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High-pressure-promoted Fmoc-aminoacylation of *N*-ethylcysteine: preparation of key devices for the solid-phase synthesis of peptide thioesters

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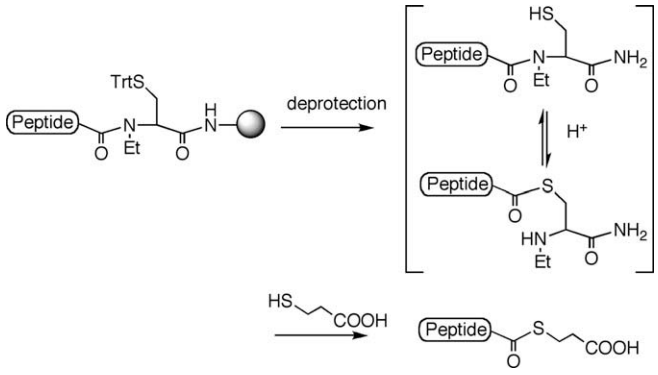
ABSTRACT

In this study synthesis of Fmoc-aminoacyl-*N*-ethyl-*S*-triphenylmethylcysteine, a new *N*→*S* acyl migratory device for the preparation of peptide thioesters by Fmoc solid-phase peptide synthesis (SPPS) is described. Condensation of Fmoc-aminoacyl fluoride and *N*-ethyl-*S*-triphenylmethylcysteine allyl ester, readily prepared from known *S*-triphenylmethylcysteine allyl ester, was efficiently promoted in CH_2Cl_2 under high-pressure (800 MPa). When the reaction was performed with the additive DIEA, considerable epimerization at the chiral centers occurred, affording a mixture of diastereomers. When the preparation procedure for *N*-ethyl-*S*-triphenylmethylcysteine allyl ester was changed and the additive DIEA in the high-pressure reaction was excluded, Fmoc-aminoacyl-*N*-ethyl-*S*-triphenylmethylcysteines was obtained as a single stereoisomer without epimerization. The Fmoc-L-leucine adduct thus prepared was deallylated and used for the SPPS of a known decapeptide. A remarkable increase (44%) in the overall yield of the decapeptidethioester was achieved, compared to the 7% obtained by the stepwise on-resin Leu-Cys condensation method.

Peptide thioester is a crucial chemical motif in ligating peptide segments by the thioester method[1] and by native chemical ligation.[2] Therefore, concise solid-phase synthesis of peptide thioesters especially by the Fmoc protocol is currently of interest to reseachers.[3] In a previous paper, we disclosed our new strategy for realizing post-SPPS thioesterification via an efficient *N*→*S* acyl migration at the C-terminal *N*-alkylcysteine moiety (NAC-assisted thioesterification).[4] Treatment with Reagent K detached the Fmoc peptide assembled on the *N*-alkylcysteine-linked amide resin from the resin,[5] and the acyl migration followed by thiol exchange with the additive 3-mercaptopropionic acid (see Scheme 1) exclusively converted the released peptide to a peptide thioester.

In this method, the decapeptide thioester carrying a C-terminal Gly residue (EVTGHRWLKG-SCH_2CH_2COOH) was synthesized in a good overall yield (34%). Synthesis of the other decapeptide thioesters with a chiral amino acid at the C-terminus was also performed with minimum epimerization, but the yields were lower (4.4–7.0%). This remarkable decrease of the yield can be attributed to insufficient loading of the C-terminal amino acid on the sterically hindered *N*-ethylcysteine site. Even in the case of the least stereo-demanding glycine, a large excess of Fmoc-glycine**1** (10 equiv × 2) and a highly reactive agent were necessary to fulfil the aminoacylation. Therefore, we set out to study the preformation of Fmoc-aminoacyl-*N*-ethylcysteines to eliminate this difficult on-resin aminoacylation step.

The first experiment was designed for direct aminoacylation of *N*-ethyl-*S*-triphenylmethylcysteine**7**[4] in solution. When Fmoc-Gly-OH **1** (1.5 equiv) was activated with HATU (1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo(4,5-*b*)pyridinium 3-oxide hexafluorophosphate) and DIEA (*N*,*N*-diisopropylethylamine) in DMF and then reacted with **7** at room temperature, the coupling reaction took place within 30 min to give dipeptide **9** in a 75% yield. However,



Scheme 1. Post-SPPS thioesterification using *N*-ethylcysteine device.

