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Fmoc resin cleavage protocols
Fmoc resin cleavage and deprotection

Having successfully synthesized a protected peptide, one is confronted with a difficult task of having to simultaneously detach the peptide from the resin support and remove all the side-chain protecting groups of the amino acid residues to yield the desired peptide. In Fmoc SPPS, this step is normally carried out by treating the peptidyl resin with TFA. During this process, highly reactive cationic species are generated from the protecting groups [1] and the handles on the resin [2], and these can, unless trapped, react with, and hence modify, those residues which contain nucleophilic functional groups: Trp, Met, Tyr and Cys. To prevent this, various nucleophilic reagents (known as scavengers) are added to the TFA to quench these ions.

A number of universal cleavage mixtures have been advocated, the most popular of which is Reagent K (TFA/water/phenol/thioanisole/EDT [82.5:5:5:5:2.5]). However, with recent advances in protecting group and linker technology, particularly the introduction of Fmoc-Trp(Boc) and Fmoc-Arg(Pmc/Pbf) derivatives, such complex mixtures containing toxic and malodorous reagents are no longer necessary, except in exceptional circumstances.

Most problems can be ameliorated by the appropriate choice of protected amino acid derivative and resin (Table 1). If these recommendations are followed, the use of TFA/TIS/water (95:2.5:2.5) will suffice for most sequences. There are, of course, sequences, especially those which contain cysteine and numerous t-butyl protected residues, for which this mixture does not give satisfactory results; in these cases, the addition of EDT to the mixture or the use of reagent K is recommended. Nevertheless, as a general, non-malodorous cleavage cocktail, this mixture has proved remarkably effective.

For those who do not wish to use the recommendations given in Table 1, the flow-chart shown in Figure 1 will aid in the selection of the most appropriate mixture.

1 Removal of N-terminal Fmoc group

Before acid cleavage of the peptidyl resin can be performed, the N-terminal Fmoc group must be removed using piperidine. Check with the instruction manual of your synthesizer; many synthesizers will automatically program the removal of the N-terminal Fmoc-group as a last step in the synthesis.

2 Preparing peptide resin for cleavage

The peptide resin should be thoroughly washed, especially when DMF is used during synthesis as it is nonvolatile and residual basic DMF can have a marked inhibitory effect on TFA-acidolysis. For PEG and polyacrylamide-based supports, washing with a mildly acidic reagent, such as acetic acid which does not cause release of the peptide, is desirable since these types of resin have a tendency to hold onto DMF [3]. Thorough washing and drying must be effected before cleavage (Method 1).

Note: Acetic acid should not be used for washing of extremely acid-labile Rink acid, TGT or 2-chlorotrityl resins.

Method 1: Preparing peptide resin for cleavage

1. Place the peptide resin in a sintered glass funnel and apply some suction.
2. Wash with DMF, acetic acid, then with DCM several times. Wash further with MeOH (polystyrene) or ether (polyacrylamide) to shrink the resin.
3. Remove the peptide resin and dry under high vacuum for 4 h, or preferably o/n, over KOH.

3 TFA cleavage and deprotection

Optimum cleavage conditions are very much dependent on the individual amino acid residues present, their number and sequence, the side-chain protecting groups, and the type of linker attached to the resin.

Due to the variability in the behaviour of different peptidyl resins, it is recommended that a preliminary small scale cleavage of peptide resin using 20–50 mg sample is carried out to determine the optimum cleavage conditions, such as the choice of scavenger(s) and length of reaction.

<table>
<thead>
<tr>
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This will enable the extent of cleavage (e.g. by quantitative analysis of the reference amino acid attached to the linker, where appropriate) and the quality of the crude cleaved peptide (by HPLC and amino acid analysis) to be determined. For the majority of peptides, provided the recommendations given in Table 1 are followed, cleavage can be effected with TFA/TIS/water (95:2.5:2.5). In cases where problems do occur, the use of Reagent K, or the addition of EDT to the above mixture, will generally provide a satisfactory solution.

