



Peptide Thioester Formation by an *N* to *S* Acyl Shift Reaction at the Cysteinyl Prolyl Cysteine Position

Toru Kawakami,* Sakiko Shimizu, and Saburo Aimoto*

Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871

Received January 6, 2010; E-mail: kawa@protein.osaka-u.ac.jp, aimoto@protein.osaka-u.ac.jp

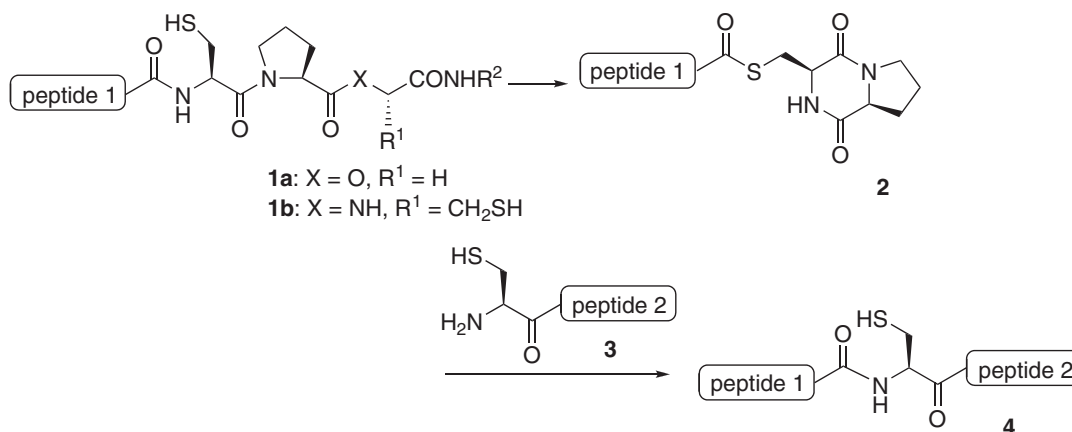
In protein synthesis, peptide thioesters are frequently used as key building blocks for the ligation strategy. We report herein on the synthesis of a peptide thioester using an *N* to *S* acyl shift reaction. A peptide containing a Cys–Pro–Cys (CPC) sequence was transformed into a peptide diketopiperazine (DKP) thioester under acidic conditions. In this reaction, the CPC moiety undergoes a tandem *N* to *S* acyl shift, followed by DKP formation to give the desired thioester. Since proteins containing a CPC sequence can be prepared by recombinant DNA techniques, the CPC peptide represents a new method for preparing peptide thioesters as recombinant proteins for protein synthesis.

The peptide thioester is one of key building blocks in protein synthesis using the ligation strategy.¹ The focus of our research has been on the use of the *N* to *S* acyl shift reaction for preparing peptide thioesters.^{2–4} A thiol-containing auxiliary,^{2,5} such as a 4,5-dimethoxy-2-mercaptobenzyl group, and a cysteine residue^{3,4,6} mediate the *N*–*S* acyl shift reaction, and can be used to produce peptide thioesters. We previously reported that a peptide containing a cysteinyl prolyl ester (CPE) moiety (CPE peptide **1a**) is spontaneously transformed into a peptide diketopiperazine (DKP) thioester **2** via an *N*–*S* acyl shift, followed by the formation of a DKP derivative (Scheme 1).⁴ The CPE peptide **1a** can be directly utilized in a ligation reaction with a peptide containing a cysteine residue at the N-terminus (Cys-peptide **3**) in a one pot reaction to give the ligated peptide **4**. In this reaction, the ester group plays a crucial role as a leaving group during DKP formation. The CPE peptide can be readily prepared by 9-fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis (SPPS) and, quite recently, a reconstituted cell free protein expression system enabled us to genetically produce the CPE peptide, which can be further transformed into a cyclic peptide.⁷ The next step was

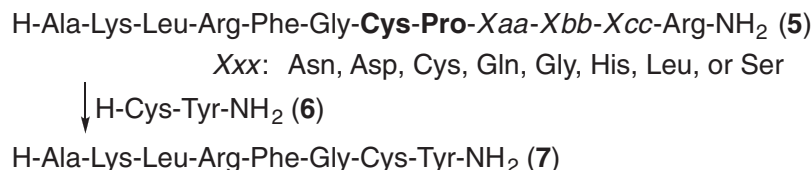
to design a novel structure without an ester group, which would make it possible to use genetically engineered proteins as peptide thioester precursors, without the need for a special translation system. We report herein on the synthesis of a peptide DKP thioester using a peptide containing a Cys–Pro–Cys sequence (CPC-peptide **1b**).

