SYNTHESIS OF THIOREDOXIN PARTIAL SEQUENCES ON A HYDROPHILIC CROSSLINKED POLYMERIC SUPPORT

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Certificate

This is to certify that the thesis entitled **Synthesis of Thioredoxin Partial Sequences on a Hydrophilic Crosslinked Polymeric Support** *is an authentic record of the research work carried out by* **Ms. Jaya T. Varkey** *under my supervision and guidance during the period July 1992 to November 1996 in partial fulfilment of the requirements for the award of the degree of* **Doctor of Philosophy** *under the faculty of science of the Mahatma Gandhi University. The work presented in this thesis has not been submitted for any other degree or diploma earlier. It is also certified that Ms. Jaya T. Varkey has fulfilled the course requirements and passed the qualifying examination for the Ph.D. degree of this University.*

VNPillai

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22nd January 1997

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Declaration

I hereby declare that the thesis entitled **Synthesis of Thioredoxin Partial Sequences on a Hydrophilic Crosslinked Polymeric Support** *is an authentic record of the research work carried out by me under the supervision and guidance of Dr. V. N. Rajasekharan Pillai, Professor, School of Chemical Sciences and Vice-Chancellor, Mahatma Gandhi University, Kottayam. No part of this thesis has been presented for any other degree or diploma earlier.*

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Contents

Abbreviations

Abbreviations and nomenclature used for the amino acids and peptides are in agreement with the recommendations of the IUPAC–IUB commission on Biochemical Nomenclature, *Biochem. J.*, 219, 345 (1984); *Eur. J. Biochem.*, 138, 9 (1984). In addition, the following abbreviations were used.

Anal.	–	Analytical
Acm	–	Acetamidomethyl
Anh.	–	Anhydrous
Boc	–	t-Butyloxycarbonyl
Boc–ON	–	(1–butyloxycarbonyloximino)–2–phenyl acetonitrile
Bz_2O_2	–	Benzoyl peroxide
Bzl	–	Benzyl
C	–	Celsius
CD	–	Circular dichroism
2ClZ	–	2–chlorobenzyloxycarbonyl
CHex	–	Cyclohexyl ester
CP/MAS	–	Cross polarised–magic angle spin
DCC	–	Dicyclohexylcarbodiimide
DCM	–	Dichloromethane
DCU	–	Dicyclohexylurea
DMF	–	Dimethylformamide
DMSO	–	Dimethylsulfoxide
DVB	–	Divinylbenzene
EtOH	–	Ethanol
ε	–	Molar extinction coefficient
Fmoc	–	9-Fluorenylmethyloxycarbonyl
FPLC	–	Fast protein liquid chromatography
HOBt	–	1–Hydroxybenzotriazole
HPLC	–	High performance liquid chromatography

IR	–	Infrared
MeOH	–	Methanol
mmol	–	Millimole
Mol. wt.	–	Molecular weight
NMP	–	N–methyl–2–pyrrolidone
NMR	–	Nuclear magnetic resonance
PS	–	Poly(styrene)
PVA	–	Polyvinylalcohol
Prep.	–	Preparative
$(\theta)_M$	–	Molar ellipticity
RPC	–	Reverse phase chromatography
SEM	–	Scanning electron microscopy
TEA	–	Triethylamine
TFA	–	Trifluoroacetic acid
TFE	–	Trifluoroethanol
THF	–	Tetrahydrofuran
TLC	–	Thin layer chromatography
Z	–	Benzyloxycarbonyl
	–	Crosslinked poly(styrene)

CHAPTER 1

INTRODUCTION AND OBJECTIVES

The continued and rapid discoveries of new peptides with interesting biological functions have created an unprecedent demand for the chemical synthesis of peptides required for structure–function correlations.[1] Several strategical improvements have been suggested and tested to meet the demand for peptides in high purity and quantity. The polymer–supported solid phase approach of peptide synthesis developed by R. B. Merrifield in 1963 has helped to overcome many of the preparative difficulties encountered in the conventional classical solution phase method of peptide synthesis.[2] This novel and widely used method continues to be the method of choice for the synthesis of biologically active peptides and protein sequences.

The fundamental premise of solid phase technique is that amino acids can be assembled into a peptide of any desired sequence while one end of the chain is anchored to an insoluble support. After the desired sequence of amino acids has been linked together on the support a reagent can be applied to cleave the chain from the support and to release the finished peptide into solution. All the reactions involved in the synthesis can be carried out in quantitative or near–quantitative yields so that a homogeneous target peptide can be obtained. The

virtue of using a solid support is that all the laborious purifications at intermediate stages in the synthesis is substituted by simple washing and filtration of the polymer supported species.

In the stepwise synthesis of peptides on crosslinked macromolecular supports it was generally believed that the support would act only as an inert, passive solid carrier. However innumerable investigations during the last two decades dealing with the quantitative aspects of polymer–supported reactions have shown that the insoluble support does have a significant dynamic influence on the bound substrates. An efficient polymeric support for peptide synthesis should have optimum hydrophobic–hydrophilic balance compatible with the peptide being synthesised. Moreover the conformational changes occurring in the growing peptide after each amino acid attachment are also to be considered since the changing conformations can have a dynamic influence on the physical and chemical properties of the growing peptide. The success of solid phase synthesis depends on the swelling characteristics of the polymer and the solvation of the peptidyl resin in different solvents.[3] Systematic studies on polymer supported reactions have shown that the use of a compatible and flexible polymer support enhances the reactivity due to its enhanced solvation characteristics. Based on this idea, polystyrene (PS) crosslinked with 1,6–hexanediol diacrylate (HDODA) possessing a hydrophobic–hydrophilic balance optimised based on the extent of crosslinking has been developed in our laboratory for the synthesis of peptides.[4] From the systematic investigations on swelling and reactivity studies of this support the 2% crosslinked system was found to be suitable for the synthesis of peptides.[5] The synthetic applications of this support are illustrated by the preparation of the partial sequences of thioredoxin.

Thioredoxin is a naturally occurring sulphur reducing protein containing 108 amino acid residues (Figure 1.1).[6] It is rich in secondary structures

(approximately 38% α–helix and 28% β–structure).[7] Thioredoxin was isolated in 1964 as a small dithiol protein (Mr 12,000) that acts as a hydrogen donor for ribonucleotide reductase.[8] This essential enzyme reduces the four common ribonucleotides to the corresponding deoxyribonucleotides which are required for deoxyribonucleotide synthesis. The total synthesis of this small protein has been a challenging problem to peptide and protein chemists for the last 3 decades. Complete synthesis of thioredoxin has not been achieved yet, even though a few partial sequences have been synthesised with reasonable success.[9,10]

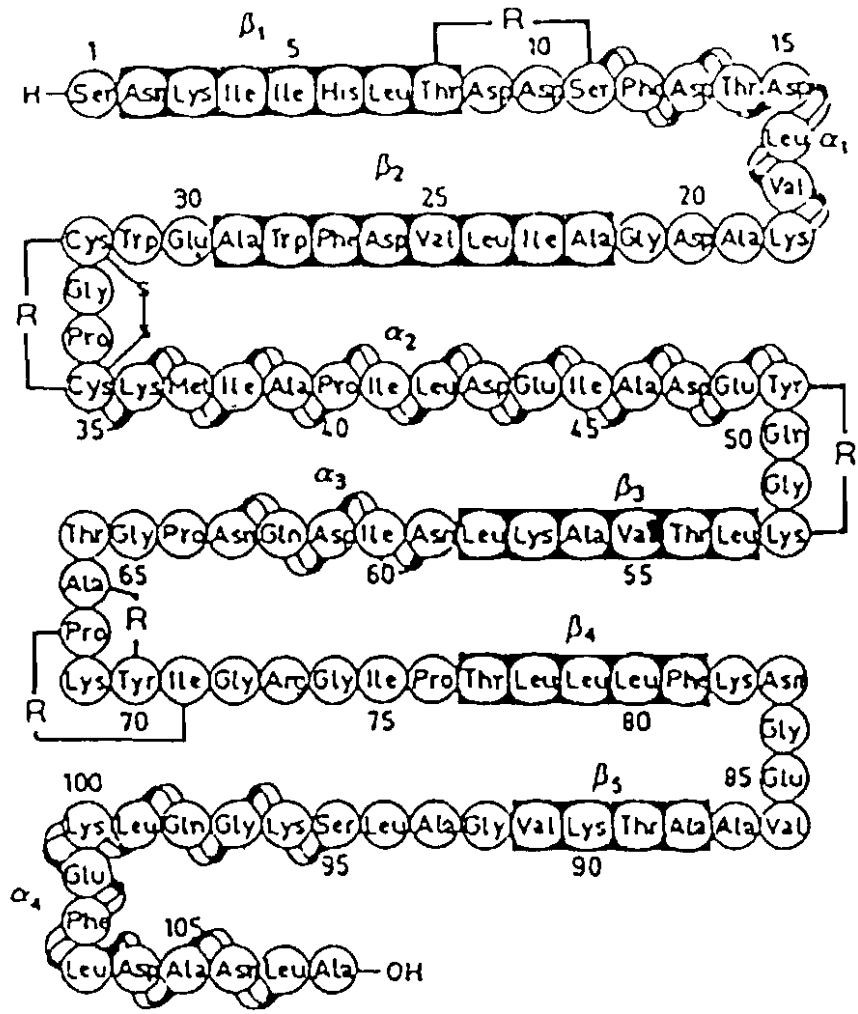


Figure 1.1 Amino acid sequence of E.Coli thioredoxin.

1.1 Objectives of the thesis

In the present study, synthesis of some of the partial sequences of thioredoxin are taken as illustrative examples for the application of the resin. The work involves:

1. Preparation of HDODA–crosslinked polystyrene supports.
2. Functionalisation and characterisation of the supports.
3. Selection of conformationally important partial sequences of thioredoxin by examining its complete amino acid sequence.
4. Stepwise synthesis of partial sequences.
5. Purification of peptides by HPLC and FPLC and characterisation by amino acid analysis.
6. Structural studies by making use of IR, NMR and CD techniques.

The following partial sequences of thioredoxin (T) were synthesised:

1) H–Asp–Lys–Ile–Ile–His–Leu–Thr–OH–(T 2–8).

2) H–Ser–Phe–Asp–Thr–Asp–Leu–Val–Lys–OH (T 11–18).

3) H–Ala–Ile–Leu–Val–Asp–Phe–Trp–Ala–OH–(T 22–29).

4) H–Met–Ile–Ala–Pro–Ile–Leu–Asp–Glu–Ile–Ala–Asp–Glu–Tyr–Gln–Gly–Lys–OH (T 37–52).

5) H–Leu–Thr–Val–Ala–Lys–Leu–OH–(T 53–58).

6) H–Asn–Ile–Asp–Gln–Asn–Pro–Gly–Thr–Ala–OH–(T 59–67).

7) H–Pro–Lys–Tyr–Ile–Gly–OH (T 68–72).

8) H–Ile–Gly–Arg–Gly–Ile–Pro–Thr–Leu–Leu–Leu–Phe–OH–(T 71–81).

9) H–Thr–Leu–Leu–Leu–Phe–OH–(T 77–81).

10) H–Ala–Thr–Lys–Val–OH–(T 88–91).

11) H–Gly–Ala–Leu–Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu–OH–(T 92–107).

12) H–Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu–OH–(T 95–107).

In addition to these partial sequences, two designed hairpin peptides were also synthesised on the same resin. They are

1) H–Glu–Val–Lys–Val–Dpro–Gly–Val–Glu–Val–Lys–OH

2) H–Ala–Cys(Acm)–Val–Leu–Val–Dpro–Gly–Val–Leu–Val–Cys(Acm)–Ala

These peptides were synthesised by following the conventional Boc–benzyl ester strategy of Merrifield.[11] Crude peptides were purified by HPLC and FPLC using reverse phase columns. Amino acid analyses were done after hydrolysing the samples with 6 N HCl for 22 h. The purified peptides were subjected to conformational investigations by IR, NMR and CD techniques.

1.2 Organisation of the thesis

The thesis consists of six chapters. The objectives of the present work along with a brief note on solid phase peptide synthesis are given in Chapter 1. Chapter 2 is a detailed review on the new trends in solid phase peptide synthesis comprising new supports and linkers, amino protecting and deprotecting agents, novel coupling agents and cleaving techniques. In Chapter 3, Section 3.1 deals with the preparation and functionalisation of the support. Section 3.2 is about the solid phase synthesis of peptides including all the details. Chapter 4 is the results and discussion part. Section 4.1 deals with the polymer preparation, functionalisation and characterisation. Section 4.2 is for the discussion of synthesised peptides which include their purification and characterisation by amino acid analysis, NMR and CD techniques. Chapter 5 gives a summary of the work and it is followed by a list of references.

CHAPTER 2

RECENT ADVANCES IN SOLID PHASE PEPTIDE SYNTHESIS

Peptide chemistry is currently witnessing a tremendous upswing. Recent developments in the biotechnology of new proteins, as well as advances in immunology and the introduction of pharmaceuticals based on inhibitors and antagonists, have led to immense demands for synthetic peptides. The numerous possibilities for research using synthetic peptides in solving biological problems are becoming increasingly better recognized.[12-19] No other area of organic chemistry seems so dependent on interdisciplinary cooperation as peptide chemistry. The fields of research in modern peptide chemistry include synthesis and analysis, isolation and structure determination, conformational investigations and molecular modelling. Project-oriented studies are being carried out in conjunction with research groups in pharmacology, physiology, immunology, biology and biophysics. More and more peptides with unusual amino acids,[20-26] modified peptide bonds,[27-32] linker or spacer molecules[33,34] and peptide mimetics[25-37] are being prepared. Highlights of the medicinal chemistry of agonists and antagonists of biologically active peptides and inhibitors have recently been summarised by Hirschmann.[37a] Significant progress has also been made in immunology at the molecular level regarding recognition mechanisms, using both synthetic peptides and vaccines.[38] The peptide chemist is continually

being confronted with new challenges at ever shorter intervals.[39,40] There is a greater demand for new strategies, faster synthesis,[39] better coupling reagents and protecting groups and especially methods for the simultaneous preparation and analysis of very large numbers of peptides in a short time. Peptide synthesis has proven to be indispensable for the structural elucidation of many recently isolated natural products having a peptide structure such as hormones, neuropeptides, and antibiotics which very often could be isolated in only minute quantities. Investigation of the structure-activity relationships of biologically active peptides also demands the synthesis of many analogues of a given peptide.

The story started with Fisher[41] and Curtius[42] at the turn of this century has now been developed into a full-pledged discipline of immense power and sophistication. In the following years formation of peptide bond through acid azides[43] and acid chlorides[44] became well established but a general approach for the synthesis of peptides was not yet available. A broadly applicable methodology requires a choice of readily available protecting groups as well. Introduction of the benzyloxycarbonyl group[45] in 1932 by Bergmann and Zervas laid the ground work for major endeavours in peptide synthesis. The first significant accomplishment of this method was done by du Vigneaud and his associates[46] by synthesising oxytocin.

The twentieth century has witnessed the development of a number of techniques for the assembly of amino acids to form peptides. The adaptation of mixed anhydrides[47-50] for peptide bond formation, the introduction of active esters[51-53] and the discovery of coupling reagents[54,55] followed each other in rapid succession.

With the development of new reagents and techniques, the synthesis of small peptides has been placed within easy reach by classical approach to

peptide synthesis.[56,57] However, these procedures are not ideally suited to the synthesis of long chain polypeptides because the technical difficulties with solubility and purification become formidable as the number of amino acid residues increases. The demonstration by Merrifield[2] in 1962 that peptide bond formation could be achieved efficiently when one of the reactants was attached to an insoluble polymeric support has proved to be one of the most important developments in the history of peptide synthesis. It opened the way to the design of rapid, machine-aided, procedures which with due recognition of their advantages and limitations, have now taken their place alongside with more traditional methods of peptide synthesis. The subject has been well reviewed by Erickson and Merrifield[58] and also very valuably by Meienhofer.[59,60]

The established classical methods of synthesis were too slow and laborious to cope easily with the increasing demands of pharmacology and the approach offered by Merrifield provided a quick and attractive solution.[61] Subsequent improvements of this novel technique and improvements in the segment condensation method[62,63] in combination with the introduction of the HF cleavage reaction by Sakakibara and coworkers[64] and the application of high performance liquid chromatography by Rivier[65] to the purification of peptides enabled the peptide chemists to synthesise complex peptides and proteins of about 100 amino acids rapidly and efficiently.

Stepwise peptide synthesis on polymer supports is regaining importance due to the recent improvements made in protecting group strategy,[66,67] anchoring techniques[11,67-71] and support properties.[67,68,72,73] The development of efficient separation method, especially preparative high pressure liquid chromatography (HPLC), has led in particular to an important breakthrough in solid phase synthesis. Medium sized peptides of upto 20 amino acid residues can be purified reliably by using the HPLC techniques.[66,74] Although the

synthesis of higher peptides on polymeric supports still appears to be difficult, it has often been provided valuable preliminary information about the physico-chemical and structural properties of the desired peptide. The combination of stepwise synthesis on a support and subsequent condensation of the segments, either in solution or on polymeric supports, could prove to be the best method for synthesising longer chain peptides. With great accuracy peptide fragments can be synthesised in gel phase or by conventional methods in solution as well.

The first detailed study of the preferred conformations of well characterised low molecular weight peptides was done by Goodman and Schmitt (1959).[75] In the 1960s and early 1970s, the structural analysis of biomolecules was virtually dominated by X-ray crystallography[76] and the foundations of modern structural molecular biology were just beginning to be gradually established. The realisation of the importance of the solution conformation of peptides began in the late 1960s, mainly through the development of high resolution nuclear magnetic resonance spectroscopy,[77] but was essentially limited to non-aqueous solutions of peptides. At just about the same time, circular dichroism (CD), Fourier transform infrared spectroscopy (FT-IR) and Raman spectroscopy began to emerge as structural probes of the solution conformation of peptides. As a result of these developments, a spectacular advance in our understanding of solution conformation of peptides was witnessed in the 1970s and 1980s. These experimental observations provided striking support for the discovery of α-helix and β-sheet by Pauling and Corey in the early 1950s.[78]

The application of nuclear magnetic resonance spectroscopy (NMR) in investigating peptide conformation was reviewed by Kessler.[79] Some recent reviews put an insight into the structure determination of proteins by three- and four-dimensional NMR spectroscopy.[80,81] Bandekar[82] studied IR and Raman spectroscopic results on amide bands in peptides, polypeptides and proteins and

the research group at Copenhagen[83] described the use of near-infrared (NIR) Fourier-transform (FT) Raman Spectroscopy as a new method for monitoring the secondary structure of the peptide chains during solid phase peptide synthesis. Some more examples of conformational analysis of peptides came from the laboratories of Thorntan,[84] Narita,[85] Baldwin,[86] and Ponnuswamy.[87]

2.1 Chemical synthesis of peptides

The three most important strategies for the synthesis of peptides are the classical solution phase method, solid phase peptide synthesis (SPPS) and the liquid-phase peptide synthesis.

The classical method has evolved since the beginning of the twentieth century and is characterised by the stepwise synthesis of short segments under homogeneous reaction conditions in solution.[88-92] The fragments are subsequently coupled via the segment condensation technique. The products are purified from side products and truncated sequences after each synthetic step. For this reason, products obtained via a classical method are distinguished by a high degree of purity and suitable for medical applications. On the other hand, this technique requires skilled chemists, and it is time-consuming. However the greatest limitation of this approach is the generally low solubility of medium-sized peptides. These problems are of great weight as the chain-length of the peptide increases.

The solid phase method of peptide synthesis differed from general synthetic organic methods, where one of the reactants was reversibly and covalently bound to an insoluble solid polymer support which was then reacted with the reagent to give a resin-bound product. After filtering the latter from the

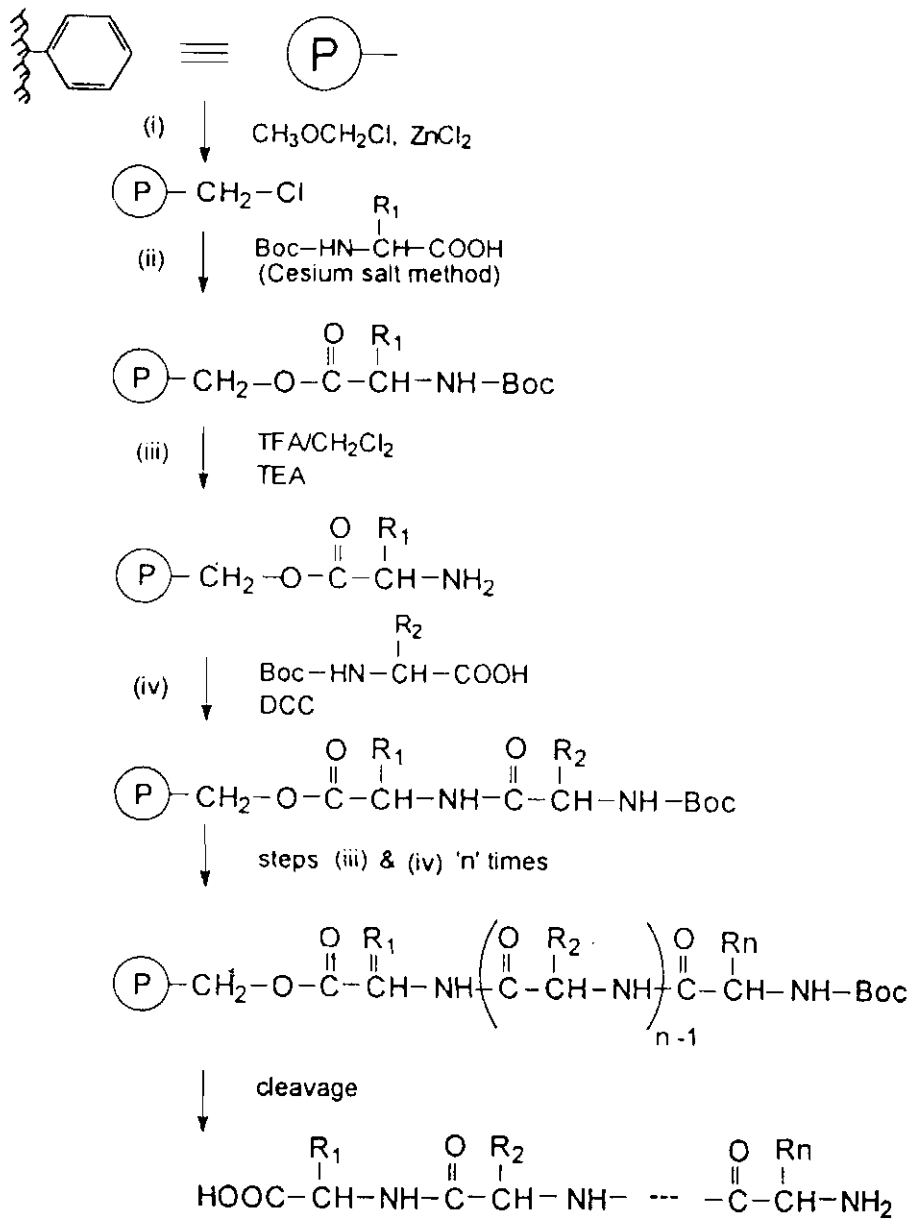
reaction mixture and after repetition of as many steps as necessary to achieve the synthesis the product was obtained by a suitable cleavage reaction.[2,93]

The liquid-phase method (LPS) developed by Mutter and Bayer[94-98] combined the advantages of polymer supported technique with those of a synthesis carried out under homogeneous reaction conditions. Unlike the solid phase method, the LPS ensures coupling and deprotection in homogeneous solution. However, reduced operational simplicity and changes in the crystallisation tendency are the two major limitations of liquid-phase peptide synthesis.

In the polymeric reagent method insoluble polymeric amino acid active esters serve as the carboxyl component for the coupling to the soluble amino terminal component. The peptide formed remains in solution from where it can be isolated and purified at each step before proceeding to the next step. An added advantage of this method is the formation of almost racemisation free peptides.

2.2 Solid phase peptide synthesis (SPPS)

The solid phase approach of peptide synthesis was conceived and elaborated by Merrifield beginning in 1959, and it has also been covered comprehensively in many reviews.[99-107] The concept of SPPS (Figure 2.1) is to retain chemistry proved in solution (protection scheme, reagents), but adding a covalent attachment step (anchoring) that links the nascent peptide chain to an insoluble polymeric support. Subsequently, the anchored peptide is extended by a series of addition (deprotection/coupling) cycles, which are required to proceed with exquisitely high yields and fidelities.



1. ***TFA*** – ***Trifluoroacetic acid***
2. ***TEA*** – ***Triethylamine***
3. ***DCC*** – ***Dicyclohexyl carbodiimide***

Figure 2.1. Typical outline of the solid phase peptide synthesis.

It is the essence of the solid phase approach that reactions are driven to completion by the use of excess soluble reagents, which can be removed by simple filtration and washing without manipulative losses. Because of the speed and simplicity of the repetitive steps, which are carried out in a single reaction vessel at ambient temperature, the major steps of the solid phase procedure are readily amenable to automation. Once chain elaboration has been accomplished, it is necessary to release protecting groups and to cleave the crude peptide from the support under conditions that are minimally destructive towards sensitive residues in the sequence. Finally, there must follow prudent purification and appropriate characterisation of the synthetic product to verify that the desired structure is indeed the one obtained. In recognition of the maturation and impact of this body of work, Merrifield was honoured with the 1984 Nobel Prize in Chemistry.[108-111]

The main advantages of solid phase peptide synthesis over classical method of synthesis are:

(a) All the reactions involved in the synthesis can be carried to 100% completion, so that a homogeneous product is obtained.

(b) All of the laborious steps of purification of intermediates in solution phase could be avoided.

(c) The entire process can be carried out in one container without any transfer of material from one vessel to another.

(d) The system was ideally suited for automatic operation.

(e) The support can be regenerated by a simple, low cost, high yield reaction.

In spite of all these advantages, solid phase method is not devoid of disadvantages. Major limitations of this techniques have been well reviewed.[112,113] The major short comings of this method are:

(a) Non-compatibility of the support resin with the growing peptide chain.

(b) Non equivalence of functional groups attached to the polymer support.

(c) racemisation leading to optically impure products.

(d) Formation of error peptides from deletion and truncated sequences.

Later SPPS received new impulses by,

(a) The development of new supports with superior swelling properties permitting an improved solvation of both matrix and growing peptide chain.

(b) The design of novel and more versatile anchoring groups (multidetachable anchors) enhancing the flexibility of the synthetic strategy.

(c) Progress in the field of chromatographic techniques such as preparative and semi-preparative HPLC.

2.3 Improvements in the original solid phase peptide synthesis

Ever since its inception in 1963, the solid phase peptide synthesis has become one of the most important tool in the synthesis of peptides, protein sequences and nucleotides. Although the earlier solid phase chemistry was very useful for making small peptides and even small proteins, it was clear that there was a need for improvement in several areas. The original technique employed by Merrifield has undergone a series of modifications and improvements. Novel improved supports such as 'isocyano' resin, [114] 'Rink' resin,[115] 5[4(9-Fmoc) amino methyl 3,5-dimethoxy phenoxy] valeric acid (PAL) resin,[116] tertiary alcohol resin,[117] carboxyl amide terminal resin (CAT),[118] 2-chlorotrityl chloride resin,[119] polyoxyethylene-polystyrene graft copolymeric support (POE-PS),[120]

polyacrylate-DVB copolymer,[121] polyamide-kieselgur[122] support have been introduced. New hydrophilic matrices[123] for the synthesis of small peptides by either batch or continuous flow methods, NPE resin[124] (2-(2-nitrophenyl ethyl) for the synthesis of protected peptides and oligonucleotides and polyethylene glycol-polystyrene resin[125] are some of the recent developments in the field of polymer supported peptide synthesis. Bis-2-acrylamidoprop-1-yl polyethyleneglycol crosslinked dimethyl acrylamide (PEGA) has been introduced as a hydrophilic, biocompatible and flexible solid support in peptide synthesis[126] and recently a new method for preparation of high capacity PEGA resins with well defined loading of functional groups has been described for continuous flow SPPS by Meldal and co-workers.[127]

5,9-(9-fluorenyl methyloxy carbonyl amino xanthen-2-oxy) valeric acid (XAL) has been introduced as an acid labile handle for Fmoc-based peptide amide synthesis.[128] Dimethoxy acido-labile linker (DAL),[129] 4-hydroxymethyl phenoxy acetic acid and 3-methoxy-4-hydroxymethyl phenoxyacetic acid[130] are some of the acid labile peptide resin linkage agents for use in solid phase peptide synthesis. A report from the second Japan peptide symposium[131] dealt with the development of novel acid-labile peptide amide linkers. Progress on handles and supports for solid phase peptide synthesis has been reviewed by Barany and Albericio.[132] Substituted benzhydrol derivatives are also used as linkers in solid phase[133] peptide synthesis.

Recently use of an oxidation labile phenylhydrazide group as a linker for solid phase synthesis was reported.[134] A new type of matrix specific linker was recently introduced by Hauske and Dorff.[135] Cleavage of finished peptide under mild conditions can be achieved by photolysis in cases were photolabile anchoring groups are employed.[136] Development of new photo removable protecting groups like Menpoc (α-methyl nitropiperonyloxy-carbonyl) and

Menvoc (α-methyl nitroveratryloxy carbonyl) were reported in the 13th American peptide symposium.[137] A new α-nitrobenzyl photolabile linker based on α-methyl-6-nitroveratrylamine was described for the generation of peptides by solid phase approach.[138] An efficient versatile linker for solid phase peptide synthesis based upon dibenzocyclohepta-1,4-diene system has been developed by McInnes and co-workers.[139] New carboxyl protecting groups like 2-(1-adamantyl)-propanol-2 esters (Adp) removable under mild acid treatment[140] and 2-bromoethyl and 2-iodoethyl esters which can be deprotected by Samarium diiodide found place in solid phase peptide synthesis.[141] Anpe [2-(4-acetyl-2-nitrophenyl)ethyl is a new base-labile carboxyl protecting group introduced by Robles and co-workers.[142]

Base-sensitive amino groups such as 2-chloro-3-indenylmethyloxy carbonyl (CLIMOC) and Benz inden-3-yl methyloxy carbonyl (BIMOC) similar to the 9-fluorenyl methyloxy carbonyl (Fmoc) group have been introduced.[143] Another newly developed base labile α-amino protecting group is 2-(4-nitro phenyl)sulphonylethoxycarbonyl (NSc).[144] 4-Methylsulphenyl benzyloxycarbonyl (Msz) group is introduced as a new class of amino protecting group removable by reductive acidolysis.[145] Several modifications to the classical tert-butyloxycarbonyl (Boc) group are also introduced.[146-148] Some newly developed side chain protecting groups for amino acids include, S-phenylacetoamidomethyl (Phacm) for cysteine,[149] 2-Adamantyloxy carbonyl group (2-Adoc) for the ε-amino group of lysine[150a] and for the imidazole function of Histidine,[150b] 2,4-dinitrophenyl (Dnp) group for the protection of hydroxyl function of tyrosine,[151] p-(methylsulphinyl)benzyl group for serine,[152] 2,2,4,6,7-pentamethyl dihydrobenzofuran-5-sulphonyl group (Pbf) for arginine[153] and polyethyleneglycol (PEG) bound benzyl and fluorenyl side chain protection for lysine and glutamic acid.[154]

New Boc deprotecting agents like chlorotrimethyl silane-phenol have been prepared which may replace the conventional ones.[155] Another newly developed reagent for the deprotection of t-butyloxycarbonyl group[156] and N^{α}-benzyloxy carbonyl group[157] is iodotrichlorosilane obtained from silicon tetrachloride and sodium iodide. A new stepwise deprotection methodology using reductive acidolysis is effective to suppress the side reactions at aspartic acid residue.[158] A report from the 22nd European Peptide Symposium (1992) is about the optimised deprotection procedure for peptides containing Arg (mtr), Cys (Acm), Trp and Met residues.[159] N-allyloxycarbonyl (Alloc) protecting group could be efficiently removed using sodium borohydride in the presence of catalytic amount of palladium (0).[160] 21st European Peptide Symposium reported on the use of trimethyl silyl triflate/trifluoro acetic acid/pentamethyl benzene for simultaneous resin cleavage and tert-butyloxycarbonyl (Boc) and benzyl deprotection in solid phase peptide synthesis.[161] Selective removal of N-Boc protecting group in the presence of tert-butyl ester and other acid sensitive groups by dry HCl in ethyl acetate at room temperature is described by Rapoport and co-workers.[162]

Novel activating agents like Benzotriazol-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP),[163] benzotriazolyloxy tri(pyrrolidine) phosphoneium hexafluorophosphate (PyBOP),[164] 2-trifluoroacetyl-thiopyridine-1-hydroxy benzotriazole, Bis[4-(2,2-dimethyl 1,3-dioxolyl) methyl]-carbodiimide (BDDC),[165] bromo-tris(pyrrolidino)-phosphonium hexa-fluorophosphate (PyBrop)[166] have been introduced. A mixture of 2-trifluoroacetylthiopyridine with the sodium salt of HOBt (NaOBt) was found to be a highly effective coupling reagent.[167] Enhancement of peptide couplings was recommended by a combination of 4-dimethylaminopyridine (DMAP)-dicyclohexylcarbodiimide (DCC) in the case of hindered amino acid residues.[168] In 1993, Carpino reported the use of 1-hydroxy-7-azabenzotriazole as an efficient peptide coupling

additive.[169] In the same year Carpino's group reported the use of Bis (Boc) amino acid fluorides as reactive peptide coupling reagents.[170] 3-Dimethylphosphinothioyl-2(3H)-oxazolone (MPTO), was introduced as a promising new reagent for racemisation free couplings.[171] Fmoc amino acid chloride coupling can be conveniently carried out in the presence of KOBt (potassium salt of 1-hydroxybenzotriazole).[172] A new hybrid that combines the structural features of dicyclohexylcarbodiimide (DCC) and N,N'-diisopropylcarbodiimide (DIC), i.e., N-cyclohexyl-N-isopropyl carbodiimide (CIC) was proposed as an efficient coupling reagent in peptide synthesis.[173] Bis[4-(2,2-dimethyl-1,3-dioxolyl)methyl carbodiimide (BDDC),[174] 2-(benzotriazol-1-yl)oxy-1,3-dimethylimidazolidinium hexafluoro-phosphate (BOI),[175] 2-(IH-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU),[176] Tris(pyrrolidino) phosphonium reagent, a phosphorous based reagent,[177] are some of the recent additions to the field of coupling reagents. Second Chinese Peptide Symposium reported the use of novel cyclic organophosphorous compounds as coupling reagents in peptide synthesis.[178] 1-β-Naphthalenesulfonyloxybenzotriazole (NSBt)[179] and 6-Nitro-1-β-Naphthalenesulfonyloxybenzotriazole[180] were the two recently introduced activating agents in peptide synthesis. Some of the other newly developed activating agents include di-tert-butyl pyrocarbonate in the presence of pyridine and ammonium hydrogen carbonate,[181] tetramethylfluoroformamidinium hexafluorophosphate, for solution and solid phase synthesis,[182] (F_3^- NO_2–PyBOP (1-hydroxy-4-nitro-6-(trifluoromethyl)benzotriazole containing phosphonium salt for *in situ* coupling of N-methylated amino acids,[183] 1-hydroxy-7-azabenzotriazole (HOAt) and its corresponding uronium and phosphonium salts for the automated synthesis of peptides containing hindered amino acids.[184] ToppipU[185] (2-(2-oxo-1(2H)-pyridyl)-1,1,3,3-bispentamethylene uronium

tetrafluoroborate) and FDP[186] (pentafluorophenyl diphenylphosphate) are the two new additions to the field of coupling agents in peptide synthesis.

Regarding the cleaving agents a mixture of hexafluoroisopropanol–dichloromethane (1:4 v/v) acts as a fast, effective and convenient reagent for cleaving protected peptide fragments with a minimal amount of racemisation from a 2-chlorotrityl chloride resin,[187] hydrolysis of polystyrene-bound protected peptides by using dimethyl amino ethanol or triethanolamine, DMF and aqueous sodium hydroxide with accelaration by ultrasound,[188] use of HF for cleavage and deprotection of peptides synthesised using a Boc/Bzl strategy[189] were reported recently.

