CHAPTER 4

SYNTHESIS OF NEUROTENSIN PEPTIDE ANALOGUES ON HYDROPHILIC POLYMER SUPPORT
4.1 Introduction

The history of neurotensin covers some 25 years since Carraway and Leeman's initial isolation of neurotensin from bovine hypothalamic extracts, it has proven to be one of the more remarkable neuropeptide discoveries outside of the classic hypothalamic releasing factors. The tridecapeptide structure of neurotensin places it among the intermediate-sized neuropeptides. The midportion of the neurotensin molecules, being highly enriched in basic amino acids, confers some of its unusual properties. Neuromedin (NMN) is a hexapeptide which has significant C-terminal sequence similarity with neurotensin and is contained in the pro-hormone precursor neurotensin molecule, where it is separated from the neurotensin sequence by a single pair of dibasic amino acids. Neuromedin shares many properties with neurotensin and binds to the neurotensin receptor with similar affinity, but is less potent and apparently more easily degraded than neurotensin. Another neuropeptide derived from extracts of frog skin that shares some sequence homology with neurotensin is xenopsin and two neurotensin related, structurally homologus peptides from chicken intestinal extracts have also been described. More recently, a new, 25 amino acid gut peptide named xenin has been isolated from human gastric mucosa and shown to bind to gut neurotensin receptors thus joining the xenopsin/neurotensin gene family.
Peptides composed of as few as three to 50 amino acids play an important role in a large number of diverse biological processes. The chemical synthesis has been accepted as a method to generate biologically active peptides and their analogues.\textsuperscript{1,2} The solution phase methods have been widely used for the synthesis of peptides, while the current method of choice to obtain peptides is by solid phase method. Solid phase protocols have also been adopted for simultaneous synthesis of peptides and peptide libraries.\textsuperscript{3,4} Synthetic peptides are essential tools for research into molecular mechanism of these neuropeptides. The present chapter describes the synthesis of neuropeptides on a novel class of polymer supports in high yield and purity.

### 4.2 Results and Discussion

#### 4.2.1 Synthesis of Neurotensin Peptide Analogues on PS-HDODA and PS-TTEGDA Resins Using Boc-Chemistry

**4.2.1.1 Boc-chemistry of peptide synthesis**

The hydrophilic polymer supports such as PS-HDODA and PS-TTEGDA with various cross-linking densities were successfully used for the synthesis of biologically active peptides. Even high capacity resin can accommodate the growing peptide chain because of the high flexibility and hydrophilicity of the cross-linker. HPLC and amino acid analysis of these peptides showed that all acylation reactions were completed in a single coupling.
The 2,5-dioxopiperazine (diketopiperazine, DKP) formation from the N-terminal residue of the peptide chain commonly occurs as a disturbing reaction in the synthesis and long term storage of peptides. The nitrogen atom of the N-terminal deprotonated amino group can attack the carbonyl carbon atom of the second residue causing a break down of the chain and formation of DKP. The coupling of third amino acid was followed immediately after the deprotection of the second amino acid can eliminate the formation of DKP.

The synthetic steps of the solid phase assembly of amino acid to peptide are illustrated in Scheme 4.1. The C-terminal amino acid was attached to the solid support by an ester bond using cesium salt method. Deprotection of Boc group was achieved by 30% TFA/DCM. After the deprotection, the resin was washed thoroughly with DCM and NMP. Acylation reactions were carried out in minimum quantity of NMP by using 2.5 equiv. excess of Boc-amino acids, DCC, HOBt and DIEA with respect to the amino capacity of the C-terminal amino acid attached resin. The coupling of each amino acids was monitored by Kaiser test.

The peptide was cleaved from the resin by treating with TFA and suitable scavengers like thioanisole, phenol and water at room temperature. After 8 h, the reaction mixture was filtered, washed
with TFA and the filtrate was evaporated to get an oily residue. The peptide was precipitated by adding ice-cold ether and washed with ether to remove the scavengers. The crude peptide was passed through a sephadex column and the peptidyl fractions were collected and lyophilized.

Scheme 4.1: PS–HDODA and PS–TTEGDA supports based SPPS using Boc-chemistry
4.2.2 Synthesis of neurotensin (1-13) on PS-HDODA and PS-TTEGDA supports (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH)

The peptide was assembled on the polystyrene based polymer supports by manual solid phase strategy. The C-terminal amino acid Leu scaffolded to the chloromethylated resin by Gisin’s cesium salt method. The substitution level of Leu was determined by picric acid method and was found to be 1.55 mmol NH₂ gm. The Boc group was removed by exposing the peptidyl resin to 30% TFA in DCM followed by neutralization with 5% DIEA in DCM. The subsequent amino acids were incorporated by the DCC mediated coupling. The extent of coupling reaction was monitored by the Kaiser test. For all the coupling steps double coupling was employed to ensure the completion of the reaction. A two fold molar excess of the Boc amino acid was used for the first stage and one molar for the second stage. The peptide was deprotected from the support using TFA in presence of thioanisole. The yield of the crude peptide obtained was 96% as calculated on the basis of first amino acid substitution. The purity of the peptide was checked by TLC in the solvent system n-butanol: acetic acid: water (4:1:1) and a single spot was obtained (Rt = 0.78). The purity was further confirmed by HPLC and MALDI TOF MS.
Fig. 4.1: HPLC profile of neurotensin (1-13) obtained from (a) PS-HDODA support (b) PS-TTEGDA (c) MALDI TOF MS of neurotensin (1-13)

