INVESTIGATIONS ON ENZYME-CATALYZED PEPTIDE SYNTHESIS

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ABBREVIATIONS

Z	benzyloxycarbonyl
BOC	tert-butyloxycarbonyl
Ac	acetyl
Bz	benzoyl
Bzl	benzyl
Bu	butyl
Ph	phenyl
Et	ethyl
Me	methyl
NMe	methylamide
Mca	monochloroacetyl
FMOC	9-fluorenylmethyloxycarbonyl
OTMB	trimethylbenzyl ester
ATOH	N-acetyl-L-tyrosine
ATEE	ethyl N-acetyl-L-tyrosinate
THF	tetrahydrofuran
CDI	carbonyldiimidazole
MTPA	(-)-2-methoxy-2-trifluoromethylphenylacetyl
PEG	polyethylene glycol
DMF	dimethylformamide
DMSO	dimethylsulfoxide
EEDQ	N-ethyloxycarbonyl-2-ethyloxy-1,2-dihydroquinoline
TFA	trifluoroacetic acid
CT	chymotrypsin
CDP-Y	carboxypeptidase Y
PPL	pig pancreatic lipase
NAD(P)H	dihydronicotinamideadenine dimucleotide(phosphate)
HLADH	horse liver alcohol dehydrogenase
RNA	ribonucleic acid
NCYC	national collection of yeast cultures
TMS	tetramethylsilane
IgE	immunoglobulin E
NMR	nuclear magnetic resonance
ppm	parts per million
J	coupling constant
s	singlet
d	doublet
t	triplet
q	quartet
b	broad
NOE	nuclear Overhauser enhancement
IR	infrared
[α]	specific rotation
c	concentration (g/100ml)
MP	melting point
BP	boiling point
GLC	gas-liquid chromatography
HPLC	high performance liquid chromatography
hrs	hours
TLC	thin-layer chromatography

MS	mass spectrometry
FAB	fast atom bombardement
CID	collision induced decomposition
ee	enantiomeric excess
de	diastereoisomeric excess
k_{cat}	maximal velocity per unit of enzyme concentration
Km	substrate concentration at half-maximal velocity
Ki	inhibition constant
Vsyn	initial velocity for peptide bond formation
Vhyd	initial velocity for hydrolysis

Notations of the standard amino acids :

alanine	Ala	tyrosine	Tyr
aspartic acid	Asp	cysteine	Cys
leucine	Leu	tryptophan	Trp
glycine	Gly	histidine	His
lysine	Lys	ornithine	Orn
serine	Ser	valine	Val
arginine	Arg	threonine	Thr
proline	Pro	methionine	Met
phenylalanine	Phe		

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DECLARATION

The work described in this thesis is the original work of the author except where acknowledgement has been made to results and ideas previously published. It was carried out in the Department of Chemistry, University of Warwick between October 1987 and September 1990 and has not been submitted previously for a degree at any institution.

PUBLICATION

Part of the research described in this thesis has appeared in the scientific literature as follows :

Peptide synthesis catalyzed by chymotrypsin in organic solvents. J.M. Ricca, D.H.G. Crout, J. Chem. Soc., Perkin Commun., 2126 (1989).

SUMMARY

Enzymes have been found to be catalytically active in organic solvents. Chymotrypsin was used to synthesize a wide range of peptides when suspended in organic solvents. This method overcame such problems as secondary hydrolysis and poor solubility in aqueous mixtures, and allowed processes to occur that were impossible in water. Syntheses involving D-amino acid derivatives were possible under these conditions. Molecular modelling studies have been carried out and structure-reactivity relationships have been drawn by using hydrolytic reactions catalyzed by chymotrypsin suspended in organic solvents with low water content.

Kinetic studies of chymotrypsin suspended in organic solvents have shown that the enzyme does not have a classical Michaelis-Menten behaviour, but shows cooperative effects with respect to the binding of the substrates. The role of the essential water has been investigated.

The use of the different chymotrysins in peptide synthesis has been investigated and γ-chymotrypsin, inactive in water, has been found fully active when suspended in organic solvents.

The enzymatic synthesis of leucine-enkephalinamide was carried out and tandem mass spectrometry was used to determine the composition of the reaction mixture. The last coupling step used an enzyme in an organic solvent.

Potential competitive inhibitors of proteases have been designed and synthesized. Several methods for the synthesis of 3-keto esters were investigated and diastereoselective reductions of 3-keto esters derived from amino acids were achieved.

GENERAL INTRODUCTION

1- Introduction

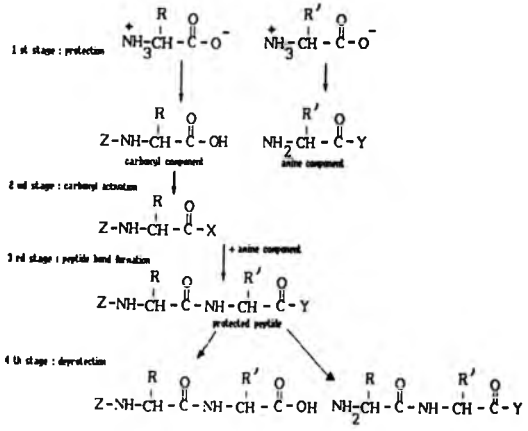
Tremendous advances in peptide synthetic chemistry have been made since the day when Emil Fischer defined the peptide bond as the amide-like linkage between amino acids and gave the reasons why he chose the term "peptide" [1].

Peptides and proteins exhibit the largest structural and functional variations of all classes of biological macromolecules. They are of prime importance in the regulation and maintenance of all biological processes. The essential structural features of peptide and protein molecules are chains of amino acids linked to one another by amide bonds. Important aspects and considerations are the polymeric nature of peptides and proteins and their wide range of properties. The polymeric character requires the use of special approaches to synthesis. The spectrum of peptide properties - from very basic to very acidic, from highly hydrophilic to totally hydrophobic, from easily soluble to completely insoluble - invariably presents surprises and obstacles during synthesis of these molecules.

Modern synthetic peptide chemistry really started in 1953 with the chemical synthesis of the nonapeptide hormone oxytocin by du Vigneaud and his collaborators [2].

The most frequently used methods of peptide synthesis are those of a chemical nature. The chemical formation of a peptide bond in principle, can be reduced to four steps (Scheme 1).

Scheme 1 :General scheme of peptide synthesis. Z, amine protecting group; Y, carboxyl protecting group; X, activating substituent.



Side chains (R) of certain amino acids contain functionalities which need protection that is maintained throughout the synthesis and therefore termed semi-permanent protection. In the second stage, the carboxyl group of the N-protected carboxyl component is activated. "Coupling reagents" [3] conveniently effect selective activation of the carboxyl component in the presence of the amine component. Peptide bond formation constitutes the third stage. An efficient condensation procedure should provide for rapid peptide bond formation, minimal racemization, few side reactions, ready work-up, and high yields. The routine laboratory scale peptide synthesis by solution techniques with up to 15 amino acid residues, can be considered as a realistic goal. Segment condensation has been successfully applied to the synthesis of many peptide hormones with up to about 60 amino acid residues. This limit will probably be extended by further refinements or existing methods and developments of novel approaches such as "the four component segment condensation" [4] or "the amine capture method" [5].

Following B. Merrifield's ingenious innovation of covalently binding the growing peptide chain to an insoluble polymeric support [6], solid-phase methodology has become increasingly popular during the last 20 years. In this strategy, excess reagents and by-products from the synthetic cycles can be removed by simple filtration and washing steps. A reappraisal of solid-phase peptide synthesis in the early 1970s [7] led to the conclusion that the original reaction conditions devised by

Merrifield were not necessarily optimal. Higher reaction yields might be obtained if the special nature of the chemical environment within the polymer matrix was considered. These considerations led to the development of a new variant of solid-phase peptide synthesis, namely the FMOC-polyamide method [8]. The most attractive improvement brought about by this procedural simplification is the time-saving and convenient mode of operation and consequently, the prospect of fully automated synthesis [9].

Nevertheless, the considerable shortcomings of these methods still impose an undiminished challenge upon synthetic peptide chemistry. These limitations arise mainly from the fact that the individual steps of the synthetic pathway are relatively unspecific in nature. Consequently, the success of many syntheses is jeopardized by the appearance of undesired by-products.

2- Use of enzymes in peptide synthesis.

2-1. Introduction

Enzymes have been widely used as catalysts in organic synthesis [10]. Recent experiments in several laboratories have signaled the emergence of proteolytic enzymes as effective tools

offering several advantages over chemical methods for the formation of peptide bonds for synthesis and semisynthesis.

The capacity of proteases to effect peptide bond synthesis stereospecifically and without the need of side chain protection was recognized early in the study of these enzymes as a natural result of their catalytic nature [11]. Although these early successes occurred largely from their being driven by precipitation of synthesized products, a more controlled type of protease-catalyzed peptide bond formation was observed in the ability of carboxypeptidases A and B, trypsin, and chymotrypsin to re-form specific peptide bonds in trypsin inhibitors [12].

2-2. CHRONOLOGY

The concept of peptide synthesis by reversal of mass action in protease-catalyzed reactions dates back to 1898, when J.H. Van't Hoff supposed that the protease "trypsin" could be endowed with the inherent capacity to catalyze the synthesis of proteins from degradation products originally generated by its own proteolytic action [13].

The rationale behind this idea involved the applicability of the law of mass action to enzyme controlled reactions and their reversibility arising from the presumed catalytic nature of the process.

During the first decades of the present century many biochemists believed that a biochemical process that required free energy to take place could be accomplished with the greatest efficiency by living organisms. As a consequence, it was generally assumed that, for instance, the catabolic pathways of biological macromolecules were inversely equal to the anabolic ones.

Moreover, this view implicitly suggested the possibility of preparing proteins by "hydrolysis in reverse" via protease catalysis. This idea of protein biosynthesis by "reversible enzymic hydrolysis" had long been considered to be supported by the phenomenon of the so-called "plastein reaction". As early as 1901, Savjalov described plastein formation correctly as the outcome of a "proteosynthetic" process, namely as the reverse of the already known "proteolytic" action of proteases [14]. Due to their complexity, however, the chemical nature of the plasteins could not be exactly characterized by the methods of that time. After simplifying the experimental conditions, Bergmann's group was the first to describe the enzymatic syntheses of well-defined dipeptides via papain [15] and α-chymotrypsin catalysis [16]. However, the concept of protein biosynthesis by reversal of enzymatic proteolysis, which was accepted as plausible until the beginning of the 1940's was put in question by thermodynamic data on peptide bond hydrolysis. It could be shown that the synthesis of peptide bonds represents a strongly endergonic process under physiological conditions. Therefore, Borsook concluded [17] :

"peptide bonds cannot be synthesized to any significant extent merely by mass action reversal of hydrolysis".

The demise of the concept of protease-controlled protein biosynthesis finally coincided with the recognition of the genetic code and the decisive role played by mRNAs and tRNAs during the process of in vivo protein synthesis.

Besides their primary role in peptide synthesis, proteases have also been successfully applied to oligomerization [18], semisynthesis [19], and protecting group chemistry [20].

2-3. Thermodynamically controlled peptide synthesis.

The thermodynamically controlled formation of peptide bonds represents the direct reversal of the catalytic cleavage of peptides by proteases [21].

Since, however, concentrations are used in the description below instead of activities, this is not an exact thermodynamic treatment. In contrast to the hydrolysis, the synthesis of a peptide bond is an endergonic process, i.e., proceeds with loss of entropy and is energetically so unfavorable that the equilibrium constant Ksyn for the coupling of two unprotected aminoacids is 10^{-5}. Since the ionic forms of the two substrates

are unreactive, for the thermodynamic approach, two equilibria have to be taken into account :

$$R\text{-}COO^- + {}^+H_3N\text{-}R' \overset{Kion}{\rightleftharpoons} R\text{-}COOH + H_2N\text{-}R \overset{Kinv}{\rightleftharpoons} R\text{-}CONH\text{-}R' + H_2O$$

Preceding the "inversion equilibrium" Kinv between the uncharged substrates and the product is an "ionization equilibrium" Kion. Taking the concentration of water into the equilibrium constant, the total process is given by :

$$Ksyn=Kion.Kinv=(RCONHR')((RCOO^-)({}^+H_3HNR'))^{-1}$$

For any given pair of substrates and known pH, Kion and Kinv are fixed. The only function of the protease is to accelerate the attainment of the equilibrium for the formation of the peptide. Therefore, reaction conditions should be chosen to ensure a high catalytic activity of the protease. The pH optimum of the synthesis lies apart from pepsin catalyzed couplings, in the pH range between the pK of the α-carboxyl group and that of the amino group of the substrates, i.e., normally between pH 6 and 7. There are two principal ways by which one can further influence thermodynamically controlled peptide formation :

- increasing Kion by alteration of the pK values of the substrates, and

- increasing the concentration of the peptide product via manipulation based on the law of mass action.

2-3.1. Increase in Kion

For a given set of reaction conditions, there are two ways to decrease the difference in pK values between amino and carboxyl components in order to increase Kion. This leads in turn, to an increase in Ksyn = Kion . Kinv

2-3.1.1. Use of water-miscible organic solvents

Water-miscible organic solvents decrease the acidity of the α carboxy group of the carboxy component, whereas they only marginally influence the pK value of the amino group of the nucleophile. For example, the pK value of acetylglycine in water is 3.60, while in 80% (v/v) dimethylsulfoxide it is 6.93 ; the pK values of $GlyNH_2$ 8.20 and 8.10, respectively, remain almost constant. A variety of other cosolvents can be used to change the pK value by one to two units, resulting in a significant increase in Ksyn. This approach, however, is problematic in so far as the catalytic activity of proteases decreases with increasing concentration of the cosolvent ; thus, the time required to reach the inversion of the equilibrium increases. Only polyalcohols, which act as enzyme stabilizers, may be used at high concentrations without inactivation of the enzyme [22].

2-3.1.2. Use of biphasic systems

In systems consisting of an aqueous phase and a non miscible phase (non-polar organic solvent), pK values are influenced in such a way that Kion increases [23]. Since the enzyme is localized in the aqueous phase, the activity can be influenced only by the saturation concentration of the organic solvent in water, and the enzyme is therefore inhibited far less than by solvents miscible with water. This advantage, however, is counteracted by the prolonged time required to reach equilibrium. Solubility of the substrates in the nonpolar organic phase limits the general use of biphasic systems for the enzymatic peptide synthesis.

2-3.2. Influence on product formation based on the law of Mass Action

The first positive experimental results for the use of a reversal of protease-catalyzed peptide hydrolysis were based on the limited solubility of the products, which thus are removed from the inversion equilibrium (see page 20)and accumulate [11]. The product can also be removed from the equilibrium by extraction or specific complexation.

2-3.2.1. Formation of insoluble products

If sufficiently high concentrations of substrates are employed, such that in the equilibrium Kinv it is possible to have a

peptide concentration that lies above the maximal saturation concentration, precipitation occurs and thus the product of the synthesis accumulates. The apparent equilibrium constant, for a reaction in which some of the product precipitates due to its limited solubility in the system, is greater the higher the starting concentration of substrates and the lower the solubility of the product in this system. Using one component in excess may result in almost quantitative reaction of the other substrate. This method is very popular in practice since the condition of low solubility of the product compared to that of the substrates frequently holds.

2-3.2.2. Extraction of products

In biphasic systems, the product is removed from the equilibrium if, owing to a favorable position of the equilibrium, it is extracted and thus accumulates in a nonpolar phase that is not miscible with water. In most cases, however, the product is only marginally soluble in the organic phase ; it precipitates and is thus removed from the equilibrium.

2-3.2.3. Specific complexation of the product

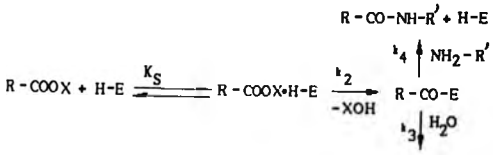
If compounds are available that can form specific complexes with the product, the latter can be removed from the equilibrium by adding such a complex-forming material [24].

2-4. Kinetically controlled syntheses.

Investigation of the catalytic mechanism of serine and cysteine proteases using chymotrypsin and papain as examples revealed that, in the presence of nucleophiles, acyl enzymes intermediates RCO-E are deacylated competitively by water and the nucleophile H_2N-R [25].

If the nucleophile is an aminoacid or peptide derivative, then a new peptide is formed during the aminolytic deacylation (Scheme 2).

Scheme 2.



Especially suitable carboxyl components for this type of enzymatically catalyzed peptide synthesis are acylamino acid alkyl esters, if they match the substrate specificity of the particular protease, since in general they fulfill the condition $k_2 \gg k_3+k_4$. Furthermore, k_2 of the ester substrate is substantially greater than k_2 of the peptide formed, which results in a maximum for product formation before the slower hydrolysis of the product starts to become important.

The area in which kinetically controlled synthesis can be used in practice is limited to serine and cysteine proteases which prefer acylamino acid esters as the carboxy component. The reactions are characterized by short reaction times and low enzyme requirements.

2-4.1. Influence of the reaction medium

Since only the non-protonated form of the nucleophile reacts in the aminolytic deacylation of the acyl-enzyme, it is necessary to take the effective concentration of nucleophile instead of the total concentration into account. This can be calculated from the pK value of the nucleophile and the pH of the medium. Since the pK values of α-amino groups of amino acid and peptide derivatives lie around 8 it is advisable to carry out kinetically controlled synthesis at pH 8.

2-5. Major developments in enzyme-catalyzed peptide bond formation.

2-5.1. Enzymatic reactions in aqueous-organic media

Recently, the area of enzymatic reactions in the presence of organic solvents, either miscible or immiscible with water, has been rapidly growing and drawing much attention. These reactions are of great interest with respect to both basic studies on the medium effects on enzyme catalysis and the application of enzymatic reactions to organic synthesis. For example, hydrolytic enzymes such as α-chymotrypsin, have been employed as catalysts for various ester syntheses in aqueous-organic two-phase systems [26]. It has been considered that in this system the decreased amount of water and the low solubility of the products in the aqueous phase shift the reaction equilibrium towards ester synthesis. However, when a hydrophobic (water-immiscible) organic solvent is used, the main drawback of the method is that if one of the reactant RCOOH such as amino acids is sparingly soluble in the organic phase, it would be concentrated in the aqueous phase. This often causes a substrate inhibition of the enzyme, leading to low yields of the esters [27,28]. The synthetic reactions by hydrolytic enzymes in water-hydrophilic (water-miscible) organic solvents have also been reported [29,30]. By using limited amounts of water in solvents, the equilibrium of the reaction can be shifted to favor synthesis. However, the catalytic activity of

the enzymes is often impaired at high concentrations of organic solvents, and the yields of products have been rather limited. For example, it has been reported that N-acetyl-L-tyrosine ethyl ester was obtained in 25-30% yields by chymotrypsin-catalysed reactions of N-acetyl-L-tyrosine with ethanol in 50-60% water, but that chymotrypsin was inactivated at lower concentrations of water [31]. However, the dipeptide derivatives Z-L-Tyr-L-LeuNH$_2$ and Mca-L-Tyr-L-LeuNH$_2$ were synthesized by α-chymotrypsin-catalyzed coupling reactions in solvent systems consisting of buffer and ethyl acetate [32]. In comparison to a pure aqueous medium, in which only insignificant synthesis takes place, product formation is greatly enhanced in a biphasic medium owing to the extraction of the dipeptide into the organic phase.

2-5.2. Immobilized proteases

Proteases can be immobilized without loss of function, and the potential of immobilized proteolytic enzymes for peptide synthesis has been demonstrated [33,34]. Peptide coupling reactions can be carried out on a preparative scale in which immobilized proteases can be used with the advantage of avoiding reaction conditions which are normally required for chemical condensation. The simplified work-up procedure that becomes possible when immobilized proteases are used, the long-term stability of the immobilized enzyme preparations, and successful reutilisation is among the advantages of such an approach. The essential

advantages in the application of covalently bound proteases can be summarized as follows : the immobilized protease can easily be recovered from the reaction mixture ; the peptides synthesized are free from contamination by proteolytic activities and denatured protein ; due to the increased stability in the presence of organic solvents, higher concentrations of such solvents can be used to influence the position of the thermodynamic equilibrium.

Immobilization of chymotrypsin, trypsin and thermolysin to various carriers has been described [35]. The possibility of using immobilized trypsin for kinetically controlled peptide bond formation was investigated by A. Könnecke et al. [34]. With the serine type enzyme trypsin, excellent product yields were obtained starting with ester carboxyl components. Covalently immobilized trypsin catalyzed the formation of peptide bonds with nearly the same efficiency as the soluble protease and could be re-used successfully for further coupling experiments.

One of the most interesting aspect of using immobilized proteases is the use of organic solvents as the reaction medium. Papain entrapped in Amberlite XAD-8 was fully active in 4-methylpentan-2-one and was used to catalyze dipeptide synthesis [36].

2-5.3. Polyethylene glycol modified enzymes

The hydroxyl groups of monomethoxypolyethylene glycols ($HO-(CH_2-CH_2-O)_nCH_3$) may be activated by cyanuric chloride and several other reagents and then coupled to lysine ε-amino groups of proteins.

This chemical modification of proteins and enzymes with polyethylene glycol (PEG) has become an approach applicable to the solution of various problems in biological sciences. The production of IgE caused by protein allergens such as ovalbumin and ragweed pollen was suppressed by the treatment with respective proteins modified with PEG [37]. Modification of Escherichia coli asparaginase [38], yeast uricase [39] and snake venom batroxobin [40] with PEG decreased their immunoreactivity towards antibodies against respective proteins.

It was demonstrated that polyethylene glycol-modified enzymes might become soluble in organic solvents such as benzene, toluene and chlorinated hydrocarbons, and exhibit high enzymic activities in these organic solvents. The modified catalase catalyzed [41] decomposition of hydrogen peroxide and peroxidase catalyzed [42] oxidation reactions, respectively, in transparent organic solvents. A similarly modified lipase was also soluble in various organic solvents and had the ability to catalyze ester synthesis and aminolysis in benzene [43,44].

The first successful attempt to form a peptide bond by aminolysis in benzene was described by Inada in 1984 [45]. Chymotrypsin was modified in the zymogen form with 2,4-bis(O-methoxypolyethyleneglycol)-6-chloro-s-triazine (activated PEG_2), followed by activation with trypsin. The modified enzyme was soluble in benzene and retained its enzymic activity. Acid-amide bond formation by the modified enzyme proceeded efficiently in benzene : benzoyl-tyrosine-(oligo)-phenylalanine ethyl esters were formed from N-benzoyl-L-tyrosine ethyl ester and L-phenylalanine ethyl esters.

Since this original publication, the use of PEG-enzymes has dramatically increased and the major proteases, chymotrypsin [46], papain [47], thermolysin [48], subtilisin and trypsin [49], have been successfully employed for peptide synthesis in organic solvents.

In a comprehensive study on the kinetics and specificity of serine proteases in peptide synthesis catalyzed in organic solvents [49], H. Gaertner and A. Puigserver showed that the enzymatic synthesis obeyed Michaelis-Menten kinetics and was consistent with a ping-pong mechanism modified by a hydrolytic shunt. A minimal water concentration was required for the catalytic activity of modified chymotrypsin in water-immiscible solvents. However, the use of PEG-modified enzymes suffers some shortcomings including the necessity of using hydrophobic solvents with polar compounds, and the difficulty of reisolating the catalyst for further use.

2-5.4. Reverse micelles

The solubilization of enzymes via reverse micelles provides a method for the catalytic biotransformation of water-insoluble material. Reverse micelles are formed by amphiphilic molecules (surfactants) in organic solvents ; the polar groups (heads) of the surfactants molecules are directed towards the interior of the spheroidal aggregate, forming a polar core and the aliphatic chains are directed towards the organic solvent. This is the "reverse" of the situation in normal micelles in water. Water can be solubilized in the polar core of reverse micelles, forming the water pool. The chemists are interested in reverse micelles as versatile microreactors in which guest molecules can be brought to reaction with novel chemical properties [50]. Interest from biotechnologists has increased over the last few years because enzymes can be hosted in reverse micelles without loss of activity. The possibility of stabilizing water-soluble enzymes against the inactivating action of organic solvents by means of surfactants has been widely studied. Several enzymes, chymotrypsin [51], trypsin [52], pyrophosphatase [53], peroxidase [54] were used to demonstrate that enzymes can be entrapped into reverse micelles formed by surfactants in an organic solvent. The enzymes solubilized in this way retain their catalytic activity and substrate specificity.

Proteases in reverse micelles have been used for the synthesis of water insoluble peptides by Luisi [55]. This example described the

synthesis of Z-Ala-Phe-$LeuNH_2$ starting from Z-Ala-Phe-OMe and $LeuNH_2$ using chymotrypsin as catalyst. Z-Ala-Phe-OMe is soluble both in water and in isooctane, and the product is much more soluble in isooctane than in water. Ac-Phe-$LeuNH_2$ has also been synthesized using proteases in reverse micelles [55]. A hollow fiber reactor has been utilized for these peptide syntheses. This system works well but is not suitable for preparing large amounts of material.

More recently, Lattes [56] reported the first successful enzymatic transesterification in a micellar medium. A new type of microemulsion system (Aerosol-OT, water, hexanol) was used to entrap α-chymotrypsin in a reverse micelle. N-acetyl-L-tyrosine ethyl ester can be solubilised in this medium to give N-acetyl-L-tyrosine hexyl ester in a good yield.

2-5.5 Direct solubilisation of enzymes in organic solvents.

Recently, it was shown that preparations of enzymes in organic solvents might be prepared directly. Crown ethers and cryptands were found to be selective complexing agents for a number of proteins, allowing their dissolution in non-aqueous solvents [57]. Bovine insulin and cytochrome C were readily solubilized in methanol by weak complexation with 18-crown-6 ether. However, the ratio protein-crown ether was found to be critical, large amounts

of the complexing agent had to be used, and no information concerning the catalytic integrity of the enzymes was given. A direct application of this concept was obtained by the preparation of a lipid-coated lipase which was soluble in organic solvents such as benzene [58]. Nonionic and cationic lipids formed complexs with lipases, and high activity was obtained in hydrophobic solvents.

2-5.6. Heterogeneous catalysis in anhydrous organic solvents

In 1966, Dastoli first observed enzymatic activities of crystalline chymotrypsin in anhydrous nonpolar organic solvents [59]. Since then, several enzymatic transformations in nonpolar organic solvents have been reported [60,61]. Klibanov and Zaks have examined the role of water in enzymatic reactions in a number of anhydrous polar and nonpolar organic media and concluded that, in general, enzymes needed a thin layer of water on the protein surface to retain their catalytically active conformation in anhydrous media [60,62]. The most adequate nonaqueous media are hydrophobic solvents that do not strip the essential water from the enzymes. Water-immiscible solvents containing water below the solubility limit are suitable for dry enzymes. Within this range of water content, the enzymatic activity in an appropriate organic solvent can be optimized, and the catalysis follows Michaelis-Menten kinetics. Recent findings indicate that the essential water can be substituted, to some degree, by other

compounds. When water is stripped from the enzyme by a solvent, areas of the protein which normally interact with water become exposed. The addition of compounds which could mimic the interaction of water with the protein should restore, to some degree, enzymatic activity. The presence of 1% formamide increased the activity of alcohol dehydrogenase in butyl acetate (0.4% water) 15-fold over the activity in the absence of the additive [63]. Enzymatic peptide synthesis in the presence of water mimics, such as formamide, ethylene glycol, or methanol, was possible using thermolysin in tert-pentyl alcohol [64]. Partial replacement of water with water-mimicking cosolvents was found to be beneficial for enzymatic fragment coupling by combining high reaction rates and the absence of side reactions such as hydrolysis. This general effect was not limited to compounds interacting with the protein through hydrogen bonding. Crown ethers considerably enhanced the rate of the chymotrypsin-catalyzed transesterification of N-acetyl-L-phenylalanine ethyl ester with 1-propanol in n-octane [65]. It was suggested that complexation of the ammonium, guanidinium, and potassium cations at the outside of the enzyme, by the crown ether added, rendered the enzyme more soluble in the apolar solvent.

Suspending enzymes in organic solvents can dramatically alter a number of their fundamental properties. It was found that the enzyme remembered the pH of the last aqueous solution it has been exposed to. The enzyme's ionogenic groups acquire the

corresponding ionisation states which then remain both in the solid state and in organic solvents [62].

The substrate specificity of chymotrypsin and subtilisin were significantly modified by placing them in organic solvents [66]. It was postulated that the major driving force of substrate binding is hydrophobic interactions between the side chain of the amino acid residue and the binding pocket of the enzyme. For that reason, the substrate specificity of chymotrypsin in organic solvents was reversed and there was a significant increase in reactivity towards hydrophilic substrates.

Enzymes were found to be extremely thermostable in water-restricted environments [67]. Whereas lipase in water at 100°C is inactivated almost instantly, the half life of the enzyme at this temperature in tributyrin was greater than 12 hours.

It was also reported that the lyophilisation of chymotrypsin from aqueous solutions containing ligands (such as N-acetyl-L-phenylalanine) had a significant effect on the activity of the enzyme in organic solvents [62]. It was postulated that the activation was the result of the ligand locking the enzyme in its active conformation.

A dramatic change of stereoselectivity was also observed by suspending subtilisin in anhydrous organic solvents [68]. The enzyme readily incorporated D-amino acid residues as donor esters in dry tert-pentyl alcohol. Klibanov explained this result by recognizing that when a substrate interacts with the enzyme, water must be excluded from the interface between them [69]. The

productive binding of the L-ester to the active site of subtilisin results in the release of more water molecules from the hydrophobic pocket of the enzyme than that of the D-isomer. The process of water release is less favourable in hydrophobic media compared with water. Thus, the reactivity of the L-ester in hydrophobic media decreases substantially and the discrimination between the D- and L-esters is diminished.

The change in free energy during enzyme-substrate (ES) complexation in water is considered to require , in addition to other changes, the disruption of a number of hydrogen bonds associated with the substrate and the enzyme active site [70]. A transition from aqueous to organic solvents for the complexation of polar substrates with their natural enzymes may result in a tight binding of either the substrate or the product to the enzyme, and a severe substrate or product inhibition or both could occur. This argument was used to explain why several carbohydrate-converting enzymes have been reported to be inactive in organic solvents [70].

Novel enzymatic reactions in gases and supercritical fluids have been exploited using the heterogeneous catalysis concept [71]. This latter approach has the merit of facilitating the recovery of the products and is environmentally acceptable.

Hydrophilic organic solvents such as DMF tend to strip water from enzymes and inactivate them. By site-directed mutagenesis, it was possible to prepare a subtilisin mutant which was several hundred times more stable than the wild type in aqueous solution [72] and

50 times more stable in dry DMF [73]. This mutant was used by C.H. Wong for the synthesis of dipeptides [73].

2-5.7. catalytic antibodies and artificial enzymes.

The idea of transition state binding led to an experimental approach toward the design and synthesis of immunogenic transtion state analogs for the hydrolysis of esters and peptides [74]. For application in synthesis, the most interesting developments are the demonstrations of stereospecific transesterifications, lipase-like hydrolysis, and aminolysis in water [75].

A novel and interesting variant of enzymatic peptide synthetic methodology was suggested by Nakajima [76]. He used aminoacyl-t-RNA synthetases, enzymes which are involved in ribosome-mediated protein synthesis and which demonstrated high specificity for their cognate amino acids, to prepare a series of dipeptides. Unfortunately, this method is far from the preparative scale.

Sasaki reported the design of an artificial catalyst for the synthesis of peptide bonds [77]. He used a crown ether as scaffold to which two thiol groups were fixed as catalytic functions. The educts were covalently linked by chemical means to the enzyme mimic via thioester bonds. Intramolecular aminolysis resulted in the formation of a peptide bond with the growing peptide still bound to the carrier through a thioester linkage.

The peptidyltransferase, a ribosomal protein that catalyses peptide bond formation *in vivo* should be an ideal choice to serve this function *in vitro*. However, its proteosynthetic activity is dependent upon the presence of other ribosomal helper proteins, so, when isolated from its environment, the enzyme cannot preserve its original functions. Attempts have been made to design and synthesize synthetase mimics [78], but it is too early to evaluate the scope of the strategy.

CHAPTER I .APPLICATIONS OF CHYMOTRYPSIN SUSPENDED IN ORGANIC SOLVENTS

I-1. PEPTIDE SYNTHESIS CATALYZED BY CHYMOTRYPSIN IN ORGANIC SOLVENTS.

I-1.1. Introduction.

As illustrated in the introduction, great interest has been shown in the use of proteases to catalyze peptide bond formation. The drawbacks inherent to catalysis in an aqueous environment (unfavourable thermodynamic equilibrium, narrow substrate specificity, and undesirable proteolysis of the peptide) have been overcome by the use of proteases in biphasic aqueous organic mixtures [32], reverse micelles [55] or chemically modified enzymes in organic solvents [45]. However, recently it has been found that enzymes can be catalytically active in anhydrous organic solvents and, under these conditions, show a new range of properties, e.g. relaxed stereospecificity and modified substrate specificity [62,66,79,80,81]. Although the use of chymotrypsin as a suspension in organic solvents for esterification [82] and transesterification [62], is well documented, the enzyme has never

been used to catalyze peptide bond formation under these conditions.

That the internal configuration of protein molecules could be radically altered by organic solvents has been shown [83,84]. In the early sixties, some interest was shown in catalytic properties of protein crystals. Carboxypeptidase [85], ribonuclease-S [86], and chymotrypsin [87,88] have all been reported to show typical catalytic activities when suspended in aqueous sulfate solutions.

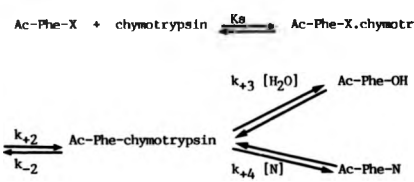
In 1966, Dastoli reported the chymotrypsin-catalyzed hydrolysis of Ac-L-TyrOEt in methylene chloride containing 0.25% water [59]. Crystals recovered from suspension in dichloromethane for up to 5 days showed no measurable loss in reactivity upon assay with acetyl-L-tyrosine ethyl ester in aqueous solution.

Based on this initial observation, we postulated that, if chymotrypsin is able to catalyze hydrolytic reactions in organic solvents, peptide synthesis was envisageable by aminolysis of esters.

Substantial evidence indicates that chymotrypsin-catalyzed hydrolyses proceed in two steps [89]. The first reaction is acylation of serine-195 of the enzyme and the second is a subsequent deacylation. Water is not unique in the latter reaction; replacement by amine or alcohol derivatives

(nucleophiles N) for example may result in changes in the rates and products (Scheme 1). Therefore, peptides are expected to be synthesized enzymatically when amino acid derivatives, peptides or their derivatives are used as nucleophiles N in Scheme 1.

Scheme 1.



If water is restricted in the reaction mixture, one can expect high yields of peptides to be obtained

I-1.2. Materials and methods.

Enzyme.

Crystalline bovine pancreatic α-chymotrypsin (EC 3.4.21.1) (type II) was purchased from Sigma as a lyophilized powder with specific activity of 51 units/mg protein (one unit will hydrolyze

1.0 μmol of benzoyl-L-tyrosine ethyl ester (BTEE) per minute at pH 7.8 at 25°C). The amount of bound water was determined by the Karl-Fischer method and found to be 5.4% (w/w) for the native enzyme and 1.5% (w/w) for the enzyme extensively dried in a desiccator over P_2O_5. The enzyme was used in this study without further purification.

Amino acid derivatives, peptides and their derivatives.

Ac-L-TyrOEt, Bz-L-TyrOEt, Ac-L-PheOEt, Ac-L-TrpOEt, Bz-L-AlaOEt were obtained from Sigma; amino acid amides were purchased from Bachem AG.

Esterification of the substrates was carried out with saturated solutions of hydrochloric acid in the given alcohol. Various Z-amino acid esters were prepared by reaction of the ester with N-(benzyloxycarbonyloxy)succinimide in chloroform. BOC-derivatives were prepared by reaction of the ester with di-tert-butyl dicarbonate in aqueous medium or in organic solvents. Protection of the amino groups of the substrates by an acetyl group was carried out by reaction with neat acetic anhydride in the presence of a base or by acetyl chloride.

Z-dipeptide substrates were prepared by reaction of the protected Z-amino acid and the amino acid ester with EEDQ in dry dichloromethane.

β-alanine amide, L-phenyllactic amide, and α-aminoisobutyramide (α-methyl alaninamide) were prepared from

their BOC-derivatives according to a procedure developed by Muramatsu [90].

Enzymatic synthesis of peptides.

In a typical experiment, to a solution of the protected amino acid ester and the amino acid amide (40 mM, 1:1 molar ratio) in anhydrous dichloromethane (distilled over calcium hydride immediately prior to use) was added 1 mg/ml of chymotrypsin, followed by the addition of 0.25% (v/v) of water. The resulting suspension was then stirred at room temperature for a certain period of time. In all cases investigated, the desired compound, if formed, precipitated during the course of the reaction. The solvent was then evaporated under reduced pressure, the residue was thoroughly washed with water, and the product recrystallized from hot methanol.

If hydrochloride or trifluoroacetate salts of the amino acid amides were to be used as substrates, the salt was first neutralized by the addition of one equivalent of triethylamine.

Computer graphics.

The computer-assisted molecular modelling systems RIMG and MOLOC have been developed at Hoffmann-LaRoche, Basel. There are built around two novel generally applicable united-atom force field methods, which are complementary to each other. One method uses automatic referencing to input structures, the other is based on a small generic set of fixed external parameters. Both modelling systems provide interactive modelling and nonlinear optimisation techniques in a well balanced and highly functional combination. They contain algorithms for different graphic representations of molecular structures and packing as well as efficient procedures for structure refinement, energy evaluation, and conformation analysis. They incorporate extensive tools for complex geometrical as well as logical manipulations of molecular structures and provide facilities for fast on-line storage and retrieval of molecular structures and structural fragments.

The two specially designed relational data base systems ROCSD (Roche Cambridge Structural Data Base), containing the converted data of the Cambridge Crystallographic Data Files, and ROPDB (Roche Protein Structural Data Base), holding the converted data of some 300 protein structures from the Brookhaven Protein Data Files, were used during this study.

I-3. Results and discussion.

The procedure was carried out on a 0.4-4 mmol scale. No activity was found with less than 0.2% of water, indicating that a minimal amount of water is absolutely essential for enzymatic catalysis. This essential water presumably allows the enzyme to maintain its native conformation, as well as sufficient flexibility, and hence to retain its catalytic activity [79,80].

The role of the essential water will be extensively discussed in Chapter II, all the literature in this area have reported the effect of water in terms of total water or in terms of added water. In fact, one can distinguish two different states of water in this type of non-aqueous enzymology, i.e. the water bound to the enzyme powder and the water freely dissolved in the organic solvent. This differentiation is useful because only the enzyme-bound water seems to decide its catalytic activity. Lysozyme has been used in most studies in this area [91]. Using a variety of experimental techniques, it has been shown that the hydration of a protein consists of a number of distinct stages : below 0.07 g/g (water per protein) water predominantly interacts with charged groups. Between 0.07 and 0.25 g/g, water forms clusters which grow and cover most of the surface. Within the range of hydration, for lysozyme, there is a significant increase in the mobility of the protein matrix. The enzymatic activity of lysozyme becomes detectable at as low as 0.2 g/g (at which only about half of the protein surface is covered by water) and goes

in parallel with the protein's motional properties. At 0.38 g/g (about 300 water molecules per protein molecule) the protein molecule is fully hydrated and the enzymatic activity reaches 10% of that in water. The amount of water necessary for an enzyme to function in organic solvents is one of the major questions of non-aqueous enzymology. The fact that the distribution of water between the enzyme and the organic solvent governs enzymatic activity implies that enzymes suspended in hydrophobic solvents require substantially less water for activity than those suspended in hydrophilic ones.

Zaks and Klibanov [63], by studying the dependence on the amount of water bound to the enzyme, showed that in hydrophobic solvents water tends to partition into the enzyme so that even very small concentrations of water in the solvent (<1%) yield up to 30% water on a protein. In a similar study concerning lipase-catalyzed intramolecular esterification in benzene, Yamane [92] established that the degree of hydration of enzyme molecules necessary to exhibit full activity had a saturation level. At a higher free water content, the enzyme powder phase contains more water than the saturated level. In fact, the optimal water content is a function not only of the organic solvent, but of the enzymes as well, and to a lesser extent of the enzymatic process.

In our study, dichloromethane rapidly established itself as the most suitable solvent. With peptide substrates, a compromise has to be reached between the need for a highly hydrophobic solvent (to decrease the water content) and the highly hydrophilic

character of the substrates. The best established solvents like tert-pentyl alcohol and isooctane could not be used because of the insolubility of the substrates.

If the importance of water for enzymatic activity is well understood, only sparse reports have been published to explain firstly the absence of enzymatic activity in anhydrous solvents which could provide valuable insights into protein structure, folding and dynamics, and second the effect of the solvent on the protein structure. Solid-state NMR spectroscopy was used to show that the catalytic triad of α-lytic protease was intact when the enzyme was suspended in acetone and isooctane [93]. Even if this reveals that part of the catalytic site remains intact, this technique does not provide information about the structural integrity of the enzyme.

If the molecules of solvents can diffuse into the crystal lattice, they might then interact strongly with the structure by, for example, disturbing hydrophobic clusters, reinforcing hydrogen bond networks, or directly interacting with the binding of the substrates. The tridimensional structure of the enzyme can therefore be modified and slight changes in the conformation of selected residues might have profound consequences for the reactivity and specificity of the enzyme.

This concept was demonstrated by experiments carried out with γ-chymotrypsin which showed that an enzyme inactive in water can become active when suspended in organic solvents due to the direct intimate effect of the solvent on the protein structure.

This example will be discussed in Chapter III.

In order to provide support for this hypothesis, the behaviour of chymotrypsin in organic solvents was investigated and by combining the experimental results with molecular modelling, a qualitative analysis of the tridimensional structure was attempted.

In no cases was any peptide formation detected in the absence of water. Under the aforementioned conditions, no hydrolysis of the final peptide was observed, indicating that the added water is not available for hydrolysis.

To examine specificity at the P_1' position (notation of Schechter and Berger [94]), experiments were performed with various amino acid derivatives as nucleophiles. The results are summarized in Table 1.

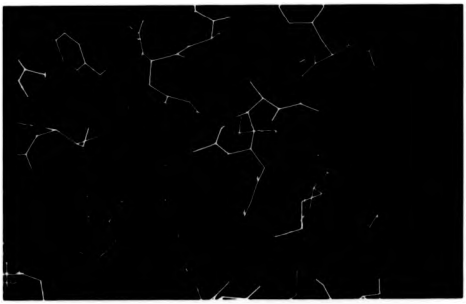
Table 1 : Effect of the nucleophile specificity on synthesis.

Donor ester	Nucleophile	Product	Yield (%)	Reaction time (hrs)
Ac-L-TyrOEt	L-PheNH$_2$	Ac-L-Tyr-L-PheNH$_2$	96	6
"	L-LeuNH$_2$	Ac-L-Tyr-L-LeuNH$_2$	95	6
"	L-LeuOMe	-	0	72
"	L-ValNH$_2$	Ac-L-Tyr-L-ValNH$_2$	92	18
"	L-AlaNH$_2$	Ac-L-Tyr-L-AlaNH$_2$	84	18
"	L-MetNH$_2$	Ac-L-Tyr-L-MetNH$_2$	86	12
"	L-ProNH$_2$	-	0	72
"	β-AlaNH$_2$	-	0	72
"	L-phenyllactic amide	-	0	72

Hydrophobic and bulky amino acid amides as nucleophiles were found to be suitable substrates for peptide synthesis, but the imino acid derivative, L-prolinamide, was not accepted as a substrate under any of the conditions tested.

It is worth noting that amino acid esters (e.g. methyl L-leucinate) were not readily accepted as nucleophiles. The explanation for this observation was provided by molecular modelling experiments. Picture 1 shows a color-coded display of putative hydrogen bonds for Ac-L-Tyr-L-AlaNMe, covalently bound to serine-195 (tetrahedral oxyanion intermediate) of chymotrypsin.

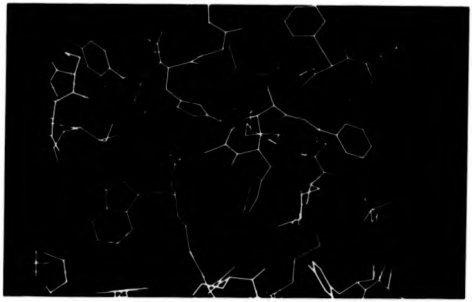
Picture 1



The oxyanion is well engaged in the oxyanion hole (two hydrogen bonds to backbone NH). The C-terminal peptide-NH of the amide engages in a strong hydrogen bond to the backbone-CO of phenylalanine-41. The N-terminal peptide-CO forms an intramolecular hydrogen bond to the NH group of the scissile peptide bond.

