# Application of Proteases for the Total Enzymatic Synthesis of the Cholecystokinin Octapeptide (CCK-8) using Benzoyl-Arginine as an Enzymatically Cleavable N-Terminal Protecting Group

# Anwendung von Proteinen für die vollenzymatische Synthese des Cholecystokininoctapeptids (CCK-8) unter Verwendung von Benzoylarginin als enzymatisch spaltbare N-terminale Schutzgruppe

**DISSERTATION**

der Fakultät für Chemie und Pharmazie
der Eberhard-Karls-Universität Tübingen

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

2007

vorgelegt von

**Rajendra Joshi**

Gedruckt mit Unterstützung des Deutschen Akademischen Austauschdienstes

# Application of Proteases for the Total Enzymatic Synthesis of the Cholecystokinin Octapeptide (CCK-8) using Benzoyl-Arginine as an Enzymatically Cleavable N-Terminal Protecting Group

# Anwendung von Proteinen für die vollenzymatische Synthese des Cholecystokininoctapeptids (CCK-8) unter Verwendung von Benzoylarginin als enzymatisch spaltbare N-terminale Schutzgruppe

**DISSERTATION**

der Fakultät für Chemie und Pharmazie
der Eberhard-Karls-Universität Tübingen

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

2007

vorgelegt von

**Rajendra Joshi**

| | |
|---|---|
| Tag der mündlichen Prüfung | 04.04.2007 |
| Dekan: | Prof. Dr. Lars Wesemann |
| 1. Berichterstatter: | Prof. Dr. Heiner Eckstein |
| 2. Berichterstatter: | Prof. Dr. Günter Häfelinger |

This scientific research work was carried out from April 2004 to January 2007 at the Institute of Organic Chemistry, Faculty of Chemistry and Pharmacy, Eberhard-Karls-Universität, Tübingen, Germany, under the guidance of Prof. Dr. Heiner Eckstein.

I would like to express my sincere thanks to Prof. Dr. Heiner Eckstein for his excellent guidance during my Ph. D. period in Tübingen. Special appreciation is expressed for his permanent help, generous advices and constructive feedbacks.

A special debt of thanks is due to Prof. Dr. Günter Häfelinger, who critically evaluated the entire thesis and made several useful suggestions, which have been incorporated here.

I would like to acknowledge the financial support provided by DAAD (Deutscher Akademischer Austausch Dienst), without which this thesis would not have been a reality. I thank DAAD for providing me an opportunity not only to achieve my academic goal but also for giving me a chance to experience German culture and history.

I thank Dr. Liping Meng for helpful discussions and advices, Mrs. Margit Wizemann for her valuable technical assistance. Special thanks goes to Mr. H. Bartholomä, Mr. R. Müller and Mr. G. Nicholson for the MS measurements. Similarly Mr. Anurag Mishra and Ms. Deepti Jha equally deserve acknowledgement for their help during the use of the LC-ESIMS at the Max Planck Institute, Tübingen.

Last but not least, I would like to acknowledge and mention with sincere gratitude the efforts and encouragement made by my family members. I thank my wife Sunita for the correction of typing errors during the preparation of manuscript. I also thank my son Devansh for not disturbing me while putting things together.

*To My Parents and Family*

# Contents

# Abbreviations

| | |
|---|---|
| Ac | Acetyl |
| ACN | Acetonitrile |
| $AcONH_4$ | Ammonium acetate |
| Boc | *t*-Butyloxycarbonyl |
| Bz | Benzoyl |
| Bzl | Benzyl |
| C18 | Octadecyl |
| Cam | Carboxamidomethyl |
| Cbo or Z | Benzyloxycarbonyl |
| CCK | Cholecystokinin |
| α-CHY | α-Chymotrypsin |
| DCC | Dicyclohexylcarbodiimide |
| DCU | Dicyclohexylurea |
| DCM | Dichloromethane |
| DMF | Dimethylformamide |
| EDTA | Ethylenediaminetetraacetic acid |
| EtOAc | Ethyl acetate |
| EtOH | Ethanol |
| ESI | Electron spray ionization |
| FAB | Fast atom bombardment |
| Fmoc | 9-Fluorenylmethyloxycarbonyl |
| h | Hour |
| HPLC | High pressure liquid chromatography |
| HOBt | N-Hydroxybenzotriazole |
| Me | Methyl |
| MeOH | Methanol |
| m. p. | Melting point |
| min | Minute |
| MPLC | Middle pressure liquid chromatography |

| | |
|---|---|
| MS | Mass spectrometry |
| OAl | Allyl ester |
| OEt | Ethyl ester |
| OMe | Methyl ester |
| OPfp | Pentaflurophenyl ester |
| PE | Petroleum ether |
| PGA | Penicillin G amidase |
| Ph | Phenyl |
| Phac | Phenylacetyl |
| $R_f$ | Retention factor |
| RP | Reversed phase |
| T | Temperature |
| TPCK | L-1-Tosylamido-2-phenylethyl chloromethyl ketone |
| tert. | Tertiary |
| $t_R$ | Retention time |
| TFA | Trifluoroacetic acid |
| TEA | Triethylamine |
| TLC | Thin layer chromatography |
| TLN | Thermolysin |
| Tris | Tris-(hydroxymethyl)-aminomethane |
| U | Units |
| v | Volume |

Abbreviations of the common amino acids are used in accord with the IUPAC-IUB conventions (Eur. J. Biochem. 138, 9-37 (1984)). Amino acids, except glycine, are of the L-configuration.

# 1 Introduction

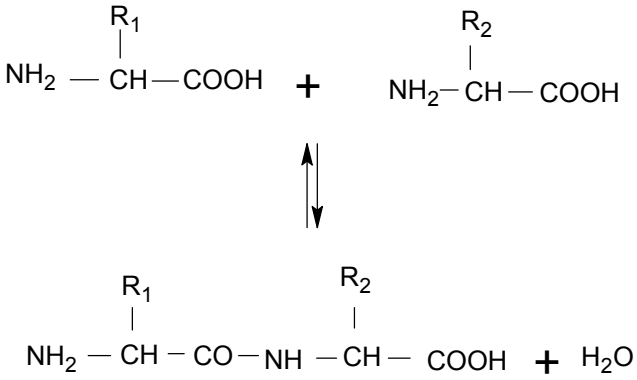
Peptides are amino acid polymers in which the individual amino acids are connected by amide linkages from the carboxyl group of one unit to the amino group of another. Peptides have the most diverse functions. Their diverse nature encompasses sweeteners, toxins, antibiotics as well as growth factors. Peptides can also act as stimulators and inhibitors of hormone release. Over 200 new peptide based drugs are under different stages of development with 50% of them under clinical trials are prior to approval (Marder and Albericio, 2003). Due to the importance of peptides, it has become a challenge for synthetic chemists to develop methodologies to construct peptides over the past few decades.

The synthesis of the first simple peptide derivatives by Curtius (1881) and then by Fischer and Fourneau (1901) marked the beginning of the peptide synthesis. The difficulties experienced in the synthesis of simple peptides stimulated a continuous effort toward improvements in the methodology of peptide synthesis. Introduction of the easily removable amino protecting benzyloxycarbonyl group (Bergmann and Zervas, 1932) was a major breakthrough in the field of peptide synthesis. The isolation of oxytocin in pure form (Pierce et al., 1952), the determination of its structure (du Vigneaud et al., 1953[a]; Tuppy, 1953) and its total synthesis (du Vigneaud et al., 1953[b]) gave a great impetus to the development of synthetic procedures. The introduction of dicyclohexylcarbodiimide, a still unsurpassed coupling reagent (Sheehan and Hess, 1955) had a major impact on the methodology of peptide bond formation and further refinement was brought about by the development of active esters (Schwyzer, 1953; Bodanszky, 1955). Long series of blocking groups which are cleaved under mild conditions were developed. Especially valuable are the tertiary butyloxycarbonyl (Boc) group (Carpino, 1957) and 9-fluorenylmethyloxycarbonyl (Fmoc) group (Carpino and Han, 1970) which are acid and base labile respectively. Originally all synthetic peptides were routinely prepared by conventional solution methods that is, both reactants were soluble in the reaction media. The invention of solid phase peptide synthesis (Merrifield, 1963) has simplified the operational procedures and opened the prospect of fully automated synthesis.

As a consequence of van't Hoff's concept of the equilibrium constant in a reversible chemical reaction (van't Hoff, 1898), it was concluded about 110 years ago that the hydrolytic action of enzymes should be reversible and the formation of the peptide bond should also be possible.

In 1938, Bergmann's group reported the first enzymatic synthesis of a defined peptide (Bergmann and Fraenkel-Conrat, 1938). Today the use of enzymes in peptide synthesis plays a promising role as a complement to the chemical method, in synthesizing peptides for research, clinical, and industrial interest (Mccoy, 2004).

The general condensation reaction of the peptide synthesis describes the connection of amino acids with the removal of water which is outlined in scheme 1.1.



**Scheme 1.1** Basic principle of peptide synthesis. $R_1$ and $R_2$ = side chain functionalities.

### 1.1 Chemical peptide synthesis

Chemical peptide synthesis takes place via three major steps which are described as follows.

**(1) First step**

As the amino acid contains at least two functional groups, the carboxyl component and the amino component of the non reacting end have to be protected. If the side chain has a reactive functional group, then it also needs to be protected. In synthesis of a larger peptide, a careful planning is required to match the coupling methods and protecting

groups. The most important protecting groups applied in chemical peptide synthesis are described as follows.

***Amino protecting groups***

***Cbo or Z group.*** The benzyloxycarbonyl group (Bergmann and Zervas, 1932) is an important amino protecting group which smoothly undergoes homolytic fission on catalytic hydrogenation. The process of hydrogenation is generally carried out in presence of a palladium catalyst deposited on charcoal. Other methods, for instance reduction with sodium in liquid ammonia (Sifferd and du Vigneaud, 1935) and acidolysis (Ben Ishai and Berger, 1952), particularly by HBr in acetic acid are also used.

***Boc group.*** The tert. butyloxycarbonyl group was introduced by Carpino (1957). The Boc group is similar to the Cbo group but the benzyl group is replaced by the tert. butyl group. The +I inductive effect of the three methyl groups of the Boc group gives rise to a stable cation, hence in contrast to the Cbo group, the Boc group can be easily cleaved by trifluoroacetic acid.

***Fmoc group.*** The 9-fluorenylmethyloxycarbonyl group was introduced by Carpino and Han (1970). This group is removed from the amino group by proton abstraction with secondary amines. Piperidine and diethylamine are the recommended bases.

***Carboxyl protecting groups***

The general approach for carboxyl protection is esterification.

***Methyl ester and ethyl ester.*** These esters are readily available through esterification, that is, by the introduction of HCl into an alcoholic suspension of the amino acid (Curtius and Goebel, 1888) or by the addition of the amino acid to a cold mixture of thionylchloride and methanol or ethanol (Brenner and Huber, 1953). Generally, methyl and ethyl esters are good blocking groups, because they can be cleaved easily by alkaline hydrolysis.

***tert. Butyl ester.*** The tert. butyl ester is prepared by the addition of isobutene to the carboxyl group (Roeske, 1959) or transesterification of carboxylic acid esters with tert.butyl acetate (Taschner et al., 1961). It is generally resistant against nucleophilic attack, base catalyzed hydrolysis and weak acids. However, the tert. butyl group is removed with moderately strong acids, such as diluted solutions of HCl in acetic acid or trifluoroacetic acid in dichloromethane.

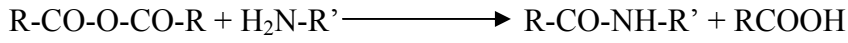
**(2) Second step**

Formation of an amide bond between two amino acids is an energy-requiring reaction. In order to avoid high temperature to form a peptide bond, either the carboxyl or the amino group must be activated. So far, no practical activation of the amino group has been found. The usual approach is activation of the carboxyl group, that is, the hydroxyl group of the carboxyl group is replaced by an electron withdrawing substituent (X). The X group can enhance the polarization of the carbonyl group and thereby the electrophilicity of its carbon atom. Thus, the nucleophilic attack by the amino group is greatly enhanced. The peptide bond is formed by coupling the carboxyl component and the amino component via an α-amide linkage. The most important practical coupling methods are specified below.

***Azide method.*** The activation in the form of acid azides (Curtius 1902) remains even today an important and practical approach for the synthesis of peptides. Hydrazinolysis of alkyl esters and then conversion of hydrazides to acid azides with the help of nitrous acid or alkyl nitrites are still practiced for the activation. The direct conversion of carboxylic acids to acid azides with the help of diphenylphosphorylazide has become an alternative way in recent years. This method is important because there is negligible racemization and therefore it can be used for fragment condensation. The major drawback of this method is the Curtius rearrangement of acid azides to isocyanates.

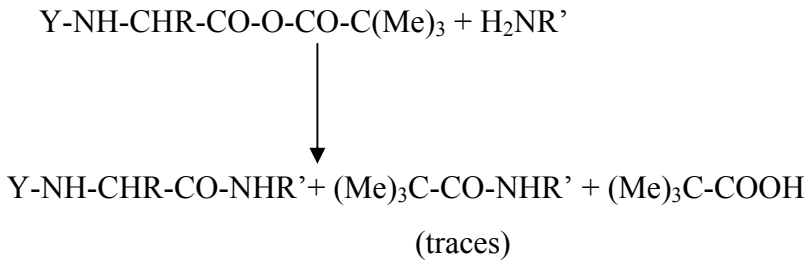
***Anhydride method.*** The treatment of amines with anhydrides of carboxylic acids leads to acylation. Reacting a symmetric anhydride of a peptide or an N-protected amino acid with the amino component of an amino acid or peptide would result in the loss of half of

the carboxylic unit of the anhydride because only half of it is used for peptide bond formation as shown in following reaction.



$$R\text{-}CO\text{-}O\text{-}CO\text{-}R + H_2N\text{-}R' \longrightarrow R\text{-}CO\text{-}NH\text{-}R' + RCOOH$$

Therefore, certain unsymmetrical anhydrides which react with the amino component only at the carbonyl of the N-protected aminoacyl component are used (Chantrenne, 1947). The reaction of the amino component only at a particular carbonyl group takes place because of the difference of electrophilic character between two carbonyl groups. The reaction of the unsymmetrical anhydride with an amino component is given below where Y is the amino protecting group.



$$Y\text{-}NH\text{-}CHR\text{-}CO\text{-}O\text{-}CO\text{-}C(Me)_3 + H_2NR'$$

$$\downarrow$$

$$Y\text{-}NH\text{-}CHR\text{-}CO\text{-}NHR' + (Me)_3C\text{-}CO\text{-}NHR' + (Me)_3C\text{-}COOH$$

(traces)

***Carbodiimide method.*** This method was introduced by Sheehan and Hess (1955). The coupling reagent, dicyclohexylcarbodiimide (DCC) is added to the mixture of the carboxyl component and the amino component. Thus, activation and coupling proceed concurrently. Amines also react with carbodiimide but the rate of this reaction is negligible, when compared with the rapid rate observed in the addition of carboxylic acids to one of the double bonds of a carbodiimide. Addition of carboxylic acid to carbodiimide results in the formation of O-acyl isoureas as an intermediate. The N=C group in O-acyl-isoureas provides activation which leads to coupling. The insoluble by-product, N, N'-dicyclohexylurea (DCU) formed in this reaction can be removed easily by filtration. In some cases, both racemization and N-acyl urea formation takes place. Both of them can be suppressed to some extent by the addition of auxiliary nucleophiles such as 1-hydroxybenzotriazole (HOBt) as proposed by König and Geiger (1970).

***Active ester method.*** The carboxyl group is activated by the formation of an active ester in which an electron withdrawing group is present. The exclusive application of *p*-nitrophenyl esters for the synthesis of oxytocin (Bodanszky and du Vigneaud, 1959) demonstrates that active esters are particularly well suited for the stepwise elongation of a peptide chain by the addition of single amino acid residues.

**(3) Third step**

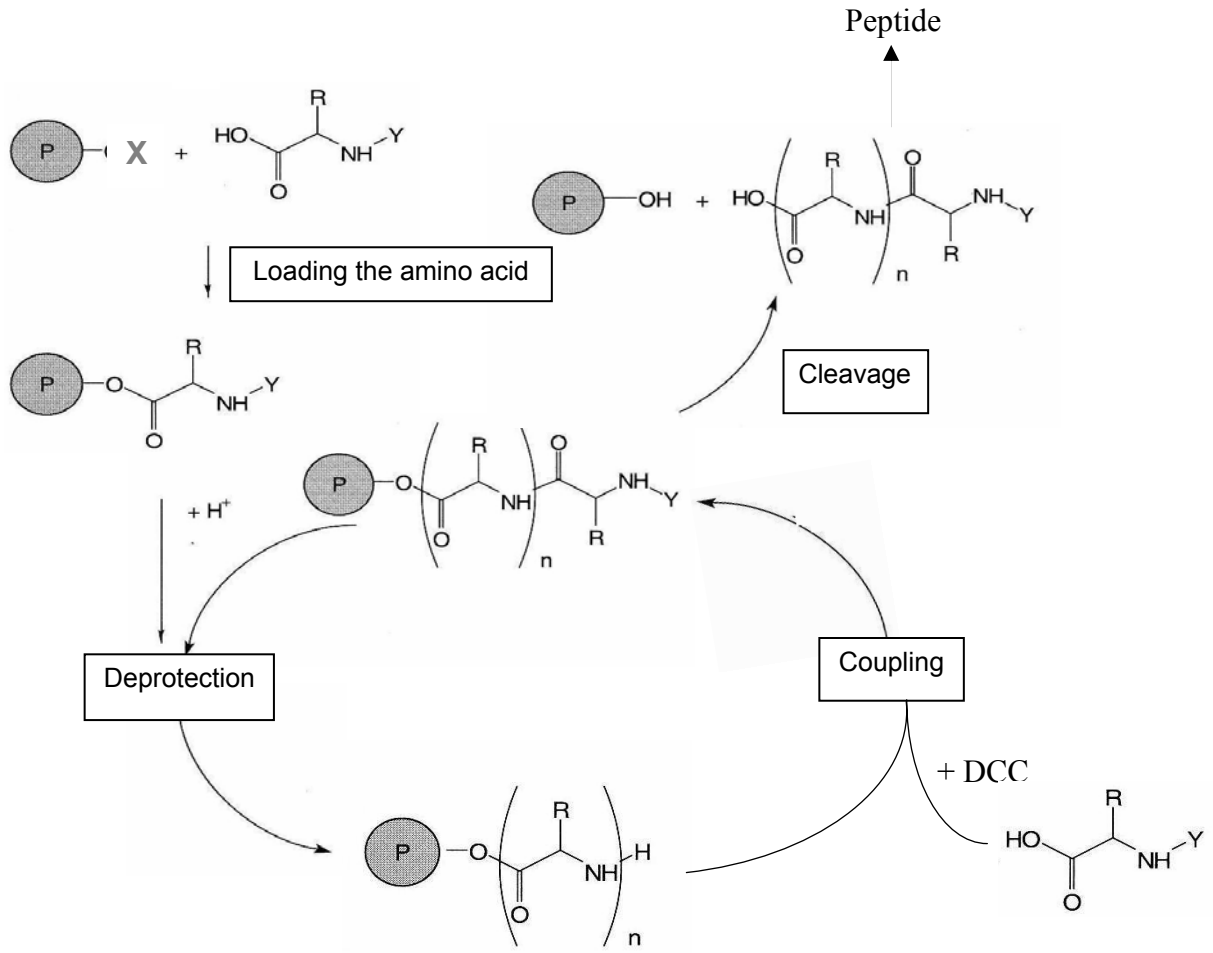
After the synthesis is complete, the protecting groups must be totally removed in order to release the free target peptide. If the synthesis is to be continued, temporary protecting groups must be removed prior to each elongation step of the growing peptide chain.

### 1.1.1 Classical peptide synthesis

Both reacting components are mixed in homogenous solution. After each coupling step, the purification of the product is done, generally through crystallization or chromatographic methods.

### 1.1.2 Solid phase peptide synthesis

This method was introduced by Merrifield (1963). The basic plan is illustrated in Scheme 1.2. An amino acid with protected amino group is bound covalently to a synthetic polymer that bears reactive groups (X) (e.g. chloromethylated polystyrene). Peptide bond formations are carried out by repeating deprotection and coupling step. The peptide of the desired sequence is assembled on the polymer support. Finally, the peptide is cleaved from the support with the appropriate reagent.




**Scheme 1.2** Reaction scheme of the solid phase peptide synthesis. Y= α-amino protecting group; X= reactive group; P= polymer.

The essential advantage of using a solid support is that all the laborious purifications at intermediate steps in the synthesis are eliminated. Only filtration and washing procedures are necessary and automatization is easy. However, one of the major disadvantages of the solid phase synthesis is the occurrence of failure and truncated sequences, if the yield of every coupling step is less than 100% (Bayer et al., 1970).

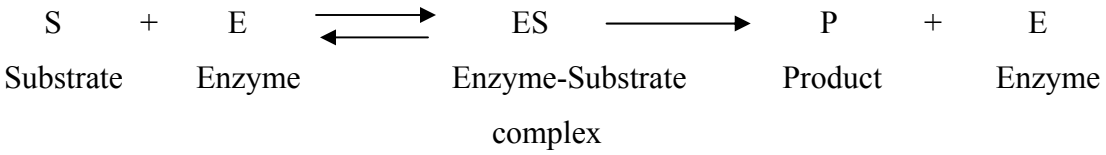
## 1.2 Enzymatic peptide synthesis

During the chemical peptide synthesis, the limitations arise mainly from the many possible side reactions (Bodanszky et al., 1979; Bodanszky and Martinez, 1981). Racemization has been a major problem in the chemical peptide synthesis throughout its history. To circumvent these problems, an increasing number of peptide syntheses are conducted under the catalysis of enzymes (Jakubke et al., 1985; Isowa et al., 1979; Jakubke et al., 1996).

### 1.2.1 Enzymes

In order to achieve the high reaction rates observed in living organisms, enzymes are required to lower the activation energy of the reaction. Some enzymes are purely protein. In other cases, the functional enzyme consists of two parts, collectively called a holoenzyme, an apoenzyme (the protein portion) and a cofactor. Depending on the enzyme, the cofactor may be an ion of a metal such as copper, iron, zinc or an organic molecule needed to assist the reaction in some particular way.

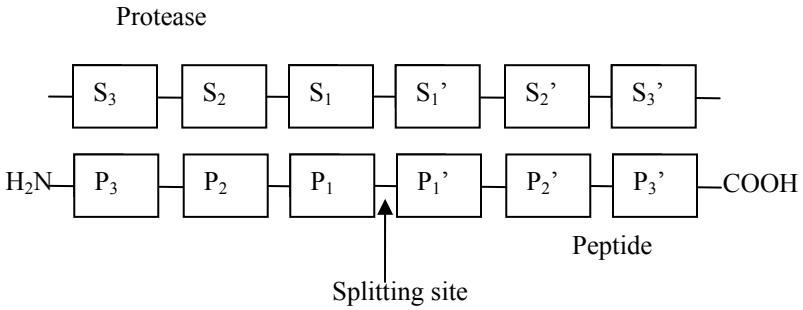
In the first step, an enzyme must come into contact with reactants, which are called substrates in the case of enzyme–mediated reactions. The enzyme combines with the substrate to form an enzyme substrate complex, which breaks down to release products and enzymes. The reaction between enzyme and substrate can be written as




At the end of the reaction, the enzyme is free to undergo the same reaction with additional substrate molecules. The overall effect is to accelerate the conversion of a substrate into the product, the enzyme acting as a catalyst. The region of the enzyme, to which the substrate binds, is known as the enzyme's active site. Each enzyme is highly specific. On the basis of the catalyzed reactions, enzymes can be classified into oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Among them, hydrolases have been the subject of more intensive study than any other.

**(1) Proteases**

Proteases are those sorts of hydrolases, which cleave peptides within their chain into smaller peptide chains (endoproteases) or hydrolyze peptides from the end to release single amino acids at each reaction cycle (exoproteases). The reversal of the proteolytic cleavage of the peptide bond by proteases leads to the enzymatic peptide synthesis. The shorthand notation of the specificity of protease, proposed by Schechter and Berger is represented in Scheme 1.3. According to this model, peptides undergoing cleavage are designated $P_1$, $P_2$, $P_3$ etc. in the N-terminal direction from the cleaved bond. Likewise, the residues in C-terminal direction are designated $P_1$', $P_2$', $P_3$' etc. In the same way, active site of the protease is named as $S_1$, $S_2$ or $S_1$', $S_2$' corresponding to $P_1$, $P_2$ or $P_1$', $P_2$' of peptide respectively.




**Scheme 1.3** Schematic representation of substrate-protease interactions (Schechter and Berger, 1967).

There are four main types of endoproteases.

***Serin proteases (e.g. Chymotrypsin, Trypsin, Elastase).*** They are characterized by a highly reactive serine residue in the active site. In the covalent substrate enzyme complex, an acyl ester is formed between the carboxylic acid of the bond being split and the serin hydroxyl function.

***α–Chymotrypsin.*** It hydrolyzes amides and esters as well as peptides and shows a marked preference for links involving aromatic amino acid residues, such as phenylalanine, tyrosine or tryptophan ($P_1$ site) (Bergmann and Fruton, 1937; 1938). The primary specificity of α-chymotrypsin, which is definitely associated with the $P_1$ position, may be extended, if the esterase activity of the enzyme is also considered (Kloss and Schröder,

1964). The secondary specificity of the enzyme is expressed to a minor degree, so that there exists a certain preference for hydrophobic amino acid residues in the $P_2$ and $P_1$' position of the chymotryptic substrates (Bauer, 1978).

***Trypsin.*** It is also a serin endopeptidase whose action is strictly confined to peptide linakge, the carbonyl group of which is contributed by a basic amino acid such as lysine or arginine (Keil, 1971). The aspartate residue located in the catalytic pocket ($S_1$) of trypsin is responsible for attacking and stabilizing positively charged lysine or arginine. Trypsin catalyzed peptide synthesis proceeds under kinetic control and trypsin have an optimal operating pH of about 8 and optimal operating temperature of about 50° C.

***Cysteine protease (e.g. Papain, Ficin).*** The catalytic mechanism involves a thioester linkage with the carbonyl moiety of the peptide bond and the side-chain of a cysteine in the active-site.

***Papain***. Papain is isolated from *Carica papaya.* It displays rather broad substrate specificity, but nevertheless, it exhibits a preference for bulky hydrophobic amino acid residues in the $P_2$ position of a given substrate (Fruton, 1982). Thus, the $P_1$ site of the substrate, associated with the secondary specificity of papain, can be occupied by a variety of amino acids (Brubacher and Zaher, 1979). Due to the structural stability of papain over a wide pH range, papain-controlled peptide synthesis has been performed within a broad pH spectrum, which ranges from 4.7 (Milne et al., 1957) to 9.5 (Mitin et al., 1984).

***Acid proteases (e.g. Pepsin, Chymosin).*** The active site of acid proteases relies on two acidic aspartate amino acids. The acid proteases have the pH optimum in the low acid range.

***Metalloproteases (e.g. Thermolysin).*** They possess metal ions in the active centre. An example is thermolysin, which is mainly used in thermodynamically controlled reactions.

***Thermolysin.*** The neutral metalloprotease, isolated from *Bacillus thermoproteolyticus* has frequently been used in enzymatic peptide synthesis. The enzyme binds one zinc ion which is essential for catalytic activity and four calcium ions which are required for its thermostability. Thermolysin exhibits a strong preference for peptide bonds, in which $P_1$' is a bulky hydrophobic amino acid residue except in the case of tryptophan. Because of its stability at elevated temperature, the reactions can be catalyzed at higher temperature with increased solubility of the educts.

**(2) Amidases**

One of the sub-groups of hydrolases comprises a number of enzymes hydrolysing –CO-NH- links which are not concerned with protein breakdown.

***Penicillin G Amidase (PGA).*** The enzyme from *Escherichia coli* cleaves not only the phenylacetyl group of benzylpenicillin (Penicillin G), but also a variety of substituted and unsubstituted phenylacetamides that are not related to benzylpencillin (Margolin et al., 1980). The $S_1$-subsite specificity of PGA is mainly restricted to phenylacetic acid and its derivatives (Virden, 1990). The exploitation of the hydrolytic potential of amidases other than proteases for the enzymatic removal of $N^{\alpha}$-blocking groups in peptide synthesis has the advantage that it does not cleave peptide bonds. In this connection, Widmer et al., (1983) and Brtnik et al., (1981) suggested the use of PGA as a catalyst for $N^{\alpha}$-deprotecting steps.

### 1.2.2 Enzyme immobilization

Here, immobilization is understood as the attachment of enzymes on insoluble support materials mostly polymers. Since enzymes are sometimes not stable in the presence of high concentrations of organic solvents or in the presence of hydrophobic interfaces of nonmiscible solvents (Kasche et al., 1987), immobilization can be used to reduce enzyme denaturation. Because immobilized enzyme molecules are not able to autolyze and aggregate due to full dispersion, immobilized enzymes show better stability than that of the free enzymes (Klibanov, 1979; Capellas et al., 1996[a]; 1996[b]).

Besides the higher stability, the immobilisates have many other advantages, e.g. the reaction can be performed continuously in a reactor and controlled easily (Eckstein and Renner, 1995). Repeated use of the immobilisates might be important from the view of economy, especially in industrial processes. The immobilized enzymes can be easily and completely separated from the reaction system and do not contaminate the synthesized peptides, which is of importance in case of pharmaceutical use of peptides. The immobilized enzyme can be recovered by a simple filtration step.

There are several methods for the immobilization of enzymes.

- By inclusion using gel or membrane
- By adsorption on ion exchangers
- By deposition onto solid materials (e.g. Celite)
- By chemical covalent bonds on polymers. It can take place either by cross-linking reaction of the enzymes or by preferentially binding the enzymes onto the outer shell of modified carrier materials with preselected properties (Horvath and Engasser, 1973).

### 1.2.3 Advantages of the enzymatic peptide synthesis

The most obvious advantage of enzymatic synthesis results from one of the most impressive biological phenomena, namely, the pronounced capacity of an enzyme to catalyze chemical reactions with an otherwise unattainable specificity.

Proteases usually demonstrate an absolute preference for the L-enantiomeric form of an amino acid residue. Hence, the enzymatically catalyzed peptide bond formation results in an optically pure product, even if the starting material might have been contaminated with some D-enantiomer.

The proteases are only associated with the α-carboxyl and the α-amino group of the sensitive peptide bonds. Therefore, in a protease catalyzed reaction, generally the side chains and other functional groups are not needed to be protected in contrast to the chemical peptide synthesis.

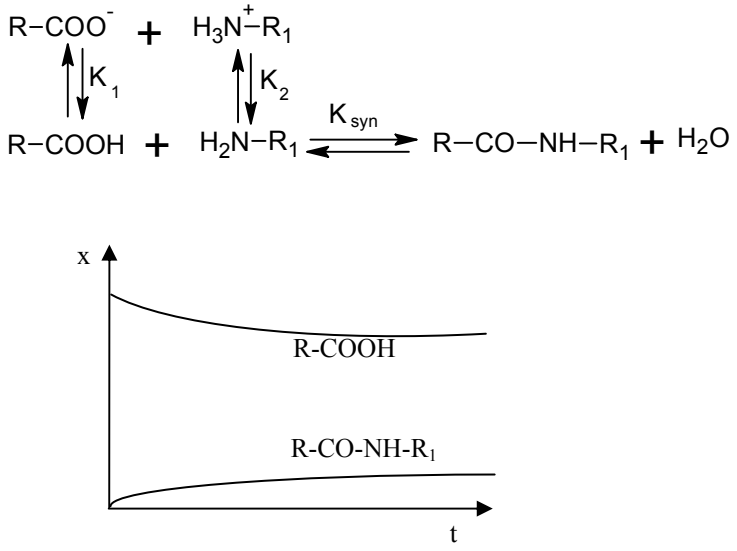
In addition, enzymatic peptide synthesis can be achieved under mild conditions with commercially available proteases which are easy to handle and, due to their application in catalytic amounts only, the costs are mostly insignificantly higher in comparison with chemical coupling reagents. By avoiding hazardous chemicals and less critical reaction steps, this method is a good example of "green chemistry". A very good example is the synthesis of aspartame on a large scale in industry, starting from L-Cbo-Asp-OH and D, L-Phe-OMe (Isowa et al., 1979).

### 1.2.4 Methods of enzymatic peptide synthesis

The enzyme-catalyzed peptide bond formation can be divided into two approaches namely thermodynamic and kinetic approaches.

#### 1.2.4.1 Thermodynamically controlled peptide synthesis

In this approach, peptide bond forms by the free carboxyl terminus which is a direct reversion of the protein hydrolysis. This is illustarted in scheme 1.4




. **Scheme 1.4** Thermodynamically controlled peptide bond formation. R = acyl residue of the carboxyl component; $R_1$ = residue of the amino component; x = relative concentration (%); t = time.

It has to be noticed that only non-ionized substrates (amino acids or peptides) participate in the peptide bond formation (Linderstrom–Lang (1962) and Carpenter (1960)). In practice, due to the amphoteric nature of the amino acid or peptide, they are ionized over the entire pH range. They can behave either as cations in an acidic environment or as anions in a basic environment. Thus, the predominant contribution to the energetic barrier to proteosynthesis is accounted for by ionization-neutralization effects. There are two ways to shift equilibrium towards the desired peptide coupling:

**(1) Shift of ionic equilibria**

The main obstacle to peptide bond synthesis, which comes in the form of the energy required for the proton transfer, depends crucially on the ionization equilibria of the reactants. An increase of the $pK_1$ value and/or a decrease of the $pK_2$ value of a given pair of educts, will reduce the energy consumption of the proton transfer and will, as a result, cause the equilibrium shift in favour of peptide synthesis.

***Cancelling the zwitterionic character of the reactants.*** The highly endergonic characters of the proton transfer can be accounted for by the strong acidity and basicity of the α-carboxyl and α-amino groups of the reactants which in turn, can be attributed to the zwitterionic nature of free amino acids and peptides. Consequently, a favourable pK shift of the ionogenic groups may be achieved by curtailing the zwitterionic character of the respective educts. This can be readily done by the introduction of α-amino and α-carboxyl protecting groups, a procedure which is routinely used in peptide synthesis.

***Effects of organic co-solvents.*** Another technique to shift ionic equilibria in favour of peptide synthesis is to add organic solvents to the reaction mixture. By lowering the dielectric constant of the reaction medium, the organic co-solvent diminishes the hydration of ionic groups. This effect predominantly affects the pK value of those groups whose ionization represents a separation of charge (Michaelis and Mizutani, 1925), most notably carboxyl functions. Thus, the acidity of the α-carboxyl group and to a lesser extent, the basicity of the α-amino group can be reduced (Mizutani, 1925; Homandberg et al., 1978).

**(2) Shift of chemical equilibria**

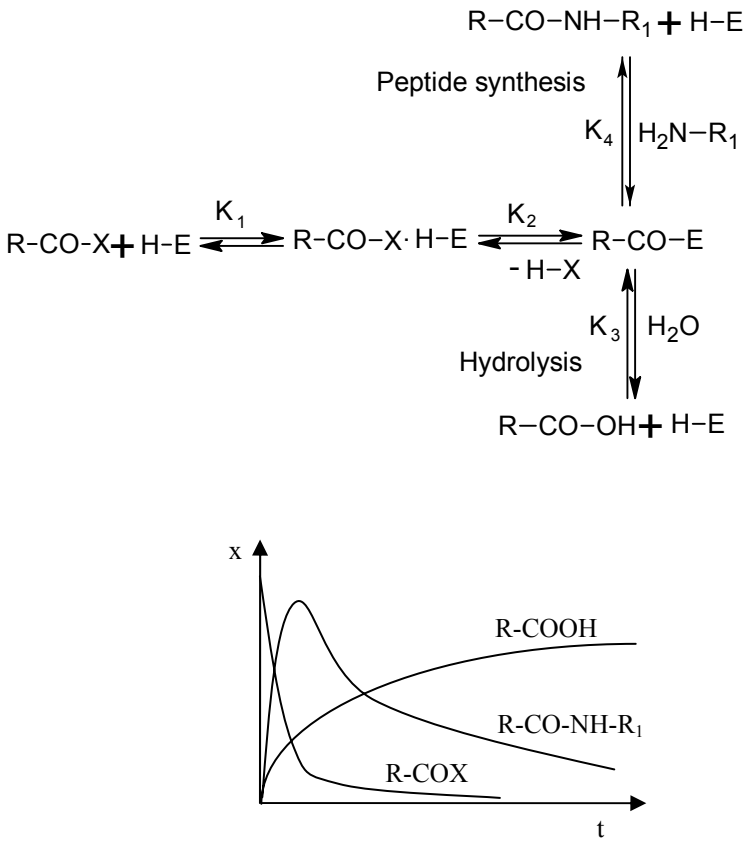
***Solubility-controlled synthesis.*** This technique is based on the removal of the newly generated products from equilibrium by precipitation (Bergmann and Fraenkel, 1938; Bergmann and Fruton, 1938)) or selective extraction in a biphasic system (Basso et al., 2000; Semenov et al., 1981).

***Effects of concentration.*** According to the law of mass action, the unfavourable equilibrium can be shifted towards synthesis by the means of the highest possible concentrations of the educts, especially of the amino component or by the means of using organic co-solvents to reduce the water concentration (Calvet et al., 1996).

***Temperature-dependent equilibrium shifts.*** The endothermic process of peptide bond formation (Borsook, 1953) will be enhanced with rising temperature, as governed by the Le Chatelier's principle.

### 1.2.4.2 Kinetically controlled peptide synthesis

In the kinetic approach the carboxyl component is employed in an activated form, mainly as an ester derivative, and the synthesis occurs under kinetic control. The rapidly formed acyl enzyme intermediate can transfer the acyl moiety either in an aminolysis reaction to the added amino component forming the desired peptide product (R-CO-$NHR_1$), or in a hydrolysis step with water forming R-CO-OH (Scheme 1.5).




**Scheme 1.5** Kinetically controlled peptide bond formation. R= acyl residue of the carboxyl component; $R_1$= residue of the amino component; H-E= protease; X= ester (leaving group); x= relative concentration (%); t= time.

The optimal peptide yield depends on both the concentration and reactivity of the nucleophile as well as the rate of hydrolysis of the acyl-enzyme. Hence, it is important to favour the synthesis of the peptide bond by working at high concentration of the nucleophile and at low water concentration to minimize the proteolytic activity. As shown in scheme 1.5, the concentration of the product $RCONHR_1$ is at its maximum at a particular time. The reaction has to be stopped at the "kinetic" optimum. It means if the reaction is terminated well before the equilibrium is established, the products which accumulate are those that are produced most rapidly. The progress of the reaction has to be controlled preferably by HPLC.

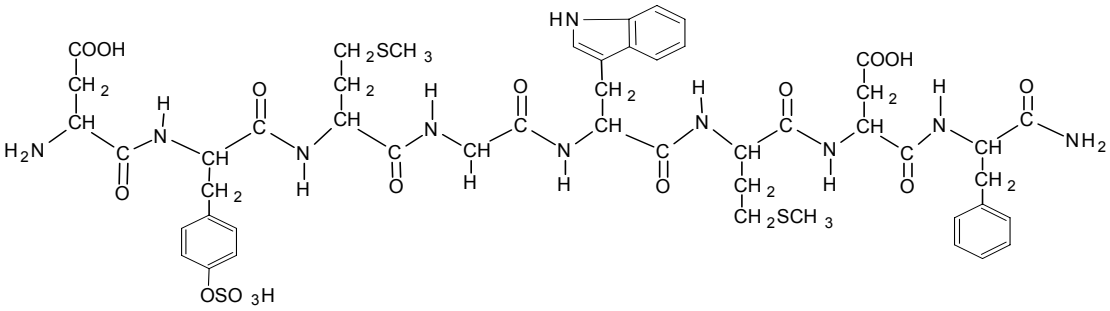
# 2 General

## 2.1 State of the art

Cholecystokinin (CCK) is a polypeptide hormone of 33 amino acid residues with a key role in biological processes involved in the food absorption and digestion (Kissileff et al., 1981; Schwartz et al., 1991). It can stimulate extensively the liberation of insulin and glucagons from the Langerhans islets (Verspohl et al., 1986). Besides these, it is a very important neurotransmitter as well (Tirassa et al., 2005). Full activity is retained in the C-terminal cholecystokinin octapeptide (CCK-8), which is used to treat intestinal paralysis and is also a potential drug candidate for the treatment of type 2 diabetes (Ahren et al., 2000), obesity (Volkoff et al., 2005) and epilepsy (Tirassa et al., 2005).

