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RESEARCH ARTICLE

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Application of 2,2′-dipyridyl disulfide-mediated thiazolidine ring-opening reaction to glycoprotein synthesis: Total chemical synthesis of evasin-3

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Thiazolidine ring-opening reaction is one of the key steps in protein chemical synthesis via sequential native chemical ligation strategy. We recently developed a novel thiazolidine ring-opening reaction with 2,2′-dipyridyl disulfide (DPDS). In order to investigate the applicability of this reaction to glycoprotein synthesis, we synthesized evasin-3, a cysteine-rich glycoprotein with chemokine-binding ability originally found in tick saliva. The sequence of evasin-3 was divided into three segments, and these segments were separately synthesized with the ordinary solid-phase peptide synthesis method. After the first ligation of middle and C-terminal segments, thiazolidine used as a protecting group of Cys residue at the N-terminus of the middle segment was converted to Cys with DPDS. In this thiazolidine ring-opening reaction, DPDS treatment did not affect the *N*-linked glycan moiety. After the second ligation with the N-terminal segment and the refolding reaction, evasin-3 could be obtained in good yield. The synthetic evasin-3 showed the binding ability specifically to CXCL chemokines. These results clearly indicate that this DPDS method is useful for glycoprotein synthesis.

KEYWORDS
2,2′-dipyridyl disulfide, evasin-3, native chemical ligation, thiazolidine

1 | INTRODUCTION

Protein chemical synthesis is an indispensable tool to promote biological and biochemical researches. Because the ordinary solid-phase peptide synthesis (SPPS) method developed by Merrifield is usually limited to the length of 40–50 amino acid residues,[1] ligation methods of peptides that are prepared with SPPS are widely used for protein chemical synthesis. In the native chemical ligation (NCL), one of the peptide ligation methods, a peptide α-thioester and an N-terminally cysteinyl peptide can be site-specifically condensed without any protecting group.[2] The NCL method provides an efficient means to generate proteins, and many proteins have been synthesized by this method.[3]

When three or more peptide segments are condensed through NCL strategy, an N-terminally cysteinyl peptide α-thioester should be used as a middle segment. To prevent the self-cyclization of the middle segment, the N-terminal Cys residue or C-terminal thioester functionality should be protected. Among the Cys protecting groups, thiazolidine-4-carboxilic acid (Thz) has been widely used as a Cys surrogate.[4] It is well known that the N-terminal Thz can be easily converted to Cys with the treatment of methoxyamine under weakly acidic conditions, and the newly generated N-terminal Cys can be afforded to the NCL with the N-terminal segment having α-thioester functionality.[4] However, the complete conversion of Thz to Cys with methoxyamine generally requires a reaction time over 8 h. To shorten the reaction time, water-soluble palladium(II) complex-mediated ring-opening method was developed. In this method, $[Pd(allyl)Cl]_2$ or $PdCl_2$ was used in the presence of 4-mercaptophenylacetic acid (MPAA) and tris(2-carboxyethyl)phosphine (TCEP), and the Thz could be completely converted to Cys within 15 min.[5] On the other hand, it is problematic that palladium(II) complexes also removed other Cys protecting groups such as acetamidomethyl (Acm) and

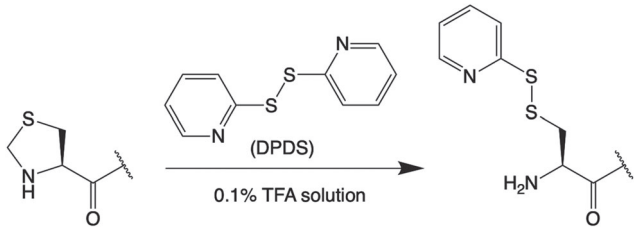

FIGURE 1 Ring-opening reaction of thiazolidine (Thz) derivative with 2,2′-dipyridyl disulfide (DPDS)

allyloxycarbonylaminomethyl (Allocam) groups.[6,7] More recently, copper-mediated Thz deprotection method has been reported,[8] whereas the compatibility of this method with the other Cys protecting groups still remains unclear.

In our previous study, we reported that 2,2′-dipyridyl disulfide (DPDS) was a good reagent for the Thz ring-opening reaction (Figure 1).[9] This reaction proceeds under weakly acidic conditions, and the reaction mechanism is presumed as follows: the sulfur atom of Thz is protonated, and the ring is opened; the generated sulfanyl group is immediately pyridylsulfenylated with DPDS, and then the

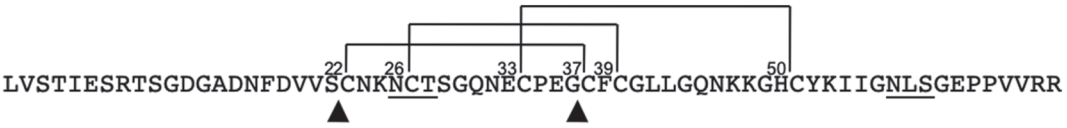

FIGURE 2 Primary structure of evasin-3. Solid lines and arrowheads indicate presumed disulfide bonds and the ligation site, respectively. Numbers represent the positions of Cys residues

