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# SOLID PHASE PEPTIDE SYNTHESIS: A SIMPLE ESTERIFICATION PROCEDURE

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In solid phase peptide synthesis, the C-terminal amino acid is usually esterified to the chloromethyl resin by the procedure originally developed by Merrifield, reflux with 0.9 equivalents triethylamine in a suitable solvent (1). Although several alternative procedures have been suggested recently (2), most of the peptides which have been synthesized by the solid phase method have been begun with the reflux procedure. Boc-Asn*, the C-terminal residue of the A-chain of insulin (3), undergoes side reactions when it is esterified under reflux. In ethanol, significant amounts of Boc-Asp(OEt)-O-Resin are formed (4); in other refluxing solvents (ethyl acetate or dioxane), large amounts of Boc-Asp-O-Resin are produced.

I present here a simple method for esterifying Boc-Asn to the chloromethyl resin which allows complete preservation of the β-amide. In addition, this new method permits esterification of all the Boc-amino acids tested to the readily available chloromethyl resin, and moreover it allows charging the resin with a desired amount of these amino acids in a predictable fashion.

In a typical experiment, 2.0 g chloromethylated polystyrene-divinylbenzene resin is suspended in 12 ml DMF (5) containing 2.0 mmols Boc-Asn and 1.8 mmols $Et_3N$. The mixture is shaken for 24 h at 25 C in a tightly stoppered vessel. It is then removed from the vessel, filtered, and the resin is washed with DMF, EtOH, HOAc, EtOH and $CH_2Cl_2$ (about 350 ml each g resin) and dried. A 50 mg aliquot is hydrolyzed in 6 N HCl/dioxane (6) for 24 h and the aspartic acid liberated is quantitated on an amino acid analyzer (7) (Table I).

To ascertain whether the β-amide is indeed intact after the esterification, a 50 mg aliquot is treated with anhydrous HF (8) or with HBr/TFA (1). The amount of Asp relative to Asn released by HF or HBr was shown to reflect only the deamidation which has occurred during

esterification, and the quantities in the filtrate after release from the resin are determined on the amino acid analyzer. Less than 0.3% Asp was detected in Boc-Asn esterifications by the new method, in contrast to Asp resulting from the reflux procedure (15-50%). Boc-Asp(ONBzl) gave about 6% conversion to Boc-Asp in the reflux procedure but was stable under the new conditions of 25° in DMF. Boc-Gln and Boc-Glu(ONBzl) were essentially stable (1-2% Glu) to either procedure.

Table I: Esterification of Boc-Asn-OH to chloromethyl resin[a] in DMF[b] at 25° in the presence of 0.9 equivalents of $Et_3N$.[c]

| Resin (g) | Boc-Asn (mmol) | Reaction time (h) | Result (mmol/g) |
|---|---|---|---|
| 2.0 | 1.0 | 24 | 0.11 |
| 2.0 | 2.0 | 24 | 0.20 |
| 2.0 | 1.0 | 72 | 0.16 |
| 2.0 | 2.0 | 72 | 0.32 |
| 1.0 | 1.0 | 24 | 0.15 |
| 1.0 | 2.0 | 24 | 0.20 |
| 1.0 | 4.0 | 24 | 0.32 |

a. 2% crosslinked, Cl = 2.0 mmol/g.
b. All volumes 12.0 ml. For the 1%-crosslinked resin, optimum results were obtained with 1.0 g resin/10 ml DMF and multiples thereof. These volumes seem to be the minimum needed to provide homogeneous wetting of the resin.
c. In DMF in the absence of $Et_3N$, or in $CH_2Cl_2$ or EtOH with $Et_3N$ at room temperature, less than 0.01 mmol/g Boc-amino acid was esterified. Diisopropylethyl amine promotes esterification poorly (0.07 mmol/g even when a tenfold excess of the base is used).

