Chapter 5

Acid Cleavage/Deprotection in Fmoc/*t*Bu Solid-Phase Peptide Synthesis

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1. Introduction

In general, a solid-phase peptide synthesis (SPPS) consists of the assembly of a protected peptide chain on a polymeric support (=synthetic step) and the subsequent cleavage/deprotection to release the crude, deprotected peptide from the solid support (=cleavage step). Usually, these two steps are followed by chromatographic purification of the crude peptide (*see* Chapters 1, 2, 4, and 5, *PAP*). The techniques to synthesize protected peptides or peptide fragments are discussed in Chapter 14. This chapter describes exclusively the cleavage step in the Fmoc/*t*Bu-SPPS with TFA as cleavage reagent leading to fully deprotected peptides (*see* Note 1) *(1)*. All of the cleavage procedures that are exemplified in Section 3. of this chapter hold for peptides synthesized under the following conditions:

1. Resins used:
   a. Sasrin resin™ (2-Methoxy-4-alkoxybenzyl alcohol resin) leads to peptide acids. It is also used to synthesize protected peptide fragments (*see* Note 2).
   b. Wang resin (4-Alkoxybenzyl alcohol resin) leads to peptide acids (*see* Note 2).
   c. Fmoc-amide resin (2,4-Dimethoxy-4'-[carboxymethyloxy]-benzhydrylamine linked to amino methyl resin) leads to peptide amides (*see* Note 3).
   d. Others with similar properties to a–c, specifically relative to the stability of the peptide-resin bond.

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2. N-terminal-protecting group: Boc or none.
3. Side-chain-protecting groups: *t*Bu for Asp, Glu, Ser, Thr, and Tyr; Boc for Lys; Pmc for Arg (Note 4); Trt, Mtt *(2)*, or none for Asn and Gln; Trt for His; Trt or Acm for Cys (*see* Note 1); none for Met and Trp.

Thirty-seven years have elapsed since the introduction of the Boc-protecting group by Carpino *(3)* and its application as a TFA-labile amino-protecting group in peptide chemistry *(4)*. During this time, Boc and other *t*Bu-type-protecting groups (esters and ethers) have proven to be very useful *(5)*. Carpino again, together with Han, introduced the Fmoc-protecting group in 1970 *(6,7)*. The combined application of the orthogonal Fmoc/*t*Bu-pair in a new SPPS strategy followed in 1978 by Atherton et al. *(8)* and independently by Chang and Meienhofer *(9)*. Only in recent years, however, has the Fmoc/*t*Bu-SPPS really been established as an extremely valuable alternative to the Boc/Bzl-SPPS *(1)*.

During the cleavage step, highly reactive species (*t*Bu-cations and *t*Bu-trifluoroacetate, among others) are generated that can undergo undesired side reactions with sensitive amino acids, such as Cys, Met, Trp, and Tyr. These reactive species have to be trapped chemically by the addition of appropriate scavengers to the actual cleavage agent TFA. Table 1 shows a choice of possible scavengers widely used in Fmoc/*t*Bu-SPPS. For a broader discussion, *see* refs. *1* and *10*, and literature cited therein.

Whenever an SPPS (including purification) leads to the target peptide without severe problems, the peptide chemist assumes that every single step has proceeded satisfactorily. In the case of "difficult peptides," on the other hand, it is not necessarily very obvious whether the synthetic step or the cleavage step is mainly responsible for the difficulties. A poor synthetic step cannot be saved even by an optimal cleavage step, but in the opposite case, an easy synthesis can be ruined by inappropriate cleavage conditions. It is therefore strongly recommended to perform small-scale trial cleavage runs with 10–50 mg of peptide resin and different cleavage cocktails, as well as variations in reaction time and reaction temperature (with HPLC analysis of the resulting different crude peptides) to determine optimum cleavage conditions prior to the main cleavage run.

2. Materials

1. All reagents required (TFA, scavengers listed in Table 1) are commercially available and used as such. TFA may be distilled under normal pressure if colored.
2. Cleavage cocktails should always be prepared freshly.