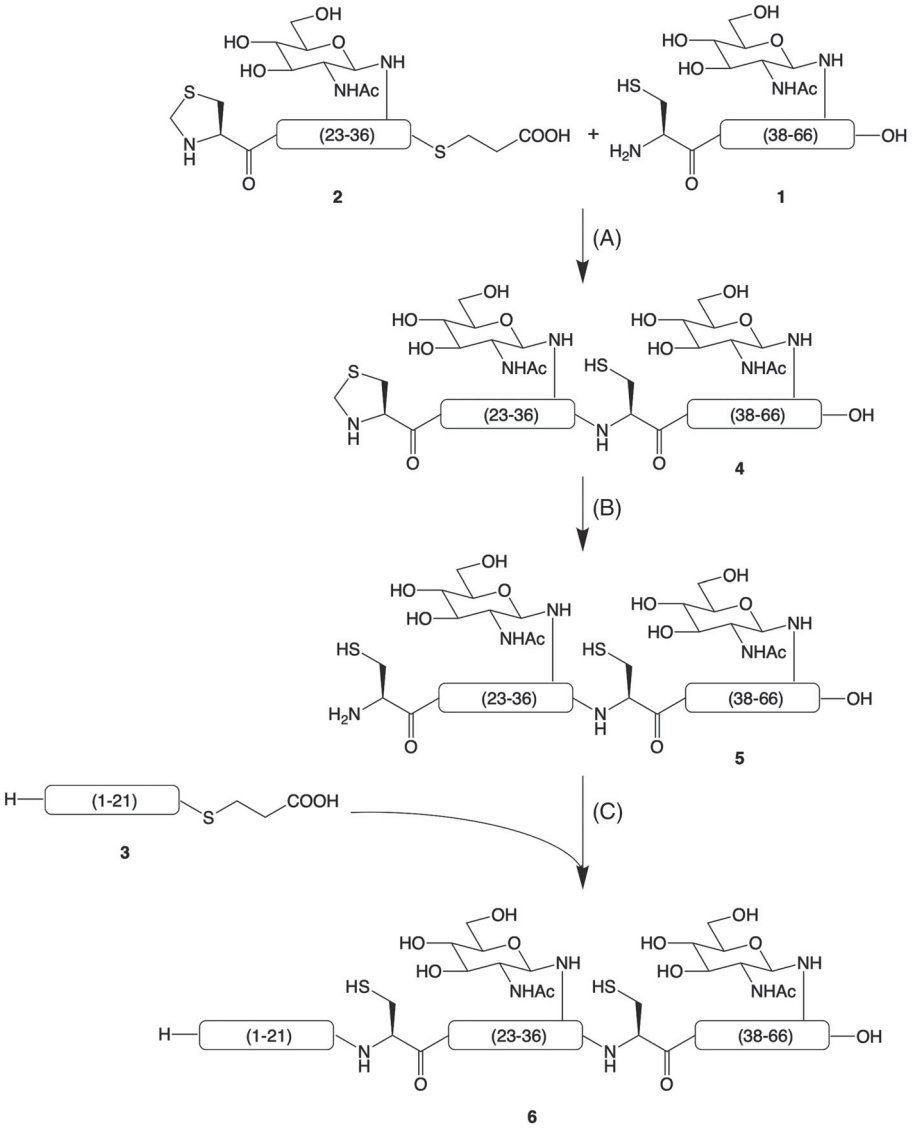

FIGURE 3 Synthetic procedure of linear evasin-3 (**6**). Reaction conditions: A, NCL buffer, room temperature, 2 h; B, (1) 2,2′-dipyridyl disulfide (DPDS), 50°C, 2 h; (2) TCEP; C, NCL buffer, room temperature for 7 h

imine structure is hydrolyzed.[9] It has been shown that this deprotection method does not affect the Cys protecting groups such as *p*-methoxybenzyl group and disulfide bond and is complete within 2 h. Thus, this method might be an alternative deprotection method for Thz for protein chemical synthesis. On the other hand, it still remains unclear whether this method can be applicable to the chemical synthesis of modified proteins such as glycoproteins.

Evasin-3 is a small glycoprotein found in tick saliva and consists of 66 amino acid residues and two *N*-linked glycan moieties (Figure 2).[10] Evasin-3 has six Cys residues at the positions of 22, 26, 33, 37, 39, and 50, forming three disulfide bonds connected between 22 and 37, 26 and 39, and 33 and 50.[11,12] Evasin-3 could bind specifically to vertebrate CXCL proteins, suppressing inflammation.[11] Chemical syntheses of a truncated evasin-3 (17–56) and of a fluorophore-labeled full-length evasin-3 have been reported,[12–14] although the evasin-3 having *N*-linked glycans has not yet been synthesized. In this paper, we show that the DPDS-mediated Thz deprotection method is applicable to glycoprotein synthesis through the total chemical synthesis of glycosylated evasin-3.

2 | RESULTS AND DISCUSSION

2.1 | Chemical synthesis of evasin-3 with the refolding method

At first, to examine the applicability of the DPDS method for the chemical synthesis of Cys-rich glycoprotein, we tried to synthesize evasin-3 without Cys protecting group. The synthetic procedure is shown in Figure 3. Evasin-3 sequence was divided into three segments (**1**–**3**) corresponding to 37–66, 22–36, and 1–21 of evasin-3, and these segments were prepared by the 9-fluorenylmethoxycarbonyl (Fmoc)-based SPPS method. To prepare the C-terminal thioester functionality, we used the *N*-alkyl-cysteine (NAC)-assisted method.[15] At the Cys[22] position, Thz was used as the Cys surrogate. Recent progress of chemical glycoprotein synthesis enables the chemoenzymatic transglycosylation reaction using *N*-acetylglucosaminylated asparagine [Asn(GlcNAc)] residue and sialylglycopeptide (SGP) as glycosyl acceptor and donor, respectively.[16] Therefore, GlcNAc-attached evasin-3 was synthesized as a prototype of glycoprotein, and the Asn(GlcNAc) derivative with acid-labile protecting groups developed in our previous study was used at the putative *N*-glycosylated Asn positions.[17]

Peptides **1** and **2** were condensed with the NCL method, giving evasin-3 (22–66) fragment (**4**) in 83% yield (Figure S1). To open the N-terminal Thz ring of peptide **4**, DPDS was treated in a 50% acetonitrile aqueous solution containing 0.1% trifluoroacetic acid (TFA) at 50°C. After 2 h, the reaction mixture was analyzed with reversed-phase (RP)-high-performance liquid chromatography (HPLC) and mass spectrum measurement. On the HPLC chromatogram, many peaks were observed (Figure 4). In the mass spectral analysis, all these peak materials gave molecular ion peaks corresponding to Thz ring-opened peptide having two or four pyridylsulfenyl groups, indicating that the

