Total Enzymatic Synthesis of the Cholecystokinin Octapeptide (CCK_{26-33})

Vollenzymatische Synthese des Cholecystokininoctapeptids (CCK_{26-33})

DISSERTATION

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

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

2006

vorgelegt von

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Tag der mündlichen Prüfung	10. Juli 2006
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This scientific research work was carried out from April 2003 to April 2006 at the Institute of Organic Chemistry, Faculty of Chemistry and Pharmacy, Eberhard-Karls-Universität, Tuebingen, Germany, under the guidance of Prof. Dr. Heiner Eckstein.

I would like to express deep and sincere gratitude to my supervisor Prof. Dr. Heiner Eckstein for providing the opportunity to study in the perfect working conditions. Heartfelt appreciation is also expressed for his kind assistance, fruitful suggestions, excellent instructions and limitless patience throughout the entire research project of my doctoral dissertation.

I am especially grateful to Prof. Dr. Guenter Haefelinger for his precious time to read and appreciate my written thesis.

Special thanks go to Mr. H. Bartholomä and Mr. R. Müller for the FABMS-measurements of samples and to Mr. Wu Su (Max Planck Institute for Biological Cybernetics, Tuebingen, Germany) for the operation of LC-ESIMS. I also thank Mrs. Margit Wizemann for her assistance during my laboratory work and to Mr. Rajendra Joshi for helpful discussion and advices.

My acknowledgements are due to the Pharmacy school, Tongji medical college of Huazhong university of science and technology, China for supporting my study.

Above all, I am indebted to my parents and family members for their permanent love, constant care, unconditional support and encouragement during the whole tenure of my research.

To my family

for all of the love

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
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EtOAc	Ethyl acetate
EtOH	Ethanol
ESI	Electron spraying 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
MS	Mass spectrometry
OAl	Allyl ester
OEt	Ethyl ester
OMe	Methyl ester
OPfP	Pentaflurophenyl ester
PA	Penicillin amidase
PE	Petroleum ether
PGA	Penicillin G amidase
Ph	Phenyl
Phac	Phenyl acetyl
PIM	Positive ion mode
R_f	Retention factor
RP	Reversed phase
T	Temperature
tert.	tertiary
t_R	retention time
TFA	Trifluoroacetic acid
TEA	Triethylamine
TLC	Thin layer chromatography
TLN	Thermolysin
Tris	Tris-(hydroxymethyl)-aminomethane
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

Nature applies peptides, a short string of amino acids linked via amide bonds, for a great variety of specific functions. Peptides therefore can act as chemical messengers, neurotransmitters, as highly specific stimulators and inhibitors, regulating various life-processes. Peptides also gained importance in our everyday life. For example, a dipeptide derivative, aspartam, is used as sweetener; oxytocin and vasopressin have been available for medical purposes for many years. More recently the discovery of a long series of biologically active peptides together with the major application of peptide hormones in medicine, such as calcitonin and secretin, has given new impetus to synthetic peptide chemistry.

The earliest modes of peptide bond formation pioneered by Curtius (1881) and Fischer (1901) yielded impressive but not yet practical results. Introduction of the amino-protecting benzyloxycarbonyl group (Bergmann and Zervas, 1932) led to a new era of peptide synthesis. Improvement in the methods of peptide bond formation, particularly the application of carbonic acid mixed anhydrides (Wieland and Bernhard, 1951; Boissonnas, 1951) gave new impetus to peptide synthesis. 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). At least equally important improvements could be noted in the methods of protection: acid labile blocking groups were built on the stability of the tert. butyl cation (Carpino, 1957), such as the tert. butyloxycarbonyl (Boc) group; base sensitive blocking in the form of the 9-fluorenylmethyloxycarbonyl (Fmoc) group was introduced (Carpino and Han, 1970). Originally all synthetic peptides were routinely prepared by conventional solution methods, so-called classical peptide synthesis, that is, both reactants were soluble in the reaction media. Yet, one of the

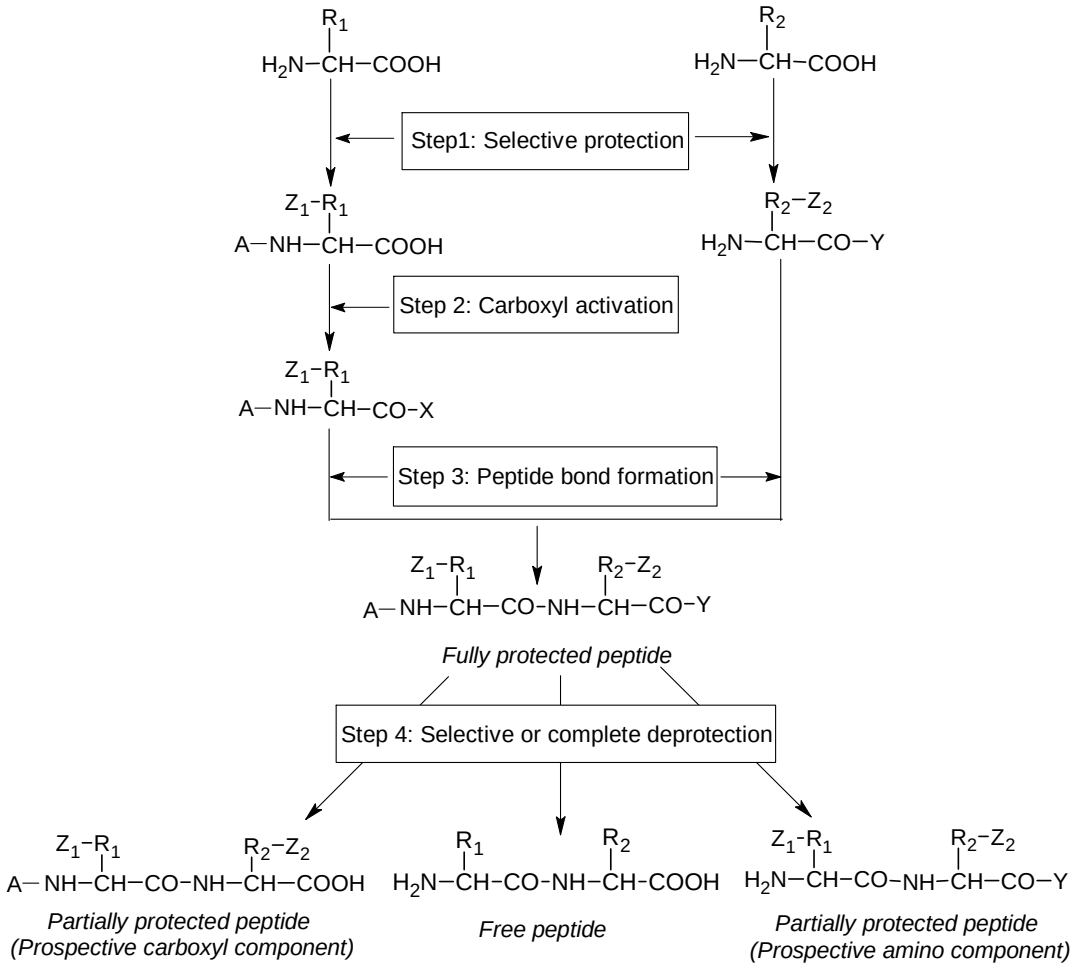
most conspicuous milestones in the history of peptide synthesis is the invention of the solid phase peptide synthesis by Merrifield (1963).

Around 110 years ago, as a consequence of van't Hoff's concept of the equilibrium constant in a reversible chemical reaction (van't Hoff, 1898) and Ostwald's definition of catalysis (Ostwald, 1901), it was concluded that the hydrolytic action of enzymes should be reversible and the formation of the peptide bond should be possible as well. In 1938, Bergmann's group reported the first enzymatic synthesis of a 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).

1.1 Chemical peptide synthesis

1.1.1 Principles

The typically chemical formation of a peptide bond is outlined in Scheme 1.1. In principle, it can be reduced to four steps.



Scheme 1.1 Basic scheme of chemical peptide synthesis. R_1, R_2, side-chain functionalities; A, α-amino protecting group; Y, α-carboxyl protecting group; X, activating substituent.

(1) The first step

To ensure the synthesis of well-defined peptides, the functional groups in main-chain and side-chain, which are not involved in the reaction, must be selectively protected. In synthesis of a larger peptide, a careful planning is required to match the coupling methods and protection 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 practised.

Boc group. The *t*-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 give 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

Through ester or amide, the carboxyl group can be protected successfully. The general approach for carboxyl protection is esterification.

Methyl ester (-OMe) and Ethyl ester (-OEt). 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 the addition of the amino acid to a cold mixture of thionylchloride and methanol or alcohol (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 (-OBu^t). 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 the attack of a nucleophile and also resistant to base catalyzed hydrolysis and weak acids. However, the tert-butyl group is removable with moderately strong acids, such as diluted solutions of HCl or trifluoroacetic acid.

(2) The 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 carbonyl group and thereby the electrophilicity of its carbon atom. Thus, the nucleophilic attack by the amino group is greatly enhanced.

(3) The third step

The peptide bond is formed by coupling the carboxyl component and the amino component via an α amide linkage.

The 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. Nitrous acid, diphenylphosphorylazide, alkyl nitrites etc. can be used for activation. The azide process remains one of the truly valuable assets of peptide chemists, mainly because

in most cases coupling via azides is accompanied with only negligible racemization. It is a valuable tool for fragment coupling.

Mixed anhydride method. To use the symmetric anhydride of a peptide or a N-protected amino acid would be wasteful of that component, since only half of it would be exploited for peptide bond formation. However, certain unsymmetrical anhydrides, which react with amino components only at the carbonyl of the N-protected aminoacyl component, are used instead (Chantrenne, 1947). For peptide synthesis, mixed anhydrides of the carboxyl component with ethyl- or isobutylcarbonic acid are employed (Vaughan, 1951).

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, N-acyl urea as a by-product and racemization arise. Both racemization and N-acyl urea formation can be suppressed to some extent by the addition of auxiliary nucleophiles such as 1-hydroxybenzotriazole (HOBt) 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.

(4) The fourth step

After the synthesis is complete, the protecting groups must be totally removed, or selectively cleaved, if the synthesis is to be continued.

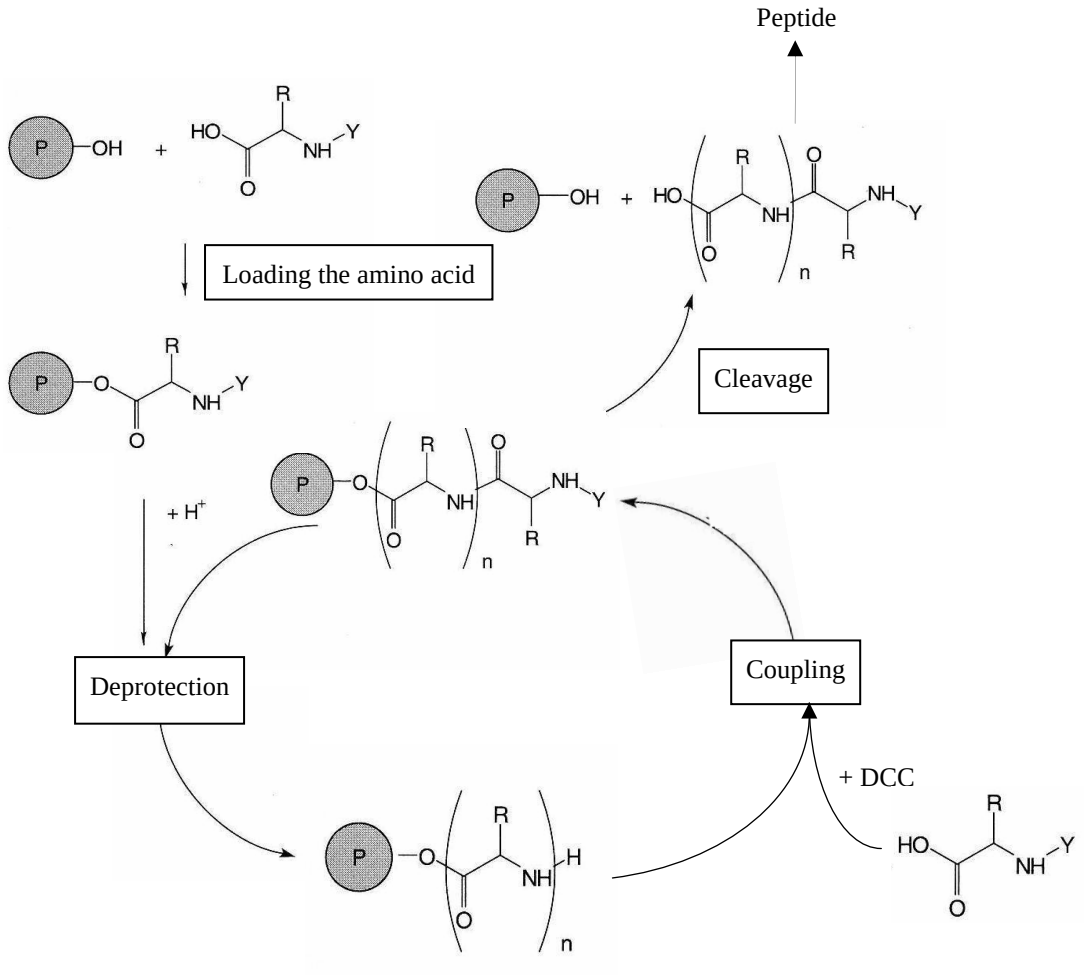
In the chemical peptide systhesis, attention should be paid to how to assemble the peptide chain. Stepwise synthesis by repeated couplings at the carboxyl end of a growing peptide chain (N→C strategy) is impractical, because racemization might occur when the carboxyl group is activated and diketopiperazines might be formed. Fragment condensation can be adopted to avoid racemization only by choosing fragments to end in carboxyl-terminal glycine or proline. The most practical and useful way is the stepwise chain elongation from the C-terminus (C→N strategy).

1.1.2 Classical peptide synthesis

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

1.1.3 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-amine 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 (Gross and Meienhofer, 1979). To circumvent these problems, an increasing number of peptide syntheses are conducted under the catalysis of enzymes (Jakubke et al., 1985; Bodanszky, 1993; Isowa et al., 1979; Jakubke et al., 1996).

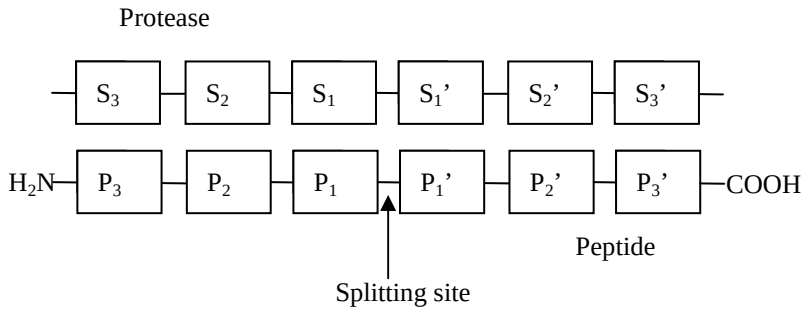
1.2.1 Enzymes

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 proteins. In other cases, the functional enzyme consists of two parts, collectively called 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. The region of the enzyme, to which the substrate binds, is known as the enzyme's active site. The topology of the region provides the basis for the enzyme's chemical specificity. On the basis of the catalyzed reactions, enzymes can be classified into oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Hydrolases have been the subject of more intensive study than any other.

(1) Proteases

A sort of hydrolases, are those enzymes 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. The peptide substrate is named as

P or P' on the basis of the splitting site of the peptide. Similarly, the active site of the protease is named as S or S'. The counting begins at the splitting site with P in direction to the amino end and with P' in direction to the carboxyl end of the peptide.



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

With respect to the catalysis mechanism four main types of endoproteinases are differentiated:

Serin proteases (e.g. Trypsin, Chymotrypsin, 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. The key amino acids at the active site of chymotrypsin constitute a "charge relay system" comprising the side-chains of Asp_{102}, His_{57} and Ser_{195} (Blow, 1976). 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).

Cysteine proteases (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 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.

Pepsin. Pepsin found in the gastric juice, is the best known member of the family of so called ‘acid proteases’. Since the ß–carboxylate functions of two aspartic acid residues (Asp_{32} and Asp_{215}) are involved in its catalytic mechanism, pepsin is designated as an aspartate protease and exhibits a pH optimum ranging from 2 to 3. Pepsin elicits a striking preference for those substrates whose P_1 and P_1’ position are occupied by a phenylalanine and an aromatic amino acid residue, respectively (Fruton, 1970).

Metalloproteases (e.g. Thermolysin). They possess metal ions in the active centre. An example is the 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. Thus, thermolysin represents a rare case, where the primary specificity of a protease is predominantly determined by structural features of the P_1' site of the substrates. 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^{α}-blocking groups in peptide synthesis has the advantage that it does not cleave peptide bonds. In this connection, Widmer et al. (1981) and Brtnik (1983) suggested the use of PGA as catalyst for N^{α}-deblocking steps.

1.2.2 Methods of enzyme immobilization

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.

Immobilization methods are often classified as follows by the type of the chemical reaction used for binding (Kennedy and Melo, 1990).

(1) Inclusion

Inclusion into membranes: By this technique the enzyme is retained within a membrane such as a hollow fibre or microcapsule, so-called “encapsulation”.

Incorporation into polymeric networks: The enzyme becomes entrapped in a fine polymeric network. The mesh width must be narrow enough to prevent movement of the enzyme out of the net, but large enough to allow free diffusion of substrates and products.

(2) Non-covalent binding

Adsorptive or ionic binding onto carrier materials: It is the simplest method of immobilization and very useful in non-aqueous system, in which desorption can be neglected owing to the low solubility of enzymes in these solvents. Conventional ion-exchange materials like diethylaminoethyl (DAEA) or carboxymethyl (CM) cellulose can be used as carriers; likewise, celite, calcium phosphate or Bio-Gel P-2 as

adsorption materials.

(3) Covalent binding

Cross-linking of enzymes: This covalent cross-linking reaction of enzymes can be achieved in the presence of bifunctionally cross-linking reagents, e.g. glutaraldehyde.

Binding onto modified carrier materials: Covalent fixation of enzymes to solid supports is the most important immobilization method. This method enables enzymes binding preferentially onto the outer shell of the modified carrier materials with preselected properties (Horvath and Engasser, 1973; Carleysmith et al., 1980).

Besides these, combined methods of immobilization have been described, namely, croos-linking following entrapment into gel or encapsulation, covalent entrapment into polymeric gels, etc.

1.2.3 Advantages of 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. One can distinguish between three levels of specificity, each of which individually contributes to the merits of the enzymatic peptide synthesis.

(1) Structural specificity

Structural specificity enables the proteases to select a substrate with distinct structural feature.

(2) Regiospecificity

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 have not to be protected in contrast to the chemical peptide synthesis.

(3) Stereospecificity

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.

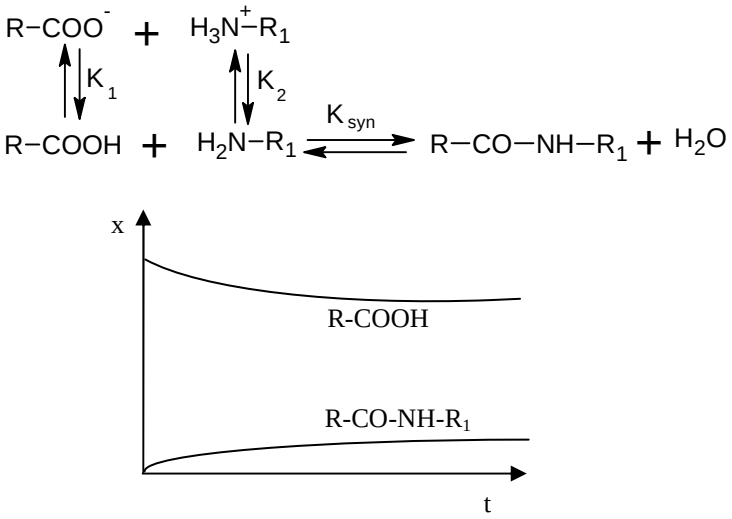
In addition, enzymatic peptide synthesis can be achieved under mild conditions with commercially available proteases which are easy to handle and, due to their application in catalytic amounts only, the costs are mostly insignificantly higher in comparison with chemical coupling reagents. By avoiding 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

According to the type of the carboxyl component used, the methods of enzyme-catalysed peptide bond formation generally can be classified into two basic strategies: the thermodynamic and the kinetic approach.

1.2.4.1 Thermodynamically controlled peptide synthesis

In the thermodynamic approach, this component has a free carboxyl terminus, and the peptide bond formation occurs under thermodynamic control, which is a direct reversion of the protein hydrolysis, according to Scheme 1.4.



Scheme 1.4 Thermodynamically controlled peptide bond formation. R = acyl residue of the carboxy 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)). 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 during chemical 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).

Variation of temperature and pH. Ionic equilibria can also be perturbated in favour of peptide synthesis by elevation of the reaction temperature. The degree of ionization of the α-amino groups of the amino acids varies significantly with temperature (pK_2 value decrease with increasing temperature) whereas that of the α-carboxyl function depends only slightly on temperature. The ionization properties are also influenced by the hydrogen ion concentration of the reaction medium. However, any pH variation is a double-edged sword, since any favourable shift of the ionic equilibrium of one educt is accompanied by an unfavourable shift of the ionic equilibrium of the other educt.

Influence of the chain length of the reactants. The acidity and the basicity of a peptide decrease with increasing chain length. Hence, the energy required for the proton transfer will also decrease and the prospects of peptide synthesis will generally improve with growing chain legth of the reactants.

(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; Martinek et al., 1981; Martinek and Semenov, 1981).

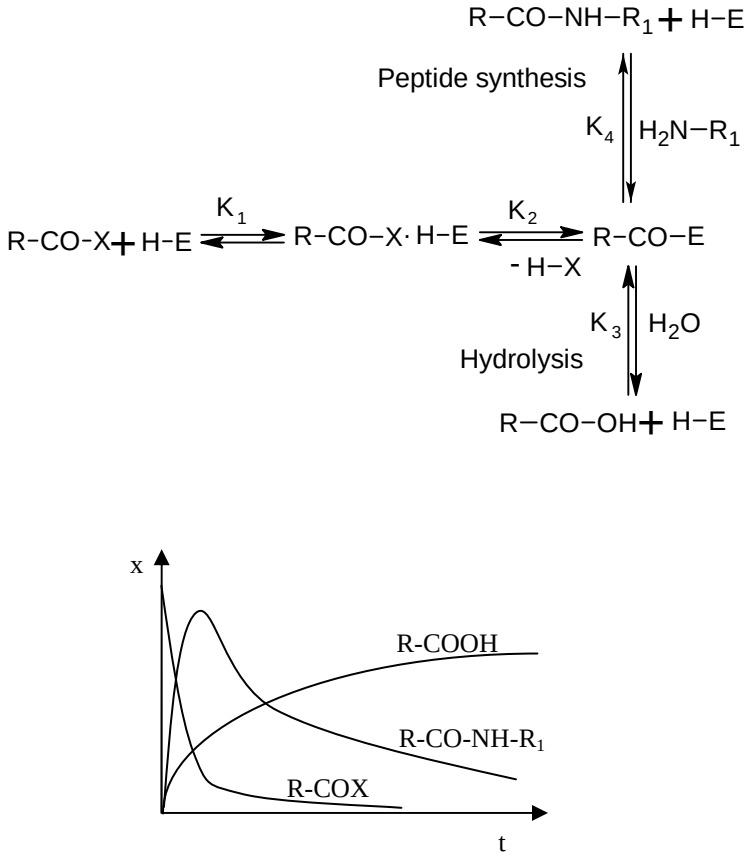
Molecular traps. A synthesized peptide is noncovalently bound (trapped) by another peptide or protein to form a stable complex. Any substance capable of forming an intra or intermolecular complex with the reaction product, but not with the educts, may act as a scavenger (Chaiken 1981).

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 goverened 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 nuclophilic amino component forming the desired peptide product (R-CO-NHR_1), or in a hydrolysis step to water (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; HE = serine or cysteine 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 acylenzyme. 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 $RCONHR_1$ has a maximum at a definite time. The reaction has to be stopped at the “kinetic” optimum. The progress of the reaction is permanently controlled by HPLC.

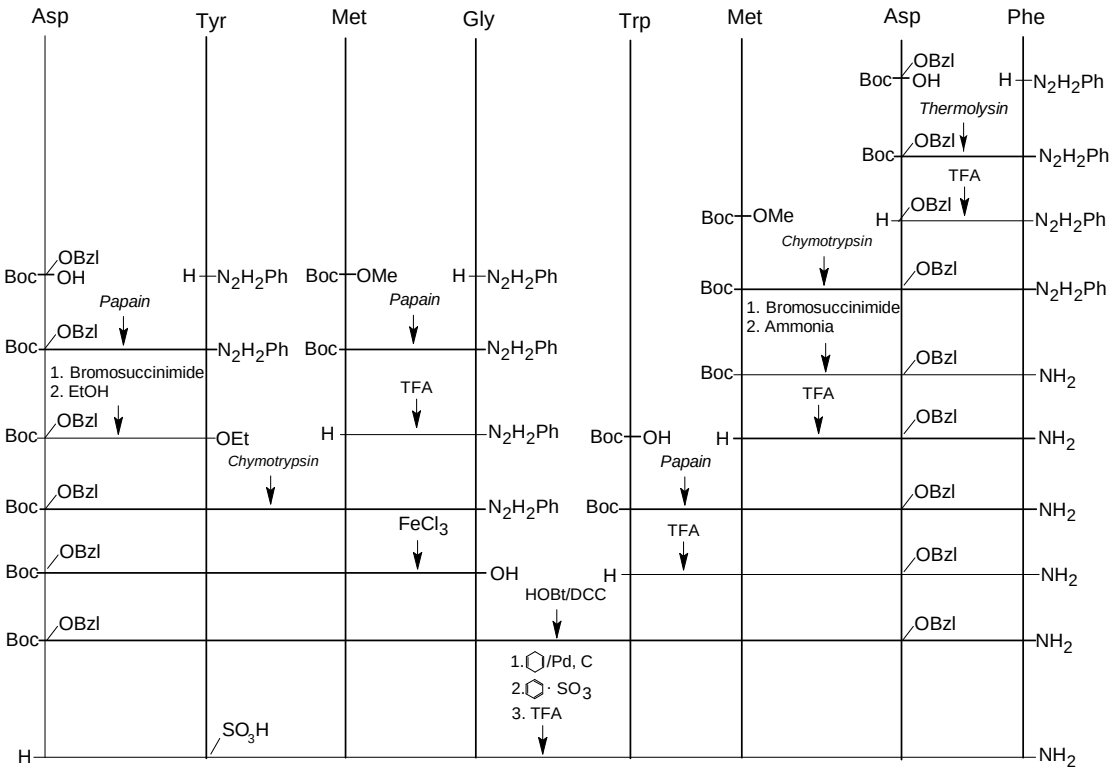
2 State of the art and aim of this research

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 (CCK), a gut and brain peptide hormone with 33 amino acid residues, is widely distributed in the central nervous system and the gastrointestinal tract and shows multiple biological activity. It can stimulate extensively the liberation of insulin and glucagon from the Langerhans islets (Verspohl, 1986). Besides, 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). CCK-8 has the amino acid sequence ($CCK_{26\text{-}33}$), that is, Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2 (Mutt and Jorpes, 1968).

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.

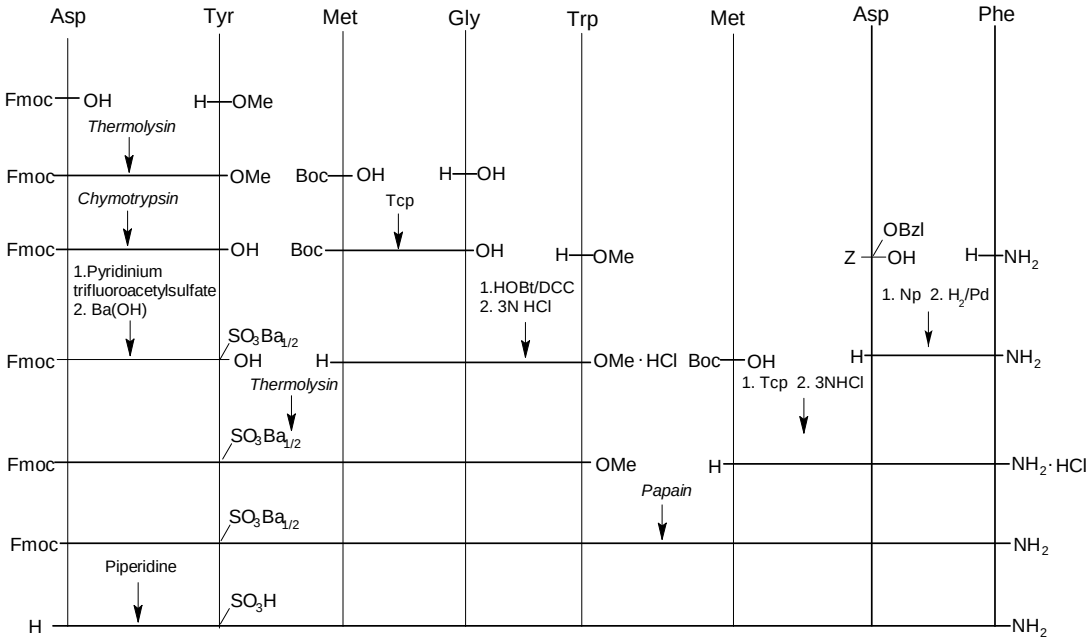
In 1982, Kullmann (1982) reported the first preparation of CCK-8 using enzymatic peptide bond formation as far as possible.The octapeptide was finally assembled by the chemical condensation of two tetrapeptide segments which have been synthesized through the concerted actions of several proteases of 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. Furthermore, the N- and C-terminal tetrapeptides could not be coupled by either papain-, thermolysin-, ficin-, or bromelain-catalysis, because at least one of the tetrapeptide was subject to proteolytic degradation before the synthesis of the desired peptide bond could be completed.



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

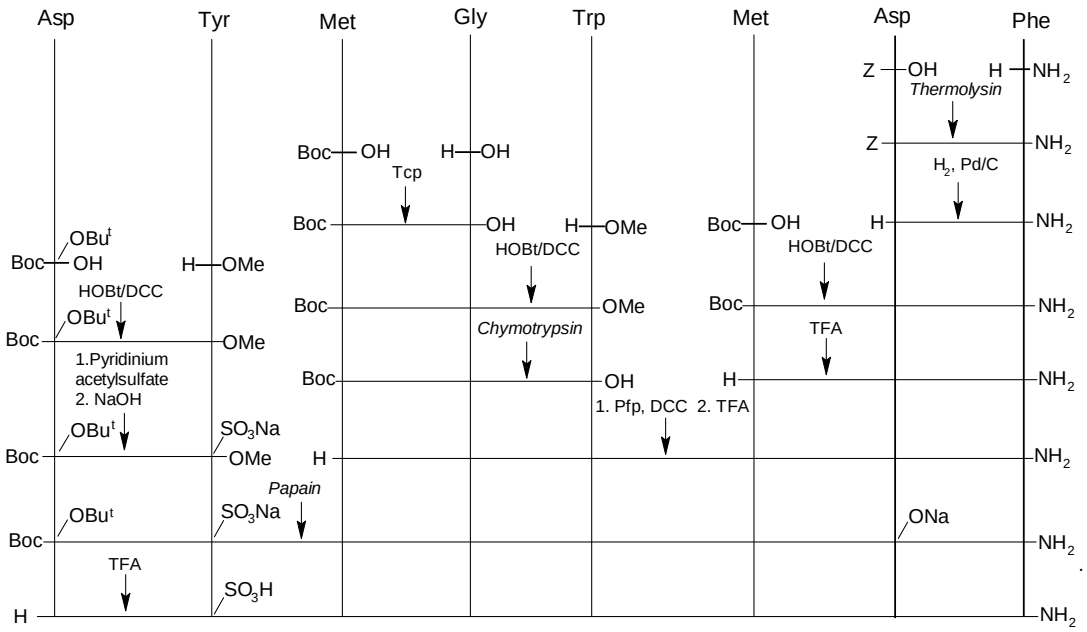
Sakina and his associates (1988) described another pathway to obtain CCK-8, which is shown in Scheme 2.2. In an abbreviated form it can be described as (2 + 3) + 3, that is, a pentapeptide yielded from a di- with a tripeptide is coupled to a tripeptide that had been previously synthesized.



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.

In this synthetic procedure, both tripeptides were obtained via the chemical way. N-α protecting group of acyl-donors were Fmoc-, Boc- and Z-group, respectively.

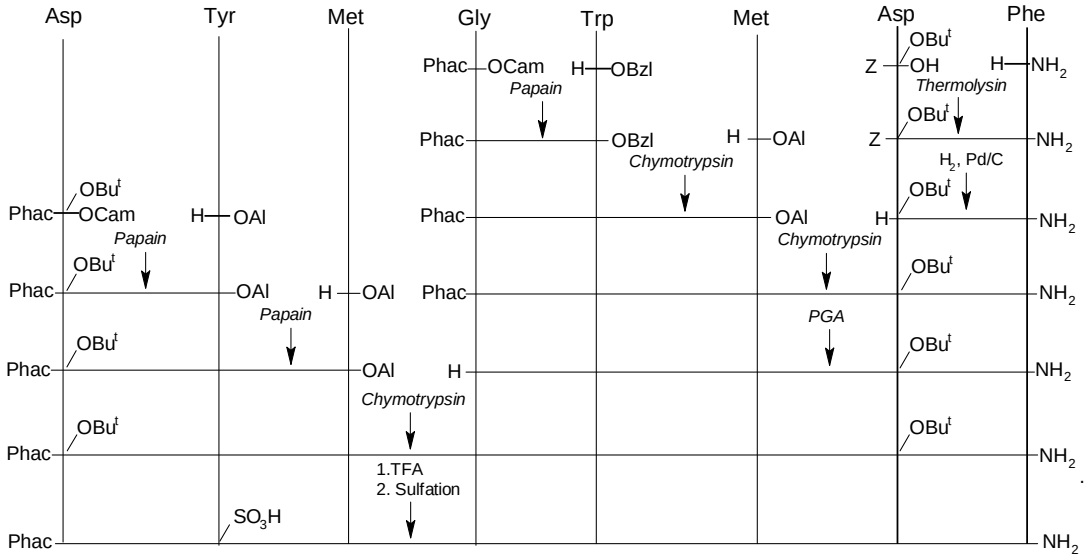
In the same year, an alternate synthetic route was presented by Cerovsky's group (1988), as shown in Scheme 2.3. CCK-8 was synthesized by 2 + (3 +3) fragment condensations, where the tripeptides were coupled via the chemical way. The attempted enzymatically catalyzed condensation between Boc-Met-Gly-Trp-OMe and H-Met-Asp-Phe-NH_2 with α-chymotrypsin (at pH 10), subtilisin (at pH 9) or proteinase K (at pH 10) were tried in an aqueous buffer containing DMF, or in biphasic systems or only in a mixture of organic solvents. In all cases the very rapid enzymatically catalyzed hydrolysis of the α-methyl ester predominated and the formed Boc-Met-Gly-Trp-OH did not react further, and the hexapeptide obtained only to the extent of 15%. Boc and Z groups were utilized to protect the α-amino groups of the acyl-donors.



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

Later Clapes and his co-workers performed a series of studies on enzymatic synthesis of fragments of CCK-8, such as dipeptides, the C-terminal tripeptide and the C-terminal pentapeptide. In the synthesis of dipeptide fragments, Z-, Boc- and Fmoc-group as N-α protecting groups were tested, N-α protected amino acid allyl, carboxamidomethyl, and methyl ester derivatives were assayed as acyl donors in kinetically controlled processes, and C-α amides and esters were used as nucleophiles. It was found that generally amino acid N-α benzyloxycarbonyl, C-α carboxamidomethyl ester derivatives as acyl-donors gave the highest yields. When the added buffer solution was reduced from 4 to 1%, in chymotryptic couplings, a decrease in the reaction rate combined with an increase of the dipeptide yield was observed. In papain-catalyzed reactions, however, loss of activity was the main effect. The best enzymes for each dipeptide synthesis were: Papain, Asp-Tyr and Gly-Trp; α-chymotrypsin, Tyr-Met and Met-Gly; thermolysin, Asp-Phe and Asp-Tyr; papain and α-chymotrypsin, Trp-Met and Met-Asp (Calvet et al., 1996). This research group synthesized Z-Gly-Trp-Met-OX (X=H, Et, Cam or Al) via stepwise chain-lengthening starting with the N-terminal under the catalysis of enzymes, which were deposited on

solid support materials. The obtained Z-Gly-Trp-Met-OX was condensed with the dipeptide H-Asp(OBu^t)-PheNH_2 via immobilized α-chymotrypsin to obtain the protected pentapeptide. The free pentapeptide was achieved by deprotection. The influence of structures and concentration of acyl-donors and nucleophiles, reaction media and support materials used to adsorb enzymes, on both the product yield and enzymatic activity was investigated (Capellas et al., 1996[a]; 1996[b]; 1997; Ruiz et al., 1997). A disadvantage of using the Z-protecting group in synthesizing CCK-8 was that CCK-8 has two methionine residues in which the sulfhydryl groups poison the catalyst and made the finial deprotection steps by means of catalytic hydrogenation rather difficult. Alternatively, the Z-group was deprotected by liquild hydrogen fluoride, an efficient but dangerous method and difficult to scale-up. Therefore, this research group changed to Phac-protecting group and finally obtained Phac-CCK-8. The strategy for Phac-CCK-8 synthesis is described as follows (Scheme 2.4) and in an abbreviated form it can be described as 3+ (3 + 2). However, it is not reported whether the phenylacetyl group of Phac-CCK-8 can be cleaved to afford the free CCK-8.



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

In summary of all above mentioned enzymatically controlled couplings, the reaction systems can be classified as follows:

(1) Aqueous system

The enzymatic coupling was conducted in water or buffer. Generally, this thermodynamic controlled reaction with unfavourable equilibrum can be shifted towards synthesis by the means of product precipitation.

