CHAPTER III

EXPERIMENTAL

MATERIALS AND METHODS

III.1. Source of Chemicals

All L-amino acids, t-butyl carbazate, 2-(t-butoxycarbonyloxyimino) 2-phenylacetonitrile (Boc-ON), dicyclohexylcarbodiimide and HOBt were obtained from Sigma chemical company, U.S.A. The side chain protected Boc-amino acids were purchased from Peninsula Laboratories, U.S.A. DIEA, cesium carbonate and thioanisol were obtained from Fluka AG, Switzerland. TTEGDA, 2-bromopropionyl chloride, p-toluic acid, TFA and stannic chloride were obtained from Aldrich Chemical Company Inc., U.S.A. NBS and styrene were obtained from E.Merck, Germany. All the solvents used were purchased from E.Merck, (India), BDH (India) and SISCO Chemicals (Bombay). The solvents were of reagent grade and further purified following literature procedures.

III.2. Physical Measurements

IR Spectra were recorded on a Shimadzu 470 A
spectrophotometer using KBr pellets. UV spectra were recorded using Hitachi double beam spectrophotometer. Fast protein liquid chromatography was carried out on a Pharmacia FPLC system comprising of 200 P solvent delivery pump, 214 UV absorbance detector and injection system. The HPLC analyses were performed using a Shimadzu LC-6A liquid chromatograph equipped with a SPD 6A UV spectrophotometric detector and C-R 6A chromatopac electronic plotter. Scanning electron micrographs of the copolymer beads were taken on WAX 2A Cambridge instrument. The $^{13}$C CP-MAS solid state NMR measurements were conducted on a Bruker 300 MSL CP-MAS instrument operating at 75.47 MHz. The amino acid analysis were carried out on 4151 Alpha plus amino acid analyzer. CD Spectrum was recorded on a

a) Center for Cellular and Molecular Biology, Hyderabad.
b) Molecular Biophysics Unit, Indian Institute of Science, Bangalore.
c) Material Research Lab, Indian Institute of Science, Bangalore.
d) Sophisticated Instrumentation Facility, Indian Institute of Science, Bangalore.
Jasco J 500 A spectropolarimeter attached with a Jasco DP-501 N data processor using quartz cell b 0.5 mm or 1 mm path length. A solution of concentration 0.2 to 1 mg/mL was used. CD data are expressed in molar ellipticities \( [Q_M] \),

\[
Q_M = \frac{Q_{\text{obs}} \times S \times 100}{c \times L}
\]

\( [Q_M] \) is molar ellipticity in deg. cm\(^2\) d mol\(^{-1}\)

\( Q_{\text{obs}} \) is observed ellipticity.

\( S \) is the sensitivity in degree.

\( c \) is the concentration in mole/litre.

\( L \) is the path length in cm.

\(^1\)H-NMR Spectra were recorded on a Bruker MSL 270 NMR instrument. Edman degradation of peptides was performed on Applied Biosystem gas phase protein sequencer Model 470 A.

III.3 Preparation of Reagents

a) 4N HCl-dioxane

Dioxane (500 mL) was purified on a basic Al\(_2\)O\(_3\) column and placed in a graduated pressure equalizing separatory funnel (1L). Dry HCl gas was bubbled into the dioxane till saturation.
The normality of the reagent was determined by diluting an aliquot of the HCl-dioxane with water (1:10) and titrating 1 mL of this dilute acid with 0.1N NaOH using phenolphthalein indicator.

b) Chloromethylmethylether

Methanol (66 mL) and formaldehyde (126 mL) were placed in a 2L R.B. flask fitted with a reflux condenser and a glass tube reaching nearly to the bottom of the flask. A rapid stream of dry HCl gas was run into the mixture which is cooled with running water. After about 4 h chloromethylmethylether begins to appear as a layer. The stream of HCl was continued for 2 to 3 hours longer until the solution was saturated. The layer of chloromethylmethylether was separated. The remaining aqueous layer was separated, saturated with calcium chloride and more ether was separated. This was added to the main portion and distilled at 55-60°C to yield 75 mL of chloromethylmethylether.

c) Boc Azide

t-Butyl carbazate (30 g Sigma) was dissolved in a mixture of glacial acetic acid (27 mL) and
water (37.5 mL). NaN\textsubscript{2}O\textsubscript{2} (17.4 g) was added to this in small portions over a period of 15 min. During the addition of NaN\textsubscript{2}O\textsubscript{2} the solution was stirred vigorously while maintaining the temperature at 0°C. After 90 min., the oily upper layer was separated from the aqueous layer. The aqueous layer was extracted with ether (3 x 20 mL). The ether extracts were combined with the oily layer, washed with water and IN NaHCO\textsubscript{3} and dried over Na\textsubscript{2}SO\textsubscript{4}. On evaporation of ether under reduced pressure, Boc azide was obtained as a golden yellow liquid. It was used directly without further purification.

d) Preparation of Palladium Black\textsuperscript{112}

Palladium Chloride (0.1 g) was suspended in a mixture of 6N HCl (1 mL) and water (10 mL). The suspension was heated on a boiling water bath. After a few minutes, when a clear solution was formed, formic acid (0.04 mL) was added to it, followed by 5N KOH till a pH of 10 was obtained. Formic acid was again added to bring the pH to 7. The Palladium black obtained was granular. These granules were washed with distilled water and stored under water as these are pyrophoric.
III.4 Preparation of Amino acid methyl/ethyl esters

Amino acid methyl/ethyl esters were prepared by thionylchloride-methanol/ethanol procedure as follows:

Absolute methanol/ethanol (10 mL) was cooled to -10°C in ice-salt bath. Thionyl chloride (1 mL) was added to it dropwise with shaking. The amino acid (10 m mol) was added to this and the reaction mixture slowly allowed to come to room temperature. After 10-12 h methanol was evaporated, the residue dissolved in water, saturated with Na₂CO₃, extracted with CHCl₃ (3 x 20 mL) and dried over Na₂SO₄. On evaporation of CHCl₃ under reduced pressure, the oily amino acid methyl/ethyl ester (free base) was obtained which was used immediately.

III.5 Synthesis of Boc Amino Acids

a) Schnabel Method

The amino acid (10 m mol) was suspended in 1:1 dioxane-water (10 mL) and Boc-azide (1.6 mL, 10 m mol) was added to it. The mixture was stirred at room temperature maintaining the pH in the alkaline
range with 4N NaOH. Particulars of the individual Boc-amino acids are given in Table III.1.

Table III.1 Physical Characteristics of Boc-Amino acids

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Melting point (°C)</th>
<th>tlc (Rf)*</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>84</td>
<td>0.62</td>
<td>0.31</td>
</tr>
<tr>
<td>Gln</td>
<td>118</td>
<td>0.36</td>
<td>0.00</td>
</tr>
<tr>
<td>Gly</td>
<td>90</td>
<td>0.65</td>
<td>0.21</td>
</tr>
<tr>
<td>Leu</td>
<td>81</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Ile</td>
<td>61</td>
<td>0.68</td>
<td>0.42</td>
</tr>
<tr>
<td>Phe</td>
<td>87</td>
<td>0.71</td>
<td>0.38</td>
</tr>
<tr>
<td>Pro</td>
<td>137</td>
<td>0.64</td>
<td>0.30</td>
</tr>
<tr>
<td>Val</td>
<td>79</td>
<td>0.74</td>
<td>0.40</td>
</tr>
</tbody>
</table>

* A. Chloroform (85)-Methanol (10)-Acetic acid (5)
B. Chloroform (95)-Acetic acid (5).

After 24 h of reaction, water (15 mL) was added and the solution extracted with ether (10 mL). The aqueous layer was cooled in ice bath, acidified with 2N HCl, and extracted with ethylacetate (ether in the case of Boc-Leu). The
solvent was evaporated on a vacuum rotary evaporator to obtain the respective Boc-amino acid.

b) 2-((t-Butoxycarbonyloxyimino)-2-phenyl acetonitrile (Boc-ON) method

The amino acid (10 m mol), Boc-ON (2.71 g, 11 m mol) and triethylamine (2.10 mL, 15 m mol) in 50% aqueous dioxane (12 mL) were stirred at room temperature for 12 hours. The reaction mixture was diluted with water (20 mL) and washed with ethyl acetate (2 x 15 mL). The aqueous layer was cooled to 0°C, acidified with 1N HCl and extracted with ethyl acetate (3 x 15 mL). The organic layer was dried over Na₂SO₄ and evaporated to obtain the Boc amino acid.

c) Preparation of Boc-Arg (Mts)-OH

i) Preparation of N-Carbobenzyloxy L-Arg

L-Arg (42.2 g) was added to a suspension of NaHCO₃ (63 g) in water (250 mL) and the mixture was vigorously stirred at room temperature. Carbobenzoxy chloride (37.4 g) was added to this mixture in five equal portions over a period of 30
min. The stirring was continued for 1 h and the pH adjusted to 8.5 with concentrated aqueous ammonia and subsequently stored for 2 h at 4°C. The crystalline precipitate formed was filtered off and washed with cold water. This residue was recrystallised from boiling water to which few drops of ammonia were added. Yield: 58.6 g; mp: 175°C.

ii) Preparation of Z-Arg(Mts)-OH

Z-Arg-OH (3.14 g) was dissolved in a mixture of 4N NaOH (10 mL) and acetone (80 mL). The mixture was cooled to 0°C and a solution of 2-mesitylene sulfonyl chloride (3.5 g) in acetone (15 mL) was added dropwise with vigorous stirring for 2 h at 0°C and the mixture was acidified to pH 5. The acetone was removed by evaporation in vacuo and the residue was extracted with ether (2x8 mL) to remove the excess mesitylene sulfonyl chloride. The product was extracted with ethyl acetate (40 mL) and after washing with water, the product was extracted with 5% bicarbonate. After acidification to pH 3 the material was extracted with 40 mL of ethyl acetate and the extract washed with 2N HCl followed by water. This extract was dried over Na₂SO₄, the solvent evaporated in vacuo leaving a nearly
colourless syrup which solidified when allowed to stand over $\text{P}_2\text{O}_5$ (yield: 2.5 g). The dried sample was used in the next step.

iii) Removal of Benzyloxy carbonyl (Z) group:
Preparation of Boc-Arg(Mts)-OH

Z-Arg(Mts) (2 g) was dissolved in methanol (5 mL) and palladium black (100 mg) was added to this followed by formic acid (0.5 mL). The reaction mixture was stirred for 5½ h and the Pd black was filtered off. Evaporation of the solvent yielded a gummy product (yield: 1.8 g) which was converted to Boc-Arg(Mts) by the Boc-ON method.

