Synthetic studies on CXCL14 and its derivatives for their biological evaluation

Thesis Presented in Partial Fulfillment of the Requirement for
the Degree of Doctor at Tokushima University
(Pharmaceutical Sciences)

Kohei Tsuji

Abbreviations

Ac	acetyl
ANP	atrial natriuretic peptide
APC	allophycocyanin
Boc	*tert*-butoxycarbonyl
BSA	bovine serum albumin
^{t}Bu	*tert*-butyl
CD	circular dichroism
COMU	1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium hexafluorophosphate
DIPCDI	*N*,*N′*-diisopropylcarbodiimide
DIPEA	*N,N*-diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DMEM	Dulbecco's modified Eagle's medium
DMF	*N*,*N*-dimethylformamide
EDT	1,2-ethanedithiol
EDTA	ethylenediaminetetraacetic acid
ESI-TOF	electrospray ionization-time-of-flight
Et	ethyl
FACS	fluorescence activated cell sorting
Fmoc	9-fluorenylmethoxycarbonyl
Fr	fragment
GFP	green fluorescent protein
Gn·HCl	guanidine·hydrochloride
HATU	*O*-(7-aza-1H-benzotriazol-1-yl)-*N*,*N*,*N′*,*N′*-tetramethyluronium hexafluorophosphate
HBTU	*O*-(1H-benzotriazol-1-yl)-*N*,*N*,*N′*,*N′*-tetramethyluronium hexafluorophosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEPPS	3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid
HOBt	1-hydroxybenzotriazole
HPLC	high-performance liquid chromatography
HRMS	high resolution mass spectrometry

IR	infrared spectroscopy
ivDde	1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl
MBHA	4-methylbenzhydrylamine
MBom	methoxybenzyloxymethyl
Me	methyl
MeCN	acetonitrile
$MeONH_2$·HCl	methoxyamine·hydrochloride
MPAA	4-mercaptophenylacetic acid
MS	mass spectrometry
NCL	native chemical ligation
NMP	*N*-methylpyrrolidone
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
SEAlide	*N*-sulfanylethylanilide
SPPS	solid-phase peptide synthesis
TCEP·HCl	tris(2-carboxyethyl)phosphine·hydrochloride
Tf	trifluoromethanesulfonyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Thz	thiazolidine carboxylate
TMS	trimethylsilyl
Trt	triphenylmethyl
UV	ultraviolet

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

A	(Ala)	Alanine
C	(Cys)	Cystein
D	(Asp)	Aspartic acid
E	(Glu)	Glutamic acid
F	(Phe)	Phenylalanine
G	(Gly)	Glycine
H	(His)	Histidine
I	(Ile)	Isoleucine
K	(Lys)	Lysine
L	(Leu)	Leucine

M	(Met)	Methionine
N	(Asn)	Asparagine
P	(Pro)	Proline
Q	(Gln)	Glutamine
R	(Arg)	Arginine
S	(Ser)	Serine
T	(Thr)	Threonine
V	(Val)	Valine
W	(Trp)	Tryptophan
Y	(Tyr)	Tyrosine

Contents

Preface···1

1. Synthesis of CXCL14 via C–to–N- or N–to–C-directive sequential native chemical ligation

1.1 CXCL14: a binder for CXCR4 and an inhibitor for the CXCL12-CXCR4 signaling axis···2

1.2 Chemical syntheses of CXCL14 and its derivatives using sequential native chemical ligation in a C–to–N- or N–to–C-directive manner and their biological evaluation···2

1.3 One-pot/sequential native chemical ligation strategy for syntheses of CXCL14 and its derivatives using *N*-sulfanylethylanilide peptide···9

1.4 Biological evaluation of the biotinylated CXCL14 derivative···15

1.5 Conclusion···16

2. Development of a novel inhibitor of the CXCL12-CXCR4 signaling axis

2.1 Exploration of the interaction region of CXCL14 with CXCR4···18

2.2 Synthesis and biological evaluation of a dimerized CXCL14 C-terminal fragment···20

2.3 Synthesis and biological evaluation of the dimer of mutated CXCL14 C-terminal fragment···21

2.4 Identification of dimer peptide residues indispensable for inhibitory activity to CXCL12-mediated chemotaxis···28

2.5 Conclusion···33

3. Conclusions···35

Experimental section

General···37

Chapter 1···38

Chapter 2···50

References···73

Acknowledgements······76

List of publications······77

Preface

Chemokines are signaling proteins that have important roles in both innate and adaptive immune systems. They function as chemoattractants to induce the migration of immune cells dependent on their concentration. Some chemokines are involved in angiogenesis, tumor metastasis, bacterial infection and other pathological phenomenon. For example, CXCL12 is a CXC-type chemokine and is related to tumor growth, metastasis, and HIV infection. Therefore, regulation of the CXCL12 signaling pathway by CXCR4, a CXCL12 receptor, would be an attractive therapeutic strategy for cancer and AIDS. AMD3100 is a CXCR4 antagonist that inhibits the CXCL12-CXCR4 signaling pathway, and has been approved by the FDA for patients of non-Hodgkin's lymphoma and multiple myeloma. CXCL14 is another variant of the CXC-type chemokine and is involved in immunosurveillance, suppression of tumor growth, exacerbation of obese diabetes and prevention of bacterial infection. Compared with other chemokines, there have been relatively few studies of CXCL14 presumably because of a lack of knowledge regarding its receptors and modulators of its functions. This study attempted to chemically synthesize CXCL14 and its derivatives for the development of molecules to modulate CXCL14 activities and to identify CXCL14 receptors.

In Chapter 1, the syntheses of CXCL14 and its derivatives by C–to–N- or N–to–C- directive sequential native chemical ligation are described. Biological evaluation of these proteins revealed that CXCL14 binds to CXCR4 and inhibits the CXCL12-induced chemotaxis of CXCR4-expressing THP-1 cells. In Chapter 2, dimer peptides derived from the CXCL14 C-terminal region were developed as novel peptide inhibitors of the CXCL12-CXCR4 signaling axis. The structure-activity relationship of the peptide inhibitors was also studied. Findings obtained in this study suggest that our modulator of CXCL14 activity might be a potential novel therapeutic agent that targets the CXCL12-CXCR4 signaling axis.

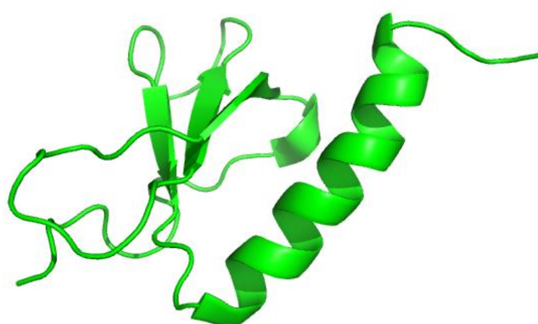


Figure. Structure of CXCL14 (PDB ID codes 2HDL).

Chapter 1

Synthesis of CXCL14 via C–to–N- or N–to–C-directive sequential native chemical ligation

1.1 CXCL14: a binder for CXCR4 and an inhibitor for the CXCL12-CXCR4 signaling axis

CXCL14 was initially isolated as a novel chemokine in 1999 by Hromas et al. from normal breast and kidney tissues and whose expression was down-regulated in cancer cell lines (Figure 1. 1).[1]

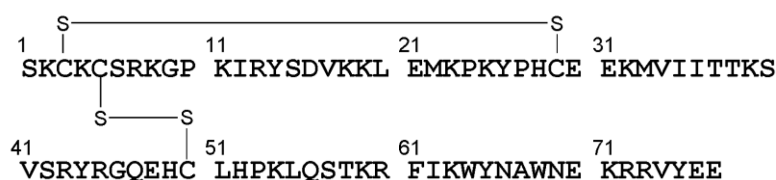
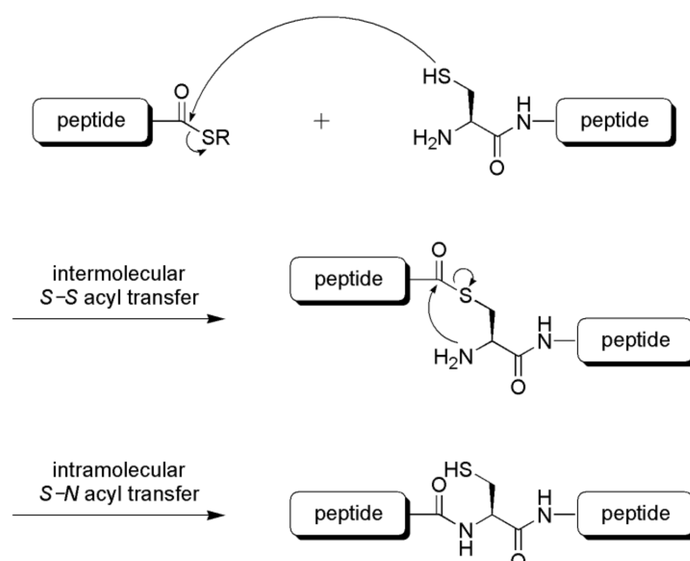


Figure 1.1. The primary aminoacid sequence of CXCL14

Although various functions of CXCL14, such as immunosurveillance,[2] tumor suppression,[3] metabolic regulation[4] and blocking of microbial infection,[5] have been revealed, receptors of CXCL14 have remained obscure. We recently reported that CXCL14 binds to CXCR4, a CXCL12 receptor, expressed on THP-1 cells, with high affinity and inhibits CXCL12-mediated chemotaxis.[6] This suggests that CXCR4 is a potential receptor for CXCL14, which is a natural inhibitor of the CXCL12-CXCR4 signaling axis. Based on this observation, we attempted to develop a molecular probe by the chemical syntheses of CXCL14 and its derivatives to further investigate the detailed involvement of CXCL14 in the CXCL12-CXCR4 signaling axis.

1.2 Chemical syntheses of CXCL14 and its derivatives using sequential native chemical ligation in a C–to–N- or N–to–C-directive manner and their biological evaluation

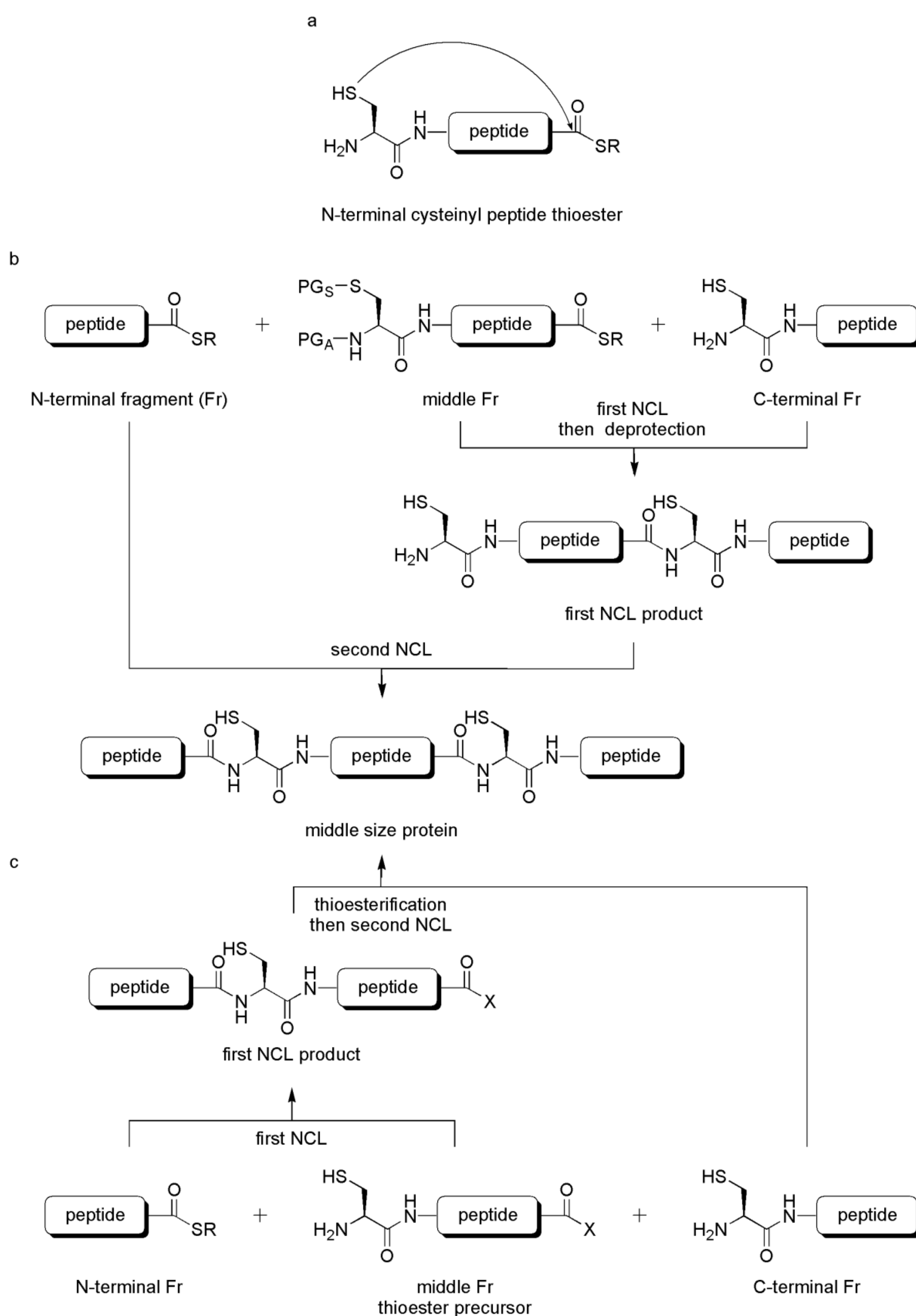
For the chemical synthesis of peptides and proteins, native chemical ligation (NCL) is the most widely used ligation method (Scheme 1. 1).[7] It enables chemoselective ligation between a thioester and an N-terminal cysteine residue. Although conventional NCL is applicable to the binding of two peptide fragments such as peptide thioester and



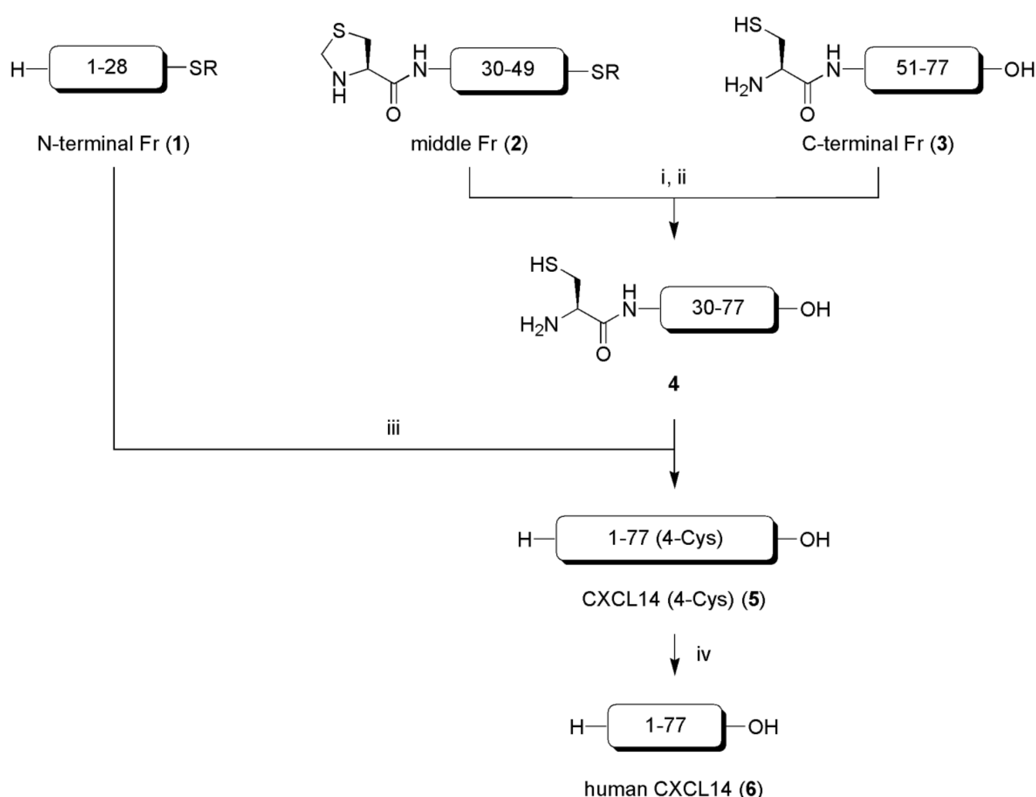
Scheme 1. 1. Native chemical ligation (NCL) to assemble two unprotected peptide fragments.

N-terminal cysteinyl peptide, and provides small proteins of about 80–100 residues, the NCL-mediated condensation of more than two fragments to produce middle sized proteins over 100 residues is difficult to achieve. The requirement of more than two fragments for the synthesis of the middle-sized proteins is attributed to the fact that the reliability of solid-phase peptide synthesis (SPPS) for fragment preparation is generally limited to proteins to 40 residues in size. Furthermore, the difficulty in achieving this by NCL is explained by the use of an N-terminal cysteinyl thioester fragment as a middle fragment. That is, the cysteinyl thioester fragment has an intrinsic tendency to be converted to a cyclic peptide via intramolecular NCL (Scheme 1. 2-a). Therefore, special concern is required when performing NCL using more than two fragments.[8] One potential method is to use C–to–N-directive sequential NCL using an *N* and/or *S*-protected N-terminal cysteinyl thioester as a middle fragment, which facilitates the diversification of the N-terminal portion of target proteins (Scheme 1. 2-b). Alternatively, diversification of the C-terminal portion is more easily achieved by use of N–to–C-directive sequential NCL (Scheme 1. 2-c).

To synthesize various CXCL14 proteins possessing diversity in the N- or C-terminal portion, we developed a three-fragment sequential NCL protocol using either the C–to–N- or N–to–C-directive sequential NCL with an N-terminal protected cysteinyl peptide thioester or N-terminal cysteinyl peptide thioacid as a middle fragment, respectively.[9] CXCL14 was divided into three fragments (Fr), 1–28, 29–49 and 50–77 (termed N-terminal Fr, middle Fr and C-terminal Fr, respectively) and the fragments were assembled in both C–to–N and N–to–C directions.



Scheme 1. 2. N-Terminal cysteinyl peptide thioester required for sequential NCL using more than two fragments. (a) N-Terminal cysteinyl peptide thioester causes undesired intramolecular NCL. (b) Protection of the N-terminal cysteine residue in the middle fragment is required for C–to–N-directive sequential NCL. PG_S: protecting group of thiol; PG_A: protecting group of amine. (c) N-terminal unprotected cysteinyl thioester precursor is used as the middle fragment for N–to–C-directive sequential NCL. X: an auxiliary that produces a corresponding peptide thioester.



Scheme 1. 3. Synthesis of human CXCL14 (**6**) in the C–to–N direction. (i) First NCL in 6 M Gn·HCl-0.2 M sodium phosphate buffer (pH 6.8) in the presence of 1% (v/v) thiophenol. (ii) Addition of 0.2 M $MeONH_2$·HCl to open the thiazolidine ring. (iii) Second NCL in 6 M Gn·HCl-0.2 M sodium phosphate buffer (pH 6.8) in the presence of 1% (v/v) thiophenol. (iv) Oxidation with air in 3 M Gn·HCl-0.1 M sodium phosphate buffer (pH 7.7). R = -$(CH_2)_2$-CO-Ala-NH_2.

In the C–to–N-directive strategy, N-terminal Fr **1** and N-terminal thiazolidine carboxylic peptide thioester **2** as middle Fr were prepared using standard *tert*-butoxycarbonyl (Boc)-SPPS (Scheme 1. 3 and Figure 1. 2). For the preparation of C-terminal Fr **3** standard 9-fluorenylmethoxycarbonyl (Fmoc)-SPPS was employed. The first NCL of middle Fr **2** and C-terminal Fr **3** proceeded within 4 h in 6 M guanidine·HCl (Gn·HCl)-0.2 M sodium phosphate buffer (pH 6.8) containing 1% (v/v) thiophenol to produce the desired ligated product. Then, 0.2 M methoxyamine·hydrochloride ($MeONH_2$·HCl) was added to the mixture to open the thiazolidine ring and produce the N-terminal unprotected ligation product **4**. After HPLC purification, **4** was subsequently ligated with N-terminal Fr **1** in 6 M Gn·HCl-0.2 M sodium phosphate buffer (pH 6.8) containing 1% (v/v) thiophenol. After completion of the second NCL, the reduced form of CXCL14 (CXCL14 (4-Cys)) **5** was isolated by HPLC purification. The resulting CXCL14 (4-Cys) **5** was subjected to a refolding procedure under air oxidation conditions[10] in 3 M Gn·HCl-0.1 M sodium phosphate buffer (pH 7.7) and the desired folded human CXCL14 **6** was obtained after purification

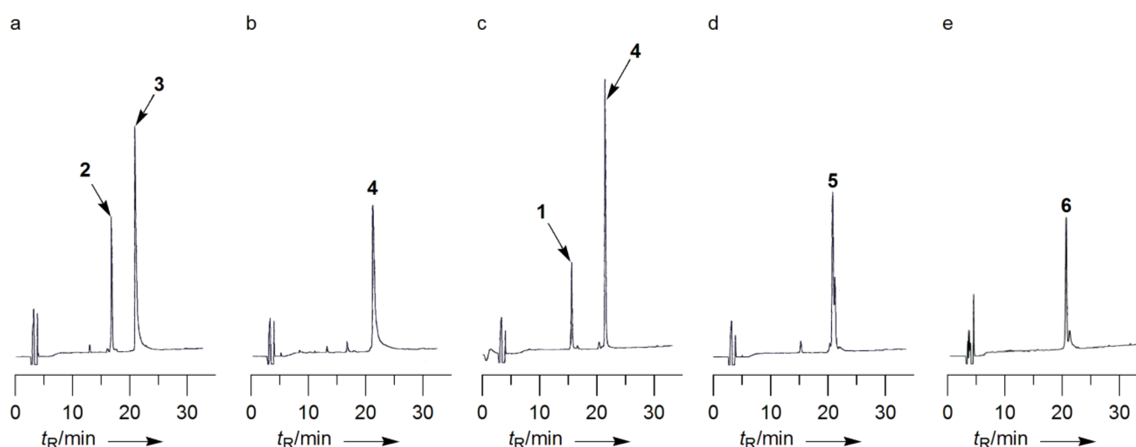
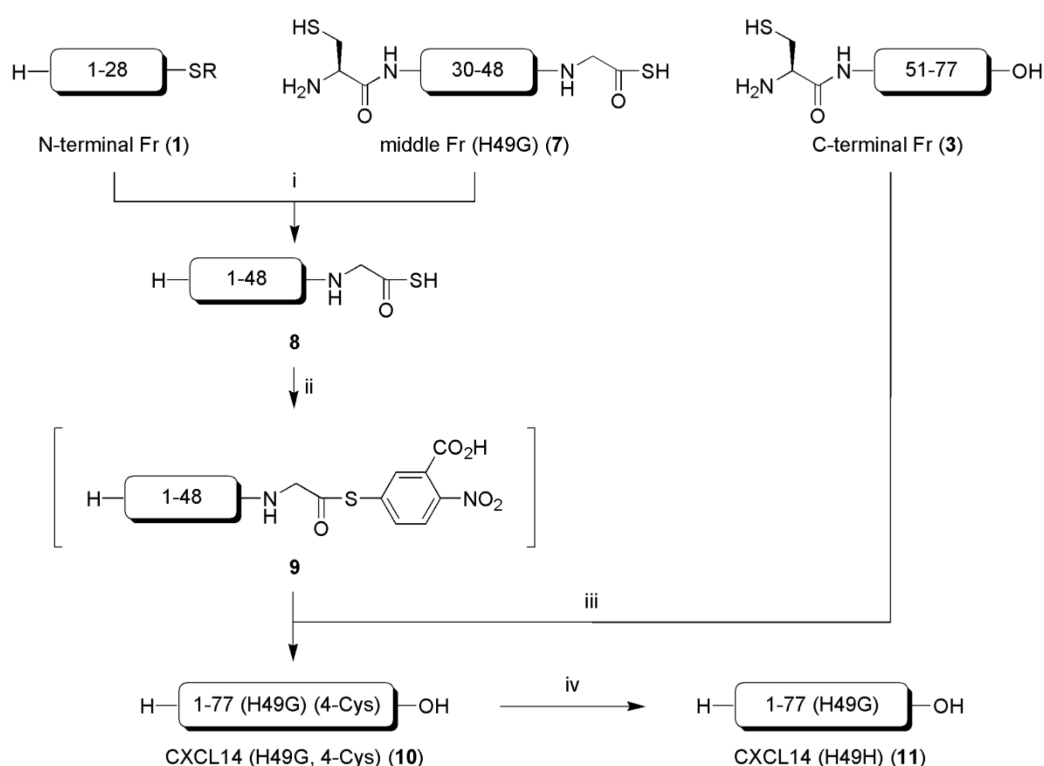


Figure 1. 2. HPLC monitoring of the C–to–N directive sequential NCL for synthesis of human CXCL14 (**6**). (a) First NCL (t < 1 min). (b) First NCL (t = 4 h) followed by addition of 0.2 M $MeONH_2$·HCl. (c) Second NCL (t < 1min). (d) Second NCL (t = 6 h). (e) Oxidation (t = 6.5 h). HPLC conditions: Cosmosil $5C_{18}$ AR-II column (4.6 × 250 mm) with a linear gradient of 0.1% TFA-MeCN/0.1% TFA aq. (5:95–45:55 over 30 min) at a flow rate of 1.0 mL/min, detection at 220 nm.

by HPLC in 13% overall yield (3 steps).

