1. Introduction

Development of appropriate resin linker combinations for solid-phase peptide synthesis (SPPS) has allowed rapid access to (fully) protected peptide fragments with a free C-terminal carboxyl moiety. These fragments may be assembled either in solution or on resin—an approach that has some intrinsic advantages compared to the stepwise methodology (see Chapter 15). Approaches to synthesize such fragments usually employ “orthogonal” (see Note 1) $N^\alpha$-protection/side-chain protection. The orthogonal combination Fmoc/tBu can, however, only be used if the peptide resin bond is cleavable either by acids weak enough to leave tBu groups intact or by a method employing neither acids nor bases, such as photolysis (1) or catalysis by a Pd(0) complex (2).

A few very acid-labile resins have been developed. Only a part of them is commercially available. Some are labile even toward acetic acid in TFE/DCM (3), which means that the acidity of the protected amino acids and HOBt has to be taken into account (or else premature cleavage may decrease the yield). If, on the other hand, too strong an acid (or too high a concentration) is needed for cleavage, $N^\alpha$-Boc groups of lysine or tBu ether groups of tyrosine may be partially cleaved, which is not acceptable. A good compromise is reached when cleavage can be carried out with 0.5−1% TFA in DCM (4,5) (see Chapter 5). Fully Boc/t-butyl-protected fragments can be obtained under these conditions, e.g., from the commercially available Sasrin™ resin (6). This chapter will deal explicitly with the synthesis on and the conditions for cleavage of fragments.
from Sasrin resin. Sample cleavage from Sasrin may also serve as a tool for SPPS monitoring (see Note 2).

Solid-phase synthesis using Fmoc strategy (e.g., on Wang resin) has already been dealt with in detail in preceding chapters of this book. The methods described there can also be applied to Sasrin resin, but, obviously, acids stronger than N-protected amino acids have to be avoided (just as prolonged treatment with piperidine) to avoid premature cleavage of the peptide from the resin. $N^\alpha$- and side-chain protecting groups have to withstand treatment with 1% TFA in DCM. The most useful protecting groups for synthesis of protected fragments on Sasrin resin and their limitations are listed below:

- for $N^\alpha$: Fmoc, Boc, Z
- Asn, Gln: Mtt, Trt, none
  (protection of the amide group is strongly recommended)
- Asp, Glu: OtBu
- Arg: Pmc, Mtr (see Chapter 5)
- Cys: Trt, Acm (polar), SrBu (see Note 3)
- His: none, Trt (see Note 4)
- Lys: Boc
- Ser, Thr, Tyr: tBu
- Met: none, sulfoxide (polar, see Note 5)
- Trp: none, Boc (see Note 6)

All these protecting groups influence the solubility of the fragment; sufficient solubility is a crucial requirement for efficient fragment coupling (see Chapter 15). If necessary, various fragments with different combinations of protecting groups have to be prepared and checked for solubility.

If possible, Gly or Pro are chosen as C-terminal amino acid of the fragment with the free carboxyl moiety to avoid racemization during coupling later on, but when synthesizing fragments with C-terminal Pro, the formation of diketopiperazine (see Note 7) has to be circumvented by coupling, e.g., Fmoc dipeptides instead of the penultimate Fmoc amino acid at the risk of concomitant racemization. Recently, other deblocking procedures to suppress diketopiperazine formation have been published (7). Unfortunately, nature has conceived most of its peptides not in the manner making the peptide chemist’s work easy by putting glycine into appropriate positions. Therefore, coupling with other C termini cannot be avoided, and appropriate coupling conditions with minimal racemization have to be worked out. Coupling fully protected peptide azides is an old but, still valu-
able method known for minimal concomitant racemization (see Note 8). These acid azides are usually generated in situ from the corresponding hydrazides. Fully protected peptide hydrazides can also be obtained rapidly by SPPS on Sasrin resin followed by cleavage with hydrazine hydrate (8). This chapter will deal exclusively with acidolytic cleavage leading to protected peptide fragments with a free C-terminal carboxyl group.