In the case of the Rink Amide resin, the phenyl benzyl ether bond, which links the handle to the resin, is acid sensitive and can be broken, especially when product release is sluggish during the cleavage reaction, resulting in colored by-products which are not easily removed from the product by simple washes. This can be avoided using the 2-step procedure outlined in Method 3, or better by using silane scavengers. These steps are not necessary with resins incorporating the more stable modified Rink linker, such as the Rink Amide AM, Rink Amide MBHA resin, and NovaSyn™ TGR resins.

Methionine, cysteine and tryptophan are extremely susceptible to alklylation by cations produced during the cleavage process. Reaction of tryptophan, methionine or cysteine with t-butyl cations results in modification of the product peptide; reaction with the linker cation gives irreversible reattachment of the peptide to the resin [3]. With methionine, further reaction can occur giving rise to homoserine and fragmentation of the peptide chain. By adding scavengers to the cleavage mixture, these side reactions can be largely suppressed. One exception is sulfonation of tryptophan by the products formed on cleavage of Mtr, Pmc and Pbf protected arginine residues [4]. Fortunately, this side reaction can be eliminated by using Fmoc-Trp(Boc) [5–8]. This derivative also suppresses reattachment of C-terminal Trp residues to the cation generated at the resin linker. Sulfonyl-based protecting groups have also been shown to be associated with the formation of N-sulfonated Arg [9] and O-sulfonated Ser and Thr [10].
The most commonly used scavenger is EDT. Not only is it an extremely good scavenger for t-butyl cations, but it assists in the removal of the trityl protecting group from cysteine and is particularly effective in preventing acid catalyzed oxidation of tryptophan residues.

Suppression of acid-catalyzed Met oxidation can be effected by including ethylmethyl sulfide (EMS), EDT or thiouanisole into the scavenger mixture; although methionine sulfoxide formation can be minimized by carrying out the cleavage reaction under nitrogen, ensuring only peroxide-free ether is used for product precipitation, and that all solvents are thoroughly degassed before use. Thiouanisole is also known to accelerate Arg(Mtr/Pmc/Pbf) removal in TFA; however, it is advisable to exercise care when using this reagent as there is evidence to suggest that it can cause partial removal of Acm, tButhio or tBu protecting groups from Cys residues [11].

Phenol is thought to offer some protection to Tyr and Trp residues [1]. Trialkylsilanes, such as TIS and TES, have been shown to be effective, non-odorous substitutes for EDT [12], particularly for peptides containing Arg(Pmc) and Trp(Boc) [5, 8]. These reagents are also very efficient at quenching highly stabilized cations liberated on cleavage of Trt [12], Tmorb [13] and the Rink Amide linker, and therefore their use is strongly recommended when these moieties are present.

When several of the less acid-labile protecting groups are present in a peptide or the peptide is long and therefore contains numerous protecting groups, cleavage time usually needs to be extended significantly. The Mtr group is less acid-labile than Pmc or Pbf groups, and its complete removal can take as long as 24 hours. In such cases where Trp is present with several Mtr protecting groups, it is extremely useful to be able to optimize the cleavage conditions by monitoring removal of this protecting group by HPLC. A compromise needs to be made between partially tryptophan-modified peptide and incomplete deprotection of Arg(Mtr). Therefore, with peptides containing Trp, the use of Trp(Boc)-derivatives is strongly recommended to avoid modification of the tryptophan side chain.

With long peptides, it can be necessary to use an extended cleavage time to completely remove all side-chain protection. If complete deprotection is not achieved in 6 hours, the peptide should be precipitated with ether, and the cleavage repeated with fresh reagents. Test cleavages should be performed to find the optimum cleavage regime. Incomplete side-chain deprotection is often overlooked as the cause for failure in the synthesis of long peptides.

Problems have been observed with sluggish deprotection of N-terminal Asn(Trt) residues. These can easily be overcome by extending the cleavage time to 4 hours or using Asn(DmcP) in place of Asn(Trt).

Fig. 1: Flow-chart for selecting cleavage cocktail for Fmoc SPPS.

Method 2: General TFA cleavage

CAUTION: TFA is an extremely corrosive liquid; great care must be taken when using this reagent. Proper eye protection, lab coat, and gloves are mandatory. Follow local, state/provincial and federal safety regulations. Use in an efficient fume hood.

