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Direct On-Resin Synthesis of Peptide-$^{\alpha}$Thiophenylesters for Use in Native Chemical Ligation

Duhee Bang,[†,‡] Brad L. Pentelute,[†,‡] Zachary P. Gates,[†,‡] and Stephen B. Kent*[,†,‡,§]

Institute for Biophysical Dynamics, Department of Chemistry, and Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, Illinois 60637

skent@uchicago.edu

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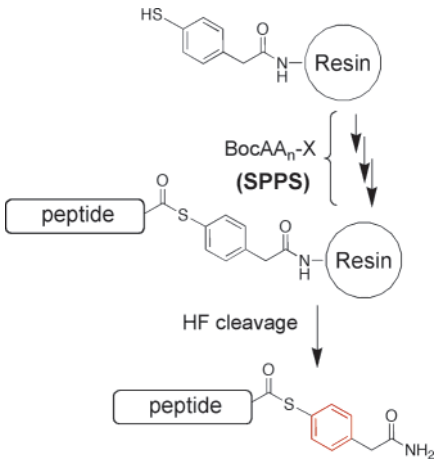
ABSTRACT



A peptide-$^{\alpha}$thiophenylester is a key reactant in native chemical ligation. Preformation of the peptide-$^{\alpha}$thiophenylester could be useful for enhancing the ligation reaction. We report the direct on-resin preparation of preformed peptide-$^{\alpha}$thiophenylesters using a simple and efficient method. The peptide-$^{\alpha}$thiophenylester reacted extremely rapidly with a Cys-peptide when compared to the peptide-$^{\alpha}$thioalkylester.

In native chemical ligation,[1] an unprotected peptide-$^{\alpha}$thioester is reacted with a second peptide containing an N-terminal cysteine residue to give a near quantitative yield of a single product linked by an amide bond (Scheme 1A). Native chemical ligation has led to the practical chemical syntheses of a wide variety of different proteins.[2] Until now, peptide-thioesters were routinely synthesized in the form of peptide-SCH_2CH_2CO-Leu (i.e. alkyl) thioester,[3] and exogenous thiophenol was added to the native chemical ligation reaction mixture to generate a more reactive peptide-$^{\alpha}$thiophenylester by transthioesterfication (Scheme 1A).[4]

Recently, we have successfully used *preformed* peptide-$^{\alpha}$thiophenylesters for convergent chemical protein syntheses by kinetically controlled ligation.[5] We correctly anticipated that the preformed peptide-$^{\alpha}$thiophenylester could be used for rapid ligation by eliminating the thiophenol exchange

[†] Institute for Biophysical Dynamics.
[‡] Department of Chemistry.
[§] Department of Biochemistry and Molecular Biology.

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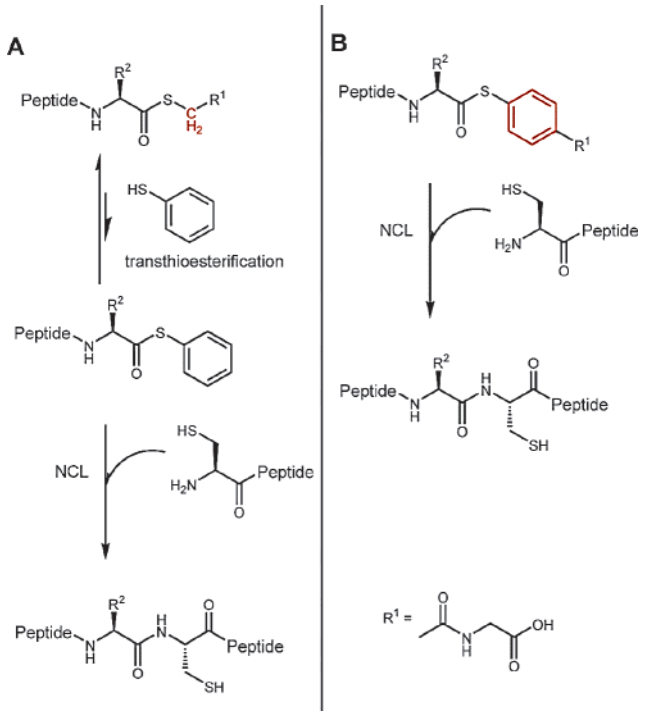
Scheme 1. (A) Native Chemical Ligation with Peptide-$^{\alpha}$Thio*Alkyl*Ester through Thiol Exchange during the Ligation Reaction and (B) Native Chemical Ligation with a Preformed Peptide-$^{\alpha}$thio*phenyl*ester

