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The use of a cysteinyl prolyl ester (CPE) autoactivating unit in peptide ligation reactions

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ABSTRACT

A peptide containing a cysteinyl prolyl ester (CPE) moiety at the C-terminus (CPE peptide) is spontaneously transformed into a diketopiperazine thioester via an intramolecular *N–S* acyl shift reaction, followed by diketopiperazine formation. The CPE peptide can be ligated with a Cys-peptide in a one-pot procedure. The peptide diketopiperazine thioester can also be transformed into a peptide thioester by intermolecular thiol–thioester exchange with external thiol compounds such as sodium mercaptoethanesulfonate. Since CPE peptides can be prepared by standard Fmoc solid-phase synthesis, it is a versatile alternative to the peptide thioester, providing a flexible ligation strategy that promises to be useful in polypeptide synthesis.

1. Introduction

Chemical ligation methodology is a powerful and useful strategy for protein synthesis. The method involves the use of a peptide thioester as a building block, which can be prepared by either chemical or biological methods.[1] In the thioester method, a partially protected peptide thioester is used as a building block and condensed in the presence of silver ions as an activating reagent for the thioester.[2] Native chemical ligation permits chemoselective ligation, in which an unprotected peptide thioester can be ligated with a cysteinyl peptide in an aqueous buffer solution.[3] In the extended chemical ligation strategy, a thiol auxiliary, attached to the N-terminal amino group, is used instead of a cysteine residue and an unprotected peptide thioester is used as a building block, thus retaining the advantageous features of the native chemical ligation reaction.[4,5]

Although these ligation methods were originally developed for use in conjunction with a peptide thioester, improved methods for preparing such thioesters would be highly desirable. Peptide thioesters can be prepared in a straightforward manner using *tert*-butoxycarbonyl (Boc) solid-phase peptide synthesis (SPPS) methodology. Starting from a thioester resin, such as Boc-Gly-SCH_2CH_2CO-β-Ala-MBHA resin, the peptide chain is elongated and cleaved by acidolysis by treatment with an acid, such as anhydrous hydrogen fluoride to give the peptide thioester.[2,6] While a peptide thioester can be readily prepared by the Boc SPPS method, the preparation of glycosylated[7] or phosphorylated[8] thioesters remains a formidable task. In addition, the preparation of a peptide thioester using the 9-fluorenylmethoxycarbonyl (Fmoc) SPPS method continues to be a challenge, because the thioester decomposes in the presence of piperidine, a reagent that is commonly used to remove the Fmoc group. Weak nucleophilic bases can be used in a direct synthesis: a mixture of 1-methylpyrrolidine, hexamethylenimine, and 1-hydroxybenzotriazole (HOBt) can be used to remove the Fmoc group, thus permitting the isolation of the intact thioester. Using this approach, a peptide thioester can be prepared in a straightforward manner,[9] but the chiral amino acid residue at the thioester position is racemized to some extent during the reaction.[10] Indirect methods have also been developed. For example, a peptide chain can be elongated on the sulfonamide linker, and the linker can then be activated and replaced by an external thiol.[11] This procedure is in widespread use, but has some drawbacks. For example, the sulfonamide moiety can undergo acetylation during the capping cycle[12] and methionine residues are frequently alkylated during the activation step.[13] A number of synthetic methods for preparing peptide thioesters based on the Fmoc SPPS procedure have been reported. These methods include the use of a Wang linker, which is replaced by a thiol,[14] an aryl hydrazine linker,[15] an in situ O to *S* acyl shift reaction,[16] trithioortho esters,[17] and an *O*-aminoanilide moiety, which is activated to give the *N*-acylurea, and thiolysis to give

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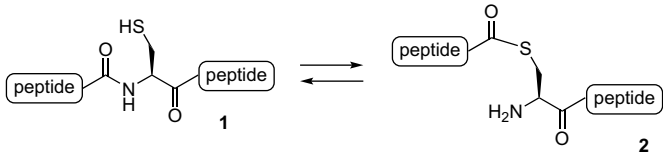
a thioester.[18] Each method may overcome some of the difficulties associated with peptide thioester synthesis by the Fmoc SPPS procedure.

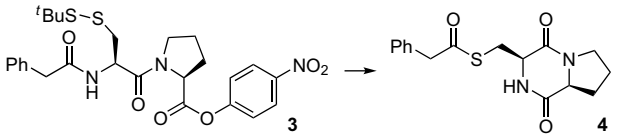
In our strategy, the basis for preparing a peptide thioester involves an *N* to *S* acyl shift reaction. We previously reported on a method for preparing peptide thioesters, based on an *N*–*S* acyl shift reaction mediated by a 4,5-dimethoxy-2-mercaptobenzyl (Dmmb) group,[19] which was originally reported as a removable auxiliary for extended chemical ligation.[4c] The Dmmb group, when attached to an amide, can be removed by treatment with a strong acid, such as trifluoromethanesulfonic acid, whereas an *S*-peptide (thioester intermediate) is formed under weakly acidic conditions, such as in the presence of trifluoroacetic acid (TFA), as the result of an intramolecular *N*–*S* acyl shift reaction.[20] The *S*-peptide can be transformed into the corresponding stable peptide thioester by an intermolecular thiol–thioester exchange reaction.[19–21] The *N*–*S* acyl shift reaction was also used to prepare peptide thioesters by other groups.[22]

The Cys-containing peptide **1** can also be converted into *S*-peptide **2** by an intramolecular *N*–*S* acyl shift reaction under acidic conditions such as in the presence of TFA (Scheme 1).[20] This *S*-peptide can easily be transformed into the original peptide even under weakly acidic conditions such as a 0.1% TFA solution, which is frequently used in RP-HPLC eluents.[20] These findings suggest that the amide and thioester forms of the Cys-containing peptide are present in equilibrium and that the amide form is the dominant species. If the equilibrium could be controlled by blocking the amino group, which forms as the result of the *N*–*S* acyl shift reaction, it would be possible to produce a peptide thioester based on an *N*–*S* acyl shift reaction at the Cys residue.