The syntheses of biologically active peptides have been widely investigated because the market of peptides is continuously growing, especially in the food and pharmaceutical industries (Vivien, 2005).

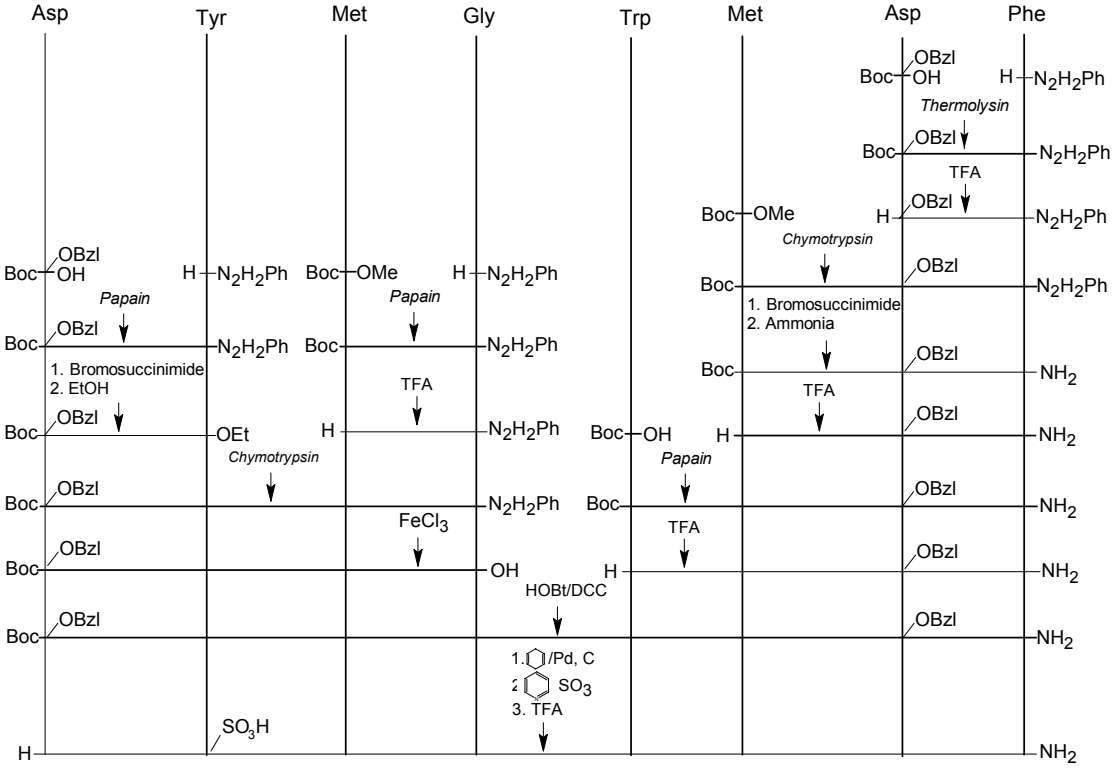
Cholecystokinin-8 ($CCK_{26-33}$) has the amino acid sequence as given below



(H-Asp-Tyr($SO_3H$)-Met-Gly-Trp-Met-Asp-Phe-$NH_2$)

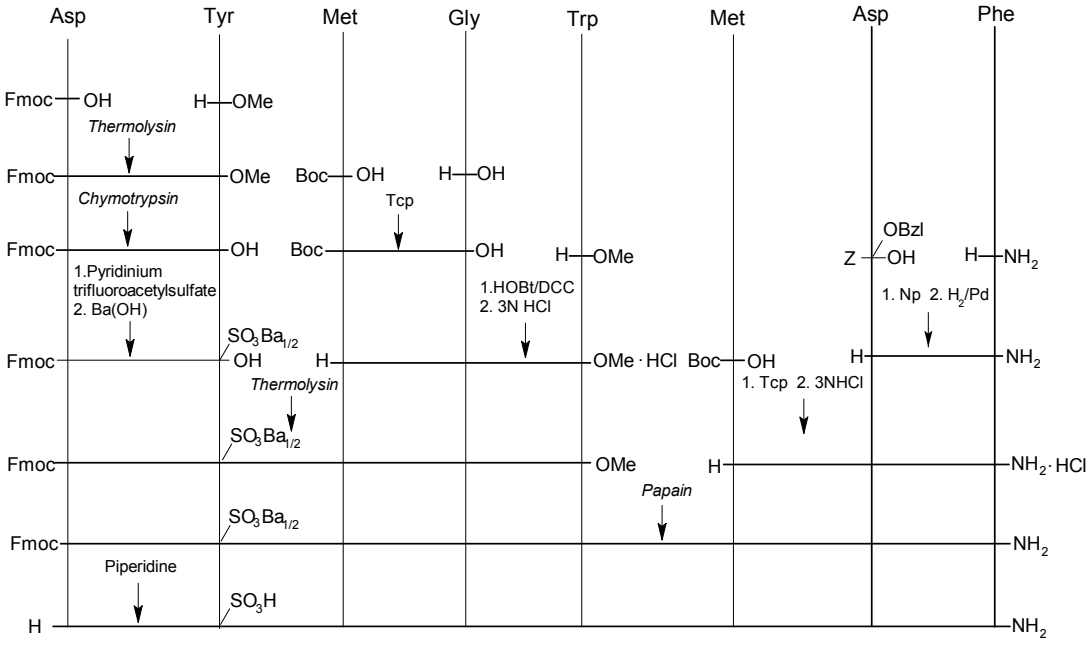
Many efforts have been devoted to the synthesis of CCK-8, its derivatives and analogues (Ondetti et al., 1970; Toth et al., 1985; Penke and Rivier, 1987). Among the different methods, enzymatic peptide synthesis is investigated by several groups as an important complement to the chemical approach. The chemical synthesis of CCK-8 is difficult because it contains many functional amino acids prone to side reaction.

In 1982, Kullmann (1982) reported the first preparation of CCK-8 using enzymatic peptide bond formation as far as possible. The octapeptide was finally assembled by the chemical condensation of two tetrapeptide segments which have been synthesized through the concerted actions of several proteases of different specificity, namely by papain-, thermolysin- and α-chymotrypsin catalysis. The design of the synthetic strategy is outlined in scheme 2.1 and in an abbreviated form it can be described as [(2 + 2) + 4]. Boc-groups were employed as N-α protecting groups for all acyl-donors. The results of these preliminary studies indicated that fragments containing more than four amino units were not attainable exclusively by enzymatic means. Protecting groups applied in this scheme are those of the chemical synthetic strategy.



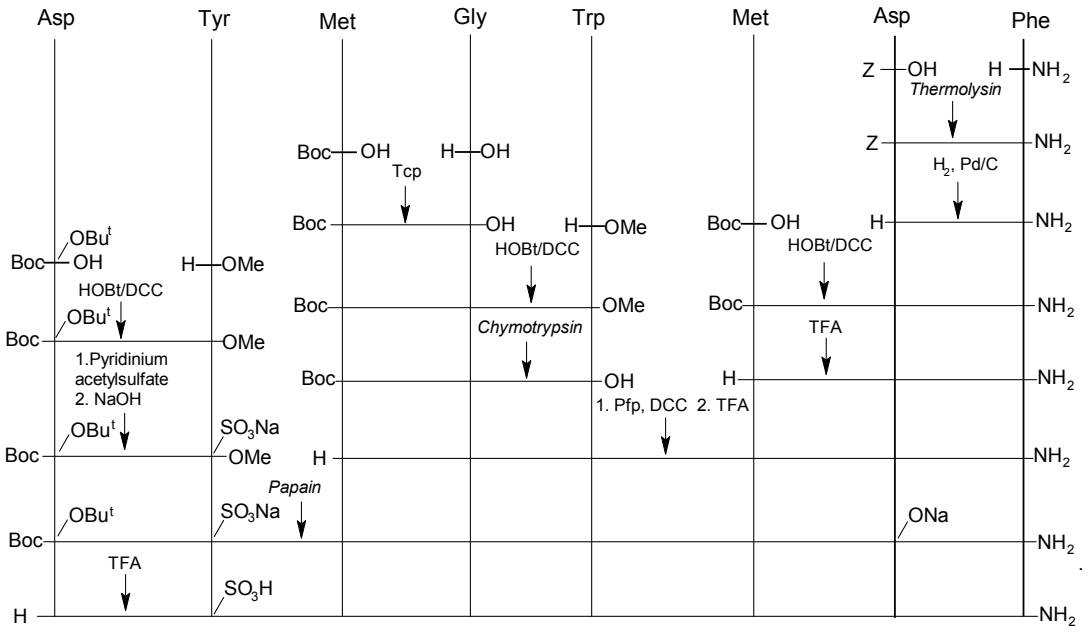

**Scheme 2.1** Synthesis of the C-terminal octapeptide amide of CCK-8 (Kullmann, 1982); -$N_2H_2Ph$ = phenylhydrazide.

Another scheme to synthesize CCK-8 was presented by Sakina et al., (1988) in which a pentapeptide synthesized from dipeptide and tripeptide was coupled with a tripeptide. In an abbreviated form it can be written as [(2 + 3) + 3]. In this procedure, N-α protecting groups of acyl donors were Fmoc, Boc and Z which could be cleaved only by chemical way. The tripeptides (Met-Gly + Trp and Met + Asp-Phe) were also synthesized chemically. The detail procedure is outlined in the scheme 2.2




**Scheme 2.2** Synthesis of the C-terminal octapeptide amide of cholecystokinin (CCK-8) (Sakina et al., 1988). Np = p-Nitrophenol; Tcp = 2, 4, 6-trichlorophenol.

Cerovsky et al., (1988) described another pathway to synthesize CCC-8 by [2 + (3 + 3)] fragment condensation. In this scheme, both tripeptides were synthesized chemically. The reaction between Boc-Met-Gly-Trp-OMe and H-Met-Asp-Phe-$NH_2$ with α-chymotrypsin did not yield a satisfactory result because of the hydrolysis of α methyl ester of Boc-Met-Gly-Trp-OMe. In this scheme also, protecting groups are those of the chemical synthetic strategy. The detail scheme is presented in scheme 2.3




**Scheme 2.3** Synthesis of the C-terminal octapeptide amide of cholecystokinin (CCK-8) (Cerovsky et al., 1988). Pfp = pentaflurophenol; Tcp = 2, 4, 6-trichlorophenol.

Later on different fragments of CCK-8 were reported to be synthesized by enzymatic way. Capellas et al., (1996[a]) reported the enzymatic synthesis of the tripeptide derivative Z-Gly-Trp-Met-OEt. The effects of organic solvent, acyl–donor ester structure, the C-α protecting group of nucleophile, enzyme and substrate concentration were studied.

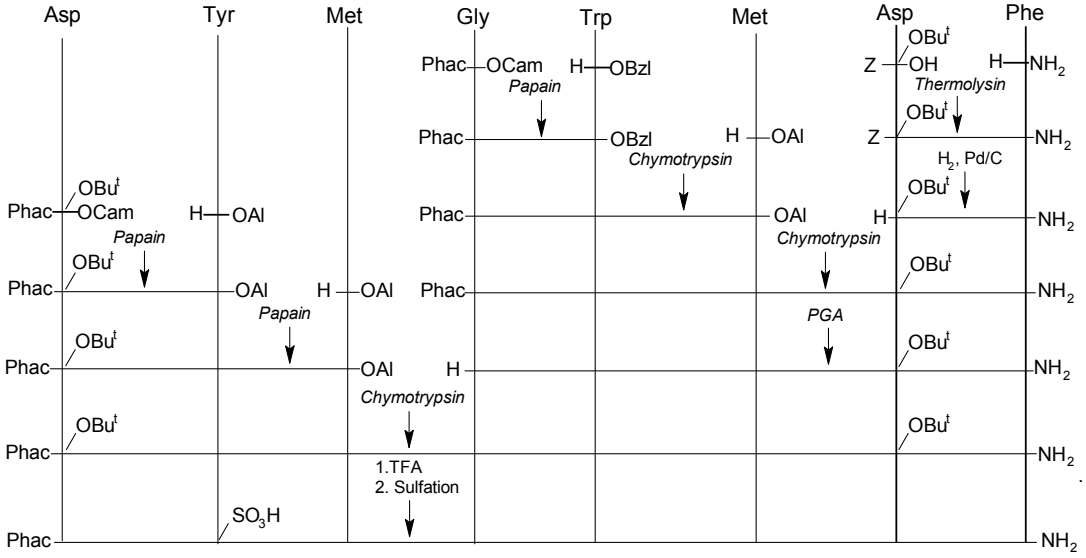
The kinetically controlled condensation of Z-Gly-Trp-OMe and H-Met-OEt catalyzed by α-chymotrypsin in organic media was reported. The influence of water activity and the support material used to adsorb α-chymotrypsin on both product yield and enzymatic activity was investigated (Capellas et al., 1996[b]).

The kinetically controlled condensation reaction of Z-Gly-Trp-Met-OX (X = Et, Al, Cam) and H- Asp(OR)-Phe-$NH_2$ (R = H, tert. butyl) catalyzed by α-chymotrypsin deposited onto polyamide in organic media was studied (Capellas et al., 1997). Substrate concentration, reaction medium, acyl donor, nucleophile structure on both enzymatic

activity and pentapeptide yield was investigated. Cam ester was found to be the best acyl-donor. The highest yield was obtained in acetonitrile as solvent.

Calvet et al., (1996) synthesized all the dipeptide fragments of CCK-8 enzymatically and the reactions conditions were optimized. The best enzyme for each dipeptide synthesis was: papain, Asp-Tyr and Gly-Trp; α-chymotrypsin, Trp-Met and Met-Gly; thermolysin, Asp-Phe and Asp-Tyr; papain and α-chymotrypsin, Trp-Met and Met-Asp.

Taking into account the information gained in previous fragment synthesis, Fite et al., (2002) described the total enzymatic synthesis of CCK-8. The Phac group was taken as N- α protecting group. The strategy for synthesis of Phac-CCK-8 is outlined in scheme 2.4 which can be summarized as [3 + (3 + 2)] condensation. In this paper, it is not mentioned whether the Phac group can be cleaved from the protected CCK-8.

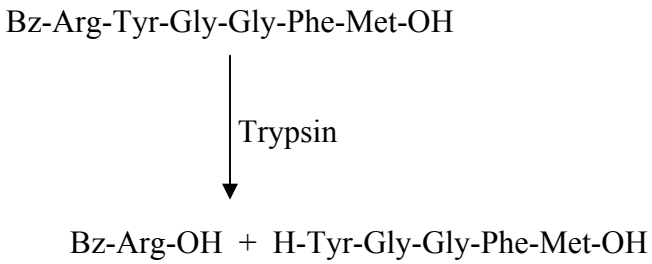



**Scheme 2.4** Synthesis of Phac-CCK-8 (Fite et al., 2002).

The main disadvantage drawn from the above schemes is the limited use of enzymes. Many reactions were not feasible during enzymatic synthesis and sometimes the yields were very poor. Clape's group could improve the enzymatic pathways of CCK-8 synthesis but the Z- group had to be cleaved chemically. The Z- group is not suitable for chemical deprotection because the sulfhydryl group of methionine poisons the catalyst

during catalytic hydrogenation. Boc is also not suitable as it gives a stable carbocation during deprotection which can attack the indole ring of tryptophan. Therefore, there is a need for an alternative N- α protecting group, which can be cleaved enzymatically.

Widmer et al., (1981) proposed the enzymatic synthesis of Met-enkephalin where the N-α protection of the amino terminal tyrosine residue was provided by the trypsin labile benzoylarginine moiety. The free Met-enkephalin was finally obtained by the action of trypsin in protected Met-enkephalin as shown in scheme 2.5.




**Scheme 2.5** Cleavage of the Bz-Arg group by trypsin during the synthesis of Met-enkephalin.

The trypsin labile protecting group was also used by Glass (1987) to synthesize oxytocin, deaminooxytocin and oxypressin. If there are no basic amino acids like arginine or lysine in the target peptide, then trypsin labile protecting group can be employed. In the current research, there is no basic amino acid in the CCK-8 sequence therefore Bz-Arg can be used as an amino protecting group which can be introduced and cleaved by trypsin.

## 2.2 Aim of the research

The aim of this research is to circumvent the drawbacks mentioned in the state of art (2.1) and to obtain a better way to synthesize free CCK-8. The chemical structure of free CCK-8 is outlined in figure (2.1).

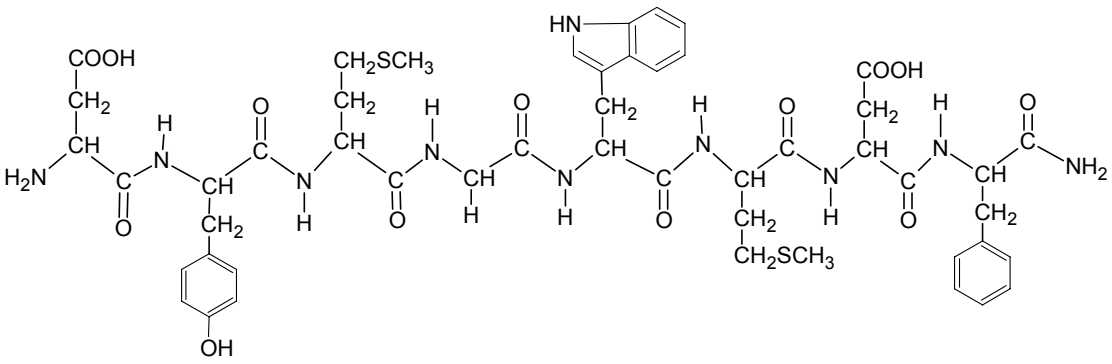


**Figure 2.1** Structure of the target octapeptide of cholecystokinin.

To obtain free CCK-8, the following points will be taken into account.

(1) In the literature of CCK-8 syntheses, to the best of my knowledge, I have not found any literature mentioned about a suitable N- α protecting group which can be removed enzymatically. Either the protecting group has been cleaved chemically (Kullmann, 1988; Cerovsky et al., 1988) or the protecting group has not been cleaved at all (Fite et al., 2002). Therefore, there is a need for a N- α protecting group which can be cleaved enzymatically. Moreover, the protecting group should be easy to be introduced, should be inert to the condition prevailing during reaction steps and should be easy to be removed at the end of the reaction. The introduction of the protecting group is also an essential requirement for ensuring the synthesis of well defined peptides and for suppressing the zwitterionic character of the reactants. Therefore in this work, another protecting group (Bz-Arg) besides Phac will be exploited for N- α protection during enzymatic synthesis. According to our experience, the longer the peptide chain, the more difficult it is to cleave the Phac group. Therefore, Bz-Arg will be investigated as a useful protecting group for the synthesis of the protected CCK-8. It was investigated in our laboratory that the aspartate unit having a methyl ester side chain was better than the free carboxylic acid

side chain during the synthesis of cholecystokinin tetrapeptide (CCK-4). With the ester side chain in aspartate, the solubility was higher in organic solvent, fewer by-products were formed and better yield was obtained. Moreover, the introduction and removal of ethyl and methyl ester is also simple. Therefore, in this work, ethyl and methyl ester will be applied for the side chain protection of aspartic acid.

(2) Enzymatic synthesis of different fragments of CCK-8 will be carried out so that there could be different possibilities of fragment condensation for the final assembly of CCK-8. Different fragments like Bz-Arg-Asp(OEt)-Tyr-Met-OH/OAl, H-Gly-Trp-OMe, Phac-Gly-Trp-Met-OH/OAl, H-Asp(OMe)-Phe-$NH_2$ will be synthesized. The influence of structures and concentrations of acyl-donors and nucleophiles, reaction media and carriers used to immobilize enzymes on both the product yield and enzymatic activity will be assayed. After the synthesis of protected CCK-8, N- α protecting group and side chain protecting group of aspartic acid will be cleaved to enable the successful synthesis of free CCK-8.

The following two pathways will be explored to obtain the target octapeptide.

(i) Bz-Arg-Asp(OEt)-Tyr-Met-OH/OAl + H-Gly-Trp-OMe

↓ α-Chymotrypsin

Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-OMe

H-Met-Asp(OMe)-Phe-$NH_2$ ↓ α-Chymotrypsin

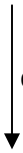
Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$

↓ Trypsin

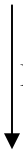
↓ NaOH

H-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-$NH_2$

(ii) Phac-Gly-Trp-Met-OH/OAl + H-Asp(OMe)-Phe-$NH_2$




Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$




H-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$

Bz-Arg-Asp(OEt)-Tyr-Met-OH/OAl + H-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$


Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$


H-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-$NH_2$

(3) During the enzymatic synthesis, the enzymes could cleave at different positions of peptides and can assemble in an unexpected way so it is relatively difficult to be sure for the product formed during an enzymatic way. Therefore it is always good to have a reference product with which the progress of the reaction can be monitored in HPLC. For this reason, reference substances will be prepared using the chemical strategy in case reference substances are not already available in our laboratory.

(4) If possible, immobilized enzymes will be utilized in place of free enzymes. The advantages of immobilized over free enzymes arise from their enhanced stability in organic solvents, ease of separation from reaction media, easy recovery and repeated use. The immobilized enzyme can be completely removed from the solution. This is especially important for the therapeutical application of peptides. When the peptide is contaminated with enzymes, it can show immunological reactions if it is used in therapeutical application. Different methods will be utilized to immobilize enzymes and the activity of the immobilized enzymes will be assayed.

(5) Different reaction systems will be applied for optimizing the reaction. The reaction will be carried out either in aqueous system (i.e. the reaction in plain water or in buffer) or in co-solvent system (water miscible solvents like alcohols or DMF can be applied as organic solvent) or in bi-phasic system (water and water-immiscible organic solvent are taken, if the acyl donor and nucleophile are soluble in water whereas the product is better soluble in the organic phase) or in the solvent free system (water needed for the enzyme will be provided by the crystalline water of sodium or potassium salts). The reaction in the solvent free system is done in the solid state.

### 2.3 Characterization of the products

Many analytical methods can be applied to confirm the structure of a product. In this research, elemental analysis is not very useful because all the peptides will have the same elements like C, H, O and N. As a result the values determined by elemental analysis will not largely differ from peptide to peptide. The information gained in this method will not give convincing proof in the structure confirmation.

Thin layer chromatography is optimal and easy to perform. Besides detecting the UV active substances by TLC with the UV lamp, the UV inactive substances could be detected with the ninhydrin test followed by the chlorine/tolidine test.

HPLC is highly useful for following the kinetics of a reaction. RP-HPLC examination of the peptides allows rapid separation and quantification of the components in a mixture.

If reference substances were available, the products were compared with the properties of the reference samples in terms of melting point, retention time in RP-HPLC and $R_f$ value in TLC. The final structure was determined by MS analysis. If the molecular mass is as calculated, the peptide sequence will be assumed to be according to the schedule of the synthesis.

In this work, the purity of the sample is determined by HPLC. If there is a single symmetrical peak of correct retention time, one can assume that the sample is pure.

Racemization occurs either in strongly acidic or strongly basic condition or during the activation of the carboxyl group. In this work, enzymatic methods are used for coupling and consequently there is no question of optical purity as there is no danger of racemization. Even if the substrate is racemized to some extent, the enzymes are highly specific and selective to couple only the L-isomer for the next coupling steps. Therefore, optical purity was not measured in this study.
As a conclusion, it can be said that the structure determination was done by MS analysis and the purity of the product was checked by HPLC.

# 3 Results and discussion

## 3.1 Enzyme immobilization and activity test

### (1) Immobilization

Before carrying out the activity test of a particular enzyme, the immobilization of trypsin, papain and α-chymotrypsin on different carriers was done. In this work, immobilization was done in two ways. Either the enzyme was just deposited on Celite-545 without any chemical bond or the enzyme was attached to VA-Epoxy or Eupergit C with a chemical bond. The epoxy polymer (VA-Epoxy) is formed by refluxing the VA-Hydroxy-Biosynth together with epichlorohydrin. However, Eupergit C is an already epoxydised acryl resin. Then the amino group of the enzyme attacks the epoxy group of VA-Epoxy or of Eupergit C and forms a covalent binding to the corresponding polymer.

The details regarding the immobilization and their stability has already been published by our group (Eckstein and Renner, 1992; Eckstein et al., 1991)

The activity of immobilized enzymes was tested prior to its use. The activity was also checked after multiple uses or prolonged storage of the immobilized enzyme. The detailed study regarding individual immobilized enzymes is mentioned below.

### (2) Activity test of immobilisates

***Trypsin.*** It was immobilized on Eupergit C (Trypsin/Eupergit C). The activity of immobilized trypsin was checked using Bz-Arg-OEt·HCl as substrate. In presence of phosphate buffer (pH 7.5), the trypsin converted the substrate to Bz-Arg-OH. The conversion of Bz-Arg-OEt to Bz-Arg-OH was followed in HPLC.

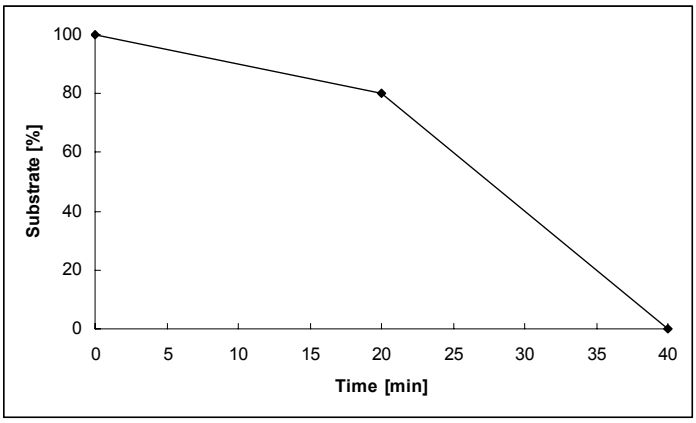



**Figure 3.1** Hydrolysis of Bz-Arg-OEt with trypsin/Eupergit C in phosphate buffer (pH 7.5).

As shown in figure 3.1, the activity of trypsin is good. Within 40 min, immobilized trypsin completely cleaved Bz-Arg-OEt to Bz-Arg-OH. Trypsin/Eupergit C was used to synthesize Bz-Arg-Asp(OEt)-OEt and to cleave Bz-Arg from both Bz-Arg-Asp(OEt)-Tyr-Met-OH and Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$.

The activity of immobilisate was checked after multiple uses during a one year period. The result of the activity test (figure 3.2) shows that immobilized trypsin can be effectively used for three times. Even after the third application, the activity was still high but when this immobilisate was used to synthesize Bz-Arg-Asp(OEt)-OEt, the by-product (Bz-Arg-Asp(OEt)-OH) was formed more than the product. The probable reason could be that after multiple uses, protease activity of trypsin decreased but esterase activity was still there so the ester was cleaved. The next possibility could be because of the impurity (mainly α-chymotrypsin) present in the trypsin. When the catalytic activity of trypsin decreased after multiple uses, the catalytic activity of α -chymotrypsin cleaved the ester. In conclusion, the immobilisate could be used three times in a period of one year. The high stability of trypsin is caused by the covalent binding between enzyme and carrier. The multiple use of immobilisate is useful both in purifying the product and reducing the cost of the reaction.

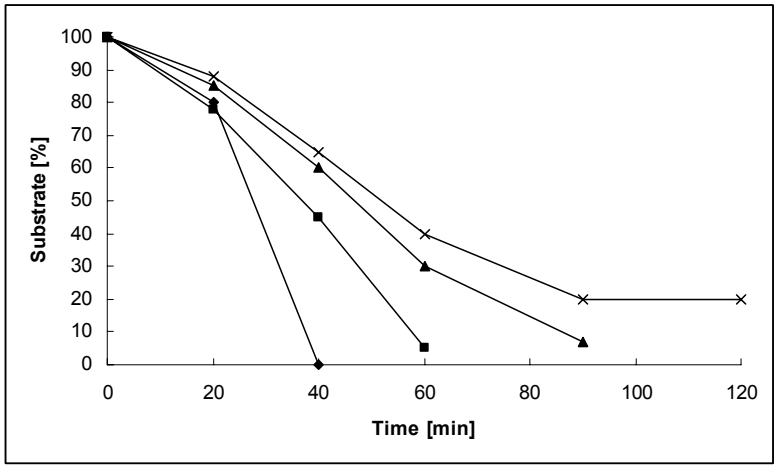



**Figure 3.2** Hydrolysis of Bz-Arg-OEt with Trypsin/Eupergit C. Activity directly after immobilization (♦), after the first application (■), after the second application (▲) and after the third application (×).

***α-Chymotrypsin.*** The activity of α-chymotrypsin immobilized on Eupergit C (α-CHY/Eupergit C) and on Celite-545 (α-CHY/Celite-545) was tested taking Ac-Tyr-OEt as substrate. The conversion of Ac-Tyr-OEt to Ac-Tyr-OH was followed in HPLC.

As shown in figure 3.3, α-CHY/Celite-545 was able to hydrolyze the given substrate in 10 min while α-CHY/Eupergit C required around 1 h. The activity of α-CHY/Celite-545 was better but it could not be used after the first application where as α-CHY/Eupergit C was successful in synthesizing Phac-Gly-Trp-Met-OEt even after the first use of the immobilisate. The possible explanation could be that there is just physical adsorption on Celite whereas there is chemical covalent bond between enzyme and Eupergit C so α-CHY/Eupergit C could retain its activity even after working up procedure. In some cases like synthesis of protected octapeptide (Bz-Arg-Asp(OEt)-Tyr-Met-OAl + Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$), α-CHY/Eupergit C was superior than α-CHY/Celite-545 while in another synthesis of Bz-Arg-Asp(OEt)-Tyr-Met-OAl (Bz-Arg-Asp(OEt)-Tyr-OH + Met-OAl·PTS) the opposite was found true. In economic point of view and keeping enzyme's contamination away from the product, α-CHY/Eupergit C is superior than α-CHY/Celite-545.

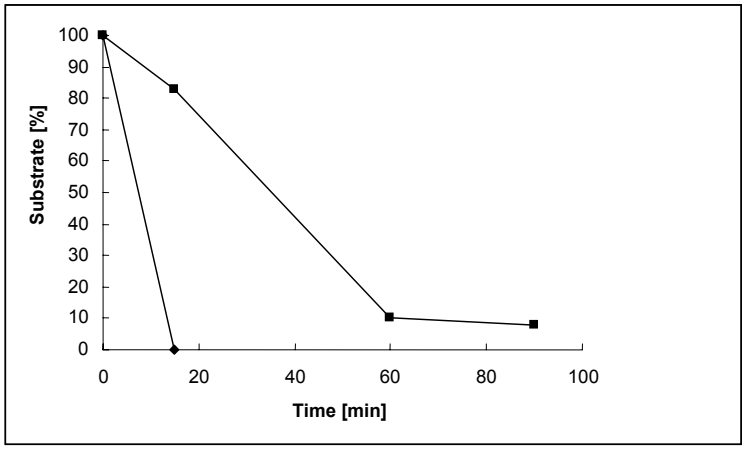



**Figure 3.3** Hydrolysis of Ac-Tyr-OEt with α-CHY/Celite-545 (♦), and with α-CHY/ Eupergit C (■) in 0.01 M Tris-HCl buffer (pH 8.1).

***Papain.*** Papain was immobilized on VA-Epoxy (papain/VA-Epoxy) and on Celite-545 (papain/Celite-545). The activity of immobilized papain was tested taking Bz-Arg-OEt as substrate. The conversion of Bz-Arg-OEt to Bz-Arg-OH was followed in HPLC.

As shown in figure 3.4, both immobilisates had almost the same activity so papain/VA-Epoxy was taken for the synthesis of Phac-Gly-Trp-OMe. Papain/VA-Epoxy was better than papain/Celite-545 because of the covalent binding between papain and VA-Epoxy which could retain the activity even after the working up procedure.

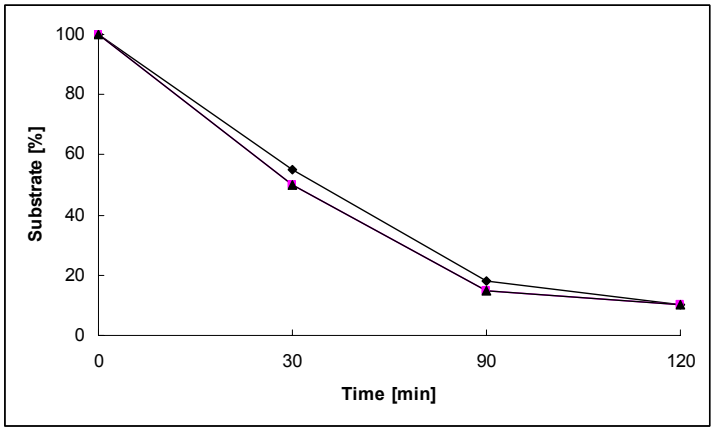
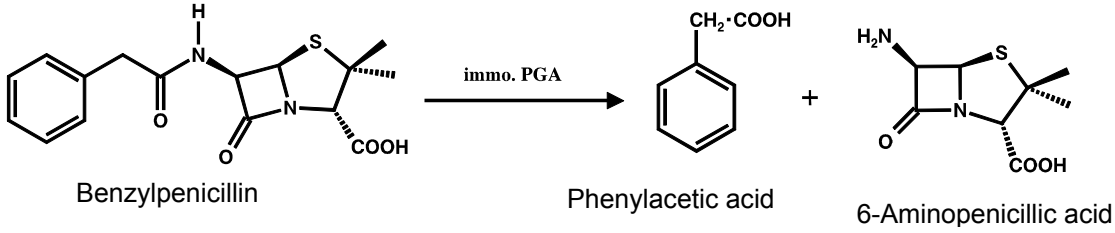



**Figure 3.4** Hydrolysis of Bz-Arg-OEt with papain/Celite-545 (♦) and with papain/VA-Epoxy (▲) in 0.5 M $KH_2PO_4$ buffer (pH 6.0) in presence of EDTA and 2-mercaptoethanol.

Papain is a cysteine protease which binds to the active site of the substrate through a thioester linkage. Papain loses its activity even by aerial oxidation that is why the papain mediated reactions were performed in presence of 2-mercaptoethanol. 2-Mercaptoethanol can activate papain and maintain its activity even in the presence of air. EDTA is also added during the reaction mediated by papain whose main function is to complex any heavy metal ion, which could otherwise deactivate the –SH group of papain.

***PGA/Eupergit C (Fluka).*** Benzylpenicillin was used as the substrate for the activity test of PGA/Eupergit C. The conversion of benzylpenicillin to phenylacetic acid and 6-aminopenicillanic acid was determined with HPLC.




**Scheme 3.1** Conversion of benzylpenicillin to phenylacetic acid and 6-aminopenicillic acid by immobilized PGA.

The activity of the immobilized PGA was found effective as there was no starting material detected in HPLC after 30 min (figure 3.5). PGA/Eupergit C was used to cleave the protecting group Phac from Phac-Gly-Trp-OMe and Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$.

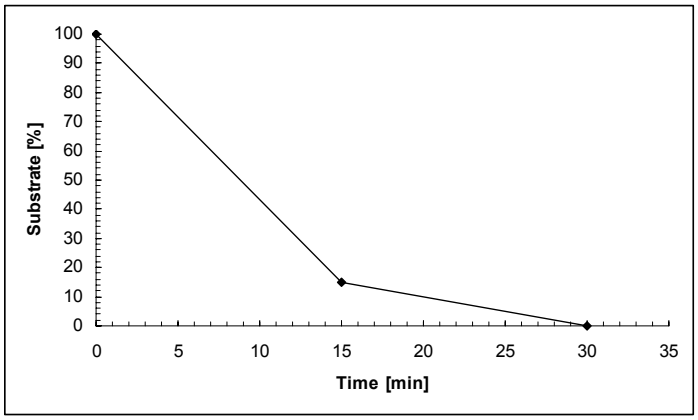



**Figure 3.5** Hydrolysis of benzylpenicillin with PGA/Eupergit C in 1 M $KH_2PO_4$ buffer (pH 7.5).

### 3.2 Synthetic strategy

There are three possible strategies for the synthesis of peptides.

**(1) Fragment condensation**

Fragment condensation in the synthesis of peptides is possible in the enzymatic way because there is no danger of racemization. During the condensation of peptides in chemical way, there might occur racemization while activating the carboxyl group of the peptide. In this work, fragment coupling between Bz-Arg-Asp(OEt)-Tyr-Met-OAl and H-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ was done successfully with the help of α-CHY/Eupergit C.

.

**(2) N ⟶ C strategy**

Starting with the N-terminal and adding one amino acid each time is the easy way of synthesizing peptides. To shift the equilibrium of the enzyme catalyzed peptide synthesis, highest possible concentration and excess of one fragment are necessary. Excess of the carboxyl group containing fragment may lead to product inhibition of the enzyme so it is better to apply amino acid ester or amide in excess which makes the synthesis cheap and operating procedure easy. This is also possible only in enzymatic way. In chemical way, this is not possible because even if the blocking group (urethane type) protects the first residue against racemization, in subsequent steps blocked peptides are activated and their activated C-terminal residues are exposed to the racemizing effect by the leaving group. In this work, Bz-Arg-Asp(OEt)-Tyr-Met-OAl and Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ were synthesized from N terminus to C terminus.

**(3) C⟶ N strategy**

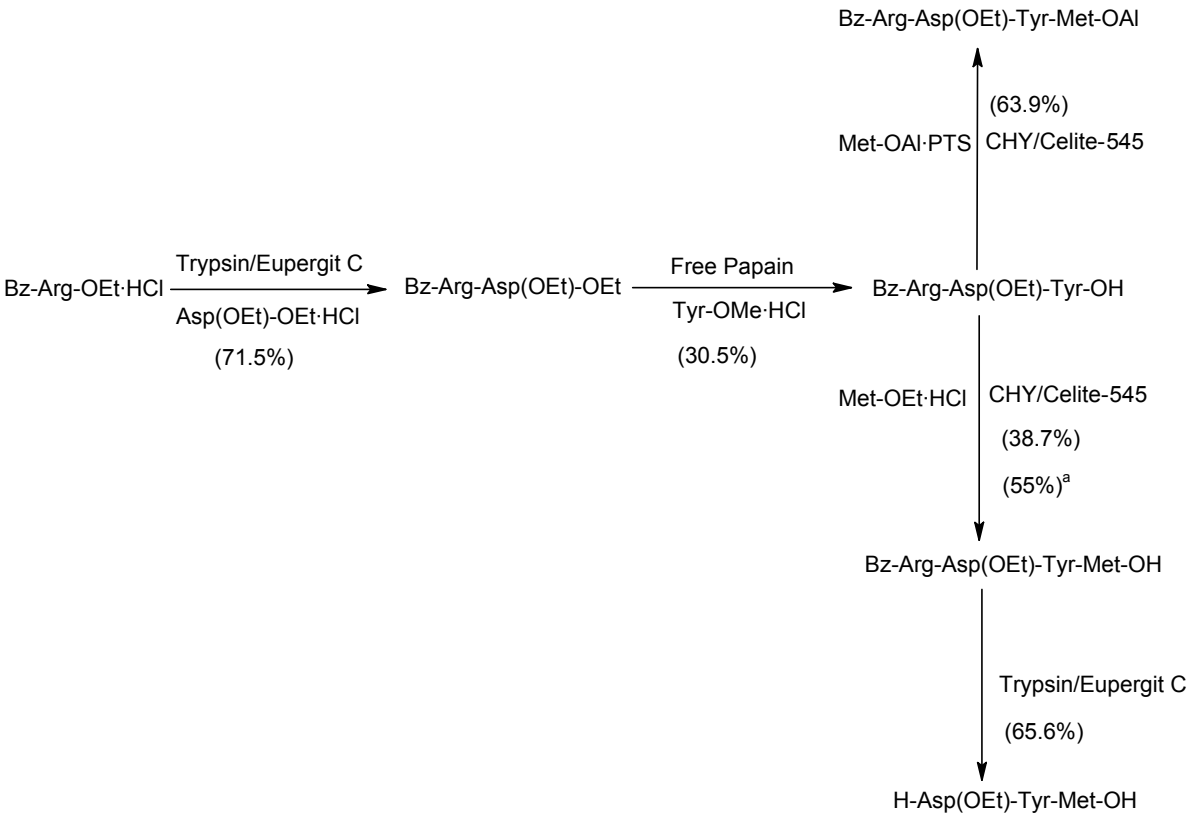
The synthesis of the peptides according to this strategy is laborious for practical purposes. In this strategy, each amino acid is added in the blocked and activated form and in a second step the amino blocking group is removed after each coupling. If each added new amino acid is protected by a blocking group of the urethane type, there is no substantial risk of racemization. In this work, C-terminal pentapeptide was synthesized from C to N terminus using the Fmoc as the N α-protecting group.

