A: THE PRIMARY STRUCTURE OF HEMOGLOBINS
FROM TWO CARNIVORA
1) Lion (Panthera leo)
2) Tiger (Panthera tigris)

B: CLASSICAL SYNTHESIS OF THYMOSIN β9 (1-4, 22-24, 33-41)

THESIS SUBMITTED IN FULFILMENT
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

By

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H.E.J. RESEARCH INSTITUTE OF CHEMISTRY
UNIVERSITY OF KARACHI
JULY 1988
In the Name of Allah, the Beneficent, the Merciful
Dedicated to

My parents
    for their affection

My sisters and brother
    for their love

My husband
    for his understanding

My child
    for her tolerance
ACKNOWLEDGEMENT

It is a great pleasure for me to express the deepest sense of gratitude to Prof. Dr. Zafar H. Zaidi for his guidance and cooperation throughout this work.

My thanks are due to Prof. Dr. Wolfgang Voelter, Physiologisch Chemisches Institute, University of Tuebingen, for his guidance and advise during my stay in Tuebingen, where part of my synthetic work was completed. I am also grateful to all his co-workers, especially Dr. H. Echnner, Dr. H. Kallacher and Dr. Stock for their enlightening suggestions and help.

I am grateful to Prof. Dr. Gerhard Braunitzer, Department of Protein Chemistry, Max-Planck Institute for Biochemistry, Munich, West Germany for his expert guidance, help and encouragement and providing all the laboratory facilities during my stay in Munich.

Special thanks are due to Prof. Dr. Salimuzzaman Siddiqui, F.R.S., Director of Institute, for providing the facilities to work in the field of Protein Chemistry.

I gratefully acknowledge Prof. W. Schaefer, Max-Planck Institute for Biochemistry for performing mass spectra of the blocked peptide and B. Sohrank, R. Mentele, R. Gantsch and E. Wottawa for the technical assistance.
This work would not have been possible without the financial assistance of the Ministry of Science and Arts, Baden Württemberg Stuttgart and Max-Planck Gesellschaft, West Germany to work in the laboratories of Prof. Dr. W. Voelter and Prof. Dr. G. Braunitzer respectively.

I take this opportunity to thank the technical staff of the H.E.J. Research Institute of Chemistry for their cooperation and to all my friends and laboratory fellows in Pakistan as well as in Germany who have assisted me during the course of this work.

I am thankful to Mr. Zahir Shah Akhunsada and Dr. Sabira Naqui for proof reading. Finally I would like to thank Mr. Mohammad Afzal for his efforts in composing this thesis.

MEENO JABAN
ABBREVIATIONS

AcOH = acetic acid
Boc = tert.-butyloxycarbonyl
CM = Carboxymethyl
DCC = N,N'-dicyclohexylcarbodiimide
DCHA = dicyclohexylamine
DEAE = diethylamino ethyl
DMSO = dimethylsulfoxide
DTE = dithioerythritol
EDTA = ethylenediamine tetraacetic acid
E-rosette = a rosette with sheep erythrocytes
EtOAc = ethyl acetate
FAB = fast atom bombardment
FCS = fetal calf serum
GvH = graft versus host
Hb = hemoglobin
HOBT = 1-hydroxybenzotriazol
HOSu = N-hydroxysuccinimide
MAF = macrophage activating factor
MeOH = methanol
MIF = migration inhibiting factor
MLC = mixed-lymphocyte-culture
OBu = tert.-butyl ester
OMe = methyl ester
ON\(_p\) = p-nitrophenyl ester
PBS = phosphate buffered saline
PTH = phenylthiohydantoin
RPMI = Rosewell Park Memorial Institute
RP-HPLC = reversed phase high-performance liquid chromatography
TdT = terminal nucleotidyl transferase
TFA = trifluoro acetic acid
TFV = thymosin fraction V
THF = tetrahydrofuran
tlc = thin-layer chromatography
Top-phe-CH\(_2\)Cl = (N-Tosyl-L-Phenylalanyl) chloromethane
Tp = Tryptic peptide
Z = Benzyloxyacetyl
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SUMMARY

The complete primary structure of hemoglobin from lion (Panthera leo) and tiger (Panthera tigris) has been determined. Globin of both animals contain two components identified to be HbI and HbII. The \( \beta \) chains of the two animals are identical while they differ in their \( \alpha \) chains.

Native globin chains were separated on ion-exchange and RP-HPLC column. The sequence of the globin chains as well as tryptic peptides was studied by automatic Edman degradation methods using liquid-phase sequencer and gas-phase sequencer. The intact subunits were digested by enzymatic cleavage into tryptic peptides. The sequences were compared for homology with human hemoglobin. The \( \beta \) chain of the major components (\( \beta I \)) has a blocked N-terminal residue, identified as acetylated serine, whereas that of the minor component (\( \beta II \)) is free glycine. The two hemoglobin components of lion as well as tiger have identical \( \alpha \) chains and differ with respect to their \( \beta \) chains at the following positions (\( \beta I/\beta II \)): M1 Ac-Ser/Gly, B11 Ser/Thr, B17 Ser/Asn and B21 Arg/Lys. The structural and functional aspects of these exchanges are discussed.

Homologous alignment of the structure with 14 other animals belonging to different families of order Carnivora discloses many interesting points.
1. INTRODUCTION
1.1: GENERAL INTRODUCTION

Hemoglobin was described by L.J. Henderson, as one of the most interesting substance in the world [1]. However, hemoglobin is not a single substance but a group of related globins to which the same prosthetic group, heme is attached.

Those hemoglobins that exist in nature today represent the end result of a very long period of evolution and are superbly adapted to the specialized and varied functions that they undertake in the cells of even the lowest animal.

Hemoglobin was the first protein isolated in crystalline form by Reichert [2]. Pflueger associated it with its specific physiological function [3]. Immunological studies by Nuttall [4] and crystallographic studies by Reichert [5] on hemoglobins provided the first indication for a protein structure which was specific to the species. Hemoglobin was one of the first proteins of which the molecular weight and amino acids composition could be established accurately by Adair [6]. The primary structure, i.e. the amino acid sequence of Hb-A was determined by Braunitzer et al. [7] and its spatial configuration was determined through X-ray structure analysis by Perutz et al. [8].

1.2: STRUCTURE OF HEMOGLOBIN

Mammalian hemoglobin is a globular protein with a maximal diameter of 6.4nm and a molecular mass of about 64,500. It is composed of
are having 146 amino acids. 63 identical residues of the \( \alpha \) and \( \beta \) chains occupy common positions when those chains are placed side-by-side on introducing gaps named as Braunitzer gaps, at appropriate intervals along them. When the primary structures of more than 20 mammalian hemoglobins are compared, 75 residues of the \( \alpha \) chain and 64 residues of the \( \beta \) chain are invariant. It is known that these invariant residues occupy the sites that are of vital importance for the maintenance of normal structure and function.

1.2.2: Three dimensional structure of Hemoglobin

Amino acid chains are more stable in the coil, or helical configuration. The configuration was first proposed by Pauling and Corey in 1950 [11]. One type of coil is called the \( \alpha \) helix in which 3.6 amino acids are formed per turn of the helix with a 5.4\( \text{Å} \) pitch. The helix is stabilized by hydrogen bonding between the carbonyl group of each residue and the amide hydrogen four residues away. The helical structure is the secondary structure. The helices are named \( \text{A to H} \) and the non helical segment \text{NA, AB} \rightarrow \text{HC} \) from the amino to the carboxyl terminus.

The peptide chains are folded forming a compact structure. The three-dimensional structure of a protein subunit is called the tertiary structure. It stabilized by van der Waals interactions between the non-polar residues, by hydrogen bonds between Ser and Thr residues.
filling the interior space and by hydrogen bonds between polar residues at the surface. The heme group is inserted in a pocket between the E and F helices.

Its three-dimensional structure was elucidated by Perutz [12] using X-ray crystallography. The heme-globin linkage is stabilized by a covalent bond between the iron atom and N, of F8 His and by many non-polar residues lining the pocket.

1.3: FUNCTION OF HEMOGLOBIN

The principal function of hemoglobin is to transport oxygen from the lungs to the tissues and to facilitate the transport of carbon dioxide from the tissues to the lungs, "his dual function is achieved through a reversible change of structure which was discovered for the first time by Hauro witz [13]. The bond between oxygen and heme iron is so weak that the equilibrium between them can be shifted by a change in the chemical potential of oxygen, consequently by a change of concentration of oxygen in the physiological range. 15g of hemoglobin, which is contained in 100ml of blood can combine with 20.7ml of oxygen.

The oxygen unloaded from Hb travels by diffusion to myoglobin which has a higher affinity for oxygen than hemoglobin and finally to cytochrome oxidase, which is the ultimate enzyme of the electron transfer system and has a higher oxygen affinity than myoglobin.
Hemoglobin plays a dominant role not only in oxygen transport but also in carbon dioxide transport. The reactions involved in the gas transport is as follows:

\[
\begin{align*}
\text{Hb} \quad 
\text{H}^+ + \text{O}_2 & \rightarrow \text{HbO}_2 + \text{H}^+ \\
\text{Hb} \quad 
\text{CO}_2 + \text{O}_2 & \rightarrow \text{HbO}_2 + \text{CO}_2 \\
\text{Hb} \quad 
\text{DPG} + \text{O}_2 & \rightarrow \text{HbO}_2 + \text{DPG} \\
\text{H}^+ + \text{HCO}_3^- & \rightarrow \text{CO}_2 + \text{H}_2\text{O}
\end{align*}
\]

Increasing the concentration of hydrogen ions, CO$_2$ or DPG (2,3-diphosphoglycerate) lowers the oxygen affinity of hemoglobin. The oxygen affinity of hemoglobin is determined by the relative stabilities of the oxy and deoxy structures, and the deoxy structure has a low oxygen affinity, than the oxy structure.

The Bohr effect is a lowering of the oxygen affinity of hemoglobin with decreasing pH. Most of the Bohr effect is caused by an interaction between positively charged weak bases and negatively charged groups in the deoxy structure. Carbon dioxide binds to hemoglobin by reacting reversibly with the \(\alpha\) amino group to form carbamino groups in the deoxy structure.

Benesches et al. [14] measured the binding of DPG to hemoglobin and found only one mole of DPG bound per deoxyhemoglobin tetramer, with an association constant of about \(10^5\) M$^{-1}$. The binding site in
deoxy hemoglobin was located by a combination of chemical [15] and crystallographic [16,17] work.

One molecule of DPG is able to form salt bridges with an array of positively charged groups in a cavity between the two \( \beta \) chains. There are two small anion in the DPG binding site positioned between the \( \beta \) chain alpha amino group and epsilon amino group of \( \beta \)-82 Lys [17]. These anions are probably expelled when either DPG or CO\(_2\) is bound in the cavity.

1.4: EVOLUTION AND PHYLOGENY

Structural studies on molecular evolution began in 1959, when Perutz examined the low resolution X-ray structure of horse methemoglobin [18]. Subsequently a number of research groups investigated the phylogeny and evolution of hemoglobin by comparison of homologous proteins with more or less related animals [19]. The similarity of hemoglobin molecules was first discovered on comparison of sequence of \( \alpha \)- and \( \beta \)-chains of human hemoglobins with human myoglobins [20]. For this relationship two processes may be possible in principle: either divergence or convergence. In divergence, a common precursor gene has to be assumed from which the successor genes evolve in the course of evolution through gene duplication, mutation and selection into its present constitution and multiplicity. In contrast, in the opposite process, non-homologous genes are assumed as precursors
which have again reached the present degree of relationship through processes of similar nature during the evolution. In order to understand the evolution of hemoglobin, the primary structure of phylogenetically more or less related hemoglobins has to be determined, because only the primary structure provides an exact information about the degree of relationship. The data so far support the divergence theory. Through computer analyses of several homologues proteins, genealogical tree have already been prepared hemoglobin by Zuckerkandl [21] and Goodman et al. [22], cytochrome c by Fitch and Margoliash [23] and immunoglobins by Bakker et al. [24]. The time period for the appearance of a mutation (mutation interval) is different for different proteins and this cause a different periodic dissipation of the individual genealogical trees.

Hemoglobin consists of α and β chains, which are present in pairs in all vertebrates except the Agnatha (hay fishes and lampreys). Hemoglobin has advantage over other proteins because it consists of two types of chains and these different chains are homologous among each other which can therefore be compared not only with the related chain types but also the α-chain with β-chain and with the likewise homologous myoglobin.

Zuckerkandl and Pauling [25] calculated that $14.5 \times 10^6$ years were required for the substitution of one amino acid of hemoglobin for
another. Later, Zuckerkandl and Pauling [26] calculated that $7 \times 10^6$ years were required for the substitution of one amino acid for another during the evolution of mammalian hemoglobins.

Kimura calculated the rate of change ($k_{aa}$: Changes per year per site) for each amino acid position in the molecular evolution of a protein and found a value of $1 \times 10^{-9}$ per year for hemoglobin [27,28]. Dickerson in 1971 defined the period needed for a 1% change in a protein within 1 million years as a "UEP" (m.y.) unit [29]. In the case of hemoglobin, the period needed for a 1% change is 5.8 m.y. These calculations were done on the assumption that the rate of molecular evolution of each protein is almost uniform.

Goodman et al. [30,22,32] Barnabas et al. [33] and Moore et al. [34,35] have constructed genealogical trees using the maximum parsimony method and have calculated the rate of molecular evolution of hemoglobins. The mean value obtained by Goodman et al. was 31 NR%. The unit "NR%" indicates the rate of change of nucleotides per 100 codons during $1 \times 10^8$ years.

Analogously, the rapid and slow periods in the rates of molecular evolution of homologous globin chains of hemoglobin were distinguished. It is assumed that the period of rapid evolution corresponds to the period when the structure of hemoglobin molecule was being formed,
and the slow period corresponds to the time when its structure had become stabilized.

The rates (NR%) of molecular evolution of hemoglobins in mammals after eutherian ancestors is supposed to have emerged $90 \times 10^6$ years ago. Comparing $\alpha$ and $\beta$ chains, the $\beta$ chain show generally more rapid evolution in mammalian hemoglobins.

1.4.1: Evolution in carnivora

The family tree of Carnivores was deduced from the fossil evidence and comparative morphology by Homer [36] and Young [31]. They estimated that the cat (Felidae) and dog (Canidae) families separated from one another in the late Eocene, approximately 40 million years ago. During Miocene, which began 20-25 million years ago, the bear (Ursidae) and raccoon (Procyonidae) families branched off from the dog family. Wurster [37], on the basis of Karyotypic similarities, believe that modern species of Felidae are derived from a common ancestor as recently as five million years ago.

1.5: MAMMALIAN HEMOGLOBIN

There are interesting and meaningful differences in the functional behavior of hemoglobins among mammals. Unlike red cells of all other vertebrates, mammalian red cells lack nuclei. All mammalian hemoglobins have a substantial Bohr effect [38]. The residues that contribute the
alkaline Bohr effect in human hemoglobin are highly conserved in other mammals. There is a small degree of variability in the Bohr effect among mammals, owing in part to differential proton binding that accompanies their interaction with organic phosphate and in part to other intrinsic differences among hemoglobins.

The negatively charged groups of 2,3-DPG form electrostatic bonds with specific positively charged residue on the β-chains of deoxy-hemoglobin A; N-terminal Val, NA2 His, EF6 Lys, and H21 His [49].

A comparison of the primary structure of the globin chains of more than 50 mammals shows that these residues are highly conserved [39,50].

Perutz [51,40] has observed that the mammalian hemoglobins with intrinsically low oxygen affinity have hydrophobic residues at position β,NA2, whereas mammals with high affinity hemoglobin has a hydrophilic residues at βNA2. Hydrophobic residues at this position are directed into the interior of the molecule in such a way that the deoxy conformation is stabilized leading to lowered oxygen affinity.

1.5.1: Carnivora hemoglobin

A comparative study of carnivora hemoglobins was done by Seal [41] with the aid of gel electrophoresis. He examined that all members of family Canoidea had a major hemoglobin component of identical mobility, and concluded that hemoglobins had not diversified since the Eocene.
Studies on tryptic peptide compositions as reported by Brimhall [42] show that such diversity does indeed exist and that hemoglobins of different carnivora species contain many amino acids differences which do not involve changes in electrophoretic mobility.

The family tree of carnivores was deduced from fossil evidence and comparative morphology by Romer [35] and Young [37]. The structure of 13 carnivore hemoglobin has been studied so far [43], including two from Mustellidae (ferret, badger), one from Procyonidae (raccoon), five from Ursidae (Bear), two from Canidae (dog, coyote), two from Felidae and one from Phocidae (Harbor seal).

1.5.2: Felidae Hemoglobin

Lion and tiger belong to the family Felidae which together with the family Canidae form the genus panthera of the order carnivora. They are terrestrial nocturnal hunters with short but high skull and large eyes, and occur mostly in the tropics but have a wide range of distribution, except in Australia and Madagascar.

Electrophoresis of the hemoglobins of three genera, Felis (puma, wild cat and domestic cat), Acinonyx (cheetah) and Panthera (Bengal tiger and snow leopard) on starch gel and cellulose acetate is reported [44,52]. Hemoglobins of the three members of genus Panthera (Cat, Amur leopard, Jaguar) have already been sequenced [107,45,46].
2,3-Diphosphoglycerate (2,3-DPG) is most effective modulator of oxygen affinity in mammals. However, it has been demonstrated that members of the family Felidae possess hemoglobins with little or no response towards 2,3-DPG, Taketa [47]. Furthermore members of this family uniformly possess blocked N-terminal, Taketa [48], serine for glycine, occurs in the NH₂-terminal position of the β-chain, and the amino group of the serine is blocked with an acetyl group accounts for a unit charge difference between the two β-chains.
1.6: AIM OF THE PRESENT STUDY

It had long been expected that the amino acid sequences of proteins and naturally occurring peptides would show species variations. This was first shown for the hemoglobins [53].

There are enough informations about the primary structure of globin subunit from a large variety of animals. These informations have been used to deduce probable sequences of ancestor subunits that provide independent determinations of phylogenetic lineages.

The aim of the present study was to determine the primary structure of hemoglobins from lion (Panthera leo) and tiger (Panthera tigris). The detection of such species variations could contribute information to the relationship between structure and function and provide a powerful independent information not only on phylogenetic branch points but also on the rate of molecular evolution.
2. EXPERIMENTAL
2.1: COLLECTION OF BLOOD SAMPLES

Blood samples from both adult lion (Panthera leo) and tiger (Panthera tigris) were kindly provided by Dr. R. Goeltenboth, Zoologischer Garten, Berlin (F.R.Germany).