In the initial attempt of N–to–C-directive strategy, an N-terminal cysteinyl thioacid was employed as the middle fragment because the N-terminal cysteinyl moiety does not react intramolecularly with the thioacid as a latent thioester unit (Scheme 1. 4 and Figure 1. 3).[7, 11] Middle Fr **7** was prepared according to a method developed by our group.[12] Briefly, the protected *N*-sulfanylethylanilide (SEAlide)[13] peptide of CXCL14 (29–49) with an N-terminal Fmoc group was synthesized on aminomethyl ChemMatrix resin using standard Fmoc-SPPS, and the acid-labile protecting groups on side chains were removed by treatment with TFA/thioanisole/*m*-cresol/H_2O/1,2-ethanedithiol (EDT)/Et_3SiH (80:5:5:5:5:5 (v/v)). The resulting peptide-attached resin was then treated with 4 M HCl/DMF in the presence of 1% (w/v) tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) to give the corresponding peptide thioester resin via on-resin-intramolecular *N–S* acyl transfer. Treatment of the thioester resin with 120 mM NaSH in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 9.2) provided the peptide thioacid, which was then converted to the middle Fr **7** by piperidine-mediated Fmoc removal. The mutation of H49G was incorporated because of the instability of thioester species derived from histidyl thioacid moieties. The first NCL of N-terminal Fr **1** and middle Fr **7** in 6 M Gn·HCl-0.2 M sodium phosphate buffer (pH 6.8) containing 1% (v/v) thiophenol at 37 °C provided the ligated thioacid **8** (Scheme 1. 4 and Figure 1. 3). During the first NCL, the intramolecular NCL product was not observed. HPLC-purified thioacid **8** was then treated with an excess amount of Ellman's reagent[14] in the presence of $KHCO_3$ in 20% (v/v) DMF aq. to afford peptide thioesters. Then, the cysteine residues were converted to the disulfide species by the action of Ellman's



Scheme 1. 4. Synthesis of CXCL14 (H49G) (**11**) in N–to–C direction. (i) First NCL in 6 M Gn·HCl-0.2 M sodium phosphate buffer (pH 6.8) in the presence of 1% (v/v) thiophenol. (ii) Thioesterification by treatment with Ellman's reagent and $KHCO_3$ in 20% (v/v) DMF aq., then addition of TCEP·HCl. (iii) Second NCL in 20% DMF aq. in the presence of 1% (v/v) thiophenol. (iv) Oxidation with air in 3 M Gn·HCl-0.1 M sodium phosphate buffer (pH 7.7). R = -$(CH_2)_2$-CO-Ala-NH_2.

reagent. To reduce the resulting disulfide species, and more importantly to quench the excess Ellman's reagent that inhibits successive NCL, TCEP·HCl, a reducing agent, was added to the reaction mixture to yield desired thioester **9**. Then, C-terminal Fr **3** and 1% (v/v) thiophenol was added to the mixture at 37 °C to initiate the second NCL. The second NCL was completed within 2 h, and following HPLC purification, it produced CXCL14 (4-Cys, H49G) **10**. The obtained peptide **10** was folded under air oxidation conditions in 3 M Gn·HCl-0.1 M sodium phosphate buffer (pH 7.7) to obtain folded CXCL14 (H49G) **11**. After completion of the folding, CXCL14 (H49G) **11** was purified by HPLC and was obtained in 1% overall yield (3 steps). The inhibitory activities of synthetic CXCL14 proteins **6** and **11** to CXCL12-mediated chemotaxis were evaluated by standard chemotaxis assays using human leukemia-derived THP-1 cells (Figure 1. 4).[15] The number of THP-1 cells in the upper chamber in the presence of 100 nM CXCL14 that migrated toward the lower chamber containing 10 nM CXCL12 was counted. Both synthetic CXCL14 proteins **6** and **11** showed inhibitory activity against CXCL12-mediated chemotaxis comparable with that of recombinant one. As mentioned here, synthetic CXCL14 proteins obtained by the conventional C–to–N- and N–to–C-directive protocols undoubtedly afforded biologically active materials; however, the

chemical yield of the N–to–C-directive method was unsatisfactory. Therefore, an efficient N–to–C-directive method is urgently required in the field of chemical protein synthesis. In this context, we attempted to develop an *N*-sulfanylethylanilide (SEAlide) peptide-mediated N–to–C-directive strategy for efficient CXCL14 preparation.

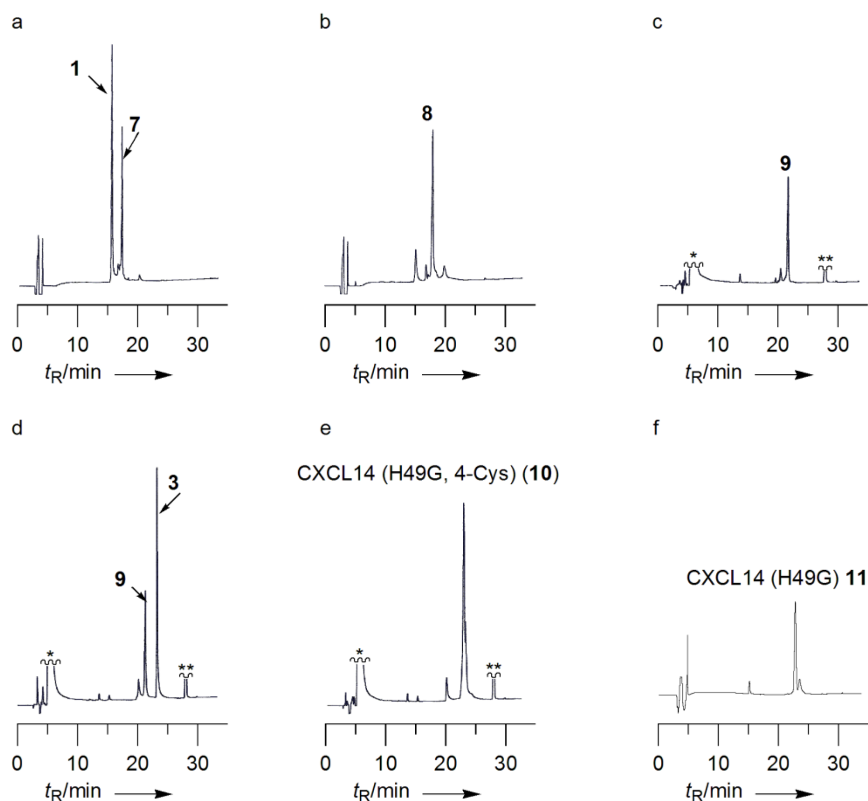


Figure 1. 3. HPLC monitoring of the N–to–C directive sequential NCL for the synthesis of CXCL14 (H49G) (**11**). (a) First NCL (t < 1 min). (b) First NCL (t = 4 h). (c) Thioesterification of thioacid **8** to corresponding aryl thioester **9** by treatment with Ellman's reagent and $KHCO_3$ in 20% (v/v) DMF aq. for 1 h followed by the addition of TCEP·HCl. (d) Second NCL (t < 1 min): addition of C-terminal fragment **3** to the thioesterification reaction mixture and then, the pH of the mixture was adjusted to approximately 7.5. (e) Second NCL (t = 2 h). (f) Oxidation (t = 22 h). HPLC conditions: Cosmosil $5C_{18}$ AR-II column (4.6 × 250 mm) with a linear gradient of 0.1% TFA-MeCN/0.1% TFA aq. (5:95–45:55 over 30 min) at a flow rate of 1.0 mL/min, detection at 220 nm. *DMF. **5-mercapto-2-nitrobenzoic acid.

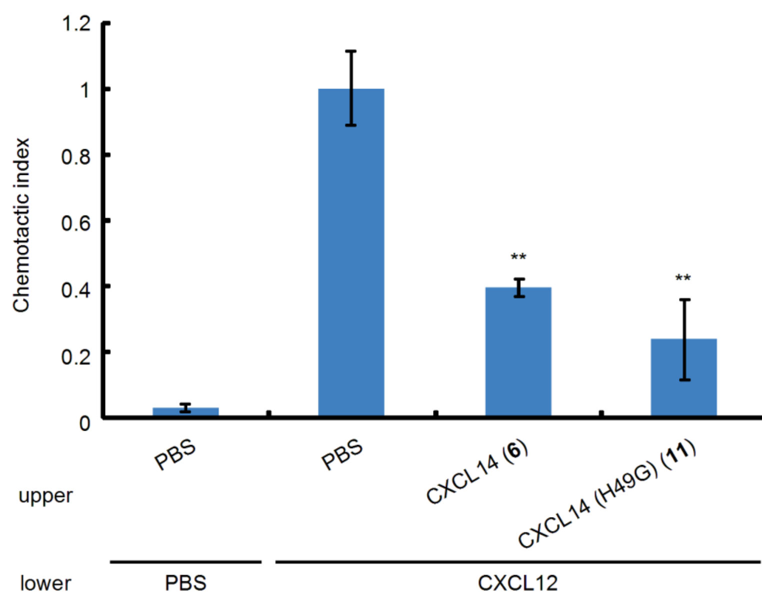
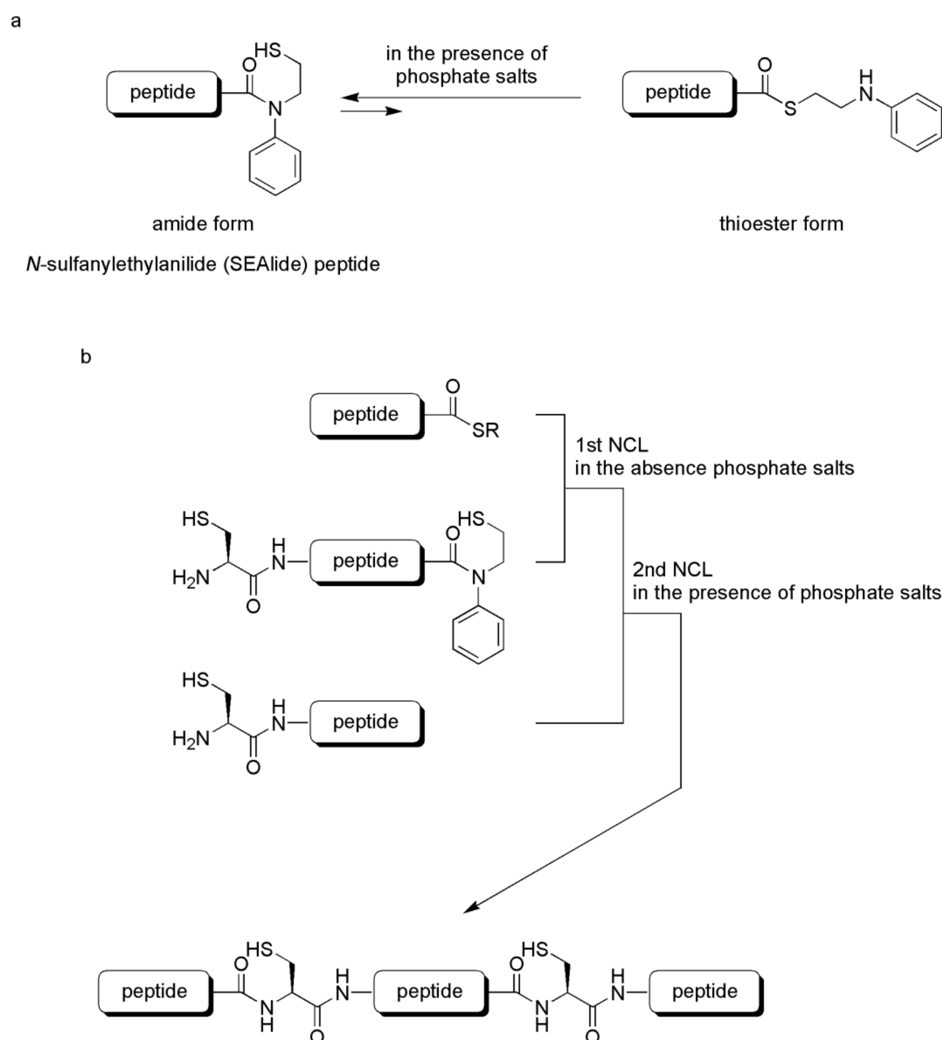


Figure 1. 4. Inhibitory activity of CXCL14 proteins to CXCL12-mediated chemotaxis. The cell number of THP-1 cells that migrated toward 10 nM CXCL12 in the absence or presence of 100 nM CXCL14 proteins was counted (n = 3). Data are shown as the chemotactic index: the migrated cell number to CXCL12 divided by migrated cell number to medium alone. **$P < 0.01$ compared with the PBS (upper)/CXCL12 (lower) control.

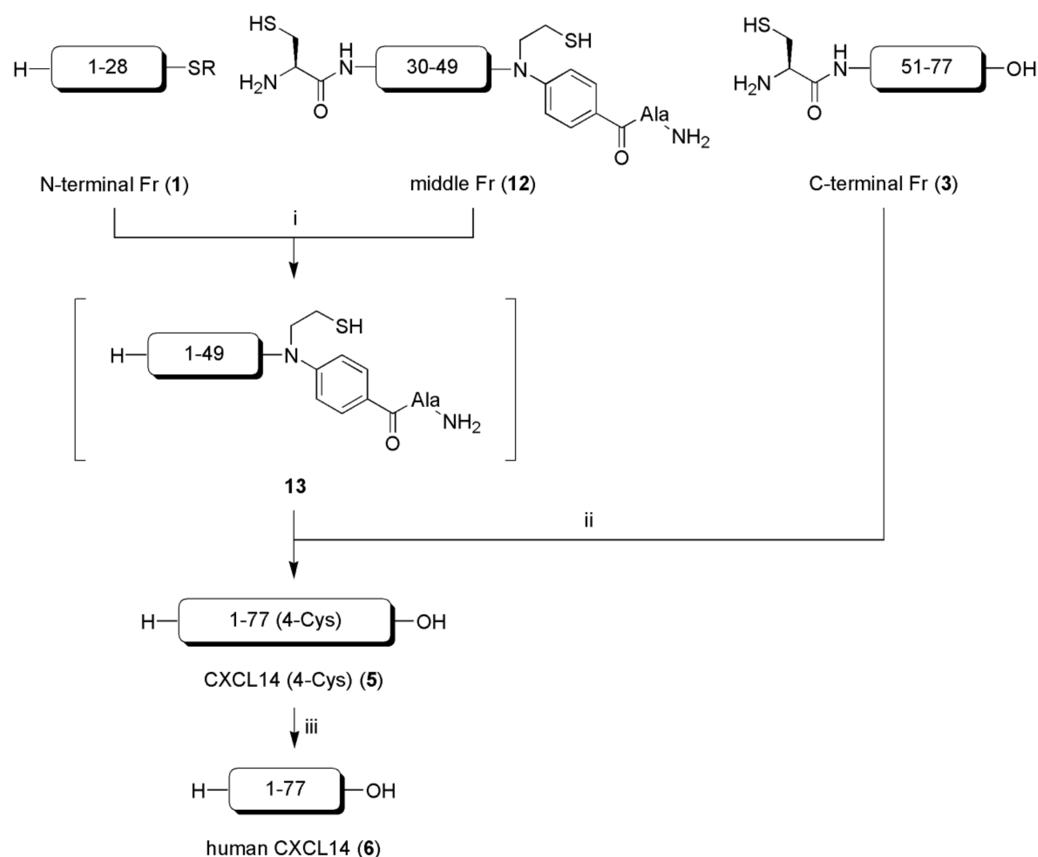
1.3 One-pot/sequential native chemical ligation strategy for syntheses of CXCL14 and its derivatives using N-sulfanylethylanilide peptide

We previously reported the use of three-fragment/one-pot sequential NCL for the synthesis of human atrial natriuretic peptide (ANP) using an N-terminal cysteinyl SEAlide peptide as a middle fragment (Scheme 1. 5).[16] The sequential NCL was achieved by controlling the reactivity of the SEAlide peptide that functions as a thioester equivalent only in the presence of phosphate salts. To achieve a greater efficiency, we applied the SEAlide peptide as a middle fragment for the synthesis of CXCL14 using the one-pot method (Scheme 1. 6). To prepare the middle fragment, Fmoc-histidine-coupled *N*-sulfanylethylaniline linker, 4-[{Fmoc-His(Fmoc)-2-tritylsulfanylethyl}amino]benzoic acid **14** was synthesized as shown in Scheme 1. 7. The protection of the histidyl imidazole group is associated with its racemization during the coupling of histidine species with the aniline linker **15**. We found that an Fmoc group on a τ-nitrogen atom of the imidazole (Fmoc-His(Fmoc)-OH) suppressed the racemization comparable with that of a previously reported methoxybenzyloxymethyl (MBom) group on the π-nitrogen atom of the imidazole.[17]



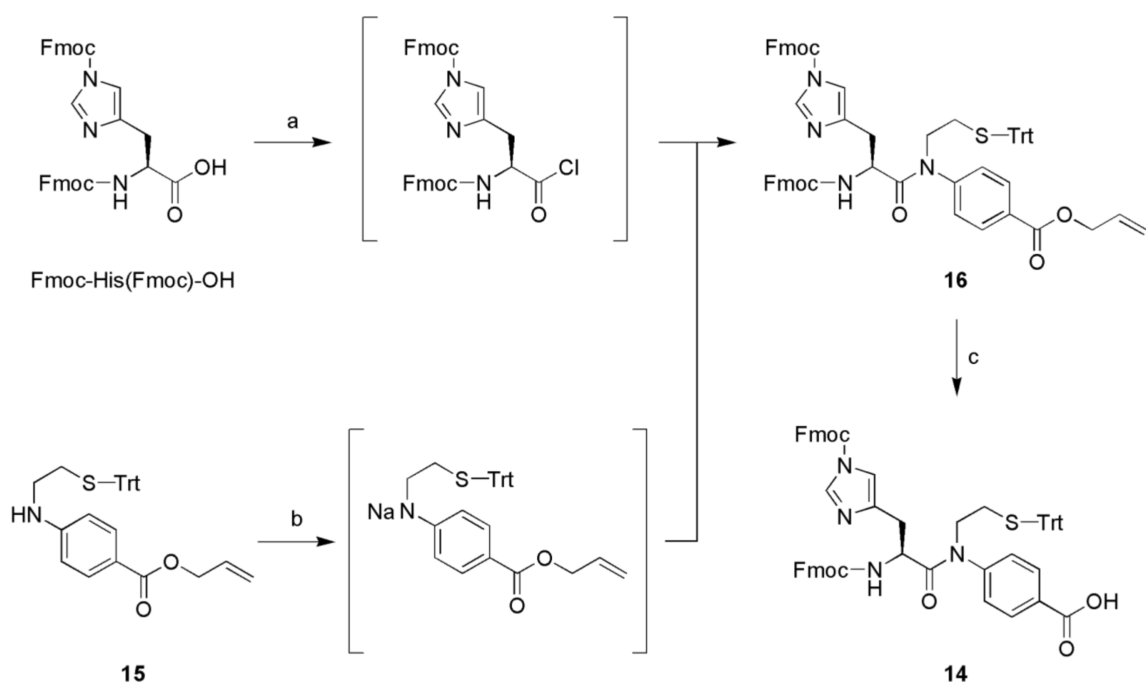
Scheme 1. 5. The character of *N*-sulfanylethylanilide (SEAlide) peptide and its application. (a) SEAlide peptide as a peptide thioester equivalent in the presence of phosphate salts. (b) A SEAlide peptide is applicable to one-pot sequential NCL by controlling concentration of phosphate salts.

The incorporation of Fmoc-Ala-OH onto NovaSyn® TGR resin and subsequent coupling of compound **14** with the resulting resin were achieved to give the SEAlide unit-incorporated resin. The protected peptide chain was elongated on the resin by standard Fmoc SPPS. The completed resin was deprotected with TFA-thioanisole-*m*-cresol-EDT-H_2O (80:5:5:5:5, v/v) at room temperature for 1.5 h, and the resulting TFA mixture was cooled to 0 °C. Ammonium iodide (NH_4I) and dimethyl sulfide (Me_2S) were subsequently added to the mixture to reduce the methionine oxide formed during chain elongation.[18] The desired SEAlide peptide **12** was obtained by HPLC purification. Once the requisite fragments for the construction of CXCL14 were obtained, the synthesis of CXCL14 by one-pot/sequential NCL method using the SEAlide peptide as a middle fragment was attempted. The first NCL between



Scheme 1. 6. Synthesis of human CXCL14 (**6**) by N–to–C directive one-pot/sequential NCL using SEAlide peptide. (i) First NCL in 6 M Gn·HCl-0.2 M HEPPS buffer (pH 6.6), 50 mM MPAA and 30 mM TCEP·HCl, then (ii) addition of C-terminal fragment **3** in 1 M sodium phosphate buffer (pH 6.6), (second NCL). (iii) Oxidation with air in 3 M Gn·HCl-0.1 M sodium phosphate buffer (pH 7.7). R = -$(CH_2)_2$-CO-Ala-NH_2.

N-terminal Fr **1** and middle Fr **12** was conducted at 25 °C in 6 M Gn·HCl-0.2 M HEPPS buffer (pH 6.6), 50 mM MPAA and 30 mM TCEP·HCl, and then C-terminal Fr **3** in 1 M sodium phosphate buffer (pH 6.6) was added to the mixture to initiate the second NCL (Figure 1. 5). The N–to–C-directive one-pot/sequential strategy yielded reduced CXCL14 **5** in 38% isolated yield. The resulting CXCL14 (4-Cys) **5** was oxidized as described in a previous section to give folded CXCL14 **6** in 39% isolated yield. This method successfully improved the overall yield (15%, 2 steps), compared with the N–to–C-directive sequential NCL using peptide thioacid mentioned above (1%, 3 steps). The reason for this improvement might be explained by the easy experimental manipulations and reduction of the number of purification step when using the one-pot procedure. Next, we attempted to develop a molecular probe based on CXCL14 to investigate the involvement of CXCL14 in the CXCL12-CXCR4 signaling axis using sequential NCL strategies. Initially, CXCL14 derivative **26** possessing an Alexa fluor® 488 hydroxyamine **25** moiety on its C-terminal portion was prepared using the



Scheme 1. 7. Synthesis of Fmoc-His(Fmoc)-SEAlide linker **14**. (a) $SOCl_2$, cat. DMF, CH_2Cl_2, r.t., then removal of the solvent *in vacuo*. The obtained crude mixture without purification was reacted with sodium amide in THF that was afforded in step (b). (b) NaH, THF, r.t.. (c) *N*-methylaniline, $Pd(PPh_3)_4$, THF, r.t., 65% (2 steps)

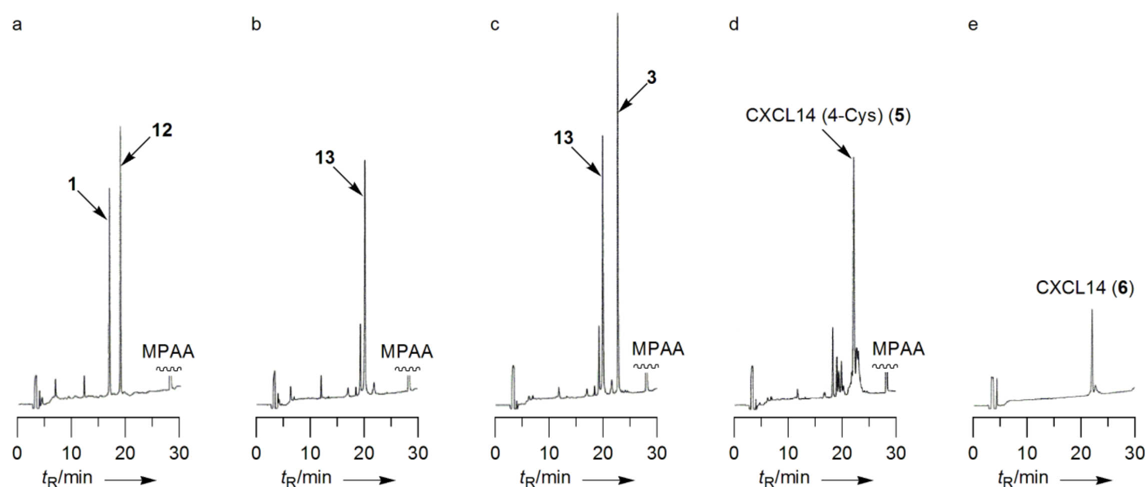
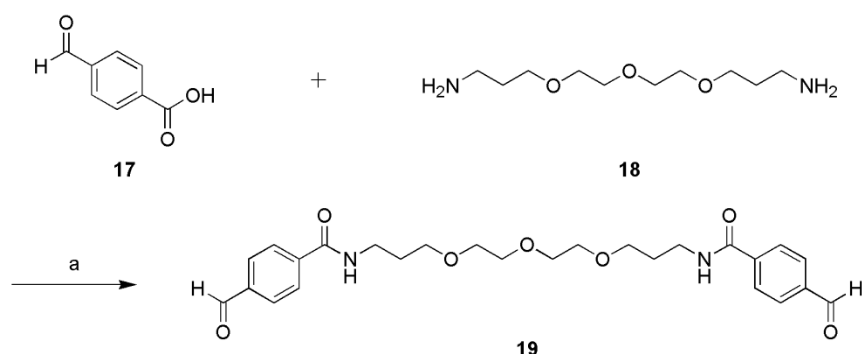