2. Materials

1. All reagents and solvents are commercially available. TFA and pyridine should be colorless or else they must be distilled. The 1% TFA/DCM solution can be stored in a tightly closed dark bottle for a few weeks. Piperidine solutions in DMF are not stable and may only be kept for a few days in a closed container.

2. DCM should be dry and acid-free, but the commercially available solvent usually fulfills these requirements, if it is properly stored. DMF has to be freed from basic impurities, such as dimethylamine, e.g., by treating it with acidic aluminum oxide or by distilling it in vacuo from ninhydrin. DMF should be kept in a dark bottle and the pH checked from time to time. Ethers must be free of peroxides especially when Cys- or Met-containing fragments are to be treated (see Note 9). Check, e.g., with Merck Quant Peroxid-Test strips 10.011 from Merck (Darmstadt, Germany). Methyl t-butyl ether is less prone to peroxide formation than diethyl or diisopropyl ether, and less volatile. The other solvents need no additional purification.

3. Fmoc-amino acid Sasrin resins are commercially available from BACHEM AG, Bubendorf, Switzerland.

4. The solutions for the Kaiser test are relatively stable; b and c have to be colorless:
   a. 5 g ninhydrin in 100 mL abs. ETOH;
   b. 80 g phenol in 20 mL abs. ETOH; and
   c. 2 mL 0.001M aq. KCN—add pyridine to 100 mL.

5. Equipment for TLC and HPLC analysis (see Chapters 1-5, PAP).

6. FAB-MS and other methods to confirm the structure of the fragment (see Chapters 6 and 7, PAP).

3. Methods

3.1. General Considerations

3.1.1. Instrumental

Synthesis and cleavage can be performed manually on a fritted-glass funnel or in any commercially available synthesizer suitable for Wang resin (some modifications may be necessary for the cleavage procedure). A shaker with a vessel equipped with a sintered-glass bottom is a simple,
but versatile apparatus for synthesis and cleavage. It may be operated manually or automatically. Equipment specifically designed for continuous flow should not be used for SPPS on Sasrin resin. During the whole procedure, the peptide resin never needs to be transferred to another vessel or funnel. Filtration can be performed by suction or, preferentially, by inert gas pressure. This way the peptide resin is protected from oxygen and moisture, and the solvents are removed rapidly, but gently. Many fully automated synthesizers apply this method. On the other hand, the simple "fritted-glass funnel method" is suitable for the rapid manual synthesis and cleavage using small amounts of resin or, especially, for cleaving small samples to monitor a synthesis.

3.1.2. Solid-Phase Synthesis of Protected Fragments

Solid-phase synthesis according to the Fmoc strategy is described elsewhere in this book. For the convenience of the reader and to avoid any misunderstanding, a brief description of a standard protocol for SPPS on Sasrin resin is given in Section 3.2.1.

3.1.3. Pretreatment of the Peptide Resin Before Cleavage

The fully protected peptide fragment may be cleaved from the resin directly after the N-terminal amino acid has been coupled, but first the coupling reagents have to be washed out carefully with the solvent used for the preceding coupling step. To remove this solvent, the resin is washed with isopropanol (see Note 10), which will shrink it (this step is optional). Then the resin has to be washed thoroughly with DCM. These washes are extremely important to remove remainders of polar solvents (DMF and alike) completely. Polar contaminants "consume" TFA. They are protonated as well as the amide groups of the peptide under the anhydrous conditions of cleavage (compare [5]). Only after this "neutralization" will the cleavage proceed smoothly.

The peptide resin may now be cleaved or dried first to determine the weight gain of the peptide resin, or if one wishes to cleave only a part of the resin and store the remainder. Such a dried peptide resin has to be washed several times with DCM for proper swelling prior to cleavage.

