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# Direct preparation of peptide thioesters using an Fmoc solid-phase method

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**Abstract**

Aiming at the direct preparation of peptide thioesters by an Fmoc solid-phase method, we searched a new deblocking reagent, which efficiently removed Fmoc groups while keeping the thioester intact. The deblocking reagent, which contains 1-methylpyrrolidine, hexamethyleneimine and HOBt in a one to one mixture of NMP and DMSO, realized the preparation of peptide thioesters by an Fmoc solid-phase method in a yield equivalent to that obtained by a Boc solid-phase method. 

*Keywords: Peptides and polypeptides; Thioesters; Deblocking; Solid-phase synthesis*

The thioester method, in which partially protected peptide *S*-alkyl thioesters are used as building blocks, has been shown to be useful for polypeptide syntheses [1-3]. Typically, such building blocks are prepared by a Boc solid-phase method [4,5]. Although the fluoren-9-ylmethoxycarbonyl (Fmoc) solid-phase method [6,7] constitutes a widely accepted method for peptide synthesis, it could not be employed for the preparation of peptide thioesters, since thioesters are easily decomposed by aminolysis during the removal of Fmoc groups by treatment with piperidine, which is involved in standard Fmoc chemistry. The Fmoc solid-phase method requires neither the repetitive use of trifluoroacetic acid nor strong acid treatment in order to obtain free peptides. These features are advantageous not only to the preparation of simple peptides, but also to the preparation of conjugated peptides such as phosphopeptides and glycopeptides. As a result, a new route for the preparation of a wide variety of polypeptides by the thioester method and chemical ligation method [8-11] is possible, provided conditions for the preparation of peptide thioesters by the Fmoc solid-phase method can be defined.

In order to prepare the peptide thioesters directly by an Fmoc solid-phase method, we carried out detailed study of the conditions for the removal of Fmoc groups while keeping the thioester intact. Using a model peptide thioester, Fmoc-Phe-Leu-Ala-Cys(Acm)-His-Gly-$SCH_2CH_2CONH_2$ (**1**), which was prepared by a Boc solid-phase method [12], we evaluated the effect of a series of amines on the rates of the removal of Fmoc groups and the aminolysis of thioester moieties in 1-methyl-2-pyrrolidinone (NMP) [13], which is one of the standard solvents for the preparation of deblocking reagents in the Fmoc solid-phase method. Peptide **1**

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was dissolved in 1-methyl-2-pyrrolidinone (NMP) at a concentration of 1 mg $ml^{-1}$ (about $1x10^{-3}$ M) and the solution (80 µl) was mixed with each amine (20 µl) at room temperature for 20 min. The reaction mixture was then analyzed by reversed phase HPLC using an ODS column and the separated peaks were subjected to MALDI-TOF mass analysis. The deblocking of Fmoc groups and the decomposition of thioester moieties were estimated by the peak areas on the HPLC.

Piperidine, cyclohexylamine, 4-aminomethylpiperidine and morpholine completely removed the Fmoc group, but the thioester moiety was also completely decomposed by aminolysis as well. Hexamethyleneimine and heptamethyleneimine completely removed the Fmoc group while 20 to 25% of the thioester moieties remained intact. Dicyclohexylamine, *cis*-2,6-dimethylpiperidine and diisopropylethylamine did not decompose the thioester moiety, but were not sufficiently strong to remove the Fmoc group within 20 min. Neither 1-methylpiperidine nor 1-methylpyrrolidine destroyed the thioester moiety. The Fmoc group was not removed by 1-methylpiperidine even after one hour treatment, while 1-methylpyrrolidine removed the Fmoc group completely within 20 minutes. 1-Hydroxybenzotriazole (HOBt) was added to the reaction mixture to lower the nucleophilicity of the amines. This is based on the fact that HOBt is known to suppress the cyclization of the aspartyl residue during base treatment [14,15]. In our hands, HOBt suppressed the aminolysis of the thioester. When peptide **1** was treated with a solution of NMP containing hexamethyleneimine (20%, *v/v*) and HOBt monohydrate (23%, *w/v*), the aminolysis of peptide **1** was reduced to 20%. Under the same treatment conditions, only 15% of the thioester in Fmoc-Phe-Leu-Ala-Cys(Acm)-His-Gly-$SC(CH_3)_2CH_2CONH_2$ (**2**) was decomposed by aminolysis. The *S*-tertiary alkyl thioester was slightly more stable than *S*-primary alkyl thioester. The presence of HOBt had no effect on aminolysis with piperidine.