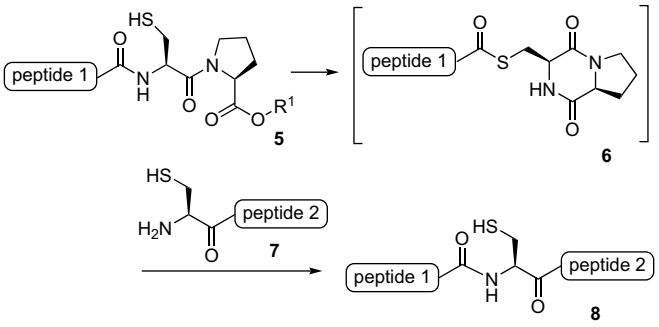
Scheme 1. Equilibrium between amide and thioester at the cysteine residue.

In 1985, Zanotti et al. reported on a reaction in which the phenylacetyl cysteinyl proline *p*-nitrophenyl active ester **3** is converted into the diketopiperazine (DKP) thioester **4** under reductive conditions (Scheme 2).[23] The reaction would be predicted to proceed via the reduction of the disulfide, a subsequent *N*–*S* acyl shift reaction, followed by diketopiperazine formation with the proline active ester. As a result, a peptide thioester is spontaneously produced by an intramolecular reaction. This reaction would be ideal for blocking the amino group, since it would stabilize the thioester and prevent the *S*–*N* acyl shift reaction from occurring.



Scheme 2. Formation of the thioester of DKP.[23]

To test this hypothesis, we designed a cysteinyl prolyl ester (CPE) unit, without thioester and active ester moieties in the peptide (Scheme 3).[24] We expected that this CPE peptide **5** would be spontaneously transformed into the C^{α}-DKP thioester **6** by an intramolecular reaction, and would then react with a cysteinyl peptide **7** in the same manner as occurs in the case of native chemical ligation. The CPE peptide is an alternative to the peptide thioester, and can be prepared by standard Fmoc (SPPS) because it



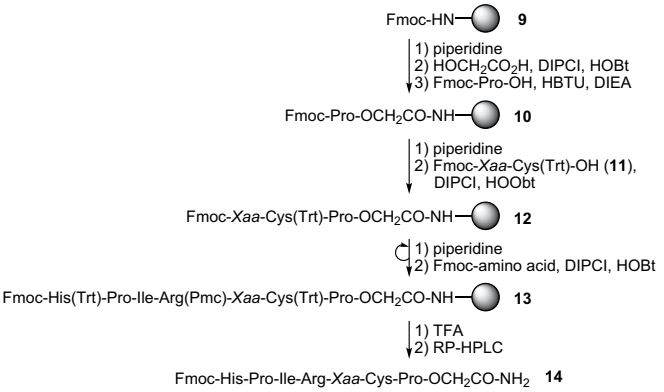
Scheme 3. Thioester formation and ligation of CPE peptide.

contains no thioester moiety itself. Moreover, the autoactivating function of the CPE unit can be quenched by introducing a thiol blocking group in the Cys residue, which would prevent inter- and intramolecular ligation itself, and the direction of ligation at the N or C terminus could be controlled,[25] thus providing a flexible ligation strategy for polypeptide synthesis using multi-component peptide building blocks. In this report, we provide a detailed description of such thioester formation and ligation using the CPE autoactivating unit.

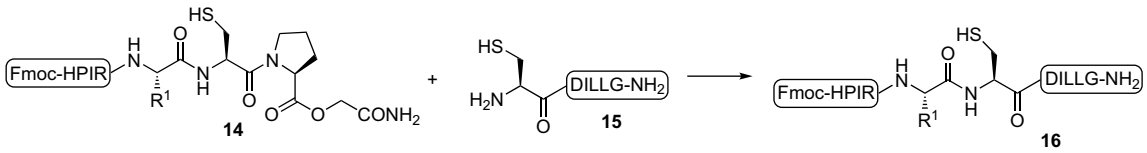
2. Results and discussion

2.1. Preparation of a peptide containing CPE unit

A peptide containing the CPE unit can be readily prepared by standard Fmoc SPPS because no thioester bond is present during the chain elongation cycles. A typical procedure is shown in the model peptide synthesis (Scheme 4). Glycolic acid was first attached to the Rink amide resin **9**, and Fmoc-Pro-OH was then introduced. It was necessary to introduce the dipeptide, Fmoc-*Xaa*-Cys(Trt)-OH (**11**), in order to avoid DKP formation from Cys-Pro ester residues. Dipeptide **11** was conveniently prepared by one-pot reaction of Fmoc-*Xaa*-OSu, which was formed by mixing Fmoc-*Xaa*-OH, *N*-hydroxysuccinimide (HOSu), and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, with H-Cys(Trt)-OH in the presence of *N*,*N*-diisopropylethylamine (DIEA) in a mixed solvent of dichloromethane and *N*,*N*-dimethylformamide (DMF) (1:1). When dipeptide **11** was introduced using 1-hydroxybenzotriazole (HOBt) in combination with diisopropylcarbodiimide (DIPCI), the model peptide contained ca. 40% D-Cys residues. The racemization was reduced to less than 10%, when 3-hydroxy-1,2,3-bezotriazin-4(3*H*)-one (HOObt)[26] was used in place of HOBt. The peptide chain was then elongated by standard Fmoc chemistry to give the protected peptide resin, Fmoc-His(Trt)-Pro-Ile-Arg(Pmc)-



Scheme 4. Preparation of CPE peptide.