(2) Cosolvent system

The enzymatic coupling steps were performed in monophasic aqueous-organic systems in order to induce a favorable shift of the ionic equilibra and to enhance the solubility of the initial reactants in the respective incubation mixtures. Water soluble solvents such as alcohols or dimethylformamide (DMF) can be employed. Nevertheless the activity of free or immobilized proteases in this medium will be affected by the presence of the organic solvent.

(3) Biphasic systems

This approach can be used when both acyl-donor and nucleophile are soluble in water and the product is mainly soluble in the organic phase. The reaction takes place in the aqueous phase and the enzyme can be used either free or immobilized by covalent attachment. This system largely prevents the enzymes from coming into unfavorable contacts with organic solvents which may cause denaturation of the proteases. However, mass transfer limitation and deactivation of proteases at the interphase are the major drawbacks of this reaction system. This problem can be overcome by using appropriate surfactants to reduce the interfacial tension (Feliu et al., 1995).

(4) Low-water content system

Proteases are active in reaction media with low water concentration. They can be employed either as enzyme powder or deposited onto a support (Mattiasson and Adlercreutz, 1991; Klibanov, 1986). The thermodynamic water activity (a_w) in this system is directly related with the enzyme activity and it also influences the ratio

synthesis/hydrolysis in the kinetically controlled synthesis. Low-water content systems such as organic media have a wide range of applications in oligopeptide synthesis, because they are normally compatible with the solubility requirement of amino acid and peptide derivatives (Wartchow et al., 1995).

The main disadvantage is drawn from the enzymatic strategies proposed by Kullmann (1982), Sakina et al. (1988) and Cerovsky et al. (1988) that many chemical couplings were used because of either poor yields or failure of the enzymatic steps. Even though Clapes' group improved coupling steps and achieved all peptide bond formations via enzymatically controlled couplings, there remains the problem that Z-group had to be cleaved chemically. Another drawback is that two additional chemical reaction steps were necessary to obtain an OCam ester and therefore the overall yield decreased. Additionally the activation of the acyl-donors might cause racemization.

The aim of this study is to circumvent such above-mentioned shortcomings and obtain a better strategy for the total enzymatic synthesis of the octapeptide (CCK_{26-33}).

During enzymatic peptide synthesis, the introduction of protecting groups is an essential requirement for ensuring the synthesis of well-defined peptides, for cancelling the zwitterionic character of the reactants and for further purpose insofar as it ensures a suitable solubility differential between educts and products. Therefore, in this work, N^{α}-protection will be achieved by the PGA-liable Phac-group and methyl ester will be applied for aspartic acid residue due to its simplest introduction and easy removal.

Based on our experience in the enzymatic synthesis of fragments of cholecystokinin (14-18) in the last years, it was planned to assemble the target octapeptide via fragment condensation between a tripeptide and a pentapeptide, that is, 3+ 5 in an abbreviated form. Both of the two fragments will be prepared separately by stepwise coupling of successive amino units starting from N-terminal residue.

In order to achieve this goal, the following five major objectives were set.

(1) Immobilization of enzymes

The advantages of immobilized over soluble enzymes arise from their enhanced stability in organic solvents, ease of separation from reaction media, easy recovery and repeated use. In the first objective, different methods will be utilized to immobilize enzymes and the activity of the immobilized enzymes will be assayed.

(2) Enzymatic synthesis of the N-terminal tripeptide

(Phac-Asp(OMe)-Tyr-Met-OAl)

The stepwise chain-elongation starting with the N-terminal residue (N→C strategy) will be applied for the synthesis of the tripeptide. Different synthetic pathways will be investigated. 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.

(3) Chemical synthesis of reference substances

Chemical synthesis using Fmoc-protected amino acid pentaflurophenyl esters as acyl donors via the C→N strategy will be carried out to obtain the C-terminal pentapeptides Gly-Trp-Met-Asp(OMe)-Phe-NH_2 and Gly-Trp-Met-Asp-Phe-NH_2 (CCK-5) as the reference substances for the following enzymatic synthesis of the C-terminal pentapeptides. Other Phac-protected peptides will be prepared according to the carbodiimide method.

(4) Enzymatic synthesis of the C-terminal pentapeptides

The pentapeptides will be enzymatically synthesized stepwise from the N-terminus (N→C strategy). Different synthetic routes will be tested. 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 investigated.

(5) Synthesis of the octapeptide (Phac-Asp(OMe)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-NH_2)

This objective will emphasize on the fragment condensation between the N-terminal tripeptide and the C-terminal pentapeptide.

3 Results and discussion

3.1 Enzyme immobilization

3.1.1 Immobilization of the enzymes

In this work, immobilized papain, immobilized α-chymotrypsin and immobilized thermolysin on different carriers were prepared as follows.

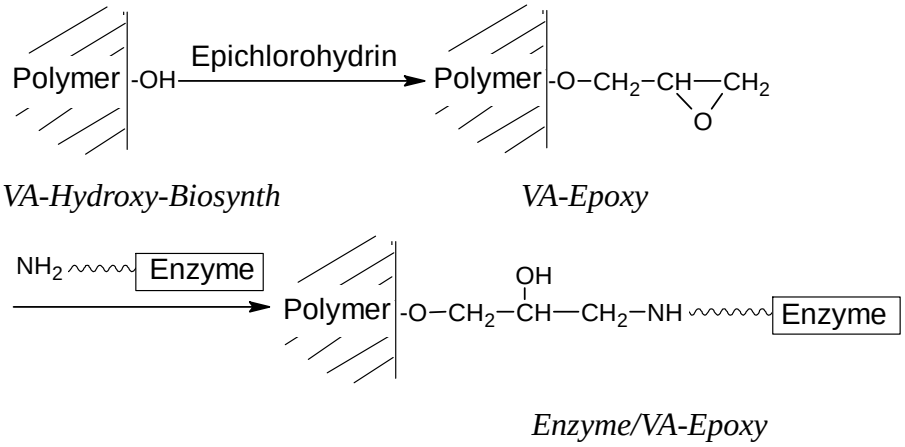
（1）Depositing on Celite-545 or Bio-Gel P-2

The enzyme is deposited on the solid support via physical effect.

(2) Covalent binding to carrier

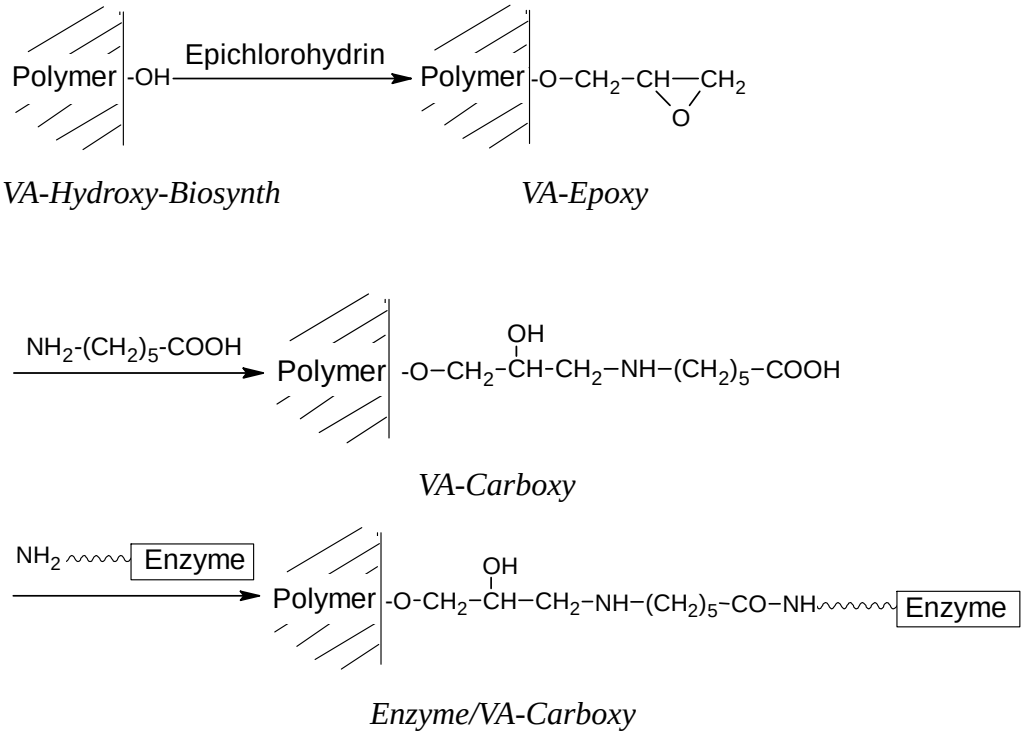
Derivatization of the VA-Hydroxy-Biosynth: The polymer VA-Hydroxy-Biosynth is a vinyl acetate resin containing hydroxyl groups. By refluxing the VA-Hydroxy-Biosynth with epichlorohydrin (Burg et al., 1988), the epoxidized polymer (VA-Epoxy) is formed (see Scheme 3.1). The Epoxy-polymer reacted with equal amounts (by weight) of 6-aminohexanoic acid (at pH 10 for 48 h), the carboxy-polymer (VA-Carboxy) was obtained (Eckstein, 1991) (see Scheme 3.2).

Covalent binding to VA-Epoxy: The amino group of enzyme attacks the epoxy group of VA-Epoxy and forms covalent binding to the polymer (Scheme 3.1).



Scheme 3.1 Epoxidation of VA-Hydroxy-Biosynth and immobilization of the enzyme.

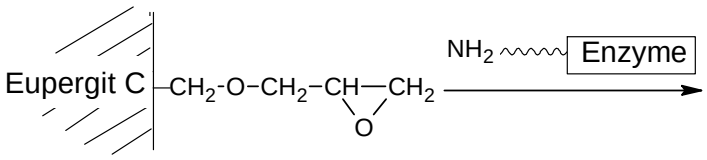
Covalent binding to VA-Carboxy: The amino group of enzyme attacks the carboxyl group of VA-Carboxy and forms covalent binding to the polymer (Scheme 3.2).



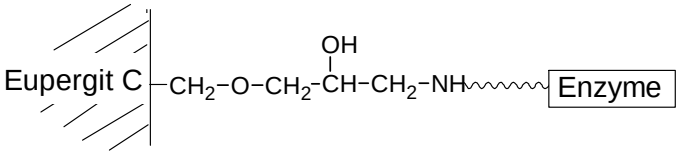
Scheme 3.2 Derivatization of the VA-Hydroxy-Biosynth and immobilization of the enzyme.

Covalent binding to Eupergit C: Eupergit C is an epoxydised acryl resin, which is copolymerized from methacrylamide, N, N'–methylen-bis- (methacrylamide) and /or allylglycidylether. The epoxy group as a functional group of the polymer can react

with the amino group of enzyme and immobilize the enzyme via covalent binding (Scheme 3.3).



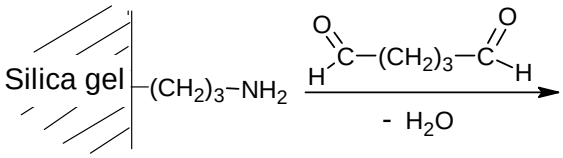
Eupergit C



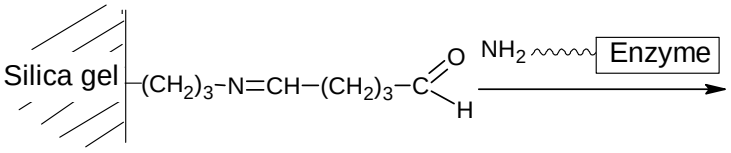
Enzyme/Eupergit C

Scheme 3.3 Immobilization of the enzyme on Eupergit C via covalent binding

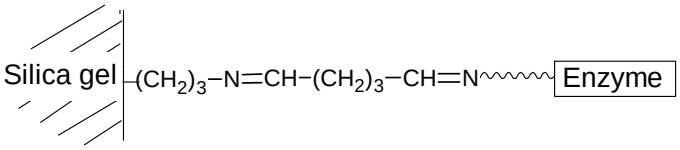
Covalent binding to Polygosil60-10NH₂: Polygosil60-10NH_2 is an aminosilica gel. This modification of the carrier with glutardialdehyde was carried out according to Cramer and Horvath (1989). The enzyme is immobilized via an amino group on the activated carrier as a Schiff's base (Scheme 3.4).



Polygosil60-10NH_2



Modified Polygosil60-10NH_2



Enzyme/modified Polygosil60-10NH_2

Scheme 3.4 Modification of Polygosil60-10NH_2 with glutardialdehyde and immobilization of enzyme.

3.1.2 Activity test of the immobilized enzymes

The activities of immobilized papain, immobilized α-chymotrypsin and immobilized thermolysin on different carriers have been investigated.

(1) Activity test of the papain-immobilisates

Using the above mentioned methods, papain was immobilized on VA-Epoxy (papain/VA-Epoxy), VA-Carboxy (papain/VA-Carboxy) and Celite-545 (papain/Celite-545) respectively. The activity of immobilized papain is tested according to Koennecke et al. (1984) with Bz-Arg-OEt as substrate. The conversion of Bz-Arg-OEt to Bz-Arg-OH was determined with HPLC.

As shown in Figure 3.1 the highest activity is observed in the case of papain/VA-Epoxy, which promises a great potential for papain-catalyzed peptide synthesis. Therefore, in this work papain/VA-Epoxy was chosen as the catalyst to synthesize Phac-Asp(OMe)-Tyr-OAl, Phac-Asp(OMe)-Tyr-OH, Phac-Asp-Tyr-Met-OAl, Phac-Gly-Trp-OMe, Phac-Gly-Trp-Met-Asp(OMe)-OMe and Phac-Gly-Trp-Met-Asp(OMe)-OH.

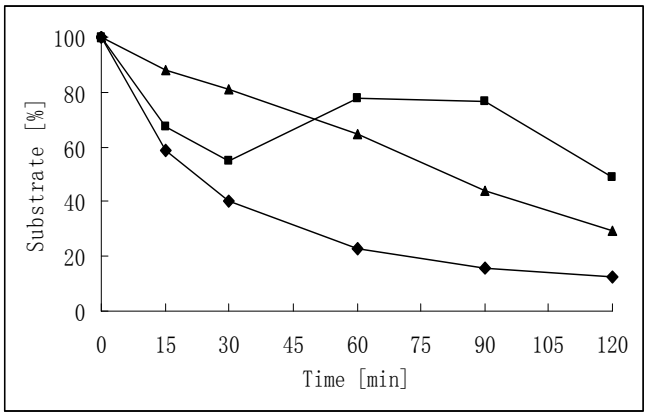


Figure 3.1 Hydrolysis of Bz-Arg-OEt with 50mg papain/VA-Epoxy (♦), papain/VA-Carboxy (■) or papain/Celite-545 (▲) in 0.5 ml 0.5 M KH_2PO_4 buffer (pH 6.0) with 0.1 mg EDTA and 0.01ml 2-mercaptoethanol.

Since papain is a thiolprotease and it looses its activity by oxidation, the peptide synthesis was performed in the presence of 2-mercaptoethanol, which can activate papain and maintain its activity in the presence of air. EDTA is used to complex any heavy metal ion, which could otherwise deactivate the –SH group of papain.

The stability of papain/VA-Epoxy was also tested after its multiple uses in synthesizing Phac-Gly-Trp-Met-Asp(OMe)-OH in aqueous media. The results of activity tests (Figure 3.2) show that the stability of the immobilized papain is reasonably high and after one application the activity almost retains. Even after the third appilication, the activity decreased only to a small extend. It has to be mentioned that after the second application, the activity test was carried out without preactivation of the immobilized papain. Hence, the activity decreased a lot. As a consequence, it is necessary to preactivate papain prior to reusage of immobilized papain for peptide synthesis (Eckstein et al. 1991). After the fourth application, the catalysis of papain/VA-Epoxy was no more efficient enough. In conclusion, this immobilisate could be used at least four times in a period of several months.

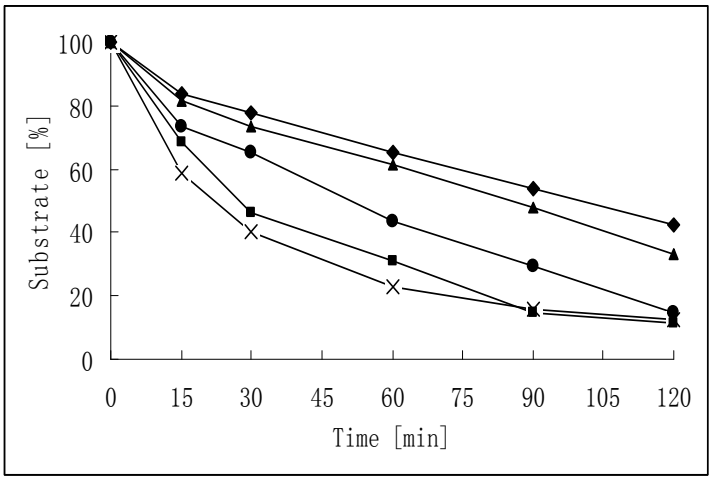


Figure 3.2 Hydrolysis of Bz-Arg-OEt with papain/VA-Epoxy. Activity directly after immobilization(×), after the first application (■), after the second application without preactivation(▲), after the third appilication (●) and after the fourth application (♦).

(2) Activity test of the α-chymotrypsin-immobilisates

The activity of α-chymotrypsin immobilized on Eupergit C (α-CHY/Eupergit C), on Celite-545 (α-CHY/Celite-545) and on Bio-Gel P-2 (α-CHY/P-2) was tested with Ac-Tyr-OEt as substrate, respectively. The conversion of Ac-Tyr-OEt to Ac-Tyr-OH was determined with HPLC.

As shown in Figure 3.3, the substrate Ac-Tyr-OEt was completely hydrolyzed to Ac-Tyr-OH with α-CHY/Celite-545 within 10 minutes, which shows the highest activity. The activity of α-CHY/Eupergit C was high enough to obtain around 80% yield of Ac-Tyr-OH within 10 minutes, though the compete hydrolysis of the substrate needed more than 2 hours. However, α-CHY/P-2 had no significant activity.

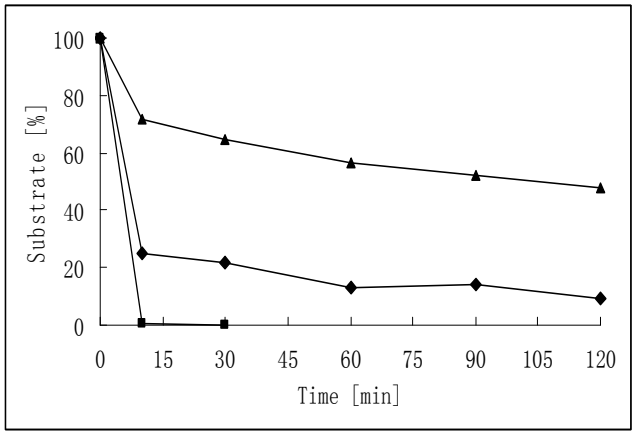


Figure 3.3 Hydrolysis of Ac-Tyr-OEt with 20mg α-CHY/Eupergit C (♦), α-CHY/Celite-545 (■) or α-CHY/P-2 (▲) in 0.5 ml 0.01 M HCl-Tris buffer (pH 8.1).

The stability of α-CHY/Eupergit C was compared to that of α-CHY/Celite-545 after its multiple uses in synthesizing Phac-Gly-Trp-Met-OEt in the solvent free system (Figure 3.4). The activity test results show that the stability of α-CHY/Eupergit C is reasonably high and after two applications no significant change in the activity was observed. However, α-CHY/Celite-545 lost its activity dramatically. One reason might be that the α-chymotrypsin is only deposited on Celite-545 and is adsorbed by weak physical effects. The enzyme can be desorbed in the aqueous solution during the

work-up procedure. Consequently, in point of economic view and keeping enzyme's contamination away from the product as well, α-CHY/Eupergit C is superior to α-CHY/Celite-545.

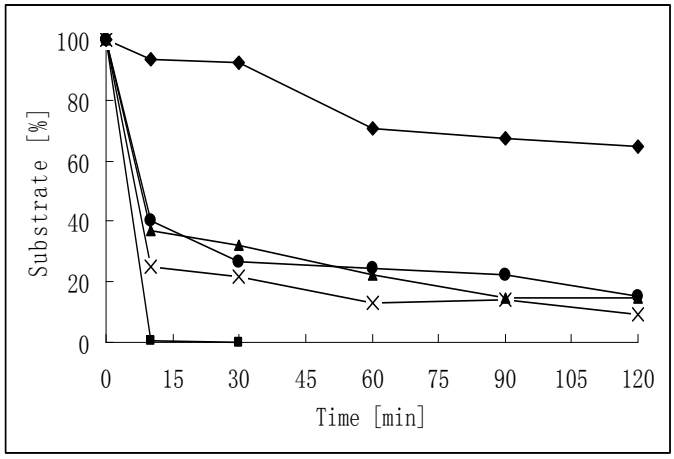


Figure 3.4 Hydrolysis of Ac-Tyr-OEt with immobilized α- chymotrypsin. Activity of α-CHY/Eupergit C directly after immobilization (×), after the first application (▲), after the second application (●); activity of α-CHY/Celite-545 directly after immobilization (■), after the first application (♦).

(3) Activity test of the thermolysin-immobilisates

Immobilization of thermolysin was investigated on different support materials, such as Eupergit C, Celite-545 and polygosil60-10NH_2. The activity test of immobilized thermolysin was carried out with Cbo-Asp-Phe-NH_2 as substrate. The conversion of Cbo-Asp-Phe-NH_2 to Cbo-Asp-OH and Phe-NH_2 was determined with HPLC.

In Figure 3.5 the activities of the thermolysin-immobilisates, thermolysin on Eupergit C (TLN/Eupergit C), on Celite-545 (TLN/Celite-545) and on modified polygosil60-10NH_2 (TLN/modified polygosil60-10NH_2) (42mg/g) are compared. TLN/Celite-545 has the highest activity with more than 80% substrate hydrolyzed within 15 minutes. According to a previous report (Eckstein et al., 1996), the activity of TLN/modified polygosil60-10NH_2 is higher than that of TLN/Eupergit C, if the activity test is carried out directly after immobilization. Here, the data shows that

TLN/polygosil60-10NH_2 after being stored at -20°C for 7 years still has the similar activity as the fresh immobilized thermolysin on Eupergit C. Besides, the activity of TLN/Eupergit C does not decrease after being stored at -20°C for 8 years. But the greatest drop in activity is observed after one application of TLN/Eupergit C in the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 in high concentration of acetonitrile with low water content. Therefore, it can be concluded that the immobilized thermolysin either on modified polygosil60-10NH_2 or on Eupergit C is definitely stable when stored at low temperature but is less stable when it is in contact with organic solvents as is the case of TLN/Eupergit C.

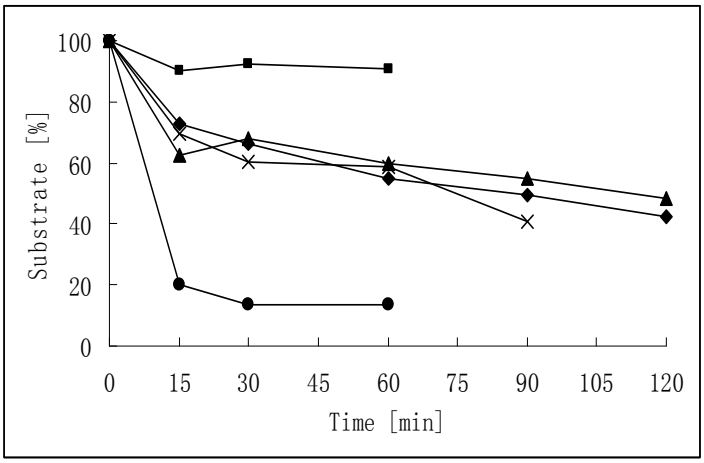


Figure 3.5 Hydrolysis of Cbo-Asp-Phe-NH_2 with 50 mg immobilized thermolysin in a0.5 ml 0.5 M HCl-Tris buffer (pH 8.5). Activity of TLN/Eupergit C directly after immobilization (♦), after the first application (■); activity of TLN/Eupergit C after being stored at -20°C for 8 years (▲); activity of TLN/modified polygosil60-10NH_2 after being stored at -20°C for 7 years (×); activity of TLN/Celite-545 directly after immobilization (●).

(4) Activity test of PGA/Eupergit C (Fluka)

Benzylpenicillin-Na 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.

The activity of the immobilized PGA was found effective as there was no starting material detected in HPLC after 30 min (Figure 3.6).

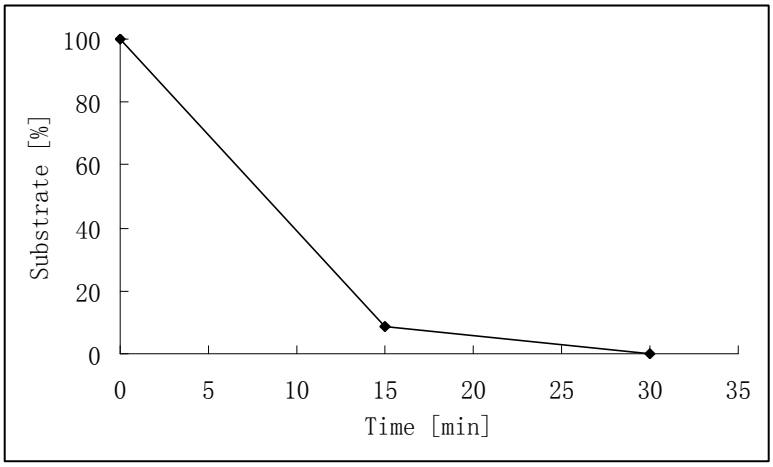


Figure 3.6 Hydrolysis of benzylpenicillin-Na with 50 mg PGA/Eupergit C in 0.5 ml 1 M KH_2PO_4 buffer (pH 7.5).

3.2 Enzymatic synthesis of the N-terminal tripeptide (Phac-Asp(OMe)-Tyr-Met-OAl)

For the synthesis of the N-terminal tripeptide fragment of CCK-8, there was little knowledge about the synthetic strategy and reaction conditions (Fite et al., 2002). Hence, in this work, two different pathways to synthesize the derivative with protease catalysts were investigated. The effect of the concentration and structure of substrate, reaction media, the enzyme and the carrier on the tripeptide derivative synthesis were studied.

Two strategies (Scheme 3.5; Scheme 3.6) were applied for synthesizing the tripetide fragment with stepwise chain-elongation from the N-terminal residue. Phac-amino acid or peptide esters were used as carboxyl components. The methionine derivative, Met-OAl, was chosen as nucleophile in the second enzymatic coupling, because the tripeptide fragment obtained can be used directly as acyl-donor in the next enzymatic coupling. Therefore, the tripeptide fragment can be easily integrated into the final assembly of the octapeptide (CCK_{26-33}).

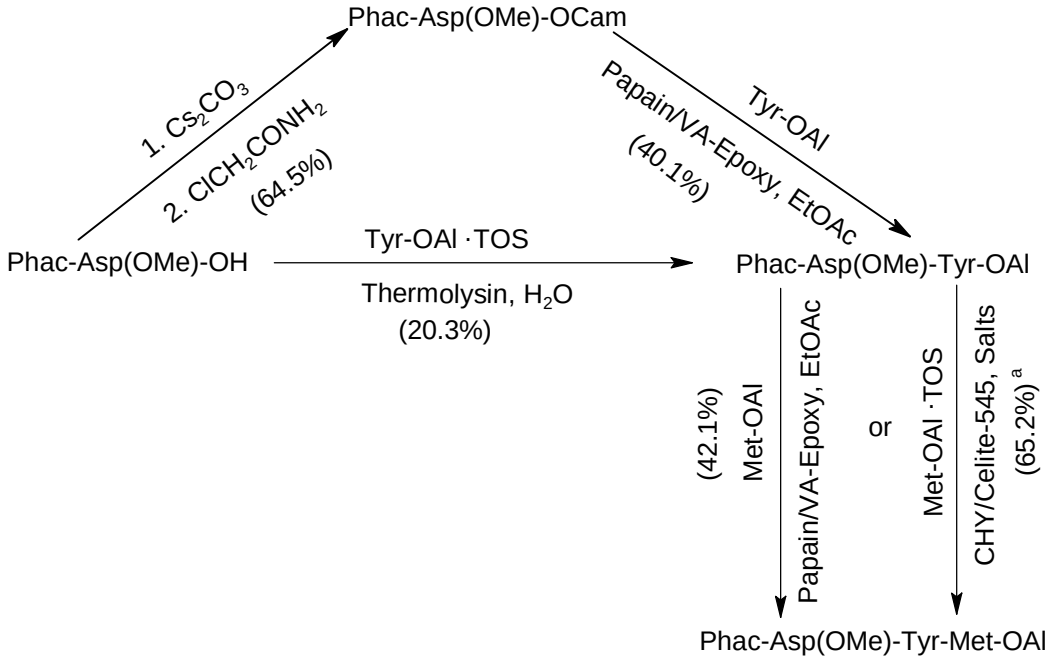
In the thermodynamic approach, the unfavourable equilibrium was shifted towards synthesis by the means of the highest possible concentrations of the educts, especially of the amino component. Beyond this, in aqueous system, the equilibrium could be also shifted towards synthesis by the precipitation of the product.

Under kinetic control several undesired side reactions can happen: the hydrolysis of the acyl enzyme, secondary hydrolysis of the formed peptide bond or other undesirable proteolytic cleavage of protease-labile bonds in the starting fragments and in the product, respectively. Therefore, the progress of the reaction had been controlled with RP-HPLC and stopped at the “kinetic” optimum. The above mentioned side reactions could be minimized or eliminated by optimising the leaving

group of the acyl donor ester, the enzyme or the medium (e.g. pH value, solvent). A solvent free system, where the necessary water is supplied by salt hydrates, was the preferred tool in optimising the reaction medium. Another option was carrying out the synthesis in organic solvents containing minimum amounts of water.

3.2.1 The first synthetic strategy

The first strategy to obtain the target N-terminal tripeptide fragment is outlined in Scheme 3.5. It includes two approaches for synthesizing Phac-Asp(OMe)-Tyr-OAl, which can couple with methionine allyl ester in two different reaction media under catalysis of different enzymes.



Scheme 3.5 The first strategy for synthesizing the N-terminal tripeptide fragment. The isolated yields of products are shown in parentheses.

[a] The yield was calculated after the unreacted educts were recycled for a repeated conversion.

3.2.1.1 Enzymatic synthesis of Phac-Asp(OMe)-Tyr-OAl

(1) Synthesis in low-water content system

Since Calvet et al. (1996) pointed out that papain was the best enzyme for the peptide bond formation of Asp-Tyr and generally N^{α}-Z protected amino acid Cam esters as acyl-donors gave the highest yields, Phac-Asp(OMe)-OCam was prepared from Phac-Asp(OMe)-OH following the procedure already described (Martinez et al., 1983). After 7 days the reaction was stopped as there was no substantial improvement in the conversion and afforded 64.5% isolated yield. The obtained 1.6 equivalents of active ester were coupled with 1 equivalent of Tyr-OAl in EtOAc containing 0.2% (v/v) 0.1 M borax buffer (pH 6.9) under the catalysis of papain/VA-Epoxy in a kinetic approach. The maximum HPLC yield (57.3%) of the dipeptide allyl ester was reached after 6 h and resulted in 40.1% isolated yield. However, the overall isolated yield of the dipeptide allyl ester was 25.9% caused by multiple synthetic steps. Oligomerization of the Tyr moiety occurred in this system, and Phac-Asp(OMe)-Tyr-Tyr-OAl was isolated as the main by-product in 9.0% yield.

(2) Synthesis in aqueous system

Phac-Asp(OMe)-OH as acyl-donor reacted with 2 equivalents of Tyr-OAl·TOS at 40^{o}C in water at pH 7 using thermolysin as catalyst in a thermodynamically-controlled peptide synthesis. The isolated yield of the dipeptide allyl ester was 20.3%. The result of the low yield is in accordance with the report (Isowa and Ichikawa, 1979) that the tyrosine residue was a poor acyl group acceptor in thermolysin-catalyzed synthesis due to the “aversion” of thermolysin for tyrosine residues in the P_1’ site.

In terms of the yield of Phac-Asp(OMe)-Tyr-OAl, both of the pathways were not efficient.

3.2.1.2 Enzymatic synthesis of Phac-Asp(OMe)-Tyr-Met-OAl

(1) Synthesis in low-water content system

Phac-Asp(OMe)-Tyr-OAl and 3 equivalents of Met-OAl were incubated with papain/VA-Epoxy in EtOAc containing 0.2% (v/v) 0.1 M borax buffer (pH 6.9). The kinetically controlled synthesis was monitored by RP-HPLC. The HPLC yield (43.1%) of the tripeptide allyl ester was reached after 3 h. Beyond this point, the product yield decreased because of the oligomerization of the Met moiety and hydrolysis of the product to Phac-Asp(OMe)-Tyr-Met-OH. After the work-up procedure, the isolated yield of the tripeptide fragment was 42.1%. The kinetic process of this synthesis is shown in Figure 3.7.

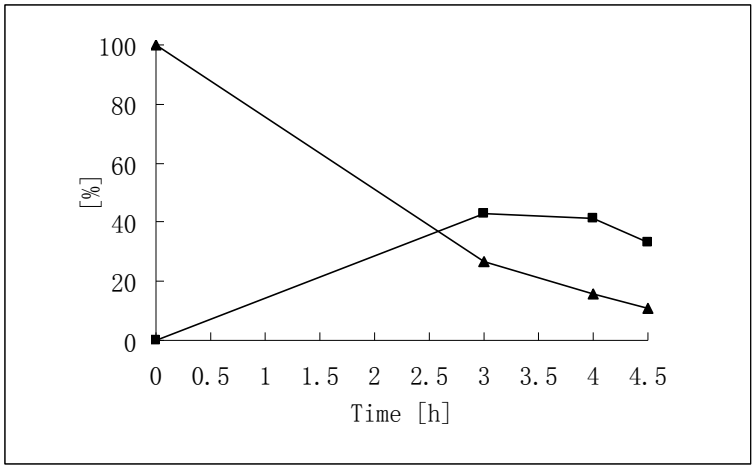


Figure 3.7 Kinetic process of synthesis of Phac-Asp(OMe)-Tyr-Met-OAl (■) from 0.25 mmol Phac-Asp(OMe)-Tyr-OAl (▲) and 0.75 mmol Met-OAl in 7.5 ml EtOAc containing 15 µl 0.1 M borax buffer (pH 6.9), 10 µl β-mercaptoethanol and 1 mg EDTA with 1.0 g papain/VA-Epoxy.

(2) Synthesis in solvent-free system

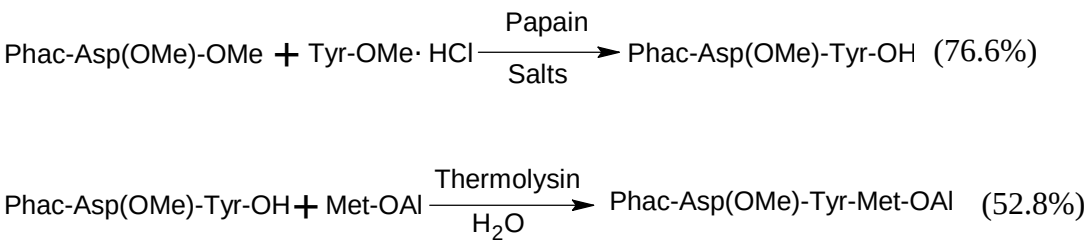
In 1992, Cerovsky (1992) reported a new method that protease-catalyzed peptide synthesis in the presence of $Na_2CO_3 \cdot 10\ H_2O$, the so-called solvent-free system, was completed within in a few hours giving satisfactory yields of the desired peptide products. Later Erbeldinger et al. (2000) applied basic salts, such as $KHCO_3$, $NaHCO_3$ and K_2CO_3 to the thermoase-catalyzed peptide synthesis. Our previous work (Lu et al.,

2004; Xiang et al., 2004) demonstrated that immobilized enzymes could also be applied in this system.

Here, the synthesis of Phac-Asp(OMe)-Tyr-Met-OAl was investigated in the solvent-free system. Phac-Asp(OMe)-Tyr-OAl reacted with 4 equivalents of Met-OAl · TOS in the presence of 2 equivalents of $KHCO_3$ and 1.6 equivalents of $Na_2CO_3 \cdot 10\ H_2O$ under the catalysis of α-CHY/Celite-545. Because of very limited water in this system, no hydrolysis by-product Phac-Asp(OMe)-Tyr-Met-OH was observed in the course of the reaction. After 5 h the reaction was stopped as there was no substantial improvement in the conversion. The isolated yield of Phac-Asp(OMe)-Tyr-Met-OAl was 42.1%. The unreacted starting material Phac-Asp(OMe)-Tyr-OAl was recycled for a repeated conversion. This methodology increased the yield to 65.2%.

3.2.2 The second synthetic strategy

Scheme 3.6 shows the the second synthetic strategy for synthesizing Phac-Asp(OMe)-Tyr-Met-OAl, where the intermediate Phac-Asp(OMe)-Tyr-OH obtained from Phac-Asp(OMe)-OMe and Tyr-OMe·HCl was an acyl-donor for the coupling of Met-OAl to afford the tripeptide fragment.



Scheme 3.6 The second strategy for synthesizing the N-terminal tripeptide fragment. The isolated yields of products are shown in parentheses.

3.2.2.1 Enzymatic synthesis of Phac-Asp(OMe)-Tyr-OH

This enzymatic coupling between Phac-Asp(OMe)-OMe and 1.5 equivalents of Tyr-OMe·HCl was performed in 20 equivalents of $KHCO_3$ and 1.5 equivalents of $Na_2CO_3 \cdot 10\ H_2O$ with free papain. In this reaction, free papain catalyzed not only the peptide bond formation but also the hydrolysis of Phac-Asp(OMe)-Tyr-OMe to the corresponding free C-terminal dipeptide simultaneously. The pure dipeptide fragment Phac-Asp(OMe)-Tyr-OH was obtained efficiently in 76.6% isolated yield.