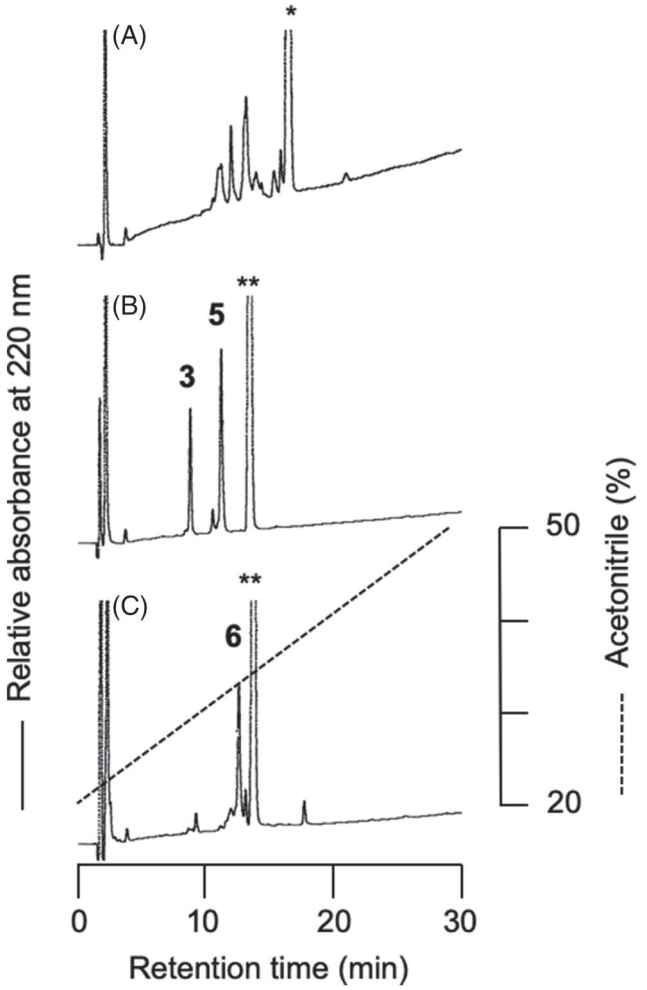

FIGURE 4 Reversed-phase HPLC chromatograms in the synthesis of linear evasin-3 (**6**). A, After DPDS treatment at 50°C for 2 h; B, initial reaction mixture of second NCL; C, the reaction mixture after second NCL (7 h). Elution conditions: column, Inertsil ODS-3 (4.6 mmϕ × 150 mm) at a flow rate of 1 ml/min. *, DPDS; **, MPAA

N-terminal Thz was completely converted to Cys, intrachain disulfide bond(s) were randomly connected in the evasin-3 (22–66) segment, and the remaining Cys residues were pyridylsulfenylated. This mixture was confirmed to be converted to the linear peptide **5** with TCEP treatment, and no significant side product was observed on the RP-HPLC chromatogram (Figure S2). Therefore, the mixture was directly used to the next NCL reaction without purification.

After lyophilization, an equimolar amount of peptide **3** was added to the mixture and dissolved in the NCL buffer. As expected, intrachain disulfide bonds were immediately reduced, and a single peak corresponding to the (22–66) segment (**5**) was observed on the RP-HPLC chromatogram. The peptide condensation reaction with NCL was almost complete within 7 h. During the reaction, no significant side reaction was observed, and the desired full-length linear evasin-3 (**6**) was obtained in 53% yield. Finally, the disulfide bonds were formed in a redox buffer containing reduced and oxidized forms of glutathione. After the reaction at 4°C for 24 h, the folded evasin-3

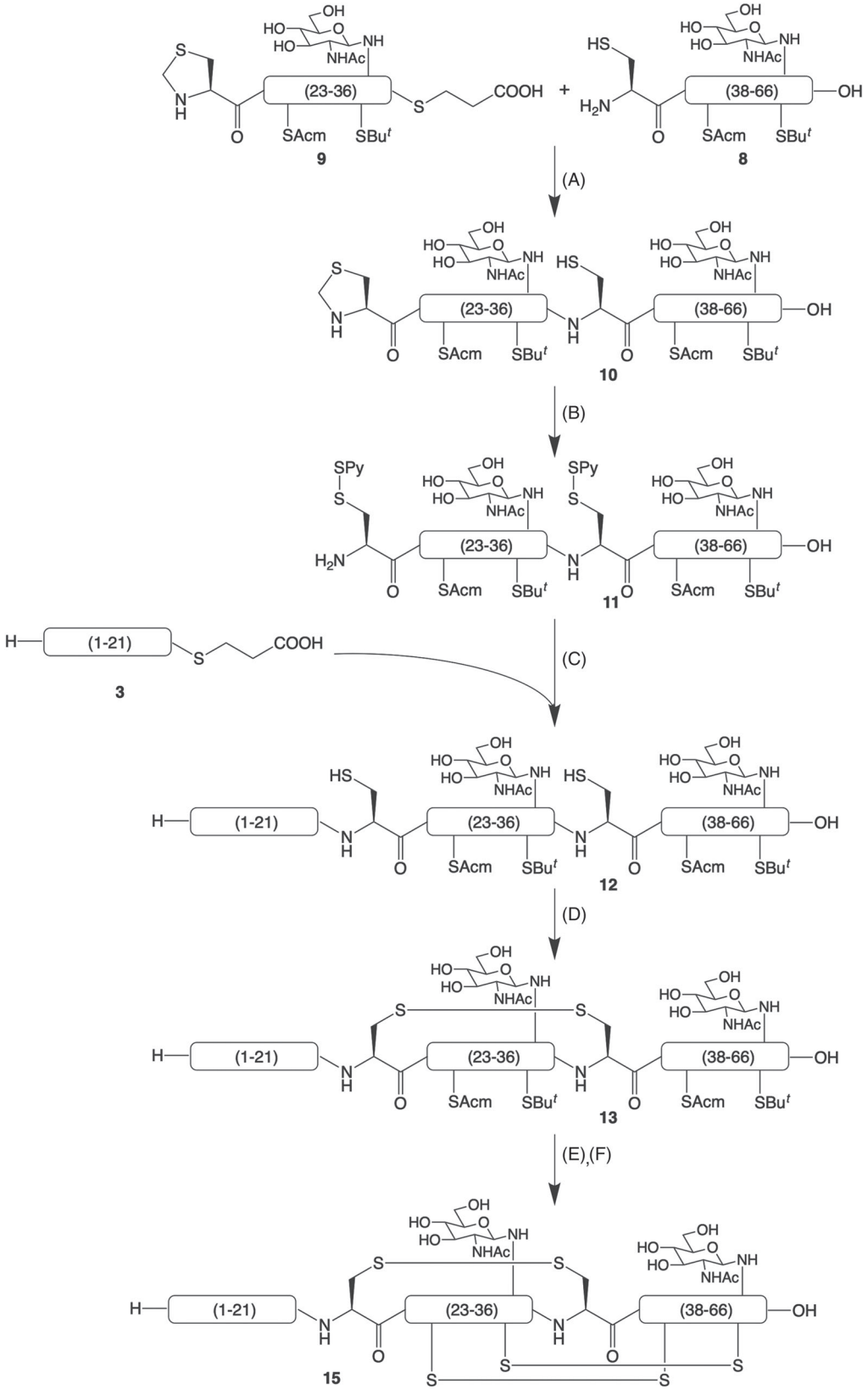

FIGURE 5 Synthetic procedure of evasin-3 (**15**) with regioselective disulfide formation reactions. Reaction conditions: A, NCL buffer, room temperature, 2 h; B, (1) 2,2′-dipyridyl disulfide (DPDS), 50°C, 2 h; (2) TCEP; C, NCL buffer, room temperature for 20 h; D, 10% DMSO/50 mM phosphate buffer (pH 7.0), room temperature, 24 h; E, I_2/HCl/CH_3OH/H_2O, room temperature, 1 h; F, 5% DMSO/TFA, room temperature, 30 min

(**7**) was obtained in 81% yield (Figure S3). Thus, the Thz ring-opening reaction with DPDS was shown to be applicable to the chemical synthesis of Cys-rich glycoprotein through three-segments sequential NCL method.