III.6 Analysis of Protected Amino Acids

Purity of all the protected amino acids was checked by tlc on silica gel using CHCl$_3$-MeOH-acetic acid (85:10:5) solvent system. Amino acids were visualized by ninhydrin after 10 min exposure to HCl vapour. All the protected amino acids were stored at room temperature. Oily materials were stored as stock solution in CH$_2$Cl$_2$ at 4°C.
III.7 Estimation of Halogen Content in Functionalized Tetraethyleneglycol Diacrylate (TTEGDA)-Crosslinked Polystyrene Resin: Volhard's Method

An aliquot of the resin (300 g) was digested with pyridine (5 mL) in a Kjeldahl digestion flask for 3 h at 100°C. It was quantitatively transferred to a 125 mL conical flask with 50% acetic acid (30 mL). Concentrated HNO₃ (5 mL) was added followed by slow addition of standard (0.1N) AgNO₃ solution (10 mL) with magnetic stirring. Water (50 mL) was added followed by sufficient toluene to form a ⅛ inch layer of toluene over water surface. The suspension was mixed well and the excess AgNO₃ was back titrated with standard ammonium thiocyanate solution using ferric alum as indicator. A calibration titration was carried out with standard NaCl solution. From the titre values the halogen capacity of the resins was calculated.

III.8 Attachment of the First Amino Acid to the Resin

The first amino acid was attached to the resin by the cesium salt method. The Boc amino
acid (2 m mol) was dissolved in ethanol:water mixture (3:1) and neutralized with a saturated solution of cesium carbonate. Ethanol was evaporated under vacuum, and the residue was dried by coevaporating with dry benzene (3x40 mL) as an azeotrope under vacuum. The white powdery cesium salt of Boc amino acid was dried over P₂O₅ overnight. Chloromethyl TTEGDA-crosslinked polystyrene resin (1 m mol Cl) was swelled in DMF (10 mL) and the cesium salt of the Boc amino acid dissolved in DMF was added to this. The suspension was kept at 60°C for 24 h. The resin was filtered and washed successively with DMF (20 mL), H₂O (20 mL), DMF (20 mL), methanol (50 mL) and CH₂Cl₂ (50 mL) and dried under vacuum.

III.9 Estimation of Amino Groups by Picric Acid Method

Free amino resin or peptidyl (free base) resin (5 mg) was treated with 0.1M picric acid (3x2 mL, 2 min) in a 5 mL sintered funnel and washed with CH₂Cl₂ (3x2 mL, 2 min) to remove unbound picric acid. The picrate bound to the free amino group was eluted with 5% TEA or DICEA in CH₂Cl₂ (4x2 mL, 2 min), followed by CH₂Cl₂ (4x2 mL, 2 min). 0.2 mL of
picrate eluent was diluted to 2.0 mL with 95% ethanol and the optical density (OD) was measured at 358 nm. From the extinction coefficient ($\varepsilon$) of the picrate solution, the free amino groups were estimated and the substitution levels or coupling efficiencies were monitored. (for picrate $\varepsilon_{358} = 14500$).

III.10 General Procedure for the Solid Phase Synthesis of Peptides

Solid phase peptide synthesis was carried out manually in a silanized glass reaction vessel clamped to a mechanical shaker. The Boc amino acids, were coupled to the resin by the symmetrical anhydride procedure$^{117}$. In a typical procedure Boc amino acid (3 equiv) was dissolved in $\text{CH}_2\text{Cl}_2$ and the solution was cooled to 0°C. A solution of DCC (1.5 equiv) in $\text{CH}_2\text{Cl}_2$ was added and stirred for 1 h at 0°C. The white precipitate of DCU was removed by filtration and the symmetric anhydride solution was added to the resin suspended in $\text{CH}_2\text{Cl}_2$. The coupling time was 1 h for most of the cases. A second coupling was performed if necessary. The extent of coupling was monitored by the ninhydrin test$^{43}$. Boc group was deprotected using 4N-HCl-dioxane or 30%
TFA-CH$_2$Cl$_2$ for 30 min. The neutralization was effected by treatment with 5% DIEA in CH$_2$Cl$_2$ or 10% TFA/CH$_2$Cl$_2$. In the case of photocleavable resin, in-situ neutralization was conducted for first two cycles$^{119}$.

In the DCC coupling procedure equimolar amounts of DCC and the Boc amino acid were directly added to the peptide resin. A 0.1M solution of DCC in CH$_2$Cl$_2$ was used. After the coupling step DCU was removed by washing with 33% EtOH in CH$_2$Cl$_2$. In the case of active ester coupling equimolar amount of DCC, Boc Amino acid and hydroxybenzotriazole were used and DMF was used as the solvent.

III.11 General Procedure for the Photolytic Cleavage

The peptide resin (1.5 g) was suspended in a mixture of 30% TFE in CH$_2$Cl$_2$ (150 mL) and placed in an immersion-type photochemical reactor. The suspension was deaerated for 1 h with dry nitrogen and irradiated with philips HPK 125 W medium-pressure mercury lamp at 340-350 nm for 18-24 h. A saturated solution of CuSO$_4$ was circulated through the outer jacket of the
photochemical reactor to filter out wavelengths below 320 nm. After the photolysis, the spent resin was removed by filtration, washed with EtOH and CH₂Cl₂. The combined filtrate and washings were evaporated on a vacuum rotary evaporator. The residue was collected and purified by chromatography on silica gel or Sephadex LH-21 column using appropriate solvents.

III.12 Column Chromatography

Silica gel 60 (Merek, 70-200 mesh size) was used for column chromatography. Sephadex G-15 obtained from Pharmacia Sweden was used for gel filtration.

III.13 Thin Layer Chromatography (tlc)

Silica gel (tlc grade) was used for thin layer chromatography. tlc of the peptides was done on silica gel plates. The following solvent systems in indicated volume ratios were used.

B: 1-Butanol : acetic acid : water (6:1:5)
C: 1-Butanol : acetic acid : water (4:1:1)
E: Chloroform : methanol (9:1)
F: Acetonitrile : water (3:1)

The following techniques were used to detect the spots on the TLC plate. One or more of the following reagents were used to visualize the spots.

i) Ninhydrin

The Boc group was removed by exposing the plate in a chamber containing conc. HCl and this was heated at 105°C for 10 min. The spots were developed by spraying ninhydrin reagent (0.1% w/v solution in acetone) and heating in an oven at 80-90°C for 5 minutes. Violet spots were observed in the case of free amino groups.

ii) Chlorine-Starch-Potassium Iodide Reagent
(Rydon's Reagent)

The plates were exposed to chlorine for approximately 10 seconds and sprayed with a mixture containing equal volumes of 1% (w/v) aqueous starch
and potassium iodide solutions. White spots over blue background were observed.

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 guanidino group of arginine peptides. The tlc plates were cooled in the refrigerator for 10 min before spraying with these reagents.

Sakaguchi A: A 0.1% solution of 8-hydroxyquinoline in acetone

Sakaguchi B: A solution of bromine (0.6 mL) in 10 mL 1M NaOH

After developing the plates in suitable solvent systems, the plates and solution were cooled in the deep-freezer for half an hour. The
plates were then sprayed with Sakaguchi A reagent followed by Sakaguchi B reagent. Arginine containing peptides gave bright orange spots within 10 seconds.

III.14 Amino Acid Analysis

The following procedure was used for the hydrolysis of peptide-resins. Approximately 10 mg of the peptide resin was accurately weighed and transferred to a sample tube. Propionic acid (0.5 mL) was added to it and the tube was evacuated to remove the air from resin beads. 12N HCl (0.5 mL) was added and the tube was fused under nitrogen and kept in a preheated (130°C) heating block for 6 h. After cooling the resin was removed by filtration and the contents were quantitatively transferred to a volumetric flask with distilled water. This was diluted with buffer and applied to the amino acid analyzer.

Amino acid analysis was used for the characterization as well as quantification of peptides. A known amount of peptide (1 mg) was taken in a sample tube and freshly prepared mixture of 4N HCl (150 µl) and TFA (50 µl) was added to it. The
tube was sealed under vacuum and kept at 120°C for 15 h. Samples were dried in a desiccator and the residue was dissolved in amino acid analysis loading buffer and 5-10 mole sample (based on peptide concentration) was subjected to analysis.

For the quantitative amino acid analysis, an aliquot of stock solution of peptide was taken in a sample tube and known amount of norleucine was added to it. The solvent was dried, and after adding 6N HCl (150 μl), TFA (50 μl), the same procedure as described above was followed. Peptide concentrations were calculated based on norleucine recovery after the analysis.

III.15 Preparation of Polymeric Supports

i) Solution copolymerisation of styrene and tetraethyleneglycol diacrylate; Preparation of tetraethyleneglycol diacrylate-crosslinked polystyrene

Styrene and tetraethyleneglycol diacrylate (TTEGDA) were shaken with 1% NaOH to remove the stabilizer, washed twice with distilled water and dried over anhydrous calcium chloride. A mixture of styrene (36.3 mL), TTEGDA (4.5 mL) and
benzoyl peroxide (0.5 g) dissolved in CHCl₃-MeOH (3:2) (25 mL) was heated on a water bath maintained at 80°C. After 1 h when gelation occurred, excess benzene (50 mL) was added and heating continued for 6 more hours. The amorphous product was filtered by suction and washed with benzene (50 mL x 6), acetone (50 mL x 6) and finally with methanol (50 mL x 6). The crosslinked polymer so obtained was Soxhleted with acetone for 72 h and the product polymer was dried at 60°C under vacuum. Yield: 24.3 g.

ii) Preparation of tetraethyleneglycol diacrylate-crosslinked polystyrene supports by suspension polymerisation

Styrene and TTEGDA were freed of inhibitors by washing with 1% NaOH solution and distilled water and dried over calcium chloride. In a typical experiment a four-necked reaction vessel equipped with a thermostat, teflon-bladded stirrer, water condenser and nitrogen inlet was used. Polyvinyl alcohol (0.5 g) dissolved in double distilled water (200 mL), calcium sulphate (5 mg) and calcium phosphate (10 mg) were added to the vessel. A mixture of styrene (25.5 g), tetraethyleneglycol diacrylate (1.5 g) and benzoyl peroxide (0.5 g) dissolved in benzene (20 mL) was added to the vessel by stirring the aqueous solution at 800 rpm. The
temperature was maintained at 80°C under a slow stream of nitrogen. After 20 hours the solvent-embedded copolymer beads were washed free of stabilizer and the unreacted monomers by treating with distilled water, acetone, chloroform and methanol. Isolation of the required beads from contaminants was then achieved by magnetically stirring the mixture overnight in acetone, when the less dense fragments formed a separate upper layer. This was readily removed by careful pipetting. Finally the beads were dried at 50°C in a vacuum oven for 24 h to yield 20.5 g of polymer beads in the size range 30-85 British Standard Mesh (185-500 μm). (Table IV.1)

IR (KBr): 1720, 1490 cm\(^{-1}\) (ester); 1150 cm\(^{-1}\) (ether); 690 and 755 cm\(^{-1}\) (aromatic).

