BIOMOLECULES
SEM-5, CC-12
PART-9, PPT-29

Part-9: Peptides-VI, Synthesis-III

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BIOMOLECULES (PART-9, PPT-29)

Peptides-VI, Synthesis-III

Solid-Phase Peptide Synthesis (SPPS)

R. B. Merrifield has introduced the 'solid-phase' method (1964) in which an amino acid or a peptide is bound chemically to an insoluble synthetic resin and then the chain is built up, one amino acid at a time (stepwise peptide synthetic method), at the free end. When the desired peptide has been synthesized, it is liberated from the solid support. The method works so well that the method has been automated, i.e., each addition of the appropriate amino acid is carried out automatically at a predetermined rate.

In this method, the carboxy-terminal amino acid is covalently anchored to an *insoluble* polymer (resin), and the peptide is "grown" by adding one amino acid residue at a time to this polymer. SPPS hinges on synthesis of the peptide residue by residue while one end of the peptide remains attached to an insoluble plastic bead. Protecting groups and other reagents are still necessary, but because the peptide being synthesized is anchored to a solid support, by-products, excess reagents, and solvents can simply be rinsed away between each synthetic step without need for intermediate purification.

Solutions containing the appropriate reagents are shaken with the polymer (resin). At the conclusion of each step, the polymer containing the peptide is simply filtered away from the solution, which contains soluble by-products and impurities. The completed peptide is removed from the polymer by a reaction that breaks its bond to the resin. The advantage of this method is the ease with which the peptide is separated from soluble by-products of the reaction. After the very last step the polypeptide is cleaved from the polymer support and subjected to a final purification by HPLC.

Solid-phase peptide synthesis begins with attachment of the first amino acid by its carboxyl group to the polymer bead, usually with a linker or spacer molecule in between. Each new amino acid is then added by formation of an amide bond between the *N*-terminal amino group of the peptide growing on the solid support and the new amino acid's carboxyl group as shown in Figure 1.

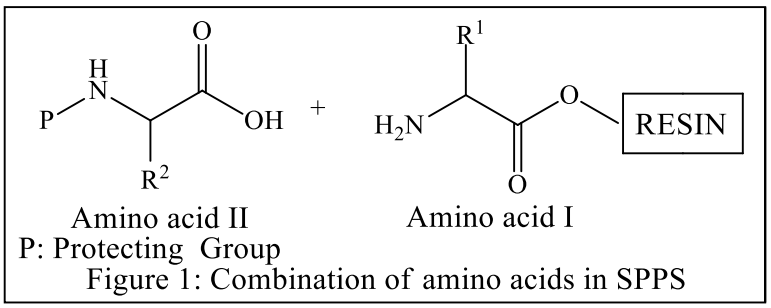


Figure 1: Combination of amino acids in SPPS

Dicyclohexylcarbodiimide (DCC) or diisopropylcarbodiimide (DIC) is used as the amide bond-forming reagent (condensing agent). To prevent undesired reactions as each new residue is coupled; a protecting group is used to block the amino group of the residue being

added. Once the new amino acid has been coupled to the growing peptide and before the next residue is added, the protecting group on the new *N*-terminus is removed, making the peptide ready to begin the next cycle of amide bond formation.

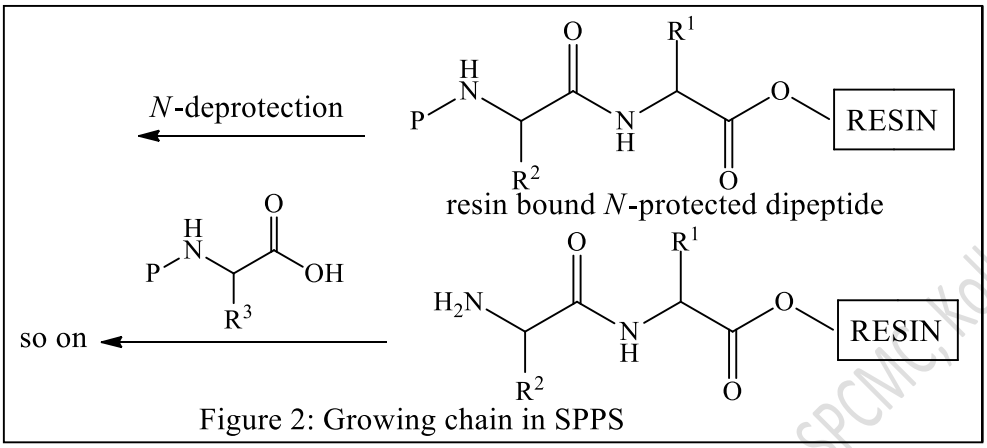


Figure 2: Growing chain in SPPS

The great advantage of solid-phase peptide synthesis is that purification of the peptide at each stage involves simply rinsing the beads of the solid support to wash away excess reagent, by-products, and solvents. Furthermore, having the peptide attached to a tangible solid during the synthesis allows all of the steps in the synthesis to be carried out by a machine in repeated cycles. Some outstanding advantages of this solid phase method are:

1. Because of the use of the insoluble solid support, purification of products is not necessary, excess of reagents being removed by thorough washing with suitable solvents;
2. High yields;
3. The time has been considerably shortened for synthesizing peptides (and proteins).

The resin is a copolymer of styrene and divinylbenzene (about 2%) are chloromethylated using chloromethyl methyl ether and tin (IV) chloride. It contains about 10% of the aromatic rings with -CH_2Cl groups. This results in the formation of 'benzyl chloride groups' through which the 'first' amino acid becomes attached as the benzyl ester.

