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# Peptide Synthesis and Applications

Edited by

John Howl

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Edited by

**John Howl**

*Research Institute in Healthcare Science,*
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# 1

# Fundamentals of Modern Peptide Synthesis

**Muriel Amblard, Jean-Alain Fehrentz, Jean Martinez, and Gilles Subra**

## Summary

The purpose of this chapter is to delineate strategic considerations and provide practical procedures to enable non-experts to synthesize peptides with a reasonable chance of success. This chapter focuses on Fmoc chemistry, which is now the most commonly employed strategy for solid phase peptide synthesis (SPPS). Protocols for the synthesis of fully deprotected peptides are presented, together with a review of linkers and supports currently employed for SPPS. The principles and the different steps of SPPS (anchoring, deprotection, coupling reaction, and cleavage) are all discussed, along with their possible side reactions.

**Key Words:** Solid phase peptide synthesis; side reaction coupling; anchoring; deprotection; cleavage; linker.

## 1. Introduction

Nowadays, "peptide synthesis" includes a large range of techniques and procedures that enable the preparation of materials ranging from small peptides to large proteins. The pioneering work of Bruce Merrifield *(1)*, which introduced solid phase peptide synthesis (SPPS), dramatically changed the strategy of peptide synthesis and simplified the tedious and demanding steps of purification associated with solution phase synthesis. Moreover, Merrifield's SPPS also permitted the development of automation and the extensive range of robotic instrumentation now available. After defining a synthesis strategy and programming the amino acid sequence of peptides, machines can automatically perform all the synthesis steps required to prepare multiple peptide samples. SPPS has now become the method of choice to produce peptides, though solution phase synthesis can still be useful for large-scale production of a given peptide.

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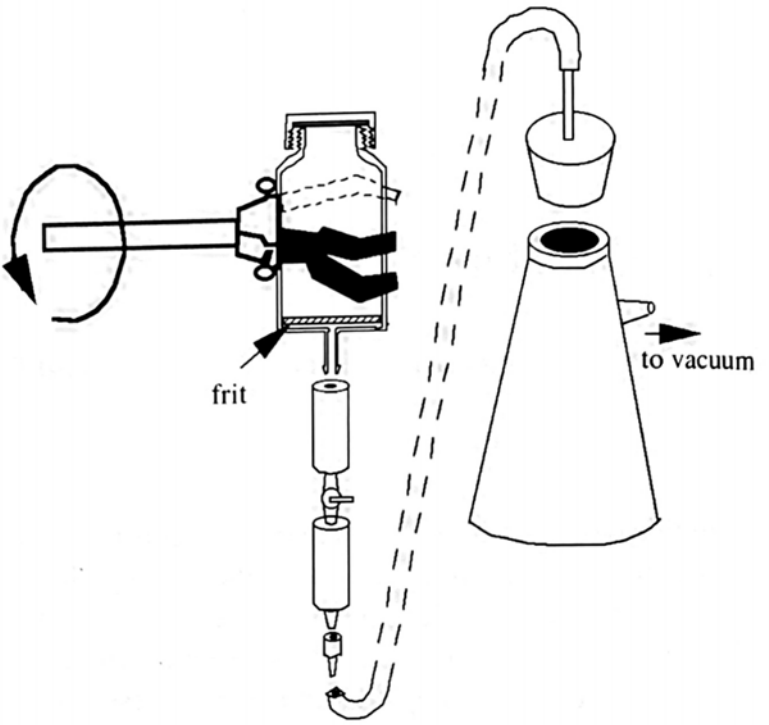



Fig. 1. Basic equipment for SPPS.

## 2. Materials

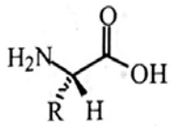
1. Reaction vessel (**Fig. 1**).
2. Polytetrafluoroethylene (PTFE) stick (15 cm length, 0.6–0.8 cm diameter).
3. Rotor.
4. Filtration flask.
5. Porous frit.
6. Lyophilizer.
7. HPLC equipped with reverse phase $C_{18}$ column.
8. pH-Indicating paper.
9. Solvents (*N,N*-dimethylformamide [DMF], methanol [MeOH], dichloromethane [DCM]) in wash bottles.
10. Diisopropylethylamide (DIPEA).
11. Piperidine solution in DMF (20:80).
12. Kaiser test solutions (ninhydrin, pyridine, phenol) (*see* **Note 1**).
13. Fmoc-amino-acids with protected side-chains (*see* **Table 1**).
14. Trifluoroacetic acid (TFA).
15. Triisopropylsilane (TIS).
16. *tert*-butyl methyl ether (MTBE).

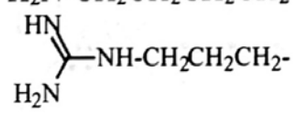
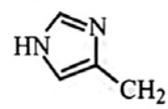
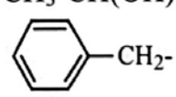
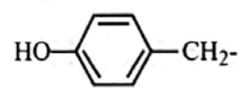
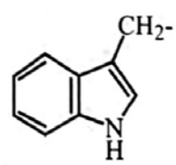
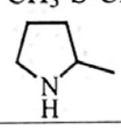
## 3. Methods

### 3.1. Principles of SPPS

As peptide synthesis involves numerous repetitive steps, the use of a solid support has obvious advantages. With such a system a large excess of reagents at

**Table 1**
**Proteinogenic Amino Acids**




| Amino acid | Three-letter code | One-letter code | Side-chain |
|---|---|---|---|
| Glycine | Gly | G | H- |
| Alanine | Ala | A | $CH_3$- |
| Valine | Val | V | $(CH_3)_2CH$- |
| Leucine | Leu | L | $(CH_3)_2CHCH_2$- |
| Isoleucine | Ile | I | $CH_3CH_2(CH_3)CH$- |
| Aspartic acid | Asp | D | $HOOC-CH_2$- |
| Asparagine | Asn | N | $H_2NOC-CH_2$- |
| Glutamic acid | Glu | E | $HOOC-CH_2CH_2$- |
| Glutamine | Gln | Q | $H_2NOC-CH_2CH_2$- |
| Lysine | Lys | K | $H_2N-CH_2CH_2CH_2CH_2$- |
| Arginine | Arg | R |  |
| Histidine | His | H |  |
| Serine | Ser | S | $HO-CH_2$- |
| Threonine | Thr | T | $CH_3-CH(OH)$- |
| Phenylalanine | Phe | F |  |
| Tyrosine | Tyr | Y |  |
| Tryptophan | Trp | W |  |
| Cysteine | Cys | C | $HS-CH_2$- |
| Methionine | Met | M | $CH_3-S-CH_2CH_2$- |
| Proline | Pro | P |  |

Note: All the 20 DNA-encoded or proteinogenic α-amino acids are of L stereochemistry.

high concentration can drive coupling reactions to completion. Excess reagents and side products can be separated from the growing and insoluble peptide simply by filtration and washings, and all the synthesis steps can be performed in the same vessel without any transfer of material.

