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Some Mechanistic Aspects on Fmoc Solid Phase Peptide Synthesis

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Abstract Peptides are biomolecules that may have several biological activities which makes them important to the environment in which they operate. Sometimes it is necessary for larger amounts of peptides to carry out some studies, like biological tests, NMR structural research or even interaction studies between peptides with other molecules. Expression can be an alternative for that. However, synthesis is specially useful when unnatural modifications or introduction of site specific tags are required. Synthetic peptides have been used for different studies such as cell signaling, development of epitope-specific antibodies, in cell-biology, biomarkers for diseases etc. Many different methodologies for peptide synthesis can be found in the literature. Solid phase peptide synthesis (SPPS) has been largely used and can be an excellent alternative to achieve larger quantities of these biomolecules. In this mini review, we aim to describe the SPPS and explain some of the mechanistic aspects and reagents involved in all phases of the synthesis: the use of resin, the ninhydrin test, some of the protecting groups, coupling reagents for peptide bond formation and the cleavage process.

Keywords Peptides · SPPS · Fmoc · Chemical mechanisms

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Introduction

Peptides are widespread molecules in nature, and they have several biological activities such as antimicrobial (Hancock and Scott 2000; Zasloff 2002), antithrombotic (Menezes et al. 2011), opioid (Goldberg 2010), antioxidant (Zhang et al. 2011) and so on. Therefore, peptides are important to the environment in which they operate and also as an object for the development of new drugs (Brogden 2005). Peptides are also becoming increasingly important in materials science due to their self-assembling properties (Bong et al. 2001; Chen 2005).

Usually, the amount of peptide that can be naturally extracted from plants, animals and microorganisms is too small, allowing only a few studies, as the initial identification and characterization of the biomolecule. To carry out some other studies, such as biological tests, NMR structure determination and interaction studies, it is necessary to acquire larger amounts of the molecule, which can be difficult to achieve by extractions from the natural source. Expression is a good choice if no changes in the structure are required. If this is the situation, a solution to this problem can be peptide synthesis.

The peptide synthesis may be carried out both in liquid (Takahashi and Yamamoto 2012; Gravert and Janda 1997) and solid-phase (Fields and Noble 1990; Chan and White

2000; Benoiton 2005; Schnolzer et al. 2007). The solid-phase synthesis has been the most widely used (Naidoo and Rautenbach 2012; Elsawy et al. 2012; Zhang et al. 2012; Hibino and Nishiuchi 2012; Aucagne et al. 2012; Amblard et al. 2006), as it has some advantages (such as better overall yield and fewer by-products) and it has greater acceptance when compared to the liquid-phase synthesis. More recently, the use of ionic liquids in peptide synthesis need also to be considered as a medium both for solid-phase and liquid-phase synthesis (Plaquevent et al. 2008; Tietze et al. 2012).

The solid-phase peptide synthesis starts with a resin which is insoluble under the conditions of the synthesis, usually a copolymer of polystyrene with 1 % divinylbenzene sometimes grafted with polyethylene glycol (Zalipsky et al. 1994), and which must also have an anchor for the synthesis to be carried out in the solid support (Merrifield 1963; Albericio 2004; Marquardt and Eifler-Lima 2001). In this synthesis, a process of washing and filtration of the sample is performed at the end of each reaction step. Thus, excess of reagents, solvents and any other compounds not bound to the resin are removed (Marquardt and Eifler-Lima 2001). This procedure can prevent unwanted reactions, allowing higher yields and producing cleaner synthesis (Marquardt and Eifler-Lima 2001).

Among the strategies for the synthesis of peptides on solid-phase, Fmoc (fluorenylmethyloxycarbonyl) and Boc (tert-butyloxycarbonyl) are most used (Miranda and Alewood 1999; Hudson 1988). In this work, our focus is the Fmoc strategy for solid phase peptide synthesis (SPPS).

Different information about the SPPS can be found in the literature (Jensen, 2013; Naidoo and Rautenbach 2012; Elsawy et al. 2012; Zhang et al. 2012; Hibino and Nishiuchi 2012; Merrifield 1963; Najera 2002; Isidro-Llobet et al. 2009), including reviews that describe details of the experimental procedures on SPPS (Shelton and Jensen 2013; Amblard et al. 2005, 2006). In all of them the main purposes are more related to practical procedures. This short review presents an overview of solid-phase peptide synthesis, describing the reagents involved throughout the chemical steps and the reaction mechanisms related to each part of the synthesis. Our focus is not on the details of the experimental procedure, but on the mechanistic aspects, describing basic mechanisms of the reactions involved in the synthesis.

Resin and Protecting Groups

In the synthetic Fmoc methodology, all amino acid derivatives used during the synthesis have the 9-fluorenylmethyloxycarbonyl (Fmoc, Fig. 1) as a protecting group. It is a temporary protecting group of the amino function, removed at each step of the synthesis, being a base labile group (Isidro-Llobet et al. 2009; Carpino and Han 1972; Carpino 1987; Machado et al. 2004), which is normally removed by piperidine or the most recently added alternative to piperidine: 4-methylpiperidine (same efficiency in Fmoc group removal as piperidine) (Hachmann and Lebl 2006). 4-methylpiperidine has become an alternative to piperidine since the latter is a controlled substance and its distribution is carefully monitored (Hachmann and Lebl 2006). Fmoc can also be removed by stronger organic bases such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), but in a condition more prone to side reactions (Mergler and Dick 2005). This lability is due to the fact that the removal of the acidic hydrogen of the Fmoc (highlighted in red in Fig. 1) generates a conjugated base stabilized by resonance (Isidro-Llobet et al. 2009). In addition to the temporary protecting group, there are also the permanent protecting groups of the amino acid residue side chains—the *t*-butyl, *O*-*t*-butyl, trityl (Trt), 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf), t-butyloxycarbonyl (Boc) groups (structures shown in Fig. 2), which remain linked throughout all stages of the synthesis, being removed only in the last step, the cleavage of the peptidyl-resin linkage.

Both permanent and temporary protectors will ensure the reaction of Fmoc-amino acids only in the site of interest

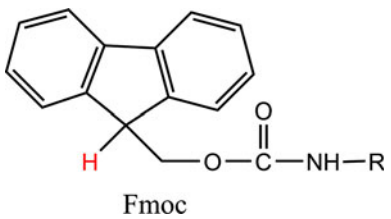


Fig. 1 Structure of Fmoc (Color figure online)

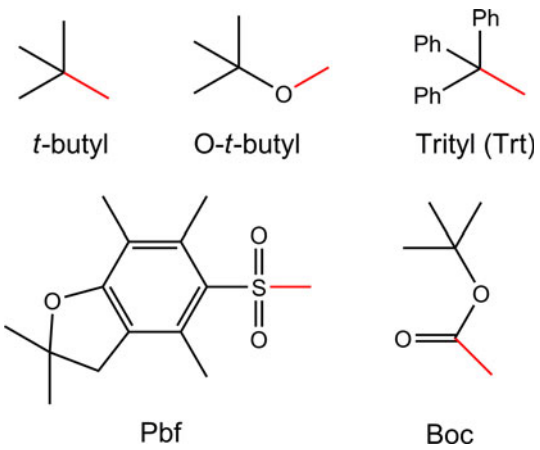


Fig. 2 Structures of *t*-butyl, O-*t*-butyl, Trt, Pbf and Boc (permanent protecting groups for the amino acids side chain). In *red* the carbon that is attached to the amino acid (Color figure online)

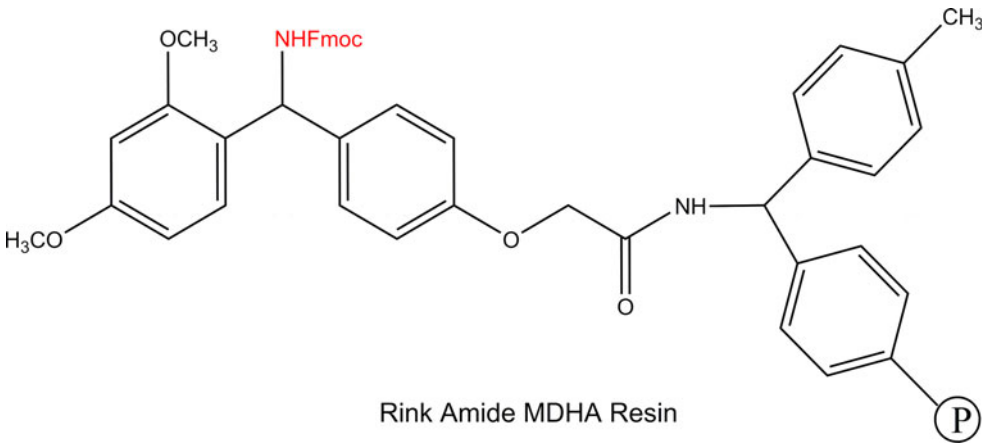


Fig. 3 Structure of the resin used throughout the synthesis. The amino group is shown in *red* and *P* is the polymer chain from the resin (Color figure online)