2.2 | Chemical synthesis of evasin-3 with regioselective disulfide formation reactions

In order to investigate whether our DPDS method could be applicable to the glycoprotein synthesis with the regioselective disulfide formation reactions, we tried to synthesize evasin-3 by the alternative synthetic procedure using Cys protecting groups. The synthetic procedure is shown in Figure 5. The C-terminal and middle segments (**8** and **9**) were synthesized by the Fmoc-SPPS. To achieve the regioselective formation of disulfide bonds, the Acm group was used at Cys^{26} and Cys^{39}, and Cys^{33} and Cys^{50} were protected with the *tert*-butyl (Bu^t) group.

Peptides **8** and **9** were condensed with the NCL method, giving evasin-3 (22–66) fragment (**10**) in 64% yield (Figure S4). To open the N-terminal Thz ring of peptide **10**, DPDS was treated in 50% acetonitrile aqueous solution containing 0.1% TFA at 50°C. Thz was almost completely converted to *S*-pyridylsulfenylated Cys residue within 2 h. During this reaction, the side chain of $Cys^{37,}$ which had no protecting group, was also *S*-pyridylsulfenylated, giving peptide **11**. Unfortunately, peptide **11** appeared as a shoulder of the peak corresponding to DPDS on the RP-HPLC chromatogram (Figure 5), which was confirmed by mass spectral analysis. At the same time, a small amount of

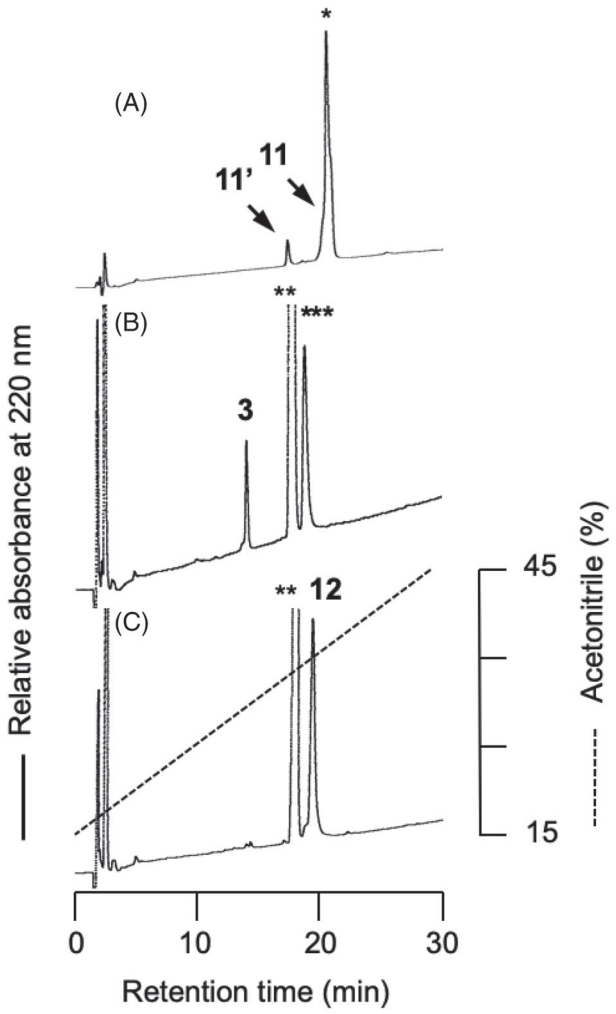

FIGURE 6 Reversed-phase HPLC chromatograms in the synthesis of linear evasin-3 (**12**). A, After DPDS treatment at 50°C for 2 h; B, initial reaction mixture of second NCL; C, the reaction mixture after second NCL (20 h). Elution conditions: column, Inertsil ODS-3 (4.6 mmϕ × 150 mm) at a flow rate of 1 ml/min. *, DPDS; **, MPAA; ***, evasin-3 (22–66) fragment without *S*-pyridylsulfenyl group

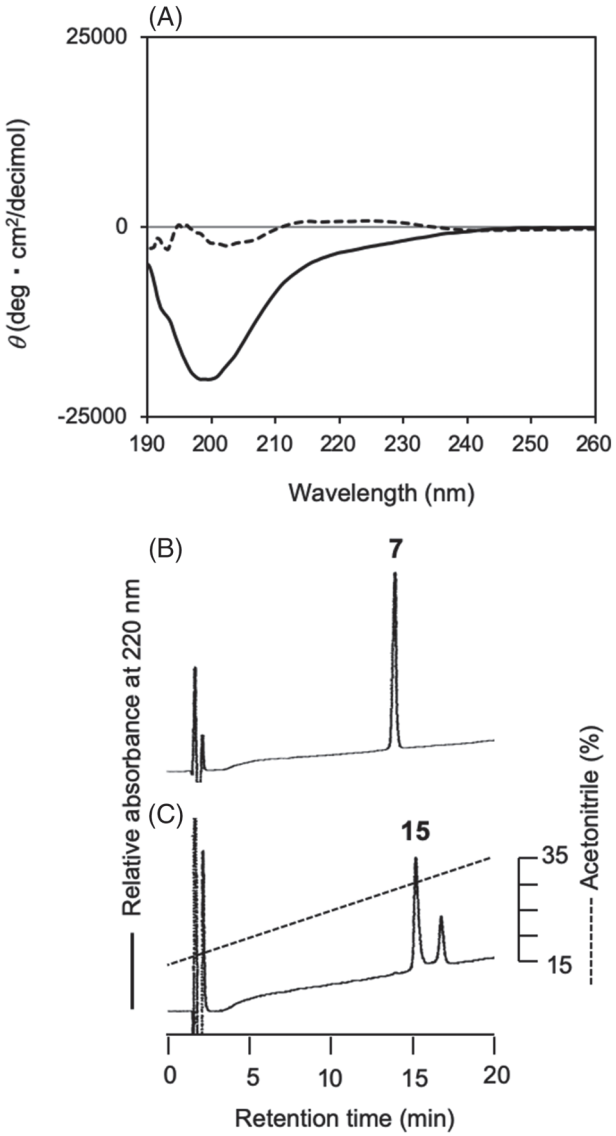

FIGURE 7 Comparison of two synthetic evasin-3 (**7** and **15**). A, Circular dichroism spectra of synthetic evasin-3 proteins. Dashed and solid lines indicate the spectra of refolded evasin-3 **7** and regioselectively disulfide-formed evasin-3 **15**, respectively. B, C, Reversed-phase HPLC elution profiles of the synthetic evasin-3. Elution conditions: column, Inertsil ODS-3 (4.6 mmϕ × 150 mm) at a flow rate of 1 ml/min

peptide **11**′ having an intrachain disulfide bond between Cys^{22}-Cys^{37} was observed on the RP-HPLC chromatogram. Different from the Pd-mediated deprotection method, which was known to remove Acm groups,[6] no deprotection of the other Cys protecting groups were observed during the Thz ring-opening reaction with DPDS, indicating that the DPDS method was compatible with Acm and Bu^t groups.