Scheme 5. CPE ligation.

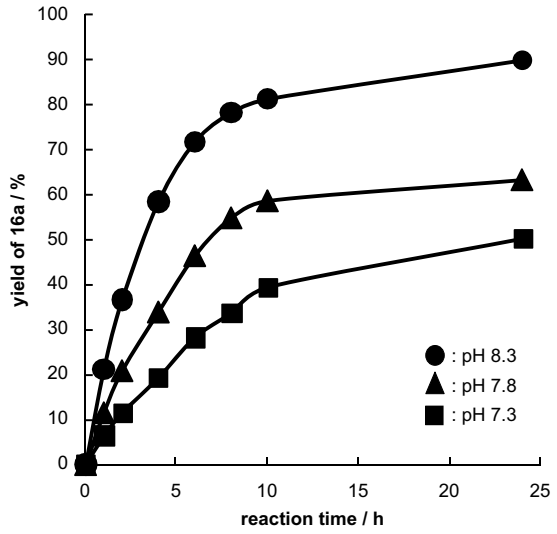


Figure 1. Time course for the ligation of Fmoc-His-Pro-Ile-Arg-Gly-Cys-Pro-OCH_2CONH_2 (**14a**) with Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (**15**) in the range of pH 7.3–8.3 at 37 °C to yield Fmoc-His-Pro-Ile-Arg-Gly-Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (**16a**). pH: 8.3 (●), 7.8 (▲), 7.3 (■).

Xaa-Cys(Trt)-Pro-OCH_2CONH-resin (**13**). Finally, the CPE peptide, Fmoc-His-Pro-Ile-Arg-*Xaa*-Cys-Pro-OCH_2CONH_2 (**14**) (*Xaa*=**a**, Gly; **b**, Ala; **c**, Val; **d**, Ser) was obtained by treatment with TFA, followed by RP-HPLC purification. Isolated yields were 30% (**14a**), 22% (**14b**), 20% (**14c**), 26% (**14d**), respectively, based on the Fmoc content of the Fmoc-Pro-OCH_2CO-NH-resin (**10**).

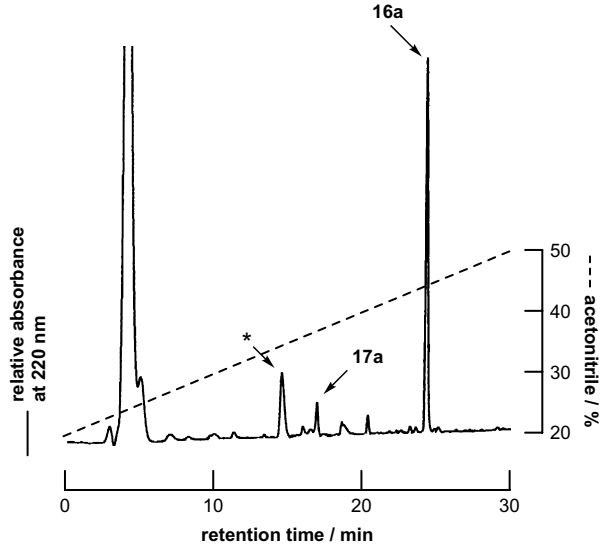


Figure 2. RP-HPLC elution profile of a reaction mixture of Fmoc-His-Pro-Ile-Arg-Gly-Cys-Pro-OCH_2CONH_2 (**14a**) and Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (**15**) at pH 8.3 and 37 °C for 24 h giving Fmoc-His-Pro-Ile-Arg-Gly-Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (**16a**). * non-peptidic compound. Column: YMC-Pack ProC18 (4.6×150 mm), eluent: 0.1% TFA in aqueous acetonitrile, 1.0 mL/min.

2.2. Direct ligation of CPE peptide with Cys-peptide

We assumed that the peptide containing the CPE unit would be spontaneously transformed into the DKP thioester in neutral buffer solutions, and when mixed with a Cys-peptide, the ligation reaction would occur in one pot. The CPE peptide, Fmoc-His-Pro-Ile-Arg-Gly-Cys-Pro-OCH_2CONH_2 (**14a**), was reacted with a Cys-peptide, Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (**15**) in sodium phosphate buffer solutions in the range of pH 7.3–8.3 at 37 °C (Scheme 5). The ligated product, Fmoc-His-Pro-Ile-Arg-Gly-Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (**16a**) was produced. The time course for product yield is shown in Figure 1. The rate of the reaction increased at higher pH. At pH 8.3, the yield of ligated peptide reached 90% after 24 h (Fig. 2). A small peak (5%), corresponding to the hydrolysis product, Fmoc-His-Pro-Ile-Arg-Gly-OH (**17a**) was observed on the RP-HPLC elution profile.

Ligations of different amino acid residues, Gly, Ala, Val, and Ser, were carried out under similar reaction conditions. Table 1 shows data on the yields of products after a 24-h reaction at pH 8.4. Ligated products **16b**–**d** were obtained in good yields similar to that for **16a**. In the case of ligation at chiral amino acid residues, small amounts of epimerized product were observed. The following D-amino acid-containing ligated peptides were observed: 1% (D-Ala), 2% (D-Val), or 5% (D-Ser), respectively. Epimerization of the Ser residue during the ligation was slightly higher than for amino acids containing aliphatic side chains. It has also been reported that the highly reactive His-thioester undergoes slight epimerization during ligation,[5f,27] but that this can be suppressed by conducting the reaction at a lower pH (vide infra).