3.2.2.2 Enzymatic synthesis of Phac-Asp(OMe)-Tyr-Met-OAl

Phac-Asp(OMe)-Tyr-OH and 2 equivalents of Met-OAl were suspended in a small amount of water. The minimal amount of ACN was used to achieve complete solution. The thermolysin-catalyzed synthesis was carried out at pH 7 in a water-bath at 40^{o}C. The poorly soluble product Phac-Asp(OMe)-Tyr-Met-OAl was formed in an isolated yield of 52.8% within half an hour. The product precipitated from the reaction system, which induced a favorable shift of the chemical equilibrium.

3.2.3 Influences on the enzymatic syntheses

Unfortunately there is no general protocol available for enzymatic peptide synthesis. In order to suppress the competing hydrolysis reaction, the undesired oligomerization and product cleavage, each coupling step has to be optimized. A series of reaction parameters were investigated to find the best conditions above mentioned for each peptide bond formation, such as optimal acyl donor, nucleophile, reaction media and enzyme.

(1) Influence of the concentration and structure of substrate

Substrate concentration. To shift the equilibrium of the enzyme catalyzed peptide bond formation, both is necessary, an excess of one reactant and the highest possible

concentration. In the cases of the synthesis of Phac-Asp(OMe)-Tyr-OH and Phac-Asp(OMe)-Tyr-Met-OAl, it is better to apply the easily available nucleophile (amino acid ester) in excess, which is more economic as well, because the carboxyl component inherits the danger of a possible "product inhibition" of the enzyme. But in the synthesis of Phac-Asp(OMe)-Tyr-OAl, an excess of Phac-Asp(OMe)-OCam with 1.6-fold molar ration to the nucleophile Tyr-OAl was applied to minimize the formation of the oligomer Phac-Asp(OMe)-Tyr-Tyr-OAl.

Structure of acyl-donor. Capellas (Capellas et al., 1996[a]) reported the reactivity difference among carboxamidomethyl (OCam), benzyl (OBzl) and methyl (OMe) esters of Z-Gly-Trp in coupling with Met-OEt in organic media. It was found that the methyl ester was the least reactive acyl-donor with the lowest yield of product but the highest yield of by-products obtained. In this study, it was also observed that Phac-Asp(OMe)-OMe could not be coupled with Tyr-OAl with immobilized papain in EtOAc. As expected, the OCam ester was necessary to obtain Phac-Asp(OMe)-Tyr-OAl in organic media. However, in the solvent free system, Phac-Asp(OMe)-OMe, the methyl ester was active enough to be coupled with Tyr-OMe·HCl and Phac-Asp(OMe)-Tyr-OH was isolated in a good yield (76.6%). With this strategy not only two reaction steps for synthesizing the Cam ester, but also a possible racemization during this activation could be avoided.

In the synthesis of the dipeptide Phac-Asp(OMe)-Tyr-OAl, the Cam ester can be avoided, if Phac-Asp(OMe)-OH or Phac-Asp(OH)-OH could couple with Tyr-OAl under the catalysis of thermolysin. The condensation between Phac-Asp(OH)-OH with the free β-carboxy group and Tyr-OAl·TOS with thermolysin was tried, but no coupling could be observed neither in aqueous nor in organic media at pH 7.0. By way of contrast, as mentioned earlier, the condensation between Phac-Asp(OMe)-OH, where the β-carboxy group is protected with a methyl group, and Tyr-OAl·TOS or Tyr-OAl could be achieved in water at pH 7.0 with thermolysin. The isolated yield of Phac-Asp(OMe)-Tyr-OAl was 20.3%.

As acyl-donor to the tripeptide Phac-Asp(OMe)-Tyr-Met-OAl both dipeptides Phac-Asp(OMe)-Tyr-OAl and Phac-Asp(OMe)-Tyr-OH were good and the yields of both reactions in different reaction systems with different enzymes were the same (42%). The yield increased to 65.2% with further conversion of the recycled unreated educts.

Structure of nucleophile. In organic media, for Phac-Asp(OMe)-OCam, both Tyr-OMe and Tyr-OAl were good nucleophiles for the corresponding synthesis of Phac-Asp(OMe)-Tyr-OMe and Phac-Asp(OMe)-Tyr-OAl in EtOAc containing low-water content with papain/VA-Epoxy. The HPLC yields of the reactions were above 25%. However, generally, the selection of the C-α terminal protecting group was made taking into account its reactivity as well as the possibility that the product of one reaction will be the acyl-donor for the next one. As mentioned above, it has been already demonstrated by Capellas (Capellas et al., 1996[a]) that the methyl ester was the least reactive acyl-donor for the next coupling step. Consequently, for the strategy 1, Tyr-OAl was chosen as the nucleophile to afford Phac-Asp(OMe)-Tyr-OAl (see Scheme 3.5).

In the solvent free system, however, in contrast to Tyr-OAl, Tyr-OMe was chosen as the nucleophile for the synthesis of Phac-Asp(OMe)-Tyr-OH. Tyr-OMe·HCl is much cheaper than Tyr-OAl·TOS and it also can be easily synthesized. More important is that the end product of the coupling reaction is the free carboxyl group, which is necessary for the next coupling step with thermolysin (see Scheme 3.6).

The coupling reaction between Phac-Asp(OMe)-Tyr-OAl and Met-OEt, Met-OBzl or Met-OAl in the low water content system (EtOAc containing small amounts of buffer) with papain/VA-Epoxy was investigated. It was found that the product yields (HPLC yields 55.2%, 29.5%, 64.4%) were obtained after 3-4 h. In the solvent free system, using Met-OEt as the nucleophile, the HPLC yield was 11.0%, while using Met-OAl, it could reach 46.3%. In both systems the highest active nucleophile was Met-OAl.

(2) Influence of the reaction media

Solvent plays an important role in enzymatic peptide synthesis in organic media (Dordick, 1989; Cassells and Halling, 1989). Previous fundamental studies by Kise 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.

However, there were no coupling reaction between Phac-Asp(OMe)-OH and Tyr-OAl·TOS with thermolysin/Celite 545 in acetonitrile with low buffer content. It succeeded with free thermolysin in water and Phac-Asp(OMe)-Tyr-OAl could be obtained in an isolated yield of 20.3%. The condensation between Phac-Asp(OMe)-OCam and Tyr-OAl with papain/VA-Epoxy in acetonitrile with low buffer content, the HPLC yield of Phac-Asp(OMe)-Tyr-OAl was only 14.9%, while in ethyl acetate with low buffer content, the HPLC yield reached 57.3% with an isolated yield of 40.1%.

The coupling between Phac-Asp(OMe)-OCam and Tyr-OAl in EtOAc was investigated in 0.1 M borax buffers with three different pH values, 5.9, 6.9 and 8.2, respectively. As shown in Table 3.1, at the highest pH (pH 8.2) the reaction rate was the fastest and the HPLC yield of over-reacted byproduct was the highest, which was opposite to the case with the lowest pH (pH 5.9). The highest dipeptide allyl ester, the desired product, was obtained at pH 6.9. Interestingly, when this coupling was carried out at the optimal pH, namely, pH 6.9, the isolated yield of the dipeptide allyl ester also changed with the content of ACN in the reaction media: 33.8% (without ACN), 40.1% (0.8% ACN) and 26.1% (7.7% ACN). The explanation therefore is that a small amount of ACN is needed to increase the solubility of reactants but a higher concentration of ACN denatures papain.

Table 3.1 pH effect on the synthesis of Phac-Asp(OMe)-Tyr-OAl from Phac-Asp(OMe)-OCam and Tyr-OAl [a].

pH	Time [h]	Components [%][b]	
		Product	By-product
5.9	168	24.3	7.3
6.9	6	57.3	9.4
8.2	3	30.1	27.1

[a] Reactions conditions: EtOAc, containing 0.2% (v:v) 0.1 M borax buffer, 0.1% (v/v) β-mercaptoethanol and EDTA, 41.1 mM Tyr-OAl, 65.8mM Phac-Asp(OMe)-OCam, papain/VA-Epoxy.
[b] The concentrations at the "kinetic" optimum were determined by HPLC; product = Phac-Asp(OMe)-Tyr-OAl, by-product = Phac-Asp(OMe)-Tyr-Tyr-OAl

In the tripeptide synthesis, the HPLC yields of Phac-Asp(OMe)-Tyr-Met-OAl from Phac-Asp(OMe)-Tyr-OAl and Met-OAl in buffered ethyl acetate with papain/VA-Epoxy were slightly different with the pH value of the 0.1 M borax buffer: 67.5% at pH 5.9, 64.4% at pH 6.9 and 62.9% at pH 8.0.

(3) Influence of the enzyme and the carrier

Free papain and papain/VA-Epoxy, containing the same amount of papain, were applied for the coupling of Phac-Asp(OMe)-OMe and Tyr-OMe·HCl in the solvent free system. With papain/VA-Epoxy, only Phac-Asp(OMe)-Tyr-OMe and over-reacted esters were formed. With free papain, however, Phac-Asp(OMe)-Tyr-OH was the main product, which can be used directly in the following coupling step. Using immobilized papain, the resulting ester has to be hydrolyzed in a second step.

After having investigated many parameters in the different pathways for synthesizing the tripeptide fragment, the best option is the one outlined in Scheme 3.6. In respect to the easily available Tyr-OMe·HCl, this strategy is more economic and the overall yield is 45%. This is much higher than the overall yield of strategy 1 of ca. 17%.

3.3 Chemical synthesis of reference substances

Reference substances are useful for the identification of the product peak during the monitoring of enzymatic reactions by RP-HPLC.

Since it is difficult to selectively protect the phenolic hydroxyl group of the Tyr residue in the chemical synthesis of the aforementioned N-terminal tripeptide Phac-Asp(OMe)-Tyr-Met-OAl, in this work the chemical synthesis of the tripeptide Phac-Asp(OMe)-Tyr-Met-OAl as reference substance was not carried out. However, all fragments as reference substances for monitoring enzymatic synthesis of the C-terminal pentapeptides were obtained in chemical way and described as follows.

3.3.1 Chemical synthesis of Phac-peptides

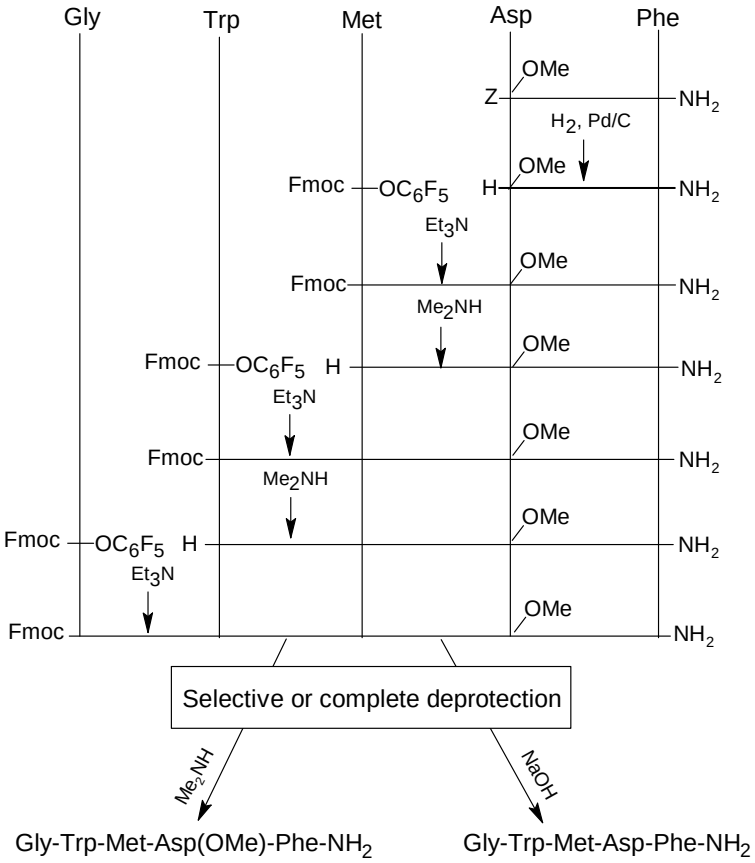
The chemical synthesis of peptides should be carried out from the C-terminus of the peptide chain. Here the needed reference Phac-peptides had been synthesized via the N→C strategy, because all necessary amino acid derivatives had been already available in the laboratory. A possible racemization and formation of diketopiperazines, however, did not influence their use as reference substances.

Equimolar amounts of HOBt and of the free C-terminus N^{α}-Phac carboxyl component were mixed with a small excess of DCC. After the activation phase, DCU was filtered off and the amino component 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 peptide bond. Phac-Gly-Trp-OMe, Phac-Gly-Trp-Met-OEt, and Phac-Gly-Trp-Met-Asp(OMe)-OMe were prepared as reference substances successively in this method using Trp-OMe·HCl, Met-OEt·HCl and Asp(OMe)-OMe·HCl as the amino component, respectively. But the HOBt/DCC method was a failure in synthesizing Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2. This

reference substance was successfully synthesized from Phac-Gly-Trp-Met-Asp(OMe)-OH and 5 equivalents of Phe-NH_2·HCl in DMF with a small excess of DCC as coupling reagent, which was added in two portions. All the reference substances of the Phac-peptide derivatives were characterized by FABMS.

3.3.2 Chemical synthesis of the C-terminal pentapeptides

The chemical synthesis of the C-terminal pentapeptide fragments was carried out in solution 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 pentafluorophenyl esters as activation of the carboxyl group. The active ester method of chemical synthesis of the C-terminal pentapeptide fragments via the C→N strategy is presented in Scheme 3.7.



Scheme 3.7 The chemical synthesis of the C-terminal pentapeptide fragments**.**

3.3.2.1 Chemical synthesis of Fmoc-amino acides pentafluorophenyl esters

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

3.3.2.2 Chemical 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 90.6% 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.

3.3.2.3 Deprotection of Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-NH_2

The partially protected pentapeptide Gly-Trp-Met-Asp(OMe)-Phe-NH_2 was obtained by cleaving the Fmoc-group with 10% dimethylamine in DMF with the isolated yield of 50.0%.

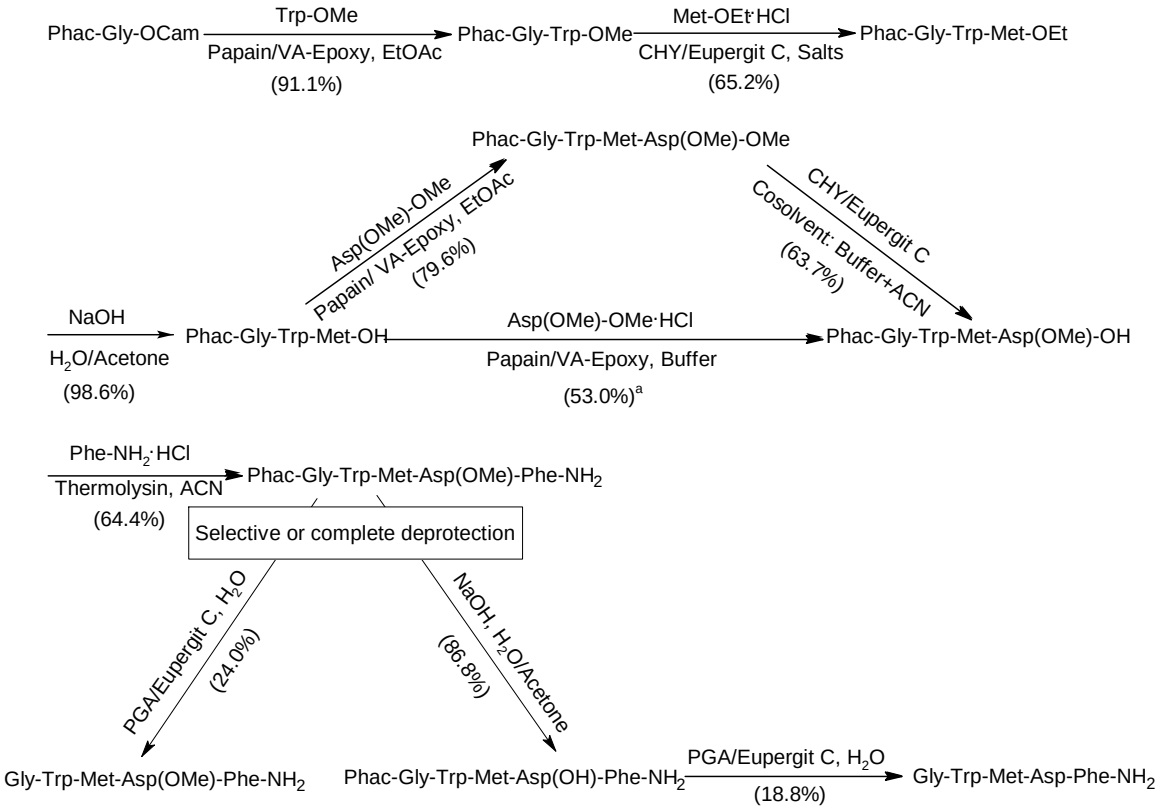
The fully deprotected pentapeptide Gly-Trp-Met-Asp(OH)-Phe-NH_2 was achieved by hydrolysis with 5% NaOH in H_2O/actone (v/v 1:1) with the isolated yield of 25.3%.

3.4 Enzymatic synthesis of the C-terminal pentapeptides

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 that 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 from Phac-Gly-Trp-Met-OH to Phac-Gly-Trp-Met-OCam and another one for the catalytic hydrogenation of Cbo-Asp(OMe)-Phe-NH_2 to obtain H-Asp(OMe)-Phe-NH_2.

In order to optimize the strategy further by avoiding the OCam ester at the tripeptide fragment and the catalytic hydrogenation of the Cbo-Asp(OMe)-Phe-NH_2, in this work the stepwise elongating the peptide chain of the pentapeptide fragments from the amino to the carboxyl end by using immobilized enzymes except thermolysin for the formation of all peptide bonds is reported. Phac-Gly-OCam, Phac-Gly-Trp-OMe as carboxyl components and Trp-OMe, Met-OEt·HCl as the corresponding amino components were coupled in EtOAc with limited water content and solvent free conditions via the kinetic approach, respectively. Phac-Gly-Trp-Met-Asp(OMe)-OMe, Phac-Gly-Trp-Met- Asp(OMe)-OH and Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 were achieved via thermodynamically-controlled coupling in EtOAc with limited water content, buffer and water system using Asp(OMe)-OMe, Asp(OMe)-OMe·HCl and Phe-NH_2·HCl as nucleophiles, respectively. Considering the total enzymatic synthesis of CCK-8, the C-terminal pentapeptides Gly-Trp-Met-Asp(OMe)-Phe-NH_2 and Gly-Trp-Met-Asp-Phe-NH_2 (CCK-5) can be chosen to couple with the N-terminal tripeptide, respectively. Therefore, the obtained Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 could be selectively deprotected by cleaving the Phac-group with immobilized PGA to give Gly-Trp-Met-Asp(OMe)-Phe-NH_2. In addition, it also could be fully

deprotected by alkaline hydrolysis of the β-methyl ester of the aspartic acid residue and cleavage of the Phac-group with immobilized PGA to yield the free CCK-5. The synthetic steps are outlined in Scheme 3.8.



Scheme 3.8 The enzymatic synthesis of the C-terminal pentapeptides via the stepwise N→C strategy. The isolated yields of products are shown in parentheses.
[a] The yield was calculated after the unreacted educts were recycled for a repeated conversion.

To achieve a high yield, the carboxy protected amino acid derivative was applied in excess for the individual elongation step. This is important, because a too high concentration of the carboxy fragment could lead to product inhibition. In order to suppress the competing hydrolysis reactions, the undesired over-reactions and product cleavage, a series of reaction parameters were investigated to obtain the following optimized condition for each peptide bond formation, such as optimal acyl donor, nucleophile, reaction media and enzyme.

3.4.1 Enzymatic synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OH

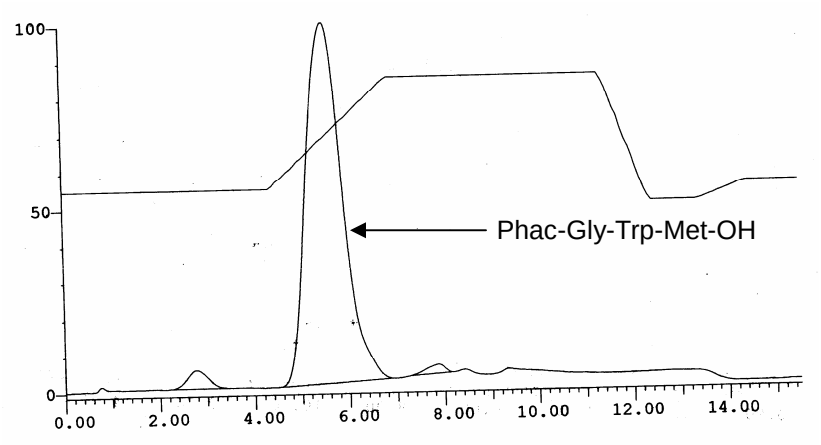
The synthesis of the dipeptide Phac-Gly-Trp-OMe was carried out in a low-water content system. Phac-Gly-OCam and 1.5 equivalents of Trp-OMe were incubated in EtOAc containing 5% (v/v) buffer with papain/VA-Epoxy. After 3 h the reaction was complete and the HPLC yield of Phac-Gly-Trp-OMe was 97% and the isolated yield was 88.4%.

The dipeptide was converted to the tripeptide Phac-Gly-Trp-Met-OEt in the solvent-free system. The coupling of Phac-Gly-Trp-OMe and 4 equivalents of Met-OEt·HCl was performed in the presence of 20 equivalents of $KHCO_3$ and 4 equivalents of $Na_2CO_3 \cdot 10\ H_2O$ with α-CHY/Eupergit C. As could be seen in HPLC, the starting material Phac-Gly-Trp-OMe almost vanished after 2 h giving the desired tripeptide (70.6% HPLC yield), the by-product Phac-Gly-Trp-OH due to the hydrolysis of the starting material (24.7% HPLC yield), the by-product Phac-Gly-Trp-Met-Met-OEt due to oligomerization (3.2 % HPLC yield) and the tripeptide with the free carboxy group Phac-Gly-Trp-Met-OH (1.5 % HPLC yield). The isolated yield of Phac-Gly-Trp-Met-OEt was 65.2%

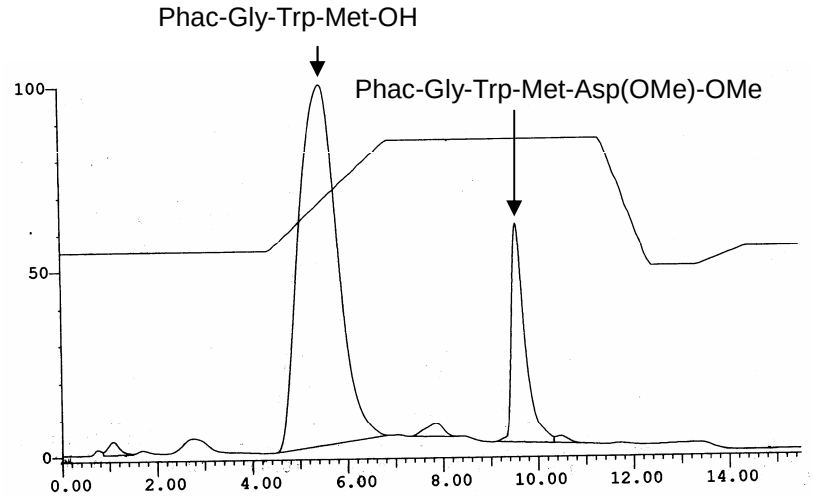
For the extension of the tripeptide to the tetrapeptide Phac-Gly-Trp-Met-Asp(OMe)-OH, the free carboxy group of the acyl-donor was necessary. Quite in contrast to the general knowledge, the alkylester even the OCam ester is here not a useful substrate for papain. Therefore, the Phac-tripeptide ester was hydrolyzed with NaOH.

The tetrapeptide Phac-Gly-Trp-Met-Asp(OMe)-OH was achieved by two approaches. In the first approach, Phac-Gly-Trp-Met-Asp(OMe)-OH was obtained in two steps (Meng et al., 2006). In the first step, Phac-Gly-Trp-Met-OH and 1.7 equivalents of Asp(OMe)-OMe were coupled under thermodynamic control in ethyl acetate with a low water content (2.0% by volume) and papain/VA Epoxy. After 75 h, the high

conversion yield of 89.1% was observed in RP-HPLC (Figure 3.8). The isolated yield was 79.6%.



(a)



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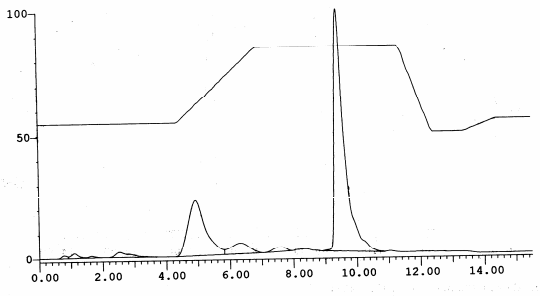
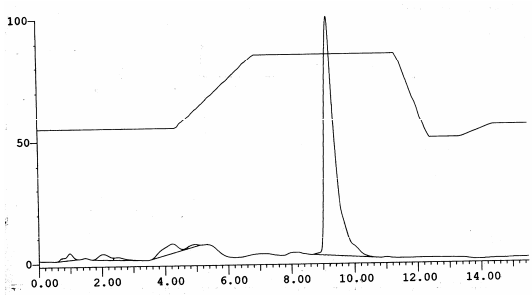


(c)



(d)

Figure 3.8 HPLC-chromatograms (HPLC system 6) of the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OMe from Phac-Gly-Trp-Met-OH and Asp(OMe)-OMe in EtOAc containing 2% buffer with papain/VA-Epoxy after the reaction time of 0 h (a), 24 h (b), 50 h (c) and 75 h (d).

In the second step, the tetrapeptide ester had to be converted to the tetrapeptide carboxylic acid. In this case, the hydrolysis could not be performed by alkali, because the β-ester of the aspartic acid residue would have been removed simultaneously. Therefore the hydrolysis was conducted with α-chymotrypsin. It has been known since 1948 that in particular α-chymotrypsin, in addition to its proteolytic potential, is capable of cleaving ester linkages (Kaufman et al., 1948; Schwert et al., 1948). In the case of aspartic- and glutamic-acid diesters, only the α-carboxyl esters were removed (Kloss and Schroeder, 1964). Here, the α-chymotrypsin-mediated removal of the α-methyl ester from the C-terminal aspartic acid residue of Phac-tetrapeptide dimethyl ester was performed in a cosolvent system containing HCl-Tris buffer and acetonitrile (27:12 v/v) at pH 7.0. The addition of acetonitrile improved the solubility of the Phac-tetrapeptide dimethyl ester. After 32 h, the selective hydrolysis was completed (Figure 3.9). The potentially α-chymotrypsin-labile, Trp-Met bond was split in around 10%. The isolated yield of Phac-Gly-Trp-Met-Asp(OMe)-OH was 63.7%.

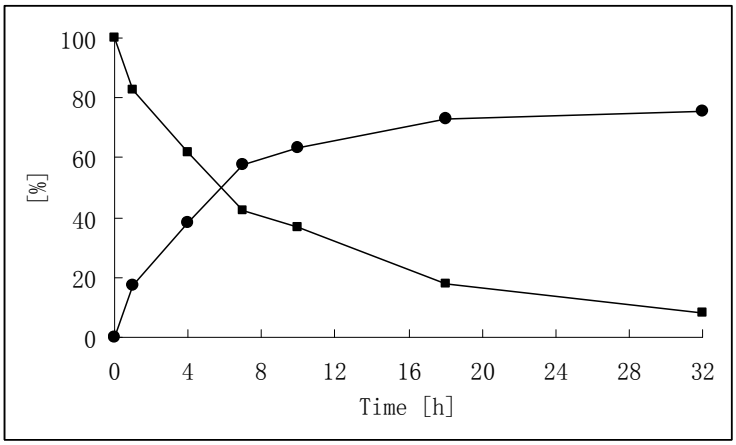
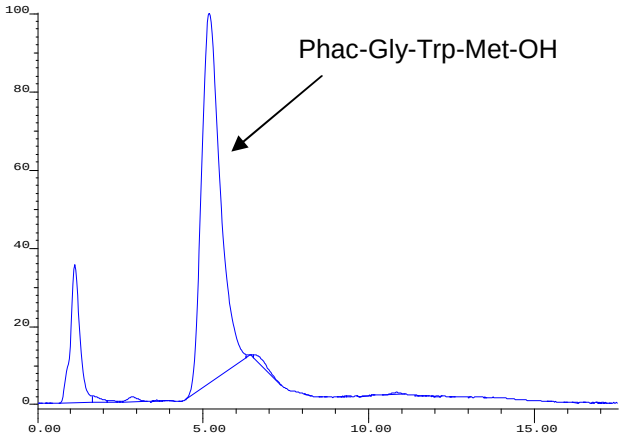
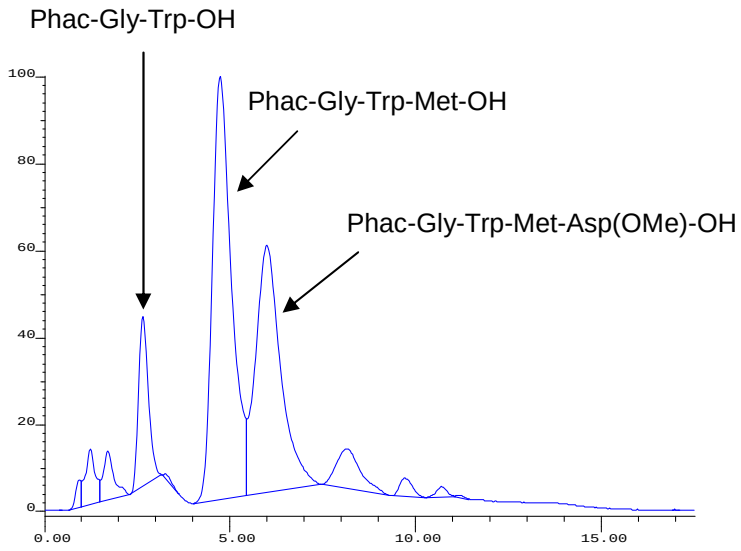


Figure 3.9 Kinetic process of the hydrolysis of Phac-Gly-Trp-Met-Asp(OMe)-OMe (■) to Phac-Gly-Trp-Met-Asp(OMe)-OH (●) in HCl-Tris and ACN (27:12, v/v) with α-CHY/Eupergit C at pH 7.0.

The second approach commenced with Phac-Gly-Trp-Met-OH and 5 equivalents of Asp(OMe)-OMe·HCl at pH 5.9 in buffer at 37°C with papain. Phac-Gly-Trp-Met-Asp(OMe)-OH could be obtained in only one step taking advantage of the proteolytic and hydrolytic potential of papain. As shown in the comparison of the chromatograms (Figure 3.10), the starting material decreased and the product peak increased, simultaneously the peak of Phac-Gly-Trp-OH increased. After some time the product peak did not increase anymore, despite the fact that there was still a considerable amount of the starting material present. At this point the reaction was stopped and the starting material and product were isolated. The recovered starting material could be used for another conversion. In doing this the yield could be increased from 37.4% to 53.0%.



(a)



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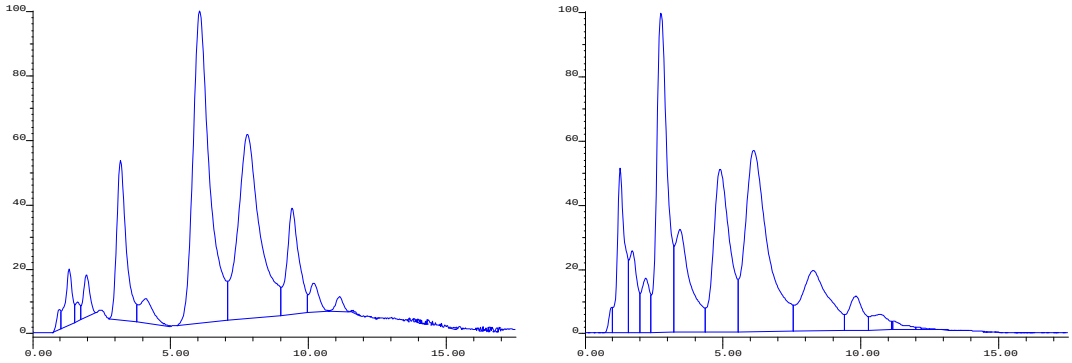


(c) (d)

Figure 3.10 HPLC-chromatograms (HPLC system 6) of the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OH from Phac-Gly-Trp-Met-OH and Asp(OMe)-OMe·HCl in 0.2 M KH_2PO_4 buffer with papain/VA-Epoxy at pH 5.9 and 37°C after the reaction time of 0 h (a), 4 h (b), 7 h (c) and 28 h (d).

3.4.2 Enzymatic synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2

Two solvent systems were investigated for the synthesis of the pentapeptide, in aqueous solution at room temperature and in a low-water content system at 40°C.

In water at pH 7.0 and room temperature, the condensation between Phac-Gly-Trp-Met-Asp(OMe)-OH and 5 equivalents of Phe-NH_2·HCl was performed with thermolysin in a thermodynamically controlled reaction. It was observed that the Trp-Met bond in the starting fragment and in the product was thermolysin-labile, because it was cleaved by thermolysin into Phac-Gly-Trp-OH, which was converted to the by-product Phac-Gly-Trp-Phe-NH_2 (Figure 3.11; Figure 3.12). The isolated yield of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 was 45.2%.

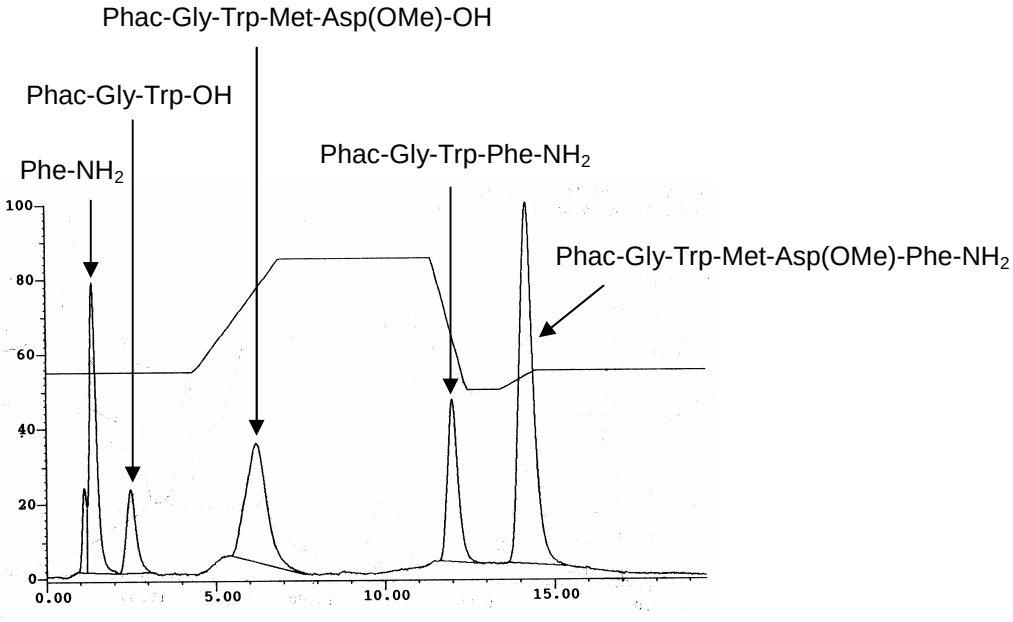


Figure 3.11 HPLC-chromatogram (HPLC system 6) of the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 from Phac-Gly-Trp-Met-Asp(OMe)-OH and Phe-NH_2·HCl in H_2O with thermolysin at pH 7.0 at room temperature after the reaction time of 8.5 h.

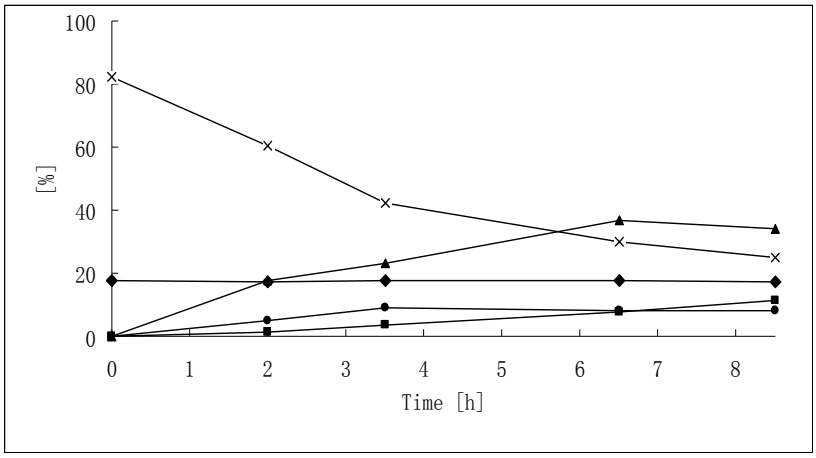


Figure 3.12 Kinetic process of the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 (▲) and the corresponding by-product Phac-Gly-Trp-OH (●), Phac-Gly-Trp-Phe-NH_2 (■) from 0.2 mmol Phac-Gly-Trp-Met-Asp(OMe)-OH (×) and 1.0 mmol Phe-NH_2·HCl (♦) in 1.1 ml H_2O with 300 µg thermolysin at pH 7.0 at room temperature.

The condensation between PhAc-Gly-Trp-Met-Asp(OMe)-OH and 5 equivalents of Phe-NH_2·HCl could be also performed in ACN containing 4% HCl-Tris buffer (v/v) at pH 7.0 and 40°C with thermolysin. Because of the high temperature and good acivity

of thermolysin at 40°C, the cleaved product Phac-Gly-Trp-OH was immediately converted to the by-product Phac-Gly-Trp-Phe-NH_2. Therefore the concentration of Phac-Gly-Trp-OH was too low to be detected in HPLC. The kinetic process is shown in Figure 3.13. The isolated yield of Phac-Gly-Trp-Met-Asp(OMe)- Phe-NH_2 was 64.4%.

