CHAPTER-3

EXPERIMENTAL
3.1 Preparation and Functionalisation of Polymer Support

3.1.1 Materials and methods

The polymer supports used were synthesised and characterized in this laboratory. IR Spectra were recorded on a Shimadzu IR-470 spectrophotometer using KBr pellets.

3.1.2 Source of chemicals

Styrene, HDODA and polyvinyl alcohol (Mol. wt. 72000) were purchased from Sigma Chemical Company, USA.

3.1.3 Polymer synthesis

3.1.3.1 Preparation of polystyrene crosslinked with 2% HDODA by suspension polymerization

Styrene was destabilized by washing with 1% sodium hydroxide solution (20 ml x 3) and then washed with distilled water (20 ml x 3). A mixture of styrene (22.45 ml, 98 mmol), HDODA (0.896 ml, 2 mmol), toluene (8 ml) as inert solvent and benzoyl peroxide (1 g) was prepared. It was then suspended in a solution of polyvinyl alcohol (mol. wt. 72000, 3.2 g) dissolved in water (320 ml) and kept mechanically stirred at 80°C. Polymerization was completed after 6 h. The beads were collected by filtration, washed with hot water, acetone (30 ml, 3x3 min), methanol (30 ml, 3x3 min) and drained. The product was soxhlet
extracted using acetone for 80 h to remove linear polymers and low mol. wt. impurities and dried in the oven at 80 °C. Beads having 200-400 mesh size were collected.

3.1.3.2 Chloromethylation of PS-HDODA resin using chloromethylmethylether (CMME)

The dry resin beads (2 g, 200-400 mesh size) were preswollen in a dry 2-necked round bottom flask using dry dichloromethane (20 ml). Excess dichloromethane was removed. And a solution of anhydrous zinc chloride (1 M, 0.2 ml) dissolved in THF was added to CMME (12 ml). This solution was slowly added to the resin under anhydrous condition with shaking. The mixture was refluxed with CaCl₂ guard tube for 5 h. The mixture was cooled and filtered through a sintered glass funnel (G-2) washed with THF (30 ml, 3 x 10 min), THF/4N HCl (30 ml, 3 x 3 min), THF/H₂O (30 ml, 3 x 3 min), THF (30 ml, 3 x 3 min), DCM (30 ml, 3 x 3 min), and finally methanol (30 ml, 3 x 3 min). Dried in the oven.

a. Preparation of 1M anhydrous ZnCl₂ in THF

Anhydrous ZnCl₂ (1.5 g) was placed in a beaker and con. HCl (3 drops) and distilled water (5 drops) were added. The contents were stirred and heated until the solid dissolved. Temperature was raised to evaporate the water and to leave a
crust of solid which was then melted by strong heating. When ZnCl₂ became a clear mobile liquid with no further evolution of bubbles, the beaker was placed in a dessicator and allowed to cool. The resulting white powder was dissolved in freshly distilled THF (10 ml).

b. **Determination of chlorine capacity by pyridine fusion method**\(^{229}\)

50 mg of the resin was fused with pyridine (5 ml) in a boiling tube at 110 °C for 5 h. The resin was washed quantitatively with acetic acid/water (1:1 v/v, 30 ml) and diluted with distilled water (50 ml) con. HNO₃ (5 ml), AgNO₃ solution (0.1 N, 5 ml) and ferric alum (0.1M, 5 ml) were added. It was titrated against standard ammonium thiocyanate solution (0.1M). A blank was also performed.

### 3.2 Synthesis of Peptides

#### 3.2.1 Source of chemicals

All side-chain protected amino acids were synthesised by standard procedures. Dicyclohexyl carbodiimide, 1,-hydroxy benzotriazole, trifluoroacetic acid, thioanisole, 1, 2,-ethanedithiol and cesium carbonate were purchased from Aldrich, USA. The amino acids were Boc protected using ditertiarybutyl pyrocarbonate. The amino acids were purified by column
chromatography wherever necessary. All solvents were purified by distillation.

3.2.2 Physical measurements

HPLC was done with C18 reverse phase column (analytical) on a Brooker instrument. IR spectra were recorded on a Nicolet impact 410 FTIR spectrometer. NMR spectra was recorded on a Brooker 200 MHz instrument & UV Spectra on a JASCO-VS-550 UV spectrometer.

3.2.3 Purification of reagents & solvents

All the solvents were purified before use.

DCM was dried over P2O5 and kept over CaH2.

TEA was refluxed over ninhydrin for 1 h and distilled.

TFA was kept over indole for 24 h.

3.2.4 Identification of the peptides using TLC

The methanol solution of the peptide was spotted on the plate and developed in a suitable solvent system.

Solvent systems used are

Butanol : CH3COOH : H2O (6 : 1 : 5)

Ethyl acetate : pyridine : acetic acid : water (30 : 15 : 3 : 2)

Methanol : Chloroform (1 : 9)

Ninhydrin spray was used for the visualisation of the spots.
3.2.5 Protection of side chain functions of amino acids

3.2.5.1 Blocking the sulphydryl group of cysteine

\[
\begin{align*}
\text{CH}_2\text{-SH} \\
\text{Cl-H}_3\text{N}^+\text{-CH- COOH} + \text{HO-CH}_2\text{-NH-CO-CH}_3 \xrightarrow{\text{CF}_3\text{COOH}} \\
\text{S-Acetamidomethyl cysteine}
\end{align*}
\]

\[
\begin{align*}
\text{Cl- H}_3\text{N}^+\text{-CH- COOH} \\
\text{CH}_2\text{-S-CH}_2\text{-NH-CO-CH}_3
\end{align*}
\]

Preparation of N-hydroxymethyl acetamide

Acetamide (10 g) was hydroxymethylated by adding it to a solution of K\textsubscript{2}CO\textsubscript{3} (1 g) in formaldehyde (12.3 g of a 41\% solution). The mixture was heated on a steam bath for about 3 min and then allowed to stand at room temperature overnight. The solution was saturated with CO\textsubscript{2} and evaporated in vacuo, the residue treated with anhydrous Na\textsubscript{2}SO\textsubscript{4} and extracted with acetone. The acetone extracts were further dried with Na\textsubscript{2}SO\textsubscript{4} and evaporated to dryness. The product, a colourless oil, solidifies on standing to a crystalline mass (m.p. 50 – 52 °C).