Results and Discussion

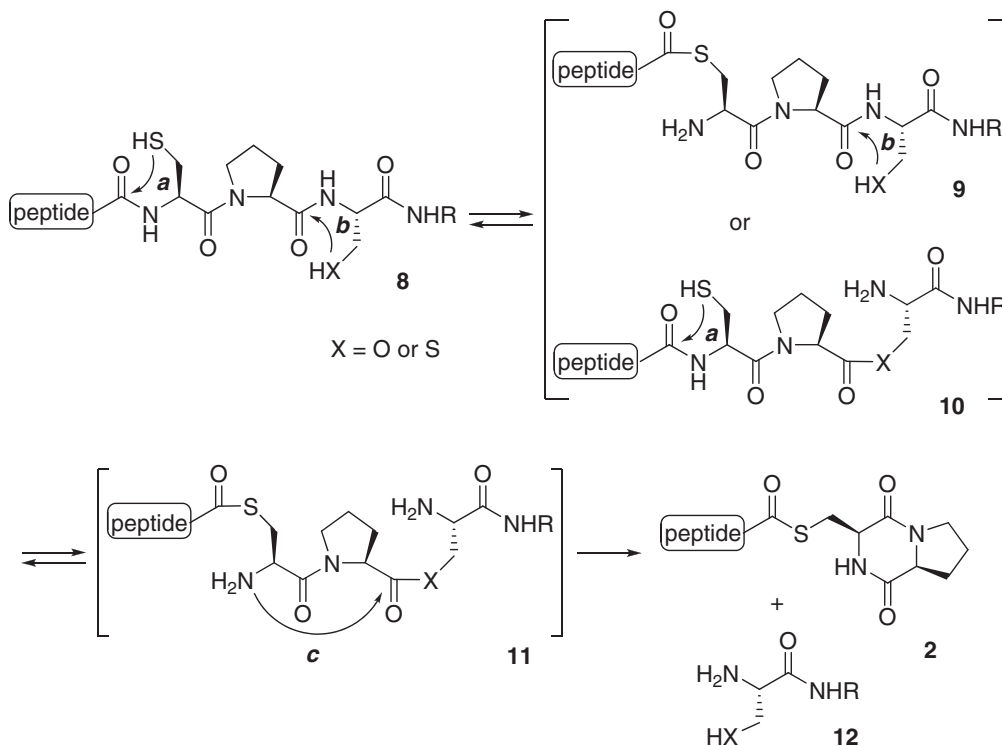
We initially prepared a peptide library containing a H–Ala–Lys–Leu–Arg–Phe–Gly–**Cys–Pro**–*Xaa–Xbb–Xcc*–Arg–NH₂ (**5**) structure, in which the Cys–Pro sequence (bold) was fixed and in which *Xaa–Xbb–Xcc* represents random sequences of 3 amino acid residues consisting of Asn, Asp, Cys, Gln, Gly, His, Leu, or Ser (Scheme 2). The library was treated with an excess amount of H–Cys–Tyr–NH₂ (**6**) in a carbonate buffer at pH 8.2. A ligated product, H–Ala–Lys–Leu–Arg–Phe–Gly–Cys–Tyr–NH₂ (**7**), would be expected to be produced, if the active sequence is contained in the library. A library, in which the *Xaa* residue was fixed with 8 different amino acid residues consisting of Asn, Asp, Cys, Gln, Gly, His, Leu, or Ser, respectively, and *Xbb* and *Xcc* contained random residues, was



Scheme 1. Peptide DKP thioester formation from CPE and CPC peptides and ligation with a Cys-peptide.



