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A novel method for sequence independent incorporation of activated/protected cysteine in Fmoc solid phase peptide synthesis

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Abstract

In conventional stepwise Fmoc solid phase peptide synthesis, incorporation of cysteine with nitro-pyridine sulfenyl (Npys) or pyridine sulfenyl (Pys) groups which act as an activation/protection of the thiol function has been accomplished in a sequence independent manner.

Peptides are attractive targets for drug discovery because they have been shown to be diagnostically and therapeutically important in many areas of biomedical research.[1] With the rapid advancement of combinatorial chemistry and high throughput screening the lead drug discovery process has undergone dramatic changes. Consequently, in recent times, interest in the solid phase peptide synthesis has increased significantly. In this connection the protection/activation of the thiol function in cysteine with nitro-pyridine sulfenyl (Npys) or pyridine sulfenyl (Pys) group has found increasing use in unsymmetrical disulfide formation,[2] protein–peptide covalent linkage[3] and for the synthesis of cyclic peptides.[4]

Both Npys and Pys groups are base labile and do not survive under Fmoc removal condition in stepwise solid phase peptide synthesis.[2,5] Therefore, one cannot have Cys(Npys) or Cys(Pys) at non N-terminal position in a peptide while performing Fmoc chemistry. However, at the N-terminal position, Boc-Cys(Npys) could be coupled to a peptide sequence which has been assembled by using Fmoc strategy. The only option left to introduce Cys(Npys) into non N-terminal position of a resin bound fully assembled peptide made by Fmoc chemistry is to replace thioether type protecting groups by Npys. This needs to react resin bound peptide with Npys-Cl. Unfortunately this method has several serious limitations.[2,4] It depends on the type of resin used, and works only for very small peptides. In addition, Npys-Cl is highly hygroscopic and the reaction is very slow. Any attempt to increase the reaction rate by using higher proportion of Npys-Cl leads to unidentified side product.[2,6]

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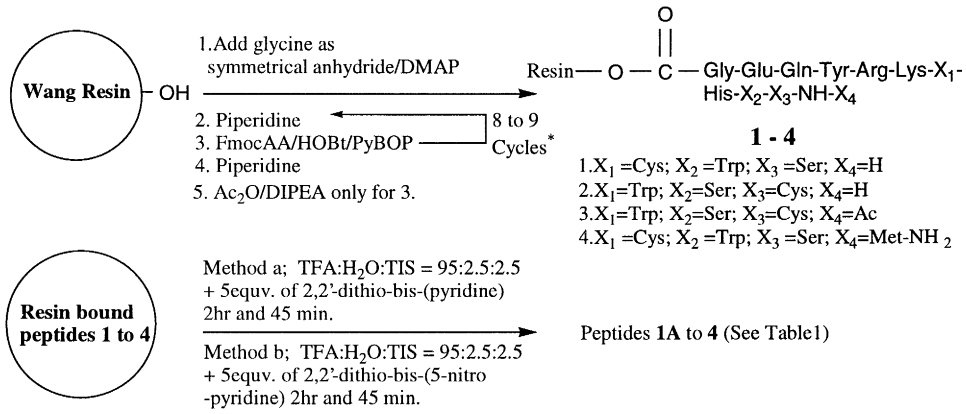
In this communication, we wish to report a very convenient and high yielding method to introduce activated/protected cysteine at either an N-terminal or a non N-terminal position in a peptide sequence assembled by Fmoc strategy. In our approach, we first synthesize the peptide sequence on resin. While cleaving the peptide from resin, we use 4–5 equivalents of 2,2′-dithio-bis-(pyridine) (or the 5-nitro substituted analogue) in the cleavage mixture (Scheme 1), since disulfide bond exchange can be conducted under acidic conditions.[7] We have designed four peptide sequences (**1**–**4** in Scheme 1 and Table 1) to test the generality of our procedure. These peptide sequences cover all common protected side chains in Fmoc peptide synthesis. This is to ensure that our procedure is not only compatible with those protection groups, but also compatible to each of those reactive side chains. In addition, sequences **1** and **2** test the sequence independence of our procedure, with cysteine at either the middle or the N-terminal of the peptide chain. Sequence **3** demonstrates the ability to modify the N-terminal of a peptide chain in our approach, which is not achievable by using Npys/Pys protected cysteine in a conventional approach. Sequence **4** tests our procedure in the presence of both cysteine and methionine.