4.2.3 Synthesis of Neuromedin on PS–HDODA and PS–TTEGDA supports (Tyr–Ile–Lys–Ile–Pro–Leu–OH)

The peptide was synthesized manually on chloromethylated resins using a disposable syringe fitted with a sintered ware filter
at the bottom. The C-terminal amino acid Leu scaffolded to the chloromethylated resin by Gisin’s cesium salt method. The successive amino acids were assembled using DCC/HOBt coupling. After the synthesis the Boc protection was removed by treating with TFA in DMF. The peptide resin was treated with TFA in presence of scavengers to cleave the peptide from the resin. The peptide formed was washed thoroughly with ice-cold ether and lyophilized. The crude peptides obtained in 94 mg (%) yield. The HPLC profiles (Figure-2) showed that the peptides synthesized were highly pure. The pure peptides were characterized by amino acid analysis and MALDI TOF MS (Figure 4.2).
The peptide was synthesized manually on chloromethylated resin using a disposable syringe fitted with a sintered ware filter at the bottom. The peptide was synthesized as per the protocol shown in scheme 4-1. The peptide formed was washed thoroughly with ice-cold ether and lyophilized. The crude peptides obtained in 94% yield. The HPLC profiles (Figure-4.3) showed that the peptides synthesized were highly pure. The identity of peptides were confirmed by amino acid analysis and MALDI TOF MS (Figure-3).
Fig. 4.3: HPLC profile of xenopsin obtained from (a) PS-HDODA support (b) PS-TTEGDA (c) MALDI TOF MS of xenopsin

4.3 Synthesis of Neurotensin Peptide Analogues by using Fmoc Chemistry

The respective amino acids incorporation with 100% efficiency on resins is still a problem and is one of the challenging problems
encountered by polymer chemists.\textsuperscript{1} Over these years this problem was overcome to an extent by the judicious selection of polymer supports, coupling reagents and solvents. A 2% GDMA-PMMA supports were successfully used for the synthesis of biologically active peptides.\textsuperscript{2} The extraordinary swelling capability of GDMA-PMMA supports in various solvents is found to have a positive impact in facilitating the attachment of respective amino acids to the resins. The introduction of suitable linkers to the resins found to improve the coupling reaction rate and also helps the cleavage of the target peptide in the acid form or C-terminal modified form within a short time. Various linkers such as 4(4-hydroxy- methyl-3-methoxyphenoxy)butyric acid,\textsuperscript{5} were incorporated to 2% GDMA-PMMA supports for peptide synthesis. The acylation reaction proceeds smoothly in a short time single coupling reaction using HBTU coupling method and the side reactions such as racemisation and diketopiperazine formation were not observed in any of these synthesis.

The new GDMA-PMMA can be successfully employed for the synthesis of peptides using Fmoc-amino acids. The resin is extremely stable under the conditions of peptide synthesis. The protocol for the synthesis of peptides using HMPB is shown in Scheme-4.1. The linkers used were suitable for Fmoc-amino acids. The C-terminal amino acid was attached to the resin through an ester bond or an amide bond. MSNT method was used for C-terminal amino acid incorporation to the resin.
The hydroxymethyl GDMA-PMMA resin was also used for the synthesis of peptides. C-terminal amino acid was attached to the resin via an ester linkage using MSNT method. The reaction time was 1 h and quantitative conversion was observed by amino estimation. Deprotection of Fmoc group was achieved by 20% piperidine in DMF. After washing the resin with DMF, acylation reactions were carried out in minimum quantity of DMF by using 2.5 equiv excess of Fmoc amino acids and HBTU, 5 equiv. excess of HOBT and 2.5 equiv DIEA with respect to the amino capacity of the C-terminal amino acid. The acylation reactions were completed in single coupling as shown by Kaiser’s test.

The peptide was cleaved from the resin by treatment with TFA and suitable scavengers at room temperature for 3-5 h. The reaction mixture was filtered and washed with TFA and DCM. The combined filtrate and washings were evaporated under pressure. The peptide was precipitated by the addition of ice-cold ether and washed thoroughly with ether to remove the scavengers. The peptide was dissolved in 1-2% acetic acid-water mixture, passed through a sephadex column, and then lyophilized. Purity of the peptides was analyzed using a Pharmacia LKB HPLC system having a P-500 pump (X2) rapid spectral detector UV-M-II.
The general protocol for the synthesis of peptides on the new resin using Fmoc-amino acids is illustrated in Scheme-4.2.

Scheme 4.2: General protocol for SPPS using Fmoc-amino acids
4.4  Synthesis of Aminomethyl GDMA-PMMA

4.4.1 Chloro-2%GDMA-PMMA resin

The 2%GDMA-PMMA resin (0.15 mmol/g, 2g) was swollen in DCM (50 mL). After 1 h, the excess DCM was filtered off, SOCl$_2$ (6 mmol, mL) was added dropwise to the swollen resin with occasional swirling at 50 °C. After 3 h, the resin was filtered, washed with DCM (3 × 50 mL), DMF (3 × 50 mL), dioxane (3 × 50 mL), ethanol (3 × 50 mL) and methanol (3 × 50 mL) and dried in vacuum. The amount of chlorine substituted was determined by Volhardt’s method.

