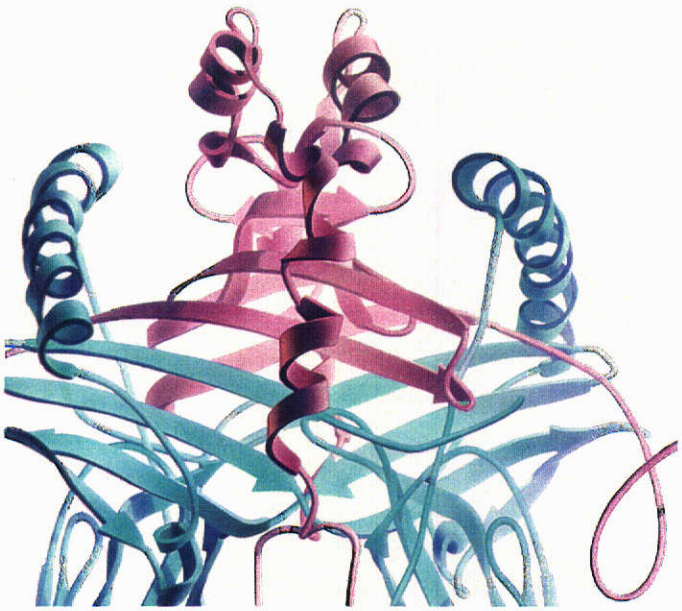
Introduction to Cleavage Techniques

2nd Edition



Strategies in Peptide Synthesis

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Introduction to Cleavage Techniques

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Introduction

This guide presents an introduction to cleavage techniques used with Fmoc- and Boc-derived peptides. The most common cleavage methods, and the ones we present in this booklet, use acid to remove both the peptide chain from the solid support and the side-chain protecting groups from the amino acids. Reaction time and temperature, the choice of side-chain protected amino acids and scavengers are all variables that can affect your final peptide product.

Some of the techniques discussed here may influence your synthesis strategy so we recommend that you refer to this guide before beginning chain assembly of your peptide. Flow charts at the beginning of both the Fmoc and Boc sections of this booklet direct you to the procedures best suited for your peptide. Cleavage and deprotection procedures are described on the pages following the flow chart.

Next, we discuss potential problems to keep in mind when choosing side-chain protected amino acids. At each step of the process of peptide assembly, the side-chain protecting group must be stable to the reagent used to remove the α-protecting group, yet it must also be easily removed by your cleavage cocktail. We provide a list of compatible side-chain protected amino acids for both Fmoc and Boc strategies.

When side-chain protecting groups are cleaved by acidolysis, the liberated carbonium ions that result can attack certain residues in the peptide. Scavengers trap carbonium ions and thereby prevent them from undergoing further reactions. We discuss the advantages and disadvantages of a number of possible scavenger mixtures and how reaction time and temperature can dramatically affect the purity of your peptide. You may consult the list of references at the end of this booklet for sources of further information.

This guide is only a starting point. Many peptide chemists have their own personal recipes; scavenger mixtures are as numerous and diverse as the chemists who invent them. We hope you find this guide to be a valuable reference that provides a foundation for your own explorations and discoveries in cleavage chemistry.

Chemical Abbreviations

Acm	acetamidomethyl
Boc	t-butyloxycarbonyl
Bom	benzyloxymethyl
Br-Z	2-bromobenzyloxycarbonyl
Bzl	benzyl
CH_3CN	acetonitrile
CHO	formyl
ClZ	2-chlorobenzyloxycarbonyl
Cl_2Bzl	dichlorobenzyl
DCM	dichloromethane
D.I. H_2O	deionized water
DMF	dimethylformamide
DMS	dimethyl sulfide
Dnp	2,4-dinitrophenyl
EDT	1,2-ethanedithiol
EMS	ethyl methyl sulfide
Et_2O	diethyl ether
Fmoc	9-fluorenylmethyloxycarbonyl
HCOOH	formic acid
HF	hydrofluoric acid
HMP resin	p-hydroxymethylphenoxymethyl-polystyrene resin; p-alkoxybenzyl alcohol resin; or Wang resin
HOAc	acetic acid
HOBt	1-hydroxybenzotriazole
Mbh	dimethoxybenzhydryl
MBHA resin	4-methylbenzhydrylamine resin
MBS	p-methylbenzenesulfonyl
Mbzl	4-methoxybenzyl
MeBzl	4-methyl benzyl
Met(O)	methionine sulfoxide
MeOH	methanol
MMA	N-methylmercaptoacetamide
MTBE	methyl t-butyl ether
Mtr	4-methoxy-2,3,6-trimethylbenzene sulfonyl
Mts	mesitylene-2-sulfonyl
NH_4OH	ammonium hydroxide
NH_4HCO_3	ammonium bicarbonate
NMP	N-methylpyrrolidone, N-methyl-2-pyrrolidone
OBzl	benzyl ester

OcHex	cyclohexyl ester
OtBu	tert-butyl ester
Pam resin	phenylacetamidomethyl resin
Pmc	2,2,5,7,8-pentamethyl-chroman-6-sulfonyl
S-tBu	sulfur-tert-butyl
t-Bu	tert-butyl
TFA	trifluoroacetic acid
TFMSA	trifluoromethane sulfonic acid
Tmob	2,4,6-trimethoxybenzyl
TMSBr	trimethylsilyl bromide
TMSOTf	trimethylsilyl trifluoromethanesulfonate
Tos	4-toluenesulfonyl (tosyl)
Trt	trityl
Z	benzyloxycarbonyl

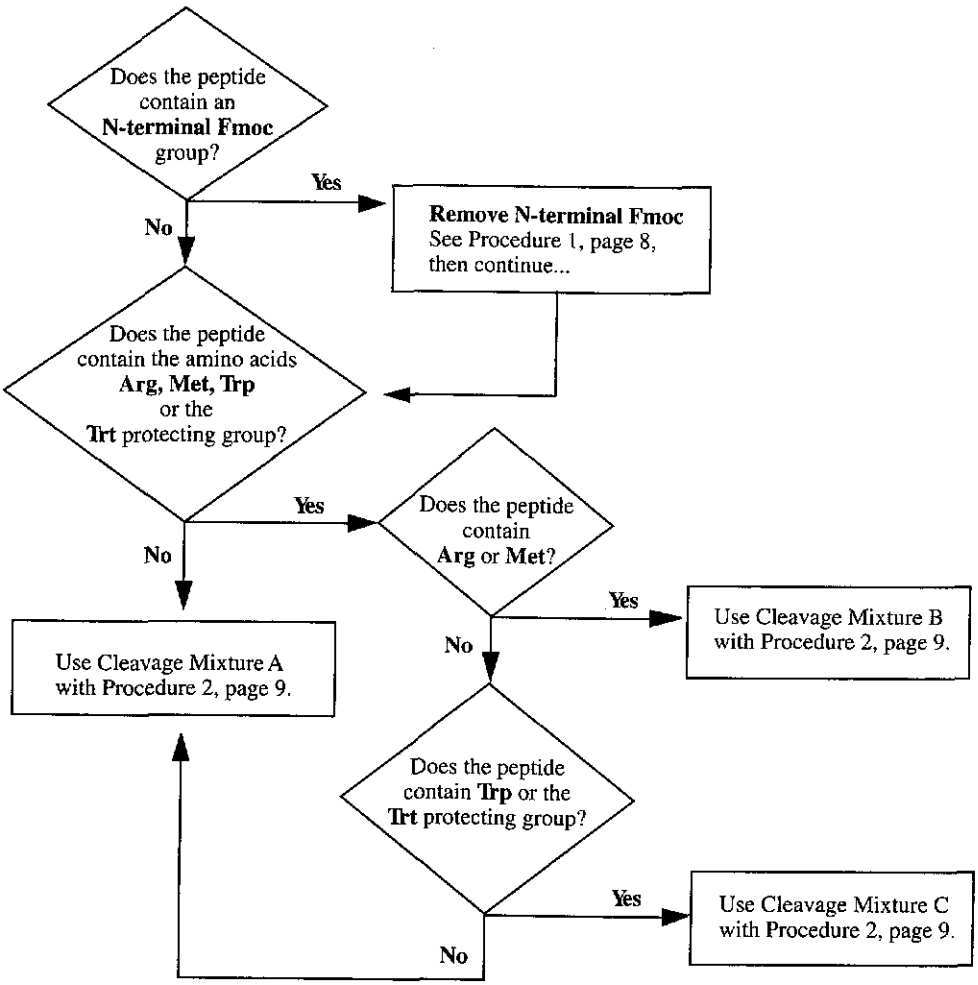
Reagent Warnings

WARNING **Some chemicals used in the following procedures are hazardous. Hazards are displayed prominently on the labels of bottles and cylinders. In addition, Material Safety Data Sheets (MSDSs) are provided by Perkin-Elmer or by the manufacturer or distributor of those chemicals. MSDSs give information on physical characteristics, hazards, precautions, first aid, spill clean-up and disposal procedures. Familiarize yourself with the information and precautions in the MSDSs before attempting to store, use or dispose of the reagents. Additional copies of each MSDS are available from Perkin-Elmer or from the chemical manufacturer or distributor.**

WARNING **Some chemical waste from these procedures may be hazardous and require special handling. Handle and dispose of waste carefully. Wear the appropriate protective apparel, gloves and eye protection when handling waste. Avoid inhalation and skin contact. Always handle waste in an area with adequate ventilation. Place the waste in an appropriately labelled container. Dispose of waste in accordance with all applicable local, state and federal government health, safety and environmental regulations.**

WARNING **All cleavage procedures require the use of extremely dangerous, corrosive, or foul-smelling chemicals. Perform these procedures in a well-ventilated, properly functioning fume hood. Wear the appropriate protective apparel, gloves and eye protection when performing cleavage procedures.**

Fmoc Cleavage Flow Chart



Note *See page18 for Fmoc cleavage with TMSBr/Thioanisole/TFA, a procedure that uses a stronger acid to remove side protecting groups, such as Mtr, that are more difficult to remove.*
See page 48 for deprotection of Cys(Acm) and Cys(t-Bu).

Fmoc-Compatible Amino Acid Derivatives

Note *The following amino acid side-chain protecting groups may be used with TFA or TMSBr cleavage protocols, although no derivative is compatible with every synthesis protocol. Select amino acid derivatives carefully.*

*	Arg(Mtr)	Gln(Mtt)	Ser(tBu)
†	Arg(Pmc)	Gln(Tmob)	Thr(tBu)
		Gln(Trt)	Trp(Boc)
	Asn(Mtt)	Glu(OtBu)	Tyr(tBu)
	Asn(Tmob)		
	Asn(Trt)	His(Boc)	
	Asp(OtBu)	His(Trt)	
**	Cys(Trt)	Lys(Boc)	

* When using Arg(Mtr), extend cleavage times to 3 or more hours with TFA cleavages. With TMSBr cleavages, less time is required.

† When using 5 or more Arg(Pmc), cleavage may require up to 3 hours with TFA cleavages. With TMSBr cleavages, less time is required.

** The use of Cys side-chain protecting groups other than Cys(Trt), may yield inferior products under TFA conditions. Cys(Acm) and Cys(t-Bu) require additional deprotection conditions (see page 48).

Remove the Fmoc protecting group from the N-terminus BEFORE you proceed with any cleavage.

