Chapter – IV

Experimental

CHAPTER IV

EXPERIMENTAL

4.1 PREPARATION AND FUNCTIONALISATION OF HEXANEDIOL DIACRYLATE - CROSSLINKED POLYSTYRENE

4.1.a Materials and Methods

The polymers under study were synthenized and characterized in this laboratory. IR spectra were recorded on a Shimadzu 1R-470 spectrophotometer using KBr pellets.

4.1.b. Source of Chemicals

The monomers and regents required for synthesis were purchased from the following sources

	Chemicals	***Sources***
1.	*Styrene*	*Sigma Chemical Company, USA*
2.	*1,6 - Hexanediol diacrylate*	*Sigma Chemical Company, USA*
3.	*Polyvinylalcohol (mol.wt.72,000)*	*Sigma Chemical Company, USA*
4.	*Benzoyl peroxide (recrystallised before use).*	*Sisco, Bombay*

4.1.c Preparation of 2% HDODA-crosslinked Polystyrene by Suspension Polymerisation

Styrene was destabilized by washing with 1% sodium hydroxide solution (20mlx3) and distilled water (20mlx3) and dried over anhydrous calcium chloride. A 1% solution of polyvinylalcohol (mol.wt. 72,000; 3.46g) in water (346 ml) was prepared and kept mechanically stirred in a cylindrical polymerisation vessel under nitrogen atmosphere on a water bath at 80°C. A mixture of HDODA (0.9ml, 2mmol), styrene (22.4ml, 98mmol), toluene (20ml) as inert diluent, and benzoyl peroxide (1g) was prepared and flushed with nitrogen gas. This mixture was then added to PVA solution kept at 80°C with stirring. The polymerization was allowed to proceed for 6h. The white shiny beads obtained were collected by filtration through a sintered funnel (G-2) and washed thoroughly with hot water (20ml x 3, 3min), acetone (20ml x 3, 3min), methanol (20ml x 3, 3min) and drained.

The resin obtained was soxhleted using acetone to remove all the low molecular weight impurities and linear polymers and dried in the oven at 50°C. Beads were sieved into different sizes using standard sieves.

4.1.d. Chloromethylation of HDODA-crosslinked Polystyrene: [192] General Procedure

The resin beads (200-400 mesh, 2g) was allowed to swell in dry dichloromethane (20ml) in a round bottomed flask. Chloromethyl methyl ether (12 ml) and a solution of anhydrous zinc chloride in THF (IM , 0.4 ml) was added

to the swollen resin under anhydrous conditions slowly with shaking. The mixture was refluxed at 50°C for 5h. It was then cooled and filtered through a sintered glass funnel (G-2), washed with THF (20 ml x 3, 3 min), finally with methanol and drained. It was soxhletted using THF and dried.

i. Preparation of chloromethyl methyl ether[193]

To a mixture of formaldehye (60 ml) and methanol (33 ml), kept at 0°C, a constant stream of dry HCl gas was passed. The formation of ether was indicated by the appearance of an oily layer, after one hour. Administration of HCl was continued for half an hour more till the ether was separated clearly from the aqueous phase. The oily layer was separated and dried over calcium chloride. Yield : 45 ml. This was used further without purification.

ii. Preparation of 1M anyhydrous $ZnCl_2$ in THF

Anhydrous $ZnCl_2$ (1.5 g) was placed in an Erlenmeyr flask. Conc. HCl (3 drops) and water (5 drops) were added and the contents stirred and heated until the solid dissolved completely. Temperature was gradually raised till a solid mass of $ZnCl_2$ was left which was melted on further heating. When it became a mobile liquid the flask was kept in a desiccator, and allowed to cool. The solid was dissolved in THF (10 ml) and kept sealed.

iii. Estimation of chlorine capacity by pyridine fusion method[194] : General prodecure

The cholоromethyl resin (200 mg) was fused with pyridine (5 ml) in a

boiling tube at 110°C for 5 h. The solution was quantitatively transferred with acetic acid/water (1:1, 30 ml) and diluted with water (25ml), conc. HNO_3 (7ml) and $AgNO_3$ (1N, 10ml) were added to this solution and titrated against standard ammonium thiocyanate solution (0.1N) using ferric alum as indicator. A blank titration was also performed.

4.2 SOLID PHASE PEPTIDE SYNTHESIS

4.2.a Source of Chemicals

The special chemicals required for the solid phase peptide synthesis were purchased from the following firms. All side chain protected L-amino acids, t-butyl carbazate, dicyclohexylcarbodiimide, t-butyloxycarbonyloximino-2-phenyl acetonitrile (Boc-ON), thioanisole, 1,2-ethanedithiol and cesium carbonate were purchased from Sigma Chemical Company, USA. Boc-Gly, Boc-Ala, Boc-Leu, Boc-Ile, Boc-Pro and Boc-Val were prepared in the laboratory following Schnabel's procedure and Boc-ON method. All solvents were of regent grade and were obtained form E. Merck, India.

All (L) amino acids were Boc protected. The various side chain protecting groups are :

Protecting group	*Amino acid*
2 Cl (Z)	Lys
Mts	Arg
Z	Glu, Asp, Ser, Thr, Tyr

4.2.b Purification of Solvents and Reagents

All the solvents used for peptide synthesis were purified before use. *Dichloromethane (DCM)* : DCM was dried by adding fused calcium chloride and kept over night.

N-Methyl-2-Pyrrolidone (NMP) : NMP was kept over molecular sieve (2A°) in amber coloured bottle.

Tetrahydrofuran (THF) : THF was freed from peroxide by shaking with alumina, dried using sodium wire, till all the water was removed, distilled and kept in amber coloured bottle.

Diisopropyl ethylamine (DIEA) : Distilled over ninhydrin and kept in amber coloured bottle.

Benzene : Dried using sodium wire and distilled.

Diethyl ether : Dried over fused calcium chloride over night.

Ethyl acetate : Commercial ethyl acetate was distilled and used for extraction purposes.

4.2.c Preparation of Reagents and Amino acid Derivatives

i. Preparation of Boc-azide from t-butyl carbazate[196]

Boc-azide was prepared from t-butyl carbazate by the method of Carpin.Tertiary butylcarbazate (30g) was dissolved in glacial acetic acid (27 ml)

and water (37.5 ml). Sodium nitrite (17.4 g) was added to it in small portions over a period of 15 min. During the addition of sodium nitrate, the solution was stirred vigorously while maintaining the temperature at 0°C. After 90 min, the oil layer was separated form the aqueous layer. This layer was extracted with ether (20ml x 3). The ether extracts were mixed with oil layer. Washed with water and sodium bicarbonate (1M) and dried over anhydrous sodium sulphate. On evaporating ether under reduced pressure Boc-azide was obtained as a golden yellow liquid. It was stored in the refrigerator and used directly without further purification. Yield : 0.25ml.

ii. Synthesis of Boc-amino acids - Schnabels Method : General Procedure[196]

The amino acid (10 mmol) was suspended in dioxane/water (1:1, 10 ml) and Boc-azide (1.6 ml, 10 mmol) was added to it. The mixture was stirred at room temperature, maintaining the pH in the alkaline range with 4N NaOH. 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 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 addition of petroleum ether is needed after the evaporation of ethyl acetate to get the Boc-amino acid as a white power.

In the case of leucine, the acidified aqueous layer was extracted with ether (25 ml x 3) instead of ethyl acetate. The pH to be maintained during preparation and yields obtained for the various amino acids are given in Table IV.1.

Table IV.1. Details of Boc-amino acids prepared by Schnable's method

Amino acid derivative	pH	Yield %
Boc - Gly	10	87
Boc – Ala	10	90
Boc – Leu	10	95
Boc – Ile	10	94
Boc – Pro	8-9	75
Boc – Val	10	70

iii. Boc-ON method : General procedure[197]

Amino acid (10 mmol), 4-t-butyloxycarbonyl oximino-2-phenyl acetonitrile (Boc-ON) (2.71g, 11mmol) 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 (2 x 12.5 ml). The organic layer was dried over anhydrous sodium sulphate, rotary evaporated to remove ethyl acetate. On adding petroleum ether, Boc-amino acid was obtained. A list of Boc-amino acids prepared by the Boc-ON method are given in Table IV.2.