The mixture of peptides **11** and **11**′ was directly used to the second NCL reaction. An equimolar amount of peptide **3** was added to the mixture and dissolved in the NCL buffer. The *S*-pyridylsulfenyl groups were immediately removed by TCEP, and a single peak corresponding to the (22–66) segment was observed on the RP-HPLC chromatogram (Figure 6). The second NCL reaction was almost complete within 20 h, and the desired linear evasin-3 (**12**) was obtained in 77% yield.

The three disulfide bonds were regioselectively formed. The first disulfide bond between Cys^{22} and Cys^{37} was formed by treating dimethyl sulfoxide (DMSO) in an aqueous buffer, giving peptide **13** in 75% yield (Figure S5). Next, Acm groups at Cys^{26} and Cys^{39} were oxidatively removed by iodine oxidation under acidic conditions, forming the second disulfide bond, and the desired peptide **14** was obtained in 80% yield. The final disulfide bond was formed with the treatment of 5% DMSO/TFA.[18] Unexpectedly, on the RP-HPLC chromatogram of crude material, two peaks showing a molecular ion peak corresponding to the desired product in the mass spectral analysis were found. Only the major peak with shorter retention time was collected, and the evasin-3 having three disulfide bonds (**15**) was obtained in 32% yield.

In contrast to the refolded evasin-3 (**7**), which gave a single peak on RP-HPLC, the purified peptide **15** gave two peaks with different retention times from that of **7** (Figure 7), demonstrating that purified peptide **15** consisted of two tautomeric conformers. The truncated evasin-3 composed of 17–56 region has been synthesized with the oxidative folding method,[12] and the disulfide bond arrangement in this peptide was determined to be the same as that in the full-length evasin-3.[14] These findings supported that the refolded evasin-3 protein **7** obtained in this study had disulfide bonds at the desired positions and may have different conformation from that of peptide **15**.

2.3 | Conformational and functional analyses of evasin-3

To check the conformation of peptides **7** and **15**, the circular dichroism (CD) spectra of these peptides were measured. As shown in Figure 7, these peptides showed the spectral patterns completely different from each other. Using the CD data, the secondary structure contents were estimated with BeStSel.[19,20] The refolded evasin-3 (**7**) was estimated to be rich in β-structure (38.7%) but to have no α-helix.

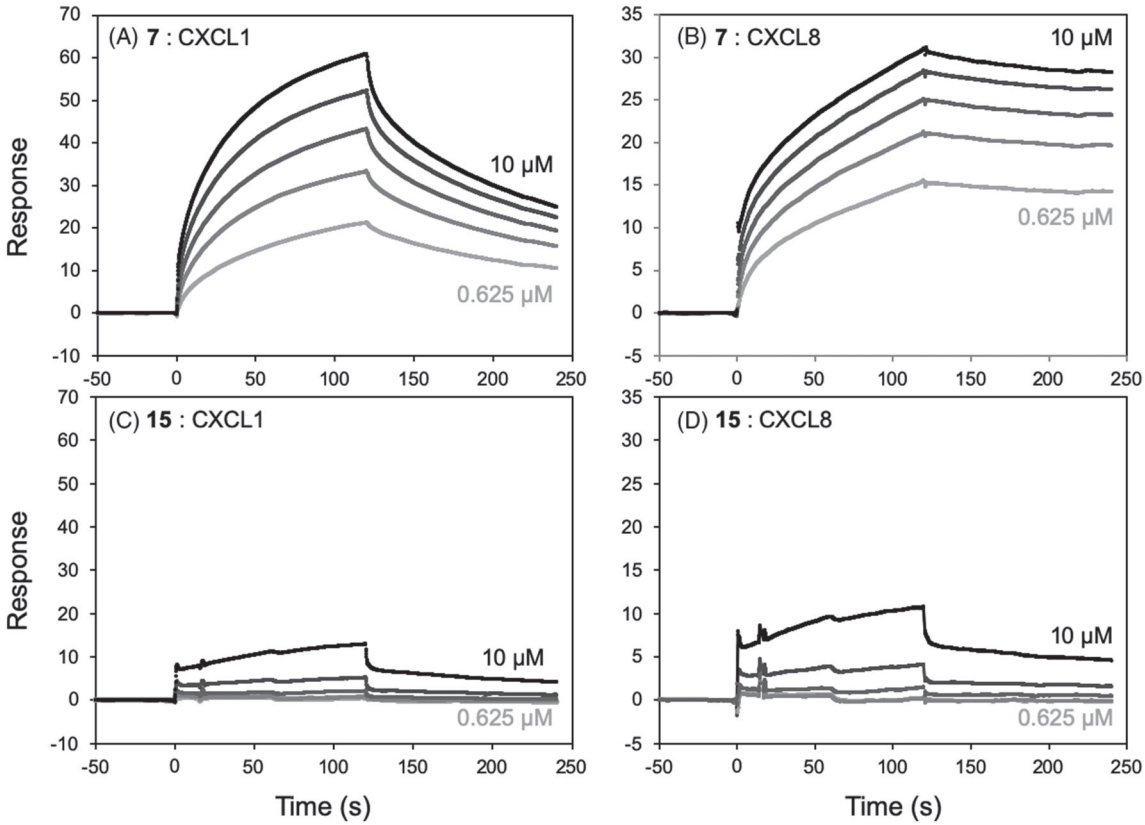

FIGURE 8 Surface plasmon resonance biosensor analysis of evasin-3 proteins (**7** and **15**) upon binding to immobilized human CXCL chemokines. A, **7** and CXCL-1; B, **7** and CXCL-8; C, **15** and CXCL-1; D, **15** and CXCL-8. Concentration series with twofold dilution, raging 10 to 0.625 μM, were examined

On the other hand, the estimated secondary structure contents of synthetic evasin-3 with regioselective disulfide bond formation reactions (**15**) were 8.4% α-helix and 26.0% β-structure. These results indicated that the two synthetic evasin-3 (**7** and **15**) have tertiary structures different from each other. The X-ray crystal structure of evasin-3 revealed that the secondary structure composition of evasin-3 is 6.1% α-helix and 36.4% β-structure.[14] These values do not coincide well with the estimated ones of peptides **7** and **15**, although the β-structure content peptide **7** (38.7%) was close to that of the native one.

