BIOMOLECULES
SEM-5, CC-12
PART-8, PPT-28

Part-8: Peptides-V, Synthesis-II

CONTENTS

1. *Carboxyl Group Protection*
2. *Activation of the Carboxyl Group*
3. *Synthesis of the Peptide, Ala-Leu*
4. *Role of DCC in Peptide Synthesis*
6. *Synthesis of Peptides: Phe-Gly, Ala-Phe-Gly and Phe-Gly-Ala*
7. *Synthesis of Higher Peptides*

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BIOMOLECULES (PART-8, PPT-28)

Peptides-V, Synthesis-II

Carboxyl Group Protection: Introduction

Because amino acids have multiple reactive groups, peptide synthesis must be carefully performed to avoid side reactions that can reduce the length and cause branching of the peptide chain. To facilitate peptide formation with minimal side reactions, chemical groups have been developed that bind to the amino acid reactive groups and block, or protect, the functional group from nonspecific reaction.

Purified, individual amino acids used to synthesize peptides are reacted with these protecting groups prior to synthesis, and then specific protecting groups are removed from the newly added amino acid (a step called deprotection) just after coupling to allow the next incoming amino acid to bind to the growing peptide chain in the proper orientation. Once peptide synthesis is completed, all remaining protecting groups are removed from the nascent peptides.

The amino acid *N*-termini are protected by groups that are termed "temporary" protecting groups, because they are relatively easily removed to allow peptide bond formation. Two common *N*-terminal protecting groups are *tert*-butoxycarbonyl (Boc) and 9-fluorenyl-methoxycarbonyl (Fmoc), and each group has distinct characteristics that determine their use.

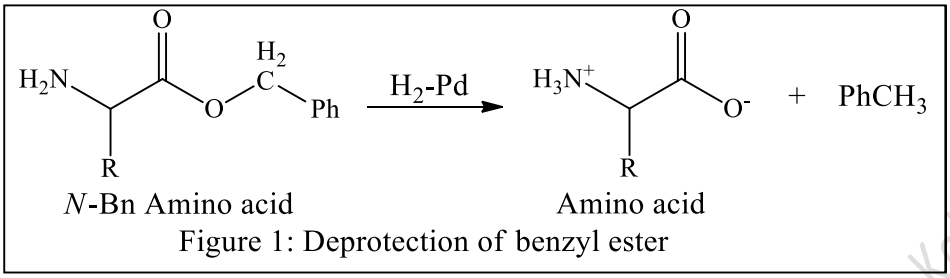
Boc requires a moderately strong acid such as trifluoracetic acid (TFA) to be removed from the newly added amino acid, while Fmoc is a base-labile protecting group that is removed with a mild base such as piperidine. Prior to peptide synthesis, the *N*-termini and amino acid side chains are "protected" with suitable chemical groups that block nonspecific reaction during synthesis. The *C*-terminus of the *C*-terminal amino acid of the peptide is also protected to facilitate peptide extension in the correct orientation.

The use of a *C*-terminal protecting group depends on the type of peptide synthesis used; while liquid-phase peptide synthesis requires protection of the *C*-terminus of the first amino acid (*C*-terminal amino acid), solid-phase peptide synthesis does not, because a solid support (resin) acts as the protecting group for the only *C*-terminal amino acid that requires protection.

Table 1: *C*-Protecting Groups and their Removal

	Me_3C- *tert*-Butyl	Ph_3C- Trityl	$PhCH_2$- Benzyl
Introduction	Isobutene	Ph_3C-Cl	Bn-Cl, Bn_2O
Removal	90% TFA	1% TFA in CH_2Cl_2	HF, H_2, etc.
Stable	Basic conditions, Hydrogenation	Basic conditions, Hydrogenation	Basic Conditions

Carboxyl group of an amino acid can be protected as the methyl or ethyl ester. A difficulty here is that alkaline hydrolysis of the peptide ester may cause racemization. This difficulty may be avoided by use of use of benzyl esters, since these can be split by catalytic hydrogenolysis (H_2-Pd) to give toluene.



N-Bn Amino acid Amino acid

Figure 1: Deprotection of benzyl ester

tert-Butyl esters are also useful since they may readily be prepared by the action of isobutene on the amino acid in the presence of small amount of concentrated sulfuric acid. Furthermore, the *tert*-butyl group is easily removed by treating the ester with anhydrous trifluoroacetic acid or with dry hydrogen chloride.