III.16 Functionalization of Tetraethyleneglycol Diacrylate-Crosslinked Polystyrene Support

i) Chloromethylation of tetraethyleneglycol diacrylate-crosslinked polystyrene support

Preparation of 2M ZnCl\(_2\) Solution in THF:
ZnCl\(_2\) (1.5 g) was dissolved in a mixture of 3 drops of conc. HCl and 5 drops of water and heated over flame till all the water get evaporated. The molten liquid was cooled in a desiccator and the solid mass
dissolved in THF (10 mL). 1 mL of this solution was titrated against std. AgNO₃ using fluorescein as indicator.

TTEGDA-crosslinked polystyrene resin (10 g), ClCH₂OCH₃ (60 mL) and CH₂Cl₂ (58 mL) were stirred for 5 min in a 500 mL round-bottomed flask. 1M ZnCl₂ in THF (2 mL) was then added and the suspension was stirred at 50°C for 7 h. The resin was filtered on a sintered glass funnel and washed with THF (4 x 100 mL), THF/water (3:1) (3 x 100 mL), THF/3N HCl (3:1) (3 x 100 mL), THF/water (3:1) (3 x 100 mL), THF (5 x 100 mL) and methanol (3 x 100 mL). The resin was dried overnight under high vacuum at room temperature (11.5 g) and the chlorine content was determined by Volhard's method (2.05 m mol Cl/g resin).

IR (KBr): 1720 and 1480 cm⁻¹ (ester); 1258 cm⁻¹ (CH₂Cl) 1150 cm⁻¹ (ether).

ii) Preparation of aminomethyl tetraethyleneglycol diacrylate-crosslinked polystyrene resin (5)

a) Phthalimide method: Chloromethyl resin (10 g, 1.1 m mol Cl/g) was suspended in DMF (100 mL). Potassium phthalimide (9.9 g, 54 m mol) was added and the reaction mixture was heated at 80°C for 12 h with occasional stirring. The resin was filtered
and washed with DMF, dioxane, ethanol and methanol. The dry resin was suspended in ethanol (100 mL) and hydrazine hydrate (99%) (4.7 mL, 140 mol) was added. The reaction mixture was refluxed for 8 h. The resin was collected by filtration and washed with hot ethanol (50 mL x 4) and methanol (50 mL x 4). The product resin was dried under vacuo, yield: 9.85 g. Picric acid titration indicated 1.01 mol of NH$_2$/g.

IR (KBr): 3400 cm$^{-1}$ (NH); 1720 and 1490 cm$^{-1}$ (ester); 1150 cm$^{-1}$ (ether).

b) Hexamine method: Chloromethyl TTEGDA-polystyrene (5 g, 2.6 mol Cl/g) resin was suspended in DMF (75 mL). Hexamethylenetetramine (7.5 g, 27 mol) and potassium iodide (8.96 g, 27 mol) were added to this suspension and heated with occasional stirring at 80°C in an oil bath for 8 h. The reaction mixture was filtered, washed with DMF, DMF-water (1:1), ethanol, ethanol-HCl (1:1) (75 mL x 5 min x 3 times), water and methanol. The amine hydrochloride resin so obtained was neutralized with 10% TEA in CH$_2$Cl$_2$ and amino group was estimated by picric acid titration (2.6 mol NH$_2$/g). Yield of the polymer 4.95 g.

IR (KBr): 3400 cm$^{-1}$ (NH$_2$); 1720 and 1490 cm$^{-1}$ (ester); 1150 cm$^{-1}$ (ether).
iii) **Preparation of 4-chloromethyl-3-nitro tetraethyleneglycol diacrylate polystyrene (3)**

The chloromethyl resin (5 g, 2.5 mol Cl/g) was added to fuming nitric acid (50 mL) at 0°C in a round-bottomed flask. The reaction mixture was stirred at 0°C for 2 h and then poured into water (150 mL) containing crushed ice. The resin was collected by filtration, washed with water, water-dioxane, DMF, ethanol and finally with methanol, drained and dried at 60°C under vacuum to yield 5.5 g resin. The chlorine capacity of the resin was estimated by Volhard's method (1.8 mol Cl/g).

IR (KBr): 1535 and 1350 cm\(^{-1}\) (NO\(_2\)); 1720 and 1490 cm\(^{-1}\) (ester); 1150 cm\(^{-1}\) (ether).

iv) **Preparation of \(\alpha\)-bromopropionyl tetraethylene glycol diacrylate-crosslinked polystyrene resin (6)**

To a suspension of anhydrous AlCl\(_3\) (21 g, 157.4 mol) in CH\(_2\)Cl\(_2\) (50 mL) \(\alpha\)-bromopropionyl chloride (13.5 g, 78.7 mol) was added slowly with gentle stirring. The light brown liquid thus obtained was added carefully to a suspension of TTEGDA-crosslinked polystyrene in CH\(_2\)Cl\(_2\) (50 mL). The reaction mixture was stirred for 9 h at room temperature. The acylated resin thus obtained was
collected by filtration and washed successively with 
\( \text{CH}_2\text{Cl}_2 \), 4N HCl-dioxane (1:1), dioxane-water (1:1), 
water, methanol and dried under vacuo. Yield: 13.6 g. Bromine content of the resin was estimated by 
Volhard's method (2.64 m mol/g). IR (KBr): 1685 cm\(^{-1}\) (C=O); 1720 and 1490 cm\(^{-1}\) (ester); 1150 cm\(^{-1}\) (ether).

v) Preparation of \( \alpha \)-aminopropionyl resin (7)

Hexamine method: \( \alpha \)-Bromopropionyl resin (7.5 g, 2.69 m mol Br/g) was suspended in DMF (100 mL) and a mixture of hexamine (11.3 g, 40.5 m mol) and potassium iodide (13.4 g, 40.5 m mol) in DMF (40 mL) was added. The reaction mixture was stirred at room temperature for 1 h followed by heating at 80°C for 8 h. The resin was collected by filtration and thoroughly washed with DMF, DMF-water, EtOH, MeOH and dried. The hexamine salt obtained from the above step was added to a mixture of ethanol (100 mL) and conc. HCl (16.5 mL) at 60°C for 8 h. The resin was collected by filtration and washed with water, dioxane, EtOH and MeOH. A small portion of the amine hydrochloride resin was neutralized with 10% TEA-\( \text{CH}_2\text{Cl}_2 \) and the amino group was estimated by the picric acid titration method. Amino capacity: 2.6 m mol/g. Residual bromine in the resin was estimated by Volhard's method (0.09 m mol Br/g).
IR (KBr): 3400 cm\(^{-1}\) (NH); 1685 cm\(^{-1}\) (C=O); 1720 and 1490 cm\(^{-1}\) (ester); 1150 cm\(^{-1}\) (ether).

vi) **Preparation of 4-bromomethyl benzoic acid**

To a suspension of p-toluic acid (13.6 g, 160 mmol) in dry benzene (100 mL), N-bromosuccinimide (17.8 g, 100 mmol) and benzoyl peroxide (0.2 g) were added. The mixture was refluxed for 24 h. The solvent was removed under vacuum and the residue was suspended in boiling water (100 mL). The crude product was recrystallised from hot methanol to give 4-bromomethyl benzoic acid (16.7 g, 80%); mp 226-229\(^{\circ}\)C.

IR (KBr): 2800-2400, 1690 cm\(^{-1}\) (COOH); 1560 cm\(^{-1}\) (aromatic).

NMR (CDCl\(_3\)DMSO-d\(_6\)):\(\delta 4.61 (s, 2H), 7.8 (8.4, J=8\text{Hz}), 10.4 (s, 1H)\).

Found: C, 44.51; H, 3.18; Br, 37.06%

Anal. calc. for \(\text{C}_8\text{H}_7\text{BrO}_2\): C, 44.18; H, 3.28; Br, 37.15%

vii) **Preparation of 4-bromomethyl-3-nitrobenzoic acid**

Fuming HNO\(_3\) (100 mL) was cooled to \(-10^{\circ}\)C and the bromomethylbenzoic acid (10 g) was added in small portions. The suspension was stirred for 24 h at \(-10^{\circ}\)C. The solution was poured into crushed
ice. The precipitate was collected by filtration and washed with ice-cold water until the washings were neutral. The crude 4-bromomethyl-3-nitro benzoic acid was crystallized from CH$_2$Cl$_2$-hexane; mp, 125-126°C.

IR (KBr): 2800-2300 cm$^{-1}$; 1670 cm$^{-1}$(COOH); 1600 cm$^{-1}$ (aromatic); 1540 cm$^{-1}$ and 1310 cm$^{-1}$(NO$_2$).

Found: C, 37.15; H, 2.40; N, 5.42 5.42; Br, 30.95%

Anal. calc. for C$_8$H$_6$NBrO$_4$: C, 36.95; H, 2.32; N, 5.38; Br, 30.73%

viii) Preparation of 4-bromomethyl-3-nitro benzamidomethyl tetraethyleneglycol diacrylate-crosslinked polystyrene resin (8)

4-Bromomethyl-3-nitro benzoic acid (3.6 g, 13.75 m mol) was dissolved in CH$_2$Cl$_2$ (5 mL) with stirring in ice bath for 30 min and at room temperature for 1 h. DCU was filtered off and the symmetric anhydride solution was added to the pre-swelled aminomethyl resin (5 g, 1.1 m mol NH$_2$/g) in CH$_2$Cl$_2$ (30 mL), followed by pyridine (0.6 mL). The suspension was filtered and washed with MeOH and CH$_2$Cl$_2$. The resin gave no blue colour with ninhydrin reagent indicating complete coupling. The bromine content of the resin was found to be 0.48 m mol/g by Volhard's method. Yield: 5.3 g.

IR (KBr): 1650 cm$^{-1}$(NH-C-); 1340 and 1540 cm$^{-1}$(NO$_2$).
ix) Preparation of 4-aminomethyl-3-nitrobenzamidomethyl tetraethyleneglycol diacrylate polystyrene resin (9)

4-Bromomethyl-3-nitrobenzamidomethyl resin (4.5 g, 0.45 mmol/g) was suspended in DMF (75 mL). Hexamethylenetetramine (2.8 g, 10 mmol) and potassium iodide (3.4 g, 10 mmol) were added to this suspension and heated with gentle stirring at 80°C in an oil bath for 10 h. The reaction mixture was filtered, washed with DMF, DMF-water, ethanol, ethanol-HCl (1:1) (50 mL x 5 min x 3 times) and water. This was then stirred with 10% TEA-CH₂Cl₂ for 2 h, filtered and washed with CH₂Cl₂, DMF and methanol, drained and dried under vacuo. Amino group capacity was determined by picric acid method; (0.43 mmol NH₂/g).