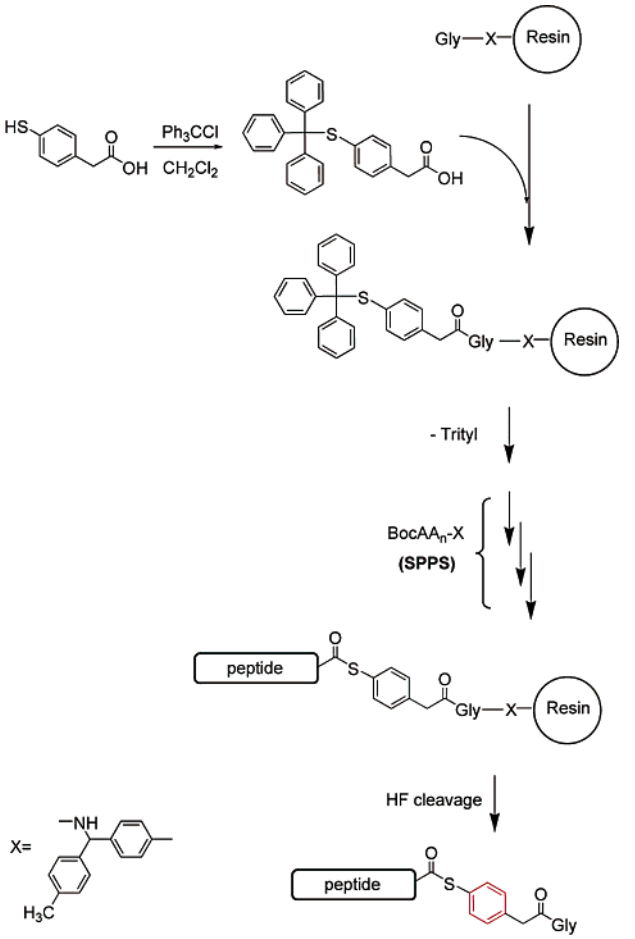


step during the native chemical ligation reaction (Scheme 1B). The peptide-$^{\alpha}$thiophenylesters were generated by exchange of a peptide-thioalkylester with a large excess of thiophenol in aqueous buffer, and then purified by reverse-phase HPLC. However, this method of generating the peptide-$^{\alpha}$thiophenylester was slow and sometimes incomplete. Consequently, we felt this exchange method would limit the potential use of the peptide-$^{\alpha}$thiophenylester for chemical protein synthesis. Here we report a method to directly prepare peptide-$^{\alpha}$thiophenylesters.

A simple and efficient chemistry for the generation of a preformed peptide-$^{\alpha}$thiophenylester was developed. A resin linker was designed for the synthesis of peptide-$^{\alpha}$thiophenylesters with a wide range of C-terminal amino acids (Scheme 2). For the synthesis of peptide-$^{\alpha}$thiophenylesters, we adapted Dawson's peptide-$^{\alpha}$thioalkylester synthesis method using a Boc chemistry-solid-phase peptide synthesis (SPPS) protocol.[3] The *S*-tritylmercaptophenylacetic acid was prepared by treating 4-mercaptophenylacetic acid with trityl chloride. (see the Supporting Information). Starting with a *p*-methylbenzhydrylamine (MBHA) resin, glycine was coupled followed by *S*-tritylmercaptophenylacetic acid. After removal of the trityl protecting group, the resulting mercaptophenylacetyl glycine-resin was used for polypeptide chain assembly

(5) We have shown that, in the absence of added thiophenol, reaction of a peptide1-thiophenylester with a Cys-peptide2-thioalkylester gives a single product, the peptide1-Cys-peptide2-thioalkylester (Bang, D.; Pentelute, B.; Kent, S. B., unpublished data). This kinetically controlled ligation principle has so far been used in the convergent chemical synthesis of proteins containng 46, 70, and 99 residues..

Scheme 2. Synthetic Strategy for the On-Resin Preparation of a Peptide-$^{\alpha}$Thiophenylester



by the use of Boc chemistry in situ neutralization SPPS protocols[6] (Scheme 2).

