found: C, 52.93; H, 4.05; N, 7.62.

Synthesis of allyl 4-[(2-nitrobenzensulfonyl)-(2-tritylsulfanylethyl)amino]benzoate (40)

To a stirred mixture of compound **38** (10 g, 28 mmol), compound **39** (9.7 g, 30 mmol) and PPh_3 (8.0 g, 30.3 mmol) in THF (200 mL) was added DEAD (14 mL, 30 mmol) dropwise at 0 °C. After stirred at room temperature for overnight, the reaction mixture was concentrated. The residue was purified by silica gel column chromatography (5:2 = *n*-hexane/EtOAc) followed by recrystallization from DMF/EtOH to afford compound **40** (13 g, 70 %) as a light yellow needle: mp 139-141 °C; ^{1}H NMR ($CDCl_3$) δ 2.37 (t, J = 7.3 Hz, 2H), 3.58 (t, J = 7.3 Hz, 2H), 4.84 (dd, J = 5.6 and 1.5 Hz, 2H), 5.32 (dt, J = 10.5 and 1.5 Hz, 1H), 5.43 (dt, J = 17.1 and 1.5 Hz, 1H), 6.04 (ddt, J = 17.1, 10.5 and 5.6 Hz, 1H), 7.09-7.21 (m, 11H), 7.30-7.32 (m, 6H), 7.41-7.64 (m, 4H), 7.92-7.94 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 30.8, 51.2, 65.9, 67.1, 118.6, 123.9, 126.7, 127.9, 128.9, 129.5, 129.8, 130.6, 131.1, 131.5, 131.8, 132.0, 133.8, 135.0, 141.9, 144.5, 165.3. HRMS (ESI-TOF) *m*/*z* calcd for $C_{37}H_{32}N_2NaO_6S_2$ ([M + Na]$^+$) 687.1600, found 687.1570. anal. calcd for $C_{37}H_{32}N_2NaO_6S_2$: C, 66.85; H, 4.85; N, 4.21. found: C, 66.58; H, 5.05; N, 4.16.

Synthesis of allyl 4-(2-tritylsulfanylethylamino)benzoate (41)

Compound **40** (1.0 g, 1.5 mmol) in DMF (25 ml) was treated with $LiOH \cdot H_2O$ (0.68 g, 17 mmol) and thioglycolic acid (0.63 ml, 9.0 mmol). The reaction was stirred at room temperature for 3 h, and then diluted with 5% aqueous $NaHCO_3$. The aqueous layer was extracted with EtOAc. Combined organic layer was washed with brine, dried over $MgSO_4$ and concentrated. The residue was purified by silica gel column chromatography (3:1 = *n*-hexane/EtOAc) followed by recrystallization from EtOAc/*n*-hexane to afford compound **41** (0.70g, 97%) as a white needle: mp 98-100 °C; ^{1}H NMR ($CDCl_3$) δ 2.52 (t, J = 6.8 Hz, 2H), 3.04 (t, J = 6.8 Hz, 2H), 4.18 (br, 1H), 4.77 (dt, J = 5.6 and 1.5 Hz, 2H), 5.25 (dt, J = 10.5 and 1.5 Hz, 1H), 5.38 (dt, J = 17.1 and 1.5 Hz, 1H,), 6.02 (ddt, J = 17.1, 10.5 and 5.6 Hz, 1H), 6.35 (d, J = 8.5 Hz, 2H), 7.19-7.29 (m, 9H), 7.40-7.42 (m, 6H), 7.82 (d, J = 8.5 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 31.4, 42.0, 64.8, 67.0, 111.5, 117.5, 118.5, 126.8, 128.0, 129.6, 131.6, 132.9, 144.6, 151.3, 166.3. HRMS (ESI-TOF) *m*/*z* calcd for $C_{31}H_{29}NNaO_2S$ ([M+ Na]$^+$) 502.1817, found 502.1825. anal. calcd for $C_{31}H_{29}NNaO_2S$: C, 77.63; H, 6.09; N, 2.92. found: C, 77.41; H, 6.14; N, 2.99.