Several investigations are going on to increase the efficiency of peptide synthesis by solid phase approach. Some of the recent publications provide a glance into it. Procedures to improve difficult couplings,[190] new solvent systems for difficult sequences,[191,192] optimised solid phase synthesis of large peptides utilising Fmoc-amino acids,[193] accelerated protocols for solid phase peptide synthesis at elevated temperatures,[194] new apparatus for synthesis,[195-198] a general strategy for the synthesis of large peptides by combining the solution phase and solid phase approach[199] and the development of an apparatus for cleaving peptides from resin supports[200] were a few of them. Progress in SPPS is well reviewed recently by Kiyoshi, John and Botand.[201-203] Thus many dramatic advances have occurred in recent years in the field of solid phase peptide synthesis.

The chemical synthesis of longer peptide chains is still a central problem of protein chemistry despite the considerable progress that has been made in the strategy of various synthetic methods and protecting group techniques. The physicochemical incompatibility of the growing peptide chain and the insoluble crosslinked polymeric support has been one of the major problems associated

with the polymer-supported method of peptide synthesis.[68,204,205] Development of polymer supports which swell in both polar and non-polar solvents facilitating the different types of organic reactions employed in repetitive stepwise peptide synthesis have therefore been a challenge to organic and polymer chemists for the past two decades.[206-209] The concept of optimum hydrophobic-hydrophilic balance serves as a guideline for the development of effective supports for peptide synthesis. Structure-reactivity and structure-property correlations in polymeric systems can be made use of in the design of such supports with optimum reactivity characteristics, mechanical stability and other essential requirements of a polymeric support useful for the stepwise synthesis involving a multitude of synthetic operations under widely varying conditions.

Attempts have been made in our laboratory to develop some new polymeric supports based on polyacrylamide and polystyrene for solid phase peptide synthesis. Polystyrene supports include tetraethyleneglycol diacrylate (TTEGDA), triethyleneglycol dimethacrylate (TEGDMA), and 1,6-hexanediol diacrylate crosslinked polystyrene supports. Their stability and solvation characteristics compared to the Merrifield resin (PS-DVB) is better and that resulted the increased use of these resins in solid phase peptide synthesis. Here we used polystyrene crosslinked with 1,6-hexanediol diacrylate as the flexible polymer support, which can be easily prepared and functionalised.

2.4 Synthesis of hydrophobic peptides

Peptides that have polar side chains are hydrophilic in nature and having non polar side chains are hydrophobic in nature. The apparent hydrophobicities of the amino acid side chains vary enormously, depending primarily on whether or not polar groups are present.

The hydrophobicities of the individual amino acid side chains have been measured experimentally in a variety of ways, using the free amino acids, amino

acids with the amino and carboxyl groups blocked, and side-chain analogues with the backbone replaced by a hydrogen atom and using a variety of non polar solvents including ethanol, octanol, dioxane and cyclohexane.[210]

Table 2.1. Relative hydrophilicities and hydrophobicities of amino acid side chains

Residue	Hydrophi-licity (Kcal/mol)[211]	Hydrophobicity (Kcal/mol)			
		Side-chain analogues[211]	Amino acids[212]	N-acetyl amide[213]	Calcu-lated[214]
Arg	-22.31	15.86	3.0	1.01	3.95
Asp	-13.34	9.66	2.5	0.77	3.81
Glu	-12.63	7.75	2.5	0.64	2.91
Asn	-12.07	7.58	0.2	0.60	1.91
Lys	-11.91	6.49	3.0	0.99	2.77
Gln	-11.77	6.48	0.2	0.22	1.30
His	-12.66	5.60	-0.5	-0.13	0.64
Ser	-7.45	4.34	0.3	0.04	1.24
Thr	-7.27	3.51	-0.4	-0.26	1.00
Tyr	-8.50	1.08	-2.3	-0.96	-1.47
Gly	0	0	0	0	0
Pro	-	-	-1.4	-0.72	-0.99
Cys	-3.63	-0.34	-1.0	-1.54	-0.25
Ala	-0.45	-0.87	-0.5	-0.31	-0.39
Trp	-8.27	-1.39	-3.4	-2.25	-2.13
Met	-3.87	-1.41	-1.3	-1.23	-0.96
Phe	-3.15	-2.04	-2.5	-1.79	-2.27
Val	-0.40	-3.10	-1.5	-1.22	-1.30
Ile	-0.24	-3.98	-1.8	-1.80	-1.82
Leu	-0.11	-3.98	-1.8	-1.70	-1.82

Molecules that have polar groups can appear to be more hydrophobic than they really are. The more hydrophobic molecules have the more negative hydrophobicities.

Synthesis of hydrophobic peptides is a difficult process because of the non-polar side chains and because of the coiling nature of the peptides.[215] Peptides that have substantial hydrophobic character also trend to aggregate with increasing concentration.[216]

In the present study, it is proposed to synthesise some of the partial sequences of thioredoxin—a naturally occurring sulphur reducing protein. Thioredoxin contains sequences of varying hydrophobicity-hydrophilicity patterns. Here, most of the sequences synthesised are hydrophobic in nature. These partial sequences were synthesised on a 2% HDODA-crosslinked polystyrene by following the standard solid phase methodology.

CHAPTER 3

EXPERIMENTAL

3.1 Preparation of polymer supports and functionalisation

3.1.1 Materials and methods

The polymer supports under study were synthesised and characterised in this laboratory. IR spectra were recorded on a Shimadzu IR–470 spectrophotometer using KBr pellets. ^{13}C CP/MAS NMR was recorded at 75.47 MHz on a Bruker 300 MSL CP/MAS instrument at Sophisticated Instrumentation Facility, Indian Institute of Science, Bangalore. Scanning electron micrographs were taken on a CAMBRIDGE S–360 instrument at Materials Research Centre, Indian Institute of Science, Bangalore.

3.1.2 Source of chemicals

Styrene, HDODA and polyvinyl alcohol (Mol. wt. 75000) were purchased from Sigma Chemical Company, USA. Benzoyl peroxide was procured from SISCO, Bombay and was recrystallised before use.

3.1.3 Polymer synthesis

Preparation of polystyrene crosslinked with 2% HDODA by suspension polymerisation

Styrene was destabilised by washing with 1% sodium hydroxide solution (20 ml x 3) and then washed with distilled water (20 ml x 3). HDODA was used as such. A mixture of styrene (22.45 ml; 98 mmol; 2 equiv.), HDODA (0.896 ml, 2 mmol; 2 equiv.), toluene (8 ml, 2 equiv.) as inert solvent and benzoyl peroxide (1 g) was prepared. It was then suspended into a solution of polyvinyl alcohol (mol. wt. 75,000, 3.2 g) dissolved in water (320 ml) and kept mechanically stirred at 80°C. Polymerisation was completed after 6 h. The beaded product was filtered, washed with hot water, acetone (30 ml, 3 x 3 min), methanol (30 ml, 3 x 3 min) and drained. The product resin was soxhlet extracted using acetone for 70 h to remove linear polymers and low molecular weight impurities and dried in the oven at 80°C. Yield 80%. Beads were meshed into 4 different sizes using standard sieves. Polymers of 1,3,4,5 and 6% HDODA crosslinks were prepared by adjusting the relative amounts of the monomers.

3.1.4. Functionalisation of PS–HDODA support with chloromethyl groups: general procedure.[217]

Chloromethyl functional group was introduced into the resin by chloromethyl methylether in the presence of anhydrous $ZnCl_2$ as a catalyst. The dry resin beads (2 g, 200–400 mesh size) were preswollen in a dry 2–necked round bottomed flask using dry dichloromethane (12 ml). A solution of anhydrous zinc chloride (1 M, 0.2 ml) dissolved in THF was added to chloromethyl methylether (12 ml) and this solution was slowly added to the resin under anhydrous conditions, with shaking. The mixture was refluxed at 50°C with calcium chloride guard tube for 5 h. The mixture was cooled and filtered carefully through a sintered glass funnel (G–2), washed with THF (30 ml,

3 x 10 min), THF/4N HCl (30 ml; 3 x 3 min), THF/water (30 ml; 3 x 3 min), THF (30 ml; 3 x 3 min), DCM (30 ml; 3 x 3 min) and finally methanol (30 ml; 3 x 3 min), drained and dried in the oven.

(a) Preparation of 1 M anh. $ZnCl_2$ in THF

Anhydrous zinc chloride (1.5 g) was placed in a 25 ml Erlenmeyer flask and conc. hydrochloric acid (3 drops) and distilled water (5 drops) were added and the contents stirred and heated until the solid dissolved completely. Temperature was gradually raised to evaporate the water and to leave a crust of solid which was then melted by stronger heating. When zinc chloride became a clear, mobile liquid with no further evolution of bubbles, the flask was placed in a desiccator and allowed to cool. The resulting mass was dissolved in freshly distilled THF (10 ml).

(b) Preparation of chloromethyl methylether[218]

A mixture of methanol (33 ml) and formaldehyde (63 ml) was placed in a 2–necked round bottomed flask, fitted with calcium chloride guard tube. The flask was cooled by placing in an ice–bath. Another 2 necked flask fitted with a dropping funnel and side tube containing conc. sulphuric acid, kept stirred magnetically. The side tube was placed in a glass washing bottle containing conc. sulphuric acid. Conc. hydrochloric acid was slowly added to the sulphuric acid kept stirred in the flask. The hydrogen chloride gas dried by conc. sulphuric acid in the drying bottle was bubbled through the methanol formaldehyde mixture. The slow stream of HCl was continued for 4 h, when the first layer of chloromethyl methylether (CMME) began to appear. The turbidity which appeared first gradually disappeared separating the aqueous and ethereal layers. HCl gas was passed for 4h more until the solution got saturated. CMME formed

as the upper organic layer. The lower aqueous layer was separated from the organic portion. The ether was dried by using anhydrous calcium chloride and stored in sealed bottles. Yield 40 ml.

(c) Estimation of chlorine capacity by pyridine fusion[219] method: general procedure

A known weight of the chloromethyl resin (200 mg) was fused with pyridine (5 ml) in a boiling tube at 110°C for 5 h. The resin was removed by filtration, washed with acetic acid/water (1:1, 30 ml), and the filtrate with the washings were acidified with 5 ml conc. nitric acid. This solution was titrated against standard ammonium thiocyanate solution (0.1 M) using ferric alum as indicator (modified Volhard's method).[220] A blank was also performed.

3.2 Peptide synthesis

3.2.1 Source of chemicals

All side chain protected L–amino acids, t–butyl carbazate, dicyclohexylcarbodiimide, 1–hydroxybenzotriazole (HOBt), trifluoroacetic acid, thioanisol, 1, 2–ethane dithiol and cesium carbonate were purchased from Sigma Chemical Company, USA. Boc–Gly, Boc–Ala, Boc–Leu, Boc–Phe, Boc–Ile and Boc–Val were prepared in the laboratory following Schnabel's method. All solvents were of reagent grade and were obtained from E.Merck (India). Whenever necessary, they were purified by literature procedure.

3.2.2 Physical measurements

An LKB BROMA high performance liquid chromatograph with C18 reverse phase column (preparative) was used for the purification of peptides. For checking the fractions a Shimadzu C–R6A liquid chromatograph with C18

reverse phase column (analytical) was used. Amino acid analyses were performed on a pharmacia LKB Alpha plus amino acid analyser. All NMR studies were carried out on a Bruker AMX–400 spectrometer. CD spectra were recorded on a JASCO–J–500 spectropolarimeter using 1 mm path length cells.

3.2.3 Purification of reagents and solvents

All the solvents used for peptide synthesis were purified before use. Following procedures were employed for purification.

Diethyl ether (E.Merck) was dried over fused calcium chloride overnight.

Dichloromethane (E.Merck) was dried by adding fused calcium chloride and kept overnight.

Ethanol was double distilled and stored in airtight bottles.

Triethylamine was refluxed over ninhydrin for 1 h, distilled and stored in amber–coloured bottles. Methanol was distilled before use.

3.2.4 Detection

TLC was used to monitor the progress of the reaction and to check the purity of the final product. TLC was performed on glass plates precoated with silica gel containing calcium sulphate binder and activated by heating for 4 h at 100°C and cooled just before use.

3.2.5 Identification of the peptides on TLC

This is an inexpensive technique and is useful for detecting small and medium range peptides. The methanol solution of the peptide is spotted on the plate and developed in suitable solvent mixture of appropriate composition. The solvent systems used include,

(a) Butanol–1: acetic acid:water:ethylacetate (1:1:1:1)
(b) Butanol–1: acetic acid:water (6:1:5)
(c) Butanol–1:acetic acid:water (4:1:1)
(d) Ethylacetate: pyridine: acetic acid:water (30:15:3:2)
(e) Methanol: chloroform (1:9)

3.2.6 Visualisation

The developed chromatogram was visualised by the following methods;

1. Ninhydrin spray detects the presence of free amino groups. A 0.2% pure ninhydrin in acetone was sprayed on the developed chromatogram and heated in an air oven at 80–100°C for 15 min Pink colour was developed by free amino groups. (N–terminal proline gives an yellow colour).
2. Iodine vapours: The TLC plates were exposed to iodine vapours in a closed chamber. Brown spots of amino acids and peptides were observed.

3.2.7 Preparation of amino acid derivatives

(a) Preparation of Boc–azide from t–butyl carbazate

Boc–azide was prepared from t–butyl carbazate following the method of Carpino and co-workers.[221] t–Butyl carbazate (20 g) was dissolved in a mixture of glacial

acetic acid (17.6 ml) and water (25 ml). Sodium nitrite (11.6 g) was added in small portions in about 15 min. During the addition the solution was stirred vigorously and maintained at 0°C. After 90 min, the oily layer was separated from the aqueous layer. The aqueous layer was extracted with ether (3 x 10 ml). The ether extracts were mixed with the oily layer, washed with water, 1 M sodium bicarbonate ($NaHCO_3$) and dried over sodium sulphate. On evaporating ether under reduced pressure, Boc–azide was obtained as a golden yellow oily liquid. It was stored in refrigerator and used directly without further purification. Yield: 18 ml.

(b) Synthesis of Boc amino acids: Schnabel's method:[222] *general procedure*

The amino acid was suspended in dioxane/water (1:1) mixture and Boc–azide was added to it. The mixture was stirred at room temperature, maintained the pH in the alkaline range using 4N sodium hydroxide. Water (15 ml) was added to the solution after 24 h and extracted with ether. The aqueous layer was cooled in an ice–bath, acidified with 2N HCl, saturated with sodium chloride and extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulphate and rotary evaporated to get the Boc–amino acid. In most cases, the Boc–amino acid was precipitated by adding dry petroleum ether followed by trituration. In certain cases, seeding by a trace of fresh Boc–amino acid followed by trituration. The Boc–amino acid was precipitated as white powder.

In the case of leucine, the acidified aqueous layer was extracted with ether. Purity of Boc–amino acid was tested on pre–coated TLC plate. Solvent for the development of the chromatogram includes a mixture of chloroform, methanol and glacial acetic acid in the ratio 85:10:5 (v/v). The chromatogram was visualised by ninhydrin spray which gave pink spot.

(c) Preparation of Boc–glycine

Glycine (1.5 g, 20 mmol) was suspended in 1:1 dioxane: water mixture (20 ml) and Boc azide (3.2 ml, 20 mmol) was added to it. The mixture was stirred at room temperature maintaining the pH in the alkaline range with 4N sodium hydroxide. After 24 h, water (15 ml) was added and the solution extracted with ether (10 ml). The aqueous layer was cooled in an ice bath, acidified with 2N HCl, saturated with NaCl and extracted with ethyl acetate (3 x 20 ml). It was dried over anhydrous sodium sulphate and ethylacetate was removed by rotary evaporation. Petroleum ether was added in excess, seeded with a trace of fresh and pure Boc–Gly to induce precipitation and triturated. The white powdery Boc–Gly precipitated was washed with fresh petroleum ether and vacuum dried. Yield: 1.2 g (80%).

Boc–Ala, Boc–Leu, Boc–Phe, and Boc–Ile were prepared following Schnabel's method.

Table 3.1. Preparation of Boc–amino acids.

Amino acid derivative	Yield (%)	M. P. observed (°C)	M. P. literature (°C)
Boc–Gly	80	84–85	86–88
Boc–Ala	85	76–78	79–81
Boc–Leu	95	72	70–73
Boc–Phe	83	77–79	79–80
Boc–Ile	80	64	66–69

(d) Boc–ON method: general procedure[223]

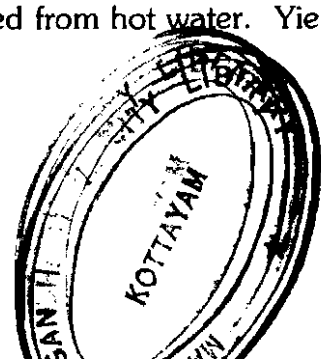
Amino acid (10 mmol), 4–tertiary butyloxy carbonyl oxgimino 2–phenyl acetonitrile (Boc–ON) (2.71 g, 11 mmol) and triethylamine (2.1 ml, 15 mmol) in 50% aqueous dioxane (12 ml) were stirred at room temperature for 12 h. The reaction mixture was diluted with water (20 ml) and washed with ethylacetate (15 ml x 2). The aqueous layer was cooled to 0°C, acidified with 1N HCl and extracted with ethylacetate (15 ml x 3). The organic layer was dried over anhydrous sodium sulphate, and rotary evaporated to remove ethylacetate. On adding petroleum ether the Boc amino acid was obtained.

(e) Purity of Boc–amino acids

Purity of all Boc amino acids were tested by TLC on precoated silica gel plates using chloroform/methanol/acetic acid (85:10:5) solvent system. Amino acids were visualised by ninhydrin spray after exposure to hydrogen chloride vapours for 10 min. All protected amino acids were stored at room temperature.

3.2.8 Preparation of 1–Hydroxybenzotriazole (HOBt)[224]

O–Chloronitrobenzene (32 g) was dissolved in ethanol (100 ml). Hydrazine hydrate (30 g) was added and the solution refluxed for 5 h. After distilling off the ethanol, the residue was diluted with water (100 ml) and extracted with ether (3 x 20 ml). The aqueous layer was acidified with conc. HCl, then HOBt precipitated. It was recrystallised from hot water. Yield: 20 g (80%), M. P. = 156°C.



3.2.9 General procedure for solid phase peptide synthesis

Manual solid phase peptide synthesis was done in a glass reaction vessel. The first amino acid of the C-terminal portion of the peptide was esterified to the resin via a benzyl ester linkage by triethyl ammonium salt or by the cesium salt of the Boc–amino acid. Boc group was deprotected by 30% TFA in DCM and neutralisation was effected by 5% TEA in DCM. Second Boc amino acid was coupled to the aminoacyl resin by DCC coupling method or by active ester procedure. Dichloromethane or N–methyl–2–pyrrolidone was used as the solvent and the coupling time was usually 1 h. The same procedure was adopted for the coupling of all the amino acids. Progress of coupling was monitored at every stage by semiquantitative ninhydrin test. In all couplings a 3–fold molar excess of the Boc–amino acid was used and double coupling was done to ensure completion of reaction. Final cleavage of peptide from the support was obtained by TFA in the presence of acid scavengers.

3.2.10 Attachment of first amino acid to the resin

(a) Merrifield's method (TEA method): general procedure

To the chloromethyl resin (1 mol of Cl), Boc amino acid (1 mol) and triethylamine (0.9 mol, 0.14 ml per mmol, sp. gravity 0.723) were added and refluxed gently in ethyl acetate (5 ml/g of resin) for 48 h. The suspension was filtered through a sintered glass funnel, washed successively with ethyl acetate (20 ml, 3 x 3 min), water (20 ml, 3 x 3 min), methanol (20 ml, 3 x 3 min) and DCM (20 ml, 3 x 3 min). The resin was vacuum dried and yield noted.

(b) Gisin's cesium salt method: general procedure

Boc amino acid was dissolved in minimum quantity of ethanol in a round bottomed flask. This was neutralised with an aqueous saturated solution of cesium carbonate (1–3 drops). The solution was kept stirred for 30 min and evaporated from dry, freshly distilled benzene in a rotary evaporator. The process was continued till a dry powder of cesium salt of Boc amino acid was obtained.

The cesium salt was dissolved in minimum quantity of freshly distilled DMF. The resin was suspended in it and the suspension was stirred at 50–60°C in an oil bath. The resin was filtered, washed with DMF (10 ml x 3), DMF/water (1:1, 10 ml x 3), DMF (10 ml x 3), MeOH (10 ml x 3) and DCM (10 ml x 3) and dried under vacuum.

3.2.11 Estimation of amino groups by picric acid method

The Boc–aminoacyl resin (10 mg) was deprotected using 30% TFA/DCM (30 min). The resin was washed thoroughly with DCM (6 times) to get rid of TFA completely and neutralised with 5% TEA in DCM for 10 min and again washed thoroughly with DCM (5 ml x 1 min) and dried. From the deprotected resin exactly 5 mg was taken in a Gisin's tube and treated with 0.1 M picric acid (2 x 5 min). All the unbound picric acid was washed off with multiple DCM washings. The resin–bound picrate was then carefully eluted with 5% TEA in DCM till the elute was clear. It was then made up to a definite volume (15 ml) using 95% ethanol. A definite volume of this solution (0.5 ml) was diluted to 5 ml with 95% ethanol. The optical density (OD) of this solution was measured at 358 nm. From the OD value, the weight of the resin taken, and the extinction coefficient of picrate (ε_{358}=14,500), the substitution level of the first amino acid was estimated.

3.2.12 Deprotection procedure

(a) Removal of t–butyloxy carbonyl group[225]

The t–butyloxy (t–Boc) protection of amino acids and peptides can be deprotected using anhydrous TFA in DCM (30%). For deprotection, the protected amino acid or peptide was treated with the above solution at room temperature for 30 min. Excess TFA was removed by filtration followed by washing with DCM and the salt thus obtained was neutralised with TEA in DCM (5%).

3.2.13 Methods of activation and coupling

Mainly two methods were used for the activation of carboxyl group by dicyclohexylcarbodiimide (DCC). (a) In the conventional DCC coupling[226] method a dichloromethane solution of 3–fold excess of the Boc–amino acid was treated with the aminoacyl resin along with the calculated quantity of DCC in DCM. The precipitated DCU was washed with methanol–dichloromethane mixture (MeOH–DCM, 33:67 v/v). The extent of coupling was followed by the semiquantitative ninhydrin test. If the test was positive, a second coupling was done and again the completion of the reaction was monitored. In some cases a third coupling was necessary to ensure the completion of reaction. (b) Certain difficult couplings were performed by the active ester method. This method were of two types. Active ester can be preformed or can be prepared in situ. In the HOBt active ester method,[227] the Boc amino acid dissolved in NMP (N–methyl 2–pyrrolidone), HOBt was added and equilibrated with the aminoacyl resin. This was followed by the addition of DCC in NMP and stirred for 1 h. The precipitated DCU was removed by washing with 33% methanol in DCM. This method was found to be useful for activating Asn, Gln and Arg.

3.2.14 Cleavage of the peptide from the resin: TFA/thioanisol method[228]

The peptidyl resin (100 mg) was suspended in TFA (10 ml) and to this thioanisol (0.1 ml), m–cresol (0.1 ml) and 1,2–ethanedithiol (0.1 ml) were added. The reaction mixture was left for 24 h at room temperature. It was filtered and the TFA solution was rotary evaporated to remove TFA. The peptide was then precipitated with ice cold dry ether and washed thoroughly with ether, centrifuged (10–15 times) and dried.

3.2.15 Purification

(a) Thin layer chromatography (TLC)

Silica gel (TLC grade) was used for thin layer chromatography. TLC of the peptides were done on silica gel plates. Aqueous or methanolic solution of the peptide was spotted on the silica plate and developed in a suitable solvent mixture of appropriate composition. The following solvent system in indicated volume ratios were used.

1. Butanol–1: acetic acid: water (6:1:5)
2. Chloroform: methanol: water: acetic acid (7:4:1:1)
3. Methanol: chloroform (1:9)

Identification sprays

The following reagents were used to detect the spots on the TLC plate.

(i) Ninhydrin spray

The plate was exposed to vapours of conc. HCl contained in a chamber for the removal of Boc group. The spots were developed by spraying ninhydrin

reagent and heating in an oven for 5–10 min. Violet spots were observed in the case of free primary amino groups (N–terminal proline gives an yellow colour).

(ii) Rydon's reagent (chlorine–starch–potassium iodide reagent)

This method can be used for the detection of protected peptides which are not visible with ninhydrin. The plates were exposed to chlorine gas for approximately 10 seconds and sprayed with a mixture containing equal volumes of 1% (w/v) aqueous starch and potassium iodide solutions. Blue black spots over blue background were observed. This test is given by almost all compounds containing–NH groups.

(iii) Iodine

The plates were exposed to iodine vapours in a closed chamber. Brown spots were observed in the case of amino acids and peptides.

(iv) Sakaguchi reagent

This reagent produces an orange red colour with compounds containing free guanidine group of arginine peptides. The plates were thoroughly cooled and sprayed with Sakaguchi reagent A. Sakaguchi A is 0.1% solution of β–hydroxy quinoline or naphthol in acetone. It was then sprayed with 2.5N sodium hydroxide solution, dried well and sprayed with Sakaguchi B. Sakaguchi B is NaOBr solution prepared by dissolving 0.67 ml Br_2 in 100 ml 1N NaOH.

(b) High performance liquid chromatography

Development of HPLC has caused a revolution in peptide purification and analysis and has brought about great increase in resolving power and speed of operation of column chromatography. This method is very helpful in establishing the homogeneity of peptides.

HPLC analyses were carried out using a Shimadzu two pump system equipped with a controller unit. On the Shimadzu system a Vydac C18 column 218 TP (particle size 5 μm) and an injection loop of 20 ml, 0.8 ml/min flow rate and detection at 226 nm were used.

Peptides were purified wherever necessary on an LKB HPLC system. The column used was C18 (4 x 250 mm, particle size = 10 μm) and an injection loop of 50 ml and a flow rate of 1.5 ml/min was used. Repetitive injections of 1 mg per run was carried out, and collection of the desired fractions, effected by the use of a LKB superrac fraction collector. Details of gradients used were indicated at appropriate places. Purified peptides were reinjected on the Shimadzu HPLC to confirm their homogeneity. Detections were done in both cases at 226 nm.

3.2.16 Amino acid analysis

Amino acid analysis is an essential requirement in determining the purity of a peptide. In the case of peptidyl resin and free peptides amino acid analysis were done after hydrolysing the samples. The following procedure was used for the hydrolysis of peptide–resins.[229] Approximately 10 mg of the peptide resin was taken in a sample tube. Propionic acid (0.5 ml) was added to it and the tube evacuated to remove the air from resin beads. Conc. HCl (0.5 ml) was added and then the tube was fused under nitrogen. It was heated at 130°C for 6 h, cooled, the resin was removed by filtration and the contents were quantitatively collected in a standard flask with distilled water. The solution was diluted with buffer and applied to amino acid analyser.

Amino acid analysis was used for the characterisation as well as quantification of peptides. The free peptide (1 mg) was taken in a sample tube

and a mixture of TFA–6NHCl (1:2) was added to it. The tube was sealed under vacuum and heated at 110°C for 24 h. The residue was dried and dissolved in the amino acid loading buffer. This was then applied to the amino acid analyser.

3.2.17 Synthesis of partial sequences of thioredoxin

(a) Synthesis of Asp–Lys–Ile–Ile–His–Leu–Thr (T 2–8)

Attachment of Boc–Thr to the chloromethyl resin

To the 2% chloromethyl HDODA–PS resin (100 g, 2.07 mmol Cl/g) in ethyl acetate (6 ml), Boc–Thr (0.1201 g) and TEA (0.026 ml) were added and refluxed for 48 h. It was then filtered through a sintered glass funnel (G–2), washed with ethyl acetate (20 ml, 3 x 3 min), water (20 ml, 3 x 3 min), methanol (20 ml, 3 x 3 min) and DCM (20 ml, 3 x 3 min) and dried overnight in a vacuum desiccator. Weight increment = 25 mg. Substitution level of Boc amino acid was found to be 1 mmol/g by picric acid method.

Synthesis of T2–8

The remaining amino acids were assembled on Boc–Thr resin (100 mg) by following the schedule given below. All Boc–amino acids without side chains were prepared by the Schnabel's method. Boc–Lys (2ClZ) and Boc–Asp (CHex) were the other amino acids used. Dicyclohexylcarbodiimide coupling method in NMP was employed for the attachment of all amino acids except Boc–Asp and Boc–His. In these cases a preformed HOBt active ester was prepared in NMP. The protocol followed for the above synthesis is shown below.

Table 3.2. Protocol used for the synthesis of Asp–Lys–Ile–Ile–His–Leu–Thr.

Ope–ration No.	Steps involved	Reagent/Solvent	Time (min)
1	Wash	DCM (10 ml)	1
2	Deprotection	30% TFA/DCM	30
3	Wash	DCM x 6	1.5
4	Neutralise	5% TEA/DCM x 1	10
5	Wash	DCM (10 ml x 4)	1.5
6	Wash	NMP (10 ml x 2)	1.5
7	Coupling	1:1 DCC–Boc amino acid in NMP	60
8	Wash	33% MeOH/DCM x 3	2
9	Wash	DCM x 3	2
10	Steps 5–9 repeated for second coupling. For Asp and His 1:1:1 DCC–HOBt–AA in NMP		120

Boc–Thr (OBzl) resin (100 mg, 0.25 mmol) was deprotected using 30% TFA in DCM in a solid phase reaction vessel. This was followed by neutralisation using TEA. Boc–Asp and Boc–His were coupled by the HOBt active ester method in NMP for 2 h. Coupling efficiency was monitored by semiquantitative ninhydrin test.[230] Double coupling was done at every stage so that the reaction is complete. After the attachment of all the seven residues the peptide resin was washed thoroughly with DCM and dried. Weight of peptidyl resin = 160 mg.

Cleavage and purification

The peptidyl resin (100 mg) was treated with a mixture of TFA (10 ml), thioanisol (0.1 ml) and 1,2–ethanedithiol (0.1 ml) at room temperature for 48 h. The resin was removed by filtration and TFA was evaporated from the filtrate by rotary evaporation. To the oily residue thus obtained, ice–cold ether was added to precipitate the peptide. The precipitated crude peptide was washed with ether (15–20 times) to get rid of TFA and scavengers, dried and yield noted.

The cleaved peptide was subjected to hydrogenation to remove the benzyl group. For this a solution of peptide in MeOH was treated with palladium charcoal under hydrogen atmosphere for 48 h. It was filtered and MeOH was evaporated. The residue obtained was purified by HPLC on a prep. rpc C18 column using CH_3CN/H_2O system.

Amino acid analysis

The peptidyl resin was hydrolysed using a mixture of propionic acid and conc. HCl (1:1 v/v) and heated to 120°C for 6 h. The resin was removed by filtration and the solution was quantitatively transferred to a standard flask with distilled water. This solution was then diluted with buffer and applied to the amino acid analyser.

*(b) Synthesis of Ser–Phe–Asp–Thr–Asp–Leu–Val–Lys (**T**11–18)*

Attachment of Boc–Lys to the functionalised resin

Boc–Lys was esterified to the chloromethyl resin (500 mg, chlorine capacity 1.1 mmol/g) following Gisin's cesium salt method. Boc–Lys (2ClZ) was dissolved in minimum quantity of ethanol and a saturated solution of cesium

carbonate was added into it till the pH reached 7. The reaction mixture was stirred for 30 min and again pH checked. Solvents were removed completely by repeated rotary evaporation using dry benzene. The white powdery cesium salt of Boc–Lys obtained was dried under vacuum. It was dissolved in minimum quantity of NMP. Chloromethyl resin was added to the above solution and the mixture was heated in an oil bath at 50–60°C for 60h with occasional shaking. The resin was filtered through a sintered funnel, washed with NMP (10 ml, 3 x 3 min), NMP/Water (10 ml, 3 x 3 min), NMP (10 ml, 3 x 3 min), methanol (10 ml, 5 x 5 min). The product was drained and dried in a vacuum desiccator. Final weight = 710 mg. The substitution level of Boc–Lys (2ClZ) was determined by picric acid method and was found to be 0.8 mmol/g.

Synthesis of T11–18

All the simple amino acids were prepared by Boc–azide method. Boc–Ser (OBzl), Boc–Asp (CHex), Boc–Thr (OBzl) were the other amino acids used for the synthesis. Boc–Lys (2ClZ) resin (100 mg. 0.24 mmol) was used for the synthesis. For each coupling a three fold molar excess of Boc–amino acids were used to ensure completion of coupling. A second coupling was carried out in all the cases. Completion of coupling was checked by ninhydrin. One complete synthetic cycle consisted of the following steps.

1. Wash with dichloromethane (10 ml, 1 x 5 min)
2. Deblocking using 30% TFA in dichloromethane (10 ml x 30 min)
3. Wash with DCM (10 ml, 6 x 1.5 min)
4. Prewash using 5% TEA in DCM (5 ml x 2 min)
5. Neutralisation using 5% TEA in DCM (10 ml x 10 min)

6. Wash with DCM (10 ml, 4 x 1.5 min)

7. Wash with NMP (10 ml, 2 x 1.5 min)

8. Coupling of Boc–amino acids in the presence of DCC and HOBt in NMP (60 min)

9. Washing off dicyclohexyl urea (DCU) with 33% methanol in DCM (10 ml, 3 x 2 min)

10. Wash with DCM (10 ml, 2 x 1.5 min)

11. Wash with NMP (10 ml, 2 x 1.5 min)

12. Repetition of steps 6, 7, 8 and 9 to ensure completion of coupling tested by semiquantitative ninhydrin test.

After the attachment of all the amino acids the peptide resin was washed with DCM and dried. weight increase = 85 mg.

Cleavage and purification

The finished peptide was cleaved from the peptidyl resin following the method suggested by Bodanszky and Bodanszky.[228] The resin bound peptide (100 mg) was treated with a mixture of TFA (10 ml), thioanisol (0.1 ml), m–cresol (0.1 ml) and 1,2–ethanedithiol (0.1 ml) at room temperature for 48 h. The resin was filtered off and TFA was removed from it by rotary evaporation and the crude peptide was precipitated using ice cold ether. It was then washed several times with ether (15–20 times) to remove TFA and scavengers.

The cleaved peptide was subjected to hydrogenation. Peptide was dissolved in MeOH/CH_3COOH mixture and treated with palladium charcoal under hydrogen atmosphere for 24 h. The residue was filtered off and MeOH was evaporated.

Amino acid analysis

Peptidyl resin was hydrolysed using a mixture of propionic acid and conc. HCl (1:1 v/v) and heated to 120°C for 6 h. The resin was filtered off and the solution was quantitatively transferred to a standard flask with distilled water. This solution was diluted with buffer and applied to amino acid analyser.

*(c) Synthesis of Ala–Ile–Leu–Val–Asp–Phe–Trp–Ala (**T**22–29)*

First amino acid attachment to the chloromethylated resin

Boc–Ala was attached to the chloromethylated 2% HDODA–PS resin (1.91 mmol/g) by the cesium salt procedure. Boc–Ala (0.451 g, 2.38 mmol) was dissolved in ethanol and the solution was neutralised with saturated solution of cesium carbonate. It was then rotary evaporated to remove ethanol and benzene was added to co–evaporate water as an azeotrope. The dry white powder of cesium salt of Boc–Ala was kept overnight in a vacuum desiccator.

To the cesium salt dissolved in DMF chloromethyl resin (500 mg, 0.96 mmol) was added and heated for 48 h at 50–60°C in an oil bath with occasional shaking. The product resin was filtered, washed with DMF (20 ml, 3 x 3 min), DMF/water (20 ml, 3 x 3 min), water, methanol (20 ml, 3 x 3 min) and DCM (20 ml, 3 x 3 min) and dried under vacuum. Final weight = 776 mg. The first amino acid capacity was determined by picric acid test and was found to be 1.8 mmol/g.

Synthesis of **T**22–29

200 mg of Boc–Ala resin was used for the synthesis of this sequence. All Boc–amino acids without side chains were prepared by the Schnabel's method. Boc–Asp (CHex) and Boc–Trp (CHO) were the other amino acids used. DCC

coupling in NMP was employed for all at the attachments, and for Asp and Trp, HOBt active ester was used. The protocol followed for the synthesis is shown below.

Table 3.3. Protocol used for the synthesis of Ala–Ile–Leu–Val–Asp–Phe–Trp–Ala.