IR (KBr): 3400 cm⁻¹ (NH₂), 1650 cm⁻¹ (NH=O), 1340 and 1540 cm⁻¹ (NO₂)

x) Preparation of 4-hydroxymethyl phenoxymethyl tetraethyleneglycol diacrylate-crosslinked polystyrene resin(10)

Chloromethyl resin (2 g, 6 mmol) [swelled in dimethylacetanide (25 mL)] was allowed to react with 4-hydroxybenzylalcohol (2.8 g, 32 mmol) and sodium methoxide (1.2 g, 22.4 mmol) at 50°C for 8 h. The
resin was filtered and washed with DMA, dioxane, CH$_2$Cl$_2$ and methanol and dried under vacuum. Yield: 2.9 g. Hydroxyl capacity of the resin was estimated by acetylation method: 2.5 m mol OH/g. IR (KBr): 1720 cm$^{-1}$(ester); 1220 cm$^{-1}$(ether)

III.17 Solvent Imbibition Measurements of Tetraethyleneglycol Diacrylate-Crosslinked Polystyrene Resin

Solvent imbibition of the various resins was determined by centrifuge method. A sample of the resin (1 g) was placed in a glass sintered stick (porosity 3) and the latter immersed in the solvent for 1 h. The stick was then transferred to a centrifuge tube where it was held in position. Excess solvent was removed by centrifuging for 15 min. The stick and the contents were then weighed. Immersion in the solvent was continued for 5 minutes. These operations were repeated until a constant weight increase was achieved. Finally a similar blank experiment was performed using the empty sintered stick. The data was expressed as the volume of solvent absorbed by unit weight of dry resin (mL/g). In addition the volume occupied by unit weight of dry resin in its solvent swollen state (mL/g) was measured by noting the volume resulting when a definite weight of dry resin was
added to a known volume of solvent in a small measuring cylinder.

III.18 Stability Studies of Tetraethyleneglycol Diacrylate-Crosslinked Polystyrene Resin

500 mg each of the resin samples was separately stirred for 36 h with the following reagents: (i) 4N HCl-dioxane (10 mL) (ii) 10% diisopropylethylamine (DIEA)/TEA in CH₂Cl₂ (10 mL) (iii) 50% trifluoroacetic acid in CH₂Cl₂ (10 mL) and (iv) 50% piperidine in DMF (10 mL). The resin samples were filtered, washed thoroughly with ethanol, water, acetone, CH₂Cl₂, dioxane, and finally with methanol, dried, and weighed (500 mg). IR (KBr): 1720 cm⁻¹, 1480 cm⁻¹ (ester); 1150 cm⁻¹ (ether); 690 and 755 cm⁻¹ (aromatic).

III.19. Synthesis of Protected Peptides Using 4-Chloromethyl-3-nitro Tetraethyleneglycol Diacrylate-Crosslinked Polystyrene Supports (2)

1) Synthesis of Boc-Met-Leu-Ala-OMe

Ala-OMe·HCl (0.85 g, 6 mol) was dissolved in THF-water mixture (5:1 v/v, 5 mL) and neutralized using TEA. A mixture of 4-chloromethyl-3-nitro TTEGDA-crosslinked polystyrene resin (1) (0.5 g, 2.4
m mol of Cl/g) in THF (10 mL) and TEA (0.17 mL, 1.2 m mol) was added to this and refluxed for 30 h. The resin was filtered and washed with THF (5 mL x 3), DMF (5 mL x 3), MeOH (5 mL x 3) and dried under vacuum at 60°C. The product resin (0.58 g) gave intense blue colour with ninhydrin reagent. Picric acid estimation indicated incorporation of 1.6 m mol of Ala-OMe/g of resin. There was no residual chlorine after the reaction.

IR (KBr): 1530 and 1340 cm\(^{-1}\) (NO\(_2\)), 1710 cm\(^{-1}\) (ester), 3400 cm\(^{-1}\) (NH).

Boc-Leu anhydride (0.58 g, 2.5 m mol) was prepared following the general procedure. This was added to the Ala-OMe-Resin (0.5 g, 0.8 m mol) followed by addition of pyridine (0.1 mL) and shaken for 2 h. The product resin was filtered and washed with CH\(_2\)Cl\(_2\) (5 mL x 3), MeOH (5 mL x 3), and dioxane (5 mL x 3). Boc-Ala-OMe-Resin was deprotected using 4N HCl-dioxane, washed with dioxane, CH\(_2\)Cl\(_2\) and neutralised using 10% TEA-CH\(_2\)Cl\(_2\). Boc-Met anhydride (0.5 g, 2.5 m mol) was added to this resin, shaken for 2 h, filtered and washed. A second coupling was carried out using the symmetric anhydride of Boc-Met (0.3 g, 1.5 m mol). The resin was washed with CH\(_2\)Cl\(_2\) (5 mL x 4), MeOH (5 mL x 4), CH\(_2\)Cl\(_2\) (5 mL x 4) and dried. Yield of the resin: 0.709 g. The peptide resin was photolysed in CH\(_2\)Cl\(_2\)-MeOH mixture (1:1).
following the general procedure to release the crude product peptide. The peptide was purified by column chromatography on a silica gel column using CHCl₃-MeOH mixture and recrystallised from chloroform-ether mixture; yield: 201 mg; mp 197-199°C. Rf(A): 0.66, Rf(E): 0.56. Amino acid analysis: Leu, 1.01 (1); Ala, 0.95 (1); Met, 0.93 (1).

ii) **Preparation of Boc-Ala-Val-Gly-OEt**

4-Chloromethyl-3-nitro TTEGDA-crosslinked polystyrene resin (1) (1 g, 2.3 m mol C1/g) was swelled in THF (10 mL) and Gly-OEt (1.1 g, 11.5 m mol) dissolved in THF-water (5:1) mixture (10 mL) was added to this followed by TEA (0.32 mL, 2.3 m mol). The mixture was refluxed for 30 h. The resin was filtered and washed with THF (10 mL x 6), CH₂Cl₂ (10 mL x 3), MeOH (10 mL x 3) and dried under vacuum to yield 1.45 g Gly-OEt-resin. 1.15 m mol Gly-OEt was incorporated per gram resin as estimated by the picric acid method. 1 g of this resin was used for further synthesis. Boc-Ala (0.47 g, 2.5 m mol) and Boc-Val (0.54 g, 2.5 m mol) were added as described earlier. The Boc-Ala-Val-Gly-OEt resin was filtered, washed with CH₂Cl₂ (15 mL x 4), MeOH (15 mL x 3), DMF, (15 mL x 3) and CH₂Cl₂ (15 mL x 4) and dried under vacuum. Yield: 1.23 g. The protected
peptide was cleaved from the resin (1.1 g) by photolysis for 30 h in CH$_2$Cl$_2$-MeOH (3:1) mixture following the general procedure. The peptide was purified on a silica gel column using CHCl$_3$-MeOH as eluent. Yield: 210 mg; Rf (A): 0.65, Rf (B): 0.84. Amino acid analysis: Ala, 1.05 (1); Val, 0.98 (1); Gly, 1.01 (1).

iii) Preparation of Boc-Met-Leu-Phe-OMe

Nitro resin (1) (1.2 g, 2.2 m mol Cl/g) was swelled in THF (10 mL) and a solution of Phe-OMe (1.6 g, 5 m mol) in dioxane-water (5:1) (5 mL) was added. TEA (0.37 mL, 2.7 m mol) was added to this mixture and heated at 70°C for 30 h. The product resin was filtered, washed with THF (10 mL x 6), dioxane (10 mL x 6), MeOH (10 mL x 6) and dried under vacuum. 1.1 m mol Phe-OMe was incorporated as estimated by picric acid method. The resulting resin (0.5 g, 0.55 m mol) was swelled in CH$_2$Cl$_2$. Boc-Leu symmetric anhydride (0.35 g, 1.5 m mol) was added and shaken for 2 h. After deprotection Boc-Met (0.37 g, 1.5 m mol) was coupled by the symmetric anhydride procedure. The resin (0.71 g) was washed with CH$_2$Cl$_2$DMF and MeOH and the fully protected peptide was cleaved by photolysis (24 h) in CH$_2$Cl$_2$EtOH (3:1) (100 mL). The crude peptide was purified on a silica gel column using CHCl$_3$-MeOH.
mixture as eluent. Yield: 175 mg.

Rf(A): 0.68, Rf(B): 0.84, Rf(C): 0.75.
Amino acid analysis: Leu, 1.2 (1); Met, 0.95 (1); Phe, 1.01 (1).

iv) **Synthesis of Boc-Val-Gly-OEt**

Nitro resin (1) (0.5 g, 2 m mol Cl/g) was swelled in THF-water mixture (5:1 v/v, 15 mL) and glycine ethyl ester (0.71 g, 5 m mol) and triethylamine (0.5 mL) were added to this and refluxed for 30 h. The resin (0.65 g) was filtered, washed with THF (5 mL x 6), DMF (5 mL x 3) and MeOH (5 mL x 6). There was no detectable amount of residual chlorine in the product resin as estimated by Volhard's method. 1.1 m mol Gly-OEt/g was incorporated as estimated by amino acid analysis. Boc-Val (1.05 g, 3 m mol) was added to the resin by the symmetric anhydride procedure in the presence of pyridine (0.1 mL). The Boc-Val-Gly-OEt was cleaved from the support (0.75 g) by photolysis in CH$_2$Cl$_2$-ethanol (3:1) for 24 h. The crude product was purified by column chromatography on a silica gel column, yield: 126 mg. A single spot was obtained by tlc and the product was compared with Boc-Val-Gly-OEt prepared by classical method.

Rf(A): 0.85, Rf(E): 0.81
Amino acid analysis: Gly, 1.01 (1); Val, 0.99 (1).
v) **Synthesis of Boc-Val-Gly-OEt by DCC coupling of Boc-Val and Gly-OEt**

Boc-Val (0.10 g, 0.5 m mol) was dissolved in THF in an ice bath. DCC (0.103 g, 0.5 m mol) was added to this followed by Gly-OEt.HCl (0.07 g, 0.5 m mol) which was neutralized by TEA. The mixture was stirred for 1 h at 0°C and then at room temperature for 4 h. The solution was filtered to remove the DCU and the solvent was evaporated. The residue was dissolved in CH$_2$Cl$_2$ (5 mL), washed with saturated solution of NaHCO$_3$ (5 mL x 2) and then with water (4 mL x 2). The organic layer was washed with 2N HCl (5 mL x 2) and water (5 mL x 2) and dried over sodium sulphate. Evaporation of the solvent yielded the pure dipeptide (0.13 g).

R$_f$(A) : 0.85, R$_f$(B) : 0.80.

vi) **Preparation of Boc-Phe-Gly-OEt**

To the Gly-OEt resin (0.7 g, 0.77 m mol) in CH$_2$Cl$_2$ (10 mL) Boc-Phe symmetric anhydride (0.9 g, 3 m mol) in CH$_2$Cl$_2$ was added and shaken for 1 h. A second coupling was carried out using Boc-Phe symmetric anhydride (0.45 g, 1.1 m mol) in the presence of catalytic amount of pyridine (0.05 mL). The product resin (0.81 g) was photolysed for 22 h in CH$_2$Cl$_2$ methanol mixture (2:1) to yield 76% Boc-
Phe-Gly-OEt which was purified on a silica gel column using CHCl₃-MeOH mixture as eluent, yield: 100 mg.

Rf(A): 0.75, Rf(B): 0.85.

Amino acid analysis: Gly, 1.05 (1); Phe, 0.98 (1).