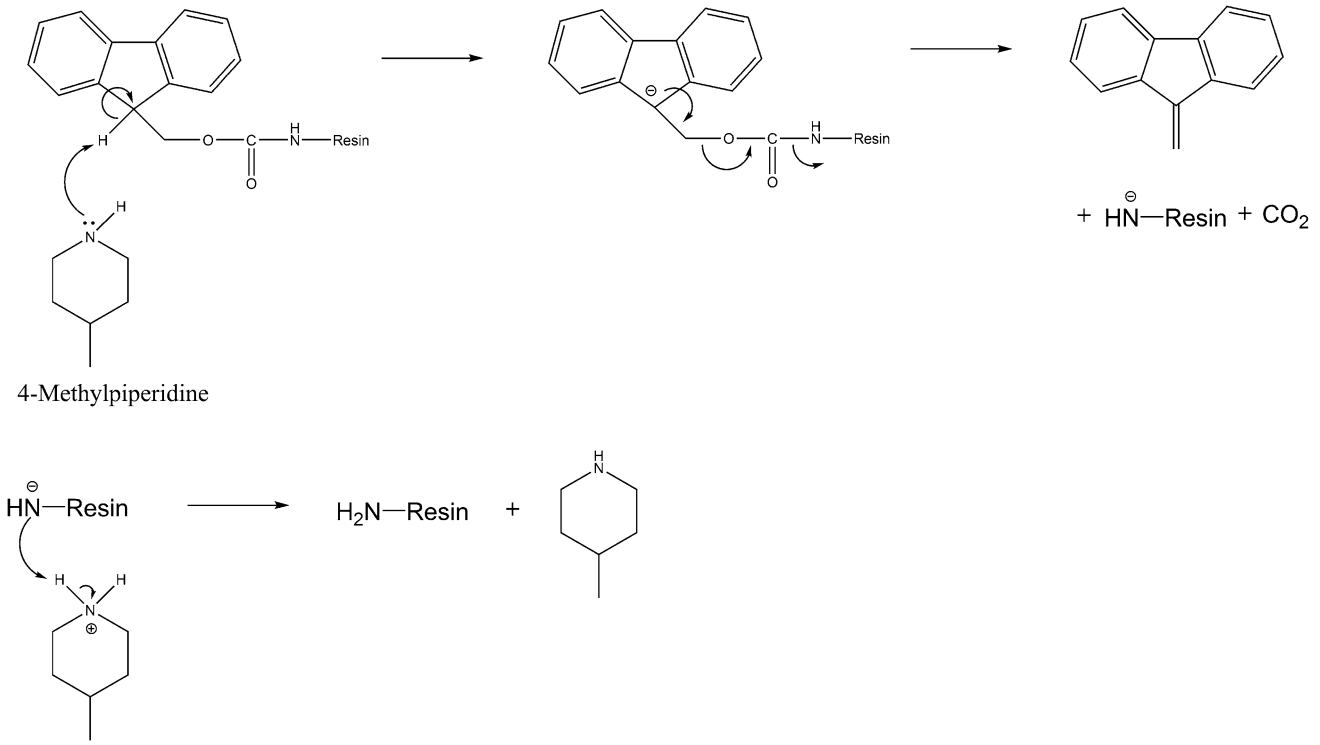


Fig. 4 Removal of the Fmoc group by 4-methylpiperidine for the formation of a free $-NH_2$ (Chan and White 2000)

for the formation of the peptide bond. It helps to prevent secondary reactions, such as the incorporation of dipeptide derivatives instead of an amino acid derivative. In addition, it may help in the final purification of the product due to smaller amount of undesired products.

The synthesis can be performed in a syringe with a filter to retain the resin that is made either of polypropylene or of a sintered glass material. As reported before, the resin is an insoluble and inert support. Nevertheless, it is derivatized with a linker to furnish proper acid lability to the peptidyl-resin linkage by the presence of electron-releasing groups. Also, the linker defines the C-terminal functional group. A Rink amide linker can be used as a support for the solid phase, as well as a Pal linker and a Sieber linker, which led to C-terminal peptide amides (Howl 2005). On the other hand, the Wang, Sasrin, 4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid (HMPB), and 2-chlorotritryl linkers can be used for the synthesis of free C-terminal peptide acids (Howl 2005; Gońgora-Benítez et al. 2013). The sasrin and the 2-Chlorotrityl chloride resin are appropriate for the synthesis of side-chain protected peptide fragments by being labile to dilute acids. Also, the 2-Chlorotrityl

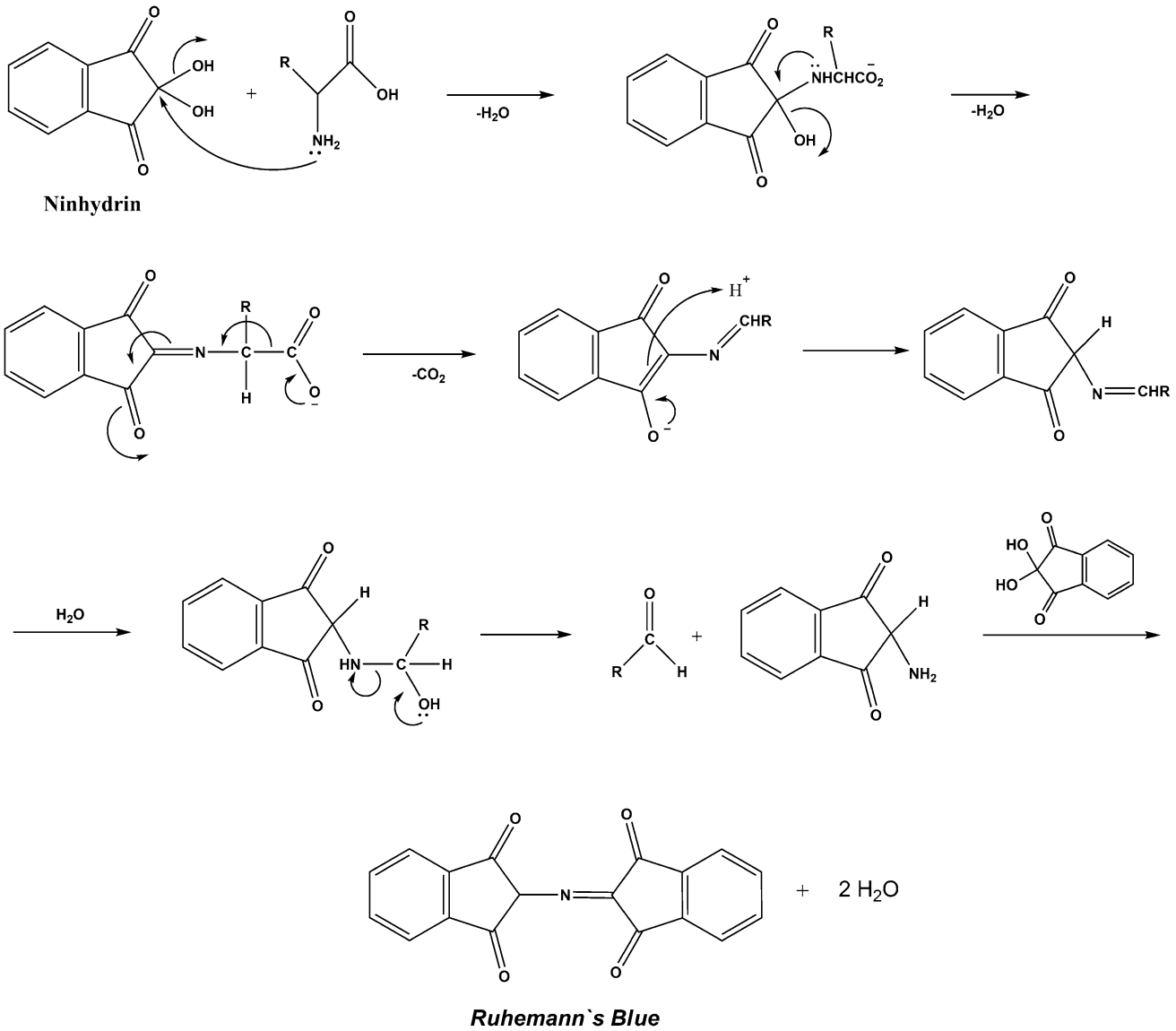


Fig. 5 Mechanism for the ninhydrin reaction with an α-amino acid with a primary amino group to form the *Ruhemann's blue* (Friedman 2004; McCaldin 1959)

chloride resin is employed for the synthesis of C-terminal peptide acids, which may be important as modified peptide drugs, such as the somatostatin-analogue octreotide (Tailhades et al. 2010).

In this work, the synthesis will be exemplified by using the Rink amide resin (MDHA), whose linker structure is shown in Fig. 3. Since many peptides are naturally found in the amidated form and due to the fact that C-terminal amides are generally more stable to degradation than C-terminal acids, a resin with an $–NH_2$ group (as Rink Amide) is very widely employed. The coupling of the first Fmoc-amino acid will occur at the amino group of the resin, forming an amide bond and it will be described together to the peptide bond formation.

