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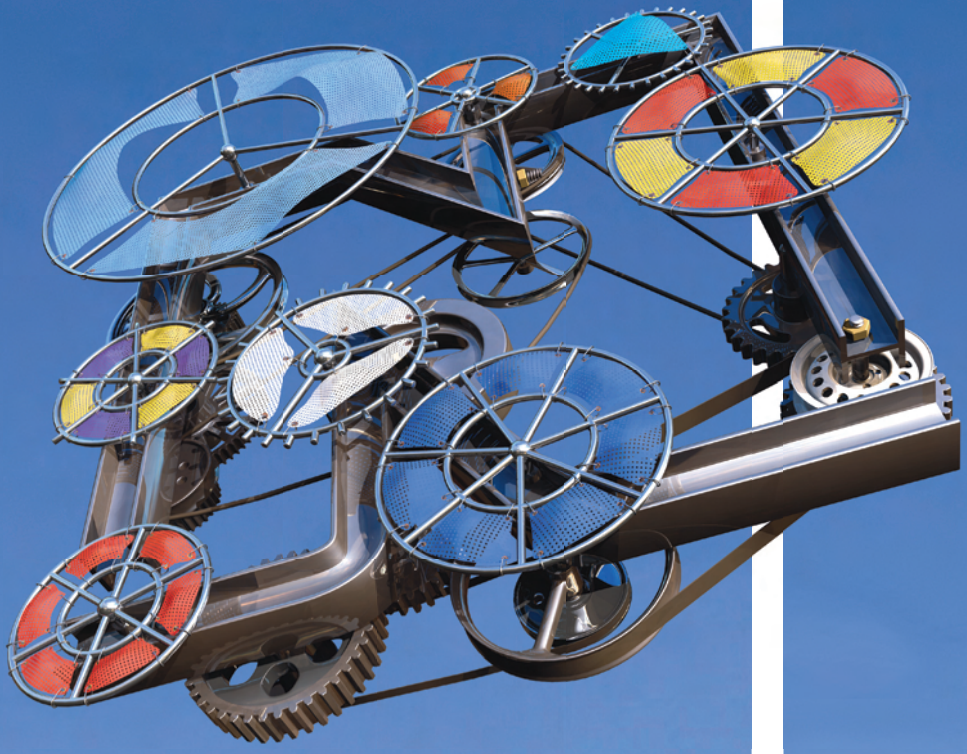
Boc resin cleavage and deprotection



Boc resin cleavage and deprotection

1. Introduction

The most popular reagent for cleavage of peptides from Boc-based resins is anhydrous HF. Of all the cleavage procedures HF appears to be the most versatile and least harmful to a wide variety of peptides synthesized on Boc-based resins. The major drawback of this procedure remains its highly toxic and reactive nature which necessitates the use of expensive HF-resistant fume hoods and cleavage apparatus.

Other strong acids such as TFMSA and trimethylsilyl trifluoromethanesulfonate (TMSOTf) can be used as alternatives to HF for cleavage from PAM and MBHA resins. Although less reactive than HF, it should be noted, however, that both TFMSA and TMSOTf are extremely corrosive and great care must be taken when using either.

In addition to acid cleavage, several resin types such as oxime can be cleaved using a variety of different methods to yield peptide hydrazides and analogs of protected fragments. Indeed, some resins such as brominated Wang and Br-PPOA resins are even cleavable by light. For the purposes of this discussion we will concentrate on the most common acid cleavage methods currently in use. Please refer to the literature cited with each resin for details on cleavage and deprotection specific for that resin type.

Table 1: Boc-compatible amino acid derivatives.

HF compatible

Arg(Mts)	Arg(NO_2)	Arg(Tos)	Asp(OBzl)
Asp(OcHx)	Cys(Acm)[1]	Cys(pMeBzl)	Cys(pMeOBzl)
Glu(OBzl)	Glu(OcHx)	His(Bom)	His(Dnp)
His(Tos)	His(Z)	Lys(2-Cl-Z)	Ser(Bzl)
Met(O)	Trp(For)[2]	Tyr(2-Br-Z)	

TMSOTf compatible

Asp(OcHp)	Asp(OBzl)	Arg(Mts)	Cys(Acm)[1]
Glu(OBzl)	Glu(OcHp)	His(Bom)	Lys(2-Cl-Z)
Met(O)[3]	Ser(Bzl)	Tyr(Bzl)	Tyr(2-Br-Z)
Tyr(Bzl)	Tyr(di-Cl-Bzl)	Trp(For)	Trp(Mts)