The growing peptide is anchored to this polymer, and excess reagents, impurities, and byproducts are removed by thorough washing after each operation. This greatly simplifies the purification of intermediates. Figure 3 shows a section of polystyrene with one of the benzene rings modified by chloromethylation. Individual polystyrene chains in the resin used in solid-phase peptide synthesis are connected to one another at various points (cross-linked) by adding a small amount of *p*-divinylbenzene to the styrene monomer.

The chloromethylation step is carried out under conditions such that only about 10% of the benzene rings bear -CH_2Cl groups.

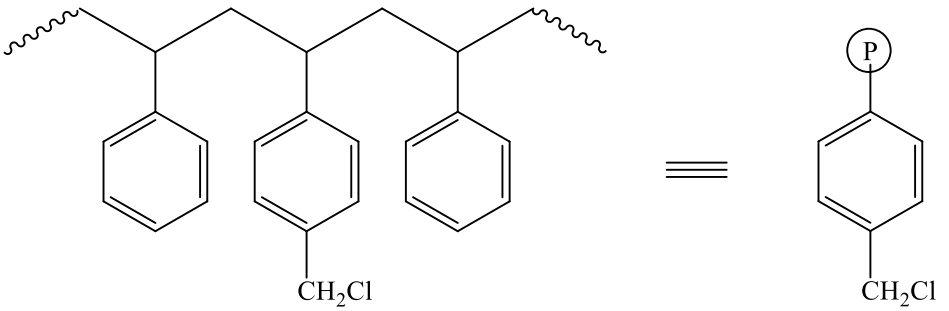
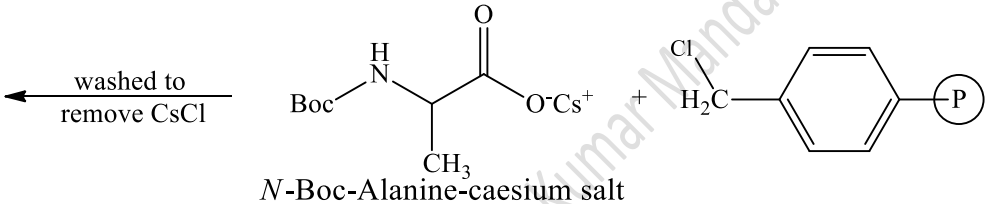


Figure 3: A section of Merrifield resin

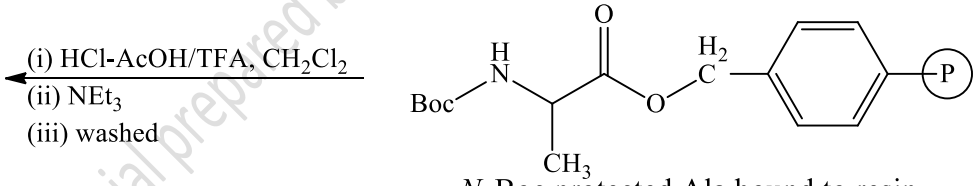
Figure 4 illustrates the peptide synthesis by the solid-phase method using *t*-butyloxycarbonyl (Boc) as the *N*-protecting group. Amino acid residues are then attached sequentially beginning at the *C*-terminus.

Step 1: The Boc-protected amino acid is anchored to the resin. Nucleophilic substitution of the benzylic chloride by the carboxylate anion gives an ester.



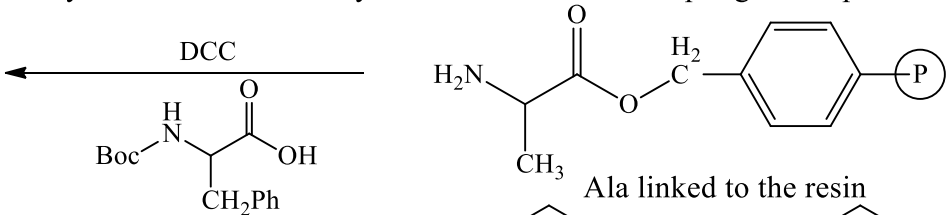
N-Boc-Alanine-caesium salt

Step 2: The Boc protecting group is selectively removed by treatment with hydrochloric acid in dilute acetic acid and the hydrochloride of the amino group is converted into the free-amino group by the addition of excess of triethylamine. After the resin has been washed, the *C*-terminal amino acid is ready for coupling.



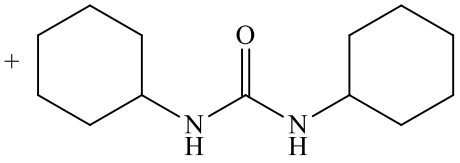
N-Boc protected Ala bound to resin

Step 3: The resin-bound *C*-terminal amino acid is coupled to an *N*-protected amino acid by using *N*, *N*-dicyclohexylcarbodiimide (DCC). Excess reagent and *N*,*N*-dicyclohexylurea are washed away from the resin after coupling is complete.



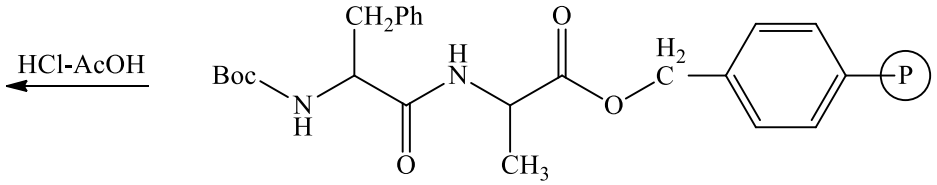
N-Boc protected Phe

Ala linked to the resin



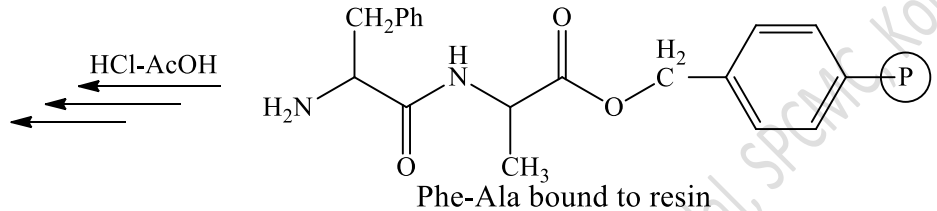
N,*N*-dicyclohexylurea

Step 4: The Boc protecting group is removed as in step 2. if desired, steps 3 and 4 may be repeated to introduce as many amino acid residues as desired.