Removing the Temporary Protecting Group Fmoc

The Fmoc group must be removed to furnish free amino groups that will react with activated carboxyl components for peptide bond formation. For this purpose, the resin must be first washed with 2-propanol and *N,N*-dimethylformamide, DMF, for the removal of soluble impurities (Chan and White 2000; Benoiton 2005; Marquardt and Eifler-Lima 2001). Both the 2-propanol and DMF can be removed from the reaction vessel by filtration at reduced pressure. For removing the Fmoc protecting group from the resin, the preferable organic base now is 4-methylpiperidine, as previously explained. According to standard procedures deprotection can be conducted in a solution of

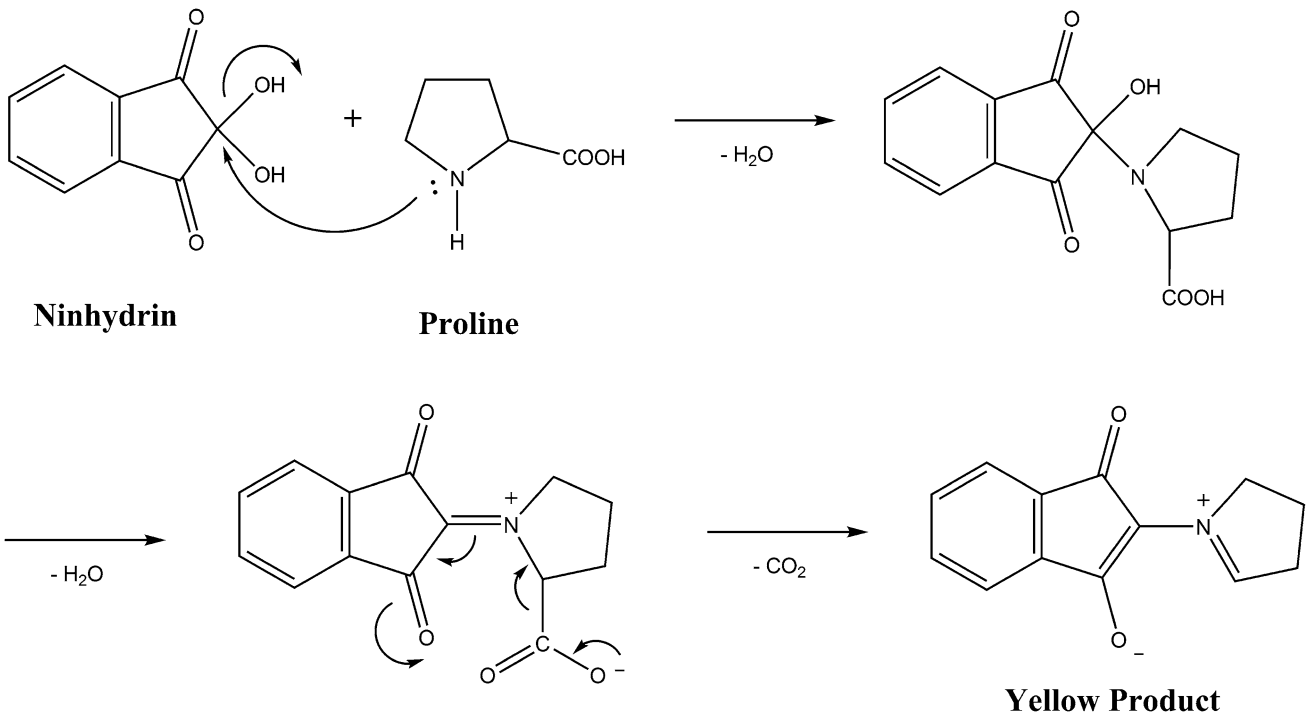


Fig. 6 Mechanism for the ninhydrin reaction with proline (Kaupp et al. 2002; McCaldin 1959)

4-methylpiperidine in DMF (20–30 % by volume) for 1–5 min (2 times). Then, the resin can be washed again with 2-propanol and DMF. Finally, the resin has to be washed with dichloromethane (DCM) to easily dry the grains. Alternatively, it can be washed with a mixture of methanol/dichloromethane (MeOH/DCM), which helps to prevent losses of resin sticking to the glass or plastic syringe. Fmoc deprotection by piperidine or by 4-methylpiperidine is usually fast and clean. Slow kinetics and low Fmoc deprotection yields are clear indications of peptide aggregation (Remuzgo et al. 2009).

The reaction mechanism for the removal of Fmoc by 4-methylpiperidine is shown in Fig. 4. Initially the acidic proton from the Fmoc group is removed by the organic base. Then, there is a beta elimination step, with formation of CO_2. The resin without the Fmoc group, formed in the previous step, is then protonated, resulting in the final product of resin with a free $–NH_2$ group (Fig. 4).

This process of deprotection is performed at each stage of the synthesis for the formation of a free $–NH_2$ group, at which the amino acid derivative coupling will occur for peptide bond formation.

Ninhydrin Test

To verify the previously described deprotection and the coupling of each amino acid derivative during all stages of synthesis, a colorimetric assay for the presence of the amino function group must be conducted. Many assays have been used such as the formation of a salt of the amino group in the resin with picric acid (2,4,6-trinitrophenol) followed by the release of the corresponding phenolate with diisopropylethylamine (Gisisn 1972) or the nucleophilic substitution of halo-1,4-naphtoquinones or nitro, halo-1,4-naphtoquinones with the amino groups linked to solid-phase resins followed by release of 2-amino-3-halo-1,4-naphtoquinone by acidolysis (Blackburn 2005). However, there is no assay with so widespread use as the ninhydrin reaction (Shelton and Jensen 2013; Kaiser et al. 1970; Troll and Cannan 1952). This test consists of the reaction of a primary or a secondary amine with ninhydrin, yielding a dark blue compound known as *Ruhemann's blue* in the former case or a brown compound corresponding to specific non-classic products for secondary amines

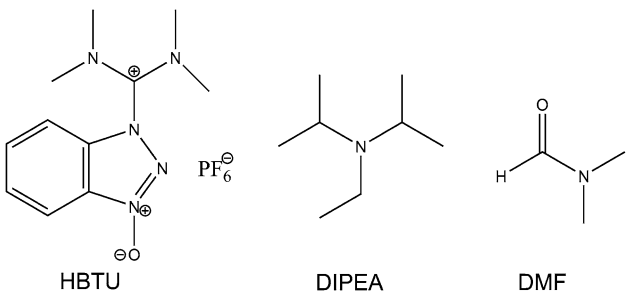


Fig. 7 Structures of HBTU, DIPEA and DMF

(Friedman 2004; McCaldin 1959). In order to perform the test, after deprotection, 1–5 mg of the obtained product are added to a test tube, then one drop of a solution made with 2 mL of KCN (1 mmol L^{-1}) and 98 mL of pyridine is added, followed by two drops of a 80 % (by weight) phenol solution in ethanol and by one drop of a 5 % (by weight) ninhydrin solution in ethanol (Kaiser et al. 1970; Troll and Cannan 1952). The mixture is heated at 110 °C for 3–5 min. A color change of the grain sample from faint yellow to blue or brown, for primary and secondary amines, respectively, indicates the presence of a free amino group, and consequently, the deprotection of the resin or peptidyl-resin (Kaiser et al. 1970; Troll and Cannan 1952).

The ninhydrin test can also be used in a quantitative way. For that, as described by Amblard et al. (2006), 5–10 mg of the resin and 1.0 mL of 20 % piperidine/DMF are added in a tube, stirred for 20 min and then centrifuged. 100 µL of this solution is transferred to a tube containing 10 mL of DMF and mixed. The resulting solution can be observed by UV spectrometer at 301 nm. The substitution of the resin can be calculated thus: [101 × (Absorbance)]/[7.8 × (weight in mg)].

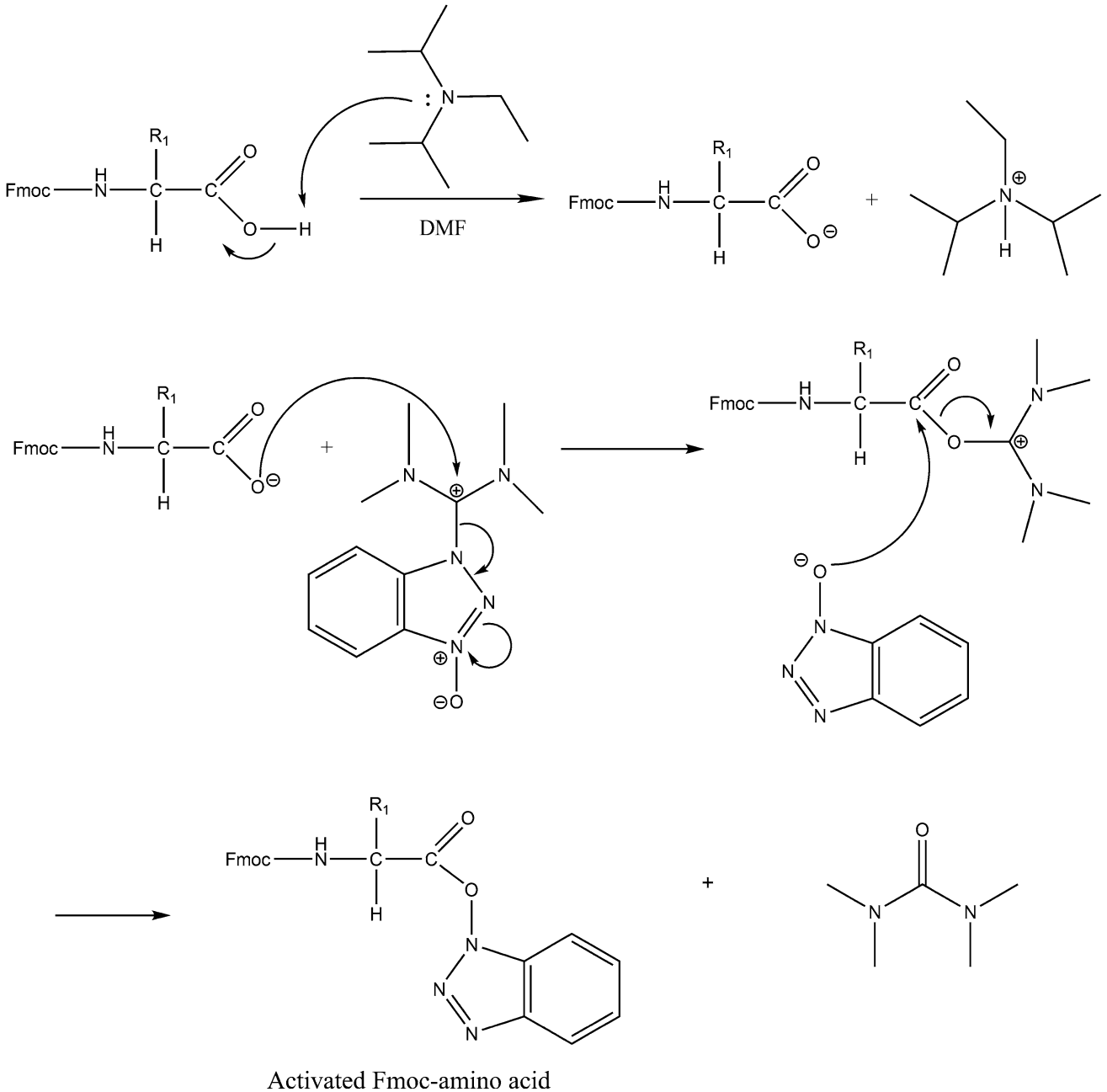


Fig. 8 Mechanism for the activation reaction of the Fmoc-amino acid with HBTU, wherein R_1 is the side chain of an amino acid (Najera 2002)