During model peptide syntheses, we found that two major byproducts were formed. First, we had 10−20% of byproduct from slow first amino acid coupling to the mercaptophenylacetyl-glycine-resin, even with the use of 1 h coupling in the in situ neutralization protocol. Second, we had 20−30% byproduct from the formation of diketopiperazine that resulted in a two amino acid deletion at the C-terminus.[7] We effectively prevented these side reactions by the use of a modified in situ neutralization protocol (see the Supporting Information for the synthesis of peptide-$^{\alpha}$thiophenylester).

Model ligation of a peptide-$^{\alpha}$thiophenylester and a Cys-peptide was performed under standard native chemical ligation conditions (aqueous buffer, 2 mM peptide concentration, and 1% thiophenol), and the model ligation was compared with the ligation of a standard peptide-$^{\alpha}$thioalkylester under identical conditions. For comparison, we prepared Phe-Leu-Leu-$^{\alpha}$thiophenylester and Phe-Leu-Leu-$^{\alpha}$thioalkylester, and we used Cys-Phe-Arg-Ala-Asn-Gly as a Cys-peptide.

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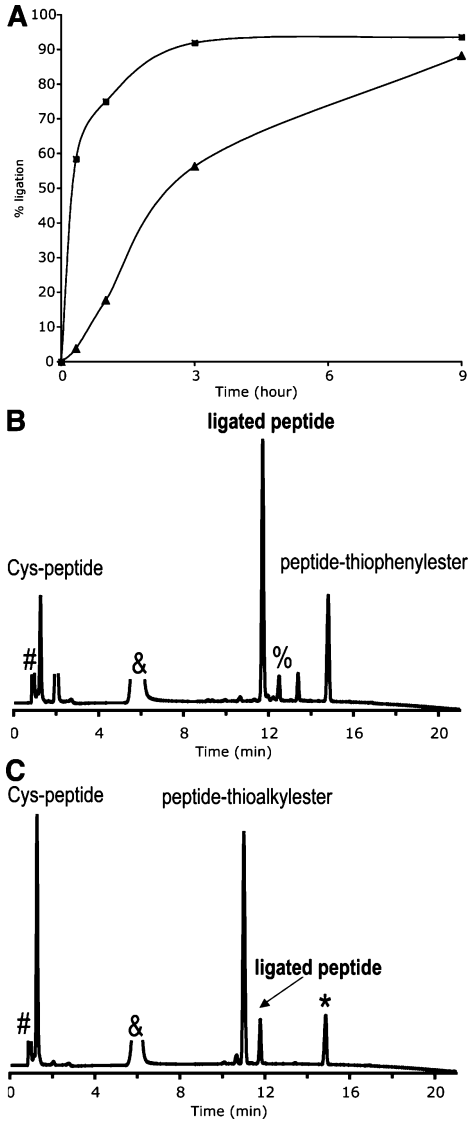

Figure 1. Model native chemical ligation of peptide-$^{\alpha}$thiophenylester (Phe-Leu-Leu-mercaptophenylacetic acid-Gly, ■) or peptide-$^{\alpha}$thioalkylester (Phe-Leu-Leu-mercaptopropionic acid-Gly, ▲) and CFRANG. (A) Observed FLL-CFRANG model-peptide formation monitored by the use of HPLC-MS analysis. At each time point during the ligation reactions (1/3, 1, 3, 9 h), an aliquot (20 μL) from the ligation reaction was quenched by the addition of 5% aqueous trifluoroacetic acid (8 μL). The aliquot was characterized by analytical HPLC. Analysis of the ligated species was done by integrating the areas from analytical HPLC profiles at each time point {area of product peptide/(area of product peptide + area of Cys-peptide)}. (B) Ligation reaction (1 h) of Phe-Leu-Leu-thiophenylester and Cys-Phe-Arg-Ala-Asn-Gly was monitored by analytical HPLC. (C) Ligation reaction (1 h) of Phe-Leu-Leu-thioalkylester and Cys-Phe-Arg-Ala-Asn-Gly was monitored by analytical HPLC. The UV profile at 214 nm is shown. The chromatographic separations were performed on a narrow-bore analytical HPLC C4 column, using a linear gradient (1−61%) of buffer B in buffer A over 15 min (buffer A = 0.1% trifluoroacetic acid (TFA) in water; buffer B = 0.08% TFA in acetonitrile) with a flow rate of 0.5 mL/min. # = residual TFA peak from acid quenching; & = thiophenol peak; % = Phe-Leu-Leu-mercaptophenylacetic acid-Gly; * = peptide-thiophenylester from thiol exchange with thiophenol.

