Development of practical procedures for protein synthesis with their application to the elucidation of CXCL14 function

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Thesis by
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Abbreviations

AA	amino acid
Ac	acetyl
Acm	acetamidomethyl
Alloc	allyloxycarbonyl
aq.	aqueous solution
Boc	*tert*-butoxycarbonyl
Bom	benzyloxymethyl
BSA	bovine serum albumin
Bzl	benzyl
CpG	cytosine-phosphate-guanine
CuAAC	copper-catalyzed azide–alkyne cycloaddition
$CuSO_4$	copper(II) sulfate
Cy3	cyanine 3
DIPCDI	*N,N'*-diisopropylcarbodiimide
DIPEA	*N*,*N*-diisopropylethylamine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDT	1,2-ethanedithiol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immuno sorbent assay
ESI	electrospray ionization
Et	ethyl
Et_2O	diethyl ether
FACS	fluorescence-activated cell sorting
Fmoc	9-fluorenylmethyloxycarbonyl
Gn	guanidine
HATU	*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate
HBTU	*O*-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate

Hepes	2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
HEPPS	3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
ivDde	1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl
KO	knockout
LCMS	liquid chromatography-mass spectrometry
MBHA	4-methylbenzhydrylamine
MBom	4-methoxybenzyloxymethyl
Me	methyl
MESNa	sodium 2-mercaptoethanesulfonate
MPA	3-mercaptopropionic acid
MPAA	4-mercaptophenylacetic acid
Mts	2-mesitylenesulfonyl
NCL	native chemical ligation
NMP	*N*-methylpyrrolidone
ODN	oligodeoxynucleotide
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
Pd	palladium
PG	protecting group
PI	propidium iodide
RPMI	roswell park memorial institute medium
PRRs	pattern recognition receptors
R_{side}	side chain
RT	retention time
rt	reaction time
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEA	*N*-sulfanylethylaniline
SEAlide	*N*-sulfanylethylanilide
Sec	selenocysteine
Sez	selenazolidine
SPPS	solid-phase peptide synthesis
t-Bu	*tert*-butyl
TCEP	tris(2-carboxyethyl)phosphine
TES	triethylsilane
Tf	trifluoromethanesulfonyl

TFA	trifluoroacetic acid
THPTA	tris(3-hydroxypropyltriazolylmethyl)amine
Thz	thiazolidine
TIPS	triisopropylsilane
TLR9	toll-like receptor 9
TMS	trimethylsilyl
TNF	tumor necrosis factor
TOF	time of flight
Trt	triphenylmethyl
UV	ultraviolet
VA-044	2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride
WT	wild type
Z	benzyloxycarbonyl

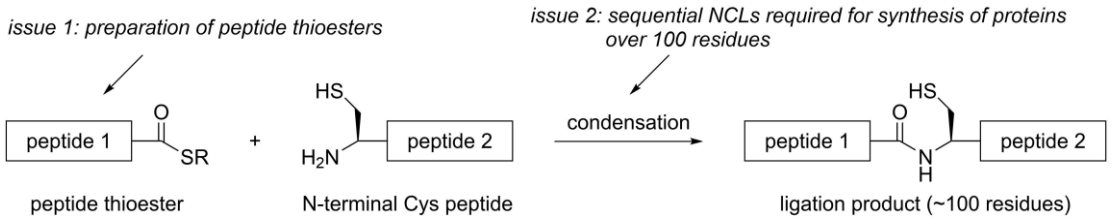
The commonly used one- and three-letter abbreviations for amino acids:

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

Contents

Preface

Proteins are indispensable for the control of biological and physiological phenomena in living organisms. Elucidation of their functions unambiguously contributes a huge impact on various life sciences fields including drug development, together with an understanding of the function of proteins relevant to the onset of diseases. Here, a broad spread of protein science technologies is required, in which technologies for preparing protein molecules have figured prominently. Preparation of proteins has been achieved by genetic or chemical protocols. Compared with the genetic protocols, synthetic methodologies allow for the facile incorporation of unnatural structural units into proteins. Artificial proteins made in this way have served as useful probe molecules which enable a precise analysis of protein function. Thus, synthetic methodologies have attracted much attention as the technology which can resolve unachievable issues by genetic protocols. This trend has been supported by the native chemical ligation (NCL) developed by Kent et al. in 1994. NCL is a powerful synthetic technique for preparing peptides/proteins through chemoselective condensation of a peptide thioester and an N-terminal cysteine (Cys) peptide. Synthesis of many proteins has been achieved by NCL with practical applications of synthetic materials to a wide range of research fields; however, there is much room for improvement in areas of NCL including: (1) preparation of peptide thioesters, and (2) use of sequential NCLs that are required for synthesis of proteins of over 100 residues (Scheme 1).



Scheme 1. Issues for chemical synthesis of proteins by NCL.

In this thesis, I attempted to develop practical synthetic procedures to address the aforementioned issues. One is a methodology for thioester preparation using a resin-bound *N*-sulfanylethylanilide (SEAlide) peptide; a second is a sequential NCL procedure using copper-mediated ring-opening of the thiazolidine derivative as an *N,S*-protected

cysteine unit in N-terminal cysteine peptides. I then applied the newly developed procedures to the syntheses of chemokine CXCL14s and several variant peptides. Then, on the basis of successful synthesis of CXCL14 derivatives, CXCL14 domains responsible for the activation of immune system were identified.

Development of the methodology for thioester preparation using resin-bound SEAlide peptide and the copper-mediated deprotective procedure for thiazolidine derivatives are described in Chapters 1 and 2, respectively. In addition, practical application of the developed procedures to the synthesis of CXCL14 is also described in these chapters.

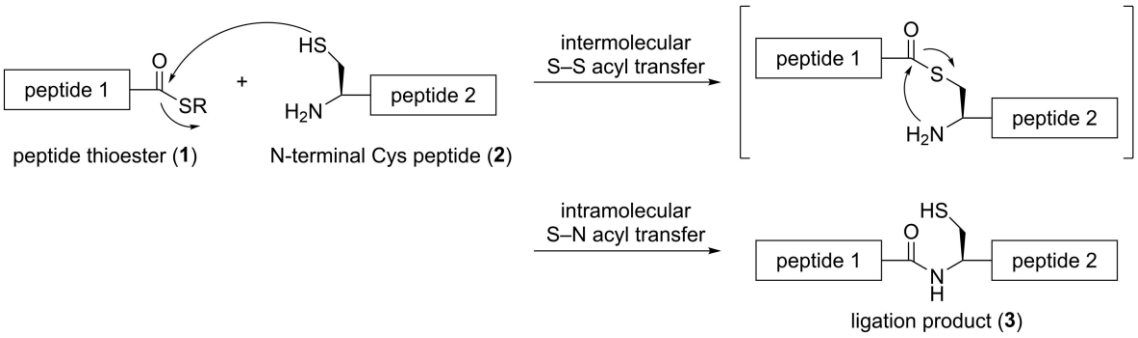
In Chapter 3 the elucidation of domains of CXCL14 responsible for the CpG DNA-mediated Toll-like receptor 9 (TLR9) activation is described.

Chapter 1

N–S-Acyl-transfer-based preparation of peptide thioesters using resin-bound *N*-sulfanylethylanilide peptide

1.1 Introduction

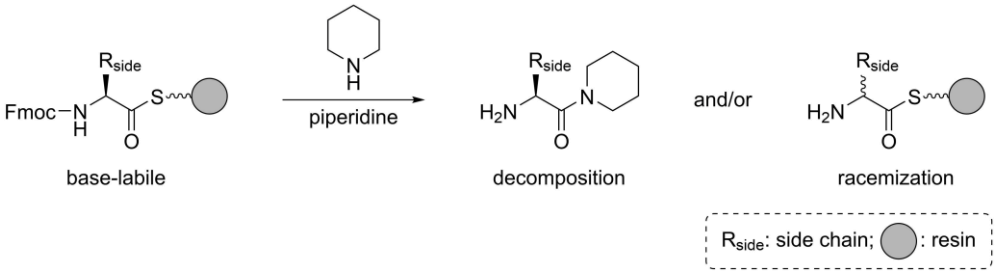
Native chemical ligation (NCL) is a powerful strategy for the preparation of proteins, including artificially modified proteins as a useful research tool with which to elucidate protein function.[1] In this protocol, a peptide thioester **1** as a key substrate chemoselectively reacts with an N-terminal cysteinyl (Cys) peptide **2** to afford a ligation product **3** via intermolecular S–S and subsequent intramolecular S–N acyl transfers (Scheme 1.1).[2]



Scheme 1.1. Reaction mechanism of NCL.

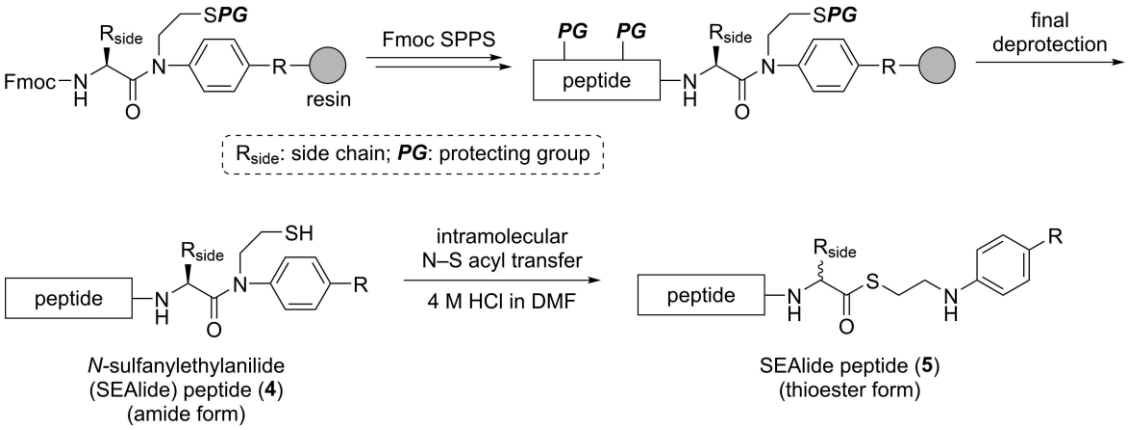
Generally, peptide thioesters can be synthesized by solid-phase peptide synthesis (SPPS) protocols. Such protocols are either *tert*-butoxycarbonyl (Boc)[3]- or 9-fluorenylmethyloxycarbonyl (Fmoc)[4]-based, and use different N^{α}-amino protections. Repeated trifluoroacetic acid (TFA) treatments for removal of the Boc group as a temporal N^{α}-protection followed by neutralization of the resulting TFA salt make the conventional Boc-based SPPS a laborious synthetic protocol.[3b] In addition, the final deprotection step for removal of side chain protecting groups along with release of peptides from the resin under harsh acidic conditions is unsuitable for the synthesis of a wide variety of post-translationally modified peptides including phosphorylated- and glycosylated-peptides.[5] From this viewpoint, operationally simple Fmoc protocols which do not require the use of harsh acidic reagents have gained popularity in the chemical synthesis of peptides. This

was not the case however for chemical synthesis of peptide thioesters because the piperidine treatment required in the Fmoc removal leads to decomposition of the thioester linkage and the racemization of the thioester-linked C-terminal amino acids. Consequently, this most straightforward strategy associated with the use of thioester-linked resins cannot be utilized for preparation of peptide thioesters by the Fmoc method (Scheme 1.2).[4]



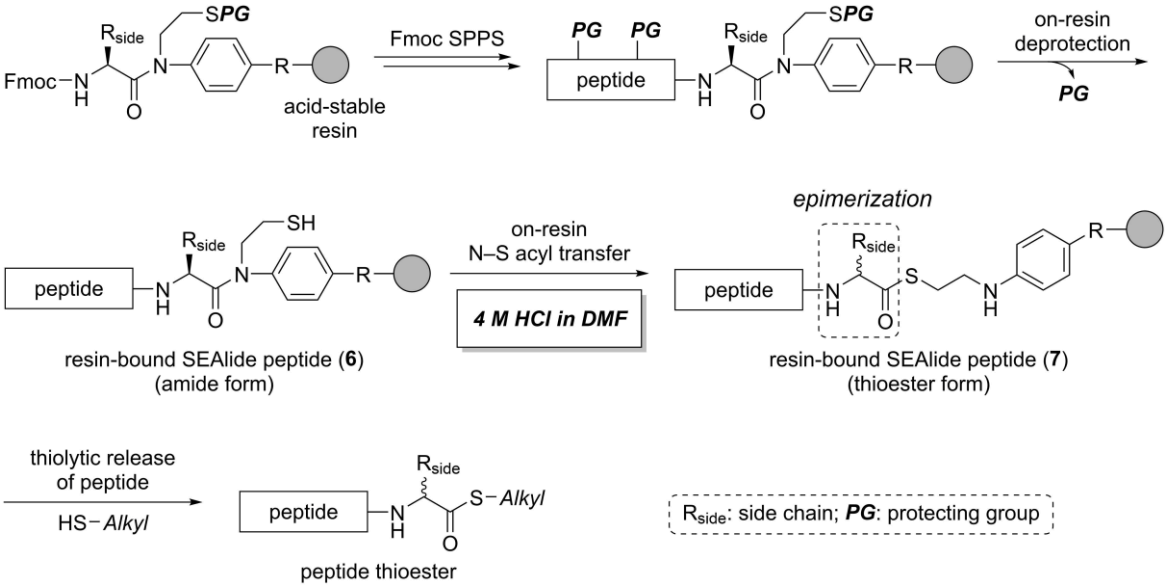
Scheme 1.2. Problems in synthesis of peptide thioesters by Fmoc SPPS.

In this context, Fmoc-chemistry-compatible thioester equivalents have been developed as alternatives to the thioester scaffold.[6, 7] Among these, various thioester equivalents, featured by peptide chain elongation on the amide linkage between the peptide and linkers and subsequent N–S-acyl-transfer-mediated conversion of the amide to thioester, have been reported.[8, 9] My laboratory has demonstrated that the *N*-sulfanylethylanilide (SEAlide) peptide **4**, which is easily obtainable using the Fmoc method, can be converted to the corresponding thioester **5** by treatment with acids such as 4 M HCl in DMF by intramolecular N–S acyl transfer (Scheme 1.3).[10]



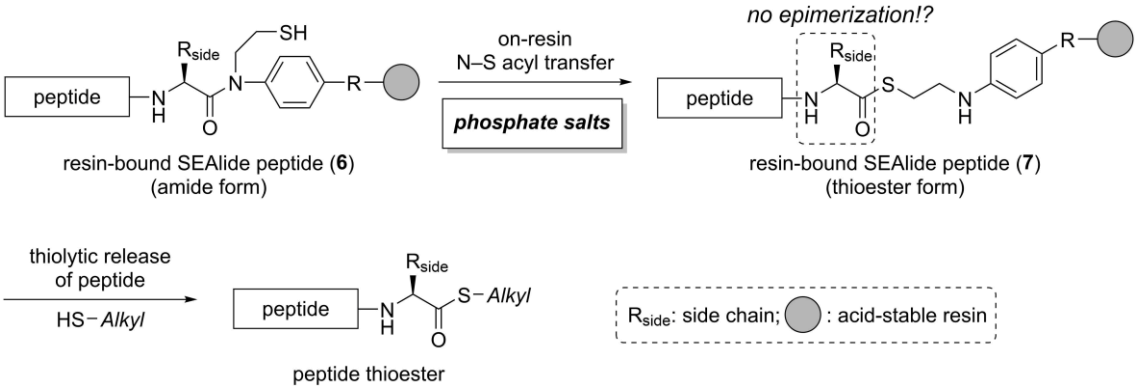
Scheme 1.3. Synthesis of peptide thioesters using SEAlide peptides.

This early finding was applied to the thiolytic release of a thioester peptide from the acid-stable resin-bound SEAlide peptide with a large excess quantify of alkyl-thiols under aqueous neutral buffer conditions. However, epimerization of C-terminal amino acids accompanied the acidic treatment involved in this protocol (Scheme 1.4).



Scheme 1.4. Synthesis of peptide thioesters under acidic conditions using a resin-bound SEAlide peptide.

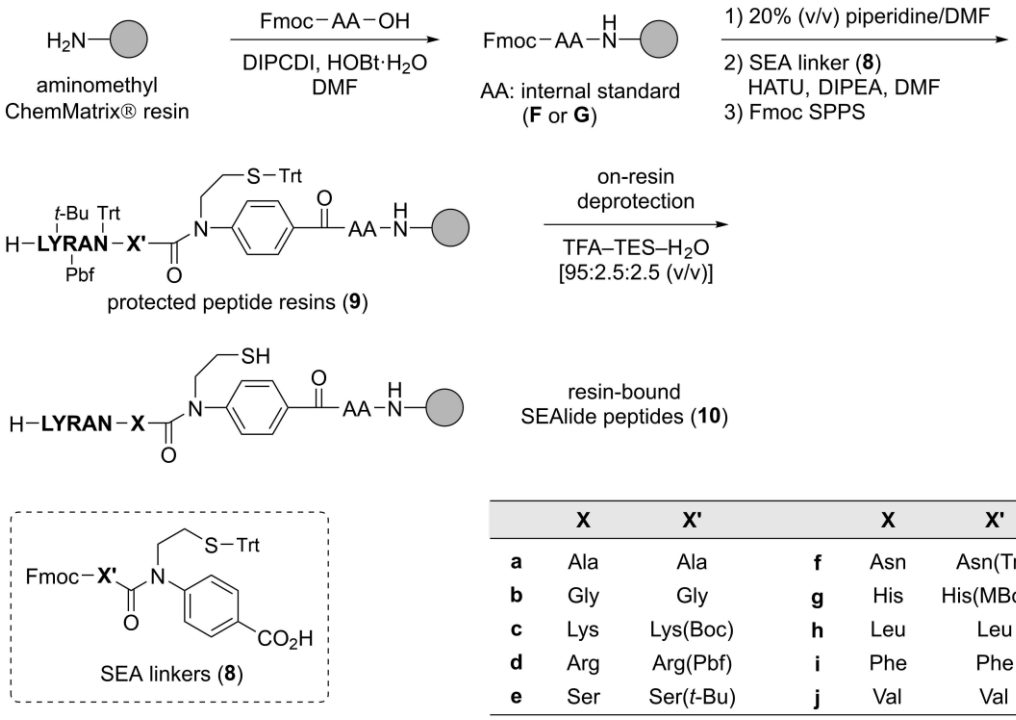
Following earlier studies on the SEAlide peptide, Sato et al. found that the SEAlide peptide functions as a thioester in the presence of phosphate salts, and participates in an NCL reaction.[11] On the basis of the unique character of the SEAlide peptide, my group established a one-pot/three-fragment ligation method and applied it successfully to the chemical syntheses of a 162-residue GM2 activator protein[12] and the chemokine CXCL14.[13] In this protocol, the epimerization was hardly confirmed during the N–S acyl transfer under neutral conditions. Accordingly, I reexamined the epimerization-free N–S-acyl-transfer-mediated conversion of the resin-bound SEAlide peptide to the corresponding peptide thioesters (Scheme 1.5).



Scheme 1.5. Synthetic strategy for synthesis of peptide thioesters using resin-bound SEAlide peptides under neutral conditions.

1.2 Optimization of reaction conditions for the preparation of peptide thioesters using model resin-bound SEAlide peptides

Resin-bound 6-residue model SEAlide peptides **10** were prepared on an aqueous buffer-compatible aminomethyl ChemMatrix® resin using standard Fmoc SPPS.[14] Internal standard amino acid (Phe or Gly) and Fmoc-aminoacyl-*N*-sulfanylethylaniline (SEA) linkers **8**[15] were successively coupled with the ChemMatrix® resin with the aid of *N,N'*-diisopropylcarbodiimide (DIPCDI)–1-hydroxybenzotriazole (HOBt) and *O*-(7-azabenzotriazole-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluoro-phosphate (HATU)–*N,N*-diisopropylethylamine (DIPEA) in DMF. Chain elongation of the resulting resin was performed using standard Fmoc protocols to give protected peptide resins **9**. Each protected resin formed in this way was treated with TFA–triethylsilane (TES)–H_2O [95:2.5:2.5 (v/v)] at room temperature for 2 h to give the corresponding SEAlide peptide-linked resins **10** (Scheme 1.6).

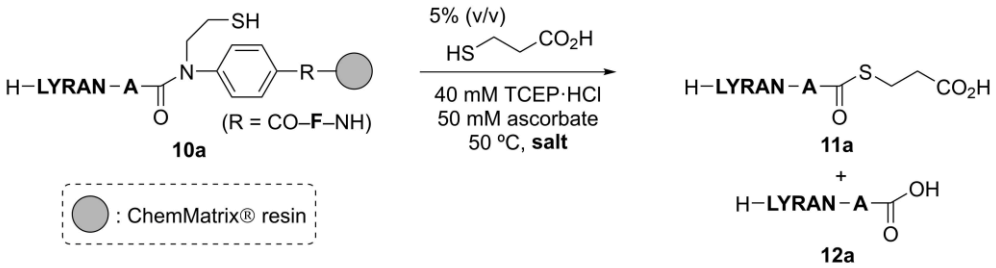


	X	X'		X	X'
a	Ala	Ala	**f**	Asn	Asn(Trt)
b	Gly	Gly	**g**	His	His(MBom)
c	Lys	Lys(Boc)	**h**	Leu	Leu
d	Arg	Arg(Pbf)	**i**	Phe	Phe
e	Ser	Ser(*t*-Bu)	**j**	Val	Val

Scheme 1.6. Synthesis of resin-bound SEAlide peptides.

Initially, the resin-bound model SEAlide peptide (H-**LYRAN**-**A**-SEAlide-resin, **10a**) was suspended in 0.2 M phosphate buffer in the presence of 5% (v/v) 3-mercaptopropionic acid (MPA),[9d] 40 mM tris(2-carboxyethyl)phosphine (TCEP) and 50 mM ascorbate[16] at 50 °C for 12 h under several different pH conditions. Suspension of **10a** allowed for the thiolytic release and gave the desired MPA-thioester peptide **11a** in 45−55% isolated yields (Table 1.1, entries 1–3). The efficacy of the release does not depend on the reaction pH but the ratio of hydrolyzed product **12a** to desired thioester **11a** decreased in response to the decrease in the pH of the reaction. From these results, the optimal pH for thiolytic release was set at 4.0 (Table 1.1, entries 1–3). The addition of guanidine (Gn)·HCl, a widely used denaturant, had no effect on the reaction (Table 1.1, entries 3 vs 4). On the other hand, the reaction efficiency depends on the reaction temperature (Table 1.1, entries 3 vs 5). As mentioned above, a SEAlide peptide in phosphate buffer solution functions only as a thioester in the NCL protocol and the reaction efficiency increases with the concentration of phosphate;[11] however, the efficacy of the thiolytic release of **11a** from the resin was not unequivocally dependent on the phosphate concentration (Table 1.1, entries 3 vs 6 and 7). Furthermore, no matter whether phosphate or 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid (HEPPS) was used, no significant difference was observed (Table 1.1, entries 3 vs 8).

Table 1.1. Examination of conditions for thiolytic release.



entry	salt (M, pH)	content of amine[a] (mmol/g)	ratio[b] (**11a**:**12a**)	yield[c] (%)	yield[d] (%)
1	phosphate (0.2, 6)	0.43	89:11	45	60
2	phosphate (0.2, 5)	0.43	90:10	55	76
3	phosphate (0.2, 4)	0.43	93:7	53	66
4[e]	phosphate (0.2, 4)	0.43	95:5	49	–
5[f]	phosphate (0.2, 4)	0.43	100:0	3.5	7.9
6	phosphate (0.5, 4)	0.43	92:8	47	–
7	phosphate (1.0, 4)	0.43	92:8	46	–
8	HEPPS (0.2, 4)	0.43	92:8	51	67
9	HEPPS (0.2, 4)	0.64	93:7	55	–
10	HEPPS (0.2, 4)	0.27	93:7	31	–

[a]Determined by quantitative spectrophotometric monitoring following piperidine deprotection of the internal standard-incorporated resin. [b]Peak ratio on HPLC analysis. [c]Isolated yield. [d]Determined by amino acid analysis. [e]In the presence of 3 M Gn·HCl. [f]25 °C.

I hypothesized that this unexpected experimental result, which the thioester peptide is efficiently released from the resin even in the absence of phosphate salts could be attributed to the highly concentrated nature of resin-bound peptides (Figure 1.1). Compared to the peptide on the resin, a peptide in solution exists under diluted conditions and consequently, a SEAlide peptide in solution participates in a unimolecular reaction which reversibly converts it from the amide to the thioester form. Furthermore, the amide form exists preferentially in equilibrium between amide and thioester forms. Since resin-bound peptides are likely to exist at higher concentrations, the partially formed thioester can interact with the amide form in a bimolecular reaction to generate the resin-bound *N,S*-dipeptidyl SEAlide peptide **14**. Finally, thiolytic release occurs upon addition of excess alkyl thiol (MPA) to the dipeptidyl resin **14** to afford the desired thioesters in preference to the inter-chain reverse reaction (**14** to **13**) affording the peptide resin **13**.

Based on above hypothesis, I considered that the "concentration effect" was important in the thioesterification reaction of the resin-bound SEAlide peptide and I confirmed the influence of the peptide resin content on the reaction in HEPPS buffer. As the result, the yield of the desired thioester decreased as the content decreased (Table 1.1, entries 8–10). The importance of the proposed "concentration effect" was partly confirmed from these experimental results.

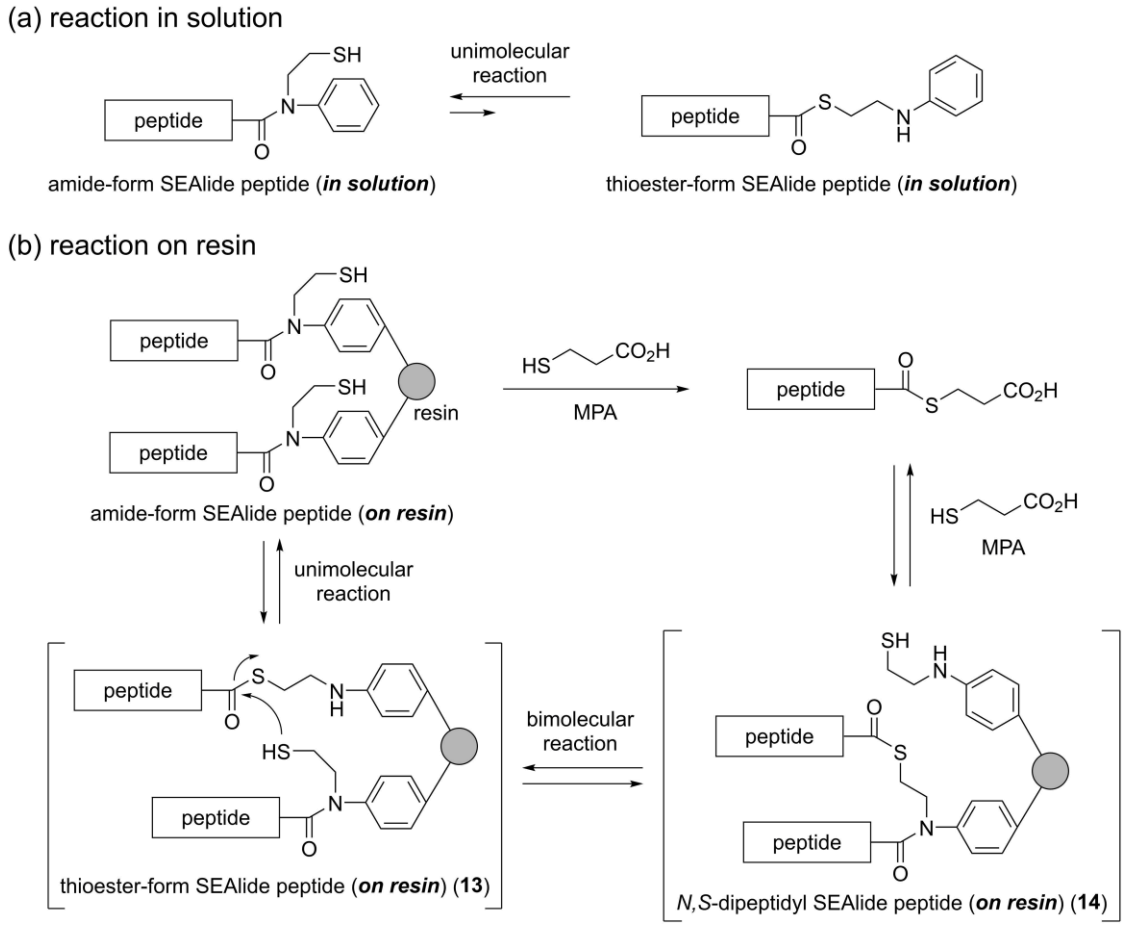
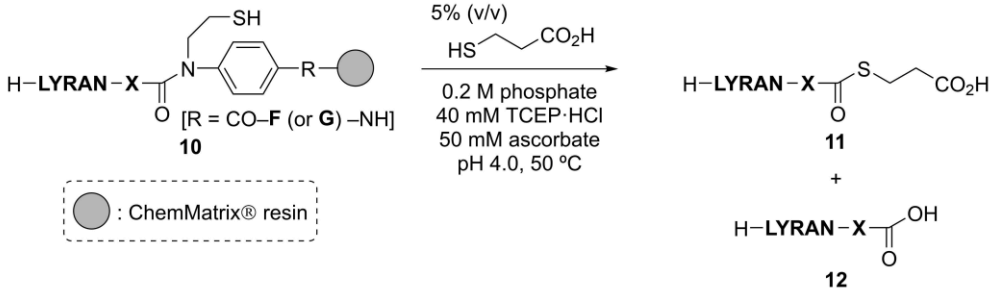


Figure 1.1. Putative mechanism for N–S acyl transfer of a SEAlide peptide in solution or on resin.