Evasin-3 is known to have a binding ability specifically to CXCL chemokine.[10] In order to evaluate the function of two synthetic evasin-3 (**7** and **15**), the binding abilities to CXCL-1 and CXCL-8 were examined with the surface plasmon resonance (SPR) biosensor analysis. As a result, the refolded peptide **7** could bind to CXCL-1 and CXCL-8 with K_D values of 7.76×10^{-7} M and 8.76×10^{-9} M, respectively (Figure 8). These values were larger than those shown in the previous report.[10] The difference in these values probably resulted from the difference in the method of SPR experiments. In contrast, peptide **15** did not bind to CXCL chemokines. These results indicated that the synthetic evasin-3 with refolding reaction (**7**) had the native conformation and that the synthetic evasin-3 with regioselective disulfide bond formation reactions (**15**) did not fold properly. Evasin-3 might not be able to be synthesized by the regioselective disulfide bond formations, at least in this formation order, but could be prepared with the refolding method in good yield.

3 | CONCLUSION

To investigate the applicability of DPDS-mediated thiazolidine ring-opening reaction to glycoprotein synthesis, we synthesized evasin-3. DPDS treatment did not affect the *N*-linked glycan moiety, and evasin-3 could be obtained by the sequential NCL reaction followed by a refolding reaction in a redox buffer. The synthetic evasin-3 possessed a binding ability to CXCL chemokines. On the other hand, we also tried to synthesize evasin-3 with regioselective disulfide bond formation reactions. Cys protecting groups such as Acm and *tert*-butyl groups were retained in thiazolidine ring-opening reaction with DPDS. Unfortunately, evasin-3 might be unsuitable for the synthetic model on the sequential disulfide bond formation reactions and the synthetic evasin-3 did not have the proper conformation, but the applicability of the DPDS method to sequential disulfide bond formation could be shown.

4 | EXPERIMENTAL PROCEDURE

4.1 | General

Fmoc-Asn[GlcNAc(benzylidene, Boc)]-OH,[17] Fmoc-Ser(Bu^t)-(Et)Cys(Trt)-OH,[21] and Fmoc-Gly-(Et)Cys(Trt)-OH[21] were prepared as described previously. MALDI-TOF mass spectra were recorded using an Autoflex spectrometer (Brucker, Germany) or a JMS-S3000 spectrometer (JEOL, Tokyo, Japan). The synthetic peptides were quantified with amino acid analysis, which was performed using a LaChrom amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis with a 6 M HCl solution at 150°C for 2 h in a vacuum-sealed tube. CD spectra were measured with a Jasco J-820 spectropolarimeter (JASCO, Tokyo, Japan) at room temperature with a 2-mm path length cell using a phosphate buffer (50 mM, pH 7.0) as a solvent.

4.2 | Evasin-3 (37–66) 1

Fmoc-Arg(Pbf)-Wang resin (0.28 mmol/g, 0.179 g, 0.05 mmol) was swelled in 1-methyl-2-pyrrolidinone (NMP) for 30 min and was treated with 20% piperidine/NMP for 5 and 15 min. After washing with NMP, Fmoc-Arg(Pbf)-OBt, which was prepared by mixing Fmoc-Arg (Pbf)-OH (0.20 mmol), 1 M *N*,*N*′-dicyclohexylcarbodiimide (DCC)/NMP (0.3 ml), and 1 M 1-hydroxybenzotriazole (HOBt)/NMP (0.3 ml) at room temperature for 30 min, was added and the reaction mixture was mixed with a vortex at 50°C for 1 h. The resin was washed with NMP and 50% dichloromethane/methanol, treated with 10% acetic anhydride (Ac_2O)/5% *N*,*N*-diisopropylethylamine (DIEA)/NMP for 5 min, and washed with NMP. The peptide chain was elongated in essentially the same manner as described above, and H-Cys(Trt)-Phe-Cys(Trt)-Gly-Leu-Leu-Gly-Gln(Trt)-Asn(Trt)-Lys(Boc)-Lys(Boc)-Gly-His(Trt)-Cys(Trt)-Tyr(Bu^t)-Lys(Boc)-Ile-Ile-Gly-Asn[GlcNAc(benzylidene, Boc)]-Leu-Ser(Bu^t)-Gly-Glu(OBu^t)-Pro-Pro-Val-Val-Arg(Pbf)-Arg(Pbf)-resin (0.449 g) was obtained. A part of the resin (21 mg) was treated with a TFA cocktail (TFA/H_2O/phenol/thioanisole/triisopropylsilane, 82.5/5/5/5/2.5, 0.3 ml) at room temperature for 2 h, and then the peptide was precipitated with diethyl ether. After washing twice with ether, the precipitate was dried in vacuo. The crude peptide was purified by RP-HPLC on an Inertsil ODS-3 column (GL Science, Tokyo, Japan) with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **1** (804 nmol, 34% yield). MALDI-TOF mass, found: *m/z* 3491.1, calcd: 3492.1 for $(M + H)^+$. Amino acid analysis: $Asp_{2.00}Ser_{0.81}Glu_{2.04}Pro_{1.88}Gly_5Val_{1.80}Ile_{1.58}Leu_{2.99}Tyr_{1.01}Phe_{1.04}Lys_{3.00}His_{1.00}Arg_{2.04}$.