2.2: ISOLATION OF HEMOGLOBIN

Hemoglobin from heparinized blood was isolated using the procedure of Drabkin [54]. Blood samples were centrifuged at 4000rpm for 15min. The supernatant plasma was discarded. Erythrocytes were washed thrice with physiological saline (0.9% NaCl), lysed with bidistilled water (1:1 V/V) and shaken vigorously for 5min. The mixture was kept for 1h in the cold. The cell debris were separated by centrifugation at 4000rpm for 10min. The supernatant containing hemoglobin was transferred and dialyzed against bidistilled water for 5h.

2.3: PREPARATION OF GLOBIN

Globin was prepared according to the method of Anson and Mirsky [55]. A solution of 2% HCl in acetone was cooled to -20°C. Hemolysate was added dropwise in cooled acidified acetone solution with constant flask shaking. The precipitated globin was centrifuged and washed twice with cooled acetone. Globin was dissolved in water and acetone was evaporated in vacuo. The solution was dialyzed against bidistilled water for 5h, and lyophilyzed.
2.4: ELECTROPHORESIS

2.4.1: Polyacrylamide gel disc electrophoresis

Disc electrophoresis of the hemolysate and purified fraction of hemoglobin components was carried out in 10% polyacrylamide gel according to Davis [56].

Composition of solutions and buffers

Sol.A: 36.3g Tris, 48ml 1N HCl, 0.46ml TEMED to 100ml water pH 8.9.
Sol.B: 25g acrylamide, 0.4g bisacrylamide in 100ml water.
Sol.C: 1g % ammoniumperoxo-disulphate in water.

Gel was prepared by mixing the following solutions: 2ml sol.A, 6.4ml sol.B, and 7.1ml water. The solution was mixed and degased. 0.5ml of sol.C was added to the solution and poured gently in glass tubes (0.5x7.5 cm). About 0.02-0.04mg hemoglobin was dissolved in transformation solution (0.16% KCN and 0.16% K₃(FeCN)₆) and one drop of saturated sucrose solution added to it. Tris/Glycine at pH 8.3 was used as electrode buffer. Electrophoresis was carried out for 2h at a constant current 3.5mA/tube. The gels were stained with 1% amido black-10B in 7% acetic acid and destained with 7% acetic acid.
2.4.2: Triton polyacrylamide gel electrophoresis

Electrophoresis of native globin and that of separated chains was carried out in 12% polyacrylamide gel in the presence of dissociating agent 8M urea and a nonionic detergent Triton X-100 according to Rovera [57] and Alter [58].

Solution for 12% gel

Sol.A: 30g acrylamide, 0.2g bisacrylamide in 100ml water.

Sol.B: 2g ammonium peroxo-disulphate in 100ml water.

Mixing ratio for gel

9.6g urea
8ml solution A
1ml acetic acid
100.1 N,N,N',N'-Tetramethyl ethylene diamine (TEMED).
400.1 Triton X-100
Total volume made to 20ml with water.

The solutions were mixed and 0.5ml of solution B was added and poured quickly in glass tubes (0.5x7.5cm).

Pre-electrophoresis was carried out at 200V (constant voltage) for 1h. The globin was dissolved in sample buffer (48.5g urea, 10ml acetic
acid, 10ml β-mercaptoethanol with water to 100ml). The globin 0.5-1mg was loaded on gel. Separation was carried out in 5% acetic acid as electrode buffer. A potential of 140V (constant) was applied for 4h. Gels were stained with 1% amido black 10B prepared in 7% acetic acid solution and destained with 7% acetic acid.

2.5: SEPARATION OF GLOBIN CHAINS

2.5.1: Ion-exchange chromatography

Separation of globin chains was carried out on the column of CM-cellulose microgranular CM-52, (Whatman) according to the method of Clegg [59]. A column (2.6x15cm) was packed with CM-cellulose and equilibrated in starting buffer 8M urea, 0.025M sodium acetate and 0.2% β-mercaptoethanol pH 5.7. Globin was dissolved in starting buffer and reduced with DTE under nitrogen for 3h and adjusted to pH 5.7. Sample was applied on the column and eluted with a linear gradient of NaCl (0.02-1M). Flow rate was maintained at 25ml/h. Absorbance was recorded at 280nm.

Separated globin chains were pooled and desalted by gel filtration on a column of Sephadex-G25 (2.5x150cm), equilibrated with 0.1N acetic acid. Absorbance was monitored at 280nm.
2.5.2: Separation of globin chains by RP-HPLC

Reverse phase HPLC was used for the separation of globin chains. All HPLC separations were carried out on a Beckman gradient liquid chromatograph Model 334, controller 421, pump Model 110A. U.V. detector model BT3030 Biotronik, injector from Altex and integrator model CRI-A Shimadzu. The stainless steel column (4.6x250mm), supplied by Knauer (Bad Homburg) were filled in our laboratories by Shandon column packing machine Gynkotheek (Munich). Two different materials were used.

1. A column filled with reversed phase material LiChrosorb-RP2 (E.Merck) was equilibrated with 50mM ammonium acetate containing 12% formic acid. Globin from lion dissolved in distilled water was subjected to the column. Elution was carried out with a gradient of acetonitrile 0-35% in 2min., followed by 35-60% in 60min. at a flow rate of 1ml/min. Absorbance was recorded at 280nm.

2. Hemolysate from tiger was separated on a column of Nucleosil-C4 (Macherey and Nagel) equilibrated with 0.1% trifluoro acetic acid (TFA). Gradient program was the same as used for LiChrosorb-RP-2 column. Absorbance was monitored at 230nm.
2.6:  **OXIDATION OF GLOBIN CHAINS**

Purified globin chains were oxidized with performic acid by the method of Hirs et al. [60]. Globin chain 5-10mg was treated with 200μl of a cold solution containing 4.5ml formic acid and 0.5ml hydrogen peroxide. Reaction was stopped after 15 min. by addition of bidistilled water. Acid was removed in vacuo and chain was lyophylized.

2.7:  **ENZYMATIC DIGESTION OF GLOBIN CHAINS**

The oxidized globin chains were digested with trypsin (EC 3.4.21.4) Tos-Phe-CH₃Cl-treated, (Worthington). According to the method of Hirs [61], the oxidized chain was dissolved in distilled water and the pH was adjusted to 10.5 with 12% ammonia. Digestion was initiated by the addition of trypsin in enzyme/substrate ratio of 5:100 for 1h, followed by digestion at pH 9.5 for another 2h. Reaction was stopped by titrating it to pH4 with 2M acetic acid. The mixture was centrifuged at 4000rpm for 10min. Soluble part was subjected to reversed phase HPLC. In case of lion hemoglobin, mixture of tryptic peptides was prefraccionated by gel filtration.

2.8:  **SEPARATION OF TRYPIC PEPTIDES**

2.8.1:  **Gel chromatography of tryptic peptides**

Prefractionation of tryptic peptides was carried out on a column (2.6x150cm) packed with Sephadex G-25 fine (Pharmacia). Tryptic
digest was applied on the column and eluted with 0.1M acetic acid at a flow rate of 20ml/h. Absorbance was recorded at 230nm.

2.8.2: **Separation of tryptic peptides by RP-HPLC**

Separation of tryptic peptides was carried out by reversed phase HPLC according to the method of Kratzin et al. [62]. A column (4.6x250nm) of LiChrosorb-RP2 (E.Merck), equilibrated with 50mM ammonium acetate buffer pH 6.00. Peptides were eluted with a linear gradient of acetonitrile 0-60% in 60 min. at a flow rate of 1ml/min. Absorbance was monitored at 230nm. The contaminated peptides were further purified by rechromatography on a Vydac-C18 column (Machery and Nagel) with a 0.1% TFA/acetonitrile system.

2.9: **Thin Layer Electrophoresis**

Purity of separated peptides was checked by thin layer electrophoresis on a pre-coated cellulose plate without indicator, thickness 1mm (E.Merck). Isolated peptides were dissolved in 0.1M acetic acid and applied on plate and air dried. Plate was sprayed smoothly with electrode buffer (5% pyridine, 5% acetone in water). The pH of buffer was adjusted with acetic acid to pH 5.4. A potential of 450V (constant) was applied for 90min. The plate was dried for 10min. at 90°C. Peptide spots were visualized with ninhydrin reagent (0.1% ninhydrin, 60% ethanol, 20% acetic acid and 8% collition) for 10min at 80°C.
2.10: AMINO ACID ANALYSIS [63]

About 2-8nmol of tryptic peptides were hydrolyzed in boiling 5.7N HCl under vacuum for 19-20h, at 110°C. The samples were dried in a vacuum concentrator. Tryptophan containing peptides were hydrolyzed in the presence of 6% thioglycolic acid [64]. The peptides with methionine and cysteine were determined after performic acid oxidation. The sample was analyzed in an automatic amino acid analyzer Model LC5000, Biotronik GmbH. The sample to be analyzed was dissolved in sodium citrate buffer pH2.2. Elution of amino acid was carried out by applying a gradient of pH 3.5-10.5 and temperature from 48°C to 70°C. Amino acids were detected after reaction with ninhydrin reagent. Absorbance was recorded at 570nm and 440nm.

2.11: FAB-MASS SPECTROSCOPY OF BLOCKED PEPTIDE

Amino acid sequence of blocked peptides were studied by FAB-mass spectroscopy [65]. The peptide FAB spectra of 2KV was recorded on a mass spectrometer CH7A and MAT 312, Varian (W.Germany) connected with data system SS200/NS.
2.12: SEQUENCE DETERMINATION OF GLOBIN CHAINS

2.12.1: Automatic amino acid sequencing

N-terminal amino acid sequence of the native chains up to 42 amino acid residues and of tryptic peptides was determined by automatic Edman degradation [66] in a liquid phase sequencer Model 890B, 890C, 890CII, Beckman Instrument, Palo Alto, (U.S.A.) and also in a non commercial gas phase sequencer [67]. In liquid phase sequencer, two different programmes were employed.

1. Quadrol program [68]

0.25M Quadrol (N,N,N′N′-tetrakis (2hydroxypropyl) ethylene diamine was used as a coupling buffer for the sequencing of the intact chain, large peptides and lysine peptides. The peptides were sequenced after modification with a reagent IV 7-(isothiocyanato) naphthalene-1,3,5-trisulfonic acid, trisodium salt [69].

2. Propyne program [70]

3-(dimethyl amino) propyne (1.25M) was used as a coupling buffer for the sequencing of long hydrophobic peptides and lysine peptides having hydrophobic character. Long hydrophobic peptides were reacted with reagent-I (1-(isothiocyanato)benzene-4-sulfonic acid, sodium salt) [71] prior to sequence. Conversion into phenylthiohydantoin derivatives of amino acid was performed in an auto converter sequmat PG (Kontron Technik), with 3M TFA at 60°C.
2.12.2: Identification of PTH-amino acid

About 1n mol of PTH derivatives of amino acid was analysed on HPLC liquid chromatographed Hewlett Packard. Two different programs were used for the identification of PTH-amino acid.

1. Stepwise gradient elution [72]

A column 4.6x250mm filled with ODS-hypersil-RP18 (Shandon) was equilibrated with 10mM ammonium acetate pH 8.4. Sample was eluted with a gradient of acetonitrile.

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Detection was monitored at 265nm and 330nm.

2. Isocratic elution [73]

PTH-amino acid also eluted isocratically on ODS-Hypersil-RP18 column. Elution was carried out with a buffer containing 1M sodium acetate (pH5.3) - acetonitrile - 0.5% dichloroethane - water (9:330:7:670). Detection was carried out at 265nm, serine and threonine were identified as their dehydro derivatives at 330nm.
3.1: LION (Panthera leo) HEMOGLOBINS

3.1.1: Electrophoresis

Disc electrophoresis of hemolysate revealed the presence of two hemoglobin components in 1:1 ratio (Fig. 3.1). The native globin checked by electrophoresis in the presence of dissociating agents Triton X-100 and 8M urea showed three chains namely α, β I, β II (Fig. 3.1).

3.1.2: Chromatography on CM-cellulose

Separation of three globin chains α, β I, β II was achieved by chromatography on the column of CM-Cellulose in the presence of 8M urea. The separation profile is presented in Fig. 3.2. Since β II was contaminated with α chain thus further purified by RP-HPLC (Fig. 3.3).

3.1.3: Reversed phase HPLC of globin chains

The native globin subjected to chromatography on column LiChrosorb-RP2, resulted in the resolution of three purified chains (Fig. 3.4).

3.1.4: Prefractionation of tryptic peptides

The prefractionation of tryptic peptides on column Sephadex-G25 (fine), gave some of the peptides in pure form (Fig. 3.5a,b). The contaminated peptides obtained was purified by HPLC.
3. RESULTS
Fig. 3.1: Polyacrylamide gel electrophoresis of Lion hemolysate.

a) Disc-electrophoresis at pH 8.3

b) Under dissociating condition, 8M urea and Triton X-100
Fig. 3.2: Separation profile of crude globin on CM-cellulose column eluted with 8M urea-25mM sodium acetate-0.2% mercaptoethanol and a gradient of 0.02-0.08M NaCl.
Fig. 3.3: Electrophoretic pattern of crude globin and fractions from CM-cellulose chromatography on polyacrylamide gels under dissociating conditions.

a) Crude globin    b) βI-chain    c) βII and α-chains
   d) α-chain
Fig. 3.4: Elution pattern of panthera leo globin chains on Lichrosorb RP-2 (7μm).
Buffer: 12% formic acid/50mM ammonium acetate; gradient 0-35% acetonitrile in 2 min, followed by 35-60% acetonitrile in 60 min; flow rate 1ml/min.
Fig. 3.5a: Prefractionation of the tryptic peptides from α-globin chain on a Sephadex G-25 column (fine 2.6x150 cm), eluted with acetic acid. Flow rate, 20ml/h.
Fig. 3.5b: Prefractionation of the tryptic peptides from β-globin chain on a Sephadex G-25 column (fine 2.6x750 cm), eluted with acetic acid flow rate, 20 ml/h.
3.1.5: Reversed phase HPLC of tryptic peptides

The contaminated peptides obtained by gel filtration were rechromatographed by reversed phase HPLC on column LiChrosorb-RP2 and pure peptides were obtained. Elution profile of some of the peptides of α and β chains purified on HPLC presented in Fig.3.6a,b.

3.1.6: Amino acid analysis

Amino acid composition of tryptic peptides from α,βI and βII chains are presented in table 3.1-3.3.

3.1.7: FAB mass spectroscopy of blocked peptide

By FAB-mass spectroscopy N-acetylserine detected as an N-terminal amino acid of βI chain (Fig.3.7).

3.1.8: Amino acid sequence of globin chains

The complete amino acid sequence of α,βI and βII is presented in Fig.3.8. The sequence comparison with that human Hb-A revealed 23(19.8%) differences in α chain, 29(19.8%) in βI and 28(19.1%) in βII chain/ Between two β-chains only two differences were found at βI/II: 1(NA1) Ac-Ser/Gly, and β144(HC1) Arg/Lys.

The exchanges which were distributed over the entire length of the hemoglobin molecule resulted in the alteration of four α1β1 contact
Fig. 3.6a: Rechromatography of prefractioned tryptic peptides of α-chain from Sephadex G-25 on RP-HPLC column: Lichrosorb RP 2; buffer 50mM ammonium acetate pH 6.0; gradient 0-60% acetonitrile in 60 min; flow rate 1 ml/min.
Fig. 3.6b: Rechromatography of prefractiooned tryptic peptides of β-chain from Sephadex G-25 on RP-HPLC column.
Table 3.1
Amino acid composition of peptides from α chain of Lian.

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* Determined after performic acid oxidation.
Numbers in parentheses denote amino acid residue found during sequencing.
Table 3.2

Amino acid composition of peptides from B1 chain of Lion.

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* Determined after performic acid oxidation.

Numbers in parentheses denote amino acid residue found during sequencing.
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* Determined after performic acid oxidation.
Numbers in parentheses denote amino acid residue found during sequencing.
Fig. 3.7: The exact masses of acetylated N-terminal βI(TpI) by FAB mass spectroscopy
Table 3.8

Amino acid sequence of Lion (P1) globin chains in alignment with the corresponding chain of human (Hb). In case of human hemoglobin only the exchanges are given. The Hb-11 differs from the Hb-1 at the following positions:

P1/P1: BNAL Ac-Ser/Gly, BHC1 Arg/Lys.
points: $\alpha_{34}(B15)$ Leu/Cys, $\alpha_{11}(G18)$ Ala/Cys, $\beta_{123}(H1)$ Thr/Asn, $\beta_{125}(H3)$ Pro/Gln. One $\alpha_{1}(B2)$ contact point at $\beta_{43}(CD2)$ Glu/Gln, and one heme contact point at $\beta_{70}(B14)$ Ala/Ser. Among the 2,3-diphosphoglycerate binding sites, two exchanges were observed at $\beta_1$ (NA1) Val/Ser and $\beta_{11}$ (NA2) His/Phe.
3.2: TIGER (Panthera tigris) HEMOGLOBINS

3.2.1: Electrophoresis

The hemoglobin of tiger consist of two components as verified by polyacrylamide gel disc electrophoresis (Fig.3.9a). Electrophoresis of crude globin on polyacrylamide gel under dissociating condition in the presence of 8M urea and Triton X-100 showed three globin chains α, β1, and βII (Fig.3.9b).

3.2.2: Reversed phase HPLC of hemoglobin

The hemolysate subjected on a reversed phase HPLC, on column Nucleosil-C4 resulted in the separation of three globin chains. The elution profile (Fig.3.10) shows the elution of heme followed by α, βII and βI chains.

