Application of a novel thioesterification reaction to the synthesis of chemokine CCL27 by the modified thioester method

Hironobu Hojo,*[a] Yuichi Murasawa,[a] Hidekazu Katayama,[a] Tsuyoshi Ohira,[b] Yuko Nakahara[a] and Yoshiaki Nakahara*[a]

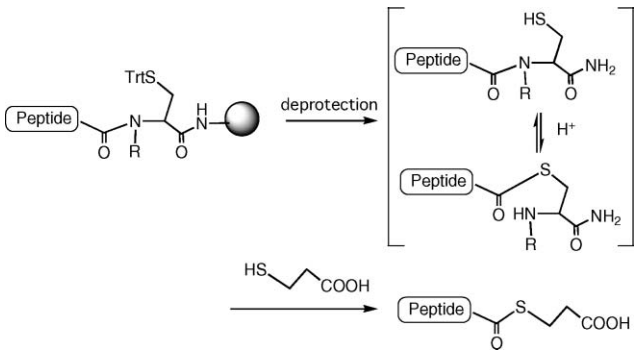
Received 17th January 2008, Accepted 3rd March 2008
First published as an Advance Article on the web 31st March 2008
DOI: 10.1039/b800884a

Aryl thioesters of peptide segments were prepared by the conventional 9-fluorenylmethoxycarbonyl (Fmoc) strategy using a novel *N*-alkyl cysteine (NAC)-assisted thioesterification reaction. The peptide carrying NAC at its *C*-terminus was prepared by the Fmoc strategy and converted to the aryl thioester by 4-mercaptophenylacetic acid (MPAA) treatment without significant side reactions. The peptide thioester was used for the efficient preparation of 95-amino acid (AA) chemokine CCL27 by an Ag^+-free thioester method.

Introduction

Peptide thioesters have been widely used as a key intermediate for the chemical synthesis of proteins in the thioester method[1] as well as for native chemical ligation.[2] The preparation of peptide thioesters has mainly been performed by the *tert*-butoxycarbonyl (Boc) strategy using an established preparation method.[1,3] In contrast, the application of the 9-fluorenylmethoxycarbonyl (Fmoc) strategy to thioester synthesis has several difficulties, which mainly arise from the fact that the piperidine used to remove the Fmoc group decomposes the thioester linkage. Recently, various Fmoc methods have been developed to avoid this issue.[4,5] The most widely used method is post-solid-phase peptide synthetic (SPPS) thioesterification using a sulfonamide linker.[5] In this method, the peptide thioester can be obtained by the activation of the sulfonamide group at the peptide's *C*-terminus using an alkylating reagent, followed by thiolysis. However, alkylation by iodoacetonitrile is reported to cause methionine modification.[6] Unverzagt *et al.* reported that the capping reaction by acetic anhydride unexpectedly acetylates the nitrogen atom in the sulfonamide linker, which results in cleavage of the peptide chain during the solid-phase synthesis.[7] Other Fmoc methods used for peptide thioester synthesis also have potential drawbacks.

Recently, we developed a novel post-SPPS thioesterification reaction, which realizes the effective preparation of peptide thioesters by the Fmoc strategy.[8] First, *N*-alkyl cysteine (NAC) is introduced at the *C*-terminus of a peptide. The chain is then elongated by the conventional Fmoc strategy. After deprotection, the peptide is treated with a thiol compound, such as 3-mercaptopropionic acid (MPA), to perform thioesterification assisted by the NAC as shown in Scheme 1. This method is fully compatible with the conventional Fmoc strategy and gives a good yield of peptide thioesters. We found that the more reactive thiophenyl ester could also be prepared by this method. Recently, preformed aryl thioester has attracted much attention for use in a kinetically controlled ligation[9] in which the peptide aryl thioester can be ligated with a bifunctional cysteinyl peptide alkyl thioester using the large difference in the reactivity of *S*-alkyl and *S*-aryl thioesters. Preformed aryl thioester is synthesized by the Boc strategy,[10] which cannot be applied to the preparation of peptide thioesters carrying acid-sensitive moieties, such as carbohydrates. Since our method provides an efficient access to aryl thioester by the Fmoc method, we could realize efficient synthesis of glycosylated and phosphorylated proteins.



Scheme 1 Novel thioesterification reaction using NAC.

The thioester method is advantageous over native chemical ligation regarding Cys-free segment coupling. In return, it requires amino and thiol protection during coupling. The combination of Boc for amino protection, acetamidomethyl (Acm) group for thiol protection and AgCl as an activator of the thioester group is the optimized procedure for the thioester method.[11] However, careful manipulation of the peptide is required during coupling and its work-up, since the Acm group is easily removed by Ag^+ under basic conditions. We found that the use of preformed aryl thioester eliminates the use of silver ions for the activation, which realizes perfect retention of the protected thiol group throughout coupling reactions. To demonstrate the usefulness of our method, a 95-residue chemokine CCL27[12] was prepared by the Ag^+-free thioester method.