I subsequently examined the applicability of the thiolytic release protocol to peptides possessing different C-terminal amino acids, such as H-**LYRAN**-**X**-SEAlide **10** (Table 1.2). With the exception of asparagine and histidine, resin-bound SEAlide peptides were converted to the corresponding thioester peptides in reasonable isolated yields. The low yield of asparagine peptide, such as H-**LYRAN**-**N**-SEAlide **10f** can probably be attributed to the facile imide cyclization of released asparagine thioester.[17] In the case of histidine peptide H-**LYRAN**-**H**-SEAlide **10g**, the ratio of epimerization of the released histidine thioester increased over time, and the reaction was terminated before completion (1 h).[17] The epimerization of the highly racemizable C-terminal Ser remained below 5% under optimized conditions, and I concluded that the optimized methodology for preparation of thioester using the resin-bound SEAlide peptide is an effective method for the preparation of a wide range of peptide thioesters.

Table 1.2. Thiolytic release of various thioester from SEAlide resins.



entry	X	rt[a] (h)	ratio[b] (**11**:**12**)	yield[c] (%)	epimerization (%)
1	Ala (**10a**)	12	93:7	53	1.5
2	Gly (**10b**)	6	91:9	57	–
3	Lys (**10c**)	12	96:4	58	–
4	Arg (**10d**)	12	95:5	63	–
5	Ser (**10e**)	12	92:8	72	4.8
6	Asn (**10f**)	12	89:11	33	–
7	His (**10g**)	1	91:9	33	3.0
8	Leu (**10h**)	24	96:4	43	–
9	Phe (**10i**)	24	89:11	52	–
10	Val (**10j**)	72	96:4	43	–

[a]rt = reaction time. [b]Peak ratio on HPLC analysis. [c]Isolated yield.

1.3 Chemical synthesis of CXCL14 via N–to–C-directed sequential ligation

The synthetic utility of my method was examined through the synthesis of human CXCL14 protein. This consists of 77 amino acid residues including four cysteine residues that form two disulfide bonds (Figure 1.2a). The one-pot/N–to–C-directed sequential ligation strategy for the synthesis of CXCL14 is shown in Figure 1.2b. That is, the synthesis of CXCL14 was performed by condensin/g three peptide fragments including N-terminal fragment prepared by my method using SEAlide-based one-pot/N–to–C-directed sequential NCL, and then folding the condensed peptide.

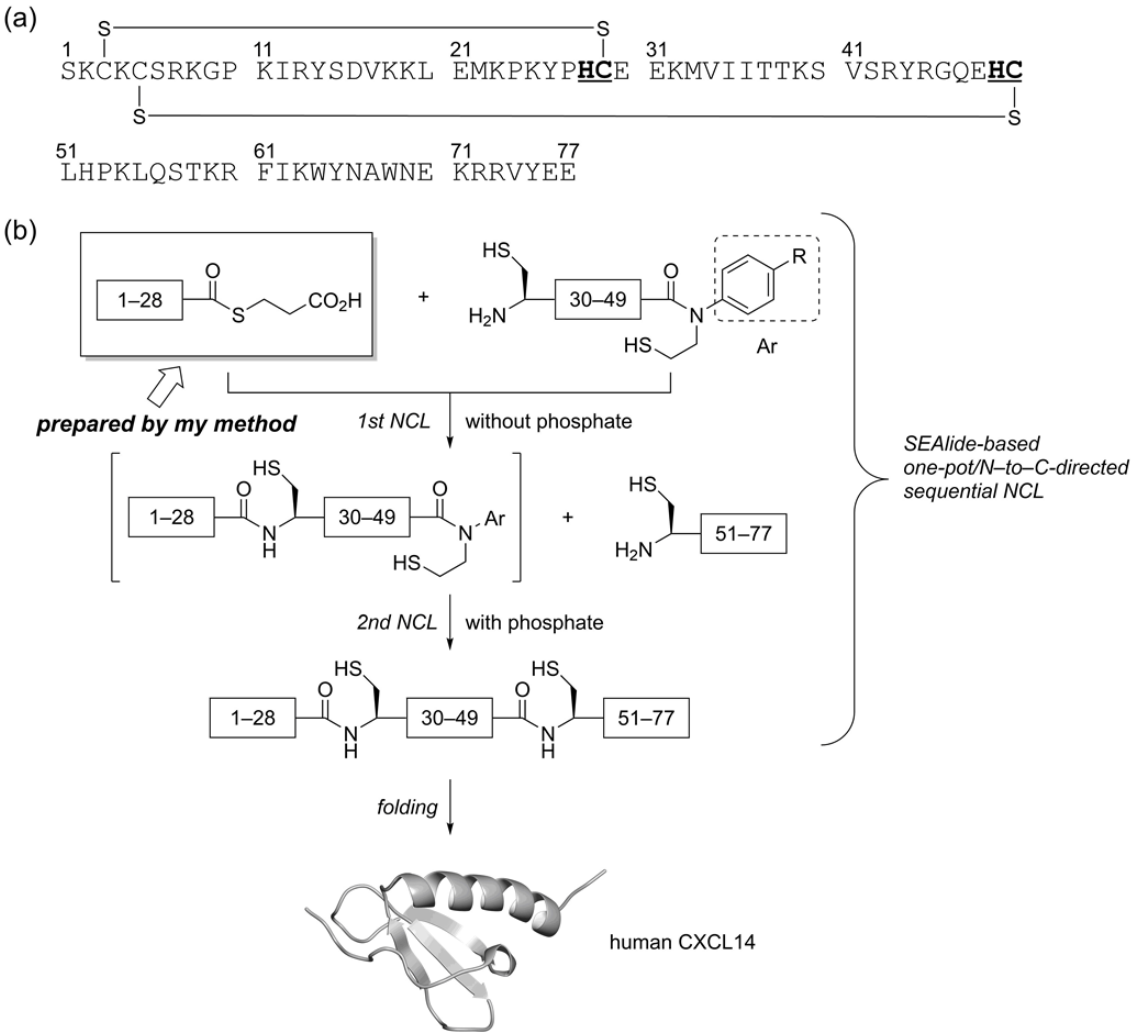


Figure 1.2. (a) Primary sequence of human CXCL14. (b) Synthetic pathway for synthesis of human CXCL14 using one-pot/N–to–C-directed sequential NCL.

Standard Fmoc SPPS protocols on Fmoc-His(MBom)-OH[18] incorporated-SEA linker ChemMatrix® resin, followed by on-resin deprotection with TFA–*m*-cresol–1,2-ethanedithiol (EDT)–thioanisole–H_2O [80:5:5:5:5 (v/v)], gave the resin-bound SEAlide peptide **15**. The resulting resin **15** was then treated with 0.2 M phosphate buffer (pH 4.0) in the presence of 5% (v/v) MPA, 40 mM TCEP and 50 mM ascorbate at 50 ºC for 1 h to give the requisite C-terminal His thioester peptide as the CXCL14 N-terminal fragment **16** in 17% isolated yield (Figure 1.3). The middle fragment **17** and C-terminal fragment **19**, were also prepared by Fmoc strategy according to previously reported protocols.[13] Having the three fragments necessary for NCL-mediated assembly, I conducted the one-pot/N–to–C-directed sequential NCL reactions for the synthesis of human CXCL14. The first NCL of **16** with **17** in 6 M Gn·HCl–0.2 M HEPPS buffer (pH 6.7) in the presence of 50 mM 4-mercaptophenylacetic acid (MPAA)[19] and 30 mM TCEP at 37 ºC for 3 h afforded the ligated amide-type SEAlide peptide **18** (Figure 1.4a and 1.4b). Then, the addition of **19** in 1 M phosphate buffer (pH 6.6) into the reaction mixture initiated the second NCL of the thioester form of **18** with the cysteine fragment **19** to give fully ligated peptide **20** in 31% isolated yield in a one-pot reaction (Figure 1.4c). Air oxidation of the resulting cysteine peptide **20** was conducted in 3 M Gn·HCl–0.1 M phosphate buffer (pH 7.7) to give human CXCL14 in 50% isolated yield (Figure 1.4d).

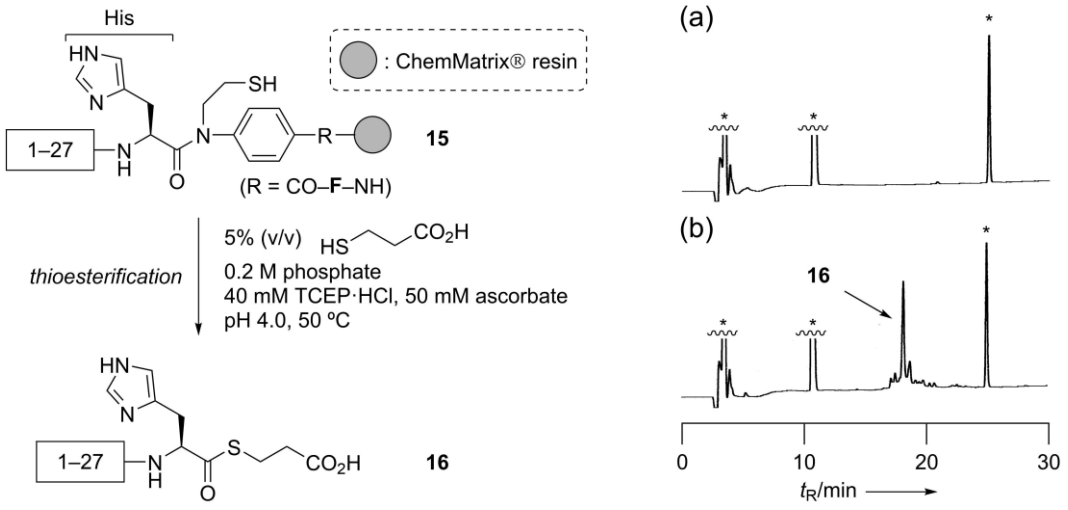


Figure 1.3. HPLC monitoring of the thiolytic release of CXCL14 N-terminal fragment **16** from resin-bound SEAlide peptide **15**: (a) thioesterification (t < 5 min); (b) thioesterification (t = 1 h). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 45% over 30 min. *Non-peptidic compounds derived from additives.

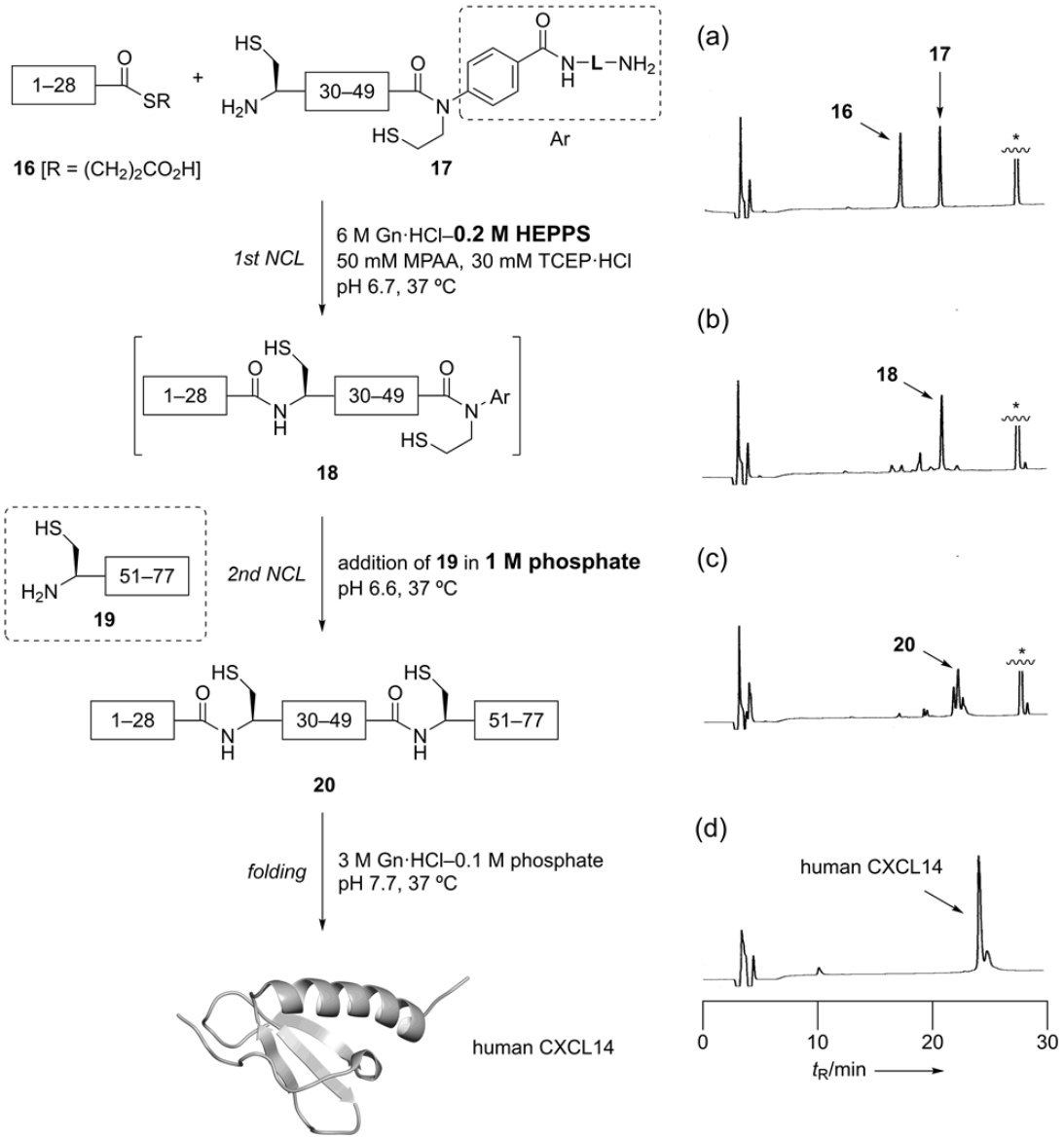


Figure 1.4. HPLC monitoring of the synthesis of CXCL14 via N–to–C-directed sequential NCL: (a) first NCL (t < 5 min); (b) first NCL (t = 3 h); (c) second NCL (t = 24 h); (d) oxidation with air (t = 24 h). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 45% over 30 min. *MPAA.

1.4 Application of the different chemical behavior of resin-bound and soluble SEAlide peptides to one-pot/three-fragment ligation

As described previously, the one-pot/N–to–C-directed sequential three-fragment ligation method which is based on the reactivity of the SEAlide peptide which is controllable by phosphate salts, serves as a widely applicable technique for protein synthesis; however, precise control of stoichiometry of employed fragments is required for efficient production and purification of the resulting ligated product. To prevent the synthetic inconvenience associated with the use of soluble SEAlide peptides, I envisioned that application of both resin-bound and soluble SEAlide peptides to the N–to–C-directed sequential three-fragment ligation should allow for an operationally simpler protocol than the conventional SEAlide-mediated protocol. Here, I noted that the resin-bound SEAlide peptide, but not the soluble SEAlide peptide functions as a thioester in the absence of phosphate salts.

Initially, the resin-bound SEAlide peptide **10a** was subjected to the first NCL step with the N-terminal cysteinyl SEAlide peptide **21** in 6 M Gn·HCl–0.1 M HEPPS buffer (pH 6.9) in the presence of 40 mM MPAA and 40 mM TCEP at 37 ºC for 12 h (Figure 1.5a and 1.5b). Under these reaction conditions, only the resin-bound SEAlide peptide worked as thioester and afforded the ligated SEAlide peptide **22**. Completion of the first NCL required at least five equivalents of **10a**, but only the ligated product **22** was detected as peptide material on HPLC analysis of the reaction. Because excessive released highly reactive aryl thioester should react with the remaining SEA unit on the resin to regenerate the resin-bound SEAlide, filtration gave a solution containing ligated product **22** with no thioester contaminant. Next, addition of the N-terminal cysteinyl peptide **23** in 0.5 M phosphate buffer (pH 6.6) in the presence of 40 mM TCEP to the filtrate allowed the soluble amide-type SEAlide unit **22** to participate in the second NCL step, thereby yielding the fully ligated peptide **24** in 40% isolated yield in a one-pot manner (Figure 1.5c).

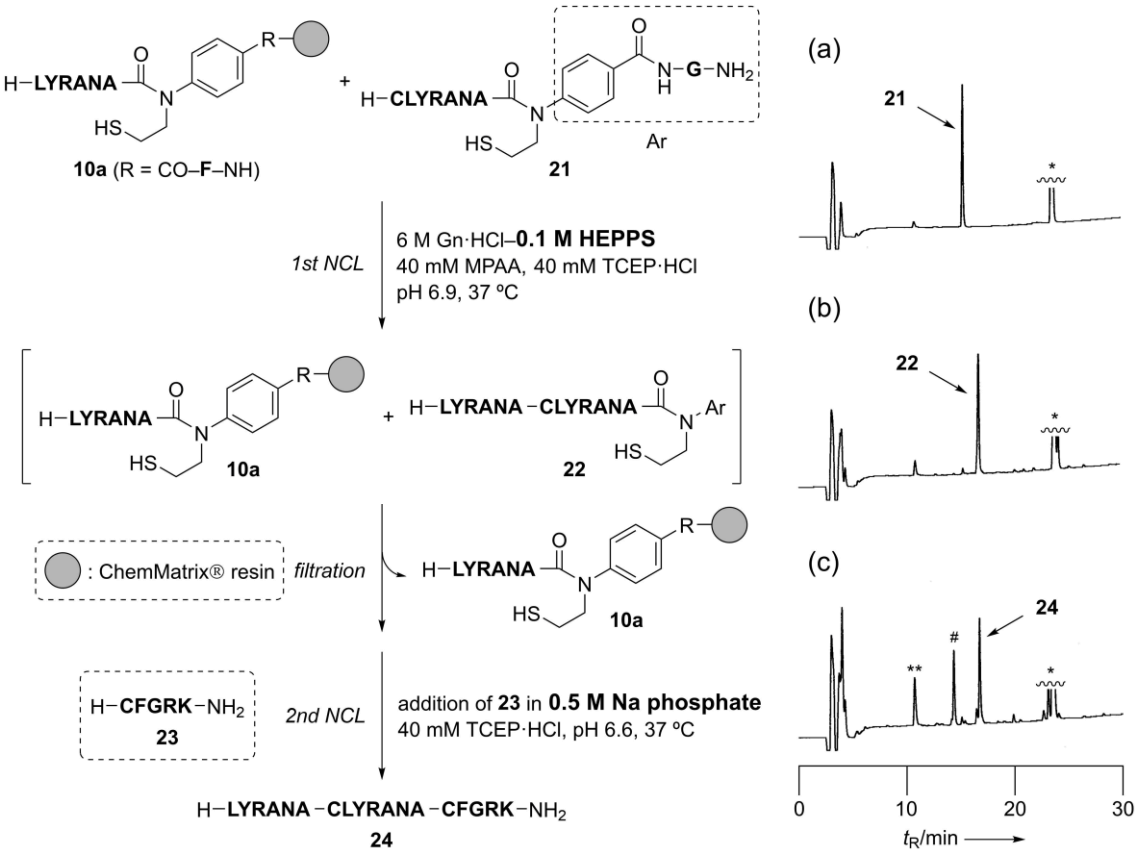


Figure 1.5. HPLC monitoring of the one-pot/three-fragment ligation using resin-bound and soluble SEAlide peptides: (a) first NCL (t < 5 min); (b) first NCL (t = 12 h); (c) second NCL (t = 24 h). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 45% over 30 min. *MPAA, **non-peptidic compounds derived from additives, #released SEAlide unit.

1.5 Conclusion

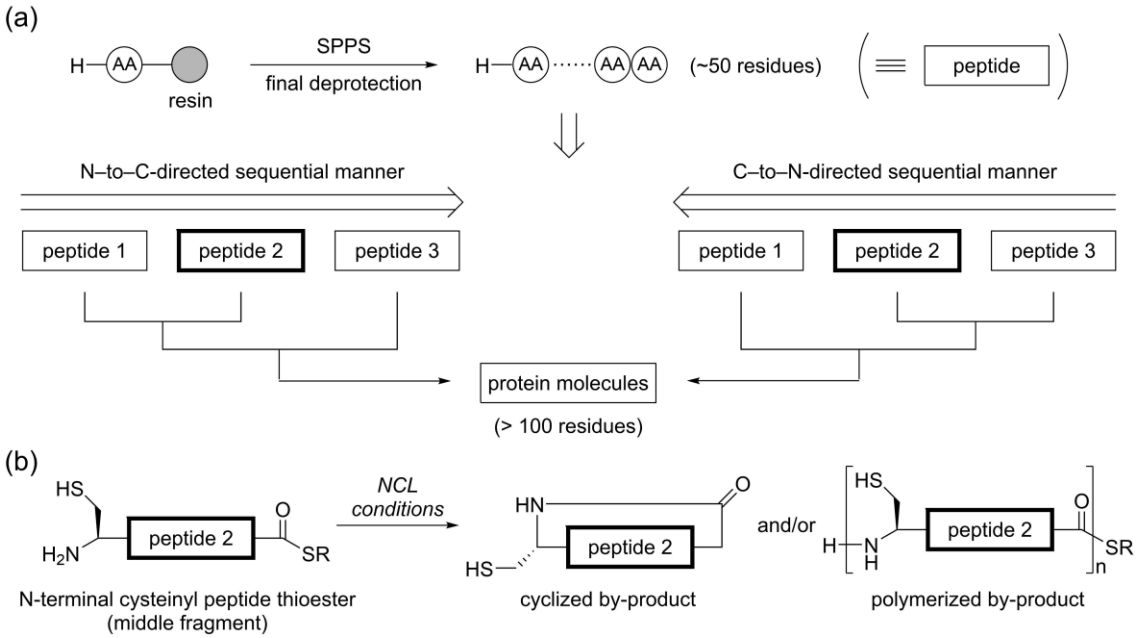
I have succeeded in establishing the epimerization-less-prompting methodology for the synthesis of peptide thioesters using resin-bound SEAlide peptides. This method can be applied to the synthesis of a 28-residue CXCL14 N-terminal fragment and its use in the synthesis of CXCL14 in the one-pot/N−to−C-directed sequential manner. Furthermore, I developed a new version of the one-pot/three-fragment ligation method with attention to the experimental observation that in the absence of phosphate salts, the resin-bound and soluble SEAlide peptides as crypto-thioesters, behave differently.

Chapter 2

Copper-mediated ring-opening reactions of thiazolidine and selenazolidine derivatives for sequential ligation

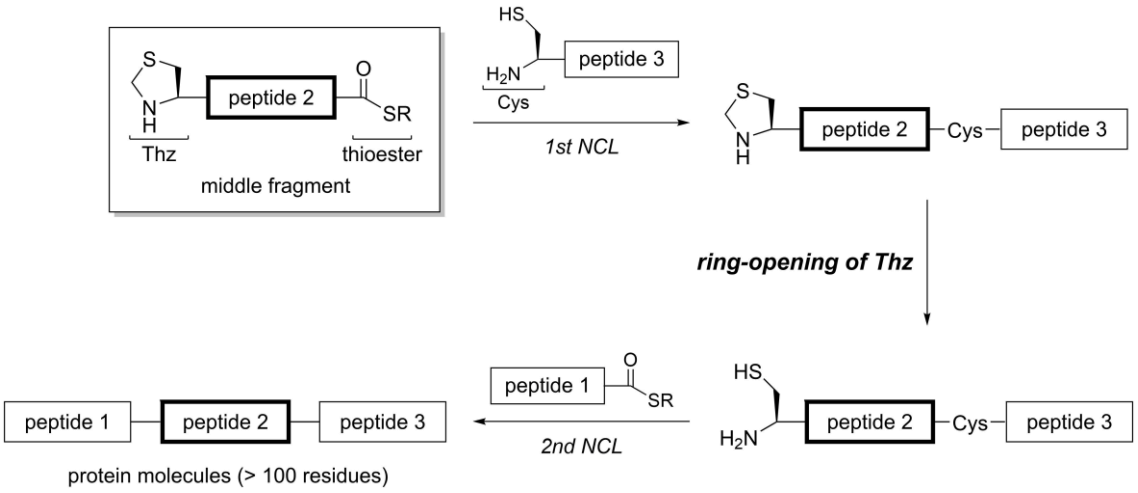
2.1 Introduction

As mentioned in the previous chapter, NCL can be used to chemoselectively connect thioester peptides and N-terminal cysteinyl peptides.[2] However, the accessible range of protein size that can be directly synthesized by one NCL coupling is limited to up to about 100 residues because the chain length of peptide that can be directly prepared by SPPS is approximately 50 residues. Consequently, sequential NCL protocols using more than two fragments have been developed for the synthesis of proteins.[20] No matter whether the N–to–C- or C–to–N-directed sequential NCL protocol is employed, the middle fragments require cysteine and thioester units at their N- and C-termini respectively, which can be converted to polymerized and/or cyclized products during NCL protocol without masking the reactivity of the N- or C-terminus (Scheme 2.1).



Scheme 2.1. (a) N–to–C- or C–to–N-directed sequential NCL for synthesis of proteins. (b) Side reaction of intra- and/or intermolecular NCL in a middle fragment.

Among N-terminus-reactivity-masking units used in the C–to–N protocol, a thiazolidine (Thz) derivative, as a protected N-terminal cysteine residue has gained popularity in this protocol (Scheme 2.2).[21, 22] The thiazolidine ring can be opened by treatment for several hours (> 8 h) with excess methoxyamine under slightly acidic conditions and the resulting cysteine units can be brought to successive NCL reactions.[21] Recently, Brik et al. reported the ring-opening reaction of thiazolidine under NCL conditions using water-soluble palladium(II) complexes such as $[Pd(allyl)Cl]_2$ or $PdCl_2$.[23] In this protocol, the reaction proceeds faster than the methoxyamine reaction but excessive amounts of expensive palladium reagents are necessary. Therefore, development of novel protocols for the ring-opening reaction of thiazolidine is wanted.

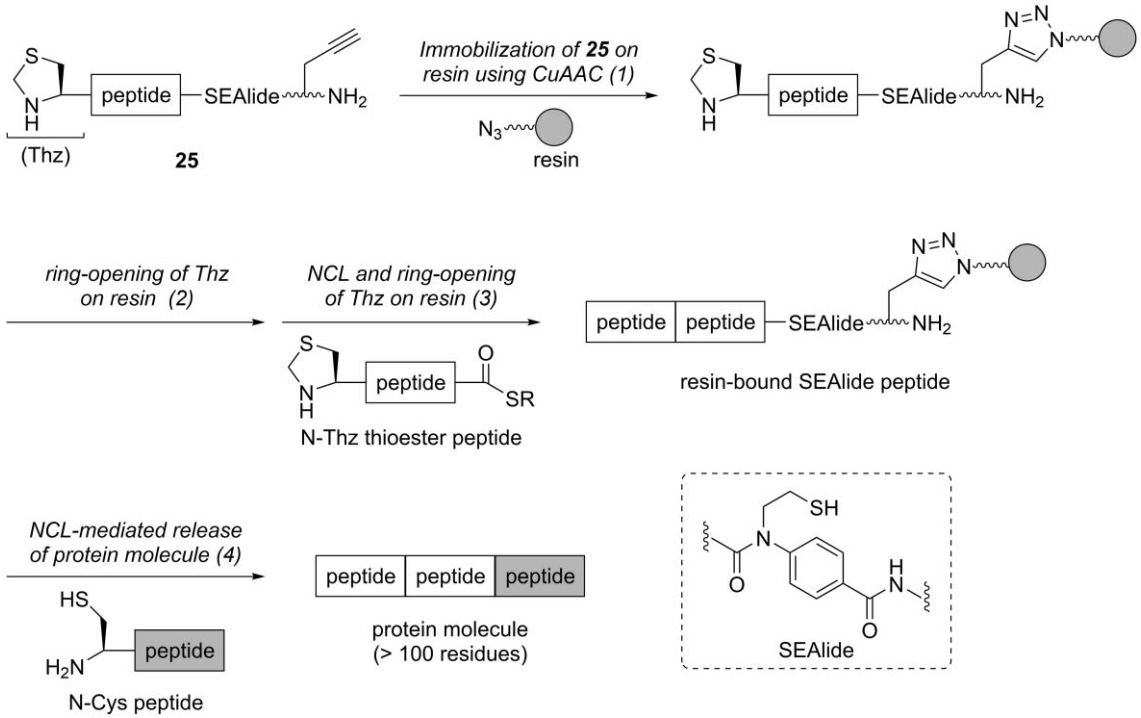


Scheme 2.2. C–to–N-Directed sequential NCL using N-terminal thiazolidine in a middle fragment.

