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Preparation of Peptide Thioesters using Fmoc-Solid-Phase Peptide Synthesis and its Application to the Construction of a Template-Assembled Synthetic Protein (TASP)

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Abstract: Preparation of peptide thioesters was conducted through peptide chain construction with Fmoc-solid-phase peptide synthesis on a 2-chlorotrityl resin followed by coupling with HS-$(CH_2)_2$-COOEt and deprotection with 95% aqueous CF_3COOH. The peptide thioester corresponding to a transmembrane segment of the calcium channel (S4 in repeat IV) thus obtained was introduced onto a peptide template to give an artificial four-helix-bundle protein. © 1997 Elsevier Science Ltd.

The distinctive feature of peptide synthesis using the S-alkyl thioester of a partially protected peptide segment[1] is its feasibility to utilize HPLC-purified peptide segments for the segment condensation to obtain highly homogenous peptides comprising as many as 100 amino acid residues. This feature seems promising to construct template-assembled synthetic protein (TASP)[2] molecules because difficulty in purification of TASP molecules has sometimes been encountered.[2b,c] Though this approach has great potential for peptide synthesis, only preparation of the thioester segments using the Boc-solid-phase peptide synthesis (SPPS) (Boc = *t*-butyloxycarbonyl) has been reported. Recently, Fmoc-SPPS[3] (Fmoc = 9-fluorenylmethyloxycarbonyl) has become more popular than the Boc method because of its easier handling. Direct preparation of the S-alkyl thioester by the Fmoc method is, however, difficult because piperidine used for the removal of the Fmoc moiety attacks the carbonyl moiety of the resin-bound thioester to liberate the peptide from the resin. If thioester segments can be prepared through Fmoc-SPPS, the thioester method should gain wider popularity in peptide synthesis. Here we report our new approaches to the preparation of S-alkyl thioester segments with the aid of Fmoc-SPPS. Application of the thioester method to the preparation of a TASP molecule composed of helices corresponding to a transmembrane segment of the calcium channel is also reported.

Our approach for the preparation of the partially protected peptide thioester involves (i) construction of protected peptide fragments on a 2-chlorotrityl (Cl-Trt) resin[4] by Fmoc-SPPS where the 2-chlorobenzyloxycarbonyl (Cl-Z) group was employed for the side-chain-protecting group of Lys, and TFA-removable protecting groups for those of other amino acids necessary for side-chain protection, (ii) coupling of the fragments with HS-$(CH_2)_2$-COOEt, and (iii) removal of the protection groups other than Lys(Cl-Z) by a TFA-treatment (Approach 1., Fig. 1a). Employment of the TFA-stable Cl-Z group can avoid the re-protection step for Lys residues in the original Boc-based thioester method.[1] In order to examine the practicability of the

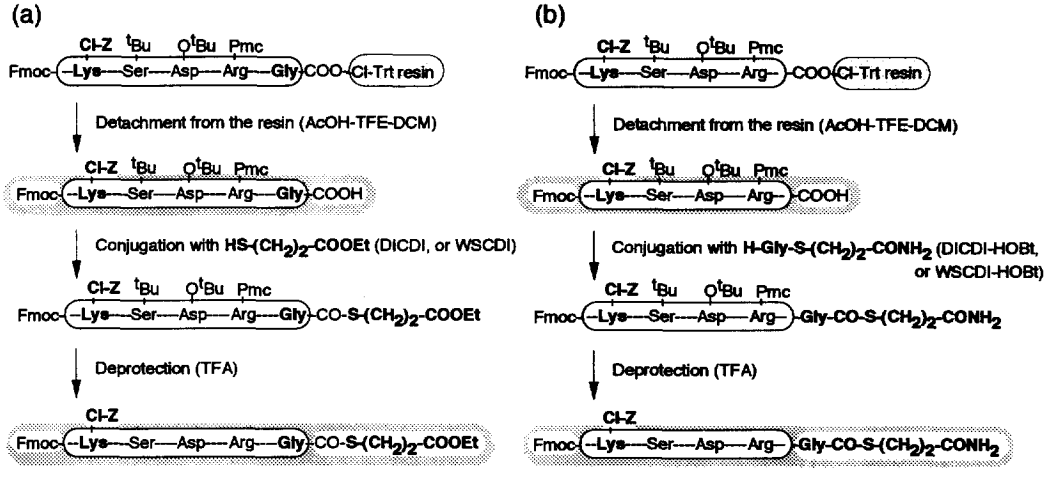

Fig. 1. General Scheme for the Preparation of Peptide Thioester Segments with Fmoc-Solid-Phase Peptide Synthesis *via* (a) Conjugation of Protected Peptide Fragments with HS-$(CH_2)_2$-COOEt (Approach 1), and (b) Conjugation of Protected Peptide Fragments with H-Gly-S-$(CH_2)_2$-$CONH_2$ (Approach 2).

