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Practical Synthesis Guide to Solid Phase Peptide 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.

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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.

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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.

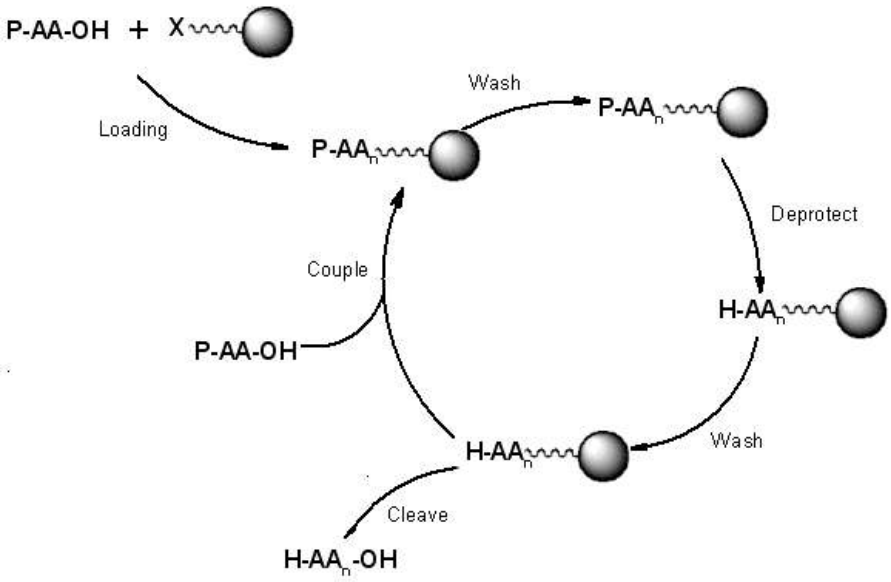

Figure 1 – General Solid Phase Peptide Synthesis Cycle

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.

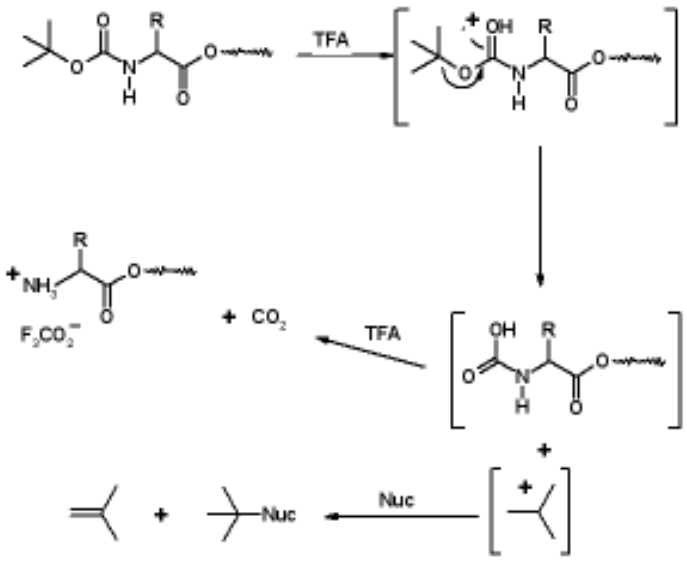

Figure 2 – Boc Deprotection Mechanism

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

[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.

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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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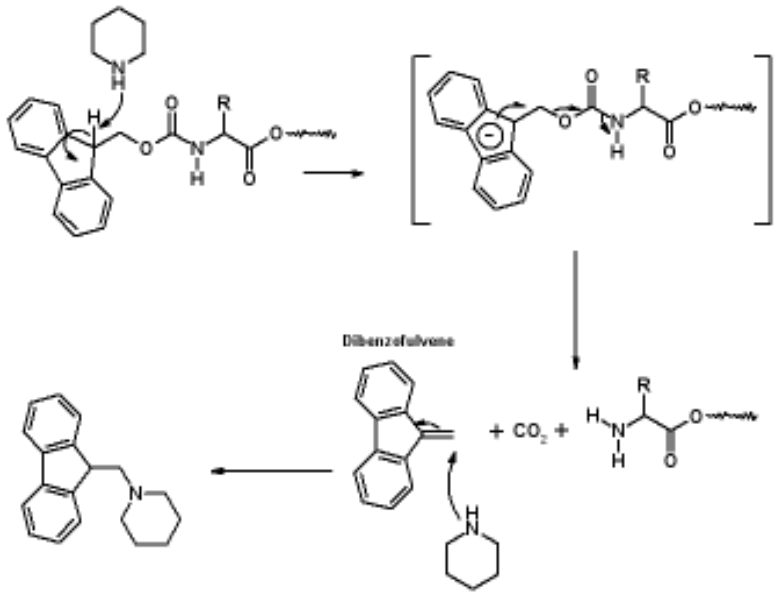

Figure 3 – Fmoc-Deprotection Mechanism

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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[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.

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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 aapptec 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_2O**	3.2
Toluene	5.3	**CH_3CN**	4.7
CH_2Cl_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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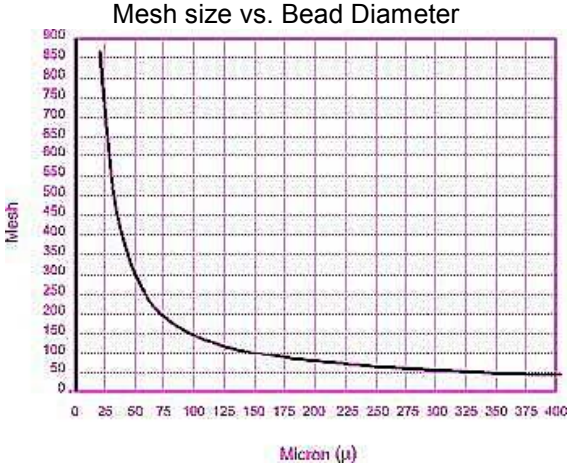
commonly used resin sizes are 100-200 mesh and 200-400 mesh (75-150 microns and 35-75 microns respectively).

Mesh size vs. Bead Diameter



Size distribution is a critical factor for high quality synthesis supports; especially those used to generate combinatorial libraries. Since each bead is essentially a microreactor, large differences in size between beads will result in unequal quantities of product in the final mixture. Such heterogeneity can lead to screening errors due to over or under representation of individual components of the mixture.[18] Bead size is also an important element in libraries since the number of possible compounds is limited to the number of beads used in the synthesis. Obviously larger libraries can be made from the same weight of resin by using smaller beads, but the quantity of each individual compound is sacrificed. The approximate number of beads per gram of resin can be calculated with the following formula:

$$\text{Number of beads per gram of resin} = \frac{1}{4/3\ \pi\ (1/2D)^3 \times \text{resin density}}$$

where D= the mean bead diameter in cm

resin density = g/ cm^3

In addition to the standard sizes listed previously, aapptec carries polystyrene in a variety of bead sizes ranging from 75-100 mesh (150-225 micron) to 200-300 mesh (50-75 micron). Size ranges not listed in this catalog can be custom manufactured upon request.

Merrifield Resin

The most fundamental substituted polystyrene resin is chloromethylated polystyrene, commonly called Merrifield resin after the Nobel Laureate who pioneered its use in peptide synthesis.[19] Substrates are attached to Merrifield resin by nucleophilic displacement of chlorine. The resulting resin-substrate bond generally is acid stable and requires strong acid conditions for cleavage. Although carboxylic acid substrates are not easily cleaved from Merrifield resin under acidic conditions, other cleavage methods including saponification,[20] transesterification[21] and cyclization-release[22] have proven effective. Since the

[18] For a discussion of possible screening errors, see Thompson, L. A.; Ellman, J. A. *Chem. Rev.* **1996**, *96*, 555-600.
[19] Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149-2154.
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[21] Marquais, S.; Arlt, M. *Tetrahedron Lett.* **1996**, *37*, 5491-5494; Pulley, S. R.; Hegedus, L. S. *J. Am. Chem. Soc.* **1993**, *115*, 9037-9047; Tortolani, D. R.; Biller, S. A. *Tetrahedron Lett.* **1996**, *37*, 5687-5690.

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Merrifield resin-substrate bond is stable to most reaction conditions in solid phase synthesis, a wide array of synthesis resins,[23] scavenger resins,[24] and polymer-supported reagents[25] have been prepared by attaching appropriate linkers to Merrifield resin.

Merrifield resin, as well as many other substituted resins, is generated by one of two methods: direct incorporation of the substrate onto the polymer core through an electrophilic aromatic substitution reaction or copolymerization of the substituted monomer with styrene. Generally, substituted resins are prepared by direct incorporation of the substrate, which invariably results in a mixture of isomers. Merrifield resin, for instance, typically has a 70:30 mixture of para-and meta-chloromethyl substitutents. Copolymerization, on the other hand, allows the use of purified monomers, enabling the preparation of resins with up to 98% para substituent. Additionally, adjusting the relative proportions of styrene and substituted monomer can precisely control the degree of substitution of the resin.

Hydroxymethyl Resin

Although Merrifield resin is the foundation of many popular resins, incomplete coupling of the substrate can lead to unreacted chloromethyl sites on the resin. If such sites will interfere with the proposed chemistry, it is possible to obtain chlorine free resin through the use of hydroxymethyl polystyrene.[26] Substrates are attached to the resin by reaction of an electrophile, such as an activated carboxylic acid, with the resin or by Mitsunobu reactions.[27] Unreacted core sites can be acetylated (end-capped) by reaction with excess acetic anhydride in pyridine.

Amino Core Resins

Aminomethyl (AM) resin has long been used in solid phase peptide synthesis as a core resin to which various linkers could be attached through a stable amide bond.[28] 4-Methylbenzhydryl amine (MBHA)[29]

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[22] Hanessian, S.; Yang, R.-Y. *Tetrahedron Lett.* **1996**, *37*, 5835-5838; Le Hetet, C.; David, M.; Carreaux, F.; Carboni, B.; Sauleau, A. *Tetrahedron Lett.* **1997**, *38*, 5153-5156; Park, K.-H.; Abbate, E.; Najdi, S.; Olmstead, M. M.; Kurth, M. J. *Chem. Commun.* **1998**, 1679-1680.

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[24] Coppola, G.M. *Tetrahedron Lett.* **1998**, *39*, 8233-8236.

[25] Adamczyk, M.; Fishpaugh, J.R.; Mattingly, P.G. *Tetrahedron Lett.* **1999**, *40*, 463-466; Andersen, J.-A.M.; Karodia, N.; Miller, D.J.; Stones, D.; Gani, D. *Tetrahedron Lett.* **1998**, *39*, 7815-7818; Dodd, D.S.; Wallace, O.B. *Tetrahedron Lett.* **1998**, *39*, 5701-5704; Vidal-Ferran, A.; Bampos, N.; Moyano, A.; Pericàs, M.A.; Riera, A.; Sanders, J.K.M. *J. Org. Chem.* **1998**, *63*, 6309-6318.

[26] Martin, G. E.; Sambhu, M.; Shakhshir, S. R.; Digens, G. A.; *J. Org. Chem.* **1978**, *43*, 4571-4574; Fréchet, J. M. J.; de Smet, M. D.; Farrall, M. J. *Polymer* **1979**, *20*, 675-680.

[27] Dodd, D. S.; Wallace, O. B. *Tetrahedron Lett.* **1998**, *39*, 5701-5704; Nicolaou, K. C.; Watanabe, N.; Li, J.; Pastor, J.; Winssinger, N. *Angew. Chem. Int. Ed. Engl.* **1998**, *37*, 1559-1561.

[28] Mitchell, A. R.; Erickson, B. W.; Ryabtsev, M. N.; Hodges, R. S.; Merrifield, R. B. *J. Am. Chem. Soc.* **1976**, *98*, 7357-7362; Mitchell, A. R.; Kent, S. B. H.; Engelhard, M.; Merrifield, R. B. *J. Org. Chem.* **1978**, *43*, 2845-2852; Giralt, E.; Andreu, D.; Pons, M.; Pedroso, E. *Tetrahedron* **1981**, *37*, 2007-2010.

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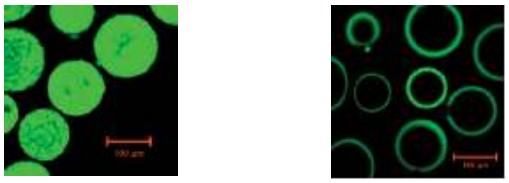
resins, however, were originally developed for the formation of peptide amides using the Boc-N protection/TFA deprotection strategy. These resins form very stable amide or amine linkages to either carboxylic or electrophilic alkyl substrates. Generally, strong acid conditions are required to cleave substrates from these resins; therefore they are also used as base resins for anchoring linkers.[30] These resins are generated through electrophilic aromatic substitution and the substitution ranges can be difficult to control. As a result, despite optimized reaction protocols, variance may be seen when purchasing these resins. Impurities on the resin can include unreacted carbonyl groups as a result of incomplete amination or leachant of substrates and byproducts during manufacture. aapptec has identified the factors leading to such impurities and has eliminated them through its proprietary manufacturing techniques, in process testing and final quality analysis.

SURE™ (SUrface-active REsin)

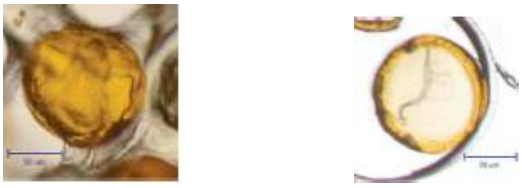
aapptec's Aminomethyl Surface Active Resin is a new and unique type of resin support. Unlike other aminomethyl resins where the reactive aminomethyl groups are distributed throughout the resin bead, the reactive sites of the Surface Active Resins are confined to a thin layer on the surface of the resin. This has been demonstrated by coupling standard aminomethyl polystyrene (AM PS) and amino methyl Surface Active Resin (AM SURE™) with fluorescein isothiocyanate (FITC). Confocal fluorescence imaging of the coupled resins clearly shows fluorescence on the surface of the AM SURE beads but is evenly distributed throughout the AM PS resin beads. Visual microscopy of cross-sectioned beads also illustrates that the reactivity is only in the surface layer of the AM SURE resin.

AM PS **AM SURE™**



Confocal fluorescence images of FITC coupled resins

AM PS **AM SURE™**



Optical microscopy of cross-sectioned FITC-coupled beads

[29] Christensen, M.; Schou, O.; Pedersen, V. S. *Acta Chem. Scand. B* **1981**, *35*, 537-581; Matsueda, G. R.; Stewart, J. M. *Peptides* **1981**, *2*, 45-50.
[30] Story, S. C.; Aldrich, J. V. *Int. J. Peptide Protein Res.* **1992**, *39*, 87-92; Hutchins, S. M.; Chapman, K. T. *Tetrahedron Lett.* **1996**, *37*, 4869-4872.

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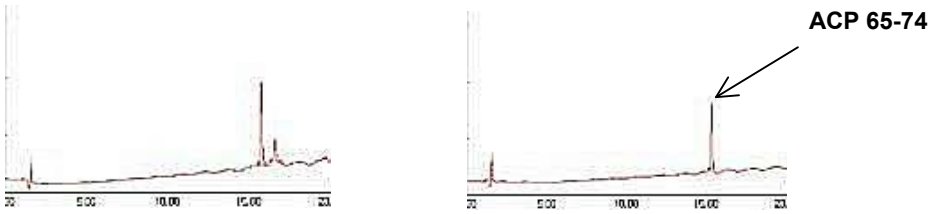
In standard polystyrene resins the restricted space surrounding the interior reactive sites inhibits free movement of the attached peptide chain and can result in deletion or chain terminated peptide impurities. The reactive sites on Surface Active Resins are unencumbered, resulting in peptides with higher purity and yield. This is clearly demonstrated in the syntheses of ACP (65-74). While the crude peptide prepared on standard resin is only 60.7% pure, the crude peptide prepared on SURE™ resin is over 90% pure.

Synthesis of ACP (65-74)

	AM PS	AM SURE™
Loading (mmol/g)	0.41	0.42
Purity (HPLC)	60.7%	90.5%

AM PS **AM SURE™**



Tentagel™ Resins

Tentagel resins consist of functionalized polyethyleneglycols (PEG) grafted onto polystyrene beads.[31] This structure imparts properties to TentaGel resins that provide advantages in solid phase peptide synthesis and in solid phase organic synthesis. In peptide synthesis, especially of large peptides, unfavorable folding of the growing peptide on the resin can cause coupling difficulties. The molecular environment of the TentaGel resins more closely resembles that of the peptide than the highly hydrophobic environment of polystyrene does. This promotes better solvation of the peptide-resin and reduces aggregation, allowing better coupling.

The resins are available in either electrophilic (Br) or nucleophilic (OH, NH_2 and SH) form as well as derivatized with various linkers. aapptec supplies these resins with very narrow size distributions, which are excellent for preparing combinatorial libraries.

Linked Resins

Linkers

[31] Bayer, E. *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 113-216.

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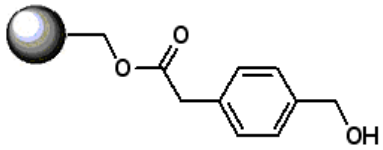
Linkers are chemical entities used to "link" a compound to a resin bead during solid phase synthesis. The nature of the linker determines the kind of chemistry that can be performed, and the conditions under which products can be cleaved from (i.e. removed from) the resin.

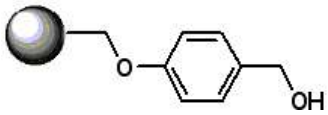
There are a wide variety of linkers for polystyrene core resins. Peptide chemists can usually focus on just a few: Wang, Rink, and derivatives of each. Wang linkers produce COOH products, and Rink linkers produce amide products. Both Wang and Rink resins are available with amino acids preloaded.

PAM Resin



PAM resin is widely used for solid phase synthesis of peptides utilizing the Boc strategy. The numerous Boc deprotection reactions with trifluoroacetic acid (TFA) required in the synthesis of large peptides leads to significant losses of peptide from Merrifield resin.[32] PAM resin provides better stability to TFA,[33] but the finished products are harder to cleave. Since typical cleavage conditions require a strong acid such a HF, this resin has found limited use in solid phase organic chemistry.[34]

Wang Resin



Wang resin is the most widely used solid phase support for acid substrates. The linker attached to the polystyrene core is a 4-hydroxybenzyl alcohol moiety.[35] The linker is bound to the resin through a phenyl ether bond and the substrate is generally attached to the linker by a benzylic ester or ether bond. This linkage has good stability to a variety of reaction conditions, but can be readily cleaved by moderate treatment with an acid, generally trifluoroacetic acid. Impurities can form if a portion of the linker is attached to the resin through the benzylic position leaving a reactive phenolic site. This can occur during attachment of the linker if exact reaction conditions are not maintained.[36]

Addition of the substrate is generally accomplished by coupling the nucleophilic resin with a desired electrophile or by a Mitsunobu reaction.[37] Care should be taken when loading optically active substrates,

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[32] Gutte, B.; Merrifield, R. B. *J. Biol. Chem.* **1971**, *246*, 1922-1941.
[33] Mitchell, A. R.; Erickson, B. W.; Ryabtsev, M. N.; Hodges, R. S.; Merrifield, R. B. *J. Am. Chem. Soc.* **1976**, *98*, 7357-7362; Mitchell, A. R.; Kent, S. B. H.; Engelhard, M.; Merrifield, R. B. *J. Org. Chem.* **1978**, *43*, 2845-2852.
[34] For examples of solid phase organic chemistry performed on PAM resin, see: Pulley, S. R.; Hegedus, L. S. *J. Am. Chem. Soc.* **1993**, *115*, 9037-9047; Smith, J.; Liras, J. L.; Schneider, S. E.; Anslyn, E. V. *J. Org. Chem.* **1996**, *61*, 8811-8818.
[35] Wang, S. *J. Am. Chem. Soc.* **1973**, *95*, 1328-1333.
[36] Lu, G.; Mojsov, S.; Tam, J. P.; Merrifield, R. B. *J. Org. Chem.* **1981**, *46*, 3433-3436.
[37] Fancelli, D.; Fagnola, M. C.; Severino, D.; Beoleschi, A. *Tetrahedron Lett.,* **1997**, *38*, 2311-2314.