A mixture of L-cysteine hydrochloride (1.58 g, 10 mmol), N-hydroxymethylacetamide (0.89 g, 10 mmol) and trifluoroacetic acid (10 ml) were stirred at room temperature for about 30 min. The trifluoroacetic acid was removed in vacuo, the residue
dissolved in 1N HCl and evaporation repeated. The crude hydrochloride was crystallised from 2-propanol, washed with ether and dried. The product (1.62 g) melts with decomposition at 155°C. Recrystallised from 2-propanol.

3.2.5.2 Protection of ε-amino group of lysine

**N^\epsilon-benzyloxy carbonyl lysine**

\[
\text{H}_2\text{N} \quad \text{CH} \quad \text{COOH} \quad \xrightarrow{\text{CuCO}_3\text{Cu(OH)}_2} \quad \text{H}_2\text{N} \quad \text{CH} \quad \text{COO}\text{C} \quad \text{NH}_2 \quad \text{Cu} \quad \text{OOC} \quad \text{NH}_2 \quad \text{CH} \quad \text{COO}\text{C} \quad \text{NH}_2 \quad \text{CH} \quad \text{COO}\text{C} \quad \text{NH}_2 \quad \text{CH} \quad \text{COO}\text{C} \quad \text{NH}_2 \quad \text{CH} \quad \text{COO}\text{C} \quad \text{NH}_2
\]
A solution of lysine monohydrochloride (18.3 g, 100 mmol) in water (1 lit) was heated to reflux while cupric carbonate (basic, 30 g) was added with caution. After 2 hrs of boiling the undissolved cupric carbonate was removed from the hot mixture by filtration and washed with hot water (100 ml). The combined filtrate and washings were cooled to room temperature. Cooled in an ice water bath and added benzyloxycarbonyl chloride (16.6 g, 120 mmol) over a period of 30 min with stirring. Adjusted the pH in the range 8 to 9. The reaction mixture was stirred for an additional h. The precipitated product was collected by filtration.
and washed on the sintered funnel with water, ethanol and ether. This copper complex of N\textsuperscript{ε}-benzyloxycarbonyl lysine was suspended in warm (50 °C) water (400 ml) containing con. HCl (2.7 ml) and H\textsubscript{2}S gas was passed through it with stirring and heating for 1 h. The heating was maintained for an additional h. The precipitated copper sulphide was removed by filtration through a celite filter bed. The clear and colourless filtrate was adjusted to pH 6-7 with con. NH\textsubscript{4}OH and the product benzyloxycarbonyl lysine crystallised out. Filtered and recrystallised from hot water. Dried in a dessicator.

3.2.5.3 Protection of hydroxyl group of threonine

O - benzyl threonine
Thr (18 g, 100mmol) was dissolved in 1 N NaOH (100ml). A solution of CuSO₄·5H₂O in water (50 ml) was added. A precipitate formed and dissolved. The mixture was heated to 60°C and cooled to room temperature; Water was evaporated, dried in a dessicator and the solid copper complex was suspended in a solution of dry DMSO and NaI which was heated under nitrogen for 1h at 60 °C. The mixture was cooled to 25 °C and benzyl bromide (13 ml, 100mmol) was added with stirring. The mixture was vigorously
stirred for 3 h. DMSO was removed in vacuo. The copper complex was suspended in warm water (50 °C) containing con. HCl. H₂S gas (prepared by dropping 4N H₂SO₄ into ferrous sulphide) was passed through it with stirring and heating for 1 h. The solution was filtered through a celite filter bed. The clear and colourless solution was adjusted to pH 6-7 using ammonium hydroxide. The precipitated O-benzyl-Thr was collected on a filter, washed with water and dried.

3.2.5.4 Blocking the hydroxyl group of tyrosine

O-benzyl-tyrosine
Tyr (18.1 g, 100 mmol) was dissolved in 2N NaOH (100 ml). A solution of CuSO₄·5H₂O (12.5 g, 50 mmol) in water was added. A precipitate formed and dissolved. The mixture was heated to 60 °C and cooled to room temperature, diluted with methanol (350 ml) and made more alkaline with 2N NaOH (15 ml). This was followed by the addition of benzyl bromide (13 ml, 100 mmol).

The mixture was vigorously stirred at room temperature for 2 h. The purple blue precipitate was collected on a filter, washed
with a mixture of methanol (50 ml) and water (175 ml) then with methanol (25 ml) and dried.

The well disintegrated copper complex was triturated and washed with 1N HCl (5 times, 50 ml each time), with distilled water (twice, 25ml each time), with 1.5N NH₄OH (5 times, 25 ml each time) and finally with water (twice, 25 ml each time). Dried in a dessicator.