Operation No.	Steps involved	Reagent/Solvent	Time (min)
1	Wash	DCM	2 x 3
2	Deprotection	30% TFA/DCM	1 x 30
3	Wash	DCM	6 x 1.5
4	Neutralise	5% TEA/DCM	1 x 5
5	Wash	DCM	6 x 1.5
6	Coupling	1:1 DCC–Boc amino acid (2.5 equiv. in NMP)	60
7	Wash	33% MeOH/DCM	3 x 1.5
8	Wash	DCM	3 x 1.5
9	Steps 6–8 repeated for second coupling		
	For Trp and Asp coupling	1:1:1 DCC–HOBt–AA (2.5 equiv.) in NMP	120

The coupling time for the attachment of each amino acid was 60 min and it was increased by 5 min for every amino acid. Double coupling was done at every stage so that the reaction is complete which was monitored by semiquantitative ninhydrin test. After the attachment of all the residues the peptide resin was washed thoroughly with DCM and dried. Weight increase = 350 mg.

Cleavage and purification

The finished peptide was cleaved from the resin by using TFA. The peptidyl resin (100 mg) was treated with a mixture of TFA (100 ml), thioanisol (0.1 ml), m–cresol (0.1 ml) and 1,2–ethanedithiol (0.1 ml) at room temperature for 48 h. The resin was removed by filtration and the solution obtained was rotary evaporated to remove TFA. The peptide was precipitated by adding ice cold dry ether and was washed with ether (15–20 times), every time centrifuging to remove the ethereal solution containing impurities and finally dried to get the peptide as a white powder. Yield: 65 mg.

The crude peptide was purified by HPLC. A prep. rpc C_{18} column was used. The solvent system used was CH_3CN/H_2O containing 0.1% TFA and H_2O containing 0.1% TFA. The major peak was collected, solvent evaporated from it and lyophilised.

Amino acid analysis

The peptidyl resin was hydrolysed using a mixture of propionic acid and conc. HCl (1:1 v/v) and heated up to 120°C for 6 h. The resin was removed by filtration and the solution was quantitatively transferred to a standard flask with distilled water. This was then diluted with buffer and applied to amino acid analyser.

(d) Synthesis of Met–Ile–Ala–Pro–Ile–Leu–Asp–Glu–Ile–Ala–Asp–Glu–Tyr–Gln–Gly–Lys (T37–52)

Boc–Lys (2ClZ) was attached to the chloromethyl resin (500 mg, 1.1 mmol/g) by following Gisin's cesium salt method. The substitution level of Boc–Lys (2ClZ) was determined by picric acid method and was found to be

0.8 mmol/g. The remaining amino acids were attached to this resin (100 mg) by solid phase technique.

Synthesis of (T37–52)

All the Boc amino acids without side chains were prepared by Schnabel's method. Boc–Asp (CHex), Boc–Glu (OBzl), Boc–Tyr (Z) were the other amino acids used. All the coupling reactions were done via the HOBt active ester method and NMP was the solvent used. A 3 fold molar excess of Boc–amino acids were used to ensure completion of coupling. The protocol used for the synthesis is given below.

1. Wash with DCM (10 ml, 2 x 1.5 min)
2. Deblocking using 30% TFA/DCM (10 ml x 30 min)
3. Wash with DCM (10 ml, 6 x 1.5 min)
4. Prewash using 5% TEA/DCM (10 ml x 1.5 min)
5. Neutralisation using 5% TEA/DCM (10 ml x 10 min)
6. Wash with DCM (10 ml, 4 x 1.5 min)
7. Wash with NMP (10 ml, 2 x 1.5 min)
8. Coupling with 1:1:1 DCC–Boc amino acid–HOBt (1 ml, 1 x 60 min) in NMP.
9. DCU washed off with 33% MeOH/DCM (10 ml, 3 x 1.5 min)
10. DCM wash (10 ml, 2 x 1.5 min)
11. NMP wash (10 ml, 2 x 1.5 min)
12. Steps 8–11 repeated to ensure completion of the reaction.

Double coupling was performed in all the cases and in the case of Boc–Tyr (Z) a third coupling was also performed. After the attachment of all the amino acids the peptidyl resin was washed with DCM and dried. Weight increase = 235 mg.

Cleavage and purification

Cleaving of the peptide from the resin was carried out using TFA and scavengers. 100 mg of peptidyl resin was suspended in TFA (10 ml), thioanisol (0.1 ml), m–cresol (0.1 ml) and 1,2–ethanedithiol (0.1 ml) for 24 h at room temperature. The solution was collected by filtering off the resin and TFA was removed from it by rotary evaporation. The peptide was precipitated by the addition of ice cold ether and washed thoroughly with cold ether (15–20 times). On removal of ether the peptide obtained as a white powder.

(e) Synthesis of Leu–Thr–Val–Ala–Lys–Leu (T53–58)

Synthesis of this hexapeptide was performed on a 2% PS–HDODA resin. (2.01 mmol/g). Boc–Leu was attached to the by cesium salt method and the substitution level (1.7 mmol/g) was determined by picric acid method. The remaining amino acids were attached to the Boc–Leu resin (400 mg) using the standard solid phase strategy. The synthetic cycle consisted of the following operations:

1. DCM wash (10 ml, 2 x 1.5 min)
2. Deprotection by 30% TFA in DCM (10 ml, 1 x 30 min)
3. DCM wash (10 ml, 6 x 1.5 min)
4. Neutralisation with 5% TEA in DCM (10 ml; 1 x 10 min)
5. DCM wash (10 ml, 6 x 1.5 min)
6. Coupling with 1:1 DCC–Boc amino acid in DCM (10 ml; 1 x 60 min)
7. Removal of DCU by washing with 33% ethanol in DCM (10 ml; 3 x 1.5 min)
8. DCM wash (10 ml; 3 x 1.5 min)
9. Repetition of steps 6–8 to ensure completion of coupling reaction.

All the remaining amino acids in the sequence were attached following the same protocol and finally the peptidyl resin was washed with DCM and dried. Final weight of peptidyl resin = 720 mg.

Cleavage

The peptide was cleaved from the support by treating the peptidyl resin (100 mg) with TFA, thioanisol and m–cresol (10 ml, 0.1 ml and 0.1 ml) for 24 h at room temperature. The TFA solution was collected by filtration through a sintered funnel and rotary evaporated to remove the excess TFA. The peptide was precipitated with ice cold dry ether and it was washed thoroughly with ether (15–20 times). On centrifugation the peptide was obtained in 80% yield. It was checked in FPLC and gave a single peak.

Amino acid analysis

The peptidyl resin was hydrolysed using a mixture of propionic acid and conc. HCl (1:1 v/v) and heated to 120°C for 6 h. The resin was removed by filtration and the solution was quantitatively transferred to a standard flask with distilled water. This solution was then diluted with buffer and applied to the amino acid analyser.

(f) Synthesis of Asn–Ile–Asp–Gln–Asn–Pro–Gly–Thr–Ala (T59–67)

This synthesis was carried out on 100 mg of Boc–Ala resin having a substitution level of 1.8 mmol/gm. Boc–Ala, Boc–Gly and Boc–Ile were synthesised by Schnabel's method. Boc–Asn, Boc–Gln, Boc–Pro, Boc–Asp (CHex), Boc–Thr (OBzl) were the other amino acids used. DCC coupling in

NMP was employed in all the additions and for Asn, Asp and Gln, HOBt method was used. The steps involved in the synthesis are given below.

1. DCM wash (10 ml, 2 x 1.5 min)
2. Deprotection by 30% TFA in DCM (10 ml, 1 x 30 min)
3. DCM wash (10 ml, 6 x 1.5 ml)
4. Prewash with 5% TEA (5 ml, 1 x 1 min)
5. Neutralisation with 5% TEA in DCM (10 ml, 1 x 10 min)
6. DCM wash (10 ml, 4 x 1.5 min)
7. NMP wash (10 ml, 2 x 1.5 min)
8. Coupling with 1:1 DCC–Boc amino acid in NMP (1 ml, 1 x 60 ml)
9. DCU removal by washing with 33% MeOH in DCM (10 ml, 3 x 1.5 min)
10. DCM wash (10 ml, 3 x 1.5 min)
11. Repetition of steps 7 to 10 for the second coupling.

The remaining amino acids were attached by using the same protocol. A second coupling was carried out in all cases to ensure complete coupling. After the synthesis, the peptidyl resin was washed with DCM and dried under vacuum. Weight of peptidyl resin = 140 mg.

Cleavage and purification

The resin bound peptide was cleaved off from the support by treating 100 mg peptide resin with TFA, thioanisol and 1,2–ethanedithiol mixture in the ratio 10:0.1:0.1 at room temperature for 18 h. The TFA solution was collected by filtration. After removing TFA ice cold ether was added into it. The precipitated peptide was washed with ether (15–20 times) and dried to get a white powder.

The peptide obtained was subjected to hydrogenation to remove benzyl groups. For this the peptide was dissolved in MeOH and one drop of acetic acid

was added. After adding palladium charcoal the reaction mixture was stirred in hydrogen atmosphere for 48 h. It was filtered and MeOH was evaporated from it. The product was purified by HPLC by using CH_3CN/H_2O containing 0.1% TFA system.

Amino acid analysis

The peptidyl resin was hydrolysed using a mixture of propionic acid and conc. HCl (1:1 v/v) and heated to 120°C for 6 h. Resin was filtered off and the solution diluted with buffer and applied to amino acid analyser.

*(g) Synthesis of Pro–Lys–Tyr–Ile–Gly (**T**68–72)*

Attachment of first amino acid to the chloromethyl resin

Cesium salt of Boc–Gly was attached to the chloromethyl resin following the method of Gisin. To the cesium salt of Boc–Gly dissolved in minimum quantity of freshly distilled DMF, chloromethyl resin (500 mg) was added, and heated at 50–60°C in an oil bath for 45 h. It was filtered through a sintered funnel, washed with DMF (15 ml, 3 times), DMF/water (1:1, 15 ml, 3 times) DMF (15 ml, 3 times), MeOH (15 ml, 3 times) and finally with DCM (15 ml, 3 times) and dried in a vacuum desiccator. Final weight = 720 mg. The glycine substitution level was found to be 1.7 mmol/g as determined by the picric acid method.

Synthesis of **T**68–72

Synthesis was carried out on Boc–Gly resin (100 mg, 1.7 mmol/g) simple Boc–amino acids were prepared by Boc–azide method. A 3–fold molar excess

of Boc–amino acids were used for each coupling. The entire synthesis was carried out in NMP. Each synthetic cycle consisted of the following steps.

Table 3.4. Protocol used for the synthesis Pro–Lys–Tyr–Ile–Gly.

Operation No.	Steps involved	Reagent/Solvent	Time (min)
1	Wash	DCM	2 x 3
2	Deblocking	30% TFA/DCM	1 x 30
3	Wash	DCM	6 x 1.5
4	Neutralisation	5% TEA/DCM	1 x 5
5	Wash	DCM	4 x 1.5
6	Wash	NMP	2 x 1.5
7	Coupling	1:1 DCC–Boc amino acid (3 equiv. in NMP)	60
8	Wash	33% MeOH/DCM	3 x 1.5
9	Wash	DCM	3 x 1.5
Steps 6–9 repeated for second coupling			

The coupling time for the attachment of each amino acid was 60 min. Double coupling was done at every stage so that the reaction is complete which was monitored by Kaiser test. After the attachment of all the residues the peptide resin was washed with DCM and dried. Final weight of peptidyl resin = 170 mg.

Amino acid analysis

The peptidyl resin was hydrolysed using a mixture of propionic acid and con. HCl (1:1 v/v) and heated up to 120°C for 6 h. The resin was removed by filtration and the solution was quantitatively transferred to a standard flask with distilled water. This was then diluted with buffer and applied to amino acid analyser.

*(h) Synthesis of Ile–Gly–Arg–Gly–Ile–Pro–Thr–Leu–Leu–Leu–Phe (**T**71–81)*

Attachment of Boc–Phe to the functionalised resin

Boc–Phe was esterified to the chloromethylated resin by Gisin's cesium salt method. For this chloromethylated 2% PS–HDODA resin (2.01 mmol/g) was used. Boc–Phe (0.94 g) was dissolved in ethanol. This was neutralised (pH=7) by a saturated solution of cesium carbonate. The solution was rotary evaporated to remove water as an azeotrope. The dry white powder of cesium salt of Boc–Phe was vacuum dried.

The cesium salt of Boc–Phe was dissolved in minimum quantity of DMF and chloromethyl resin (700 mg) was suspended in it. This was heated at 50–60°C in an oil bath with occasional shaking. The product resin was filtered through a sintered funnel washed successively with DMF (20 ml x 3), DMF: water (20 ml x 3), DCM (20 ml x 3) and drained and dried under vacuum. Final weight = 930 mg.

The Boc–Phe substitution level was estimated by the picric acid test and was found to be 1.9 mmol/g.

Synthesis of (**T**71–81)

All the simple Boc–amino acids were prepared by following the Boc–azide procedure. Boc–Arg (CBZ), Boc–Thr (OBzl) and Boc–Pro were the other amino acids used. Boc–Phe resin (100 mg, 0.19 mmol) was used for the synthesis. For each coupling a 2.5 fold molar excess of Boc–amino acids were used to ensure completion of coupling. One complete synthetic cycle consisted of the following steps.

1. Wash the resin with DCM (10 ml, 2 x 1.5 min)
2. Boc–deblocking using 30% TFA/DCM (10 ml x 30 min)
3. Wash with DCM (10 ml, 6 x 1.5 min)
4. Prewash using 5% TEA/DCM. (10 ml x 1.5 min)
5. Neutralisation using 5% TEA/DCM (10 ml x 10 min)
6. Wash with DCM (10 ml, 4 x 1.5 min)
7. Wash with NMP (10 ml, 2 x 1.5 min)
8. Coupling with 1:1:1 DCC–Boc amino acid and HOBt (1 ml, 1 x 60 min) in NMP.
9. DCU washed off with 33% MeOH/DCM (10 ml, 3 x 1.5 min)
10. DCM wash (10 ml, 2 x 1.5 min)
11. NMP wash (10 ml, 2 x 1.5 min)
12. Steps 8–11 repeated to ensure completion of reaction.

In all the cases double coupling was performed, except in the case of arginine where the reaction was completed after the 4th coupling. The HOBt–active ester method was used for the coupling of Arg. After the attachment of all the residues the peptidyl resin was dried and yield noted.

Cleavage

The finished peptide was cleaved from the support by neat TFA. 100 mg of peptidyl resin was suspended in a mixture of 10 ml TFA, 0.1 ml thioanisol and 0.1 ml m–cresol for 48 h at room temperature. TFA was removed by rotary evaporation after filtration and the crude peptide was precipitated by the addition of ice cold ether. It was then washed several times with ether to get 50 mg of crude peptide. The was checked in an analytical HPLC using CH_3CN/H_2O system and only one major peak was obtained. The peptide was subjected to NMR analysis and CD measurements.

Amino acid analysis

The peptidyl resin (10 mg) was hydrolysed using a mixture of propionic acid and conc. HCl (1:1 v/v) and heated to 120° for 6 h. The resin was removed by filtration and the solution was quantitatively transferred to a standard flask with distilled water. This solution was then diluted with buffer and applied to the amino acid analyser.

*(i) Synthesis of Thr–Leu–Leu–Leu–Phe (**T**77–81)*

This synthesis was carried out on 500 mg of Boc–Phe resin having an amino capacity of 1.9 mmol/g. Boc–Leu and Boc–Phe were prepared by Schnabel's method and Boc–Thr (OBzl) was used. The protocol used for the synthesis is given below.

Table 3.4 . Steps involved in the synthesis of Thr–Leu–Leu–Leu–Phe.

Ope–ration No.	Steps involved	Reagent/Solvent	Time (min)
1	Wash	DCM	2 x 3
2	Boc–deprotection	30% TFA/DCM	1 x 30
3	Wash	DCM	6 x 1.5
4	Neutralise	5% TEA/DCM	1 x 5
5	Wash	DCM	6 x 1.5
6	Coupling	1:1 DCC–Boc amino acid (2.5 equiv. in NMP)	40
7	Wash	33% MeOH/DCM	3 x 1.5
8	Wash	DCM	3 x 1.5
9	Steps 6–8 repeated for second coupling		

A second coupling was carried out in all the cases in order to ensure complete coupling. When the sequence was completed the peptidyl resin was washed thoroughly with DCM and dried. Final weight of peptidyl resin = 760 mg.

Cleavage of the finished peptide from the resin

The peptidyl resin (100 mg) was treated with a mixture of TFA (10 ml), thioanisol (0.1 ml) and m–cresol (0.1 ml) at room temperature for 24 h. The resin was filtered off and TFA was removed from the solution. The peptide was precipitated by adding ice cold ether and centrifuged (15–20 times) with ether. The white powder obtained was dried to get the peptide in 80% yield. The purity was checked in FPLC and a single peak was obtained. The peptide was subjected to NMR analysis (270 MHz) and CD measurements.

Amino acid analysis

The peptidyl resin was hydrolysed using a mixture of propionic acid and conc. HCl (1:1 v/v) and heated to 120°C for 6 h. The resin was removed by filtration and the solution was quantitatively transferred to a standard flask with distilled water. The solution was then diluted with buffer and applied to the amino acid analyser.

(j) Synthesis of Ala–Thr–Lys–Val (T88–91)

Synthesis of this tetrapeptide was carried out on a Boc–Val resin (300 mg) with an amino capacity of 1.91 mmol/g. A 2.5 molar excess of Boc–amino acids were used for each coupling and the entire synthesis was carried out in DCM. A double coupling was carried out in all the cases to ensure complete reaction. The efficiency of coupling was monitored in each step by semiquantitative ninhydrin test. The protocol used for the above synthesis is shown below.

Table 3.5. Protocol used for the synthesis of Ala–Thr–Lys–Val.

Operation No.	Steps involved	Reagent/Solvent	Time (min)
1	Wash	DCM x 2	1.5
2	Deprotection	30% TFA/DCM x 1	30
3	Wash	DCM x 6	1.5
4	Neutralise	5% TEA/DCM x 1	5
5	Wash	DCM x 6	1.5
6	Coupling	1:1 DCC–Boc amino acid (2.5 equiv. in NMP)	45
7	Wash	33% MeOH/DCM x 3	1.5
8	Wash	DCM x 3	1.5
9	Steps 6–8 repeated for second coupling		

The coupling time given for the attachment of second amino acid was 45 min and it was increased by 5 min for every amino acid. After the attachment of 4 residues the peptide resin was washed and dried. Weight increase = 200 mg.

Cleavage of the peptide from the resin

100 mg of peptidyl resin was treated with 10 ml TFA and 0.1 ml thioanisol. This was kept at room temperature for 20 h. After 20 h the resin was filtered out and TFA removed from it. The white precipitate of peptide was

obtained by adding ice cold dry ether. It was then washed several times with ether and centrifuged to get an yield of 90% crude peptide.

Purity of the crude peptide was checked in FPLC and a single peak obtained. It was then subjected to NMR and CD analysis.

Amino acid analysis

The peptidyl resin was hydrolysed using a mixture of propionic acid and conc. HCl (1:1 v/v) and heated to 120°C for 6 h. The resin was removed by filtration and the solution was quantitatively transferred to a standard flask with distilled water. This solution was then diluted with buffer and applied to the amino acid analyser.

(k) Synthesis of Gly–Ala–Leu–Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu (T92–107)

Boc–Leu was attached to a chloromethylated resin (700 mg, 2.01 mmol/g by cesium salt method. The substitution level of Boc–Leu was determined by picric acid method (1.7 mmol/g).

Synthesis of (T92–107)

200 mg of Boc–Leu substituted resin was used for the synthesis. All Boc–amino acids without side chains were prepared by Schnabel's method. Boc–Asp(Bzl), Boc–Glu(OBzl), Boc–Lys(2ClZ), Boc–Ser(OBzl) were the other amino acids used. DCC coupling method in NMP was employed for the attachment of amino acids and for Boc–Ser, Boc–Lys, Boc–Gln, Boc–Glu, Boc–Asp and Boc–Asn, HOBt active ester method was performed. The procedure for one synthetic cycle is given below.

1. DCM wash (10 ml, 2 x 1.5 min)
2. Boc deprotection by 30% TFA in DCM (10 ml, 1 x 30 min)
3. DCM wash (10 ml, 6 x 1.5 min)
4. Neutralisation with 5% TEA in DCM (10 ml, 1 x 10 min)
5. DCM wash (10 ml, 4 x 1.5 min)
6. NMP wash (10 ml, 2 x 1.5 min)
7. Coupling with 1:1:1 DCC–Boc amino acid and HOBt in NMP (1 ml, 1 x 45 min)
8. DCU washed off with 33% MeOH/DCM (10 ml, 2 x 1.5 min)
9. DCM wash (10 ml, 2 x 1.5 min)
10. NMP wash (10 ml, 2 x 1.5 min)
11. Steps 7–10 repeated to ensure completion of reaction.

A double coupling was carried out in all the cases and the completion of coupling was checked by semiquantitative ninhydrin test. In the case of Boc–Asp (CHex) and Boc–Asn a third coupling was also performed.

Cleavage and purification

The finished peptide was cleaved from the support by following the method of Bodanszky and Bodanszky.[228] For this the peptidyl resin (100 mg) was treated with a mixture of TFA (10 ml), thioanisol (0.1 ml), m–cresol (0.1) and 1,2–ethanedithiol (0.1 ml) at room temperature for 48 h. The resin was filtered off from it. After removing TFA by rotary evaporation, the crude peptide was precipitated by the addition of ice–cold ether. It was then washed free of scavengers and other impurities to get 70% of crude peptide.

The cleaved compound was subjected to hydrogenation to remove the benzyl groups. For this the peptide was dissolved in MeOH and a drop of acetic acid was added. This was stirred with palladium charcoal under hydrogen atmosphere for 48 h. This was filtered and methanol evaporated from it. It was purified in HPLC using a C18 rp column using CH_3CN/H_2O system containing 0.1% TFA. The purified compound was subjected to NMR and CD analysis.

Amino acid analysis

The peptidyl resin was hydrolysed using a mixture of propionic and conc. HCl (1:1 v/v) and heated to 120°C for 6 h. The resin was removed by filtration and the solution was quantitatively transferred to a standard flask with distilled water. This solution was then diluted with buffer and applied to the amino acid analyser.

(I) Synthesis of Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu (T95–107)

This synthesis was carried out on 200 mg of Boc–Leu resin having a substitution value of 1.7 mmol/g. A 2.5 fold molar excess of Boc–amino acids were used for each coupling.

The schedule for the coupling of Boc–amino acids by DCC method is given in Table below.

Table 3.6. Schedule for the coupling of Boc amino acids by DCC method.

Operation No.	Steps involved	Reagent/Solvent	Time (min)
1	Wash	DCM x 2	1.5
2	Deprotection	30% TFA/DCM x 1	30
3	Wash	DCM x 6	1.5
4	Neutralise	5% TEA/DCM x 1	5
5	Wash	DCM x 4	1.5
6	Coupling	1:1 DCC–Boc amino acid (2.5 equiv. in NMP)	60
7	Wash	33% MeOH/DCM x 6	1.5
8	Wash	NMP x 2	1.5
9	Steps 6–8 repeated for second coupling		

A double coupling was carried out in all the cases and the completion of coupling was checked by semiquantitative ninhydrin test. In the case of Boc–Asp (CHex) and Boc–Asn a third coupling was also performed.

Cleavage and purification

The finished peptide was cleaved from the support by using TFA. 100 mg of peptidyl resin was kept in TFA, thioanisol, m–cresol and 1,2–ethanedithiol in the ratio 10:1:1:1 at room temperature for 20 h. The resin was removed by

filtration and TFA was removed from it. Peptide was precipitated by the addition of ice cold ether and the precipitate washed thoroughly with ether and dried.

The cleaved compound was subjected to hydrogenation to remove the benzyl groups. For this the peptide was dissolved in MeOH and a drop of acetic acid was added. This was stirred with palladium charcoal under hydrogen atmosphere for 48 h. It was filtered and methanol was evaporated from it. The residue was purified in HPLC using a C18 rp column using CH_3CN/H_2O containing 0.1% TFA system. The purified compound was subjected to NMR and CD analysis.

Amino acid analysis

The peptidyl resin was hydrolysed using a mixture of propionic acid and conc. HCl (1:1 v/v) and heated to 120°C for 6 h. The resin was removed by filtration and the solution was quantitatively transferred to a standard flask with distilled water. This solution was then diluted with buffer and applied to the amino acid analyser.

3.2.18 Synthesis of hairpin peptides

(a) Synthesis of Glu–Val–Lys–Val–Dpro–Gly–Val–Glu–Val–Lys

Boc–Lys (2ClZ) was attached to the chloromethyl resin by cesium salt method and the amino capacity was found to be 0.8 mmol/g. The remaining amino acids were attached to this resin (200 mg) by DCC method. Boc–Val and Boc–Dpro were prepared by Boc–ON method. All the coupling reactions were done by the HOBt active ester method and NMP was the solvent system used. A three–fold molar excess of amino acids were used to ensure complete coupling. The steps used for the synthesis are given below.

1. Wash the resin with DCM (10 ml, 2 x 1.5 min).
2. Deblocking using 30% TFA/DCM (10 ml x 30 min).
3. Wash with DCM (10 ml, 6 x 1.5 min).
4. Prewash using 5% TEA/DCM (10 ml x 1.5 min)
5. Neutralisation using 5% TEA/DCM (10 ml x 10 min).
6. Wash with DCM (10 ml, 4 x 1.5 min).
7. Wash with NMP (10 ml, 2 x 1.5 min).
8. Coupling with 1:1:1 DCC–Boc amino acid, HOBt in NMP (1 x 60 min).
9. DCU washed off with 33% MeOH/DCM (10 ml, 3 x 1.5 min).
10. DCM wash (10 ml, 2 x 1.5 min).
11. NMP wash (10 ml, 2 x 1.5 min).
12. Kaiser test to check the extent of coupling.
13. Steps 8–11 repeated to ensure completion of the reactions.

Most of the couplings were completed by the first coupling itself and a double coupling was carried out in all the cases. After the attachment of all the amino acids the peptidyl resin was washed with DCM and dried. Final weight of the peptidyl resin = 280 mg.

Cleavage and purification

Final cleavage of finished peptide from the resin was carried out using 5% aqueous TFA. 100 mg of peptidyl resin was suspended in TFA (9.5 ml) and water (0.5 ml) for 12 h. The resin was filtered off and TFA was evaporated from the solution. The peptide was precipitated by the addition of ice cold ether and washed thoroughly with ether. The white powder obtained was subjected to

hydrogenation. For this the crude peptide was dissolved in MeOH containing little acetic acid. After adding palladium charcoal the reaction mixture was stirred in hydrogen atmosphere for 24 h. It was filtered and MeOH evaporated from it. The hydrogenated peptide was obtained in >95% purity. This was further purified by HPLC. The pure peptide was subjected to NMR analysis.

Acm *Acm*

(b) Synthesis of Ala–Cys–Val–Leu–Val–Dpro–Gly–Val–Leu–Val–Cys–Ala

This peptide was synthesised on a 2% chloromethylated PS–HDODA resin having a chlorine capacity 1.91 mmol/g. Boc–Ala was attached to this by cesium salt method and the substitution level was found to be 1.8 mmol/g. 150 mg of Boc–Ala resin was used for this synthesis. Boc–Val and Boc–Dpro were synthesised by Boc–ON method and all other simple amino acids were synthesised by Schnabel's method. Boc–Cys was the side chain protected amino acid used. HOBt active ester method was used for coupling and NMP was the solvent system. The protocol followed for the synthesis is shown below.

Kaiser test was carried out to check the coupling. Steps 8–11 repeated for second coupling. Double coupling was carried out in all the cases to ensure complete coupling. After the attachment of all the residues the peptide resin was washed thoroughly with DCM and dried.

Table 3.7. Protocol used for the above synthesis.

Operation No.	Steps involved	Reagent/Solvent	Time (min)
1	Wash the resin	DCM	2 x 3
2	Boc–deprotection	30% TFA/DCM	1 x 30
3	Wash	DCM	6 x 1.5
4	Prewash	5% TEA/DCM	10 ml x 1.5
5	Neutralisation	5% TEA/DCM	10 ml x 10
6	Wash	DCM	4 x 1.5
7	Wash	NMP	2 x 1.5
8	Coupling	1:1:1 DCC–Boc–amino acid–HOBt (3 equiv. each in NMP	60
9	Wash	33% MeOH/DCM	3 x 1.5
10	Wash	DCM	4 x 1.5
11	Wash	NMP	2 x 1.5

Cleavage and purification

The 12 residue peptide was cleaved from the support by 5% aqueous TFA. 100 mg of peptidyl resin was suspended in 9.5 ml TFA and 0.5 ml H_2O. The crude peptide was obtained in high purity with the Acm group on cysteine. This was further purified on HPLC using CH_3CN/H_2O containing 0.1% TFA. The pure peptide was subjected to conformational analysis.

CHAPTER 4

RESULTS AND DISCUSSION

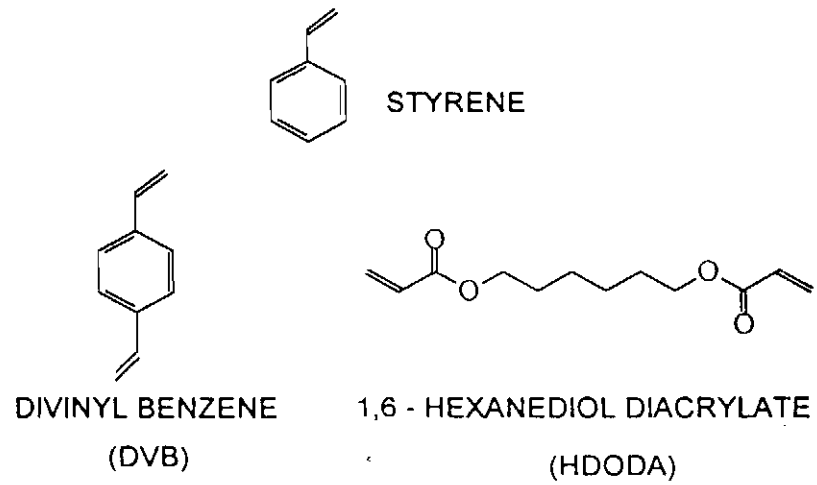
4.1. Preparation of polymer supports and functionalisation

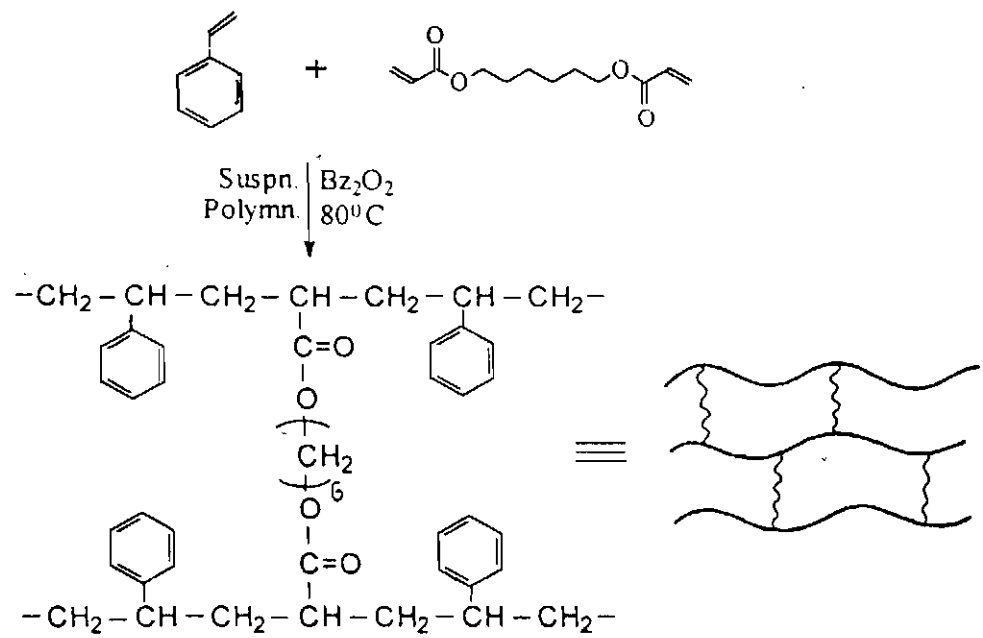
4.1.1 Preparation of polystyrene–crosslinked hexanediol diacrylate supports for solid phase peptide synthesis

Design and development of an effective polymeric support with optimum hydrophobic–hydrophilic balance for multistep peptide synthesis has been a challenge to the peptide chemists for the last three decades. Polystyrene crosslinked with 1,6–hexanediol diacrylate has been developed in our laboratory as a new system for solid phase peptide synthesis. A set of hydrophobic peptides and two designed β–hairpin peptides were synthesised using this newly developed support.

4.1.2 Polymer synthesis

HDODA–crosslinked polystyrene resins of varying crosslink densities were prepared by suspension copolymerisation of the monomer in the presence of an inert diluent, toluene at 80°C. Polymerisation was carried out in polyvinylalcohol medium and benzoyl peroxide was used as the initiator. Polymers of varying crosslink densities were prepared by adjusting monomer ratio.





Scheme 4.1 Preparation of HDODA–crosslinked polystyrene by suspension polymerisation.

Table 4.1. Preparation of HDODA–crosslinked polystyrene.

HDODA (mol%)	Amount of monomers		Yield (g)
	Styrene (ml)	HDODA (ml)	
1	11.34	0.22	8.7
2	11.20	0.40	9.8
3	11.11	0.67	9.2
4	11.00	0.90	8.3
5	10.88	1.12	9.5

These resins were characterised by IR (Figure 4.1), solid state ^{13}C CP/MAS NMR spectroscopy (Figure 4.2) and SEM (Figure 4.3). IR (KBr) 1720 cm^{-1} (ester carbonyl), 3015 cm^{-1} (aromatic CH). Solid state ^{13}C CP/MAS NMR spectrum exhibited an intense peak at 128.33 ppm (C_b) corresponding to aromatic polystyrene carbons and a small peak at 146.6 ppm arising from the styrene carbon (C_a). The backbone carbons of the polymer gave peaks at 24.78 and 40.83 ppm (C_c, C_d). The peat at 180.04 ppm corresponding to ester carbonyl (C_e) and at 76.21 ppm for the CH_2 attached to the oxygen of HDODA (C_f). Scanning electron micrograph revealed the smooth and porous nature of the polymer.

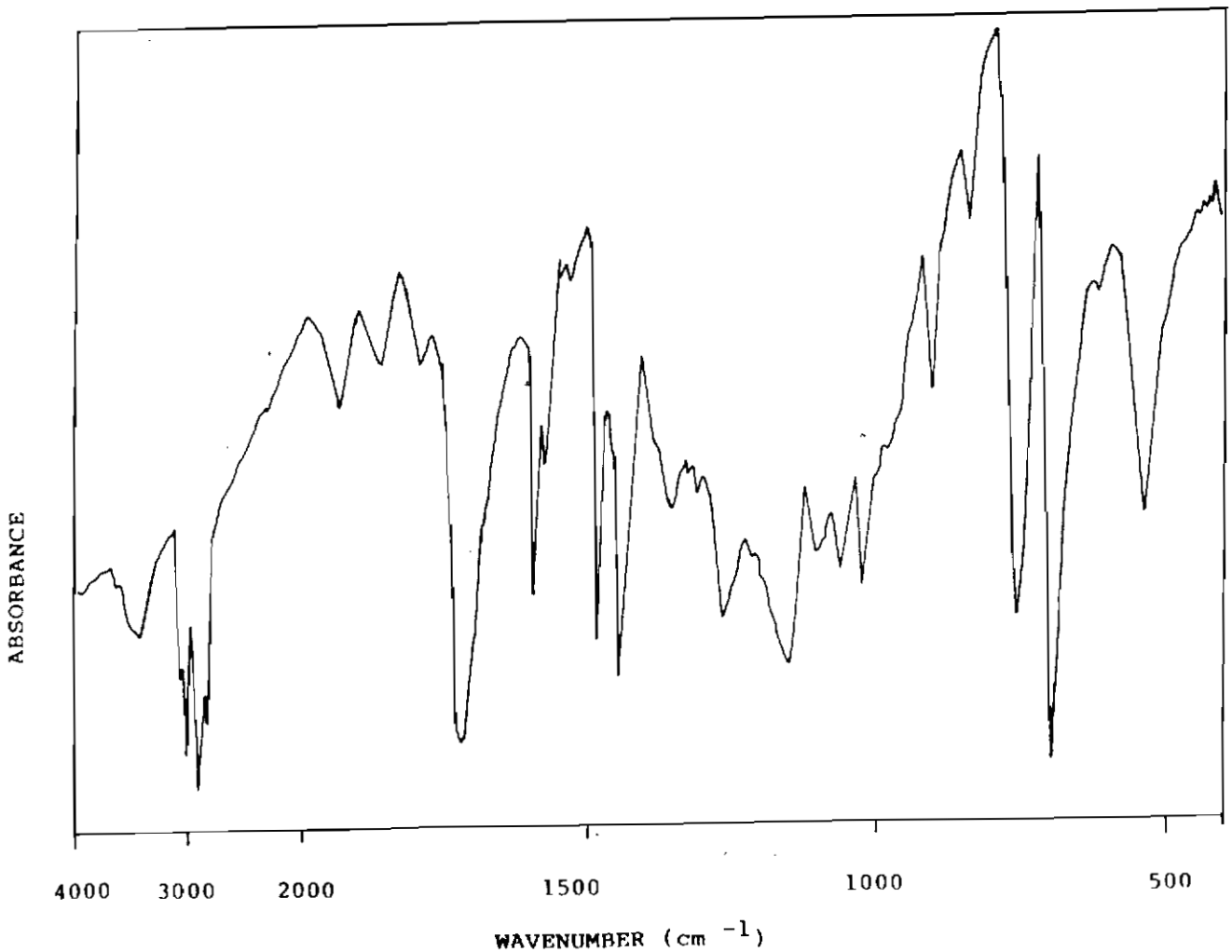


Figure 4.1. IR spectrum of PS-HDODA resin.