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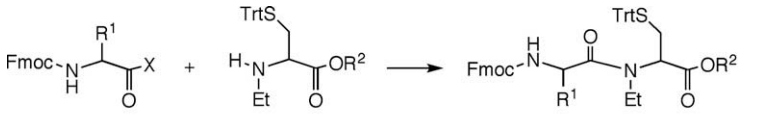

1: R^1 = H, X = OH
2: R^1 = CH_3, X = OH
3: R^1 = $CH_2CO_2Bu^t$, X = OH
4: R^1 = $CH_2CH(CH_3)_2$, X = F
5: R^1 = $CH_2CO_2Bu^t$, X = F
6: R^1 = $CH_2CH_2CONHTrt$, X = F

7: R^2 = H
8: R^2 = -$CH_2CH{=}CH_2$ (All)

9: R^1 = H, R^2 = H
10: R^1 = H, R^2 = All
11: R^1 = $CH_2CH(CH_3)_2$, R^2 = All
12: R^1 = $CH_2CH(CH_3)_2$, R^2 = H
13: R^1 = $CH_2CO_2Bu^t$, R^2 = All
14: R^1 = $CH_2CH_2CONHTrt$, R^2 = All

Scheme 2. Preparation of Fmoc-aminoacyl *N*-ethylcysteines.

the same reactions using chiral Fmoc-amino acids, **2** and **3**, produced a complex mixture from which the desired pure Fmoc-L-aminoacylated *N*-ethyl-*S*-triphenylmethyl-cysteine was isolated only in a low yield after cumbersome chromatographic purification (see Scheme 2).

The second experiment was carried out with *N*-ethyl-*S*-triphenylmethyl-cysteine allyl ester **8** to improve the preparation and isolation of the dipeptidyl product. *S*-Triphenylmethyl-cysteine allyl ester, prepared by the reported procedure,[6] was N-ethylated with acetaldehyde and $NaBH_3CN$ to give allyl ester **8** in a 75% yield. Coupling of Fmoc-glycine **1** and **8** was readily promoted by HATU to produce **10** in a 90% yield. However, using the same procedure, chiral Fmoc-amino acids poorly produced the desired coupling products. Enhanced conditions are required to facilitate amide formation with such poor nucleophiles as *N*-alkyl amino acid.[7] After several experiments to screen conditions, we found that a high-pressure reaction[8] (800 MPa, 3 h) using Fmoc-aminoacyl fluorides (1.5 equiv) and **8** in CH_2Cl_2 in the presence of DIEA (1.0 equiv) produced high yields of the coupling products. We tested Fmoc-Leu-F **4**, Fmoc-Asp(OBu^t)-F **5** and Fmoc-Gln(Trt)-F **6** in this reaction. Among the coupling products, **11** and **14** were obtained as a separable mixture of major and minor components. NMR and mass spectral data indicated that the major and minor products were diastereomers of each other. Table 1 (entries 1–3) shows the yield and the specific rotation values of each coupling product.

To determine the stereostructure by transformation into a known oligopeptide, the Leu-derived major and minor products **11a** and **11b** were individually deallylated with $Pd(Ph_3P)_4$ and dimedone in THF to form the carboxylic acids **12a** and **12b**. The compound **12b** was obtained as crystals. Recrystallization of **12b** from CH_3CN made its specific rotation value almost null, and afforded a sample suitable for X-ray analysis. The crystallographic data showed that the sample was a racemate of Fmoc-L-Leu-D-(Et)-Cys(Trt)-OH and Fmoc-D-Leu-L-(Et)Cys(Trt)-OH.[9] Therefore, it became clear that epimerization occurred in part at both the chiral carbons of the Leu and Cys residues during this synthetic process, and accordingly the major diastereomer **12a** (**11a**) might have been contaminated with its enantiomer to some extent.

Table 1
Dipeptides prepared by high-pressure reaction

Entry	Fluoride	Cys	DIEA	Major product (%)	$[\alpha]_D$[a]	Minor product (%)	$[\alpha]_D$[a]
1	**4**	**8**	+	**11a** (70)	−28.7	**11b** (21)	+10.1
2	**5**	**8**	+	**13ab** (74)	−33.9		
3	**6**	**8**	+	**14a** (67)	−17.7	**14b** (25)	+8.9
4	**4**	**8**	–	**11a′** (68)	−32.2	**11b′** (14)	+36.8
5	**4**	**8′**	–	**11c** (94)	−33.5		
6	**5**	**8′**	–	**13c** (77)	−56.1		
7	**6**	**8′**	–	**14c** (76)	−20.2		

[a] Specific rotation values were measured at 20 ± 2 °C for solutions in $CHCl_3$.

Definitive evidence on the epimerization was gained by a peptide thioester synthesis with **12a** and **12b**.The known decapeptide-ethioester (ATEVTGHRWL-$SCH_2CH_2CO_2H$) **15** was synthesized as a synthetic model since both the peptide and its C-terminally epimerized peptide had been synthesized and readily distinguished from each other by HPLC.[4] Dipeptide **12a** (2 equiv) was used to attach to the CLEAR-amide resin by activation with DCC–HOBt.[10] Subsequently, the resin was subjected to chain assembly in an automated peptide synthesizer under the FASTMOC program (HBTU–HOBt, NMP).