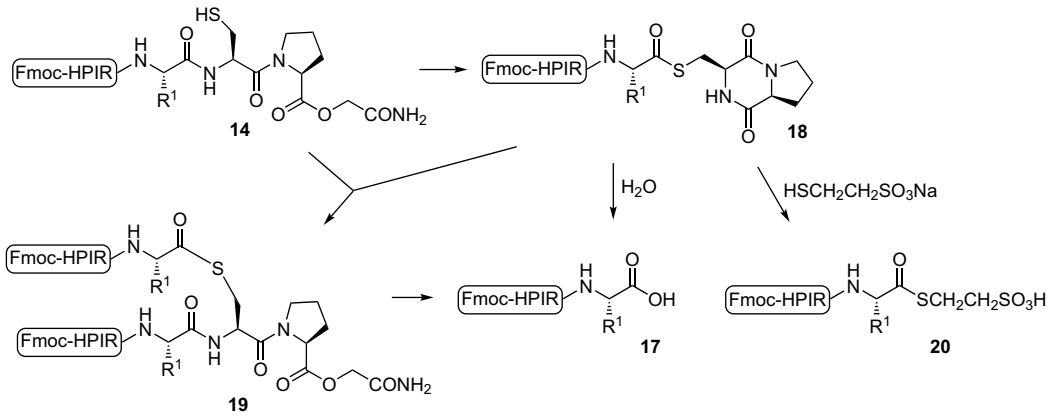
2.3. Transformation of CPE peptide into peptide thioester

In order to investigate the reaction of the CPE peptide in more detail, the CPE peptide **14a** was treated without a Cys-peptide in a sodium phosphate buffer (pH 8.0), and subjected to RP-HPLC and mass analysis. On the RP-HPLC, a small peak, corresponding to the DKP thioester **18a** (R^1=H) was observed, which further reacted with peptide **14a** to give a branched dimeric thioester **19a** (R^1=H) (Scheme 6, Fig. 3). The hydrolysis product **17a** was produced in the prolonged reaction and, after a 24-h reaction, **17a** was produced as the main product. When an excess of sodium

Table 1
CPE ligation yields for different amino acid residues[a]

Entry	Peptide (*Xaa*)	Yield[b,c]/%	Epimerization D-*Xaa*[b]/%
1	**16a** (Gly)	82	—
2	**16b** (Ala)	72	1
3	**16c** (Val)	74	2
4	**16d** (Ser)	75	5

[a] Fmoc-His-Pro-Ile-Arg-*Xaa*-Cys-Pro-OCH_2CONH_2 (**14**) was ligated with Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (**15**) at pH 8.4 and 37 °C for 24 h to give Fmoc-His-Pro-Ile-Arg-*Xaa*-Cys-Asp-Ile-Leu-Leu-NH_2 (**16**).
[b] Determined by RP-HPLC.
[c] Excluding epimerized products.



Scheme 6. Thioester formation from the CPE peptide.

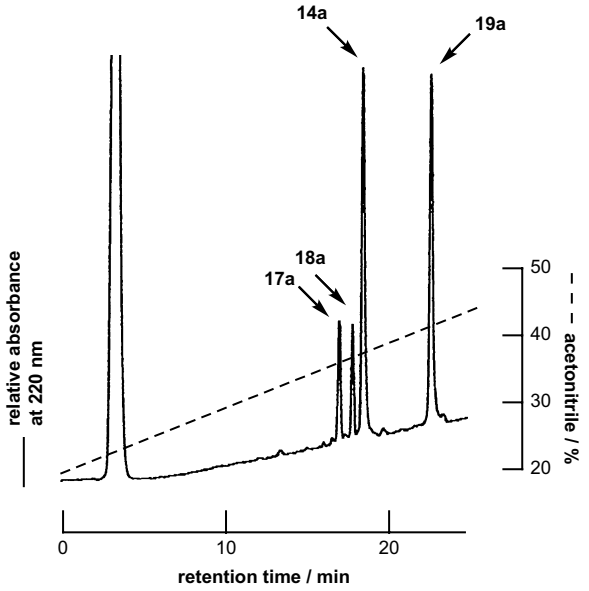


Figure 3. RP-HPLC elution profile for the reaction of Fmoc-His-Pro-Ile-Arg-Gly-Cys-Pro-OCH_2CONH_2 (**14a**) in sodium phosphate buffer (pH 8.0) for 8 h. The DKP thioester **18a** was produced along with the branched dimeric thioester **19a**, a hydrolysis product **17a**, and DKP, *cyclo*(-Cys-Pro-). Column: YMC-Pack ProC18 (4.6×150 mm), eluent: aq acetonitrile containing 0.1% TFA, flow rate: 1.0 mL/min.

2-mercaptoethanesufonate was added to the reaction mixture, the peptide thioester, Fmoc-His-Pro-Ile-Arg-Gly-$SCH_2CH_2SO_3H$ (**20a**) was formed in 72% yield, after a 6-h reaction at pH 8.8 and 37 °C (Fig. 4). When the reaction was continued, the thioester was gradually hydrolyzed. The time course for the reaction is shown in Figure 5(A). When the pH of the reaction buffer was reduced to below 8, thioester formation was slower, but the thioester that was formed, was relatively stable to hydrolysis (Fig. 5(B)).