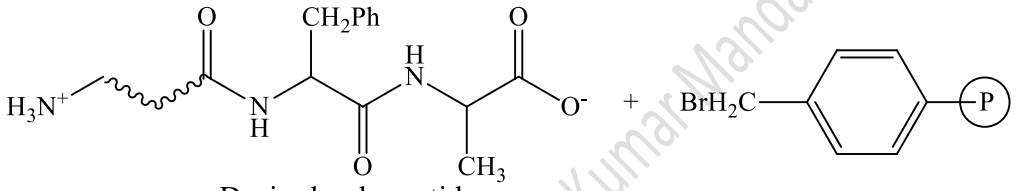


N-Boc protected Phe-Ala linked to the resin

Step *n*: When the desired peptide completely assembled, it is removed from the resin by treatment with dry hydrogen bromide in trifluoroacetic acid.



Phe-Ala bound to resin



Desired polypeptide

Figure 4: Peptide synthesis by the solid-phase method

Although Merrifield's initial method for solid-phase peptide synthesis used the Boc (*t*-butyloxycarbonyl) group to protect the *α*-amino group of residues being coupled to the growing peptide, several advantages of the Fmoc (9-fluorenylmethoxycarbonyl) group have made it the group of choice. The reasons have mainly to do with excellent selectivity for removing the Fmoc group in the presence of other protecting groups used to block reactive side chains along the growing peptide and the ability to monitor the progress of the solid-phase synthesis by spectrophotometry as the Fmoc group is released in each cycle.

Let us discuss the choice of protecting groups further. As noted*basic conditions* (piperidine in DMF) are used to remove the Fmoc group. On the other hand, protecting groups for the side chains of the peptide residues are generally blocked with *acid labile* moieties. The base-labile Fmoc groups and acid-labile side-chain protecting groups are said to be orthogonal protecting groups because one set of protecting groups is stable under conditions for removal of the other, and vice versa.

Another advantage of Fmoc as compared to Boc groups for protecting the *α*-amino group of each new residue is that repetitive application of the acidic conditions to remove Boc groups from each new residue slowly sabotages the synthesis by prematurely cleaving some peptide molecules from the solid support and deprotecting some of the side chains. The basic conditions for Fmoc removal avoid these problematic side reactions.

Use of Protecting Groups and the Use of Active Esters in SPPS

The purpose of an amino protecting group is to block an amine from reacting as a nucleophile at some point in the synthesis. The amino protecting group that can be used in SPPS is the (9-fluorenyl)methyloxycarbonyl group, known as the Fmoc group. *Active esters* are more reactive toward nucleophiles than ordinary alkyl esters. One widely used active ester is *N*-hydroxysuccinimide (NHS). This compound has N-O bond. This "alcohol" has very low p$K_a$value that is closer to the pK_a values of carboxylic acids than ordinary alcohols.

Weak bases generally are good leaving groups in carbonyl substitution reactions, just as they are in S_N2 reactions, because the same property that makes them weak bases - their electron-withdrawing character-stabilizes the transition state for substitution.

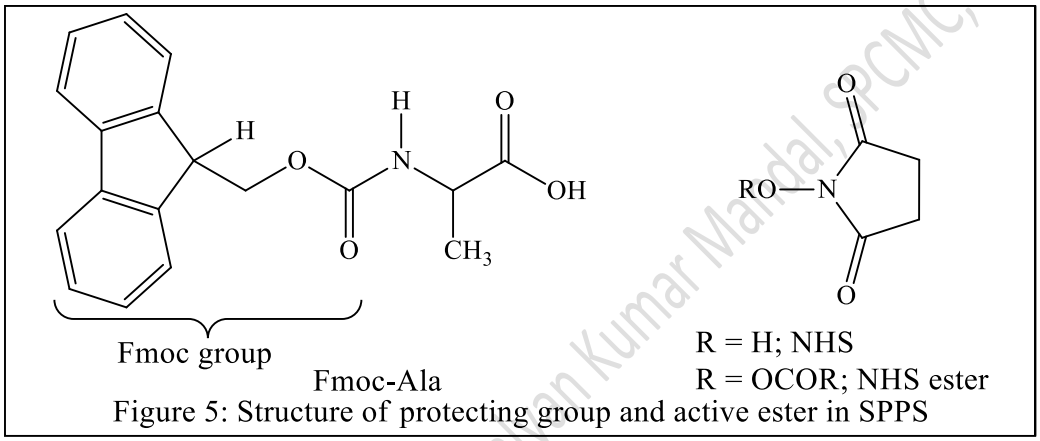


Figure 5: Structure of protecting group and active ester in SPPS

NHS ester is much more reactive in nucleophilic substitution reactions than alkyl esters. For example, an Fmoc-protected alanine and an active ester have the structures shown in Figure 5. This reaction illustrates the superior leaving-group properties of the NHS group. Fmoc-NHS is a carbonate ester; potentially, two alcohols could be leaving groups. Because of its low p*K*a, NHS is a much better leaving group than (9-fluorenyl)methanol.

SPPS using Fmoc Protecting Group

Consequently, the NHS group is displaced and the (9-fluorenyl)-methyl group remains intact as a carbamate-ester protecting group.