Table IV. 2 Details of the Boc-amino acids prepared by Boc-ON method

Amino acid derivative	Yield %
Boc – Gly	87
Boc – Ala	90
Boc – Leu	90
Boc – Ile	90
Boc – Pro	70
Boc – Val	85

4.2.d. Purity of Boc-Amino Acids

Purity of all Boc-amino acids was checked by TLC on pre-coated silica gel plates using chloroform/methanol/acetic acid (85:10:5) as the solvent system. Amino acids were visualized by ninhydrin vapour after 10 min exposure to HCl vapour. All the Boc-amino acids which were solids were stored at room temperature and oily derivatives were stored in refrigerator.

4.2.e. Preparation of 1-Hydroxybenzotriazole (HOBt)

HOBt was prepared following the procedure adopted by Konig and Geiger.[198] O-chloronitrobenzene (32g) was dissolved in ethanol (100 ml). Hydrazine hydrate (30 g) was added and the solution was refluxed for 5h. After distilling off ethanol, the residue was diluted with water (100 ml) and extracted

with ether (20 ml x 3). The aqueous layer was acidified with concentrated HCl and the HOBt got precipitated. It was recrytallised from hot water. Yield : 20g (80%) ; Melting point : 155°C.

4.2.f Solid Phase Synthesis : General Procedure

Manual solid phase peptide synthesis using Boc strategy was done in a glass reaction vessel. The C-terminal amino acid was esterified to the resin via a benzyl ester linkage by the cesium salt of the Boc-amino acid. Boc group was deprotected by 33% TFA in DCM and neutralisation effected by 5% DIEA in DCM or 5% TEA in DCM. The second Boc-amino acid was coupled to the free amino acyl resin using DCC/HOBt, active ester method. Either DCM or NMP was used as solvent. The same procedure was adopted for the coupling of all amino acids. Progress of coupling was monitored at every stage by semi quantitative ninhydrin test. In all couplings, 2.5 fold molar excess of Boc-amino acid was used and double coupling was done to ensure completion of the reaction. Final cleavage of peptide form the support was obtained by TFA in the presence of acid scavengers.

4.2.g Attachment of First Amino Acid to the Resin

i. Cesium salt method[199,200] *: General procedure*

The Boc-amino acid (2.5 mmol) was dissolved in ethanol (10 ml) and neutralized with saturated solution of cesium carbonate (slow addition till the pH reached 7). Ethanol was evaporated under vacuum and water was removed

by azeotropic distillation with dry benzene, till white powder of cesium salt of amino acid was obtained.

The cesium salt of Boc-amino acid was dissolved in minimum quantity of NMP and the dried cholromethylated resin (1g, 1mmol) was added. The suspenstion was kept at 50°C in an oil bath, with occasional shaking for 48 hours, The resin was filtered, washed with NMP, NMP/water (1:1), methanol, DCM and dried under vacuum and the weight was noted.

ii. Estimation of first amino acid substitution:[201] *: General procedure*

The Boc - amino acid resin (10 mg) was kept for deprotection in 33% TFA in DCM for 30 min. Filtered, washed with DCM (6 times) to get rid of TFA completely. Neutralized with 5% DIEA in DCM for 5 min and again washed with DCM and dried. From this deprotected resin, 5 mg, was taken in 5 ml sintered funnel or pasteur pipet with cotton at the narrow end. It was treated with 0.1M DCM solution of picric acid for 5 min. The unbound picric acid was washed off with DCM. The picric acid bound to the resin was separated and collected with 5% TEA in DCM (2 x 2ml, 3min) followed by DCM wash (3 x 2ml, 3 min). 0.5ml of the elute was made up to 5ml with 95% ethanol, and the absorbance at 358 nm was measured. TEA-picrate complex in ethanol has $\varepsilon_{\lambda_{358}}$ = 14,500. From the OD value and weight of the resin taken, the amount of substituted amino acid on the resin was calculated.

4.2.h Deprotection of t-Boc Group[202] : General Procedure

When the amino acid or peptides contains t-butyloxy carbonyl group (t-Boc) as their amino protecting group, it can be deprotected using anhydrous TFA in DCM (33%). For this 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 wahing with DCM and the salt thus obtained was neutralized with DIEA in DCM (5%)

4.2.i Methods for Activation and Coupling

Symmetrical anhydride method and HOBt active ester method was used for the formation of peptide bond.

i. Anhydride method

A mixture of 2mmol of amino acid and 1 mmol of dicyclohexyl-carbodiimide (DCC) was added to 1mmol of amino resin in DCM. The mixture was shaken for half an hour, and washed with MeOH-DCM mixture (33:65, 5ml x 3, 3min) and DCM (5ml x 3, 2min).

ii. HOBt - active ester method

Active esters of amino acids were prepared by adding 2.5 mequiv of HOBt, 2.5 mequiv of DCC and 2.5 mequiv of amino acid in NMP. This was stirred for 45 min and the HOBt ester of amino acid was added to the amino

resin/peptidyl resin (1.0 mequiv). The mixture was shaken for 45 minutes. Filtered off the solution and washed the resin with 33% MeOH/DCM (5mlx3, 2min) DCM (5ml x 3, 2 min) and NMP (5ml x 3, 2 min).

4.2.j Cleavage of Peptide from the Resin by TFA/Thioanisole Method

The peptidyl resin (100 mg) was suspended in TFA (10 ml) and to this thioanisole (0.1ml) and in cresol (0.1ml) and 1,2-ethanedithol (0.1ml) were added. The reaction mixture was kept for 12h at room. It was filtered and TFA sloution was rotary evaporated to remove TFA. The peptide was then precipitated by addition of ice cold ether and washed thoroughly with ether, centrifuged (8-10 times) and dried.

4.2.k Purification and Analysis

i. Purification by TLC

TLC was used for estimating the purity both of starting materials for SPPS and of synthesized short peptides. Aqueous or methanolic solution of the peptide was spotted on the TLC plate and developed in a suitable solvent mixture of appropriate composition. The solvent system used were:

a. Butanol - 1 : Acetic acid : Water (4 : 1: 5)

b. Chloroform : Methanol : Water : Acetic acid (7 : 4: 1: 1)

Identification sprays

The following reagent were used to visualize the spots on the plate. *Ninhydrin spray* : The Boc group was removed by exposing the plate to

vapours of conc. HCl contained in a chamber. The spots were developed by spraying ninhydrin reagent and kept in an oven for 5 min. Violet spots were observed in the case of free primary amino groups.

Chlorine gas/starch spray/KI reagent (Rydon's reagent): For protected peptides which are not visible with ninhydrin test, this spray can be used. This test is given by almost all compounds containing -NH groups. Appear as blue black spots on a faint blue back ground. The plate was exposed to chlorine gas and sprayed with a mixture containing equal volumes of 1% aqueous starch and pottasium iodide solutions.

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

ii. *High performance liquid chromatography*

Development of HPLC has caused a revolution in peptide purification and analysis and has brought about great increases in resolving power and speed of operation of column chromatography. HPLC analyses were done using a gradient solvent system consisting of (A) 0.1% TFA and (B) 50% CH_3 CN in A, on a ultropac column (Lictrosorb, RP 18, 5µm, 4x 250mm).

