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# Studies on Peptides. CLXV.[1,2] Combination of a New Amide-Precursor Reagent and Trimethylsilyl Bromide Deprotection for the 9-Fluorenylmethyloxycarbonyl-Based Solid-Phase Synthesis of Chicken Antral Peptide

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A 36-residue peptide amide corresponding to the entire amino acid sequence of chicken antral peptide was synthesized by the 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase synthesis, for which a new amide precursor reagent, 3-(α-Fmoc-amino-4-methoxybenzyl)-4-methoxyphenylpropionic acid, was employed in combination with thioanisole-mediated trimethylsilyl bromide deprotection. A homogeneous standard sample prepared by the solution-phase method was used to check the effectiveness of the purification step.

**Keywords**——new amide precursor reagent; modified benzhydrylamine resin; hard acid deprotection; thioanisole-mediated deprotection; trimethylsilyl bromide deprotection; Fmoc-based solid phase synthesis; amide peptide synthesis

Recently we introduced a new amide precursor reagent, 3-(α-Fmoc-amino-4-methoxybenzyl)-4-methoxyphenylpropionic acid,[3] for the Fmoc-based solid-phase synthesis.[4] This acid-sensitive modified dimethoxybenzhydryl type reagent, can be prepared easily and introduced smoothly by DCC[5] onto the aminomethylated polystyrene resin (type A resin) or the PAM resin (type B resin) through the propionic acid side chain as a handle. In addition, we introduced a new deprotecting procedure with TMSBr in TFA,[6] based on a hard acid principle. This reagent has an ability to cleave readily the benzyl and *tert*-butyl type protecting groups, as well as phenylsulfonyl type protecting groups, with the aid of a soft nucleophile, such as thioanisole. TMSBr is a volatile reagent. This attractive feature is judged to be particularly useful for a deprotecting reagent in solid-phase peptide synthesis, since this property makes it easier to separate deprotected peptides from the resin employed, compared to the use of nonvolatile deprotecting reagents, such as TFMSA[7] or TMSOTf.[8]

We wish to report, as an example, that a relatively complex 36-residue peptide amide, chicken antral peptide (cAP),[9] can be prepared successfully on the resin by using the combination of the above new precursor resin and a new deprotecting procedure (Fig. 1). An homogeneous sample of chicken antral peptide prepared by the solution method,[1] was used as a standard to monitor the effectiveness of the purification step of the present synthesis, since the solid-phase method often gives a product contaminated with many partially amino acid-deleted peptides.

After removing the Fmoc group from the resin A by 20% piperidine treatment,[10] the first C-terminal residue, Fmoc-Phe-OH, was loaded on the resin by the Pfp active ester procedure.[11] Fmoc-amino acids, including derivatives, except for Arg(Mtr),[12] bearing protecting groups based on *tert*-butanol were employed. The combination of piperidine treatment and Pfp ester condensation served to elongate the peptide chain manually, but in

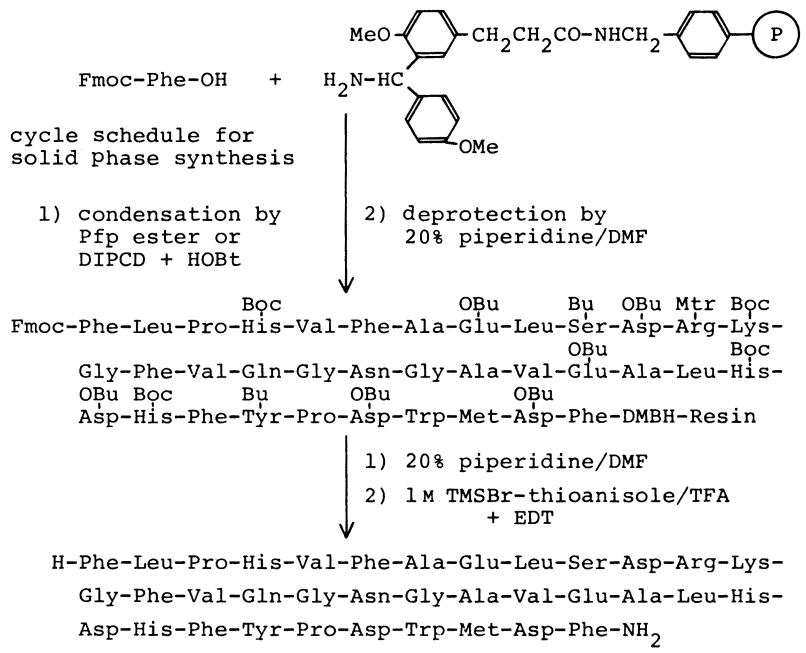


Fig. 1. Fmoc-Based Solid-Phase Synthesis of Chicken Antral Peptide

principle, according to the automated program proposed by Sheppard *et al.*[4] A few amino acid residues, Val, Ser(*t*Bu) and Leu, were condensed by the DIPCD plus HOBt procedure[13] to ensure complete coupling. After the condensations of 20 residues, double coupling was performed until the resin became negative to the ninhydrin test.