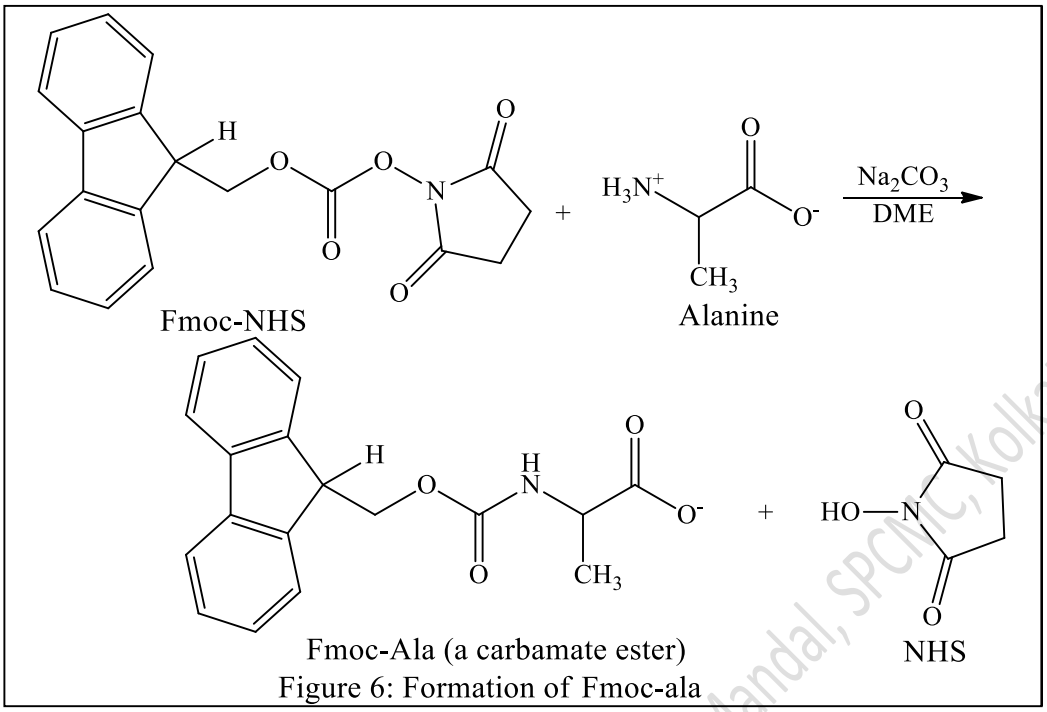


Figure 6: Formation of Fmoc-ala

The Fmoc-Ala is anchored onto an insoluble solid polymeric support, called a *resin*, using the reactivity of its free carboxylate group (Figure 7). It's enhanced reactivity is generally associated with benzylic halides. An S_N2 reaction between the cesium salt of Fmoc-Ala and the chloromethyl group of the resin results in the formation of an ester linkage to the resin by alkylation of the carboxylate ion.

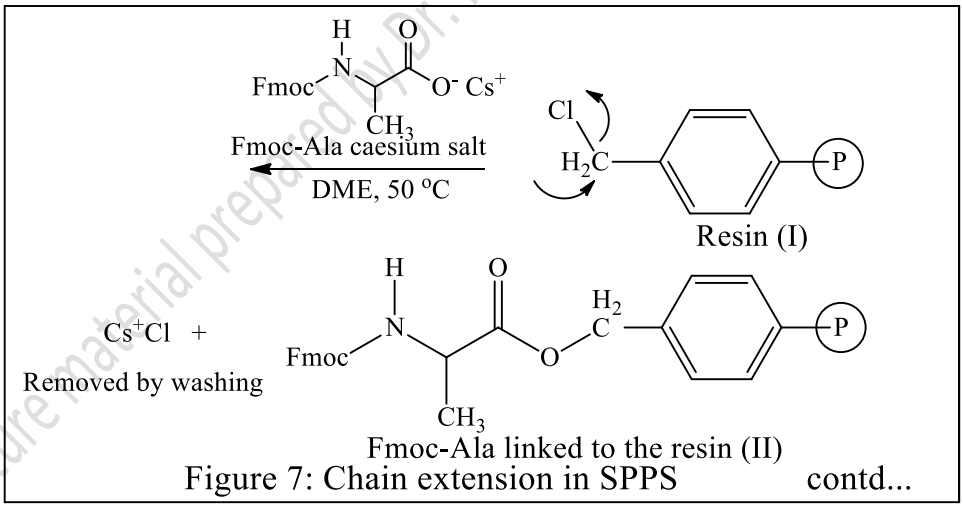


Figure 7: Chain extension in SPPS contd...

The role of the Fmoc protecting group in this reaction is to keep the amino group from competing with the carboxylate group as a nucleophile for the benzylic halide group on the resin. Once the Fmoc-amino acid is anchored to the resin, the Fmoc protecting group is removed by treatment with piperidine, an amine base (Figure 8).

