Practical Guide to Solid Phase Peptide Synthesis Chemistry

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aapptec Practical Guide to Solid Phase Peptide Synthesis

Introduction

The purpose of this guide is to provide practical information for planning and executing successful solid phase peptide syntheses. The procedures included were found to be generally applicable, but they may not be optimal in every synthesis. Various factors, including the production scale, peptide sequence and length of the peptide might require modification of these procedures for best results. In critical applications, if time and materials permit, small-scale tests are recommended. Before preparing any peptide on a large scale, it should be synthesized on a small scale first to identify and rectify potential problems.

Many books covering the theory and practice of solid phase synthesis have been published. The following are a few of the recent publications.

Methods of Enzymology, 289, Solid Phase peptide Synthesis, (G. B. Fields Ed.) Academic Press, 1997.

Chemical Approaches to the Synthesis of Peptides and Proteins, P. Lloyd-Williams, F. Albericio, and E. Giralt Eds), CRC Press, 1997.

Fmoc Solid Phase Peptide Synthesis, A Practical Approach, (W. C. Chan, P. D. White Eds), Oxford University Press, 2000.

Solid Phase Synthesis, A Practical Guide, (S. F. Kates, F Albericio Eds), Marcel Dekker, 2000.

P. Seneci, Solid-Phase Synthesis and Combinatorial Technologies, John Wiley & Sons, 2000.

Houben-Weyl E22a, Synthesis of Peptides and Peptidomimetics (M. Goodman, Editor-in-chief, A. Felix, L. Moroder, C. Tmiolo Eds), Thieme, 2002, p. 665ff.

N. L. Benoiton, Chemistry of Peptide Synthesis, CRC Press, 2005.

J. Howl, Methods in Molecular Biology, 298, Peptide Synthesis and Applications, (J. Howl Ed) Humana Press, 2005.

Brief Outline and History of Solid Phase Peptide Synthesis

History

Bruce Merrifield developed, and was awarded the Nobel Prize for, solid phase peptide synthesis. By anchoring the C-terminal amino acid of the peptide to be synthesized to an insoluble resin support, he was able to use reagents in large excess to drive reactions to completion, then cleave the peptide from the support in relatively pure form. Utilizing a resin support also allowed him to automate the peptide synthesis process. These advances made it practical to synthesize larger, more complex peptides. The easy availability of synthetic peptides has revolutionized research in biology, biochemistry, microbiology, medicinal chemistry and new drug development.

SYNTHESIS NOTES

Some of the significant events are listed below:

1963	Merrifield developed solid phase peptide synthesis on crosslinked polystyrene beads.
1964	Merrifield introduces the Boc/Bzl protection scheme in peptide synthesis.
1967	Sakakibara introduces HF cleavage.
1968	First automated solid phase synthesizer
1970	Pietta and Marshall introduce BHA resin for preparing peptide amides, Carpino and Han introduce the base labile Fmoc protecting group.
1973	Wang develops p-alkoxybenzyl alcohol resin (Wang resin)
1976	Burgus and Rivier utilize preparative reverse phase HPLC to purify peptides synthesized by solid phase methodology.
1977	Barany and coworkers develop the concept of orthogonal protection.
1978	Fmoc/tBu strategy utilizing Wang resin is developed by Meienhofer and coworkers,
1983	First production solid phase peptide synthesizer with preactivation of amino acids.
1985	Simultaneous parallel peptide synthesis, synthesis of peptide libraries.
1987	Rink introduces a TFA labile resin (Rink resin) for preparing peptide amides by Fmoc protocols, Sieber introduces xanthenyl linker (Sieber resin) for preparing fully protected peptide amides by Fmoc protocols.
1987	First commercial multiple peptide synthesizer
1988	First commercial large-scale synthesizer; Barlos and coworkers introduce 2-Chlorotritylchloride resin for preparing fully protected peptide acids by Fmoc protocols.
1988	Introduction of split-mix synthesis for preparation of large combinatorial peptide libraries.
1992	Kent and Alewood develop the Fast Boc protocol.
1993	Solid phase organic synthesis and combinatorial chemistry for rapid preparation of small molecule libraries.
2003	Stepwise preparation of long peptides (approximately 100 AA) by Fmoc protocols.

Overview of Solid Phase Peptide Synthesis

General Solid Phase Peptide Synthesis Scheme

The general process for synthesizing peptides on a resin starts by attaching the first amino acid, the C-terminal residue, to the resin. To prevent the polymerization of the amino acid, the alpha amino group and the reactive side chains are protected with a temporary protecting group. Once the amino acid is attached to the resin, the resin is filtered and washed to remove byproducts and excess reagents. Next, the N-alpha protecting group is removed in a deprotection process and the resin is again washed to remove byproducts and excess reagents. Then the next amino acid is coupled to the attached amino acid. This is followed by another washing procedure, which leaves the resin-peptide ready for the next coupling cycle. The cycle is repeated until the peptide sequence is complete. Then typically, all the protecting groups are removed and the peptide resin is washed, and the peptide is cleaved from the resin.
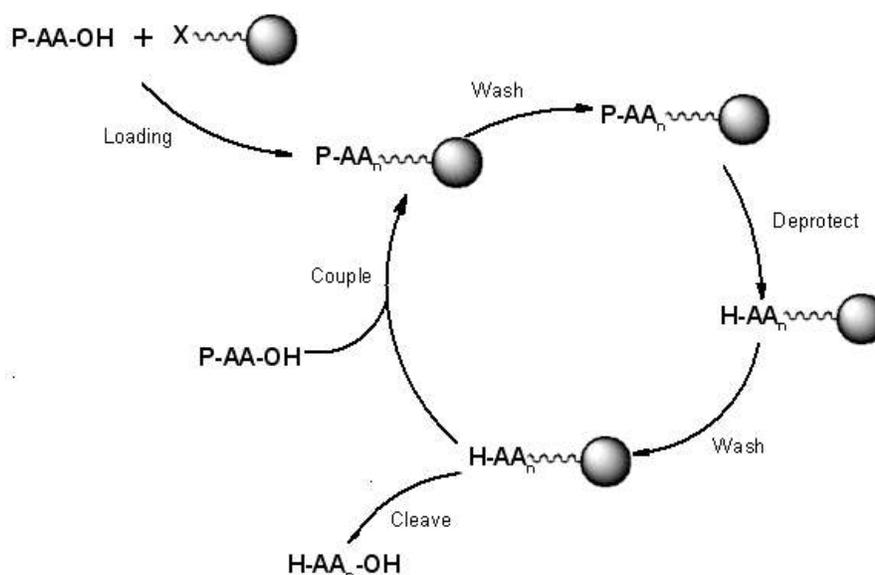
Figure 1 – General Solid Phase Peptide Synthesis Cycle

Selective Protection

The side chains of many amino acids are reactive and may form side products if left unprotected. For successful peptide synthesis, these side chains must remain protected despite repeated exposure to N alpha deprotection conditions. Ideally, the N alpha protecting group and the side chain protecting groups should be removable under completely different conditions, such as basic conditions to remove the N alpha protection and acidic conditions to remove the side chain protection. Such a protection scheme is called "orthogonal" protection.