Scheme 2. Search for ligation activity in the CP library.



Scheme 3. Proposed mechanism for the formation of DKP thioesters from CPC or CPS peptides.

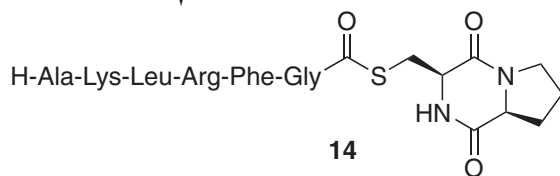
reacted with the Cys-peptide **6**. The ligated product **7** was observed as a small but distinct peak by RP-HPLC and mass spectroscopy (MS), when a Ser or Cys residue was located at the Xaa position (data not shown). When a Ser residue was located at the Xaa position and the Xbb or Xcc position was fixed with the 8 different amino acid residues, respectively, the results were similar; a signal corresponding to peptide **7** was observed in the majority of cases, although the ligation efficiency was low. The results indicate that only a Ser or Cys residue at the Xaa position is required for activation of the peptide for ligation, whereas the reaction appears to proceed in low yield under neutral conditions.

We hypothesized that this ligation reaction proceeds via the formation of the peptide DKP thioester **2** from a peptide **8** containing Cys-Pro-Ser or Cys-Pro-Cys sequences (Scheme 3). In this scenario, an N-S/O acyl shift reaction at the second Cys or Ser residue (path **b**) would result in the production of a (thio)ester structure **10**, which is structurally similar to the CPE peptide. Once the CPE structure is constructed, the ligation reaction would be expected to proceed via an N-S acyl shift reaction at the first Cys residue (path **a**) followed by DKP formation (path **c**), liberating the C-terminal Ser/Cys-peptide **12**. The order of reactions “a” and “b” would not be critical for the success of the overall reaction.

Since the equilibrium between a thioester and an amide forms at a Cys site shifts in favor of the thioester under the acidic conditions,³ in a subsequent experiment, some of the selected peptides, H-Ala-Lys-Leu-Arg-Phe-Gly-Xaa-Xbb-Xcc-Y (**13**), shown in Table 1, were treated with a 0.1 M hydrochloric acid solution at 110 °C in an evacuated, sealed tube for 2 h. In the reaction of H-Ala-Lys-Leu-Arg-Phe-Gly-Cys-Pro-Cys-NH₂ (**13a**), the mass number corresponding to the DKP derivative of thioester **14** was observed. The other major peaks were the CPC peptide **13a**, the C-terminally hydrolyzed CPC peptide, H-Ala-Lys-Leu-Arg-Phe-Gly-Cys-Pro-Cys-OH (**13a'**), and the hydrolysis product, H-Ala-Lys-Leu-Arg-Phe-Gly-OH (**15**), respectively (Figure 1A). The proportions of these compounds, based on peak areas, are shown in Table 1. The proportion of DKP thioester **14** (17%) was in good agreement with its isolated yield (12%). When a Gly residue was added after the CPC sequence (peptide **13c**), the reaction proceeded quite similarly to the reaction without the Gly residue, indicating that additional residues can be attached at the C-terminus of the CPC sequence (Entry 3). The CPC peptides with a C-terminal carboxylic acid **13b** and **13d**, which are analogs of **13a** and **13c**, respectively, gave almost the same results (Entries 2 and 4). Furthermore, the CPC peptide **13i**, which contains 5 additional amino acid residues

Table 1. Formation of DKP Thioester **14** from Peptide **13**H-Ala-Lys-Leu-Arg-Phe-Gly-*Xaa*-*Xbb*-*Xcc*-Y (**13**)