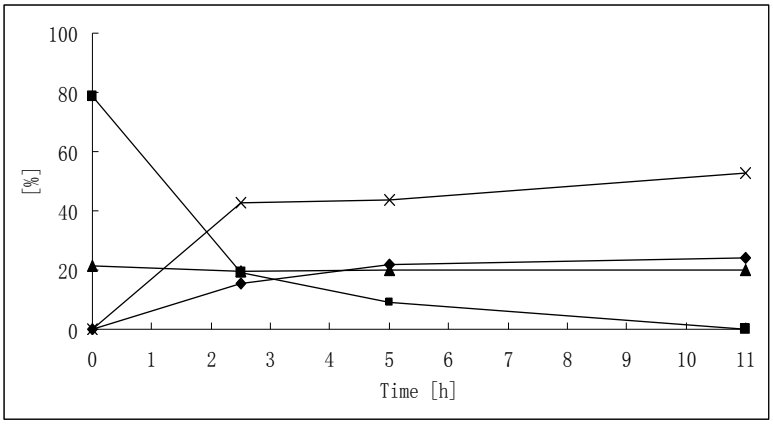


Figure 3.13 Kinetic process of the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 (×) and the corresponding by-product Phac-Gly-Trp-Phe-NH_2 (♦) from 0.16 mmol Phac-Gly-Trp-Met-Asp(OMe)-OH (■) and 0.78 mmol Phe-NH_2·HCl (▲) in 4.2 ml ACN containing 4% (v/v) 0.05M HCl-Tris buffer with 4 mg thermolysin at pH 7.0 at 40°C.

3.4.3 Deprotection of the Phac-pentapeptide

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 split 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 with NaOH in acetone/water. After acidification and washing, Phac-Gly-Trp-Met-Asp(OH)-Phe-NH_2 (Phac-CCK-5) could be obtained in the isolated yield of 86.8%. In the second step, the removal of the Phac-group from

Phac-CCK-5 with PGA/Eupergit C was carried out in water at pH 7.6 and 35°C with PGA/Eupergit C. After 24 h, CCK-5 was obtained in the isolated yield of 18.8%, which is lower than the yield of 57.4% previously reported by our group (Xiang et al. 2004). Probably the case was caused by the different batches of PGA/Eupergit C.

The selective deprotection of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 was conducted in one step to remove the Phac-group. The cleavage of the Phac-group from Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 in the cosolvent system with PA/Eupergit C or PGA/Eupergit C was investigated as follows.

Due to the poor solubility of the starting material Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2, a mixture of EtOH/ACN (1:1 v/v) containing 0.1 M Na_2HPO_4 buffer (pH 8.0) was applied with PA/Eupergit C at 35°C. After 25 h, the HPLC showed that the β-methyl ester of Asp residue was hydrolyzed but no cleavage of Phac-group was observed. However, using PGA/Eupergit C instead of PA/Eupergit C in 20% EtOH in 1 M KH_2PO_4 buffer (pH 7.5), the removal of Phac-group from Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 was successful (Figure 3.14). The isolated yield of Gly-Trp-Met-Asp(OMe)-Phe-NH_2 was 24.0%. Therefore, PGA was used to remove the Phac-group.

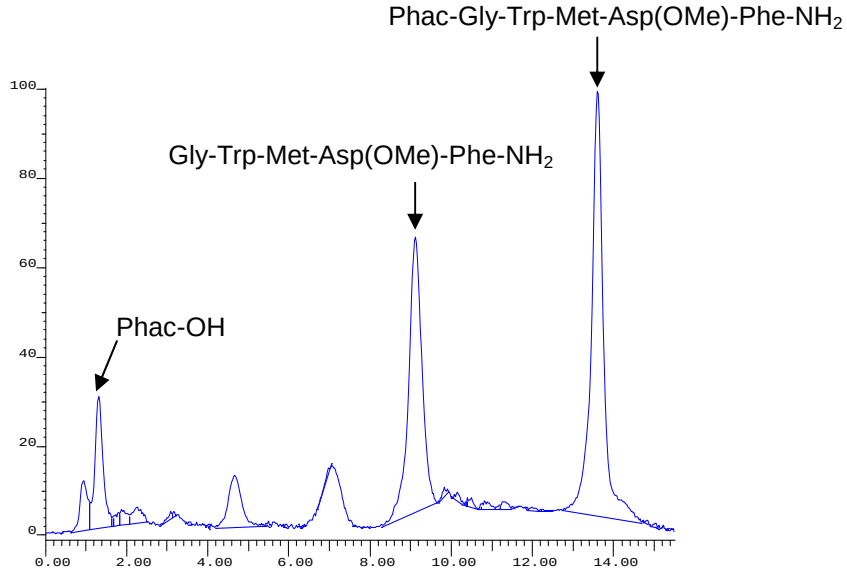


Figure 3.14 HPLC-chromatogram (HPLC system 6) of the selective deprotection of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 to Gly-Trp-Met-Asp(OMe)-Phe-NH_2 and Phac-OH with PGA/Eupergit C in 20% EtOH in 1 M KH_2PO_4 buffer (pH 7.5) at 35°C after the reaction time of 24 h.

3.4.4 Influences on the enzymatic syntheses

The reaction conditions above mentioned were the result of optimization studies regarding the enzymatic activity and yield. Valuable information was provided by systematic studies on every coupling step. The following variables had been modified for these enzymatic couplings: reaction media and temperature, substrate concentration and structure, type of protease and support for enzyme immobilization. Their effects on the condensation reactions are given below.

(1) Influence of substrate structure and concentration

Structure of acyl-donor. The chemical nature of the ester moiety of the acyl-donor has a strong influence on enzyme activity and product yield in kinetically controlled synthesis (Schellenberger et al., 1991). When the reactivity of the enzyme towards the acyl-donor is high, the formation of by-products is greatly minimized during the reaction. Furthermore, the structure of the ester may influence the relative rates of hydrolysis and aminolysis of the acyl-enzyme complex.

In organic media, it was reported that carboxamidomethyl ester (Cam) always gave the best results and methyl and ethyl esters had the lowest reactivity (Capellas et al, 1996; 1997). In this work, in spite of the low reactivity of methyl and ethyl esters, they were the first choice in practical, due to their availability. Only Phac-Gly-OMe could not be coupled with Trp-OMe with immobilized papain as catalyst. As expected, the OCam ester was necessary and the dipeptide was isolated in good yield (91.1%).

In the solvent-free system, the methyl and ethyl ester of the dipeptide were active enough to be coupled with Met-OEt·HCl. There was no different reactivity between the two dipeptide esters and both afforded the similar isolated yield of the tripeptide

methyl ester. This tripeptide ester obtained via the catalysis of α-CHY/Celite 545 could be isolated in 71.4% yield. With this strategy three reaction steps could be avoided in comparison to the published method by Capellas et al. (1996).

From kinetics of papain-catalyzed peptide synthesis, esterified acyl donors should be preferable to acyl donors having a free α-carboxyl group (Oka and Morihara, 1978). However, Phac-Gly-Trp-Met-OEt could not be coupled with Asp(OMe)-OMe·HCl or Asp(OMe)-OH·HCl in buffer system, cosolvent system and solvent-free system, respectively, neither with papain nor α-chymotrypsin. There is also no coupling between Phac-Gly-Trp-Met-OAl or Phac-Gly-Trp-Met-OCam and Asp(OMe)-OMe in EtOAc containing 2% buffer. On the contrary, Phac-Gly-Trp-Met-OH could be easily coupled with Asp(OMe)-OMe·HCl under the catalysis of papain either in buffer to yield Phac-Gly-Trp-Met-Asp(OMe)-OH (53.0% isolated yield) or in EtOAc containing 2% buffer to yield Phac-Gly-Trp-Met-Asp(OMe)-OMe (79.6% isolated yield).

Two acyl-donors, Phac-Gly-Trp-Met-Asp(OMe)-OH with β-carboxyl group protected as β-methyl ester and Phac-Gly-Trp-Met-Asp(OH)-OH with free β-carboxyl group were used to couple with Phe-NH_2·HCl in water with thermolysin, respectively. In the former coupling reaction, Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 was obtained in an isolated yield of 51.0%. However, if the acyl donor was Phac-Gly-Trp-Met-Asp(OH)-OH with the free β-carboxyl group, the product Phac-Gly-Trp-Met-Asp(OH)-Phe-NH_2 was less than 6% HPLC yield even when it reacted with a 8-fold excess of Phe-NH_2·HCl. Simultaneously, the amounts of the by-products, Phac-Gly-Trp-OH and Phac-Gly-Trp-Phe-NH_2 increased to more than 33%.

Structure of nucleophile. The C-α group of the nucleophile is also of great importance for the enzymatic synthesis of oligopeptides. In the present work, Trp-OMe was a good nucleophile for the synthesis of Phac-Gly-Trp-OMe with a very good isolated yield of 91.1%, catalyzed by papain/VA-Epoxy. And the product Phac-Gly-Trp-OMe can be used directly without any further chemical modification in the next enzymatic step.

Met-OEt·HCl was a better nucleophile than Met-OAl·TOS to attack the carboxyl component Phac-Gly-Trp-OMe. In the α-CHY/Celite 545 catalyzed peptide bond formation, PhAc-Gly-Trp-Met-OEt was obtained in a good isolated yield of 71.4% within 2 hours and the by-product Phac-Gly-Trp-$(Met)_n$-OEt, n=2, could be kept below 5%. Under the same reaction condition, Phac-Gly-Trp-Met-OAl was obtained in an isolated yield of 58.5%.

The coupling of Phac-Gly-Trp-Met-OH with different nucleophiles was investigated in 0.2 M KH_2PO_4 buffer with free papain at 37°C and pH 5.9. Using Asp(OEt)-OEt·HCl as the nucleophile, Phac-Gly-Trp-Met-Asp(OEt)-OH was obtained in HPLC yield of 21.1%, while using Asp(OMe)-OMe·HCl as the nucleophile, Phac-Gly-Trp-Met-Asp(OMe)-OH was obtained in HPLC yield of 46.1%. Therefore, the OMe ester was used for protecting the Asp residues.

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

The hydrolysis of Phac-Gly-Trp-Met-Asp(OMe)-OMe with α-CHY/Eupergit C to obtain Phac-Gly-Trp-Met-Asp(OMe)-OH could be improved from 52.2% to 63.7% (isolated yield) with lowering the concentration of Phac-tetrapeptide dimethyl ester from 76.9 mM to 25.6 mM.

(2) Influence of the reaction media and temperature

The coupling reaction between Phac-Gly-OCam (10 mmolar) and Trp-OMe (15 mmolar), catalyzed by papain/VA-Epoxy, was investigated in both solvents, acetonitrile and ethyl acetate. In this coupling step, ethyl acetate was superior (HPLC yield 97.0%) to acetonitrile (HPLC yield 67.0%) (Xiang et al., 2004).

The condensation reaction between Phac-Gly-Trp-OMe and Met-OEt·HCl was studied in organic solvent systems with α-CHY/Celite 545. In this case no

tripeptide was found in either acetonitrile or ethyl acetate. However, using solvent free conditions, peptide bond formation was observed. Different inorganic salt systems with crystal water were investigated to optimize the pH of the reaction. Three systems of inorganic salts were studied and the results are shown in Figure 3.15. The lowest conversion rate, 23.9%, was observed with the mixture of sodium sulfate and sodium carbonate decahydrate. A better yield, 40.2%, could be obtained with sodium carbonate and its decahydrate. With 96.0% HPLC yield the system potassium hydrogen carbonate / sodium carbonate decahydrate was clearly superior. Applying the last salt system with a 4 fold excess of the nucleophile Met-OEt the tripeptide ethyl ester was obtained in an isolated yield of 71.4%. At a higher excess of the nucleophile the Met-Met formation increased considerably.

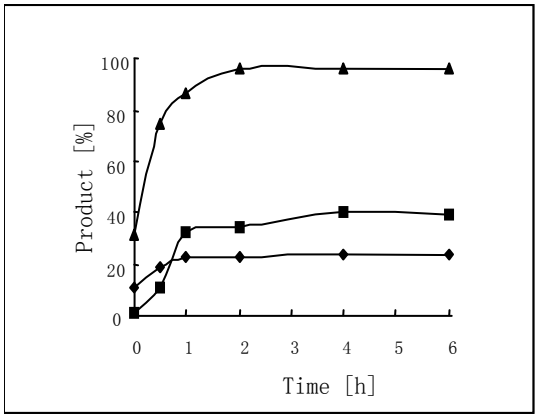


Figure 3.15 Effect of different inorganic salt systems $Na_2SO_4/Na_2CO_3 \cdot 10H_2O$ (♦), $Na_2CO_3/Na_2CO_3 \cdot 10H_2O$ (■), $KHCO_3/Na_2CO_3 \cdot 10H_2O$ (▲) in the synthesis of Phac-Gly-Trp-Met-OEt (Xiang and Eckstein, 2005).

The synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OMe, via the catalysis of papain/VA-Epoxy in the low-water content system, was studied in EtOAc or ACN as the organic media. The better isolated yield (66.3%) was obtained in EtOAc system than that (21.7%) in the ACN system, where more hydrolysis product Phac-Gly-Trp-Met-Asp(OMe)-OH was observed in the HPLC chromatogram.

In the biocatalytic hydrolysis of Phac-Gly-Trp-Met-Asp(OMe)-OMe, the pH value of the reaction media affected the reaction rate as well as the yield (Figure 3.16). At the

optimal pH value of 7, the hydrolysis with free α-chymotrypsin had a maximum HPLC yield of 52.0%.

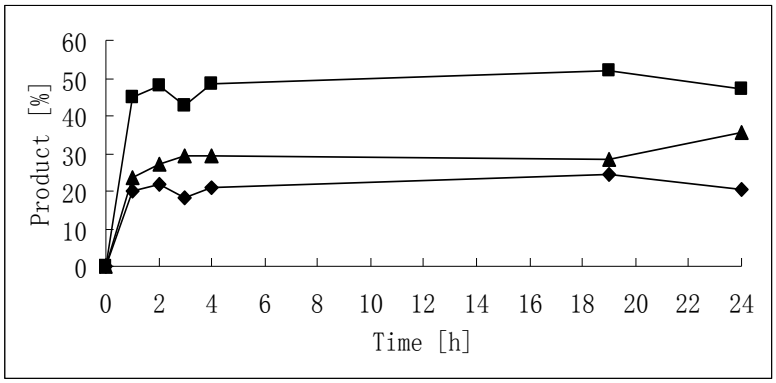


Figure 3.16 Effect of the pH on the hydrolysis of 0.05 mmol Phac-Gly-Trp-Met-Asp(OMe)-OMe in 0.2 ml ACN, 0.45 ml 0.05M HCl-Tris buffer and 3 µl Et_3N with 10 mg free α-chymotrypsin (pH 5.6 (●), pH 7.0 (■), pH 8.0 (▲)).

For the one step synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OH, the reaction of Phac-Gly-Trp-Met-OH and Asp(OMe)-OMe·HCl was investigated at different pH values, reaction media and temperatures. As shown in Figure 3.17, the optimal pH value range in the aqueous phase is 5.5 to 6.0 with the HPLC yield (33%) of Phac-Gly-Trp-Met-Asp(OMe)-OH.

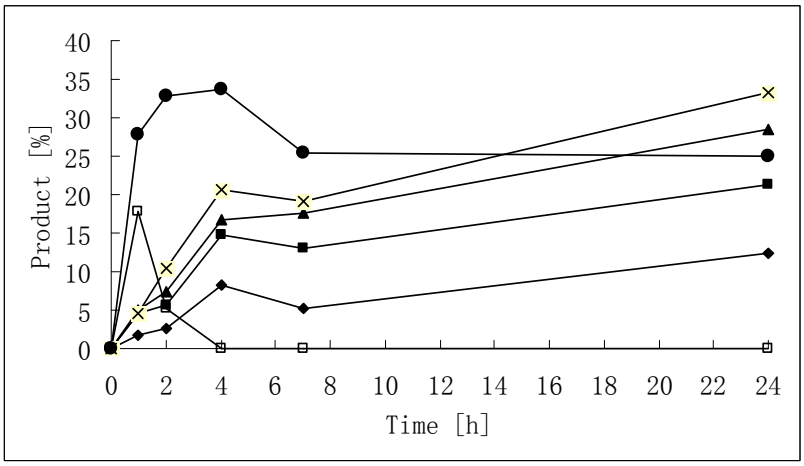


Figure 3.17 Effect of the pH on the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OH from 0.6 mmol Phac-Gly-Trp-Met-OH and 3 mmol Asp(OMe)-OMe·HCl in 10 ml 0.2M KH_2PO_4 buffer at room temperature with 30 mg free papain (pH 5.0 (□), pH 5.5 (●),pH 6.0 (×), pH 6.5 (▲), pH 7.0 (■), pH 7.5 (♦)).

At the optimal pH different systems were applied for this reaction of Phac-Gly-Trp-Met-OH and Asp(OMe)-OMe·HCl at room temperature. The results are shown in Table 3.2. In the solvent-free system, there was no reaction. In the biphasic system, EtOAc and buffer (1:1 or 1:2.3, v/v), the reaction was not efficient. Comparing the yields in the cosolvent system to those in the aqueous system, the highest yield (38.6%) was obtained in the phosphate buffer with the highest possible concentration of educts.

Table 3.2 Effect of the reaction system on the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OH from 0.2 mmol Phac-Gly-Trp-Met-OH and 1.0 mmol Asp(OMe)-OMe·HCl [a].

Reaction System	Reaction Media	Time	HPLC Yield
Solvent-free system	0.4 mmol $KHCO_3$ + 0.8 mmol $Na_2CO_3 \cdot 10H_2O$	3 d	0.0%
Biphasic system	0.75 ml EtOAc + 0.75 ml 0.2M KH_2PO_4	5 d	12.7%
	0.45 ml EtOAc + 1.05 ml 0.2M KH_2PO_4	5 d	11.3%
Cosolvent system	0.75 ml ACN + 0.75 ml 0.2M KH_2PO_4	5 d	4.7%
	0.9 ml ACN + 2.1 ml 0.2M KH_2PO_4	3 d	28.9%
Aqueous system	3 ml 0.2 M KH_2PO_4	22.5 h	24.8%
	3 ml 0.2 M HCl-Tris	22.5 h	25.8%
	1.5 ml 0.2 M KH_2PO_4	8 h	38.6%
	1.5 ml 0.2 M HCl-Tris	8 h	28.1%

[a] Reactions were carried out at room temperature in the presence of 12 µl β-mercaptoethanol and 1 mg EDTA. In the two-phase system, co-solvent system and aqueous system, the pH of the reaction was 5.9 and 200 mg of papain/VA-Epoxy were used. In the solvent free system, 8 mg of free papain were used.

Therefore these conditions with the highest possible concentration of educts in phosphate buffer at pH 5.9 were used to investigate further the influence of the reaction temperature on the reaction of Phac-Gly-Trp-Met-OH and Asp(OMe)-OMe·HCl. As shown in Table 3.3, increasing the temperature, the reaction

rate increased as well as the side reaction. The yield could not be improved that way. The highest yield (38.6%) was obtained at room temperature, however, the reaction time 8 hours was the longest.

Table 3.3 Effect of the reaction temperature on the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OH from 0.2 mmol Phac-Gly-Trp-Met-OH and 1.0 mmol Asp(OMe)-OMe·HCl [a].

Temperature	Time	Components [b] [%]		
[°C]	[h]	Product	By-product	Unreacted Substrate
25	8	38.6	9.3	37.9
30	6	28.1	13.0	44.5
37	4.3	34.5	20.8	41.9

[a] Reactions were carried out in the presence of 1.5 ml 0.2 M KH_2PO_4, 12 µl β-mercaptoethanol, 1 mg EDTA at pH 5.9 and 200 mg papain/VA-Epoxy.
[b] The concentrations at the optimum were determined by HPLC; product = Phac-Gly-Trp-Met-Asp(OMe)-OH, by-product = Phac-Gly-Trp-OH, unreacted substrate = Phac-Gly-Trp-Met-OH.

For synthesizing Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2, the condensation of Phac-Gly-Trp-Met-Asp(OMe)-OH with Phe-NH_2·HCl was investigated in three different reaction systems. In the solvent free system, the condensation reaction failed completely. After 22.5 h, the product HPLC yield was only 1.7% with 27.9% HPLC yield of Phac-Gly-Trp-OH, 9.9% HPLC yield of Phac-Gly-Trp- Phe-NH_2 and 39.5% HPLC yield of the unreacted starting material Phac-Gly-Trp-Met-Asp(OMe)-OH. However, in water at reaction pH 7.0, Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 could be obtained in an isolated yield of 45.2%. In the low-water content system, ACN containing 4% (v/v) 0.05M HCl-Tris buffer at pH 7.0, Phac-Gly-Trp-Met-Asp(OMe)-OH could condense with Phe-NH_2·HCl to give the product Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 in the HPLC yield from 24.9% to 52.5% depended on the reaction termperature.

The effect of the reaction temperature on the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 in the low-water content system are shown in Table 3.4. Increasing the reaction temperation from 25°C to 40°C, the reaction rate of coupling Phac-Gly-Trp-Met-Asp(OMe)-OH with Phe-NH_2·HCl increased very much and the product yield could be improved. However, at 50°C, the reaction rate slowed down probably due to the fact that thermolysin lost some activity at such a high temperature and the optimal product yield was the lowest. Hence, this condensation should be carried out in the low-water content system at an optimal temperature of 40°C.

Table 3.4 Effect of the reaction temperature on the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 [a]. The concentrations at the optimum are shown in the second line.

Temperature	Time [h]	Components[b] [%]			
		Product	By-product 1	By-product 2	Unreacted Substrate
25 °C	2.5	16.6	17.6	11.3	31.6
	24	32.5	2.5	42.1	0
30 °C	2.5	33.5	1.6	22.9	19.2
	4	42.8	0	34.4	0
40 °C	2.5	42.7	0	15.4	19.1
	11	52.5	0	24.3	0
50 °C	2.5	21.3	14.6	12.5	28.8
	7	24.9	14.1	17.3	20.9

[a] Reactions were carried out at pH 7.0 with 4 mg thermolysin, 0.78 mmol Phe-NH_2·HCl and 0.156 mmol Phac-Gly-Trp-Met-Asp(OMe)-OH in 4.032 ml ACN containing 0.168 ml 0.05 M HCl-Tris buffer.

[b] The concentrations were determined by HPLC; product = Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2, by-product 1 = Phac-Gly-Trp-OH, by-product 2 = Phac-Gly-Trp-Phe-NH_2.

(3) Influence of enzyme and carrier

The character of the enzyme, the chemical nature of the carrier and its interaction with enzyme and /or substrate play an important role in the yield.

Two α-chymotrypsin immobilisates, α-CHY/Celite 545 and α- CHY/Eupergit C, were used for the coupling of Phac-Gly-Trp-OMe with Met-OEt·HCl in a solvent free system respectively and the corresponding isolated yields of Phac-Gly-Trp-Met-OEt were 71.4% and 65.2%. If using Met-OAl·TOS as the nucleophile instead of Met-OEt·HCl in this coupling, the corresponding isolated yield of Phac-Gly-Trp-Met-OAl was 58.5% and 42.0%. Despite of the lower yield in the case of Eupergit C, this immobilisate was preferred, since the enzyme is linked covalently in contrast to celite, where the enzyme is only deposited and could be desorbed and contaminate the final peptide.

In the enzymatic hydrolysis of Phac-Gly-Trp-Met-Asp(OMe)-OMe, papain could cleave the α-methyl ester at pH 6.0 in 0.2 M KH_2PO_4 buffer to 36.2% HPLC yield after 4 h. In the cosolvent system, three different types of α-chymotrypsin, free α-chymotrypsin, α-CHY/Celite-545 and α-CHY/Eupergit C were tested in this selective hydrolysis. As shown in Table 3.5, α-CHY/Eupergit C has the advantage over free α-chymotrypsin and α-CHY/Celite-545 that it simplifies the purification procedure, because of the lower amount of the by-product Phac-Gly-Trp-OH.

Table 3.5 Effect of the type of α-chymotrypsin on the hydrolysis of 0.05 mmol Phac-Gly-Trp-Met-Asp(OMe)-OMe [a]. The concentrations at the optimum are shown in the second line.

Catalyst	Time	Components[b] [%]		
	[h]	Product	By-product	Unreacted Substrate
10 mg α-CHY	1	44.3	14.1	39.8
	5	44.7	15.4	37.7
100 mg α-CHY/Celite	5	44.8	16.9	36.4
545	1	45.8	14.2	39.9
100 mg	1	45.7	0	49.4
α-CHY/Eupergit C	5	49.2	2.8	43.0

[a] Reactions were carried out at pH 7.0 in 0.6 ml ACN and 1.35 ml 0.05 M HCl-Tris buffer (pH 9.0) containing 10 µl Et_3N.
[b] The concentrations were determined by HPLC; product = Phac-Gly-Trp-Met-Asp(OMe)-OH, by-product = Phac-Gly-Trp-OH, unreacted substrate = Phac-Gly-Trp-Met-Asp(OMe)-OMe.

However α-chymotrypsin is not suitable in the second approach for synthesizing Phac-Gly-Trp-Met-Asp(OMe)-OH in one step from Phac-Gly-Trp-Met-OH and Asp(OMe)-OMe·HCl since there was no coupling was observed in phosphate buffer at reaction pH 7.3. But the cleavage of the chymotrypsin-labile bond, Trp-Met to the by-product Phac-Gly-Trp-OH was obtained.

The condensation of Phac-Gly-Trp-Met-Asp(OMe)-OH and Phe-NH_2·HCl was studied with free thermolysin, TLN/Eupergit C and TLN/modified Polygosil60-10NH_2 in ACN with low-water content. The results are shown in Table 3.6. The highest HPLC yield of the pentapetide was observed in the reaction catalyzed by free thermolysin. TLN/Eupergit C was not efficient for catalyzing the coupling with around 25% yield of product and the highest yield of by-products. By increasing the amount of TLN/Eupergit C, the initial coupling rate was increased but without influence on the product yield. The catalytic ability of TLN/modified Polygosil60-

$10NH_2$ was the lowest, even though the activity of TLN/ modified Polygosil60-$10NH_2$ was similar to that of TLN/Eupergit C as shown in Figure 3.5. Consquently, free thermolysin was the optimal enzyme for this coupling in organic media.

Table 3.6 Effect of the type of thermolysin on the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 in the low-water content system.[a] The concentrations at the optimum are shown in the second line.

Catalyst	Time [h]	HPLC Yield [%]			
		Product	By-product 1	By-product 2	Unreacted Substrate
4 mg TLN	2.5	42.7	0	15.4	19.1
	11	52.5	0	24.3	0
150 mg	25	16.9	22.0	8.8	33.5
TLN/Eupergit C	48	24.8	22.4	16.3	17.3
1 g TLN/Eupergit C	3.5	16.1	9.9	0	53.7
	47	25.8	29.3	9.4	14.9
1 g TLN/modified	7	2.8	0	0	77.7
Polygosil60-$10NH_2$	22.5	5.0	0	0	75.3

[a] Reactions were carried out at pH 7.0 and 40°C in 4.032 ml ACN and 0.168 ml 0.05 M HCl-Tris buffer (pH 8.5) with 0.78 mmol Phe-NH_2·HCl 0.16 mmol Phac-Gly-Trp-Met-Asp(OMe)-OH.

[b] The concentrations were determined by HPLC; product = Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2, by-product 1 = Phac-Gly-Trp-OH, by-product 2 = Phac-Gly-Trp-Phe-NH_2, unreacted substrate = Phac-Gly-Trp-Met-Asp(OMe)-OH.

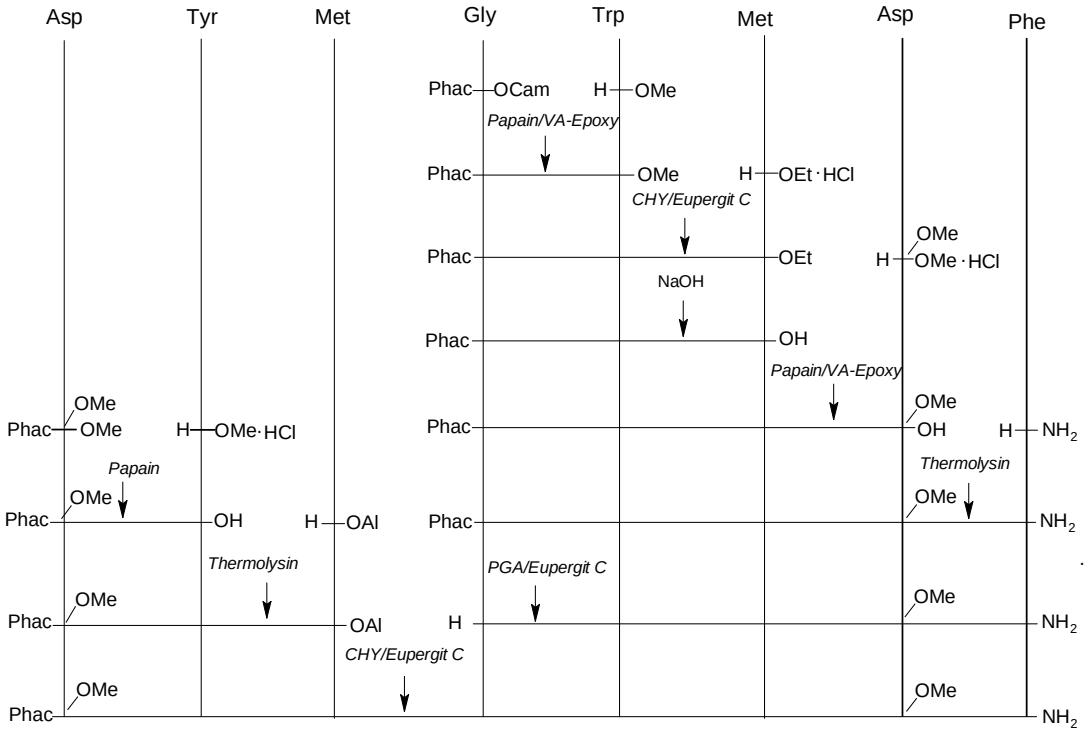
After having investigated many parameters in different pathways for synthesizing the C-terminal pentapeptides, the one-step synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OH was superior to that of two-steps including the enzymatic hydrolysis of Phac-Gly-Trp-Met-Asp(OMe)-OMe in terms of cheaper and less hazardous solvents, and higher yield as well (see Scheme 3.8).

Compared to the fully deprotection of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 in two steps, the selective deprotection of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 is preferred. Thereofore Gly-Trp-Met-Asp(OMe)-Phe-NH_2 was used for the final fragment condensation to obtain the octapeptide (CCK_{26-33}).

3.5 Synthesis of the octapeptide

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

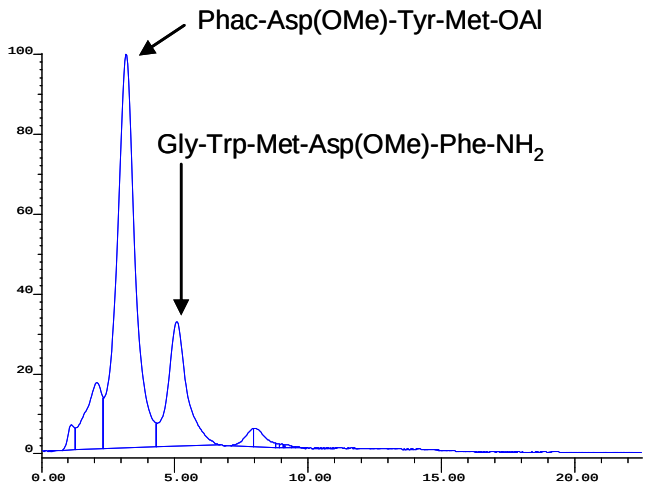
The octapeptide (CCK_{26-33}) was assembled via fragment condensation between the obtained tripeptide Phac-Asp(OMe)-Tyr-Met-OAl and pentapeptide Gly-Trp-Met-Asp(OMe)-Phe-NH_2 in the presence of α-CHY/Eupergit C. Based on the optimal strategies for synthesizing the tripeptide and the pentapeptides, the strategy for the total enzymatic synthesis of the octapeptide (CCK_{26-33}) is outlined in Scheme 3.9. This convergent synthetic strategy can be described as 3 + 5 in an abbreviated form.



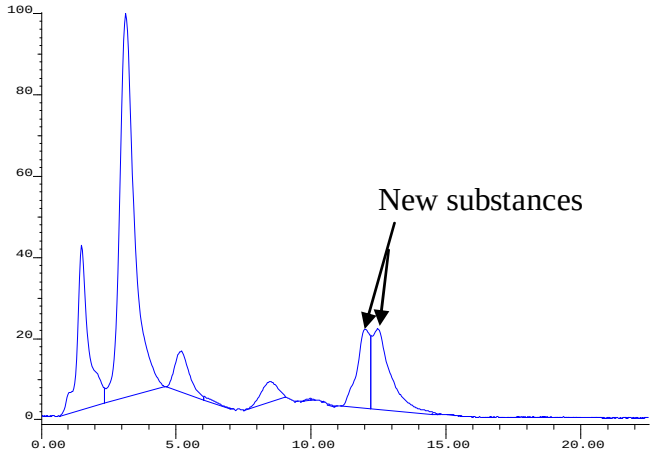
Scheme 3.9 The strategy for the total enzymatic synthesis of the octapeptide (CCK_{26-33})

The fragment coupling of the tripeptide and the pentapeptide was conducted as follows. Gly-Trp-Met-Asp(OMe)-Phe-NH_2 was condensed with 2 equivalents of Phac-Asp(OMe)-Tyr-Met-OAl in ACN containg 0.5% 0.05 M HCl-Tris buffer (pH 9.0) and 0.5% Et_3N under the catalysis of α-CHY/Eupergit C. The reaction was detected

by HPLC. Some new peaks were observed. The total HPLC yield of the new stubstances was 25.7% after 63 h (Figure 3.18).



