Review Article

Side reactions in peptide synthesis: An overview

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ABSTRACT
Peptide synthesis involves condensation of two or more amino acids which seems to be easier but requires specialized techniques. Since all the amino acids have basic skeleton but vary in their side chains, and their nature such as acidic, basic or neutral depending on the presence or absence of functional groups, these side chains are prone to side reactions during the process of synthesis either due to interaction with the solvent used for synthesis or during the process of the deprotection of the specific groups. It could also occur due to lower rate of reaction where the amino acids reacting forms byproducts leading to the lesser yield of the desired peptide chain. In the present document, the side reactions that occur are described in brief with their mechanism. All the images of the reactions in this document were drawn using ChemDraw software and have been made strong efforts to make the explanations to be clear and easier to understand.

Keywords: Peptide synthesis, functional groups, side reactions, mechanisms.

INTRODUCTION
Peptides are the sequence of amino acids which are formed by the condensation of two amino acids in which a peptide bond is formed between a carbonyl group of one amino acid and the amino group of another amino acid [1]. These are synthesized either by solid phase [2] or solution phase [3] in which one of the groups are protected using specific protecting agents such as o-Boc, Fmoc, etc whereas the other group reacts with another amino acid to form the peptide bond. During peptide synthesis, solid phase or solution phase, the side chain present in the amino acid skeleton are prone to many side reactions either due to interaction with the solvent or by an acid or a base during deprotection of the specific groups [4]. This results in formation of electrophile or a nucleophile that leads to inactivation of the peptide by forming racemization [5] or cyclic molecules [6], or may prevent the chain to form peptide bonds further with other amino acids. These reactions in peptide may be possible by abstraction of a proton [5] or protonation to form a carbanion [7] or carbocation [8] which results in loss of their chiral nature. Sometimes, this may be also due to overactivation [9] where the functional side chain of the amino acid forms other compounds such as anhydrides [10] or azlactones [11]. In some cases, there might not be a functional side chain but reaction is seen due to individual amino acids [12]. The possible side reactions that can occur during peptide synthesis and their mechanism are described below.

Mechanism of side reactions
By proton abstraction
Abstraction of acidic proton in presence of a base from carboxyl group results in carboxylate anion which prevents the formation of another anionic ester at α-carbon. Therefore, this anion prevents the elongation of peptide chain due to the absence of carboxyl group to form a peptide bond [5]. This reaction is shown in the figure 1 in which glycine when treated by a base is taken as an example.

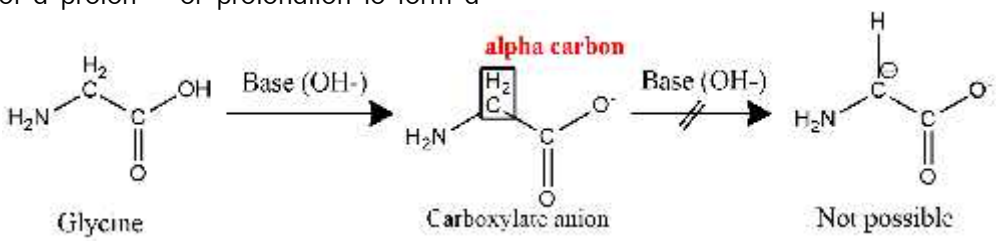

Fig:1 Formation of carboxylate anion when treated with a base

Racemization
In esters, electron withdrawing forces present in the activating group (X) enhance the activity of α-Hydrogen abstraction that leads to the formation of carbanion which results in total or partial loss of chiral purity resulting in irreversible racemization [7]. This type of racemization is shown in the following reaction where the activating group of the carboxylic group present in the glycine derivative enhances the abstraction of α-Hydrogen resulting in the formation of carbanion with the change in the orientation of the molecule [13]. The reaction of formation of carbanion is shown in the figure 2.

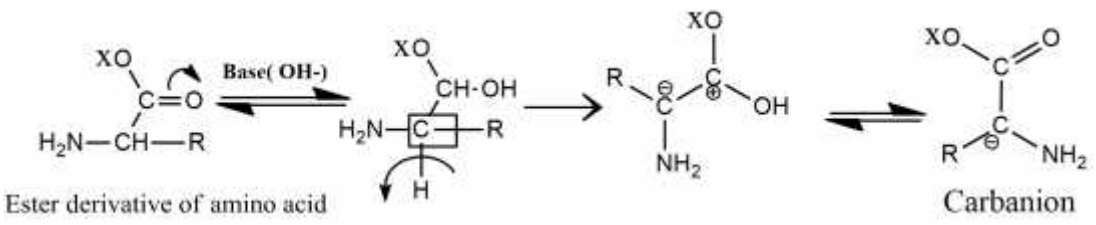

Fig: 2 Formation of carbanion in esters

In a peptide chain, due to amide bond, proton abstraction does not occur at the α-carbon but occurs at the amide nitrogen of acyl amino acid. This is due to presence of lone pair of electrons at 'N'. When amide bond, in presence of an acid, undergoes proton abstraction, the abstracted proton leaves the Nitrogen atom retaining its electrons. As a result, the amide Nitrogen acts as a nucleophile and is very much prone to cyclization of amino acids due to electron rich nature as it poses a lone pair of electron and an excess pair resulted due to proton abstraction. This cyclization changes the chiral nature of the amino acids resulting in formation of succinic acid derivative which affects the peptide stereochemistry [14] [15]. Formation of nucleophile and its cyclization is depicted in the figure 3.