Automated removal of the N-terminal Fmoc group

ABI 430A Chemistry	End Cycle
NMP-HOBt	**rdefmoc** (**rdefmoc r** if resin sampling)

ABI 431A & 433A Chemistry	Modules
FastMoc™	BIDc
Fmoc/HOBt/DCC	bdc

Manual removal of the N-terminal Fmoc group

Recommended Equipment and Chemicals:

round-bottom flask	piperidine
magnetic stir bar	NMP
stir plate	DMF
fritted glass funnel (medium porosity)	DCM
vacuum desiccator	MeOH
vacuum pump	

1. Add the peptide-resin to a round-bottom flask that contains a magnetic stir bar.
2. Add enough 20% (v/v) piperidine in NMP (or DMF) to allow the resin to stir well—approximately 10 mL per gram peptide-resin. Stir at room temperature for 20 to 30 minutes.
3. Filter the resin through the fritted glass funnel. Wash well, first with NMP or DMF several times and then with DCM/MeOH (3:2) several times. Use DCM for the final wash.
4. Dry the peptide-resin in a vacuum desiccator at least 3 hours or overnight, if possible.

The peptide-resin is now ready for cleavage.

Procedure 2: TFA Cleavage Procedure (1 g scale)

WARNING **TFA is an extremely dangerous and corrosive liquid. Perform this procedure in an efficient, properly functioning fume hood. Wear appropriate eye protection, lab coat and gloves.**

> ***Remove the Fmoc group from the peptide-resin* BEFORE *beginning this procedure. (See Procedure 1, preceding page)***

In the first four steps of the TFA cleavage procedure presented here, the peptide is deprotected and removed from the resin support. Following step 4, you may use either filtration (method **I**), centrifugation (method **II**), or diethyl ether extraction (method **III**) to isolate the peptide from the TFA solution.

The following procedures are designed for cleavage of 0.1 to 1.5 g of peptide-resin. We strongly suggest that you first use only 10-15 mg of peptide-resin to perform a small-scale trial run with any of these procedures. Analyze the crude material that results from the small-scale trial run to determine the optimum cleavage conditions for your peptide, then apply them to the remaining peptide-resin.

Cleavage mixtures (for 0.1-1.5 g peptide-resin):

A:	**B[1]:**	**C:**
0.5 mL D.I. H_2O	0.75 g crystalline phenol	0.25 mL EDT
9.5 mL TFA	0.25 mL EDT	0.25 mL D.I. H_2O
	0.5 mL thioanisole	9.5 mL TFA
	0.5 mL D.I. H_2O	
	10 mL TFA	

Note *When the peptide has an N-terminal glutamic acid, omit the water in the cleavage mixture, to prevent the conversion of the N-terminal glutamic acid to a pyroglutamyl residue.*

Cleavage steps

Recommended Equipment and Chemicals:

round-bottom flask (10-50 mL) with stopper
micro stir bar
stir plate
ice bath
micropipettor
pipet tips
appropriate cleavage mixture

TFA

1. Prepare the appropriate cleavage mixture—either A, B, or C—as described on page 9 and indicated by the Fmoc Cleavage Flow Chart on page 6.

Note *Cleavage mixture B has been used successfully with peptides containing up to four Arg(Pmc). With five or more Arg(Pmc), more deprotection time may be required. However, do not extend this reaction to more than three hours.*

2. Place the dried peptide-resin in a round-bottom flask that contains a micro stir bar. Cool the flask in an ice bath.
3. Cool the cleavage mixture in an ice bath, then add the volume specified in Table 1 to the cooled peptide-resin. After the cleavage mixture has been added, remove the flask from the ice bath and allow it to warm to room temperature.

Table 1. Volume of Cleavage Mixture Added by Peptide-Resin Weight

Peptide-Resin (mg)	Cleavage Mixture (mL)
100-250	1.5
250-500	2.5
500-1000	6.0
1000-1500	10.0

4. Stopper the flask and stir the reaction mixture at room temperature for 1.5 hours. To isolate the peptide from the TFA solution, continue with either method I, method II, or method III.

I: Preliminary peptide isolation by filtration

Recommended Equipment and Chemicals:

round-bottom flask (10-50 mL) with stopper
2 fritted glass funnels (medium and fine porosities)
vacuum filtration flask or disposable filter tubes
rotary evaporator
ice bath
vacuum desiccator
vacuum pump

cold Et_2O
DCM

5. After the reaction time has elapsed, vacuum filter the reaction mixture through a medium-porosity, fritted glass funnel into another round-bottom flask to separate the resin support from the peptide solution.

   For small-scale cleavages, you may use disposable filter tubes to remove the solid support from the peptide solution.

6. Add approximately 1 mL TFA to the reaction flask to wash any remaining resin. Filter this mixture through the funnel arrangement in step 5.
7. Rinse the flask and funnel with 5–10 mL DCM. Allow the DCM wash to combine with the TFA filtrate.
8. If necessary, concentrate the filtrate on a rotary evaporator to a volume of approximately 1 to 2 mL. Maintain the temperature of the water bath below 40 °C to prevent damage to the peptide that may be caused by heat.
9. Add 50 mL (or more) cold Et_2O to the concentrated reaction mixture to precipitate the peptide.
10. Collect the peptide by filtering the mixture through a fine-porosity, fritted glass funnel. The precipitate may be dried by putting the funnel in a desiccator under high vacuum.
11. After drying, the peptide may be dissolved in an aqueous solvent for lyophilization. Refer to page 52 to determine the appropriate solvent.

Note *For complete removal of the Boc side-chain protecting group from Trp(Boc), let the dissolved peptide sit in the aqueous solution at room temperature for one hour prior to lyophilization.*

II: Preliminary peptide isolation by centrifugation

Recommended Equipment and Chemicals:

round-bottom flask (10–50 mL) with stopper	MTBE
vacuum filtration flask	HOAc
micropipettor	DCM
pipet tips	
ice bath	
vacuum desiccator	
vacuum pump	
centrifuge tubes (40–50 mL size) with Teflon™-lined screw caps	
bench-top centrifuge, capable of at least 2000 x g	

5. Vacuum filter the TFA solution through the medium-porosity filter directly into 30 mL (or more) cold MTBE.
6. Add approximately 1 mL TFA to the reaction flask to wash any remaining resin. Filter this mixture through the funnel arrangement into the cold MTBE suspension from the previous step.
7. Transfer the MTBE suspension to a centrifuge tube. Tightly screw on a Teflon-lined cap and centrifuge for 10 minutes at 2000 x g at room temperature.
8. Discard the supernatant by aspiration and resuspend the precipitate in 30 mL MTBE. Centrifuge again, as described in step 7.
9. Repeat step 8 at least 2 more times.
10. The peptide may now be dissolved in an aqueous solvent for lyophilization. Refer to page 52 to determine the appropriate solvent.

Note *For complete removal of the Boc side-chain protecting group from Trp(Boc), let the dissolved peptide sit in the aqueous solution at room temperature for one hour prior to lyophilization.*

III: Preliminary peptide isolation by extraction[2]

In this isolation method, the peptide dissolves in water, while the scavengers go into an ether layer.

Recommended Equipment and Chemicals:

fritted glass funnel, medium porosity	D.I. H_2O
round-bottom flask (10–50 mL) with stopper	cold Et_2O
rotary evaporator	DCM
pasteur pipet	
separatory funnel	

5. After the reaction time has elapsed, vacuum filter the reaction mixture through a medium-porosity, fritted glass funnel into another round-bottom flask to separate the peptide solution from the resin support.
6. Add approximately 1 mL TFA to the reaction flask to wash any remaining resin. Filter this mixture through the funnel arrangement in step 5.
7. Rinse the flask and funnel with 5–10 mL DCM, allowing the DCM wash to combine with the TFA filtrate.
8. Concentrate the filtrate on a rotary evaporator to a volume of approximately 1 to 2 mL. Maintain the temperature of the water bath below 40 °C to prevent damage to the peptide that may be caused by heat.
9. Dilute the residual solution in the flask with a tenfold excess of D.I. H_2O. Extract the scavengers with an equivalent amount of cold Et_2O. Use a separatory funnel to remove the Et_2O layer. Repeat the extraction at least two more times with the same volume of cold Et_2O. Save the Et_2O until the peptide has been isolated.

Note *For small-scale extractions, add cold Et_2O directly to the flask. Stopper and shake the flask to extract the scavengers from solution. Remove the Et_2O with a pasteur pipet.*

10. The peptide may now be dissolved in an aqueous solvent for lyophilization. Refer to page 52 to determine the appropriate solvent.

Note *For complete removal of the Boc side-chain protecting group from Trp(Boc), let the dissolved peptide sit in the aqueous solution at room temperature for one hour prior to lyophilization.*

TFA Cleavage Recommendations[3]

Fmoc solid-phase synthesis uses TFA for cleavage and side-chain deprotection. TFA is a relatively weak acid when compared with HF and TFMSA used in Boc synthesis strategies and is therefore considered to be a desirable alternative to those acids.

TFA Cleavage Scavengers

When peptide residues are protected by either Boc or t-butyl groups, cleavage of those peptides produces t-butyl cations and t-butyl trifluoroacetate, which can bring about t-butylation of Trp, Tyr, and Met, an undesirable side-reaction. The use of cleavage scavengers can minimize t-butylation.

Scavengers are reagents that trap cations and thereby prevent them from undergoing further reactions. EDT has been shown to be the most efficient scavenger for t-butyl trifluoroacetate, when compared to anisole, phenol, ethyl methyl sulfide, thioanisole, 2-mercaptoethanol, thiophenol, or the amino acids Trp and Met when they are used as scavengers.[4] However, EDT alone does not completely prevent Trp butylation; a second scavenger is necessary to suppress alkylation. A mixture of TFA/$H_2$0 (95:5) works best when there are only t-butyl or Boc side-chain protecting groups and Trp is not present.

Arginine

The Pmc group is preferred for side-chain protection of arginine during Fmoc peptide synthesis, especially in sequences containing multiple Arg residues.[5] Its lability in TFA is similar to that of both the t-butyl derivatives and the trityl protections for cysteine and histidine. Thioanisole accelerates Arg(Pmc) deprotection by TFA.

On deprotection with anhydrous TFA, the Pmc group may be transferred to the side-chain of tryptophan or tyrosine, and form sulfonated compounds. Large amounts of scavenger are necessary for optimal suppression of Pmc substitution products in the presence of tryptophan.[6] Most of these by-products can be avoided by the addition of water, which has proven to be an essential scavenger.[1,6]

When a peptide contains both Trp and Arg(Pmc) residues, use the more effective Cleavage Mixture B (listed in Procedure 2, page 9) to remove the Pmc group and minimize side reactions. It is composed of 82.5% TFA/ 5% phenol/5% thioanisole/ 2.5% 1,2-ethanedithiol/5% $H_2$0. Figure 1 shows HPLC elution profiles of a peptide after cleavage with this mixture and three other cleavage mixtures.

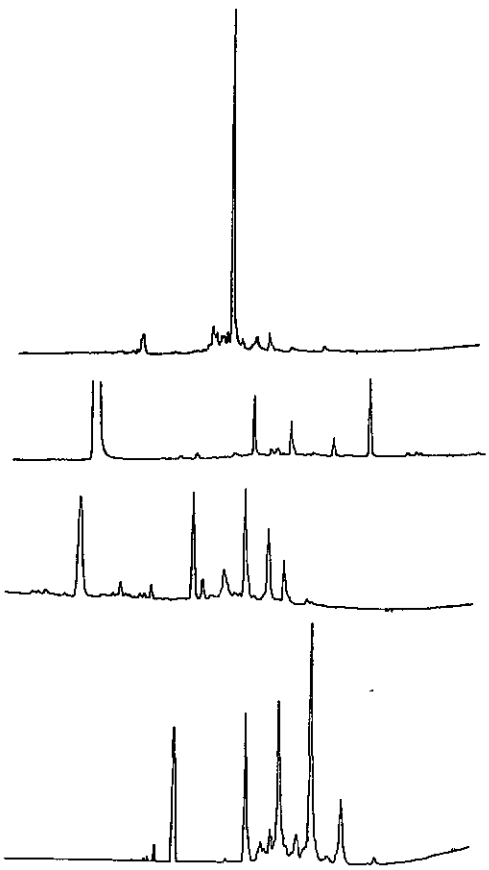


Figure 1a. Cleavage mixture B = 82.5% TFA/5% phenol/5% H_2O/ 5% thioanisole/ 2.5% ethanedithiol

Figure 1b. 95% TFA/3% anisole/ 1% EMS/1% EDT

Figure 1c. 92.5% TFA/5% H_2O/ 2.5% EMS/1 mg/mL indole

Figure 1d. 91.5% TFA/5% H_2O/ 2.5% thioanisole/ 1% dimethylaminophenol

Figure 1. Analytical HPLC elution profile of Fmoc-Cys-Pro-Asp-Phe-Gly-His-Ile-Ala-Met-Glu-Leu-Ser-Val-Arg-Thr-Trp-Lys-Tyr after cleavage with different cleavage mixtures.

