Chapter 2

Linkers, Resins, and General Procedures for Solid-Phase Peptide Synthesis

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Abstract

This chapter describes the basic protocols for solid-phase peptide synthesis using the Fmoc group as the N^{α}-protecting group (Fmoc-SPPS). The chapter introduces resins and their handling, choice of linkers, and the most common methods for peptide chain assembly. The proper choice of resins and linkers for solid-phase synthesis is a key parameter for successful peptide synthesis. This chapter provides an overview of the most common and useful resins and linkers for the synthesis of peptides with *C*-terminal amides, carboxylic acids, and more. The chapter finishes with robust protocols for general solid-phase peptide synthesis, i.e., the standard operations.

Key words Solid-phase peptide synthesis, SPPS, TentaGel, ChemMatrix, PEGA, Polystyrene, HBTU, HATU, DIC, COMU, HOBt, HOAt, Oxyma

1 Introduction

In the past decades, numerous resins and linkers have been developed and commercialized, which have enabled a wide range of applications. The resin determines the physical properties, e.g., swelling of the peptidylresin and may also limit the conditions and chemistries under which the resins are stable [1, 2]. The linker determines not only the conditions, which can be used during peptide chain assembly, but also the conditions required for release of the peptide and, finally, also the nature of the *C*-terminal functionality.

The proper choice of resin is important for the efficient and economical preparation of a peptide. The first resin to be used by Merrifield in the 1960s was polystyrene and this efficient type of resin is still widely used today [3]. Over the years, a limited number of resins, which contain polyethylene glycol (PEG) linked to polystyrene, have been developed. Also, resins made solely from PEG and a cross-linker have become commercially available. However, companies which specialize in reagents for solid-phase peptide synthesis tend to use their own names for resins and linkers as well as

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Table 1
Overview of the three subgroups of resins for Fmoc-based SPPS and their swelling properties in selected solvents [26]

Resin subgroups	Commercial name	Initial loading (mmol/g)	Approximate swelling (mL/g)						
			DCM	DMF (NMP)	Ether	Water	TFA	THF	MeOH
PS (1 % DVB)	Aminomethylated PS	0.4–1.5	7	4	4	N.A	2	9	1.6
PEG-PS	Amino TentaGel (TG)[a]	0.15–0.3	6	5	2	3.6	N.A.	5.0	3.6
PEG based	Amino PEGA[b]	0.2–0.4	13	11	N.A	16	N.A.	13	13
	ChemMatrix (CM)	0.4–0.6	11	8	N.A	11	14	N.A.	9

[a]The swelling volumes are for standard grade TG resins
[b] The swelling properties listed are for 0.2 mmol/g resins

for premade linker-resins. This can make it difficult to survey the commercially available linkers and resins.

Linkers in SPPS provide a reversible linkage between the peptide chain and the solid support (resin). Furthermore, in most cases, the linker provides protection and blockage of the *C*-terminal α-carboxyl group during synthesis. An exception here is the use of linkers which do not attach to the α-carboxylic acid but leave the *C*-terminal free for modifications. This can be achieved by side-chain anchoring or by a backbone amide linker (Chapter 9). Furthermore, the choice of linker determines which chemistry can be used during peptide chain assembly, as well as release of the peptide in the final step (Chapter 3). The strategies for Boc- and Fmoc-SPPS require different linkers, due to different conditions for repetitive removal of the N^{α}-protecting groups [4]. For further information on linkers for SPPS using the Boc-strategy, *see* Chapter 4, as the following chapter mainly will focus on linkers for Fmoc-based solid-phase chemistry.

1.1 Resins

The varieties of resins for SPPS may seem bewildering. However, there are only three different subgroups of resins depending on what type of material they contain (Table 1). The most common classes of resins are the classic polystyrene (PS) resins, the PS-functionalized polyethylene glycol (PEG) resins, and pure cross-linked PEG resins. Conventional PS resins have in numerous cases proven successful in the synthesis of short- to medium-length peptides. However, PEG-based resins often outperform the PS-based resins in the synthesis of medium-length and long peptides as well as peptides which contain "difficult sequences." This has to be balanced with the fact that PEG-containing resins in

general also are more costly. The length of the PEG as well as its percentage of the total resin, the amount of cross-linking, as well as possible batch-to-batch variation will influence how the resin performs during Fmoc-SPPS. Thus, when synthesizing medium to long peptide sequences, choosing the optimal resin is likely to be a very important factor.

