BIOMOLECULES
SEM-5, CC-12
PART-7, PPT-27

Part-7: Peptide-IV: Synthesis-I, Protecting Groups

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BIOMOLECULES (PART-7, PPT-27)

Synthesis-I, Protecting Groups

Synthesis of Peptides: Methods of Peptide Synthesis

Peptides are linear sequence of amino acid residues. Therefore, principally a peptide can be synthesized sequentially through *stepwise condensation* resulting in the formation of peptide bonds between the carboxylic acid group of one amino acid and amino group of the other amino acid. This stepwise condensation of amino acids is illustrated in Figure 1.

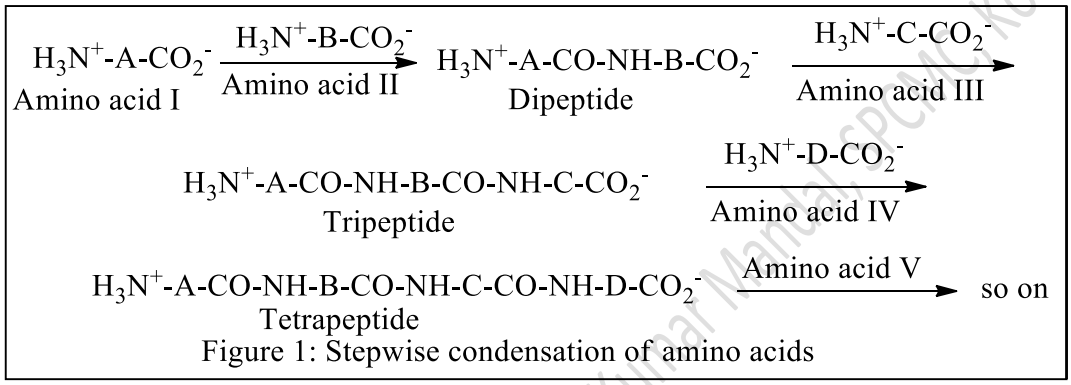


Figure 1: Stepwise condensation of amino acids

Another protocol that may be used in the synthesis of larger peptides is to synthesize small or medium-sized peptide fragments by stepwise condensation, purify them and then join these fragments to make the required larger peptides. This protocol, commonly known as *fragment condensation*, is illustrated in Figure 2.

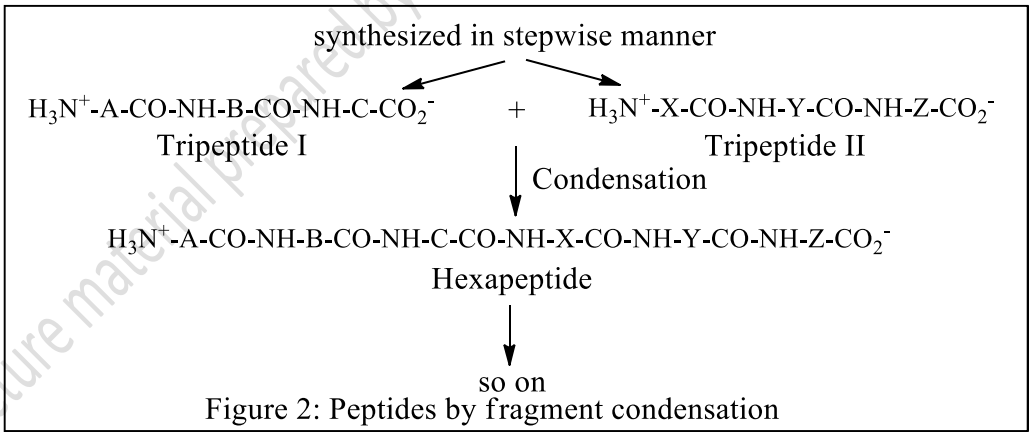


Figure 2: Peptides by fragment condensation

Peptide linkage is actually an amide (-CO-NH-) bond. Therefore, peptide synthesis essentially means the formation of amide bonds. The synthesis of an amide linkage is a relatively simple one. The carboxyl group of an acid is activated by converting it to an anhydride or acid chloride and then allow it to react with an amine. Generally, an amide bond is formed using the technique shown in Figure 3.

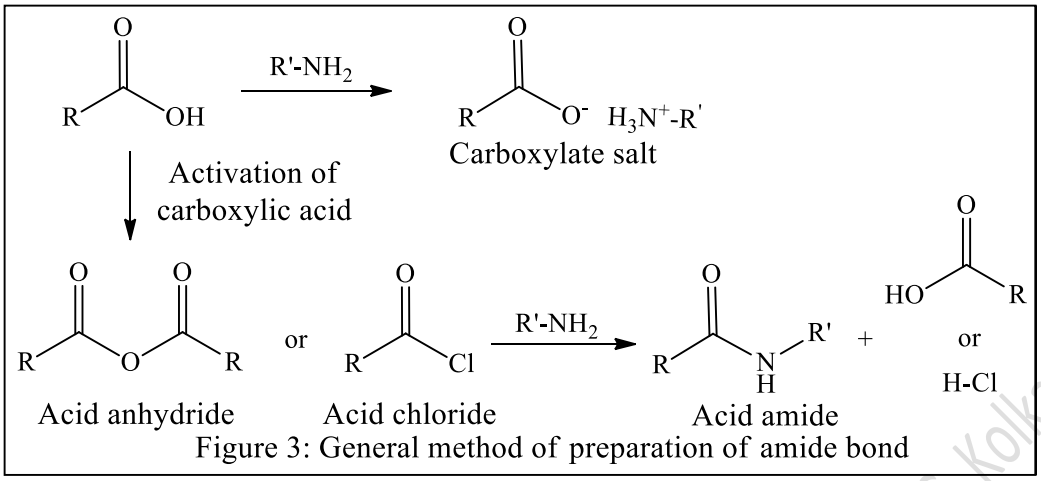


Figure 3: General method of preparation of amide bond

Problems in Peptide Synthesis

The problem starts in peptide bond formation when both the acid (-CO_2H) group and the amino (-NH_2) group are part of the same molecule, as they are in an amino acid, especially when the goal is the synthesis of a naturally occurring polyamide where the sequence of different amino acids is all important. Let us consider, the synthesis of the dipeptide alanylglycine, A-G. The strategy is to activate the carboxyl group of alanine by converting it to an acid chloride, and then allow it to react with glycine. Unfortunately, under this condition it is not possible to prevent alanyl chloride from reacting with itself.