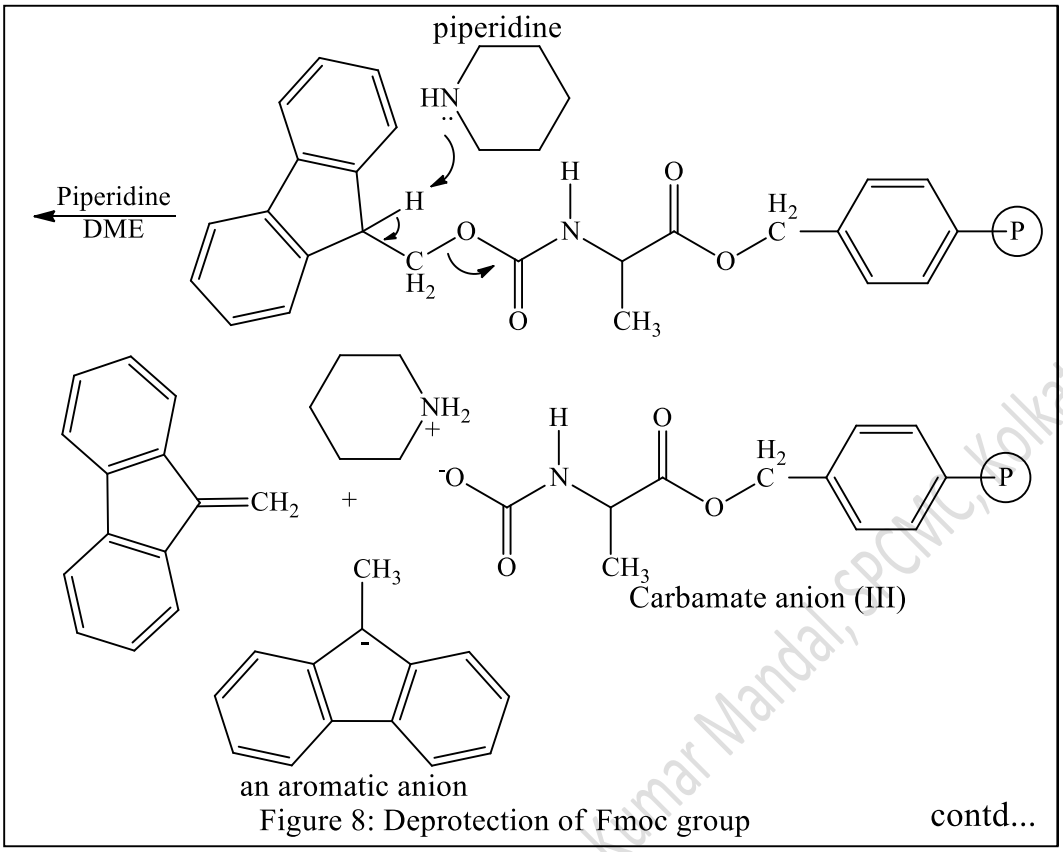
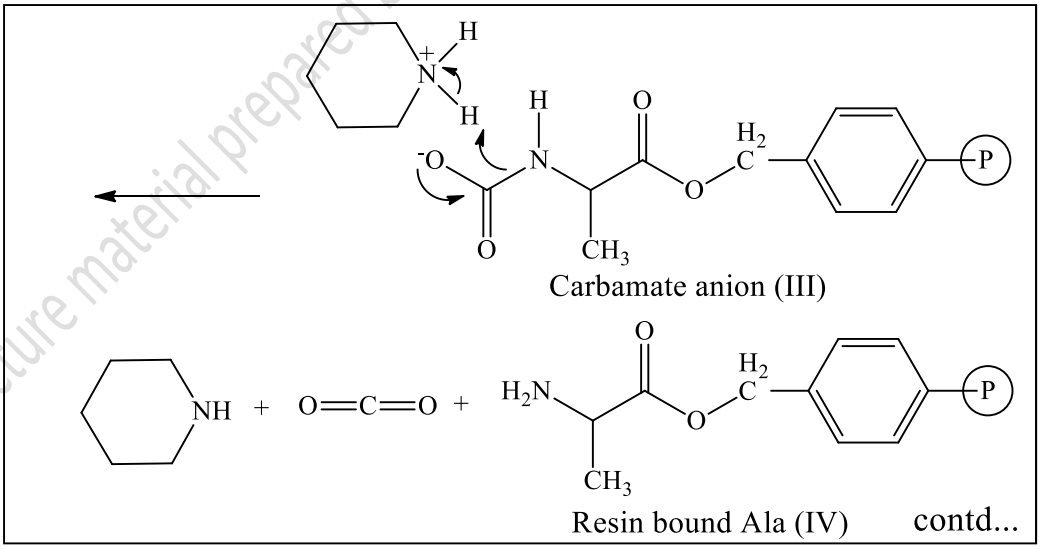
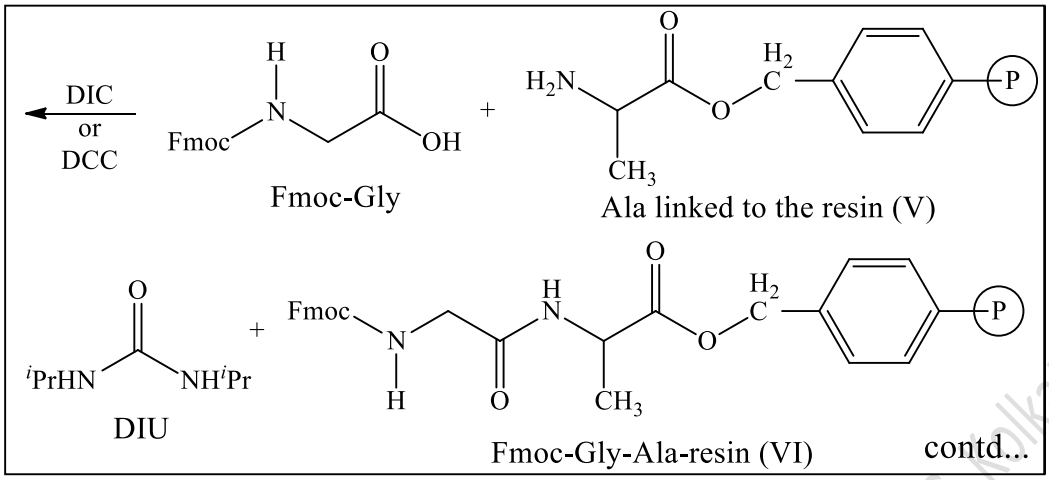