3.2.3: Separation of tryptic peptides

The globin chain digested with TPCK-trypsin subjected directly on column LiChrosorb-RP2 resulted in the separation of tryptic peptides. The contaminated peptides were rechromatographed on Vydac-C18 column yielding pure peptides. The elution profile of tryptic peptides from α and β chains are presented in Fig.3.11-3.12.
Fig. 3.9a,b: Polyacrylamide gel electrophoresis of Tiger hemolysate.

a) Disc-electrophoresis at pH 8.3

b) Under dissociating condition, 8M urea and Triton X-100.
Fig. 3.10: Separation of the hemoglobin chains from a crude Tiger hemolysate on RP-HPLC column: Nucleosil-C-4 buffer 0.1% TFA: gradient 0-35% acetonitrile in 2 min. followed by 35-60% in 60 min. flow rate 1 ml/min.
Fig. 3.11: Separation pattern of tryptic peptides from the oxidized β-chain of Tiger. Column: LiChrosorb-RP2; buffer 50 mM ammonium acetate pH 6.0; gradient 0-60% acetonitrile in 70 min; flow rate 1 ml/min.
Fig. 3.12: RP-HPLC pattern of tryptic peptides from oxidized β-chain of Tiger

Column: Lichrosorb-RP 2; buffer 50mM ammonium acetate pH 6.0 gradient 0-60% acetonitrile in 70 min; flow rate 1 ml/min.
3.2.4: Amino acid analysis

Amino acids composition of tryptic peptides from \( \alpha, \beta_1 \) and \( \beta_{11} \) chain are presented in table 3.4-3.6.

3.2.5: Amino acid sequence

The complete primary structure of the hemoglobin was established in part by sequencing the N-terminal part of the native chain up to 42 residues, and mainly by sequencing the tryptic peptides.

The amino acid sequence of the globin chains are presented in Fig. 3.13. The sequence aligned with that of human Hb-A showed 23 amino acid exchanges occur in the \( \alpha \) chain, 29 in \( \beta_1 \) and 28 in \( \beta_{11} \) chains. Within the two \( \beta \) chain only two exchanges have been located at \( \beta_1/\beta_{11}:\beta_1(NA1) \) Ac-Ser/Gly and \( \beta_1 144(HC1) \) Arg/Lys. Among the functionally important positions, eight substitutions were observed; four in \( \alpha_{1}\beta_{1} \), one in the \( \alpha_1 \beta_2 \) and one in the home contact point. Two exchanges were located at binding sites for 2,3-diphosphoglycerate, \( \beta_1(NA1) \) and \( \beta_2(NA2) \) with Ac-Ser and Phe respectively.
Table 3.4
Amino acid composition of peptides from α chain of Tiger.

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* Determined after performic acid oxidation.
Numbers in parentheses denote amino acid residue found during sequencing.
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* Determined after performic acid oxidation.

Numbers in parentheses denote amino acid residue found during sequencing.
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</table>

*Determined after performic acid oxidation.

Numbers in parentheses denote amino acid residue found during sequencing.
Fig. 3.13

Amino acid sequence of Tiger (Pt) globin chains in alignment with the corresponding chain of human (Hu). In case of human hemoglobin only the exchanges are given. The Hb-II differs from the Hb-I at the following positions:

HbI/HbII: ΔMA1 Ac-Ser/Gly, ΔRCl Arg/Lys.
4. DISCUSSION
4.1: CARNIVORA HEMOGLOBINS

Amino acids sequence provide information about the primary structure of a protein. It is still not possible to determine the tertiary structure merely on the basis of amino acids sequence data, but a comparison with other homologous proteins of known tertiary structures enables one to make attempts on structural interpretation.

Family Felidae (cat and allies) has two living subfamilies, the Felinae and Acinonychinae. The subfamilies Proailurinae, Nimravinae, Machairondotinae and Hyainailourinae are extinct.

The subfamily Felinae has two genera. The genus Uncia is represented by one specie Snow leopard (Uncia uncia) whereas the genus Panthera comprise four species leopard (Panthera pardus), jaguar (Panthera onco), tiger (Panthera tigris) and lion (Panthera leo).

Hemoglobins from the order Carnivora in general and the family Felidae in particular have not been investigated in detail. In the present study we have characterized the globin chains from lion and tiger, representative of the family Felidae.

Hemoglobins from both animals are heterogenous and contain two components namely Hb-I and Hb-II and show identical mobility on disc electrophoresis, in contrast only one component is reported in other
carnivores. Triton gel electrophoresis revealed that the hemolysate contains three globin chains. The structural differences in the two hemoglobins components are confined to the β chains.

Separation of globin chains by chromatography on CM-cellulose in the presence of 8M urea resulted in the separation of three peaks. The peak which correspond to β II was found to be contaminated with the α chain. Reversed phase HPLC resulted in the resolution of three purified chains. These results are in agreement with Triton electrophoresis.

4.2: HOMOLOGOUS CORRELATION OF LION AND TIGER WITH HUMAN HEMOGLOBIN

The sequences of both α and β chains from lion and tiger hemoglobins when compared for homology with human Hb-A revealed alteration of 23 residues in the α 29 in β I and 28 in the β II chain.

The exchanges which are distributed over the entire length of the molecule have resulted in the alteration of 10 functionally important positions, α1β1, α1β2, heme contact points, DPG binding sites and the amino acid which are responsible for the Bohr effect.
4.2.1: Binding sites for deoxy-hemoglobin

The heme binding sites for the hemoglobin (Table 4.1) in the $\alpha$ and $\beta$ chain are present inside the molecule and the amino acid residues are mostly hydrophobic. In lion and tiger $\beta 70$ (E14) alanine is replaced by serine; this substitution is found frequently in the mammalian hemoglobin, the rest are similar to human $\beta$ chain. The binding sites in the $\alpha$ chain correspond to human hemoglobin.

The $\alpha_1 \beta_2$ binding sites (Table 4.2) which are responsible for the construction of tetrameric structure, found to be invariant in many species. Substitution at these sites alter the heme-heme interaction. In the $\beta$ chain of lion and tiger one substitution is located at $\beta 43$ (CD2) where a polar amino acid is replaced by nonpolar amino acid (Glu/Gln). The binding sites in the $\alpha$ chain is identical with human hemoglobin.

For the dimeric structure $\alpha_1 \beta_1$ contact (Table 4.3) is very important. Four exchanges have been located in lion and tiger hemoglobins at $\alpha 34$ (B13) Leu/Cys, $\alpha 111$ (G18) Ala/Cys and in the $\beta$ chain $\beta 123$ (H1) Thr/Asn and $\beta 125$ (H3) Pro/Gln.

Bunn [74] has divided mammalian hemoglobins in to two groups on the basis of substitution at these binding sites.
Table 4.1: Heme binding sites for deoxy-hemoglobin.

Exchanges are given for lion (Pl) and tiger (Pt) hemoglobins only.

<table>
<thead>
<tr>
<th>α</th>
<th>Hb-Human</th>
<th>Pl-δ</th>
<th>Pt-δ</th>
<th>Hb-Human</th>
<th>β</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>C7</td>
<td>Tyr</td>
<td></td>
<td>Phe</td>
<td>C7</td>
</tr>
<tr>
<td>43</td>
<td>CD1</td>
<td>Phe</td>
<td></td>
<td>Phe</td>
<td>CD1</td>
</tr>
<tr>
<td>45</td>
<td>CD3</td>
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<td></td>
<td>His</td>
<td>E7</td>
</tr>
<tr>
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<td>Phe</td>
<td></td>
<td>Lys</td>
<td>E10</td>
</tr>
<tr>
<td>58</td>
<td>E7</td>
<td>His</td>
<td></td>
<td>Val</td>
<td>E11</td>
</tr>
<tr>
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<td>E10</td>
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<td>Ser</td>
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<td>Ala</td>
</tr>
<tr>
<td>62</td>
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<td>Val</td>
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<td>Phe</td>
<td>E15</td>
</tr>
<tr>
<td>66</td>
<td>E15</td>
<td>Leu</td>
<td></td>
<td>Phe</td>
<td>F1</td>
</tr>
<tr>
<td>80</td>
<td>F1</td>
<td>Leu</td>
<td></td>
<td>Leu</td>
<td>F4</td>
</tr>
<tr>
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<td>Leu</td>
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<td>Leu</td>
<td>F7</td>
</tr>
<tr>
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<td>His</td>
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<tr>
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<td>Leu</td>
<td>FG3</td>
</tr>
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<td>Leu</td>
<td></td>
<td>Val</td>
<td>FG5</td>
</tr>
<tr>
<td>93</td>
<td>FG5</td>
<td>Val</td>
<td></td>
<td>Asn</td>
<td>G4</td>
</tr>
<tr>
<td>97</td>
<td>G4</td>
<td>Asn</td>
<td></td>
<td>Phe</td>
<td>G5</td>
</tr>
<tr>
<td>98</td>
<td>G5</td>
<td>Phe</td>
<td></td>
<td>Leu</td>
<td>G8</td>
</tr>
<tr>
<td>101</td>
<td>G8</td>
<td>Leu</td>
<td></td>
<td>Val</td>
<td>H15</td>
</tr>
<tr>
<td>132</td>
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<td>Val</td>
<td></td>
<td>Leu</td>
<td>H19</td>
</tr>
<tr>
<td>139</td>
<td>H19</td>
<td>Leu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\alpha)</td>
<td>Hb-Human</td>
<td>P1-(\beta)</td>
<td>P2-(\beta)</td>
<td>Hb-Human</td>
<td>(\beta)</td>
</tr>
<tr>
<td>---</td>
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<td>---</td>
<td>---</td>
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</tr>
<tr>
<td>37</td>
<td>C2</td>
<td>Pro</td>
<td>His</td>
<td>HC3</td>
<td>146</td>
</tr>
<tr>
<td>38</td>
<td>C3</td>
<td>Thr</td>
<td>Tyr</td>
<td>HC2</td>
<td>145</td>
</tr>
<tr>
<td>40</td>
<td>C5</td>
<td>Lys</td>
<td>Leu</td>
<td>G7</td>
<td>105</td>
</tr>
<tr>
<td>41</td>
<td>C6</td>
<td>Thr</td>
<td>Asn</td>
<td>G4</td>
<td>105</td>
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<td>C7</td>
<td>Tyr</td>
<td>Glu</td>
<td>G3</td>
<td>101</td>
</tr>
<tr>
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<td>Pro</td>
<td>Asp</td>
<td>G1</td>
<td>99</td>
</tr>
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<td>88</td>
<td>F9</td>
<td>Ala</td>
<td>Val</td>
<td>FG5</td>
<td>58</td>
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<tr>
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<td>Arg</td>
<td>Gln</td>
<td>Gln</td>
<td>Glu</td>
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<td>Phe</td>
<td>C7</td>
<td>41</td>
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<td>G2</td>
<td>Pro</td>
<td>Arg</td>
<td>C6</td>
<td>40</td>
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<td>G3</td>
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<td>Trp</td>
<td>C3</td>
<td>37</td>
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<td>Pro</td>
<td>C2</td>
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<tr>
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<td>Tyr</td>
<td>Tyr</td>
<td>C1</td>
<td>35</td>
</tr>
<tr>
<td>141</td>
<td>HC3</td>
<td>Arg</td>
<td>Val</td>
<td>B16</td>
<td>34</td>
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</tbody>
</table>
Table 4.3: $\alpha_1^B_1$ binding sites for human deoxy hemoglobin.

Exchanges are given for lion (Pl) and tiger (Pt) hemoglobins.

<table>
<thead>
<tr>
<th>$\alpha$</th>
<th>Hb-human</th>
<th>Pl $\alpha$</th>
<th>Pt $\alpha$</th>
<th>Pl $\beta$</th>
<th>Pt $\beta$</th>
<th>Hb-human</th>
<th>$\beta$</th>
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<td>31</td>
<td>B12</td>
<td>Arg</td>
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</tr>
<tr>
<td>34</td>
<td>B15</td>
<td>Leu</td>
<td>Cys</td>
<td>Cys</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>B16</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>C1</td>
<td>Phe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Cys</td>
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<td></td>
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<tr>
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<tr>
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<td>Phe</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>118</td>
<td>H1</td>
<td>Thr</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>119</td>
<td>H2</td>
<td>Pro</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>122</td>
<td>H5</td>
<td>His</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>123</td>
<td>H6</td>
<td>Ala</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>126</td>
<td>H9</td>
<td>Asp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
a) The hemoglobin with low intrinsic oxygen affinity show no effect from 2,3-DPG organic phosphate. In this group $\beta$ (NA1) is deleted and $\beta$ (NA2) is replaced with methionine e.g. bovine [75].

b) The hemoglobin with high intrinsic oxygen affinity modulated with 2,3-diphosphoglycerate. Representative of this group are the hemoglobin from human and most of the other mammalian hemoglobin.

Our results show that the hemoglobins from lion and tiger have two alterations at these key positions. The $\beta$ I which has blocked N-terminus and Phe in place of His at $\beta$ (NA2), while the $\beta$ II chain has free Gly as N-terminal residue and substitution at $\beta$ (NA2) is identical with that of $\beta$I, both these sites play a key role in determining the ease of loading and unloading of oxygen.

Substitution of hydrophylic residue $\beta$ (NA2) His with hydrophobic Phe should result in alteration of the secondary structure, since Phe would tend to take an interior position in the molecule Perutz and Imai [76]. Table 4.4 summarizes the alterations observed at these four binding sites in different mammalian hemoglobins.

Though mutation for the phosphate binding sites mostly at $\beta$ NA2 resulted in the reduction of phosphate-hemoglobin interaction. The other heterotrophic ligands, $H^+$ Lohr effect, $Cl^-$ and $CO_2$ also decreases the oxygen affinity of the hemoglobin molecule.
Table 4.4: Mammalian hemoglobins with alteration at contact points for 2,3-diphosphoglycerate.

<table>
<thead>
<tr>
<th>Mammal</th>
<th>β 1(NA1)</th>
<th>β 29(NA2)</th>
<th>β 82(EF6)</th>
<th>β 143(H21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Val</td>
<td>His</td>
<td>Lys</td>
<td>His</td>
</tr>
<tr>
<td>Mountain Zebra</td>
<td>Val</td>
<td>Gln</td>
<td>Lys</td>
<td>His</td>
</tr>
<tr>
<td>Low Land Tapir</td>
<td>Val</td>
<td>Glu</td>
<td>Lys</td>
<td>His</td>
</tr>
<tr>
<td>Armadillo</td>
<td>Val</td>
<td>Asn</td>
<td>Lys</td>
<td>His</td>
</tr>
<tr>
<td>Ring tailed Lemur</td>
<td>Thr</td>
<td>Phe</td>
<td>Lys</td>
<td>His</td>
</tr>
<tr>
<td>Brown Lemur</td>
<td>Thr</td>
<td>Leu</td>
<td>Lys</td>
<td>His</td>
</tr>
<tr>
<td>Cat (βA)</td>
<td>Gly</td>
<td>Phe</td>
<td>Lys</td>
<td>His</td>
</tr>
<tr>
<td>Cat (βB)</td>
<td>Ac-Ser</td>
<td>Phe</td>
<td>Lys</td>
<td>His</td>
</tr>
<tr>
<td>Lion (βI)</td>
<td>Ac-Ser</td>
<td>Phe</td>
<td>Lys</td>
<td>His</td>
</tr>
<tr>
<td>Lion (βII)</td>
<td>Gly</td>
<td>Phe</td>
<td>Lys</td>
<td>His</td>
</tr>
<tr>
<td>Tiger (βI)</td>
<td>Ac-Ser</td>
<td>Phe</td>
<td>Lys</td>
<td>His</td>
</tr>
<tr>
<td>Tiger (βII)</td>
<td>Gly</td>
<td>Phe</td>
<td>Lys</td>
<td>His</td>
</tr>
<tr>
<td>Bovine</td>
<td>-</td>
<td>Met</td>
<td>Lys</td>
<td>His</td>
</tr>
<tr>
<td>Grand Galago (βI)</td>
<td>Val</td>
<td>His</td>
<td>Cys</td>
<td>His</td>
</tr>
<tr>
<td>European Hedgehog</td>
<td>Val</td>
<td>His</td>
<td>Lys</td>
<td>Ala</td>
</tr>
</tbody>
</table>
4.3: BLOCKED N-TERMINAL CHAIN

The occurrence of two types of β chains one with blocked acetylated N-terminus and other with free N-terminus appear to be a common feature in hemoglobin from family Felidae, Taketa et al. [77]. This has been reported in the large members of the genera Felis, Acinonyx and Panthera Taketa [44]. In vitro, experiments are suggestive of the presence of an acetyl transferase in the intermediate stages or translation as a possible clue to N-terminal acetylation, Kaston-Jolly et al. [78]. It shows that the enzyme has a high degree of specificity, as only N-terminal serine residue has been acetylated whereas free glycine is found as the N-terminus of β II globin chain. An interesting result is that, the major β chain (β I) is found to be with blocked N-terminal while in the cat, a member of the same family, the minor beta chain (β II) has acetylated serine and the major beta chain (β I) with free N-terminal glycine.

4.4: EVOLUTIONARY RELATIONSHIP

About 425-500 million years ago, the original globin gene was duplicated and 300-500 million years ago separated into α and β chains of different chromosomes Goodman et al. [79-80] and mammals separated from reptile and birds from that time. Duplication of α gene took place about 300 million years ago.
Divergent evolution occurred at constant rate Zuckerkandl and Pauling [25]. Goodman gave a period of 300 million years for a constant rate on "molecular clock" and suggested that mutation of one amino acid required about 1.5 million years. Kimura [81] gave a period of one million year, and Wilson [82] predicted a different evolution rate for the \( \alpha \) and \( \beta \) chains. For \( \alpha \) chain 1.2 million and for the \( \beta \) chain 1.1 million years. In the reported data of the mammalian hemoglobin, the substitutions are mostly in the \( \beta \) chain.

4.4.1: Molecular interpretation of sequences

Comparison of the amino acids sequence with that of other available sequences of the family Felidae revealed a high degree of homology both in \( \alpha \) chain and \( \beta \) chain. By taking the mutation rate as given by Goodman [80] 1.5 million years per amino acid residue. The values obtained for the different Felidae are given in figure 4.1.

4.5: PRIMARY STRUCTURE OF GLOBIN CHAIN

By comparing two polypeptide \( \alpha \)-chains from lion and tiger hemoglobins 3 amino acids are found to be altered. Two of these are replacements by closely related amino acids at \( \alpha 57(\text{Ala}/\text{Thr}) \) and \( \alpha 71(\text{Val}/\text{Ala}) \). Variation from this general trend occurs only in position \( \alpha 57(\text{Asp}/\text{Asn}) \), where a polar amino acid residue is replaced by a non-polar residue. These data might allow to gain more information about tertiary structure of Felidae hemoglobins.
Fig. 4.1: Estimated values for the separation of human and members of the family Felidae in million years.