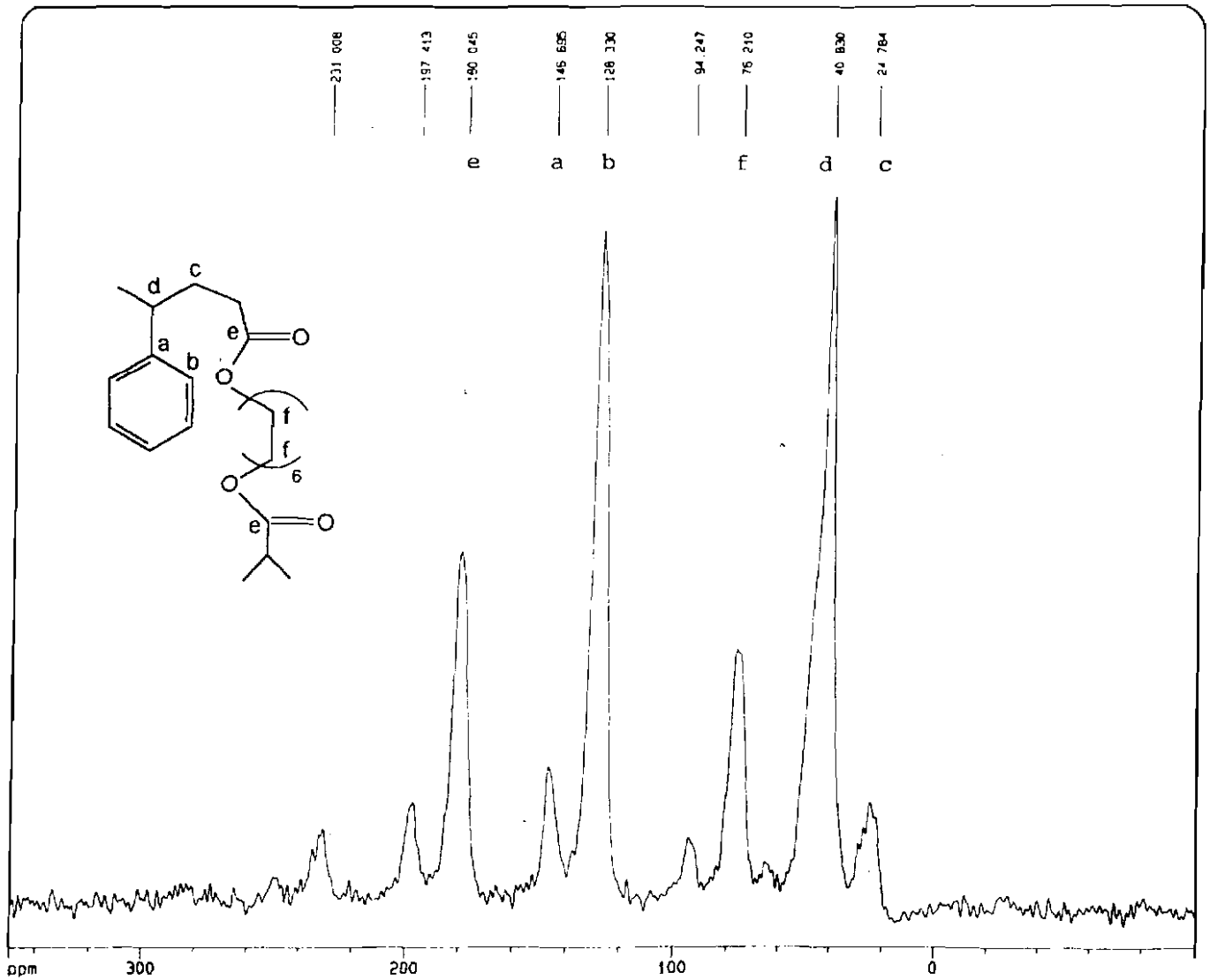


Figure 4.2. ^{13}C-NMR of PS-HDODA resin.

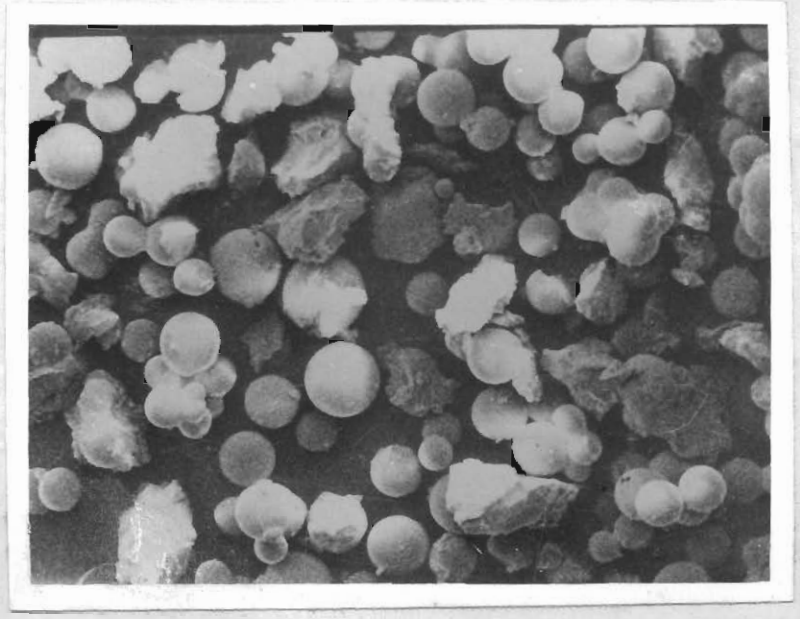
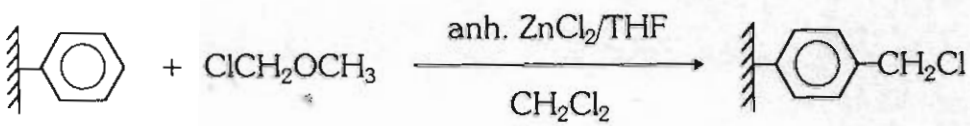


Figure 4.3. SEM of PS–HDODA resin beads.

4.1.3 Functionalisation of HDODA–crosslinked polystyrene by chloromethylation

Chloromethyl groups were introduced into the crosslinked polystyrene using anhydrous $ZnCl_2$ in THF and chloromethyl methylether.



Scheme 4.2. Chloromethylation of HDODA–crosslinked polystyrene resin.

Friedel–Crafts chloromethylation has been used to introduce chloromethyl functional group into the resin. Capacity of the resin can be varied by adjusting the reaction conditions such as time and temperature. 2% crosslinked resin was chloromethylated at different conditions. The chloromethylated resin was characterised by IR (Figure 4.4) and ^{13}C CP/MAS NMR spectroscopy. IR (KBr) 680 cm^{-1} (C–Cl str.). ^{13}C spectrum showed peak at 46.09 ppm corresponding to C_g. This is a clear indication of chloromethylation on benzene ring. The spectrum showed other peaks that corresponding to the crosslinker and polymer backbone (Figure 4.5).

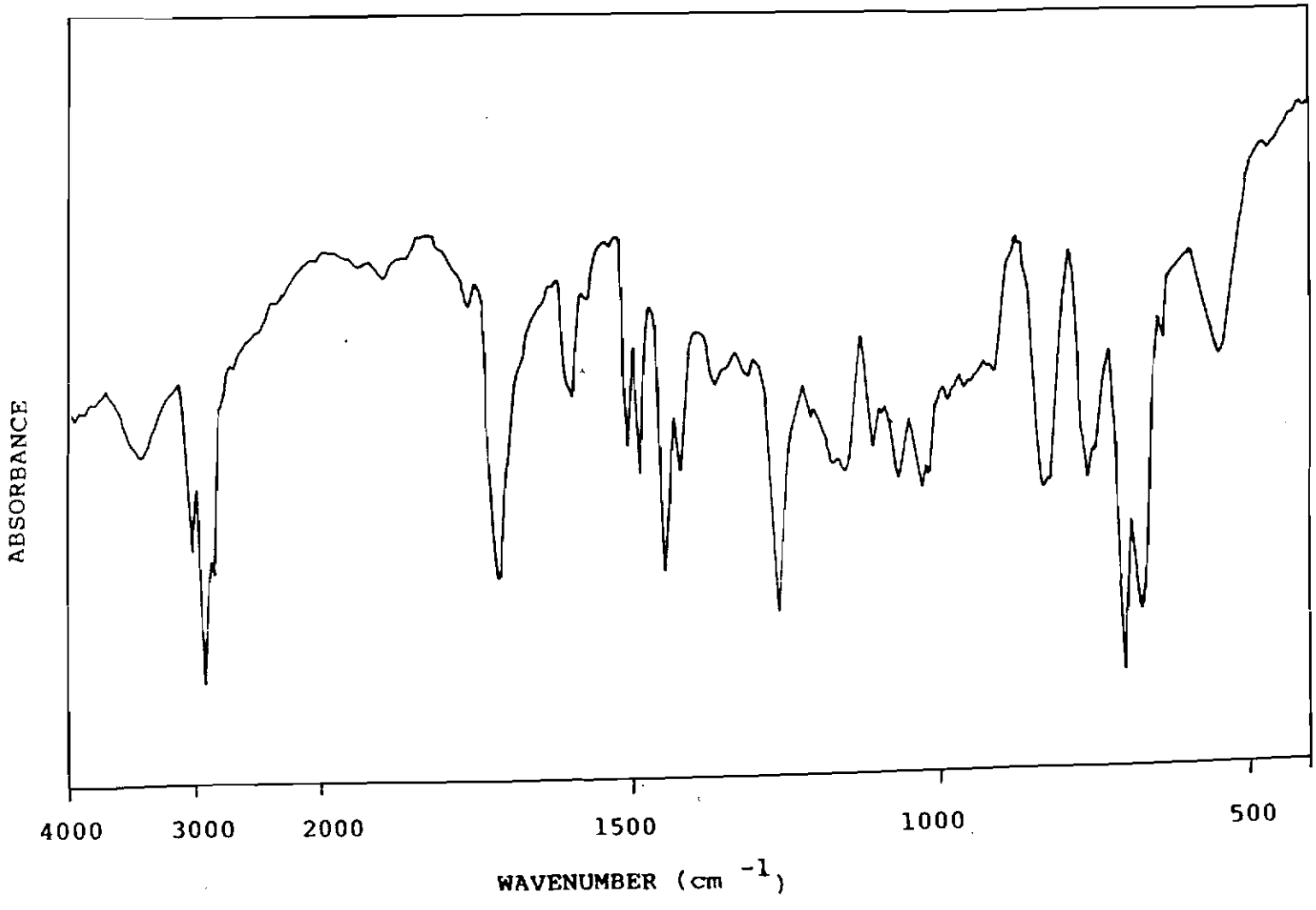


Figure 4.4. IR spectrum of chloromethylated PS-HDODA resin.

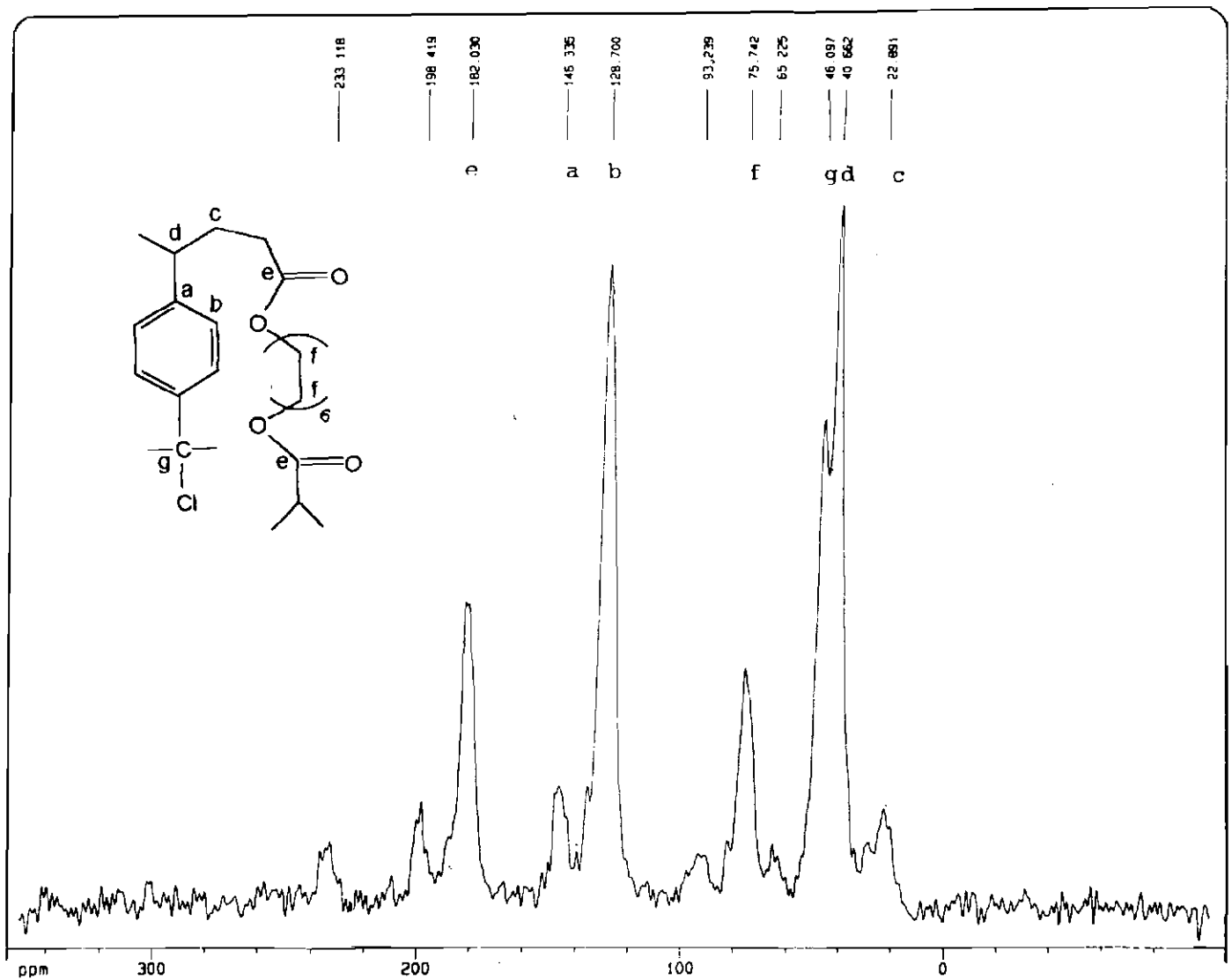


Figure 4.5. ^{13}C-NMR of chloromethylated PS-HDODA resin.

4.1.4 Functional group analysis

Several methods have been developed for the analysis of functional groups in polymers. The chlorine capacity values of the chloromethyl resins were determined titrimetrically by modified Volhard's method is shown in Table 4.2.

Table 4.2. Chlorine Capacities of 2% HDODA-crosslinked poly(styrene)s.

Time (h)	Temperature (°C)	Wt. of resin after functionalisation* (g)	Chlorine capacity (mmol/g)
4	40	2.10	1.10
5	50	2.30	1.91
5	50	2.05	2.01

* In all the cases, 2 g of resin was used for functionalisation.

4.2. Peptide synthesis

4.2.1 Synthesis of thioredoxin partial sequences using HDODA–crosslinked polystyrene support.

The present work deals with the synthesis and characterisation studies of some of the partial sequences of thioredoxin (T). Thioredoxin contains 108 amino acid residues and it is rich in secondary structures. Most of the sequences are hydrophobic in nature. The following sequences were synthesised on a 2% crosslinked PS–HDODA support.

1. H–Asp–Lys–Ile–Ile–His–Leu–Thr–OH (T2–8)
2. H–Ser–Phe–Asp–Thr–Asp–Leu–Val–Lys–OH (T11–18)
3. H–Ala–Ile–Leu–Val–Asp–Phe–Trp–Ala–OH (T22–29)
4. H–Met–Ile–Ala–Pro–Ile–Leu–Asp–Glu–Ile–Ala–Asp–Glu–Tyr–Gln–Gly–Lys–OH (T37–52)

5. H–Leu–Thr–Val–Ala–Lys–Leu–OH (T53–58)

6. H–Asn–Ile–Asp–Gln–Asn–Pro–Gly–Thr–Ala–OH (T59–67)

7. H–Pro–Lys–Tyr–Ile–Gly–OH (T68–72)

8. H–Ile–Gly–Arg–Gly–Ile–Pro–Thr–Leu–Leu–Leu–Phe–OH (T71–81)

9. H–Thr–Leu–Leu–Leu–Phe–OH (T77–81)

10. H–Ala–Thr–Lys–Val–OH (T88–91)

11. H–Gly–Ala–Leu–Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu–OH (T92–107)

12. H–Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu–OH (T95–107)

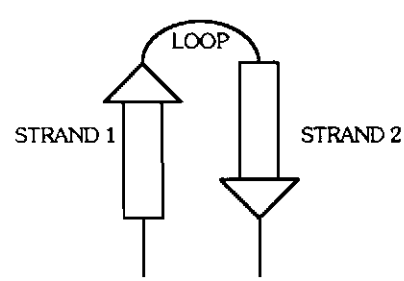
These peptides were synthesised by DCC activation method. Crude peptides were purified by high performance liquid chromatography (HPLC) using reverse phase columns. Amino acid analyses were done after hydrolysing the samples with 6N HCl for 22 h. The purified peptides were subjected to conformational investigations by NMR and CD techniques.

Besides these partial sequences, two designed β–hairpin peptides were also synthesised on the same resin. The sequences are,

1. Glu–Val–Lys–Val–Dpro–Gly–Val–Glu–Val–Lys.

2. Ala–Cys(Acm)–Val–Leu–Val–Dpro–Gly–Val–Leu–Val–Cys(Acm)–Ala.

A β–hairpin is a two–stranded segment of antiparallel β–sheet in which the strands are connected by a loop. Employing small peptides to study sheet structure has proven difficult because of



their propensity to self-associate into large, generally insoluble, quaternary β–sheet structures with a few exceptions. So it is difficult to observe isolated β–hairpins. So we are interested in β–hairpin formation by a designed peptide. These secondary structure elements act as initiation sites for folding.

The synthesised peptides were further purified by HPLC and subjected to NMR and CD analysis.

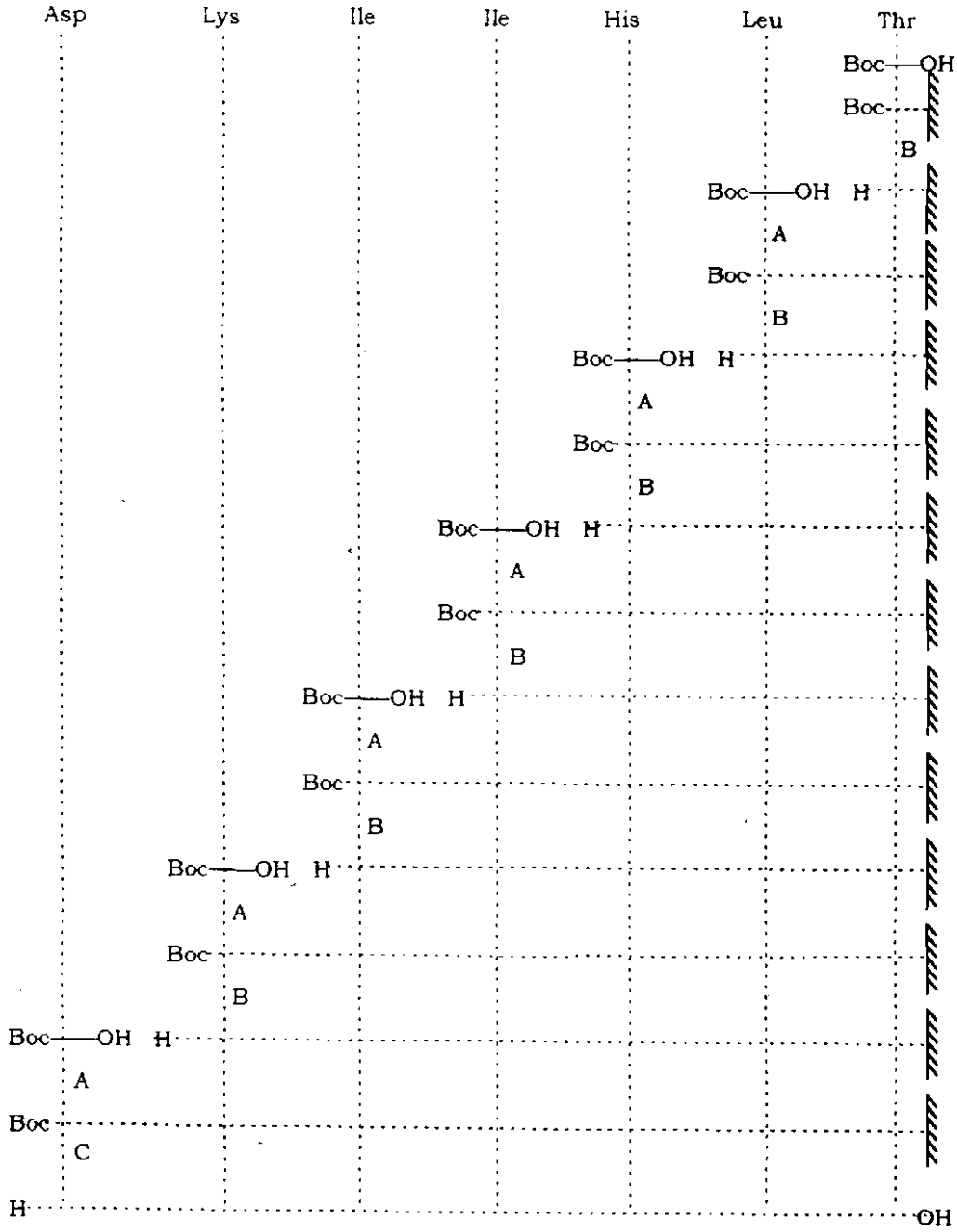
The synthesis methodology and conformational studies of each of the sequences are described below.

4.2.2 Synthesis of thioredoxin partial sequences

(a) Asp–Lys–Ile–Ile–His–Leu–Thr (T2-8)

The first amino acid Boc–Thr was esterified to the 2% chloromethylated resin by TEA method and the substitution level of Boc–amino acid was found to be 1 mmol/g. The Boc protecting group was then removed by treating with 30% TFA/DCM followed by neutralisation with 5% TEA/DCM. It was then washed thoroughly with DCM and tested for the presence of free amino groups by ninhydrin. The second amino acid Boc–Leu was then coupled to the aminoacyl resin in NMP at room temperature in the presence of DCC as coupling agent. 33% MeOH/DCM was used to remove the precipitated DCU. Subsequent amino acids were assembled following the same procedure. The final peptide was cleaved from the resin support using neat TFA in the presence of thioanisol. The peptide was precipitated by ice–cold ether and isolated. Hydrogenation gave peptide free from side chain protecting groups.

The synthetic procedure is represented in Scheme 4.3.



Scheme 4.3. Solid phase synthesis of Asp–Lys–Ile–Ile–His–Leu–Thr on a 2% HDODA–PS chloromethyl resin.

A – DCC coupling

B – Boc deprotection using 30% TFA/DCM followed by neutralisation with 5% TEA/DCM

C – Cleavage of peptide.

The purity of hydrogenated peptide was checked on an analytical HPLC and was found to be >90% pure. (HPLC trace is shown in Figure 4.6). NMR spectrum was recorded in DMSO (Figure 4.7) and circular dichroism spectra was recorded in MeOH and TFE (Figure 4.8). The peptide showed negative bands at 203 and 222 nm in TFE, a well known helix promoter,[231] indicating the formation of a helix peptide.

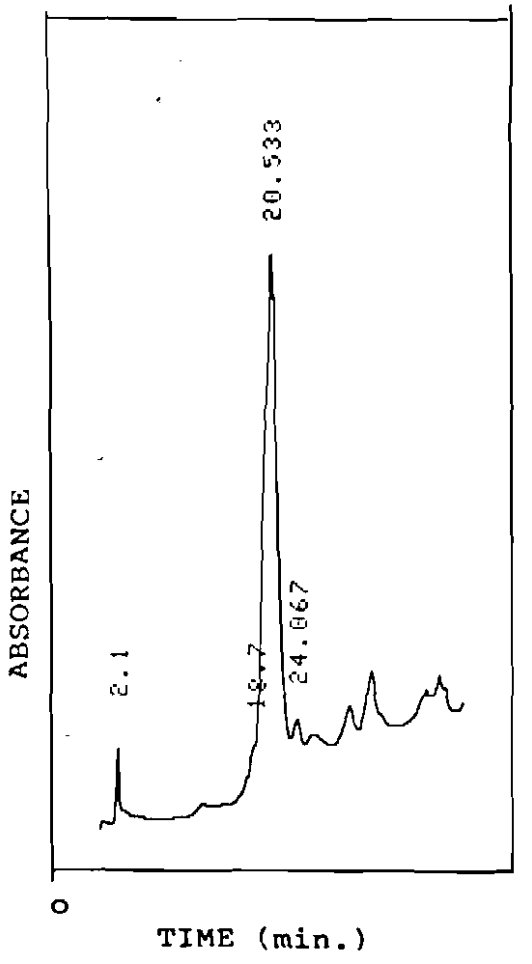


Figure 4.6. HPLC trace of crude peptide Asp-Lys-Ile-Ile-His-Leu-Thr. Solvent system: (a) CH_3CN/H_2O containing 0.1% TFA, (b) H_2O containing 0.1% TFA. Gradient used: 5 to 45% CH_3CN/H_2O in 40 min

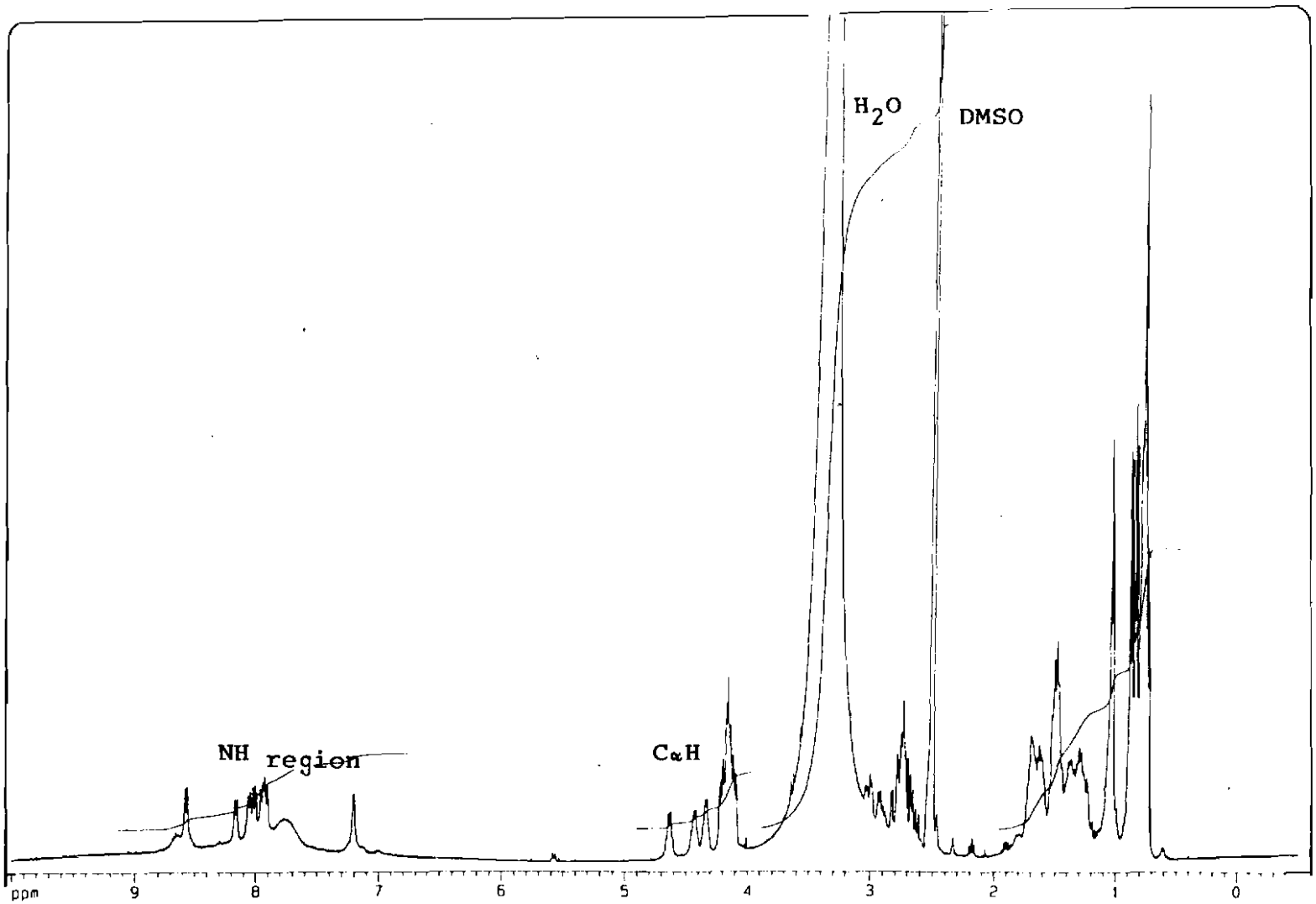


Figure 4.7. NMR spectrum of Asp-Lys-Ile-Ile-His-Leu-Thr in DMSO at 400 MHz.

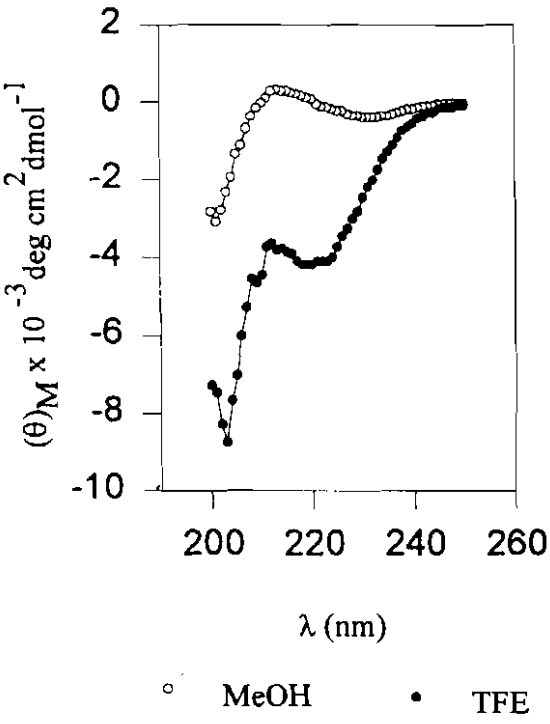
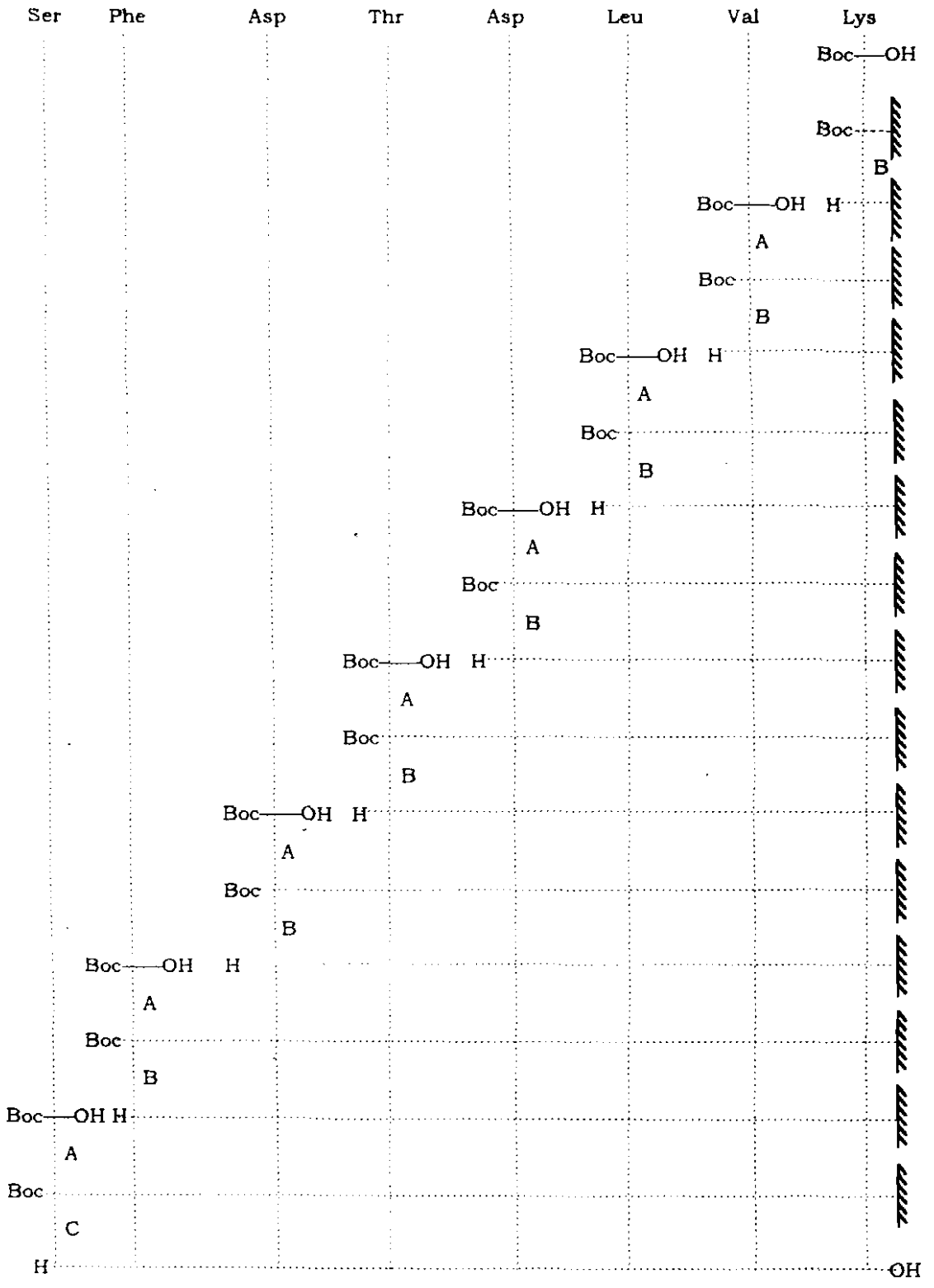


Figure 4.8. CD spectra of Asp-Lys-Ile-Ile-His-Leu-Thr.

(b) Synthesis of Ser–Phe–Asp–Thr–Asp–Leu–Val–Lys (T11-18)

Boc–Lys was attached to the chloromethyl resin by Gisin's cesium salt method. Amino capacity was found to be 0.8 mmol/g. The next amino acid Boc–Val was coupled to the aminoacyl resin, obtained after deprotection and neutralisation, using DCC in DCM. After removal of the precipitated DCU a second coupling was done to ensure completion of reaction. The remaining amino acids were similarly attached. When the assembly of amino acids were over, the peptide was cleaved from the support using neat TFA in presence of m–cresol and thioanisol. The synthetic protocol is depicted in Scheme 4.4.



Scheme 4.4. Solid phase synthesis of Ser–Phe–Asp–Thr–Asp–Leu–Val–Lys on a 2% chloromethylated HDODA–PS resin.