The principles of SPPS are illustrated in **Fig. 2**. The *N*-protected C-terminal amino acid residue is anchored via its carboxyl group to a hydroxyl (or chloro)

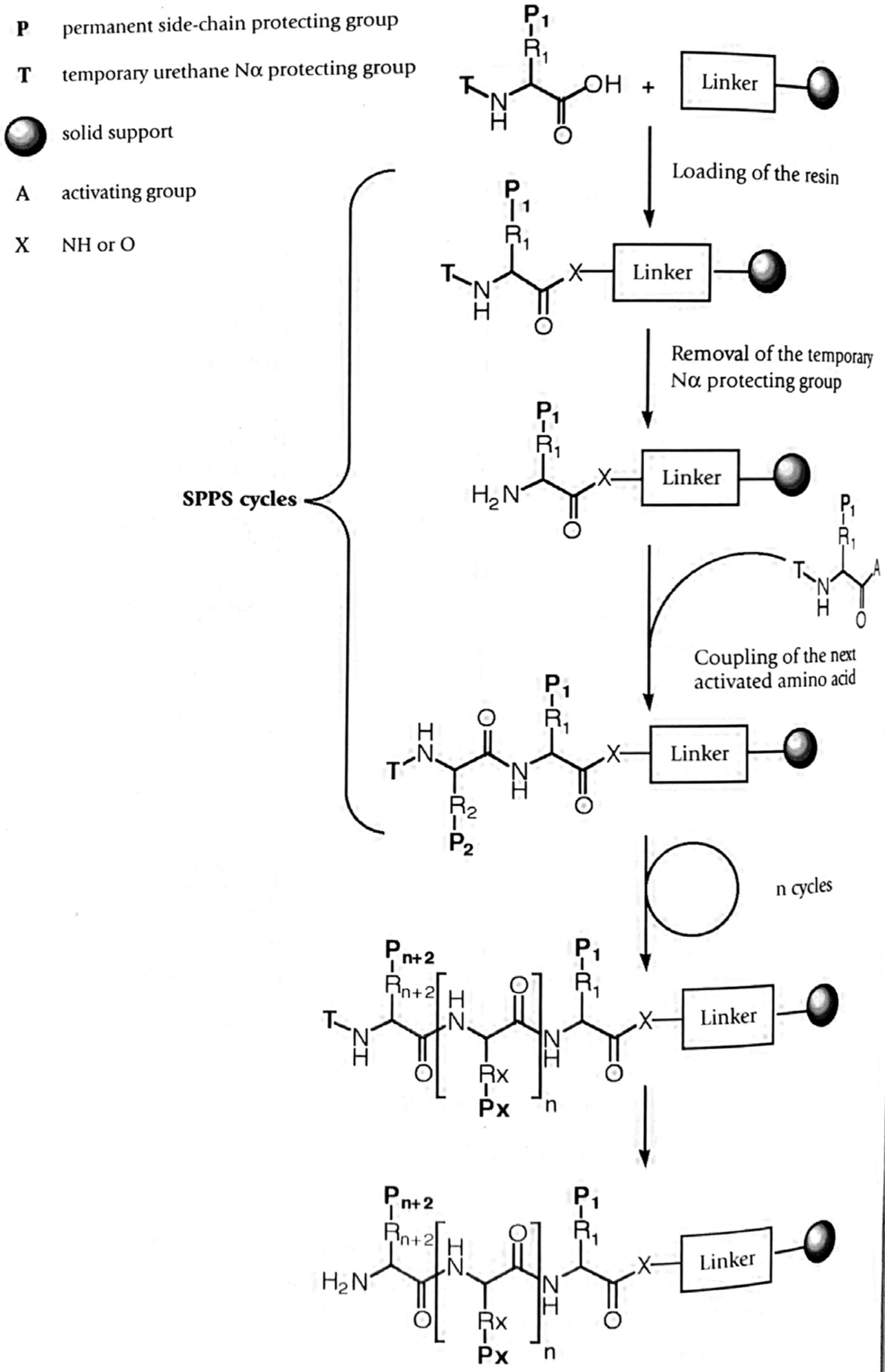



Fig. 2. Principles of SPPS.

or amino resin to yield respectively an ester or amide linked peptide that will ultimately produce a C-terminal acid or a C-terminal amide peptide. After loading the first amino acid, the desired peptide sequence is assembled in a linear fashion from the C-terminus to the N-terminus (the C → N strategy) by repetitive cycles of $N^{\alpha}$ deprotection and amino acid coupling reactions.

Side-chain functional groups of amino acids must be masked with permanent protecting groups ($P_n$) that are stable in the reaction conditions used during peptide elongation. The α-amino group is protected by a temporary protecting group (T) that is usually a urethane derivative. The temporary protecting group (T) can be easily removed under mild conditions that preserve peptide integrity and reduce the rate of epimerization, which can occur via 5(4*H*)-oxazolone formation of the activated amino acid during the coupling step ***(2,3)*** as indicated in **Fig. 3**. The protective role of urethanes against epimerization also explains the predominance of the C → N strategy.

After coupling, the excess of reactants is removed by filtration and washings. The temporary N-terminal protecting group is removed allowing the addition of the next *N*-urethane protected amino acid residue by activation of its α-carboxylic acid. This process (deprotection/coupling) is repeated until the desired sequence is obtained. In a final step, the peptide is released from the resin and the side-chain protecting groups ($P_n$) concomitantly removed.

### *3.2. Fmoc/tBu SPPS*

In SPPS, two main strategies are used: the Boc/Bzl and the Fmoc/tBu approaches for T/$P_n$ protecting groups. The former strategy is based on the graduated acid lability of the side-chain protecting groups. In this approach, the Boc group is removed by neat TFA or TFA in dichloromethane, and side-chain protecting groups and peptide-resin linkages are removed at the end of the synthesis by treatment with a strong acid such as anhydrous hydrofluoric acid (HF). While this method allows efficient syntheses of large peptides and small proteins, the use of highly toxic HF and the need for special polytetrafluoroethylene-lined apparatus limit the applicability of this approach to specialists only. Moreover, the use of strongly acidic conditions can produce deleterious changes in the structural integrity of peptides containing fragile sequences.

The Fmoc/*t*Bu method ***(4)*** is based on an orthogonal protecting group strategy. This approach uses the base-labile *N*-Fmoc group for protection of the α-amino function, acid-labile side-chain protecting groups and acid-labile linkers that constitute the C-terminal amino acid protecting group. This latter strategy has the advantage that temporary and permanent orthogonal protections are removed by different mechanisms, allowing the use of milder acidic conditions for final deprotection and cleavage of the peptide from the resin. For all these reasons, Fmoc-based SPPS is now the method of choice for the routine synthesis of peptides.