Picture 2 shows the color-coded display of hydrogen bonding between Ac-L-Tyr-L-AlaOBzl and the active site domain of chymotrypsin.

Picture 2



One can note the lack of any hydrogen bond between the benzyl ester and chymotrypsin.

The loss of one strong hydrogen bonding interaction to the peptide-CO of phenylalanine explains why amino acid esters are not suitable nucleophiles. This loss may account for a loss in the free binding energy between 2-4 kcal/mol in water, and due to the new organic environment where hydrophobic interactions will be diminished but hydrogen bonding greatly enhanced, the figure might well be much higher.

The importance of the hydrogen bonding between the amide moiety and the backbone-CO of Phe-41 was further demonstrated by using β-alaninamide as a nucleophile. β-Alaninamide having one more methylene unit should therefore lose this stabilising interaction. As shown in Table 1, no reaction was observed when β-alaninamide was used as a nucleophile.

The specificity of α-chymotrypsin for hydrophobic amino acid nucleophiles is often explained in terms of hydrophobic interactions between the side-chain of the nucleophile and the peptide backbone. If that explanation were correct, one might then assume that by replacing water with another reaction medium, one would change the substrate specificity. This is not experimentally observed as shown by the results given in Table 1.

One can consider the suspended enzyme in organic solvents as being shielded from the bulk of the solvent. Firstly, the enzyme-bound water will protect the enzyme molecule from any detrimental effect of the solvent. Second, the enzyme being in a quasi-crystalline state, the penetration of the solvent into the crystal lattice will be controlled by diffusion factors.

Therefore, one might assume that if molecules can diffuse into the enzyme molecule, no direct solvation phenomenon will be observed, but rather the solvent will coordinate with the enzyme matrix at specific binding sites such as hydrophobic clusters, isolated hydrophobic side-chains etc.

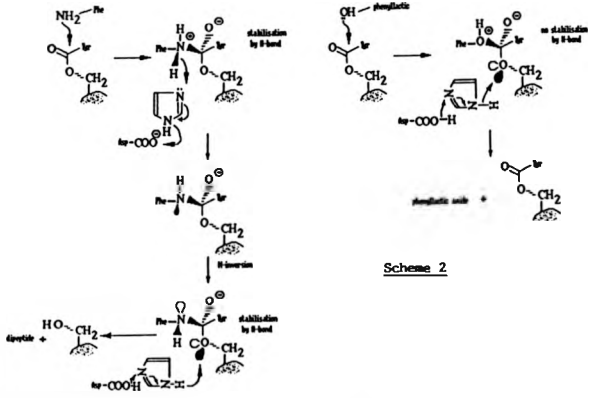
The enzyme will then still remain in a highly organized state, the only difference with an aqueous environment being the presence at specific points of solvent molecules. According to Dewar [69], the proper substrate of an enzyme is generally believed to fit its active site closely. If so, adsorption of the substrate in the active site will necessarily lead to the expulsion of all molecules of solvents (i.e. water) from between them, leaving bare substrate in contact with bare enzyme. Any subsequent reaction between the enzyme and the adsorbed substrate will then take place in the absence of solvent, i.e., as it would in the gas phase, no water separating the groups that are directly involved in the reaction. Recent work has shown that gas phase chemistry differs greatly from solution chemistry. In particular, many reactions that take place slowly in solution take place with little or no activation, and hence extremely rapidly, in the gas phase. According to Dewar, this suffices to explaining the high rates observed in enzymatic reactions. But, more interestingly, a substrate too big to fit into the active site of an enzyme cannot react with it, while a substrate which is too small cannot squeeze out all the molecules of solvent. Therefore, the residual molecules of solvent inhibit the

reaction. According to this concept, one has no problem in explaining why the enzyme prefers large hydrophobic nucleophiles, such as phenylalanine, instead of smaller ones like alanine.

One could argue that the release of water molecules into the reaction medium will be thermodynamically unfavourable. This is shown by lower rates (k_{cat}/Km) for the reaction in organic solvents compared to the same reaction in aqueous medium (see Chapter II). But, this does not take into account the molecules of organic solvent present in the active site. On the other hand, the so-called release of water will involve only a small number of molecules which can well rearrange themselves in the vicinity of the active site.

No reaction was observed when L-phenyllactic amide was used as nucleophile (Table 1). L-Phenyllactic amide is a structural analogue of L-phenylalaninamide. Whereas the reaction with the latter substrate proceeded very efficiently, no transesterification reaction could be observed with L-phenyllactic amide. If one can assume that the two substrates will bind with the same efficiency, a direct measure of the nucleophilicity of the substrates can be estimated. Nitrogen, being more polarizable and less electronegative than oxygen, displays a greater reactivity towards the nucleophilic attack of the acyl-enzyme intermediate. Nevertheless, the complete absence of reactivity of the phenyllactic derivative remains surprising. If the reaction were to take place in an unsolvated state, the more electronegative atom, oxygen, should display the faster

rate. In water, it was found that for structurally related compounds the reactivity of both amines and alcohols with the acyl-enzyme intermediate, furoyl-chymotrypsin, shows little or no sensitivity to the basicity of the nucleophile [95]. In water, the reactive species is the anion of the alcohol, whereas this possibility does not exist in organic solvents. In this case, the amino group exhibits a more nucleophilic character than its hydroxy counterpart. Furthermore, Picture 1 shows the existence of an intramolecular hydrogen bond between the N-terminal peptide-CO and the NH group of the scissile peptide bond. This stabilizing effect does not exist in the case of the phenyllactic derivative. Scheme 2 shows a potential mechanism for explaining the absence of reactivity of the phenyllactic derivative.



Scheme 2

As shown in Scheme 2, the formation of the intermediate is favoured in the case of the amino derivative due to its greater nucleophilicity and the existence of an intramolecular hydrogen bond. Furthermore, if the spatial arrangement of the triad Ser-195/His-57/Asp-102 has been slightly modified due to the new organic environment, the transfer of the N-ε-proton of the imidazole system to the potential good leaving group, Phe-$\overset{+}{O}$H-Ser, might lead to a non-productive reaction. In the case of L-phenylalaninamide, no such possibility exists due to the very difference between the amino group and the Ser-195 moiety.

When lysine was used as a nucleophile, only the natural linkage (involving the α-NH_2 of lysine) was formed (Table 2).

Table 2 : diamino acid derivatives as substrates

donor ester	nucleophile	dipeptide	yield(%)	reaction time(hrs)
Ac-L-TyrOEt	L-LysOBu^t	Ac-L-Tyr-α-LysOBu^t	86	18
Ac-L-TyrOEt	L-OrnOBu^t	-	0	72

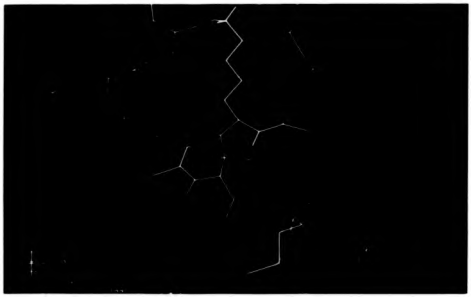
The dipeptide was characterized using ^1H NMR (400 MHz) spectroscopy. Formation of the peptide bond through the α-NH_2 resulted in a downfield shift of the α-proton from 3.45 ppm (in the free amino acid derivative) to 4.50 ppm in the dipeptide. No change in the chemical shift of the ε-protons (2.95 ppm) was

observed. This result can be contrasted with the synthesis of similar derivatives using subtilisin suspended in anhydrous tert-pentyl alcohol in which only the unnatural ε-linkage was obtained [96]

The regioselectivity of α-chymotrypsin is not modified compared to that in water [97].

Picture 3 shows the explanation for the stringent regioselectivity of α-chymotrypsin.

Picture 3



Picture 3 shows the hydrogen-bonding pattern between the covalently bound Ac-L-Tyr-L-LysNMe and chymotrypsin. The lysine side-chain fits nicely into a groove formed by His-57 to the

left, the disulfide bridge in the back, and the peptide backbone of Phe-41/Cys-42 to the right. The lysine side-chain can adopt an all-antiperiplanar conformation, and the terminal amino group forms a perfect hydrogen bond with the backbone-CO of Cys-58. If this explanation for the regioselectivity is correct, then shortening the side-chain of the nucleophile will result in the loss of this hydrogen bond, and therefore loss in reactivity. When the reaction was carried out with the ornithine derivative, no peptide bond was formed (Table 2). This result explicitly demonstrates that a single hydrogen bond can have a profound effect on the outcome of the reaction.

This phenomenon will be further emphasized when the reaction is carried out in organic solvents. If one can expect hydrophobic interactions to play a lesser role in organic solvents, hydrogen bonding and ionic interactions will be greatly re-inforced.

It was also found that tyrosine derivatives were far the best substrates for the enzyme at the P_1-subsite (Table 3). Other amino acid esters gave the desired dipeptides in high yield, but at a much lower rate. This indicates the importance of the phenolic group for the binding of the substrate to the active site.

Table 3 : Effect of the nature of the donor ester.

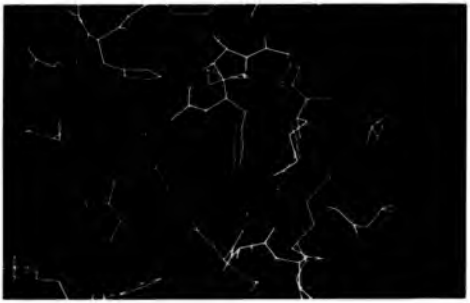
Donor ester	Nucleophile	Product	Yield (%)	Reaction time(hrs)
Ac-L-TyrOEt	L-PheNH$_2$	Ac-L-Tyr-L-PheNH$_2$	96	6
Z-L-TyrOEt	"	Z-L-Tyr-L-PheNH$_2$	94	6
Bz-L-TyrOEt	"	Bz-L-Tyr-L-PheNH$_2$	86	12
Ac-L-PheOEt	"	Ac-L-Phe-L-PheNH$_2$	90	24
Boc-L-PheOEt	"	Boc-L-Phe-L-PheNH$_2$	0	72
Ac-L-TrpOEt	"	Ac-L-Trp-L-PheNH$_2$	88	48
Z-L-DopaOEt	"	Z-L-Dopa-L-PheNH$_2$	90	12
Bz-L-AlaOEt	"	Bz-L-Ala-L-PheNH$_2$	68	48

Picture 4 shows the covalently bound Ac-L-Tyr-L-AlaNMe relaxed together with the five water molecules observed in the specificity pocket. This is a color-coded display of the putative hydrogen-bonding network. Compared with a similar Picture showing only the water molecules, the tyrosine side-chain replaces one water molecule, whereas the four other molecules may simply rearrange themselves so as to improve the hydrogen-bonding network. The tyrosine-OH is nicely engaged in hydrogen-bonding through surrounding water molecules.

To explain the difference in rates between tyrosine and phenylalanine, one can argue that the side-chain of phenylalanine, by binding in the specificity pocket, will have to displace the same number of water molecules but will not be able to provide a counterbalancing favourable effect through hydrogen-

bonding. As long as one might expect the hydrophobic interactions with the side-chain of Met-192 to be diminished due to the suspected presence of solvent molecules, the interaction of the substrates with the water cluster will be of paramount importance. Disturbing the water cluster will be thermodynamically unfavourable because of the crystal-like structure. By not being able to provide stabilizing counterbalancing interactions, the binding of phenylalanine will be less thermodynamically favourable than its tyrosine counterpart.

Picture 4.



This effect was confirmed by the use of N-benzyloxycarbonyl-3,4-dihydroxy-L-phenylalanine ethyl ester as a donor ester, which led to the synthesis of the corresponding dipeptide at a much greater rate than with the corresponding phenylalanine ester (Table 3).

Surprisingly, BOC-protected acyl donors were never involved in synthesis indicating that the conformation of the P_1-subsite might have been modified. It is worth noting that, when the reaction between BOC-L-PheOEt and L-LeuNH_2 was carried out in 0.2M carbonate buffer pH 9.2, the resulting dipeptide was obtained in 90% yield in less than 20 minutes.

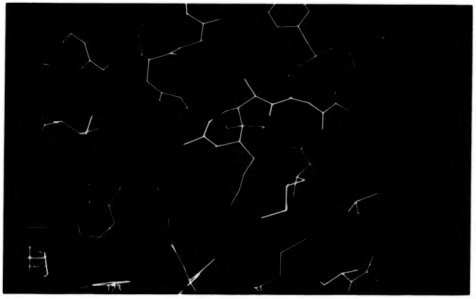
When the binding of BOC-L-Tyr-L-Ala-GlyNMe was modelled, the BOC-group was found to fall short of interacting with the peptide backbone. But the molecular modelling strategy is using X-ray structures of chymotrypsin obtained from an aqueous environment. Therefore, the absence of reactivity of BOC-derivatives in organic solvents indicates clearly that part of the active site has been modified. This was further demonstrated by the use of dipeptides (as acyl donors) which led to negative results (Table 4).

Table 4 : fragment condensation.

Donor ester	nucleophile	yield(%)	reaction time(hrs)
Z-L-TyrOEt	Gly-GlyOEt	88	18
Z-L-Leu-L-PheOEt	L-PheNH_2	0	72
Z-L-Leu-L-LeuOMe	L-PheNH_2	0	72

On the other hand, dipeptides could be successfully used as nucleophiles. Picture 5 shows a color-coded display of hydrogen bonds between the covalently docked Ac-L-Tyr-L-Ala-GlyNMe and chymotrypsin.

Picture 5



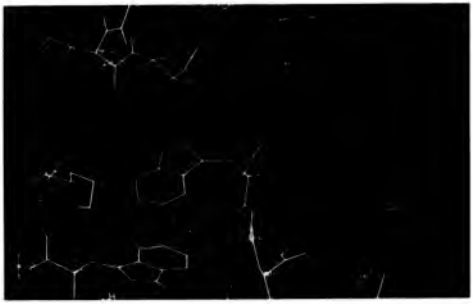
Both peptide-NH groups of the C-terminal unit of the substrate can engage in hydrogen bonds to the backbone-CO of His-40/Phe-41 in an antiparallel pleated sheet.

The failure of BOC-protected and dipeptide to function as donor esters led us to examine very closely the structure of the P-subsite. The P_1-P_2 subsites are constituted by the specificity

pocket, the backbone-CO of Ser-214 which is involved in binding the NH-moiety of the substrate, and a 'grease spot' which is formed by the pile-up of hydrophobic side-chains and accommodates the protecting group or the peptide residue On the top of this 'grease spot' sits the side-chain of Trp-215.

Picture 6 shows a color-coded display of the putative hydrogen bonding network in chymotrypsin (including tightly bound water molecules around Trp-215).

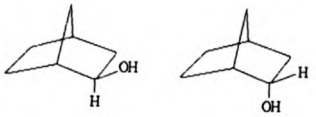
Picture 6



One can note that the indole-NH is not solvated, but water molecules form a nice hydrogen bond network from Ser-214 (conserved residue, hydrogen-bonded to Asp-102) to a tricoordinated water (one hydrogen bond to backbone-CO of Ile-99), to a tetracoordinated water (hydrogen bonds to the backbone-NH of Asn-101 and Asp-102 and backbone-CO of Ala-179). In no X-ray structures of chymotrypsin is the Trp-215 indole ring involved in hydrogen bonding.

One can then postulate that, if molecules of solvent have been able to diffuse into the crystal lattice, hydrophobic clusters will be the first to be disturbed by the new environment. Therefore, it seems reasonable to suggest that the side-chain of Trp-215, which is kept in place only by hydrophobic interactions, might well adopt a new conformation. If the new conformation leads only to a partial obstruction of the P-subsite, this might have a profound effect on the binding of the substrates. Several factors could force the Trp-215 side-chain to adopt a new conformation in the new organic environment. Firstly, as we previously saw, the indole nucleus is not solvated but just kept in place by hydrophobic contacts. Second, the endo conformation of the residue (see below) is thermodynamically unfavourable and might constitute the driving force in the conformational change (Scheme 3).

Scheme 3 : endo- and exo conformations.

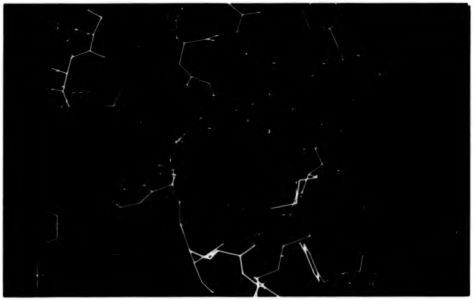


exo-2-norborneol endo-2-norborneol

And finally, if one considers the free energy values for the transfer of the side-chain from water to ethanol at 25°C, the tryptophan side-chain possesses by far the lowest value compared to the other amino acids. This indicates that it would be very favourable for the solvent molecules to solvate the side-chain of Trp-215 in its new conformation.

The molecular modelling strategy provided us with the possibility of verifying the validity of this postulate. Picture 7 shows the covalently docked Z-L-Tyr-L-Ala-GlyNMe (red structure) with the altered conformation of Trp-215 (yellow).

Picture 7



This Picture was obtained by allowing the enzyme structure to relax. One can see that the Trp-215 adopts a strainless, exo conformation compared to the endo conformation (blue). The new position of the Trp-215 side-chain is obstructing heavily the P_2-subsite, and therefore, if this model is valid, it could explain the absence of rectivity of the BOC-protected and dipeptide donor esters lacking strong interactions with the specificity pocket.

The stereoselectivity of the enzyme suspended in dichloromethane was found to be modified as shown in Table 5.

Table 5: Stereoselectivity of chymotrypsin suspended in dichloromethane.

Donor ester	Nucleophile	Product	Yield (%)	Reaction time (hrs)
Ac-L-TyrOEt	D-PheNH$_2$	Ac-L-Tyr-D-PheNH$_2$	94	12
"	D-LeuNH$_2$	Ac-L-Tyr-D-LeuNH$_2$	93	12
Ac-L-TyrOEt	D,L-PheNH$_2$	Ac-L-Tyr-L-PheNH$_2$	48	12
		Ac-L-Tyr-D-PheNH$_2$	47	
Ac-L-TyrOEt	α-methyl alanine	-	0	72
Ac-D-TyrOEt	L-PheNH$_2$	-	0	72
Ac-D-PheOEt	"	-	0	72
Ac-D-TrpOEt	"	-	0	72

Involvement of D-amino acid derivatives as nucleophiles has been reported in reactions catalyzed by chymotrypsin under alkaline conditions, but the reaction was slow [98] (approximately 10% as fast as that using L-amino acid derivatives). In another report [79], D-LeuNH$_2$ was used for synthesis in aqueous medium, although the reaction was much slower than that of the corresponding L-form. Thus, the resolution of D,L-LeuNH$_2$ was possible.

Results depicted in Table 5 indicate that no resolution was possible when using chymotrypsin in organic solvents. even if the rate of reaction of the D-nucleophiles is slower, with $[k_{cat}/Km]_L/[k_{cat}/Km]_D$ estimated to be around 3, D-amino acid amides can be used efficiently as nucleophiles. The modification,

to some extent, of the stereoselectivity for enzymes suspended in organic solvents, is a rather general phenomenon [68,99]. However, no satisfactory explanation for it has so far been given.

Picture 8 shows a color-coded display of torsional strain in covalently docked Ac-L-Tyr-D-AlaNMe.

Picture 8

The accommodation of the substrate induces some 3 kcal/mol of torsional strain into the peptide backbone of D-alanine. This can be compared with an almost strainless fit of the standard Ac-L-Tyr-L-AlaNMe substrate (Picture 9).

Picture 9

This shows the juxtaposition of covalently docked Ac-L-Tyr-L-AlaNMe (left) and Ac-L-Tyr-D-AlaNMe (right).

If the additional torsional strain in the backbone of D-alanine, explains the stereoselectivity in aqueous medium, this does not provide a satisfactory explanation for the absence of stereoselectivity observed in organic solvents.

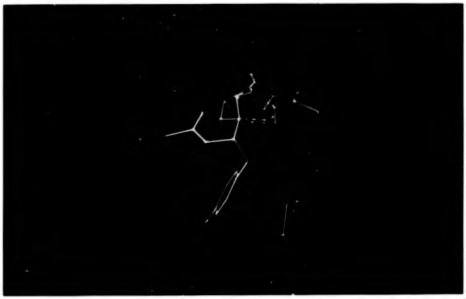
One can propose several explanations to account for the experimental results. Firstly, the tridimensional structure might have been modified by the presence of the new organic environment. This conformational change would not have to be dramatic to release some of the torsional strain in the D-

substrates. Second, as described previously, the binding of the L-substrate results in the release of a number of water molecules. If the D-enantiomer binds to the active site, it might well release fewer molecules of water than its L-counterpart. Therefore, the reactivity of the L-enantiomer should be diminished to a greater extent [68]. But, this explanation can be contradicted by the concept elaborated by Dewar [69] of pseudo-gas phase reactions. According to this concept, the perfect binding of the L-enantiomer will squeeze out the molecules of solvents, should they be water or organic solvent, and , if this might be thermodynamically unfavourable (for water, but not for the organic solvent) therefore the reaction will take place at a much faster rate than for the D-enantiomer which will leave in place residual solvent molecules. If one examines the X-ray structures of chymotrypsin with the inclusion of the experimentally observed water molecules, one can see that only one of these sits in the binding site of the nucleophile. Furthermore, this water molecule will have to be displaced whatever the configuration of the nucleophile.

We cannot provide a definite explanation for the apparent relaxed stereoselectivity observed at the P_1'-subsite. Nevertheless, slight changes in the conformation of the protein backbone could well influence the binding of the substrates. But, without knowing the location of the molecules of solvents and the spatial arrangement of the P'-subsite, no valid argument can be put forward.

The molecular modelling studies suggested that replacing D-alanine by α-amino isobutyramide (Aib) would result in a further displacement of the C-terminal peptide unit away from Phe-41-CO with concomitant relief of some torsional strain in the backbone (Picture 10).

Picture 10



Picture 10 shows the juxtaposition of covalently docked Ac-L-Tyr-L-AlaNMe (yellow), Ac-L-Tyr-D-AlaNMe (red), and Ac-L-Tyr-AibNMe (blue).

As indicated in Table 5, no reaction was observed when the $AibNH_2$ was used as a nucleophile. If the displacement of the C-terminal unit away form the peptide backbone occurs, one of the methyl

groups might be in conflict with the tetrahedral intermediate as indicated in Picture 10.

As indicated in Table 5, no change in the sterospecificity of the enzyme for the donor ester was observed. D-amino acid esters were never involved in synthesis. This stringent stereospecificity contradicts experimental results obtained with transesterification reactions catalyzed by chymotrypsin suspended in organic solvents [68]. The ratio of specificity constants $[k_{cat}/Km]_L/[k_{cat}/Km]_D$ which reflects the enantioselectivity of the enzyme was found to decrease from 710 in water to 3.2 in butyl ether. However, as stated above, we never observed any involvement of D-amino acid esters in synthesis.

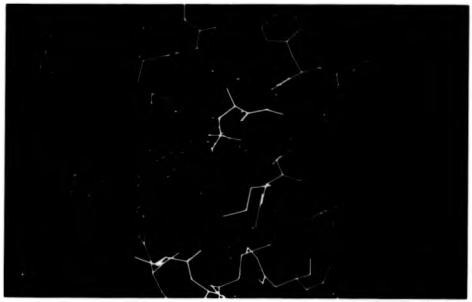
Picture 11 shows a color-coded display of torsional strain in docked Ac-D-Tyr-L-AlaNMe.

Picture 11

If this docking may look reasonable, torsional strain in the backbone of D-tyrosine amounts to some 8 kcal/mol and is quite prohibitive. To relieve the torsional strain, one might postulate an alternative docking mode for the substrate, with the protecting group occupying the specificity pocket and the tyrosine side-chain sitting at an approximate P_2-position.

Picture 12 shows such an alternative docking mode.

Picture 12



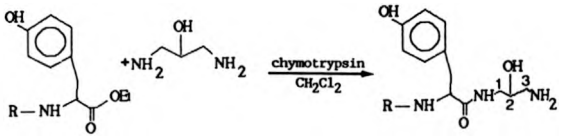
The benzyloxycarbonyl moiety of Z-D-Tyr-L-AlaNMe (red) reaches deeply into the specificity pocket (Ac-D-Tyr-L-AlaNMe (blue) in the conventional docking mode). The Z-protecting group by going

deeply in the specificity pocket competes with several tightly bound water molecules, at least three, thus isolating the top from the bottom two water molecules. As long as no counterbalancing stabilizing effect, such as hydrogen bonding, is provided, this binding mode is thermodynamically highly unfavourable, and therefore might well be prohibited.

The theory according to which the enantioselectivity of an enzyme decreases because the D-enantiomer releases less water than its L-counterpart [68], is not valid in this case, because it does not take into account the torsional strain induced by the binding of the substrates. The alternative binding mode (Picture 12) would require the release of even more water molecules than the binding of the L-enantiomer.

The stereoselectivity of chymotrypsin was further investigated by using a prochiral substrate as nucleophile, 1,3-diamino-2-hydroxy-propane (Scheme 4).

Scheme 4.



R=Ac, 1
R=Z , 12

31

32 88%
33 92%

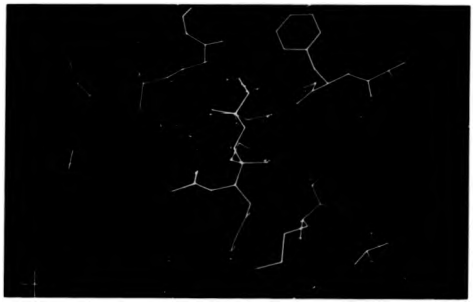
1,3-diamino-2-hydroxy-propane being a prochiral compound, coupled with a chiral amino acid derivative, the resulting peptide derivative will exist as a mixture of two diastereoisomers, (S,S) and (S,R), unless the enzyme is able to discriminate between the enantiotopic groups during the coupling process. As depicted in Scheme 4, the reaction proceeds efficiently, giving rise to the desired peptide in high yields. After purification, the compound **33** was analyzed using high field NMR.

The ^{1}H NMR spectrum (400 Mhz) shows a signal at 3.24 ppm corresponding to the protons at C-3. This signal appears as a triplet which is in fact the combination of two doublets coming from each of the diastereoisomers. Integration of the signals indicated that no diastereoisomer had been preferentially formed.

This was further confirmed by the ^{13}C NMR spectrum (100.62 MHz) of the derivative **33**. Identification of the individual carbon atoms was provided by using the ^{13}C [DEPT] technique which enabled us to distinguish between CH, CH_2, and CH_3. The signals corresponding to C-1 (42.5 ppm) and C-3 (37 ppm) appeared as doublets in the full NMR spectrum. No diastereoisomeric excess was therefore observed.

Under our conditions, the enzyme was not able to discriminate between the two enantiotopic faces. Picture 13 shows the superposition of (S)- and (R)-diaminopropanol derivatives of Ac-L-Tyrosine (red for (S), yellow for (R)).

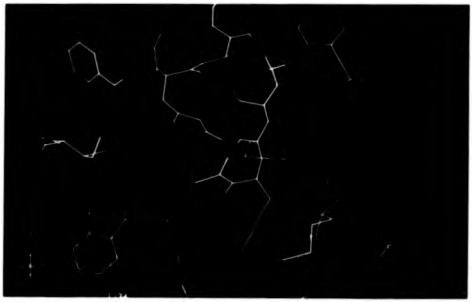
Picture 13



It is worth noting that the (R)-diaminopropanol unit can be nicely placed into the groove formed by His-57 to the left, the disulfide bridge in the back, and the peptide backbone of Phe-41/Cys-42 to the right. Furthermore, the diaminopropanol unit adopts a fully staggered conformation in which both N-C-C-O moieties adopt gauche conformations. In this 'up-position' the terminal amino group hydrogen bonds to the Cys-58 backbone-CO, whereas the carbinol-OH hydrogen bonds to the backbone-CO of His-57.

Picture 14 shows the color-coded display of hydrogen bonds between covalently docked (R)-diaminopropanol derivative and chymotrypsin.

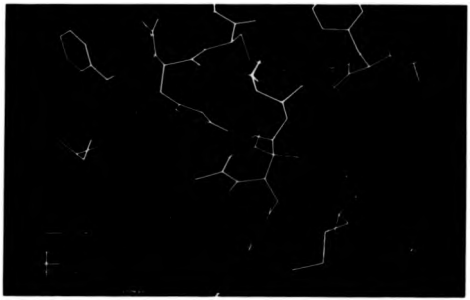
Picture 14



One can see that the hydrogen bond (substrate)OH...OC(His-57) is relatively weak due to the large O...O distance of 3.1 Å (preferred 2.6-2.8 Å).

Picture 15 shows the hydrogen bonding pattern for the covalently bound (S)-isomer.

Picture 15

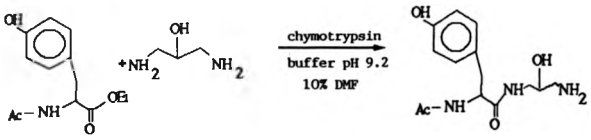


It is worth noting the strong hydrogen bonds of the aminopropanol unit to the two backbone-CO's of His-57 and Phe-41.

In order to clarify this apparent absence of diastereoselectivity of chymotrypsin in organic solvents, the synthesis of the diaminopropanol derivative was carried out in aqueous medium (Scheme 5).

After two days, the reaction was stopped and the products were purified using preparative thin-layer chromatography. No attempt was made to separate the diastereoisomeric mixture.

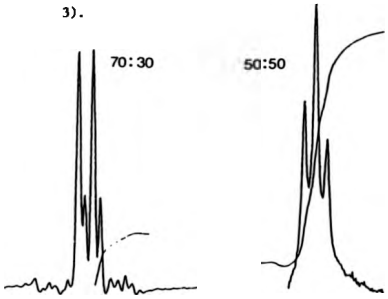
Scheme 5.



1 **31** **32** **48%**

The purified diaminopropanol derivative was then analyzed using ^{1}H NMR (400 MHz). The NMR spectrum revealed the presence of two sets of doublets at 2.85 ppm, corresponding to the protons at C-3 (Fig. 1).

Figure 1 : 400 MHz NMR spectrum of the diaminopropanol derivative (signals at 2.85 ppm corresponding to C-3).



carbonate buffer pH 9.2 — CH_2Cl_2 0.25% H_2O

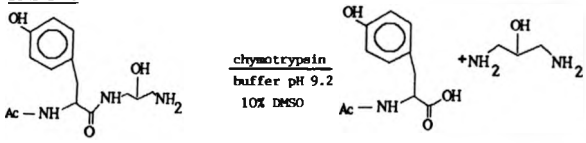
After integration of the signals, it was estimated that the diaminopropanol derivative was composed of a mixture of two diastereoisomers in a ratio 72:28 (de=44%). Every attempt to separate the two diastereoisomers failed, thus it was impossible to assign the absolute configuration at C-2 for both diastereoisomers.

This result indicates clearly that the stereoselectivity of the enzyme has been modified through the transition from an aqueous to an organic environment. In water, the enzyme is able to discriminate between the two enantiotopic groups, yielding a major diastereoisomer in 44% de.

This stereoselectivity is dramatically reduced when the enzyme is suspended in organic solvent to the extent that no major diastereoisomer is preferentially formed. However, one has to remain cautious in analyzing these results. When the reaction is carried out in aqueous medium, the desired compound 32 is isolated in a rather disappointing yield (48%) and the hydrolyzed acid, Ac-L-TyrOH, is isolated as a byproduct. Therefore, the diastereoisomeric excess observed for this reaction might come from the stereoselectivity for the synthesis, but also, if the product is synthesized without discrimination, the enzyme might well exert some stereoselectivity in the hydrolysis of the product. At first, it seems unlikely because the product, once formed, immediately precipitates. But, even if it is scarcely soluble, it might still be a substrate for the enzyme.

In order to clarify this point, the hydrolysis of the diaminopropanol derivative **32** (as a 1:1 diastereoisomeric mixture) was attempted (Scheme 6).

Scheme 6.



After reaction overnight, the reaction mixture was freeze-dried and the remaining diaminopropanol derivative **32** recrystallized from methanol.

The ^1H NMR spectrum (400 MHz) shows the presence of two sets of doublets at 2.85 ppm corresponding to the protons at C-3. Integration of the signals afforded the diastereoisomeric excess (de=30%). The major diastereoisomer is the same as that observed when the synthetic reaction was carried out in aqueous medium. In other words, this diastereoisomer is the fastest to be synthesized but the slowest to be hydrolyzed. The diastereoisomeric excess observed when the synthetic reaction was carried out in water, might then arise from a preferential

hydrolysis of one of the diastereoisomers, both being synthesized at the same rate.

If a final answer to those questions cannot be provided here, one can see that it is theoretically possible to obtain one pure diastereoisomer by combining synthesis and subsequent hydrolysis in water. No information concerning the absolute configuration at C-2 could be obtained and the molecular modelling experiments showed that there was no obvious reason for the preferential formation of one particular diastereoisomer.

In summary, chymotrypsin suspended in hydrophobic organic solvents permitted preparation of diverse peptides. The isolation of the desired compounds was made very simple and the enzyme could eventually be recovered without significant loss of activity.

The stereoselectivity of the enzyme has not been drastically altered. Nevertheless, various D-amino acid derivatives were readily incorporated at the P_1'-subsite. The specificity of the P_1 subsite becomes more stringent towards single N-protected L-amino acid esters, and high specificity for hydroxylated aromatic amino acid esters was shown.

Molecular modelling proved to be a very valuable tool for correlating the experimental results to the peptide structure in terms of substrate specificity and enantioselectivity.

Computer graphics allowed a direct comparison between the behaviour of the enzyme in organic solvents to that in water, thus, permitting the formulation of reasonable predictions concerning the influence of the new environment on the protein structure.

I-2. HYDROLYTIC REACTIONS CATALYZED BY CHYMOTRYPSIN SUSPENDED IN ORGANIC SOLVENTS WITH LOW WATER CONTENT.

I-2.1. Introduction.

The primary in vivo role of chymotrypsin is to catalyze the hydrolysis of amide bonds of proteins adjacent to the carbonyl groups of aromatic L-amino acids such as phenylalanine, tyrosine, and tryptophan. Most of the specificity data relevant to the use of chymotrypsin for resolution and in asymmetric synthesis have come from studies with ester and amide substrates. Esters are hydrolyzed much more rapidly than the corresponding amides. As a result, the structure-specificity relationships of the enzyme have been largely developed by using ester substrates in aqueous medium. The transition from an aqueous to an organic environment usually results in novel properties being exhibited by the enzyme. This may provide valuable insights in our understanding of the enzyme, as well as enabling us to carry out new reactions which are impossible in water. Our previous studies dealing with the application of powdered enzyme suspended in organic solvent have already shown that the enzyme structure was modified [100], allowing for example the use of D-amino acid nucleophiles for

peptide synthesis. But the overall impression is that the enzyme seems to be even more restricted in terms of specificity than its counterpart in water.

In order to establish structure-reactivity relationships, hydrolytic reactions provide a better model system because only part of the active site is involved. But if chymotrypsin is able to hydrolyze selected substrates when suspended in dichloromethane containing 0.25% water, the reactions are so slow, even with good substrates such as tyrosine derivatives, that this approach is impracticable for the screening of a large number of substrates.

Fortunately, such a model was discovered when we studied the potential stereospecific synthesis of primary amides. Primary amides of amino acids can be easily synthesized according to a method described by Muramatsu [90] (Scheme 7).

Scheme 7.

$$R\text{-}COOH \xrightarrow[NH_4HCO_3]{EEDQ} R\text{-}CONH_2$$

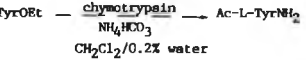
where R is a N-protected amino acid.

This method affords efficient preparation of primary amides with yields usually over 90%.

Ammonium bicarbonate acts as an efficient ammonium donor in organic solvents and therefore could be used in an enzymatic

reaction as a nucleophile, the coupling reagent EEDQ being substituted by the action of an enzyme (Scheme 8).

Scheme 8.



When the reaction depicted in Scheme 8 was carried out using 5 equivalents of ammonium bicarbonate, the isolated product was found to be, not the desired amide Ac-L-TyrNH$_2$, but the hydrolyzed acid, Ac-L-TyrOH. Control reactions showed that no hydrolysis was observed in the absence of either the salt, the enzyme, or the added water.

This system of hydrolytic reactions with low water content provided us with a perfect model to study structure-reactivity relationships. Active site mapping was possible and valuable information about the structure of the enzyme in the organic solvent could be obtained by the screening of simple substrates.

I-2.2. Materials and methods.

Enzyme.

Crystalline bovine pancreatic α-chymotrypsin (EC 3.4.21.1) (type II) was purchased from Sigma as a lyophilized powder with

specific activity of 51 units/mg protein. The enzyme was used without further purification.

Substrates and reagents.

Ammonium bicarbonate was purchased in the highest purity available. The salt was extensively washed with anhydrous solvents (THF, CH_2Cl_2) to remove any contaminants prior to drying under high vacuum.

Dichloromethane was obtained through continuous distillation over calcium hydride.

Ester substrates were synthesized by esterification with a concentrated solution of hydrochloric acid in the appropriate alcohol. Hydroxy esters were prepared by reduction with sodium borohydride of the corresponding ketoesters. N-protected amino acid esters and peptides were prepared by conventional methods [101].

Assays.

The standard reaction mixture in dichloromethane consisted of 40 mM of the substrate, 16 μM enzyme, 5 equivalents of ammonium bicarbonate. The reaction was then started by the addition of 0.2% (v/v) water. The reaction was followed by thin-layer

chromatography (dichloromethane/methanol 0-10%). At a given time, the reaction mixture was filtered, the solvents were evaporated, and the remaining residue was dissolved in the appropriate NMR solvent (D_2O/acetone-d^6, $CDCl_3$/TFA). In most cases, the unreacted ester was isolated (via extraction or chromatography on silica gel), analyzed by ^1H NMR (220 MHz), and its optical rotation and melting point were compared with commercially available samples.

I-2.3. Results and discussion.

Recent findings indicate that the essential water can be substituted to some degree by other compounds. When water is stripped from the enzyme by the solvent, it was suggested that areas of the protein which normally interact with water become exposed. This exposure has a detrimental effect on activity. If this is the case, then addition of compounds which can mimic the interaction of water with the protein (e.g., by forming hydrogen bonds) should to some degree restore the activity. A.M. Klibanov found that the presence of 1% formamide increased the activity of alcohol dehydrogenase in butyl acetate (0.4% water) [63]. This strategy was employed for enzymatic peptide synthesis via segment condensation in the presence of water mimics [64].

To try to explain the actual nature of the ammonium bicarbonate effect, several control experiments were carried out. Firstly, no

hydrolysis of Ac-L-TyrOEt was observed in the absence of the added salt. Second, no reaction took place in the absence of the enzyme.

In order to estimate the effect of the salt on the protein structure, experiments were carried out under anhydrous conditions. The model reaction was the synthesis of the dipeptide Ac-L-Tyr-L-PheNH$_2$ catalyzed by chymotrypsin suspended in anhydrous dichloromethane containing 3 equivalents of ammonium bicarbonate. After 3 days at room temperature, the desired dipeptide was isolated in 83% yield. The remaining starting material, Ac-L-TyrOEt, was recovered (84% conversion) and traces of the hydrolyzed acid, Ac-L-TyrOH, were detected by thin-layer chromatography. If the reaction proceeded at a rate several orders of magnitude slower than the reaction with 0.25% water, it proved nonetheless that peptide synthesis under anhydrous conditions was possible.

The effect was highly specific to ammonium bicarbonate compared to other inorganic salts (Table 6).

Formamide did not give any effect, thus invalidating the simple hypothesis of hydrogen bonding

Table 6.

Ac-L-TyrOEt —chymotrypsin, CH_2Cl_2/0.2% water, 5 eq. of salt→ Ac-L-TyrOH

Salt	Yield (%)	Reaction time (hrs)
none	0	48
NH_4HCO_3	100	12
$NaHCO_3$	<10	48
$(NH_4)_2SO_4$	<10	48
$NH_4H_2PO_4$	<10	48
formamide	<10	48

Two explanations can be proposed to try to explain the effect of ammonium bicarbonate. Ammonium bicarbonate shows a slight solubility in the organic phase. If the theory of the stripping of water from the enzyme by the solvent is correct, dissolution of the salt in the organic phase could decrease the overall solubility of water in the organic solvent, thereby making it available for hydrolysis.

Furthermore, if the salt limits the solubility of water in the organic phase, the essential water will be conserved, and the enzyme will then exhibit some activity even under anhydrous conditions, as shown by its ability to catalyze the synthesis of dipeptides in anhydrous dichloromethane. The second possibility could be a binding effect by the salt. By replacing water

molecules on the protein molecule, the salt could help to keep the enzyme in its active form. But the negative effect observed with formamide indicates that this could not occur via hydrogen bonding. A more specific effect can be envisaged if, by binding to the active site, the salt is able to trigger the enzyme in its active conformation. Such an effect was observed with L-PheNH_2 as a nucleophile and will be discussed in Chapter II.

The results shown in Table 7 demonstrate that the system can be used for the resolution of racemates of amino acid derivatives.

Table 7 : hydrolysis of aromatic amino acid esters*.

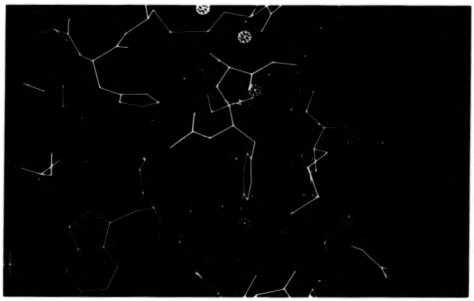
substrate		reaction time (hrs)	yield (%)	ee (%)
Ac-L-TyrOEt	1	8	100	-
Ac-L-PheOEt	35	12	100	-
Ac-L-TrpOEt	37	21	45	-
Ac-D,L-PheOEt	39	18	100	100(ester)
L-TyrOEt	40	12	100	-
L-TyrOMe	42	15	100	-
D,L-TyrOMe	43	15	85	65(ester)
L-PheOEt	45	36	100	-
L-PheOMe	47	48	100	-

* Details of the characteristics of the different products are given in the experimental part.

Tyrosine appears to be the best substrate for hydrolysis, far better than the phenylalanine and tryptophan equivalents.

Picture 16 shows the covalently bound tetrahedral intermediate Ac-L-Tyr-L-AlaNMe with inclusion of the water molecules.

Picture 16



One can see that the oxyanion replaces one water molecule. The tyrosine side-chain of the substrate replaces one water molecule, whereas the other four water molecules may simply rearrange themselves so as to improve the hydrogen bonding network (Picture 4). The phenylalanine derivative 35 cannot provide any

hydrogen bonds with the water molecules inside the specificity pocket. Therefore, as long as the unfavourable effect of displacing water molecules from the specificity pocket cannot be counterbalanced by the creation of hydrogen bonds, the overall effect will be that phenylalanine esters will be hydrolyzed slower than their tyrosine counterparts.

The very slow hydrolysis of the tryptophan ester **37** cannot be readily explained. By binding, the indole side-chain will displace the same number of water molecules as the tyrosine and phenylalanine derivatives. In water, the indolyl residue of tryptophan affords a near perfect fit to the specificity pocket (Ac-L-TrpOMe : Km=0.095mM and $k_{cat}/Km=2.9x10^5 M^{-1}.s^{-1}$ [122]). To explain the low reactivity of tryptophan esters, one could postulate that the tridimensional structure of the protein could have been altered by the new organic environment. If such modifications occurred near the active site, the binding of the bulkier tryptophan derivative will be more affected than the monocyclic side-chains of tyrosine and phenylalanine. Without knowing the actual structure of the enzyme in an organic solvent, no conclusive answer can be provided, but, if any conformational changes do occur, they will have to affect the binding segment which is constituted by the hydrophobic cavity with Met-192 residue as the lid.