### 3.3 Enzymatic synthesis of the fragments

The syntheses of several fragments of CCK-8 are described below.

#### 3.3.1 Bz-Arg-Asp(OEt)-Tyr-Met-OH/OAl

This tetrapeptide, Bz-Arg-Asp(OEt)-Tyr-Met-OH/OAl is the N-terminal tripeptide fragment of CCK-8. The synthesis was started from the N-terminus and was continued adding one amino acid ester each time. For the synthesis of this tetrapeptide (N-terminal tripeptide fragment of CCK-8), there was little knowledge about the synthetic strategy and reaction conditions (Fite et al., 2002). In this work, benzoylarginine (Bz-Arg) was taken as an amino protecting group. As this group was new for the synthesis of CCK-fragments, the effect of substrate structure, reaction media, enzyme and its carriers were studied in detail. The synthetic pathway is outlined in scheme 3.2. The synthesis of individual steps is described below.




**Scheme 3.2** The N ⟶ C strategy for the synthesis of N-terminal tripeptide fragment of CCK-8. The isolated yields are shown in parentheses. [a]The yield is calculated after the unreacted educts were recycled for a repeated conversion.

***Synthesis of Bz-Arg-Asp(OEt)-OEt.*** Bz-Arg as the protecting group can be introduced easily to Asp(OEt)-OEt and removed at the end of the synthesis with trypsin. Enzymatic introduction and cleavage of protecting groups offer advantages over chemical methods due to their selectivity, specificity and mild operation condition. The reaction between Bz-Arg-OEt·HCl and Asp(OEt)-OEt·HCl to yield Bz-Arg-Asp(OEt)-OEt was kinetically controlled. The optimized reaction was conducted between 1 equivalent Bz-Arg-OEt·HCl and 2 equivalents Asp(OEt)-OEt·HCl under solvent free conditions similar to the method described by Cerovsky (1992). The system was made alkaline by adding solid $Na_2CO_3$ and solid NaOH. The water needed for the enzyme was supplied by $Na_2CO_3 \cdot 10H_2O$. In this condition, the isolated yield of Bz-Arg-Asp(OEt)-OEt was 71.5% in 4 hours. If the reaction was not terminated at its optimum point, side reactions of immobilized trypsin (trysin/Eupergit C) were significant. The two by-products were achieved by the esterase activity of trypsin in which trypsin hydrolyzed the esters of both edduct (Bz-Arg-OEt·HCl) and product (Bz-Arg-Asp(OEt)-OEt) giving rise to Bz-Arg-OH and Bz-Arg-Asp(OEt)-OH respectively. The formation of Bz-Arg-OH is also possible from the proteolytic action of trypsin in Bz-Arg-Asp(OEt)-OEt. Both the by-products could be kept below 10% by monitoring the kinetics of the reaction in HPLC. The immobilized enzyme could be used three times without significant loss of activity which is one of the major advantages of immobilization.

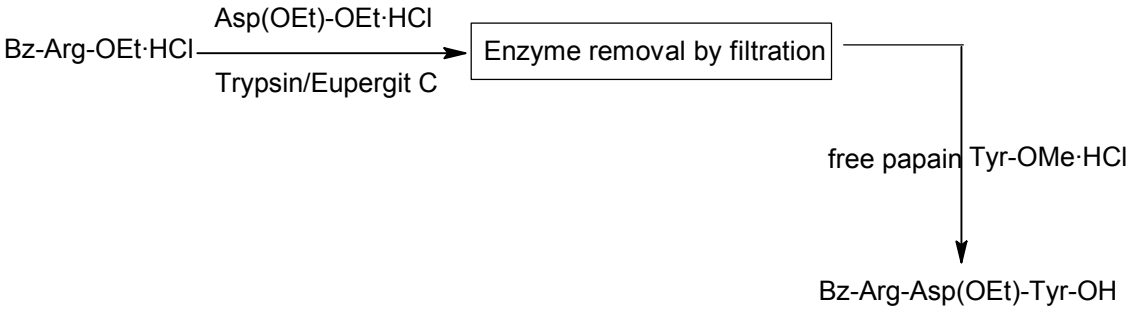
This synthesis was carried out in Tris-HCl buffer (0.1 M, pH 8.0) with the same immobilisates. In this case, only the by-products Bz-Arg-OH and Bz-Arg-Asp(OEt)-OH were obtained.

***Synthesis of Bz-Arg-Asp(OEt)-OH.*** The synthesis of this peptide was carried out via two methods. In the first method, Bz-Arg-Asp(OEt)-OEt was synthesized as mentioned above and in analogy to our earlier reported findings ( Meng et al., 2006), already synthesized Bz-Arg-Asp(OEt)-OEt was selectively hydrolysed by α-chymotrpsin to yield Bz-Arg-Asp(OEt)-OH. The second method to synthesize Bz-Arg-Asp(OEt)-OH was the reaction between Bz-Arg-OEt·HCl and Asp(OEt)-OEt·HCl in water with immobilized trypsin.
The isolated yield of the product in first and second method was 38.8% and 41.2%

respectively. The second method was superior to first method because the isolated yield was better and the numbers of reaction steps were less.

Bz-Arg-Asp(OEt)-OH was tried to extend to Bz-Arg-Asp(OEt)-Tyr-OMe. In our earlier reported findings (Meng et al., In Press), free carboxylic acid in Phac-Asp(OMe)-OH reacted with Tyr-OAl·TOS to yield Phac-Asp(OMe)-Tyr-OAl in presence of thermolysin. However, the free carboxylic acid in Bz-Arg-Asp(OEt)-OH could not be reacted with Tyr-OMe·HCl in presence of thermolysin.

***Synthesis of Bz-Arg-Asp(OEt)-Tyr-OH.*** The optimized coupling between Bz-Arg-Asp(OEt)-OEt and 1.5 equivalents Tyr-OMe·HCl was performed in 20 equivalents of $KHCO_3$ and 0.25 equivalents of $Na_2SO_4 \cdot 10H_2O$ in presence of free papain. The isolated yield was 30.5%. In the synthesis of this peptide, it was possible to convert Bz-Arg-OEt·HCl to Bz-Arg-Asp(OEt)-Tyr-OH in a one pot reaction without purification and isolation of Bz-Arg-Asp(OEt)-OEt as illustrated in scheme 3.3.




**Scheme 3.3** Two steps reactions in a one pot reaction, illustrated by converting Bz-Arg-Asp(OEt)-Tyr-OH from Bz-Arg-OEt·HCl without isolating the intermediate Bz-Arg-Asp(OEt)-OEt.

When the reaction between Bz-Arg-Asp(OEt)·HCl and Asp(OEt)-OEt·HCl had its highest HPLC conversion, the product was dissolved in 80% ethanol and the immobilized trypsin was separated. The resulting product was concentrated and Tyr-OMe· HCl was added in the ratio mentioned above and the reaction was again performed under solvent

free conditions. Thus by using the immobilized enzyme, a three step reaction could be performed in two steps.

***Synthesis of Bz-Arg-Asp(OEt)-Tyr-Met-OH.*** Bz-Arg-Asp(OEt)-Tyr-OH and 5 equivalents Met-OEt·HCl were suspended in Tris-HCl buffer (0.1 M, pH 8.1) containing 10% acetonitrile. The product Bz-Arg-Asp(OEt)-Tyr-Met-OH was formed in an isolated yield of 38.7% in 6 hours. The unreacted starting material Bz-Arg-Asp(OEt)-Tyr-OH was recovered and recycled for a repeated conversion. With this method, the isolated yield of product could be increased to 55%.

***Synthesis of Bz-Arg-Asp(OEt)-Tyr-Met-OAl.*** The activity of the OCam ester is the highest among the benzyl, methyl and Cam esters (Capellas et al., 1996[a]) but Bz-Arg-Asp(OEt)-Tyr-Met-OCam could not be prepared from Bz-Arg-Asp(OEt)-Tyr-Met-OH via the chemical strategy because the tyrosine residue in this sequence was not protected. The next approach was to use Met-OAl as a nucleophile and to end up in allyl ester (Bz-Arg-Asp(OEt)-Tyr-Met-OAl) so that the resulting peptide can be used as an acyl donor without any modification in the next fragment coupling. The use of allyl ester in the synthesis of protected CCK-8 was reported by Fite et al., (2002).

Bz-Arg-Asp(OEt)-Tyr-OH and 2.5 equivalents Met-OAl·PTS were suspended in acetonitrile containing 3.75% Tris-HCl buffer (0.1 M, pH 8.1). The reaction was catalyzed by α-CHY/Celite-545. At 48 hours, the reaction was stopped and the isolated yield of the pure Bz-Arg-Asp(OEt)-Tyr-Met-OAl was 63.9%. During this reaction, the hydrolyzed product (Bz-Arg-Asp(OEt)-Tyr-Met-OH) was also formed but it could be kept below 5% by optimizing the conditions. In this reaction, the amount of buffer played a crucial role to direct the reaction pathway either towards the target product or towards the hydrolyzed product.

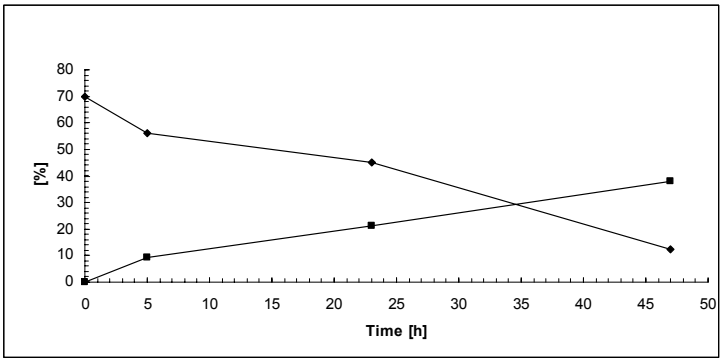



**Figure 3.6** Kinetic process of the synthesis of Bz-Arg-Asp(OEt)-Tyr-Met-OAl (■) from 0.3 mmol Bz-Arg-Asp(OEt)-Tyr-OH (♦) in 8 ml acetonitrile containing 3.75% Tris-HCl buffer in presence of α-CHY/Celite-545.

As shown in figure 3.6, the product yield increased steadily up to 48 h and then there was no improvement in the yield. Instead, the product was hydrolyzed to Bz-Arg-Asp(OEt)-Tyr-Met-OH. The reaction was terminated at this point. In the figure 3.7, HPLC chromatograms of this synthesis at time 0 and 48 hours are shown. At 48 hours, the peak of Bz-Arg-Asp(OEt)-Tyr-OH nearly vanished and the target product as well as the by-product was formed.

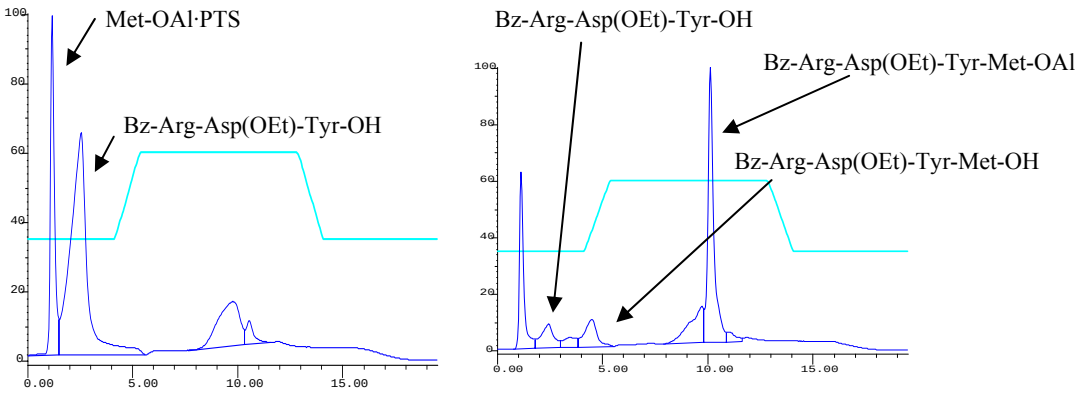



**Figure 3.7** HPLC-chromatograms of the synthesis of Bz-Arg-Asp(OEt)-Tyr-Met-OAl from Bz-Arg-Asp(OEt)-Tyr-OH and Met-OAl·PTS at 0 min (a) and 48 hrs (b).

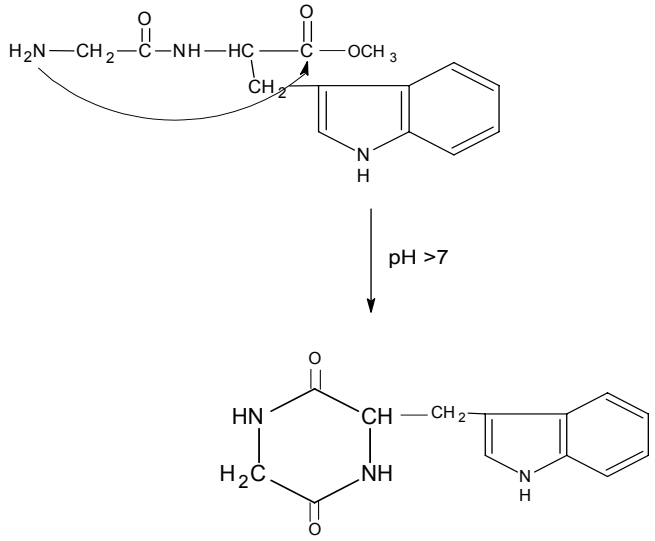
### 3.3.2 H-Gly-Trp-OMe

This dipeptide was synthesized in order to investigate the fragment coupling between Bz-Arg-Asp(OEt)-Tyr-Met-OH/OAl and Gly-Trp-OMe. The synthesis of Phac-Gly-Trp-OMe and the cleavage of its Phac group is described below.

***Phac-Gly-Trp-OMe.*** The enzymatic reaction between Phac-Gly-OH and Trp-OMe·HCl to synthesize Phac-Gly-Trp-OMe was unsuccessful. However the condensation to Phac-Gly-Trp-OMe was successful when Phac-Gly-OCam was employed as an acyl donor and Trp-OMe was employed as a nucleophile in EtOAc in presence of papain/VA Epoxy. Making the Cam ester is a lengthy and laborious process.

Glycine is an optically inactive amino acid so there is no danger of racemization during carboxyl activation of glycine in chemical synthetic strategy. That is why, Phac-Gly-Trp-OMe was better synthesized using the DCC method and the isolated yield was 74%.

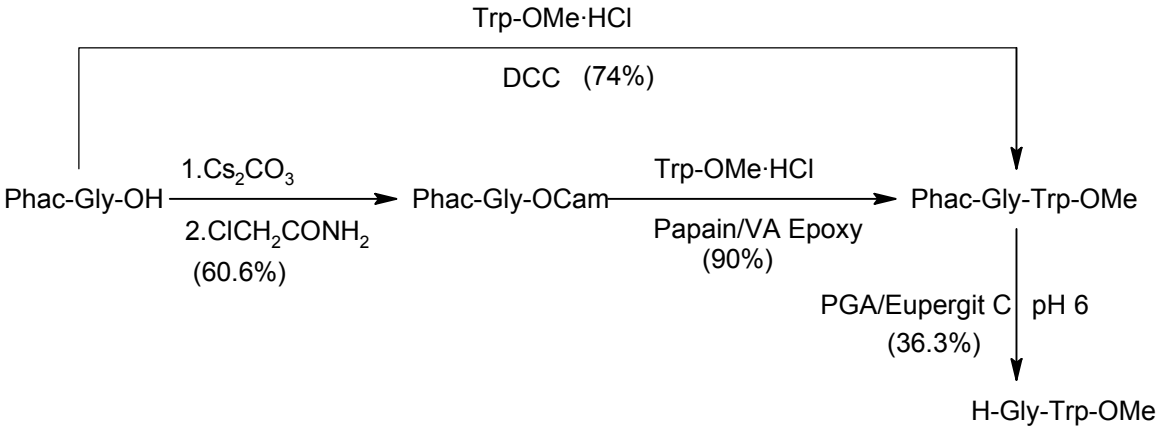
This dipeptide was treated with PGA in order to remove the Phac group. PGA was added to the suspension of dipeptide in water (pH 7.6). In this reaction, after 80 hours, 32.6% diketopiperazines and only 8.4% free Gly-Trp-OMe was observed in HPLC. As soon as free Gly-Trp-OMe was formed, there was internal nucleophilic attack from N of the amino group to the C of the carboxyl group to make 6-membered stable diketopiperazines. The reaction is shown in the scheme 3.4.




**Scheme 3.4** Formation of diketopiperazines from H-Gly-Trp-OMe at pH 7.6.

If the nucleophilicity of the $NH_2$ group can be reduced by protonating it, then the rate of internal nucleophilic attack would reduce which in turn reduce the diketopiperazines formation. Therefore, the reaction was investigated in different acidic pH values. The higher temperature also favors diketopiperazine formations in this reaction, therefore the working up procedure (solvent removal) was done at 25º C. The isolated yield of the free dipeptide could be increased to 36.3% at pH 6.0.

The scheme of synthesis of H-Gly-Trp-OMe is outlined in scheme 3.5.




**Scheme 3.5** Enzymatic and chemical synthesis of Phac-Gly-Trp-OMe and the cleavage of Phac from Phac-Gly-Trp-OMe. The isolated yields are shown in parentheses.

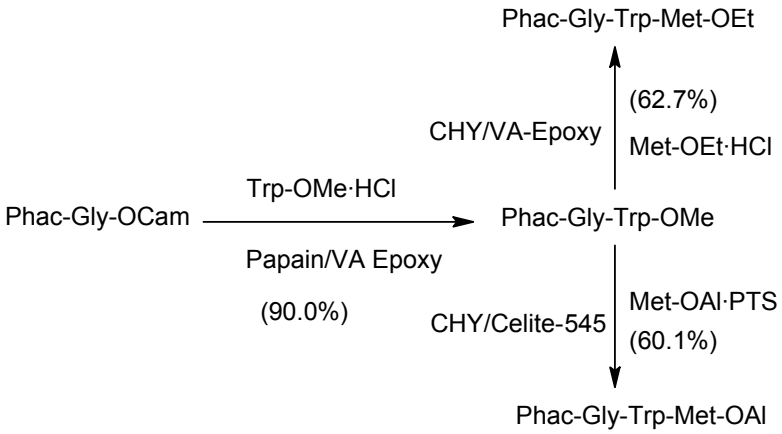
The results of effect of pH in the synthesis of free Gly-Trp-OMe are mentioned in table 3.1. As can be seen in this table, there was highest conversion at pH 6.0 and pH below 5.0 was not good for the enzymatic activity.

**Table 3.1** Effect of pH in the cleavage of Phac from Phac-Gly-Trp-OMe.

| pH | Time [h] | HPLC conversion (%) |
|---|---|---|
| 4.5 | 24 | 23.4 |
| 5.0 | 24 | 10.6 |
| 5.5 | 24 | 32.5 |
| 6.0 | 20 | 47.8 |
| 6.5 | 20 | 35.2 |

### 3.3.3 Phac-Gly-Trp-Met-OEt/OAl

Phac-Gly-Trp-OMe was converted to two different tripeptide esters, Phac-Gly-Trp-Met-OEt and Phac-Gly-Trp-Met-OAl. Phac-Gly-Trp-Met-OEt was obtained by reacting one equivalent of Phac-Gly-Trp-OMe, 4 equivalents of Met-OEt·HCl, 20 equivalents of $KHCO_3$ and 4 equivalents of $Na_2CO_3 \cdot 10H_2O$ in the presence of α-CHY/Eupergit C. The isolated yield of Phac-Gly-Trp-Met-OEt was 62.7%. The major by-product (Phac-Gly-Trp-OH) formed by the hydrolysis of the educt was 26% (HPLC yield). The other by-products like the Met-Met oligomer (Phac-Gly-Trp-Met-Met-OEt), hydrolysed tripeptide (Phac-Gly-Trp-Met-OH) were kept below 5% by monitoring the reaction in HPLC. The synthesis of Phac-Gly-Trp-Met-OAl was done in analogy to that of Phac-Gly-Trp-Met-OEt. Basic, neutral and acidic solvent free systems were created by changing the salts and then the reaction was investigated in each system for the optimization. The highest isolated yield (60.1%) was obtained at 4 hours when one equivalent of Phac-Gly-Trp-OMe, 1.5 equivalents of Met-OAl·PTS, one equivalent of $KHCO_3$ and 0.3 equivalents of $Na_2CO_3 \cdot 10H_2O$ were incubated in presence of α-CHY/Celite-545. The following scheme shows the reaction pathways leading to Phac-Gly-Trp-Met-OEt and Phac-Gly-Trp-Met-OAl.




**Scheme 3.6** Synthesis of Phac-Gly-Trp-Met-OAl and Phac-Gly-Trp-Met-OEt .The isolated yields of products are shown in parentheses.

Figure 3.8 shows the chromatograms of the synthesis of Phac-Gly-Trp-Met-OAl at 0 and 4 hours. As can be seen in figure 3.8 (b), Phac-Gly-Trp-OMe nearly disappears within 4 hours and the only one by-product (Phac-Gly-Trp-OH) appears in a small peak at a retention time of 4.5 min. Therefore, the reaction was terminated at 4 hours.

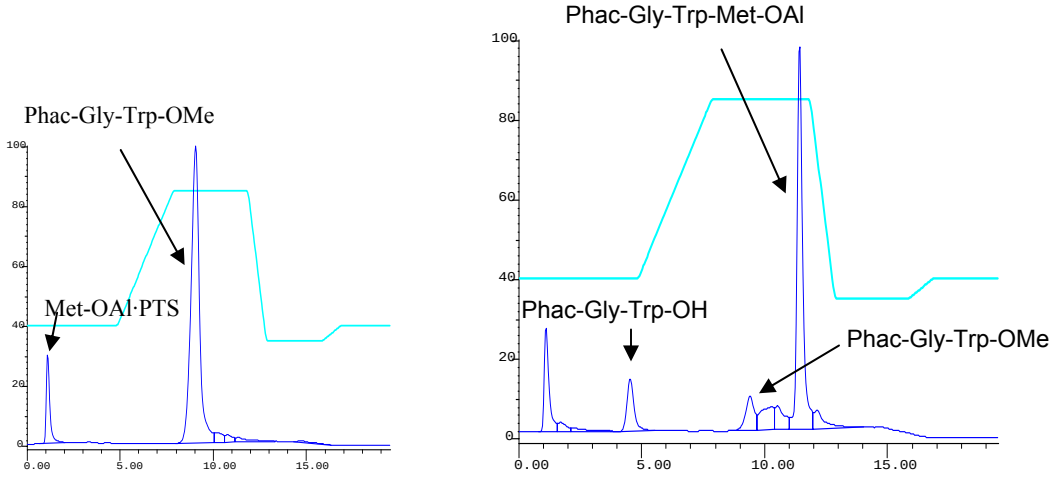
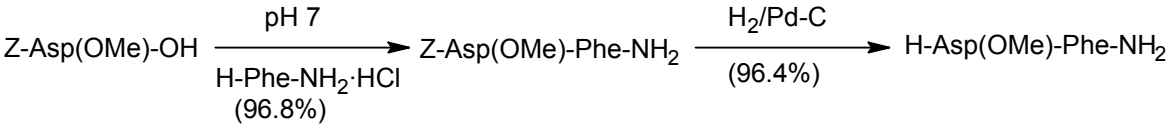



**Figure 3.8** Synthesis of Phac-Gly-Trp-Met-OAl from Phac-Gly-Trp-OMe and Met-OAl·PTS at 0 min (a) and 4 hrs (b).

### 3.3.4 H-Asp(OMe)-Phe-$NH_2$

***Z-Asp(OMe)-Phe-$NH_2$.*** The Z group was chosen to protect the N α-amino group because the Z group could be cleaved by catalytic hydrogenation in 15 min with more than 95% isolated yield. One equivalent Z-Asp(OMe)-OH and 1.3 equivalents H-Phe-$NH_2$·HCl were suspended in water at pH 7. The reaction was carried out in presence of thermolysin at 40 ºC, the optimal temperature for this enzyme. The isolated yield of this dipeptide was 96.8%.

The protected dipeptide amide was subjected to hydrogenation in presence of Pd/C in order to remove Z group. The isolated yield at this step was 96.4%.




**Scheme 3.7** Synthesis of Z-Asp(OMe)-Phe-$NH_2$ and the cleavage of Z group from Z-Asp(OMe)-Phe-$NH_2$. The isolated yield of the products are shown in parentheses.

### 3.3.5 Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ (3+2)

One strategy to synthesize Gly-Trp-Met-Asp-Phe-$NH_2$ (CCK-5) has been already published by our group (Xiang et al., 2004). In the reported strategy Gly-Trp-Met-Asp-Phe-$NH_2$ was obtained by coupling Phac-Gly-Trp-Met-OCam and H-Asp(OMe)-Phe-$NH_2$ in acetonitrile with α-chymotrypsin, immobilized on Celite-545, followed by basic hydrolysis of the β-methyl ester, and removal of the Phac group with PGA. It should be mentioned that there were two chemical reaction steps necessary to transfer the ethyl ester of Phac-Gly-Trp-Met-OEt to the OCam ester of Phac-Gly-Trp-Met-OCam.

In order to optimize the strategy by avoiding the OCam ester at the tripeptide fragment, in this work the fragment condensation between tripeptide and dipeptide is reported. The earlier mentioned allyl ester (Phac-Gly-Trp-Met-OAl) was reacted with 2.5 equivalents of H-Asp(OMe)-Phe-$NH_2$ to give Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$. The reaction

was carried out in ethyl acetate containing both 6.6% Tris-HCl buffer (0.1 M, pH 8.1) and 4% Borax buffer (0.1 M, pH 8.2) in presence of α-CHY/Celite-545. The isolated yield of the product was 54%. The by-product Phac-Gly-Trp-Met-OH was ca.2% (HPLC yield). Thus, by using the allyl ester, the earlier procedure of the synthesis of the pentapeptide could be improved by reducing the number of synthetic steps.

Figure 3.9 (b) shows the HPLC chromatogram of the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ at 95 hours. After this time, the target product started to decrease so the reaction was stopped at this point.

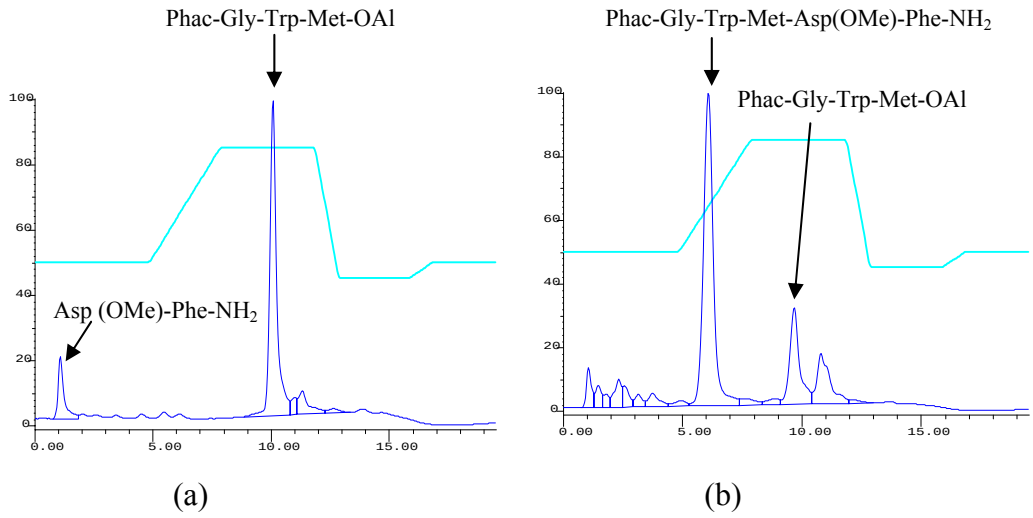
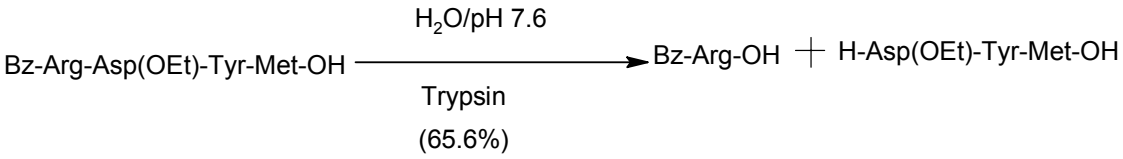



**Figure 3.9** HPLC chromatograms of the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ from Phac-Gly-Trp-Met-OAl and Asp(OMe)-Phe-$NH_2$ at 0 min (a) and 95 hrs (b).

### 3.4 Cleavage of Bz-Arg from Bz-Arg-Asp(OEt)-Tyr-Met-OH

The Bz-Arg protecting group was considered for the N α-amino protection of the final CCK-8. Therefore it was important to check whether it can be cleaved from the tripeptide or not. If it can be cleaved from the tripepetide, there is a high chance that it will also be cleaved from the octapeptide. The deprotection is outlined in scheme 3.8.

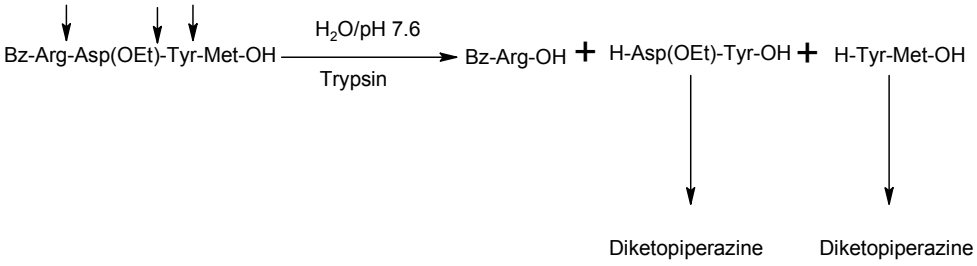



**Scheme 3.8** Cleavage of Bz-Arg from Bz-Arg-Asp(OEt)-Tyr-Met-OH.

The reaction was carried out in water in presence of free trypsin or trypsin/Eupergit C. In fact, both free enzyme and immobilized enzyme had the same effect for deprotection so immobilized trypsin was chosen as it can be separated from the solvent just by filtration. Moreover, the enzyme does not contaminate the product. The yield of H-Asp(OEt)-Tyr-Met-OH was 65.6% after isolation.

The reaction was investigated at two different pH values to optimize the reaction conditions. At pH 7.6, the isolated yield was 65.6% after 24 hours and at acidic pH 5.5, the HPLC conversion was only 12.5% after 48 hours.

Three different products were verified by FAB-MS when the trypsin immobilisate obtained earlier and from another company was used.




**Scheme 3.9** Cleavage of Arg-Asp, Asp-Tyr- and Tyr-Met bonds by trypsin leading to the formation of diketopiperazines.

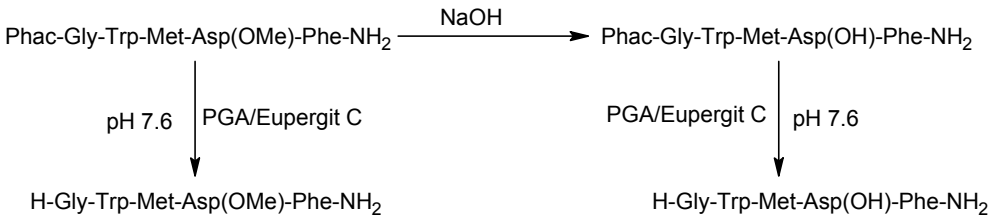
The trypsin was found to cleave at three different positions, Arg-Asp, Asp-Tyr and Tyr-Met. Two six member stable rings (diketopiperazine) formed from two individual fragments because of the internal nucleophilic attack from amino end to carboxyl end as shown in scheme 3.9. This unusual cleavage is probably caused by the impurities present in the trypsin. α-Chymotrypsin is the most common impurity in trypsin. Therefore, in later deprotection steps, TPCK trypsin was used in which the catalytic action of α-chymotrypsin is blocked.

### 3.5 Deprotection of the C-terminal pentapeptide (Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$)

The Phac-pentapeptide Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ contains two protecting groups: one is the β-methyl ester of the Asp residue, which could be easily removed by alkaline hydrolysis; the other is the N α-Phac group, which could be removed enzymatically by PGA/Eupergit C.

The fully deprotection of Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ was conducted in two steps. In the first step, the Phac-pentapeptide with the free β-carboxyl group in the aspartic acid residue was achieved through hydrolysis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ by NaOH in acetone/water (1:1). After acidification and washing, Phac-Gly-Trp-Met-Asp(OH)-Phe-$NH_2$ could be obtained with the isolated yield of 80.4%. In the second step, the removal of the Phac group from Phac-CCK-5 was carried out in water at pH 7.6 and 35º C with PGA/Eupergit C. After 24 hours, free CCK-5 was obtained. This isolated yield is lower than previously reported by our group (Xiang et al., 2004). One reason for the lower yield could be because a different batch of PGA was used.

The selective deprotection of Phac from Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ was conducted in one step. The reactant was suspended in water at pH 7.6 and PGA/Eupergit C was added. The yield of the reaction was low. This could be possible because of low solubility of the reactant in water.




**Scheme 3.10** Selective or complete deprotection of Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$. NaOH was used to cleave the methyl ester and immobilized PGA to cleave the Phac group.

The free pentapeptide was needed to condense with the tripeptide segment to synthesize CCK-8. Because of the poor yield of the Phac-deprotection step, it was difficult to make enough free pentapeptide for the further condensation. Therefore, the free pentapeptide was synthesized chemically using Fmoc as protecting group. The chemical synthesis of CCK-5 from C-terminus to N-terminus is outlined in section 3.7. The next alternative for the synthesis of free CCK-5 would have been the use of Fmoc or Bz-Arg as N α-protecting group and to continue from N-terminus to C-terminus by enzymatic catalysis.

## 3.6 Factors affecting the enzymatic synthesis of CCK-8 fragments

### 3.6.1 N-terminal tripeptide fragment

There is no general protocol available for enzymatic peptide synthesis. This is one of the drawbacks of enzymatic peptide synthesis. In order to suppress the competing hydrolysis reaction, the undesired oligomerization and the hydrolytic cleavage of the already formed peptide bonds, each coupling step has to be optimized. A series of reaction parameters such as optimal acyl-donor, nucleophile, reaction media, enzyme were investigated to find the best conditions for each reaction step.

**(1) Influence of the concentration and substrate structure**

***Substrate concentration.*** It is well known that under normal condition, the equilibrium in protease catalyzed reactions is in the direction of proteolysis and not proteosynthesis. To shift the equilibrium of the enzyme catalyzed synthesis, an excess of one reactant and the highest possible concentration is needed besides other parameters which can shift the

equilibrium. In the stepwise elongation from Bz-Arg-OEt·HCl to Bz-Arg-Asp(OEt)-Tyr-Met-OAl, it is better to apply the easily available nucleophile (amino acid ester) in excess. Beyond this, this is more economic also. The excess concentration of the carboxyl component may lead to 'product inhibition' of the enzyme. On the other hand, higher concentration of nucleophile may generate oligomer products as in the synthesis of Bz-Arg-Asp(OEt)-Tyr-OH. When the ratio of Bz-Arg-Asp(OEt)-OEt and Tyr-OMe·HCl was 1:4, the HPLC conversion of Bz-Arg-Asp(OEt)-$(Tyr)_n$-OH (where n= 2, 3) was above 10% and when the ratio was 1:1.5, the oligomer was kept below 5%. Therefore, it was important to optimize both, the concentration of acyl-donor and nucleophile.

***Structure of both, acyl-donor and nucleophile.*** As far as possible, ethyl and methyl esters were chosen as the acyl-donors in this work. These esters are cheap and easy to prepare in comparison to Cam ester, although the Cam ester has been reported as the most effective acyl-donor (Capellas et al., 1996[a]). For the synthesis of Bz-Arg-Asp(OEt)-OEt, Bz-Arg-OEt·HCl was chosen as an acyl donor. The isolated yield of the condensation between Bz-Arg-OEt·HCl and Asp(OEt)-OEt·HCl was 71.5% in solvent free condition under the catalytic influence of immobilized trypsin.

For the extension of this sequence to Bz-Arg-Asp(OEt)-Tyr-OH, two different acyl-donors were used in solvent free condition under the catalytic influence of papain. The first acyl-donor Bz-Arg-Asp(OEt)-OH did not react with Tyr-OMe·HCl but the second one Bz-Arg-Asp(OEt)-OEt could react to give 30.5% product. This is generally the case in peptide synthesis where peptide esters react better than peptides with a free carboxylic acid. From the kinetics of papain-catalyzed peptide synthesis, esterified acyl-donors should be preferable than acyl-donors having a free carboxyl group (Oka and Morihara, 1978). When the sequence was further extended to Bz-Arg-Asp(OEt)-Tyr-Met-OAl/OH with α-chymotrypsin/Celite 545, the free carboxylic acid (Bz-Arg-Asp(OEt)-Tyr-OH) could react with both Met-OEt·HCl and Met-OAl·PTS to form Bz-Arg-Asp(OEt)-Tyr-Met-OH (isolated yield 65.6%) and Bz-Arg-Asp(OEt)-Tyr-Met-OAl (isolated yield 63.9%) respectively. Synthesis by free carboxylic acid as acyl-donor is not so common in peptide synthesis but this case was observed many times in our laboratory.

For the synthesis of Bz-Arg-Asp(OEt)-Tyr-Met-OH/OAl, both nucleophiles, Met-OEt·HCl and Met-OAl·PTS could react. But the selection of the amino group was made taking into account its reactivity as well as the possibility that the product of this reaction will be the acyl-donor for the next one. In this case, Met-OAl·PTS was superior to Met-OEt·HCl because with Met-OAl·PTS, the obtained product Bz-Arg-Asp(OEt)-Tyr-Met-OAl could be used directly for the next coupling step.

**(2) Influence of the reaction media**

The solvent plays an important role in enzymatic peptide synthesis in organic media (Dordick, 1989; Cassells and Halling, 1989). Previous fundamental studies by Kisse et al., (1990; 1988) showed that both acetonitrile and ethyl acetate were the best solvents providing high enzymatic activities and product yields in some enzymatic dipeptide syntheses.

The coupling between Bz-Arg-Asp(OEt)-OEt and Tyr-OMe was investigated in different reaction media. In acetonitrile containing Tris-HCl buffer and ethyl acetate containing borax buffer, neither α-chymotrypsin nor papain could catalyze the reaction. There was only hydrolysis of the reactant i.e. Bz-Arg-Asp(OEt)-OH was formed. The solvent free system, however worked well for this coupling. Different concentrations of $KHCO_3/Na_2CO_3{\cdot}10H_2O$ and $KHCO_3/Na_2SO_4{\cdot}10\ H_2O$ were investigated for optimizing the yield of the reaction. The highest yield (30.5%) was obtained when one equivalent of Bz-Arg-Asp(OEt)-OEt was reacted with 1.5 equivalents of Tyr-OMe·HCl, 10 equivalents of $KHCO_3$ and 0.25 equivalents of $Na_2SO_4{\cdot}10H_2O$ in presence of free papain. When the reaction was carried out in neutral solvent free condition by adding $Na_2SO_4{\cdot}10\ H_2O$ in place of $KHCO_3$ and $Na_2CO_3$, there was no reaction at all.