3.1.4. The Cleavage

The prewashed peptide resin is now treated several times with 1% TFA/DCM (see Note 11). The amide moieties of the peptide fragment
will “bind” TFA, thus reducing the amount of acid. The protonation of
the amide bonds may be responsible for the good solubility of the frag-
ment during cleavage (see Note 12). Repetitive short treatments with 1%
TFA/DCM with immediate subsequent neutralization of the peptide-con-
taining filtrates as described by Flörshermer and Riniker (5) minimize
the actual time of exposure to acid during cleavage. The first (with con-
comitant decrease of TFA concentration; see Section 3.1.3.) to fourth
treatment will cleave most of the fragment, if no polar contaminants have
been present, but treatments should be continued until no peptide is
cleaved anymore (checked by TLC; see Note 13). Normally the resin
will turn deeply violet as the cleavage proceeds (see Note 14). This color
change may serve as an indicator for cleavage, but not for determining
its “end point.” Concomitantly, the volume of the resin decreases signifi-
cantly. Inertization of the cleavage vessel is optional (see Note 15).

3.2. General Procedures

3.2.1. General Synthetic Procedure

1. Swell the dry Sasrin resin (with the appropriate Fmoc amino acid attached)
by treating (see Note 16) it several times with DMF. Swelling will take
some time. Other solvents, especially NMP, have been applied success-
fully in fully automated SPPS (see Chapter 3).

2. Treat the swollen resin with 20% piperidine/DMF for 5 min, suck off, and
repeat the treatment for 10 min. If piperidine/DMF leads to incomplete
Fmoc cleavage, it may be replaced by 20% piperidine/DMF containing 2–
5% diazabicycloundecene.

3. Wash thoroughly with DMF, until the washes are neutral because the base
has to be removed completely (traces of base may cause premature Fmoc
cleavage during the coupling!). Washing can be sped up by shrinking,
after a few DMF washes, with isopropanol (two to three washes) and swell-
ing again with DMF (three to four washes; check pH). In most cases, the
base will be removed after 10 washes.

4. Take a small sample. The Kaiser test (9) has to be positive, i.e., deeply blue or
red (with proline) (see Chapter 8). With 2,4,6-trinitrobenzenesulfonic acid
(TNBS) (10), the beads turn red if free amino groups are present (see Notes
17 and 18). These two tests only show the presence of free amino groups in
a qualitative manner. Quantitative information may be gained either via
UV monitoring of the Fmoc cleavage or by completely cleaving a sample
of peptide resin followed by HPLC analysis (see Chapter 3, PAP).

5. The coupling: First the Fmoc amino acid has to be activated, e.g., with
DCC/HOBt (see Notes 19 and 20) or TBTU/DIPEA (see Note 21). Fmoc
amino acids and coupling reagents have to be used in excess (e.g., threefold) to drive the coupling to completion. As HOBt is liberated during the coupling, the suspension will turn slightly acidic. Adjust to pH 7–7.5 by adding small amounts of DIPEA, but avoid an excess of base.

6. After 15–30 min take a small sample, wash it with DMF, and perform the Kaiser test (and, optionally, the TNBS test; see Chapter 8). In both cases, the beads should be nearly colorless and the supernatant yellowish. The sensitivity of the TNBS test can be increased by using a microscope to inspect the beads. Many other methods for monitoring SPPS have been developed, but none of them reached the popularity of the Kaiser test.

7. If the tests show (e.g., Kaiser test bluish or greenish) that the coupling is not yet completed even after 1 h, suck off and wash five times with DMF. Repeat the Kaiser test since impurities may have been the cause of a slightly positive test before. If not, repeat the coupling.

8. Eventually the tests will be virtually negative (see Note 17). Wash thoroughly with DMF to remove the reagents. Again, shrinking and reswelling are optional. To block free amino groups that have not been detected and thus to avoid deletions, the resin may be treated with a “capping” reagent, e.g., a large excess of acetic anhydride/pyridine in DMF for 10 min. Again wash carefully with DMF to remove the reagents.