The use of organic solvents in the ninhydrin test, such as alcohols, pyridine and phenol, helps to accelerate the appearance of the color (Troll and Cannan 1952). The visible spectrum of Ruhemann's compound in pyridine is blue-shifted when compared to other solvents like DMF and DMSO. The heating of the mixture near to 100 °C for up to 5 min gives a quantitative yield for the appearance of color when reacting with a $–NH_2$ of the resin or of the amino acid residue (Troll and Cannan 1952). The reaction mechanism between the primary amino group of an α-amino acid and ninhydrin to form the *Ruhemann's blue* is shown in Fig. 5.

This test can be successfully used for all amino acids, except for proline. In this case, another colorimetric test, also using ninhydrin, can be used, giving a yellow product. The mechanism for the reaction of ninhydrin with proline is shown in Fig. 6.

Coupling of the First Amino Acid Derivative and Peptide Bond Formation

After deprotection and its confirmation by quantitative nynhidrin test yielding 90–100 % of theoretical value (Kilk and Langel 2006), the synthesis can be started and the first amino acid derivative can be coupled to the resin. Since the synthesis is carried towards the N-terminus (Lloyd-Williams et al. 1993), the nucleophilic group of the resin linker reacts with the acid group of the amino acid derivative while its amino group is protected. For this reason, the last amino acid derivative is added first in the reaction vessel for coupling to the resin. The method and the mechanism of coupling of the Fmoc-amino acid derivative to the resin depend upon the nature of the bond being formed. For the ester bond formation to linkers such as the Wang resin and the HMPB resin it is necessary to use a base as catalyst, for which 4-dimethylaminopyridine is usually employed, with a carbodiimide as the carboxyl-activating reagent (Chan and White 2000). Other coupling reagents that furnish good yield for the esterification were also introduced, such as the chloro derivative 2-chloro, 1,3-dimethylimidazolidinium hexafluorophosphate (CIP) (Akaji et al. 1992). For the very acid-labile 2-chlorotrityl chloride linker, the carboxyl group of the amino acid derivative attacks the methyl carbon and chloride is the leaving group, which leads to the linkage of Fmoc-amino acids to the 2-chlorotrityl chloride resin (Barlos et al. 1989; James 1999). The ideal linker would fulfill a number of important criteria like cost, availability, attachment of starting material should be readily achieved in high yield, stable to the chemistry used in synthesis, cleavage would be efficient under conditions that do not damage the final product(s) and should not introduce impurities that are difficult to remove (James 1999). The choice of the linker requires careful consideration and readers must refer to the James (1999) review for a complete description of the linkers used in solid-phase

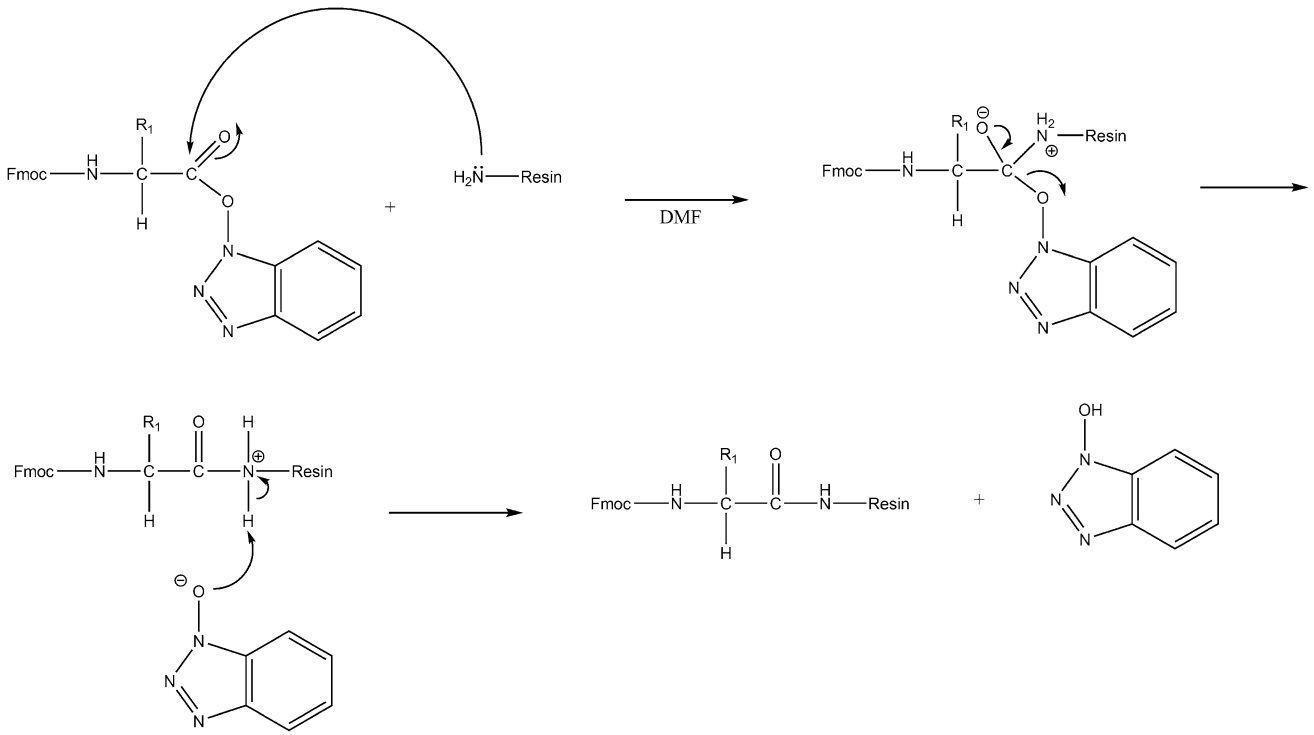


Fig. 9 Mechanism for the reaction of coupling the activated Fmoc-amino acid to the resin. R_1 is the side chain of an amino acid (Montalbetti and Falque 2005)