A – DCC coupling

B – Boc deprotection using 30% TFA/DCM followed by neutralisation with 5% TEA/DCM

C – Cleavage from the resin using TFA.

The peptide was hydrogenated using 5% palladium charcoal. The hydrogenated peptide was checked on HPLC using CH_3CN/H_2O system containing 0.1% TFA and was found to be >95% pure. The HPLC profile is shown in Figure 4.9. CD spectra of the peptide was recorded in MeOH and TFE (Figure 4.10). In both cases, the CD spectra showed negative bands at 204 and 222 nm indicating the formation of helix. In the original structure also this fragment is showing a helix structure.

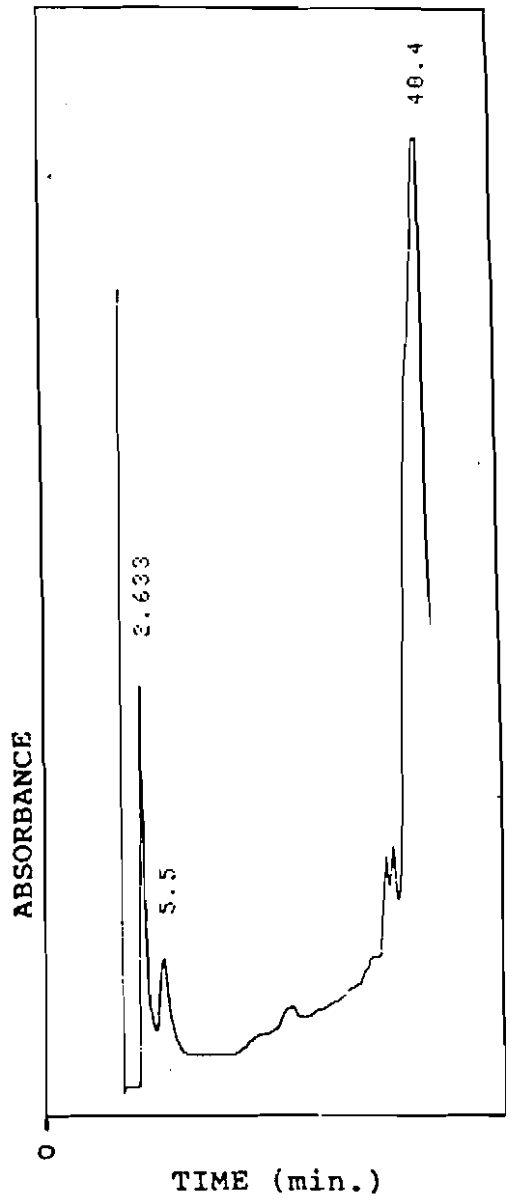


Figure 4.9. HPLC profile of crude peptide Ser-Phe-Asp-Thr-Asp-Leu-Val-Lys. Gradient used: 5 to 45% in 40 min CH_3CN/H_2O containing 0.1% TFA.

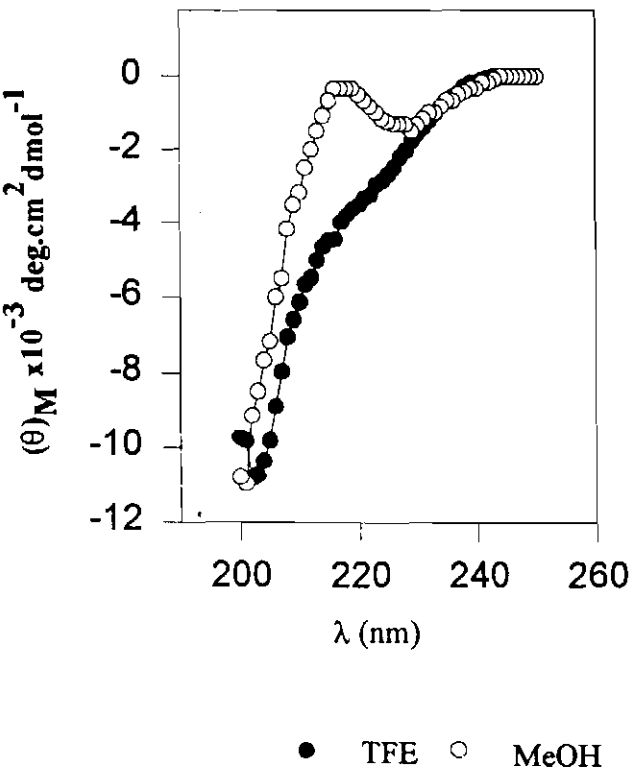
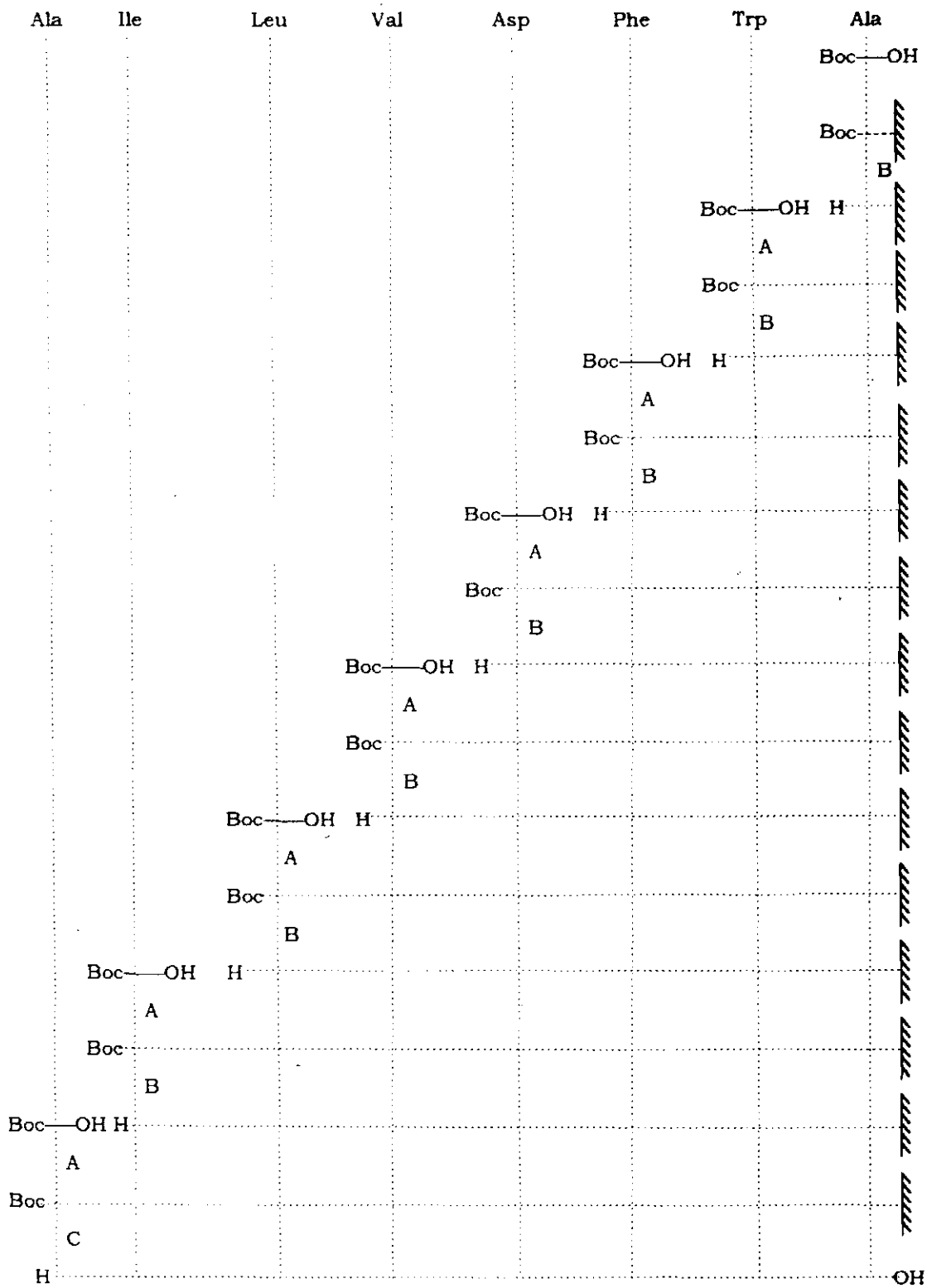


Figure 4.10. CD spectra of Ser-Phe-Asp-Thr-Asp-Leu-Val-Lys.

(c) Synthesis of Ala–Ile–Leu–Val–Asp–Phe–Trp–Ala (T22-29)

Chloromethylated 2% HDODA–PS resin with a chlorine capacity of 1.91 mmol/g was used for the synthesis of this peptide. The amino capacity was found to be 1.8 mmol/g. DCC coupling was used for the attachment of the next seven amino acids. NMP was used as the solvent. In all the cases a double coupling was performed to ensure complete coupling.

The finished peptide was cleaved off from the support by TFA/thioanisol method. The synthetic protocol is outlined in Scheme 4.5.



Scheme 4.5. Solid phase synthesis of Ala–Ile–Leu–Val–Asp–Phe–Trp–Ala.
A – Coupling mediated by DCC.
B – Removal of Boc group using 30% TFA/DCM and neutralisation using 5% TEA/DCM.
C – Cleavage from the resin using TFA.

The FPLC profile of the peptide showed two peaks (Figure 4.11). The major peak was collected and identified as the right compound by amino acid analysis. Ala 2.2 (2.0), Ile 0.9 (1.0), Leu 0.8 (1.0), Val 1.0 (1.0), Asp 1.2 (1.0), Phe 1.1 (1.0). Trp was lost during hydrolysis.

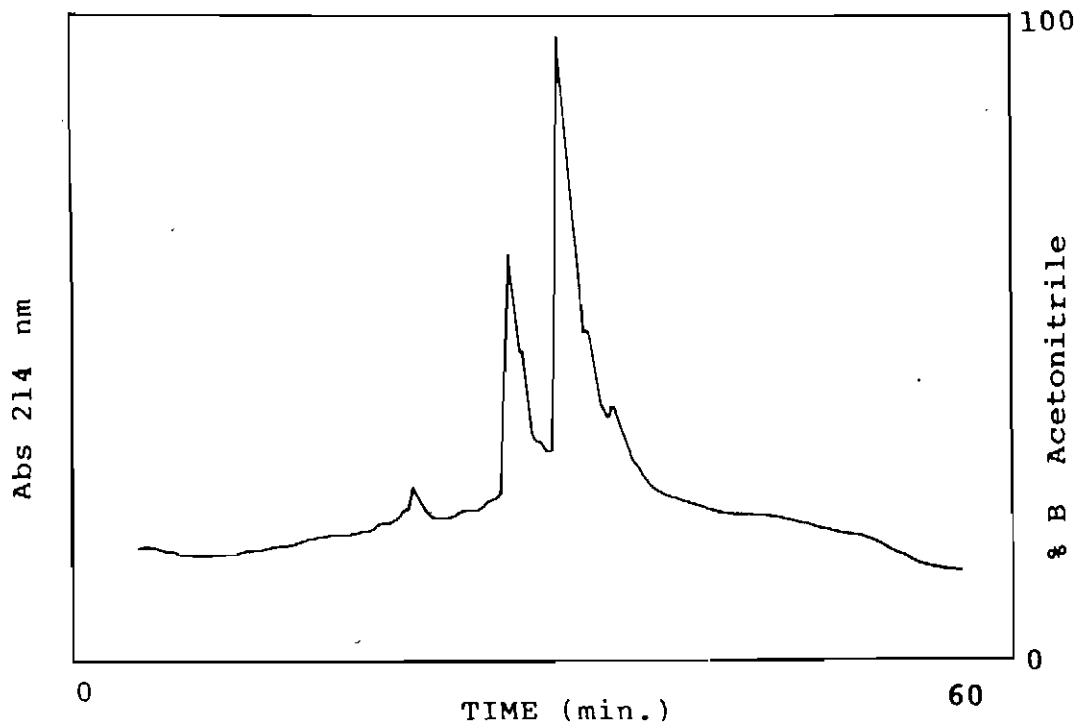
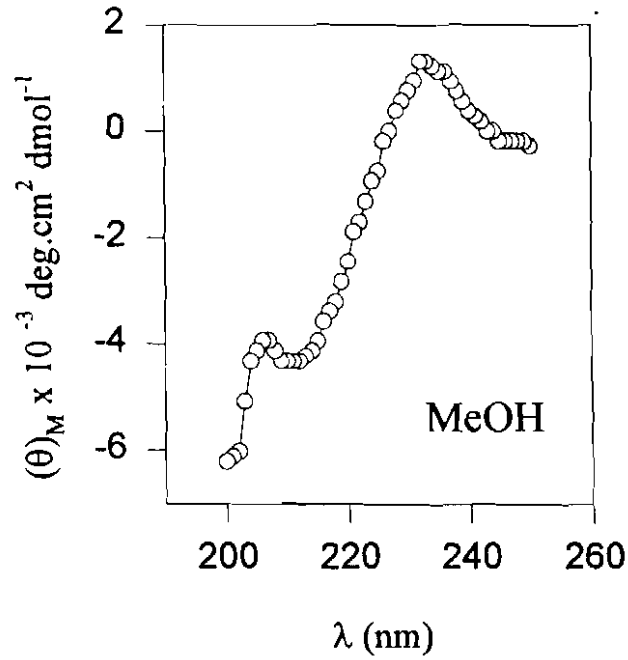


Figure 4.11. FPLC profile of Ala-Ile-Leu-Val-Asp-Phe-Trp-Ala.
Conditions: Solvent A water containing 0.1% TFA; solvent B acetonitrile containing 0.1% TFA; flow rate 0.5 ml/min; detection 214 nm.

The peptide was subjected to CD analysis. CD spectrum was recorded in MeOH and TFE (Figure 4.12). CD showed negative band at 214–216 nm in TFE, which is a characteristic of β–sheet peptide. But in MeOH it is not showing β–sheet structure. But on addition of TFE, the β–structure is regained.



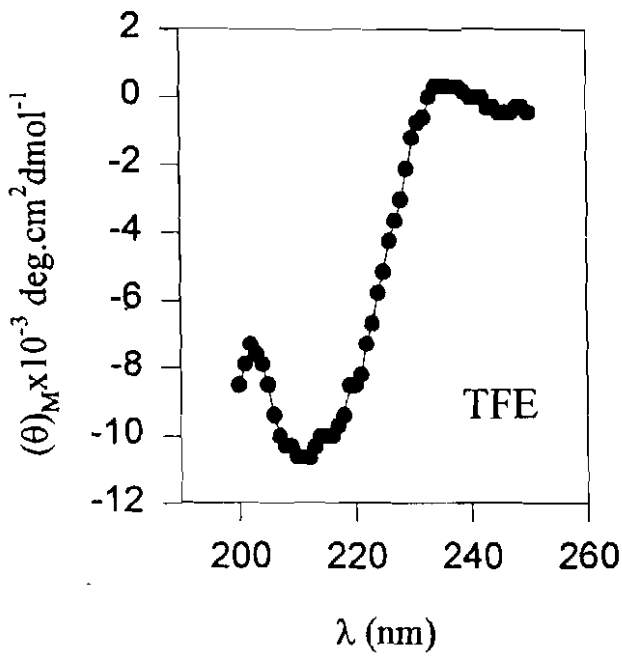


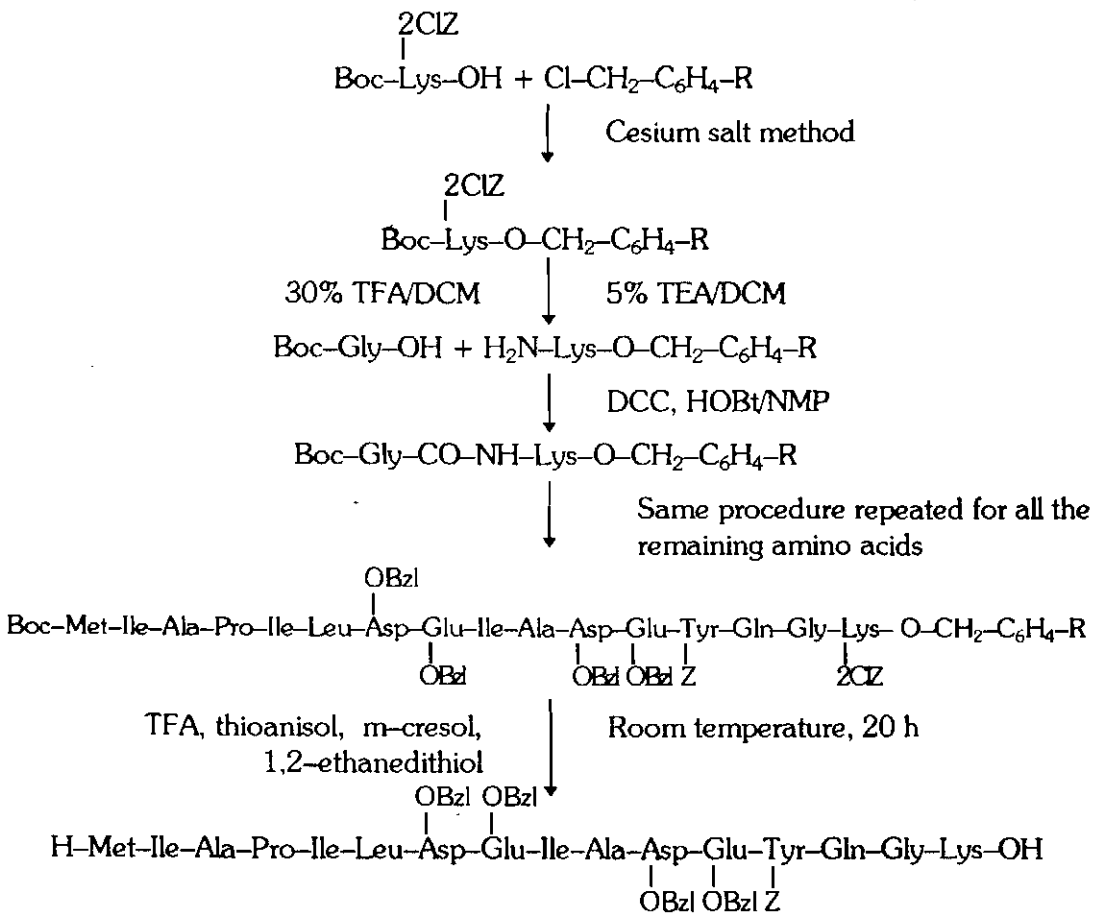
Figure 4.12. CD spectra of Ala-Ile-Leu-Val-Asp-Phe-Trp-Ala.

(d) Synthesis of Met–Ile–Ala–Pro–Ile–Leu–Asp–Glu–Ile–Ala–Asp–Glu–Tyr–Gln–Gly–Lys (T37-52)

Boc–Lys (2ClZ) was anchored on to the chloromethyl resin by cesium salt method and the amino capacity was found to be 0.8 mmol/g. The remaining

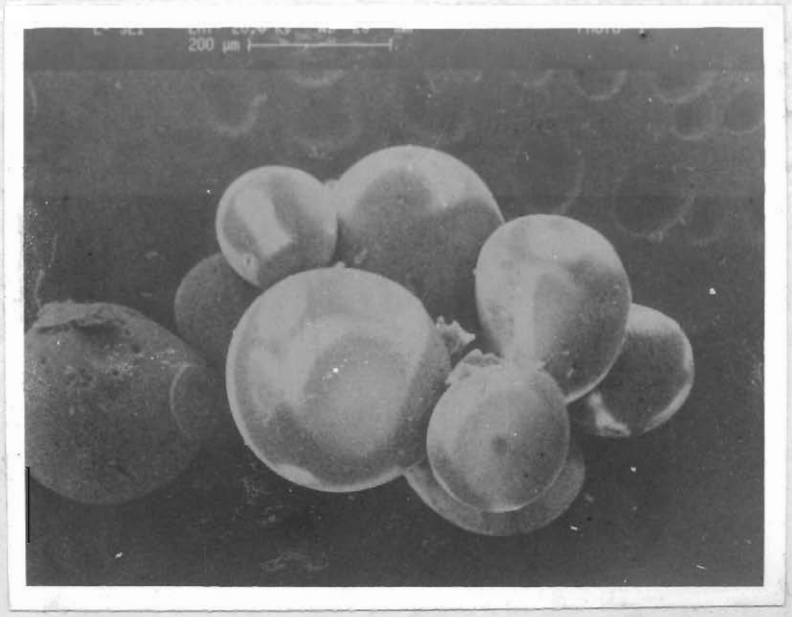
amino acids were attached to this by HOBt active ester method. HOBt active ester was prepared by mixing DCC, HOBt and amino acid in 1:1:1 ratio in NMP and the by-product DCU obtained was filtered off. This active ester was added to the free aminoacyl resin, obtained after deprotection and neutralisation. Coupling was allowed to proceed for 45 min. In all the cases a double coupling was carried out to ensure complete coupling.

After the attachment of the next amino acid, the peptidyl resin was washed with 33% MeOH/DCM to remove any remaining DCU and finally washed with DCM. The same procedure was repeated for the attachment of all the fifteen amino acids. The coupling time was increased by 5 min for each amino acid. An outline of the synthesis procedure is shown in Scheme 4.6.

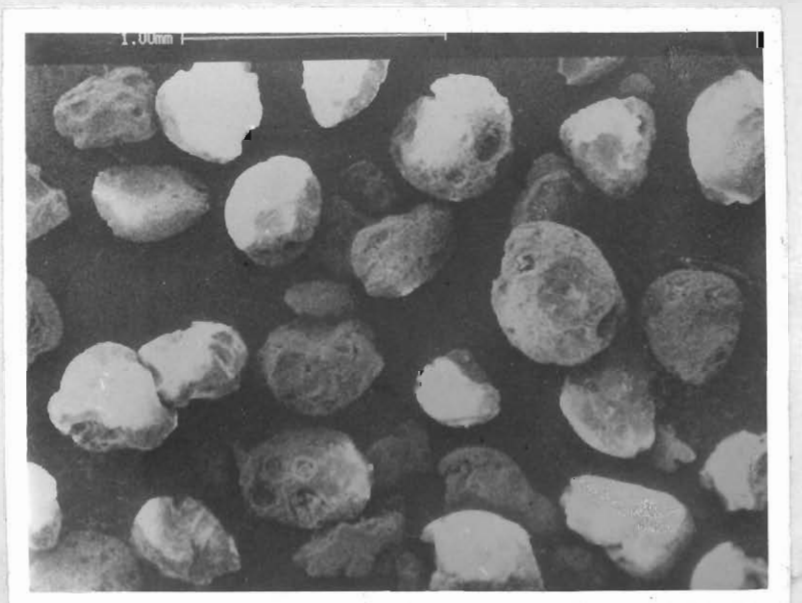


Scheme 4.6. Solid phase synthesis of Met–Ile–Ala–Pro–Ile–Leu–Asp–Glu–Ile–Ala–Asp–Glu–Tyr–Gln–Gly–Lys.

The scanning electron micrograph of (a) the chloromethylated resin and (b) the peptide bearing resin are shown in Figure 4.13. The micrographs reveal the significant changes in the surface characteristics brought about by the attachment of the peptide chain. The smooth resin surface became rough after the synthesis.



(a)



(b)

Figure 4.13. The scanning electron micrographs of (a) the chloromethylated resin and (b) the peptide bearing resin.

The cleaved peptide was checked on HPLC (Figure 4.14) using the solvent system CH_3CN/H_2O containing 0.1% TFA and H_2O containing 0.1% TFA. It is clear from the HPLC profile that the compound is >90% pure.

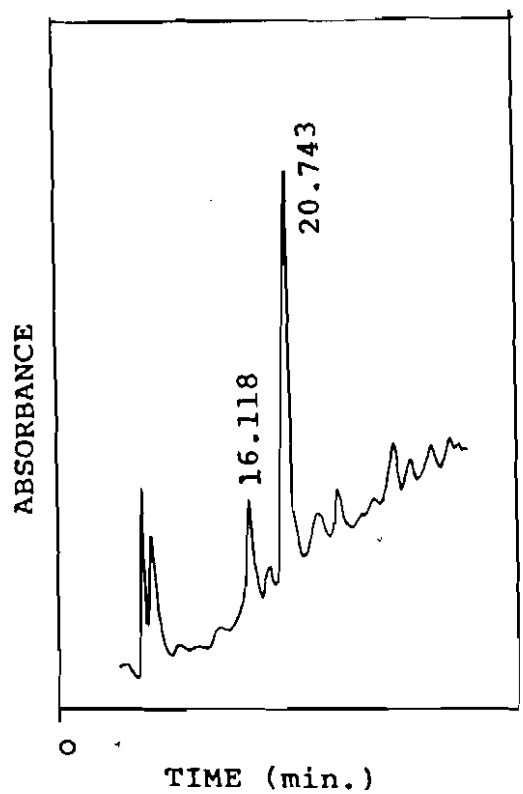


Figure 4.14. HPLC profile of crude Met–Ile–Ala–Pro–Ile–Leu–Asp–Glu–Ile–Ala–Asp–Glu–Tyr–Gln–Gly–Lys. Gradient used: 5 to 45% CH_3CN/H_2O in 40 min

The 16–residue peptide was subjected to NMR and CD analysis. NMR spectrum was recorded in DMSO and the peaks were found to be merging (Figure 4.15). CD spectrum was recorded in MeOH and TFE (Figure 4.16). In both cases, the spectra are showing the formation of a helix. The isolated peptide corresponding to 37-52 region of thioredoxin is showing the same conformation as that of it in the complete structure.

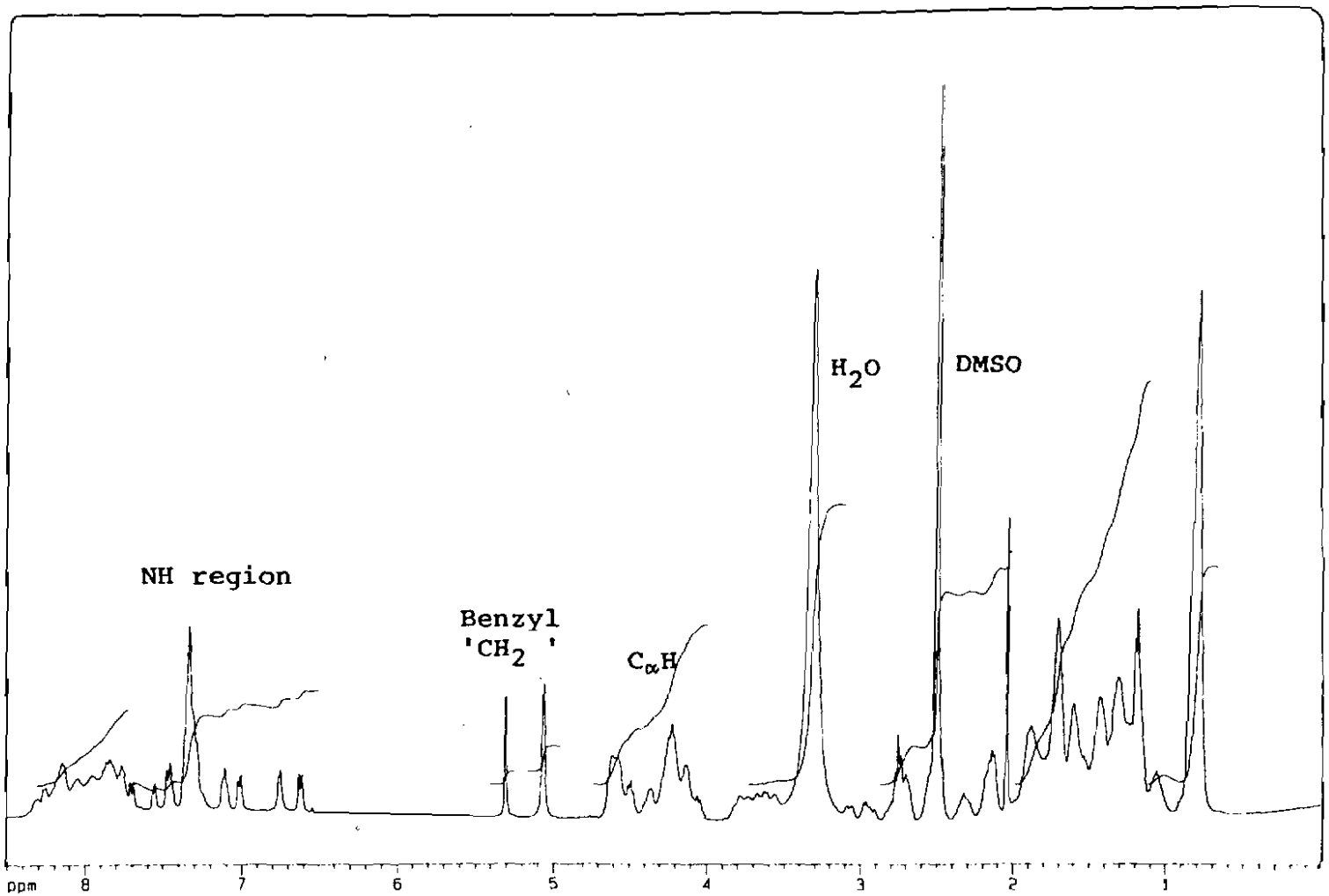


Figure 4.15. NMR spectrum of Met–Ile–Ala–Pro–Ile–Leu–Asp–Glu–Ile–Ala–Asp–Glu–Tyr–Gln–Gly–Lys in DMSO at 400 MHz.

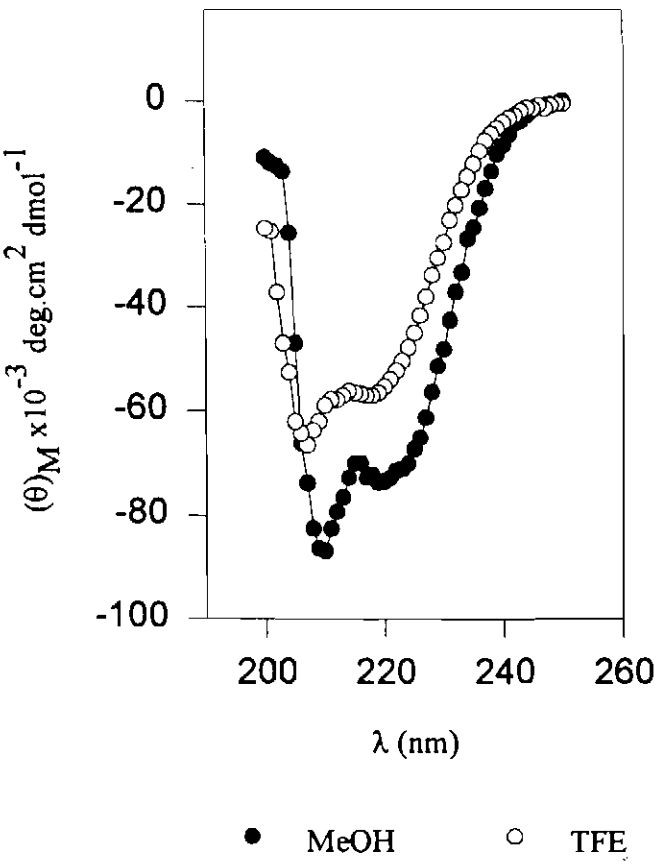
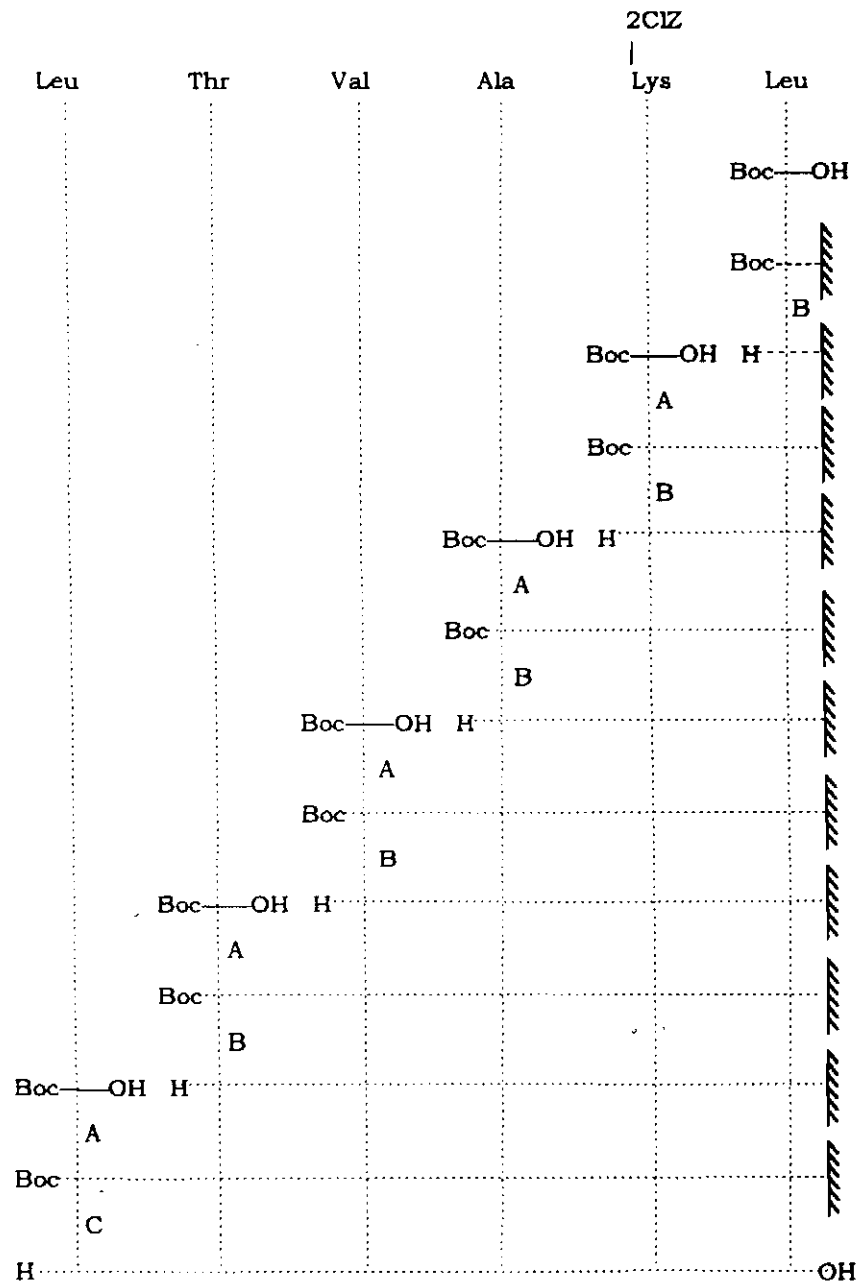


Figure 4.16. CD spectra of Met–Ile–Ala–Pro–Ile–Leu–Asp–Glu–Ile–Ala–Asp–Glu–Tyr–Gln–Gly–Lys.

(e) Synthesis of Leu–Thr–Val–Ala–Lys–Leu (T53-58)

Synthesis of this hexapeptide was carried out on a 2% HDODA-crosslinked polystyrene resin. Boc–Leu was anchored on this resin by cesium salt method and the amino capacity was found out by picric acid method (1.7 mmol/g). The remaining amino acids were coupled to the Boc–Leu resin by the standard solid phase strategy. After the attachment of all the amino acids, the peptidyl resin was washed with DCM and dried. The final peptide was cleaved from the resin by TFA/thioanisol method. The crude peptide was checked on FPLC and a single peak was obtained. The protocol used for the above synthesis (Scheme 4.7) and the FPLC diagram (Figure 4.17) are given below.



Scheme 4.7. Solid phase synthesis of Leu–Thr–Val–Ala–Lys–Leu

A – DCC coupling

B – Boc group removal by 30% TFA/DCM and neutralisation using 5% TEA/DCM.

C – TFA cleavage.