Figure 8: Deprotection of Fmoc group

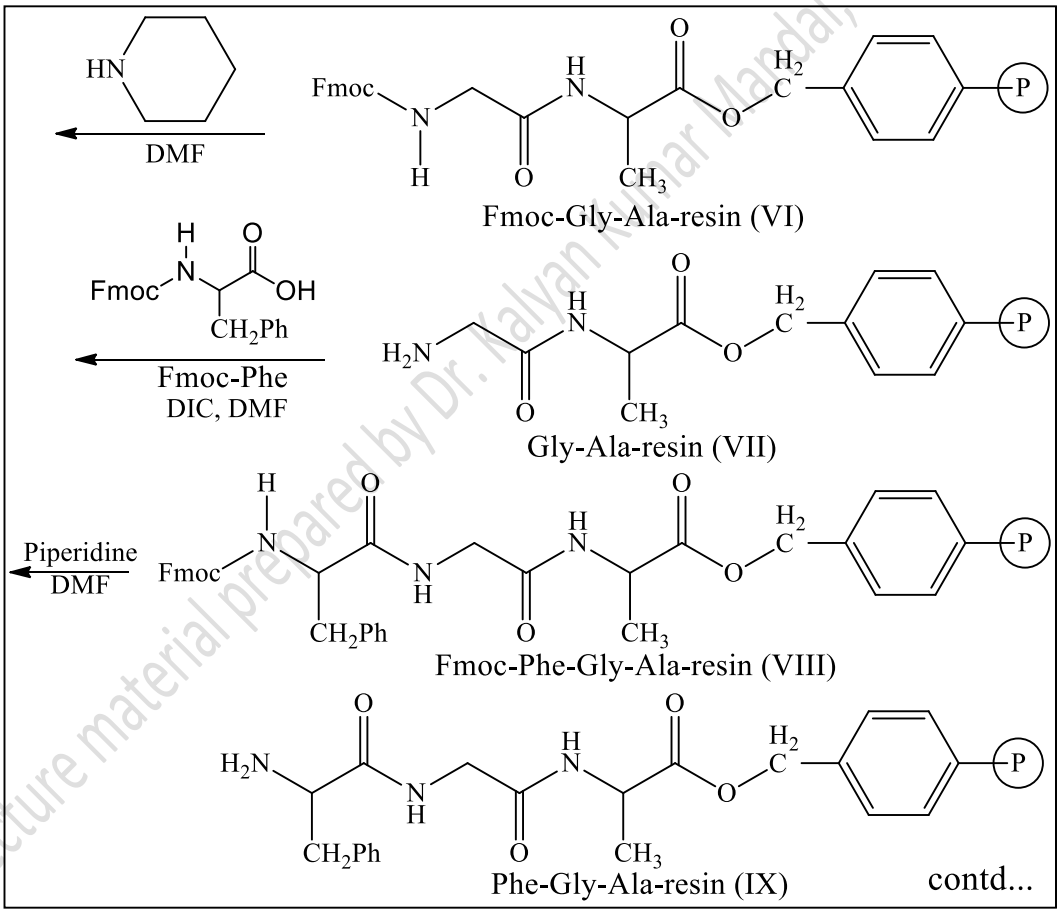
This is an E2 reaction. The *β*-hydrogen of this group (Fmoc) is particularly acidic because the anion that would be formed by removal of this hydrogen as a proton is *aromatic* and therefore particularly stable. Because the product of the *β*-elimination is a carbamate anion (III), it decarboxylates under the reaction conditions.



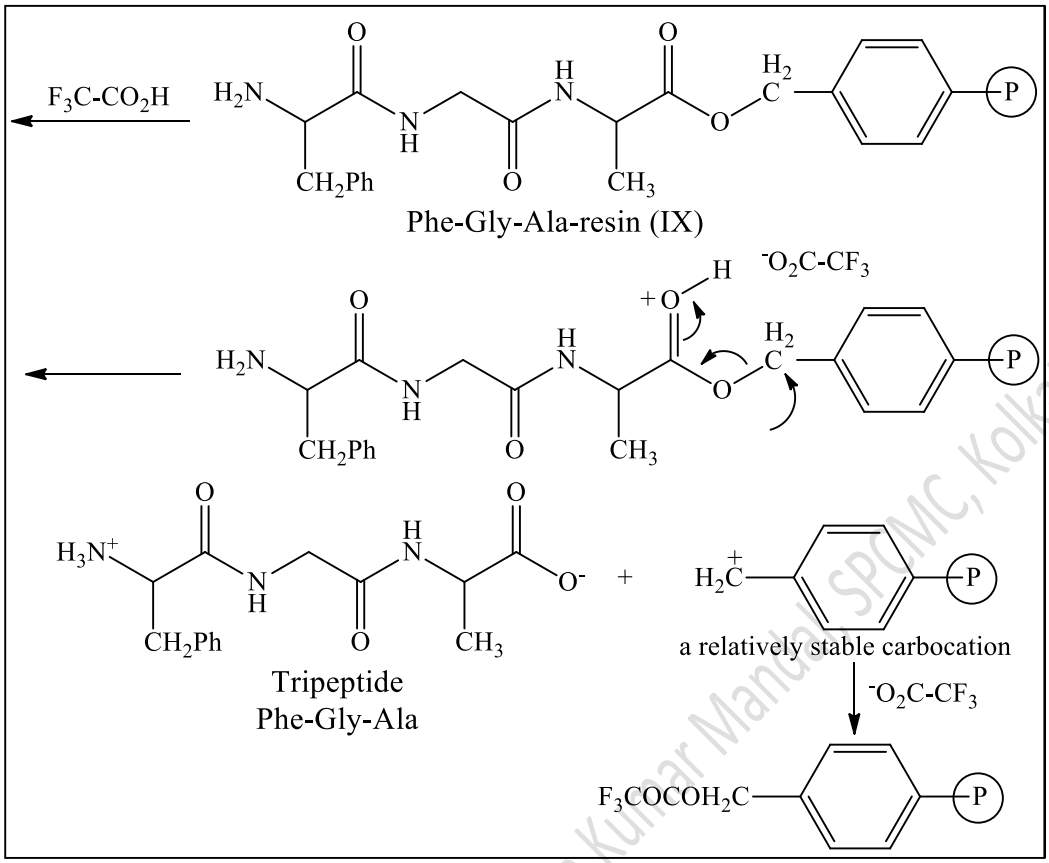
This reaction exposes the amino group of the resin-bound amino acid. This amino group serves as a nucleophile in the next reaction. Next comes the formation of the first peptide bond. Coupling of Fmoc-glycine to the free amino group of the resin-bound Ala is effected by the reagent 1,3-diisopropylcarbodiimide (DIC) or DCC.