Synthesis of allyl 4-[(Boc-L-leucyl-L-alanyl-2-tritylsulfanylethyl)amino]benzoate (42a)

To a stirred solution of compound **41** (1.0 g, 2.1 mmol) in THF (20 mL) was added NaH (0.10 g, 2.3 mmol) dropwise at 0 °C. After stirred for 30 min at room temperature, Fmoc-L-Ala-Cl (1.72 g, 5.21 mmol) was added. The reaction mixture was stirred for 2 h, then quenched with 5% aqueous $NaHCO_3$. The aqueous layer was extracted with EtOAc. The obtained EtOAc layer was washed with brine, dried over MgSO4 and concentrated. The resulting residue was purified by silica gel column chromatography (5:2 = *n*-hexane/EtOAc) to afford Fmoc-L-alanylanilide (1.3 g, 80%) as a white amorphousness: $[\alpha]^{23}{}_D$ 92.5 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.07 (d, J = 6.8 Hz, 3H), 2.26-2.61 (m, 2H), 3.28-3.61 (m, 2H), 4.13-4.24 (m, 2H), 4.30 (d, J = 6.8 Hz, 2H), 4.86 (d, J = 5.6 Hz, 2H), 5.33 (d, J = 10.5 Hz, 1H), 5.44 (d, J = 17.1 Hz, 1H), 5.54 (d, J = 7.6 Hz, 1H), 6.06 (ddt, J = 17.1, 10.5 and 5.6 Hz, 1H), 7.09-7.41 (m, 21H), 7.58 (d, J = 6.8 Hz, 2H), 7.76 (d, J = 8.1 Hz, 2H), 8.06 (d, J = 8.1 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 18.8, 29.2, 47.1, 47.6, 48.9, 65.9, 66.8, 66.9, 118.6, 119.9, 125.1, 126.6, 127.0, 127.6, 127.8, 128.2, 129.5, 131.3, 131.9, 141.2, 143.7, 143.9, 144.5, 144.8, 155.4, 165.2, 172.4. HRMS (ESI-TOF) *m*/*z* calcd for $C_{49}H_{44}N_2NaO_5S$ ([M + Na]$^+$) 795.2833, found 795.2869.

The obtained L-Ala derivative (0.20 g, 0.26 mmol) was treated with 20%

piperidine/DMF (1.0 mL) at room temperature. After 30 min, solvents including piperidine were completely evaporated. Boc-L-Leu derivative in DMF (1.5 mL) (preactivated from Boc-Leu-OH (71 mg, 0.29 mmol), HBTU (0.11 g, 0.29 mmol) and DIEA (47 μL, 0.26 mmol) for 10 min) was added to the residue. After stirred at room temperature for 30 min, the mixture was diluted with 5% aqueous $NaHCO_3$ and extracted with EtOAc. After usual work-up procedure, the crude was purified by silica gel column chromatography (5:2 = *n*-hexane/EtOAc) to afford compound **42a** (0.18 g, 91%, for 2 steps) as a white amorphousness: $[\alpha]^{25}{}_{D}$ 46.9 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.93 (d, *J* = 6.8 Hz, 6H), 1.06 (d, *J* = 6.8 Hz, 3H,), 1.42 (s, 9H), 1.55-1.71 (m, 3H), 2.08 (br, 1H), 2.25-2.52 (m, 2H), 3.29-3.57 (m, 2H), 4.07 (br, 1H), 4.32 (br, 1H), 4.86 (d, J = 5.6 Hz, 2H), 5.33 (dq, *J* = 10.5 and 1.5 Hz, 1H), 5.44 (dq, *J* = 17.1 and 1.5 Hz, 1H), 6.05 (ddt, *J* = 17.1, 10.5 and 5.6 Hz, 1H), 6.63 (d, *J* = 7.1 Hz, 1H), 7.08-7.35 (m, 17H), 8.05 (d, *J* = 8.6 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 18.4, 21.8, 23.1, 24.6, 28.3, 29.1, 41.4, 46.1, 48.9, 52.9, 65.9, 66.9, 80.9, 118.6, 126.6, 127.8, 128.2, 129.5, 130.0, 131.2, 131.9, 144.5, 144.7, 155.5, 165.2, 171.7, 171.9. HRMS (ESI-TOF) *m*/*z* calcd for $C_{45}H_{53}N_3NaO_6S$ ($[M + Na]^+$) 786.3553, found 786.3536.