III.20 Assembly of Boc-(Ala-Leu-Ala)₄-OMe Peptide Chain On High Capacity Chloromethyl TTEGDA-Crosslinked Polystyrene Resin

The first amino acid was attached to the resin by cesium salt procedure following the general procedure. A resin with 1.8 mmol of Boc-Ala/g was used for the synthesis. Substitution was estimated by the picric acid method. Boc-Leu and Boc-Ala were coupled by the DCC procedure. Each coupling was performed twice. The couplings were monitored by Kaiser's semiquantitative ninhydrin test. Solid phase reactions were carried out on a mechanical shaker in a glass vessel equipped with a fritted disc and a stopcock. One cycle of synthesis based on 0.46 g of starting resin consisted of the following operations: (1) CH₂Cl₂ wash, 10 mL, 3 x 1 min (2) deprotection: anhydrous formic acid, 15 mL, 30 min (3) CH₂Cl₂ wash, 10 mL, 3 x 2 min (4) DMF wash, 10 mL x 3 x 2 min (5) pre-wash 5% DIEA/DMF; 10 mL, 1x2 min (6) neutralisation: 5% DIEA/DMF 10 mL, 1x10 min (7) DMF wash, 10 mL, 3 x 2 min (8) CH₂Cl₂
wash 10 mL, 3x2 min (9) equilibration with Boc-amino acid (2.5 m mol) in 7 mL, CH₂Cl₂ 10 min (10) DCC in 5 mL, CH₂Cl₂ (2.5 m mol), 45 min (11) 30% Ethanol/CH₂Cl₂ wash, 10 mL, 3 x 2 min (12) CH₂Cl₂ wash, 10 mL, 3 x 2 min. At the end of the synthesis, the resin was washed with methanol and dried. 1.5 g of dry peptide resin was obtained at the end of the synthesis. About 100 mg of the resin was consumed for the ninhydrin test during the course of the synthesis.

i) Cleavage of Boc(Ala-Leu-Ala)₄OMe from the Solid Support by Trans-esterification

A suspension of the peptide-resin (0.7 g) in 30 mL of anhydrous methanol and 4 mL of triethylamine was stirred under reflux for 4 h. The resin was filtered and the methanol solution evaporated to yield the crude peptide. Two cycles of trans-esterification were carried out to ensure complete recovery of the peptide. 360 mg of peptide was obtained from 700 mg of the peptide resin. The crude peptide was partially soluble in warm DMSO. The peptide was purified by reprecipitation from DMSO-diethylether mixture. A single spot was obtained on tlc.

Rf(A): 0.76, Rf(B): 0.66, Rf(E): 0.89.

Amino acid analysis: Ala, 2.05 (2); Leu, 0.98 (1);
NMR (DMSO-$d_6$, 270 MHz), $\delta$ 1.44 (s, 9H Boc); 0.95 (t, 24 H, C$_5$H Leu); 1.37-152 (m, C$_6$H and C$_8$H, Leu); 1.25 (d, 24H, C$_{24}$H Ala); 3.6 (3H, OCH$_3$); 4.13-4.35 (8H C$_{24}$H Ala, 4H, C$_{24}$H Leu); 7.5-7.92 (8H, -C-NH Leu-Ala) 6.65 (1H, Boc NH Ala).

III.21 Synthesis of Model Peptides on 4-Bromomethyl-3-nitro Tetraethyleneglycol Diacrylate-Crosslinked Polystyrene Support

i) Esterification of Boc-Leu with Bromomethyl resin (9)

4-Bromomethyl-3-nitrobenzamidomethyl resin (7) (0.5 g, 1.1 m mol Br/g) was added to a solution of Boc-Leu (0.92 g, 4 m mol) in ethyl acetate (10 mL). TEA (0.56 mL, 4 m mol) was added to this and the suspension was refluxed at 75°C for 25 h. The resin was collected by filtration, washed with ethyl acetate, MeOH, CH$_2$Cl$_2$ and MeOH (10 mL x 3 x 2 min) and dried under vacuum to yield 0.52 g of the Boc-Leu-resin. Picric acid titration indicated a capacity of 0.9 m mol Boc Leu/g resin.

ii) Solid Phase Synthesis of Boc-Leu-Leu-Leu-Leu-Leu

Boc-Leu resin (0.45 g, 0.40 m mol) was deprotected by shaking with 4N HCl-dioxane (7 mL) for 30 min. After neutralization with 10% TEA
CH₂Cl₂ the resin was washed with CH₂Cl₂ (7 mL x 3 x 2 min). A solution of symmetric anhydride of Boc-Leu (0.29 g) in CH₂Cl₂ (7 mL) was added and the resin was shaken for 1 h. The resin was filtered, washed with CH₂Cl₂ (10 mL x 3 x 3 min), methanol (10 mL x 3 x 3 min) and CH₂Cl₂ (10 mL x 2 x 2 min). During the third cycle, in-situ neutralization was followed and finally Boc-Leu was coupled by the symmetric anhydride procedure. The tetrapeptide was cleaved from the resin (0.95 g) by photolysis in CH₂Cl₂-EtOH (3:1) mixture. The crude product after purification on silica gel column using chloroform-methanol as eluent afforded the peptide (350 mg, 80% yield).
Rf(A): 0.69, Rf(D): 0.63, Rf(E): 0.71

iii) Solid Phase Synthesis of Boc-Gly-Val-Ala-Leu

Boc-Leu-OH was attached to the bromo resin (7) by the esterification process as described above. Boc-Leu-resin (0.45 g, 0.9 m mol Leu/g) was shaken with 4N HCl-dioxane (7 mL x 2 x 3 min) and CH₂Cl₂ (7 mL x 3 x 3 min). The washed product was neutralized with 10% TEA-CH₂Cl₂ (10 mL x 1 x 10 min) and washed thoroughly with CH₂Cl₂ (15 mL x 3 x 3 min). The deprotected resin thus obtained was suspended in CH₂Cl₂ (3 mL) and a solution of preformed symmetric anhydride of Boc-Ala-OH (0.2 g, 1.05 m mol) was added. The reaction mixture was
shaken for 1 h, filtered and washed with CH$_2$Cl$_2$ (7 mL x 3 x 3 min), MeOH (7 mL x 3 x 13 min) and CH$_2$Cl$_2$ (7 mL x 1 x 3 min). The efficiency of the coupling was checked by the ninhydrin test. Since the coupling was not complete, a second coupling was performed with the symmetric anhydride of Boc-Ala (0.1 g, 0.5 mmol in 30 min). In the third cycle after the Boc deprotection, symmetric anhydride of Boc-Val (0.22 g, 1.05 mmol) followed by TEA (0.1 mL) was added and shaken for 1 h. After deprotection and neutralization Boc-Gly (0.18 g, 1.105 mmol) was coupled by the symmetric anhydride procedure. Boc-Gly-Val-Ala-Leu-resin (10) was filtered and washed with CH$_2$Cl$_2$ (7 mL x 6 x 2 min) and MeOH (7 mL x 6 x 2 min).

The peptide resin (620 mg) was suspended in absolute ethanol-CH$_2$Cl$_2$ (30% v/v, 150 mL) and photolysed for 2 h following the general procedure. The crude peptide after silica gel chromatography (CHCl$_3$-MeOH system) and crystallization from EtOH-Et$_2$O afforded the pure peptide (130 mg, 71% yield).

Amino acid analysis: Leu, 1.09 (1); Ala, 1.05 (1); Val, 1.01 (1); Gly, 1.00 (1).
III.22 Synthesis of Boc-Val-Glu(OBzl)-Ala-Leu-Tyr(Bz1)-Leu-NH$_2$ on 4-Aminomethyl-3-nitro Benzamidomethyl Tetraethyleneglycol Diacrylate-Crosslinked Polystyrene Resin (11)

4-Aminomethyl-3-nitrobenzamidomethyl resin (11) (1g, 0.43 m mol NH$_2$/g) was placed in a reaction vessel and CH$_2$Cl$_2$ (10 mL) was added. Symmetric anhydride solution of Boc-Leu (0.44 g, 2 m mol) (prepared using DCC, 0.21 g, 1 m mol) was added to this and shaken for 2 h. This resin was filtered and second coupling was carried out for 30 min. The product resin was collected by filtration, washed and dried in vacuum (1.45 g). Boc-Leu-resin was used for further synthesis. Boc-Tyr(Bz1) (1 m mol) was coupled followed by Boc-Leu (1 m mol), Boc-Ala (1 m mol) Boc-Glu(OBzl) (1 m mol), and Boc-Val (1 m mol), all by the symmetric anhydride procedure. The product peptide resin (1.4 g) was photolysed in a mixture of TFE-CH$_2$Cl$_2$(1:1) (100 mL) for 25 h to get crude peptide which was purified on a silica gel column (210 mg, 45% yield). Rf(A): 0.78, Rf(B): 0.81, Rf(D): 0.71, Rf(E): 0.57. Amino acid analysis: Val, 0.98 (1); Gly, 1.05 (1); Ala, 1.04 (1).
III.23 Solid Phase Synthesis of Boc-Met-Phe-Leu Using α-Bromopropionyl Tetraethyleneglycol Diacrylate-Crosslinked Polystyrene Support (13)

α-Bromopropionyl resin (0.5 g, 1.2 m mol Br/g) was added to a solution of Boc-Leu (0.92 g, 4 m mol) in ethyl acetate (10 mL). TEA (0.56 mL, 4 m mol) was added and the suspension was refluxed for 25 h. The resin was filtered, washed with ethyl acetate, MeOH, CH₂Cl₂ and MeOH (10 mL x 3 x 2 min) and dried in vacuum. Yield: 0.65 g. Picric acid estimation indicated incorporation of 0.82 m mol of Boc-Leu/g.

The Boc-Leu-resin (0.4 g, 0.33 m mol) was deprotected with 4N HCl-dioxane (10 mL, 30 min) washed with dioxane, CH₂Cl₂, MeOH and CH₂Cl₂. The symmetric anhydride of Boc-Phe (0.53 g, 1.1 m mol) was added followed by TEA (0.24 mL, 1 m mol). A second coupling with Boc-Phe (0.27 g, 0.55 m mol) was carried out for 1 h. The resin was washed with CH₂Cl₂, MeOH and CH₂Cl₂. After deprotection of Boc group, Boc-Met (0.5 g, 1.1 m mol) was incorporated in presence of TEA. The Boc-Met-Phe-Leu-OH was removed from the peptide resin (14) by photolysis for 30 h in CH₂Cl₂EtOH (1:1). The crude product (150 mg) after crystallisation from ethyl acetate/ether afforded the peptide (130 mg, 80% yield).
IR(KBr): 1650 cm$^{-1}$ (amide), 1710 cm$^{-1}$ (Urethane).

Rf(E): 0.63, Rf(F): 0.75.

Amino acid analysis: Leu, 1.01 (1); Met, 0.95 (1); Phe, 0.1 (1).