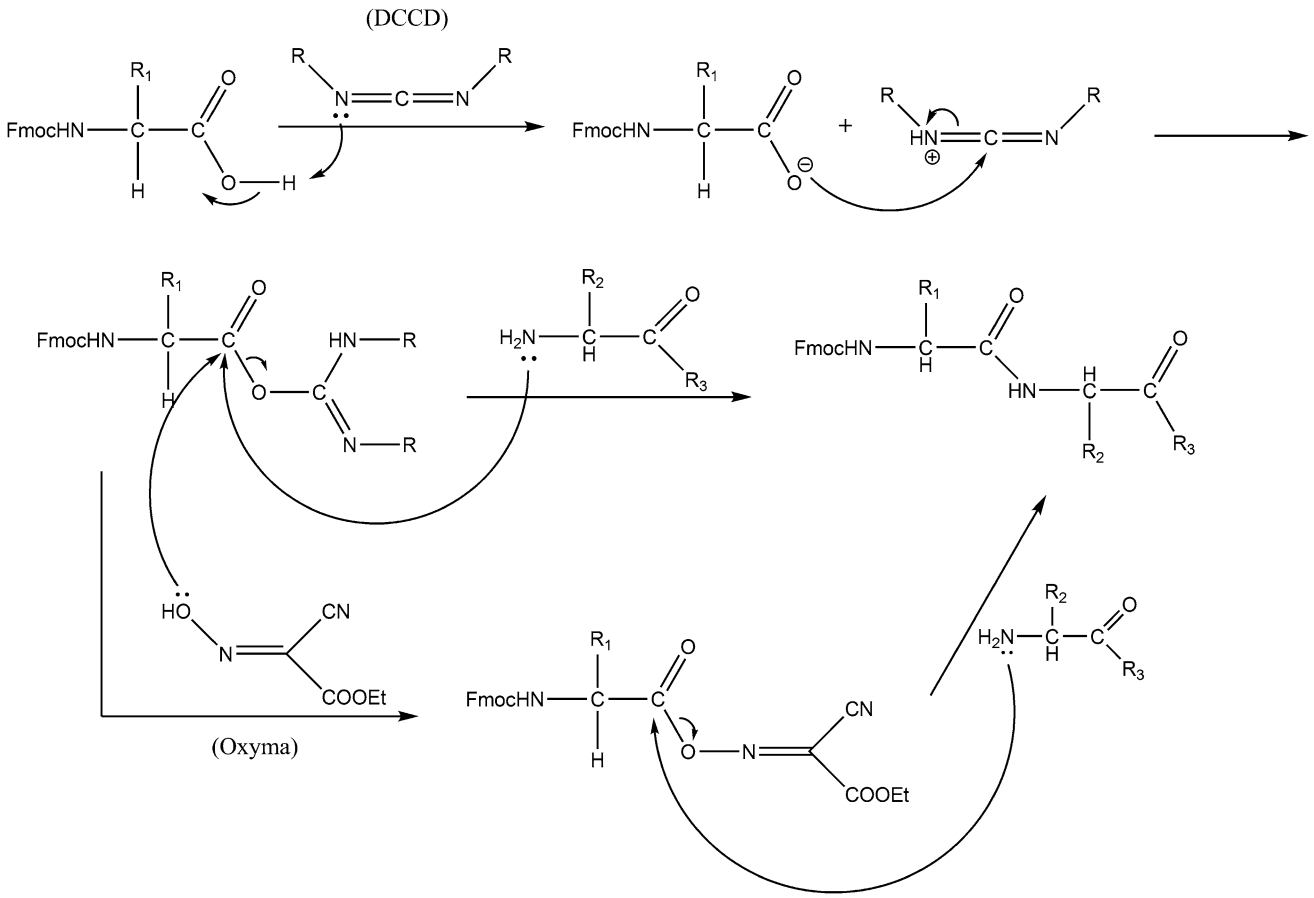


Fig. 10 Mechanism for the activation reaction by DCCD/Oxyma and the coupling of the activated Fmoc-amino acid to another amino acid. R_1 is the side chain of the amino acid to be activated, R is radical group of DCCD (isopropyl, DIC, for example), R_2 is the side chain of the second amino acid to be coupling with the first and R_3 is a peptide chain. (Benoiton, 2005; Al-Warhi et al. 2012; Subirós-Funosas et al. 2009)

synthesis of peptides. For the synthesis of amidated peptides the anchorage of Fmoc-amino acid to resins and the peptide bond formation are very similar reactions and the mechanistic aspects of the most common methods employed are discussed in this section.

To achieve the coupling of the amino acid derivative, the use of aminium or phosphonium salts are the methods of choice. HBTU ([benzotriazol-1-yloxy(dimethylamino)methylidene]-dimethylazanium-hexafluorophosphate) is employed as a prototype of a aminium-type reagent (Al-Warhi et al. 2012). For conducting the anchorage of Fmoc-amino acid to linkers like the Rink amide resin and for performing the peptide coupling reaction during the synthesis, N,N-diisopropylethylamine (DIPEA), DMF and HBTU (structures shown in Fig. 7) are added beyond the Fmoc-amino acid to be coupled. DIPEA is used to deprotonate the acid group of the Fmoc-amino acid and DMF is the solvent for the reaction (Montalbetti and Falque 2005). For the amide bond formation, it is necessary to introduce an activating group in the Fmoc-amino acid to be coupled to reduce the free energy difference between the reagents and the activated complex to kinetically favor the peptide bond formation (Najera 2002). The high kinetic barrier for peptide bond formation has as a counterpart, this is the very high stability of the peptide bond formed (Wolfenden 2006). Thus, the formation of the peptide bond and the amide bond to linkers are usually very stable even to some harsh conditions of synthesis. The added activating group is also a good leaving group that favors the reaction kinetically (Montalbetti and Falque 2005).

The suggested mechanism for the reaction of the activation of the amino acid derivative with the aminium-type reagent HBTU and the proton removal by DIPEA is shown in Fig. 8. Initially, the acidic proton of the Fmoc-amino acid is removed by DIPEA or other organic base. Then, the deprotonated Fmoc-amino acid is added to HBTU, generating the elimination of a triazole derivative. Finally, this hydroxybenzotriazole derivative is added to the carbonyl group of the Fmoc-amino acid, leading to the formation of the final product, the activated Fmoc-amino acid, by nucleophilic substitution. Very similar mechanisms are valid for other aminium type reagents like TBTU ([benzotriazol–1–yloxy(dimethylamino) methylidene]-dimethylazanium trifluoroborane fluoride) and HATU

Table 1 Molecular weight of Fmoc-amino acids with protecting groups, free amino acids and amino acids within a peptide

	MW (g/mol) Fmoc-amino acid	MW (g/mol) Free-amino acid	MW (g/mol) Amino acid within a peptide
Fmoc-Ala-OH	311.3	89.1	71.1
Fmoc-Arg(Pbf)-OH	648.8	174.2	156.2
Fmoc-Asn(Trt)-OH	596.7	132.1	114.1
Fmoc-Asp(O*t*Bu)-OH	411.5	133.1	115.1
Fmoc-Cys(Trt)-OH	585.7	121.2	103.2
Fmoc-Glu(tBu)-OH	443.5	147.1	129.1
Fmoc-Gln(Trt)-OH	610.7	146.1	128.1
Fmoc-Gly-OH	297.3	75.1	57.1
Fmoc-His(Trt)-OH	619.7	155.1	137.1
Fmoc-Ile-OH	353.4	131.2	113.2
Fmoc-Leu-OH	353.4	131.2	113.2
Fmoc-Lys(Boc)-OH	468.5	146.2	128.2
Fmoc-Met-OH	371.5	149.2	131.2
Fmoc-Pro-OH	337.4	115.1	97.1
Fmoc-Phe-OH	387.4	165.2	147.2
Fmoc-Ser(*t*Bu)-OH	383.4	105.1	87.1
Fmoc-Thr(*t*Bu)OH	397.5	119.1	101.1
Fmoc-Trp(Boc)-OH	526.6	204.2	186.2
Fmoc-Tyr(*t*Bu)-OH	459.6	181.2	163.2
Fmoc-Val-OH	339.4	117.1	99.1

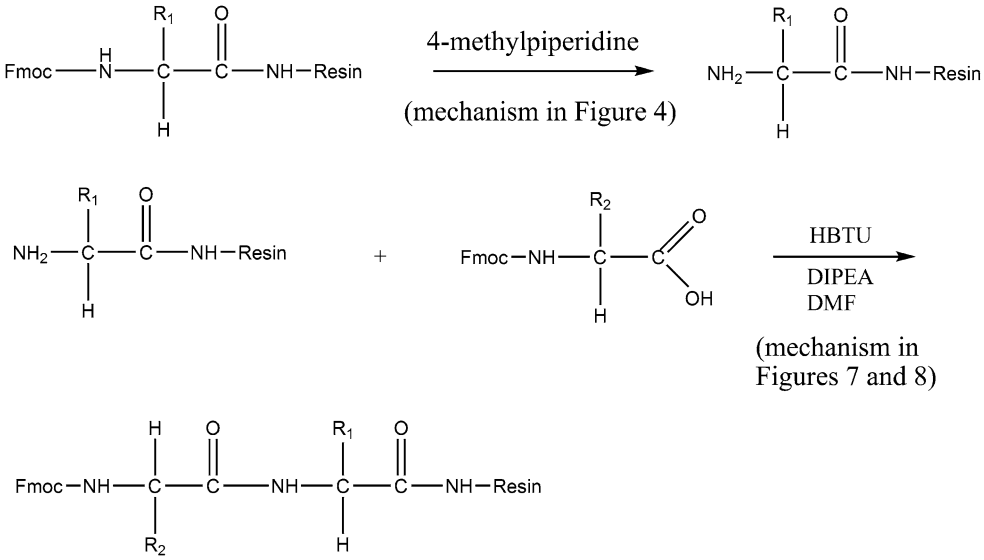


Fig. 11 Scheme for coupling an Fmoc-amino acid to another amino acid residue already attached to the resin. R_1 and R_2 are side chains of two different amino acids being linked

([dimethylamino(triazolo[4,5-b]pyridin-3-yloxy)methylidene]-dimethylazanium hexafluorophosphate) and phosphonium-type BOP (benzotriazol-1-yloxy-tris(dimethylamino)phosphanium hexafluorophosphate) and PyBOP (benzotriazol-1-yloxy(tripyrrolidin-1-yl)phos
phanium hexafluorophosphate).

Another method for coupling reaction can be performed by esterification of the carboxylic acid with 2,4,6-mesitylene-sulfonyl-3-nitro-1,2,4-triazol (MSNT) in the presence of 1-methylimidazol (Melm) (Blankemeyer-Menge et al. 1990). This procedure for anchoring Fmoc-amino acids to hydroxyl solid supports can achieve high yields and prevent the formation of dipeptide and racemization (Blankemeyer-Menge et al. 1990).

With the activated Fmoc-amino acid, it is possible to carry out the coupling of this activated compound to the resin or to another protected amino acid. The mechanism for the coupling reaction of an activated Fmoc-amino acid

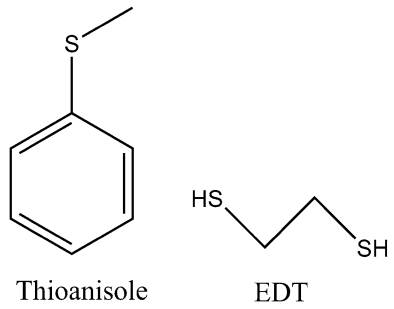


Fig. 12 Structures of thioanisole and EDT

to the resin is shown in Fig. 9. At first it takes place the nucleophilic substitution: the $-NH_2$ group of the resin is added to the acyl carbon of the activated Fmoc-amino acid, followed by the elimination of 1-hydroxybenzotriazole after a proton transfer from the aminium group of the resin linker. As a result, there is the coupling of the Fmoc-amino acid to the resin. An analogous mechanism occurs for the peptide bond formation.