3.2.5.5 Protection of indol nitrogen in tryprotophan

Trp (N-CHO)

Dry HCl gas (prepared by dropping con. H₂SO₄ into NaCl and dried by passing through con. H₂SO₄) was bubbled into a solution of Trp (20.4 g, 100 mmol) in formic acid (300 ml). At about hourly intervals samples were taken from the mixture diluted with water and their UV Spectra recorded. A 0.5 ml sample was diluted with water to 10ml and 1ml of the dilute solution was further diluted to 100 ml. The maximum at 278 nm, characteristic for tryptophan, gradually decreased and a new peak emerged at 298 nm. Formylation was completed when there was no increase in the absorption at 298 nm. The solvent was removed in vacuo, ether was added to the remaining syrup and
the crystals were collected by filtration. They were washed with ether and dried in air.

3.2.6 Preparation of Boc-amino acids

\[
\begin{align*}
\text{CH}_3 & \quad \text{C} - \text{O} - \text{CO} - \text{O} - \text{CO} - \text{O} - \text{C} - \text{CH}_3 + \text{H}_2\text{N} - \text{CHR} - \text{COOH} \\
& \quad \text{CH}_3 \\
\text{CH}_3 & \quad \text{C} - \text{O} - \text{CO} - \text{NH} - \text{CHR} - \text{COOH} + \text{CO}_2 + \text{HO} - \text{C} - \text{CH}_3 \\
& \quad \text{CH}_3
\end{align*}
\]

A solution of the amino acid (10 mmol) was dissolved in a mixture of dioxane (20 ml) and water (10 ml). It was cooled in an ice water bath, 1N NaOH (10ml) solution was added and stirred. Di-tertiarybutyl pyrocarbonate (2.4 g, 11mmol) was added and stirring continued at 0 °C for 4 h and at room temperature overnight. The solution was concentrated in vacuo to about 10 ml, cooled in an ice water bath and acidified with citric acid solution. The precipitated product was extracted with ethyl acetate. The extracts were washed with water (twice) and brine (twice). Dried over anhydrous sodium sulphate and ethyl acetate was removed in rotavapor. The residue was recrystallised with a suitable solvent.
3.2.7 Procedure for solid phase peptide synthesis

The first amino acid of the C-terminal portion was esterified to the resin via a benzyl ester linkage by the cesium salt of Boc amino acid. Boc group was then removed by 30% TFA in DCM. It was neutralised by 5% TEA in DCM. Second amino acid was coupled to the aminoacyl resin by DCC coupling method or by active ester procedure. DCM and NMP were used as the solvents. Coupling time was 1 h. By the same method all the amino acids were coupled to the resin. Coupling and deprotection were monitored by ninhydrin test. A three fold molar excess of the Boc amino acids were used for coupling. When the desired sequence of amino acids has been assembled on the resin, the peptide was cleaved by TFA in the presence of acid scavengers.

3.2.7.1 Attachment of first amino acid to the resin: Gisins cesium salt method\textsuperscript{230,231}

Boc amino acid was dissolved in minimum quantity of ethanol in RB flask. This was neutralised with an aqueous saturated solution of cesium carbonate (1 –3 drops). The solution was stirred for 30 minutes and the solvent removed in vacuo. Freshly distilled dry benzene was added and evaporated in a rotavapor. The process was continued till a dry powder of cesium salt of Boc amino acid was obtained.
The cesium salt was dissolved in minimum quantity of freshly distilled DMF. The resin was suspended in it and the suspension was stirred at 50-60 °C in an oil bath. The resin was filtered, washed with DMF (10 ml x 3), DMF/water (1:1, 10 ml x 3), DMF (10ml x 3), methanol (10ml x 3) and DCM (10ml x 3) and dried under vacuum.

3.2.7.2 Estimation of amino groups by picric acid method

The Boc-amino acyl resin (10 ml) was deprotected using 30% TFA/DCM (30 min.). The resin was washed with DCM (7 times) to get rid of TFA completely and neutralised with 5% TEA in DCM for 10 min. The resin was washed with DCM (7 times) and dried in a dessicator. Exactly 5 mg. of the dry resin was taken in a Gisin’s tube and treated with 0.1 M picric acid (2 ml) for 5 min. The unbound picric acid was washed off with DCM (10 times). The resin bound picrate was then eluted with DCM till the elute was clear. It was made up to a certain volume (15 ml) using 95% ethanol. A definite volume of this solution (0.5 ml) was diluted to 5 ml, with 95% ethanol. The optical density (OD) of this solution was measured at 358nm. From the optical density, weight of the resin taken and the extinction coefficient of picrate (ε_{358} = 14500), the substitution level of the first amino acid was estimated.
3.2.7.3 Deprotection procedure: Removal of Boc group

The Boc group can be removed using anhydrous TFA in DCM (30%) for 30 min at room temperature. TFA was washed off with DCM and the amino acid salt thus obtained was neutralised with triethylamine in DCM (5%) for 10 min.

3.2.7.4 Activation and coupling

Preformed active ester method was used for the activation of the Boc amino acid. 3 molar excess of Boc amino acid, HOBT and DCC were used for coupling.

To the Boc amino acid (3 mmol) dissolved in a suitable solvent (NMP, DMF or DCM), HOBT (3 mmol) dissolved in DMF was added and stirred. Chilled to 0°C and added DCC (3 mmol). Warmed to room temperature and kept stirred for 30 min. This active ester solution was added to the resin (capacity 1 mmol per gram) and shaken for 1h. The precipitated DCU was removed by washing with 30% methanol/DCM.

3.2.7.5 Cleavage of the peptide from the resin

The peptidyl resin was suspended in TFA (10 ml) and added thioanisole (0.1 ml), m-cresol (0.1 ml) and 1, 2-ethane dithiol (0.1 ml). It was kept at room temperature for 24 h. Filtered and the TFA solution was removed in vacuo. The peptide was then
precipitated with ice cold dry ether. Centrifuged and washed with dry ether (15 times) and dried.