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Jaguar</th>
<th>Leopard</th>
<th>Lion</th>
<th>Tiger</th>
<th>Cat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<tr>
<td>Leopard</td>
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<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lion</td>
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<td>1.5</td>
<td>0</td>
<td>0</td>
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<td></td>
</tr>
<tr>
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<td>120.0</td>
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<td>3.0</td>
<td>4.5</td>
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<td>Cat</td>
<td>112.5</td>
<td>28.5</td>
<td>28.5</td>
<td>30</td>
<td>31.5</td>
<td>0</td>
</tr>
</tbody>
</table>
4.5.1: Identical $\beta$-chains

Our study on the primary structure of hemoglobin from lion and tiger revealed that the two $\beta$-chains are identical in their amino acids sequence. Among the order carnivora two examples of identical sequences have already been reported, i.e. hemoglobin from Asiatic black bear, Polar bear and Malyan sun bear [83,84] of the family Ursidae and secondly in family Canidae from domestic dog and coyote [85,86]. In support of our results, a list of presently known hemoglobins having identical $\beta$-chains are presented in Table 4.5.

4.6: Variations and Conserved Amino Acids

A comparison of primary structures of $\alpha$ and $\beta$ chains from 16 carnivores hemoglobins as shown in Table 4.6A. and 4.6B entails the following 6 families of the order carnivora.

Felidae (cat and allies), Canidae (dogs), Mustelidae (badgers), Phocidae (true seals), Procyonidae (raccoons) and Ursidae (bears).

Among all the carnivores families, only Felidae hemoglobins has been found with two components which are composed of three globin chains, two $\beta$ and one $\alpha$, however, other carnivores hemoglobins have only one component.

Another characteristic feature of Felidae hemoglobins is the blocked N-terminal $\beta1$ chain (major component) due to acetylation.
Table 4.5: Identical β-globin chain found in different species.

<table>
<thead>
<tr>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo sapiens, Pan paniscus, Pan troglodytes [87]</td>
</tr>
<tr>
<td>Cebus capucinus [88], Cebus apella [89]</td>
</tr>
<tr>
<td>Ateles geoffroyi, Ateles belzebuth, Ateles paniscus [90]</td>
</tr>
<tr>
<td>Saguinus oedipus oedipus [91], Saguinus nigricollis [92]</td>
</tr>
<tr>
<td>Macaca sepeciosa cuvier [93], Macaca fuscata fuscata [94]</td>
</tr>
<tr>
<td>M. nemestrina [95], Macaca fascicularis [96]</td>
</tr>
<tr>
<td>Camelus dromedarius, Camelus ferus bactrianus [97]</td>
</tr>
<tr>
<td>Lama glama, Lama vicugna, Lama pacos [98]</td>
</tr>
<tr>
<td>Bos taurus (A-Allele) [75, 99], Bos grunniens [100]</td>
</tr>
<tr>
<td>Equus hemionus kulan, Equus zebra [101]</td>
</tr>
<tr>
<td>Ursus tibetanus, Ursus maritimus, Helarctos malayanus [83]</td>
</tr>
<tr>
<td>Panthera onco [46], Panthera pardus [102]</td>
</tr>
<tr>
<td>Panthera leo [103], Panthera tigris [104]</td>
</tr>
<tr>
<td>Branta canadensis, Cygnus olor [105]</td>
</tr>
<tr>
<td>Gyps rueppellii, Aegypius monachus [106]</td>
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</tbody>
</table>
From Table 4.6A the following mutations can be seen in α-chain which appear to be characteristics of the Felidae: position α4 (Pro/Ser), α13 (Thr/Cys), α15 (Asp/Gly), α19 (Gly/Ser), α26 (Gly/Ala), α56 (Lys/Gln), α60 (Lys/Gln), and α82 (Ala/Asp). At position 23 and 30 all carnivores have Glu, as do most other animals, but dog and its more recently diverged relative, coyote, has Asp in these two positions. Furthermore residue α36 and α111 are also interesting, as these two positions are involved in α31 contacts. In Harbor seal 36 is mutated with Ala whereas other members of the order carnivora have Phe. α111 residue in Ursidae is substituted by Ser, but in other carnivores this position is occupied by Cys. Two heme binding sites (position 80 and 101) are mutated, at position α80, Leu is found in all carnivores except in coyote where this position is occupied by Met and residue α101 is occupied by Leu in all carnivore families, except in Ursidae where Leu is mutated by Ser. Among the Felidae members α71 is occupied by Ala, only lion has Val at this position. Residue α75 is also interesting because in all carnivores this position is occupied by Asp whereas in tiger, it is mutated by Asn which is a non polar residue.

In cat, hemoglobin differences between β-chains are found at position β1 Gly/As-Ser, β4 Thr/Ser, β139 Asn/Ser and β144 Lys/Arg, while in all members representing the genus panthera (lion, tiger leopard and jaguar), only two differences have been reported, one at N-terminal position β(1NA) where Ac-Ser is mutated with free N-terminal Gly and the other position is β144 HC', where Arg is replaced by Lys.
Table 4.6A. Amino acid differences between globin chains of lion and other carnivores which has been determined by amino acid sequencing.

Carnivore α-chains

<table>
<thead>
<tr>
<th>Residue No.</th>
<th>4 8 10 12 13 15 17 19 23 26 30 34 36 50 56 57 60 68</th>
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<tbody>
<tr>
<td>Lion</td>
<td>S N V A C G I S E A E C F H Q A Q K T</td>
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<tr>
<td>Tiger</td>
<td>T</td>
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<tr>
<td>Lion</td>
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<tr>
<td>Smur leopard</td>
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<td>Jaguar</td>
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<td>Cat</td>
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<td>Persian leopard</td>
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<tr>
<td>Lesser panda</td>
<td>P T S T D L G G A P K K L</td>
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<tr>
<td>Giant panda</td>
<td>P T T D G G A P K K T</td>
</tr>
<tr>
<td>Asiatic black bear</td>
<td>P S T D G G A P K K T</td>
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<tr>
<td>Polar bear</td>
<td>P S T D G G A P K K T</td>
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<tr>
<td>Baliyan bear</td>
<td>P S T D G G A P K K T</td>
</tr>
<tr>
<td>Domestic dog</td>
<td>P T I S T D G D G D Q P K K T</td>
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<tr>
<td>Coyote</td>
<td>P T I S T D G D G D Q P K K T</td>
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<tr>
<td>Badger</td>
<td>P A I T D G G A K G K N</td>
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<tr>
<td>Raccoon</td>
<td>P A I T D G G A P K K L</td>
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<tr>
<td>Harbor seal</td>
<td>P T T D G G T A K K T</td>
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V 70 71 72 74 75 78 80 82 89 100 101 111 115 129 130 diff.
Table 4.6B: Carnivore β-chain

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<th>Residue No.</th>
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<th>43</th>
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<tr>
<td>Lion</td>
<td>Ac-S</td>
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54  56  58  73  78  80  84  90  104  120  121  123  130  139  144  diff.

<table>
<thead>
<tr>
<th>ISADIDADRDHENFSR0 0 0</th>
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<tbody>
<tr>
<td>E D N K 9</td>
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<tr>
<td>V N P E L N T E K K T Y N K 25</td>
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<tr>
<td>V N P D L N T E K K T Y N K 25</td>
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<tr>
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</table>
Comparison of the sequences of \( \beta \)-chain in Table 4.6B shows the following residues which appear to be characteristic of the Felidae. Residue \( \beta 78 \) (Leu/Ile), \( \beta 80 \) (Asn/Asp), \( \beta 84 \) (Thr/Ala), \( \beta 90 \) (Glu/Asp), \( \beta 120 \) (Lys/His), \( \beta 123 \) (Thr/Asn), \( \beta 130 \) (Tyr/Phe), \( \beta 139 \) (Asn/Ser) and \( \beta 144 \) (Lys/Arg).

Table 4.6B shows changes in \( \beta \)-chains depicting that most of the carnivores have N-terminal Val in \( \beta I \) except the Felidae members where the genus Panthera (lion, tiger, jaguar and leopard) mutated by Ac-Ser and in \( \beta II \) by free Gly, but it is interesting that cat, belonging to the same family, \( \beta I \) has free N-terminal Gly, whereas \( \beta II \) is blocked (Ac-Ser).

His is found at position \( \beta 2 \) in all carnivores except in Felidae where only cat has Thr, but all other Felidae have Phe in this position.

At the position \( \beta 1 \) and \( \beta 21 \) Glu and Asp are found in all carnivores except in raccoon, where alterations are observed in Glu/Asp and Asp/Glu respectively.

Residue \( \beta 41 \), which is involved in heme-binding and \( \alpha_1 \beta_2 \) contact, is occupied by Phe in all carnivores except in badger where Tyr is found, as well as in position \( \beta 123 \), which is \( \alpha_1 \alpha_1 \) contact point, all Felidae have Asn as compared to other carnivores.
Another residue Asp at position $\beta 43$, involved in $\alpha_1 \beta_2$ contact, is found in all carnivores except in genus Panthers, where Asp is mutated to a non-polar Gln, but in cat and raccoon it is altered with Glu.

The carnivores globin chains in this study differ from one another by 29 or less residues, as shown in both Tables 4.6A and 4.6B giving minimum $\alpha$ and $\beta$ chains differences among 16 carnivores.
5. REFERENCES


PART-B
Thymosin B9, identified as a component of the thymosin fraction V, was isolated from calf thymus. It is composed of 41 amino acid residues. The NH₂ terminus of the polypeptide is blocked by an acetyl group. Three fragments of thymosin B9, Ac-Ala-Asp-Lys-Pro-OH (position 1-4), Thr-Gln-Glu-OH (position 22-24), and Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-Lys-OH (position 33-41) were synthesized by the conventional method, using tert.-butyl side chain protection.

The fragment 33-41 was synthesized by assembling two peptide fragments in solution followed by deprotection with trifluoroacetic acid – anisole (molar ratio 1:1). Optimum conditions were found for the final DCC/HOBt coupling of the two fragments; pentapeptide and tetrapeptide. The product was shown to be homogenous by thin layer-chromatography. Elemental analysis, amino acid analysis, optical rotation, ¹³C-NMR spectroscopy confirmed the structure of desired fragments.

The biological activity of fragments was determined in the rosette assay, fragment 33-41 exhibited activity in vivo.
1. INTRODUCTION
1.1: GENERAL DESCRIPTION

Experimental and clinical research have shown that the role of the thymus involves immune response in various diseases, like the primary and secondary immunodeficiency disease, cancer and auto-immune diseases [1-4, 27]. Studies on thymus began with the reports that neonatal thymectomy in mice and in rabbits [5, 6] resulted in the failure of normal postnatal growth and lymphoid tissue development and maturation. This arose interest in the field and results by several workers [7-9] led to an understanding of the role of the thymus in the development of lymphoid system and in the maintenance of the immune balance. The thymic functions in mammals include primary transplant and tumor immunity, as well as viral, mycobacterial, fungal and protozoal immunity. It is also understood that subpopulations of the T cells influence the activity of B cells populations that produce antibodies.

1.2: GENERAL INTRODUCTION

1.2.1: The thymus as an endocrine gland

In mammals, the thymus (Gk. thymos, "warty excrescence") is a bilobed pinkish-gray lymphoid organ situated in the mediastinum.

The thymus has at least two functions:

1. Production of lymphoid cells, that are exported to the peripheral tissues, notably lymph nodes and spleen for immunologic responsiveness.
2. Synthesis and secretion of hormones that control the rate of development and maturation of selected populations of lymphoid cells.

The thymus functions via a hormonal mechanism [10] and for the last two decades, research efforts are being directed towards isolation and characterization of thymic factors or hormones responsible for the physiological actions of thymus [12,28,29].

In the period between 1961-1966, most studies in this field were confined to analyses of the effects of thymectomy and restoration of thymic function by implanting thymus tissue, or T cells [11,26].

1.2.2: Thymic Hormones

A partially purified, biologically active, thymic extract termed, thymosin fraction V, from bovine calf thymus [13], has been demonstrated to be effective in reconstituting immune functions in thymic deprived or immuno-deprived animals [1,13], as well as human with primary immunodeficiency disease [15,27], and in immuno suppressed cancer patients [16,30]. Studies on thymosin indicate that the peptides in, fraction V, consist of three groups of molecules as under:

1. Thymic hormones
2. Lymphokine-like molecules
3. non-specific peptides
Thymosin fraction V, is composed of heat stable acidic polypeptides ranging in molecular weight from 1,000 to 15,000 D [67]. It has been found to induce T cell differentiation and enhance immunological functions in animal models as well as in human [17].

Adequate biological data has been reported for thymosin fraction V, both in vitro and in vivo systems [18,19]. Four bioassay systems, the TdT (terminal nucleotidyl transferase) assay [20], the migration inhibiting factor MILF (microphage migration inhibitory factor) assay [21], the MLR assay [22], and the TCGF (T Cell growth factor) assay [23], have been routinely used.

According to the proposed nomenclature, thymosin peptides, based on their isoelectric points, have been divided into three regions. The α region consists of polypeptides with isoelectric points below 5.0; the β region 5.0-7; and the γ region, above 7.0 [17]. The subscript numbers α₁, α₂, β₁, β₂, etc. are assigned to the peptides in each region on the basis of time at which the peptides have been isolated.

1.2.3: Immunity

Immunology is a science which deals with all the phenomena resulting from the specific interaction of the cells of the immune system with antigen. As a consequence of such interaction, cells appear that mediate cellular immune responses, as well as, cells that synthesize and secrete one of the several classes of immunoglobulins [24].
Two types of acquired immunity occur in the body. 1) Humoral immunity and 2) Cellular immunity.

When the body develops circulating antibodies, capable of attacking the invading agent, the immunity is humoral whereas cellular immunity is achieved through the formation of large number of highly specialized lymphocytes that are specifically sensitized against the foreign agent. These sensitized lymphocytes have the special ability to attach and destroy the invader. Figure 1.1 illustrates the two separate lymphocyte systems. Though all the lymphocytes of the body originate from the lymphocytic stem cells of the embryo, these stem cells are themselves incapable of forming either sensitized lymphocytes or antibodies.

Most of the preprocessing of the T lymphocytes of the thymus gland occurs shortly before birth. Therefore, this period of time, removal of the thymus usually will not seriously impair the T lymphocytic immune system.

However, at prenatal stage, thymectomy can completely prevent development of all cellular immunity.

Most invading organisms are phagocytized by the macrophages, and the antigenic products are liberated from the invader. It is believed that these antigens then pass directly from the macrophages to the lymphocytes to stimulate the specific lymphocytic clones. Close physical
Fig. 1.1: Schematic representation of the immune system.
association between macrophages and lymphocytes is required for the expression of a variety of physiological processes of lymphoid cells. Macrophage-lymphocyte cooperation is integrally involved in the development of specific immune phenomena, such as, in vitro, initiation of immune lymphocyte proliferation [31-43]. The initial excitation of T lymphocytes can lead to secondary excitation of B lymphocytes so that both sensitized lymphocytes and antibodies can be formed against the invading agent [25].

Obviously, if a person should become immune to his or her own tissues, the process of acquired immunity would destroy the individual's own body. During the processing of lymphocytes in the thymus and in the B lymphocytes processing area, all those clones of lymphocytes that are specific for the body's own tissues are self-destroyed because of their continual exposure to the body's antigens.

1.2.4: Thymic peptides

Thymosin fraction V is active in all three major compartments of the lymphoid system (the bone marrow, thymus and peripheral lymphoid tissue) in influencing the maturation and differentiation of helper T cells [49] and suppressor T cells. Figure 1.2 illustrates the role of thymosin fraction V and sites of action of thymosin peptides in T-cell maturation.
Fig 1.2: The role of fraction V and sites of action of thymosin peptides in T cell maturation.
It is now known that several purified peptides, from fraction V, have selective sites of action. For instance, α₁ induces helper T-cells [49,50], and phenotypic T-cell markers [51], whereas thymosin α₇ is an inducer of suppressor cells [49]. Thymosin β₃ and β₄ appear to act at an earlier stage in T-cell differentiation, particularly at low doses [52,53]. The isolation, chemical characterization and biological properties of the following hormones and peptides have already been determined. Thymosin α₁ [54], thymosin α₁₁ [55], thymosin β₄ [56], thymosin β₈ [57], thymosin β₉ [57], thymosin β₁₀ [58,59], thymosin β₁₁ [60], polypeptide β₁ [50], thymic humoral factor (THF) [61], thymopoietin [62], ubiquitin [63], thymulin (FTS-Zn) [64] and thymic factor X [65].

1.2.4.1: Thymosin fraction V (TF V)

TF V is the parent preparation of thymus extract from calf thymus [67-71] that has been used for further purification and characterization of most of the hormone-like peptides. It is a mixture of a group of 30-40 polypeptides and its active component is called thymosin [67]. TF V is known to be effective in inducing differentiation of specific subclasses of T-lymphocytes and certain cell markers (TdT, Thy-1, and Lyt)) and functional expression of lymphocyte maturation including enhancement of lymphokine activity. TF V is biologically active in E-Rosette test [4], in "Graft Versus Host"-(GVH)-test [14] and in "Mixed lymphocytes culture "MLC"-test [15,22].
1.2.4.2: Thymosin $\alpha_1$

Thymosin $\alpha_1$, is a highly acidic N-acetyl octacosapeptide (28 amino acid residues), with a molecular weight of 3108 and an isoelectric point of 4.2. It has been isolated from calf thymus gland [72] and characterized by sequence analysis [54]. It has been synthesized by classical procedures in solution [74-76] and by solid phase [77,78] methods. Wetzel et al. has synthesized thymosin $\alpha_1$ utilizing recombinant DNA procedures [73].

1.2.4.3: Thymosin $\alpha_7$

Thymosin $\alpha_7$ has been isolated from thymosin fraction V with molecular weight of 2200 D and an isoelectric point of 3.5. Ahmed et al. has identified it as an inducer of suppressor cells [49].

1.2.4.4: Thymosin $\alpha_{11}$

Thymosin $\alpha_{11}$ is a polypeptide of 35 residues. It has identical sequence to thymosin $\alpha_1$ with a C-terminal extension G-P-E-A-P-A-N and has similar biological activity as thymosin $\alpha_1$ in protecting mice against opportunistic infections with Candida albicans [55].

1.2.4.5: Thymosin $\beta_3$

Thymosin $\beta_3$ is composed of 48 amino acid residues, with a molecular mass of 5500 and an IP of 5.2 [99]. It is biologically active in the induction of TdT in bone marrow cells [19].
1.2.4.6: Thymosin \( \beta_4 \)

Thymosin \( \beta_4 \) has been isolated from calf thymosin fraction V by Low et al. and have determined its amino acid sequence [56,79]. It is composed of 43 amino acid residues with a molecular mass of 4982D and an isoelectric point of 5.1. Its sequence is given in Figure 1.3.