1. Place dry resin in a flask and add TFA solution containing appropriate scavengers (10-25 ml/g resin, Figure 3-33). NOTE: a calculation should be made to ensure sufficient scavenger is present for the quality of peptide and protecting groups present. Stopper the flask and leave to stand at rt with occasional swirling. Reaction time depends upon the sequence (see “Monitoring the cleavage reaction”, below).

2. Remove the resin by filtration under reduced pressure. Wash the resin twice with TFA. Combine filtrates, and add (drop-wise) an 8-10 fold volume of cold ether. Sometimes it is necessary to evaporate most of the TFA to achieve a good precipitation of the crude peptide. The ether can be cooled with ice to further assist precipitation.

3. Isolate the peptide as described in Method 13.

Method 3: Two-stage procedure for detachment/deprotection of Rink amide resin

1. Slurry the resin in 10% TFA in DCM and pour into a glass funnel with a fine sinter.

2. Allow the solvent to percolate slowly through the resin bed. Wash the resin with 5% TFA, allowing it to pass through the resin bed slowly. The detachment is an acid-catalyzed equilibrium, so it is important to continually remove the detached peptide by using this flow method. Carrying out the reaction in a flask will not achieve complete detachment. Yields can be improved by the addition of 1:5% TIS to the cleavage mixture.

3. Remove the excess TFA/DCM under reduced pressure and complete the deprotection with 95% TFA plus scavengers, according to the amino acid composition (see Figure 13).

Monitoring the cleavage reaction

The presence of Mtr protected arginine in a peptide necessitates protracted reaction times varying from 3 to 6 hours depending upon the choice of scavengers used (Figure 3-33). Multiple arginine residues can require an extension of reaction times up to 24 hours. In such cases it is extremely useful to be able to optimize the cleavage conditions by monitoring removal of this protecting group by HPLC.

4. General protocols involving strong acids

As an alternative to TFA, for rapid deprotection of less acid-labile side-chain protecting groups such as Arg(Mtr/Pmc/Pbf), Asn(Mbo) and Gln(Mbo), stronger acids can be used with appropriate scavengers with no report of side reactions.
HBF₄ in TFA
Several papers [14, 15, 16] have described the use of HBF₄ (1 M) in TFA as an alternative to TFA cleavage and deprotection. This reagent completely removes the tBu protecting-group from Ser, Thr, Tyr, Asp and Glu residues, Boc from Lysine, and the Mbh from Gln and Asn.

Both Cys(pMeOBzl) and Cys(tBu) residues are deprotected by this procedure. However, Cys(pMeBzl) deprotection is incomplete and Cys(Acm) remains intact even after the addition of thioanisole.

The cleavage of Wang and Rink Amide resins is complete within 30-60 minutes at 4°C, while MBHA resin requires more than 90 minutes of cleavage at 25°C.

The main advantage of this method over TFA alone is that cleavage with HBF₄ in TFA cleaves the Mtr group of arginine in under an hour, thus greatly reducing the risk of Trp alkylation. EDT should be used as a second scavenger when Trp residues are present.

Method 4: Cleavage and deprotection of peptide resins using HBF₄ in TFA
CAUTION: HBF₄ is an extremely strong acid; proper eye protection, face shield, rubber apron and rubber gloves are mandatory. Follow local, state/provincial and federal safety and environmental codes and regulations. Always use in an efficient fume hood. Vapor is harmful if inhaled.

1. Add 10 mmole of HBF₄-diethylether complex to 10 mmole thioanisole dissolved in TFA and adjust the total volume to 10 ml.
2. Treat the peptide resin (15-25 mg) using the above reagent in the presence of m-cresol and EDT at 0°C for 60 min. For MBHA resins cleave for 90 min at 25°C.
3. Remove the resin by filtration under reduced pressure. Wash the resin twice with clean TFA. Combine filtrates, and add (drop-wise) an 8-10 fold volume of cold ether.
4. Isolate the peptide as described in Method 13.