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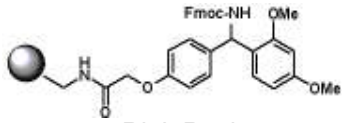
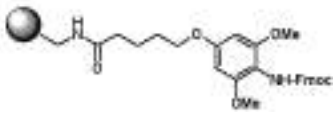
such as α-amino acid derivatives, because the activation step can lead to racemization. Many techniques have been developed to minimize this problem.[38] Solvents such as DMF and DCM are commonly used because of the large swelling factors associated with these solvents. In situations where the substrate has base labile protecting groups such as Fmoc, it is important to either use amine free DMF or avoid the use of this solvent altogether. Partial deprotection during the coupling process leads to oligomerization and subsequent impurities in the final product. aapptec has researched this chemistry extensively to manufacture the finest quality Wang resin available.

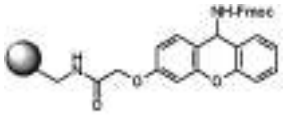
Amide/Amine Forming Resins for Fmoc/tBu



Rink Resin



PAL Resin



Sieber Resin

The most popular solid phase supports for the formation of amide products include Rink and PAL resins. All of these resins were originally developed for peptide amide synthesis using the Fmoc strategy. These resins are favored due to their higher acid lability; cleavage can be performed under conditions as mild as 1% TFA.[39] In solid phase organic chemistry, these resins have been used to produce amines by reductive alkylation.[40] To provide stability on storage, each of these resins is supplied with the amine protected by a Fmoc group, therefore pretreatment with piperidine is required to render the free amine. Acids can be coupled using standard amide forming conditions such as DIC/HOBt, HBTU or BOP.

Like the Wang linker, the Rink linker is bonded to the polystyrene matrix through an ether linkage.[39] Rink and PAL resins exhibit similar characteristics with respect to cleavage conditions and the type of products formed. Rink resin, however, has been more widely utilized. PAL is somewhat more acid labile.[41] PAL resin has been found to give cleaner products with long peptide sequences.[42]

Sieber Amide Resin is useful for preparing amides and amines and fully protected peptide amide fragments. Products can be cleaved under mild conditions,[43] using 1%TFA in DCM. This resin is less sterically hindered than Rink resin and thus allows for higher loading in sterically demanding applications than Rink resins. This is illustrated in a recent synthesis of secondary amides.[44]

MBHA Resin

[38] Van Nispen, J. W.; Polderdijk, J. P.; Greven, H. M. *Recl. Trav. Chim. Pays-Bas* **1985**, *104*, 99-100; Sieber, P. *Tetrahedron Lett.* **1987**, *28*, 6147-6150; Grandas, A.; Jorba, X.; Giralt, E.; Pedroso, E. *Int. J. Peptide Protein Res.* **1989**, *33*, 386-390; Bernatowicz, M. S.; Kearney, T.; Neves, R. S.; Köster, H. *Tetrahedron Lett.* **1989**, *30*, 434-444; Kundu, B.; Srivastava, A.; Devadas, B.; Mathur, K. B. *Indian J. Chem.* **1989**, *28B*, 604-605; Harth-Fritschy, E.; Cantacuzène, D. *J. Peptide Res.* **1997**, *50*, 415-420.
[39] Rink, H. *Tetrahedron Lett.* **1987**, *28*, 3787-3790.
[40] Purandare, A. V.; Poss, M. A. *Tetrahedron Lett.* **1998**, *39*, 935-938.
[41] Bernatowicz, M.S.; Daniels, S.B.; Köster, H. *Tetrahedron Lett.* **1989**, *30*, 4645-4648.
[42] Fyles, T. M.; Leznoff, C. C. *Can. J. Chem.* **1976**, *54*, 935-942.
[43] Sieber, P. *Tetrahedron Lett.* **1987**, *28*, 2107-2110.
[44] Chan, W.C.; Mellor, S. L. *J. Chem. Soc., Chem. Commun.*, **1995**, 1475-1477.

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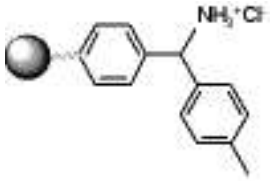
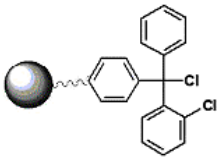


MBHA (methylbenzhydryl amine) is an amide-forming resin structurally similar to Rink. Although it is not Fmoc protected it must be activated by treatment with base (e.g. diisopropylethylamine).

The method of attaching the first amino acid is the same as Rink; ordinary amide bond forming conditions. Coupling the first amino acid is no different from coupling the rest.

In contrast, conditions for cleaving peptide products from MBHA are much harsher than from Rink. MBHA requires treatment with hydrofluoric acid or trifluoromethanesulfonic acid (TFMSA).

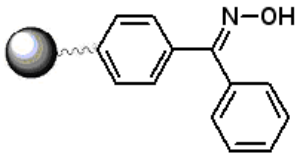
Trityl and 2-Chlorotrityl Resins



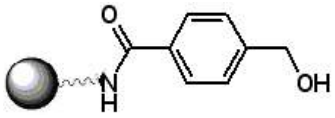
Trityl resins have been widely used in both solid phase organic and peptide chemistry. These resins are very acid labile and can be cleaved with acetic acid.[45] Protected peptides can be cleaved with 1:4 v/v hexafluoroisopropyl alcohol/dichloromethane[46] with all sidechain protecting groups intact, even trityl groups on sulfhydryl function of homocysteine.[47] These resins are particularly useful when less acid labile protecting groups are required on the substrate following cleavage, or in cases where the substrate can cyclize on the anchoring linkage causing premature cleavage. The bulky triphenylmethyl group prevents such attack through steric hindrance. In addition to being used to immobilize acids and alcohols, trityl resins can also be used to immobilize amines or thiols.

Barlos has reported that the 2-chlorotrityl resin has better stability during peptide synthesis than the trityl resin.[45] The 2-chlorotrityl resins are available in the chloride form. The chloride form is exceedingly moisture sensitive and must be handled and stored under inert conditions. Should the resin become deactivated, treatment with thionyl chloride immediately before use restores the activity.

Base Labile Resins



Oxime Resin



HMPA Resin

[45] Barlos, K.; Gatos, D.; Kallitsis, J.; Papaphotiu, G.; Sotiriu, P.; Wenqing, Y.; Schäfer, W. *Tetrahedron Lett.* **1989**, *30*, 3943-3946.
[46] Bollhagen, R.; Schirdberge, M.; Barlos, K. Grell, E. *J. Chem. Soc., Chem. Commun*. **1994**, 2559-2560.
[47] Cyr, J. E.; Pearson, D. A.; Wilson, D. M.; Nelson, C. A.; Guaraldi, M. Azure, M. T.; Lister-James, J. Dinkelborg, L. M.; Dean, R. T. J. Med. Chem. 2007, 50, 1354-1364.

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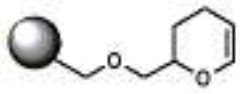
Both oxime resin and hydroxymethyl benzoic acid linked resin (HMBA resin) can be cleaved with a variety of nucleophilic agents [ammonia or primary amines (amides),[48] hydrazine (hydrazides),[49] methanol/triethylamine (methyl esters),[50] sodium borohydride (alcohols), sodium hydroxide (acids)] to produce the wide range of products indicated from the same precursor resin. A special application of oxime resin is the formation of cyclic peptides by cyclization cleavage.[51]

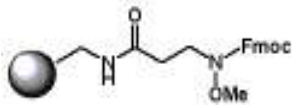
Although oxime resin is compatible with Boc chemistry, the oxime ester linkage is susceptible to TFA. Therefore the Boc group is removed with 25% TFA in DCM during synthesis and end-capping should be performed after each coupling to block any active sites on the resin that may have been exposed. For best results, the peptide should not exceed 10 residues.

DHP Resin



DHP resin was developed as a solid phase support for alcohols. The DHP linker is less sterically hindered than the trityl linkers are, hence it is preferred for sterically bulky substrates such a s secondary alcohols. Alcohols are attached to the resin under anhydrous catalytic acid conditions, forming an acetal. The bound alcohols can be cleaved from the resin under acidic conditions.[52]

Weinreb Aminomethyl Resin



This resin was developed by Fehrentz and coworkers to prepare aldehydes by solid phase methodology.[53] An amino acid or carboxylic acid is attached to the resin by standard coupling procedures. Reduction with $LiAlH_4$ releases the product aldehyde. Reacting the resin-substrate with Grignard reagents produces ketones.[54]

Substrate-Attached Resins

---

[48] Story, S. C.; Aldrich, J. V. *Int. J. Peptide Protein Res.* **1992**, *39*, 87-92; Niu, J.; Lawrence, D. S. *J. Biol. Chem.* **1997**, *272*, 1493-1499; Mohan, R.; Chou, Y.-L.; Morrissey, M.M. *Tetrahedron Lett.* **1996**, *37* , 3963-3966.

[49] DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1980**, *45*, 1295-1300.

[50] Pichette, A.; Voyer, N.; Larouche, R.; Meillon, J.-C. *Tetrahedron Lett.* **1997**, *38*, 1279-1282; Hutchins, S. M.; Chapman, K. T. *Tetrahedron Lett.* **1996**, *37*, 4869-4872.

[51] Dutton, F. E.; Lee, B. H. *Tetrahedron Lett.* **1998**, *39*, 5313-5316; Lee, B. H. *Tetrahedron Lett.* **1997**, *38*, 757-760; Paulitz, C.; Steglich, W. *J. Org. Chem.* **1997**, *62*, 8474-8478.

[52] Thompson, L.A.; Ellman, J.A. *Tetrahedron Lett.* **1994**, *35*, 9333-9336.

[53] Fehrentz, J.A.; Paris, M.; Heitz, A.; Velek, J.; Liu, C.-F.; Winternitz, J.; Martinez, J. *Tetrahedron Lett.*, **1995**, *36*, 7871-7874.

[54] Dinh, T.Q.; Armstrong, R.W. *Tetrahedron Lett.*, **1996**, *37*, 1161-1164.

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Attaching a substrate unit onto a resin is a step crucial to the success of solid phase syntheses, especially library syntheses. One problem often encountered in this step incomplete reaction of the reactive sites on the resin. Unless they are blocked, these unreacted sites can react with other reagents in later steps of the synthesis and generate impurities that may be difficult to remove. Inefficient attachment of the substrate also lowers the useful substitution, reducing the overall yield and efficiency of the solid phase synthesis. Partial racemization of optically active substrates, such as protected amino acids, is another problem that can occur during the loading reaction.

Numerous catalysts, activating agents, additives and reaction conditions have been tested to find the optimum conditions for the preparation of substrate-attached resins. As may be expected, these conditions vary for the different resins. aapptec substrate-attached resins are prepared by proven procedures that ensure good substitution and minimize racemization of the substrates. Each substituted resin is end-capped to block any remaining unreacted active resin sites that could interfere later in the synthesis.

Solvents

Methylene chloride (CH_2Cl_2)is often used in Boc chemistry protocols because it readily dissolves most Boc-proceted amino acids, it produces good swelling of polystyrene-based resins, and it is unreactive to TFA. It is seldom used in Fmoc chemistry, though because it slowly reacts with piperdine forming an insoluble crystaline solid.

N-methylpyrrolidone (NMP) is the solvent of choice for most peptide chemists. Most common peptide reagents are very soluble in NMP. Further, NMP can be obtained free of reactive amines that might interfere with coupling reactions.

In contrast, dimethylformamide (DMF) tends to spontaneously break down over time, releasing dimethylamine impurities. Dimethylamine is reactive toward the Fmoc protecting group and may remove it. This would result in excess couplings, and accordingly impure products.

DMF is cheaper than NMP, so some peptide chemists use it in spite of its tendency to release reactive amines. They limit impurities by degassing their DMF just prior to use.

Amino Acid Derivatives

Arginine

Common arginine side chain protecting groups used in Boc chemistry are NO_2 and Tos. The NO_2 group is removed during HF cleavage of the peptide from the resin, but it can undergo side reactions during cleavage leading to ornithine residues.[55] The NO_2 is stable to TFMSA, TMSOTf and HBr/AcOH and is useful for preparing protected peptide fragments for further condensation reactions. If the NO_2 group is not removed during cleavage, it can be removed with stannous chloride[56] or by hydrogeolysis.[57]

[55] Yamashiro, D.; Blake, J.; Li, C. H. *J. Amer. Chem. Soc.* **1972**, *94*, 2855-2859.
[56] Hayakawa, T.; Fujiwara, Y.; Noguchi, J. *Bull. Chem. Soc. Jpn.* **1967**, *40*, 1205-1208.
[57] ElAmin, B.; Anantharamaiah, G. M.; Royer, G.; Means, G. E. *J. Org. Chem.* **1979**, *44*, 3442-3444; Anwer, M. K.; Spatola, A. F. *Synthesis* **1980**, 929-932.

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The Tos protecting group is also removed during HF cleavage from the resin, but it is not susceptible to side reactions the NO_2 protecting group is prone to. During cleavage the released Tos group can modify trytophan residues. This side reaction can be avoided by adding thioanisole in the cleavage mixture and using the N^{in}-formyltryptophan derivative to introduce the tryptophan residues.

In Fmoc chemistry, the common arginine sidechain protecting groups are Mtr, Pmc, and Pbf. The Mtr group is acid labile and can be removed with TFA/thioanisole.[58] When there are multiple arginine residues in the peptide, complete removal of all Mtr groups becomes difficult. The prolonged reaction times or elevated reaction temperatures required can lead to undesired side reactions.[59] The Pmc group is more acid labile than the Mtr group and is useful in preparing peptides with multiple arginine residues. The Pbf group is the most labile of these protecting groups and is especially useful in preparing peptides containing many arginine residues. During cleavage, side products can result from these protecting groups reattaching to tryptophan residues, though the Pbf group may be less prone to this side reaction than the other groups. One example reported in the literature showed a 3 hour cleavage and deprotection treatment with TFA resulted 46% of the desired peptide when Arg(Pmc) was used versus 69% when Arg(Pbf) was used.[60] These side reactions can be suppressed by using N^{in}-Boc protected tryptophan and thioanisole to scavenge the free sulfonyl groups.

Aspartic Acid and Glutamic Acid

In Boc chemistry, the side chains of these amino acids are often blocked in the form of benzyl esters. These residues can form sideproducts by cyclization, however. N-terminal glutamic acid residues can cyclize to pyroglutamte residues. Aspartic acid residues can cyclize to aminosuccinate moieties that in turn can reopen to produce a mixture of the desired α-coupled product and undesired β-coupled isomer. Aminosuccinate formation is especially prevalent when an aspartic acid reside comes after glycine, serine, or phenylalanine. These undesired cyclizations could be minimized by blocking the side chains as cyclohexyl esters.

In Fmoc chemistry, these amino acid side chains are typically protected as *tert*-butyl esters. Allyl esters may be utilized if the aspartic acid or glutamic acid sidechain needs to be selectively deprotected, as for cyclization of the peptide. Aspartamide formation can occur, especially in strongly basic conditions. DBU, which is sometimes utilized in Fmoc-deprotection, often promotes aspartimide formation and should not be used if the peptide-resin contains aspartic acid residues.

Asparagine and Glutamine

Asparagine and glutamine can be used in either Boc or Fmoc chemistry without sidechain protection. There is some risk, however, of the amide moieties in the side chains reacting with carbodiimide reagents to form nitriles. This side reaction becomes a problem mostly in preparing long peptides where the asparagine or glutamine residue is repeatedly exposed to coupling reagents. This side reaction is minimized with a protecting group on the amide nitrogen. In Boc chemistry, the xanthyl (Xan) group is commonly used, while in Fmoc chemistry, the trityl (Trt) group is preferred. An added benefit of protecting the asparagine and glutamine sidechains is that it improves the solubility characteristics of the protected asparagine and glutamine derivatives.

Cysteine, Penicillamine

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The cysteine sidechain must be protected during synthesis to prevent oxidation to form disulfide bonds. A number of cysteine protecting groups have been developed to allow selective disulfide cyclization between multiple cysteine residues, pre-cleavage cyclization and post-cleavage cyclization. In Boc chemistry, the acetamidomethyl (Acm), *tert*-butyl (But), benzyl (Bzl), 4-methylbenzyl (4-MeBzl), 4-methoxybenzyl (4-MeOBzl), trityl (Trt) and 9-fluorenylmethyl (Fm) groups are used. The Trt group may be removed with trifluoroacetic acid (TFA) and triisopropylsilane (TIS) and thus is useful in on-resin cyclization methods. The Trt group can also be removed, and at the same time cyclized, with iodine. The Acm group is cleaved by iodine, too, and can be used with Trt-protected cysteines in selective on-resin cyclization strategies. The Acm group is stable under peptide cleavage conditions so it is also used in post-cleavage cyclization schemes. The Acm group may also be cleaved without disulfide formation using mercury (II) acetate. The Fm group likewise is stable to peptide cleavage conditions, but is removed with piperidine. The Fm group is used in selective post-cleavage cyclizations. The tBu group is cleaved during HF cleavage and TMSOTf cleavage, but is only partially removed with1M trifluormethansulfonic acid (TFMSA). The Bzl, 4-MeBzl, and 4-MeOBzl groups are removed during HF cleavage. The 4-MeOBzl group is also removed during TFMSA cleavage and the 4-MeBzl group is removed in HBr cleavage conditions.

The major cysteine sidechain protecting groups used in Fmoc chemistry include Acm group, the *tert*-butyl (tBu) group, the *tert*-butylthio (t-Buthio) group, 4-MeOBzl group, Trt group and the 4-methoxytrityl (Mmt) group. Like the Trt group, the Mmt group can be cleaved with TFA or iodine. The Mmt group is more acid labile; it can be removed with 1% TFA in dichloromethane/TIS (95:5 v/v). This group is useful in selective on-resin cyclizations and for producing peptides with a free cysteine sidechain.[61] The tBu group is stable to iodine oxidation and TFA cleavage conditions. Hence it is useful in selective deprotection and post-cleavage cyclization. The tBu group is removed using TFMSA, mercury (II) acetate, or TFA/dimethylsulfoxide/anisole.[62] Recently, trimethylsilylbromide (TMSBr)-thioanisole/TFA was reported to cleave the But protecting group.[63] The 4-MeBzl group is not removed in TFA cleavage and was used with But-protected cysteine residues in an elegant one-pot regioselective synthesis of the disulfide bridges in α-conotoxin SI.[62] The t-Buthio group is acid stable but cleaved under reducing conditions, making this group useful for preparing peptides with a free cysteine residue. The t-Buthio group can also undergo exchange with thiols producing new disulfides.

Penicillamine is less widely used; only a few derivatives are available. For Boc chemistry, the thiol moiety in the penicillamine side chain is protected with the 4-MeBzl group. In Fmoc chemistry, the Trt group is used to protect the sidechain.

Histidine

Histidine residues cause two problems in peptide synthesis. The imidazole moiety in the histidine sidechain, if unprotected, can react with activated acid moieties to form acylimidazoles during coupling. This seldom introduces sideproducts, for the acylimidazoles are reactive and the acyl group is removed from the histidine sidechain by the next coupling step. This reaction reduces the amount of activated acid available for coupling, however, so that more equivalents of acid are required to ensure rapid complete coupling. The more serious problem is that histidine is very prone to racemization during coupling and produces mixtures of enantiomeric peptides. The free N^{π} in the imidazole moiety of the histidine sidechain catalyzes epimerization of the activated amino acid.

Sidechain acylation is prevented and racemization reduced by protecting the histidine side chain.