The polystyrene resin is a polymer cross-linked with 1 % of divinylbenzene (DVB) and with a loading of 0.2–1.2 mmol/g. This type of support is compatible with DMF and DCM but not compatible with water (*see* Table 1). The most well-established PEG-functionalized PS resin today is TentaGel (TG) reported by Bayer and Rapp [5, 6]. TG resins are prepared by grafting of PEG (50–70 %) to low cross-linked polystyrene by an ether linkage. The TG resins have excellent swelling properties in most solvents compatible with PEG, such as DCM, DMF, NMP, water, ethanol, and tetrahydrofuran (THF) (*see* Table 1). When changing the solvent from an organic media to a water-based media, it is recommended to use a solvent gradient by going from DCM to THF or ethanol and then to water. This will maintain the optimal swelling properties of the TG resins. There are other PEG-PS-based resins developed and commercialized under a variety of brand names. The TG resins have evolved over 20 years and have been used in many scientific studies, making it a well-tested and reliable solid support. This type of support is highly suited for the synthesis of longer peptides.

The pure cross-linked PEG resins contain no polystyrene. This group includes the poly(ethylene glycol)-poly-(*N*,*N*-*dimethylacrylamide*) copolymer (PEGA) developed by Meldal [7] and the more recent ChemMatrix (CM) resin which was reported by Albericio and co-workers in 2006 [8]. A unique advantage of PEGA resins is that they swell very well in water and that peptide substrates anchored to PEGA thus can be used in interaction studies with proteins up to 35–70 kDa. PEGA resins are supplied swollen in ethanol, due to the very sticky nature of the resin beads. The beads are easily damaged when shrunk or dried and are therefore best handled in a swollen state. The newer CM resins are fully PEG based and contain primary ether bonds and are reported to be chemically robust. CM resins have the advantage compared to the PEGA-type resins that they are easily handled in a dry state and therefore in regard to handling are more comparable to the PS- or PEG-PS-based resins. The CM resins have found usage in the synthesis of longer peptides and even small proteins due to its excellent swelling properties, which is beneficial for diffusion and for accessibility of reactive sites. They are used with the same procedures as for polystyrene and TG; however, they swell more in most solvent, in particular TFA (*see* Table 1). It should be mentioned that the CM resins are still relatively new compared to the TG family of resins.

Most of the abovementioned resins are commercially available either as amino-functionalized resins or with a variety of different linkers attached. The amino-functionalized resins allow for anchoring of specific linkers by a standard amide bond.

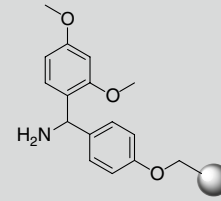
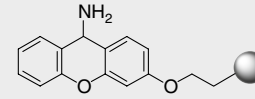
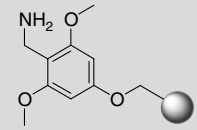
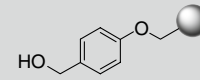
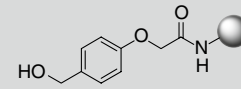
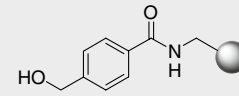
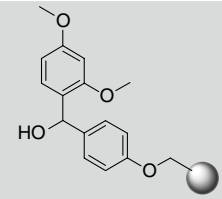
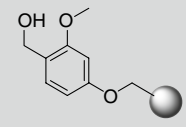
1.2 Linkers for Fmoc-Based SPPS

A large variety of linkers suitable for Fmoc-SPPS are available [9, 10], and many resins can be purchased with the linker preinstalled. A focused table with the linkers divided into subclasses is included in this chapter (*see* Table 2), and Chapters 1 and 3 give further guidelines regarding cleavage conditions. Most linkers release the peptide upon TFA treatment as the *C*-terminal acids and amides, the classic examples being the Wang and Rink-amide linkers, respectively [11]. The Rink-amide linker and other aminomethyl-based linkers can be installed on the resin by coupling of the Fmoc-protected linker to the resin using standard couplings procedures. Another class of linkers are trityl based, where a classic example is the 2-chlorotrityl chloride resin which is used in the production of peptide acids [12]. Often 2-chlorotrityl resins are attached to polystyrene resins by direct on-resin synthesis. However, premade trityl linkers can also be coupled to a variety of resins, typically amino-functionalized base resins. Specialized linkers which release the peptide as esters, secondary amines, or thioesters have also been developed, e.g., the safety-catch linker and aryl hydrazide linkers [13, 14]. The safety-catch linker is cleaved by alkylation of the sulfonamide which enables release of the modified peptides upon treatment with different nucleophiles (Chapter 8). The aryl hydrazide linker is cleaved by oxidation to an acyldiazene that enables release with suitable nucleophiles. Another class of handles which provides *C*-terminal modified peptides is the backbone amide linker (BAL) (Chapter 9) [15]. Here the peptide is anchored through a backbone amide, typically of the *C*-terminal residue. These linkers leave the *C*-terminal free to be modified, and peptide esters, aldehydes, as well as thioesters have been synthesized by this method [16, 17].