9. Cleave the Fmoc group as described in step 2.

10. Coupling of the next Fmoc amino acid: follow 3–8 (see Note 22) and so on.

11. For washings after the last coupling step, see Section 3.1.3.

### 3.2.2. General Cleavage Procedure

Before cleavage, the peptide resin has been carefully washed and (optionally) dried (compare Section 3.1.3.).

1. The dry resin is weighed into a sufficiently large (mind the swelling!) fritted-glass funnel (preferably G4), where it remains throughout the whole procedure, e.g., a 100-mL funnel can be used for 5–10 g peptide resin.

2. The resin has to be washed at least five times with DCM (10–20 mL/g resin, contact time not <5 min). If the resin is not used in dried form, but directly after the last coupling step, it should be washed the same way, but contact time can be reduced to about 1 min. Prolonged suction should be avoided, and the resin must never be sucked to dryness. Slow stirring of the resin suspension is optional.

3. After placing the funnel on a clean vessel, the resin is treated with 1% TFA/DCM (10–15 mL/g) for 2–5 min. All the solvent is sucked into a vessel containing pyridine (>1.2 mL/mL TFA), and thus, the filtrate is immediately neutralized. Pyridine (200 mL/g resin) in methanol (2 mL/g resin) has also been recommended (5). Again, prolonged suction and suck-
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ing-through of air should be avoided (see Note 23). The resin should have changed its color slightly to distinctly (for exceptions, see Note 14).

4. This treatment is repeated with further portions of 1% TFA/DCM until it can be assumed that all the peptide has been cleaved from the resin, which by then should have turned deeply violet (see Note 14). Normally, three to six treatments will be sufficient.

5. The neutralized filtrates should be kept in separate vessels and analyzed, e.g., by TLC. Only the fractions containing a significant amount of peptide are pooled and subjected to work-up (see Note 24). Often the second and/or third fraction will contain most of the peptide.

6. To determine the cleavage yield (from weight loss of the resin), the resin has to be washed thoroughly with alcohol and ether, and dried to constant weight. The resin will be discolored rapidly by these washings.

7. When cleaving small amounts of peptide resin, e.g., for monitoring a synthesis (see Note 2), the filtrates need not be collected separately to avoid losses.

3.3. Work-Up Procedures

At first, the peptide fragment has to be freed from contaminating pyridinium trifluoracetate (cf Section 3.2.2., step 3). Utmost care has to be taken to remove remainders of trifluoroacetic acid completely before using the fragment for subsequent coupling. During work-up, Met has to be protected from oxidation. Depending on the solubility of the fragment, work-up varies. A protected fragment may precipitate during cleavage or during neutralization, or it may be precipitated thereafter or extracted. The following paragraphs deal with the different work-up procedures.

1. Neutralization of the cleavage fractions already leads to precipitation of the peptide fragment (even though TLC checks should not be omitted since only a part of the peptide may have precipitated). Diethylether or methyl t-butyl ether is added to the pooled fractions to complete precipitation (at least the same volume, up to 5 vol of solvent). Stirring is recommended, and then the precipitate should be left to settle. Often the pyridinium trifluoroacetate crystallizes in long needles. The precipitate is filtered off and washed with ether. Since fully protected peptide fragments usually are hardly soluble in water, the precipitate may be triturated with water (until the washes are neutral) to remove the salt. Then it is washed with ether, dried, and weighed. The mother liquor and washes should be checked for peptide before discarding them. Polar impurities may be removed by dissolving the fragment in DMF (or another water-miscible solvent—the amount should be kept as low as possible) and precipitating it by adding water or
0.5N aqueous KH$_2$SO$_4$ \((\text{see Note 25})\). The precipitate is filtered off, washed with water until the washings are neutral, dried carefully, and weighed.

2. The neutralized fractions become extremely viscous, or a gel precipitates. Then ether (5–10 vol) has to be added in small portions. The gel should be stirred. Such precipitates may cause problems during filtration, and they are prone to form inclusions. Thus, they should be redissolved and precipitated if possible \((\text{see step 1})\).