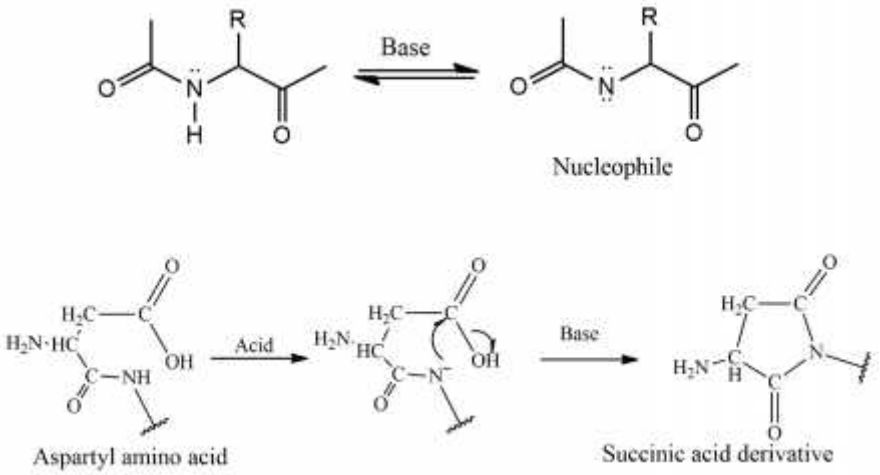

Fig: 3 Formation of nucleophile and cyclization of the amino acid derivative

Racemization resulting in loss of chiral purity may occur in either of the pathways which are described below:

Direct abstraction of α-proton:

When an amino acid which is attached with a protecting group (Y), is treated with a base, proton abstraction occurs at α-carbon resulting in the formation of carbanion which can be attacked by any electrophile resulting in undesired reaction which changes the stereochemistry of the amino acid [7].

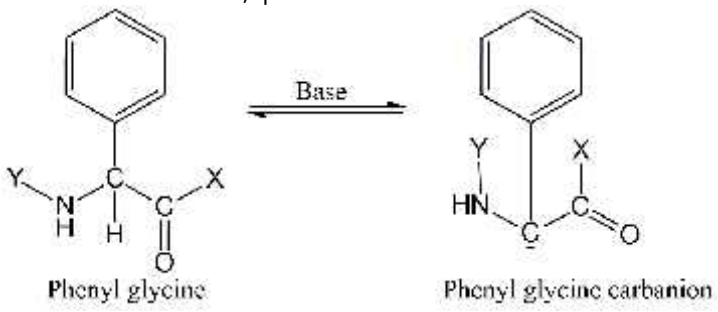

Fig: 4 Racemization by direct abstraction of proton

By forming azlactones

The keto group of the amide bond undergoes keto-enol tautomerism to form a hydroxyl group which upon treatment with a base, abstracts a proton from the hydroxyl group resulting in formation of negatively charged oxygen. This initiates the activating group (X) to leave the carboxylic end. As a result, the carbon holding the keto group gets positive charge on it. Since the carbon now has deficient of electrons, and the oxygen with excess of electrons, a bond is formed between the carbon and the oxygen, and therefore forms an azlactone ring [16] [17].

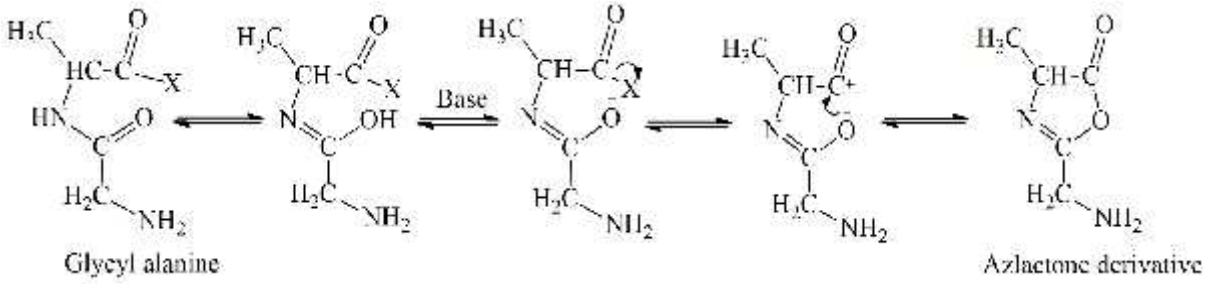

Fig: 5 Racemization by forming of azlactone

Due to presence of unsaturation in the azlactone, and upon treating with a base leads to the abstraction of proton at α-carbon, which results in a total of three resonating structures [18]. Therefore, the resonance stabilized structure forms the carbanion

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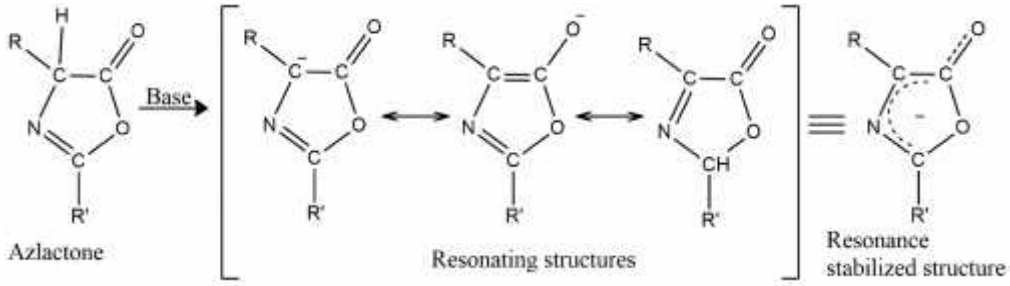


Fig: 6 Resonating structures of azlactones

Formation of azlactones is better explained by considering Benzoyl L-leucine-p-Nitrophenol, in which, the carboxyl and amino groups are protected by p-Nitrophenol and Benzoyl group respectively. This when treated with a basic solvent (tertiary amine), it results in formation of azlactone.