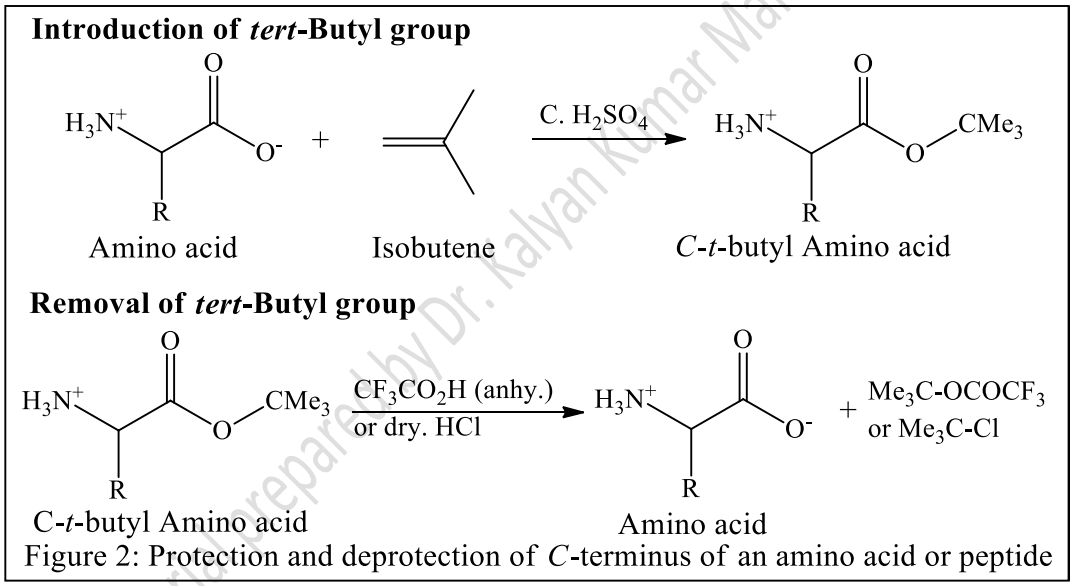
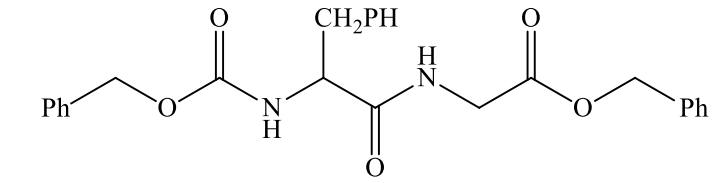


Figure 2: Protection and deprotection of *C*-terminus of an amino acid or peptide

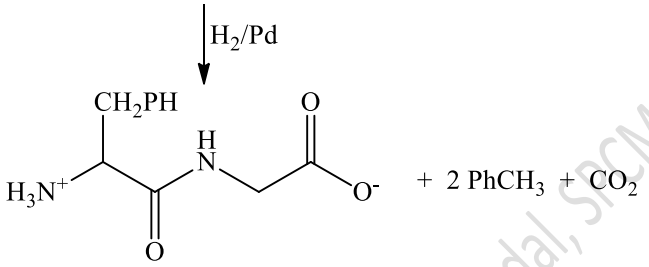
Since racemization is always possible in some of the methods described for peptide synthesis, it is desirable to be able to ascertain whether this has happened. One way is to attempt hydrolysis of the synthetic peptide with enzymes which are highly stereospecific. Carboxylic groups of amino acids and peptides are normally protected by converting the carboxyl acid group into an ester. Generally, a mixture of the amino acid and a large excess of an alcohol is treated with dry HCl. The ester is so chosen that it must get hydrolyzed under weakly alkaline condition as then the peptide linkage remains unaffected. Methyl and ethyl esters are prepared by Fischer esterification.

However, more selective methods are available for the removal of certain esters. Deprotection of methyl and ethyl esters is accomplished under weakly alkaline conditions, whereas *t*-butyl esters are deprotected by dry trifluoroacetic acid and benzyl or the more acid resistant 4-nitrobenzyl esters are removed via hydrogenolysis.

Thus, a synthetic peptide, protected at both its *N*-terminus with a benzyloxycarbonyl (Cbz) group and at its *C*-terminus as a benzyl ester, can be completely removed in a single operation.



N-Benzyloxycarbonylphenylalanylglycine benzyl ester

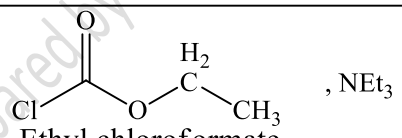


Phenylalanylglycine

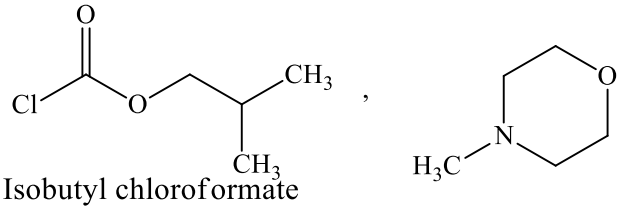
Figure 3: Peptide synthesis using cbz as the protecting groups at both *N*- and *C*-terminals

Activation of the Carboxyl Group

For a successful coupling leading to the formation of a desired peptide, the carboxylic acid end (*C*-terminus) of an *N*-protected amino acid must be activated. The commonly used activating groups are shown in Figure 4.

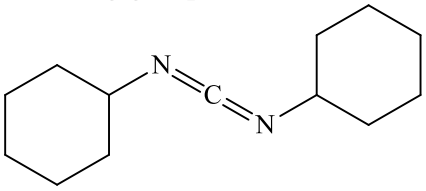


Ethyl chloroformate



Isobutyl chloroformate

Isobutyl chloroformate is better than ethyl chloroformate as a *C*-activating group of an amino acid or peptide



Dicyclohexylcarbodiimide (DCC)

Figure 4: *C*-activating groups of an amino acid or peptide

Perhaps the most obvious way to activate a carboxyl group is to convert it to an acyl chloride. This method was used in early peptide syntheses, but acyl chlorides are actually more reactive than necessary. As a result, their use leads to complicating side reactions. A much better method is to convert the carboxyl group of the "protected" amino acid to a mixed anhydride using ethyl chloroformate (Figure 5). Isobutyl chloroformate is superior because yields are reported to be better than with ethyl chloroformate.