2.2 The history of development of copper-mediated deprotective procedures for thiazolidine derivatives

Because resin-bound SEAlide peptides function as thioesters, as mentioned in Chapter 1, I attempted the application of such a resin-bound crypto-thioester to the synthesis of proteins using SEAlide-based solid-phase chemical ligation consisting four steps (Scheme 2.3): 1) immobilization of alkyne-containing peptide **25** on azide-loading resin using a copper-catalyzed azide–alkyne cycloaddition (CuAAC) click reaction, 2) ring-opening of thiazolidine on resin, 3) NCL and then ring-opening of thiazolidine on resin, 4) NCL-mediated release of protein molecule from the resin. Initially, I tried to immobilize the model N-terminal thiazolidine SEAlide peptide **26** on the azide-loading resin **27** using a CuAAC click reaction. However, I unexpectedly found that the thiazolidine was efficiently deprotected under these reaction conditions (Figure 2.1).

This phenomenon has already been reported by Kent et al.,[24] but further investigation of the copper-mediated ring-opening of the thiazolidine in peptide synthesis including NCL chemistry was necessary and I examined the copper-mediated opening of the thiazolidine ring in peptide synthesis.



Scheme 2.3. SEAlide-based solid-phase chemical ligation for synthesis of proteins.

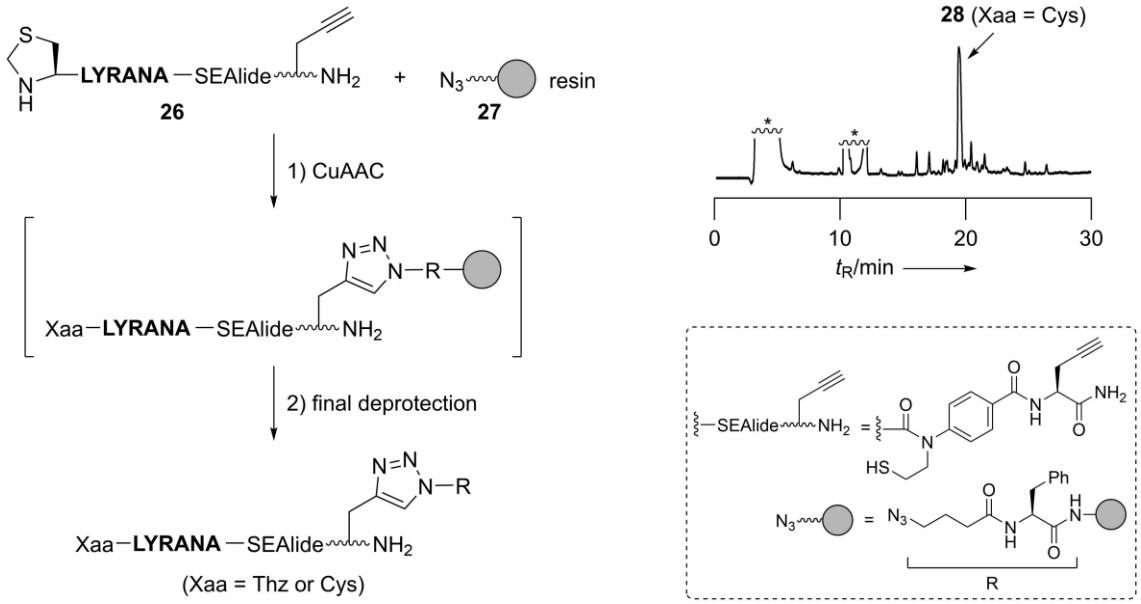


Figure 2.1. Initial examination for SEAlide-based solid-phase chemical ligation. CuAAC: 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.0) in the presence of 20 mM tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), 20 mM $CuSO_4$ and 30 mM ascorbate at 37 ºC for 6 h. Final deprotection: TFA–H_2O [95:5 (v/v)], at room temperature for 1 h. For HPLC analysis, the crude material was treated with 6 M Gn·HCl in the presence of 80 mM TCEP·HCl and 100 mM ascorbate at 37 ºC for 1 h. Analytical HPLC conditions: linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 45% over 30 min. *Non-peptidic compounds derived from additives.

2.3 Optimization of reaction conditions for ring-opening of thiazolidine derivatives using copper reagents

As mentioned above, the thiazolidine is commonly used in a middle fragment and its deprotection is generally performed on NCL-product peptides containing the free-cysteine residues. I evaluated the effect of buffers and additives on deprotection of thiazolidine with $CuSO_4$ under aerobic condition using a cysteine-containing thiazolidine peptide, **29** (Thz-**GALYR**-Cys-**FG**-NH_2). In order to investigate suitable conditions for the ring-opening reaction, the influence of peptide–copper complex formation was excluded by adding excess $CuSO_4$ (40 mM) to 1 mM peptide. After completion of the reaction, quenching with dithiothreitol (DTT) or ethylenediaminetetraacetic acid (EDTA) was performed to remove excess copper salts.

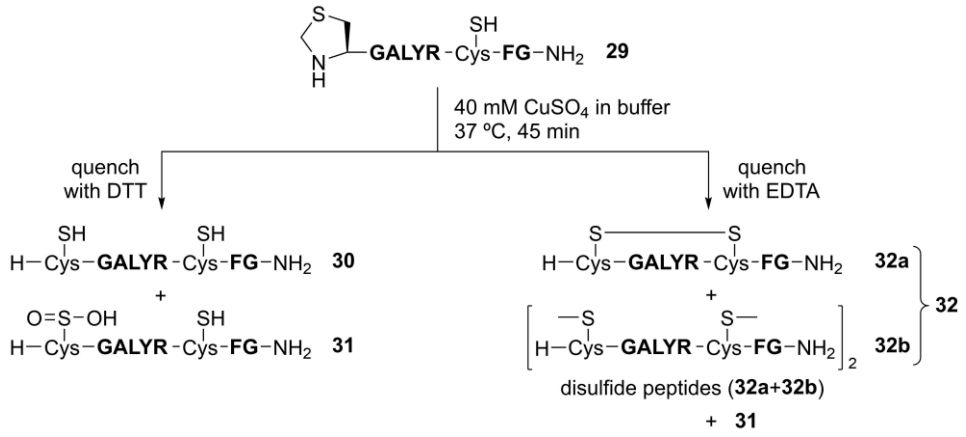
Initially, the thiazolidine peptide **29** was dissolved in 6 M Gn·HCl–0.1 M phosphate or HEPPS buffer (pH 7.0) in the presence of 40 mM $CuSO_4$ and 40 mM ascorbate (Table 2.1, entries 1 and 2, and Figure 2.2). In the case of phosphate buffer, a precipitate appeared in the blue reaction solution. The conversion yield varied between 56 and 95%. When the buffer was replaced by HEPPS, the reaction solution became clear and pale blue and **30** was obtained in 95% yield. No epimerization of the N-terminal cysteine residue was observed.

Entries 3 and 4 report the examination of whether guanidine and/or ascorbate are necessary for the deprotection. In the absence of guanidine (Table 2.1, entry 3) the reaction generated insoluble materials in a turbid yellow suspension, and peptide-derived peaks could not be detected in the filtrate after quenching with DTT. On the other hand, the presence or absence of ascorbate failed to affect the completion of thiazolidine deprotection although compounds with an additional mass of 32 corresponding to the sulfinic acid derivative **31** were observed in the absence of ascorbate (Table 2.1, entries 2 vs 4, and Figure 2.2). The reason for such reaction outcomes seems to be the difference in the copper species involved in the reactions where its presence and absence allow Cu(I) and Cu(II) species to participate in the ring opening, respectively.

The quenching with DTT not only removed copper salts but also reduced S–S bonds, and the actual experimental results could not be detected by HPLC analysis. Therefore, in order to analyze the results of the actual reaction, HPLC analysis was performed immediately after quenching with EDTA, which has no reducing ability (Table 2.1, entries 5 and 6, and Figure 2.2). As the result, a mixture of intramolecular **32a** and intermolecular **32b** bridging peptides was detected by HPLC analysis in the presence or absence of ascorbate.

In the light of these results, I propose a mechanism for copper-mediated ring-opening of thiazolidine as shown Figure 2.3. Coordination of the sulfur atom in the thiazolidine ring to Cu ions, as a Lewis acid, results in the opening of the thiazolidine ring and the release of formaldehyde. Disulfide bond formation observed in the presence of Cu(I) or Cu(II) promotes the deprotection by inhibiting the reverse reaction which would regenerate the thiazolidine ring.

Table 2.1. Deprotection of cysteine-containing thiazolidine peptide **29** with copper salts.



entry[a]	buffer	ascorbate (mM)	quench	product conversion[d] (%)
1	6 M Gn·HCl–0.1 M phosphate	40	DTT[b]	**30** (56–95)
2	6 M Gn·HCl–0.1 M HEPPS	40	DTT[b]	**30** (>95)
3	0.1 M HEPPS	40	DTT[b]	–
4	6 M Gn·HCl–0.1 M HEPPS	0	DTT[b]	**30** (>95), **31** (<5)[e]
5	6 M Gn·HCl–0.1 M HEPPS	40	EDTA[c]	**32** (>95)
6	6 M Gn·HCl–0.1 M HEPPS	0	EDTA[c]	**32** (>95), **31** (<5)[e]

[a]$CuSO_4 \cdot 5H_2O$ was added to peptide (1.0 mM) in buffer (pH 7.0) in the presence or absence of ascorbate (40 mM), and the reaction was continued for 45 min at 37 °C at pH 5. [b]Quenched with DTT for 15 min at 37 °C. [c]Quenched with EDTA followed immediately by HPLC analysis. [d]Peak ratio on HPLC analysis. [e]A small amount of **31** (< 5%) was formed.

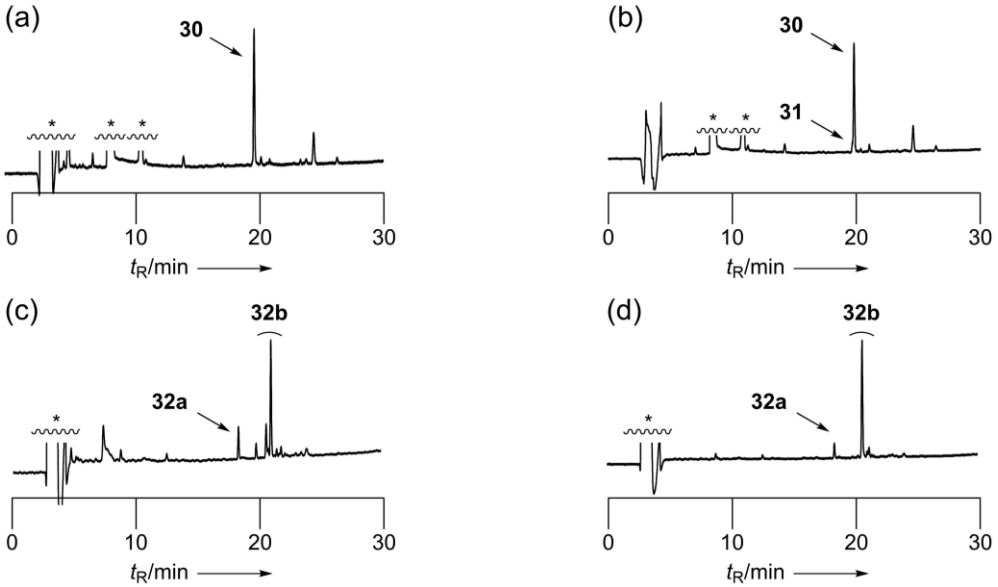


Figure 2.2. Analytical HPLC charts of crude reaction materials: (a) Table 2.1, entry 2, 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.0) with 40 mM $CuSO_4$ and 40 mM ascorbate at 37 ºC for 45 min, then quenched with DTT at 37 ºC for 15 min followed by HPLC analysis; (b) Table 2.1, entry 4, 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.0) with 40 mM $CuSO_4$ at 37 ºC for 45 min, then quenched with DTT at 37 ºC for 15 min followed by HPLC analysis; (c) Table 2.1, entry 5, 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.0) with 40 mM $CuSO_4$ and 40 mM ascorbate at 37 ºC for 45 min, then quenched with EDTA followed immediately by HPLC analysis; (d) Table 2.1, entry 6, 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.0) with 40 mM $CuSO_4$ at 37 ºC for 45 min, then quenched with EDTA followed immediately by HPLC analysis. Analytical HPLC conditions: linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 45% over 30 min. *Non-peptidic compounds derived from additives.

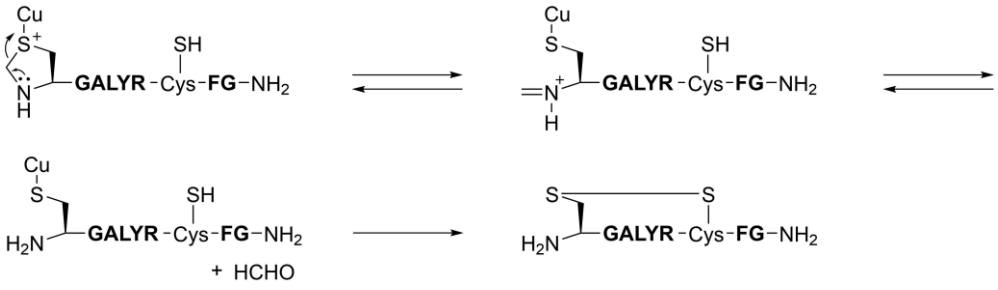


Figure 2.3. Disulfide bond formation facilitates the ring-opening of thiazolidine.

Disulfide bond formation in the two cases is thought to proceed via different mechanisms. N-Terminal cysteine residues are known to be more easily oxidized in the presence of metal salts than an internal cysteine residue.[25] After Cu(II)-mediated ring-opening of thiazolidine, oxidation of the regenerated N-terminal cysteine by the action of Cu(II) ions gives, for example, a sulfenic acid derivative **33** electrophilically acceptable to thiols, resulting in the formation of a disulfide bond as shown in Figure 2.4.

The remaining sulfenic acid unit of **33** derived from its failure to interact with the free cysteine was provably further oxidized to a sulfinic acid **31** and a sulfonic acid **34**. In contrast, no further oxidized materials were observed in reactions in the presence of ascorbate, which implies that disulfide-producing species such as **33** formed under the Cu(II) conditions are not formed in the reaction with Cu(I). Possible explanations for the disulfide bond formation under Cu(I) conditions include oxidative addition of the thiol to a Cu(I) complex or a dehydroascorbate-mediated oxidation, but other possibilities cannot be ruled out.

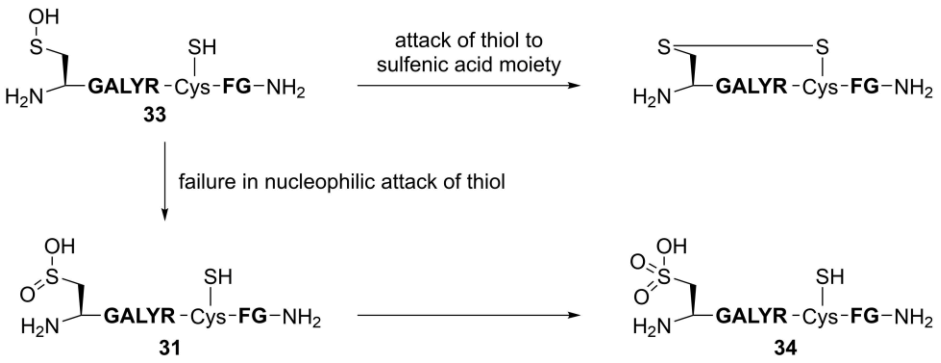


Figure 2.4. Plausible mechanism for the formation of disulfide and overoxidized peptides followed by the ring-opening under Cu(II) conditions.

Next, to further investigate the influence of disulfide bond formation in copper-mediated reactions, the reaction was carried out using **35** (Thz-**GALYR**-Ala-**FG**-NH_2), a thiazolidine peptide lacking an internal cysteine. Ring-opening reaction of **35** with $CuSO_4$ (40 mM) in 6 M Gn·HCl–0.1 M HEPPS (pH 7.0) in the presence 40 mM ascorbate was conducted at 37 ºC for 45 min (Figure 2.5). As the result, oxidized peptides **37** and **38** were not detected, the starting material **35** remained with a conversion yield of 38% and **36** was formed in 62% yield. The reaction was completed in the absence of ascorbate, but over-oxidized peptides **37** and **38** were confirmed [conversion yields **37** (41%); **36** + **38**

(combined 59%)]. These reaction outcomes are different from those obtained in reactions of a cysteine-containing peptide **29** (Table 2.1, entries 5 and 6), and this indicates the importance of the disulfide bond formation.

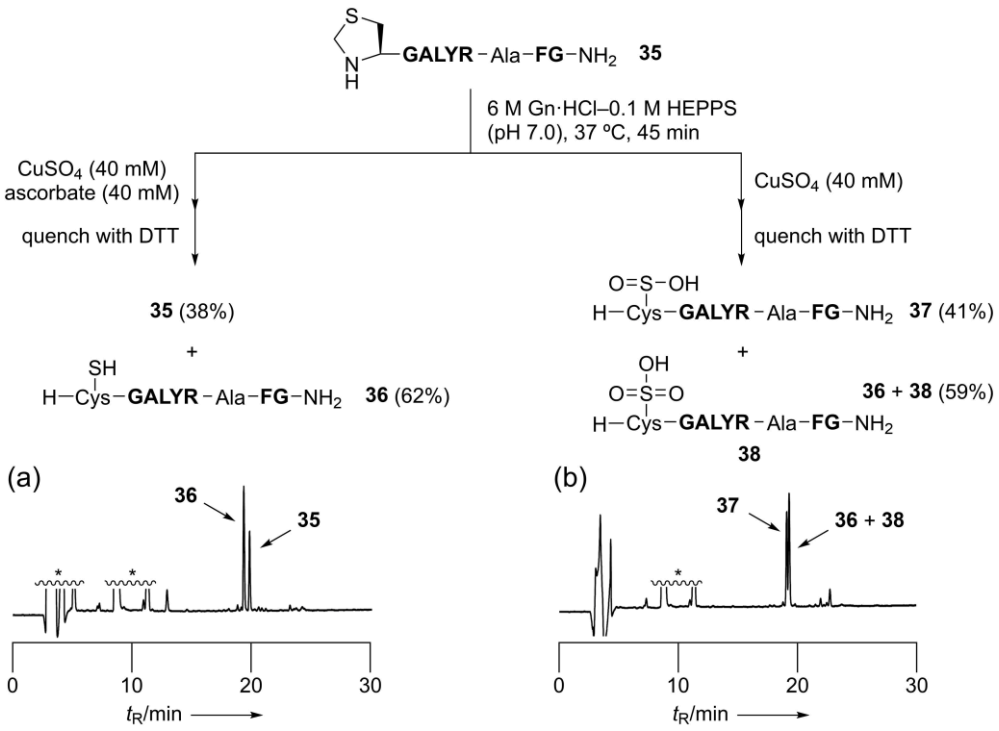


Figure 2.5. Analytical HPLC charts of crude reaction mixtures: (a) 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.0) with 40 mM $CuSO_4$ and 40 mM ascorbate at 37 ºC for 45 min, then quenched with DTT at 37 ºC for 15 min followed by HPLC analysis; (b) 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.0) with 40 mM $CuSO_4$ at 37 ºC for 45 min, then quenched with DTT at 37 ºC for 15 min followed by HPLC analysis. Analytical HPLC conditions: linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 45% over 30 min. *Non-peptidic compounds derived from additives.

As shown in Figure 2.4 and Figure 2.5, disulfide bond formation is indispensable for the facile and clean conversion of thiazolidine peptides. I next examined the efficacy of disulfide bond formation by Cu(I) or Cu(II) using a bis-cysteine peptide **30** (Cys-**GALYR**-Cys-**FG**-NH_2) (Figure 2.6). Disulfide-bond formation reaction of **30** with $CuSO_4$ (40 mM) in the absence of ascorbate was completed in 10 min to afford a mixture of disulfide peptides **32** [conversion yields **32** (>95%)]. The oxidation reaction of **30** in

the presence of ascorbate (40 mM) was not completed in 10 min [conversion yields **30** (44%); **32** (56%)]. Comparing the above results, it could be seen that disulfide bond formation, which contributes to inhibition of the reverse reaction leading to regeneration of the thiazolidine ring, proceeds more rapidly with Cu(II) than with Cu(I). However, since no significant difference was observed in the ring-opening reaction after 1 h in both the Cu(I)- and Cu(II)-mediated reactions, the condition with ascorbate was deemed suitable for ring-opening of thiazolidines.

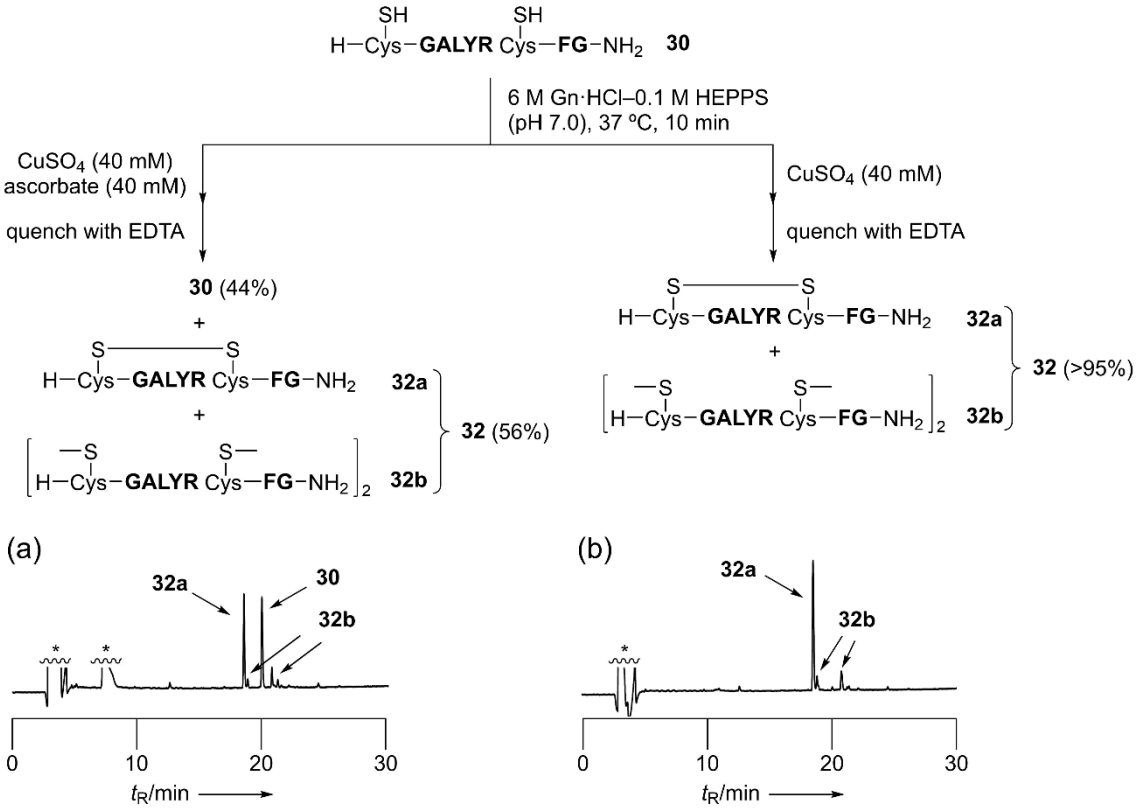
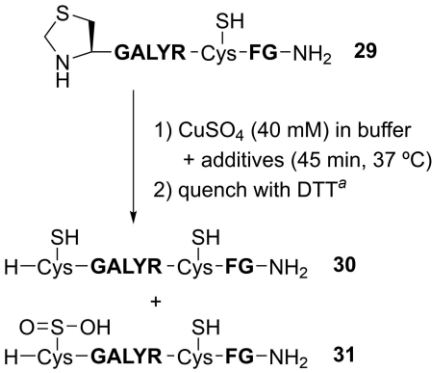


Figure 2.6. Analytical HPLC charts of crude reaction materials: (a) 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.0) with 40 mM $CuSO_4$ and 40 mM ascorbate at 37 ºC for 10 min, then quenched with EDTA followed immediately by HPLC analysis; (b) 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.0) with 40 mM $CuSO_4$ at 37 ºC for 10 min, then quenched with EDTA followed immediately by HPLC analysis. Analytical HPLC conditions: linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 45% over 30 min. *Non-peptidic compounds derived from additives.

Since ring-opening of thiazolidine is generally carried out in a one-pot reaction after NCL, I next examined whether the copper-mediated reaction progresses in the presence of NCL additives such as MPAA and/or TCEP. The copper-mediated ring-opening reaction of **29** proceeded in the presence of MPAA or TCEP (Table 2.2, entries 1 and 2); however, the reaction became slightly slower in reaction mixtures containing equimolar amounts of MPAA and $CuSO_4$ (Table 2.2, entry 1). Surprisingly, the conversion yield decreased significantly under conditions in which 40 mM MPAA and 20 mM TCEP were added to 40 mM $CuSO_4$ (Table 2.2, entry 3). Since MPAA and TCEP appeared to coordinate with copper ions and inhibit the progress of the reaction, I examined the condition in which an excess amount of $CuSO_4$ was added to the NCL additives. This reaction proceeded quantitatively for 45 minutes when 20 mM MPAA and 10 mM TCEP were added to the reaction containing 40 mM $CuSO_4$ (Table 2.2, entry 4). Additionally, the presence of ascorbate effectively suppressed the formation of over-oxidized products such as **31** (Table 2.2, entries 4 vs 5). From the above results, it was concluded that the ring-opening of thiazolidine with copper salts can be performed in a one-pot reaction after NCL.

Table 2.2. Deprotection of thiazolidine peptide **29** with copper salts under NCL conditions.



entry	additives (mM)			product conversion[b] (%)
	MPAA	TCEP	ascorbate	
1	40	0	40	**30** (95), **29** (5)
2	0	20	40	**30** (> 95)
3	40	20	40	**30** (30), **29** (70)
4	20	10	40	**30** (> 95)
5	20	10	0	**30** (83), **31** (17)

[a]Quenched with DTT for 15 min at 37 °C. [b]Peak ratio on HPLC analysis.

2.4 Chemical synthesis of CXCL14 via C–to–N-directed sequential ligation

As mentioned in Chapter 1, I succeeded in the synthesis CXCL14 via N–to–C-directed sequential NCL. In this chapter, the synthesis of CXCL14 using thiazolidine derivatives as the middle fragment in C–to–N-directed sequential NCL was examined (Figure 2.7).

The first NCL between N-terminal thiazolidine thioester peptide **39** and N-terminal cysteinyl peptide **19** was performed in 6 M Gn·HCl−0.1 M HEPPS buffer (pH 7.0) in the presence of 20 mM MPAA and 10 mM TCEP at 37 °C for 6 h to afford the N-terminal thiazolidine peptide **40** (Figure 2.7a and 2.7b). Then the ring-opening reaction of **40** was conducted by addition of $CuSO_4$ (40 mM) and ascorbate (40 mM) to the reaction mixture. After 45 min, the crude reaction was quenched with DTT at 37 °C for 15 min followed by HPLC purification to give the ligated N-terminal cysteinyl peptide **41** in 52% isolated yield over two steps (Figure 2.7c). The resulting compound **41** was subjected to the second NCL step with thioester peptide **42** in 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.0) in the presence of 40 mM MPAA and 30 mM TCEP at 37 ºC for 8 h to afford a 77-residue ligated and reduced form of CXCL14 **20** in 65% isolated yield (Figure 2.7d). Finally, air oxidation of this product **20** was conducted in 3 M Gn·HCl–0.1 M phosphate buffer (pH 7.7) to give human-CXCL14 in 69% isolated yield (Figure 2.7e).