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In Boc chemistry, the commonly used protecting groups are Boc, 2,4-dinitrophenyl (Dnp), Tos and benzyloxymethyl (Bom). The sidechain Boc group is removed when the N-terminal Boc group is removed, so Boc-His(Boc)-OH is mainly useful to prepare short peptides or to introduce a histidine residue near the N-terminal of a peptide. The Tos group is removed by HOBt, which is often added in coupling reactions to reduce racemization and is generated as a byproduct in coupling with BOP, HBTU and TSTU. Glycine insertion through N^{im}-N^{α} transfer on the deprotected histidine residue has been reported.[64] Therefore, Boc-His(Tos)-OH is most useful for preparing short peptides or introducing histidine residues near the N-terminus of peptides.

The Dnp group is stable to most reaction and cleavage conditions, thus it is useful in preparing larger peptides. It may also be used to prepare protected peptide fragments for fragment coupling, but in typical peptide synthesis the Dnp group is removed prior to cleaving the peptide from the resin.

The previous protecting groups attach to the π-nitrogen of the sidechain imidazole and suppress racemization to varying degrees, but racemization remains a problem. The Bom group, which is attached at the τ-nitrogen of the imidazole moiety, is very effective in suppressing racemization. Boc-His(Bom)-OH is more difficult to prepare than the other histidine derivatives and hence is more costly. This is why it is not commonly used in general peptide synthesis. It is invaluble, though, when racemization of histidine residues is a significant problem.

Histidine side chain protecting groups used in Fmoc chemistry include Fmoc and the trityl-based protecting groups, Trt, Mmt, and 4-methyltrityl (Mtt). The sidechain Fmoc group is removed when the N-terminal Fmoc protecting group is removed, so this histidine derivative is most useful in preparing short peptides and introducing a histidine residue near the N-terminal of a peptide. The trityl protecting groups are all acid labile. The general order of lability is Trt>Mtt>Mmt. The Trt protecting group is typically removed with 90% TFA and it can be used with 2-chlorotrityl resins to prepare protected peptide fragments. The Mtt and Mmt groups are completely removed with 15% TFA. Under the mild acetic acid conditions (1:1:8 acetic acid: trifluoroethanol: dichlormethane) used to cleave peptides from 2-chlorotritylchloride resins, 75% to 80% of the Mmt groups are cleaved within 30 minutes while only 3% to 8% of the Mtt groups are removed.[65]

The trityl based protecting groups do not prevent racemization during coupling.[66] Modified coupling protocols have been developed, however, that minimize the extent of racemization.[67]

Lysine, Ornithine, 2,3-Diaminopropionic Acid, 2,4-Diaminobutanoic Acid

The sidechain protecting groups used in lysine derivatives need to withstand repeated N-terminal deprotection cycles to prevent branched peptide sideproducts from forming during peptide synthesis. In Boc chemistry, some of the lysine sidechain protecting groups are benzyloxycarbonyl (Z), 2-chlorobenzyloxycarbonyl (2-ClZ) and Fmoc. The 2-ClZ protected derivative is the lysine commonly used in peptide synthesis by Boc chemistry. It is stable in 50% TFA, but is removed under the standard peptide cleavage conditions (e.g. HF, TFMSOTf, TFSMA, HBr/AcOH). The Fmoc group is acid stable and Boc-Lys(Fmoc)-OH is used to prepare protected peptide fragments for fragment coupling. It can also be selectively removed while the peptide is still attached to the resin, allowing selective modification of lysine residues (e.g. biotinylation or fluorescent labeling) on resin.

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For Fmoc chemistry, lysine derivatives are available with the following protecting groups: allyloxycarbonyl (Aloc), Boc, Mtt, Dde, ivDde and Z. Fmoc-Lys(Boc)-OH is the commonly used lysine derivative. The Boc group is removed when the peptide is cleaved from Wang resin, but is not removed under the milder cleavage conditions used with trityl chloride resins, Sieber resin and PAL resin. The tert-butyl carbonium ion that is generated in Boc deprotection can react with tyrosine and tryptophan residues if scavengers are not added. When this is a problem, the Mtt group can be used. Aloc and Z are stable to the acid conditions used to cleave peptides from Wang and Rink resins, so these groups are very useful for preparing protected peptide fragments on the resins. The Aloc group is removed with a palladium catalyst and a hydrogen donor. Z is removed by hydrogeolysis.

The Dde group is utilized when a lysine residue is to be selectively modified, as in a cyclization or a dye labelling. The Dde group is removed with hydrazine which does not affect t-butyl based protection groups.[68] The deprotection byproduct is a strong UV absorption and the deprotection reaction reaction can be monitored photometrically.

Hydrazine will remove Fmoc groups however, so the peptide should have an N-terminal Boc group before the Dde group is removed. The N-terminal Boc group can be introduced by removing the N-terminal Fmoc group, then treating the peptide-resin with Boc anhydride or Boc-ON. Alternatively, the final N-terminal residue can be incorporated as an appropriate Boc-protected amino acid.

Problems with Dde migration and premature loss have been reported.[69] The more sterically hindered ivDde overcomes most of these problems and is often used in place of Dde.[70]

Ornithine derivatives use the same sidechain protecting groups as lysine. In Boc chemistry, the Z and 2-ClZ groups are popular. For Fmoc chemistry, ornithine derivates with Aloc, Boc, Z, and 2-ClZ groups are available.

2,3-Diaminoproanoic acid and 2,4-diaminobutanoic acid derivatives are commercially available. In Boc chemistry, Boc-Dpr(Fmoc)-OH and Boc-Dbu(Fmoc)-OH are available. In Fmoc chemistry, the Aloc and Boc sidechain protected derivatives of these unusual amino acids are commonly used.

Methionine

Methionine derivatives are usually used in Boc and Fmoc chemistry without sidechain protection. Methionine residues can oxidize to the sulfoxide during cleavage, however. The oxidation can be prevented if scavengers such as dimethylsulfide are added to the cleavage mixture. If oxidation does occur, the methionine sulfoxide moieties can be converted back to methionine by a post-cleavage reduction. In Boc chemistry, oxidation may occur during synthesis, too, leading to a mixture of oxidized and reduced peptides. Methionine sulfoxide is sometimes used in Boc synthesis. The peptide can be purified before the methionine residues are reduced; assuring that the peptide does not oxidize during isolation and purification.

Methionine can be replaced with the isosteric analog norleucine. The norleucine side chain has nearly the same size and polarity as the methionine sidechain, but is not subject to oxidation. Peptide analogs in which methionine has been replaced with norleucine generally retain biological activity and are easier

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to isolate and purify. In addition, replacing the oxidizable methionine residues can increased shelf life of the peptide.

Serine, Threonine, and 4-Hydroxyproline

Serine and threonine can be incorporated into short peptides or the N-terminal of peptides without protection of the sidechains, but these amino acids are normally used with side chain protection. In Boc chemistry, the serine and threonine sidechains are protected with Boc or most commonly as the benzyl ether. In Fmoc chemistry, these amino acids are side chain protected as *tert*-butyl ethers or trityl ethers. The *tert*-butyl ether is removed under the conditions for cleaving peptides from Rink resin or Wang resin, but are stable under the mild conditions used to cleave peptides from 2-chlorotrityl resins and may be used to prepare protected peptide fragments. The trityl-protected derivatives can be selectively deprotected on resin, which is useful for preparing phosphoserine- and phosphothreonine-containing peptides by global phosphorylation methodology.

Boc and Fmoc hydroxyproline derivatives are available with or without protecting groups on the alcohol moiety. As with serine and threonine, Bzl is the protecting group used in Boc chemistry whereas the *tert*-butyl ether is used in Fmoc chemistry.

Tryptophan

In peptide synthesis by Boc and Fmoc chemistry, tryptophan can be used without protecting the indole moiety of the sidechain. The tryptophan residue can be oxidized or can be modified by cationic species during cleavage, most notably the sulfonyl moieties released from arginine moieties. These problems can be greatly reduced by protecting the indole nitrogen. In Boc chemistry, tryptophan is protected with a formyl group on the indole nitrogen. The formyl group is removed during HF cleavage, but it must be removed prior to cleavage with other cleavage reagents. In Fmoc chemistry, the tryptophan side chain is protected with a Boc group on the indole nitrogen. When used with Fmoc-Arg(Pbf)-OH, sulfonyl modification of tryptophan residues are nearly eliminated.

When the indole-Boc group is cleaved with TFA, the tert-butyl moiety leaves first leaving an indole-carboxy moiety which protects the trytophan sidechain from alkylation. This intermediate subsequently decarboxylates upon further treatment with dilute acetic acid.

Tyrosine

In short peptides, tyrosine can be incorporated without side chain protection. The unprotected tyrosine sidechain can be acylated in coupling reactions, which could lead to side products and require more activated carboxylic acid to ensure complete coupling. In addition, the unprotected tyrosine side chain can be modified by cationic moieties that are released during deprotection and cleavage steps.

In most peptide synthesis applications, the tyrosine side chain is protected. Derivatives with the side chain protected as the Bzl ether are used in both Boc and Fmoc chemistry. The Bzl group is partially removed by TFA, so this side chain protection is more useful in Fmoc chemistry, although Boc-Tyr(Bzl)-OH is useful for preparing moderate size peptides. Two protecting groups with greater acid stability are very useful in Boc chemistry. 2,6-Dichlorobenzyl (2,6-Cl_2Bzl) ether and 2-bromobenzylcarbonate (2-BrZ) are stable in 50% TFA and are readily removed in HF as the peptide is cleaved from the resin. The 2,6-Cl_2Bzl group is also compatible with TMSOTf cleavage. The 2-BrZ group is compatible with TFMSA cleavage and HBr cleavage. The 2,6-Cl_2Bzl protecting group may also be used in Fmoc chemistry to produce fully protected peptide fragments on Wang resin for fragment condensation synthesis. The 2-

BrZ is removed by piperidine[71] hence its use in Fmoc chemistry is limited to preparing small to medium peptides and to introducing a tyrosine residue near the N-terminal of peptides. In Fmoc chemistry, the preferred protecting group for the tyrosine sidechain is the *tert*-butyl (But) ether. The But group is removed when the peptide is cleaved from Wang resin or Rink resin. Used with more acid labile resins such as Pal resin and 2-chlorotrityl resins, Fmoc-Tyr(But)-OH may be used to prepare protected peptide fragments. Boc-Tyr(But)-OH is useful in both Boc and Fmoc chemistries as a derivative to attach N-terminal tyrosine residues to peptides for ^{125}I labeling.

Coupling Reagents

Carbodiimides

Dicyclohexylcarbodiimide (DCC) and diisopropylcarbodiimide (DIC) are commonly used to prepare amides, esters and acid anhydrides from carboxylic acids. These reagents can also convert primary amides to nitriles, which can be useful in organic synthesis but is a troublesome side reaction of asparagine and glutamine residues in peptide synthesis. Dicyclohexylurea, the byproduct formed from DCC, is nearly insoluble in most organic solvents and precipitates from the reaction mixture as the reaction progresses. Hence DCC is very useful in solution phase reactions, but is not appropriate for reactions on resin. DIC is used instead in solid phase synthesis since the urea byproduct is more soluble and will remain in solution. In certain applications, such as modifying proteins, ethyl-(N',N'-dimethylamino)propylcarbodiimide hydrochloride (EDC) is used. This carbodiimide reagent and its urea byproduct are water soluble, so the byproduct and any excess reagent are removed by aqueous extraction.

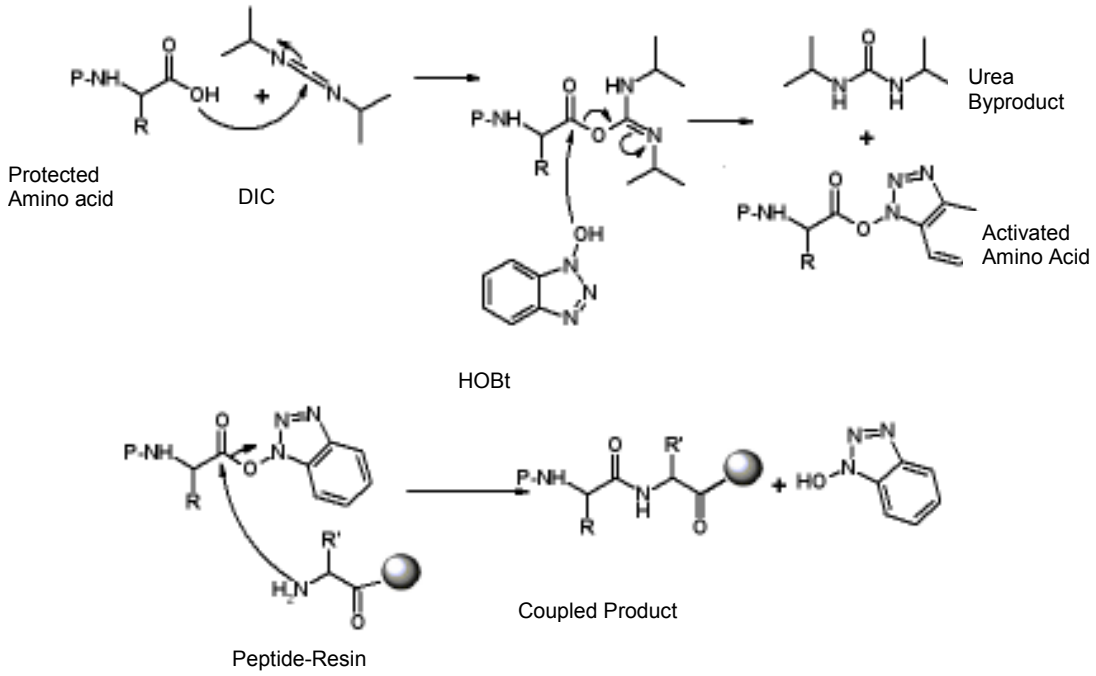

Figure 4 – DIC/HOBt Coupling

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Carbodiimide activation of amino acid derivatives often causes a partial racemization of the amino acid. In peptide synthesis, adding an equivalent of 1-hydroxybenzotriazole (HOBt) minimizes this problem. The OBt esters that form as intermediates couple with primary amines with little racemization, although certain residues such as histidine may be troublesome. Coupling an amino acid derivative to a hydroxy-functionalized resin requires a catalytic amount of 4-(N,N-dimethylamino)pyridine (DMAP). The basic DMAP can produce undesirable levels of racemization, so no more than 0.15 equivalents should be used.

Recently, a low-racemization protocol for linking the unusual amino acid derivative 4,4,4-trifluoro-N-Fmoc-O-tert-butyl-threonine to resin was reported. The protocol utilizes DIC/HOBt coupling with a small excess of HOBt and one equivalent of $CuCl_2$ added.[72]

Phosphonium-Based Reagents

To avoid the racemization and side reactions that can occur with carbodiimide reagents, many alternative reagents were developed to generate OBt esters *in situ*. (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) is one of the first reagents developed.[73] BOP does not generate asparagine and glutamine dehydration byproducts and racemization is minimal. BOP is also useful for preparing esters under mild conditions.[74] It must be handled with caution as highly carcinogenic hexamethylphosphoramide is formed as a byproduct in coupling reactions.

(Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate couples amino acids as efficiently as BOP, but the byproducts are less hazardous. Coupling reactions are rapid, being nearly complete within a few minutes. (Benzotriazol-1-yloxy)tripyrrolidino-phosphonium hexafluorophosphate may be used in place of BOP in peptide synthesis without loss of coupling efficiency.

Bromotripyrrolidinophosphonium hexafluorophosphate is a more reactive coupling reagent. It is used in difficult coupling, such as coupling N-methylamino acids or α,α-dialkylglycines, where other coupling reagents are inefficient.

Aminium-Based Reagents

Two other popular coupling reagents are O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) and O-(Benzotriazol-1-yl)- N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU). As their names reflect, these reagents were believed to have a uronium structure, but crystal and solution structure studies revealed that these reagents actually have aminium structure.[75] Both are very efficient peptide coupling reagents with little racemization. Coupling reactions are complete in as little as six minutes and when HOBt is added, racemization can be reduced to insignificant levels.[76] This makes these the reagents of choice in critical applications. TBTU was very effective, for instance, in key macrocyclization and coupling steps in the total synthesis of the macrocyclic peptide cyclotheonamide B.[77]

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O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) is similar to HBTU, but reacts faster with less epimerization during coupling. HATU is preferred to HBTU in most rapid coupling protocols. HATU is utilized in the same manner as HBTU. As with HBTU, HATU should not be used in excess because it can react with the unprotected N-terminal and block further chain elongation.

O-(6-Chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HCTU) remains colorless through long synthesis sequences and presumably has greater stability. It is reported to be less allergenic than other coupling reagents, but nonetheless it should be handled cautiously. DiFenza and Rovero have reported that HCTU showed reduced rates of racemizatin compared to BOP.[78]

Park and coworkers recently reported a fast coupling protocol utilizing HCTU in which coupling times are five minutes or less.[79]

In the coupling of peptide fragments to form SK&F 107647, TDBTU was shown to produce significantly less epimerization than PyBOP, HBTU, HATU, and many other common coupling reagents.[80] TDBTU was utilized in the large scale synthesis of over 2 kg of SK&F 107647.[80]

These reagents should used in equal molar amounts relative to the carboxylic acid component of the coupling reaction. Excess HBTU and TBTU can react with the unprotected N-terminal of the peptide and form a guanylidine moiety that blocks further elongation of the peptide.

Uronium Based Reagents

Coupling reagents in this class include O-(N-Suc-cinimidyl)-1,1,3,3-tetramethyl-uronium tetrafluoroborate (TSTU), O-(5-Norbornene-2,3-dicarboximido)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TNTU), O-[(Ethoxycarbonyl)cyanomethylenamino]-N,N,N',N'-tetramethyluronium tetrafluoroborate (TOTU) and O-(1,2-Dihydro-2-oxo-1-pyridyl0-N,N,N',N'-tetramethyluronium tetrafluoroborate (TPTU). Unlike HATU, HBTU and HCTU, these are not based on 1-hydroxybenzotriazole or its analogs and actually have a uronium structure. These reagents are not are not commonly utilized, but are useful in special applications. TSTU, for example, is used to form activated N-hydroxysuccinimide esters and to perform coupling reactions in aqueous solutions. It is quite useful coupling glycopeptides[81] and conjugating oligosaccharides to proteins.[82] Recently, TSTU was used to couple highly water-soluble dyes to a beta-alanine linker, forming amino reactive fluorescent labels for biological microscopy.[83] In addition TSTU, used with N-hydroxysuccinimide and $CuCl_2$, is reported to eliminate the racemization during coupling of peptides containing a carboxy-terminal N-methyl amino acid.[84]

TNTU is a uronium compound derived from N-hydroxy-5-norbornene-2,3-dicarboxylic acid imide. TNTU has been reported to produce little racemization during coupling.[85] Like TSTU, TNTU can be utilized for

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coupling reactions in aqueous solvents and was recently tested in solid phase peptide synthesis in water.[86] One special application of TNTU is coupling biomolecules to 11-mercapto-undecanoiuc acid which, in turn, bind to gold electrodes forming highly sensitive sensors for binding studies and assays.[87]

TOTU has been used to couple cyclam to the decapeptide HOE140 to produce a technetium-99m labelled reagent utilized as a bradykinin B_2 receptor imaging agent[88] and to assemble Mtr-Asp(Peg)-D-Adf-Pip, a potent, long-lasting thrombin inhibitor.[89] Recently, TOTU was used to couple Boc-glycine to a secondary amine.[90] In similar applications, TOTU was used to prepare PNA monomers by coupling functionalized carboxylic acids to 2-aminoethylglycine derivatives.[91]

Other Coupling Reagents

3-(Diethylphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one (DEPBT) is a coupling reagent that causes very little epimerization during coupling[92]. It is especially useful for coupling easily epimerized amino acids such as arylglycines.[93] DEPBT was also shown to be a superior reagent for head-to-tail cyclization of linear peptides.[94]

Carbonyldiimidazole (CDI) is useful for forming amides, esters and thioesters. It is not commonly used in routine peptide synthesis, but is quite useful for coupling peptide fragments to form large peptides and small proteins. One unique application of CDI is the preparation of urea dipeptides.[95]

Monitoring of Coupling and Capping

Monitoring of the coupling steps is critical in successful peptide synthesis and a number of qualitative tests have been developed. One commonly used historically and today is the Kaiser test based on the reaction of ninhydrin with amines. Primary amines produce an intense blue color while secondary amines, such as N-terminal proline, produce a less intense red-brown color. In critical applications, an alternative test for N-terminal proline is recommended. Two such tests are the isatin test and the chloranil test, which both produce a blue color with unprotected N-terminal proline.