(a)



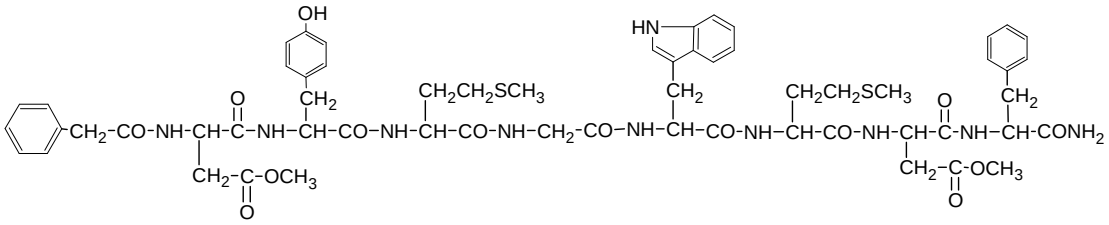
(b)

Figure 3.18 HPLC-chromatograms (HPLC system 7) of the synthesis of Phac-Asp(OMe)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 from Phac-Asp(OMe)-Tyr-Met-OAl and Gly-Trp-Met-Asp(OMe)-Phe-NH_2 in ACN containing 0.5% 0.05 M HCl-Tris buffer (pH 9.0) and 0.5% Et_3N with α-CHY/Eupergit C after the reaction time of 0 h (a), 63 h (b)

LC-ESIMS/PIM confirmed that the coupling between the tripeptide fragment and the pentapeptide fragment was successful. As can be seen from the HPLC chromatogram of LC-ESIMS (Figure 3.19), ESIMS electronic curve (Figure 3.20) and ESIMS spectra, peak **1** with t_R 15.253 in HPLC (44.9%) and 14.0 min in the ESIMS electronic curve was the product Phac-Asp(OMe)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-

Phe-NH_2 (Compound **1**), which was identified with the ESIMS spectrum (Figure 3.21) m/z 1209.6 [M+H^+], m/z 1231.7 [M+Na^+], $C_{59}H_{72}N_{10}O_{14}S_2$ requires 1208.9. The product was obtained in a HPLC yield of 11.5%. The chemical formula for the product (Compound **1**) is as follows:

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



Product (Compound **1**)

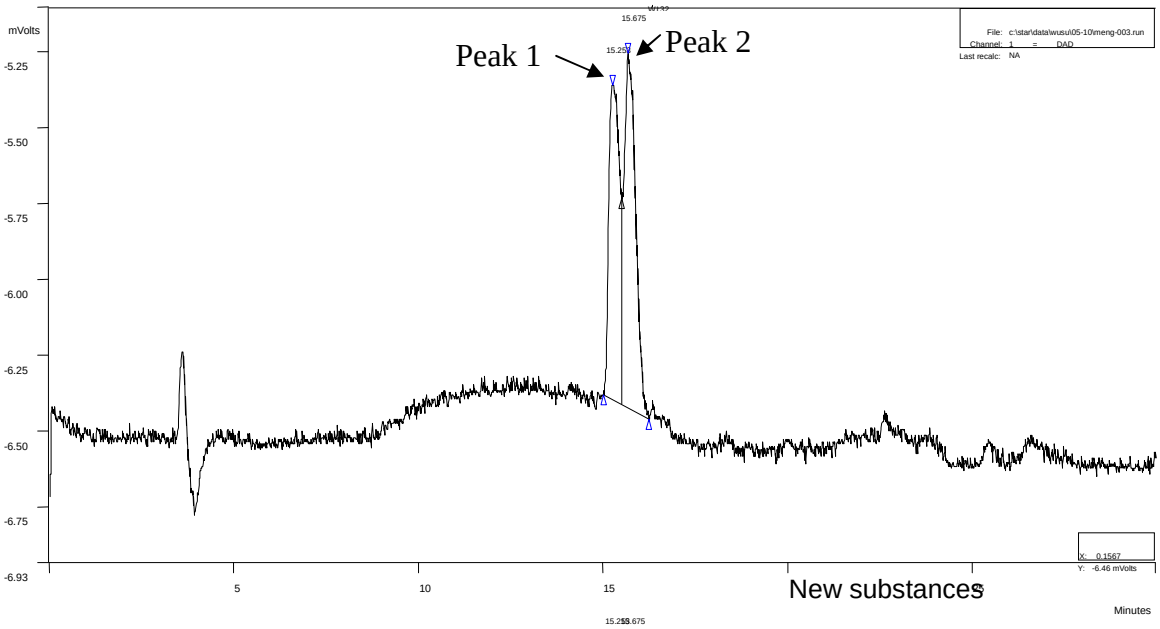


Figure 3.19 HPLC chromatogram (HPLC system in LC-ESIMS) of the crude products in the synthesis of Phac-Asp(OMe)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-NH_2

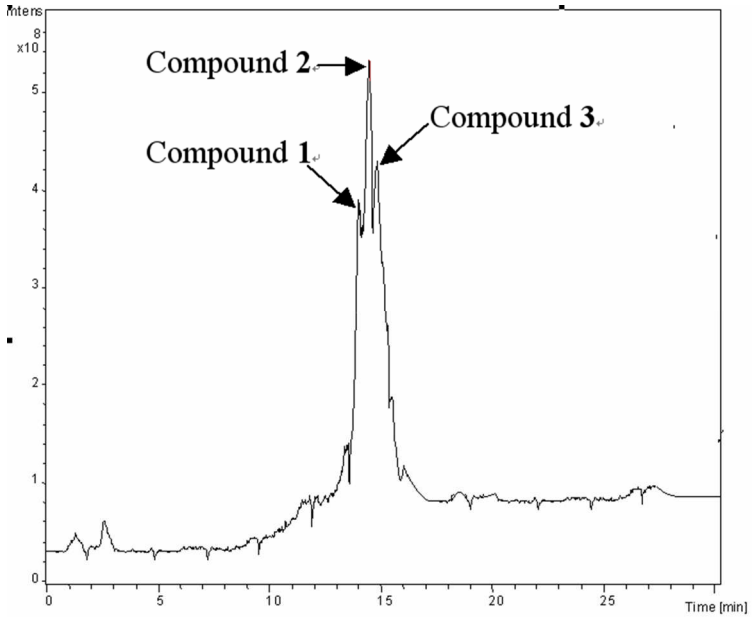


Figure 3.20 ESIMS electronic curve of the crude products in the synthesis of Phac-Asp(OMe)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-NH_2

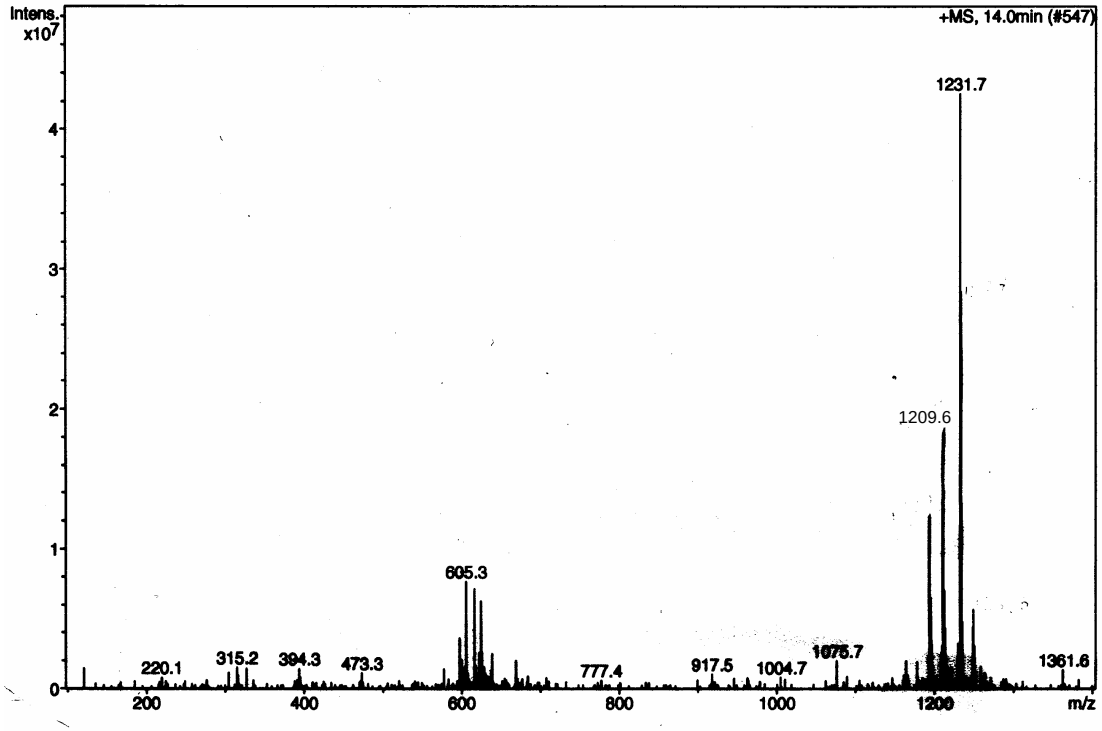
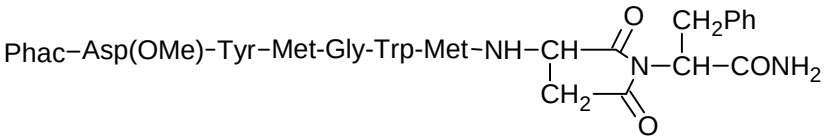


Figure 3.21 ESIMS spectrum of the product Phac-Asp(OMe)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 (Compound **1**)

The second peak (Peak **2**) with t_R 15.675 in HPLC (55.1%) contains compound **2** with 14.5 min in the ESIMS electronic curve and compound **3** with 14.8 min in the ESIMS

electronic curve. As shown in the ESIMS spectra (Figure 3.22 and Figure 3.23), compound **2** shows m/z 1177.5 [M+H$^+$], m/z 1199.5 [M+Na$^+$]. its molecular mass is then 1176, which is 32 less than the mass of the product Phac-Asp(OMe)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-NH_2. Probably, one MeOH is released by an intramolecular nucleophilic attack in the peptide which contains the aspartyl residue with the side chain carboxyl group protected in the frorm of methyl ester according to the literature (Bodanszky, 1993[b]). The fragments m/z 916.4 and 785.2 were matched to Phac-Asp(OMe)-Tyr-Met-Gly-Trp-Met$^+$ and Phac-Asp(OMe)-Tyr-Met-Gly-Trp$^+$, therefore this substance might be the compound **2** with one succinimide ring.



Compound **2**

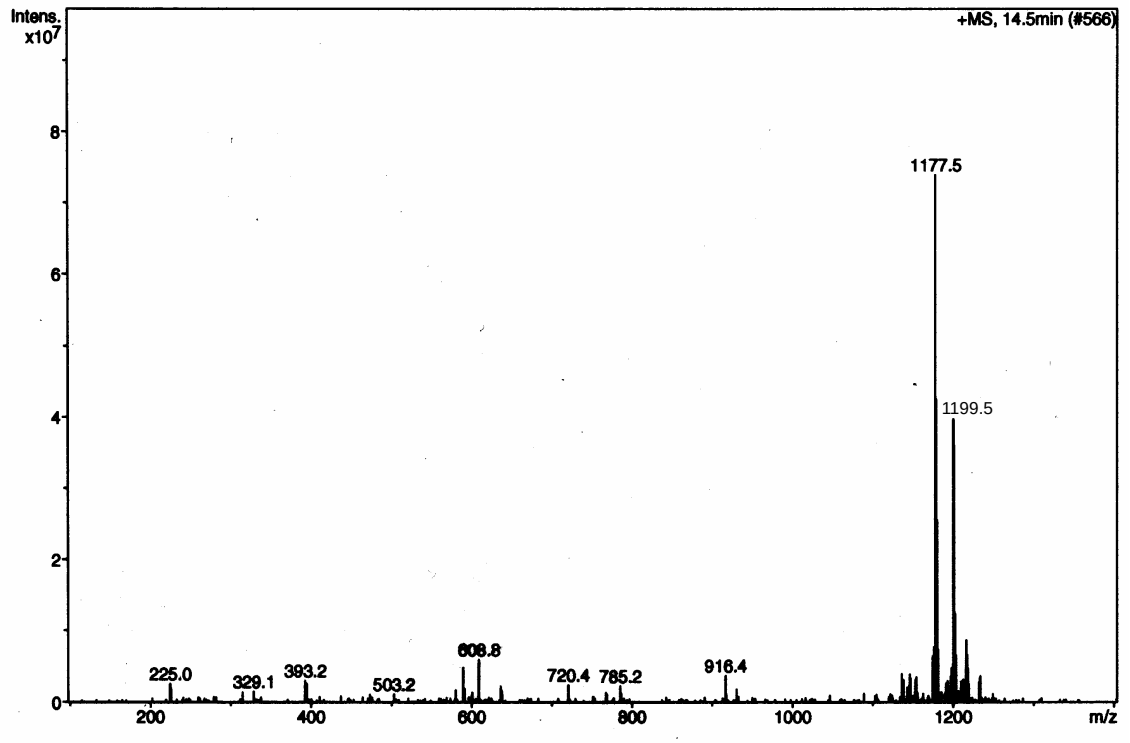
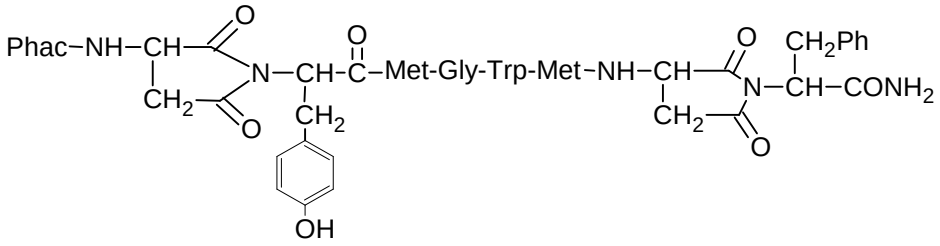


Figure 3.22 ESIMS spectrum of compound **2**.

Compound **3** shows m/z 1145.5 [M+H$^+$], m/z 1167.6 [M+Na$^+$]. Its molecular mass is then 1144, which is 32 mass units less than in the case of the compound **2**. It might release one MeOH again from the other Asp residue with β-methyl ester protecting group and form the compound **3** with two succinimide rings.



Compound **3**

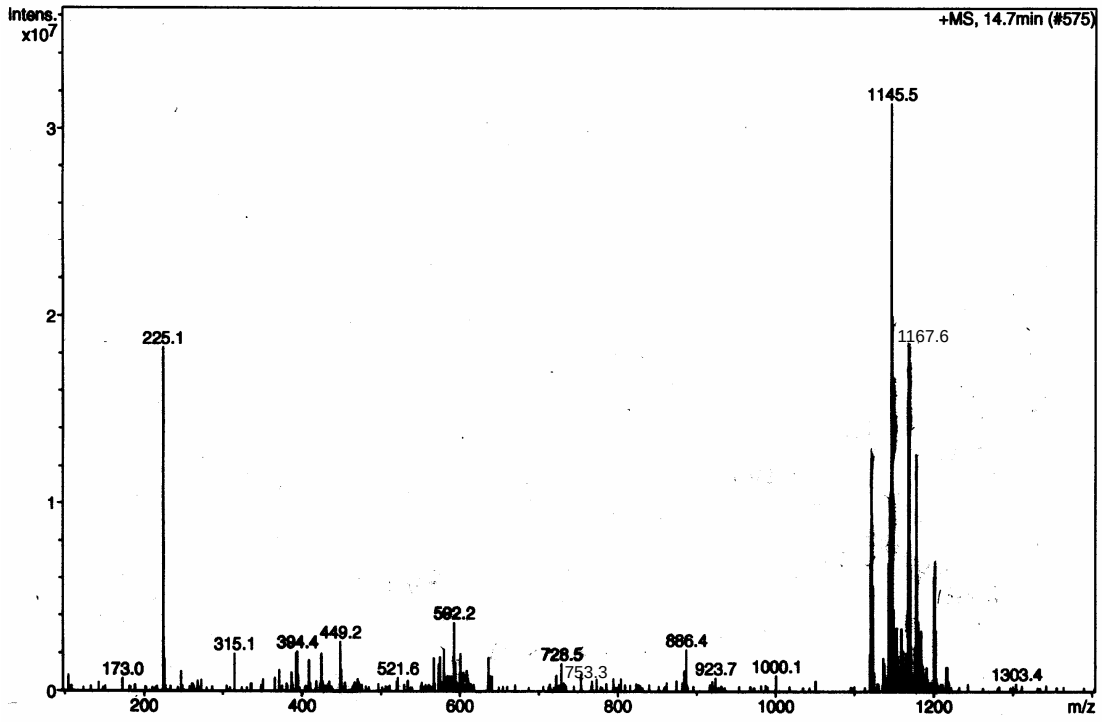


Figure 3.23 ESIMS spectrum of compound **3**.

In conclusion, under this reaction condition, the two fragments could be successfully coupled to the product Phac-Asp(OMe)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-NH_2. Further investigations are needed to avoid the β-methyl aspartyl residue succinimide formation. Considering that the methyl ester of aspartyl residue is subject to ring closure, the alternative approach is to use Gly-Trp-Met-Asp(OH)-Phe-NH_2 with the

free β-carboxy group in aspartyl residue to couple with the N-terminal tripeptide fragment. The further research on the synthesis of CCK-8 will be carried out in our group.

4 Experimental section

4.1 Apparatus

(1) Analytical HPLC

Pumps: 305 and M307 (Gilson); Software: 712 HPLC system control software (Gilson). UV Detector: L-7400 (Merck Hitachi LaChrom), set to 260 nm; Column: Nucleosil C18 (Macherey-Nagel), 5µm, 100x2 mm;

(2) Preparative HPLC system

Pumps: Metering pumps Model I/III (ConstaMetric); UV Detector: ISCO Model UA-6, UV/Vis detector, set to 254 nm; Column: Nucleosil, C18, 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: ISCO Model UA-5, Absorbance/Fluorescence detector, set to 254 nm, and Model 1132 multiplexer-expander; Fraction collector: Model-328 (Instrumentation Specialties Company); Column: Polygosil C18 (50-60µm), 30×4 cm.

(4) Size exclusion chromatography

UV Detector: ISCO Model UA-5, set to 260 nm, Absorbance/Fluorescence detector and Model 1132 multiplexer-expander; Fraction collector: Model-328 (Instrumentation Specialties Company); Column: Sephadex LH 20 (25-100 µm), 90×4 cm.

(5) LC-ESIMS

1100 Series LC/MSD Trap (Agilent); Column: Nucleosil C18 (Macherey-Nagel), 5µm (100Å), 250x4.6 mm.

(6) FABMS

TSQ 70 Finnigan MAT, positive ion mode

(7) Centrifuge

Universal 16 A (Hettich)

(8) pH-Meter

E 512 (Metrohm Herisau)

(9) UV lamp

Universal-UV-lamp CAMAG (Berlin)

(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. Tyrosin allyl ester *p*-tosylate (Tyr-OAl·TOS) was purchased from Senn Chemicals AG. Methionine allyl ester

p-tosylate (Met-OAl·TOS) and Trp-OEt·HCl were from Fluka. Fmoc-Gly-OH, Fmoc-Trp-OH and Fmoc-Met-OH were obtained from Novabiochem, Merck. Pentafluorophenol was from Merck (Darmstadt, Germany). Benzyl penicillin sodium was obtained from Fluka. N-Acetyl-L-tyrosin 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. 1.5L DMF, 180ml benzene and 70ml water were mixed and distilled under normal pressure until the temperature remained constant at 153^{o}C. Then calcium hydride (10 mg) was added slowly and kept overnight. Then it was distilled in vacuum under nitrogen and excusion of light with maintaining the temperature between 60 and 100^{o}C. HPLC grade methanol and HPLC grade 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 (Darmstadt, Germany). 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 (St. Louis, MO, U.S.A.). Penicillin G Amidase (PGA), immobilized on Eupergit C (109U/g) was obtained from Fluka.

(3) Carriers for immobilization of enzymes

Celite-545 (particle size 20-45 µm) was obtained from Fluka (Buchs, Switzerland). Eupergit C and VA-Hydroxy-Biosynth were from Roehm Pharma (Darmstadt). Polygosil60-10 NH_2 (particle size 10 µm) was from Macherey-Nagel. Bio-Gel P-2 polyamide was obtained from Bio-Rad.

(4) Materials for chromatography

Nucleosil C18 (5 µm) and Polygosil C18 (60-80µm) were from Macherey-Nagel (Düren, Germany). 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 (Sweden).

4.3 Methods of chromatography

(1) Analytical HPLC

Solvent system. Eluent A: 0.05M $AcONH_4$ (pH 6.5), which was prepared from 2 M $AcONH_4$ (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 $AcONH_4$ (pH 6.5), 800 ml methanol and 175 ml water and degassed by evacuation.

Flow rate. 0.3 ml/min.

HPLC system 1. Isocratic eluent 50% B. This method was used for the activity test of the papain-immobilisates and PGA-Eupergit C.

HPLC system 2. Isocratic eluent 40% B. This method was used for the activity test of the α-chymotrypsin-immobilisates.

HPLC system 3. Isocratic eluent 65% B. This method was used for the activity test of the thermolysin-immobilisates.

HPLC system 4. Gradient elution 40%B to 85%B over 10 min: 0-4.5 min, 40% B; 4.5-7 min, 40% B to 85% B; 7-11.5 min, 85% B; 11.5-12.5 min, 85% to 35% B; 12.5-13.5 min, 35% B; 13.5-14.5 min, 35% B to 40% B; 14.5-17.5 min, 40% B. This method was used during the synthesis of the N-terminal tripeptide fragment from amino acid derivaties.

HPLC system 5. Isocratic eluent 90% B. This method was used during the chemical synthesis of the C-terminal pentapeptides.

HPLC system 6. Gradient elution 55% B to 85% B over 10 min: 0-4.5 min, 55% B; 4.5-7 min, 55% B to 85% B; 7-11.5 min, 85% B; 11.5-12.5 min, 85% to 50% B; 12.5-13.5 min, 50% B; 13.5-14.5 min, 50% B to 55% B; 14.5-19.5 min, 55% B. This method was used during the enzymatic synthesis of the C-terminal pentapeptides.

HPLC system 7. Gradient elution 70% B to 90% B over 10 min: 0-4.5 min, 70% B; 4.5-7 min, 70% B to 90% B; 7-11.5 min, 90% B; 11.5-12.5 min, 90% to 65% B; 12.5-13.5 min, 65% B; 13.5-14.5 min, 65% B to 70% B; 14.5-22.5 min, 70% B. This method was used during the enzymatic synthesis of the octapeptide (CCK_{26-33}).

(2) Preparative HPLC

Solvent system. Eluent A: 0.05 M $AcONH_4$ (pH 6.5); Eluent B: 80% MeOH/0.05 M $AcONH_4$.

Flow rate. 3 ml/min.

(3) MPLC

Solvent system. Eluent A: 0.05 M $AcONH_4$ (pH 6.5); Eluent B: 80% MeOH/0.05 M $AcONH_4$.

Elution. Gradient elution 30% B to 80% B.

Flow rate. 5 ml/min.

(4) Size exclusion chromatography

Elution. MeOH

Flow rate. 5 ml/min.

(5) LC-ESIMS

Solvent system. Eluent A: 0.1% HCOOH in H_2O; Eluent B: 0.1% HCOOH in ACN.

HPLC System. Gradient elution 30% B to 90% B over 30 min: 0-5 min, 30% B; 5-15 min, 30% B to 90% B; 15-30 min, 90% B.

Flow rate. 1 ml/min

MS condition. ESI, positive ion mode; dry temperature: 350^{o}C; dry gas: 10.0 l/min; nebulizer 50.0 psi.

(6) Thin layer chromatography

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

TLC system 2. Dichloromethane/methanol 7:3 (v/v)

TLC system 3. Dichloromethane/methanol 9:1 (v/v)

Spots were detected as follows:

UV-Test. The UV active substances were detected in 254 nm.

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. The positive ninhydrin substances showed the violet spots.

Chlorine/Tolidine reaction. The plates were exposed to chlorine gas for about 5 minutes. Then tolidine solution was sprayed on these plates. 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 protected amino acids and the peptides showed a greenish yellow colour.

4.4 Characterization of the products

There are many ways to confirm the structure of a product. In this work, elemental analysis is not very useful, because the values determined by elemental analysis will not largely differ from peptide to peptide due to their abundant C, H, O and N elememts.

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 kinetic of a coupling reaction. RP-HPLC examination of the peptides allows rapid separation and quantitation of the components in a mixture.

If reference substances are 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 determination was made by MS analysis.

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 and 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.

4.5 Enzyme immobilization

(1) Derivatization of the VA-Hydroxy-Biosynth

Preparation of VA-Epoxy. 6 g VA-Hydroxy-Biosynth in 60 ml epichlorohydrin were refluxed for 4 h, and then sucked and washed with acetone. The VA-Epoxy was stored at -20^{o}C.

Preparation of VA-Carboxy. 0.5 g VA-Epoxy and 0.5 g 6-aminohexanoic acid were suspended in 10 ml H_2O. The pH value was adjusted by 4 M NaOH to pH10. After

stirring for 48 h, it was filtered and the product carboxy-polymer (VA-Carboxy) was washed with 1 M HCl and water successively, dried in vacuum and stored at -20^{o}C.

(2) 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 h, the suspension was lyophilized and stored at -20^{o}C.

Immobilizing papain on VA-Epoxy. 4 g VA-Epoxy washed with water and then 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 pain were added and mixed for 24 h. It was filtered and the papain/VA-Epoxy was washed with water and stored at -20^{o}C.

Immobilizing papain on VA-Carboxy. 500 mg VA-Carboxy was incubated with 333 mg N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in 13 ml water (pH adjusted to 5) for 30 min, then 200 mg papain was added. After rotating for 24 h, it was filtered and the papain/VA-Carboxy was completely washed with water and stored at -20^{o}C.

(3) 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 HCl-Tris buffer (pH 9.0) was mixed with 10 g Celite-545 rods. After thorough mixing for 24 h, the suspension was lyophilized and stored at -20^{o}C.

Adsorbing α-chymotrypsin on Bio-Gel P-2. A solution of α-chymotrypsin in 5 ml water and 5 ml 0.05M HCl-Tris buffer (pH 9.0) was mixed with 1g Bio-Gel P-2 polyamide. After thorough mixing for 24 h, the solvent was sucked off and the α-CHY/P-2 was dried under vacuum overnight.

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°C.

(4) Immobilization of thermolysin

Adsorbing thermolysin on Celite-545. The solution of 50 mg thermolysin in 30 ml water was mixed with 2 g Celite-545 rods. After thorough mixing for 24 h, the suspension was lyophilized and stored at -20°C.

Immobilizing thermolysin on Eupergit C. 1 g Eupergit C washed with 1 M KH_2PO_4 buffer (pH 7.5) were added to the solution of 45 mg thermolysin in 15 ml 1 M KH_2PO_4 buffer (pH 8.0). After rotating for 24 h, the solvent was sucked off and the TLN/Eupergit C was washed with 1 M KH_2PO_4 buffer (pH 7.5), 1 M HCl-Tris buffer (pH 7.5) and water successively and then stored at -20°C.

Immobilizing thermolysin on Polygosil 60-10 NH_2. 600 mg aminosilica gel was washed with water and then 0.01 M KH_2PO_4 buffer (pH 6.0). This silica gel was activated with 36 ml 6% gluardialdehyde (50% in water)/ 0.01 M KH_2PO_4 buffer (pH 6.0) (that is, 4.3 ml glutardialdehyde (50% in water) and 31.7 ml 0.01 M KH_2PO_4 buffer (pH 6.0)). After stirring for 30 min, the solution was sucked off and the activated Polygosil60-10NH_2 was washed with water and then the same buffer in order to be equilibrated. To the solution of 25 mg thermolysin in 3.6 ml 0.01 M KH_2PO_4 buffer (pH 6.0), the activated Polygosil60-10NH_2 was added and mixed for 75 min at 4°C. After sucking the solvent off and washing with water, the filter cake was rotated in a 10 ml vial with 0.5 M HCl-Tris buffer for 30 min at 10°C. At the end, it was filtered and the TLN/modified Polygosil60-10NH_2 was washed with water and 0.01 M KH_2PO_4 buffer (pH 6.0), and then stored at -20°C.

4.6 Activity test of immobilized enzymes

The enzyme-activity was determined as μmol conversion of test substrate per mg immobilized enzyme after 1 hour reaction time.

(1) Papain-immobilisates

Preactivation of papain-immobilisate. 800 mg papain-immobilisate in 8 ml 0.5 M KH_2PO_4 buffer (pH 6.0), 1mg EDTA and 12 μl mercaptoethanol were mixed for 1 h, then filtered. The papain-immobilisate was washed with 0.5 M KH_2PO_4 buffer (pH 6.0) (3x8 ml).

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 520 min and analyzed by HPLC with HPLC system 1.

Activity of papain-immobilisates.

Papain/Celite-545 (30mg/g): 0.65 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

Papain/VA-Epoxy (200mg/g) (fresh): 1.41 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

Papain/VA-Epoxy (200mg/g) (after the first application)): 1.26 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

Papain/VA-Epoxy (200mg/g) (after the second application without preactivation): 0.70 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

Papain/VA-Epoxy (200mg/g) (after the third application): 1.04 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

Papain/VA-Epoxy (200mg/g) (after the fourth application): 0.61 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

Papain/VA-Carboxy (400mg/g): 0.41 $\mu mol \cdot h^{-1} \cdot mg^{-1}$

(2) α-Chymotrypsin-immobilisates

Activity test. To 0.5 ml test solution (20 mg Ac-Tyr-OEt in 2 ml 0.01 M HCl-Tris 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 120 min and analyzed by HPLC with HPLC system 2.

Activity of α-chymotrypsin-immobilisates.

α-CHY/Celite-545 (10mg/g)(fresh): 1.00 μmol·h^{-1}·mg^{-1}

α-CHY/Celite-545 (10mg/g)(after the first application): 0.29 μmol·h^{-1}·mg^{-1}

α-CHY/P-2 (30mg/g): 0.43 μmol·h^{-1}·mg^{-1}

α-CHY/Eupergit C (60mg/g)(fresh): 0.87 μmol·h^{-1}·mg^{-1}

α-CHY/Eupergit C (60mg/g)(after the first application): 0.77 μmol·h^{-1}·mg^{-1}

α-CHY/Eupergit C (60mg/g)(after the second application): 0.75 μmol·h^{-1}·mg^{-1}

(3) Thermolysin-immobilisates

Activity test. To 0.5 ml test solution (180 mg Cbo-Asp-Phe-NH_2 in 10 ml 0.5 M HCl-Tris buffer (pH 8.5)), 50 mg immobilized α-chymotrypsin were added. Aliquots (10 μl) were withdrawn from the reaction mixture at different times from 0 min to 120 min and analyzed by HPLC with HPLC system 3.

Activity of thermolysin-immobilisates.

TLN/Celite-545 (25mg/g): 0.39 μmol·h^{-1}·mg^{-1}

TLN/Eupergit C (45mg/g) (fresh): 0.20 μmol·h^{-1}·mg^{-1}

TLN/Eupergit C (45mg/g) (after the first application): 0.09 μmol·h^{-1}·mg^{-1}

TLN/Eupergit C (42mg/g) (after being stored for 8 years): 0.18 μmol·h^{-1}·mg^{-1}

TLN/Polygosil60-10 NH_2 (42mg/g) (after being stored for 7 years): 0.19 μmol·h^{-1}·mg^{-1}

(4) PGA/Eupergit C

Activity test. To the solution of 6.4 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 35min and analyzed by HPLC with HPLC system 1.

Activity of PGA/Eupergit C.

PGA/Eupergit C (120U/g): 0.36 μmol·h^{-1}·mg^{-1}

4.7 Preparation of amino acid derivatives

(1) Preparation of Phac-Gly and Phac-Asp (Mardle et al., 1968; Suyama et al., 1965)

To a stirred solution of 0.26 mol of amino acid in 260 ml of 2 M NaOH, 35 ml (0.26 mol) of phenylacetylchlorid and 65 ml of 4 M NaOH were added dropwise simultaneously within 1 h. After 2 h, the reaction mixture was acidified with 6 M HCl to pH 2 in an ice-bath. The product was precipitated, filtered and recrystallized with methanol. The yield and analytical data are given in Table 4.1.

(2) Preparation of the methyl or ethyl ester hydrochlorids of tyrosine, tryptophan, methionine, asparatic acid, phenylalanine and Phac-glycine (Brenner et al., 1950; Fles and Markovac-Prpic, 1957)

To a stirred solution of 0.15 mol amino acid or Phac-Gly in 300 ml of absolute MeOH (or EtOH) cooled at -5^{o}C, 73 ml (1 mol) of thionyl chloride were added dropwise and the reaction temperature was kept below 0^{o}C. After adding two third of thionyl chloride, another 100 ml of MeOH was 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 of 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 of MeOH (or EtOH) at -5^{o}C and the above precudure was repeated. After removing the solvent, of MeOH (or EtOH) (3x150 ml) added into the residue and removed in vacuo. At the end the residue was recrystallized in 50ml of MeOH (or EtOH) and 150 ml of diethyl

ether. The crystals were filtered and washed with 50 ml of diethyl ether and dried in vacuo. The yield and analytical data are given in Table 4.1.

(3) Preparation of Asp(OBzl)-Bzl·TOS

13.3 g (0.1 mol) of aspartic acid, 43.2 ml of benzyl alcohol, 22.8 g of *p*-toluenesulfonic acid and 300 ml of benzene were mixed and refluxed for 10 h with separating water by azeotropic distillation. After cooled to room temperature, the precipitate was filtered off and recrystallized with ether/petroleum ether (1:1 v/v). The yield and analytical data are given in Table 4.1.

(4) Preparation of Met-OBzl·TOS

7.46 g (0.05 mol) of methionin, 80 ml of benzyl alcohol, 9.75 g (0.051 mol) of *p*-toluenesulfonic acid monohydrate and 100 ml of benzene were mixed and refluxed with separating water by azeotropic distillation. Refluxing was continued for another 2 h after no more water was produced. Then benzene and the excess of benzyl alcohol were distilled off under reduced pressure (bath temperature$<45^{o}$C), and the residue was solidified by trituration with ether. The solid was recrystallized with ethanol/ether. The yield and analytical data are given in Table 4.1.

(5) Preparation of free amino acid esters

To 1.4 g (5 mmol) of $Na_2CO_3 \cdot 10H_2O$ in 20 ml of 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 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^{o}C. The free amino acid ester was used immediately for the coupling reaction.

(6) Preparation of Asp(OMe)-OH·HCl

To 208 ml of absolute MeOH cooled to -10^{o}C, 31 ml of $SOCl_2$ and 40 g (0.3 mol) of aspartic acid were added. A clear solution was obtained on slowly warming to room temperature. After standing 25 min at room temperature, 600 ml of 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. The yield and analytical data are given in Table 4.1.

(7) Preparation of Phe-NH_2·HCl

To a stirred, cooled to -5°C solution of 65 g (0.3 mol) of Phe-OMe·HCl in 342 ml of absolute MeOH, ammonia gas was passed through. The saturated solution was placed at room temperature overnight. Then the solvent was removed, 2 M HCl and water were added to the residue. After removal of the solvent, 2 M HCl and water were added to the residue again. The crude product was obtained after removing the solvent and recrystallization with EtOH. The yield and analytical data are given in Table 4.1.

(8) Preparation of Phac-Asp(OMe)-OH and Z-Asp(OMe)-OH (Schwarz et al., 1957)

9.6 g (52.3 mmol) of Asp(OMe)-OH·HCl were dissolved in a solution of 90 ml of water, 12 ml of 4 M NaOH (48 mmol) and 8.7 g (103 mmol) of $NaHCO_3$. Under stirring at room temperature 66.6 mmol phenylacetylchloride (or benzylchloroformate) was added over a 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/petroleum ether. The yield and analytical data are given in Table 4.1.

(9) Preparation of Phac-Asp(OMe)-OMe (according to Martinez et al., 1983; 1985)

5 g (0.02 mol) of Phac-Asp was suspended in 100 ml of absolute MeOH and cooled to -5°C. 5.5 ml of $SOCl_2$ was added to the mixture over 30 min with rapid stirring. Then the reaction mixture was kept at room temperature for 2 h. After removal of MeOH

under vacuum (bath temperature＜45°C), the residue was treated twice with 5.5 ml $SOCl_2$ in 100 ml absolute MeOH as mentioned above, but at the second time, the reaction was kept at room temperature overnight. After removal of MeOH, the residue was transferred to 100 ml of EtOAc, washed with 20 ml cold 5% $NaHCO_3$, and then washed with 5% NaCl until pH 7.0. The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo to dryness. The yield and analytical data are given in Table 4.1.

(10) Preparation of Phac-Gly-OCam and Phac-Asp(OMe)-OCam

Phac-Gly-OH (5.0 g, 26 mmol) [Phac-Asp(OMe)-OH (4.24 g, 16mmol)] was dissolved in 50 ml of MeOH/water (4:1) [296 ml of MeOH/water (2:1)] and 20 ml [13.64 ml] of 20% Cs_2CO_3 was 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. The white crystals (Phac-Gly-OCs) [Phac-Asp(OMe)-OCs] were dried overnight under high vacuum. The cesium salt was dissolved in 60ml [240 ml] absolute DMF. In this solution α-chloroacetamide (3.5 g, 37.5mmol) [2.24 g, 24 mmol] was added. After the mixture was stirred for 4 d at 35°C, the solution was evaporated to dryness under vacuum. The residue was dissolved in 100ml 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. A white solid was obtained. The yield and analytical data are given in Table 4.1.

PhAc-Asp(OMe)-OCam: FABMS *m/z* 323.0 [$M+H^+$], *m/z* 345.1 [$M+Na^+$], $C_{15}H_{18}N_2O_6$ requires 322.2.

(11) 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 with 20 ml DMF or 100 ml EtOAc, dicyclohexylcarbodiimide (20 mmol) was added and stirring was continued for 1 h at 0°C and for 1 h at room temperature. Dicyclohexylurea was filtered off and the

solvent was evaporated in vacuo at 40°C. The residue was recrystallized in EtOAc/petroleum ether (for Fmoc-Trp-OPfp, Fmoc-Met-OPfp) or EtOAc (for Fmoc-Gly-OPfp). The yield and analytical data are given in Table 4.1.

Table 4.1 Isolated yield, melting point, HPLC retention time and R_f value of obtained amino acid derivative

Derivative	Isolated Yield [%]	m.p. [°C]	HPLC System (3-6)/t_R [min]	TLC System (1-3)/R_f
Phac-Gly	53.3	139-141	6/1.19	1/0.69
Phac-Asp	51.8	129-133	6/0.94	1/0.68
Phac-Gly-OMe	65.3	81-83	6/2.18	1/0.81
Tyr-OMe·HCl	88.9	178-180	4/ 1.95	1/0.53
Trp-OMe·HCl	78.3	210-212	6/3.33	1/0.55
Phe-OMe·HCl	93.7	158-160	6/3.41	1/0.57
Met-OEt·HCl	91.2	82-84	UV-inactive	1/0.63
Asp(OEt)-OEt·HCl	80.0	104-106	UV-inactive	1/0.54
Asp(OMe)-OMe·HCl	94.3	114-115	UV-inactive	1/0.43
Asp(OBzl)-OBzl·TOS	90.2	154-157	UV-inactive	1/0.67
Met-OBzl·TOS	33.6	129-131	UV-inactive	1/0.58
Phe-NH_2·HCl	85.0	230-235	6/1.19	1/0.54;
Asp(OMe)-OH·HCl	66.0	193-195	UV-inactive	1/0.28
Phac-Asp(OMe)-OH	49.7	100-102	4/1.13	1/0.65
Z-Asp(OMe)-OH	65.1	95-96	6/1.96	3/0.2
Phac-Asp(OMe)-OMe	93.2	71-73	4/7.29	1/0.74
Phac-Gly-OCam	85.6	128-129	6/1.57	3/0.65
Phac-Asp(OMe)-OCam	64.5	100-101	4/3.86	1/0.71
Fmoc-Gly-OPfp	87.5	160-161	5/2.10	1/0.82
Fmoc-Trp-OPfp	91.9	185-186	5/4.45	1/0.91
Fmoc-Met-OPfp	99.5	101-103	5/4.99	1/0.85

4.8 Peptide synthesis

4.8.1 Chemical synthesis of reference peptides

4.8.1.1 Chemical synthesis of Phac-peptides

(1) Chemical synthesis of Phac-dipeptide, -tripeptide and –tetrapeptide fragments

20 mmol of HOBt were dissolved in 50 ml anhydrous THF and 20 mmol of Phac-Gly-OH or Phac-peptidyl acid in 60 ml DMF. 22 mmol of 3 M DCC in DCM were added. After stirring for 1 h, the DCU was removed by filteration. The filtrate was added into a solution of 20 mmol of amino acid ester hydrochloride 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 $NaSO_4$, filtration and concentration, the residue (Phac-peptidyl ester) was crystallized (Table 4.2).

To obtain Phac-peptidyl acid, 7 mmol of the corresponding Phac-peptidyl ester were dissolved in water/acetone (140 ml, 1:1 v/v) and treated with 4 ml 4 N NaOH. The reaction was controlled by TLC (TLC system 2). After 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 $NaSO_4$, filtration and concentration, the product (Phac-peptidyl acid) was obtained (Table 4.2).

Phac-Gly-Trp-Met-OH: FABMS *m/z* 511.1 [$M+H^+$], *m/z* 533.2 [$M+Na^+$], $C_{26}H_{30}N_4O_5S_1$ requires 510.6.

Table 4.2 Analytical data of the synthesized Phac-peptide fragments.

