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Tetrahedron Letters
Tetrahedron Letters 48 (2007) 1903–1905

Sequential peptide ligation by using a controlled cysteinyl prolyl ester (CPE) autoactivating unit

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Received 19 December 2006; revised 15 January 2007; accepted 18 January 2007
Available online 23 January 2007

Abstract—A peptide building block containing a cysteinyl prolyl ester (CPE) autoactivating unit was ligated with a cysteinyl peptide under native chemical ligation conditions. The CPE autoactivating function can be controlled by the protection of the thiol group, permitting the selective ligation of the peptide building block at either the C or N terminus.

The chemical ligation of peptide segments, prepared by either chemical or biological methods, is a widely used technique in polypeptide synthesis.[1] Ligation chemistry has been developed based on the use of peptide thioesters. In the thioester method, partially protected peptide thioesters are used as building blocks and are condensed in the presence of silver ion as an activating reagent for the thioester.[2] Native chemical ligation permits perfect chemoselective ligation, in which an unprotected peptide thioester is 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 side chain non-protected peptide thioesters are used as building blocks, thus maintaining the advantageous features of the native chemical ligation reaction.[4]

The sequential ligation procedure can be used to prepare polypeptides containing over 100 amino acid residues by using more than three peptide building blocks, and is performed stepwise in the carboxy terminal to the amino terminal (C to N) direction.[1] An N-terminally-protected peptide thioester is used to prevent intramolecular ligation (cyclization) or oligomerization via intermolecular ligation reactions. We wish to describe herein, an alternative strategy in which a cysteinyl prolyl ester (CPE) is used as an autoactivating unit,[5] in which the direction of ligation is regulated by the controlled CPE unit.

We previously reported on *N* to *S* acyl shift reactions at a thiol containing residue such as a cysteine residue and a thiol auxiliary for peptide ligation under acidic conditions.[6,7] On the other hand, in 1985 Zanotti et al. reported that a diketopiperazine thioester, *cyclo*-(–Cys($COCH_2Ph$)-Pro-), is produced when a *p*-nitrophenyl (Np) ester, $PhCH_2CO$–Cys(S^{t}Bu)-Pro-ONp, was treated with tributylphosphine under aqueous conditions.[8] Based on these observations, we designed a CPE autoactivating unit for peptide ligation. A peptide, containing a CPE unit, can be prepared by standard Fmoc solid phase peptide synthesis (SPPS), because it does not contain a thioester moiety, and the CPE unit can be used to activate the peptide for ligation with a cysteinyl peptide. CPE peptide **1** reacts with cysteinyl peptide **3** to give ligated peptide **4** via a diketopiperazine thioester **2**, which is spontaneously formed in the reaction (Scheme 1, $R^2 = H$). The autoactivating function of the CPE unit is quenched by introducing a protecting group to the thiol group (Scheme 1, R^2 = protecting group).[5] Therefore the C-terminal CPE peptide would be applied to ligation in both directions, C to N and N to C, by controlling the CPE function.

The strategy for the sequential ligation is shown in Scheme 2. When the thiol group in the CPE unit is protected, the ligation reaction proceeds at the N terminus of peptide **6** (first ligation of **5** and **6**). When the protecting group is removed, ligation can be carried out at the C terminus (second ligation of **9** and **7**).

The sequential ligation using the CPE peptide was demonstrated by the synthesis of a model peptide, H-Leu-Lys-Asn-Thr-Ser-Val-Leu-Gly-Ala-Ala-Cys-His-

Keywords: CPE autoactivating unit; Controlled sequential ligation; Peptide ligation, Peptide thioester.
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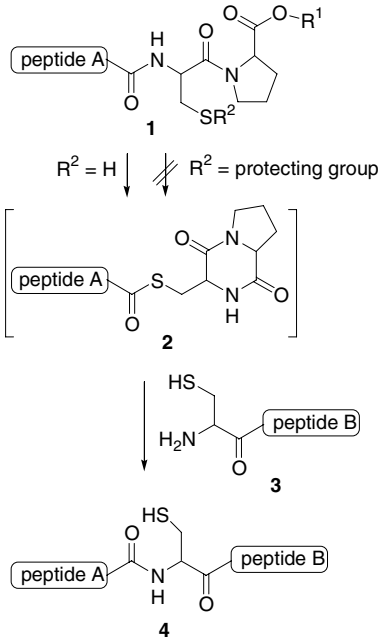
doi:10.1016/j.tetlet.2007.01.086



Scheme 1. Peptide ligation using a cysteinyl prolyl ester (CPE) as an autoactivating unit.