The CPE peptides containing four different amino acid residues, Gly, Ala, Val, and Ser at the thioesterification sites were applied to the formation of the peptide thioesters **20** by reaction with sodium 2-mercaptoethanesufonate (Fig. 6). At pH 8.2 and 37 °C, the rates of thioester formation were very similar for all of the different amino acid residues used, and the yield of thioester reached about 70% after 6 h. The thioesters of Gly, Ala, and Ser underwent gradual hydrolysis with increasing reaction time, but the Val-thioester was stable after a 24-h reaction. A thioester with a bulky side chain group would likely be more resistant to hydrolysis. Epimerization was observed to some extent in cases of chiral amino acid residues of thioesters. The extent of epimerization was as follows: 5% (**20b**, Ala), 1% (**20c**, Val), and 26% (**20d**, Ser), respectively, after a 6-h reaction. The extent of epimerization of the Ser residue was exceptionally high and, when examined more closely, it was found to increase with reaction time. The epimerization of Ser-thioester **20d** increased from 12% (2 h) to 26% (6 h). The isolated peptide thioester of L-Ser **20d** underwent epimerization when treated at pH 8.2. These findings clearly indicate that epimerization occurs after thioester formation. The α-proton of the thioester is acidic, is deprotonated in the presence of a base and, as a result, the amino acid undergoes racemization.[28] The order of racemization was in the order of Ser>Ala>Val, of the amino acid residues tested in this study, which is in good agreement with results observed for the alkaline hydrolysis of peptides.[29] Amino acid residues with aliphatic side chains, such as Val residue, would be expected to be more resistant to deprotonation, and, in the case of an electron-withdrawing group such as a Ser residue, deprotonation would be accelerated. When the CPE peptide **14d** was reacted with $HSCH_2CH_2SO_3Na$ at pH 7.3, Ser-thioester **20d** was formed in 65% yield after 10 h and 80% after 24 h, and the degree of epimerization was 9% and 16%, respectively (Fig. 6). Under lower pH conditions, the thioester was relatively stable to hydrolysis, and epimerization

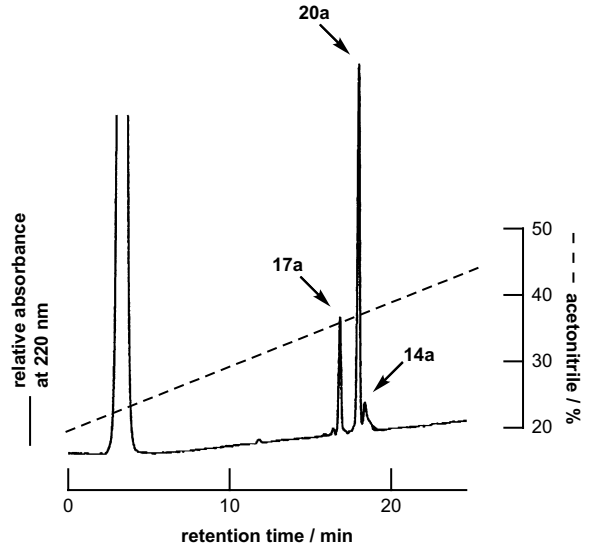


Figure 4. RP-HPLC elution profile for the reaction of a mixture of Fmoc-His-Pro-Ile-Arg-Gly-Cys-Pro-OCH_2CONH_2 (**14a**) and $HSCH_2CH_2SO_3Na$ at pH 8.8 and 37 °C for 6 h to give Fmoc-His-Pro-Ile-Arg-Gly-$SCH_2CH_2SO_3H$ (**20a**). Column: YMC-Pack ProC18 (4.6×150 mm), eluent: 0.1% TFA in aqueous acetonitrile, 1.0 mL/min.

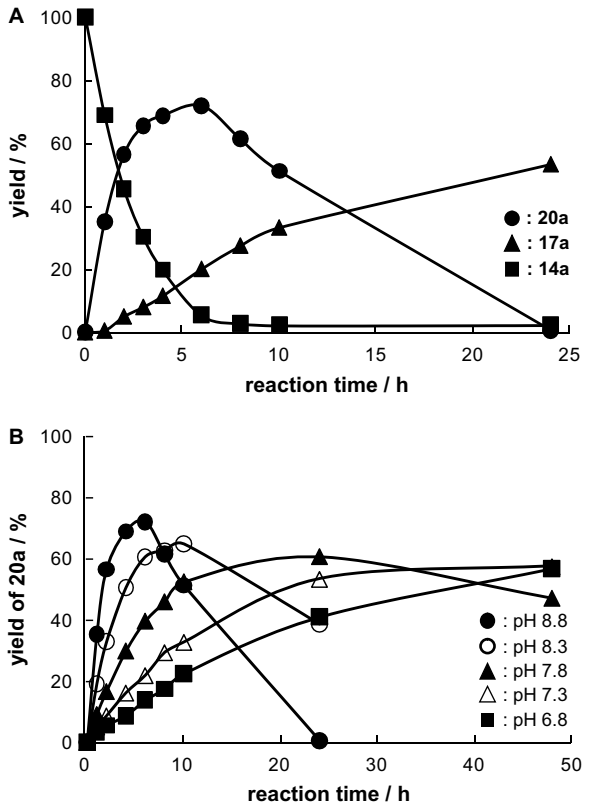


Figure 5. Time course for the reaction of the CPE peptide, Fmoc-His-Pro-Ile-Arg-Gly-Cys-Pro-OCH_2CONH_2 (**14a**) with $HSCH_2CH_2SO_3Na$ at 37 °C to yield Fmoc-His-Pro-Ile-Arg-Gly-$SCH_2CH_2SO_3Na$ (**20a**). (A) Reaction at pH 8.8: **20a** (●); **17a** (▲); **14a** (■). (B) Yields of **20a** in the reaction at the pH of 8.8 (●), 8.3 (○), 7.8 (▲), 7.3 (△), and 6.8 (■).

was suppressed somewhat, even in the case of the sensitive Ser-thioester. Furthermore, when the peptide thioester is immediately reacted with a Cys-peptide, these undesirable reactions are suppressed to minimum levels, and the desired ligated product is produced in good yield (vide supra).