Chlorine capacity = 0.14 mmol/g

4.4.2 Amino-2%GDMA-PMMA resin

The chloro-2%GDMA-PMMA resin (0.14 mmol Cl/g, 2 g) was swollen in DMF (50 mL). After 2 h, the resin was filtered and stirred with potassium phthalimide (mg, 2.8 mmol) in DMF (20 mL) at 120 °C for 12 h. The resin was filtered, washed with DMF (3 × 50 mL), dioxane (3 × 50 mL), ethanol (3 × 50 mL) and methanol (3 × 50 mL). The dried resin was suspended in ethanol (20 mL) and refluxed with hydrazine hydrate (mL, mmol). After 8 h, the resin was filtered, washed with hot ethanol (3 × 50 mL) and methanol (3 × 50 mL) and dried in vacuum. The amino capacity of the resin = 0.12 mmol/g as estimated by picric acid method.
4.4.3 Esterification of Fmoc-amino acid to polymer support using MSNT

The cross-linked resin (1 equiv) was swelled in dry DCM. Dry Fmoc-amino acid (2 equiv) in a septum-stoppered flask was dissolved in dry DCM using appropriate volume of dry THF. This solution was transferred to a stoppered flask containing MSNT (2 equiv) and methyl imidazole(1.5equiv). The mixture was immediately added to the swollen resin. After 30 min, the reactants were washed off with DCM (6 × 30 mL), DMF (6 × 30 mL), MeOH (5 × 25 mL) and ether (5 × 25 mL). The Fmoc-protection was removed by 20% piperidine/DMF and coupling yield was determined from the UV absorption of dibenzofulvene-piperidine adduct at 290 nm.

4.4.4 Synthesis of 4-(4-hydroxymethyl-3-methoxyphenoxy) butylamidomethyl 2% GDMA–PMMA support

HMPB-resins were prepared by treating aminomethyl GDMA-PMMA resins with 4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid linker in presence of HOBT /HBTU /DIEA for 1 h (Scheme-4.3).
Scheme 4.3: Preparation of 4-(4-hydroxymethyl-3-methoxyphenoxy)butylamidomethyl 2% GDMA–PMMA support

The amino acid corresponding to the C-terminal region of the peptide was attached to the resin by an ester bond. This ester linkage was extremely stable under repeated treatment of 20% piperidine in DMF (reagent used for the deprotection of Fmoc group). The synthesized peptide was cleaved from the support using TFA and scavengers at room temperature for 2 hrs.

4.4.5 Fmoc-deprotection

The study of Fmoc-removal using 5, 10, 15, 20 and 25% piperidine in DMF from the C-terminal amino acid of the resin showed that the percentage cleavage increases with base concentration. The results showed that 20 and 25% base concentrations are suitable for the Fmoc-removal. But 20% base concentration is preferred due to avoid the side reactions during higher base concentration. The rate of cleavage of Fmoc group with 20% piperidine in DMF was
determined by measuring the optical density of the dibenzofulvene-piperidine adduct. The GDMA-PMMA resin required 15 min whereas PS-HDODA, PS-TTEGDA resin required 20 min for quantitative removal. The results show that the reaction rate in the GDMA-PMMA resin is more than PS-HDODA and PS-TTEGDA. The higher solvation and swelling characteristics of the GDMA-PMMA resin in the reaction medium may enhance the free interaction of the protected amino acids and cleavage reagent improving the reaction rate.

4.4.6 Synthesis of neurotensin (1—13) (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH)

35mg of Fmoc-Leu was attached to hydroxymethyl 2% GDMA-PMMA support by MSNT method in presence of methyl imidazole. The quantitative reaction was observed from the Fmoc-estimation by UV absorbance method. After removing the Fmoc-protecting group by 20% piperidine in DMF, the resin was washed thoroughly with DMF and the consecutive amino acids were incorporated by HBTU/HOBt method.

The peptide was synthesized manually on GDMA-PMMA-HMPB resin using a disposable syringe fitted with a sintered glass ware filter at the bottom. Respective Fmoc-amino acid (2.5 molar excess) were attached to C-terminal amino acid bound polymer,
HOBt (2.5 equiv) and HBTU (2.5 equiv) were used for the coupling reaction. A 40 min coupling was required for the quantitative reaction on the resin. After the synthesis the Fmoc protection was removed by treating with 20% piperidine in DMF and the resin was washed with DMF and lyophilized. The peptide resin was treated with TFA in presence of scavengers to cleave the peptide from the resin. The peptide formed was washed thoroughly with ice-cold ether and lyophilized. The crude peptides obtained in 96 mg (%), yield. The HPLC profiles showed that the peptides synthesized were highly pure. The pure peptide was characterized by amino acid analysis and MALDI TOF MS.
Fig. 4.4: HPLC profile of neurotensin obtained from (a) GDMA-PMMA support (b) MALDI TOF MS of neurotensin

4.4.7 Synthesis of Neuromedin (Tyr-Ile-Lys-Ile-Pro-Leu-OH)

The peptide was synthesized manually on GDMA-PMMA-HMPB resin using a disposable syringe fitted with a sintered ware filter at the bottom. Fmoc-amino acid attached to the polymer by using MSNT in presence of methyl imidazole (2.5 molar excess) along with HBTU/HOBt in of DIEA. A 40 min coupling was required for the quantitative reaction on the resin. After the synthesis the Fmoc protection was removed by treating with 20%
piperidine in DMF and the resin was washed with DMF and lyophilized. The peptide resin was treated with TFA in presence of scavengers to cleave the peptide from the resin. The peptide formed was washed thoroughly with ice-cold ether and lyophilized. The crude peptides obtained in 94 mg (%) yield. The HPLC profiles showed that the peptides synthesized were highly pure. The pure peptide was characterized by amino acid analysis and MALDI TOF MS.
4.4.8 Synthesis of Xenopsin on GDMA-PMMA Support \((Glu-Gly-Lys-Arg-Pro-Trp-Ile-Leu-OH)\)

The peptide was synthesized manually on GDMA-PMMA-HMPB resin using a disposable syringe fitted with a sintered ware filter at the bottom. Fmoc-amino acid attached to the polymer by using MSNT in presence of methyl imidazole (2.5 molar excess) along with HBTU/HOBt in DIEA. A 40 min coupling was required for the quantitative reaction on the resin. After the synthesis the Fmoc protection was removed by treating with 20% piperidine in DMF and the resin was washed with DMF and lyophilized. The peptide resin was treated with TFA in presence of scavengers to cleave the peptide from the resin. The peptide formed was washed thoroughly with ice-cold ether and lyophilized. The crude peptides obtained in 94 mg (%)
yield for peptide Xenopsin as revealed by the amino acid analysis of the residual resin after cleavage of the peptide. The HPLC profiles showed that the peptides synthesized were highly pure. The pure peptide was characterized by amino acid analysis and MALDI TOF MS.