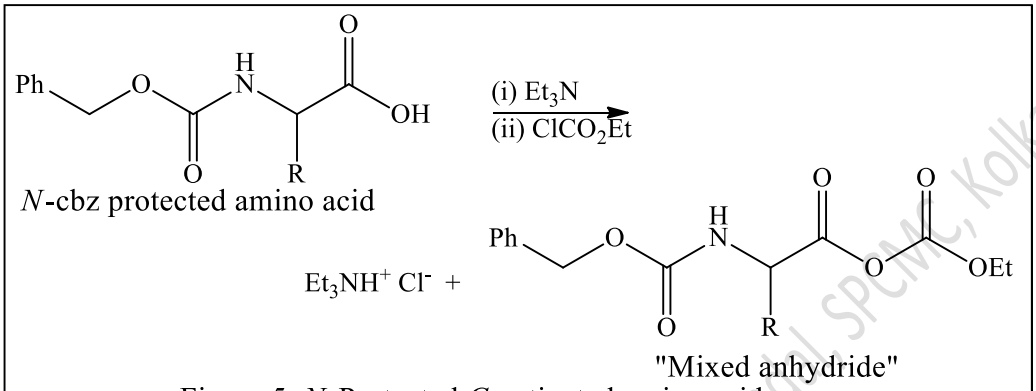


Figure 5: *N*-Protected *C*-activated amino acid

The mixed anhydride method of coupling used in peptide synthesis involves reaction of a protected amino acid or peptide with an alkyl chloroformate in the presence of a tertiary amine base to give the mixed carboxylic acid - carbonic acid anhydride (Figure 5). The anhydride is then reacted with an amine nucleophile, which is either an amino acid ester (a *C*-protected amino acid) or a peptide ester, giving the protected peptide as shown in Figure 6.

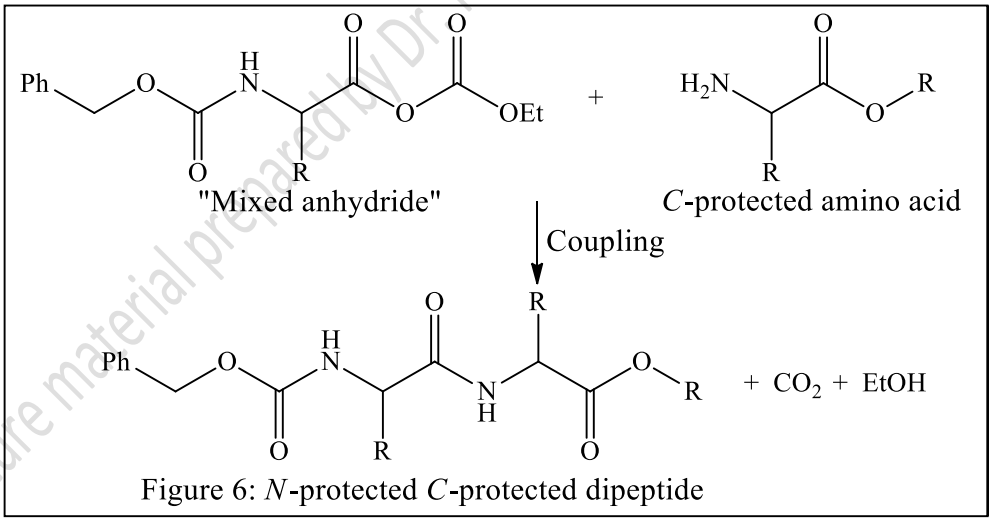


Figure 6: *N*-protected *C*-protected dipeptide

- There are other ways in which the peptide synthesis may be carried out with *p*-nitrophenyl ester method (Figure 7).