For the next coupling reaction between Bz-Arg-Asp(OEt)-Tyr-OH and Met-OEt·HCl, the solvent free system in presence of α-chymotrypsin resulted in only 5% conversion of Bz-Arg-Asp(OEt)-Tyr-Met-OH. When the same reaction was carried out in Tris-HCl buffer (0.1 M, pH 8.1) containing 10% acetonitrile in presence of α-CHY/Celite-545, the isolated yield of the product was 38.7%. By recycling the unreacted educt, the isolated yield could be increased to 55%. When the concentration of organic solution was

increased from 10% to 80% by mixing EtOH and ACN (EtOH:ACN= 5:3) in Tris-HCl buffer (0.1 M, pH 8.1), the product yield decreased from 38.7% to 31.1%. The idea to increase the organic solvent was to make the reactants more soluble in the system but the lower yield could have been probably caused by the denaturation of enzymes by organic solvent mixture.

In the analogous way, Bz-Arg-Asp(OEt)-Tyr-OH and Met-OAl·PTS reacted in the acetonitrile containing Tris-HCl buffer (0.1 M, pH 8.1). The concentration of the buffer was very important in this reaction to direct the reaction to Bz-Arg-Asp(OEt)-Tyr-Met-OAl or to Bz-Arg-Asp(OEt)-Tyr-Met-OH. When the reaction was conducted in Tris-HCl buffer (0.1 M, pH 8.1) containing 10% acetonitrile, there was only Bz-Arg-Asp-OEt-Tyr-Met-OH formed however when the concentration of acetonitrile was increased from 10% to 96.2% under similar conditions, the isolated yield of Bz-Arg-Asp(OEt)-Tyr-Met-OAl was 63.9%.

**(3) Influence of the enzyme and carrier**

As far as possible, immobilized enzymes were used instead of free enzymes. The reaction between Bz-Arg-Asp(OEt)-OEt and Tyr-OMe·HCl failed when free papain was replaced by papain/VA-Epoxy.

### 3.6.2 C-terminal pentapeptide fragment

As done in the N-terminal tripeptide fragment of CCK-8, every coupling step was studied in detail for the synthesis of C-terminal pentapeptide. Different parameters for e.g. solvent, temperature, pH, acyl-donor structure, nucleophile structure, enzymes were taken into account for optimizing the yield in every step.

**(1) Influences of substrate structure and concentration**

***Structure of acyl-donor and nucleophile.*** The chemical nature of the ester moiety of the acyl-donor has a strong influence on enzymatic activity and product yield in kinetically controlled synthesis (Schellenberger et al., 1991). When the formation of acyl-enzyme complex between acyl-donor and enzyme is high, the formation of other by-products is greatly minimized during the reaction.

In this work, Phac-Gly-OMe did not react with Trp-OMe in the presence of immobilized papain. The reaction however was possible with very good isolated yield (90%) only when the structure of acyl-donor was changed from methyl to Cam ester.

The methyl ester in Phac-Gly-Trp-OMe was active enough to couple with both Met-OEt·HCl and Met-OAl·PTS under solvent free condition. The reaction with Met-OEt was catalyzed by α-CHY/Eupergit C and with Met-OAl was catalyzed by α-CHY/Celite-545. The isolated yield of both the products was almost the same (ca. 61%). The structure of the nucleophile was not really important for the yield of this step but the structure of the nucleophile was important for the further extension of the sequence.

Both acyl-donors, Phac-Gly-Trp-Met-OEt and Phac-Gly-Trp-Met-OAl were tried for fragment condensation with H-Asp(OMe)-Phe-$NH_2$. The ethyl ester of Phac-Gly-Trp-Met-OEt was not active enough to couple with H-Asp(OMe)-Phe-$NH_2$ and this condensation was carried out earlier in our laboratory by converting the ethyl ester into the Cam ester (Xiang et al., 2004). In this work, by choosing another nucleophile (Met-OAl·PTS), it was possible to get Phac-Gly-Trp-Met-OAl directly. There was no need of any chemical modification in the allyl ester for fragment condensation with H-Asp(OMe)-Phe-$NH_2$. The isolated yield in the present work is 54%. The isolated yield (73%) in our earlier reported work becomes 60.4% if the final yield is taken into account by calculating the yield in every steps converting from ethyl ester to free carboxylic acid to Cam ester and finally to the target product. In this way, the synthesis of the pentapeptide was improved by reducing the number of steps as well as the required time of the reaction maintaining nearly the same yield.

***Substrate concentration.*** Substrate concentration affects both enzymatic activity and yield in protease catalyzed peptide synthesis. In all coupling reactions, the nucleophile exceeded the acyl-donor by at least fifty percent in a molar ratio.

**(2) Influence of reaction media and temperature**

The influences of the reaction media for the synthesis of Phac-Gly-Trp-OMe was reported already by our group (Xiang et al., 2004). The extension of this dipeptide to Phac-Gly-Trp-Met-OAl was investigated in different reaction media. First, the condensation reaction between Phac-Gly-Trp-OMe and Met-OAl·PTS was performed in ethyl acetate containing 0.2% (Tris-HCl buffer, 0.1 M, pH 8.1,). α-Chymotrypsin was chosen for this coupling because there is an aromatic side chain of tryptophan at the $P_1$ position which is the primary specificity of α-chymotrypsin. There was only 3% product (Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$) and 20% by-product (Phac-Gly-Trp-OH).

Then the reaction was performed in a solvent free condition using α-CHY/Celite-545. One equivalent of Phac-Gly-Trp-OMe was reacted with 4 equivalents of Met-OAl·PTS, 2 equivalents of $KHCO_3$ and 0.4 equivalents of $Na_2CO_3 \cdot 10H_2O$. The HPLC conversion of the product and by-product was 40.3% and 8.1% respectively. In another investigation, one equivalent of Phac-Gly-Trp-OMe was reacted with 4 equivalents of Met-OAl·PTS, 20 equivalents of $KHCO_3$ and 4 equivalents of $Na_2CO_3 \cdot 10H_2O$. The HPLC conversion of the product and by-product was 17.8% and 17.9%, respectively. This result showed that higher amount of basic salts and higher amount of crystalline water is not so suitable for this coupling therefore slightly basic, neutral and acidic conditions were prepared to optimize the yield of the reaction.

Three different types of solvent free systems were made by choosing different salt systems. The basic solvent free system was prepared by taking 2 equivalents of $KHCO_3$ and 0.4 equivalents of $Na_2CO_3 \cdot 10\ H_2O$. The neutral solvent free system was prepared by taking 1 equivalent of $Na_2SO_4 \cdot 10\ H_2O$. Similarly, 0.5 equivalents of $Na_2SO_4 \cdot 10\ H_2O$ and 1 equivalent of $KHSO_4$ were mixed for the acidic solvent free system. When one equivalent of Phac-Gly-Trp-Met-OAl and 4 equivalents of Met-OAl·PTS were mixed to all three systems separately in presence of α-CHY/Celite-545, the basic system gave the highest yield. The results are shown in table 3.2.

**Table 3.2** The HPLC yield of product, by-product and reactant observed when Phac-Gly-Trp-OMe and Met-OAl·PTS were mixed in three different solvent free systems differing in pH.

| Reaction system (solvent free) | HPLC yield (%) | | |
|---|---|---|---|
| | Phac-Gly-Trp-Met-OAl | Phac-Gly-Trp-OH | Phac-Gly-Trp-OMe |
| Basic | 44.4 | 6.5 | 14.2 |
| Neutral | 2.5 | 9.8 | 60.2 |
| Acidic | - | - | 73.8 |

Therefore, further optimization was carried out in the basic system to improve the yield. The best result was obtained when one equivalent of Phac-Gly-Trp-OMe, 1.37 equivalents of Met-OAl·PTS, one equivalent of $KHCO_3$ and 0.3 equivalents of $Na_2CO_3 \cdot 10\ H_2O$ were mixed in presence of α-CHY/Celite-545. Under this condition, the isolated yield of the product (Phac-Gly-Trp-Met-OAl) was 60.1%.

For synthesizing Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$, the condensation of Phac-Gly-Trp-Met-OAl with Asp(OMe)-Phe-$NH_2$·HCl was investigated in different reaction systems which is outlined in table 3.3. α-CHY/Celite-545 was used in all systems. The reaction was unsuccessful under the solvent free and co-solvent systems. There was no effect in the reaction when the amount of enzyme and Tris-HCl buffer was increased to some extent in both systems. However, the coupling was observed in bi-phasic systems. The HPLC yield of Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ was 24.3% when the reaction was carried out in ethyl acetate containing 0.35% Tris-HCl buffer (0.1 M, pH 8.1). The HPLC yield increased to 34.2% when the Tri-HCl buffer (0.1 M, pH 8.1) was replaced by borax buffer (0.1 M, pH 8.2) and $Et_3N$ was added. The best result (HPLC yield 66.7%) was obtained when one equivalent of tripeptide reacted with 2.5 equivalents of dipeptide in ethyl acetate containing 6.6% Tris-HCl buffer (0.1 M, pH 8.1), 4% borax (0.1 M, pH 8.2) buffer and 0.3% $Et_3$ N. After 95 h, the reactant, Phac-Gly-Trp-Met-OAl was completely finished and the isolated yield of target product was 54%. By optimizing the conditions, the by-product (Phac-Gly-Trp-Met-OH) could be kept at 2%.

**Table 3.3** HPLC yield of product (Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$), by-product (Phac-Gly-Trp-Met-OH) and acyl-donor (Phac-Gly-Trp-Met-OAl) when acyl-donor and nucleophile were mixed in different reaction systems. α-CHY/Celite-545 was used to catalyze the reaction. * 10 µl $Et_3N$ were added. ** 20 µl $Et_3N$ were added.

| Reaction System | Reactants | Time [h] | HPLC yield of (%) | | |
|---|---|---|---|---|---|
| | | | Product | By-product | Acyl-donor |
| Solvent free ($KHCO_3$: 0.2 mmol $Na_2CO_3 \cdot 10H_2O$: 0.04 mmol) | Acyl-donor: 0.1mmol Nucleophile: 0.2 mmol | 72 | - | 15.3 | 42.9 |
| Co-solvent* (ACN: 2 ml Tris-HCl: 10 µl, 0.1 M, pH 8.1) | Acyl-donor: 0.1 mmol Nucleophile: 0.2 mmol | 72 | - | - | 82.9 |
| Bi-phasic (EtOAC: 2ml Tris-HCl: 7 µl, 0.1 M, pH 8.1) | Acyl-donor: 0.05mmol Nucleophile: 0.1 mmol | 96 | 24.3 | 12.0 | 36.8 |
| Bi-phasic* (EtOAc: 2 ml Borax: 8 µl, 0.1 M, pH 8.2) | Acyl-donor: 0.1mmol Nucleophile: 0.2 mmol | 72 | 34.2 | 9.5 | 3.9 |
| Bi-phasic** (EtOAc: 6 ml Borax: 240 µl, 0.1 M, pH 8.2 Tris-HCl: 400 µl, 0.1 M, pH 8.1) | Acyl-donor: 0.2 mmol Nucleophile: 0.5 mmol | 95 | 66.7 | 2.0 | - |

The kinetics of tripeptide, dipeptide, by-product and product is shown in figure 3.10. As shown in the figure, the product yield increased sharply after 70 hours and after 96 hours, it decreased. At 70 hours, more immobilisate was added which caused the sharp rise in the product yield. The reaction was stopped at 96 hours.

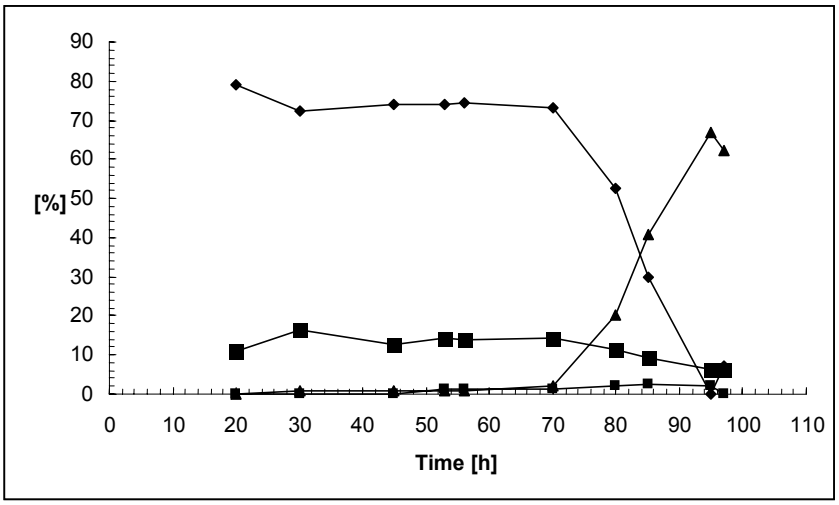



**Figure 3.10** Kinetic process of the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$(▲) and the by-product Phac-Gly-Trp-Met-OH (▪) from 0.2 mmol Phac-Gly-Trp-Met-OAl (♦) and 0.5 mmol H-Phe-$NH_2$·HCl (▄) in 6 ml ethyl acetate containing 4% borax buffer (0.1 M, pH 8.2), 6.6% Tris-HCl buffer (0.1 M, pH 8.1), 0.3% $Et_3N$ with α-CHY/Celite-545.

### 3.7 Chemical synthesis

Chemical synthesis was performed either to obtain references substances or to generate enough starting materials. Reference substances are necessary for the identification of the product peak while monitoring the enzymatic reactions in HPLC. In enzymatic peptide synthesis, the newly formed product is not always the target product. Many times, cleaved products are also formed so it is difficult to follow the reaction if there is no reference substance. However, in the chemical approach, the product is reliably formed if the reaction is done according to the standard well known procedures. There might be racemization and diketopiperazine formation during the reaction but it does not matter if the substances are used as reference materials. Most of the reference substances were

already available in our laboratory and the lacking substance ( Phac-Gly-Trp-Met-OAl) was synthesized prior to the enzymatic investigation.

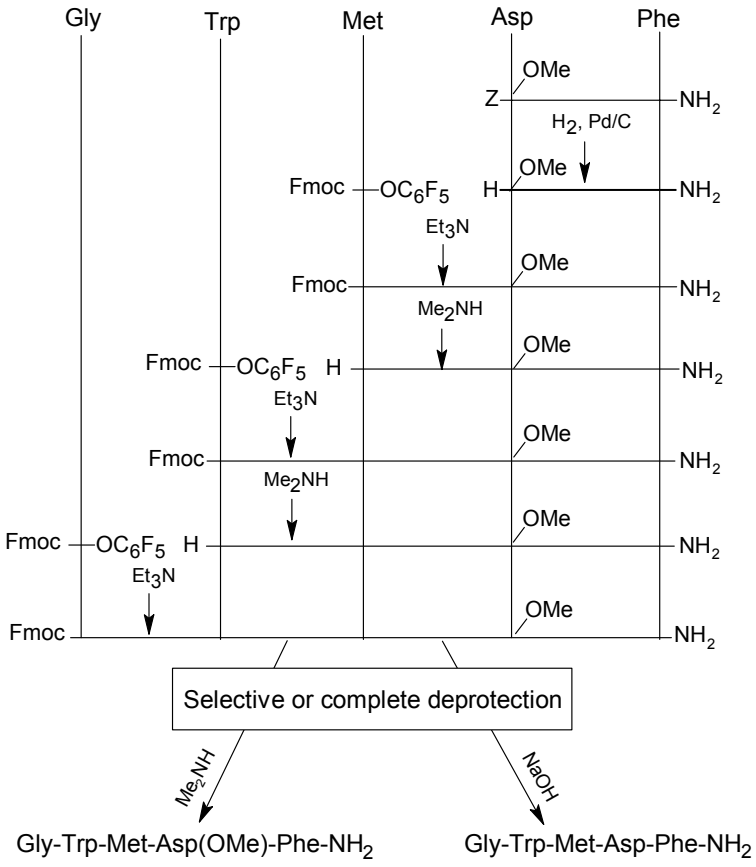
### 3.7.1 Synthesis of Phac-Gly-Trp-Met-OAl

For the synthesis of Phac-Gly-Trp-Met-OAl, first of all, Phac-Gly-Trp-OMe was synthesized as described in 3.3.2. The chemical synthesis of Phac-Gly-Trp-Met-OAl was done by mixing the equimolar amounts of HOBt and Phac-Gly-Trp-OH with 10% excess of DCC. After the activation, DCU was filtered off and Met-OAl·PTS was added to the solution. Attack of the amino component on the activated carbonyl C-atom of carboxyl component resulted in the formation of the Phac-Gly-Trp-Met-OAl.

### 3.7.2 Synthesis of C-terminal pentapeptide

Chemical synthesis of C-terminal pentapeptide was performed because the yield of free pentapeptide obtained by cleavge of Phac from Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ was not good enough. High amount of free pentapeptide is mandatory to carry out the further steps of CCK-8 synthesis. There could have been another possibility to replace the Phac group by other protecting groups like Fmoc or Bz-Arg in enzymatic synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ but the C $\longrightarrow$ N way of chemical synthesis was performed because the reaction time was short (ca. 20 min) and the isolated yield was good in every step. However, the main problem observed during this chemical synthesis was the side reaction created by the ring closure of the side chain of aspartic acid. The details regarding the ring closure is given in 3.7.2.3.

The chemical synthesis of the C-terminal pentapeptide fragments was carried out in solution phase in analogy to the literature (Kisfaludy and Schoen, 1983). The base-labile 9-fluorenylmethyloxycarbonyl group (Fmoc) was applied for the protection of the amino group in acyl-donors and the pentafluorophenylester was used for the activation of the carboxyl group. The cleavage of the Fmoc group can take place by the basicity of the amino group to be acylated. Therefore, highly reactive pentafluorophenylesters was used to increase the rate of coupling and to prevent the side reaction created by Fmoc cleavage. The chemical synthesis of the C-terminal pentapeptide fragment via the C $\longrightarrow$ N strategy is presented in scheme 3.11.




**Scheme 3.11** Chemical synthesis of the C-terminal pentapeptide fragments.

### 3.7.2.1 Synthesis of Fmoc amino pentaflurophenyl esters

Fmoc-amino acid pentafluorophenyl esters were easily obtained in high yield (≥87.5%) from the equal molar ratio of Fmoc-amino acid and pentafluorophenol in EtOAc or a mixture of EtOAc and DMF using DCC as the coupling reagent.

### 3.7.2.2 Synthesis of Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$

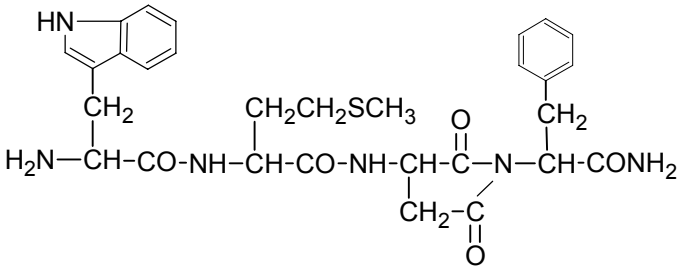
Z-Asp(OMe)-Phe-$NH_2$, easily obtained from thermolysin-controlled peptide synthesis in 96.8% isolated yield, was hydrogenated with the Pd/C catalyst to afford Asp(OMe)-Phe-$NH_2$ in quantitative yield. The free dipeptide amide was condensed with an excess of Fmoc-Met-$OC_6F_5$ in DMF with addition of triethylamine. The obtained Fmoc-Met-Asp(OMe)-Phe-$NH_2$ was treated with 10% dimethylamine solution in DMF to remove the Fmoc-group. The peptide chain was elongated by repeating the condensation and

deprotection cycle until Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ was achieved. The isolated yields were around 80% in every coupling and every deprotection step. FABMS was used to characterize the product in every step.

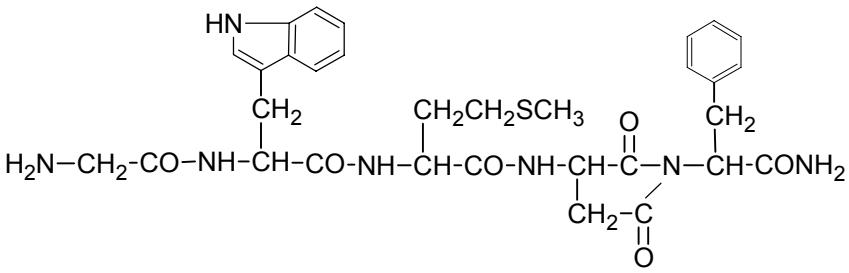
### 3.7.2.3 Deprotection of Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$

The fully deprotected pentapeptide Gly-Trp-Met-Asp(OH)-Phe-$NH_2$ was achieved by hydrolysis with 5% NaOH in $H_2O$/acetone in one step. When the reaction was finished, the pH was adjusted to 7.0. The acetone was removed in vacuum. After removal of the acetone, the product precipitated from water.

The partially protected pentapeptide Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ was obtained by cleaving the Fmoc group with 10% diethylamine. After the cleavage, other by-products were separated in MPLC. The isolated peak of Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ from MPLC seemed to be homogeneous by HPLC in the methanol system but in the ACN system, this single peak was resolved into four peaks. All the four peaks were separated by preparative HPLC and found that the first peak was Trp-Met-Asp(OMe)-Phe-$NH_2$ followed by Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$. The next two successive peaks were the products formed by the ring closure of the Asp side chain of Trp-Met-Asp(OMe)-Phe $NH_2$ (Compound 1) and Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ (Compound 2), respectively. The chemical formulae of these two compounds are as follows:




**Compound 1**



**Compound 2**

To investigate further the ring closure of the free tetra and pentapeptide, a purified free tetrapeptide was used. The chromatogram of the free tetrapeptide is shown in figure 3.11 (a). When the tetrapeptide dissolved in DMF was mixed with dimethyl amine, a new peak appears in figure 3.11 (b) which is the cyclic product of the tetrapeptide. When the mixture was heated at 50ºC just for couple of minutes, the concentration of the cyclic product increased rapidly as can be seen in figure 3.11 (c). Therefore, the removal of solvents during working up procedure was carried out at 25ºC to reduce the rate of ring formation. It can be concluded that ring formation takes place during the cleavage of the protecting group by dimethyl amine and the concentration of this by-product increases during the working up procedure.

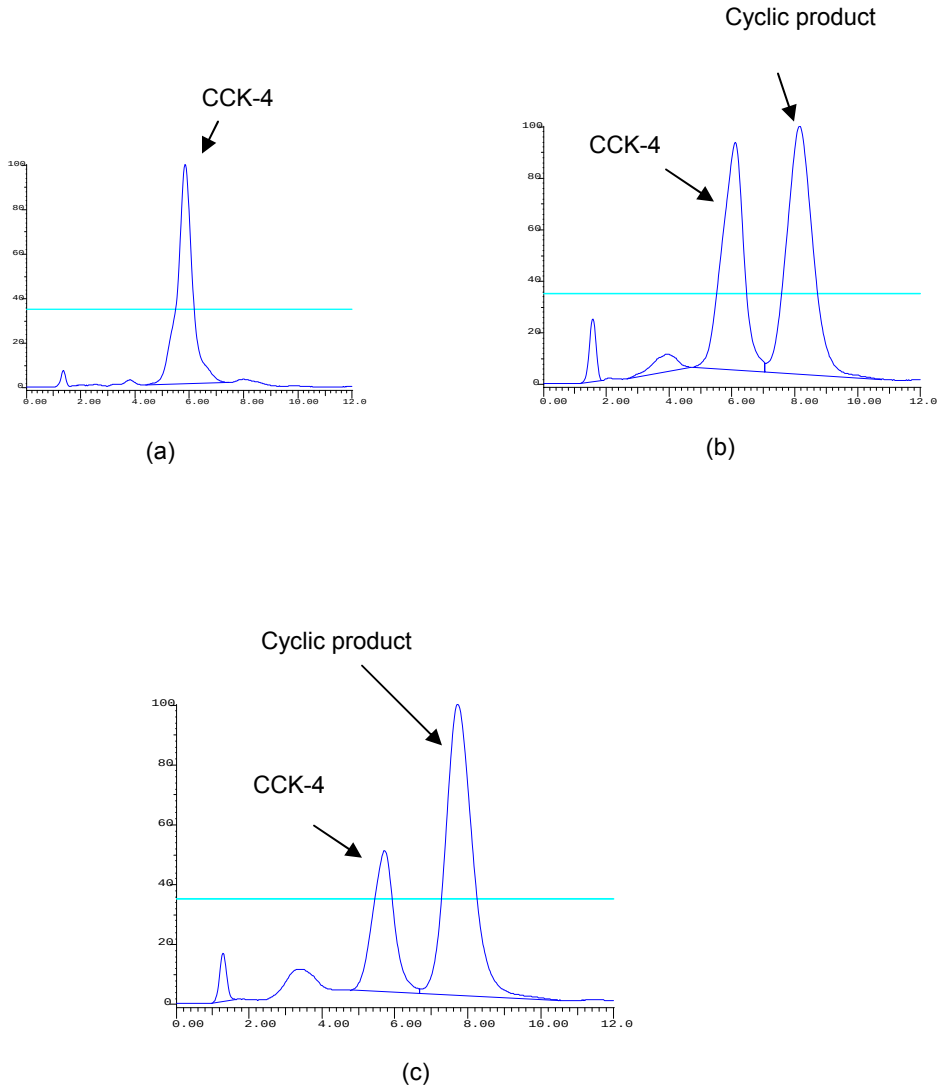
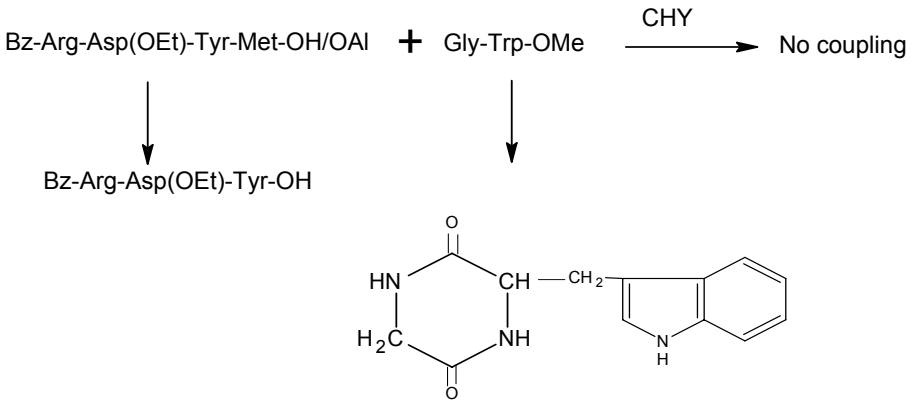



**Figure 3.11** HPLC chromatogram of free tetrapeptide (a), after addition of dimethyl amine in tetrapeptide (b) and after heating the solution of dimethyl amine tetrapeptide (c).

### 3.8 Enzymatic synthesis of the protected CCK-8 [3+ (3+2)]

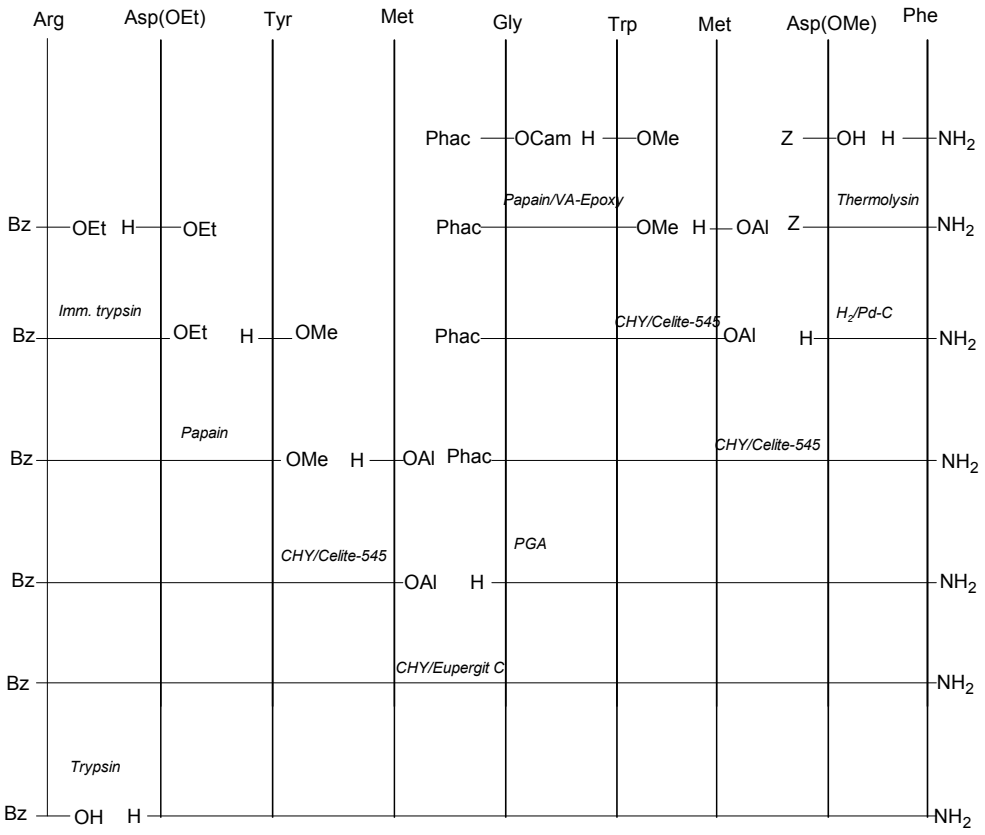
Two different strategies were explored for the final assembly of the octapeptide ($CCK_{26-33}$). In the first strategy, it was planned to couple Bz-Arg-Asp(OEt)-Tyr-Met-OH/OAl and Gly-Trp-OMe and then Met-Asp(OMe)-Phe-$NH_2$ in order to synthesize Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$. The strategy can be described as [(3+2) +3] in an abbreviated form.




**Scheme.3.12** Side reactions during the planned synthesis of Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$.

During the coupling of Bz-Arg-Asp(OEt)-Tyr-Met-OH/OAl with Gly-Trp-OMe, no target product could be detected because of two side reactions. First, the free Gly-Trp-OMe underwent rapid internal nucleophilic attack to form diketopiperazine. Second, the acyl-donor peptide was cleaved by α–chymotrypsin to form Bz-Arg-Asp(Tyr)-OH. The side reactions are outlined in scheme 3.12. The fragment coupling neither worked in acetonitrile solvent containing Tris-HCl buffer nor in ethyl acetate containing borax buffer.

The second strategy, however, was successful. The synthesis of CCK-8 commenced with the α-CHY/Eupergit C controlled coupling of Bz-Arg-Asp(OEt)-Tyr-Met-OAl and Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$. The free pentapeptide Gly-Trp-Met-Ap(OMe)-Phe-$NH_2$ was obtained by coupling Phac-Gly-Trp-Met-OAl and H-Asp(OMe)-Phe-$NH_2$ in ethyl acetate with α-CHY/Celite-545 followed by removal of the Phac group by immobilized PGA. Based on the many coupling reactions, the optimal strategy is outlined in scheme 3.13 which can be described as [3+ (3+2)].




**Scheme 3.13** The second strategy for the enzymatic synthesis of the octapeptide.

The crucial fragment coupling between Bz-Arg-Asp(OEt)-Tyr-Met-OAl and H-Gly-Trp-Met-Asp(OMe)-Phe$NH_2$ was performed as follows. The deprotected H-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ was reacted with 2 equivalents of Bz-Arg-Asp(OEt)-Tyr-Met-OAl in ACN containing 0.65% Tris-HCl buffer (pH 7.0, 0.05 M) and 0.45% $Et_3N$ where the condensation took place with α-CHY/Eupergit C. After 118 hours, the isolated yield of protected CCK-8 was 17.1%. Different parameters were investigated in order to increase the yield. The isolated yield could not be improved by changing the reaction systems and the enzyme carriers as compiled in table 3.4.

**Table 3.4** Synthesis of protected CCK-8 in different reaction media under the influence of different enzyme carrier. Tris-HCl buffer was 0.5%, 0.05 M, pH 7.0. Borax buffer was 0.4%, pH 8.2, 0.05 M.

| Reaction system | Enzyme | Isolated yield |
|---|---|---|
| Solvent free | α-CHY/Celite-545 | - |
| ACN containing Tris-HCl buffer | α -CHY/Celite-545 | < 5% |
| ACN containing Tris-HCl buffer | Free α-chymotrypsin | - |
| ACN containing Tris-HCl buffer | α-CHY/Eupergit C | 17% |
| Ethyl acetate containing borax buffer | α-CHY/Eupergit C | 9% |

As can be seen in the table, free α-chymotrypsin could not catalyze the reaction, however, when the same enzyme was immobilized on Eupergit C, the best result was obtained. This result is surprising because immobilized enzyme could catalyze the condensation which free enzyme failed to do so. This abnormal behavior could not be studied during this period. α-CHY/Celite-545 was also not suitable for this reaction. The reaction in acetonitrile containing 0.5% Tris-HCl buffer was better than in ethyl acetate containing borax buffer.

The main cause for the low yield of the reaction was the hydrolysis of the allyl ester of Bz-Arg-Asp(OEt)-Tyr-Met-OAl to Bz-Arg-Asp(OEt)-Tyr-Met-OH. When the kinetic was analyzed, it was found that the rates of both condensation and hydrolysis were nearly the same. It means that both the target product and the hydrolysis product were formed at the same rate. Next, it was tried to lower the hydrolytic action of the enzyme by lowering the water content in the system. The amount of buffer was already low so the main source of water in the system was from α-CHY/Eupergit C, because it was always stored in the wet form after immobilization. This water content in the enzyme was reduced by washing the enzyme with ACN containing 0.5% Tris-HCl buffer. The result showed that there was no reaction at all. Therefore the best investigated way so far was to use α-CHY/Eupergit C.

Unlike in other coupling steps, the acyl-donor in this reaction was added in double molar ratio to the nucleophile. The reason for this is that it is comparatively easier to prepare Bz-Arg-Asp(OEt)-Tyr-Met-OAl than H-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$.

Figure 3.12 represents the synthesis of protected CCK-8 during the reaction period [(a) and (b)] and after purification (c)]. The reaction was slow and when more enzyme was added to speed up the reaction, the by-product (Bz-Arg-Asp(OEt)-Tyr-Met-OH) was formed faster than the target protected octapeptide.

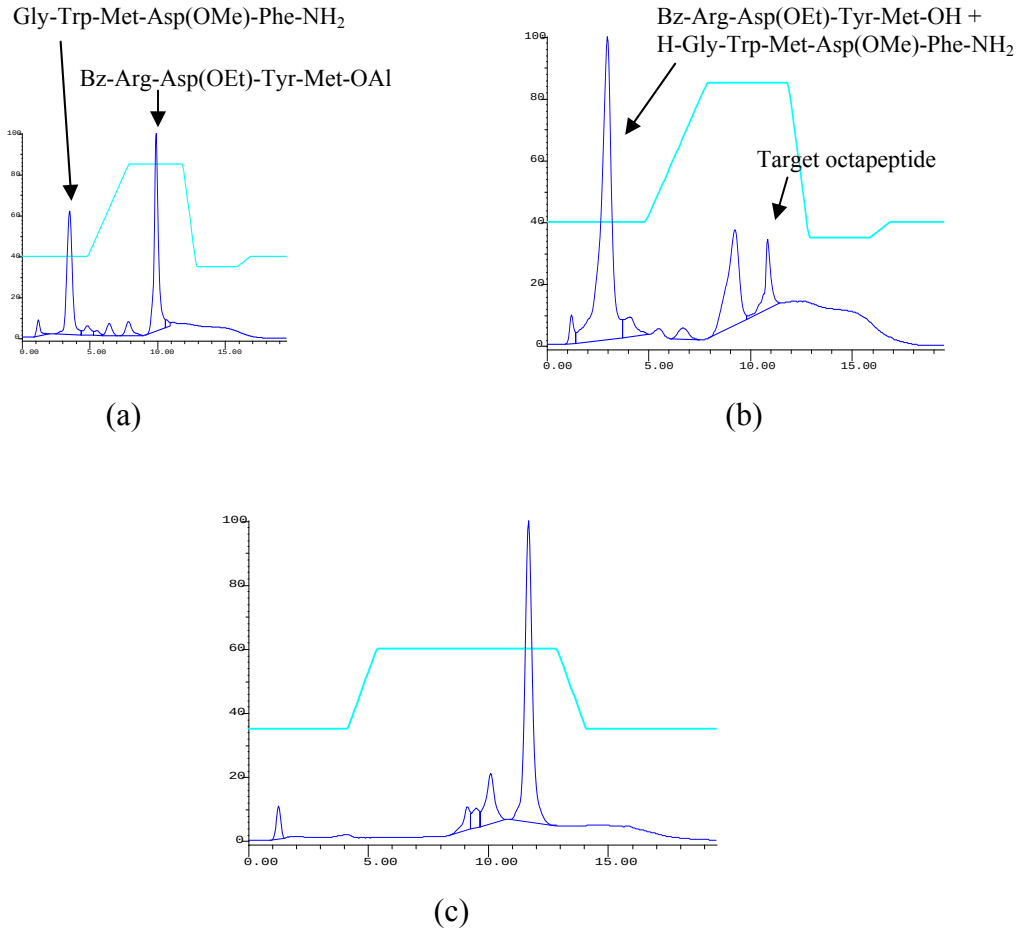



**Figure 3.12** Synthesis of Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ at 0 min (a), after 90 hrs (b) and after isolation (c).

### 3.9 Cleavage of Bz-Arg from protected CCK-8

The action of trypsin is restricted to the linkage of the carboxyl group of arginine or any other basic amino acid. Therefore, trypsin should split exclusively the Arg-Asp bond. To be sure that all other peptide bonds except Arg-Asp were inert to the action of trypsin; a test experiment was done prior to the cleavage of Bz-Arg. In the test experiment, trypsin was fed to the aqueous suspension of Z-Gly-Trp-Met-Asp(OMe)-OMe. There was no

change up to 36 hours which is a confirmation that trypsin indeed does not cleave any peptide bond in this tetrapeptide.