**Fig. 4.6:** HPLC profile of Xenopsin obtained from (a) GDMA-PMMA support (b) MALDI TOF MS of Xenopsin
4.5 Experimental

4.5.1 Materials

Cesium carbonate, styrene, t-butyl carbazate, dicyclohexyl carbodiimide (DCC), diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), thioanisole, ethanedithiol and phenol were purchased from Aldrich Chemical Co., USA and Boc-amino acids, Fmoc amino acids, HMPB, HOBt, HBTU and DIEA were purchased from Novabiochem Ltd., UK. All solvents used were of HPLC grade purchased from E. Merck (India) and SRL (India).

IR spectra were recorded on a Shimadzu IR 470 spectrometer using KBr pellets. The $^{13}$C CP-MAS solid-state NMR measurements were conducted on a Bruker 300 MSL CP-MAS instrument operating at 75.47 MHz. HPLC was done on a Pharmacia Akta purifier instrument using C-18 reverse phase semi prep. HPLC column. The amino acid analysis was carried out on an LKB 4151 Alpha plus amino acid analyzer. For this the peptide was hydrolyzed using 6 N HCl in a Pyrex glass tube fused under nitrogen for 15 h at 130 °C. Mass spectra of peptides were obtained with a Kratos PC-Kompact MALDI TOF MS instrument.
4.5.2. Preparation of amino acid derivatives

4.5.2.1 Boc-amino acids (Schnabel’s method):

L-amino acids (10 mmol) were suspended in 1:1 dioxane-water (10 mL) and Boc-azide (1.6 mL, 10 mmol) was added to it. The mixture was stirred at room temperature maintaining the pH in the alkaline range with 4 N NaOH. After 24 h, water (25 mL) was added and the solution was extracted with ether (10 mL). The aqueous layer was cooled in an ice bath, acidified with 2 N HCl and extracted with ethyl acetate (3 × 20 mL). For Boc-Leu, ether was used for extraction. It was then dried over anhydrous Na₂SO₄ and the solvent was evaporated under vacuum.

Purity of the protected amino acids was monitored by tlc on silica gel using CHCl₃-MeOH-Acetic acid (85:10:5) as solvent system. The tlc plate was exposed to HCl vapour for 10 min, and ninhydrin was sprayed to the plate forming blue spots.
Table 1

Preparation of Boc-amino acids

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<th>Yield (%)</th>
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<td>0.56</td>
</tr>
</tbody>
</table>

A: Chloroform (85)-Methanol (10)-Acetic acid (5)

B: Chloroform (95)-Acetic acid (5)

4.5.2.2 Preparation of 1-Hydroxybenzotriazole (HOBt)

O-Chloronitrobenzene (32 g) was dissolved in ethanol (100 mL). Hydrazine hydrate (30 g) was added and refluxed for 5 h. Ethanol was distilled off and the residue obtained was diluted with water (100 mL) and extracted with ether (4 × 50 mL). The aqueous layer was acidified with conc.HCl and HOBt precipitated was recrystallized from hot water. (Yield=20 g, 80%) mp = 157 °C.
4.5.3 General procedure for solid phase peptide synthesis

The following steps are involved in the synthesis of peptides using Boc-chemistry.

The C-terminal Boc-amino acid (3 mmol excess than the capacity of the resin) was dissolved in minimum amount of ethanol and a saturated solution of Cs$_2$CO$_3$ was added till the pH become 7. Ethanol was evaporated under pressure and water was removed by azeotropic distillation with dry benzene. The white powdery cesium salt of Boc-amino acid was dissolved in minimum amount of NMP and added to the pre-swollen chloromethyl resin (200 mg, 0.136 mmol Cl) in NMP. The reaction mixture was kept at 50 °C for 24 h. The resin was washed with NMP (6 × 30 mL), NMP: water (1:1, 6 × 30 mL), methanol (5 × 25 mL), DCM (5 × 25 mL), ether (5 × 25 mL) and dried under vacuum. The Boc protection was removed by 30% TFA in DCM. The resin was washed with DCM (6 × 30 mL) and neutralized with 5% DIEA in DCM and again washed with DCM (5 × 25 mL) and NMP (5 × 25 mL). The extent of incorporation of amino acid was estimated by picric acid method.

The stepwise incorporation of amino acids to the resin was carried out manually in a specially designed silanised glass vessel clamped to a mechanical shaker. The successive amino acids in the target sequence were coupled to the resin as their HOBT active
ester. After 30 min DMSO (2.4 μL) was added and shaken for 5 min and then added one drop of DIEA and kept for 5 min. Each step of the coupling reactions was monitored by Kaiser’s test. After the synthesis, the peptidyl resin was washed with NMP (5 x 25 mL), methanol: DCM (33:67, 10 x 25 mL), DCM (5 x 25 mL), ether (5 x 25 mL) and dried in vacuum. The peptide was cleaved by suspending in TFA/scavenger mixture for 8 h. The reaction mixture was filtered and the filtrate was evaporated till an oily residue obtained. The peptide was precipitated by adding ice-cold ether and the precipitate was washed with ether until the scavengers were removed and dried in vacuum. A small amount of peptide was dissolved in water, injected to C-18 RPC column, and eluted with 0.1% TFA in water (A) and 0.1% TFA in acetonitrile: water (80:20) (B) in a linear gradient.

