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An improved deblocking agent for direct Fmoc solid-phase synthesis of peptide thioesters

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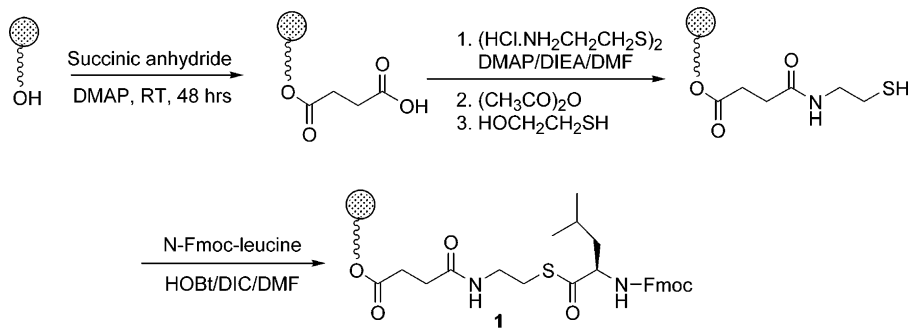
Abstract—To synthesize peptide thioesters directly on a solid support for use as substrate analogues for thioesterases in non-ribosomal peptide synthases, we modified a reagent compatible with Fmoc solid-phase peptide synthesis that efficiently removes the protecting group while keeping the thioester intact. The deprotecting reagent, consisting of DBU and HOBt, was successfully used to prepare a decapeptide in high yield. © 2002 Elsevier Science Ltd. All rights reserved.

Peptide thioesters have been useful in cyclic peptide synthesis[1] and native chemical ligation.[2] They are base-labile and are usually synthesized using Boc solid-phase methods.[3,4] Direct synthesis of peptide thioesters using the Fmoc solid-phase method is hampered by their sensitivity towards aminolysis under most of the deblocking conditions employed. For most purposes, these activated peptides can also be obtained indirectly from thiol esterification of peptides synthesized by a standard or modified Fmoc method.[5,6] However, in our attempt to mimic thiol-template synthesis of cyclic peptides by non-ribosomal peptide synthases (NRPS),[7,8] we needed to synthesize directly the peptide thioesters on a solid-support with a linker similar to pantetheine. Since the Boc method requires a special experimental set-up, we aimed to find a deblocking agent suitable for direct parallel synthesis of peptide thioesters, substrate analogues for thioesterases in NRPS, via the Fmoc solid-phase method.

A few successful methods to prepare peptide thioesters directly by Fmoc solid-phase synthesis have been reported.[9–11] Aimoto and co-workers[10] used a mixture of 25% 1-methylpyrrolidine, 2% hexamethylene imine, and 2% HOBt in *N*-methylpyrrolidinone and dimethylsulfoxide (1:1) as the deblocking agent and successfully synthesized a 25-mer peptide thioester in high yield. However, the necessitated use of a tertiary thioester as a linker in this method was not suitable for our purpose in which a primary thioester ester has to be synthesized to mimic the wild-type pantetheinyl linker. Wade and co-workers[11] reported another deblocking agent consisting of 1% DBU and 1% HOBt in DMF (v/w/v) to prepare primary peptide thioesters for native chemical



Scheme 1. Addition of a cysteamine linker to TentaGel and coupling of the first amino acid leucine.

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ligation. Following this method, we prepared TentaGel (TG) resin with a primary thiol linker as shown in Scheme 1 and used it to synthesize the pentapeptide, Boc-Phe(D)-Val-Pro-Orn-Leu-S-linker-TG, a substrate analogue of gramicidin thioesterase,[7,8] in a batch-wise manner. After Boc removal and complete cleavage of the peptides from the resin with 20% piperidine, the product was lyophilized and analyzed by RP-HPLC and LC–MS. Surprisingly, although a small amount of the expected peptide (<5%) was obtained, the major components in the mixture were piperidine adducts of dibenzofulvene and leucine, products of the Fmoc deblocking reaction and aminolysis of leucine thioester linked to the TG resin, respectively. This result indicated that a significant amount of the Fmoc protecting group of the first amino acid leucine remained intact after repetitive deblocking treatment. Therefore, the deblocking agent used in this method is not efficient enough to remove Fmoc for high-yield production of peptide thioesters.