To study the suitability of this reaction for the cleavage of Bz-Arg, TPCK treated trypsin was added to 5 mg protected CCK-8 suspended in water (pH around 8). As mentioned earlier, TPCK treated trypsin blocks the activity of α-chymotrypsin present in trypsin. Unlike in proteosynthesis, dilute solution is preferred for proteolysis. After 2 hours, the sample mixture was checked in ESIMS. Three peaks belonging to Bz-Arg-OH (Mol. Mass 278), H-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ (Mol. Mass 1104) and unreacted reactant, Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ (Mol. Mass 1364) were clearly seen in the ESIMS spectrum. (see figure 3.13). From these results it can be concluded that enzymatic removal of Bz-Arg from Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ to obtain H-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ was successful. The Bz-Arg protecting group in contrast to Phac group proved to be useful in longer peptide sequences. The enzymatic removal of the protecting group from protected CCK-8 is a significant improvement because in the literature of CCK-8 synthesis, either the protecting group has not been cleaved at all (Fite et al., 2002) or is removed via chemical way (Kullmann, 1982; Sakina et al., 1988; Cerovsky et al., 1988)

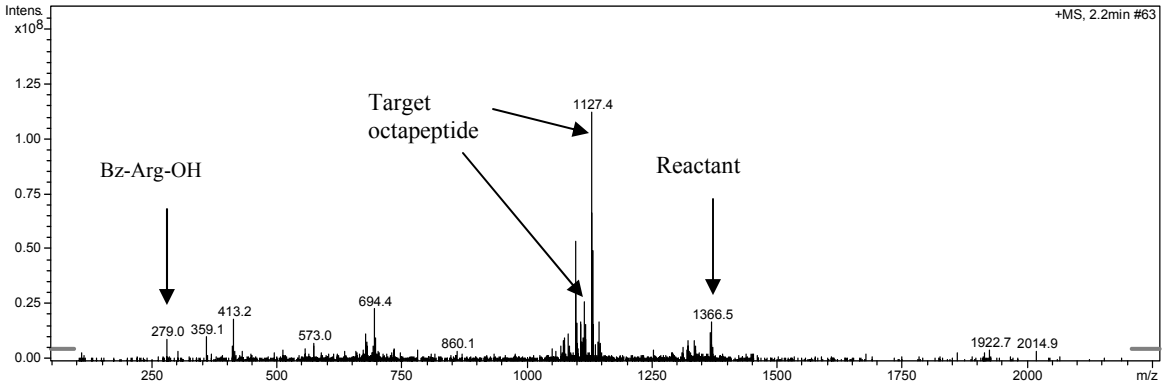
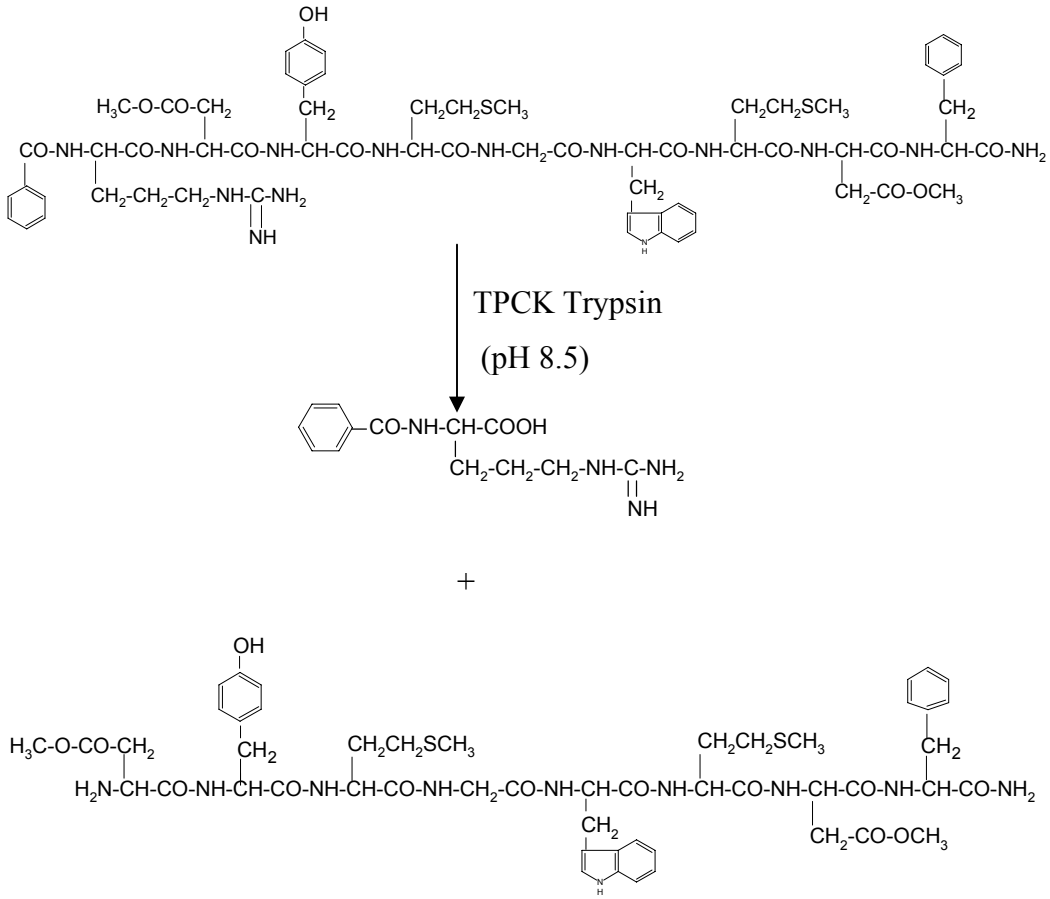



**Figure 3.13** ESIMS of reaction mixture of aqueous suspension of protected CCK-8 and TPCK trypsin at pH around 8.

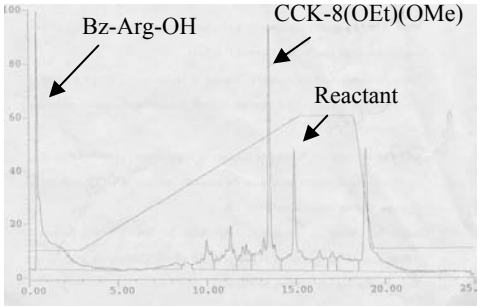
The reaction of deprotection is elucidated in scheme 3.14



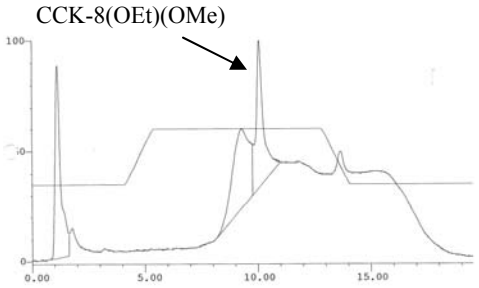

**Scheme 3.14** Cleavage of Bz-Arg from the protected CCK-8 (Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$) by the action of TPCK trypsin at pH 8.5.

To be able to isolate the deprotected CCK-8 peptide, a higher amount of the protected peptide (15 mg) was used. Because under alkaline conditions, cyclisation in the aspartate residue was observed earlier, the pH of the residue was adjusted to pH 7. Cyclisation in Asp residue with the side chain carboxyl group protected in the form of ethyl, methyl ester was also reported by Bodanszky (1993[a]).

There was no reaction at this pH up to 48 h. When the pH was increased to 8.5, the reactant vanished completely in 4 hours and the target product (ethyl, methyl ester of CCK-8) was formed in an isolated yield of 16.5%. Figure 3.14 illustrates the deprotection of the protected CCK-8 peptide. Figure 3.14 (a) represent the chromatogram at the end of the reaction and (b) represent the chromatogram of deprotected CCK-8 after purification.




(a)




(b)

**Figure 3.14** N-terminal deprotection of the protected CCK-8. Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ before purification (a) and after purification (b).

As can be seen in figure 3.14 (a), at the end of the reaction, only ca. 30% reactant remained unreacted and ca. 70% reactant converted to target product and Bz-Arg-OH. In this regard, the isolated yield should have been significantly high. However, the amount of the reactant taken was not enough for an efficient working up procedure. Therefore, the yield was low. It can be concluded that this deprotection reaction goes smoothly with trypsin and the yield will be far better than the present yield if the amount of reactant is more.

An alternative approach to increase the yield could be the use of thrombin instead of trypsin for the cleavage of Arg-Asp bond as reported by Bodanszky (1993[b]).

The deprotected CCK-8 after purification [3.14 (b)] was checked in ESIMS. The result is presented in figure 3.15. In the figure, the molecular mass of the target peptide is 1104.

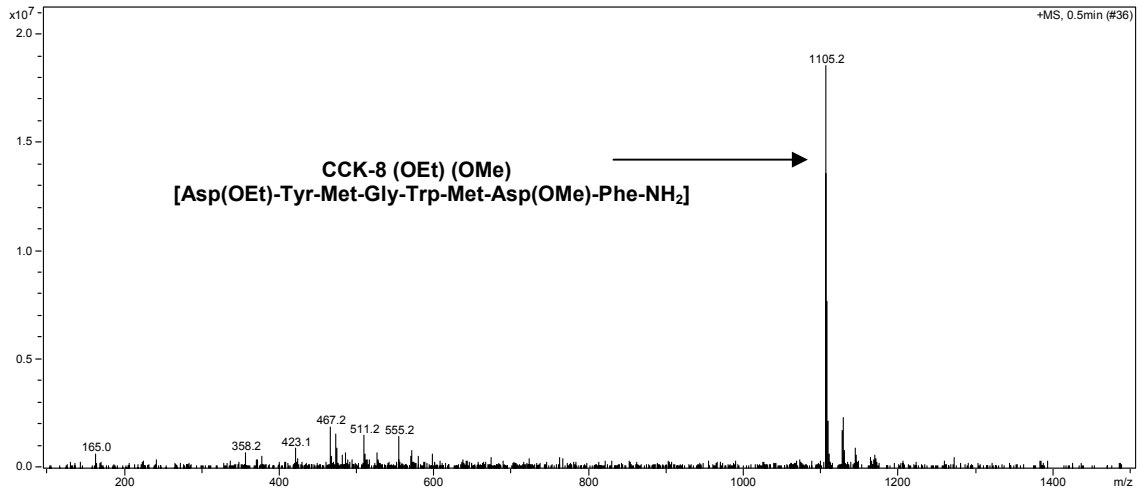



**Figure 3.15** ESIMS of Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$.

M+1 (1105) and M+23 (1127) of the target peptide in the ESIMS spectrum clearly demonstrates that the isolated octapeptide is absolutely pure. No other significant peaks except some low molecular masses can be seen in the MS spectrum. Beyond that, no cyclisized product could be detected; neither in HPLC (figure 3.14) nor in ESIMS (figure 3.15). It could also be demonstrated that the Bz-Arg group is superior to other used N-terminal protecting groups in CCK-8 syntheses, because it can be cleaved easily with trypsin.

# 4 Experimental section

## 4.1 Apparatus

**(1) Analytical HPLC**

Pumps: 305 and M 307 and Software 712 HPLC system control software (Gilson). UV Detector: L-7400 (Merck Hitachi LaChrom) set to 260 nm; Column: Nucleosil C18 (Macherey-Nagel), 5 µm, 100x2 mm.

**(2) Preparative HPLC**

(i) Pumps: Metering pumps Model I/III (ConstaMetric); UV Detector: Model UA-6, UV/Vis detector (ISCO), set to 254 nm; Column: Nucleosil C18 (Macherey-Nagel), 7 µm, 250×8 mm.

(ii) Pumps: 305 and M 307 and Software 712 HPLC system control software (Gilson). UV Detector: L-7400 (Merck Hitachi LaChrom) set to 260 nm; Column: Nucleosil C18 (Macherey-Nagel), 7 µm, 250×8 mm.

**(3) Preparative chromatography (MPLC)**

Pumps: Peristaltic pump P-1 (Pharmacia Fine Chemicals) and ProMinent electronic E-0803 pump; UV Detector: Model UA-5, Absorbance/Fluorescence detector (ISCO), set to 254 nm; Fraction collector: Model-328 (ISCO); Column: Polygosil C18 (Macherey-Nagel), 50-60 µm, 30×4 cm.

**(4) Size exclusion chromatography**

UV Detector: Model UA-5, Absorbance/Fluorescence detector (ISCO), set to 260 nm, Fraction collector: Model-328 (ISCO); Column: Sephadex LH 20 (Pharmacia), 25-100 µm, 90×4 cm.

**(5) LC-ESIMS**

1100 Series LC/MSD Trap (Agilent); Column: Nucleosil C18 (Macherey-Nagel), 3 µm, 30×4 mm.

**(6) FABMS**

TSQ 70 (Finnigan MAT), positive ion mode

**(7) Centrifuge**

Universal 16 A (Hettich)

**(8) pH-Meter**

E 512 (Metrohm)

**(9) UV lamp**

Universal-UV-lamp (CAMAG)

**(10) Ultrasound**

Transsonic 310 (ELMA)

**(11) Ultra-pure water system**

Milli-Q plus 185 (Millipore)

**(12) Melting point apparatus**

Modell SMP-20 (Büchi)

**(13) Lypohilization machine**

Cryostat: Unicryo MC 2L-60$^{o}$C (Uniequip); Vacuum pump: Type 109021 Chemvac 6 DP-101 (ILMVAC)

**4.2 Materials**

**(1) Amino acids and chemicals**

All L-amino acids were gifts from Degussa. Methionine allyl ester *p*-tosylate (Met-OAl·TOS) was from Fluka. Fmoc-Gly-OH, Fmoc-Trp-OH and Fmoc-Met-OH were obtained from Novabiochem, Merck. Pentafluorophenol was from Merck. Benzyl penicillin sodium was obtained from Fluka. N-Acetyl-L-tyrosine ethyl ester was from Sigma. Bz-Arg-OEt·HCl and Z-Asp-Phe-$NH_2$ were prepared by standard procedures in

our laboratory. 10% Pd-C was a gift from Degussa. Absolute DMF was prepared according to the following procedure which was published in Organikum (1996). 1.5 L DMF, 180 ml benzene and 70 ml water were mixed and distilled under normal pressure until the temperature remained constant at 153°C. After cooling down to room temperature, calcium hydride (10 g) was added slowly and kept overnight. After that, it was distilled in vacuum under nitrogen avoiding light. The vacuum was set in a way that the temperature of the distillation was between 60 to 80ºC. HPLC grade methanol and acetonitrile were obtained from Fischer chemicals. All other chemicals used were of analytical grade.

**(2) Enzymes**

Papain (EC 3.4.22.2) from *Carica papaya* (water-soluble, 30000 USP-U/mg using casein as substrate) and α-chymotrypsin (EC 3.4.21.1) from bovine pancreas (Type II, 3xcrystallized, lyophilized powder, 350 U/mg using N-acetyl-L-tyrosine ethyl ester (ATEE) as substrate) were obtained from Merck. Thermolysin (EC 3.4.24.2) from *bacillus thermoproteolyticus rokko* (Protease X, lyophilized powder containing calcium and sodium buffer salts, 50 U/mg protein, casein assay) was from Sigma. Trypsin (EC 3.4.21.4) from porcine pancreas (crystallized, lyophilized, 40 U/mg using N-benzoyl-L-argininethylester (BAEE) as substrate) was obtained from Merck. TPCK treated trypsin (EC 3.4.21.4) from bovine pancreas (10138 U/mg) and Penicillin G Amidase (PGA) immobilized on Eupergit C (109 U/g) were obtained from Fluka.

**(3) Carriers for immobilization of enzymes**

Celite-545 (particle size 20-45 μm) was obtained from Fluka. Eupergit C and VA-Hydroxy-Biosynth were from Roehm Pharma.

**(4) Materials for chromatography**

Nucleosil C18 (5 μm) and Polygosil C18 (50-60 μm) were from Macherey-Nagel. The HPLC columns were packed in our laboratory according to the high viscosity method. Silica gel plates, $F_{254}$ were obtained from Merck. Sephadex LH 20 (25-100 μm) was from Pharmacia Fine Chemicals.

## 4.3 Chromatographic methods

### (1) Analytical HPLC

***Methanol solvent system.*** Eluent A: 0.05 M aqueous $AcONH_4$ (pH 6.5), which was prepared from 2 M $AcONH_4$ of the stock soltion (pH 6.5, filtered through 4 μ-frit) and degassed by evacuation; Eluent B: 80% MeOH/0.05 M $AcONH_4$, which was prepared by mixing 25 ml 2 M $AcONH_4$ of the stock solution, 800 ml methanol and water was added to make volume 1 L and degassed by evacuation. The flow rate was 0.3 ml/min.

***HPLC i.*** Isocratic eluent 40% B.

***HPLC ii.*** Isocratic eluent 90% B.

***HPLC iii.*** Gradient elution from 40% B to 55% B over 24 min.

| Time (min) | % of B |
|---|---|
| 0-3.60 | 40 |
| 3.60-7.50 | 40-55 |
| 7.50-16.50 | 55 |
| 16.50-17.20 | 55-40 |
| 17.20-24.00 | 40 |

***HPLC iv.*** Gradient elution from 40% B to 85% B over 19.50 min.

| Time (min) | % of B |
|---|---|
| 0-5.00 | 40 |
| 5.00-8.00 | 40-85 |
| 8.00-12.00 | 85 |
| 12.00-13.00 | 85-35 |
| 13.00-16.00 | 35 |
| 16.00-17.00 | 35-40 |
| 17.00-19.50 | 40 |

***HPLC v.*** Gradient elution from 50% B to 70% B over 14 min

| Time (min) | % of B |
|---|---|
| 0-4.00 | 50 |
| 4.00-5.00 | 50-70 |
| 5.00-10.00 | 70 |
| 10.00-11.00 | 70-50 |
| 11.00-14.00 | 50 |

***HPLC vi.*** Gradient elution from 50% B to 85% B over 19.50 min

| Time (min) | % of B |
|---|---|
| 0-5.00 | 50 |
| 5.00-8.00 | 50-85 |
| 8.00-12.00 | 85 |
| 12.00-13.00 | 85-45 |
| 13.00-16.00 | 45 |
| 16.00-17.00 | 45-50 |
| 17.00-19.50 | 50 |

***HPLC vii.*** Gradient elution from 55% B to 85% B over 22 min

| Time (min) | % of B |
|---|---|
| 0-4.00 | 55 |
| 4.00-7.00 | 55-85 |
| 7.00-11.50 | 85 |
| 11.50-12.50 | 85-50 |
| 12.50-13.51 | 50 |
| 13.51-14.50 | 50-55 |
| 14.50-22.00 | 55 |

***Acetonitrile solvent system.*** Eluent A: 0.1% aqueous TFA which was degassed by evacuation; Eluent B: 80% ACN/0.1% TFA, which was prepared by mixing 1 ml TFA, 800 ml ACN and water was added to make the volume 1 L and degassed by evacuation. The flow rate was 0.3 ml/min.

***HPLC i.*** Gradient elution from 35% B to 60% B over 19.50 min

| Time (min) | % of B |
|---|---|
| 0-4.30 | 35 |
| 4.30-5.50 | 35-60 |
| 5.50-13.00 | 60 |
| 13.00-14.20 | 60-35 |
| 14.20-19.50 | 35 |

***HPLC ii.*** Gradient elution from 40% B to 85% B over 19.50 min

| Time (min) | % of B |
|---|---|
| 0-5.00 | 40 |
| 5.00-8.00 | 40-85 |
| 8.00-12.00 | 85 |
| 12.00-13.00 | 85-35 |
| 13.00-16.00 | 35 |
| 16.00-17.00 | 35-40 |
| 17.00-19.50 | 40 |

***HPLC iii.*** Gradient elution from 50% B to 85% B over 19.50 min

| Time (min) | % of B |
|---|---|
| 0-5.00 | 50 |
| 5.00-8.00 | 50-85 |
| 8.00-12.00 | 85 |
| 12.00-13.00 | 85-45 |
| 13.00-16.00 | 45 |
| 16.00-17.00 | 45-50 |
| 17.00-19.50 | 50 |

**(2) Preparative HPLC**

***Methanol solvent system.*** Eluent A: 0.05 M aqueous $AcONH_4$ (pH 6.5); Eluent B: 80% MeOH/0.05 M $AcONH_4$. The flow rate was 4 ml/min.

***Acetonitrile solvent system.*** Eluent A: 0.1% aqueous TFA; Eluent B: 80% ACN/0.1% TFA. The flow rate was 4 ml/min.

**(3) MPLC**

The substance to be separated in MPLC was dissolved in particular percentage of aqueous methanol. The particular percentage of aqueous methanol was prepared as written in individual steps. The eluent was 20 to 80% methanol containing 0.05 M $AcONH_4$. In all cases, the starting percentage of the eluent was same to that of sample prepared. The concentration of the eluent was changed depending upon the elution of the product. The flow rate was 5 ml/min.

**(4) Size exclusion chromatography**

***Eluent.*** MeOH. The flow rate was 5 ml/min.

**(5) Thin layer chromatography**

***TLC i.*** n-Butanol/acetic acid/water 3:1:1 (v/v/v)

***TLC ii.*** Dichloromethane/methanol 7:3 (v/v)

***TLC iii.*** Dichloromethane/methanol 9:1 (v/v)

Spots were detected as follows:

***UV-Test.*** The UV active substances were detected at 254 nm with the UV lamp.

***Ninhydrin reaction.*** 1.5 g ninhydrin and 15 ml acetic acid were mixed in 450 ml ethanol. The solution was sprayed on the plates and heated to 110$^{o}$C. Free amino group of protected amino acids or of peptides showed violet spots.

***Chlorine/tolidine reaction.*** This test was performed according to the procedure published by Pataki (1996). The tolidine solution was prepared by dissolving 240 mg tolidine, 2 g potassium iodide in 69 ml acetic acid and then the volume was brought to 1000 ml with water. The plates were exposed to chlorine gas for about 5 minutes. After about 5 minutes, the tolidine solution was sprayed on these plates. The protected amino acids and the peptides showed greenish yellow colour.

### 4.4 Enzyme immobilization

The rotation during the immobilization refers to mixing in a covered glass bottle held by a clamp which was rotated with a horizontally mounted stirrer motor as shown in figure 4.1.

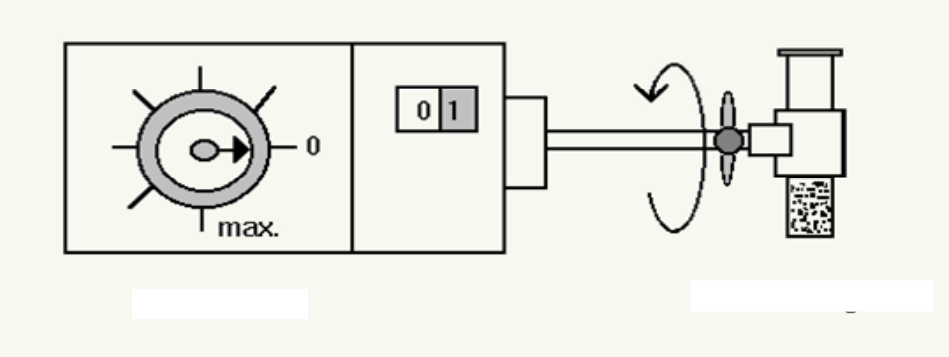



**Figure 4.1** Rotating apparatus during the use of immobilized enzyme

Wherever the immobilized enzyme was used, the rotation refers to the mixing as described above. This type of mixing prevents the polymer from damage.

**(1) Immobilization of trypsin**

***Immobilizing trypsin on Eupergit C.*** 1 g Eupergit C washed with 1 M $KH_2PO_4$ buffer (pH 7.5) was added to the solution of 300 mg trypsin in 10 ml 1 M $KH_2PO_4$ buffer (pH 7.5). After rotating for 24 hours, the solvent was sucked off and the immobilisate was washed with water before storing at -20$^{o}$C.

**(2) Immobilization of α-chymotrypsin**

***Adsorbing α-chymotrypsin on Celite-545.*** The solution of 100 mg α-chymotrypsin in 15 ml water and 15 ml 0.05 M Tris-HCl buffer (pH 9.0) was mixed with 10 g Celite-545 rods. After thorough mixing for 24 hours, the suspension was lyophilized and stored at -20$^{o}$C.

***Immobilizing α-chymotrypsin on Eupergit C.*** 10 g Eupergit C washed with 1 M $KH_2PO_4$ buffer (pH 8.0) were added to the solution of 600 mg α-chymotrypsin in 100 ml 1 M $KH_2PO_4$ buffer (pH 8.0). After rotating for 3 days, 100 ml 1 M NaCl were added to the suspension. The solvent was sucked off and the α-CHY/Eupergit C was washed with 0.05 M $KH_2PO_4$ buffer (pH 8.0) and stored at -20$^{o}$C.

**(3) Immobilization of papain**

***Adsorbing papain on Celite-545.*** The solution of 60 mg papain in 30 ml water was mixed with 2 g Celite-545 rods. After thorough mixing for 24 hours, the suspension was lyophilized and stored at -20$^{o}$C.

***Immobilizing papain on VA-Epoxy.*** Prior to the immobilization of papain on VA-Epoxy, the VA-Hydroxy polymer had to be epoxidised. 6 g VA-Hydroxy-Biosynth suspended in 60 ml epichlorohydrin were refluxed for 4 hours, and then sucked and washed with acetone. The resulting VA-Epoxy was stored at -20$^{o}$C.

4 g VA-Epoxy washed first with water and then with 1 M $KH_2PO_4$ buffer (pH 7.5) were suspended in 40 ml 1 M $KH_2PO_4$ buffer (pH 7.5). To this solution, 800 mg papain were added and mixed for 24 hours. It was filtered and the immobilisate was washed with water and stored at -20$^{o}$C.

**4.5 Activity test of immobilized enzymes**

The activity of the enzyme was determined as μmol conversion of test substrate per mg immobilized enzyme after one hour reaction time. The conversion rates were not the same throughout the reaction time. The rates were higher at the beginning of the reaction and lower after some time. Therefore, one hour was used as a reference time at which all the conversions were calculated.

**(1) Trypsin-immobilisate**

***Activity test.*** To the solution of 62 mg Bz-Arg-OEt·HCl in 1 ml 0.5 M $KH_2PO_4$ (pH 7.5), 100 mg trypsin/Eupergit C were added. Aliquots (10 μl) were withdrawn from the reaction mixture at different times from 0 min to 120 min and analyzed by *HPLC i* (methanol system).

***Activity of trypsin-immobilisate.***

Trypsin/ Eupergit C (300 mg/g) (fresh): 1.81 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

Trypsin/ Eupergit C (300 mg/g) (after the first application): 1.72 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

Trypsin/ Eupergit C (300 mg/g) (after the second application): 1.26 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

Trypsin/ Eupergit C (300 mg/g) (after the third application): 1.08 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

**(2) α-Chymotrypsin-immobilisate**

***Activity test.*** To 0.5 ml of the test solution (20 mg Ac-Tyr-OEt in 2 ml 0.01 M Tris-HCl buffer (pH 8.1)), 20 mg immobilized α-chymotrypsin were added. Aliquots (10 µl) were withdrawn from the reaction mixture at different times from 0 min to 100 min and analyzed by *HPLC i* (methanol system).

***Activity of α-chymotrypsin-immobilisate.***

α-CHY/Celite-545 (10 mg/g)(fresh): 3.98 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

α-CHY/Eupergit C (60 mg/g)(fresh): 3.58 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

**(3) Papain-immobilisate**

***Activity test.*** To 0.5 ml test solution (300 mg Bz-Arg-OEt·HCl in 5 ml 0.5 M $KH_2PO_4$ buffer (pH 6.0), 1 mg EDTA and 100 µl mercaptoethanol), 50 mg immobilized papain were added. Aliquots (10 µl) were withdrawn from the reaction mixture at different times from 0 min to 120 min and analyzed by *HPLC i* (methanol system).

***Activity of papain-immobilisate.***

Papain/Celite-545 (30 mg/g) (fresh): 10.51 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

Papain/VA-Epoxy (200 mg/g) (fresh): 11.03 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

**(4) PGA/Eupergit C**

***Activity test.*** To the solution of 6 mg benzylpenicillin-Na in 0.5 ml 1 M $KH_2PO_4$ buffer (pH 7.5), 50 mg PGA/Eupergit C were added. Aliquots (10 µl) were withdrawn from the reaction mixture at different times from 0 min to 30 min and analyzed by *HPLC i* (methanol system).

***Activity of PGA/Eupergit C.***

PGA/Eupergit C (120 U/g): 0.36 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

### 4.6 Preparation of amino acid derivatives

**(1) Preparation of Bz-Arg-OH** (Bergmann, M. and Zervas, L., 1932; Greenstein J. P. and Winitz M., 1961)

To a stirred and ice cooled solution of 0.3 mol arginine dissolved in 250 ml water and 80 ml ether, 35 ml benzoylchloride and 150 ml 2 M NaOH were added dropwise simultaneously. The pH of the system should be around 11. At the end of the reaction, crystallization of the product started. The product (Bz-Arg-OH) was recrystallized in ca. 800 ml water containing a drop of ammonia solution (m. p. 284-286ºC, yield 47%, $t_R$ 1.94 min in *HPLC iv* (methanol system), $R_f$ 0.34 in *TLC i*).

**(2) Preparation of Phac-Gly-OH**

To a stirred solution of 0.26 mol amino acid in 260 ml 2 M NaOH, 35 ml (0.26 mol) phenylacetylchloride and 65 ml 4 M NaOH were added dropwise simultaneously within 1 hour. After 2 hours, the reaction mixture was acidified with 6 M HCl to pH 2 in an ice-bath. The product was precipitated, filtered and recrystallized in methanol (m. p. 139-141 ºC, yield 53.3%, $t_R$ 1.22 min in *HPLC vii* (methanol system), $R_f$ 0.69 in *TLC i*).

**(3) Preparation of the methyl and ethyl ester hydrochlorides of tyrosine, tryptophan, methionine, aspartic acid, phenylalanine, phenylacetyl glycine and benzoyl arginine** (Brenner et al., 1950; Fles and Markovac-Prpic, 1957)

To a stirred solution of 0.15 mol amino acid or Phac-Gly or Bz-Arg in 300 ml absolute MeOH (or EtOH) cooled at -5$^o$C ( for Bz-Arg, -25º C) , 73 ml (1 mol) thionyl chloride were added dropwise and the reaction temperature was kept below 0$^o$C. After adding two third thionyl chloride, another 100 ml MeOH were added to the reaction mixture. When all thionyl chloride was added, the reaction mixture was kept at room temperature overnight. The mixture was concentrated in vacuum and the crude product was dissolved in 150 ml MeOH (or EtOH), and then the MeOH (or EtOH) was removed completely. For synthesizing the diester of aspartic acid, another 73 ml (1 mol) of thionyl chloride were added dropwise to the residue dissolved in 200 ml MeOH (or EtOH) at -5$^o$C and the above procedure was repeated. After removing the solvent, MeOH (or EtOH) (3x150 ml) was added into the residue and removed *in vacuo*. At the end, recrystallization was

performed in MeOH (or EtOH)/ diethyl ether. The crystals were filtered and washed with diethyl ether and dried *in vacuo*. The yield and analytical data are given in table 4.1

**Table 4.1** Isolated yields and analytical data of Tyr, Trp, Met, Asp, Phe, Phac-Gly and Bz-Arg derivatives.

| Derivative | Isolated yield (%) | m. p.[ºC] | HPLC (MeOH system), $t_R$ [min] | *TLC i,* $R_f$ |
|---|---|---|---|---|
| Tyr-OMe·HCl | 88.9 | 178-180 | *HPLC iii*/ 2.5 | 0.53 |
| Trp-OMe·HCl | 78.3 | 210-212 | *HPLC vii/* 3.45 | 0.55 |
| Met-OEt·HCl | 91.2 | 82-84 | UV-inactive | 0.63 |
| Asp(OEt)OEt·HCl | 80.0 | 104-106 | UV-inactive | 0.54 |
| Phe-OMe·HCl | 93.7 | 158-160 | *HPLC vii/* 3.44 | 0.57 |
| Phac-Gly-OMe | 65.3 | 81-83 | *HPLC vii/* 2.30 | 0.81 |
| Bz-Arg-OEt·HCl | 89.0 | 131-133 | *HPLC iv/* 6.39 | 0.38 |

**(4) Preparation of free amino acid ester by removing hydrochloride**

To 1.4 g (5 mmol) of $Na_2CO_3 \cdot 10H_2O$ in 20 ml water in an ice-bath, 10 mmol of the hydrochloride amino acid ester were added in portions. The mixture was stirred for 15 min at a pH, which was above 8. It was extracted with $CH_2Cl_2$ (3x15 ml). The organic layer was washed with saturated NaCl, dried over anhydrous $Na_2SO_4$ and concentrated *in vacuo* at 30°C. The free amino acid ester was used as soon as it was prepared.

**(5) Preparation of Phe-$NH_2$·HCl**

Ammonia gas was passed through a stirred solution of 65 g (0.3 mol) Phe-OMe·HCl in 342 ml absolute MeOH, cooled to -5°C. After saturation the solution was kept at room temperature overnight. Then the solvent was removed *in vacuo*. The residue was dissolved in 2 M HCl as far as possible. After removal of the solvent *in vacuo*, water was added to the residue again. The crude product was obtained after removing the solvent *in vacuo* and recrystallization was performed in EtOH (m. p. 230-233ºC, yield 85%, $t_R$ 1.50 min in *HPLC vii* (methanol system), $R_f$ 0.54 in *TLC i*).

**(6) Preparation of Phac-Gly-OCam**

Phac-Gly-OH (5.0 g, 26 mmol) was dissolved in 50 ml MeOH/water (4:1) and 20 ml of 20% aqueous $Cs_2CO_3$ were added dropwise in 1 h. The solution was evaporated to dryness under vacuum (bath temperature<45°C) and the residue was evaporated 3 times with 50 ml toluene each time. The white crystals (Phac-Gly-OCs) were dried overnight under high vacuum. The cesium salt was dissolved in 60 ml absolute DMF. In this solution α-chloroacetamide (3.5 g, 37.5 mmol) was added. After the mixture was stirred for 4 days at 35°C, the solution was evaporated to dryness under vacuum. The residue was dissolved in 100 ml EtOAc, washed with water (3x100 ml), 5% (w/v) $NaHCO_3$ (3x25 ml) and saturated NaCl solution (3x100 ml), dried over anhydrous $Na_2SO_4$ and finally evaporated to dryness under vacuum. Plac-Gly-OCam was obtained as a white solid (m. p. 128-130ºC, yield 85.6%, $t_R$ 1.88 min in *HPLC vii* (methanol system), $R_f$ 0.56 in *TLC i*).

**(7) Preparation of Asp(OMe)-OH·HCl**

To 208 ml absolute MeOH, cooled to -10°C, 31 ml $SOCl_2$ and 40 g (0.3 mol) aspartic acid were added. A clear solution was obtained on slowly warming to room temperature. After standing 25 min at room temperature, 600 ml absolute ether were added. After cooling in an ice-bath, the precipitate was filtered off and recrystallized with methanol/ether containing a small amount of HCl (m. p. 193-195ºC, yield 66.0%, UV-inactive in HPLC, $R_f$ 0.28 in *TLC i*).

**(8) Preparation of Z-Asp(OMe)-OH**

9.6 g (52.3 mmol) Asp(OMe)-OH·HCl were dissolved in a solution of 90 ml water, 12 ml 4 M NaOH (48 mmol) and 8.7 g (103 mmol) $NaHCO_3$. Under stirring at room temperature, 66.6 mmol benzylchloroformate were added over 2 hour period. Then 4 M NaOH was added and the pH was kept at 8 via a pH stat. The reaction was finished when no more NaOH was consumed. The mixture was extracted with ether (3x20 ml) and the ether extracts were discarded. The aqueous phase was acidified to pH 2.0 with concentrated HCl, and then extracted with EtOAc (3x50 ml). The combined EtOAc layers were washed with water (2x20 ml), dried over anhydrous $Na_2SO_4$ and concentrated *in vacuo*.

The residue was recrystallized with EtOAc/PE (m. p. 95-96ºC, yield 65.1%, $t_R$ 2.06 min in *HPLC vii* (methanol system), $R_f$ 0.64 in *TLC i*).

**(9) Preparation of Fmoc-Gly-OPfp, Fmoc-Trp-OPfp and Fmoc-Met-OPfp**

To a stirred, ice-cold solution of the Fmoc-amino acid (20 mmol) and pentafluorophenol (20 mmol) in 80 ml EtOAc containing 20 ml DMF or 100 ml EtOAc, dicyclohexylcarbodiimide (20 mmol) was added and stirring was continued for 1 hour at 0°C and for 1 hour at room temperature. Dicyclohexylurea was filtered off and the solvent was evaporated *in vacuo* at 40°C. The residue was recrystallized in EtOAc/PE (for Fmoc-Trp-OPfp, Fmoc-Met-OPfp) or EtOAc (for Fmoc-Gly-OPfp). The yield and analytical data are given in table 4.2. The products were verified by FABMS.

**Table 4.2** Isolated yields and analytical data of pentafluorophenyl esters of Fmoc-Gly, Fmoc-Trp and Fmoc-Met. *HPLC was performed with methanol system.

| Derivative | Isolated yield (%) | m. p. [ºC] | $t_R$ [min] HPLC* | FABMS | $R_{f,}$ *TLC i* |
|---|---|---|---|---|---|
| Fmoc-Gly-OPfp | 91.4 | 146-148 | 1.99/*HPLC ii* | m/z 464.0 [M+H$^+$], $C_{23}H_{14}F_5N_1O_4$ requires 463 | 0.82 |
| Fmoc-Trp-OPfp | 92.4 | 174-176 | 3.66/*HPLC ii* | *m/z* 593.0 [M+H$^+$], $C_{32}H_{21}F_5N_2O_4$ requires 592.5 | 0.91 |
| Fmoc-Met-OPfp | 96.8 | 98-100 | 3.20/*HPLC iii* | *m/z* 537.9 [M+H$^+$], *m/z* 560 [M+Na$^+$] $C_{26}H_{20}F_5N_1O_4S_1$ requires 537 | 0.85 |

## 4.7 Synthesis of peptides

### 4.7.1 Enzymatic synthesis of peptides

During the synthesis of the peptides mentioned below, the progress of each reaction was monitored by RP-HPLC. The reaction was stopped at its kinetic optimum. Melting point was noted only for the peptides which crystallized and the decomposition point of lyophilized product has no significance for characterizing the product.

**(1) Synthesis of Bz-Arg-Asp(OEt)-OEt**

The synthesis of this dipeptide was carried out under solvent free conditions. 2.052 g (6 mmol) Bz-Arg-OEt·HCl, 2.7 g (12 mmol) Asp(OEt)-OEt·HCl, 0.8 g (2.8 mmol) $Na_2CO_3 \cdot 10H_2O$, 0.8 g (7.6 mmol) $Na_2CO_3$ and 0.34 g (8.4 mmol) NaOH were mixed. 4 g trypsin/Eupergit C were added and stirred manually every 20 min. The reaction was monitored by *HPLC iv* (methanol system). When the reaction was finished, the mixture was diluted with 15 ml 80% ethanol containing 2 ml acetic acid. The pH of the system was around 7. This suspension was transferred to a sintered glass frit and washed with 80% EtOH until the product was extracted completely. The filtrate was evaporated to dryness under vacuum. The residue left after removal of the solvent was dissolved in 40% aqueous methanol and separated by MPLC. The pooled fractions of Bz-Arg-Asp(OEt)-OEt were lyophilized twice yielding a white powder (1.925 g, 71.5%, $t_R$ 11.83 min in *HPLC iv* (methanol system), $R_f$ 0.55 in *TLC i*).

**(2) Synthesis of Bz-Arg-Asp(OEt)-OH using trypsin**

85 mg (0.25 mmol) Bz-Arg-OEt·HCl and 115 mg (0.5 mmol) Asp(OEt)-OEt·HCl were dissolved in 1.5 ml water containing 100 µl 0.5 M phosphate buffer (pH 8.0). To this solution, 60 mg trypsin/Eupergit C were added and the mixture was rotated for 5 hours. The reaction was monitored by *HPLC iv* (methanol system). The reactant completely vanished in 5 hours and the mixture was filtered to remove the immobilized trypsin. The immobilisate was washed with 80% EtOH until the product was extracted completely. The combined filtrates were concentrated *in vacuo* and the residue was dissolved in 35 % aqueous MeOH and separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder of Bz-Arg-Asp(OEt)-OH ( 43 mg, 41.18%, $t_R$ 3.67 min in *HPLC*

*iv* (methanol system), FABMS *m/z* 422.2 [M+H$^+$], *m/z* 444.2 [M+Na$^+$], $C_{19}H_{27}N_5O_6$ requires 421.4).

**(3) Synthesis of Bz-Arg-Asp(OEt)-OH using α-chymotrypsin**

220 mg (0.49 mmol) Bz-Arg-Asp(OEt)-OEt were dissolved in 2.4 ml ACN containing 1 ml 0.1 M Tris-HCl buffer (pH 8.1). To this solution, 120 mg α-CHY/Celite-545 were added and rotated at room temperature. The reaction was monitored by *HPLC iv* (methanol system). At 65 hours, the reaction was stopped and α-CHY/Celite-545 was removed by filtration. The filter cake was washed with water until the product was transferred completely to the filtrate. The combined filtrates were concentrated *in vacuo* and the residue was dissolved in 35% MeOH and separated by MPLC. The pooled fractions of Bz-Arg-Asp(OEt)-OH were lyophilized twice yielding a white powder (80 mg, 38.8%).