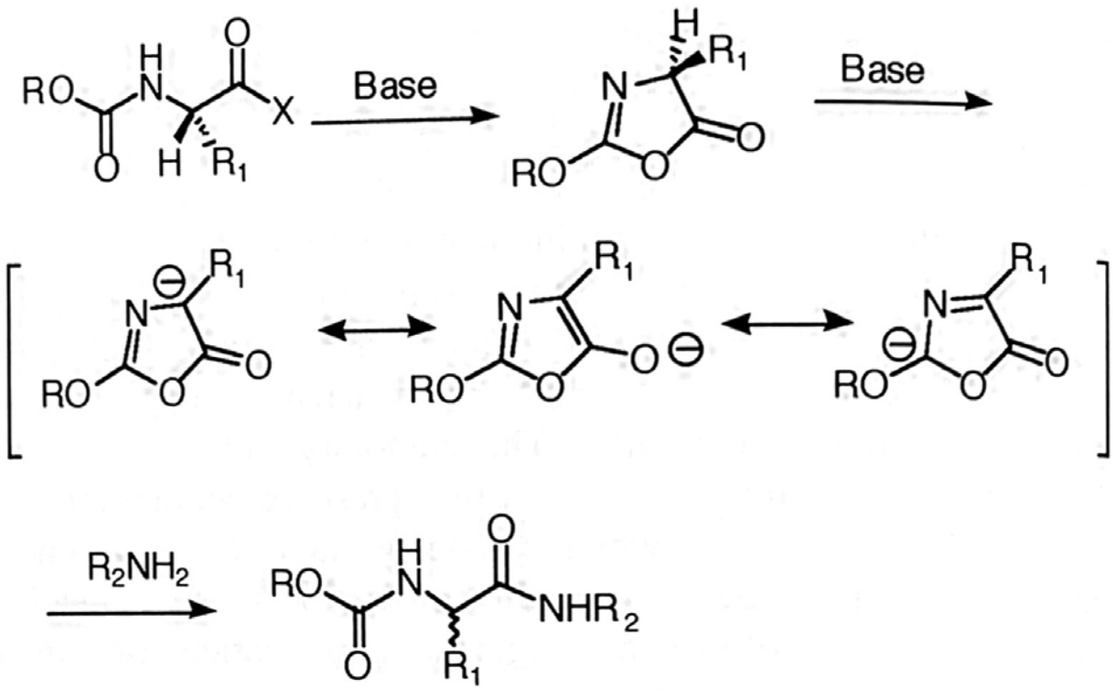



Fig. 3. Epimerization by oxazolone formation.

### 3.3. Solid Supports

The matrix polymer and the linker can characterize solid supports *(5)*. Often the term "resin" is improperly used in place of the linker system, ignoring the fact that the matrix polymer is as important in supported chemistry as the solution phase *(6)*. As hundreds of different resins are commercially available, some of them carrying the same linkers, special care should be taken to properly choose the most suitable linker for the synthesis.

#### 3.3.1. Matrix Polymers

Cross-linked polystyrene (PS)-based resins are most commonly used for routine SPPS. Beads of 200 to 400 mesh size distribution (corresponding to a diameter of about 50 μm) and a loading of 0.5 to 0.8 mmol/g present good characteristics for polymer swelling in solvents such as DMF and DCM, diffusion of reactants into the polymer matrix, and accessibility of linker sites buried into the bead. For larger peptides (more than 25 amino acids) or more difficult sequences, a lower loading is required (0.1–0.2 mmol/g).

Cross-linked polyamide (PA)-based resins and composite PS-polyethylene glycol (PEG)-based resins are much more hydrophilic supports exhibiting physical properties different from PS resins at microscopic and macroscopic levels *(7)*. These supports, often with a lower loading capacity, may represent an alternative to standard cross-linked PS resins for the synthesis of difficult sequences and large peptides.

**Table 2**
**Type of SPPS Reaction Vessels**

| Vessel length (cm) | Vessel diameter (cm) | Max. resin weight (g) | Working volume (mL) |
|---|---|---|---|
| 5 | 2 | 0.5 | 10 |
| 11 | 2.6 | 2 | 40 |
| 15 | 3.4 | 4 | 90 |

## 3.4. Resin Handling

### 3.4.1. SPPS Reaction Vessels

SPPS can be performed in classical glass reaction vessels that can be made by glassblowers or purchased from manufacturers (**Fig. 1**). Alternatively, syringes equipped with PTFE or glass frits may also be used.

Reaction vessel size should be in relation to the amount of resin used, according to **Table 2**.

### 3.4.2. Solvents

As 99% of coupling sites are not at the surface but inside the resin beads, swelling of beads carrying the growing peptide chain is essential for the optimal permeation of activated *N*-protected amino acids within the polymer matrix, thus improving coupling yields. Before starting the solid phase synthesis, the resin has to be swollen in an adequate solvent such as DCM or DMF for 20 to 30 min (**Protocol 1**). For cross-linked polystyrene beads used in SPPS, DCM presents optimal swelling properties. For coupling steps, polar aprotic solvents such as DMF or *N*-methylpyrrolidone (NMP) are preferred to improve solubility of reactants. Alcohols and water are not adequate solvents for PS resins (*see* **Note 2**). Nevertheless, methanol or isopropanol can be used during the washing steps (**Protocol 2**) to shrink PS resin beads. This shrinking will efficiently remove reactants in excess. After such treatment, PS beads should be swollen in DCM or DMF.

### 3.4.3. Stirring and Mixing

It is not necessary to agitate the reaction vessel vigorously, as diffusion phenomena dictate the kinetic reaction in SPPS. Moreover, most types of resin beads used for peptide synthesis are fragile, so magnetic stirring is not recommended. An old rotary evaporator rotor can be used for stirring during coupling and deprotection steps or alternately any apparatus enabling smooth agitation by rocking

or vortexing is appropriate. As beads usually stick to glass, the important condition is that all surfaces of the reactor must be in contact with the reaction mixture during stirring.

### 3.4.4. Washing

Washing steps are essential to remove soluble side products and the excess of reactants used during coupling and deprotection steps. Filling the reactor with solvent contained in a wash bottle and emptying it under vacuum is an appropriate and simple method. If necessary, stirring and mixing of the resin in the washing solvent can be performed with a PTFE stick.

PROTOCOL 1. RESIN SWELLING

1. Place the dry resin in the appropriate reaction vessel (*see* **Subheading 3.4.1.**).
2. Fill the reactor with DCM until all resin beads are immersed. Resin suspension can be gently mixed with a PTFE stick.
3. Leave for 20 to 30 min.
4. Remove DCM by filtration under vacuum.

PROTOCOL 2. STANDARD WASHING PROCEDURES

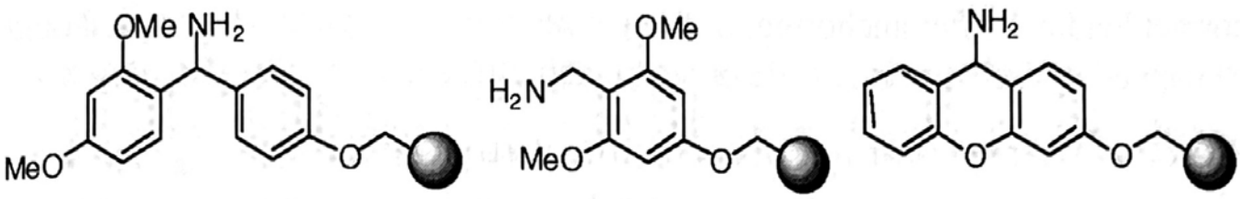
1. Fill the reaction vessel with DMF.
2. Leave for 10 s and remove the solvent by filtration.
3. Carefully wash the screw cap and the edge of the reactor with DMF.
4. Repeat **steps 1** and **2** twice with DMF.
5. Repeat **steps 1** and **2** with MeOH.
6. Repeat **steps 1** and **2** with DCM.
7. Repeat **steps 1** and **2** with DMF.