The other very widespread method for amino acid derivative activation is by means of carbodiimide, usually in the presence of a hydroxylamine derivative. There are

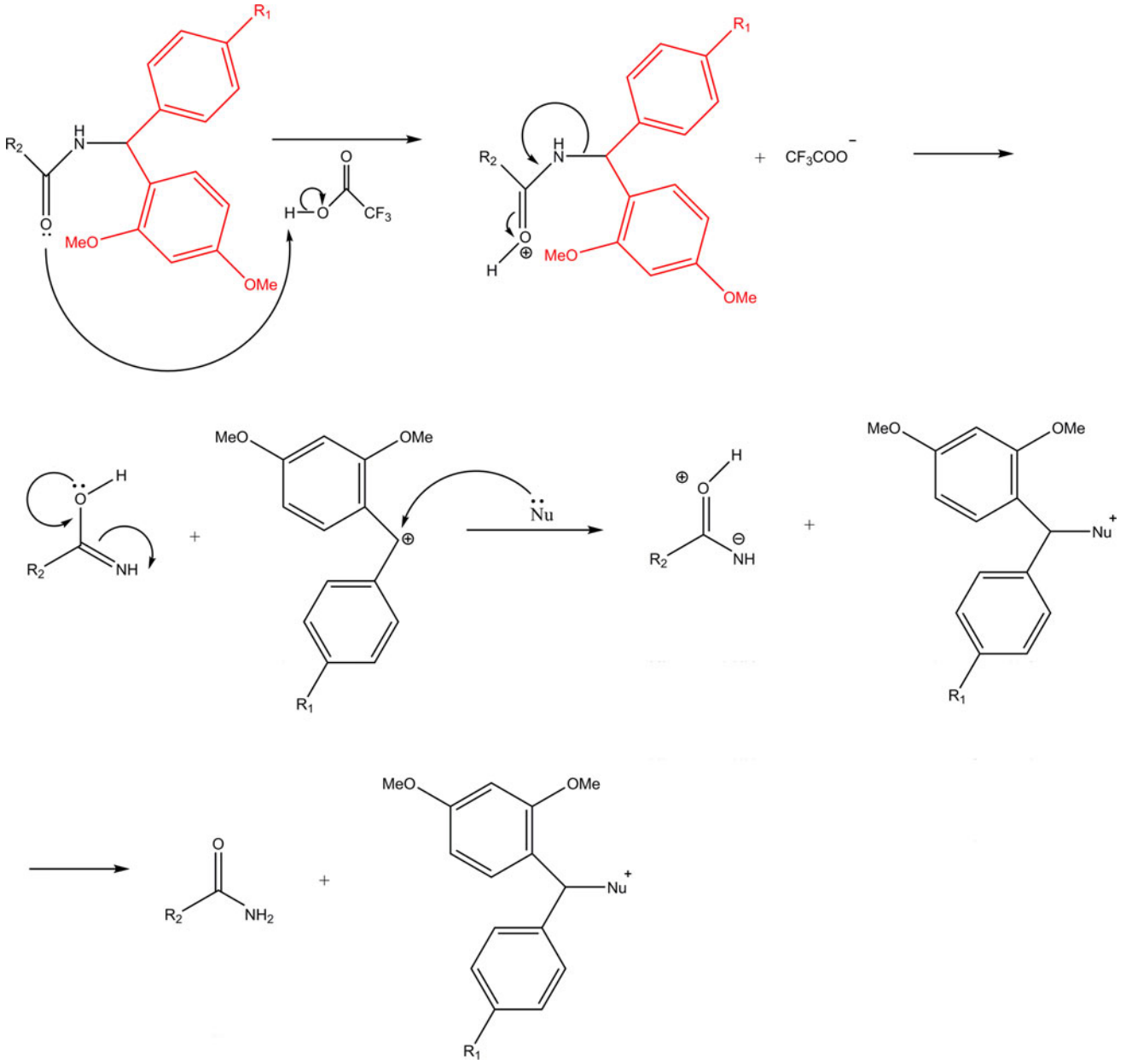


Fig. 13 Reaction mechanism to remove the resin by TFA (Brady et al. 1977). In *red*, the linker structure to be removed by cleavage for the formation of amidated peptide. R_1 is the resin chain, R_2 is a peptide chain and Nu is a nucleophilic compound (see Fig. 10) present in the cleavage process (Color figure online)

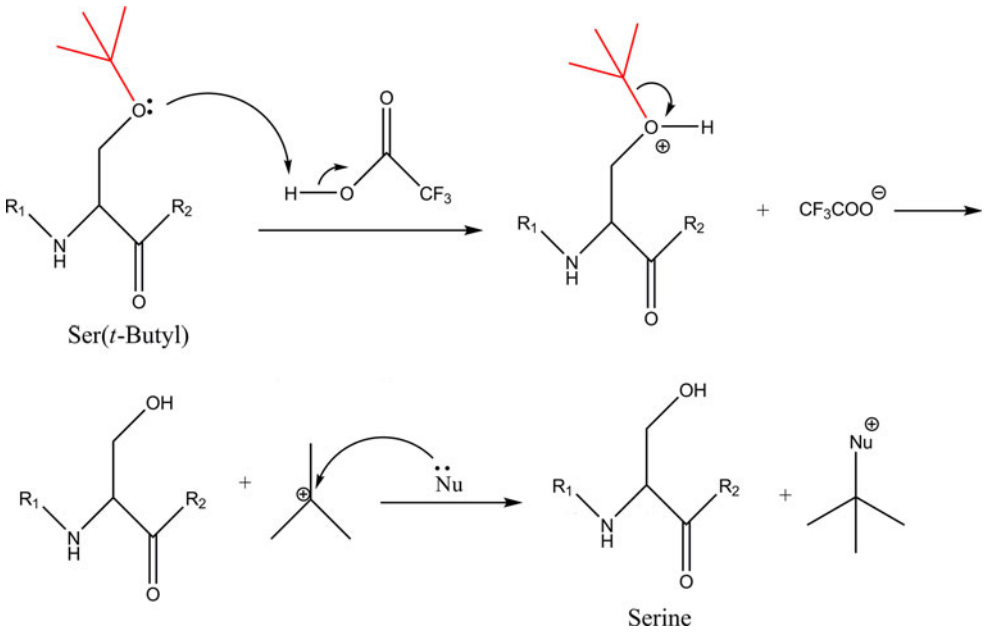


Fig. 14 Removal of *t*-butyl group of serine. In *red*, the group to be removed. R_1 and R_2 are peptide chains and Nu is a nucleophile compound present in the cleavage process (Brady et al. 1977) (Color figure online)

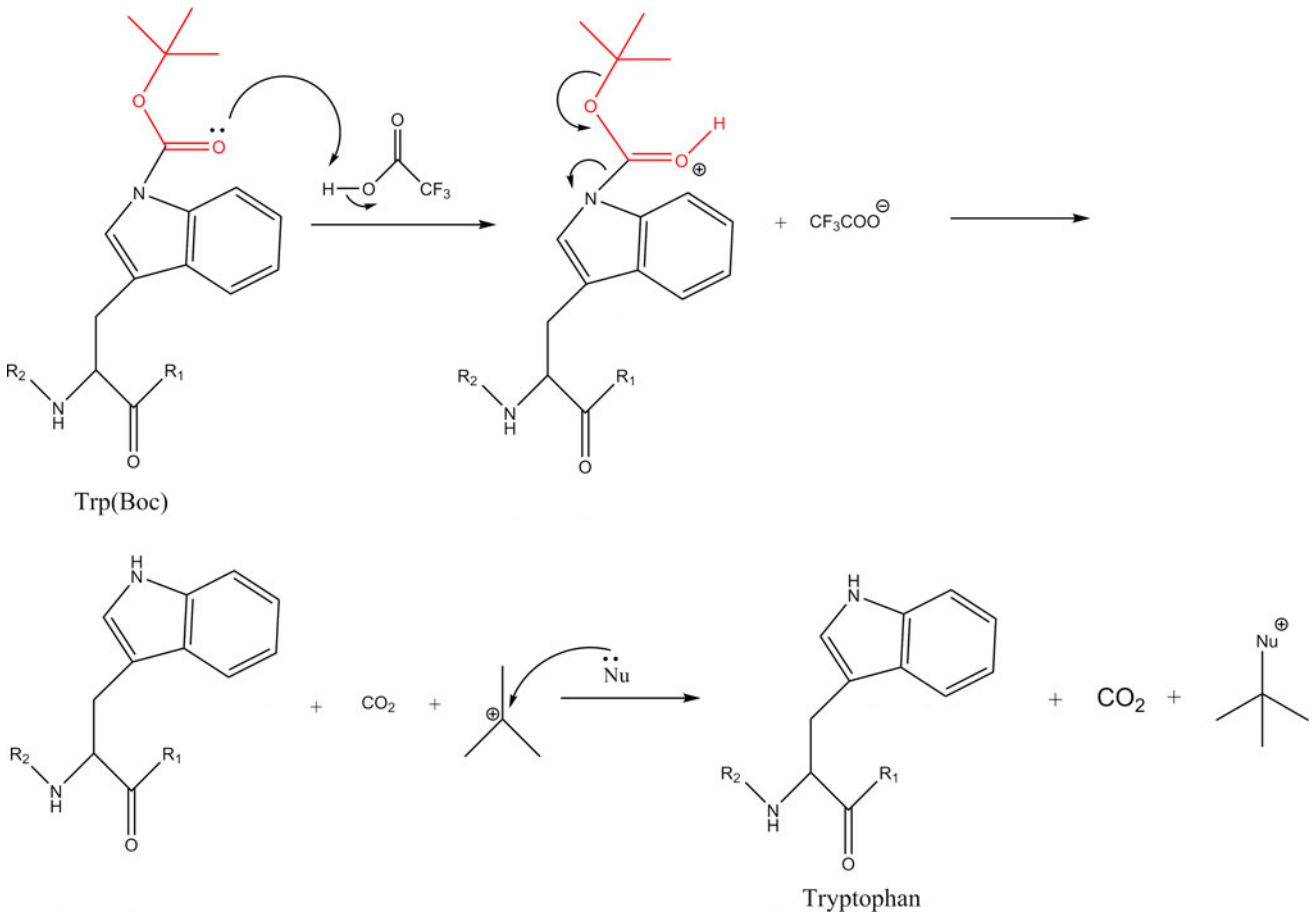
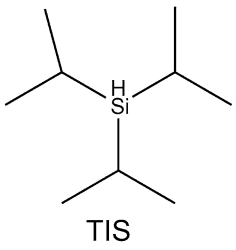