**(4) Synthesis of Bz-Arg-Asp(OEt)-Tyr-OH**

450 mg (1.0 mmol) Bz-Arg-Asp(OEt)-OEt, 350 mg (1.5 mmol) Tyr-OMe·HCl, 1.0 g (10 mmol) $KHCO_3$, 75 mg (0.25 mmol) $Na_2SO_4 \cdot 10H_2O$, 50 µl EDTA and 60 µl β-mercaptoethanol were mixed. Free papain (80 mg) was added and stirred manually every 20 min. The reaction was monitored by *HPLC iii* (methanol system). When the reaction was finished, 20 ml water was added to the mixture, transferred to a sintered glass frit and washed with water until the filtrate was neutral. The remaining filter cake was washed with pure methanol and the filtrate was collected. The filtrate was evaporated to dryness under vacuum. The obtained residue was dissolved in 35% aqueous methanol and separated by MPLC. The pooled fractions of Bz-Arg-Asp(OEt)-Tyr-OH were lyophilized twice yielding a white powder (179 mg, 30.5%, $t_R$ 7.0 min in *HPLC iii* (methanol system), FABMS *m/z* 585.2 [M+H$^+$], *m/z* 607.2 [M+Na$^+$], $C_{28}H_{36}N_6O_8$ requires 584.6).

By-products were isolated and characterized by FABMS as Bz-Arg-Asp(OEt)-Tyr-Tyr-OH (13 mg, 1.7%, $t_R$ 9.0 min in *HPLC iii* (methanol system), FABMS *m/z* 748.2 [M+H$^+$], *m/z* 770.3 [M+Na$^+$], $C_{37}H_{45}N_7O_{10}$ requires 747.8) and Bz-Arg-Asp(OEt)-Tyr-Tyr-Tyr-OH ($t_R$ 11.0 min in *HPLC iii* (methanol system), FABMS *m/z* 911.5 [M+H$^+$], *m/z* 933.7 [M+Na$^+$], $C_{46}H_{54}N_8O_{12}$ requires 910.9).

**(5) Synthesis of Bz-Arg-Asp(OEt)-Tyr-OH from Bz-Arg-OEt·HCl without isolating the intermediate**

1.026 g (3 mmol) Bz-Arg-OEt·HCl, 1.350 g (6 mmol) Asp(OEt)-OEt·HCl, 400 mg (3.8 mmol) $Na_2CO_3$, 400 mg $Na_2CO_3$·10 $H_2O$ (1.4 mmol), 170 mg NaOH (4.2 mmol) and 300 mg trypsin/Eupergit C were mixed under solvent free conditions. The reaction was monitored by *HPLC iv* (methanol system). When the highest HPLC yield was obtained, the reaction was stopped and the mixture was diluted with 15 ml 80% ethanol. The immobilized trypsin was removed by filtration and the immobilisate was washed with 80% EtOH until the product was extracted completely. The filtrate was concentrated under vacuum. The solid left after concentration was used for the next coupling. To this solid, 700 mg (3 mmol) Tyr-OMe·HCl, 4 g $KHCO_3$ (40 mmol), 1.150 g $Na_2CO_3$·10$H_2O$ (4 mmol), 100 µl EDTA, 120 µl β-mercaptoethanol and 200 mg free trypsin were mixed under solvent free conditions. The reaction was checked in *HPLC iii* (methanol system). When the reaction was finished, 20 ml water were added to the mixture, transferred to a sintered glass frit and washed with water until the filtrate was neutral. The filter cake was washed with pure methanol and the filtrate was collected. The filtrate was evaporated to dryness under vacuum. The remaining residue was dissolved in 35% aqueous methanol and separated by MPLC. The pooled fractions of Bz-Arg-Asp(OEt)-Tyr-OH were lyophilized twice yielding a white powder (460 mg, 26.2%).

**(6) Synthesis of Bz-Arg-Asp(OEt)-Tyr-Met-OH**

234 mg (0.4 mmol) Bz-Arg-Asp(OEt)-Tyr-OH and 428 mg (2 mmol) Met-OEt·HCl were dissolved in 4 ml 0.1 M Tris-HCl buffer (pH 8.1) containing 400 µl ACN. To this solution, 50 mg free α-chymotrypsin were added and thoroughly mixed by rotation. The reaction was monitored by *HPLC v* (methanol system). The highest HPLC yield (39.3%) was obtained after 5 h 30 min. The reaction was stopped by adding few drops of acetic acid. The mixture was filtered and washed with methanol. The filtrate was evaporated to dryness under vacuum. The residue was dissolved in 40% aqueous methanol and separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder of Bz-Arg-Asp(OEt)-Tyr-Met-OH (111 mg, 38.7%, $t_R$ 9.11 min in *HPLC v* (methanol system), FABMS *m/z* 716.3 [M+$H^+$], *m/z* 738.2 [M+$Na^+$], $C_{33}H_{45}N_7O_9S_1$ requires 715.8).

The unreacted Bz-Arg-Asp(OEt)-Tyr-OH was also collected from the MPLC fractions, lyophilized and used again in this reaction. By this procedure, another 47 mg of the product (Bz-Arg-Asp(OEt)-Tyr-Met-OH) were obtained and the isolated yield increased to 55.1%.

**(7) Synthesis of Bz-Arg-Asp(OEt)-Tyr-Met-OAl**

180 mg (0.3 mmol) Bz-Arg-Asp(OEt)-Tyr-OH and 272 mg (0.75 mmol) Met-OAl·PTS were dissolved in 8 ml ACN containing 300 µl 0.1 M Tris-HCl buffer (pH 8.1). To this solution, 500 mg α-CHY/Celite-545 were added and the mixture was rotated. The reaction was monitored by *HPLC i* (acetonitrile system). After 47 hours, the reaction was stopped by adding a few drops of acetic acid and was filtered to remove α-CHY/Celite-545. The filter cake was washed with pure methanol. The filtrate was collected and evaporated to dryness under vacuum. The residue was dissolved in 45% aqueous methanol and separated by MPLC. The pooled fractions were lyophilized yielding a white powder of Bz-Arg-Asp(OEt)-Tyr-Met-OAl (290 mg, 63.9%, $t_R$ 10.09 min in *HPLCi*

(acetonitrile system), FABMS *m/z* 756.2 [M+H$^+$], *m/z* 778.2 [M+Na$^+$], $C_{36}H_{49}N_7O_9S_1$ requires 755.9).

**(8) Cleavage of Bz-Arg from Bz-Arg- Asp(OEt)-Tyr-Met-OH**

72 mg (0.1 mmol) Bz-Arg-Asp(OEt)-Tyr-Met-OH were suspended in 3.5 ml water and the pH was adjusted to 7.6 by 1 M NaOH. To this suspension, 60 mg trypsin/Eupergit C were added and thoroughly mixed by rotation. The reaction was checked in *HPLC iv* (methanol system). There was no increase in the HPLC yield of the product after 22 h. At 48 h, the reaction was stopped by adding few drops of acetic acid. The mixture was filtered and the filter cake was washed with 80% methanol thoroughly. The filtrate was evaporated to dryness under vacuum. The left residue was dissolved in 27% aqueous methanol and separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder of Asp(OEt)-Tyr-Met-OH (30 mg, 65.6%, $t_R$ 2.30 min in *HPLC iv* (methanol system), FABMS *m/z* 456.1 [M+H$^+$], *m/z* 477.9 [M+Na$^+$] $C_{20}H_{29}N_3O_7S_1$ requires 455.5).

**(9) Synthesis of Phac-Gly-Trp-OMe**

This dipeptide was synthesized according to the method described by Xiang et al., 2004. 125 mg (0.5 mmol) Phac-Gly-OCam and 164 mg (0.75 mmol) H-Trp-OMe were dissolved in 50 ml EtOAc containing 270 µl 0.2 M borax buffer (pH 8.5), 30 µl β-mercaptoethanol, and 1 mg EDTA. To this solution 300 mg papain/VA-Epoxy were added and thoroughly mixed by rotation. After 3 hours the reaction was complete and the HPLC yield was 95% in *HPLC vii* (methanol system). The mixture was filtered to remove the immobilized enzyme which was washed with a mixture of 40 ml ethyl acetate and 10 ml water. The combined filtrates were extracted successively with 1M citric acid (3x100 ml), 5% $NaHCO_3$ (3x100 ml), and saturated sodium chloride (1x100 ml). The organic phase was dried over anhydrous $Na_2SO_4$ and concentrated under vacuum. The residue was lyophilized yielding a white solid of Phac-Gly-Trp-OMe (177 mg, 90.0%, m. p. 126-128ºC, $t_R$ 10.30 min in *HPLC vii* (methanol system)).

**(10) Cleavage of Phac from Phac-Gly-Trp-OMe**

120 mg (0.3 mmol) Phac-Gly-Trp-OMe and 250 mg PGA/Eupergit C were suspended in water and the pH was adjusted to 6.0 with 1 M HCl. The mixture was rotated. The reaction was monitored in *HPLC vi* (methanol system) and was stopped after 24 hours. The mixture was filtered to remove the immobilized enzyme and the filter cake was washed with 80% ethanol. The filtrate was evaporated to dryness under vacuum. The residue was dissolved in 45% aqueous methanol and separated by MPLC. The pooled fractions were lyophilized yielding a white solid (30 mg, 36.3%, $t_R$ 5.72 min in *HPLC vi* (methanol system), FABMS *m/z* 276.2 [$M+H^+$], *m/z* 298.1 [$M+Na^+$], $C_{14}H_{17}N_3O_3$ requires 275.3). The results caused by pH change during the cleavage of Phac from Phac-Gly-Trp-OMe are mentioned in table 3.1

**(11) Investigations of the synthesis of Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-OMe**

Bz-Arg-Asp(OEt)-Tyr-Met-OH/OAl and Gly-Trp-OMe were stirred in different reaction conditions in presence of different enzymes but no product could be observed. The detail conditions and the amount of acyl donor and nucleophile are presented in table 4.3.

**Table 4.3** Conditions employed during the investigation of synthesis of Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-OMe; Tris-HCl buffer was 0.1 M (pH 8.1) and borax buffer was 0.1M (pH 6.9).

| Reactants | Reaction media | Catalyst | HPLC yield |
|---|---|---|---|
| Bz-Arg-Asp(OEt)-Tyr-Met-OH: 35 mg<br>Gly-Trp-OMe: 20 mg | ACN: 300 µl<br>Tris-HCl: 600 µl | 10 mg<br>α-CHY | 0 |
| Bz-Arg-Asp(OEt)-Tyr-Met-OH: 22 mg<br>Gly-Trp-OMe: 42 mg | ACN: 600 µl<br>Borax: 5 µl ;EDTA: 5 µl<br>β-mercaptoethanol: 5 µl | 300mg<br>Papain/VA-Epoxy | 0 |
| Bz-Arg-Asp(OEt)-Tyr-Met-OH: 22 mg<br>Gly-Trp-OMe: 12 mg | $Na_2CO_3$·10 $H_2O$: 20 mg<br>$KHCO_3$: 25 mg<br>EDTA: 5 µl<br>B-mercaptoethanol: 5 µl | 8 mg papain | 0 |
| Bz-Arg-Asp(OEt)-Tyr-Met-OH: 22 mg<br>Gly-Trp-OMe: 12 mg | $Na_2CO_3$·10 $H_2O$: 20 mg<br>$KHCO_3$: 25 mg | 8 mg α-CHY | 0 |
| Bz-Arg-Asp(OEt)-Tyr-Met-OAl: 19 mg<br>Gly-Trp-OMe: 28 mg | ACN: 2 ml<br>Tris-HCl: 15 µl | 100 mg α-CHY/Celite-545 | |
| Bz-Arg-Asp(OEt)-Tyr-Met-OAl: 19 mg<br>Gly-Trp-OMe: 28 mg | ACN: 1 ml<br>Tris-HCl: 1 ml | 50 mg α-CHY/Celite-545 | 0 |
| Bz-Arg-Asp(OEt)-Tyr-Met-OAl: 19 mg<br>Gly-Trp-OMe: 28 mg | EtOAc: 2.5 ml<br>Borax buffer: 15 µl<br>$Et_3N$: 25 µl | 50 mg α-CHY/Celite-545 | 0 |

In most cases, Gly-Trp-OH was formed which finally converted to diketopiperazines. When α-CHY was used, Bz-Arg-Asp(OEt)-Tyr-OH was formed from Bz-Arg-Asp(OEt)-Tyr-Met-OH. Similarly, Bz-Arg-Asp(OEt)-Tyr-Met-OAl was also hydrolysed to Bz-Arg-Asp(OEt)-Tyr-Met-OH.

**(12) Synthesis of Phac-Gly-Trp-Met-OEt**

The synthesis was performed under solvent free conditions. 158 mg (0.4 mmol) Phac-Gly-Trp-OMe, 343 mg (1.6 mmol) Met-OEt·HCl, 80 mg (0.8 mmol) $KHCO_3$ and 458 mg (1.6 mmol) $Na_2CO_3$·10$H_2O$ were mixed. 500 mg α-chymotrypsin/Eupergit C were added

and stirred manually every 20 min. The reaction was monitored in *HPLC vii* (methanol system). After the completion of the reaction, the mixture was washed with water until the filtrate was neutral. Most of the peptide was extracted with 100 ml 80% EtOH. The residue was then sonicated to extract the product completely. The combined filtrates were evaporated to dryness under vacuum. The tripeptide ester Phac-Gly-Trp-Met-OEt was obtained by recrystallization with EtOH as a white solid (135 mg, 62.7%, m. p. 172-174ºC, $t_R$ 13.51 min in *HPLC vii* (methanol system)).

**(13) Synthesis of Phac-Gly-Trp-Met-OAl**

158 mg (0.4 mmol) Phac-Gly-Trp-OMe, 200 mg (0.55 mmol) Met-OAl·PTS, 40 mg (0.4 mmol) $KHCO_3$ and 35 mg (0.12 mmol) $Na_2CO_3 \cdot 10H_2O$ were mixed. 150 mg α-chymotrypsin/Celite-545 were added and stirred manually every 20 min. The reaction was monitored in *HPLC ii* (acetonitrile system). When the reaction was finished, the mixture was washed with water until pH 7.0 was reached, then was diluted with 100 ml warm 80% EtOH and then sonicated to extract the tripeptide. After removal of the immobilized enzyme by filtration, the solvent was evaporated to dryness under vacuum. The tripeptide ester Phac-Gly-Trp-Met-OAl was obtained by recrystallization with EtOH as a white solid (134 mg, 60.1%, m. p. 164-167ºC, $t_R$ 11.63 min in *HPLC ii* (acetonitrile system), FABMS *m/z* 551.2 [$M+H^+$], *m/z* 573.1 [$M+Na^+$], $C_{29}H_{34}N_4O_5S_1$ requires 550). The effect of different salts in the yield of the product is mentioned in table 3.2.

**(14) Synthesis of Z-Asp(OMe)-Phe-$NH_2$**

4.0 g (14.2 mmol) Z-Asp(OMe)-OH and 3.68 g (18.3 mmol) H-Phe-$NH_2$·HCl were suspended in 64 ml $H_2O$ and the pH was adjusted to 7.0 with 1 M NaOH. To this solution 10 mg thermolysin were added and stirred at 40°C. After 7 hours, the reaction was complete. The reaction was monitored in *HPLC vii* (methanol system). The precipitated white product Z-Asp(OMe)-Phe-$NH_2$ was isolated by filtration, washed with 5% cold citric acid (3x100 ml) and cold water (3x100 ml) successively. Finally, the product was air dried. (5.87 g, 96.8%, m. p. 180-183°C, $t_R$ 11.57 min in *HPLC vii* (methanol system)).

**(15) Synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$**

110 mg (0.2 mmol) Phac-Gly-Trp-Met-OAl and 165 mg (0.5 mmol) Asp(OMe)Phe-$NH_2$·HCl were dissolved in 6 ml EtOAc containing 240 µl 0.1 M borax buffer (pH 8.2), 400 µl 0.1 M Tris-HCl buffer (pH 8.1) and 20 µl $Et_3N$. To this solution, 600 mg α-CHY/Celite-545 were added and rotated. The reaction was monitored in *HPLC iii* (acetonitrile system). After 95 hours, the reactant Phac-Gly-Trp-Met-OAl was completely converted and the HPLC yield of the target product was 66.7%. After this time, the concentration of the product started to decrease. The reaction was stopped by adding a few drops of acetic acid. The mixture was filtered and the filter cake was washed with 80% methanol followed by EtOAc/$H_2O$ (4:1) until the product was extracted completely. The combined filtrates were dried under vacuum and the crude product was dissolved in pure methanol and separated by a Sephadex LH 20 column. Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ was eluted first and the pooled fractions were collected and dried under vacuum yielding a white solid (85 mg, 54.0%, $t_R$ 6.11 min in *HPLC iii* (acetonitrile system), FABMS *m/z* 786.2 [M+$H^+$], $C_{40}H_{47}N_7O_8S_1$ requires 785.9). The yield of the product obtained in different reaction systems is given in table 3.3.

**(16) Cleavage of Phac from Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$**

112 mg (0.2 mmol) Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ was suspended in 10 ml water and the pH was adjusted to 7.6 with 1 M NaOH. After addition of 350 mg PGA/Eupergit C, the mixture was stirred at 35ºC for 30 hours. The reaction was monitored in *HPLC ii* (acetonitrile system). To extract the product, the mixture was diluted with 20 ml 80% EtOH, sonicated and filtered. The filtrate was evaporated to dryness. The residue was separated by preparative HPLC. The pooled fractions were lyophilized twice yielding white powder (9 mg, 6.7%, $t_R$ 3.85 min in *HPLC i* (acetonitrile system), FABMS *m/z* 668.2 [M+$H^+$], $C_{32}H_{41}N_7O_7S_1$ requires 667.7)

**(17) Cleavage of Phac and methyl ester from Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$**

This synthesis was repeated according to the procedure reported elsewhere (Xiang et al., 2004). The reaction was monitored in *HPLC vii* (methanol system). (isolated yield

10.8%, $t_R$ 6.72 min in *HPLC vii* (methanol system), FABMS, *m/z* 676.1 [M+$Na^+$], $C_{31}H_{39}N_7O_7S_1$ requires 653.7).

**(18) Synthesis of Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$**

46 mg (0.06 mmol) Bz-Arg-Asp(OEt)-Tyr-Met-OAl and 21 mg (0.03 mmol) Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ were dissolved in 4 ml ACN containing 18 µl 0.05 M Tris-HCl buffer (pH 8.0) and 18 µl $Et_3N$. To this mixture, 70 mg α-CHY/Eupergit C were added and rotated at room temperature. The reaction was monitored in *HPLC ii* (acetonitrile system). After 118 hours, the reaction was stopped and filtered to remove immobilized enzyme. The filter cake was washed with 80% ACN in which the solubility of reactants and product was higher in comparison to other solvents like MeOH, EtOH and EtOAC. The combined filtrates were dried in vacuum and the residue was dissolved in 50% MeOH and separated by MPLC. The pooled fractions were lyophilized twice yielding a white solid (7 mg, 17.1%, $t_R$ 11.02 min in *HPLC ii* (acetonitrile system), FABMS *m/z* 1365.4 [M+$H^+$], $C_{65}H_{84}N_{14}O_{15}S_2$ requires 1364). The effect of different reaction media and different enzyme carriers in the yield of the product is given in table 3.4.

**(19) Cleavage of Bz-Arg from Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$**

15 mg (0.01 mmol) Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ were suspended in 3.5 ml water and pH was adjusted to 7 with 1 M NaOH. 30 mg trypsin/Eupergit C were added and the mixture was rotated at room temperature. The reaction was monitored in *HPLC i* (acetonitrile system). There was no reaction up to 48 hours although more immobilized enzyme and free enzymes (TPCK treated, 2 mg) were added in between. Then the pH was increased to 8.5 and the reactant completely vanished in 4 hour after the pH was 8.5. The reaction was stopped by shifting the pH to pH 6 of the mixture with acetic acid. The mixture was filtered to remove enzyme. The filter cake was washed with 80% ACN till the product was completely transferred to the filtrate. The combined filtrates were evaporated to dryness under vacuum and the residue was dissolved in 52.5% ACN and separated by preparative HPLC in the acetonitrile system. The pooled fractions were lyophilized yielding a white powder (2 mg, 16.5%, $t_R$ 10.09

min in *HPLC i* (acetonitrile system), ESIMS *m/z* 1105.2 [M+$H^+$], *m/z* 1127.4 [M+$Na^+$], $C_{52}H_{68}N_{10}O_{13}S_2$ requires 1104).

### 4.7.2 Chemical synthesis of peptides

#### 4.7.2.1 Synthesis of Phac-peptides

***Phac-Gly-Trp-OMe.*** 20 mmol HOBt were dissolved in 50 ml anhydrous THF and 20 mmol Phac-Gly-OH were dissolved in 60 ml DMF. After mixing these two solutions, 22 mmol 3 M DCC in DCM were added. After stirring for 1 hour, the DCU was removed by filtration. The filtrate was added into a solution of 20 mmol Trp-OMe·HCl in 60 ml DMF. The pH was adjusted to 8 by adding N-methylmorpholine. The mixture was stirred overnight, filtered and evaporated *in vacuo* to dryness. The residue was dissolved in 200 ml EtOAc and extracted with 1 M citric acid (3x50 ml), 10% $Na_2CO_3$ (3x50 ml) and saturated NaCl (3x50 ml). After drying over anhydrous $Na_2SO_4$, filtration followed by concentration, the residue Phac-Gly-Trp-OMe was crystallized (table 4.4).

***Phac-Gly-Trp-OH.*** To obtain Phac-Gly-Trp-OH, 7 mmol Phac-Gly-Trp-OMe were dissolved in water/acetone (140 ml, 1:1 v/v) and treated with 4 ml 4 M NaOH. The reaction was controlled by *TLC i*. When the reaction was completed, acetone was removed *in vacuo*. The solution was acidified with 6 M HCl to pH 3.0, extracted with 110 ml EtOAc and washed with saturated NaCl to pH 7.0. After drying over anhydrous $Na_2SO_4$, filtration and concentration, Phac-Gly-Trp-OH was obtained. The yield after isolation and analytical data are given in table 4.4.

***Phac-Gly-Trp-Met-OAl.*** 20 mmol HOBt were dissolved in 50 ml anhydrous THF and 20 mmol Phac-Gly-Trp-OH were dissolved in 60 ml DMF. After mixing these two solutions, 22 mmol 3 M DCC in DCM were added. After stirring for 1 hour, the DCU was removed by filtration. The filtrate was added into a solution of 20 mmol Met-OAl·PTS in 60 ml DMF. The pH was adjusted to 8 by adding N-methylmorpholine. The mixture was stirred overnight, filtered and evaporated *in vacuo* to dryness. The residue was dissolved in 200 ml EtOAc and extracted with 1 M citric acid (3x50 ml), 10% $Na_2CO_3$ (3x50 ml) and saturated NaCl (3x50 ml). After drying over anhydrous $Na_2SO_4$, filtration followed by concentration, the residue Phac-Gly-Trp-Met-OAl was crystallized (table 4.4).

**Table 4.4** Solvents used for recrystallization, isolated yields and analytical data of Phac-peptides. * HPLC methanol system. ** HPLC acetonitrile system.

| Derivative | Recrystalized in | Isolated yield (%) | m. p. [ºC] | $t_R$(min), HPLC system | TLC i $R_f$ |
|---|---|---|---|---|---|
| Phac-Gly-Trp-OMe | EtOAc/PE | 74 | 120-123 | 10.30/*HPLC vii** | 0.79 |
| Phac-Gly-Trp-OH | - | 90 | - | 3.01/*HPLC vii** | 0.68 |
| Phac-Gly-Trp-Met-OAl | EtOH | 52 | 164-167 | 11.63/*HPLC ii*** | 0.80 |

### 4.7.2.2 Synthesis of the C-terminal pentapeptides

**(1) Hydrogenation of Z-Asp(OMe)-Phe-$NH_2$**

4 g (9.3 mmol) Z-Asp(OMe)-Phe-$NH_2$ were completely dissolved in 700 ml warm MeOH followed by addition of 1.7 ml 6 M HCl, and then 200 mg of 10% Pd-C were added. The mixture was divided into two parts. Half of the mixture was poured into a 500 ml shaked-bottle and gaseous hydrogen was led in. The reaction was checked in *HPLC vii* (methanol system). The reaction was complete in 20 min. The remaining portion of the mixture was also hydrogenated as done before. The combined reaction mixture was filtered and the filtrate was concentrated *in vacuo* to yield a white solid of Asp(OMe)-Phe-$NH_2$·HCl (2.97 g, 96.4%, m. p. 155-158$^oC$, $t_R$ 2.10 min in *HPLC vii* (methanol system)).

**(2) Fmoc-Met-Asp(OMe)-Phe-$NH_2$**

To a stirred solution of Asp(OMe)-Phe-$NH_2$·HCl (3.96 g, 12 mmol) in DMF (55 ml), Fmoc-Met-OPfp (6.48 g, 12 mmol) and $Et_3N$ (3.36 ml, 24 mmol) were added and stirring was performed for 20 min, then the solution was concentrated *in vacuo*. The residue was crystallized by trituration with ether, giving Fmoc-Met-Asp(OMe)-Phe-$NH_2$ (7.4 g, 95.3%, m. p. 170-171$^oC$, $t_R$ 5.65 min in *HPLC ii* (methanol system), FABMS *m/z* 647.0 [$M+H^+$], *m/z* 669.2 [$M+Na^+$], $C_{34}H_{38}N_4O_7S_1$ requires 646.8).

**(3) Fmoc-Trp-Met-Asp(OMe)-Phe-$NH_2$**

Fmoc-Met-Asp(OMe)-Phe-$NH_2$ (7.4 g, 11.4 mmol) was treated with 10% dimethylamine solution in DMF (6 ml dimethylamine + 54 ml DMF) for 10 min, then the solution was concentrated *in vacuo*. The residue was triturated with petroleum ether. Met-Asp(OMe)-Phe-$NH_2$ was obtained by filtration. (4.6 g, 95.1%, m. p. 148-150°C, $t_R$ 9.16 min in *HPLC ii* (methanol system), FABMS *m/z* 424.9 [$M+H^+$], $C_{19}H_{28}N_4O_5S_1$ requires 424.5).

To a stirred solution of this compound (5.4 g, 12.7 mmol) in 50 ml DMF, Fmoc-Met-OPfp (7.5 g, 12.6 mmol) and $Et_3N$ (1.82 ml, 13 mmol) were added and stirring was performed for 15 min, then the solution was concentrated *in vacuo*. The residue was crystallized by trituration with EtOAc, giving Fmoc-Trp-Met-Asp(OMe)-Phe-$NH_2$ (10.0 g, 94.6%, m.p. 182-183°C, $t_R$ 8.33 min in *HPLC ii* (methanol system), FABMS *m/z* 833.2 [$M+H^+$], *m/z* 855.5 [$M+Na^+$], $C_{45}H_{48}N_6O_8S_1$ requires 832.9).

**(4) Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$**

Fmoc-Trp-Met-Asp(OMe)-Phe-$NH_2$ (9.2 g, 11.0 mmol) was treated with 10% dimethylamine solution in DMF (5 ml dimethyl amine + 45 ml DMF) for 20 min, then the solution was concentrated *in vacuo*. The residue was triturated with petroleum ether. Trp-Met-Asp(OMe)-Phe-$NH_2$ was obtained by filtration. (6.5 g, 96.4%, decomposition at 169°C, $t_R$ 8.91 min in *HPLC ii* (methanol system), FABMS *m/z* 611.1 [$M+H^+$], $C_{30}H_{38}N_6O_6S_1$ requires 610.7).

To a stirred solution of this compound (6.1 g, 10 mmol) in DMF (50 ml), Fmoc-Gly-OPfp (4.6 g, 10 mmol) and $Et_3N$ (1.4 ml, 10 mmol) were added and stirring was performed for 20 min, then the solution was concentrated *in vacuo*. The residue was crystallized by trituration with EtOAc, giving Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ (8.0 g, 89.9%, m. p. 166-170°C, $t_R$ 6.79 min in *HPLC ii* (methanol system), FABMS *m/z* 890.2 [$M+H^+$], $C_{47}H_{51}N_7O_9S_1$ requires 889.9).

**(5) Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$**

Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ (8.5 g, 9.6 mmol) was treated with 10% dimethylamine solution in DMF (7 ml dimethyl amine + 63 ml DMF) for 15 min, then the solution was concentrated *in vacuo*. The residue was triturated with petroleun ether, then filtered off to give Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ (6.0 g). This free pentapetide was found impure when checked in HPLC. This crude pentapeptide was purified by MPLC with gradient elution of methanol starting with 50%. The concentration of methanol was increased depending upon the elution of the product. The first, second and third peak was collected, concentrated and lyophilized before carrying out the MS analysis. The FABMS showed that only first peak contained the free pentapeptide. When this pentapeptide was checked in *HPLC i* (acetonitrile system), the single peak in methanol system was resolved into four peaks. All four peaks were separated and isolated by preparative HPLC in the acetonitrile system with 35% B. The first peak was found to be pure Trp-Met-Asp(OMe)-Phe-$NH_2$ (1.1 g, 18.8%, $t_R$ 3.70 min in *HPLC i* (acetonitrile system), FABMS *m/z* 611.2 [$M+H^+$], *m/z* 633.3 [$M+Na^+$], $C_{30}H_{38}N_6O_6S_1$ requires 610.7) followed by pure Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ (1.9 g, 29.6%, $t_R$ 3.85 min in *HPLC i* (acetonitrile system), FABMS *m/z* 668.3 [$M+H^+$], *m/z* 690.0 [$M+Na^+$], $C_{32}H_{41}N_7O_7S_1$ requires 667.8). The next two successive peaks were cyclic products formed by the ring closure of side chain of asparatic acid of both tetrapeptide (390 mg, 7.0%, $t_R$ 3.87 min in *HPLC i* (acetonitrile system), FABMS *m/z* 579.2 [$M+H^+$], *m/z* 601.2 [$M+Na^+$], $C_{29}H_{34}N_6O_5S_1$ requires 578) and pentapeptide respectively (870 mg, 14.3% $t_R$ 3.95 min in *HPLC i* (acetonitrile system), FABMS *m/z* 636.1 [$M+H^+$], *m/z* 658.2 [$M+Na^+$], $C_{31}H_{37}N_7O_6S_1$ requires 635).

**(6) Gly-Trp-Met-Asp(OH)-Phe-$NH_2$**

Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ (356 mg, 0.4 mmol) was suspended in water/acetone (40 ml, 1:1 v/v) and treated with 1 ml 4 M NaOH for 1 hour. Then the solution was concentrated *in vacuo* to remove acetone. The remaining solution was neutralized with 1 M HCl. A white solid (Gly-Trp-Met-Asp(OH)-Phe-$NH_2$) precipitated. It was isolated by filtration and dried (52 mg, 19.9%, decomposition at 230$^oC$, $t_R$ 6.73 min in *HPLC vii* (methanol system), FABMS *m/z* 654 [$M+H^+$], *m/z* 676.1 [$M+Na^+$], $C_{31}H_{39}N_7O_7S_1$requires653.7).

# 5 Summary

Enzymes can be used often favorably in organic syntheses, because they can be applied at room or slightly elevated temperature and in aqueous phase. Therefore, enzymatic reactions are economically and environmentally superior to classical organic reactions. Moreover, many side reactions, especially racemization occurring in the chemical peptide synthesis can be avoided and it is not necessary to protect all the side chains of amino acids involved in the coupling. However, there is no general protocol available for the enzymatic reactions therefore each step has to be optimized taking into account substrate structure, concentration, reaction media, temperature and the type of protease.

The target octapeptide (CCK-8) is the minimum active sequence with the same biological activity as naturally occurring cholecystokinin and is a potential therapeutic agent in the control of gastrointestinal functions. CCK-8 also acts as a neurotransmitter.

The objective of this thesis is to synthesize N-terminal protected and deprotected CCK-8. In former investigations the enzymatically cleavable Phac group was used. But in our group it was found that the cleavability depends on the peptide sequence and sometimes can not be cleaved at all. As an alternative Bz-Arg was used as the N-terminal protecting group as suggested by Glass (1987). This is possible because of the high specificity of trypsin towards basic amino acids in the $P_1$ position and because there is no basic amino acid in the target peptide sequence. This protecting group could be introduced and removed easily with trypsin in test peptides and also in the octapeptide. The enzymatic removal of Bz-Arg from protected CCK-8 is a significant improvement because in the literature of CCK-8 syntheses, either the protecting group (Phac) has not been cleaved at all (Fite et al., 2002) or is removed chemically (Kullmann, 1982; Sakina et al., 1988; Cerovsky et al., 1988) by using the Boc or Fmoc group.

Different fragments of CCK-8 were synthesized, because it was not known in advance which fragment condensation will be successful. Two possible strategies were considered [(3+2) +3] and [3+ (2+3)]. The fragments were synthesized using enzymatic methods exclusively. Whenever it was possible, immobilized enzymes were applied. This is

important for the large scale synthesis and therapeutical application of peptides. Except the Asp-Tyr and Asp-Phe couplings, all other couplings were performed using immobilized enzymes.

The catalytic mechanism of cysteine or serine proteases involves the formation of an acyl-enzyme complex at the transition state. It is mentioned in the literature that the acyl-donor needs an ester group to form the enzyme substrate complex. In contradiction with this statement, we had found quite often and also in this work that the free acyl-donor (e.g. Bz-Arg-Asp(OEt)-Tyr-OH) could couple effectively with a nucleophile (Met-OAl·PTS) to yield the peptide (Bz- Arg-Asp(OEt)-Tyr-Met-OAl).

In chemical peptide synthesis, where racemization is always a danger during the activation of the carboxyl component, especially in the case of peptides, the sequence is synthesized from the C-terminal to the N-terminal (C ⟶ N strategy). For the enzymatic synthesis the opposite, N⟶C strategy is superior. The N-terminal tripeptide fragment of CCK-8 (Bz-Arg-Asp(OEt)-Tyr-Met-OH/OAl) was achieved by the coupling of the individual amino acid derivative towards the C-terminal end of the growing peptide chain. This fragment was used to investigate whether the Bz-Arg group can be cleaved from Bz-Arg-Asp(OEt)-Tyr-Met-OH or not. The result showed that Bz-Arg could be removed easily from Bz-Arg-Asp(OEt)-Tyr-Met-OH with trypsin in a good isolated yield (65.6%). Even after a prolonged exposure of the peptide to trypsin no attack of any of the other peptide bonds could be detected.

For the [(3+2) +3] strategy Phac-Gly-OCam and Trp-OMe were coupled. The cleavage of the Phac group could also be performed successfully with PGA. However, this dipeptide is prone for diketopiperazine formation at higher pH (>7). This side reaction could be suppressed completely by carrying out the reaction at pH 6. However, the 3+2 coupling could not be performed at this pH. On the other hand, the diketopiperazine formation at higher pH values exceeded by far the peptide bond formation. Therefore, the [(3+2) +3] strategy was interrupted at this stage.

For the alternative strategy [3+(3+2)], the synthesis of the tripeptide fragment Phac-Gly-Trp-Met-OEt could be performed in analogy to Capellas et al. (1996[a]), however, prior to the coupling of Phac-Gly-Trp-OMe with Met-OEt·HCl, the dipeptide methyl ester had to be converted in a laborious way to the more active Cam ester. This synthesis could be greatly improved performing the coupling of the dipeptide methyl ester with Met-OEt·HCl under solvent free conditions. Thus three reaction steps could be avoided. For the following 3+2 coupling, the tripeptide allyl ester was superior to the ethyl ester. Phac-Gly-Trp-Met-OAl was synthesized by stepwise N⟶C strategy and Asp(OMe)-Phe-$NH_2$ was achieved by catalytic hydrogenation of Z-Asp(OMe)-Phe-$NH_2$. The C-terminal pentapeptide fragment (CCK-5) was prepared by α-chymotrypsin mediated fragment condensation of Phac-Gly-Trp-Met-OAl and Asp(OMe)-Phe-$NH_2$. In an earlier CCK-5 synthesis in our laboratory (Xiang, et al., 2004), Phac-Gly-Trp-Met-OEt was first converted to the Cam ester by using three chemical steps and was then coupled with Asp(OMe)-Phe-$NH_2$. Met-OAl could be used as a nucleophile in the extension of the dipetide and was effective enough as an acyl-donor for the 3+2 fragment coupling. With this strategy the earlier method has been improved by reducing the number of steps.

The final fragment coupling in the [3+ (3 +2)] approach was successful by coupling Bz-Arg-Asp(OEt)-Tyr-Met-OAl with Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$. This coupling failed when free α-chymotrypsin was used, however, when the enzyme was immobilized on Eupergit C, the reaction had 17.1 % product yield. The reason for this quite surprising behavior could not be investigated in this work.

As assumed, the cleaving of Bz-Arg from Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ with trypsin at pH 8.5 was successful and the isolated product was pure as can be seen in HPLC (figure 3.14 (b) ). In ESIMS (figure 3.15) there was only one significant peak of the target peptide. Therefore there is no doubt, that the octapeptide with the free N-terminus was indeed homogeneous.

In this thesis it could be demonstrated that the fully enzymatic peptide synthesis even of longer peptides is possible if all available condensation methods, coupling in aqueous phase, co-solvents, bi-phasic and solvent free conditions are applied. It could also be demonstrated that the Bz-Arg group is superior to other used N-terminal protecting groups in CCK-8 syntheses, because it can be cleaved easily with trypsin. The enzymatic technology, the "Green Chemistry" is a versatile alternative to the chemical peptide synthesis.