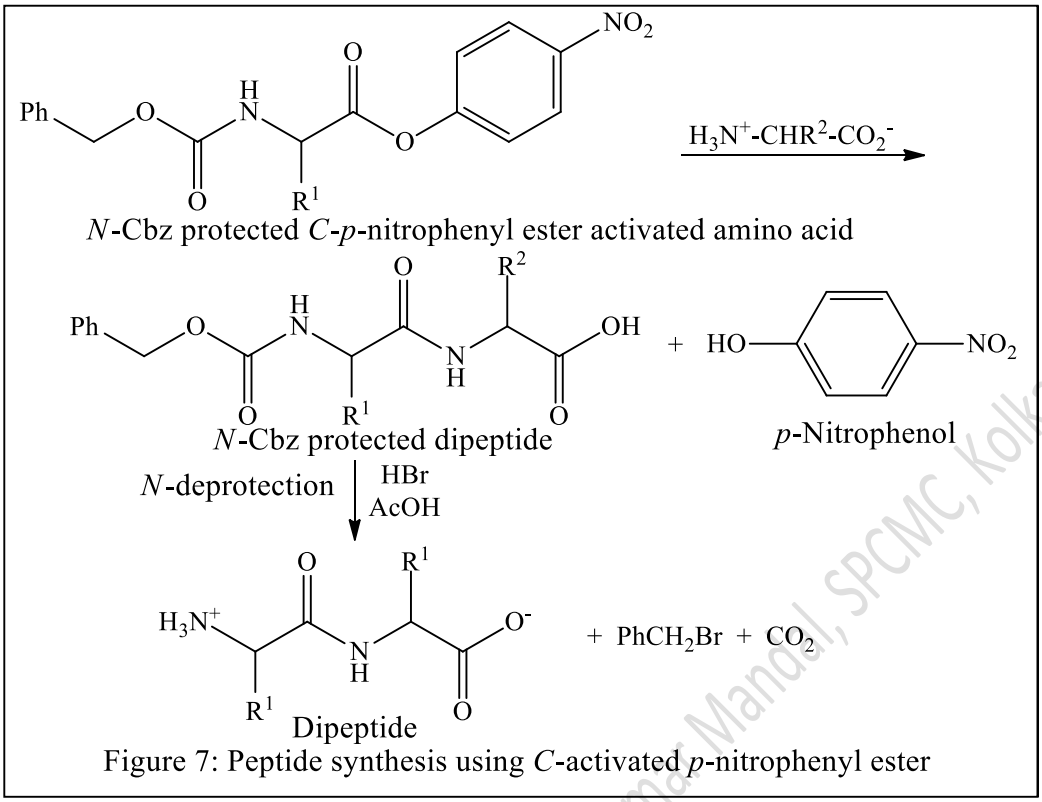


Figure 7: Peptide synthesis using *C*-activated *p*-nitrophenyl ester

The use of the *p*-nitrophenyl ester, an activated ester, depends on the fact that it readily reacts with an amino group. The corresponding ethyl ester combines much more slowly. *p*-Nitrophenyl esters are prepared in high yield by the addition of dicyclohexylcarbodiimide (DCC), a dehydrating agent, to a solution of the benzyloxycarbonyl derivative of the amino acid and *p*-nitrophenol in ethyl acetate.

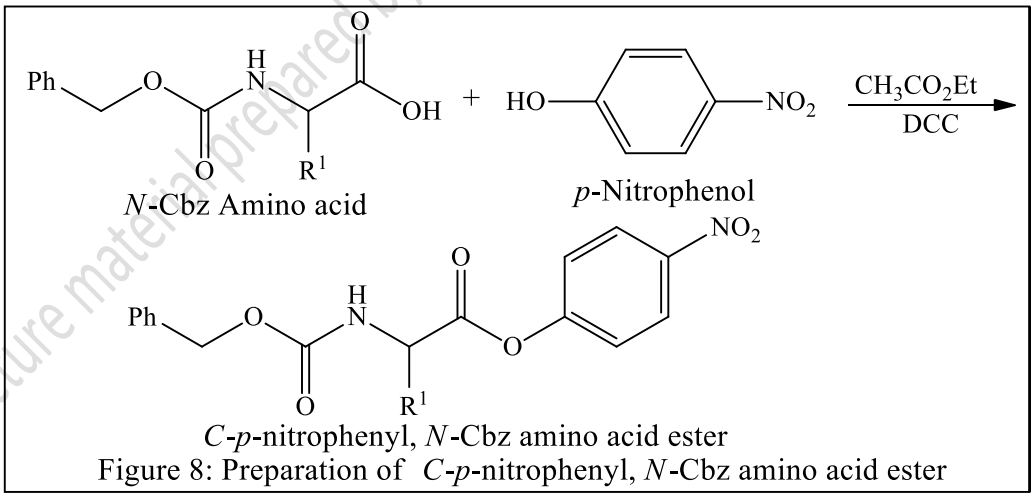


Figure 8: Preparation of *C*-*p*-nitrophenyl, *N*-Cbz amino acid ester

On the other hand, the azide method may be used instead of acid chlorides or esters (Figure 9). Reaction of *N*-protected amino acid ester with hydrazine gives the corresponding hydrazides. Subsequent reaction with nitrous acid generates the azide ester. The azide synthesis is not accompanied by racemization.