Boc/Bzl Protection

In the Boc/Bzl protection scheme, Boc protecting groups are used to temporarily protect the N alpha nitrogen groups of the amino acids and benzyl-based protecting groups provide more permanent protection of sidechains. Boc and benzyl-based protecting groups are both acid labile, so Boc/Bzl is not a true orthogonal protection scheme. It is practically utilized though, because the Boc group is removed

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under moderate conditions (50% TFA in DCM) while benzyl-based protection groups require very strong acids, such as HF or TFMSA, to remove them.

Boc Deprotection Mechanism

As shown in the mechanism below, *tert*-butyl carbonium ions are formed during Boc-deprotection. These cations react further with nucleophiles, forming isoprene or *tert*-butyl adducts. Tryptophan (Trp), cysteine (Cys) or methionine (Met) residues within a peptide can react with tert-butyl carbonium ions and produce undesired peptide side products. Adding 0.5% dithioethane (DTE) to the cleavage solution scavenges the tert-butyl cations and prevents the formation of peptide side products.
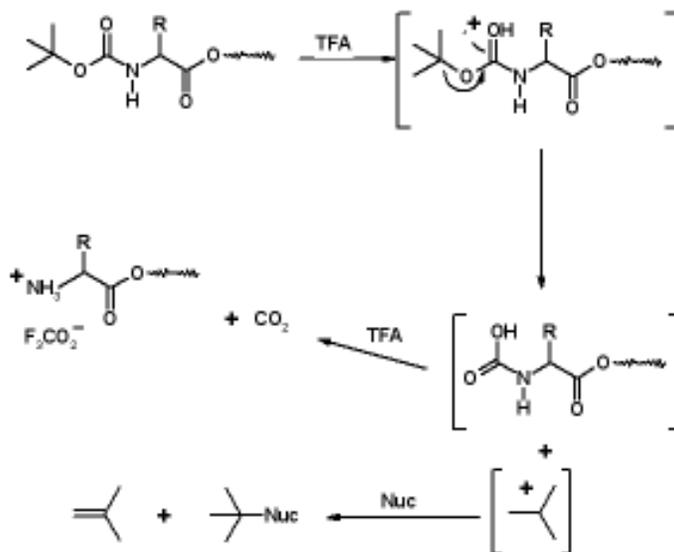

Figure 2 – Boc Deprotection Mechanism

After the Boc group has been removed by treatment with TFA, the deprotected amine is in the form of a TFA salt. The salt must be converted to the free amine before the next amino acid can be coupled. Typically this is achieved by treating the resin-peptide with a 50% solution of diisopropylethylamine (DIEA) in dichloromethane (DCM), followed by several washes.

Castro and coworkers have reported using an in situ neutralization procedure with BOP/DIEA.[1] Kent and Alewood have developed in situ neutralization with HATU or HBTU coupling.[2] In addition to saving time through eliminating the separate neutralization and washing procedures, in situ neutralization can improve coupling yields when aggregation causes problems. Since aggregation occurs mainly in the neutral resin-peptide, in situ neutralization presumably minimizes aggregation by minimizing the period of time that the deprotected resin-peptide is in the neutral state.

Fmoc/tBu Protection

In this protection scheme, the alpha nitrogen of the amino acids is protected with the base labile Fmoc group and the side chains are protected with acid labile groups based either on the tert-butyl protecting

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[1] Le-Nguyen, D; Heitz, A; Castro, B, *J. Chem Soc., Perkin Trans 1*, **1987**, 1915-1919.

[2] Schnölzer, M; Alewood, P; Jones, A; Alewood, D; Kent, SBH, *Int. J. Peptide Protein Res*., **1992**, *40*, 180-193.

group or the trityl (triphenylmethyl) group. This is an orthogonal protection system, since the side chain protecting groups can be removed without displacing the N-terminal protection and visa versa. It is advantageous when sidechains need to be selectively modified, as when the peptide is selectively labeled or cyclized through the side chain.

Fmoc Deprotection Mechanism

The Fmoc group is removed when a base abstracts the relatively acidic proton from the fluorenyl ring system, leading to β-elimination and the formation of dibenzofulvene and carbon dioxide. Dibenzofulvine is a reactive electrophile and would readily attach irreversibly to the deprotected amine unless it was scavenged. Secondary amines such as piperidine add to dibenzofulvene and prevent deleterious side reactions. Hence piperidine is typically used to remove the Fmoc group and also scavenge the dibenzofulvene by-product.

A report on utilizing 5% piperidine solution to remove Fmoc protecting groups from resin bound amino acids was recently published.[3] In the prepararation of a poly-alanine peptide, the time required to remove the the Fmoc group from the first five alanine residues was between 20 and 30 minutes. For the next five alanine residues (Ala6 through Ala10) the deprotection time jumped to 100 to 170 minutes, probably due to aggregation. Recently reported ooptimized fast Fmoc protocols utilize piperdine deprotection of three minutes or less.[4]

1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) removes the Fmoc protecting group much faster than piperidine.[5] When Fmoc deprotection during a peptide synthesis is slow or incomplete, replacing piperidine with DBU can improve the deprotection yield and thus increase the yield of desired peptide[6]. Since DBU is non-nucleophilic and will not react with the fulvulene byproduct, piperidine is often added just to react with this byproduct.[7] DBU should not be used when aspartic acid (Asp) residues are part of the peptide-resin for DBU catalyzes aspartimide formation with subsequent reaction with piperidine.