The 36-residue peptide-DMBH-resin thus obtained was treated with 1 M TMSBr–thioanisole/TFA in the presence of two additional scavengers, *m*-cresol and EDT, in an ice-bath for 2 h to remove all protecting groups employed and at the same time to cleave the peptide from the resin as an amide. The deprotected peptide was incubated with 2-mercaptoethanol for 5 h to reduce the Met(O) partially formed during manipulations, then purified by gel-filtration on Sephadex G-15, followed by high-performance liquid chromatography (HPLC) on a Cosmosil 5C18 column using gradient elution with MeCN in 0.1% TFA. The isolation yield was 11.3% based on the first Phe loaded on the resin. HPLC examination of the gel-filtered sample revealed the presence of a main peak having a retention time identical with that of the standard sample mentioned above (Fig. 2), indicating that the stepwise additions of amino acid residues progressed in fairly good yields, and deprotection of the peptide amide proceeded well, as predicted from our previous model experiment.[3] The treated resin was subjected to 6 N HCl hydrolysis and from the amounts of amino acid detected, approximately 77% of the peptide was judged to be cleaved from the resin under the conditions employed.

Arg(Mtr) is a selected Arg-derivative for the Fmoc-based solid-phase synthesis in combination with thioanisole-mediated TFA deprotection.[14] However, it has been claimed occasionally that this group can not be cleaved satisfactorily from relatively large peptides.[15] In our present synthesis, Arg (Mtr) was satisfactorily cleaved by the TMSBr reagent. From these experimental results, we conclude that in order for the Fmoc based solid-phase procedure to gain wider acceptance in the synthesis of Arg-containing peptides, our thioanisole-mediated TMSBr/TFA deprotecting procedure could play a very important role. In addition, for the synthesis of peptide amides, our amide precursor reagent offers some advantageous

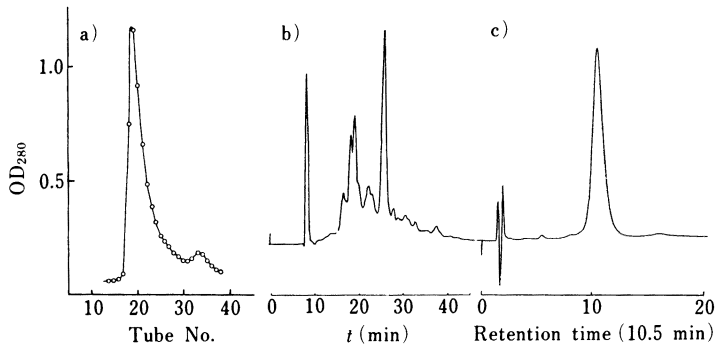


Fig. 2. Gel-Filtration and HPLC of Synthetic Chicken Antral Peptide

a) Gel-filtration. b) HPLC of the gel-filtered sample. c) HPLC of a mixture of the synthetic sample and the authentic sample (1 : 1).

features over the previous ones, as regards its easy preparation and acid lability. As reported in the preceding paper,[1] the solution-phase synthesis of cAP suffered some difficulty in chain elongation, *i.e.*, condensation of the N-terminal portion of peptide fragments, presumably due to steric effects of the amino component employed. This difficulty is judged to have been overcome in the present solid-phase synthesis by performing the double coupling reaction, as described above.

## Experimental

**Solid-Phase Synthesis**——Solid-phase synthesis was carried out manually according to the principle of Sheppard *et al.*[4] (Table I). First Fmoc-DMBH-resin (A) (172 mg; amine content 0.1 mmol) was treated 3 times with 20% piperidine in DMF (3 ml) for 5 min, then washed 6 times with DMF (3 ml). The following Fmoc amino acids and derivatives were used as the corresponding Pfp ester (2.5 eq each); *i.e.*, Phe, Pro, Ala, Gly, Gln, Asn, Trp, Leu, Val, Met, Lys(Boc), Glu(O*t*Bu), Asp(O*t*Bu), Tyr(*t*Bu), Ser(*t*Bu), His(Boc), Arg(Mtr). The active ester condensation was performed in NMP in the presence of HOBt (2.5 eq). The following Fmoc-amino acids (2.5 eq each) were condensed by DIPCD (2.5 eq) in the presence of HOBt (2.5 eq); *i.e.*, Val, Leu, Ser(*t*Bu). Every reaction was continued until the resin became negative to the Kaiser test. Single coupling was effective to elongate the peptide chain to the stage of the Gln residue (position 17). Beyond this 20-residue peptide chain, double coupling was employed. Amino acid ratios in a 6 N HCl hydrolysate of protected antral peptide-resin were: Asp 5.00 (5), Ser 0.76 (1), Glu 2.99 (3), Gly 3.24 (3), Ala 2.79 (3), Val 2.69 (3), Met 0.95 (1), Leu 2.40 (3), Tyr 1.13 (1), Phe 4.36 (5), Lys 0.95 (1), His 2.64 (3), Trp N.D. (1), Arg 0.95 (1), Pro 1.66 (2).