TFMSA compatible

Arg(Mts)	Asp(OBzl)	Cys(Acm)1	Cys(pMeOBzl)
Glu(OBzl)	His(Bom)	His(Dnp)	His(Tos)
His(Z)	Lys(2-Cl-Z)	Met(O)	Ser(Bzl)
Thr(Bzl)	Trp(For)[2]	Tyr(2-Br-Z)	

HBr compatible

Arg(Mts)	Asp(OBzl)	Cys(Acm)[1]	Cys(pMeBzl)
Glu(OBzl)	His(Bom)	His(Dnp)	His(Tos)
His(Z)	Lys(2-Cl-Z)	Met(O)	Ser(Bzl)
Thr(Bzl)	Trp(For)[2]	Tyr(2-Br-Z)	

1. Cys(Acm) is cleaved with cyclization by iodine or without cyclization by mercury (II) acetate.
2. Trp(For) should be deprotected using the low-high HF method. Alternatively, it should be treated with 10% piperidine in DMF (Method 2) before treatment with strong acid.
3. Met(O) is not quantitatively reduced by TMSOTf; Low-High HF or TFMSA are preferred.

2. Preparing the resin for cleavage

Proper preparation of the peptidyl resin prior to cleavage is important in preventing side reactions and incomplete cleavage and deprotection of the peptide. The choice of cleavage and deprotection method is dependent not only on the resin used, but also on the sequence and choice of amino acid side-chain protection. Before beginning your synthesis, please ensure that the resin and side-chain protection used is compatible with the cleavage method. All resins should be properly washed and dried before cleavage.

2. 1 Removal of the Dnp protecting group of His

If your peptide contains His(Dnp), the histidine must be deprotected before removal of the N-terminal Boc-group [1]. For specific details on the removal of the Dnp group of histidine and alternative histidine derivatives, please see Method 1 below.

Method 1: Removal of Dnp protecting groups

1. Swell the resin in the minimum volume of DMF.
2. Treat with a 20-fold molar excess of thiophenol for 1-2 h at rt (this reaction can be left o/n).
3. Transfer the resin to a sintered glass funnel and wash sequentially with DMF, methanol and diethyl ether before treating with liquid HF or TFMSA.

2.2 Removal of the N-Terminal Boc-group

Before HF cleavage can be performed the N-terminal Boc-group must be removed using TFA. This not only prevents possible *t*-butylation of susceptible residues during HF cleavage, but also removes any Boc-amino acid held on the resin by ion exchange [2]. Removal of the N-terminal Boc-group is not required for TFMSA cleavage. Check with the instruction manual of your synthesizer; many synthesizers automatically program the removal of the N-terminal Boc-group as a last step in the synthesis.

Manual removal of the N-terminal Boc-group can be accomplished by placing the resin in a round bottom flask and washing with 50% (v/v) TFA/DCM for 15 minutes at room temperature with constant mixing.

After removal of the N-terminal Boc-group the peptide resin can then be transferred to a sintered glass funnel and washed twice (5 volumes) with DCM, twice with MeOH and a final couple of washes with DCM. Avoid excessive sucking of air as moisture in the air will absorb onto the cold resin and may be difficult to remove. Moisture can be very harmful in HF cleavage [2].

After washing, the peptide resin should be dried under high vacuum for 4 hours, or preferably overnight over KOH or P_2O_5.

If you are using TFMSA cleavage without removal of the N-terminal Boc-group use the same washing and drying steps as above.

2.3 Deformylation of Trp(For)-containing peptide resins

The formyl group is stable to acid cleavage reagents and can be removed prior to cleavage using standard HF protocols (Method 2). Although alkylation of the unprotected indole ring of tryptophan by *t*-butyl carbonium ions is a problem during cleavage with HF, scavengers such as indole [2] have been used to successfully protect Trp-containing peptides from alkylation. However, indole can also undergo acid catalyzed dimerization with tryptophan and irreversibly modify the indole ring [3].

Method 2: Piperidine deformylation of Trp(For)-containing peptide resins

1. Place piperidine: DMF 1:10 (v/v) in a round bottom flask and cool with an ice bath to 0°C.
2. Add the peptide resin (1 g per 10 ml) and stir for 2 h maintaining the temperature at 0°C.
3. Filter the resin twice with DMF (5 volumes), twice with DCM and twice with MeOH.
4. Dry under high vacuum as above for at least 4 h prior to cleavage using HF.