Resin cleavage using trimethylsilyl bromide [17]
The long cleavage times often found necessary for the complete deprotection of peptide containing multiple Arg(Mtr) residues can lead to serious degradation in product quality. In particular, prolonged exposure of tryptophan-containing peptides to EDT in TFA can lead to modification of tryptophan residues due to dithioketal formation. Cleavage with trimethylsilyl bromide (TMSBr) eliminates these problems since this reagent has been shown to cleanly deprotect up to 4 Arg(Mtr) residues in 15 minutes. Furthermore, this method has been found to completely suppress formation of sulfonation by-products, even when unprotected tryptophan is used [18].

Method 5: Cleavage with TMSBr
1. Add TMSBr (1.32 ml) to a solution of EDT (0.50 ml), m-cresol (0.1 ml) and thioanisole [1.17 ml] in TFA (7.5 ml) cooled to 0°C. Add the peptide resin (200 mg) and allow the mixture to stand for 15 min under a blanket of N₂ at 0°C.
2. Remove the resin by filtration under reduced pressure. Wash the resin twice with clean TFA. Combine filtrates, and add (drop-wise) an 8-10 fold volume of cold ether. Sometimes it is necessary to evaporate most of the TFA to achieve a good precipitation of the crude peptide. The ether can be cooled with ice to further assist precipitation.
3. Isolate the peptide as described in Method 13.
NOTE: Occasionally an additional treatment of the peptide with ammonium fluoride is required to reverse any silylation which may have occurred.

5 Cleavage from very acid-sensitive resins
The Rink Acid resin [19], 2-chlorotrityl [20], HMPB [21], NovaSyn TGT [22] and Sieber resins [23] contain highly acid-sensitive linkers and are suitable for the synthesis of protected peptides.

HMPB resins & Sieber amide resins
Fully protected peptide acids can be generated from the HMPB linker [21] and protected amides from the Sieber amide resin [23]. However, careful experimentation is essential if premature loss of side-chain protecting groups is to be avoided. Repetitive treatment of peptidyl resin with a solution of 1% solution of TFA in dichloromethane in tandem with minimum reaction times will give the best results.

Ideally, the cleavage should be carried out in a sealable sintered glass funnel to prevent evaporation of the highly volatile DCM, and the filtration should be carried out by applying nitrogen pressure rather than by the use of a vacuum.

If the peptide contains Met or Trp, 1% EDT should be added to the cleavage mixture to prevent reattachment of the peptide. If the peptide contains a C-terminal Trp residue, the use of Trp(Boc) is strongly recommended [24].

In this batch-wise procedure, the acid strength increases step-wise, as determined by the amount of TFA-buffering groups present. The maximal concentration of peptide may be contained in the first or in one of the later washes, depending on the buffering capacity of the amide bonds and other functional groups present. The side-chain protecting groups of the t-buty1 type as well as Trt (on Asn and Gln) remain completely intact during this process [21].

Method 6: Cleavage with dilute TFA
1. Pre-swell the dry resin (1 g) with DCM in a sealable sintered glass funnel and remove excess DCM.
2. Add 1% TFA in dry DCM (10 ml), seal funnel and shake for 2 min. Filter solution by applying nitrogen pressure into a flask containing 10% pyridine in methanol (2 ml).
3. Repeat step 2 up to 10 times, wash the residual protected peptide from the resin with 3 x 30 ml DCM, 3 x 30 ml MeOH, 2 x 30 ml DCM, 3 x 30 ml MeOH, and check filtrates by TLC or HPLC.
4. Combine filtrates which contain product and evaporate under reduced pressure to 5% of the volume. Add water (40 ml) to the residue and cool mixture with ice to aid precipitation of the product.
5. Isolate product by filtration through a sintered glass funnel. Wash product three times with fresh water. Dry sample in a desiccator under vacuum over KOH, and later over P₂O₅.

2-Chlorotrityl, NovaSyn® TGT and NovaPEG Trt resins
2-Chlorotrityl [20], NovaSyn® TGT [22], and NovaPEG Trt resins can be cleaved with 1% TFA, as described above, or under milder conditions with AcOH or TFE [25] to produce protected peptides. When preparing protected peptides, the use of Fmoc-His(Clt)-OH is particularly recommended for introduction of histidine. This helps avoid partial side-chain deprotection of histidine which can occur when His(Trt) is used.