4.5.4 Synthesis of Neurotensin on PS-HDODA and PS-TTEGDA supports (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH)

Cesium salt of Boc-Leu was prepared by adding a saturated solution of cesium carbonate to Boc-Leu dissolved in minimum amount of ethanol till the pH become 7.0. Ethanol was evaporated under reduced pressure and water was removed by azeotropic distillation with benzene. The cesium salt was dissolved in minimum volume of NMP and added to the pre-swollen chloromethyl resin (200 mg, 0.14 mmol) in NMP, the mixture was kept at 50 °C for
24 h. The resin was washed with NMP (3 x 30 mL), 1:1 NMP/water (3 x 30 mL), MeOH (3 x 30 mL), DCM (3 x 30 mL), ether (3 x 30 mL) and dried under vacuum. Boc-protection was removed by treating with 30% TFA in DCM. The resin was washed with DCM (5 x 50 mL) and then neutralized with 5% DIEA in DCM and again washed thoroughly with DCM and NMP. Boc-Ile (60 mg, 0.34 mmol), Boc-Tyr (78.7 mg, 0.34 mmol), Boc-pro and remaining protected amino acids were coupled successively to the resin as their HOBT active esters. The active ester was prepared by mixing Boc-amino acid with HOBT (46 mg, 0.34 mmol) and DCC (70 mg, 0.34 mmol) in NMP. DCU formed was filtered off and the active ester was added to the resin. After the synthesis, the peptide resin was washed with NMP (3 x 40 mL), MeOH (3 x 40 mL), DCM (3 x 40 mL), ether (3 x 40 mL) and dried in vacuum.

The peptide was cleaved from the resin by adding TFA (2.85 mL) and water (150 µL) for 8 h. The polymeric material was filtered off and the filtrate was concentrated to get an oily residue. By adding ice-cold diethyl ether, the peptide was precipitated as white powder and was washed thoroughly with ether. The purity of the peptide was checked by injecting an aqueous solution of peptide to C-18 RPC column and eluted using 0.1% TFA in water (A) and 0.1% TFA in
The HPLC profile gave a single peak corresponding to the target peptide.

Boc-Leu (88 mg, 0.4 mmol) was attached to the chloromethylated resin by cesium salt method. After removing the Boc group by 30% TFA/DCM and neutralization with 5% DIEA/DCM, Boc-Lys(Cl-Z) (142 mg, 0.34 mmol), HOBt (46 mg, 0.34 mmol) and DCC (70 mg, 0.34 mmol) in NMP were added and shaken for 30 min. DMSO (2.4 μL) was added, shaken for 5 min followed by the addition of a drop of DIEA and kept for 5 min. After washing with 33% MeOH/DCM (5 × 25 mL), DCM (5 × 25 mL), NMP (5 × 25 mL), a second coupling was performed using the same procedure. The remaining amino acids Boc-Ile (100 mg, 0.34 mmol), Boc-Tyr (90 mg, 0.34 mmol), Boc-Pro (79 mg, 0.34 mmol) and Boc-Arg (85 mg, 0.34 mmol) (twice) were successively added till the target sequence was formed. After the synthesis, the peptidyl resin was suspended in anhydrous methanol (15 mL) in presence of DIEA (1.75 mL) for 8 h with occasional stirring at 50 °C. The polymeric material was filtered off and washed with dry methanol and DCM. The resin was subjected to three cycles of trans-esterification for complete recovery of peptide. The filtrate was evaporated to get an oily residue and was precipitated as a white powder by the addition of cold ether (41 mg). The peptide was washed thoroughly with ether to remove
DIEA and passed through a Sephadex G-10 column, the peptidyl fractions were collected and lyophilized. TLC analysis showed a single spot corresponding to the peptide. HPLC analysis gave a single peak corresponding to the peptide. The solvent system used in HPLC were 1% TFA/water (A) and 1% TFA/acetonitrile (B).

Amino acid analysis Glu, 2.18 (2); Leu, 2.11 (2); Tyr, 1.89 (2); Asp, 0.93 (1); Lys, 0.95 (1); Pro, 1.92 (2); Arg, 1.83 (2); Ile, 1.05 (1). (Asn is hydrolysed to Asp) MALDI TOF MS: m/z 1689.90 [(M+H)+, 100%], C_{28} H_{121} N_{21} O_{20} requires M+ 1690.41