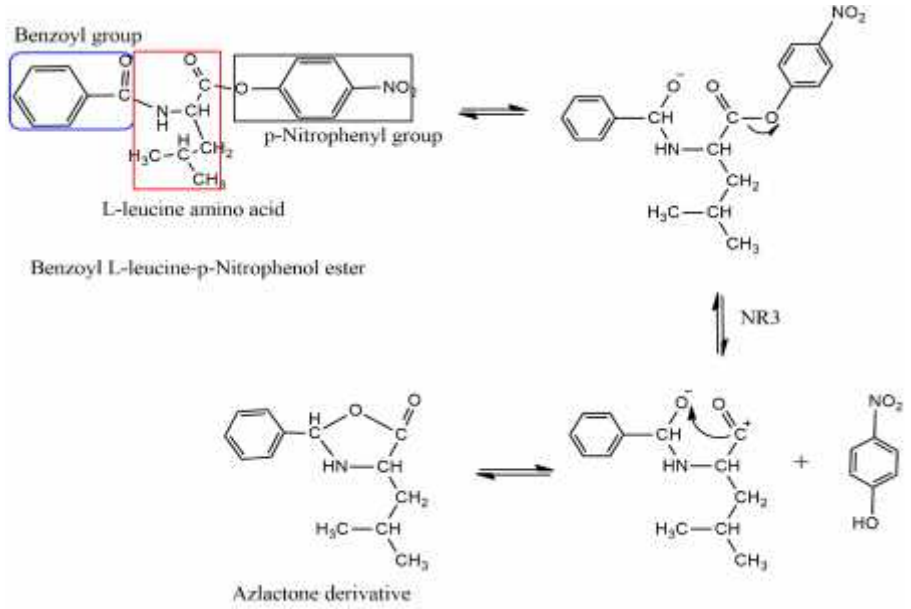


Fig: 7 Formation of azlactone in Benzoyl L-leucine-p-Nitrophenol

Most of the racemization occurs through azlactones only and rarely by direct abstraction [19]. The Factors that affect racemization through azlactones are:

i. Nature of amino acid involved
ii. Solvent used in reaction
iii. Presence of tertiary amine.

When stability aspect of anion produced by proton abstraction through azlactones is considered, it is enhanced by electron withdrawing effects in acyl groups. Therefore, methyl azlactone is less stable when compared to phenyl azlactone. This is so, as aryl azlactone has more resonating structures than alkyl azlactone.

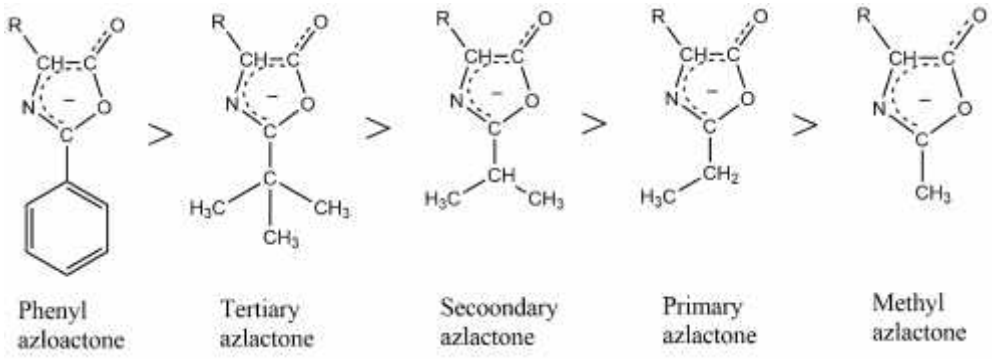


Fig: 8 Order of stability of azlactones

Cyclization

During peptide synthesis (solid phase), presence of benzyl ester can cause premature cleavage of the chain from insoluble support. The esters formed upon cleavage, undergoes cyclization to form ketopiperzines [20] [21]. This cyclic compound, when subjected to hydrolysis, leads to amide bond cleavage, as a result, the dipeptide is obtained with a different stereochemistry making it inactive [22].