2 Materials

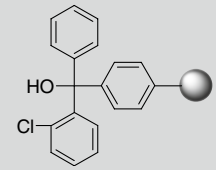
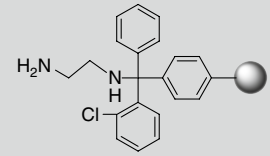
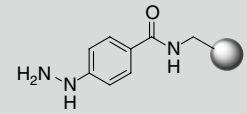
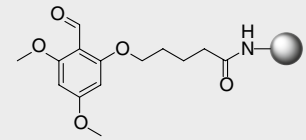
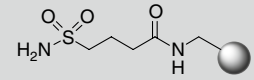
1. Selection of commercially available resins:
   (a) Aminomethylated polystyrene (PS) resins:
      - Sold as either high loading (HL, 0.5–1.5 mmol/g) or low loading (LL, 0.3–0.5 mmol/g) from many distributors, however, in varying qualities.
   (b) Amino PS-PEG (TentaGel TG) resins:
      - TentaGel® resins sold as standard grade (S) loading approx. 0.25 or research grade (R) loading approx. 0.15 mmol/g from Rapp Polymere.

Table 2
Overview linkers for Fmoc-based SPPS

Linker types	Name	Final *C*-terminal functionality	Linker structure	Described in this book
Aminomethyl				
	Rink-amide linker	Peptide amides		Chapter 2
	Sieber linker	Peptide amides		
	PAL linker	Peptide amides		
Hydroxymethyl				
Wang-type resins:	Wang/PHB linker	Peptide acids		Chapter 2
	HMPA linker	Peptide acids		Chapter 2
	HMBA linker	Protected peptide acids, amides, alcohols, hydrazides		Chapter 3
Variants	Rink acid linker	Peptide acids		
	SASRIN linker	Peptide acids		

(continued)

Table 2
(continued)

Linker types	Name	Final *C*-terminal functionality	Linker structure	Described in this book
2-Chlorotrityl				
		Peptide acids		Chapter 2
		Peptide amines		
Others				
	Aryl hydrazide linker	Peptide amines or esters		
BAL	*ortho*-PALdehyde linker (o-BAL)	Peptide acids, aldehydes, thioesters, among others		Chapter 9
Safety catch	4-sulfamylbutyryl/ Kenner safety-catch linker	Peptide thioesters		Chapter 8

- Alternatively, there is NovaSyn® TG with a loading of approx. 0.2–0.3 mmol/g from Novabiochem®.

(c) Poly(ethylene glycol)-poly(acryl amide)copolymer (PEGA, Polymer Laboratories, now Agilent) resins:

- Available with loadings of 0.2–0.4 mmol/g.

(d) ChemMatrix® (CM) resins:

- Sold from PCAS BioMatrix Inc with a loading of approx. 0.46 mmol/g. Furthermore, this resin is sold from distributors, such as Biotage AB, and from Novabiochem®, where it is sold under the trade name NovaPEG.

2. Solvents:

(a) Dichloromethane (DCM).

(b) *N*,*N*-Dimethylformamide (DMF).

(c) *N*-Methyl-2-pyrrolidinone (NMP).
(d) Methanol (MeOH).
(e) Tetrahydrofuran (THF).
(f) Trifluoroacetic acid (TFA).
(g) Piperidine.
(h) Pyridine.
(i) Acetic anhydride.

3. Coupling reagents:
   (a) Diisopropylcarbodiimide (DIC).
   (b) 1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholinomethylene)]methanaminium hexafluorophosphate (COMU).
   (c) *N*-[(Dimethylamino)-1H-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate *N*-oxide (HATU).
   (d) *N*-[(1*H*-Benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU).
   (e) 1-Hydroxybenzotriazole (HOBt).
   (f) Ethyl(2-cyano-2-(hydroxyimino)acetate) (Oxyma).
   (g) 1-(Mesitylene-2-sulfonyl)-3-nitro-*1H*-1,2,4-triazole (MSNT).
4. Other reagents:
   (a) *N*,*N*-Diisopropylethylamine (DIEA).
   (b) 4-(*N*,*N*-Dimethylamino)pyridine (DMAP).
   (c) 1-methylImidazole (MeIm).
5. Equipment:
   (a) Polypropylene syringes equipped with a polyethylene filter.
   (b) A shaking table or equivalent.
   (c) A "Teflon" block design for multiple filtrations or an equivalent setup for manual SPPS.
6. Building blocks:
   (a) Fmoc-protected amino acids.
   (b) Fmoc-protected pentafluorophenyl esters, if needed.
   (c) Fmoc-protection pseudoproline dipeptides, if needed.
   (d) Fmoc-protected *N*-Hmb amino acids.
   (e) Fmoc-protected *N*-Dmb amino acids.
7. Kaiser test: ninhydrin, phenol, ethanol, pyridine, potassium cyanide (KCN).