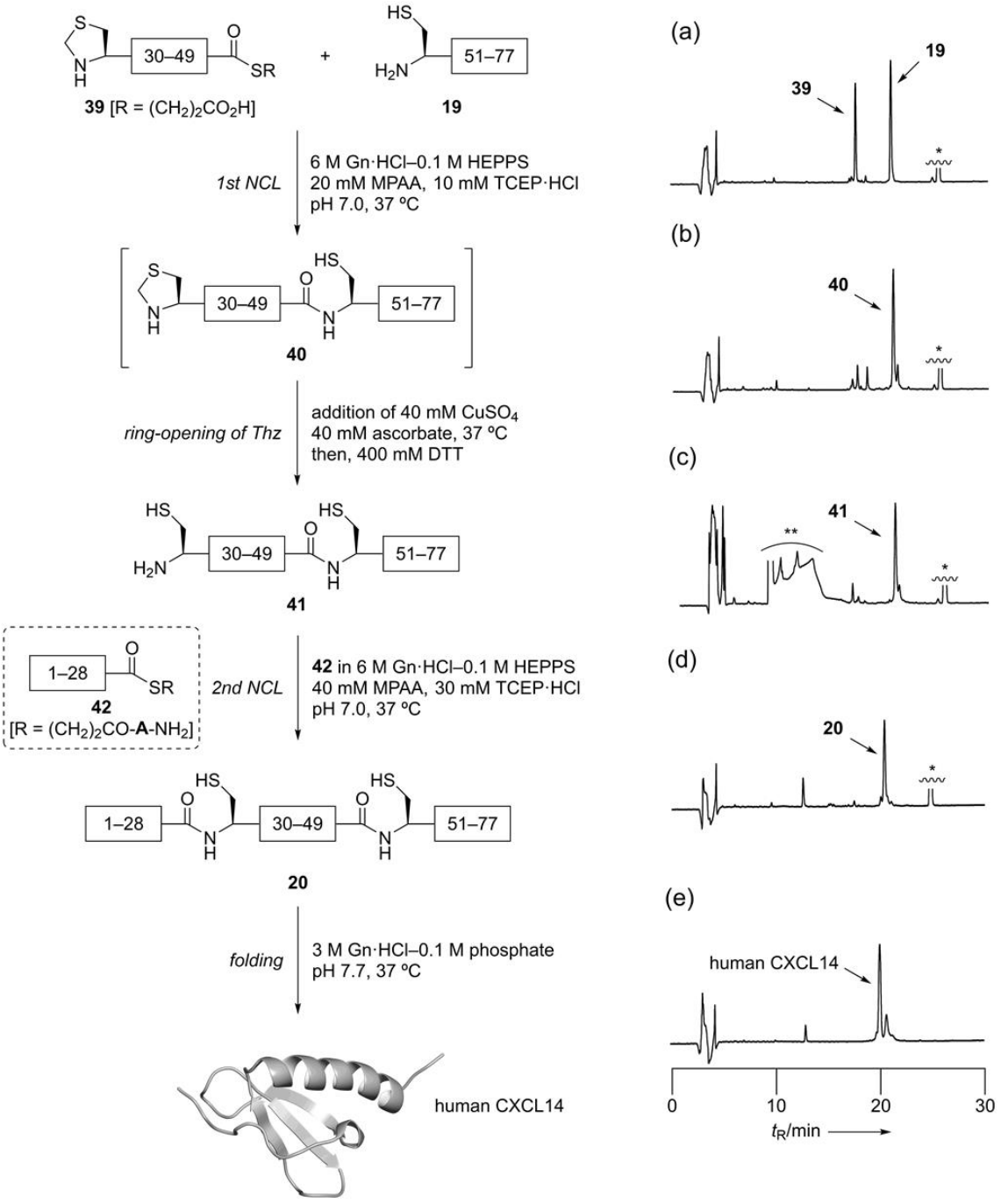
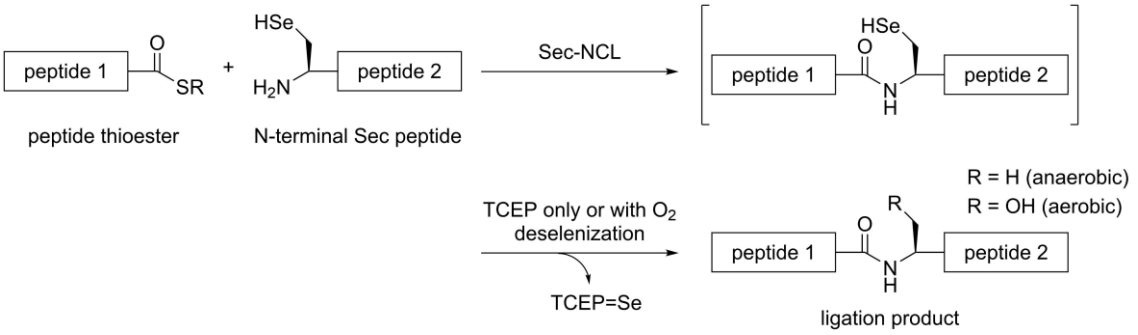


Figure 2.7. HPLC monitoring of the synthesis of CXCL14 via C–to–N-directed sequential NCL: (a) first NCL (t < 5 min); (b) first NCL (t = 6 h); (c) ring-opening of thiazolidine (t = 1 h); (d) second NCL (t = 8 h); (e) oxidation with air (t = 24 h). Analytical HPLC conditions: linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 45% over 30 min. *MPAA, **non-peptidic compounds derived from additives.

2.5 Application of a copper-mediated deprotection procedure to ring-opening of selenazolidine derivatives for sequential ligation

NCL is a very reliable chemical technology for preparation of proteins; however, the fundamental requirement of cysteine residues at the ligation sites is often troublesome because cysteine has a low occurrence in nature.[26] Many naturally occurring proteins contain the cysteine residues nor the cysteine residues suitable for NCL. Therefore NCL-based methodologies enabling condensation at non-cysteine sites are necessary.

Recently, the use of selenocysteine (Sec) as an approach to solve this problem has been rapidly expanding.[27] Selenocysteine as a substitution for the N-terminal cysteine allows facile construction of Xaa–Ala or Xaa–Ser junctions with radical or oxidative deselenization, respectively (Scheme 2.4).[28] Moreover, the use of selenazolidine (Sez) as a protected N-terminal selenocysteine residue enables the implementation of selenocysteine-mediated C–to–N-directed sequential NCL.[29] In comparison with the thiazolidine peptides, ring-opening of a selenazolidine achieved by the action of methoxyamine requires a longer reaction time.[21, 29] Furthermore, the palladium-mediated protocol effective for the deprotection of the thiazolidine has been reported to be ineffective for selenazolidines.[23a] I therefore decided to investigate whether the copper-mediated protocol can be applied to ring-opening of selenazolidines.



Scheme 2.4. The NCL at selenocysteine and deselenization reactions.

The ring-opening reaction using a model selenazolidine peptide **43** (Sez-**GALYRCFG**-NH_2) in 6 M Gn·HCl−0.1 M HEPPS buffer (pH 7.0) in the presence of 40 mM $CuSO_4$ and 40 mM ascorbate was conducted at 37 °C under an ambient atmosphere. After 45 min, the reaction mixture was quenched with aqueous EDTA and this was followed immediately by HPCL analysis to afford a mixture of intra- and inter-molecular bridging peptides **44** and **45** (Figure 2.8).

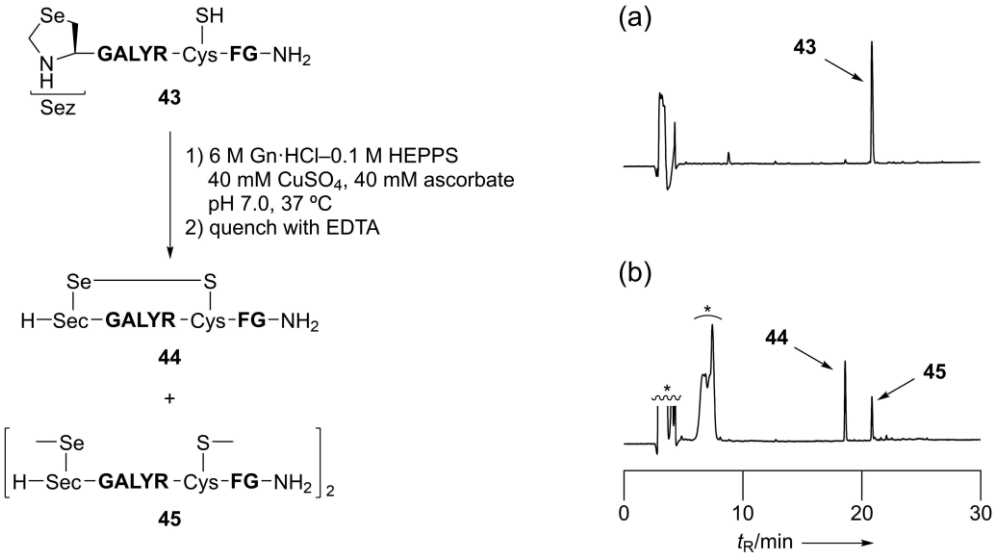


Figure 2.8. HPLC monitoring of the ring-opening of a selenazolidine: (a) ring-opening of selenazolidine (t < 5 min); (b) ring-opening of selenazolidine (t = 45 min). Analytical HPLC conditions: linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 45% over 30 min. *Non-peptidic compounds derived from additives.

From the above study, it was seen that copper-mediated ring-opening reaction of purified selenazolidine peptide is easily performed. I evaluated whether the ring-opening reaction of selenazolidine progresses under conventional NCL conditions. The ring-opening reaction of **43** with $CuSO_4$−ascorbate (each at 40 mM) in 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.0) in the presence of 20 mM MPAA and 10 mM TCEP proceeded smoothly, and various S–Se-linked peptides **44** and **45** were detected by HPLC analysis. Although HPLC purification was attempted after reduction of the S–Se bridge with TCEP or DTT, it became difficult to purify the products because a mixture of peptides of oxidized and reduced forms was obtained.

Thus, I turned my attention to examination of the reaction conditions which allow a one-pot/direct NCL followed by the ring-opening of the selenazolidine ring. Suppression of deselenization to give Ala (or Ser) during the following NCL step was also examined. In comparison with the ring-opening of thiazolidine, less copper salt (2 mM) is needed to achieve the opening of the selenazolidine ring. Furthermore, the use of a 2−5-fold excess amount of ascorbate to TCEP was found to be effective for the suppression of deselenization during NCL.[28b] On the basis of these results, I devised a synthetic route in which NCL and deselenization are carried out in one-pot protocol

without intermediate HPLC purification after the ring-opening of the selenazolidine. Initially, the ring-opening reaction of **43** was performed in 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.0) in the presence of 2 mM $CuSO_4$ and 2 mM ascorbate at 37 ºC to afford the ring-opened peptides **44** and **45** (Figure 2.9a). Then, addition of **47** in 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.0) in the presence of 100 mM MPAA, 40 mM TCEP and 100 mM ascorbate to the reaction mixture gave the ligated product **48** (Figure 2.9b and 2.9c). Finally, the reaction mixture was subjected to deselenization by addition of 0.25 M solid TCEP to afford the deselenized alanine product **49** in 74% isolated yield in a one-pot manner (Figure 2.9d).

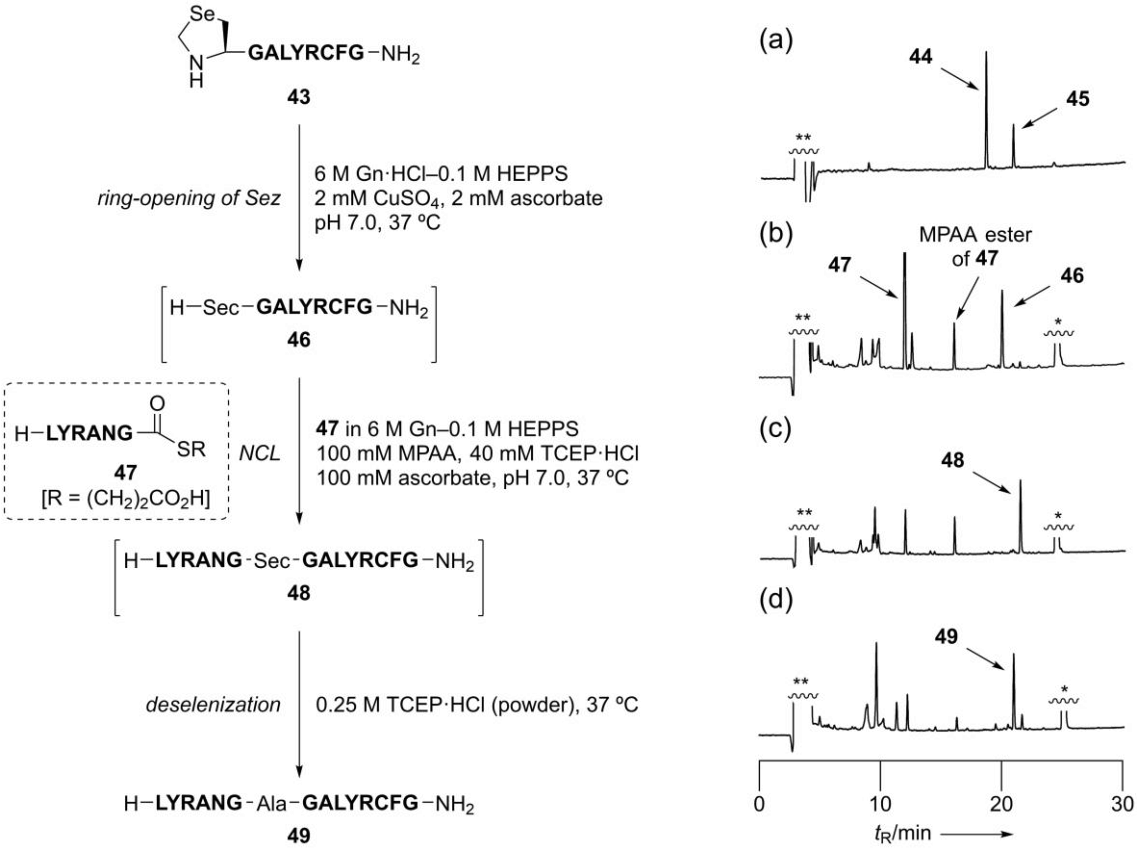


Figure 2.9. HPLC monitoring of the ring-opening of selenazolidine, NCL and deselenization in one-pot protocol: (a) ring-opening of selenazolidine (t = 45 min); (b) NCL (t < 5 min); (c) NCL (t = 1 h); (d) deselenization (t = 48 h). For HPLC analysis, reaction (a) was quenched with EDTA; (b)–(d) were quenched by the addition of 6 M Gn·HCl in the presence of 20 mM TCEP·HCl and 100 mM ascorbate. Analytical HPLC conditions: linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 45% over 30 min. *MPAA, **non-peptidic compounds derived from additives.

2.6 Conclusion

Towards the synthesis of proteins including CXCL14 by C–to–N directed sequential NCL, I developed a practical ring-opening reaction of thiazolidines with inexpensive copper salts. This protocol has advantages in that the ring-opening reaction is complete within 1 h and can be carried out under aerobic conditions.

This copper-mediated protocol was successfully applied to the deprotection of selenazolidine. Ring-opening of selenazolidine, followed by NCL and deselenization was achieved in a one-pot protocol. As this work was proceeding, Metanis et al. independently reported a deprotection protocol of the selenazolidine with its application to the one-pot chemical synthesis of a protein.[30]

Chapter 3

Elucidation of the domains of CXCL14 responsible for CpG DNA-mediated Toll-like receptor 9 activation

3.1 Introduction

In 1999, CXCL14 was isolated as a CXC-type chemokine consisting of 77 amino acids,[31] and has been reported to be involved in multiple immunological functions.[32] Although "classical" chemokines have chemotactic activity through which they exert immunological and inflammatory functions, whether or not CXCL14 plays a physiological role in mobilization of leukocytes has been a mystery because it has a very low chemotactic function[33] and there is no change in leukocyte distribution in CXCL14-knockout (KO) mice.[34] My group found that the C-terminal region of CXCL14 binds to CXCR4, the known receptor for CXCL12, and inhibits the chemotactic activity of CXCL12.[35] However, these characteristics alone do not explain the original physiological functions of CXCL14 such as activation of tumor immunity[36] and progression of chronic inflammation in obese mice.[37]

The innate immune response is a biological defense mechanism that plays important roles in pathogen recognition, induction of inflammatory cytokines and activation of the adaptive immune response.[38, 39] Pattern recognition receptors (PRRs) expressed on or in dendritic cells cause activation of downstream signaling pathways after recognizing pathogen-associated molecular patterns. Among PRRs, Toll-like receptor 9 (TLR9) is localized within the endosome/lysosome and recognizes the unmethylated cytosine-phosphate-guanine-containing DNA (CpG DNA) present in the microbial environment, thereby inducing activation of the innate immune system.[40, 41] Thus, unmethylated CpG DNA acts as a sensor for TLR9-mediated immunosurveillance.[42–45] Experimentally, several types of CpG oligodeoxynucleotides (ODN) have been identified as strong agonists for TLR9. For instance, ODN2395, a synthetic phosphorothioate oligodeoxynucleotide with 22-mer long is a potent activator for secretion of inflammatory cytokines and interferon responses that have been used in my study.[46]

In 2017, Tanegashima et al., in my research group, found that CXCL14 enhances the immune response to CpG DNA-mediated TLR9 activation by promoting intracellular uptake of CpG DNA.[47] CXCL14 forms a complex with CpG DNA with high affinity (Figure 3.1: I), and the CXCL14–CpG DNA complex is then transported into the

endosome/lysosome (Figure 3.1: II). The CXCL14–CpG DNA complex is disrupted by acidic conditions in the endosome/lysosome, and the released CpG DNA mediates TLR9 activation (Figure 3.1: III). Thus, CXCL14 behaves as a carrier molecule transporting CpG DNA from extracellular locations to the TLR9 present in the intracellular endosome/lysosome milieu. Since CXCR4-KO cells also exhibited this immune activation, the cellular uptake of CXCL14–CpG DNA was not mediated by CXCR4. Since CXCL14 enhances binding of CpG DNA in dendritic cells, it was estimated that CpG DNA was endocytosed via receptor X bound to the CXCL14–CpG DNA complex (Figure 3.1: II). However, domain structures of CXCL14 that are important for the binding of CpG DNA and receptor X have not been identified yet.

CXCL14 has two characteristic domain structures: an anti-parallel β-sheet at the N-terminal region (residues 1–50) and an α-helix at the C-terminal region (50–77).[48] To elucidate the domains required for the CpG DNA-mediated TLR9 activation, i.e. the DNA-binding domain and the receptor recognition domain, I synthesized a series of CXCL14 derivatives and evaluated their synergistic behavior with CpG DNA.

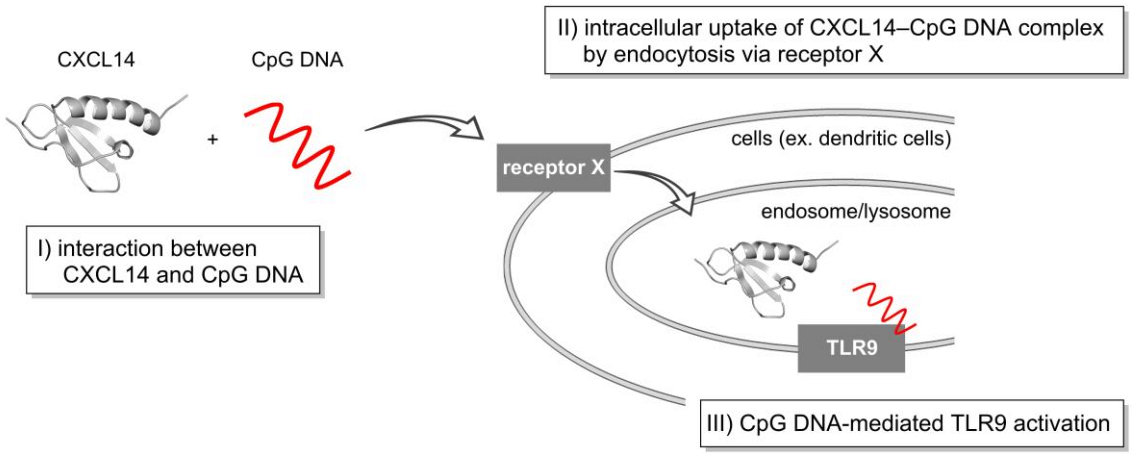


Figure 3.1. Putative mechanism for TLR9 activation by the CXCL14–CpG DNA complex.

3.2 Synthesis and biological evaluation of CXCL14 derivatives: splitting the 1–77 region into regions 1–50 and 50–77

Along with domain structure, I planned to split the 1–77 region of CXCL14 into 1–50 and 50–77 regions, and synthesized the 1–50 region peptide with disulfides **50a**, the 50–77 region peptide **51a** and their biotinylated peptides (**50b** and **51b**) (Table 3.1).

Table 3.1. Sequence of CXCL14 derivatives: the 1–77 region split into 1–50 and 50–77 fragments.

Region	Compound name	Sequence
1–77[a,c]	WT: **B** = CO_2H WT-bio: **B** = Lys(bio)-$CONH_2$	SK**C**K**C**SRKGPKIRYSDVKKLEMKPKYPH**C**EEKMVIITTKS VSRYRGQEH**C**LHPKLQSTKRFIKWYNAWNEKRRVYEE–**B**
1–50[a,c]	**50a**: **X** = Gly-$CONH_2$ **50b**: **X** = Lys(bio)-$CONH_2$	SK**C**K**C**SRKGPKIRYSDVKKLEMKPKYPH**C**EEKMVIITTKS VSRYRGQEH**C**–**X**
50–77[c]	**51a**: **B** = CO_2H **51b**: **B** = Lys(bio)-$CONH_2$	**C**LHPKLQSTKRFIKWYNAWNEKRRVYEE–**B**
50–77[c]	**52a**: **B** = CO_2H	**A**LHPKLQSTKRFIKWYNAWNEKRRVYEE–**B**
50–77[b,c]	**53a**: **B** = CO_2H	*C**C**LHPKLQSTKRFIKWYNAWNEKRRVYEE–**B**

[a]Disulfide bonds between Cys^3 and Cys^{29}, Cys^5 and Cys^{50}. [b]Disulfide bond between $Cys^{50}$and Cys^{50}. [c]N-Terminal: NH_2.

The synthesis of 1–50 region peptide **50a** is shown in Figure 3.2. NCL of the thioester peptide Cys^5(Acm) **54** and the N-terminal cysteinyl peptide Cys^{50}(Acm) **55a** was performed in 6 M Gn·HCl–0.1 M phosphate buffer (pH 6.8) in the presence of 50 mM MPAA and 50 mM TCEP at 37 ºC for 3 h, and this was followed by HPLC purification to obtain the ligated peptide **56a** in 71% isolated yield. This peptide **56a** was subjected to air oxidation in 3 M Gn·HCl–0.1 M phosphate buffer (pH 7.7) at 37 ºC for 44 h to afford the single S–S bridged peptide **57a** in 63% isolated yield after HPLC purification. The Acm groups on **57a** were removed by the action of AgOTf–anisole in TFA at 4 ºC for 6 h. Then 50% (v/v) DMSO/1 M HCl aq. was added to the reaction and the resulting mixture was incubated at 25 ºC for 6 h.[49, 50] After HPLC purification, the doubly S–S bridged peptide **50a** was obtained in 37% isolated yield over two steps. For the synthesis of **50b**, the C-terminal glycine residue was replaced with a biotinylated-lysine residue, using the same procedure as was used for **50a** and mentioned in Figure 3.2.

The synthesis of the 50–77 region peptide **51a** and its biotinylated peptide **51b** were performed on Fmoc-Glu(O*t*-Bu)-Wang resin (0.29 mmol/g) and NovaSyn® TGR resin (0.24 mmol/g),[13] and their peptide chains were elongated by standard Fmoc-SPPS protocol as described in Chapters 1 and 2.

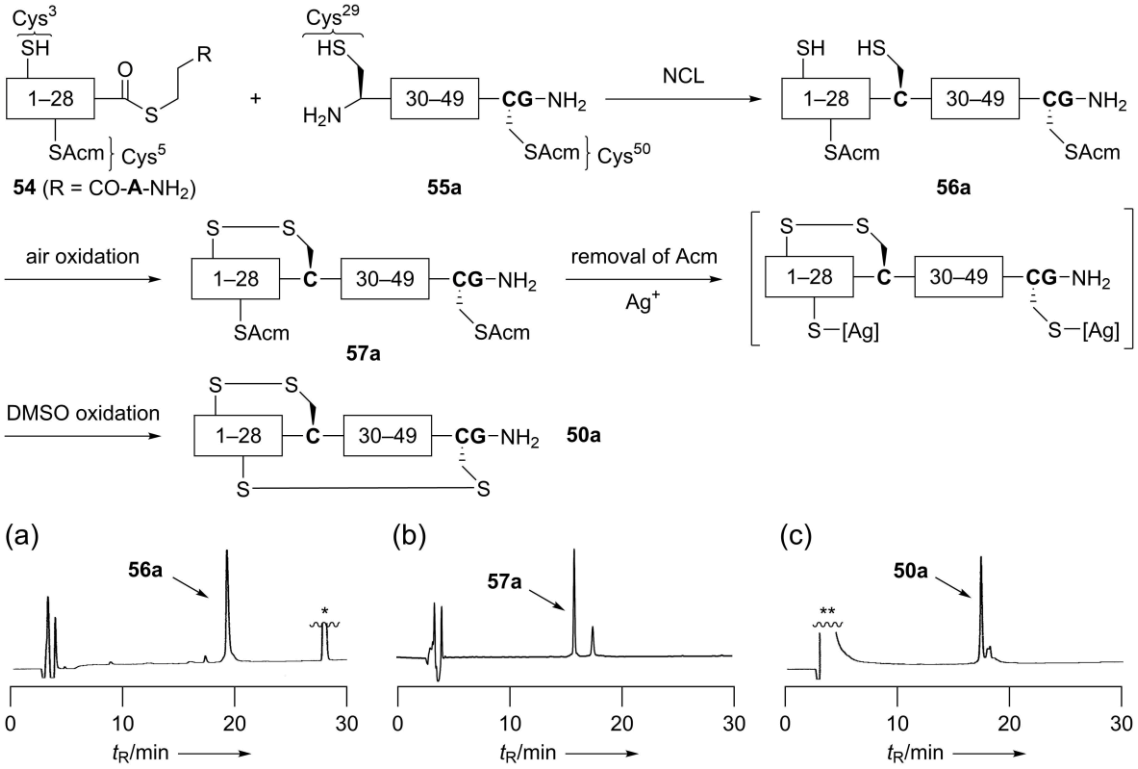


Figure 3.2. HPLC monitoring of the synthesis of the 1–50 region peptide **50a**: (a) NCL (t = 3 h); (b) air oxidation (t = 44 h); (c) DMSO oxidation (t = 6 h). Analytical HPLC conditions: linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 10% to 40% over 30 min. *MPAA, **non-peptidic compounds derived from additives.

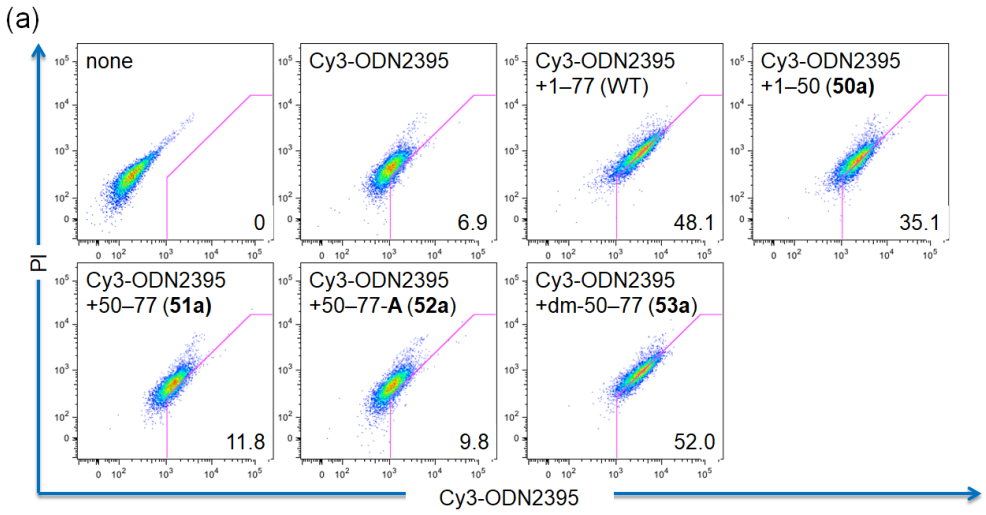
I next determined whether the synthetic peptides (**50** and **51**) exhibited CpG DNA-mediated TLR9 activation. Initially, I examined by FACS analysis whether various synthetic peptides enhance the cellular uptake of Cy3-labeled ODN2395 (Cy3-ODN2395), which is one of the representative types of CpG ODN. Raw 264.7 cells were incubated with 30 nM Cy3-ODN2395 and 300 nM of peptides (**50a** and **51a**) at 37 ºC for 1 h (Figure 3.3a and 3.3b). Although the 1–50 region peptide **50a** significantly enhanced the cellular uptake of Cy3-ODN2395, the 50–77 region peptide **51a** showed lower activity than that of the 1–50 region peptide **50a**. I also found that the C-terminal cysteine

dimer peptide **53a** (dm-50–77) enhanced the activity of CpG DNA uptake, but the C-terminal alanine peptide **52a** (50–77-**A**) which is completely monomeric, failed to do so. These results suggest that the subtle enhancing activity of monomer peptide **51a** was caused by dimer peptide **53a** which was produced by oxidation of N-terminus cysteine in the cells. The activity of the dimeric 50–77 region peptide **53a** was not detected in other cell types (data not shown), suggesting cell type-specific effects, which were distinct from the action of WT CXCL14. Taken together, the intact 50–77 region peptide **51a** was concluded to be inactive.

The evaluation of cytokine induction by the CpG DNA-mediated TLR9 activation with synthetic peptides was performed. Raw 264.7 cells were treated with 30 nM ODN2395 in the presence or absence of 300 nM of various peptides (**50a** and **51a**) at 37 ºC. After 6 h, culture supernatants were subjected to ELISA for TNF-α (Figure 3.3c). The 1–50 region peptide **50a**, but not the 50–77 region peptide **51a**, induced strong TNF-α production in the presence of 30 nM ODN2395.