Phac-peptide Fragment	Solvent for Recrystallizati-on	Isolated Yield [%]	m.p. [°C]	HPLC System (3-6)/t_R[min]	TLC System (1-3)/R_f
Phac-Gly-Trp-OMe	EtOAc/PE	67.3	126-128	6/10.27	1/0.79
Phac-Gly-Trp-OEt	EtOAc/PE	70.8	192-195	6/11.77	1/0.80
Phac-Gly-Trp-OH	----------	93.0	72-75	6/2.78	1/0.68
Phac-Gly-Trp-Met-OEt	EtOH	58.0	174-176	6/13.32	3/0.8
Phac-Gly-Trp-Met-OH	----------	98.6	180-182	6/5.15	3/0.15
Phac-Gly-Trp-Met-Asp (OMe)-OMe	EtOAc/PE	39.8	118-120	6/9.42	1/0.77
Phac-Gly-Trp-Met-Asp (OH)-OH	----------	79.9	125-127	6/1.17	1/0.65

(2) Chemical synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 and Phac-Gly-Trp-Met-Asp(OH)-Phe-NH_2

96 mg (0.15 mmol) of Phac-Gly-Trp-Met-Asp(OMe)-OH and 171 mg (0.75 mmol) of Phe-NH_2·HCl were dissolved in as less as possible DMF. 26 µl 3 M DCC in DCM were added and the pH was adjusted to 8 by adding N-methylmorpholine. After stirring for 1 h, another 26 µl 3 M DCC in DCM were added. The mixture was stirred overnight, filtered and distillated in vacuo to dryness. The residue was dissolved in MeOH and prepared for size exclusion chromatography. The product fractions were pooled after checked by HPLC and concentrated. The crude product was dissolved in 30 ml of 60% MeOH in water (v/v) and chromatographed with MPLC. The pure product (Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2) was lypholized twice after concentration of the pooled fractions and 52 mg (44.2%) of a white solid were obtained (decomposition at 220°C, HPLC system 6 with t_R 13.95, FABMS m/z 786.1 [M+H^+], m/z 808.1 [M+Na^+], $C_{40}H_{47}N_7O_8S_1$ requires 785.9).

0.4 g (0.51)mmol of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 were dissolved in 100 ml water/acetone (1:1 v/v) and treated with 1 ml 4 N NaOH. After 1 h the acetone was removed in vacuo. The product was precipitated by acidification with 1M HCl to pH 3.0 and filtered. The residue was washed with 50 ml water. After drying over anhydrous $NaSO_4$, filtration and concentration, the product Phac-Gly-Trp-Met-Asp(OH)-Phe-NH_2 was obtained as a white solid (0.34 g, 86.8%, m.p. 214-217^{0}C, HPLC system 6 with t_R 10.88, FABMS m/z 772.9 [M+H^+], m/z 794.2 [M+Na^+], $C_{39}H_{45}N_7O_8S_1$ requires 771.9).

4.8.1.2 Chemical synthesis of the C-terminal pentapeptides

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

2 g (4.7 mmol) of Z-Asp(OMe)-Phe-NH_2 were completely dissolved in 320 ml of MeOH and 0.8 ml of 6 M HCl, and then 100 mg of 10% Pd-C were added. After sucking the air out of the flask with water pump, gaseous H_2 was led into it. The reaction was fininshed in 30 min. The reaction mixture was filtered and the filtrate was concentrated in vacuo to yield a white solid of Asp(OMe)-Phe-NH_2·HCl (1.53g, 99.0%, m.p. 155-158^{o}C, HPLC system 6 with t_R 1.85).

(2) Chemical synthesis of Fmoc-Met-Asp(OMe)-Phe-NH_2

To a stirred solution of Asp(OMe)-Phe-NH_2·HCl (1.98 g, 6 mmol) in DMF (30 ml), Fmoc-Met-OPfp (3.23 g, 6 mmol) and Et_3N (1.68 ml, 12 mmol) were added and stirring was contiuned for 20 min, then the solution was concentrated in vacuo. The residue crystallized by trituration with ether, giving Fmoc-Met-Asp(OMe)-Phe-NH_2 (3.09 g, 80.5%, m.p. 169-171^{o}C, HPLC system 5 with t_R 6.28). An analytical sample was recrystallized from methanol (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) Chemical synthesis of Fmoc-Trp-Met-Asp(OMe)-Phe-NH_2

The above protected tripeptide amide (2.912 g, 4.5 mmol) was treated with a 10% dimethylamine solution in DMF (27 ml) for 10 min, then the solution was concentrated in vacuo. The residue was triturated with petroleum ether, then filtered off to give Met-Asp(OMe)-Phe-NH_2 (1.89 g, 99.1%, m.p. 148-150^{o}C, HPLC system 5 with t_R 9.29, FABMS m/z 425.0 [M+H^+], m/z 447.1 [M+Na^+], $C_{19}H_{28}N_4O_5S_1$ requires 424.5).

To a stirred solution of this compound (2.035 g, 4.8 mmol) in DMF (24 ml), Fmoc-Met-OPfp (2.846 g, 4.8 mmol) and Et_3N (0.672 ml, 4.8 mmol) were added and stirring was contiuned for 20 min, then the solution was concentrated in vacuo. The residue crystallized by trituration with EtOAc, giving Fmoc-Trp-Met-Asp(OMe)-Phe-NH_2 (3.75 g, 93.8%, m.p. 182-183^{o}C, HPLC system 5 with t_R 8.92, FABMS m/z 833.0 [M+H^+], m/z 855.0 [M+Na^+], $C_{45}H_{48}N_6O_8S_1$ requires 832.9).

(4) Chemical synthesis of Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-NH_2

The above protected tetrapeptide amide (3.744 g, 4.5 mmol) was treated with a 10% dimethylamine solution in DMF (28.125 ml) for 10 min, then the solution was concentrated in vacuo. The residue was triturated with petroleum ether, then filtered off to give Trp-Met-Asp(OMe)-Phe-NH_2 (2.74 g, 99.8%, decomposition at 169^{o}C, HPLC system 5 with t_R 9.08, FABMS m/z 611.0 [M+H^+], m/z 633.0 [M+Na^+], $C_{30}H_{38}N_6O_6S_1$ requires 610.7).

To a stirred solution of this compound (3.05 g, 5 mmol) in DMF (30 ml), Fmoc-Gly-OPfp (2.315 g, 5 mmol) and Et_3N (0.7 ml, 5 mmol) were added and stirring was contiuned for 20 min, then the solution was concentrated in vacuo. The residue crystallized by trituration with EtOAc, giving Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 (3.26 g, 73.7%, m.p. 166-170^{o}C, HPLC

system 5 with t_R 7.27, FABMS m/z 890.3 [M+H$^+$], m/z 912.3 [M+Na$^+$], $C_{47}H_{51}N_7O_9S_1$ requires 889.9).

(5) Removal of Fmoc from Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-NH_2

Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 (535 mg, 0.6 mmol) was treated with a 10% dimethylamine solution in DMF (5 ml) for 10 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 (200 mg, 50.0%, decomposition at 210^{o}C, HPLC system 5 with t_R 1.36, FABMS m/z 668.0 [M+H$^+$], m/z 690.2 [M+Na$^+$], $C_{32}H_{41}N_7O_7S_1$ requires 667.8).

(6) Removal of Fmoc and OMe from Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-NH_2

Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 (178 mg, 0.2 mmol) was suspended in water/acetone (10 ml, 1:1 v/v) and treated with 0.5 ml 4 N NaOH for 1 h, then the solution was concentrated in vacuo to remove acetone. The residue was neutralized to pH 7.0, white solid (Gly-Trp-Met-Asp(OH)-Phe-NH_2) came out, was filtered off and dried (33 mg, 25.3%, decomposition at 230^{o}C, HPLC system 6 with t_R 6.73, FABMS m/z 654.2 [M+H$^+$], m/z 676.2 [M+Na$^+$], $C_{31}H_{39}N_7O_7S_1$ requires 653.7).

4.8.2 Enzymatic synthesis of peptides

(1) Synthesis of Z-Asp(OMe)-Phe-NH_2 with thermolysin

5.0 g (17.8 mmol) Cbo-Asp(OMe)-OH and 4.6 g (23.0 mmol) H-Phe-NH_2·HCl were suspended in 80 ml H_2O and the pH was adjusted to 7.0 with 1N NaOH. To this solution 25 mg thermolysin were added and stirred at 40°C. After 7h the reaction was complete and no starting material was detected by TLC. The precipitated white product Z-Asp(OMe)-Phe-NH_2 was isolated by filtration, washed with cold 5% citric acid (3x100 ml) and cold water (3x100 ml) successively, and then dried in vacuum (6.88 g, 90.6%, m.p. 185-186^{o}C, HPLC system 6 with t_R 11.46, FABMS *m/z* 428.1 [M+H$^+$], *m/z* 450.1 [M+Na$^+$], $C_{22}H_{25}N_3O_6$ requires 427.5. TLC system 3 with R_f = 0.5).

(2) Synthesis of Phac-Asp(OMe)-Tyr-OMe with papain/VA-Epoxy in low-water content system

16 mg (0.068 mmol) Tyr-OMe·HCl and 32 mg (0.1 mmol) Phac-Asp(OMe)-OCam were dissolved in 1.5 ml EtOAc, 3 µl 0.1 M borax buffer (pH 8.2), 2 µl β-mercaptoethanol and 0.2 mg EDTA. To this solution 200 mg papain/VA-Epoxy were added and thoroughly mixed by rotating at room temperature. The reaction was monitored by HPLC. After 2 h the HPLC yield was 25.5%. The mixture was filtered to remove the immobilized enzyme which was washed with a mixture of 40 ml ACN and 10 ml water. The combined filtrates were evaporated to dryness in vacuo and the residue was dissolved in 40% MeOH and separated by preparative HPLC with 55% eluent B. The pooled fractions were lyophilized twice yielding a white powder Phac-Asp(OMe)-Tyr-OMe (7 mg, 23.3%, m.p. 130-133ºC, HPLC system 4 with t_R 10.96, FABMS *m/z* 443.1 [M+H^+], *m/z* 465.1 [M+Na^+], $C_{23}H_{26}N_2O_7$ requires 442.5).

A by-product was isolated (6 mg, 14.6%) and characterized as Phac-Asp(OMe)-Tyr-Tyr-OMe by FABMS *m/z* 606.1 [M+H^+], *m/z* 628.1 [M+Na^+], $C_{32}H_{35}N_3O_9$ requires 605.6, HPLC system 4 with t_R 13.78, decomposition at 220ºC.

(3) Synthesis of Phac-Asp(OMe)-Tyr-OAl with papain/VA-Epoxy

0.5 mmol Tyr-OAl or Tyr-OAl·TOS and 256 mg (0.8 mmol) Phac-Asp(OMe)-OR (R = Me, Cam) were dissolved in 12 ml organic solvent, 24 µl 0.1 M borax buffer, 16 µl β-mercaptoethanol and 1.6 mg EDTA. To this solution papain/VA-Epoxy was added and thoroughly mixed by rotating at room temperature. The reaction was monitored by HPLC. After reaching the “kinetic” optimum, the mixture was filtered to remove the immobilized enzyme which was washed with a mixture of 40 ml ACN and 10 ml water. The combined filtrates were evaporated to dryness in vacuo and the residue was dissolved in 40% MeOH and separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder Phac-Asp(OMe)-Tyr-OAl (m.p. 91-93ºC, HPLC system 4 with t_R 12.88, FABMS *m/z* 469.1 [M+H^+], *m/z* 491.0 [M+Na^+], $C_{25}H_{28}N_2O_7$ requires 468.3). The experimental data are shown in Table 4.3.

Table 4.3 Experimental data for synthesis of Phac-Asp(OMe)-Tyr-OAl with papain/VA-Epoxy.

R	Amino Component	Catalyst [g]	Reaction Media	pH Value of Borax Buffer	HPLC Yield [%]	Isolated Yield [%]
Me	Tyr-OAl·TOS	1.6	EtOAc + 7 mg NaOH	8.2	0	0
Cam	Tyr-OAl·TOS	1.6	ACN + 7 mg NaOH	8.2	14.9	------
Cam	Tyr-OAl·TOS	1.6	EtOAc + 7 mg NaOH	8.2	27.9	22.2
Cam	Tyr-OAl	1.6	EtOAc	8.2	25.2	22.6
Cam	Tyr-OAl	1	EtOAc + 100µl ACN	8.2	30.1	24.0
Cam	Tyr-OAl	1	EtOAc + 100µl ACN	5.9	24.3	17.8
Cam	Tyr-OAl	1	EtOAc	6.9	40.7	33.8
Cam	Tyr-OAl	1	EtOAc + 100µl ACN	6.9	57.3	40.1
Cam	Tyr-OAl	1	EtOAc + 1ml ACN	6.9	30.5	26.1

The HPLC yields of the by-product were shown in Table 3.1. The by-product was isolated and characterized as Phac-Asp(OMe)-Tyr-Tyr-OAl by FABMS *m/z* 632.2 [M+H$^+$], *m/z* 654.2 [M+Na$^+$], $C_{34}H_{37}N_3O_9$ requires 631.3, HPLC system 4 with t_R 13.78, m.p. 108-110ºC.

(4) Synthesis of Phac-Asp(OR)-Tyr-OAl with free or immobilized thermolysin

265 mg (1 mmol) Phac-Asp(OR)-OH (R = H, Me) and 2 mmol Tyr-OAl·TOS or Tyr-OAl were dissolved in the reaction media and the pH was adjusted to 7.0 with 1M

NaOH. To this solution free or immobilized thermolysin was added and thoroughly mixed by rotating. After 5 d, the mixture was washed with ACN and filtered. The filtrate was evaporated to dryness under vacuum. The residue was dissolved in 30% MeOH and separated by MPLC. The pooled fractions of Phac-Asp(OR)-Tyr-OAl were lyophilized twice yielding a white powder. The experimental data are shown in Table 4.4.

Table 4.4 Experimental data for synthesis of Phac-Asp(OR)-Tyr-OAl via the catalysis of free or immobilized thermolysin .

R	Amino Component	Catalyst	Reaction Media	Temperature [^{o}C]	Isolated Yield [%]
H	Tyr-OAl·TOS	400 mg TLN/Celite-545	3.6 ml ACN + 200 µl 0.05 M HCl-Tris	25	0
H	Tyr-OAl·TOS	10 mg TLN	4 ml 0.05 M HCl-Tris	25	0
Me	Tyr-OAl·TOS	400 mg TLN/Celite-545	3.6 ml ACN + 200 µl 0.05 M HCl-Tris	25	0
Me	Tyr-OAl·TOS	100 mg TLN	1.5 ml H_2O	40	20.3
Me	Tyr-OAl	100 mg TLN	1.5 ml H_2O + 40 µl ACN	40	15.4

(5) Synthesis of Phac-Asp(OMe)-Tyr-OH with papain and Phac-Asp(OMe)-Tyr-OMe with papain/VA-Epoxy in the solvent-free system

556 mg (2.4 mmol) Tyr-OMe·HCl, 3.2 g (32 mmol) $KHCO_3$ and 686 mg (2.4 mmol) $Na_2CO_3 \cdot 10H_2O$ were mixed. 446 mg (1.6 mmol) Phac-Asp(OMe)-OMe and 240 mg papain or 1.2 g papain/VA-Epoxy were added and stirred manually from time to time. After 1 d, the mixture was washed with ACN and filtered. The filtrate was evaporated

to dryness under vacuum. The residue was dissolved in 30% MeOH and separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder.

Phac-Asp(OMe)-Tyr-OH: reaction via the catalysis of free papain, 328 mg, 76.6% (isolated yield), m.p. 158-159ºC, HPLC system 4 with t_R 3.38, FABMS *m/z* 429.1 [M+H$^+$], *m/z* 451.1 [M+Na$^+$], $C_{22}H_{24}N_2O_7$ requires 428.2.

Phac-Asp(OMe)-Tyr-OMe: reaction via the catalysis of papain/VA-Epoxy, 260 mg, 36.8% (isolated yield), m.p. 130-133ºC.

(6) Synthesis of Phac-Asp(OMe)-Tyr-Met-OR with papain/VA-Epoxy

Met-OR (1.0 mmol Met-OEt, 1.0 mmol Met-OBzl or 0.75 mmol Met-OAl) and 113 mg (0.25 mmol) Phac-Asp(OMe)-Tyr-OAl were dissolved in 7.5 ml EtOAc, 15 µl buffer, 10 µl β-mercaptoethanol and 1 mg EDTA. To this solution 1 g papain/VA-Epoxy was added and thoroughly mixed by rotating at room temperature. After reaching the “kinetic” optimum within 3-4 h, the mixture was filtered to remove the immobilized enzyme which was washed with a mixture of 40 ml ACN and 10 ml water. The combined filtrates were evaporated to dryness in vacuo and the residue was dissolved in 40% MeOH and separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder.

Product Phac-Asp(OMe)-Tyr-Met-OEt: Reaction in 0.1 M borax buffer (pH 6.9), HPLC yield 55.2%, isolated yield 55 mg, (37.5%), m.p.141-143ºC, HPLC system 4 with t_R 11.49, FABMS *m/z* 588.2 [M+H$^+$], *m/z* 610.2 [M+Na$^+$], $C_{29}H_{37}N_3O_8S_1$ requires 587.2. A by-product was isolated (5 mg, 13.9%) and characterized as Phac-Asp(OMe)-Tyr-Met-Met-OEt by FABMS *m/z* 719.2 [M+H$^+$], *m/z* 741.2 [M+Na$^+$], $C_{34}H_{46}N_4O_9S_2$ requires 718.2, HPLC system 4 with t_R 12.56, decomposition at 180ºC.

Product Phac-Asp(OMe)-Tyr-Met-OBzl: Reaction in 0.1 M borax buffer (pH 6.9), HPLC yield 29.5%, isolated yield 15 mg, (9.2%), m.p. 90-92ºC, HPLC system 4 with

t_R 15.31, FABMS *m/z* 650.4 [M+H^+], *m/z* 672.4 [M+Na^+], $C_{34}H_{39}N_3O_8S_1$ requires 649.2. When the reaction was carried out for 21 h, the main product was Phac-Asp(OMe)-Tyr-Met-OH: Isolated yield 31 mg (22.4%), decomposition at 230ºC, HPLC system 4 with t_R 7.32, FABMS *m/z* 560.2 [M+H^+], *m/z* 582.1 [M+Na^+], $C_{27}H_{33}N_3O_8S_1$ requires 559.2.

Product Phac-Asp(OMe)-Tyr-Met-OAl: reaction in 0.1 M borax buffer (pH 6.9), HPLC yield 64.4%, isolated yield 63 mg, (42.1%); reaction in 0.1 M borax buffer (pH 5.9), HPLC yield 67.5%; reaction in 0.1 M borax buffer (pH 8.0), HPLC yield 62.9%; m.p. 161-163ºC, HPLC system 4 with t_R 14.36, FABMS *m/z* 600.3 [M+H^+], *m/z* 622.2 [M+Na^+], $C_{30}H_{37}N_3O_8S_1$ requires 599.2. A by-product (HPLC yield around 10%) was isolated and characterized as Phac-Asp(OMe)-Tyr-Met-Met-OAl by FABMS *m/z* 731.2 [M+H^+], *m/z* 753.2 [M+Na^+], $C_{35}H_{46}N_4O_9S_2$ requires 730.2, HPLC system 4 with t_R 16.37, decomposition at 188ºC;.

(7) Synthesis of Phac-Asp(OMe)-Tyr-Met-OEt with α-CHY/Celite-545

23 mg (0.05 mmol) Phac-Asp(OMe)-Tyr-OAl, 42.8 mg (0.2 mmol) Met-OEt·HCl, 10 mg (0.1 mmol) $KHCO_3$ and 24 mg (0.08 mmol) $Na_2CO_3 \cdot 10H_2O$ were mixed. 150 mg of α-CHY/Celite-545 were added and stirred manually every 20 min. The reaction was monitored by HPLC. The HPLC yield of Phac-Asp(OMe)-Tyr-Met-OEt was 11.0%.

(8) Synthesis of Phac-Asp(OMe)-Tyr-Met-OAl with α-CHY/Celite-545

113 mg (0.25 mmol) Phac-Asp(OMe)-Tyr-OAl, 361 mg (1 mmol) Met-OAl·TOS, 50 mg (0.5 mmol) $KHCO_3$ and 120 mg (0.4 mmol) $Na_2CO_3 \cdot 10H_2O$ were mixed. 750 mg of α-CHY/Celite-545 were added and stirred manually every 20 min. The reaction was monitored by HPLC. After 5 h, the HPLC yield was 46.3%. The mixture was washed with EtOAc and filtered. The filtrate was evaporated to dryness under vacuum. The residue was dissolved in 40% MeOH and separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder of Phac-Asp(OMe)-Tyr-

Met-OAl (63 mg, 42.1%, m.p. 161-163ºC). To increase the yield, unreacted substates were recycled for a repeated conversion and an overall yield of 65.2% could be obtained.

(9) Synthesis of Phac-Asp(OMe)-Tyr-Met-OAl with thermolysin

220 mg (0.5 mmol) Phac-Asp(OMe)-Tyr-OH and 189 mg (1 mmol) Met-OAl were dissloved in 750 µl H_2O with 20 µl ACN and the pH was adjusted to 7.0 with 1N NaOH. After addition of 30 mg thermolysin, the mixture was stirred at 40°C. After 30 min, the reaction was complete and the HPLC yield was 81.9%. The mixture was filtered and washed with ACN. The filtrate was evaporated to dryness under vacuum. The residue was dissolved in 30% MeOH and separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder Phac-Asp(OMe)-Tyr-Met-OAl (174 mg, 58.2 %, m.p. 161-163ºC).

(10) Synthesis of Phac-Gly-Trp-OMe with papain/VA-Epoxy

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 rotating. After 3 h the reaction was complete and the HPLC yield was 97%. 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 sodium sulfate and concentrated under vacuum. The residue was lyophilized yielding a white solid of Phac-Gly-Trp-OMe (174 mg, 88.4%, m.p. 126-128ºC, HPLC system 6 with t_R 10.27, FABMS *m/z* 394.1 $[M+H^+]$, $C_{22}H_{23}N_3O_4$ requires 393.5).

(11) Synthesis of Phac-Gly-Trp-Met-OEt with immobilized α-chymotrypsin

5.0 mmol Phac-Gly-Trp ester, 4.28 g (20.0 mmol) Met-OEt·HCl, 10 g (100 mmol) $KHCO_3$ and 5.72 g (20.0 mmol) $Na_2CO_3 \cdot 10H_2O$ were mixed. Immobilized α-chymotrypsin was added and stirred manually every 20 min. The reaction was

monitored by HPLC (system 6). After the reaction was finished, the mixture was washed with water until a pH of 7.0 was reached, diluted with 100 ml 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-OEt was obtained by recrystallization with EtOH as a white solid (m.p. 174-176ºC, HPLC system 6 with t_R 13.32, FABMS *m/z* 539.1 [M+H^+], *m/z* 561.2 [M+Na^+], $C_{28}H_{34}N_4O_5S_1$ requires 538.6). The experimental data are shown in Table 4.5.

Table 4.5 Experimental data for synthesis of Phac-Gly-Trp-Met-OEt with immobilized α-chymotrypsin.

Carboxyl Component	Amino Component	Catalyst	Isolated Yield [%]
Phac-Gly-Trp-OMe	Met-OEt·HCl	2.0 g α-CHY/Celite-545	71.4
Phac-Gly-Trp-OMe	Met-OEt·HCl	2.5 g α-CHY/Eupergit C	65.2
Phac-Gly-Trp-OEt	Met-OEt·HCl	2.5 g α-CHY/Eupergit C	65.3

(12) Synthesis of Phac-Gly-Trp-Met-OAl with immobilized α-chymotrypsin

474 mg (1.2 mmol) Phac-Gly-Trp-OMe, 600 mg (1.66 mmol) Met-OAl·TOS, 120 mg (1.2 mmol) $KHCO_3$ and 105 mg (0.367 mmol) $Na_2CO_3 \cdot 10H_2O$ were mixed. Immobilized α-chymotrypsin was added and stirred manually every 20 min. The reaction was monitored by HPLC (system 6). After the reaction was finished, to the mixture 20 ml water was added, transferred to a sintered glass frit and washed with water until the filtrate showed a pH of 7.0. The filter cake was diluted with 100 ml 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 (m.p. 176-178ºC, HPLC system 6 with t_R 14.51, FABMS *m/z* 551.1 [M+H^+], $C_{29}H_{34}N_4O_5S_1$ requires 550.6). The experimental data are shown in Table 4.6.

Table 4.6 Experimental data for synthesis of Phac-Gly-Trp-Met-OAl via immobilized α-chymotrypsin.

Carboxyl Component	Amino Component	Catalyst	Isolated Yield [%]
Phac-Gly-Trp-OMe	Met-OAl·TOS	1.5 g α-CHY/Celite-545	58.5
Phac-Gly-Trp-OMe	Met-OAl·TOS	1.5 g α-CHY/Eupergit C	42.0

(13) Synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OMe with papain/VA-Epoxy

0.31 g (0.6 mmol) Phac-Gly-Trp-Met-OH, and 0.16 g (1 mmol) Asp(OMe)-OMe were dissolved in 10 ml organic solvent, 0.2 ml 0.2 M borax buffer (pH 8.1), 0.02 ml β-mercaptoethanol and 0.2 mg EDTA. To this solution 1.0 g papain/VA-Epoxy were added and thoroughly mixed by rotating. The reaction was monitored by HPLC (system 6). After reaction, 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 (2x10 ml), 5% $NaHCO_3$ (2x10 ml), and saturated sodium chloride (3x10 ml). The organic phase was dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was lyophilized yielding a white solid Phac-Gly-Trp-Met-Asp(OMe)-OMe (79.6%, mp. 118-120ºC, HPLC system 6 with t_R 9.42, FABMS *m/z* 654.0 [M+H^+], *m/z* 676.8 [M+Na^+], $C_{32}H_{39}N_5O_8S_1$ requires 653.8). The experimental data are shown in Table 4.7.

Table 4.7 Experimental data for the synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OMe with papain/VA-Epoxy from Phac-Gly-Trp-Met-OH and Asp(OMe)-OMe.

Organic Solvent	Reaction Time [h]	HPLC Yield [%]	Isolated Yield [%]
EtOAc	75	89.0	79.6
ACN	84	36.6	21.7

(14) Investigation of synthesizing Phac-Gly-Trp-Met-Asp(OMe)-OMe from Phac-Gly-Trp-Met-OEt

54 mg (0.1 mmol) Phac-Gly-Trp-Met-OEt, and 100 mg (0.5 mmol) Asp(OMe)-OMe·HCl were stirred in different reaction system under the catalysis of papain or α-chymotrypsin. When papain was used, the reaction media contained 6 μl β-mercaptoethanol and 0.5 mg EDTA. The reaction was monitored by HPLC (system 6). The experimental data are shown in Table 4.8.

Table 4.8 Experimental data for investigation of synthesizing Phac-Gly-Trp-Met-Asp(OMe)-OMe from Phac-Gly-Trp-Met-OEt and Asp(OMe)-OMe·HCl .

Reaction System	Reaction Media	Catalyst	Reaction pH	Reaction Time [d]	HPLC Yield [%]
Solvent-free system	0.2 mmol $KHCO_3$ + 0.4 mmol $Na_2CO_3 \cdot 10H_2O$	4 mg Papain	-----	1	0
		2 mg α-Chymotrypsin	-----	1	0
Buffer system	2 ml 0.2 M KH_2PO_4	4 mg Papain	5.9	3	0
Cosolvent system	0.45 ml ACN +1.05 ml 0.2 M KH_2PO_4	4 mg Papain	5.9	3	0

(15) Investigation of synthesizing Phac-Gly-Trp-Met-Asp(OMe)-OMe from Phac-Gly-Trp-Met-OAl or Phac-Gly-Trp-Met-OCam

0.6 mmol Phac-Gly-Trp-Met-OR (R=Al or Cam), and 160 mg (1 mmol) Asp(OMe)-OMe were dissolved in 10 ml EtOAc, 0.2 ml 0.2 M borax buffer (pH 8.1), 0.02 ml β-mercaptoethanol and 0.2 mg EDTA. To this solution 1.0 g papain/VA-Epoxy were added and thoroughly mixed by rotating. The reaction was monitored by HPLC (system 6). After 22 h, no coupling reaction was observed.

(16) Selective hydrolysis of Phac-Gly-Trp-Met-Asp(OMe)-OMe with α-CHY/Eupergit C

264 mg (0.4 mmol) Phac-Gly-Trp-Met-Asp(OMe)-OMe were dissolved in 4.8 ml ACN with 10.8 ml 0.05 M Tris-HCl buffer and 80 µl triethylamine. After adjusting the pH to 7.0, 800 mg α-CHY/Eupergit C was added and thoroughly mixed by rotating. The reaction was completed after 32 h. To extract the product, the mixture was diluted with 3x50 ml 80% MeOH, sonicated, and filtered. The filtrate was evaporated to dryness under vacuum. 30 ml water was added to the residue, which was extracted with 3x30 ml EtOAc. The aqueous layer was acidified to pH 3.0 with 6 M HCl and extracted with 3x30 ml EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The residue was recrystallized with EtOH to yield the product Phac-Gly-Trp-Met-Asp(OMe)-OH (163 mg, 63.7%, m.p. 150-152^oC, HPLC system 6 with t_R 6.02, FABMS *m/z* 640.0 [$M+H^+$], *m/z* 662.1 [$M+Na^+$], $C_{31}H_{37}N_5O_8S_1$ requires 639.7).

In another experiment, 1.6 ml acetonitrile with 3.6 ml 0.05 M Tris-HCl buffer was used instead of 4.8 ml acetonitrile with 10.8 ml 0.05 M Tris-HCl buffer. The isolated yield of Phac-Gly-Trp-Met-Asp(OMe)-OH was 52.2%.

(17) Selective hydrolysis of Phac-Gly-Trp-Met-Asp(OMe)-OMe with papain

261 mg (0.4 mmol) Phac-Gly-Trp-Met-Asp(OMe)-OMe were suspended in 11 ml 0.2 M KH_2PO_4 buffer (pH 7.5), 16 µl β-mercaptoethanol and 1.6 mg EDTA. After adjusting the pH to 6 with 1 M HCl, 15 mg papain was added and mixed by stirring. The reaction was monitored by HPLC (system 6). The HPLC yield was 36.2% after 4 h.

(18) Selective hydrolysis of Phac-Gly-Trp-Met-Asp(OMe)-OMe with α-chymotrypsin

33 mg (0.05 mmol) Phac-Gly-Trp-Met-Asp(OMe)-OMe were dissolved in 0.2 ml acetonitrile, 0.45 ml 0.05 M Tris-HCl buffer and 3 µl triethylamine. 10 mg α-chymotrypsin was added and thoroughly mixed by rotating. The reaction was monitored by HPLC (system 6). The experimental data are shown in Table 4.9 and Figure 3.16.

Table 4.9 Experimental data for selective hydrolysis of Phac-Gly-Trp-Met-Asp(OMe)-OMe with α-chymotrypsin.

Reaction pH	Reaction Time [h]	HPLC Yield [%]
5.6	19	24.4
7.0	19	52.0
8.0	24	35.5

(19) Selective hydrolysis of Phac-Gly-Trp-Met-Asp(OMe)-OMe with different types of α-chymotrypsin

33 mg (0.05 mmol) Phac-Gly-Trp-Met-Asp(OMe)-OMe were dissolved in 0.6 ml acetonitrile, 1.35 ml 0.05 M Tris-HCl buffer and 10 µl triethylamine. After adjusting the pH to 7.0, α-chymotrypsin or immobilized α-chymotrypsin was added and thoroughly mixed by rotating. The reaction was monitored by HPLC (system 6). The experimental data are shown in Table 3.5.

(20) Synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OH with α-chymotrypsin

100 mg (0.2 mmol) Phac-Gly-Trp-Met-OH and 240 mg (1.2 mmol) Asp(OMe)-OMe·HCl were suspended in 3 ml of 0.2 M Na_2HPO_4 buffer (pH 9.3). After adjusting the reaction mixture to pH 7.3, 20 mg α-chymotrypsin was added and the reaction mixture was stirred at room temperature. The reaction was monitored by HPLC (system 6). No coupling reaction was obseverd but Phac-Gly-Trp-OH was obtained in HPLC yield of 30.7% after 8 h.

(21) Synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OH with papain

300 mg (0.6 mmol) Phac-Gly-Trp-Met-OH and 600 mg (3 mmol) Asp(OMe)-OMe·HCl were suspended in 10 ml of 0.2 M KH_2PO_4 buffer, 36 µl β-mercaptoethanol and 3 mg EDTA. After adjusting the reaction mixture to different pH values, 30 mg papain were added and the reaction mixture was stirred at room temperature. The reaction was monitored by HPLC (system 6). The experimental data are shown in Table 4.10 and Figure 3.17.

Table 4.10 Experimental data for synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OH with papain from Phac-Gly-Trp-Met-OH and Asp(OMe)-OMe·HCl.

Reaction pH	Reaction Time [h]	HPLC Yield [%]
5.0	1	17.9
5.5	4	33.7
6.0	24	33.3
6.5	24	28.4
7.0	24	21.2
7.5	24	12.4

(22) Synthesis of Phac-Gly-Trp-Met-Ap(OMe)-OH with papain in solvent-free system

100 mg (0.2 mmol) Phac-Gly-Trp-Met-OH, 200 mg (1 mmol) Asp(OMe)-OMe·HCl, 40 mg (0.4 mmol) $KHCO_3$, 229 mg (0.8 mmol) $Na_2CO_3 \cdot 10H_2O$, 12 µl β-mercaptoethanol and 1 mg EDTA were mixed. 8 mg papain were added and stirred manually from time to time. The reaction was monitored by HPLC (system 6). After 3 days, no coupling reaction was observed.

(23) Synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OH with papain/VA-Epoxy in different reaction systems

100 mg (0.2 mmol) Phac-Gly-Trp-Met-OH and 200 mg (1 mmol) Asp(OMe)-OMe·HCl were suspended in different reaction media with 12 µl β-mercaptoethanol and 1 mg EDTA. After adjusting the reaction mixture to pH 5.9, 200 mg papain/VA-Epoxy were added and the mixture was rotated at room temperature. The reaction was monitored by HPLC (system 6). The experimental data are shown in Table 3.2.

(24) Synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OH with papain/VA-Epoxy at different temperatures

100 mg (0.2 mmol) Phac-Gly-Trp-Met-OH and 200 mg (1 mmol) Asp(OMe)-OMe·HCl were suspended in 1.5 ml of 0.2 M KH_2PO_4 buffer (pH 8.5), 12

µl β-mercaptoethanol and 1 mg EDTA. After adjusting the reaction mixture to pH 5.9, 200 mg papain/VA-Epoxy was added into it and the mixture was rotated at different temperatures. The reaction was monitored by HPLC (system 6). The experimental data are shown in Table 3.3.

(25) Synthesis of Phac-Gly-Trp-Met-Asp(OMe)-OH with papain/VA-Epoxy

500 mg (1.0 mmol) Phac-Gly-Trp-Met-OH and 1 g (5.1 mmol) Asp(OMe)-OMe·HCl were suspended in 7.5 ml of 0.2 M KH_2PO_4 buffer (pH 8.5), 60 µl β-mercaptoethanol and 5 mg EDTA. After adjusting the reaction mixture to pH 5.9, 1 g papain/VA-Epoxy was added and the reaction mixture was rotated at 37°C. The reaction was monitored by HPLC (system 6) and stopped when the reaction reached its optimum. The mixture was filtered and washed with 3x50 ml 80% MeOH. The filtrate was concentrated to dryness. The residue was dissolved in 40% MeOH and separated by MPLC. The pooled fractions of Phac-Gly-Trp-Met-Asp(OMe)-OH were lyophilized twice yielding a white powder (239 mg, 37.4 %, m.p. 150-152ºC). The pooled fractions of the staring material Phac-Gly-Trp-Met-OH were lyophilized twice and were used for a repeated conversion, which allowed to increase the yield from 37.4% to 53.0%.

(26) Synthesis of Phac-Gly-Trp-Met-Asp(OR)-OH with papain

200 mg (0.4 mmol) Phac-Gly-Trp-Met-OH and 2 mmol Asp(OR)-OR·HCl (R = Me, Et) were suspended in 6 ml of 0.2 M KH_2PO_4 buffer, 24 µl β-mercaptoethanol and 2 mg EDTA. After adjusting the reaction mixture to pH 5.9, 16 mg papain was added and the reaction mixture was stirred at 37°C. The reaction was monitored by HPLC (system 6) and stopped the reaction at its optimal point. The mixture was filtered and washed with 3x50 ml 80% MeOH. The filtrate was concentrated to dryness. The residue was dissolved in 40% MeOH and separated by MPLC. The pooled fractions of Phac-Gly-Trp-Met-Asp(OR)-OH were lyophilized twice yielding a white powder. The experimental data are shown in Table 4.11.

Table 4.11 Experimental data for synthesis of Phac-Gly-Trp-Met-Asp(OR)-OH via papain from Phac-Gly-Trp-Met-OH.

R	Product	Reaction Time [h]	HPLC Yield [%]	Isolated Yield [%]
Me	Phac-Gly-Trp-Met-Asp(OMe)-OH	2.5	46.1	24.0
Et	Phac-Gly-Trp-Met-Asp(OEt)-OH	1	21.1	5.9

Phac-Gly-Trp-Met-Asp(OEt)-OH: decomposition at 155ºC, HPLC system 6 with t_R 10.05, FABMS *m/z* 654.2 [M+H$^+$], *m/z* 676.1 [M+Na$^+$], $C_{32}H_{39}N_5O_8S_1$ requires 653.2.

(27) Synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 with thermolysin in water

128 mg (0.2 mmol) Phac-Gly-Trp-Met-Asp(OMe)-OH and 200 mg (1.0 mmol) Phe-NH_2·HCl were suspended in 1.1 ml water. After adjusting the reaction mixture to pH 7.0 with 1 M NaOH, 0.3 mg thermolysin were added and the reaction mixture was stirred at room temperature. After 6.5 h, the mixture was filtered and washed with 3x50 ml MeOH. The filtrate was concentrated to dryness. The residue was dissolved in 50% MeOH and separated by MPLC. The pooled fractions of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 were lyophilized twice yielding a white powder (71 mg, 45.2%, decomposition at 220ºC, HPLC system 6 with t_R 13.95, FABMS m/z 786.1 [M+H$^+$], m/z 808.1 [M+Na$^+$], $C_{40}H_{47}N_7O_8S_1$ requires 785.9).

A by-product was isolated (10 mg, 9.5%) and characterized as Phac-Gly-Trp-Phe-NH_2 by FABMS *m/z* 526.2 [M+H$^+$], *m/z* 548.1 [M+Na$^+$], $C_{30}H_{31}N_5O_4$ requires 525.6, HPLC system 6 with t_R 11.99, m.p. 188-190ºC.