[a]*Department of Applied Biochemistry, Institute of Glycotechnology, Tokai University, Hiratsuka, Kanagawa, 259-1292, Japan. E-mail: hojo@keyaki.cc.u-tokai.ac.jp, yonak@keyaki.cc.u-tokai.ac.jp; Fax: +81 463 50 2075; Tel: +81 463 58 1211*
[b]*Department of Biological Sciences, Faculty of Science, Kanagawa University, Hiratsuka, Kanagawa, 259-1293, Japan. E-mail: ohirat-bio@kanagawa-u.ac.jp; Fax: +81 463 58 9684; Tel: +81 463 59 4111*

Results and discussion

Synthesis of CCL27

The amino acid sequence of CCL27 and the coupling sites of the segments are shown in Fig. 1. To examine the efficiency of the new method, the *N*-terminal and intermediate peptide thioesters were synthesized by both the Fmoc strategy using NAC-assisted thioesterification and the conventional Boc strategy.[3] In the case of the Boc strategy, a Leu residue was inserted between the thioester linker and the resin to increase the acid stability of the linker during SPPS.[3] The NAC-assisted procedure for intermediate segment **6** is shown in Fig. 2. Fmoc-*N*-ethyl-*S*-trityl-cysteine (Fmoc-(Et)Cys(Trt)-OH) was introduced to CLEAR amide resin by the *N*,*N*′-dicyclohexylcarbodiimide (DCC)–1-hydroxybenzotriazole (HOBt) method. After the Fmoc removal, Fmoc-Gly was introduced by *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU)–*N*,*N*-diisopropylethylamine (DIEA) at 50 °C. The Gly residue was introduced quantitatively after double coupling. Then, the chain elongation was performed using an ABI 433A peptide synthesizer by the Fmoc strategy. After the cleavage from the resin and deprotection by TFA cocktail, the crude peptide was dissolved in aq. acetonitrile containing 6 M urea and 2% 4-mercaptophenylacetic acid (MPAA). As shown in Fig. 3, the reaction was almost completed within 2 d giving the desired peptide thioester **6** in high purity. The yield of **6** after HPLC purification was 5.0%. The peptide segments prepared for CCL27 synthesis are shown in Table 1. The yields of peptide thioesters

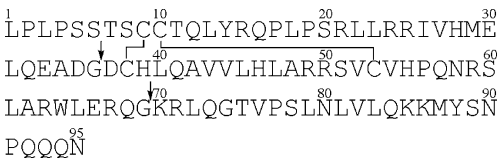


Fig. 1 Structure of CCL27. Arrows indicate sites of segment coupling.

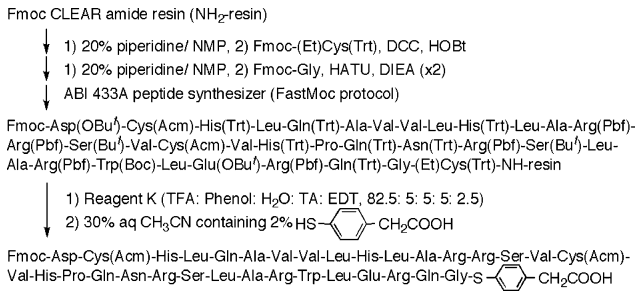


Fig. 2 Synthetic route for peptide thioester **6**.

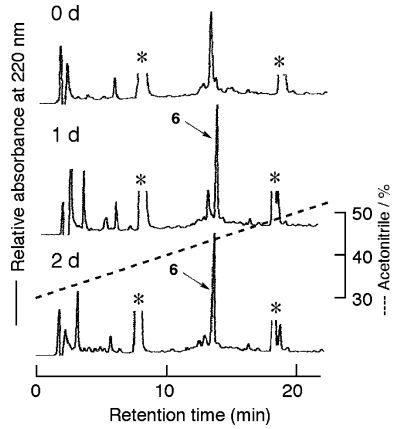


Fig. 3 RPHPLC profile of thioesterification to obtain **6**. The asterisks show the peaks derived from MPAA. Elution conditions: column, Mightysil RP-18 GP (4.6 × 150 mm, Kanto, Japan) at a flow rate of 1 ml min^{-1}; eluent, A, 0.1% TFA, B, acetonitrile containing 0.1% TFA.

5 and **6** obtained by the new method were comparable to those of peptide thioesters **8** and **9** obtained by the conventional Boc strategy, demonstrating that the new method provides a simple and efficient method for Fmoc peptide thioester synthesis.

The segment coupling was performed as shown in Fig. 4. For comparison, the coupling was also achieved by the conventional Ag^+-assisted thioester method using peptides **7**, **8** and **9** (see Experimental). Peptides **6** and **7** were dissolved in DMSO containing 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (HOOBt).

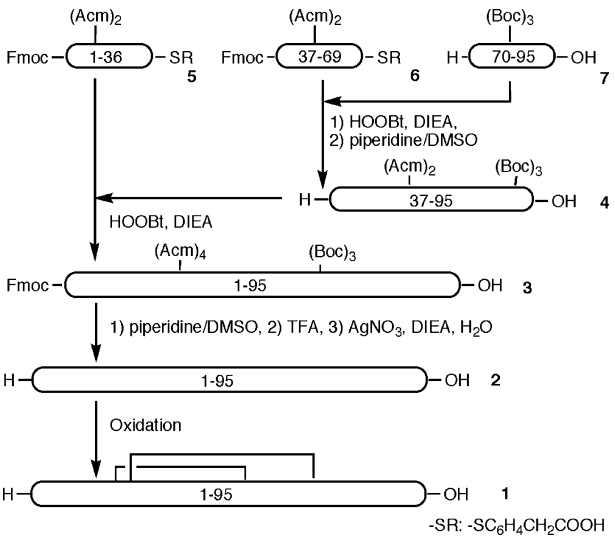


Fig. 4 Synthetic route for CCL27 **1**.