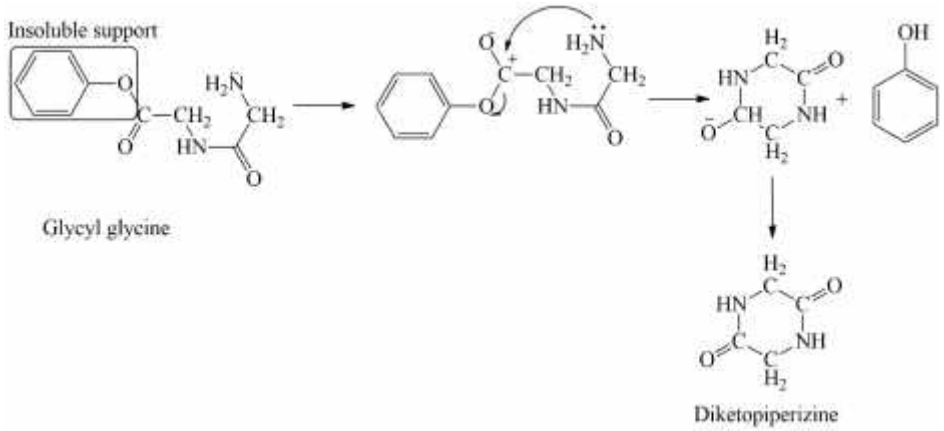


Fig: 9 Formation of diketopiperazine in glycyl glycine

O – Acylation

When an amino acid is treated with a base such as tertiary amine, it abstracts the proton and converts alcohols or phenols to alcoholates or phenolates respectively which is shown in figure no 10. The formed alcoholate/phenolate, then reacts with an acylating agent and facilitates acylation at the electron rich oxygen atom. Since the acylation occurs at the nucleophile (O^-), the reaction is named as O-Acylation [23] [24]. In the figure 11, p-Hydroxy alanine (tyrosine) is treated with a tertiary amine which acts as proton abstractor, and also with p-Nitrophenyl ester, as a result, the acylated product p-Acyl oxy phenyl alanine (Acyl tyrosine) is formed along with p-Nitrophenol.

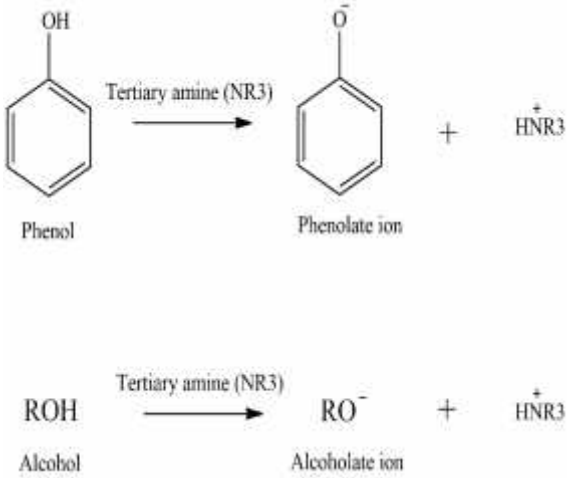


Fig: 10 Formation of alcoholate/phenolate ion

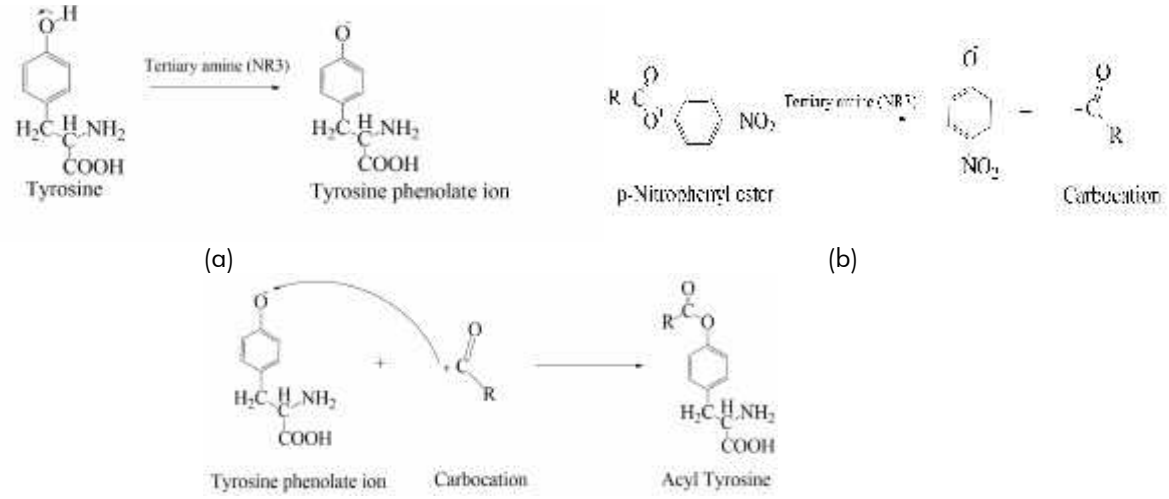


Fig: 11 (a) Formation of tyrosine phenolate (b) Formation of carbocation (c) Acylation of Tyrosine

It can also occur in coupling reactions mediated by carbonyldiimidazole with alcohols when tertiary amine is absent [25] [26] [27]. Since, imidazole acts as proton abstractor; it mediates the abstraction of proton from alcohols. The mechanism of the entire reaction is shown in figure 12.

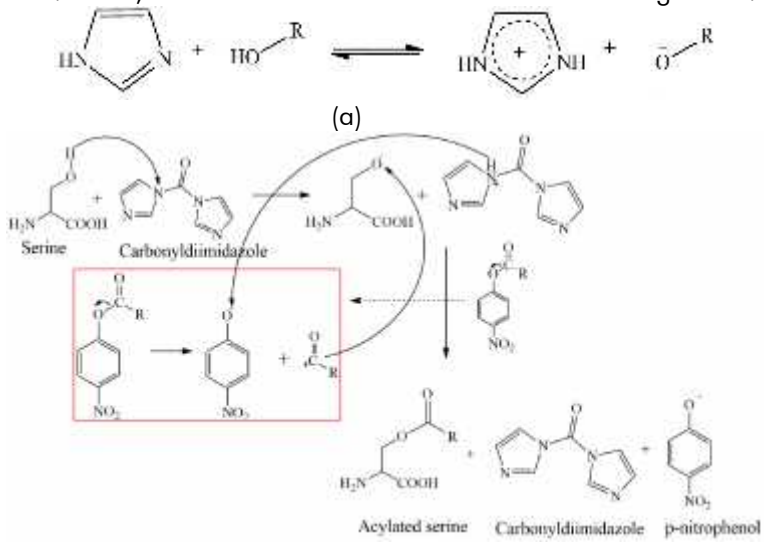


Fig: 12 (a) Formation of alcoholate (b) Overall reaction of O-Acylation in alcohols

Side reactions initiated by protonation

Racemization

It is an acid catalyzed reaction involving protonation of carbonyl oxygen resulting in the formation of a carbocation [8]. Proton abstraction then occurs at the adjacent carbon next to carbocation and therefore forms a double bond by sharing the electrons as shown in figure 13. This produces enolized products which do not retain their stereochemistry and lose their chiral purity [28].It requires a strong acid as protonation does not occur with weak acids. Racemization by protonation occurs during the process of deprotection of the groups using strong acids like HB or HF resulting in loss of chirality [29] [30].

