CHAPTER II

PEPTIDE SYNTHESIS

2.1 Introduction

The first peptide was synthesized by Emil Fischer in the early 20^{th} century , but the modern era of peptide synthesis began in 1932 with the introduction by Max Bergmann and Leonidas Zervas of the carbobenzozy derivative for the protection of the α-amino group of amino acids and peptides (30). Today we have numerous other very useful protecting agents, and new and widely applicable coupling methods have been developed. However, peptide synthesis is essentially the same procedure as it was in 1932.

Now there are two standard methods used for peptide synthesis :

(a) Classical method

(b) Solid phase method

The classical method is also known as the liquid method. By using this method, reactive groups of the amino acids or peptides which are not to participate in the coupling reaction are protected. The protected components are coupled, either by prior activation of the carboxyl group or by the use of various coupling reagents. When the peptide chain has reached the desired length, all protecting groups are removed. With improved reagents such as coupling agents, protecting groups etc., the classical method is still one of the most widely used methods in peptide synthesis, particularly when large amounts are needed.

Solid phase method is a more modern technique which now competes with the classical method in the area of speed and efficiency. This method was developed by R.B. Merrifield in 1963 (31), and involves the attachment of the first amino acid of the chain to a solid polymer by a covalent bond, the addition of the succeeding amino acids one at a time in a stepwise manner until the desired sequence is assembled, and finally the removal of the peptide from the solid support. The solid support is usually polystyrene with the peptide being attached at its C-terminus, with the material retained within a glass column fitted with a sintered filter (32). The great advantages of the solid phase technique are the ease of operation and the high overall yield. Almost all synthetic peptides are now made by the solid phase method.

For this study, all peptide synthesis were carried out by the classical method.

2.2 Protecting Groups

Each amino acid residue has at least two reactive functional groups, amino group and carboxyl group . The reactive groups of the amino acids or peptides which are not to participate in the coupling reaction are protected. A suitable protecting group must fulfill the following criteria (33) :

1. The protecting group must be easy to introduce into the molecule.

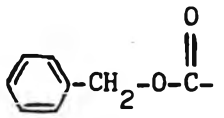
2. It must protect the functional group under conditions of amide formation.

3. It must be removable under conditions that leave the

newly created amide link intact.

2.2.1 Amino-Protecting Groups

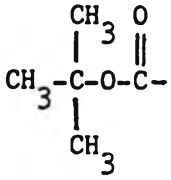
1. Benzyloxycarbonyl group (Carbobenzoxy or CBZ)



CBZ group

Carbobenzoxy-amino acids were prepared by the method of Schwartz et al. (34). The procedure involved the addition of benzylchloroformate to the unprotected amino acid at 0°C in aqueous alkaline solution. This protecting group is usually stable towards alkaline hydrolysis, but it can be removed by catalytic hydrogenation or hydrogenation with hydrogen bromide in glacial acetic acid or other organic solvent (35-38).

2. tert-Butyloxycarbonyl group (t-BOC group)



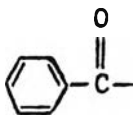
t-BOC group

This protecting group has found wide use since its introduction into peptide synthesis by McKay and Albertson (39) and by Anderson and McGregor (40) in 1957. The t-BOC group is introduced by treating the amino acid with t-butoxycarbonyloximinophenylacetonitrile (33). This group can be removed under mildly acidic conditions by treating the protected amino acid or peptide with anhydrous acid, such as trifluoroacetic acid (41) or hydrogen chloride in acetic acid (39). On the other hand, it is stable to hydrogenation, sodium in liquid ammonia and aqueous alkali.

3. Acyl-type protecting groups

The use of acyl-type protecting groups in peptide synthesis has been fairly extensive. The acyl amino acids, such as the acetyl and benzoyl amino acids, tend to undergo racemization and have found little use. Until the development of more convenient groups, such groups as the formyl and tosyl groups were used extensively for protection of the ε-amino group of lysine. Other acyl-type groups, including the trifluoroacetyl and O-nitrophenyl-sulfenyl groups, because of their ease of removal under specialized conditions, have found important applications.

In this work, the amino protecting groups used in all the experiments are either the carbobenzozy or the benzoyl group.



Benzoyl group

2.2.2 Carboxyl-Protecting Groups

As is the case with amino-protecting groups, a variety of carboxyl-protecting groups are available that can be selectively removed by various methods. It is thus possible to remove the carboxyl-protecting groups without affecting amino-protecting groups, or vice versa, and to choose different protecting groups for carboxyl groups so that one carboxyl-protecting group can be removed without affecting the others.

In this work, the carboxyl group of the amino acids were protected by the formation of their methyl esters. These esters are prepared by the method of Brenner and Huber (42). Using this method, thionyl chloride is first added to the alcohol followed by

the amino acid. The removal of methyl ester groups is usually accomplished by alkaline hydrolysis at room temperature. Various organic solvents in combination with aqueous alkali may be used. These include acetone, dioxane, methanol, ethanol and dimethylsulfoxide (43-46).