Therefore, the reaction would give not only AG but also AA. It could also lead to AAA and AAG, and so on. The yield of the desired product would be low, and also there is a problem in separating the dipeptides, tripeptides, and higher peptides. The reaction is illustrated in Figure 4.

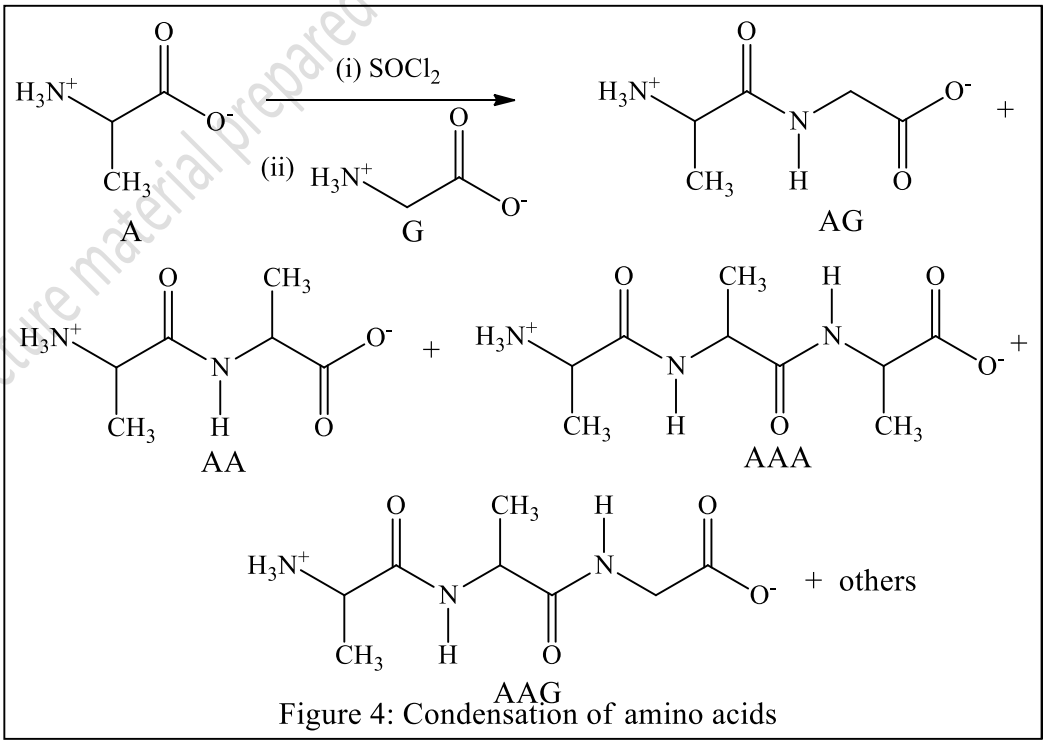


Figure 4: Condensation of amino acids

The general principles may be illustrated by condensation of the synthesis of a dipeptide. It is seen that two different amino acids, L-A (≡ H_3N^+-A-CO_2^-) and L-B (≡ H_3N^+-B-CO_2^-), may be combined in two different ways to give two dipeptides.

Dipeptide I: H_3N^+-A-CO-NH-B-CO_2^-

Dipeptide II: H_3N^+-B-CO-NH-A-CO_2^-

Therefore, the real difficulty lies in ensuring that the correct sequence is obtained. Consequently, to get the desired dipeptide it is necessary to direct the course of the reaction so that only the desired peptide is obtained exclusively. Thus, the objective in peptide synthesis may be simply stated as to connect amino acids in a prescribed sequence by amide bond (-CO-NH-) formation between them.

Planning in Peptide Synthesis

To prepare dipeptide I (H_3N^+-A-CO-NH-B-CO_2^-), the amino group of A must be protected and the carboxyl group of A must be activated so that it readily reacts with the free amino group of B. Similarly, to prepare dipeptide II (H_3N^+-B-CO-NH-A-CO_2^-), the amino group of B must be protected and the carboxyl group of B must be activated so that it readily reacts with the free amino group of A. Therefore, peptide synthesis generally involves the following steps:

Step I: Protection of *N*-T-AA residue of amino acid I
Step II: Activation of *C*-T-AA residue of amino acid I
Step III: Protection of *C*-T-AA residue of amino acid II
Step IV: Condensation of two residues
Step V: Deprotection of *N*-, and *C*-terminals to get the desired dipeptide

Figures 5 illustrates the synthesis of AB type dipeptide (I) using P and R as the protecting groups and Z as the activating group.