(28) Synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 with thermolysin in the solvent-free system

100 mg (0.16 mmol) Phac-Gly-Trp-Met-Asp(OMe)-OH, 156 mg (0.78 mmol) Phe-NH_2·HCl, 390 mg (3.9 mmol) $KHCO_3$, 223 mg (0.78mmol) Na_2CO_3·$10H_2O$, were mixed. 4 mg thermolysin were added and stirred manually from time to time.

The reaction was monitored by HPLC (system 6). After 22.5h, the HPLC yield of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 was 1.7%.

(29) Synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 with thermolysin in ACN at different temperatures

100 mg (0.16 mmol) Phac-Gly-Trp-Met-Asp(OMe)-OH and 156 mg (0.78 mmol) Phe-NH_2·HCl were suspended in 4.2 ml acetonitrile containing 168 µl 0.05 M Tris-HCl buffer (pH 8.5). After adjusting the reaction mixture to pH 7.0 with 4 M NaOH, 4 mg thermolysin were added and the reaction mixture was stirred at different temperatures. For the reaction at 40°C, it was completed after 11 h. The mixture was filtered and washed with 3x50 ml 80% MeOH. The filtrate was concentrated to dryness. The residue was dissolved in 50% MeOH and separated by MPLC. The pooled fractions of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 were lyophilized twice yielding a white powder (81 mg, 64.4%, decomposition at 220°C). The experimental data with yields are shown in Table 3.4.

(30) Synthesis of Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 with immobilized thermolysin in ACN

100 mg (0.16 mmol) Phac-Gly-Trp-Met-Asp(OMe)-OH and 156 mg (0.78 mmol) Phe-NH_2·HCl were suspended in 4.2 ml acetonitrile containing 168 µl 0.05 M Tris-HCl buffer (pH 8.5). After adjusting the reaction mixture to pH 7.0 with 4 M NaOH, immobilized thermolysin was added and the reaction mixture was stirred at 40°C. The reaction was monitored by HPLC (system 6). The experimental data with yields are shown in Table 3.6.

(31) Synthesis of Phac-Gly-Trp-Met-Asp(OH)-Phe-NH_2 with thermolysin in water

62 mg (0.1 mmol) Phac-Gly-Trp-Met-Asp(OH)-OH and 160 mg (0.8 mmol) Phe-NH_2·HCl were suspended in 0.36 ml water. After adjusting the reaction mixture to pH 7.0 with 4 M NaOH, 0.06 mg thermolysin were added into it and the reaction mixture was stirred at room temperature. The reaction was monitored by HPLC

(system 6). After 1 day, the HPLC yield of Phac-Gly-Trp-Met-Asp(OH)-Phe-NH_2 was 5.5%.

(32) Investigation of cleaving Phac-group from Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 with PA/Eupergit C

39 mg (0.05 mmol) Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 were dissolved in 3 ml EtOH, 3 ml ACN and 8 ml 0.1 M Na_2HPO_4 buffer (pH 8.0). After addition of 850mg PA/Eupergit C, the mixture was rotated at 35°C for 25 h. No cleavage of Phac-group was observed but Phac-Gly-Trp-Met-Asp(OH)-Phe-NH_2 was obtained in an isolated yield of 40.2%.

(33) Cleavage Phac-group from Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 via PGA/Eupergit C

80 mg (0.1 mmol) Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 were suspended in 10 ml 1 M KH_2PO_4 buffer (pH 7.5) and 2.4 ml EtOH. After addition of 200 mg PGA/Eupergit C, the mixture was rotated at 35°C for 24 h. To extract the product, the mixture was diluted with 50 ml 80% MeOH, sonicated, and filtered. The filtrate was evaporated to dryness under vacuum. The residue was separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder Gly-Trp-Met-Asp(OMe)-Phe-NH_2 (16 mg, 24.0%, decomposition at 210^{o}C, HPLC system 6 with t_R 9.13, FABMS m/z 668.0 [M+H^+], m/z 690.2 [M+Na^+], $C_{32}H_{41}N_7O_7S_1$ requires 667.8).

(34) Cleavage Phac-group from Phac-Gly-Trp-Met-Asp(OH)-Phe-NH_2 via PGA/Eupergit C (Xiang et al. 2004)

0.2g (0.26 mmol) Phac-Gly-Trp-Met-Asp(OH)-Phe-NH_2 were suspended in 10 ml H_2O and the pH was adjusted with 1M NaOH to 7.6. After addition of 0.4 g PGA/Eupergit C, the mixture was rotated at 35°C for 24 h. To extract the product, the mixture was diluted with 20 ml 80% EtOH, sonicated, and filtered. The filtrate was evaporated to dryness under vacuum. The residue was separated by preparative HPLC with an eluent of 60% B. The residue was dissolved in 5 ml 60% B and the desired

product was eluted as the second peak. The pooled fractions were lyophilized twice yielding a white powder Gly-Trp-Met-Asp-Phe-NH_2 (32 mg, 18.8%, decomposition at 230ºC, HPLC system 6 with t_R 6.73, FABMS m/z 654.2 [M+H^+], m/z 676.2 [M+Na^+], $C_{31}H_{39}N_7O_7S_1$ requires 653.7).

(35) Synthesis of Phac-Asp(OMe)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 via α-CHY/Eupergit C

To a solution of 60 mg (0.1 mmol) Phac-Asp(OMe)-Tyr-Met-OAl and 31 mg (0.046 mmol) Gly-Trp-Met-Asp(OMe)-Phe-NH_2 in 5 ml ACN containing 25 µl 0.05 M Tris-HCl buffer (pH 9.0) and 25 µl Et_3N, 100 mg α-CHY/Eupergit C were added. The reaction mixture was rotated at room temperature. The reaction was monitored by HPLC (system 6). After 63 h, the total HPLC yield (25.8%) of a group of new peaks with t_R from11.99 to 12.47 (HPLC system 7) was obtained. To extract the products, the mixture was diluted with 50 ml 80% MeOH, sonicated, and filtered. The filtrate was evaporated to dryness under vacuum. The residue was separated by preparative HPLC with an eluent of 85% B. The pooled fractions of the new substances were lyophilized twice yielding a white powder (14 mg, 25.2%). LC-ESIMS was used to investigate the mixture of new substances (LC-ESIMS: compound **1** (product) Phac-Asp(OMe)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-NH_2, HPLC system in LC-ESIMS with t_R 15.253 (44.9%); ESIMS: 14.0 min, m/z 1209.6 [M+H^+], m/z 1231.7 [M+Na^+], $C_{59}H_{72}N_{10}O_{14}S_2$ requires 1208.9. Presumed compound **2** and compound **3,** HPLC system in LC-ESIMS with t_R 15.675 (55.1%). ESIMS of presumed compound **2**: 14.5 min, m/z 1177.5 [M+H^+], m/z 1199.5 [M+Na^+], $C_{58}H_{68}N_{10}O_{13}S_2$ requires 1176.6. ESIMS of presumed compound **3**:14.8 min, m/z 1145.5 [M+H^+], m/z 1167.6 [M+Na^+], $C_{57}H_{64}N_{10}O_{12}S_2$ requires 1145.6 (Figure 3.18 – 3.21). The product Phac-asp(OMe)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 was obtained in a HPLC yield of 11.5%.

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 these enzymatically catalyzed reactions are economically and environmentally superior to classical organic reactions. The objective of this thesis is to develop a synthetic path to the cholecystokinin octapeptide CCK-8 using exclusively enzymatic methods. The fragment CCK-8 has nearly the full biological activity of cholecystokinin and its therapeutic potential against type 2 diabetes, obesity and epilepsy is studied intensively.

During the coupling process, many side reactions observed in the chemical peptide synthesis can be avoided in the enzymatic peptide synthesis. However, each coupling reaction has to be optimized. That means the optimal strategy, substrate structure and concentration, reaction media and temperature, type of the protease and its support for enzyme deposition.

In the synthesis of the N-terminal tripeptide fragment Phac-Asp(OMe)-Tyr-Met-OAl, two strategies (Scheme 3.5, Scheme 3.6) with stepwise peptide chain elongation from the N-terminus to the C-terminus were investigated. Because of less reaction steps, higher overall yield and more economic starting materials, the second strategy was superior to be integrated in the final CCK-8 assembly. Compared to the reported synthesis of the tripeptide fragment Phac-Asp(OBu^t)-Tyr-Met-OAl (Fite et al., 2002) in the second strategy two reaction steps could be avoided to synthesize an OCam ester and an overall yield of 45% of Phac-Asp(OMe)-Tyr-Met-OAl could be obtained.

The pentapeptide fragment CCK-5 was synthesized by a stepwise N→C coupling strategy using the enzymes papain, α-chymotrypsin, thermolysin and penicillin G amidase.

In comparison with earlier developed syntheses of CCK-5 in our laboratory (Xiang et al., 2004) and in another group (Fite et al., 2002) several chemical reaction steps could be circumvented. But still one OCam ester was necessary. In the coupling with Met-OEt·HCl as nucleophile in a solvent free system, the OMe ester of Phac-Gly-Trp-OMe could be used directly as acyl-donor. Thus three reaction steps, otherwise necessary for the preparation of an OCam ester as reported (Capellas et al., 1996), could be avoided.

Except of the Asp-Phe coupling using free thermolysin, all other peptide couplings and the cleavage of the Phac-group at the end of the synthesis could be performed with covalently immobilized enzymes. This is important for the large scale synthesis of peptides and for therapeutical applications.

According to the literature, it is necessary that acyl-donors need to carry an ester group to form the acyl-enzyme with serine or cysteine proteases. However, in this work and also in earlier investigations in our group we observed that quite often peptide fragments with free carboxyl groups are good acyl-donors for couplings with papain and α-chymotrypsin. For the elongation of Phac-Gly-Trp-Met-OH to the tetrapeptide Phac-Gly-Trp-Met-Asp(OMe)-OH, the free carboxyl group of the acyl-donor was necessary. Neither an alkyl ester nor the usually most reactive OCam ester was a useful substrate for papain.

The final fragment condensation of the tripeptide Phac-Asp(OMe)-Tyr-Met-OAl and the pentapeptide Gly-Trp-Met-Asp(OMe)-Phe-NH_2 could be achieved with α-chymotrypsin/Eupergit C. According to LC-ESIMS the condensation reaction was quite effective. The protected CCK-8 peptide was formed in the HPLC yield of 11.5%. A by-product was an octapeptide with 32 mass units less. Most likely one of the ß-esters of the aspartic acid residue underwent a ring closure reaction, quite often observed as one of the most common side reactions of aspartic acid moieties under basic conditions. Another by-product with 64 mass units less was probably formed

when both aspartic acid residues underwent ring closure reactions. It will need further investigation to avoid these side reactions.

6 References

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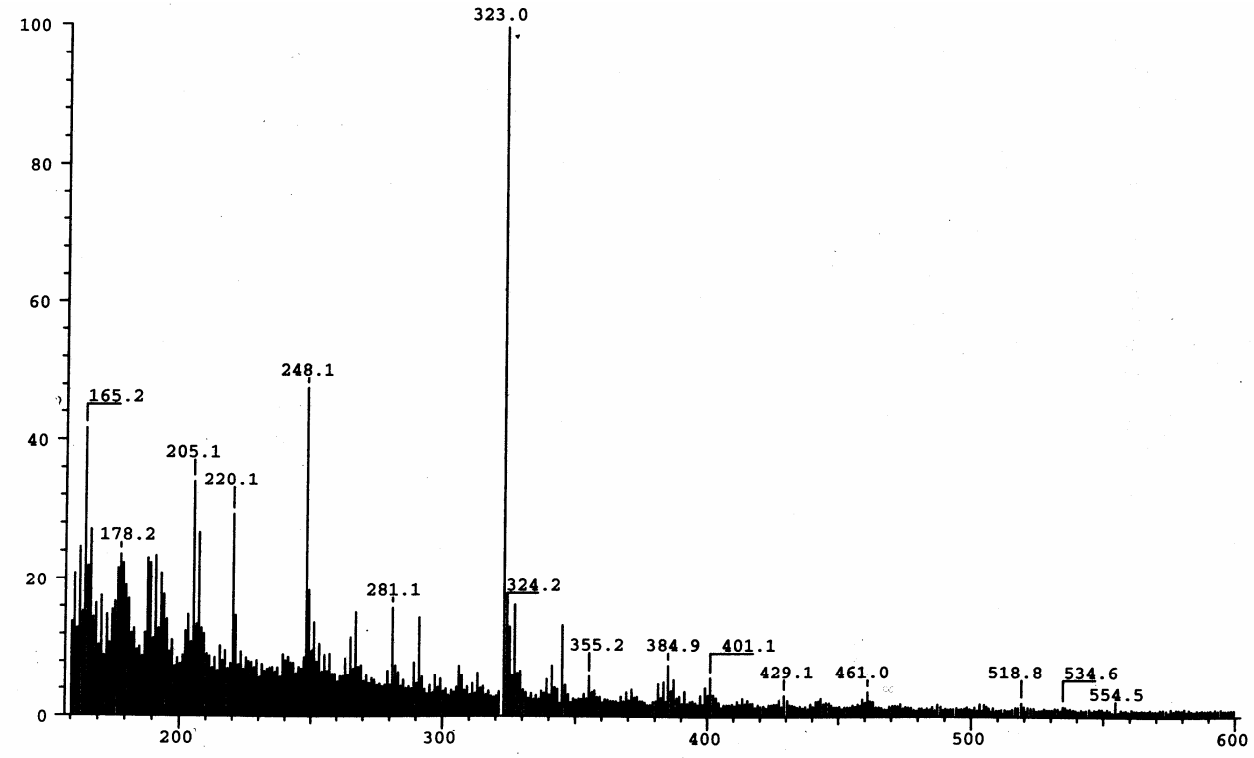
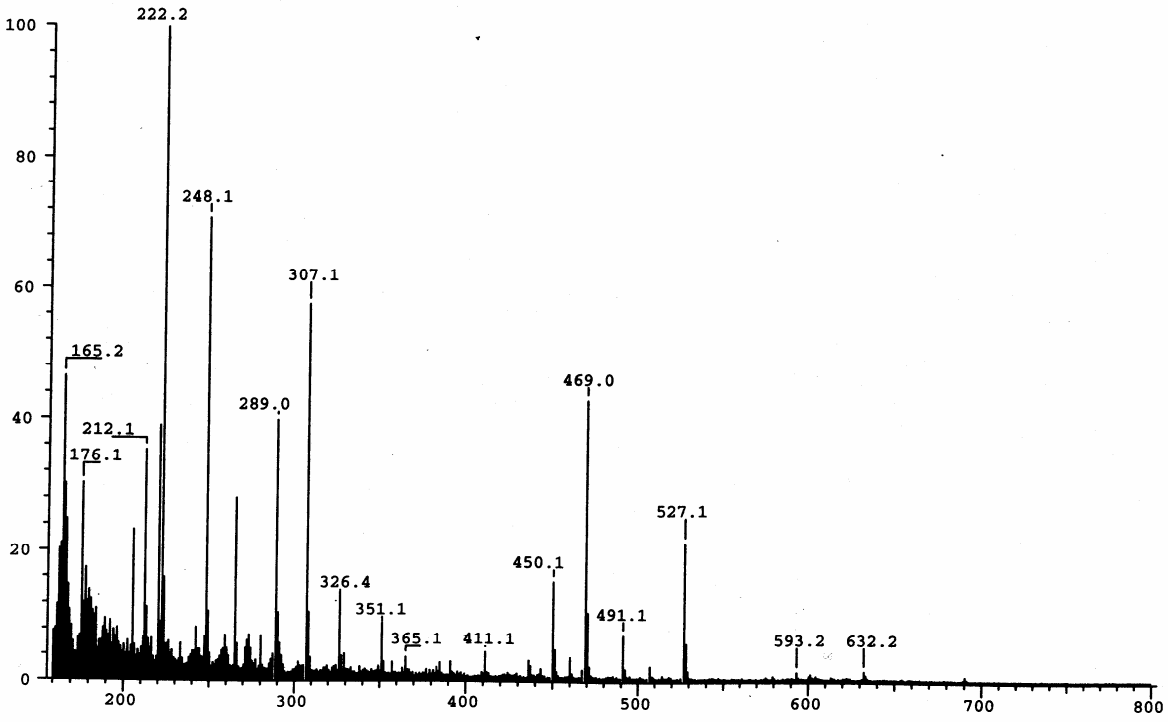
7 Appendix

FABMS spectra

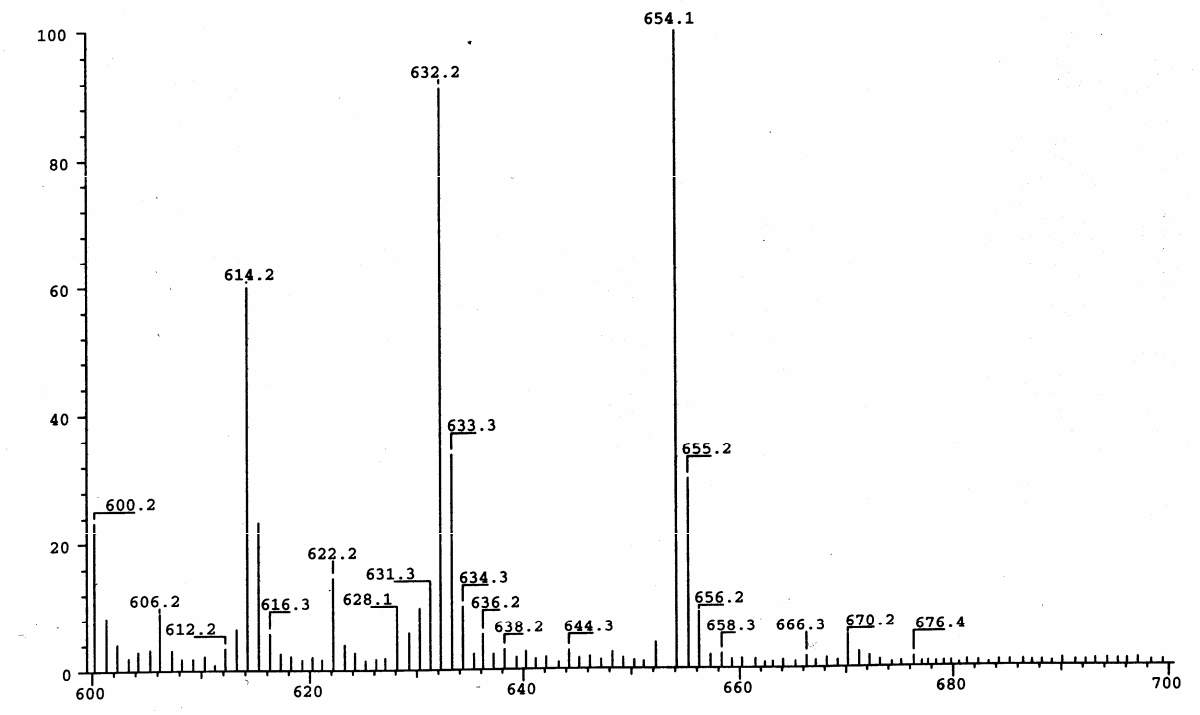
(1) Phac-Asp(OMe)-OCam, [M+H$^+$]=323.0, [M+Na$^+$]=355.2



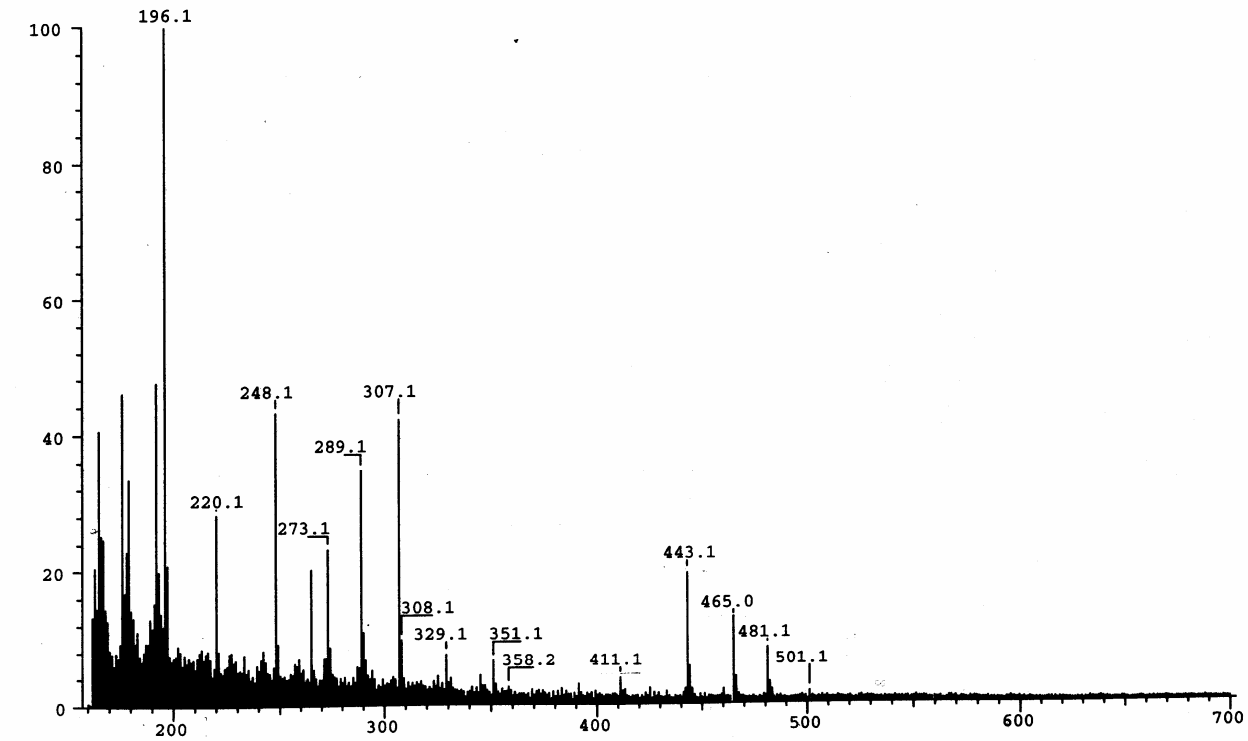
(2) Phac-Asp(OMe)-Tyr-OAl, [M+H$^+$]=469.0, [M+Na$^+$]=491.1



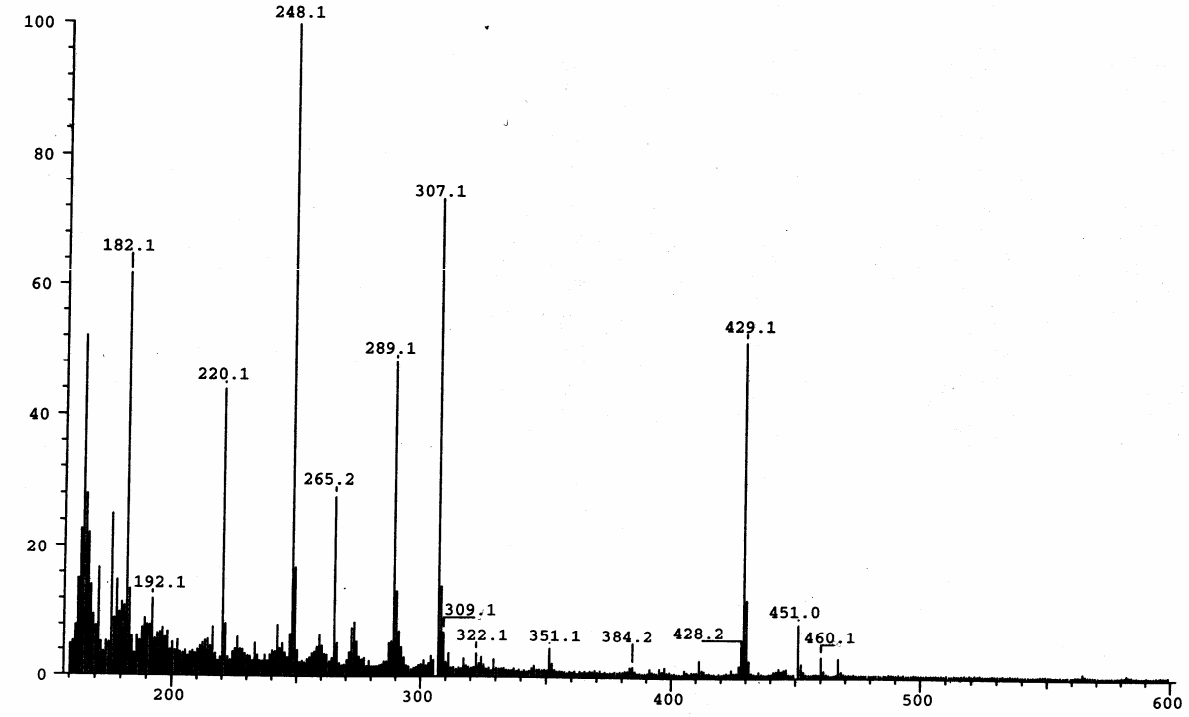
(3) Phac-Asp(OMe)-Tyr-Tyr-OAl, [M+H$^+$]=632.2, [M+Na$^+$]=654.1



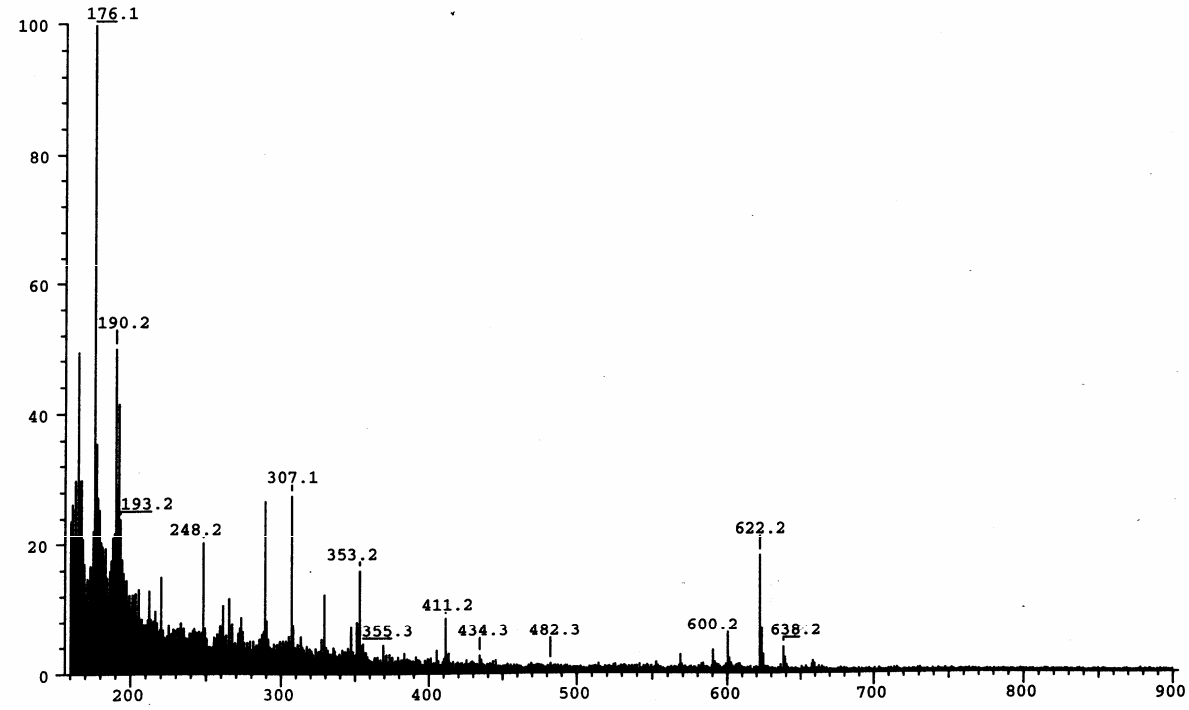
(4) Phac-Asp(OMe)-Tyr-OMe, [M+H$^+$]=443.1, [M+Na$^+$]=465.0



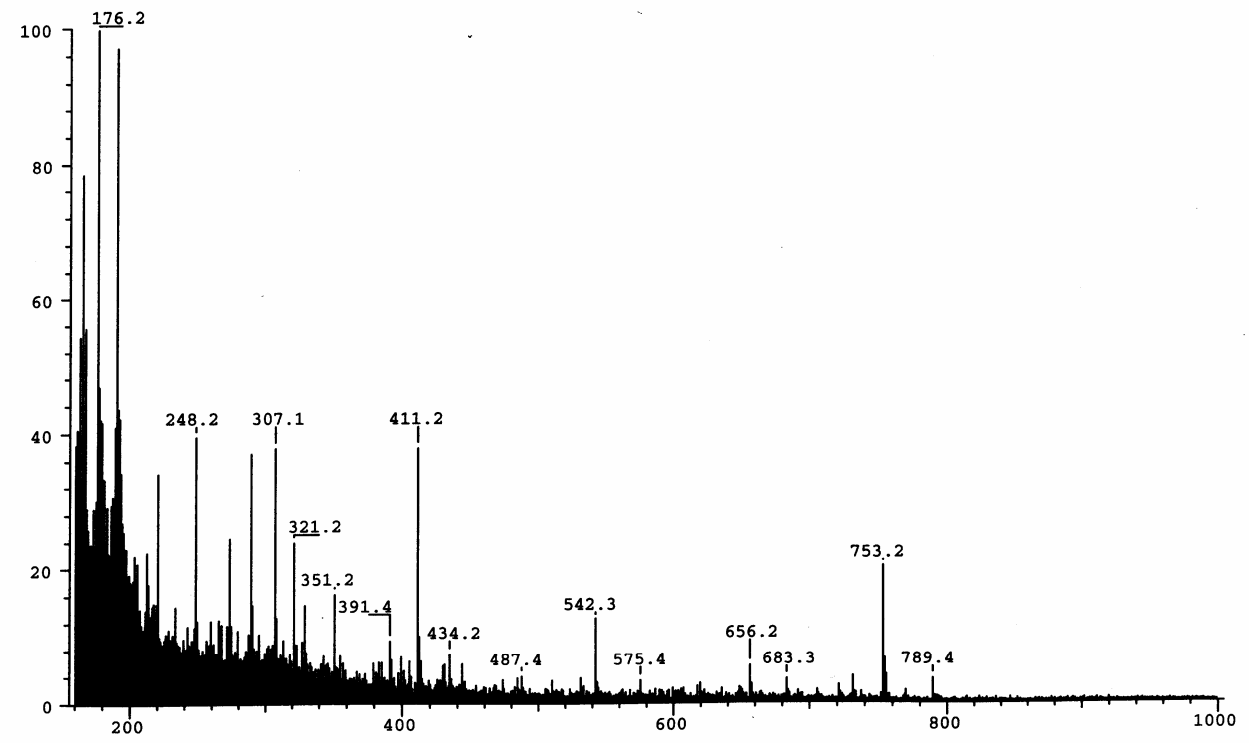
(5) Phac-Asp(OMe)-Tyr-OH, [M+H$^+$]=429.1, [M+Na$^+$]=451.0



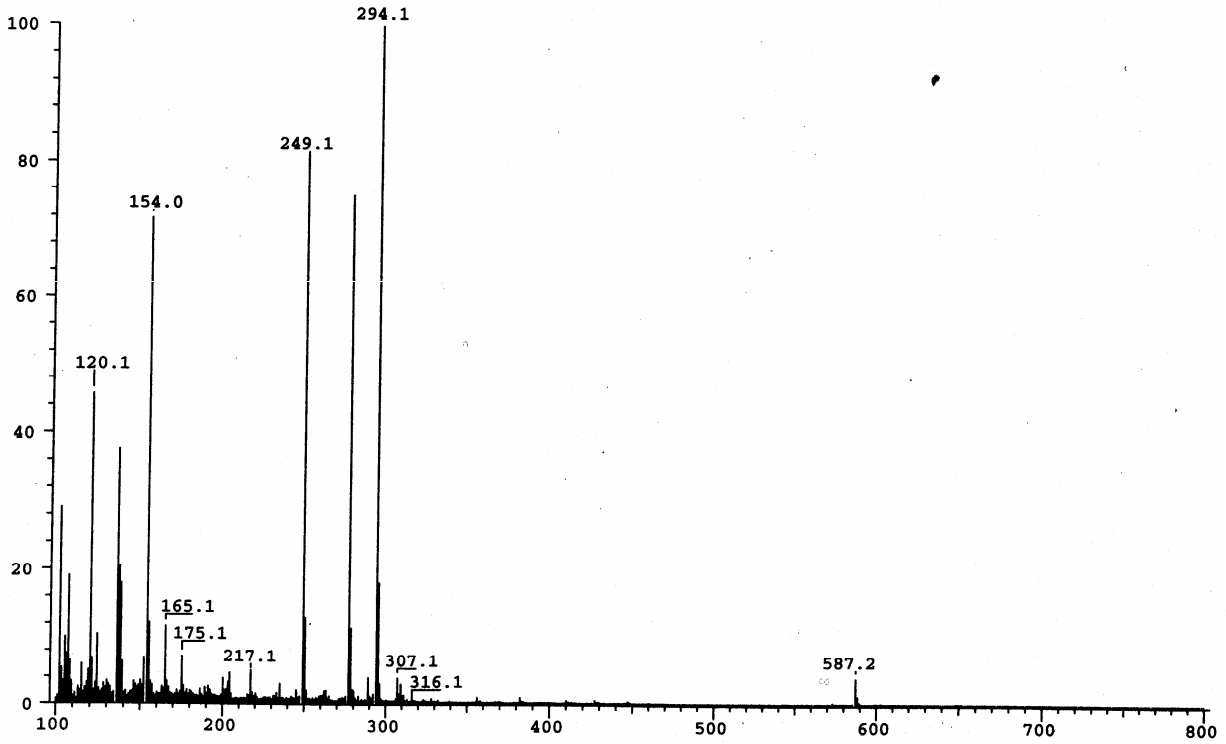
(6) Phac-Asp(OMe)-Tyr-Met-OAl, [M+H$^+$]=600.2, [M+Na$^+$]=622.2



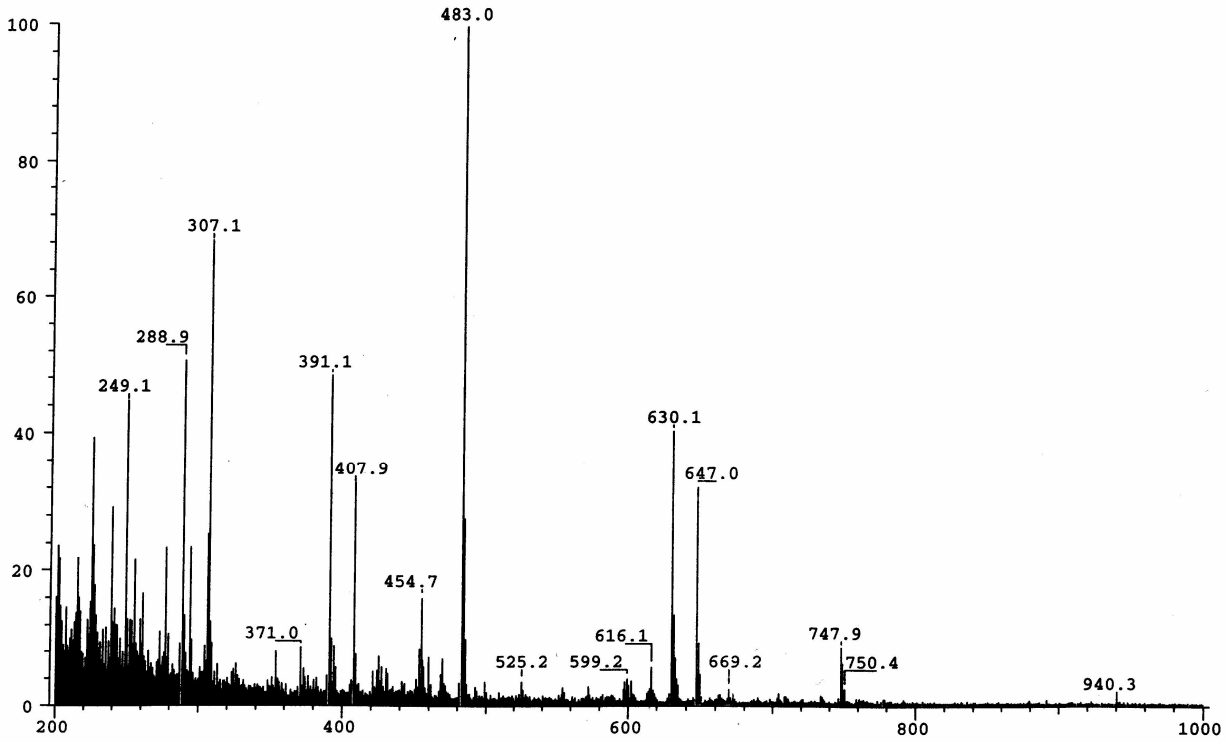
(7) Phac-Asp(OMe)-Tyr-Met-Met-OAl, [M+H$^+$]=731.2, [M+Na$^+$]=753.2



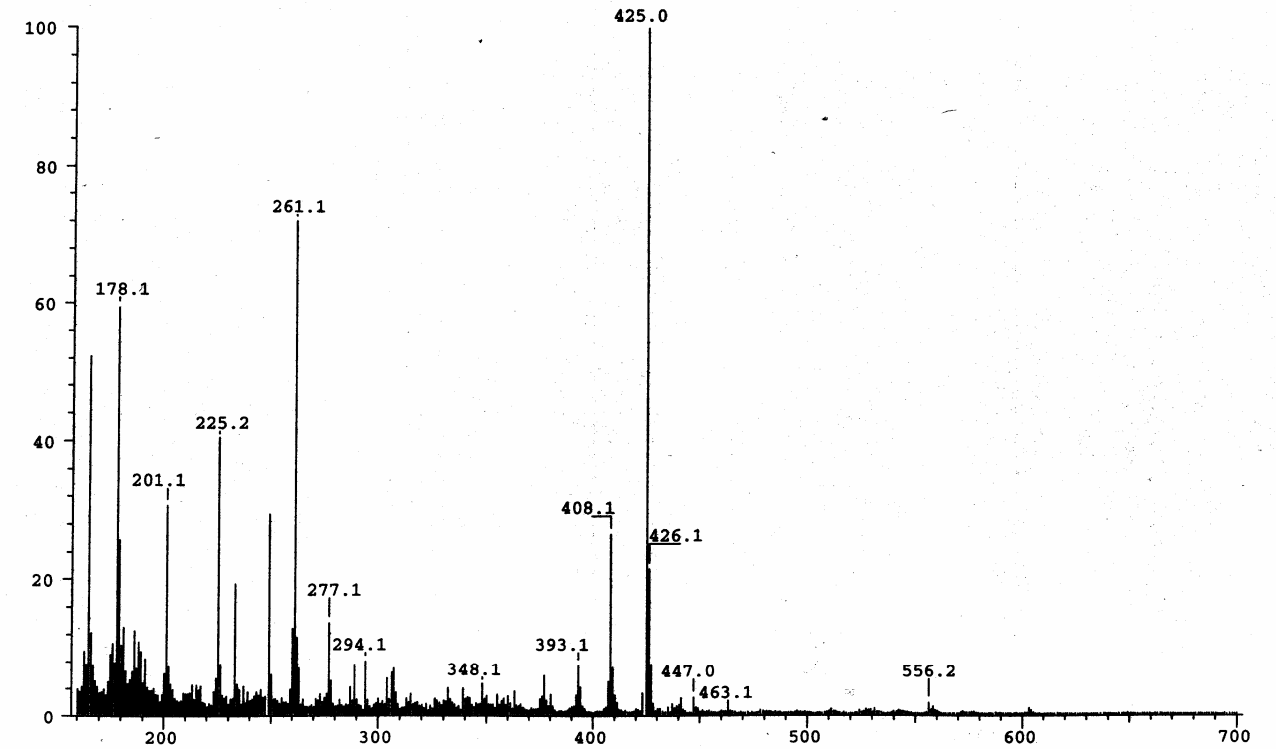
(8) Asp(OMe)-Phe-NH$_2$, [M+H$^+$]=294.1, [M+Na$^+$]=316.1



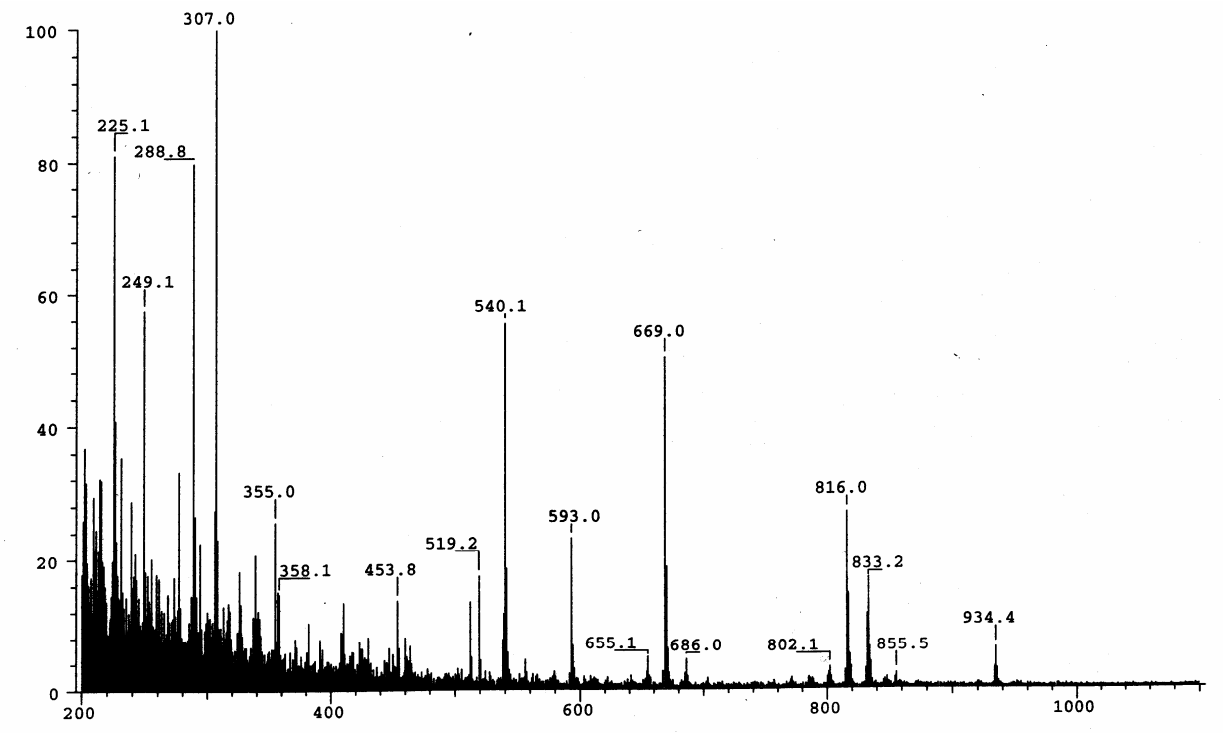
(9) Fmoc-Met-Asp(OMe)-Phe-NH_2, [M+H^+]=647.0, [M+Na^+]=669.2.