2.3 Peptide Bond Formation

Less than 20 years ago, only two general methods for forming the peptide bond were available, these being the acid chloride and azide procedures. During the past two decades several new general methods and a great variety of specific new reagents have been developed. It has become difficult, if not impossible, for each individual chemist to test all suggested methods.

Almost all methods that have assumed importance involve activation of the carboxyl group of an N-protected amino acid or peptide. Some derivatives, such as the acid chlorides, azides, and mixed anhydrides, are unstable and are prepared *in situ* or used immediately after preparation. On the other hand, most of the activated esters are practically indefinitely stable, and are isolated and stored in crystalline form. In other methods, the protected carboxyl and amino components are mixed and coupled by the addition of stable reagents, such as the ester phosphites, carbodiimides, Woodward 's reagent and carboxyldiimidazole.

All coupling methods involve the possibility of side reactions. Careful control of temperature, time, solvent and pH may be used to minimize these side reactions. The possibility of racemization is an ever present danger, and intermediates, solvents,

and activating reagents must be so chosen to minimize or eliminate this side reaction.

Some methods of peptide bond formation are shown below :

2.3.1 Acid Chloride Method

Acid chlorides, first used by Fischer for peptide synthesis, are rarely used today. Most amino acid chloride derivatives are unstable. The acid chloride procedure has been eclipsed by more convenient methods. Earlier, however, this procedure was used to prepare a variety of peptides (47-49).

2.3.2 The Azide Procedure

Curtins introduced the azide method at about the time Fischer began use of acid chloride procedure. Unlike the acid chloride method, however, the azide procedure is still in wide use. As previously indicated, the azide method is the only method which is free of racemization when the carboxyl group of a protected peptide is activated. In addition, the azide procedure is still the method of choice in the preparation of histidyl and seryl peptides (50-51).

Hydrazides are most often prepared by the reaction of hydrazine hydrate with methyl or ethyl esters, or, less often, with benzyl ester (52-53). Alcohol is frequently used as solvent, although dimethylformamide may be used where solubility problems are encountered. The hydrazides are treated with nitrous acid to give the reactive azides [Eq. (1)].

$$R\text{-}NH\text{-}\underset{\displaystyle R_1}{CH}\text{-}COOCH_3 \xrightarrow{NH_2NH_2} R\text{-}NH\text{-}\underset{\displaystyle R_1}{CH}\text{-}CO\text{-}NHNH_2 \xrightarrow{HNO_2} R\text{-}NH\text{-}\underset{\displaystyle R_1}{CH}\text{-}CON_3 \qquad (1)$$

The azides react with the amino component with release of gaseous NH_3 [Eq. (2)].

$$R\text{-}NH\text{-}\underset{\displaystyle R_1}{CH}\text{-}CON_3 + H_2N\text{-}\underset{\displaystyle R_2}{CH}\text{-}COOR_3 \longrightarrow R\text{-}NH\text{-}\underset{\displaystyle R_1}{CH}\text{-}CONH\text{-}\underset{\displaystyle R_2}{CH}\text{-}COOR_3 + NH_3 \quad (2)$$

The reaction may be carried out in anhydrous solvents or under Schotten-Bauman conditions.

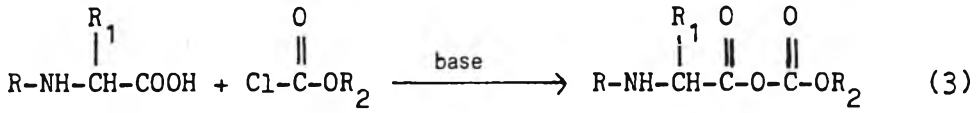
Side reactions are sometimes encountered during preparation of hydrazides. It has been reported that benzyl mercaptan may be split off during formation of hydrazides of S-benzyl-cysteine derivatives (54). Threonine- and serine-containing peptides suffer slight racemization. To suppress side reactions, the nitrite is added to the solution of the hydrazide at -5 to -10 °C. The azide, which forms within minutes, is extracted into organic solvents, dried briefly after washing, and used immediately for coupling. When dimethylformamide is used as solvent, the solution may be neutralized with a tertiary base and the amino component added directly to this solution (55).

2.3.3 Mixed Anhydride Method

Mixed anhydride methods were introduced by Wieland and co-workers (56). Mixed anhydrides between N-protected amino acids and a wide variety of organic and inorganic acids have been used in peptide synthesis. Those that have found the greatest application have been the anhydrides with monoesters of carbonic acid and with organic acids. The discussion here will be limited to these two types.