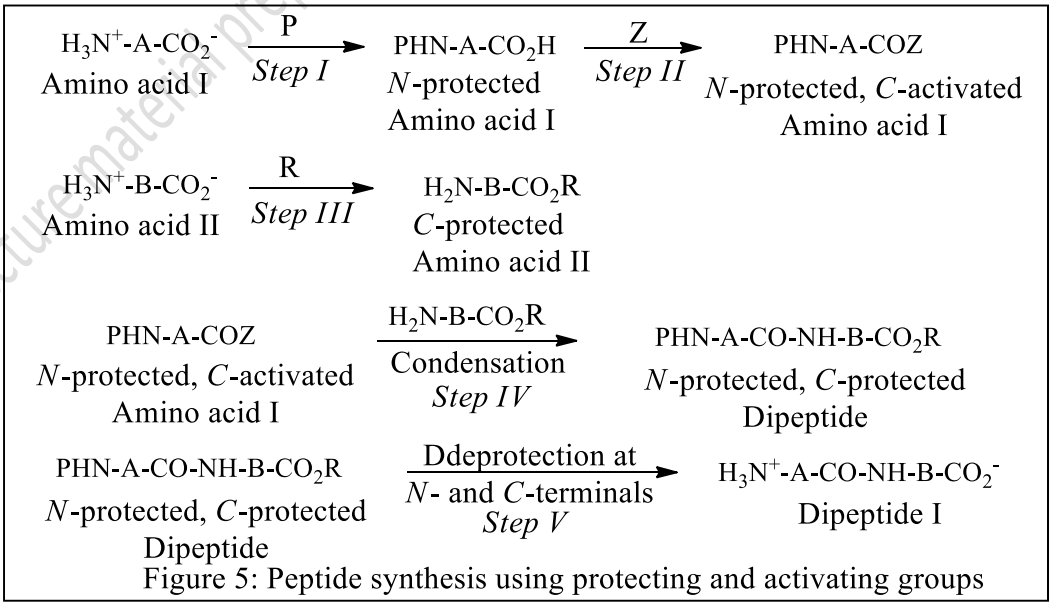


Figure 5: Peptide synthesis using protecting and activating groups

Figures 6 illustrates the synthesis of BA type dipeptide (II) using and R as the protecting groups and Z as the activating group.

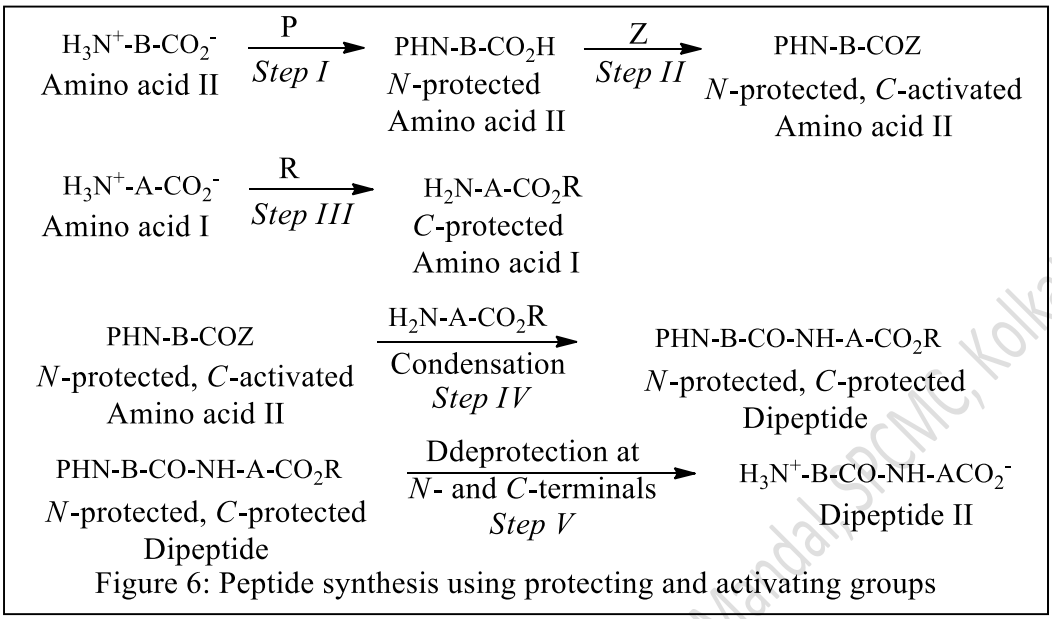


Figure 6: Peptide synthesis using protecting and activating groups

To extend the length of the peptide chain, one of the protecting groups in the dipeptide is selectively removed and the peptide is built up from this end. Thus, a peptide chain may be extended, one amino acid residue at a time, from either end (*N*- or *C*-) of its precursor, as illustrated in Figure 7.

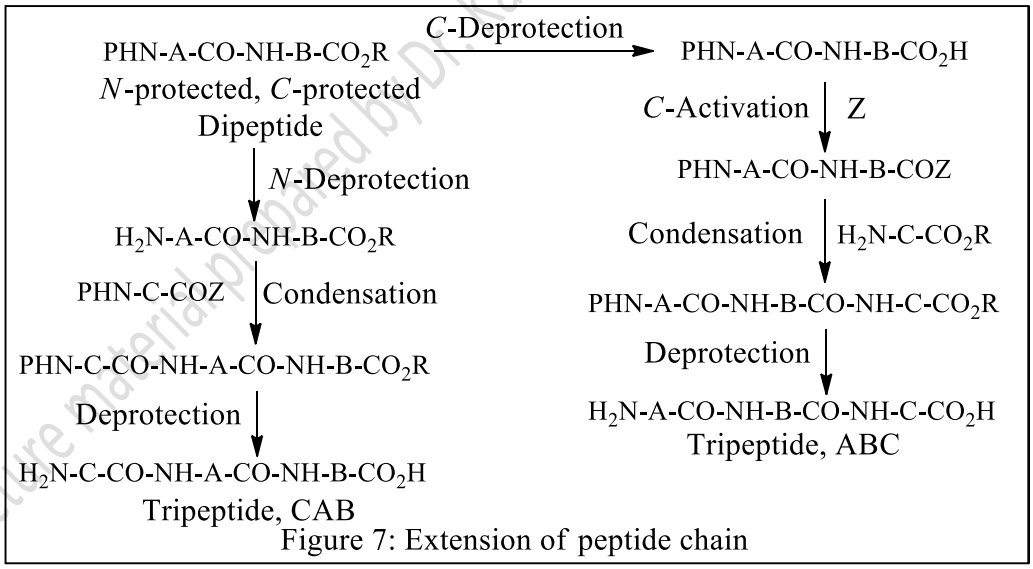


Figure 7: Extension of peptide chain

On the other hand, a number of suitable simple peptides may be synthesized, these then linked together to give the required protected peptide (or protein), from which the protecting groups are finally removed. The pictorial representation of the synthesis of a dipeptide and longer peptides are shown in Figure 8.