## 3.5. Linkers and Resins for Fmoc-Based SPPS

The first step of SPPS is the anchoring (or loading) of the *N*-protected C-terminal amino acid residue to the solid support via an ester or an amide bond depending on the C-terminal functional group of target peptide (respectively acid or amide). Most of the linkers are commercially available anchored on the different matrices (PS, PA, PEG-PS). The bead (ball) symbol used in the following paragraph is generic and does not refer to a particular matrix.

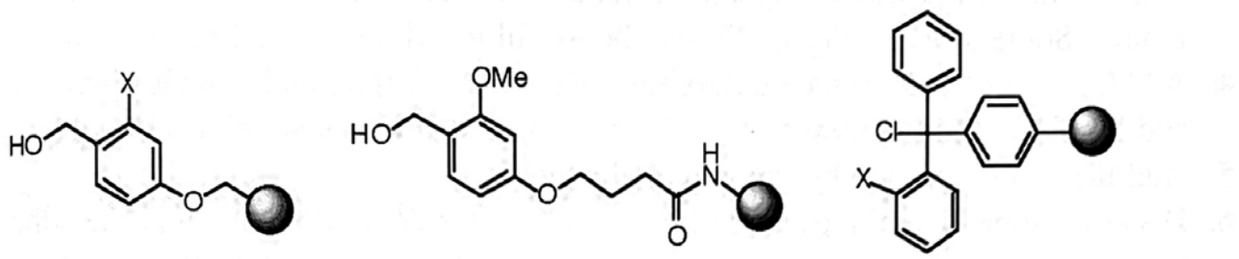
### 3.5.1. Peptide Amides

For the synthesis of C-terminal peptide amides, the commonly used resins are 1 to 3 (**Fig. 4**) ***(8–10)***. These resins are compatible with Fmoc chemistry and final TFA cleavage. For attachment of the first urethane *N*-protected residue, standard peptide coupling procedures (**Protocol 5**) can be used. These resins are usually supplied Fmoc-protected and should be deprotected before incorpora-




1: Rink amide resin 2: Pal resin 3: Sieber resin

Fig. 4. Resins for peptide amide synthesis.




4: X = H, Wang resin
5: X = OMe, Sasrin resin

6: HMPB resin

7: X = H, Trityl chloride resin
8: X = Cl, 2-Chlorotrityl chloride resin

Fig. 5. Resins for peptide acid synthesis.

tion of the first residue. With bulky C-terminal amino acids, a double coupling step can be necessary.

### 3.5.2. Peptide Acids

The anchoring of an amino acid to the solid support by esterification is often more difficult, and even hazardous, for some residues and can lead to epimerization, dipeptide formation, and low substitution. Thus, we recommend the purchase of resins preloaded with the first C-terminal *N*-protected amino acid; these are commercially available from various manufacturers.

Commonly used resins in Fmoc/tBu strategy for the synthesis of C-terminal peptide acid are reported in **Fig. 5** ***(11–14)***. Anchoring reactions must be performed in an anhydrous medium and amino acids containing water should be dried before use.

### 3.5.3. Hydroxymethyl-Based Resins

For hydroxymethyl-based resins 4 to 6, formation of the ester linkage is easier with unhindered resins such as Wang resin 4 compared with resins possessing withdrawing methoxy groups 5 and 6. The most commonly used esterification process is the symmetrical anhydride method (**Protocol 3**). Determination of the loading can be performed by Fmoc release measurement (*see* **Note 3**). In the case of difficult anchoring, the esterification step can be repeated with fresh reactants. Arginine derivatives can need three esterification steps to achieve

correct loading. After anchoring, unreacted resin-bound hydroxyl groups should be capped by benzoic anhydride or acetic anhydride (*see* **Protocol 3, step 8**).

PROTOCOL 3. ATTACHMENT TO HYDROXYMETHYL-BASED RESIN

1. Place the resin in the appropriate SPPS reactor.
2. Swell the resin as described in **Protocol 1**.
3. The desired Fmoc amino acid (10 eq relative to resin substitution) is placed in a dry, round-bottom flask with a magnetic stirrer and dissolved in dry DCM at 0°C (3 mL/mmol). Some drops of dry DMF may be useful to achieve complete dissolution.
4. Add DIC (5 eq) and stir the mixture for 10 min at 0°C. If a precipitate is observed, add DMF until dissolution and stir for 10 min longer.
5. Add the solution to the hydroxylmethyl resin.
6. Dissolve dimethylaminopyridine (DMAP) (0.1–1 eq) in DMF and add the solution to the reaction mixture.
7. After 1 h stirring, wash the resin with DMF (three times) and finally with DCM.
8. Dry the resin *in vacuo* for 18 h before performing Fmoc release measurement on a sample (*see* **Note 3**). When the loading is less than 70% repeat the esterification step.
9. When the desired substitution is achieved, cap the remaining hydroxyl groups by adding benzoic or acetic anhydride (5 eq) and pyridine (1 eq) in DMF to the resin (previously swelled) and stir for 30 min.
10. Wash the resin (**Protocol 2**) and start classical elongation with *N*-protected amino acid after Fmoc deprotection (**Protocol 7**).

### *3.5.4. Trityl-Based Resins*

Trityl-based resins are highly acid-labile. The steric hindrance of the linker prevents diketopiperazine formation and the resins are recommended for Pro and Gly C-terminal peptides. Extremely mild acidolysis conditions enable the cleavage of protected peptide segments from the resin. These resins are commercially available as their chloride or alcohol precursors. The trityl chloride resin is extremely moisture-sensitive, so reagents and glassware should be carefully dried before use to avoid hydrolysis into the alcohol form. It is necessary to activate the trityl alcohol precursor and it is highly recommended to reactivate the chloride just before use (*see* **Note 4**). After activation, attachment of the first residue occurs by reaction with the Fmoc amino acid derivative in the presence of a base. This reaction does not involve an activated species, so it is free from epimerization. Special precautions should be taken for Cys and His residues that are particularly sensitive to epimerization during activation (**Table 2**).

PROTOCOL 4. ATTACHMENT TO TRITYL-BASED RESIN

1. Place 1 g of trityl-based resin (1.0–2.0 mmol chloride/g resin) in an SPPS reaction vessel.

2. Swell the resin as described in **Protocol 1**.
3. Add a solution of 3 eq of Fmoc amino acid and 7.5 eq of DIPEA in dry DCM (10 mL/g resin). When a lower substitution resin is desired, reduce the amount of amino acid.
4. Stir the mixture for 30 to 60 min at room temperature.
5. Wash the resin with DMF (two to three times).
6. Add 10 mL of a mixture of DCM/MeOH/DIPEA (80:15:5) to cap any remaining reactive chloride group.
7. Mix for 15 min and filter.
8. Wash the resin three times with DMF and DCM. After drying *in vacuo*, the substitution can be measured from Fmoc release (*see* **Note 3**).