[3] Zinieris, N.; Leondiadis, L.; Ferderigos, N. *J. Comb. Chem*. **2005**, *7*, 4 – 6.
[4] Hood, C. A.; Fuentes, G.; Patel, H.; Page, K.; Menakuru, M.; Park, J. H. *J. Pept. Sci.*, **2008,** *14,* 97-101.
[5] Villain, M.; Jackson, P. L.; Krishna, N.R.; Blalock, J.E. in "Frontiers of Peptide Science, Proceedings of the 15th American Peptide Symposium Nashville, TN,. 1997" (J.P. Tam and P.T.P. Kaumaya, Eds.)., Kluwer Academic Publishers, Dordrecht, 1999, 255-256.
[6] J. Wade, et al. *Pept. Res*., **1991**, *4*, 194; Dettin, M.,et al., *J. of Pept. Res.* **1997**, *49*, 103.
[7] Fields et al. *J. Biol. Chem.,* **1993**, *268*, 14153.

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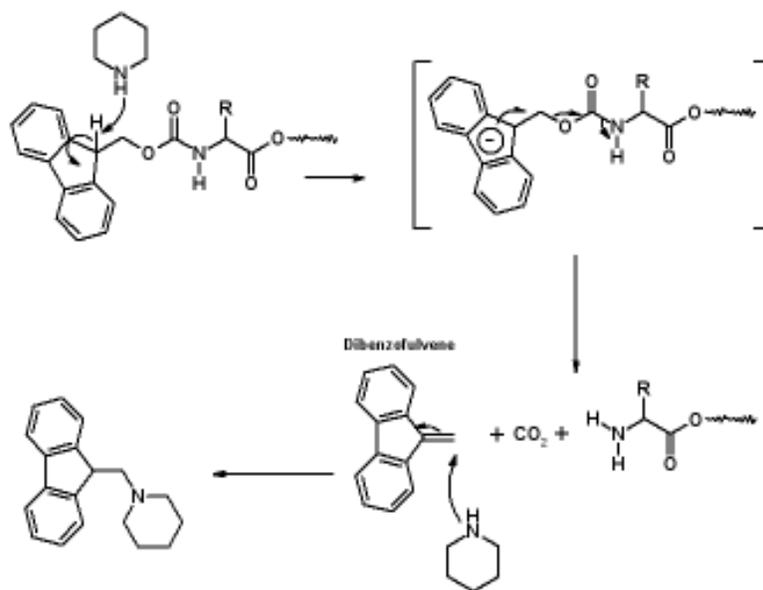**Fax: 502.986.3338   info@aapptec.com**

Figure 3 – Fmoc-Deprotection Mechanism

Ligation and Fragment Condensation

Although solid phase peptide synthesis methodology has improved to the point where preparing peptides of up to 100 amino acids is feasable,[8] larger peptides and small proteins, as yet, are not accessable by solid phase peptide synthesis alone. Much larger products can be assembled by coupling protected peptide segments in solution. The synthesis of the 238-amino acid precursor of green fluorescent protein is an outstanding example.[9] This technique is often hampered by insolubility of the protected peptide segments.

Native chemical ligation is a method for coupling unprotected peptide segments in aqeous solution. In this methodolgy, a peptide with an unprotected N-terminal cysteine reacts with a peptide thioester forming an S-acyl intermediate which then undergoes S-N acyl shift to form a standard peptide bond.[10] Native chemical ligation has proven very useful for preparing large peptides[11] or complex peptides such as glycopeptides.[12] The requirement for cysteine residues in appropriate positions within the target product

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[8] White, P.; Keyte, J. W.; Bailey, K.; Bloomberg, G. *J. Pept. Sci.*, **2003**, *10*, 18-26; Kakizawa, T.; Koide-Yoshida, S.; Kimura, T.; Uchimura, H.; Hayashi, Y.; Saito, K.; Kiso, Y. *J. Pept. Sci.*, **2008**, *14*, 261-266.

[9] Nishiuchi, Y.; Inui, T.; Nishio, H.; Bódi, J.; Kimura, T.; Tsuji, F. I.; Sakakibara, S*. Proceed. Natl. Acad. Sci. U.S.A.*, **1998**, *95*, 13549-13554.

[10] Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. *Science*, **1994**; *266*, 776 - 779.

[11] Li, X.; de Leeuw, E.; Lu, W. *Biochemistry*, **2005**, *44*, 14688 –14694; Durek, T.; Torbeev, V.Y.; Kent, S. B. H. *Proceed. Natl. Acad. Sci. U.S.A.*, **2007**, *104*, 4846-4851.

[12] Shin, Y.; Winans, K. A.; Backes, B. J.; Kent, S. B. H.; Ellman, J. A.; R. Bertozzi, C. R. *J. Am. Chem. Soc.*, **1999**, *121*, 11684 –11689; Dudkin, V. Y.; Miller, J. S.; Danishefsky, S. J. *J. Am. Chem. Soc.,* **2004**, *126*, 736 –738; Yang, Y.-Y.; Ficht, S.; Brik, A.; Wong, C.-H. *J. Am. Chem. Soc.*, **2007**, *129*, 7690-7701.

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currently is a limitation of native chemical ligation, but a number of methods are being studied to extend the utility of the method.[13]

Staudinger ligation is another promising method for assembling peptide segments. The Staudinger ligation couples a peptide thioester with an azide via a phosphinothioester intermediate.[14] To illustrate the potential of Staudinger ligagation and native chemical ligation, both methods were used together to assemble functional ribonuclease A.[15]

Equipment for Solid Phase Peptide Synthesis

Manual Synthesis

Manual solid phase peptide synthesis can be carried out with standard laboratory glassware (i.e. round bottom flasks, sintered glass funnels, etc.) that has been treated with a silylating agent to prevent the resin from sticking to the glass surfaces. Orbital shakers, wrist-action shakers or overhead mechanical stirrers may be used to agitate the resin suspensions. Magnetic stirrers should not used because the resin beads can be damaged if they come between the stirring bar and the inside surface of the flask.

While manual solid phase peptide synthesis can be performed with standard laboratory glassware, the repeated transfers required for filtering and washing in each amino acid coupling cycle are time consuming and may lead to resin loss from spillage or incomplete transfer of the resin. A variety of specialized reactors for manual peptide synthesis are available. Generally, they incorporate a glass frit for filtering and washing resin without transferring it from the vessel and ports for added reaction solutions while maintaining an inert gas atmosphere. As a rule, these reactors are fitted into a wrist action shaker to provide agitation.

Manual synthesizers like the aapptec LabMate™ incorporate reactors and a shaker into a convenient, compact unit. Most manual synthesizers have a number of reactors that allow the user to prepare multiple peptides at the same time.