Completion of the tripeptide synthesis requires deprotection of the dipeptide-resin and a final coupling step with Fmoc-Phe and DIC.



Once all the peptide bonds in the desired tripeptide are assembled, the completed peptide must be removed from the resin. The ester linkage that connects the peptide to the resin, like most esters, is more easily cleaved than the peptide (amide) bonds. The particular ester linkage used in this case is broken by a carbocation mechanism using 50-60% trifluoroacetic acid (TFA) in dichloromethane.



Detachment of Peptides from the Resin

The acidic conditions promote breaking of the ester linkage by an S_N1 mechanism. Protonation of the peptide carbonyl converts this group into a good leaving group because it is the conjugate acid of a very weak base. The S_N1 cleavage yields a benzylic carbocation that is resonance-stabilized, not only by the benzene ring but also by the para oxygen.

In some cases $ClCH_2C_6H_4$- group is attached to the resin bead via a *para*- -OCH_2- group. The reason for inclusion of the *para* -OCH_2- group in the design of the resin is that it accelerates ester cleavage, and thus release of the peptide by acid. As a result of this reaction, the peptide is liberated into solution, from which it can be readily isolated. It is to be noted that the conditions of peptide synthesis and deprotection do *not* affect the ester group by which the peptide is linked to the resin.

Benzylic esters undergo aminolysis very sluggishly with secondary amines such as piperidine because of steric hindrance between the phenyl hydrogens and the amine. Furthermore, the piperidine treatment required for removal of the Fmoc group takes only one minute. This is too brief a time for aminolysis of the ester to occur. However, the ester is cleaved by acidic conditions because of the ease with which it forms a relatively stable carbocation. The method of solid-phase peptide synthesis just discussed, which employs the Fmoc group as the amino-terminal protecting group, is one of two major methods in common use today. The other important method involves a conceptually similar stepwise approach but employs a different protecting group, the *tert*-butoxycarbonyl (Boc) group, which can be removed by anhydrous acid.

Mechanism of *N*-Boc Deprotection

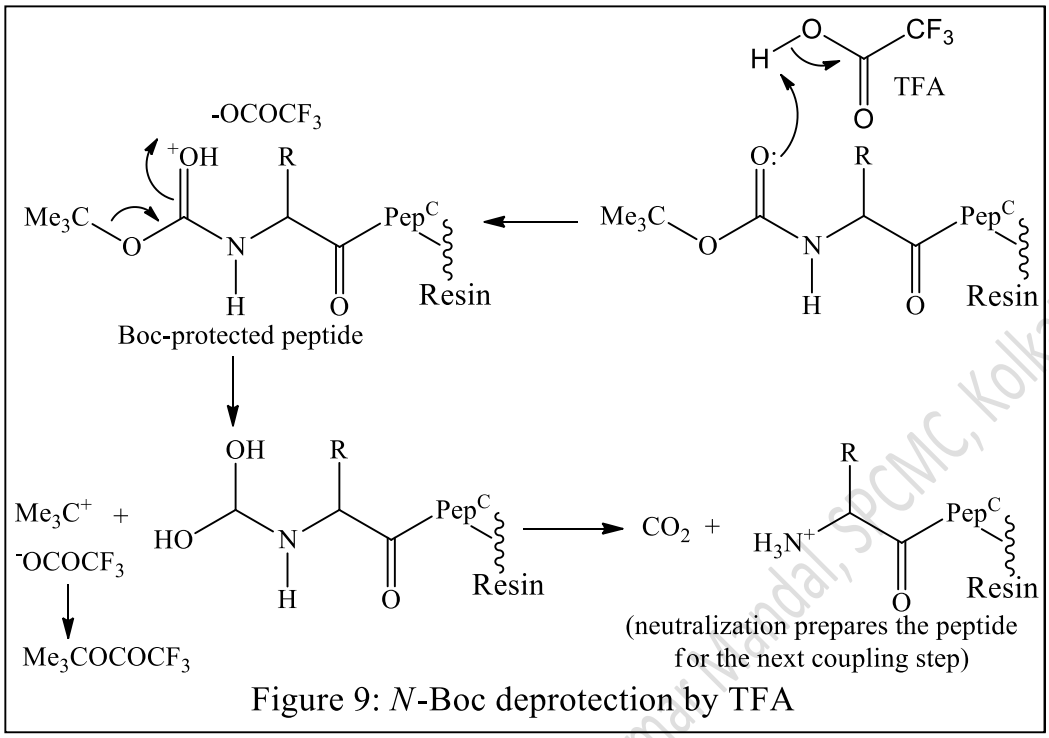


Figure 9: *N*-Boc deprotection by TFA

Deprotection of *N*- and *C*-Terminals

This deprotection scheme relies on the formation of a relatively stable *tert*-butyl cation by an S_N1 mechanism. Because an *acid* (TFA) is used for deprotection, the linkage of the peptide to the resin must be stable to treatment with TFA. Hence, a different type of resin linkage is used when Boc protection is employed. Contrary to the resin used in Fmoc chemistry, there is no *para* oxygen in the resin used in the case when Boc is used as a *N*-protecting group, although both are benzylic esters.