### 3.6 Side-Chain Protecting Groups

We will limit the description of side-chain protecting groups *(5)* to those that have been found most effective for the preparation of a large number of classical peptide sequences in Fmoc SPPS and are commercially available from most of the protected amino acid providers. For routine synthesis, TFA-labile protecting groups are usually used. However, for selective modifications of a particular residue on the solid support (e.g., side-chain cyclized peptides, biotinylated peptides), special orthogonal protecting groups are needed *(15)*. Some of the most commonly used side-chain protecting groups are reported in **Table 3**.

### 3.7. Coupling Reaction

The most simple and rapid procedure for the stepwise introduction of *N*-protected amino acids in SPPS is the *in situ* carboxylic function activation (**Protocol 5**). A large excess of the activated amino acid is used (typically 2–10 times excess compared to the resin functionality, which is provided by the manufacturer or empirically determined; *see* **Note 3**). This excess allows a high concentration of reactants (typically 60–200 m*M*) to ensure effective diffusion. The time required for a complete acylation reaction depends on the nature of the activated species, the peptide sequence that is already linked to the resin, and the concentration of reagents. This last parameter must be as high as possible and is in connection with the volume of the reaction vessel and the resin substitution (**Table 2**). The preferred coupling reagents for *in situ* activation are benzotriazol-1-yl-oxytripyrrolidino phosphonium hexafluorophosphate (PyBOP) *(16)* for phosphonium-based activation and *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) *(17)* or *N*-[1H-benzotriazol-1-yl)(dimeth-ylamino)methylene]-*N*-methyl-methanaminium hexafluorophosphate *N*-oxide (HBTU) *(18)* for aminium/uronium-based activation (**Fig. 6**). These coupling reagents convert *N*-protected amino acids into their corresponding OBt esters. A tertiary amine (generally diisopropylethylamine) is required to produce

**Table 3**
**Side-Chain Protecting Groups in Fmoc-Based SPPS**

| Amino acid and side-chain functionality | Protecting groups | Removal condition | Remarks and side reactions |
|---|---|---|---|
| **Arg** | Pmc | 95% TFA | Presence of thiols may accelerate the cleavage. |
| | Pbf | 95% TFA | |
| **Asp/Glu** | OtBu | 95% TFA | Aspartimide formation (*see* **Subheading 12.2.**). |
| | OAll[a] | $Pd(Ph_3P)_4/PhSiH_3$ | |
| **Asn/Gln** | Trt | 95% TFA | Protections avoid dehydration of the carboxamide side-chain during activation and help to solubilize Fmoc-Asn-OH and Fmoc-Gln-OH. pyrGlu formation for N-terminal glutamine peptides (*see* **Note 5**). |
| **Cys** ~~SH | Trt | 95% TFA | High level of epimerization can occur during activation (*see* **Note 6**). Participate in the folding of peptides and proteins by disulfide bridge formation (*see* **Subheading 3.11.**). |
| **His** | Trt (NHτ) | TFA | |
| | Mtt[a] | 1% TFA | Even with protection of the imidazole ring, problems of epimerization can occur during activation. |
| **Lys** ~~$NH_2$ | Boc | TFA | |
| | Mtt[a] | 1% TFA | |
| | Aloc[a] | $Pd(Ph_3P)_4/PhSiH_3$ | |
| **Ser/Thr/Tyr** ~~OH | tBu | TFA | |
| | Trt[a] | 1% TFA | |
| **Trp** | Boc | TFA | Trp can be used unprotected. However, if Arg(Pmc) or Arg(Pbf) is present in the sequence, side-reaction can occur. |

[a]Protection used for on-resin derivatization.

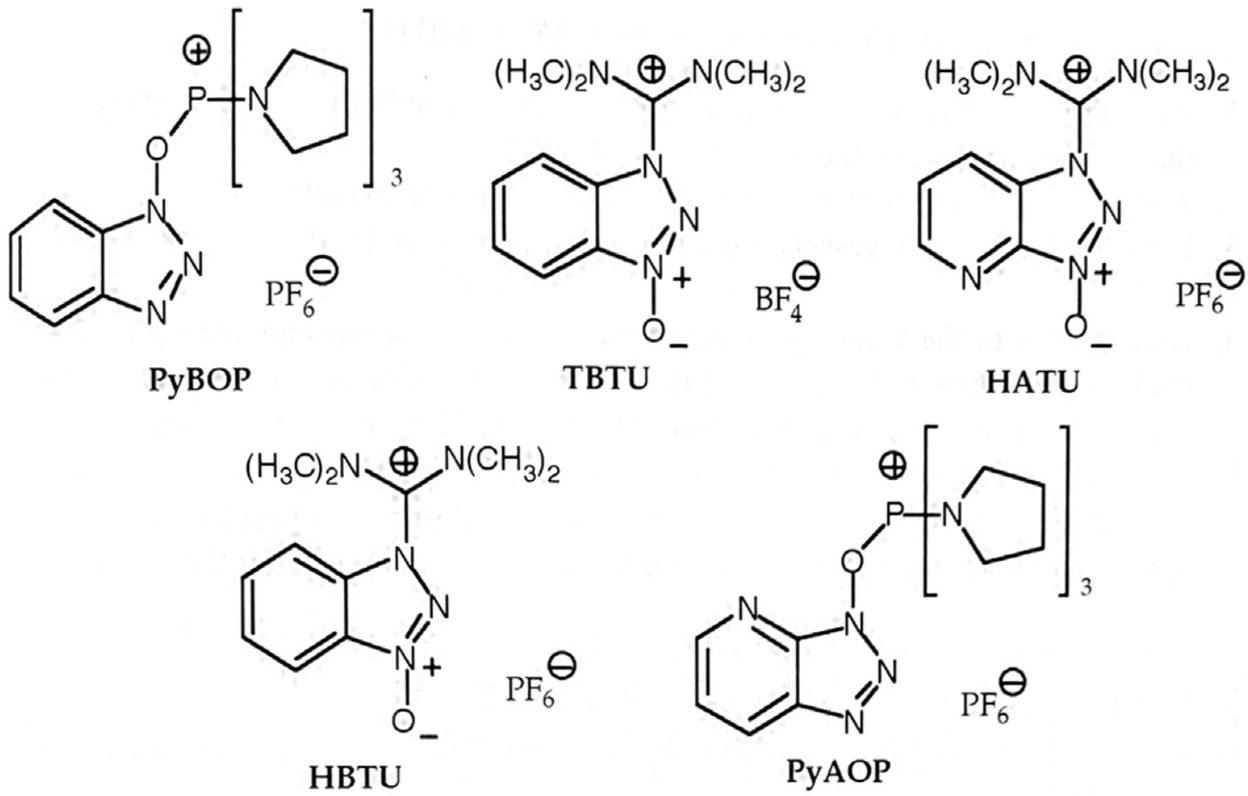



Fig. 6. Phosphonium and uronium/aminium coupling reagents.