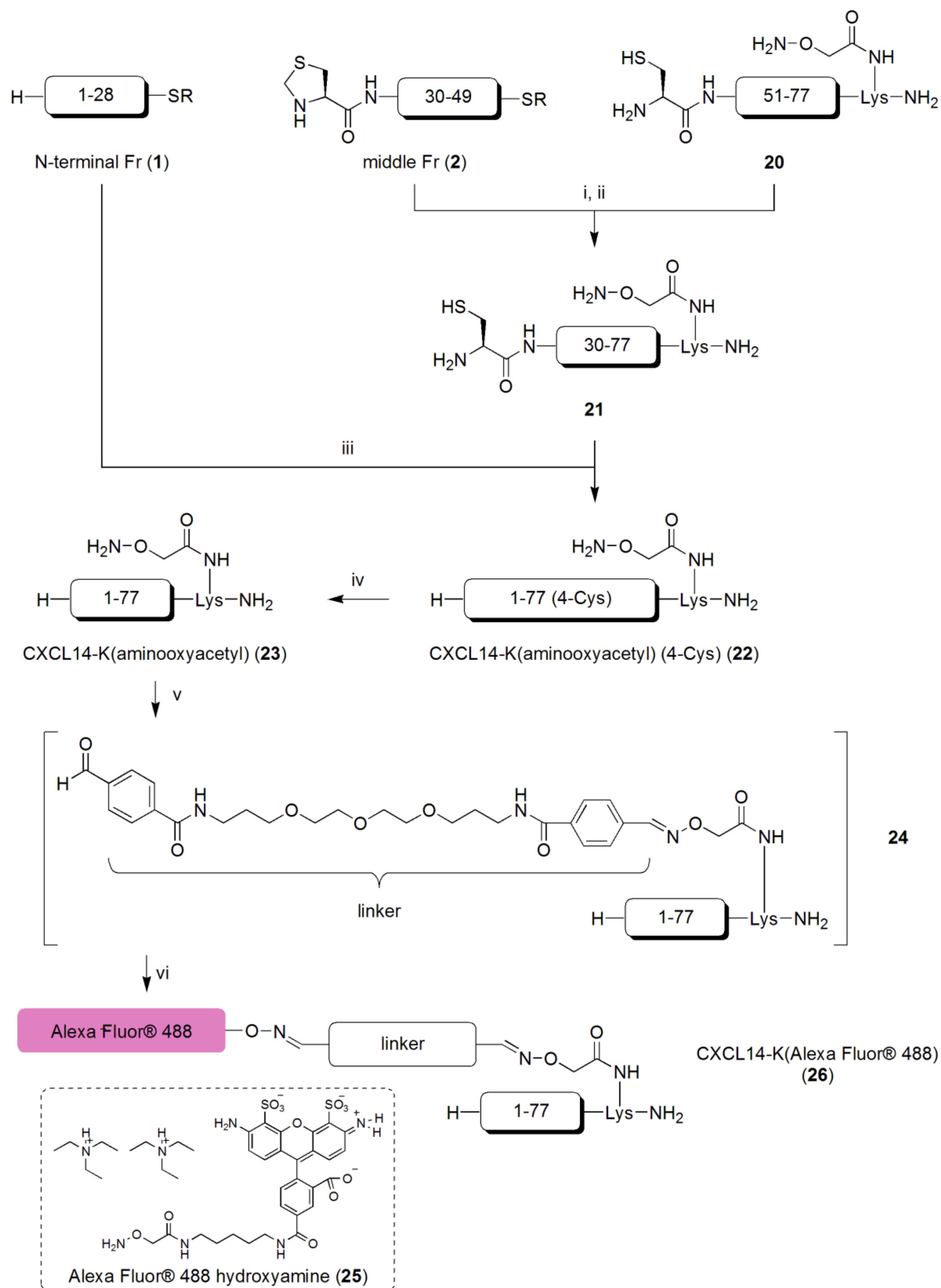
Figure 1. 5. HPLC monitoring of the N–to–C directive one-pot/sequential NCL for synthesis of human CXCL14 (**6**). (a) First NCL ($t < 1$ min) in 6 M Gn·HCl-0.2 M HEPPS buffer (pH 6.6), 50 mM MPAA and 30 mM TCEP·HCl. (b) First NCL ($t = 5$ h). (c) followed by addition of C-terminal fragment **3** in 1 M sodium phosphate buffer (pH 6.6) (second NCL, $t < 1$ min). (d) Second NCL ($t = 48$ h) (e) Oxidation with air ($t = 23$ h). HPLC conditions: Cosmosil $5C_{18}$ AR-II column (4.6 × 250 mm) with a linear gradient of 0.1% TFA-MeCN/0.1% TFA aq. (5:95–45:55 over 30 min) at a flow rate of 1.0 mL/min, detection at 220 nm.

conventional C–to–N-directive strategy (Scheme 1. 8 and 1. 9). To conjugate CXCL14 and Alexa fluor® 488 by oxime linkage, we initially synthesized a dialdehyde linker **19**.[19] *p*-Formylbenzoic acid **17** was coupled with *O*,*O'*-bis(3-aminopropyl)diethylene glycol **18** to afford dialdehyde linker **19**. Then, CXCL14 derivative **23** possessing an

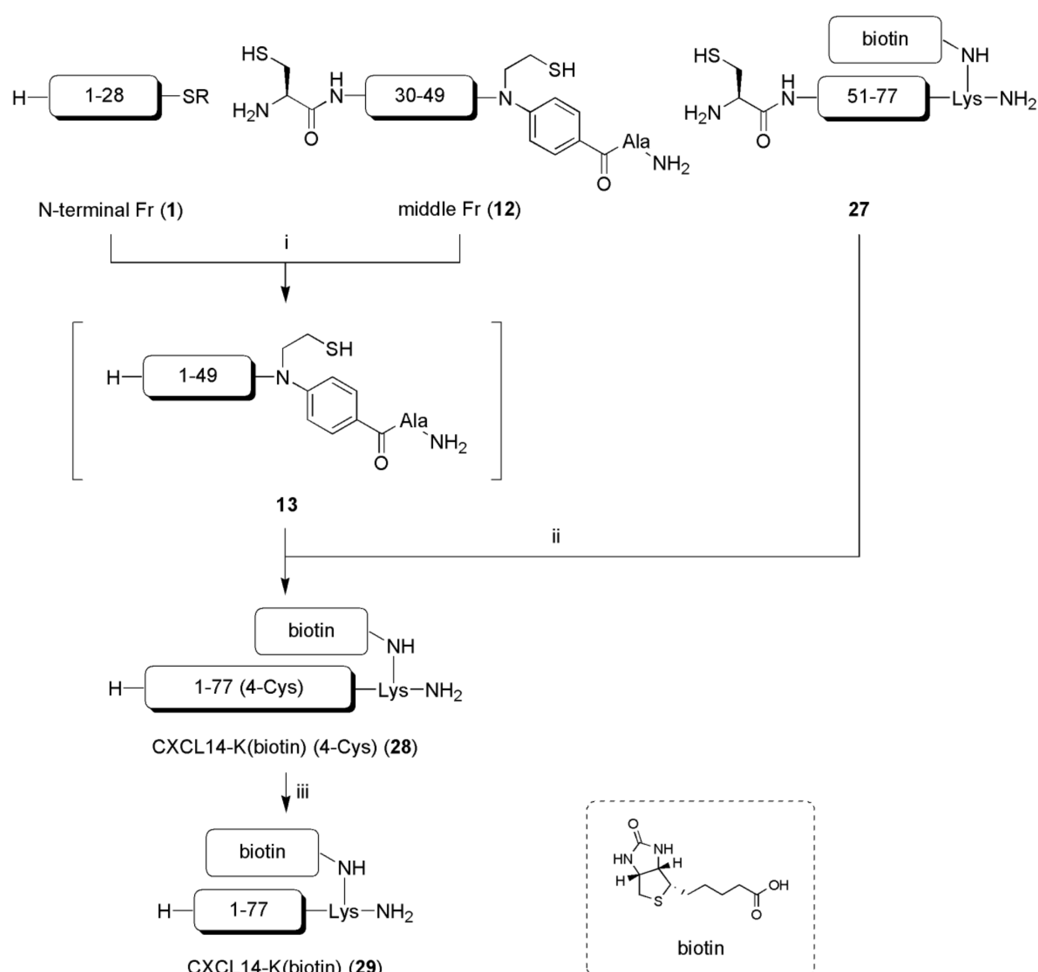


Scheme 1. 8. Synthesis of dialdehyde linker **19**. (a) DIPCDI, DMAP, CH_2Cl_2, r.t., 84%

additional lysine residue at the C-terminus was synthesized. A side chain amino group of the additional residue was modified by an aminooxyacetyl group to conjugate the aldehyde unit of linker **19**. The requisite aminooxyacetylated fragment **20** was prepared by Fmoc SPPS using N^{ε}-ivDde-protected lysine followed by on-resin-selective deprotection of ivDde and coupling of Boc-aminooxyacetic acid with the resulting N^{ε}-amino group. Fragment couplings using C-terminal Fr **20** bearing aminooxyacetylated lysine at its C-terminus were performed by conventional C–to–N-directive NCL similar to that employed for CXCL14 **6**. Resulting linear protein **22** was folded under air-oxidation conditions to give the folded CXCL14 derivative **23**, which was then treated with an excess amount of linker **19** in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 5.8) and 0.5 mM EDTA to generate linker-incorporated CXCL14 derivative **24** through oxime bond formation. After completion of the reaction, excess linker **19** was washed out by CH_2Cl_2 extraction, and then Alexa fluor® 488 hydroxyamine **25** in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 4.0) and 0.5 mM EDTA was subsequently added to the mixture to afford the desired Alexa fluor® 488-incorporated CXCL14 derivative **26**. Unfortunately, synthesized derivative **26** was not suitable for practical use in fluorescent imaging due to its low fluorescence intensity. Therefore, a CXCL14 derivative with a biotin unit (CXCL14-K(biotin)) **29**, which is highly fluorescent upon treatment with streptavidin-fluorescent (allophycocyanin (APC)) conjugate, was synthesized by the SEAlide peptide-mediated N–to–C-directive one-pot/sequential NCL method similar to that employed for CXCL14 **6** using C-terminal Fr **27** with additional C-terminal biotinylated lysine residue (Scheme 1. 10). Requisite biotinylated fragment **27** was synthesized by an Fmoc protocol similar to that employed for **20**. The N–to–C-directive one-pot/sequential NCL followed by folding successfully produced the desired biotinylated CXCL14 analog **29** that was fluorescent with the aid of a streptavidin-APC conjugate. Next, we attempted to confirm the binding of CXCL14-K(biotin) **29** to CXCR4.



Scheme 1. 9. Synthesis of human CXCL14-K(Alexa Fluor® 488) **26** (i) First NCL in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 6.8) and 0.5 mM EDTA in the presence of 1% (v/v) thiophenol. (ii) Addition of 0.2 M $MeONH_2$·HCl to open the thiazolidine ring. (iii) Second NCL in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 6.8) and 0.5 mM EDTA in the presence of 1% (v/v) thiophenol. (iv) Oxidation with air in 3 M Gn·HCl-0.1 M sodium phosphate buffer (pH 7.7). (v) Oxime ligation in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 5.8) and 0.5 mM EDTA, then excess linker **19** was washed out by CH_2Cl_2. (vi) Addition of Alexa Fluor® 488 hydroxyamine in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 4.0) and 0.5 mM EDTA to the resulting crude solution of **24**. R = -$(CH_2)_2$-CO-Ala-NH_2.



Scheme 1. 10. Synthesis of CXCL14-K(biotin) (**29**) in N–to–C directive one-pot/sequential NCL using SEAlide peptide. (i) First NCL in 6 M Gn·HCl-0.2 M HEPPS buffer (pH 6.6), 50 mM MPAA and 30 mM TCEP·HCl, then (ii) addition of C-terminal fragment **27** in 1 M sodium phosphate buffer (pH 6.6), (second NCL). (iii) Oxidation with air in 3 M Gn·HCl-0.1 M sodium phosphate buffer (pH 7.7). R = -$(CH_2)_2$-CO-Ala-NH_2.

1.4 Biological evaluation of the biotinylated CXCL14 derivative

To confirm whether biotinylated CXCL14 **29** maintained its inhibitory activity for CXCL12-mediated cell migration, standard chemotaxis assays using THP-1 cells were conducted (Figure 1. 6). CXCL14-K(biotin) **29** exhibited an inhibitory activity comparable with that of the expressed and chemically synthesized CXCL14. This suggests that the biotin derivative **29** could be used as a molecular probe to evaluate the binding potency of CXCL14. The binding of CXCL14-K(biotin) **29** to CXCR4 was assessed by fluorescence activated cell sorting (FACS) analysis using human cervical carcinoma-derived HeLa cells transfected with a pCS2+*GFP* vector for HeLa-GFP cells or a pCS2+*CXCR4-GFP* vector for HeLa-CXCR4-GFP cells (Figure 1. 7). Initially, to

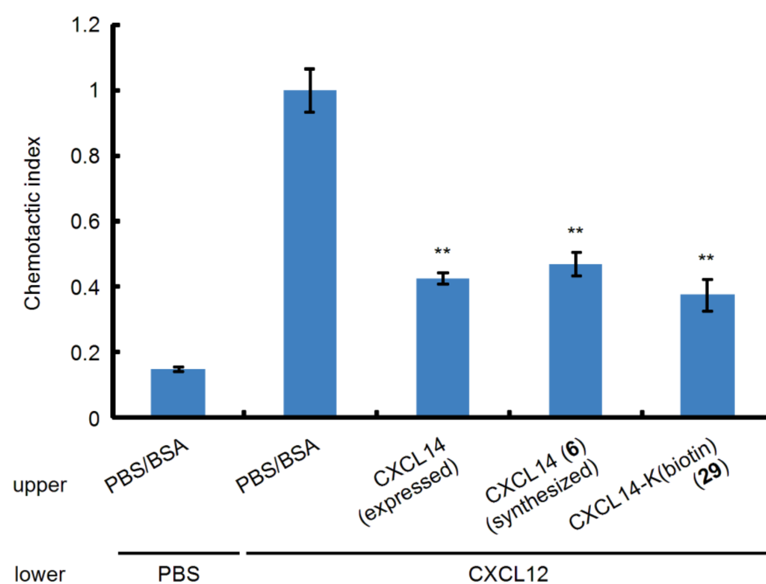


Figure 1. 6. Inhibitory activity of CXCL14 proteins to CXCL12-mediated chemotaxis. Numbers of migrating THP-1 cells toward 10 nM CXCL12 in the absence or presence of 100 nM CXCL14 proteins were counted (n = 3). Data are shown as the chemotactic index: the migrated cell number to CXCL12 divided by migrated cell number to the medium alone (PBS/BSA). **$P < 0.01$ compared to the (PBS/BSA)/CXCL12 control.

confirm the expression of CXCR4, these cells were analyzed by FACS analysis using APC-conjugated anti-human CXCR4 antibody (anti-CXCR4-APC) (Figure 1. 7-a, b). The expression of CXCR4 on HeLa-GFP cells was slightly detected due to the presence of endogenous CXCR4 on the cells (Figure 1. 7-a). In HeLa-CXCR4-GFP cells, the expression level of CXCR4 was markedly enhanced (Figure 1. 7-b). Thus, we evaluated the binding of CXCL14-K(biotin) **29** to CXCR4 on these HeLa cell lines by FACS analysis and found that CXCL14-K(biotin) **29** had potent binding to HeLa-GFP cells (Figure 1. 7-c) and a higher binding potency to HeLa-CXCR4-GFP cells (Figure 1. 7-d). In the presence of high concentrations of unlabeled CXCL14, the binding of CXCL14-K(biotin) **29** to the cells was significantly reduced (Figure 1. 7-c, d). These results proved that CXCL14-K(biotin) **29** specifically binds to CXCR4 on HeLa cells. The reason for the partial inhibition of binding by excess CXCL14 might be explained by the presence of low affinity binding site for basic CXCL14 such as heparan sulfate on the cell surface.[20]

1.5 Conclusion

A synthetic platform for CXCL14 was developed using C–to–N- or N–to–C-directive sequential NCL protocols. In particular, the N–to–C-directive protocol using a SEAlide peptide as a middle fragment allowed one-pot sequential ligation with easy

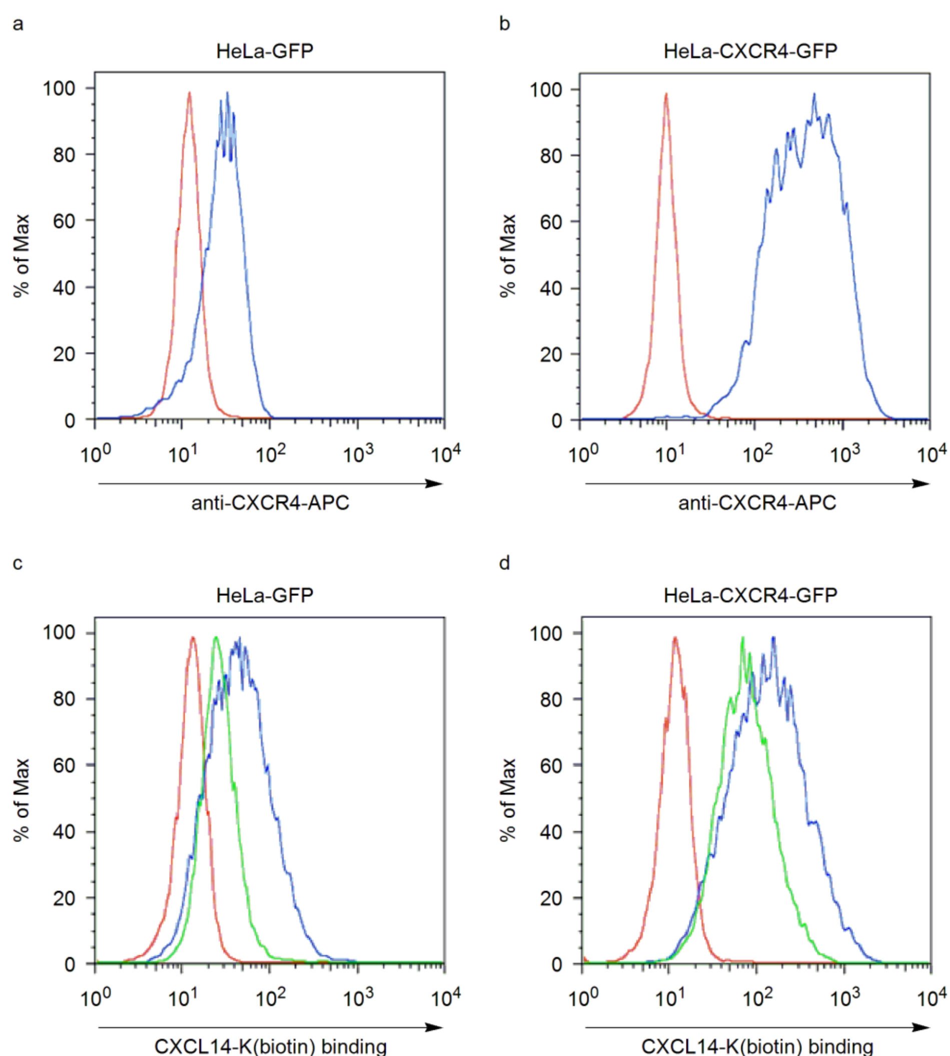


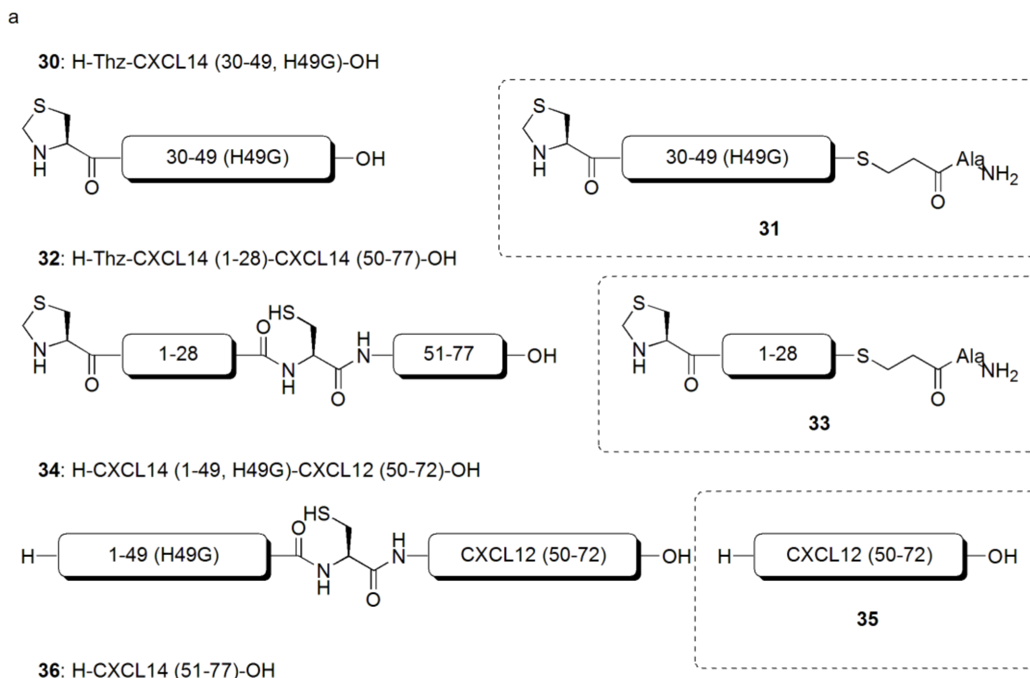
Figure 1. 7. (a, b) FACS analysis of CXCR4 expression (a) HeLa-GFP cells or (b) fusion protein of CXCR4 and GFP (HeLa-CXCR4-GFP). Anti-CXCR4-APC bound on the cells was detected by FACS analysis. The cells were untreated (red) or treated with anti-CXCR4-APC (blue), respectively. (c, d) FACS analysis of CXCL14-K(biotin) **29** binding (c) HeLa-GFP or (d) HeLa-CXCR4-GFP. CXCL14-K(biotin) bound on the cells was stained with APC-streptavidin, and then detected by FACS analysis. The cells were treated with 100 nM biotin (red), 100 nM CXCL14-K(biotin) **29** (blue) or 100 nM CXCL14-K(biotin) **29** + 4 µM CXCL14 (green), respectively.

experimental manipulations. This protocol provided better results in overall yield and purity than the protocol employing a peptide thioacid which required multi-step manipulation and intermediate purification. The binding experiment of biotinylated CXCL14 derivative **29** against CXCR4-expressing HeLa cells clearly indicated that CXCL14 binds to CXCR4, a receptor for CXCL12.

Chapter 2

Development of a novel inhibitor of the CXCL12-CXCR4 signaling axis

2.1 Exploration of the interaction region of CXCL14 with CXCR4



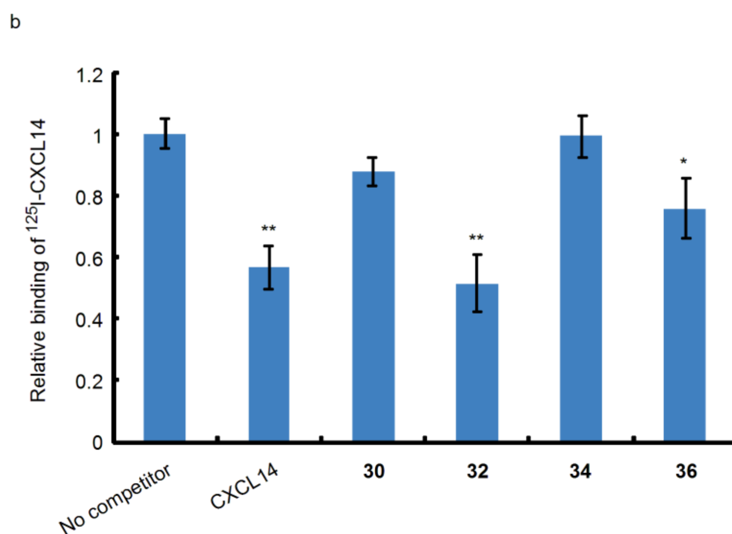


Figure 2. 1. Competitive binding assay of CXCL14 derivatives. (a) Synthesized CXCL14 derivatives. (b) Competitive inhibition of ^{125}I-CXCL14 binding by CXCL14 derivatives. CXCR4 transfected THP-1 cells were incubated with 10 nM ^{125}I-CXCL14 in the presence or absence of 100 nM CXCL14 derivatives, and cell-associated radioactivity was measured (n = 6). Relative binding of ^{125}I-CXCL14 to no competitor is shown. *P < 0.05, **P < 0.01 compared with no competitor, respectively.

Most CXC-type chemokines possess flexible N-terminal regions that are important for their receptor binding,[21] whereas CXCL14 lacks a corresponding region. Therefore, we hypothesized that CXCL14 binds to its receptor in a manner different from other CXC-type chemokines, presumably using a C-terminal α-helical region. To investigate the critical region of CXCL14 that is involved in interaction with CXCR4, the binding affinities of various CXCL14-derived peptides, listed in Figure 2. 1, were evaluated. The binding potency of CXCL14-derived peptides was examined by competitive inhibition of ^{125}I-labeled CXCL14 (^{125}I-CXCL14) binding using CXCR4 transfected THP-1 cells (Figure 2. 1b).[15] Middle fragment-deleted analog **32** without folded structure (i.e. reduced form) showed binding affinity to CXCR4-expressing cells comparable with that of CXCL14. Furthermore, C-terminal Fr **36** also showed binding potency to CXCR4-expressing cells, whereas middle Fr **30** and CXCL14-CXCL12 chimera **34** with CXCL14 (51–77) replaced by CXCL12 (50–72) did not show any binding inhibition. These results suggested that the C-terminal α-helical region of CXCL14 (CXCL14 (51–77)) was essential for binding to CXCR4 expressing THP-1 cells. This region is highly conserved among numerous species from elephant shark to humans and possesses unique conserved aromatic amino acid residues on one surface of the α-helix (Figure 2. 2).[22] In our recent study, chemical crosslinking experiments of CXCR4 on THP-1 cells with CXCL14 indicated that a dimeric form of CXCL14 is associated with the interaction with CXCR4 in addition to the monomeric form.[6] It was also reported that other chemokines such as CXCL8 and CXCL12 form homodimers that have unique activities.[23] Therefore, we attempted to synthesize and evaluate dimeric peptides of this region.

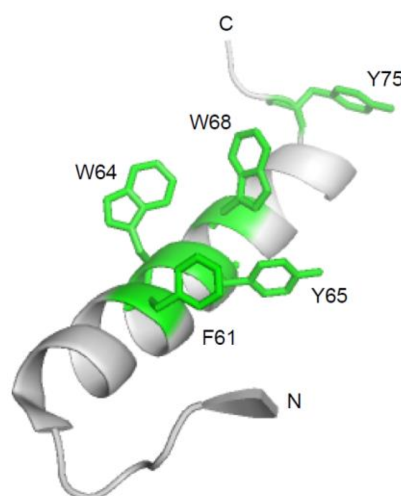
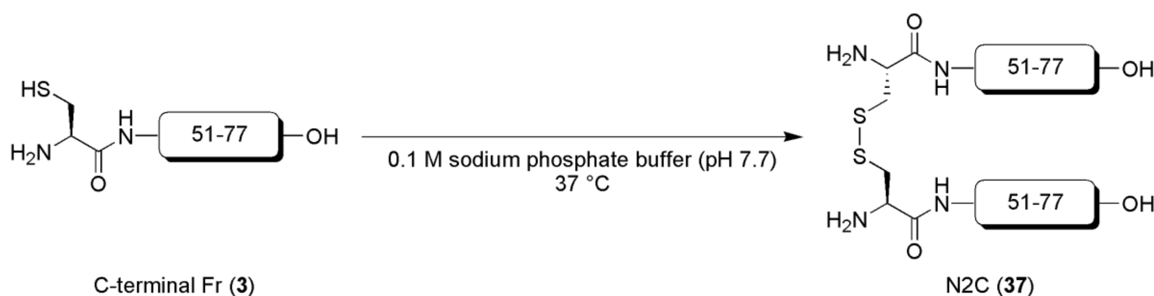


Figure 2. 2. Structure of the CXCL14 (50–77) (PDB ID codes 2HDL). Aromatic residues are shown.