3. Neutralization does not cause any visible effect.
   a. After pooling the fractions, the peptide may be precipitated with ether. Precipitation can be enhanced by removing a part of the DCM on a rotavap beforehand. A small sample is taken and treated with 10 vol of ether. If the peptide precipitates, it can be isolated by this method; the mother liquor has to be checked for peptide. For further treatment, \textit{see} step 1
   b. The peptide cannot be precipitated with an ether, so the DCM has to be removed \textit{in vacuo}. It can be (discontinuously) replaced directly with ethyl acetate \((\text{see Note 26})\). High concentrations of pyridinium trifluoracetate have to be avoided if the fragment contains very acid-sensitive moieties, such as Tyr($r$Bu) or His(Trt). The resulting solution in ETOAc (which may contain small amounts of DCM) has to be extracted several times with water (the phase separation may take some time) and brine. The last aqueous washes should be neutral. All phases should be checked by TLC and reextracted with ETOAc, when necessary. The organic phase is dried with sodium sulfate and evaporated. The residue may be triturated with an appropriate solvent, e.g., an ether \((\text{see Note 27})\).
   c. The DCM has been removed, but the residue turns out to be insoluble in ethyl acetate (a rather unusual case). At first, a sample should be treated with DMF \((\text{see step 1})\) and, in case of dissolution, precipitated with water. Normally, dissolving and precipitation are more effective than just triturating the insoluble residue with water.

4. The fragment is scarcely soluble in 1% TFA/DCM, yet it is cleaved from the resin (as can be deduced from the color change). Therefore, samples of the resin should be extracted with DMF, DMA, NMP, and similar solvents, or mixtures thereof, until a suitable solvent system is found \((\text{see Note 28})\). (For further treatment, \textit{see} step 1.) Such a behavior can be expected when cleaving fragments containing ions, e.g., short fragments containing the Ba-salt of sulfated tyrosine.

### 3.4. Purification Procedures

Normally, the fully protected peptide fragments cleaved from Sasrin resin and worked up as described turned out to be sufficiently pure
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according to TLC and HPLC. Their structures have to be confirmed by FAB-MS (see Chapter 7, PAP), amino acid analysis, and other methods. The formation of side products during cleavage seems to be rather unlikely because of the mild conditions, but it cannot be ruled out during synthesis. The well-established methods for the purification of unprotected peptides, such as RP-HPLC (see Chapter 3, PAP) and ion-exchange chromatography (see Chapters 2 and 5, PAP), usually cannot be applied because of the lack of fragment solubility in aqueous systems. The fragments are rather unpolar. Hence, they often are soluble in common organic solvents such as chloroform or methanol. Thus extraction, precipitation, and (flash) chromatography on silica may be suitable methods. Recently, purification methods for fully protected fragments using preparative chromatography have been developed by Lloyd-Williams et al. (I) and by Riniker et al. (II).

3.5. Examples

1. Fmoc-Gly-Val-Val-Lys(Boc)-Asn(Trt)-Asn(Trt)-Phe-Val-Pro-Thr(tBu)-Asn(Trt)-Val-Gly-OH—This fragment represents the sequence 21–33 of α-h CGRP. An example for a large-scale synthesis and cleavage is: SPPS starting with 125 g Fmoc-Gly-Sasrin (=81.2 mEq). Peptide resin (after the last coupling step) is carefully washed with isopropanol and then with DCM. It is treated with 1% TFA/DCM (12 × 1000 mL, 10 min). Each fraction is neutralized with 15 mL pyridine (no precipitation). Fractions are pooled and DCM removed in vacuo. Oily residue is triturated with water (2 L) leaving a white precipitate which is filtered, washed with water (five times) and ether (eight times), and dried. Yield is 176 g (89%). Purity is (TLC) >90%.