Pro-Ile-Arg-Leu-Cys-Asp-Ile-Leu-Leu-Gly-NH_2 (**10**), as shown in Scheme 2.[9] A peptide containing the protected CPE unit, H-Cys-His-Pro-Ile-Arg-Leu-Cys(4-MeOBzl)-Pro-OCH_2CONH_2 (**6**),[10] was first ligated at the N terminus, with a peptide thioester, H-Leu-Lys-Asn-Thr-Ser-Val-Leu-Gly-Ala-Ala-SCH_2CH_2CO-Leu-NH_2 (**5**) in sodium phosphate buffer (pH 7.2) containing 6 M guanidine (Gdn) and 2% 4-trimethylsilylthiophenol (v/v).[11] The RP-HPLC of the reaction mixture after 24 h is shown in Figure 1a. The ligated product, H-Leu-Lys-Asn-Thr-Ser-Val-Leu-Gly-Ala-Ala-Cys-His-Pro-Ile-Arg-Leu-Cys(4-MeOBzl)-Pro-OCH_2CONH_2 (**8**), was isolated in 72% yield. The 4-methoxybenzyl group of peptide **8** was then removed by treatment with 1 M trifluoromethanesulfonic acid (TFMSA) in trifluoroacetic acid (TFA) containing 1 M thioanisole on an ice bath for 1 h (Fig. 1b). Peptide **9**, purified by RP-HPLC, was isolated in 90% yield. The next ligation at the C terminus

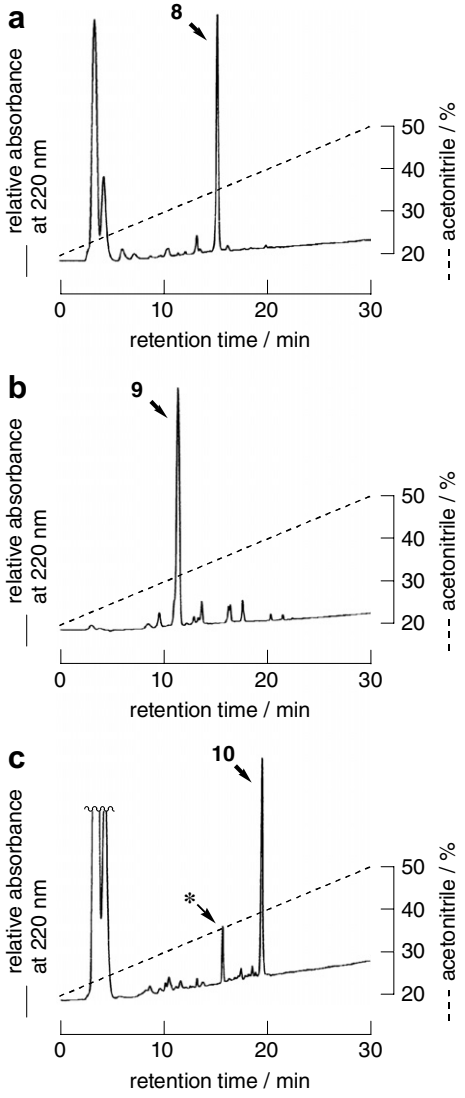
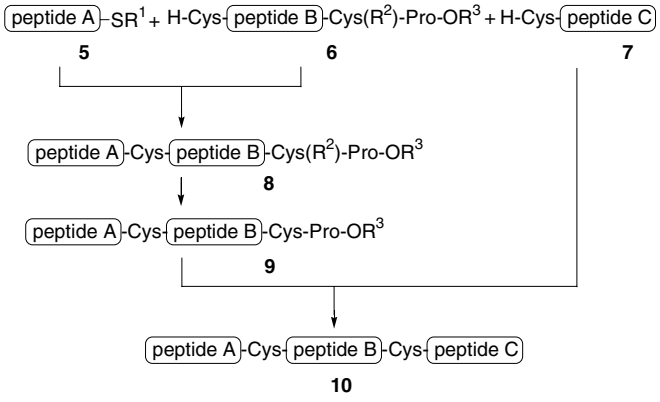