# 6 References


Ahren, B. O., Holst, J. J. and Efendic, S. (2000) *J. Clin. Endocr. Metab.* **85**, 1043

Basso, A., Martin, L. D., Ebert, C., Gardossi, L. and Linda, P. (2000) *Chem. Commun.* **3**, 467

Bayer, E., Eckstein, H., Haegele, K., Koenig, W. A., Bruening, W., Hagenmaier, H. and Parr, W. (1970) *J. Amer. Chem. Soc.* **92**, 1735

Bauer, C. A. (1978) *Biochemistry* **17**, 375

Ben Ishai, D. and Berger, A. (1952) *J. Org. Chem.* **17**, 1564

Bergmann, M. and Fraenkel-Conrat, H. (1938) *J. Biol. Chem.* **124**, 1

Bergmann, M. and Fruton, J. S. (1937) *J. Biol. Chem.* **118**, 405

Bergmann, M. and Fruton, J. S. (1938) *J. Biol. Chem.* **124**, 321

Bergmann, M. and Zervas, L. (1932) Ber. *Dtsch. Chem. Ges.* **65**, 1192

Bodanszky, M. (1955) *Nature* **175**, 685

Boanszky, M. (1993[a]) *Peptide Chemistry - A Practical Textbook (2nd Edition)*, Springer-Verlag, 109

Bodanszky, M. (1993[b]) *Peptide Chemistry - A Practical Textbook (2nd Edition)*, Springer-Verlag, 31

Bodanszky, M., Deshmane, S. S. and Martinez, J. (1979) *J. Org. Chem.* **44,** 1622

Bodanszky, M. and du Vigneaud, V. (1959) *Nature* **183**, 1324; *J. Amer. Chem. Soc.* **81**, 5688

Bodanszky, M. and Martinez, J. (1981) *Synthesis* **5**, 333

Borsook, H. (1953) *Adv. Pot. Chem.* **8**, 127

Brenner, M. and Huber, W. (1953) *Helv. Chim. Acta* **36**, 1109

Brenner, M., Mueller, R. H. and Pfister, W., R., (1950) *Helv. Chim. Acta* **33**, 568

Brtnik, F., Barth, T. and Jost, K. (1981) *Collect. Czech. Chem. Commun.* **46**, 1983

Brubacher, L. J. and Zaher, M. R. (1979) *Can. J. Biochem.* **57,** 1064

Calvet, S., Torres, J. L. and Clapes, P. (1996) *Biocatal. Biotransform.* **13**, 201

Capellas, M., Benaiges, M. D., Caminal, G., Gonzalez, G., Lopez-Santin, J. and Clapes, P. (1996[a]) *Biotech. Bioeng.* **50**, 700

Capellas, M., Benaiges, M. D., Caminal, G., Gonzalez, G., Lopez-Santin, J. and Clapes, P. (1996[b]) *Biocatal. Biotransform.* **13**, 165

Capellas, M., Caminal, G., Gonzalez, G., Lopez-Santin, J. and Clapes, P. (1997) *Biotech. Bioeng.* **56**, 456

Cassells, J. M. and Halling, P. S. (1989) *Biotechnol. Bioeng.* **33**, 1489

Carpenter, F. H. (1960) *J. Am. Chem. Soc* **82**, 1111

Carpino, L. A. (1957) *J. Amer. Chem. Soc.* **79**, 4427

Carpino, L. A. and Han, G. Y. (1970) *J. Amer. Chem. Soc.* **92**, 5748

Cerovsky, V., Hlavacek, J., Slaninova, J. and Jost, K. (1988) *Collection Czechoslovak Chem. Commun.* **53**, 1086

Cerovsky, V. (1992) *Biotechnol. Tech.* **6**, 155

Chantrene, H. (1947) *Nature* **160**, 603

Curtius, T. (1881) *J. Prakt. Chem.* **24**, 239

Curtius, T. (1902) *Ber. Dtsch. Chem. Ges.* **35**, 3226

Curtius, T. and Goebel, F. (1888) *J. Prakt. Chem.* **37**, 150

Dordick, J. S. (1989) *Enzyme Microb. Technol.* **11**, 194

du Vigneaud V., Ressler C., Trippett S. (1953 [a]) *J. Biol. Chem.* **205**, 949

du Vigneaud V., Ressler C., Swan J. M., Roberts C. W., Katsoyannis P. G. and Gordon S. (1953 [b]) *J. Amer. Chem. Soc. **75***, 4879

Eckstein, H. and Renner, H.-J. (1992) In *Peptides* 1992, Proceedings 22nd European Peptide Symposium, (Schneider, C. H., Eberle, A. N.,Eds.), Escom Science Publishers, Leiden, Holland, 1993, 403

Eckstein, H. and Renner, H.-J. (1995) In *Peptides* 1994, Proceedings 23rd European Peptide Symposium, (H. L. S. Maja, Eds.), Escom Science Publishers, Leiden, Holland, 1995, 230

Eckstein, H., Renner, H.- J. and Brun, H. (1991) *Biomed. Biochim. Acta* **50,** 114

Fischer, E. (1903) *Ber. Dtsch. Chem. Ges.* **36**, 2094

Fischer, E. and Fourneau, E. (1901) *Ber. Dtsch. Chem. Ges***. 34**, 2868

Fite, M., Clapes, P., Lopez-Santin, J., Benaiges, M. D. and Caminal, G. (2002) *Biotech. Prog.* **18**, 1214

Fles, D. and Markovac-Prpic, A. (1957) *Croatia Chemica Acta* **29**, 79

Fruton, J. S. (1982) *Adv. Enzymol. Relat. Areas Mol. Biol.* **53**, 239

Glass, J. D. (1987) *The Peptides* **9**, 167

Greenstein, J. P., Winitz, M. (1961) *Chem. Am. Acids Vol II*, John Wiley & Sons, Inc., New York, London, 9241

Homandberg, G. A., Mattis, J. A. and Laskovski, M. (1978) *J. Biochemistry* **17**, 5220

Horvath, C. and Engasser, J-M. (1973) *Ind. Eng. Chem. Fundam.* **12,** 229

Isowa, Y., Ohmori, M., Ichikawa, T., Mori, K., Nonaka, Y., Kihara, K., Oyama, K., Satoh, H. and Nishimura, S. (1979) *Tetrahedron Lett.* **28***,* 2611

Jakubke, H. D., Eichhorn, U., Haensler, M. and Ullmann, D. (1996) *Biol. Chem.* **377**, 455

Jakubke, H. D., Kuhl, P. and Koennecke, A. (1985) *Angew.Chem.Int. Ed. Engl.* **24**, 85

Kasche, V., Haufler, U. and Riechman, L. (1987) *Methods in Enzymol.* **136**, 280

Keil, B. and Boyer, P. D. (1971) *Ed. Academic Press, New York (3rd Ed., Part III)* 249

Kisfaludy, L. and Schoen, I. (1983) *Synthesis* **4**, 325

Kisse, H., Hayakawa, A. and Noritomi, H. (1990) *J. Biotechnol.* **14**, 239

Kisse, H., Fujimoto, K. and Noritomi, H. (1988) *J. Biotechnol*. **8**, 279

Kissileff, H. R., Pi Sunyer, F. X., Thornton, J. and Smith, G. P. (1981) *Am. J. Clin. Nutr.* **34**, 154

Klibanov, A. M. (1979) *Anal. Biochem.* **93**, 1

Kloss, G. and Schröder, E. (1964) *Hoppe-Seyler's Z. Physiol. Chem.* **336**, 248

König, W. and Geiger, R. (1970) *Chem. Ber.* **103,** 788, 2024

Kullmann, W. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 2840

Linderstrom-Lang, K. (1962) *Biological synthesis of proteins, Lane medical lectures 1951*, in Selected Papers, Academic Press, New York, 448

Marder, O. and Albericio, F. (2003) *Chem. Today* **21,** 35

Margolin, A. L., Svedas, V., K. and Berezin, I. V. (1980) *Biochim. Biophys. Acta* **616**, 283

Mccoy, M. (2004) *Chem. Engin*. **8,** 23

Meng, L. P., Joshi, R. and Eckstein, H. (2006) *Chem. Today* **24**, 50

Meng, L. P., Joshi, R. and Eckstein, H. *Amino acid,* In Press

Merrifield, R. B. (1963) *J. Amer. Chem. Soc.* **85**, 2149

Michaelis, L. and Mizutani, M. (1925) *Z. Phys. Chem. (Leipzig)* **116,** 135

Milne, H. B., Halver, J. E., Ho, D. S. and Mason, M. S. (1957) *J. Am. Chem. Soc.* **79**, 637

Mitin, Y. V., Zapevalova, N. P. and Gorbunava, E. Y. (1984) *Int. J. Peptide Protein Res.* **23,** 528

Mizutani, M. (1925) *Z. Phys. Chem. (Leipzig)* **116**, 350

Oka, T. and Morihara, M. (1978) *J. Biochem. (Tokyo)* **84**, 1277

Ondetti, N. A., Pluscec, J., Sabo, E. P., Sheehan, J. T. and Williams, N. (1970) *J. Am. Chem. Soc.* **92**, 195

Organikum (1996) Johann Ambrosius Barth Verlag Heidelberg, Leipzig, 693

Pataki, G. (1996), *Dünnschichtchromatographie in der Aminosäure- und Peptidchemie*, de Gruyter, Berlin

Pierce J. G., Gordon S. and du Vigneaud V. (1952) *J. Biol. Chem.* **199**, 929

Penke, B. and Rivier, J. (1987) *J. Org. Chem.* **52**, 1197

Roeske, R. (1959) *Chem. Ind.* (London), 1121

Sakina, K., Kawazura, K., Morihara, K. and Yajima, H. (1988) *Chem. Pharm. Bull.* **36**, 3915

Schechter, I. and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157

Schwartz, G. J., Netterville, L. A., McHugh, P. R. and Moran T. H. (1991) *Am. J. Physol.* **261**, 1141

Schellenberger, V., Goerner, A., Koennecke, A. and Jakubke, H. D. (1991) *Peptide Res*. **4**, 265

Schwyzer, R. (1953) *Helv. Chim. Acta* **36**, 414

Semenov, A. N., Berezin, J. V. and Martinek, K. (1981) *Biotechnol. Bioeng.* **23**, 355

Sheehan, J. C. and Hess, G. P. (1955) *J. Amer. Chem. Soc.* **77,** 1067

Sifferd, R. H. and du Vigneaud V. (1935) *J. Biol. Chem.* **108**, 753

Taschner, E., Chimiak, A., Bator, B. and Sokolowska, T. (1961) *Liebigs Ann. Chem.* **646**, 134

Tirassa, P., Costa, N. and Aloe, L. (2005) *Neuropharmacol.* **48**, 732

Toth, G. K., Penke, B., Zarandi, M. and Kovacs, K. (1985) *Int. J. Peptide Protein Res*. **26**, 630

Tuppy H. (1953) *Biochim. Biophys. Acta.* **11**, 449

Van't Hoff, J. H. (1898) *Z. Anorg. Chem*. **18**, 1

Verspohl, E. J., Ammon, H. P. T., Williams. J. A. and Goldfine, I. D. (1986) *Diabetes* **35**, 38

Virden, R. (1990) *Biotechnol. Gen. Eng. Rev.* **8**, 189

Vivien, M. (2005) *Chem. Engin.* **14**, 17

Volkoff, H., Canosa, L. F., Unniappan, S., Cerda-Reverter, J. M., Bernier, N. J., Kelly, S. P. and Peter, R. E. (2005) *Ger. Comp. Endocrinol.* **142**, 3

Widmer, F., Breddam, K. and Johansen, J. T. (1981) In *Peptides* Proceedings 16th European Peptide Symposium, (Brunfeldt, K., Eds.), Scriptor, Kopenhagen., 46

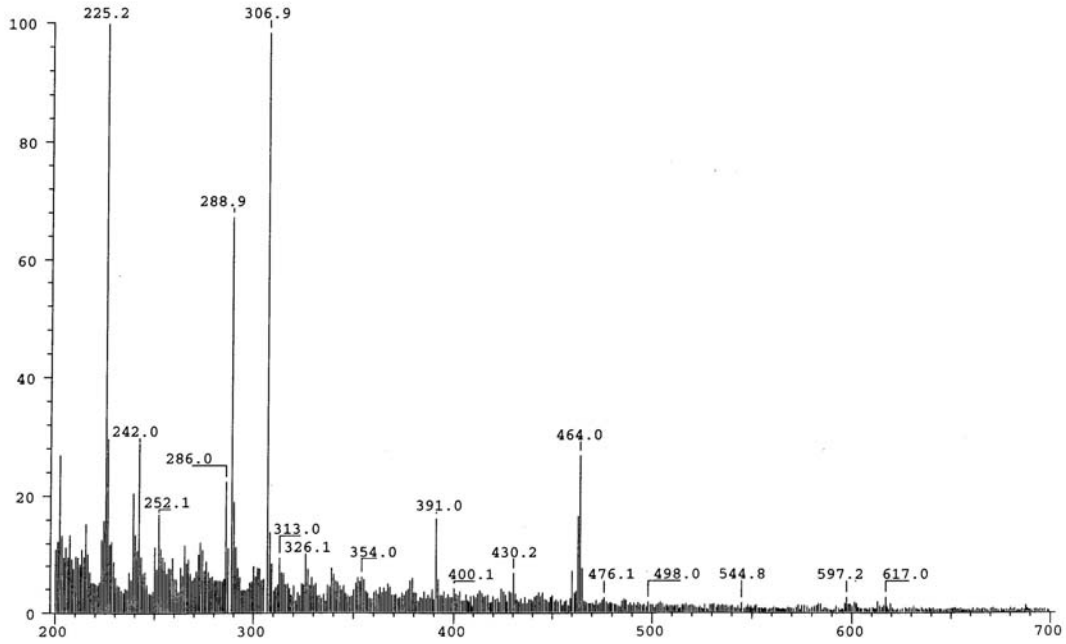
Widmer, F., Ohno, M., Smith, M., Nelson, N. and Anfinsen, C. B. (1983) In *Peptides* 1982, Proceedings 17th European Peptide Symposium, (Blaha, K and Malon, P. Eds.), Walter der Gruyter, Berlin, 375

Xiang, H., Xiang, G. Y., Lu, Z. M., Guo, L. and Eckstein, H. (2004) *Amino Acids* **27**, 285

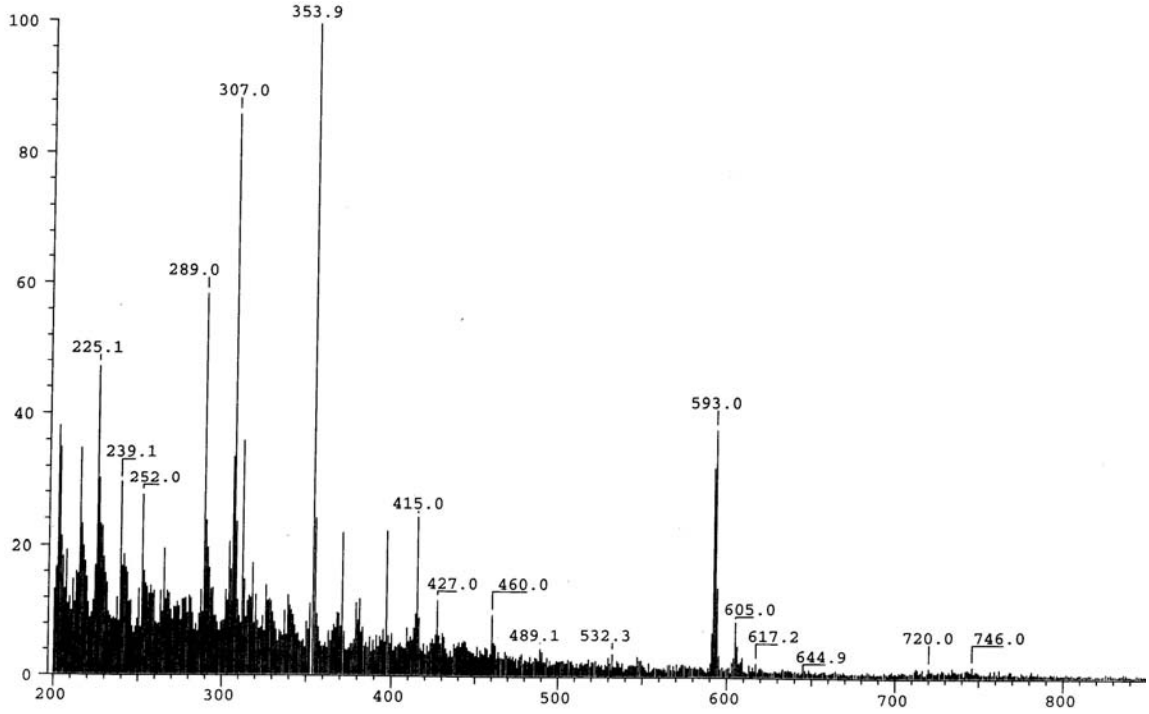
# 7 Appendix

## FABMS spectra

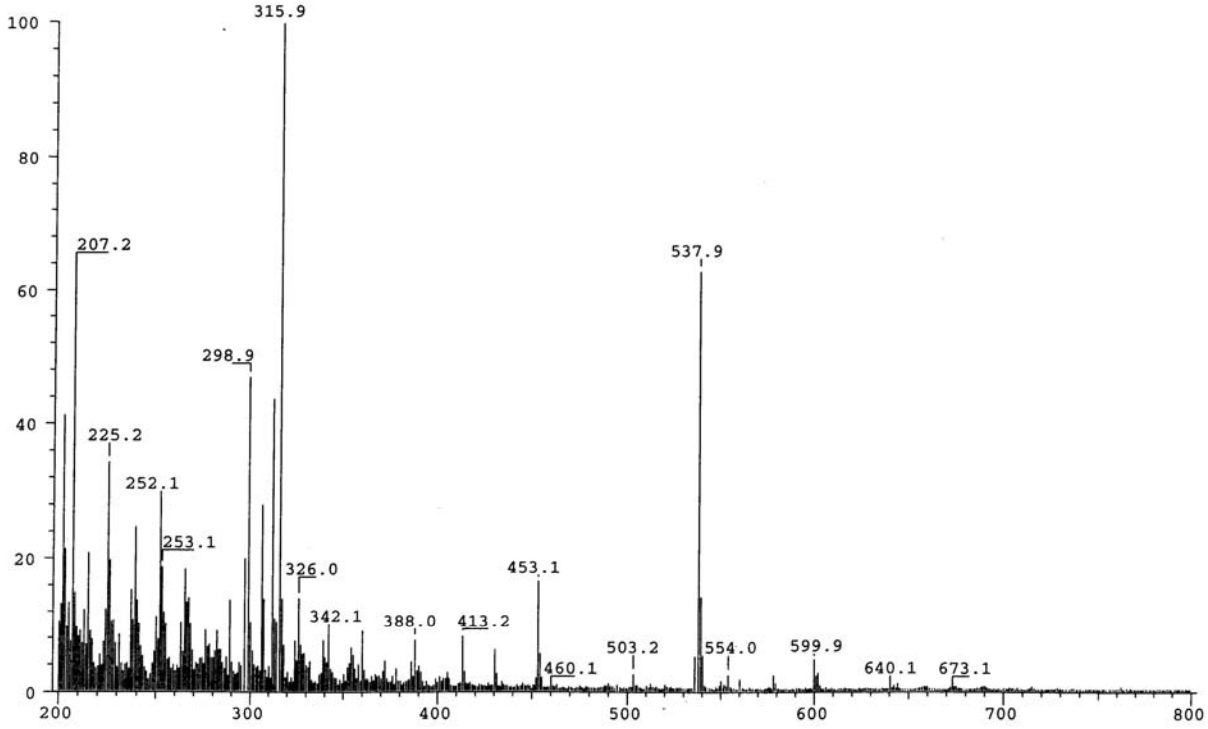
### (1) Fmoc-Gly-OPfp, [M+H$^+$]=464.0



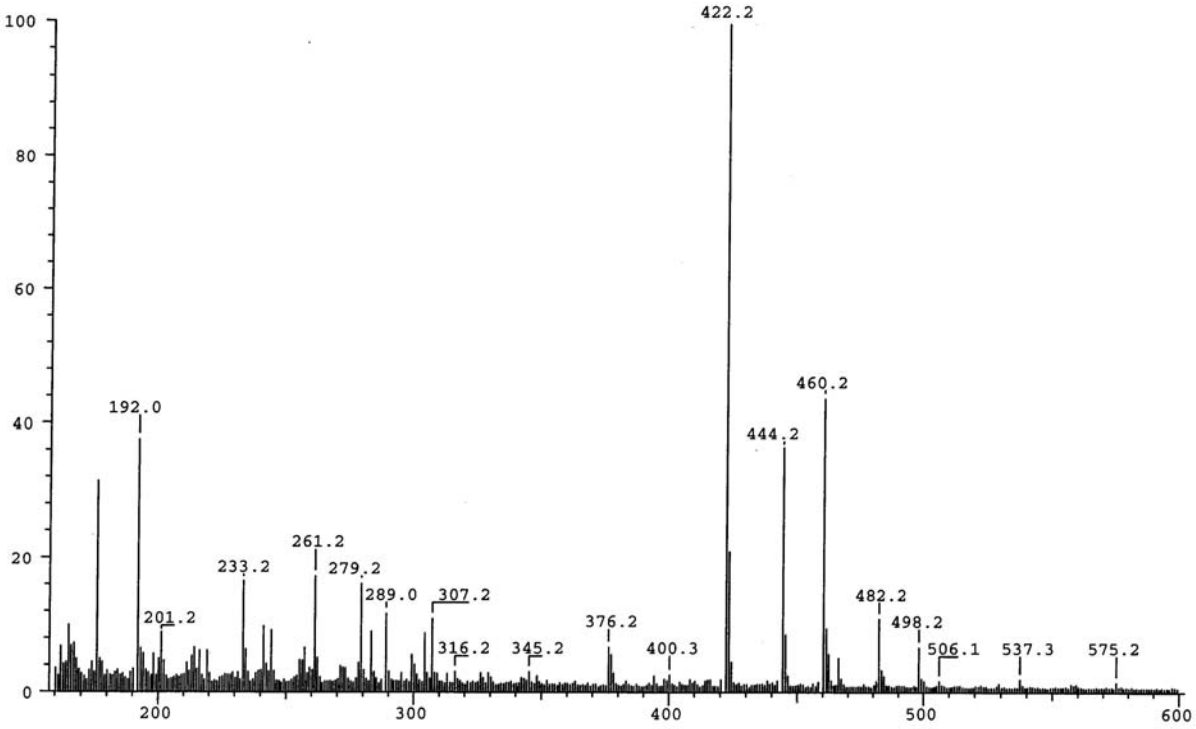

### (2) Fmoc-Trp-Opfp, [M+H$^+$]=593.0



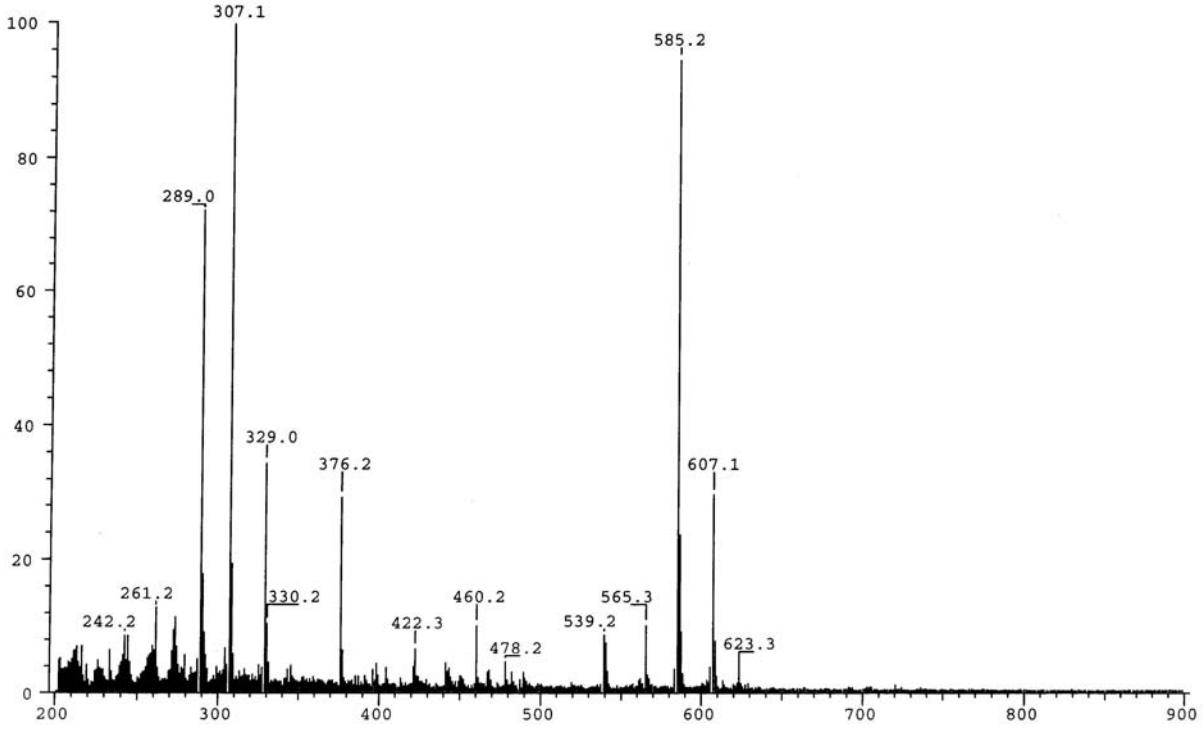
**(3) Fmoc-Met-OPfp, [M+H$^+$]=537.9, [M+Na$^+$]=599.9**



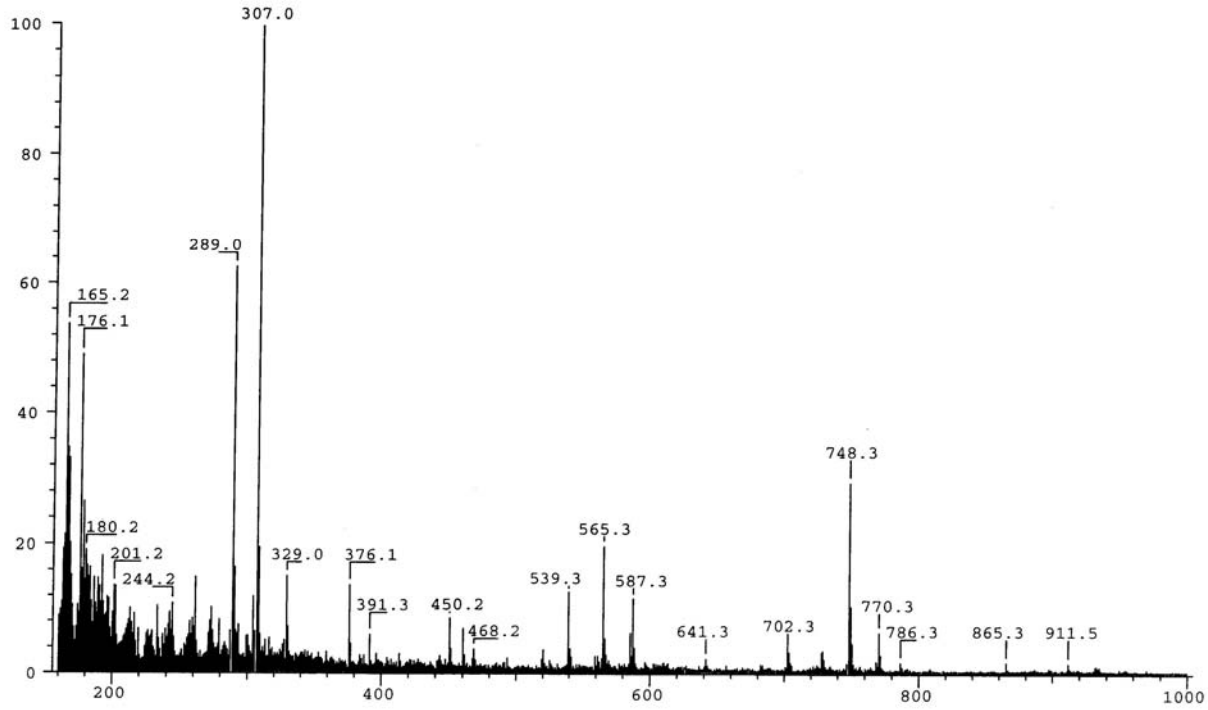

**(4) Bz-Arg-Asp(OEt)-OH, [M+H$^+$]=422.2, [M+Na$^+$]=444.2**



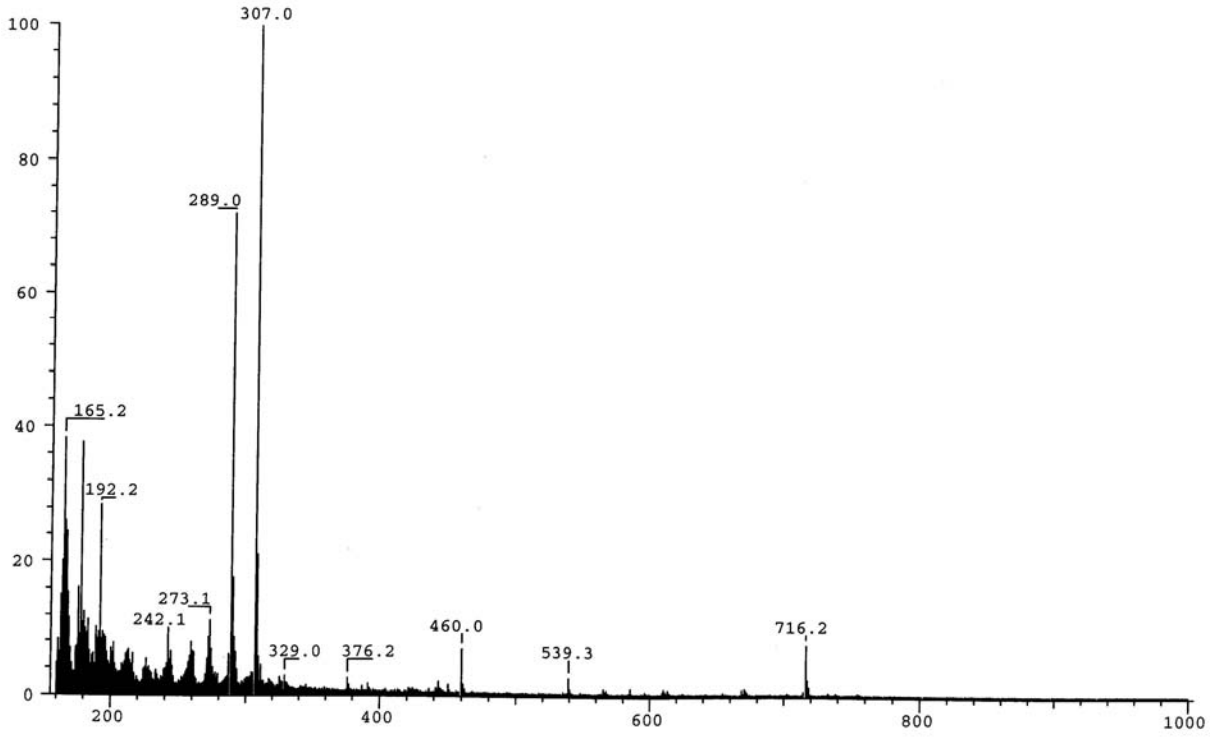
**(5) Bz-Arg-Asp(OEt)-Tyr-OH, [M+H$^+$]=585.2, [M+Na$^+$]=607.1**



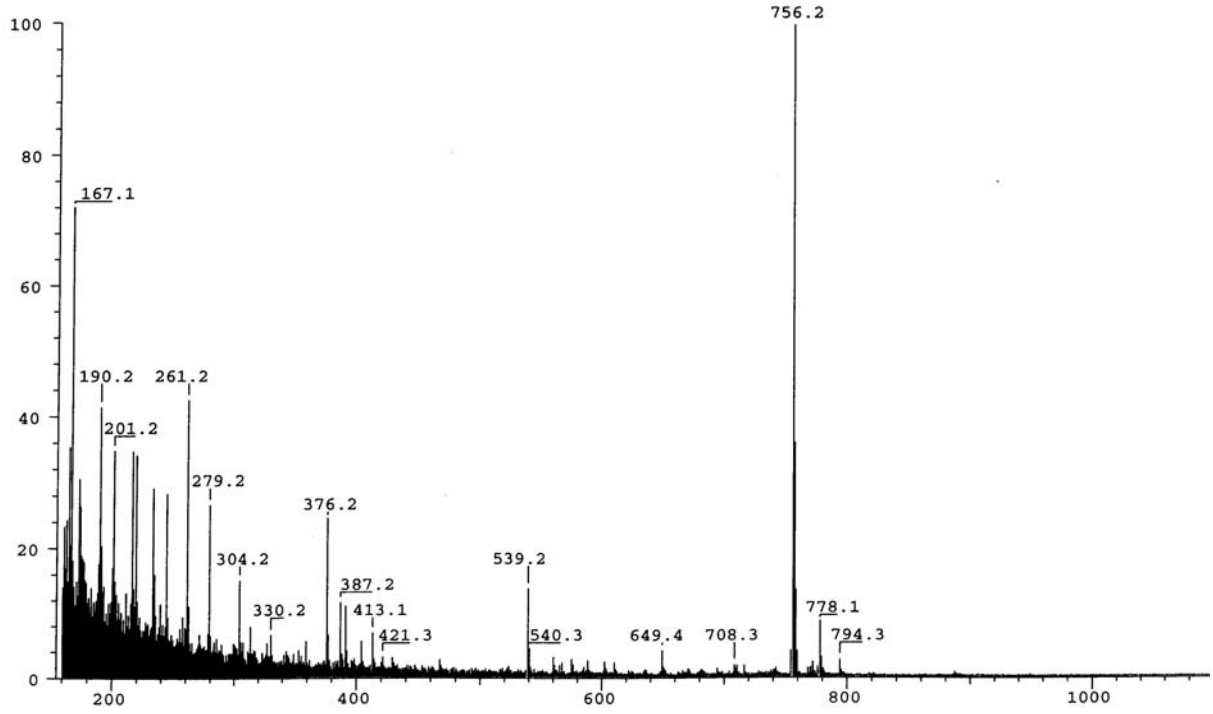

**(6) Bz-Arg-Asp(OEt)-(Tyr)$_n$-OH, [M+H$^+$]=748.3, [M+Na$^+$]=770.3 when n=2; [M+H$^+$]=911.5 when n=3**



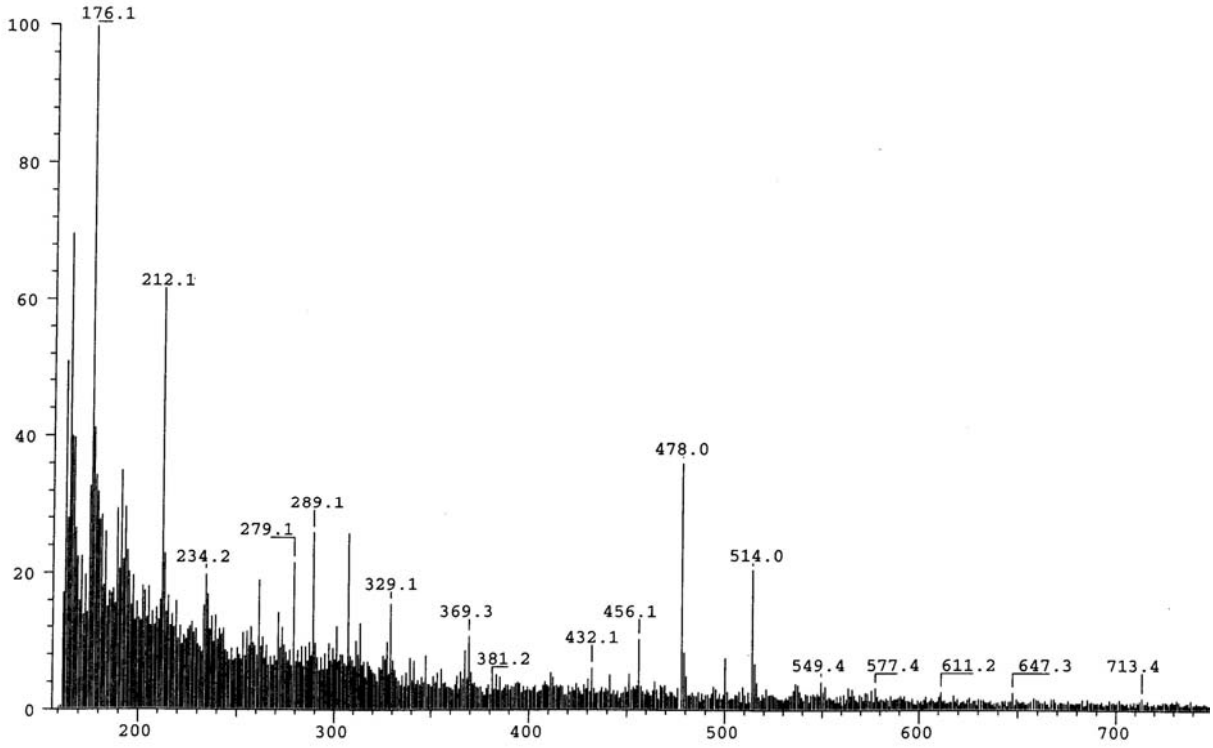
**(7) Bz-Arg-Asp(OEt)-Tyr-Met-OH, [M+H$^+$]=716.2**



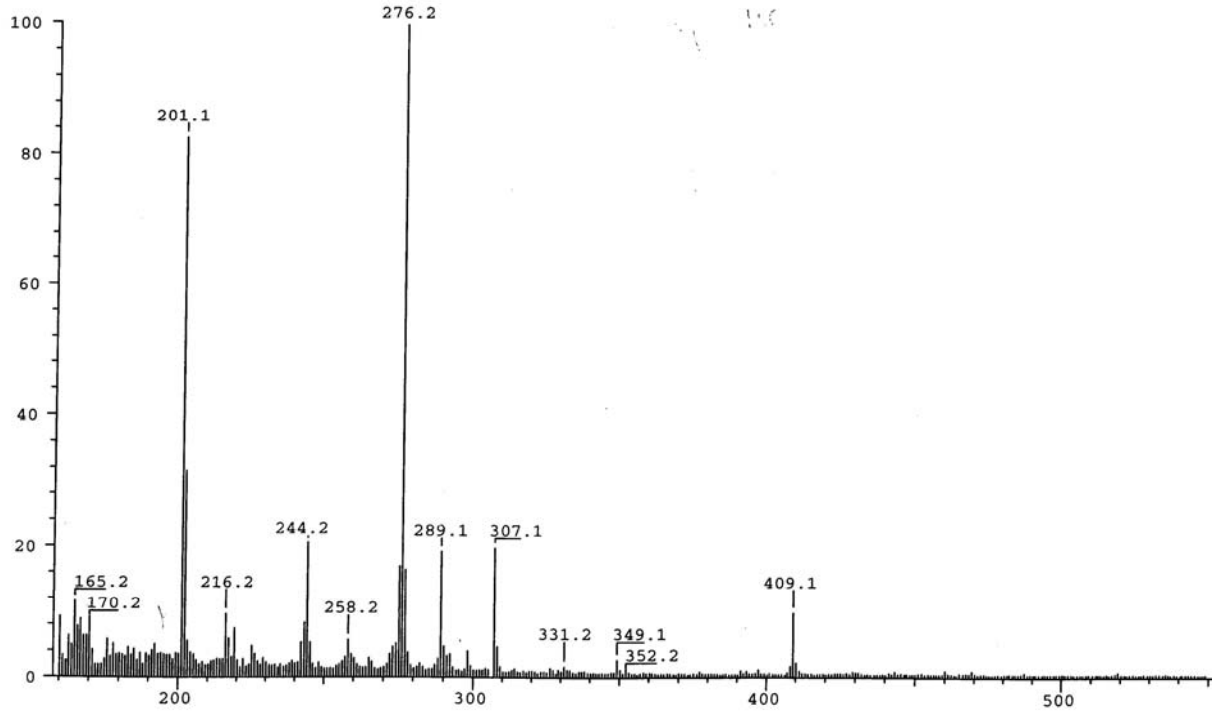

**(8) Bz-Arg-Asp(OEt)-Tyr-Met-OAl, [M+H$^+$]=756.2, [M+Na$^+$]=778.1**



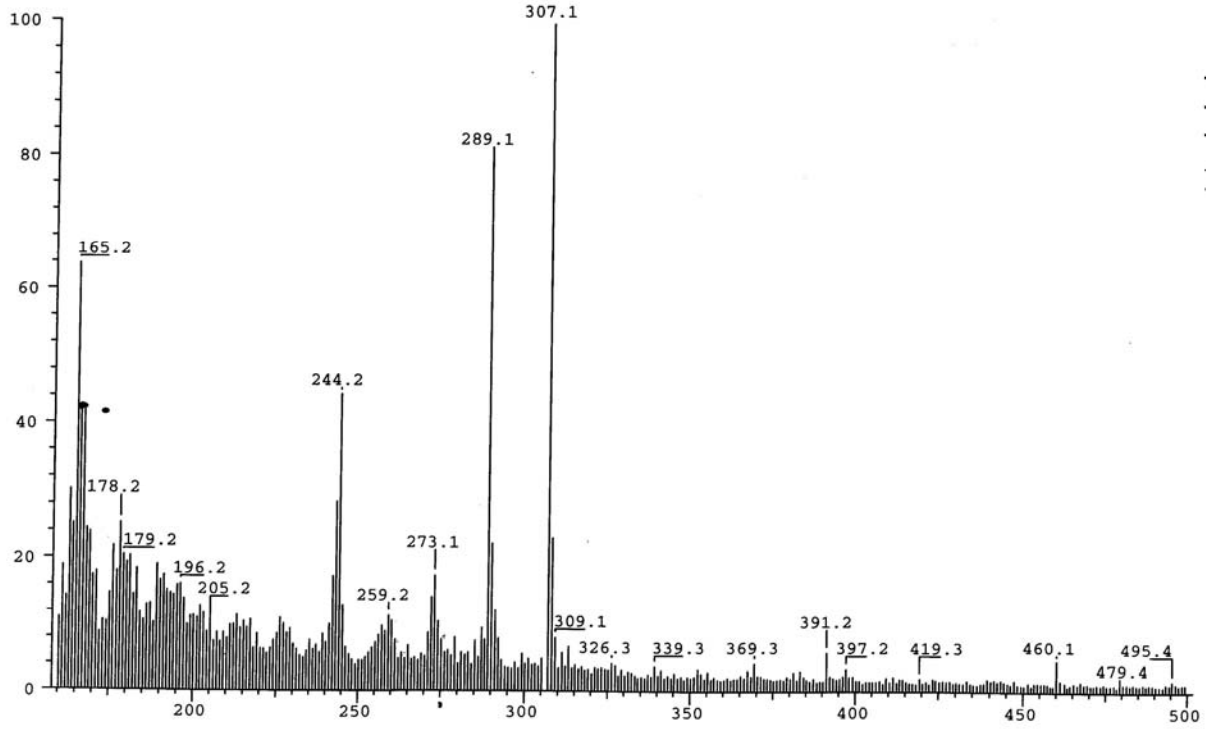
**(9) Asp(OEt)-Tyr-Met-OH, [M+H$^+$]=456.1, [M+Na$^+$]=478.0**