above approach, two peptide thioester segments, Ac-Ile-Ser-Ile-Thr-Phe-Phe-Arg-Leu-Phe-Arg-Val-Met-Arg-Leu-Val-Lys(Cl-Z)-Leu-Leu-Ser-Arg-Gly-Glu-Gly-S-$(CH_2)_2$-COOEt **1** and Ac-Leu-Pro-Leu-Ala-Leu-Ala-Gln-Leu-Val-Leu-Gly-Leu-Leu-Pro-Val-Leu-Leu-Glu-Gln-Phe-Gly-S-$(CH_2)_2$-COOEt **2** were prepared. The former peptide corresponds to one of the transmembrane segments (S4 in repeat IV) of the rabbit skeletal muscle calcium channel,[5] and the latter to an alamethicin analog.[6]

As shown in Fig. 2, the protected peptide fragment corresponding to **1** was prepared by Fmoc-SPPS on a Cl-Trt resin[4] followed by the detachment from the resin with acetic acid (AcOH)-trifluoroethanol (TFE)-dichloromethane (DCM) (1:1:8) at r. t. for 1h. Coupling of the fragment with HS-$(CH_2)_2$-COOEt (20 eq) was then conducted in dimethylformamide (DMF) using diisopropylcarbodiimide (DICDI, 20 eq) at. r. t. for 48 h.[7]

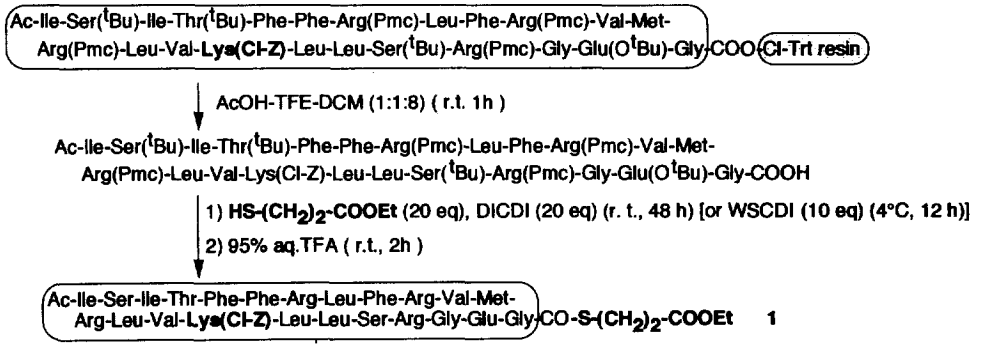

Fig. 2. Preparation of a Peptide Thioester Segment **1** Corresponding to a Transmembrane Sequence of the Calcium Channel (S4 in Repeat IV).

Treatment of the protected thioester segment with 95% aqueous TFA (r. t., 2 h) followed by purification by HPLC afforded the desired segment **1** in an acceptable yield (16%). The use of water-soluble carbodiimide (WSCDI, 10 eq) (4°C, 12 h) gave a slightly better yield (20%). The fidelity of the product was confirmed by time of flight mass spectrometry (TOFMS) [calcd. 3066.0, found 3066.7 $(M+H)^+$]. Segment **2** was also obtained in the same manner as **1** using DICDI in a good yield [59%, TOFMS: calcd. 2399.9, found 2400.3 $(M+Na)^+$].[8] The yields of the thioester segments obtained here were comparable to those obtained by Boc-SPPS (11-26%).[1]

An alternative approach was developed for the preparation of the thioester segment, where an N-terminal protected peptide was conjugated with H-Gly-S-$(CH_2)_2CONH_2$ through an amide bond formation (Approach 2., Fig. 1b). To estimate the practicability of this approach, the thioester segments **1'** [Ac-Ile-Ser-Ile-Thr-Phe-Phe-Arg-Leu-Phe-Arg-Val-Met-Arg-Leu-Val-Lys(Cl-Z)-Leu-Leu-Ser-Arg-Gly-Glu-Gly-S-$(CH_2)_2$-$CONH_2$] and **2'** [Ac-Leu-Pro-Leu-Ala-Leu-Ala-Gln-Leu-Val-Leu-Gly-Leu-Leu-Pro-Val-Leu-Leu-Glu-Gln-Phe-Gly-S-$(CH_2)_2$-$CONH_2$] having identical amino acid sequences with segments **1** and **2**, respectively, were prepared. A protected peptide, Ac-Ile-Ser(^{t}Bu)-Ile-Thr(^{t}Bu)-Phe-Phe-Arg(Pmc)-Leu-Phe-Arg(Pmc)-Val-Met-Arg(Pmc)-Leu-Val-Lys(Cl-Z)-Leu-Leu-Ser(^{t}Bu)-Arg(Pmc)-Gly-Glu(O^{t}Bu)-OH (Pmc=N^G-2,2,5,7,8-pentamethylchroman-6-sulfonyl), which was prepared by Fmoc-SPPS on a Cl-Trt resin, was coupled with H-Gly-S-$(CH_2)_2$-$CONH_2$[9] in DMF in the presence of DICDI and 1,2-hydroxybenzotriazole (HOBt) (20 eq, each) at r. t. for 48 h.[7] Treatment of the sample with 95% aqueous TFA (r. t., 2 h) afforded the desired thioester **1'** in a 13% yield.[10] The use of WSCDI (10 eq) and HOBt (1eq) (4°C, 12 h) resulted in a comparable yield (14%). The segment **2'** was obtained in a similar fashion in a 51% yield.[8] The ratios of racemization of Glu (position 22) in **1'** and Phe (position 20) in **2'** after the coupling of the protected peptides with H-Gly-S-$(CH_2)_2$-$CONH_2$ were determined by gas chromatography using the capillary of a Chirasil-Val column as reported,[11] and judged to be negligible (<2 % for Glu in **1'** and not detected for Phe in **2'**, respectively). This approach was thus evaluated to be also practical.