Automated Synthesizers

Automated synthesizers may be classified as one of three types: batch synthesizers, continuous flow synthesizers, and parallel synthesizers. Batch synthesizers such as the aapptec Endeavor 90™ can only prepare one or two peptides at a time, but can prepare them on a large scale. Depending on the design of the instrument, batch synthesizers can prepare peptides in up to kilogram scales. These instruments use inert gas bubbling or mechanical agitation to provide mixing.

Continuous flow synthesizers use pumps to recirculate the process solution through a column containing the resin. These synthesizers typically require special resins with low swelling. Usually some automatic

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[13] Crich, D.; Banerjee, A. *J. Am. Chem. Soc.*, **2007**, *129,* 10064 –10065; Pentelute, B. L.; Kent, S. B. H. *Org. Lett.*, **2007**, *9*, 687 –690; Tchertchian, S.; Hartley, O.; Botti, P. *J. Org. Chem.*, **2004**, *69*, 9208 – 9214; Yan, L. Z.; Dawson, P. E. *J. Am. Chem. Soc.*, **2001**, *123*, 526 –533; Canne, L. E.; Bark, S. J.; Kent, S. B. H. *J. Am. Chem. Soc.*, **1996**, *118*, 5891 –5896.

[14] Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.,* **2000**, *2*, 1939 –1941; Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.,* **2001**, *3*, 9 –12; Soellner, M. B.; Tam, A.; Raines, R. T. *J. Org. Chem.* **2006**, *71,* 9824 –9830.

[15] Nilsson, B. L.; Hondal, R. J.; Soellner, M. B.; Raines, R. T. *J. Am. Chem. Soc.*, **2003**, *125,* 5268 –5269.

monitoring method is used to track the progress of the reactions. When the monitoring indicates the reaction is complete, the next process step is initiated. Since the reaction solution constantly flows through the resin, continuous flow synthesizers do not require a mechanical means of agitation.

Parallel synthesizers like the ααρτες Apex 396™ and Matrix 384™ excel in preparing numerous peptides in small scale simultaneously. The Apex 396 can prepare up to 96 individual peptides at once and the Matrix 384 can prepare up to 384 different peptides at a time. Parallel synthesizers often are utilized to prepare peptides for epitope mapping and structure-activity studies. Parallel synthesizers can also be used to prepare larger quantities of peptide by running parallel syntheses of the same sequence.

Resins for Solid Phase Synthesis

Core Resins

Polystyrene

Polystyrene is the most common core resin in solid phase chemistry, but other core matrices include polyacrylate, polyacrylamide, and polyethylene glycol.[16] Uncrosslinked, or linear, polystyrene will dissolve in hydrophobic solvents and precipitate in protic solvents. This interesting property has been exploited by Janda in a synthesis of prostaglandin $F_{2\alpha}$.[17] Most polystyrene supports used in solid phase chemistry, however, contain 1% or 2% divinylbenzene (DVB) as a crosslinking agent. These cross-linked polystyrenes are insoluble in all common solvents. Typically, these resins are prepared and utilized as small, spherical beads.

Even though the cross-linked polystyrene resins are insoluble in organic solvents, they are solvated and swollen by aprotic solvents such as toluene, dimethylformamide (DMF), and dichloromethane (DCM). One gram of 1% DVB cross-linked resin will swell 4 to 6 times its original volume in DCM. In contrast, one gram of 2% DVB cross-linked resin swells only 2 to 4 times its original volume in DCM. The swelling factor is important in solid phase synthesis, since reaction kinetics is diffusion controlled. Consequently, resin that swells more will have a higher diffusion rate of reagents into the core of the matrix, resulting in shorter reaction times and more complete chemical conversions. Typical swelling factors of 1% crosslinked polystyrene in a selection of common solvents is listed below.

Swelling factor of 1% crosslinked polystyrene in various solvents (mL/g of resin):

THF	5.5	**Et$_2$O**	3.2
Toluene	5.3	**CH$_3$CN**	4.7
CH$_2$Cl$_2$	5.2	**EtOH**	5.0
Dioxane	4.9	**MeOH**	1.8
DMF	3.5	**Water**	1.0 (no swelling)

Polystyrene beads are available in sizes ranging from less than a micron to 750 microns in diameter. Reaction kinetics is generally faster using smaller beads due to the higher surface area to volume ratio. In practice, however, too small a bead can lead to extended filtration times. Beads in the range of 75 to 150 microns in diameter offer a good balance of reaction kinetics versus reliability. Bead size is commonly reported in Tyler Mesh size, which is inversely proportional to the nominal diameter. Two

[16] García-Martín, F.; Quintanar-Audelo, M.; García-Ramos, Y.; Cruz, L. J.; Gravel, C.; Furic, R.; Côté, S.; Tulla-Puche, J.; Albericio, F. *J. Comb. Chem.,* **2006**, *8,* 213 –220.

[17] Chen, S.; Janda, K. D. *Tetrahedron Lett.* **1998**, *39*, 3943-3946.

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Cleavage with Sodium Methoxide (NaOMe)[196]

1. Prepare a saturated solution of NaOMe in MeOH (5 mL/ g resin).
2. Use the saturated NaOMe solution to prepare a 4:1 (v/v) mixture of THF and saturated NaOMe/MeOH. Prepare approximately 25 mL of this solution per gram of resin.
3. Suspend the resin in the THF/MeOH/NaOMe mixture for 3 hours at room temperature.
4. Filter the resin. Acidify the filtrate with anhydrous HCl in THF.
5. Evaporate the organic solvents. Mix the residue with ethyl ether (Et_2O). Separate the Et_2O solution from the insoluble inorganic salts. Evaporate the Et_2O to recover the product.

Cleavage Cocktails

"Odorless" Cleavage Cocktail (Reagent B)[197]

In place of highly odorous ethane dithiol and thioanisole, triisopropylsilane is used in Reagent B to scavenge cationic species. Reagent B is especially useful when the resin-product contains trityl-based protecting groups. Reagent B will not prevent oxidation of methionine residues during cleavage.

Composition of Reagent B

    trifluoroacetic acid (88% v/v)
    phenol (5% v/v)
    water (5% v/v)
    triisopropylsilane (2% v/v)

    Use immediately after preparation

Cleavage with Reagent B

1. If necessary, remove any Fmoc-protecting groups using the standard Fmoc deprotection procedure.
2. Wash the resin with dichloromethane.
3. Suspend the resin in Reagent B (100 μL/mg of peptide resin)
4. Stir the mixture for 1 hour at room temperature.
5. Filter and wash the resin with a little additional Reagent B.
6. Cool the combined filtrates to 4°C and add cold methyl t-butyl ether (3 times the volume of the combined filtrates) to precipitate the crude peptide.