Table 1 Yields of peptide and peptide thioesters prepared for CCL27 synthesis

Peptide and peptide thioester	R	R′	Name	Yield[a]
R-[Cys(Acm)^{9,10}]-CCL27(1–36)-R′	Fmoc	$SC_6H_4CH_2COOH$	**5**	11
	Boc	SCH_2CH_2CO-Leu-NH_2	**8**	10[b]
R-[Cys(Acm)^{38,53}]-CCL27(37–69)-R′	Fmoc	$SC_6H_4CH_2COOH$	**6**	5.0
	Fmoc	SCH_2CH_2CO-Leu-NH_2	**9**	3.7
R-[Lys(Boc)^{70,85,86}]-CCL27(70–95)-R′	H	OH	**7**	11[c]

[a] Yields are calculated based on the initial loading of *C*-terminal amino acids on resin. [b] The overall yield of SPPS followed by Boc protection of terminal amino groups. [c] The overall yield of SPPS, introduction of the Boc group followed by the Fmoc removal.

The coupling was initiated by adding DIEA. In spite of the absence of silver ions, the reaction was almost completed within 12 h without considerable side reactions, the speed of which is comparable with Ag^+-assisted segment condensation. Fmoc removal was easily accomplished by adding piperidine to the reaction mixture to obtain peptide **4** (Fig. 5a). In the case of Ag^+-assisted coupling of **7** and **9**, the trapping of Ag^+ prior to Fmoc removal was essential, as the Acm group was cleaved by Ag^+ even with the use of highly insoluble AgCl as an activator. After the purification by gel filtration chromatography (GFC), peptide **4** was condensed with an *N*-terminal peptide thioester **5** in the same manner. The desired peptide **3** was obtained in high purity within 12 h as shown in Fig. 5b. Then, Fmoc and Boc groups were removed by piperidine and TFA treatments, respectively, and the thiol-protected form of CCL27 was obtained. The Acm groups were removed by $AgNO_3$ in the presence of DIEA and water without considerable side reactions as shown in Fig. 5c. After purification by reversed-phase (RP) HPLC, the peptide carrying free thiol group **2** was oxidized in the presence of DMSO as shown in Fig. 5e. The main peak was isolated by RPHPLC to give the folded CCL27 **1** in high purity.

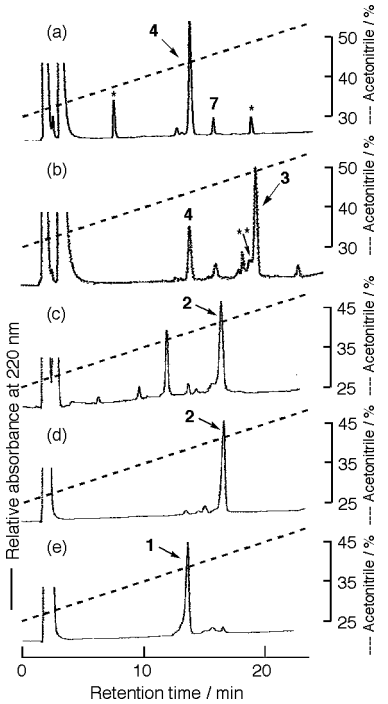


Fig. 5 RPHPLC profiles for the synthesis of CCL27 (a) Reaction mixture for the synthesis of peptide **4**, (b) crude peptide **3**, (c) crude peptide **2**, (d) purified peptide **2**, (e) crude peptide **1**. The asterisks in (a) are for non-peptide components. The double asterisks in (b) are for the elution position of peptide **5**. Elution conditions are the same as described in Fig. 3.

Determination of the disulfide pairing

To determine the mode of disulfide bonds, the synthetic CCL27 was digested with trypsin at 37 °C for 15 h and the fragments were separated by RPHPLC. One of the fragments showed a mass number of 4925.0 (average), which is close to the value of the structure in Fig. 6. Edman degradation of this fragment showed that cystine appeared at cycles 10 and 13, not at cycle 9, indicating that the

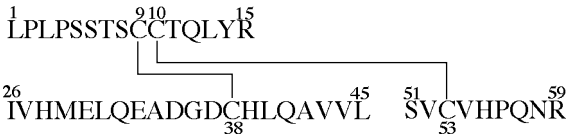


Fig. 6 Disulfide pairing of the tryptic digest of CCL27.

disulfide bond pairing is Cys9/38 and Cys10/53. Thus, the mode of disulfides was determined as shown by the structure in Fig. 1, which is identical with other chemokines prepared previously.[13]

Conclusions

A novel post-SPPS thioesterification reaction was successfully applied to prepare reactive peptide aryl thioesters, demonstrating that the preparation of peptide thioesters by the Fmoc method has become practical with regard to yield and simplicity. Using these peptide thioesters, synthesis of 95-AA chemokine, CCL27, was successfully prepared by the Ag^+-free thioester method. Work is now continuing on the synthesis of the glycosylated version of CCL27. We are also applying the method at a non Gly-X site to extend the general applicability of our method.

Experimental

Fmoc-(Et)Cys(Trt)-OH was prepared by the previously described method.[8] MALDI-TOF mass spectra were recorded with a Voyager-DE PRO spectrometer (Applied Biosystems, Foster City, CA). Amino acid composition was determined with a LaChrom amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis with 6 M HCl at 150 °C for 2 h in an evacuated sealed tube. Amino acid sequence analysis was performed with a protein sequencer 491 (Applied Biosystems).