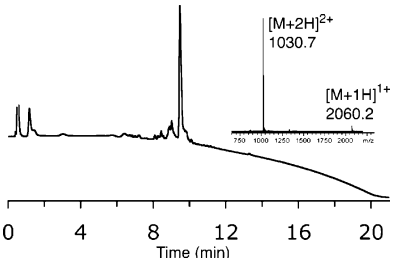

Figure 2. Crude peptide (Thz-Ala-Leu-Gln-Ile-Val-Ala-Arg-Leu-Lys-Asn-Asn-Asn-Arg-Gln-Val-mercaptophenylacetic acid-Gly) was analyzed by HPLC. The UV profile at 214 nm is shown. The chromatographic separations were performed as describe in Figure 1. The observed mass of the highest peak (t = 9.7 min) was 2059.4 ± 0.2 Da; the calculated mass of the peptide molecular structure with use of average isotopes was 2059.3 Da.

The model ligation reactions between 2.2 mM concentrations of peptide-$^{\alpha}$thioesters and 2 mM of Cys-peptide were carried out in pH 6.8 aqueous buffer (containing 200 mM sodium phosphate, 6 M guanidine hydrochloride, and 20 mM Tris(2-carboxyethyl)phosphine hydrochloride) in the presence of 1% thiophenol for 9 h (Figure 1). We checked the pH after 9 h and found that the pH dropped to 6.3 for both reaction mixtures. Model ligation rates were monitored by HPLC analysis. The analysis showed that the preformed peptide-thiophenylester reacted significantly faster (>10× initial rate under these conditions) than peptide-$^{\alpha}$thioalkylester with a Cys-peptide.

The generality[8] of direct on-resin syntheis of peptide-thiophenylesters was tested by using another peptide sequence (Thz-Ala-Leu-Gln-Ile-Val-Ala-Arg-Leu-Lys-Asn-

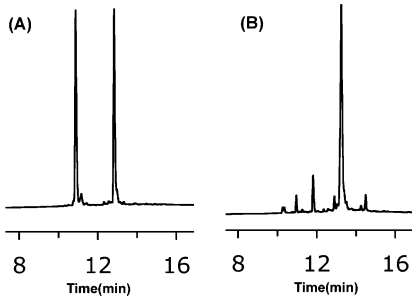

Figure 3. Native chemical ligation of peptide-Val-$^{\alpha}$thiophenylester and Cys-peptide: (A) at reaction time = 0 h, Thz-Ala-Leu-Gln-Ile-Val-Ala-Arg-Leu-Lys-Asn-Asn-Asn-Arg-Gln-Val-mercaptophenylacetic acid-Gly (t = 10.5 min; obsd 2059.2 ± 0.2 Da, calcd 2059.3 Da) and Cys-Ile-Asp-Pro-Lys-Leu-Lys-Trp-Ile-Gln-Glu-Tyr-Leu-Glu-Lys-Ala-Leu-Asn (t = 12.8 min; obsd 2004.4 ± 0.2 Da, calcd 2004.6 Da); (B) at reaction time = 17 h, ligation was essentially done and the product (Thz-Ala-Leu-Gln-Ile-Val-Ala-Arg-Leu-Lys-Asn-Asn-Asn-Arg-Gln-Val-Cys-Ile-Asp-Pro-Lys-Leu-Lys-Trp-Ile-Gln-Glu-Tyr-Leu-Glu-Lys-Ala-Leu-Asn) is eluted at 13.5 min (obsd 4038.8 ± 0.3 Da, calcd 4038.6 Da) The UV profile at 214 nm is shown. The chromatographic separations were performed as described in Figure 1.