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The testing protocol should be followed carefully. Excess heating can cause loss of Fmoc protecting groups through reaction with pyridine (contained in the test reagents), resulting in a false positive result.

If the monitoring test indicates that there is still unreacted N-terminal amine following a coupling reaction, then a second coupling step should be performed. If the monitoring test indicates that a significant amount of unreacted amine remains, the peptide is likely aggregating. Changing the coupling conditions, such as switching to a different solvent (NMP, DMSO, or DCM/DMF instead of DMF) or a different coupling reagent (HATU or HCTU instead of HBTU) may be beneficial. If unreacted amine is still present after the second coupling, acetic anhydride should be used to cap it. Capping prevents deletion peptide impurities from forming by blocking any further reactions at the unreactive sites. Capping can also make purification of the crude peptide product easier. The desired peptide will have different HPLC retention characteristics than the capped impurities, making it easier to isolate.

Aggregation, Difficult Sequences and Side Reactions

Aggregation

Aggregation of the peptide-resin can cause slow or incomplete deprotection and incomplete coupling. One indication that aggregation is occurring is that the peptide-resin fails to swell. It has been attributed to self-association of the peptide through hydrogen bonding.[96] Aggregation can not be predicted reliably from sequence data, although hydrophobic sequences are more prone to aggregation and aggregation is not likely before the fifth or sixth residue or after the twenty-first residue.[97]

If aggregation becomes troublesome, a number of different steps can be taken to disrupt the hydrogen bonding causing the aggregation. Some actions that can be taken include:

- If Fmoc deprotection is slow or incomplete, switch to DBU in the deprotection reagent.
- Switch to in situ neutralization protocols if Boc/Bzl protection is employed.[98]
- Switch to N-methylpyrrole (NMP) or add dimethylsulfoxide (DMSO) to the solvent[99]
- Sonicate the reaction mixture[100]
- Couple at a higher temperature[101]
- Add chaotropic salts such as CuLi, $NaClO_4$ or KSCN[102]
- Add nonionic detergents or ethylene carbonate (Magic Mixture).[103]

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- Utilize microwave irradiation[104]

If these measures fail to significantly improve coupling, then resynthesizing the peptide on a low substitution resin or on a different resin such as TentaGel or SURE™ may be beneficial.

Esterification on serine or threonine residues efficiently disrupts aggregation. If the peptide contains either of these residues, then it may be possible to prepare the corresponding depsipeptide and with mild base rearrange it to the desired peptide.[105] Protocols have been recently adapted to allow for fully automated syntheses of long-chain depsipeptides, including the esterification procedures.[106]

Utilizing backbone-protecting groups such as 2-hydroxy-4-methoxybenzyl (Hmb)[107] on the nitrogen will prevent hydrogen bonding. Incorporation of a Hmb moiety every six to seven residues will effectively disrupt aggregation.[108]

Since proline in a peptide sequence is known to disrupt aggregation, utilizing pseudoprolines derived from threonine and serine is another strategy for disrupting aggregation.[109] The TFA treatment to cleave the peptide from the resin also converts the pseudoprolines to the corresponding serine or threonine residue.

Pseudoprolines have been shown to be quite effective in disrupting aggregation[110] and have made the stepwise synthesis of long peptides feasible.[111]

Racemization

Activation of the protected amino acid can result in some degree of racemization. The epimerization occurs through the mechanism illustrated below[112]. Adding HOBt or HOAt suppresses the racemization.[113] Histidine and cysteine are especially prone to racemization. Protecting the π imidazole nitrogen in the histidine side-chain with the methoxybenzyl group greatly reduces racemization. A number of reduced-racemization protocols for coupling cysteine residues have been evaluated and compared.[114]

Copper (II) chloride with HOBt has been utilized in solution phase coupling of peptide segments to suppress racemization.[115] Recently $CuCl_2$ has been reported to be effective in solid phase synthesis utilizing the unusual amino acid 4,4,4-trifluoro-N-Fmoc-O-tert-butyl-threonine.[116]

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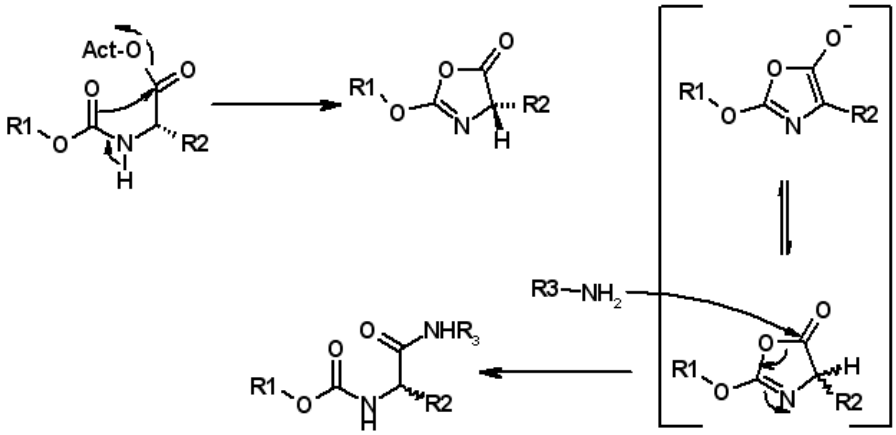

Figure 5 – Racemization Mechanism

Side Reactions

Diketopiperadine Formation

This side reaction occurs at the dipeptide stage and is more likely in Fmoc-based syntheses. Diketopiperazine formation is especially prevalent when proline is one of the first two residues.

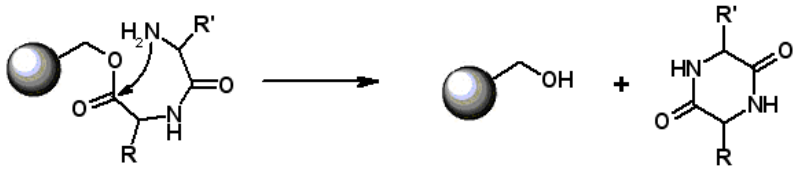

Figure 6 – Diketopiperizine Formation

In Boc-based synthesis, diketopiperazine formation can be suppressed by utilizing in situ neutralization protocols.[117]

If the Fmoc/tBu protection strategy is utilized, performing the synthesis on 2-chlorotrityl chloride resin is preferred when proline, pipecolic acid or TIC is one of the first two amino acids. The steric bulk of the 2-chlorotrityl moiety inhibits formation of diketopiperazines.

A second alternative is to add the second and third amino acid residues as a dipeptide unit, thus avoiding the dipeptide-resin intermediate. This strategy is limited by the availability of the appropriate dipeptide. A third alternative is to couple an N-trityl protected amino acid in the second position.[118] The trityl group is then removed with dilute TFA, resulting in a protonated dipeptide-resin, which can then be coupled by in situ neutralization protocols.

[117] Nguyen, D. L.; Heitz, A.; Castro, B. *J. Chem. Soc., Perkin Trans. 1* **1987**, 1915-1919; Gairí, M.; Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Tetrahedron Lett.*, **1990**, *31*, 7363-7366.
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Aspartimide Formation

Aspartimide formation is especially prevalent in peptides containing Asp-Gly, Asp-Ala or Asp-Ser sequences. This side reaction can occur acidic or basic conditions. The aspartimide can reopen producing a mixture of alpha and beta coupled peptides. In Fmoc-based syntheses, piperidine can open the asartimide to yield piperidides.[119] Adding HOBt to the piperidine deprotecting solution will reduce aspartimide formation. A special cleavage protocol has been developed that reduces aspartimide formation[120]

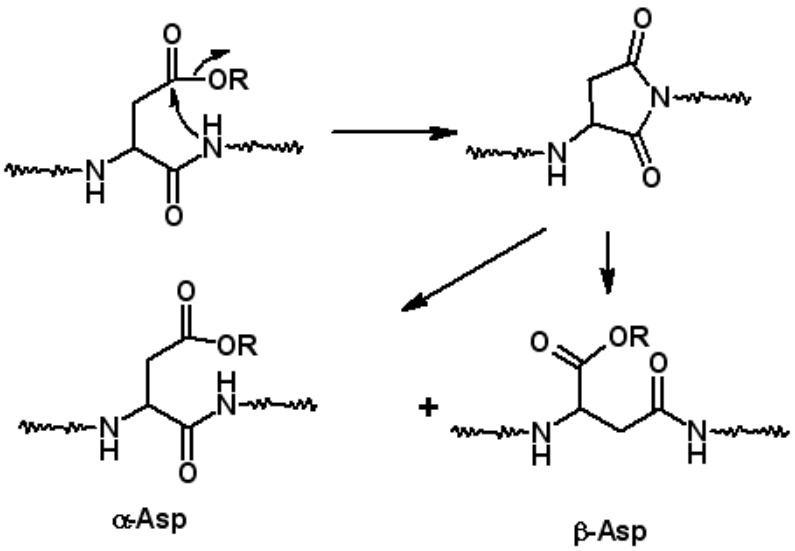

Figure 7 – Aspartimide Formation and Opening

In Boc synthesis, using the beta cyclohexyl ester instead of the beta benzyl ester of aspartic acid significantly lowers the amount of aspartimide formed.[121]

Pyrogluamate Formation

N-terminal glutamine residues may undergo base-catalyzed cyclization to form pyroglutamate.[122] As with aspartic acid derivatives, adding HOBt to the deprotection solution suppresses this side reaction.

3-(1-Piperidinyl)alanine Formation

[119] R Dolling, M. Beyermann,J. Haenel, F. Kernchen, E. Krause, P. Franke, M. Brudel, M. Bienert in "3rd International Symposium on Innovation and Perspectives in SPPS, Oxford UK," R. Epton Ed., Mayflower Scientific Ltd, Birmingham 1994, 489; Yang, Y.; Sweeney, W. V.; Schneider, K.; Thornqvist, S.; Chait, B. T.; Tam, J. P. *Tetrahedron Lett.*, **1994**, *35*, 9689-9692; Lauer, J. L.; Fields, C. G.; Fields, G. B. *Lett. Pept. Sci.* **1995**, *1*, 197-205.

[120] Mergler, M.; Dick, F.; Sax, B.; Stähelin, C. Vorherr, T. *J. Pept. Sci.*, **2003**, *9*, 518-526.

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This side product forms when peptides containing a C-terminal cysteine are prepared by Fmoc/tBu protocols. Base catalyzed elimination of the protected sulfhydryl group produces a dehydroalanine residue, which, in turn, adds piperidine.[123] This side product can be confirmed by mass spectroscopy as a mass shift of +51. Utilizing the sterically bulky trityl protecting group will minimize, but not eliminate, this side product.

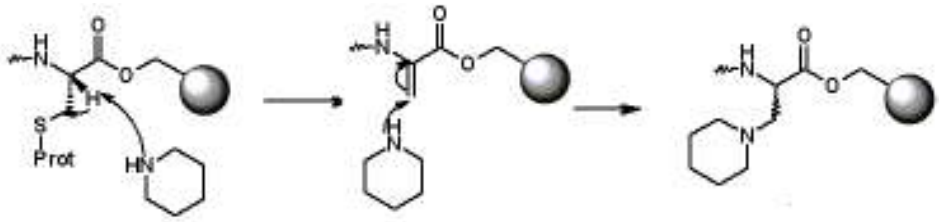

Figure 8 - 3-(1-Piperidinyl)alanine Formation

Guanidinylation

Uronium/aminium coupling reagents will react with the unprotected N-terminal of a peptide-resin to form a guanidine moiety, which irreversibly terminates the peptide chain. Guanidinylation can be avoided by preactivating the protected amino acids with a stoichiometric amount of the coupling reagent prior to adding the solution to the peptide-resin. In situ neutralization suppresses guanidinylation in Boc-protection based protocols.

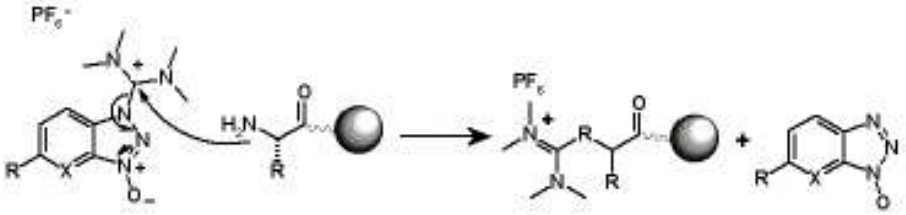

Figure 9 - Guanidinylation

Transfer of Sulfonyl Protecting Groups from Arg to Trp

Sulfonyl protecting groups can transfer from arginine residues to trytophan residues during the final cleavage/deprotection of the peptide-resin. The amount of byproduct formed depends on the protecting group[124] and the distance between tryptophan and arginine residues.[125] Using a cleavage cocktail containing scavengers can reduce the amount of byproducts formed. Reagent K (see page 61) and Reagent R (see page 62) are a couple of examples of the cleavage cocktails used when arginine residues are present.

The most effective way to prevent migration of sulfonyl protecting groups to tryptophan during cleavage is to utilize indole protected tryptophan derivatives: Boc-Trp(For)-OH in Boc/Bzl based synthesis and Fmoc-Trp(Boc)-OH in Fmoc/tBu based synthesis.

[123] Lukszo, J.; Patterson, D.; Albericio, F.; Kates, S. A. *Lett. Pept. Sci.,* **1996**, *3*, 157-166.
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Oxidation of Methionine

The thioether of the methionine sidechain is readily oxidized to sufoxide under acidic conditions. Adding dithiothreitol (DTT) to the cleavage mixture will suppress oxidation. Alternatively, the oxidized peptide can be reduced to the desired peptide following cleavage.[126]

N–O Shift

Peptides containing serine or threonine residues can undergo acid catalyzed acyl N–O shift.[127] Treatment with base, aqueous ammonia for example, reverses the reaction.

Side Reactions during HF Cleavage

Homoserine Lactone Formation

Tert-butyl cations, formed in the deprotection of tert-butyl based protecting groups, can alkylate the thioether sidechain of C-terminal methionine, which subsequently cyclizes to produce homoserine lactone.[128] This side reaction can be prevented by removing all tBu-based protecting groups prior to HF cleavage.

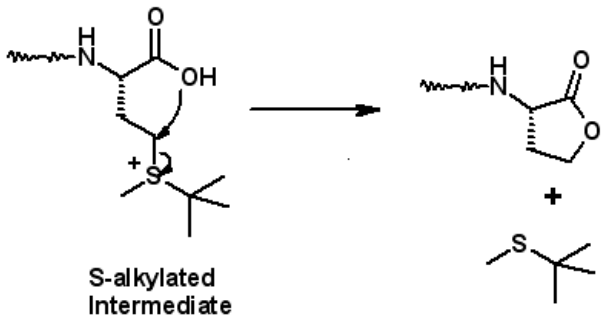

Figure 10 – Homoserine Lactone Formation

Glutamic Acid Side Reactions

Deprotection of glutamic acid residues during HF cleavage can result in the formation of an acylium ion from the HF protonation and dehydration of the unprotected carboxyl moiety. The acylium ion can cyclize to produce a pyroglutamine residue or it can react with scavengers such as anisole to form an aryl ketone.[129]

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[126] Nicolás, E.; Vilaseca, M.; Giralt, E. *Tetrahedron*, **1995**, *51*, 5701-5710.
[127] Sakakibara, S.; Shin, K. H.; Hess, G. P. *J. Am. Chem. Soc.*, **1962**, *84*, 4921-4928; Carpino, L. A.; Krause, E.; Sferdean, C. D.; Schümann, M.; Fabian, H.; Bienert, M.; Beyermann, M. *Tetrahedron Lett.*, **2004**, *45*, 7519-7523.
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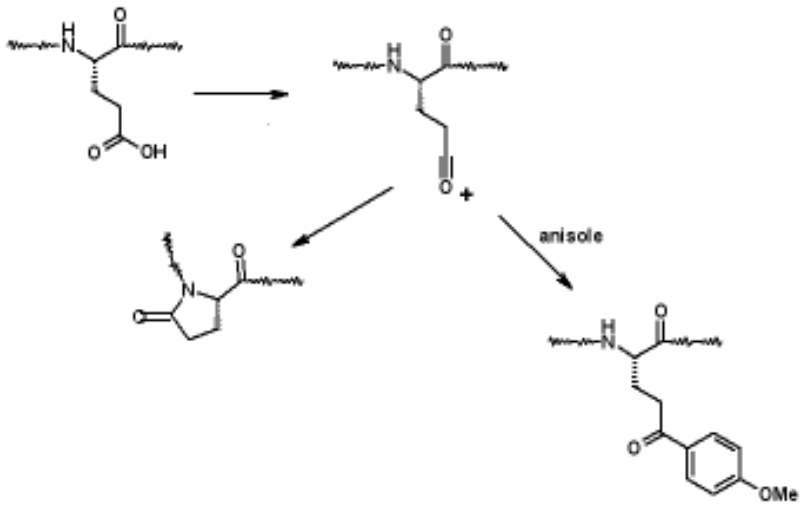

Figure 11 – Glutamic Acid Side Reactions

Asp-Pro Cleavage
Cleavage of this bond during HF cleavage has been reported.[130]

Peptide Modification

Some peptides require additional modification to better mimic the native peptide or protein fragment they were modeled on or to introduce elements that enhance their later application. Most modifications can either be incorporated post-synthetically or during the peptide synthesis by utilizing appropriately derivatized amino acids. Some of the common modifications are listed:

- Cyclization
- N-Methylation of the peptide backbone
- Phosphorylation
- Myristilation
- Farnesylation
- PEGylation
- Biotinylation
- Fluorescent Labeling
- Caged Peptides
- MAP Peptides
- Thioesters

Cyclization

Many natural peptides with interesting biological activity are cyclic. Cyclization is also used in synthetic peptides to impose a desired conformation in the peptide, especially when the peptide is based on a portion of a much larger peptide or protein. Cyclic peptides are formed in several ways: sidechain-to-sidechain, terminus-to-sidechain and terminus-to-terminus. In each case, cyclization typically is performed after the linear peptide has been synthesized.

[130] Wu, C. R.; Wade, J. D.; Tregear, G. W. *Int. J. Pept. Protein Res.*, **1988**, *31*, 47-57.

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The most common type of sidechain-to-sidechain cyclization is disulfide bridging of cysteine residues. This cyclization is introduced by deprotecting a pair of cysteine residues and oxidizing to form the disulfide bond. Multiple cycles can be selectively formed if selectively removable protecting sulfhydryl protecting groups are utilized. Cyclization can be performed either in solution following cleavage from the resin or on-resin before cleavage. On-resin cyclization may be less effective than cyclization in solution, since the resin-bound peptide may not as easily achieve a favorable conformation for cyclization. Analogs of disulfide bridges have been prepared utilizing two allylglycine residues cyclized by ring closing metathesis.[131]

Another type of sidechain-to-sidechain cyclization is amide formation between and asparic acid or glutamic acid residue and one of the basic amino acids, i.e. lysine (Lys), ornithine (Orn), 2,4-diaminobutyric acid (Dab) and 2,3-diaminopropionic acid (Dap). These cyclizations require side chain protecting groups that can be selectively removed while the peptide is still attached to the resin or after cleavage.

A third form of sidechain-to-sidechain cyclization is through biaryl ethers of tyrosine or hydroxyphenylglycine. Natural products with this type of cyclization are exclusively microbial products and often have interesting and desirable pharmaceutical properties. Preparing these compounds requires unique reactions and conditions and currently not a matter of routine peptide synthesis.

