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A New Stepwise Deprotection Method using Reductive Acidolysis Followed by Fluoride Ion in Solid Phase Peptide Synthesis[1]

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Abstract: A new stepwise deprotection method using reductive acidolysis followed by fluoride ion in solid phase peptide synthesis has been found to minimize aspartimide formation; this new method has been successfully applied to the synthesis of thymic humoral factor γ 2, which contains an Asp-Gly sequence.

The major side reaction during the synthesis of aspartic acid-containing peptides is aminosuccinimide formation under acidic or basic condition. We now report that the stepwise deprotection methodology using reductive acidolysis followed by fluoride ion is effective to suppress the side reaction at Asp residue.

In the course of our investigation on a deprotecting method using fluoride ion,[2-4] we have observed that practically no succinimide peptide was formed when a peptide having unprotected β-carboxyl group of Asp was treated with fluoride ion. Since the succinimide formation is a serious unresolved problem especially in the solid phase synthesis of Asp-containing peptides, this finding prompted us to examine the stepwise deprotection method in which the protecting group of β-carboxyl group of Asp is removed before the final deprotection with fluoride ion.

In this stepwise method, we employed the safety-catch type of protecting groups, *i.e.*, 4-methylsulfinylbenzyl (Msob)[5] and 4-methylsulfinylbenzyloxycarbonyl (Msz)[6] groups, for the protection of the side chain functional groups including β-carboxyl group of Asp. Msob and Msz groups show high stability under acidic and basic conditions, but can be smoothly removed by a one-pot reaction involving reductive acidolysis using tetrachlorosilane-trifluoroacetic acid (TFA)-scavengers,[6] which is mild acidolysis system

Table 1 Percentage of Side Reactions in the Synthesis of AaH II(1-6)

run	β-carboxyl protecting groups	α-form	β-form	suc.-form
1	Msob [1)]	98.4	1.6	N.D.
2	cHex [2)]	97.6	N.D.	2.4
3	Bzl [2)]	93.0	0.2	6.8
4	*tert*-Bu [3)]	98.3	N.D.	1.7
5	Fm[4)]	56.6	43.4	N.D.

1) $SiCl_4$-TFA-*m*-cresol/DCM(r.t., 30min) and then 0.1M TBAF/DMF(r.t., 1h);
2) HF-*m*-cresol(0°C, 1h); 3) TFA-thioanisole(r.t., 1h); 4) 0.1M TBAF/DMF(r.t., 1h);
N.D.: not detected

having strong reductivity. The fluoride ion labile semi-permanent protecting groups such as diphenylphosphinothioyl (Ppt)[2] group for Trp or diphenylphosphinyl (Dpp)[4] group for Tyr were also employed, if necessary. Following the reductive acidolysis, the C-terminal phenacyl (Pac) ester linkage[7] to the resin support and remaining fluoride ion labile protecting groups were removed by the simple treatment with tetra-*n*-butylammonium fluoride trihydrate (TBAF).

The amount of succinimide peptide formed during above stepwise deprotection procedure was examined using a hexapeptide fragment of toxin II of the scorpion *Androctonus australis* Hector [AaH II(1-6); H-Val-Lys-Asp-Gly-Tyr-Ile-OH].[8] This peptide contains an Asp-Gly sequence, which is known to be one of the most labile sequences for succinimide formation.[9] The protected peptide resin was synthesized on a Pac resin using the amino acid derivatives bearing the safety-catch type and fluoride ion labile protecting groups, *i.e.*, Asp(OMsob), Lys(Msz) and Tyr(Dpp) (run 1). After stepwise deprotection of the protected peptide resin, the amounts of the desired α-form peptide and by-products, the succinimide peptide (suc.-form) and the rearranged β-form peptide, were examined using HPLC before purification of the crude product.[10] For comparison, the same peptide was synthesized by the conventional Boc-based (benzyl type protection-HF deprotection; run 2, 3) or Fmoc-based (t-butyl type protection-TFA deprotection; run 4) solid phase peptide synthesis. Single fluoride ion deprotection of the protected peptide-Pac resin, prepared using Asp(OFm),[11] Lys(Fmoc)[12] and Tyr(Dpp), was also conducted (run 5). As summarized in Table 1, the best recovery (98 %) of the desired α-form peptide was obtained by the combination of Msob group and stepwise deprotection method. Similar recovery (98 %) was obtained by the use of t-butyl group and the acidolytic removal with TFA. The worst result (56.6 % of desired α-form peptide and 43.4 % of rearranged β-form peptide) arose from the use of Fm group and the removal with fluoride ion.