HPLC conditions: Aquapore RP-300 C-8 reverse phase column (2.1 x 100 mm, 7 μ particle size, 300 Å pore size) with detection at 220 nm. Samples were eluted with a linear gradient of 0-100% B in 60 minutes, where A was 0.1% aqueous TFA and B was 0.08% TFA/CH_3CN.

Tryptophan

Trp alkylation occurs with Arg(Pmc) although to a lesser extent than the alkylation that occurs with Arg(Mtr) when identical scavenger combinations are used.[5,6,7] In the presence of Trp, both Mtr and Pmc can alkylate the indole ring of Trp at the 2' position and hinder deprotection. This alkylation can be minimized by the use of Fmoc-Trp(Boc) during the chain assembly of the peptide. Trp can also be modified by the Fmoc Asn/Gln protecting groups Tmob and Mbh. Cleavage Mixture B has been shown to prevent these modifications.[1]

TFA cleavage of carboxyl-terminal Trp from the p-alkoxybenzyl ester linker may result in indole modification. During cleavage, a resin-bound benzyl cation species is generated that irreversibly alkylates the indole ring. The same scavengers previously suggested for t-butyl cations and t-butyl trifluoroacetate suppression also minimize this alkylation reaction.

Methionine

Methionine sulfoxide formation can be prevented by scavengers such as EMS or thioanisole, however, thioanisole can partially remove the non-acid-labile protecting groups of Cys (t-Bu, S-t-Bu) and should not be used in their presence. N-methylmercaptoacetamide can reverse methionine sulfoxide formation (see page 47). Lyophilization reverses t-butyl modifications of methionine-containing peptides.[8] Other modifications, such as those found with Trt, Mtr or Pmc groups, have not been thoroughly investigated.

Cysteine

TFA alone does not remove the tBu or Acm protecting groups on Cys. Thallium (III) trifluoroacetate in combination with TFA has been used as a weak acid for deprotection of Cys side chains.[9] The Trt group is easily removed during acid cleavage with 95% TFA, but it tends to reattach to the free sulfhydryl if a thiol scavenger is not present. We recommend EDT as the thiol scavenger of choice, although thioanisole or thiophenol may also be used. When thioanisole is used, Cys(t-Bu) and Cys(S-tBu) may be modified during TFA cleavage. The use of a scavenger mixture that contains phenol has been reported to prevent Cys modification in some cases.[3]

The Acm group can be used with both Fmoc and Boc chemistry although additional cleavage steps are required to remove it. However, when constructing multiple disulfide bridges, Cys(Acm) is particularly useful for differential side-chain protection of multiple cysteines. Mercury (II) acetate deprotects both Cys(Acm) and Cys(t-Bu) (see page 48). In the case of the peptide Somatostatin, it was found that a minimum of five hours is necessary to remove the t-Bu group on Cys when using mercury (II) acetate for

deprotection.[10] The same report found that TFMSA deprotected 80% of Cys(t-Bu). After deprotection with mercury (II) acetate, it may be difficult to completely remove mercuric ions from the peptide.

Iodine also deprotects Cys(Acm), but according to the earlier cited study of Somatostatin, even after several days exposure to Cys(tBu) it does not effectively remove the t-Bu group.[10] An important side reaction of iodine deprotection is the iodination of other side chains, such as the indole ring of tryptophan[10] or the benzyl ring on tyrosine.

TFA Cleavage: Temperature and Time

Cleavage and side-chain deprotection of peptides synthesized by the Fmoc strategy is typically performed at room temperature (22 °C). A lower cleavage temperature (-20 °C) can be used to suppress side-reactions, but as a consequence of the low temperature, the desired cleavage reactions will also be significantly slower. Scavengers offer a more beneficial way to minimize side-products than reduced temperatures.

In general, most Fmoc compatible side-chain protecting groups are removed in 95% aqueous TFA at room temperature, and in 1.5 to 2.5 hours. However, Mtr is a notable exception. Deprotection and cleavage of Arg(Mtr) requires 3 or more hours, which is more time than that required with other acid-labile, side-chain protecting groups. Peptides with multiple Arg(Mtr) groups may undergo cleavage for 6 hours and still have attached Mtr groups. TMSBr/thioanisole/TFA is an alternate cleavage mixture (see page 18) that can remove Mtr more effectively.

Procedure 3: TMSBr/Thioanisole/TFA Cleavage

WARNING **TMSBr is corrosive and flammable. Extreme care must be taken when using it. Always wear protective equipment, eyewear, lab coat and gloves when handling TMSBr and work in a well-ventilated fume hood.**

An efficient fume hood has a minimum face velocity of 0.50 meters/second (100 linear feet/minute) as the average velocity of the open area. This should be measured by individuals knowledgeable in industrial hygiene or air flow monitoring. TMSBr exhaust may require additional treatment as mandated by local, state or federal regulatory agencies.

Yajima and coworkers[11] have reported that an Fmoc-synthesized peptide-resin can be cleaved and deprotected with 1M TMSBr/TFA in the presence of thioanisole, m-cresol, and EDT for 60 minutes at 0 °C. The protecting groups used were Mtr, Boc, and tBu. A good yield of cleaved peptide was obtained with apparently complete deprotection of the Mtr group.

When removing Mtr from Arg, Funakoshi[12] demonstrated that the deprotection rate can be increased by using 1M TMSBr/thioanisole/TFA instead of 1M thioanisole/TFA. TMSBr/TFA deprotects most side-chain protecting groups used in Fmoc synthesis, with the exception of Cys(Acm). The intermediate trimethylsilylated compounds can be hydrolyzed easily with H_2O or ammonium fluoride[11] to complete deprotection.

The following procedure is designed for 0.1-1.0 g of peptide resin.

Recommended Equipment and Chemicals

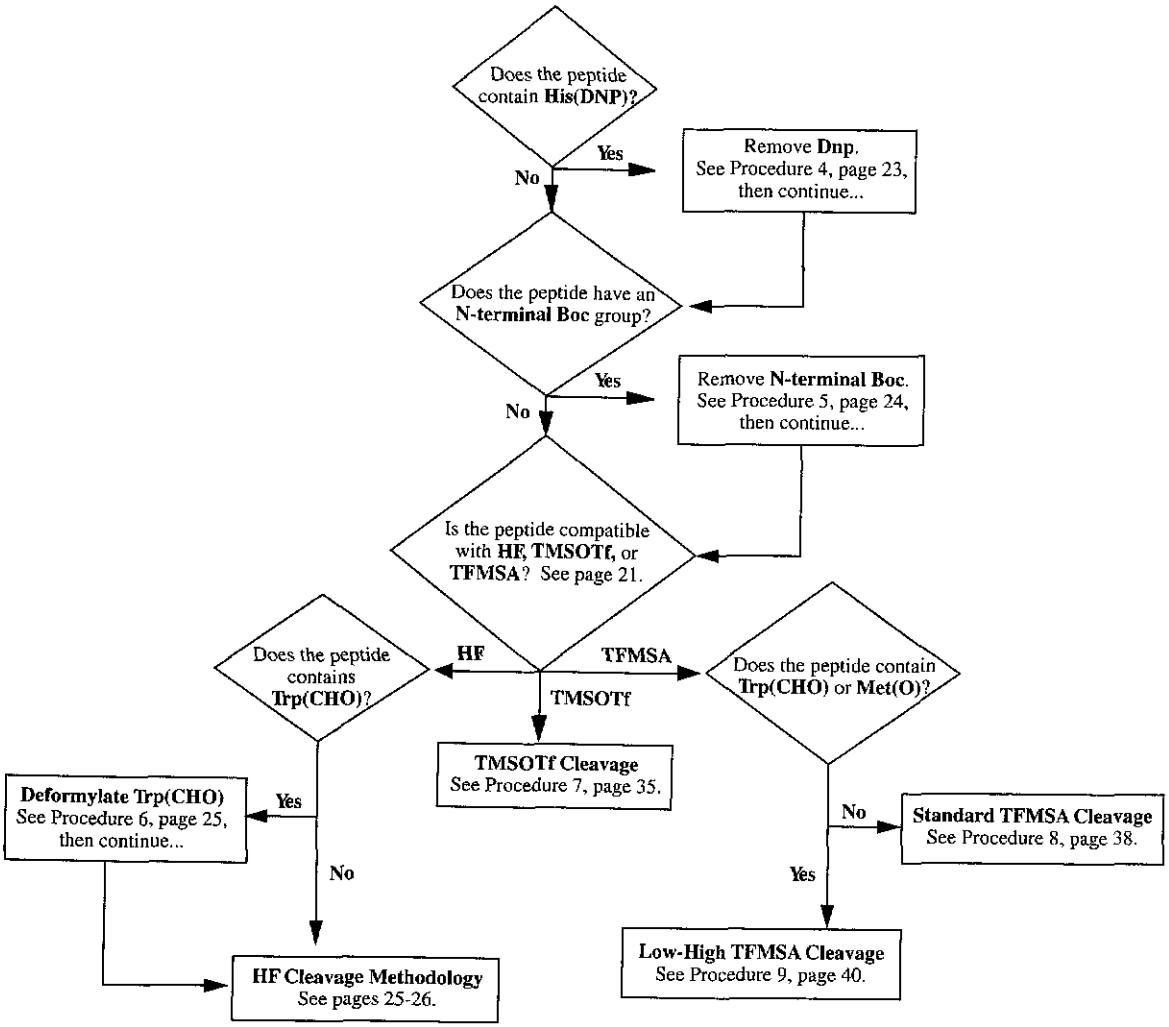
round bottom flask (50 mL)
stir bar
stir plate
ice bath
micropipettor
pipet tips
TMSBr
TFA
scavenger mixture
[thioanisole/EDT/m-cresol
(1.2 mL:0.6 mL:0.20 mL)]

TMSBr/Thioanisole/TFA Cleavage Mixture

1.35 mL	TMSBr
2.00 mL	scavenger mixture
7.48 mL	TFA

1. Place the dried peptide-resin in a round-bottom flask that contains a stir bar. Cool the flask in an ice bath.
2. Cool the prepared cleavage mixture and add it to the cooled peptide-resin. Maintain the temperature of the reaction flask at 0 °C with an ice bath for 1 hour.
3. To isolate the peptide from the solution, proceed with either method I, II, or III on pages 12–14. After you have isolated the peptide, desalt it by gel filtration (see page 46).