We reasoned that the slow Fmoc deblocking reaction was due to the overall acidity of the reagent (molar ratio DBU:HOBt = 0.88:1). To test this theory, the amount and the ratio of DBU and HOBt were varied to find the best conditions under which Fmoc could be removed efficiently while keeping the thioester intact. Fmoc deblocking was performed under a nitrogen atmosphere by immersing 100 mg of the swollen modified TG resin with leucine (**1**) in 1 mL of a selected deblocking mixture for exactly 1 min. The deprotection reagent was completely removed by filtration and the resin was immediately washed with 3×10 mL of dry DMF. After setting aside an aliquot of about 10 mg of resin for analysis, the deprotection was repeated four times. Aliquots of resin from each deprotection cycle were dried overnight by vacuum evaporation, weighed, swollen again in DMF and finally analyzed. Residual Fmoc was determined by complete cleavage of the blocking group in 20% piperidine, and by measuring the absorbance (290 nm) of the filtrate.[12] The extent of aminolysis was determined by measuring the amount of free amine using the ninhydrin method.[13] After normalizing the resulting absorbance with the amount of resin, the extent of Fmoc deprotection and amount of free amine after each wash are shown in Fig. 1. As expected, the most efficient Fmoc deprotection and least aminolysis (most free amine) were achieved simultaneously when the deprotection reagent was kept slightly basic at an appropriate concentration (0.080 mM DBU/74 mM HOBt/DMF). All other combinations of DBU and HOBt either demonstrated low deprotection ability leading to incomplete Fmoc removal or excessive aminolysis that would eventually fail peptide synthesis. Using this mixture, the best results were achieved by three 1 min deprotection cycles.

Based on the deprotection study, 80 mM DBU/74 mM HOBt/DMF was the best deblocking mixture for direct Fmoc solid-phase synthesis of thioester peptides. We used this reagent to synthesize the tyrocidine substrate[7,8] analogue Boc-Phe(D)-Pro-Phe-Phe(D)-Asn-Gln-Tyr(^{t}Bu)-Val-Orn (Boc)-Leu-S-linker-TG starting from 300 mg of resin (**1**) (0.26 mmol/g). The synthesis followed the standard procedure in a batch-wise manner with the Fmoc deprotected by 3×1 min washes with the deblocking mixture for the first two residues. Considering the peptide chain has protecting effects on the thioester,[11] deprotection was changed to 4×1 min for more efficient Fmoc removal starting from the third amino acid, valine. After the synthesis and removal of the Boc and *t*-butyl protecting groups using trifluoroacetic acid, 40 mg of the vacuum-dried resin was treated with 1 mL of aqueous ammonia for 1 h at room temperature to cleave the peptide from the resin for characterization. After two 1.5 mL washes of the resin, the resulting aqueous solutions were combined and dried in a vacuum. The residue was washed once with 5 mL cold ether and the solvent was evaporated to obtain 5 mg of a crude white material.

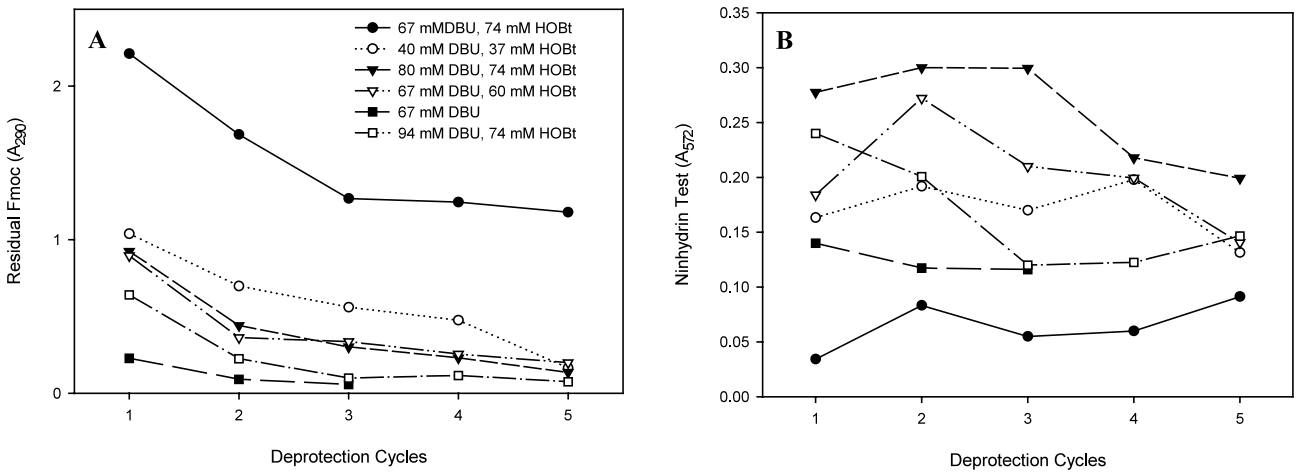

Figure 1. Optimization of Fmoc deprotection conditions for solid-phase peptide thioester synthesis. (A) Amount of residual Fmoc per milligram resin after each deprotection cycle. (B) Amount of free amino group per milligram resin after each deprotection cycle. The absorbance values have been normalized to represent that of 1 mg of dry resin. Each symbol and line pattern denotes one deblocking mixture as specified in the legend of (A).