2. Boc-Ala-Cys(Acm)Asp(OtBu)-Thr(tBu)-Ala-Thr(tBu)-Cys(Trt)-Val-Thr(tBu)-His-Arg(Pmc)-Leu-Ala-Gly-OH—This fragment represents the sequence 1–14 of α-h CGRP. After cleavage, it is oxidized with I₂.

An example for work-up by precipitation (cf Section 3.3., step 3a) is: SPPS starting with 70 g Fmoc-Gly-Sasrin (49 mEq). Peptide resin (after the last coupling step) is washed with isopropanol (seven times) and DCM (seven times). This is treated with 1% TFA/DCM (8 × 1000 mL, 10 min). Fractions are neutralized with pyridine and checked by TLC. The fragment does not precipitate. Fractions 1–7 are pooled and DCM removed in vacuo until ca 2000 mL are left. Five liters of diisopropyl ether are added to precipitate the peptide under stirring. The precipitate is filtered off, washed with ether (four times) and dried. Yield is 89.4 g (78%). Purity is (TLC) >80%.
3. Boc-His(Boc)-Lys(Boc)-Thr(tBu)-Asp(OtBu)-Ser(tBu)-Phe-Val-Gly-OH—This fragment represents the sequence 1–8 of substance K. An example for work-up by trituration (cf Section 3.3., step 1) is: SPPS starting with 15 g Fmoc-Gly-Sasrin (10.5 mEq). Peptide resin (after the last coupling step and washes) is washed with DCM six times. This is treated with 1% TFA/DCM (5 × 300 mL, 5 min). Fractions are neutralized with pyridine, and fractions 2–5 are pooled. DCM is removed _in vacuo_ leaving a gel, which was triturated with 200 mL diisopropyl ether. Precipitate is triturated with diisopropyl ether (3 × 100 mL), dried, and stirred in 300 mL water for 0.5 h, filtered off, washed with water, dried, triturated with ether, and dried. Yield is 11.68 g (82%). Purity is >96% (HPLC).

4. Fmoc-Ser(tBu)-Pro-Lys(Boc)-Met-Val-Gln(Mtt)-Gly-OH—This fragment represents the sequence 1–7 of hBNP. An example for work-up by precipitation (cf Section 3.3., step 3c and Section 3.3., step 1) is: After SPPS, resin is washed and dried; 26.6 g peptide resin (9.4 mEq peptide) are prewashed with DCM. This is treated with 1% TFA/DCM (6 × 280 mL, 5 min). Each fraction is neutralized with 4.8 mL pyridine. All fractions are pooled and DCM removed in vacuo. Residue is dissolved in 140 mL DMA at ca. 40°C. The resulting solution is slowly poured into water (1.2 L). Precipitate is filtered off, washed with water (3 × 500 mL), and dried. Yield is 10.8 g (83%). Purity is >87% (deprotected, HPLC).

5. Fmoc-Asn(Mtt)-Lys(Boc)-His-Thr(tBu)-Phe-Pro-Gln(Mtt)-Thr(tBu)-Ala-Ile-Gly-OH—This fragment represents the sequence 17–28 of human calcitonin, a “small-scale” cleavage. An example for work-up by extraction (cf Section 3.3., step 3b) is: 1.67 g peptide resin (0.49 mEq) is prewashed with DCM (5 × 20 mL) and cleaved with 1% TFA/DCM (5 × 15 mL, 5 min). Peptide does not precipitate when neutralizing the fractions with pyridine. All fractions are pooled and DCM is removed _in vacuo_. Residue is triturated with diisopropyl ether and dried. It is dissolved again in EtOAc and extracted with water (5 × 10 vol%) and brine. EtOAc is removed, and residue is triturated with ether. Yield is 706 mg (67%). Purity is 65% (HPLC, after cleavage of the protecting groups).