The crude peptides were purified by reversed phase HPLC on a preparative column (Biorad, Hi-pore RP-318, 25 x 2.15 cm) using a gradient system consisting of 0.1% (W/V) TFA in water and acetonitrile at a flow rate of 2 ml/min was used. Peptides after purification were checked on reversed analytical HPLC. Detections were done at 230 and 280nm.

iii. *Amino acid analysis*

Amino acid analysis was used for the characterisation of peptides. Amino acid analysis of free peptides were done after hydrolysing the samples. The free peptides were dissolved in 1ml of 50% CH_3COOH. From that 100µl was taken in a sample tube and 1 ml of conc. HCl. was added to it. The tube was sealed under vacuum and heated at 100°C for 24h. The residue was dried and made up to 250µl by loading buffer (sodium citrate, pH 2.2). Certain µl of this solution which should be equivalent to 20 nmoles of peptide was injected to the amino acid analyzer (Shimadzu column, CTU 10A, temp. 55°C, Spectroflourimetric detector, Styrene-DVB cation exchange resin).

Buffer used : (1) Buffer A : pH 2.20
Sodium citrate, 70% Alcohol, Perchloric acid

(2) Buffer B : pH 10
Sodium citrate, Boric acid

(3) Buffer C : 2M NaOH

Reagents used : (1) A - Sodium hypochlorite

(2) B - Orthophthalaldehyde (OPA)

iv. Mass spectroscopy

Sequencing a peptide by the recording of a single spectrum is a very attractive proposition which stimulated considerable research. Mass spectrometric analysis can be used for the accurate determination of molecular weight of proteins, peptides and oligonucleotides.

Electrospray ionisation has recently emerged as a powerful technique for producing intact ions in vacuo from large and complex species in solution. To an extent greater than has previously been possible with the more familiar *soft ionisation* methods, this technique makes the power and elegance of mass spectrometric analysis applicable to large biomolecules such as polypeptides. The idea of using ES dispersion of an analytic solution in gas to produce solute ions for mass analysis originated with Dole *et al.*[203]

In the electrospray ionisation process, a flow of sample solution is pumped through a narrow bore metal capillary held at a potential of a few kilovolts relative to a counter electrode. Charging of the liquid occurs and as a result, it sprays (aerosol) from the capillary orifice as a mist of very fine, charged droplets. Solvent is striped away by a heated inert gas. The spraying process takes place at atmospheric pressure in a specially designed chamber, outside the vacuum region of mass spectrometer. The whole ionisation process is, in fact a form of atmospheric pressure ionisation (API). A number of small droplets are formed from charged droplets which undergo reduction in size and became unstable and explode due to solvent evaporation. Because of excess charge, field created is large enough to cause the desorption of ionized sample molecules from the droplet. These ions, which are field desorbed are then sampled through a series of skimmers and lenses focus the ions in to a beam. After pumping away the drying gases. These ions are transferred into the vacuum system for mass analysis.

ESI spectra records the molecular ions as multiple charged, $(M + nH)^{n+}$ in the positive ion mode or $(M-nH)^{n-}$ in the negative ion made and also cover a

range of charge states. On average one charge is added per 1000 Da in mass. As a result, mass assignment can be made with greater precision and more confidence than is possible with spectra of singly charged ions.

Electrospray ionisation makes possible the mass analysis of proteins with molecular weight upto 130,000 Da.[204] McLafferty and colleagues[205] proved the compatibility of ES with Fourier Transform Ion Cyclotron Resonance (FTICR) to provide very high resolution in the analysis of very large ions promises to be a fruitful union. ES/MS is sensitive to Femtogram to picogram of sample.

4.2.1 Synthesis of Model Peptides

i. Synthesis of Ala-Pro-Ala

Attachment of Boc-Ala to chloromethyl resin Boc-Ala (80.75mg; 0.475 mmol) was dissolved in ethanol and converted to cesium salt by adding in saturated solution of cesium carbonate till the solution becomes neutral. Ethanol was evaporated under pressure and water removed by azeotropic distillation with benzene. The cesium salt was dissolved in minimum volume of NMP and chloromethyl resin (100 mg; 19 mmol) was added and kept at 50°C for 48h. The resin was washed with NMP (20 ml x 3), 1:1 NMP - water (20 ml x 3), water - methanol (20 ml x 3), DCM (20 ml x 3) and dried under vacuum.

Synthesis of Ala-Pro-Ala

The deprotection of Boc-Ala was done with 33% TFA in DCM for 30 minutes and the resin was washed with DCM (10ml x 6, 2min) and neutralized

with 5% DIEA in DCM (1ml x 2, 2min) and NMP (10ml x 2, 2min). This Boc-Pro (81.7mg, 38 mmol) and Boc-Ala (71.82, 38 mmol) were coupled successively by HOBt active ester method. Active ester was prepared by shaking the respective amino acid with HOBt (51.3mg, 0.38mmol) and DCC (78.24 mg, 38 mmol) in NMP. DCU formed was filtered off and the active ester was added to the resin. After the synthesis the peptidyl resin was washed with NMP (5 ml x 2, 2 min), 33% MEOH in DCM (10 ml x 3, 2 min), DCM (10 ml x 3, 2 min) and dried in vacuum. The details of one synthetic cycle is given below in Table IV.3

Table IV.3. Details of a synthetic cycle in SPPS

Step	Reagent	Time
1	DCM	5 ml x 4, 2 min
2	33% TFA in DCM	1 ml, 30 min
3	DCM	10 ml x 6, 2 min
4	DIEA (5% in DCM)	1 ml x 2, 2 min
5	DCM	10 ml x 2, 2 min
6	NMP	10 ml x 4, 2 min
7	HOBt active ester coupling 1:1:1 (Boc amino acid, DCC, HOBt)	5 ml, 45 min
8	NMP	5 ml x 2, 2 min
9	DCM	5 ml x 1, 2 min
10	33% MeOH in DCM	10 ml x 3, 2 min
11	DCM	10 ml x 3, 2 min
12	Repetition of steps 6 - 11	
13	Kaiser test	

Cleavage of the peptide from the resin

50 mg of the peptidyl resin was treated with TFA (5 ml), thioanisole (0.05 ml) and 1,2 - ethanedithiol (0.05 ml) at room temperature for 18 h. The resin was removed by filtration and TFA removed by rotary evaporation. The peptide was precipitated by additions of ice cold ether, to get rid of TFA and scavengers, dried and the yield was noted (12 mg). The purity of the peptide was checked by TLC.

ii. Synthesis of Ala-Ala-Pro-Ala

Attachment of Boc-Ala to chloromethylated resin

A 2% HDODA-PS resin was used for this synthesis. Boc-Ala (89.75 mg, 0.475 mmol) was dissolved in ethanol. Saturated solution of cesium carbonate was added to make the solution neutral. It was stirred for sometime and rotary evaporated to remove ethanol. Water was removed by azeotropic distillation with benzene. This was repeated to get white powder of cesium salt and dried under vacuum. Cesium salt of Boc-Ala was dissolved in NMP, chloromethyl resin (100 mg, 0.19mmol) was added and heated at 50°C for 48 h. The resin was filtered, washed with NMP (10ml x 3, 2min), NMP-water (10ml x 3, 2 min), water, methanol (10ml x 3, 2min), DCM (10ml x 3, 2min), drained and dried under vacuum. The substitution level of Boc-Ala was determined.

Synthesis of Ala-Ala -Pro-Ala

Deprotection of Boc-Ala resin was done using 33% TFA in DCM for 30 minutes. The resin was washed with DCM (10ml x 6, 2min) and neutralised with 5% DIEA in DCM (1ml x 2, 5 min). The resin was washed with DCM (10ml x 4, 2min) and NMP (10ml x 2, 2min). Then Boc-Pro (81.7mg, 0.38 mmol) Boc-Ala (71.82, 0.38mmol) were coupled succesively by HOBt active ester method. Active ester was prepared by shaking the respective amino acid with HOBt (51.3mg, 0.38mmol) and DCC (78.24mg, 0.38mmol) in NMP. DCU formed was filtered off and filtrate was added to the resin. The mixture was shaken for 45 minutes. After synthesis, peptidyl resin was washed with NMP (5ml x 3, 2 min) DCM (5 ml x 1, 2 min) 33% MeOH in DCM (10 ml x 3, 2in), DCM (10ml x 3, 2min) and dried in vacuum. The protocol for a cycle of operation is given below.