As indicated in Table 7, there is no need for a protecting group on the α-amino moiety. Results from the molecular modelling experiments indicated that the hydrogen bond between the amino

moiety and the peptide backbone-CO of Ser-214 was of primary importance. This hydrogen bond is important in the orientation of the substrates at the active site. Unprotected amino acid esters were readily hydrolyzed with a marked preference for the tyrosine derivatives. In water, the difference between the two derivatives is hardly noticeable (Ac-L-PheOMe : Km=1.25mM and $k_{cat}/Km=4.2x10^4$ $M^{-1}.s^{-1}$ and Ac-L-TyrOMe, 0.7mM and $3x10^5$ $M^{-1}.s^{-1}$, respectively [122]). In organic solvents, there is approximately a four-fold difference in rates of hydrolysis. This , once again, emphasizes the importance of the phenolic group in the tyrosine derivative **40**.

The ester functionality of the substrate in chymotrypsin-catalyzed hydrolyses exhibits a limited influence on the reaction rates. Ethyl esters appear to be better than their methyl counterparts. The difference between methyl and ethyl esters becomes very noticeable for the phenylalanine derivatives.

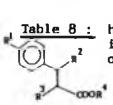
As shown in Table 7, the resolution of racemic amino acid esters was possible. The L-enantiomer of the substrate **35** was readily hydrolyzed whereas the D-enantiomer **29** was kept untouched. The lower rates of hydrolysis are due to the competitive inhibition of the reaction by the D-enantiomers. To explain the stereoselectivity of the enzyme towards L-enantiomers, we have seen previously that the binding of the D-ester induces a significant torsional strain which amounts to some 8 kcal/mol in the case of D-tyrosine derivatives (Picture 11). This is sufficient in itself to explain the stereospecificity of the

enzyme. But one can add to that factor that, in the D-enantiomer, no possibility of hydrogen bonding between the amino group and the peptide backbone-CO of Ser-214 exists.

In order to draw structure-reactivity relationships, we investigated the hydrolysis of aromatic amino acid analogues bearing new functionalities at the α- and β-positions (Table 8).

We have seen that the hydroxyl group of the tyrosine derivative can be part of a network of hydrogen bonds with the water molecules in the specificity pocket (Picture 16). When the aromatic ring was substituted at the para position by a chloro group **58**, no hydrolysis could be observed. The p-chloro derivative **58** goes deeper into the specificity pocket than its phenylalanine counterpart. Therefore, the network of water molecules at the bottom of the pocket is heavily disturbed. If this explanation seems reasonable on thermodynamic grounds, the possibility of a modification of the tridimensional structure of the enzyme cannot be ruled out. In water, substitution at the para-position is usually not detrimental [122].

Table 8 : hydrolysis of aromatic amino acid analogues * (unless indicated the reaction time corresponds to the completion of the reaction).



	Configuration	Hydrolysis	Reaction time(hrs)	products
43 R^1=OH, R^2=H R^3=NH_2, R^4=CH_3	D,L	+	15	L-acid 41(opt. pure) D-ester 44(opt. pure)
47 R^1=R^2=H R^3=NH_2, R^4=CH_3	L	+	48	L-acid 46
48 R^1=R^2=H R^3=OH, R^4=Et	L	+slow	96	L-acid 49
50 R^1=R^2=R^3=H R^4=Et	-	-	72	non substrate
52 R^1=R^3=OH R^2=H, R^4=Me	D,L	+	60	D,L-acid 53
55 R^1=OH, R^2=R^3=H R^4=Me	-	+	18	acid 54 (24% yield)
57 R^1=R^3=H, R^2=OH R^4=Et	D,L	-	48	non substrate
58 R^1=Cl, R^2=H R^3=NH_2, R^4=Et	D,L	-	48	non substrate
59 R^1=OH, R^2=H R^3=NHBOC, R^4=Me	L	+slow	30	acid 60 (35% yield)
62 R^1=R^3=NH_2, R^2=H R^4=Me	D,L	+	26	L-acid 63(opt. pure) D-ester 64(opt. pure)
17 R^1=R^2=H R^4=Et R^3=NHBOC	L	-	48	-

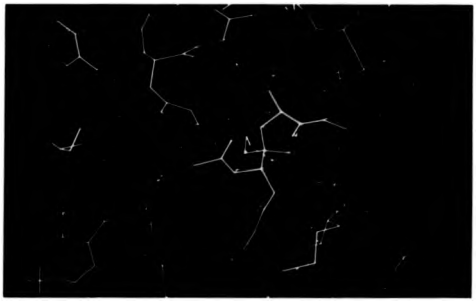
* Full details of the characteristics of the different products are available in the experimental part.

As shown in Table 8, substitution at the para-position by an amino group **62** immediately restored the activity. The amino group can be involved in hydrogen bonding with the water molecules in the specificity pocket, and therefore proved to be a much better substrate than the phenylalanine equivalent. The reaction is stereospecific and the optically pure L-enantiomer can be obtained.

By replacing the α-amino group by a hydroxyl group, the substrate reactivity is diminished to a great extent as shown for ethyl L-phenyllactate **48**. With the tyrosine analogue **52**, low substrate reactivity and loss of stereoselectivity was observed. No resolution was possible and the racemic D,L-acid was obtained. In water, the hydrolysis of phenyllactate derivatives is stereoselective, the L-enantiomer being hydrolyzed more rapidly than its D-counterpart [102].

When the reaction is carried out in organic solvents, one can clearly see that the enzyme has lost its stereospecificity for α-hydroxy esters. Picture 17 shows the superposition of covalently docked Ac-L-Tyr-L-AlaNMe and L-Phelac-L-AlaNMe.

Picture 17



From the molecular modelling experiments, one can see that not much difference in the conformations of the bound substrates is to be expected. The lactate OH sits at the position of the N-terminal amino group of Ac-L-Tyr-L-AlaNMe. If Picture 17 provides an explanation for the stereoselectivity observed in aqueous medium, it does not explain the loss of stereoselectivity for α-hydroxy esters when the reaction is carried out in organic solvents. The orientation of the L-enantiomer at the active site is provided by the existence of hydrogen bond between the amino group and the peptide backbone-CO of Ser-214 [103]. If this hydrogen bond is lost, then no stereoselectivity is to be observed. In an aqueous environment, this hydrogen bond is

established by the α-amino- and α-hydroxyl groups. To explain the loss of stereoselectivity for α-hydroxyl esters, one can suggest that the capacity of forming a strong hydrogen bond has been lost in the case of the α-hydroxy derivative, but is conserved for the amino acid derivative.

We have seen previously that a modification of the tridimensional structure of the enzyme due to the organic environment was envisageable (Picture 7). This modification affects chiefly the P-subsite, and consequently the environment of Ser-214. Any modification around Ser-214 will have a direct effect on the capacity of Ser-214 to hydrogen bond with the substrate, and therefore, on the stereoselectivity of the reaction. If one cannot define more precisely the nature of these modifications in the protein structure, one can see nevertheless that slight variations can have dramatic consequences on the outcome of the reaction.

The average distances of hydrogen bonding for O-H...O(carbonyl) and N-H...O(carbonyl) are 2.7 Å [104] and 3.04 Å [105] respectively. It is therefore not inconceivable that only the amino group will be able to establish a strong hydrogen bond with the backbone-CO of Ser-214. But this argument also suggests that a high level of rigidity has to exist in the protein structure. If the enzyme structure was highly flexible, the hydrogen bond might be established between the hydroxyl group and Ser-214. If this possibility does not exist, it is an indication of the rigidity of the protein, rigidity induced either by the new organic

environment, or by some strain induced by the binding of the substrates.

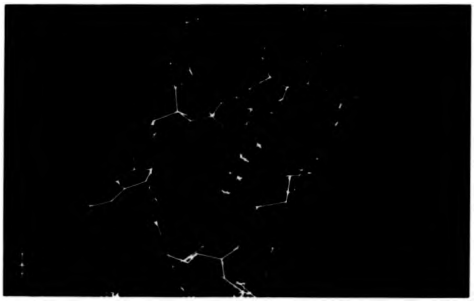
If the α-functionality is replaced by a hydrogen atom, the direct effect of the binding of the aromatic substituent can be measured. Once again, one can observe a striking difference between the tyrosyl **55** and the phenylalanyl **50** analogues. The ester **55** is hydrolyzed to the acid in 24% yield over 18 hours. This rate is slow compared to the natural tyrosine derivative **43**. For the phenylalanine analogue **50**, the rate is so low that no reaction could be observed. These results emphasize the importance of the hydrogen bond between the α-functionality and the carbonyl of Ser-214 in the orienting effect and in restricting the rotational mobility of the substrates. The orienting role of Ser-214 is more effective for groups such as amino- which can form a strong hydrogen bond. This could lead to a high k_{cat} value, but not exclusively when the substrate binds strongly overall.

Previous results indicated that the overall structure of the P-subsite might have been modified as indicated by the fact that BOC-derivatives and dipeptides are not suitable substrates in peptide synthesis catalyzed by chymotrypsin suspended in organic solvents (Table 4). As indicated by the marked difference in reactivity between phenylalanine and tyrosine derivatives, we carried out the hydrolysis of BOC-L-PheOEt **17** and BOC-L-TyrOMe **59** (Table 8). Despite a perfect interaction with the specificity pocket, BOC-L-TyrOMe **59** is only very slowly hydrolyzed, whereas

for the phenylalanine derivative **17**, no reaction was observed. Picture 18 shows a color-coded display of hydrophobic interactions between the covalently docked BOC-L-Tyr-L-Ala-GlyNMe and chymotrypsin.

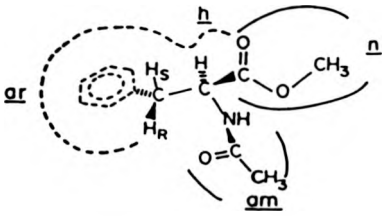
The BOC-unit occupies the space between His-57 and Trp-215 its altered conformation (this conformational change is believed to be possible due to the fact that in no X-ray structures is the Trp-215 indole ring involved in hydrogen bonding (Pictures 6 and 7)).

Picture 18



The BOC-protecting group does not interact strongly with the Trp-215 side-chain. But one has to bear in mind that only a very slightly unfavourable interaction would be responsible for the absence of reactivity. The apparent modification of the P-subsite is further emphasized by the result obtained with the ester 57. The resolution of the hydroxy ester 57 was not possible and no hydrolysis was observed. In water, separating the chiral centre bearing the hydroxyl group from the ester causes a reversal in stereoselectivity [103]. This type of inversion of stereoselectivity is readily explained in terms of the active site model shown in Fig. 2 [106].

Figure 2 : The active site of chymotrypsin according to the model of Cohen [106] (Ac-L-PheOMe as an interacting substrate). Ar represents the hydrophobic pocket, am the amide binding site (hydrogen bond with Ser-214), n the nucleophilic site, and h site which does not contribute directly to binding or orientation but its restricted volume plays a role in the specificity of chymotrypsin.



For the preferred D-enantiomers, the hydroxyl group will occupy the H_R position of the acetyl-L-phenylalanine structure. Interaction of these groups with the ar-site is possible in this position, and orientations favouring hydrolysis are thus possible. On the other hand, the OH functionality of the less easily hydrolyzed L-enantiomers will occupy the more distant and conformationally opposed H_S location, virtually precluding effective association with the ar-site.

Our results indicate clearly that this wrong binding is not permitted. This indicates that at the β -position, any substituent but hydrogen cannot be accommodated in this part of the ar-site, once again pinpointing the evidence of a modification of the protein structure at the P-substite.

The result of the hydrolysis of BOC-L-TyrOMe 59 has led us to examine closely the structure of the P_2- and P_3-subsites. If the P_2-subsite is not totally obstructed (as indicated by the peptide synthesis experiments), and if one provides a perfect interaction with the specificity pocket (tyrosine derivatives), one might be able to get some reactivity towards dipeptide derivatives. The results are summarized in Table 9.

Table 9 : hydrolysis of dipeptide derivatives (the chemical yields were determined by 220 Mhz NMR of the crude reaction mixture).

Dipeptide	hydrolysis	reaction time (hrs)	yield(%)
Z-L-Leu-L-PheOEt **23**	-	24	0
Z-L-Leu-L-LeuOMe **24**	-	24	0
Z-L-Leu-L-TyrOEt **65**	+	24	35
Z-L-Ala-L-TyrOEt **67**	+	24	100
Z-D-Ala-L-TyrOEt **69**	+	24	20

The two dipeptides Z-D-Ala-L-TyrOH and Z-L-Leu-L-TyrOH could not be isolated due to the low chemical yields.

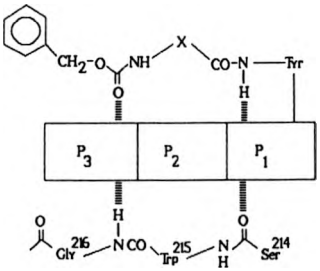
According to Segal [107] on the basis of an X-ray crystallographic study of the covalent complex formed between α-chymotrypsin and a series of amino acid-L-phenylalanine chloromethyl ketone inhibitors, an antiparallel β-type interaction exists between the amino acyl chain and an extended peptide chain at the enzyme surface adjacent to the primary specificity site. The protein residues involved in the interaction are Ser-214, Trp-215, and Gly-216. As indicated by the results shown in Table 9, dipeptides lacking a strong interaction with the specificity pocket, **23** and **24**, are not hydrolyzed by chymotrypsin. But when tyrosine occupied the C-terminal unit, hydrolysis could occur.

When L-alanine occupies the P_2-position, complete hydrolysis of the dipeptide **67** was observed. But with the bulkier

L-leucine, 65, a slow hydrolysis took place which was fractionally faster than for the dipeptide 68 where D-alanine occupied the P_2-position.

In accordance with the antiparallel β-configuration proposed by Segal [107], all acyl-enzymes have a hydrogen bond between the NH-group of tyrosine and the CO-group of Ser-214. There are no hydrogen bonds between the P_2-residue and Trp-215, but there are two possible hydrogen-bonded interactions at Gly-216 (Scheme 9).

Scheme 9 : Schematic diagram of the subsite binding in N-amino-L-tyrosyl chymotrypsins. The hydrogen bonds involved in the antiparallel β-configuration with the residues 214-216 of the enzyme are shown (dotted lines).



According to X-ray structures and molecular modelling experiments, the P_2-subsite is formed by a groove constituted by the side-chain of Trp-215 on one side, and Asp-102/His-57 on the other. The driving force for the binding at the P_2-subsite is provided by hydrophobic interactions to the hydrophobic cluster onto which Trp-215 is lying (Picture 19).

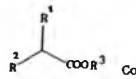
Picture 19 : structure of the P_2-subsite with dotted Van der Walls surface of active site domain (yellow/blue pattern for hydrophobic and hydrophilic surface domains with Ac-L-Tyr-L-LysNMe as docked substrate)

Therefore, one might predict that hydrophobic residues at the P_2-subsite should be good substrates. However, results from Table 9 indicate that L-alanine is preferred to L-leucine at the P_2-position. This result is an indication that the leucine side-chain is in conflict with part of the protein structure, and according to aforementioned results, the Trp-215 indole nucleus seems to be once again involved. If the Trp-215 side-chain were to adopt the new altered conformation (Picture 18), it will interact more strongly with the leucine derivative than with the L-alanine one. If D-alanine is used at the P_2-position, the antiparallel β-type structure will put the side-chain of D-alanine in direct conflict with Trp-215, whatever the conformation of the latter residue.

In order to provide more information about the structure of the enzyme in an organic environment, hydrolytic reactions involving amino acid analogues were carried out (Table 10).

Phenylglycine derivatives **71** and **72** are not substrates under these conditions despite that acetyl-D,L-phenylglycine methyl ester is readily hydrolyzed in water, yielding the L-acid whereas the D-enantiomer is kept untouched [122].

Table 10 : hydrolysis of amino acid analogues *.



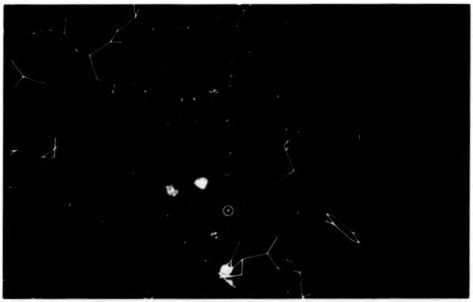
	Configuration	Hydrolysis	Reaction time(hrs)	Products
71 R^1=Ph, R^2=NH_2, R^3=Me	D,L	-	48	non substrate
72 R^1=Ph, R^2=NH-Z, R^3=Me	D,L	-	48	non substrate
73 R^1=Me, R^2=NH_2, R^3=Me	L	+slow	72	L-acid 74
75 R^1=Me, R^2=Br, R^3=Et	D,L	-	72	non substrate
76 R^1=Me, R^2=OH, R^3=Et	D,L	-	48	traces
77 R^1=CH_2COOMe R^2=NH_2, R^3=Me	L	+	18	monoester 78 50% yield
79 R^1=CHOH-CH_3 R^2=H, R^3=Et	D,L	-	72	non substrate
80 R^1=CH_2COOBzl R^2=NH-Z, R^3=Bzl	L	-	72	non substrate

* details of the characteristics of the compounds are available in the experimental part.

The optimum binding and orientation appear to occur when two methylene units separate the aromatic ring from the ester carbonyl. Substrates which exceed these dimensions are not hydrolyzed as shown with the aspartyl derivative **80**. In water, the presence of the β-benzyl moiety is not detrimental and N-maleyl-L-asp(OBzl)-OBzl was readily hydrolyzed by chymotrypsin (Km = 0.85 mM) leading to the β-benzyl monoester **108**. All attempts to hydrolyze this compound with chymotrypsin in organic solvent failed.

Picture 20 shows the covalently bound Ac-L-Asp(OBzl)-L-AlaNMe with inclusion of selected water molecules observed in the specificity pocket of chymotrypsin.

Picture 20



One can see that the benzyl group reaches deeply into the specificity pocket and thus competes with at least three tightly bound water molecules of the water cluster in the pocket, thus isolating the top from the two bottom water molecules. Removal of water molecules will be thermodynamically unfavourable in the organic environment, and this might well explain the absence of reactivity of this derivative. It was then postulated that by changing the ester moiety from benzyl to methyl, some reactivity could be obtained. This was demonstrated for the regioselective hydrolysis of the dimethyl aspartate **77** where the monoester could be obtained in 50% yield in 18 hours.

The reactivity of substrates lacking a strong binding interaction with the specificity pocket was also investigated (Table 10). The results indicate that replacing the α-amino group leads to a complete loss of reactivity. Ethyl D,L-2-bromo-propionate **75** and ethyl D,L-lactate **76** did not react under these conditions. Even the alanine derivative **73** reacted with a very low rate.

The absence of reactivity of the lactate derivative **76** results from the combination of weak ar-CH_3 interaction and the ability of the hydroxyl group to fit both the am- and h-sites (Fig. 2). According to the same model, the bromopropionate bears a substituent much too large for a h-interaction and unable to form a hydrogen bond with am. Separating the chiral centre bearing the hydroxyl group from the ester by a methylene, **79**, once again resulted in a loss of reactivity.

In conclusion, hydrolytic reactions catalyzed by chymotrypsin suspended in dichloromethane in the presence of ammonium bicarbonate provided an excellent model for the study of the structure-reactivity relationships. By direct comparison with the binding of the substrates in aqueous medium through molecular modelling, valuable insights into the tridimensional structure of the enzyme in organic medium could be gained. The structure of the active site of the enzyme seems to have been modified via conformational changes during the transition form an aqueous to an organic environment. The enzyme exhibits a stringent specificity for tyrosyl residues at the C-terminus. If the hydrolysis is stereospecific for amino acid derivatives, no stereoselectivity was observed for α-hydroxy esters.

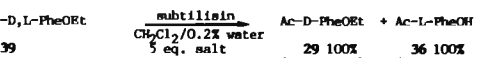
The absence of reactivity of β-substituted derivatives will greatly limit future applications of the system to chiral building blocks. A very valuable clue could be provided by obtaining the X-ray structure of the enzyme in an organic environment. This would give a clear Picture of the tridimensional structure of the protein, and if it was to be confirmed that conformational changes have occurred, site-directed mutagenesis could be an ideal tool to design a catalyst to specific features of the substrates.

We have suggested that conformational changes have occurred leading to a newly designed active site. If these changes are drastic, this could result in a complete inactivation. Despite much effort, we were never able to find trypsin, a very closely

related protease to chymotrypsin, active as a suspension in organic solvent. But if these changes involve only restricted parts of the active site, this could result in a new range of properties, explaining for example with chymotrypsin, why D-amino acid derivatives can be used as nucleophiles in synthesis and why no stereoselectivity was found for α-hydroxy esters.

Chymotrypsin is not the sole enzyme to exhibit enzymatic activity in organic solvents. Preliminary studies have shown that this approach could be extended to another protease subtilisin (EC 3.4.21.14 from Bacillus licheniformis) (Scheme 10).

Scheme 10 : hydrolytic reactions catalyzed by subtilisin suspended in dichloromethane containing 0.2% water in the presence of ammonium bicarbonate.



Nevertheless, the resolution of the phenylalanine derivative proceeded less efficiently than with chymotrypsin, 96 hours being required to reach the completion of the reaction.

A more fascinating aspect of subtilisin-catalyzed reactions was provided by the hydrolysis of unusual substrates (Scheme 11).

Scheme 11 .

Ac-L-PheOEt + L-LysOBu^t $\xrightarrow[CH_2Cl_2/0.25\%\ water]{subtilisin}$ Ac-L-PheOEt + L-LysOH

35 10 35 100% 81 100%

Instead of the expected dipeptide, only the hydrolyzed acid, L-lysine, was isolated, whereas the phenylalanine ester was kept untouched. This result indicates a complete reversal of specificity. Aromatic amino acid derivatives are the best substrates for subtilisin in aqueous medium, but when the enzyme is suspended in organic solvents, it seems to prefer derivatives able to give favourable interactions when bound to the specificity pocket. This point was evoked to explain the selectivity of chymotrypsin for tyrosine derivatives and could well apply for subtilisin.

Nevertheless, subtilisin suspended in dichloromethane containing 0.25% water is a very efficient catalyst for the hydrolysis of α,ω-diamino acid esters (Scheme 12).

Scheme 12.

$\xrightarrow[CH_2Cl_2/0.25\%\ water]{subtilisin}$
24 hours

x=4 10 81 95%

x=3 12 82 100%

This effect is very specific to the structure of the substrates. When D,L-serine methyl ester was used as substrate, no hydrolysis was observed. This tends to indicate the importance of the interaction with tightly bound water molecules as observed with chymotrypsin.

Hydrolytic reactions in organic solvents appeared as a method of choice for the study of the properties of an enzyme in an organic environment. The amount of water is controlled and can be adjusted to the optimal level. The procedure allows easy recovery of the products, and can be used for hydrolyzing substrates exhibiting very low water solubility, thus avoiding the use of cosolvents that may be difficult to remove.

By providing a simple model, hydrolytic reactions can be used to screen the activity of enzymes in organic solvents and the acquisition of kinetic parameters is envisageable.

CHAPTER II . KINETIC OF α-CHYMOTRYPSIN CONTROLLED SYNTHESES OF PEPTIDE BONDS IN ORGANIC SOLVENTS

II-1. Introduction.

In recent years, much effort has been devoted to the search for conditions under which enzymes could retain most if not all their catalytic activity in organic media.

The evaluation of the kinetic parameters of proteases in organic solvents is of prime importance for assessing enzyme specificity and catalytic efficiency, as well as for gaining insight into enzymatic catalysis in organic media in general.

Kinetic parameters such as the Michaelis constant, Km, (substrate concentration at half-maximal velocity) and the catalytic constant, k_{cat} or k_0 (maximal initial velocity per unit enzyme concentration)[11] could provide a reliable insight into specificity and efficiency of the proteases in organic media.

However, though the kinetics of the protease-catalyzed hydrolysis of peptide-, ester-, and amide bond have been extensively investigated, reports of kinetic studies dealing with the protease-catalyzed synthesis of peptide bonds are relatively sparse.

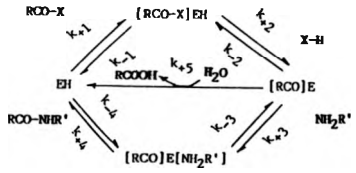
In the early 60s, Gawron et al. [109] carried out a kinetic analysis of the first well-defined chymotryptic peptide bond synthesis performed by Bergmann and Fruton[110].

The initial reaction velocities of the synthesis of Bz-Tyr-Gly-NH-Ph from Bz-Tyr-OH and H-Gly-NH-Ph indicated both a Michaelis-Menten relationship between the rate of product formation and the concentration of the first substrate, Bz-Tyr-OH, and a linear dependency of the rate of product formation on the concentration of the second substrate, H-Gly-NHPh. The authors concluded from these results that the molecular mechanism of the protease-catalyzed synthesis was the reverse of the hydrolytic reaction.

In a comprehensive review[111], W. Kullmann focused on the kinetic aspects of enzymatic syntheses of enkephalins. These investigations were particularly aimed at the numerical evaluation of kinetic constants in order to provide a measure of the specificity and catalytic efficiency of papain- and α-chymotrypsin in peptide bond formation in aqueous medium.

The kinetic analysis of the chymotrypsin-mediated synthesis of the dipeptide Boc-Tyr-Gly-N_2H_2-Ph revealed that the system fits a Michaelis-Menten law for a two-substrate reaction. The kinetic data were consistent with a ping-pong mechanism modified by a hydrolytic branch (Scheme 1).

Scheme 1 : Reaction scheme for concurrent peptide bond formation and hydrolysis as catalyzed by chymotrypsin.



According to the proposed mechanism, the same acyl-enzyme complex was involved in both aminolysis and hydrolysis. The initial-velocity ratios of peptide bond formation to hydrolysis were found to be linearly dependent on the concentration of the individual acyl-acceptor, but were independent of the acyl-donor concentration.

Consequently, the protease-controlled reactions could be shifted in favour of peptide synthesis by increasing the concentration of the acyl-acceptor.

However, the picture for enzymatic catalysis in organic solvents might be very different. Water no longer plays the role of the solvent (55M). If the fast, kinetically controlled reaction in water-rich reaction media is used for peptide synthesis it is

essential to monitor the reaction and stop it when the optimal amount of peptide is formed. Otherwise, the yield rapidly decreases owing to hydrolysis of the product. However, if water-poor media are used for peptide synthesis, close monitoring of the reaction is not necessary because hydrolysis of the peptide is suppressed because of the low water activity of the system.

The kinetics and specificity of serine proteases in peptide synthesis catalyzed in organic solvents were comprehensively studied by H. Gaertner and A. Puigserver[49].

Initial rates of peptide bond synthesis catalyzed by poly(ethylene glycol)-modified chymotrypsin in benzene were determined using high-performance liquid chromatography. A minimal water concentration was required for the catalytic activity of modified chymotrypsin in water-immiscible organic solvents. The catalytic activity of modified chymotrypsin was dependent on both water concentration and type of organic solvent. Enzymatic synthesis of Bz-Tyr-$PheNH_2$ from Bz-Tyr-OEt and $PheNH_2$ was found to obey Michaelis-Menten kinetics and to be consistent with a ping-pong mechanism modified by a hydrolytic branch.

Though hydrolysis was considerably decreased in water-restricted environment as compared to enzymatic synthesis in aqueous buffer, its level became significant when only a low excess of the amine nucleophile was used.

In a detailed investigation concerning solid enzymes dispersed in organic solvents, A. Zaks and A.M. Klibanov[62] studied subtilisin

and α-chymotrypsin as catalysts in a variety of dry organic solvents. Enzymatic transesterifications in organic solvents followed Michaelis-Menten kinetics, and the values of Vm/Km roughly correlated with solvent's hydrophobicity.

Nevertheless, to be catalytically active, α-chymotrypsin needed to be activated either by freeze-drying from a buffer containing N-acetyl-L-Phenylalanine or by adding 0.1% (V/V) water. However, these results showed only a partial picture in the extent that only the P-1 subsite (acyl donor) could be studied in terms of catalytic parameters.

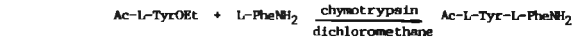
No information relevant to peptide synthesis or hydrolysis could be drawn. The alcohol, n-propanol, used for transesterification reactions plays no part in the binding to the P'1-subsite (the nucleophile site) and the use of an excess (1M alcohol for 2-12mM substrate) might well have an influence on the kinetic parameters.

It is against that background that we decided to study in detail enzymatic peptide synthesis in non aqueous solvents in order to try to understand its basic features and characteristics.

This could help us to provide a valuable insight into issues such as the role of water in enzyme catalysis, catalytic parameters, conformational stabilities of enzymes in organic solvents, and protein intramolecular interactions and flexibility.

As a model system we have studied protease-catalyzed peptide synthesis in an organic solvent (Scheme2).

Scheme 2.



1 2

The hydrolyzed starting ester, Ac-TyrOH (ATOH), is also produced during the course of the reaction owing to the presence of water. The effect of water content in the reaction medium on protease-catalyzed peptide synthesis was studied in the range 0-1% (v/v) water. The kinetic parameters were obtained for a water concentration of 0.2% with concentrations of the starting materials ranging from 15 mM to 150 mM.

To investigate the effect of water on enzymatic catalysis, a series of experiments was carried out using D-$PheNH_2$ as a substrate either with the enzyme in a suspended state, or under biphasic conditions.

II-2. Methods and experimental procedures.

α-Chymotrypsin as a suspension in organic solvent links the system to heterogeneous catalysis in which diffusion constraints have to be taken into account.

The newly formed dipeptide and the hydrolyzed ester, ATOH, are released as insoluble precipitates which do not interfere with the overall reaction. Therefore, the last steps (Scheme 1) are considered to be irreversible ($k_{+4} \gg k_{-4}$).

Owing to the special nature of the system, a method of analysis had to be designed. None of the standard techniques for kinetic measurements, such as potentiometry, spectrophotometry, GLC, or HPLC, were of any use because of the presence of a heterogeneous reaction mixture. To get a complete picture of the reaction mixture, ^1H NMR appeared to be suitable, even if the technique presented some drawbacks, such as the need for one-point experiments with their tendency to lack of reproducibility.

- Materials.

Crystalline bovine pancreatic α-chymotrypsin (EC 3.4.21.1) (type II) was purchased from Sigma as a lyophilized powder with a specific activity of 51 units/mg of protein. The amount of bound water was determined by the Karl-Fischer method and was found to be 5.4% (w/w) for the native enzyme and 1.5% (w/w) for the enzyme extensively dried in a desiccator over P_2O_5. This represents 75

molecules of water per molecule of enzyme and 21 molecules per 1 molecule of enzyme respectively. N-acetyl-L-tyrosine ethyl ester and L-phenylalaninamide (free base) were obtained commercially. The solvent, dichloromethane, was obtained from a continuous distillation from calcium hydride and considered to contain less than 0.02% (v/v) water.

- Assays.

The ability of α-chymotrypsin to synthesize peptides in organic media was determined by estimating the amount of condensation products formed during the reaction. The standard reaction mixture in dichloromethane (5 ml) consisted of given concentrations of the starting materials, 0.4 mg/ml of enzyme (16 μM). The reaction was started by the addition of 0.2% (v/v) of water (110 mM). The reaction was performed at 20°C for a given time, the reaction mixture was filtered, and the enzyme was washed with warm methanol.

After evaporation of the solvents, the dried residue was dissolved in a mixture of deuterochloroform - trifluoroacetic acid (1:1) and the composition of the reaction mixture was analyzed by ^{1}H NMR (220 MHz). Each experiment was repeated until three similar results were obtained (estimation of the standard error of about 5% taking into account weighing, reproducibility and measurements of NMR integrals)

Both Km and k_{cat} apparent values were derived from linear least-square regression analysis in the double-reciprocal plot of V versus [S]. It was assumed that the concentration of active enzyme in dichloromethane was the same than that in water.

Due to the low activity of the enzyme in organic solvents, initial velocities are expressed in $mM.mn^{-1}$ instead of katal ($mol.s^{-1}$). The straight conversion is :

$$1 \text{ unit } (mM.mn^{-1}) = 60 \text{ mkat.} l^{-1}.$$

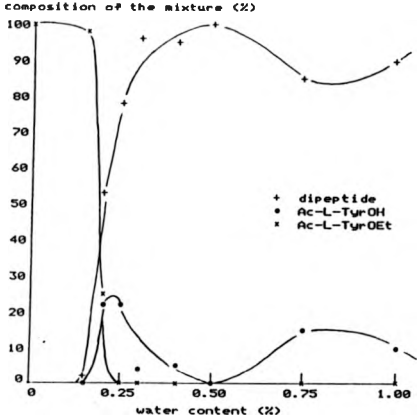
II-3.Results and discussion.

II-3.1. Effect of the water content.

A series of experiments was carried out with a water content ranging from 0-1% (40 mM of the starting materials, reaction time 1 hour). In a control experiment, the enzyme was dissolved in water first (0.5%) to provide biphasic conditions.

The results shown in Fig. 1 indicate clearly that a minimal water concentration is required for catalytic activity. The water content of the reaction mixture has a strong influence on peptide synthesis.

Figure 1 : effect of the water content (40 mM starting materials, 1 hour)



Though the reaction proceeds in a water-restricted environment, the hydrolysis of the starting ester reaches a significant level for low water concentrations. When the water content attains 0.3%, an almost quantitative yield in dipeptide is obtained and the synthetic reaction proceeds very efficiently up to water content of 0.6% where an increase in the hydrolytic reaction is observed. It can be seen from Fig. 1 that the activity of chymotrypsin in dichloromethane correlates with the amount of

water up to 0.3% water : the more water, the greater the enzymatic activity. It has been suggested that a given solvent has the ability to partition the essential water from the enzyme [62]. The loss of the essential water has a strong detrimental effect on enzymatic activity.

Under our conditions, one can correlate the amount of water needed for enzymatic activity (around 0.2%) to the data of the solubility of water in dichloromethane which appears to be 0.198% (w/w) at 25°C [112]. This correlates well with the fact that no enzymatic activity is detected below 0.15% due to the stripping of the essential water from the enzyme by the solvent.

Results shown in Fig. 1 also afford information about the conformational stability and the flexibility of the protein in organic solvents. The existence of a significant level of the hydrolyzed product, ATOH, at low water concentrations (0.2%), a level which tends to decrease as the water content increases, may be an indication of the rigidity of the protein molecule. The active site of chymotrypsin is constituted of two subsites, P and P', responsible for the binding of the donor ester and the nucleophile respectively. A relatively high level of hydrolysis compared to peptide formation, indicates a direct effect of the water content on the binding of the substrates, and on the binding of the nucleophile to the P'-subsite in particular.

According to this interpretation, increasing the water content would allow more flexibility in the protein structure. Therefore,

effective binding to the P'-subsite is restored allowing the nucleophile to compete efficiently with water.

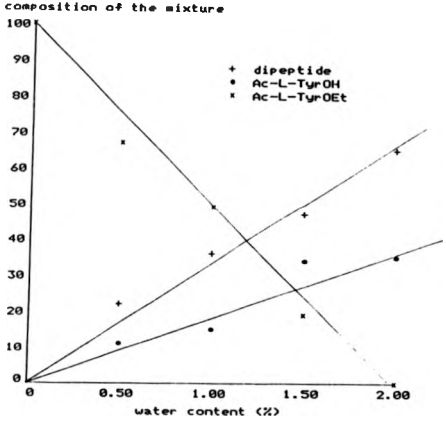
The break in the graph for water content over 0.6% (Fig. 1) could mark the transition point between the suspended and biphasic states. One cannot observe any difference between the control reaction (biphasic) and the experimental value for 0.5% water. To estimate the transition point between suspended and biphasic systems, we decided to take full advantage of the dramatic change in enzyme stereoselectivity when suspended in organic solvents.

The results from Chapter I show that D-aminoacid amides can be efficiently used as nucleophiles. Chymotrypsin in aqueous medium is known to be a poor catalyst in the synthesis of dipeptides using D-aminoacid derivatives as nucleophiles [79]. Therefore, one can expect a low stereoselectivity for the enzyme suspended in the organic solvent, and a return to high stereoselectivity for the enzyme under biphasic conditions.

Chymotryptic peptide syntheses in biphasic systems have been performed successfully by Martinek and co-workers [113,114] and, in particular, by Jakubke and collaborators [115,116]. Kuhl et al. were able to demonstrate the efficient synthesis via chymotrypsin catalysis of the tripeptide Ac-Leu-Phe-LeuNH_2 in 92% yield in a biphasic system composed of carbonate buffer, pH 10, 98% (v/v) trichloroethylene, and only 2% (v/v) water [116]. However, the stereoselectivity of the enzyme under these conditions was not investigated; it was assuming that the enzyme solubilized in an aqueous phase would keep its inherent properties.

We therefore investigated the chymotrypsin-catalyzed synthesis of Ac-L-Tyr-D-PheNH$_2$ under heterogeneous catalysis and biphasic conditions with water contents ranging from 0-2% (v/v). Fig. 2 shows the composition of the reaction mixture for different water contents after a reaction time of one hour (biphasic conditions).

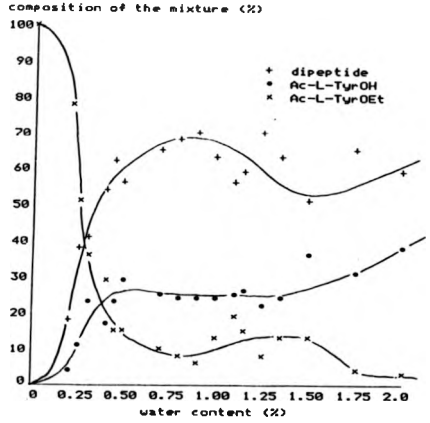
Figure 2 : effect of the water content (biphasic conditions, 40 mM, 1 hour)



It was at first very surprising to observe an efficient peptide synthesis due to the fact that chymotrypsin is known to be highly stereoselective for L-nucleophiles. A set of straight lines was

obtained and one can see that synthesis always prevails over hydrolysis in the range of water concentrations investigated. Despite the fact that the enzyme is solubilized in an aqueous phase, the organic solvent has a dramatic effect on the stereoselectivity of the enzyme. When the variation of the composition of the reaction mixture was studied for different water concentrations under heterogeneous catalysis, a different picture was obtained (Fig. 3).

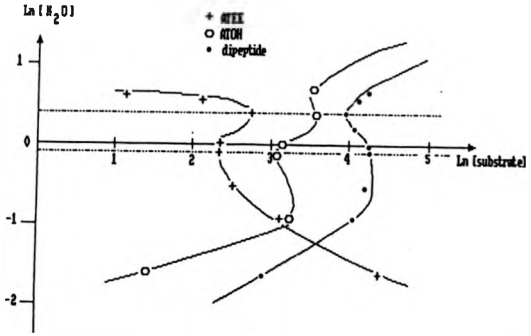
Figure 3 : effect of the water content (heterogeneous catalysis, 40mM, 1 hour)



The variations of the composition of the reaction mixture are not linear any more, reaching a maximum for the synthesis of the dipeptide at a water content of 0.9%, then slowly decreasing to reach a minimum at 1.5% water. From then, the variations are consistent with those under biphasic conditions.

For visualization purposes, the variation in product composition as a function of $Ln[H_2O]$ was studied (Fig. 4).

Figure 4 : effect of the water content (heterogeneous catalysis, 40mM, 1 hour)



The three S-shaped curves show the existence of two inflexion points along the y-axis for water contents of 0.9 and 1.5%

indicated by the horizontal broken lines. The minimum for 1.5% marks the point where the system reaches true biphasic conditions. The existence of a minimum for water content of 0.9% could be correlated to the optimum water content for the reaction studied under heterogeneous catalysis.

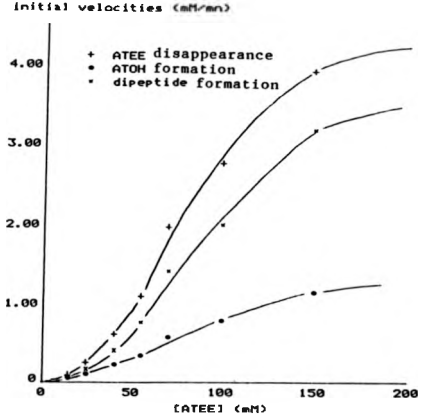
The results discussed above, and in particular the comparison of the data recorded in Fig. 2 and Fig. 3, indicate clearly that the transition from heterogeneous catalysis to biphasic conditions occurs for water contents greater than 1%, despite the fact that the enzyme is clearly soluble in lower amounts of water.

The results shown in Fig. 2 demonstrate that D-aminoacid derivatives can be used efficiently as nucleophiles even when the enzyme is solubilized in an aqueous phase. Under very low water contents (below 2%), the aqueous phase is saturated with the organic solvent (the solubility of dichloromethane in water is 1.30% (w/w) at 25°C [112]), and the relaxed stereoselectivity for D-aminoacid derivatives may be related to the direct effect of the organic solvent on the structure of the enzyme.

II-3.2. Kinetics of chymotrypsin-catalyzed synthesis of N-acetyl-L-tyrosyl-L-phenylalaninamide in dichloromethane.

The initial velocities of synthesis of the dipeptide and hydrolysis of the starting ester were determined with concentrations of the starting materials (1:1 molar ratio) ranging from 15 to 150 mM. Fig. 5 shows the initial velocity pattern as a function of the concentration of the starting ester.

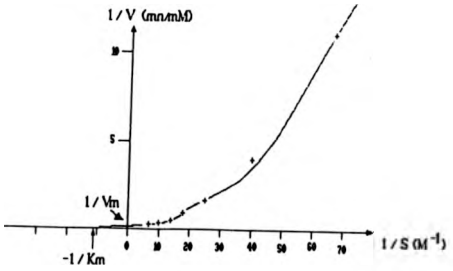
Figure 5 : initial velocities pattern as a function of [ATEE]



Saturation of the enzyme is obtained for concentrations of 150 mM and extrapolation of the curve affords the maximum velocity Vm^S for synthesis estimated to be 3.9 mM/mn. The S-shape of the curve shows a striking difference with the conventional shape observed in Michaelis-Menten kinetics. The Michaelis constant Km^A for Ac-L-TyrOEt (ATEE) was derived from this curve and estimated to be approximately 85 mM. The conventional double-reciprocal Lineweaver-Burk plot (Fig. 6) shows a straight line for saturation concentrations continued by a hyperbolic segment reflecting the S-shape of Fig. 5. The same patterns were obtained for the initial velocities of the ester hydrolysis.

Figure 6.

Lineweaver-Burk plot : initial velocity pattern as a function of [ATEE]



The hyperbolic shape of Fig. 6 indicates that the order of the reaction for the substrate is greater than one. The sigmoid pattern of Fig. 5 shows that there is a range of substrate concentration (0-20 mM) in which the enzymatic activity is only slightly dependent on the concentrations of substrates. Beyond that level of concentration, the enzymatic activity varies with small variations of the concentrations of the substrates.

The Michaelis-Menten Km^B for the nucleophile was estimated to be 95 mM. The maximum velocity of hydrolysis Vm^h was derived from Fig. 5 and estimated to be 1.4 mM/mn.

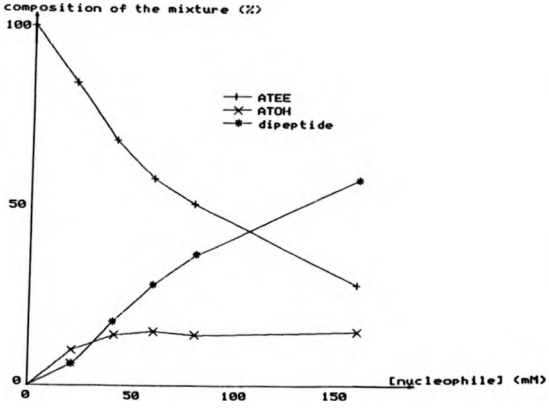
In order to clarify the observations from Fig. 6, experiments were carried out with various concentrations of the nucleophile (ranging from 0-160 mM) and fixed amounts of ATEE (80 mM) and water (0.2%, 110 mM) in dichloromethane. The results are shown in Fig. 7.

No enzymatic activity was detected in the absence of the nucleophile. In a further control experiment, only traces of the hydrolyzed product, ATOH, were detected after 4 days of reaction for concentrations of the nucleophile below 10 mM.

These observations reflected in the S-shaped curve of Fig. 5 tend to indicate a cooperative effect of the binding of the nucleophile on the enzymatic activity. The catalytic behaviour of chymotrypsin suspended in organic solvent is drastically different to the similar reaction in aqueous media. These results dismiss the classical kinetic scheme which would involve the formation of a non covalent enzyme-acyl donor complex, which is

then transformed into an acyl-chymotrypsin intermediate with the concomitant release of the alcohol product. If this were the case, the acyl-chymotrypsin would interact with water yielding the acid and the free enzyme. Our results indicate that the presence of the nucleophile is crucial for enzymatic activity.