↓ 0.1 M HCl, 110 °C, 2 h



Entry	13	<i>Xaa</i>	<i>Xbb</i>	<i>Xcc</i>	<i>Y</i>	Proportion of peak area on RP-HPLC / % ^{a)}		
						13 (13') ^{b)}	14	15 ^{d)}
1	13a	Cys	Pro	Cys	NH ₂	21 (17)	17 (2) ^{c)}	26
2	13b	Cys	Pro	Cys	OH	32 (—)	19 (1) ^{c)}	25
3	13c	Cys	Pro	Cys	Gly-NH ₂	2 (34)	17 (2) ^{c)}	23
4	13d	Cys	Pro	Cys	Gly-OH	36 (—)	16 (2) ^{c)}	23
5	13e	Cys	Pro	Ser	NH ₂	33 (39)	1	16
6	13f	Cys	Pro	Gly	NH ₂	2 (61)	4	20
7	13g	Cys	Ala	Cys	NH ₂	23 (28)	4 ^{d)}	24
8	13h	Ser	Pro	Cys	NH ₂	29 (30)	7 ^{e)}	16

a) The proportions of **13**, **13'**, **14**, and **15** were determined by absorbance at 220 nm. b) In bracket the proportion of the hydrolysis product at the C-terminal amide, H-Ala-Lys-Leu-Arg-Phe-Gly-*Xaa*-*Xbb*-*Xcc*-OH (**13'**), is shown. c) In bracket the proportion of DKP thioester with an epimerized DKP moiety. d) DKP [*cyclo*(-Cys-Ala-)] thioester **17**. e) DKP [*cyclo*(-Ser-Pro-)] ester **18**. f) H-Ala-Lys-Leu-Arg-Phe-Gly-OH (**15**).

after the CPC moiety, was also transformed into the DKP thioester **14** in an isolated yield of 11% with the CPC peptide recovered in 23%, and the cleaved C-terminal peptide, H-Cys-Ala-Pro-Ala-Arg-Gly-OH (**16**) was isolated in a yield of 30% (Scheme 4). This result supports the proposed reaction mechanism shown in Scheme 3. Furthermore, the production of a peptide thioester via recombinant DNA techniques is quite promising, because the C-terminal structure after the CPC sequence is flexible and proteins containing the CPC sequence can easily be prepared as recombinant proteins.

On the other hand, when the *Xbb* or *Xcc* position of the Cys-Pro-Cys sequence was substituted with other residues, the yields of DKP thioester were decreased substantially, although similar levels of the hydrolysis product **15** was observed (Entries 5–7). This indicates that the *S*-peptide, corresponding to structure **9**, is directly hydrolyzed prior to the formation of the DKP derivative. When the Cys residue at the *Xaa* position was substituted with Ser, the corresponding DKP ester **18** was obtained (Entry 8). In comparison with the substitution at the *Xcc* position with Ser (Entry 5), a thioester might be superior to an ester (structure **11** in Scheme 3), in terms of producing a DKP structure under the acidic conditions used in this reaction.

In the reaction of CPC peptide **13a** in 0.1 M hydrochloric acid, extensive hydrolysis was observed while the CPC peptide continued to be present. Mass numbers corresponding to the DKP, *cyclo*(-Cys-Pro-), and H-Cys-Pro-Cys-OH were observed in the reaction mixture, which indicated that the hydrolysis occurred before and after the formation of the