3.2.8 Hydrogenation of the peptide

The side chain protected peptides were dissolved in 50% MeOH/acetic acid and treated with palladium charcoal (10%) under hydrogen atmosphere in Parr hydrogenation apparatus for 48 h. Filtered and removed the solvent.

3.2.9 Cyclisation of the peptide via disulphide formation

Pure linear peptide was dissolved in 50% methanol/acetic acid. Nitrogen gas was bubbled in to the solution for 10 min. Stirred at room temperature under nitrogen and added a solution of iodine in methanol dropwise till a brown colour persisted. After 1 h the reaction mixture was chilled to 0 °C and the reaction was quenched by adding ascorbic acid in water to decolourise the solution.

3.2.10 Purification

Thin layer chromatography

Silica gel was used for tlc. TLC of side chain protected amino acids, Boc amino acids and peptides were done using suitable solvent systems.
**Column chromatography**

Columns were prepared using silica gel in chloroform. Purification of the side chain protected and Boc protected amino acids were achieved using these columns in appropriate solvent systems.

**High performance liquid chromatography**

HPLC spectra were recorded on a Brooker instrument using a rp Bondapak C18 column.

### 3.3 Synthesis Of Somatostatin Analogues On 2% PS - HDODA Resin

#### 3.3.1 Synthesis of octreotide

\[
\text{D-phe} - \text{Cys} - \text{Phe} - \text{D-Trp} - \text{Lys} - \text{Thr} - \text{Cys} - \text{Thr} \]

\[\text{s} \quad \text{-----------------------} \quad \text{s}\]

#### 3.3.1.1 Attachment of Boc-Thr (O-Bzl) to the chloromethyl resin

1 g of Boc-Thr (O-Bzl) was dissolved in minimum amount of ethanol in a r.b. flask. pH was adjusted to 7 by adding 2M aq. Cs$_2$CO$_3$ (1-3 drops). Solution was kept stirred for 30 min. Dry freshly distilled benzene was added and the solvent removed in rotavapor. The process was repeated to get a dry powder of cesium salt of Boc-Thr (O-Bzl).
The cesium salt of Boc-Thr (O-Bzl) (1mmol, 0.44g) was dissolved in minimum quantity of freshly distilled DMF. The resin was suspended in it and the suspension was stirred at 50-60 °C in an oil bath for 12 h. The resin was filtered, washed with DMF (10 ml x 3), DMF /water (1:1, 10 ml x 3), DMF (10ml x 3), methanol (10ml x 3) and DCM (10ml x 3) and dried under vacuum.

3.3.1.2 Coupling of the subsequent amino acids

Rest of the amino acids were coupled by the standard procedure. Boc-S-Acm-Cys, Boc-Thr (O-Bzl), Boc-Lys (Z) and Boc-D-Trp (CHO) were the side chain protected amino acids used. HOBt active ester method was used for the coupling of all amino acids.

<table>
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<th>Sl. No.</th>
<th>Operation used</th>
<th>Reagent/Solvent</th>
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<tr>
<td>9</td>
<td>Washing</td>
<td>DCM</td>
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Table 3.1. Protocol used for the synthesis of D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr
Boc-Thr (O-Bzl) resin (100 mg,) was deprotected using 30% TFA/DCM. Neutralised by 5% TEA/DCM. Washed with DCM and coupled the subsequent amino acids by the HOBt active ester method. After the attachment of final residue the resin was washed thoroughly with DCM and dried.

3.3.1.3 Cleavage of the peptide from the resin

The peptidyl resin (100 mg) was suspended in TFA (10 ml) and added thioanisole (0.1 ml), m-cresol (0.1 ml) and 1, 2-ethane dithiol (0.1 ml). It was kept at room temperature for 52 h. Filtered and the TFA solution was removed in vacuo. The peptide was then precipitated with ice cold dry ether. Centrifuged and washed with dry ether (15 times) and dried. Purity was checked by tlc using chloroform: methanol: acetic acid (98: 1: 1) solvent system. Purified by preparative tlc to remove the remaining scavenger impurities.

HPLC was recorded on a rp C$_{18}$ column using 20% CH$_3$CN/H$_2$O system. The pure peptide was subjected to hydrogenation using 10 % Pd/charcoal in methanol/acetic acid to remove the side chain protecting groups. Deprotection was completed in 72 h as shown by the change in Rt in HPLC before and after hydrogenation. Filtered and removed the solvent. The residue obtained was dried in a dessicator and yield noted.
3.3.1.4 Cyclisation of the peptide via disulphide formation

Pure linear peptide (50mg)

D-Phe—Cys—phe—D-Trp—Lys—Thr—Cys—Thr

was dissolved in 50% methanol/acetic acid. Nitrogen gas was bubbled in to the solution for 10 min. Stirred at room temperature under nitrogen and a solution of iodine in methanol was added dropwise till a brown colour persisted. After 1 h the reaction mixture was chilled to 0 °C and the reaction was quenched by adding ascorbic acid in water to decolourise the solution.

The solution was concentrated and tlc was checked using butanol : acetic acid : water (6 : 1 : 6) solvent system. Preparative tlc was used to remove ascorbic acid. HPLC of the cyclised peptide was recorded.

3.3.2 Synthesis of TOC

D-Phe—Cys—Tyr—D-Trp—Lys—Val—Cys—Thr

was dissolved in 50% methanol/acetic acid. Nitrogen gas was bubbled in to the solution for 10 min. Stirred at room temperature under nitrogen and a solution of iodine in methanol was added dropwise till a brown colour persisted. After 1 h the reaction mixture was chilled to 0 °C and the reaction was quenched by adding ascorbic acid in water to decolourise the solution.