Scheme 1. *Fmoc amino acids: Glu(OtBu)OH, Gln(Trt)OH, Tyr(OtBu)OH, Arg(Pbf)OH, Lys(BOC)OH, Cys(Trt)OH, His(Trt)OH, Trp(BOC)OH, Ser(OtBu)OH, MetOH. Only Cys was coupled as symmetrical anhydride and all others with 3 equiv. HOBt, 3 equiv. PyBOP, 6 equiv. DIPEA in DMA for 40 to 60 mins

The actual synthesis was straightforward and the results were excellent with our procedure. In a typical experiment, for example, after the synthesis of peptide sequence **1** on Wang resin, the peptide was cleaved with a mixture of TFA:H_2O:TIS (95:2.5:2.5., TIS: triisopropylsilane) which contains 5 equivalents of 2,2′-dithio-bis-(pyridine). After the removal of resin and most of the solvent, the crude peptide **1A** was obtained by ether precipitation. Analysis by electrospray mass spectrometry (ES-MS) and analytical HPLC of this crude sample shows mostly one compound (Scheme 2). In this crude mixture, no unmodified peptide with free cysteine was detected (by ES-MS or HPLC), indicating that the formation of Cys-(Pys) was quantitative. Preparative HPLC purification of this ether precipitated crude product afforded 66% of pure peptide **1A** (Table 1). Subsequent experiments using 2,2′-dithio-bis-(5-nitro-pyridine) in cleavage mixture instead of 2,2′-dithio-bis-(pyridine) produced similar results (**1B** and **2B** in Table 1). According to our procedure, one should be able to freely modify the N-terminal amino group. We have carried out acetylation to give an excellent yield of **3**. We also tested our procedure on a peptide sequence containing more than one cysteine. A mixture of products with intramolecular disulfide bond and bis-Npys modification were obtained. We are currently investigating modified procedures which would lead to selective formation of either the cyclic product or the bis-Npys (bis-Pys) modified peptide.

Methionine suffers easy acid catalyzed oxidation of thioether during cleavage. In order to prevent oxidation ethyl methyl sulfide or thioanisole has been routinely used in cleavage mixture. To prove the

Table 1

Peptides	Peptide Sequences	Method	Mass Spectrum Calcd[c]	Mass Spectrum Obsd[d]	HPLC Rt.in Minutes[e]	Yield[f]
1A	NH_2-Ser-Trp-His-Cys(Pys)-Lys-Arg-Tyr-Gln-Glu-Gly-OH	a	1402.6	1402.6	26.5	66%
1B	NH_2-Ser-Trp-His-Cys(Npys)-Lys-Arg-Tyr-Gln-Glu-Gly-OH	b	1447.6	1447.4	31.5	60%
2A	NH_2-Cys(Pys)-Ser-His-Trp-Lys-Arg-Tyr-Gln-Glu-Gly-OH	a	1402.6	1402.5	23.3	68%
2B	NH_2- Cys(Npys)-Ser-His-Trp-Lys-Arg-Tyr-Gln-Glu-Gly-OH	b	1447.6	1447.4	28.8	60%
3	AcNH-Cys(Npys)-Ser -His-Trp-Lys-Arg-Tyr-Gln-Glu-Gly-OH	b	1489.6	1489.2	26.3	54%
4	NH_2-Met-Ser-Trp-His-Cys(Npys)-Lys-Arg-Tyr-Gln-Glu-Gly-OH	b	1578.8	1578.3	25.1	65%

[a] Method a: see Scheme 1. [b] Method b: see scheme 1. [c] Theoretical molecular weight. [d] Observed molecular weight from ES-MS. [e] Reversed-Phase HPLC(C-18, 10 μm, 4.6 x 250 mm, λ=220 nm) retention time (min). Each compound was found to be more than 98% pure after purification. [f] Yield (not optimized) is reported after preparative reversed-phase HPLC (C-18, 10-15 μm, 22 x 250 mm, λ=220 nm) and with respect to the loading of the first amino acid to resin. Pys or Npys modification of Cys was quantitative.

generality of our method we applied it (under nitrogen, with 2,2′-dithio-bis-(5-nitro-pyridine) but without the addition of other thio-scavengers) to a resin bound peptide **4,** containing both cysteine and methionine. Analysis of crude sample after ether precipitation by ES-MS showed no evidence of oxidized product. Methionine oxidation may have been prevented by the combined effects of a nitrogen atmosphere and the presence of 2-thio-5-nitropyridine, a by-product formed during the cleavage reaction. After preparative HPLC we obtained more than 60% yield of pure peptide **4**.