Figure 7 composition of the mixture with various concentrations of PheNH2



A possible explanation for these observations relates to the induced-fit hypothesis developed by D.E. Koshland [117] . He postulated that the essential functional groups on the active site of the free enzyme are not in their optimal positions for promoting catalysis when the active site is unoccupied but that when the substrate molecule is bound to the enzyme, the binding affinity forces the enzyme molecule into a conformation in which the catalytic groups assume a favourable geometrical position to form the transition state; i.e. there is an induced fit. The enzyme molecule is unstable in its active conformation and tends to revert to its free form in the absence of substrate. This hypothesis might help to explain our observations.

One can speculate that the tridimensional structure of the enzyme is different to that in water, either because it is too rigid to accommodate the substrate owing to the low water content, or because the geometry of the active site has been modified by the new organic environment. Molecules of solvent can diffuse in the crystal lattice and therefore perturb the overall tridimensional structure. The binding of the nucleophile would induce the transition to an active conformation according to the induced-fit hypothesis.

To evaluate the nature of the cooperative effect shown by the nucleophile, we carried out an analysis according to the model of Monod, Wyman and Changeux [118] .

According to their model, applied to allosteric systems, the enzyme is supposed to exist as an equilibrium between two states:

$$R \text{ (relaxed)} \rightleftharpoons T \text{ (tense)}$$

Scheme 3

If the T-state has a great affinity for the substrate, and the R-state a low affinity, in the absence of the substrate, the R-state is predominantly abundant. If the concentration of the substrate is increased, the equilibrium is displaced towards the T-state.

By using the representation of Hill [119], one can estimate the nature of the cooperative effect and measure the interaction between the sites.

$$E + nS \rightleftharpoons ES_n \rightleftharpoons E + P$$

$V = Vm.S^h / (Km + S^h)$ where h measures the interaction between the sites

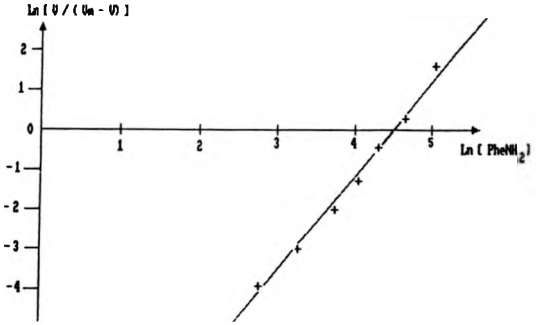
$V.Km + V.S^h = Vm.S^h$

$V.Km = Vm.S^h - V.S^h = (Vm - V).S^h$

$V / (Vm - V) = S^h / Km$ or $Ln\ V/(Vm - V) = h.Ln\ S - Ln\ Km$

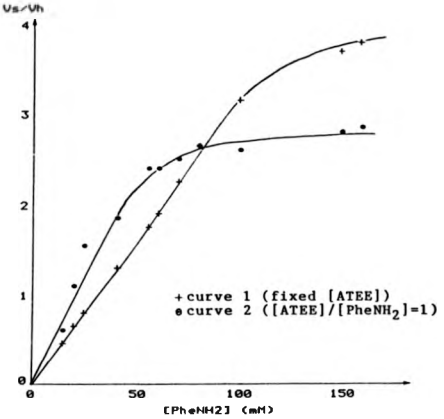
When the variation of $Ln\ V^s/(Vm^s-V^s)$ is plotted as a function of $Ln[PheNH_2]$, a straight line is obtained (Fig. 8) and the slope (2.4>1) indicates that the substrate is an activator.

Figure 8. Variations of Ln[V/(Vm−V)] against Ln[PheNH2]



Further evidence for a non-classical mechanism was obtained by plotting the initial velocity ratio of peptide bond formation to hydrolysis Vsyn/Vhyd as a function of substrate concentrations (Fig. 9).

Figure 9. Initial velocity ratio Vs/Vh as a function of [PheNH2]



The ratio of Vsyn to Vhyd was found not to be proportional to the concentration of the nucleophile over the range of concentrations, and to be dependent upon the concentration of the acyl donor. The critical point where synthesis prevails over hydrolysis is also dependent upon the ratio ATEE/$PheNH_2$.

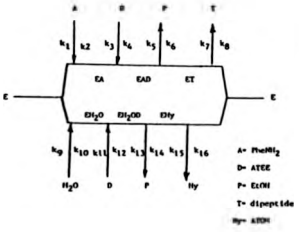
An excess of the nucleophile (curve 1) does improve the ratio Vsyn/Vhyd but at the expense of the overall rate of reaction (Vm^{app} of 2.6 mM/mn instead of 3.6 mM/mn for a molar ratio of 1). Consequently, the protease-controlled reactions can be shifted in favour of peptide synthesis by increasing the concentration of the nucleophile.

The results shown in Fig. 9 are not compatible with a ping-pong mechanism modified by a hydrolytic shunt (Scheme 1).

In such mechanism in which the acyl donor binds first, the ratio Vsyn/Vhyd is directly proportional to the concentration of the nucleophile, and independent of the concentration of the acyl donor [120]. The existence of a cooperative effect shown by the nucleophile further emphasizes these observations.

An alternative mechanism could be envisaged in which the nucleophile binds first to the enzyme. This ordered pathway mechanism F-1 [120] is described in Scheme 4 by using the graphical representation of Cleland [121].

Scheme 4 : ordered pathway mechanism F-1.



In such mechanism, the ratio Vsyn/Vhyd is a function of the concentrations of the nucleophile and the acyl donor, according to

$$\frac{Vsyn}{Vhyd} = \frac{(A)[\ f + g(D)]}{h + i(D)}$$

where f, g, h, i are combinations of the individual kinetic constants of both the hydrolysis and the transfer pathways.

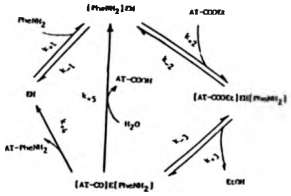
With this mechanism F-1, the nucleophile may be an activator or an inhibitor depending upon the values of various constants and of the concentrations of D.

We have not been able to establish a similar mathematical relation in the case of peptide synthesis catalyzed by chymotrypsin suspended in organic solvents.

Nevertheless, from these preliminary results, one can propose a possible reaction scheme for peptide bond formation and hydrolysis catalyzed by chymotrypsin in organic solvent (Scheme 5).

The ordered pathway mechanism is slightly different from the one depicted in Scheme 4. This difference relates to the actual role of water.

Scheme 5



The existence of a binary enzyme-substrate complex as a central feature is based on the observation that no activity is observed in the absence of the nucleophile (Fig. 7). This is further emphasized by the fact that the variations of Vsyn/Vhyd are not independent of the concentration of the acyl donor. This indicates that the nucleophile binds first to the enzyme.

Because no activity is found in the absence of water, one cannot rule out the existence of a tertiary enzyme-substrates complex involving water, the acyl-group being transferred either to water (hydrolysis) or to the nucleophile (aminolysis).

The role of water in non-aqueous enzymology, which is one of the major questions, cannot be investigated in detail owing to the

multiple function of water, which affects the structural properties of the catalyst but also contributes to the overall reaction.

Under our conditions, the chemical activity of water is not equal to its concentration and it is impossible to measure the amount of water directly involved in the chemical reaction. For water contents below 0.15% (Fig. 1), no activity was detected. By increasing the water content, there is a significant increase in the mobility of the protein and full enzymatic activity is recovered for water levels of about 0.3%. The actual water concentration available for the hydrolytic reaction is in fact much below 110 mM, and the model would have to be corrected by terms taking into account the actual water concentration and reflecting the cooperative effect of water on protein dynamics, i.e. the changes that may occur in the tertiary structure of the enzyme molecule.

Nevertheless, the kinetic data are consistent with the proposed mechanism (Scheme 5). In order to define more precisely the nature of the observed mechanism, it would be necessary to study the initial velocity patterns, obtained for a series of dipeptide syntheses using the acyl donor as the variable substrate in the presence of different fixed concentrations of the nucleophile, and that for different water concentrations (because it might well be that the enzyme would recover a Michaelis-Menten behaviour for higher concentrations of water owing to an increase in flexibility). These data, together with a study of the initial

velocity of the release of ethanol, would afford a complete picture of the mechanism involved.

II--4. Conclusions.

The kinetics of chymotrypsin-catalyzed peptide synthesis with a suspended enzyme show that the enzyme does not follow the classical Michaelis-Menten scheme. The binding of the nucleophile triggers the enzyme into the active conformation. This shows that the enzyme structure has been altered through the transition from water to an organic environment. Though hydrolysis was considerably decreased in a water-restricted environment as compared to enzymatic synthesis in aqueous buffer, its level became significant when a small excess of the amine nucleophile was used. Moreover, one can relate the amount of water to the rigidity of the enzyme. The extent of hydrolysis is greater at low water concentrations (0.2%) reflecting the apparent role of the water in inducing flexibility, in other words by a direct effect on the binding of the substrates.

This can be described by an equilibrium similar to that depicted in Scheme 3 :

$$E \underset{}{\overset{H_2O}{\rightleftharpoons}} E'$$

(inactive) (active)

For water contents below 0.15%, the equilibrium lies strongly to the left. Increasing the water content displaces the equilibrium to the right towards the form of the enzyme with great affinity for the substrates.

This is similar to an allosteric transition because in this respect one can consider chymotrypsin as being an allosteric enzyme owing to the existence of binding sites for water outside the catalytic site itself. Although this theoretical approach is highly speculative, it has the merit of providing grounds for further investigations.

On the practical side, Fig. 1 shows that it is worth working with water contents greater than 0.2% where full activity is obtained. Hydrolysis is almost suppressed and quantitative yields can be obtained.

It is interesting to compare the kinetic parameters obtained with the suspended chymotrypsin with some obtained from different systems (table 1).

From the data shown in Table 1, one can see that the heterogeneous catalysis approach affords the less efficient result in terms of kinetic features. Despite those unfavourable kinetic parameters, the system still provides an efficient peptide synthesis as shown by the value of k_{cat}/Km.

Table 1 : comparison of the suspended chymotrypsin system with other chymotryptic systems.

	Km_A (mM)	Km_B (mM)	k_{cat}/Km ($M^{-1}.s^{-1}$)	Vm/Km (mn^{-1})
Heterogeneous Catalysis (0.2% water)	85	95	56	$5.4x10^{-2}$
Hydrolysis in buffer [122]	0.7	—	$3x10^5$	—
Synthesis in buffer [111]	38 (BOC-TyrOEt)	81 ($GlyN_2H_2Ph$)	684	5.5
Transesterification in octane (APEE) [62]	—	—	—	$4.8x10^{-5}$
PEG-CT in benzene (0.18% water) [49]	6.8	—	3,029	—

The results shown in Table 1 concerned enzymes freely dissolved, except for the transesterification reactions, where diffusion constraints will play no part. During the course of the study, we have not been able to detect any diffusion limitation. A lapse of few minutes was necessary to reach the linear part of the curve, only when low substrate concentrations were used, and this does not provide a concrete evidence for the existence of diffusion limitations.

CHAPTER III . CHYMOTRYPSINOGEN AND CHYMOTRYPSINS AS CATALYSTS FOR PEPTIDE SYNTHESIS.

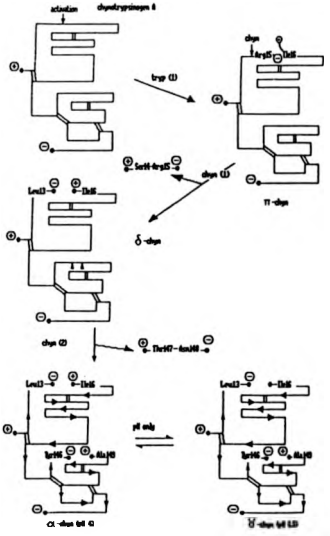
Chymotrypsin is secreted into the gastrointestinal tract as an inactive precursor, or zymogen, chymotrypsinogen. Chymotrypsinogen has a single polypeptide chain of 245 residues held together by five intrachain disulfide bridges.

Small amounts of trypsin slowly activate chymotrypsinogen into α-chymotrypsin. The latter undergoes a slow transformation into β and γ chymotrypsins. β-Chymotrypsin is formed as a result of limited autolysis and γ-chymotrypsin arises as a result of a pH-induced transition. A fast activation of α-chymotrypsinogen yields two new forms of chymotrypsin, a very unstable form π, and a fairly stable form δ, which are more active than α chymotrypsin [123]. The activation process depends on the cleavage of the cyclic molecule of α chymotrypsinogen by trypsin, and by chymotrypsin itself (autolysis) (Fig. 1).

The activation of α chymotrypsinogen to α chymotrypsin is triggered by the tryptic cleavage of the Arg-15 and Ile-16 bond [124]. It appears that it is specifically the formation of a new terminal amino group, rather than any "unblocking" reaction, which is responsible for promoting enzymic activity [125]. The role of the newly formed amino group of Ile-16 in stabilising the enzymatically active conformation of α-chymotrypsin has been

investigated and it was shown that the negatively charged β-carboxylate group of Asp-194 and the positively charged α-amino group of Ile-16 form an ion-pair in a region of presumably low dielectric constant.

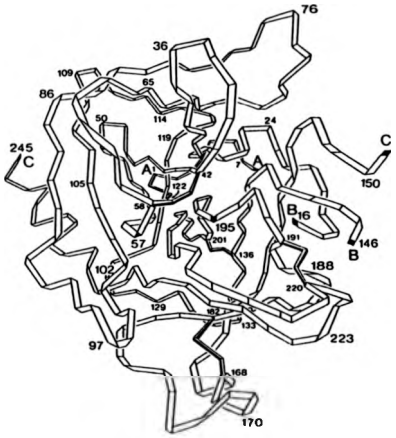
Figure 1 : activation scheme for the chymotrypsins.



The overall tertiary structure of the zymogen is similar to that of the active enzyme. There is no gross reorganisation in the folding of the main chain nor a significant helix-coil transition.

The position of the chain termini created during the activation and subsequent autolysis are consistent with the cleavage of two dipeptides from the surface of a zymogen but in each case leaving the protein in the same conformation [127] (Fig. 2).

Figure 2 : A view of the complete polypeptide chain of α-chymotrypsin (from Birktoft J.J. and Blow D.M. [128]).



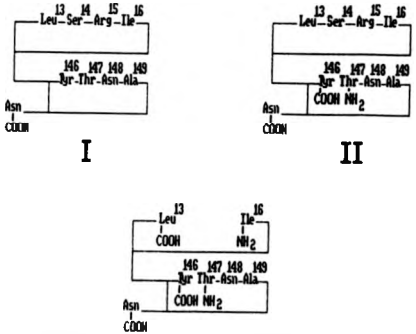
The stereochemistry of the activation of the zymogen is paralleled in the enzyme by a pH-dependent structural transition. In both transitions activity depends upon the integrity of the ion-pair between Ile-16 and Asp-194. A pH-dependent structure transition occurs in the native enzyme between an "active" and an "inactive" form. The active form dominates the equilibrium below pH 8 and is characterized functionally as the form which binds specific substrates in a productive mode [129]. The other conformational state, the "inactive" form is favoured at high pH and is unable to bind specific substrates and inhibitors in a productive mode. This inability is considered to be the basis for the fall-off in enzymatic activity at pH $>$ 8. Since the optical rotation is different from that of the active form, but similar to that of the zymogen, the inactive form is felt to resemble α-chymotrypsinogen in its tertiary structure.

The group with pK_a 8 to 9 which controls this transition has been identified as the α-amino group of N-terminal Ile-16 [125]. By treating the zymogen with acetic anhydride prior to activation, these workers prepared a derivative, acetyl-δ-chymotrypsin, in which all amino groups except this one were acetylated. This derivative was found to be fully active at neutral pH, and to undergo the transition to an inactive form at high pH. Reacetylation of the derivative, so as to block the amino group, inactivated the enzyme.

This fact was latter contradicted by P. Valenzuela and M.L. Bender in a study on δ-chymotrypsin [130]. Their results

indicated that the deprotonation of the isoleucine 16- amino group causes only a minor effect on the binding ability of this enzyme. This led them to suggest that the peculiar behaviour of α-chymotrypsin at alkaline pH may be caused by the ionization of the phenolic group of tyrosine 146 or the amino group of alanine 149, which are present as chain termini in α-chymotrypsin but not in δ-chymotrypsin. They investigated the preparation, characterisation and kinetic properties at alkaline pH of a new stable and active form of chymotrypsin which possesses threonine 147 instead of alanine 149 as the amino terminal group of the C-chain. This enzyme was called α_1-chymotrypsin (Scheme 1).

Scheme 1: Schematic representation of the structures of chymotrypsinogen A (I), thr-neochymotrypsinogen (II), and α_1-chymotrypsin (III).



They found that the kinetic properties of α_1-chymotrypsin in the neutral and alkaline pH regions strongly resemble those of δ-chymotrypsin. Their results strongly implicated the amino-terminus of alanine 149 as a participant in the reversible inactivation of α-chymotrypsin at high pH. Observations by H. Kaplan and H. Dugas [131] seem to confirm the postulated involvement of alanine 149. Studying the properties of the isoleucine-16 amino group by a competitive labeling technique, Kaplan found that above pH 9.8, α-chymotrypsin undergoes an additional conformational change not controlled by the isoleucine 16 terminus. Furthermore, experiments with the alanine-149 amino group suggested that this group is involved in a conformational change on deprotonation of the isoleucine-16 amino terminus.

Despite these results, it seems that the active conformation is stabilized by the ion-pair formed by Asp-194 and Ile-16. The existence of the ion-pair accounts for the anomalously high pK_a of the α amino group of Ile-16, by comparison with the pK_a of less than 8 which is usual for peptide α amino groups. If the positive charge is removed from the amino group, the high potential created by a negative charge in a medium of low dielectric constant within the molecule would require the carboxylate ion to seek an alternative orientation [132] in which it would point into the solvent. The model suggests that this could be accomplished by a movement of the carboxylate group into the vicinity of the side group of Ser-195 and His-57. Although the exact nature of the disruption of the active site cannot be

predicted, it is suggested that at high pH the conformation in the region of the active centre reverts to that of the zymogen. This could account for the absence of enzymatic activity in both proteins.

It is against that background that we found it would be interesting to investigate the behaviour of the different forms of chymotrypsin and their ability to catalyze peptide synthesis when suspended in organic solvent.

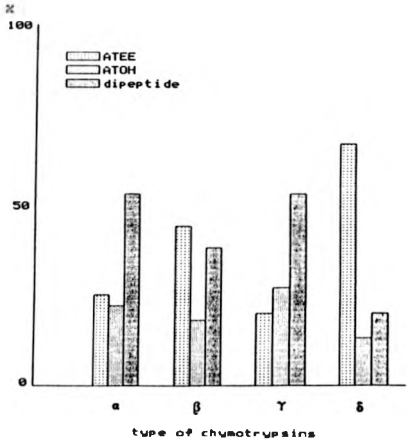
Peptide bond formation involves the two subsites S and S' [94] of chymotrypsin, and therefore would give more information about the tridimensional structure of the enzyme than studies of hydrolytic reactions in which only the S-subsite is concerned. The ability of synthesizing peptide bonds by the different forms of chymotrypsin could help to provide a valuable insight into molecular recognition, and a study of the behaviour of these enzymes in organic solvents could yield information about the intimate effect of the new environment on the molecular structure.

The ability of the different forms of chymotrypsin to synthesize peptides in organic media was determined by estimating the amount of condensation products formed during the reaction between N-acetyl-L-tyrosine ethyl ester and L-phenylalaninamide in dichloromethane.

The experimental procedure was the following : to a solution of N-acetyl-L-tyrosine ethyl ester and L-phenylalaninamide (40mM, 1:1 molar ratio) in dry dichloromethane was added the appropriate

form of chymotrypsin. To the remaining suspension was then added 0.2% (v/v) of water. The suspension was then stirred 1 hour at room temperature, filtered, and the residue was washed with warm methanol. After evaporation of the solvents, the content of the reaction mixture was analysed by ^1H NMR (220MHz, $CDCl_3$/TFA 1:1). The results are depicted in Fig. 3.

Figure 3. *Composition of the reaction mixture after 1 hour*



In the case of α-chymotrypsinogen (Sigma, containing 0.3 units/mg), no activity was detected for 0.2% water. Therefore, the water content was increased to 0.25% (Table 1).

	1 hr	18 hr
ATEE	90	66
ATOH	5	13
dipeptide	5	21

Table 1 : Dipeptide synthesis catalyzed by α-chymotrypsinogen suspended in organic solvent.

A control reaction was carried out to check for the presence of chymotrypsin activity (hydrolysis of ATEE in phosphate buffer pH 7.8):

Overnight : 85% ATEE

15% ATOH

For the latter result, it appeared difficult to relate the results of peptide synthesis to the sole activity of α-chymotrypsinogen.

Nevertheless, it is surprising that no activity was detected with a water content of 0.2%.

If further studies would be necessary to estimate the chemical integrity of chymotrypsinogen and the amount of contamination by chymotrypsin, the necessity of a higher water content for the reaction catalyzed by α-chymotrypsinogen (which is an indication of the flexibility of the protein structure) might well be a manifestation of the capacity of the zymogen to synthesize peptide bonds in organic solvent. Unequivocal evidence for the activity of the zymogen will be extremely difficult to obtain owing to the high activity of the contaminant.

The results depicted in Fig. 3 with the four different forms of chymotrypsin show that they are all efficient catalysts when suspended in organic solvent. Even the "inactive" form γ shows the same activity as the native α chymotrypsin. The transient form δ shows the lower activity under these conditions. Apart the α form, δ is the only form which has been described as a catalyst for peptide synthesis [133]. Enzymatic fragment condensation with δ chymotrypsin (which differs from chymotrypsin in that the B- and C- chains are still connected) could be successfully performed. Starting with N-acetylated, α-carboxyl-methylated acyl-group donors and α-carboxyl-aminated components the resulting tetra-, penta- and hexapeptides could be obtained in good yields. In an additional study on δ-chymotrypsin-assisted peptide bond formation, Bizzozero et al. employed Z-TyrOH and Z-PheOH, respectively, as carboxyl

components and amino acid or dipeptide amides as amine components to prepare Z-protected di- and tripeptide amides [133].

Our results indicate that δ chymotrypsin is the less effective catalyst under these conditions. This could be related to the molecular structure of δ chymotrypsin.

In δ chymotrypsin, the B- and C-chains are still connected. Knowing that the δ form has been described as more active than the native α form (in terms of hydrolysis in water), it could be deduced that the poor results obtained with the δ form reflect the contribution of the part of the structure in the region of the P'-subsite of the enzyme. We demonstrated earlier (chapter II) that the binding of the amine component triggers the enzyme into the "active" form. If part of the structure obstructs the region of the P'-subsite, the binding of the nucleophile will be less effective, and consequently, the outcome will be that the overall reaction will be less productive as shown by the results. On a molecular level, α-chymotrypsin results from a chymotryptic cleavage of the dipeptide Thr-147-Asn-148 between Tyr-146 and Ala-149. Fig. 2 which shows the view of the complete polypeptide chain of α-chymotrypsin indicates that the newly formed chain termini are situated in the region of the S'-subsite.

After the chymotryptic cleavage leading to the α, β, and γ forms, full activity is recovered. Due to the fact that only partial information is available on the β form, no conclusive

evidence can be found to relate the molecular structure to the enzymatic activity.

The β form is nevertheless an efficient catalyst but does not match the activities of α and γ forms.

The striking result obtained with the γ form (the "inactive" form) leads us to concentrate on the nature and importance of the ion-pair between Asp-194 and Ile-16. γ Chymotrypsin exhibits the same efficiency than the α form in the synthesis of peptide bonds under these conditions. The ion-pair is known to stabilise the enzymatically active conformation of the α form and disappears at high pH values leading to the inactivation. Under our conditions, it is impossible to have an idea of the local pH conditions and ionisation states. It has been suggested above that , in the γ form, if the positive charge is removed from the amino group, the high potential created by the negative charge (Asp-194) in a medium of low dielectric constant within the molecule would require the carboxylate anion to seek an alternative orientation, in which it would point out into the solvent. Examination of the X-ray structures of the active site shows that this would disrupt the active site, hence leading to inactivation. But, under our conditions, the high dielectric constant medium has been replaced by a hydrophobic solvent of low dielectric constant. Then it would become highly unfavourable for the carboxylate anion to point out into the solvent. The position of the carboxylate anion would be determined by the balance of unfavourable interactions. The exact position of this residue

cannot be determined, but the experimental results indicate that it does not interfere with the enzymatic activity. In addition, it is interesting to relate our results showing a relaxed stereospecificity on the P'_1-subsite to those obtained under high pH conditions. D-amino acid derivatives are known to be poor nucleophiles in peptide synthesis catalyzed by α chymotrypsin. J.B. West and C.H. Wong [98] have shown that it was possible to use D-amino acid derivatives as nucleophiles under high pH conditions (0.2M carbonate buffer- DMF 1:1). Although the reaction was slow (ca. 10% as fast as that using L-amino acid derivatives) and the enzyme was deactivated quickly at room temperature, D-amino acid residues were readily incorporated. The stability of the enzyme was greatly improved by selectively modifying the Met-residue at position 192 into methionine sulfoxide. In the native chymotrypsin, Met-192 forms the backbone of the hydrophobic pocket which helps to bind aromatic residues.

These high pH conditions apparently relaxed the stereoselectivity of the P'_1-subsite and the presence of the methionine sulfoxide 192 greatly enhances the stability of the enzyme. The apparent pH of the medium can be expected to be 1-2 units higher in the presence of organic cosolvent. At high pH, the predominant conformation of chymotrypsin is inactive reflecting the transition $\alpha \rightleftharpoons \gamma$. The modification of methionine apparently greatly reduces the extent of the transition leading to greater stability.

According to X-ray structures [134], the distance between the side chains of Met-192 and Asp-194 is relatively small. The newly introduced sulfoxide group could account for the stability of the enzyme to high pH conditions. We have seen previously that the inactivation process was apparently due to the movement of the carboxylate anion of Asp-194 seeking a more favourable environment after the positive charge had been removed from Ile-16. Therefore, to try to explain the new stabilisation of chymotrypsin at high pH, one can postulate that the sulfoxide group of Met-192 helps in stabilising the structure either by physically forbidding any movement of the Asp-194 side chain or by establishing stabilising interactions.

In conclusion, this study shows that the different forms of chymotrypsin are able to catalyze peptide bond formation when suspended in organic solvent, with the exception of chymotrypsinogen (we have not been able to prove unequivocally whether the activity was coming from the zymogen itself or from traces of chymotrypsin). Further investigations are needed to relate the poor activity of the β form to its molecular structure.

The δ form which is a more active enzyme than the α form (in terms of hydrolysis in water) showed poor activity in synthesizing peptide bond. This has been related to the part of the structure obstructing part of the P'-subsite, hence limitating the binding of the nucleophile.

γ -Chymotrypsin, which is inactive against peptides in an aqueous medium, has been shown to be active when suspended in an organic solvent. This demonstrates that the new environment has a dramatic effect on the molecular structure of the enzyme. This is a indication that the organic solvent is directly in contact with the enzyme molecule which is not protected from the external medium by water molecules. This represents one of the first example of the actual effect of organic solvents on protein structures. In this case a dramatic effect whereby an inactive enzyme in water is tuned into an active catalyst when suspended in an organic solvent.

CHAPTER IV .ENZYMATIC SYNTHESIS OF LEUCINE-ENKEPHALINAMIDE

The potent effect of morphine and its agonists and antagonists have been known for centuries. Pharmacologists have, for some time, explained these effects in terms of interactions at one or more specific opioid receptors, but the nature of the natural factors interacting with such receptors was not discovered until 1975. In that year, Hughes and Kosterlitz [135] isolated two pentapeptides from brain tissue with potent opiate-like activity. These were named enkephalins. Their structures were determined (using very small quantities) by mass spectrometry :

methionine enkephalin : H-Tyr-Gly-Gly-Phe-Met-OH

leucine enkephalin : H-Tyr-Gly-Gly-Phe-Leu-OH

Although extraction of these peptides from brain tissue is difficult, their chemical synthesis is relatively easy, and synthesis of substantial quantities has paved the way for a variety of studies on their chemistry and biology.

The biological actions of enkephalins, endorphins ('endogenous morphines', 31-residue peptides derived from lipotropin), and related peptides are similar to those of morphine, though important differences between them have been described. The

original assays used to isolate enkephalins utilized the ability of these substances, like morphine, to inhibit the electrically stimulated contraction of guinea pig ileum or mouse vas deferens. The activities of the peptides in these assays is considerably greater than morphine; [Leu]-enkephalin is rather less potent than [Met]-enkephalin.

Like morphine, enkephalins and β-endorphin induce analgesia (resistance to pain) in cats and rats. They are far more potent in this respect if injected intraventricularly (i.e. into the ventricules of the brain) than if injected intravenously, presumably because they cannot easily pass the blood-brain barrier.

β-Endorphin and the enkephalins injected intraventricularly also stimulate release of pituitary growth hormone and prolactin, a response presumably mediated by hypothalamic factors, although similar effects on prolactin release may be produced also by direct action at the pituitary [136].

Like morphine, β-endorphin and [Met]- (although possibly not [Leu]-) enkephalin can induce tolerance and dependence. Rats treated for prolonged periods with β-endorphin become resistant to the analgesic effect and show withdrawal symptoms when administration of the peptide is stopped or when they are treated with the morphine-antagonist naloxone. Rats rendered tolerant (resistant) to morphine are also unresponsive to β-endorphin, and vice-versa.

Receptors which bind labelled morphine, morphine agonists, and in some cases, antagonists such as naloxone have been known for some time. They are found in the brain and other morphine target tissues. β-Endorphin and the enkephalins will displace labelled ligands from such receptors, and show greater affinity for them than morphine itself [137]. Labelled β-endorphin or enkephalins will also bind specifically to such receptors.

It is now clear that these receptors can be classified into at least three types : μ, κ , and δ. The enkephalins possess a preferential affinity for δ receptors; dynorphins and α-neoendorphin bind preferentially to κ receptors; while β-endorphin has a high affinity for all three types of receptors.

The opioid peptides in general appear to have predominantly inhibitory effects on their target cells. A consequence of the binding of enkephalins to receptors on neuronal membranes within the brain is inhibition of action potential discharges in individual nerve cells, possibly as a result of opening of K^+ channels.

Enkephalins were first isolated from the brain, and have subsequently been detected, by immunoassay and immunocytochemistry, in many regions of that organ. In the rat, their concentration varies from about 5 nmoles/g tissue in the hypothalamus and corpus striatum to 0.01 nmoles/g tissue in the cerebellum. The ratio of [Met]-enkephalin : [Leu]-enkephalin varies from about 2:1 to 10:1 [138].

To the organic chemist, the structure and chemistry of a naturally occurring protein or polypeptide is of limited interest. The interest of such compounds lies mainly in their biological activities. Since the publication of the structures of the enkephalins by Hughes et al. [135], there have been several reviews of the correlation between structure and function in analogs of endorphins and enkephalins.

Over the past few years, the field of opioid peptides has taken on such importance that a complete volume of the collection 'The peptides' has been devoted to their biology, chemistry, and genetics [139].

Alteration of the structure of a polypeptide by chemical or enzymic means can provide analogues whose biological and other properties may be altered.

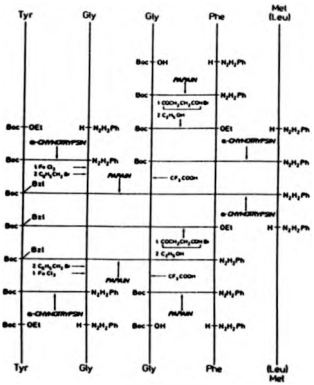
Peptide chemists are able to synthesize polypeptide hormones of moderate size. The method allows, in principle, the construction of any analogue of a peptide, so here alteration of unreactive amino acid side-chains is as easy as alteration of reactive ones. Solid-phase peptide synthesis [140] has proved very valuable for the synthesis of small and medium size peptides like enkephalins and their analogues.

The reports on protease-catalyzed syntheses of model peptides dealt predominantly with the exploration of the proteosynthetic potential and the specificity of certain proteases for the formation of individual peptide bonds. Owing to the model character of these studies, interest was largely aimed at the

synthesis of only a single peptide bond via protease catalysis. In that respect, [Leu]- and [Met]-enkephalins constitute a good model for the synthetic applicability of proteases to the preparation of naturally occuring peptides.

W. Kullmann was the first to design two synthetic pathways for the enzymatic synthesis of enkephalins [141,142] whose peptide bonds were prepared exclusively either by papain- or α-chymotrypsin catalysis (Scheme 1).

Scheme 1 : enzymatic syntheses of [Leu]- and [Met]-enkephalin.



The peptide bond-forming steps were solubility-controlled. Hence the poor solubility of the resulting products firstly induced a favorable shift of the chemical equilibria and second greatly simplified the purification procedures. During the syntheses of the enkephalins, amino- and carboxyl-protection was achieved respectively by t-butyloxycarbonyl- and phenylhydrazide moieties, using, for example, via papain catalysis [143], BOC-amino acid- or peptide ethyl esters were used as pre-activated carboxyl components during α-chymotrypsin catalysis.

Although all the possible dipeptide subunits of both [Leu]- and [Met]-enkephalin were readily obtainable as their BOC-dipeptide-phenylhydrazides either by papain- or by chymotrypsin catalysis, only the synthetic routes outlined in Scheme 1 led successfully to the desired opiate-pentapeptides. Truncated peptides resulted from papain transpeptidation reactions. The initial design of a route to the protease-catalyzed synthesis of [Leu]-enkephalin required a final fragment condensation of BOC-Tyr(Bzl)-Gly-Gly-OH and H-Phe-Leu-N_2H_2Ph in the presence of papain. However, instead of yielding the targeted pentapeptide, the reaction resulted in the formation of the tetrapeptide, BOC-Tyr(Bzl)-Gly-Phe-Leu-N_2H_2Ph. Apparently, the COOH-terminal glycine residue of the tripeptide had been proteolytically removed, followed by papain-mediated peptide bond formation between the resulting dipeptide BOC-Tyr(Bzl)-Gly-OH and the original amine component, the dipeptide H-Phe-LeuN_2H_2Ph.

The last step of the first synthetic route comprised the coupling, via papain catalysis, of the dipeptide BOC-Tyr(Bzl)-Gly-OH with the tripeptides H-Gly-Phe-Leu-N_2H_2Ph and H-Gly-Phe-Met-N_2H_2Ph, respectively at pH 6.1. These reactions furnished the fully protected [Leu]- and [Met]-enkephalin in 82 and 73% yield respectively.

The above component, BOC-Tyr(Bzl)-Gly-OH was prepared as follows : BOC-Tyr-OEt and H-Gly-N_2H_2Ph were incubated at pH 10.1 in the presence of α-chymotrypsin, giving BOC-Tyr(Bzl)-Gly-N_2H_2Ph in 72% yield. The resulting dipeptide was treated successively with ferric chloride to remove the phenylhydrazide moiety and with benzyl bromide to benzylate the phenolic group of the tyrosyl unit. This 'tactical' introduction of a benzyl group was in fact indispensable, since the preparation of the desired enkephalin pentapeptides turned out to be impracticable unless BOC-Tyr-Gly-OH, which had been initially considered as acyl group donor, was replaced by BOC-Tyr(Bzl)-Gly-OH. Evidently, the benzylation of the tyrosine side-chain provided the driving force for the papain-catalyzed synthesis by lowering the solubility of the resulting pentapeptides.

The N-acylated forms of the prospective amine components could be synthesized by incubation of BOC-Gly-OH and H-Phe-N_2H_2Ph at pH 4.8 in the presence of papain (yield 80%). The newly formed dipeptide phenylhydrazide BOC-Gly-Phe-N_2H_2Ph was treated in succession with bromsuccinimide and ethanol to give the corresponding ethyl ester BOC-Gly-Phe-OEt which was then coupled

with H-Leu-N_2H_2Ph or H-Met-N_2H_2Ph at pH 9.95 (yields 70 and 61% respectively).

The second enkephalin synthesis commenced with a papain-controlled fragment condensation of BOC-Tyr(Bzl)-Gly-OH and H-Gly-Phe-N_2H_2Ph at pH 6.0 (Scheme 1). In an analogous manner to the procedure described above, the outcoming tetrapeptide phenylhydrazide (yield 71%) was subsequently rearranged to give the corresponding tetrapeptide ester. Incubation of the latter with H-Leu-N_2H_2Ph or H-Met-N_2H_2Ph in the presence of α-chymotrypsin gave the target peptides in 50 and 45% yield respectively. The above tetrapeptide ethyl ester, BOC-Tyr(Bzl)-Gly-Gly-Phe-OEt, was obtained in moderate yield (35%). This shortcoming presumably resulted from the treatment of the precursor tetrapeptide with bromosuccinimide, which is known to be potentially hazardous to tyrosine-containing peptides.

The C-terminal elongation of the tetrapeptide ethyl ester by leucine- or methionine phenylhydrazide was completed in moderate yields by using increased enzyme concentration and an incubation period prolonged to 40 hours. After completion of the syntheses and deprotection, the resulting free enkephalin peptides were purified and the enzymatically prepared enkephalins displayed naxolone-reversible opiate-like activities equivalent to their natural analogues.

Papain was exclusively used to catalyze the formation of all peptide bonds during an enzymatic synthesis of [Leu]-enkephalin

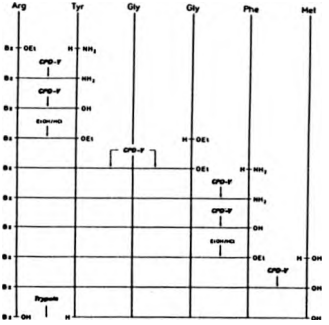
reported by Zapevalova et al. [144]. The opioid pentapeptide was assembled by a stepwise mode in conjunction with a C ⟶ N coupling schema, and monodirectional chain growth proceeded via repeated incorporation of successive protected amino acid units. Z-protected amino acid methyl esters functioned as carboxyl components while leucine t-butyl ester and, with growing chain length, free di-, tri-, and tetrapeptides, respectively, served as amine components. In order to prevent secondary hydrolysis of the preexisting peptide bonds, the papain-mediated coupling steps were conducted within an alkaline environment (pH = 8.1 to 9.6). The product yields ranged from 74 to 79%. The final coupling reaction, however, gave the target pentapeptide in a rather low yield (23%).

A different strategy for the enzymatic synthesis of [Met]-enkephalin was developed by Widmer et al. [145]. This approach was based principally on carboxypeptidase-Y-catalyzed condensation and desamidation steps (Scheme 2).

Carboxypeptidase Y (CDP-Y) is an exopeptidase which specifically acts on the carboxyl terminus of a given substrate. Consequently, the enzymatic synthesis proceeds by the consecutive addition of the individual amino acid derivatives to the C-terminal end of the growing peptide chain. Due to the ability of CDP-Y to catalyze in an orderly fashion the dimerization of H-Gly-OEt [146], the dipeptide H-Gly-Gly-OEt was incorporated through a single coupling step (Scheme 2). The semipermanent 'protection' of the

amino-terminal tyrosine residue was provided by the trypsin-labile benzoyl-arginine moiety, which could be finally removed by trypsin catalysis.

Scheme 2 : Carboxypeptidase Y-catalyzed synthesis of [Met]-enkephalin.



N-benzoylated amino acid or peptide ethyl esters served as acyl group donors, whereas H-Tyr-NH_2, H-Gly-OEt, H-Phe-NH_2, and

H-Met-OH were used in the given order as amine components. The reactions were performed in aqueous solutions in the pH range of 8.0 to 9.6 and coupling yields were between 60 and 90%. Prior to elongation of the peptide chain the amide groups were removed by CDP-Y catalysis and subsequently replaced by an ethyl ester group. The CDP-Y approach to enkephalin synthesis took advantage of a salient feature of the peptidase, namely that it lacks the capacity to cleave internal peptide bonds. However, this picture is darkened by transpeptidation phenomena, which may occur during each elongation step.

In connection with the design and synthesis of an opiate-receptor mimetic peptide, a series of chemically modified [Leu]-enkephalin peptides were required to explore the binding characteristics of the artificial receptor molecule [147].

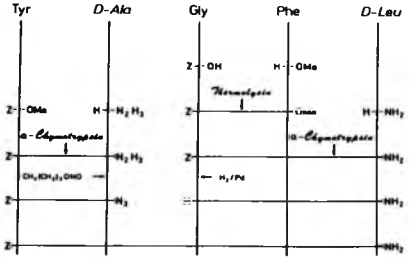
The synthesis of three analogues of [Leu]-enkephalin in which the N-terminal tyrosine moiety was replaced by phenylalanine, phloretic acid, or hydrocinnamic acid, was accomplished by enzymic and mixed enzymochemical procedures.

Stoineva and Petkov reported a synthetic pathway leading to [D-Ala_2 and D-Leu_5]-enkephalinamide [148] (Scheme 3).

In this case, the desired peptide was finally assembled via azide fragment condensation of the dipeptide Z-Tyr-D-Ala-NH-NH_2 and the tripeptide H-Gly-Phe-D-Leu-NH_2. The former was prepared at pH 9.3 in 80% yield from Z-Tyr-OMe and H-D-Ala-NH-NH_2 via chymotrypsin

catalysis. The N-acylated form of the latter was obtained by thermolysin condensation of Z-Gly-OH and H-PheOMe (yield 65%, pH 6.8) followed by the chymotrypsin coupling of the resulting dipeptide with H-D-Leu-NH_2 (yield 85%, pH 9.3).

Scheme 3 : enzymo-chemical synthesis of (D-Ala^2, D-Leu^5)-enkephalinamide.



Over the last few years, there has been an increased development of non-aqueous enzymology. It was shown that enzymes can function as catalysts in organic solvents instead of water, and when

placed in this unnatural environment they exhibit novel properties [67].

Enkephalins were once again chosen as model peptides to investigate the potentiality of the new strategies.

In a study on the papain-catalyzed synthesis of dipeptides containing D-amino acid and derivatives, Wong and Barbas [36] were able to enhance greatly the relative esterase activity of the enzyme by using a large proportion of organic solvent (40% methanol) and high pH (pH 9). Under these conditions, the coupling of a Z-amino acid ester and an amine component (the nucleophile) under papain catalysis was much faster than the enzyme-catalyzed hydrolysis of amide bonds, if the activity of water is reduced. Stepwise synthesis of [Leu]-enkephalinamide illustrated the feasibility of this approach towards polypeptide synthesis (Scheme 4).

Z-Tyr-Gly-Gly-Phe-Leu-NH_2

70% 87% 86% 73%

Scheme 4 : synthesis of [Leu]-enkephalinamide (figures correspond to isolated yields.

Crude papain was first entrapped in Amberlite XAD-8 and the biocatalyst was extensively dried. The reaction mixture consisted of multimolar solutions of the starting components in 4-methyl-pentan-2-one containing traces of 2-mercaptoethanol. [Leu]-

enkephalinamide was synthesized from C to N terminus with the coupling of Z-Phe-OMe and H-Leu-NH_2, hydrogenolysis of the amino protecting group and the coupling of the resulting N-free dipeptide with the subsequent Z-amino ester, hydrogenolysis, etc as in Scheme 4.

In these syntheses, each of the first two couplings involved two equivalents of the amine components while each of the last two couplings involved two equivalents of the ester components. The use of immobilized enzyme allows regeneration of the catalyst which can then be reused for the next coupling step. The great advantage of using a protease in organic solvent is that after coupling, the resulting peptide is safe from subsequent hydrolysis by the enzyme owing to the absence of water.

In a study on the incorporation of D-amino acid into peptides via enzymatic condensation in organic solvents, Klibanov et al. [99] found that the stereoselectivity of subtilisin was dramatically reduced when suspended in anhydrous organic solvents. The synthesis of the dipeptide L-Tyr-D-Ala-NH-$(CH_2)_3$-Ph which is known to possess an enkephalin-like activity was accomplished by the subtilisin-catalyzed coupling of Z-L-Tyr-OEtCl and H-D-Ala-NH-$(CH_2)_3$-Ph in dry tert-amyl alcohol. If the synthesis of the naturally occuring opioid peptides was not investigated, this method could help in overcoming some of the shortcomings of protease-catalyzed reactions in aqueous medium. Nevertheless, the solubility of highly polar compounds in dry hydrophobic solvents

is a major problem which will considerably reduce the scope of applications to bioactive peptides synthesis.