1. Washed the resin with DCM (5ml x 4, 2 min)
2. Added 33% TFA in DCM for deprotection (1ml, 30 min)
3. Filtered off the TFA solution
4. Washed the resin with DCM (10 ml x 6, 2 min)
5. Added 5 % DIEA in DCM for neutralisation (1ml x 2, 5 min) with DCM (10 ml x 4, 2 min)
6. Washed the resin with DCM (10 ml x 4, 2 min)
7. Washed the resin with NMP (10 ml x 2, 2 min)
8. Added active ester of Boc - amino acids
   (Prepared by shaking 0.38 mmol each of Boc - amino acids, HOBt and DCC in NMP).
9. Filtered off the solution, washed with NMP (5ml x 2, 2min)
10. Washed the resin with DCM (5ml x 1, 2 min)

11. Washed the resin with 33% MeOH in DCM (10 ml x 3, 2 min)
12. Washed the resin with DCM (10 ml x 3, 2 min)
13. Repeat Steps 7-12 for completion of reaction
14. Kaiser test

Cleavage of peptide from the resin

50 mg of the peptidyl resin was treated with TFA (5ml), thioanisole (0.05ml) and 1,2 - ethanedithiol (0.05ml) at room temperature for 18 h. The resin was removed by filtration and TFA was removed by rotary evaporation. The peptide was precipitated by the addition of ice-cold ether. The precipitated peptide was washed several times with ether to remove TFA and scavengers, dried and weight was noted (16 mg).

iii. Synthesis of Ala-Ala -Ala-Ala -Ala

Attachment of Boc-Ala to the chloromethyl resin

To Boc-Ala (89.75 mg, 0.475 mmol) in ethanol (5 ml), a saturated solution of cesium carbonate was added till the pH of the solution reached 7.0. The solution was stirred for 2h. Ethanol was rotary evaporated under reduced pressure. Water was removed by azeotropic distillation with dry benzene. The white powder of cesium salt was dried under vacuum. The salt was dissolved in NMP (3 ml), and to this 100 mg of chloromethylated resin of capacity 1.9 mmol/g was added. The mixture was kept at 50°C for 48 h. The resin was filtered, washed with NMP (5ml x 3, 3 min), NMP - water (5ml x 3, 3 min), water, methanol (10 ml x 3, 2 min), DCM (10ml x 3, 2 min), drained and dried under vacuum. The substitution level of Boc-Ala was determined.

Synthesis of Ala-Ala-Ala-Ala-Ala

The Boc-Ala protection was removed by 33% TFA in DCM. The resin was washed with DCM (10 ml x 6, 2 min) and 5% DIEA in DCM (5ml x 2, 5min) was used for neutralization. The resin was washed with DCM (10 ml x 4, 2 min) NMP (10 ml x 2, 2min). The successive Boc-Ala were coupled by active ester method. For this Boc-amino acid (0.38 mmol), was dissolved in NMP. HOBt (0.38 mmol) and DCC (0.38mmol) were added to it and stirred for 45 minutes. DCU formed was filtered off and the filtrate was added to the resin. After the synthesis, the peptide resin was washed with NMP (5 ml x 2, 2 min), DCM (5 ml x 1, 2 min), 33% MeOH/DCM (10 ml x 3, 2min) DCM (10 ml x 3, 2 min) and dried under vacuum. The protocol for synthesis is given below in Table IV.4.

Table IV.4 : Steps involved in the SPPS of Ala-Ala-Ala-Ala-Ala

Operation No.	Steps involved	Reagent/solvent	Time(min)
1	Wash	DCM	5ml x 4, 2 min
2	Boc-deblocking	33% TFA/DCM	1 ml, 30 min
3	Wash	DCM	10 ml x 6, 2 min
4	Neutralisation	5%DIEA/DCM	1 ml x 2, 5 min
5	Wash	DCM	10 ml x 4, 2 min
6	Wash	NMP	10 ml x 2, 2 min
7	Coupling	Boc-amino acid :HOBt : DCC in NMP (1:1:1)	45 min
8	Wash	NMP	5ml x 2, 2min
9	Wash	DCM	5 ml x 2, 2 min
10	Wash	33% MeOH in DCM	10 ml x 3, 2min
11	Wash	DCM	10 ml x 3, 2min
12	Steps 6-11 repeated for further couplings		
13	Kaiser test		

Cleavage of the peptide from the resin

Peptidyl resin (50 mg) was treated with TFA (5ml), thioanisole (0.05ml) and 1,2-ethanedithiol (0.05ml) at room temperature for 18 h. The resin was removed by filtration and TFA by rotary evaporation. The peptide was precipitated by the addition of ice cold ether. The precipitated peptide was washed several times with ether to remove TFA and scavengers, dried and weight was noted (23 mg).

iv. Synthesis of Ala-Ala -Pro

Attachment of Boc-Pro to Chloromethyl resin

A 2% HDODA-PS resin was used for this synthesis. Boc-Pro (102.12 mg, 0.475 mmol) was dissolved in ethanol and converted to cesium salt by adding a saturated solution of cesium carbonate till the pH reached 7. Ethanol was evaporated under pressure and water was removed by azeotropic distillation with benzene. The cesium salt was dissolved in minimum volume of NMP and chloromethyl resin (100 mg, 0.19 mmol) was added and kept at 50°C for 48 h. The resin was washed with NMP (5ml x 3, 3 min), NMP - water (10 ml x 3, 3min), water- methanol (10 ml x 3, 2 min), DCM (10ml x 3, 2min), drained and dried under vacuum.

Synthesis of Ala-Ala -Pro

Deprotection of Boc-Pro was done using 33% TFA in DCM for 30

minutes. The resin was washed with DCM (10 ml x 6, 2 min) and neutralised with 5% DIEA is DCM (1ml x 2, 5 min). The resin was washed with DCM (10 ml x 4, 2 min) and NMP: (10ml x 2, 2 min). The successive Boc-alanines were coupled by HOBt active ester method. HOBt active ester was prepared by dissolving respective Boc - amino acid (0.38 mmol) in NMP. HOBt (0.38 mmol) and DCC (ratio of Boc - amino acid : HOBt : DCC is 1:1:1) were added to this and shaken well for 45 minutes. After the synthesis, the peptide was washed with NMP, (5ml x 2, 2 min), DCM (5ml x 1, 2 min), 33% MeOH/DCM (10 ml x 3, 2 min) and dried under vacuum. The protocol for synthesis is given below.

1. Washed the resin with DCM (5 ml x 4, 2min)
2. Added 33% TFA/DCM for deprotection (1 ml, 30 min)
3. Filtered off the TFA solution
4. Washed the resin with DCM (10 ml x 6, 2 min)
5. Added 5% DIEA/DCM for neutralization (1 ml x 2, 5 min)
6. Filtered off the solution and washed with DCM (10 ml x 4, 2 min).
7. Washed the resin with NMP (10 ml x 2, 2 min)
8. Added active ester of Boc - amino acids
   (Prepared by shaking Boc amino acid, HOBt and DCC in NMP in 1:1:1 ratio for 45 minutes).
9. Filtered off the solution, washed with NMP (5 ml x 2, 2 min)
10. Washed the resin with DCM (5 ml x 1, 2 min)
11. Washed the resin with 33% MeOH / DCM (10 ml x 3, 2 min)
12. Washed the resin the DCM (10 ml x 3, 2 min)
13. Repeated steps 6-12
14. Kaiser test

Cleavage of peptide from the resin

The peptidyl resin (50 mg) was treated with TFA (5 ml), thioanisole (0.05ml) and 1,2-ethanedithiol (0.05ml) at room temperature for 18 h. The resin was removed by filtration and the filtrate was subjected to rotary evaporation to remove TFA. The peptide was precipitated by adding ice-cold ether. The precipitated peptide was washed several times with ether to remove TFA and scavengers, dried and weight was noted (28 mg).

e. *Synthesis of Ala-Ala-Ala-Pro-Ala*

Attachment of Boc-Ala to chloromethylated resin.