Synthesis of allyl 4-[(Boc-L-leucyl-D-alanyl-2-tritylsulfanylethyl)amino]benzoate (42b)

To a stirred solution of compound **41** (0.85 g, 1.8 mmol) in THF (20 mL) was added NaH (87 mg, 2.0 mmol) dropwise at 0 °C. After stirred for 30 min at room temperature, Fmoc-D-Ala-Cl (1.46 g, 4.4 mmol) was added. The reaction mixture was stirred for 2 h, then quenched with 5% aqueous $NaHCO_3$. The aqueous layer was extracted with EtOAc. The obtained EtOAc layer was washed with brine, dried over MgSO4 and concentrated. The resulting residue was purified by silica gel column chromatography (5:2 = *n*-hexane/EtOAc) to afford Fmoc-D-alanylanilide (1.1 g, 83%) as a white amorphousness: $[\alpha]^{25}{}_{D}$ -98.5 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.07 (d, *J* = 6.8 Hz, 3H), 2.26-2.61 (m, 2H), 3.28-3.61 (m, 2H), 4.13-4.24 (m, 2H), 4.30 (d, *J* = 6.8 Hz, 2H), 4.86 (d, *J* = 5.6 Hz, 2H), 5.33 (d, *J* = 10.5 Hz, 1H), 5.44 (d, *J* = 17.1 Hz, 1H), 5.54 (d, *J* = 7.6 Hz, 1H), 6.06 (ddt, *J* = 17.1, 10.5 and 5.6 Hz, 1H), 7.09-7.41 (m, 21H), 7.58 (d, *J* = 6.8 Hz, 2H), 7.76 (d, *J* = 8.1 Hz, 2H), 8.06 (d, *J* = 8.1 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 18.8, 29.2, 47.1, 47.6, 48.9, 65.9, 66.8, 66.9, 118.6, 119.9, 125.1, 126.6, 127.0, 127.6, 127.8, 128.2, 129.5, 131.3, 131.9, 141.2, 143.7, 143.9, 144.5, 144.8, 155.4, 165.2, 172.4. HRMS (ESI-TOF) *m*/*z* calcd for $C_{49}H_{44}N_2NaO_5S$ ($[M + Na]^+$) 795.2833, found 795.2867.

The obtained D-Ala derivative (0.20 mg, 0.26 mmol) was treated with 20%

piperidine/DMF (1.0 mL) at room temperature. After 30 min, solvents including piperidine were completely evaporated. Boc-D-Leu derivative in DMF (1.5 mL) (preactivated from Boc-Leu-OH (71 mg, 0.29 mmol), HBTU (0.11 g, 0.29 mmol) and DIEA (47 μL, 0.26 mmol) for 10 min) was added to the residue. After being stirred at room temperature for 30 min, the mixture was diluted with 5% aqueous $NaHCO_3$ and extracted with EtOAc. After usual work-up procedure, the crude was purified by silica gel column chromatography (5:2 = *n*-hexane/EtOAc) to afford compound **42b** (0.17 g, 85%, for 2 steps) as a white amorphousness: $[\alpha]^{24}_{D}$ -99.5 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.91 (d, *J* = 6.4 Hz, 3H), 0.92 (d, *J* = 6.4 Hz, 3H), 1.06 (d, *J* = 6.8 Hz, 3H), 1.45 (s, 9H), 1.55-1.71 (m, 3H), 2.25-2.55 (m, 2H), 2.58 (br, 1H), 3.28-3.58 (m, 2H), 4.10 (br, 1H), 4.32 (br, 1H), 4.86 (d, *J* = 5.6 Hz, 2H), 5.33 (dq, *J* = 10.5 and 1.5 Hz, 1H), 5.44 (dq, *J* = 17.1 and 1.5 Hz, 1H), 6.05 (ddt, *J* = 17.1, 10.5 and 5.6 Hz, 1H), 6.79 (br, 1H), 7.08-7.35 (m, 17H), 8.05 (d, *J* = 8.6 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 18.1, 21.6, 23.0, 24.6, 28.2, 29.0, 41.4, 46.1, 48.9, 52.9, 65.8, 66.9, 80.0, 118.5, 126.6, 127.8, 128.2, 129.4, 130.0, 131.2, 131.8, 144.4, 144.6, 155.5, 165.1, 172.0, 172.1. HRMS (ESI-TOF) *m*/*z* calcd for $C_{45}H_{53}N_3NaO_6S$ ($[M + Na]^+$) 786.3553, found 786.3563.