The obtained decapeptide-bound resin had a reasonable increase in weight. Then treatment with Reagent K released the peptide, and ether precipitated it. The crude peptide was converted into the thioester through *N*→*S* acyl migration and thiol exchange with 3-mercaptopropionic acid (MPA) according to the reported procedure. We next synthesized the decapeptide using **12b** in place of **12a**. Figure 1a and b compares the HPLC data of peptide thioesters produced from **12a** and **12b**. Peak 1 ($M+H^+$: *m/z* 1257.92) and peak 2 ($M+H^+$: *m/z* 1257.93) represent the decapeptides carrying C-terminal L-Leu (**15**) and D-Leu, respectively. Both isomers were separated to determine their respective yields by amino acid analysis. A distinct improvement in the yield of **15** (43%) was established over the previous one (7%).[4] The C-terminal isomer corresponding to peak 2 was isolated in a 2% derived from the **12a** experiment. In contrast, the product derived from **12b** demonstrated peaks 1 and 2 with an approximately equal intensity. The ratio of the stereoisomers in the products appeared to reflect the enantiomeric purity of **12a** and **12b**, given that no additional epimerization occurred in the acyl migration and thiol exchange stages.

Therefore, the high-pressure reaction of **4** and **8** in the absence of the additive base was reinvestigated taking into account the potential participation of DIEA or generated conjugate base F^- in the above epimerization. The reaction was promoted with no damage to the acid-labile *S*-Trt group to again produce a mixture of two stereoisomers in a high yield. However, the proportion shifted more to the major isomer than the previous experiment. In addition, the separated minor isomer (**11b′**, see Table 1, entry 4) displayed a marked increase in its value of specific rotation (+36.8), suggesting its high enantiomeric purity.[11] Therefore, it is speculated that excluding DIEA minimized the epimerization and that **11b′** arose from the D-isomer contaminated in reactant **8**. However, we were unable to determine whether or not the epimerization at Cys had occurred before exposure to the high-pressure conditions. An attempt to determine the enantiomeric purity of **8** by chromatographic analysis with chiral columns was unsuccessful.

Then *S*-triphenylmethyl-cysteine allyl ester was alternatively synthesized by allylation of a cesium salt[12] of Fmoc-*S*-triphenylmethyl-cysteine with allyl bromide and then by N-deprotection. Though difficult to discuss its precise enantiomeric purity with such a small value range, a higher enantiomeric purity of the newly synthesized sample (**8′**) was expected, owing to the slight increase in its specific rotation value (+7.0, cf. **8**: +5.5). Thus, high-pressure

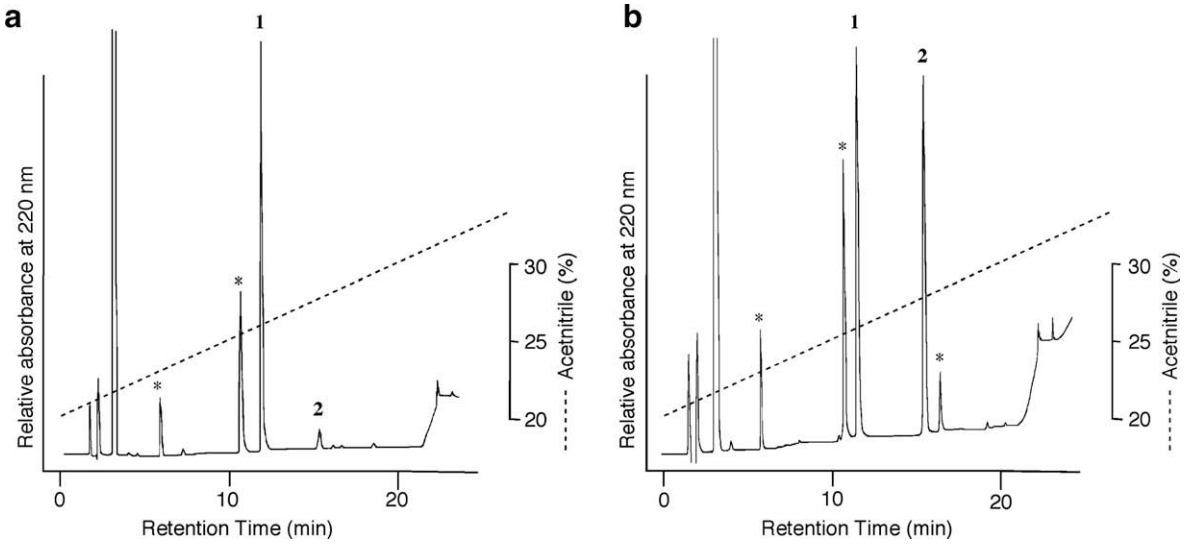

Figure 1. HPLC profiles of the synthetic peptide thioesters synthesized using Fmoc-Leu-(Et)Cys(Trt)-OH, (a) **12a** and (b) **12b**: Peaks 1 and 2 correspond to the peptide thioesters carrying C-terminal L-Leu and D-Leu, respectively. Conditions: column, Mightysil RP-18, 4.6 × 150 mm (5 μm); eluent A, distilled water containing 0.1% TFA, eluent B, acetonitrile containing 0.1% TFA, flow rate, 1 ml/min. * Non-peptidyl contaminants.