2.2 *Synthesis and biological evaluation of a dimerized CXCL14 C-terminal fragment*

Initially, a disulfide dimer peptide of CXCL14 C-terminal Fr **3** (termed N2C **37**) was synthesized (Scheme 2. 1). The abbreviation "N2C" represents a cystine-containing (C) dimerized (2)-native sequence (N). C-Terminal Fr **3**, the synthetic intermediate requisite for the preparation of CXCL14 as an N-terminal cysteinyl fragment, was incubated in 0.1 M sodium phosphate buffer (pH 7.7, peptide concentration: 1 mM) to form an intermolecular disulfide bond and N2C **37** was obtained after HPLC purification (Figure 2. 3). Of note, N2C **37** showed competitive inhibition of the binding of I^{125}-CXCL14 to CXCR4 and inhibitory activity of CXCL12-mediated chemotaxis that was comparable with that of CXCL14 (Figure 2. 4).



Scheme 2. 1. Synthesis of the disulfide dimer N2C **37**.

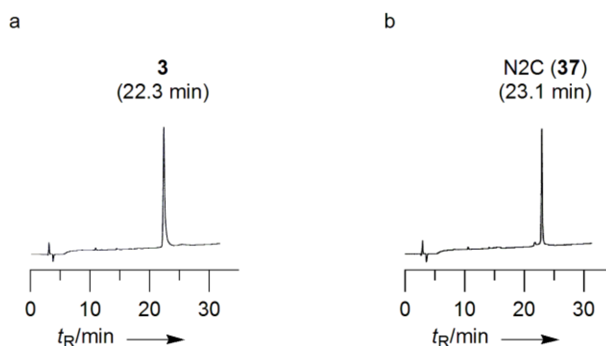


Figure 2. 3. HPLC monitoring of the synthesis of N2C **37**. (a) Disulfide bond formation (t < 1 min). (b) Disulfide bond formation (t = 12 h). HPLC conditions: Cosmosil $5C_{18}$ AR-II column (4.6 × 250 mm) with a linear gradient of 0.1% TFA-MeCN/0.1% TFA aq. (5:95–45:55 over 30 min) at a flow rate of 1.0 mL/min, detection at 220 nm.

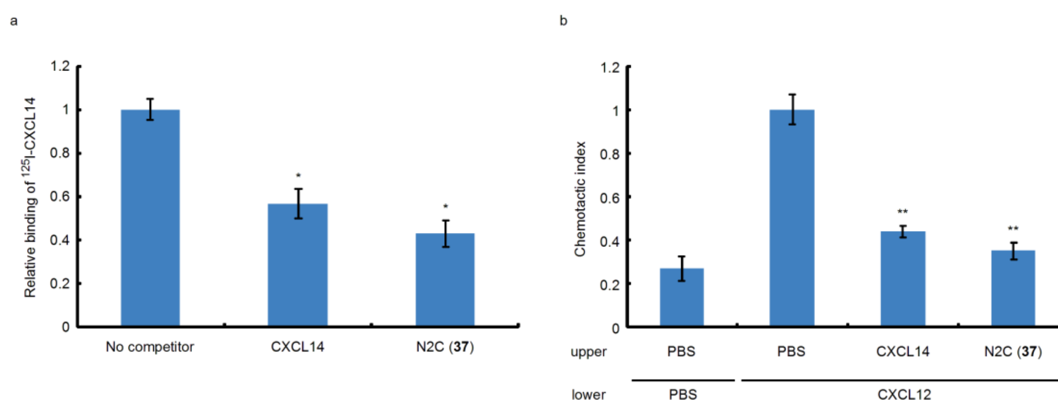


Figure 2. 4. Biological evaluation of N2C **37**. (a) Competitive inhibition of ^{125}I-CXCL14 binding by N2C **37**. CXCR4 transfected THP-1 cells were incubated with 10 nM 125I-CXCL14 in the presence or absence of 100 nM competitors, and cell-associated radioactivity was measured (n = 6). Relative binding of ^{125}I-CXCL14 to no competitor is shown. (b) Inhibition activity of N2C **37** against CXCL12-mediated chemotaxis. Numbers of migrating THP-1 cells toward 10 nM CXCL12 in the absence or presence of 100 nM CXCL14 derivatives were counted (n = 3). Data are shown as the chemotactic index: the migrated cell number to CXCL12 divided by migrated cell number to the medium alone (PBS). *P < 0.05, **P < 0.01 compared with no competitor or PBS/CXCL12, respectively.

2.3 Synthesis and biological evaluation of the dimer of mutated CXCL14 C-terminal fragment

N2C **37** is considered to form an α-helical structure and its characteristic structure is likely to be responsible for its inhibitory activity against CXCL12-induced chemotaxis through its CXCR4 binding. Thus, we speculated that the α-helicity of peptides would be relevant to inhibitory potency.[24] Furthermore, the α-helical wheel representation of the CXCL14 C-terminal region shows that conserved aromatic residues accumulate on one α-helical face composed of *a*, *d* and *e* positions (Figure 2. 2, 2. 5). This prompted us to envision that an appropriate positioning of such aromatic

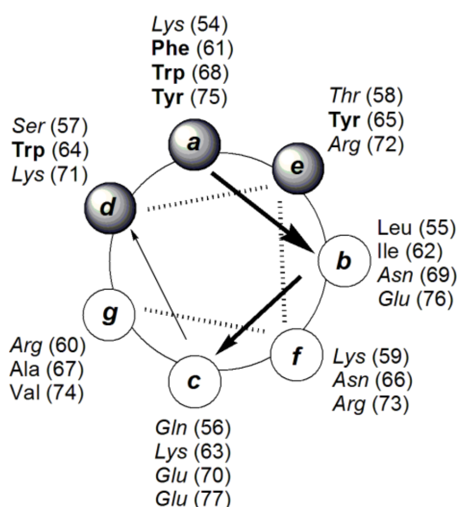


Figure 2. 5. Helical wheel representation of C-terminal portion of CXCL14 (54-77).

residues on one face of an α-helix would be responsible for CXCR4-binding and inhibitory activity. Taking these speculations into consideration, we attempted to incorporate a substitution of Glu and Lys in an *i* to *i+4* relationship manner for *b*,*f*, *c*,*g* or *f*,*c* pairing (termed *b*,*f*-EK, *c*,*g*-EK or *f*,*c*-EK, respectively) (Table 2. 1).[24] Incorporation of Glu-Lys pairing in *i* to *i+4* relationship has been well documented to enhance α-helicity of peptides through intrahelical salt bridge formation.[25]

Additionally, limited substitutions to *b*, *c*, *f*, and *g* positons was thought to result in the appropriate positioning of the aromatic residues in *a*, *d*, and *e* positions. Disulfide bridge-formed dimer peptides (BF2C **38** (*b*,*f*-EK), CG2C **40** (*c*,*g*-EK) and FC2C **42** (*f*,*c*-EK): substitution pattern is shown in parenthesis) were synthesized by a

Table 2. 1. Synthesized mutants of N2C **37**.

Mutated positions	Sequence	Compound number	Dimerized compound number (Label)
b,*f*-EK	(50) H-CLHPK***E***QSTKRF***E***KWY***K***AW***E***EKR***K***VYEE-OH (77)	**39**	**38** (BF2C)
c,*g*-EK	H-CLHPKL***E***STK***K***FI***E***WYN***K***WNEKRR***K***YEE-OH	**41**	**40** (CG2C)
f,*c*-EK	H-CLHPKL***K***ST***E***RFIKWY***E***AWN***K***KR***E***VYE***K***-OH	**43**	**42** (FC2C)

Lines over peptide sequences indicate the introduced *i* to *i* + 4 Glu-Lys arrangement.

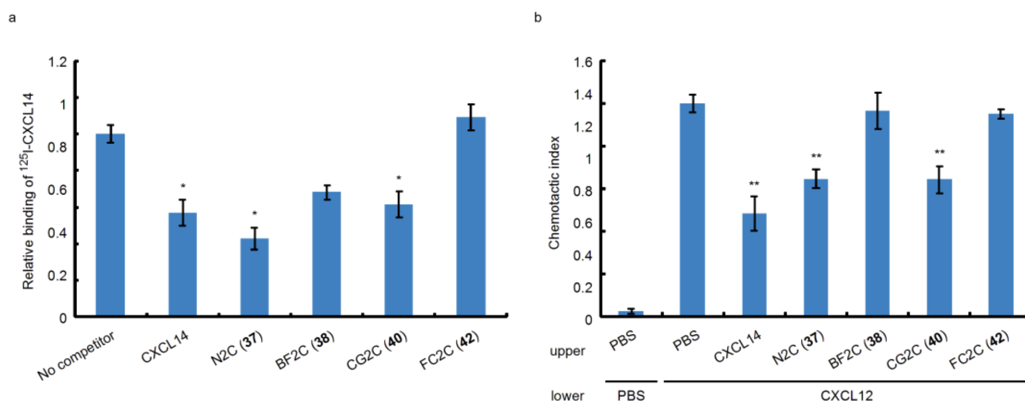


Figure 2. 6. Biological evaluation of mutants of N2C **37**. (a) Competitive inhibition of ^{125}I-CXCL14 binding by mutants of N2C **37**. CXCR4 transfected THP-1 cells were incubated with 10 nM ^{125}I-CXCL14 in the presence or absence of 100 nM competitors, and cell-associated radioactivity was measured (*n* = 6). Relative binding of ^{125}I-CXCL14 to no competitor is shown. (b) Inhibition activity of the mutants of N2C **37** to CXCL12-mediated chemotaxis. Numbers of migrating THP-1 cells toward 10 nM CXCL12 in the absence or presence of 100 nM CXCL14 derivatives were counted (*n* = 3). Data are shown as chemotactic index: the migrated cell number to CXCL12 divided by migrated cell number to the medium alone (PBS). **P* < 0.05, ***P* < 0.01 compared with no competitor or PBS/CXCL12, respectively.

method similar to that employed for N2C **37**. Biological evaluations of synthetic materials were performed by a competitive binding assay using the CXCR4-transfected THP-1 cells and ^{125}I-CXCL14 and a standard chemotaxis assay (Figure 2. 6). Among the mutated peptides, CG2C **40** functioned as the most effective competitive binder and antagonized CXCL12-induced chemotaxis with an efficacy similar to that of native CXCL14 or N2C **37**. CD measurement of synthetic peptides indicated that CG2C **40** had the best-organized α-helical structure (Figure 2. 7). Furthermore, mutated peptides containing the substitution of Glu and Lys for *g* and *d* (GD2C **44**), *d* and *a* (DA2C **46**), *a* and *e* (AE2C **48**) or *e* and *b* (EB2C **50**) showed no significant inhibiting activity against CXCL12-mediated chemotaxis (Table 2. 2 and Figure 2. 8). These results suggested that the accumulation of aromatic residues (F61, W64, Y65, W68) on the

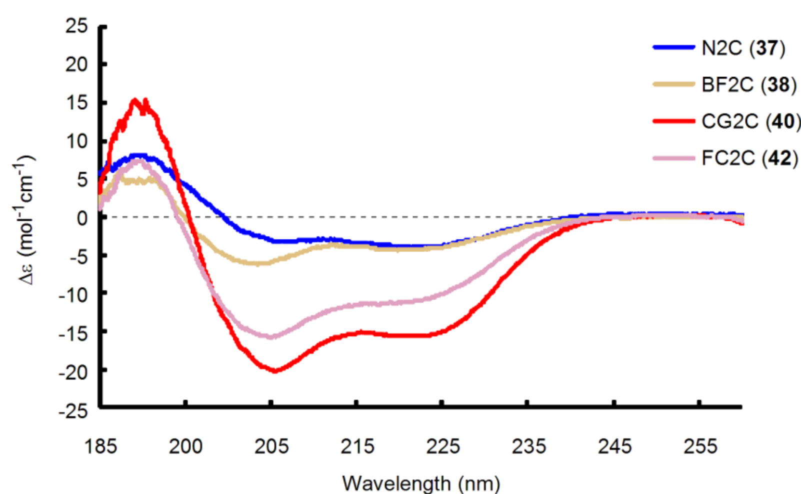


Figure 2. 7. CD spectrum of dimer peptides.

Table 2. 2. Synthesized mutants of N2C **37** with mutation of *a*, *d* and *e*-positions..

Mutated positions	Sequence	Compound number	Dimerized compound number (Label)
g,*d*-EK	H−CLHPKLQ***K***TK***E***FIK***K***YN***E***WNEKRR***E***YEE−OH (50 … 77)	**45**	**44** (GD2C)
d,*a*-EK	H−CLHPKLQ***E***TKR***K***IK***E***YNA***K***NE***E***RRV***K***EE−OH	**47**	**46** (DA2C)
a,*e*-EK	H−CLHP***E***LQS***K***KR***E***IKW***K***NA***E***NEK***K***RV***E***EE−OH	**49**	**48** (AE2C)
e,*b*-EK	H−C***E***HPK***K***QS***E***KRF***K***KW***E***NAW***K***EK***E***RVY***K***E−OH	**51**	**50** (EB2C)

Lines over peptide sequences indicate the introduced *i* to *i* + 4 Glu-Lys arrangement.

α-helical surface through helix formation is an indispensable event for the antagonistic activity of CXCL14 against the CXCL12-CXCR4 signaling axis.

As mentioned above, although N2C **37** and CG2C **40** are effective inhibitors of the CXCL12-CXCR4 signaling axis, physiological instability due to the presence of the

Figure 2. 8. Inhibition activity of the mutated dimers to CXCL12-mediated chemotaxis. Numbers of migrating THP-1 cells toward 10 nM CXCL12 in the absence or presence of 100 nM CXCL14 derivatives were counted ($n = 3$). Data are shown as chemotactic index: the migrated cell number to CXCL12 divided by migrated cell number to the medium alone (PBS). $^{**}P < 0.01$ compared with PBS/CXCL12.

Scheme 2. 2. Synthesis of the oxime dimers **53** and **55**. Excess amounts of the monomer peptide were incubated with linker **19** in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 7.6) and 0.05 mM EDTA.

disulfide bridge is an issue. Thus, we planned to synthesize a new type of dimer connected by oxime linkage. The oxime bond is more stable under physiological conditions compared with the disulfide bond.[26] Dimers with oxime linkage were successfully synthesized using dialdehyde linker **19** and N-terminal additional aminooxyacetyl group on the monomeric peptide (Scheme 2. 2). Oxime versions (**53** and **55**) corresponding to disulfide dimers N2C **37** and CG2C **40**, respectively, were synthesized as shown below. Incubation with an excess amount of the N-terminal aminooxyacetylated C-region peptides (**52** and **54**) in the presence of aldehyde linker **19** in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 7.6) and 0.05 mM EDTA, followed by HPLC purification, yielded oxime dimers **53** and **55** corresponding to N2C **37** and CG2C **40**, respectively (Scheme 2. 2 and Figure 2. 9). The abbreviations including O

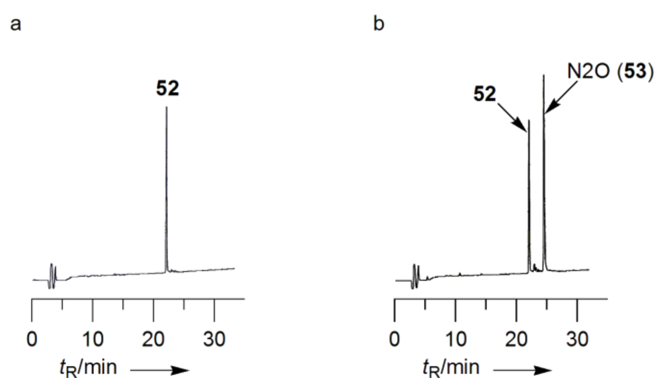


Figure 2. 9. HPLC monitoring of the synthesis of N2O **53**. (a) Oxime ligation (dimerization) (t < 1 min). (b) Oxime ligation (dimerization) (t = 3 h). HPLC conditions: Cosmosil 5C$_{18}$ AR-II column (4.6 × 250 mm) with a linear gradient of 0.1% TFA-MeCN/0.1% TFA aq. (5:95–45:55 over 30 min) at a flow rate of 1.0 mL/min, detection at 220 nm.

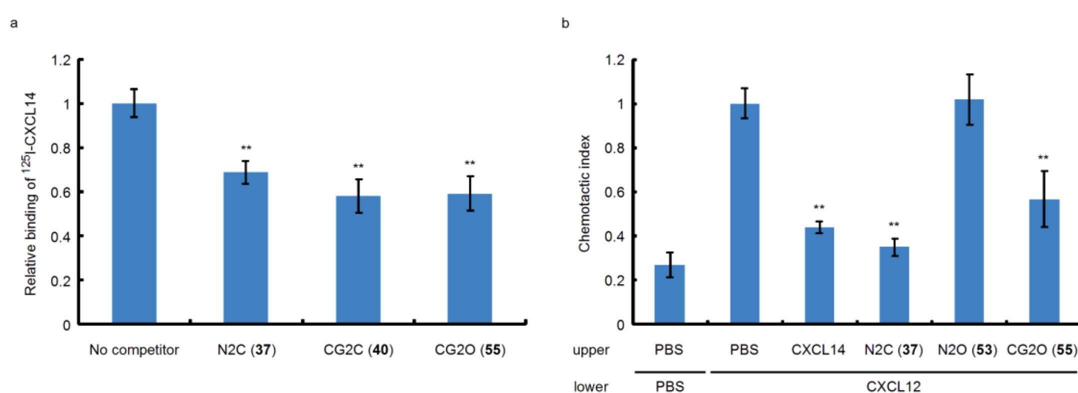
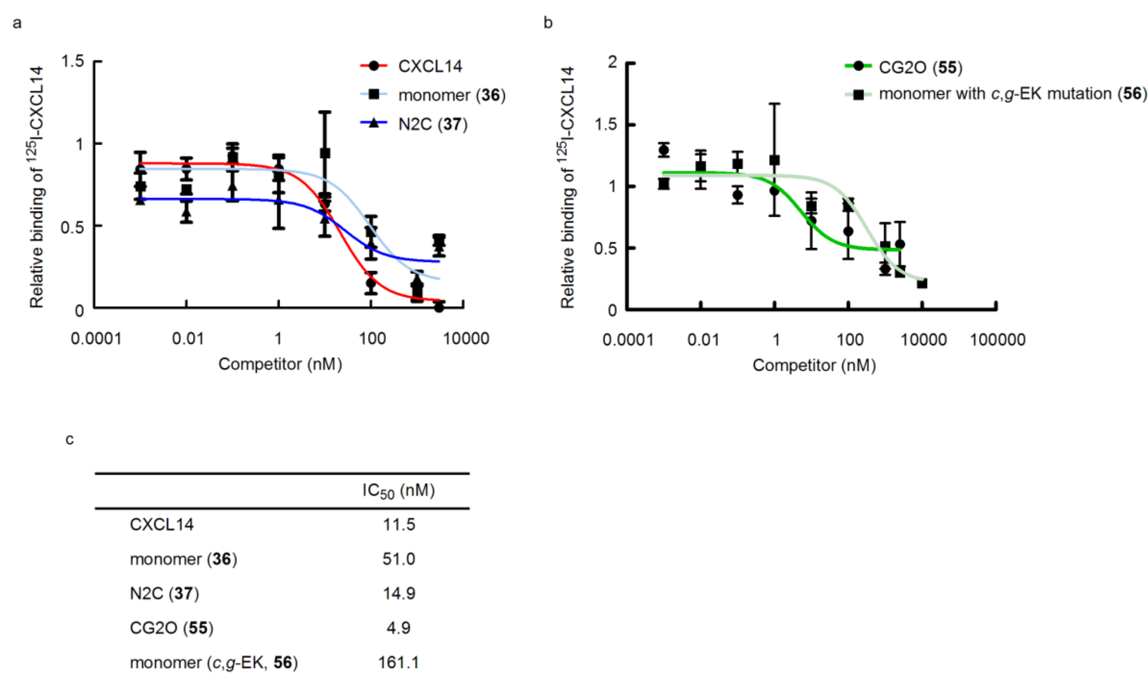


Figure 2. 10. Biological evaluation of oxime dimers. (a) Competitive inhibition of ^{125}I-CXCL14 binding by oxime dimers. *CXCR4* transfected THP-1 cells were incubated with 10 nM ^{125}I-CXCL14 in the presence or absence of 100 nM competitors, and cell-associated radioactivity was measured (n = 6). Relative binding of ^{125}I-CXCL14 to no competitor is shown. (b) Inhibition activity of oxime dimers to CXCL12-mediated chemotaxis. Numbers of migrating THP-1 cells toward 10 nM CXCL12 in the absence or presence of 100 nM CXCL14 derivatives were counted (n = 3). Data are shown as chemotactic index: the migrated cell number to CXCL12 divided by migrated cell number to the medium alone (PBS). **P < 0.01 compared with no competitor or PBS/CXCL12.

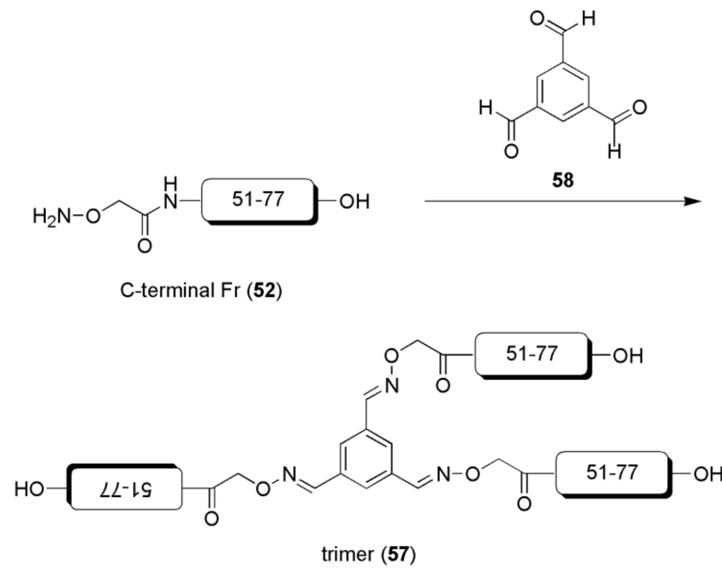
instead of C, such as N2O **53** and CG2O **55**, were used to indicate oxime linkage (O). The binding potency and inhibitory activity of the oxime dimers were assessed by competitive binding assay and standard chemotaxis assay similar to those employed for disulfide dimers (Figure 2. 10). CG2O **55** showed a binding potency to CXCR4 expressing THP-1 cells and inhibitory activity to CXCL12-mediated chemotaxis comparable with that of N2C **37**. Next, the IC_{50} value of inhibition of ^{125}I-CXCL14 binding to THP-1 cells expressing CXCR4 was determined (Figure 2. 11). The IC_{50} value of CG2O **55** was 4.9 nM, and was superior to that of CXCL14 (11.5 nM) and N2C **37** (14.9 nM), whereas, the IC_{50} value of monomer peptide **36** and monomer peptide with mutation *c*,*g*-EK **56** were 51.0 nM and 161.1 nM, respectively. These data demonstrated the importance of dimerization of C-terminal fragments for inhibitory activity. Furthermore, to confirm the importance of dimerization, we synthesized a trimer of the CXCL14 C-terminal fragment **57** (Scheme 2. 3). Excess amounts of monomer peptide **52** were incubated with the commercially available trialdehyde linker **58** in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 5.5) and 0.5 mM EDTA. After completion of the reaction, trimer **57** was purified by HPLC. Evaluation of inhibitory activity of trimer **57** to CXCL12-mediated chemotaxis and measurement of its CD spectrum revealed that trimerization of this fragment was not effective either inhibitory



	IC_{50} (nM)
CXCL14	11.5
monomer (**36**)	51.0
N2C (**37**)	14.9
CG2O (**55**)	4.9
monomer (*c*,*g*-EK, **56**)	161.1

Figure 2. 11. Effective competition of ^{125}I-CXCL14 binding by (a) a disulfide dimer and (b) an oxime dimer mutated to *c*,*g*-EK. CXCR4 transfected THP-1 cells were incubated with 10 nM ^{125}I-CXCL14 in the presence of various concentration of competitors, and cell-associated radioactivity was measured (*n* = 4 (a) and *n* = 3 (b), respectively). Relative binding of ^{125}I-CXCL14 to no competitor is shown. (c) Calculated IC_{50} values of the CXCL14 derivatives.

activity or the formation of an α-helix structure (Figure 2. 12).



Scheme 2. 3. Synthesis of a trimer of CXCL14 C-terminal Fr. Excess amounts of monomer peptide **52** were incubated with linker **58** in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 5.5) and 0.5 mM EDTA.

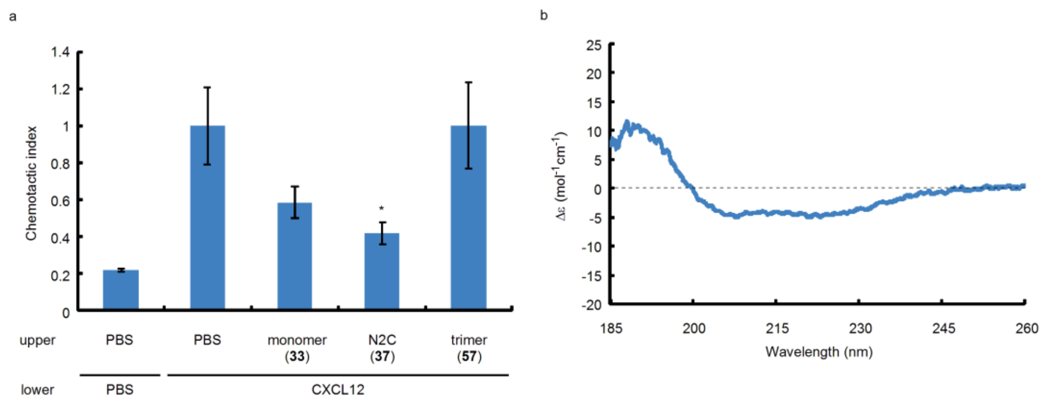


Figure 2. 12. (a) Inhibition activity of trimer **57** to CXCL12-mediated chemotaxis. Numbers of migrating THP-1 cells toward 10 nM CXCL12 in the absence or presence of 100 nM CXCL14 derivatives were counted (n = 3). Data are shown as chemotactic index: the migrated cell number to CXCL12 divided by migrated cell number to the medium alone (PBS). *P< 0.05 compared to PBS/CXCL12. (b) CD spectrum of the trimer **57**.