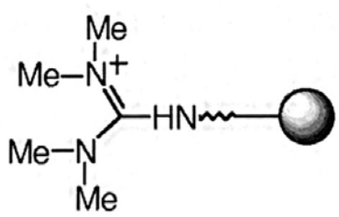


Fig. 7. N-terminal tetramethylguanidinated peptides.

the carboxylate of *N*-protected amino acids which reacts with coupling reagents. More recently, *N*-[1H-benzotriazol-1-yl(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate (HATU) ***(19)*** and 7-azabenzotriazol-1-yl-oxytris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP) ***(20)***, that generate OAt esters, have been reported to be more efficient and to reduce epimerization.

Uronium/aminium-based reagents (HBTU, HATU, TBTU) are thought to mechanistically function in a similar way to their phosphonium analogs but unlike them, they can irreversibly block the free N-terminal amino function of the peptide-resin by forming tetramethylguanidinium derivatives (**Fig. 7**) ***(21)***. To avoid this side-reaction, it is recommended to generate the carboxylate of the amino acid before adding these coupling reagents at a slightly reduced equivalent compared to the amino acid.

PROTOCOL 5. STANDARD COUPLING PROCEDURE WITH HBTU

1. After Fmoc deprotection and washings (or properly conditioning and swelling when the resin is dry), wash the resin once with DMF.
2. Add the *N*-α Fmoc-protected amino acid as a powder (usually 3 eq).
3. Fill the SPPS reaction vessel (at least 2/3 of volume) with DMF and stir with a PTFE stick for 10–20 s.
4. Add DIPEA to the vessel (usually slight excess, 3.5–4 eq) and stir with a PTFE stick until complete dissolution of the *N*-protected amino acid occurs. Other tertiary amines can be used such as *N*-methyl morpholine or triethylamine.
5. Add HBTU (2.9 eq compared with 3 eq of amino acid), screw the cap, and stir for 30 min. For difficult sequences it is recommended to preactivate the *N*-protected amino acid before coupling to the free $N^{\alpha}$-amino function to limit the guanylation side-reaction.
6. Remove the coupling solution by filtration.
7. Wash the resin properly according to **Protocol 2**.
8. Perform qualitative monitoring of the coupling reaction using a colorimetric test (*see* **Note 1**).

In some cases coupling is not complete and a colorimetric test will reveal the presence of free amino groups. On these occasions a double coupling with fresh reagents must be performed. When acylation is incomplete after a second coupling, a capping procedure through acetic anhydride (**Protocol 6**) can be performed to stop the elongation of these less-reactive amino groups. When the colorimetric test still reveals the presence of free amine functions after capping, aggregation can be suspected.

PROTOCOL 6. CAPPING

1. Swell the resin in DCM.
2. Remove the DCM by filtration.
3. Fill the SPPS reactor (at least 2/3 of volume) with a 50/50 DCM/acetic anhydride solution (in case of trityl linker, *see* **Note 7**) and mix for 3 min.
4. Remove the capping solution by filtration and repeat **step 3** for 7 min.
5. Wash three times with DCM.
6. Check the disappearance of free amino groups by a convenient colorimetric test (*see* **Note 1**) and repeat the operation if necessary.

### 3.8. Difficult Sequences and Aggregation

When $N^{\alpha}$-deprotection reactions and amino acid coupling steps do not go to completion or proceed in low yields, repeated or prolonged reaction times should overcome these problems. Nevertheless, this is not always sufficient. The cause of this failure is thought to be self-association of the peptide chain by hydrogen bond formation leading to aggregation. This aggregation results in incomplete

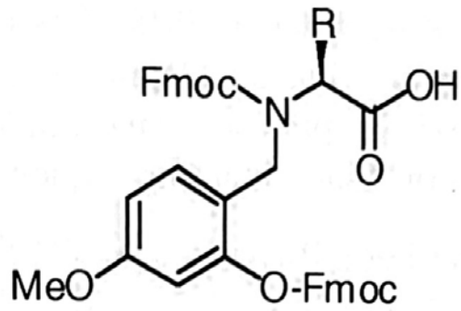



Fig. 8. Fmoc-(FmocHmb) amino acid.

solvation of the peptide-resin and inaccessibility of the reagents to the N-terminal amino group. This phenomenon is sequence-dependent with particular propensity for sequences containing high proportions of hydrophobic residues and can start from the fifth coupled residue. Two general methods can be used to disrupt the formation of secondary structures. The first consists of changing the peptide environment by adding chaotropic salts such as potassium thiocyanate or lithium chloride at a 0.4 *M* concentration, detergent solvents at 1% (v/v), or solvents such as DMSO, trifluoroethanol, or hexafluoroisopropanol to the reaction medium *(22–28)*. The second approach involves structural changes in the peptide backbone itself by the introduction of protecting groups to selected amide bonds. This is usually achieved by using the 2-hydroxy-4-methoxybenzyl (Hmb) derivative of glycine. Other amino acids can also be incorporated as their *N,O-bis*(Fmoc)-*N*-(Hmb) derivatives approximately every 6–7 residues (**Fig. 8**) *(29, 30)*. The incorporation of (Hmb) amino acids can be performed through their corresponding pentafluorophenyl esters, which are commercially available, or by standard coupling methods. For optimal *N*-acylation of the terminal Hmb residue that can be slow, it is recommended to use powerful coupling methods such as symmetrical anhydrides or preformed acid fluorides in DCM. The O-Fmoc protection of the Hmb derivatives is cleaved under piperidine treatment and the Hmb group in the final TFA cleavage. For Cys-, Ser-, and Thr-containing peptides their pseudoproline derivatives, also known to disrupt aggregation, can also be used *(31–33)*.

### 3.9. Fmoc Deprotection

Removal of the temporary Fmoc protecting group from the N-terminus of the peptidyl-resin is normally achieved by short treatment with 20% piperidine in DMF (**Protocol 7**). The reaction is generally complete within 10 min, but can be longer in some cases. For safe removal a 20-min deprotection time is recommended. The deprotection results in formation of a dibenzofulvene-piperidine adduct that strongly absorbs in the UV range (*see* **Note 3**). Fmoc removal can be monitored by UV spectroscopy. This is a common procedure with automatic

synthesizers but not in standard manual SPPS. When incomplete deprotection is suspected, the use of 20% piperidine containing 1 to 5% DBU in DMF is recommended. However, DBU can promote aspartimide formation, thus its use should be avoided in Asp- or Asn-containing sequences.

PROTOCOL 7. REMOVAL OF $N^{\alpha}$ FMOC PROTECTION

1. After coupling reaction and washings, resin is washed once with DMF.
2. Fill the SPPS reaction vessel (at least 2/3 of volume) with a 80:20 DMF/piperidine solution and stir for 20 min (*see* **Note 8**).
3. Remove the DMF/piperidine solution by filtration.
4. Wash the resin properly according to **Protocol 2**.