Table 1
Scavengers in Fmoc/*t*Bu–SPPS

	Protection* of		
Scavenger	Met	Trp	Tyr
Phenol	o	o	+
p-Cresol	o	o	+
Anisole	–	–	–
Thioanisole	–	o	+
Ethanedithiol	+	o	–
Dithioerythritol	+	o	–
Dimethylsulfide	+	o	o
2–Methylindole	o	+	–
Water**	o	o	o

*+ = good, o = fair, – = poor
** Indispensable if Arg(Pmc) is present.

3. Solvents, such as dichloromethane, diethylether, diisopropylether, and methyl-*tert*-butylether, are commercially available, too, and used as such. Ethers must be free of peroxides if an oxidizable peptide (containing Met and/or Cys) is to be treated (check, e.g., with test strips Merckoquant 10011, from Merck).
4. Equipment for HPLC analysis (*see* Chapter 3, *PAP*).
5. Further analytical tools to check peptides for purity and identity, as needed (*see* Chapters 6, 7, and 9, *PAP*).

3. Methods

3.1. General Considerations

3.1.1. Pretreatment of the Peptide Resin

After completion of the synthetic step, the peptide resin is usually thoroughly washed with DCM or any of the ethers mentioned above, and subsequently dried in a vacuum desiccator. Such a peptide resin is ready for cleavage. In some cases, however, it can be advisable to preswell the dry peptide resin for 10 min in DCM (the remaining DCM in the resin dilutes the subsequently added cleavage cocktail and can smoothen the cleavage reaction). This means, on the other hand, that drying after a final DCM wash is not strictly necessary.

3.1.2. Cleavage Cocktails

The four cleavage cocktails given below are just a selection. Other scavengers (cf Table 1) or scavenger ratios may give better results in a

Table 2
Peptide Composition and Cleavage Cocktail of Choice

	Cleavage cocktail
The peptide contains	
Any amino acid, except Met, Ser, Thr, Trp, Tyr, or Cys(Trt)	1 or 2
Any amino acid, except Met, Trp, or Cys(Trt)	2
Any amino acid, including Met, Trp, and/or Cys(Trt)	3 or 4

particular case. Trial runs are recommended as mentioned in Chapter 4. Quantities to cleave 1 g of peptide resin (dry wt) follow:

1. 9.5 mL of TFA are mixed with 0.5 mL of water.
2. 9 mL of TFA are mixed with 0.5 g of *p*-cresol and 0.5 mL of water.
3. 7.5 mL of TFA are mixed with 1.5 mL of EDT, 0.5 g of *p*-cresol, and 0.5 mL of water.
4. 8.3 mL of TFA are mixed with 0.25 mL of EDT, 0.5 g of phenol, 0.5 mL of thioanisole, and 0.5 mL of water (Reagent K *[10]*).

3.1.3. How to Choose a Cleavage Cocktail

The choice of an appropriate cleavage cocktail depends on the amino acid composition of the peptide to be cleaved and can be made according to Table 2.

3.1.4. General Cleavage Procedure

The cleavage should be performed under an inert gas.

1. One gram of peptide resin (dry wt) is placed in a round-bottom flask with a magnetic stirring bar and cooled in an ice bath.
2. Ten milliliters of precooled (ice bath), freshly prepared cleavage cocktail are added.
3. The reaction mixture is allowed to warm up to room temperature and stirred for 90 min (*see* Note 5).
4. After this time, the suspension is filtered through a fritted-glass funnel of medium porosity.
5. The resin is washed on the filter with 1–2 mL of neat TFA and 5–10 mL of DCM in several portions (*see* Note 6).
6. The combined filtrates are concentrated quickly to a volume of approx 2 mL on a rotatory evaporator (temperature of the water bath below 40°C) (for a milder alternative, *see* Note 7).
7. The concentrate is added dropwise to 100 mL of ice-cold ether with good stirring (*see* Note 8).

8. The precipitated peptide is collected by filtering the suspension from step 7 through a fritted-glass funnel of fine porosity and subsequent washing (two to three times) with ether (*see* Notes 8–10).
9. Finally, the wet, crude peptide is dried in a vacuum desiccator and weighed (*see* Note 11).
10. The peptide is now ready for HPLC analysis. In case of incomplete cleavage of protecting groups, steps 2, 3, and 6–9 can be repeated accordingly with the crude peptide (*see* Note 12).