Boc-Ala (89.75 mg, 0.475 mmol) was dissolved in ethanol (3 ml), to this saturated solution of cesium carbonate was added drop by drop for making the solution neutral. Stirred this for 2 h. Ethanol was removed under reduced pressure and water was removed by azeotropic distillation with benzene. The white powder obtained was dried under vacuum. This cesium salt of Boc - amino acid was dissolved in NMP (2 ml) and added the solution to weighed resin (100 mg, 0.19 mmol), kept this for 48 h at 50°C. Then resin was washed with NMP (5ml x 3, 3 min), NMP water (5 ml x 3, 3 min), water, methanol (10 ml x 2 min), DCM (10ml x 3, 3min), drained and dried under vacuum.

Synthesis of Ala-Ala-Ala-Pro-Ala

Added 33% TFA/DCM, for the deprotection of Boc-Ala. The resin was washed with DCM (10 ml x 6, 2 min) and neutralized with 5% DIEA/DCM

(1ml x 2, 5 min). The resin was washed with DCM (10 ml x 4, 2 min) and NMP (10 ml x 2, 2 min). Boc-Pro (81.7 mg, 0.38 mmol) and successive Boc-Alanines (71.82 mg, 0.38 mmol) were coupled by active ester method. Active ester of HOBt was prepared by dissolving Boc-amino acid, HOBt and DCC in 1:1:1 ratio (0.38 mmol each) in NMP and stirred for 45 minutes. DCU formed was removed by filtration and filtrate was added to the resin. After synthesis peptidyl resin was washed with NMP (5 ml x 2, 2 min), DCM (5 ml x1, 2 min), 33% MeOH/DCM (10 ml x 3, 2 min), DCM (10 ml x 3, 2 min) and dried under vacuum. The synthetic protocol is given in Table IV.5.

Table IV.5 : Synthetic protocol for the SPPS of Ala-Ala-Ala-Pro-Ala

Operation No.	Steps involved	Reagent / Solvent	Time (min)
1.	Wash	DCM	5ml x 4, 2 min
2.	Deprotection	33% TFA / DCM	1ml x 30 min
3.	Wash	DCM	10ml x 6, 2 min
4.	Neutralization	5% DIEA/DCM	1ml x 2, 5 min
5.	Wash	DCM	10ml x 4, 2 min
6.	Wash	NMP	10ml x 2, 2 min
7.	Coupling	Active ester (Boc-amino acid : DCC: HOBt, 1:1:1 in NMP)	45 min
8.	Wash	NMP	5ml x 2, 2 min
9.	Wash	DCM	5ml x 1, 2 min
10.	Wash	33% MeOH/DCM	10ml x 3, 2 min
11.	Wash	DCM	10ml x 3, 2 min
12.	Repeat steps 6-11 for further couplings to ensure completion of reaction		
13.	Kaiser test		

Cleavage of peptide from the resin

To 50mg of peptidyl resin, 5 ml TFA, 0.05ml thioanisole and 0.05ml 1,2-ethanedithiol were added and kept at room temperature for 48 h. The resin was removed by filtration and the filtrate was subjected to rotary evaporation. The peptide was precipated by adding ice cold ether. The precipitated peptide was washed several times with ether to remove TFA and scavengers, dried and weight was noted (20 mg).

vi. Ala-Pro-Gly-Pro-Arg

Attachment of Boc-Arg to chloromethylated resin

Boc-Arg (0.216 g, 0.475 mmol) was dissolved in minimum amount of ethanol. Then, saturated solution of cesium carbonate was added drop by drop to it, till the pH reached 7. It was stirred for 2 h and rotary evaporated to remove ethanol. Water was removed by azeotropic distillation with benzene. The white powder obtained was dried under the vacuum. Cesium salt of Boc-Arg was dissolved in minimum amount of NMP, kept for 48 h at 50°C. The resin was washed with NMP (5ml x 3, 3 min), NMP-water (5 ml x 3, 3 min), drained and dried under vacuum.

Synthesis of Ala-Pro-Gly-Pro-Arg

Deprotection of Boc-Arg was done by 33% TFA/DCM for 30 minutes. The resin was washed with DCM (10 ml x 6, 2 min) and neutralized with 5% DIEA (10ml x 4, 2 min) and NMP (10 ml x 2m, 2 min).

Then Boc-Pro (81.7 mg, 0.38 mmol), Boc-Gly (66.5 mg, 0.38 mmol), again Boc-Pro and Boc-Ala (71.82 mg, 0.38 mmol) were coupled successively to the resin as their HOBt active esters. Active ester was prepared by shaking the respective Boc-amino acid with HOBt (51.3 mg, 0.38 mmol) and DCC (78.24 mg, 0.38 mmol) in NMP. DCU formed was filtered off and active ester was added to the resin. After the synthesis, the peptide was washed with NMP (5ml x 2, 2 min), DCM (5 ml x 1, 2 min), 33% MeOH / DCM (10 ml x 3, 2 min) and dried. The details of synthesis is given below.

1. Washed the resin with DCM (5 ml x 4, 2 min)
2. Added 33% TFA/DCM for deprotection (1ml, 30 min)
3. Filtered off TFA solution
4. Washed the resin with DCM (100 ml x 6, 2 min)
5. Added 5% DIEA/DCM for neutralization (1 ml x 2, 5 min)
6. Filtered off the solution, washed with DCM (10 ml x 4, 2 min)
7. Washed with NMP (5ml x 2, 2 min)
8. Added active ester of Boc-amino acids
   (Prepared by shaking Boc-amino acid, HOBt, and DCC in 1:1:1 ratio in NMP) (45 minutes)
9. Filtrated off the solution, washed with NMP (5ml x 2, 2 min)
10. Washed with DCM (5 ml x 1, 2 min)
11. Washed with 33% MeOH/DCM (10ml x 3, 2 min)
12. Washed with DCM (10ml x 3, 2 min)
13. Repeat steps 6-12 for second coupling to ensure completion of reaction
14. Kaiser test

Cleavage of peptide from the resin

To 50 mg of peptidyl resin, TFA (5ml), thioanisole (0.05ml) and 1,2-ethanedithiol (0.05ml) were added kept at room temperature for 48 h. The resin was removed by filtration and the filtrate was subjected to rotary evaporation. The peptide was precipitated by adding ice cold ether. The precipitated peptide was washed several times with ether to remove TFA and scavengers, dried and weight was noted (20 mg).

4.2.m Synthesis of Short Fragments of Rubber Elongation Factor (REF) Protein

i. Synthesis of Gln-Gln-Gly-Gln-Gly (7-11)

Attachment of Boc-Gly to the chloromethyl resin

Boc-Gly (83.125 mg, 0.475 mmol) was dissolved in ethanol and the cesium salt was prepared by adding a saturated solution of cesium carbonate till the solution is neutral. Ethanol was evaporated under vacuum and water was removed by azeotropic distillation with benzene. The cesium salt was dissolved in minimum volume of NMP and chloromethyl resin (100mg, 1.9 mmol/g) was added and kept at 50°C for 48h. The resin was washed with NMP (10ml x 2, 2min), 1:1 NMP-water (10ml x 2, 2min), water, methanol (10ml x 2, 2min), water, methanol (10ml x 2, 2min), DCM (10ml x 3, 2 min) and dried under vacuum.