reactions with **8′** were performed using the fluorides **4**, **5** and **6** by excluding DIEA. Every reaction produced a single coupling product as shown in Table 1 (entries 5–7: **11c**, **13c**, and **14c**). These results supported the high enantiomeric purity of **8′** and the lack of epimerization in the high-pressure reactions.

Finally, peptide thioester **15** using **12c**, derived by deallylation of **11c**, was synthesized. The result was in accord with our expectations as shown in Figure 2, in which **15** appeared as a single stereoisomer. The production of the C-terminal isomer was not observed. Therefore, it was concluded that no epimerization occurred during the post-SPPS thioester synthesis. The yield of isolated **15** was 44%.

In summary, solid-phase synthesis of peptide thioester was remarkably improved by the use of Fmoc-L-aminoacyl-*N*-ethyl-*S*-triphenylmethyl-L-cysteine, which we efficiently prepared through a high-pressure reaction. The enantiomerically pure L-leucine derivative was attached to resin with DCC–HOBt, and then nine amino acids were condensed on the resin by the Fmoc protocol with an automated peptide synthesizer. Cleavage of the peptide from the resin with Reagent K followed by thioester exchange with the additive 3-mercaptopropionic acid produced a stereochemically pure decapeptide thioester in 44% yield. Thus, the thioester synthesis including *N*→*S* acyl migration and thiol exchange steps exclusively proceeds with configurational retention of the C-terminal amino acid. As a consequence, the method demonstrated here extends the scope of NAC-assisted synthesis of peptide thioesters, which should allow us to design the peptide segments usable in chemical ligation more freely.

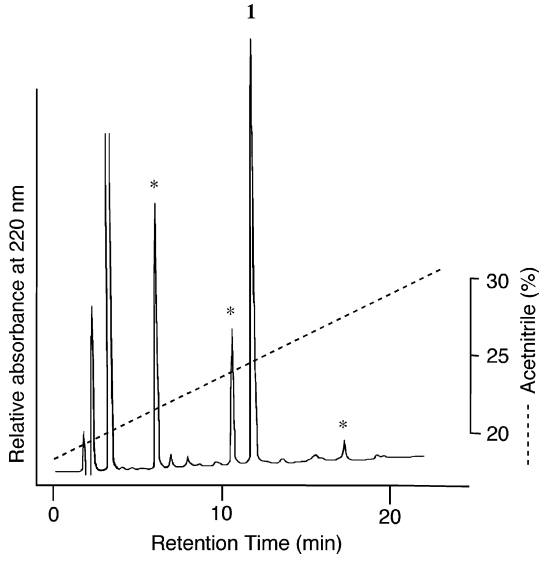

Figure 2. HFLC profile of the peptide thioester synthesized using Fmoc-Leu-(Et)Cys(Trt)-OH **12c**: Peak 1 corresponds to the peptide thioester **15**. Conditions: column, Mightysil RP-18, 4.6 × 150 mm (5 μm); eluent A, distilled water containing 0.1% FFA; eluent B, acetonitrile containing 0.1% TFA; flow rate, 1 ml/min. * Non-peptidyl contaminants.

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Supplementary data

Supplementary data (experimental procedures, NMR and MS of **8**, **8′**, **9**, **10**, **11a**, **11b**, **11a′**, **11b′**, **11c**, **12a**, **12b**, **12c**, **13ab**, **13c**, **14a**, **14b**, **14c** and **15**) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.11.034.

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9. Fmoc-Leu-(Et)Cys(Trt)-OH **12 b** was crystallized with equimolar CH_3CN, and the crystal was determined as space group: $P2_1/n$. The 3D drawing is shown in the online Supplementary data.
10. When using HATU as an activating agent in place of DCC–HOBt, a decapeptidyl by-product carrying C-terminal Leu-NH_2 was ultimately produced in a substantial quantity (8%).
11. The $[\alpha]_D$ value was in fair agreement with that (+35.2) of the major dipeptide separately synthesized by high-pressure coupling of **4** and D-(Et)Cys(Trt)-OAll which was analogously prepared from Fmoc-D-Cys(Trt)-OH and allyl alcohol with HOBt, HBTU, and DIEA (see Ref. 6).
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