It induces TdT, both in vivo and in vitro, in bone marrow cells from normal and athymic mice; in vivo, induction of TdT in thymocytes of immunosuppressed mice [25]. Thymosin \( \beta_4 \) has been synthesized by solid phase methods [80,81] and by classical procedures in solution [82].

1.2.4.7: Thymosin \( \beta_8 \) and \( \beta_9 \)

In 1982, Hannappel et al.-[57] isolated two polypeptides, thymosin \( \beta_8 \) and thymosin \( \beta_9 \), from calf thymosin fraction V, and established their amino acid sequences. Thymosin \( \beta_9 \) is identical to thymosin \( \beta_8 \) except for the presence of an additional dipeptide -Ala-Lys-OH at the C-terminus. They concluded that thymosin \( \beta_9 \) is the natural form of the peptide and that it is converted to thymosin \( \beta_8 \) during the preparation of fraction V. Thymosin \( \beta_9 \) has a mass of 4717D and 32 of its 41 amino acid residues are identical to those of thymosin \( \beta_4 \) [57]. The amino acid sequences of thymosins \( \beta_8 \), \( \beta_9 \), and \( \beta_4 \) are shown in Figure 1.3. There is a significant homology between \( \beta_8 \), \( \beta_9 \), and \( \beta_4 \). The presence of the two very similar peptides, thymosin
$\beta_4$ and thymosin $\beta_9$, in calf thymus extracts raises interesting questions regarding their possible role in the regulation of the immune response.

Figure 1.3. Comparison of Amino Acid Sequences of Thymosin $\beta_9$, Thymosin $\beta_8$ and Thymosin $\beta_4$. Identical sequences are shown in boxes.
It has been reported that Lupus nephritis patients have a defect of cell-mediated immunity [100] and decrease of T cells [100,101].

In vitro, thymosin $\beta_8$ and $\beta_9$ can restore the low E-rosette-forming cells in cases of Lupus nephritis. Thymosin $\beta_8$ and $\beta_9$ have been synthesized by Abiko et al. [101,102] via classical solution methods.

1.2.4.8: Thymopoietin I, II and III (TP1, II, and III)

The three polypeptides, thymopoietin I, II and III, have identical sequences except for the amino acid residues at 1, 2, 34 and 43.

TP I and TP II have been isolated and shown to be active in induction of early T-lymphocyte differentiation [83,84] and modulation of mature lymphocytes [85]. Goldstein et al. has elucidated the primary structure of thymopoietin II [62] and III [87]. TP I and TP III have been synthesized by classical methods [88,89].

1.2.4.9: Thymulin

Thymulin (Facteur thymique serique) is a nonapeptide which has been isolated from pig serum [90,91], with a molecular mass of 859D and an isoelectric point of 7.5 [91,92] and its sequence is Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn [91]. Thymulin is active on most T-cell markers and functions [92], and its use in clinics has been reported [93]. It
has specific binding sites on some lymphoblastoid T-cell lines [94,95]. Several analogues of thymulin have been synthesized to determine the structure-activity relationships [86,96-98].

1.3. Objective of the Work

In last few years, research in the field of thymology has proved the importance of thymus in the development and maintenance of immune system.

Some purified, physiologically active peptides isolated from thymus extract, have been used in several immunological, clinical problems, auto immune disorders and tumor formation.

The most important contribution of thymic hormone research resides in its application to the clinical management of thymus-dependent diseases.

In the studies of structure-function relationships of biologically active hormones, a number of identification problems occur.

First, at the analytical level, when structures should be established, some segments can be difficult to analyze. Similarly, in relation to functional aspects, important regions or particular portion need identification. At all levels, synthetic peptides are valuable tools in analyses of naturally occurring peptides, and they can be used for comparison in identification.
Thymosin \( \beta_9 \) has been synthesized by classical procedures in solution [102] where benzyl-derived side chain protecting groups were used, which required final deprotection with liquid HF. We synthesized thymosin \( \beta_9 \) by a method using tert.-butyl side chain protection for the present synthesis, the TFA-anisole deprotecting procedure was employed instead of the hydrogen fluoride deprotecting procedure. Optimum conditions were found for the final DCC/HOBt coupling of the two fragments.
2. DISCUSSION
2.1 Tactics and Strategy in Synthesis of Thymosin $\beta_9$

2.1.1 Tactics in Synthesis of Fragments A, B, C, and D.

All fragments of Thymosin $\beta_9$ are linear peptides with the following primary structure:

$\text{Ac-L-Ala-L-Asp-L-Lys-L-Pro-CH} \ (A) \ (\text{position} \ 1-4)$

$\text{H-L-Thr-L-Gln-L-Glu-OH} \ (B) \ (\text{position} \ 22-24)$

$\text{H-L-Thr-L-Ile-L-Glu-L-Gln-L-Glu-OH} \ (C) \ (\text{position} \ 33-37)$

$\text{H-L-Lys-L-Gln-L-Ala-L-Lys-OH} \ (D) \ (\text{position} \ 38-41)$

Since fragment A, B, C and D served as starting materials for an investigation of their suitability as building blocks for synthesis of larger segments of Thymosin $\beta_9$, it was important to prepare gram quantities of these intermediates in a high state of purity within a reasonable span of time. The stepwise procedure [103] was selected for this purpose. Hydrogenolysis removed the benzyleoxy carbonyl protecting group with formation of an "amino component" in terms of peptide terminology [104].

The peptide chains were extended from the amino end in a stepwise manner (Scheme I-IV) using the benzyleoxy carbonyl group for $\alpha$-amino
Scheme I: Synthetic scheme for Ac-Ala-Asp(OBt)-Lys(Boc)-Pro-OH(A)
protection. Hydrogenolysis over spongy palladium was used exclusively to remove amino protecting groups.

In the side chain protection, tert.-butyloxy carbonyl was used to protect the ε-amino group of lysine 3,38, and 41 [105] tert.-butyl esters protected the β-carboxyl of aspartic acid 2 [106] and γ-carboxyl of glutamic acids 24,35 and 37 [107], and tert.-butyl ether protected the α-hydroxyl of threonine 22 and 33 [108].

2.1.2 Strategy in synthesis of fragments A,B,C and D.

The protected peptides, such as fragments A,B,C and D (positions 1-4, 22-24, 33-37 and 38-41 respectively), are desirable intermediates for construction of large peptides via fragments condensation [109]. Detailed information about the applied coupling and cleavage methods for the synthesis of the thymosin β9 fragments is seen from Schemes I-IV, which demonstrate the strategies for the syntheses of the thymosin β9 A,B,C and D fragments.

The 13C assignments, presented in Figure 2.1-2.12 were made by comparison with the established data for closely related peptides [112].

Dicyclohexylcarbodiimides in the presence of 1-hydroxy benzotriazole (DCC/HOBt) [110] served as the carboxyl activating reagent for incorporation of sequence 40-41 into fragment D. Other amino acids
Fig. 2: Z-Asp(OBu)\textsuperscript{t}-Lys(Boc)-Pro-OH
Fig. 2.3: Ac-Ala-Asp(Obu⁺)-Lys(Boc)-Pro-OH
Scheme II: Synthetic scheme for Z-Thr(But)-Gln-Glu (OBut)-OH(B)
Fig. 2.4: Z-Gln-Glu(OBu\textsuperscript{t})-OH
Fig. 2.5: Z-Thr-Gln-Glu(Obu\textsuperscript{t})-OH
residues were introduced in the form of benzylxycarbonyl N-hydroxy-
succinimido esters [128] in fragments A, B, C and D. Certain sequences
such as 39-40, 36-37 and 23-24, in fragments, B, C and D were
introduced via p-nitrophenyl active ester method [133].

Solubility problems arose during the synthesis of fragment C and it
was found necessary to employ hydrogenolysis in solvent mixture,
Methanol/DMF/H₂O (3:2:2), instead of only methanol and washed the
catalyst repeatedly with hot water, to remove the benzylxycarbonyl
group following the coupling step.

2.2 Purification and Assessment of Homogeneity of Fragment A,
B, C and D.

In many instances, the crude coupling products were distributed between
aqueous citric acid or acetic acid and a suitable organic solvent to
remove unreacted amino component, and the material from the organic
phase was purified by solvent precipitation. Crystallization was
employed to purify intermediates, whenever possible, however, only a
limited number of the protected peptides were obtained in crystalline
form.

Protected intermediates were characterized by melting point, optical
rotation, elemental analysis and tlc. The amino acids composition of
the acid hydrolysates of a number of protected intermediates were
also determined.
The progressive accumulation of impurities was particularly apparent during the synthesis of fragment A, which contains aspartic acid residue in position 2. It should be noted that the amino acid ratio in the acid hydrolysates of protected fragment A was in good agreement with theory. No attempt was made to identify the nature of the various contaminants.

The amino acids composition of the acid hydrolysates agreed closely with that observed in the crude product. This result supports the conclusion that amino acids analysis of the acid hydrolysates of deprotected synthetic peptides which were not shown to be "pure" by other methods, provide little support for homogeneity. Amino acids analyses in agreement with theoretical values are good indicators but not sufficient criterion of purity.

Reagent dicyclohexylcarbodiimides (DCC) in the presence of 1-hydroxybenzotriazole [110] served as the carboxyl-activating reagent for incorporation of alanine 40 into fragment D. Fragment A, B and C were prepared via the N-hydroxysuccinimide active ester method [128].

Attempts to introduce the proline-α-benzylester 4 (position 3-4) to 9-hydroxysuccinimide ester of protected lysine 3 into fragment A were unsuccessfull; however, the sodium salt of proline was used for coupling.
Scheme III: Synthetic scheme for Z-Thr(Bu<sup>t</sup>)-Ile-Glu(OBu<sup>t</sup>)-Gln-Glu(OBu<sup>t</sup>)OH(C)
Fig. 2.6: Z-Glu(OBu')-Gln-Glu(OBu')-OH
Fig. 2.7 : Z-Ile-Glu(OBu')-Gln-Glu(OBu')OH
2.3 Assembly of fragment C and D.

For the synthesis of fragment CD (position 33-41), fragment D was deprotected by hydrogenolysis over palladium on charcoal in DMF and the ensuing deprotected tetrapeptide was coupled with fragment C (position 33-37) by the dicyclohexylcarbodiimide-N-hydroxy succinimide (DCC/HOSu) procedure [110]. Following completion of the coupling reaction, the product was precipitated by cooling in (or addition of) ice-cool water. The desired fragment CD was in homogenous form in solvent system n-butanol/acetic acid/water (3:1:1). The overall yield of chromatographically pure fragment was 93%. Hydrogenation of fragment CD over 10% palladium on charcoal in solvent system MeOH/DMF/water (3:2:2) removed the benzyloxy carbonyl group.

The hydrogenation product was pure on tlc and the deprotected N-terminal nonapeptide (CD) was treated with TFA-anisole to remove the t-butyl and t-butyloxy carbonyl side chain protecting group. The crude product was purified by chromatography on a Sephadex G-15 column. Determination of the ratios of amino acids residues in a peptide, which was obtained by condensation of two homogenous fragments, provides a valid criterion for assessment of purity of the coupling product.
Scheme IV: Synthetic scheme for Z-Lys(Boc)-Gln-Ala-Lys(Boc)-OBu^t(D)
Fig. 2.9: Z-Ala-Lys(Boc)-Cbè
Fig. 2.10: Z-Glu-Ala-Lys(Boc)OBu^t
Fig. 2.11: Z-Lys(Boc)-Gln-Ala-Lys(Boc)-OBu^t
Fig. 2.12: Z-Thr(Bu^t)-Ile-Glu(OBu^t)-Gln-Glu(OBu^t)-Lys(Boc)-Gln-Ala-Lys(Boc)-OBu^t
In the course of this investigation, tlc of the some protected peptides in fragments A and D revealed the presence of contaminants. The impurities accumulated with progressive chain elongation, and thus repeated precipitation failed. The impurities do not necessarily appear in the form of distinct spots but create streaking effect on the plates. Despite the fact that tlc demonstrated the presence of contaminants, elemental analyses and amino acids compositions of the acid hydrolysates afforded values which agreed within experimental error, with those expected by theory. It follows that these analytical tools are not adequate to establish homogeneity, or the compound slowly decomposes on tlc plates.

The experiments presented have shown that fragments B and C, synthesized by the stepwise method, were pure. Chromatographically purified materials were homogenous, as judged by tlc, and their acid hydrolysates exhibited, within the experimental error, the correct amino acids composition. However, it should be recognized that the analytical procedures used are not sensitive enough to detect minor degree of racemization.
3. EXPERIMENTAL
3.1 Spectroscopic Methods

3.1.1 NMR Spectroscopy

$^{13}$C-NMR Spectra were recorded on Bruker HFX-90-Multikerngeraet (22.628 MHz for $^{13}$C) and WP-80-Spectrometer (20.115 MHz), Bruker, (W.Germany), operating in the Fourier transform mode and linked to a BNC-28 computer. The sweep width was 5000 Hz and the pulse width 5μs. Chemical shifts were measured relative to $(\text{CH}_3)_4\text{Si}$ (TMS) as an internal standard. Every substance was measured app. 200-300 mg dissolved in 1.0-1.5 ml of the corresponding solvent.

3.1.2 Optical rotation

Optical rotations were measured with a digital-polarimeter OLD 5, Zeiss. Measurements were carried out with a mercury lamp at 578, 546, 436, 405 and 365 nm. The $[\alpha]_D$ values were determined by graphical extrapolation. Some rotations were carried out with a Perkin-Elmer 141 polarimeter and $[\alpha]_D$ values were measured directly.

3.2 Chromatographic methods

Reaction mixtures were examined by the thin layer chromatography on silica gel 60 F$_{254}$ (Merck, W.Germany). Solvent systems for ascending tlc were the following:

A: n-Butanol/Acetic acid/Water 3:1:1

B: Hexane/tert. Butanol/Pyridine 5:1:1
C: Chloroform/Methanol/Acetic acid/Water  60:45:6:14
D: Chloroform/Methanol/Benzene/Water  8:8:8:1
E: Chloroform/Methanol  98:2
F: Chloroform/Methanol/25% Ammonia solution  12:9:4
G: Butanol/Pyridine/AcOH/H₂O  30:20:6:24
H: Butanol/Acetic acid/ Water/Pyridine  60:6:24:20
I: Chloroform/Methanol  9:4

Spots were visualized by ninhydrin and Cl₂-Tolidine reagents having the following composition:

Ninhydrin solution [113,114].

300 mg Ninhydrin was dissolved in 100ml n-butanol and 3 ml acetic acid. The solution was stored at 4°C and used as a spraying reagent.

Chloro-Tolidine solution [115].

80 o-Tolidine, 15ml acetic acid and 0.55g potassium iodide were dissolved in 250 ml water. TLC plates were kept in Cl₂ chamber for 10mins., dried with air and sprayed with reagent.
3.3 Biological Assay [39,116]

Human T-lymphocytes form rosettes with sheep erythrocytes. The formation of rosettes may be inhibited by α-amantin or theophylline. The inhibition is reversible in presence of immunologically active peptides and is used to determine their biological activity. The isolation of human T lymphocytes is surveyed in Figure 3.1. Experimental details for the rosette assay are given in Figure 3.2.

Several synthetic pure and characterized peptides were tested according to Fig.3.2. Incubation of $5 \times 10^5$ lymphocytes/ml with 1.0 g α-amantin caused an inhibition of rosette formation of 40-80% depending on the lymphocytes preparation.

3.4 Miscellaneous machines

All melting points are uncorrected and taken on Dr. Tottoli Type Buechi 510), Buechi, Flavil, Switzerland.

A combination of pH-Meter E 512 and impulsomat E 73, Metrohm, Switzerland, was used to calculate pH.

The amino acid compositions of acid hydrolysates were determined with Biotronik Model LC 6000 amino acid analyzer according to the method of S. Moore [117]. Acid hydrolysis was performed in 5.7 N HCl at 110°C for 24h. in evacuated tubes and acid was removed on rotary
HUMAN BLOOD (WITH HEPARIN)

MIXTURE WITH PBS (1:2)

TO TUBE WITH FICOLL, CENTRIFUGE, (400G, 30')

PELLET (ERYTHROCYTES)

INTER PHASE (LYMPHOCYTES, MACROPHAGES)

SUPERNATANT (SERUM)

INTERPHASE, 2 X WITH PBS, CENTRIFUGED AT 150-200 G, 5 MIN.

WHITE PELLET, SUSPENDED IN RPMI/20% FCS AND BROUGHT TO PETRI DISH

INCUBATION, 37°C (MACROPHAGES ARE ADSORBED ON GLASS SURFACE)

CENTRIFUGE AT 150-200 G, 10 MIN.

PELLET, SUSPENDED IN RPMI 1640/FCS (20%), 5 X 10^6 LYMPHOCYTES/ML

Fig. 3.1: Survey of the isolation of human T-lymphocytes (PBS: phosphate buffered saline; RPMI: RPMI 1640 medium; FCS: fetal calf serum).
**ROSETTE ASSAY**

<table>
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<tr>
<th>Step</th>
<th>Details</th>
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<tr>
<td>100μL LYMPHOCYTE SUSPENSION</td>
<td>INCUBAT.</td>
</tr>
<tr>
<td>(5x10⁶ L/ML + 20 μl FCS)</td>
<td>(37°C, 9h)</td>
</tr>
<tr>
<td>+ 10 μl α-CAMANITIN (=1.0 G)</td>
<td></td>
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<tr>
<td>+ 50 μl PEPTIDE SOLUTION</td>
<td>INCUBAT.</td>
</tr>
<tr>
<td>(= 10μG PROTEIN)</td>
<td>(37°C, 1h)</td>
</tr>
<tr>
<td>+ 50 μl SRBC (1x10⁹ S/ML)</td>
<td>INCUBAT.</td>
</tr>
<tr>
<td></td>
<td>(4°C, 5')</td>
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</table>

**Diagram:**
- CENTRIFUG. (40 G, 5')
- PELLET (ROSETTES) + SUPERNATANT (4°C, 5h)
- RESUSPEND
- COUNT ROSETTES

**Fig. 3.2:** Experimental details for the rosette assay (L: human T-lymphocytes; SRBC = S: sheep red blood cells).
evaporator. The racemate tests were carried out by proper hydrolysis of peptide. \( N(0,S) \text{-pentafluoropropionyl} \) amino acid esters were separated on a glass capillary coated with Chirasil-Vat, dimension 20mm x 0.25mm. Carrier gas was hydrogen at an inlet pressure of 38K Pa; injector and detector temp. were at 250°C. The gas chromatograph was done on a Fractovap Mod.G1. Carlo Erba, Milano and a Fractovap 2101 AC, Carlo Erba.