In conclusion, a novel method for sequence independent incorporation of Npys and Pys activated/protected cysteine in Fmoc solid phase peptide synthesis has been developed. Some unique merits of our method include: operational simplicity, no need to consider dry reaction condition, fast and efficient reaction, and the use of commercially available and non-moisture sensitive reagents. Modification of N-terminal amino group could be done if required. We believe our procedure would be a beneficial addition to the peptide chemistry.

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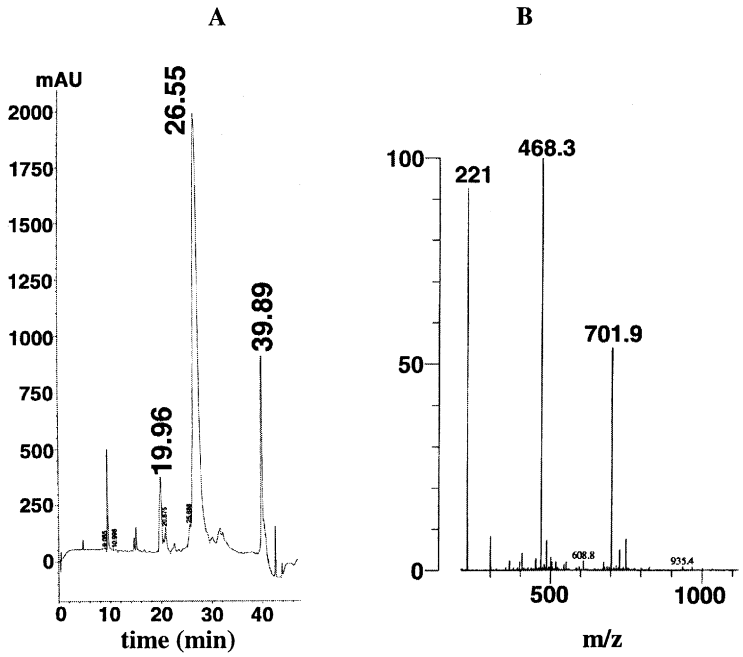

Scheme 2. A: HPLC profile of the crude **1A** from ether precipitation shows mainly the product at 26.5 min. B: Electrospray mass spectrum of crude **1A** with the following main peaks: *m/z* 468.3 ((**1A**+3H)$^{3+}$), 701.9 ((**1A**+2H)$^{2+}$), and 221 (2,2′-dithio-bis-(pyridine)). The peaks for unmodified peptide (*m/z* 1294 or its multiple charged forms at 647.5 or 432.2) were undetected. The internal standard showed a down-shift of 0.3 mass units during measurement. Therefore, the observed mass for **1A** is 1402.6, an average of the triply charged peak at 468.6 and the doubly charged peak at 702.2

References

1. (a) Gordon, E. M.; Barrett, R. W.; Dower, W. J.; Fodor, S. P.; Gallop, M. A. *J. Med. Chem.* **1994**, *37*, 1385; (b) Hocart, S. J.; Jain, R.; Murphy, W. A.; Taylor, J. E.; Coy, D. H. *J. Med. Chem.* **1999**, *42*, 1863–1871.
2. Simmonds, R. G.; Tupper, D. E.; Harris, J. R. *Int. J. Pept. Protein Res.* **1994**, *43*, 363–366.
3. Drijfhout, J. W.; Perdijk, E. W.; Weijer, W. J.; Bloemhoff, W. *Int. J. Pept. Protein Res.* **1988**, *32*, 161.
4. Matsueda, R.; Higashida, S.; Ridge, J. R.; Matsueda, G. *Chem. Lett.* **1982**, 921–924.
5. Ploux, O.; Chassing, G.; Marquet, A. *Int. J. Pept. Protein Res.* **1987**, *29*, 62–169.
6. Ghosh, A. K.; Fan, E., unpublished results.
7. Carlsson, J.; Drevin, H.; Axen, R. J. *Biochemistry* **1978**, *173*, 723–737.