The solution was concentrated and tlc was checked using butanol : acetic acid : water (6 : 1 : 6) solvent system. Preparative tlc was used to remove ascorbic acid. HPLC of the cyclised peptide was recorded.
3.3.2.1 Attachment of Boc-Thr (O-Bzl) to the chloromethyl resin

1 g of Boc-Thr (O-Bzl) was dissolved in minimum amount of ethanol in a r.b. flask. pH was adjusted to 7 by adding 2M aq. Cs₂CO₃ (1 - 3 drops). Solution was kept stirred for 30min. Dry freshly distilled benzene was added and the solvent removed in rotavapor. The process was repeated to get a dry powder of cesium salt of Boc-Thr (O-Bzl).

The cesium salt of Boc-Thr (O-Bzl) (1mmol, 0.44g) was dissolved in minimum quantity of freshly distilled DMF. The resin was suspended in it and the suspension was stirred at 50-60 °C in an oil bath for 12 h. The resin was filtered, washed with DMF (10 ml x 3), DMF/water (1:1, 10 ml x 3), DMF (10ml x 3), methanol (10ml x 3) and DCM (10ml x 3) and dried under vacuum.

3.3.2.2 Coupling of subsequent amino acid units

Side chain protected amino acids used were Boc-Cys (S-Acm), Boc-Lys(Z), Boc-D-Trp(CHO) and Boc-Tyr(O-Bzl).

Couplings were done using HOBT and DCC in NMP. 30% TFA in DCM was used for deprotection of Boc group and 5% TEA in DCM for neutralisation. Washed with DCM.
3.3.2.3 Cleavage of the peptide from the resin

The peptidyl resin (100mg) was suspended in TFA (10 ml) and added thioanisole (0.1 ml), m-cresol (0.1 ml) and 1, 2-ethane dithiol (0.1 ml). It was kept at room temperature for 48 h. Filtered and the TFA solution was removed in vacuo. The peptide was then precipitated with ice cold dry ether. Centrifuged and washed with dry ether (15 times) and dried. Purity was checked by tlc using chloroform: methanol: acetic acid (98:1:1) solvent system. Purified by preparative tlc to remove the remaining scavenger impurities.

3.3.2.4 Hydrogenation of the peptide

The side chain protected peptides were dissolved in 50% MeOH/acetic acid and treated with palladium charcoal (10%)

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<td>Washing</td>
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Table 3.2 Schedule for the synthesis of D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr
under hydrogen atmosphere in Parr apparatus. Completion of deprotection was checked by HPLC. The reaction was over in 60 h. Filtered and removed the solvent.

3.3.2.5 Cyclisation of the peptide via disulphide formation

Pure linear peptide, (50 mg)

D-phe—Cys—Tyr—D-Trp—Lys—Val—Cys—Thr
   | S-Acm                  | S-Acm

was dissolved in 50% methanol/acetic acid. Nitrogen gas was bubbled in the solution for 10 min. Stirred at room temperature under nitrogen and added a solution of iodine in methanol dropwise till a brown colour persisted. After 1 h the reaction mixture was chilled to 0 °C and the reaction was quenched by adding ascorbic acid in water to decolourise the solution.

The solution was concentrated and tlc was checked using butanol: acetic acid: water (6 : 1 : 6) solvent system. Preparative tlc was used to remove ascorbic acid. HPLC of the cyclised peptide was recorded. Linear peptide was separated from cyclic peptide by HPLC.

3.3.3 Synthesis of RC 160

D-Phe—Cys—Tyr—D-Trp—Lys—Val—Cys—Trp
   | S----------------------S
3.3.3.1 Coupling of Boc-Trp(N-CHO) to the chloromethylated resin

1 g of Boc-Trp (CHO) was dissolved in minimum amount of ethanol in a r.b. flask. pH was adjusted to 7 by adding 2M aq. Cs₂CO₃ (1 – 3 drops). Solution was kept stirred for 30 min. Dry freshly distilled benzene was added and the solvent removed in rotavapor. The process was repeated to get a dry powder of cesium salt of Boc-Trp(CHO).

The cesium salt of Boc-Trp(CHO), (1mmol, 0.492g) was dissolved in minimum quantity of freshly distilled DMF. The resin was suspended in it and the suspension was stirred at 50-60 °C in an oil bath for 12 h. The resin was filtered, washed with DMF (10 ml x 3), DMF/water (1:1, 10 ml x 3), DMF (10ml x 3), methanol (10ml x 3) and DCM (10ml x 3) and dried under vacuum.

3.3.3.2 Coupling of remaining amino acids

Side chain protected amino acids used were Boc-Cys (S-Acm), Boc-Lys(Z), Boc-D-Trp(CHO) and Boc-Tyr(O-Bzl).