Alternatively, the formyl-group may be removed by thiolytic cleavage using the "low-high" protocol (HF [4] or TFMSA [5]) with DMS, *p*-thiocresol or thiophenol. Under this protocol the low cleavage conditions remove most of the benzyl groups prior to the thiolytic cleavage of the Trp(For) under the high conditions. Removal of the *t*-butyl carbonium ions during the low cleavage greatly minimizes alkylation of the indole ring of tryptophan during the high procedure [4].

Another option, although possibly less desirable, involves the removal of the formyl group after high HF cleavage using aqueous base such as 0.03 M hydroxylamine at pH 9.0 for two hours [6].

3. HF cleavage

CAUTION: *Anhydrous HF is an extremely toxic, corrosive and volatile (b.p.19°C) liquid. All procedures requiring HF must be performed in HF-resistant apparatus in a fume hood equipped with a scrubber. DO NOT USE GLASS APPARATUS as HF will dissolve glass in an extremely rapid and exothermic reaction. Proper eye protection, face shield, rubber apron and rubber gloves are mandatory when using HF. Do not take chances. Follow local, state/provincial and federal safety and environmental codes and regulations. Breathing HF can cause death.*

For an excellent description of HF apparatuses and their use, please refer to Solid Phase Peptide Synthesis by Stewart & Young [2]. Always follow the information provided by the manufacturer of the HF cleavage apparatus you are using.

3.1 Standard HF cleavage

HF cleavage is generally performed at temperatures of 0-5°C for a period 30-60 minutes. Peptides containing Arg(Tos) may require longer cleavage times. Problems may be encountered if the peptide contains one or a combination of the problem residues such as Trp, Met, Asp, Glu, Cys and Tyr. Several strategies for dealing with these potential problems can be found in the 2006 Novabiovhem catalog, chapter 4, section 1, page 4.1.

Both time and temperature play a key role in minimizing possible side reactions during cleavage and deprotection. Peptide resins containing Asp/Glu (OBzl) or Asp/Glu (OcHx) should be cleaved at 5°C or lower to reduce aspartimide formation [7] and anisylation of the Glu respectively [8]. Lowering the temperature affects the rate of removal of the side-chain protecting groups. Cleavage of peptide resins containing Arg(Tos), Cys(pMeBzl) and Lys(2-Cl-Z) at temperatures lower than 5°C can be very slow and impractical. Peptide resins with Arg(Tos) could require cleavage times up to 2 hours at 5°C. When His(Dnp) and/or Trp(For) is used, pre-cleavage treatment is required.

Scavengers play a key role in reducing the possibility of side reactions. Anisole remains one of the most widely used scavengers for HF cleavage and prevents alkylation of tryptophan by t-butyl and benzyl cations. In combination with DMS and p-thiocresol, anisole will prevent alkylation of Met and Cys [9].

Avoid the use of thioanisole if your peptide contains tryptophan. Thioanisole cation adducts can alkylate the nitrogen of the indole ring of Trp.

Method 3: Standard HF cleavage

0.2 mmole scale

1. Place the peptide resin, a Teflon-coated stirring bar and the scavenger mixture into the reaction vessel. For peptides containing Cys most researchers use HF/anisole/DMS/*p*-thiocresol (10:1:1:0.2). For other peptides not containing Cys use HF/DMS/anisole (10:1:1).
2. Screw the cap on the reaction vessel and cool in a dry ice methanol bath for at least 5 min before proceeding with the cleavage.
3. Distil 10 ml of HF into the flask following the manufacturer's instruction (maintaining the temperature between -5°C and 0°C). For peptides containing Arg(Tos) the reaction will require up to 2 h.
4. At the end of the reaction time, evaporate the HF and DMS under a stream of N_2.
5. Extract the resin with TFA to remove the peptide from the resin matrix.
6. 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.
7. Isolate the peptide as described in Method 8.

3.2 Low-high HF procedure

The low-high procedure of Tam, *et. al.* [4] uses low concentrations of HF in a large amount of scavenger such as DMS (1:3 by volume). Under low conditions the cleavage mechanism changes from the usual S_N1 (where carbonium and nitronium ions are produced) to S_N2. This procedure prevents alkylation of tyrosine by benzyl and *t*-butyl cations, formation of succinimide peptides from Asp-Gly sequences and acylation of scavenger molecules by glutamyl side chains. At low

concentrations of HF and in the presence of DMS, Met(O) will be reduced to Met.
If the low procedure is followed by a standard HF cleavage, Arg(Tos) and Arg(NO_2) will be deprotected. The low procedure alone may not be sufficient to cleave peptides from the BHA resins. The major drawbacks of this procedure are the additional time required for cleavage, the use of large quantities of DMS and the formation of thiol scavenger adducts which have very strong and offensive odors.