Boc Cleavage Flow Chart



Note *To produce a peptide with a C-terminal ethylamide, see the Ethylamine Cleavage procedure, page 45.*

Boc-Compatible Amino Acid Derivatives

Note *No derivative is compatible with every synthesis protocol. Select amino acid derivatives carefully.*

HF Compatible

Arg(Mts)	Cys(4-MeBzl)	** His(Dnp)	Thr(Bzl)
†Arg(Tos)	Cys(Mbzl)	His(Tos)	††Trp(CHO)
Asp(OBzl)	Glu(OBzl)	His(Z)	Tyr(Br-Z)
Asp(OcHex)	Glu(OcHex)	Lys(ClZ)	
* Cys(Acm)	His(Bom)	Ser(Bzl)	

TMSOTf Compatible

Arg(Mts)	Glu(OBzl)	Ser(Bzl)	Tyr(Bzl)
Arg(MBS)	Glu(OcHex)	Thr(Bzl)	Tyr(Cl_2Bzl)
Asp(OBzl)	His(Bom)	Trp(CHO)	
Asp(OcHex)	Lys(ClZ)	Trp(Mts)	
* Cys(Acm)	* Met(O)	Tyr(Br-Z)	

Note *Other derivatives, such as His(Tos) and His(Z), have not been fully investigated for use with TMSOTf.*

TFMSA Compaible

Arg(Mts)	His(Bom)	Met(O)
Asp(OBzl)	** His(Dnp)	Ser(Bzl)
* Cys(Acm)	His(Tos)	Thr(Bzl)
Cys(Mbzl)	His(Z)	Trp(CHO)
Glu(OBzl)	Lys(Cl-Z)	Tyr(Br-Z)

Note *The use of side-chain protecting groups other than those described above for Arg or Cys, will yield substantially inferior products under TFMSA conditions.*

† When Arg(Tos) is used, cleavage reaction time must be extended.

* Post-cleavage treatment is required when Met(O) is used with TMSOTf and whenever Boc-Cys(Acm) is used.

** When His(Dnp) is used, pre-cleavage treatment is required.

†† When Trp(CHO) is used with HF, pre-cleavage treatment is required.

Procedure 4: Removal of Dnp Protection from Boc-His(Dnp)

The Dnp protecting group of histidine is stable to strong-acid cleavages such as HF or TFMSA. A separate step is required to remove Dnp protection from the peptide-resin.

Removal of DNP group from the peptide-resin before cleavage with thiophenol in DMF

When working with His(Dnp) containing peptide-resins, you must remove Dnp from the peptide while it is still attached to the resin and before removing the Boc group. If the N-terminus is unprotected, the liberated Dnp groups can attach to the unprotected amine group and become very difficult to remove.

> ***DO NOT REMOVE THE BOC GROUP UNTIL AFTER YOU HAVE USED THIS PROCEDURE***

Recommended Equipment and Chemicals:

glass solid-phase reaction vessel	DMF
round-bottom flask	thiophenol
stir bar and stir plate	EtOH
fritted glass funnel (medium porosity)	DCM
vacuum filtration flask	

1. Suspend the Boc-protected peptide-resin in DMF for several minutes in a glass solid-phase reaction vessel, as described by Stewart and Young.[13] As an alternative, use a round-bottom flask equipped with a stir bar, then filter the mixture through a medium-porosity, fritted glass funnel.
2. Drain the resin. Add fresh DMF at a ratio of 5 mL DMF per gram of peptide-resin.
3. Add 20 mmol thiophenol (0.102 mL/mmol, or 2 mL) for each mmol of His(Dnp) present.
4. Stir (or use a shaker to shake) the resin for one to four hours at room temperature. The solution should turn bright yellow.

5. Drain and thoroughly wash the resin three times with DMF, followed by washes with H_2O, EtOH, and finally, DCM. A small amount of intensely yellow Dnp-thiophenol may be adsorbed to the resin. After standard purification procedures, a slight yellow color may persist.
6. Now the N-terminal Boc group can be removed with 50% TFA/DCM before HF cleavage. You do not need to remove the N-terminal Boc group if you are going to use TFMSA.

Removal of Dnp group from free peptides after cleavage

Once you have deprotected and cleaved the peptide from the resin, attempts to remove Dnp protection groups on the free peptide are not only time consuming, but also likely to result in a peptide that is difficult to purify. When the Dnp group is removed, it can irreversibly attach to any free amines, including the unprotected N-terminus. Some researchers have used 2–mercaptoethanol to remove Dnp from free peptides, though we have not thoroughly investigated this procedure. For best results, we recommend re-synthesizing the peptide.

Procedure 5: Removal of the Boc Group Prior to Cleavage

Remove the Boc protecting group from the N-terminus before you proceed with an HF cleavage. You can leave the Boc protection on the peptide if you use TFMSA.

Automated removal of the N-terminal Boc group

ABI Model 430A Chemistry	End Cycle
DMF	**endnh2** (**endnh2 r** if resin sampling)
NMP-HOBt	**rnh211** (**rnh211 r** if resin sampling)
DMF (small scale)	**rnh221** (**rnh221 r** if resin sampling)

ABI Model 431A & 433A Chemistry	Modules
Boc/HOBt/DCC	**bcdc**

Manual removal of the N-terminal Boc group

Recommended Equipment and Chemicals:

round-bottom flask
magnetic stir bar
fritted glass funnel (medium porosity)
stir plate
vacuum desiccator
vacuum pump

TFA
DCM
MeOH

1. Add the peptide-resin to a round-bottom flask that contains a magnetic stir bar.
2. Add 50% (v/v) TFA/DCM (30 mL/g resin) and let sit for one minute to vent the CO_2 that is liberated during the deprotection. Cap the flask and let stir at room temperature for 15 minutes.
3. Filter the resin through a fritted glass funnel. Wash twice with DCM, then twice with MeOH, and finally twice with DCM again.
4. Dry the peptide-resin in a vacuum desiccator for at least 3 hours or overnight, if possible.

The peptide-resin is now ready for HF cleavage (see pages 26-27).

HF Cleavage Methodology

Procedure 6: Piperidine Deformylation of TRP(CHO)-Containing Peptide-Resins

Use this procedure to deformylate the peptide-resin prior to cleavage with HF.

Recommended Equipment and Chemicals:

round-bottom flask
stir bar
stir plate
ice bath
fritted glass funnel (medium porosity)
vacuum filtration flask
vacuum desiccator
vacuum pump

piperidine
DMF
DCM
MeOH

1. Prepare a solution of 1 mL piperidine in 10 mL DMF for 0.5–1.5 g peptide-resin in a round-bottom flask equipped with a stir bar. Cool the solution to 0 °C.
2. Add the peptide-resin and stir for 2 hours. The solution must be maintained at 0 °C throughout this reaction time.
3. Filter the peptide-resin. Wash twice with DMF, twice with DCM, and then twice with MeOH.
4. Dry the peptide-resin in a vacuum desiccator for at least 3 hours or overnight, if possible.

The peptide-resin is now ready for HF cleavage (see pages 26-27).

WARNING **HF is a highly corrosive and toxic material. All procedures involving HF must be performed with HF-resistant apparatus, in an efficient fume hood that is preferably equipped with a scrubber. Use of proper eye protection, rubber gloves, rubber apron and a face shield are mandatory. Always use HF in accordance with local, state and federal safety and environmental codes and regulations.**

WARNING **Any pressurized cylinder must be safely attached to the wall or fume hood by means of approved brackets, chains or clamps. Failure to do so could cause the cylinders to fall over and explode, which could result in physical harm.**

HF Cleavage Conditions and Scavenger Mixtures

In general, HF cleavage reactions are performed at –5 °C to 0 °C for 60 minutes. If the peptide contains Arg(Tos), cleavage reaction time should be extended to 2 hours.

The following two cleavage mixtures have been tested in our laboratory and found to decrease the occurrence of side reactions.

For peptides containing Cys:	*For other peptides:*
HF/anisole/*DMS/p-thiocresol (10:1:1: 0.2)	HF/*DMS/anisole (10:1:1)

* In the past we have recommended adding DMS to scavenger mixtures, however we now no longer use it with HF cleavage procedures, due to the offensive odor of the generated DMS adduct.

Sources of HF Cleavage Apparatus

A number of companies distribute cleavage apparatus. Because Perkin-Elmer does not distribute HF cleavage apparatus, we recommend that you follow the guidelines established by the manufacturer of your cleavage apparatus for the appropriate HF handling procedures. You may contact one of the following vendors for HF cleavage apparatus.

Immuno-Dynamics, Inc
Division of Peptide Chemistry
P.O. Box 766
La Jolla, Ca 92038
(619) 452-1270

Peninsula Laboratories, Inc
611 Taylor Way
Belmont, CA 94002
In California: (415) 592-5392
Outside California: (800) 922-1516

Peptides International
P.O. Box 24658
Louisville, KY 40224
In Kentucky: (502) 266-8787
Outside Kentucky: (800) 777-4779

After the peptide-resin has reacted in HF at –5 °C to 0 °C for 60 minutes, evaporate the HF completely with the aid of nitrogen gas flow. To prevent side reactions during this process, it is important to maintain the temperature of the reaction vessel at –5 °C to 0 °C.

After removal of the HF, add ether to the reaction vessel and stir the peptide-resin-scavenger mixture for about 30 seconds. Filter the ether solution through a fritted glass funnel. Repeat this ether wash two more times to remove most of the scavengers.

Extract the peptide from the peptide-resin mixture by stirring the mixture in 30% (v/v) acetic acid. Approximately 30 mL of 30% acetic acid is used for one gram of peptide-resin. The acetic acid extract is filtered through the same fritted glass funnel used in the ether extraction, but into a different filter flask. To ensure complete extraction of the peptide, repeat the extraction procedure twice, using approximately 30 mL of 30% acetic acid per gram of peptide-resin each time. For those peptides that are not soluble in 30% acetic acid, use a higher concentration of acetic acid or see page 52 to determine the appropriate solvent.

Before lyophilizing the peptide in solution, add water to dilute the acetic acid to a less than 10% concentration. This dilute solution of acetic acid in water remains frozen during lyophilization, while a more concentrated solution of acetic acid may melt.

Under acidic conditions, an N→O acyl shift occurs in peptides containing serine and threonine residues when the acyl moiety migrates from the α-amino group to the hydroxyl group. This reaction can be reversed by either neutralizing the acetic acid extract with NH_4OH or by redissolving the lyophilized peptide in a weak base, such as 5% (w/v) NH_4HCO_3.

CAUTION *When redissolving peptides that contain cysteine, use **cold** 5% (w/v) NH_4HCO_3 as the weak base.*

Boc: HF Cleavage Recommendations

Sakakibara et al. originally applied HF for final removal of protecting groups on peptides that had been synthesized in solution.[14,15] HF has also been used to remove side-chain protecting groups and to cleave peptides from the solid support after preparation by solid-phase synthesis.

Typically, HF cleavage is performed at 0 °C for 60 minutes with anisole as the scavenger, employing a ratio of peptide-resin/HF/anisole of 1:10:1. However, you may encounter problems if the peptide contains one or a combination of the following amino acids: tryptophan, methionine, cysteine, aspartic acid, glutamic acid and tyrosine. Strategies for these potential problems are detailed by amino acid in the sections that follow.

Benzyl- and t-butyl-cations formed during HF cleavage can alkylate tryptophan,[16,17] methionine,[18] cysteine and tyrosine[19] (Figure 2a-2d). To prevent further alkylation by t-butyl cations during HF cleavage, remove the N-α-Boc group with TFA before HF cleavage (see page 24 for this procedure).

HF Cleavage Scavengers

When side-chain protecting groups, such as the benzyls, are removed, they exist as benzyl cation intermediates. These intermediates can cause side reactions and alkylate other amino acids. Scavengers trap cations to prevent them from undergoing further reactions.