It was recently demonstrated that proteases may be modified to have only esterase activities and yet retain their native binding properties [149]. In this case, the amidase-deficient proteases may catalyse the aminolysis reaction through an acyl-enzyme intermediate. α-Chymotrypsin has been modified and methyl-chymotrypsin, prepared by selective methylation at $N\varepsilon_2$ of the active site histidine, became a weak esterase without amidase activity and catalyzed the aminolysis of N-protected peptide methyl or cyanomethyl esters.

This approach was successfully applied to the last coupling step in the synthesis of [Leu]-enkephalinamide (Scheme 5).

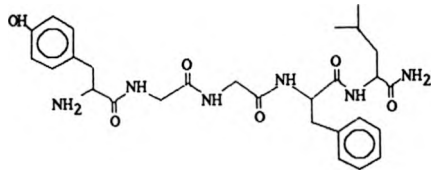
Z-Tyr-Gly-Gly-Phe-OCH_2CN (1 eq) + Leu-NH_2

methylchymotrypsin
50% DMF, pH 8.81
Z-[Leu]-enkephalinamide (99%)

Scheme 5 : Synthesis of [Leu]-enkephalinamide catalyzed by methylchymotrypsin.

During the course of our study on α-chymotrypsin as a catalyst in organic solvents, [Leu]-enkephalinamide was found to be a good candidate to test the potential of our strategy.

The endogenous peptide [Leu]-enkephalinamide shows several features which make it a good model peptide for establishing an enzymatic procedure :



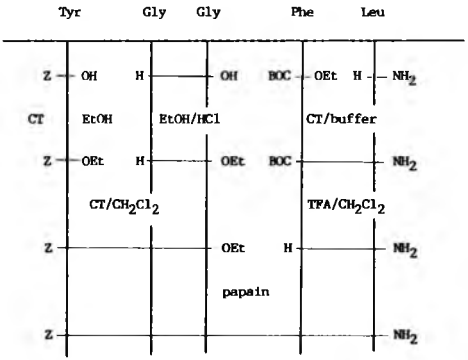
Scheme 6 : structure of [Leu]-enkephalinamide.

A heterogenous amino acid composition offers the possibility of using different proteases; aromatic amino acids like tyrosine and phenylalanine allow the use of chymotrypsin, and the presence of two glycine residues constitutes a good case for papain-catalyzed peptide bond synthesis. Solubility of protected [Leu]-enkephalinamide and precursors in aqueous medium is a major problem which could be overcome by the enzymes in a suitable organic solvent.

Finally, if the pentapeptide does not constitute a target in itself, its synthesis might give opportunities to test the scope of applications of enzymatic synthesis in organic solvents.

A synthetic pathway for the synthesis of the desired opioid peptide was elaborated in which peptide bonds would be prepared either by papain- or chymotrypsin catalysis (Scheme 7).

Scheme 7 : enzymatic synthesis of [Leu]-enkaphalinamide.



Because of the model character of this study, our interest was largely aimed at the synthesis of only a single bond via protease-catalysis. As a consequence, the synthesis often started

from partially protected amino acid residues and did not proceed beyond the level of the protected pentapeptide. Where protected peptides served as educts, they had to be prepared by chemical means prior to their use in enzymatic synthesis.

The synthetic pathway depicted in Scheme 7 involves the final fragment condensation of Z-Tyr-Gly-Gly-OEt **85** and H-Phe-Leu-NH_2 **86** in the presence of papain. In a previously described synthesis of [Leu]-enkephalinamide [141], this synthetic concept had to be discarded due to the formation of a tetrapeptide composed of Tyr-Gly-Phe-Leu via a transpeptidation reaction.

Despite what could appear to be a dead end, we decided to push ahead with the final fragment condensation for several reasons. Firstly, the structures of the peptide precursors were different from those described in the previously mentioned synthesis [141,142]. The nature of the protecting groups (Z instead of BOC, and $CONH_2$ instead of N_2H_2Ph) might have an effect on the outcome of the reaction. In the synthetic pathway depicted in figure 4, the side-chain of tyrosine had to be protected by a benzyl group for specificity or solubility reasons. In the present case, temporary protection of the phenolic moiety was not required and this might well exert an influence on the binding of the precursor peptide to the active site of the enzyme. Second and more importantly, the final coupling step was to be carried out in organic solvents according to the procedure developed by Wong [36]. It is of primary importance to investigate if the

behaviour of a particular enzyme is modified by the new environment and therefore, the detection of the truncated peptide alongside the desired opoid peptide will reveal any influence of the organic solvent on the outcome of the reaction.

Owing to the unpredictable nature of the papain-catalyzed reaction, a critical evaluation of the chemical nature of the resultant peptides is indispensable to avoid confusion. The elaboration of a rapid and reliable method of analysis would allow a screening by which a series of enzymes could be used to catalyze the final fragment condensation. This method of analysis would have to be extremely sensitive and be able to assess the chemical nature of a large number of fragments in a complicated mixture. Tandem mass spectrometry was elected as a method of choice, because of its high sensitivity and the possibility of characterizing fully peptide sequence through the identification of specific fragments.

The major drawback of this analytical approach is that no quantitative assessment would be possible. As it turned out, attempts to quantify the different peptide fragments by using high field NMR proved to be very difficult because the presence of numerous peptide structures. Nevertheless, tandem mass spectrometry was elected as a method of choice to help in defining the optimal conditions under which the enzymatic reactions might take place.

Finally, during the course of the synthesis, several attempts would be made to profit from new developments in non-aqueous enzymology.

Firstly, esterification reactions catalyzed by α-chymotrypsin suspended in ethanol would be investigated according to the observation of Noritomi et al. [82]. Mild esterification methods are of considerable interest in the field of peptide chemistry owing to the chemical sensitivity of the protecting groups. Esterification by conventional means, i.e. saturated solution of hydrochloric acid in ethanol or ion-exchange resins in ethanol, would cleave traditional semipermanent protecting groups like BOC and Z.

By suspending α-chymotrypsin in ethanol containing small amounts of buffer solutions, Noritomi et al. [82] were able to synthesize N-acetyl-L-tyrosine ethyl ester from the free acid in 88% yield. We decided it would be interesting to investigate this approach, and apply it to the synthesis of one of the precursor of [Leu]-enkephalinamide, Z-L-Tyr-OEt **13**. At the same time, the specificity of the enzyme would be investigated and the reaction optimized.

Second, the use of immobilized enzymes is of major interest in biotransformations. The many potential attractions of this approach include short reaction times, high volumetric efficiency, continuous processing, ready separation of the products and recovery of the biocatalyst. This technology offers

economical methods of recovery and purification; scale-up can be relatively straightforward.

In the approach using free enzymes in suspension in organic solvents, the biocatalyst can be recovered by simple filtration but is often contaminated by some of the reaction products and the regeneration of the catalyst could be made difficult due to the low concentrations of enzymes, especially with highly purified enzymes like chymotrypsin, generally used. Therefore, it would be of considerable interest to develop a biocatalyst consisting of the enzyme immobilized on a suitable support.

The ideal immobilization matrix would have to display the following properties :

- the loss of activity of the biocatalyst on immobilization should be low.
- the biosupport should have high mechanical strength.
- the support should be thermally and chemically stable.
- regeneration of the support should be possible.
- the biosupport should offer a high contact area with the surrounding medium.
- substrates and products should be able to diffuse freely through the biosupport.

and more specifically for enzymatic catalysis in organic solvents.

- the biosupport would have to be obtained in an almost anhydrous state without loss of properties.

- the biosupport would have to be able to withstand repeated washings with ethanol.

Many of the requirements listed above could be met by the use of a porous inorganic support that combines high strength with a structure containing pores of appropriate dimensions.

A range of biosupports, which meet many of criteria set above, have recently been developed. The Biofix range (English China Clays International) consists of four materials (table 1), two for cell support (C_1 and C_2) and two for immobilization of enzymes (E_1 and E_2). All four products are derived from the clay mineral kaolinite, though the methods of manufacture differ from grade to grade.

Table 1 : Typical technical properties of the Biofix range.

Grade	C1	C2	E1	E2
Product form	Hollow porous microspheres	Hollow porous microspheres	Porous microspheres	Porous granules
Particle size	20 - 50 µm	50 - 75 µm	20 - 50 µm	0.5 - 1.5 mm
Cavity entrance	10 µm	20 µm	—	—
Mean pore size	900 Å	900 Å	800 Å	300 Å
Surface area/m^2g^{-1}	5.0	9.5	4.0	25
Bulk density/g cm^{-1}	0.55	0.50	1.0	0.72
% Void volume	22 wall 65 cavity	22 wall 70 cavity	50	50
pH stability	1 - 14	1 - 14	1 - 14	1 - 14
Thermal stability	1000°C	1000°C	1000°C	1000°C
Crush strength	> 55 MPa	> 55 MPa	> 55 MPa	> 55 MPa

Because of the organic environment, the enzyme does not need to be covalently linked to the Biofix support. Hydrophilic interactions maintain the coherence of the biocatalyst, and this provides a very simple and efficient method of preparation by simple evaporation of a solution of the enzyme mixed with the appropriate support. The biocatalyst can then be extensively dried to an almost anhydrous state.

The N-protected C-activated tyrosyl derivative, Z-L-Tyr-OEt **13**, was prepared by the chymotrypsin-catalyzed esterification reaction. Chymotrypsin suspended in ethanol containing 2% of water was able to catalyze the formation of the ethyl ester under thermodynamic control. The optimized yield reached 44% over a period of 24 hours.

In their original observation, Noritomi et al. [82] recommended the use of a phosphate buffer pH 7.8. As far as we could observe, no difference was noted by using pure distilled water instead of a buffer solution. Furthermore, the salts constituting the buffer immediately precipitated after their addition to the alcoholic solution. The actual effect of the buffer is impossible to assess and we decided to use pure water for reproducibility purposes.

This approach was applied to several N-protected amino acids to investigate the specificity of the enzyme under these conditions. The results are summarized in Table 2.

Table 2 : Esterification with chymotrypsin in ethanol containing 2% water.

Substrates	Yield (%)	reaction time (hrs)
Z-L-TyrOH	44	24
Z-L-PheOH	34	24
Z-L-AlaOH	0	72
Z-L-LeuOH	Traces	72
Z-L-MetOH	0	72
Z-L-LysOH	0	72
Z-L-ProOH	0	72
Z-L-AspOH	0	72
Z-L-Asp(OBu^t)OH	0	72

The specificity of the enzyme is strictly limited to aromatic amino acid derivatives which is in accordance with the known specificity of α-chymotrypsin.

Despite this limitation, this approach is valuable for the synthesis on a preparative scale of esters of amino acid derivatives bearing highly labile protecting groups.

Due to the model character of the study, we had no immediate interest in the dipeptide fragment H-Gly-Gly-OEt **22**. This partially protected dipeptide was chemically synthesized by a conventional esterification of the commercially available dipeptide by HCl in dry ethanol. The reaction proceeds

efficiently in 93% yield, giving the ethyl ester which is recrystallized from methanol-diethyl ether.

The chymotryptic condensation of Z-L-Tyr-OEt **13** and H-Gly-Gly-OEt **22** was considered to be a central point in the synthesis of [Leu]-enkephalinamide. This is a direct application of the strategy using α-chymotrypsin suspended in dichloromethane. This approach applied to the synthesis of the tripeptide fragment constituted a great improvement compared to previously reported syntheses of [Leu]-enkephalinamide. It allows the use of equimolar quantities of the starting components, reduces the extent of hydrolysis by the low water content, makes the purification of the peptide extremely easy, and facilitates the recovery of the biocatalyst. Furthermore, the use of the dipeptide C-protected as an ethyl ester avoids subsequant deprotection and activation steps for the papain-catalyzed fragment condensation.

The coupling of the dipeptide H-Gly-Gly-OEt **22** with Z-Tyr-OEt **13** was catalyzed by α-chymotrypsin suspended in dichloromethane containing 0.25% water. The immediate formation of a precipitate indicated the appearance of the desired tripeptide. After two days of reaction, the tripeptide Z-Tyr-Gly-Gly-OEt **85** was isolated by simple filtration. After recrystallisation, the tripeptide was obtained in 88% yield.

In order to improve the recovery of the biocatalyst, to facilitate purification and to allow the scale-up of the

reaction, chymotrypsin was immobilized on a ceramic support, Biofix E_1 (Table 1).

The immobilization procedure for α-chymotrypsin consisted in the preparation of a solution of the enzyme (100 mg) in water (1.5 ml). Biofix E_1 (1 gm) was added to this solution and the water removed under reduced pressure. The biocatalyst was then extensively dried in a desiccator over P_2O_5.

One of the advantages of using a solid support is to greatly increase the surface area and avoid the aggregation of enzyme molecules in organic solvents. Electron micrographs were taken of the Biofix E_1 and Biofix E_1/chymotrypsin surfaces. Unfortunately, the picture of the Biofix E_1 alone appeared to be so badly encrusted with presumably amorphous silica that direct comparison of the two pictures was difficult. Nonetheless, it seemed fairly clear that there were no large (>0.1 μm) chymotrypsin crystals on the Biofix surface.

Aggregation of enzyme particles is therefore greatly reduced and a large surface area can be obtained. Aggregation in organic solvents was observed by A.M. Klibanov who used ultrasonication of α-chymotrypsin suspended in dry octane [66]. It reduced the average enzyme particle size from 270 μm to 5 μm. Biofix E_1/chymotrypsin possesses the same properties as the free enzyme in organic solvents. This was verified by the synthesis of the dipeptide Ac-L-Tyr-D-Phe-NH_2 from Ac-L-Tyr-OEt and D-Phe-NH_2. The reaction proceeds efficiently reaching 95% yield in 3 hours. The relaxed stereospecificity of chymotrypsin suspended in organic

solvents is a characteristic feature which is also observed for the Biofix E_1/chymotrypsin biocatalyst.

Gram quantities of the tripeptide Z-Tyr-Gly-Gly-OEt were then synthesized in an average 84% yield of the purified compound. After completion of the reaction, the reaction mixture was filtered and the precipitate was washed with dichloromethane to remove any traces of the starting components. The precipitate was then dissolved in warm ethanol and separated from the biocatalyst. After a single recrystallization, the pure dipeptide 85 was obtained. The biocatalyst was easily recovered and after drying could be reused without noticeable loss of activity. The entire operation can take place in a peptide synthesis apparatus which limits the number of individual manipulations.

Unfortunately, it was impossible to design a column reactor. Even by reducing the concentrations of the starting materials and increasing the ratio Biofix/enzyme to 50:1, the tripeptide precipitated during the course of the reaction, thus blocking the column.

The dipeptide fragment BOC-Phe-Leu-NH_2 84 was synthesized via chymotrypsin-catalysis. The tert-butyloxycarbony group was readily introduced by the reaction of di-tert-butyl dicarbonate with the ethyl ester of phenylalanine.

Incubation of BOC-Phe-OEt with H-Leu-NH_2 in the presence of chymotrypsin in a carbonate buffer pH 9.2 gave the target

dipeptide 84 in 90% yield in 20 minutes. It was shown earlier that BOC-amino acid derivatives were unsuitable substrates for chymotrypsin suspended in organic solvents (chapter I), and therefore this approach was not suitable in this particular case. Nevertheless, preliminary experiments showed that the synthesis of the dipeptide Z-Phe-Leu-NH_2 was possible via suspended chymotrypsin in organic solvents. But this latter strategy did not prove itself as efficient as the synthesis involving peptide bond formation in water. This proves that despite very recent developments, peptide synthesis in aqueous medium might still be the ideal strategy in specific cases.

The synthesis of BOC-Phe-Leu-NH_2 was solubility-controlled. The poor solubility of the resulting dipeptide firstly induces a favorable shift of the chemical equilibrium and second greatly facilitates the purification procedure.

The acid-labile BOC group was then cleaved by treatment by a solution of trifluoroacetic acid in dry dichloromethane, yielding the trifluoroacetate salt 86 in an almost quantitative yield.

The last step of the synthetic route comprised the coupling via papain-catalysis, of the tripeptide Z-Tyr-Gly-Gly-OEt 85 with the dipeptide H-Phe-Leu-NH_2 86. Solubility problems played rapidly a major role. The tripeptide Z-Tyr-Gly-Gly-OEt is insoluble in aqueous solutions even with large concentrations of cosolvents

such as DMF, DMSO, ethanol, ... Therefore, a coupling step in an organic solvent was to be designed. A compromise had to be reached and the ideal organic solvent would have to be able to solubilize the starting components and allow enzymatic activity. Acetonitrile was elected as a suitable reaction medium due to its ability to solubilize both starting components. Furthermore, water being soluble in acetonitrile allows the formation of a homogenous phase.

Crude papain (EC 3.4.22.2, from Sigma, purified from papaya latex, 1.5-3.5 units per mg solid) was immobilized in Amberlite XAD-8 (a neutral cross-linked poly(methylmethacrylate) from Sigma) according to the method developed by Wong and Barbas [36]. Crude papain (1 gram) and XAD-8 (4 grams) were stirred in 50 ml 0.1M phosphate buffer pH7 for 6 hours. Papain entrapped in XAD-8 was filtered and dried under reduced pressure over potassium hydroxide for 12 hours.

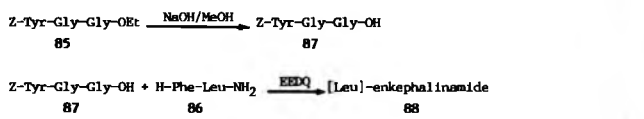
Preliminary experiments showed that water was required to observe enzymatic activity in acetonitrile. 3% added water was found to be the upper limit. Amounts of water greater than 3% immediately precipitated the starting component, Z-Tyr-Gly-Gly-OEt.

In a typical experiment, the trifluoroacetate salt of the dipeptide H-Phe-Leu-NH_2 (3 mmol) in acetonitrile (5 ml) was neutralized by the addition of one equivalent of triethylamine. The acyl component, Z-Tyr-Gly-Gly-OEt (1 mmol), the immobilized enzyme (500 mg), and 2-mercaptoethanol (15 μl) as the enzyme activator, were then added. The reaction was then started by the

addition of 0.1M phosphate buffer pH 7.8 (3%, v/v). The mixture was stirred for 4 days at 40°C until all the starting ester, Z-Tyr-Gly-Gly-OEt was consumed as monitored by TLC. The immobilized enzyme was removed by filtration and the solvents evaporated under reduced pressure. The dry residue was then washed with water, 0.1M HCl, 0.5M sodium hydrogen carbonate solution, and water, and dried under reduced pressure yielding a dry powder. TLC analysis of the residue showed a complicated mixture of several compounds, some of them bearing a free amino group as indicated by reaction with ninhydrin.

The crude mixture was then analyzed using tandem mass spectrometry. In order to make the identification of the structures easier, a pure sample of [Leu]-enkephalinamide was synthesized according to Scheme 8.

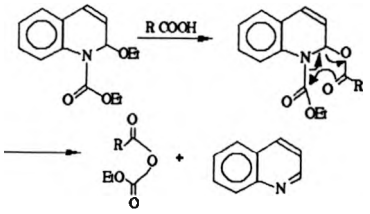
The tripeptide Z-Tyr-Gly-Gly-OEt was saponified using an aqueous solution of sodium hydroxide in methanol. 73% conversion was reached after stirring at room temperature for 24 hours. After acidification and extraction, the free tripeptide Z-Tyr-Gly-Gly-OH **87** was obtained in 59% yield.



Scheme 8 : chemical synthesis of [Leu]-enkephalinamide.

The coupling reagent, N-ethyloxycarbonyl-2-ethyloxy-1,2-dihydroquinoline (EEDQ), was used for the final coupling step between the tripeptide Z-Tyr-Gly-Gly-OH and the dipeptide H-Phe-Leu-NH_2. This reagent was introduced by Belleau and Malek [150] who proposed that it reacts with the carboxyl component to form a mixed anhydride intermediate and quinoline as shown in Scheme 9.

Scheme 9 : Mechanism of the coupling reaction involving EEDQ.



The anhydride reacts with the amine component to generate the peptide bond with liberation of ethanol and carbon dioxide. The advantage of the reagent consists mainly in the convenience of its use as a coupling reagent, i.e. in the simple addition to a mixture of the carboxyl and amine components at ambient

temperature. The mixed anhydride is generated slowly but reacts rapidly, thus limiting the risk of side reactions. If racemization can occur, it is generally limited. Quinoline is the only by-product which makes the method very attractive.

The trifluoroacetate salt of H-Phe-Leu-NH_2 (1 mmol) in acetonitrile was neutralized using triethylamine, and Z-Tyr-Gly-Gly-OH (1 equivalent) and EEDQ (1.1 equivalent) were then added. After stirring at room temperature overnight, the protected [Leu]-enkephalinamide was isolated in 85% yield and purified by preparative thin-layer chromatography.

The pentapeptide [Leu]-enkephalinamide was analyzed by mass spectrometry following the strategy described below, using a four sector mass spectrometer. The molecular weights of both [Leu]-enkephalinamides obtained by chemical and enzymatic syntheses, were determined by FAB MS (fast atom bombardement) where the $[M+H]^+$ ion (protonated peptide molecule) were mass-analyzed in the first section of a tandem mass spectrometer (MS 1). Then, in order to obtain sequence information of both peptides, the MS/MS technique was used. The Kratos CONCEPT four sector mass spectrometer ($E_1B_1E_2B_2$ geometry) used in this study allows this technique. This instrument is a combination of two double focusing mass spectrometers. Between the two "magnets" (MS 1 and MS 2) is a collision cell, device which allows ions of a particular m/z (parent ion) to be selected. Collision with a neutral gas such as argon, produces fragments (daughter ions). The resulting fragment ions are accelerated from the collision

cell into the second mass spectrometer for analysis. The resulting spectrum is called a Collision Induced Decomposition (CID) spectrum [151,152].

Experimental :

CID mass spectra were recorded with the four sector mass spectrometer from sample introduced directly into a standard KRATOS FAB source. The sample was bombarded with a beam of xenon atoms having a kinetic energy of 8 KeV. The instrument was operated at an accelerating voltage of 8KV.

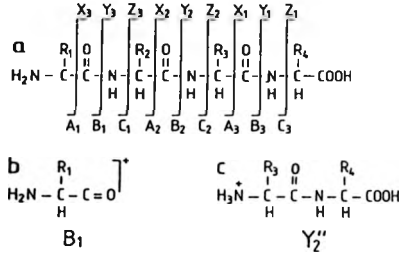
In MS/MS mode, only the ^{12}C component of the $[MH]^+$ ion is selected by MS 1 and transmitted into the collision region. Argon is introduced into the collision cell which is floated at 2 KV at such a pressure that 30% of the precursor ion intensity is transmitted and its decomposition recorded to give the CID spectra. The accuracy of the mass assignment is at least of + 0.3 a.m.u.

The common nomenclature used for free as well as derivatized peptides independent of the ionization method employed is depicted in Scheme 10 [153,154].

The three cleavage points of the peptide backbone are called A,B, and C when the charge is retained at the N-terminal fragment of the peptide, and X,Y, and Z when the charge is retained by the C-terminal fragment. The numbering indicates which peptide bond is

cleaved counting from the N- and C-terminus respectively, and thus also the number of amino acid residues in the fragmentation.

Scheme 10.



The number of hydrogens transferred to or lost from the fragment is indicated with apostrophes to the right and the left of the latter respectively. Thus, the acylium ion is named B_n (Scheme 10 (b)) and the most common C-terminal fragment ion in both FAB and CI, Y_n'' (Scheme 10 (c)).

This nomenclature has been applied to [Leu]-enkephalinamide and the results are described in Table 3.

Table 3.

N-benzyloxycarbonyl-[Leu]-enkephalinamide

Z-Tyr-Gly-Gly-Phe-Leu-NH_2 M = 688.3372, MH^+ = 689.3452

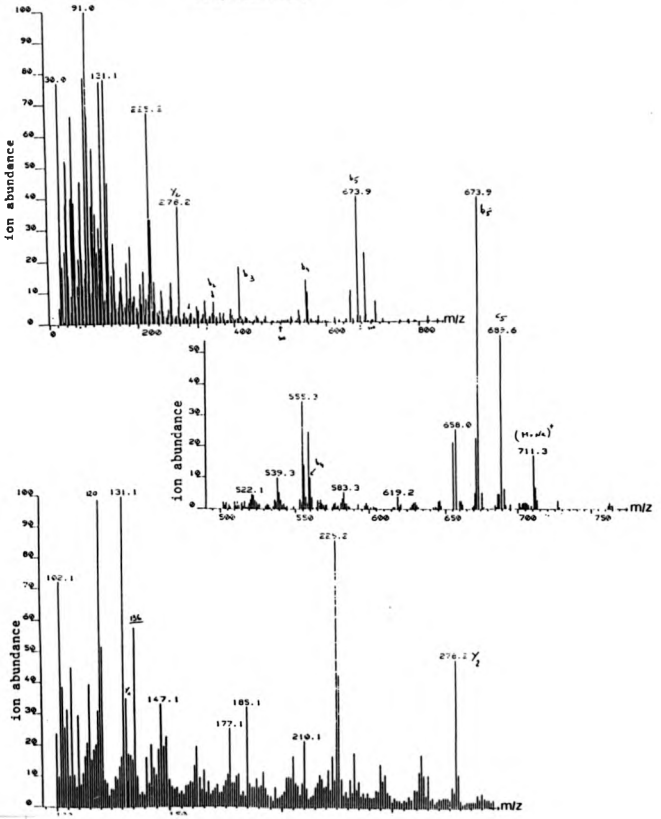
	A	B	C
5	645.3303	672.3262	689.3452
4	532.242	560.2379	576.2569
3	385.1697	413.1656	429.1846
2	328.1454	356.1413	372.1603
1	271.1211	299.1170	315.136
	108.0529	136.0488	152.0678

	X	Y	Z
5		689.3452	672.3182
4	418.2171	392.2372	375.2102
3	361.1928	336.2129	318.1859
2	304.1685	278.1886	261.1616
1	157.0962	132.1163	114.0893

The fragments b_4, b_3, b_5, y_2, b_2, together with $(M + Na)^+$ were detected in the full mass spectrum. Alongside these well-defined fragmentations of the peptide backbone, several more specific cleavages were identified (Fig. 1).

The advantage of tandem mass spectrometry is that it makes it possible to follow the fragmentation of a particular ion. The fragments arising from the selected ions are called daughter ions

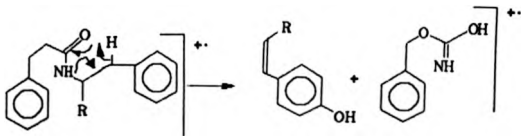
Figure 1 : Full mass spectrum of synthetic [Leu]-enkephalinamide.



and are specific to the selected ions. By using this method, peptide sequencing is possible, and furthermore it makes it possible to isolate a particular ion in a mixture and determine its structure. However, the quality of the analysis depends upon the intensity of the parent ion. The structure of the protected [Leu]-enkephalinamide was confirmed by this method and the 'fingerprints' of the peptide were then available to help its determination in mixtures (Fig. 2).

The mixture obtained from the papain-catalyzed reaction was analyzed using the same technique. The presence of N-benzyloxycarbonyl-[Leu]-enkephalinamide was confirmed by the fragments c_5, b_5, a_2, y_2, and $(M + Na)^+$. Furthermore, more specific fragmentations such as the loss of the protecting group (m/z = 555) and the occurence of a primary rearrangement fragmentation as depicted in Scheme 11 were identified (Fig. 3).

Scheme 11.



m/z 538.18 m/z 151

Figure 2 : CID mass spectrum of synthetic [Leu]-enkephalinamide.

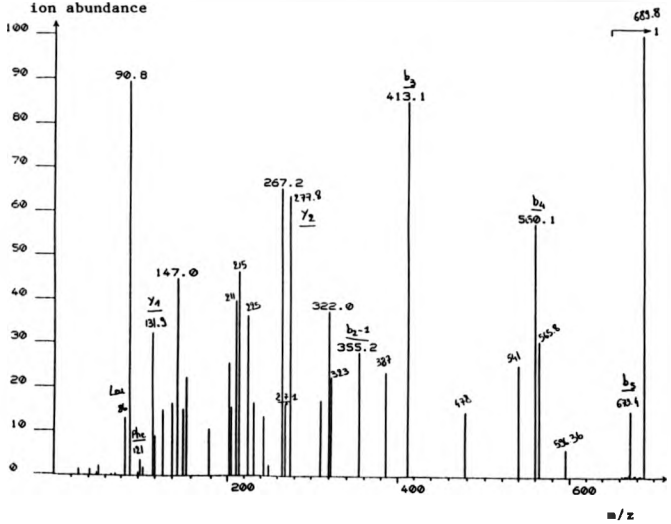
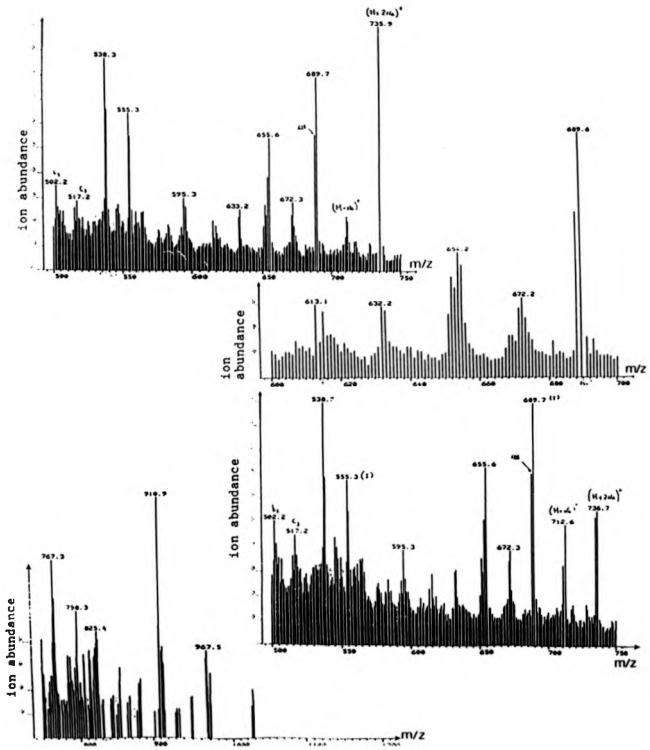


Figure 3 : Full mass spectrum of the enzymatically-synthesized [Leu]-enkephalinamide.



The expected transpeptidation product, Z-Tyr-Gly-Phe-$LeuNH_2$ was also identified by the fragments c_4 (MH^+), c_3, and b_3. The fragment with m/z 632.2 does not come from fragmentation of the native pentapeptide. Examination of the full mass spectrum of [Leu]-enkephalinamide synthesized chemically does not show the presence of this particular fragment.

Alongside these two expected peptides, one can detect the presence of several other peptides. A consideration of the potential behaviour of the enzyme, in particular its specificity, might help to identify these unknown peptide structures. Besides the synthesis of [Leu]-enkephalinamide and the transpeptidation reaction, papain is also able to catalyze several other reactions. An obvious one is the polycondensation of the nucleophile, H-Phe-$LeuNH_2$, yielding H-$(Phe-Leu)_n$-NH_2 where n is greater than one.

Two peptides where n=2 (MH^+=538.708) and n=3 (MH^+=798.035) can be detected in the full mass spectrum. Unfortunately, no specific fragments could be identified and the CID technique was not envisageable because of the low abundance of the ions.

The presence of higher peptide fragments can also be detected (m/z 967.5, 910.9, 825.4, 767.3) but it proved impossible to establish the structures of these particular fragments.

A more interesting feature in the full mass spectrum was found for m/z 688. This ion which was not detected in the case of the chemically synthesized [Leu]-enkephalinamide presents one mass unit difference with the fragment corresponding to [Leu]-

enkephalinamide (MH^+=689.3452). The difference being -1, it cannot be an isotopic effect. It was possible to follow the specific fragmentation of this particular ion using the CID technique .

The ion with m/z 689.3 which corresponds to [Leu]-enkephalinamide (MH^+) was also analyzed by this technique and a spectrum identical to that obtained with the chemically synthesized compound was obtained, then confirming the chemical integrity of the enzymatically synthesized peptide (figure 4).

The spectrum corresponding to the ion at m/z 688 shows similar features compared with that corresponding to the native peptide (figure 5).

The peak at m/z 278 corresponds to a type y fragment revealing the presence of a dipeptide fragment [Phe-Leu]. The presence of phenylalanine is further confirmed by a peak at m/z 120 which corresponds to $Ph-CH_2-CH=\overset{+}{N}H_2$. A very low abundance of a peak at m/z 672 which corresponds in the native peptide to a fragment b_5, is observed which tends to indicate that the peptide fragment at m/z 688 might be a fragmentation peptide itself, of type C, i.e., $Z\text{-Tyr-Gly-Gly-Phe-Leu-CONH}]^+$.

The ion at m/z 163.8 could correspond to $Ph-CH_2-C(=NH_2)-CONH_2]^+$ from an intramolecular cleavage.

The ion at m/z 596.4 corresponds to a loss of 91 being either $Ar-CH_2^{\cdot}$ or $Ar-CH_2^+$. The ion at m/z 535.9 might correspond to a type Z fragmentation involving the loss of the Z-group and NH_3.

Figure 4 : CID mass spectrum corresponding to the ion at m/z 689.3.

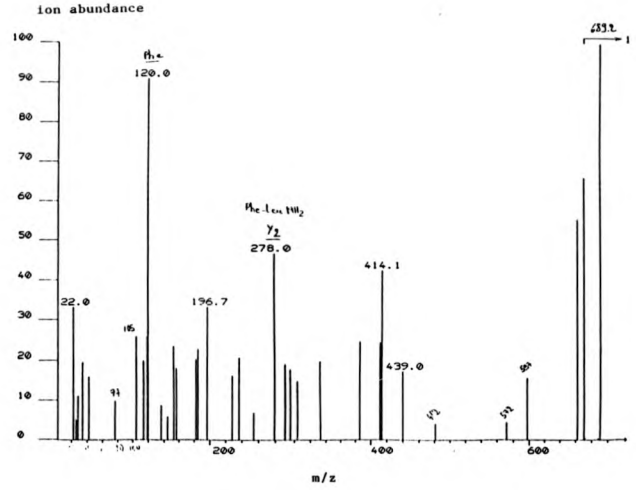
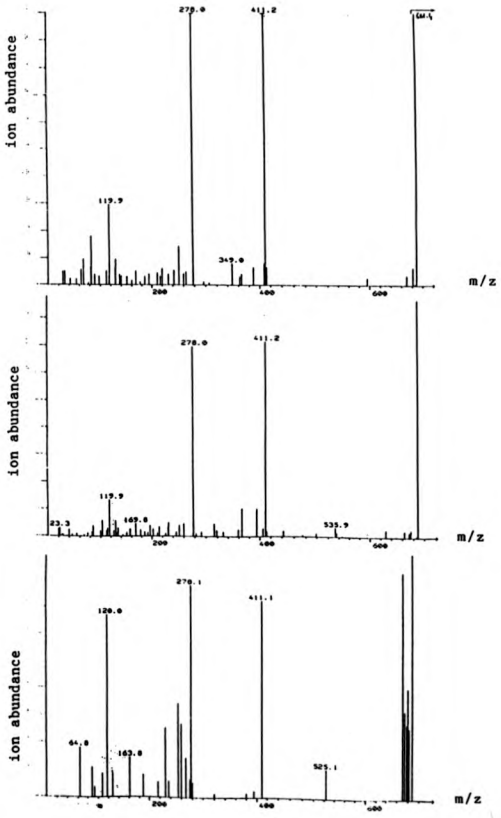
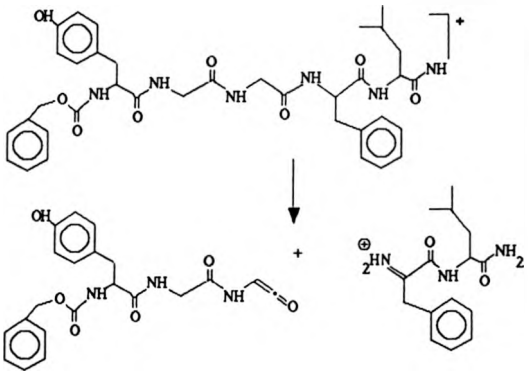


Figure 5 : CID mass spectrum corresponding to the ion at m/z 688.



A problem arises with the interpretation of the ion at m/z 411.2. In the native pentapeptide, the ion at m/z 413 corresponds to a fragment type b_3. Two mass units separate the two types of ions. One of the possible structures for the ion at m/z 411 is depicted in Scheme 12.

Scheme 12.



If this explanation is correct, it means that higher peptides than [Leu]-enkephalinamide are formed during the papain-catalyzed reaction. It was impossible to complete the structure

corresponding to the ion at m/z 688. An obvious possibility was the heptapeptide Z-Tyr-Gly-Gly-Phe-Leu-Phe-$LeuNH_2$. The molecular ion (MH^+, m/z 933.089) could not be formally identified. An ion at m/z 798 which was previously assigned to the fragmentation c_6 in the peptide H-$(Phe-Leu)_3$-NH_2 could correspond as well to the loss of the protecting group from the molecular ion of Z-Tyr-Gly-Gly-Phe-Leu-Phe-$LeuNH_2$. The formation of the heptapeptide via papain catalysis is not unlikely but due to the low abundance of the ion at m/z 688, further experimentation proved impossible.

In conclusion, the peptides identified by tandem mass spectrometry are depicted in table 4.

Table 4 : structures of the peptides formed during the papain-catalyzed reaction of Z-Tyr-Gly-GlyOEt and H-Phe-$LeuNH_2$.

Z-Tyr-Gly-Gly-Phe-$LeuNH_2$	MH^+ = 689.3
Z-Tyr-Gly-Phe-$LeuNH_2$	MH^+ = 632
and possibly	
H-Phe-Leu-Phe-$LeuNH_2$	MH^+ = 536
H-Phe-Leu-Phe-Leu-Phe-$LeuNH_2$	MH^+ = 795
Z-Tyr-Gly-Gly-Phe-Leu-Phe-$LeuNH_2$	

No cyclic peptides of the type $\overline{HN-[Phe-Leu]_n-CO}$ (where n=1,2 or 3) could be detected. Another likely papain-catalyzed reaction could have been the cleavage of the protecting group from a nucleophilic attack of the sulfhydryl group on the acyl group of the benzyloxycarbonyl moiety. If the ion at m/z 555 was detected, it was impossible to distinguish between a type Y cleavage from

the molecular ion of the desired pentapeptide and a papain-catalyzed cleavage of the Z-group. One can even go further and envisage the aminolysis of the acyl-enzyme intermediate Ar-O-CO-S-papain by the nucleophile H-Phe-LeuNH_2. This would give rise to the synthesis of the protected dipeptide Z-Phe-LeuNH_2 (M= 411.499). An ion at m/z 411 is detected in the full mass spectrum but it is indistinguishable from type B fragments arising from the molecular ion of the native pentapeptide. Such transamidation reactions have been reported [155]. In the coupling of Z-PheOH with H-Phe-NH-NHPh, the principal product was not Z-Phe-Phe-NH-NHPh, but Z-Phe-NH-NHPh formed by transamidation.

Concerning transpeptidation reactions, Kullmann [141] found that the condensation of BOC-Tyr(Bzl)-Gly-GlyOH with H-Phe-LeuOTMB gave the tetrapeptide BOC-Tyr(Bzl)-Gly-Phe-LeuOTMB; he attributed this to the hydrolytic cleavage of the Gly-Gly bond, but a papain-catalyzed transamidation reaction was also a strong possibility. In view of Kullmann's observation, the report by Wong [156] that he effected the papain-catalyzed condensation of BOC-Tyr(Bzl)-Gly-GlyOH with H-Phe-LeuOTMB to give the desired pentapeptide in 66% yield is surprising, especially since their experimental conditions appeared to be very similar to those used by Kullmann. Our results using immobilized papain in organic solvents indicate clearly that the transpeptidation reaction occurs alongside the synthesis of the desired pentapeptide.

Unfortunately, our strategy makes any quantitative assessment impossible.

In the original paper by Kullmann [33], it was not established if the BOC-Tyr(Bzl)-Gly-papain complex was directly aminolyzed, i.e., whether a direct transpeptidation reaction took place, or alternatively if the acyl-papain complex was hydrolyzed prior to the coupling of the newly emerged product BOC-Tyr(Bzl)-GlyOH to H-Phe-Leu-NH-NHPh. Under our conditions using low water conditions, the hydrolysis of the Z-Tyr-Gly-papain complex is unlikely. We have not tried to detect in the reaction mixture the presence of Z-Tyr-Gly-GlyOH and Z-Tyr-GlyOH. Their presence was not detected by tandem mass spectrometry, but due to extensive washings with a bicarbonate solution, one can assume that the free carboxylic acids would have disposed of. More positive evidence is provided by the fact that if Z-Tyr-Gly-GlyOEt is incubated alone with papain under our previously described conditions, no hydrolysis could be observed. This method was investigated in order to improve the synthesis of Z-Tyr-Gly-GlyOH for the chemical synthesis of [Leu]-enkephalinamide. Therefore, one can postulate that the formation of the transpeptidation product, Z-Tyr-Gly-Phe-$LeuNH_2$, arises from a direct aminolysis of the acyl-enzyme complex by the nucleophile H-Phe-$LeuNH_2$.

In conclusion, immobilized papain in XAD-8 was able to catalyze the synthesis of [Leu]-enkephalinamide. Although no quantitative assessment was possible, the nature of the by-products was

elucidated using tandem mass spectrometry. Tandem mass spectrometry proved to be a method of choice applied to enzymatic peptide synthesis, allowing the peptide chemist to get a full picture of a particular reaction.

The identification of the by-products is of primary importance because it helps to understand the behaviour of an enzyme under particular conditions. This is particularly valuable in the case of non-aqueous enzymology in which the enzyme might exhibit novel properties. The behaviour of papain has not been modified by the transition from an aqueous to an organic environment. When using papain in an aqueous medium, Kullmann reported the exclusive formation of the transpeptidation product [141]. Our results, using immobilized papain in organic solvents, indicate clearly that the native pentapeptide is formed alongside the transpeptidation product. This cannot be classified as a major modification in the behaviour of the enzyme in the light of the results of Wong [156]. Determination of the ratio of the different products would help us to establish if any modification in the behaviour of the enzyme has taken place. Owing to the complex character of the mixture, no obvious method can be proposed. Preparative HPLC could help by providing well defined fractions of the reaction mixture, but it seems unlikely that a direct quantification would be possible.

An alternative solution would be to change the conditions under which the coupling takes place. The nature of the solvent, the water content, the nature of the protecting group, in particular

the nature of the activated ester, and finally the enzyme itself, could have profound effects on the outcome of the reaction. The amount of the transpeptidation product could certainly be reduced by changing the ester moiety to a more activated form such as the chloroethyl or cyanoethyl ester. This would greatly enhance the rate of formation of the acyl-enzyme intermediate, therefore limiting the extent of transpeptidation.

Changing the enzyme itself is probably the brightest prospect. Enzymes lacking peptidase activity but possessing high esterase activity would limit side-reactions. Lipases, in contrast to proteolytic enzymes have a broad specificity and do not catalyze secondary hydrolysis of peptides [157]. Recently, it was found that lipases can act as catalysts in anhydrous solvents and under these conditions, catalyze processes impossible in water, e.g., the reaction between carboxylic esters and aliphatic amines [158]. Peptide synthesis catalyzed by porcine pancreatic lipase (PPL) in organic solvents was also investigated [80]. By using PPL as a suspension in organic solvents, various amino acid residues could be incorporated into either side of the dipeptides.