4.5.5 Synthesis of Neuromedin on PS-HDODA and PS-TTEGDA supports (Tyr-Ile-Lys-Ile-Pro-Leu-OH)

Cesium salt of Boc-Leu (126 mg, 0.4 mmol) was prepared by treating with a saturated solution of Cs2CO3 till the pH become 7. The solvent was removed by azeotropic distillation with dry benzene and the cesium salt of Boc-Leu formed was dried over P2O5 under vacuum. The cesium salt was dissolved in minimum amount of NMP and chloromethyl resin (200 mg, 0.14 mmol Cl) was added and kept at 50 °C for 24 h with occasional shaking. The resin was washed with NMP (5 × 25 mL), NMP:water (1:1, 5 × 25 mL), DCM (5 × 25 mL), methanol (5 × 25 mL), ether (5 × 25 mL) and dried in vacuum. The amount of Leu attached to the resin was estimated by picric acid method and was observed as 0.56 mmol/g.
The Boc protection was removed by 30% TFA/DCM and after neutralization with 5% DIEA/DCM, Boc-Ile (74 mg, 0.34 mmol), HOBt (46 mg, 0.34 mmol) and DCC (70 mg, 0.34 mmol) were added and shaken for 30 min. DMSO (2.4 μL) was added, shaken for 5 min followed by the addition of a drop of DIEA and kept for 5 min. After washing with 33% methanol/DCM (5 × 25 mL), DCM (5 × 25 mL), NMP (5 × 25 mL) a second coupling was performed using the same procedure. The remaining amino acids Boc-Tyr (90 mg, 0.34 mmol), Boc-Pro (79 mg, 0.34 mmol), Boc-Arg (84.7 mg, 0.34 mmol), Boc Arg (73 mg, 0.34 mmol) were successively coupled using HOBt active ester method. After the synthesis the peptide was cleaved from the resin by suspending in anhydrous methanol (15 mL) and DIEA (1.75 mL). The suspension was kept at 50 °C with occasional stirring for 8 h. The polymeric material was filtered and washed with dry methanol and DCM. The peptidyl resin was subjected to three cycles of trans-esterification for quantitative removal of the peptide. The filtrate and washings were evaporated under vacuum till an oily residue obtained. It was then precipitated by adding ice cold-ether (113 mg). The peptide formed was washed thoroughly with ether to remove DIEA. The crude peptide was passed through a sephadex G-10 column, the peptidyl fractions were collected and lyophilized. tlc analysis gave a single spot corresponding to the peptide. HPLC analysis using 1% TFA/water (A) and 1% TFA/acetonitrile (B) as eluent, a single peak was observed
corresponding to the peptide. Amino acid analysis: Lys, 0.93 (1); Ile, 2.03 (2); Pro, 0.98 (1); Tyr, 0.86 (1); Leu, 1.02(1). MALDI TOF MS: m/z 745.95. [(M+H)⁺, 100%], C₃₈H₇₅N₇O₁₆ equirers M⁺ 747.1.

4.5.6 Synthesis of Xenopsin on PS-HDODA and PS-TTEGDA supports (Glu-Gly-Lys-Arg-Pro-Trp-Ile-Leu-OH)

Boc-Leu (88 mg, 0.4 mmol) was dissolved in minimum amount of ethanol, a saturated solution of Cs₂CO₃ was added with stirring till pH become 7. The ethanol was evaporated and the water was removed by azeotropic distillation with dry benzene. The white powder obtained was kept over P₂O₅ under vacuum. Cesium salt was dissolved in minimum amount of NMP and chloromethyl resin (200 mg, 0.14 mmol Cl⁻) was added to it and kept at 50 °C for 24 h with occasional shaking. The resin was washed with NMP (5 × 25 mL), NMP:water (1:1, 5 × 25 mL), DCM (5 × 25 mL), methanol (5 × 25 mL), ether (5 × 25 mL) and dried under vacuum. The extent of incorporation of Boc-Leu was estimated by picric acid method and was observed to be 0.59 mmol/g.

After the deprotection of Boc group with 30% TFA/DCM, followed by neutralization with 5% DIEA/DCM, Boc-Ile (79 mg, 0.34 mmol), HOBt (46 mg, 0.34 mmol) and DCC (70 mg, 0.34 mmol) in NMP were added and shaken for 30 min. DMSO (2.4 µL) was added, shaken for 5 min followed by addition of a drop of DIEA and kept for 5
After washing with 33% methanol/DCM (5 x 25 mL), DCM (5 x 25 mL) and NMP (5 x 25 mL), a second coupling was conducted using the same procedure. The remaining amino acids Boc-Tyr (90 mg, 0.34 mmol), Boc-Pro (79 mg, 0.34 mmol), Boc-Arg (84.7 mg, 0.34 mmol), and Boc-Arg (74 mg, 0.34 mmol) were successively attached by HOBT active ester coupling method. After the synthesis, the peptide was cleaved from the resin by suspending in dry methanol (15 mL) and DIEA (1.75 mL). The reaction mixture was kept at 50 °C with occasional shaking for 8 h. The polymeric material was filtered off and washed with methanol and DCM. The filtrate along with the washings was evaporated under vacuum to obtain an oily residue. The peptide was precipitated by the addition of cold ether and was washed thoroughly with ether to remove DIEA (105 mg). The crude peptide was passed through a sephadex G-10 column and the tlc analysis showed a single spot corresponding to the peptide. The HPLC analysis of the peptide using 1% TFA/water (A) and 1% TFA/acetonitrile (B) showed a single peak supporting the extra purity of the peptide. Amino acid analysis: Glu, 0.94 (1); Gly, 1.12 (1); Lys, 0.89 (1); Arg, 0.82 (1); Pro, 0.98 (1); Trp, 0.87 (1); Ile, 1.1 (1); Leu 1.03 (1). MALDI TOF MS: m/z 998.35, [(M+H)+, 100%], C_{47}H_{91}N_{13}O_{19} requires M^* 999.51.
4.6 Synthesis of Neurotensin Based on Fmoc-Chemistry

4.6.1 Preparation of 4-(4-hydroxymethyl-3-methoxyphenoxy) butyl amidomethyl resin

4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid (0.052 g, 0.22 mmol), HBTU (0.082 g, 0.22 mmol), HOBT (0.029 g, 0.22 mmol) and DIEA (0.028 g, 10.22 mmol) were added to pre-swollen aminomethyl resin (0.500 g, 0.12 mmol/g) in DMF and the reaction mixture was kept at room temperature for 1 h with occasional swirling. The resin was filtered, washed with DMF (3 x 30 mL), dioxane:H₂O (1:1, 3 x 30 mL), MeOH (3 x 30 mL) and ether (3 x 30 mL). The resin was collected and dried in vacuum. IR (KBr): 3420 cm⁻¹ (NH), 3400 cm⁻¹ (OH), 1680 cm⁻¹ (ester), 1640 cm⁻¹ (NHCO).