Fmoc-[Cys(Acm)^{9,10}]-CCL27(1–36)-SC$_6$H$_4$CH$_2$COOH 5

Fmoc-CLEAR amide resin (0.21 g, 0.10 mmol) was reacted with 20% piperidine–1-methyl-2-pyrrolidinone (NMP) for 5 min × 1 and 15 min × 1. After washing with NMP (×5), Fmoc-(Et)-Cys(Trt)-OBt, which was prepared by mixing Fmoc-(Et)Cys(Trt)-OH (0.25 g, 0.41 mmol), 1 M DCC–NMP (0.5 ml) and 1 M HOBt–NMP (0.5 ml) for 30 min at room temperature, was added and the reaction mixture was vortexed for 1 h at 50 °C. The resin was washed with NMP five times and the remaining amino groups were acetylated using 10% Ac_2O–5% DIEA–NMP for 5 min. After NMP washing, removal of the Fmoc group was performed as described above, and the resin was reacted with Fmoc-Gly (0.30 g, 1.0 mmol), HATU (0.36 g, 0.95 mmol) and DIEA (0.35 ml, 2.0 mmol) at 50 °C for 1 h. The reaction was repeated with fresh reagents. Then the resin was subjected to automated synthesis by an Applied Biosystems (ABI) 433A peptide synthesizer using the FastMoc protocol and Fmoc-Leu-Pro-Leu-Pro-Ser(Bu^t)-Ser(Bu^t)-Thr(Bu^t)-Ser(Bu^t)-Cys(Acm)-Cys(Acm)-Thr(Bu^t)-Gln(Trt)-Leu-Tyr(Bu^t)-Arg(Pbf)-Gln(Trt)-Pro-Leu-Pro-Ser(Bu^t)-Arg(Pbf)-Leu-Leu-Arg(Pbf)-Arg(Pbf)-Ile-Val-His-(Trt)-Met-Glu(OBu^t)-Leu-Gln(Trt)-Glu(OBu^t)-Ala-Asp(OBu^t)-Gly-(Et)Cys(Trt)-NH-resin (0.80 g) was obtained. The resin (0.16 g, 21 μmol) was treated with Reagent K (TFA : phenol : H_2O : thioanisole (TA) : 1,2-ethanedithiol (EDT), 82.5 : 5 : 5 : 5 : 2.5)[14] (3.0 ml) at room temperature for 2 h. TFA was

removed under a nitrogen stream and the peptide was precipitated with ether, washed twice with ether and dried *in vacuo*. The crude peptide was dissolved in 10 ml of 30% aq. acetonitrile containing 6 M urea and 2% MPAA, and the solution was kept at room temperature for 2 d. MPAA was extracted with ether (×3) and the crude peptide was purified by RPHPLC using aq. acetonitrile containing 0.1% TFA to obtain peptide thioester **5** (11 mg, 2.3 μmol, 11% yield). MALDI-TOF mass: found *m/z* 4625.4 $(M + H)^+$ (average), calcd 4625.4 $(M + H)^+$. Amino acid analysis: $Asp_{1.02}Thr_{1.72}Ser_{2.87}Glu_{4.74}Pro_{3.46}Gly_{1.04}Ala_1Val_{0.58}$ $Met_{1.07}Ile_{0.53}Leu_{6.33}Tyr_{0.77}His_{0.84}Arg_{3.57}$.

Boc-[Cys(Acm)^{9,10}]-CCL27(1–36)-SCH$_2$CH$_2$CO-Leu-NH$_2$ 8

MBHA-resin (NH_2-resin) hydrochloride (0.15 g, 0.1 mmol) was washed with 5% DIEA–NMP (×2) and NMP (×5). Boc-Leu-OBt, which was prepared by mixing Boc-Leu hydrate (100 mg, 0.4 mmol), 1 M DCC–NMP (0.4 ml) and 1 M HOBt–NMP (0.4 ml) for 30 min, was added to the resin. The mixture was vortexed for 1 h and the resin was washed with NMP (×5) and DCM (×4). Then the resin was treated with 50% TFA–DCM (5 min × 1 and 15 min × 1) and washed with DCM (×3) and NMP (×2). The resin was neutralized with 5% DIEA–NMP (×2) and washed with NMP (×5). Boc-Gly-SCH_2CH_2COOBt, which was prepared by mixing Boc-Gly-SCH_2CH_2COOH (53 mg, 0.2 mmol), 1 M DCC/NMP (0.2 ml) and 1 M HOBt–NMP (0.2 ml) for 30 min, was added and the mixture was vortexed for 1 h. The resin was washed with NMP (×5) and treated with 10% Ac_2O–5% DIEA–NMP for 5 min. After washing with NMP, the resin was subjected to automated synthesis using an ABI 433A peptide synthesizer with the 0.1 mmol scale Boc–HOBt–DCC protocol and Boc-Leu-Pro-Leu-Pro-Ser(Bzl)-Ser(Bzl)-Thr(Bzl)-Ser(Bzl)-Cys(Acm)-Cys(Acm)-Thr(Bzl)-Gln-Leu-Tyr(BrZ)-Arg(Tos)-Gln-Pro-Leu-Pro-Ser(Bzl)-Arg(Tos)-Leu-Leu-Arg(Tos)-Arg(Tos)-Ile-Val-His(Bom)-Met-Glu(OBzl)-Leu-Gln-Glu(OBzl)-Ala-Asp(O*c*Hex)-Gly-SCH_2CH_2CO-Leu-NH-resin (0.57 g) was obtained. The resin (0.26 g) was treated with HF (5 ml) containing 10% anisole at 0 °C for 1.5 h. After evaporation of HF under reduced pressure, the residue was washed three times with ether. The crude peptide was extracted with 50% aq. acetonitrile containing 0.1% TFA and lyophilized. The crude peptide was purified by RPHPLC to obtain [Cys(Acm)^{9,10}]-CCL27(1–36)-SCH_2CH_2CO-Leu-NH_2 (20 mg, 4.5 μmol, 10% yield). [Cys(Acm)^{9,10}]-CCL27(1–36)-SCH_2CH_2CO-Leu-NH_2 (20 mg, 4.5 μmol) was dissolved in DMSO (0.1 ml), and Boc-OSu (3.9 mg, 18 μmol) and DIEA (3.1 μl, 18 μmol) were added. After the solution was stirred for 1 h, peptide was precipitated with ether, washed with ethyl acetate twice, and lyophilized from dioxane suspension to give Boc-[Cys(Acm)^{9,10}]-CCL27(1–36)-SCH_2CH_2CO-Leu-NH_2 **8** (quantitative). MALDI-TOF mass: found *m/z* 4550.4 $(M + H)^+$, calcd 4550.4 $(M + H)^+$. Amino acid analysis: $Asp_{0.94}Thr_{1.60}Ser_{2.65}Glu_{4.18}Pro_{3.20}Gly_1Ala_{0.95}Val_{0.70}Met_{0.96}$ $Ile_{0.63}Leu_{7.32}Tyr_{0.90}His_{0.94}$ $Arg_{3.95}$.