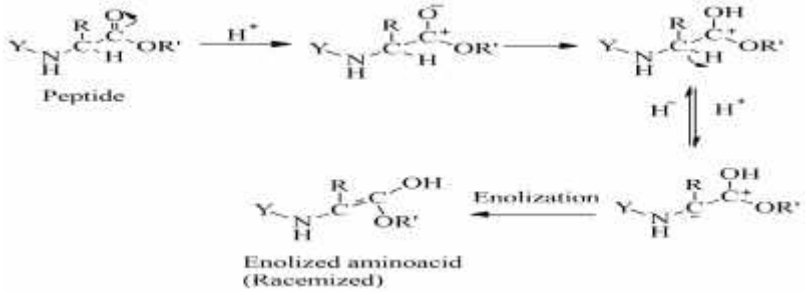


Fig: 13 Racemization through protonation

Cyclization

The products obtained by cyclization via protonation are same as that of products obtained by cyclization via proton abstraction. The only difference is that, former occurs in presence of acids where as latter occurs in the presence of the base [31]. The mechanism is explained in the figure 14 taking dipeptide (Aspartyl glycine) of which carboxylic acid end of aspartic acid is protected by oxy benzyl group. In the resulting products, the protecting group leaves as hydroxy toluene and the dipeptide forms a cyclic molecule which is a succinamide derivative [32].

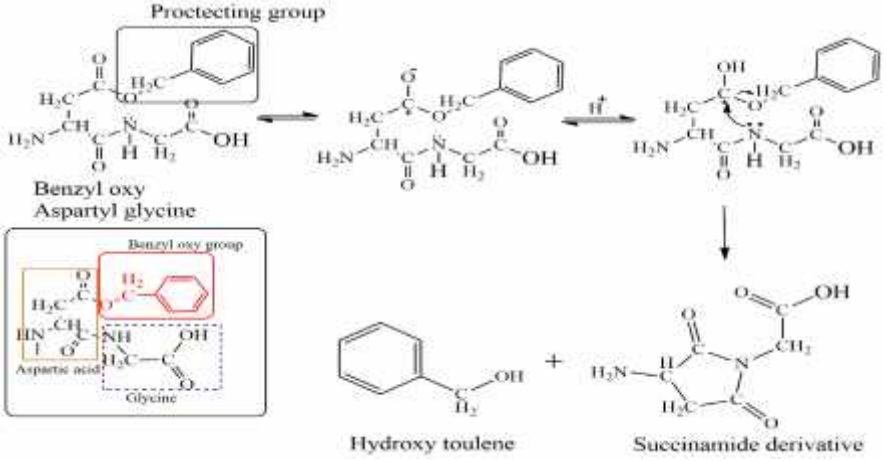


Fig: 14 Cyclization by protonation

Alkylation

Formation of carbocation is the general step during the removal of protecting groups from amino acids in presence of an acid [33]. These carbocations then act as alkylating agent to any nucleophilic centers and undergo intramolecular rearrangement to form the alkylated amino acid. The rearrangement reaction is shown in figure 15. Sometimes, the carbocations formed also react with the solvent surrounding them and form a better alkylating agent and act by electrophilic substitution reaction. Alkylation in tyrosine occurs only at *-ortho* position to hydroxyl group and not at *-meta* position due to steric hindrance by the bulkiness of the amino acid skeleton [34].