Fig. 15 Removal of Boc group from the protected tryptophan. In *red*, the group that will be removed. R_1 and R_2 are peptide chains and Nu is a nucleophile compound present in the cleavage process (Brady et al. 1977) (Color figure online)

Fig. 16 Structure of TIS



many carbodiimides available in the literature, but the most widely used is 1,3-diisopropylcarbodiimide, whose side product is a urea that is soluble in DMF and DCM. Nevertheless, it is not advisable to couple amino acid derivatives only in the presence of the carbodiimide since this usually leads to a high level of enantiomerization through an oxazolone intermediate (Montalbetti and Falque 2005; Wolfenden 2006; Benoiton 1996) stabilized by resonance. In fact, 2-phenyl-5-oxazolone (Yalcin et al. 1995), which is a useful reference compound for peptide fragmentation in mass spectrometry, is being prepared by activation with a carbodiimide, which clearly shows a straightforward formation of oxazolones by carbodiimide. The mechanism of carboxyl group activation is shown in Fig. 10. The carboxyl group performs a nucleophilic attack of the electrophilic carbon of carbodiimide, which occurs together with a proton transfer. Since the carbodiimide has a basic nitrogen capable of accepting the carboxyl proton, one of the advantages of this reagent compared to aminium or phosphonium salts is that it is not necessary to add an organic base to generate the carboxylate. Nevertheless, the *O*-acyl isourea formed is activated (i.e. it has a very good leaving group and a high free energy content), which favours the oxazole formation and amino acid enantiomerization. A hydroxylamine is usually added to prevent enantiomerization by converting the *O*-acyl isourea into a less active intermediate. 1-hydroxybenzotriazole and other triazole derivatives such as 1-hydroxy-7-azabenzotriazole (Chan and White 2000; Carpino 1993) are very efficient suppressors of racemization, but triazoles may be explosive under certain conditions, which made the international transport of those chemicals difficult. Fortunately, the reagent ethyl (2Z)-2-cyano-2-hydroxyiminoacetate (the so-called Oxyma) was recently introduced as a safe and efficient suppressor of enantiomerization (Subirós-Funosas et al. 2009). Like the benzotriazoles, oxyma has an hydroxylamine function which performs a nucleophilic attack on the *O*-acyl isourea amino acid derivative forming an active ester (Fig. 10).

As the first amino acid residue bound to the resin or the amino acid residue introduced has the temporary protecting group, in our case the Fmoc group, the deprotection should be carried out again with 4-methylpiperidine/DMF (1/4,

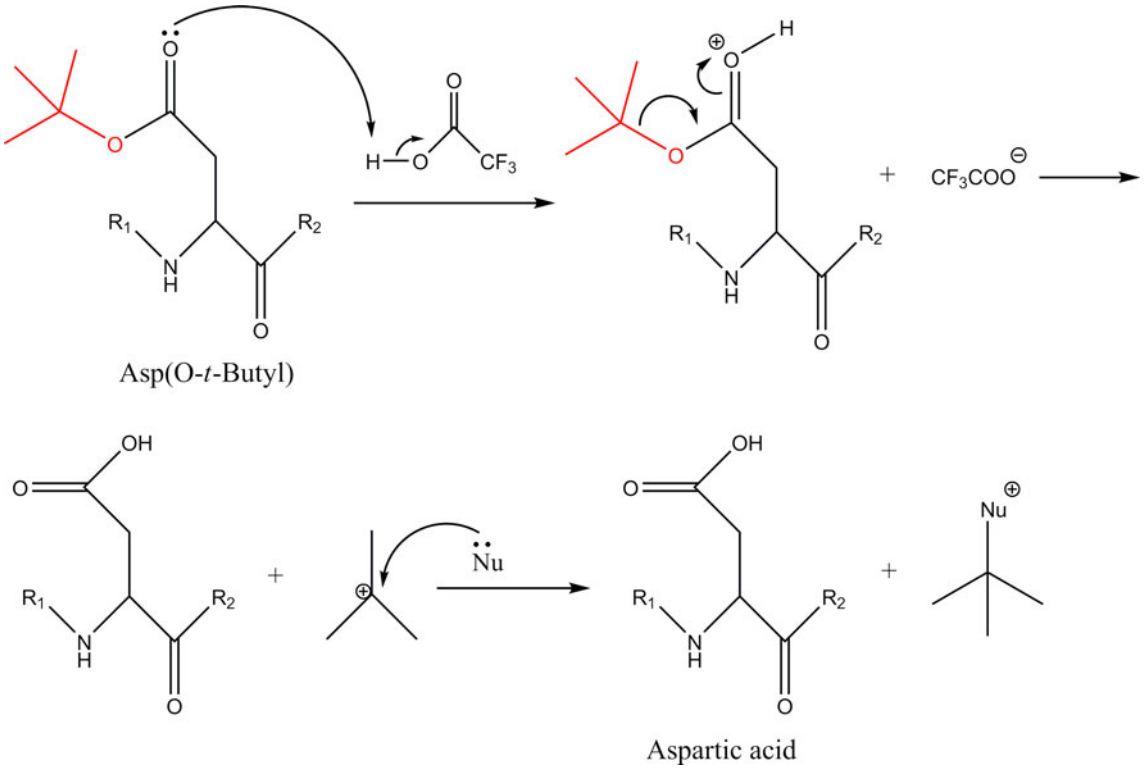


Fig. 17 Removal of O-*t*-butyl group from the protected aspartic acid. In *red*, the group that will be removed. R_1 and R_2 are peptide chains and Nu is a nucleophilic compound present in the cleavage process (Color figure online)

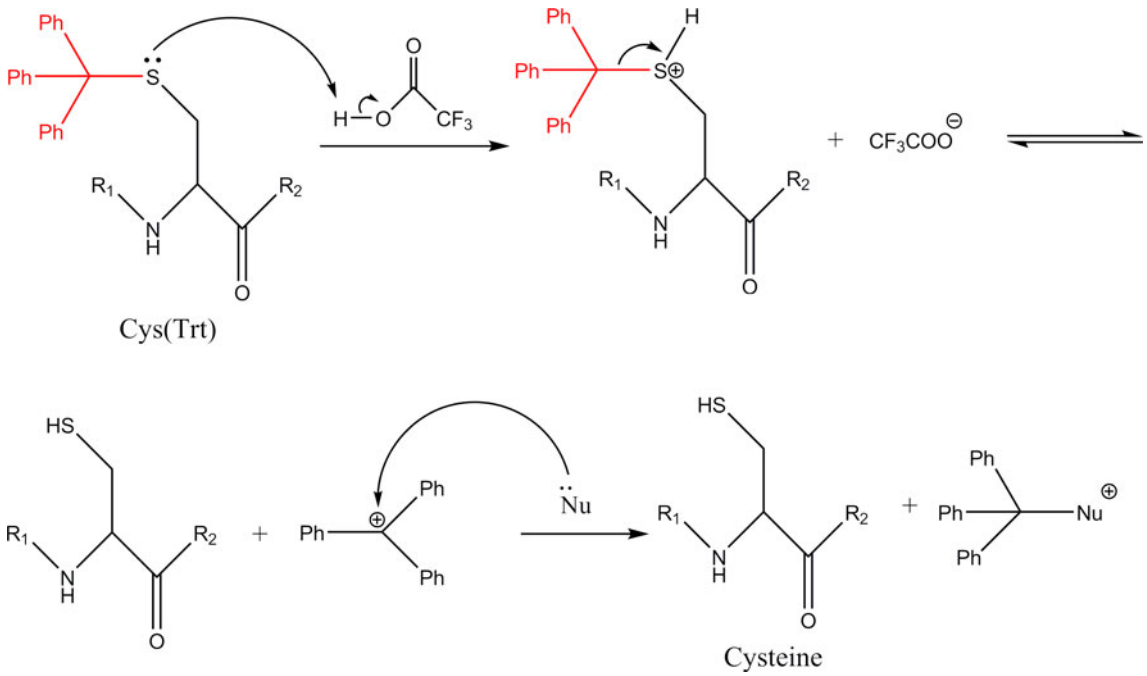


Fig. 18 Removal of Trt group from the protected cysteine. The group that will be removed is represented in *red*. R_1 and R_2 are peptide chains and Nu is a nucleophilic compound present in the cleavage process that scavengers the carbocation (Benoiton 2005) (Color figure online)

v/v) for the removal of this group (as previously described) and making it possible to continue the synthesis and the coupling of the following Fmoc-amino acid. To check if the amino acid residue is without the Fmoc group (i.e. with a free amino group), the ninhydrin test must be held again.