III.24 Synthesis of Boc-Leu-Ala-Gly-Val-NH$_2$

using $\alpha$-Aminopropionyl Tetraethyleneglycol Diacrylate-Crosslinked Polystyrene Resin (15)

The $\alpha$-aminopropionyl resin (0.8 g, 2.65 m mol NH$_2$/g) was placed in a reaction vessel and CH$_2$Cl$_2$(10 mL) was added. In a separate experiment the symmetric anhydride of Boc-Val (0.99 g, 4.16 m mol) was prepared using DCC (0.41 g, 2.08 m mol). The Boc-Val anhydride was added to the resin and shaken for 2h. The resin after filtration was again coupled with Boc-Val anhydride (0.45 g, 2.08 m mol) for 1 h. The product resin was collected by filtration, washed with CH$_2$Cl$_2$, (10 mL x 3 x 2 min), MeOH (10 mL x 3 x 2 min), and dried under vacuum, yield: 1.1 g, capacity: 2.61 m mol Boc-Val/g as estimated by picric acid method.

Boc-Val resin (0.1 g, 0.26 m mol Val) was subjected to the following cycles of operations:

i) treatment with 4N HCl-dioxane for 30 min.

ii) wash with CH$_2$Cl$_2$(5 mL x 3 x 2 min)
iii) neutralization with 10% TEA/CH₂Cl₂
iv) wash with CH₂Cl₂(5 mL x 3 x 2 min)
v) treatment with Boc-Gly symmetric anhydride (1 mol) for 1 h.
vi) wash with CH₂Cl₂ (5 mL x 1 x 2 min)
vii) Second coupling with Boc-Gly-symmetric anhydride (0.5 mol) for 1 h.
viii) wash with CH₂Cl₂(5 mL x 3 x 3 min)
ix) wash with MeOH (5 mL x 2 x 2 min)
x) wash with CH₂Cl₂ (5 mL x 1 x 1 min)
xi) coupling with Boc-Ala anhydride (1 mol) for 1 h.
xii) wash with CH₂Cl₂ (5 mL x 3 x 3 min)
xiii) wash with MeOH (5 mL x 3 x 3 min)
xiv) repetition of deprotection, neutralization and washing
xv) coupling with Boc-Leu (1 mol) anhydride for 1 h.
xvi) wash with CH₂Cl₂(10 mL x 3 x 3 min)
xvii) wash with MeOH (5 mL x 2 x 2 min)

The Boc-Leu-Ala-Gly-Val-Resin (200 mg) was suspended in CH₂Cl₂-EtOH (1:1) (100 mL) and photolysed by the general procedure for 30 h. The crude peptide (75% yield) was purified on a sephadex LH-20 column. mp 136-138°C
Rf(A): 0.88 , Rf(D): 0.75 , Rf(E): 0.61.
Amino acid analysis: Leu, 1.00 (1); Ala, 0.95 (1); Gly, 1.02 (1); Val 0.97 (1).
i) **Boc-Phe-Phe-Gly-Leu-Met-NH₂**

Boc-Met (0.99 g, 4 m mol) and HOBt (0.54 g, 4 m mol) were dissolved in THF-CH₂Cl₂ mixture (1:1 v/v, 10 mL) followed by addition of DCC in CH₂Cl₂ (0.83 g, 4 m mol). The mixture was stirred in an ice bath for 15 min and at room temperature for 1 h. The DCU was filtered off and the filtrate was added to α-amino propionyl resin (1 g, 1 m mol NH₂) in CH₂Cl₂ (10 mL) and shaken for 2 h. The resin was filtered and recoupled with Boc-Met (0.5 g, 2 m mol), HOBt (0.27 g, 2 m mol) and DCC (0.41 g, 2 m mol) by the same procedure for 1 h. The product resin was filtered and washed with CH₂Cl₂ (10 mL x 6 x 2 min), THF (10 mL x 6 x 2 min), MeOH (10 mL x 6 x 2 min) and dried under vacuum.

Boc-Leu (3 m mol), Boc-Gly (3 m mol) and Boc-Phe (3 m mol) were successively incorporated into the Boc-Met-resin using HOBt double coupling procedure as described earlier. After the solid phase assembly the peptide was removed from the support (1.7 g) by photolysis for 22 h in CH₂Cl₂-EtOH (1:1) mixture. The crude peptide (600 mg) was purified on a silica gel column using CHCl₃-MeOH mixture as the eluent to yield 420 mg pure peptide Rf(A): 0.63, Rf(D): 0.71, Rf(E): 0.58.

Amino acid Analysis: Phe, 1.97 (2); Gly, 0.98 (1);
Leu, 1.01 (1); Met, 1.1 (1).

III.25 Synthesis of Biologically Important Peptides Using Chloromethyl TTEGDA-Crosslinked Polystyrene Resin (4)

i) Synthesis of Deltorphin: Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH₂

a) Boc-Gly-O-CH₂-C₆H₄-Resin

Boc-Gly (0.52 g, 3 mol) and TEA (0.38 mL, 2.7 mol) were added to a suspension of chloromethyl tetraethyleneglycol diacrylate-crosslinked polystyrene resin (4) (0.5 g, 2.1 mol Cl/g) in DMF (15 mL) and the mixture was kept at 80°C for 24 h. The esterified resin was washed, dried and weighed (0.78 g). The Gly content of the resin was estimated by picric acid method (1.6 mol Gly/g of resin).

b) Fmoc-D-Ala-Phe-Asp(Bzl)-Val-Val-Gly-O-CH₂-C₆H₄-Resin

Boc-Gly-OCH₂-C₆H₄-Resin (0.7 g) was taken in a 40 mL solid phase synthesis vessel and 30% TFA-CH₂Cl₂(10 mL) was added and mixed for 30 min. The resin was then washed with CH₂Cl₂(3 x 10 mL) and DMF (3 x 10 mL) before and after treatment with 10% TEA-
DMF (10 mL x 10 min) to convert the amino acid salt to free amino group. Introduction of each amino acid moiety was carried out by the following sequence of operations: (1) washing with CH₂Cl₂ (3 x 2 min); (2) coupling with Fmoc-amino acid (3 m mol) in presence of DCC (3 m mol), HOBt (1 m mol) in CH₂Cl₂-DMF (1:1) (1 x 60 min); (3) washing with 33% EtOH in CH₂Cl₂ (4 x 2 min); (4) washing with DMF (2 x 2 min); (5) Deprotection with 50% piperidine in DMF (1 x 10 min); (6) washing with DMF (3 x 2 min); (7) washing with dioxane-water (2:1) (2 x 5 min); (8) washing with isopropanol (2 x 12 min). After the synthesis the resin was finally washed with ether and dried over KOH pellets and P₂O₅ under vacuum, yield: 1.1 g.

c) Z-Tyr(Bz1)-D-Ala-Phe-Asp(Bz1)-Val-Val-Gly-O-\(\text{CH}_2\text{C}_6\text{H}_4\)Resin (18)

Fmoc-D-Ala-Phe-Asp(Bz1)-Val-Val-Gly-O-\(\text{CH}_2\text{C}_6\text{H}_4\)resin was treated with 50% piperidine in DMF (10 mL) for 10 min. The resin was washed and a solution of Z-Tyr(Bz1)OTCP (3 m mol) and HOBt (0.04 g, 1 m mol) in DMF-CH₂Cl₂ (1:1, 10 mL) was added and shaken for 40 min. The protected heptapeptide-resin (18) was finally washed with ether and dried under vacuum. Yield: 1.2 g.
d) \( Z\text{-Tyr(Bzl)-D-Ala-Phe-Asp(Bzl)-Val-Val-Gly-NH}_2 \)

The protected peptide resin (18) (1.2 g) was suspended in dry methanol (100 mL) saturated with ammonia (distilled over Na) and stirred at 0°C for 72 h under anhydrous conditions. The resin was filtered and washed with hot MeOH. The combined filtrate was evaporated under vacuum and the residue was washed with ether to yield 400 mg of crude protected heptapeptide amide.

e) \( \text{Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH}_2 \)

To a solution of the protected peptide amide (110 mg, 0.1 mol) in 85% HCOOH (1.2 mL) freshly prepared palladium-black catalyst (0.15 g) was added and stirred at room temperature for 3 h. The removal of protecting groups was monitored by tlc. The catalyst was filtered, washed several times with hot MeOH. The combined filtrate was evaporated under vacuum and reprecipitated from MeOH-ether; yield: 80 mg (95%).

The crude peptide was subjected to gel filtration on a sephadex G-15 column (1.6 x 160 cm) in 3N acetic acid. The heptapeptide \( \text{Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH}_2 \) was isolated by lyophilysation of fractions comprising a single symmetrical peak. Yield: 60 mg (80%), mp. 226-228°C.
Rf(B): 0.51, Rf(c): 0.48, Rf(D): 0.41.

Amino acid analysis: Gly, 0.98 (1); Val, 2.10 (2); Asp, 1.1 (2); Phe, 0.98 (1); D-Ala, 1.01 (1); Tyr, 1.14 (1).

ii) Synthesis of cytoadhesive inhibitory peptide: Gly-Arg-Gly-Asp-Ser-Pro

Boc-Pro (1.5 g, 7 m mol) was attached to chloromethyl resin (4) (1 g, 2.5 m mol Cl/g) by the cesium salt method following the general methods. The resulting resin contained 1.8 m mol Boc-Pro/g as indicated by picric acid estimation. A portion of this resin (0.25 g, 0.45 m mol) was used for the synthesis of hexapeptide Gly-Arg-gly-Glu-Ser-Pro. Boc-Ser(Bz1) (0.295 g, 1 m mol), Boc-Asp(OBz1) (0.32 g, 1 m mol) and Boc-Gly (0.175 g, 1 m mol) were successively incorporated. The coupling steps were monitored by ninhydrin reaction. Boc-Arg(Mts) (0.45 g, 1 m mol) was added in presence of HOBt (0.15 g, 1 m mol) and DCC (0.206 g, 1 m mol) for 2 h. Finally Boc-Gly (0.175 g, 1 m mol) was coupled in the presence of DCC (0.206 g, 1 m mol). The peptide resin was washed with 33% EtOH-CH2Cl2 (10 mL x 6 x 2 min), MeOH (10 mL x 6 x 2 min) and CH2Cl2 (10 mL x 6 x 2 min) and dried under vacuum. Yield: 0.63 g.
iii) **Cleavage of Gly-Arg-Gly-Asp-Ser-Pro from the peptide resin** (22) **using trifluoroacetic acid**

To the peptide resin (0.60 g), TFA (12 mL), thioanisole (1.2 mL) and m-cresol (1.2 mL), were added and kept at 40 °C for 22 h under anhydrous conditions. The resin was removed by filtration and washed with TFA (3 mL x 2). The filtrate was evaporated to dryness to give an oily residue. This was reprecipitated from MeOH-ether mixture. The precipitate was thoroughly washed with cold ether (10 mL x 10) to yield 360 mg peptide. The product was dissolved in MeOH and injected to a C-18 FPLC (RPC) and eluted using 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B) in a linear gradient 0-45% B in 20 min. mp, 140-145°C.