4.6.2 Estimation of hydroxyl group in HMPB resin

200 mg of the resin was acetylated with measured amount of acetic anhydride-piperidine mixture (1:4, 3 mL) for 6 h. 10 mL distilled water was added and the reaction mixture was refluxed for 3 h. The mixture was cooled, filtered and acetic acid formed was back titrated with standard (0.1N) NaOH. A blank titration was also carried out. From the titre values, hydroxyl capacity of the resin can be calculated.

Capacity = 0.11 mmol, OH/g
4.6.3 Preparation of Fmoc-Leu-O-CH₂-C₆H₅(OCH₃)₂-O-(CH₂)₃-NHCO-CH₂-C₆H₄-resin

Fmoc-Leu (0.187 g, 0.6 mmol), dissolved in minimum volume of DCM was mixed with DCC (0.062 g, 0.3 mmol) and the mixture was stirred well for 1 h. DCU formed was filtered off, evaporated the DCM and the Fmoc-Leu anhydride formed was dried in vacuum.

The HMPB resin (500 mg, 0.06 mmol) swollen in DMF (10 mL) for 1 h and the excess DMF was decanted. Fmoc-Leu anhydride was dissolved in minimum volume of DMF and added to the swollen resin. DMAP (7.3 mg, 0.06 mmol) was added to the mixture and shaken for 1 h. The resin was filtered, washed with DMF (3 x 40 mL), isoamyl alcohol (3 x 30 mL), diethyl ether (3 x 30 mL) and dried under vacuum.

4.6.4 Esterification of Fmoc-amino acid to polymer support using MSNT

The cross-linked resin (1 equiv) was swelled in dry DCM. Dry Fmoc-amino acid (2 equiv) in a septum-stoppered flask was dissolved in dry DCM using appropriate volume of dry THF. This solution was transferred to a stoppered flask containing MSNT (2 equiv). The mixture was immediately added to the swollen resin. After a 30 min, the reactants were washed off with DCM (6 x 30 mL), DMF (6 x 30 mL), MeOH(5 x 25 mL) and ether (5 x 25 mL). The Fmoc-protection was removed by 20% piperidine/DMF and coupling yield
was determined from the UV absorption of dibenzofulvene-piperidine adduct at 290 nm.

4.6.5 Estimation of amino group in the resin

Fmoc-Leu-\text{-O-CH}_2-C_6H_3(OCH_3)-O-(CH_2)_3-NHCO-CH_2-C_6H_4-resin (10 mg) was suspended in 20% piperidine in DMF (3 mL) for 30 min and then the OD of the solution was measured at 290 nm. From the OD values, the extent of Leu attached to the resin can be calculated [10 mg Fmoc-Leu resin suspended in a solution of 20% piperidine in DMF (3 mL) for 30 min has an optical density 1.65 at 290 nm, if the amino capacity of the resin is 0.1 mmol/g]. Amino capacity of Leu-HMPB resin = 0.11 mmol/g resin.

4.6.6 Synthesis of Neurotensin on GDMA-PMMA support by Fmoc chemistry(Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH)

Fmoc-Leu-O-CH_2-C_6H_4-resin (150 mg, 0.2 mmol OH/g) was swelled in DMF for 1 h. Fmoc protection was removed by using 20% piperidine in DMF (1 \times 10 \text{ mL}, 20 \text{ min}), wash the resin with DMF (6 \times 10 \text{ mL}). Fmoc-Ile (26.50 mg, 0.1 mmol), DCC (21 mg, 0.1 mmol) and HOBT (14 mg, 0.1 mmol) dissolved in DMF was added to the reaction mixture and kept at room temperature. The resin was filtered after 40 min and washed thoroughly with DMF (6 \times 10 \text{ mL}). The remaining amino acids, Fmoc-Tyr (46.8 mg, 0.1 mmol), Fmoc-Pro (33.7 mg, 0.1 mmol), Fmoc-Arg (Mtr) (60.8 mg, 0.1 mmol),
Fmoc-Arg(Mtr) (29.7 mg, 0.1 mmol), Fmoc-Lys (46.8 mg, 0.1 mmol), Fmoc-Asn (59.6 mg, 0.1 mmol), Fmoc-Glu (42.5 mg, 0.1 mmol) and Fmoc-Leu (35.3 mg, 0.1 mmol) were successively incorporated by treating the Fmoc-removed resin with DCC (21.6 mg, 0.1 mmol) and HOBt (14 mg, 0.1 mmol). The resin was washed with DMF (6 × 10 mL). All acylation reactions were performed twice for confirming the quantitative conversion. The Fmoc-deprotection and extent of coupling in each cycle were monitored by Kaiser test. After the attachment of all amino acids, Fmoc-protection was removed and the resin was washed with DMF (6 × 10 mL), ether (6 × 10 mL) and dried in vacuum.

The peptide was cleaved from the resin by suspending in TFA (2.7 μL), water (150 μL), thioanisole (150 μL) and ethanedithiol (75 μL) for 6 h at room temperature. The resin was filtered, washed with TFA and DCM and the combined filtrate was evaporated. The peptide was precipitated by adding ice-cold ether. The peptide was washed thoroughly with ether to remove the scavengers added. The yield of crude peptide is 49 mg (96%). The peptide was dissolved in 1% acetic acid in water and passed through a sephadex G-15 column. The peptidyl fractions were collected and lyophilized. Amino acid analysis: Glu, 2.18 (2); Leu, 2.11 (2); Tyr, 1.89 (2); Asp, 0.93 (1); Lys, 0.95 (1); Pro, 1.92 (2); Arg, 1.83 (2); Ile,
1.05 (1). (Asn is hydrolysed to Asp). MALDI TOF MS: m/z 1689.90
[(M+H)+, 100%], C_{78} H_{121} N_{21} O_{20} requires M^+ 1690.41.