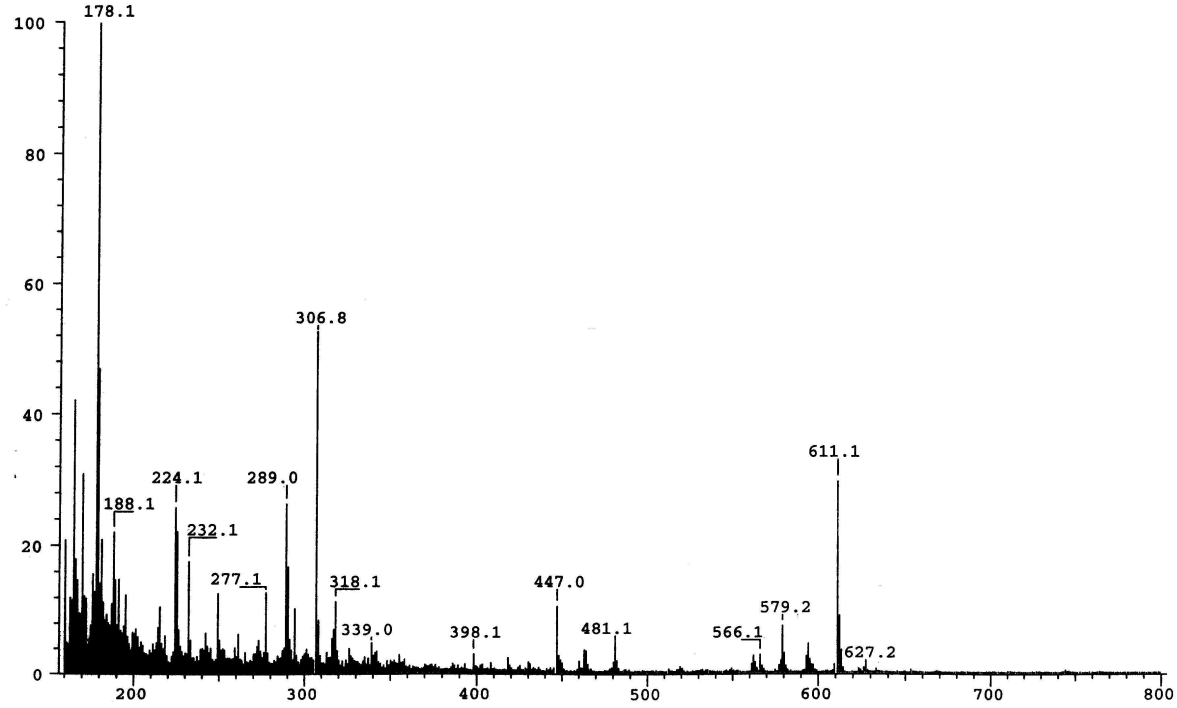
(10) Met-Asp(OMe)-Phe-NH_2, [M+H^+]=425.0, [M+Na^+]=447.0



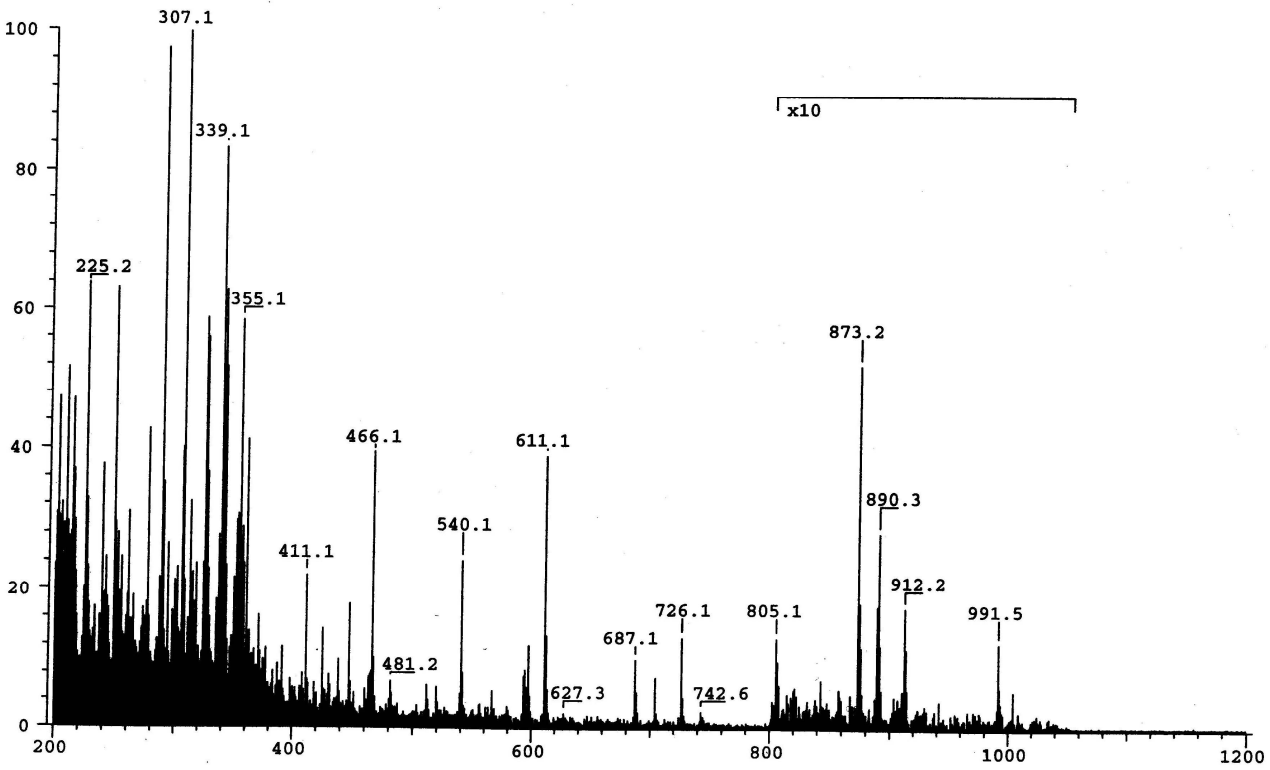
(11) Fmoc-Trp-Met-Asp(OMe)-Phe-NH_2, $[M+H^+]$=833.2, $[M+Na^+]$=855.5



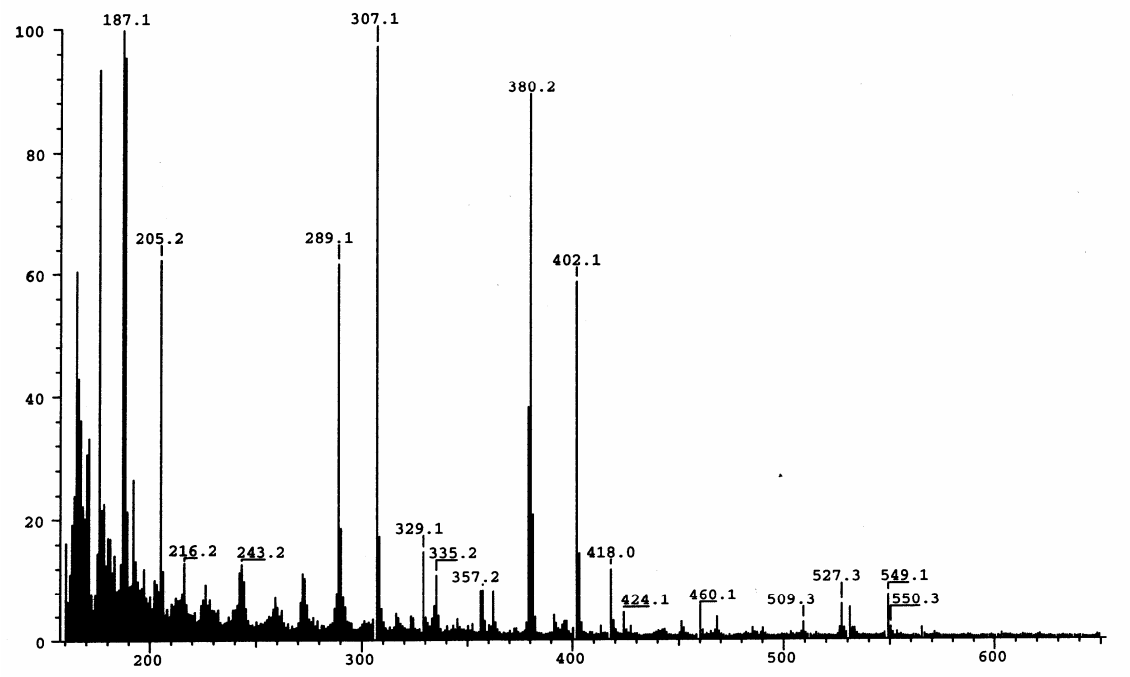
(12) Trp-Met-Asp(OMe)-Phe-NH_2, $[M+H^+]$=611.1



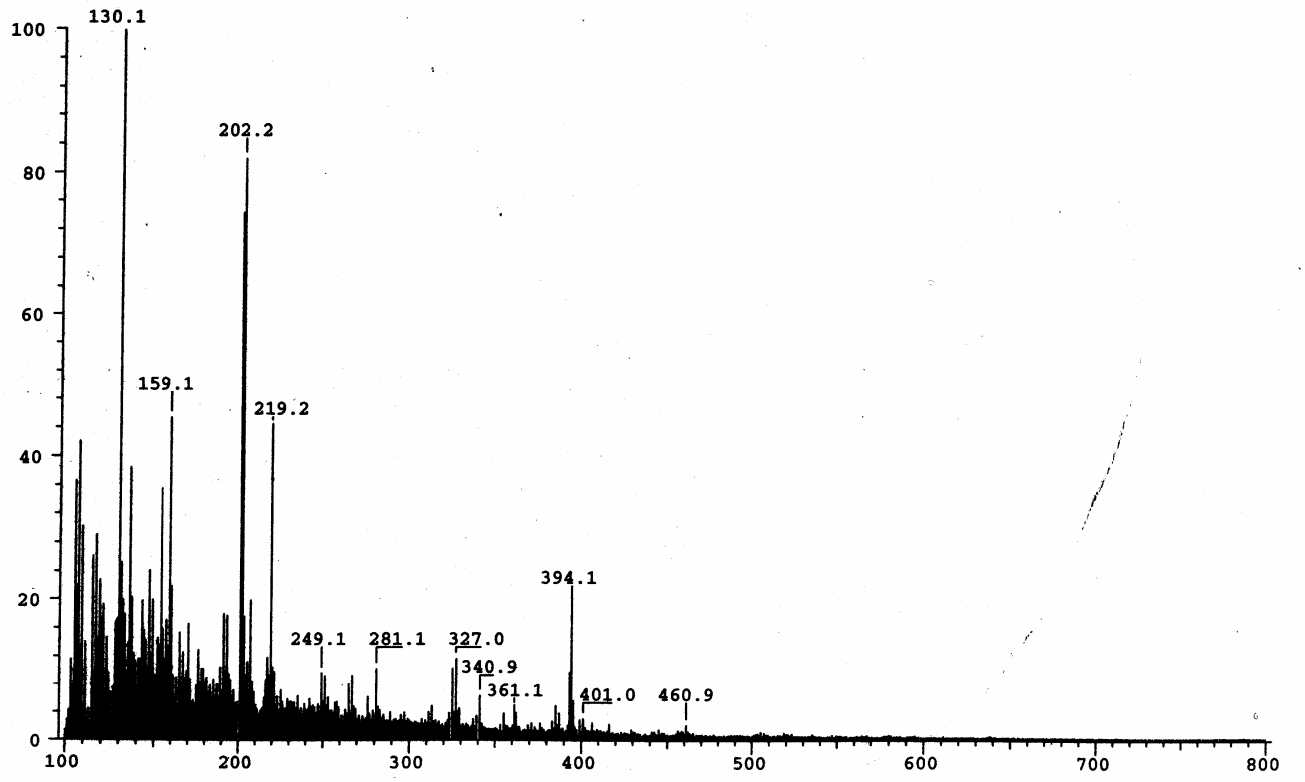
(13) Fmoc-Gly-Trp-Met-Asp(OMe)-Phe-NH_2, $[M+H^+]$=890.3, $[M+Na^+]$ =912.2



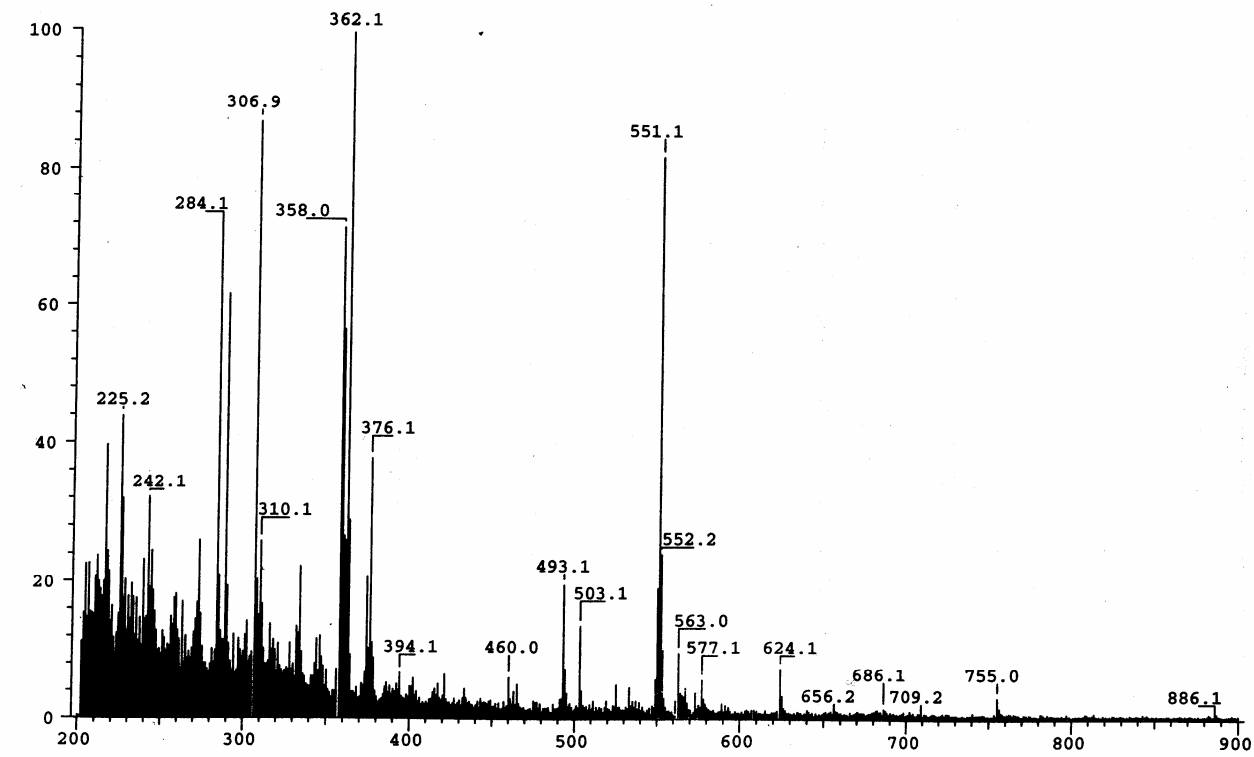
(14) Phac-Gly-Trp-OH, $[M+H^+]$=380.2, $[M+Na^+]$=402.1



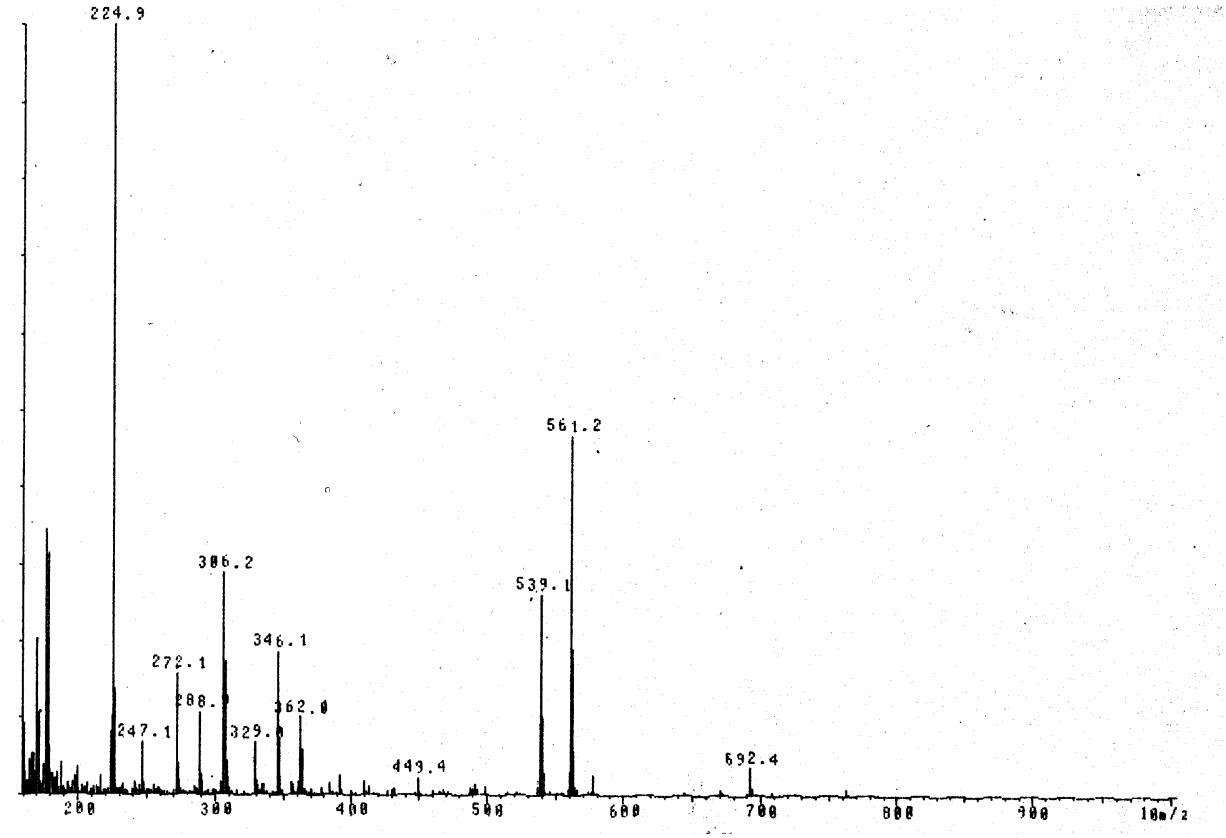
(15) Phac-Gly-Trp-OMe, [M+H$^+$]=394.1



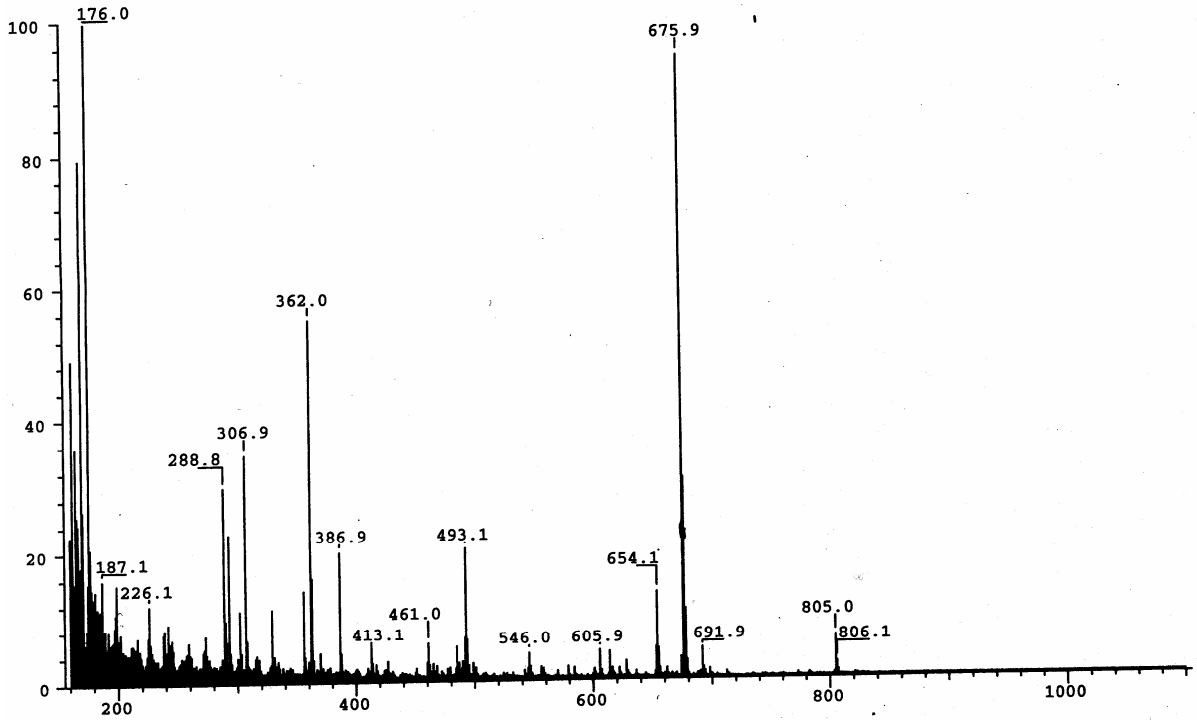
(16) Phac-Gly-Trp-Met-OAl, [M+H$^+$]=551.1



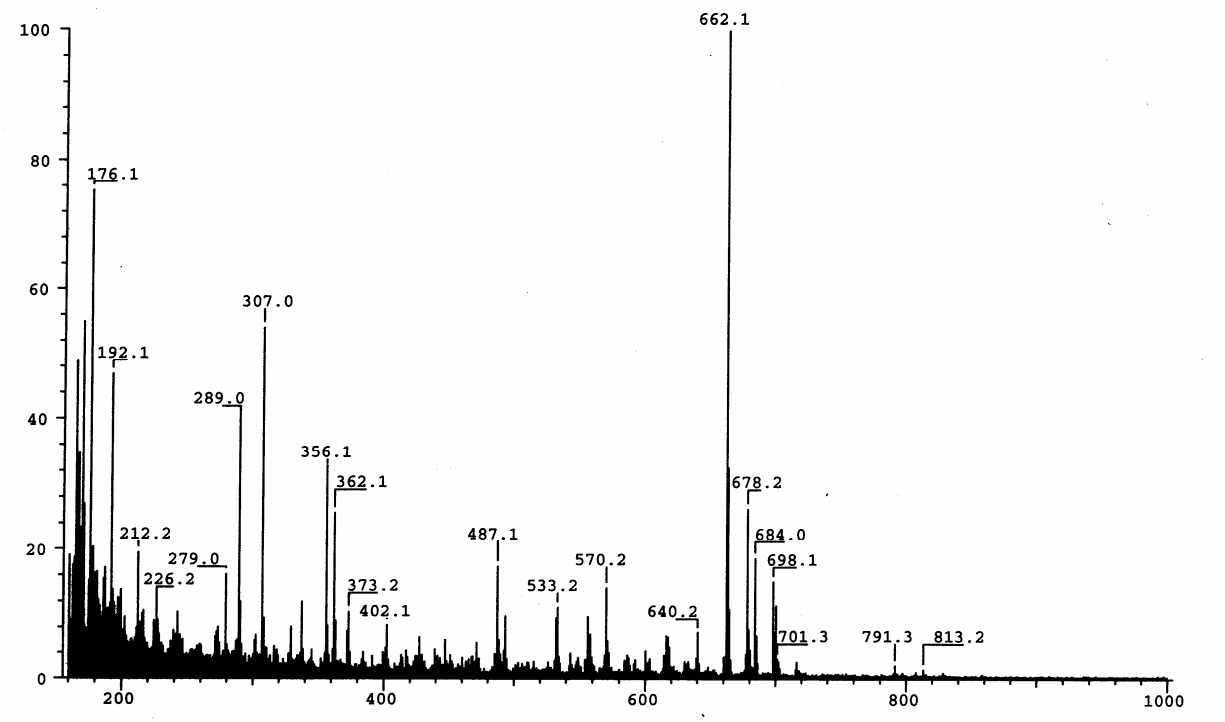
(17) Phac-Gly-Trp-Met-OEt, [M+H$^+$]=539.1, [M+Na$^+$]=561.2



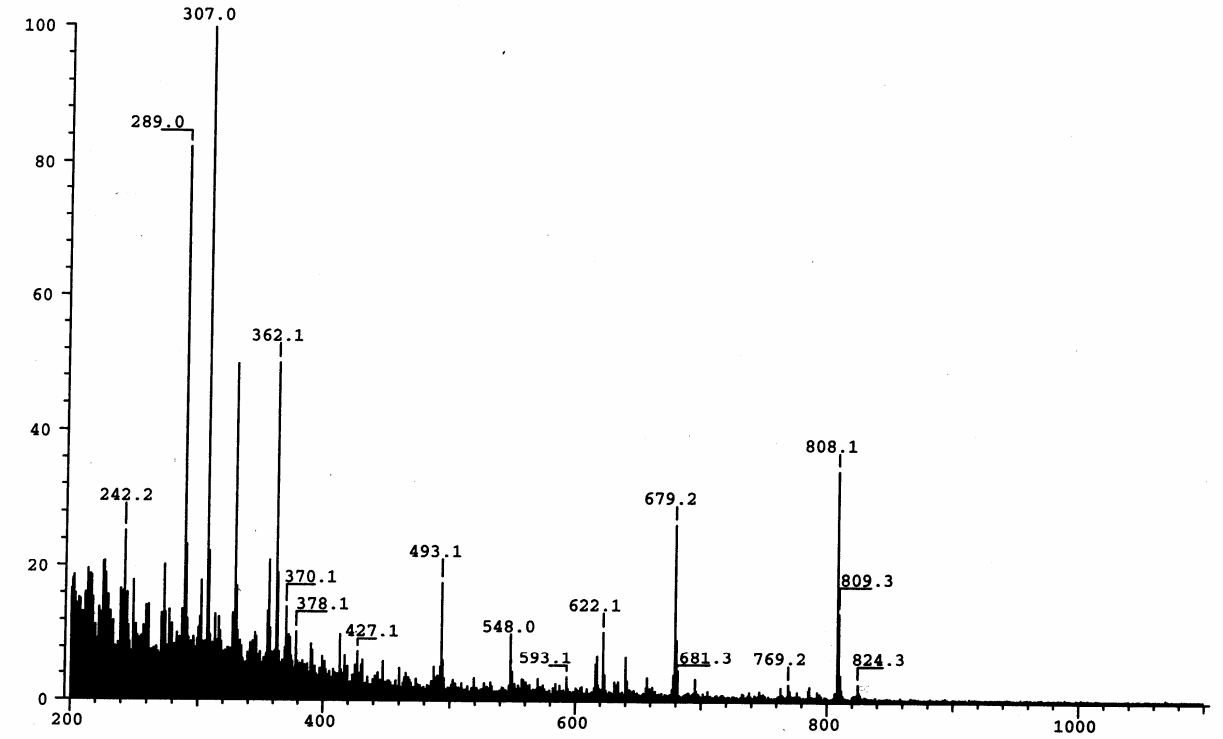
(18) Phac-Gly-Trp-Met-Asp(OMe)-OMe, [M+H$^+$]=654.1, [M+Na$^+$] =676.0



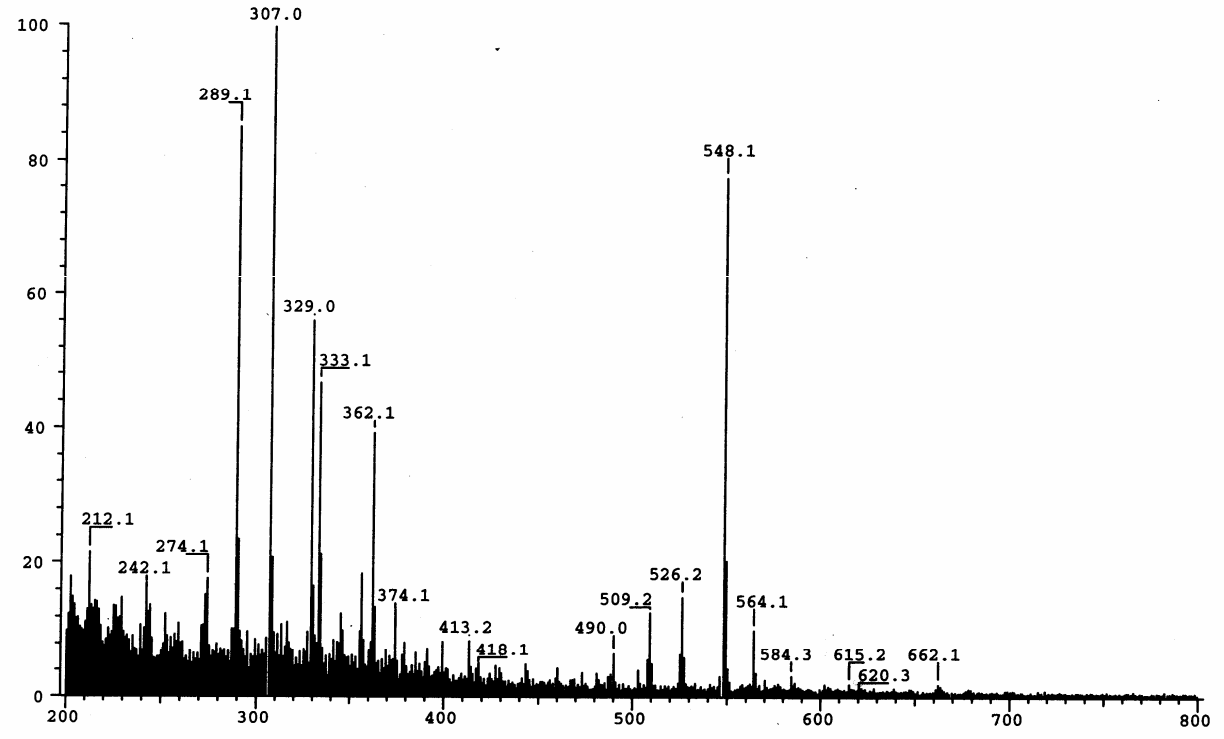
(19) Phac-Gly-Trp-Met-Asp(OMe)-OH, [M+H$^+$]=640.2, [M+Na$^+$]=662.1



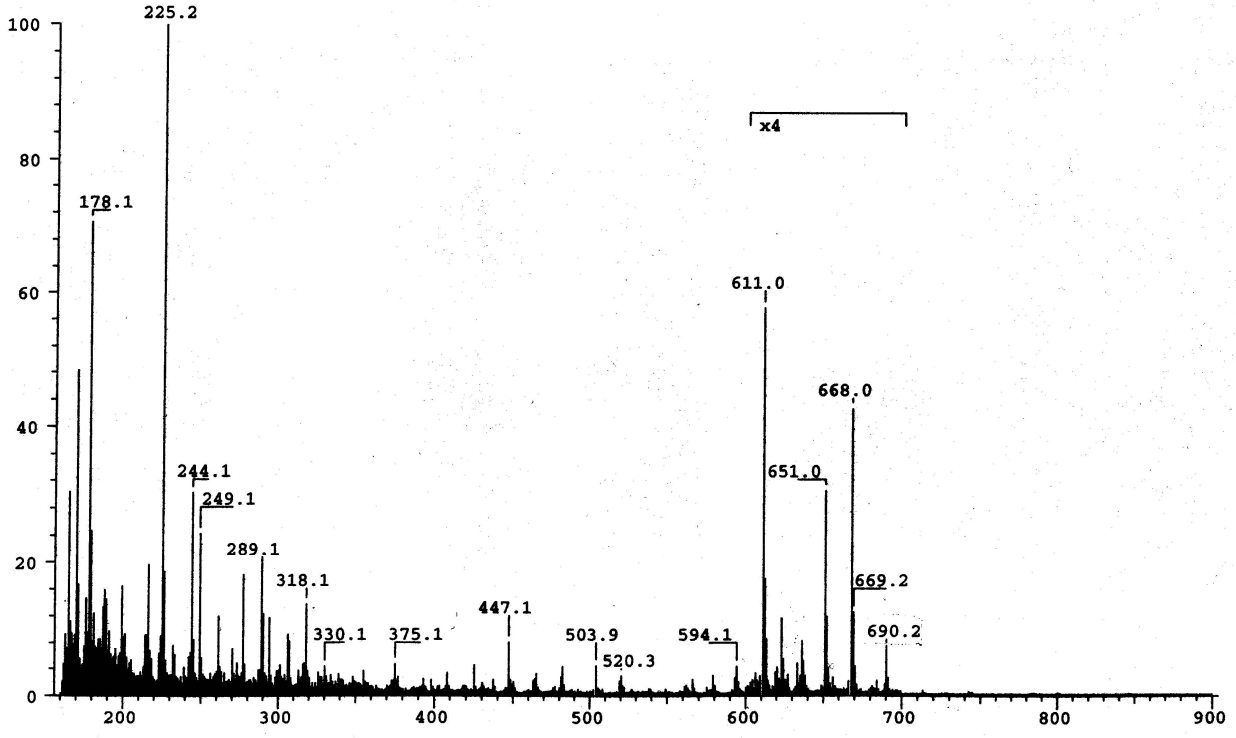
(20) Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH2, [M+Na$^+$]=808.1



(21) Phac-Gly-Trp-Phe-NH2, [M+H$^+$]=526.2, [M+Na$^+$]=548.1



(22) Gly-Trp-Met-Asp(OMe)-Phe-NH2, [M+H$^+$]=668.0, [M+Na$^+$]=690.2



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