Synthesis of allyl 4-[(Fmoc-glycyl-2-tritylsulfanylethyl)amino]benzoate (43)

To a stirred solution of compound **41** (0.43 g, 0.92 mmol) in THF (9.0 mL) was added NaH (44 mg, 1.0 mmol) dropwise at 0 °C. After 30 min at room temperature, Fmoc-Gly-Cl (0.72 g, 2.3 mmol) was added. The reaction mixture was stirred for 2 h, and then quenched with 5% aqueous $NaHCO_3$. The aqueous layer was extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$ and concentrated. The resulting residue was purified by silica gel column chromatography (2:1 = *n*-hexane/EtOAc) to afford compound **43** (0.66 g, 94%) as a white amorphousness: ^{1}H NMR ($CDCl_3$) δ 2.41 (t, *J* = 7.3 Hz, 2H), 3.50 (t, *J* = 7.3 Hz, 2H), 3.57 (s, 2H), 4.18 (t, *J* = 7.1 Hz, 1H), 4.30 (d, *J* = 7.1 Hz, 2H), 4.87 (d, *J* = 5.6 Hz, 2H), 5.33 (d, *J* = 10.5 Hz, 1H), 5.44 (d, *J* = 17.1 Hz, 1H), 5.62 (br, 1H), 6.06 (ddt, *J* = 17.1, 10.5 and 5.6 Hz, 1H), 7.06 (d, *J* = 8.1 Hz, 2H), 7.13-7.41 (m, 19H), 7.57 (d, *J* = 7.6 Hz, 2H), 7.75 (d, *J* = 7.6 Hz, 2H), 8.05 (d, *J* = 8.1 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 29.4, 43.4, 47.1, 48.7, 65.9, 67.0, 67.2, 118.6, 119.9, 125.1, 126.7, 127.0, 127.7, 127.9, 128.1, 129.5, 130.5, 131.4, 131.9, 141.3, 143.8, 143.9, 144.5, 156.2, 165.1, 168.0. HRMS (ESI-TOF) *m*/*z* calcd for $C_{48}H_{42}N_2NaO_5S$ ($[M + Na]^+$) 781.2712, found 781.2708.

Synthesis of ally 4-[(Fmoc-glycyl[^{13}C]-2-tritylsulfanylethyl)amino]benzoate (43')

To a stirred solution of compound **41** (0.48 g, 1.0 mmol) in THF (10 mL) was

added NaH (44 mg, 1.0 mmol) dropwise at 0 °C. After 30 min at room temperature, Fmoc-Gly(^{13}C=O)-Cl (0.47 g, 1.5 mmol) was added. The reaction mixture was stirred for 2 h, and then quenched with 5% aqueous $NaHCO_3$. The aqueous layer was extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$ and concentrated. The resulting residue was purified by silica gel column chromatography (2:1 = *n*-hexane/EtOAc) to afford compound **43'** as a white amorphousness (0.71 g, 93%): ^{1}H NMR (CDCl3) δ 2.41 (t, J = 7.3 Hz, 2H), 3.50 (t, J = 7.3 Hz, 2H), 3.57 (s, 2H), 4.18 (t, J = 7.1 Hz, 1H), 4.30 (d, J = 7.1 Hz, 2H), 4.87 (d, J = 5.6 Hz, 2H), 5.33 (d, J = 10.5 Hz, 1H), 5.44 (d, J = 17.1 Hz, 1H), 5.63 (br, 1H), 6.06 (ddt, J = 17.1, 10.5 and 5.6 Hz, 1H), 7.06 (d, J = 8.1 Hz, 2H), 7.13-7.41 (m, 19H), 7.57 (d, J = 7.6 Hz, 2H,), 7.75 (d, J = 7.6 Hz, 2H), 8.05 (d, J = 8.1 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 29.3, 43.4 (d, J = 54 Hz), 47.1, 48.6,65.9, 67.0, 67.1, 118.6, 119.9, 125.1, 126.7, 127.0, 127.7, 127.9, 128.1, 129.5, 130.4, 131.4, 131.9, 141.2, 143.8, 143.9, 144.4, 156.1, 165.1, 167.7. HRMS (ESI-TOF) *m*/*z* calcd for C_{47} $^{13}CH_{42}N_2NaO_5S$ ([M + Na]$^+$) 782.2746, found 782.2769.