The 20 most common Fmoc-amino acids that can be used on solid-phase synthesis are listed in Table 1, showing also the permanent protecting group (i.e., side chain) and their molecular weight. The side chain permanent protecting group is only removed in the final cleavage step that is discussed in the next session. If the side chain protecting group remains bound to the peptide, the mass addition can easily be detected by mass spectrometry (Table 1).

A scheme for coupling an Fmoc-amino acid to another amino acid residue already attached to the resin is shown in Fig. 11.

Cleavage

After performing the coupling of all Fmoc-amino acids of the peptide sequence, the next step is the process of cleavage for removing the resin and the side chains protecting groups (Fig. 2) and thus obtain the desired peptide, free of any protecting group or structural modification, except for special cases when a side chain protected peptide is necessary, as in the fragment condensation, orthogonal protection or modification and folding, among others. Our discussion will be focused on the methods applied to acid-labile linkers compatible with the Fmoc strategy. For the cleavage and removal of a permanent protecting group, the experimental procedure consists of adding to a tube the peptidyl-resin and a solution containing trifluoroacetic acid (TFA) in the presence of carbocation scavengers. When the peptide contains side chains prone to alkylation, such as sulfhydryl, thioether, indole and phenol, the use of a mixture of different nucleophilic scavengers is recommended (King et al. 1990). Examples of popular mixtures are 81.5 % TFA, 5 % thioanisole, 5 % phenol, 5 % water, 2.5 % ethanedithiol (EDT), and 1 % TIS (by volume) or 92.5 % TFA, 2.5 % water, 2.5 % TIS and 2.5 % EDT (Fields and Noble 1990; Chan and White 2000; Benoiton 2005). The mixture is allowed to react under stirring for 90 min. After completing the reaction, nitrogen gas is bubbled into the solution to evaporate most of the solvents. With positive pressure via nitrogen, the cleaved peptide is then washed, preferably with cold diisopropyl ether. Then, the peptide is extracted with an aqueous solution of acetonitrile (50 % by volume + 0.1 % TFA, for keeping the peptide under slight acidic condition) and freeze-dried. Upon lyophilization, the synthetic peptide can be characterized by mass spectrometry (Karas and Hillenkanp 1988; Fen et al. 1989; Biemann and Papayannopoulos 1994).

The process of cleavage by TFA leads to the formation of carbocations that are highly reactive intermediates. They may specifically react with side chains of tyrosine,

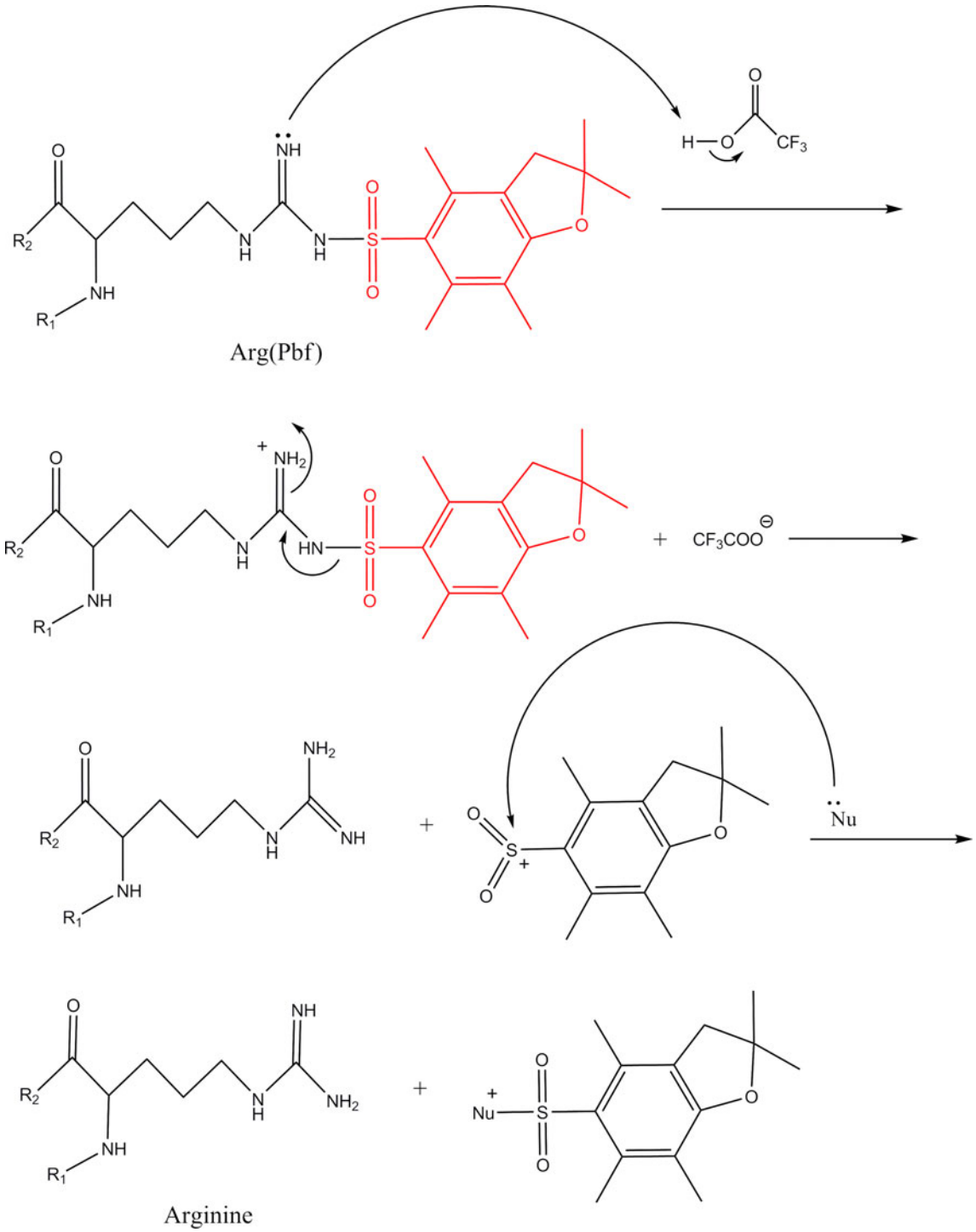


Fig. 19 Removal of Pbf group from the protected arginine. In *red*, the group that will be removed. R_1 and R_2 are peptide chains and Nu is a nucleophilic compound present in the cleavage process (Carpino et al. 1993) (Color figure online)

tryptophan, methionine and cysteine (which are rich in electrons) generating undesirable products (Chan and White 2000). To prevent this, good nucleophilic species are added to the cleavage reaction, such as thioanisole (methylsulfanylbenzene) and 1,2-ethanedithiol (EDT) (Chan and White 2000), whose structures are shown in Fig. 12. The reaction mechanism for the cleavage by acidolysis of a Rink-amide type peptidyl-resin linkage is shown in Fig. 13.

Fmoc-amino acids such as serine, threonine, and cysteine derivatives, among others, have not only alkyl groups in their side chain but also contain reactive functional groups that need permanent protection (Fig. 2) in order to

prevent unwanted reactions in these groups throughout the synthesis. These protecting groups must be removed in this final process of cleavage.

The cleavage of *t*-butyl and Boc protecting groups (Fig. 2) leads to the formation of *t*-butyl cations by a S_N1 reaction. The proposed mechanisms are shown in Figs. 14 and 15, respectively. The t-butyl carbocation is one of the most stable among common alkyl or aryl groups (March 1992). Thus, despite still being an transient specie, it has a sufficiently long lifetime to alkylate electron-rich peptide moieties. This means these carbocations may react with tryptophan, leading to its alkylation (Fields and Noble 1990). To avoid this reaction, EDT is added to the cleavage process to sequester *t*-butyl cations. To completely prevent alkylation of tryptophan, thioanisole (methylsulfanylbenzene) is also very efficient and thus it is employed as a second scavenger (Fields and Noble 1990).

The cleavage of the Trt group from cysteine is reversible without the presence of carbocation receptors (Fields and Noble 1990). Triphenylmethyl (or Trt) is a well-studied carbocation that has a non-planar structure with a central carbon with a high charge density accordingly to its NMR carbon chemical shift data (March 1992). To avoid this reversibility, phenol and triisopropylsilane (tri(propan-2-yl)silicon, TIS (structure shown in Fig. 16) are added to the cleavage process (Fields and Noble 1990). The mechanisms for the removal of the protecting groups *O*-*t*-butyl, Trt and Pbf are shown in Figs. 17, 18 and 19, respectively.

Although (Carpino et al. 1993) gives evidences of a S_N1 reaction for the removal of Pbf group, Han et al. (1996) suggests that some scavengers are necessary to remove this group, indicating a possibility of a S_N2 reaction.

All the mechanisms showed before for the process of the cleavage for the removal of the side-chain protecting groups (Figs. 13, 14, 15, 17, 18, 19) involves the acceptance of the acidic proton from TFA by the most basic electron-pair of the protected amino acid. This leads to the elimination of the protecting group as a highly reactive carbocation, which promptly reacts with the nucleophiles scavenger present in the reaction medium (e.g. EDT, thioanisole and TIS).

Conclusion

SPPS is the method of choice to introduce non-natural peptides.

This paper presents all the stages of the synthesis using the Fmoc strategy, while also addressing the mechanisms involved in each step, which can enable a better understanding of this process. This short review can also be used in basic organic chemistry courses for biology students, contextualizing and exemplifying some organic reactions in the synthesis of peptides.

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Conflict of interest The authors declare that they have no conflict of interest.

Compliance With Ethics This article does not contain any studies with human or animal subjects.

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