Cleavage Cocktail for Methionine Containing Peptides (Reagent H)

A cleavage cocktail containing trifluoroacetic acid, phenol, thioanisol, 1,2-ethanedithiol, dimethylsulfide, ammonium iodide and water has been shown to prevent methionine oxidation during cleavage.[198] Dimethylsulfoxide and iodine are generated from the reduction of methionine sulfoxide, so peptides containing Cys(Trt) residues can be isolated as linear peptides or as cyclized products upon extended treatment with the cleavage cocktail.

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[196] Based on procedures in Kurth, M.J.; Ahlberg, L.A.; Takenouchi, K.J. *J.Org. Chem*. **1996**, *61*, 8755-8761.
[197] Solé, N. A.; Barany, G. *J. Org. Chem.* **1992**, *57*, 5399-5403.
[198] Huang, H.; Rabenstein, D. L. *J. Peptide Res.* **1999**, *53*, 548-553.

Composition of Reagent H

        trifluoroacetic acid (81% w/w),
        phenol (5% w/w)
        thioanisole, (5% w/w)
        1,2-ethanedithiol (2.5% w/w)
        water (3% w/w)
        dimethylsulfide (2% w/w)
        ammonium iodide (1.5% w/w)

Peptide Cleavage (Without Disulfide Formation)

1. If necessary, remove Fmoc-protecting groups using the standard Fmoc removal procedure.
2. Suspend the peptide resin in the cleavage cocktail (30 mL/g resin).
3. Allow the mixture to stand three hours at room temperature under inert gas.
4. Filter and wash the resin with trifluoroacetic acid.
5. Combine the filtrates and add methyl *tert*-butyl ether to precipitate the crude product.

Peptide Cleavage with Disulfide Formation

1. Suspend the peptide resin in the cleavage cocktail (30 mL/g resin).
2. Allow the mixture to stand ten hours at room temperature.
3. Filter and wash the resin with trifluoroacetic acid.
4. Combine the filtrates and add methyl *tert*-butyl ether to precipitate the crude product.

Cleavage Cocktail for Peptides Containing Cys, Met, Trp and Tyr Residues (Reagent K)[199]

This cleavage cocktail is commonly used to cleave peptides containing combinations of sensitive residues such as cysteine, methionine, tryptophan and tyrosine. Since this reagent is suitable to most of the sensitive amino acid residues, it is often used as a general cleavage reagent. This reagent is recommended when peptides prepared on PAL or BAL resins contain tryptophan residues.

Composition of Reagent K

        trifluoroacetic acid (82.5% v/v)
        phenol (5% v/v)
        water (5% v/v)
        thioanisole (5% v/v)
        1,2-ethanedithiol (2.5% v/v)

 Cleavage and Deprotection with Reagent K

1. If necessary, remove and Fmoc protecting groups using the general Fmoc removal procedure.
2. Suspend the resin in Reagent K (10 mL to 40 mL per gram of peptide resin)
3. Stir at room temperature for 1 to 2.5 hours. Peptides containing multiple arginine residues may require longer for complete deprotection.
4. Filter and rinse the resin with trifluoroacetic acid.
5. Concentrate the combined filtrates to a syrup under reduced pressure.
6. Dissolve the syrup in a minimum volume of trifluoroacetic acid.

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[199] King, D.; Fields, C. G.; Fields, G. B. *Int. J. Peptide Protein Res.* **1990**, *36*, 255-266.

7.   Add the solution to methyl t-butyl ether (approximately 300:1 v/v methyl t-butyl ether: peptide solution).
8.   Filter or centrifuge to collect the crude peptide.

Low Odor Cleavage Cocktail (Reagent L)[200]

In this cleavage cocktail, dithiothreitol (DTT) replaces the pungent scavengers ethanedithiol (EDT) and thioanisole. Additionally, unlike EDT, DTT does not react readily with the benzophenone moiety of 4-benzoylphenylalanine (Bpa), making Reagent L a preferred cleavage cocktail for Bpa containing peptides.

Composition of Reagent L

    trifluoroacetic acid (TFA, 88% v/v)
    triisopropylsilane (TIS, 2% v/v)
    dithiothreitol (5% w/v)
    water (5% w/w)

    Use immediately after preparation

Cleavage and Deprotection with Reagent L

1.   If necessary, remove Fmoc-protecting groups using the standard Fmoc removal procedure.
2.   Suspend the resin in Reagent L (5 ml to 10 mL/g resin).
3.   Allow the mixture to stand at room temperature for 90 minutes.
4.   Filter the resin.
5.   Wash the resin with TFA (5 mL to 10 mL/g resin).
6.   Combine the filtrates and add cold ether to precipitate the crude peptide.

Cleavage Cocktail for Peptides Containing Arg Residues (Reagent R)[201]

Reagent R is especially suited for cleaving and deprotecting peptides that contain arginine residues protected with sulfonyl protecting groups. This reagent also is recommended for tryptophan-containing peptides prepared on PAL or BAL resin, for it minimizes reattachment of the peptide to the linker at the tryptophan residue.

Composition of Reagent R

    trifluoroacetic acid (90% v/v)
    thioanisole (5% v/v)
    1,2-ethanedithiol (3% v/v)
    anisole (2% v/v)

    Use immediately after preparation

Cleavage and Deprotection with Reagent R

1.   If necessary, remove Fmoc-protecting groups using the standard Fmoc removal procedure.

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[200] Bonner, A. G.; Udell, L. M.; Creasey, W. A.; Duly, S. R.; Laursen, R. A. *J. Peptide Res.* **2001**, *57*, 48-58.
[201] Albericio, F.; Kneib-Cordonier, N.; Biancalana, S.; Gera, L.; Masada, R.I.; Hudson, D.; Barany, G. *J.Org.Chem.* **1990**, *55*, 3730-3743.

2.   Suspend the resin in Reagent R (10 mL/g of peptide resin).
3.   Allow the mixture to stand at room temperature for 2 hours.
4.   Filter the resin.
5.   Wash the resin with Reagent R (2 x 5 mL /g resin).
6.   Combine the filtrates and add cold ether to precipitate the crude peptide.