3 Methods

3.1 Resin Swelling, Washing, and Drying

Many resins benefit from an initial swelling procedure in order to increase the final peptide yield. The standard procedures described here (*see* Subheading 3.1.1) are suited for aminomethylated resins and preloaded hydroxy-functionalized resins. A more elaborate swelling procedure is recommended for the ChemMatrix resins in order to obtain higher final yields (*see* Subheading 3.1.2) [18]. Furthermore, standard resin washing and drying are described in the following section.

3.1.1 Standard Resins Swelling

1. Place the resin in a syringe equipped with a polypropylene filter.
2. Add DCM until the resin is completely covered. The volume of solvent depends on the type of resin used.
3. Empty the syringe by applying vacuum and repeat the DCM treatment.
4. Cover the resin in DMF and leave for 15–30 min.
5. Remove the DMF by applying vacuum. The resin is now ready for synthesis. N.B. As many resins are purchased in the Fmoc-protected form, a N^{α}-deprotection should be performed first!

3.1.2 ChemMatrix Resin Swelling

1. Place the resin in a syringe equipped with a polystyrene filter.
2. Add MeOH until the resin is covered and leave for 1 min.
3. Remove MeOH by applying vacuum and repeat treatment with MeOH.
4. In a similar manner, wash with DMF (2 × 1 min), DCM (3 × 1 min) TFA-DCM (1:99) (3 × 1 min) DIEA-DCM (1:19) (3 × 1 min), DCM (3 × 1 min), DMF (3 × 1 min).
5. The resin is now ready for synthesis. N.B. As many resins are purchased in the Fmoc-protected form, a N^{α}-deprotection should be performed first!

3.1.3 Washing of the Resin

1. Add DMF (approx. 5 mL/0.5 mmol) to the peptidyl-resin.
2. Leave for 1 min before removing the solvent by vacuum filtration.
3. Repeat three to five times depending on previously performed chemistry.

3.1.4 Drying of Standard PS, TG, or CM Resins

Some resins such as PEGA require a more specific washing procedure. It is therefore always recommended to check the supplier's recommendation. The method described below can be used for standard PS, TG, or CM resins loaded with a variety of linkers.

1. Perform a thorough resin wash with DMF (*see* Subheading 3.1.3)
2. Wash in a similar manner with ethanol (3 × 1 min) and diethyl ether (2 × 1 min).
3. Apply vacuum for 10 min.
4. (a) If the next step is a peptide cleavage step (*see* Chapter 3), leave the resin for 1–2 h at room temperature.
   (b) If the next step is a resin loading test (*see* Subheading 3.7), place the resin in a desiccator under vacuum for 12–18 h prior to the loading test.

3.2 Loading of Amino-Functionalized Resins

For amino-functionalized resins, anchoring of the first residue to the solid support is performed using standard amide coupling reactions. This linkage will upon cleavage yield a *C*-terminal amide, and if there is no specific requirement for the *C*-terminal functionality, this functionality is the preferred. The most common amino-functionalized linker is the Rink-amide linker, however PAL handle is also very reliable and may have a higher acid-lability (*see* Table 2) [19, 20]. Standard coupling procedures are described in Subheading 3.5.

3.3 Loading of Hydroxy-Functionalized Resins

For anchoring of the first amino acid onto hydroxymethyl-based resin, which upon cleavage provides a *C*-terminal carboxylic acids, it is recommended to use a protocol without the use of tertiary bases, such as DIEA. This type of protocol is designed to minimize the degree of self-acylation, hence, double incorporation, and racemization of the first residue. The most common hydroxymethyl-based resins are Wang-type linkers (*see* Table 2). Two different protocols are described below for loading of hydroxymethyl-based resins: (1) the symmetrical anhydride method and (2) the MSNT/MeIm method. The MSNT/MeIm method is recommended for difficult situations, which includes the attachment of amino acids that are prone to epimerization. For the synthesis of *C*-terminal acids where the first residue is either Cys or Pro, it is recommended to use the trityl-based resins. Many of the resins with a hydroxymethyl linker can be obtained with the first amino acid already preloaded.