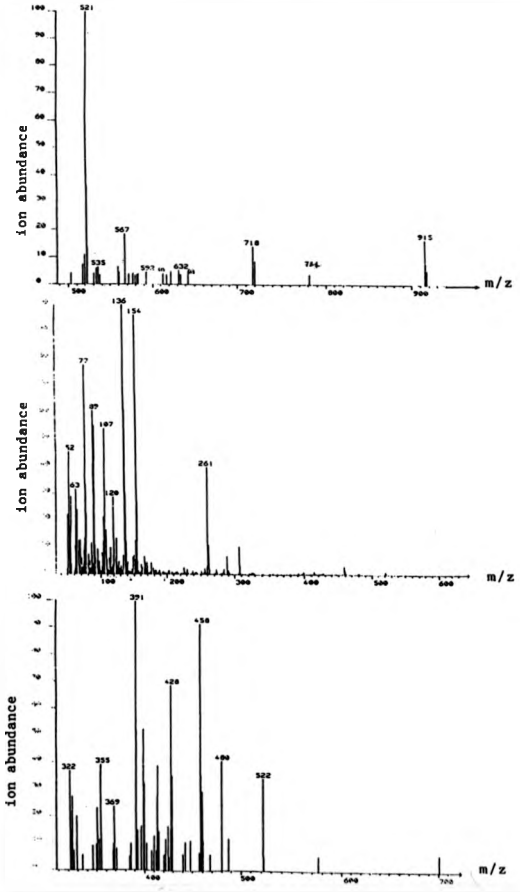
We carried out preliminary experiments to investigate the potential use of PPL in the synthesis of [Leu]-enkephalinamide. In a typical experiment, crude PPL (EC 3.1.1.3, type II from porcine pancreas) was added to a solution of the starting components Z-Tyr-Gly-GlyOEt and H-Phe-LeuNH$_2$ (2 equivalents) in dry acetonitrile. The reaction mixture was then stirred at 40°C for 8 days. The enzyme was removed by filtration and the solvent

evaporated. Two fractions were collected by preparative thin-layer chromatography and analyzed by FAB mass spectrometry and high field NMR (figure 6).

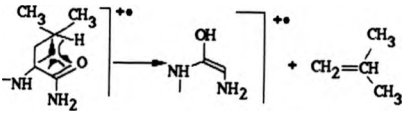
The ^1H NMR spectra (400 MHz) show the presence of the starting materials in both fractions and leucine-containing peptides could be detected.

More positive evidence for the presence of the desired pentapeptide could be obtained from FAB mass spectrometry. The presence of the desired [Leu]-enkephalinamide could be positively identified in one of the fractions. If the molecular ion could not be detected, the ion $[(M+H) + \text{glycerol}]^+$ at m/z 781, and several characteristic fragments such as y_4 (m/z 392), ions at m/z 322, 355, and 522 were identified. From the information obtained from tandem mass spectrometry, those ions are specific to [Leu]-enkephalinamide and cannot be confused with the ions arising from fragmentations of the starting materials. An ion at m/z 633 could also be detected and, as the previous results of papain catalysis have indicated, could correspond to the transpeptidation product. This is extremely unlikely due to the inherent inability of lipases to cleave peptide bonds. However, crude PPL preparations display many activities, including protease activities, and, therefore, such possibility cannot be ruled out. The ion at m/z 633 could correspond to the desired pentapeptide according to the mechanism depicted in Scheme 13.

Figure 6 : FAB mass spectrum of PPL-catalyzed synthesis of [Leu]-enkephalinamide.



Scheme 13 : proposed mechanism for the formation of the ion at m/z 633 from [Leu]-enkephalinamide.



m/z 633.2372 m/z 56.108

This particular fragmentation was not observed in the tandem mass spectrometric analysis of papain-catalyzed coupling because of the different experimental conditions.

FAB analysis of the other fraction isolated by thin-layer chromatography does not reveal the presence of the desired pentapeptide. It consisted of a mixture of the two starting materials.

These preliminary results indicate that PPL could be a valuable catalyst for the final coupling step in the synthesis of [Leu]-enkephalinamide. The conversion appears to be low and this could be related to the anhydrous conditions. The selectivity of the reaction seems to be high, because no peptides of general sequence $H-(Phe-Leu)_n-NH_2$ could be detected. Investigating the role of the water content and the use of surfactants might well

provide valuable information for the optimisation of this coupling step. This study could be greatly facilitated by the coupling with tandem mass spectrometry.

The preparative synthesis, via protease catalysis, of naturally occuring peptides such as [Leu]-enkephalinamide provides convincing proof of the capabilities of the enzymatic approach to peptide synthetic chemistry. Owing to the inherent protease stereospecificity, the enzymatic procedure provides a way of avoiding the risk of racemization which is frequently encountered during chemical fragment condensation. Furthermore, the regiospecific action of proteases facilitates the preparation of complex peptides, whose syntheses by chemical means is often impeded by sequence-dependent side-reactions.

Besides these promising features, the synthesis of a bioactive peptide provides a good ground for the investigation of new techniques such as non-aqueous enzymology. Due to the model character of the study, a particular coupling step could be investigated in great detail and the potential of new methods of analysis, such as tandem mass spectrometry of peptide mixtures, could be evaluated.

The preparation of a biologically active peptide therefore provides a good opportunity to demonstrate the capabilities of enzyme-mediated peptide synthesis. As far as we were concerned, the synthesis of [Leu]-enkephalinamide was a good model for the exploration of the synthetic potential of enzymes in organic

solvents. If the final coupling step of [Leu]-enkephalinamide remains to be investigated and optimized, we have devised a very efficient synthesis of the precursor fragment by combining aqueous and non-aqueous enzymology.

CHAPTER V . DESIGN OF COMPETITIVE INHIBITORS OF PROTEASES

V-1. Introduction.

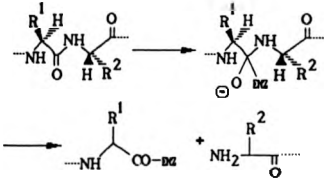
One can distinguish two broad classes of enzyme inhibitors, reversible and irreversible. Reversible inhibitors have been studied almost from the time of the 'lock and key' hypothesis of E. Fischer. They are usually substrate analogues which bind to the enzyme active site and compete with the normal substrate. They can either be refractory to the enzyme catalytic act or undergo it. The affinity of this type of inhibitor is usually similar to that of the substrate. As in chemical reactions, a reaction catalyzed by an enzyme proceeds via a high-energy transition state. The energy is provided by minimizing enzyme-substrate interactions. When considering the design of potential inhibitors, maximum binding to the enzyme is therefore likely to be achieved by arranging for the substrate to mimic as closely as possible the structure of the transition state [159]. If the guess of the structure of the transition state has been correct and if a stable molecule approaching the geometry of this transition state can be synthesized, one can gain several orders of magnitude in binding constant. When this binding constant reaches

10^{-9}-10^{-10}M or even lower, the notion of reversibility becomes almost irrelevant and the frontier with irreversible inhibitors very fragile.

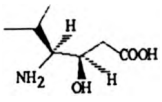
Nature and chemists have devised various ways of stabilizing peptide bonds against the hydrolytic action of proteases. For instance, peptides in which one or several of the normal L-amino acids have been replaced by their D-counterparts or by an α-alkyl amino acid may retain their pharmaceutical activity in vitro and be more active than the natural peptide in vivo owing to resistance to proteolytic breakdown [160].

The cleavage of a peptide bond is believed to occur by formation of a tetrahedral intermediate as depicted in Scheme 1.

Scheme 1 : cleavage of a peptide bond by hydrolytic enzymes.



Pepstatin, a pentapeptide first isolated by H. Umezava [161], contains the unusual Y-amino acid statine [(3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid] (Scheme 2). Statine and its analogues are central building blocks for many highly active, specific aspartyl protease inhibitors, including even renin inhibitors [162].



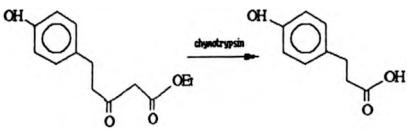
Scheme 2 : structure of statine.

It is believed that the extremely strong binding of pepstatin to pepsin ($K_I=10^{-11}M$) is due to the fact that statin resembles the tetrahedral transition state. Kinetic studies have shown that the carbon atom bearing the hydroxyl group in statines must be have the S-configuration, but the carbon chain at C-4 can be chemically modified [163].

Our interest in 3-keto- and 3-hydroxy-esters derived from amino acids was stimulated by a report by D.G. Doherty [164] on the hydrolysis of carbon-carbon bonds by α-chymotrypsin.

The enzymatic hydrolysis of ethyl 5-(p-hydroxyphenyl)-3-ketovalerate by α-chymotrypsin to yield p-hydroxyphenyl propionic acid was claimed as shown in Scheme 3.

Scheme 3 : cleavage of carbon-carbon bonds by α-chymotrypsin.



The point made [164] was that, if the carbon-methylene bond could be sufficiently polarized by the addition of electrophilic groups, its activation energy might be lowered to the extent that the enzyme could bring about the scission of the carbonyl to carbon linkage. Polarization of this bond was effected by the addition of a carboethoxy group to form a 3-keto ester. Such compounds would have the necessary requisites for binding on the active site of chymotrypsin together with a labilized carbonyl-carbon bond.

When the substrate 3-keto ester was submitted to the action of α-chymotrypsin, a very fast hydrolysis was observed and it was

claimed that the compound p-hydroxyphenyl propionic acid could be isolated. The same conditions were applied to a similar 3-keto ester, ethyl 5-phenyl-3-ketovalerate, and the hydrolysis of the carbon-carbon bond was also observed.

This report immediately triggered our interest for several reasons. Firstly, the 3-keto ester system is an ideal system to provide data on the upper limit of bond strengths susceptible to enzyme action. Second, results from molecular modelling studies on chymotryptic substrates would enable us to design model compounds with the perfect requisites for binding at the active site of the enzyme. Third, the development of non-aqueous enzymology, and in particular the hydrolytic system in organic solvents described earlier in this thesis (Chapter I), could help to solve the solubility problems reported in the aforementioned paper. Finally, and more importantly, the reverse reaction, i.e. enzymatically controlled carbon-carbon bond synthesis, was an intriguing possibility.

In parallel with this mainstream strategy, it was proposed to develop a synthetic approach to potential protease inhibitors, namely 3-hydroxy esters derived from amino acids (Scheme 4).

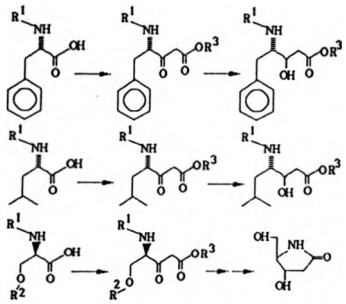
3-Hydroxy esters derived from L-phenylalanine and L-leucine are potential protease competitive inhibitors. The amino sugar derived from D-serine is an interesting target. It is an analogue of 2-deoxyribose and might exhibit antiviral properties.

The strategy outlined in Scheme 4 requires a diastereoselective reduction of the 3-keto esters yielding the 3-hydroxy esters.

Chemical methods such as metal hydride reduction and biotransformations (e.g. yeast reductions) were to be used to provide stereoselective reduction of the keto group.

Scheme 4 : synthetic route to selected 3-hydroxy esters.

(R^1=Ac,Z,BOC; R^2=Bzl; R^3=Et,^{t}Bu)

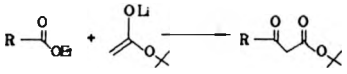


3-Keto esters are central building blocks for many natural products. Claisen [165] first reported the ester condensation procedure, and subsequently, numerous other methods have been described [166]. However, mixtures of products are sometimes obtained and the reaction is often not useful synthetically. If

only one of the esters has an α-hydrogen atom, the mixed reaction is frequently satisfactory. Several synthetic methods have been introduced to overcome some of the drawbacks encountered in cross-Claisen reactions.

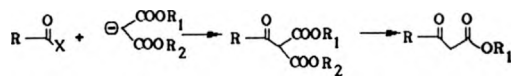
Rathke [167] described a synthesis of 3-keto esters by direct treatment of the ethyl esters of the building blocks with the stable lithium enolate of tert-butyl acetate which afforded the desired 3-ketoesters acording to Scheme 5.

Scheme 5 : Synthesis of 3-keto esters using lithio tert-butyl acetate.



This method presents several advantages which makes it very attractive for the synthesis of 3-keto esters. Firstly, the lithium enolate of tert-butyl acetate is a stable solid which can be stored. Handling is therefore very convenient, and the reaction occurs simply by mixing the two components in the appropriate solvent. Second, the acid-labile tert-butyl carboxyl protecting group is easily cleaved under mild conditions for further reactions at the carboxyl end.

The problem of product mixtures, as usually obtained in cross-Claisen condensations, can be overcome by the malonic ester method (Scheme 6).



X : Cl, O(CO)OEt, $PO(C_6H_5)_2$, imidazole
R^1: alkyl
R^2: alkyl, benzyl, silyl, H

Scheme 6 : malonic acid synthesis.

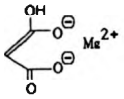
Magnesium, as introduced by Lundt, is usually the most suitable counterion [168]. As the acylating agent any form of activated carboxylic acid derivative can be used. Examples include acyl chlorides, mixed anhydrides, phosphine oxides, imidazolides etc. However, this route suffers from the requirement for selective ester hydrolysis of one of the two malonic ester groups. If both of them are hydrolyzed, double decarboxylation leads to the formation of a methyl ketone as a side product [169].

The use of mixed malonates (R^2: alkyl or benzyl) can partially overcome this problem, especially when the two ester groups react to selective cleavage conditions, such as hydrogenolysis. The use

of ethyl trimethylsilyl malonates offers an attractive modern alternative and gives 3-keto esters in good yields [170].

Since all mixed malonates are synthesized from the corresponding ethyl hydrogen malonate [171] (R^1: Et; R^2: H), this compound itself has been extensively investigated. It has been shown that it can be used indeed as its magnesium chelate in tetrahydrofuran, together with mild acylation species such as imidazolides [172]. The great advantage of this procedure is the instant decarboxylation upon acidic workup, which permits a one-pot synthesis of 3-keto esters.

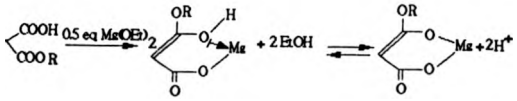
The magnesium enolate of the malonate acid monoester can be generated by two different methods. In the first method [173,174] the enolate is generated by reaction of the Grignard reagent, isopropyl magnesium bromide with the monoalkyl hydrogen malonate. If one equivalent of magnesium is used, this leads to the formation of a basic compound (Scheme 7).



Scheme 7 : Basic magnesium malonate.

In the second strategy [175], the half malonic esters are treated with magnesium ethoxide (Scheme 8).

Scheme 8 : preparation of the magnesium enolate.

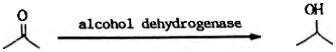


These reagents have a distinct advantage over previously reported magnesium salts and should allow virtually neutral reaction conditions.

The second problem which needed to be solved concerned the stereoselective reduction of the 3-keto esters. In our strategy, the use of a chiral amino acid precursor could provide for a asymmetric induction in the diastereoselective reduction of the 3-keto ester. Because of our interest in biotransformations, a diastereoselective reduction of the 3-keto ester with baker's yeast was envisaged.

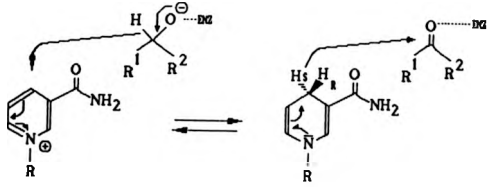
Saccharomyces cerevisiae (baker's yeast) is a microorganism displaying oxidoreductase activity which can be used in biotransformations. The enzyme responsible for the bioreduction with yeasts is an alcohol dehydrogenase which requires a cofactor, NAD(P)H (Scheme 9).

Scheme 9 : reaction catalyzed by alcohol dehydrogenases in yeast.



The stereoselectivity of the reaction catalyzed by alcohol dehydrogenase comes from a direct hydride transfer from the nicotinamide ring of the cofactor as shown in Scheme 10 for the case of the alcohol dehydrogenase from horse liver HLADH.

Scheme 10 : reaction mechanism of HLADH.



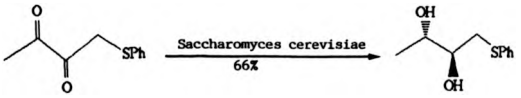
It was discovered that the hydride transfer between the substrate and NAD^+ is stereospecific, and that HLADH uses the pro-R hydrogen of NADH as indicated in Scheme 10 and transfers the hydride ion to the re-face of NAD^+.

In 1976, Ridley [176] established that the 3-keto esters and 3-keto acids were particularly good substrates for reduction with S. cerevisiae. In 1980, Fischli [177] reviewed the state of the art, but since 1980 interest in biotransformations with baker's yeast and other yeasts has grown tremendiously.

One of the best investigated biotransformations with S. cerevisiae is the reduction of 3-keto esters to the corresponding 3-hydroxy esters [178].

One new application of yeast reductions is the enantio- and diastereoselective reduction which makes it possible to obtain one of the stereoisomers in one step. One example of this strategy is shown in Scheme 11.

Scheme 11 : Diastereoselective yeast reduction [179].

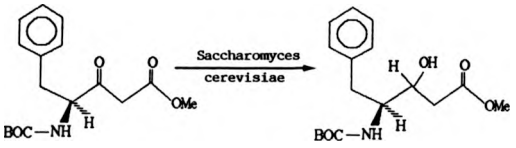


Many examples of diastereoselective yeast reductions have been reported [180,181,182] to the extent that it was possible to draw up a model for the diastereoselective reduction with S. cerevisiae [183].

In our strategy outlined in Scheme 4, the configuration at C-4 is fixed and no kinetic resolution by an enantioselective reduction as in Scheme 11 would be needed.

In a synthesis of (3S,4S)-4-amino-3-hydroxypentanoic acids, Raddatz [184] used a reduction with yeast cells to provide the desired diastereoisomer (Scheme 12).

Scheme 12.



The attempt with commercially available yeast (S. cerevisiae) resulted in incomplete reaction (30-50%) but the desired (3S,4S)-diastereoisomer was formed in 60% de. By screening a large number

of yeast strains, it was possible to select five of 74 yeast strains which reduced completely the 3-keto ester. Four strains formed the (3S,4S)-diastereoisomer with de=92% whereas one formed predominantly the (3R,4S)-diastereoisomer (90% de).

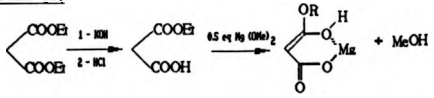
Alongside the biotransformation approach, chemical reductions with metal hydrides were to be attempted. The presence of a chiral centre at C-4 led us to expect a diastereoselective reduction of the 3-keto ester even with simple metal hydrides such as sodium borohydride.

V-2. Hydrolysis of carbon-carbon bonds by chymotrypsin.

The three 3-keto ester substrates were synthesized using the acylation version of the malonic acid synthesis mentioned in the introduction.

The malonate enolate of the malonic acid monoethyl ester was synthesized from the corresponding malonic ester by partial hydrolysis, and the half malonic ester was treated with magnesium methoxide (Scheme 13).

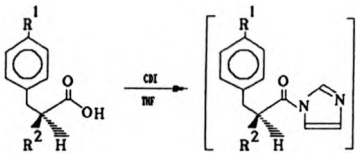
Scheme 13.



89 90

Evaporation and drying under reduced pressure overnight afforded the magnesium salt.

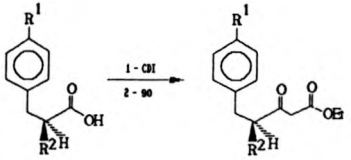
The starting acids were then activated with carbonyldiimidazole, CDI [185], according to Scheme 14.



R^1-R^2-H	92
R^1-OH, R^2-H	54
R^1-H, R^2-NH-Ac	36

Scheme 14 : activation with CDI.

The chain extension proceeded smoothly in anhydrous THF at room temperature. The acid was first treated with one equivalent of CDI and the resulting imidazolide reacted *in situ* with the magnesium salt. Acidic workup resulted in decarboxylation and the products could be purified either by flash chromatography or recrystallization (Scheme 15).



R^1=R^2=H	92	93	93%
R^1=OH, R^2=H	54	94	56%
R^1=H, R^2=NH-Ac	36	95	88%

Scheme 15 : synthesis of 3-keto esters.

The presence of the phenolic group of 3-(p-hydroxyphenyl)-propionic acid showed a detrimental effect on the outcome of the reaction as indicated by the rather disappointing yield. However, the presence of a labile hydrogen on the nitrogen atom of the phenylalanine derivative did not influence the reaction presumably owing to its relative low acidity.

The potential cleavage of the carbon-carbon bond in the keto ester **94** by chymotrypsin was then investigated according to the procedure described in the aforementioned paper [164].

The extent of hydrolysis was followed potentiometrically at 25°C in 0.02M phosphate buffer pH 8. The activity of chymotrypsin was checked using N-acetyl-L-tyrosine ethyl ester as a substrate. Since the substrate possessed limited solubility in water it was necessary to carry out all enzymatic determinations in 30% ethanol.

No measurable hydrolysis was observed in 3 hours. This negative result strongly contradicts the observations made by D.G. Doherty. The author, under the same experimental conditions, reported that the addition of α-chymotrypsin to the weakly buffered solution of the ethyl (p-hydroxyphenyl)-3-keto valerate brought about an immediate progressive decrease in pH. The substrate was rapidly hydrolyzed (15-30% in 10-15 minutes). We were not able to make such observations.

We then decided to vary the experimental conditions. Experiments were carried out in mixtures consisting of ethanol (30%) and 0.1M phosphate buffer pH 7.3-8.1. In all cases, no hydrolysis was observed over periods of time up to 24 hours. NMR analysis (^1H, 220 MHz) confirmed the chemical integrity of the starting materials. Enzymes other than chymotrypsin, such as papain and subtilisin, were tested with similar negative outcomes.

In order to overcome the solubility problems, it was decided to study the potential hydrolysis of the 3-keto ester substrates in

organic solvents according to the general strategy outlined in Chapter I.

Millimolar solutions of the substrates in dichloromethane containing ammonium bicarbonate (5 equivalents) and water (2%, v/v) were submitted to the action of α-chymotrypsin as a suspension. In all cases, the reaction mixture was worked up and the composition of the mixture analyzed by ^{1}H NMR (220 MHz). No hydrolyzed products were detected for reaction times up to 48 hours.

It is not possible to provide a definite answer about those negative results.

One possibility is that the substrates are powerful competitive inhibitors of α-chymotrypsin. It was not possible to make kinetic measurements on the 3-keto ester substrates themselves for solubility reasons. Determination of the inhibition constant Ki was possible for the 3-hydroxy ester, ethyl (4S)-acetamido-3-hydroxy-5-phenyl pentanoate, synthesized by a diastereoselective reduction of the 3-keto ester **106**. The diastereoisomeric mixture was later (see below) found to consist of the (3R,4S) and (3S,4S) in a ratio 70:30. Spectrophotometric measurements afforded a value for Ki of about 1 mM.

If direct comparison between the 3-hydroxy- and 3-keto ester, in terms of competitive inhibition, is difficult, one can nevertheless postulate that the 3-hydroxy ester which mimics the

transition state of the enzymatic reaction, should be a better competitive inhibitor than the 3-keto ester. An inhibition constant Ki value of 1 mM is not low enough to explain the complete absence of reactivity of the substrates with chymotrypsin.

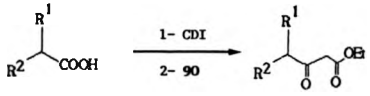
A better explanation might be given in terms of the thermodynamics of the postulated reaction. The approximate pKa value (relative to water) of the ester, $ROOC-CH_2-R'$, is 24.5 [186]. It seems probable that the cleavage of the carbon-carbon bond would be thermodynamically unfavourable.

In view of these negative results, it was decided to abandon this subject and concentrate on the chemistry of the 3-keto esters. Firstly, the synthesis of the 3-keto esters was to be investigated to provide a mild, high yielding procedure.

Second, the diastereoselective reduction of the 3-keto esters to yield 3-hydroxy esters as potential protease inhibitors, would be studied.

V-3. Synthesis of 3-keto esters derived from amino acids.

The malonic acid synthesis strategy was applied to several amino acid derivatives as indicated in Scheme 16.



R^1=Bzl, R^2=NH-Z	(S) 96	97	92%
R^1=iBu, R^2=NH-BOC	(S) 98	99	5%
R^1=iBu, R^2=NH-Z	(S) 100	101	12%
R^1=CH_2OBzl, R^2=NH-Z	(R) 91	102	84%

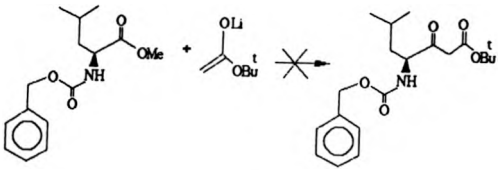
Scheme 16 : synthesis of 3-keto esters.

Although the reaction proceeds very efficiently with the phenylalanine and serine derivatives, a peculiar result is observed with the compounds deriving from L-leucine. In the latter case, the formation of the transient imidazolide occurs as shown by the evolution of carbon dioxide. However, the acylation step does not seem to occur efficiently. This might be due to the conformation of the leucine derivatives in solution. Steric

hindrance might prevent the formation of the intermediate as reflected by the low yields observed. In both cases, alongside the small quantities of the desired 3-keto esters, only the starting acid derivative and monoethyl hydrogen malonate could be isolated. This case of substrate dependence is not isolated and has been reported in similar syntheses of natural product precursors [187].

In order to overcome this problem, N-benzyloxycarbonyl-L-leucine methyl ester was reacted with the lithium enolate of tert-butyl acetate according to Scheme 17.

Scheme 17.



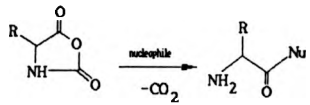
103

Treatment of the methyl ester **103** with two equivalents of the lithium enolate of tert-butyl acetate in THF did not afford the 3-keto ester. This negative result might be a complementary argument to explain the absence of reactivity of the leucine derivatives in the malonate synthesis.

Enhancement of the reactivity of the donor ester could be achieved by reaction of the acyl chloride or imidazolide of the leucine derivative.

In order to find a more convenient synthetic route to 3-keto esters derived from amino acids, the behaviour of selected 2,5-oxazolidinediones towards nucleophiles was investigated (Scheme 18).

Scheme 18.



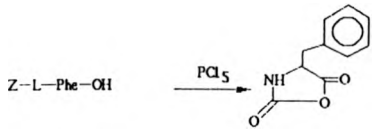
These N-carboxy or Leuch's anhydrides of amino acids are N-protected and C-activated derivates. Leuch's anhydrides have been extensively studied by peptide chemists [188].

N-carboxy- α -amino acid anhydrides undergo rapid decomposition in the presence of a large excess of water with the evolution of carbon dioxide and the generation of the parent amino acid. If only a little water is employed, however, a polymeric material is formed instead of the free amino acid.

Polypeptides of considerable size may result from such spontaneous build-up, and this method has been extensively utilized for the production of a wide variety of amino acid polymers.

The N-carboxy anhydride of L-phenylalanine was synthesized by reaction of benzyloxycarbonyl-L-phenylalanine with phosphorus pentachloride in dry dichloromethane (Scheme 19).

Scheme 19.



96 104 95%

The Leuch's anhydride **104** gave satisfactory elemental analysis and was fully characterized by its melting point and NMR spectrum. As traces of contaminants such as moisture may cause the carboxyanhydride to undergo polymerization, only reactants and solvents which are carefully dried and extensively purified may be used.

The reactivity of the N-carboxy anhydride of L-phenylalanine with various acylating agents was investigated and the results summarized in Table 1.

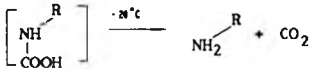
Table 1 : reactivity of the Leuch's anhydride **104** with various acylating agents.

Nucleophile	conditions	outcome
di-(hydrogen monoethyl malonate) magnesium	THF/20°C	polymerization
lithium enolate of tert-butyl acetate	THF/20°C	polymerization
	THF/-40°C	5% 3-ketoester

As indicated in Table 1, no reaction but polymerization was observed when di-(hydrogen monoethyl malonate) magnesium was used as a nucleophile. A more promising result was obtained with the reaction of the lithium enolate of tert-butyl acetate. If

polymers constituted the bulk of the resulting mixture, the ^1H NMR (220 MHz) clearly showed the distinctive quartet (AB system) at 2.5 ppm for the protons at C-2.

The control of the temperature seems to be critical because of the formation of a transient carbamic acid which decarboxylates upon warming-up over -30°C, thus initiating the polymerization (Scheme 20).

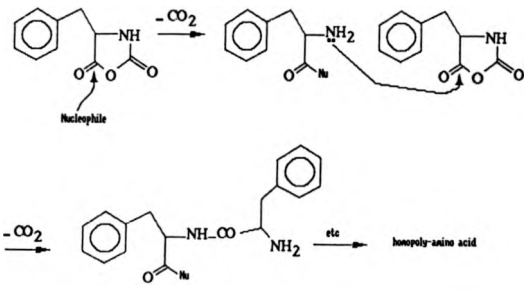


Scheme 20.

Another mechanism can be proposed to explain the extent of polymerization even at low temperatures (Scheme 21).

From Scheme 21, it seems clear that, if one wants the acylation reaction to overcome the polymerization, firstly, working at low temperatures is vital, and second, a strong nucleophile should be used. The lithium enolate of tert-butyl acetate might not be nucleophilic enough. Thus, the rate of polymerization will still prevail over the acylation reaction.

Scheme 21 : Polymerization initiated by the nucleophile.



Nevertheless, this strategy using N-carboxy anhydrides of amino acids is very promising and might lead to the shortest synthetic route to 3-keto esters derived from amino acids. Its main advantages are that it does not require the protection of the amino group, it provides activation of the carboxyl group, and finally, the Leuch's anhydride can be synthesized directly from the free amino acid by reaction with phosgene.

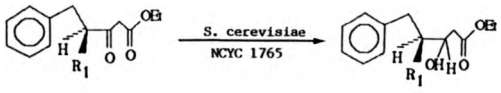
V-4. Diastereoselective reductions of 3-keto esters.

A satisfactory synthetic route to the 3-keto esters derived from amino acids having been found through the malonic acid synthesis, we concentrated our efforts on the diastereoselective reduction to 3-hydroxy esters.

A baker's yeast strain was bought from the local Sainsbury superstore and deposited as NCYC strain 1765 [175].

Bioreduction of the 3-keto ester substrates was carried out with the resting cells of S. cerevisiae NCYC 1765 (Scheme 22).

Scheme 22.

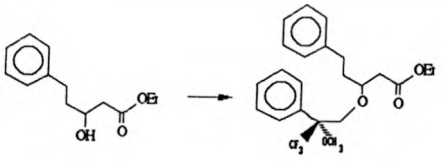


R^1=H	**93**	**105**	48%
R^1=NH-Ac	**95**	**106**	9%

In the reduction of the 3-keto ester **93**, the reaction was stopped after 4 hours and the products were isolated by filtration, extraction, and flash chromatography on silica gel. A small

amount of the starting material was recovered (92% conversion) and the desired 3-hydroxy ester **105** was obtained in 48% yield. The enantiomeric purity was determined by making the Mosher-ester derivative **107**. It was prepared from the corresponding (S)-(-)-2-methoxy-2-trifluoro-methyl-2-phenylacetic acid chloride, as described by Mosher [189] (Scheme 23).

Scheme 23.



105 **107**

The Mosher-ester derivative was analyzed by ^1H NMR (400 MHz) in benzene-d^6. With TMS as reference, the signal corresponding to the methoxy group in one enantiomer appeared at 3.47 ppm and that due to the other enantiomer at 3.55 ppm. Integration of the two respective singlets indicated an enantiomeric excess of 46%.

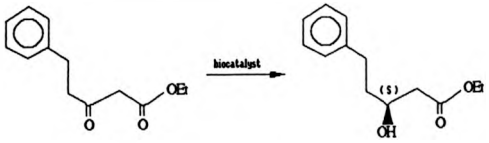
In the reduction of the 3-keto ester **95** derived from L-phenylalanine, only a low conversion was observed after 24 hours. The reaction was stopped and the products were purified by

preparative thin-layer chromatography. The starting material was recovered (36% conversion) and the desired 3-hydroxy ester **106** was obtained in a low yield (9%).

The diastereoisomeric purity of the 3-hydroxy ester **106** was determined using ^{1}H NMR (400 MHz) based on the signals corresponding to the acetate protons at 2.0 ppm. After integration, the diastereoisomeric excess (de) was found to be 40%.

It was not possible to assign directly the absolute configuration at C-3 for the unsubstituted 3-hydroxy ester **105**. However, in previously described bioreductions of similar 3-keto esters with *S. cerevisisae* NCYC 1765 [190], the S-enantiomer of the 3-hydroxy ester was obtained predominantly. By analogy, the S-configuration at C-3 for the 3-hydroxy ester can be tentatively assigned (Scheme 24).

Scheme 24.



93 **105** 46% ee

To explain the low conversion and yield obtained in the yeast reduction of the substituted 3-keto ester **95**, it can be suggested by analogy with Van Middlesworth [183], that there is one enzyme responsible for the reduction, with different affinities for the enantiomers of the starting materials. In our case, the starting 3-keto ester **95** could well be the least preferred enantiomer for the enzyme, explaining the low conversion and yield. On the other hand, this heavily substituted compound might have a low affinity for the enzyme for steric reasons. Finally, the possible involvement of the substrate in the general metabolism of the microorganism cannot be ruled out and this might explain the low selectivity (chemical yield/conversion).

Because the yeast reduction did not appear to provide an efficient synthetic route to 3-hydroxy esters, it was decided to investigate chemical reductions with metal hydrides (Scheme 25).

Scheme 25 : chemical reduction of 3-keto esters.

95 **106**

The presence of a defined chiral centre at C-4 which could serve as chiral inductor, and the acetamido moiety which could lead to complexation with a metal atom, led us to experiment with a variety of conditions for the reduction with sodium borohydride. The use of the sterically bulky hydride, lithium aluminium tri-tert-butoxy hydride [191] was also studied. The results are summarized in Table 2.

Table 2 : Diastereoselective reduction of the 3-hydroxy ester 95 by various chemical methods.

Method	reaction time (hrs)	%yield	%de
S. Cerevisiae	24	9	40
$NaBH_4$,THF,0°C	0.5	95	40
$NaBH_4$,THF/iPrOH=4/1,-20°C	4	95	14
$LiAlH(OBu)_3$,THF,-20°C	4	20	5

The product mixtures were analyzed by ^1H NMR (220 MHz). In all cases, the same major diastereoisomer was identified. As outlined in Table 2, reduction by sodium borohydride in THF afforded the same diastereoisomeric excess as that observed with the yeast reduction. The use of cosolvents resulted in a sharp fall in the diastereoisomeric excess. This indicates the presence of a coordination between the metal and the 3-keto ester substrate. The introduction of cosolvents possibly disrupts this

complexation and therefore reduces the observed diastereoisomeric excess.

Neither of the diastereoisomers is preferentially formed with the sterically bulky hydride, $LiAlH(OBu)_3$ (de=5%). Two explanations are possible. Firstly, complexation is disfavoured for steric reasons. Second, the approach of the hydride occurs from the least hindered face of the molecule. This suggests that with sodium borohydride, the nucleophilic attack occurs from the most hindered face of the molecule. Without knowing the conformation of the molecule in solution, it is impossible to identify these diastereotopic faces, but one can nevertheless suggest that complexation with the metal atom has to be heavily involved.

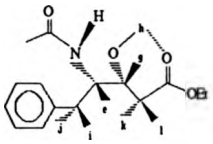
It proved possible to separate the two diastereoisomers by preparative thin-layer chromatography. Unfortunately, it was impossible to obtain crystals of suitable characteristics for X-ray analysis.

Therefore, a NMR analysis was attempted in order to bring information about the possible absolute configurations of the two diastereoisomers. It was postulated that, if a pure diastereoisomer could be analyzed by molecular modelling to provide a favoured conformation in solution (PC Model, from Serena Software, works ultimately on the Allinger force field but was extensively developed by Gayevski and Gilbert), it might be possible to obtain geometrical experimental parameters by high-field NMR and Nuclear Overhauser Enhancement (NOE). The NOE effect gives information about molecular geometry, particularly,

on proximities of protons in a given molecule. If the experimental results established by double resonance experiments were to confirm those obtained by molecular modelling, the absolute configuration at C-3 could be determined with reasonable certainty. The results of the molecular modelling and NOE experiments are summarized in Tables 3a and 3b.

Table 3a : compound **108** (major isomer)

Proposed favoured conformation in solution



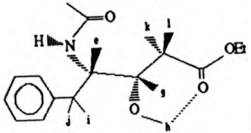
Calculated distances in A with observed strong, small, possible NOEs.

	NH	e	g	h	i	j	kl
e	2.98						
g	3.00	2.47					
h	3,24	4.04	2.19				
i	3.80	2.46	3.90	4.34			
j	2.70	3.09	3.80	3.53	1.79		
kl	4.39	2.59	2.53	3.04	2.34	2.83	
Ph	3.00	2.66	5.08	5.29	2.58	2.44	4.64

Note the strong NOEs between e and g, h and g, and i and e; but the small NOE for h and e, and h and NH. These data are in accordance with the proposed favoured conformation in solution.

Table 3b : compound **109** (minor isomer)

proposed favoured conformation in solution



Calculated distances in A with observed strong, small, possible NOEs.

	NH	e	g	h	ij	k	l
e	2.99						
g	3.70	2.57					
h	3.39	4.07	2.25				
ij	2.63	2.48	2.52	3.44			
k	3.10	3.09	3.11	2.96	4.58		
l	4.06	2.44	2.54	3.60	4.26	1.80	
Ph	2.90	2.71	4.44	5.36	2.46	5.79	4.97

Note the strong NOEs between e and g, and g and l; the NOE for h and k, but not for h and l; and the absence of NOE for g and k. These data are in accordance with the proposed favoured conformation in solution.

From the data shown in Tables 3a and 3b, the relative configuration for the major diastereoisomer **108** can be assigned as (3R,4S) or (3S,4R), and for the minor isomer **109** as (3S,4S) or (3R,4R). The absolute configurations (3R,4S) and (3S,4S) follow

from the known absolute configuration of the L-phenylalanine used in the synthesis of the substrates.

These results are not in agreement with previous reports of yeast reductions of similar 3-keto esters [184] (Scheme 12). The (3S,4S)-diastereoisomer was obtained by reduction with S. cerevisiae, but the method of assigning the absolute configuration was not described. Despite this apparent contradiction, previous studies of yeast reductions of 3-keto esters [190] showed that S. cerevisiae NCYC 1765 afforded predominantly the L-enantiomer, whilst other species studied reduced the starting materials to the corresponding D-enantiomers. This underlines the fact that the outcome of a microbial biotransformation may depend not only on the species, but also on the particular strain used. In that respect, our results do not contradict the aforementioned bioreduction in so far as no direct comparison is possible.

Nevertheless, results from reductions of 4-substituted acetoacetates with S. cerevisiae NCYC 1765 obtained in our laboratory [175,190] are in accordance with our proposed absolute configuration of the major diastereoisomer **108**.

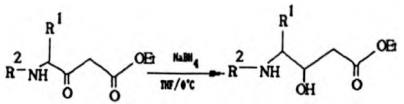
The low conversion and yield obtained in the biotransformation could be explained by the fact that the substrate is disubstituted at C-4, thus steric hindrance might well prevent strong binding to the active site of the enzyme.

It would have been interesting to study the yeast reduction of the enantiomer of the 3-keto ester derived from D-phenylalanine together with the screening of other yeast species with opposite

stereoselectivities such as Candida guilliermondi. However, since this was outside the scope of this thesis, no such experiments were carried out.

The optimal conditions for the reduction with sodium borohydride were applied to the 3-keto esters derived from amino acids (Table 4).

Table 4 : Diastereoselective reduction of 3-keto esters by sodium borohydride.



Compound		configuration at C-4	Product	Reaction time (hrs)	Yield (%)	de (%)
R^1=Bzl, R^2=Ac	95	S	106	0.5	95	40
R^1=Bzl, R^2=Z	97	S	110	1	95	72
R^1=iBu, R^2=Z	101	S	111	3	90	50
R^1=CH_2OBzl, R^2=Z	102	R	112	2	95	60

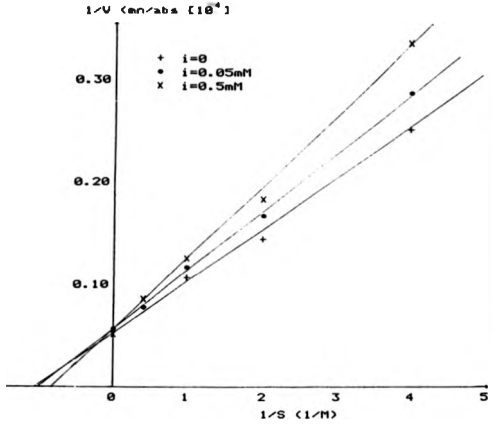
The results depicted in Table 4 show that the introduction of a bulkier protecting group, Z instead of acetyl, resulted in an increase of the diastereoisomeric excess. The same isomer is predominantly formed in both cases as shown by the ^{1}H NMR pattern.

3-Hydroxy esters derived from L-leucine **111** and D-serine **112** could be obtained in high yields and high diastereoisomeric excesses, but the absolute configuration at C-3 was not determined.

Although more stereoselective methods of reduction could be envisaged, such as that published by Narasaka [192] which involves chelation by tri-isobutylborane before reduction with sodium borohydride, the results described above show that high stereoselectivity can be obtained by using cheap and readily available reducing agents such as sodium borohydride.

The possibility of the 3-hydroxy esters being protease inhibitors was investigated spectrophotometrically. the potential competitive inhibition by the 3-hydroxy ester **106** derived from acetyl-L-phenylalanine on the hydrolysis of N-benzoyl-L-tyrosine ethyl ester by α-chymotrypsin was studied according to a method published by Hummel [193]. The results are summarized in Fig. 1.

FIGURE 1 : *Lineweaver–Burk plot showing the competitive inhibition.*



The 3-hydroxy ester 106 was composed of a mixture of the two diastereoisomers (3R,4S) and (3S,4S) in a ratio 7:3. The effect of varying the substrate concentration [S] on the initial

velocity V was determined at a fixed concentration of the inhibitor **106**. This experiment was then repeated with a different concentration of inhibitor. In the conventional double-reciprocal Lineweaver-Burk plot [194] (figure 1), a family of straight lines was obtained. The lines are intersecting at a common intercept on the 1/V axis. The features observed in Fig. 1 are characteristic of a competitive inhibition, where the presence of a competitive inhibitor increases the apparent Michaelis constant Km of the enzyme for the substrate. From the plot of the uninhibited reaction, the Michaelis constant Km for benzoyl-L-tyrosine ethyl ester was calculated to be 0.9 mM. The competitive inhibitor characteristically does not affect the maximal velocity Vm as shown by the common intercept on the 1/V axis. The inhibition constant Ki of the enzyme for the 3-hydroxy ester **106** was derived from Fig. 1. In the inhibited reaction, the Michaelis constant Km for the substrate is increased by a factor of 1 + [I]/Ki where [I] is the concentration of the inhibitor and Ki the inhibition constant. From the set of inhibited reactions, Ki was estimated to be 1-1.3 mM.

This constant corresponds to the inhibitory effect exerted by a particular mixture of the two diastereoisomers. The individual effect of one isomer is not reflected by this value of Ki.

According to the literature [195], an inhibitor can be arbitrarily classified as a good one if its Ki << 10 mM. The most potent inhibitors reported to date have Ki < 0.1 mM.

The individual weight of each diastereoisomer in Ki cannot be estimated directly. In the study of pepsin inhibitors [163], Rich reported that changing the chirality of the (3S)-hydroxy group to 3R diminished the binding to pepsin over 100-fold. The inhibitor with the 3S-configuration is then supposed to have the most 'transition state-like' structure. If the mechanisms of pepsin and chymotrypsin are different, kinetic measurements with the pure (3S,4S)-diastereoisomer might well reveal much lower Ki values.

In conclusion, despite the failure of the concept concerning the cleavage of carbon-carbon-bonds, 3-keto- and 3-hydroxy esters derived from amino acids appeared to be valuable compounds for several reasons. Firstly, their potential inhibition action on proteases makes them very attractive compounds in drug design. Second, their synthesis provides a good ground for the investigations of synthetic methods such as diastereoselective reductions, carbon-carbon bond synthesis.

We have demonstrated that the classical malonic acid synthesis proved very valuable in the synthesis of these compounds from readily available starting materials such as amino acids. If this synthetic strategy proved to be substrate-dependent, it was shown that a new route involving the Leuch's anhydrides could be elaborated.

Microbial reductions were used to provide the desired 3-hydroxy esters. If neither the yields nor the conversions were

satisfactory, the reduction of 3-keto esters derived from amino acids by biological systems could provide a good case for the gathering of information concerning the intimate mechanism of the reductions.

And finally, reduction of the 3-keto esters with sodium borohydride provided on a preparative scale the desired 3-hydroxy esters with a good control of the stereochemistry.

EXPERIMENTAL DETAILS

All chemicals, including solvents were purified according to literature methods [196].