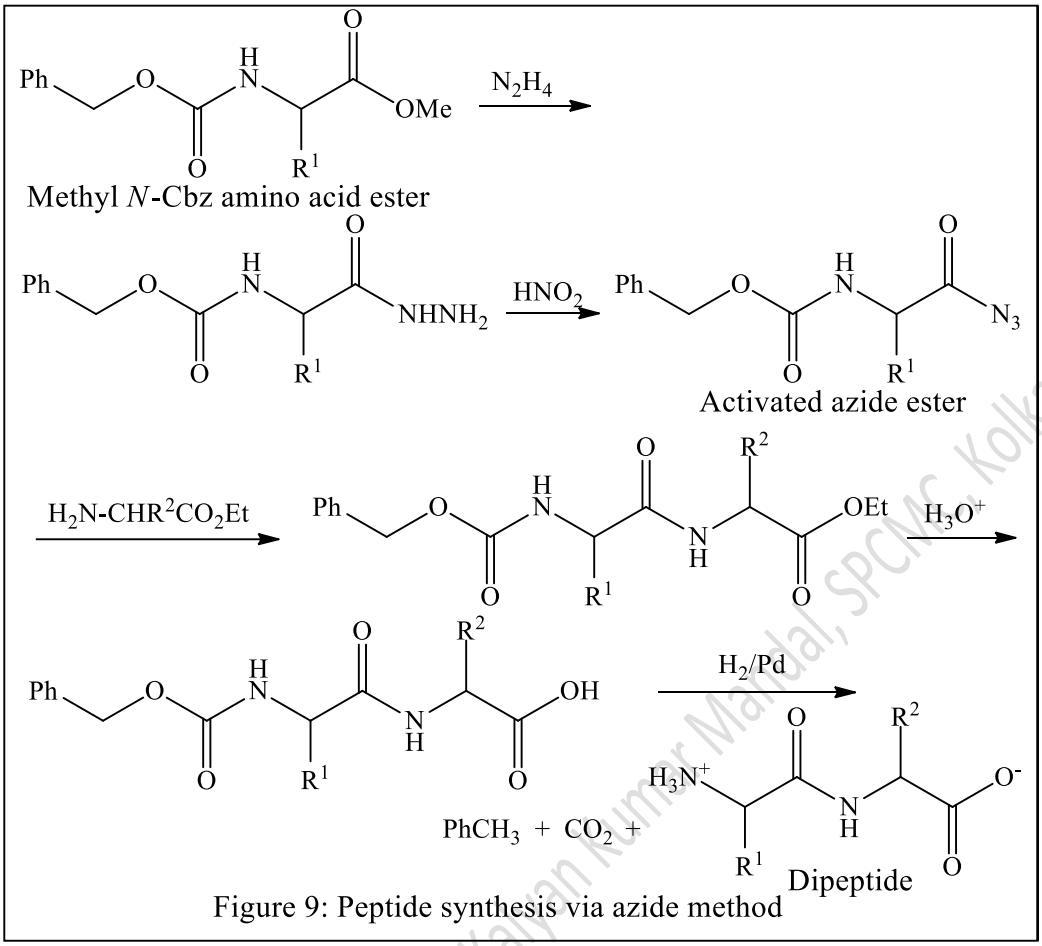


Figure 9: Peptide synthesis via azide method

Synthesis of the Peptide, Ala-Leu

Let us examine now how these reagents are used in the preparation of the simple dipeptide alanylglycine (A-L). The principles involved here can, of course, be extended to the synthesis of much longer polypeptide chains.

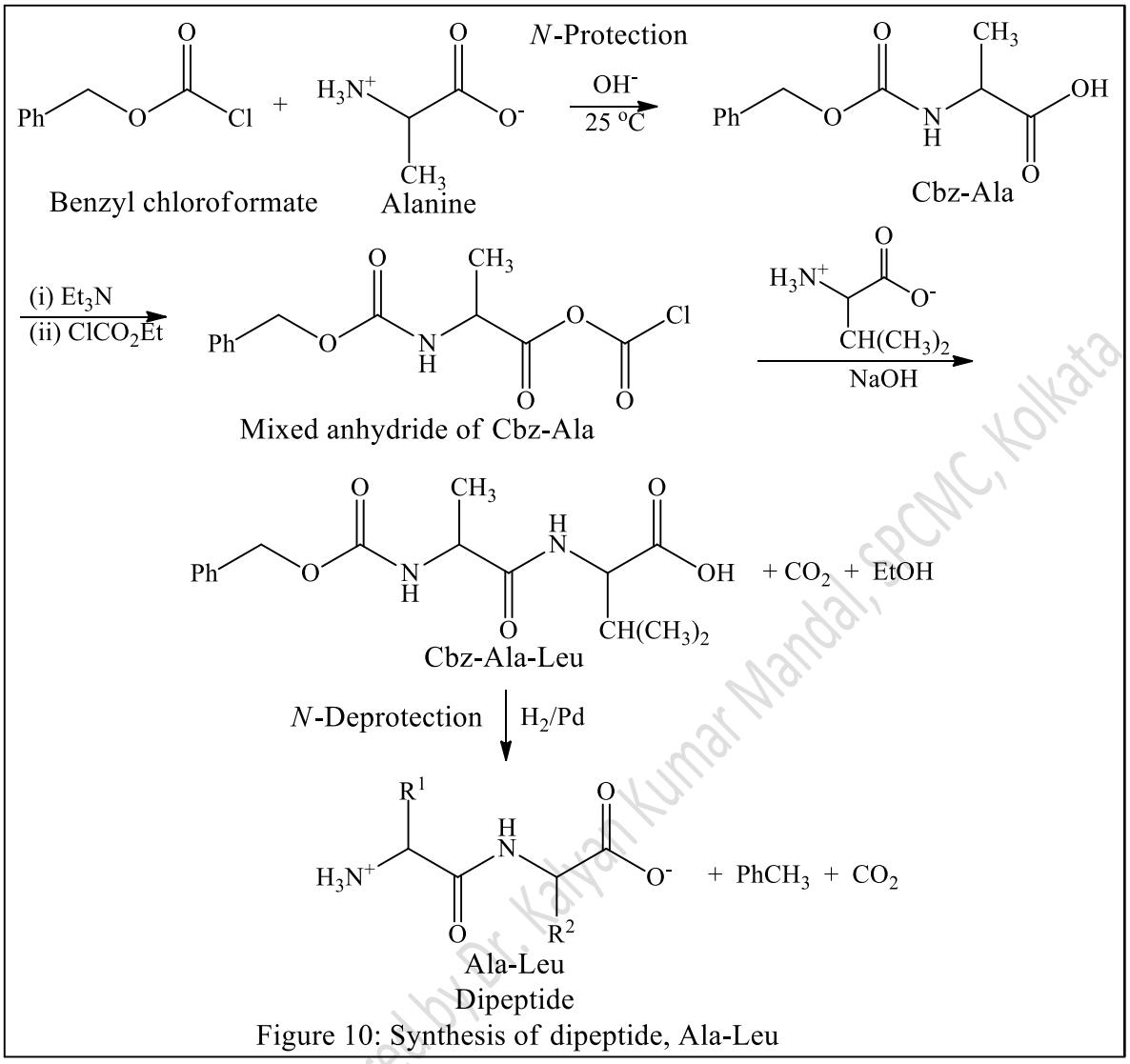


Figure 10: Synthesis of dipeptide, Ala-Leu

Role of DCC in Peptide Synthesis

Treatment of a solution containing the *N*-protected and the *C*-protected amino acids with *N*,*N*-dicyclohexylcarbodiimide (DCC) in an inert solvent (methylene chloride, THF, etc.) leads directly to peptide bond formation.