Table I also illustrates that, under given reaction conditions, the esterified proportion of the amino acid is rather constant. In my hands this has allowed planning a desired degree of substitution more precisely than under the reflux conditions.

Extension of this method to some of the other Boc-amino acids is summarized in Table II. Boc-Met could not be esterified efficiently as such, apparently because of competing S-alkylation (9). Boc-Methionine sulfoxide [Boc-Met(O)] was esterified successfully as judged by amino acid analysis (6, 7, 10), Schiff base determination of the free, neutralized amino group (11), and direct sulfur analysis of the Boc-Met(O)-O-Resin (12).

Table II: Results of DMF esterifications with various Boc-amino acids.[a]

| Amino acid | Amount esterified (mmol/g) |
|---|---|
| Arg($NO_2$) | 0.11[b] |
| Arg(Tos) | 0.18 |
| Gln | 0.22 |
| Lys(Z)[c] | 0.22 |
| Met(O) | 0.24 |
| Gly | 0.27 |
| Phe | 0.27 |
| Ala | 0.29 |
| Pro[c] | 0.31 |
| Val | 0.34 |
| Leu | 0.34 |

a. Resin (2% crosslinked, Cl = 2.0 mmol/g) 2.0 g, Boc-amino acid 2.0 mmol, $Et_3N$ 1.8 mmol, DMF 12 ml, 24 h.
b. Amount of esterification could be increased to 0.18 mmol/g when 4.0 mmols Boc-Arg($NO_2$) was used with 1.0 g chloromethyl resin in 12 ml DMF.
c. Esterified by V.A. Najjar, Division of Protein Chemistry, Tufts University School of Medicine.

To test the applicability of the new procedure for the synthesis of peptides, the A- and B-chains of insulin were newly synthesized (1, 3). The C-terminal tetrapeptide of the B-chain [Boc-Thr(Bzl)-Pro-Lys(Z)-Ala-O-Resin] was examined in detail at a sample substitution of 0.38 mmol/g Boc-Ala. Cleavage by HBr/TFA-$CH_2Cl_2$ (1): H-Thr-Pro-Lys-Ala-OH (yield 89%), homogeneous (13) by tlc ($R_f^1$ 0.02, $R_f^2$ 0.14), paper electrophoresis at pH 5.0 ($R_{Lys}$ 0.55), cation-exchange chromatography (amino acid analyzer, 5 cm column, pH 5.25, sodium citrate 0.35 M: emergence 20 ml, $C_{Leu}$ 1.04; less than 0.3% contamination with H-Pro-Lys-Ala-OH, which emerges at 27 ml elution in the same system). Cleavage by $NH_3$/MeOH (14): H-Thr(Bzl)-Pro-Lys(Z)-Ala-$NH_2$ (yield 81%), homogeneous by tlc ($R_f^1$ 0.33, $R_f^2$ 0.62) and electrophoresis ($R_{Lys}$ 0.37). Tryptic digestion of the HF-deblocked (15) peptide-amide indicated that the exclusive N-terminus was Ala-$NH_2$ All these peptides gave the expected amino acid ratios (7) after hydrolysis in 6 N HCl for 20 h.

*Abbreviations: Amino acid residues and protecting groups are abbreviated as recommended by IUPAC-IUB (J. Biol. Chem. 241, 2491 (1966). Other abbreviations: DMF, dimethylformamide; TFA, trifluoroacetic acid; Boc-Asp(ONBzl), Boc-Aspartic acid-β-p-nitrobenzyl ester; Boc-Glu(ONBzl), Boc-Glutamic acid-γ-p-nitrobenzyl ester.

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14. 400 mg of Boc-peptide-resin was suspended in anhydrous MeOH, which was saturated with $NH_3$ at 0 C. The sealed suspension was stirred at 25 C for 40 h, cooled and opened, filtered and washed with MeOH and DMF. The combined filtrates were evaporated (rotary evaporator, bath temperature 45 C, water aspirator) and the product was lyophilized from water.

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