To confirm the binding of split peptides to CpG DNA, pull-down assays were performed with biotinylated peptides (**50b** and **51b**) in the presence of different concentrations of Cy3-ODN2395, and their dissociation constants (K_d) were calculated by Scatchard plot analysis (Figure 3.3d–3.3f). All peptides showed the ability to bind to DNA, and K_d values of the 1–50 region peptide **50b** and the 50–77 region peptide **51b** were 103 nM and 217 nM, respectively. These K_d values are higher than that of biotinylated WT CXCL14 (K_d = 27 nM).

These results suggest that the N-terminal region (1–50) of CXCL14 is an important region for the CpG DNA binding, receptor X-mediated intracellular uptake, and TLR9 activation (Figure 3.1: I, II and III), while the C-terminal region (50–77) of CXCL14 is involved only in the CpG DNA binding (Figure 3.1: I).



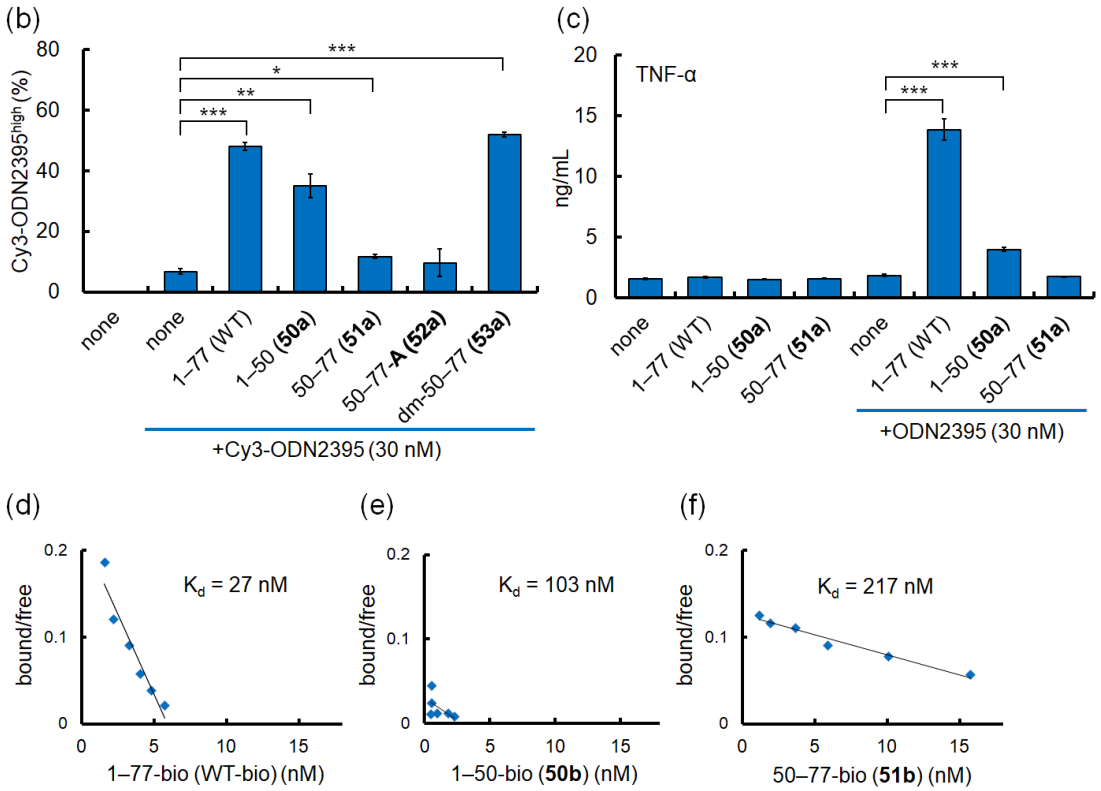


Figure 3.3. Biological evaluation of synthetic peptides on the CpG DNA uptake and activity. (a) FACS analysis of Raw 264.7 cells incubated with 30 nM Cy3-ODN2395 in the presence or absence of 300 nM peptide at 37 ºC for 1 h. The Propidium Iodide (PI) negative fraction was gated to select live cells. (b) Percentages of the Cy3-ODN2395^{high} cell fraction were calculated from FACS profiles shown in (a). (c) Raw 264.7 cells were treated with 30 nM ODN2395 in the presence or absence of 300 nM peptide at 37 ºC for 6 h. Culture media were collected and analyzed by ELISA for TNF-α. (d–f) Scatchard plot analyses. Various concentrations of Cy3-ODN2395 were pulled-down with biotinylated peptides in streptavidin-agar at 4 ˚C for 1 h. The Cy3-fluorescence of each bound and free fraction was quantified. In (b) and (c), each value represents the mean ± SE (n = 3), and P-value ($^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001, otherwise not significant) compares with the condition treated with Cy3-ODN2395 alone. All statistical analyses were performed using Microsoft Excel and an unpaired Student's t-test.

3.3 Synthesis and biological evaluation of CXCL14 derivatives: a strategy to divide the 1–50 region into 15-residue fragments

As shown in the previous section, the N-terminal region (1–50) of CXCL14 is involved in the CpG DNA binding and the CpG DNA-mediated cytokine production via TLR9. To determine responsible domains for the DNA binding and receptor recognition for TLR9 activation, the 1–50 region was divided into 15-residue fragments and these peptides were synthesized (Table 3.2).

Synthesis of the 15-residue peptides (**58**–**65**) was performed on Rink-amide ChemMatrix® resin (0.48 mmol/g), and their peptide chains were elongated by a standard Fmoc-SPPS protocol as shown in Chapters 1 and 2 and described briefly in Scheme 3.1. Since it was considered that synthesis and biological evaluation of peptides would be difficult due to the formation of disulfides when free cysteine was present, four cysteine residues (Cys^3, Cys^5, Cys^{29} and Cys^{50}) were replaced by alanine residues. I confirmed that 1–50 region peptide, in which four-cysteine residues have been replaced by alanine residues, possesses an internalization-enhancing activity of ODN2395 similar to that of the 1–50 region peptide **50**.

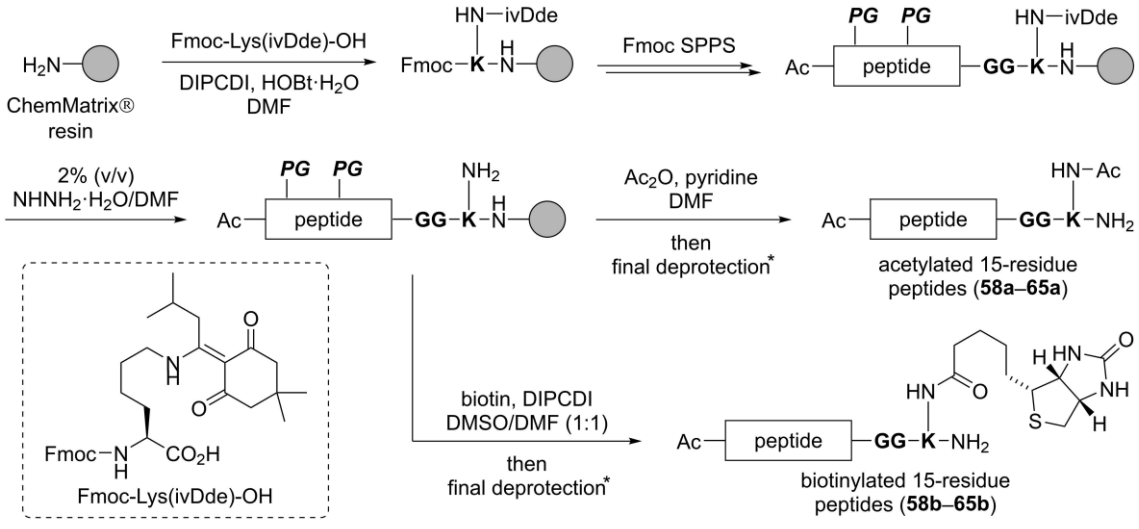
The synthetic 15-residue peptides (**58**–**65**) were subjected to biological evaluation experiments including DNA binding assay, FACS-based internalized assay, and TNF-α production assay. However, all peptides failed to enhance the internalization of ODN2395 and TNF-α secretion (Figure 3.4).

These results suggest that the three-dimensional conformation and/or molecular size of CXCL14 are necessary for the internalization of CpG DNA.

Table 3.2. Sequence of CXCL14 derivatives: the 1–50 region is divided into 15-residue peptides.

Region	Compound name	Sequence
1–50[a,b]	**50a**: **X** = Gly-$CONH_2$ **50b**: **X** = Lys(bio)-$CONH_2$	^{1}SK**C**K^{3}**C**^{5}SRKGPKIRYSDVKKLEMKPKYPH^{29}**C**EEKMVIITTKS VSRYRGQEH^{50}**C**–**X**
1–15[c]	**58a**: **Z** = Lys(Ac)-$CONH_2$ **58b**: **Z** = Lys(bio)-$CONH_2$	^{1}SK**A**K**A**SRKGPKIRYS^{15}–GG**Z**
6–20[c]	**59a**: **Z** = Lys(Ac)-$CONH_2$ **59b**: **Z** = Lys(bio)-$CONH_2$	^{6}SRKGPKIRYSDVKKL^{20}–GG**Z**
11–25[c]	**60a**: **Z** = Lys(Ac)-$CONH_2$ **60b**: **Z** = Lys(bio)-$CONH_2$	^{11}KIRYSDVKKLEMKPK^{25}–GG**Z**
16–30[c]	**61a**: **Z** = Lys(Ac)-$CONH_2$ **61b**: **Z** = Lys(bio)-$CONH_2$	^{16}DVKKLEMKPKYPH**A**E^{30}–GG**Z**
21–35[c]	**62a**: **Z** = Lys(Ac)-$CONH_2$ **62b**: **Z** = Lys(bio)-$CONH_2$	^{21}EMKPKYPH**A**EEKMVI^{35}–GG**Z**
26–40[c]	**63a**: **Z** = Lys(Ac)-$CONH_2$ **63b**: **Z** = Lys(bio)-$CONH_2$	^{26}YPH**A**EEKMVIITTKS^{40}–GG**Z**
31–45[c]	**64a**: **Z** = Lys(Ac)-$CONH_2$ **64b**: **Z** = Lys(bio)-$CONH_2$	^{31}EKMVIITTKSVSRYR^{45}–GG**Z**
36–50[c]	**65a**: **Z** = Lys(Ac)-$CONH_2$ **65b**: **Z** = Lys(bio)-$CONH_2$	^{36}ITTKSVSRYRGQEH**A**^{50}–GG**Z**

[a]Disulfide bonds between Cys^3 and Cys^{29}, Cys^5 and Cys^{50}. [b]N-Terminal: NH_2. [c]N-Terminal: Ac.



*final deprotection: TFA–*m*-cresol–EDT–TES–thioanisole–H_2O [80:5:2.5:2.5:5:5 (v/v)]

Scheme 3.1. Synthesis of 15-residue peptides (**58**–**65**).

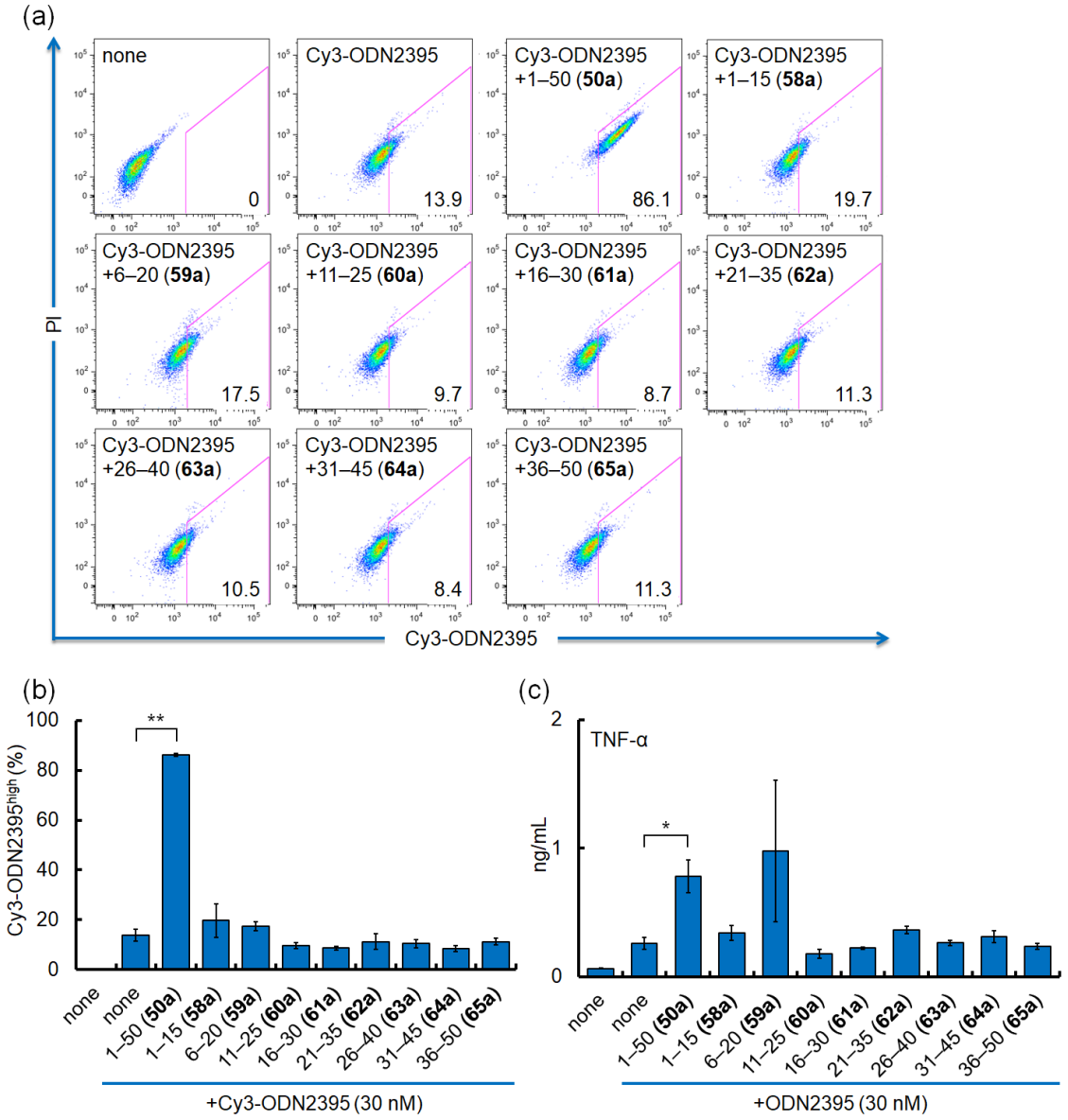


Figure 3.4. Biological evaluation of synthetic peptides on the CpG DNA uptake and activity. (a) FACS analysis of Raw 264.7 cells incubated with 30 nM Cy3-ODN2395 in the presence or absence of 300 nM peptide at 37 ºC for 1 h. The PI negative fraction was gated to select live cells. (b) Percentages of the Cy3-ODN2395^{high} cell fraction were calculated from FACS profiles shown in (a). (c) Raw 264.7 cells were treated with 30 nM ODN2395 in the presence or absence of 300 nM peptide at 37 ºC for 6 h. Culture medium were collected and analyzed by ELISA for TNF-α. In (b) and (c), each value represents the mean ± SE (n = 3), and P-value ($^{*}P$<0.05, $^{**}P$<0.01, otherwise not significant) compares with the condition treated with Cy3-ODN2395 alone. All statistical analyses were performed using Microsoft Excel and an unpaired Student's t-test.

3.4 Synthesis and biological evaluation of CXCL14 derivatives: a strategy to delete the 1–50 region from N- or C-terminus

As mentioned in Chapters 3-2 and 3-3, the N-terminal region (1–50) of CXCL14 was important for the internalization-enhancing activity of CpG DNA, but the shorter peptides failed to retain the activity. I therefore worked on the synthesis of peptides by disrupting domain structures as described above.[48] Experimentally, I deleted the N-terminal loop (1-12 region), the middle region of the loop structure (41-47 region), and the third β-sheet (48-50 region) (Table 3.3).

The synthetic result of a 1–47 region peptide **66a** (deleting the third β-sheet domain) is shown Figure 3.5. NCL of the SEAlide peptide **69**[10, 11] and the N-terminal cysteinyl peptide **70a** was performed in 6 M Gn·HCl–0.5 M phosphate buffer (pH 6.8) in the presence of 40 mM MPAA and 30 mM TCEP at 37 ºC for 15 h, and this was followed by HPLC purification to isolate the ligated peptide **71a** in 71% isolated yield. This product **71a** was subjected to the desulfurization step under 6 M Gn·HCl–0.1 M phosphate buffer (pH 3.5) with 40 mM VA-044, 40 mM glutathione and 100 mM TCEP at 37 ºC for 3 h. After HPLC purification, the desired peptide **66a** was obtained in 63% isolated yield. The synthesis of other deletion peptides **67a** in which the middle region of loop domain was deleted, **68a** in which the N-terminal loop domain was deleted, and biotinylated peptides (**66b**, **67b** and **68b**) was conducted with basically the same procedure as was used for **66a** and mentioned in Figure 3.5.

Table 3.3. Sequence of CXCL14 derivatives: deletion of the 1–50 region from N- or C-termini.

Region	Compound name	Sequence
1–50[a,b]	**50a**: **X** = Gly-$CONH_2$ **50b**: **X** = Lys(bio)-$CONH_2$	^{1}SK**C**K**C**SRKGPKIRYSDVKKLEMKPKYPH**C**EEKMVIITTKS^{29} VSRYRGQEH**C**–**X**^{50}
1–47[b]	**66a**: **X** = Gly-$CONH_2$ **66b**: **X** = Lys(bio)-$CONH_2$	^{1}SK**A**K**A**SRKGPKIRYSDVKKLEMKPKYPH**A**EEKMVIITTKS VSRYRGQ–**X**^{47}
1–40[b]	**67a**: **X** = Gly-$CONH_2$ **67b**: **X** = Lys(bio)-$CONH_2$	^{1}SK**A**K**A**SRKGPKIRYSDVKKLEMKPKYPH**A**EEKMVIITTKS^{40}–**X**
13–50[b]	**68a**: **X** = Gly-$CONH_2$ **68b**: **X** = Lys(bio)-$CONH_2$	^{13}RYSDVKKLEMKPKYPH**A**EEKMVIITTKSVSRYRGQEH**A**^{50}–**X**

[a]Disulfide bonds between Cys^3 and Cys^{29}, Cys^5 and Cys^{50}. [b]N-Terminal: NH_2.

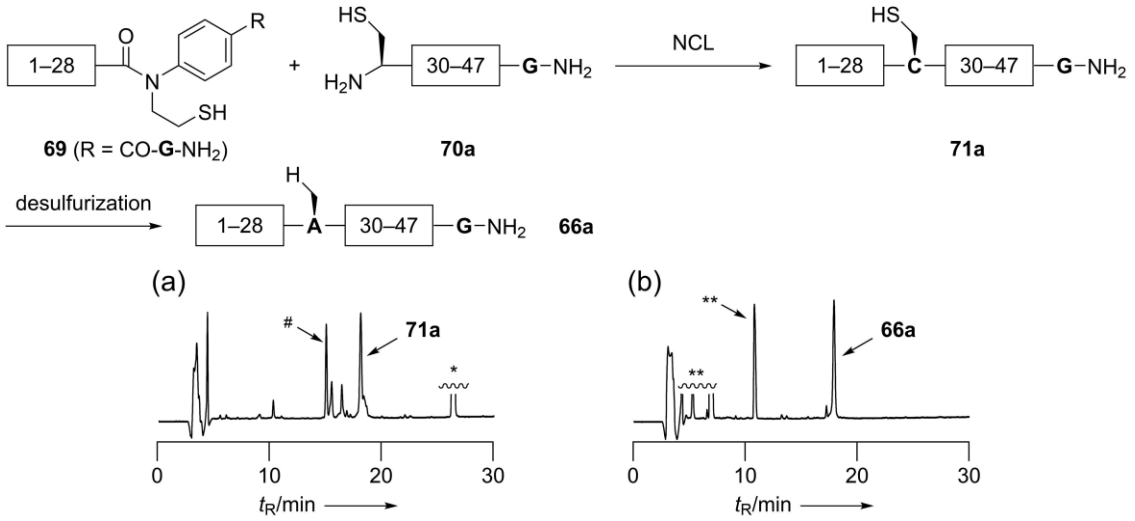


Figure 3.5. HPLC monitoring of the synthesis of the 1–47 region peptide **66a**: (a) NCL (t = 15 h); (b) desulfurization (t = 3 h). Analytical HPLC conditions: linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 45% over 30 min. *MPAA, **non-peptidic compounds derived from additives, #released SEAlide unit.

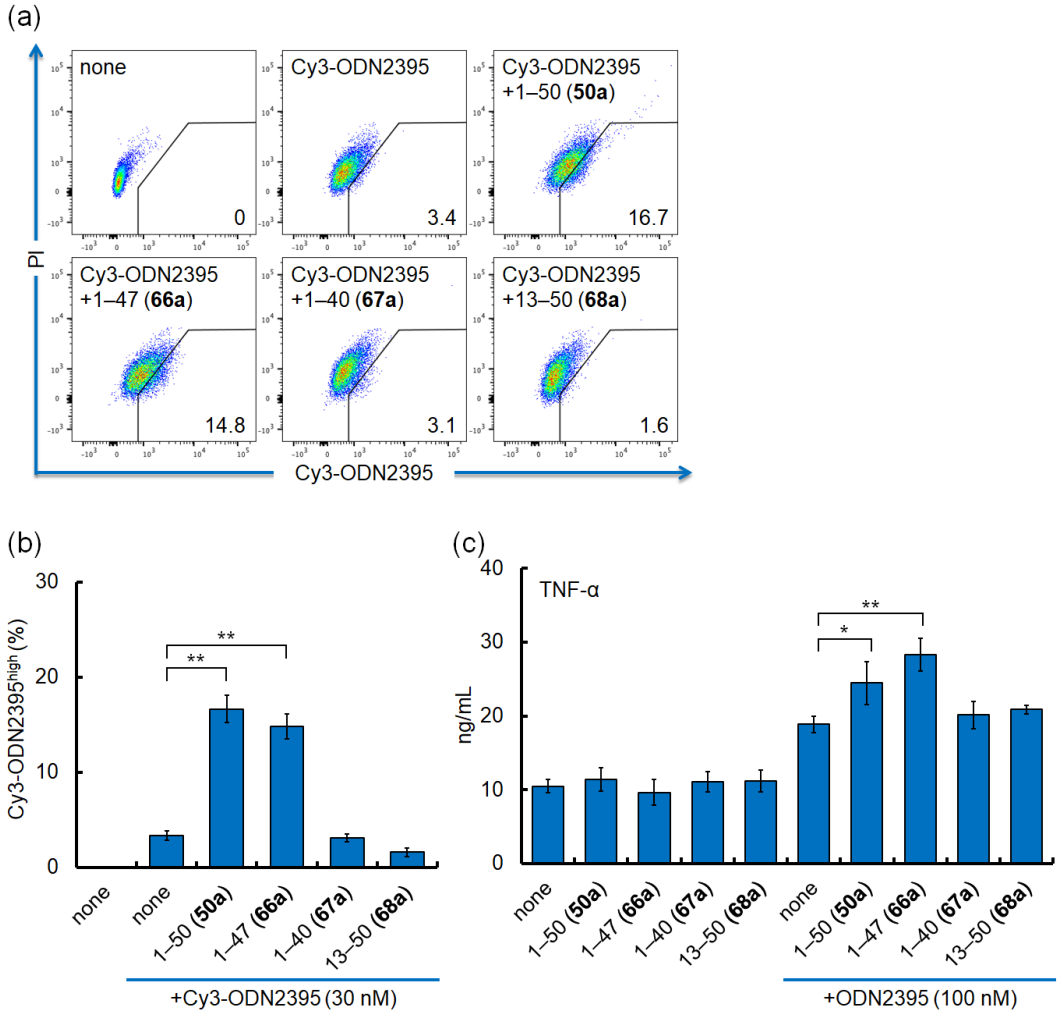
I next conducted the biological assays using synthesized deletion peptides (**66**, **67** and **68**). Initially, the FACS-based internalization assay was performed with Cy3-ODN2395 and synthetic peptides. Raw 264.7 cells were incubated with 30 nM Cy3-ODN2395 and 300 nM of peptides (**66a**, **67a** and **68a**) at 37 ºC for 1 h (Figure 3.6a and 3.6b). The 1–47 region peptide **66a** showed the same intracellular uptake activity of Cy3-ODN2395 as the 1–50 region peptide **50a**. In contrast, the enhancement of intracellular uptake was not observed in the 1–40 region peptide **67a** or the 13–50 region peptide **68a**.

Evaluation of the cytokine production by TLR9 activation was performed with synthetic peptides. Raw 264.7 cells were treated with 100 nM ODN2395 in the presence or absence of 300 nM of peptides (**66a**, **67a** and **68a**) at 37 ºC. After 6 h, culture supernatants were subjected to ELISA for TNF-α (Figure 3.6c). The 1–47 region peptide **66a** induced the production of TNF-α in the presence of ODN2395, but the 1–40 region **67a** and 13–50 region peptides **68a** failed to do so.

Next, I checked whether the deletion peptides were directly bound to CpG DNA. Biotinylated peptides (**66b**, **67b** and **68b**) (100 pmol) were coupled with streptavidin-agarose, and then treated with 100 nM of Cy3-ODN2395 at 4 ºC for 1 h. The precipitate containing Cy3-ODN2395 and peptide was visualized by gel electrophoresis (SDS-PAGE) (Figure 3.6d). Cy3-ODN2395 was pulled-down with the 1–47 or the 1–40 region

peptide (**66b** and **67b**). The 13–50 region peptide **68b** was not bound to Cy3-ODN2395. In particular, the K_d value calculated by Scatchard plot analysis of the 1–47 region peptide **66b** was 175 nM (Figure 3.6e), which was comparable to that of the 1–50 region peptide [K_d: 103 nM (**50b**)].

To summarize these results, the 1–12 region, the loop domain of CXCL14 is responsible for the CpG DNA binding (Figure 3.1: I), and the 41–47 region, the loop domain of CXCL14 is responsible for activation of TLR9 by CpG ODN uptake through receptor X (Figure 3.1: II and III).



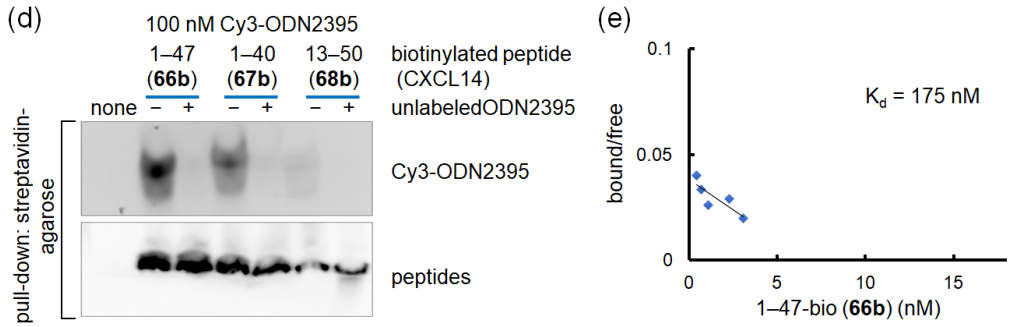


Figure 3.6. Biological evaluation of synthetic peptides on the CpG DNA uptake and activity. (a) FACS analysis of Raw 264.7 cells incubated with 30 nM Cy3-ODN2395 in the presence or absence of 300 nM peptide at 37 ºC for 1 h. The PI negative fraction was gated to select live cells. (b) Percentages of Cy3-ODN2395^{high} cell fraction were calculated from FACS profiles shown in (a). (c) Raw 264.7 cells were treated with 100 nM ODN2395 in the presence or absence of 300 nM peptide at 37 ºC for 6 h. Culture media were collected and analyzed by ELISA for TNF-α. (d) Cy3-ODN2395 (100 nM) was incubated with 100 pmol of biotinylated peptide coupled with streptavidin-agarose in the presence or absence of unlabeled 3 μM of ODN2395 at 4 ºC for 1 h. Unlabeled ODN2395 blocked binding to the peptides, indicating the specific binding. ODN2395 and peptide in the precipitate were visualized by gel electrophoresis and Western blotting. (e) Scatchard plot analyses. Various concentrations of Cy3-ODN2395 were pulled-down with biotinylated peptides and streptavidin-agar at 4˚C for 1 h. Cy3-fluorescence of each bound and free fraction was quantified. In (b) and (c), each value represents the mean ± SE (n = 3), and P-value ($^{*}P$<0.05, $^{**}P$<0.01, otherwise not significant) compares with the condition treated with Cy3-ODN2395 alone. All statistical analyses were performed using Microsoft Excel and an unpaired Student's t-test.

3.5 Conclusion

The internalization-enhancing activity of CpG DNA was observed only in the N-terminal region (1–47) of CXCL14, which is the smallest unit. The C-terminal region (50–77) of CXCL14 had no ability to enhance the internalization of CpG DNA, but assisted the formation of CXCL14–CpG DNA complex with high affinity.