Sidechain-to-terminal cyclization usually involves the C-terminus and the amine function of a lysine or ornithine sidechain or the N-terminal and an aspartic acid or glutamic acid sidechain. Some cyclic peptides are formed through an ester bond between the C-terminal and a serine or threonine sidechain. Although forming the cyclic structure by forming the ester bond may seem to be the obvious way to prepare these compounds, higher cyclization yields are usually obtained when the cyclization occurs by forming a peptide amide bond elsewhere in the cyclic peptide.

The third form of cyclization is a terminal-to-terminal, or head-to-tail cyclization. The linear peptide can be cyclized in solution[132] or while attached to resin through a sidechain.[133] Cyclization in solution should be performed at low concentrations of the linear peptide to prevent oligomerization of the peptide.

The yield of head-to-tail cyclized peptide from linear peptide can depend the sequence of the linear peptide. In a recently published optimization of the protocol for preparing cyclic gramicidin S, the sequence of the linear peptide had a signifcant influence on the success of the cyclization reaction.[134] Before preparing head-to-tail cyclized peptides on a large scale, a library of possible linear precursor peptides should be prepared and cyclized to find the sequence that provides optimum results.

N-Methylation of the Peptide Backbone

N-Methylation occurs in natural peptides and is introduced in to synthetic peptides to disrupt normal hydrogen-bonding and to make the peptide more resistant to biodegradation and elimination. The N-methylated backbone is synthesized into the peptide by incorporating the appropriate N-methylated

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[131] Mollica, A.; Guardidani, G.; Davis, P.; Ma, S.-W., Porreca, F.; Lai, J.; Marrina, L.; Sobolev, A. P.; Hruby, V. J. *J. Med. Chem.*, **2007**, *50*, 3138-3142.

[132] For a recent example see Cyr, J. E.; Pearson, D. A.; Wilson, D. M.; Nelson, C. A.; Guaraldi, M.; Azure , M. T.;m Lister-James, J.; Dinkelborg, L. M.; Dean, R. T. *J. Med. Chem.* **2007**, *50*, 1354-1364.

[133] Dartois, V.; Sanchez-Quesada, J.; Cabezas, E.; Chi, E.; Dubbelde, C.; Dunn, C.; Granja, J.; Gritzen, C.; Weinberger, D.; Ghadiri, M. R.; Parr, T. R., Jr. *Antimicrob. Agents Chemother.*, **2005**, *49*, 3302-3310; Berthelot, T.; Gonçalves, M.; Laïn, G.; Estieu-Gionnet, K.; Deleris, G.. *Tetrahedron*, **2006**, *62*, 1124-1130; Xiao, Q. Pei, D. *J. Med. Chem.*, **2007**, *50*, 3132-3137.

[134] Wadhwani, P.; Afonin, S.; Ieronimo, M.; Buerck, J.; Ulrich, A. S. *J. Org. Chem.*, **2006**, *71,* 55-61.

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amino acid derivatives. An alternative method involving a Mitsunobu reaction with an N-(2-nitrobenzenesulfonyl) peptide-resin intermediate and methanol has recently been employed in the preparation of a library of cyclic peptides containing N-methylated amino acids.[135]

Phosphorylation

Phosphorylated peptides play a regulatory role with most protein kinases. Phosphorylation occurs on tyrosine, threonine and serine residues. Phosphotyrosine, phosphoserine and phosphothreonine derivatives can be incorporated into a peptide during synthesis or can be formed after the peptide is synthesized. Phosphoarmidite reagents are preferred for introducing phosphate groups post-synthetically.[136] Selective phosphorylation can be achieved by using selectively removable protecting groups on the serine, threonine or tyrosine residues to be phosphorylated.

Myristylation/Palmitylation

Acylating the N-terminal with a fatty acid causes peptides and proteins to associate with plasma membranes. Protein kinases in the src family and retroviral Gag proteins are targeted to plasma membranes by a myristylated sequence at the N-terminal. Myristylation is also necessary in protein kinase C inhibitors based on substrate analogs.[137]

The acids can be coupled using to the resin-peptide N-terminal by standard coupling protocols.[138] The resulting lipopeptide can be cleaved under standard conditions and purified by RP-HPLC on a C4 column.[139]

Glycosylation

Glycopeptides like vancomycin and teicoplanin are important antibiotics used to treat bacterial infections that are resistant to other antibiotics. Other glycopeptides are utilized because they can stimulate the immune system. Studies with these peptides may lead to improved treatment of infections for many microbial antigens are glycosylated. Glycopeptides may also play an important role in the study of cancer and the immune defense against tumors, because cancer cells display an abnormal glycosylation of the proteins in the cellular membrane.[140]

Glycopeptides are prepared by Fmoc/tBu protocols. The glycosylated residues, typically serine or threonine are incorporated into the peptide by means of active pentafluorophenol esters of the Fmoc-protected glycosylated amino acid.[141]

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Isoprenylation (Farnesylation/Gernaylgeranylation)

Isoprenylation of proteins promotes membrane association and contributes to protein-protein interactions. Farnesylated proteins include tyrosine phosphatases, small GTPases, cochaperones, nuclear lamina, and centromere-associated proteins.[142] Isoprenylation occurs on the sidechaine cysteine residues in the proximity of the C-terminal.

Farnsylated peptides can be prepared by on-resin farnesylation[143] or by incorporating farnyslyated cysteine derivatives.[144] A novel method involving the SN2 displacement of bromine from a bromoalanine residue by farnesyl mercaptan has been reported. Characteristic partial structures of human Ras peptides were reported to have been prepared in good yield by this methodology.[145]

PEGylation

Attaching polyethyleneglycol (PEG) chains to peptides can improve their pharmacological profiles. The bulky PEG inhibits degradation of the peptide by proteolytic enzymes. The hydrodynamic radius of a PEGylated peptide is greater than the normal cross section glomerular capillaries, which greatly impedes renal clearance.[146] These factors combined increase the effective half-life of the peptide in the body. Therefore a lower, less frequent doses are required to maintain therapeutic levels of the peptide in the body.

PEGylation can have negative effects, too. The PEG bulk that prevents enzymes from degrading the peptide can also reduce the binding of the peptide to the targeted receptor. The lower affinity of the PEGylated peptide is usually offset by its longer pharmokinetic half-life. By remaining in the body longer, there is a greater probability that the PEGylated peptide will be taken up by its target tissue. The size of the PEG polymer should be optimized for the best results.[147]

Due to their reduced renal clearance, PEGylated peptides can accumulate in the liver, leading to macromolecular syndrome. Hence, the PEGylation has to be carefully designed if the peptide is to be utilized in drug testing.

The PEG polymers are available in sized from 1kDa up to 40kDa. The PEG polymers may be monodisperse or polydisperse. Monodisperse PEG polymers have a uniform length and molecular weight. Polydisperse PEG polymers however have a distribution of lengths and molecular weights.

A number of PEG derivatives have been developed for PEGylating peptides and proteins. PEG derivatives with acid or activated carbonate functions can be coupled to N-terminal amines or lysine side chains. Amine functionalized PEG can be coupled to aspartic acid or glutamic acid side chains. PEG functionalized with malimide will couple to the free thiol moiety of fully deprotected cysteine side chains.[148]

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Biotinylation

Biotin binds to avidin or streptavidin with strength that almost approaches a covalent bond. Biotin-labeled peptides can be used for affinity labeling and affinity chromatography. Labeled anti-biotin antibodies can also be used to bind biotinylated peptides.

Biotin labels are usually attached to lysine side chains or N-termini. Often a spacer such as 6-aminohexanoic acid is applied between the peptide and the biotin. The spacer is flexible and allows better binding to substrates in sterically hindered cases.

Fluorescent Labeling

Fluorescent tags are useful for tracing peptides within living cells. Fluorescent tags are also very useful in studying enzymes and mechanisms. Tryptophan is fluorescenct and seldom occurs more than once in common peptides. Therefore it may be utilized as an intrinsic label. The emission wavelength of tryptophan is dependent on the surrounding enviroment; the emission wavelength decreases as the solvent polarity decreases[149]. This property is useful for probing peptide structure and binding.[150] Tryptophan fluorescence can be quenched by protonated Asp and Glu residues, which may limit its utility.

The Dansyl group, when attached to an amine, is highly fluorescent and is commonly used as a fluorescent lable on amino acids and proteins.[151]

Excitation and Emission Wavelengths of Common Fluorescent Labels

	Excitation Wavelength	Emission Wavelength
Trp	280 nm	300-350 nm
2-Abz	315 nm	400 nm
AMC	365-380 nm	430-460 nm
Dansyl	335-339 nm	490-500 nm
EDANS	335 nm	493 nm
Carboxyfluorescein	494 nm	518 nm
Carboxytetramethylrhodamine	555 nm	580 nm

Florescence resonance energy transfer (FRET) is quite useful for studying enzymes. For FRET applications, the substrate peptide contains a fluorescent label and a fluorescence quenching moiety. The fluorescence of the tag is quenched by the quencher through a non-photonic energy transfer. When the peptide is cleaved by the enzyme being studied, the tag fluoresces. Recently an inverted FRET strategy was used to study crenellation of a GTPase.[152]

FRET Donor-Acceptor Pairs

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Donor	Acceptor
IAEDANS (1)	DDPM (2)
Dansyl	FITC
Dansyl	Octadecylrhodamine
CF (3)	Texas Red
Fluorescein	Tetramethylrhodamine
FITC	Eosin Thiosemicarbazide
EDANS (4)	Dabsyl
Mca (5)	Dnp
Tryptophan	Dansyl[153]
2-Abz	Tyr(3-NO_2)
2-Abz	Dnp

(1) 5-(2-iodoacetylaminoethyl)aminonaphthalene-1-sulfonic acid
(2) N-(4-dimethylamino-3,5-dinitrophenyl)maleimide
(3) carboxyfluorescein succinimidyl ester
(4) 5-(2-laminoethyl)aminonaphthalene-1-sulfonic acid
(5) 7-methoxycoumarin

Caged Peptides

Caged peptides have photoremovable protecting groups that block their affinity for a receptor. The peptide can be activated by UV irradiation. Because the photoactivation can be controlled in terms of time, amplitude and localization, caged peptides are used to investigate reactions occurring in intracellular pathways.[154]

The protecting groups most frequently used for caged peptides are based on 2-nitrobenzyl moieties. These are incorporated by utilizing appropriately protected amino acid derivatives during the peptide synthesis. Lysine, cysteine, serine, and tyrosine derivatives have been developed. A recent attempt to utilize 4,5-dimethoxy-2-nitrobenzyl esters on the side chains of aspartic acid and glutamic acid was unsuccessful. These derivatives proved to be highly prone to cyclization during peptide synthesis and cleavage.[155]

MAP Peptides

Short peptides usually are not immunogenic and must be conjugated to a carrier protein to prepare antibodies. Multiple Antigenic Peptides (MAP) are an alternative to preparing peptide-carrier protein conjugates. MAPs consist of multiple copies of a peptide attached to a lyseine core and typically produce highly efficient immunogens.[156]

MAP peptides can be prepared by step-wise solid phase peptide synthesis on a MAP resin. However, incomplete coupling can produce deletion or truncation chains on some of the branches, resulting in a crude MAP peptide that may be difficult to characterize. Alternatively, the peptide can be prepared and purified separately, then coupled to the MAP core.[157] The peptide sequence attached to the MAP core is unambiguous and the peptide can be readily characterized by mass spectroscopy.

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Thioesters

Peptide thioesters are reactive intermediates used to form cyclic peptides and to prepare large peptides and small proteins through native chemical ligation. Peptide thioesters can be readily prepared on thiol-linked resins utilizing Boc protocols.[158] When Fmoc chemistry is used, the thioester has to be incorporated last since piperdine will react with thioesters to form the piperdine amide. A number of strategies have been developed, from coupling a thiol to the C-terminal while the peptide is anchored to the resin through an amino acid side chain[159] to using a safety-catch resin and displacing the peptide from the resin with the C-terminal amino acid thioester.[160]

Recently N-alkyl cysteine-assisted thioesterification was reported which utilizes N to S migration.[161] Peptides with a C-terminal N-alkyl cyteine are in an acid catalysed equilibrium between the amide and thioester forms as illustrated. Addition of 3-mercaptoproionic acid cleanly converts the peptide to the thioester of 3-mercatopropionic acid.

Figure 12 – N – S Migration

Planning a Peptide Synthesis

N- and C-Terminal Functionality

Synthetic peptides are usually prepared to mimic naturally occurring peptides or segments of peptides or proteins. If the synthetic peptide is based on a naturally occurring peptide, then the N- and C-terminals probably will not require modification. If the synthetic peptide is mimicking a segment of a larger peptide or protein, then the basic N-terminal and the acidic C-terminal may need to be modified to approximate the neutral amide functions of the native peptide or protein.

- If the peptide sequence is from the N-terminal, then the C-terminal should be an amide.
- If the peptide sequence is from the C-terminal, the N-terminal may need to be acylated.
- If the peptide sequence is from the interior portion of the model, both ends of the peptide may need to be modified.

C-terminal amides are most conveniently prepared on an amide-forming resin such as MBHA, Rink, PAL or Sieger resins. C-terminal amides can also be formed by cleaving the peptide from the resin by amminolysis. While amminolysis can be performed on many standard resins such as Merrifield and Wang resins, Oxime and HMBA resins are preferred.

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N-terminal acetylation is easily achieved by adding a capping step to the peptide synthesis protocol.

Boc vs. Fmoc

The first step in planning a successful peptide synthesis is to choose the protection scheme. Boc/Bzl protection, when utilized with in situ neutralization, can provide superior results for long or difficult peptide sequences. Cleaving the peptide product from the resin requires strong acids such as TFMSA or HF. HF is toxic and reacts with ordinary laboratory glassware. It requires special apparatus, which can make scaling up difficult if a laboratory is not specially equipped for large-scale peptide production.

Fmoc/tBu protection typically does not require reagents stronger than 50% TFA to remove side-chain protecting groups and cleave the peptide from the resin support, hence it can be scaled up easily in the laboratory. The side chains can be deprotected while the N-terminal Fmoc remains in place, allowing side chain modification. In addition, there are a variety of other side chain protecting groups available which allow selective deprotection at a specific site.

Aggregation tends to be more of a problem when Fmoc/tBu protection is utilized, since the peptide-resin is always in a neutral state. This is not significant with short sequences, but can become problematical when large peptides are synthesized. Fmoc deprotection, which is rather rapid in small peptides, often becomes slower in longer peptides due to aggregation.

In general, Boc/Bzl protection is best for long or difficult sequences and base sensitive peptides while Fmoc/tBu protection is best for acid sensitive peptides and peptides with sidechain modifications.

	Boc/Bzl	**Fmoc/tBu**
N-alpha Deprotection	50% TFA	40% Piperidine
Final Cleavage	HF, special equipment	50% TFA, plain glassware
Synthetic Steps	deprotect, wash, neutralize, wash, couple, wash	deprotect, wash, couple, wash
Scale	Limited by HF cleavage apparatus	Any scale
Recommended for:	Difficult sequences prone to aggregation, base sensitive peptides	Acid sensitive peptides, peptides labeled or modified on the sidechains

Selecting a Resin for Peptide Synthesis

Before choosing a resin to use in a peptide synthesis, you must answer several questions first:

1) Will Boc-amino acids or Fmoc-amino acids be used to synthesize the peptide?
2) Will the product be a peptide acid or a peptide amide?
3) How large is the peptide?
4) Will the product peptide be unprotected (all protecting groups removed) or protected for further applications (fragment condensation, ligation, etc.)?

There are additional questions that may need to be considered, but these are the main questions that must be answered before choosing a resin.

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Will Boc-amino acids or Fmoc-amino acids be used to synthesize the peptide?

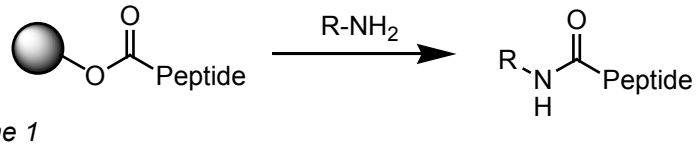
In synthesizing peptides, the N-protecting group of the previous amino acid has to be removed before the next amino acid is coupled. The Boc protecting group is usually removed with 50% trifluoroacetic acid (TFA) in dichloromethane (DCM), so the peptide-linker bond has to be stable in these conditions. Merrifield, Hydroxymethyl polystyrene, PAM, MBHA and BHA resins are the resins most commonly used for peptide synthesis with Boc-amino acids.

Using Fmoc-amino acids, you can prepare peptides under neutral or basic conditions, so most of resins used in Fmoc-peptide synthesis can be cleaved under relatively mild acid conditions. Wang, 2-chlorotrityl, Rink, Seiber and PAL resins are some of the resins commonly used with Fmoc-amino acids.

Will the product be a peptide acid or a peptide amide?

Most peptides have either a carboxylic acid group (–COOH) or an amide group (–CONH2) at the C-terminal. The common resins used for preparing acid peptides are Merrifield, hydroxymethyl polystyrene, Wang, and 2-chlorotrityl resins. The common resins for preparing peptide amides are MBHA, BHA, Rink, Knorr, DCHD and PAL resins.

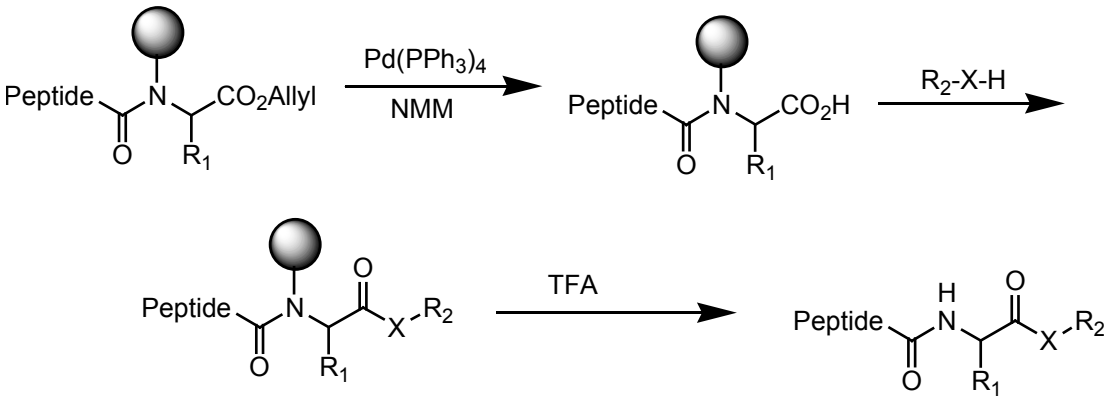
A few peptides and peptide fragments have other functional groups at the C-terminal. Alcohols (–CH_2OH), methyl amides (–$CONHCH_3$) and ethyl amides (–$CONHCH_2CH_3$) are the most common alternatives. There are several methods, and resins, for preparing these peptides. A common method for preparing these products is to synthesize the peptide on an appropriate resin, then to displace the peptide from the resin with an amino or amino acid (Scheme 1). Some of the resins used in this application are



Scheme 1

phenol resin and oxime resin, used for Boc peptide synthesis; HMBA-MBHA resin, used for Fmoc peptide synthesis; and Aliphatic Safety Catch resin, also used for Fmoc peptide synthesis.

Another resin that you can use to prepare peptides with non-standard C-terminals is BAL resin. With this resin, the peptide is attached to the resin linker at one of the “backbone” amide groups instead of the C-terminal. The C-terminal is protected by an ester as the peptide is synthesized, then is deprotected and modified (Scheme 2).



Scheme 2

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How large is the peptide?

In preparing large peptides, aggregation of the growing peptide chains can cause difficulty during coupling reactions. Generally, using a low-substitution resin will reduce this difficulty. If the peptide you will synthesize is very large (30 to 50 amino acids), then a resin with a low substitution (0.1 to 0.4 mmol/g) is best. For peptides of 10 to 20 amino acids, you can usually use a resin with standard substitution (0.5 to 1.2 mmol/g). To prepare a short peptide (<10 amino acids), a higher substitution resin (1.3-2.0 mmol/g) may be utilized.