Fmoc-[Cys(Acm)^{38,53}]-CCL27(37–69)-SC$_6$H$_4$CH$_2$COOH 6

Fmoc-CLEAR amide resin (0.21 g, 0.10 mmol) was reacted with 20% piperidine–NMP for 5 min × 1 and 15 min × 1. After washing with NMP (×5), Fmoc-(Et)Cys(Trt)-OBt, which was prepared by mixing Fmoc-(Et)Cys(Trt)-OH (0.25 g, 0.41 mmol), 1 M DCC–NMP (0.5 ml) and 1 M HOBt–NMP (0.5 ml) for 30 min at room temperature, was added and the reaction mixture was vortexed for 1 h at 50 °C. The resin was washed with NMP five times and the remaining amino groups were acetylated using 10% Ac_2O–5% DIEA–NMP for 5 min. After NMP washing, the removal of the Fmoc group was performed as described above, and the resin was reacted with Fmoc-Gly (0.30 g, 1.0 mmol), HATU (0.36 g, 0.95 mmol) and DIEA (0.35 ml, 2.0 mmol) at 50 °C for 1 h. The reaction was repeated with fresh reagents. Then the resin was subjected to automated synthesis by the ABI 433A peptide synthesizer using the FastMoc protocol and Fmoc-Asp(OBu^t)-Cys(Acm)-His(Trt)-Leu-Gln(Trt)-Ala-Val-Val-Leu-His(Trt)-Leu-Ala-Arg(Pbf)-Arg(Pbf)-Ser(Bu^t)-Val-Cys(Acm)-Val-His(Trt)-Pro-Gln(Trt)-Asn(Trt)-Arg(Pbf)-Ser(Bu^t)-Leu-Ala-Arg(Pbf)-Trp(Boc)-Leu-Glu(OBu^t)-Arg(Pbf)-Gln(Trt)-Gly-(Et)Cys(Trt)-NH-resin (0.68 g) was assembled. An aliquot of the resin (92 mg) was treated with Reagent K (2 ml) at room temperature for 2 h. TFA was removed under a nitrogen stream, and the peptide was precipitated with ether, washed twice with ether and dried *in vacuo*. The crude peptide was dissolved in 10 ml of 30% aq. acetonitrile containing 6 M urea and 2% MPAA and the solution was kept at room temperature for 2 d. MPAA was extracted with ether (×3) and the crude peptide was purified by RPHPLC using aq. acetonitrile containing 0.1% TFA to obtain peptide thioester **6** (0.68 μmol, 5.0% yield). MALDI-TOF mass: found *m/z* 4365.8 $(M + H)^+$ (average), calcd 4365.1 $(M + H)^+$. Amino acid analysis: $Asp_{2.03}Ser_{1.91}Glu_{4.09}Pro_{0.91}Gly_{1.14}Ala_3Val_{2.85}Leu_{4.98}His_{2.73}$ $Arg_{4.96}$.