Synthesis of Gln-Gln-Gly-Gln-Gly (7-11)

The Boc protection was removed by 33% TFA in DCM. The resin was washed with DCM (10ml x 6, 2min) and neutralized with 5% DIEA in DCM. The resin was washed with DCM (10ml x 4, 2min) and NMP (10ml x 2, 2min). Then Boc-Gln (93.48, 0.38mmol), Boc-Gly (66.5, 0.38mmol), Boc-Gln (93.48, 0.38mmol), Boc-Gln (93.48, 0.38mmol) were successively coupled to the resin as their HOBt active esters. Active ester was prepared by shaking in respective Boc-amino acid with HOBt (51.3mg, 0.38mmol) and DCC (78.28mg, 0.38mmol) in NMP. DCU formed was filtered off and the active ester was added to the resin. After the synthesis, the peptidyl resin was washed with NMP (5ml x 2, 2min), DCM (5ml x 1, 2min) 33% MeOH/DCM (10ml x 3, 3min), DCM (10ml x 3, 3min) and dried under vacuum. The steps involved in the synthesis are given below

1. Washed the resin with DCM (10ml x 2, 2min)
2. Added 33% TFA/DCM (1ml, 30min) for deprotection
3. Filtered off the TFA solution
4. Washed the resin with DCM (10ml x 6, 2min)
5. Added 5% DIEA/DCM (1ml x 2, 5min) for neutralization
6. Filtered off DIEA solution, washed with DCM (10ml x 4, 2min)
7. Washed with NMP (10ml x 2, 2min)
8. Added active ester of Boc-amino acid (45min) (Boc amino acid : DCC : HOBt - 1:1:1 in NMP)
9. Filtered off the solution, washed with NMP (5ml x 2, 2min)

10. Washed with DCM (5ml x 1, 2min)

11. Washed with 33% MeOH / DCM (10ml x 3, 2min)

12. Washed with DCM (10ml x 3, 2min)

13. Repeat steps 7-12 for second coupling

14. Kaiser test

Cleavage and purification

100mg of the peptidyl resin was treated with TFA (10ml), thionisole (0.1ml), 1,2-ethanedithiol, (0.1ml) at room temperature for 20h. The resin was removed by filtration and TFA by rotary evaporation. The peptide was precipitated by the addition of ice-cold ether. The precipitated peptide was washed several times with ether to get rid of TFA and scavengers, dried and yield noted (21mg). A small amount of the peptide was dissolved in 50% CH_3COOH and injected to analytical reversed phase HPLC using a gradient solvent system of (A) 0.1% TFA/water and (B) 50% CH_3CN in 0.1% TFA. The peptide was then purified by reverse phase HPLC on a preparative column (Biorad, Hi-Pore, RP 318, 25 x 2.15cm) using a gradient system consisting of 0.1% (w/v) TFA in water and acetonitrile. Homogeneity of the peptide was checked in analytical reversed phase HPLC. The purified peptide was characterized by amino acid analysis.

ii. ***Synthesis of Val-Gln-Asp-Ala-Ala-Thr-Tyr-Ala (20-27)***

Attachment of Boc-Ala to chloromethyl resin

To Boc Ala (89.75mg, 0.475mmol) in ethanol (2ml), a saturated solution of cesium carbonate was added till the pH of the solution reached 7.0. The

solution was stirred for 2h, ethanol was evaporated under reduced pressure. Water was removed by azeotropic distillation with dry benzene. The white powder of cesium salt of Boc-Ala was dried under vacuum. The salt was dissolved in NMP (2ml), and to this added 100mg of chloromethyl resin (capacity 1.9mmol/g). The mixture was kept at 50°C for 48h. The resin was filtered, washed with NMP (5ml x 2, 2min), 1:1, NMP-water (10ml, 2, 2min), water, methanol (10ml x 2, 2min) and DCM (10ml x 3, 3min). Dried under vacuum and substitution level of Boc-Ala was noted (1.8mmol/g).

Synthesis of Val-Gln-Asp-Ala-Ala-Thr-Tyr-Ala (20-27)

The Boc-Ala protection was removed by 33% TFA/DCM. The resin was washed with DCM (10ml x 6, 2min) and neutralized with 5% DIEA/DCM (1ml x 2, 5min). The resin was washed with DCM (10ml x 4, 2min) and NMP (10ml x 2, 2min). Then Boc-Tyr.OBzl (141.93mg x 38mmol), Boc-Thr.OBzl (117.56mg, 0.38mmol), Boc-Ala (71.82mg, 0.38mmol), Boc-Ala (71.82mg, 0.38mmol), Boc-Asp.OBzl (123.99mg, 0.38mmol), Boc-Gln (93.48mg x 38mmol) and Boc-Val (82.54mg, 0.38mmol) were coupled to the resin as their HOBt active esters. Active ester was prepared by shaking the respective Boc-amino acids with HOBt (51.3mg, 0.38mmol) and DCC (78.28mg, 0.38mmol) in NMP. DCU formed was filtered off and the active ester was added to the resin. After the synthesis, the peptidyl resin was washed with NMP (5 ml x 2, 2min), DCM (5 ml x 1, 2min), 33% MeOH/DCM (10 ml x 3, 3min), DCM (10 ml x 3, 3 min) and dried in vacuum. The protocol for synthesis is given below in Table IV.6.

Table IV.6 The protocol adopted for the synthesis of Val-Gln-Asp-Ala-Ala-Thr-Tyr-Ala

Operation No.	Steps involved	Reagent/solvent	Time (min)
1.	Washing the resin	DCM	10 ml x 2, 2min
2.	Boc-deprotection	33% TFA/DCM	1ml, 30min
3.	Wash	DCM	10ml x 6, 2min
4.	Neutralization	5% DIEA/DCM	1ml x 2, 5min
5.	Wash	DCM	10ml x 4, 2min
6.	Wash	NMP	10ml x 2, 2min
7.	Coupling	Boc-amino acid: HOBt:DCC (1: 1:1) in NMP	45min
8.	Wash	NMP	5ml x 1, 2min
9.	Wash	DCM	5ml x 1, 2min
10.	Wash	33% MeOH/DCM	10ml x 3, 3min
11.	Wash	DCM	10ml x 3, 3min
12.	Repeat steps 7 to 11 for second coupling		
13.	Kaiser test		

Cleavage and purification of the peptide

100mg of the peptidyl resin was treated with TFA (10ml), thioanisole (0.1ml), 1,2-ethanedithiol (0.1ml) at room temperature for 20h. The resin was removed by filtration and TFA removed by rotary evaporation. The peptide was precipitated by the addition of ice-cold ether. The precipitated peptide was washed several times with ether to get rid of TFA and scavengers, dried and yield was noted (80mg). A small amount of the peptide was dissolved in 50% CH_3COOH and injected to analytical reversed phase HPLC using a gradient solvent system of (A) 0.1% TFA/water and (B) 50% CH_3CN in 0.1% TFA. The

peptide was then purified by reverse phase HPLC on a preparative column (Biorad, Hi-pore, RP 318, 25x2.15cm) using a gradient system consisting of 0.1% (w/v) TFA in water and acetonitrile. Homogeneity of the peptide was checked in analytical reversed phase HPLC. The purified peptide was characterized by amino acid analysis.

iii. Synthesis of Pro-Leu-Gln-Pro-Gly-Val-Asp-Ile-Ile-Glu-Gly-Pro (44-55)

Attachment of Boc-Pro to chloromethyl resin

To Boc-Pro (102.17mg, 0.475 mmol) in ethanol (2ml), a saturated solution of cesium carbonate was added till the pH of the solution reached 7.0. Ethanol was evaporated under vacuum and water removed by azeotropic distillation with benzene. The cesium salt was dissolved in NMP (3ml) and chloromethyl resin (100mg, 19mmol) was added and kept at 50°C for 48h. The resin was washed with NMP (10ml x 2, 2min) NMP-water (1:1, 5ml x 2, 2min), methanol (10ml x 3, 3min), DCM (10ml x 3, 3min) and dried. The substitution level of Boc-Pro was determined (1.7mmol/g).