Merrifield resins

For Merrifield-based resins, Tam, *et al.* [4] recommend cleavage using HF/DMS/*p*-cresol, 25:65:10, v/v at 0°C for 2 hours. If the peptide contains Arg(Tos) or other functionalities stable to low HF they recommend first cleaving with HF/DMS/*p*-cresol 25:65:10, v/v for 2 hours at 0°C, followed by the high procedure, which involves evaporating all the HF and DMS *in vacuo* at 0°C and then recharging the vessel with anhydrous HF and cleaving for 30-60 minutes at 0°C to 10°C depending on the sequence.
If Trp(For) is present in the peptide, the formyl group can be removed under low HF by replacing the *p*-cresol with *p*-thiocresol or thiophenol [4].

PAM and MBHA resins

For peptides synthesized on PAM or MBHA resins Tam, *et al.* [4] recommend treatment under the low conditions for 1-2 hours to deprotect the side chains. After deprotection, evaporate the HF and DMS and wash the resin with DCM or EtOAc to remove sulfonium salts, dimethylsulfoxide and thiol derivatives and suction dry. The peptide can then be cleaved using HF/*p*-cresol 9:1(v/v) for 30-60 minutes at 0°C which will remove all remaining protecting groups and cleave the peptide from the resin.

4. TFMSA cleavage

CAUTION: *TFMSA 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.*
TFMSA is an alternative to HF cleavage. The main advantage of this procedure is that it can be performed using standard laboratory glassware. Unlike HF, TFMSA is not volatile and is therefore difficult to remove by evaporation [2]. The peptide must be precipitated from solution using a dry solvent such as ethyl ether.
TFMSA-cleaved peptides are susceptible to salt and scavenger association. The precipitated peptides [9] should be neutralized and the salts removed either by ion exchange or by using Sephadex columns before further purification.
Similar to HF, TFMSA can be used either with a standard protocol or a "low-high" protocol depending on the sequence. Please note that this reagent will not deprotect Arg(NO_2) or Arg(Tos) groups, if present [10]. His(Dnp) requires pre-cleavage treatment.

4.1 Standard TFMSA cleavage

The reaction time for PAM resins is typically between 30-60 minutes at room temperature. MBHA resins will require cleavage times between 90 minutes and two hours at room temperature (Method 4).
Asp(OcHx) and Cys(MeBzl) groups are not efficiently removed by this procedure. Peptide resins containing Trp(For) should be cleaved using the low-high procedure with EDT (Method 5).

Method 4: Standard TFMSA cleavage

250 mg scale

1. Place 250 mg dried resin in a round bottom flask with a stirring bar. Add 750 µl of thioanisole/EDT (2:1).
2. Chill the round bottom flask in an ice bath and add 5 ml of TFA and stir for 5-10 min.
3. Add (slowly) 500 µl of TFMSA drop-wise with vigorous stirring to dissipate the heat generated.
4. Allow the reaction to continue at rt for the desired length of time.
5. 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.
6. Isolate the peptide as described in Method 8.

4.2 TFMSA low-high cleavage

The low-high procedure initially uses low concentration of TFMSA in a large volume of the scavenger DMS. Under low conditions the cleavage mechanism changes from S_N1 (where carbonium and nitronium ions are produced) to S_N2. If your peptides contain Met, use the Met(O) derivative for your synthesis. Under low conditions in the presence of DMS the Met(O) will be reduced to Met [11]. The addition of EDT to the cleavage cocktail under the high conditions will deprotect Trp(For) *via* thiolytic cleavage [5]. EDT will also minimize the formation of dimers via disulfide bridges when the peptide contains Cys residues.
In the low phase of the low-high cleavage protocol it is important to maintain low temperatures of 0°C to 5°C during the entire reaction.

Method 5: Low-high TFMSA cleavage [9]

Low cleavage

1. Place the peptide resin (250 mg) in a round bottom flask with a stirring bar and cool in an ice bath to between 5°C and 0°C.
2. Add 250 µl of m-cresol, 750 µl DMS, and 1.25 ml of TFA, add 250 µl of TFMSA drop-wise to the reaction mixture with stirring to dissipate any heat produced.
3. For peptides containing Trp(For) add 50 µl of EDT to the cleavage cocktail.
4. Allow the mixture to react for 3 h, keeping the temperature between 0°C and 5°C.
5. Transfer the contents of the flask to a medium sintered glass funnel and wash the resin with several volumes of Et_2O and suction dry.
6. Dry the resin under high vacuum for a minimum of 4 h over P_2O_5 or KOH.