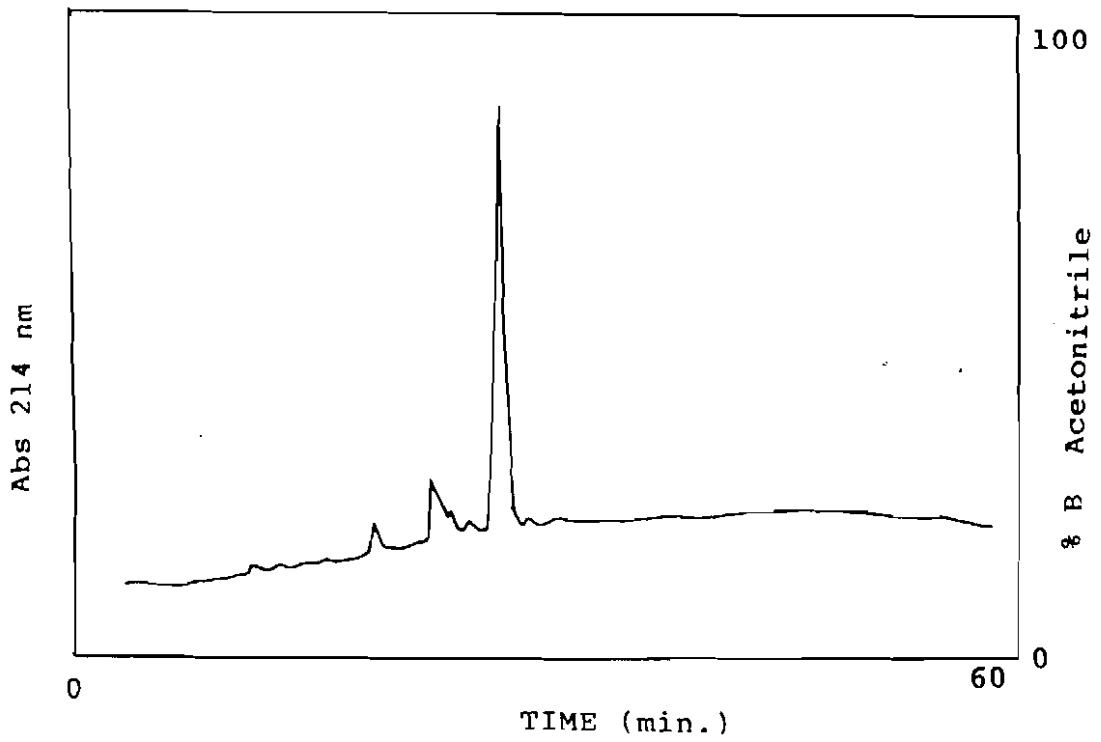


Figure 4.17. FPLC profile of Leu-Thr-Val-Ala-Lys-Leu.

Conditions: Solvent A water containing 0.1% TFA; solvent B acetonitrile containing 0.1% TFA; flow rate 0.5 ml/min; detection 214 nm.

Amino acid analysis: Amino acid analysis of the peptidyl resin gave the following results. Leu 2.10 (2), Thr* 0.60 (1), Val 1.00 (1), Ala 0.89 (1), Lys 0.92 (1).

*Thr was lost during hydrolysis.

The hexapeptide was subjected to CD and NMR analysis. The circular dichroism spectrum was recorded in MeOH and TFE and is not showing any structure (Figure 4.18). NMR spectrum was recorded in DMSO at 270 MHz (Figure 4.19).

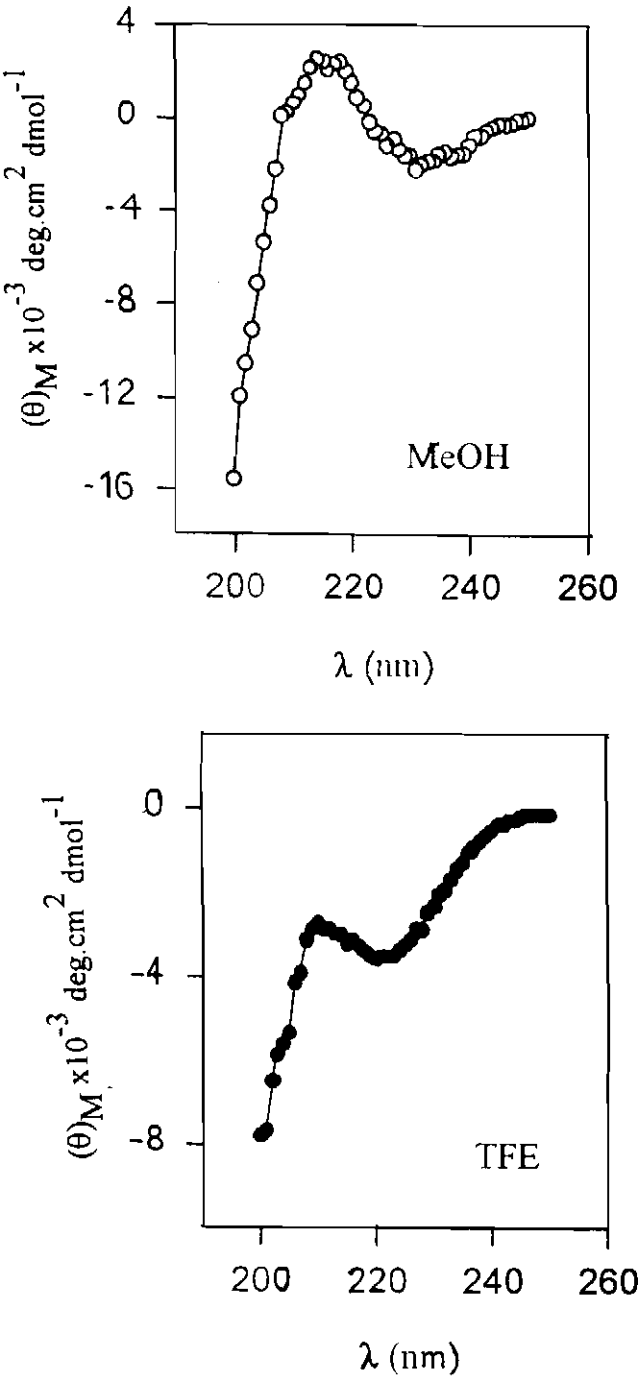


Figure 4.18. CD spectra of Leu–Thr–Val–Ala–Lys–Leu.

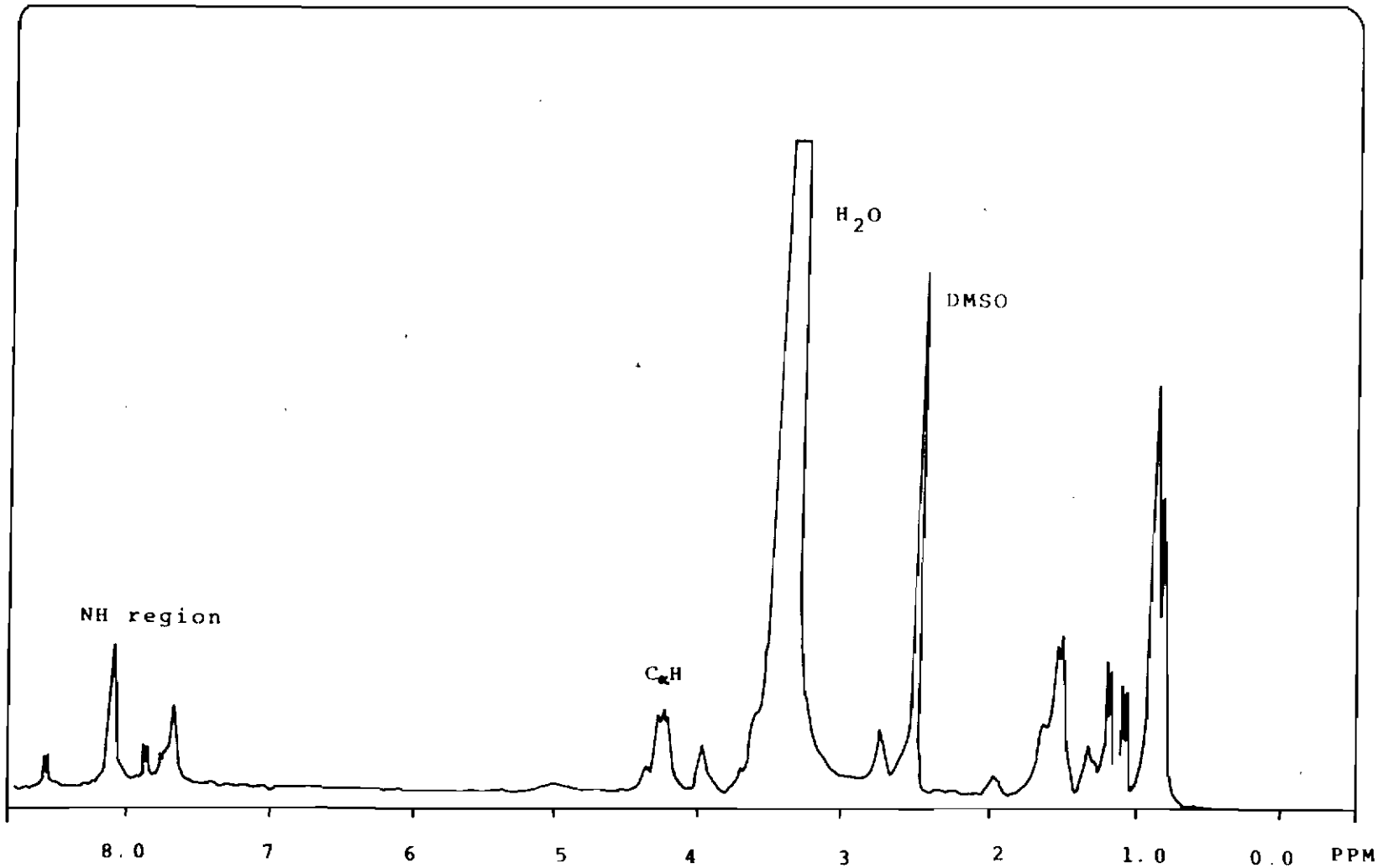
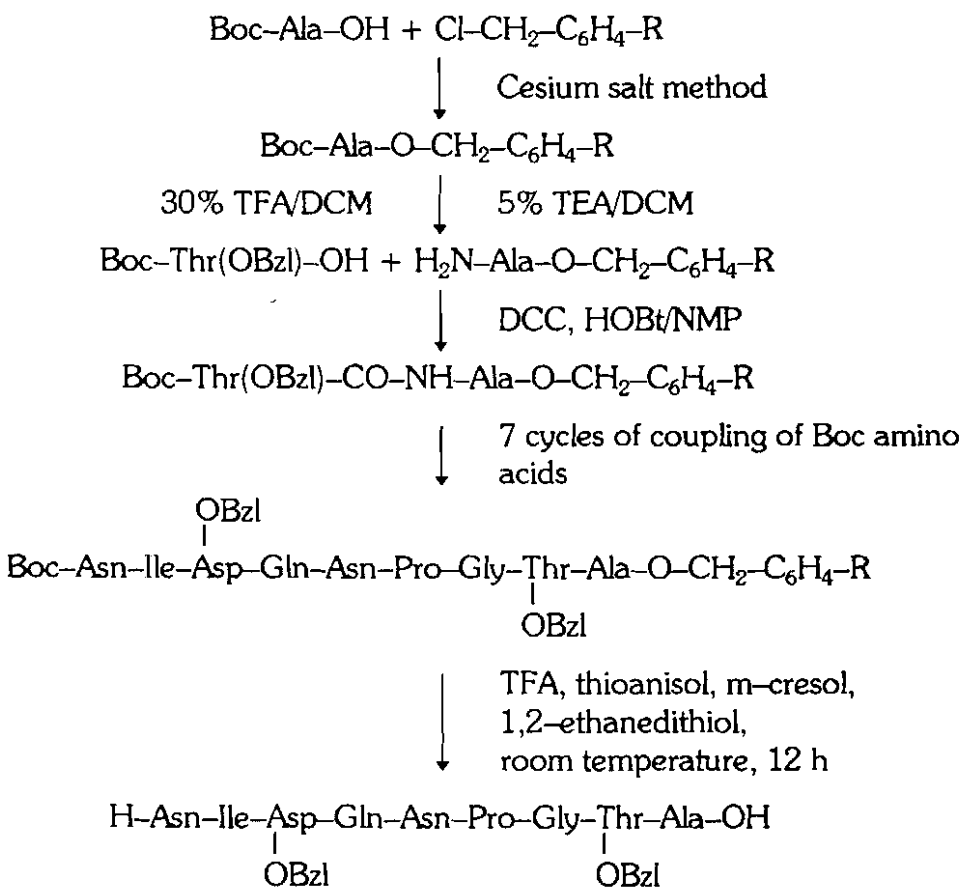


Figure 4.19. NMR spectrum of Leu–Thr–Val–Ala–Lys–Leu in DMSO at 270 MHz.

(f) Synthesis of Asn–Ile–Asp–Gln–Asn–Pro–Gly–Thr–Ala (T59-67)

Boc–Ala was anchored on a 2% PS–HDODA resin by cesium salt method and the amino capacity was found to be 1.8 mmol/g. Boc group was removed by 30% TFA/DCM and neutralisation was carried out by 5% TEA/DCM. The remaining amino acids were attached to the aminoacyl resin by HOBt active ester method. A second coupling was also carried out to ensure completion of reaction. The same procedure was repeated for the attachment of all the remaining amino acids. Completion of coupling was checked by ninhydrin. After the attachment of all the amino acids, the peptide was cleaved from the support by using TFA/thioanisol. The protocol used for the synthesis is given below (Scheme 4.8).



Scheme 4.8. Solid phase synthesis of Asn–Ile–Asp–Gln–Asn–Pro–Gly–Thr–Ala.

The cleaved peptide was hydrogenated to remove benzyl side chain protecting groups. Hydrogenation was carried out by using activated palladium charcoal in MeOH in hydrogen atmosphere. The hydrogenated peptide was purified on HPLC using CH_3CN/H_2O containing 0.1% TFA and H_2O containing 0.1% TFA. HPLC profile of the hydrogenated peptide is shown in Figure 4.20.

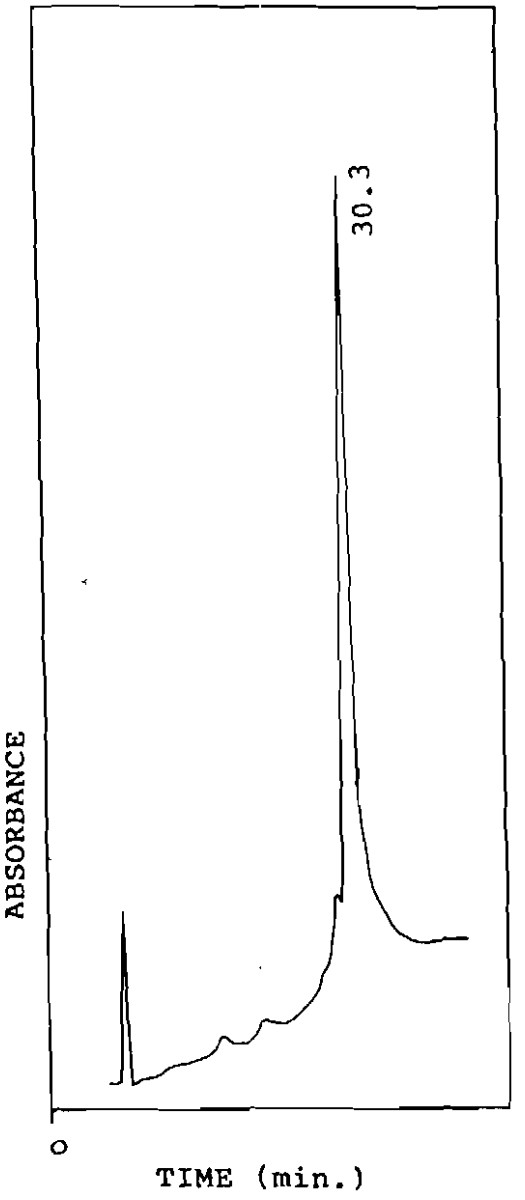


Figure 4.20. HPLC profile of Asn–Ile–Asp–Gln–Asn–Pro–Gly–Thr–Ala. Gradient used 5 to 45% CH_3CN/H_2O containing 0.1% TFA in 40 min time.

The circular dichroism spectra of the peptide was recorded in MeOH and TFE (Figure 4.21). It showed negative bands at 203 and 220 nm in MeOH and 204 and 220 nm in TFE, which are characteristic bands of a helix. This fraction of thioredoxin has helical conformation.

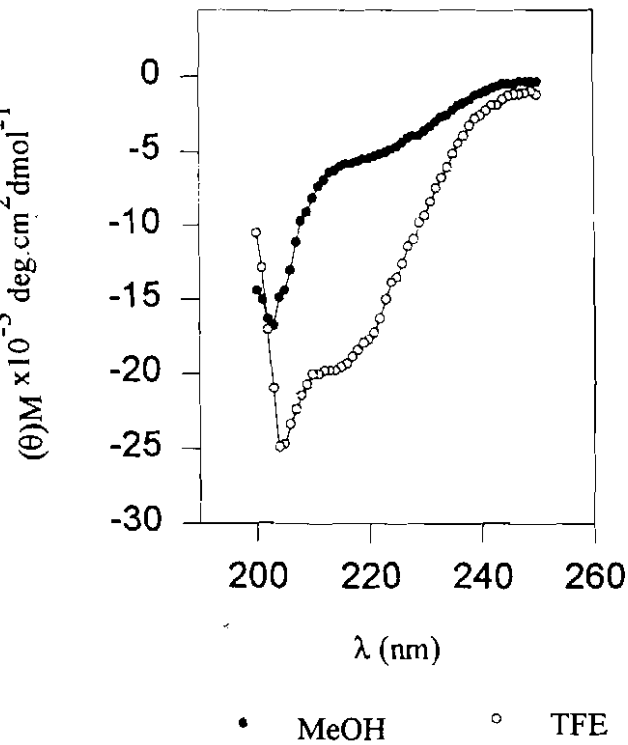
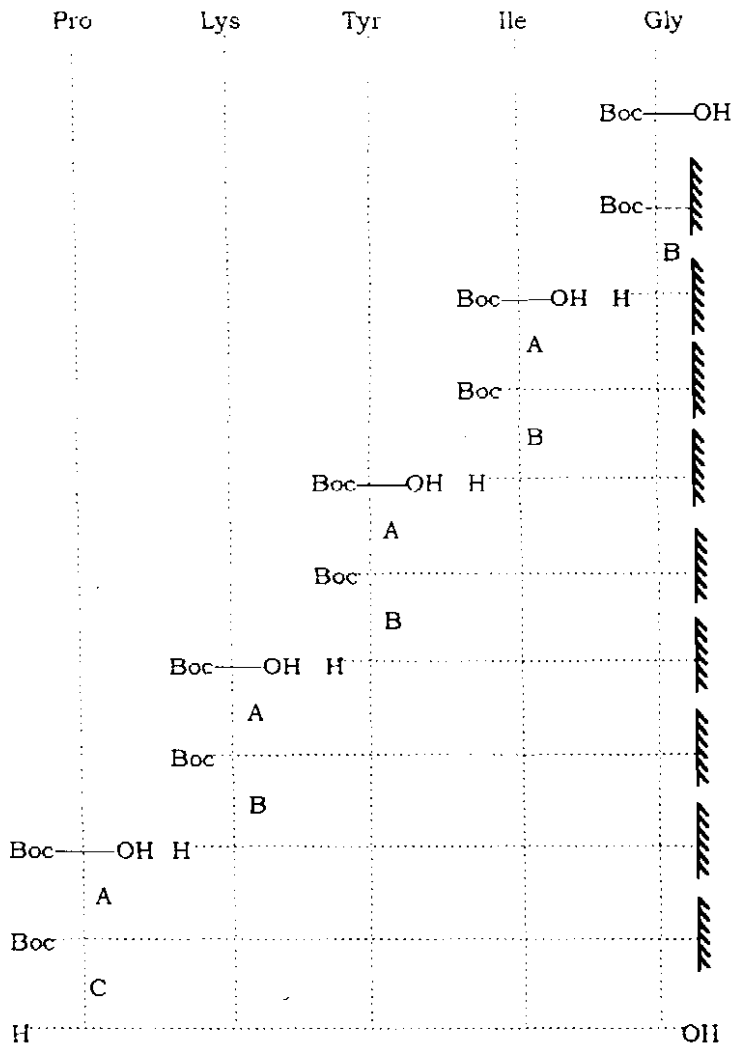


Figure 4.21. CD spectrum of Asn–Ile–Asp–Gln–Asn–Pro–Gly–Thr–Ala.

(g) Synthesis of Pro–Lys–Tyr–Ile–Gly (T68-72)

Boc–Gly was anchored on a 2% PS–HDODA resin and the amino capacity was found to be 1.7 mmol/g. DCC along with catalytic amount of HOBt was used for the coupling all the remaining amino acids. NMP was the solvent system used. The various stages of the synthesis are shown below in Scheme 4.9.



Scheme 4.9. Solid phase synthesis of Pro–Lys–Tyr–Ile–Gly
A – DCC coupling
B – Deprotection using 30% TFA/DCM and neutralisation by 5% TEA/DCM
C – TFA cleavage.

Amino acid analysis: Amino acid analysis of the peptidyl resin gave the following results.

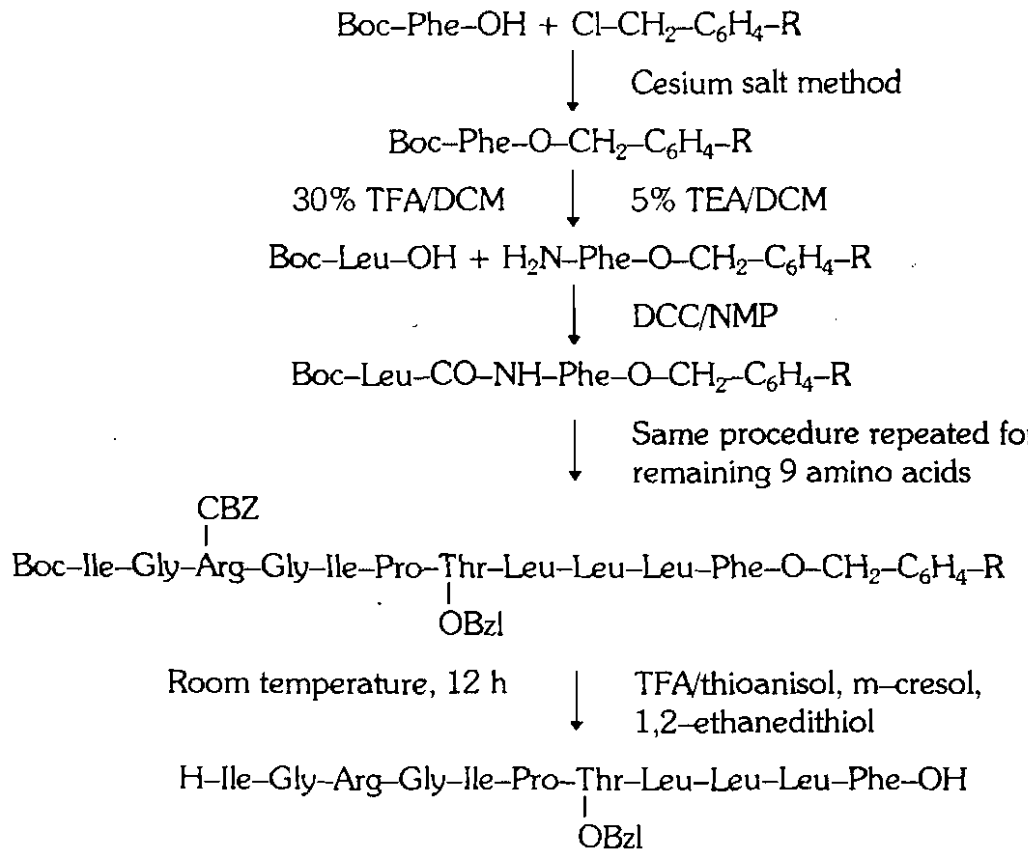
Pro 0.98 (2.0), Lys 0.95 (2.0), Tyr* 0.3 (1.0) Ile 0.89 (1.0), Gly 1.01 (1.0).

*Tyr undergoes degradation under hydrolytic conditions.

(h) Synthesis of Ile–Gly–Arg–Gly–Ile–Pro–Thr–Leu–Leu–Leu–Phe (T71-81)

Chloromethylated 2% HDODA–crosslinked PS resin with a chlorine capacity of 2.01 mmol/g was used for the synthesis of this hydrophobic sequence. After attaching Boc–Phe to the resin the substitution level obtained was 1.9 mmol/g. DCC method was used for the coupling of all the remaining amino acids. NMP was the solvent used in all the attachments and a double coupling was performed.

The finished peptide was cleaved from the support using TFA/thioanisol. The synthetic procedure is outlined in Scheme 4.10.



Scheme 4.10. Protocol used for synthesis of Ile–Gly–Arg–Gly–Ile–Pro–Thr–Leu–Leu–Leu–Phe.

Amino acid analysis was performed using the peptidyl resin after hydrolysing it with a mixture of propionic acid and con. HCl (1:1 v/v). The following results were obtained.

Ile 2.30 (2), Gly 1.67 (2), Arg 0.90 (1.0), Pro 0.98 (1.0), Thr* 0.40 (1), Leu 2.89 (3), Phe 1.0 (1).

*Thr was lost during hydrolysis.

HPLC trace of crude peptide is shown in Figure 4.22. CD spectra of this 11–residue was recorded in MeOH and TFE and is not showing any structure (Figure 4.23). The peptide was subjected to NMR analysis in DMSO at 400 MHz (Figure 4.24).

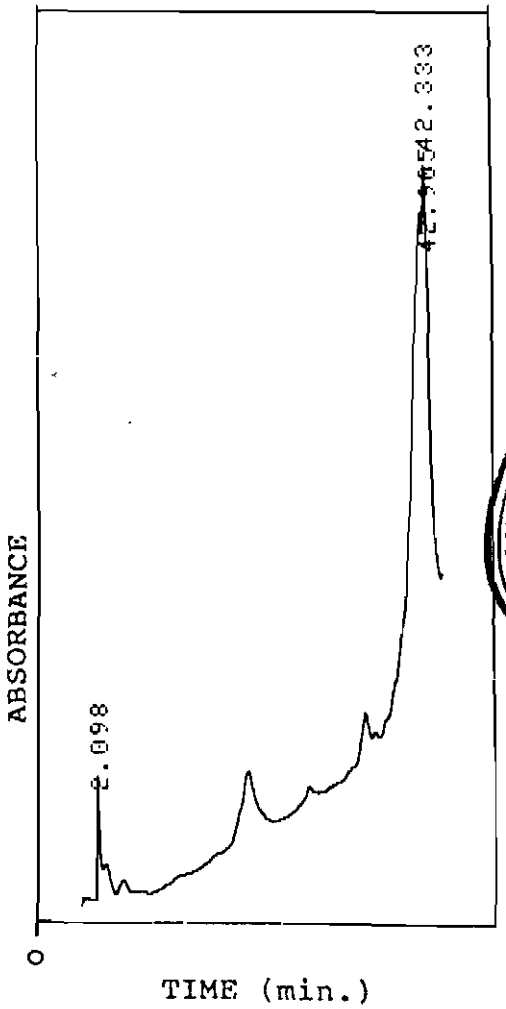


Figure 4.22. HPLC trace of 11-residue (Ile–Gly–Arg–Gly–Ile–Pro–Thr–Leu–Leu–Leu–Phe) hydrophobic peptide (crude). Gradient used: 5 to 45% CH_3CN/H_2O/0.1% TFA system in 45 min time.

This 11–residue hydrophobic peptide is obtained in high yield and from the amino acid analysis and NMR spectra it is clear that the peptide formed is the desired one. This is an evidence for the application of this new resin for the synthesis of hydrophobic peptides.

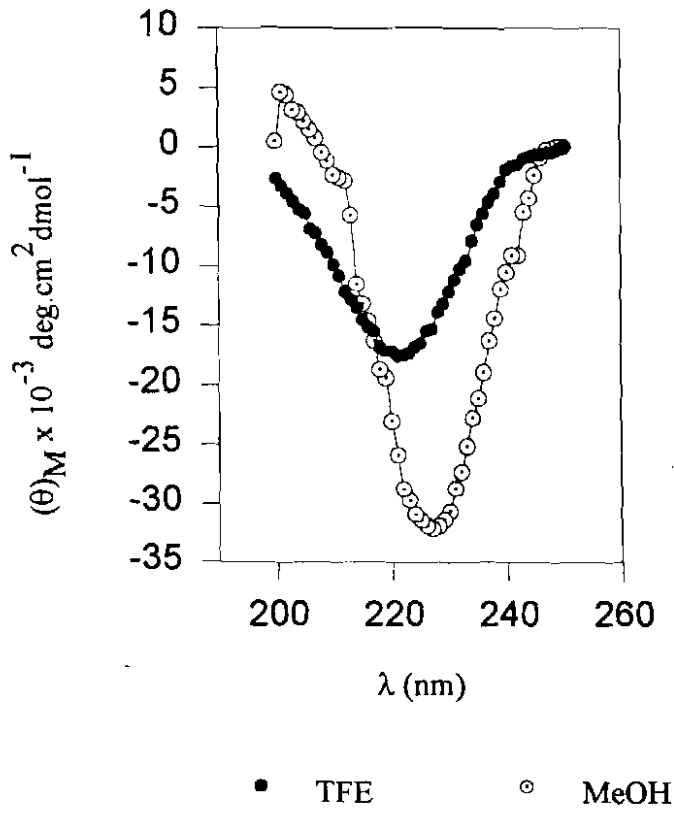


Figure 4.23. CD spectra of Ile–Gly–Arg–Gly–Ile–Pro–Thr–Leu–Leu–Leu–Phe.

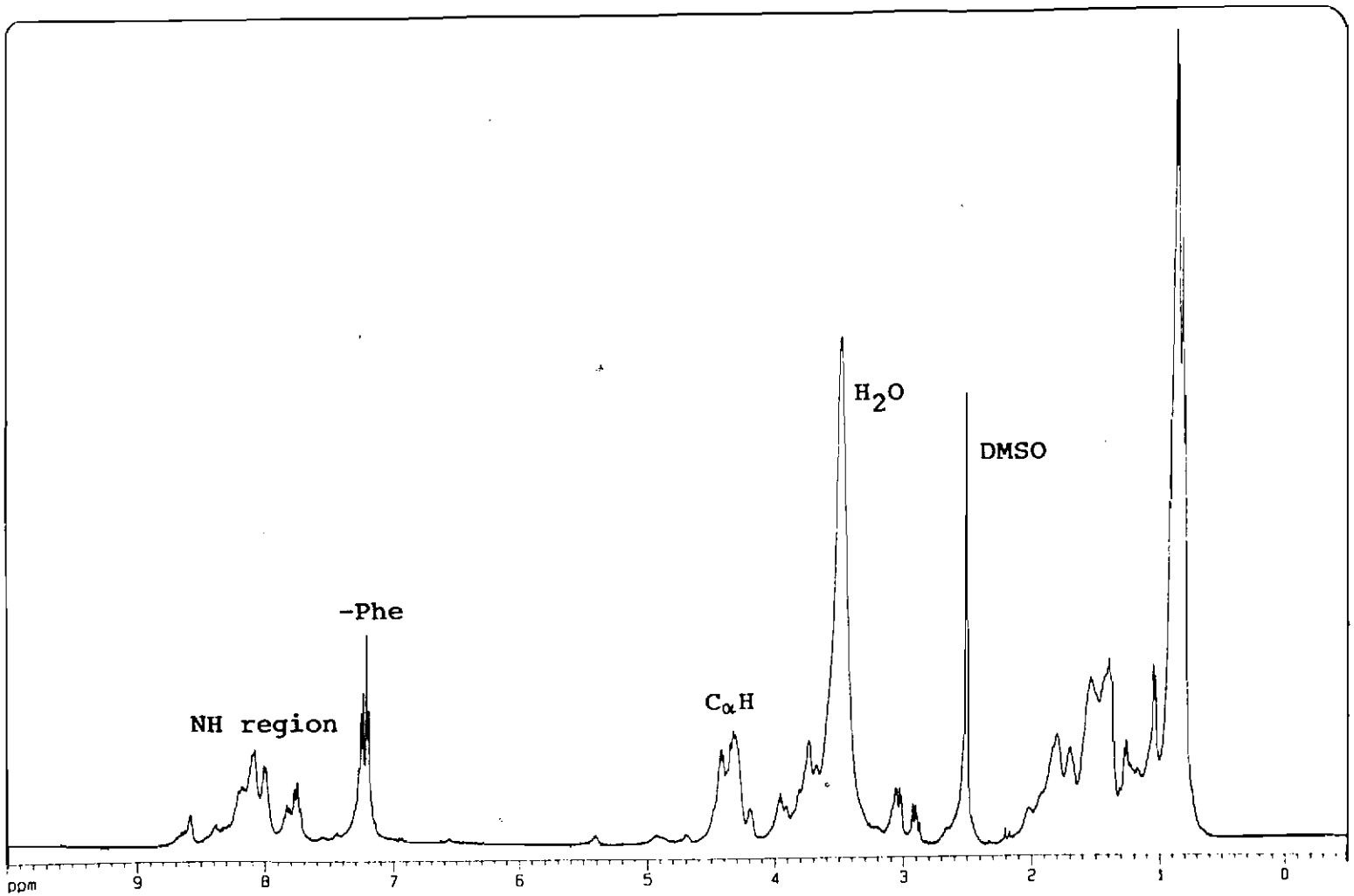
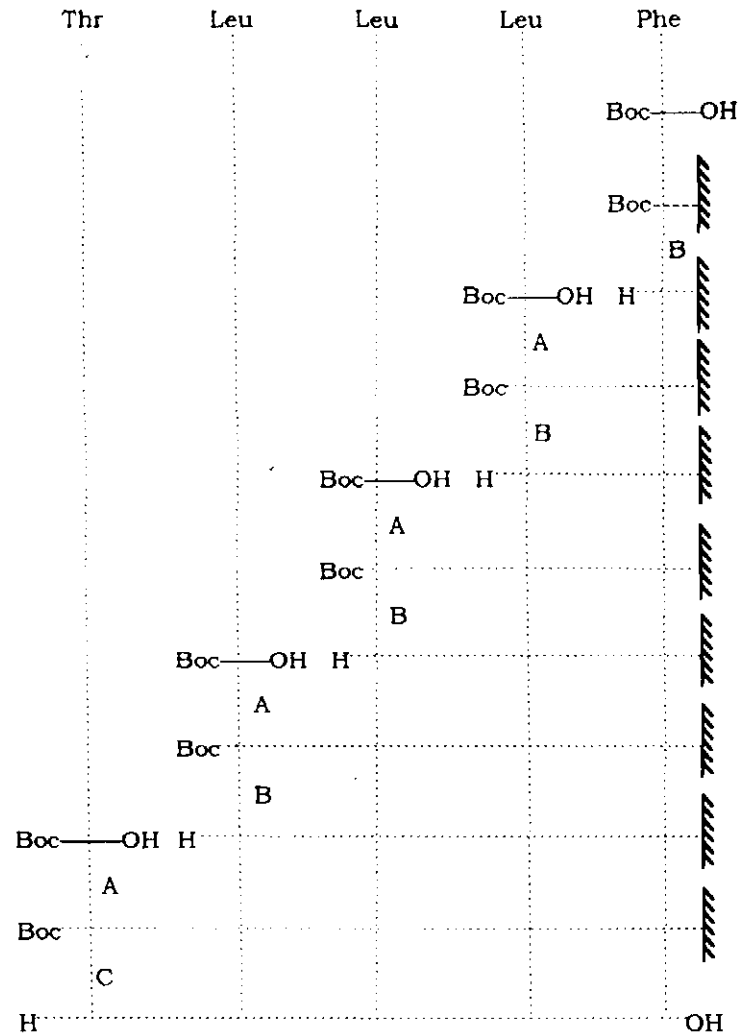


Figure 4.24. NMR spectrum of Ile–Gly–Arg–Gly–Ile–Pro–Thr–Leu–Leu–Leu–Phe in DMSO at 400 MHz.

(i) Synthesis of Thr–Leu–Leu–Leu–Phe (T77-81)

Boc–Phe was attached to the 2% chloromethylated PS–HDODA resin by cesium salt method. Amino capacity was found to be 1.9 mmol/g. Deblocking was carried out using 30% TFA/DCM. DCC coupling method was used for the attachment of all the remaining amino acids and DCM was used as the solvent. After the attachment of all the amino acids the peptide was cleaved from the support by using TFA/thioanisol. The synthetic procedure is depicted in Scheme 4.11.



Scheme 4.11. Solid phase synthesis of Thr–Leu–Leu–Leu–Phe.
A – DCC coupling.
B – Boc group removal by 30% TFA/DCM and neutralisation using 5% TEA/DCM.
C – TFA cleavage.

Amino acid analysis

The peptidyl resin was hydrolysed with a mixture of propionic acid and con. HCl (1:1 v/v) and subjected to amino acid analysis. The following results were obtained. Thr* 0.51 (1.0), Leu 2.85 (3.0), Phe 0.86 (1.0).

*Thr was lost during hydrolysis.