Anisole is commonly used as a scavenger, but it can anisylate glutamyl residues (see Figure 7). Other scavengers that have been employed in acid cleavage reactions include: thioanisole,[20] dimethyl sulfide,[21] ethyl methyl sulfide,[22] 2-mercaptoethanol,[23] 1,2-ethanedithiol,[23] methionine,[24] dithiothreitol,[25] 2-mercaptopyridine,[26] indole,[27] p-cresol and p-thiocresol.[21]

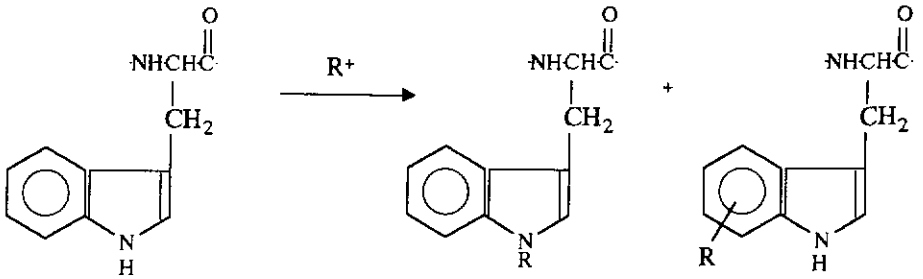


Figure 2a. Alkylation of tryptophan

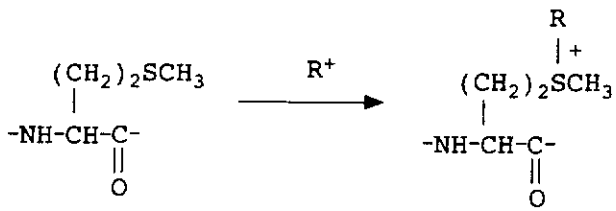


Figure 2b. Alkylation of Methionine

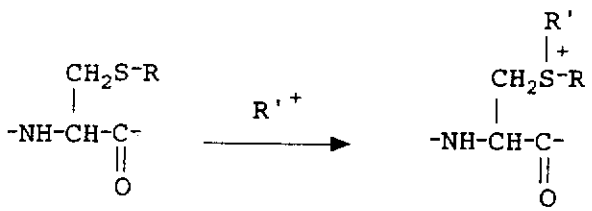


Figure 2c. Alkylation of Cysteine

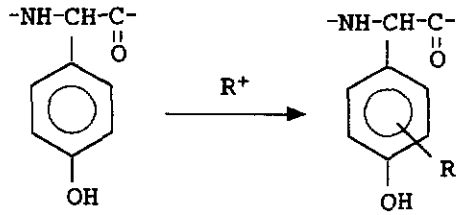


Figure 2d. Alkylation of Tyrosine

Tyrosine

Thioanisole is preferable to anisole for preventing the c-alkylation of tyrosine. However, the thioanisole-cation adducts are not stable and can undergo reversible reactions to alkylate other amino acids, such as methionine and tryptophan.

Methionine

Thioanisole should not be used as a scavenger when methionine is present because the unstable thioanisole-cation adducts alkylate methionine. DMS and EMS prevent the methionine residue from being alkylated by competing with methionine for cations.[22] When peptides containing methionine undergo HF cleavage, either of these two scavengers can minimize the oxidation of methionine to methionine sulfoxide. It has also been reported that either DMS or EMS can reduce methionine sulfoxide to methionine.[28]

Yamashiro reports that when 2-mercaptopyridine is used in HF cleavage, it reduces methionine sulfoxide to methionine.[26] He treated a peptide containing methionine sulfoxide in HF in the presence of the standard scavenger anisole for one hour at 0 °C, after which time no detectable methionine-peptide was formed. The same treatment, with the addition of ten equivalents of 2-mercaptopyridine, after ten minutes produced a quantitative yield of methionine with only a trace of methionine sulfoxide remaining.

Tryptophan

Avoid the use of thioanisole during HF cleavage if your peptide contains tryptophan. Thioanisole cation adducts are not stable and can undergo reversible reactions to alkylate the nitrogen of the indole ring if tryptophan is not protected. Tryptophan[29] readily undergoes irreversible oxidation, as shown in Figure 3. Indole has been used as a scavenger to minimize oxidation and alkylation of tryptophan residues. However, indole can undergo acid-catalyzed dimerization with tryptophan and irreversibly modify the tryptophan indole ring.[30]

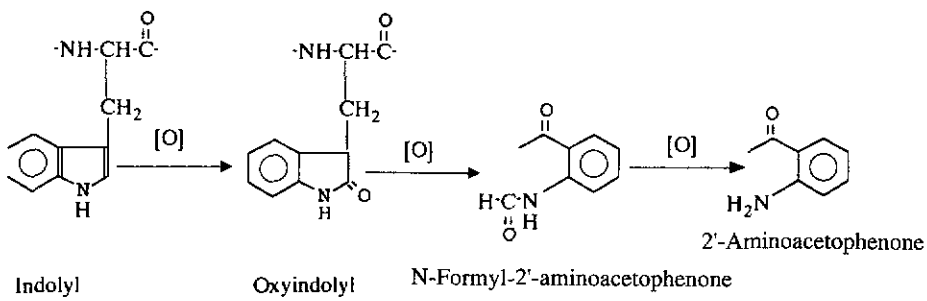


Figure 3. Oxidation of Tryptophan during HF cleavage

Cysteine

For peptides containing cysteine, in addition to a mixture of anisole/DMS, a third scavenger is needed to obtain a purer HF-cleavage product. In one study,[31] we found that a scavenger mixture of anisole/DMS/p-thiocresol (1:1:0.2) with HF cleavage, improved the purity of the peptide Val-Asp-Cys-Ser-Glu-Tyr-Pro-Ala-Cys-Thr-Leu (see Figure 4).

It is possible, but not easy, to reverse the oxidation of cysteine. We recommend that you take steps to prevent cysteine oxidation. Minimize the peptide-resin's exposure to air during cleavage. After the peptide has been lyophilized, store it in an air-tight container, away from light and at 0 °C or below.

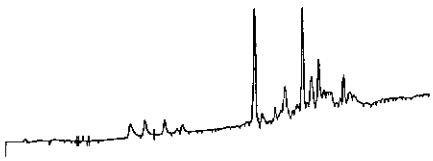


Figure 4a. Anisole/DMS (1:1)

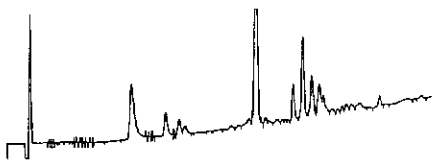


Figure 4b. DMS/p-thiocresol (1:1)

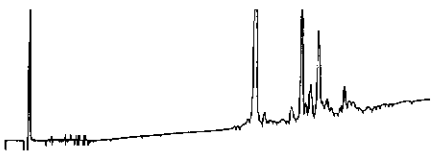


Figure 4c. Anisole

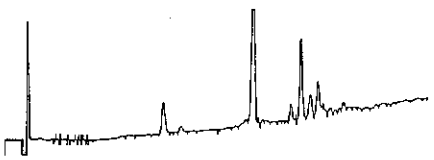


Figure 4d. Anisole/DMS/p-thiocresol (1:1:0.2)

Figure 4. HPLC profiles of HF-cleaved peptide yields with four different scavengers.

The peptide Val-Asp-Cys-Ser-Glu-Tyr-Pro-Ala-Cys-Thr-Leu was cleaved with HF, using four different scavenger mixtures. The HPLC profiles above illustrate how choice of scavengers affects purity of peptide yield. HPLC conditions: Brownlee, C8 column (300 Å, 7 μ, 22 cm x 4.6 mm) at 1 mL/min with detection at 220 nm. Samples were eluted at 0 - 100% B in 45 minutes; A was 0.1% TFA/H_2O, B was 0.1% TFA/60% CH_3CN-40% H_2O.

Best results occur when the peptide-resin is reacted with HF at temperatures below 0 °C; a common mistake is the removal of the ice bath from the reaction reservoir during HF evaporation. Temperature affects the rate of removal of the side-chain protecting groups. At –20 °C, the removal rate is slow and impractical for the following protecting groups:

- the tosyl group on arginine
- 2-chlorobenzyloxycarbonyl (Cl-Z) on lysine
- p-methylbenzyl (4-MeBzl) on cysteine

However, treatment of the peptide-resin with HF for sixty minutes at –5 °C to 0 °C facilitates cleavage of protecting groups, including the groups described above.

Aspartic Acid

The side-chain ester of aspartic acid can be cyclized to form aspartimide, a five-membered succinimide ring, which on exposure to water reopens to form a mixture of α- and β-branched peptides[32] (see Figure 5). Aspartimide formation is difficult to avoid in peptide synthesis, because it can occur under a wide range of acid and base concentrations.[33] However, as long as the reaction temperature is maintained at –5 °C or lower, aspartimide formation with benzyl-protected Asp is significantly decreased. The cyclohexyl ester of aspartic acid offers an even more effective protection at low temperatures. In one study, under standard cleavage conditions for one hour, 6.5% aspartimide formation occurred with benzyl at –15 °C compared to 1.7% with the cyclohexyl protection.[33]

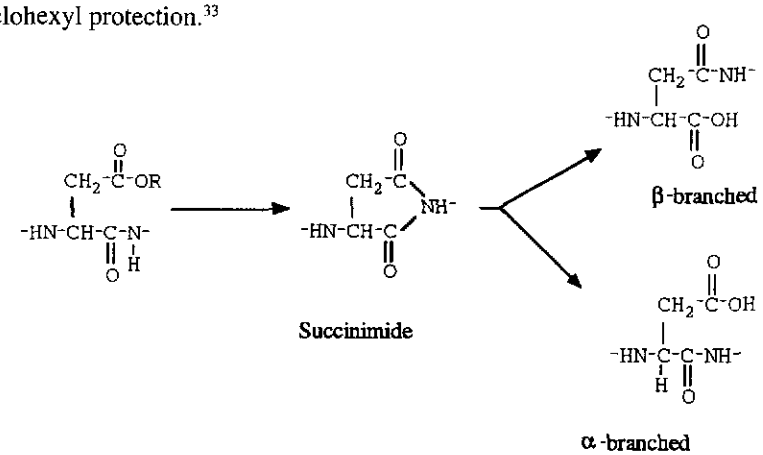


Figure 5. Formation of succinimide and branched aspartic acid residues during HF reaction

Glutamic Acid

If your peptide contains Glu, dehydration of the protonated side-chain ester of glutamyl residue followed by anisylation of the resulting acylium ion, as shown in Figure 6, can occur when the reaction temperature rises to 0 °C or more.[34] However, the formation of glutarimide, a six-membered ring, is usually minimal, regardless of whether the protecting group for Glu is cyclohexyl or benzyl.[33]

To prevent the formation of anisylated glutamyl peptide, decrease the temperature to between –5 °C and 0 °C with a salt-ice slurry.

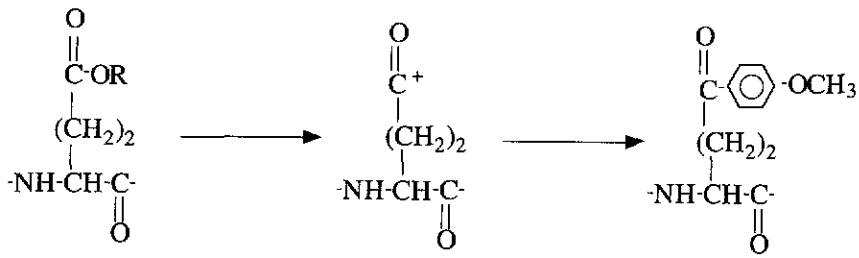


Figure 6. Formation of anisylated glutamic acid during HF reaction

Cyclization of N-terminal glutamine residues to pyroglutamyl residues (Figure 7) can cause chain termination in peptide synthesis. This reaction is catalyzed by weak acids such as the Boc amino acids.[13] To effectively minimize pyroglutamic acid formation, couple in a polar solvent using the preformed symmetric anhydride of the amino acid.[35]

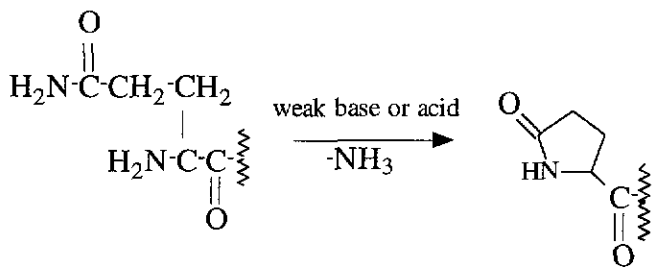


Figure 7. Cyclization of N-terminal glutamine to pyroglutamyl residue

Procedure 7: TMSOTf Cleavage Procedure

WARNING **TMSOTf is corrosive and flammable. Extreme care must be taken when using it. Always work in a well-ventilated fume hood and wear protective equipment, eyewear, lab coat and gloves when handling TMSOTf.**

An efficient fume hood has a minimum face velocity of 0.50 meters/second (100 linear feet/minute) as the average velocity of the open area. This should be measured by individuals knowledgeable in industrial hygiene or air flow monitoring. TMSOTf exhaust may require additional treatment as mandated by local, state or federal regulatory agencies.