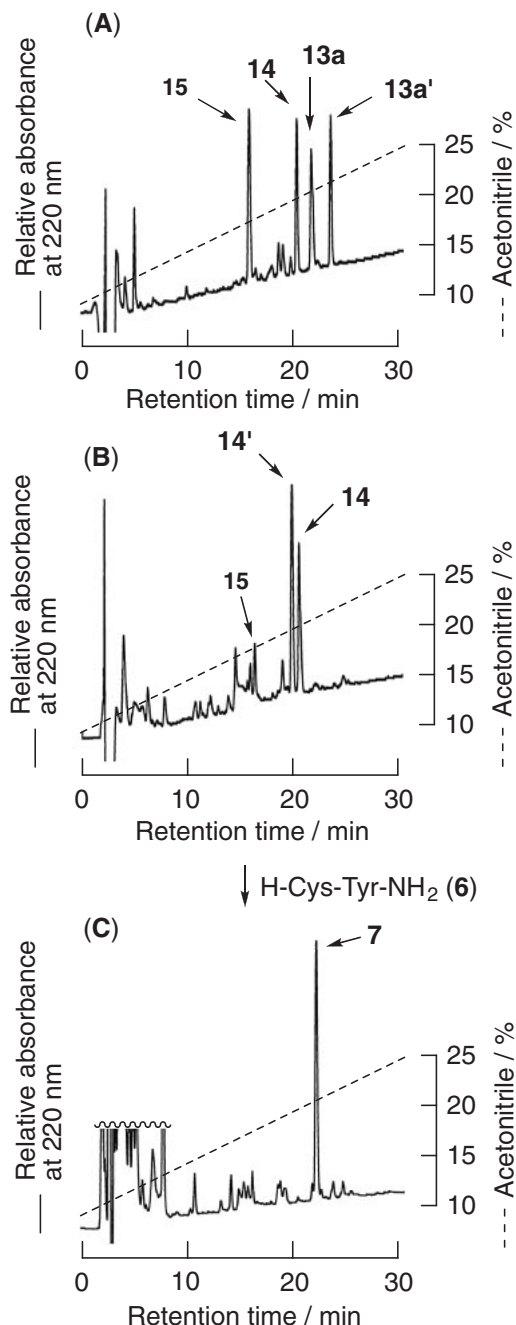


Figure 1. RP-HPLC elution profiles of reaction mixtures. DKP thioester formation from CPC peptide **13a** (A) in 0.1 M HCl at 110 °C for 2 h and (B) in HFBA at 110 °C for 4 h. (C) Ligation of the crude DKP thioester, prepared in HFBA, and Cys-peptide **6**. Column: YMC-Pack ProC18 (4.6 × 150 mm). Eluent: 0.1% TFA in aq acetonitrile, 1.0 mL min⁻¹.

DKP thioester. To reduce the extent of hydrolysis, the reaction was carried out in heptafluorobutyric acid (HFBA) vapor at 110 °C in an evacuated, sealed tube. After a 4-h reaction, the CPC peptide **13a** had disappeared and the extent of hydrolysis was reduced (Figure 1B). A peak corresponding to DKP thioester **14** was observed and a new peak, indicated as **14'** in the figure, appeared, whose mass number was identical to that

H-Ala-Lys-Leu-Arg-Phe-Gly-Cys-Pro-Cys-Ala-Pro-Ala-Arg-Gly-OH (**13i**)**14** + H-Cys-Ala-Pro-Ala-Arg-Gly-OH (**16**)**Scheme 4.** DKP thioester formation and cleavage of the C-terminal portion from the CPC peptide.

for DKP thioester **14**. DKP thioester **14'** was determined to be an epimer of **14** in the DKP moiety, *cyclo*-(Cys(*R*)-Pro-), which was confirmed by RP-HPLC, in which product **14'** co-eluted with the epimers in the DKP moiety, although *cyclo*-(D-Cys(*R*)-Pro-) and *cyclo*-(Cys(*R*)-D-Pro-) were not separated on a C18 column. Although DKPs are known to be epimerized easily in the presence of a base,⁸ it is not clear at this stage that the DKP was epimerized in the formation in HFBA. The crude mixture of DKP peptides **14** and **14'** was reacted with an excess amount of Cys-peptide **6** to give a single ligated isomer **7** in a yield of 17% based on CPC peptide **13a** (Figure 1C).

Conclusion

In conclusion, a peptide containing a Cys-Pro-Cys (CPC) sequence can be transformed into a peptide DKP thioester and a C-terminal peptide with the N-terminal Cys residue. The reaction required acidic conditions and a high temperature, and current efforts are directed toward optimizing the reaction. It is noteworthy that recombinant proteins containing the CPC sequence would be transformed into the thioester, since proteins containing a CPC sequence can be prepared by recombinant DNA techniques. So far, the peptide thioesters are prepared, via recombinant DNA techniques, by the use of intein domains, which catalyze protein splicing reactions.⁹ The CPC peptide will provide an alternate approach for preparing recombinant peptide thioesters.