Nuclear magnetic resonance spectra were recorded using a Bruker WH 400 (^{1}H 400.13 MHz, ^{13}C 100.62 MHz) and a Perkin-Elmer R 34 (^{1}H 220 MHz). Chemical shifts, unless otherwise stated, are quoted in ppm downfield from a tetramethylsilane internal reference.

Mass spectra were recorded using a Kratos MS 80 spectrometer. Chemical ionisation (CI) mass spectra were recorded using ammonia as reactant gas. Accurate mass determinations were performed using Electron Impact (EI) mass spectrometry. Unless otherwise stated, the data correspond to the molecular ion $M^{+\cdot}$. Fast Atom Bombardment (FAB) mass spectra were recorded in a glycerol matrix using argon as the reactant gas. Optical rotations were measured on an "Optical Activity Ltd" AA-1000 polarimeter at 589 nm in a 2 dm path length cell. Infrared spectra were recorded using a Pye-Unicam SP3-100 IR spectrophotometer. Melting points were determined using a Gallenkamp apparatus and are quoted uncorrected. Elemental analysis were carried out by Medac Ltd. Thin-layer chromatography was performed on Merck Kieselgel F_{254} precoated aluminium plates. Spot detection was effected by conventional methods [197]. Flash chromatography was performed on Merck Kieselgel 60 silica gel (230-400 mesh).

General procedure for the synthesis of peptides using chymotrypsin suspended in dichloromethane.

To a solution of the N-protected amino acid ester and the amino acid derivative used as nucleophile (40 mM, 1:1 molar ratio) in anhydrous dichloromethane (distilled over calcium hydride immediately prior to use) was added 1mg/ml of chymotrypsin, followed by the addition of 0.25% (v/v) of water. The resulting suspension was then stirred at room temperature until completion of the reaction. The solvent was then evaporated under reduced pressure, the residue was thoroughly washed with water, and the product recrystallized from hot methanol.

N-Acetyl-L-tyrosyl-L-phenylalaninamide 2

Yield 96%

MP = 258-260°C

$[\alpha]_D^{25}$ = -24.0° (c=0.1, DMF)

Rf = 0.68 (CH_2Cl_2/MeOH 13%

Elemental analysis : Found C 64.1 H 6.25 N 11.1%

$C_{20}H_{23}N_3O_4$ requires C 63.9 H 6.50 N 10.9%

^1H NMR (220 MHz, $CDCl_3$/TFA 1:1) 2.0 (s, 3H, acetyl), 2.8 (m, 2H, βPhe), 3.1 (m, 2H, ^2J = 15.9 Hz, βTyr), 4.4 (m, 1H, α), 4.6 (m, 1H, α), 6.35 (bs, 1H, NH), 6.7 (d, 2H,

J = 9.8 Hz, H-aromatic Tyr), 6.9 (d, 2H, J = 7.3 Hz, H-aromatic Tyr), 6.95-7.25 (m, 7H, H-aromatic Phe and amide), 7.6 (d, 1H, J = 3.7 Hz, OH).

N-Acetyl-L-tyrosyl-L-leucinamide 3

Yield 95%

MP = 248-250°C

$[\alpha]_D^{25}$ = -11.5° (c=0.12, DMF) (Lit.[80] 245-247°C and -7.7° (c=0.33, MeOH) respectively)

Rf = 0.67 (CH_2Cl_2/MeOH 13%)

MS Acc. Mass $C_{17}H_{25}N_3O_4$ 335.185 +1.4 ppm

Elemental analysis : Found C 59.95 H 7.6 N 12.3%

$C_{17}H_{25}N_3O_4$ requires C 59.8 H 7.75 N 12.0%

^1H NMR (220 MHz, $CDCl_3$/TFA 1:1) 0.8 (t, 6H, J = 3.5 Hz, CH_3 Leu), 1.5 (m, 3H, β, γ Leu), 2.1 (s, 3H, acetyl), 3.0 (m, 2H, β Tyr), 4.5 (bq, 1H, α Leu), 4.7 (bq, 1H, α Tyr), 6.85 (bd, 4H, H-aromatic and amide), 7.05 (d, 2H, H-aromatic), 7.3 (d, 1H, J = 5 Hz, NH), 7.6 (bs, 1H, OH), 7.8 (d, 1H, J = 5 Hz, NH).

N-Acetyl-L-tyrosyl-L-valinamide 4

Yield 92%

MP = 257-258°C

$[\alpha]_D^{25}$ = +20.5° (c=0.1, DMSO)

Rf = 0.59 (CH_2Cl_2/MeOH 13%)

Elemental analysis : Found C 56.2 H 7.0 N 12.5%

$C_{16}H_{23}N_3O_4$ requires C 56.6 H 7.4 N 12.4%

^1H NMR (220 MHz, DMSO-d^6) 0.8 (t, 6H, J = 4.9 Hz, CH_3), 1.75 (s, 3H, acetyl), 1.95 (m, 1H, β Val), 2.75 (m, 2H, J = 14.6 Hz, β Tyr), 4.15 (m, 1H, α Val), 4.5 (m, 1H, α Tyr), 6.7 (d, 2H, J = 7.3 Hz, H-aromatic Tyr), 7.1 (d, 2H, J = 7.3 Hz, H-aromatic Tyr), 7.75 (d, 1H, J = 9.8 Hz, NH Val), 8.1 (d, 1H, J = 9.5 Hz, NH Tyr), 9.2 (s, 1H, OH).

N-Acetyl-L-tyrosyl-L-alaninamide 5

Yield 84%

MP = 207°C d

$[\alpha]_D^{25}$ = +19.0° (c=0.1, DMSO)

Rf = 0.45 (CH_2Cl_2/MeOH 13%)

Elemental analysis : Found C 56.4 H 6.5 N 14.2%

$C_{14}H_{19}N_3O_4$ requires C 56.35 H 6.5 N 14.3%

^1H NMR (220 MHz, $CDCl_3$/TFA 1:1) 0.95 (d, 3H, J = 6 Hz, β Ala), 2.25 (s, 3H, acetyl), 3.1 (m, 2H, β Tyr), 4.6 (m, 1H, α Ala), 4.9 (q, 1H, α Tyr), 6.75 (bs, 2H, amide), 6.95 (d, 2H, J = 7.3 Hz, H-aromatic), 7.15 (d, 2H, J = 7.3 Hz, H-aromatic), 7.5 (d, 1H, J = 5 Hz, NH), 7.6 (bs, 1H, OH), 8.05 (d, 1H, J = 5 Hz, NH).

N-Acetyl-L-tyrosyl-L-methioninamide 6

Yield 86%

MP = 236-238°C

$[\alpha]_D^{25}$ = -29.0° (c=0.13, DMSO)

Rf = 0.62 (CH_2Cl_2/MeOH 13%)

Elemental analysis : Found C 53.9 H 6.6 N 11.8%

$C_{16}H_{23}N_3O_4S$ requires C 54.2 H 6.55 N 11.85%

^1H NMR (220 MHz, $CDCl_3$/TFA 1:1) 2.15 (m, 2H, ϵ Met), 2.2 (s, 3H, CH_3 Met), 2.3 (s, 3H, acetyl), 2.65 (m, 2H, γ Met), 3.15 (m, 2H, β Tyr), 4.85 (m, 2H, α Met and Tyr), 6.9 (bs, 2H, amide), 7.0 (d, 2H, J = 7.2 Hz, H-aromatic), 7.2 (d, 2H, J = 7.3 Hz, H-aromatic), 7.6 (bs, 1H, OH), 7.75 (d, 1H, J = 5 Hz, NH), 7.95 (d, 1H, J = 5 Hz, NH).

N -tert-Butyloxycarbonyl-β-alaninamide 7

To a solution of BOC-β-alanine (5g, 26.4 mmol) in dry dichloromethane (80 ml) was added 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ, 7.18g, 29 mmol) and ammonium bicarbonate (10.435g, 132 mmol). The remaining suspension was stirred at room temperature overnight, the salt filtered, and the solution washed with water, 0.5M aqueous hydrochloric acid and 1M aqueous sodium hydrogen carbonate. The organic phase was then dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was crystallized from ethyl acetate / petroleum ether 40-60°C.

Yield 82%

MP = 151-153°C

Elemental analysis : Found C 51.00 H 8.55 N 14.82%

$C_8H_{16}N_2O_3$ requires C 51.05 H 8.57 N 14.88%

^1H NMR (220 MHz, $CDCl_3$) 1.5 (s, 9H, BOC), 2.5 (t, 2H, J = 6.7 Hz, α), 3.45 (q, 2H, J = 6.7 Hz, β), 5.9 (bs, 2H, amide).

β-Alaninamide trifluoroacetate 8

The N-BOC-protected derivative 7 (3g, 15.9 mmol) was added to a solution of trifluoroacetic acid in dry dichloromethane (50% v/v, 50 ml). The solution was stirred at room temperature for 30 minutes and the solvents removed under reduced pressure.
The free derivative (as a trifluoroacetate) was then extensively dried under high vacuum, dissolved in the minimal amount of methanol and precipitated with diethyl ether.

Yield 95%

MP = 87-89°C

Elemental analysis : Found C 29.75 H 4.98 N 13.71%

$C_5H_9N_2O_3F_3$ requires C 29.71 H 4.49 N 13.86%

^1H NMR (220 MHz, CD_3OD) 3.2 (t, 2H, J = 6 Hz, α), 3.75 (bt, 2H, J = 6 Hz, β).

L-Phenyllactic amide 9

To a suspension of L-phenyllactic acid (1g, 6 mmol) in dry dichloromethane (30 ml) was added 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ, 1.637g, 6.6 mmol) and ammonium bicarbonate (2.372g, 30 mmol). The remaining suspension was stirred at room temperature for 24 hours, the salt filtered, and

the solution washed with water, 0.5M aqueous hydrochloric acid and 1M aqueous sodium hydrogen carbonate. The organic phase was then dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was crystallized from ethyl acetate / petroleum ether 40-60°C.

Yield 86 %

MP = 104-106°C

$[\alpha]_D^{25}$ = -67.2° (c=0.9.25, MeOH)

Rf = 0.50 (CH_2Cl_2/MeOH 10%)

Elemental analysis : Found C 65.69 H 7.03 N 8.71%

$C_9H_{11}NO_2$ requires C 65.44 H 6.71 N 8.48%

^1H NMR (220 MHz, CD_3OD) 2.8 and 3.15 (2q, 4H, ^2J = 14 Hz, ^3J = 9 Hz, β), 4.3 (q, 1H, J = 9 Hz, α), 7.35 (s, 5H, H-aromatic).

Isobutyl L-lysinate dihydrochloride 10

Acetyl chloride (5 ml, 70.3 mmol) was added to isobutyl alcohol (50 ml) cooled in an ice-water bath. L-Lysine monohydrochloride (5g, 27.3 mmol) was then added and the resulting suspension was refluxed for 4 days. The mixture was filtered and the solvent evaporated under reduced pressure. The dry residue was dissolved in the minimal amount of methanol and the remaining L-Lysine

dihydrochloride was precipitated out. The filtrate was evaporated under reduced pressure and the residue dried at 60°C under high vacuum.

Yield 76%

$[\alpha]_D^{25}$ = +13.8° (c=0.6, MeOH)

Elemental analysis : Found C 43.35 H 8.89 N 10.06 Cl 25.48%

$C_{10}H_{24}N_2O_2Cl_2$ requires C 43.64 H 8.79 N 10.18 Cl 25.76%

MS (CI) m/z 203 (M-2HCl), 186, 130 ($^+NH_2$-CH-COOBu^i), 101 (NH_2-$(CH_2)_3$-CH=$^+NH_2$), 84 (3,4,5,6-tetrahydro-pyridinium), 56 (isobutene).

^{1}H NMR (220 MHz, CD_3OD) 0.95 (d, 6H, CH_3), 1.35-2.05 (m, 7H, β, γ, δ, and CH ester), 2.65 (t, 2H, ε), 3.45 (t, 1H, α), 3.90 (m, 2H, OCH_2).

Isobutyl N-acetyl-L-tyrosyl-α-L-lysinate 11

For experimental procedure, see page 250. Two equivalents of triethylamine were used to neutralize the salt.

Yield 86%

MP = 264-266°C

$[\alpha]_D^{25}$ = +33.1° (c=0.1, DMSO)

Rf = 0.48 (CH_2Cl_2/MeOH 13%)

Elemental analysis : Found C 56.4 H 7.75 N 9.4%

$C_{21}H_{33}N_3O_5$ requires C 56.9 H 7.5 N 8.7%

^{1}H NMR (400 MHz, CD_3OD) 1.0 (d, 6H, J = 7 Hz, CH_3), 1.5 (m, 2H, γ), 1.7 (m, 2H, δ), 1.95 (m, 3H, CH ester and β), 1.95 (s, 3H, acetyl), 2.85 and 3.05 (2q, 2H, β Tyr), 2.95 (t, 2H, J = 8 Hz, ε), 3.95 (m, 2H, OCH_2), 4.45 (q, 1H, α Lys), 4.55 (q, 1H, α Tyr), 6.75 and 7.1 (2d, 4H, J = 7.3 Hz, H-aromatic).

Isobutyl L-ornithinate dihydrochloride 12

Acetyl chloride (5 ml, 70.3 mmol) was added to isobutyl alcohol (50 ml) cooled in an ice-water bath. L-ornithine hydrochloride (5g, 29.6 mmol) was then added and the resulting suspension was refluxed for 4 days. The mixture was filtered and the solvent was evaporated under reduced pressure. The dry residue was dissolved in the minimal amount of methanol and the remaining L-Lysine dihydrochloride was precipitated out. The filtrate was evaporated under reduced pressure and the residue dried at 60°C under high vacuum.

Yield 53%

$[\alpha]_D^{25}$ = +30.1° (c=1.275, MeOH)

MS FAB 189(MH^+), 172(MH^+-NH_3), 154(MH^+-$2NH_3$), 116(MH^+-BuOH), 70, 44.

^1H NMR (220 MHz, D_2O) 0.95 (d, 6H, J = 5 Hz, CH_3), 1.8-2.05 (m, 5H, CH ester, β, γ), 3.1 (t, 2H, J = 7 Hz, δ), 4.1 (d, 2H, J = 5 Hz, OCH_2), 4.25 (t, 1H, α).

Ethyl N-benzyloxycarbonyl-L-tyrosinate **13**

Chymotrypsin (10 mg) was added to a solution of N-benzyloxycarbonyl-L-tyrosine (126 mg, 0.4 mmol) in ethanol (10 ml). Water (200 µl, 2% v/v) was added and the remaining suspension was stirred at room temperature for 24 hours. The reaction mixture was then filtered and the solvent evaporated under reduced pressure. Ethyl acetate (5 ml) was added to the dry residue and the organic phase was then washed with water (2 ml), 0.5M aqueous sodium hydrogen carbonate (2 ml), and water (2 ml). After drying over magnesium sulfate, the solvent was evaporated under reduced pressure and the residue recrystallized from ethyl acetate / petroleum ether 40-60°C.

Yield 44%

MP = 86-88°C (Lit.[198] 88-91°C)

$[\alpha]_D^{25}$ = -10.5° (c=0.5, MeOH)

Rf = 0.63 (CH_2Cl_2/MeOH 5%)

Elemental analysis : Found C 66.95 H 5.98 N 4.98%

$C_{19}H_{21}NO_5$ requires C 66.66 H 5.89 N 4.09%

^{1}H NMR (220 MHz, acetone-d^6) 1.35 (t, 3H, J = 5 Hz, CH_3), 3.1 (q, 1H, β), 3.2 (q, 1H, ^{2}J = 13 Hz, β), 4.3 (q, 2H, ^{2}J = 13 Hz, OCH_2), 4.6 (q, 1H, α), 5.25 (s, 2H, OCH_2-Ar), 6.7 (d,1H, NH), 7.0 (d, 2H, H-aromatic), 7.3 (d, 2H, H-aromatic), 7.55 (s, 6H, H-aromatic and OH).

N-Benzyloxycarbonyl-L-tyrosyl-L-phenylalaninamide 14

For the experimental procedure, see page 250

Yield 94%

MP = 191-193°C

$[\alpha]_D^{25}$ = -18.0° (c=0.1, DMF)

Rf = 0.70 (CH_2Cl_2/MeOH 13%)

Elemental analysis : Found C 67.95 H 5.90 N 9.11%

$C_{26}H_{27}N_3O_5$ requires C 67.66 H 5.90 N 9.10%

^{1}H NMR (220 MHz, DMSO-d^6) 2.7-3.3 (m, 4H, β Tyr and Phe), 4.5 (m, 1H, α Phe), 4.9 (m, 1H, α Tyr), 5.35 (s, 2H, OCH_2-Ar), 7.0 (d, 2H, J = 7 Hz, H-aromatic Tyr), 7.4-7.8 (m, 16 H, H-aromatic Phe, Tyr, and Z, NH, OH, and amide), 8.4

(d, J = 5 Hz, NH).

N-Benzoyl-L-tyrosyl-L-phenylalaninamide **15**

For experimental procedure, see page 250.

Yield 86%

MP = 255-257°C d

$[\alpha]_D^{25}$ = -32.0° (c=0.1, DMSO)

Rf = 0.66 (CH_2Cl_2/MeOH 13%)

Elemental analysis : Found C 69.1 H 5.8 N 9.7%

$C_{25}H_{25}N_3O_4$ requires C 69.6 H 5.85 N 9.75%

^1H NMR (220 MHz, $CDCl_3$/TFA 1:1) 3.1 (m, 4H, β Tyr and Phe), 5.0 (m, 2H, α Tyr and Phe), 6.9 (d, 2H, J = 7 Hz, H-aromatic Tyr), 7.05 (d, 2H, J = 7 Hz, H-aromatic Tyr), 7.25-7.7 (m, 15H, H-aromatic, NH, OH, and amide).

N-Acetyl-L-phenylalanyl-L-phenylalaninamide **16**

For experimental procedure, see page 250.

Yield 90%

MP = 258-260°C (Lit.[79] 266-268°C)

$[\alpha]_D^{25}$ = -15.6° (c=0.2, MeOH)

Rf = 0.73 (CH_2Cl_2/MeOH 13%)

Elemental analysis : Found C 65.45 H 6.35 N 11.0%

Calc. for $C_{20}H_{21}N_3O_3$ C 65.8 H 6.6 N 11.0%

^{1}H NMR (220 MHz, $CDCl_3$/TFA 3:1) 2.2 (s, 3H, acetyl), 3.3 (m, 4H, β Phe), 4.6 (m, 1H, α Phe), 5.05 (q, 1H, α Phe), 7.1-7.5 (m, 12H, H-aromatic, NH, amide).

Ethyl N-tert-butyloxycarbonyl-L-phenylalaninate 17

To a solution of ethyl L-phenylalaninate hydrochloride (10 mmol) in dry dichloromethane (20 ml) was added triethylamine (10 mmol) and di-tert-butyl dicarbonate (11 mmol). The solution was stirred at room temperature overnight, washed with water (5 ml) and 0.5M aqueous hydrochloric acid (5 ml). The organic phase was dried over magnesium sulfate and evaporated under reduced pressure. The residue was purified by flash column chromatography (dichloromethane).

yield 74%

MP = 25-27°C

$[\alpha]_D^{25}$ = +40.2° (c=1.3, $CHCl_3$)

Rf = 0.33 (CH_2Cl_2)

Elemental analysis : Found C 65.73 H 7.98 N 4.66%

$C_{16}H_{23}NO_4$ requires C 65.51 H 7.90 N 4.77%

^1H NMR (220 MHz, $CDCl_3$) 1.2 (t, 3H, J = 5 Hz, CH_3), 1.4 (s, 9H, BOC), 3.1 (m, 2H, β), 4.15 (q, 2H, J = 5 Hz, OCH_2), 4.55 (m, 1H, α), 5.15 (bd, 1H, NH), 7.3 (m, 5H, H-aromatic).

N-Acetyl-L-tryptophyl-L-phenylalaninamide 18

For experimental procedure, see page 250.

Yield 88%

MP = 198-200°C

$[\alpha]_D^{25}$ = -81.0° (c=0.1, DMSO)

Rf = 0.55 (CH_2Cl_2/MeOH 13%)

Elemental analysis : Found C 65.5 H 6.05 N 13.85%

$C_{22}H_{24}N_4O_3$ requires C 65.1 H 6.6 N 13.2%

MS Acc. Mass $C_{22}H_{24}N_4O_3$ 392.1914 +16.7 ppm

^1H NMR (220 MHz, $CDCl_3$/TFA 1:1) 2.2 (s, 3H, acetyl), 2.95 (m, 2H, β Trp), 3.3 (m, 2H, β Phe), 4.7 (m, 1H, α Phe), 4.9 (m, 1H, α Trp), 7.0-7.45 (m, 11H, H-aromatic), 7.6 (d, 1H, J = 5 Hz, NH), 7.9 (d, 1H, J = 5 Hz, NH).

Ethyl N-benzyloxycarbonyl-3,4-dihydroxy-L-phenylalaninate **19**

3,4-dihydroxy-L-phenylalanine (5 g, 25 mmol) was added to a concentrated solution of hydrochloric acid in anhydrous ethanol. The solution was refluxed for 4 hours and the solvent evaporated under reduced pressure. The dry residue, ethyl 3,4-dihydroxy-L-phenylalaninate hydrochloride, was suspended in dry dichloromethane (50 ml) and triethylamine (3.5 ml, 25 mmol) was added. N-(benzyloxycarbonyloxy) succinimide (6.23 g, 25 mmol) was added to the remaining solution. After stirring at room temperature for 18 hours, the organic phase was washed with water, 0.1M aqueous hydrochloric acid, 0.5M aqueous sodium hydrogen carbonate, and dried over magnesium sulfate. After evaporation of the solvent, the oily residue was purified by flash column chromatography (dichloromethane / methanol 15%).

yield 57%

$[\alpha]_D^{25}$ = +25.5° (c=0.25, $CHCl_3$)

Rf = 0.63 (CH_2Cl_2/MeOH 10%)

MS Acc. Mass $C_{19}H_{21}NO_6$ 359.1353 -4.4 ppm

^1H NMR (220 MHz, $CDCl_3$) 1.25 (t, 3H, J = 5 Hz, CH_3), 3.0 (m, 2H, β), 4.2 (q, 2H, J = 5 Hz, OCH_2), 4.6 (q, 1H, α), 5.15 (s, 2H, OCH_2-Ar), 6.0 (d, 1H, NH), 6.6 (d, 1H, H-aromatic), 6.85 (m, 2H, H-aromatic), 7.4 (s, 5H, H-aromatic).

N-Benzyloxycarbonyl-3,4-dihydroxy-L-phenylalanyl-L-phenylalaninamide 20

For experimental procedure, see page 250.

Yield 90%

MP = 228-229°C

$[\alpha]_D^{25}$ = -16.0° (c=0.1, DMSO)

Rf = 0.46 (CH_2Cl_2/MeOH 13%)

Elemental analysis : Found C 63.40 H 5.60 N 8.55%

$C_{26}H_{27}N_3O_6$ requires C 63.65 H 6.10 N 8.25%

^{1}H NMR (220 MHz, $CDCl_3$/TFA 1:1) 2.85-3.1 (m, 4H, β Phe and DOPA), 4.5 (m, 1H, α Phe), 4.85 (m, 1H, α DOPA), 5.2 (s, 2H, OCH_2-Ar), 6.6-6.95 (m, 4H, H-aromatic and amide), 7.15 (bm, 2H, OH), 7.3-7.55 (m, 8H, H-aromatic, NH).

N-Benzoyl-L-alanyl-L-phenylalaninamide 21

For experimental procedure, see page 250.

Yield 68%

MP = 164°C d

$[\alpha]_D^{25}$ = -12.0° (c=0.1, DMSO)

Rf = 0.35 (CH_2Cl_2/MeOH 13%)

Elemental analysis : Found C 65.15 H 6.8 N 10.5%

$C_{19}H_{21}N_3O_3$ requires C 65.65 H 6.8 N 11.3%

^1H NMR (220 MHz, D_2O) 1.45 (d, 3H, J = 6 Hz, CH_3 Ala), 3.2 (m, 2H, β Phe), 4.1 (t, 1H, α), 7.5 (m, 10H, H-aromatic).

Ethyl glycyl-glycinate hydrochloride 22

Glycyl-glycine (5g, 38 mmol) was added to a solution of hydrochloric acid in anhydrous ethanol. The solution was refluxed overnight, the solvent evaporated and the dried residue crystallized from methanol / diethyl ether.

yield 96%

MP = 176-178°C

Rf = 0.17 (CH_2Cl_2/MeOH 5%)

Elemental analysis : Found C 36.73 H 6.59 N 14.20 Cl 18.03%

$C_6H_{13}N_2O_3Cl$ requires C 36.65 H 6.66 N 14.25 Cl 18.03%

^1H NMR (220 MHz, D_2O) 1.3 (t, 3H, CH_3), 4.0 (s, 2H, CH_2 Gly), 4.2 (s, 2H, CH_2 Gly), 4.35 (q, 2H, OCH_2).

General procedure for the coupling with EEDQ

The coupling reagent (2.47 g, 10 mmol) is added to a solution of the N-protected amino acid (10 mmol) and the amino acid ester (10 mmol, if the hydrochloride is to be used, it is first neutralized by the addition of 1 equivalent of triethylamine) in dry dichloromethane (30 ml) and the mixture is stirred at room temperature for 24 hours. The solution is then extracted with 1M aqueous hydrochloric acid, 0.5M aqueous sodium hydrogen carbonate and water. The organic phase is dried over magnesium sulfate and evaporated to dryness under reduced pressure. The residue is recrystallized from ethyl acetate / petroleum ether 40-60°C.

Ethyl N-benzyloxycarbonyl-L-leucyl-L-phenylalaninate 23

Yield 92%

MP = 76-78°C

$[\alpha]_D^{25}$ = -25.0° (c=0.5, MeOH)

Elemental analysis : Found C 68.03 H 7.24 N 6.89%

$C_{25}H_{32}N_2O_5$ requires C 68.16 H 7.32 N 6.36%

^1H NMR (220 MHz, $CDCl_3$) 0.95 (d, 6H, J = 5 Hz, CH_3 Leu), 1.25 (t, 3H, CH_3 ester), 1.4-1.8 (m, 3H, β, γ Leu), 3.15 (m, 2H, β Phe), 4.2 (q, 2H, OCH_2), 4.85 (q, 1H, α Leu), 5.15

(s, 2H, OCH_2-Ar), 5.25 (m, 1H, α Phe), 6.55 (bd, 1H ,NH), 7.1-7.4 (m, 11H, H-aromatic and NH).

Methyl N-benzyloxycarbonyl-L-leucyl-L-leucinate 24

Yield 84%

MP = 86-88°C

$[\alpha]_D^{25}$ = -24.7° (c=1, $CHCl_3$)

Elemental analysis : Found C 64.09 H 8.20 N 7.22%

$C_{21}H_{32}N_2O_5$ requires C 64.26 H 8.22 N 7.14%

^1H NMR (220 MHz, $CDCl_3$) 0.95 (m, 12H, CH_3 Leu), 1.65 (m, 6H, β,γ Leu), 3.8 (s, 3H, OCH_3), 4.3 (m, 1H, α), 4.7 (m, 1H, α), 5.2 (s, 2H, OCH_2-Ar), 5.5 (d, 1H, NH), 6.7 (d, 1H, NH), 7.5 (s, 5H, H-aromatic).

Ethyl N-benzyloxycarbonyl-L-leucyl-L-tyrosinate 65

Yield 75%

$[\alpha]_D^{25}$ = +20.0° (c=1.2, $CHCl_3$)

MS Acc. Mass $C_{25}H_{32}N_2O_6$ 456.537 +3.4 ppm

^{1}H NMR (220 MHz, $CDCl_3$) 0.9 (bq, 6H, δ Leu), 1.2 (t, 3H, J = 5 Hz, CH_3), 1.45-1.75 (m, 3H, β, γ Leu), 3.0 (m, 2H, β Tyr), 4.15 (q, 2H, J = 5 Hz, OCH_2), 4.35 (m, 1H, α), 4.8 (m, 1H, α), 5.1 (q, 2H, OCH_2-Ar), 6.05 (d, 1H, J = 5 Hz, NH), 6.8 and 7.0 (2d, 4H, J = 7 Hz, H-aromatic Tyr), 7.35 (s, 6H, H-aromatic and NH).

Ethyl N-benzyloxycarbonyl-L-alanyl-L-tyrosinate **67**

Yield 88%

MP = 133-135°C

$[\alpha]_D^{25}$ = +29.2° (c=0.9, $CHCl_3$)

Elemental analysis : Found C 63.11 H 6.31 N 6.55%

$C_{22}H_{26}N_2O_6$ requires C 63.76 H 6.32 N 6.76%

^{1}H NMR (220 MHz, $CDCl_3$) 1.3 (t, 3H, J = 5 Hz, ester), 1.35 (d, 3H, CH_3 Ala), 3.05 (m, 2H, β), 4.25 (q, 2H, J = 5 Hz, OCH_2), 4.3 (bm, 1H, α), 4.85 (m, 1H, α), 5.15 (q, 2H, OCH_2-Ar), 5.65 (d, 1H, J = 5 Hz, NH), 6.8 (bd, 3H, H-aromatic Tyr and NH), 7.0 (d, 2H, J = 7 Hz, H-aromatic), 7.45 (s, 5H, H-aromatic).

Ethyl N-benzyloxycarbonyl-D-alanyl-L-tyrosinate 69

Yield 93%

$[\alpha]_D^{25}$ = +44.0° (c=1, $CHCl_3$)

Elemental analysis : Found C 63.67 H 6.35 N 6.53%

$C_{22}H_{26}N_2O_6$ requires C 63.76 H 6.32 N 6.76%

^1H NMR (220 MHz, $CDCl_3$) 1.35 (m, 6H, β Ala and ester), 3.1 (m, 2H, β Tyr), 4.3 (q, 2H, OCH_2), 4.35 (m, 1H, α), 4.9 (m, 1H, α), 5.2 (q, 2H, OCH_2-Ar), 5.8 (d, 1H, J = 5 Hz, NH), 6.85 (d, 2H, J = 7 Hz, H-aromatic Tyr), 7.05 (m, 3H, H-aromatic and NH), 7.5 (s, 5H, H-aromatic), 7.65 (bs, 1H, OH).

N-Acetyl-L-tyrosyl-D-phenylalaninamide 25

For experimental procedure, see page 250.

Yield 94%

MP = 206-208°C

$[\alpha]_D^{25}$ = +32.5° (c=0.1, DMSO)

Rf = 0.58 (CH_2Cl_2/MeOH 13%)

Elemental analysis : Found C 63.4 H 6.2 N 10.95%

$C_{20}H_{23}N_3O_4$ requires C 62.85 H 6.7 N 10.50%

^{1}H NMR (220 MHz, $CDCl_3$/TFA 1:1) 2.2 (s, 3H, acetyl), 3.05-3.2 (m, 4H, β Tyr and Phe), 4.85 (m, 2H, α Phe and Tyr), 6.6 (bs, 1H, OH), 6.9 (d, 2H, H-aromatic Tyr), 7.05-7.5 (m, 10H, H-aromatic Tyr and Phe, NH, amide), 7.85 (d, 1H, J = 5 Hz, NH).

N-Acetyl-L-tyrosyl-D-leucinamide **26**

For experimental procedure, see page 250.

Yield 93%

MP = 210°C d

$[\alpha]_D^{25}$ = +14.0° (c=0.1, DMSO)

Rf = 0.55 (CH_2Cl_2/MeOH 13%)

Elemental analysis : Found C 60.85 H 7.5 N 12.35%

$C_{17}H_{25}N_3O_4$ requires C 60.9 H 7.5 N 12.50%

^{1}H NMR (220 MHz, $CDCl_3$/TFA 1:1) 0.95 (t, 6H, J = 4 Hz, CH_3 Leu), 1.65 (m, 3H, β, γ Leu), 2.25 (s, 3H, acetyl), 3.1 (m, 2H, β Tyr), 4.65 (m, 1H, α Leu), 4.85 (m, 1H, α Tyr), 6.95 (bd, 4H, H-aromatic and amide), 7.15 (d, 2H, J = 7 Hz, H-aromatic Tyr), 7.4 (d, 1H, J = 5 Hz, NH), 7.7 (bs, 1H, OH), 7.85 (d, 1H, J = 5 Hz, NH).

α-Amino-isobutyramide trifluoroacetate **27**

To a solution of N-BOC-methylalanine (1g, 4.9 mmol) in dry dichloromethane (15 ml) was added 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ, 1.338g, 5.4 mmol) and ammonium bicarbonate (1.937, 24.5 mmol). The remaining suspension was stirred at room temperature overnight, the salt filtered, and the solution washed with water, 0.5M aqueous hydrochloric acid and 1M aqueous sodium hydrogen carbonate. The organic phase was then dried ($MgSO_4$) and the solvent was removed under reduced pressure. The oily residue (450mg) was added to a solution of trifluoroacetic acid in dry dichloromethane (50% v/v, 5 ml). The solution was stirred at room temperature for 30 minutes and the solvents were removed under reduced pressure. The free derivative (as a trifluoroacetate) was then extensively dried under high vacuum, dissolved in the minimal amount of methanol and precipitated with diethyl ether / pentane (1:1).

Yield 22%

MP = 197-200°C

Elemental analysis : Found C 33.37 H 5.29 N 13.20%

$C_6H_{11}N_2O_3F_3$ requires C 33.34 H 5.13 N 12.96%

^1H NMR (220 MHz, D_2O) 1.65 (s, 6H, CH_3).

Ethyl N-acetyl-D-tyrosinate 28

D-Tyrosine (1g, 5.5 mmol) was dissolved in 1N aqueous solution of sodium hydroxide (11.2 ml) and acetic anhydride (4 ml, 42.4 mmol) was added. The solution was heated overnight at 60°C. The calculated amount of 6N aqueous solution of sulfuric acid to neutralize the sodium hydroxide (0.93 ml) was added and the solution evaporated under reduced pressure to a thick syrup and again concentrated with two portions of water. The residue was dissolved in acetone and evaporated. The residue was then dissolved in water (1 ml) and 2N aqueous solution of sodium hydroxide (4 ml) was added to adjust the reaction mixture to pH 10 to 11. After stirring at room temperature for 30 minutes, 6N aqueous solution of sulfuric acid (0.67 ml) was added and the mixture concentrated to a syrup. N-acetyl-D-tyrosine was separated from sodium sulfate with wet acetone. The mixture was filtered and the solvent was evaporated under reduced pressure. The oily residue solidified upon washing with petroleum ether 40-60°C. TLC analysis revealed the presence of a mixture of mono- and di-acetates. The residue was purified by flash column chromatography (chloroform / methanol 10%).

The dried residue was added to a solution of concentrated hydrochloric acid in anhydrous ethanol (10 ml) and the mixture refluxed for 2 hours. The solvent was evaporated under reduced pressure and the residue recrystallized from ethyl acetate / petroleum ether 40-60°C.

Overall yield 25%

MP = 80-82°C

$[\alpha]_D^{25}$ = -8.0° (c=1, MeOH)

Rf = 0.85 (CH_2Cl_2/MeOH 13%)

Elemental analysis : Found C 61.67 H 6.57 N 6.18%

$C_{13}H_{17}NO_4$ requires C 62.13 H 6.82 N 5.57%

^{1}H NMR (220 MHz, $CDCl_3$) 1.25 (t, 3H, CH_3), 2.2 (s, 3H, acetyl), 3.05 (m, 2H, β), 4.25 (q, 2H, OCH_2), 4.9 (q, 1H, α), 6.25 (bd, 1H, NH), 6.85 (d, 2H, J = 7 Hz, H-aromatic), 7.1 (d, 2H, J = 7 Hz, H-aromatic), 7.25 (bs, 1H, OH).

Ethyl N-acetyl-D-phenylalaninate 29

Acetyl chloride (0.6 ml, 8.4 mmol) was added to anhydrous ethanol (10 ml) cooled in an ice-water bath. N-acetyl-D-phenylalanine (1g, 4.8 mmol) was then added and the mixture refluxed overnight. The solvent was evaporated under reduced pressure and ethyl acetate (10 ml) was added. The organic phase was washed with 0.5M aqueous solution of sodium bicarbonate (3 ml) and water (5 ml). After drying over magnesium sulfate, the solvent was evaporated under reduced pressure and the residue recrystallized from ethyl acetate / diethyl ether.

Yield 89%

MP = 92-94°C

$[\alpha]_D^{25}$ = -87.7° (c=1, $CHCl_3$)

Rf = 0.45 (CH_2Cl_2/MeOH 4%)

Elemental analysis : Found C 66.58 H 7.35 N 5.83%

$C_{13}H_{17}NO_3$ requires C 66.36 H 7.28 N 5.95%

^1H NMR (220 MHz, $CDCl_3$) 1.25 (t, 3H, CH_3), 2.0 (s, 3H, acetyl), 3.15 (m, 2H, β), 4.2 (q, 2H, OCH_2), 4.9 (m, 1H, α), 6.2 (bd, 1H, NH), 7.2 (m, 2H, H-aromatic), 7.35 (m, 3H, H-aromatic).

Ethyl N-acetyl-D-tryptophanate 30

Acetyl chloride (2 ml, 28.1 mmol) was added to anhydrous ethanol (10 ml) cooled in an ice-water bath. N-acetyl-D-tryptophan (3g, 12.2 mmol) was added and the mixture refluxed for 5 hours. After evaporation of the solvent under reduced pressure, ethyl acetate (20 ml) was added to the yellow residue, and the organic phase was washed with 0.5M aqueous solution of sodium bicarbonate (5 ml) and water (10 ml). The organic phase was dried over magnesium sulfate and the solvent evaporated under reduced pressure. The oily residue was triturated with petroleum ether 40-60°C to give a crystalline residue which was recrystallized from ethyl acetate / petroleum ether 40-60°C.

Yield 74%

MP = 102-104°C

$[\alpha]_D^{25}$ = -34.3° (c=1, $CHCl_3$)

Rf = 0.53 (CH_2Cl_2/MeOH 5%)

Elemental analysis : Found C 65.60 H 6.46 N 10.32%

$C_{15}H_{18}N_2O_3$ requires C 65.68 H 6.61 N 10.21%

^1H NMR (220 MHz, $CDCl_3$) 1.25 (t, 3H, CH_3), 2.0 (s, 3H, acetyl), 3.4 (m, 2H, β), 4.2 (m, 2H, OCH_2), 5.0 (m, 1H, α), 6.15 (d, 1H, J = 5 Hz, NH), 7.05-7.65 (m, 5H, H-aromatic), 8.5 (bs, 1H, NH indole).

1-(N-Acetyl-L-tyrosyl)-amido-2-(R,S)-hydroxy-3-amino propane 32

For experimental procedure, see page 250.

Yield 88%

de 0%

Rf = 0.49 (CH_2Cl_2/MeOH/AcOH 18:6:1)

Elemental analysis : Found C 56.00 H 7.24 N 13.76%

$C_{14}H_{21}N_3O_4$ requires C 55.05 H 7.70 N 12.83%

^1H NMR (400 MHz, CD_3OD) 1.9 (s, 3H, acetyl), 2.35-2.6 (m, 2H, ^1C), 2.85 (2d, 2H, ^3C), 3.0-3.15 (m, 2H, β), 3.55 (m, 1H, ^2C), 4.3 (m, 1H, α), 6.7 (d, 2H, H-aromatic), 7.0 (d, 2H, H-aromatic).

^{13}C NMR (100.62 MHz, CD_3OD) 22.4 (acetyl), 37 (^3C), 43 (^1C), 44 (β), 56.4 (α), 70.4 (^2C), 117, 127, and 131 (Aromatic), 158 (acetyl), 175 (amide).

1-(N-Benzyloxycarbonyl-L-tyrosyl)-amido-2-(R,S)-hydroxy-3-amino propane **33**

For experimental procedure, see page 250.

Yield 92%

de 0%

Rf = 0.58 (CH_2Cl_2/MeOH/AcOH 18:6:1)

Elemental analysis : Found C 60.70 H 6.42 N 10.56%

$C_{20}H_{25}N_3O_5$ requires C 60.13 H 6.96 N 10.01%

^1H NMR (400 MHz, CD_3OD) 2.55 (m, 2H, ^1C), 2.8 (m, 1H, β), 3.05 (m, 1H, β), 3.25 (t, 2H, ^3C), 3.6 (bq, 1H, ^2C), 4.3 (m, 1H, α), 5.1 (q, 2H, OCH_2-Ar), 6.75 (d, 2H, H-aromatic Tyr), 7.1 (d, 2H, H-aromatic Tyr), 7.35 (m, 5H, H-aromatic).

^{13}C NMR (100.62 MHz, DMSO) 37 (^3C), 42.5 (^1C), 45.2 (β), 56.6 (α), 65.2 (OCH_2-Ar), 70.7 (^2C), 115 (Aromatic Tyr), 127-130 (Aromatic Tyr and Z), 137 (Aromatic Tyr), 155.7 (CO Z), 171.8 (CO amide).

MS FAB m/z 388 (MH^+), 136 (Tyr), 107 (Tyr), 91 and 77 (aromatic).

N-Acetyl-L-tyrosine 34

MP = 150-152°C

$[\alpha]_D^{25}$ = +62.0° (c=1, MeOH) (Lit.[199] 152-154°C and +60.5°(c=4, EtOH) respectively)

^1H NMR (400 MHz, CD_3OD) 2.9 (q, 1H, ^2J = 14 Hz, β), 3.15 (q, 1H, ^2J = 14 Hz, β), 4.5 (q, 1H, α), 6.7 (d, 2H, J = 9 Hz, H-aromatic), 7.15 (d, 2H, J = 9 Hz, H-aromatic).

Ethyl L-3-phenyllactate 48

Acetyl chloride (5 ml, 70.3 mmol) was added to anhydrous ethanol (50 ml) cooled in an ice-water bath. L-phenyllactic acid (1g, 6 mmol) was then added and the remaining solution was refluxed overnight. The solvent was then evaporated under reduced pressure and ethyl acetate was added (25 ml). The organic phase was washed with 0.5M aqueous solution of sodium bicarbonate (5 ml) and water (5 ml). After drying over magnesium sulfate, the solvent was evaporated under reduced pressure to give an oil which solidified on standing.

Yield 94%

MP = 26-28°C

$[\alpha]_D^{25}$ = -19.3° (c=0.75, $CHCl_3$)

Elemental analysis : Found C 68.00 H 7.16%

$C_{11}H_{14}O_3$ requires C 68.02 H 7.27%

^1H NMR (220 MHz, $CDCl_3$) 1.3 (t, 3H, J = 7 Hz, CH_3), 2.85 (d, 1H, J = 5 Hz, OH), 3.05 (q, 1H, ^2J = 14.7 Hz, β), 3.15 (q, 1H, ^2J = 14.7 Hz, β), 4.3 (q, 2H, J = 7 Hz, OCH_2), 4.55 (q, 1H, α), 7.4 (m, 5H, H-aromatic).

Methyl 3-(4-hydroxyphenyl)-propionate 55

Acetyl chloride (5 ml, 70.3 mmol) was slowly added to anhydrous methanol (50 ml) cooled in an ice-water bath. 3-(4-hydroxyphenyl) propionic acid (1g, 6 mmol) was then added and the remaining solution was refluxed overnight. The solvent was then evaporated under reduced pressure and ethyl acetate was added (25 ml). The organic phase was washed with 0.5M aqueous solution of sodium bicarbonate (5 ml) and water (5 ml). After drying over magnesium sulfate, the solvent was evaporated under reduced pressure. The oily residue was purified by flash column chromatography (dichloromethane / methanol 2.5%).

Yield 89%

MP = 24-26°C

Rf = 0.83 (CH_2Cl_2/MeOH 10%)

MS Acc. Mass $C_{10}H_{12}O_3$ 180.203 -2.5 ppm

^1H NMR (220 MHz, $CDCl_3$) 2.65 (t, 2H, J = 7.3 Hz, C-2), 2.9 (t, 2H, J = 7.3 Hz, C-3), 3.7 (s, 3H, CH_3), 6.8 (d, 2H, J = 9.8 Hz, H-aromatic), 7.1 (d, 2H, J = 9.8 Hz, H-aromatic).

Methyl (p-amino)-D,L-phenylalaninate dihydrochloride **63**

Acetyl chloride (5 ml, 70.3 mmol) was added to anhydrous methanol (50 ml) cooled in an ice-water bath. 4-amino-L-phenylalanine hydrate (1g, 5.5 mmol) was then added and the remaining solution was refluxed overnight. The solvent was then evaporated under reduced pressure. The dried residue was dissolved in the minimal amount of methanol and crystallisation was induced by the addition of diethyl ether.