Resins that provide a more polar, peptide-like environment than polystyrene are also helpful for reducing aggregation. TentaGel is one example. TentaGel resins incorporate polyethylene glycol chains between the polystyrene bead and the linker.

SURE™ resins are another option for preparing large peptides. The reactive sites are confined to a thin layer on the surface of the beads so reagents do not have to diffuse to the core of the beads. In addition, the peptide chains attached to SURE™ resin have conformational freedom and are not constrained by the volume of the pores within the resin. The N-terminal of the peptides attached to the resin remain readily accessible for deprotection and coupling, thus improving the yield in each coupling step and minimizing deletions.

Will the product peptide be unprotected or protected for further applications?

In standard solid-phase synthesis protocols, the peptide is cleaved from the resin and at the same time the side-chain protecting groups are removed. If you are preparing small protected peptides to couple together to form large peptides or small proteins, though, you want to cleave the peptides from the resin without removing the side-chain protecting groups. 2-Chlorotrityl chloride resin is an acid labile resin commonly used for this purpose.

Oxime resin and phenol resin may also be used to prepare short, protected peptide segments for fragment condensation. The protected peptide is cleaved from these resins with hydrazine, producing the peptide hydrazide which, in turn, can couple to the unprotected N-terminal of another peptide.

The choice of resin can have a large effect in the success of the peptide synthesis. By considering these questions when planning a synthesis, the chemist will be able to choose a resin that will best suit the requirements and yield good results.

Chemistry	Peptide Product	Suitable Resins	Coupling Methods	Cleavage Reagents
Boc	Peptide acid	Merrifield resins	Cesium salt of amino acid; Potassium fluoride method	HF TFMSA TFSOTf HBr/AcOH
		Hydroxymethyl Polystyrene	DIC/HOBt/ DMAP catalyst Mitsunobu Coupling	HF TFMSA TFSOTf HBr/AcOH
		Preloaded Merrifield resins	-	HF TFMSA TFSOTf HBr/AcOH

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		PAM resin	DIC/HOBt/ DMAP catalyst	HF
		Preloaded PAM resins	-	HF
		TentaGel S OH	DIC/HOBt/ DMAP catalyst	$NaOH/H_2O$
		TentaGel S Br	Cesium salt of amino acid	$NaOH/H_2O$
	Peptide amides	MBHA resin	Desalt then DIC/HOBt	HF TFMSA TFSOTf HBr/AcOH
Fmoc	Peptide Acids	Wang resin	DIC/HOBt/ DMAP catalyst Mitsunobu Coupling	50% TFA
		Preloaded Wang resins	-	50% TFA
		TentaGel S PBH	DIC/HOBt/ DMAP catalyst Mitsunobu Coupling	50% TFA
	Peptide amides	Rink resin	Remove Fmoc then DIC/HOBt	20% to 50% TFA
		Knorr resin	Remove Fmoc then DIC/HOBt	30% to 50% TFA
		PAL resin	Remove Fmoc then DIC/HOBt	30% to 50% TFA
		TentaGel S AM resin	Remove Fmoc then DIC/HOBt	30% to 50% TFA
		TentaGel S AC	DIC/HOBt/ DMAP catalyst Mitsunobu Coupling	1% to 2% TFA
	Protected peptide acid fragments	2-Chlorotrityl Chloride resin	Fmoc-amino acid plus DIPEA	1% TFA AcOH
		2-Chlorotrityl Alcohol resin	Activate with acetyl chloride or thionyl chloride, then Fmoc-amino acid plus DIPEA	1% TFA AcOH

Chemistry	Peptide Product	Suitable Resins	Coupling Methods	Cleavage Reagents
Fmoc	Protected peptide acid fragments	Preloaded 2-Chlorotrityl resins	-	1% TFA AcOH
	Protected peptide amide fragments	TentaGel S Trt-OH	Activate with acetyl chloride or thionyl chloride, then Fmoc-amino acid plus DIPEA	1% TFA AcOH
		Sieber resin	Remove Fmoc then DIC/HOBt	1-2% TFA

	Peptide acids Peptide amides Peptide esters Cyclic peptides	TentaGel S HMB	DIC/HOBt/ DMAP catalyst Mitsunobu Coupling	NaOEt then NaOH: acids Amine: amides NaOR: esters N-deprotect: cyclic peptides
	Peptide aldehydes	Weinreb resin	DIC/HOBt	$LiAlH_4$
	Peptide ketones	Weinreb resin	DIC/HOBt	Grignard reagents
	Hydroxamic acids	Fmoc-Hydroxylamine 2-Chlorotrityl resin	DIC/HOBt	1% to 2% TFA AcOH

Selecting Amino Acid Derivatives

Careful selection of the amino acid derivatives used in the synthesis of a long peptide sequence can minimize the formation of by-products, increase the yield of peptide and ease the purification of the isolated product. In preparing short peptides, the selection of side chain protecting groups usually isn't critical, they just need to prevent major side reactions. On the other hand, the choice of protecting groups is more nuanced when a long peptide is synthesized. The amino acid residues near the C-terminal, since they are incorporated first, are exposed to many deprotection and coupling cycles. Even if there is a very small percentage of side reaction in each cycle, the cumulative total can become significant. Side chain protecting groups with greater stability should be considered for the amino acids in the early portion of the peptide synthesis.

Problems can arise when the peptide is deprotected and cleaved from the resin. Reactive intermediates formed during the cleavage of the protecting groups can react with vulnerable moieties in the peptide. Again, this usually in not a big problem with short peptide sequences, as short sequences are less probable to have two interacting species. Long peptide sequences are more likely to have at least one vulnerable residue and choosing appropriate protecting groups and cleavage additives is becomes critical. The following tables list some of the common amino acid side chain protecting groups, the standard conditions used to remove them, and comments about their applications.

Boc Amino Acid Derivatives

Amino Acid	Protecting Groups	Deprotection	Comments
Arg	NO2	HF or hydrogeolysis	Prone to side reactions, seldom used
	Tos	HF	Can react with Trp residues during peptide cleavage, use Trp(For) and add thioanisole during peptide cleavage.
Amino Acid	**Protecting Groups**	**Deprotection**	**Comments**
Asp/Glu	OBzl	HF	Standard protection, acid catalyzed cyclization byproducts possible
	OcHx	HF	Suppresses aspartimide and pyroglutamate formation, recommended in long peptide sequences

Asn/Gln	Xan	HF	Suppresses dehydration and increases solubility of the protected amino acid
Cys	Bzl	HF	Standard
	MeOBzl	HF, TFMSA	Standard
	MeBzl	HF, HBr/HOAc	Standard
	Acm	Hg(II), I_2	Iodine forms S-S bond during deprotection. Stable to cleavage conditions, useful for preparing protected peptides
	Trt	TFA, HF, I_2	Can be used with Cys(Acm) for on resin cyclization
His	Boc	TFA	Temporary protection
	Bom	HF	Suppresses racemization
	Dnp	Thiolysis	Remove protecting group before peptide cleavage
	Tos	HOBt	Suppresses racemization
	Trt	TFA, HF	Temporary protection
Lys/Orn	Boc	TFA	Temporary protection
	2-Cl-Z	HF, TFMSOTf, TFSMA, HBr/HOAc	Standard
	Fmoc	Piperidine	Used for preparing protected peptide fragments and on-resin derivatization of the side chain
	Z	HF, Hydrogenolysis	Standard
Met	Sulfoxide	Mercaptoethanol	Oxidized form of methionine, simplifies purification of crude peptide when a mixture of oxidized and reduced forms occur.
Ser/Thr	Bzl	HF	Standard
	tBu	TFA, HF	Temporary protection
Amino Acid	**Protecting Groups**	**Deprotection**	**Comments**
Trp	For	HF	Standard
Tyr	tBu	TFA	Temporary protection
	Bzl	HF	Can for side products during cleavage
	2-Br-Z	HF	Standard
	2,6-Cl_2Bzl	HF	Standard

Fmoc Amino Acid Derivatives

Amino Acid	Protecting Groups	Deprotection	Comments
Arg	Mtr	TFA at 35°C	Best in small peptides with only one Arg residue
	Pmc	TFA	Standard
	Pbf	TFA	Standard, Less likely to react with Trp residues during cleave than Pmc
Asp/Glu	OtBu	TFA	Standard
	OBzl	H_2/Pd, HF	Seldom used
	OcHx	HF	Seldom used
Asn/Gln	Trt	TFA	Standard, suppresses dehydration and increases solubility of the protected amino acid
Cys	Acm	Hg (II), I_2	Iodine forms S-S bond during deprotection. Stable to cleavage conditions, useful for preparing protected peptides
	tBu	TFSMA, Hg(II), TFA/DMSO/Anisole	Useful in selective formation of multiple disulfide bridges
	pMeOBzl	TFMSA	Useful in selective formation of multiple disulfide bridges
	Mmt	1% TFA	On resin modification
	Trt	TFA, I_2	Standard
His	Fmoc	Piperidine	Temporary
	Trt	90% TFA/DCM	Standard
	Mtt	15% TFA/DCM	Standard
Lys/Orn	Boc	TFA	Standard
	tfa	TFA	Does not form side products during cleavage

Amino Acid	Protecting Groups	Deprotection	Comments
Lys/Orn	Trt	TFA	Fewer side reactions during cleavage
	Mtt	1% TFA/DCM	On-resin modification
	Dde, ivDde	Hydrazine	N-terminal Fmoc protecting group must be replaced with Boc before hydrazine treatment.
	Fmoc	Piperidine	Temporary
Ser/Thr	tBu	TFA	Standard

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	Trt	1% TFA/DCM	On resin modification
	Bzl	H_2/Pd, HF	Seldom used
Trp	Boc	TFA	Greatly reduces by-products formed during cleavage
Tyr	tBu	TFA	Standard
	Bzl	H_2/Pd, HF	Seldom used

Planning the Synthesis of Peptides Containing Multiple Disulfide Bridges

Peptides that contain multiple disulfide bridges are especially challenging synthetically. In some instances, all of the cysteine residues can be deprotected and simple air oxidation will form primarily the correctly bridged peptide.[162] When substantial mispairing makes this approach impractical, selective deprotection and stepwise bridge formation are required.

A peptide containing two disulfide bridges requires two pairs of differentially protected cysteines. One pair can have protecting groups that are removed at the same time as the peptide is cleaved from the resin while the other pair are protected with groups, such as Acm, that remain in place during cleavage. After the peptide has been synthesized and cleaved from the resin, the first disulfide bridge is formed, then the remaining pair of cysteine residues are deprotected and the second disulfide bridge is formed.

Preparing a peptide with three disulfide bonds requires three pairs of cysteine residues that can be independently deprotected. In this case, Fmoc chemistry is preferred over Boc chemistry because there is a greater selection of independently deprotected cysteine derivatives available. The set of Cys(Trt), Cys(Acm) and Cys(pMeOBzl) is example that has been employed successfully in the synthesis of several peptides, including defensins, relaxin, and sapecin.[163] The preparative sequence is outlined in the table below.

Cycle	**Cysteine Protection**	**Deprotection**	**Cyclization**
Cycle 1	Cys(Trt)	TFA/H_2O/Scavengers	Air oxidation
Cycle 2	Cys(Acm)	Cleavage and cyclization with I_2	
Cycle 3	Cys(pMeOBzl)	TFSMA/TFA/anisole	air oxidation or I_2

Storing and Using Amino Acid Solutions

Dissolved amino acids have a finite life span and will need to be replaced if they are not used soon after preparation.

Amino acid solutions are typically made up 0.5 M in an HOBt solution that is itself 0.5 M. This preparation can typically sit open to air at room temperature throughout the course of a synthesis. Unused solution can be refrigerated for weeks awaiting later use. When the solution becomes discolored it should be discarded.

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[162] Moroder, L.; Besse, D.; Musiol, H. J.;, Rudolph-Böhmer, s.; Siedler, F. *Biopolymers*, **1996**, *40*, 207-234.

[163] Durieux, J. P.; Nyfeler, R., in "Peptides 1994, Proceedings of the 23rd European Peptide Synposium, Braga 1994", Maia, H. L. S. Ed., ESCOM Publishers, Leiden 1995, 165.; Büllesbach, E. E.; Schwabe, C. *J. Biol. Chem.*, **1991**, *266*, 10754-10761.; Mergler, M.; Nyfeler, R., in "Proceedings of the 4th international Symposium on Innovation and Perspectives in SPPS, Edinburgh 1995", Epton, R. Ed., Mayflower Scientific Ltd, Birmingham 1996, 42.

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Certain amino acids are particularly prone to oxidation and should not be kept longer than a week in solution. These include cysteine, methionine, tryptophan, and histidine.

Technical Notes

The following are general procedures. Although these procedures generally produce good results, they may not be applicable to all resins or substrates.

Attaching the First Residue to a Resin

Merrifield Resin

Cesium Salt Method (Gisin Method)[164]

1. Dissolve the carboxylic acid in methanol (5 mL/mmol) and add water (0.5 mL/mmol). Titrate the solution to pH 7.0 with a 20% aqueous solution of cesium carbonate. Evaporate the mixture to dryness. Add DMF (2.5 mL/mmol) and evaporate to dryness (45°C). Add a second portion of DMF (2.5 mL/mmol) and evaporate to dryness (45°C).
2. Set up a flask with a heating mantle and thermometer on an orbital shaker.
3. Swell the resin in DMF (6-8 mL per gram of resin). Add the dry carboxylic acid cesium salt (1.0 equivalent based on the chlorine substitution of the resin). The cesium salt must be completely dry to obtain satisfactory results.
4. Shake the mixture at 50°C for 24 hrs.
5. Filter the resin. Wash the resin thoroughly with DMF, then 50% (v/v) aqueous DMF, then 50% (v/v) aqueous methanol, and finally methanol. Dry the resin *in vacuo* to a constant weight.

Potassium Fluoride Method[165]

1. Set up a flask with a heating mantle and thermometer on an orbital shaker.
2. Dissolve the Boc-amino acid (1.5 equivalents based on the chlorine substitution of the Merrifield resin) in DMF (6 mL/g resin) and add it to the flask.
3. Add the Merrifield resin (1 equivalent) and anhydrous potassium fluoride (3 equivalents based on the chlorine substitution of the Merrifield resin).
4. Shake the mixture at 50°C for 24 hrs.
5. Filter the resin. Wash the resin thoroughly with DMF, then 50% (v/v) aqueous DMF, then 50% (v/v) aqueous methanol, and finally methanol. Dry the resin *in vacuo* to a constant weight.

Activation of Trityl Alcohol Resins

1. Suspend the resin in DCM (approximately 10 mL per gram of resin).
2. Cool the suspension in an ice bath.
3. Add 1.2 equivalents of thionyl chloride (SO_2Cl_2) and 2.4 equivalents of pyridine. Caution: Thionyl chloride is corrosive and reacts with moisture to release HCl. Wear safety goggles and chemical resistant gloves. Perform this reaction in an efficient fume hood.
4. Heat at reflux for 2 to 4 hours.
5. Filter the resin. Wash it 6 times with DCM. Dry the resin *in vacuo* over KOH.

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[164] Geison, B. F.; *Helv. Cheim. Acta*, **1973**, *56*, 1476-1482.
[165] Based on procedures in Yajima, H.; Fujii, N.; Funokoshi, S.; Watanabe, T.; Murayama,E.; Otaka, A. *Tetrahedron* **1988**, *44*, 805-819.

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Attachment of Amino Acids to Trityl Chloride Resins[166]

1. Use 1.0 equivalent of the protected amino acid. If a lower substitution resin is required, reduce the amount of acid. Dissolve the amino acid in DCM (approximately 10 mL per gram of resin). If the acid does not dissolve in completely, add a small amount of DMF.
2. Add the amino acid solution to the resin. Add 1.0 equivalent (respective to the acid) of DIPEA. Agitate the mixture with a shaker for 5 minutes, then add 1.5 equivalents (respective to the acid) of DIPEA. Agitate the mixture vigorously for 30 to 60 minutes.
3. To endcap any remaining reactive trityl groups, add HPLC grade methanol, 0.8 mL per gram of resin, and mix for 15 minutes. Filter the resin and wash it three times with DCM, using approximately 10 mL per gram of resin. Wash the resin twice with DMF, twice with DCM, and three times with methanol. Dry the resin in vacuo. The substitution of the resin can be estimated from the weight gain.

Attachment of Alcohols and Phenols to Trityl Chloride Resins[167]

1. Dissolve 2 equivalents (relative to the resin substitution) of the alcohol or phenol in pyridine (8 mL per gram of resin).
2. Add the resin to this solution and mix at room temperature. Reaction may take up to two days.
3. Filter the resin and wash it 4 times DCM. Wash the resin 4 times with dry ether.
4. Dry *in vacuo* to a constant weight. The substitution of the resulting resin can be estimated from the weight gain of the resin.

Attachment of Carboxylic Acids to Hydroxy-Substituted Resins

1. In a round bottom flask suspend the resin in 9:1 v/v CH_2Cl_2/DMF (approximately 15 mL per gram of resin).
2. In a separate flask dissolve 1.5 to 2.5 equivalents (relative to the resin) of the carboxylic acid in a minimum amount of DMF. Add the same equivalency of HOBt. Stir the mixture until the HOBt dissolves. If the HOBt doesn't dissolve completely, add DMF to bring it into solution. Add this solution to the resin.
3. In a separate flask dissolve 0.1 equivalent (relative to the resin) of DMAP in a minimum amount of DMF.
4. Add 1.0 equivalent (relative to the carboxylic acid) of DIC to the resin mixture then add the DMAP solution. Equip the flask with a drying tube.
5. Agitate the mixture with a mechanical shaker for 2 to 3 hours at room temperature. Add 2 equivalents (relative to the resin) of acetic anhydride and pyridine to the reaction flask and mix an additional 30 minutes at room temperature to end cap any unreacted hydroxyl groups on the resin.
6. Filter the resin in a fine porosity sintered glass funnel and wash it 3 times with DMF, then 3 times with DCM, and finally 3 times with methanol. In each wash use enough solvent to slurry the resin. After the final methanol wash, dry the resin in vacuo to a constant weight.

Mitsunobu Coupling to Hydroxy-Substituted Resins[168]

1. Dissolve the acid or phenol (5 equivalents based on resin substitution) in THF (50 mL/g resin). Add the resin and flush the flask with argon.

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[166] Based on Barlos, K.; et al. *Tetrahedron Lett.* **1989**, *30*, 3943-3946.
[167] Fyles, T. M.; Leznoff, C. C. *Can. J. Chem.* **1976**, *54*, 935-942.
[168] Fancelli, D.; Fagnola, M. C.; Severino, D.; Bedeschi, A. *Tetrahedron Lett.* **1997**, *38*, 2311-2314.

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2. Add triphenylphosphine (10 equivalents based on resin substitution).

3. Add 10 equivalents (based on resin substitution) of a 2M solution of diethyldiazodicarboxylate in THF dropwise at 5 ºC.

4. Shake the mixture with a mechanical shaker for 4 hours at room temperature. Filter the resin and wash it with THF, MeOH, and Et_2O. Dry the resin under vacuum to a constant weight. The coupling can be repeated to improve coupling yield.

Attachment of Fmoc-Amino Acids to Rink, PAL, or Sieber Resins

1. In a round bottom flask suspend the resin in 20% v/v piperidine/DMF (approximately 15 mL per gram of resin).
2. In a separate flask dissolve 1.5 to 2.5 equivalents (relative to the resin) of the Fmoc-amino acid in a minimum amount of DMF. Add the same equivalency of HOBt. Stir the mixture until the HOBt dissolves. If the HOBt doesn't dissolve completely, add DMF to bring it into solution.
3. Add 1.0 equivalent (relative to the amino acid) of DIC to the Fmoc-amino acid/HOBt mixture. Equip the flask with a drying tube. Let the mixture stand at room temperature for 10 minutes.
4. Add the activated amino acid solution to the resin and equip the flask with a drying tube. Agitate the mixture with a mechanical shaker for 2 to 3 hours at room temperature.
5. Remove a small sample of the resin and wash it with DCM. Test for free amino groups using the Kaiser test. If there are free amino groups, add 1 equivalent of acetic anhydride and pyridine to the reaction flask and mix for 30 minutes.
6. Filter the resin in a fine porosity sintered glass funnel and wash it 3 times with DMF, then 3 times with DCM, and finally 3 times with methanol. In each wash use enough solvent to slurry the resin. After the final methanol wash, dry the resin *in vacuo* to a constant weight. The substitution of the resin can be estimated from the weight gain of the resin.