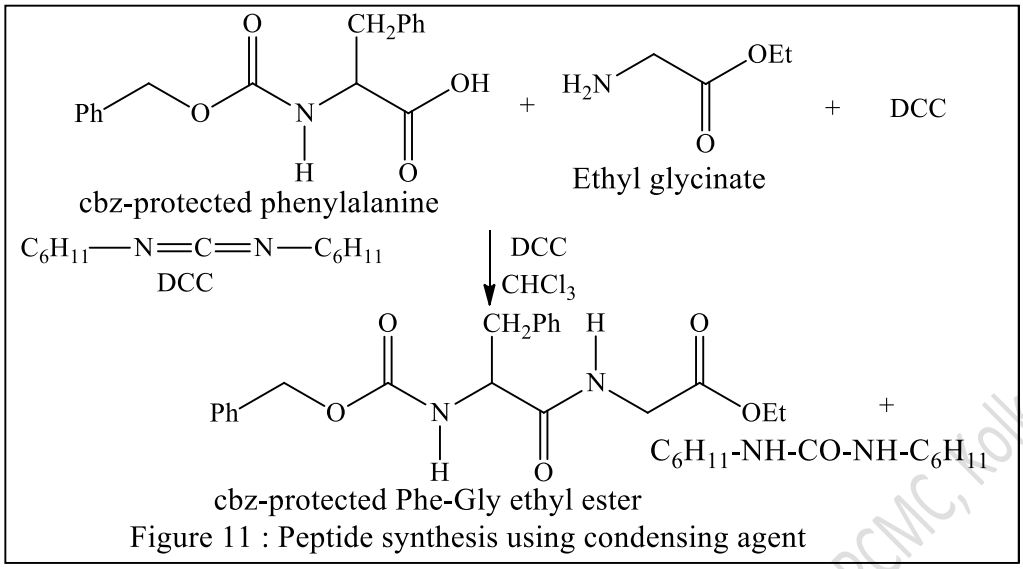


Figure 11 : Peptide synthesis using condensing agent

The mechanism by which DCC promotes the condensation of an amine and a carboxylic acid to give an amide is outlined in Fig. 12.

Mechanism with DCC

Step 1: In the first stage of the reaction, the carboxylic acid adds to one of the double bonds of DCC to give an *o*-acylisourea.

Step 2: Structurally, *o*-acylisoureas resemble carboxylic acid anhydrides and are powerful acylating agents. In the reaction's second stage the amine adds to the carbonyl group of the *o*-acylisourea to give a tetrahedral intermediate.

Step 3: the tetrahedral intermediate dissociates to an amide and *N*,*N*-dicyclohexylurea.

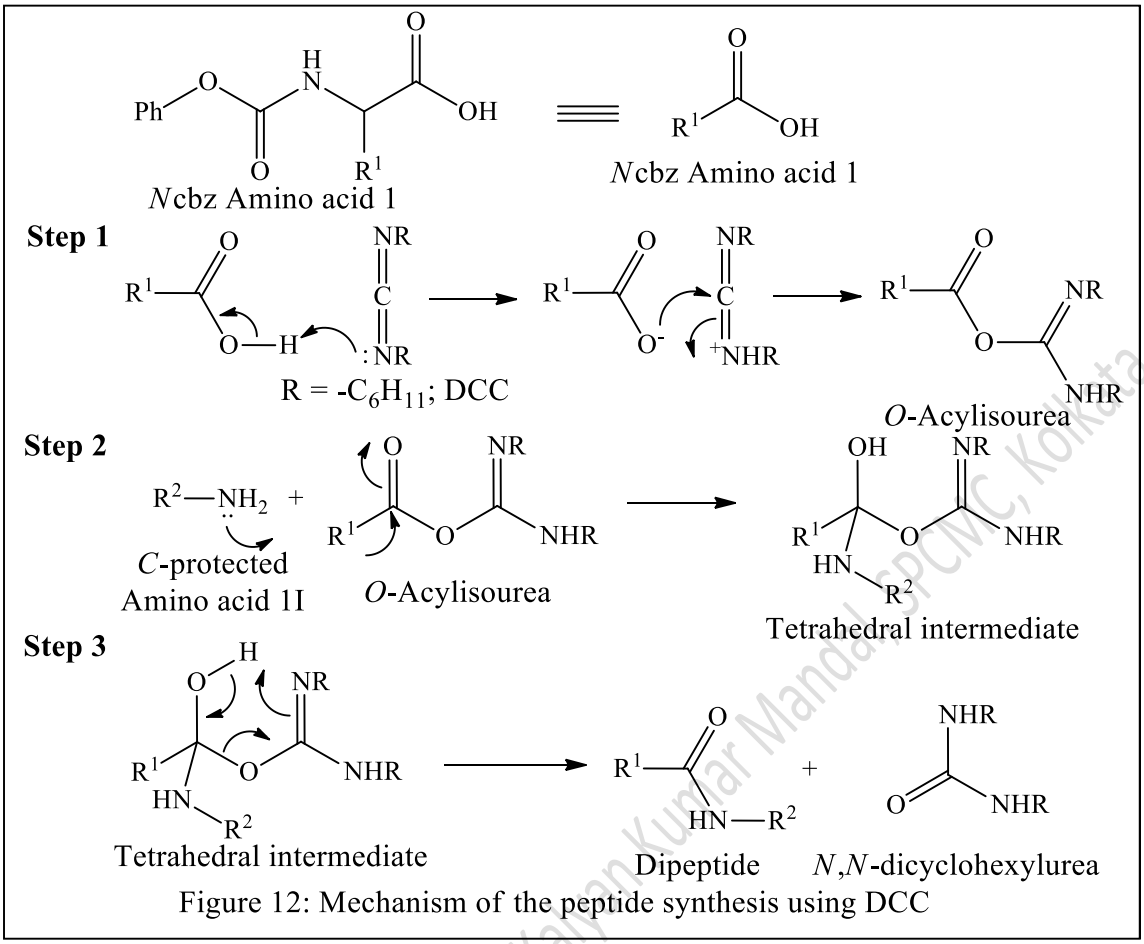


Figure 12: Mechanism of the peptide synthesis using DCC

Peptide Bond Formation

In the second major method of peptide synthesis the carboxyl group is activated by converting it to an active ester, usually a *p*-nitrophenyl ester. *p*-Nitrophenyl esters are much more reactive than methyl and ethyl esters in these reactions because *p*-nitrophenoxide is a better (less basic) leaving group than methoxide and ethoxide. Simply by allowing the active ester and a *C*-protected amino acid to stand in a suitable solvent is sufficient to bring about peptide bond formation by nucleophilic acyl substitution.