Taking these results into account, the solid-phase synthesis of the partial sequence of Vero-toxin [16] was carried out using a peptide synthesizer model 433A (Applied Biosystems Inc., Foster City, CA) to establish conditions for the practical solid-phase synthesis of peptide thioesters by Fmoc strategy. Based on the standard protocol of *FastMoc* 0.10 mmol, the deprotection module of the Fmoc group was slightly modified. A pre-mixed deblocking reagent was introduced to the reaction vessel and the peptide resin was treated with the deblocking reagent for 2.9 min and then 18 min. Starting resin, Fmoc-Gly-$SC(CH_3)_2CH_2CONH$-SAL-β-Ala-MBHA resin (Gly: 0.20 mmol/g resin), was prepared by condensing Fmoc-Gly-$SC(CH_3)_2CH_2CO_2H$ and 4-(2',4'-dimethoxyphenyl-aminomethyl)-phenoxyacetoamido-β-alanyl-*p*-methyl-benzhydrylamine resin ($NH_2$-SAL-β-Ala-MBHA resin) [17] in the presence of dicyclohexylcarbodiimide and HOBt. Each Fmoc-amino acid (1 mmol) was introduced to the resin after activation by 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU) (0.9 mmol), HOBt (0.9 mmol) and *N,N*-diisopropylethylamine (2.0 mmol) in a mixed solvent of DMF and NMP. Excess reagents were washed with NMP throughout the chain elongation cycle. Each obtained peptide resin, Tyr($Bu^t$)-Thr($Bu^t$)-Lys(Boc)-Tyr($Bu^t$)-Asn(Trt)-Asp($OBu^t$)-Asp($OBu^t$)-Asp($OBu^t$)-Thr($Bu^t$)-Phe-Thr($Bu^t$)-Val-Lys(Boc)-Val-Gly-$SC(CH_3)_2CH_2CONH$-SAL-β-Ala-resin (50 mg) was treated with reagent K [18] (1 ml) for 3 hours at room temperature. The resulting cleaved peptide was precipitated by adding cold

ether. The precipitate was then washed 3 times with ether, extracted with 50% aq. acetonitrile, and lyophilized. The crude product thus obtained was analyzed by reversed-phase HPLC and each isolated peak was subjected to MALD-TOF mass analysis to characterize the product. The isolated yields of the desired product, Tyr-Thr-Lys-Tyr-Asn-Asp-Asp-Asp-Thr-Phe-Thr-Val-Lys-Val-Gly-$SC(CH_3)_2CH_2CONH_2$ (Vero-toxin(11–25)-$SC(CH_3)_2CH_2$-$CONH_2$ (3)), were calculated based on the Gly content in the starting resin and quantitative amino acid analysis data for peptide 3, and are summarized in Table 1.

**Table 1.**
Synthesis of Vero-toxin(11–25)-$SC(CH_3)_2CH_2CONH_2$ (3) using different deblocking reagents

| deblocking reagent[a] | yield of peptide 3 (%) |
|---|---|
| piperidine (25%) in NMP | 0 |
| piperidine (25%), HOBt (2%) in NMP | 0 |
| hexamethyleneimine (25%) in NMP | 1.9 |
| hexamethyleneimine (25%), HOBt (2%) in NMP | 3.3 |
| 1-methylpyrrolidine (25%) in NMP | 5.0 |
| 1-methylpyrrolidine (25%), HOBt (2%) in NMP | 6.8 |
| 1-methylpyrrolidine (25%), hexamethylene imine (2%), HOBt (2%) in NMP | 14 |
| 1-methylpyrrolidine(25%), hexamethylene imine (2%), HOBt (2%) in NMP-DMSO (1:1) | 24 |

[a]Percentage of piperidine, hexamethyleneimine, and 1-methylpyrrolidine is shown in *v/v*, and percentage of HOBt is in *w/v*, where 2% *w/v* is equal to 2 g in 100 ml of solution.