In order to examine the applicability of the thioester method to the construction of TASP molecules, the above-obtained thioester segment **1** (12 eq) was taken as an example of the trial the introduction onto a Mutter-type template, Ac-Lys-Ala-Lys-Pro-Gly-Lys-Ala-Lys-Gly-NH_2[2] in the presence of HOBt (36 eq), $AgNO_3$ (24 eq) and diisoproylethylamine (DIEA) (36 eq) in dimethylsulfoxide (DMSO) at 37°C for 48 h (Fig. 3). After DMSO was removed by evaporation, the sample was treated with 1M $(CH_3)_3SiBr$-thioanisole in TFA

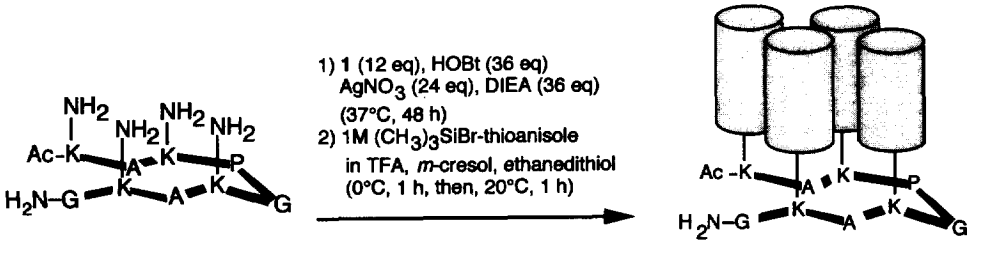

Fig. 3. Construction of a TASP Molecule Using a Partially Protected Peptide Thioester Segment
(K = Lys, A = Ala, P = Pro, G = Gly)

in the presence of *m*-cresol and ethanedithiol[12] to remove the remaining Cl-Z group followed by purification on HPLC to afford the desired four-helix-bundle protein **3** in a 19% yield (for the two steps) [TOFMS: calcd. 11978.5; found 11979.1 $(M+H)^+$]. Using **1'**, the identical protein was also obtained without difficulty (yield: 18%). The thioester R-CO-S-$(CH_2)_2$-COOEt was thus judged as effective as the conventional thioester R-CO-S-$(CH_2)_2$-$CONH_2$ (R denotes a partially protected peptide segment). Also, the obtained yields were comparable with that of a TASP molecule obtained by the reaction of a peptide thiocarboxylic acid (peptide-COSH) with a template bearing four bromoacetylated Lys side chains.[13]

In conclusion, we have developed two approaches to preparation of the S-alkyl thioester of a partially protected peptide segment using Fmoc-SPPS. Also, our results should open the way to construction of TASP molecules using peptide thioesters. The ion channel activity of **3** is under investigation in our laboratory.

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7. The reaction was almost complete in 3 h as judged by HPLC, but the reaction was continued further with the expectation of obtaining a better yield.
8. The yields of the thioester seemed to be highly dependent on the purity of the protected peptide fragments constructed on the 2-chlorotrityl resin. Also, a notable amount of the acylurea (Sheehan, J. C.; Goodman, M.; Hess, G. P. *J. Am. Chem. Soc.* **1956**, *78*, 1367) and the methionine sulfoxide was formed during conjugation of the protected fragment with HS-$(CH_2)_2$-COOEt in the preparation of **1**.
9. Prepared by HCl-dioxane treatment of Boc-Gly-S-$(CH_2)_2$-$CONH_2$ that was obtained by the reaction of Boc-Gly-S-$(CH_2)_2$-COOH[1a] with ammonium hydroxide through a mixed anhydride method (Vaughan, J. R. Jr. *J. Am. Chem. Soc.* **1951**, *73*, 3547).
10. The oxidation of the methionine and the partial hydrolysis of the thioester were observed during the coupling of the protected fragment with H-Gly-S-$(CH_2)_2$-$CONH_2$ in the preparation of **1'**.
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