4.6.7 Synthesis of Neuromedin on GDMA-PMMA support by Fmoc
chemistry (Tyr-Ile-Lys-Ile-Pro-Leu-OH)

Fmoc-Leu-O-CH₂-C₆H₄-resin (150 mg, 0.2 mmol/g) was
swelled in DMF for 1 h. Fmoc-protection was removed by using 20%
piperidine in DMF (1 × 10 mL, 20 min). Wash the resin thoroughly
with DMF (6 × 10 mL) and coupling reactions were carried out in a
minimum volume of DMF as solvent. Fmoc-Leu (35.3 mg, 0.1 mmol)
was attached to the resin in presence of DCC (21 mg, 0.1 mmol) and
HOBT (14 mg, 0.1 mmol) dissolved in DMF and the reaction mixture
was kept at room temperature. The resin was filtered after 40 min
and washed thoroughly with DMF (6 × 10 mL). The remaining amino
acids in the target sequence, Fmoc-Ile (35.3 mg, 0.1 mmol), Fmoc-
Tyr (46.8 mg, 0.1 mmol), Fmoc-Lys (46.8 mg, 0.1 mmol) and Fmoc-Pro
(33.7 mg, 0.1 mmol) were successively incorporated by treating the
Fmoc removed resin with DCC (21.6 mg, 0.1 mmol) and HOBT (14 mg,
0.1 mmol) for 40 min. After 40 min the resin was washed with DMF
(6 × 10 mL). All acylation reactions were performed twice for confirming
the quantitative conversion. Each coupling and deprotection step was
monitored by Kaiser test. After the synthesis, Fmoc-protection was
removed and the resin was washed with DMF (6 × 10 mL), ether (6 × 10 mL) and dried in vacuum.

The peptidyl resin was suspended in TFA (2.7 mL) and a mixture of scavengers thioanisole (150 µL), water (150 µL) and ethanedithiol (75 µL) for 8 h at room temperature. The resin was filtered, washed with TFA and DCM. The combined filtrate was evaporated under pressure to obtain an oily residue. The peptide was precipitated by adding ice-cold ether to the oily residue. The peptide formed was washed thoroughly with ether to remove the scavengers and was dissolved in 1% acetic acid in water. The peptide solution was passed through a sephadex G-15 column and the peptidyl fractions were collected and lyophilized. The yield of crude peptide is 50 mg (95%). Amino acid analysis: Lys, 0.93 (1); Ile, 2.03 (2); Pro, 0.98 (1); Tyr, 0.86 (1); Leu, 1.02(1). MALDI TOF MS: m/z 745.95. [(M+H)+, 100%], C_{38}H_{75}N_{10}O_{16} requires M^+ 747.1

4.6.8 Synthesis of Xenopsin on GDMA-PMMA support by Fmoc chemistry (Glu-Gly-Lys-Arg-Pro-Trp-Ile-Leu-OH)

Fmoc-Leu-O-CH₂-C₆H₄-resin (150 mg, 0.2 mmol OH/g) was swelled in DMF for 1 h. Fmoc protection was removed by using 20% piperidine in DMF (1 × 10 mL, 20 min), wash the resin with DMF (6 × 10 mL). Fmoc-Leu (35.3 mg, 0.1 mmol), DCC (21 mg, 0.1 mmol) and HOBt (14 mg, 0.1 mmol) dissolved in DMF was added to the
reaction mixture and kept at room temperature. The resin was filtered after 40 min and washed thoroughly with DMF (6 × 10 mL). The remaining amino acids, Fmoc-Ile (35.3 mg, 0.1 mmol), Fmoc-Tyr (46.8 mg, 0.1 mmol), Fmoc-Pro (33.7 mg, 0.1 mmol), Fmoc-Arg(Mtr) (60.8 mg, 0.1 mmol), Fmoc-Lys (46.8 mg, 0.1 mmol), Fmoc-Gly (29.7 mg, 0.1 mmol) and Fmoc-Glu (42.5 mg, 0.1 mmol) were successively incorporated by treating the Fmoc-removed resin with DCC (21.6 mg, 0.1 mmol) and HOBt (14 mg, 0.1 mmol). The resin was washed with DMF (6 × 10 mL). All acylation reactions were performed twice for confirming the quantitative conversion. The Fmoc-deprotection and extent of coupling in each cycle were monitored by Kaiser test. After the attachment of all amino acids, Fmoc-protection was removed and the resin was washed with DMF (6 × 10 mL), ether (6 × 10 mL) and dried in vacuum.

The peptide was cleaved from the resin by suspending in TFA (2.7 μL), water (150 μL), thioanisole (150 μL) and ethanedithiol (75 μL) for 6 h at room temperature. The resin was filtered, washed with TFA and DCM and the combined filtrate was evaporated. The peptide was precipitated by adding ice-cold ether and was washed thoroughly with ether to remove the scavengers added. The yield of crude peptide is 49 mg (96%). The peptide was dissolved in 1% acetic acid in water and passed through a Sephadex G-15 column.
The peptidyl fractions were collected and lyophilized. Amino acid analysis: Glu, 0.94 (1); Gly, 1.12 (1); Lys, 0.89 (1); Arg, 0.82 (1); Pro, 0.98 (1); Trp, 0.87 (1); Ile, 1.1 (1); Leu, 1.03 (1). MALDI TOF MS: m/z 998.35, [(M+H)+, 100%], C_{47}H_{91}N_{13}O_{19} requires M* 999.51.
References