Attachment of Boc-Amino Acids to BHA or MBHA Resins

1. In a round bottom flask suspend the resin in 10% v/v DIPEA/DMF (approximately 15 mL per gram of resin). Agitate the suspension with a mechanical shaker for 5 minutes. Filter the resin.
2. Wash the resin with DMF (10 ml of DMF per gram of resin) and filter. Repeat 2 more times.
3. In a separate flask dissolve 1.5 to 2.5 equivalents (relative to the resin) of the Boc-amino acid in a minimum amount of DMF. Add the same equivalency of HOBt. Stir the mixture until the HOBt dissolves. If the HOBt doesn't dissolve completely, add DMF to bring it into solution.
4. Add 1.0 equivalent (relative to the amino acid) of DIC to the Boc-amino acid/HOBt mixture. Equip the flask with a drying tube. Let the mixture stand at room temperature for 10 minutes.
5. Add the activated amino acid solution to the resin and equip the flask with a drying tube. Agitate the mixture with a mechanical shaker for 2 to 3 hours at room temperature.
6. Remove a small sample of the resin and wash it with DCM. Test for free amino groups using the Kaiser test. If there are free amino groups, add 1 equivalent of acetic anhydride and pyridine to the reaction flask and mix for 30 minutes.
7. Filter the resin in a fine porosity sintered glass funnel and wash it 3 times with DMF, then 3 times with DCM, and finally 3 times with methanol. In each wash use enough solvent to slurry the resin. After the final methanol wash, dry the resin *in vacuo* to a constant weight. The substitution of the resin can be estimated from the weight gain of the resin.

Attaching Amines to Aldehyde Resins by Reductive Amination

1. Suspend the resin in DMF (approximately 8 mL per gram of resin).

2. Dissolve 10 equivalents of the amine (relative to the resin) and 10 equivalents (relative to the resin) of sodium cyanoborohydride ($NaBH_3CN$) in DMF (approximately 800 μL per gram resin).
3. Add the solution of amine and $NaBH_3CN$ to the resin suspension.
4. Shake the mixture at room temperature. The reaction will take 1 to 24 hours depending on the structure of the amine.

Attachment of Amines to Trityl Chloride Resins

1. Dissolve 2 to 4 equivalents (relative to the resin substitution) of the amine in dry THF (10 to 12 mL per gram of resin).
2. Add the trityl chloride resin to the solution. Mix with a mechanical shaker for 2 hours.
3. Filter the resin and wash it 3 times with 17:2:1 DCM/MeOH/DIPEA (v/v/v). Wash the resin 3 times with DCM.
4. Dry *in vacuo* to a constant weight. The substitution of the resulting resin may be estimated from the weight gained by the resin.

Alcohol Coupling to Carboxypolystyrene

Carbodiimide Activation

1. In a round bottom flask suspend the resin in DMF (approximately 15 mL per gram of resin).
2. In a separate flask dissolve 1.5 to 2.5 equivalents (relative to the resin) of the compound to be attached in a minimum amount of DMF. Add the same equivalency of HOBt. Stir the mixture until the HOBt dissolves. If the HOBt doesn't dissolve completely, add just enough DMF to bring it into solution. Add this solution to the resin.
3. In a separate flask dissolve 0.1 equivalent (relative to the resin) of DMAP in a minimum amount of DMF.
4. Add 1.0 equivalent (relative to the amino acid) of DIC to the resin mixture then add the DMAP solution. Equip the flask with a drying tube.
5. Agitate the mixture with a mechanical shaker for 4 to 6 hours at room temperature.
7. Filter the resin in a fine porosity sintered glass funnel and wash it 3 times with DMF, then 3 times with DCM, and finally 3 times with methanol. In each wash use enough solvent to slurry the resin. After the final methanol wash, dry the resin in vacuo to a constant weight. The substitution of the resin can be estimated from the weight gain of the resin.

Oxalyl Chloride/Thionyl Chloride Activation[169]

1. Suspend the resin in dry benzene (15 - 20 mL/g resin).
2. Add oxalyl chloride or thionyl chloride (4.0 equivalents based on resin substitution).
3. Heat the mixture at reflux for 12 hours.
4. Filter the resin and wash it with dry benzene under nitrogen. Dry the resin *in vacuo* to a constant weight.
5. Suspend the resin in DCM (10 mL/g resin). Add 1.5 equivalents (based on starting resin substitution) of the alcohol, phenol, or amine to be attached. Add 1.5 equivalents (based on starting resin substitution) of triethylamine and 0.1 equivalent of DMAP.
6. Attach a drying tube to the flask and shake the mixture with a mechanical shaker at room temperature for 6 to 48 hours. The progress of the reaction can be monitored by the disappearance of the acid chloride C=O stretching band in the IR spectrum.

[169] Based on the procedure in Panek, J.S.; Zhu, B. *Tetrahedron Lett.* **1996**, *37*, 8151-8154.

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7. When the reaction is complete, filter the resin and wash it with DCM, then with 50% (v/v) DCM/methanol, and finally with methanol. Dry the resin *in vacuo* to a constant weight. The resin substitution can be approximated from the weight gain of the resin.

Alkylation with Primary Alkyl Halides[170]

1. Suspend the resin in N-methylpyrrolidone (NMP, 10 mL/g resin).
2. Add cesium carbonate (2.5 equivalents based on resin substitution), potassium iodide (0.1 equivalents based on resin substitution) and 1,5-diazabicyclo[4.3.0]non-5-ene (DBN, 0.1 equivalents based on resin substitution).
3. Add the alkyl halide (4.0 equivalents based on resin substitution).
4. Shake the mixture at 70 °C for 72 hours.
5. Filter the resin, then wash it with NMP, 1:1v/v NMP/water, NMP, DCM and methanol.
6. Dry the resin *in vacuo* to a constant weight.

Measuring Substitution of Fmoc–Amino Acid Resins[171]

1. Accurately weigh approximately 10 mg of resin into a 2 mL microcentrifuge tube.
2. Add 1 mL of 20% (v/v) piperidine in DMF.
3. Vortex briefly and agitate 1 hour at room temperature on a rotary shaker.
4. Vortex the tube and allow the resin to settle for 2 minutes.
5. Dilute 50 microliters of the supernate to 5 mL with DMF (dilution factor = 0.01).
6. Prepare a blank by diluting 50 microliters of 20% (v/v) piperidine to 5 mL with DMF.
7. Measure the absorbance of the sample versus the blank at 278 nm.
8. Calculate the absorbance using the following formula:

$$S(\text{mmol/g})=(1000\cdot A)/(M\cdot 7800\cdot D)$$

S=substitution of the resin in mmol/g
A=absorbance of the sample-absorbance of the blank
M=mass of the resin used (in milligrams)
D=dilution factor

[170] Veerman, J.J.N.; et al. *Eur.J.Org.Chem.* **1998**, 2583-2589.
[171] *Biotech. Bioeng. (Comb. Chem.),* **1998**, *61*, 55-60.

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Standard Coupling Procedures

N-Terminal Deprotection

Standard Removal of Boc Protecting Group

1. Suspend the resin in 50% (v/v) TFA/dichloromethane (DCM), using 1 mL of TFA/DCM per gram of resin.
2. Shake the resin at room temperature for 30 minutes.
3. Filter the resin.
4. Wash the resin three times with DCM (1 mL/gm resin).
5. Wash the resin three times with 5% (v/v) diisopropylethylamine (DIPEA)/DCM) (1 mL/gm resin) to remove TFA.

Standard Removal of Fmoc Protecting Group

1. Place the resin in a round bottom flask and add 20% (v/v) piperidine in DMF (approximately 10 mL/gm resin).
2. Shake the mixture at room temperature for 30 minutes.
3. Filter the resin and wash it with several portions of DMF.

Removal of Fmoc Protecting Group with DBU

DBU-piperidine Deprotection Reagent

Dissolve 2.0 g of DBU and 2.0 g of piperidine in 96 grams of DMF or NMP.

Deprotection Procedure

1. Place the resin in a round bottom flask and add 20% (v/v) piperidine in DMF (approximately 10 mL/gm resin).
2. Shake the mixture at room temperature for 30 minutes.
3. Filter the resin and wash it with several portions of DMF.

Converting DCHA and CHA Salts to Free Acids

1. Dissolve the DCHA or CHA salt in dichloromethane (DCM).
1. Extract the DCM solution with ice-cold aqueous $KHSO_4$ solution 3 times.
2. Dry the organic layer over $MgSO_4$ and filter.
4. Evaporate the solvent under reduced pressure to obtain the free acid.

Coupling

Standard DIC/HOBt Coupling

1. Remove the N-terminal protecting group by standard deprotection protocols.
2. Suspend the resin in dichloromethane (DCM, 10 mL per gram resin)
3. Dissolve 5 equivalents (based on resin substitution) in DMF (approximately 1 mL per gram) of amino acid derivative.
4. Dissolve 5.5 equivalents (based on resin substitution) of HOBt in DMF (minimum volume necessary for complete solution).

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5. Add the amino acid solution and the HOBt solution to the resin suspension.
6. Add 5.5 equivalents (based on resin substitution) of DIC.
7. Shake the mixture at room temperature under inert gas. Monitor the reaction using the ninhydrin test. When the ninhydrin test is negative, filter and wash the resin three times with DMF, three times with DIC, then three times with either methanol or DCM. If the ninhydrin test is not negative within four hours, repeat the coupling procedure.

Coupling with EDC

1. Dissolve the N-protected amino acid and the amino acid ester to be coupled in dichloromethane (DCM).
2. Cool the mixture in an ice bath.
3. Add 1.2 equivalents of EDC and stir the mixture.
4. When the reaction is complete, wash the mixture with water to remove excess EDC and urea by-product.
5. Dry the organic phase over sodium sulfate, filter, and evaporate to obtain the crude product.

Coupling with BOP Reagent[172]

1. Remove the N-protecting group using standard deprotection protocols.
2. Dissolve 2.0 equivalents (based on resin substitution) of the protected amino acid in DMF (5 mL/g of resin) and add to the resin.
3. Add 2.0 equivalents (based on resin substitution) of 1.0 M BOP solution and 4.0 equivalents (based on resin substitution) of diisopropylethylamine (DIPEA). 2.0 equivalents (based on resin substitution) of 0.5 M HOBt solution in DMF can be added to suppress racemization.
4. Mix for 10-60 minutes until the Kaiser test is negative.

Coupling with PyBOP[173]

1. Remove the N-protecting group using standard deprotection protocols.
2. Dissolve 1.1 equivalents (based on resin substitution) of the protected amino acid in DMF (5 mL/g of resin) and add to the resin.
3. Add 1.1 equivalents (based on resin substitution) of 1.0 M PyBOP solution and 2.2 equivalents (based on resin substitution) of diisoproplyethylamine (DIPEA). 1.1 equivalents (based on resin substitution) of 0.5 M HOBt solution in DMF can be added to suppress racemization.
4. Mix for 10-60 minutes until the Kaiser test is negative.

Coupling N-Methyl Amino Acids with Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate[174]

1. Remove the N-protecting group from the resin peptide using standard procedures.
2. Suspend the resin in DCM (10 mL/gram resin).
3. Dissolve 2 equivalents (based on resin substitution) of the protected amino acid in DCM or DMF. Add the solution to the resin.
4. Add 2 equivalents (based on resin substitution) of PyBroP®. Cool the mixture to 0 °C.

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[172] Rovero, P.; Quartara, L.; Fabbri, G. *Tetrahedron Lett.* **1991**, *32*, 2639-2642.
[173] Based on the procedure in Hoeg-Jensen, T.; Jakobsen, M. H.; Holm, A. *Tetrahedron Lett.* **1991**, *32*, 6387-6390.
[174] Based on procedure of Coste, J.; Frérot, E.; Jouin, P.; Castro, B. *Tettrhedron Lett.* **1991**, *32*, 1967-1970.

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5. Add 6 equivalents of diisopropylethylamine (DIPEA). Mix 1 minute cold and 1 hour at room temperature.
6. Filter the resin and wash with DCM.

Coupling with HATU,HBTU, HCTU or TBTU

1. Remove the N-protecting group using standard deprotection protocols.
2. Dissolve 2.0 equivalents (based on resin substitution) of the protected amino acid in DMF (5 mL/g of resin) and add to the resin.
3. Add 2.0 equivalents (based on resin substitution) of 1.0 M HBTU solution and 4.0 equivalents (based on resin substitution) of diisoproplyethylamine (DIPEA). 2.0 equivalents (based on resin substitution) of 0.5 M HOBt solution in DMF can be added to suppress racemization.
4. Mix for 10-60 minutes until the Kaiser test is negative.
5. Filter and wash the resin with DMF.

Coupling with TSTU in Aqueous Solvent Mixtures[175]

1. Dissolve the acid in a 2:2:1 mixture of DMF/dioxane/water.
2. Add 3 equivalents of diisopropylethylamine and 1.3 equivalents of TSTU.
3. After the formation of the -OSu ester is complete, add 1.5 equivalents of the amine.
4. After the reaction is complete, the solvents are removed and the crude product is isolated.

Fragment Coupling with TDBTU[176]

1. Dissolve the peptide ester in DMF (N,N-dimethylformamide) (approximately 16 ml per mmole of peptide ester).
2. Add 1.25 equivalents of the N-protected peptide fragment.
3. Cool the solution to -25˚ C.
4. Add 2.25 equivalents of DIEA (diisopropylethylamine).
5. Add 1.05 equivalents of TDBTU.
6. Stir at –25˚ C for one hour.
7. Stir overnight at room temperature
8. Pour the mixture into water (10 times the volume of DMF) containing 8% w/w sodum carbonate.
9. Stir for 30 minutes
10. Collect the solid by filtration, wash with water, and dry in vacuo to obtain the crude product.

Solid Phase Coupling with DEPBT [177]

1. Suspend the resin in dichloromethane (DCM) or N,N-dimethylformamide (DMF) (approximately 10 ml per gram of resin).
2. Add 1.5 equivalents (based on resin substitution) of the protected amino acid.
3. Add 3 equivalents of DIPEA or Et_3N.
4. Add 1.5 equivalents of DEPBT and shake the mixture at room temperature for 1-2 hours.
5. Filter the resin.
6. Wash the resin 3 times with DMF then 3 times with DCM.

[175] Bannwarth, W.; Knorr, R. *Tetrahedron Lett.* **1991**, *32*, 1157-1160.
[176] Hiebl, J; et al. *J. Peptide Res.*, **1999**, *54*, 54-65.
[177] Tang, Y.-C.; Xie, H.-B.;Tian, G.-L.; Ye, Y.-H. *J. Peptide Res.* **2002**, *60*, 95-103.

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Solution Phase Coupling with DEPBT [178]

11. Dissolve the protected amino acid and amino acid ester in THF (tetrahydrofuran) (approximately 2 ml per mmole of amino acid ester).
12. Add 1.1 –1.2 equivalents of DEPBT and 2.0 equivalents of triethylamine (Et_3N) or diisopropylethylamine (DIPEA). If the coupling involves amino acids highly susceptible to epimerization, cool the mixture to 0˚ C before adding the DEPBT and use sodium carbonate in place of triethylamine.
13. Stir for 1 to 2 hours.
14. Filter to remove any solid. Rinse solid with a little THF.
15. Evaporate *in vacuo* to obtain the crude peptide.

Cyclization with DEPBT [134]

1. Prepare a $1x10^{-3}$ M to $2x10^{-3}$ M solution of the linear peptide in DMF.
2. Cool the solution in an ice bath and add 4 equivalents of Et_3N and 2 equivalents of DEPBT.
3. Stir 30 minutes at 0˚ C, then warm to room temperature and stir for 24 hours.
4. Evaporate the DMF *in vacuo*.
5. Purify the residue by column chromatography to obtain the crude peptide.

Tests for Monitoring Solid Phase Reactions

The **Kaiser Test** is a very sensitive test for primary amines. It is commonly utilized in solid phase peptide synthesis to determine if coupling reactions are complete. Ninhydrin reacts with the deprotected N-terminal amine group of the peptide-resin to produce an intense blue color. The Kaiser test is not reliable for detecting secondary amines. Thus, if the N-terminal amino acid is proline, pipecolic acid, or tetrahydroisoquinoline-3-carboxylic acid, another test such as the **Isatin Test** or the **Chloranil Test** is used.

Kaiser Test (Ninhydrin Test)[179]
Kaiser Test Solutions

Reagent A:

1. Dissolve 16.5 mg of KCN in 25 mL of distilled water.
2. Dilute 1.0 mL of above solution with 49 mL of pyridine (freshly distilled from ninhydrin).
3. Pour it into a small reagent bottle and label it “A”.

Reagent B:

1. Dissolve 1.0 g of ninhydrin in 20 mL of n-butanol.
2. Pour into a small reagent bottle and label it as “B”.

Reagent C:

1. Dissolve 40 g of phenol in 20 mL of n-butanol.
2. Pour it into a small reagent bottle and label it “C”.

[178] Boger, D.L.; Kim, S.H.; Miyazaki, S.; Strittmatter, H.; Weng, J.-H.; Mori, Y.; Rogel, O.; Castle, S.L.; McAtee, J.J. *J. Am. Chem. Soc.* **2000**, *122*, 7416-7417.
[179] Wellings, D. A.; Atherton, E. “Methods in Enzymology Volume 289: Solid-Phase Peptide Synthesis” Ed. Fields, G. B. Academic Press, San Diego, 1997, p. 54.

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Kaiser Test Procedure:

1. Take 10-15 beads of resin in a test tube and label it S.
2. Take tube S and another empty tube designated R (reference)
3. To each tube add:
   2 to 3 drops of Reagent A
   2 to 3 drops of Reagent B
   2 to 3 drops of Reagent C
4. Heat both the tubes at 110°C for 5 minutes.
5. Compare the color with reference.

Colorless or faint blue color: complete coupling, proceed with synthesis
Dark blue solution but beads are colorless: nearly complete coupling, extend coupling or cap unreacted chains
Solution is light blue but beads are dark blue: coupling incomplete, recouple
Solution is intense blue and all beads are blue: failed coupling, check amino acid, reagents, then recouple

Isatin Test for Unprotected Proline[180]

Isatin Test Solution

1. Add 2 g of isatin to 60 mL of benzyl alcohol.
2. Stir at room temperature for 2 hours.
3. Filter to remove any undissolved isatin.
4. Dissolve 2.5 g of Boc-Phe-OH in the filtrate.

Isatin Test Procedure

Place a small amount of the test sample (4-5 mg of resin-peptide) in a small test tube. Add 2 to 3 drops of the isatin solution and heat at 100 °C for 5 minutes. If the beads turn blue, the coupling reaction is incomplete.

Chloranil Test for Secondary Amines[181]

Chloranil Test Solutions

Reagent A

Mix 1 mL of acetaldehyde with 49 mL of N,N-dimethylformamide (DMF).

Reagent B

Dissolve 1 g of p-chloranil in 49 mL of DMF.

Chloranil Test Procedure

1. Place 1 to 5 mg of resin in a small test tube.
2. Add 1 drop of Reagent A.

[180] Wellings, D. A.; Atherton, E. "Methods in Enzymology Volume 289: Solid-Phase Peptide Synthesis" Ed. Fields, G. B. Academic Press, San Diego, 1997, pp. 54-55.
[181] Vojkovsky, T. *Pept. Res.,* **1995**, *8,* 236-237.