3.3.1 The Symmetrical Anhydride Method

1. Place the hydroxy-functionalized resin in a dry flask and add dry DMF until the resin is completely covered (*see* **Note 1**).
2. Let the resin swell for 30 min before applying vacuum to remove the DMF.
3. Place the desired Fmoc-protected amino acid (10 equiv. relative to the resin loading) in a dry round-bottomed flask containing a magnetic stirrer.
4. Add dry DCM (approx. 3 mL/mmol amino acid derivative) to dissolve the Fmoc-protected amino acids. A few drops of DMF may be needed to aid complete dissolution.

5. Prepare a solution of DIC (5 equiv. relative to the resin loading) in dry DCM (approx. 1 mL/mmol DIC) and add to the flask containing the dissolved Fmoc-protected amino acids.
6. Stir the mixture for 15 min at 0 °C, keeping the reaction mixture free of moisture with a calcium chloride drying tube. If any precipitation is observed, add more DMF (dropwise) and leave stirring for an additional 10 min (*see* **Note 2**).
7. Remove the DCM by evaporation using a rotary evaporator.
8. Redissolve in a minimum volume of DMF and add to the swelled hydroxy-functionalized resin prepared in **steps 1** and **2**. The resin should be completely covered.
9. Prepare a solution of DMAP (0.1 equiv. relative to resin loading) in DMF (approx. 1 mL/mmol) and add to the resin mixture.
10. Apply a stopper to the flask and leave for 1 h at room temperature with occasional swirling (*see* **Note 3**).
11. Remove excess reagent by filtration and wash the resin with DMF (×5).
12. Perform a loading test as described in Subheading 3.8.
13. If the loading is less than 70 % of the theoretical, the procedure described above should be repeated.

3.3.2 The MSNT/ Melm Method

1. Place the hydroxy-functionalized resin in a dry reaction vessel and swell the resin in DCM
2. Remove DCM by applying vacuum and add fresh DCM until the resin is completely covered.
3. Flush the vessel with nitrogen and seal with a septum.
4. Place the desired Fmoc-protected amino acid (5 equiv. relative to the resin loading) in a dry round-bottomed flask containing a magnetic stirrer.
5. Add dry DCM (approx. 3 mL/mmol amino acid derivative) to dissolve the Fmoc-protected amino acids. A few drops of THF may be needed to aid complete dissolution.
6. Add MeIm (3.75 equiv. relative to the resin loading) followed by MSNT (5 equiv. relative to the resin loading). Flush the flask with nitrogen and seal with a septum. The mixture is stirred until the MSNT has dissolved.
7. Using a syringe, transfer the amino acids solution to the vessel containing the swelled hydroxy-functionalized resin prepared in **steps 1–3**. Leave the resin mixture to react for 1 h at room temperature applying occasional swirling (*see* **Note 3**).
8. Remove the septum and remove excess reagent by filtration and wash the resin with DCM (×3).

9. Perform a loading test as described in Subheading 3.8.
10. If the loading is less than 70 % of the theoretical, the procedure described above should be repeated.

3.4 Loading of Chlorotrityl Resins

The last protocol which will be described in the following is loading of the trityl-based linkers to yield *C*-terminal carboxylic acids upon cleavage. This resin is a good alternative to hydroxymethyl-based resins due to the absence of epimerization during loading of the first amino acids. It is recommended in particular for *C*-terminal His, Cys, Pro, Met, and Trp which are highly prone to give unwanted side reaction when using the symmetrical anhydride method for loading of hydroxymethyl-based resins. This resin can in a similar manner as described below be used for synthesis of a large variety of *C*-terminal functionalities, however, it may require a trityl linker with different substituents to tune its properties. Peptide release from this type of resin is described in Chapter 3.

1. In a Falcon tube, dissolve the Fmoc-protected amino acids (1.2 equiv. relative to the resin loading) and DIEA (5 equiv. relative to the resin loading) in dry DCM (10 mL/g resin) (*see* **Note 4**).
2. If necessary, add a small amount of DMF to aid dissolution of the mixture.
3. Add the mixture to the resin and stir for approx. 2 h at room temperature.
4. Wash the resin with DCM/MeOH/DIEA (17:2:1) (3 × 1 min), DCM (3 × 1 min), DMF (2 × 1 min), and DCM (2 × 1 min).
5. Perform a loading test as described in Subheading 3.8.

3.5 Standard Coupling Procedures

The following section describes three general protocols for standard amide bond formation in SPPS: (1) the activation using aminium or phosphonium salts, (2) the DIC/HOBt method, and the use of (3) preformed active esters [21]. The first two procedures are the most common whereas the latter is used in more specialized situations.