3.5 Chemicals

All the solvents for the coupling reaction were dried according to the known procedures [118]. The anhydrous solvents store over molecular sieves (Merck, 3-4Å).

All amino acids employed in the synthesis were of the L-configuration and obtained from Merck. \( N \text{-Hydroxy succinimide} \) was purchased from Serva (Switzerland). \( 1 \text{-Hydroxy benzotriazol} \) from Fluka (Switzerland), Benzylchloroformate from Merck (W.Germany).

Solutions were concentrated in a rotary evaporator under reduced pressure at a temp. of 30-40°C. The free peptides were dried at 70°C in high vacuum over phosphorus pentoxide, other compounds were dried at room temperature.
Reported yields are those obtained after crystallization or purification. t-butyl and t-butyloxy carbonyl groups of the protected peptides were removed by TFA-anisole treatment [119].

Mol. weights were calculated using following atomic weights.

\[ C = 12.011, \quad H = 1.008, \quad Cl = 35.453, \quad F = 18.998, \quad N = 14.007 \]
\[ O = 15.998, \quad \text{and} \quad S = 32.064 \]

3.6 Synthesis of Thymosin \( \beta_\text{9}((1-4) \text{ Acetyl-alanyl-asparagyl} \)
\( \text{ (} \beta\text{-tert.buty l ester)} - N^\text{c} \text{-tert.buty loxy carbonyl-lysyl-proline} \)
\( \text{ (Ac-Ala-Asp(OBu})^\text{t} ) - \text{Lys(Boc)-Pro-OH} \]

3.6.1 \( N^\text{c} \text{-Benzylkoxy carbonyl-} N^\text{c} \text{-tert.-butyloxy carbonyl-lysine-N-}
\text{hydroxysuccininimide ester (Z-Lys(Boc)-OSu)} \)

3.6.1.1 \( N^\text{c} \text{-Benzylidene-L-Lysine (H-L-Lys(Bal)-OH).} \)

131 g (0.72 mole) of L-lysine hydrochloride was dissolved under \( N_2 \)
with stirring in 360 ml of 2N LiOH solution. The mixture was cooled
to \( 1^\circ C \) and 76.4 ml (0.74 mole) of benzaldehyde (freshly distilled) was
added dropwise at a rate such that the temperature remained at \( 4^\circ C \).
Precipitation occurred during this time and stirring became difficult.
The reaction mixture was kept at 3-5\(^\circ\)C over night, diluted with 200
ml cold ethanol and filtered. Solid was washed with 30 ml cold
ethanol, triturated with stirring at 3-5\(^\circ\)C with 100 ml cold ethanol and
again filtered. The solid product was washed with cold ethanol and
dried. Yield: 105.24 g (65%).
m.p. : 198-199°C, Lit. 120 : 206-208°C, Lit. 121 : 182-186°C

Lit. 105 : 203-206°C.

\( \text{(C}_{13}\text{H}_{18}\text{N}_{2}\text{O}_{2} (234.3).} \) Anal. Calcd.: C 66.64 H 7.74 N 11.96

Found: 65.97 7.69 11.82

3.6.1.2 \( \text{N}^\alpha\text{-Benzyl oxycarbonyl-L-lysine (Z-L-lys-OH)} \)

A stirred mixture of 448 ml 1N NaOH and 448 ml of ethanol was cooled to -30°C. To this solution was added 105 g (0.448 M\text{ol}) finely ground \( \text{N}^\alpha\text{-Benzylidene-L-lysine}. \) Immediately, in the above solution benzyl oxycarbonyl chloride was added from jacketed addition funnel kept at -20 to -10°C. Simultaneously, a precooled (-20°C) mixture of 1112 ml 1N NaOH and 862 ml ethanol was added. During this time the bath temperature rose to 25°C. The reaction mixture was stirred with cooling until the internal temperature began to drop and then without cooling until the temperature reached -5.5°C. 111.5 ml (1.34 M\text{ol}) of precooled conc. HCl was added. The mixture was warmed to 150°C and then distilled. The residue was washed three times with 227 ml ether and brought from pH 1 to pH 6.2 by addition of 2N NaOH. The solution was kept at 3-5°C overnight. Filtered and the supernatant was concentrated. The resultant slurry was stored at 3°C overnight and filtered. The solid was washed with cold water and dried to give 59.29 g (yield: 47%).

Rf$_A$: 0.46, Rf$_D$: 0.13

$[\alpha]_D^{23}$ : 11.0 (c=1.00, 0.2N HCl)

$[\alpha]_D^{26}$ : Lit.122 : -12.0 (c=2.00, 0.2N HCl)

C$_{14}$H$_{20}$N$_2$O$_4$ (280.3). Anal. Calcld.: C 59.99 H 7.19 N 10.00

Found: 59.90 7.18 10.10

3.6.1.3 $^\alpha$-Benzyloxy carbonyl-$^\beta$-tert-butyloxycarbonyl-L-lysine
dicyclohexylamine salt(Z-L-lys(Boc)-OH.DCHA)

To a stirred mixture of 59.29 g (0.21 Mol) of $^\alpha$-benzyloxy carbonyl-L-lysine in 106 ml DMSO was added 24.41 g (0.211 Mol) of tetramethylguanidine and 45.47 g (0.233 Mol) of tert.butylyphenylcarbonate. The mixture was heated at 50°C for 5 h., cooled and poured into 75 ml ice-water. The solution was brought from pH 7.0-9.3 to pH 3 by the addition of 10% H$_2$SO$_4$, washed with ether and cooled. The oily suspension was extracted with ether. The ether extract was washed with water, dried over Na$_2$SO$_4$ and evaporated to give 86 g. of Z-Lys(BOC)-OH. A solution of this material in 411 ml of ethylacetate was stirred under N$_2$ and treated with 42.1 ml (.212 Mol) of dicyclohexylamine for 1 h. The suspension was cooled to 3°C filtered,
washed with ether and dried to give 86.51 g (yield: 70%).


$R_f_A : 0.83, R_f_D : 0.48$

$[\alpha]_{D}^{23} : +7.1$ (c=1.00, EtOH)

$[\alpha]_{D}^{20}$ Lit. : +7.8 (c=1.00, EtOH)


3.6.1.4 $N^\epsilon$-Benzyloxy carbonyl-$N^\alpha$-tert.-butyloxy carbonyl-L-lysine-$N^\epsilon$-hydroxysuccinimidester (Z-Lys(Boc)-OSu)

$N^\alpha$-Benzyloxy carbonyl-$N^\epsilon$-tert.-butyloxy carbonyl-L-lysine dicyclohexyl amine salt was prepared by the method of Scott and Parrish [105]. 8 g of this salt (142 mM) was ground in mortar, then stirred in 50% aqueous ethyl acetate, and brought to pH 2 with 10% citric acid. The solution was extracted with ethyl acetate and the organic phase was washed with water and dried over Na$_2$SO$_4$. The solvent was removed in a rotary evaporator to give $N^\alpha$ benzyloxy carbonyl-$N^\epsilon$-tert.-butyloxy carbonyl-L-lysine as an oil. The product obtained in 75% (51.15 g) yield, was ninhydrin negative and was pure in chromatography in system D. The oil (39.54 g, 103 mM) was dissolved in 185 ml of a
mixture of ethyl acetate and dioxane (1:4) together with 11.82 g (103 mM) of N-hydroxysuccinimide. The solution was cooled in an ice bath, 21.17 g (103 mM) of DCC was added, and the mixture was stirred overnight at 5°C. Dicyclohexyl-urea was removed by filtration. The filtrate was evaporated and the residue was crystallized from isopropanol-petroleum ether to give 34.42 g (yield: 70%).


[α]$_D^{25}$ : -14.1° (c=3.5, dioxane)

[α]$_D^{27}$ : Lit. : -15.9° (c=3.5, dioxane)

C$_{23}$H$_{31}$N$_3$O$_8$ (477.472). Anal. Calcd.: C 57.85 H 6.54 N 8.80

Found: 57.78 6.50 8.81

3.6.2 N$^\alpha$-Benzylxoycarbonyl-$N^\epsilon$-tert.-butyloxy carbonyl-L-lysine-L-proline (Z-L-Lys-(Boc)-L-Pro-OH)

A solution of 2.86 g (0.006 mole) of the N-hydroxysuccinimide ester of benzylxoycarbonyl-$N^\epsilon$-tert.-butyloxy carbonyl-L-lysine in 10 ml of DMF was added to a solution of 0.748 g (0.0065 mole) of proline and 0.58 g (0.007 mole) of NaHCO$_3$ in 20ml DMF:H$_2$O (4:18) with stirring at room temp. A few ml of DMF was added to maintain a clear solution. After 5 h. the solution was concentrated under vacuum to
remove DMF. The aqueous reaction mixture was acidified to pH 2 with 2N citric acid and extracted with ethyl acetate. The extract was washed with cold water and dried over Na₂SO₄, evaporated in rotary evaporator and the concentrated extract was taken up in a small volume of ethyl acetate. Precipitation was obtained by dropwise addition of the above solution to a mixture of ether/hexane (1:4) at -10°C with constant stirring. The precipitate was filtered and dried under reduced pressure 1.91 g (yield: 67%).

m.p. : 43-44°C

[α]₂⁵ᵩ : -1.39° (c = 1, CHCl₃)

Rᶠ D : 0.44

C₂₄H₃₅O₇N₃ (477.513). Anal. Caled.: C 60.30 H 7.38 N 8.78

Found: 60.00 7.38 8.36

Amino acid analysis: Lys Pro

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0.98 0.85

3.6.2.1 N⁶-tert.-Butyloxycarbonyl-Lysyl-Proline (H-L-Lys(Boc)-L-Pro-OH)

The protected dipeptide (7.2 g, 0.015 mole) in 100 ml. MeOH was hydrogenated over 10% Pd/charcoal for 6 h. The catalyst was removed
by filtration and the filtrate was evaporated.

m.p. : 174-177°C

\[ \alpha \]_D^{25} : 1.32° (c=0.5, MeOH)

R_f D : 0.22

C_{16}H_{28}N_{3}O_{5} (342.38). Anal. Calcd. C 56.12 H 8.23 N 12.27

Found: 56.20 8.33 12.22

3.6.3  \text{N}^{\alpha}-\text{Benzyl oxy carbonyl-L-aspartic acid (6-tert.-butylester)-N-hydroxysuccinimidester (Z-L-Asp(OBu')-OSu)}

3.6.3.1 \text{N}^{\alpha}-\text{Benzyl oxy carbonyl-L-aspartic acid (Z-L-Asp-OH)}

Z-L-Asp-OH was synthesized according to Wuensch and Zwick [125] in 80% yield, purity was checked in system A and D.


\[ \alpha \]_D^{20} : 9.65° (c=2, AcOH), Lit. : 9.25 (c=2, MeOH).

R_f D : 0.19, R_f A : 0.71.

C_{12}H_{13}NO_{6} (267.24). Anal. Calcd.: C 53.93 H 4.90 N 5.24

Found: 53.94 4.87 5.29
3.6.3.2 (S)-3-Benzylxocarbonyl-5-oxo-4-oxazolidine acetic acid [106]
A mixture of α-benzylxocarbonyl-L-aspartic acid (58 g), paraformaldehyde (10.32 g) and p-toluenesulfonic acid (2 g), in benzene (1400 ml), was refluxed for 10 h., the liberated water being removed azeotropically using a Dean-stark trap. The benzene solution was washed with water and extracted with 5% NaHCO₃ solution. The aqueous layer was acidified with 6N HCl on ice-cooling and extracted with EtOAc. The extract was washed with water, dried over MgSO₄. Evaporation of the solvent gave a pale yellow syrup (54.5 g, 90% yield).

C₁₃H₁₃NO₆ (279.24). Anal. Calcd.: C 55.91 H 4.70 N 5.02
Found: 55.60 4.61 5.10

3.6.3.3 tert.-Butyl(S)-3-benzylxocarbonyl-5-oxo-4-oxazolidine acetate [106]
59 g (6.3.2) of above product was dissolved in dry CH₂Cl₂ (403 ml) containing concd. H₂SO₄ (2.68 ml) as a catalyst. Isobutene was added to the solution at -10°C. The solution was kept in a stoppered flask at room temp. for 4 days. After addition of EtOAc, the solution was washed with 5% NaHCO₃ solution, then with water, and dried, over Na₂SO₄. Solvents were removed to give tert. butyl (S)-3-benzylxocarbonyl-5-oxo-4-oxazolidine acetate as a pale yellow syrup. 62.67 g, (89% yield).
3.6.3.4 Benzyloxy carbonyl-L-aspartic acid(β-tert. butylester) dicyclohexylammonium salt (Z-L-Asp(OBu)\(^\text{t}\)-OH.DCHA) [106]

A solution of 6.3.3 product (47 g) in ethanol (400 ml) was treated with 1N NaOH (135 ml) at room temp. for 8 h. The reaction mixture was neutralized with 1N HCl, and ethanol was removed under reduced pressure. The product dissolved in EtOAc was extracted with 5% Na\(_2\)CO\(_3\) soln. The extract was washed once with fresh EtOAc and acidified with 6N HCl. The acidified soln. was extracted with EtOAc. The organic layer was washed with water and dried over Na\(_2\)SO\(_4\). Evaporation of solvent gave a syrupy product. Upon adding dicyclohexylamine (21 g) to a solution of the above product in ether (100 ml), 32 g, (45%) of crystalline salt was obtained.

m.p.: 126-127°C, Lit. [106]: 128-129°C, Lit. [126]: 123-124°C, Lit. [127]: 125-126.5°C.

\([\alpha]\)\(_D\)\(^{22}\) : +12.9° (c=2, EtOH).

\([\alpha]\)\(_D\)\(^{24}\) Lit. [12]: 6.5° (c=1.72, 90% AcOH)

\([\alpha]\)\(_D\)\(^{25}\) Lit. [17]: 7.7° (c=1, 95% AcOH)

\([\alpha]\)\(_D\)\(^{25}\) Lit. [18]: 5.5 ± 1° (c=1, 90% AcOH)

C\(_{28}\)H\(_{44}\)N\(_2\)O\(_6\) (504.66). Anal. Calcd.: C 66.64 H 8.78 N 5.55

Found: 66.69 8.88 5.50
3.6.3.5 Benzyloxy carbonyl-L-aspartic acid (β-tert. butyl ester)-N-hydroxysuccinimide ester (Z-L-Asp(BOBu⁺)-OSu)

The active ester was synthesized according to Anderson et al. [128] from Z-L-Asp(BOBu⁺)-OH, HOSu and DCC (Z-L-Asp(BOBu⁺)-OH.DCHA salt was treated with 10% citric acid). 30 g (79%) yield.

m.p. : 150-153°C, Lit. [129] : 151-152.5°C {129}

RfD : 0.69

[a]D²³ : -19.0° (c=1, dioxane)

[a]D²⁰ Lit. [129] : 19.4 ± 0.5° (c=1.12, dioxane)

C₂₀H₂₄N₂O₈ (420.4). Anal. Calcd.: C 57.4 H 5.75 N 6.66

Found: 57.61 5.77 6.57

3.6.4 Benzyloxy carbonyl( β-tert.-butylester)-L-aspartyl-NE-tert.-butyloxy carbonyl-L-lysyl-Proline (Z-L-Asp(BOBu⁺)-L-Lys (Boc)-L-Pro-OH)

3.78 g (0.009 mole) of Z-Asp(BOBu⁺)-OSu was dissolved in 10 ml DMF, and added to a solution of H-Lys(Boc)-Pro-OH (3.25 g 95 mM) in DMF (12 ml) and water (44 ml) containing NaHCO₃ (0.79 g, 95 mole). The mixture was allowed to stand for 15 h at room temp., and the
bulk of the solvent was removed. The residue was dissolved in EtOAc, acidified to pH 2 with 2N citric acid and extracted with EtOAc. The soln. was washed three times with cold water and once with saturated NaCl, and dried over sodium sulfate. EtOAc was evaporated to a small vol., and the product precipitated by dropwise addition of EtOAc into a large volume of rapidly stirred hexane (-10°C) and filtered. The precipitate was collected and dried 2.83 g (50% yield).

m.p. : 72-75°C.

[α]$_D^{28}$ : - 28.8 (c=1, DMF)

R$_f$$^D$ : 0.40

C$_{32}$H$_{48}$N$_4$O$_{10}$ (648.692). Anal. Calcd.: C 59.25 H 7.40 N 8.60

Found: 59.30  7.41  8.59

3.6.4.1 L-Aspartyl( β-tert.-butylester)-N$_F$-tert.-butyloxycarbonyl-L-lysyl-L-Proline (-L-Asp(OBu$_t^\dagger$)-L-Lys(Boc)-L-Pro-OH)

1.5 g Z-L-Asp(OBu$_t^\dagger$)-L-Lys(Boc)-L-Pro-OH was hydrogenated in (20 ml) EtOH in the presence of 10% Pd-charcoal for 2 h. After the removal of catalyst by filtration with the aid of celite, the filtrate was evaporated to dryness, and dissolved in a small volume of MeOH.
Solid residue was obtained by adding ether and filtered under vacuum. Yield 1.06 g (90%).

m.p. : 90–94°C.

R_f D : 0.19

C_{24}H_{40}N_{4}O_{8} (512.528). Anal. Calcd.: C 56.24 H 7.80 N 10.90

Found: 56.33 7.89 10.80

3.6.5 N-Acetyl-L-alanine (Ac-L-Ala-OH) [130]

44.5 g (0.5 M) L-alanine was suspended in 450 ml glacial AcOH and boiled. The mixture was cooled up to 15°C. 51.1 g (0.5 M) freshly distilled acetic anhydride was added dropwise. The resulting solution was boiled for 2 min., and then allowed to cool to 25°C.

m.p. : 134–135°C : Lit. [130] : 125°C.