3.2. Examples

3.2.1. Sex Pheromone Inhibitor iPD 1 (11)

- Sequence: H-Ala-Leu-Ile-Leu-Thr-Leu-Val-Ser-OH.
- Characteristics: 8-mer; neutral, rather hydrophobic; no Tyr, Cys, Met, Trp; in spite of (anticipated) hydrophobicity, considered to be a peptide that is rather easy to make (because short).
- Resin: Wang (*see* Section 1.).
- Cleavage: 1.93 g of peptide resin, 20 mL of cleavage cocktail 2, 90 min at room temperature.
- Crude: 1.05 g (>100% of theory, contains scavenger) with a purity of 56% (HPLC).
- Remarks: Amino acid analysis and FAB-MS (both after purification) in accordance with theory. The peptide is not an easy one as expected, mainly because of its low solubility.

3.2.2. Angiotensin II (Human)

- Sequence: H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-OH.
- Characteristics: 8-mer; slightly basic; no Cys, Met, Trp; considered to be a peptide that is rather easy to make.
- Resin: Wang (*see* Section 1.).
- Cleavage: 863 mg of peptide resin, 8 mL of cleavage cocktail 2, 90 min at room temperature.
- Crude: 463 mg (>100% of theory, contains scavenger) with a purity of 87% (HPLC; *see* Chapter 3, *PAP*).
- Remarks: Amino acid analysis (after purification) in accordance with theory. Easy peptide.

3.2.3. α-MSH Agonist (12)

- Sequence: Ac-Cys-Glu-His-D-Phe-Arg-Trp-Cys-Lys-Pro-Val-NH_2.
- Characteristics: 10-mer; both termini blocked, slightly basic; contains two Cys (anticipated) (bridged) and Trp; could be sensitive.
- Resin: Fmoc-amide resin (*see* Section 1.).

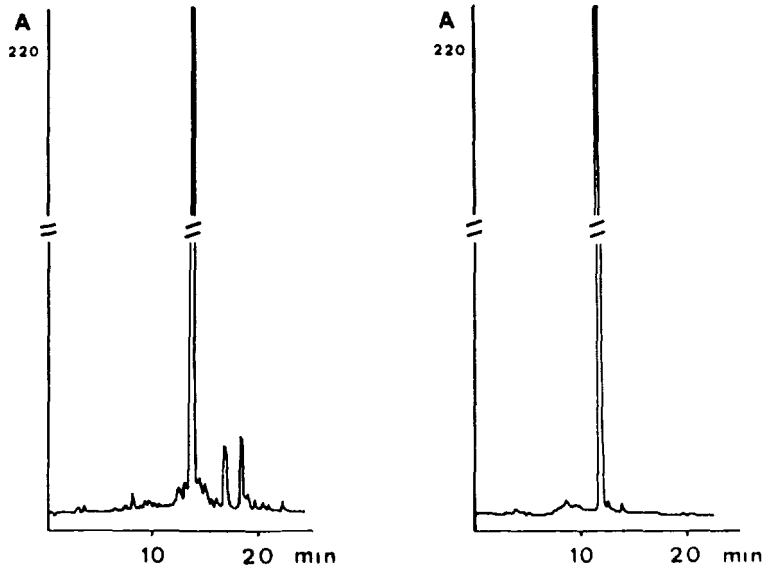

Fig. 1. RPHPLC gradient elution profile of *3*, crude: left, purified: right.

- Cleavage: 1.0 g of peptide resin, 10 mL of cleavage cocktail 3, 90 min at room temperature, resin cleaved for a second time.
- Crude: 365 mg (73% of theory) of peptide in the reduced state, i.e., with free sulfhydryl groups, both crops of equal purity (82%, HPLC; *see* Fig. 1, left).
- Remarks: Amino acid analysis and FAB-MS (both after oxidation and purification) in accordance with theory (HPLC; *see* Fig. 1, right; FAB-MS [*see* Chapter 7, *PAP*]; *see* Fig. 2). Difficulties below expectations.