Fmoc-[Cys(Acm)^{38,53}]-CCL27(37–69)-SCH$_2$CH$_2$CO-Leu-NH$_2$ 9

Boc-Gly-SCH_2CH_2CO-Leu-NH-resin (0.1 mmol), which was prepared by the same procedure as described for peptide **8**, was subjected to the automated synthesis using the 0.1 mmol FastBoc protocol and Boc-Cys(Acm)-His(Bom)-Leu-Gln-Ala-Val-Val-Leu-His(Bom)-Leu-Ala-Arg(Tos)-Arg(Tos)-Ser(Bzl)-Val-Cys(Acm)-Val-His(Bom)-Pro-Gln-Asn-Arg(Tos)-Ser(Bzl)-Leu-Ala-Arg(Tos)-Trp(For)-Leu-Glu(OBzl)-Arg(Tos)-Gln-Gly-SCH_2-CH_2CO-Leu-NH-resin was obtained. The Boc group was removed by 50% TFA–CH_2Cl_2 treatment for 5 min × 1 and 15 min × 1, followed by 5% DIEA–NMP treatment (2 min ×2). Then, Fmoc-Asp(OBu^t)-OBt, which was prepared by mixing Fmoc-Asp(OBu^t)-OH (0.41 g, 1.0 mmol), 1 M HOBt–NMP (1.0 ml) and 1 M DCC–NMP (1.0 ml) for 30 min, was added to the resin. After the mixture was vortexed for 1 h, the resin was washed with NMP followed by DCM and dried *in vacuo*. A part of the resin obtained (0.25 g out of 0.44 g) was treated with HF (5.4 ml) containing anisole (0.40 ml) and 1,4-butanedithiol (0.75 ml) at 0 °C for 1.5 h. After HF was removed *in vacuo*, the residue was washed with ether three times. Crude peptide was extracted with aq. acetonitrile containing 0.1% TFA and lyophilized. The obtained powder was purified by RPHPLC to give **9** (9.3 mg, 2.1 μmol. 3.7%). MALDI-TOF mass: found *m/z* 4414.6 $(M + H)^+$ (average), calcd 4415.2 $(M + H)^+$. Amino acid analysis: $Asp_{1.86}Ser_{1.83}Glu_{4.29}Pro_{0.94}Gly_{1.15}Ala_3Val_{2.72}$ $Leu_{5.70}His_{2.62}Arg_{5.17}$.

[Lys(Boc)^{70,85,86}]-CCL27(70–95) 7

Starting from Fmoc-Asn(Trt)-CLEAR Acid Resin (0.54 g, 0.15 mmol), the peptide chain was elongated by the synthesizer

to obtain Fmoc-Lys(Boc)-Arg(Pbf)-Leu-Gln(Trt)-Gly-Thr(Bu^t)-Val-Pro-Ser(Bu^t)-Leu-Asn(Trt)-Leu-Val-Leu-Gln(Trt)-Lys(Boc)-Lys(Boc)-Met-Tyr(Bu^t)-Ser(Bu^t)-Asn(Trt)-Pro-Gln(Trt)-Gln-(Trt)-Gln(Trt)-Asn(Trt)-CLEAR Acid Resin (1.4 g). A part of the resin (0.35 g) was treated with Reagent K (5 ml) for 2 h at room temperature. TFA was removed by a nitrogen stream and the product was precipitated with ether, washed with ether twice and dried *in vacuo*. The peptide was extracted with aq. acetonitrile containing 0.1% TFA and lyophilized. The obtained powder was purified by RPHPLC to give Fmoc-CCL27(70–95) (24 mg, 7.3 μmol, 20% yield). This peptide was dissolved in DMSO (360 μl) containing Boc-OSu (11 mg, 51 μmol) and DIEA (11 μl, 63 μmol). After the solution was kept at room temperature for 5 h, the product was precipitated with ether, washed with ethyl acetate twice and lyophilized from a dioxane suspension. The peptide obtained was dissolved in DMSO (300 μl) containing piperidine (15 μl) and the reaction mixture was kept for 1.5 h. The product was precipitated with ether, washed with ether twice and dried *in vacuo*. The precipitate was purified by RPHPLC to give peptide **7** (14 mg, 4.2 μmol). MALDI-TOF mass: found *m/z* 3313.2 $(M + H)^+$, calcd 3312.8 $(M + H)^+$. Amino acid analysis: $Asp_{2.98}Thr_{0.94}Ser_{1.84}Glu_{5.11}Pro_{2.02}Gly_1Val_{1.93}Met_{1.05}Leu_{3.92}Tyr_{0.90}Lys_{2.98}Arg_{1.08}$.

[Cys(SH)^{9,10,38,53}]-CCL27(1–95) 2

Peptide **6** (2.6 mg, 0.60 μmol) and **7** (2.4 mg, 0.72 μmol) were dissolved in DMSO (140 μl) containing HOOBt (2.9 mg, 18 μmol) and DIEA (2.1 μl, 12 μmol). After the solution was kept at room temperature overnight, piperidine (7 μl) was added and the reaction mixture was kept at room temperature for 1 h. The product was precipitated with ether, washed with ether twice and dried *in vacuo*. The precipitate was purified by GFC using G3000PW$_{XL}$ using 50% aq. acetonitrile containing 0.1% TFA to give peptide **4**. Peptide **4** obtained and peptide **5** (1.9 mg, 0.42 μmol) were dissolved in DMSO (100 μl) containing HOOBt (2.4 mg, 15 μmol) and DIEA (1.7 μl, 9.8 μmol). The solution was kept at room temperature overnight. Piperidine (7.5 μl) was added and the solution was kept for 1 h at room temperature. Ether was added to precipitate the product, which was washed with the same solvent twice and dried *in vacuo*. The powder was dissolved in 5% 1,2-ethanedithiol–TFA (200 μl) at room temperature for 10 min. TFA was removed by a nitrogen stream and the product was precipitated with ether, washed with the same solvent twice and dried. The residue was dissolved in 80% DMSO (0.5 ml) containing silver nitrate (5.1 mg, 30 μmol) and DIEA (0.26 μl, 1.5 μmol), and the solution was kept in the dark at 50 °C for 1 h. Dithiothreitol (DTT) (50 mg, 0.32 mmol) was added to the solution, which was acidified by 0.5 M HCl (0.25 ml). The product was purified by RPHPLC to obtain peptide **2** (0.15 μmol). MALDI-TOF mass: found *m/z* 10934 $(M + H)^+$ (average), calcd 10934 $(M + H)^+$. Amino acid analysis: $Asp_{5.82}Thr_{2.69}Ser_{6.89}Glu_{13.65}Pro_{6.73}Gly_3Ala_{4.12}Val_{4.34}Met_{1.97}Ile_{0.43}Leu_{15.15}Tyr_{1.53}Lys_{2.69}His_{3.64}Arg_{9.93}$.