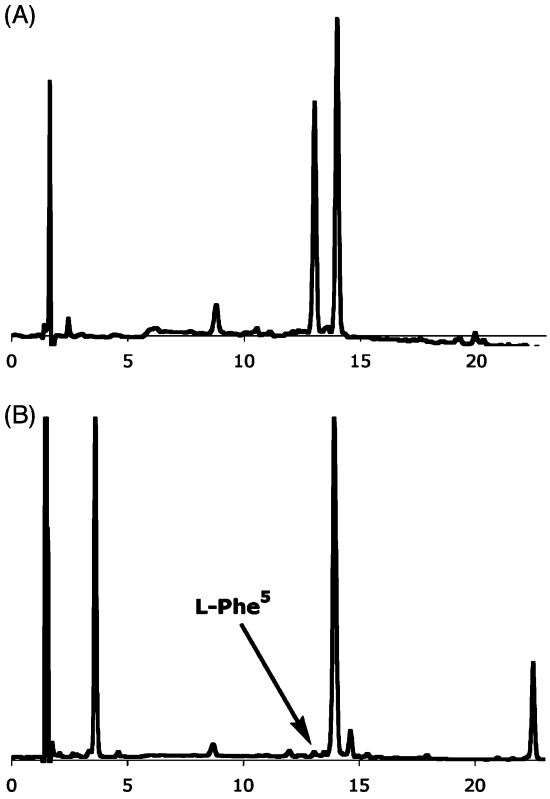

Figure 4. Evaluation of racemization in the synthesis and use of peptide-$^{\alpha}$thiophenylesters. (A) Analytical HPLC separation of the diastereomeric peptides Ala-Leu-Phe-Ala-L-Phe-Cys-Gly-Pro-Ala-Ser (t = 13.2 min) and Ala-Leu-Phe-Ala-D-Phe-Cys-Gly-Pro-Ala-Ser (t = 14.5 min). (B) Analysis after 30 min of the native chemical ligation reaction of Ala-Leu-Phe-Ala-D-Phe-$^{\alpha}$thiophenylester with Cys-Gly-Pro-Ala-Ser at pH 6.8, 2 mM for each peptide. The arrow indicates the low level of L-Phe^5 byproduct formed during the reaction. Analytical HPLC conditions are described in the Supporting Information.

Asn-Asn-Arg-Gln-Val-mercaptophenylacetic acid-Gly) from SDF1-α (Thz = 1,3-thiazolidine-4-carboxylic acid). This sequence was chosen to demonstrate the ease of preparation of a peptide-Val-$^{\alpha}$thiophenylester, because β-branching amino aicds (Val, Thr, and Ile) as a C-terminal residue are exceptionally difficult to exchange by transthioesterification in aqueous solution. A crude peptide-$^{\alpha}$thiophenylester was successfully obtained after HF cleavage, and analyzed by analytical HPLC (Figure 2).

We ligated the peptide-Val-$^{\alpha}$thiophenylester with a Cys-peptide (Figure 3). The ligation was essentially done in 17 h. In contrast, peptide-Val-$^{\alpha}$thioalkylester is known to ligate very slowly (~60% completion after 48 h) with a Cys-peptide (as shown by Hackeng et al.).[3]

Occurrence of racemization in the synthesis and use of a peptide-$^{\alpha}$thiophenylester in native chemical ligation with a Cys-peptide was evaluated as follows. A model pentapeptide Ala-Leu-Phe-Ala-D-Phe-$^{\alpha}$thiophenylester was prepared by the modified in situ neutralization SPPS protocols. This peptide was reacted with Cys-Gly-Pro-Ala-Ser, and the ligation reaction was analyzed by reverse-phase HPLC under analytical conditions that separated the diastereomeric reaction products that contained either −L-Phe^5− or −D-Phe^5− (see Supporting Information). The results are shown in Figure 4. Formation of the diastereomeric −L-Phe^5− reaction byproduct was barely detectable (<2%), in agreement with the low racemization levels reported for the use of peptide-$^{\alpha}$thiophenylesters in native chemical ligation.[3]

In conclusion, we have developed a novel method for the direct on-resin preparation of preformed peptide-$^{\alpha}$thiophenylesters. The resulting peptide-$^{\alpha}$thiophenylesters reacted faster when compared to the widely used peptide-$^{\alpha}$thioalkylester under native chemical ligation conditions. This stragtegy may be useful in kinetically controlled convergent ligation[5] and for the reaction of slowly ligating peptide-Xxx-thioesters (Xxx = Ile, Val, Thr) with Cys-peptide.[9]

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Supporting Information Available: Experimental procedures for synthesis and characterization of *S*-trityl mercaptophenyl acetic acid, for peptide synthesis, and for evaluation of racemization. This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) We have used the modified in situ neutralization protocol to prepare peptide-$^{\alpha}$thiophenylesters ranging from 3 to 31 residues. We observed unidentified byproduct peaks apart from our desired peaks. The peptides were easily purified and used in chemical protein synthesis. These data will be published elsewhere.

(9) Previously, because of their extremely slow reaction,[3] beta branched amino acid ligation sites were largely avoided or mutated to other amino acid residues for the design of chemical protein synthesis.