Synthesis of 4-[(Fmoc-glycyl-2-tritylsulfanylethyl)amino]benzoic acid (44)

To a stirred mixture of compound **43** (0.2 g, 0.26 mmol) in THF (4.0 mL), *N*-methylaniline (0.28 mL, 2.6 mmol) and $Pd(PPh_3)_4$ (30 mg, 26 μmol) were added. The reaction mixture was stirred at room temperature for 1 h. The solvent was evaporated and the product was purified by silica gel column chromatography (1:1 = *n*-hexane/EtOAc) to afford compound **44** (0.19 g, 80%) as a white amorphousness: ^{1}H NMR ($CDCl_3$) δ 2.41 (t, *J* = 7.3 Hz, 2H), 3.52 (t, *J* = 7.3 Hz, 2H), 3.68 (s, 2H), 4.19 (t, *J* = 6.8 Hz, 1H), 4.30 (d, *J* = 6.8 Hz, 2H), 5.83 (s, 1H), 7.06 (d, *J* = 7.6 Hz, 2H), 7.13-7.41 (m, 19H), 7.58 (d, *J* = 7.6 Hz, 2H), 7.75 (d, *J* = 7.6 Hz, 2H), 8.01 (d, *J* = 8.1 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 30.2, 44.4, 47.9, 49.5, 67.9, 68.3, 120.9, 126.0, 127.6, 128.0, 128.6, 128.8, 129.0, 130.3, 130.7, 132.7, 142.1, 144.6, 144.8, 145.3, 157.4, 168.5, 169.0. HRMS (ESI-TOF) *m*/*z* calcd for $C_{45}H_{38}N_2NaO_5S$ ([*M* + Na]$^+$) 741.2399, found 741.2392.

Synthesis of allyl 4-[(Fmoc-glycyl[^{13}C]-2-mercaptoethyl)amino]benzoate (45)

Compound **43'** (54 mg, 71 μmol) was treated with TFA/TES (0.97 mL/30 μL) for 15 min. The solvent was removed using N_2 stream followed by purification on silica gel column chromatography (1:1 = *n*-hexane/EtOAc) to afford compound **45** (36 mg, 78%) as a white amorphousness: ^{1}H NMR ($CDCl_3$) δ 1.40 (t, *J* = 7.3 Hz, 1H), 2.69 (q, *J* = 7.3 Hz, 2H), 3.71 (s, 2H), 3.92 (t, *J* = 7.3 Hz, 2H), 4.20 (t, *J* = 7.1 Hz, 1H), 4.34 (d, *J* = 7.1 Hz, 2H), 4.86 (d, *J* = 5.6 Hz, 2H), 5.32 (d, *J* = 10.5 Hz, 1H), 5.43 (d, *J* = 17.1 Hz,

1H), 6.04 (ddt, J = 17.1, 10.5 and 5.6 Hz, 1H), 7.27-7.41 (m, 6H), 7.58 (d, J = 7.3 Hz, 2H), 7.75 (d, J = 7.3 Hz, 2H), 8.17 (d, J = 8.1 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 22.1, 43.5 (d, J = 54 Hz), 47.0, 52.2, 65.9, 67.1, 118.5, 119.8, 125.0, 127.0, 127.6, 128.0, 129.3, 131.6, 131.7, 141.1, 143.6, 144.0, 156.0, 164.8, 168.1. HRMS (ESI-TOF) m/z calcd for $C_{28}{}^{13}CH_{28}N_2NaO_5S$ ($[M + Na]^+$) 540.1650, found 540.1611.

Synthesis of the peptide thioester (51)

On a NovaSyn® TGR resin (Rink amide type: 0.10 g, 25 μmol) was coupled Fmoc-Leu-OH (26 mg, 75 μmol) with the aid of DIC (12 μL, 75 μmol) and HOBt·H_2O (12 mg, 0.075 mmol) in DMF at room temperature for 2 h followed by Fmoc removal by 20% piperidine/DMF to give H-Leu-NovaSyn® TGR resin. Compound **44** (36 mg, 50 μmol) was coupled with the resulting resin using HATU (19 mg, 50 μmol) and DIEA (9.0 μL, 50 μmol) to yield the resin **48**. The peptide-chain elongation was performed using a KMS-3 on obtained resin **48** according to the general manual Fmoc-SPPS. Resulting resin **49** (20 mg) was treated with TFA/TES/H2O (v/v, 95:2.5:2.5) at room temperature for 1.5 h to afford crude product **46.** Analytical HPLC: Rt, 24.7 min (5–30 % CH_3CN/0.1% TFA for 30min); ESI MS calcd (average isotopes) 878.5, found 878.4. Peptide **46** was then treated with 4 M HCl/DMF (1.0 ml) in the presence of TCEP (1%, (w/v)) at 37 °C for 8 h and purified by semi-preparative HPLC to afford desired peptide thioester **50** (2.1 mg, 33%). HPLC: Rt, 25.0 min (5–30 % CH_3CN/0.1% TFA for 30min); ESI MS calcd (average isotopes) 878.5, found 878.4. Purified thioester **50** was treated with 2-mercaptoethanesulfonate (1%, (w/v)) in a phosphate buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, pH 7.3) at room temperature for 1 h to afford peptide thioester **51** in virtually complete conversion. Analytical HPLC condition: HPLC: Rt, 10.1 min (5–30% CH_3CN/0.1% TFA for 30min); ESI MS calcd (average isotopes) 711.3, found 711.3.