Couplings were done using HOBt and DCC in NMP. 30% TFA in DCM was used for deprotection of Boc group and 5% TEA in DCM for neutralisation.
<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Operation used</th>
<th>Reagent/Solvent</th>
<th>No. of times x Duration(min)</th>
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</thead>
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</tr>
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<td>Deprotection</td>
<td>30% TFA/DCM</td>
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</tr>
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<td>Washing</td>
<td>DCM</td>
<td>6 x 2</td>
</tr>
<tr>
<td>4</td>
<td>Neutralisation</td>
<td>5% TEA/DCM</td>
<td>1 x 10</td>
</tr>
<tr>
<td>5</td>
<td>Washing</td>
<td>DCM</td>
<td>4 x 2</td>
</tr>
<tr>
<td>6</td>
<td>Washing</td>
<td>NMP</td>
<td>2 x 2</td>
</tr>
<tr>
<td>7</td>
<td>Coupling</td>
<td>HOBt : DCC : Boc-amino acid (1:1:1) (3 equivalents) in NMP</td>
<td>1 x 60</td>
</tr>
<tr>
<td>8</td>
<td>Washing</td>
<td>33% MeOH/DCM</td>
<td>3 x 2</td>
</tr>
<tr>
<td>9</td>
<td>Washing</td>
<td>DCM</td>
<td>3 x 2</td>
</tr>
</tbody>
</table>

**Table 3.3.** Steps involved in the synthesis of D-Phe-Cys-Tyr-D-Trp -Lys-Val-Cys-Trp

### 3.3.3.3 Cleavage of the peptide from the resin

The peptidyl resin (100mg) was suspended in TFA (10 ml) and added thioanisole (0.1 ml), m-cresol (0.1 ml) and 1, 2-ethane dithiol (0.1 ml). It was kept at room temperature for 48 h. Filtered and the TFA solution was removed in vacuo. The peptide was then precipitated with ice cold dry ether. Centrifuged and washed with dry ether (15 times) and dried. Purity was checked by tlc using chloroform: methanol: acetic acid (98:1:1) solvent system. Purified by preparative tlc to remove the remaining scavenger impurities.
3.3.3.4 Hydrogenation of the peptide

The side chain protected peptides were dissolved in 50% MeOH/acetic acid and treated with palladium charcoal (10%) under hydrogen atmosphere in Parr apparatus. Completion of deprotection was checked by HPLC. The reaction was over in 60 h. Filtered and removed the solvent. Yield noted.

3.3.3.5 Cyclisation of the peptide via disulphide formation

Pure linear peptide, (50 mg)

\[
\text{D-Phe} \rightarrow \text{Cys} \rightarrow \text{Tyr} \rightarrow \text{D-Trp} \rightarrow \text{Lys} \rightarrow \text{Val} \rightarrow \text{Cys} \rightarrow \text{Trp} \\
\downarrow \quad \downarrow \\
\text{S-Acm} \quad \text{S-Acm}
\]

was dissolved in 50% methanol/acetic acid. Nitrogen gas was bubbled in to the solution for 10 min. Stirred at room temperature under nitrogen and a solution of iodine in methanol was added dropwise till a brown colour persisted. After 1 h the reaction mixture was chilled to 0 °C and the reaction was quenched by adding ascorbic acid in water to decolourise the solution.

The solution was concentrated and tlc was checked using butanol: acetic acid: water (6 : 1 : 6) solvent system. Preparative tlc was used to remove ascorbic acid. HPLC of the cyclised peptide
was recorded. Linear peptide was separated from cyclic peptide by HPLC.

3.4 Synthesis of Somatostatin Analogues on 2% PS-DVB Resin

3.4.1 Synthesis of octreotide

\[
\text{D-Phe} - \text{Cys} - \text{Phe} - \text{D-Trp} - \text{Lys} - \text{Thr} - \text{Cys} - \text{Thr} \\
\text{S} \quad \text{S}
\]

3.4.1.1 Attachment of Boc-Thr (O-Bzl) to the chloromethyl resin

1 g of Boc-Thr (O-Bzl) was dissolved in minimum amount of ethanol in a r.b. flask. pH was adjusted to 7 by adding 2M aq. Cs₂CO₃ (1–3 drops). Solution was kept stirred for 30 min. Dry freshly distilled benzene was added and the solvent removed in rotavapor. The process was repeated to get a dry powder of cesium salt of Boc-Thr (O-Bzl).

The cesium salt of Boc-Thr (O-Bzl) (1mmol, 0.44g) was dissolved in minimum quantity of freshly distilled DMF. The resin was suspended in it and the suspension was stirred at 50-60 °C in an oil bath for 12 h. The resin was filtered, washed with DMF (10 ml x 3), DMF/water (1:1, 10 ml x 3), DMF (10ml x 3),
methanol (10ml x 3) and DCM (10ml x 3) and dried under vacuum.

### 3.4.1.2 Coupling of the subsequent amino acids

Rest of the amino acids were coupled by the standard procedure. Boc-S-Acm-Cys, Boc-Thr (O-Bzl), Boc-Lys (Z) and Boc-D-Trp (CHO) were the side chain protected amino acids used. HOBt active ester method was used for the coupling of all amino acids.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Operation</th>
<th>Reagent/Solvent</th>
<th>No. of times x Duration(min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Washing</td>
<td>DCM</td>
<td>3 x 3</td>
</tr>
<tr>
<td>2</td>
<td>Deprotection</td>
<td>30% TFA/DCM</td>
<td>1 x 30</td>
</tr>
<tr>
<td>3</td>
<td>Washing</td>
<td>DCM</td>
<td>6 x 2</td>
</tr>
<tr>
<td>4</td>
<td>Neutralisation</td>
<td>5% TEA/DCM</td>
<td>1 x 10</td>
</tr>
<tr>
<td>5</td>
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</tr>
<tr>
<td>6</td>
<td>Washing</td>
<td>DMF</td>
<td>2 x 2</td>
</tr>
<tr>
<td>7</td>
<td>Coupling</td>
<td>HOBt : DCC : Boc-amino acid (1:1:1) [3 equivalents] in DMF/DCM</td>
<td>1 x 60</td>
</tr>
<tr>
<td>8</td>
<td>Washing</td>
<td>33% MeOH/DCM</td>
<td>3 x 2</td>
</tr>
<tr>
<td>9</td>
<td>Washing</td>
<td>DCM</td>
<td>3 x 2</td>
</tr>
</tbody>
</table>

**Table 3.4.** Protocol used for the synthesis of D-Phe-Cys-Phe-Trp-Lys-Thr-Cys-Thr

Boc-Thr (O-Bzl) resin (100 mg,) was deprotected using 30% TFA/DCM. Neutralised by 5% TEA/DCM. Washed with DCM and coupled the subsequent amino acids by the HOBt active ester
method. After the attachment of final residue the resin was washed thoroughly with DCM and dried.