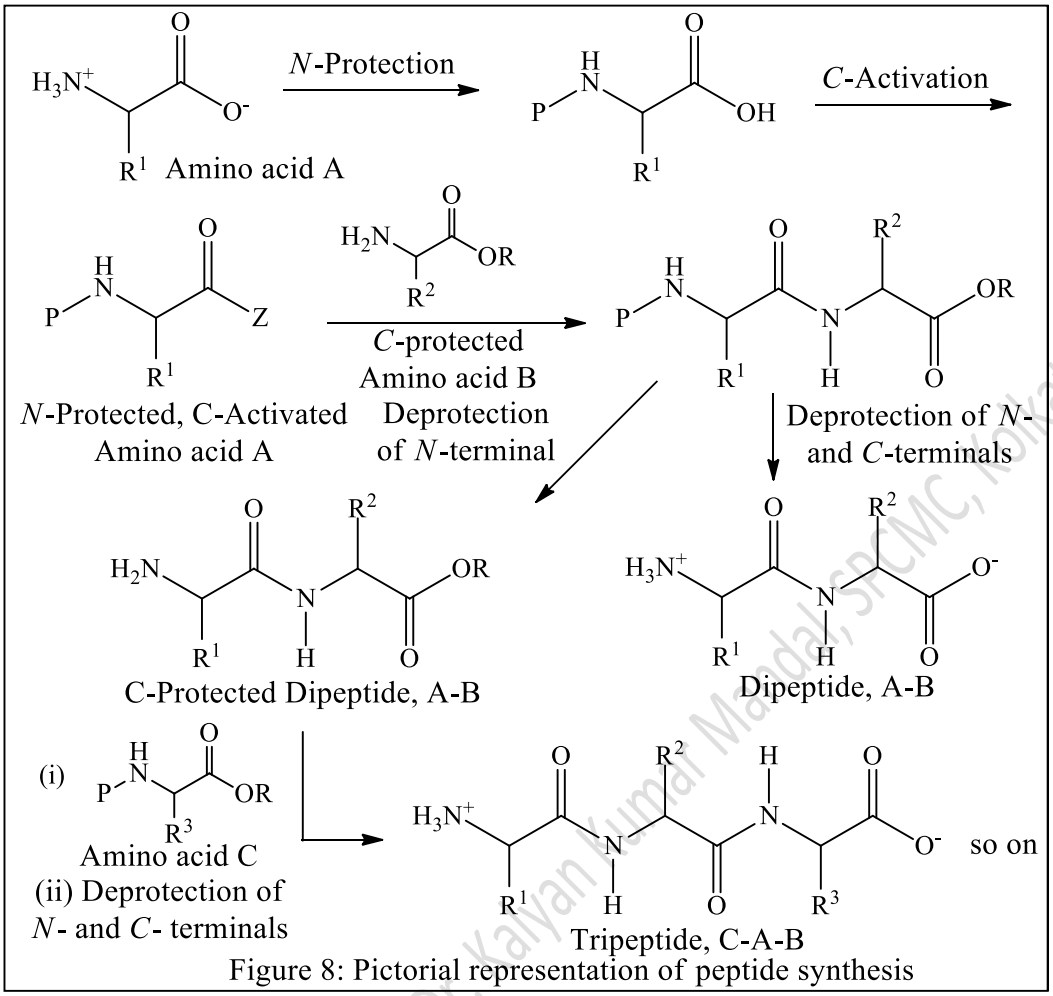


Figure 8: Pictorial representation of peptide synthesis

One other point that requires consideration in the peptide synthesis is that if the amino acid side chain contains reactive groups, these must be protected. Such reactive groups are, e.g., amino (lysine), carboxyl (aspartic acid, glutamic acid), hydroxyl (tyrosine), thiol (cysteine). Since peptide synthesis involves protection of reactive *N*- and *C*-terminals (and also reactive side chains), it is necessary to use protecting groups which can be selectively removed one at a time.

Criteria of a good protecting group: It is also important that protecting groups should be easily introduced and should be removable under sufficiently mild conditions that the peptide bond is not hydrolyzed and that no racemization or rearrangement occur during the course of the reaction.

N-Protecting Groups in Peptide Synthesis

The solution to the problem of getting undesired peptide is to "protect" the amino group of the first amino acid before activating it and allow it to react with the second. By protecting the amino group, it means that the said group is converted it to some other group of low nucleophilicity - *one that will not react with a reactive acyl derivative.* The protecting group must be carefully chosen because after the synthesis of the amide linkage between the first

amino acid and the second, the protecting group must be able to remove without disturbing the new amide bond.

A number of reagents have been developed to meet these requirements. Three that are often used are benzyl chloroformate, di-*tert*-butyl dicarbonate (sometimes abbreviated Boc_2O, where Boc stands for *tert*-butyloxycarbonyl), and 9-fluorenylmethyl chloroformate (Figure 9). Benzyl chloroformate provides benzyloxycarbonyl (carbobenzyloxy, Cbz) group, di-*tert*-butyl dicarbonate (sometimes abbreviated Boc_2O, where Boc stands for *tert*-butyloxycarbonyl) provides *t*-butyloxycarbonyl (Boc) group, and 9-fluorenylmethyl chloroformate provides 9-fluorenylmethyloxycarbonyl (Fmoc) group.