In order to demonstrate the usefulness of this stepwise deprotection method, we have synthesized thymic humoral factor γ 2 (THF γ 2; H-Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu-OH) (Fig. 1.).[13] The protected peptide

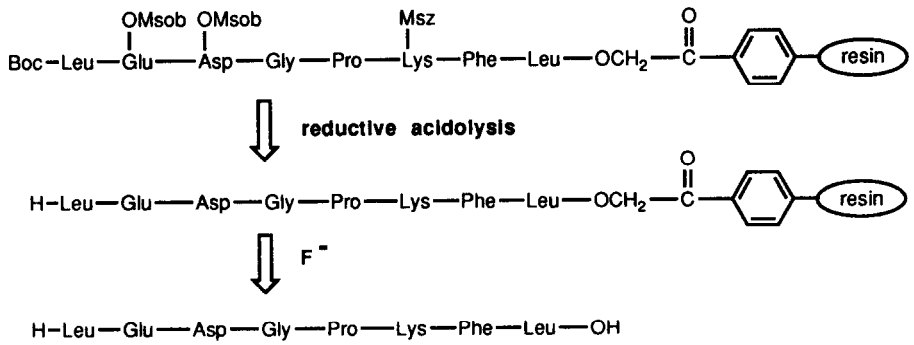

Fig. 1. Synthesis of THFγ2 by stepwise deprotection methodology

resin was constructed automatically using a Biosearch 9500 synthesizer according to the schedule of efficient solid phase peptide synthesis.[4,14] For the first step of deprotection, the protected peptide resin was treated with $SiCl_4$-TFA-*m*-cresol to remove the Msob and Msz groups and then, as the second deprotection step, with 0.1 M TBAF to cleave the peptide from the resin. Subsequent purification was carried out by fast protein liquid chromatography (FPLC, Pharmacia). The homogeneous peptide was obtained with 65 % yield (based on the starting C-terminal residue) and showed the same elution time on HPLC as that of the commercial sample (purchased from Peptide Institute Inc., Osaka, Japan). The content of the β-form peptide in the crude product obtained after the stepwise deprotection was 0.4 %, whereas the content of the aspartimide form was 10.9 % when the same peptide was synthesized by conventional Boc strategy using Asp(OBzl).[15]

These results show the potential of our stepwise deprotection methodology especially for the solid phase synthesis of Asp-containing peptides.

Experimental procedure for deprotection and purification of THF γ2. Boc-Leu-Glu(OMsob)-Asp(OMsob)-Gly-Lys(Msz)-Phe-Leu-OPac resin (65 mg) was treated with $SiCl_4$ (57 μl)-30 % TFA/DCM (5 ml)-*m*-cresol (138 μl) for 30 min at 25 °C. After washing with DCM (5 ml x 5) and DMF (5 ml x 5), the resin was treated with TBAF (155 mg) in DMF (5 ml) for 1h at 25 °C. The mixture was filtered and the resin was washed with 50 % aq. AcOH. The combined filtrate was concentrated *in vacuo* and the residue was treated with ether (10 ml) containing TFA (100 μl) to precipitate the product. The crude peptide was dissolved in 1N AcOH (ca. 3 ml) and the solution was subjected to a column (1.6 x 50 cm) packed with YMC gel ODS-AQ 120A S-50. The product was eluted using a gradient of 60 % acetonitrile (0-100 %, 400 min) in 0.1 % aq. TFA at the flow rate of 3.0 ml/min. The eluate corresponding to the main peak, monitored by measuring the UV absorption at 254 nm, was collected and lyophilized to give a white fluffy powder; yield 9.7 mg (65 %).

Abbreviations

Boc=t-butoxycarbonyl, Fmoc=9-fluorenylmethoxycarbonyl, Fm=9-fluorenylmethyl, cHex=cyclohexyl, Bzl=benzyl, *tert*-Bu=t-butyl, DMF=dimethylformamide, DCM=dichloromethane.

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15. HPLC [YMC AM 303, (4.6 x 250 mm), 20.7 % MeCN in 10 mM H_3PO_4, 50 mM Na_2SO_4, 0.8 ml/min], retention time; α-form: 16.48 min, β-form: 14.5 min, suc.-form: 23.7min.

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