Figure 1. RP-HPLC of sequential peptide ligation reaction mixtures.[9] (a) First ligation at the N terminus of peptide **6**. (b) Removal of the protecting group of peptide **8**. (c) Second ligation at the C terminus of peptide **9**. (*) A compound derived from peptide **7**. Column: YMC-Pack ProC18 (4.6 × 150 mm), eluent: aq acetonitrile containing 0.1% TFA, flow rate: 1.0 mL/min.



Scheme 2. Sequential peptide ligation strategy in the C to N direction, followed by the N to C direction. $-R^1 = -CH_2CH_2CO$-Leu-NH_2, $-R^2 = -CH_2C_6H_4OCH_3$-*p*, $-R^3 = -CH_2CONH_2$, peptide A = Leu-Lys-Asn-Thr-Ser-Val-Leu-Gly-Ala-Ala, peptide B = His-Pro-Ile-Arg-Leu, peptide C = Asp-Ile-Leu-Leu-Gly-NH_2.

was carried out with peptide **7** in a 0.1 M sodium bicarbonate solution (pH 8.3) containing 10 mM tris-(hydroxypropyl)phosphine (THP) and 50% acetonitrile.[12] After 24 h, product **10** was isolated by RP-HPLC (Fig. 1c) in 64% yield.

A kinetically controlled convergent native chemical ligation procedure has recently been reported, in which the ligation of cysteinyl peptide thioesters was controlled by taking advantage of the difference in reactivities between *S*-alkyl and *S*-aryl thioesters.[13] The CPE unit can be inactivated by the thiol protecting group safely without cyclization or oligomerization, and is ready for ligation, after removal of the protecting group. Thus, our approach represents a promising alternative for achieving ligation in either direction.

In summary, a peptide containing a CPE unit is used as a building block for ligation with a cysteinyl peptide in a manner similar to that used in native chemical ligation. The autoactivating function of the CPE unit can be quenched by introducing a protecting group to prevent inter- and intramolecular self ligation, and the direction of ligation at the N or C terminus can be controlled, thus providing a flexible ligation strategy in polypeptide synthesis using multi-component peptide building blocks. Modifications, such as phosphorylation, glycosylation, methylation, acylation, and stable isotope labeling, would be introduced at the final ligation step at either the N- or C-terminal part of the peptides selectively.