Synthesis of Pro-Leu-Gln-Pro-Gly-Val-Asp-Ile-Ile-Glu-Gly-Pro

The Boc-protection was removed by 33% TFA in DCM (1ml, 30min) and then washed with DCM (10ml x 6, 2min). 5% DIEA/DCM was used for neutralization. Filtered off the solution and again washed with DCM (10ml x 4, 2min). Washed the resin with NMP (5ml x 2, 2min). Boc-Gly (66.5mg, 0.38mmol), Boc-Glu.OBzl (128.2mg, 0.38mmol), Boc-Ile (87.856mg, 0.38mmol), Boc-Ile (87.856mg, 0.38mmol), Boc-Asp. OBzl (123.99mg, 0.38mmol) Boc-Val (82.54mg, 0.38mmol), Boc-Gly (66.5mg, 0.38mmol), Boc-Pro (81.73mg,

0.38mmol), Boc-Gln (93.48mg, 0.38mmol), Boc-Leu (87.856mg, 0.38mmol) and Boc-Pro (81.73mg, 0.38mmol) were successively coupled to the resin as their HOBt active esters. HOBt active esters were prepared by dissolving respective Boc-amino acids, HOBt, and DCC in 1:1:1 ratio in NMP. DCU formed was removed by filtration. Filtrate was added to the resin. After the synthesis, the resin was washed with NMP (5ml x 2, 2min), DCM (5ml x 1, 2min), 33% MeOH/DCM (10ml x 3, 3min), DCM (10ml x 3, 3min) and dried in vacuum. The steps involved in the synthesis are given below in Table IV.7.

Table IV.7 : The protocol adopted for the synthesis of Pro-Leu-Gln-Pro-Gly-Val-Asp-Ile-Ile-Glu-Gly-Pro

Operation No.	Steps involved	Reagent/solvent	Time (min)
1.	Washing the resin	DCM	10 ml x 2, 2min
2.	Boc-deprotection	33% TFA/DCM	1ml, 30min
3.	Wash	DCM	10ml x 6, 2min
4.	Neutralization	5% DIEA/DCM	1ml x 2, 5min
5.	Wash	DCM	10ml x 4, 2min
6.	Wash	NMP	10ml x 2, 2min
7.	Coupling	Boc-amino acid: HOBt:DCC (1: 1:1) in NMP	45min
8.	Wash	NMP	5ml x 1, 2min
9.	Wash	DCM	5ml x 1, 2min
10.	Wash	33% MeOH/DCM	10ml x 3, 3min
11.	Wash	DCM	10ml x 3, 3min
12.	Repeat steps 7 to 11 for second coupling		
13.	Kaiser test		

Cleavage of peptide from the resin

The resin bound peptide (100mg) was treated with a mixture of TFA, (10ml), thioanisole (0.1ml) and 1,2-ethanedithiol (0.1ml) at room temperature for 22h. TFA was removed by rotary evaporation after filtration and the crude peptide was precpitated by the addition of ice-cold ether. It was washed to remove of TFA and scavengers.

Purification of crude peptide was done by reverse phase HPLC on a preparative column (Biorad, Hi-pore RP 318, 25x2.15cm) using a gradient system of 0.1% TFA (w/v) in water and CH_3CN. Homogeneity of the purified peptide was checked in analytical reverse phase HPLC using binary solvent system (A) 0.1% TFA/water and (B) 50% CH_3CN in A. Distinct peak observed in HPLC profile was collected and rotor evaporated to remove solvents and were hydrolysed using 6N HCl at 110°C for 24h. This was then subjected to amino acid analysis.

iv. Synthesis of Val-Lys-Asn-Val-Ala-Val-Pro (56-62)

Attachment of Boc-Pro to chloromethyl resin

Boc-Pro (102.17mg, 0.475mmol) was dissolved in 1ml ethanol. The pH of the solution was brought to 7 by slow addition of saturated solution of cesium carbonate. The solution was stirred for one hour maintaining the pH at 7. Ethanol was removed by rotary evaporation and water by azeotropic distillation with benzene. White powder of cesium salt of Boc-Pro thus obtained was kept in vacuum desiccator, chloromethylated HDODA-PS resin (0.1gm, added to the

dried cesium salt of Boc-Pro dissolved in NMP (2ml). The mixture was gently stirred at 50°C for 48h. The resin was filtered through a sintered funnel (G_2), washed with NMP (5ml x 3, 3min), NMP-water (1:1, 5ml x 3, 3min), methanol (5ml x 3, 3min), DCM (5ml x 3, 3min). The resin was kept in vacuum desiccator containing P_2O_5 overnight.

Synthesis of Val-Lys-Asn-Val-Ala-Val-Pro

100mg of Boc-Pro resin was used for the synthesis of this sequence. 33% TFA/DCM was used for Boc-deprotection. The resin was washed with DCM (10ml x 2, 2min) and then neutralized with 5% DIEA/DCM (1ml x 2, 5min). The resin was washed with DCM (10ml x 4, 2min) and NMP (10ml x 2, 2min). Active esters of Boc-Val (73.814mg, 0.34mmol), Boc-Ala (64.294mg, 0.34mmol), Boc-Asn (78.914mg, 0.34mmol), Boc-Lys.Cl-Z (141.06mg, 0.34mmol), Boc-Val (73.814mg, 0.34mmol) were coupled successively to the resin as their HOBt active esters. Active esters of amino acids were prepared by dissolving respective Boc-amino acids, HOBt, DCC in 1:1:1 ratio in NMP. After the synthesis of desired sequence, the peptidyl resin was washed with NMP (5ml x 2, 2min), DCM (5ml x 1, 2min), 33% MeOH/DCM (10ml x 3,3min), DCM (10ml x 3, 3min) and dried in vacuum. The synthetic protocol is given below in Table IV.8.

Table IV.8 : Protocol adopted for the synthesis of Val-Lys-Asn-Val-Ala-Val-Pro

Operation No.	Steps involved	Reagent/solvent	Time (min)
1.	Washing the resin	DCM	10 ml x 2, 2min
2.	Boc-deprotection	33% TFA/DCM	1ml, 30min
3.	Wash	DCM	10ml x 6, 2min
4.	Neutralization	5% DIEA/DCM	1ml x 2, 5min
5.	Wash	DCM	10ml x 4, 2min
6.	Wash	NMP	10ml x 2, 2min
7.	Coupling	Boc-amino acid: HOBt:DCC (1: 1:1) in NMP	45min
8.	Wash	NMP	5ml x 1, 2min
9.	Wash	DCM	5ml x 1, 2min
10.	Wash	33% MeOH/DCM	10ml x 3, 3min
11.	Wash	DCM	10ml x 3, 3min
12.	Repeat steps 7 to 11 for second coupling		
13.	Kaiser test		

Cleavage of peptide from the resin

The peptidyl resin (100mg) was treated with a mixture of TFA (10 ml), thioanisole (0.1ml) and 1,2-ethanedithiol (0.1ml) at room temperature for 22h. It was then filtered through a 5ml sintered funnel, and the solution was rotary evaporated to remove TFA. Then ice-cold ether was added to precipitate the peptide which was washed several times with ether, every time centrifuging to

remove the etherial solution containing scavengers and TFA and dried under vacuum.

The crude peptide was dissolved in 50% CH_3COOH and was purified by reverse phase HPLC on a preparative column (Biorad, Hi-Pore RP 318.25 x 2.15cm) using a gradient system of 0.1TFA/water (w/v) and CH_3CN. The major peak was collected separately and rotary evaporated to remove solvents. Purified peptide was characterized by amino acid analysis. The peptide obtained after HPLC was hydrolysed using 6N HCl at 110°C for 24h. This was then subjected to amino acid analysis on a Shimadzu column, CTU 10A (Styrene-DVB cation oxchange resin, column temperature 55°C).

v. Synthesis of Tyr-Ile-Pro-Asn-Gly-Ala-Leu-Lys-Phe-Val-Asp-Ser-Thr-Val-Val-Ala (69-84)

Attachment of Boc-Ala on chloromethylated resin

Boc-Ala (179.55mg, 0.95mmol) was dissolved in ethanol (5ml). By the slow addition of saturated solution of cesium carbonate the pH of the solution was brought to 7. The ethanol was evaporated under vacuum and water was removed by azeotropic distillation with dry benzene, till white powder of cesium salt of amino acid was obtained.