Method 7: Cleavage with TFE/DCM
1. Treat the peptide resin at rt with TFE/DCM (2:8) for 3 x 1 h.
2. After the appropriate time, remove resin by filtration, wash 3 times with cleavage mixture.
3. Evaporate solution to dryness and precipitate protected peptide with ether. The detached, fully protected peptide will be very hydrophobic and may require to be extracted further from the resin with DMF, DMSO. The completeness of the cleavage can be checked by TLC of the filtrates. Purify by low pressure chromatography on silica gel, HPLC on phenyl silica or by recrystallization.

6 Peptides attached by the HMBA and oxime linker
For the synthesis of peptide carboxamides, the HMBA linker has been largely superseded by those of the Rink Amide type. Nevertheless, this...
linker is still one of the most flexible of the peptide-resin linkers. Peptides can be released from linker with a number of different nucleophiles to yield peptides with a variety of functional groups at the C-terminus [3, 27-30] (Table 2). These protocols are also compatible with the oxime linker used in Boc SPPS.

Table 2: Products from cleavage of HMBA linker.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Product</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄/MeOH</td>
<td>Peptide carboxamide; Method 3-32</td>
<td></td>
</tr>
<tr>
<td>NH₄H₂O</td>
<td>Peptide hydrazide; Method 3-33</td>
<td></td>
</tr>
<tr>
<td>aq.NaOH</td>
<td>Peptide acid; Method 3-34</td>
<td></td>
</tr>
<tr>
<td>MeOH/DIPEA</td>
<td>Peptide methyl ester; Method 3-35</td>
<td></td>
</tr>
<tr>
<td>NaN₃/EOH</td>
<td>Peptide alcohol; Method 3-36</td>
<td></td>
</tr>
</tbody>
</table>

Before cleaving the peptide from HMBA derivatized resins, it is important that the side-chain protecting groups are removed, especially if the peptide contains Asp or Glu. This is achieved by treating the peptide resin with 95% aq. TFA. If the peptide contains Arg(Mtr), the Arg residue is best deprotected after cleavage of the peptide from the resin in an additional step by treatment with reagent K.

Method 8: Methanolic ammonia cleavage to give peptide amides

1. Place dry resin in a flask and add 95% aqueous TFA (20 ml/g). Stopper the flask and leave to stand at rt for 1 h with occasional swirling.
2. Isolate the resin by filtration under reduced pressure and wash with TFA. Discard the filtrate. Wash the resin with DCM, 10% DIPEA in DCM, DCM. Dry the resin under vacuum over P₂O₅.
3. Place the dried resin in a clean, dry pressure vessel and sufficient DMF to swell the resin. Place dry resin in a flask and add 95% aqueous TFA (20 ml/g). Stopper the flask and leave to stand at rt for 1 h with occasional swirling.
4. Seal the flask and let it warm to rt. Leave to stand for 18 h and then cool the flask to 0°C again. Carefully open the cooled vessel and filter the resin through a sintered glass funnel.
5. Wash the resin first with methanol, and then with TFA into a separate flask to remove any methanol insoluble peptide.
6. Evaporate the filtrates separately to dryness on a rotary evaporator. Precipitate peptide with ether and isolate by filtration.

NOTE: If the peptide resin is not thoroughly dried prior to this cleavage procedure, peptide acid may be obtained as a by-product.

Method 9: Cleavage with hydrazine to give the C-terminal hydrazide

If required, the side-chain protecting groups should be first removed following Method 8, steps 1 & 2.
1. Suspend the peptide resin in DMF and add a solution of 5% hydrazine hydrate in DMF (20 ml/g). Leave to stand for 1 h at rt.
2. Filter the resin through a sintered glass funnel and wash the resin first with DMF, and then with TFA into a separate flask to remove any DMF insoluble peptide.
3. Evaporate the filtrates separately to dryness on a rotary evaporator. Precipitate peptide with ether and isolate by filtration.