Amino Acid Sidechain Deprotection

Deprotection of Arg(Mtr) in Fmoc Peptide Synthesis[202]

1.   If necessary, remove the Fmoc group and cleave the peptide from the resin by standard protocols.
2.   Dissolve the peptide in 5% (w/w) phenol/TFA (approximately 10 μmol/mL).
3.   Monitor the cleavage of the Mtr group by HPLC.
4.   After the cleavage is complete (approximately 7.5 hours) evaporate the solution to dryness.
5.   Partition the residue between water and dichloromethane.
6.   Wash the aqueous layer with dichloromethane (4 times).
7.   Lyophilize the aqueous layer to obtain the crude peptide.

Deprotection of Arg(Pmc) and Arg(Pbf)

This procedure will also remove *tert*-butyl based protecting groups and trityl based groups.

1.   Dissolve the peptide in 95% TFA/water (v/v, approximately 5 to 10 mL per gram of protected peptide) containing scavengers appropriate for the amino acid composition of the peptide.
2.   Stir the mixture at room temperature for 20 to 30 minutes. If the peptide contains multiple Arg residues, deprotection may take longer.
3.   Slowly add ice-cold ether, methyl tert-butyl ether, or diisopropyl ether (approximately ten times the volume of TFA).
4.   Filter the precipitate and wash it with a little cold ether to obtain the crude product.

Deprotection of Cys(Acm)

The S-acetamidomethyl group can be removed using mercury (II) acetate or silver (I) tetrafluoroborate. This group can also be removed with simultaneous oxidation to disulfides using iodine.

Removal with Mercury(II) Acetate[203]

S-t-Butyl and S-trityl protecting groups may be removed under these conditions.

1.   Dissolve the protected peptide in water or 10% (v/v) aqueous acetic acid (100 μL/mg peptide) and carefully adjust the pH to 4.0 with glacial acetic acid.
2.   Add, with stirring, 1.0 equivalent of mercury (II) acetate per S-acetamidomethyl group in the peptide.
3.   Readjust the pH of the solution to 4.0 with acetic acid or aqueous ammonia. Stir the mixture at room temperature for 1 hour under an inert atmosphere.
4.   Add beta-mercaptoethanol (0.5 mL per 100 μmol of peptide) and let the mixture stand at room temperature for 5 hours.

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[202] Atherton, E.; Sheppard, R. C.; Ward, P. *J. Chem. Soc., Perkin Trans.1* **1985**, 2065-2073.
[203] Marbach, P.; Rudinger, J. *Helv.Chim.Acta* **1974**, *57*, 403-414.

5. Centrifuge the mixture to remove the precipitate. Desalt the supernatant containing the crude peptide under an inert atmosphere then lyophilize.

Removal with Ag(I) Salts[204]

S-Trityl and S-p-methoxybenzyl groups may be partially removed by this procedure.

1.   Dissolve the protected peptide in cold (4 ºC) trifluoroacetic acid (200 μL/ μmole peptide). Add anisole (4 μL/μmol peptide) to the solution.
2.   Add 20 equivalents of silver salt (AgOTf or AgBF$_4$) per S-acetamidomethyl group.
3.   Stir the mixture at 4 ºC for 1.5 hours, then add cold ether to precipitate the peptide silver salt.
4.   Centrifuge to isolate the precipitated peptide silver salt. Suspend the precipitate in 1 M aqueous acetic acid. Add dithiothreitol (40 equivalents per acetamidomethyl group) and mix at room temperature for 3 to 4 hours.
5.   Centrifuge to remove solids. Desalt the supernatant under an inert atmosphere. Lyophilize the desalted supernatant to obtain the crude peptide.

Iodine Oxidation[205]

S-Trityl protecting groups may also be removed under these conditions.

1.   Dissolve the protected peptide in methanol (1.25 μL/ μmol).
2.   Add 0.4 M methanolic iodine solution (2.5 equivalents per acetamidomethyl group). Stir vigorously for 30 minutes.
3.   Add 1 M aqueous ascorbic acid or sodium thiosulfate solution (100 μL/μmol peptide). Evaporate under reduced pressure to approximately one third of the original volume.
4.   Desalt and lyophilize the crude peptide.

Deprotection of Cys(But)

The S-t-butyl group is stable to trifluoroacetic acid, so it can be used with either Boc or Fmoc chemistries. It can be removed with trifluoromethanesulfonic acid (TFMSA) or mercury (II) acetate. The TFMSA method is usually used with Boc chemistry to simultaneously cleave the peptide from the resin and remove the S-t-butyl group from cysteine.

Standard Trifluoromethanesulfonic Acid Procedure

1.   If the peptide contains His(Dnp), remove the Dnp group. If the peptide contains Trp(CHO), remove the N-terminal BOC group then remove the formyl group.
2.   Check that the peptide-resin has been washed and thoroughly dried.
3.   Transfer the resin into a round bottom flask equipped with a stirring bar. For every 100 mg of peptide-resin add 200 mL of thioanisole and 100 mL of ethandithiol.   Cool the flask in an ice bath and add 2 mL of TFA for every 100 mg of resin. Stir for 5 to 10 minutes.
4.   For every 100 mg of resin slowly add 200 mL of TMSFA dropwise. Stir vigorously during addition of the TFMSA to dissipate the heat generated.
5.   Let the mixture stir at room temperature for 30 to 60 minutes.
6.   Filter the resin with a fine sintered funnel. Wash the resin with a small amount of TFA. Combine the filtrates and add 8-10 times the volume of cold ether. If necessary, keep the mixture at 4°C overnight to precipitate the peptide. Filter the peptide using a fine sintered glass funnel. Wash the crude peptide with cold ether to remove cleavage scavengers.

[204] Fujii, N; Otaka, A.; Watanabe, T.; Okamachi, A.; Tamamura, H.; Yajima, H; Inagaki, Y.; Nomizu, M.; Asano, K. *J. Chem. Soc., Chem. Commun.* **1989**, 283.
[205] Kamber, B.; Hartmann, A.; Eisler, K.; Riniker, B.; Rink, H.; Sieber, P.; Rittel, W. *Helv. Chim. Acta* **1980**, *63*, 899-914.

7.   Desalt the peptide by ion exchange column.

<u>Removal with Mercury(II) Acetate</u>[206]

S-t-Butyl and S-trityl protecting groups may be removed under these conditions.

1.   Dissolve the protected peptide in water or 10% (v/v) aqueous acetic acid (100 μL/mg peptide) and carefully adjust the pH to 4.0 with glacial acetic acid.
2.   Add, with stirring, 1.0 equivalent of mercury (II) acetate per S-acetamidomethyl group in the peptide.
3.   Readjust the pH of the solution to 4.0 with acetic acid or aqueous ammonia. Stir the mixture at room temperature for 1 hour under an inert atmosphere.
4.   Add beta-mercaptoethanol (0.5 mL per 100 μmol of peptide) and let the mixture stand at room temperature for 5 hours.
5.   Centrifuge the mixture to remove the precipitate. Desalt the supernatant containing the crude peptide under an inert atmosphere.