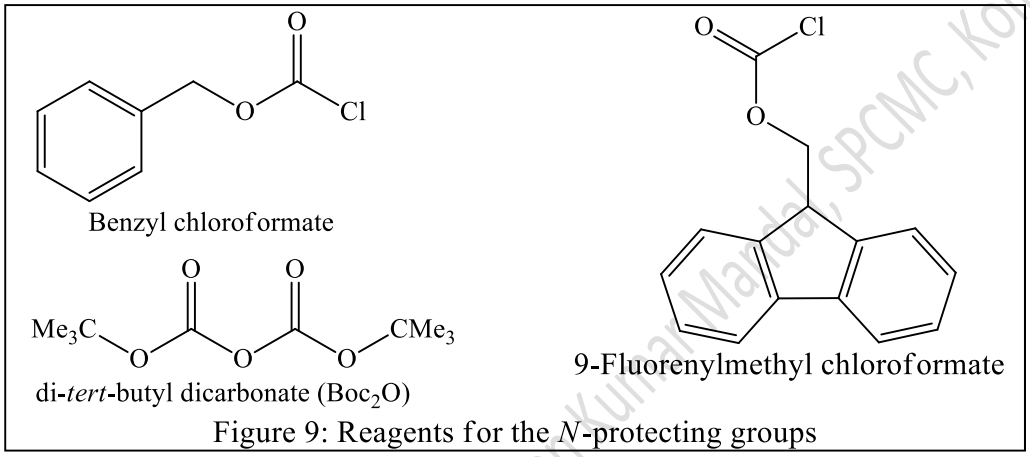


Figure 9: Reagents for the *N*-protecting groups

All these three reagents react with the amine to block it from further acylation. These derivations, however, are types that allow removal of the protecting group under conditions that do not affect peptide bonds.

The benzyloxycarbonyl group (abbreviated Cbz) can be removed with catalytic hydrogenation or cold HBr in acetic acid. The *tert*-butyloxycarbonyl group (abbreviated Cbz) can be removed with trifluoroacetic acid (CF_3CO_2H) in acetic acid. The 9-fluorenylmethoxy-carbonyl (abbreviated Fmoc) group is stable under acid conditions but can be removed under mild basic conditions using piperidine (a secondary amine).

Benzyloxycarbonyl ($PhCH_2$-O-CO-) Group

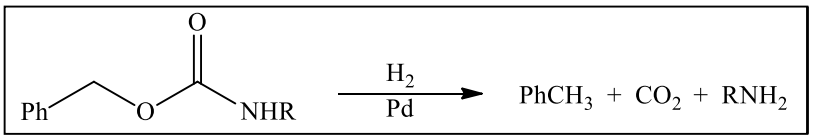
Benzyloxycarbonyl group (also known as carbobenzyloxy, abbreviated as cbz) is an amino protecting group, and this appears to be the most widely used method of protection. Benzyloxycarbonyl chloride is readily prepared by the action of carbonyl chloride on benzyl alcohol in toluene solution followed by alkaline work-up.

$$PhCH_2OH + COCl_2 \rightarrow PhCH_2OCOCl + HCl$$

The use of *N*-benzyloxycarbonyl derivatives causes no appreciable racemization.

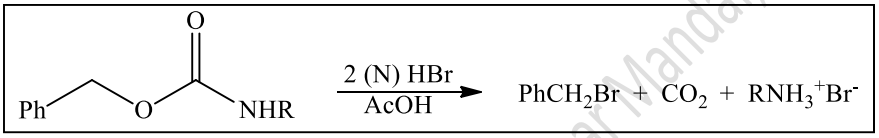
Removal of benzyloxycarbonyl group

1. *Using reducing agents:* Benzyl oxygen cleavage may be brought about reductively by hydrogen in presence of metal (catalytic hydrogenation) with the formation of toluene.



When amino caid contains sulfur atom, e.g., methionine, cysteine, etc., then catalytic hydrogenation cannot be used since sulfur poisons the catalyst. The removal of the protecting group may be successfully accomplished by means of sodium in liquid ammonia.

2. *Hydrolysis in acidic medium*: Benzyl oxygen cleavage can be done by hydrolysis in acid medium that results in the formation of benzyl halide.



N-protection amino acid residue using benzyloxycarbonyl group and subsequent *N*-deprotection of the said group is illustrated in Figure 10.

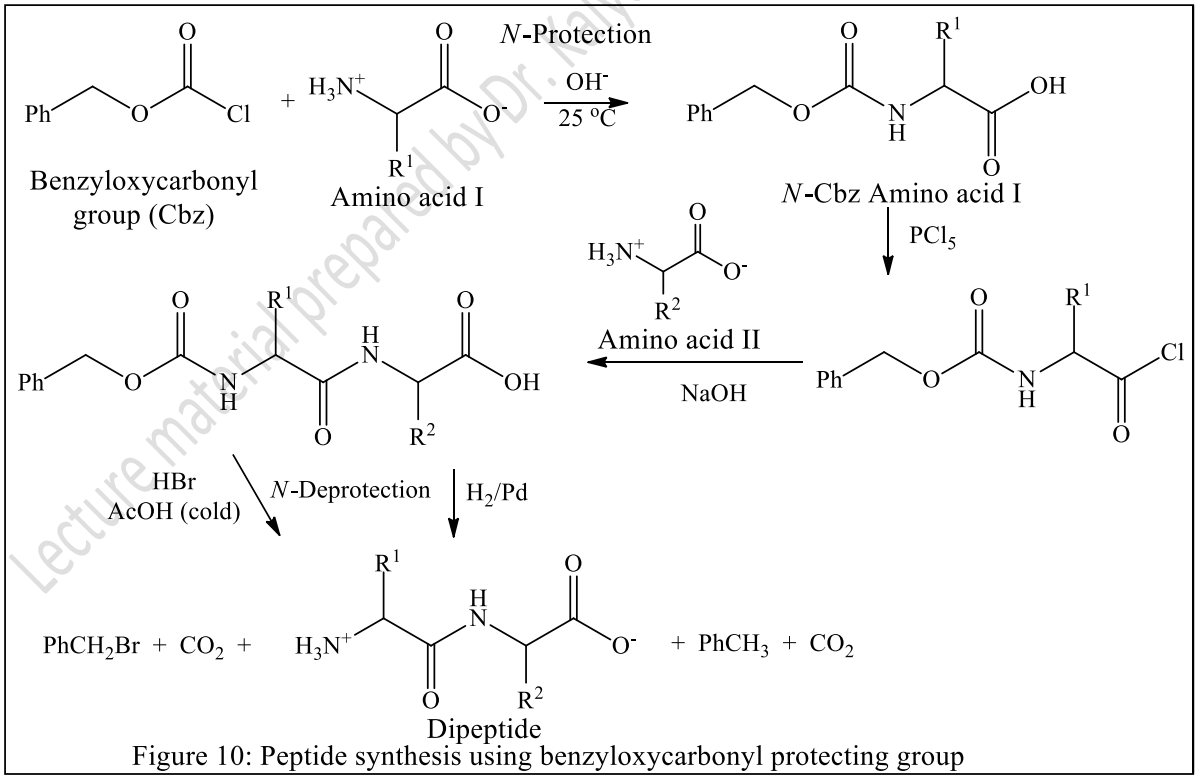
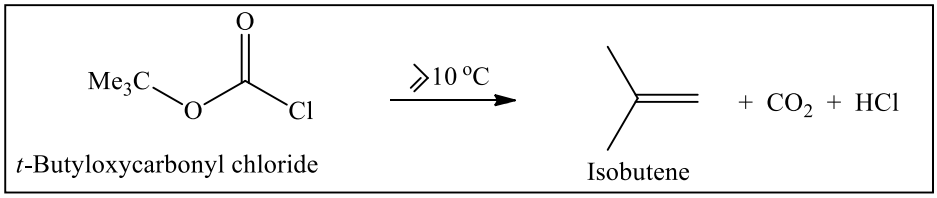