Experimental

Peptide Synthesis. Peptides were prepared by the standard Fmoc solid phase peptide synthesis (SPPS) by using an automated peptide synthesizer, ACT440Ω (aapptec, Louisville, KY), or a manual procedure by employing *N,N*-diisopropylcarbodiimide and 1-hydroxybenzotriazole as activating reagents. Peptides were cleaved from the solid support by treatment with reagent B,¹⁰ purified by RP-HPLC on a C18 column using a linear increasing gradient of acetonitrile in water/0.1% TFA, and confirmed by MALDI-TOF MS on a Bruker AutoFLEX spectrometer or ESI-MS on a Thermo Finnigan LCQ™ DECA XP spectrometer.

Preparation of Peptide Library 5. A library was prepared by the split-and-mix synthesis method.¹¹ A typical procedure for the synthesis of a peptide containing a Ser residue at the *Xaa* site is as follows: After Fmoc-Arg(Pbf)-OH was introduced to a Rink amide resin, the resin was divided into 8 aliquots. To each resin, 8 different amino acid derivatives, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O^tBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Leu-OH, or Fmoc-Ser(^tBu)-OH, were separately introduced. All resins were combined, and divided into 8 aliquots again. To each resin, 8 different amino acid derivatives were

separately introduced. After combining all of the resins again, the peptide chain was elongated by the usual SPPS to give a H-Ala-Lys(Boc)-Leu-Arg(Pbf)-Phe-Gly-Cys(Trt)-Pro-Ser(^tBu)-Xbb-Xcc-Arg(Pbf)-NH-resin, in which the *Xaa* position was fixed with a Ser residue, and both *Xbb* and *Xcc* positions contained 8 different amino acid residues. The peptides were cleaved from the resin, and partially purified by RP-HPLC, in which the peptide-fractions were combined together and lyophilized to give a library of H-Ala-Lys-Leu-Arg-Phe-Gly-Cys-Pro-Ser-Xbb-Xcc-Arg-NH₂.

Reaction of Peptide Library 5 with Cys-peptide 6. A typical reaction procedure is as follows: A peptide library **5** (0.5 mg) was treated with Cys-peptide **6** (0.5 mg) in a carbonate buffer solution (pH 8.2) (60 μL) containing 20 mM tris(2-carboxyethyl)phosphine (TCEP) at room temperature for 24 h. After adding a solution of 0.2 M dithiothreitol (DTT) (0.12 mL), followed by stirring for 1 h, the reaction mixture was analyzed by RP-HPLC with detection by absorbance at 220 and 280 nm, and the fraction containing a ligated product **7** was analyzed by MS. In the reactions in which the *Xaa* residue was fixed with a Cys or Ser residue, a ligated peptide was observed both by RP-HPLC and MS. **7**: MALDI-TOF: found *m/z* 956.7, calcd for (M + H)⁺ 956.5.

Formation of Peptide DKP Thioester 14 under Acidic Conditions. Typical Procedures: 0.1 M HCl: A solution of peptide **13** (0.20 μmol) containing 10 mM TCEP and 0.1 M HCl (0.20 mL) in an evacuated, sealed tube was heated at 110 °C for 2 h. The reaction mixture (30 μL) was directly analyzed by RP-HPLC, with detection by absorbance at 220 nm, and the fractions were applied to ESI-MS. Isolated yield was determined by quantitative amino acid analysis.

HFBA vapor:¹² A small glass tube (6 mm × 30 mm), containing lyophilized peptide **13** (0.20 μmol), was placed in a large glass tube (12 mm × 105 mm), that contained HFBA (0.10 mL), evacuated and then flame sealed. It was incubated at 110 °C for 4 h. A 50% aqueous acetonitrile solution was added to the reaction mixture, which was then lyophilized. The crude mixture was dissolved in 5% acetonitrile (0.10 mL), an aliquot of the solution (30 μL) was analyzed by RP-HPLC and the fractions were analyzed by ESI-MS.