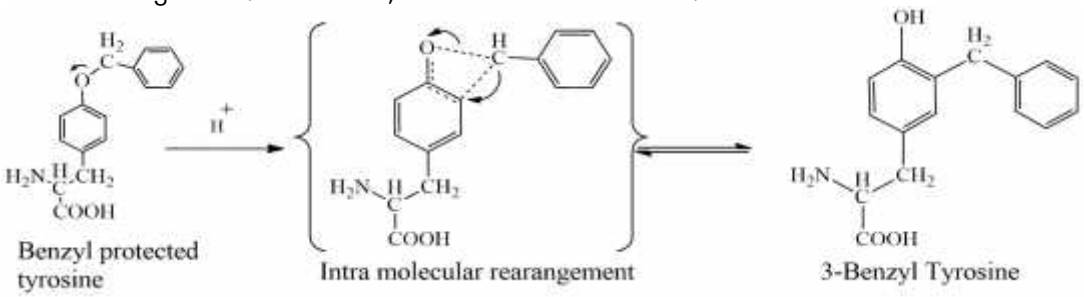


Fig: 15 Alkylation by intramolecular rearrangement

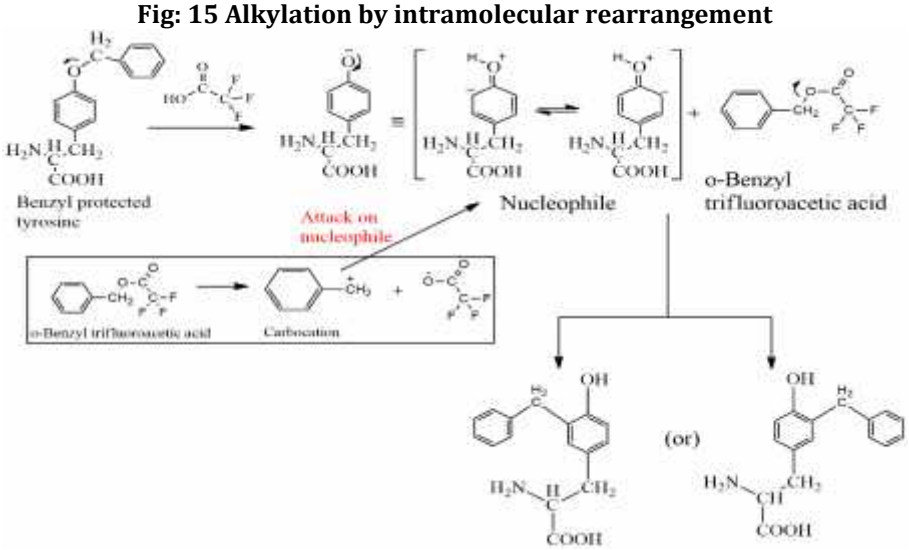


Fig: 16 Alkylation by electrophilic substitution

Chain Fragmentation

The amide bonds linking amino acids to each other to create the backbone of a peptide chains are stable enough to withstand the usual rigors of peptide synthesis. Under the influence of strong acids, an acyl group attached to the nitrogen atom of a serine residue migrates to its hydroxyl oxygen. Such an N →O shift takes place also when the acyl group is a part of a peptide chain [35] [36]. This reaction, which in all likelihood proceeds via cyclic intermediates, is easily reversed by treating the product with aqueous sodium bicarbonate but partial hydrolysis of the sensitive ester bond will lead to fragmentation of the chain [37]. The reaction of the fragmentation is shown in the figure 17 in which a dipeptide (serine and alanine) forms cyclic intermediate in presence of acid followed by acyl group attached to the nitrogen atom of serine residue shift to its hydroxyl oxygen and its hydrolysis to form two different amino acids.

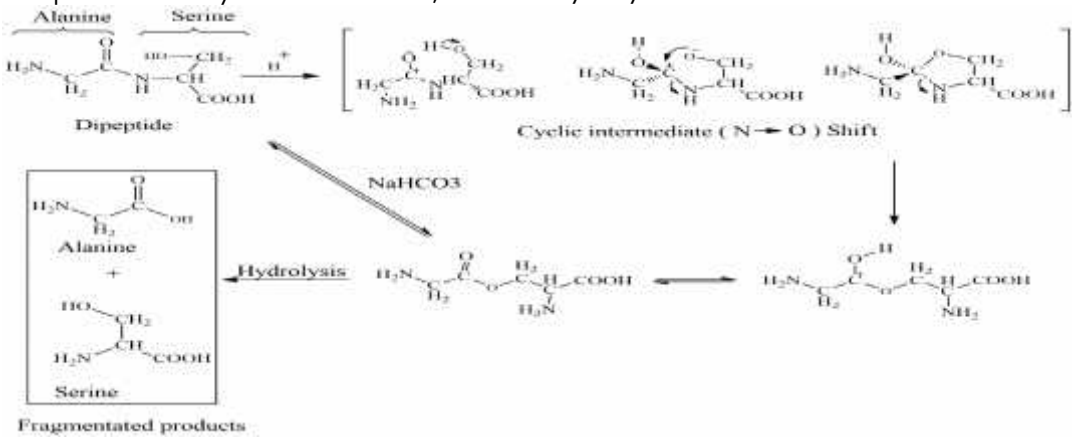


Fig: 17 Reaction showing the fragentation of peptide

Side reaction by overactivation

Overactivation occurs in the process of acylation of amino acid where the carboxyl component is too powerful to be acylated. Therefore, acylation occurs primarily at the amino group which is exposed for peptide bond formation followed by acylation of hydroxyl group of the carboxylic component.

Sometimes, during coupling of amino acids, using a coupling agent like N, N'- disubstituted carbodiimide, subtle intermediates are formed such as O-Acylisourea [38] which give rise to symmetrical anhydrides [39] [40] [41] and azlactones [9] [42] and can also undergo rearrangement to N-acylurea derivatives.