Figure 10: Peptide synthesis using benzyloxycarbonyl protecting group

t-Butyloxycarbonyl (Me_3C-O-CO-) Group

The *t*-butyloxycarbonyl reagent is not used as its chloride, since this is unstable, but is used as its *p*-nitrophenyl ester or through the corresponding acid azide.



Di-*tert*-butyl dicarbonate is used as *N*-protecting group. It is inexpensive, so it is usually purchased. Classically, this compound is prepared from *tert*-butanol, carbon dioxide, and phosgene, using DABCO as a base (Figure 11). This group is readily is readily removed by HBr-AcOH, and also by HCl-AcOH, the latter being particularly useful in that the benzyloxycarbonyl group is not removed by this reagent. The best reagent for removing the *t*-butyloxycarbonyl group appears to be trifluoroacetic acid (TFA) (Figure 12).

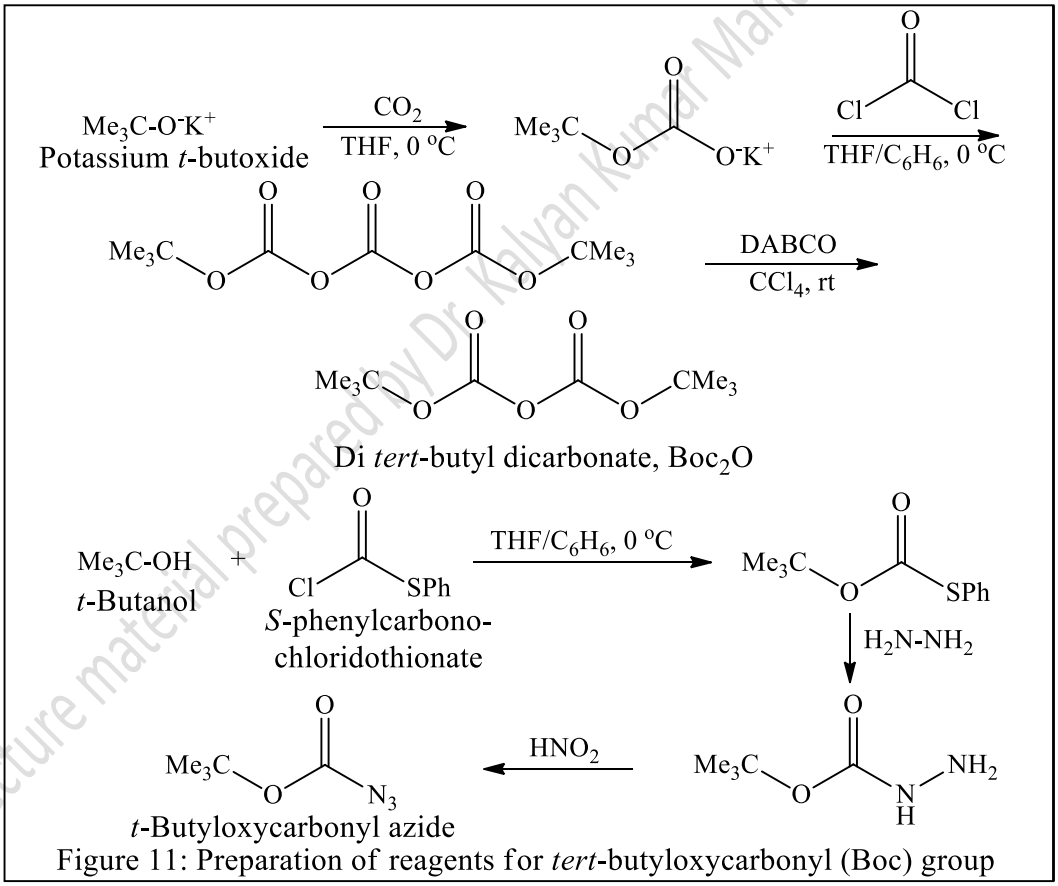