In conclusion, a peptide containing the CPE unit at the C-terminus can be spontaneously transformed into a peptide thioester through an *N–S* acyl shift reaction, followed by DKP formation. When the CPE peptide is mixed with a Cys-peptide, the ligation reaction proceeds well through thioester formation, followed by native chemical ligation, in a one-pot process. The ligation can be carried out using a variety of amino acid residues and the extent of epimerization is minimal. The CPE peptide is one of the alternatives to the peptide thioester, for use as a building block in polypeptide synthesis by ligation, and can be readily prepared by Fmoc SPPS. The CPE peptide can be also transformed into the corresponding peptide thioester via an intermolecular thiol–thioester exchange reaction after C^{α}-DKP thioester formation. Suppression of hydrolysis and epimerization of the thioester in the case of sensitive amino acid residues, such as Ser, need to be investigated further, although these are suppressed to some extent, when lower pH reaction conditions are employed. At this stage, the CPE peptide is the reagent of choice for use as a building block in one-pot thiol-mediated ligation reactions, since undesirable reactions, such as epimerization, can be avoided and yields are generally better than stepwise procedures through isolation of the peptide thioester.

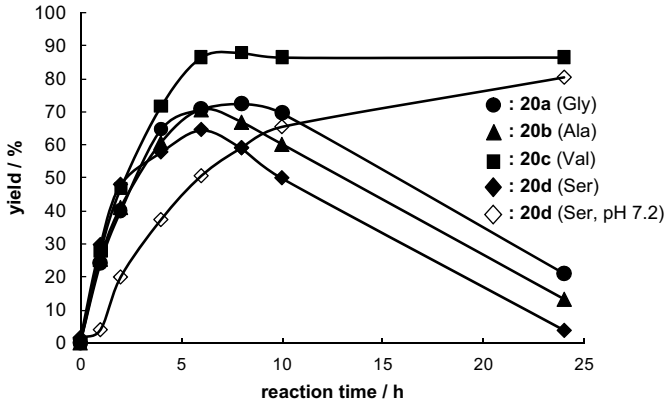


Figure 6. Time course for the reaction of the CPE peptide, Fmoc-His-Pro-Ile-Arg-*Xaa*-Cys-Pro-OCH_2CONH_2 (**14**) with $HSCH_2CH_2SO_3Na$ at pH 8.2 and 37 °C to yield Fmoc-His-Pro-Ile-Arg-*Xaa*-$SCH_2CH_2SO_3H$ (**20**). Peptide thioester (*Xaa*): **20a** (Gly, ●), **20b** (Ala, ▲), **20c** (Val, ■), **20** (Ser, ◆), and **20d** (Ser, at pH 7.2, ◇).

3. Experimental

3.1. General

The amino acid derivatives used were of the L-configuration, unless otherwise noted. Fmoc-amino acid derivatives were purchased from the Peptide Institute, Inc (Minoh, Japan). 4-(*N*-α-9-Fluorenylmethoxycarbonyl-amino-2′,4′-dimethoxybenzyl)phenoxy resin (Fmoc-Rink amide resin) were purchased from Watanabe Chemical Ind., Ltd (Hiroshima, Japan).

HPLC was carried out on a reversed phase column, YMC-Pack ProC18 (4.6×150 mm), Hydrosphere C18 (4.6×150 mm), YMC Chiral NEA(S) (4.6×150 mm), or YMC-Pack ProC18 (10×250 mm) (YMC Co., Ltd., Kyoto, Japan) using a linear increasing gradient of acetonitrile in water/0.1% TFA, and detection was by an absorbance measurement at 220 nm. ESI-MS spectra were recorded on a Thermo Finnigan LCQ™ DECA XP spectrometer. MALDI-TOF-MS spectra were recorded on a Bruker AutoFLEX spectrometer. Peptide yields were determined by amino acid analyses, unless otherwise noted, which were performed on a Hitachi L-2000 amino acid analyzer after hydrolysis with constant boiling point HCl (Nacalai Tesque) at 110 °C for 24 h in an evacuated sealed tube.

3.2. Preparation of Fmoc-His-Pro-Ile-Arg-*Xaa*-Cys-Pro-OCH_2CONH_2 (14)

*3.2.1. A typical procedure for preparation of Fmoc-His-Pro-Ile-Arg-Gly-Cys-Pro-OCH_2CONH_2 (**14a**)*

Fmoc-Rink amide resin (Fmoc-NH-resin) (**9**) (1.00 g, 0.65 mmol/g) was treated with 1-methyl-2-pyrrolidinone (NMP) (8 mL×3), followed by 20% piperidine (8 mL) for 5, 10, and 10 min. After washing with NMP (8 mL×5), the resin was treated with a solution containing glycolic acid (99 mg, 1.3 mmol), HOBt·H_2O (0.23 g, 1.5 mmol), and DIPCI (0.23 mL, 1.5 mmol) in NMP (5 mL) for 16 h. The resin was washed with NMP (8 mL×5), then treated for 8 h with a solution prepared by mixing Fmoc-Pro-OH (0.68 g, 2.0 mmol), 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate (HBTU) (0.76 g, 2.0 mmol), and DIEA (0.52 mL, 3.0 mmol) in DMF 5 mL for 2 min. The resin was washed with NMP (8 mL×3), followed by MeOH (8 mL×3), and dried in vacuo. Fmoc-Pro-OCH_2CONH-resin (**10**) was obtained in 1.14 g (Fmoc: 0.46 mmol/g).