2.4 *Identification of dimer peptide residues indispensable for inhibitory activity to CXCL12-mediated chemotaxis*

As mentioned in a previous section, both the presence of aromatic residues in *a*, *d* and/or *e* positions of α-helix and the dimerization are likely to be essential for the antagonistic activity of the CXCL14-derived peptide against CXCL12-CXCR4 axis. To obtain more precise information about the relationship between the *a*, *d* and *e*-residues and the inhibitory activity, single mutations on the aromatic residues of *a*, *d* or *e* position were performed. Dimer peptides **59** (W64A, from **60**), **61** (Y65A, from **62**), **63** (W68A, from **64**), and **65** (Y75A, from **66**) were synthesized using a method similar to

Table 2. 3. Synthesized disulfide dimer with aromatic amino acid residues mutated to alanine.

Mutated positions	Sequence	Compound number	Dimerized compound number
W64A	(50 … 77) H-CLHPKLQSTKRFIK**A**YNAWNEKRRVYEE-OH	**60**	**59**
Y65A	H-CLHPKLQSTKRFIKW**A**NAWNEKRRVYEE-OH	**62**	**61**
W68A	H-CLHPKLQSTKRFIKWYNA**A**NEKRRVYEE-OH	**64**	**63**
Y75A	H-CLHPKLQSTKRFIKWYNAWNEKRRV**A**EE-OH	**66**	**65**

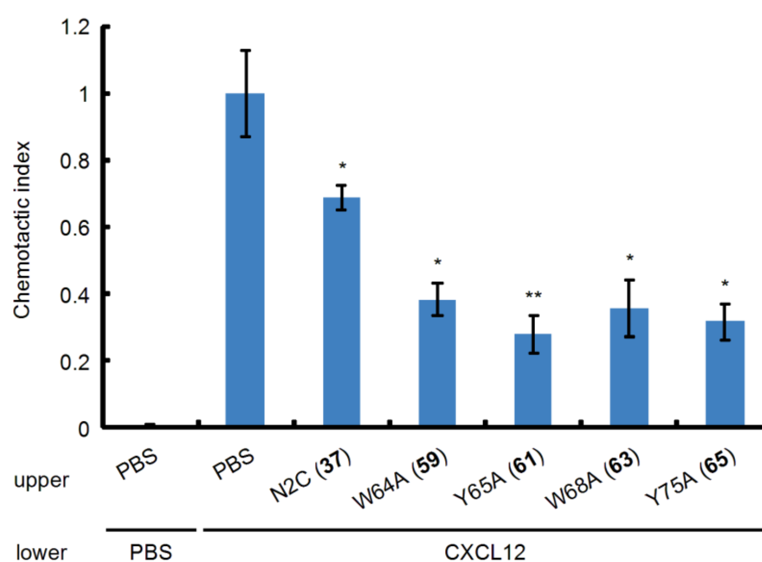


Figure 2. 13. Inhibition activity of mutated dimers to CXCL12-mediated chemotaxis. Numbers of migrating BaF/3 cells toward 10 nM CXCL12 in the absence or presence of 100 nM CXCL14 derivatives were counted (n = 3). Data are shown as chemotactic index: the migrated cell number to CXCL12 divided by migrated cell number to the medium alone (PBS). *$P < 0.05$, **$P < 0.01$ compared with PBS/CXCL12, respectively.

that for N2C **37** (Table 2. 3). In comparison with N2C **37**, the singly mutated peptides did not show a significant difference in inhibitory activity against CXCL12-mediated chemotaxis (Figure 2. 13). This indicated the cooperative involvement of aromatic residues in inhibitory activity. Although an evaluation of the cooperative involvement of aromatic residues was required, we next planned to synthesize deletion peptides sets from the C-terminal (residue 77) and N-terminal (residue 52) ends of the CXCL14 C-terminal region. Two series of deletion mutants from the C and N ends, listed in Table 2. 4 and 2. 5, were prepared as their disulfide dimer forms. Evaluation of the inhibitory activity of the deletion sets are summarized in Figure 2. 14 (for C end deletion) and Figure 2. 15 (for N end deletion). Regarding the deletion mutants from the N end (**109**, **111**, **113** and **115**), there were solubility problems that precluded the use of these peptides for biological evaluation. Consequently, a one- to five-residue deletion from the C end and one-residue deletion from the N-end were acceptable for maintenance of inhibitory activity. From these results, we tentatively concluded that residues 52–72 might function as a core sequence indispensable for inhibitory activity. Indeed, disulfide dimer peptide **117** covering residues 52–72 retained an inhibitory activity comparable with that of N2C **37** (Figure 2. 16).

Because the shorted C-terminal analog **117** had an inhibitory activity comparable with that of parent N2C **37**, we incorporated a combinatorial substitution of Ala for aromatic residues (F61, W64, Y65 and/or W68) to explore their cooperative

Table 2. 4. Synthesized disulfide dimers with deletion of the sequence from the C-terminus.

Deleted positions	Sequence	Compound number	Dimerized compound number
50-76	H-CLHPKLQSTKRFIKWYNAWNEKRRVYE-OH (50 … 76)	**68**	**67**
50-75	H-CLHPKLQSTKRFIKWYNAWNEKRRVY-OH	**70**	**69**
50-74	H-CLHPKLQSTKRFIKWYNAWNEKRRV-OH	**72**	**71**
50-73	H-CLHPKLQSTKRFIKWYNAWNEKRR-OH	**74**	**73**
50-72	H-CLHPKLQSTKRFIKWYNAWNEKR-OH	**76**	**75**
50-71	H-CLHPKLQSTKRFIKWYNAWNEK-OH	**78**	**77**
50-70	H-CLHPKLQSTKRFIKWYNAWNE-OH	**80**	**79**
50-69	H-CLHPKLQSTKRFIKWYNAWN-OH	**82**	**81**
50-68	H-CLHPKLQSTKRFIKWYNAW-OH	**84**	**83**
50-67	H-CLHPKLQSTKRFIKWYNA-OH	**86**	**85**
50-66	H-CLHPKLQSTKRFIKWYN-OH	**88**	**87**
50-65	H-CLHPKLQSTKRFIKWY-OH	**90**	**89**
50-64	H-CLHPKLQSTKRFIKW-OH	**92**	**91**

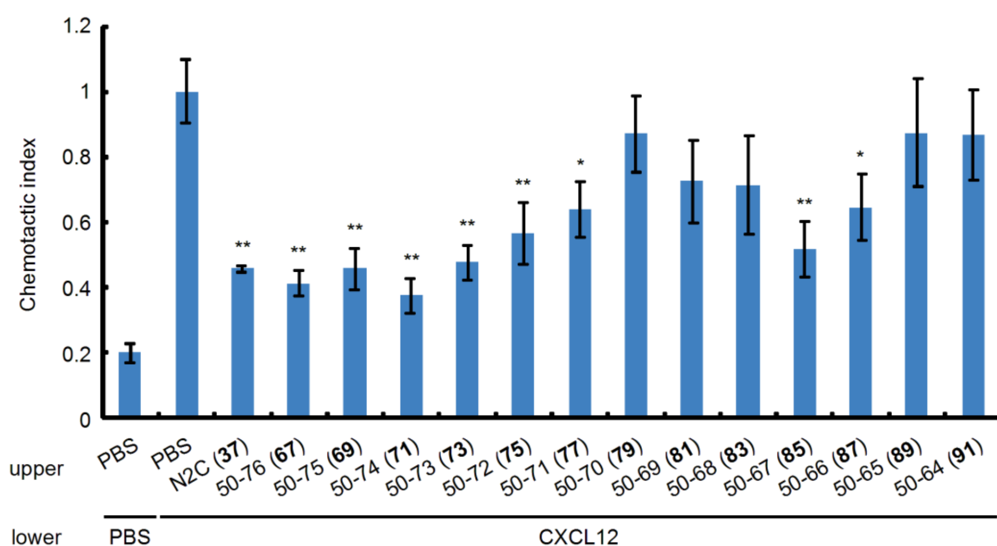


Figure 2. 14. Inhibition activity of the disulfide dimers deleted the sequence of CXCL14 C-terminal Fr from C-terminus to CXCL12-mediated chemotaxis. Numbers of migrating THP-1 cells toward 10 nM CXCL12 in the absence or presence of 100 nM CXCL14 derivatives were counted (n = 3). Data are shown as chemotactic index: the migrated cell number to CXCL12 divided by migrated cell number to the medium alone (PBS). *$P < 0.05$, **$P < 0.01$ compared with PBS/CXCL12, respectively.

Table 2. 5. Synthesized disulfide dimers with deletion of the sequence from N-terminus.

Deleted positions	Sequence	Compound number	Dimerized compound number
52-77	52 … 77 H−C−HPKLQSTKRFIKWYNAWNEKRRVYEE−OH	**94**	**93**
53-77	H−C−PKLQSTKRFIKWYNAWNEKRRVYEE−OH	**96**	**95**
54-77	H−C−KLQSTKRFIKWYNAWNEKRRVYEE−OH	**98**	**97**
55-77	H−C−LQSTKRFIKWYNAWNEKRRVYEE−OH	**100**	**99**
56-77	H−C−QSTKRFIKWYNAWNEKRRVYEE−OH	**102**	**101**
57-77	H−C−STKRFIKWYNAWNEKRRVYEE−OH	**104**	**103**
58-77	H−C−TKRFIKWYNAWNEKRRVYEE−OH	**106**	**105**
59-77	H−C−KRFIKWYNAWNEKRRVYEE−OH	**108**	**107**
60-77	H−C−RFIKWYNAWNEKRRVYEE−OH	**110**	**109**
61-77	H−C−FIKWYNAWNEKRRVYEE−OH	**112**	**111**
62-77	H−C−IKWYNAWNEKRRVYEE−OH	**114**	**113**
63-77	H−C−KWYNAWNEKRRVYEE−OH	**116**	**115**

Each peptides possess additional cysteine residue in N-terminus for dimerization via difulfide bond.

Figure 2. 15. Inhibition activity of the disulfide dimers deleted the sequence of CXCL14 C-terminal Fr from N-terminus to CXCL12-mediated chemotaxis. Numbers of migrating THP-1 cells toward 10 nM CXCL12 in the absence or presence of 100 nM CXCL14 derivatives was counted (n = 3). Data are shown as chemotactic index: the migrated cell number to CXCL12 divided by migrated cell number to the medium alone (PBS). *P < 0.05 compared with PBS/CXCL12.

Figure 2. 16. Inhibition activity of the disulfide dimer of CXCL14 (52-72) to CXCL12-mediated chemotaxis. Numbers of migrating THP-1 cells toward 10 nM CXCL12 in the absence or presence of 100 nM CXCL14 derivatives were counted (n = 3). Data are shown as chemotactic index: the migrated cell number to CXCL12 divided by migrated cell number to the medium alone (PBS). *P < 0.05, **P < 0.01 compared with PBS/CXCL12.

Table 2. 6. Synthesized disulfide dimers with mutation of aromatic amino acid residues to alanine.

Mutated positions	Sequence	Compound number	Dimerized compound number
	52 … 72		
W64A, Y65A	H-C-HPKLQSTKRFIK**AA**NAWNEKR-OH	**120**	**119**
W64A, W68A	H-C-HPKLQSTKRFIK**A**YNA**A**NEKR-OH	**122**	**121**
Y65A, W68A	H-C-HPKLQSTKRFIKW**A**NA**A**NEKR-OH	**124**	**123**
W64A, Y65A, W68A	H-C-HPKLQSTKRFIK**AA**NA**A**NEKR-OH	**126**	**125**
F61A	H-C-HPKLQSTKR**A**IKWYNAWNEKR-OH	**128**	**127**
F61A, W64A	H-C-HPKLQSTKR**A**IK**A**YNAWNEKR-OH	**130**	**129**
F61A, Y65A	H-C-HPKLQSTKR**A**IKW**A**NAWNEKR-OH	**132**	**131**
F61A, W68A	H-C-HPKLQSTKR**A**IKWYNA**A**NEKR-OH	**134**	**133**
F61A, W64A, Y65A	H-C-HPKLQSTKR**A**IK**AA**NAWNEKR-OH	**136**	**135**
F61A, W64A, W68A	H-C-HPKLQSTKR**A**IK**A**YNA**A**NEKR-OH	**138**	**137**
F61A, Y65A, W68A	H-C-HPKLQSTKR**A**IKW**A**NA**A**NEKR-OH	**140**	**139**
F61A, W64A, Y65A, W68A	H-C-HPKLQSTKR**A**IK**AA**NA**A**NEKR-OH	**142**	**141**

Each peptides possess additional cysteine residue in N-terminus for dimerization via difulfide bond.

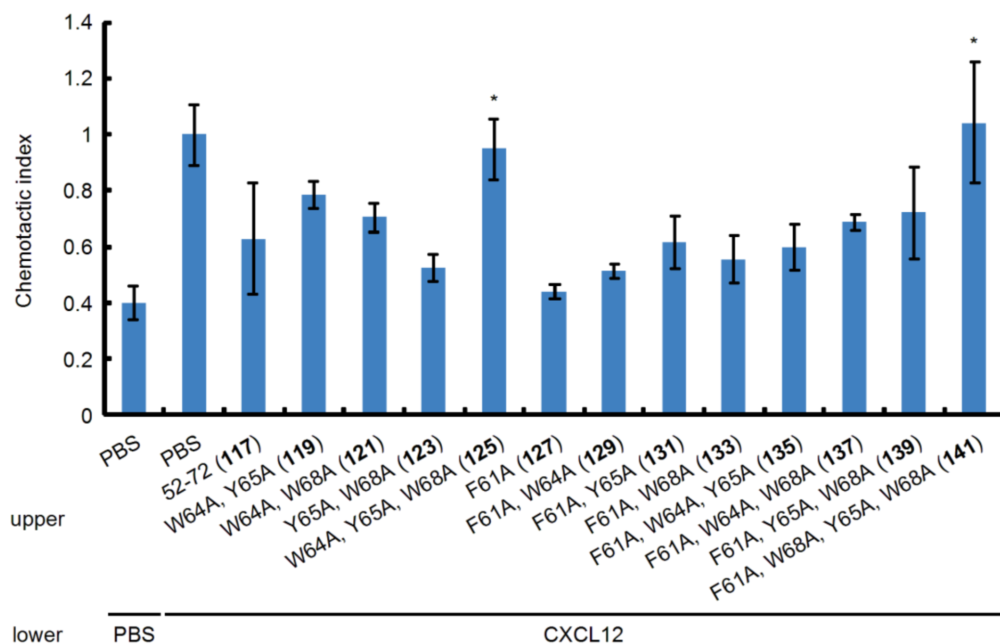


Figure 2. 17. Inhibition activity of the disulfide dimer of CXCL14 (52-72) with mutation of aromatic amino acid residues to alanine to CXCL12-mediated chemotaxis. Numbers of migrating THP-1 cells toward 10 nM CXCL12 in the absence or presence of 100 nM CXCL14 derivatives were counted (n = 4). Data are shown as chemotactic index: the migrated cell number to CXCL12 divided by migrated cell number to the medium alone (PBS). *P < 0.05 compared with no competitor (PBS/PBS) and not significant compared with PBS/CXCL12.

involvement in the inhibitory activity. Evaluation of these peptides by chemotaxis assay revealed that peptides (**125**: W64A, Y65 and W68A and **141**: F61A, W64A, Y65A and W68A) possessing multi Ala-mutations did not retain the inhibitory potency (Figure 2. 17).

Evaluation of these peptides by chemotaxis assay revealed that three aromatic residues (W64, Y65 and W68) functioned in a coordinated manner to provide the inhibitory activity. In terms of the inhibitory role of the amino acids located in *b*, *c*, *f* or *g* positions, pairwise substitution of Glu and Lys for *b* and *f* resulted in a significant decrease in inhibitory activity as mentioned in section 2.3 (Figure 2. 6). However, such mutations at *c* and *g* positions did not affect the inhibitory activity. Thus, we next examined whether only single set of mutations (*b* or *f* only) was acceptable for maintenance of the inhibitory activity. Biological evaluation of analogs of dimer peptide

Table 2. 7. Synthesized disulfide dimers with mutation of *b*- or *f*-position to alanine.

Mutated positions	Sequence	Compound number	Dimerized compound number
b to Ala	H-C-HPK**A**QSTKRF**A**KWYNAW**A**EKR-OH (52 ... 72)	**144**	**143**
f to Ala	H-C-**A**PKLQST**A**RFIKWY**A**AWNEKR-OH	**146**	**145**

Each peptides possess additional cysteine residue in N-terminus for dimerization via difulfide bond.

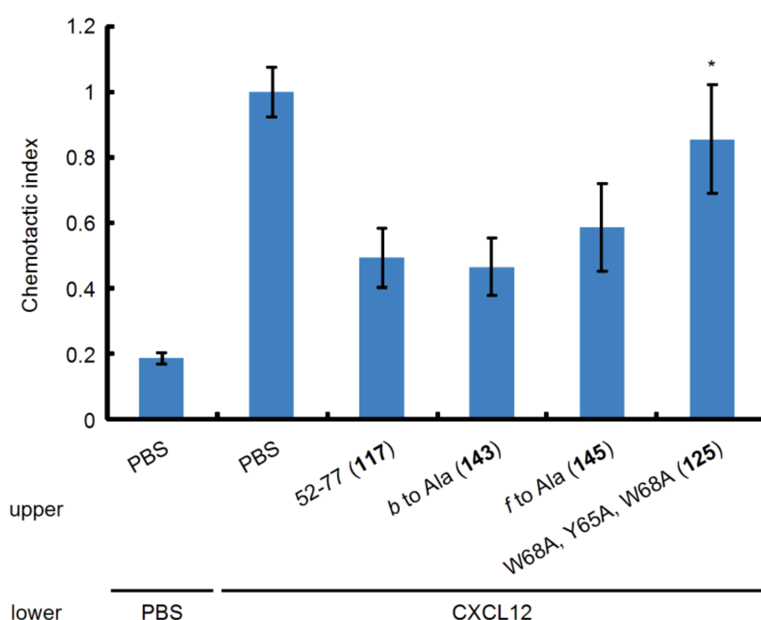


Figure 2. 18. Inhibition activity of the disulfide dimer of CXCL14 (52-72) with mutation of the *b*- or *f*-position to alanine to CXCL12-mediated chemotaxis.Numbers of migrating THP-1 cells toward 10 nM CXCL12 in the absence or presence of 100 nM CXCL14 derivatives were counted (n = 3). Data are shown as chemotactic index: the migrated cell number to CXCL12 divided by migrated cell number to the medium alone (PBS). *P < 0.05 compared with no competitor (PBS/PBS) and not significant compared with PBS/CXCL12.

117 possessing a replacement of *b*- (**143**) or *f*-residues (**145**) with Ala showed that these peptides retained their inhibitory activity (Table 2. 7, Figure 2. 18). These results indicate that the three aromatic residues in *a*, *d* and *e*-positions are critical for inhibitory activity against CXCL12-mediated chemotaxis. Additionally, *b*- and *f*-residues were proven to function in a cooperative manner to affect the inhibitory activity to a lesser extent than aromatic residues.

2.5 Conclusion

Competitive binding assay of various monomeric reduced-type CXCL14-derived peptides to CXCR4 expressing THP-1 cells using ^{125}I-CXCL14 indicated that the C-terminal region within residues 50–77 was indispensable for binding of CXCL14 to THP-1 cells expressing CXCR4. Taking into consideration the fact that chemokine molecules have a tendency to form dimer species through non-bonding protein/protein interactions, we attempted to synthesize dimer analogs derived from CXCL14 (50–77) to examine the effect of dimerization on binding to CXCR4 expressing THP-1 cells including the inhibitory activity against the CXCL12-CXCR4 signaling axis. Disulfide dimer N2C **37** possessing a native C-terminal sequence could bind to CXCR4 expressing cells and inhibit CXCL12-mediated chemotaxis with an efficiency comparable with the parent CXCL14. An α-helical structure consisting of one α-helical surface (*a*, *d* and *e* residues) with the accumulation of highly conserved aromatic residues prompted us to incorporate pairwise Glu-Lys substitutions in an *i* to *i+4* manner for residues in the other α-helical surface (*b*, *c*, *f* and *g* residues) to enhance CXCR4 binding and inhibitory activity. The salt bridge forming Glu-Lys substitution was expected to contribute to an appropriate arrangement of the conserved aromatic residues by increasing the α-helicity of mutated analogs. Among the synthesized derivatives, CG2O **55** with a *c*,*g*-EK substitution pattern and oxime-dimer structure showed a binding affinity slightly superior to CXCL14. The critical involvement of the aromatic residues in CXCL12-mediated chemotaxis was demonstrated using C-terminal dimer analogs with a shorter sequence and/or replacement of Ala with aromatic residues. Chemotaxis assays using these analogs showed that three aromatic residues (W64, Y65 and W68) are indispensable residues for inhibitory activity. Additionally, biological evaluation of *b*- and/or *f*-residue mutated dimer peptides indicated that these residues are also cooperatively involved in the inhibitory activity.

Chapter 3

Conclusions

1. The Syntheses of CXCL14 and its derivatives were achieved by C–to–N- or N–to–C-directive sequential NCL methods using protected N-terminal cysteinyl (N-Cys) peptide thioester (C–to–N), unprotected N-Cys peptide thioacid (N–to–C) or unprotected N-Cys SEAlide peptide (N–to–C) as the middle fragment, respectively. In particular, the use of SEAlide peptide as the middle fragment enabled us to synthesize CXCL14 in a one-pot manner with easy experimental manipulation. Biological evaluation of the synthetic CXCL14 proteins indicated that they exhibited inhibitory activity against CXCL12-mediated chemotaxis comparable with that of recombinant CXCL14. Furthermore, binding of biotinylated CXCL14 to HeLa cells expressing CXCR4 was also confirmed by FACS analysis.

   Findings in this study demonstrate that the use of sequential NCL methods in both C–to–N and N–to–C directions for the synthesis of CXCL14 enables the easy preparation of various CXCL14 derivatives including biotin- and fluorophore-incorporated CXCL14. As mentioned above, CXCR4 was shown to function as a cell surface binder for CXCL14; however, the identification of a putative receptor for CXCL14 has yet to be achieved. The next goal for CXCL14 research should be the identification of potential CXCL14 receptor(s) that are responsible for mediating the physiological effects of CXCL14.

2 Evaluation of various peptides derived from CXCL14 revealed that CXCL14 binds to THP-1 cells expressing CXCR4 by the C-terminal α-helical region 51-77, and that the disulfide dimer of this region functions as an inhibitor of the CXCL12-CXCR4 signaling axis. Biological activity of mutated dimers, in which substitutions of glutamic acid (E) and lysine (K) in *i* and *i+4* positions were incorporated to enhance their α-helicity, was also evaluated. The evaluation of these mutated dimers suggested that one α-helical surface (*a*, *d* and *e*) where aromatic residues (F61, W64, Y65 and W68) were accumulated are essential for the inhibitory activity. In addition, a dimer peptide with a substitution of pairwise Glu and Lys mutations for *c* and *g* positions did not

affect the inhibitory activity, whereas dimers with such a substitution for *b* and *f* or *f* and *c* positions decreased the inhibitory activity. Cooperative involvement of the aromatic residues was confirmed using shortened mutants derived from CXCL14 52-72 residues. The indispensability of this sequence for inhibitory activity was determined by deletion experiments. As a result, it was revealed that three aromatic residues (W64, Y65 and W68) are essential for the inhibitory activity and that they function cooperatively

Information about which residues are indispensable for the function of CXCL14 and their special arrangement on an α-helix structure should provide a platform for the design of small molecule CXCL14 mimics. Extensive efforts along these lines are currently underway in our laboratory.

Experimental section

General Methods

All reactions of small molecules were carried out under a positive pressure of argon. For column chromatography, silica gel (KANTO KAGAKU N-60, Kanto Chemicals, Tokyo, Japan) was employed. Thin layer chromatography was performed on precoated plates (0.25 nm, silica gel Merck Kieselgel $60F_{245}$). Exact mass spectra were recorded on Waters MICROMASS LCT PREMIER. Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker AV400N at 400 MHz frequency for ^{1}H and a JEOL JNM-AL300 at 75 MHz frequency for ^{13}C in $CDCl_3$. Chemical shifts were calibrated to the solvent signal. For HPLC separations, a Cosmosil $5C_{18}$-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1 mL/min), a $5C_{18}$-AR-II semi-preparative column (Nacalai Tesque, 10 × 250 mm, flow rate 3.0 mL/min) or a $5C_{18}$-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% TFA aqueous solution (v/v, solvent A) and 0.1% TFA in MeCN (v/v, solvent B) was used for HPLC elution. IR spectra and optical rotations were recorded with FT-IP 6200 (Jasco, Tokyo, Japan) and P-2200 polarimeter (Jasco, concentration in g/100 mL), respectively. Circular dichroism (CD) spectra were measured using a J-600 spectropolarimeter (Jasco) and a J-1500 (Jasco). Far-UV CD spectra were recorded from 185 to 265 nm at 25 °C (peptide concentration: 50 μg/mL).

All reagents that were used for chemical synthesis were purchased from either Kanto Chemicals, Sigma Aldrich Japan, Tokyo Chemical Industry, Wako Pure Chemical Industries, Peptide Institute, Inc., Watanabe Chemical Industry or Novabiochem. Peptide Synthesizer CS336 (CS Bio Co., Menlo Park, CA, USA) was used for automatic peptide synthesis.