[α]_{D}^{20} : -65.4 (c=1, H_{2}O)

R_f D : 0.16, R_f A : 0.60

C_{5}H_{9}NO_{3} (131.1). Anal. Calcd.: C 45.80 H 6.60 N 10.68

Found: 45.90 6.93 10.55
3.6.5.1 N-Acetyl-L-alanine-N-hydroxysuccinimide ester (Ac-L-Ala-OSu)

The active ester was synthesized according to Anderson et al. [128] from Ac-L-Ala-OH, HO:Su, and DCC in dimethoxyethane at -30°C, and recrystallized from isopropanol, 30 g (74% yield).


\[ \alpha_D^{22} : 24.9 \text{ (c=0.5, } H_2O) \]

\[ \alpha_D^{20} \text{ Lit. [128] : 25.8 (c=0.5, } H_2O) \]

\[ R_D : 0.64, R_E : 0.19 \]

C₉H₁₂N₂O₅ (228.2). Anal. Calcd.: C 47.37 H 5.30 N 12.28

Found: 47.44 5.55 12.27

3.6.6 Acetyl-L-alanyl-L-asparty(ε-tert.-butylester)-N-tert.-butyl-oxycarbonyl-L-lysyl-L-proline (Ac-L-Ala-L-Asp(OBuᵗ)L-Lys (Boc)-L-Pro-OH)

H-L-Asp(OBuᵗ)-L-Lys(Boc)-L-Pro-OH (1.5 g, 0.003 mole) and NaHCO₃ (0.25 g, 0.003 mole) were dissolved in 30 ml of water and 20 ml DMF. A soln. of N-hydroxysuccinimide ester of N-acetyl-L-alanine (0.445 g,
0.022 mole) in 10 ml DMF was added with stirring at room temp. This was allowed to stand at room temp. for 2 h. The solution was then acidified with 2N citric acid to pH 3 and extracted with EtOAc. The resulting solution was dried over anhydrous \( \text{Na}_2\text{SO}_4 \) anhydrous. The extract was conc. in vacuo, and poured dropwise in to ice-chilled mixture of ether and hexane (2:4) with vigorous stirring. The ppt. obtained was filtered. After thorough drying in a vacuum dessicator; 0.5 g (42%) tetra peptide was obtained.

m.p. : 60-62°C (amorphous).

\[ R_f^D : 0.36, \quad R_f^A : 0.65 \]

\[ \text{C}_{29}\text{H}_{41}\text{N}_5\text{O}_{10} \quad (627.672). \quad \text{Anal. Calcd.:} \quad \text{C} 55.44 \quad \text{H} 7.80 \quad \text{N} 11.15 \]

\[ \text{Found:} \quad 51.72 \quad 7.37 \quad 10.08 \]
3.7 Benzyloxy carbonyl-O-tert.-butyl-L-threonyl-L-glutaminyll-
L-glutamic acid (γ-tert.-butylester) (Z-L-Thr(Bu<sup>t</sup>)-L-Gln-L-
Glu(OBu<sup>t</sup>)-OH

3.7.1 L-Glutamic acid (γ-tert.-butylester) (H-L-Glu(OBu<sup>t</sup>)-OH

3.7.1.1 Benzyloxy carbonyl-L-glutamic acid (Z-L-Glu-OH)

Z-L-Glu-OH was synthesized in 89% yield using a Z-CI procedure [131].


\[ \alpha \] <sup>21</sup><sub>D</sub> : -7.5° (c=10, AcOH)

\[ \alpha \] <sup>21</sup><sub>D</sub> Lit. : -7.9° (c=10, AcOH)

C<sub>13</sub>H<sub>15</sub>NO<sub>6</sub> (281.27). Anal. Calcld.: C 55.52 H 5.38 N 4.98

Found: 55.49 5.32 4.99

3.7.1.2 (S)-3-Benzyloxy carbonyl-5-oxo-4-oxazolidinopropionic acid (1)

[106]

A mixture of Z-Glu-OH (56.2 g), paraformaldehyde (120.0 g) and
p-toluenesulfonic acid (2.0 g) in benzene (1400 ml) was refluxed for
18h. the liberated water being removed azeotropically using a Dean-stark
distilling apparatus. The benzene solution was washed with water
and extracted with 5% NaHCO<sub>3</sub> solution. The ice-cooled aqueous layer
was acidified with 6N HCl, and extracted with EtOAc. The extract was washed with water and dried over Na₂SO₄. Evaporation of the solvent gave a pale yellow syrup, 52.6 g, (90%). For analysis, a sample of 1.5 g was used for chromatographic analysis on silica gel.

C₁₄H₁₅O₆N (293.274). Anal. Calcd.: C 57.33 H 5.16 N 4.78
Found: 57.31 4.99 4.68

3.7.1.3 tert.-Butyl(S)-3-benzylxoycarbonyl-5-oxo-4-oxazolidine propionate (II)

The product (I) (30 g) was allowed to react with isobutene, as described in Section 3.6.3.3. The oily product obtained 30.8 g (88% yield) was used for further reaction without any purification.

3.7.1.4 γ-tert-Butyl-L-Glutamate (II-L-Glu(OBuᵗ)-OH)

A solution of product II (30 g) in MeOH (300 ml) was hydrogenated. Crude product ppted. from MeOH/Ether gave 7.6 g (44.1% yield).

m.p.: 184-186°C, Lit. [106]: 174-176°C.

[α]D²₂.₅: + 11.5° (c=2, H₂O)

[α]D²₃: + 20.4° (c=1.9, 95% AcOH)

C₉H₁₇O₄N (203.2). Anal. calcd.: C 53.19 H 8.43 N 6.89
Found: 53.01 8.50 6.90
3.7.2 Benzylloxycarbonyl-L-glutamine (Z-L-Gln-OH)

Z-L-Gln-OH was synthesized according to Gibian and Klieber [132]. The product, obtained in 94% yield, was found to be homogenous on tlc. in system A and D.

m.p. : 133-134°C, Lit. [132], 133-135°C.

\[ [\alpha]_{D}^{23} : -5.6^\circ \ (c=1, \text{EtOH}) \]

\[ [\alpha]_{D}^{20} \text{ Lit. [132]} : -7.1^\circ \ (c=2, \text{EtOH}) \]

C_{13}H_{16}N_{2}O_{5} \ (280.28). \ Anal. Calcd.: C 55.70 H 5.57 N 10.00

Found: 55.35 5.74 10.22

3.7.2.1 Benzylloxycarbonyl-L-glutamin-p-nitrophenyl ester (Z-L-Gln-ONp)

This ester was prepared from the benzylloxycarbonyl-L-glutamine according to the procedure of Bodanszky and Vigneaud [133] for p-Nitrophenyl esters of carbobenzyloxy amino acids. The product was obtained in 74% yield.


\[ [\alpha]_{D}^{22} : -24.2^\circ \ (c=2, \text{DMF}) \]
\[ \alpha_D^{20} \text{ Lit. [134]} : -24^\circ \ (c=2, \ DMF) \]

\[ \alpha_D^{21} \text{ Lit. [133]} : -24.4^\circ \ (c=2, \ DMF) \]

C_{19}H_{19}N_3O_7 (401.38). Anal. calcd.: C 56.86 H 4.77 N 10.47

Found: 56.67 4.91 10.51
3.7.3  \( \alpha \)-Benzyloxy carbonyl-L-glutaminyl-L-glutamic acid (\( \gamma \)-tert. butyl ester) (Z-L-Gln-L-Glu(Obut)-OH)

A solution of L-glutamic acid (\( \gamma \)-tert. butyl ester) 6 g (29 mM) and NaHCO\(_3\) (2.9 g 34 mM) was prepared in 100 ml H\(_2\)O:DMF (8:2). To this was added a solution of Z-Gln-ONP (11.85 g 29 mM) in DMF (50 ml), at room temp., the resulting solution was allowed to stand for 10 h. After evaporating the reaction mixture, EtOAc was added to the residue, acidified to pH 1.5 by the addition of 2N citric acid. The organic layer was washed with water and dried over anhydrous Na\(_2\)SO\(_4\). The filtrate was evaporated and was crystallized from EtOAc-Petroleum ether 11.1 g (yield: 80.6%).

m.p.: 112-113\(^\circ\)C.

\([\alpha]_{D}^{22}\) : -2.4 (c=1, dioxane)

\(R_f\) \(D\) : 0.28

\(C_{22}H_{33}N_{3}O_{8}\) (465.41). Anal. Calcd. C 56.76 H 6.70 N 8.90

Found: 56.88 6.75 8.88
3.7.3.1 L-Glutaminy1-L-glutamic acid (γ-tert-butyl ester) (H-L-Gln-L-Glu(OBu\textsuperscript{t})-OH)

6 g Benzyloxycarbonyl glutaminyl glutamic acid (γ-tert. butyl ester) was hydrogenated over palladium in (300 ml) MeOH:DMF: Water (3:2:2). After 30 min., the hydrogenated product was crystallized. The thick reaction mixture was filtered and the catalyst was washed several times with hot water. The filtrates were mixed together and evaporated. The residue was dissolved in a small volume of MeOH and ether was added. The ppt. was collected and dried (5g) 78.12% yield.

m.p. : 175-176°C

[α]\textsubscript{D}\textsuperscript{22} : + 1.5 (c=1, AcOH)

R\textsubscript{f}D : 0.10, R\textsubscript{f}A : 0.24

C\textsubscript{14}H\textsubscript{25}N\textsubscript{3}O\textsubscript{6} (331.335). Anal. Calcld.: C 50.75 H 7.59 N 12.68

Found: 50.59 7.48 12.28

3.7.4 Benzylxycarbonyl-O-tert.-butyl-L-threonine hydroxysuccinimide ester (Z-L-Thr(Bu\textsuperscript{t})-OSe)

3.7.4.1 L-Threonine-methyl ester (H-L-Thr-OME)

The ester was synthesized according to Vogler [135] in 90% yield and purity was checked in system D.
m.p. : 68-69°C Lit. [135] : 70-72°C

$[\alpha]_{D}^{22} : +3.7^\circ$ \(c=2, \text{MeOH}\) Lit. [135] : $+5^\circ$ \(c=3, \text{MeOH}\)

$\text{C}_5\text{H}_{11}\text{NO}_3$ (133.15). Anal. Calc.: C 45.10 H 8.33 N 10.52

Found: 45.11 8.35 10.49

3.7.4.2 Benzylloxycarbonyl-L-threonine-α-methyl ester. (Z-L-Thr-OMe)

H-L-Thr-OMe was coupled with Z-Cl according to Schroeder [136]. Z-L-Thr-OMe was pure on tlc. in system D. 20 g (89% yield).

m.p. : 89-91°C, Lit. [136] : 90°C

$[\alpha]_{D}^{22} : -17.1^\circ$ \(c=1, \text{MeOH}\)

$[\omega]_{D}^{20}$ : Lit. [136] : $-17.3^\circ$ \(c=1, \text{MeOH}\)


Found: 58.45 6.42 5.25

3.7.4.3 Benzylloxycarbonyl-O-tert.-butyl-L-threonine-α-methyl ester (Z-L-Thr(Bu')-OMe)

Z-L-Thr(Bu')-OMe was synthesized using the method of Schroeder [108] in 91% yield. The product was oil.
\[ \{\alpha\}_{D}^{23} : 4.2^\circ \text{ (c=1, DMF)} \]

\[ \{\alpha\}_{D}^{20} \text{ Lit. [108]} : 6.3^\circ \text{ (c=1, DMF)} \]

\[ \text{C}_{17}\text{H}_{25}\text{NO}_{5} \text{ (323.39). Anal. Calcd.: C 63.14 H 7.79 N 4.33} \]

\[ \text{Found: 63.10 7.77 4.31} \]

### 3.7.4.4 Benzzyloxy carbonyl-O-tert.-butyl-L-threonine (Z-L-Thr(Bu^t) -OH)

Z-L-Thr(Bu^t)-OMe was saponified using the procedure of Schroeder [108] in 70% yield.

m.p. : oil

\[ \{\alpha\}_{D}^{25} : +7.2^\circ \text{ (c=0.1, DMF)} \]

\[ \{\alpha\}_{D}^{25} : +8.4^\circ \text{ (c=1.0, DMF)} \]

\[ \text{C}_{28}\text{H}_{46}\text{N}_{2}\text{O}_{5} \text{ (309.30). Anal. Calcd.: C 62.12 H 7.49 N 4.53} \]

\[ \text{Found: 62.11 7.40 4.50} \]

### 3.7.4.5 Benzzyloxy carbonyl-O-tert.-butyl-L-threonine dicyclohexyl ammonium salt (Z-L-Thr(Bu^t)-OH.DCHA)

The salt was obtained from Z-L-Thr(Bu^t)-OH using a known method [108] in 90% yield.
m.p.: 122-126°C Lit. [108]: 126-126.5°C, 146-147°C [137]

\[[\alpha]_D^{25}\] : 7.3° (c=1, MeOH)

\[[\alpha]_D^{23}\] Lit. [108]: +5.3° (c=1, DMF)

\[[\alpha]_D^{20}\] Lit. [137]: +9.93° (c=1, EtOH)

R_f H : 0.80, R_f I : 0.87

C_{28}H_{46}N_2O_3 (490.69). Anal. Calcld.: C 68.54 H 9.45 N 5.71

Found: 68.60 9.41 5.72

3.7.4.6 Benzyloxycarbonyl-O-tert.-butyl-L-threonine-hydroxy-succinimid ester (Z-L-Thr(Bu\textsuperscript{t})-OSu) [138]

The free acid was obtained from Z-L-Thr(Bu\textsuperscript{t})-OH.DCHA salt by dissolving it in a mixture of water/EtOAc and adding 2N citric acid until the aqueous layer was acidified. Organic layer was washed with water, dried over Na_2SO_4. To an ice-cold (-15°C) solution of Z-L-Thr (Bu\textsuperscript{t})-OH and HOSu in dioxane DCC was added, and the mixture was stirred for 14 h. at 4°C and for 2 h. at room temperature. The suspension was filtered and filtrate was evaporated yielding an oil which crystallized on standing. It was recrystallized from EtOAc/PE and then isopropanol. 80.2 g (88.5% yield).
m.p. : 86-88°C Lit. [138] : 90°C

\[ \{a\}_D^{21} = -3.8^\circ \ (c=1, \text{MeOH}) \]

\[ \{a\}_D^{22} \quad \text{Lit. [138]} : -6.3^\circ \ (c=1, \text{Dioxane}) \]

C_{20}H_{26}N_2O_7 (406.44). Anal. Calcd.: C 59.10 H 6.45 N 6.89

*Found:* 59.00 6.50 6.80

3.7.5 Benzzyloxy carbonyl-0-tert.-butyl-L-threonyl-L-glutaminyl-L-glutamic acid (γ-tert.-butyl ester) (Z-L-Thr(Bu^t)-L-Gln-L-Glu(OBu^t)-OII)

Benzzyloxy carbonyl-0-tert.-butyl-L-threonine-hydroxysuccinimide ester (2.03 g, 5m mol.) was dissolved in DMF (10 ml) and mixed with a solution of H-Gln-Glu(OBu^t)-OH (1.9 g, 6m mol.) and NaHCO_3 (0.5 g, 6m mol.) in 20 ml water:DMF (8:2). The mixture was stirred at room temprature overnight, conc to a small vol. in vacuo and acidifled to pH 2 with 2N citric acid, extracted with EtOAc. The organic phase was washed with cold water, dried with MgSO_4, and crystallized from EtOAc/Pet.ether, the yield was 1.8 g (58%).

m.p. : 114-115°C

\[ \{\omega\}_D^{25} = -2.00^\circ \ (c=0.1, \text{MeOH}) \]
\[ R_{d}D : 0.28 \quad R_{d}A : 0.43 \]

\[ C_{30}H_{46}N_{4}O_{10} (622.713) \]

**Anal. Calc.**
- C 57.86
- H 7.44
- N 8.99

**Found**
- C 56.90
- H 7.30
- N 8.90

3.8  \( N^{\alpha} \)-Benzylloxycarbonyl-\( N^{\varepsilon} \)-tert.butyloxycarbonyl-L-lysyl-L-glutaminyl-L-alanyl-\( N^{\varepsilon} \)-tert.-butyloxycarbonyl-L-lysine-\( \alpha \)-tert.butyl ester \((Z\text{-Lys(Boc)}\text{-L-Gln-L-Ala-L-Lys-}(Boc)\text{-OBu}^{\dagger})\)

3.8.1  \( N^{\alpha} \)-Benzylloxycarbonyl-\( N^{\varepsilon} \)-tert.butyloxycarbonyl-L-lysine-,\( \alpha \)-tert.butyl ester \((Z\text{-Lys(Boc)}\text{-OBu}^{\dagger})\) [45]

Lys(Boc)OBu\(^{\dagger}\) was synthesized according to Wuensch et al. [45] in 73% yield. The product (oil) was used for further reaction without purification.