## 3.10. Final Cleavage

Concentrated trifluoroacetic acid is widely used for the simultaneous cleavage of the peptide from the resin and removal of side-chain protecting groups. In these conditions, side-chain protecting groups produce stabilized carbocations which can readily react with the electron rich side-chain of amino acids (Cys, Met, Tyr, Thr, Ser, Trp), leading to unwanted byproducts. To minimize this phenomenon, scavengers are added to the cleavage cocktail to trap carbocations. Numerous cocktails have been described to optimize the cleavage conditions of particular sequences. Silane-based cocktails that generally provide good results are nonodorous and less toxic than thiol-based cocktails (*34*). Special care should be taken to prepare and to use cleavage cocktails. All operations should proceed under a fume hood using gloves and safety glasses.

PROTOCOL 8. STANDARD TFA CLEAVAGE

1. Weigh the resin and place it in a round-bottom flask. When the resin is dry, swell it as described in **Protocol 1**.
2. Add 10 mL of cleavage cocktail (trifluoroacetic acid/water/triisopropyl silane 95:2.5:2.5) per 100 mg of resin.
3. Resin is cleaved for 90 min under gentle stirring (*see* **Note 9**).
4. Filter the resin on a glass frit and wash it twice with fresh cleavage cocktail. Recover the filtrate in a round-bottom flask.
5. Concentrate the cleavage cocktail *in vacuo* to approx 1/4 of its original volume.
6. Under vigorous stirring, add MTBE to precipitate the peptide. At least 10 times the initial TFA volume of MTBE should be added to precipitate the unprotected peptide. When the peptide does not precipitate, concentrate the solution in vacuo and go directly to **step 9**.
7. Filter the precipitate on a 4-porosity glass frit.
8. Triturate and wash by filtration the precipitated peptide on the frit three times with MTBE.

9. Solubilize the peptide in acetonitrile/water/TFA 50:50:0.1 and lyophilize. Solvent used for this step can be changed to increase solubility. Lyophilization can be repeated twice when scavengers are detected after reverse phase HPLC analysis of the peptide.

### 3.11. Disulfide Bridge Formation

One of the simplest ways to form disulfide bonds is oxidation of the fully deprotected linear peptide obtained after cleavage from the resin (in some cases, disulfide bridges began to form during the cleavage step) before or after preparative HPLC purification. The cyclization has to be performed using high dilution conditions to promote intramolecular disulfide bond formation versus intermolecular dimerization. Dimethyl sulfoxide is used to accelerate the oxidation reaction *(35)*.

PROTOCOL 9. STANDARD FORMATION OF DISULFIDE BRIDGES BY AIR OXIDATION

1. Dissolve the lyophilized peptide at 0.5 m*M* concentration in a 5% acetic acid solution.
2. Add 10% volume of DMSO in a large beaker. Adjust the pH to 6.0 to 7.0 if necessary with a 0.5 *M* ammonium acetate solution.
3. Stir vigorously the solution at room temperature for 12 h to incorporate atmospheric oxygen into the solution. Ideally, disulfide bridge formation should be monitored by reverse phase HPLC. Purification (**step 4**) should be performed immediately after the end of the reaction.
4. Purify the peptide by preparative reverse phase HPLC after acidification with a TFA solution (pH 2.0).
5. Lyophilize the solution containing the fractions.

For peptides having more than two cysteines, orthogonal protection of these residues can be used to allow selective and sequential disulfide bridge formation *(5)*. However, especially if the peptide sequence is a natural one, simultaneous deprotection/bridge formation can be attempted in an adequate redox buffer. The equilibrium between the different disulfide bridge arrangements during "oxidative folding" leads, in most of cases, to the native folding.

PROTOCOL 10. FORMATION OF DISULFIDE BRIDGES USING OXIDATIVE FOLDING IN REDOX BUFFER

1. Dissolve the linear peptide at a 0.05 to 0.1 m*M* concentration in a buffer (0.1–0.2 *M* Tris-HCl, pH 7.7–8.7) containing 1 m*M* EDTA and reduced (1–10 m*M*) and oxidized (0.1–1 m*M*) glutathione.
2. Stir the solution at 25 to 35°C and monitor the reaction by HPLC, normally from 16 to 48 h.
3. Lyophilize the solution after acidification with a TFA solution (pH 2.0).
4. Purify the oxidized peptide by preparative reverse phase HPLC.
5. Lyophilize the solution containing the fractions.

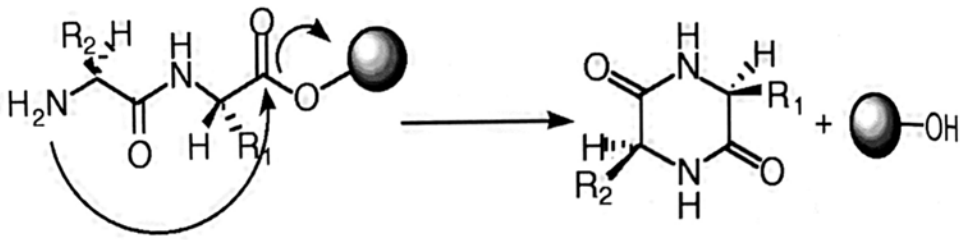



Fig. 9: DKP formation.

### 3.12. Side Reactions

Several residue- or sequence-dependent side reactions can occur during SPPS.

#### 3.12.1. Diketopiperazine Formation

Diketopiperazine formation occurs at the C-terminal deprotected dipeptide stage by intramolecular cleavage of the resin ester linkage by the free amino function of the penultimate amino acid under basic conditions. This side reaction is particularly favored in Fmoc SPPS strategy as the base-induced deprotection of the Fmoc group releases a free amino group. The extent of its formation depends on the nature of the C-terminus amino acid and the type of peptide-linker ester anchor. Peptides containing proline or glycine in the C-terminal dipeptide sequence are especially sensitive to DKP formation. In such cases, hindered trityl-based resins should be used to avoid its formation and consequently the loss of the C-terminal dipeptide from the resin (**Fig. 9**).

#### 3.12.2. Aspartimide Formation

The cyclization of aspartic acid residues to form aspartimide is the most likely side-reaction observed in routine SPPS (**Fig. 10**). This is a sequence-dependent side-reaction that occurs either during chain elongation or during final TFA cleavage when Asp(OtBu)-AA sequence (AA = Ala, Gly, Ser, Asn(Trt)) is present in the peptide. Hydrolysis of the aspartimide ring leads to a mixture of both α- and β-peptides. Its reaction with piperidine used for Fmoc removal also leads to the formation of α- and β-piperidides. Normally, in Fmoc-based SPPS, Asp(OtBu) provides sufficient protection. However, for particular sequences such as Asp(OtBu)-Asn(Trt) particularly sensitive to aspartimide formation, addition of HOBt to the piperidine solution or protection of the aspartyl amide bond with the 2-hydroxy-4-methoxybenzyl (Hmb) group should be considered (*36*).