Acknowledgement

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

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9. Procedure for the sequential ligation: A solution of peptide thioester **5** (2.7 μmol) and peptide **6** (2.2 μmol) in 0.1 M sodium phosphate buffer (pH 7.2) containing 6 M Gdn and 2% 4-trimethylsilylthiophenol (v/v) (0.60 mL) was stirred at room temperature for 24 h, and DTT was then added. Product **8** was isolated by RP-HPLC in 1.7 μmol yield (72%): MS (MALDI-TOF): found *m/z* 2070.0, calcd for $(M+H)^+$ 2070.1; amino acid analysis: $Asp_{1.0}Thr_{0.96}Ser_{0.90}Pro_{2.0}Gly_1Ala_{2.0}Cys_{nd}Val_{1.0}Ile_{0.94}Leu_{3.0}$-$Lys_{0.97}His_{1.0}Arg_{0.96}$.
Peptide **8** (0.96 μmol) was treated with 1 M TFMSA in a TFA solution containing 1 M thioanisole (0.40 mL) on an ice bath for 1 h. Peptide **9** was precipitated with ether, the resulting precipitate freeze-dried, and then purified by RP-HPLC in 0.86 μmol yield (90%): MS (MALDI-TOF): found *m/z* 1949.9, calcd for $(M+H)^+$ 1950.0; amino acid analysis: $Asp_{1.0}Thr_{0.99}$-$Ser_{0.95}Pro_{1.6}Gly_1Ala_{2.0}Cys_{nd}Val_{0.98}Ile_{0.96}Leu_{3.1}Lys_{1.0}His_{1.0}$-$Arg_{0.98}$.
Peptide **9** (0.25 μmol) was reacted with **7** (0.57 μmol) in a 0.1 M sodium bicarbonate solution (pH 8.3, 80 μL) containing 50% acetonitrile and 10 mM THP. The solution was stirred at room temperature for 24 h, and DTT was then added. Peptide **10** was isolated by RP-HPLC in 0.16 μmol yield (64%): MS (MALDI-TOF): found *m/z* 2306.2, calcd for $(M+H)^+$ 2306.3; amino acid analysis: $Asp_{2.0}Thr_{0.95}Ser_{0.89}Pro_{0.98}Gly_2Ala_{2.0}Cys_{nd}Val_{0.96}Ile_{1.89}$-$Leu_{4.9}Lys_{0.98}His_{2.0}Arg_{0.91}$.
10. Peptide **6** was prepared by standard Fmoc SPPS starting from Fmoc-Leu-Cys(4-MeOBzl)-Pro-OCH_2CO-Rink amide resin.[5] A protected peptide resin, H-Cys(Trt)-His(Trt)-Pro-Ile-Arg(Pmc)-Leu-Cys(4-MeOBzl)-Pro-O-CH_2CO-Rink amide resin, was treated with a TFA solution containing 5% phenol, 5% water, and 2.5% triisopropylsilane (v/v/v), and the peptide was purified by RP-HPLC in 23% yield based on the Leu content of the starting resin: MS (MALDI-TOF): found *m/z* 1116.3, calcd for $(M+H)^+$ 1115.6; amino acid analysis: $Pro_{2.0}Cys_{nd}Ile_{0.93}Leu_1His_{0.98}Arg_{0.92}$.
11. In the native chemical ligation reaction, an additional aryl thiol, such as thiophenol, is usually used to enhance the reactivity of the thioester by in situ transthioesterification, and several thiols were examined; Johnson, E. C. B.; Kent, S. B. H. *J. Am. Chem. Soc.* **2006**, *128*, 6640–6646; 4-Trimethylsilylthiophenol was reported as an odorless equivalent of thiophenol; Nishide, K.; Miyamoto, T.; Kumar, K.; Ohsugi, S.-I.; Node, M. *Tetrahedron Lett.* **2002**, *43*, 8569–8573. A phosphine, such as tris(2-carboxyethyl)phosphine, can be used instead of a thiol to reduce disulfide formation.[3b]
12. The ligation efficiency was dependent on the solvent used; it was slightly improved when a sodium bicarbonate buffer was used instead of a tricine buffer.[5] Thus, the isolated yield of the peptide, Fmoc-His-Pro-Ile-Arg-Gly-Cys-Asp-Ile-Leu-Leu-Gly-NH_2 or Fmoc-His-Pro-Ile-Arg-Val-Cys-Asp-Ile-Leu-Leu-Gly-NH_2, by the reaction of a peptide, Fmoc-His-Pro-Ile-Arg-Gly-Cys-Pro-OCH_2CONH_2 or Fmoc-His-Pro-Ile-Arg-Val-Cys-Pro-OCH_2CONH_2, with peptide **7** was increased from 60% to 65% or from 49% to 68%, respectively. In these ligation reactions, only a small amount of THP was added, and acetonitrile was added to dissolve peptides. Other additives, such as thiols and Gdn, could also be useful. The ligation reactions proceeded faster in a buffer solution at the pH over 8 than at that below 8.
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