Resin **10** (0.520 g, 0.24 mmol) was treated with NMP (3 mL×3), followed by an NMP solution (5 mL) containing 10% Ac_2O and 5% DIEA for 10 min. After washing with NMP (3 mL×3), the resin was treated with 20% piperidine (3 mL) for 5, 5, and 10 min, and washed with NMP (3 mL×5). The resin was treated with a solution containing Fmoc-Gly-Cys(Trt)-OH (0.32 g, 0.50 mmol), HOOBt (90 mg, 0.55 mmol), and DIPCI (0.092 mL, 0.60 mmol) in NMP (4 mL) for 15 h. The resin was washed with NMP (3 mL×3), followed by MeOH (5 mL×3), and dried in vacuo. Fmoc-Gly-Cys(Trt)-Pro-OCH_2CONH-resin (**12a**) was obtained in 0.608 g (Fmoc: 0.31 mmol/g).

Resin **12a** (0.249 g) was applied to an automated peptide synthesizer, ACT 440 Ω (aapptec, Louisville, KY). The peptide chain was elongated by the following steps: NMP wash (1 min×3); 20% piperidine in the NMP treatment (5, 5, 10 min); NMP wash (1 min×6); coupling with the Fmoc-amino acid derivative (0.50 mmol) using DIPCI and HOBt·H_2O for 1.5 h; NMP wash (1 min×3); capping with a solution containing 10% Ac_2O, 5% DIEA in NMP for 10 min. After drying, 0.335 g of the Fmoc-His(Trt)-Pro-Ile-Arg(Pmc)-Gly-Cys(Trt)-Pro-OCH_2CONH-resin (**13a**) was obtained.

Resin **13a** (0.335 g) was treated with a solution containing 2% triisopropylsilane, 5% phenol, and 5% water in TFA (4.0 mL) for 2 h. The peptide was precipitated by adding cold ether and the precipitate was washed with ether (20 mL×3). The crude material was passed through a disposable cartridge, TOYO pack ODS-M (Tosoh, Tokyo, Japan), with 50% acetonitrile, and freeze-dried to give the crude peptide (0.112 g). After purification by RP-HPLC on YMC-pack ProC18 (1×25 cm), 45 mg of Fmoc-His-Pro-Ile-Arg-Gly-Cys-Pro-OCH_2CONH_2 (**14a**) was obtained (30 μmol, 30% based on the Fmoc content in **10**). Compound **14a**: MS (MALDI-TOF): found *m*/*z* 1058.6, calcd for $(M+H)^+$ 1058.5; amino acid analysis: $Pro_{1.8}Gly_1Cys_{nd}Ile_{1.0}His_{0.84}Arg_{0.92}$.

Fmoc-His-Pro-Ile-Arg-Ala-Cys-Pro-OCH_2CONH_2 (***14b***): 22%; MS (MALDI-TOF): found *m*/*z* 1072.8, calcd for $(M+H)^+$ 1072.5; amino acid analysis: $Pro_{1.8}Ala_{1.1}Cys_{nd}Ile_{1.2}His_{0.93}Arg_1$.

Fmoc-His-Pro-Ile-Arg-Val-Cys-Pro-OCH_2CONH_2 (***14c***): 20%; MS (MALDI-TOF): found *m*/*z* 1100.9, calcd for $(M+H)^+$ 1100.5; amino acid analysis: $Pro_{2.0}Cys_{nd}Val_1Ile_{0.94}His_{0.84}Arg_{0.94}$.

Fmoc-His-Pro-Ile-Arg-Ser-Cys-Pro-OCH_2CONH_2 (***14c***): 26%; MS (MALDI-TOF): found *m*/*z* 1088.3, calcd for $(M+H)^+$ 1088.5; amino acid analysis: $Ser_{0.57}Pro_{2.4}Cys_{nd}Ile_{0.97}His_{0.87}Arg_1$.

3.3. Analysis of ligation of CPE peptide 14 with Cys-peptide 15

Peptides, Fmoc-His-Pro-Ile-Arg-*Xaa*-Cys-Pro-OCH_2CONH_2 (**14**) (3–5 mM) and Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (**15**) (6–9 mM) were dissolved in a mixture of acetonitrile (25 μL) and a 0.2 M sodium phosphate buffer (75 μL) containing 0.01 M tris(2-carboxyethyl)phosphine (TCEP), and 50 mM Boc-Lys(Cl-Z)-OH at appropriate pH. The solution was stirred at 37 °C, and an aliquot (5 μL) was removed to quench the reaction with acetic acid (8 μL) and 0.5 M DTT (8 μL). The reaction was analyzed by RP-HPLC on YMC-Pack ProC18 (4.6×150 mm) for **16a–c** or YMC CHIRAL NEA(S) (4.6×150 mm) for **16d**. The yield of peptides was calculated based on the peak area compared with that of Boc-Lys(Cl-Z)-OH as an internal standard. Epimerized products were confirmed by MS and RP-HPLC analyses in comparison with the peptides containing D-amino acid residues, which were prepared separately.

Fmoc-His-Pro-Ile-Arg-Gly-Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (***16a***): MS (MALDI-TOF): found *m*/*z* 1414.9, calcd for $(M+H)^+$ 1414.7; amino acid analysis: $Asp_{0.99}Pro_{1.1}Gly_2Cys_{nd}Ile_{1.9}Leu_{2.0}His_{0.84}Arg_{0.92}$.