Rf (A): 0.85, Rf (C): 0.81, Rf (D): 0.65

Amino acid analysis: Gly, 2.01 (2); Arg 0.93 (1); Asp 1.00 (1); Ser 0.75 (1); Pro 1.1 (1);

iv) **Synthesis of Gly-Arg-Gly-Glu-Ser-Pro**

Boc-Pro-resin (0.25 g, 0.45 m mol) was deprotected using 30% TFA-CH₂Cl₂ for 30 min and neutralized using 5% DIEA-CH₂Cl₂ for 10 min. DCC coupling of Boc-Ser(Bzl), (0.295 g, 1 m mol) Boc-Glu(OBz1) (0.337 g, 1 m mol), Boc-Gly (0.179 g, 1 m mol).
mol), Boc-Arg(Mts) (0.45 g, 1 m mol) and Boc-Gly (0.175 g, 1 m mol) was carried out as described above. 0.65 g of peptide-resin was obtained after the synthesis. The peptide was cleaved using TFA (12 mL), thioanisole (1.2 mL), m-cresol (1.2 mL) (10:1:1) to yield 360 mg of the peptide. This was recrystallised from MeOH-ether to yield 290 mg (75%) of the pure peptide.

Rf(A): 0.83, Rf(C): 0.81, Rf(D): 0.61.
Amino acid analysis: Gly, 2.1 (2); Arg, 1.01 (1); Glu, 0.98 (1); Ser 0.73 (1); Pro, 1.05 (1).

v) Synthesis of nuclear signal sequence Ser-Thr-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-Cys(Acm)

Boc-Cys(Acm) was attached to chloromethyl TTEGDA-crosslinked polystyrene resin (4% crosslinked) by the cesium salt method. To the chloromethyl resin (0.5 g, 0.8 m mol Cl/g) swelled in DMF, Boc-Cys(Acm) cesium salt (1 m mol) was added and heated at 60°C for 24 h to give 0.66 g of the Boc-Cys(Acm)-resin. Picric acid estimation indicated incorporation of 0.35 m mol Boc-Cys(Acm)/g. 0.6 g of the resulting resin was used for the stepwise synthesis of 11-residue peptide. Boc-Val (0.108 g, 0.5 m mol) and
Boc-Lys(Clz) (0.207 g, 0.5 m mol) were coupled successively by the DCC double coupling procedure. After the coupling of Boc-Arg(Mts) (0.225 g, 0.5 m mol) using HOBT (0.075 g, 0.5 m mol) and DCC (0.107 g, 0.5 m mol), the free amino groups were blocked by acetylation. The product resin was filtered, washed with DMF (10 mL x 6), CH₂Cl₂ (10 mL x 6), MeOH (10 mL x 6) and dried under vacuum. The resulting resin showed no blue colour to ninhydrin reagent. The Boc-Lys(Clz) (0.207 g, 0.5 m mol) was added on by the DCC-mediated coupling procedure, followed by Boc-Pro-OH (0.108 g, 0.5 m mol) and Boc-Thr(Bz1) (0.155 g, 0.5 m mol). Finally Boc-Ser(Bz1) (0.15 g, 0.5 m mol) was coupled by DCC method. 0.9 g of peptide resin was obtained from which the peptide was cleaved using TFA (18 mL), thioanisole (1.8 mL) and m-cresol (1.8 mL) at 40°C for 24 h; 220 mg of crude peptide was obtained. This was purified on a C-18 (RPC) FPLC column using 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). A linear gradient was used (Fig. V.6). The pure peptide was obtained in 40% overall yield.

Amino acid analysis: Ser, 0.74 (1); Thr, 0.63 (1); Pro, 2.1 (2); Lys, 3.95 (4); Arg, 0.96 (1); Val, 1.1 (1).
III.26 Synthesis of Pardaxin Sequence 16-33; 5-33 and 1-33

Boc-Glu(Obz1) (0.33 g, 1 mmol) was dissolved in 4:1 ethanol/water (7 mL) and a 1M solution of CsCO₃ was added dropwise till the pH was 7. The solvent was removed by azeotropic distillation with benzene and the resulting white solid was kept overnight over P₂O₅ under vacuum.

Boc-Glu(Obz1)-O-Cs from the above step was dissolved in DMF (7 mL) and chloromethyl resin (0.3 g, 0.45 mmol) was added. The mixture was kept at 50-60°C for 22 h with occasional shaking. The resin was washed with DMF (3 x 1 min), DMF-H₂O (9:1), (5 x 2 min), DMF-H₂O (4:6), (5 x 2 min), DMF (3 x 1 min) CH₂Cl₂ (3 x 2 min) and finally with CH₃OH. The product resin was dried under vacuum for 9 h. Yield: 0.55 g. Amino acid analysis of the resin hydrolysate indicated 1.25 mmol of Glu/g of resin.

Boc-Glu-OBzl-resin (0.2 g, 0.21 mmol) was deprotected using 30% TFA-CH₂Cl₂ and after neutralization with 5% DIEA, Boc-Gln (0.12 g, 0.5 mmol), HOBt (0.5 mmol) and DCC (0.5 mmol) in DMF (10 mL) were added and shaken for 3 h. After washing with 30% EtOH-CH₂Cl₂, a second coupling was conducted
### Table III.1 DCC Coupling of Boc-Amino Acids for the Synthesis of Pardaxin 1-33 sequence

<table>
<thead>
<tr>
<th>Amino Acid Residue</th>
<th>wt in mg</th>
<th>No. of couplings</th>
<th>Duration of coupling (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32 Boc-Gln/HOBt</td>
<td>123.0</td>
<td>Two</td>
<td>180</td>
</tr>
<tr>
<td>31 Boc-Glu(OBz1)</td>
<td>168.55</td>
<td>Two</td>
<td>45</td>
</tr>
<tr>
<td>30 Boc-Gly</td>
<td>87.6</td>
<td>Two</td>
<td>45</td>
</tr>
<tr>
<td>29 Boc-Ser(Bz1)</td>
<td>147.55</td>
<td>Three</td>
<td>45</td>
</tr>
<tr>
<td>28 Boc-Ser(Bz1)</td>
<td>147.55</td>
<td>Two</td>
<td>45</td>
</tr>
<tr>
<td>27 Boc-Ser(Bz1)</td>
<td>147.55</td>
<td>Three</td>
<td>45</td>
</tr>
<tr>
<td>26 Boc-Leu</td>
<td>115.6</td>
<td>Three</td>
<td>45</td>
</tr>
<tr>
<td>25 Boc-Ala</td>
<td>94.55</td>
<td>Two</td>
<td>45</td>
</tr>
<tr>
<td>24 Boc-Ser(Bz1)</td>
<td>147.55</td>
<td>Two</td>
<td>45</td>
</tr>
<tr>
<td>23 Boc-Gly</td>
<td>87.6</td>
<td>Two</td>
<td>45</td>
</tr>
<tr>
<td>22 Boc-Val</td>
<td>108.55</td>
<td>Two</td>
<td>45</td>
</tr>
<tr>
<td>21 Boc-Ala</td>
<td>94.55</td>
<td>Two</td>
<td>45</td>
</tr>
<tr>
<td>20 Boc-Ser(Bz1)</td>
<td>147.55</td>
<td>Two</td>
<td>45</td>
</tr>
<tr>
<td>19 Boc-Leu</td>
<td>115.6</td>
<td>Two</td>
<td>45</td>
</tr>
<tr>
<td>18 Boc-Leu</td>
<td>115.6</td>
<td>Two</td>
<td>45</td>
</tr>
<tr>
<td>17 Boc-Thr(Bz1)</td>
<td>154.6</td>
<td>Three</td>
<td>45</td>
</tr>
<tr>
<td>16 Boc-Lys(ClZ)</td>
<td>207.4</td>
<td>Two</td>
<td>45</td>
</tr>
</tbody>
</table>

Synthesis was stopped here and 1/3 of the peptide resin was removed
<table>
<thead>
<tr>
<th>15 Boc-Phe</th>
<th>106.08</th>
<th>Two</th>
<th>55</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 Boc-Leu</td>
<td>92.44</td>
<td>Two</td>
<td>55</td>
</tr>
<tr>
<td>13 Boc-Pro</td>
<td>86.04</td>
<td>Two</td>
<td>55</td>
</tr>
<tr>
<td>12 Boc-Ser(Bzl)</td>
<td>118.04</td>
<td>Two</td>
<td>55</td>
</tr>
<tr>
<td>11 Boc-Ser(Bzl)</td>
<td>118.04</td>
<td>Two</td>
<td>55</td>
</tr>
<tr>
<td>10 Boc-Ile/HOBt</td>
<td>96.08</td>
<td>Two</td>
<td>180</td>
</tr>
<tr>
<td>9 Boc-Ile/HOBt</td>
<td>96.08</td>
<td>Two</td>
<td>180</td>
</tr>
<tr>
<td>8 Boc-Lys/(ClZ)</td>
<td>165.92</td>
<td>Two</td>
<td>55</td>
</tr>
<tr>
<td>7 Boc-Pro</td>
<td>86.04</td>
<td>Two</td>
<td>55</td>
</tr>
<tr>
<td>6 Boc-Ile</td>
<td>96.08</td>
<td>Three</td>
<td>180</td>
</tr>
<tr>
<td>5 Boc-Leu</td>
<td>92.44</td>
<td>Two</td>
<td>55</td>
</tr>
</tbody>
</table>

Synthesis was stopped and 100 mg of the peptide resin (23) was removed

<table>
<thead>
<tr>
<th>4 Boc-Ala</th>
<th>75.64</th>
<th>Two</th>
<th>65</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 Boc-Phe</td>
<td>106.08</td>
<td>Two</td>
<td>65</td>
</tr>
<tr>
<td>2 Boc-Phe</td>
<td>106.08</td>
<td>Two</td>
<td>65</td>
</tr>
<tr>
<td>1 Boc-Gly</td>
<td>70.0</td>
<td>Two</td>
<td>60</td>
</tr>
</tbody>
</table>
using the same procedure. The remaining amino acid units were stepwise incorporated by DCC double coupling procedure (Table V.4). The amount of amino acid derivatives taken and duration of coupling are shown in Table III.1. The ninhydrin test was performed for the detection of incomplete coupling reaction. After the addition of Boc-Lys(ClZ) at the 16th residue, the resin was filtered, washed with CH₂Cl₂ (10 mL x 6 x 1 min), MeOH (10 mL x 6 x 1 min) and CH₂Cl₂ (10 mL x 6 x 1 min) and dried under vacuum to yield 0.52 g product resin. 0.22 g of peptide resin (22) was kept aside and the synthesis was continued using 0.3 g resin. 0.1 g resin was removed at the 5th residue. The synthesis was continued using 0.27 g of the peptide resin. At this stage 5% DIEA in DMF was used for neutralization step. After the synthesis the resin was thoroughly washed with 30% EtOH-CH₂Cl₂ (10 mL x 6 x 1 min), MeOH (10 mL x 6 x 1 min), CH₂Cl₂ (10 mL x 6 x 1 min) and dried under vacuum: Yield of the peptide resin: 0.28 g.

i) Cleavage of 16-33 pardaxin peptide from the resin (22) using trifluoroacetic acid

100 mg of the peptide resin was suspended in dry TFA (2 mL) and thioanisole (0.2 mL), m-cresol (0.2
mL) were added to this. The mixture was deaerated with nitrogen and kept at 37°C for 22 h. The polymeric material was filtered off and washed with fresh TFA. The filtrate was evaporated to obtain a thick oily residue which was precipitated as a white powder (56 mg) by the addition of cold ether. The peptide was washed thoroughly with cold ether (5 mL x 8) to remove all the scavenging reagents. The resin was kept for a second cleavage under the same conditions as above to yield an additional crop of 5 mg of the peptide. The peptide was dissolved in methanol and again reprecipitated with cold ether to yield 58 mg of the crude peptide.