3.4.1.3 Cleavage of the peptide from the resin

The peptidyl resin (100 mg) was suspended in TFA (10 ml) and added thioanisole (0.1 ml), m-cresol (0.1 ml) and 1, 2-ethane dithiol (0.1 ml). It was kept at room temperature for 52 h. Filtered and the TFA solution was removed in vacuo. The peptide was then precipitated with ice cold dry ether. Centrifuged and washed with dry ether (15 times) and dried. Purity was checked by tlc using chloroform: methanol: acetic acid (98:1:1) solvent system. Purified by preparative tlc to remove the remaining scavenger impurities.

HPLC was recorded on a rp C_{18} column using 20% CH_{3}CN/H_{2}O system. The pure peptide was subjected to hydrogenation using 10% Pd/charcoal in methanol/acetic acid to remove the side chain protecting groups. Deprotection was complete in 72 h as shown by the change in Rt in HPLC before and after hydrogenation. Filtered and removed the solvent. The residue obtained was dried in a dessicator and yield noted.

3.4.1.4 Cyclisation of the peptide via disulphide formation

Pure linear peptide, (50 mg)
was dissolved in 50% methanol/acetic acid. Nitrogen gas was bubbled in to the solution for 10 min. Stirred at room temperature under nitrogen and added a solution of iodine in methanol dropwise till a brown colour persisted. After 1 h the reaction mixture was chilled to 0 °C and the reaction was quenched by adding ascorbic acid in water to decolourise the solution.

The solution was concentrated and tlc was checked using butanol: acetic acid: water (6:1:6) solvent system. Preparative tlc was used to remove ascorbic acid. HPLC of the cyclised peptide was recorded.

3.4.2 Synthesis of Tyr$^3$-Octreotide (TOC)

3.4.2.1 Attachment of Boc-Thr (O-Bzl) to the chloromethyl resin

1 g of Boc-Thr (O-Bzl) was dissolved in minimum amount of ethanol in a r.b. flask. pH was adjusted to 7 by adding 2M aq. Cs₂CO₃ (1–3 drops). Solution was kept stirred for 30 min. Dry
freshly distilled benzene was added and the solvent removed in rotavapor. The process was repeated to get a dry powder of cesium salt of Boc-Thr (O-Bzl).

The cesium salt of Boc-Thr (O-Bzl) (1mmol, 0.44 g) was dissolved in minimum quantity of freshly distilled DMF. The resin was suspended in it and the suspension was stirred at 50-60 °C in an oil bath for 12 h. The resin was filtered, washed with DMF (10 ml x 3), DMF/water (1:1, 10 ml x 3), DMF (10ml x 3), methanol (10ml x 3) and DCM (10ml x 3) and dried under vacuum.

3.4.2.2 Coupling of subsequent amino acids

Side chain protected amino acids used were Boc-Cys (S-Acm), Boc-Lys(Z), Boc-D-Trp(CH$_2$O) and Boc-Tyr(0-Bzl).

Couplings were done using HOBT and DCC in NMP. 30% TFA in DCM was used for deprotection of Boc group and 5% TEA in DCM for neutralisation. After washing with DCM, weight of the dry peptidyl resin was noted.
Table 3.5. Steps involved in the synthesis of D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Operation</th>
<th>Reagent/Solvent</th>
<th>No. of times x Duration(min)</th>
</tr>
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<tr>
<td>1</td>
<td>Washing</td>
<td>DCM</td>
<td>3 x 3</td>
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<tr>
<td>2</td>
<td>Deprotecton</td>
<td>30% TFA/DCM</td>
<td>1 x 30</td>
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<tr>
<td>3</td>
<td>Washing</td>
<td>DCM</td>
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<td>Neutralisation</td>
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<td>DCM</td>
<td>4 x 2</td>
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<tr>
<td>6</td>
<td>Washing</td>
<td>DMF</td>
<td>2 x 2</td>
</tr>
<tr>
<td>7</td>
<td>Coupling</td>
<td>HOBt : DCC : Boc-amino acid (1:1:1) (3 equivalents) in DMF/DCM</td>
<td>1 x 60</td>
</tr>
<tr>
<td>8</td>
<td>Washing</td>
<td>33% MeOH/DCM</td>
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</tr>
<tr>
<td>9</td>
<td>Washing</td>
<td>DCM</td>
<td>3 x 2</td>
</tr>
</tbody>
</table>

3.4.2.3 Cleavage of the peptide from the resin

The peptidyl resin (100mg) was suspended in TFA (10 ml) and added thioanisole (0.1 ml), m-cresol (0.1 ml) and 1, 2-ethane dithiol (0.1 ml). It was kept at room temperature for 48 h. Filtered and the TFA solution was removed in vacuo. The peptide was then precipitated with ice cold dry ether. Centrifuged and washed with dry ether (15 times) and dried. Purity was checked by tlc using chloroform : methanol : acetic acid (98 : 1 : 1) solvent system.
Purified by preparative tlc to remove the remaining scavenger impurities.