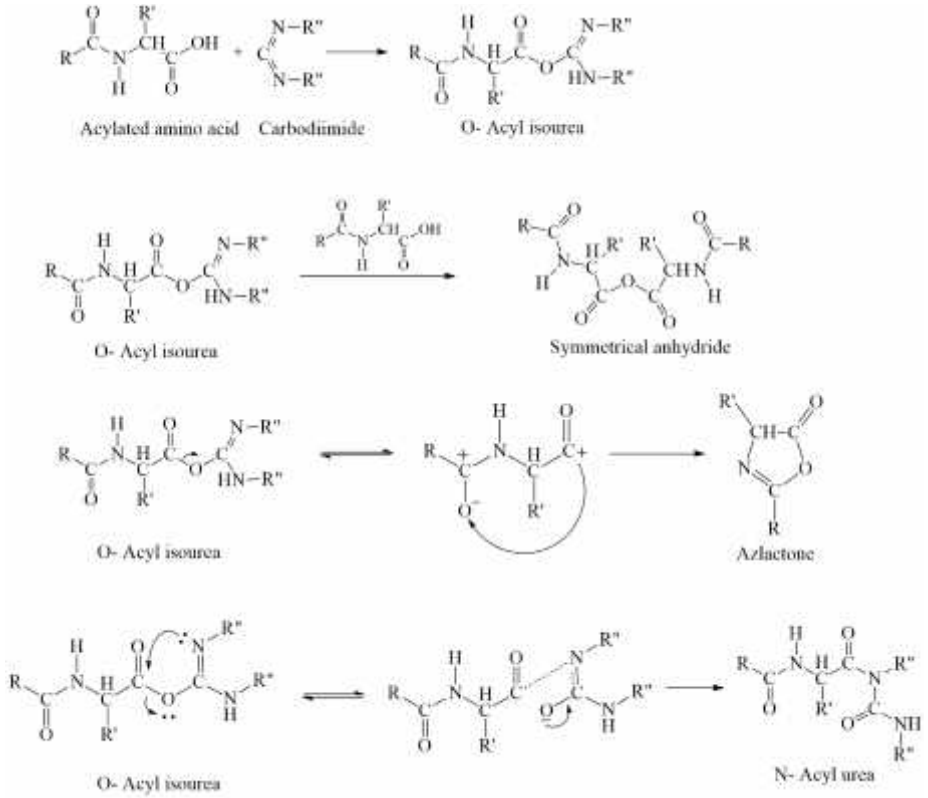
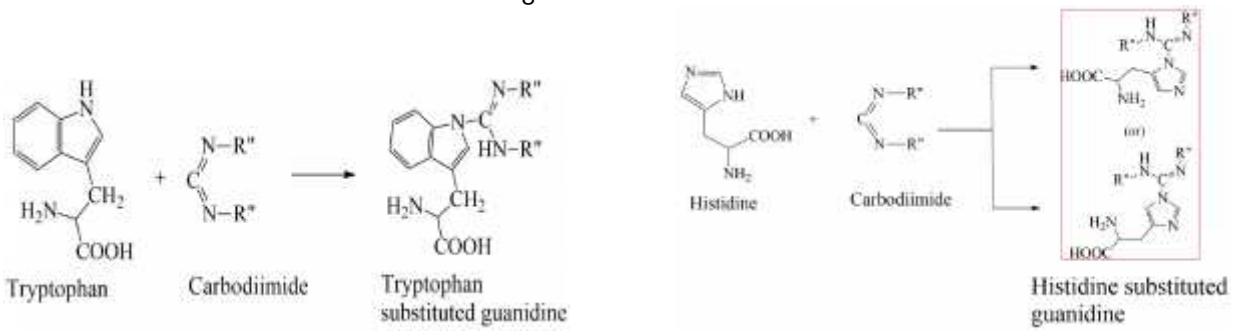


Fig: 18 Reaction showing complete overactivation

Imidazole containing amino acids such as tryptophan react with carbodiimide and forms substituted guanidine and similar is the case with that of histidine [43]. The reaction is shown in the figure 19. However, the O-Acylation or substituted guanidine side reaction that occurred can be revered by acid catalyzed methanolysis which is shown in the figure 20.



(a) (b)

Fig: 19 Formation of substituted guanidine in (a) Tryptophan (b) Histidine

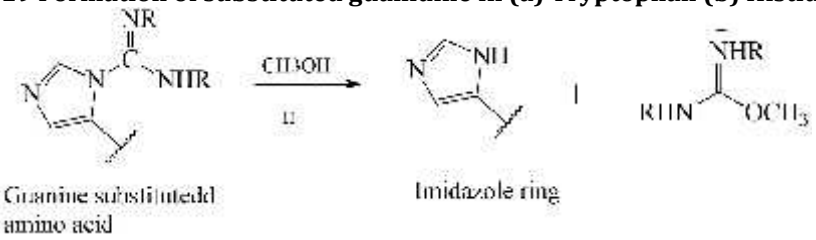


Fig: 20 Methanolysis of substituted guanidine

Side reactions related to individual amino acid residues

Amino acids with no functional side chains are not involved in side reactions which can be possible only in case of alanine and leucine as there is no side chain in alanine whereas in leucine, branching is at γ carbon which is far away from the α-carbon to undergo a side reaction. In case of valine and isoleucine, branching at β-carbon atom leads to steric hindrance which lowers the rate of coupling reaction and therefore, cause an increase in the extent of unimolecular side reactions. Figure 21 shows the condensation reaction with carbodiimide where isoleucine undergo a unimolecular side reaction (higher rate of reaction) by forming acylated intermediate (O-Acylisourea) [38] followed by ureides. However, the same O-Acylisourea when treated with a primary amine (amino acid) forms a peptide bond and has lower rate of reaction [9] [44]. Due to higher rate of reaction, formation of ureides is dominated over peptide bond formation.

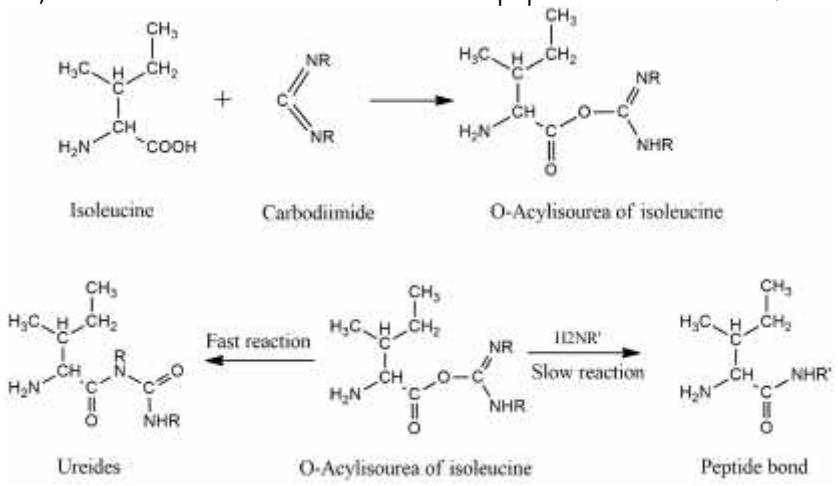


Fig: 21 Formation of ureides (left) and peptide bond (right) from O - Acylisourea