Figure 11: Preparation of reagents for *tert*-butyloxycarbonyl (Boc) group

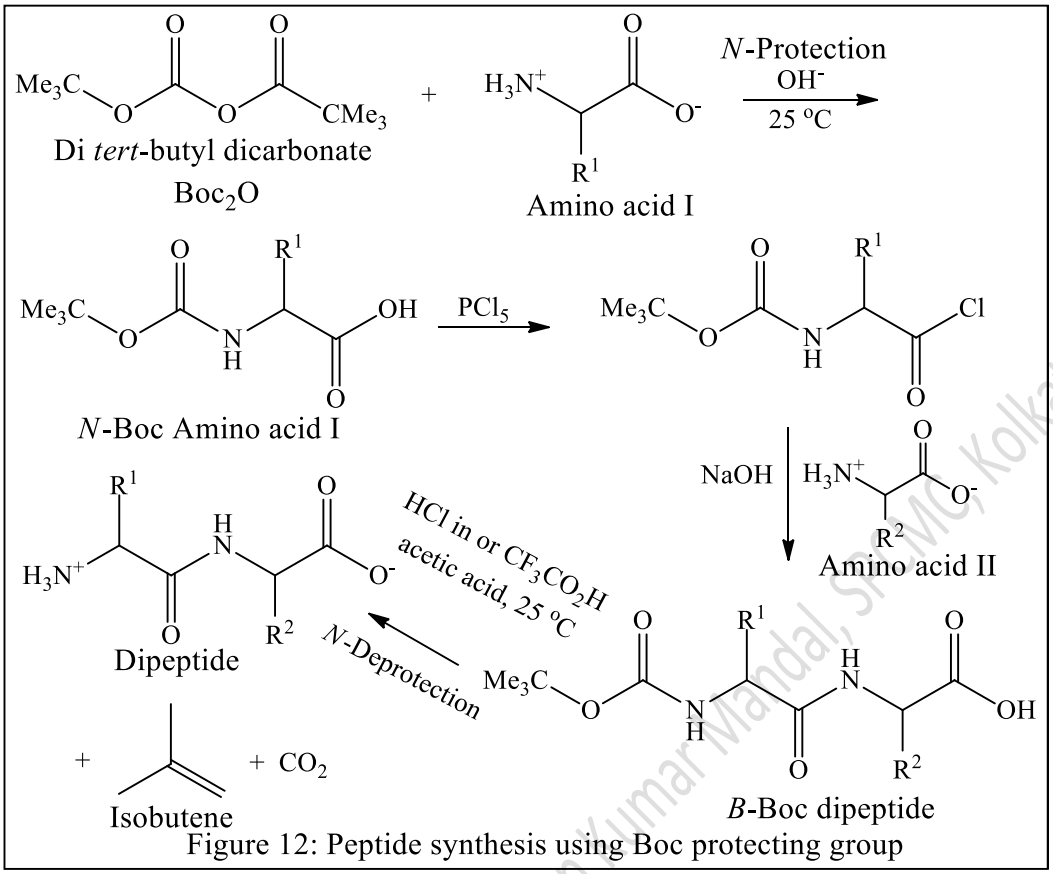
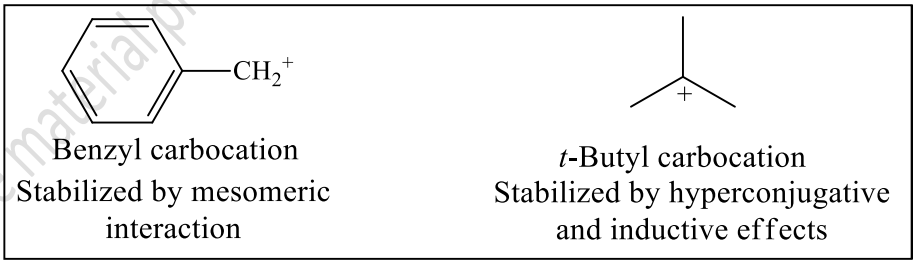


Figure 12: Peptide synthesis using Boc protecting group

Deprotection of Cbz and Boc groups

The easy removal of the benzyloxycarbonyl (Cbz) and *t*-butyloxycarbonyl (Boc) groups in acidic media results from the exceptional stability of the carbocations that are formed initially. The benzyloxycarbonyl group gives a benzyl carbocation, and the *tert*-butyloxycarbonyl group yields, initially, a *tert*-butyl cation. This is then followed by the formation of a stable by-product, isobutene, on deprotonation.



Removal of the benzyloxycarbonyl group with hydrogen and a catalyst depends on the fact that benzyl-oxygen bonds are weak and subject to hydrogenolysis at low temperatures, resulting in methylbenzene (toluene) as one product.