3.2.4. Calmodulin Antagonist (13)

- Sequence: H-Leu-Lys-Lys-Phe-Asn-Ala-Arg-Arg-Lys-Leu-Lys-Gly-Ala-Ile-Leu-Thr-Thr-Met-Leu-Ala-OH.
- Characteristics: 20-mer; basic, with accumulation of basic amino acids in the first half from the N-terminus; contains Met near the C-terminus; not an easy peptide.
- Resin: Wang (*see* Section 1.).
- Cleavage: 1.0 g of peptide resin, 10 mL of cleavage cocktail 3, 60 min at room temperature. Second cleavage of the crude peptide necessary, same conditions.
- Crude: 545 mg (65% of theory) after two cleavages, purity 31% (HPLC).
- Remarks: Amino acid analysis and FAB-MS (both after purification) in accordance with theory. A rather difficult peptide. Met easily oxidized.

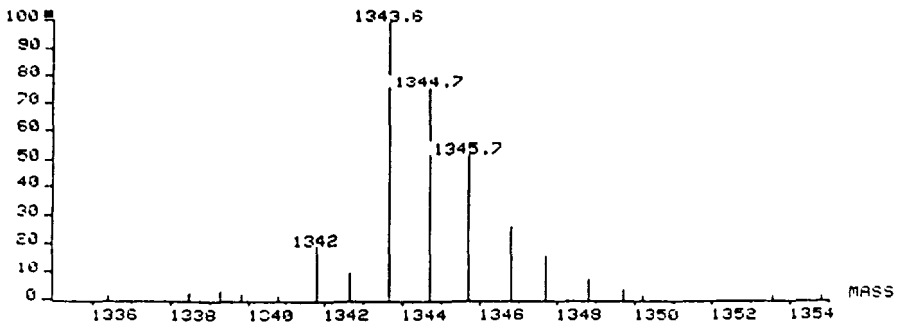

Fig. 2. FAB-MS of purified *3* $[M + H]^+$: calculated = 1343.6, found = 1343.6.

3.2.5. Dermaseptin (14)

- Sequence: H-Ala-Leu-Trp-Lys-Thr-Met-Leu-Lys-Lys-Leu-Gly-Thr-Met-Ala-Leu-His-Ala-Gly-Lys-Ala-Ala-Leu-Gly-Ala-Ala-Ala-Asp-Thr-Ile-Ser-Gln-Gly-Thr-Gln-OH.
- Characteristics: 34-mer; basic, with a marked hydrophobic domain of seven amino acids; contains two Met and one Trp; considered as difficult peptide.
- Resin: Wang (*see* Section 1.).
- Cleavage: 1.0 g of peptide resin, 10 mL of cleavage cocktail 3, 90 min at room temperature.
- Crude: 429 mg (44% of theory) with a purity of 39% (HPLC; *see* Fig. 3, left).
- Remarks: Amino acid analysis and ES-MS (both after purification) in accordance with theory (HPLC; *see* Fig. 3, right; ES-MS; *see* Fig. 4). A difficult and sensitive peptide. Prone to oxidation.

4. Notes

1. Any Cys(Acm) as part of the peptide chain on the resin remains unchanged, i.e., Cys will not be deprotected.
2. Commercially available with the first (C-terminal)-protected amino acid linked to the resin (from BACHEM Feinchemikalien AG, Switzerland).
3. Commercially available with Fmoc protection, i.e., without amino acid loading (e.g., from BACHEM Feinchemikalien AG, Switzerland).
4. Mtr as protecting group for Arg is no longer recommended (except where explicitly required), since it needs more drastic cleavage conditions, which may damage the peptide.
5. It may be necessary to optimize the reaction conditions (reaction time and/or reaction temperature).
6. The resin should not be discarded yet at this stage, but stored at –25°C for a possible second cleavage (*see* Note 11).