Deprotection of Cys(Trt) with S-S Bond Formation[207]

1.   Dissolve the protected peptide in dichloromethane (DCM) (1 mL/μmol peptide).
2.   Add a 0.1 M solution of iodine in DCM (22 μL/ μmol peptide). Stir 5 minutes at room temperature.
1.   Add 0.2 M citrate buffer containing ascorbic acid (5 mg/ mL) (Add 100 μL of buffer per μmol peptide).
4.   Isolate the peptide by chromatography on a Sephadex column.

Deprotection of His(Dnp)[208]

1.   Suspend the peptide resin in DMF (10 mL/ g of resin).
2.   Add thiophenol and triethylamine (2 mL of each/ g of resin).
3.   Shake the mixture with a mechanical shaker at room temperature for approximately 90 minutes.
4.   Filter the resin and wash it twice with DMF, twice with DCM, and twice with methanol.
1.   Dry the resin *in vacuo* to a constant weight.

Removal of Allyl Based Protecting Groups[209]

1.   Swell the substrate-resin in chloroform ($CHCl_3$).
2.   Suspend the swollen resin in $CHCl_3$ (approximately 35 mL per gram of resin).
3.   Add acetic acid (0.5 mL per gram of resin), N-methylmorpholine (2 mL per gram of resin), and $Pd(PPh_3)_4$ (3 equivalents based on resin substitution).
        Shake the mixture at room temperature for 4 hours.
4.   Filter the resin and resuspend it in $CHCl_3$ (approximately 35 mL per gram of resin).
5.   Add acetic acid (0.5 mL per gram of resin), N-methylmorpholine (2 mL per gram of resin), and $Pd(PPh_3)_4$ (3 equivalents based on resin substitution).
6.   Shake the mixture at room temperature for 12 hours. Filter and wash the deprotected resin product with dichloromethane (DCM).

[206] Marbach, P.; Rudinger, J. *Helv.Chim.Acta* **1974**, *57*, 403-414.
[207] Sieber, P.; Kamber, B.; Riniker, B.; Rittel, W. *Helv. Chim. Acta* **1980**, *63*, 2358-2363.
[208] Based of the procedure in Uhmann, R.; Bayer, E. *Liebigs Ann. Chem.* **1974**, 1955-1964.

[209] Lee, J.; Griffin, J. H.; Nicas, T. I. *J. Org. Chem.* **1996**, *61*, 3983-3986.

Removal of Dde and ivDde Groups[210]

1.   If necessary, replace the N-terminal Fmoc group with Boc.
2.   Prepare a 2% (w/v) solution of hydrazine monohydrate in DMF (25 ml/g of peptide-resin).
3.   Add the hydrazine solution to the flask containing the peptide-resin.
4.   Stopper the flask and allow the mixure to stand t room temperature for 3 minutes.
5.   Filter the resin and repeat the hydrazine treatment twice.
6.   Wash the resin with DMF.

Piperidine Deprotection of Trp(CHO)[211]

1.   Mix piperidine and DMF (1:10 v/v). Prepare 10 mL of the solution per gram of the peptide-resin. Cool the mixture to 0 °C.
2.   Add the peptide resin and stir at 0 °C for 2 hours.
3.   Filter the resin and wash it three times with DMF. Wash the resin 3 times with DCM, then 3 times with methanol. Dry the resin *in vacuo* until a constant weight is achieved.

Post-Cleavage Reduction of Met(O)[212]

Methionine can easily be oxidized to the corresponding sulfoxide and this may occur during peptide synthesis and cleavage. Fortunately, this oxidation can be readily reversed. In some cases, methionine sulfoxide derivatives are used as side-chain protected methionine in peptide synthesis and then are reduced back to methionine residues following synthesis and cleavage.

Method 1

1.   Dissolve the peptide in water (approximately 100 μL/ mg of peptide). Adjust the pH of the peptide solution to 8.0 with triethylamine.
2.   Cool the mixture in an ice bath. Add mercaptoethanol (4 μL/ mg of peptide) and 1 M ammonium fluoride (4 μL/ mg of peptide).
3.   Stir the mixture in an ice bath for 30 minutes.
4.   Lyophilize the solution to obtain the crude product.

Method 2
1.   Dissolve the peptide in 10% v/v aqueous acetic acid (approximately 200 μL to 1000 μL / mg of peptide)
2.   Add 2-10 mg of N-(methyl)mercaptoacetamide.
3.   Warm the solution at 37 °C under inert atmosphere for 24 to 36 hours. The reaction can be monitored by HPLC.
4.   Lyophilize the mixture to obtain the crude peptide.

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[210] Based on the procedures in Bycroft, B. W.; Chan, W. C.; Chhabra, S. R.; Hone, N. D. *J. Chem. Soc., Chem. Commun.* **1993**, 778-779 and Chhabra, S. R.; Hothi, B.; Evans, D. J.; White, P. D.; Bycroft, B. W.; Chan, W. C.; *Tetrahedron Lett.* **1998**, *39*, 1603-1606.

[211] Based on Chowdhury, S. K.: Chait, B. T. *Anal. Biochem.* **1989**, *180*, 387-395.

[212] Yajima, H.; Fujii, N.; Funakoshi, S.; Watanabe, T.; Murayama, E.; Otaka, A. *Tetrahedron* **1988**, *44*, 805-819.

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Post Cleavage Purification and Analysis

Precipitation and Isolation

Analytical HPLC

Most peptides are analyzed on C18 columns such as a 25 cm Spirit™ C-18 Peptide Column for routine analysis. Typical sample concentrations are ~ 1 mg/mL in water or buffer A (0.15% TFA in water). Purity is determined by calculating %purity from comparing peak areas to the total area.
Typical gradient reverse phase HPLC buffers are:

> A = 0.15% TFA in water, and
> B = 0.10% TFA in CH_3CN

Mass Spectroscopy

MW of the product is verified via mass spectroscopy. One common method used lab is direct injection on a single quad instrument with an electrospray interface. Other widely used methods include LC/MS and MALDI techniques. Some ion trapping instruments are also available on which one may obtain sequence data.