6. Boc-Arg(Mtr)-Ser(tBu)-Ser(tBu)-Cys(Acm)-Phe-Gly-Gly-Arg(Mtr)-Met-Asp(OtBu)-Arg(Mtr)-Ile-Gly-OH—This fragment represents the sequence 4–16 of α-h ANF. An example for a fragment forming a gel (cf Section 3.3., step 2) is: After SPPS, resin is washed and dried; 50 g peptide resin (14.2 mEq peptide) are prewashed with DCM. This is treated with 1% TFA/DCM (6 × 500 mL, 10 min). When neutralizing with pyridine, a part of the fractions turns into thick gels. Thus, they cannot be pooled directly: 500 mL ether are added under vigorous stirring to fraction 2–5 to precipitate the peptide. Fractions are pooled, left to settle, and filtered. The pre-
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Precipitate is washed with ether, water, and again ether, and dried. Yield is 30.7 g (89%). Purity is >90% (HPLC).

7. Pyr-Gln-Asp(OtBu)-Tyr(tBu)-Thr(tBu)-Gly-OH—This fragment represents the sequence 1–6 of cerulein. An example for precipitation owing to neutralization (cf Section 3.3., step 1) is: SPPS starting with 20 g Fmoc-Gly-Sasrin (13 mEq). Peptide resin (after the last coupling step) is carefully washed with DCM. This is treated with 1% TFA/DCM (4 x 300 mL, 5 min). Each fraction is neutralized with 5 mL pyridine, causing the peptide to precipitate (the second fraction contains most of the peptide). Fractions 2 and 3 are pooled and diluted with ca 600 mL ether. The precipitate is left to settle, filtered off, triturated with ether and water, and dried. Yield is 7.82 g (95%). Purity is >85% (TLC).

4. Notes

1. Orthogonal: “an orthogonal system has been defined as a set of completely independent classes of protecting groups, such that each class of groups can be removed in any order and in the presence of all other classes” (12).

2. The solid-phase synthesis on Sasrin resin can be monitored by cleaving samples at any stage. These samples, which only have to be washed carefully before cleavage, actually show what the resin-bound product looks like. This is an advantage of very acid-labile resins compared to, e.g., Wang resin, where protecting groups are concomitantly removed when cleaving from the resin.

3. When using Cys(SzBu), piperidine treatment has to be kept as short as possible (13).

4. His(Trt) is very acid-sensitive. Trt may be partially cleaved even with 1% TFA/DCM.

5. Met sulfoxide may be formed unintentionally during handling, especially during coupling of Met-containing fragments.

6. N'-protection of Trp is strongly recommended when Trp is “exposed,” e.g., C-terminal (11,14).

7. Diketopiperazine formation is favored when Pro is the C-terminal amino acid (see Compound 1).


9. Normally protected peptides are somewhat less sensitive toward oxidation than the corresponding free peptides, especially when many hydrophobic protecting groups are present.

10. Washes with isopropanol also remove dicyclohexyl urea (which is formed when coupling with DCC, DCC/HOBt, and so forth). This scarcely soluble compound also has to be removed carefully before cleavage since it “con-
sumes" TFA. Actually, it is dissolved by 1% TFA/DCM owing to salt formation, whereas its solubility in pure DCM is negligibly low. Dicyclohexyl urea can also be removed by treating the peptide resin with DCM/alcohol (1:1).

11. Higher yields of Trp and/or Met-containing fragments may be obtained by adding a neutral scavenger, e.g., 5% EDT. Nevertheless, indole protection, e.g., Trp(Boc), may be the best way to prevent the yield-decreasing alkylation.

12. Precipitation or low solubility of the fragment in TFA/DCM has been observed very rarely and was not found to obstruct the cleavage. On the other hand, neutralization of the TFA can cause precipitation of the protected fragment (see Section 3.3.).