4.3 | Thz^{22}-Evasin-3 (22–36)-thioester 2

Starting from Fmoc-Rink Amide MBHA resin (0.39 mmol/g, 0.128 g, 0.05 mmol), Fmoc-Arg(Pbf)-OH was introduced to the resin by DCC-HOBt method as described above. Another Fmoc-Arg(Pbf)-OH was introduced to the resin, and Fmoc-Arg(Pbf)-Arg(Pbf)-NH-resin was obtained. This rein was treated with 20% piperidine/NMP for 5 and 15 min. After washing with dichloromethane, Fmoc-Gly-(Et)Cys(Trt)-OBt, which was prepared by mixing Fmoc-Gly-(Et)Cys(Trt)-OH (0.10 mmol), *N*,*N*′-diisopropylcarbodiimide (DIC, 23 μl, 0.15 mmol), HOBt (20 mg, 0.15 mmol), and dichloromethane (0.4 ml) at room temperature for 30 min, was added, and the reaction mixture was mixed with a vortex at room temperature for 30 min. Another DIC (23 μl,

0.15 mmol) was added, and the reaction mixture was mixed with a vortex at room temperature overnight, giving Fmoc-Gly-(Et)Cys(Trt)-Arg(Pbf)-Arg(Pbf)-NH-resin. The peptide chain was further elongated with the DCC-HOBt method described above, and Boc-Thz-Asn(Trt)-Lys(Boc)-Asn[GlcNAc(benzylidene, Boc)]-Cys(Trt)-Thr(Bu^t)-Ser(Bu^t)-Gly-Gln(Trt)-Asn(Trt)-Glu(OBu^t)-Cys(Trt)-Pro-Glu(OBu^t)-Gly-(Et)Cys(Trt)-Arg(Pbf)-Arg(Pbf)-NH-resin (0.319 g) was obtained. A part of the resin (20 mg) was treated with a TFA cocktail (0.3 ml) at room temperature for 2 h, and then the peptide was precipitated with diethyl ether. After washing twice with ether, the precipitate was dried in vacuo. The crude peptide was dissolved in 50% acetonitrile/5% acetic acid aqueous solution (950 μl), and 3-mercaptopropionic acid (MPA, 50 μl) was added. The mixture was gently mixed at room temperature for 3 days and purified by RP-HPLC on an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **2** (309 nmol, 9.7% yield). MALDI-TOF mass, found: *m/z* 1886.8, calcd: 1886.7 for $(M + H)^+$. Amino acid analysis: $Asp_{2.53}Thr_{0.81}Ser_{0.83}Glu_{2.68}Pro_{0.93}Gly_2Lys_{0.91}$.

4.4 | Evasin-3 (1–21)-thioester 3

Starting from Fmoc-Rink Amide MBHA resin (0.39 mmol/g, 0.128 g, 0.05 mmol), Fmoc-Ser(Bu^t)-(Et)Cys(Trt)-Arg(Pbf)-Arg(Pbf)-NH-resin was obtained using Fmoc-Ser(Bu^t)-(Et)Cys(Trt)-OH by essentially the same manner as described above. The peptide chain was further elongated with the DCC-HOBt method described above, and H-Leu-Val-Ser(Bu^t)-Thr(Bu^t)-Ile-Glu(OBu^t)-Ser(Bu^t)-Arg(Pbf)-Thr(Bu^t)-Ser(Bu^t)-Gly-Asp(OBu^t)-(Dmb)Gly-Ala-Asp(OBu^t)-Asn(Trt)-Phe-Asp(OBu^t)-Val-Val-Ser(Bu^t)-(Et)Cys(Trt)-Arg(Pbf)-Arg(Pbf)-NH-resin (0.351 g) was obtained. A part of the resin (104 mg) was treated with a TFA cocktail (1.5 ml) at room temperature for 2 h, and then the peptide was precipitated with diethyl ether. After washing twice with ether, the precipitate was dried in vacuo. The crude peptide was dissolved in 6 M urea dissolved in 50% acetonitrile/5% acetic acid/H_2O (4.75 ml), and MPA (250 μl) was added. The mixture was gently mixed at room temperature for 7 days and purified by RP-HPLC on an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **3** (1.86 μmol, 13% yield). MALDI-TOF mass, found: *m/z* 2257.0, calcd: 2257.0 for $(M + H)^+$. Amino acid analysis: $Asp_{4.01}Thr_{1.76}Ser_{3.26}Glu_{1.04}Gly_2Ala_{1.01}Val_{2.48}Ile_{1.06}Leu_{0.94}Phe_{1.05}Arg_{1.08}$.

4.5 | Thz^{22}-Evasin-3 (22–66) 4

Peptides **1** and **2** (308 nmol each) were dissolved in 6 M guanidine-HCl/10 mM TCEP/60 mM MPAA/100 mM phosphate buffer (pH 7.4, 310 μl), and the solution was gently mixed at room temperature for 1 h. The crude peptide was separated by RP-HPLC on an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **4** (256 nmol, 83% yield). MALDI-TOF mass, found: *m/z* 5272.9, calcd: 5272.9 for $(M + H)^+$. Amino acid analysis: $Asp_{4.65}Thr_{0.82}Ser_{1.59}Glu_{4.69}Pro_{2.70}Gly_7Val_{1.88}Ile_{1.47}Leu_{3.00}Tyr_{1.09}Phe_{1.13}Lys_{3.94}His_{1.15}Arg_{2.08}$.

4.6 | Linear evasin-3 6

Peptide **4** (210 nmol) was dissolved in 20 mM DPDS/0.1% TFA/50% acetonitrile aq. (210 μl), and the solution was mixed with a vortex at 50°C for 2 h. Peptide **3** (210 nmol) was added to the solution, and the mixture was lyophilized. The residue was dissolved in 6 M guanidine-HCl/40 mM TCEP/60 mM MPAA/100 mM phosphate buffer (pH 7.4, 250 μl), and the solution was gently mixed at room temperature for 7 h. The crude peptide was separated by RP-HPLC on an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **6** (111 nmol, 53% yield). MALDI-TOF mass, found: *m/z* 7411.1, calcd: 7412.2 for $(M + H)^+$. Amino acid analysis: $Asp_{7.82}Thr_{2.48}Ser_{4.30}Glu_{5.43}Pro_{3.73}Gly_{9.74}Ala_{0.40}Val_{4.35}Ile_{2.55}Leu_4Tyr_{1.11}Phe_{2.18}Lys_{4.30}His_{1.11}Arg_{3.16}$.

4.7 | Refolded evasin-3 7

Peptide **6** (111 nmol) was dissolved in 8 M urea aq. (1.5 ml) and diluted with an ice-cooled buffer containing 0.6 M Tris/2.4 mM glutathione (reduced form) (7.5 ml). After stirring the solution at 4°C for 15 min, glutathione (oxidized form, 2.8 mg, the final concentration at 1 mM) was added. The solution was further stirred at 4°C overnight. The solution was then acidified by adding acetic acid (270 μl) and separated by RP-HPLC on an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **7** (90.2 nmol, 81% yield). MALDI-TOF mass, found: *m/z* 7406.9, calcd: 7406.2 for $(M + H)^+$. Amino acid analysis: $Asp_{8.86}Thr_{2.60}Ser_{4.65}Glu_{5.91}Pro_{3.17}Gly_{10.30}Ala_{0.44}Val_{4.56}Ile_{2.61}Leu_4Tyr_{0.92}Phe_{2.06}Lys_{4.02}His_{1.03}Arg_{3.08}$.