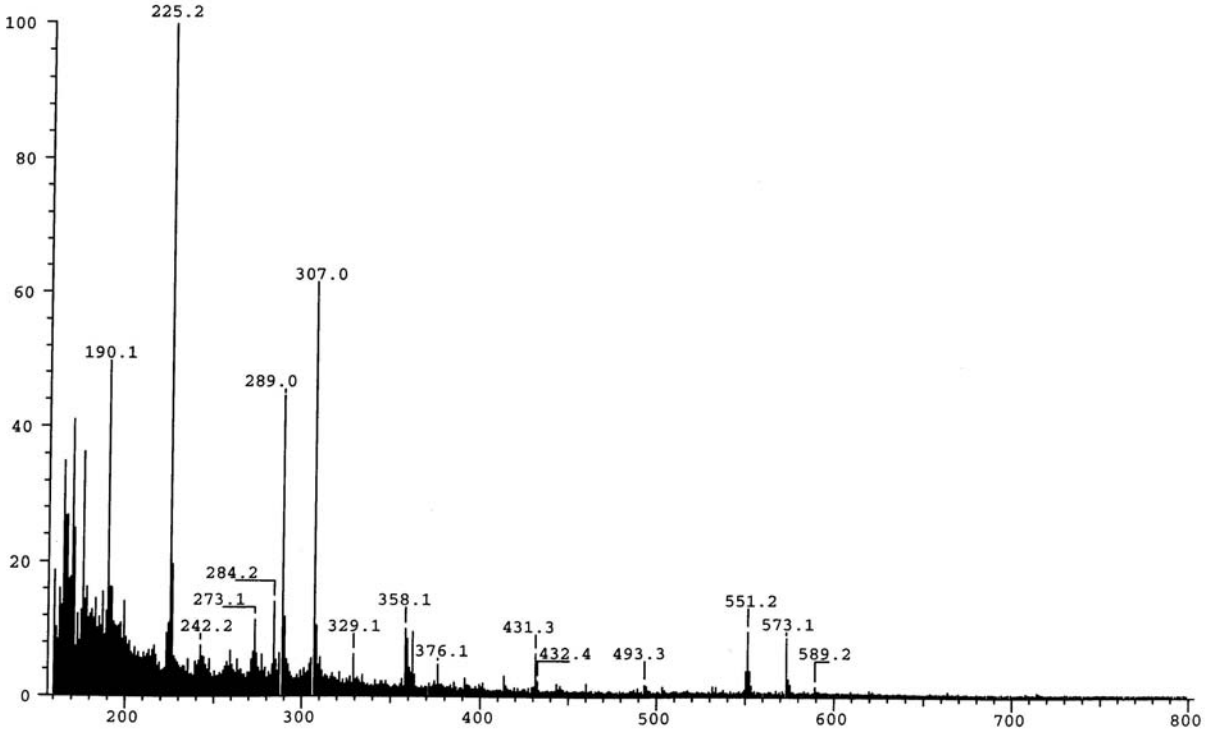

**(10) Gly-Trp-OMe, [M+H$^+$]=276.2, [M+Na$^+$]=298.0**



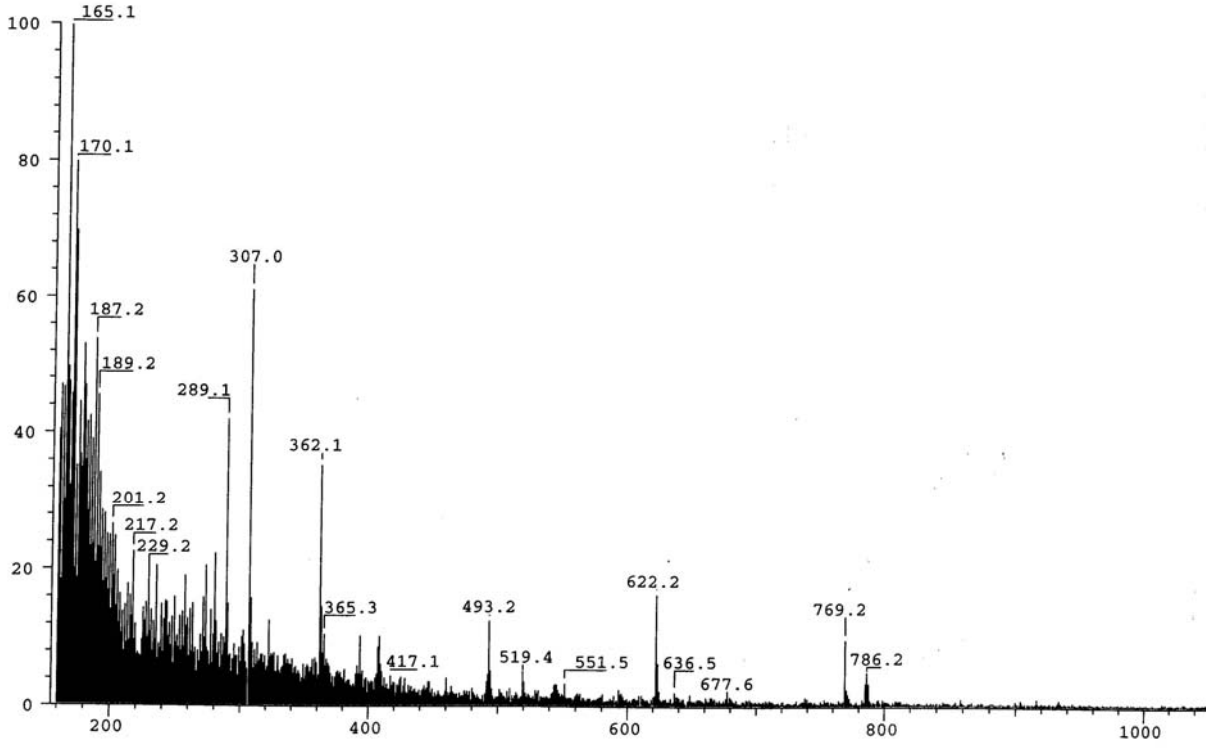
**(11) Diketopiperazine obtained from Gly-Trp-OMe, [M+H$^+$]=244.2**



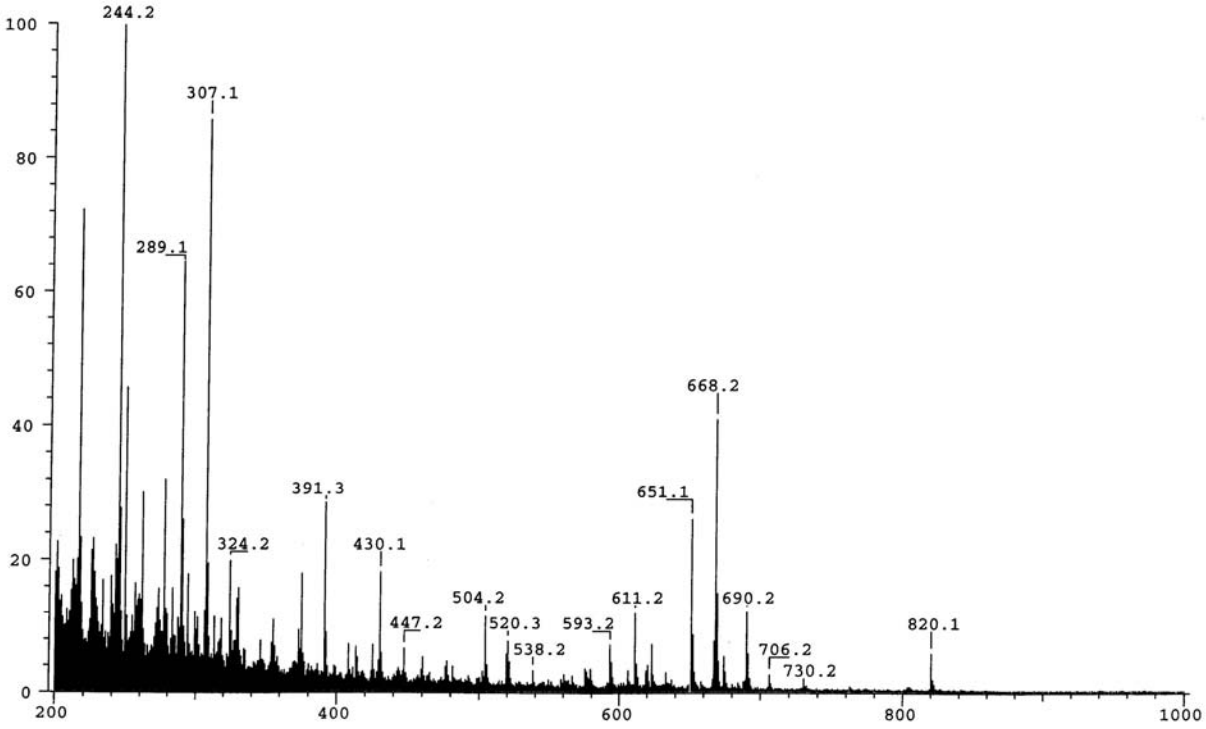

**(12) Phac-Gly-Trp-Met-OAl, [M+H$^+$]=551.2, [M+Na$^+$]= 573.1**



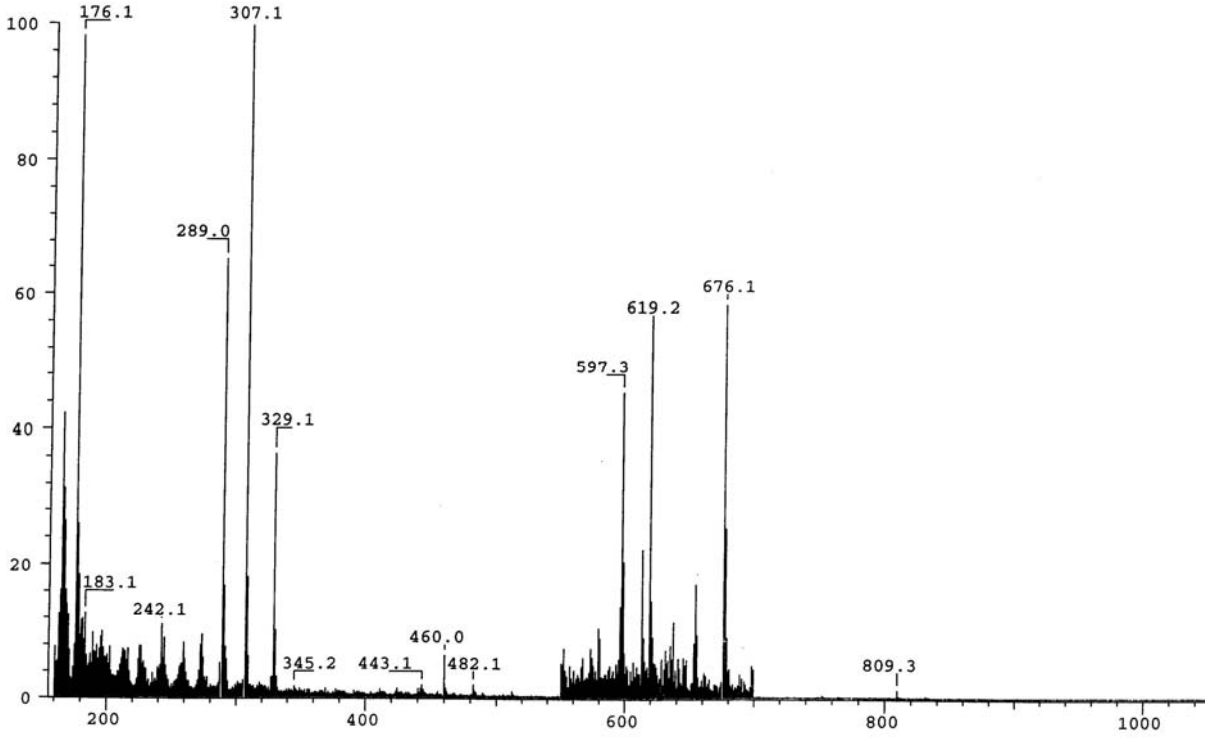
**(13) Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$, $[M+H^+]$=786.2**



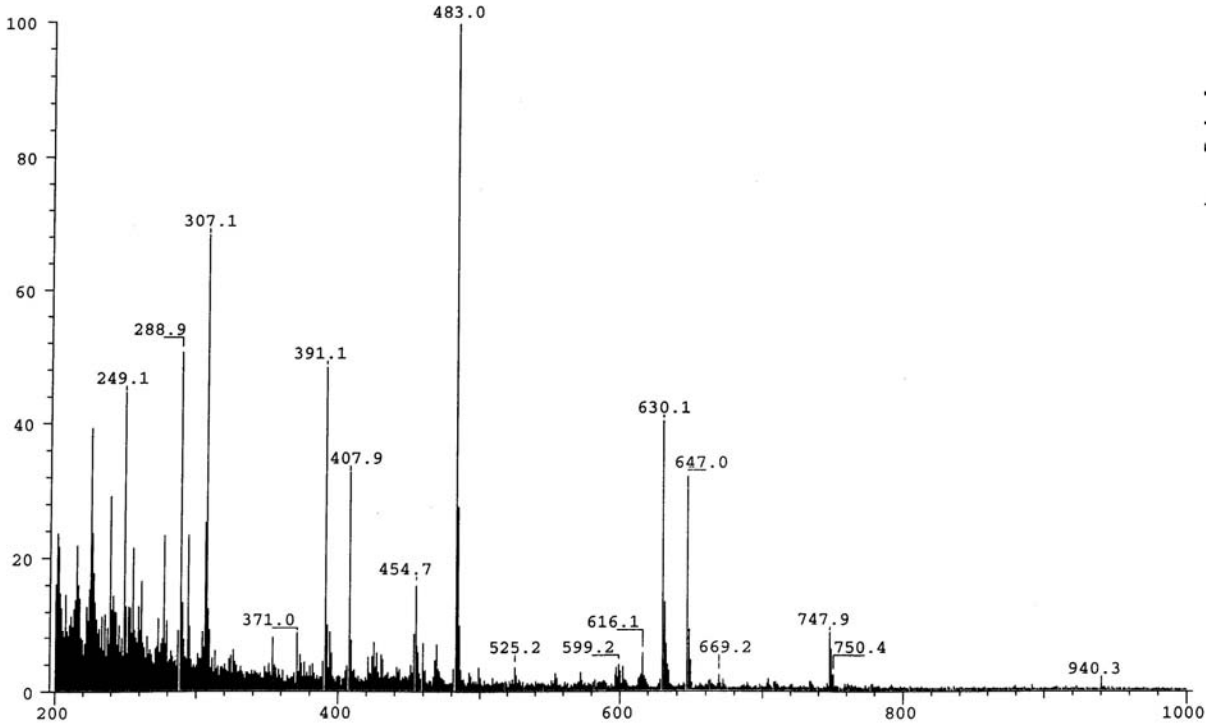

**(14) Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$, $[M+H^+]$=668.2, $[M+Na^+]$=690.2**



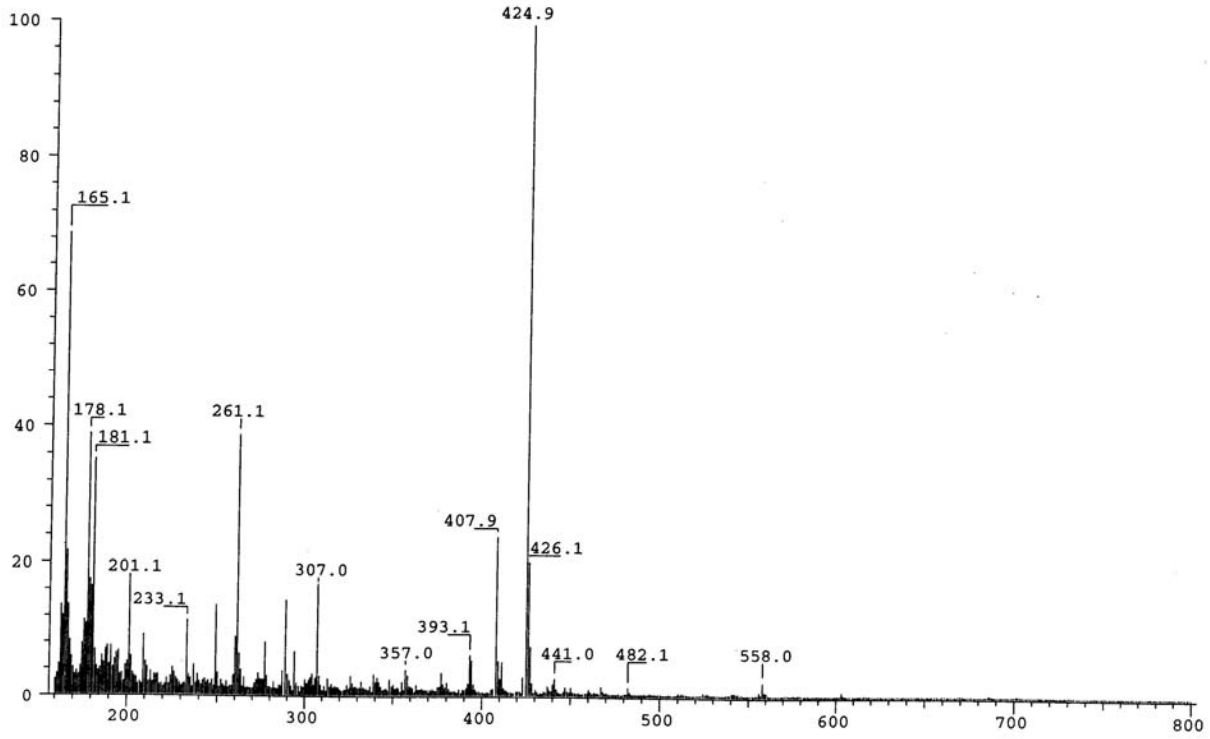
**(15) Gly-Trp-Met-Asp-Phe-$NH_2$, $[M+Na^+]$= 676.1**



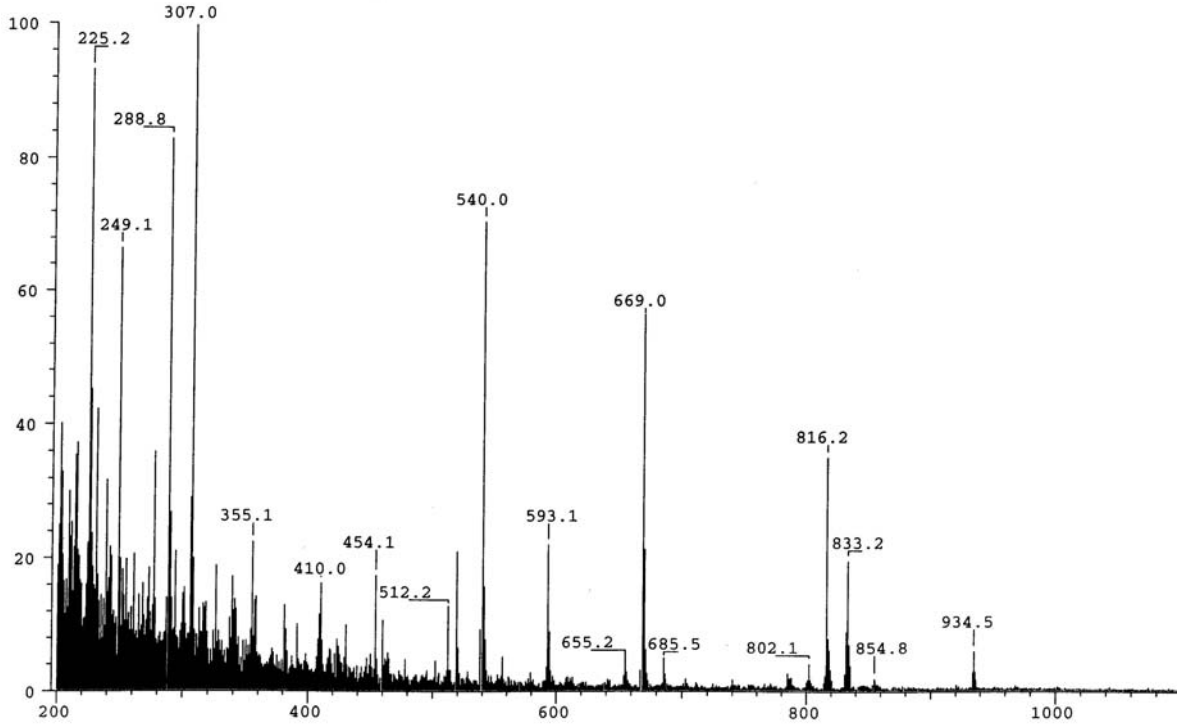

**(16) Fmoc-Met-Asp(OMe)-Phe-$NH_2$, $[M+H^+]$=647.0, $[M+Na^+]$=669.2**



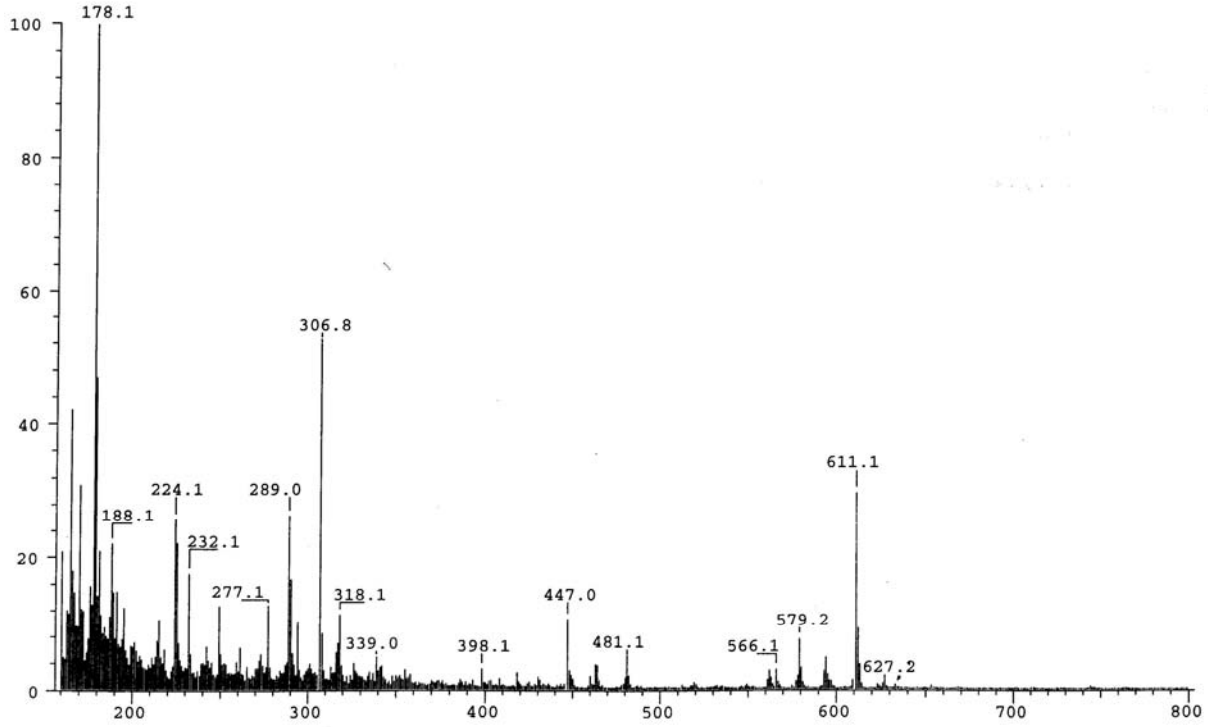
**(17) Met-Asp(OMe)-Phe-$NH_2$, $[M+H^+]$= 424.9**



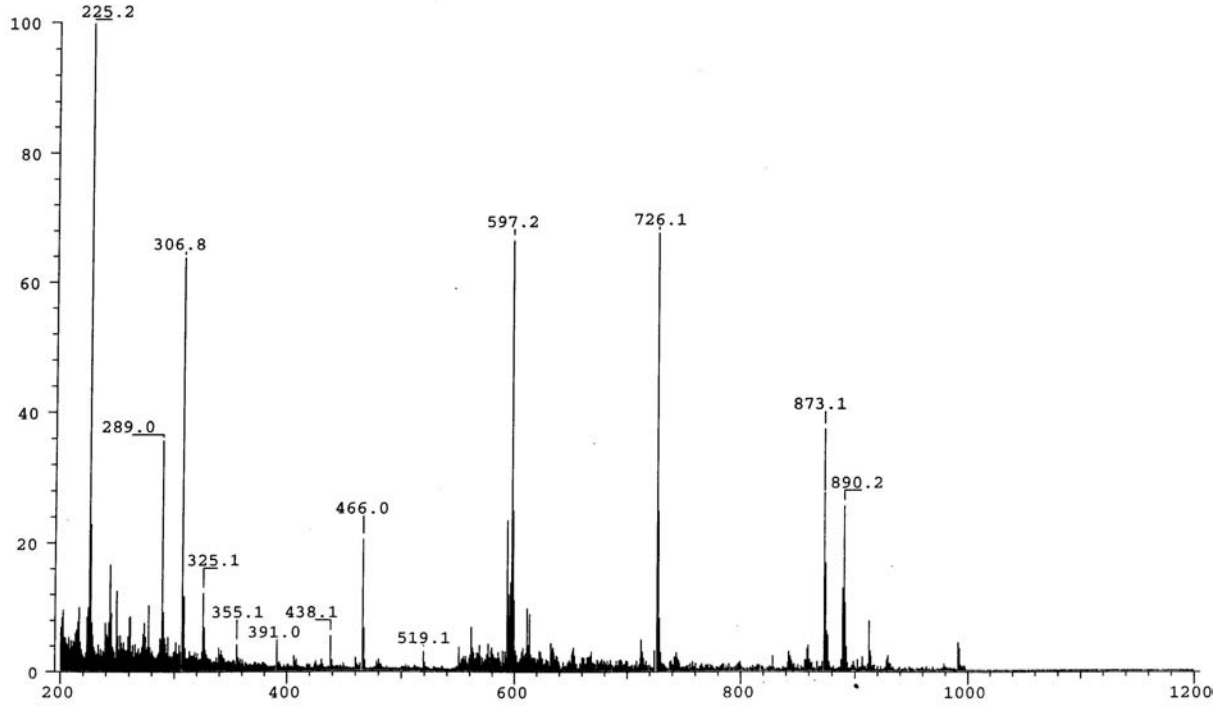

**(18) Fmoc-Trp-Met-Asp(OMe)-Phe-$NH_2$, $[M+H^+]$=833.2, $[M+Na^+]$=854.8**

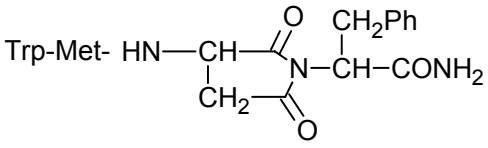


**(19) Trp-Met-Asp(OMe)-Phe-$NH_2$, $[M+H^+]$=611.1**

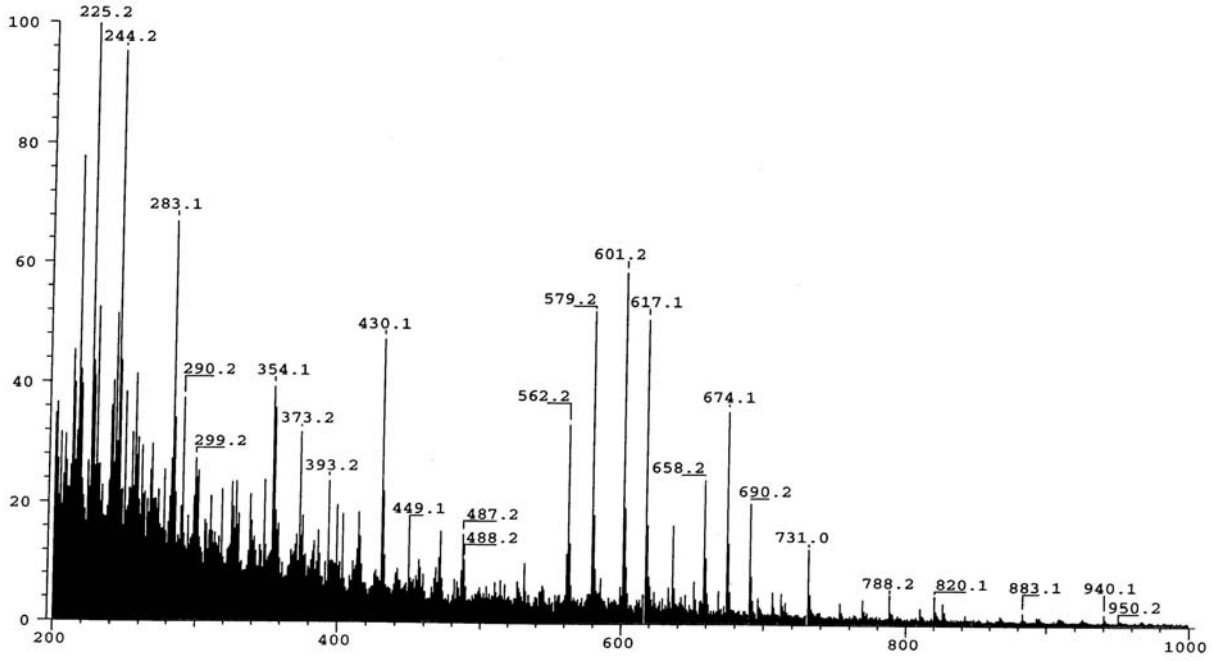



**(20) Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$, $[M+H^+]$=890.2**

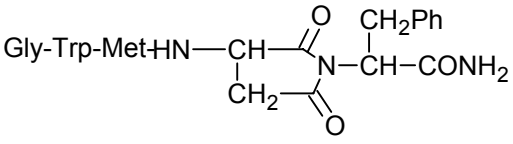




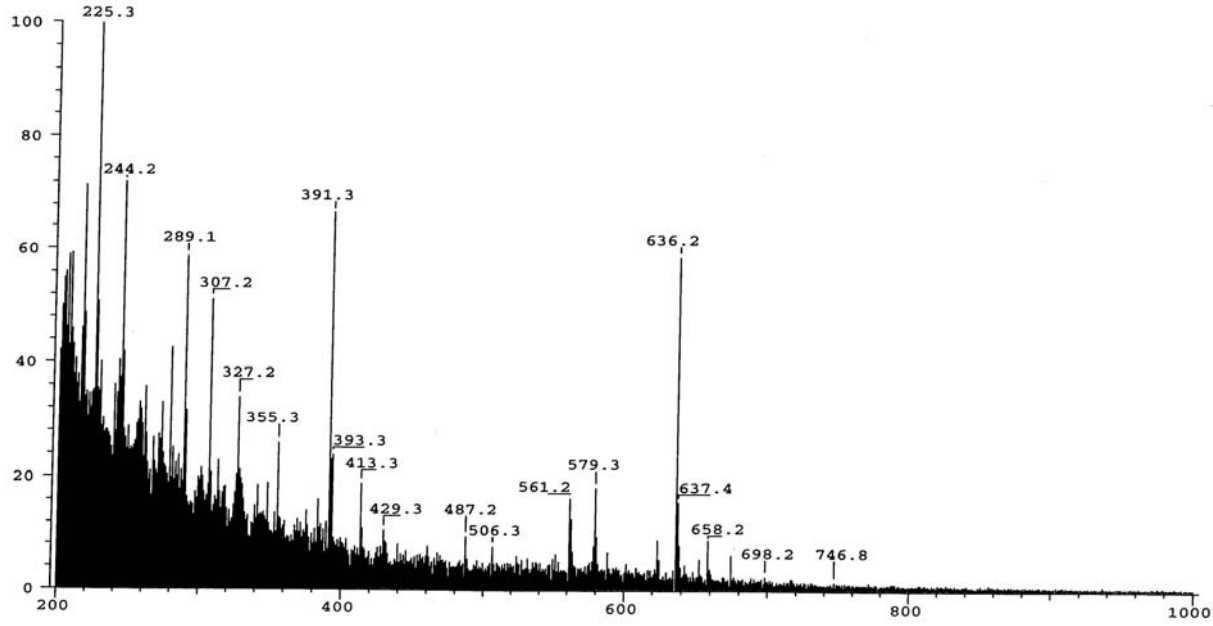

**(21)** **[M+H$^+$]=579.2, [M+Na$^+$]=601.2**




**(22)**

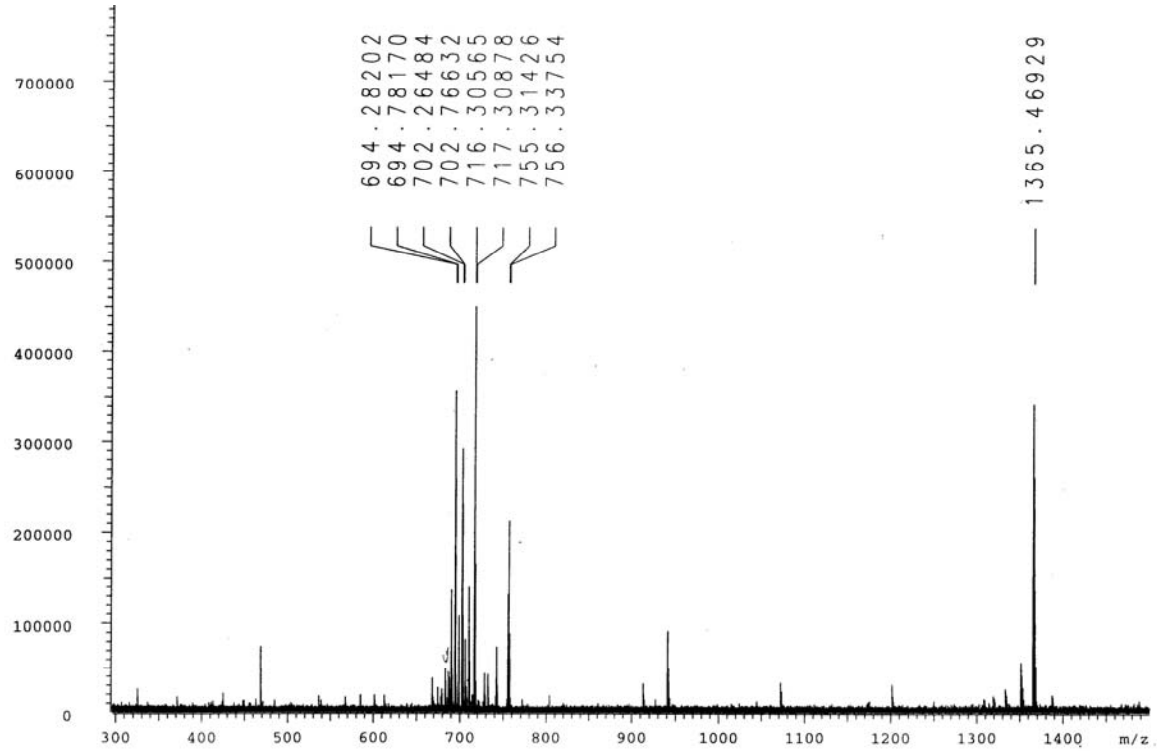



**[M+H$^+$]=636.2, [M+Na$^+$]=658.2**

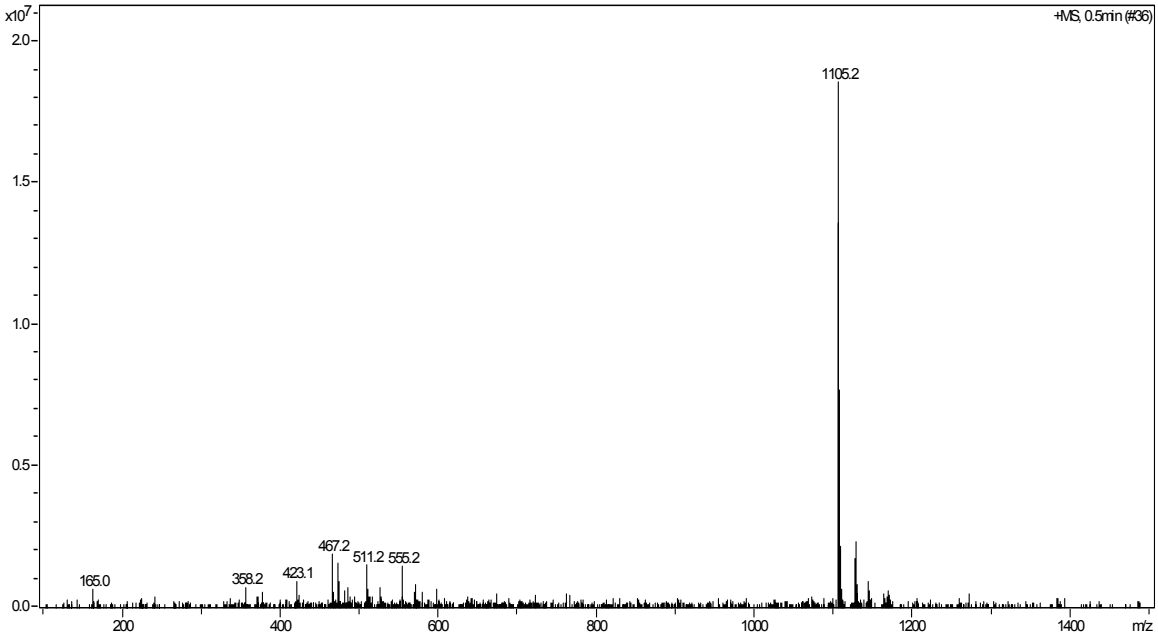


**ESIMS**

**(1) Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$, $[M+H^+]$=1365.4**




**(2) Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$, $[M+H^+]$=1105.2, $[M+Na^+]$=1127**



**My academic teachers were:**

S. P. Dhoubdel, H. Eckstein, C. L. Gajurel, M. B. Gewali, G. Häfelinger, K. Manandhar, M. Manandhar, T. P. Pathak, R. R. Pradhananga, J. K. Shrestha, D. P. Wagle

# Lebenslauf

| | |
|---|---|
| Name | Rajendra Joshi |
| Geburtsdatum | 26.10.1973 |
| Geburtsort | Bhajani-1 Kailali, Nepal |
| Staatsangehörigkeit | Nepali |
| Familienstand | Verheiratet |

| | |
|---|---|
| 1979-1985 | Mahunyal Grundschule, Bhajani, Kailali, Nepal |
| 1986-1989 | Martyrs' Memorial High School, Kathmandu, Nepal |
| 1990-1992 | Intermediate in Science am Amrit Science College, Kathmandu, Nepal |
| 1993-1995 | B. Sc. Studium an der Tribhuvan Universität, Nepal |
| 1995-1997 | M. Sc. Studium an der Tribhuvan Universität, Nepal |
| 08/1998-08/2001 | Quality Control Officer bei Colgate Palmolive, Nepal |
| 08/2001-03/2003 | Teaching Assistant an der Kathmandu Universität, Nepal |
| 10/2003-03/2004 | Diplomarbeit am Institut für Organische Chemie, Universität Tübingen unter Anleitung von Prof. Dr. Heiner Eckstein mit dem Thema: "Synthesis of a CCK-8 Tetrapeptide Fragment by the Enzymatic Method" |
| 04/2004-01/2007 | Promotion am Institut für Organische Chemie, Universität Tübingen unter Anleitung von Prof. Dr. Heiner Eckstein mit dem Thema: "Application of Proteases for the Total Enzymatic Synthesis of the Cholecystokinin Octapeptide (CCK-8) using Benzoyl-Arginine as an Enzymatically Cleavable N-Terminal Protecting Group". |