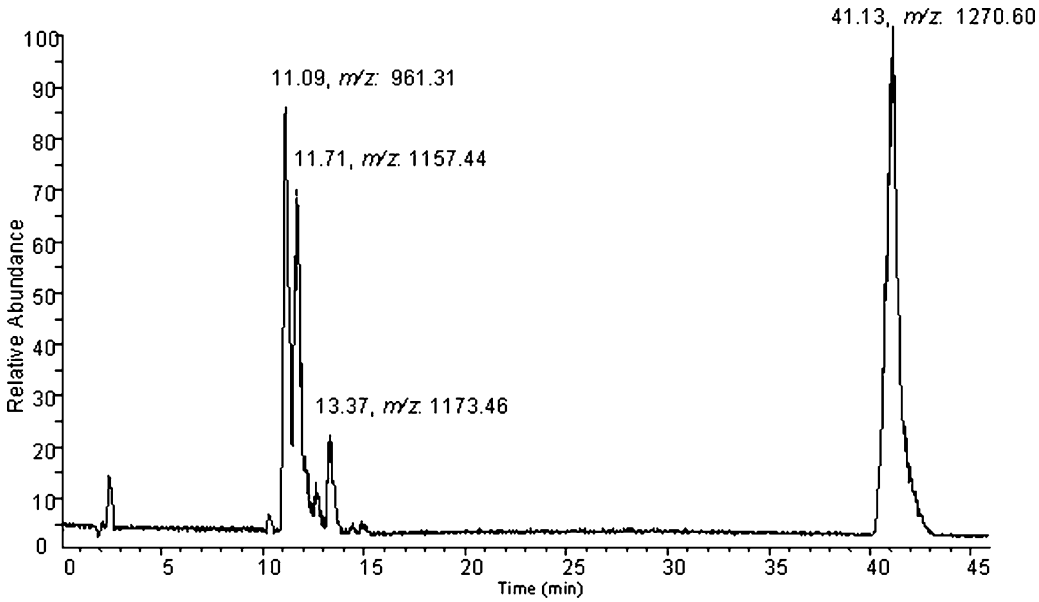

Figure 2. LC–ESI-MS chromatogram for the crude material cleaved from the resin after decapeptide thioester solid-phase synthesis. The sample was run on a ThermoQuest Hypersil Elite C18 column (2.1×100 mm, 5 μm) coupled to a Finnigan LCQ Classic mass spectrometer. HPLC conditions: flow rate at 0.2 mL/min; linear gradient from 20 to 30% acetonitrile in water within 5 min; 30% acetonitrile for the next 20 min; and finally, linear gradient from 30 to 80% acetonitrile within 25 min.

The crude product was subsequently subjected to LC–ESI-MS analysis. As shown in the chromatogram (Fig. 2), the major component with a retention time of 41.1 min was identified as tyrocidine A (obs. $[M+H]^+$: m/z 1270.60, calcd: 1270.66), the cyclic product of the expected linear peptide. However, the hydrolysis and aminolysis products of the linear peptide thioester were not found, indicating spontaneous cyclization of the peptide was complete (overall yield 25%). Meanwhile, three major impurities were identified in the crude material. Two of these were the linear aminolysis products depleted of Leu-Orn-Val (R_t 11.1 min, obs. $[M+H]^+$: 961.31, calcd: 961.07) or the first amino acid leucine (R_t 13.4 min, obs. $[M]^+$: 1173.46, calcd: 1173.60). The third side product at 11.7 min had a mass equivalent to the cyclic product of the thioester peptide which had lost the C-terminal leucine (obs. $[M+H]^+$: 1157.44; calcd: 1157.58). However, its retention time indicates that this side product has a mobility close to that of a linear structure in contrast with that for tyrocidine A (R_t 41.1 min). A reasonable explanation is that the γ-NH_2 of the ornithine cleaves the thioester to form a six-membered lactam ring in the absence of the C-terminal leucine residue.

These impurities with shortened sequences most likely originated from cleavage of the thioester in the Fmoc deblocking of the first and the third residue. Formation of Leu-depleted peptide denotes that the peptide thioester is most vulnerable to aminolysis or hydrolysis with only one amino acid residue and that its resistance to the deblocking mixture is indeed increased as the peptide chain grows, and is consistent with the previous observations.[11] On the other hand, formation of the side product depleted of Val-Orn-Leu indicates that the tripeptide thioester is not stable enough to withstand a further cycle of deprotection treatment which results in appreciable aminolysis of the thioester. Indeed, when Fmoc deblocking for the third amino acid was changed back to three 1 min washes with the deprotection mixture, the depletion product disappeared and the yield of tyrocidine A was increased accordingly.

These results show that the new deblocking reagent is useful for direct synthesis of peptide thioesters using the Fmoc solid-phase method. It may find wide application in native chemical ligation and in the preparation of cyclic peptides.

Acknowledgements

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