Recently, Yajima et al. have been investigating a procedure that uses a mixture of TMSOTf, TFA and thioanisole.[11] They have found that it easily removes most of the side-chain protecting groups commonly used in conjunction with Boc synthesis, with the exceptions of Cys(Bzl), Cys(Acm), and Arg(NO_2) (see table of Boc-Compatible Amino Acid Derivatives, page 21). The intermediate trimethylsilylated compounds can be hydrolyzed easily with H_2O or ammonium fluoride to complete deprotection.

Yajima and co-workers have found that the cleavage rate increases when 1M TMSOTf/TFA is used instead of 1M TFMSA/TFA.[11] They report that TMSOTf cleavage produces fewer side reactions and yields a product that is less hygroscopic than the peptide product of TFMSA cleavage. Desalting the peptide product of TMSOTf cleavage is recommended (see Desalting, page 46).

The following procedure is designed for 0.1–1.0 g of peptide resin.

Recommended Equipment and Chemicals

round bottom flask (50 mL)
stir bar
stir plate
micropipettor
pipet tips

TMSOTf
TFA
scavenger mixture [thioanisole/EDT/m-cresol (1.2 mL:0.6 mL:0.20 mL)]

TMSOTf/TFA Cleavage Mixture

1.94 mL TMSOTf

2.00 mL scavenger mixture

6.89 mL TFA

1. Place the dried peptide-resin in a round-bottom flask that contains a stir bar. Cool the flask in an ice bath.
2. Cool the prepared cleavage mixture and add it to the cooled peptide-resin. Maintain the temperature of the reaction flask at 0 °C with an ice bath for 2 hours.
3. To isolate the peptide from the TFA solution, proceed with either method I, II, or III on pages 11–13. After you have isolated the peptide, desalt it by gel filtration (see page 46).

TMSOTf: Cleavage Recommendations

Although TMSOTf cleavage is a relatively new procedure, results of studies by Yajima et al. suggest that it may be faster and more efficient than TFMSA cleavage, and generates fewer side-reactions and a less-hygroscopic peptide product.[11]

TMSOTf Cleavage Scavengers

To date, few scavengers have been studied in conjunction with TMSOTf. Yajima has found that thioanisole suppresses side reactions, accelerates cleavage and is a superior scavenger compared to DMS or diphenylsulfide. When added together, EDT and m-cresol protect Tyr and Trp from side reactions. EDT is also required to achieve complete deformylation of Trp(CHO).

Arginine

TMSOTf and thioanisole treatment of Z-Arg(Tos) at 0 °C for 30 minutes gave only 62% deprotection.[11] Longer cleavage times should improve removal of the tosyl group, however definite time values have not been reported.

Methionine

TMSOTf/TFA reduces 27% of Met(O) to Met. Therefore, we recommend using N-methylmercaptoacetamide to generate the reduced peptide product (see page 47 for this procedure).

Tryptophan

In the presence of EDT, Trp(CHO) is completely deprotected by TMSOTf/TFA treatment after 30 minutes at 0 °C.[36]

TMSOTf Cleavage: Temperature and Time

Yajima et al. found that TMSOTf/TFA removed most side-chain protecting groups after 30 minutes at 0 °C, although they typically cleaved peptides for 2 hours to ensure complete deprotection.[11] To minimize potential side-reactions, cold temperatures must be maintained throughout the cleavage procedure.

TFMSA Cleavage

WARNING **TFMSA is one of the strongest acids known and extreme care must be taken when using it. Always wear protective equipment, eyewear, lab coat and gloves when handling it and work in a well-ventilated fume hood.**

An efficient fume hood has a minimum face velocity of 0.50 meters/second (100 linear feet/minute) as the average velocity of the open area. This should be measured by individuals knowledgeable in industrial hygiene or air flow monitoring. TFMSA exhaust may require additional treatment as mandated by local, state or federal regulatory agencies.

Procedure 8: Standard TFMSA cleavage

The standard TFMSA cleavage procedure that follows is for a small scale reaction (100 mg), but it may be adjusted for use with larger or smaller amounts of peptide-resin, as long as the prescribed equivalents are maintained. For large-scale reactions, the reaction flask should be immersed in an ice bath prior to the addition of TFA in step 3. Remove the flask from the ice bath after all the TFMSA has been added in step 4.

The reaction time in the standard protocol is 25 minutes for Pam resins and 1.5–2 hours for MBHA resins. Yields and HPLC data indicate that TFMSA-cleaved peptides are more susceptible to salt and scavenger association than HF-cleaved crude products. For complete removal of all TFMSA ions, the peptide solution must be neutralized and then desalted by ion exchange chromatography. A procedure for desalting on a Sephadex® column is desccribed on page 46.

Standard TFMSA cleavage (100 mg)

Recommended Equipment and Chemicals:

round bottom flask (50 mL)
micro stir bar
stir plate
2 fritted glass funnels (medium and fine porosities)
vacuum filtration flasks
ice bath
micropipettor
pipet tips

thioanisole
EDT
TFA
TFMSA
Et_2O

1. Place the peptide resin in a small (50 mL) round-bottom flask that contains a micro stir bar.
2. Add 150 mL of thioanisole/EDT (2:1) and let the mixture stir for approximately 10 minutes at room temperature.
3. Add 1 mL neat TFA and let stir for 5–10 minutes.
4. Slowly add 100 mL TFMSA dropwise with vigorous stirring to dissipate the heat generated. For Pam resins, allow the mixture to react for 25 minutes at room temperature; MBHA resins require 1.5-2 hours reaction time.
5. When the reaction is complete, add cold Et_2O to fill the flask and precipitate the peptide. Let the mixture stir for approximately one minute more.
6. Transfer the contents of the reaction flask to a clean fritted glass funnel (medium porosity). Attach the funnel to a vacuum filtration flask and wash the solid with cold Et_2O several times while applying the vacuum.
7. Remove the vacuum filtration flask and replace with a clean filtration flask that contains 200 mL cold Et_2O. Dissolve the peptide on the frit with a minimum volume of neat TFA, and gently swirl or stir the contents of the funnel. The peptide should easily dissolve in the TFA, and filter into the cold Et_2O for the second precipitation.
8. Wash the resin on the frit two additional times with a minimum volume of TFA. Cool the precipitated solution of peptide and Et_2O over ice for 10 minutes.
9. Filter the precipitate through a fritted glass funnel (fine porosity) and wash the solid with cold Et_2O several times.
10. To ensure the removal of scavengers and excess TFMSA, proceed with the desalting method on page 46.

Procedure 9: Low-High TFMSA Cleavage (1 gram)

The Low-High TFMSA cleavage protocol can quantitatively reduce Met(O), deformylate Trp(CHO) and promote mechanistic deviation from S_N1 to S_N2, which theoretically reduces side-chain reactions.

The Low-High protocol that follows is based on a 1-gram scale, but it can easily be adapted to both larger and smaller reactions if the prescribed equivalents are maintained. With large scale reactions, the reaction flask should be immersed in an ice bath prior to the addition of TFA. Remove the flask from the ice bath after all the TFMSA has been added.

Three hours is the usual Low TFMSA cleavage reaction time, whether the peptide was assembled on Pam or MBHA resins.

Recommended Equipment and Chemicals:

round-bottom flask (50 mL)
micro stir bar
stir plate
ice bath (–5 °C to 0 °C)
fritted glass funnel (medium porosity)
vacuum pump
vacuum filtration flask
vacuum desiccator
round-bottom flask (100 mL)
stir bar
micropipettor
pipet tips

m-cresol
DMS
TFA
TFMSA
EDT [for Trp(CHO)]
cold Et_2O

Low Condition

1. Place 1 g of peptide resin in a 50 mL round bottom flask that contains a micro stir bar.
2. Cool the reaction flask to between –5 °C and 0 °C.
3. Add, in the following order:

   1 mL m-cresol
   3 mL DMS
   5 mL TFA
   1 mL TFMSA

For Trp (CHO) containing peptides add:

4L 200 mL EDT
4L 800 mL m-cresol
3 mL DMS
5 mL TFA
1 mL TFMSA

Allow the mixture to stir at –5 °C to 0 °C for 3 hours.

4. Transfer the flask contents to a fritted glass funnel of medium porosity, filter the resin and wash well with cold Et_2O.
5. Dry the funnel containing the resin-bound peptide. High vacuum effectively removes residual ether.
6. Transfer the dried solid into a clean 100 mL round-bottom flask that contains a magnetic stir bar.

High Condition

1. Add 1.5 mL thioanisole/EDT (2:1) and stir for 10 minutes.
2. Immerse the reaction flask in an ice bath. Add 10 mL TFA and stir for 5–10 minutes.
3. Slowly add 1 mL TFMSA dropwise. Then remove the ice bath and let the reaction proceed at room temperature for 25 minutes with Pam resins or for 1.5-2 hours with MBHA resins.
4. Continue the cleavage process with steps 5 through 9 of the Standard TFMSA Cleavage procedure described earlier on pages 38-39.
5. To ensure the removal of scavengers and excess TFMSA, proceed with the desalting method on page 46.

Boc: TFMSA Cleavage Recommendations

TFMSA is an economical alternative to HF for routine cleavage procedures. Its main advantage is that it may be used with standard laboratory glassware instead of the specialized apparatus needed for containment during an HF cleavage.

TFMSA Cleavage Scavengers

Aryl thiol ethers and thiols promote reactions and accept cations. With thioanisole we often can cleave 90–99% of the theoretically available peptide from Pam resin and 70–80% from MBHA resin, which makes it a requirement for efficient cleavage from both supports.

Methionine

Methionine should be incorporated as its sulfoxide, Met(O), to avoid alkylation during chain assembly and to minimize side products from cleavage. Normally, 10–40% of all the Met peptides are reduced under standard TFMSA cleavage of Met(O)-peptides. When certain Met(O) peptides are exposed to a TFMSA-TFA solution containing DMS, the Met(O)-peptide quantitatively reduces to Met-peptide (see "Low-High" TFMSA procedure on pages 40-41). Other methods for reduction of Met(O)-peptides include incubation with thioglycolic acid or N-methylmercaptoacetamide (see page 47).

Tryptophan

EDT has been observed to catalyze deformylation of Trp(N^{in}-CHO) residues, and therefore, is essential for the removal of formyl in peptides that contain that residue.[22,36] Thiophenols also catalyze deformylation of Trp(N^{in}-CHO) residues, but because they have deleterious effects on cysteinyl peptides, EDT is the preferred thiol scavenger.

Cysteine

EDT apparently serves as a reducing agent under strongly acidic cleavage conditions to significantly minimize the formation of dimers and other types of -S-S- aggregates in cysteinyl peptides.

Standard TFMSA cleavages are performed at room temperature (22 °C) with particular attention given to the TFMSA delivery step to avoid excessive generation of heat. For small-scale reactions, add the TFMSA slowly with vigorous stirring. For large-scale cleavages, the peptide-resin slurry may be placed in an ice bath while the TFMSA is being slowly added. After all TFMSA is added, the ice bath should be removed and the solution allowed to come to room temperature before you begin to time the reaction.