The cleaved peptide was checked on FPLC and showed a single peak. The peptide was obtained in >95% purity (Figure 4.25). CD spectra was recorded in MeOH and TFE. In both the cases the peptide was not showing any structure (Figure 4.26). NMR spectrum was recorded in DMSO at 270 MHz (Figure 4.27).

Hydrophobic pentapeptide Thr–Leu–Leu–Leu–Phe was synthesised in high yield and purity using a 2% PS–HDODA resin. From amino acid analysis and NMR, it is evident that the right compound is formed. Thus it is proved again that the new resin is suitable for hydrophobic sequences.

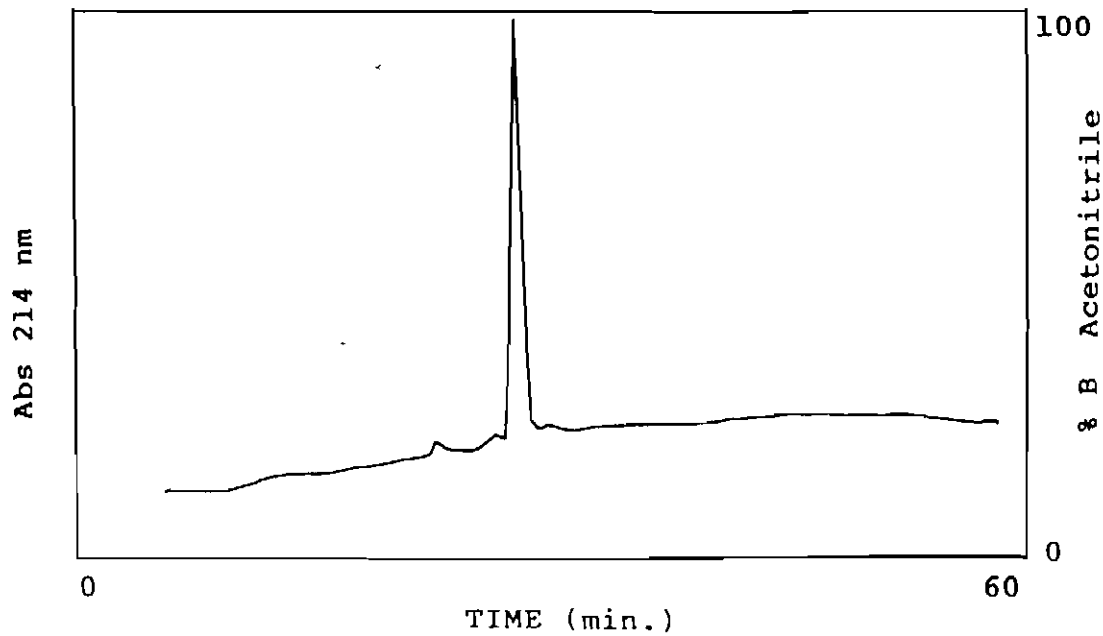


Figure 4.25. FPLC profile of Thr-Leu-Leu-Leu-Phe.

Conditions: Solvent A water containing 0.1% TFA; solvent B acetonitrile containing 0.1% TFA; flow rate 0.5 ml/min; detection 214 nm.

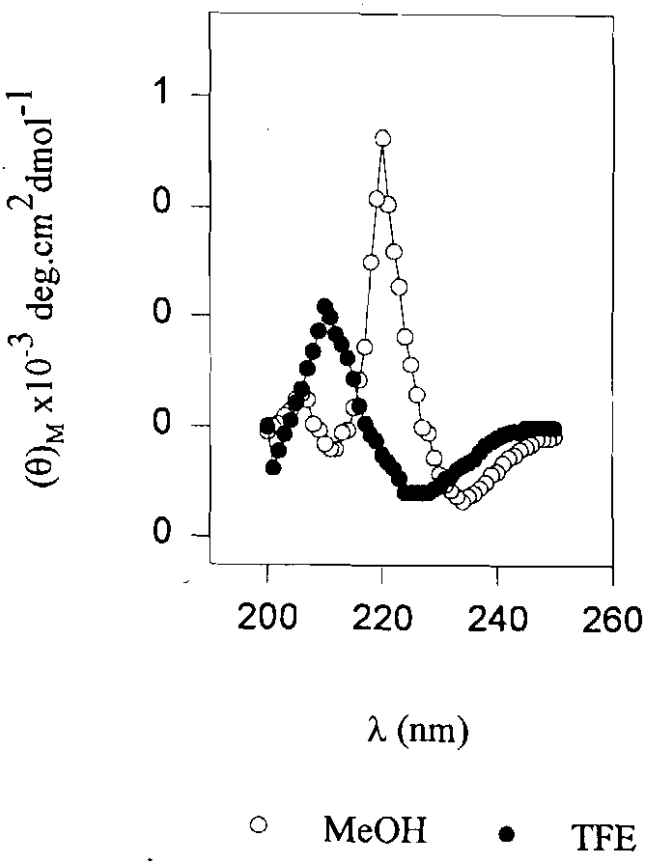


Figure 4.26. CD spectra of Thr-Leu-Leu-Leu-Phe.

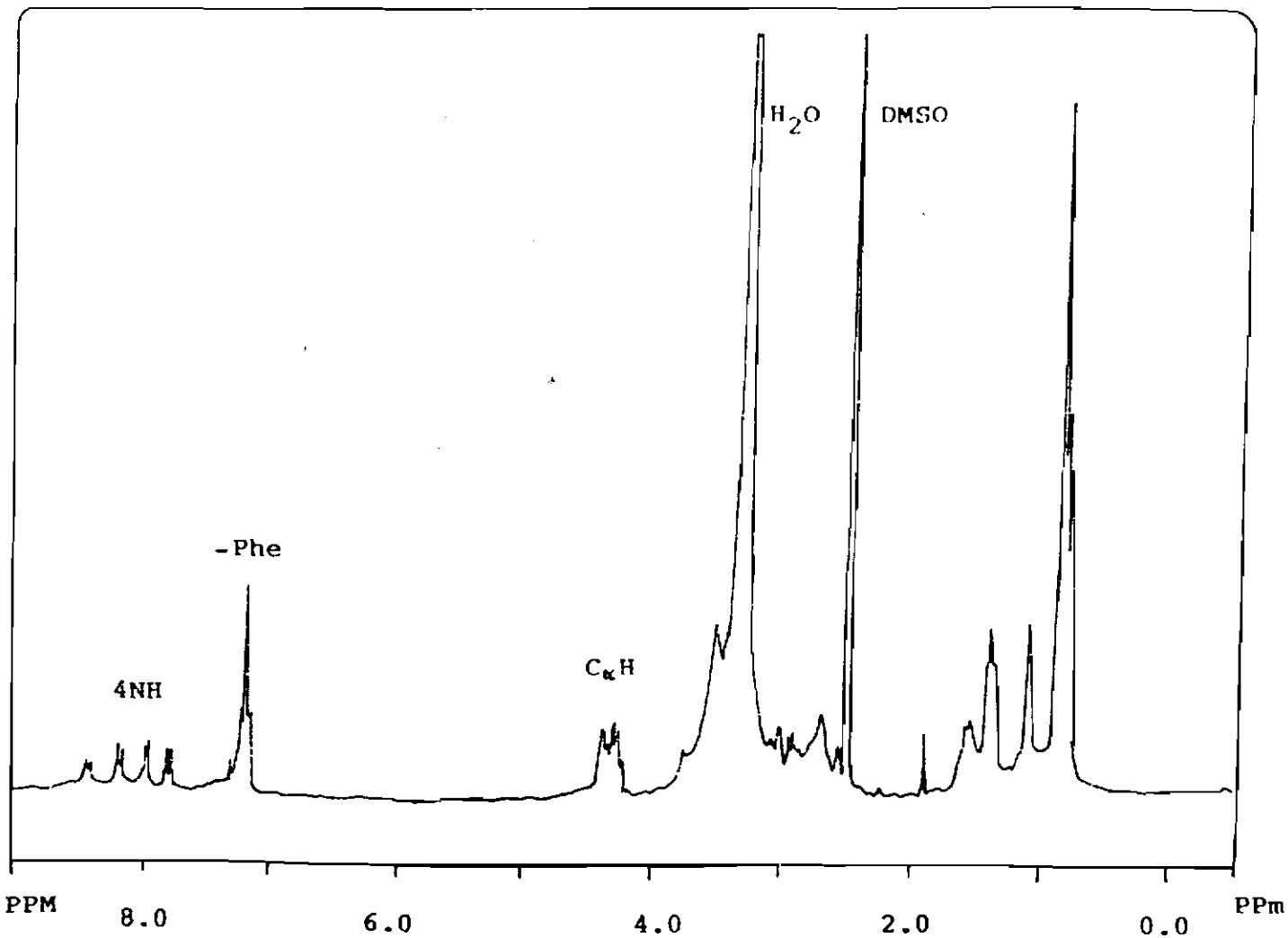
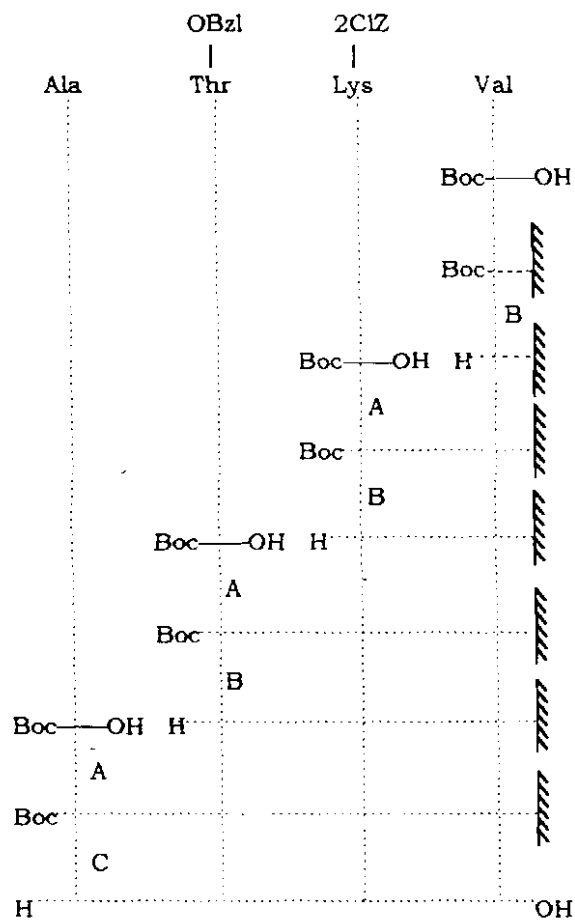


Figure 4.27. NMR spectrum of Thr-Leu-Leu-Leu-Phe in DMSO at 270 MHz.

(j) Synthesis of Ala–Thr–Lys–Val (T88-91)

This tetrapeptide was synthesised on a 2% PS–HDODA resin having a substitution level of 1.91 mmol/g. DCC mediated coupling method was used for the attachment of all the amino acids and DCM was the solvent used. A double coupling was carried out in all the cases to ensure complete reaction. The protocol used for the above synthesis is given below in Scheme 4.12.



Scheme 4.12. Solid phase synthesis of Ala–Thr–Lys–Val on a 2% HDODA-crosslinked polystyrene.

A – DCC coupling.

B – Deblocking using 30% TFA/DCM and neutralisation using 5% TEA/DCM.

C – Cleavage by TFA.

The purity of the peptide was checked on FPLC and a single peak obtained. Thus it is evident that the crude peptide is > 95% pure (Figure 4.28). Amino acid analysis was performed using the peptidyl resin after hydrolysing it with a mixture of propionic acid and con. HCl (1:1 v/v). The following results were obtained. Ala 0.85 (1.0), Thr* 0.6 (1.0), Lys 0.83 (1.0), Val 0.80 (1.0).

*Thr has low value due to loss during hydrolysis.

CD spectrum was recorded in MeOH and TFE and no definite structure was obtained (Figure 4.29).

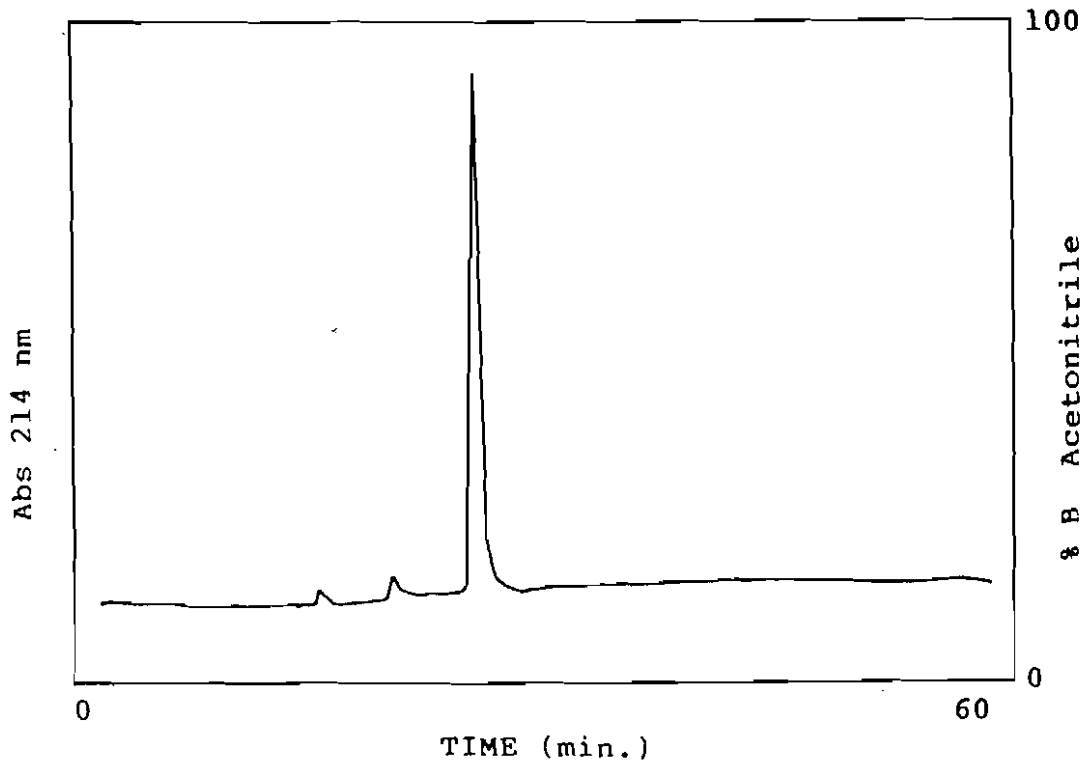


Figure 4.28. FPLC trace of Ala-Thr-Lys-Val. Conditions: Solvent A water containing 0.1% TFA; solvent B acetonitrile containing 0.1% TFA; flow rate 0.5 ml/min; detection 214 nm.

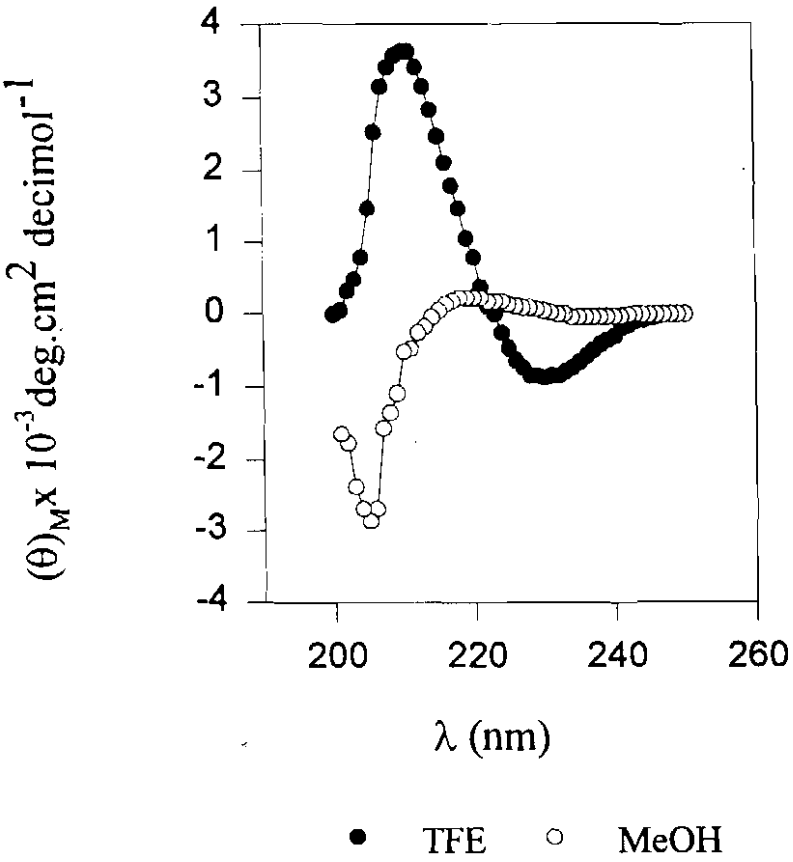
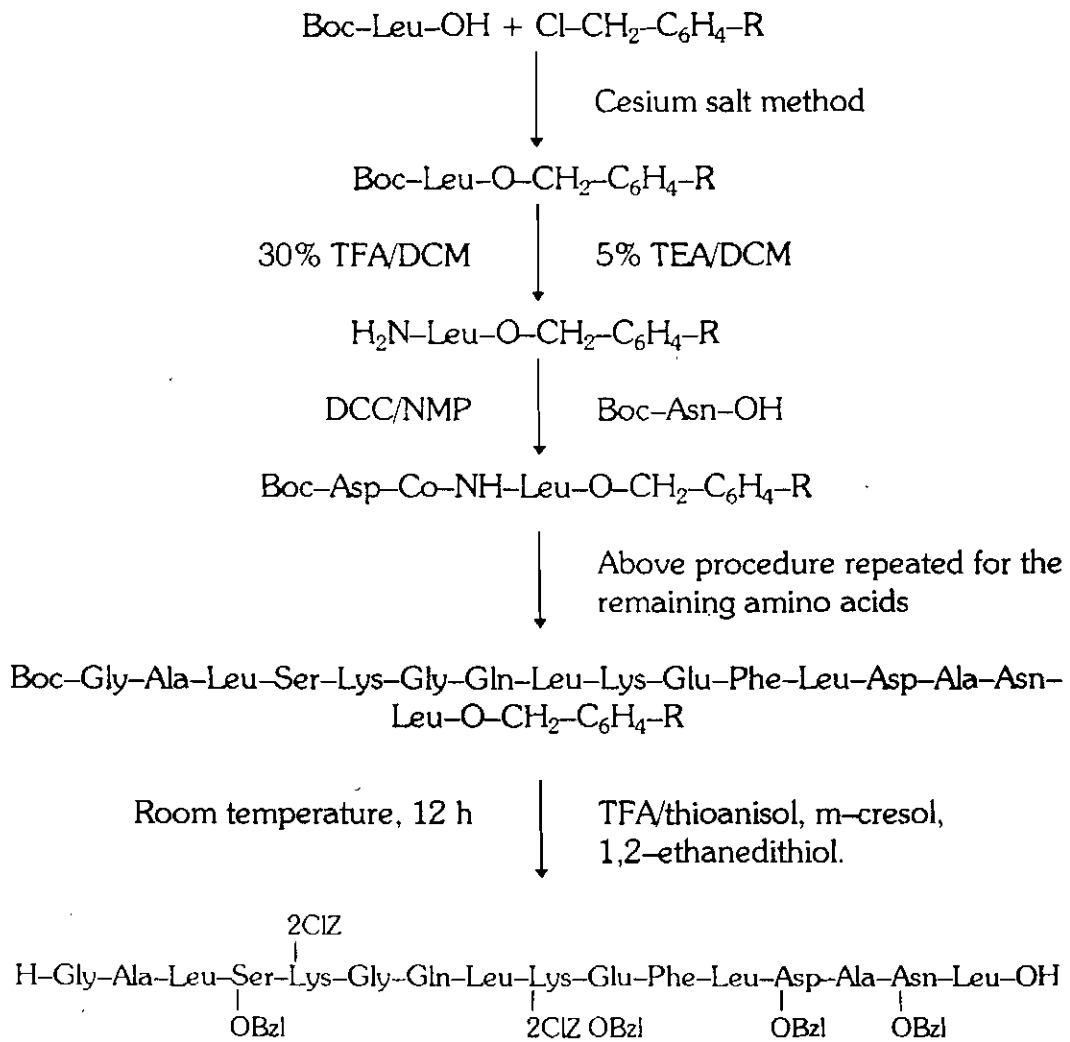


Figure 4.29. CD spectra of Ala-Thr-Lys-Val.

(k) Synthesis of Gly–Ala–Leu–Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu (T92-107)

The 16–residue peptide was synthesised on a 2% chloromethylated PS–HDODA resin having a substitution level of 1.7 mmol/g. DCC mediated

coupling method was used in all the attachments. NMP was used as the solvent. The steps used for the synthesis are shown in Scheme 4.13.



Scheme 4.13. Protocol used for the synthesis of Gly–Ala–Leu–Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu on a 2% HDODA-crosslinked polystyrene.

The peptide was injected on HPLC (analytical column) and got only one major peak (Figure 4.30). The 16–residue peptide was subjected to CD and NMR analysis. Circular dichroism spectra was recorded in MeOH and TFE. In both cases it showed negative bands at 204 and 220 nm indicating the formation

of helix, which is in good agreement with the structure of corresponding fragment of thioredoxin (Figure 4.31). NMR spectrum was recorded in DMSO at 400 MHz and the 'NH' peaks were found to be merging. An intense peak at 5.1δ indicates that benzyl protecting groups are not removed by TFA cleavage (Figure 4.32).

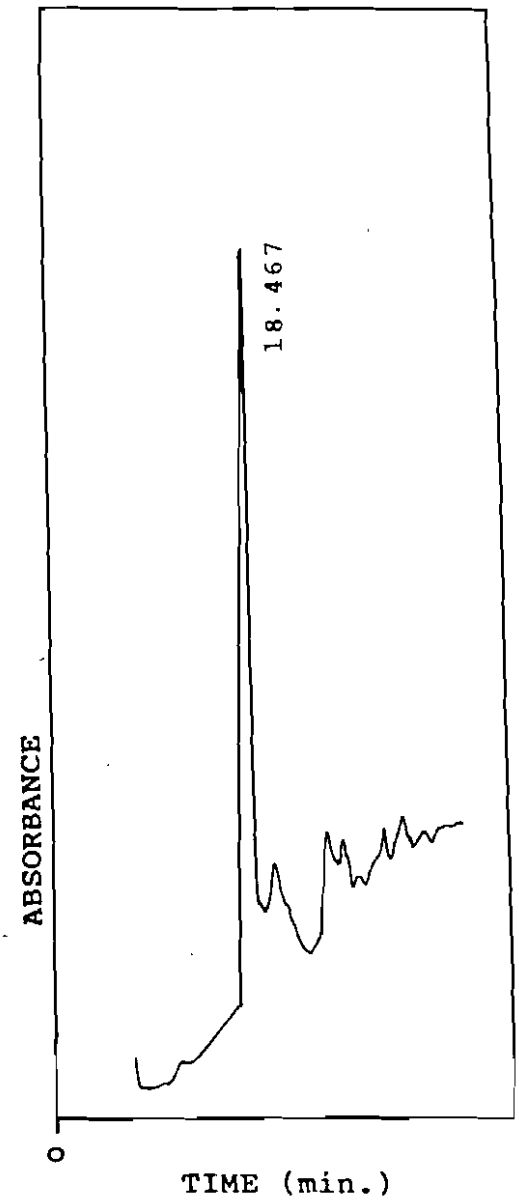


Figure 4.30. HPLC trace of 16-residue peptide (crude) Gly–Ala–Leu–Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu. Gradient used: 5 to 45% CH_3CN/H_2O containing 0.1% in 40 min time.

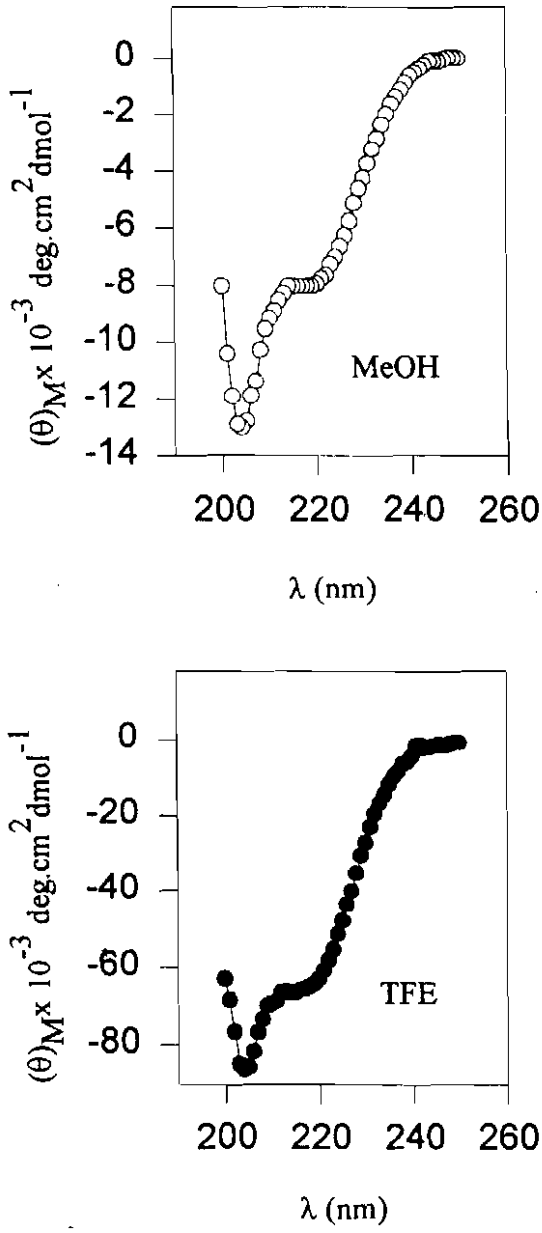


Figure 4.31. CD spectra of Gly–Ala–Leu–Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu.

(l) Synthesis of Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu (T95-107)

This sequence was synthesised using chloromethylated 2% HDODA–PS resin having a chlorine capacity of 2.01 mmol/g. Boc–Leu was attached to the resin by Gisin's cesium salt method and the amino capacity was found to be

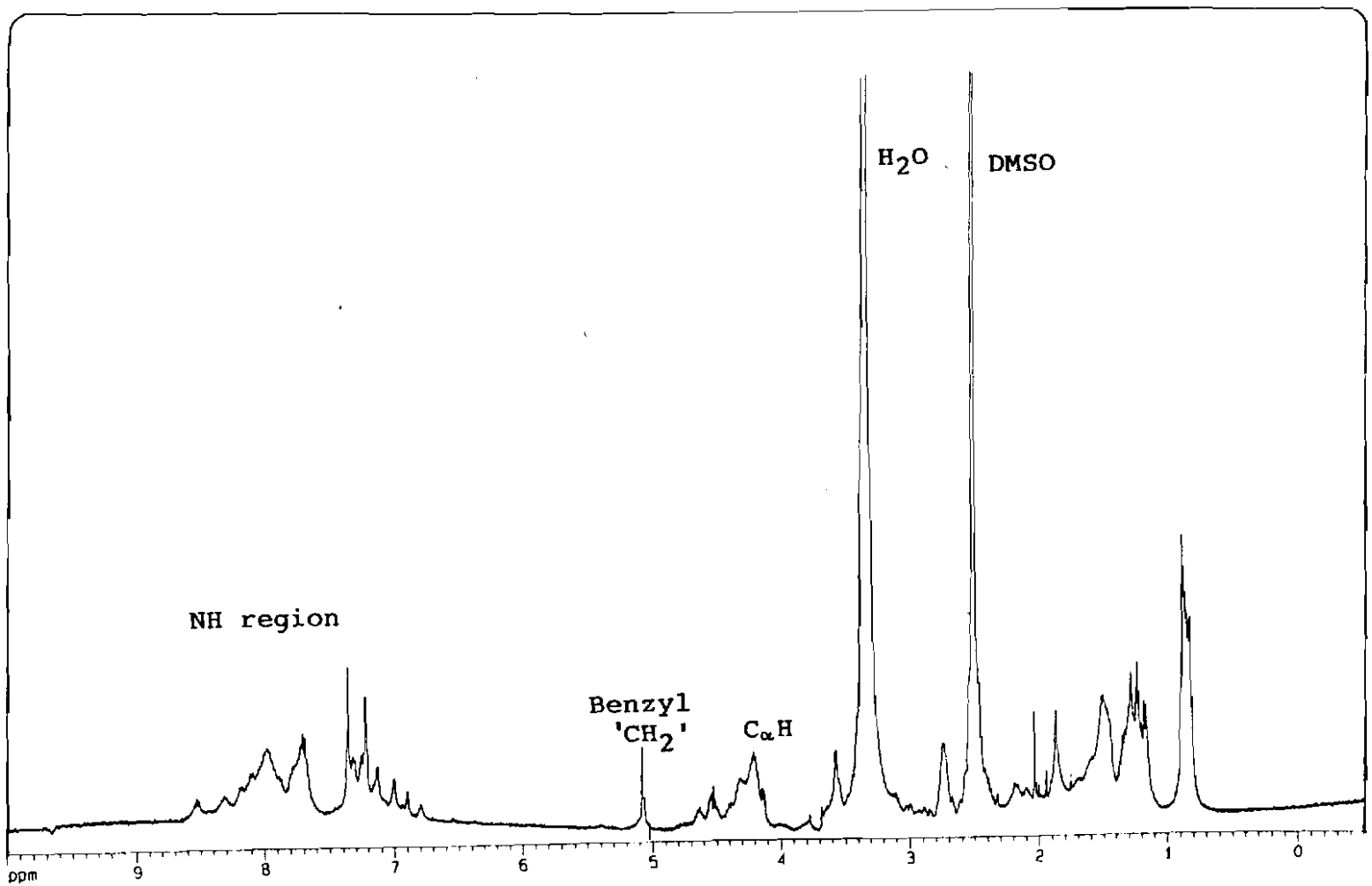
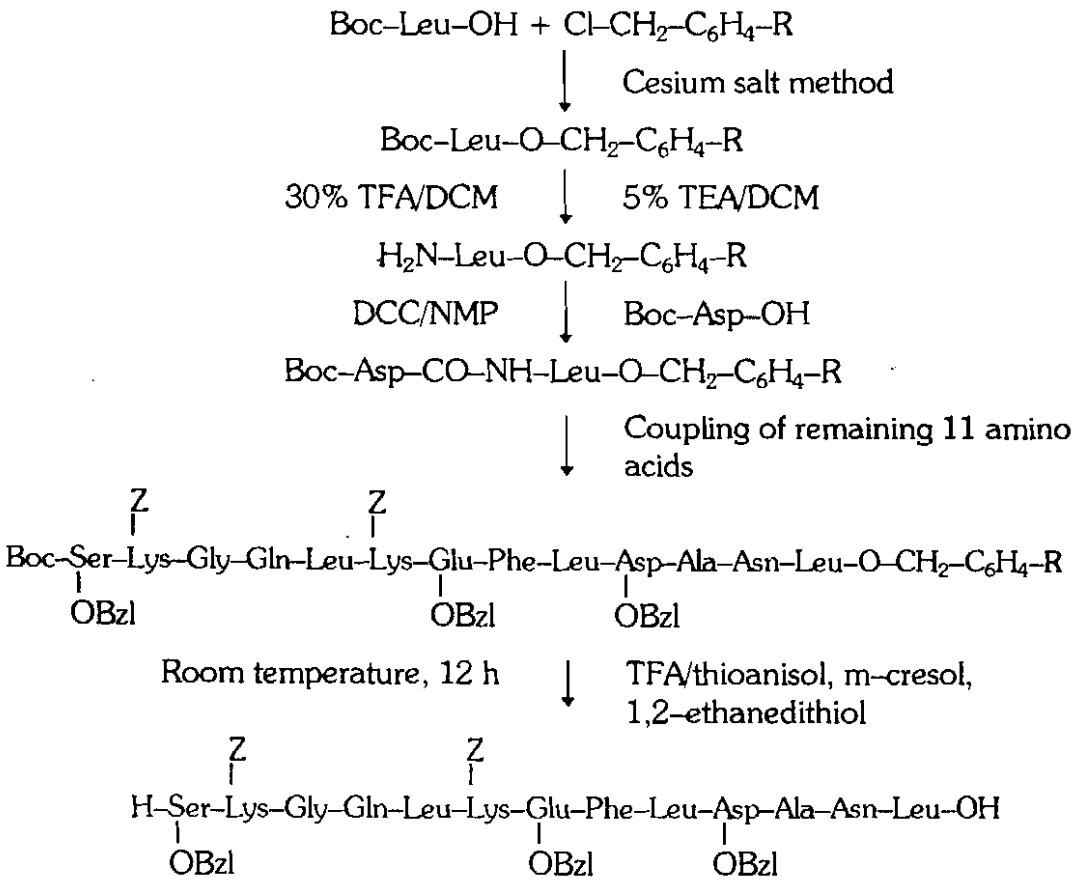


Figure 4.32. NMR spectrum of Gly–Ala–Leu–Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu in DMSO at 400 MHz.

1.7 mmol/g by picric acid method. DCC coupling in NMP was used for the attachment of remaining amino acids. The progress of the coupling reaction was monitored by semiquantitative ninhydrin test. The coupling time for the first coupling was 1 h and for each amino acid an increment of 5 min was given. The by-product DCU was then washed off with 33% MeOH/DCM mixture followed by washing with DCM. Double coupling was given at every stage for completion of reaction. After the attachment of all the amino acid residues the peptide was cleaved from the support using TFA/thioanisol method. TFA was removed by rotary evaporation and the peptide was precipitated by ice-cold ether. The impurities were removed by washing several times with ether to get the crude peptide. The entire synthetic sequence is depicted in Scheme 4.14.



Scheme 4.14. Solid phase synthesis of Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu.

Purity of the crude peptide was checked on HPLC and only one major peak was obtained (Figure 4.33).

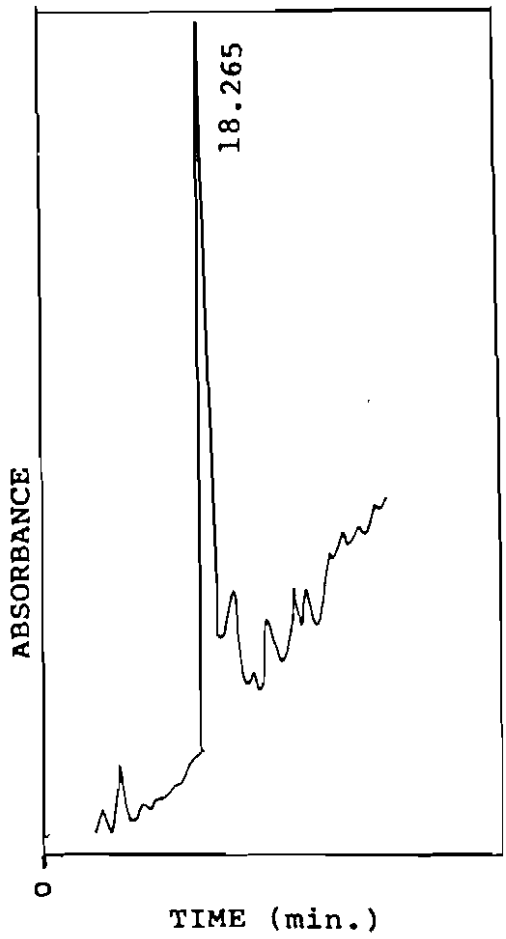


Figure 4.33. HPLC trace of (crude) 13-residue peptide, Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu. Gradient used: 5 to 45% CH_3CN/H_2O/0.1% TFA in 45 min time.

The peptide was subjected to NMR and CD analysis. CD spectra were recorded in MeOH and TFE. In MeOH the spectrum showed negative bands at 203 and 222 nm which is characteristic of helical structure. TFE, well known helix inducing solvent, showed further increase in intensity of negative bands without affecting band position. Thus it is clear that the sequence has helical conformation (Figure 4.34).