β - Carbon branching also interferes with other reactions such as alkaline hydrolysis and hydrazinolysis of alkyl esters. Alkylcarbonic mixed anhydride, which is a second acylation product (urethane), is formed as a result of coupling of valine or isoleucine [45] [46] [47] since the nucleophile has better chance to attack on the undesired carbonyl group. This causes the reaction to occur at other position rather than the desired position. In figure 22, when isoleucine is treated with trimethyl acetyl chloride, alkylcarbonic mixed anhydride is formed which upon treatment with a primary amine, undergo hydrolysis at the undesired carbonyl group rather than the desired carbonyl group. This is because of the bulkiness of isoleucine that prevents the hydrolysis at first carbonyl carbon.

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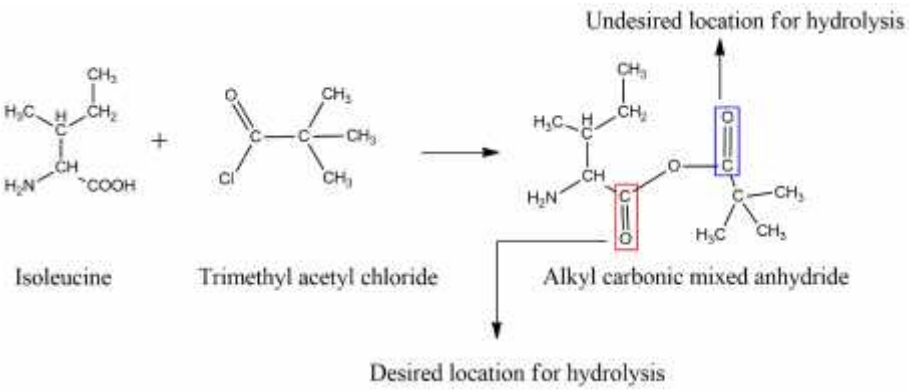

Fig: 22 Formation of Alkylcarbonic mixed anhydride

Despite forming alcoholates in presence of a base, alcoholic hydroxyls, under neutral conditions are reactive to undergo intramolecular reactions to get acylated at the carbodiimide activated carboxyl group and produce lactone [48] [49] which upon further treatment with another amino acid forms a peptide bond. Such reactions are seen in amino acids containing a hydroxyl group such as serine and threonine. Figure 23 shows the reaction of threonine forming lactone followed by a peptide bond which is similar for serine as well.

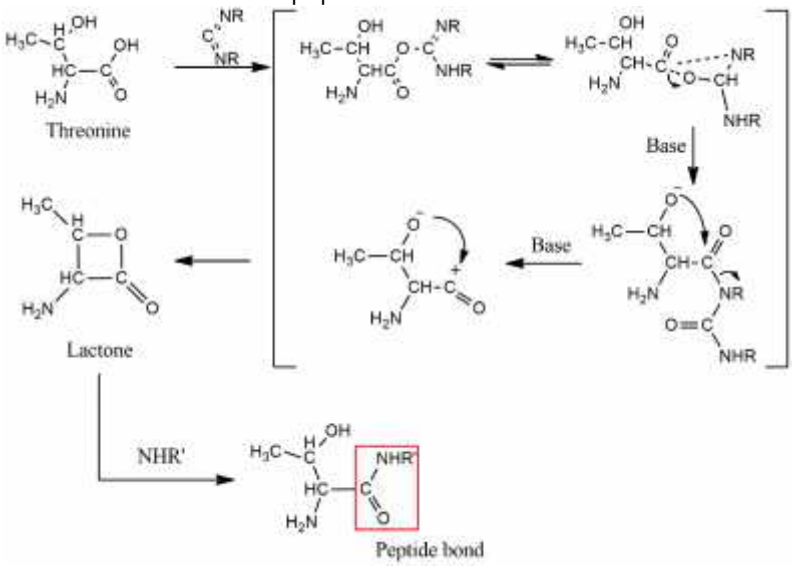

Fig: 23 Formation of lactone in threonine

In case of tyrosine, the phenolate ion formed after proton abstraction acts as an excellent nucleophile and gets acylated easily to form esters which can be later deacylated by treating with ammonia, hydrazine or hydroxylamine [50]. Inert side chain containing amino acid such as phenylalanine, do not undergo any side reaction but during catalytic hydrogenation, the aromatic ring gets saturated and gets converted to hexahydrophenylalanine (cyclohexyl alanine) [51].Glycine, which is devoid of any side chains, does not undergo any side reactions but its acylated amino group accepts second acyl group when treated with a powerful acylating agent and forms diacylamide [52] during the preparation of a dipeptide.

Conclusion

Peptide synthesis involves robust techniques as the side chains in the peptides are prone to side reactions which can degrade the amino acid or stop the peptide synthesis. Almost all the amino acids undergo side reaction due to the presence of side chains and those without side chain, forms various derivatives which are reversible and sometimes irreversible. Therefore, when peptides are being synthesized, the groups as well as the chains have to be protected and selective solvents have to be used for deprotection to get the desired peptide sequence with maximum yield.

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