THP-1 was obtained from the Human Science Research Resource Bank (JCRB0112) and maintained at 37 °C in RPMI-1640 medium (Sigma, Tokyo, Japan) containing 10% fetal calf serum (FCS) (Invitrogen, Life Technologies Japan, Tokyo, Japan), 0.5% penicillin-streptomycin (Sigma), and 50 μM β-mercaptoethanol (Sigma). Hela S3 cells were maintained at 37 °C in DMEM medium (Sigma) containing 10% FCS, 0.5% penicillin-streptomycin. Recombinant human CXCL14 and human CXCL12 were purchased from PeproTech (Rocky Hill, NJ, USA). BSA and HEPES were obtained from Sigma and Invitrogen, respectively. In chemotaxis assays, chemotaxicell filters (5 μm pore size; Kurabo, Osaka, Japan) and Diff-Quik stain (Sysmex, Kobe,

Japan) were used.

Bolton-Hunter reagent and WIZARD2 gamma counter were obtained from Perkin Elmer. Borate buffer (0.1 M, pH 8.2) containing 0.01% Tween 20 was purchased from Nacalai Tesque. For separation of ^{125}I-labeled CXCL14, D-Salt polyacrylamide desalting column (Pierce) was employed. BSA and HEPES were obtained from Sigma and Invitrogen, respectively. GraphPad Prism (GraphPad Software) was used for calculating dissociation constants of the binding assays.

Chapter 1

Preparation of CXCL14 N-terminal fragment 1

On 4-methylbenzhydrylamine (MBHA) resin (0.70 mmol amine/g), introduction of Boc-Ala-OH (5.0 equiv) in the presence of DIPCDI (5.0 equiv) and HOBt·H_2O (5.5 equiv) in DMF at room temperature for 2 h followed by Boc removal by TFA/anisole/toluene (50:2:48 (v/v), 15 min) afforded the Boc-Ala-incorporated resin. Next, treatment of the resulting resin with *S*-Trt mercaptopropionic acid (5 equiv),[27] DIPCDI (5.0 equiv), HOBt·H_2O (5.5 equiv) and DIPEA (2.0 equiv) in DMF at room temperature for 2 h followed by Trt removal by TFA/Et_3SiH/H_2O (95:2.5:2.5 (v/v), 5 min) gave HS-CH_2CH_2CO-Ala-MBHA resin. Activated Boc-His(Bom)-OH (5.0 equiv) with HBTU (4.8 equiv) and DIPEA (6.8 equiv) in DMF was coupled with the HS-CH_2CH_2CO-Ala-MBHA resin for 3 h, and the resin was subsequently subjected to Boc removal by TFA/anisole/toluene (50:2:48 (v/v), 15 min). On the resulting resin, standard *in situ* neutralization Boc SPPS (Acylation: Boc amino acid (5.0 equiv), DIPEA (2.0 equiv.), DIPCDI (5.0 equiv) and HOBt·H_2O (5.5 equiv) in DMF for 2 h; Boc removal: TFA/anisole/toluene (50:2:48 (v/v), 20 min) was performed for the chain elongation to give protected peptide resin for N-terminal Fr **1**.[28] The resulting completed resin (100 mg) was treated with 1 M TMSOTf-thioanisole in TFA and *m*-cresol (100:5 (v/v)) at 4 ^{o}C for 2 h, and then the mixture was warmed to room temperature and stirred at this temperature for 30 min. The reaction mixture was then cooled to 4 ^{o}C, and NH_4I (75 equiv) and Me_2S (75 equiv) were added to the mixture. The mixture was stirred at 4 ^{o}C for 30 min for reduction of methionine oxide formed during chain elongation. After filtration of the reaction mixture into cooled Et_2O, the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with cold Et_2O and purified by preparative HPLC to give peptide thioester **1** (11 mg, 26%).

N-Terminal Fr **1**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 15.5 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 13 to 23% over 30 min. MS (electrospray ionization-time-of-flight (ESI-TOF)) *m/z* calcd for $[M + 4H]^{4+}$ 874.0, found 873.5.

Preparation of CXCL14 middle fragment 2

Middle Fr **2** was synthesized using the Boc SPPS in a manner similar to that employed for N-terminal Fr **1**.

Middle Fr **2** (5.1 mg from 100 mg resin, 10%): Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 19.5 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 15 to 25% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 888.8, found 888.7.

Preparation of CXCL14 C-terminal fragment 3

The protected peptide resin was constructed on Fmoc-Glu(O^{t}Bu)-Alko-PEG resin (loading: 0.24 mmol amino acid/g) using standard Fmoc SPPS (Acylation: Fmoc amino acid (5.0 equiv), DIPCDI (5.0 equiv) and HOBt·H_2O (5.5 equiv) in DMF for 2 h; Fmoc removal: 20% (v/v) piperidine in DMF for 10 min). The completed resin (100 mg) was treated with TFA-thioanisole-*m*-cresol-H_2O-1,2-ethanedithiol (EDT) (80:5:5:5:5, (v/v)) at room temperature for 1.5 h. After filtration of the resin, cooled Et_2O was added to the filtrate, and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with cold Et_2O and purified by preparative HPLC to give C-terminal Fr **3** (24 mg, 62%).

C-Terminal Fr **3**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 23.8 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 906.5, found 906.5.

Synthesis of CXCL14 6 using C–to–N-directive sequential NCL

The First NCL: N-Terminal thiazolidine carboxylic (*N*-Thz) peptide thioester **2** (1.5 mg, 0.57 μmol) as middle Fr and N-terminal cysteine (*N*-Cys) peptide **3** (2.1 mg, 0.57 μmol) as C-terminal Fr were dissolved in 6 M Gn·HCl-0.2 M sodium phosphate buffer (pH 6.8) containing 1% (v/v) thiophenol to initiate the first NCL (Final concentration of each peptides: 1.0 mM). After completion of the reaction by incubation at 37 °C for 4 h, methoxyamine·hydrochloride ($MeONH_2$·HCl) was added directly to

the ligation reaction mixture at a final concentration of 0.2 M. The opening of Thz ring to convert to Cys was completed within 2 h. The crude material was purified by semi-preparative HPLC to afford ligated peptide **4** (2.6 mg, 76%).

The Second NCL: N-Terminal Fr **1** (0.95 mg, 0.27 μmol) and the ligated product **4** (1.7 mg, 0.27 μmol) were dissolved in 6 M Gn·HCl-0.2 M sodium phosphate buffer (pH 6.8) containing 1% (v/v) thiophenol (Final concentration of each peptides: 1.0 mM). After disappearance of the starting materials **1** and **4** by incubation of the mixture at 37 °C for 6 h, the crude material was purified by semi-preparative HPLC to give reduced form CXCL14 **5** (CXCL-14 (4-Cys)). Oxidation of the purified **5** with air was performed in 3 M Gn·HCl-0.1 M sodium phosphate buffer (pH 7.7, concentration of the peptide: 0.050 mM).[10] After incubation of the mixture at 37 °C for 8 h, CXCL14 **6** (0.34 mg, 17%, 2 steps) was isolated by semi-preparative HPLC.

CXCL14 (29-77) **4**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 23.6 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 15 to 45% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 1525.3, found 1525.2.

CXCL14 (4-Cys) **5**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.7 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 11H]^{11+}$ 856.7, found 856.9.

CXCL14 **6:** Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.1 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 10H]^{10+}$ 941.9, found 941.9.

Preparation of CXCL14 middle fragment (H49G) 7 with peptide thioacid moiety

On aminomethyl ChemMatrix resin (1.0 mmol amine/g) was coupled Fmoc-Ala-OH (5.0 equiv) in the presence of DIPCDI (5.0 equiv) and HOBt·H_2O (5.5 equiv) in DMF at room temperature for 2 h, and then the resulting resin was treated with 20% (v/v) piperidine in DMF for 10 min for Fmoc removal. Next, treatment of the resulting resin with Fmoc-Gly-incorporated *N*-sulfanylethylaniline linker (2 equiv),[13] HATU (1.9 equiv), and DIPEA (2.0 equiv) in DMF at room temperature for 2 h followed by Fmoc removal by 20% (v/v) piperidine in DMF for 10 min afforded the SEAlide linked resin. On the resulting resin, standard Fmoc SPPS mentioned above was performed for the chain elongation to give protected peptide resin. Deprotection of the completed resin with TFA-thioanisole-*m*-cresol-H_2O-EDT-Et_3SiH (80:5:5:5:2.5:2.5,

(v/v)) at room temperature for 1.5 h followed by the treatment with 4 M HCl/DMF in the presense of TCEP·HCl (1% (w/v)) at room temperature for 20 h gave deprotected on-resin peptide thioester. The on-resin peptide thioester was incubated with 120 mM NaSH in 6 M guanidine-0.1 M sodium phosphate buffer (pH 9.2) at 37 °C for 30 min to yield the hydrothiolytically released N^{α}-Fmoc protected peptide thioacid. Then piperidine was added to the crude mixture of the N^{α}-Fmoc protected peptide thioacid, and the Fmoc group on the peptide thioacid was completely removed to give crude materials which were then subjected to semi-preparative HPLC purification to afford the desired middle Fr **7** with peptide thioacid moiety.

Peptide thioacid **7**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 18.7 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 12 to 27% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 810.7, found 810.7.

Synthesis of CXCL14 (H49G) 11 using N–to–C-directive sequential NCL

The first NCL: Peptide thioester **1** (2.7 mg, 0.78 μmol) and *N*-Cys peptide thioacid **2** (1.8 mg, 0.76 μmol) were dissolved at a final concentration of 1 mM in 6 M Gn·HCl-0.1 M sodium phosphate buffer and 1% (v/v) thiophenol. After disappearance of the starting materials by incubation of the reaction mixture at 37 °C for 2 h, the crude material was then purified by semi-preparative HPLC to give ligated peptide thioacid **8** (1.0 mg, 22%).

The second NCL: The purified peptide thioacid **8** (0.29 mg, 0.050 μmol) was dissolved at a final concentration of 1 mM in DMF/H_2O (2:8 (v/v)) containing 6 mM Ellman's reagent and 6 mM $KHCO_3$. The resulting mixture was shaken at room temperature for 1 h. Then 1% (w/v) TCEP·HCl was added to the reaction mixture to reduce an excess Ellman's reagent and the hetero disulfide resulted from reaction of the cysteine sulfhydryl group with 5-mercapto-2-nitrobenzoic acid. The conversion of thioacid **8** to thioester **9** was confirmed by HPLC analysis. *N*-Cys fragment **3** (0.18 mg, 0.050 μmol) and 1% (v/v) thiophenol were added to the reaction mixture and then the pH of the reaction mixture was adjust around 7.5 by addition of 10% (w/v) K_2CO_3 aqueous solution at 4 °C. After disappearance of the starting materials by incubation of the mixture at 37 °C for 2 h, the crude material was purified by semi-preparative HPLC to give ligated peptide **10** (0.056 mg, 12%). Oxidation of the purified CXCL14 (H49G, 4-Cys) **10** (2.26 mg, 2.4 μmol) with air was performed in 3 M Gn·HCl-0.1 M sodium phosphate buffer (pH 7.7, concentration of the peptide: 0.050 mM). After incubation of the mixture at 37 °C for 6 h, CXCL14 (H49G) **11** (0.66 mg, 38%) was isolated by

semi-preparative HPLC.

Peptide thioacid **8**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 18.7 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 12 to 27% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1150.0, found 1150.0.

Peptide thioester **9**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.3 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 1478.5, found 1478.6.

CXCL14 (H49G, 4-Cys) **10**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.5 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 15 to 45% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 9H]^{9+}$ 1037.9, found 1038.3.

CXCL14 (H49G) **11**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 19.3 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 7H]^{7+}$ 1333.7, found 1333.8.

Synthesis of Fmoc-His(Fmoc)-incorporated *N*-sulfanylethylaniline linker 14

To a solution of aniline **15** (1.2 g, 2.5 mmol) in tetrahydrofuran (THF) (25 mL) was added 55% NaH (120 mg, 2.8 mmol) by portions at room temperature and the mixture was stirred at same temperature. In another flask, Fmoc-His(Fmoc)-OH (3.0 g, 5 mmol) was treated with $SOCl_2$ (3.7 mL, 50 mmol) in CH_2Cl_2 (60 mL) in the presence of catalytic amount of DMF (39 μL, 0.5 mmol) at room temperature. After being stirred at same temperature for 3 h, the reaction mixture was concentrated under reduced pressure. The residue was added to another flask containing THF solution of **15** and NaH, and then the mixture was stirred at room temperature overnight. The reaction was quenched with saturated $NaHCO_3$ aqueous solution (sat. $NaHCO_3$ aq.) and extracted with EtOAc. The organic layer was washed with sat. $NaHCO_3$ aq. followed by brine and dried over $MgSO_4$. After removal of the solvent under reduced pressure, the crude material was roughly purified by column chromatography on silica gel using EtOAc/*n*-hexane (= 1:2 (v/v)) and gave Fmoc-His(Fmoc)-incorporated linker **16** as a white amorphousness. Compound **16** was subsequently treated with *N*-methylaniline (10 equiv) and $Pd(PPh_3)_4$ (0.10 equiv) in THF (0.1 M) at room temperature for 2.5 h. The solvent was then removed under reduced pressure and the product was purified by column chromatography on silica gel using EtOAc/*n*-hexane (= 1:1 (v/v)) to give 1.7 g (65%, 2 steps) of compound **14** as a white amorphousness.

Allyl 4-[{Fmoc-His(Fmoc)-2-tritylsulfanylethyl}amino]benzoate **16**: $[\alpha]^{24}_{D}$ 52.4 (*c* 1.00, $CHCl_3$); IR (KBr) ν_{max}, cm^{-1}: 703, 743, 1012, 1270, 1405, 1447, 1491, 1602, 1667, 1723, 1763, 2950, 3020, 3060, 3297; ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 2.29-2.35 (m, 1H), 2.50-2.57 (m, 1H), 2.64 (dd, *J* = 6.4 and 14.8 Hz, 1H), 2.75 (dd, *J* = 5.2 and 14.8 Hz, 1H), 3.29-3.36 (m, 1H), 3.51-3.58 (m, 1H), 4.18 (t, *J* = 7.2 Hz, 1H), 4.28-4.32 (m, 3H), 4.48-4.49 (m, 1H), 4.66 (d, *J* = 6.8 Hz, 2H), 4.84 (d, *J* = 5.6 Hz, 2H), 5.31 (dd, *J* = 1.2 and 10.4 Hz, 1H), 5.43 (dd, *J* = 1.2 and 17.2 Hz, 1H), 5.86 (d, *J* = 8.4 Hz, 1H), 6.04 (ddt, *J* = 5.6, 10.4 and 17.2 Hz, 1H), 7.05-7.19 (m, 12H), 7.27-7.34 (m, 10H), 7.36-7.44 (m, 4H), 7.53-7.58 (m, 4H), 7.75 (d, *J* = 7.2 Hz, 2H), 7.78 (d, *J* = 7.6 Hz, 2H), 7.91 (s, 1H), 8.04 (d, *J* = 8.4 Hz, 2H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 29.3, 30.9, 46.7, 47.2, 49.4, 51.6, 66.0, 67.1, 67.2, 69.9, 114.6, 118.7, 120.1, 120.4, 124.9, 125.3, 126.8, 127.2, 127.5, 127.8, 128.0, 128.3, 129.6, 130.1, 131.3, 132.1, 136.7, 139.0, 141.4, 141.5, 142.8, 144.0, 144.6, 145.1, 148.3, 155.7, 165.4, 170.6; high resolution mass spectrometry (HRMS) (ESI-TOF) *m/z* calcd for $C_{67}H_{57}N_4O_7S$ ($[M+H]^+$), 1061.3948, found 1061.3962.

4-[{Fmoc-His(Fmoc)-2-tritylsulfanylethyl}amino]benzoic acid **14**: $[\alpha]^{24}_{D}$ 43.9 (*c* 1.00, $CHCl_3$); IR (KBr) ν_{max}, cm^{-1}: 703, 742, 760, 1013, 1248, 1404, 1447, 1492, 1601, 1667, 1723, 1766, 2495, 2621, 2953, 3061, 3292; ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 2.27-2.34 (m, 1H), 2.50-2.56 (m, 1H), 2.80 (dd, *J* = 7.2 and 14.0 Hz, 1H), 2.87-2.92 (m, 1H), 3.22-3.29 (m, 1H), 3.53-3.60 (m, 1H), 4.19 (t, *J* = 7.3 Hz, 1H), 4.30 (t, *J* = 7.0 Hz, 1H), 4.34 (d, *J* = 7.3 Hz, 2H), 4.64-4.69 (m, 1H), 4.65 (d, *J* = 7.0 Hz, 2H), 5.90 (br d, *J* = 8.4 Hz, 1H), 6.97 (br d, *J* = 6.8 Hz, 2H), 7.08-7.19 (m, 10H), 7.27-7.43 (m, 14H), 7.54 (d, *J* = 7.6 Hz, 2H), 7.57-7.59 (m, 2H), 7.73-7.74 (m, 2H), 7.78 (d, *J* = 7.6 Hz, 2H), 8.01 (d, *J* = 8.4 Hz, 2H), 8.24 (s, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 29.2, 31.3, 46.6, 47.2, 49.4, 51.3, 67.1, 67.3, 70.2, 115.2, 120.1, 120.4, 124.9, 125.3, 126.8, 127.2, 127.6, 127.8, 128.0, 128.4, 128.6, 128.7, 129.6, 130.6, 131.8, 132.2, 132.3, 137.6, 138.0, 141.4, 141.4, 142.7, 143.9, 144.3, 144.6, 148.1, 155.7, 167.9, 171.1; HRMS (ESI-TOF) *m/z* calcd for $C_{64}H_{53}N_4O_7S$ ($[M+H]^+$), 1021.3635, found 1021.3633.

Preparation of CXCL14 middle fragment 12 as SEAlide peptide

The protected peptide resin was constructed on NovaSyn® TGR resin (loading: 0.25 mmol/g) using Fmoc SPPS (Acylation: Fmoc amino acid (5.0 equiv), DIPCDI (5.0 equiv) and HOBt·H_2O (5.5 equiv) in DMF or **14** (2.0 equiv), HATU (1.95 equiv) and DIPEA (1.95 equiv) in DMF for 2 h; Fmoc removal: 20% (v/v) piperidine in DMF for 10 min). The completed resin (100 mg) was treated with TFA-thioanisole-*m*-cresol-H_2O-EDT (80:5:5:5:5, (v/v)) at room temperature for 1.5 h,

and then NH_4I (75 equiv) and Me_2S (100 equiv) were added to the TFA solution at 0 °C. After stirring of the mixture at same temperature for 30 min, the resin was filtered off and the filtrate was directly added to cold Et_2O to generate precipitate. The precipitate collected by centrifugation was washed with cold Et_2O and purified by preparative HPLC to give the middle fragment **12** (8.2 mg, 18%).

SEAlide peptide **12**: Analytical HPLC conditions, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 18.6 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 15 to 23% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 915.1, found 914.9.

Synthesis of CXCL14 6 using N–to–C-directive one-pot/sequential NCL with the use of the SEAlide peptide

The first NCL between N-terminal Fr **1** (1.7 mg, 0.35 μmol as **1**·12TFA) and SEAlide peptide **12** (1.2 mg, 0.35 μmol as **12**·6TFA) was performed in 6 M Gn·HCl-0.2 M HEPPS buffer containing 50 mM MPAA and 30 mM TCEP·HCl (pH 6.6, 347 μL, 1 mM each peptide) at 25 °C. The reaction was completed within 5 h. Then, C-terminal Fr **3** solution (1.6 mg, 0.35 μmol as **3**·9TFA) in 1 M sodium phosphate buffer (pH 6.6, 347 μL) was added to the reaction mixture. The second NCL proceeded at 25 °C within 48 h, and then the crude material was purified by semi-preparative HPLC to give CXCL14 (4-Cys) **5** in 38% isolated yield. The purified CXCL14 (4-Cys) **5** (1.6 mg, 0.13 μmol as **5**·25TFA) was oxidized with air in 3 M Gn·HCl-0.1 M sodium phosphate buffer (pH 7.7, concentration of the peptide: 0.05 mM) at 37 °C for 10 h. After oxidative folding followed by purification using semi-preparative HPLC, human CXCL14 **6** (0.47 mg) was obtained in 39% isolated yield.

Synthesis of dialdehyde linker 19[19]

O,*O'*-Bis(3-aminopropyl)diethylene glycol **18** (100 mg, 0.45 mmol), 4-folmylbenzoic acid **17** (545 mg, 3.6 mmol) and DMAP (44 mg, 0.36 mmol) were dissolved in CH_2Cl_2 (23 mL), and the obtained solution was cooled to 0 °C. The cold solution of DIPCI (562 μL, 3.6 mmol) in CH_2Cl_2 (4.5 mL) was added dropwise over 10 min to the reaction mixture. The obtained mixture was stirred at 0 °C for 4 h and then slowly warmed to room temperature. After 17 h, the reaction mixture was evaporated and then the residue was purified by silica gel column chromatography (eluting with $CHCl_3$-MeOH mixture, 30: 1 (v/v)) to afford 185 mg (84%) of **19** as white powder.

N-{13-[2-(4-Formylphenyl)acetamido]-4,7,10-trioxatridecyl}-4-formylbenzamide **19**; ^1H-NMR (400 MHz, $CDCl_3$) δ = 1.87 (quint, *J* = 6.0 Hz, 4H), 3.54-3.63 (m,

16H), 7.38 (s, 2H), 7.92 (d, J = 8.4 Hz, 4H), 7.98 (d, J = 8.4 Hz, 4H), 10.06 (s, 2H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 28.7, 39.0, 70.0, 70.2, 70.5, 127.7, 129.6, 137.9, 139.8, 166.1, 191.7; HRMS (ESI-TOF) *m/z* calcd for $C_{26}H_{33}N_2O_7$ ($[M+H]^+$), 485.2288, found 485.2278.

Preparation of CXCL14 (50-77)-Lys(aminooxyacetyl)-NH_2 20

On NovaSyn TGR® resin (0.25 mmol amine/g) was coupled Fmoc-Lys(ivDde)-OH (3.0 equiv) in the presence of DIPCDI (3.0 equiv) and HOBt·H_2O (3.3 equiv) in DMF at room temperature for 3 h. On the resulting resin, standard Fmoc SPPS mentioned above was performed for the chain elongation to give the protected peptide resin. Next, the resulting resin was treated with Boc_2O (5.0 equiv) in DMF for protection of α-amino group and then treated with 2% (v/v) hydrazine·H_2O/DMF at room temperature for 4 days for ivDde removal. On the resulting resin was coupled Boc-aminooxyacetic acid (5.0 equiv) in the presence of DIPCDI (5.0 equiv) and HOBt·H_2O (5.5 equiv) in DMF at room temperature for 3 h to afford the protected peptide resin. The resulting resin (200 mg) was treated with TFA-thioanisole-*m*-cresol-H_2O-EDT (80:5:5:5:5, (v/v)) at room temperature for 1.5 h. After filtration of the resin, cooled Et_2O was added to the filtrate, and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with cold Et_2O and purified by preparative HPLC to give desired CXCL14 (50-77)-Lys(aminooxyacetyl)-NH_2 **20** (9.4 mg, 13%).

CXCL14 (50-77)-Lys(aminooxyacetyl)-NH_2 **20**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 60% over 30 min, retention time = 16.6 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 956.5, found 956.5.

Synthesis of Alexa Fluor® 488-conjugated CXCL14 derivative 26 using C–to–N directive sequential NCL and oxime ligation

The First NCL: *N*-Thz peptide thioester **2** (2.7 mg, 1.0 μmol) as middle Fr and *N*-Cys peptide **20** (3.9 mg, 1.0 μmol) as C-terminal Fr were dissolved in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 6.8) containing 0.5 mM EDTA and 1% (v/v) thiophenol to initiate the first NCL (concentration of each peptides: 1 mM). After completion of the reaction by incubation at 37 °C for 4 h, $MeONH_2$·HCl was added directly to the ligation reaction mixture at a final concentration of 0.2 M. The opening of Thz ring to convert to Cys was completed within 2 h. The crude material was then purified by semi-preparative HPLC to afford ligated peptide **21** (3.2 mg, 50%).

The Second NCL: N-Terminal Fr **1** (1.8 mg, 0.51 μmol) and the ligated product **21** (3.2 mg, 0.51 μmol) were dissolved at a final concentration of 1 mM in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 6.8) containing 0.5 mM EDTA and 1% (v/v) thiophenol. After disappearance of the starting materials **1** and **21** by incubation of the reaction mixture at 37 °C for 6 h, the crude material was purified by semi-preparative HPLC to give CXCL14-K(aminooxyacetyl) (4-Cys) **22** (1.7 mg, 34%). Oxidation of the purified **22** (1.7 mg, 0.17 μmol, concentration of the peptide: 0.050 mM) with air was performed in 3 M Gn·HCl-0.1 M sodium phosphate buffer (pH 7.7). After incubation of the mixture at 37 °C for 20 h, CXCL14-K(aminooxyacetyl) **23** (0.85 mg, 51%) was isolated by semi-preparative HPLC.

Fluorescent dye conjugation: CXCL14-K(aminooxyacetyl) **23** (0.85 mg, 0.088 μmol) was incubated with excess amount of the linker **19** (10 equiv) in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 5.8) containing 0.5 mM EDTA at 37 ℃ for 1 h to afford the linker conjugated CXCL14 derivative **24**. After completion of the reaction, excess linker was washed out by extraction with CH_2Cl_2. Then Alexa Fluor® 488 hydroxyamine **26** (4.0 equiv) in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 4.0) containing 0.5 mM EDTA was added to the reaction mixture. After 7 h, **24** was disappeared and the crude material was purified by semi-preparative HPLC to give CXCL14-K(Alexa Fluor® 488) **26**.

CXCL14 (29-77)-K(aminooxyacetyl) **21**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.0 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 6H]^{6+}$ 1792.1, found 1791.9.

CXCL14-K(aminooxyacetyl) (4-Cys) **22**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.9 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 11H]^{11+}$ 874.9, found 875.3.

CXCL14-K(aminooxyacetyl) **23:** Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.4 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 7H]^{7+}$ 1373.7, found 1373.7.

Linker conjugated CXCL14 derivative **24**: Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.8 min. MS (ESI-TOF) *m/z* calcd for $[M + 6H]^{6+}$ 1680.2, found 1680.0.

CXCL14-K(Alexa Fluor® 488) **26:** Analytical HPLC condition: linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.7 min.

Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 6H]^{6+}$ 1792.4, found 1791.9.