3.4.2.4 Hydrogenation of the peptide

The side chain protected peptides were dissolved in 50% MeOH/acetic acid and treated with palladium charcoal (10%) under hydrogen atmosphere in Parr apparatus. Completion of deprotection was checked by HPLC. The reaction was over in 60 h. Filtered and removed the solvent. Yield noted.

3.4.2.5 Cyclisation of the peptide via disulphide formation

Pure linear peptide, (50mg)

\[
\text{D-Phe} \quad \text{Cys} \quad \text{Tyr} \quad \text{D-Trp} \quad \text{Lys} \quad \text{Val} \quad \text{Cys} \quad \text{Thr} \quad \text{S-Acm} \quad \text{S-Acm}
\]

was dissolved in 50 % methanol/acetic acid. Nitrogen gas was bubbled in to the solution for 10 min. Stirred at room temperature under nitrogen and added a solution of iodine in methanol dropwise till a brown colour persisted. After 1 h the reaction mixture was chilled to 0 °C and the reaction was quenched by adding ascorbic acid in water to decolourise the solution.

The solution was concentrated and tlc was checked using butanol: acetic acid: water (6:1:6) solvent system. Preparative tlc
was used to remove ascorbic acid. HPLC of the cyclised peptide was recorded. Linear peptide was separated from cyclic peptide by HPLC.

3.4.3 Synthesis of RC 160

\[
\begin{align*}
\text{D-Phe} & \quad \text{Cys} & \quad \text{Tyr} & \quad \text{D-Trp} & \quad \text{Lys} & \quad \text{Val} & \quad \text{Cys} & \quad \text{Trp} \\
S & \quad \text{S} & \quad & \quad & \quad & \quad & \quad & \quad
\end{align*}
\]

3.4.3.1 Coupling of Boc-Trp(N-CHO) to the chloromethylated resin

1 g of Boc-Trp (CHO) was dissolved in minimum amount of ethanol in a r.b. flask. pH was adjusted to 7 by adding 2M aq. Cs₂CO₃ (1 - 3 drops). Solution was kept stirred for 30min. Dry freshly distilled benzene was added and the solvent removed in rotavapor. The process was repeated to get a dry powder of cesium salt of Boc-Trp (CHO).

The cesium salt of Boc-Trp(CHO). (1mmol, 0.492g) was dissolved in minimum quantity of freshly distilled DMF. The resin was suspended in it and the suspension was stirred at 50-60 °C in an oil bath for 12 h. The resin was filtered, washed with DMF (10 ml x 3), DMF /water (1:1, 10 ml x 3), DMF (10ml x 3), methanol (10ml x 3) and DCM (10ml x 3) and dried under vacuum.
### 3.4.3.2 Coupling of subsequent amino acids

<table>
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<tr>
<th>Sl. No.</th>
<th>Operation</th>
<th>Reagent/Solvent</th>
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<td>Washing</td>
<td>DMF</td>
<td>2 x 2</td>
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<tr>
<td>7</td>
<td>Coupling</td>
<td>HOBt : DCC : Boc-amino acid (1:1:1) (3 equivalents) in DMF/DCM</td>
<td>1 x 60</td>
</tr>
<tr>
<td>8</td>
<td>Washing</td>
<td>33% MeOH/DCM</td>
<td>3 x 2</td>
</tr>
<tr>
<td>9</td>
<td>Washing</td>
<td>DCM</td>
<td>3 x 2</td>
</tr>
</tbody>
</table>

**Table 3.6.** Protocol for the synthesis of D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp

Side chain protected amino acids used were Boc-Cys (S-Acm), Boc-Lys(Z), Boc-D-Trp(CH0) and Boc-Tyr(O-Bzl).

Couplings were done using HOBt and DCC in NMP. 30% TFA in DCM was used for deprotection of Boc group and 5% TEA in DCM for neutralisation. After washing with DCM, weight of the dry peptidyl resin was noted.
3.4.3.3 Cleavage of the peptide from the resin

The peptidyl resin (100mg) was suspended in TFA (10 ml) and added thioanisole (0.1 ml), m-cresol (0.1 ml) and 1, 2-ethane dithiol (0.1 ml). It was kept at room temperature for 48 h. Filtered and the TFA solution was removed in vacuo. The peptide was then precipitated with ice cold dry ether. Centrifuged and washed with dry ether (15 times) and dried. Purity was checked by tlc using chloroform : methanol : acetic acid (98 : 1 : 1) solvent system. Purified by preparative tlc to remove the remaining scavenger impurities.

3.4.3.4 Hydrogenation of the peptide

The side chain protected peptides were dissolved in 50% MeOH/acetic acid and treated with palladium charcoal (10%) under hydrogen atmosphere in Parr apparatus. Completion of deprotection was checked by HPLC. The reaction was over in 60 h. Filtered and removed the solvent. Yield noted.

3.4.3.5 Cyclisation of the peptide via disulphide formation

Pure linear peptide, (50mg)

```
D-Phe — Cys — Tyr — D-Trp — Lys — Val — Cys — Trp
     |           |      |           |
S-Acm    |           | S-Acm|
```
was dissolved in 50% methanol/acetic acid. Nitrogen gas was bubbled in to the solution for 10min. Stirred at room temperature under nitrogen and a solution of iodine in methanol was added dropwise till a brown colour persisted. After 1 h the reaction mixture was chilled to 0 °C and the reaction was quenched by adding ascorbic acid in water to decolourise the solution.

The solution was concentrated and tlc was checked using butanol: acetic acid: water (6:1:6) solvent system. Preparative tlc was used to remove ascorbic acid. HPLC of the cyclised peptide was recorded. Linear peptide was separated from cyclic peptide by HPLC.