The cesium salt of Boc-Ala was dissolved in 5ml of NMP and the dried chloromethylated resin (0.2g, 0.38mmol) was added. The suspension was kept at 50°C in an oil bath for 48h. The resin was filtered, washed with NMP (5ml x 2,

2min), NMP-water (1:1, 5ml x 2, 2min), methanol (10ml x 2, 2min), DCM (10ml x 2, 2min) and dried under vacuum. Substitution level of Boc-Ala was determined.

Synthesis of Tyr-Ile-Pro-Asn-Gly-Ala-Leu-Lys-Phe-Val-Asp-Ser-Thr-Val-Val-Ala

Boc-protection was removed with 33% TFA/DCM. The resin was washed with DCM (10ml x 3, 2min) and then neutralized with 5% DIEA/DCM (2ml x 2, 5min). The resin was washed with DCM (10ml x 4, 2min) and NMP (5ml x 2, 2min). Active esters of Boc-Val (165.11mg, 0.76mmol), Boc-Val (165.11mg, 0.76mmol), Boc-Thr.OBzl (235.13mg, 0.76mmol), Boc-Ser.OBzl (224.4mg, 0.76mmol), Boc-Asp.OBzl (248mg, 0.76mmol), Boc-Val (165.11mg, 0.76mmol), Boc-Phe (201.6mg, 0.76mmol), Boc-Lys.Cl-Z (315.3mg, 0.76mmol), Boc-Leu (175.7mg, 0.76mmol), Boc-Ala (143.6mg, 0.76mmol), Boc-Gly (133mg, 0.76mmol), Boc-Asn (176.39mg, 0.76mmol), Boc-Pro (163.4mg, 0.76mmol), Boc-Ile (175.7mg, 0.76mmol), Boc-Tyr.OBzl (283.86, 0.76mmol) were coupled successively to the resin.

HOBt active esters of Boc-amino acids are prepared by dissolving Boc-amino acids, HOBt, DCC in 1:1:1 ratio in NMP. DCU formed was filtered off and the filtrate was added to the resin. After the synthesis peptidyl resin was washed with NMP (10ml x 2, 2min), DCM (10ml x 1, 2min), 33% MeOH/DCM (10ml x 3, 3min), DCM (10ml x 3, 3min), dried under vacuum. The synthetic protocol is given below.

1. Washed the resin with DCM (10ml x 3, 2min)
2. 33% TFA/DCM, for deprotection (2ml, 30min)
3. Washed with DCM (10ml x 6, 2min)
4. 5% DIEA/DCM for neutralization (2ml x 2, 5min)
5. Washed with NMP (10ml x 4, 2min)
6. Washed with NMP (10ml x 2, 2min)
7. Active esters of Boc-amino acids (45min) (Boc amino acid, HOBt, DCC in 1:1:1 ratio in NMP)
8. Washed with NMP (10ml x 2, 2min)
9. Washed with DCM (10ml x 1, 2min)
10. Washed with 33% MeOH/DCM (10 ml x 3, 3min)
11. Washed with DCM (10 ml x 3, 3min)
12. Repeat steps 6-11 for second coupling to ensure complete reaction
13. Kaiser test

vi. Synthesis of Gln-Thr-Lys-Ile-Leu-Ala-Lys-Val-Phe-Tyr-Gly (125-135)

Attachment of Boc-Gly to the chloromethylated resin

Boc-Gly (166.3mg, 0.95mmol) was dissolved in ethanol (5ml) and a saturated solution of cesium carbonate was added till the solution became neutral. The solution was stirred for 2h. The ethanol was evaporated under reduced pressure. Water was removed by azeotropic distillation with benzene. The white powder of cesium salt of Boc-Gly was dried under vacuum. The salt was dissolved in 5ml of NMP and to this 0.2gm of chloromethylated resin

(capacity 1.9mmol/g) was added. The mixture was kept at 50°C for 48h. The resin was filtered, washed with NMP (10ml x 3, 2min), NMP-water (1:1, 10ml x 2, 2min), MeOH (10ml x 2, 2min), DCM (10ml x 2, 2min) and dried under vacuum.

Synthesis of Gln-Thr-Lys-Ile-Leu-Ala-Lys-Val-Phe-Tyr-Gly

Boc-protection was removed by using 33% TFA/DCM. Filtered off the solution and washed the resin with DCM (10ml x 6, 2min), 5% DIEA/DCM (2ml x 2, 5min) was used for neutralization. Washed the resin with DCM (10ml x 4, 2min) and NMP (10ml x 2, 2min). Active esters of Boc-Tyr.OBzl (283.86mg, 0.76mmol), Boc-Phe (201.6mg, 0.76mmol), Boc-Val (165.11mg, 0.76mmol), (Boc-Lys.Cl-Z (315.3mg, 0.76mmol), Boc-Ala (143.6mg, 0.76mmol), Boc-Leu (175.7mg, 0.76mmol), Boc Ile (175.7mg, 0.76mmol), Boc-Lys.Cl-Z (315.3mg, 0.76mmol), Boc-Thr.OBzl (235.13mg, 0.76mmol), Boc-Gln (187.15mg, 0.76mmol) were coupled successively to the resin. Active esters of Boc-amino acids were prepared by dissolving Boc-amino acids, HOBt, DCC in 1:1:1 ratio in NMP. DCU formed was filtered off and the filtrate was added to the resin.

After the synthesis, peptide was washed with NMP (10ml x 2, 2min) DCM (10ml x 3, 3min), 33% MeOH/DCM (10ml x 3, 3min) and DCM (10ml x 3, 3min) and dried under vacuum. The synthetic protocol is given in Table IV.9.

Table VI.9 : Protocol adopted for the synthesis of Gln-Thr-Lys-Ile-Leu-Ala-Lys-Val-Tyr-Gly (125-135)

Operation No.	Steps involved	Reagent/solvent	Time (min)
1.	Washing the resin	DCM	10 ml x 2, 2min
2.	Boc-deprotection	33% TFA/DCM	1ml, 30min
3.	Wash	DCM	10ml x 6, 2min
4.	Neutralization	5% DIEA/DCM	1ml x 2, 5min
5.	Wash	DCM	10ml x 4, 2min
6.	Wash	NMP	10ml x 2, 2min
7.	Coupling	Boc-amino acid: HOBt:DCC (1: 1:1) in NMP	45min
8.	Wash	NMP	5ml x 1, 2min
9.	Wash	DCM	5ml x 1, 2min
10.	Wash	33% MeOH/DCM	10ml x 3, 3min
11.	Wash	DCM	10ml x 3, 3min
12.	Repeat steps 7 to 11 for second coupling		
13.	Kaiser test		

Cleavage of peptide from the resin

The peptide resin (200mg) was treated with a mixture of TFA (20 ml), thioanisole (0.2ml), 1,2-ethanedithiol (0.2ml) at room temperature for 20h. It was then filtered and the filtrate was subjected to rotary evaporation under reduced pressure. Then ice-cold ether was added to precipitate the peptide. Washed with ether 6-8 times, centrifuged and dried under vacuum. Yield : (230mg).

An aliquot of crude peptide was dissolved in 50% CH_3COOH and injected in analytical reversed phase HPLC using binary solvent system (A) 0.1% TFA/water, (B) 50% CH_3CN/0.1% TFA in water. The crude peptide was purified by reversed phase HPLC on a preparative column (Biorad, Hi-Pore RP 318, 25 x 2.15cm) using a gradient system consisting of 0.1% TFA (w/v) and CH_3CN. The major peak collected from HPLC was dried and subjected to amino acid analysis for characterization (Shimadzu column, CTU 10A, Styrene-DVB cation exchange resin, column temperature 55°C). The purified sample from HPLC was hydrolysed using 6N HCl at 110°C for 24h, diluted with buffer and subjected to amino acid analysis.