Method 10: Cleavage with alkali to give the free acid

The side-chain protecting groups should be first removed using Method 8, steps 1 & 2.
1. Pre-swell the resin in dioxane.
2. Cool 0.1 M NaOH dioxane (1.3, 20 ml/g) to 0°C in an ice-water bath. Add the peptide resin and leave to stand for 15 min at rt.
3. Filter the resin using a glass sintered funnel into a flask containing 0.1 M HCl (5 ml/g). This flask should be cooled in an ice bath to prevent warming as the base solution is neutralized.
4. Wash the resin with water and adjust the pH of the filtrate to 7.0. Lyophilize the filtrate and remove the sodium chloride by gel-filtration.

Method 11: Cleavage with methanol/DIPEA to give the methyl ester

The side-chain protecting groups should be first removed using Method 8, steps 1 & 2.
1. Place the resin in a clean flask and add sufficient DMF to swell the resin. Clean aJn with DIPEA/MeOD/DMF (1:5:5, 50 ml/g).
2. Wash the resin first with MeOD/DMF and then with TFA into a separate flask to remove any methanol insoluble peptide.
3. Evaporate the filtrates separately to dryness on a rotary evaporator. Precipitate peptide with ether and isolate by filtration.

If the yields are low, repeat the reaction with fresh reagents at 50°C.

Method 12: Cleavage with borohydride to give the peptide alcohol

This method is only applicable to TG and PEGA-type resins.

1. Treat the peptide resin (1.0 g) with 50% aq. EtOH, and allow to drain. Add NaBH₄ (126 mg) in 3 ml 50% aq. EtOH and gently agitate for 4 h.
2. Remove the resin by filtration and wash with 50% aq. EtOH (40 ml) to give a solution of peptide at pH~9.

References

Post-cleavage work-up

DO NOT DISCARD resin support or ether until peptide analysis is complete. Both can be stored under nitrogen or argon at 4°C to prevent oxidation.

7 Ether precipitation

Most cleavage protocols involve precipitation of the crude cleaved peptide using cold ethyl ether. The following are general procedures for post cleavage work-up.

Method 13: Post-cleavage work-up

Peptide isolation and work-up can be achieved by ether precipitation (1) or centrifugation (2). For water soluble peptides, the method in steps 3-6 can be used.

1. **Precipitation**: Filter the precipitated peptide through hardened filter paper in a Hirsch funnel under a light vacuum. Wash the precipitate further with cold ether, dissolve the peptide in a suitable aqueous buffer and lyophilize.

2. **Centrifugation**: Add a small volume of t-butyl methyl ether to the residue and triturate thoroughly until a free suspension is obtained. Transfer the suspension to a clean centrifuge tube, seal, and centrifuge. It is essential that a spark-free centrifuge is used for this process. Carefully decant the ether from the tube. Repeat the ether wash as necessary. Dissolve the residual solid in a suitable aqueous buffer and lyophilize.

3. **Water-soluble peptides**: After precipitation, add water to the residue and transfer mixture to a separating funnel. A little AcOH may be necessary to aid dissolution.

4. **Shake** the stoppered funnel well. Release the stopper and allow the two layers to separate by standing. Isolate the lower (aqueous) layer.

5. **Add** more water to the funnel and repeat step 4 three times. Remove the upper (ethereal) layer and store in a clean flask. Return the combined aqueous extracts to the separating funnel.

6. **Add** a small amount of fresh diethyl ether and repeat step 4 two or three times, each time removing the ethereal layer and returning the aqueous layer to the separating funnel. Collect the aqueous layer in a clean flask and lyophilize.

In the methods described above, the yield of peptide can often be increased if the TFA is first removed using a rotary evaporator (equipped with a CO2/acetone cold finger, oil pump and acid trap) prior to the ether precipitation step. In most cases, after adding the ether, the peptide will adhere to the sides of the reaction flask, enabling the scavengers to be quickly and easily removed by repeated ether washing. Note: cleavage mixtures containing TFMSA and HBF4 should not be evaporated to dryness.

Since peptides prepared using the low-high HF cleavage method may contain water soluble sulfonium derivatives, it is advisable to remove these immediately prior to lyophilization as, under neutral or slightly basic conditions, they may cause alkylation of methionine and cysteine residues.
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