**Deprotection with 1 M TMSBr–Thioanisole/TFA**——The protected peptide-resin (75 mg) was treated with 1 M TMSBr–thioanisole/TFA (10 ml) in the presence of *m*-cersol (100 $\mu$l) and EDT (500 $\mu$l) in an ice-bath for 2 h. The resin was removed by filtration and washed with TFA (2 ml) three times. The filtrate and the washing were combined and concentrated *in vacuo* at a bath temperature below 25 °C. Dry ether was added to precipitate the product, then ether was removed by decantation and the residue was dissolved in 0.2 M Tris–HCl buffer (pH 8.3) containing 6 M guanidine hydrochloride (3 ml). After addition of 1 M $NH_4F$ (100 $\mu$l) and 2-mercaptoethanol (200 $\mu$l), the solution was kept in an ice-bath for 5 min, then the pH was adjusted to 5.0 with 1 N AcOH. After being incubated at 37 °C for 5 h, the solution was applied to a column of Sephadex G-15 (3.8 × 140 cm), which was eluted with 1 N AcOH (Fig. 2a). The fractions corresponding to the front main peak (tube Nos. 17—25, 7 ml each, monitored by ultraviolet (UV) absorption measurement at 280 nm) were combined and the solvent was removed by lyophilization to give a powder; 20.1 mg (isolation yield 61%). The Phe content on the treated resin was 52 $\mu$mol/g, indicating that 77% of the peptide was cleaved from the resin. The HPLC elution pattern of the gel-filtered sample is shown in Fig. 2b.

The crude peptide thus obtained was purified by HPLC. A sample (0.8 mg) was applied to a Cosmosil 5C18 column (10 × 250 mm), which was eluted with a gradient of MeCN (32—40%, 45 min) in 0.1% aqueous TFA at the flow rate of 1.8 ml/min (Fig. 2b). The eluate corresponding to the main peak (retention time 25.9 min, detected by UV absorption measurement at 227 nm) was collected and the solvent was removed by lyophilization to give a fluffy white powder. The rest of the sample was similarly purified; yield 3.74 mg (11.3%, based on the Phe loaded on the resin). A mixture of the present sample and the standard sample gave a single peak (retention time 10.5 min), when an Asahipak ODP 50 HPLC column (4.6 × 150 mm) was eluted with a gradient of MeCN (32—40%, 30 min) in 0.1%

TABLE I. Manual Schedule for the Fmoc-Based Solid-Phase Synthesis

| Manipulation | Reagent | Solvent | Time/repeat |
|---|---|---|---|
| Deprotection | 20% piperidine/DMF | DMF | 5 min × 3 |
| Washing | | | 1 min × 6 |
| Coupling | Fmoc-A.A.-OPfp (2.5 eq) | DMF | 2—12 h |
| | or | | |
| | Fmoc-A.A.-OH (2.5 eq) DIPCD+HOBt | NMP | |
| Washing | | DMF | 1 min × 4 |

TFA at the flow rate of 1.0 ml/min (Fig. 2c). Single spot on TLC, *Rf* 0.40, when developed with *n*-BuOH–pyridine–AcOH–$H_2O$ (4 : 1 : 1 : 2). Amino acid ratios in a 6 N HCl hydrolysate (numbers in parentheses are theoretical): Asp 4.79 (5), Ser 1.46 (1), Glu 3.16 (3), Pro 1.93 (2), Gly 3.49 (3), Ala 3.12 (3), Val 2.89 (3), Met 0.92 (1), Leu 3.00 (3), Tyr 1.04 (1), Phe 4.16 (5), Lys 1.00 (1), His 2.99 (3), Arg 1.17 (1), Trp, N. D., (recovery of Leu 90%). Amino acid ratios in a LAP digest: Asp 3.75 (4), Gln 0.94 (1), Glu 2.06 (2), Pro 1.83 (2), Gly 2.94 (3), Ala 3.01 (3), Val 2.85 (3), Met 0.97 (1), Leu 3.00 (3), Tyr 0.98 (1), Phe 4.30 (5), Lys 1.03 (1), His 2.82 (3), Trp 0.89 (1), Arg 1.01 (1), Ser + Asn N. D. (recovery of Leu 85%).

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2) Amino acids used in this investigation are of the L-configuration. The following abbreviations are used: Boc = *tert*-butoxycarbonyl, Fmoc = 9-fluorenylmethyloxycarbonyl, *t*Bu = *tert*-butyl, Mtr = 2,3,6-trimethyl-4-methoxybenzenesulfonyl, EDT = ethanedithiol, TMSBr = trimethylsilyl bromide, TFMSA = trifluoromethanesulfonic acid, TMSOTf = trimethylsilyl trifluoromethanesulfonate, NMP = *N*-methylpyrrolidone, HOBt = *N*-hydroxybenzotriazole, DMF = dimethylformamide, TFA = trifluoroacetic acid, DIPCD = 1,3-diisopropylcarbodiimide, DCC = dicyclohexylcarbodiimide, DMBH = 2,4′-dimethoxybenzhydryl, PAM = phenylacetamidomethyl, Pfp = pentafluorophenyl.
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