Preparation of CXCL14 C-terminal fragment 27 possessing biotin on C-terminal additional lysine

On NovaSyn® TGR resin (0.25 mmol amine/g) was coupled Fmoc-Lys(ivDde)-OH (3 equiv) in the presence of COMU[29] (2.95 equiv) and DIPEA (6 equiv) in DMF at room temperature for 2.5 h. On the resulting resin standard Fmoc SPPS mentioned above was performed for the chain elongation to give corresponding protected peptide-Lys(ivDde) resin. The resulting resin was treated with Boc_2O (4.0 equiv), HOBt (4.0 equiv) and DIPEA (4.0 equiv) in DMF to protect an N-terminal α-amino group of elongated peptide. The resin was subsequently treated with 2% (v/v) hydrazine·H_2O/DMF or 2% (v/v) hydrazine·H_2O/NMP 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 (5.0 equiv) in the presence of DIPCDI (5.0 equiv) in DMF/DMSO = 1:1 (v/v) at room temperature overnight to afford protected peptide-K(biotin) resin. The completed resin (50 mg) was treated with TFA-thioanisole-*m*-cresol-H_2O-EDT (80:5:5:5:5, (v/v)) at room temperature for 1.5 h. After filtration of the resin, cooded Et_2O was added to the filtrate, and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with cold Et_2O and purified by preparative HPLC to give desired biotin-labeled C-terminal fragment **27** (1.4 mg 6% as **27**·9TFA).

Biotinylated C-terminal Fr **27**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.2 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 994.8, found 994.5.

Synthesis of biotinylated CXCL14 29 using N–to–C-directive one-pot/sequential NCL with the use of the SEAlide peptide

The first NCL between alkyl thioester peptide **1** (1.4 mg, 0.28 μmol as **1**·12TFA) and SEAlide peptide **2** (1.0 mg, 0.28 μmol as **2**·6TFA) was performed in 6 M Gn·HCl-0.2 M HEPPS buffer containing 50 mM MPAA and 30 mM TCEP·HCl (pH 6.6, 278 μL, 1.0 mM each peptide) at 25 °C. The reaction was completed within 5 h. Then, peptide **27** (1.4 mg, 0.28 mg as **27**·9TFA) in 1 M sodium phosphate buffer (pH 6.6, 278 μL) was added to the reaction mixture. The second NCL proceeded at 25 °C for 48 h, and then the crude material was purified by semi-preparative HPLC. The purified

CXCL14-K(biotin) (4-Cys) was folded by the same procedure as that employed for CXCL14 **6** and the desired CXCL14-K(biotin) (672 μg) was obtained in 25% isolated yield (2 steps).

CXCL14-K(biotin) (4-Cys) **28**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.8 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 11H]^{11+}$ 888.8, found 888.8.

CXCL14-K(biotin) **29**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.3 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 6H]^{6+}$ 1628.0, found 1628.1.

Chemotaxis assay

Chemotaxis assays were conducted as previously described.[6] Briefly, THP-1 cells or BaF/3 cells were washed and resuspended at 10^6 cells/mL in RPMI-1640 medium containing 0.1% fatty acid-free BSA (Sigma) and 20 mM HEPES buffer (pH 7.5; Invitrogen). Wells in a 24-well culture plate were filled with 550 μL of 10 nM CXCL12. Chemotaxicell filter was placed in each well and the cells (200 μL) were added to the upper chamber with or without 100nM synthetic CXCL14, recombinant CXCL14, or CXCL14-K(biotin). These plates were incubated at 37 °C for 2 h. The number of migrated cells in the lower chamber were determined using LSR Fortessa-X20 (BD Bioscience, San Jose, CA, USA) with 123 counting beads (Affymetrix Japan K.K., Tokyo, Japan) as a reference.

Fluorescence activated cell sorting analysis

HeLa cells were transfected with pCS2+*GFP* or pCS2+*CXCR4-GFP* expression vectors (*CXCR4* corresponds to human CXCR 4 transcript variant 1: NM_001008540) by using polyethyleneimine MAX (Polyscience, Warrington, PA). Twenty four hours after transfection, cells were trypsinized, and incubated with 100 nM biotin (Wako), 100 nM CXCL14-K(biotin), or 100 nM CXCL14-K(biotin) plus 4μM CXCL14 at 4 °C for 1 h. To determine the expression of human CXCR4, APC anti-human CD184 (CXCR4) antibody was used (clone 12G5: Biolegend). After incubation with APC-streptavidin (Life Technologies, Carlsbad, CA), cell surface binding of the biotinylated CXCL14 was measured by LSR Fortessa-X20. GFP positive cells were gated for the analyses of fluorescent intensity by FlowJo software (FlowJo, LLC, Ashland, OR).

Statistical analyses

All statistical analyses were performed using ANOVA repeated measures analysis (Statview J5.0, Abacus Concepts). A *p*-value of <0.05 was considered significant for the unpaired Student's *t*-test.

Chapter 2

Preparation of H-Thz-CXCL14 (30-49, H49G)-OH 30

Middle Fr **30** was prepared by hydrolysis of corresponding peptide thioester **31** which was synthesized using the Boc SPPS in a manner similar to that employed for N-terminal Fr **1**. Thioester **31** (1.9 mg, 0.73 μmol) was incubated in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 9.0) at 37 °C for 23 h (concentration of the peptide: 1.0 mM). After completion of the hydrolysis, peptide **30** (0.50 mg, 28%) was obtained after HPLC purification.

Middle Fr **30** (5.6 mg from 100 mg resin, 10%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 17.1 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 15 to 40% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 809.4, found 809.3.

Peptide thioester **31**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 17.3 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 13 to 23% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 862.1, found 861.9.

Preparation of H-Thz-CXCL14 (1-28)-CXCL14 (50-77) 32

The Δ(29-49) derivative **32** was generated as byproduct during the synthesis of other CXCL14 derivative using **33** as N-terminal Fr. Thioester **33** was prepared using the Boc SPPS in a manner similar to that employed for N-terminal Fr **1**. During the synthesis of a CXCL14 derivative using N-terminal Fr **33**, middle Fr **31**, and C-terminal Fr **3** by conventional C–to–N-directive NCL method almost identical to that employed for CXCL14 **6**, HPLC purification afforded mixture of the first ligated product (conjugates of **31** and **3**) and **3**. The resulting mixture was subjected to the second NLC with N-terminal Fr **33**. As a result, desired CXCL14 protein and the Δ(29-49) derivative **32** was isolated, respectively, after HPLC purification.

H-Thz-CXCL14 (1-28)-CXCL14 (50-77) **32**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.1 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 15 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1411.5, found 1410.9.

H-Thz-CXCL14 (1-28)-SCH_2CH_2CO-Ala-NH_2 **33** (7.4 mg from 100 mg resin, 16%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 15.5 min. Preparative HPLC condition: linear gradient of

solvent B in solvent A, 13 to 23% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 902.7, found 902.5.

Preparation of H-CXCL14 (1-49, H49G)-CXCL12 (50-72)-OH 34

H-CXCL14 (1-49, H49G)-CXCL12 (50-72)-OH **34** was synthesized from N-terminal Fr **1**, middle Fr **31**, and CXCL12 (50-72) **35** as C-terminal Fr using conventional C–to–N-directive NCL method similar to that employed for CXCL14 **6**.

The First NCL: *N*-Thz peptide thioester **31** (2.0 mg, 0.76 μmol) as middle Fr and *N*-Cys peptide **35** (2.2 mg, 0.76 μmol) as C-terminal Fr were dissolved in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 6.8) containing 0.5 mM EDTA and 1% (v/v) thiophenol to initiate the first NCL (concentration of each peptides: 1.0 mM). After completion of the reaction by incubation at 37 °C for 3 h, $MeONH_2$·HCl was added directly to the ligation reaction mixture at a final concentration of 0.2 M. The opening of Thz ring to convert to Cys was completed within 2 h. The crude material was purified by semi-preparative HPLC to afford ligated peptide (H-CXCL14 (29-49, H49G)-CXCL12 (50-72)-OH, 2.4 mg, 60%).

The Second NCL: N-terminal Fr **1** (1.6 mg, 0.46 μmol) and the resulting H-CXCL14 (29-49, H49G)-CXCL12 (50-72)-OH (2.4 mg, 0.46 μmol) were dissolved in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 6.8) containing 0.5 mM EDTA and 1% (v/v) thiophenol (concentration of each peptides: 1.0 mM). After disappearance of the starting materials by incubation of the mixture at 37 °C for 4 h, the crude material was purified by semi-preparative HPLC to give reduced form H-CXCL14 (1-49, H49G)-CXCL12 (50-72)-OH (H-CXCL14 (1-49, H49G)-CXCL12 (50-72)-OH (4-Cys), 2.2 mg, 55%). Oxidation of the resulting material (2.2 mg, 0.25 μmol) with air was performed in 2.4 M Gn·HCl-0.1 M sodium phosphate buffer (pH 7.7, concentration of the peptide: 0.050 mM). After incubation of the mixture at 37 °C for 3 d, H-CXCL14 (1-49, H49G)-CXCL12 (50-72)-OH **34** (0.47 mg, 22%) was isolated by semi-preparative HPLC.

H-CXCL14 (1-49, H49G)-CXCL12 (50-72)-OH **34**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 23.9 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 45% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 9H]^{9+}$ 956.6, found 957.2.

CXCL12 (50-72) **35** (75 mg as **35**·7TFA from 204 mg resin, 86%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 65% over 30 min, retention time = 19.5 min. Preparative HPLC condition: linear gradient of solvent B in

solvent A, 13 to 27% over 37 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 724.4, found 724.4.

H-CXCL14 (29-49, H49G)-CXCL12 (50-72)-OH: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 60% over 30 min, retention time = 19.8 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 25 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 1323.2, found 1323.2.

H-CXCL14 (1-49, H49G)-CXCL12 (50-72)-OH (4-Cys): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 60% over 30 min, retention time = 18.9 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 26 to 36% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 10H]^{10+}$ 861.5, found 861.5.

Preparation of H- CXCL14 (51-77)-OH 36

H-CXCL14 (51-77)-OH **36** was synthesized using standard Fmoc SPPS in a fashion similar to that employed for C-terminal Fr **3**.

CXCL14 (51-77) **36** (4.3 mg from 25 mg resin, 45%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 60% over 30 min, retention time = 16.7 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 1174.0, found 1173.7.

Synthesis of the disulfide dimers of CXCL14 C-terminal fragments

The requisite monomer peptides were prepared using standard Fmoc SPPS in a manner almost identical to that employed for C-terminal Fr **3**.

For synthesis of N2C **37**, C-terminal Fr **3** (3.2 mg, 0.88 μmol) was dissolved in 0.1 M sodium phosphate buffer (pH 7.7, concentration of the peptide: 1.0 mM). After disappearance of the starting materials by incubation at 37 °C for 12 h, the crude material was purified by semi-preparative HPLC to give dimerized C-terminal Fr (N2C) **37** (1.5 mg, 47%). Other mutant dimers were also synthesized using the method similar to that employed for N2C **37** with corresponding monomer peptides.

N2C 37 from 3

N2C **37**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 23.3 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1449.3, found 1449.7.

BF2C 38 from 39

BF2C **38** (0.43 mg from **39** (2.0 mg, 0.55 μmol), 22%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.5 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1462.5, found 1461.9.

CXCL14 (50-77, *b*,*f*-EK) **39** (8.6 mg from 50 mg resin, 29%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 65% over 30 min, retention time = 15.4 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 914.7, found 914.6.

CG2C 40 from 41

CG2C **40** (0.21 mg from **41** (1.1 mg, 0.30 μmol), 19%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.3 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 40% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 8H]^{8+}$ 921.2, found 921.2.

CXCL14 (50-77, *c*,*g*-EK) **41**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 65% over 30 min, retention time = 16.4 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 921.5, found 921.2.

GD2C 44 from 45

GD2C **44** (0.45 mg from **45** (2.0 mg, 0.55 μmol), 23%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 19.8 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 15 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1455.0, found 1454.0.

CXCL14 (50-77, *g*,*d*-EK) **45** (12 mg from 50 mg resin, 64%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 18.3 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 910.0, found 910.0.

DA2C 46 from 47

DA2C **46** (0.50 mg from **47** (1.3 mg, 0.36 μmol), 40%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 16.4 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 10 to 20% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 8H]^{8+}$ 874.7, found 874.4.

CXCL14 (50-77, *d*,*a*-EK) **47** (5.9 mg from 50 mg resin, 29%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 15.3 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 12 to 18% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 875.0, found 874.7.

AE2C 48 from 49

AE2C **48** (0.53 mg from **49** (1.1 mg, 0.32 μmol), 48%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 16.2 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 12 to 18% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1397.7, found 1397.1.

CXCL14 (50-77, *a*,*e*-EK) **49** (9.3 mg from 50 mg resin, 45%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 15.1 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 10 to 19% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 874.2, found 874.0.

EB2C 50 from 51

EB2C **50** (0.27 mg from **51** (0.85 mg, 0.23 μmol), 31%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 19.4 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 25% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1459.7, found 1459.0.

CXCL14 (50-77, *e*,*b*-EK) **51** (6.1 mg from 50 mg resin, 36%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 18.9 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 25% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 913.0, found 912.7.

Preparation of CXCL14 (50-77, *f*,*c*-EK) 43

The protected peptide resin was constructed on Wang resin (loading: 0.17 mmol/g)[30] using standard Fmoc SPPS mentioned above. The completed resin (25 mg) was treated with TFA-thioanisole-*m*-cresol-H_2O-EDT (80:5:5:5:5, (v/v)) at room temperature for 1.5 h. After filtration of the resin, cooled Et_2O was added to the filtrate and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with Et_2O and purified by preparative HPLC to give desired CXCL14 (50-77, *f*,*c*-EK) **43** (5.0 mg, 68%).

CXCL14 (50-77, *f*,*c*-EK) **43**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.5 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 903.2, found 903.0.

Synthesis of FC2C 42

FC2C **42** was synthesized from the corresponding monomer peptide **43** using the method similar to that employed for N2C **37**.

FC2C **42** (0.16 mg from **43** (1.9 mg, 0.53 μmol), 8%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.8 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 8H]^{8+}$ 903.0, found 903.1.

Preparation of aminooxyacety C-terminal fragment 52 and its *c*,*g*-EK mutant 54

The protected peptide resin was constructed on Fmoc-Glu(O^tBu)-Alko-PEG resin (loading: 0.24 mmol amino acid/g) using standard Fmoc SPPS mentioned above. Coupling of Boc-aminooxyacetic acid (5.0 equiv) with α-amino group on the resulting resin was subsequently subjected in the presence of DIPCDI (5.0 equiv) and HOBt·H_2O (5.5 equiv) in DMF at room temperature for 5 h to afford protected aminooxyacetyl CXCL14 (51-77) peptide resin. The completed resin was treated with TFA-thioanisole-*m*-cresol-H_2O-EDT (80:5:5:5:5, (v/v)) at room temperature for 1.5 h. After filtration of the resin, cooled Et_2O was added to the filtrate and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with Et_2O and purified by preparative HPLC to give peptide **52** or **54**, respectively.

Aminooxyacetylated CXCL14 (51-77) **52** (9.9 mg from 50 mg resin, 51%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.9 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 899.0,

found 898.7.

Aminooxyacetylated CXCL14 (51-77, *c*,*g*-EK) **54** (68 mg from 394 mg resin, 39%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.2 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 914.0, found 913.6.

Synthesis of the oxime dimers of CXCL14 C-terminal fragments

Linker **19** was added to the solution of excess amount of peptide **52** or **54** in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 7.6, concentration of the linker **19**: 0.30 mM) containing 0.05 mM EDTA. After incubation at 37 °C for 3 h, the reaction mixture was purified by semi-preparative HPLC to give dimer peptide **53** or **55**, respectively.

N2O **53** (1.6 mg from linker **19** (0.12 mg, 0.25 μmol), 85%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 24.8 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 8H]^{8+}$ 955.0, found 954.8.

CG2O **55** (13 mg from linker **19** (0.70 mg, 1.4 μmol), 92%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 23.6 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 8H]^{8+}$ 970.0, found 969.8.

Preparation of the monomer peptide with *c*,*g*-EK mutation 56

The monomer peptide with *c*,*g*-EK mutation **56** was synthesized using standard Fmoc SPPS in a manner similar to that employed for C-terminal Fr **3**.

CXCL14 (51-77, *c*,*g*-EK) **56** (3.3 mg from 25 mg resin, 31%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 65% over 30 min, retention time = 15.7 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 895.7, found 895.5.

Synthesis of the trimer of CXCL14-C-terminal fragment 57

Commercially available trialdehyde linker **58** (0.037 mg, 0.23 μmol) was added to the solution of excess amount of peptide **52** in 6 M Gn·HCl-0.1 M sodium phosphate buffer (pH 5.5, concentration of the linker **58**: 0.33 mM) containing 0.05 mM EDTA. After incubation at 37 °C for 4 h, the reaction mixture was purified by semi-preparative

HPLC to give trimerized peptide **57** (0.20 mg, 8%).

Trimer peptide **57**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 24.5 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 23 to 31% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 9H]^{9+}$ 1210.3, found 1210.6.

Synthesis of the disulfide dimers with mutation of an aromatic amino acid residue to alanine

These monomeric mutants were prepared using standard Fmoc SPPS in a manner almost identical to that employed for C-terminal Fr **3**. The mutant dimers were synthesized using the method similar to that employed for N2C **37** with corresponding monomer peptides.

The dimer of CXCL14 (50-77, W64A) 59 from 60

Dimer of CXCL14 (50-77, W64A) **59** (0.64 mg from **60** (1.9 mg, 0.55 μmol), 33%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.7 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 6H]^{6+}$ 1169.6, found 1169.6.

CXCL14 (50-77, W64A) **60** (7.4 mg from 100 mg resin, 17%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.0 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 17 to 27% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 877.7, found 877.4.

The dimer of CXCL14 (50-77, Y65A) 61 from 62

Dimer of CXCL14 (50-77, Y65A) **61** (0.73 mg from **62** (1.9 mg, 0.53 μmol), 39%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.9 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 6H]^{6+}$ 1177.3, found 1177.4.

CXCL14 (50-77, Y65A) **62** (7.9 mg from 100 mg resin, 18%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.9 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 883.5, found 883.1.

The dimer of CXCL14 (50-77, W68A) 63 from 64

Dimer of CXCL14 (50-77, W68A) **63** (0.78 mg from **64** (2.0 mg, 0.57 μmol), 39%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.4 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1403.3, found 1403.3.

CXCL14 (50-77, W68A) **64** (11 mg from 100 mg resin, 26%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.3 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 17 to 27% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 877.7, found 877.4.

The dimer of CXCL14 (50-77, Y75A) 65 from 66

Dimer of CXCL14 (50-77, Y75A) **65** (1.1 mg from **66** (2.3 mg, 0.66 μmol), 46%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 23.1 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1412.5, found 1412.4.

CXCL14 (50-77, Y75A) **66** (11 mg from 100 mg resin, 25%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.8 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 883.5, found 883.4.

Synthesis of the disulfide dimers of CXCL14 C-terminal fragments with deletion from C-terminus

The requisite monomers were prepared using standard Fmoc SPPS in a fashion almost identical to that employed for C-terminal Fr **3** (**68** and **80**) or CXCL14 (50-77, *f*,*c*-EK) **43** (**70**, **72**, **74**, **76**, **78**, **82**, **84**, **86**, **88**, **90**, and **92**), respectively. The deleted dimers were synthesized using the method similar to that employed for N2C **37** with corresponding monomer peptides.

The dimer of CXCL14 (50-76) 67 from 68

Dimer of CXCL14 (50-76) **67** (1.5 mg from **68** (2.3 mg, 0.66 μmol), 66%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.3 min. Semi-preparative HPLC condition: linear gradient of

solvent B in solvent A, 20 to 40% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1397.7, found 1397.3.

CXCL14 (50-76) **68** (13 mg from 50 mg resin, 62%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.2 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 874.2, found 874.0.

The dimer of CXCL14 (50-75) 69 from 70

Dimer of CXCL14 (50-75) **69** (1.1 mg from **70** (2.1 mg, 0.61 μmol), 55%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.3 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1346.1, found 1346.2.

CXCL14 (50-75) **70** (7.8 mg from 50 mg resin, 36%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.1 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 842.0, found 841.8.

The dimer of CXCL14 (50-74) 71 from 72

Dimer of CXCL14 (50-74) **71** (0.90 mg from **72** (2.0 mg, 0.63 μmol), 45%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.7 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1280.9, found 1280.5.

CXCL14 (50-74) **72** (5.2 mg from 50 mg resin, 33%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.3 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 15 to 25% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 801.2, found 801.0.

The dimer of CXCL14 (50-73) 73 from 74

Dimer of CXCL14 (50-73) **73** (0.82 mg from **74** (1.8 mg, 0.58 μmol), 45%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.5 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 7H]^{7+}$ 886.9, found 887.2.

CXCL14 (50-73) **74** (4.5 mg from 50 mg resin, 20%): Analytical HPLC

condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.3 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 1034.9, found 1034.4.

The dimer of CXCL14 (50-72) 75 from 76

Dimer of CXCL14 (50-72) **75** (1.0 mg from **76** (2.1 mg, 0.71 μmol), 49%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.6 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1178.8, found 1178.5.

CXCL14 (50-72) **76** (11 mg from 50 mg resin, 65%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.1 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 737.4, found 737.5.

The dimer of CXCL14 (50-71) 77 from 78

Dimer of CXCL14 (50-71) **77** (1.3 mg from **78** (2.7 mg, 0.95 μmol), 48%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.8 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1116.4, found 1116.3.

CXCL14 (50-71) **78**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.3 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 930.8, found 930.6.

The dimer of CXCL14 (50-70) 79 from 80

Dimer of CXCL14 (50-70) **79** (0.95 mg from **80** (1.9 mg, 0.71 μmol), 51%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.4 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 1331.2, found 1330.9.

CXCL14 (50-70) **80** (9.5 mg from 50 mg resin, 52%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.9 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 888.1, found 887.8.

The dimer of CXCL14 (50-69) 81 from 82

Dimer of CXCL14 (50-69) **81** (1.5 mg from **82** (2.8 mg, 1.1 μmol), 52%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.6 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1013.5, found 1013.7.

CXCL14 (50-69) **82**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.8 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 845.1, found 844.9.

The dimer of CXCL14 (50-68) 83 from 84

Dimer of CXCL14 (50-68) **83** (0.89 mg from **84** (1.9 mg, 0.77 μmol), 48%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 23.5 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 1209.6, found 1209.4.

CXCL14 (50-68) **84** (1.9 mg from 50 mg resin, 10%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.8 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 807.1, found 806.9.

The dimer of CXCL14 (50-67) 85 from 86

Dimer of CXCL14 (50-67) **85** (1.5 mg from **86** (2.8 mg, 1.2 μmol), 53%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.5 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 1116.6, found 1116.2.

CXCL14 (50-67) **86**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 19.0 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 17 to 27% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 745.1, found 745.0.

The dimer of CXCL14 (50-66) 87 from 88

Dimer of CXCL14 (50-66) **87** (0.90 mg from **88** (1.9 mg, 0.89 μmol), 47%):

Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.0 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 1081.1, found 1080.8.

CXCL14 (50-66) **88** (11 mg from 50 mg resin, 48%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 18.5 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 16 to 26% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 721.4, found 721.3.

The dimer of CXCL14 (50-65) 89 from 90

Dimer of CXCL14 (50-65) **89** (1.0 mg from **90** (2.7 mg, 1.3 μmol), 39%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.6 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 35% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 1024.1, found 1023.7.

CXCL14 (50-65) **90**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 19.8 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 17 to 27% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 683.4, found 683.3.

The dimer of CXCL14 (50-64) 91 from 92

Dimer of CXCL14 (50-64) **91** (0.82 mg from **92** (1.8 mg, 0.96 μmol), 46%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.6 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 942.5, found 942.3.

CXCL14 (50-64) **92** (12 mg from 50 mg resin, 54%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 18.9 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 15 to 25% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 629.0, found 629.0.

Synthesis of the disulfide dimers of CXCL14 C-terminal fragments with deletion from N-terminus

The requisite monomers were prepared using standard Fmoc SPPS in a manner almost identical to that employed for C-terminal Fr **3**. The deleted dimers were synthesized using the method similar to that employed for N2C **37** with corresponding

monomer peptides.

The dimer of CXCL14 (52-77) 93 from 94

Dimer of CXCL14 (52-77) **93** (0.66 mg from **94** (1.4 mg, 0.39 μmol), 48%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.7 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 21 to 31% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1404.1, found 1403.4.

CXCL14 (52-77) **94**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.5 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 878.2, found 878.0.

The dimer of CXCL14 (53-77) 95 from 96

Dimer of CXCL14 (53-77) **95** (0.64 mg from **96** (1.2 mg, 0.34 μmol), 56%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 23.1 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 21 to 31% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1349.3, found 1348.7.

CXCL14 (53-77) **96**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.0 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 1124.9, found 1124.5.

The dimer of CXCL14 (54-77) 97 from 98

Dimer of CXCL14 (54-77) **97** (0.72 mg from **98** (1.4 mg, 0.44 μmol), 51%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.5 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 21 to 31% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1310.5, found 1310.0.

CXCL14 (54-77) **98**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.9 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 1092.6, found 1092.2.

The dimer of CXCL14 (55-77) 99 from 100

Dimer of CXCL14 (55-77) **99** (0.75 mg from **100** (1.6 mg, 0.50 μmol), 47%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 23.5 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 21 to 31% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1259.2, found 1258.7.

CXCL14 (55-77) **100**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.8 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 1049.9, found 1049.6.

The dimer of CXCL14 (56-77) 101 from 102

Dimer of CXCL14 (56-77) **101** (0.40 mg from **102** (1.1 mg, 0.35 μmol), 38%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.4 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 21 to 31% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1214.0, found 1213.7.

CXCL14 (56-77) **102**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.6 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 1012.2, found 1011.9.

The dimer of CXCL14 (57-77) 103 from 104

Dimer of CXCL14 (57-77) **103** (0.53 mg from **104** (0.84 mg, 0.29 μmol), 63%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.5 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1162.8, found 1162.3.

CXCL14 (57-77) **104**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.7 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 969.5, found 969.2.