Fmoc-His-Pro-Ile-Arg-Ala-Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (***16b***): MS (MALDI-TOF): found *m*/*z* 1428.8, calcd for $(M+H)^+$ 1428.7; amino acid analysis: $Asp_{1.0}Pro_{0.98}Gly_1Ala_{1.1}Cys_{nd}Ile_{1.9}Leu_{2.0}His_{0.84}Arg_{0.96}$.

Fmoc-His-Pro-Ile-Arg-Val-Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (***16c***): MS (MALDI-TOF): found *m*/*z* 1456.6, calcd for $(M+H)^+$ 1456.8.7; amino acid analysis: $Asp_{1.0}Pro_{1.1}Gly_1Cys_{nd}Val_{0.91}Ile_{1.9}Leu_{2.0}His_{0.84}Arg_{0.93}$.

Fmoc-His-Pro-Ile-Arg-Ser-Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (***16d***): MS (MALDI-TOF): found *m*/*z* 1444.5, calcd for $(M+H)^+$ 1444.7; amino acid analysis: $Asp_{1.1}Ser_{0.65}Pro_{nd}Gly_{1.1}Cys_{nd}Ile_{1.9}Leu_2His_{0.90}Arg_{1.1}$.

3.4. Analysis of the reaction of CPE peptide 14a forming DKP thioester

Fmoc-His-Pro-Ile-Arg-Gly-Cys-Pro-OCH_2CONH_2 (**14a**) (3 mM) was dissolved in a mixture of acetonitrile (30 μL) and 0.2 M sodium phosphate buffer (70 μL) containing 0.01 M TCEP and 0.10 M Boc-Phe-OH (pH 8.0). The solution was stirred at room temperature, and an aliquot (5 μL) was removed to quench the reaction with acetic acid (8 μL). The reaction was analyzed by RP-HPLC on YMC-Pack ProC18 (4.6×150 mm).

cyclo(-Cys(Fmoc-His-Pro-Ile-Arg-Gly-)-Pro-) (***18a***): MS (MALDI-TOF): found *m*/*z* 983.4, calcd for $(M+H)^+$ 983.5; amino acid analysis: $Pro_{1.1}Gly_{1.3}Cys_{0.86}Ile_{0.85}His_{0.81}Arg_1$.

Fmoc-His-Pro-Ile-Arg-Gly-Cys(Fmoc-His-Pro-Ile-Arg-Gly-)-Pro-OCH_2-$CONH_2$ (***19a***): MS (MALDI-TOF) found *m*/*z* 1840.3, calcd for $(M+H)^+$ 1840.9; amino acid analysis: $Pro_{2.2}Gly_{2.2}Cys_{0.50}Ile_{1.8}His_{1.7}Arg_2$.

Fmoc-His-Pro-Ile-Arg-Gly-OH (***17a***): MS (MALDI-TOF): found *m*/*z* 801.3, calcd for $(M+H)^+$ 801.4; amino acid analysis: $Pro_{0.59}Gly_{1.1}Ile_{0.95}His_{0.88}Arg_1$.

cyclo(-Cys-Pro-): MS (ESI): found *m*/*z* 201.2, calcd for $(M+H)^+$ 201.1.

3.5. Analysis of formation of peptide thioester 20 from CPE peptide 14

Fmoc-His-Pro-Ile-Arg-*Xaa*-Cys-Pro-OCH_2CONH_2 (**14**) (3–5 mM) was dissolved in a mixture of acetonitrile (30 μL) and a 0.2 M sodium phosphate buffer (70 μL) containing 0.4 M $HSCH_2CH_2SO_3Na$, 0.01 M TCEP, and 0.10 M Boc-Phe-OH at appropriate pH. The solution was stirred at 37 °C, and an aliquot (5 μL) was removed to quench the reaction with acetic acid (8 μL). The reaction was analyzed by RP-HPLC on YMC-Pack ProC18 (4.6×150 mm) for **20a–c** or YMC Hydrosphere C18 (4.6×150 mm) for **20d**. The yield of peptides was calculated based on the peak area compared with that of Boc-Phe-OH as an internal standard. The epimerized products were confirmed by MS and RP-HPLC analyses in comparison with the peptides containing D-amino acid residues, which were prepared separately.

Fmoc-His-Pro-Ile-Arg-Gly-$SCH_2CH_2SO_3H$ (***20a***): MS (MALDI-TOF): found *m*/*z* 925.3, calcd for $(M+H)^+$ 925.4; amino acid analysis: $Pro_{0.60}Gly_{1.1}Ile_{0.92}His_{0.86}Arg_1$.

Fmoc-His-Pro-Ile-Arg-Ala-$SCH_2CH_2SO_3H$ (***20b***): MS (MALDI-TOF): found *m*/*z* 939.2, calcd for $(M+H)^+$ 939.4; amino acid analysis: $Pro_{nd}Ala_{1.1}Ile_1His_{0.84}Arg_{0.92}$.

Fmoc-His-Pro-Ile-Arg-Val-$SCH_2CH_2SO_3H$ (***20c***): MS (MALDI-TOF): found *m*/*z* 967.3, calcd for $(M+H)^+$ 967.4; amino acid analysis: $Pro_{0.83}Val_{1.0}Ile_{0.99}His_{0.79}Arg_1$.

Fmoc-His-Pro-Ile-Arg-Ser-$SCH_2CH_2SO_3H$ (***20d***): MS (MALDI-TOF): found *m*/*z* 955.3, calcd for $(M+H)^+$ 955.4; amino acid analysis: $Ser_{0.97}Pro_{1.2}Ile_{0.96}His_{0.88}Arg_1$.

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