[Cys(SH)^{9,10,38,53}]-CCL27(1–95) 2 (by Ag$^+$-assisted thioester method)

Peptides **9** (5.7 mg, 1.3 μmol) and **7** (4.2 mg, 1.3 μmol) were dissolved in DMSO (300 μl) containing HOOBt (6.3 mg, 39 μmol) and DIEA (4.5 μl, 26 μmol). AgCl (0.56 mg, 3.9 μmol) was added and the reaction mixture was vortexed in the dark overnight. DTT (20 mg, 130 μmol) was added to the solution to trap silver ions. Then, the Fmoc group was removed by adding piperidine (15 μl) to the solution. After the solution was kept for 1 h at room temperature, ether was added to the solution to form a precipitate, which was washed with ether twice and dried *in vacuo*. The powder was subjected to GFC using G3000SW$_{XL}$ with 50% aq. acetonitrile 0.1% TFA as an eluent to give peptide **4**. Peptides **4** and **8** (3.5 mg, 0.76 μmol) were dissolved in DMSO (200 μl) containing HOOBt (3.7 mg, 23 μmol) and DIEA (2.6 μl, 15 μmol). AgCl (0.50 mg, 3.5 μmol) was added and the reaction mixture was vortexed in the dark overnight. After AgCl was removed by centrifugation, ether was added to precipitate the product, which was washed with ether twice and dried *in vacuo*. The powder was dissolved in 5% 1,2-ethanedithiol–TFA (200 μl) at room temperature for 10 min. TFA was removed by a nitrogen stream and the product was precipitated with ether, washed with the same solvent twice and dried. The residue was dissolved in 80% DMSO (1.0 ml) containing silver nitrate (10 mg, 59 μmol) and DIEA (0.52 μl, 3.0 μmol), and the solution was kept in the dark at 50 °C for 1 h. DTT (100 mg, 0.65 mmol) was added to the solution, which was acidified by 0.5 M HCl (0.5 ml). The solution was purified by RPHPLC to obtain peptide **2** (0.32 μmol). MALDI-TOF mass: found *m/z* 10928 $(M + H)^+$ (average), calcd 10934 $(M + H)^+$. Amino acid analysis: $Asp_{5.52}Thr_{2.50}Ser_{6.05}Glu_{13.14}Pro_{6.01}Gly_3Ala_{4.04}Val_{5.68}Met_{1.90}Ile_{0.65}Leu_{15.11}Tyr_{1.51}Lys_{2.53}His_{3.79}Arg_{10.20}$.

CCL27(1–95) 1

Peptide **2** (58 nmol) was dissolved in 6 M guanidine hydrochloride (30 μl) and diluted with a 0.1 M Tris-HCl buffer containing 1 M guanidine hydrochloride (pH 8.5, 2.0 ml). DMSO (0.23 ml) was added and the solution was kept at room temperature for 2 d. The solution was purified by RPHPLC to give **1** (34 nmol). MALDI-TOF mass: found *m/z* 10928 $(M + H)^+$ (average), calcd 10930 $(M + H)^+$. Amino acid analysis: $Asp_{5.98}Thr_{2.82}Ser_{6.89}Glu_{14.20}Pro_{6.97}Gly_3Ala_{3.92}Val_{5.86}Met_{1.87}Ile_{0.63}Leu_{15.76}Tyr_{1.65}Lys_{2.95}His_{3.82}Arg_{9.99}$.

Determination of disulfide bond pairings

Synthetic CCL27(1–95) **1** (*ca.* 5 nmol) was dissolved in 0.1 M Tris-HCl (pH 6.8, 100 μl) followed by the addition of TPCK-treated trypsin (1 μg). After the solution was kept at 37 °C overnight, the reaction was stopped by adding acetic acid to the solution. The fragments were separated by RPHPLC. The peak showing the mass value of *m/z* 4925.0 was collected and subjected to Edman sequencing.

Acknowledgements

This work was partly supported by Grant-Aid for Scientific Research from the Ministry of Education, Culture, Sport, Sciences and Technology of Japan. We thank Tokai University for a grant-in-aid for high-technology research. We also thank the Japan Society for the Promotion of Science for a Grant-in-Aid for Creative Scientific Research (No. 17GS0420).

Notes and references

1 H. Hojo and S. Aimoto, *Bull. Chem. Soc. Jpn.*, 1991, **64**, 111–117; S. Aimoto, *Biopolymers*, 1999, **51**, 247–265.

2 P. E. Dawson, T. W. Muir, I. Clark-Lewis and S. B. H. Kent, *Science*, 1994, **266**, 776–779; P. E. Dawson and S. B. H. Kent, *Annu. Rev. Biochem.*, 2000, **69**, 923–960.

3 H. Hojo, Y. D. Kwon, Y. Kakuta, S. Tsuda, I. Tanaka, K. Hikichi and S. Aimoto, *Bull. Chem. Soc. Jpn.*, 1993, **66**, 2700–2706; T. M. Hackeng, J. H. Griffin and P. E. Dawson, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 10068–10073; H. Hojo, J. Watabe, Y. Nakahara, Y. Nakahara, Y. Ito, K. Nabeshima and B. P. Toole, *Tetrahedron Lett.*, 2001, **42**, 3001–3004.