On-resin thiolytic release of peptide thioesters (57)

On an aminomethyl ChemMatrix® resin (40 mg, 40 μmol) was coupled either Fmoc-Ala-OH or Fmoc-Leu-OH (0.12 mmol each) as internal standard amino acids by the action of DIC (19 μL, 0.12 mmol) and HOBt·H_2O (19 mg, 0.12 mmol) in DMF at room temperature for 2 h followed by Fmoc removal by 20% piperidine/DMF to afford internal standard-incorporated resins. In the case of synthesis of phosphopeptide **57d**, before incorporation of Fmoc-Ser(PO(OBzl)OH)-OH, protected-peptide resin was constructed using manual Fmoc-SPPS as mentioned above. The subsequent condensations of protected amino acids (0.12 mmol each) including

Fmoc-Ser(PO(OBzl)OH)-OH were performed with aid of HBTU (46 mg, 0.12 mmol), HOBt·H_2O (19 mg, 0.12 mmol) and DIEA (60 μL, 0.36 mmol) at room temperature for 2 h.[36] Compound **44** (58 mg, 75 μmol) was coupled with the resulting resin using HATU (30 mg, 75 μmol) and DIEA (14 μL, 75 μmol) to afford the resins **53**. The peptide-chain elongations were performed on resins **53** using the manual Fmoc-SPPS. The resulting resins **54** were treated with TFA/thioanisole/*m*-cresol/H_2O/EDT/TES (v/v, 80:5:5:5:2.5:2.5) at room temperature for 1.5 h to afford the side-chain-unprotected peptide resins (**55**). After washing the resins **55** with CH_2Cl_2, each resin was subjected to *N*→*S* acyl shift reaction with 4 M HCl/DMF in the presence of TCEP (1%, (w/v)) at room temperature for 12 h to afford the corresponding thioester resins **56**. The resulting thioester resins were reacted with 2-mercaptoethanesulfonate (3%, (w/v)) in phosphate buffers (6 M Gn·HCl, 100 mM Na_2HPO_4, pH 7.3) in the presence of PhSH (1%, (v/v)) at room temperature for 4 h to afford peptide thioesters **57**. After release of peptides, each treated resin was hydrolyzed with 6 M HCl in the presence of phenol (0.5%, (v/v)). The resulting hydrolysates were subjected to AAA, showing that each release yield was calculated based on the internal standard.

57a Analytical HPLC condition: HPLC: Rt, 20.6 min (2–25% CH_3CN/0.1% TFA for 30min); ESI MS calcd (average isotopes) 1010.5, found 1010.4.
57b Analytical HPLC condition: HPLC: Rt, 15.9 min (2–25% CH_3CN/0.1% TFA for 30min); ESI MS calcd (average isotopes) 937.4, found 937.4.
57c Analytical HPLC condition: HPLC: Rt, 13.1 min (2–25% CH_3CN/0.1% TFA for 30min); ESI MS calcd (average isotopes) 642.3, found 642.2.
57d Analytical HPLC condition: HPLC: Rt, 13.7 min (2–30% CH_3CN/0.1% TFA for 30min); ESI MS calcd (average isotopes) 1148.5, found 1148.5.

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List of publications

This study was published in the following papers.

1. *N*→*S* Acyl-transfer-mediated synthesis of peptide thioesters using anilide derivatives
   Shugo Tsuda, Akira Shigenaga, Kiyomi Bando, and Akira Otaka
   Org. Lett., **2009**, *11*, 823–826.

2. Synthesis of cysteine-rich peptides by native chemical ligation without use of exogenous thiols
   Shugo Tsuda, Taku Yoshiya, Masayoshi Mochizuki, and Yuji Nishiuchi
   Org. Lett., **2015**, *17*, 1806–1809.

3. Development of a sufficiently reactive thioalkylester involving the side-chain thiol of cysteine applicable for kinetically controlled ligation
   Shugo Tsuda, Masayoshi Mochizuki, Hideki Nishio, Taku Yoshiya and Yuji Nishiuchi
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