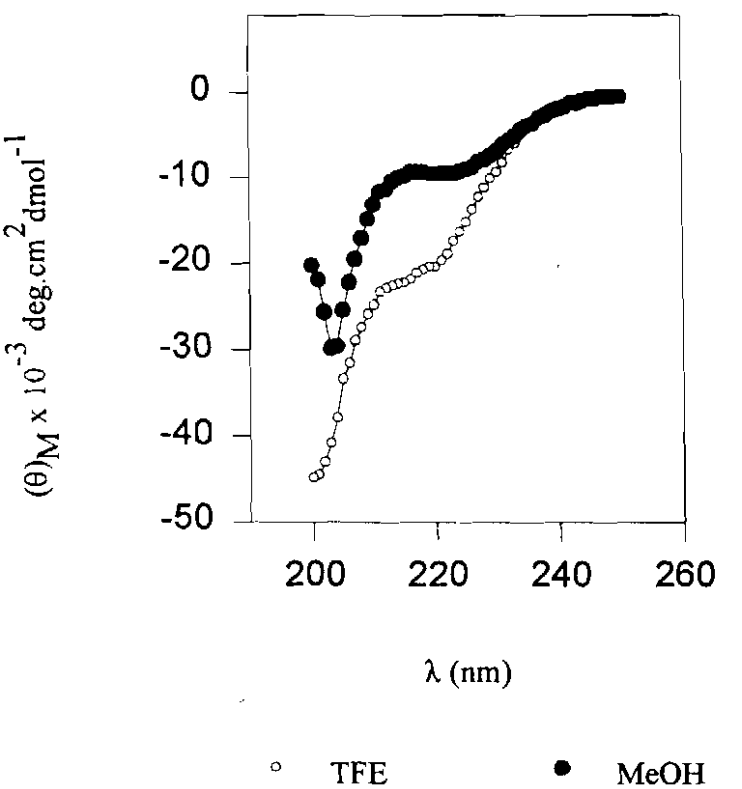


Figure 4.34. CD spectra of Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu.

NMR spectrum was recorded in DMSO at 400 MHz. The 'NH' peaks are not differentiated because this is a 13 residue peptide. The peak at 5.1 δ is characteristic of benzyl group. This may be due to the incomplete removal of Bzl group in TFA treatment (Figure 4.35).

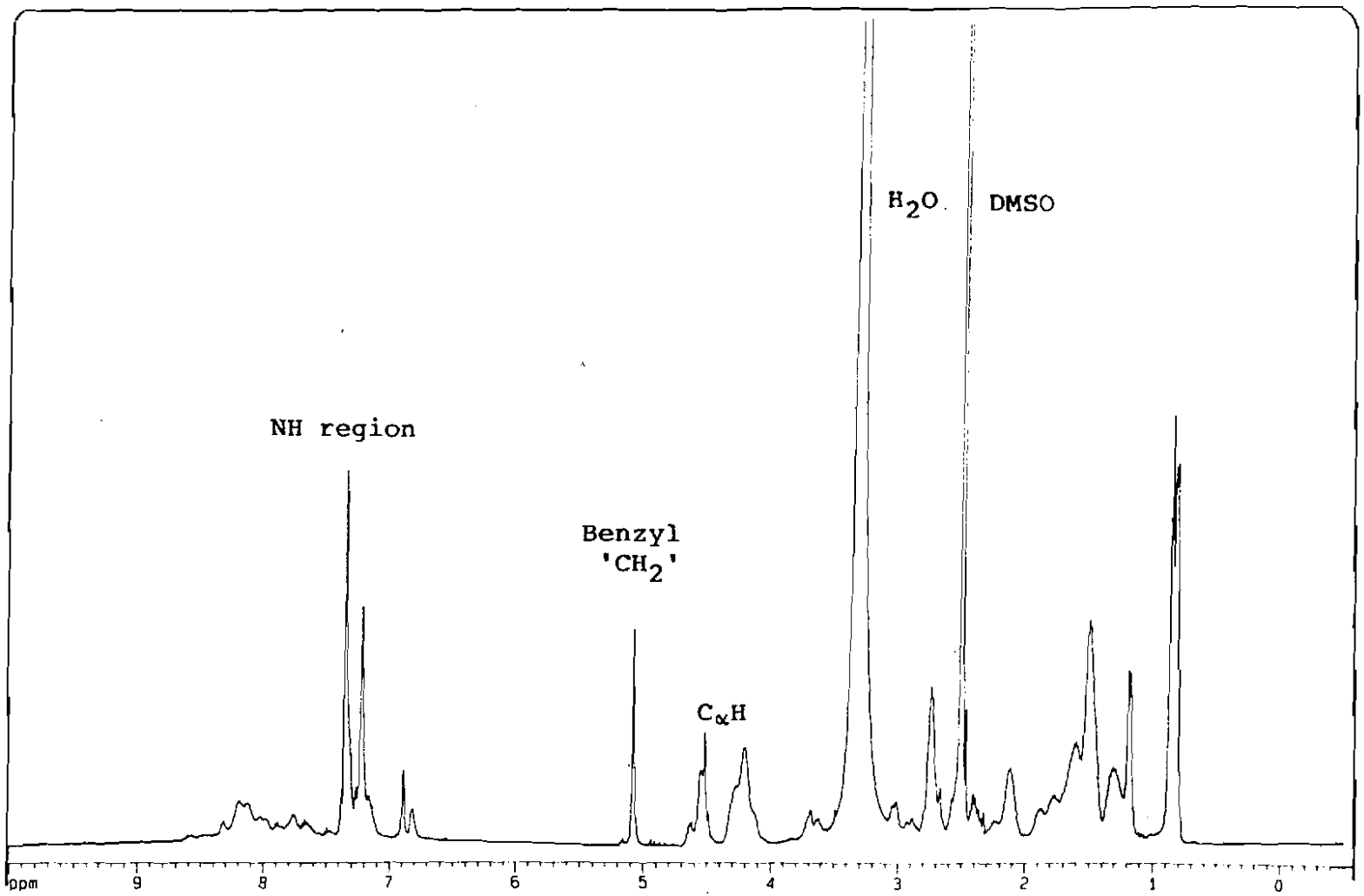


Figure 4.35. NMR spectrum of Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu in DMSO at 400 MHz.

4.2.3 Synthesis of designed β–hairpin peptides

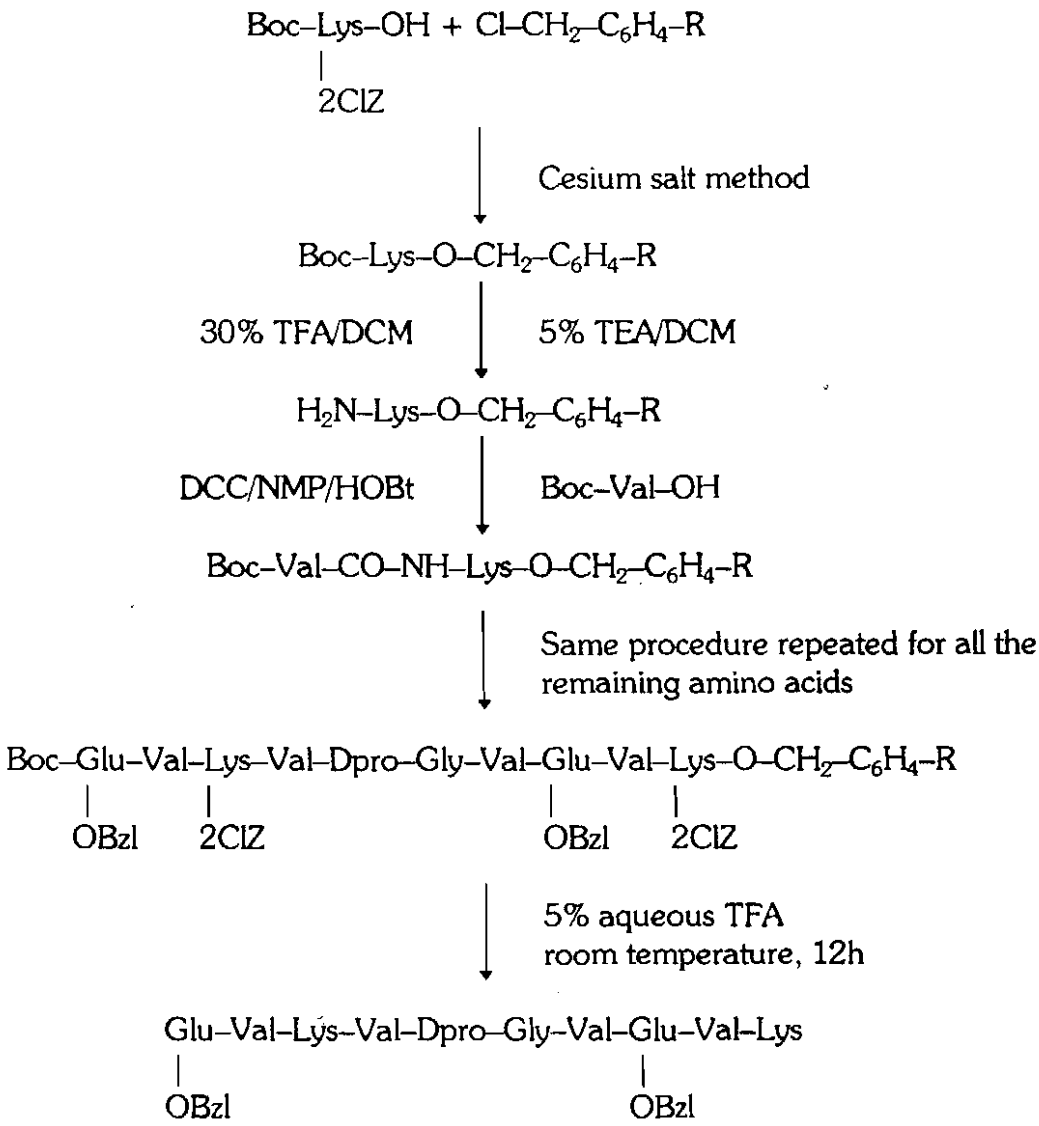
The design of short peptide sequences adopting well defined conformations is a critical element in the development of synthetic protein mimics. The rational construction of β–hairpins has attracted lesser attention, although they form one of the simplest elements of super secondary structure in globular protein. The two hairpins synthesised are,

(a)
```
Dpro–Gly
|     |
Val   Val
|     |
Lys   Glu
|     |
Val   Val
|     |
Glu   Lys
```

(b)
```
Dpro–Gly
|     |
Val   Val
|     |
Leu   Leu
|     |
Val   Val
|     |
Acm–Cys   Cys–Acm
|     |
Ala   Ala
```

(a) Synthesis of Glu–Val–Lys–Val–Dpro–Gly–Val–Glu–Val–Lys

Boc–Lys (2ClZ) was anchored to the chloromethyl resin by cesium salt method. Amino capacity was found to be 0.8 mmol/g. The Boc–group was removed by treatment with 30% TFA/CH_2Cl_2 and the resulting amine salt was neutralised with triethylamine. The peptide chain was built by sequentially extending it towards the amino terminus by stepwise addition of Boc amino acid (3 equiv.) DCC (3 equiv.) and HOBt. Most of the couplings were completed by the first coupling itself. Progress of the coupling reaction was monitored by Kaiser reagent. In all the cases a second coupling was also carried out to ensure complete reaction. The protocol used for the above synthesis is given below.



Scheme 4.15. Synthesis of Glu–Val–Lys–Val–Dpro–Gly–Val–Glu–Val–Lys.

Cleaved peptide was subjected to hydrogenation using activated palladium charcoal in MeOH under hydrogen atmosphere for 24 h. The hydrogenated peptide was loaded on HPLC and only one peak was obtained and it was further purified on reverse phase columns using CH_3CN/H_2O/0.1% TFA and water containing 0.1% TFA. Homogeneity was checked on analytical column (Figure 4.36). The purified peptide was subjected to NMR analysis in DMSO at 400 MHz (Figure 4.37).

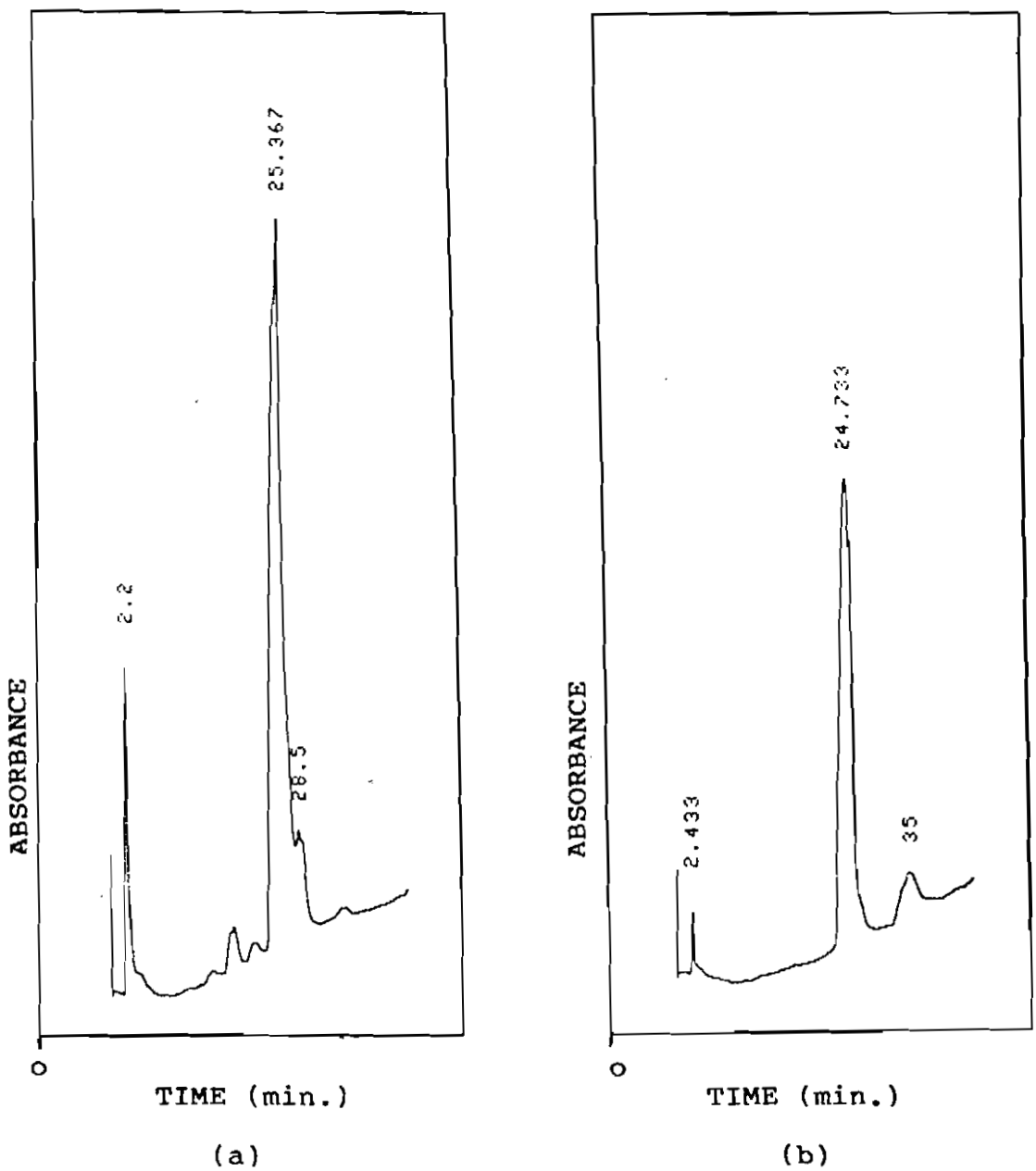


Figure 4.36. HPLC trace of (a) crude and (b) purified 10 residue peptide Glu–Val–Lys–Val–Dpro–Gly–Val–Glu–Val–Lys. Gradient used: 5 to 45% CH_3CN/H_2O containing 0.1% TFA in 45 min time.

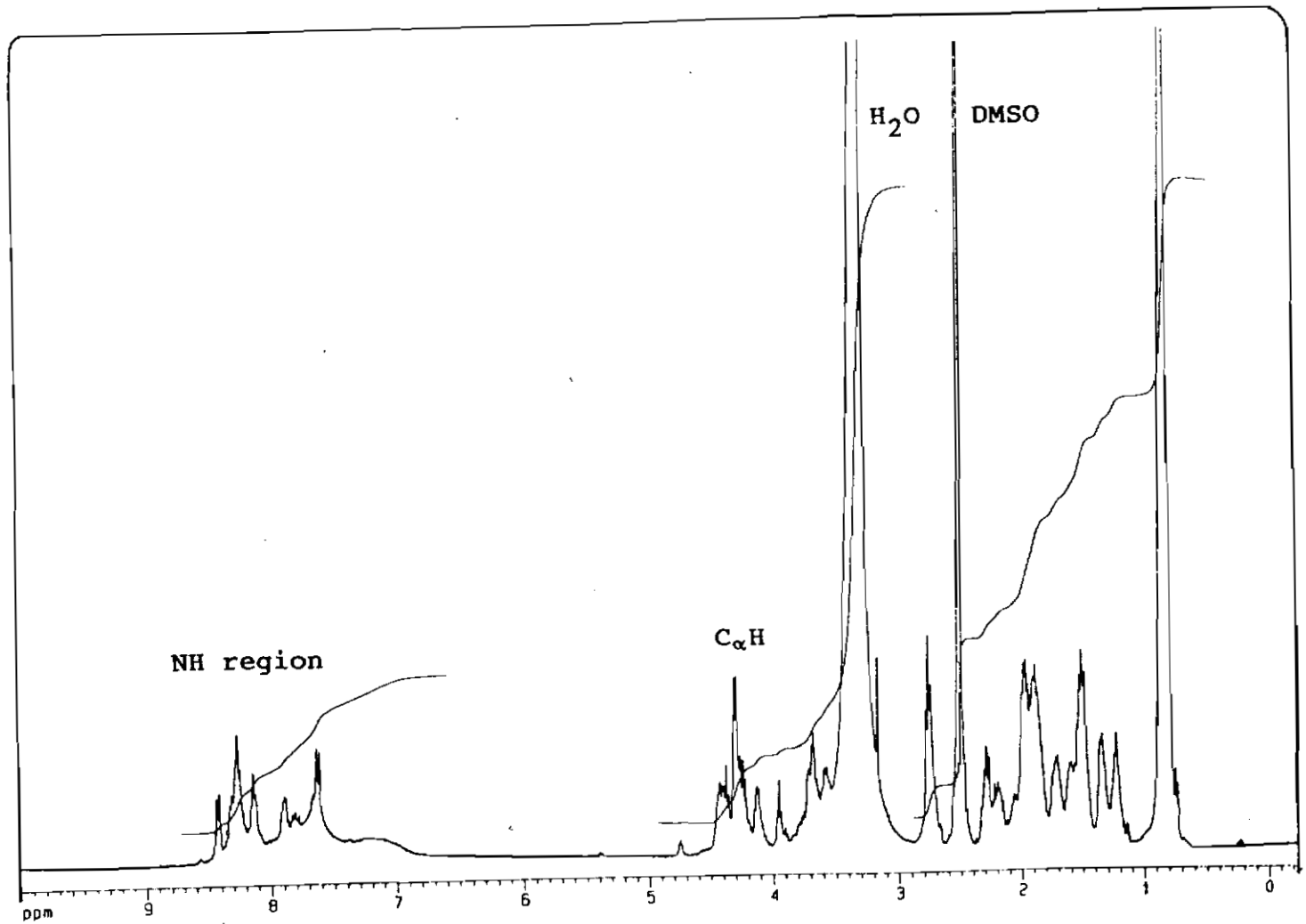
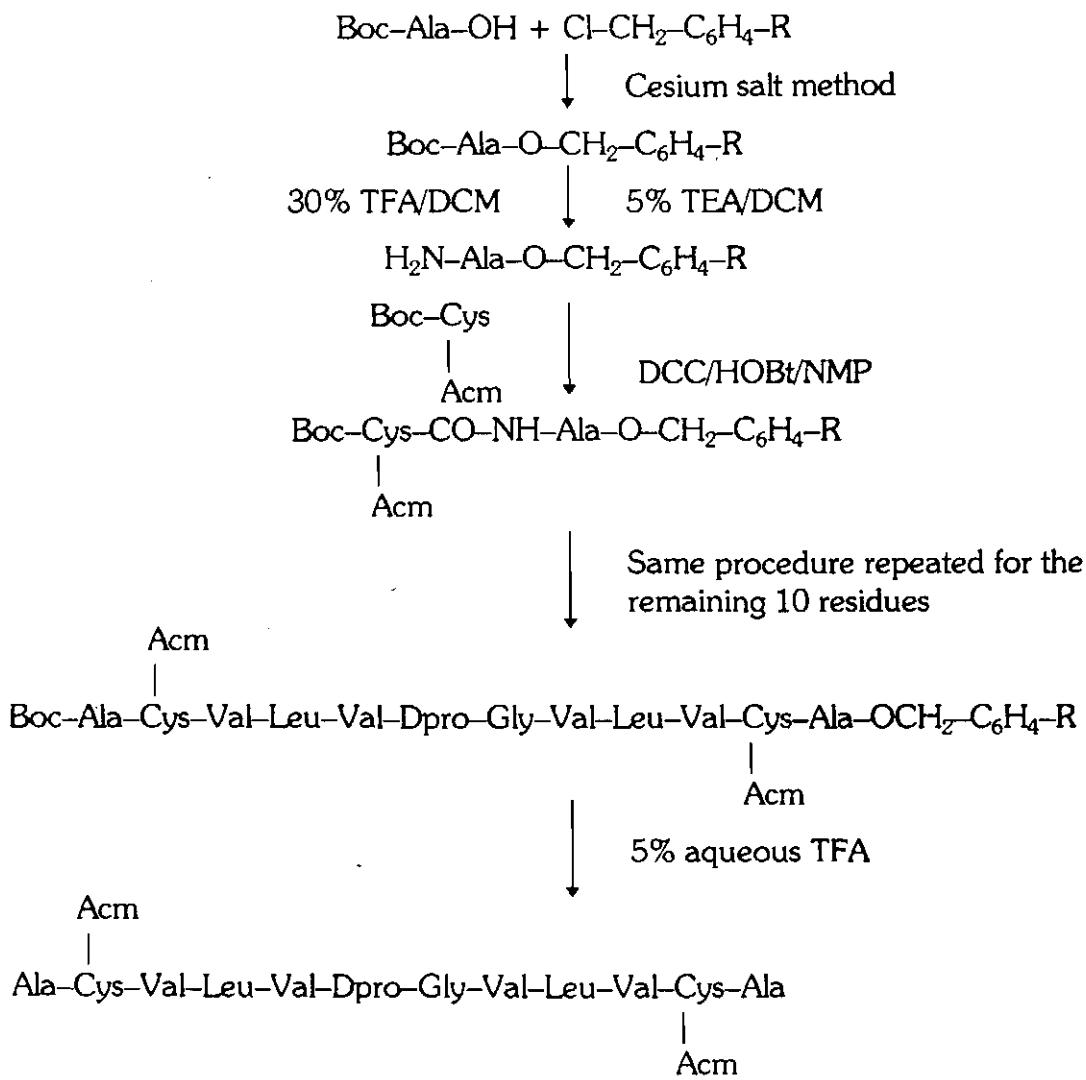


Figure 4.37. NMR spectrum of 10 residue peptide Glu–Val–Lys–Val–Dpro–Gly–Val–Glu–Val–Lys in DMSO at 400 MHz.

(b) Synthesis of Ala–Cys(Acm)–Val–Leu–Val–Dpro–Gly–Val–Leu–Val–Cys(Acm)–Ala

The 12 residue peptide was synthesised on a 2% chloromethylated PS–HDODA resin having a substitution level of 1.8 mmol/g. DCC/HOBt coupling method was used for all the attachments of amino acids and NMP was the solvent used. The synthetic steps are shown in Scheme 4.16.



Scheme 4.16. Synthesis of Ala–Cys(Acm)–Val–Leu–Val–Dpro–Gly–Val–Leu–Val–Cys(Acm)–Ala.

The progress of the coupling reaction was followed by Kaiser reagent. Most of the couplings were completed by the first coupling itself. But in all the cases a second coupling was also carried out to ensure complete reaction.

The peptide was obtained with the Acm group on cysteine. Purity was checked on HPLC (analytical column) and the HPLC profile showed only one major peak. This was further purified on a preparative column (Figure 4.38). The NMR spectrum of this 12 residue peptide was taken in DMSO at 400 MHz (Figure 4.39).

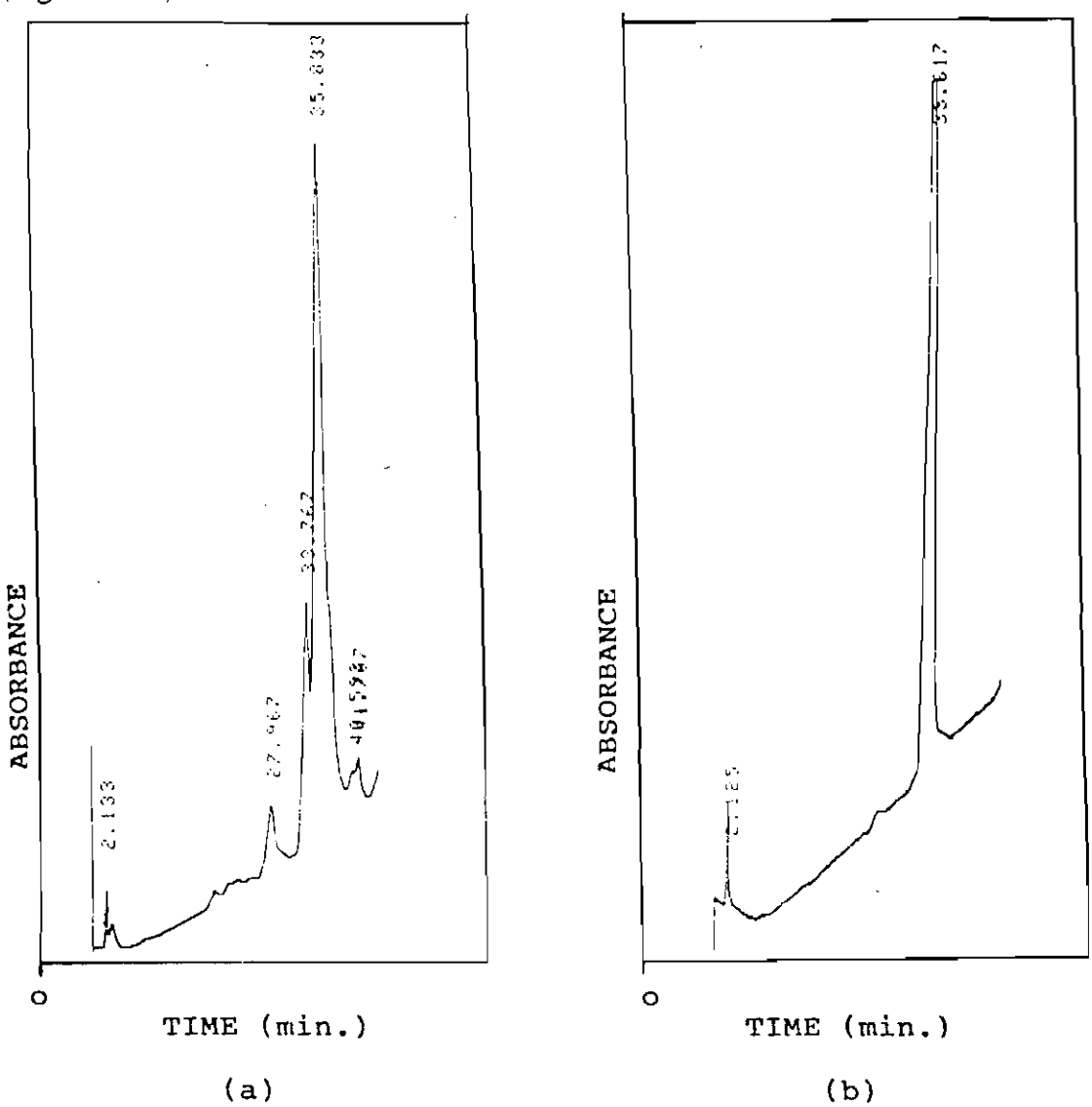


Figure 4.38. HPLC trace of (a) crude and (b) purified 12 residue peptide Ala–Cys–Val–Leu–Val–Dpro–Gly–Val–Leu–Val–Cys–Ala. Gradient used: 5 to 45% CH_3CN/H_2O containing 0.1% in 45 min time.

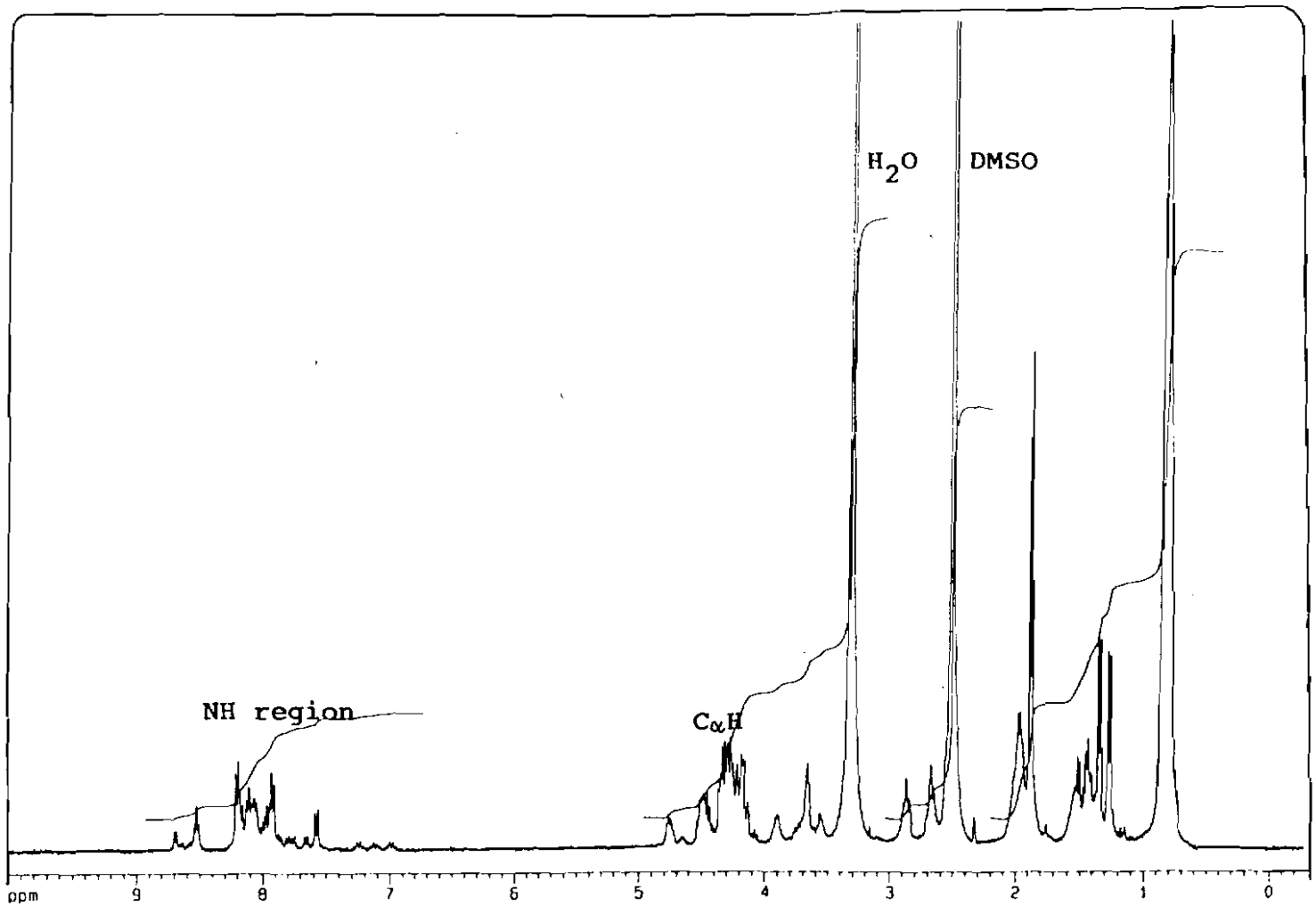


Figure 4.39. NMR spectrum of 12 residue peptide Ala–Cys–Val–Leu–Val–Dpro–Gly–Val–Leu–Val–Cys–Ala in DMSO at 400 MHz.

CHAPTER 5

SUMMARY AND CONCLUSION

Synthesis of thioredoxin partial sequences on a newly developed polymeric support is the main objective of this work. Thioredoxin contains both sequences of hydrophobic and hydrophilic nature. Most of the sequences synthesised here are hydrophobic in nature. These peptides were synthesised on a 2% 1,6-hexanediol diacrylate crosslinked polystyrene support. The optimum hydrophobicity-hydrophilicity balance of the new resin is exploited for the efficient synthesis of these peptides.

A brief account of the recent developments in solid phase peptide synthesis, including new support, deprotecting agents, coupling agents, new linkers, new types of amino protecting groups, novel cleaving agents and a description of hydrophobic peptides are covered in the review.

Experimental section has two parts, (1) dealing with the polymer preparation and functionalisation and (2) the detailed procedure for the solid phase synthesis of peptides.

The first section of the results and discussion dealt with the characterisation of the polymer support by IR, ^{13}C NMR and SEM. The synthesis of peptides was illustrated in the next part. Their purification by HPLC,

characterisation by amino acid analysis, NMR and circular dichroism techniques were explained with figures.

The list of partial sequences of thioredoxin synthesised are

1) H–Asp–Lys–Ile–Ile–His–Leu–Thr–OH–(T 2–8).

2) H–Ser–Phe–Asp–Thr–Asp–Leu–Val–Lys–OH (T 11–18).

3) H–Ala–Ile–Leu–Val–Asp–Phe–Trp–Ala–OH–(T 22–29).

4) H–Met–Ile–Ala–Pro–Ile–Leu–Asp–Glu–Ile–Ala–Asp–Glu–Tyr–Gln–Gly–Lys–OH (T 37–52).

5) H–Leu–Thr–Val–Ala–Lys–Leu–OH–(T 53–58).

6) H–Asn–Ile–Asp–Gln–Asn–Pro–Gly–Thr–Ala–OH–(T 59–67).

7) H–Pro–Lys–Tyr–Ile–Gly–OH (T 68–72).

8) H–Ile–Gly–Arg–Gly–Ile–Pro–Thr–Leu–Leu–Leu–Phe–OH–(T 71–81).

9) H–Thr–Leu–Leu–Leu–Phe–OH–(T 77–81).

10) H–Ala–Thr–Lys–Val–OH–(T 88–91).

11) H–Gly–Ala–Leu–Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu–OH–(T 92–107).

12) H–Ser–Lys–Gly–Gln–Leu–Lys–Glu–Phe–Leu–Asp–Ala–Asn–Leu–OH–(T95–107).

These peptides were synthesised on a 2% chloromethylated HDODA-PS resin using Boc-benzyl ester strategy and finally cleaved by neat TFA. DCC was the coupling agent used and NMP as the medium for coupling. The peptides were purified by making use of protein purification techniques and characterised by amino acid analysis, NMR and CD. Most of the peptides were obtained in good yield (> 80%) and purity (> 85%).

Besides these partial sequences of thioredoxin, two designed hairpin peptides were also synthesised on a chloromethylated 2% PS-HDODA resin. They are,

(a)

```
Dpro–Gly
  |     |
Val   Val
  |     |
Lys   Glu
  |     |
Val   Val
  |     |
Glu   Lys
```

(b)

```
      Dpro–Gly
        |     |
      Val   Val
        |     |
      Leu   Leu
        |     |
      Val   Val
        |     |
Acm–Cys     Cys–Acm
        |     |
      Ala   Ala
```

These peptides were synthesised by using Boc strategy and finally cleaved by 5% aqueous TFA. DCC/HOBt coupling method in NMP was used for all the attachments of amino acids. Both the hairpin peptides were obtained in high yield (> 90%) and purity (> 95%). They were further purified by HPLC and subjected to conformational analysis.

The synthesis of helices on solid support is more easy and straight forward because the chain elongation is always in one direction. But the synthesis of sheet peptides require the reversal of synthesis direction and usually goes towards the support. Acylation at this stage is very critical and results in truncated sequences and thus lower yield. But in the case of these two hairpin peptides most of the acylations were completed by the first coupling itself. Thus these two designed sequences with varying chain length have been synthesised successfully on a 2% chloromethylated PS-HDODA resin with high purity and high yield.

In the case of thioredoxin sequences also, both hydrophobic and hydrophilic sequences were synthesised without any difficulty using this new support. The conformations of most of the partial sequences obtained by CD analysis were in good agreement with the structure of corresponding fragment of thioredoxin. The flexible nature and the favourable swelling and solvation characteristics of the support facilitated effective synthesis. These studies proved that the new resin is suitable for the synthesis of hydrophobic, hydrophilic and designed β-hairpin peptides.

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