Purification of the peptide. The crude peptide (10 mg) in methanol (0.5 mL) was loaded in small portions onto a Pharmacia RP-C-18 FPLC column and eluted with a linear gradient as shown in Fig. V.7. Solvent (A) is H₂O/0.25% TFA and B is MeCN/0.25% TFA. A flow rate of 0.5 mL/min was maintained. The major peak corresponding to 37% acetonitrile was collected and evaporated to get 7 mg of pure peptide. The concentration and purity of the peptide was determined by amino acid analysis. The pure peptide was reinjected onto a Shimadzu RP-C-18 analytical HPLC column and eluted
with a linear gradient starting from 5\% to 40\% B in 40 min followed by eluting (5 min) with 40\% B, where (A) is H\textsubscript{2}O/0.1\% TFA. Retention time of the peptide under these conditions is 43.85 min (Fig. V.7).

Amino acid analysis: Thr, 0.60 (1); Ser, 4.16 (5); Glu, 3.12 (3); Gly, 2.02 (2); Ala, 2.01 (2); Val, 0.99 (1); Leu, 3.01 (3); Lys, 1.02 (1); (The values for Ser and Thr were found to be somewhat low due to some degradation during hydrolysis).

ii) **Cleavage of the 5-33 pardaxin from the resin (23) using trifluoroacetic acid**

TFA (2 mL), thioanisole (0.2 mL) and m-cresol (0.2 mL) were added to the peptide resin (0.1 g) and kept at 37\degree C. After 24 h the resin was removed by filtration and TFA was evaporated under vacuum. The peptide was precipitated using cold ether and washed thoroughly with ether (10 mL x 8); yield: 73 mg.

iii) **Cleavage of the 33-residue pardaxin peptide toxin from the resin (24) using trifluoroacetic acid**

The 33-residue peptide pardaxin was also cleaved by TFA-thioanisole-m-cresol method. 70 mg of peptide was isolated from 0.1 g of the peptide
resin 24. The cleavage yield as observed from the amino acid analysis was found to be 95%.

III.26 Manual Microsequencing of Pardaxin (1-33) and (5-33) Peptides

Manual sequencing of 2 peptide samples—pardaxin 1-33 and 5-33 along with RNA polymerase, a protein with known sequence, and a blank was carried out simultaneously according to Tarr's method 123. The following precycle was carried out before the sequencing in order to remove any hydrolysed amino acid or deprotecting agents. For this 5 mg of crude peptides and 1 mg of RNA polymerase were taken in a stoppered 3 mL centrifuge tube and TFA (50 μl) was added under nitrogen atmosphere and vortexed. The samples were incubated at 50°C for 8 min and dried under vacuum. 50 μl of methanol was added to this. After vortexing the samples were incubated at 50°C for 3 min and dried under vacuum. 400 μl heptane-ethyl acetate containing 0.5% triethylamine was added to this to yield two layers. The supernatant layer was decanted after gentle mixing and this was dried under vacuum. For the manual sequencing the following operations were carried out during each cycle.
1. **Set-up:** Dry salt-free samples (1 mg) precycled and then dried from triethylamine.

2. **Coupling:** 40 µl (50% pyridine, 30% water, 20% triethylamine) and 40 µl (20% phenylisothiocyanate (PITC) in pyridine) were added to the centrifuge tube containing samples and flushed with nitrogen. The samples were vortexed and incubated at 50°C for 3 min.

3. **Washing:** 400 µl heptane - ethyl acetate (1:1) containing 0.5% triethylamine was added to this to yield two phases. The mixture was slowly rotated in which sample precipitated as rough film. Supernatant layer was decanted and the same procedure was repeated for three times with 500 µl ethyl acetate. Finally this was vacuum-dried.

4. **Extraction:** The dried sample was extracted three times with 30 µl benzene-acetonitrile (1:1) containing 0.1% acetic acid into a 6 x 50 mm tube and vacuum-dried.

5. **Conversion:** 20 µl 50% TFA in water was added to the dried sample in 6 x 50 mm tube and incubated for 10 min at 65°C and vacuum-dried. The second cycle of sequencing was carried out using the residue in the centrifuge tube.
The residue in the small tubes (6 x 5 mm) was dissolved in 100 µl acetonitrile-water (1:1) and injected to a C-18 Reverse phase HPLC column. The column was eluted using a linear gradient 0-45% (B) in 10 min, where (A) is 30 mM NaOAc-buffer (pH 5) with acetonitrile and (B) 60% isopropanol -water mixture. The standard PTH amino acid mixture was injected to the HPLC column and eluted using the same linear gradient. By comparing the HPLC profile the amino acid sequence was assigned (Table V.6).

III.28 Synthesis of Pardaxin (1-26) Hydrophobic Segment on a High Capacity Chloromethyl TTEGDA-Crosslinked Polystyrene Resin (4)

The first amino acid residue Boc-Leu was attached to the resin by cesium salt method of Gisin. The extent of substitution in the resin as well as in the coupling were monitored by picric acid test or semiquantitative ninhydrin method. A third coupling with symmetric anhydride or active ester was carried out when necessary to reach 99.8% completion. The resin with a substitution of 1.8 mmol Boc-Leu/g was used for synthesis. One cycle of synthesis for 160 mg (0.3 mmol) resin consists of
the following operations:

(1) CH$_2$Cl$_2$ wash: (5 mL x 3 x 1 min); (2), Deprotection: 30% TFA/CH$_2$Cl$_2$ (5 mL x 1 x 30 min); (3) CH$_2$Cl$_2$ wash: (5 mL x 3 x 1 min); (4) Prewash: 5% TEA/CH$_2$Cl$_2$ wash: (5 mL x 1 x 1 min); (5) Neutralization: 5% DIEA/CH$_2$Cl$_2$ (5 mL x 1 x 10 min); (6) CH$_2$Cl$_2$ wash: (5 mL x 6 x 1 min); (7) Equilibration with Boc-amino acids (0.75 m mol) in CH$_2$Cl$_2$, followed by addition of 0.75 m mol DCC in CH$_2$Cl$_2$: total volume 7 mL, 45 min; (8) 33% ethanol/CH$_2$Cl$_2$ wash, (5 mL x 4 x 1 min); steps 7 and 8 were repeated for second coupling. At the end of the synthesis the resin was washed with CH$_2$Cl$_2$ (5 mL x 4 x 1 min), CH$_2$Cl$_2$: MeOH (1:1) (5 mL x 8 x 1 min) and MeOH (5 mL x 4 x 1 min) and dried under vacuum to yield 750 mg of the peptide resin.

i) Cleavage of 1-26 pardinin segment from the resin (25) using trifluoroacetic acid

The resin-bound peptide (300 mg) was suspended in TFA (6 mL); thioanisole (0.6 mL) and m-cresol (0.6 mL) were added and the suspension was allowed to stand in an oil bath at 40°C for 18 h. The resin was filtered, and washed with TFA (2 mL x 2). The filtrate was evaporated under reduced
pressure to remove TFA. The residue was cooled in ice bath and ice-cold dry ether was added to precipitate the free peptide completely. The peptide (130 mg) was washed thoroughly with ice-cold ether (10 mL x 8) to remove all the scavenging reagents. The resin was kept for a second cleavage under the same conditions as described above to yield an additional crop of 55 mg peptide. The peptide was dissolved in methanol and again reprecipitated with cold ether to afford 182 mg of the crude product.

ii) Purification of 1-26 pardaxin peptide

The crude peptide was purified by Fast Protein Liquid Chromatography (FPLC Pharmacia). A C-18 reverse phase Pharmacia column was used for purification. 5 mg of peptide was dissolved in 0.5 mL methanol and injected into Analytical C-18 RPC in small portions. The solvent systems used were 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). A single major peak was obtained at 58% acetonitrile (time 39 min) which was collected and evaporated to get 1 mg pure peptide. The FPLC profiles of the crude and purified peptides are given in Fig. V.9.
Amino acid analysis: Leu, 4.91 (5); Ala, 2.87 (3); Ser, 3.51 (4); Gly, 2.07 (2); Val, 0.97 (1); Thr, 0.901 (1); Lys, 2.00 (2); Phe, 3.02 (3); Pro, 2.10 (2); Ile, 2.35 (3). (The value of Ile is slightly low due to partial cleavage of Ile-Ile bond under the hydrolytic conditions used. Ser was also found to be less due to some degradation during hydrolysis).

iii) **Automatic Edman degradation of 1-26 segment of pardaxin sequence**

Edman degradation was performed on an Applied Biosystem gas phase protein sequencer Model 470 A. Solvents, reagents and phenylthiohydantoin (PTH) standards were also from Applied Biosystems. The PTH derivatives were transferred automatically from the sequencer-conversion vessel to an on-line Applied Biosystem PTH Analyzer Model 120 A. The PTH derivatives of free and side-chain protected amino acids were separated on an Applied Biosystems PTH C-18 column (2.1 mm x 29 cm) at a flow-rate of 200 μL/min at 55°C. The following solvents were used for elution: A) 5% aqueous THF containing 30 mL of 3 M sodium acetate buffer at pH 3.8 and 7 mL of 3 M sodium acetate buffer at pH 4.6/litre. B) MeCN containing 500 n mol N,N'-dimethyl-N'-phenylthiourea
(DMPTU) per litre. The elution was programmed at \( \%B \) as follows: 10\% at 0 min; 14\% at 2 min; 40\% at 20 min; 60\% at 25 min; 60\% at 45 min; 0\% at 45 min. With the pure 26-peptide the resolution was possible only up to 13 residue from the N-terminal. This appears to be due to the hydrophobic nature of the peptide which got washed off from the solid support. Therefore the resin bound pardaxin 1-26 (25) was sequenced on 470 A Applied Biosystem gas phase sequencer using the 03R REZ programme. This run is designed for sequencing resin-bound peptides synthesised by solid phase peptide synthesis method. This is carried out at a high cartridge temperature (50 °C) and flash temperature remains the same as other run (55°C). The increased temperature of the cartridge will help to promote both cleavage and extraction of PTH amino acids.