High cleavage

1. Place the dried resin from above in a round bottom flask with a stirring bar and add 250 µl of thioanisole and 75 µl of EDT and stir for 5-10 min.
2. Cool to 5°C to 0°C using an ice bath and add 2.5 ml of TFA. Mix 5-10 min followed by addition of 250 µl of TFMSA drop-wise with mixing to dissipate heat.
3. Remove the flask from the ice bath and allow to react at rt for 30 min with PAM resins. MBHA resins require 90-120 min for complete cleavage.
4. 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.
5. Isolate the peptide as described in Method 8.

5. TMSOTf Cleavage

CAUTION: *TMSOTf is an extremely corrosive and flammable liquid. 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 an efficient fume hood. The vapor is harmful if inhaled.*

TMSOTf represents an alternative to HF and TFMSA cleavage. Yajima, *et al.* [12] reported that TMSOTf cleavage produces fewer side reactions and yields a product which is less hygroscopic than peptide products from TFMSA cleavage. This procedure cleaves most of the side-chain protecting groups found in Boc synthesis. Peptides containing Cys(Bzl), Cys(Acm) and Arg(NO_2) should not be deprotected using TMSOTf since these side-chain protecting groups are stable to TMSOTf.

Peptides containing Arg(Tos) will require longer cleavage times. Unlike TFMSA, the Met(O) will not be quantitatively reduced to Met and therefore an alternative method for the reduction of Met(O) to Met is necessary [13], 2006 Novabiochem catalog, section 7.6, page 3.29. Peptides containing Trp(For) require the presence of EDT in the cleavage cocktail in order to fully remove the formyl group.

Method 6: TMSOTf cleavage

1. Place 1 g of dried resin in a round bottom flask containing a stirring bar.
2. Cool the flask to 0°C using an ice bath and add the cooled cleavage mixture to the flask (1.95 ml TMSOTf, 6.90 ml of TFA, 1.2 ml m-cresol). Maintain the temperature at 0°C for 2h with constant mixing to ensure complete deprotection.
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 8.

A new two-step hard acid deprotection/cleavage procedure for solid phase peptide synthesis involving TMSBr/TFA followed by TMSOTf/TFA has been reported by Nomizu, *et al.* [14]. Its usefulness is demonstrated by comparison with other deprotection methods in the synthesis of two different test peptides. Readers are encouraged to refer to this paper for further details.

6. Hydrogen bromide cleavage

CAUTION: *HBr in acids such as TFA, pivalic acid, isobutyric acid, isovaleric acid and acetic acid are extremely strong acids. 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 an efficient fume hood. The vapor is harmful if inhaled.*

This procedure [15] is used for cleaving peptides from MBHA-resin. The advantages of this method are its use of commercially available 30% HBr in acetic acid (about 5 M), the ability to use standard laboratory glassware and the potential for easy scale up. The cleavage mixture should contain pentamethylbenzene which accelerates the cleavage and the acidolytic removal of the protecting groups. Experiments also demonstrated that Met(O) is quantitatively reduced to Met during the HBr procedure but dimethylsulfide has to be used instead of thioanisole to prevent formation of S-methyl-methionyl peptides.

6.1 Standard HBr/acetic acid cleavage

The reaction time for MBHA-resin is typically between 60 and 90 minutes at room temperature. Boc and formyl protecting groups should be removed prior to the cleavage procedure. The procedure is compatible with Asp(OBzl), Glu(OBzl) and Lys(ClZ) protection (Method 7). Please note that the peptide resin should be washed and well dried before cleavage.

Method 7: HBr cleavage

250 mg scale

1. Place 250 mg dried resin in a round bottom flask with a magnetic stirring bar and mix with 500 µl pentamethylbenzene, 600 µl thioanisole and 10 ml TFA.
2. To this suspension add 400 µl 30% HBr/acetic acid (5 M) and stir for 60-90 min.
3. Remove the resin by filtration and evaporate the filtrate under reduced pressure at 30°C after completion of the reaction.
4. Triturate the peptide with 50 ml dry ether and isolate as described in Method 8.

7. 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.

7.1 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 8: 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 CO_2/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 HBF_4 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.

NOTE: In all the above protocols, diethyl ether can be replaced by *t*-butyl-methyl ether.

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