9-Fluorenylmethoxycarbonyl (Fmoc) Group

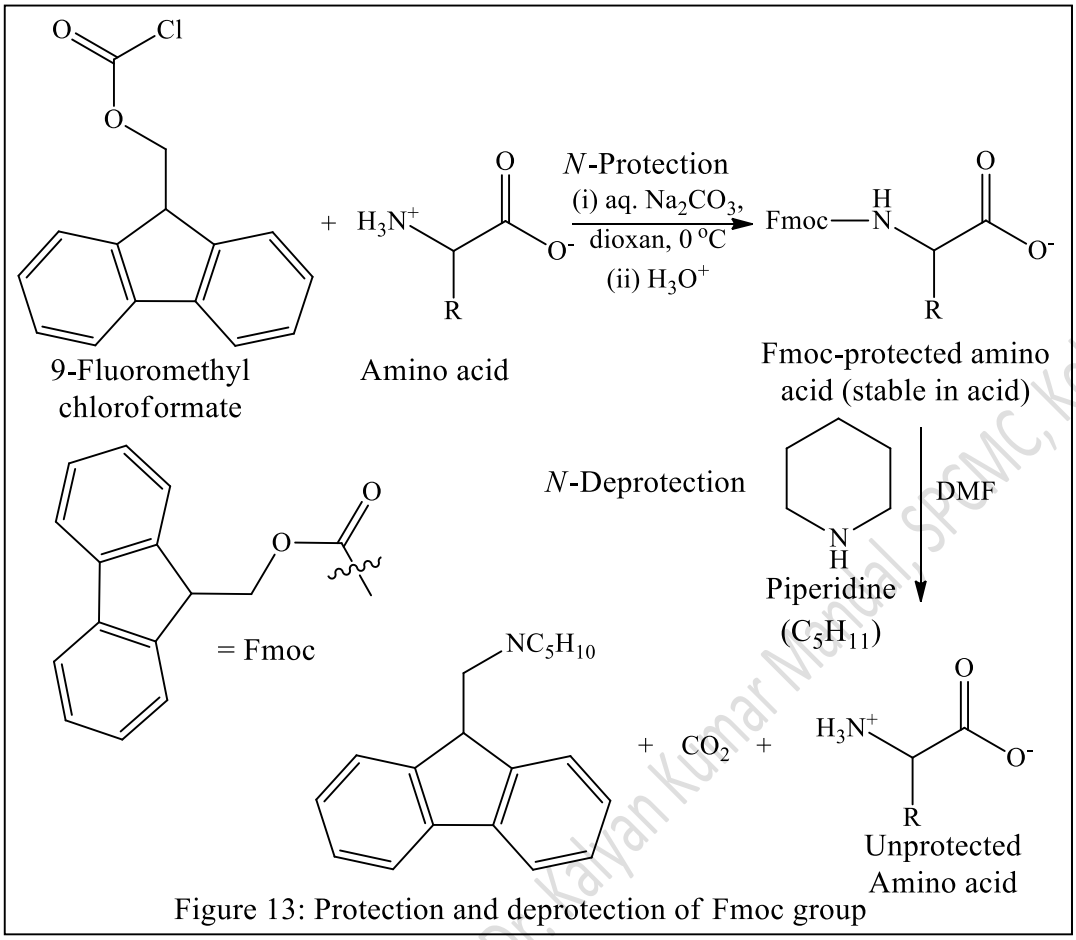


Figure 13: Protection and deprotection of Fmoc group

Phthaloyl as *N*-Protecting Group

Phthaloyl group is used as a means of protecting an end amino group. This group may be removed by hydrazinolysis (use of hydrazine) (Figure 14). Phthaloylation occurs without racemization provided the temperature does not exceed above 150 °C. On the other hand, a much milder method of phthaloylation involves the use of *N*-carbethoxyphthalimide (prepared from potassium phthalimide and ethyl chloroformate). This reagent reacts with amino acids in aqueous sodium hydrogen carbonate solution at room temperature to form the optically pure phthaloyl derivative in excellent yield.

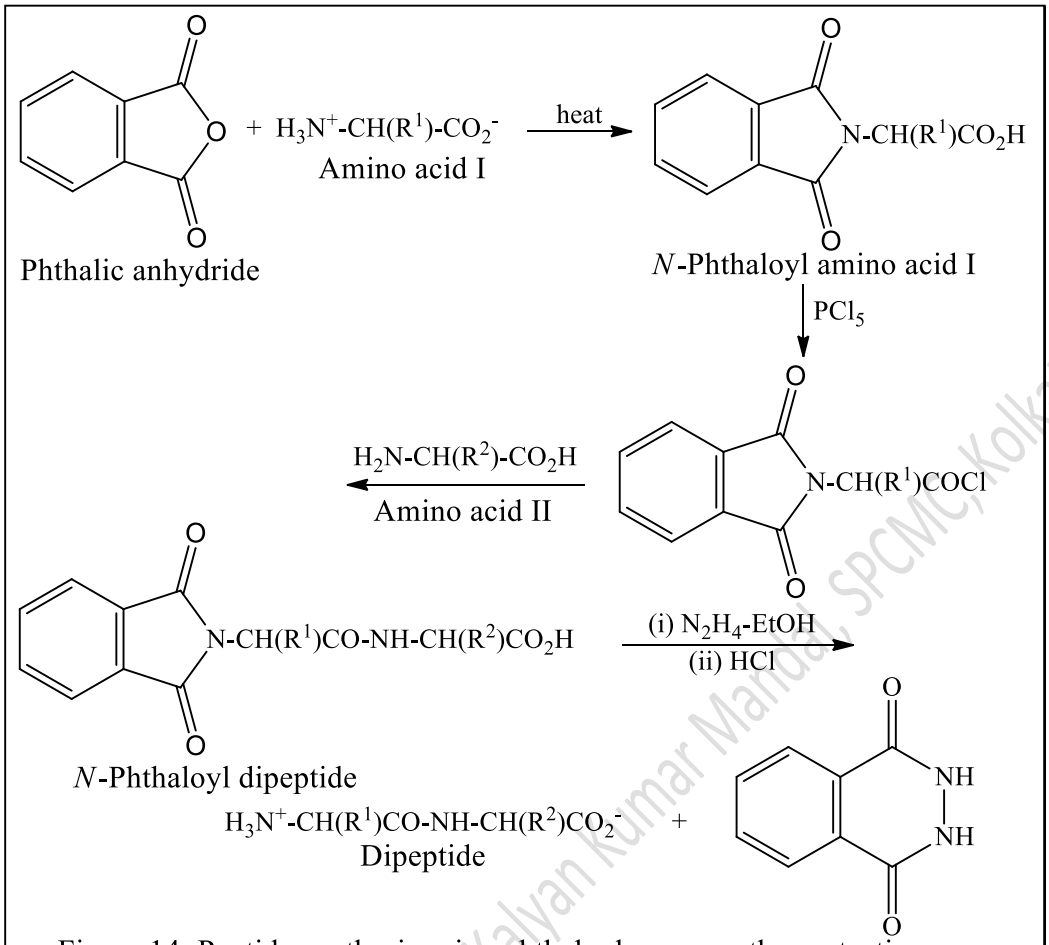


Figure 14: Peptide synthesis using phthaloyl group as the protecting group