Transfer the product from the cleavage block to centrifuge tubes. Fill each tube only ~1/10. Add 9 parts cold diethyl ether (pre-chilled in a dry ice/acetone bath or otherwise reduced to ~ -70°C). At this point the product will crash out of solution forming a fluffy white flocculent. Centrifuge for five minutes at 3300 rpm; a well-formed pellet should result. Decant the supernatant, and resuspend the product in cold ether again. Repeat this process at least 3-4 times, or until the odor of TFA is not detectable. Allow the ether to evaporate slowly overnight from the open centrifuge tube. The resulting dry product is suitable for HPLC analysis or MS.

Washing in this manner will remove TFA as well as deprotection byproducts and excess scavengers. This process tends to remove lower molecular weight impurities such as residual scavengers, and may improve %purity by a couple percentage points. Alternatively, batch scale HPLC purification can be utilized for higher purity.

Yield Calculation

Calculate % yield by comparing the dry mass of the product obtained above to the theoretical yield calculated from the following equation:

$$\text{Theoretical Yield (mg)} = s_{resin} * m_{resin} * MW_{product}$$

where
> s_{resin} = resin substitution in mmol/g
> m_{resin} = resin dry mass in g
> $MW_{product}$ = MW of the product in mg/mmol

HPLC Purification of Peptides

Depending on how the synthesized peptide will be used, the crude peptide cleaved from the resin and isolated may be sufficiently pure. The table below indicates typical applications and purity levels.

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Application	Purity
Tissue culture; ligand for affinity purification; non-quantitative antibody blocking experiments	80% or greater
In vivo studies; bioassays; markers for electrophoresis; monoclonal antibodies	90% or greater
ELISA; RIA; enzyme substrate	95% or greater
NMR; chromatography standards	98%

If the synthesized peptide requires HPLC purification, then a 30-minute gradient from 0% to 70% acetonitrile on a Spirit™ C-18 Peptide Column will usually provide peptide with satisfactory purity. The HPLC solvents should contain 0.1 % trifluoroacetic acid (TFA) which acts as an ion-pairing reagent and improves the shape of the peptide peaks. Long peptides or relatively hydrophobic peptides should be purified on a Spirit™ C-4 orC-8 column. If the crude peptide has impurities that elute close to the product, a shallower gradient, such as 0%-30% acetonitrile or 10%-40% acetonitrile can provide better separation.

The crude peptide should be dissolved in a minimal volume of 0.1% aqueous TFA. If the peptide is not soluble in dilute TFA, it may dissolve in 6M guanidine hydrochloride containing 0.1% TFA. (6M guanidine hydrochloride solution can be prepared by dissolving 1 gram of guanidine in 1 ml of water). The guanidine salts elute in the void volume of the column while the peptide elutes later. Inject the peptide solution onto the HPLC column and monitor the eluant from the column at 220 nm. Collect fractions as the peptide elutes.

Test the fractions and combine all fractions that contain only the pure peptide. The combined fractions can be lyophilized to isolate the purified peptide.

Removing Trifluoroacetic Acid (TFA) From Peptides

Trifluoroacetic acid is toxic. Depending on how the peptide will be used, it may be necessary to exchange TFA for a more biologically benign acid, such as HCl or acetic acid. Recently several common methods of exchanging or removing trifluoroacetate were evaluated.[213] The following procedures can be utilized to efficiently exchange acid anions. The trifluoroacetate, fluoride ,chloride and acetate content of the peptide can be accurately measured by ion chromatography.[214]

 TFA/HCl Exchange

1.   Dissolve the peptide in 100 mM HCl.
2.   Allow the solution to stand at room temperature for 1 minute.
3.   Freeze the solution in liquid nitrogen.
4.   Lyophilize the frozen solution to obtain the peptide hydrochloride salt.

TFA/Acetate Exchange

1.   Prepare a small column (10-fold to 50-fold excess of anion sites in the column relative to anion sites in the peptide) of strong anion exchange resin.
2.   Elute the column with a 1M solution of sodium acetate.
3.   Wash the column with distilled water to remove the excess sodium acetate.

[213] Roux, S.; Zékri, E.; Rousseau, B.; Paternostre, M.; Cintrat, J.-C.;Fay, N. *J. Pept. Sci.* **2008**, *14*, 354-359.
[214] For a sample procedure, see Dionex Application Note 115, "Determination of Trifluoroacetic Acid (TFA) in Peptides", Dionex Corporation, 2002.

4.   Dissolve the peptide in distilled water and apply it to the column.
5.   Elute the column with distilled water and collect the fractions containing the peptide.
6.   Lyophilize the combined peptide containing fractions to obtain the peptide acetate salt.

Storage and Handling of Peptides

1.   Store peptides long term as lyophilized powders in a desiccator at –20 °C. Peptides may be stored 3 months to 5 years.
2.   Peptides may be stored short term in a frozen solution at –20 °C or as a refrigerated lyophilized powder. Peptides can be stored up to 3 months in these conditions.
3.   Before opening weighing peptides, place the vials in a desiccator and allow the vials to warm to room temperature.
4.   Acidic peptides dissolve best in a basic buffer, basic peptides are best dissolved in acidic buffer.
5.   Peptides that contain cysteine, methionine, or tryptophan are sensitive to oxidation. Before dissolving these peptides, degas the buffer under reduced pressure to remove oxygen.
6.   A small amount of dimethylformamide or dimethylsulfoxide can be added to the buffer if the peptide is very hydrophobic.
7.   Use peptide solutions immediately to avoid degradation. Unused portions may be frozen in aliquots and stored short term at –20 °C if the pH of the solution is between pH 5-7. For long tern storage, lyophilize the peptide solutions and store the dry powders at –20°C in sealed vials within a desiccator.
8.   Avoid unnecessary thaw-freeze cycles of peptide solutions. Do not store frozen peptide solutions in a frost-free freezer
9.   For maximum stability, relyophilize unused peptide solutions.

Dissolving Purified Peptides

1.   Dissolve basic peptides in a small amount of 30% acetic acid, then dilute to the required concentration.
2.   Dissolve acidic peptides in a small amount of 10% ammonium bicarbonate, then dilute to the required concentration.
3.   For hydrophobic peptides that do not dissolve in water, dissolve the peptide in the minimum amount of dimethylsulfoxide (DMSO) then dilute with water to the required concentration.
4.   Peptides that aggregate can be dissolved by adding 6 M urea, 6 M urea with 20% acetic acid or 6 M guanidine hydrochloride salt, then diluting to the required concentration.

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