In the low phase of a Low-High TFMSA cleavage, it is **critical** to maintain low temperatures during the reaction. A salt-ice slurry can decrease the temperature to 0 °C and below; you must monitor the slurry to maintain the cold conditions throughout the reaction.

Aspartic Acid

Avoid using the Asp(OcHex) with TFMSA cleavages. With this protection, the residue is resistant to efficient removal with TFMSA. Figure 8 shows the result of using Asp(OcHex) and Cys(MeBzl). Note that the protecting groups (in peptides B, C, and D) were not removed by TFMSA.

Reduce the cleavage reaction temperature to suppress the incidence of aspartimide and glutarimide formation. For peptides containing Asp followed by Gly, Ser, Thr, Ala or Asn, the first 30 minutes of the cleavage reaction should be performed at –10 °C, followed by room temperature for the necessary reaction time.

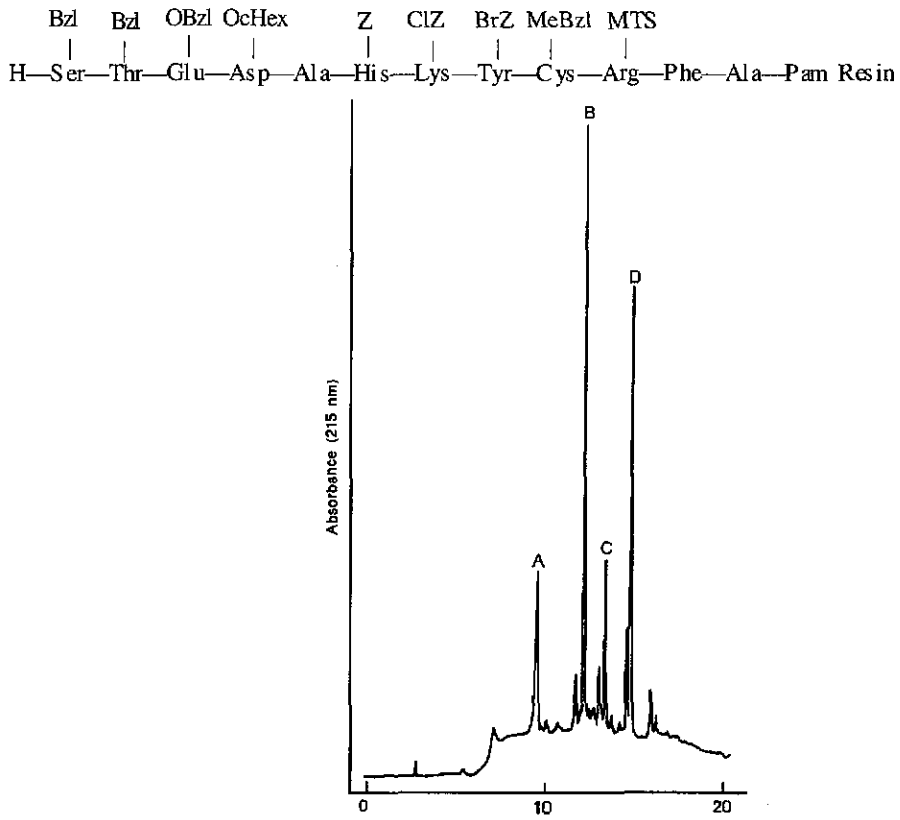


Figure 8. HPLC analysis of TFMSA cleavage of a peptide where Asp, Cys, and Arg are protected with O-cyclohexyl (OcHex), S-(MeBzl) and N^{gu}-MTS, respectively.

Peak A=authentic peptide, Peak B= peptide [Asp (OcHex)], Peak C= peptide [Cys (MeBzl)], Peak D= peptide [Asp (OcHex) Cys (MeBzl)].

Conditions: Perkin-Elmer Series 4 Liquid Chromatograph, fitted with a Perkin-Elmer ISS-100 autosampler and LCI integrator. The column was a 25 x 0.46 cm Vydac C_{18} (300 Å, 5 μ), operated at 45 °C and 1.0 mL/min flow rate. Peaks were detected at 215 nm, using a Kratos Model 773 variable wavelength detector. A ternary gradient program was used; Solvent A was 25:75 MeOH-H_2O, solvent B was 1% aqueous TFA, and solvent C was CH_3CN. The gradient was developed from 0 to 40 °C over 14 minutes, followed by a ramp to 60 °C over an additional 2 minutes while holding B constant at 10% throughout the run.

Ethylamine Cleavage of Boc-Derived Peptides

CAUTION *The ethylamine cleavage procedure should be performed in a fume hood.*

Neat ethylamine ($EtNH_2$) cleaves Boc-derived peptides from the benzyl-ester linkage of a resin, which results in peptides with a C-terminal ethylamide and protected side chains. Peptides that have C-terminal ethylamides can occur naturally or can be used in the production of peptide analogs. After $EtNH_2$ cleavage, side-chain protecting groups are removed with HF,TMSOTf, or TFMSA.

Use other amines, such as methylamine, to produce other short-chain alkylamides, such as methylamide. You can safely use a significant excess of amines that have a low-boiling point, such as $EtNH_2$, because they are easily removed by evaporation. However, with high-boiling point alkylamines, such as benzylamine, use somewhat less reagent, around 10% excess, because a slight excess can be removed easily through normal purification. Greater amounts of high-boiling point alkylamines are difficult to remove from the peptide.

This procedure can be applied to all peptides except those containing side-chain esters of aspartic acid or glutamic acid. Those side-chain esters react with amines to convert to amides.

Recommended Equipment and Chemicals:

three-neck round-bottom flask (25 mL)
dry ice trap
ice bath
stir bar and stir plate
lyophilization flask
vacuum desiccator and vacuum pump
$EtNH_2$ lecture bottle
HOAc

1. Place the peptide-resin (0.5 g) in a 25-mL three-neck, round-bottom flask. Fit the top of the flask with a dry ice trap and immerse the flask in an ice bath.
2. Condense 10 mL of neat $EtNH_2$ into the flask. Mix the peptide-resin in the $EtNH_2$ with a magnetic stirrer for four hours. Allow the ice bath to warm up to room temperature, slowly evaporating the $EtNH_2$. Place the flask under high vacuum for 3 or more hours to remove any residual $EtNH_2$.
3. Wash the protected peptide/resin mixture three times with HOAc. Lyophilize the acetic acid mixture.
4. Proceed with the appropriate cleavage procedure.

Additional Procedures

Desalting

Desalting removes salts and residual scavengers by ion exchange chromatography or reverse phase HPLC. Although desalting is not necessary after TFA and HF cleavages, data from HPLC and yield studies indicate that peptides cleaved by TFMSA, TMSBr and TMSOTf are more susceptible to salt and scavenger association than HF-cleaved crude products. To ensure the removal of scavengers and excess TFMSA, neutralize the peptide solution with NH_4HCO_3 to pH 8.5 before desalting.

CAUTION *When redissolving peptides that contain cysteine, use* ***cold*** *5% NH_4HCO_3 as a weak base.*

Perform ion exchange chromatography for complete removal of all TFMSA ions. For desalting on a Sephadex column, use the following procedure.

1. Dissolve the crude peptide in a minimum amount of H_2O and pass it through a Sephadex G-10 or G-25 column. Because this is a size exclusion column, the peptide should come off before the salts and scavengers. If the peptide is not soluble in H_2O, try dissolving it with increasing strengths of HOAc, from 10% to glacial, or refer to page 52 to determine the appropriate solvent.
2. Collect fractions and lyophilize them prior to the next purification step.

Reduction of Methionine Sulfoxide with N-Methylmercaptoacetamide (MMA)

The presence of methionine sulfoxide Met(O) in peptides containing methionine can be introduced either intentionally with the use of sulfoxide-protected methionine, or unintentionally by oxidation of methionine during chain assembly, strong acid cleavage and deprotection, or by prolonged storage and handling. The following procedure is recommended for the reduction of Met(O) in peptides containing methionine.

Recommended Equipment and Chemicals:

round-bottom flask	ethylmercaptoacetate
stir bar	methylamine (40% aqueous)
vacuum distillation apparatus	N_2
heating bath	10% (v/v)HOAc

1. MMA is commercially available, but you may prepare it yourself to ensure purity and freshness. Prepare MMA by reacting ethylmercaptoacetate (50 mL, 0.456 mol) with methylamine (90 mL, 40% aqueous solution, 1.16 mol) at 21°C overnight. Distill the resulting solution at reduced pressure (0.2 mm), at 71–72 °C. The distilled product, stored under N_2, will be a clear viscous liquid at room temperature.
2. Dissolve the peptide in 10% HOAc to a concentration between 1 and 5 mg/mL.
3. Add enough MMA to the dissolved peptide to produce a 10% (w/v) solution.
4. Incubate the peptide-MMA solution under N_2 at 37 °C for 24–36 hours.
5. Monitor the reduction on an appropriate HPLC column and solvent system. We typically use:

Column:	C-8
Buffer A:	0.1% TFA
Buffer B:	0.1% TFA/60% CH_3CN
gradient:	0–100% B in 45 minutes

The methionine peptide will have a longer retention time than the peptide containing methionine sulfoxide.

Mercury (II) Acetate Deprotection of Cys(Acm)

The following mercury (II) acetate procedure can be used to deprotect both Cys(Acm) and Cys(t-Bu). The procedure is identical for both derivatives except that for deprotection of the Cys(t-Bu), the reaction time is extended to 5 hours. Longer reaction times may be necessary for some peptides. This procedure does not form disulfide bridges. To form Cys-Cys bridges, refer to "Disulfide Bridge Formation," page 50.

Recommended Equipment and Chemicals:

round-bottom flask	30% (v/v) HOAc
stir bar	mercury (II) acetate
stir plate	2-mercaptoethanol
fritted glass funnel	Sephadex G-10 or G-25
vacuum filtration flask	

1. Dissolve the crude peptide in 30% HOAc at the highest concentration possible, dependent on the solubility characteristics of the peptide (we suggest 20 mg/mL).
2. Add two equivalents of mercury (II) acetate (MW = 318) for each equivalent of Cys. (To calculate reaction equivalents, see the example in the box on the next page). Let the reaction stir for 1 hour.
3. Add twenty equivalents of 2-mercaptoethanol (MW=78.13, d=1.114) to the reaction mixture. Stir for one additional hour. Mercuric sulfide salt, which is gray in color, should precipitate.
4. Filter the reaction mixture through a pad of Celite®, or a similar filtering agent, to remove the precipitated salts. Desalt by either gel filtration (Sephadex G-10 or G-25) or reverse-phase HPLC. At this point, if you want to form Cys-Cys bridges, see page 50.

The peptide is ready for lyophilization.

How to calculate reaction equivalents for deprotection of Cys(Acm)

In the preceding procedure, each mmol of Cys(Acm) reacts with mercury (II) acetate and 2-mercaptoethanol in a ratio of 1:2:20.

For example, 40 mg of the peptide Somatostatin [Ala-Gly-Cys(Acm)-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys(Acm)] are dissolved in 2 mL 30% HOAc.

MW Somatostatin [Cys(Acm)] = 1782.05 g/mol

40 mg of Somatostatin [Cys(Acm)] = 0.0224 mmol

Because there are two cysteine residues in this peptide,

2 x 0.0224 mmol = 0.0448 mmol Cys(Acm)

2 equivalents of mercury (II) acetate (MW =318) = 0.0896 mmol = 28 mg.

20 equivalents of 2-mercaptoethanol (MW=78.13) = 0.896 mmol = 63 μL.

Procedures for the deprotection of Cys(Acm) with iodine can be found in *Solid Phase Peptide Synthesis*, Second Edition, by J.M. Stewart and J.D. Young. Although iodine treatment is a viable method for the deprotection of this derivative, in our opinion, the mercury (II) acetate treatment produces superior results and is easier to perform. Also, Trp, Tyr and Met may be modified in the presence of iodine.