Instead of weighing out the individual reagents prior to each cycle, a stock solution of different coupling reagent and Fmoc-protected amino acids can be made. The shelf life for these solutions depends on the general storage and preparation conditions which includes the water content in the DMF used for making the solutions, the average temperature by which the solution is kept, the amount of time the solutions is open, and for how long the solutions is kept open (*see* Table 3). The majority of by-products present in the solutions of amino acids upon standing are due to loss of Fmoc or other protecting groups. The least stable protecting group is the trityl moiety and trifunctional amino acids with a

Table 3
Guidelines for storage of different Fmoc-protected amino acids in DMF/NMP

Fmoc-protected amino acids	Storage time at room temperature (DMF)	Storage time at 4 °C (DMF)
Bifunctional Fmoc-protected amino acids	<2 weeks	<4 weeks
Trifunctional Fmoc-protected amino acids, except trityl protected	<2 weeks	<4 weeks
Trityl-protected Fmoc-protected amino acids	<5 days	<10 days
Fmoc-Trp(Boc)-OH	<2 days	<5 days

Table 4
Guidelines for the storage of different coupling reagents in DMF (NMP)

Coupling reagent and other reagents	Storage time in open containers (DMF)	Storage time in closed containers (DMF)
HOBt and Oxyma	<2 weeks	–
HBTU and DIC	<1 week	<2 weeks
HATU	<1 days	<1 week
COMU	<4 h	<5 days

trityl protection group should not be kept in solution for over a week at room temperature. The other relatively unstable amino acid is Trp(Boc) which also degrades rapidly and should not be stored in solution for more than 2 days at room temperature. Otherwise, Fmoc-Aaa-OH solutions in DMF can last at least 2 weeks at room temperature and up to 4 weeks at 4 °C. The shelf life for all the solutions is increased if they are kept under nitrogen which is the case for many automated peptide synthesizer.

Some laboratories routinely add HOBt to stock solutions of Fmoc-protected amino acids. However, it appears that the presence of HOBt in the stock solutions accelerates the decomposition of the stock solutions and breakdown products can be found after only 1 week (Table 3).

The stability of different coupling reagents are also of great importance since a partially degraded stock solution of coupling reagents will have a negative impact on average amino acid incorporation (*see* Table 4). It has been reported recently that the stability of commercial COMU in open vials in comparison to HATU and HBTU is very low [22]. The key issue here is the water content of the DMF used for preparing the stock solution of the coupling reagent. It is in particular recommended to use fresh (thus relatively

anhydrous) or anhydrous DMF for preparing stock solutions of COMU. This will increase the storage time in open or closed containers. Furthermore, inert gas covers or closed systems which provide a cover of dry nitrogen are applied in many automated systems and will also aid the stability of the coupling reagent in solution. Furthermore, as is the case for solutions of Fmoc-protected amino acids, lowering the temperature will also increase stability.

Another method for improving the synthesis of difficult peptides is to incorporate either backbone-protected Dmb or Hmb derivatives or pseudoproline dipeptides (described in Chapter 1). It is recommended to incorporate Dmb or Hmb derivatives or pseudoproline dipeptides at every sixth residue if possible or before a region of hydrophobic residues. Moreover, there should be a spacing of at least two residues between Dmb- or Hmb-protected derivatives, pseudoproline dipeptides, and Pro residues. These derivatives are purchased Fmoc protected and can be used with standard coupling procedures as described below. These specialized amino acid derivatives can, however, add significantly to the cost of synthesis. Also, the removal of the Dmb and Hmb protecting groups might be slow.

3.5.1 HBTU/HATU/COMU Manual Coupling

The standard coupling reagents as described in Chapter 1 are generally applied using the same protocol. As a general rule, the coupling reagents can be ranked by their coupling efficiency as HBTU<HATU/COMU.

1. Place the resin in a syringe equipped with a polypropylene filter. If this is the beginning of a synthesis, go to Subheading 3.1 for resin swelling (*see* **Note 5**).
2. In a separate flask or Falcon tube, mix the Fmoc-protected amino acid (4 equiv. relative to the resin loading) and HATU (3.8 equiv.) and add DMF (for a scale of 0.1 mmol, approx. 2.0 mL DMF is used for an overall concentration of 0.2 M in regard to the amino acids).
3. When all is dissolved, add DIEA (7.8 equiv.).
4. Immediately after addition of the base, add the mixture to the syringe containing the resin.
5. Mix well and react while shaking for 45 min at room temperature. Sometimes longer coupling times or double couplings are required (*see* guidelines in Table 5).
6. Remove the excess reagents by filtration.
7. Wash the resin with DMF (×4).
8. Optionally, a Kaiser test can be performed to investigate whether the coupling needs to be repeated. However, the Kaiser test is not well suited for peptides with an *N*-terminal Pro (*see* Subheading 3.9).