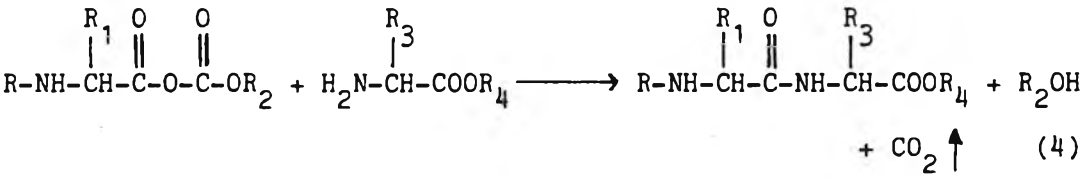
1. Mixed anhydrides with monoesters of carbonic acid

This method was introduced independently by Wieland and Bernhard (56), Boissonnas (57), and Vaughan (58). The anhydrides are formed by reaction of N-protected amino acids with various esters of chloroformic acid [Eq. (3)].



(3)

Aminolytic cleavage of the anhydride yields only CO_2 and an alcohol, in addition to the peptide [Eq. (4)].



(4)

Normally the condensation of the acyl amino acid and chloroformate require only a few minutes at -5 to -10 °C (58-59). However, temperatures as high as 10°C and reaction times up to 30 minutes have been used (60). Tetrahydrofuran is favored as a solvent, although many other solvents have been used. Triethylamine is generally used to neutralize the HCl formed.

Few side reactions are encountered with this method of bond formation. Aminolytic cleavage of the anhydride at the wrong place may occur on rare occasions (61-62). No racemization occurs when protected amino acids are coupled.

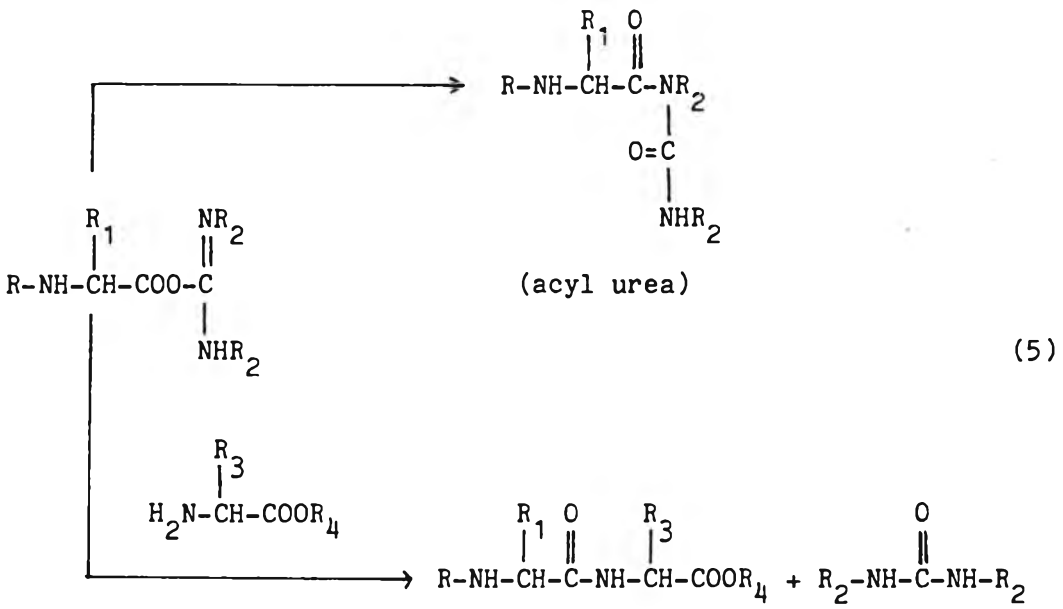
2. Mixed anhydrides with organic acids

Mixed anhydrides of protected amino acids with carboxylic acids tend to cleave in the manner that does not lead to

formation of the peptide bond (63). The organic acids that have been used with good success have been very limited. Good results have been obtained with the sterically hindered isovaleric (64) and pivalic acid (65).

2.3.4 The Carbodiimide Method

This method was introduced into peptide synthesis by Sheehan and Hess in 1955 (66). The initial reaction involves the formation of an intermidiate (67), further reaction leading either to the desired peptide or to an acyl urea [Eq. (5)].



(5)

This tendency to form acyl ureas is the main side reaction encountered with the carbodiimide method (38). Because low temperature reduces this tendency, a temperature of 0°C is generally used. The use of strong bases also favors this side reaction (68).

The first carbodiimide used for peptide synthesis, and still most commonly used, was N,N-dicyclohexylcarbodiimide. Dicyclohexylurea, the by-product of the reaction with this reagent,

is only slightly soluble in most solvents, but difficulty is sometimes encountered in removing the last traces of the urea from the product (69). However, special applicability of this method in the Merrifield solid phase procedure has added to its already wide use.

The addition to those methods which have already been discussed above, there are still many methods for peptide bond formation ; such as the active ester method, coupling via oxidation, Leuch 's anhydride method or enzymatic synthesis. The method used for peptide bond formation in this study is the mixed anhydride method, especially mixed anhydrides with monoesters of carbonic acid.