13a: MS (ESI): found *m/z* 993.7, calcd for (M + H)⁺ 993.5; amino acid analysis: Pro_{nd}Gly₁Ala_{0.91}Cys_{nd}Leu_{0.90}Phe_{0.87}-Lys_{0.98}Arg_{0.93}.

13b: MS (ESI): found *m/z* 994.6, calcd for (M + H)⁺ 994.5; amino acid analysis: Pro_{nd}Gly₁Ala_{0.97}Cys_{nd}Leu_{0.97}Phe_{0.99}-Lys_{0.97}Arg_{0.97}.

13c: MS (ESI): found *m/z* 1050.5, calcd for (M + H)⁺ 1050.5; amino acid analysis: Pro_{nd}Gly₂Ala_{0.97}Cys_{nd}Leu_{0.98}-Phe_{0.99}Lys_{1.0}Arg_{0.98}.

13d: MS (ESI): found *m/z* 1051.5, calcd for (M + H)⁺ 1051.5; amino acid analysis: Pro_{nd}Gly₂Ala_{0.99}Cys_{nd}Leu_{0.98}-

Phe_{0.99}Lys_{0.98}Arg_{0.96}.

13e: MS (ESI): found m/z 977.7, calcd for (M + H)⁺ 977.5; amino acid analysis: Ser_{0.86}Pro_{nd}Gly₁Ala_{0.98}Cys_{nd}Leu_{0.98}-Phe_{0.93}Lys_{0.98}Arg_{0.97}.

13f: MS (ESI): found m/z 947.5, calcd for (M + H)⁺ 947.5; amino acid analysis: Pro_{nd}Gly₂Ala_{1.0}Cys_{nd}Leu_{0.99}Phe_{0.91}-Lys_{1.0}Arg_{1.0}.

13g: MS (ESI): found m/z 967.7, calcd for (M + H)⁺ 967.5; amino acid analysis: Gly₁Ala_{2.0}Cys_{nd}Leu_{0.96}Phe_{0.92}Lys_{0.98}-Arg_{0.98}.

13h: MS (ESI): found m/z 977.7, calcd for (M + H)⁺ 977.5; amino acid analysis: Ser_{0.83}Pro_{nd}Gly₁Ala_{0.99}Cys_{nd}Leu_{0.97}-Phe_{0.95}Lys_{0.99}Arg_{0.97}.

13i: MS (ESI): found m/z 1446.7, calcd for (M + H)⁺ 1446.7; amino acid analysis: Pro_{nd}Gly₂Ala_{2.9}Cys_{nd}Leu_{0.98}-Phe_{1.0}Lys_{0.99}Arg_{2.0}.

14: MS (ESI): found m/z 873.5, calcd for (M + H)⁺ 873.5; amino acid analysis: Pro_{nd}Gly₁Ala_{1.0}Cys_{nd}Leu_{0.96}Phe_{0.99}-Lys_{0.97}Arg_{1.0}.

14': MS (ESI): found m/z 873.5, calcd for (M + H)⁺ 873.5; amino acid analysis: Pro_{nd}Gly₁Ala_{1.2}Cys_{nd}Leu_{0.96}Phe_{1.0}Lys_{0.96}-Arg_{0.99}.

15: MS (ESI): found m/z 691.6, calcd for (M + H)⁺ 691.4; amino acid analysis: Gly₁Ala_{1.0}Leu_{0.96}Phe_{0.99}Lys_{0.98}Arg_{1.0}.

16: MS (ESI): found m/z 574.3, calcd for (M + H)⁺ 574.3; amino acid analysis: Pro_{nd}Gly₁Ala_{2.2}Cys_{nd}Arg_{1.1}.

17: MS (ESI): found m/z 847.5, calcd for (M + H)⁺ 847.5.

18: MS (ESI): found m/z 857.7, calcd for (M + H)⁺ 857.5.