Table 5
Guidelines for standard coupling conditions at room temperature

Conditions	Coupling time
Standard	45 min
Predicted difficult coupling	90 min
After incorporation of the first 20 amino acids	2×45 min
After steric hindered amino acids, e.g., Aib	2×45 min

Furthermore, the use of microwave heating has been successful for fast and high-yielding couplings. Robust protocols are described in Chapters 16 and 17

3.5.2 DIC/HOBt Manual Synthesis

The DIC/HOBt coupling procedure differs from the latter protocol by being performed in the absence of base. This can be beneficial in many situations and minimize the risk of racemization. HOAt is a more powerful additive than HOBt, but due to their potentially explosive properties, the commercial availability of these reagents has become restricted. An alternative additive to HOBt is Oxyma Pure which is now commercially available. Oxyma, which is the nucleophile implicit in the very effective coupling reagent COMU, has in many cases been reported to be superior to HOBt but not always to HOAt.

1. Place the resin in a syringe equipped with a polypropylene filter. If this is the beginning of a synthesis, go to Subheading 3.1 for resin swelling (*see* **Note 5**).
2. In a separate flask or Falcon tube, mix the Fmoc-protected amino acid (5 equiv. relative to the resin loading) and HOBt (5 equiv.) and add the minimum amount of DMF to dissolve.
3. Add DIC (5 equiv.) and stir the mixture for 20 min.
4. Mix well and react while shaking for 45 min at room temperature. Sometimes longer coupling times or double couplings are required (*see* Table 5).
5. Remove the excess reagents by filtration.
6. Wash the resin with DMF (×4).
7. Optionally, a Kaiser test can be performed to investigate whether the coupling needs to be repeated (*see* Subheading 3.9).

3.5.3 Use of Preformed Active Esters

The majority of activated esters such as OBt esters are not stable in storage, and they are therefore prepared fresh for each step as described above. However, the pentafluorophenyl esters (OPfp) are stable preformed esters and commercially available for most common proteinogenic amino acids. They are efficient acylation agents; however, due to their higher price, they are not used except in more specific situations.

1. Place the resin in a syringe equipped with a polypropylene filter. If this is the beginning of a synthesis, go to Subheading 3.1 for resin swelling (*see* **Note 5**).
2. In a separate flask or Falcon tube, mix the Fmoc-protected amino acid pentafluorophenyl ester (5 equiv. relative to the resin loading) and HOBt (5 equiv.) and add the minimum amount of DMF to dissolve.
3. Mix well and react while shaking for 45 min at room temperature. Sometimes longer coupling time or double couplings are required (*see* Table 5).
4. Remove the excess reagents by filtration.
5. Wash the resin with DMF (×4).
6. Optionally, a Kaiser test can be performed to investigate whether the coupling needs to be repeated (*see* Subheading 3.9).

3.6 Capping or Acylation of the N-Terminal

In some cases, it can be beneficial to "cap" unreacted amino groups after the completion of a coupling reaction; three classical methods using acetic anhydride are explained below. However, this capping step is in most cases not a necessity, and it has been shown that the classic acetic anhydride method is not suited for automated peptide syntheses due to the low stability of the acetic anhydride solution. Alternative protocols have been developed using Z(2Cl)-OSu in NMP:DCM:DIEA [23]. The three methods described below can also be used for a *N*-terminal acylation of the final peptidyl-resin.

Method 1:

1. After completion of a coupling reaction, the resin is washed thoroughly with DMF.
2. Add 25 % acetic anhydride in DMF. The total volume should exceed the height of the resin threefold.
3. Leave for 2 h while shaking.
4. Wash resin with DMF (×4) and continue on to a Fmoc removal (Subheading 3.7) or for *N*-terminal acylation continue to a peptide cleavage reaction (Chapter 3).

Method 2:

1. After the completion of a coupling reaction, the resin is washed thoroughly with DMF.
2. Add 25 % acetic anhydride in DMF. The total volume should exceed the height of the resin threefold.
3. Leave for 5 min while shaking.
4. Add 1.5 equiv. of DIEA and leave for 30 min under shaking.
5. Wash resin with DMF (×4) and continue on to a *N^{α}-deprotection* (*see* Subheading 3.7) or for *N*-terminal acylation continue to a peptide cleavage reaction (Chapter 3).