4 S. Futaki, K. Sogawa, J. Maruyama, T. Asahara, M. Niwa and H. Hojo, *Tetrahedron Lett.*, 1997, **38**, 6237–6240; X. Li, T. Kawakami and S. Aimoto, *Tetrahedron Lett.*, 1998, **39**, 8669–8672; J. Alsina, T. S. Yokum, F. Albericio and G. Barany, *J. Org. Chem.*, 1999, **64**, 8761–8769; A. B. Clippingdale, C. J. Barrow and J. D. Wade, *J. Pept. Sci.*, 2000, **6**, 225–234; D. Swinnen and D. Hilvert, *Org. Lett.*, 2000, **2**, 2439–2442; A. R. Mezo, R. P. Cheng and B. Imperiali, *J. Am. Chem. Soc.*, 2001, **123**, 3885–3891; J. Brask, F. Albericio and K. J. Jensen, *Org. Lett.*, 2003, **5**, 2951–2953; J. A. Camarero, B. J. Hackel, J. J. de Yoreo and A. R. Mitchell, *J. Org. Chem.*, 2004, **69**, 4145–4151; J. D. Warren, J. S. Miller, S. J. Keding and S. J. Danishefsky, *J. Am. Chem. Soc.*, 2004, **126**, 6576–6578; P. Botti, M. Villain, S. Manganiello and H. Gaertner, *Org. Lett.*, 2004, **6**, 4861–4864; T. Kawakami, M. Sumida, K. Nakamura, T. Vorherr and S. Aimoto, *Tetrahedron Lett.*, 2005, **46**, 8805–8807; N. Ollivier, J.-B. Behr, O. El-Mahdi, A. Blanpain and O. Melnyk, *Org. Lett.*, 2005, **7**, 2647–2650; Y. Ohta, S. Itoh, A. Shigenaga, S. Shintaku, N. Fujii and A. Otaka, *Org. Lett.*, 2006, **8**, 467–470; F. Nagaike, Y. Onuma, C. Kanazawa, H. Hojo, A. Ueki, Y. Nakahara and Y. Nakahara, *Org. Lett.*, 2006, **8**, 4465–4468; T. J. Hogenauer, Q. Wang, A. K. Sanki, A. J. Gammon, C. H. L. Chu, C. M. Kaneshiro, Y. Kajihara and K. Michael, *Org. Biomol. Chem.*, 2007, **5**, 759–762.

5 Y. Shin, K. A. Winans, B. J. Backes, S. B. H. Kent, J. A. Ellman and C. R. Bertozzi, *J. Am. Chem. Soc.*, 1999, **121**, 11684–11689; R. Ingenito, E. Bianchi, D. Fattori and A. Pessi, *J. Am. Chem. Soc.*, 1999, **121**, 11369–11374.

6 R. R. Flavell, M. Huse, M. Goger, M. Trester-Zedlitz, J. Kuriyan and T. W. Muir, *Org. Lett.*, 2002, **4**, 165–168.

7 S. Mezzato, M. Schaffrath and C. Unverzagt, *Angew. Chem., Int. Ed.*, 2005, **44**, 1650–1654.

8 H. Hojo, Y. Onuma, Y. Akimoto, Y. Nakahara and Y. Nakahara, *Tetrahedron Lett.*, 2007, **48**, 25–28; H. Hojo, Y. Onuma, Y. Akimoto, Y. Nakahara and Y. Nakahara, *Tetrahedron Lett.*, 2007, **48**, 1299.

9 D. Bang, B. L. Pentelute and S. B. H. Kent, *Angew. Chem., Int. Ed.*, 2006, **45**, 3985–3988.

10 D. Bang, B. L. Pentelute, Z. P. Gates and S. B. H. Kent, *Org. Lett.*, 2006, **8**, 1049–1052.

11 T. Kawakami and S. Aimoto, *Tetrahedron Lett.*, 1998, **39**, 7901–7904.

12 J. W. Baird, R. J. B. Nibbs, M. Komai-Koma, J. A. Connolly, K. Ottersbach, I. Clark-Lewis, F. Y. Liew and G. J. Graham, *J. Biol. Chem.*, 1999, **274**, 33496–33503; J. Morales, B. Homey, A. P. Vicari, S. Hudak, E. Oldham, J. Hedrick, R. Orozco, N. G. Copeland, N. A. Jenkins, L. McEvoy and A. Zlotnik, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 14470–14475; R. Hromas, H. E. Broxmeyer, C. Kim, K. Christopherson II and Y.-H. Hou, *Biochem. Biophys. Res. Commun.*, 1999, **258**, 737–740; I. Ishikawa-Mochizuki, M. Kitaura, M. Baba, T. Nakayama, D. Izawa, T. Imai, H. Yamada, K. Hieshima, R. Suzuki, H. Nomiyama and O. Yoshie, *FEBS Lett.*, 1999, **460**, 544–548; A. Gortz, R. J. B. Nibbs, P. McLean, D. Jarmin, W. Lambie, J. W. Baird and G. J. Graham, *J. Immunol.*, 2002, **169**, 1387–1394.

13 I. Clark-Lewis, L. Vo, P. Owen and J. Anderson, *Methods Enzymol.*, 1997, **287**, 233–250.

14 D. S. King, C. G. Fields and G. B. Fields, *Int. J. Pept. Protein Res.*, 1990, **36**, 255–266.