Furthermore, based on the results of the deletion peptides, it became clear that the two loop domains, the 1–12 and 41–47 regions in the N-terminal region (1–50) of CXCL14 are involved in the DNA binding and receptor recognition, respectively.

Chapter 4

Conclusion

1. An epimerization-less-prompting procedure for the synthesis of peptide thioesters using a resin-bound SEAlide peptide was developed. This procedure can be applied to the synthesis of 28-residue N-terminal fragment of CXCL14 with its application to the synthesis of CXCL14 in the one-pot/N−to−C-directed sequential manner. Furthermore, a new version of the one-pot/three-fragment ligation method using the difference in chemical behavior of resin-bound and soluble SEAlide peptides was established.

2. A practical procedure for the copper-mediated ring-opening of thiazolidine and selenazolidine derivatives was developed. This procedure was successfully applied to the synthesis of CXCL14 via a C−to−N-directed sequential NCL protocol. The features of this procedure are the use of inexpensive copper reagents and the ability to perform reactions in a short time (< 1 h) under an ambient atmosphere.

3. The N-terminal region (1–47) of CXCL14 was identified as the smallest unit that can enhance the activity of CpG DNA, and the role of the C-terminal region (50–77) was found to assist in the formation of CXCL14–CpG DNA complexes. Two loop domains (the 1–12 and 41–47 regions) were found to function as DNA binding and receptor recognizing regions, respectively.

Taken together, these developed procedures solve issues in protein chemical synthesis and can be applied to the synthesis of CXCL14 in N–to–C- and C–to–N-directed sequential manners. Also, the developed synthesis procedures enabled efficient synthesis of CXCL14, and I have gained clues to the elucidate of the role of CXCL14. Currently, further studies are in progress in my laboratory. Finally, it is hoped that these developed procedures will further advance the field of protein chemical synthesis.

Experimental section

General information

General experimental

All commercially available chemical reagents and protected amino acids were purchased and used without further purification. NCL reactions were carried out under an atmosphere of argon. All reagents and solvents were obtained from the FUJIFILM Wako Pure Chemical Corporation, Tokyo Chemical Industry Co., Ltd., Kishida Chemical Co., Ltd., Peptide Institute, Inc., CEM Corporation, Watanabe Chemical Industries, Ltd., Nacalai Tesque, Inc., Kanto Chemical Co., Inc., CS bio Co., Merck KGaA and Sigma-Aldrich Co. LLC.

HPLC and MS

Mass spectra were recorded on a Waters MICROMASS® LCT PREMIER™ (ESI-TOF) or LCMS (Shimadzu, Japan, Prominence-I LC-2030, LCMS-2020, a Cosmosil 5C_{18}-AR-II analytical column (Nacalai Tesque, Japan, 4.6×250 mm, flow rate 1.0 mL/min, eluting products were detected by UV at 220 nm and MS). For HPLC separation, a Cosmosil 5C_{18}-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL/min), a Cosmosil 5C_{18}-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL/min), or a Cosmosil 5C_{18}-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% (v/v) TFA in H_2O and 0.1% (v/v) TFA in CH_3CN, was used for HPLC elution.

Solid-phase peptide synthesis

Manual peptide synthesis by Boc SPPS was performed on 4-methylbenzhydrylamine (MBHA) resin. The peptide chain was elongated using *in situ* neutralization protocols[3b] [acylation: Boc-amino acid–DIPCDI–HOBt·H_2O–DIPEA (4:4:4:2 equiv) in DMF for 2 h; Boc removal: TFA–anisole–toluene [50:2:48 (v/v)] for 30 min. The following Boc amino acids were employed: L-Ala-OH, D-Ala-OH, L-Cys(Acm)-OH, L-Cys(4-

MeOBzl)-OH, L-Asp(OBzl)-OH, L-Glu(OBzl)-OH, L-Phe-OH, L-Gly-OH, L-His(Bom)-OH, D-His(Bom)-OH, L-Ile-OH, L-Lys(Cl-Z)-OH, L-Leu-OH, L-Met-OH, L-Asn-OH, L-Pro-OH, L-Gln-OH, L-Arg(Mts)-OH, L-Ser(Bzl)-OH, D-Ser(Bzl)-OH, L-Thr(Bzl)-OH, L-Val-OH, L-Trp-OH, Tyr(Br-Z)-OH.

Manual peptide synthesis by Fmoc SPPS was performed on ChemMatrix®, 2-chlorotrityl chloride, NovaSyn® TGR or Wang resin. The peptide chain was elongated using SPPS [acylation: Fmoc-amino acid–DIPCDI–HOBt·H_2O (4:4:4 equiv) or Fmoc-amino acid–HBTU–DIPEA (4:3.9:8 equiv) in DMF for 2 h; Fmoc removal: 20% (v/v) piperidine in DMF for 10 min]. The following Fmoc amino acids were employed: L-Ala-OH, L-Cys(Acm)-OH, L-Cys(Trt)-OH, D-Cys(Trt)-OH, L-Asp(O*t*-Bu)-OH, L-Glu(OAllyl)-OH, L-Glu(O*t*-Bu)-OH, L-Phe-OH, L-Gly-OH, L-His(MBom)-OH,[18] L-His(Trt)-OH, L-Ile-OH, L-Lys(Alloc)-OH, L-Lys(Boc)-OH, L-Lys(ivDde)-OH, L-Leu-OH, L-Met-OH, L-Asn(Trt)-OH, L-Pro-OH, L-Gln(Trt)-OH, L-Arg(Pbf)-OH, L-Ser(*t*-Bu)-OH, L-Thr(*t*-Bu)-OH, L-Val-OH, L-Trp(Boc)-OH, L-Tyr(*t*-Bu)-OH, L-Val-Ser($\Psi^{Me,Me}$pro)-OH.

Automated peptide synthesis by Fmoc SPPS was performed on ChemMatrix®, 2-chlorotrityl chloride, NovaSyn® TGR or Wang resin using an automated peptide synthesizer (CS336X, CSBio). The peptide chain was elongated using SPPS [acylation: Fmoc-amino acid–HBTU–DIPEA (10:9.9:20 equiv) in NMP for 30 min; Fmoc removal: 20% (v/v) piperidine in NMP for 10 min]. The following amino acids were employed same as manualized method.

Experimental section

Chapter 1

S1.1 Preparation of peptide thioesters using resin-bound SEAlide peptides

Synthesis of resin-bound SEAlide peptides 10

Typical procedure: On aminomethyl ChemMatrix® resin (~1.0 mmol amine/g, 5.0 mmol) was coupled Fmoc-L-Phe-OH (5.0 equiv, 25 mmol) with the aid of DIPCDI (5.0 equiv, 25 mmol) and HOBt·H_2O (5.0 equiv, 25 mmol) in DMF at room temperature. After 24 h, content of amine of the internal standard (Phe)-incorporated resin was analyzed by quantification of the Fmoc group (0.43 mmol amine/g, 3.1 mmol). Then unreacted amino group of the resin was capping by acetic anhydride and pyridine (each at 10 equiv, 31 mmol) in DMF and subsequent Fmoc removal by 20% (v/v) piperidine in DMF afforded a Phe-incorporated resin. To vary the initial amine content of the resin, reaction conditions (equivalent of amino acid and reaction time) were changed. For the preparation of 0.27 mmol amine/g resin, 0.50 equiv of Fmoc-L-Phe-OH was coupled for 24 h. Coupling of 5.0 equiv of Fmoc-L-Phe-OH for 48 h afforded 0.58 mmol amine/g resin. The resulting resin (0.43 mmol amine/g, 0.1 mmol) was treated with **8a** (4.0 equiv, 0.40 mmol), HATU (3.6 equiv, 0.36 mmol) and DIPEA (8.0 equiv, 0.80 mmol) to yield an anilide-linked resin. The peptide chain was elongated on this resin using a manual Fmoc SPPS procedure as described in the General information. The completed resin (0.03 mmol) was treated with TFA–TES–H_2O [95:2.5:2.5 (v/v), 50 μL/1.0 mg resin] at room temperature for 2 h. The deprotected resin was washed with DMF and CH_2Cl_2 (3 times). The resulting resin was successively dried in vacuo to get the resin-bound SEAlide peptide **10a** (0.03 mmol).

Synthesis of peptide thioesters 11

Typical procedure: Resin-bound SEAlide peptide **10a** (20 mg, 6.3 μmol) was treated with 0.2 M sodium phosphate buffer (pH 4.0, 1.0 mL, 50 μL/1.0 mg resin) in the presence of 5% (v/v) MPA, 40 mM TCEP·HCl and 50 mM sodium ascorbate at 50 ºC. After 12 h, filtration of the reaction mixture followed by HPLC purification gave **11a** (3.4 μmol) in 53% isolated yield.

Table S1.1. Characterization data of model thioester peptides.

Peptide	Analytical HPLC[a]		Preparative HPLC[b]	*m/z*		Yield (%)[d]
	RT[e] (min)	Gradient (%)	Gradient (%)	Calcd	Found	
11a	18.2	5 to 35	5 to 35[c]	795.4 $[M+H]^+$	795.4	53
11b	15.6	5 to 35	5 to 35[c]	781.4 $[M+H]^+$	781.4	57
11c	14.4	5 to 35	5 to 35[c]	852.4 $[M+H]^+$	852.4	58
11d	16.0	5 to 35	5 to 35[c]	440.7 $[M+2H]^{2+}$	440.7	63
11e	15.5	5 to 35	5 to 35[c]	811.4 $[M+H]^+$	811.4	72
11f	14.8	5 to 35	5 to 35[c]	838.4 $[M+H]^+$	838.4	33
11g	14.3	5 to 35	5 to 35[c]	861.4 $[M+H]^+$	861.4	33
11h	23.3	5 to 35	10 to 35[c]	837.4 $[M+H]^+$	837.4	43
11i	25.9	5 to 35	10 to 35[c]	871.4 $[M+H]^+$	871.4	52
11j	21.9	5 to 35	10 to 35[c]	823.4 $[M+H]^+$	823.4	43

0.1% TFA in H_2O (v/v, solvent A) and 0.1% TFA in CH_3CN (v/v, solvent B) was used for HPLC elution over 30 min. [a]Cosmosil $5C_{18}$-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min. [b]Cosmosil $5C_{18}$-AR-II preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [c]Cosmosil $5C_{18}$-AR-II semi-preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [d]Isolated yield. [e]Retention time.

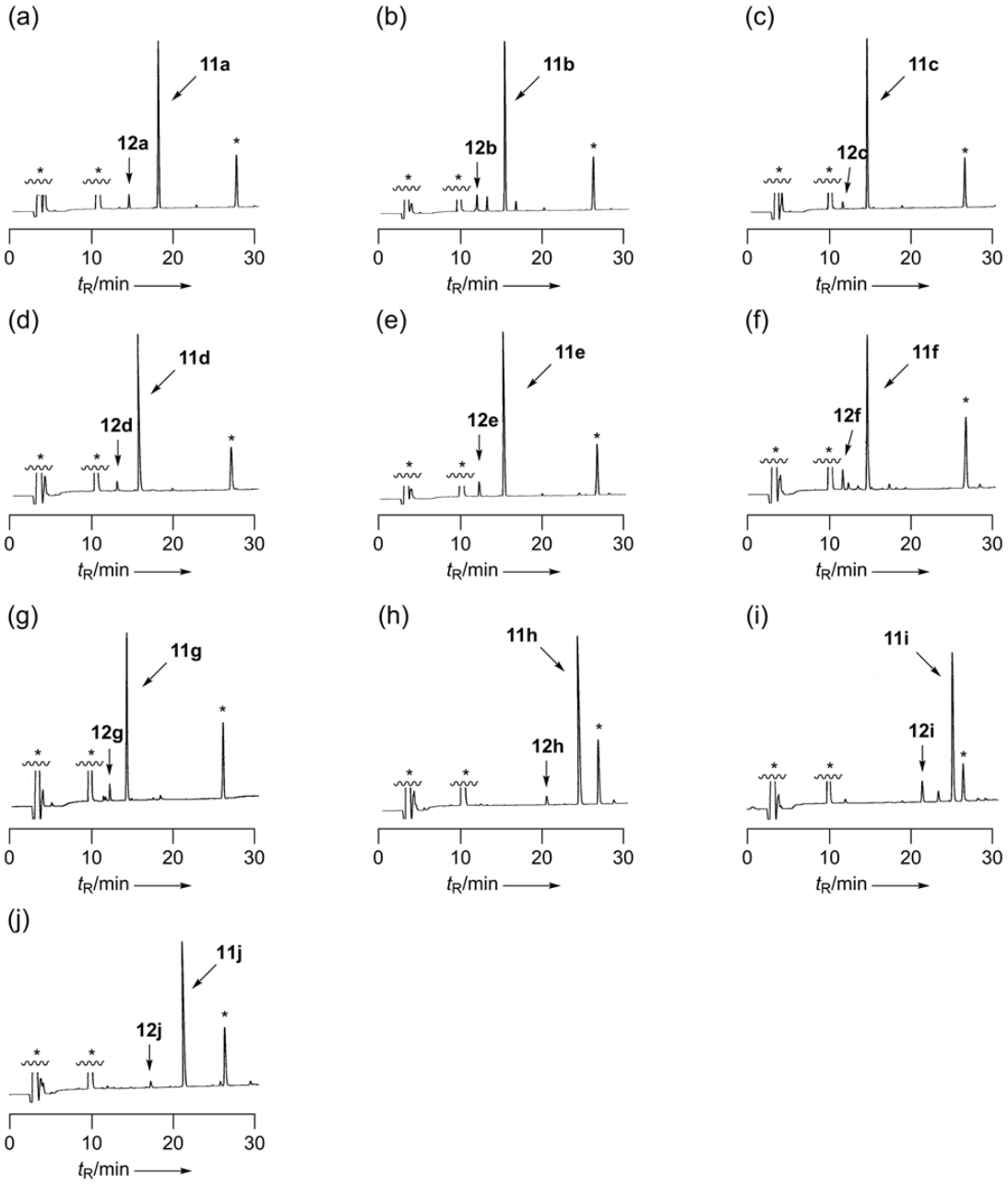
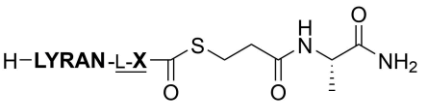


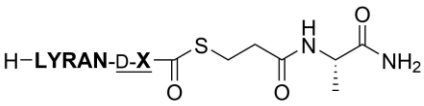
Figure S1.1. Analytical HPLC charts of crude reaction materials: (a) thioesterification of **10a** (t = 12 h); (b) thioesterification of **10b** (t = 6 h); (c) thioesterification of **10c** (t = 12 h); (d) thioesterification of **10d** (t = 12 h); (e) thioesterification of **10e** (t = 12 h); (f) thioesterification of **10f** (t = 12 h); (g) thioesterification of **10g** (t = 1 h); (h) thioesterification of **10h** (t = 24 h); (i) thioesterification of **10i** (t = 24 h); (j) thioesterification of **10j** (t = 72 h). Analytical HPLC conditions: linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 35% over 30 min. *Non-peptidic compounds derived from additives.

S1.2 Examination of epimerization during thioesterification of resin-bound SEAlide peptides

Synthesis of peptide thioesters (S1 and S2)



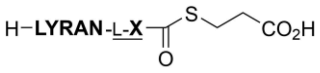
S1a (**X** = Ala), **S1e** (**X** = Ser), **S1g** (**X** = His)



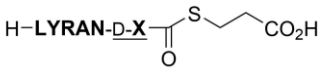
S2a (**X** = Ala), **S2e** (**X** = Ser), **S2g** (**X** = His)

Typical procedure: On MBHA resin (0.70 mmol amine/g, 0.09 mmol) was coupled Boc-L-Ala-OH (5.0 equiv, 0.45 mmol) with the aid of DIPCDI (5.0 equiv, 0.45 mmol), HOBt·H_2O (5.0 equiv, 0.45 mmol) and DIPEA (2.0 equiv, 0.18 mmol) in DMF at room temperature for 2 h and subsequent Boc removal by TFA–anisole–toluene [50:2:48 (v/v), 30 min] afforded an Ala-incorporated resin. The resin then was treated with *S*-Trt mercaptopropionic acid (5.0 equiv, 0.45 mmol), DIPCDI (5.0 equiv, 0.45 mmol), HOBt·H_2O (5.0 equiv, 0.45 mmol) and DIPEA (2.0 equiv, 0.18 mmol) in DMF at room temperature for 2 h and subsequent Trt removal by TFA–TES [95:5 (v/v), 30 min] gave a HS-CH_2CH_2CO-Ala-MBHA resin. On the resulting resin, the peptide chain was elongated using a manual Boc SPPS procedure as described in the General information. The completed resin (50 mg, 0.03 μmol) was treated with 1 M TMSOTf–thioanisole in TFA–*m*-cresol–EDT [100:5:5 (v/v), 50 μL/1.0 mg resin] at 4 °C for 2 h. The resin was filtered off, and the filtrate was directly added to cold Et_2O to generate precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O followed by lyophilization to afford **S1a**.

Thioesterification of peptide thioesters (S3 and S4)



S3a (**X** = Ala), **S3g** (**X** = His)



S4a (**X** = Ala), **S4g** (**X** = His)

Typical procedure: Thioesterification of **S1a** (1 mM) in 0.1 M HEPPS buffer (pH 4.1) in the presence of 5% (v/v) MPA was conducted at 37 °C for 1 h to give **S3a**.

Synthesis of peptides (S5 and S6)

H−**LYRAN**-L-**S**−**CYRANK**−NH_2 **S5**

H−**LYRAN**-D-**S**−**CYRANK**−NH_2 **S6**

NCL between **11e**, **S1e** or **S2e** (1 mM) and H-**CYRANK**-NH_2[9q] (2.5 mM) in 6 M Gn·HCl–0.2 M HEPPS buffer (pH 6.8) in the presence of 40 mM MPAA and 40 mM TCEP·HCl was conducted at 37 °C for 2 h to give **S5** or **S6**.

Table S1.2. Characterization data of peptides.

Peptide	Analytical HPLC[a]		Preparative HPLC[b]	*m/z*		Yield (%)[c]
	RT[d] (min)	Gradient (%)	Gradient (%)	Calcd	Found	
S1a	15.6	5 to 35	8 to 18	433.2 $[M+2H]^{2+}$	433.3	13
S1e	14.5	5 to 35	8 to 18	881.4 $[M+H]^{+}$	881.3	16
S1g	14.1	5 to 35	5 to 15	466.2 $[M+2H]^{2+}$	466.3	3
S2a	17.2	5 to 35	8 to 18	433.2 $[M+2H]^{2+}$	433.3	13
S2e	14.6	5 to 35	8 to 18	881.4 $[M+H]^{+}$	881.3	13
S2g	14.0	5 to 35	5 to 15	466.2 $[M+2H]^{2+}$	466.3	18
S3a	18.4	5 to 35	—	795.4 $[M+H]^{+}$	795.4	—
S3g	16.0	5 to 35	—	861.4 $[M+H]^{+}$	861.4	—
S4a	18.7	5 to 35	—	795.4 $[M+H]^{+}$	795.4	—
S4g	16.3	5 to 35	—	861.4 $[M+H]^{+}$	861.4	—
S5	15.8	5 to 35	—	729.4 $[M+2H]^{2+}$	729.3	—
S6	15.8	5 to 35	—	729.4 $[M+2H]^{2+}$	729.3	—

0.1% TFA in H_2O (v/v, solvent A) and 0.1% TFA in CH_3CN (v/v, solvent B) was used for HPLC elution over 30 min. [a]Cosmosil $5C_{18}$-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min. [b]Cosmosil $5C_{18}$-AR-II preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [c]Isolated yield. [d]Retention time.

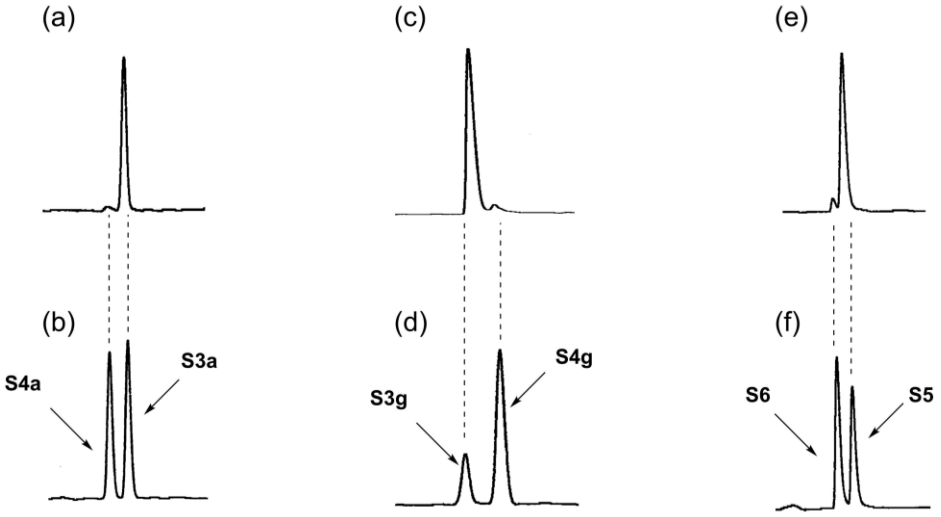


Figure S1.2. Verification of epimerization of C-terminal chiral amino acids during N–S acyl transfer mediated thioesterification: (a) crude reaction chart of thioesterification of **10a** (t = 12 h); (b) co-injection **S3a** with **S4a**; (c) crude reaction chart of thioesterification of **10g** (t = 1 h); (d) co-injection **S3g** with **S4g**; (e) crude reaction chart of NCL between **11e** and H-**CYRANK**-NH_2 (t = 2 h); (f) co-injection **S5** with **S6**. Analytical HPLC conditions of charts (a) and (b): linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 11% to 15% over 60 min. Analytical HPLC conditions of charts (c) and (d): linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 7% to 10% over 30 min. Analytical HPLC conditions of charts (e) and (f): linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 7% to 10% over 30 min. Only a critical retention time region of the HPLC charts was enlarged.

S1.3 Chemical synthesis of CXCL14 via N–to–C-directed sequential ligation

Synthesis of N-terminal fragment 16

Resin-bound SEAlide peptide **15** (30 mg, 4.1 μmol) was treated with 0.2 M sodium phosphate buffer (pH 4.0, 1.5 mL, 50 μL/1.0 mg resin) in the presence of 5% (v/v) MPA, 40 mM TCEP·HCl and 50 mM sodium ascorbate at 50 ºC. After 1 h, filtration of the reaction mixture followed by HPLC purification gave **16** (0.7 μmol) in 17% isolated yield.

Synthesis of middle fragment 17

Middle fragment **17** was prepared by standard Fmoc SPPS protocol on NovaSyn® TGR

resin (0.24 mmol amine/g) by procedures identical to those reported.[13]

Synthesis of C-terminal fragment 19

C-Terminal fragment **19** was prepared by standard Fmoc SPPS protocol on Fmoc-Glu(O*t*-Bu)-Wang resin (0.24 mmol acid/g) by procedures identical to those reported.[13]

Synthesis of human CXCL14 using one-pot/N–to–C-directed sequential NCL

The first NCL of **16** (0.84 μmol, 1 mM) with **17** (0.84 μmol, 1 mM) in 6 M Gn·HCl–0.2 M HEPPS buffer (pH 6.7, 0.84 mL) in the presence of 50 mM MPAA and 30 mM TCEP·HCl at 37 ºC for 3 h afforded the ligated the amide-type SEAlide peptide **18**. Then, the addition of cysteine fragment **19** (0.84 μmol) in 1 M sodium phosphate buffer (pH 6.6, 0.84 mL; final concentration of **18** and **19**, 0.5 mM) into the reaction mixture initiated the second NCL of thioester form of **18** with **19** at 37 ºC for 24 h to give fully ligated peptide **20** (0.26 μmol) in 31% isolated yield in one-pot manner. Air oxidation of the resulting **20** (0.26 μmol, 0.05 mM) in 3 M Gn·HCl–0.1 M sodium phosphate buffer (pH 7.7, 5.2 mL) was conducted at 37 ºC for 24 h to give human CXCL14 (0.13 μmol) in 50% isolated yield.

Table S1.3. Characterization data of CXCL14-derived peptides.

Peptide	Analytical HPLC[a]		Preparative HPLC[b]	*m/z*		Yield (%)[d]
	RT[e] (min)	Gradient (%)	Gradient (%)	Calcd	Found	
16	17.5	5 to 45	10 to 30[c]	856.5 $[M+4H]^{4+}$	856.4	17
17	22.3	5 to 45	21 to 28	1393.2 $[M+2H]^{2+}$	1393.1	11
19	20.5	5 to 45	15 to 35	1208.3 $[M+3H]^{3+}$	1208.5	74
20	22.5	5 to 45	20 to 40[c]	1569.8 $[M+6H]^{6+}$	1571.3	31
CXCL 14	20.0	5 to 45	20 to 35[c]	1569.2 $[M+6H]^{6+}$	1570.5	50

0.1% TFA in H_2O (v/v, solvent A) and 0.1% TFA in CH_3CN (v/v, solvent B) was used for HPLC elution over 30 min. [a]Cosmosil $5C_{18}$-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min. [b]Cosmosil $5C_{18}$-AR-II preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [c]Cosmosil $5C_{18}$-AR-II semi-preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [d]Isolated yield. [e]Retention time.

S1.4 Application of the different chemical behavior of resin-bound and soluble SEAlide peptides to one-pot/three-fragment ligation

Synthesis of N-terminal cysteine SEAlide peptide 21

The synthesis of **21** was performed on NovaSyn® TGR resin (0.25 mmol amine/g, 0.10 mmol) using a manual Fmoc SPPS procedure as described in the General information. The completed resin (300 mg, 53.1 μmol) was treated with TFA–*m*-cresol–EDT–thioanisole–H_2O [80:5:5:5:5 (v/v), 50 μL/1.0 mg resin] at room temperature for 2 h. The resin was filtered off, and the filtrate was directly added to cold Et_2O to generate precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide. The crude peptide was analyzed by analytical HPLC and purified by preparative HPLC.

Synthesis of peptide 24 using one-pot/three-fragment ligation

The first NCL of **10a** (16.5 mg, 5.0 μmol) with **21** (1.0 μmol, 1 mM) in 6 M Gn·HCl–0.1 M HEPPS buffer (pH 6.9, 1.0 mL) in the presence of 40 mM MPAA and 40 mM TCEP·HCl at 37 ºC for 12 h afforded the ligated the amide-type SEAlide peptide **22**. After the peptide resin was filtered out, the addition of **23** (1.0 μmol) in 0.5 M sodium phosphate buffer (pH 6.6, 1.0 mL; final concentration of **22** and **23**, 0.5 mM) into the reaction mixture initiated the second NCL of thioester form of **22** with **23**. After 24 h, the crude material was purified by semi-preparative HPLC to give **24** (0.40 μmol) in 40% isolated yield in one-pot manner.

Table S1.4. Characterization data of peptides.

Peptide	Analytical HPLC[a]		Preparative HPLC[b]	*m/z*		Yield (%)[d]
	RT[e] (min)	Gradient (%)	Gradient (%)	Calcd	Found	
21	20.8	5 to 35	13 to 28	523.2 $[M+2H]^{2+}$	523.3	43
24	17.4	5 to 55	10 to 30[c]	697.0 $[M+3H]^{3+}$	697.0	40

0.1% TFA in H_2O (v/v, solvent A) and 0.1% TFA in CH_3CN (v/v, solvent B) was used for HPLC elution over 30 min. [a]Cosmosil 5C_{18}-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min. [b]Cosmosil 5C_{18}-AR-II preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [c]Cosmosil 5C_{18}-AR-II semi-preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [d]Isolated yield. [e]Retention time.

S1.5 Examination of recycling of the resin-bound SEAlide peptide

The first round NCL of resin-bound SEAlide peptide **10a** (6.0 mg, 2.0 μmol) and N-Cys SEAlide peptide **21** (0.2 μmol, 1 mM) was performed in 6 M Gn·HCl–0.1 M HEPPS buffer (pH 6.9, 0.2 mL) in the presence of 40 mM MPAA and 40 mM TCEP·HCl at 37 ºC. After 12 h, the completion of reaction was checked by analytical HPLC. The peptide resin was filtered out and regenerated by treatment with 40 mM TCEP·HCl and 50 mM sodium ascorbate in 6 M Gn·HCl (pH 3.2) for 2 h at 37 °C. Resulting regenerated resin **10a** (6.0 mg, 1.8 μmol) was subjected to a second round NCL with **21** (0.18 μmol, 1 mM) in 6 M Gn·HCl–0.1 M HEPPS buffer (pH 6.9, 0.18 mL) in the presence of 40 mM MPAA and 40 mM TCEP·HCl at 37 ºC, and the attempted reaction was continued for 42 h to go to completion.