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3. Add 1 drop of Reagent B.
4. Let the mixture stand at room temperature for 5 minutes.
5. Observe the color of the beads. If the beads are blue, secondary amine is present.

Standard Capping Procedure

1. Filter and wash the resin several times with DMF.
2. Suspend the resin in a DMF solution containing acetic anhydride (50 equivalent based on resin substitution) and pyridine (50 equivalents based on resin substitution). DIPEA may be substituted for the pyridine.
3. Gently shake at room temperature for 30 minutes.
4. Filter and wash the resin with DMF.
5. Perform a Kaiser test. If the Kaiser test is not negative, repeat the capping procedure.

Cleavage Procedures

Cleavage from Merrifield Resin

There are many protocols for cleaving peptides from Merrifield resin. Five common protocols are standard HF, low-high HF, standard TFSMA, low-high TFSMA, and TMSOTf. In the standard HF protocol the N-terminal Boc group is removed then the peptide-resin and a mixture of scavenger are mixed with a high concentration of HF inside a special HF apparatus. With the low-high HF procedure a low concentration of HF in DMS is used to remove most of side chain protection groups followed by a standard HF cleavage. TFSMA standard and low-high procedures are alternatives to the corresponding HF procedures. The main advantage of these procedures is that they do not require special HF resistant apparatus. TFMSA-cleaved peptides are susceptible to salt and scavenger association and should be neutralized and desalted before further purification. TMSOTf is an alternative to HF and TFMSA. It does not require HF resistant apparatus, produces fewer side reactions, and the products are less hygroscopic than the TFSMA cleavage products.

In choosing a cleavage protocol, the side chain protecting groups as well as the amino acid composition of the peptide must be considered. The following chart will assist in selecting the appropriate protocol.

If peptide contains:	
His(Dnp)	The Dnp group must be removed before cleaving the peptide from the resin or removing the N-terminal Boc group of the finished peptide [Deprotection of His(Dnp)].
Trp(CHO)	In the standard HF and TFSMA cleavage protocols the peptide resin must be treated with piperidine in DMF following the removal of the N-terminal Boc group to remove the formyl group [Piperidine Deprotection of Trp(CHO)]. The formyl group can also be removed thiolytically in a “low-high” HF procedure or “low-high” TFSMA procedure where p-cresol is replaced with p-thiocresol or thiophenol. The TMSOTf procedure can remove the formyl group if EDT is added to the cleavage mixture.
Trp	Anisole should be used in the cleavage mixture to prevent alkylation of Trp by benzyl or t-butyl cations. Avoid using thioanisole in HF cleavages.
Arg(Tos)	TFSMA does not deprotect Arg(Tos). Arg(Tos) is deprotected during standard HF cleavage conditions, but may require longer reaction times. The low-high HF cleavage is recommended.
Arg(NO2)	TFMSA and TMSOTf will not deprotect this group. During “low-high” HF cleavage, it is cleaved under the “high” conditions.

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Asp(OBzl) Glu(OBzl)	The cleavage should be performed at 5°C or lower to minimize side reactions of these amino acids.
Asp(OcHx)	Cleavage should be performed at 5°C or lower to minimize aspartimide formation. TFMSA does not remove the OcHx group efficiently.
Glu(OcHx)	Cleavage should be performed at 5°C or lower to reduce anisylation of Glu.
Cys(ACM)	TMSOTf does not deprotect this group.
Cys(Bzl)	TMSOTf does not deprotect this group.
Cys(MeBzl)	TFMSA does not remove this group efficiently. Cleavage at temperatures below 5°C may be very slow.
Met(O)	This group is reduced to Met during low-high HF and low-high TFMSA cleavages. TSMOTf will not quantitatively reduce Met(O), so post-cleavage reduction is necessary [Post Cleavage Reduction of Met(O)].
Cys and Met	DMS, p-thiocresol, and anisole should be added to the cleavage mixture to prevent alkylation of these amino acids.

Standard HF Cleavage[182]

1. Place a Teflon-coated stirring bar and the peptide-resin into the reaction vessel of the HF apparatus. Add the appropriate mixture of scavengers.
2. Secure the cap onto the reaction vessel and cool it in a dry ice/methanol bath for at least 5 minutes. For every 0.2 mmol of substrate-resin, distill 10 mL of HF into the reaction vessel. Maintain the temperature between -5°C and 0°C while collecting the HF.
3. Maintain the temperature between 0°C and 5°C for 30 to 60 minutes as the cleavage mixture is stirred. If the substrate contains Arg(Tos), the cleavage may take up to 2 hours. After the end of the reaction time, evaporate the HF under a stream of nitrogen.
4. Filter the resin and wash it with a small amount of TFA. Combine the filtrates. Evaporate under reduced pressure to obtain the crude product.

Low-High HF Cleavage[183]

1. Place a Teflon-coated stirring bar and the peptide-resin into the reaction vessel of the HF apparatus. Add the appropriate mixture of scavengers. For most peptides, add 1 mL of p-cresol and 6.5 mL dimethylsulfide for every 0.2 mmol of peptide resin. If the peptide resin contains Cys add 1 mL p-cresol, 6.5 mL dimethylsulfide, and 0.2 mL of p-thiocresol for every 0.2 mmol of peptide resin.
2. Fasten the cap onto the reaction vessel and cool it in a dry ice/methanol bath for at least 5 minutes. For every 0.2 mmol of peptide-resin, distill 2.5 mL of HF into the reaction vessel. Maintain the temperature between -5°C and 0°C while collecting the HF.
3. Maintain the temperature at 0°C for 2 hours as the cleavage mixture is stirred. After the end of the reaction time, evaporate the HF and DMS *in vacuo* at 0 ºC.
4. Filter the resin; wash it with DCM or EtOAc to remove scavenger by products and suction dry.
5. Return the resin to the reaction vessel and add 1 mL of p-cresol for every 0.2 mmol of peptide-resin. If the peptide contains Trp(CHO), substitute thiocresol or thiophenol for p-cresol.
6. Fasten the cap onto the reaction vessel and cool it in a dry ice/methanol bath for at least 5 minutes. For every 0.2 mmol of peptide-resin, distill 10 mL of HF into the reaction vessel Maintain the temperature between -5°C and 0°C while collecting the HF.

[182] Based on procedures in J. M. Stewart and J. D. Young in "Solid Phase Peptide Synthesis", Pierce Chemical Company, Rockford, Illinois, 1984.
[183] Based on the procedures in Tam, J.P.; et al. *J. Am. Chem. Soc.* **1983**, *105*, 6442.

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7. Maintain the temperature between 0°C and 5°C for 30 to 60 minutes as the cleavage mixture is stirred. If the peptide contains Arg(Tos), the cleavage may take up to 2 hours. After the end of the reaction time, evaporate the HF under a stream of nitrogen.
8. Filter the resin with a fine sintered glass funnel. Wash the resin with a small amount or TFA. Combine the filtrates and add 8-10 times the volume of cold methyl t-butyl 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.

Standard Trifluoromethanesulfonic Acid Cleavage[184]

1. Check that the peptide-resin has been washed and thoroughly dried.
2. Transfer the resin into a round bottom flask equipped with a stirring bar. For every 100 mg of peptide-resin add 200 µL of thioanisole and 100 µL 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.
3. For every 100 mg of resin slowly add 200 µL of TMSFA dropwise. Stir vigorously during addition of the TFMSA to dissipate the heat generated.
4. Let the mixture stir at room temperature for 30 to 60 minutes.
5. 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.
6. Desalt the peptide by ion exchange or Sephadex columns.

Low-High TFMSA Cleavage

1. Transfer the resin to round bottom flask equipped with a stirring bar. For every 100 mg of resin add 100 mL of m-cresol and 300 mL dimethylsulfide. Cool the mixture to 0 ºC in an ice bath and 0.5 mL of TFA for every 100 mg of resin. If the peptide contains Trp(CHO), add 20 mL of EDT for each 100 mg of peptide resin.
2. Maintain the mixture at 0°C in an ice bath. For every 100 mg of peptide-resin slowly add 100 µL of TFMSA. Stir vigorously during addition of the TFMSA to dissipate the heat generated. Stir the mixture for 3 hours while maintaining the temperature between 0°C and 5°C.
3. Filter the resin in a medium sintered glass funnel. Wash the resin with several volumes of ether. Dry the resin under high vacuum over KOH or P_2O_5 for at least 4 hours.
4. Place the dried resin in a round bottom flask equipped with a stirring bar. For every 100 mg of resin add 100 mL of thioanisole and 30 mL of EDT. Cool the flask to between 0°C and 5°C using an ice bath. For every 100 mg of resin add 1.0 mL of TFA and mix for 5 to 10 minutes. For every 100 mg of resin slowly add 100 mL of TFMSA while stirring vigorously to dissipate the heat generated.
5. Warm the flask to room temperature and continue stirring for 90 to 120 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 methyl t-butyl 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.
7. Desalt the peptide by ion exchange or Sephadex columns.

[184] Based on procedures in "Introduction to Cleavage Techniques", Applied Biosystems Inc. Foster City, California, 1990.

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TMSOTf Cleavage[185]

1. Prepare the cleavage mixture. For each gram of resin mix 1.8 mL of TMSOTf, 7.0 mL of TFA, and 1.2 mL of thiocresol. If the peptide contains Trp(CHO) add 1.2 mL of EDT per gram of resin. Cool the cleavage mixture in an ice bath.
2. Place the peptide-resin in a round bottom flask equipped with a stirring bar. Cool the flask to 0°C in an ice bath, then add the chilled cleavage mixture.
3. Stir the mixture at 0°C for 1 to 2 hours.
4. Filter the resin with a fine sintered glass funnel. Wash the resin with a small amount of TFA. Combine the filtrates and add 8-10 times the volume of cold methyl t-butyl 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.

Cleavage from Wang Resin

TFA Cocktail Procedure

1. Remove the N-terminal Fmoc group before starting the cleavage procedure.
2. Slurry the resin in an appropriate cleavage cocktail.
3. Swirl the mixture occasionally during the reaction time. The reaction time will depend on the composition of the substrate. Normally, cleavage will take 1.5 to 2 hours.
4. Filter the resin in a fine sintered glass funnel. Wash the resin 3 times with small portions of TFA.
5. 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 further with cold ether.

TMSBr Procedure[186]

If the peptide contains Arg(Mtr), this procedure will cleave the peptide from the resin and remove the Mtr group more rapidly than the TFA procedure.

1. Place the resin in a round bottom flask and add 20% piperidine in DMF until the resin is just covered. Let the mixture stand for 30 minutes to remove the N-terminal Fmoc group.

2. Transfer the resin to a sintered glass funnel with fine porosity and apply vacuum. Wash the resin 3 times with DMF. Slurry the resin in DCM three times to remove the DMF.

3. For 100 mg of peptide-resin, mix 250 uL of ethanedithiol, 50 uL of m-cresol, 590 uL of thioanisole and 3.75 mL of TFA. Cool the mixture in an ice bath then add 660 ul of TMSBr. Cool the cleavage mixture to 0 ºC, then add 100 mg of the peptide resin. Allow the mixture to stand 15 minutes under nitrogen.

4. Filter the resin in a fine sintered glass funnel. Wash the resin 3 times with small portions of TFA.

5. 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. Dissolve the precipitated peptide in 20% aqueous acetic acid and lyophilize.

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[185] Based on procedures in Yagima, H.; et al. *Tetrahedron* **1988**, *44*, 805-819.
[186] Based on procedures in Guo,S.; et al. *Chem. Pharm. Bull*. **1988**, *36*, 4989; Yajima, H.; et al. *Tetrahedron* **1988**, *44*, 805-819.

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Cleavage from HMPA Resin[187]

1. Remove the N-terminal Fmoc group before starting the cleavage procedure.
2. Slurry the resin in a cleavage cocktail appropriate to the amino acid composition of the product. Allow the mixture to stand at room temperature for 1 hour.
3. Filter the resin in a fine sintered glass funnel. Wash the resin 3 times with small portions of TFA.
5. 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 further with cold ether.

Cleavage from Rink Resin[188]

1. Remove the N-terminal Fmoc group before starting the cleavage procedure.
2. Slurry the resin in a cleavage cocktail appropriate to the amino acid composition of the product.
3. Add 3% v/v 1,3-dimethoxybenzene.
4. Allow the mixture to stand at room temperature for 15 minutes. Filter and wash the resin with DCM.
5. 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 further with cold ether.

Cleavage from PAL Resin[189]

1. If necessary, remove any Fmoc protecting groups.
2. Slurry the resin in 95% TFA /anisole (v/v) containing scavengers as required by the amino acid composition of the peptide. Allow the mixture to stand at room temperature for 30 minutes.
3. Filter and wash the resin once with DCM.
4. Combine the filtrates and add 8-10 times the volume of cold methyl t-butyl 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 further with cold methyl t-butyl ether.

Cleavage from Seiber Resin to Produce Protected Peptide Amides[190]

1. Slurry the resin in 1% TFA in DCM (v/v).
2. Mix the resin at room temperature for 30 minutes.
3. Filter the resin and wash it once with DCM.
4. 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 further with cold ether.

Cleave Protected Peptides from 2-Chloro-Trityl Resin

1. Prepare a 1:1:8 by volume mixture of acetic acid/TFE/DCM (approximately 20 mL per gram of resin).

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[187] Albericio, F.; Barany, G. *Int. J. Pept. Protein Res.* **1984**, *23*, 342-349.
[188] Based on procedures reported in Stathopoulos, P.; Papas, S.; Tsikaris, V. *J. Pept. Sci*. **2006**, *12*,:227-32.
[189] Bernatowicz, M.S.; Daniels, S.B.; Köster, H. *Tetrahedron Lett.* **1989**, *30*, 4645-4648.
[190] Han, Y.; Bontems, S. L.; Hegyes, P.; Munson, M. C.; Minor, C. A.; Kates, S. A.; Albericio, F.; Barany, G. *J. Org. Chem.*, **1996,** *61*, 6326 –6339.

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2. Suspend the resin in half of this mixture. Allow the mixture to stand at room temperature for 30 minutes.
3. Filter the resin and wash it with the remaining mixture.
4. Combine the filtrates and lyophilize.

Cleave Alcohols and Phenols from Trityl Resins

1. Suspend the resin in 2% (v/v)TFA/DCM.
2. Allow the mixture to stand 30 minutes at room temperature.
3. Filter the resin and wash it with a small amount of TFA. Combine the filtrates and evaporate *in vacuo* to obtain the crude product.

Cleaving Amines from Trityl Resin

1. Prepare a 5% v/v mixture of TFA in 97:3 DCM/triethylsilane.
2. Shake the mixture at room temperature for 2 hours.
3. Filter the resin in a fine sintered glass funnel. Wash the resin 3 times with small portions of TFA.
4. Combine the filtrates and evaporate them *in vacuo* to obtain the crude product mixture.

Oxime Resin

Cleavage to Protected Peptide Acid[191]

1. Suspend the resin in 95:5 THF/H_2O (v/v).
2. Add 2 equivalents of DBU and shake the mixture mechanically for 4 hours.
3. Filter the resin and wash it several times with DCM and methanol.
4. Combine the filtrates and evaporate to dryness.
5. Dissolve the residue in DCM and wash it twice with 1N HCl.
6. Dry the DCM layer over $MgSO_4$ then evaporate to yield the crude protected peptide.

Cleavage to Protected Peptide Ester[172]

1. Suspend the resin in the alcohol or 95:5 THF/alcohol (v/v).
2. Add 2 equivalents of DBU and shake the mixture mechanically for 2 hours.
3. Filter the resin and wash it several times with DCM and methanol.
4. Combine the filtrates and evaporate to dryness.
5. Dissolve the residue in DCM and wash it twice with 1N HCl.
6. Dry the DCM layer over $MgSO_4$ then evaporate to yield the crude protected peptide.

Cleavage to Protected Peptide Amides[192]

1. Add a solution (60 mL/100 mg of resin) of ammonia in 50% (v/v) THF/methanol saturated at 0°C. Seal the flask and set it aside in a fume hood at room temperature for 16 hours.
2. In a fume hood, filter the resin and wash it with methanol.
3. Evaporate the combined filtrates to obtain the crude peptide amide.

[191] Pichette, A.; Voyer, N.; Larouche, R.; Meillon, J.-C. *Tetrahedron Lett.* **1997**, *38*, 1279-1282.
[192] Mohan, R.; Chou, Y.-L.; Morrissey, M.M. *Tetrahedron Lett.* **1996**, *37*, 3963-3966.

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Cleavage from HMBA-MBHA Resin

Cleavage to Obtain Acids[193]

1. To obtain unprotected peptide acid, remove all protecting groups using appropriate deprotection procedures.
2. Add 1 equivalent (based on resin substitution) of freshly prepared 1.0 M sodium methoxide in methanol.
3. Shake the mixture with a mechanical shaker at room temperature for 30 minutes.
4. Add water (2 mL/g of resin) to the mixture and mix it at room temperature 30 minutes.
5. Filter the resin. Acidify the filtrate and lyophilize it to obtain the peptide acid.

Amminolysis to Obtain Amides[194]

1. To obtain unprotected peptide amide, remove all protecting groups using appropriate deprotection procedures.
2. Swell the resin in DMF, then filter. To remove any acid remaining from the deprotection procedure, wash the resin three times with 10% (v/v) DIPEA in DCM.
3. Wash the resin with DMF, filter, and then transfer the resin to a round bottom flask. Add a solution (60 mL/100 mg of resin) of ammonia in methanol saturated at 0°C. Seal the flask and set it aside in a fume hood at room temperature for 16 hours.
4. In a fume hood, filter the resin and wash it with methanol. Evaporate the combined filtrates to obtain the crude peptide amide.

Cleavage from Weinreb Amide Resin Forming Aldehydes[195]

1. Suspend the resin in THF and place it in an ice bath.
2. Add two molar equivalents of $LiAlH_4$ plus one additional molar equivalent of $LiAlH_4$ for every additional amide or urethane group in the product.
3. Mix for 30 minutes.
4. Hydrolyze the reaction mixture with a 5% aqueous $KHSO_4$ solution.
5. Filter the mixture and wash the resin twice with DCM..
6. Dilute the combined filtrates with DCM. Wash with 5% aqueous KHSO4 solution, saturated aqueous NaHCO3 solution and saturated aqueous NaCl solution.
7. Dry and evaporate the organic layer to obtain the crude product.

Alcohol Cleavage from Carboxypolystyrene

Product Cleavage with K_2CO_3

1. Suspend the resin in 2:1 (v/v) MeOH/THF (50 mL/g resin). Add 5 equivalents (based on resin substitution) of solid potassium carbonate.
2. Shake the mixture at room temperature for 12 hours.
3. Filter the resin and wash it with 2:1 MeOH/THF. Extract the combined filtrates with ethyl acetate (EtOAc).
4. Separate the EtOAc layer, dry it and evaporate to obtain the crude product.

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[193] Christensen, J.W.; Peterson, M.L.; Saneii, H.H.; Healy, E.T. in "Peptides: Chemistry, Structure and Biology", Kaumaya, P.T.P.; Hodges, R.S., Eds; Mayflower Scientific Ltd. 1996, pp 141-143.
[194] Brown, E.; Sheppard, R.C.; Williams, B.J. *J. Chem. Soc., Perkin Trans. 1* **1993**, 75-83.
[195] Based on Salvino, J. M.; Mervic, M.; ason, H. J.; Kiesow, T.; Teagger, D.; Airey, J.; Labaudiniere, R. *J. Org. Chem.* **1999**, *64*, 1823-1830.

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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.

[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.

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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.

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

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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.

[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.

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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.

[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.

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

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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.

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[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.

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7. Desalt the peptide by ion exchange column.

Removal with Mercury(II) Acetate[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).

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[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.

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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:

$$\text{Theoretical Yield (mg)} = sresin * mresin * MWproduct$$

where

sresin = resin substitution in mmol/g
mresin = resin dry mass in g
MWproduct = 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.

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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.