The dimer of CXCL14 (58-77) 105 from 106

Dimer of CXCL14 (58-77) **105** (0.41 mg from **106** (1.1 mg, 0.38 μmol), 38%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.3 min. Semi-preparative HPLC condition: linear gradient of

solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 1409.7, found 1409.3.

CXCL14 (58-77) **106**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.2 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 940.5, found 940.2.

The dimer of CXCL14 (59-77) 107 from 108

Dimer of CXCL14 (59-77) **107** (0.54 mg from **108** (1.2 mg, 0.43 μmol), 45%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.2 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 1359.2, found 1358.6.

CXCL14 (59-77) **108**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.0 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 906.8, found 906.4.

The dimer of CXCL14 (60-77) 109 from 110

Dimer of CXCL14 (60-77) **109** (0.33 mg from **110** (1.2 mg, 0.47 μmol), 27%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.8 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 1295.1, found 1294.7.

CXCL14 (60-77) **110**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.3 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 864.1, found 863.8.

The dimer of CXCL14 (61-77) 111 from 112

Dimer of CXCL14 (61-77) **111** (0.27 mg from **112** (1.2 mg, 0.47 μmol), 23%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 23.8 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 1217.1, found 1216.8.

CXCL14 (61-77) **112**: Analytical HPLC condition, linear gradient of solvent B

in solvent A, 5 to 45% over 30 min, retention time = 22.9 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 812.1, found 811.9.

The dimer of CXCL14 (62-77) 113 from 114

Dimer of CXCL14 (62-77) **113** (0.60 mg from **114** (1.1 mg, 0.46 μmol), 56%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.2 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 1143.5, found 1143.1.

CXCL14 (62-77) **114**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.8 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 763.0, found 763.0.

The dimer of CXCL14 (63-77) 115 from 116

Dimer of CXCL14 (63-77) **115** (0.39 mg from **116** (1.1 mg, 0.50 μmol), 36%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.2 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 1087.0, found 1086.7.

CXCL14 (63-77) **116**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 19.9 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 16 to 26% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 2H]^{2+}$ 1087.5, found 1087.1.

Synthesis of the disulfide dimer of CXCL14 (52-72) 117 from corresponding monomer 118

The requisite monomers were prepared using standard Fmoc SPPS in a manner almost identical to that employed for CXCL14 (50-77, *f*,*c*-EK) **43**. The truncated dimers were synthesized using the method similar to that employed for N2C **37** with corresponding monomer peptides.

Dimer of CXCL14 (52-72) **117** (0.96 mg from **118** (1.6 mg, 0.56 μmol), 61%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 22.7 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$

1133.6, found 1133.3.

CXCL14 (52-72) **118** (5.4 mg from 50 mg resin, 68%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.3 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 945.2, found 944.9.

Synthesis of the disulfide dimer of CXCL14 (52-72) with mutation of their aromatic amino acid residues to alanine

These mutated monomeric peptides were constructed on Fmoc-Arg(Pbf)-Alko-PEG resin (loading: 0.22 mmol amino acid/g) using Peptide Synthesizer CS336. Fmoc SPPS (Acylation: Fmoc amino acid (4.0 equiv), HBTU (3.8 equiv) and DIEA (8.0 equiv) in DMF for 1 h; Fmoc removal: 20% (v/v) piperidine in DMF for 15 min) was performed for the chain elongation to give protected peptide resin. The completed resin was treated with TFA-thioanisole-*m*-cresol-H_2O-EDT (80:5:5:5:5, (v/v)) at room temperature for 1.5 h. After filtration of the resin, cooled Et_2O was added to the filtrate, and the resulting precipitate was collected by centrifugation. The obtained precipitate was washed with cold Et_2O and purified by preparative HPLC to give desired monomer peptides.

The dimer of CXCL14 (52-72, W64A, Y65A) 119 from 120

Dimer of CXCL14 (52-72, W64A, Y65A) **119** (0.51 mg from **120** (1.2 mg, 0.46 µmol), 42%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 17.4 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 16 to 26% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1050.8, found 1050.5.

CXCL14 (52-72, W64A, Y65A) **120** (9.0 mg from 50 mg resin, 54%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 18.6 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 657.4, found 657.3.

The dimer of CXCL14 (52-72, W64A, W68A) 121 from 122

Dimer of CXCL14 (52-72, W64A, W68A) **121** (0.51 mg from **122** (1.3 mg, 0.51 µmol), 39%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 16.4 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 12 to 22% over 30 min. MS (ESI-TOF) *m/z*

calcd for $[M + 5H]^{5+}$ 1041.6, found 1041.3.

CXCL14 (52-72, W64A, W68A) **122** (9.0 mg from 50 mg resin, 48%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 15.3 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 10 to 20% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 652.0, found 651.5.

The dimer of CXCL14 (52-72, Y65A, W68A) 123 from 124

Dimer of CXCL14 (52-72, Y65A, W68A) **123** (0.51 mg from **124** (0.87 mg, 0.33 μmol), 58%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 18.2 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 16 to 26% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1050.8, found 1050.6.

CXCL14 (52-72, Y65A, W68A) **124** (5.3 mg from 50 mg resin, 30%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 17.1 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 12 to 22% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 876.1, found 875.9.

The dimer of CXCL14 (52-72, W64A, Y65A, W68A) 125 from 126

Dimer of CXCL14 (52-72, W64A, Y65A, W68A) **125**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 15.9 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 10 to 20% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 1255.7, found 1255.2.

CXCL14 (52-72, W64A, Y65A, W68A) **126** (7.7 mg from 50 mg resin, 45%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 14.8 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 9 to 19% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 837.8, found 837.5.

The dimer of CXCL14 (52-72, F61A) 127 from 128

Dimer of CXCL14 (52-72, F61A) **127** (0.29 mg from **128** (0.95 mg, 0.34 μmol), 31%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.0 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for

$[M + 5H]^{5+}$ 1103.2, found 1102.9.

CXCL14 (52-72, F61A) **128** (8.5 mg from 50 mg resin, 50%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 18.0 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 16 to 26% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 919.8, found 919.5.

The dimer of CXCL14 (52-72, F61A, W64A) 129 from 130

Dimer of CXCL14 (52-72, F61A, W64A) **129** (0.26 mg from **130** (0.95 mg, 0.36 μmol), 27%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 17.1 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 17 to 27% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1057.2, found 1056.9.

CXCL14 (52-72, F61A, W64A) **130** (9.0 mg from 50 mg resin, 56%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 16.1 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 11 to 21% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 881.5, found 881.2.

The dimer of CXCL14 (52-72, F61A, Y65A) 131 from 132

Dimer of CXCL14 (52-72, F61A, Y65A) **131** (0.41 mg from **132** (1.0 mg, 0.38 μmol), 41%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 19.4 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 17 to 27% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1066.4, found 1066.2.

CXCL14 (52-72, F61A, Y65A) **132** (8.7 mg from 50 mg resin, 48%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 18.0 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 17 to 27% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 4H]^{4+}$ 667.1, found 667.0.

The dimer of CXCL14 (52-72, F61A, W68A) 133 from 134

Dimer of CXCL14 (52-72, F61A, W68A) **133** (0.63 mg from **134** (1.2 mg, 0.44 μmol), 55%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 17.3 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 15 to 25% over 30 min. MS (ESI-TOF) *m/z* calcd for

$[M + 4H]^{4+}$ 1321.2, found 1320.8.

CXCL14 (52-72, F61A, W68A) **134** (8.6 mg from 50 mg resin, 58%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 16.5 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 12 to 22% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 881.5, found 881.3.

The dimer of CXCL14 (52-72, F61A, W64A, Y65A) 135 from 136

Dimer of CXCL14 (52-72, F61A, W64A, Y65A) **135** (0.37 mg from **136** (1.2 mg, 0.46 μmol), 32%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 16.5 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 8 to 18% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1020.3, found 1020.2.

CXCL14 (52-72, F61A, W64A, Y65A) **136** (8.3 mg from 50 mg resin, 60%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 15.8 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 10 to 20% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 850.8, found 850.6.

The dimer of CXCL14 (52-72, F61A, W64A, W68A) 137 from 138

Dimer of CXCL14 (52-72, F61A, W64A, W68A) **137** (0.54 mg from **138** (1.2 mg, 0.47 μmol), 46%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 15.0 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 10 to 20% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1011.1, found 1011.0.

CXCL14 (52-72, F61A, W64A, W68A) **138** (5.6 mg from 50 mg resin, 44%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 14.1 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 8 to 18% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 843.1, found 843.0.

The dimer of CXCL14 (52-72, F61A, Y65A, W68A) 139 from 140

Dimer of CXCL14 (52-72, F61A, Y65A, W68A) **139** (0.48 mg from **140** (1.0 mg, 0.41 μmol), 47%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 16.8 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 12 to 22% over 30 min. MS

(ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1020.3, found 1020.2.

CXCL14 (52-72, F61A, Y65A, W68A) **140** (3.6 mg from 50 mg resin, 26%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 15.5 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 13 to 19% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 850.8, found 850.6.

The dimer of CXCL14 (52-72, F61A, W64A, Y65A, W68A) 141 from 142

Dimer of CXCL14 (52-72, F61A, W64A, Y65A, W68A) **141**: Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 14.4 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 8 to 18% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 974.3, found 974.1.

CXCL14 (52-72, F61A, W64A, Y65A, W68A) **142** (4.9 mg from 50 mg resin, 40%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 13.3 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 7 to 17% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 812.4, found 812.3.

Synthesis of the disulfide dimers of CXCL14 (52-72) with mutation of its *b*- or *f*-position to alanine

The requisite monomers were prepared using the Fmoc SPPS with the use of Peptide Synthesizer CS336 mentioned above (*b* to Ala) or standard Fmoc SPPS in a fashion similar to that employed for C-terminal Fr **3** (*f* to Ala), respectively. The mutated deletion dimers were synthesized using the method almost identical to that employed for N2C **37** with corresponding monomer peptides.

The dimer of CXCL14 (52-72, *b* to Ala) 143 from 144

Dimer of CXCL14 (52-72, *b* to Ala) **143** (0.71 mg from **144** (1.4 mg, 0.51 μmol), 51%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 21.4 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1082.7, found 1082.5.

CXCL14 (52-72, *b* to Ala) **144** (5.2 mg from 50 mg resin, 39%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 20.0 min. Preparative HPLC condition: linear gradient of solvent B in

solvent A, 18 to 28% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 902.8, found 902.6.

The dimer of CXCL14 (52-72, *f* to Ala) 145 from 146

Dimer of CXCL14 (52-72, *f* to Ala) **145** (0.18 mg from **146** (1.4 mg, 0.52 μmol), 13%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 24.5 min. Semi-preparative HPLC condition: linear gradient of solvent B in solvent A, 20 to 30% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 5H]^{5+}$ 1067.2, found 1067.0.

CXCL14 (52-72, *f* to Ala) **146** (5.5 mg from 50 mg resin, 39%): Analytical HPLC condition, linear gradient of solvent B in solvent A, 5 to 45% over 30 min, retention time = 23.2 min. Preparative HPLC condition: linear gradient of solvent B in solvent A, 19 to 29% over 30 min. MS (ESI-TOF) *m/z* calcd for $[M + 3H]^{3+}$ 889.8, found 889.5.

Binding assays with ^{125}I-labeled CXCL14

CXCL14 was labeled with ^{125}I by Bolton-Hunter reagent. Briefly, recombinant human CXCL14 (5 μg) was suspended in 50 μL of 0.1 M borate buffer (pH 8.2) containing 0.01% Tween 20. The mixture was reacted with Bolton-Hunter reagent [^{125}I] (9.25 MBq) and incubated on ice for 2 h. The reaction mixture was applied to a D-Salt polyacrylamide desalting column with a 1.8 K molecular weight cut off and highly radioactive fractions (~ 2 mL) were saved as ^{125}I-labeled CXCL14.

Binding assays were carried out according to the previously described method.[15] In brief, 5×10^5 cells were suspended in 50 μL RPMI1640 containing 0.1% fatty acid-free BSA and 20 mM HEPES (pH 7.5) and incubated on ice for 2 h with 50 μL 10 nM ^{125}I-labeled CXCL14 in the presence or absence of 100 nM competitor. Cells were centrifuged at 7,500 × *g* on a 150 μL oil cushion in a 400 μL polyethylene tube. Radioactivity in the tip fragment was measured using a WIZARD2 gamma counter.

References

1. (a) Hromas, R.; Broxmeyer, H. E.; Kim, C.; Nakshatri, H.; Christopherson, K., II.; Azam, M.; Hou, Y.-H. *Biochem. Biophys. Res. Commun.* **1999**, *255*, 703-706. (b) Frederick, M. J.; Henderson, Y.; Xu, X.; Deavers, M. T.; Sahin, A. A.; Wu, H.; Lewis, D. E.; El-Naggar, A. K.; Clayman, G. L.; *Am. J. Pathol.* **2000**, *156*, 1937-1950. (c) Starnes, T.; Rasila, K. K.; Robertson, M. J.; Brahmi, Z.; Dahl, R.; Christopherson, K.; Hromas, R. *Exp. Hematol.* **2006**, *34*, 1101-1105.
2. Shurin, G. V.; Ferris, R.; Tourkova, I. L.; Perez, L.; Lokshin, A.; Balkir, L.; Collins, B.; Chatta, G. S.; Shurin, M. R. *J. Immunol.* **2005**, *174*, 5490-5498.
3. (a) Shellenberger, T. D.; Wang, M.; Gujrati, M.; Jayakumar, A.; Strieter, R. M.; Burdick, M. D.; Ioannides, C. G.; Efferson, C. L.; El-Naggar, A. K.; Roberts, D.; Clayman, G. L.; Frederick, M. J. *Cancer Res.* **2004**, *64*, 8262-8270. (b) Schwarze, S. R.; Luo, J.; Isaacs, W. B.; Jarrard, D. F. *The Prostate* **2005**, *64*, 67-74. (c) Ozawa, S.; Kato, Y.; Komori, R.; Maehata, Y.; Kubota, E.; Hata, R. *Biochem. Biophys. Res. Commun.* **2006**, *348*, 406-412. (d) Ozawa, S.; Kato, Y.; Ito, S.; Komori, R.; Shiiki, N.; Tsukinoki, K.; Ozono, S.; Maehata, Y.; Taguchi, T.; Imagawa-Ishiguro, Y.; Tsukuda, M.; Kubota, E.; Hata, R. *Cancer Science* **2009**, *100*, 2202-2209. (e) Tessema, M.; Klinge, D. M.; Yingling, C. M.; Do, K.; Van Neste, L.; Belinsky, S. A. *Oncogene* **2010**, *29*, 5159-5170.
4. (a) Nara, N.; Nakayama, Y.; Okamoto, S.; Tamura, H.; Kiyono, M.; Muraoka, M.; Tanaka, K.; Taya, C.; Shitara, H.; Ishii, R.; Yonekawa, H.; Minokoshi, Y.; Hara, T. *J. Biol. Chem.* **2007**, *282*, 30794-30803. (b) Hara, T.; Tanegashima, K. *J. Biochem.* **2012**, *151*, 469-476.
5. Maerki, C.; Meuter, S.; Liebi, M.; Muhlemann, K.; Frederick, M. J.; Yawalkar, N.; Moser, B.; Wolf, M. *J. Immunol.* **2009**, *182*, 507-514.
6. Tanegashima, K.; Suzuki, K.; Nakayama, Y.; Tsuji, K.; Shigenaga, A.; Otaka, A.; Hara, T. *FEBS Lett.* **2013**, *587*, 1731-1735.
7. Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776-778.
8. (a) Pemtelute, B. L.; Gates, Z. P.; Dashnau, J. L.; Vanderkooi, J. M.; Kent, S. B. H. *J. Am. Chem. Soc.* **2008**, *130*, 9702-9707. (b) Durek, T.; Torbeev, V. Y.; Kent, S. B. H. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 4846-4851. (c) Johnson, E. C. B.; Durek, T.; Kent, S. B. H. *Angew. Chem. Int. Ed.* **2006**, *45*, 3283-3287. (d) Gang, D.; Kent, S. B. H. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 5014-5019. (e) Ueda, S.; Fujita, M.;

Tamamura, H.; Fujii, N.; Otaka, A. *ChemBioChem* **2005**, *6*, 1983-1986. (f) Bang, D.; Kent, S. B. H. *Angew. Chem. Int. Ed.* **2004**, *43*, 2534-2538. (g) Bang, D.; Chopra, N.; Kent, S. B. H. *J. Am. Chem. Soc.* **2004**, *126*, 1377-1383. (h) Brik, A.; Keinan, E.; Dawson, P. E. *J. Org. Chem.* **2000**, *65*, 3829-3835. (i) Hackeng, T. M.; Griffin, J. H.; Dawson, P. E. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 10068-10073.

9. Tsuji, K.; Shigenaga, A.; Sumikawa, Y.; Tanegashima, K.; Sato, K.; Aihara, K.; Hara, T.; Otaka, A. *Bioorg. Med. Chem.* **2011**, *19*, 4014-4020.
10. Clark-Lewis, I.; Vo, L.; Owen, P.; Anderson, J. *Methods Enzymol.* **1997**, *287*, 233-250.
11. (a) Bruick, R. K.; Dawson, P. E.; Kent, S. B. H.; Usman, N.; Joyce, G. F. *Chemistry & Biology* **1996**, *3*, 49-56. (b) Zhang, L.; Tam, J. P. *Tetrahedron Lett.* **1997**, *38*, 3-6. (c) Camarero, J. A.; Muir, T. W. *Chem. Commun.* **1997**, 1369-1370. (d) Canne, L. E.; Botti, P.; Simon, R. J.; Chen, Y.; Dennis, E. A.; Kent, S. B. H. *J. Am. Chem. Soc.* **1999**, *121*, 8720-8727.
12. Shigenaga, A.; Sumikawa, Y.; Tsuda, S.; Sato, K.; Otaka, A. *Tetrahedron* **2010**, *66*, 3290-3296.
13. Tsuda, S.; Shigenaga, A.; Bando, K.; Otaka. A. *Org. Lett.* **2009**, *11*, 823-826.
14. Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70-77.
15. Tanegashima, K.; Tsuji, K.; Suzuki, K.; Shigenaga, A.; Otaka, A.; Hara, A. *FEBS Lett.* **2013**, *587*, 3770-3775.
16. Sato, K.; Shigenaga, A.; Tsuji, K.; Tsuda, S.; Sumikawa, Y.; Sakamoto, K.; Otaka, A. *ChemBioChem* **2011**, *12*, 1840-1844.
17. Sakamoto, K.; Sato, K.; Shigenaga, A.; Tsuji, K.; Tsuda, S.; Hibino, H.; Nishiuchi, Y.; Otaka, A. *J. Org. Chem.* **2012**, *77*, 6948-6958.
18. (a) Fujii, N.; Watanabe, T.; Otaka, A.; Bessho, K.; Yamamoto, I.; Konishi, J.; Yajima, H. *Chem. Pharm. Bull.* **1987**, *35*, 4769-4776. (b) Huang, H.; Rabenstein, D. L. *J. Peptide Res.* **1999**, *53*, 548-553.
19. Bouillon, C.; Paolantoni, D.; Rote, J. C.; Bessin, Y.; Peterson, L. W.; Dumy, P.; Ulrich, S. *Chem. Eur. J.* **2014**, *20*, 14705-14714.
20. Tanegashima, K.; Suzuki, K.; Nakayama, Y.; Hara, T. *Exp. Cell Res.* **2010**, *316*, 1263-1270.
21. (a) Clark-Lewis, I.; Schumacher, C.; Baggiolini, M.; Moser, B. *J. Biol. Chem.* **1991**, *266*, 23128-23134. (b) Crump, M. P.; Gong, J.-H.; Loetsher, P.; Rajarathnam, K.; Amara, A.; Arenzana-Seisdedos, F.; Virelizier, J.-L.; Baggiolini, M.; Sykes, B. D.; Clark-Lewis, I. *EMBO J.* **1997**, *16*, 6996-7007. (c) Loetscher, P.; Gong, J.-H.; Dewald, B.; Baggiolini, M.; Clark-Lewis, I. *J. Biol. Chem.* **1998**, *273*, 22279-22283.

(c) Qin, L.; Kufareva, I.; Holden, L. G.; Wang, C.; Zheng, Y.; Zhao, C.; Fenalti, G.; Wu, H.; Han, G. W.; Cherezov, V.; Abagyan, R.; Stevens, R. C.; Handel, T. M. *Science* **2015**, *347*, 1117-1122.

22. Huising, M. O.; van der Meulen, T.; Flik, G.; Verburg-van Kemenade, B. M. L. *Eur. J. Biochem.* **2004**, *271*, 4094-4106.
23. (a) Rajarathnam, K.; Kay, C. M.; Clark-Lewis, I.; Sykes, B. D. *Methods Enzymol.* **1997**, *287*, 89-105. (b) Veldkamp, C. T.; Peterson, F. C.; Pelzek, A. J.; Volkman, B. F. *Protein Sci.* **2005**, *14*, 1071-1081. (c) Veldkamp, C. T.; Ziarek, J. W.; Su, J.; Basnet, H.; Lennertz, R.; Weiner, J. J.; Peterson, F. C.; Baker, J. E.; Volkman, B. F. *Protein Sci.* **2009**, *18*, 1359-1369. (d) Das, S. T.; Rajagopalan, L.; Guerrero-Plata, A.; Sai, J.; Richmond, A.; Garofalo, R. P.; Rajarathnam, K. *PLoS ONE* **2010**, *5*, e11754. (e) Gandhi, N. S.; Mancera, R. L. *J. Chem. Inf. Model.* **2011**, *51*, 335-358.
24. Otaka, A.; Nakamura, M.; Nameki, D.; Kodama, E.; Uchiyama, S.; Nakamura, S.; Nakano, H.; Tamamura, H.; Kobayashi, Y.; Matsuoka, M.; Fujii, N. *Angew. Chem. Int. Ed.* **2002**, *41*, 2937-2940.
25. (a) Marqusee, S.; Baldwin, R. L. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 8898-8902. (b) Burkhard, M.; Meier, A.; Lustig, A., *Protein Sci.* **2000**, *9*, 2294-2301.
26. (a) Canne, L. E.; Ferre- D'Amare, A. R.; Burley, S. K.; Kent, S. B. H. *J. Am. Chem. Soc.* **1995**, *117*, 2998-3007. (b) Dirksen, A.; Dawson, P. E. *Bioconjugate Chem.* **2008**, *19*, 2543-2548. (c) Tiefenbrunn, T. K.; Dawson, P. E. *PeptideScience* **2010**, *94*, 95-106.
27. Rugglesa, E. L.; Dekerb, P. B.; Hondal, R. J. *Tetrahedron* **2009**, *65*, 1257-1267.
28. (a) Schnölzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Pept. Protein Res.* **1992**, *40*, 180-193. (b) Alewood, P.; Alewood, D.; Miranda, L.; Love, S.; Meutermans, W.; Wilson, D. *Methods Enzymol.* **1997**, *289*, 14-29.
29. El-Faham, A.; Funosas, R. S.; Prohens, R.; Albericio, F. *Chem. Eur. J.* **2009**, *15*, 9404-9416.
30. Alfonso, E. P.; Martin, F. G.; Bayo, N.; Cruz, L. J.; Roca, M. P.; Samitier, J.; Errachid, A.; Albericio, F. *Tetrahedron* **2006**, *62*, 6876-6881.

Acknowledgement

I express my deepest gratitude and sincere, wholehearted appreciation to Prof. Akira Otaka (Department of Bioorganic Synthetic Chemistry, Institute of Biomedical Sciences and Graduate School of Pharmaceutical Sciences, Tokushima University) for his kind guidance, constructive support and hearty encouragement provided throughout this study. In addition, I feel honored to have been given the opportunity of being the one to study organic and peptide chemistry from the beginning.

I wish to express my sincere and heartfelt gratitude to Prof. Hisao Nemoto, Prof. Akira Shigenaga, Prof. Tsubasa Inokuma (Department of Bioorganic Synthetic Chemistry, Institute of Biomedical Sciences and Graduate School of Pharmaceutical Sciences, Tokushima University) for their kind support, constructive discussions, constant encouragement and their careful perusing of my original manuscript.

I also wish to express my gratitude to Prof. Takahiko Hara and Prof. Kosuke Tanegashima (Stem Cell Project, Tokyo Metropolitan Institute of Medical Science) for their generous encouragement and constructive discussion.

I am grateful to Mr. Yoshitake Sumikawa, Dr. Kohei Sato, Mr. Keisuke Aihara, Mr. Ken Sakamoto, Mr. Yusuke Tsuda and all other colleagues in the Department of Bioorganic Synthetic Chemistry, Graduate School of Pharmaceutical Sciences, Tokushima University for their valuable comments and for their assistance and cooperation in various experiments.

I would like to thank the Nakato Scholarship Foundation and Research Fellowship from the Japan Society for the Promotion of Science (JSPS) for financial support, and Mr. Hitoshi Iida and Mr. Syuji Kitaike (Tokushima University) for scientific analysis.

Finally, I am most grateful to my parents, Yutaka and Michiyo Tsuji, for their constantsupport— emotional, moral and of course financial — thoughout my time at the Academy. I am also grateful to my sister, Maiko, for her constant encouragement throughout my time at the Academy.

List of publications

This study was published in the following papers.

1. Application of N-C- or C-N-directed sequential native chemical ligation to the preparation of CXCL14 analogs and their biological evaluation
   Kohei Tsuji, Akira Shigenaga, Yoshitake Sumikawa, Kosuke Tanegashima, Kohei Sato, Keisuke Aihara, Takahiko Hara, and Akira Otaka
   Bioorg. Med. Chem. **2011**, *19*, 4014-4020.

2. Dimeric peptides of the C-terminal region of CXCL14 function as CXCL12 inhibitors
   Kosuke Tanegashima[#], Kohei Tsuji[#], Kenji Suzuki, Akira Shigenaga, Akira Otaka, and Takahiko Hara ([#]Equally contributed)
   FEBS Lett. **2013**, *587*, 3770-3775.

3. Efficient one-pot synthesis of CXCL14 and its derivative using an *N*-sulfanylethylanilide peptide as a peptide thioester equivalent and their biological evaluation
   Kohei Tsuji, Kosuke Tanegashima, Kohei Sato, Ken Sakamoto, Akira Shigenaga, Tsubasa Inokuma, Takahiko Hara, Akira Otaka
   Bioorg. Med. Chem. in press.