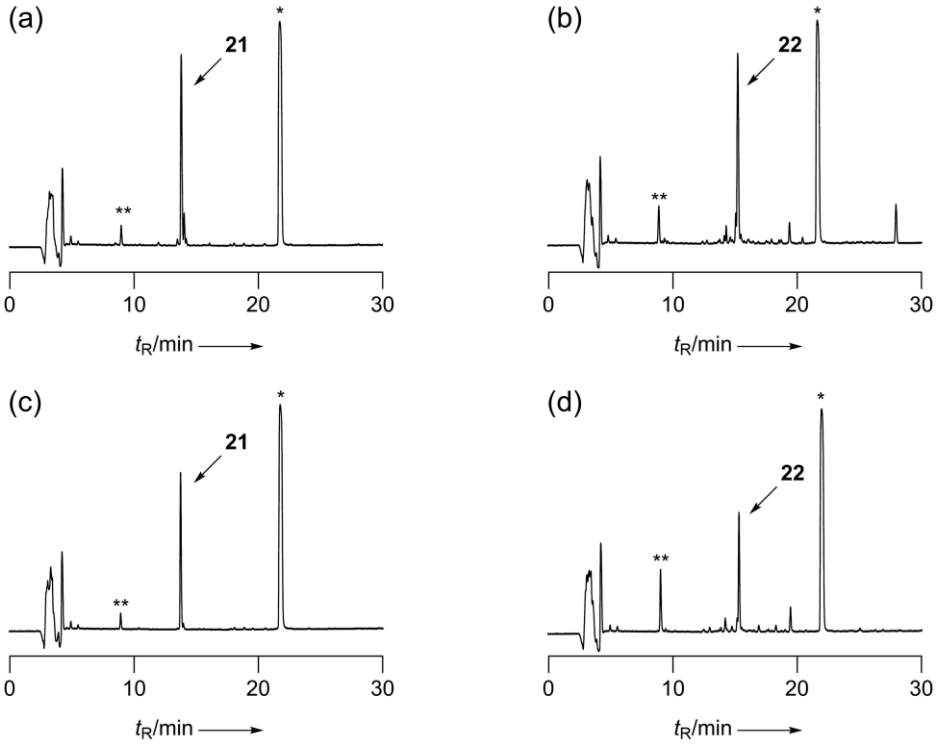


Figure S1.3. HPLC monitoring of recycling of resin-bound SEAlide peptide to NCL: (a) first round NCL (t < 5 min); (b) first round NCL (t = 12 h); (c) second round NCL (t < 5 min); (d) second round NCL (t = 42 h). Analytical HPLC conditions: linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 55% over 30 min. *MPAA, **non-peptidic compounds derived from additives.

Experimental section

Chapter 2

S2.1 Examination of SEAlide-based solid-phase chemical ligation

Synthesis of model alkine-containing SEAlide peptide 26

The synthesis of **26** was performed on NovaSyn® TGR resin (0.22 mmol amine/g, 0.05 mmol) using an automated Fmoc SPPS procedure as described in the General information. The completed resin (50 mg, 8.3 μmol) was treated with TFA–triisopropylsilane (TIPS)–H_2O [95:2.5:2.5 (v/v), 50 μL/1.0 mg resin] at room temperature for 2 h. The resin was filtered off, and the filtrate was directly added to cold Et_2O to generate precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide. The crude peptide was analyzed by LCMS and purified by preparative HPLC. For synthesis of N-terminal thiazolidine-containing peptide, Boc-Thz-OH was coupled.

Preparation of azide-loading resin 27

On Rink-amide ChemMatrix® resin (0.50 mmol amine/g, 0.10 mmol) was coupled Fmoc-L-Phe-OH (0.40 equiv, 40 μmol) with the aid of DIPCDI (0.40 equiv, 40 μmol) and HOBt·H_2O (0.40 equiv, 40 μmol) in DMF at room temperature. After 2.5 h, content of amine of the Phe-incorporated resin was checked by quantification of the Fmoc group (0.11 mmol amine/g, 0.22 mmol). Then unreacted amino group of the resin was capping by acetic anhydride and pyridine (each at 10 equiv, 2.2 mmol) in DMF and subsequent Fmoc removal by 20% (v/v) piperidine in DMF yielded a Phe-incorporated resin. The resulting resin was treated with 4-azidobutanoic acid[51] (10 equiv, 2.2 mmol), DIPCDI (10 equiv, 2.2 mmol) in DMF at room temperature. After 2 h, the completion of reaction was confirmed by Kaiser test to obtained azide-loading resin **27**.

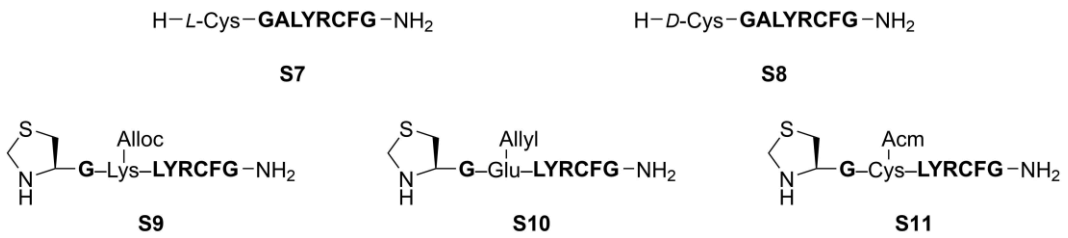
Click reaction between 26 and 27

Click reaction of alkine-containing SEAlide peptide **26** (1.1 μmol, 2.0 mM) and azide-loading resin **27** (10 mg, 1.1 μmol) was performed in 6 M Gn·HCl–0.1 M HEPPS buffer (pH 7.0, 0.51 mL) containing 20 mM tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), 20 mM $CuSO_4·5H_2O$ and 30 mM sodium ascorbate at 37 ºC for 6 h. The peptide resin was filtered out and washed by 6 M Gn·HCl aq., 0.25 M EDTA aq., H_2O

and CH_2Cl_2 (3 times). After removal of the solvent in vacuo, the resin (10 mg, 10 mg, 1.1 μmol) was treated with TFA–H_2O [95:5 (v/v), 50 μL/1.0 mg resin] at room temperature for 1 h. The resin was filtered off and the filtrate was directly added to cold Et_2O to generate precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide. The crude peptide was treated with 6 M Gn·HCl in the presence of 80 mM TCEP·HCl and 100 mM sodium ascorbate at 37 ºC for 1 h. Then, the crude peptide was analyzed by LCMS.

S2.2 Examination of copper-mediated ring-opening reaction of thiazolidine derivatives

Synthesis of model peptides (29, 35, S7, S8, S9, S10 and S11)



Typical procedure: The synthesis of model peptide **29** was performed on NovaSyn TGR® resin (0.22 mmol amine/g, 0.10 mmol) using an automated Fmoc SPPS procedure as described in the General information. The completed resin (150 mg, 41.1 μmol) was treated with TFA–TES–H_2O [95:2.5:2.5 (v/v), 50 μL/1.0 mg resin] at room temperature for 2 h. The resin was filtered off, and the filtrate was directly added to cold Et_2O to generate precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide. The crude peptide was analyzed by LCMS and purified by preparative HPLC. For synthesis of N-terminal thiazolidine-containing peptide, Boc-Thz-OH was coupled.

Optimization of reaction conditions for copper-mediated ring-opening of thiazolidine peptides (29 and 35)

Typical procedure: A solution of $CuSO_4·5H_2O$ (5.0 μL of 0.80 M; final concentration 40 mM) was added to **29** (1.0 mM) in 6 M Gn·HCl in 0.1 M HEPPS (or 0.1 M sodium phosphate) buffer (pH 7.0) in the presence of sodium ascorbate (40 mM) or in the absence of sodium ascorbate. The reaction was continued for 45 min at 37 °C and pH 5.0 under

ambient atmosphere. To quench the reaction with DTT, 400 equiv DTT (solid form) was added to the reaction with additional stirring for 15 min at 37 °C. After filtering off the precipitate, the filtrate was diluted with 10-fold of 0.1% TFA/H_2O and the resulting solution was analyzed by LCMS. Quenching with EDTA was carried out as follows: a 10-fold amount of EDTA solution (0.25 M) was added to the reaction mixture and the mixture was immediately analyzed by LCMS.

Examination of epimerization during ring-opening of thiazolidine derivative

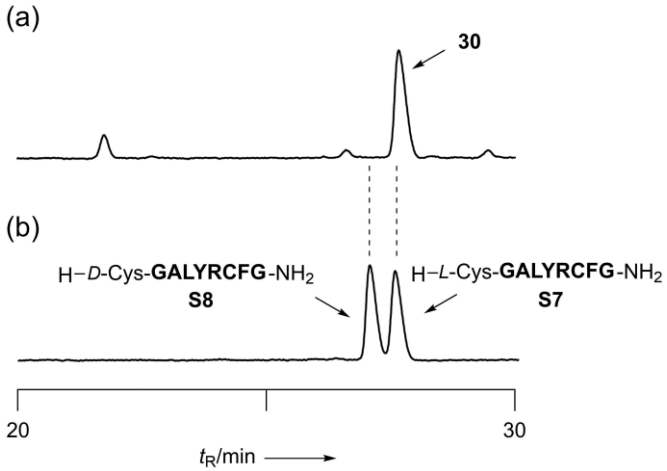


Figure S2.1. Verification of epimerization of N-terminal chiral amino acids during thiazolidine opening from model peptide **29**: (a) crude reaction chart of ring-opening of **29** (t = 1 h); (b) co-injection **S7** with **S8**. Analytical HPLC conditions: linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 15% to 25% over 30 min. Only a critical retention time region of the HPLC charts was enlarged.

Copper-mediated ring-opening of Lys(Alloc)-, Glu(OAllyl)- or Cys(Acm)-containing thiazolidine peptides (S9, S10 and S11)

Experimental procedures identical to those employed for the deprotection of peptide **29** were applied to the titled peptides (**S9, S10** and **S11**).

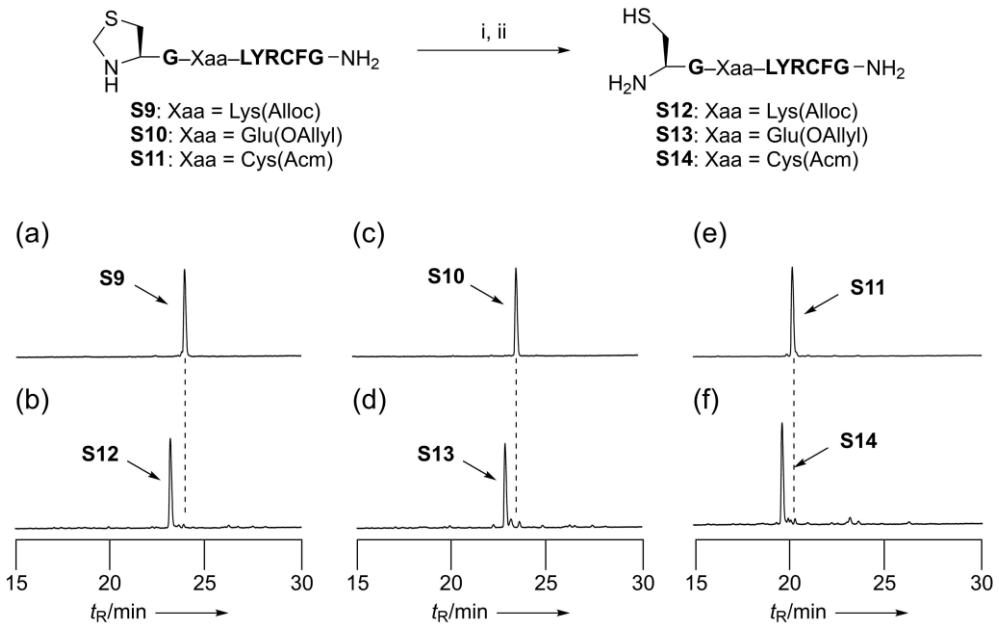


Figure S2.2. HPLC monitoring of the copper-mediated ring-opening of Lys(Alloc)-, Glu(OAllyl)- or Cys(Acm)-containing thiazolidine peptides (**S9, S10** and **S11**): (i) $CuSO_4 \cdot 5H_2O$ and sodium ascorbate (each at 40 mM) in 6 M Gn·HCl−0.1 M HEPPS buffer (pH 7.0) at 37 °C for 45 min; (ii) quenched with DTT (400 mM) at 37 °C for 15 min; (a) ring-opening of **S9** (t < 5 min); (b) ring-opening of **S9** (t = 1 h); (c) ring-opening of **S10** (t < 5 min); (d) ring-opening of **S10** (t = 1 h); (e) ring-opening of **S11** (t < 5 min); (f) ring-opening of **S11** (t = 1 h). Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 45% over 30 min. Only a critical retention time region of the HPLC charts was enlarged.

Table S2.1. Characterization data of peptides.

Peptide	Analytical HPLC[a]		Preparative HPLC[b]	*m/z*		Yield (%)[d]
	RT[e] (min)	Gradient (%)	Gradient (%)	Calcd	Found	
26	18.9	5 to 45	15 to 30	1095.5 $[M+H]^+$	1095.6	44
28	19.4	5 to 45	—	1358.6 $[M+H]^+$	1358.7	—
29	20.3	5 to 45	15 to 30	1000.5 $[M+H]^+$	1000.5	49
30	19.8	5 to 45	17 to 32	988.5 $[M+H]^+$	988.4	78
31	19.7	5 to 45	—	1020.4 $[M+H]^+$	1020.1	—
32a	18.8	5 to 45	—	986.4 $[M+H]^+$	986.5	—
32b	20.7	5 to 45	—	658.0 $[M+3H]^{3+}$	658.1	—
35	19.7	5 to 45	15 to 30	968.5 $[M+H]^+$	968.7	23
36	19.3	5 to 45	—	956.5 $[M+H]^+$	956.5	—
37	19.1	5 to 45	—	988.5 $[M+H]^+$	988.4	—
38	19.3	5 to 45	—	1004.5 $[M+H]^+$	1004.4	—
S7	19.9	5 to 45	—	988.5 $[M+H]^+$	988.9	—
S8	19.9	5 to 45	—	988.5 $[M+H]^+$	988.9	—
S9	23.6	5 to 45	20 to 35	1141.5 $[M+H]^+$	1141.6	33
S10	23.4	5 to 45	20 to 35	1098.5 $[M+H]^+$	1098.6	32
S11	20.1	5 to 45	18 to 33	1103.5 $[M+H]^+$	1103.7	46
S12	23.1	5 to 45	—	1129.5 $[M+H]^+$	1129.8	—
S13	22.8	5 to 45	—	1086.5 $[M+H]^+$	1086.7	—
S14	19.6	5 to 45	—	1091.5 $[M+H]^+$	1091.7	—

0.1% TFA in H_2O (v/v, solvent A) and 0.1% TFA in CH_3CN (v/v, solvent B) was used for HPLC elution over 30 min. [a]Cosmosil $5C_{18}$-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min. [b]Cosmosil $5C_{18}$-AR-II preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [c]Cosmosil $5C_{18}$-AR-II semi-preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [d]Isolated yield. [e]Retention time.

S2.3 Chemical synthesis of CXCL14 via C–to–N-directed sequential ligation

Synthesis of middle fragment 39

The synthesis of N-terminal thiazolidine peptide thioester **39** was performed on hydrazine-incorporated 2-chlorotrityl chloride resin (0.10 mmol/g, 0.05 mmol). The hydrazine-incorporated resin was prepared by the previously reported protocol.[52] The peptide chain was elongated using a manual Fmoc SPPS procedure as described in the General information. The completed resin (60 mg, 51 μmol) was treated with TFA–TIPS–H_2O–*m*-cresol–thioanisole [80:2.5:2.5:5:10 (v/v), 50 μL/1.0 mg resin] at room temperature.[53] After 2 h, 10 μL of 10% (w/v) $NaNO_2$ aq. was added to the mixture at −10 °C for 20 min, and cold Et_2O was added to the reaction mixture to give a precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O (3 times). To the crude product was added 2 mL of 3% (v/v) MPA in buffer (6 M Gn·HCl−0.2 M sodium phosphate, pH 7.3). After 1 h at 37 °C, the crude peptide thioester was analyzed by LCMS and purified by preparative HPLC.

Synthesis of N-terminal fragment 42

Thioester peptide **42** was synthesized by standard Boc SPPS protocol on MBHA resin (0.70 mmol amine/g) by procedures identical to those reported.[13]

Synthesis of human CXCL14 using C–to–N-directed sequential NCL

The first NCL of **39** (0.5 μmol, 2 mM) and **19** (0.25 μmol, 1 mM) in 6 M Gn·HCl−0.1 M HEPPS buffer (pH 7.0, 0.25 mL) in the presence of 20 mM MPAA and 10 mM TCEP·HCl was conducted at 37 °C for 6 h. Then $CuSO_4 \cdot 5H_2O$ solution (0.8 M, 5 μL, final concentration 40 mM) and sodium ascorbate (0.8 M, 5 μL, final concentration 40 mM) were added to open the thiazolidine ring. After 45 min of reaction, the resulting reaction was quenched by the addition of 400 equiv DTT (solid form) with additional stirring at 37 °C for 15 min. After filtration of the mixture, HPLC purification on semi-preparative HPLC gave ligated **41** (0.13 μmol) in 52% isolated yield. The second NCL of **41** (0.2 μmol, 1 mM) and **42** (0.3 μmol, 1.5 mM) in 6 M Gn·HCl−0.1 M HEPPS buffer (pH 7.0, 0.2 mL) in the presence of 40 mM MPAA and 30 mM TCEP·HCl was conducted at 37 °C for 8 h to give **20** (0.44 μmol) in 65% isolated yield. Refolding of **20** (0.44 μmol, 0.05 mM) in 3 M Gn·HCl–0.1 M phosphate buffer (pH 7.7, 8.8 mL) was conducted at 37 ºC for 24 h to give human CXCL14 (0.31 μmol) in 69% isolated yield.

Table S2.2. Characterization data of CXCL14-derived peptides.

Peptide	Analytical HPLC[a]		Preparative HPLC[b]	*m/z*		Yield (%)[d]
	RT[e] (min)	Gradient (%)	Gradient (%)	Calcd	Found	
20	20.3	5 to 45	17 to 27[c]	1569.8 $[M+6H]^{6+}$	1571.3	65
39	17.2	5 to 45	12 to 22	865.4 $[M+3H]^{3+}$	866.0	11
41	20.7	5 to 45	13 to 23[c]	1017.2 $[M+6H]^{6+}$	1017.3	52
42	15.5	5 to 45	11 to 21	874.0 $[M+4H]^{4+}$	873.8	4.9
CXCL 14	20.0	5 to 45	17 to 32[c]	1569.2 $[M+6H]^{6+}$	1570.5	69

0.1% TFA in H_2O (v/v, solvent A) and 0.1% TFA in CH_3CN (v/v, solvent B) was used for HPLC elution over 30 min. [a]Cosmosil $5C_{18}$-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min. [b]Cosmosil $5C_{18}$-AR-II preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [c]Cosmosil $5C_{18}$-AR-II semi-preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [d]Isolated yield. [e]Retention time.

S2.4 Examination of copper-mediated ring-opening reaction of selenazolidine derivatives

Synthesis of model peptides 43

The synthesis of model peptide **43** was performed on NovaSyn TGR® resin (0.22 mmol amine/g, 0.10 mmol) using an automated Fmoc SPPS procedure as described in the General information. The completed resin (150 mg, 41.1 μmol) was treated with TFA–TES–H_2O [95:2.5:2.5 (v/v), 50 μL/1.0 mg resin] at room temperature for 2 h. The resin was filtered off, and the filtrate was directly added to cold Et_2O to generate precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide. The crude peptide was analyzed by LCMS and purified by preparative HPLC. For synthesis of N-terminal selenazolidine-containing peptide, Boc-Sez-OH[29] was coupled.

Synthesis of model thioester peptide 47

The synthesis of peptide thioester **47** was performed on hydrazine-incorporated 2-chlorotrityl chloride resin (0.23 mmol/g, 0.04 mmol). The hydrazine-incorporated resin

was prepared by the previously reported protocol.[52] The peptide chain was elongated using an automated Fmoc SPPS procedure as described in the General information. The completed resin (50 mg, 10 μmol) was treated with TFA–TIPS–H_2O–*m*-cresol–thioanisole [80:2.5:2.5:5:10 (v/v), 50 μL/1.0 mg resin] at room temperature.[53] After 2 h, 10 μL of 10% (w/v) $NaNO_2$ aq. was added to the mixture at −10 °C for 30 min, and cold Et_2O was added to the reaction mixture to give a precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O (3 times). To the crude product was added 2 mL of 3% (v/v) MPA in buffer (6 M Gn·HCl−0.2 M sodium phosphate, pH 7.0). After 30 min at 37 °C, the crude peptide thioester was analyzed by LCMS and purified by preparative HPLC.

Examination of copper-mediated ring-opening of selenazolidine peptide 43

Experimental procedures identical to those employed for the deprotection of thiazolidine peptide **29** were applied to the titled peptide **43**.

Ring-opening of selenazolidine, NCL and deselenization by one-pot protocol

A $CuSO_4·5H_2O$ solution (5 μL of 0.04 M, final concentration 2 mM) was added to a solution of **43** (1.0 μmol, 1 mM) in 6 M Gn·HCl−0.1 M HEPPS buffer (pH 7.0, 1 mL) in the presence of sodium ascorbate (2 mM). The reaction was continued for 45 min at 37 °C under ambient atmosphere. The thioester peptide **47** (2.0 μmol) in 6 M Gn·HCl−0.1 M HEPPS buffer (pH 7.0, 1 mL, final concentration of **46** and **47**, 0.5 mM and 1.0 mM) in the presence of 100 mM MPAA, 40 mM TCEP·HCl and 100 mM sodium ascorbate was added to the reaction mixture. After 1 h at 37 °C, the ligation mixture was then subjected to the deselenization process by addition of solid TCEP·HCl (final concentration 0.25 M) to the mixture to yield **49** (0.74 μmol) in 74% isolated yield.

Table S2.3. Characterization data of peptides.

Peptide	Analytical HPLC[a]		Preparative HPLC[b]	*m/z*		Yield (%)[d]
	RT[e] (min)	Gradient (%)	Gradient (%)	Calcd	Found	
43	21.1	5 to 45	18 to 33	1048.4 $[M+H]^+$	1048.8	17
44	18.6	5 to 45	—	1034.4 $[M+H]^+$	1034.5	—

45	20.9	5 to 45	—	1034.4 $[M+2H]^{2+}$	1034.5	—
47	14.2	5 to 35	10 to 20	781.4 $[M+H]^{+}$	781.7	41
49	21.0	5 to 45	18 to 33[c]	815.9 $[M+2H]^{2+}$	816.0	74

0.1% TFA in H_2O (v/v, solvent A) and 0.1% TFA in CH_3CN (v/v, solvent B) was used for HPLC elution over 30 min. [a]Cosmosil 5C_{18}-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min. [b]Cosmosil 5C_{18}-AR-II preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [c]Cosmosil 5C_{18}-AR-II semi-preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [d]Isolated yield. [e]Retention time.

S2.5 Deprotection of thiazolidine and selenazolidine derivatives using less amount of copper reagents

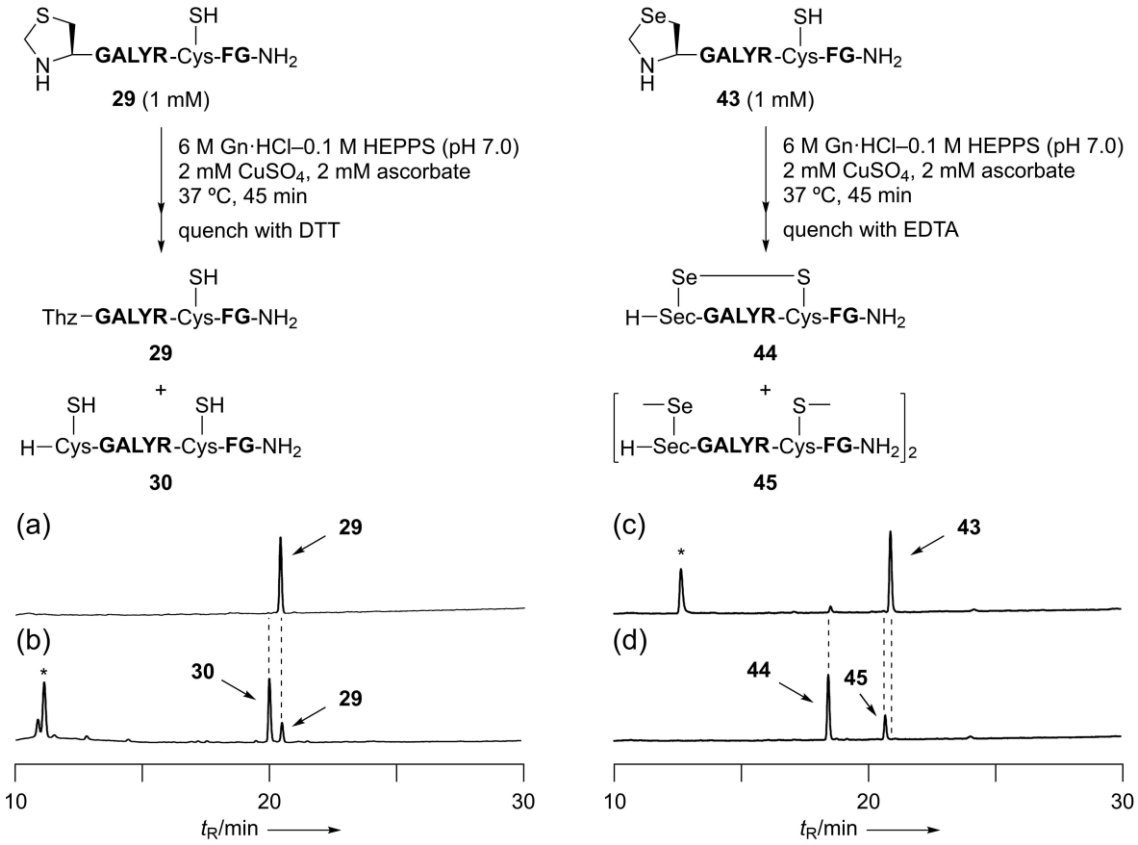


Figure S2.3. HPLC monitoring of the ring-opening reaction of **29** and **43**: (a) ring-opening of **29** (t < 5 min); (b) ring-opening of **29** (t = 1 h); (c) ring-opening of **43** (t < 5 min); (d) ring-opening of **43** (t = 45 min). Analytical HPLC conditions: linear gradient of 0.1 % TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 45% over 30 min. *Non-peptidic compounds derived from additives.

Experimental section

Chapter 3

S3.1 Synthesis of CXCL14 derivatives

Synthesis of C-terminal region peptides (51a and 51b)

The synthesis of **51a** and **51b** were performed on Fmoc-Glu(O*t*-Bu)-Wang resin (0.29 mmol acid/g) and NovaSyn® TGR resin (0.24 mmol amine/g) by procedures identical to those reported.[13]

Synthesis of 50–77 region peptide-Ala 52a

Desulfurization of **51a** (0.64 μmol, 0.5 mM) was conducted under 6 M Gn·HCl–0.1 M sodium phosphate buffer (pH 3.5, 1.3 mL) in the presence of 40 mM VA-044, 40 mM glutathione and 100 mM TCEP·HCl at 37 ºC for 16 h. After HPLC purification, the desired peptide **52a** (0.28 μmol) was obtained in 43% isolated yield.

Synthesis of dimer-50–77 region peptide 53a

Air oxidation of **51a** (1.1 μmol, 1.0 mM) was conducted under 3 M Gn·HCl–0.1 M sodium phosphate buffer (pH 7.7, 1.1 mL) at 37 ºC for 23 h to afford **53a** (0.43 μmol) in 39% isolated yield.

Synthesis of thioester peptide 54

The synthesis of **54** was conducted in the basically same procedure as that of **42** as mentioned in Experimental section Chapter 2.

Synthesis of N-terminal cysteine peptides (55a, 70a, S15a and S16a)

Typical procedure: The synthesis of N-terminal cysteine peptide **55a** was performed on NovaSyn TGR® resin (0.25 mmol amine/g, 0.025 mmol) using a manual Fmoc SPPS procedure as described in the General information. The completed resin (50 mg, 10.6 μmol) was treated with TFA–*m*-cresol–EDT–thioanisole–H_2O [80:5:5:5:5 (v/v), 50 μL/1.0 mg resin] at room temperature for 2 h. The resin was filtered off, and the filtrate was directly added to cold Et_2O to generate precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide. The crude peptide was analyzed by analytical HPLC and purified by preparative HPLC.

Synthesis of biotin-containing peptides (55b, 70b, S15b and S16b)

Typical procedure: The synthesis of biotin-containing peptide **55b** was performed on NovaSyn TGR® resin (0.25 mmol amine/g, 75 μmol) using a manual Fmoc SPPS procedure as described in the General information. The resulting resin was treated with Boc_2O (4.0 equiv, 0.30 mmol), HOBt·H_2O (4.0 equiv, 0.30 mmol) and DIPEA (4.0 equiv, 0.30 mmol) in DMF to protect an N-terminal α-amino group of elongated peptides. The resin was (150 mg, 19 μmol) subsequently treated with 2% (v/v) hydrazine·H_2O/DMF at room temperature for several times until the completion of removal of the ivDde group. The regenerating ε-amino group of the additional Lys residue was reacted with biotin (4.0 equiv, 76 μmol) in the presence of DIPCDI (4.0 equiv, 76 μmol) in DMF/DMSO = 1:1 (v/v) at room temperature overnight to afford a protected peptide-Lys(biotin) resin. The completed resin (70 mg, 9.5 μmol) was treated with TFA–*m*-cresol–EDT–thioanisole–H_2O [80:5:5:5:5 (v/v), 50 μL/1.0 mg resin] at room temperature for 2 h. The resin was filtered off, and the filtrate was directly added to cold Et_2O to generate precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide. The crude peptide was analyzed by analytical HPLC and purified by semi-preparative HPLC.