No desired product was obtained when NMP solutions containing piperidine (25%, *v/v*), or both piperidine (25%, *v/v*) and HOBt (2%, *w/v*) were used as debloking reagents. All the deblocking reagents, including NMP solutions containing hexamethyleneimine (25%, *v/v*) with or without HOBt (2%, *w/v*), and NMP solutions containing 1-methylpyrrolidine (25%, *v/v*) with or without HOBt gave poor results. The yields of the desired product were low and numerous by-products were observed in these preparations. As a result, a three-component deblocking reagent was prepared, which contained 1-methylpyrrolidine, hexamethyleneimine and HOBt. This reagent greatly improved the yields and was used as a deblocking reagent. The yield was further improved by the use of a one to one mixture of NMP and DMSO as a solvent to dissolve these three components. These results suggest that the mixed solvent composed of NMP and DMSO efficiently solvated the growing peptide chain [13], that 1-methylpyrrolidine was a sufficiently strong base to remove Fmoc groups, that hexamethyleneimine was efficient nucleophile for scavenging the reactive dibenzofulvene, and that HOBt efficiently suppressed the aminolysis caused by hexamethyleneimine.

This deblocking reagent, which contains 1-methylpyrrolidine (25% *v/v*), hexamethyleneimine (2% *v/v*) and HOBt (2% *w/v*) in a one to one mixture of NMP-DMSO, was applied to the synthesis of Thr-Pro-Asp-Cys(Acm)-Val-Thr-Gly-Lys-Val-Glu-Tyr-Thr-Lys-Tyr-Asn-Asp-Asp-Asp-Thr-Phe-Thr-Val-Lys-Val-Gly-$SC(CH_3)_2CH_2CONH_2$ ([Cys(Acm)[4]]-Vero-toxin (1–25)-$SC(CH_3)_2CH_2CONH_2$). This protected peptide resin (350 mg) was obtained from the starting resin (0.20 mmol/g resin, 200 mg). After treatment of the protected peptide resin with reagent

K, the crude product was analyzed by reversed-phase HPLC (Fig. 1) and the desired product was isolated by reversed-phase HPLC in the yield of 22% based on the Gly content in the starting resin. The product was confirmed by mass spectrometry as well as amino acid analysis; $[M+H]^+$obs.: *m/z* 2982.25, $[M+H]^+$calcd: 2982.35; $Asp_{4.92}Thr_{4.57}Glu_{1.16}Gly_{2.06}Val_{4.12}Tyr_{2.22}Phe_{(1)}$ $Lys_{2.91}Pro_{0.78}$1/2$Cys_{n.d.}$. The yield of this peptide thioester was slightly higher than that obtained by Boc strategy, by which the desired peptide was obtained in 19% yield.

This result represents an important step for the generalization of the thioester method and the preparation of conjugated polypeptides, such as phosphoproteins and glycoproteins.

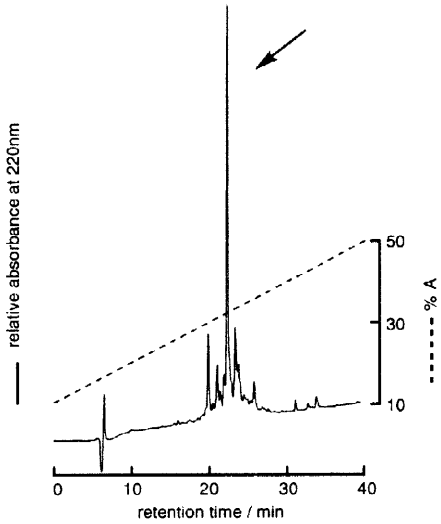


**Figure 1.** An analytical HPLC elution profile of a crude [Cys(Acm)$^4$]-Vero-toxin(1–25)-SC$(CH_3)_2CH_2CONH_2$. Reservoir A contained acetonitrile containing 0.1% TFA and reservoir B contained 0.1% aq. TFA. Peptides were analyzed by a linear gradient of 10–50% A over 40 min using Cosmosil 5$C_{18}$AR (10 x 250 mm) at a flow rate of 2.5 ml/min. An arrow indicates the peak that contains a desired product.

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