## 4. Notes

1. **Kaiser test.** Solution A: 5% Ninhydrin in ethanol (w/v). Solution B: 80% phenol in ethanol (w/v). Solution C: KCN in pyridine (2 mL 0.001 *M* KCN in 98 mL pyridine). After washings, sample a few resin beads in a small glass tube and add 2 drops of each of the above solutions. Heat to 120°C for 4–6 min. Blue resin beads indicate the presence of resin-bound free amines.

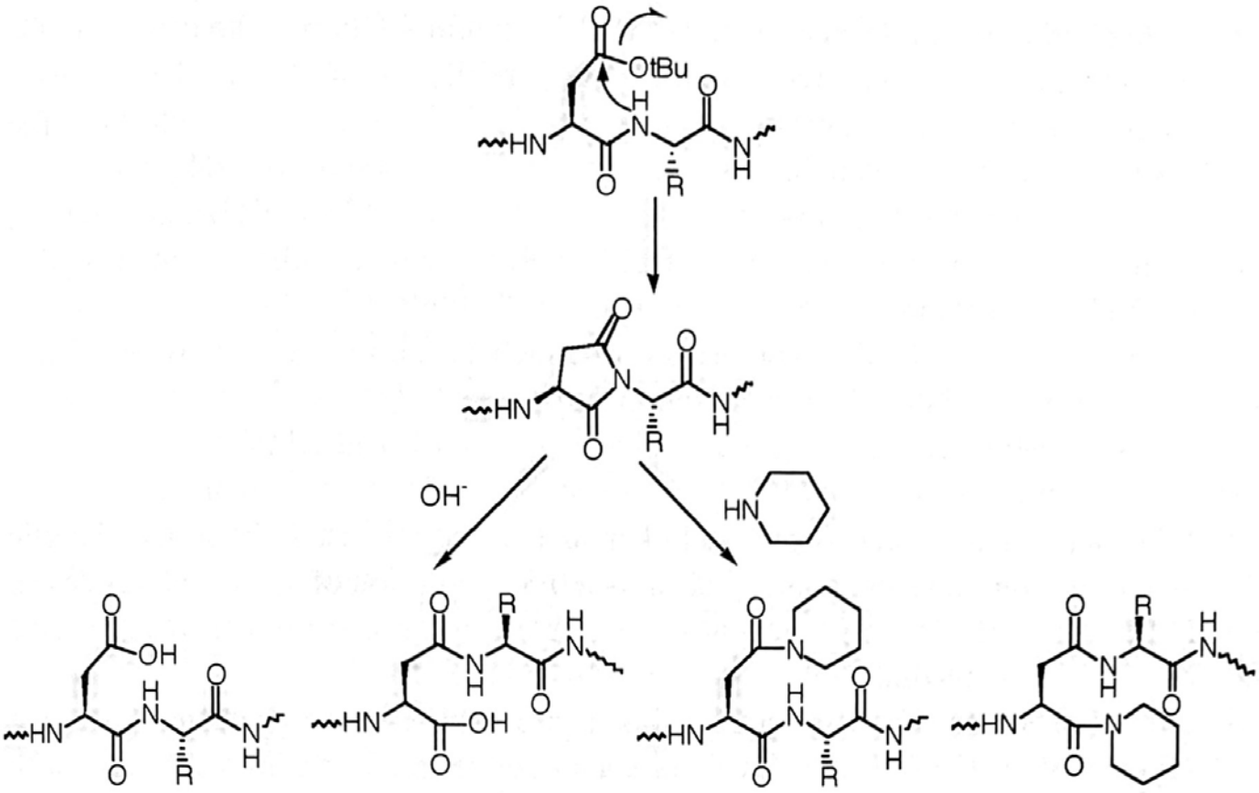



Fig. 10. Aspartimide formation and succinimide ring reopening in basic medium.

**TNBS test.** Solution A: 5% DIPEA in DMF (v/v). Solution B: 1% TNBS in water (w/v), commercially available from Fluka, St. Quentín-Fallavier, France 1% in DMF. Sample a few resin beads in a small glass tube, add 1 drop of each of the above solutions, and watch the sample under a microscope. The presence of resin-bound free amines is indicated within 10 min by yellow or red resin beads.

2. Polyamide-based and composite PEG-PS resin beads present a larger scope of solvent compatibility than PS beads, even allowing the use of water solutions. However, for routine peptide synthesis, solvents are the same than those used for PS-resin.
3. Fmoc determination: (1) Weigh duplicate samples of 5 to 10 mg loaded resin in an Eppendorf tube and add 1.0 mL 20% piperidine/DMF, stir for 20 min, and centrifuge down the resin. (2) Transfer 100 μL of the above solution to a tube containing 10 mL DMF and mix. (3) Set the spectrophotometer at 301 nm. Transfer 2 mL DMF into each of the two cells of the spectrophotometer (reference and sample cells), set the spectrophotometer to zero. Empty the sample cell, transfer 2 mL of the solution to measure, and check absorbance. (4) Substitution of the resin = [101 × (Absorbance)]/[7.8 × (weight in mg)] Check absorbance three times at 301 nm; calculate average substitution.
4. Reactivation of trityl resins: After swelling and washing the resin with toluene (five times), place the resin in a round-bottom flask and cover with toluene. Add freshly distilled acetyl chloride (1 mL/g resin) and fit a reflux condenser on the flask. Heat the reaction mixture at 60°C for 3 h. Allow to cool to room temperature, filter the resin on a frit, and wash six times with DCM. Load it immediately as described in **Protocol 4**.

5. To avoid the intramolecular cyclization of N-terminal Gln peptide leading to the formation of N-terminal pyroglutamic acid peptide, it is recommended to cleave the peptide from the support before removing the *N*-Fmoc terminal protection. The DMF/piperidine treatment has to be performed in solution followed by concentration of the mixture. The target peptide is then precipitated by MTBE and filtered.
6. To minimize epimerization during *N*-protected cysteine coupling, some coupling methods are recommended:
   a. Preferably use a hindered or weaker base such as 2,4,6-trimethylpyridine (TMP) or 2,6-di-tert-butyl-4-(dimethylamino)pyridine ***(37)***.
   b. Use 50:50 DMF/DCM coupling solvent instead of neat DMF.
   c. Avoid preactivation.
7. When a particularly acid-sensitive linker such as trityl is used, 50:50 DCM/acetic anhydride solution should be replaced by a 0.5 *M* solution of acetic anhydride and DIPEA in DMF. This will prevent premature cleavage of the linker by acetic acid released in the medium.
8. Some linkers, such as Rink amide linker, are sold *N*-Fmoc protected. The first removal of the Fmoc group may require a longer deprotection time particular with highly loaded resin (>0.9 mmol/g). In these cases it should be useful to repeat steps **2** and **3** of **Protocol 7**.
9. Longer reaction times can be required to fully deprotect bulky side-chain protecting groups (Pbf, Trt…), especially when several protecting groups are close to each other in the sequence. When uncompleted deprotection is detected, the partially deprotected peptide should be cleaved in solution using larger cocktail volume and/or longer reaction time.

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