3.8.1.1  \( N \text{-tert.} \)butyloxycarbonyl-L-lysine-\( \text{-tert.} \)butyl ester \((H\text{-L-Lys(Boc)}\text{-OBu}^{\dagger})\) [45]

The above product was deprotected according to Wuensch et al. [45] 20 g (89% yield) was obtained.

m.p. : 135–137\(^{\circ}\) C

\[ \alpha_{D}^{20} = +10.2 \pm 1^\circ : (c=1.0, \text{MeOH}) \]
3.8.2 Benzyloxy carbonyl-L-alanine (Z-L-Ala-OH)

Z-L-Ala-OH was prepared by the method of Bergmann and Zervas [44]. The product was crystallized from EtOH/Pet.ether. 120.5 g (90% yield).


\[ \alpha_D^{22} \approx -13.7° \text{ (c=2, AcOEt)} \]

Lit. [44] \[ \alpha_D^{22} : -14.3° \text{ (c=2, EtOAc)} \]

R\text{f}D : 0.48

C\text{H}_{11}\text{H}_{13}\text{O}_{4}\text{N} (223.21). Anal. Calcd.: C 59.19 H 5.87 N 6.20

Found: 59.09 5.81 6.19

3.8.3 Benzyloxy carbonyl-L-alanyl-N\text{\textsuperscript{6}-}tert.-butyloxy carbonyl-L-lysine-\text{\textalpha-}tert.-butyl ester (Z-L-Ala-L-Lys(Boc)-OBu\textsuperscript{t})

6.3 g (18.5 mM) H-Lys(Boc)OBu\textsuperscript{t}.HCl [30] and Z-Ala-OH (21 mM) were dissolved in 50 ml DMF with 2.7 g (20 mM) HOBT and N-Ethylmorpholine (2.13 g). The mixture was stirred at -10°C for 10 min, 4.33 g (21 mM) cooled DCC in small volume of DMF, was added to the mixture, stirred at -10°C for 1 h. and 12 h. at room temperature. N,N dicyclohexylurea was filtered at 0°C. The filtrate was evaporated
under reduced pressure and the residue was dissolved in EtOAc, washed with 5% NaHCO₃, 2N citric acid and water. Dried over Na₂SO₄ and crystallized from EtOAc/Pet.ether 7.6 g (80.8% yield).

m.p. : 126-127°C

[α]D²² : -64.5 (c=1, DMF)

RfD : 0.75, RfA : 0.81

Found: 61.46 8.20 8.28

3.8.3.1 L-Alanyl N°-tert.butylxocarbonyl-L-lysine-α-tert.butyl ester
(H-L-Ala-L-Lys(Boc)--OBu⁻)

7.15 g (14.1 mM) Z-Ala-Lys(Boc)--OBu⁻ was dissolved in 100 ml THF, hydrogenated in the presence of 750 mg palladium on charcoal (10%) for 4 h. The catalyst was removed by filtration, the filtrate was evaporated and the oily product was used for further coupling.

m.p. : oil

RfD : 0.59

C₁₈H₄₅N₃O₅ (383.579). Anal. Calcld.: C 56.36 H 11.82 N 10.95
Found: 56.90 11.90 10.88
3.8.4 Benzyloxy carbonyl-L-glutaminy1-L-alanyl-N^c-tert.-butyloxy carbonyl-L-lysine-α-tert. butyl ester (Z-L-Gln-L-Ala-L-Lys (Boc)-OBu^t)

The oily product, Ala-Lys(Boc)-OBu^t was dissolved in DMF, and 5.2 g (13.6 mM) Z-Gln-ONp was added. The resulting mixture was stirred for two days at room temperature. The reaction mixture was evaporated using high vacuum, dissolved to a small volume of DMF, added dropwise to an ice-cooled 2N citric acid and stirred. The colorless ppt. was filtered, washed with ice-cool water and then with ether. The product was finely ground after drying and again washed excessively with ether 7.17 g (89% yield).

m.p. : 189-191°C

R_f^A : 0.85 R_f^D : 0.78

[α]_D^{22} : - 5.25° (c=1, DMF)

C_{31}H_{49}N_{3}O_{9} (621.75). Anal. Calcd.: C 58.56 H 7.77 N 11.02

Found: 58.44 7.87 11.08

3.8.4.1 L-Glutaminy1-L-alanyl-N^c-tert. butyloxy carbonyl-L-lysine-α-tert. butyl ester (H-L-Gln-L-Ala-L-Lys(Boc)-OBu^t)

6.4 g (10.3 mM) Z-Gln-Ala-Lys(Boc)-OBu^t was hydrogenated in 100 ml DMF as described in section (3.8.3.1). The product was oil and
homogenous on tlc in system D and A. It was used for next coupling without crystallization.

m.p. : oil

$R_f D : 0.26$

$C_{23}H_{43}N_5O_7$ (501.61). Anal. Calc'd.: C 55.07 H 8.64 N 13.96

Found: 55.10 8.68 13.89

3.8.5  $N^\alpha$-Benzyloxycarbonyl-$N^\varepsilon$-tert.butyloxycarbonyl-L-lysyl-L-glutaminyL-L-alanyl-N-tert.butyloxycarbonyl-L-lysine-$\alpha$-tert.

butyl ester ($Z$-L-Lys(Boc)-L-Gln-L-Ala-L-Lys(Boc)-OBu$^t$)

4.5 g (8.9 mM) $H$-$Gln$-$Ala$-$Lys(Boc)$-OBu^t$ was dissolved in 50 ml DMF and mixed with 5.3 g (9.4 mM) Z-Lys(Boc)OSu, in DMF, at 0°C. The mixture was stirred overnight at room temperature. Solvent was evaporated and pptd. from water. The product was filtered and washed with ether, dried; yield 6.61 g (69.5%).

m.p. : 158-159°C

$\left[\alpha\right]_D^{22} : -14.9$ (c=1, DMF)

$R_f D : 0.59$
C₄₂H₆₉N₁₀O₁₂ (864.039). Anal. Calcld.:  
C 58.38  H 8.05  N 11.35  
57.88  8.10  11.38

3.8.5.1 N⁵-tert.-Butyloxycarbonyl-L-lysyl-L-glutaminyl-L-alanyl-N-tert.-butyloxycarbonyl-L-lysine-α-tert. butylester  (H-L-Lys (Boc)-L-Gln-L-Ala-L-Lys(Boc)-OBu¹)

4 g protected tetrapeptide was hydrogenated in DMF (130 ml) for 7 r. The catalyst was removed by filtration. Filtrate was concentrated to a volume (approximately 50 ml). The product was pure on tlc in system D and used for next coupling.

m.p. : 143°C

C 55.94  H 8.70  N 13.43  
55.99  8.72  13.40
3.9  Benzyloxy carbonyl-O-tert.-butyl-L-threonyl-L-isoleucyl-L-glutamyl(γ-tert. butyl ester)-L-glutaminyln-L-glutamyl (γ-tert. butyl ester) (Z-L-Thr(Bu)₄)-L-Ile-L-Glu(Obu)₄)-L-Gln-L-Glu (Obu)₄)-OH

3.9.1  Benzyloxy carbonyl-L-glutamic acid (γ-tert. butyl ester) (Z-L-Glu(Obu)₄)-OH

3.9.1.1  Benzyloxy carbonyl-L-glutamic anhydride (Z-L-Glu-O)

The anhydride of Z-Glu-OH was synthesized as described by Gibian [132] in 68% yield.


[α]D ²¹  :  -3.7° (c=1, CHCl₃)

[α]D ²⁵  Lit. :  -43° (c=10, AcOH)

C₁₃H₁₃NO₅ (263.20).  Anal. Calcd.: C 59.31  H 4.98  N 5.32

Found:  59.30  4.99  5.31
3.9.1.2 Benzylxycarbonyl-L-glutamic acid-\(\alpha\)-methyl ester (Z-L-Glu-OMe)

Z-L-Glu-\(\alpha\)-OMe was synthesized as described [46] in 60% yield. Purity was checked in system D.


\([\alpha]_{D}^{22}\) : -23.0° (c=1, MeOH)

\([\alpha]_{D}^{25}\) Lit. : -25.9° (c=1.06, MeOH)

\(\text{C}_{14}\text{H}_{17}\text{NO}_{6}\) (295.29). Anal. Calcd.: C 56.95 H 5.80 N 4.74

Found: 56.90 5.81 4.73

3.9.1.3 Benzylxycarbonyl-(\(\gamma\)-tert-butyl ester)-L-glutamic acid-\(\alpha\)-methyl ester (Z-L-Glu(\(\text{OBu}^{t}\))-OMe)

Z-L-Glu-\(\alpha\)-OMe was treated with a large excess of isobutylene in the presence of sulfuric acid as a catalyst, as described [107]. The product was obtained in 76% yield.

m.p. : oil, Lit. [107] oil.

\([\alpha]\) : 3.1° (c=1, CHCl\(_3\))
$[^{25}\alpha]_D$ Lit. [107] : $2.9^\circ$ (c=1, CHCl$_3$)


Found: 61.44 7.00 3.49

3.9.1.4 Benzylxycarbonyl (γ-tert.butyl ester)-L-glutamic acid (Z-L-Glu(OBu$^t$)-OH)

The methyl ester was saponified according to E.Klieger [107] and DCHA salt was synthesized in 60% yield.

m.p. : 137-138°C, Lit. : 139-140°C

$[^{22}\alpha]_D$ : + 7.21° (c=1.0, MeOH)

$[^{25}\alpha]_D$ Lit. : + 7.3° (c=1, MeOH)

C$_{29}$H$_{46}$N$_2$O$_6$ (518.7). Anal. Calcd.: C 67.15 H 8.94 N 5.40

Found: 66.60 9.06 5.25

3.9.1.5 L-Glutamic acid (γ-tert.butyl ester) (H-L-Glu(OBu$^t$)-OH)

The free acid was obtained from Z-Glu(OBu$^t$)-OH. DCHA salt and Z-group was removed by catalytic hydrogenation to give 74.6% yield.

m.p. : 186-188°C, Lit. : 186-187°C [107]
$[\alpha]^D_{23}$ : $+10.3^\circ$ (c=1, water)

$[\alpha]^D_{25}$ : $+10.1^\circ$ (c=1, water), $+18.1^\circ$ (c=1, 5.5% AcOH)

$C_9H_{17}NO_4$ (203.2). Anal. Calcd.: C 53.17 H 8.42 N 6.89

Found: 52.97 8.60 6.99

3.9.2 Benzylloxycarbonyl-L-glutamate (\(\gamma\)-tert. butyl)-\(\alpha\)-hydroxysuccinimide ester (Z-L-Glu(OBu\(^t\))-OSu)

The hydroxysuccinimide ester of Z-Glu(OBu\(^t\))-OH was synthesized as described [47] in 61% yield.

m.p.: 102-103°C, Lit. [47]: 101-103°C

$[\alpha]^D_{23}$ : $-28.5^\circ$ (c=2.5, EtOH)

$[\alpha]^D_{26}$ Lit : $-29.9^\circ$ (c=2.43, EtOH)

$C_{21}H_{26}N_2O_8$ (434.4). Anal. Calcd.: C 58.10 H 6.00 N 6.40

Found: 58.01 6.16 6.48
3.9.3 Benzyloxy carbonyl-L-glutamyl(γ-tert.butyl ester)-L-glutaminyl-L-glutamic acid (γ-tert. butyl ester) (Z-L-Glu(OBu\textsuperscript{t})-L-Gln-L-Glu(OBu\textsuperscript{t})-OH

3.62 g (10 mM) Z-Glu(OBu\textsuperscript{t})-OSu in 50 ml DMF was added to 3.84 g (12 mM) H-Gln-Glu(OBu\textsuperscript{t})-OH in (300 ml) DMF:water (2:8) and 1.012 g (12 mM) NaHCO\textsubscript{3}. The mixture was stirred at room temp. for 18 h. and treated as described in 3.7.3. Yield was 5.22 g (80%).

m.p. : 156-157°C

R\textsubscript{f}\textsuperscript{A} : 0.34

C\textsubscript{31}H\textsubscript{46}N\textsubscript{4}O\textsubscript{11} (650.665). Anal. Calcd.: C 57.22 H 7.11 N 8.61

Found: 57.90 7.29 8.20

3.9.3.1 L-Glutamyl (γ-tert. butyl ester)-L-glutaminyl-L-glutamic acid
(γ-tert. butyl ester) (H-Glu(OBu\textsuperscript{t})-Gln-Glu(OBu\textsuperscript{t})-OH)

The protected tripeptide (Z-Glu-(OBu\textsuperscript{t})-Gln-Glu(OBu\textsuperscript{t})-O\textsubscript{H}) 3 g was hydrogenated in 150 ml MeOH : DMF : Water (3:2:2) and treated in the same manner as described in Section 7.3.1. The solid residue obtained by addition of ether, was collected and dried; yield 1.9 g (80%).

m.p. : 181-182°C

C\textsubscript{23}H\textsubscript{40}N\textsubscript{4}O\textsubscript{9} (516.513). Anal. Calcd.: C 53.48 H 7.70 N 10.84

Found: 52.90 7.70 10.46
3.9.4 Benzyloxy carbonyl-L-isoleucine (Z-L-Ile-OH)

L-Isoleucine was coupled with Z-Cl via the known procedure [48], and obtained in 72.7% yield.

m.p. : oil, Lit. [48]: oil

\[ \text{C}_{14}\text{H}_{19}\text{O}_{4}\text{N} \] (265.285). Anal. Calcld.: C 63.38 H 7.21 N 5.27

Found: 63.44 7.24 5.22

3.9.4.1 Benzyloxy carbonyl-L-isoleucine-\(\alpha\)-hydroxysuccinimide ester (Z-L-Ile-OSu)

The ester was synthesized from Z-Ile-OH according to Anderson et al. [128] and recrystallized with isopropyl alcohol in 54% yield.


\([\alpha] \) : -15.44° (c=2, dioxane)

\([\alpha] \) Lit. [128] : -15.5° (c=2, dioxane)

\( R_f \) D : 0.76

\[ \text{C}_{18}\text{H}_{22}\text{O}_{4}\text{N}_{2} \] (330.334). Anal. Calcld.: C 59.66 H 6.12 N 7.73

Found: 58.29 6.09 7.74
3.9.5 Benzyloxycarbonyl-L-isoleucyl-L-glutamyl (γ-tert.butyl ester) -L-glutaminyl-L-glutamic acid (γ-tert.butyl ester) (Z-L-Ile-L-Glu(OBu\textsuperscript{t})-L-Gln-L-Glu(OBu\textsuperscript{t})-OH)

To a solution of deprotected tripeptide (Glu(OBu\textsuperscript{t})-Gln-Glu(OBu\textsuperscript{t})-OH) (0.77 g) and (0.134 g) NaHCO\textsubscript{3} in (200 ml) DMF:water, was added 0.432 g (1.3 mM) Z-Ile-OSu, in 50 ml DMF. The mixture was stirred for 8 h. at room temp. then treated accordingly as described in section 6.2.1. ppted. from EtOAc/Pet.ether. Yield: 0.81g (81%).

m.p. : 187-188°C

R\textsubscript{T}A : 0.41

C\textsubscript{37}H\textsubscript{57}N\textsubscript{5}O\textsubscript{12} (763.755). Anal. Calcd.: C 58.18 H 7.50 N 9.16

Found: 57.60 7.48 8.80

3.9.5.1 L-isoleucyl-L-glutamyl (γ-tert.butyl ester)-L-glutaminyl-L-glutamic acid (γ-tert. butyl ester) (-L-Ile-L-Glu(OBu\textsuperscript{t})-L-Gln-L-Glu(OBu\textsuperscript{t})-OH)

2 g of protected tetrapeptide was hydrogenated in MeOH:DMF:water (3:2:2) for 2h. After normal procedure, the residue was dissolved in a small vol. of MeOH and ppted. with ether, filtered, dried. Yield: 1.4 g (90%).

m.p. : 173-174°C
C_{28}H_{51}N_{5}O_{10} (629.64). Anal. Calcd.: C 55.32 H 8.14 N 11.12
Found: 55.10 7.90 11.10

3.9.6 Benzyloxy carbonyl-O-tert.-butyl-L-threonyl-L-isoleucyl-L-glutamyl (γ-tert.-butyl ester)-L-glutaminyl-L-glutamic acid (α-tert.-butyl ester) (Z-L-Thr(Bu')-L-Ile-L-Glu(OBu')-L-Gln-L-Glu(OBu')-OH)

0.772 g Z-Thr(Bu')-OSu in 10 ml DMF was added to a solution of 1.26 g deprotected tetrapeptide, vide supra, and NaHCO₃ (0.18 g) in 40 ml DMF:water (2:8) at room temp. The coupling was completed in 18 h. and the reaction mixture was treated in usual way, recrystallized from EtOAc/Pet.ether. The yield was 1.35 g (79%).

m.p. : 183-184°C

[α]_{D}^{22} : 84.25° (c=1, dioxane)

R_{f}D : 0.46

C_{44}H_{69}N_{6}O_{14} (905.989). Anal. Calcd.: C 58.33 H 7.60 N 9.20
Found: 58.26 7.50 9.27
3.9.6.1 H-L-Threonyl-L-isoleucyl-L-glutamyl-L-glutaminyl-L-glutamic acid (H-L-Thr-L-Ile-L-Glu-L-Gln-L-Glu-OH)

320 mg (0.35 mM) of protected pentapeptide (seq. 33-37) was hydrogenated in MeOH:DMF:Water (3:2:2) for 2.5 h. over Pd-charcoal as already described. The residue obtained was treated with TFA-anisole (2 ml : 0.4 ml) in an ice-bath for 40 min. TFA was removed by evaporation. The residue was washed with dry ether, dried over NaOH pellets in vacuo for 4 h. The unprotected peptide was dissolved in 1% AcOH (2 ml), applied to a column of Sephadex G-15 (3x90cm), and eluted with the same eluant. Fractions of 6 ml were collected per 18 min, and absorption was determined at 230 nm.

Racemate Test

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Amino acid analysis

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3.10 Benzyloxy carbonyl-L-threonyl-O-tert.butyl ester-L-isoleucyl-L-glutamyl(γ-tert.-butyl ester)-L-glutaminyl-L-glutamyl(γ-tert.butyl ester)-L-lysyl-({N\textsuperscript{\textalpha}-tert.butyl oxycarbonyl)-L-glutaminyl-L-alanyl(N\textsuperscript{\textalpha}-tert.butyl oxycarbonyl)-L-lysine-\textalpha-tert butyl ester (Z-L-Thr(Bu\textsuperscript{1})-L-Ile-L-Glu(OBu\textsuperscript{1})-L-Gln-L-Glu (OBU\textsuperscript{1})-L-Lys(Boc)-L-Gln-L-Ala-L-Lys(Boc)-OBu\textsuperscript{1})}

To a solution of 0.906 g protected pentapeptide (seq. 33-37) and 0.73 g unprotected tetrapeptide (seq. 38-41) in DMF, 0.17 g HOSu was
added. 0.226 g DCC was put in a small volume of DMF, added to the reaction mixture at 0°C and stirred. The following day urea was filtered off, filtrate concentrated and poured slowly to ice-cool water. The product was ppted in amorphous form, separated by filtration and washed with water. The product on tlc was pure. The yield: 1.5 g (93%).

m.p. : 120°C

[α]$_D^{22}$ : 74.89 (c=2, dioxane)

R$_f$A : 0.80

C$_{78}$H$_{131}$O$_{23}$N$_{13}$ (1618.807). Anal. Calcd.: C 57.87 H 8.10 N 11.24 Found: 57.96 8.37 11.12


200 mg (0.123 mM) protected thymosin 9 (33-41) was hydrogenated in 15 ml MeOH:DMF:Water (3:2:2), over 10% Pd-charcoal for 3 h. The catalyst was removed with the aid of celite. The filtrate was evaporated to dryness and treated with TFA-anisole (2 ml : 0.4 ml) in an ice bath for 40 min., TFA was removed by evaporation. The residue was
washed with dry ether, dried over NaOH pellets in vacuo for 5 h. The d blocked peptide was dissolved in 1% AcOH (2 ml), applied to a column of Sephadex G-15 (3x90 cm), eluted with the same eluant. Fraction of 6 ml were collected per 18 min., and absorption was determined at 230 nm. Purity was checked in system g and sprayed with ninhydrin. Yield: 46.2 mg (35%).

Amino acid ratios in 6N hydrolysate:

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