Synthesis of SEAlide peptides (69 and S17)

Typical procedure: The synthesis of SEAlide peptide **69** was performed on NovaSyn TGR® resin (0.22 mmol amine/g, 0.05 mmol) using an automated Fmoc SPPS procedure as described in the General information. The completed resin (200 mg, 25 μmol) was treated with TFA–*m*-cresol–EDT–thioanisole–H_2O [80:5:5:5:5 (v/v), 50 μL/1.0 mg resin] at room temperature for 2 h. The resin was filtered off, and the filtrate was directly added to cold Et_2O to generate precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide. The crude peptide was analyzed by analytical HPLC and purified by preparative HPLC.

Synthesis of acetylated 15-residue peptides (58a–65a)

Typical procedure: The synthesis of **58a** was performed on Rink-amide ChemMatrix® resin (0.48 mmol amine/g, 0.13 mmol) using a manual Fmoc SPPS procedure as described in the General information. The resulting resin was treated with acetic anhydride and pyridine (each at 10 equiv, 1.3 mmol) in DMF to protect an N-terminal α-amino group of elongated peptides. The resin was (270 mg, 0.13 mmol) subsequently treated with 2% (v/v) hydrazine·H_2O/DMF at room temperature for several times until the completion of removal of the ivDde group. The regenerating ε-amino group of the additional Lys residue

was reacted with acetic anhydride and pyridine (each at 10 equiv, 1.3 mmol) in DMF at room temperature overnight to afford a protected peptide-Lys(Ac) resin. The completed resin (50 mg, 25 μmol) was treated with TFA–*m*-cresol–EDT–TES–thioanisole–H_2O [80:5:2.5:2.5:5:5 (v/v), 50 μL/1.0 mg resin] at room temperature for 2 h. The resin was filtered off, and the filtrate was directly added to cold Et_2O to generate precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide. The crude peptide was analyzed by analytical HPLC and purified by semi-preparative HPLC.

Synthesis of biotinylated 15-residue peptides (58b–65b)

Typical procedure: The synthesis of **58b** was performed on Rink-amide ChemMatrix® resin (0.48 mmol amine/g, 0.13 mmol) using a manual Fmoc SPPS procedure as described in the General information. The resulting resin was treated with acetic anhydride and pyridine (each at 10 equiv, 1.3 mmol) in DMF to protect an N-terminal α-amino group of elongated peptides. The resin was (270 mg, 0.13 mmol) subsequently treated with 2% (v/v) hydrazine·H_2O/DMF at room temperature for several times until the completion of removal of the ivDde group. The regenerating ε-amino group of the additional Lys residue was reacted with biotin (4.0 equiv, 0.52 mmol) in the presence of DIPCDI (4.0 equiv, 0.52 mmol) in DMF/DMSO = 1:1 (v/v) at room temperature overnight to afford a protected peptide-Lys(biotin) resin. The completed resin (60 mg, 32 μmol) was treated with TFA–*m*-cresol–EDT–TES–thioanisole–H_2O [80:5:2.5:2.5:5:5 (v/v), 50 μL/1.0 mg resin] at room temperature for 2 h. The resin was filtered off, and the filtrate was directly added to cold Et_2O to generate precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide. The crude peptide was analyzed by analytical HPLC and purified by semi-preparative HPLC.

Table S3.1. Sequence of CXCL14 derivatives.

Region	Compound name	Sequence
29–50[a]	**S15a**: **X** = Gly-$CONH_2$ **S15b**: **X** = Lys(bio)-$CONH_2$	^{29}**C**EEKMVIITTKSVSRYRGQEH^{50}**C**–**X**
29–40[a]	**S16a**: **X** = Gly-$CONH_2$ **S16b**: **X** = Lys(bio)-$CONH_2$	^{29}**C**EEKMVIITTKS^{40}–**X**
13–28[a]	**S17**: **Z** = SEAlide-CO-Ala-$CONH_2$	^{13}RYSDVKKLEMKPKYPH^{28}–**Z**

[a]N-Terminal: NH_2.

Table S3.2. Characterization data of peptides.

Peptide	Analytical HPLC[a]		Preparative HPLC[b]	*m/z*		Yield (%)[d]
	RT[e] (min)	Gradient (%)	Gradient (%)	Calcd	Found	
51a	20.5	5 to 45	15 to 35	1208.3 $[M+3H]^{3+}$	1208.5	74
51b	21.3	5 to 45	15 to 30	1326.0 $[M+3H]^{3+}$	1327.1	26
52a	20.5	5 to 45	10 to 40[c]	1197.6 $[M+3H]^{3+}$	1198.1	43
53a	22.0	5 to 45	17 to 32	1208.0 $[M+6H]^{6+}$	1208.5	39
54	17.4	5 to 45	15 to 25	1188.6 $[M+3H]^{3+}$	1188.7	4
55a	18.6	5 to 45	19 to 29	908.8 $[M+3H]^{3+}$	908.9	11
55b	19.3	5 to 45	15 to 30[c]	1007.8 $[M+3H]^{3+}$	1008.3	3
58a	19.4	5 to 30	10 to 17[c]	668.1 $[M+3H]^{3+}$	668.3	13
58b	22.2	5 to 30	10 to 20[c]	729.4 $[M+3H]^{3+}$	729.7	5
59a	22.1	5 to 30	15 to 20[c]	700.7 $[M+3H]^{3+}$	700.7	6
59b	24.3	5 to 30	15 to 25[c]	762.1 $[M+3H]^{3+}$	762.4	6
60a	24.5	5 to 30	5 to 25[c]	730.1 $[M+3H]^{3+}$	730.4	15
60b	25.8	5 to 30	5 to 25[c]	791.4 $[M+3H]^{3+}$	791.8	12
61a	23.8	5 to 30	5 to 20[c]	713.4 $[M+3H]^{3+}$	713.7	12
61b	24.6	5 to 30	5 to 20[c]	774.7 $[M+3H]^{3+}$	775.1	6
62a	25.6	5 to 30	5 to 20[c]	719.0 $[M+3H]^{3+}$	719.4	9
62b	28.0	5 to 30	5 to 20[c]	780.4 $[M+3H]^{3+}$	780.8	8
63a	19.9	5 to 45	5 to 20[c]	691.4 $[M+3H]^{3+}$	691.8	6
63b	21.3	5 to 45	5 to 20[c]	752.7 $[M+3H]^{3+}$	753.1	4
64a	21.8	5 to 45	5 to 20[c]	712.7 $[M+3H]^{3+}$	713.0	12
64b	22.5	5 to 45	5 to 25[c]	774.1 $[M+3H]^{3+}$	774.3	5
65a	22.1	5 to 30	5 to 15[c]	686.7 $[M+3H]^{3+}$	687.0	10
65b	24.6	5 to 30	5 to 20[c]	748.0 $[M+3H]^{3+}$	748.3	7
69	16.3	5 to 45	11 to 26	893.2 $[M+4H]^{4+}$	893.9	9

70a	16.8	5 to 45	12 to 27	762.1 $[M+3H]^{3+}$	762.6	15
70b	17.5	5 to 45	14 to 29	861.1 $[M+3H]^{3+}$	861.5	6
S15a	18.6	5 to 45	19 to 29	885.1 $[M+3H]^{3+}$	885.2	12
S15b	19.9	5 to 45	15 to 30[c]	984.1 $[M+3H]^{3+}$	984.6	1
S16a	15.5	5 to 45	12 to 27	719.4 $[M+2H]^{2+}$	719.8	26
S16b	17.3	5 to 45	14 to 29	868.5 $[M+2H]^{2+}$	868.5	8
S17	16.0	5 to 45	12 to 27	752.1 $[M+3H]^{3+}$	752.6	6

0.1% TFA in H_2O (v/v, solvent A) and 0.1% TFA in CH_3CN (v/v, solvent B) was used for HPLC elution over 30 min. [a]Cosmosil 5C_{18}-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min. [b]Cosmosil 5C_{18}-AR-II preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [c]Cosmosil 5C_{18}-AR-II semi-preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [d]Isolated yield. [e]Retention time.

Synthesis of 1–50 region peptide 50a

NCL of **54** (1.1 μmol, 1.0 mM) and **55a** (1.1 μmol, 1.0 mM) was performed in 6 M Gn·HCl–0.1 M sodium phosphate buffer (pH 6.8, 1.1 mL) in the presence of 50 mM MPAA and 50 mM TCEP·HCl at 37 ºC for 3 h, and this was followed by HPLC purification to get **56a** (0.78 μmol) in 71% isolated yield. Resulting **56a** (0.30 μmol, 0.05 mM) was subjected to the air oxidation step under 3 M Gn·HCl–0.1 M sodium phosphate buffer (pH 7.7, 6.0 mL) at 37 ºC for 44 h to afford **57a** (0.19 μmol) in 63% isolated yield by HPLC purification. The Acm groups on the resulting **57a** (0.06 μmol) were removed by the action of AgOTf–anisole (34 mg:15 μL) in TFA (340 μL) at 4 ºC for 6 h. Then 50% (v/v) DMSO/1 M HCl aq. (1.0 mL) was added to the reaction and the resulting mixture was incubated at 25 ºC for 6 h. After HPLC purification, **50a** (0.02 μmol) was obtained in 37% isolated yield over two steps.

Synthesis of 1–50 region biotin-containing peptide 50b

NCL of **54** (0.50 μmol, 1.6 mM) and **55b** (0.31 μmol, 1.0 mM) was performed in 6 M Gn·HCl–0.1 M sodium phosphate buffer (pH 6.8, 0.31 mL) in the presence of 50 mM MPAA and 50 mM TCEP·HCl at 37 ºC for 3 h, and this was followed by HPLC purification to get **56b** (0.25 μmol) in 81% isolated yield. Resulting **56b** (0.25 μmol, 0.05 mM) was subjected to the air oxidation step under 3 M Gn·HCl–0.1 M sodium phosphate buffer (pH 7.7, 5.0 mL) at 37 ºC for 16 days to afford **57b** (0.05 μmol) in 20% isolated yield by HPLC purification. The Acm groups on the resulting **57b** (0.05 μmol) were removed by the action of AgOTf–anisole (34 mg:15 μL) in TFA (340 μL) at 4 ºC for 6 h. Then 50% (v/v) DMSO/1 M HCl aq. (1.0 mL) was added to the reaction and the resulting

mixture was incubated at 25 ºC for 6 h. After HPLC purification, **50b** (0.06 μmol) was obtained in quantitative yield over two steps.

Synthesis of 1–50 region peptide-4Ala S18a

NCL of **16** (0.90 μmol, 1.0 mM) and **S15a** (0.90 μmol, 1.0 mM) was performed in 6 M Gn·HCl–0.1 M sodium phosphate buffer (pH 6.7, 0.90 mL) in the presence of 50 mM MPAA and 50 mM TCEP·HCl at 37 ºC for 3 h, and this was followed by HPLC purification to get **S19a** (0.64 μmol) in 71% isolated yield. Resulting **S19a** (0.20 μmol, 0.5 mM) was subjected to the desulfurization step under 0.1 M sodium phosphate buffer (pH 3.5, 0.40 mL) with 40 mM VA-044, 40 mM glutathione and 100 mM TCEP·HCl at 37 ºC for 1 h. After HPLC purification, the desired peptide **S18a** (0.11 μmol) was obtained in 55% isolated yield.

Synthesis of 1–50 region biotin-containing peptide-4Ala S18b

NCL of **16** (0.20 μmol, 2.5 mM) and **S15b** (0.08 μmol, 1.0 mM) was performed in 6 M Gn·HCl–0.1 M sodium phosphate buffer (pH 6.8, 0.08 μL) in the presence of 50 mM MPAA and 50 mM TCEP·HCl at 37 ºC for 3 h, and this was followed by HPLC purification to get **S19b** (0.11 μmol) in quantitative yield. Resulting **S19b** (0.11 μmol, 0.50 mM) was subjected to the desulfurization step under 6 M Gn·HCl–0.1 M sodium phosphate buffer (pH 3.5, 0.22 mL) with 40 mM VA-044, 40 mM glutathione and 100 mM TCEP·HCl at 37 ºC for 1 h. After HPLC purification, the desired peptide **S18b** (0.05 μmol) was obtained in 45% isolated yield.

Synthesis of 1–47 region peptide 66a

NCL of **69** (1.0 μmol, 1.0 mM) and **70a** (1.5 μmol, 1.5 mM) was performed in 6 M Gn·HCl–0.5 M sodium phosphate buffer (pH 6.8, 1.0 mL) in the presence of 40 mM MPAA and 30 mM TCEP·HCl at 37 ºC for 24 h, and this was followed by HPLC purification to get **71a** (0.45 μmol) in 45% isolated yield. Resulting **71a** (0.45 μmol, 1.0 mM) was subjected to the desulfurization step under 6 M Gn·HCl–0.1 M sodium phosphate buffer (pH 3.5, 0.40 mL) with 40 mM VA-044, 40 mM glutathione and 100 mM TCEP·HCl at 37 ºC for 3 h. After HPLC purification, the desired peptide **66a** (0.27 μmol) was obtained in 60% isolated yield.

Synthesis of 1–47 region biotin-containing peptide 66b

NCL of **69** (0.49 μmol, 1.0 mM) and **70b** (0.73 μmol, 1.5 mM) was performed in 6 M Gn·HCl–0.5 M sodium phosphate buffer (pH 6.8, 0.49 mL) in the presence of 40 mM

MPAA and 30 mM TCEP·HCl at 37 ºC for 24 h, and this was followed by HPLC purification to get **71b** (0.37 μmol) in 75% isolated yield. Resulting **71b** (0.37 μmol, 1.0 mM) was subjected to the desulfurization step under 6 M Gn·HCl–0.1 M sodium phosphate buffer (pH 3.5, 0.37 mL) with 40 mM VA-044, 40 mM glutathione and 100 mM TCEP·HCl at 37 ºC for 3 h. After HPLC purification, the desired peptide **66b** (0.28 μmol) was obtained in 75% isolated yield.

Synthesis of 1–40 region peptide 67a

NCL of **69** (1.0 μmol, 1.0 mM) and **S16a** (1.5 μmol, 1.5 mM) was performed in 6 M Gn·HCl–0.5 M sodium phosphate buffer (pH 6.8, 1.0 mL) in the presence of 40 mM MPAA and 30 mM TCEP·HCl at 37 ºC for 24 h, and this was followed by HPLC purification to get **S20a** (0.75 μmol) in 75% isolated yield. Resulting **S20a** (0.37 μmol, 1.0 mM) was subjected to the desulfurization step under 6 M Gn·HCl–0.1 M sodium phosphate buffer (pH 3.5, 0.75 mL) with 40 mM VA-044, 40 mM glutathione and 100 mM TCEP·HCl at 37 ºC for 3 h. After HPLC purification, the desired peptide **67a** (0.52 μmol) was obtained in 69% isolated yield.

Synthesis of 1–40 region biotin-containing peptide 67b

NCL of **69** (0.45 μmol, 1.0 mM) and **S16b** (0.67 μmol, 1.5 mM) was performed in 6 M Gn·HCl–0.5 M sodium phosphate buffer (pH 6.8, 0.45 mL) in the presence of 40 mM MPAA and 30 mM TCEP·HCl at 37 ºC for 24 h, and this was followed by HPLC purification to get **S20b** (0.24 μmol) in 54% isolated yield. Resulting **S20b** (0.24 μmol, 1.0 mM) was subjected to the desulfurization step under 6 M Gn·HCl–0.1 M sodium phosphate buffer (pH 3.5, 0.24 mL) with 40 mM VA-044, 40 mM glutathione and 100 mM TCEP·HCl at 37 ºC for 3 h. After HPLC purification, the desired peptide **67b** (0.15 μmol) was obtained in 61% isolated yield.

Synthesis of 13–50 region peptide 68a

NCL of **S17a** (0.45 μmol, 1.1 mM) and **S15a** (0.42 μmol, 1.0 mM) was performed in 6 M Gn·HCl–0.5 M sodium phosphate buffer (pH 6.8, 0.42 mL) in the presence of 40 mM MPAA and 30 mM TCEP·HCl at 37 ºC for 24 h, and this was followed by HPLC purification to get **S21a** (0.23 μmol) in 55% isolated yield. Resulting **S21a** (0.23 μmol, 1.0 mM) was subjected to the desulfurization step under 6 M Gn·HCl–0.1 M sodium phosphate buffer (pH 3.5, 0.23 mL) with 40 mM VA-044, 40 mM glutathione and 100 mM TCEP·HCl at 37 ºC for 3 h. After HPLC purification, the desired peptide **68a** (0.16 μmol) was obtained in 71% isolated yield.

Synthesis of 13–50 region biotin-containing peptide 68b

NCL of **S17b** (0.45 μmol, 1.1 mM) and **S15b** (0.41 μmol, 1.0 mM) was performed in 6 M Gn·HCl–0.5 M sodium phosphate buffer (pH 6.8, 0.41 mL) in the presence of 40 mM MPAA and 30 mM TCEP·HCl at 37 ºC for 24 h, and this was followed by HPLC purification to get **S21b** (0.16 μmol) in 39% isolated yield. Resulting **S21b** (0.16 μmol, 1.0 mM) was subjected to the desulfurization step under 6 M Gn·HCl–0.1 M sodium phosphate buffer (pH 3.5, 0.16 mL) with 40 mM VA-044, 40 mM glutathione and 100 mM TCEP·HCl at 37 ºC for 3 h. After HPLC purification, the desired peptide **68b** (0.07 μmol) was obtained in 43% isolated yield.

Table S3.3. Sequence of CXCL14 derivatives.

Region	Compound name	Sequence
1–50[a]	**S18a**: **X** = Gly-$CONH_2$ **S18b**: **X** = Lys(bio)-$CONH_2$	^{1}SK**A**K**A**SRKGPKIRYSDVKKLEMKPKYPH**A**EEKMVIITTKS VSRYRGQEH**A**$_{50}$–**X**
1–50[a]	**S19a**: **X** = Gly-$CONH_2$ **S19b**: **X** = Lys(bio)-$CONH_2$	^{1}SK**C**K**C**SRKGPKIRYSDVKKLEMKPKYPH**C**EEKMVIITTKS VSRYRGQEH**C**$_{50}$–**X**
1–40[a]	**S20a**: **X** = Gly-$CONH_2$ **S20b**: **X** = Lys(bio)-$CONH_2$	^{1}SK**C**K**C**SRKGPKIRYSDVKKLEMKPKYPH**C**EEKMVIITTKS^{40}–**X**
13–50[a]	**S21a**: **X** = Gly-$CONH_2$ **S21b**: **X** = Lys(bio)-$CONH_2$	^{13}RYSDVKKLEMKPKYPH**C**EEKMVIITTKSVSRYRGQEH**C**^{50}–**X**

[a]N-Terminal: NH_2.

Table S3.4. Characterization data of ligation product peptides.

Peptide	Analytical HPLC[a]		Preparative HPLC[b]	*m/z*		Yield (%)[d]
	RT[e] (min)	Gradient (%)	Gradient (%)	Calcd	Found	
50a	17.5	10 to 40	15 to 35[c]	1193.8 $[M+5H]^{5+}$	1194.8	37
50b	16.9	10 to 40	15 to 35[c]	1253.2 $[M+5H]^{5+}$	1254.2	quant.
56a	19.2	10 to 40	15 to 35[c]	1019.4 $[M+6H]^{6+}$	1020.6	71
56b	19.9	10 to 40	15 to 35[c]	1282.5 $[M+5H]^{5+}$	1283.4	81

57a	17.9	10 to 40	15 to 35[c]	1222.6 $[M+5H]^{5+}$	1224.0	63
57b	17.2	10 to 40	15 to 35[c]	1602.3 $[M+4H]^{4+}$	1603.6	20
66a	17.9	5 to 45	14 to 29[c]	1101.6 $[M+5H]^{5+}$	1102.4	60
66b	18.3	5 to 45	15 to 30[c]	1161.0 $[M+5H]^{5+}$	1161.5	75
67a	16.8	5 to 45	14 to 29[c]	1165.2 $[M+4H]^{4+}$	1165.9	69
67b	17.6	5 to 45	15 to 30[c]	991.8 $[M+5H]^{5+}$	992.1	61
68a	17.9	5 to 45	14 to 29[c]	1148.1 $[M+4H]^{4+}$	1148.3	71
68b	18.3	5 to 45	15 to 30[c]	978.1 $[M+5H]^{5+}$	978.4	43
71a	17.9	5 to 45	14 to 29[c]	1120.8 $[M+5H]^{5+}$	1121.6	45
71b	18.5	5 to 45	15 to 30[c]	1180.2 $[M+5H]^{5+}$	1180.9	75
S18a	18.6	10 to 40	15 to 35[c]	1169.0 $[M+5H]^{5+}$	1169.8	55
S18b	19.5	10 to 40	15 to 35[c]	1228.5 $[M+5H]^{5+}$	1229.5	45
S19a	19.4	10 to 40	15 to 35[c]	995.7 $[M+6H]^{6+}$	996.8	71
S19b	20.1	10 to 40	15 to 35[c]	1567.3 $[M+4H]^{4+}$	1568.3	quant.
S20a	17.1	5 to 45	14 to 29[c]	1189.1 $[M+4H]^{4+}$	1190.0	75
S20b	17.9	5 to 45	15 to 30[c]	1263.4 $[M+4H]^{4+}$	1264.1	54
S21a	18.0	5 to 45	14 to 29[c]	1164.1 $[M+4H]^{4+}$	1164.5	55
S21b	18.5	5 to 45	15 to 30[c]	990.9 $[M+5H]^{5+}$	991.4	39

0.1% TFA in H_2O (v/v, solvent A) and 0.1% TFA in CH_3CN (v/v, solvent B) was used for HPLC elution over 30 min. [a]Cosmosil $5C_{18}$-AR-II analytical column was employed with a linear gradient of solvent B in solvent A over 30 min. [b]Cosmosil $5C_{18}$-AR-II preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [c]Cosmosil $5C_{18}$-AR-II semi-preparative column was employed with a linear gradient of solvent B in solvent A over 30 min. [d]Isolated yield. [e]Retention time.

S3.2 Biological evaluation of CXCL14 derivatives

Cell preparation

Raw264.7 cells were cultured in RPMI-1640 medium (Nacalai Tesque) containing 10% fetal bovine serum (Thermo Fisher Scientific) and Penicillin-Streptomycin (Sigma).

Reagents

ODN2395 (5'-TCGTCGTTTTCGGCGCGCGCCG-3') is a phosphorothioate CpG oligonucleotide, and was purchased from InvivoGen. Cy3-ODN2395 was Cy3-labeled at 5'-end, and synthesized by Eurofins genomics. Propidium Iodide (PI) was purchased from Sigma.

FACS analysis

Raw264.7 cells were plated onto 24 well plates (10^5 cells/well; Corning) and incubated for 1 h at 37 ºC with Cy3-ODN2395 in the presence or absence of CXCL14 or CXCL14 derivatives in RPMI-1640 medium containing 20 mM Hepes-NaOH, pH 7.5, and 0.1% BSA (Sigma, fatty acid free). Cells were then trypsinized, stained with PI, and analyzed by LSR-Fortessa X-20 (BD Biosciences).

Enzyme-linked immunoassay (ELISA)

Raw264.7 cells were plated onto 24-well plates (10^5 cells/well) (Corning) and incubated in RPMI-1640/20 mM Hepes-NaOH pH 7.5/0.1% BSA for 6 h at 37 ºC with or without ODN2395 in the presence or absence of CXCL14 or CXCL14 derivatives. Culture supernatants were then tested using ELISA MAX™ mouse TNF-α Kits (BioLegend).

***In vitro* binding assay**

Biotinylated CXCL14 derivatives (100 pmol) were coupled to streptavidin-agarose (Sigma). 100 nM of Cy3-ODN2395 in 100 µL of binding buffer (50 mM Hepes-NaOH pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% BSA) was added to the streptavidin-agarose coupled with biotinylated peptides for 1 h at 4 °C. Precipitates were then washed and eluted in SDS sample buffer at 70 ºC for 10 min. Cy3-ODN was separated by 15% TBE-SDS polyacrylamide gel electrophoresis, and Cy3 fluorescence was measured in a LAS-3000 (Fuji Film). Biotinylated CXCL14 derivatives were blotted onto a 0.22 mm PVDF membrane (Thermo Fisher Scientific) and incubated with peroxidase-conjugated avidin (Thermo Fisher Scientific). Chemiluminescence detection was performed using the ECL Plus detection reagent (GE Healthcare), and signals were

measured in a LAS-3000.

Scatchard plot analysis

Biotinylated CXCL14 derivatives (100 pmol) were incubated for 1 h at 4 ºC with streptavidin-agarose and Cy3-ODN2395 in 100 µL of binding buffer. The supernatant was collected and used as free Cy3-ODN. Bound Cy3-ODN was eluted by heating at 56 ºC for 1 h in 100 µL of elution buffer [Tris-HCl pH 7.5, 200 mM NaCl, 5 mM EDTA, 1% SDS, proteinase K (0.02 mg/ml)]. Cy3 fluorescence was then measured in a Varioskan Flash (Thermo Fisher Scientific). The bound/free ratio was then plotted against bound Cy3-ODN2395 (nM). The slope of the liner regression line was determined, and K_d was calculated as follows: −1 divided by the slope of the liner regression line.

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List of publications

Publications regarding this thesis

Chapter 1

Resin-bound crypto-thioester for native chemical ligation

Naoto Naruse, Kento Ohkawachi, Tsubasa Inokuma, Akira Shigenaga and Akira Otaka*

Organic Letters **2018**, *20*, 2449–2453.

Chapter 2

Copper-mediated deprotection of thiazolidine and selenazolidine derivatives applied to native chemical ligation

Naoto Naruse, Daishiro Kobayashi, Kento Ohkawachi, Akira Shigenaga and Akira Otaka*

The Journal of Organic Chemistry **2020**, *85*, 1425–1433.
JOC Special Issue "Modern Peptide and Protein Chemistry"

Chapter 3

Manuscript in preparation

Other publications

1. The total chemical synthesis of the monoglycosylated GM2 ganglioside activator using a novel cysteine surrogate

   Kohei Sato, Keisuke Kitakaze, Takahiro Nakamura, **Naoto Naruse**, Keisuke Aihara, Akira Shigenaga, Tsubasa Inokuma, Daisuke Tsuji, Kohji Itoh and Akira Otaka*

   Chemical Communications **2015**, *51*, 9946–9948.

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

   Tatsuhiko Shimizu, Rin Miyajima, Kohei Sato, Ken Sakamoto, **Naoto Naruse**, Miku Kita, Akira Shigenaga* and Akira Otaka*

   Tetrahedron **2016**, *72*, 992–998.

3. Facile preparation of peptides with C-terminal *N*-alkylamide via radical-initiated dethiocarboxylation

   Tatsuhiko Shimizu, Rin Miyajima, **Naoto Naruse**, Kosuke Yamaoka, Keisuke Aihara, Akira Shigenaga* and Akira Otaka*

   Chemical and Pharmaceutical Bulletin **2016**, *64*, 375–378.

4. One-pot/sequential native chemical ligation using photocaged crypto-thioester

   Keisuke Aihara, Kosuke Yamaoka, **Naoto Naruse**, Tsubasa Inokuma, Akira Shigenaga and Akira Otaka*

   Organic Letters **2016**, *18*, 596–599.

5. Development of an anilide-type scaffold for the thioester precursor *N*-sulfanylethylcoumarinyl amide

   Mitsuhiro Eto[†], **Naoto Naruse**[†], Kyohei Morimoto, Kosuke Yamaoka, Kohei Sato, Kohei Tsuji, Tsubasa Inokuma, Akira Shigenaga[*] and Akira Otaka[*]
   (†equal contribution)

   Organic Letters **2016**, *18*, 4416–4419.

6. Tailored synthesis of 162-residue *S*-monoglycosylated GM2-activator protein (GM2AP) analogues that allows facile access to a protein library

   Takahiro Nakamura, Kohei Sato, **Naoto Naruse**, Keisuke Kitakaze, Tsubasa Inokuma, Takatsugu Hirokawa, Akira Shigenaga, Kohji Itoh and Akira Otaka[*]

   ChemBioChem **2016**, *17*, 1986–1992.

7. CXCL14 acts as a specific carrier of CpG DNA into dendritic cells and activates toll-like receptor 9-mediated adaptive immunity

   Kosuke Tanegashima[*], Rena Takahashi, Hideko Nuriya, Rina Iwase, **Naoto Naruse**, Kohei Tsuji, Akira Shigenaga, Akira Otaka and Takahiko Hara[*]

   EBioMedicine **2017**, *24*, 247–256.

8. ProteoFind: A script for finding proteins that are suitable for chemical synthesis

   Akira Shigenaga[*], **Naoto Naruse** and Akira Otaka

   Tetrahedron **2018**, *74*, 2291–2297.