4.8 | Cys^{39}(Acm), $Cys^{50}(Bu^t)$-Evasin-3 (37–66) 8

Peptide **8** was prepared essentially according to the method for peptide **1** and obtained in 23% yield. MALDI-TOF mass, found: *m/z* 3619.1, calcd: 3619.3 for $(M + H)^+$. Amino acid analysis: $Asp_{1.99}Ser_{0.80}Glu_{1.96}Pro_{1.95}Gly_5Val_{1.78}Ile_{1.49}Leu_{3.05}Tyr_{1.04}Phe_{1.02}Lys_{2.97}His_{1.01}Arg_{2.04}$.

4.9 | Thz^{22}, Cys^{26}(Acm), $Cys^{33}(Bu^t)$-Evasin-3 (22–36)-thioester 9

Peptide **9** was prepared essentially according to the method for peptide **2**, and obtained in 16% yield. MALDI-TOF mass, found: *m/z* 2013.8, calcd: 2013.8 for $(M + H)^+$. Amino acid analysis: $Asp_{2.63}Thr_{0.82}Ser_{0.81}Glu_{2.56}Pro_{1.08}Gly_2Lys_{0.94}$.

4.10 | Thz^{22}, Cys^{26,39}(Acm), Cys^{33,50}(Bu^t)-Evasin-3 (22–66) 10

Peptides **8** and **9** (388 nmol each) were ligated in the same manner as for peptide **4**, and peptide **10** was obtained in 64% yield (250 nmol). MALDI-TOF mass, found: *m/z* 5527.1, calcd: 5527.3 for $(M + H)^+$. Amino acid analysis: $Asp_{4.86}Thr_{0.89}Ser_{1.69}Glu_{4.83}Pro_{3.00}Gly_7Val_{1.87}Ile_{1.46}Leu_{2.99}Tyr_{1.02}Phe_{1.04}Lys_{3.88}His_{0.98}Arg_{2.11}$.

4.11 | Cys^{26,39}(Acm), Cys^{33,50}(Bu^t)-Evasin-3 12

Peptide **10** (198 nmol) was treated with 20 mM DPDS/0.1% TFA/50% acetonitrile aq. (200 μl) at 50°C for 2 h. Peptide **3** (198 nmol) was added to the solution, and the mixture was lyophilized. The residue was dissolved in 6 M guanidine-HCl/40 mM TCEP/60 mM MPAA/100 mM phosphate buffer (pH 7.4, 200 μl), and the solution was gently mixed at room temperature for 20 h. The crude peptide was separated by RP-HPLC on an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **12** (153 nmol, 77% yield). MALDI-TOF mass, found: *m/z* 7666.2, calcd: 7666.5 for $(M + H)^+$. Amino acid analysis: $Asp_{8.35}Thr_{2.50}Ser_{4.19}Glu_{5.54}Pro_{3.86}Gly_{9.81}Ala_{0.48}Val_{4.23}Ile_{2.29}Leu_4Tyr_{1.02}Phe_{2.07}Lys_{4.30}His_{1.09}Arg_{3.18}$.

4.12 | Cys^{26,39}(Acm), Cys^{33,50}(Bu^t)-Evasin-3 with one disulfide bond 13

Peptide **12** (153 nmol) was dissolved in 50 mM phosphate buffer (900 μl, pH 7.0), and DMSO (100 μl) was added. The mixture was gently mixed at room temperature for 24 h and separated by RP-HPLC on an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **13** (116 nmol, 75% yield). MALDI-TOF mass, found: *m/z* 7665.0, calcd: 7664.5 for $(M + H)^+$. Amino acid analysis: $Asp_{8.97}Thr_{2.66}Ser_{4.80}Glu_{5.97}Pro_{3.05}Gly_{9.80}Ala_{0.44}Val_{4.36}Ile_{2.16}Leu_4Tyr_{1.00}Phe_{2.11}Lys_{4.23}His_{1.11}Arg_{3.11}$.

4.13 | Cys^{33,50}(Bu^t)-Evasin-3 14

Peptide **13** (115 nmol) was dissolved in H_2O (1.2 ml), and added dropwisely to methanol (4.8 ml) containing 20 mM iodine/methanol (72 μl) and conc. HCl aq. (12 μl). After mixing with a vortex at room temperature for 1 h, the reaction was quenched by adding ascorbic acid aqueous solution. Peptide **14** was obtained by RP-HPLC purification using an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA in 80% yield (91.5 nmol). MALDI-TOF mass, found: *m/z* 7520.3, calcd: 7520.4 for $(M + H)^+$. Amino acid analysis: $Asp_{8.70}Thr_{2.62}Ser_{4.78}Glu_{5.78}Pro_{3.54}Gly_{9.65}Ala_{0.47}Val_{4.23}Ile_{2.55}Leu_4Tyr_{1.10}Phe_{2.18}Lys_{4.07}His_{1.01}Arg_{3.01}$.

4.14 | Evasin-3 with three disulfide bonds 15

Peptide **14** (52.7 nmol) was dissolved in 5% DMSO/TFA (500 μl), and the solution was mixed with a vortex at room temperature for 30 min. The crude peptide was precipitated with diethyl ether, washed twice with ether, and dried under vacuum. The residue was separated by RP-HPLC on an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **15** (17.0 nmol, 32% yield). MALDI-TOF mass, found: *m/z* 7406.1, calcd: 7406.1 for $(M + H)^+$. Amino acid analysis: $Asp_{8.79}Thr_{2.56}Ser_{4.52}Glu_{5.91}Pro_{2.99}Gly_{9.74}Ala_{0.47}Val_{4.41}Ile_{2.87}Leu_4Tyr_{0.99}Phe_{2.20}Lys_{4.01}His_{0.99}Arg_{3.01}$.

4.15 | Surface plasmon resonance

Affinity assays of the synthetic evasin-3 were performed with BIAcore T200 SPR system (GE Healthcare). All assays were carried out in PBS (pH 7.4) as a running buffer. Human CXCL-1 and CXCL-8 purchased from PEPROTECH were immobilized on Sensor Chip CM5 (GE Healthcare) according to the manufacturer's protocol. Concentration series of evasin-3 with twofold dilution, raging 10 to 0.625 μM, were examined. Association and dissociation kinetics were monitored at a flow rate of 30 μl/min for 125 s each. The data were analyzed using BIAcore T200 software.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

H. K.: Conceptualization, investigation, writing-original draft, editing, funding acquisition; K. N.: investigation, editing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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