Method 3:

1. Make a fresh solution of 20 % acetic anhydride, 20 % pyridine in DMF.
2. Add this mixture to the resin after completion of the coupling step (or for *N*-terminal acylation after completion of the Fmoc removal). The total volume should exceed the height of the resin threefold.
3. Leave the resin shaking for 2 h.
4. Wash resin with DMF (×4) and continue on to a *N^{α}-deprotection* (*see* Subheading 3.7) or for *N*-terminal acylation continue to a peptide cleavage reaction (*see* Chapter 3).

3.7 Standard Fmoc Removal

After each coupling step, the Fmoc group is removed with a solution of 20 % piperidine in DMF. Most commercially available amino-functionalized resins or preloaded hydroxy-functionalized resins require an initial N^{α}-Fmoc removal before synthesis. For longer peptide sequences, an increase in deprotection time can be beneficial further into the synthesis (after 25–30 cycles). Furthermore, for certain combinations of Asp-Xxx (in particular Asp-Gly) in the sequence, a side reaction is aspartimide formation. This side reaction can be minimized by adding 0.1 M formic acid or 1 M HOBt (1 M Oxyma) to the piperidine solutions applied in the Fmoc removal steps [24]. However, only the use of Asp(OtBu)-(Dmb)Gly-OH dipeptides completely prevent aspartimide formation.

1. Add 20 % piperidine in DMF (approx. 5 mL/0.5 mmol) to the resin (placed in a syringe equipped with a polypropylene filter).
2. Leave to react for 2 min before removal of solvent by vacuum filtration.
3. Repeat the treatment with 20 % piperidine in DMF and leave standing for 15 min.
4. Remove solvent by vacuum filtration and wash the resin with DMF (×5)
5. N.B. For longer peptide sequences, it can be beneficial to repeat **steps 3–4**.

3.8 Fmoc Quantification/ Loading Test

In many cases, it is necessary to quantify the loading of the resin in order to determine the available amino groups.

1. Take out a small sample of resin (enough for 3 × 5 mg dry resin) and dry it down as described in Subheading 3.1.4.
2. Measure out three resin sample of approx. 5 mg each in an Eppendorf tube.
3. Add 1 mL of 20 % piperidine in DMF to each tube and leave for 10 min under shaking.
4. Leave the sample for 5 min without shaking (*see* **Note 6**).

5. Extract 100 µl from the top liquid (without resin) and dilute in a UV cell to 1 mL with 20 % piperidine in DMF (*see* **Note 7**).
6. Create a reference by filling 1 mL piperidine in DMF in a silica UV cuvette.
7. Measure the absorbance at 290 nm (A_{290}) where $\varepsilon_{290} = 5{,}800$ M^{-1} cm^{-1}.
8. Calculate the loading for each sample according to the following equation:

$$\text{Loading}\left(\text{mmol} / \text{g}\right) = \frac{\left(V \times A_{290\text{nm}}\right)}{\left(\varepsilon \times d \times m_{\text{sample}}\right)},$$

where V = volume in L, d is the cuvette size in cm, and $\varepsilon_{290} = 5{,}800$ M^{-1} cm^{-1}.

3.9 Kaiser Test

In order to perform the Kaiser test, three different solutions are prepared. These solutions are stable and can be used for months [25]. However, the Kaiser test is not well suited for peptides with *N*-terminal Pro residues.

Solution 1: Dissolve 5 g ninhydrin in 100 mL ethanol.

Solution 2: Dissolve 40 g phenol in 10 mL ethanol.

Solution 3: Add 2 mL of a 0.001 M aq. KCN solution to 98 mL pyridine

Procedure

1. Wash resin with DCM (×2).
2. Sample a few beads of resin in an Eppendorf tube.
3. Add one to two drops of each of the three solutions described above.
4. Mix well and heat to 120 °C for 3–5 min.
5. The presences of free resin-bound amines are indicated by blue resin beads.

4 Notes

1. This method is not suited for loading of His or Cys.
2. The total amount of DMF should not exceed 10 % of the total volume. Adding DMF will aid solubility of the mixture but will decrease reactivity.
3. It is not recommended to use magnetic stirring in combination with resins. Magnetic stirring can grind the resin and thereby ruin the resin hence the following synthesis. It is recommended to use a shaker. Automated peptide synthesizers usually use either vortex stirring or nitrogen bobbling.

4. It can be necessary to dry down the Fmoc-protected amino acids to increase the success of the reaction. Drying can be done by repeated evaporation in dioxane.
5. Remember to remove the Fmoc group from the purchased resin.
6. The resin will sink to bottom of the tube.
7. Here it is important to use either a quartz or fused silica cuvette due to the properties of DMF.

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