Disulfide Bridge Formation

Either of the following procedures may be used to form a disulfide bridge. The potassium ferricyanide method is faster; the alternative air oxidation method may take several days. Both procedures use a dilute solution of peptide in H_2O to prevent the formation of aggregates and polymers. **Deprotect the Cys residues before using either procedure.**

Disulfide Bridge Formation with Potassium Ferricyanide

Recommended Equipment and Chemicals:

large Erlenmeyer flask
stir bar
stir plate
pH meter or pH paper
buret
fritted glass funnel
vacuum filtration flask
preparative chromatography column
fraction collector
HPLC
lyophilization flask

1 M NH_4OH solution
potassium ferricyanide
10% and 50% (v/v) acetic acid
Bio-Rex® 70

1. Dissolve the peptide in a suitable solvent (such as H_2O) as a 0.1 mg/mL (or 1 mmol/ 4 liters) solution in a flask that contains a stir bar. A greater dilution is acceptable. Adjust to pH 7 with 1M NH_4OH.
2. Slowly add a solution of potassium ferricyanide [400 mg $K_3Fe(CN)_6$ in 200 mL H_2O] dropwise to the peptide solution until a pale yellow color is maintained for at least one hour.
3. Adjust the pH to 4.5 with 10% acetic acid and slowly (approximately 1 hr for 1 liter) load the solution onto a cation exchange column (such as Bio-Rex® 70).
4. To wash off salts, elute the column with an amount of H_2O equal to half the volume loaded onto the column. Run a gradient of H_2O to 50% HOAc (1 liter each) and collect fractions. Analyze fractions by HPLC.
5. Lyophilize appropriate fractions and purify by HPLC.

Air Oxidation Protocol

Dissolve the peptide in an aqueous solvent at a concentration of 0.1 mg/mL (a more dilute solution is acceptable). Adjust the pH of the solution to approximately 8.5 with NH_4OH and allow the solution to stir for several days.

Use HPLC analysis to monitor the oxidation process. The solution may be desalted (see page 46) and lyophilized.

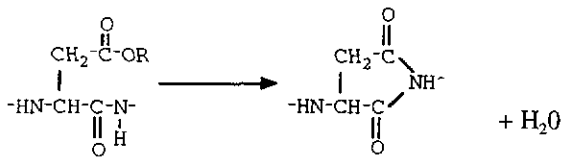
DMSO Oxidation Protocol

Dissolve the peptide in 5% acetic acid to a concentration of 0.5 to 1.0 mg/mL. Adjust the pH of the solution to approximately 6 with $(NH_4)_2CO_3$. Add DMSO, up to 20% by volume, to this solution.[37]

Use HPLC analysis to monitor the oxidation process. The solution may be desalted (see page 46) and lyophilized.

Side Reactions with Sequences Containing Asp-X

A frequent side-reaction with peptides that contain a sequence with Asp-X—where X is Ser, Thr, Gly, or Asn—is the formation of aspartmide or β-aspartyl peptides. This modification can be identified by the loss of H_2O ($M+H^+=18$), as detected by mass analysis.



Succinimide

To reverse this situation, treat the lyophilized peptide with 7% aqueous ammonia at 0 °C for 10 minutes, or until the conversion is complete, as monitored by HPLC.

How to Choose Peptide Solvents

To choose a suitable solvent for dissolving your peptide, consult the Peptide Solubility Guide on page 53 to calculate the net charge of the peptide. Once you have determined which solvent type to use, test a small sample of your peptide (approximately 0.5 mg). If your initial solvent choice does not work, start with a fresh sample of peptide before trying a new solvent. When you find the appropriate solvent, increase the amount of solution proportionately to the amount of peptide you want to dissolve.

Solvent Type 1

Basic peptides often dissolve in water. If that doesn't work, try 10% and increasing strengths of acetic acid. If the peptide still does not go into solution, add TFA (≤50 µL) to solubilize the peptide, then dilute to 1 mL with water.

Solvent Type 2

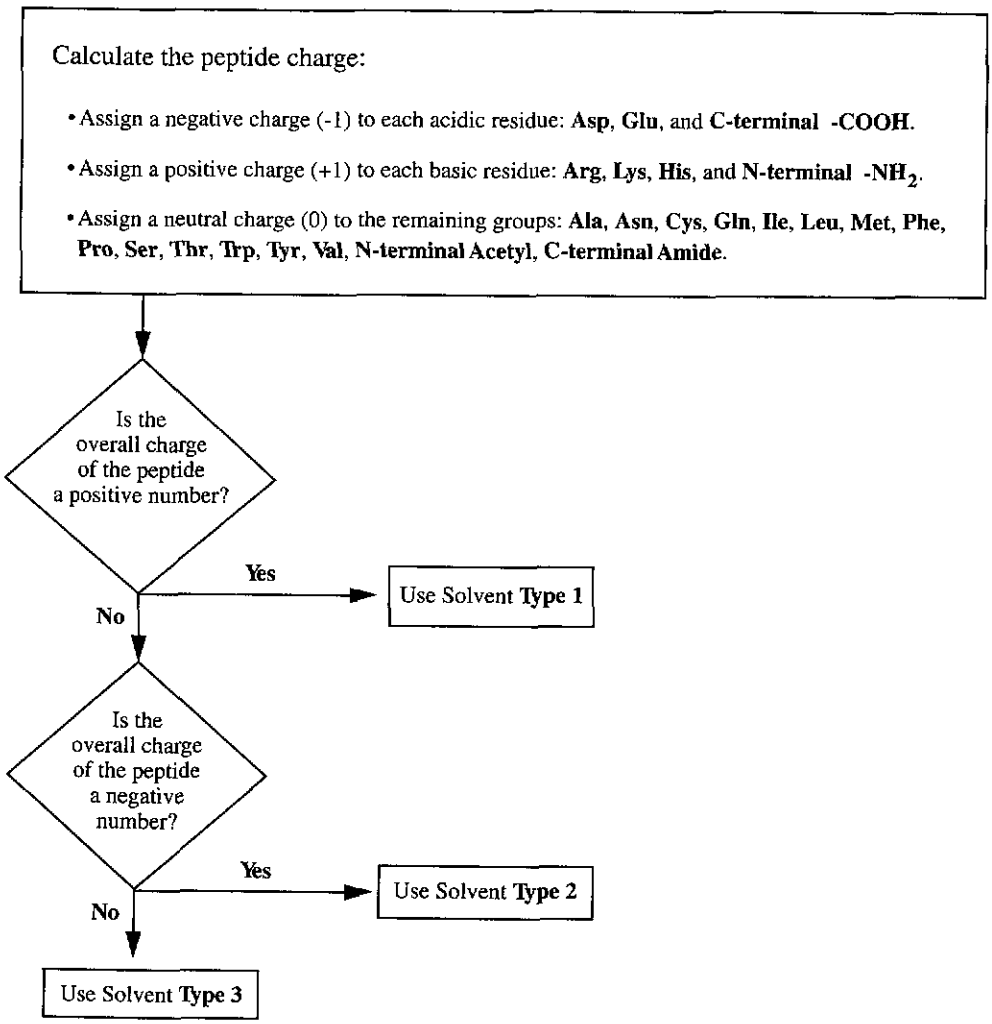
Acidic peptides may dissolve in water or acetic acid. If these fail, add NH_4OH (≤50 µL) and dilute to 1 mL with water.

Solvent Type 3

Neutral peptides may require the addition of glacial acetic acid, neat TFA, formic acid, or other organic solvents, such as methanol, isopropanol, or acetonitrile. Denaturants, such as urea or 6M guanidinium-HCl may also be required.

If your peptides are slow to dissolve, sonicate the peptide-solvent mixture for 10 minutes.

Peptide Solubility Guide



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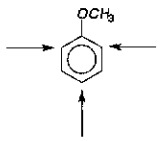
Notes

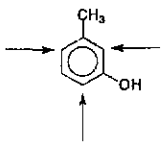
Notes

Table of Scavengers

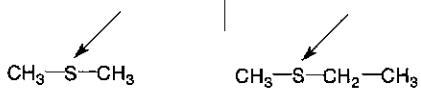
Anisole



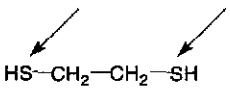
m-Cresol



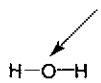
*DMS, EMS



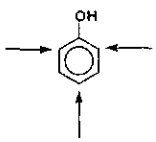
EDT



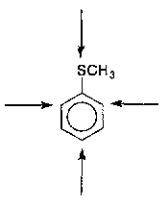
H_2O



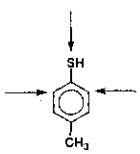
Phenol



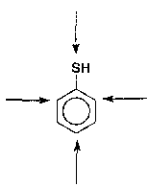
Thioanisole



p-Thiocresol



Thiophenol



*Because of its lower vapor pressure, DMS is often used instead of EMS. Both produce a highly disagreeable odor.

TFA

Not as effective as EDT. Scavenges t-butyl trifluoroacetate. A poor scavenger for t-butyl carbonium ions. Forms an adduct with benzyl and t-butyl carbonium ions.

Same as phenol but less reactive.

Prevents oxidation of Met. Scavenges t-butyl trifluoroacetate.

The most efficient scavenger for t-butyl trifluoroacetate. Scavenges Trt protecting groups. Minimizes alkylation of Trp by Arg(Mtr) and Arg(Pmc). Use with a second scavenger (i.e., H_2O) to completely prevent Trp t-butylation. Can irreversibly form an adduct with Trp and TFA.

Works well with TFA when t-butyl and/or Boc groups are the only protecting groups. Essential when Arg(Pmc) is used in the presence of Trp and Tyr. Use with EDT to scavenge t-butyl groups in the presence of Trp.

A t-butyl trifluoroacetate and t-butyl carbonium ion scavenger.

Accelerates deprotection of Arg(Pmc) by TFA. Partially deprotects Cys(t-Bu, S-t-Bu, and Acm). Prevents Met(O) formation. Can be used as a Trt scavenger, although EDT is preferred. Forms an adduct that alkylates Met and Trp. Scavenges t-butyl trifluoroacetate.

See phenol.

Scavenges t-butyl trifluoroacetate and Trt. Forms a reversible adduct with t-butyl groups.

HF

One of the most common scavengers used. Can be acylated by glutamyl residues. Effective with DMS when Cys is present.

Reportedly reduces Met(O) to Met. Prevents alkylation of Met. Minimizes formation of Met(O) during cleavage.

Acts as a reducing agent to minimize dimer aggregates of Cys.

Reacts violently with HF. Do not use!

Preferable to anisole for prevention of c-alkylation of Tyr. Thioanisole-cation adducts are not stable and can undergo reversible reactions to alkylate other amino acids (for example, Met and Trp).

Routinely used for cleavage in our labs. Use with DMS in "Low-High" procedure to deprotect Trp(CHO)[21]

Routinely used for cleavage in our labs. Use with DMS in "Low-High" procedure to deprotect Trp(CHO)[21]

Intr

TFMSA	TMSOTf
No effect when used with thioanisole.	
No effect when used with thioanisole.	Protects Tyr and Trp when used with EDT.
Reportedly reduces Met(O) to Met.	
Acts as a reducing agent to minimize dimer aggragates of Cys. Removes the formyl group of Trp under low-high conditions.	Required for deformylation of Trp(CHO). Protects Trp and Tyr when used with m-cresol.
Reacts violently with TFMSA. Do not use!	
Lowers overall cleavage yield by 25-90% when used as a substitute for thioanisole.	
Accelerates acidolysis. Required for efficient cleavage from PAM and MBHA resins.	Accelerates acidolysis. Required for efficient cleavage from PAM and MBHA resins.
Not recommended with peptides containing Cys-Cys.	
Deleterious effect on cysteinyl peptides.	

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