The *p*-nitrophenol formed as a by-product in this reaction is easily removed by extraction with dilute aqueous base. Unlike free amino acids and peptides, protected peptides are not zwitterionic and are more soluble in organic solvents than in water.

Synthesis of Peptides

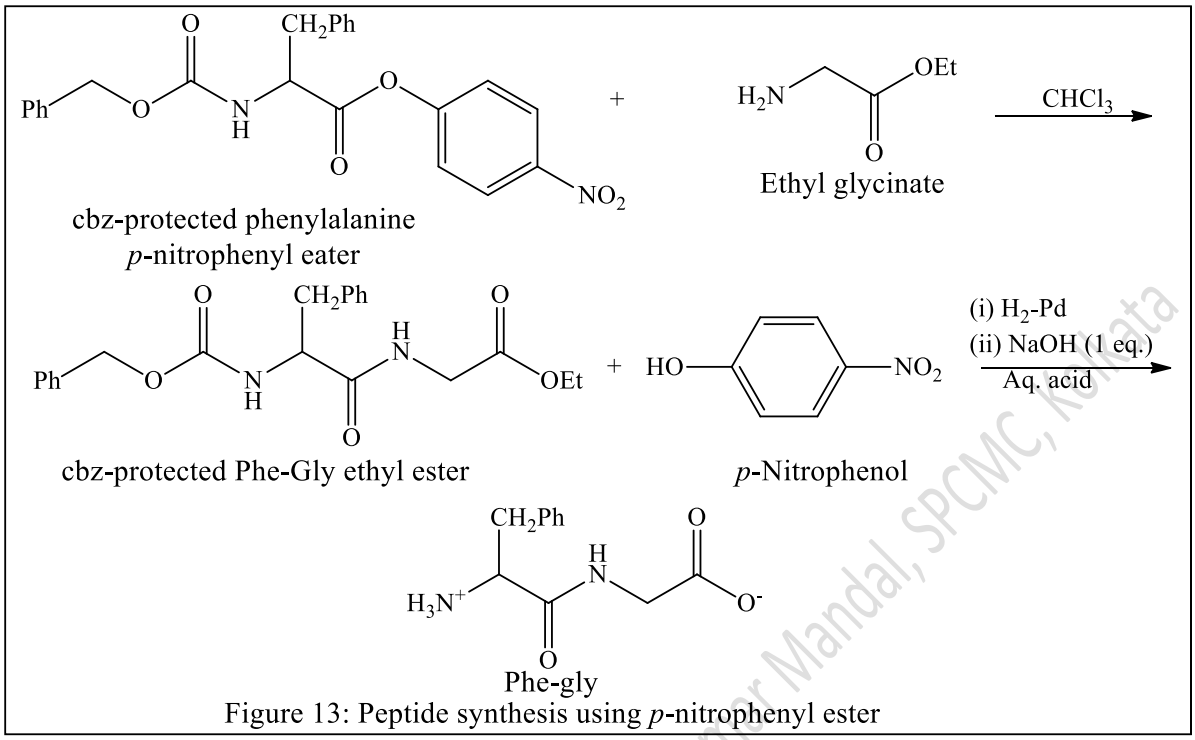


Figure 13: Peptide synthesis using *p*-nitrophenyl ester

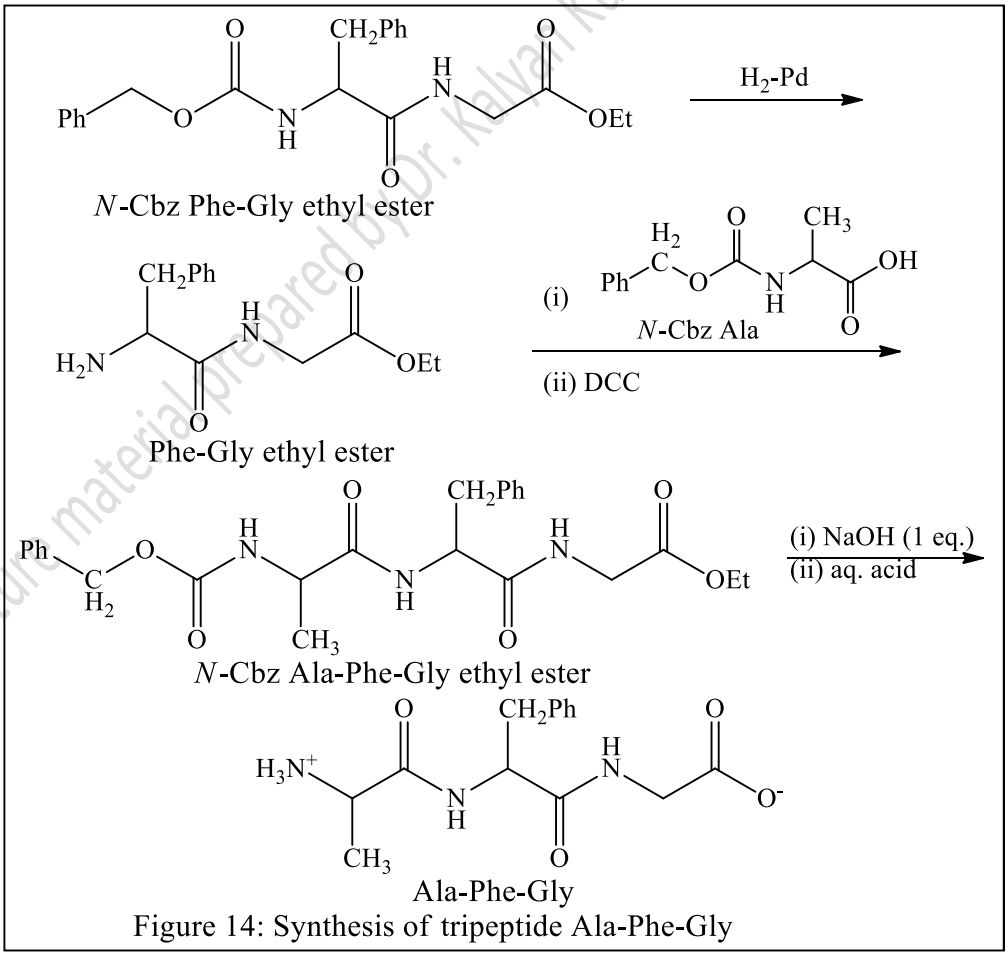


Figure 14: Synthesis of tripeptide Ala-Phe-Gly

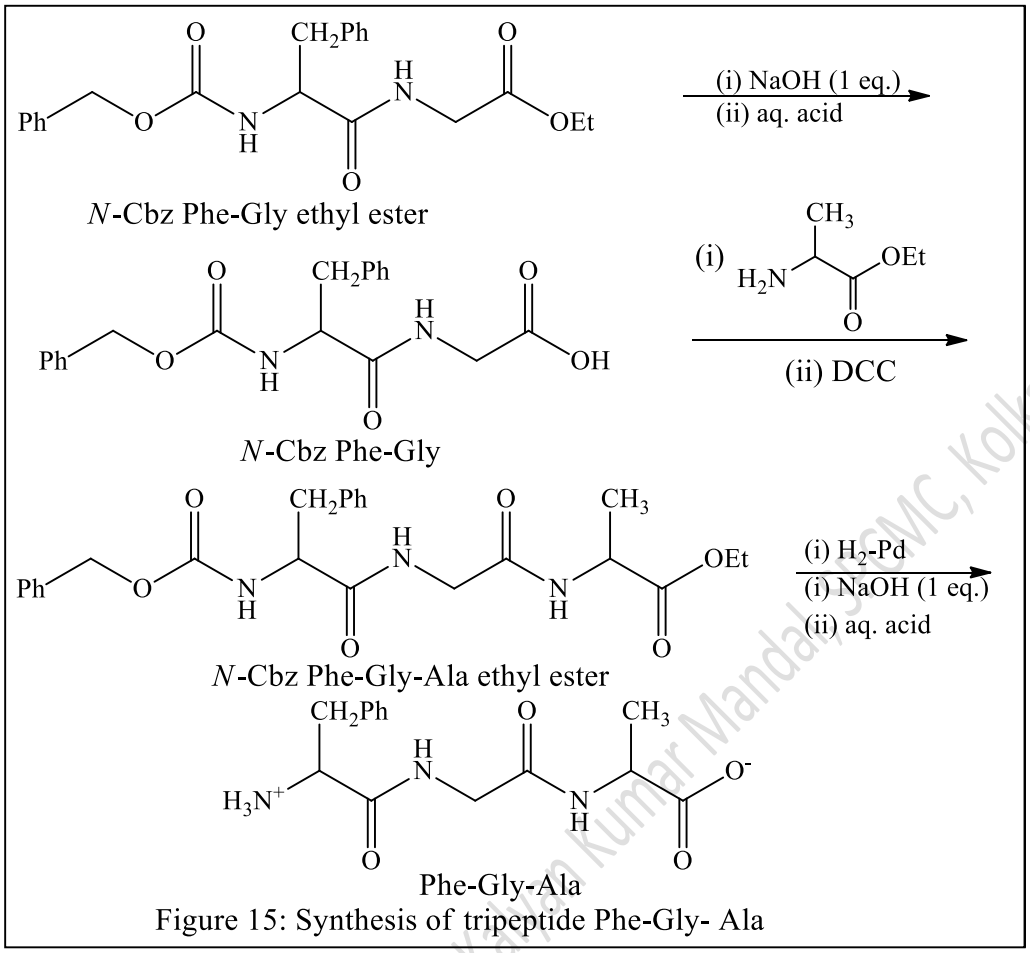


Figure 15: Synthesis of tripeptide Phe-Gly- Ala

Synthesis of Higher Peptides

Higher peptides are prepared either by stepwise extension of peptide chains, one amino acid at a time, or by coupling of fragments containing several peptides (the fragment condensation approach). An attractive feature of fragment condensation approach is that the various protected peptide fragments may be individually purified, which simplifies the purification of the final product.

In the stepwise extension approach, the starting peptide in a particular step differs from the coupling product by only one amino acid residue and the properties of the two peptides may be so similar as to make purification by conventional techniques all but impossible. A number of methods have been developed for peptide synthesis, but most widely used solid phase method overcomes many of the difficulties involved in the purification of intermediates.