This peptide linkage is therefore much more stable to acid because the carbocation S_N1 cleavage mechanism is less favorable. In fact, liquid HF is required to cleave the peptide from the resin. Although the HF cleavage procedure is relatively simple with the proper apparatus, liquid HF is extremely hazardous.

Problem in Solid-Phase Peptide Synthesis

Despite its advantages, solid-phase peptide synthesis has one unique problem. Suppose, for example, that a coupling reaction is incomplete, or that other side reactions take place to give impurities that remain covalently bound to the resin. These are then carried along to the end of the synthesis, when they are also removed from the resin and must be separated (in some cases tediously) from the desired peptide product.

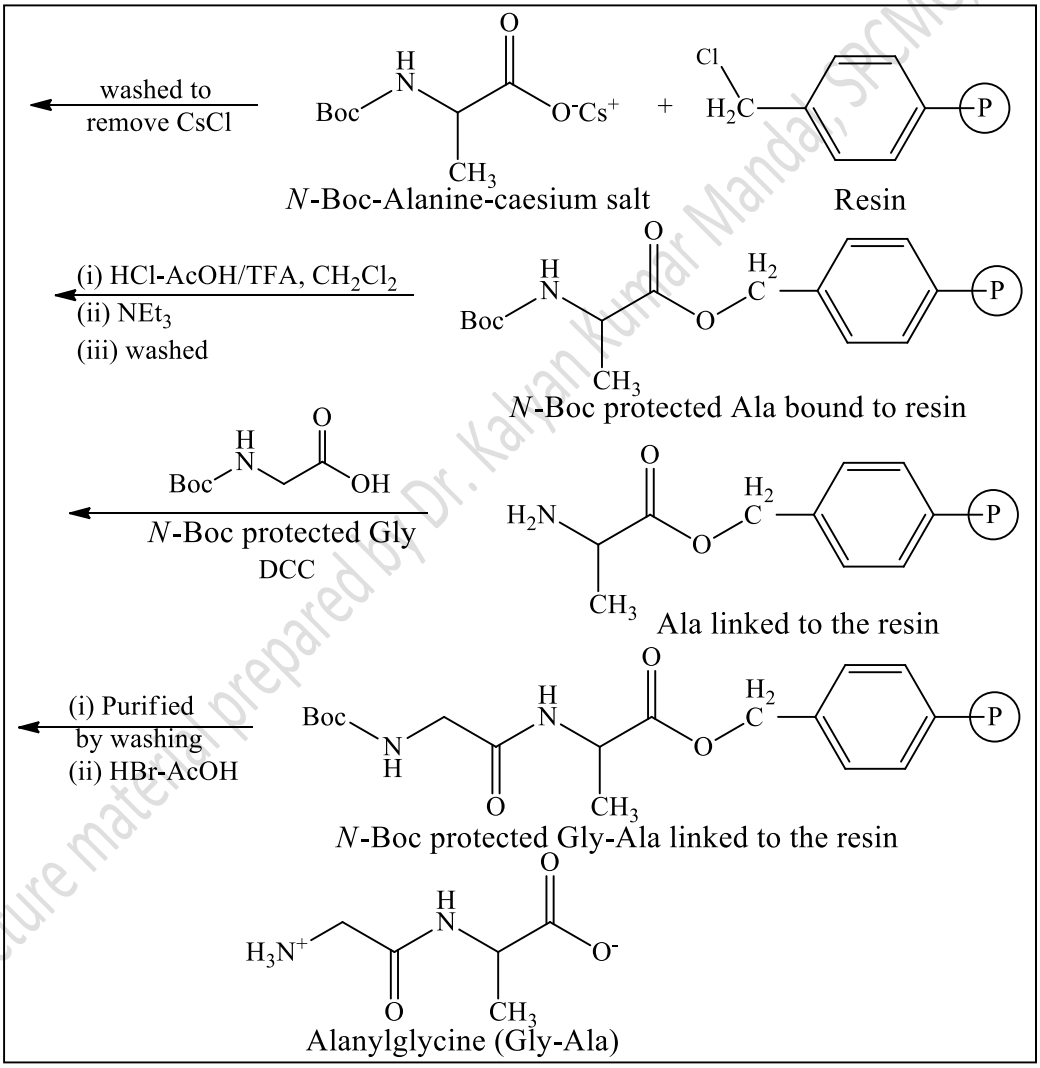
Advantages of Automated Peptide Synthesis

Automated peptide synthesizers are available that can complete one cycle in 40 min and carry out 45 cycles of unattended operation. Though not as efficient as protein synthesis in the body, where enzymes directed by DNA can catalyze assembly of a protein with 150 amino

acids in about 1 min, automated peptide synthesis is highly advantageous from the tedious process of manually synthesizing a peptide step after step.

A hallmark example of automated peptide synthesis was the synthesis of ribonuclease, a protein with 124 amino acid residues. The synthesis involved 369 chemical reactions and 11,930 automated steps - all carried out without isolating an intermediate. The synthetic ribonuclease not only had the same physical characteristics as the natural enzyme, it possessed the identical biological activity as well. The overall yield was 17%, which means that the average yield of each individual step was greater than 99%.

Synthesis of Gly-Ala by SPPS



The development of a procedure by R. B. Merrifield (Rockefeller University, dec. 2005) for automating this process was a breakthrough in peptide synthesis. For this method, Merrifield received the 1984 Nobel Prize in Chemistry.