Yield 56%

MP = 208-210°C

Elemental analysis : Found C 43.45 H 6.15 N 10.16 Cl 17.44%

$C_{10}H_{16}N_2O_2Cl_2$ requires C 44.96 H 6.04 N 10.48 Cl 17.58%

^{1}H NMR (220 MHz, D_2O) 3.2 (m, 2H, β), 3.7 (s, 3H, CH_3), 4.35 (t, 1H, α), 7.35 (s, 4H, H-aromatic).

Methyl D,L-phenylglycinate hydrochloride 71

Acetyl chloride (5 ml, 70.3 mmol) was added to anhydrous methanol (50 ml) cooled in an ice-water bath. 2-D,L-phenylglycine (5g, 33 mmol) was then added and the remaining solution was refluxed overnight. The solvent was then evaporated under reduced pressure. The residue was recrystallized from methanol / diethyl ether.

Yield 89%

MP = 205-207°C

Rf = 0.38 (CH_2Cl_2/MeOH 10%)

Elemental analysis : Found C 53.47 H 6.12 N 7.04 Cl 17.44%

$C_9H_{11}NO_2Cl$ requires C 53.61 H 5.99 N 6.94 Cl 17.58%

^{1}H NMR (220 MHz, D_2O) 3.8 (s, 3H, CH_3), 5.3 (s, 1H, α), 7.55 (m, 5H, H-aromatic).

Methyl N-benzyloxycarbonyl-D,L-phenylglycinate 72

To a solution of methyl D,L-phenylglycinate hydrochloride (5 mmol) in dry dichloromethane (20 ml) were added triethylamine

(0.7 ml, 5 mmol) and N-(benzyloxycarbonyloxy) succinimide (1.25g, 5 mmol). The solution was stirred overnight at room temperature and then washed with water (5 ml), 1M aqueous hydrochloric acid (5 ml), 0.5M aqueous sodium hydrogen carbonate (5 ml), and water (5 ml). After drying over magnesium sulfate, the solvent was removed under reduced pressure and the residue recrystallised from ethyl acetate / petroleum ether 40-60°C.

Yield 91%

MP = 69-70°C

Rf = 0.86 (CH_2Cl_2/MeOH 10%)

Elemental analysis : Found C 67.86 H 5.67 N 4.71%

$C_{17}H_{16}NO_4$ requires C 68.45 H 5.41 N 4.69%

^{1}H NMR (220 MHz, $CDCl_3$) 3.7 (s, 3H, OCH_3), 5.15 (q, 2H, ^2J = 12.2 Hz, OCH_2-Ar), 5.4 (d, 1H, J = 5 Hz, α), 5.9 (bd, 1H, NH), 7.4 (m, 10H, H-aromatic).

Ethyl D,L-lactate 76

Acetyl chloride (5 ml, 70.3 mmol) was added to anhydrous ethanol (50 ml) cooled in an ice-water bath. D,L-lactic acid (5g, 55.5 mmol) was then added and the remaining solution was refluxed overnight. The solvent was then evaporated under reduced pressure

and the residue purified by distillation under reduced pressure.
Yield 64%

BP = 53°C (18 mm Hg) (Lit.[200] 155°C (760 mmHg))

MS Acc. Mass $C_5H_{10}O_3$ 118.132 +38.5 ppm
IR (liquid film) cm^{-1} 3450 (OH), 1725 (ester).

^1H NMR (220 MHz, $CDCl_3$) 1.33 (t, 3H, CH_3 ester), 1.45 (d, 3H, J = 6.7 Hz, CH_3), 3.1 (bs, 1H, OH), 4.3 (q, 2H, OCH_2), 4.35 (q, 1H, J = 6.7 Hz, CH).

Methyl 3-(p-hydroxyphenyl)-2-D,L-hydroxy-propionate 52

3-(p-Hydroxyphenyl)-2-oxo propionic acid (2 g, 12 mmol) was added to a concentrated solution of hydrochloric acid in anhydrous methanol (25 ml). The mixture was refluxed for 2 hours. The solvent was evaporated and anhydrous dichloromethane was added. The pale yellow precipitate was filtered and the solvent removed under reduced pressure. TLC analysis (dichloromethane/methanol 5%) revealed the presence of several compounds which could not be separated by chromatography. The bright orange residue was dissolved in anhydrous THF (18 ml) and the mixture cooled to 0°C. Sodium borohydride (12 mmol) was then added and stirring was continued for 2 hours, the reaction was quenched by the addition of 0.5M aqueous hydrochloric acid and the solvent was removed

under reduced pressure. The residue was then purified by flash column chromatography (dichloromethane/methanol 5%).

Yield 34%

Rf = 0.35 (CH_2Cl_2/MeOH 5%)

MS Acc. Mass $C_{10}H_{12}O_4$ 196.202 -0.3 ppm

IR (liquid film) cm^{-1} 3400-3300 (OH), 1880 (aromatic), 1730 (ester), 1600 (aromatic), 1500 (aromatic), 820 (para aromatic).

^1H NMR (220 MHz, CD_3OD) 3.45 (q, 1H, J = 12.2 Hz, β), 3.75 (q, 1H, J = 12.2 Hz, β), 4.05 (s, 3H, CH_3), 4.85 (t, 1H, α), 7.45 (d, 2H, H-aromatic), 7.85 (d, 2H, H-aromatic).

Ethyl 3-D,L-hydroxy-3-phenyl propionate 57

Sodium borohydride (2 mmol) was added to a solution of ethyl 3-oxo-3-phenyl propionate (1 mmol) in ethanol. The solution was stirred at 0°C for 1 hour. The reaction was quenched by the addition of aqueous hydrochloric acid and the solvent was evaporated under reduced pressure. The residue was purified by flash column chromatography (dichloromethane/ methanol 2.5%).

Yield 80%

Rf = 0.63 (CH_2Cl_2/MeOH 4%)

MS Acc. Mass $C_{11}H_{14}O_3$ 194.0941 -1.0 ppm

IR (liquid film) cm^{-1} 3440 (OH), 1705 (ester), 1600 (aromatic), 1490 (aromatic), 755 (para aromatic)

^1H NMR (220 MHz, $CDCl_3$) 1.25 (t, 3H, J = 6 Hz, CH_3), 2.75 (m, 2H, β), 3.55 (d, 1H, J = 4 Hz, OH), 4.2 (q, 2H, J = 6 Hz, OCH_2), 5.15 (m, 1H, α), 7.4 (m, 5H, H-aromatic).

Methyl N-tert-butyloxycarbonyl-L-tyrosinate 59

To a solution of methyl L-tyrosinate (10 mmol) in dioxane (20 ml) cooled in an ice-water bath was added di-tert-butyl dicarbonate (2.4 g, 11 mmol) and stirring was continued at room temperature for 3 hours. The solvent was then removed under reduced pressure and the residue dissolved in ethyl acetate (20 ml). The organic phase was washed with water, 0.5M aqueous hydrochloric acid, dried over magnesium sulfate and evaporated under reduced pressure. The crystalline residue was recrystallized from ethyl acetate / petroleum ether 40-60°C.

Yield 89%

MP = 102-104°C

$[\alpha]_D^{25}$ = +5.6° (c=1, MeOH) (Lit.[198] 102-104°C and +5.4° (c=1, MeOH) respectively)

Elemental analysis : Found C 60.56 H 7.22 N 4.58%
Calc. for $C_{15}H_{21}NO_5$ C 61.00 H 7.17 N 4.74%

^1H NMR (220 MHz, CD_3OD) 1.6 (s, 9H, BOC), 3.1 (m, 2H, β), 3.9 (s, 3H, OCH_3), 4.5 (t, 1H, α), 6.95 (d, 2H, J = 7 Hz, H-aromatic), 7.25 (d, 2H, J = 7 Hz, H-aromatic).

Dibenzyl N-benzyloxycarbonyl-L-aspartate 80

Triethylamine (0.7 ml, 5 mmol) was added to a suspension of dibenzyl L-aspartate p-toluenesulfonate (5 mmol) in dry dichloromethane (20 ml). N-(benzyloxycarbonyloxy) succinimide (1.25 g, 5 mmol) was added to the remaining solution and stirring was continued at room temperature for 18 hours. The organic phase was washed with water (5 ml), 1M aqueous hydrochloric acid (5 ml), water (5 ml), and dried over magnesium sulfate. After evaporation of the solvent, the residue was recristallized from diethyl ether / pentane.

Yield 89%

MP = 61-63°C (Lit.[201] 61-63°C)
$[\alpha]_D^{25}$ = +4.6° (c=1.45, $CHCl_3$)
Elemental analysis : Found C 69.81 H 5.64 N 3.07%
Calc. for $C_{26}H_{25}NO_6$ C 69.79 H 5.63 N 3.13%

^{1}H NMR (220 MHz, $CDCl_3$) 3.0 (m, 2H, β), 4.75 (m, 1H, α), 5.15 (m, 4H, OCH_2-Ar), 5.95 (bd, 1H, NH), 7.35 (bs, 15H, H-aromatic).

General procedure for the hydrolysis of ester substrates by chymotrypsin suspended in dichloromethane in the presence of ammonium bicarbonate.

Resolution of ethyl N-acetyl-D,L-phenylalaninate 39

To a solution of ethyl N-acetyl-D,L-phenylalaninate 39 (1 mmol) in dichloromethane (25 ml) are added chymotrypsin (25 mg) and ammonium bicarbonate (5 mmol). The resulting suspension is vigourously stirred while water (50 µl, 0.2% v/v) is added.
After completion of the reaction (TLC dichloromethane/methanol 10%), the mixture is filtered. The precipitate is dissolved in methanol and crystallisation is induced by the addition of diethyl ether. N-acetyl-L-phenylalanine 36 (0.38 mmol) is obtained as a white powder (MP 168-169°C, $[\alpha]_D^{25}$ = +38.0 (c=1, MeOH).
The filtrate which contains ethyl N-acetyl-D-phenylalaninate 29 and the remaining of N-acetyl-L-phenylalanine 36, is washed with 0.5M aqueous sodium carbonate (5 ml) and water (5ml). After drying over magnesium sulfate and evaporation of the solvent, the pure ethyl N-acetyl-D-phenylalaninate 29 (0.5 mmol) is

recrystallized from ethyl acetate / petroleum ether 40-60°C (MP 92-94°C, $[\alpha]_D^{25}$ = -87.7 (c=1, $CHCl_3$).

Characteristics of the amino acid derivatives.

derivative		MP (°C)	$[\alpha]_D^{25}$
D-TyrOMe	44	133-135	-22.9° (c=2, MeOH)
(4-amino)-D-PheOMe	64	204-206	-16.0 (c=1, MeOH)
L-Asp(OMe)OH	78	176-180	+ 6.5 (c=1, MeOH)
Ac-L-TyrOH	34	150-152	+62.0° (c=1, MeOH)
Ac-L-TrpOH	38	200d	+17.8° (c=1.2, MeOH)
BOC-L-TyrOH	60	136-138	+ 4.2° (c=1.5, MeOH)

Amino acids identified by TLC by comparison with authentical samples :

amino acid		Rf (AcOEt/Pyridine/H_2O/AcOH 6:2:1:0.6)
L-TyrOH	41	0.25
L-AlaOH	74	0.11
(4-amino)-L-PheOH	63	0.11
L-OrnOH	82	0.04
L-LysOH	81	0.05

L-3-Phenyllactic acid 49

MP = 115-117°C

$[\alpha]_D^{25}$ = -23.0° (c=1, H_2O) (Lit.[202] 122-124°C and -20.8° (c=2, H_2O) respectively)

Elemental analysis : Found C 64.99 H 6.10%

Calc. for $C_9H_{10}O_3$ C 65.05 H 6.06%

^1H NMR (220 MHz, D_2O) 2.9 (q, 1H, β), 3.1 (q, 1H, β), 4.4 (q, 1H, α), 7.5 (m, 5H, H-aromatic).

3-(p-Hydroxyphenyl)-2-D,L-hydroxy propionic acid 53

MP = 133-135°C

Rf = 0.44 (CH_2Cl_2/MeOH 20%)

Elemental analysis : Found C 59.34 H 5.53%

$C_9H_{10}O_4$ requires C 59.63 H 5.83%

^1H NMR (220 MHz, D_2O) 2.75 (q, 1H, ^2J = 15 Hz, β), 2.95 (q, 1H, ^2J = 15 Hz, β), 4.15 (q, 1H, α), 6.8 (d, 2H, J = 9 Hz, H-aromatic), 7.15 (d, 2H, J = 9 Hz, H-aromatic).

3-(p-Hydroxyphenyl) propionic acid **54**

MP = 125-127°C

Elemental analysis : Found C 65.05 H 6.10%

$C_9H_{10}O_3$ requires C 65.05 H 6.06%

^1H NMR (220 MHz, D_2O) 3.45 (t, 2H, J = 7 Hz, β), 3.7 (t, 2H, J = 7 Hz, α), 7.7 (d, 2H, J = 7 Hz, H-aromatic), 8.0 (d, 2H, J = 7 Hz, H-aromatic).

N-Benzyloxycarbonyl-L-leucyl-L-tyrosine **66**

Rf = 0.55 (CH_2Cl_2/MeOH 20%)

N-Benzyloxycarbonyl-L-alanyl-L-tyrosine **68**

MP = 145-147°C

$[\alpha]_D^{25}$ = +14.0° (c=0.5, MeOH)

Rf = 0.28 (CH_2Cl_2/MeOH 20%)

MS Acc. Mass $C_{20}H_{22}N_2O_6$ 386.402 -3 ppm

^1H NMR (220 MHz, acetone-d^6) 1.4 (d, 3H, J = 7.3 Hz, CH_3 Ala), 3.0 (q, 1H, ^2J = 14.6 Hz, β Tyr), 3.1 (q, 1H, ^2J = 14.6 Hz, β Tyr), 4.4 (m, 1H, α), 4.8 (q, 1H, α), 5.2 (q, 2H, ^2J = 12.2 Hz, OCH_2-Ar), 6.9 (m, 3H, H-aromatic

and NH), 7.2 (d, 2H, J = 7.3 Hz, H-aromatic), 7.5 (m, 7H, H-aromatic, OH, and NH).

N-Benzyloxycarbonyl-D-alanyl-L-tyrosine **70**

Rf = 0.32 (CH_2Cl_2/MeOH 20%)

Ethyl N-benzyloxycarbonyl-L-phenylalaninate **83**

For experimental procedure, see page 259.

Yield 34%

$[\alpha]_D^{25}$ = +38.0° (c=1.6, $CHCl_3$)

MS CI 326 (MH^+), 282 (M-COOEt), 208 (M-COOEt-CO_2), 176 (cinnamate), 91 (benzyl).

IR (liquid film) cm^{-1} 3320 (NH), 3000 (aromatic), 1715 (ester), 1700 (amide), 1500 (aromatic), 735 and 690 (monosubstituted aromatic).

^1H NMR (220 MHz, $CDCl_3$) 1.15 (t, 3H, J = 7 Hz, CH_3), 3.05 (m, 2H, ^2J = 14.6 Hz, β), 4.1 (q, 2H, J = 7 Hz, OCH_2), 4.65 (q, 1H, α), 5.05 (s, 2H, OCH_2-Ar), 5.65 (d, 1H, J = 5 Hz, NH), 7.1-7.35 (m, 10H, H-aromatic).

N-tert-Butyloxycarbonyl-L-phenylalanyl-L-leucinamide 84

To a solution of ethyl N-benzyloxycarbonyl-L-phenylalaninate (117mg, 0.4 mmol) and L-leucinamide hydrochloride (67mg, 0.4 mmol) in 0.2M carbonate buffer pH 9.2 was added chymotrypsin (10mg). The solution was stirred at room temperature for 30 minutes and the reaction mixture filtered. The precipitate was recrystallized from methanol at room temperature.

Yield 90%

MP = 218-220°C

$[\alpha]_D^{25}$ = -17.7° (c=0.375, MeOH)

Elemental analysis : Found C 58.19 H 9.16 N 10.44%

$C_{20}H_{31}N_3O_4$ requires C 58.99 H 8.72 N 9.83%

^{1}H NMR (220 MHz, DMSO-d^6) 0.85 (t, 6H, J = 7 Hz, CH_3 Leu), 1.35 (s, 9H, BOC), 1.4-1.75 (m, 3H, β, γ Leu), 2.8 (q, 1H, ^{2}J = 14.6 Hz, β Phe), 3.0 (q, 1H, ^{2}J = 14.6, β Phe), 4.2 (bm, 1H, α), 4.3 (q, 1H, α), 7.1 (bs, 2H, amide), 7.35 (m, 6H, H-aromatic and NH), 7.9 (d, 1H, J = 7 Hz, NH).

Ethyl N-benzyloxycarbonyl-L-tyrosyl-glycyl-glycine 85

Triethylamine (0.111 ml, 0.8 mmol) was added to a suspension of ethyl glycyl-glycinate hydrochloride (0.157g, 0.8 mmol) in

dichloromethane (20 ml). Ethyl N-benzyloxycarbonyl-L-tyrosinate (0.275g, 0.8 mmol) was then added and the solution was stirred at room temperature while immobilized chymotrypsin (10mg) on Biofix E_1 (100mg) was added. After two days of reaction, the precipitate was filtered and dissolved in warm ethanol. After evaporation of the solvent, the dried residue was recrystallized from methanol.

Yield 84%

MP = 158-160°C

$[\alpha]_D^{25}$ = -5.5° (c=0.1, DMSO)

Rf = 0.28 (CH_2Cl_2/MeOH 5%)

Elemental analysis : Found C 59.45 H 6.12 N 9.20%

$C_{23}H_{27}N_3O_7$ requires C 58.89 H 6.38 N 8.58%

^1H NMR (220 MHz, CD_3CN) 1.25 (t, 3H, J = 7 Hz, ester), 2.85 (m, 2H, β Tyr), 3.85 (m, 4H, Gly), 4.15 (q, 2H, J = 7 Hz, OCH_2), 4.3 (m, 1H, α), 5.0 (q, 2H, J= 14 Hz, OCH_2-Ar), 6.15 (bs, 1H, NH), 6.8 (d, 2H, J = 9 Hz, H-aromatic), 7.1 (d, 2H, J = 9 Hz, H-aromatic), 7.2 (bs, 1H, NH), 7.4 (m, 6H, H-aromatic and OH).

L-Phenylalanyl-L-leucinamide trifluoroacetate 86

N-tert-butyloxycarbonyl-L-phenylalanyl-L-leucinamide (1g, 2.6 mmol) was added to a solution of trifluoroacetic acid in dry

dichloromethane (10 ml, 50%). After stirring at room temperature for 30 minutes, the solvent was evaporated under reduced pressure and the residue extensively dried under high vacuum. The dipeptide was recrystallized from methanol.

Yield 98%

MP = 191-193°C

$[\alpha]_D^{25}$ = +1.7° (c=0.6, MeOH)

Elemental analysis : Found C 48.43 H 5.75 N 9.79%

$C_{17}H_{24}N_3O_4F_3$ requires C 48.97 H 6.85 N 9.52%

^{1}H NMR (220 MHz, CD_3OD) 1.0 (t, 6H, J = 7 Hz, CH_3 Leu), 1.1-1.9 (m, 3H, β, γ Leu), 3.15 (q, 1H, ^2J = 14.6 Hz, β Phe), 3.35 (q, 1H, ^2J = 14.6 Hz, β Phe), 4.25 (q, 1H, α), 4.50 (t, 1H, α), 7.4 (m, 5H, H-aromatic).

N-Benzyloxycarbonyl-L-tyrosyl-glycyl-glycine 87

Ethyl N-benzyloxycarbonyl-L-tyrosyl-glycyl-glycinate (0.5g, 10.9 mmol) was added to a solution of sodium hydroxide (0.872g, 22 mmol) in methanol / water (25 ml, 1:1 (v/v)). The remaining suspension was stirred at room temperature for 24 hours. The mixture was filtered and the filtrate acidified with 1M aqueous solution of hydrochloric acid to pH 2-3. The aqueous phase was extracted with ethyl acetate (three times, 20 ml), and the

organic extracts were pooled, dried over magnesium sulfate, and the solvent was evaporated under reduced pressure. The crystalline residue was purified by recrystallisation from methanol / diethyl ether.

Yield 59%

MP = 184-186°C

$[\alpha]^{25}$ = +4.2 (c=0.3, MeOH)

MS Acc. Mass $C_{21}H_{23}N_3O_7$ 429.426 +0.2 ppm

^1H NMR (220 MHz, acetone-d^6) 2.9 (q, 1H, ^2J = 14.6 Hz, β Tyr), 3.15 (q, 1H, ^2J = 14.6 Hz, β Tyr), 4.0 (m, 4H, CH_2 Gly), 4.5 (m, 1H, α Tyr), 5.1 (q, 2H, J = 9.8 Hz, OCH_2-Ar), 6.8 (bd, 3H, J = 9 Hz, H-aromatic Tyr and NH), 7.1 (d, 1H, J = 9 Hz, NH), 7.2 (d, 2H, J = 9 Hz, H-aromatic Tyr), 7.35 (m, 5H, H-aromatic), 7.65 (bs, 1H, NH), 8.0 (bs, 1H, OH).

N-Benzyloxycarbonyl-L-tyrosyl-glycyl-glycyl-L-phenylalanyl-L-leucinamide 88

Triethylamine (0.028 ml, 0.2 mmol) was added to a suspension of L-phenylalanyl-L-leucinamide trifluoroacetate (78mg, 0.2 mmol) in dry acetonitrile (5 ml). N-benzyloxycarbonyl-L-tyrosyl-glycyl-glycine (86mg, 0.2 mmol) and EEDQ (54mg, 0.22 mmol) were added

and the solution stirred at room temperature overnight. The solvent was evaporated and the dried residue washed with 0.5M aqueous solution of hydrochloric acid, water, 0.5M aqueous solution of sodium bicarbonate, and water. The dried residue was purified by preparative thin-layer chromatography (dichloromethane / methanol 20%).

Yield 85%

MP = 169-172°C

$[\alpha]^{25}$ = -7.9° (c=0.98, MeOH)

Rf = 0.68 (CH_2Cl_2/MeOH 20%)

^{1}H NMR (220 MHz, DMSO-d^6) 1.0 (q, 6H, J = 7 Hz, CH_3 Leu), 1.8 (m, 3H, β,γ Leu), 2.8-3.3 (m, 4H, β Tyr and Phe), 3.9 (m, 4H, CH_2 Gly), 4.4 (m, 2H, α), 4.7 (m, 1H, α), 5.2 (q, 2H, J = 10 Hz, OCH_2-Ar), 5.7 (d, 1H, J = 7 Hz, NH), 6.8 (d, 2H, J = 9 Hz, H-aromatic Tyr), 7.2 (d, 2H, J = 9 Hz, H-aromatic Tyr), 7.4 (m, 10H, H-aromatic Phe and Z), 8.2 (m, 3H, NH), 8.4 (bs, 1H, NH), 9.3 (s, 1H, OH).

Monoethyl hydrogen malonate 89

A solution of potassium hydroxide (3.5g, 62 mmol) in absolute ethanol (40 ml) was added to a solution of diethyl malonate (10g, 62 mmol) in ethanol (40 ml). A white crystalline precipitate formed during the addition and stirring was continued for hours. After the mixture had stood overnight, it was heated to boiling and filtered while hot. Precipitation of the potassium ethyl malonate was completed by cooling the filtrate in an ice-water bath. The salt was collected by filtration, washed with a small amount of ether, and dried under reduced pressure. To a solution of potassium ethyl malonate (8g, 47 mmol) in water (5 ml) cooled in an ice-water bath, was added concentrated hydrochloric acid (4 ml) over 10 minutes while the temperature was maintained below 10°C. The mixture was filtered and the precipitate of potassium chloride washed with ether (10 ml). The aqueous layer of the filtrate was separated and extracted with three 10 ml-portions of ether. The combined ether solutions were dried over magnesium sulfate and the solvent was evaporated under reduced pressure. The oily residue was dried under reduced pressure overnight.

Yield 56%

MS Acc. Mass $C_5H_9O_4$ (MH^+) 133.0502 +0.9 ppm

IR (liquid film) cm^{-1} 3200-3000 (CO acid), 1710 (ester), 1660 (acid).

^1H (220 MHz, D_2O) 1.35 (t, 3H, J = 7 Hz, CH_3), 3.6 (s, 2H, CH_2), 4.3 (q, 2H, J = 7 Hz, OCH_2).

Synthesis of the magnesium salt 90

Magnesium methoxide (0.43g, 0.5 mmol) was added to a solution of hydrogen ethyl malonate (10 mmol) in dry THF (25 ml), and the mixture stirred for 2 hours. The solvent was removed under reduced pressure to give an oily residue which tended to solidify upon drying overnight under reduced pressure.

N-Benzyloxycarbonyl-O-benzyl-D-serine dicyclohexylammonium salt 91

To a solution of O-benzyl-D-serine (1g, 5.4 mmol) in 0.2M aqueous sodium hydroxide / DMF (20%) was added N-(benzyloxycarbonyloxy) succinimide (1.5g, 6 mmol). The remaining solution was stirred at room temperature for 24 hours. The mixture was acidified with 1M aqueous hydrochloric acid to pH 2-3 and extracted with ethyl acetate (10 ml, three times). The organic phase was dried over magnesium sulfate and the solvent evaporated under reduced pressure to give a colorless oil. The oily residue was dissolved in the minimal amount of methanol and dicyclohexylamine (0.98g, 5.4 mmol) was added. The dicyclohexylammonium salt was

precipitated with diethyl ether and recrystallized from methanol / ethyl acetate.

Yield 72%

MP = 86-88°C

$[\alpha]_D^{25}$ = -16.6° (c=0.5, $CHCl_3$)

Elemental analysis Found C 65.53 H 7.76 N 4.99%

$C_{30}H_{42}N_2O_5$ requires C 65.32 H 7.64 N 4.62%

^1H NMR (220 MHz, $CDCl_3$) 2.75-3.0 (m, 22H, DCHA), 3.85 (q, 1H, J = 12 Hz, β), 4.0 (q, 1H, J = 12 Hz, β), 4.6 (s, 2H, OCH_2-Ar), 4.6 (bm, 1H, α), 5.2 (s, 2H, OCH_2-Ar), 6.1 (d, 1H, J = 9 Hz, NH), 7.4 (m, 11H, H-aromatic and NH).

The finely powdered dicyclohexylammonium salt (1g, 1.96 mmol) was added to a two phase system of ethyl acetate (8 ml) and a solution of potassium hydrogen sulfate (0.386g, 2.9 mmol) in water (8 ml). The mixture was shaken until the dicyclohexylammonium salt was completely dissolved. The aqueous layer was extracted with ethyl acetate (twice with 10 ml each time) and the combined organic layers washed with water (5 ml, three times). the organic phase was then dried over magnesium sulfate and the solvent evaporated under reduced pressure.

General procedure for the synthesis of 3-keto esters via acylation.

Carbonyldiimidazole (180 mg, 1.1 mmol) was added to a solution of the acid (1 mmol) in dry THF (5 ml). After stirring at room temperature for 2 hours, the magnesium salt was added. The mixture was stirred 24 hours at room temperature and ethyl acetate (5 ml) was added. The reaction mixture was washed with 0.5M aqueous hydrochloric acid (10 ml, twice), and 1M aqueous sodium hydrogen carbonate (10 ml). The organic phase was then dried over magnesium sulfate and the solvent was removed under reduced pressure. The residue was purified either by flash chromatography or crystallisation.

Ethyl 5-phenyl-3-keto valerate **93**

Yield 93%

MS Acc. Mass $C_{13}H_{16}O_3$ 220.1095 -2.1 ppm

IR (liquid film) cm^{-1} 2900 (CH), 1715 (ester), 1695 (CO), 1600 and 1500 (aromatic), 700 and 660 (monosubstituted aromatic).

^1H NMR (220 MHz, $CDCl_3$) 1.25 (t, 3H, J = 7.4 Hz, CH_3), 2.90 (m, 4H, C-4 and C-5), 3.45 (s, 2H, C-2), 4.2 (q, 2H, J = 7.4 Hz, OCH_2), 7.3 (m, 5H, H-aromatic).

Ethyl 5-(p-hydroxyphenyl)-3-keto valerate **94**

Yield 56%

MS Acc. Mass $C_{13}H_{16}O_4$ 236.1045 -1.5 ppm

IR (liquid film) cm^{-1} 3400 (OH), 1710 (ester), 1690 (CO), 1600 and 1500 (aromatic), 820 (parasubstituted aromatic).

^1H NMR (220 MHz, $CDCl_3$) 1.5 (t, 3H, J = 7.4 Hz, CH_3), 3.0 (s, 4H, C-4 and C-5), 3.6 (s, 2H, C-2), 4.4 (q, 2H, J = 7.4 Hz, OCH_2), 7.0 (bd, 3H, J = 7 Hz, H-aromatic and OH), 7.3 (d, 2H, J = 7 Hz, H-aromatic).

Ethyl 4-(S)-acetamido-5-phenyl-3-keto valerate **95**

Yield 88%

MP = 86-87°C

$[\alpha]^{25}$ = +17.1° (c=0.5, $CHCl_3$)

Elemental analysis Found C 64.97 H 7.05 N 4.89%

$C_{15}H_{19}NO_4$ requires C 64.97 H 6.91 N 5.05%

^1H NMR (220 MHz, $CDCl_3$) 1.25 (t, 3H, J = 6.7 Hz, CH_3), 1.95 (s, 3H, acetyl), 3.0 (q, 1H, ^2J = 14 Hz, ^3J = 6 Hz, C-5), 3.15 (q, 1H, ^2J = 14 Hz, ^3J = 6 Hz, C-5), 3.5 (q, 2H,

^2J = 15 Hz, C-2), 4.2 (q, 2H, J = 6.7 Hz, OCH_2), 4.9 (q, 1H, ^3J = 6 Hz, J_{NH} = 7 Hz, C-4), 6.5 (bd, 1H, J = 7 Hz, NH), 7.3 (m, 5H, H-aromatic).

Ethyl 4-(S)-N-(benzyloxycarbonyl)-amino-5-phenyl-3-keto valerate 97

Yield 92%

MP = 71-72°C

$[\alpha]^{25}$ = +7.0° (c=1, $CHCl_3$)

Elemental analysis Found C 68.30 H 6.26 N 3.78%

$C_{21}H_{23}NO_5$ requires C 68.28 H 6.27 N 3.79%

^1H NMR (220 MHz, $CDCl_3$) 1.25 (t, 3H, J = 6.7 Hz, CH_3), 3.05 (q, 1H, ^2J = 22 Hz, C-5), 3.20 (q, 1H, ^2J = 22 Hz, C-5), 3.5 (q, 2H, ^2J = 17 Hz, C-2), 4.2 (q, 2H, J = 6.7 Hz, OCH_2), 4.75 (q, 1H, C-4), 5.1 (q, 2H, ^2J = 12 Hz, OCH_2-Ar), 5.4 (d, 1H, J = 7.4 Hz, NH), 7.3 (m, 5H, H-aromatic).

Ethyl 4-(S)-N-(tert-butyloxycarbonyl)-amino-6-methyl-3-keto heptanoate 99

Yield 5%

$[\alpha]^{25}$ = +13.4° (c=0.7, $CHCl_3$)

MS FAB m/z 302 (MH^+), 246, 202, 186, 176, 132.

MS Acc. Mass $C_{10}H_{18}O_3^{+\cdot}$ 186.1499 +1.39 ppm

^1H NMR (220 MHz, $CDCl_3$) 0.95 (d, 6H, J = 5 Hz, CH_3 Leu), 1.3 (t, 3H, J = 6.7 Hz, CH_3), 1.45 (s, 9H, BOC), 1.7 (m, 3H, C-5 and C-6), 3.65 (s, 2H, C-2), 4.25 (q, 2H, J = 6.7 Hz, OCH_2), 4.35 (bm, 1H, C-4), 5.1 (d, 1H, J = 9 Hz, NH).

Ethyl 4-(S)-N-(benzyloxycarbonyl)-amino-6-methyl-3-keto valerate 101

Yield 12%

$[\alpha]^{25}$ = +7.8° (c=0.6, $CHCl_3$)

MS CI 280 $(M-C_4H_8)^+$, 236 $(M-C_4H_8-CO_2)$, 220, 176, 91.

MS EI 220, 176, 91.

MS Acc. Mass $C_{14}H_{17}NO_5^{+\cdot}$ 229.5118 +0.10 ppm

^1H NMR (400 MHz, $CDCl_3$) 0.90 (d, 6H, J = 5 Hz, CH_3 Leu), 1.3 (t, 3H, J = 6.7 Hz, CH_3), 1.7 (m, 3H, C-5 and C-6), 3.60 (s, 2H, C-2), 4.20 (q, 2H, J = 6.7 Hz, OCH_2), 4.35 (bm, 1H, C-4), 5.0 (q, 2H, J = 12 Hz, O-CH_2-Ar), 5.1 (d, 1H, J = 9 Hz, NH), 7.35 (m, 5H, H-aromatic).

Ethyl 4-(R)-N-(benzyloxycarbonyl)-amino-5-benzyloxy-3-keto valerate 102

Yield 84%

$[\alpha]^{25} = -2.5°$ (c=1.35, $CHCl_3$)

MS Acc. Mass $C_{12}H_{10}O_4^{+\cdot}$ 218.0800 −0.4 ppm

MS EI 282 ($M-COCH_2COOEt$), 218, 115 ($COCH_2COOEt$), 108 (benzyloxy), 91.

IR (liquid film) cm^{-1} 3400 (NH), 1720 (CO), 1500 (aromatic), 740 and 710 (monosubstituted aromatic).

^1H NMR (220 MHz, $CDCl_3$) 1.25 (t, 3H, J = 6.7 Hz, CH_3), 3.6 (bs, 2H, C-2), 3.65 and 3.85 (2q, 2H, J = 9.8 Hz, C-5), 4.2 (q, 2H, OCH_2), 4.5 (bs, 2H, OCH_2-Ar), 4.6 (m, 1H, C-4), 5.15 (s, 2H, OCH_2-Ar), 5.85 (d, 1H, J = 7 Hz, NH), 7.35 (m, 10H, H-aromatic).

Methyl N-benzyloxycarbonyl-L-leucinate 103

To a solution of methyl L-leucinate hydrochloride (3g, 16.5 mmol) in dichloromethane (50 ml) was added triethylamine (2.3 ml, 16.5 mmol) and N-(benzyloxycarbonyloxy) succinimide (4.5g, 18 mmol). The remaining solution was stirred at room temperature for 18 hours and the organic phase was washed with water (10 ml), 1M aqueous hydrochloric acid (10 ml), and water (10 ml). After

drying over magnesium sulfate, the solvent was removed under reduced pressure and the oily residue was purified by flash column chromatography (dichloromethane).

Yield 93%

$[\alpha]^{25}$ = -28.2° (c=0.9, $CHCl_3$)

MS Acc. Mass $C_{15}H_{21}NO_4$ 279.1468 -1.0 ppm

IR (liquid film) cm^{-1} 3340 (NH), 1715 (ester), 1690 (amide), 1500 (aromatic), 730 and 700 (monosubstituted aromatic).

^1H NMR (220 MHz, $CDCl_3$) 0.9 (t, 3H, J = 2.5 Hz, CH_3), 2.05 (m, 3H, β,γ), 3.75 (s, 3H, OCH_3), 4.4 (m, 1H, α), 5.1 (s, 2H, OCH_2-Ar), 5.25 (d, 1H, J = 7.4 Hz, NH), 7.35 (s, 5H, H-aromatic).

N-Carboxy-L-phenylalanine anhydride **104**

To a solution of N-benzyloxycarbonyl-L-phenylalanine **96** (5g, 16.7 mmol) in dry dichloromethane (30 ml) cooled in an ice-water bath is added phosphorous pentachloride (3.5g, 16.7 mmol). After nearly all the phosphorous pentachloride was dissolved, the reaction mixture was filtered and the solution permitted to stand at room temperature for 1 hour. The solution was evaporated under

reduced pressure and the dry residue was recrystallized from anhydrous dichloromethane / diethyl ether.

Yield 95%

MP = 123-125°C (Lit.[203] 125-126°C)

Elemental analysis Found C 62.78 H 4.89 N 7.14%

$C_{10}H_{10}NO_3$ requires C 62.82 H 4.75 N 7.32%

^1H NMR (220 MHz, $CDCl_3$) 3.05 (q, 1H, ^2J = 14.7 Hz, β), 3.25 (q, 1H, ^2J = 14.7 Hz, β), 4.6 (m, 1H, α), 6.85 (bs, 1H, NH), 7.25 (m, 2H, H-aromatic), 7.4 (m, 3H, H-aromatic).

Reduction of 3-keto esters with resting cells

A suspension of fresh baker's yeast (10g) and sucrose (10g) in water (200 ml) was stirred at 30°C for 30 minutes. Then the substrate (1 mmol) in ethanol (5 ml) was added and the reaction was followed by TLC (dichloromethane / methanol 2.5%). When the reaction was complete, celite (20 g) was added and the mixture was stirred for a further 30 minutes. The suspension was then filtered and the filtrate extracted with diethyl ether (50 ml, four times), the combined organic extracts were dried over magnesium sulfate, and the solvent removed under reduced pressure. The residue was purified by flash column chromatography (dichloromethane / methanol 2%).

Ethyl 3-(R,S)-hydroxy-5-phenyl pentanoate **105**

Yield 48% (92% conversion)
ee = 46%

$[\alpha]^{25}$ = -1.5° (c=0.75, $CHCl_3$)
MS Acc. Mass $C_{13}H_{18}O_3$ 222.1259 +1.4 ppm
IR (liquid film) cm^{-1} 3450 (OH), 1715 (ester), 1600 and 1480 (aromatic), 745 and 695 (monosubstituted aromatic).

^1H NMR (220 MHz, $CDCl_3$) 1.3 (t, 3H, J = 7.4 Hz, CH_3), 1.8 (m, 2H, C-4), 2.5 (q, 2H, J = 17 Hz, C-2), 2.8 (m, 2H, C-5), 3.2 (bs, 1H, OH), 4.1 (bm, 1H, C-3), 4.25 (q, 2H, J = 7.4 Hz, OCH_2), 7.35 (m, 5H, H-aromatic).

Ethyl 3-(R,S)-hydroxy-4-(S)-acetamido-5-phenyl pentanoate **106**

Yield 9% (36% conversion)
de = 52%

Elemental analysis Found C 64.54 H 7.57 N 4.95%
$C_{15}H_{21}NO_4$ requires C 64.49 H 7.58 N 5.01%

^1H NMR (220 MHz, $CDCl_3$) 1.25 (m, 3H, CH_3), 1.9 (s, 3H, acetyl b), 2.0 (s, 3H, acetyl a), 2.25-3.05 (m, 6H, C-2, C-3, C-

5 and OH), 4.2 (m, 3H, OCH_2 and C-4), 6.25 (d, 1H, NH b), 6.4 (d, 1H, NH a), 7.35 (m, 5H, H-aromatic).

Ethyl 3-(2-methoxy-2-trifluoromethylphenylacetoxy)-5-phenyl pentanoate **107**

To dry pyridine (2 ml, 24.7 mmol) in a dry flask was added under nitrogen (S)-(-)-2-methoxy-2-trifluoromethyl-2-phenylacetic acid chloride (378 mg, 1.5 mmol), dry dichloromethane (2 ml), and ethyl 3-hydroxy-5-phenyl pentanoate (222 mg, 1 mmol). The reaction was stirred at room temperature and the formation of the products monitored by TLC. When the reaction was complete (4 hours), water was added and it was extracted with dichloromethane. The combined organic extracts were washed with 0.5M aqueous hydrochloric acid, 1M aqueous sodium hydrogen carbonate, and dried over magnesium sulfate. Evaporation of the solvent under reduced pressure, and purification by flash chromatography (dichloromethane) yielded the product.

^{1}H NMR (400 MHz, benzene-d^6) 3.47 ppm (s, 3H, OCH_3) 27%
3.55 ppm (s, 3H, OCH_3) 73%

^{13}C NMR (100.62 MHz, benzene-d^6) 60.60 ppm (s, OCH_3) 72%
60.75 ppm (s, OCH_3) 28%

General procedure for the diastereoselective reduction with sodium borohydride

To a solution of the 3-keto ester (1 mmol) in dry THF (5 ml) cooled in an ice-water bath was added solid sodium borohydride (0.5 mmol). When the reaction was complete, 1M aqueous hydrochloric acid (2 ml) was added and the reaction mixture was extracted with ethyl acetate (3 ml, three times). The combined organic extracts were dried over magnesium sulfate and the solvent was evaporated under reduced pressure.

Ethyl 3-(R)-hydroxy-4-(S)-acetamido-5-phenyl pentanoate 108

Rf = 0.3 (dichloromethane / methanol 5%)

MP = 108-110°C

$[\alpha]_D^{25}$ = -4.2° (c=0.225, $CHCl_3$)

^1H NMR (400 MHz, $CDCl_3$) 1.25 (t, 3H, J = 7 Hz, CH_3), 1.875 (s, 3H, acetyl), 2.5 (m, 2H, ^2J = 16 Hz, k and l), 2.85 (q, 1H, ^2J = 14 Hz, j), 2.95 (q, 1H, ^2J = 14 Hz, i), 3.8 (d, 1H, J = 4 Hz, h), 4.05 (m, 1H, g), 4.15 (q, 2H, J = 7 Hz, OCH_2), 4.2 (m, 1H, e), 5.55 (d, 1H, J = 8 Hz, NH), 7.2-7.3 (m, 5H, H-aromatic).

Ethyl 3-(S)-hydroxy-4-(S)-acetamido-5-phenyl pentanoate **109**

Rf = 0.35 (dichloromethane / methanol 5%)

$[\alpha]_D^{25}$ = -15.9° (c=0.6, $CHCl_3$)

^1H NMR (400 MHz, $CDCl_3$) 1.2 (t, 3H, J = 7 Hz, CH_3), 1.925 (s, 3H, acetyl), 2.35 (q, 1H, ^2J = 17 Hz, j), 2.5 (q, 1H, ^2J = 17 Hz, i), 2.9 (d, 2H, ^2J = 8 Hz, k and l), 3.75 (bd, 1H, J = 2 Hz, h), 4.0 (bdd, 1H, g), 4.10 (q, 2H, OCH_2), 4.15 (m, 1H, e), 6.0 (d, 1H, J = 10 Hz, NH), 7.25 (m, 5H, H-aromatic).

Ethyl 3-(R,S)-hydroxy-4-(S)-acetamido-5-phenyl pentanoate **106**

Yield 95%

de = 40%

Ethyl 3-(R,S)-hydroxy-4-(S)-N-(benzyloxycarbonyl)-amino-5-phenyl pentanoate **110**

Yield 95%

de = 72% (^1H and ^{13}C NMR)

Elemental analysis Found C 67.77 H 6.82 N 3.67%

$C_{21}H_{25}NO_5$ requires C 67.91 H 6.78 N 3.77%

Ethyl 3-(R,S)-hydroxy-4-(S)-N-(benzyloxycarbonyl)-amino-6-methyl heptanoate **111**

Yield 90%

de = 50% (^{1}H NMR 400 MHz)

Elemental analysis Found C 64.17 H 8.06 N 4.09%

$C_{18}H_{17}NO_5$ requires C 64.07 H 8.07 N 4.15%

Ethyl 3-(R,S)-hydroxy-4-(R)-N-(benzyloxycarbonyl)-amino-5-benzyloxy pentanoate **112**

Yield 95%

de = 60% (^{1}H NMR 400 MHz)

Elemental analysis Found C 66.24 H 6.76 N 3.41%

$C_{22}H_{25}NO_6$ requires C 66.15 H 6.31 N 3.51%

Diastereoselective reduction with lithium tributylalumino hydride

To a solution of ethyl 4-(S)-acetamido-5-phenyl-3-keto valerate 95 (277 mg, 1 mmol) in dry THF (5 ml) cooled to -20°C was added lithium tributylaluminohydride (400 mg, 2 mmol). After stirring at -20°C for 4 hours, 1M aqueous hydrochloric acid (1 ml) was added and the solution was concentrated under reduced pressure.

Ethyl acetate (10 ml) was added and the organic phase was washed with water (4 ml), 0.5M aqueous sodium hydrogen carbonate (4 ml), and water (4 ml), and dried over magnesium sulfate. The solvent was evaporated under reduced preasure and the oily residue was purified by preparative thin-layer chromatography (dichloromethane / methanol 5%).

Yield 20%

de = 5% (^{1}H NMR 220 MHz)

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