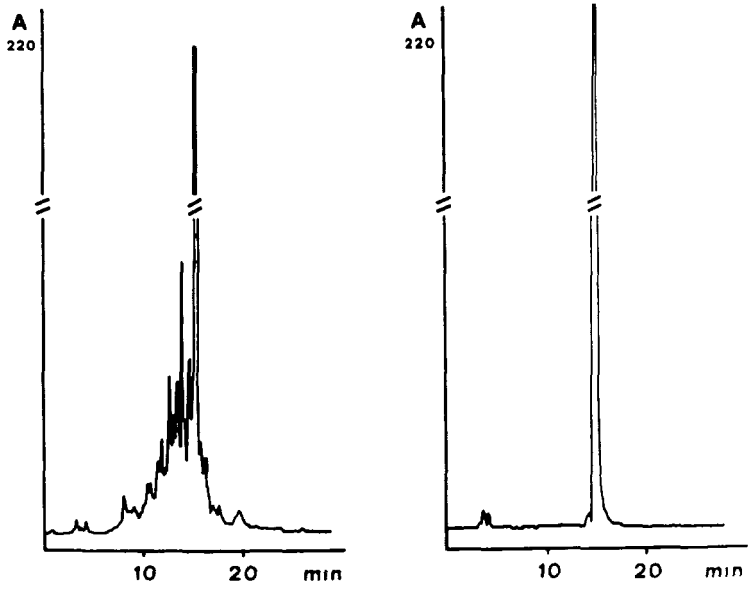

Fig. 3. RPHPLC gradient elution profile of *5*, crude: left, purified: right.

7. Step 6 can be omitted in many cases, i.e., the combined filtrates can be added directly to the cold ether (*see* Note 8) without previous concentration. It is, however, advisable to check if the peptide is precipitable by this alternative treatment by adding a few drops of the filtrate to 5–10 mL of cold ether (*see* Note 8).
8. Methyl-*tert*-butylether is preferred, since it is less prone to peroxide formation than diethylether and diisopropylether.
9. This step can be laborious and time-consuming, because many precipitated peptides are of slimy consistency. The isolation could also be done by centrifugation.
10. Mother liquors must be disposed of properly. To destroy bad-smelling components, such as mercaptans, the mother liquors are carefully neutralized with aqueous NaOH and subsequently treated with commercial laundry bleach *(15)*.
11. If the crude yield is low (<50–70% of theory), the whole procedure can be repeated with the resin (*see* Note 6) The second crop (if any) may be of poorer quality than the first one.
12. In most cases, the RPHPLC-trace shows one clear main peak. The occurrence of peaks with longer retention times than the main peak is a hint for incomplete cleavage.

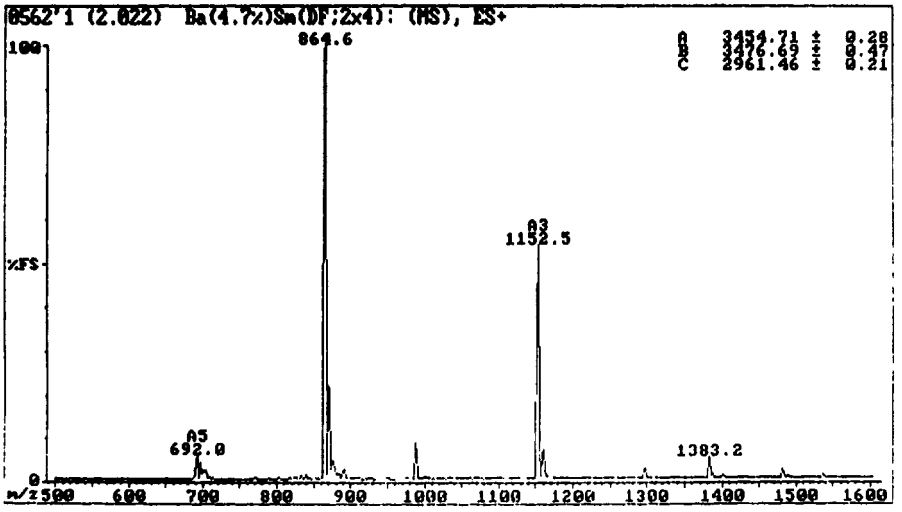

Fig. 4. ES-MS of purified *5*, M_r: calculated = 3455.1, found = 3454.7 (**A**), sodium adduct (**B**), unknown impurity (**C**).

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