13. TLC systems have to be less polar than systems for the analysis of free peptides. A few useful systems are:

- CHCl₃/MeOH/AcOH 77.5:15:7.5
- CHCl₃/MeOH/32% AcOH 15:4:1
- ETOAc/pyridine/AcOH/H₂O 6:5:1:3
- CHCl₃/MeOH/32% AcOH 5:3:1
- CHCl₃/TFE/80% AcOH 6:2:1

Detection is by, e.g., Greig-Leaback.
14. The color change is probably caused by the stable carbocation formed by cleaving the peptide-resin bond. Minor color changes occur when cleaving Trp- or Met-containing peptides or when using scavenger.

15. Inertization is recommended, but not absolutely necessary when cleaving Met- or Cys-containing peptides.

16. “Treat” here means shake or stir slowly. The swollen beads may be damaged by mechanical forces.

17. The Kaiser ninhydrin test (9) is: Put a small sample of swollen beads in a test tube. Add three drops of each solution (see Section 2.), and keep the suspension at 100°C for 5–6 min. Free amino groups are detected by a deep-blue color (red: for proline). Unfortunately, the intensity of the color depends on the N-terminal amino acid. It may be rather weak, e.g., for Asp and Ser. On the other hand, all the coded amino acids (except Pro) seem to yield the same intensity of color with TNBS, making this test a valuable additional control (see Note 18). Special care has to be taken when coupling an Fmoc amino acid to Pro (which tends to be a sluggish reaction) since the Kaiser test gives somewhat ambiguous results.

18. The TNBS test (10): Put a small sample of swollen beads into a test tube. Add a tiny amount of solid TNBA and three drops of 10% DIPEA in DMF. Vortex and wait 5 min. The supernatant should be yellow. Remove it with a Pasteur pipet or by centrifugation. The beads turn red when free amino groups are present.

19. Avoid skin contact with DCC, which is a strong allergen. When spilled, decompose with AcOH.

20. Activation with DCC yielding the Fmoc-amino acid OBt ester: Dissolve or suspend equimolar amounts of Fmoc amino acid and HOBT in DMF. Try to keep the amount of solvent low. Add a low excess (1.1X) of DCC, and stir for 0.5–1 h; the reaction is slightly exothermic, and dicyclohexyl urea will start to precipitate after a few minutes. Filter off and wash the precipitate (matted white needles) twice with small volumes of DMF. Add the combined filtrates to the resin. This method should not be applied to Fmoc-Arg derivatives. When activating Fmoc-Gly-OH, do not filter off the precipitate.

21. Activation with TBTU/DIPEA (15) is simpler. It can be performed directly before coupling (this is especially important when coupling Fmoc-Arg derivatives). Equimolar amounts of Fmoc amino acid and TBTU are suspended in DMF. On adding an equimolar amount of DIPEA, the TBTU will dissolve (the reaction is slightly exothermic), and the color may turn yellow or red. Stir and add to the resin. Try to keep the concentration of the reactants as high as possible.

22. The number of washings may have to be increased in the course of the synthesis, especially when synthesizing larger fragments. The volume of the
swollen resin should increase slowly. Sudden decreases indicate peptide aggregation. Then difficulties in Fmoc cleavage and coupling have to be expected.

23. Even water may condense on the resin surface because of strong sucking and cooling caused by the evaporation of DCM. From this point of view, pressure filtration is superior to suction.

24. When cleaving Trt-protected peptides, deeply yellow solutions may be obtained. The color disappears when neutralizing.

25. The peptide fragments may have been obtained as pyridinium salt that will be converted into the free acid by this procedure.

26. n-Butanol can also be used, when the residue cannot be dissolved in ethyl acetate.

27. An alternative "washing-protocol" removing most basic and acidic impurities follows:
   a. 5% Aqueous NaHCO₃;
   b. Water;
   c. 0.5–1 N aqueous KHSO₄; and
   d. Water and brine until the washings are neutral. The peptide fragment is converted into the free acid.

28. The solvents TFE, HFIP, and their mixtures with DCM or chloroform are also excellent solvents for fully protected peptide fragments. Since they are weakly acidic, Sasrin will turn pink when treated with them.

References