Ligation of Peptide DKP Thioester 14 with Cys-peptide

6. Peptide thioester **14** was prepared by the reaction of CPC peptide **13a** (0.2 mg, 0.20 μ mol) in HFBA vapor in an evacuated, sealed tube at 110 °C for 4 h. After the addition of aqueous acetonitrile, followed by lyophilization, the crude material was reacted with Cys-peptide **6** (0.5 mg, 1.8 μ mol) in a tricine buffer solution (pH 8.2, 0.10 mL) containing 10 mM TCEP at 37 °C for 1 h. Ligated peptide **7** was isolated by RP-HPLC and the yield was determined by quantitative amino acid analysis to be 17% based on CPC peptide **13a**. **7:** ESI-MS: found m/z 956.6, calcd for (M + H)⁺ 956.5; amino acid analysis: Gly₁Ala_{1.0}Cys_{nd}Leu_{0.98}Tyr_{0.82}Phe_{0.90}Lys_{1.2}Arg_{1.0}.

This research was supported, in part, by The Naito Foundation and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

- a) C. P. R. Hackenberger, D. Schwarzer, *Angew. Chem., Int. Ed.* **2008**, *47*, 10030. b) T. W. Muir, *Annu. Rev. Biochem.* **2003**, *72*, 249. c) S. Aimoto, *Curr. Org. Chem.* **2001**, *5*, 45. d) P. E. Dawson, S. B. H. Kent, *Annu. Rev. Biochem.* **2000**, *69*, 923. e) S. Aimoto, *Biopolymers* **1999**, *51*, 247.
- a) T. Kawakami, M. Sumida, K. Nakamura, T. Vorherr, S. Aimoto, *Tetrahedron Lett.* **2005**, *46*, 8805. b) K. Nakamura, H. Mori, T. Kawakami, H. Hojo, Y. Nakahara, S. Aimoto, *Int. J. Pept. Res. Ther.* **2007**, *13*, 191. c) K. Nakamura, T. Kanao, T. Uesugi, T. Hara, T. Sato, T. Kawakami, S. Aimoto, *J. Pept. Sci.* **2009**, *15*, 731.
- K. Nakamura, M. Sumida, T. Kawakami, T. Vorherr, S. Aimoto, *Bull. Chem. Soc. Jpn.* **2006**, *79*, 1773.
- a) T. Kawakami, S. Aimoto, *Chem. Lett.* **2007**, *36*, 76. b) T. Kawakami, S. Aimoto, *Tetrahedron Lett.* **2007**, *48*, 1903. c) T. Kawakami, S. Aimoto, *Tetrahedron* **2009**, *65*, 3871.
- a) N. Ollivier, J.-B. Behr, O. El-Mahdi, A. Blanpain, O. Melnyk, *Org. Lett.* **2005**, *7*, 2647. b) Y. Ohta, S. Itoh, A. Shigenaga, S. Shintaku, N. Fujii, A. Otaka, *Org. Lett.* **2006**, *8*, 467. c) F. Nagaïke, Y. Onuma, C. Kanazawa, H. Hojo, A. Ueki, Y. Nakahara, Y. Nakahara, *Org. Lett.* **2006**, *8*, 4465. d) H. Hojo, Y. Omura, Y. Akimoto, Y. Nakahara, Y. Nakahara, *Tetrahedron Lett.* **2007**, *48*, 25.
- J. Kang, J. P. Richardson, D. Macmillan, *Chem. Commun.* **2009**, 407.
- T. Kawakami, A. Ohta, M. Ohuchi, H. Ashigai, H. Murakami, H. Suga, *Nat. Chem. Biol.* **2009**, *5*, 888.
- G. G. Smith, R. C. Evans, R. Baum, *J. Am. Chem. Soc.* **1986**, *108*, 7327.
- a) V. Muralidharan, T. W. Muir, *Nat. Methods* **2006**, *3*, 429. b) L. Saleh, F. B. Perler, *Chem. Rec.* **2006**, *6*, 183.
- N. A. Sole, G. Barany, *J. Org. Chem.* **1992**, *57*, 5399.
- A. Furka, F. Sebestyén, M. Asgedom, G. Dibó, *Int. J. Pept. Protein Res.* **1991**, *37*, 487.
- A. Tsugita, K. Takamoto, M. Kano, H. Iwadate, *Eur. J. Biochem.* **1992**, *206*, 691.