CHAPTER V

PEPTIDE SYNTHESIS USING TETRAETHYLENEGLYCOL DIACRYLATE (TTEGDA)-CROSSLINKED POLYSTYRENE SUPPORTS

The practical applications of TTEGDA-crosslinked polystyrene supports are illustrated in this section by the synthesis of various model protected peptides, C-terminal peptide amides and biologically important peptide sequences. Solid phase synthesis of protected peptides is very significant in conformational studies\textsuperscript{192-195} and in segment condensation methods\textsuperscript{151-153}. A new strategy has been introduced for the preparation of fully protected peptide segments which can be cleaved under mild photolytic conditions\textsuperscript{154,155,178}. Conventional trans-esterification method has also been utilized for the synthesis of fully protected model peptides\textsuperscript{117,202}. The utility of TTEGDA-crosslinked polystyrene resin with photolabile 2-nitrobenzyl and \(\alpha\)-methylphenacyl anchoring groups is illustrated by the solid phase synthesis of various peptides. Further application of the new resin in the synthesis of longer biological sequences was demonstrated by synthesising these peptides on chloromethylated...
TTEGDA-crosslinked polystyrene resin and by cleavage with trifluoroacetic acid or by the ammonolysis method. All these peptides after purification were characterised by spectral data and amino acid analysis.

Results and Discussion

V.1. Synthesis of Protected Model Peptides on 4-Chloromethyl-3-nitro Tetraethyleneglycol Diacrylate (TTEGDA)-Crosslinked Polystyrene Resins

The use of photolabile 4-N-alkyl-3-nitroaminomethyl polystyrene supports in the synthesis of C-terminal N-alkyl peptide amides has been reported recently from our laboratory. Here the drastic conditions of base- or acid-treatment for cleavage of peptide amides from the resin can be avoided to obtain fully protected peptides. The 2-nitrobenzyl type of linkages are prone to formation of diketopiperazine during the solid phase synthesis and this can decrease the yield and purity of the peptides. In the present study C-protected amino acid esters are directly attached to the TTEGDA-crosslinked polystyrene resin with 4-chloromethyl-3-nitro anchoring group. This type
of linkage will avoid the formation of diketopiperazine and thereby allow smooth synthesis of peptides.

Five-fold excess amino acid esters was allowed to react with 4-chloromethyl-3-nitro resin (1) in THF-water mixture in the presence of a base like triethylamine (TEA) or diisopropylethylamine (DIEA) at 50-60°C for 10 h. Various solvents were tried for this reaction and THF-water mixture was found to be the best solvent. The resulting amino acid resin showed IR absorptions at 1350 and 1540 cm\(^{-1}\) (characteristic of the \(\text{NO}_2\) group) 1720 cm\(^{-1}\) (ester) and at 3400 cm\(^{-1}\) (broad) (NH). The amino acid incorporation of resin (2) was determined by picric acid method\(^{41}\) or amino acid analysis\(^{117}\). The possibility of the formation of tertiary and quaternary amino acid salt during the reaction of resin (1) with amino acid ester was negligible since a large excess of the amino acid was used. The possibility of this side reaction was further ruled out because of site-site isolation phenomenon in the polymer matrix.

The use of this new 3-nitro-N-methylamino acid ester TTEGDA-polystyrene resin (2) in solid phase
Scheme V.1 Synthesis of Fully Protected Peptides on 4-chloromethyl-3-nitro Photolabile TTEGDA-Crosslinked Polystyrene Resin (1)
peptide synthesis was demonstrated by the synthesis of a few dipeptides and tripeptides (Scheme V.1). The first amino acid was anchored to the solid support through the N-terminal and the peptide chain was elongated from the secondary amino group by Boc amino acid symmetric anhydride procedure. The removal of the Boc-group was effected by treatment with 4N HCl-dioxane. The progress of the coupling reaction was monitored by the semiquantitative ninhydrin test. After the solid phase synthesis, the protected peptides were removed from the support by photolysis. The photolysis was carried out in ethanol-CH₂Cl₂ and trifluoroethanol-CH₂Cl₂ mixtures under nitrogen atmosphere. The peptides after purification on silica gel column was characterized by comparison with authentic samples and amino acid analysis. The yields of the peptides are given in Table V.1.

Table V.1. Photolytic Cleavage Yield of Fully Protected Peptides from the Resin (3)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boc-Met-Leu-Ala-OMe</td>
<td>70</td>
</tr>
<tr>
<td>Boc-Ala-Val-Gly-OEt</td>
<td>77</td>
</tr>
<tr>
<td>Boc-Met-Leu-Phe-OMe</td>
<td>62</td>
</tr>
<tr>
<td>Boc-Val-Gly-OEt</td>
<td>78</td>
</tr>
<tr>
<td>Boc-Phe-Gly-OEt</td>
<td>76</td>
</tr>
</tbody>
</table>
V.2. Mechanism of Photolysis

The mechanism of photolytic cleavage of 2-nitrobenzyl and related system is well-documented\textsuperscript{171}. This involves a light-induced internal oxidation-reduction reaction of aromatic

![Chemical diagram showing the mechanism of photolytic cleavage of 2-nitrobenzyl and related system.]

Scheme V.2. Mechanism of Photolytic Cleavage of Protected Peptides from 2-nitrobenzyl Resins
nitro compounds containing a carbon-hydrogen bond ortho to the nitro group. This mechanism has been established in the case of the low molecular weight compounds. The mechanism of the photolytic cleavage of peptides from the modified peptide resin (3) is analogous to that of the low molecular weight 2-nitrobenzyl system (Scheme V.2).

The foregoing observations illustrate the applicability of the modified TTEGDA-polystyrene resins as photoremovable polymeric supports for the solid phase synthesis of fully protected peptides. This method has the unique advantage of avoiding the unwanted side reaction in 2-nitrobenzyl ester linkage, the diketopiperazine formation, thus increasing the yield of the peptide. In comparison with the trans-esterification method, photolytic cleavage can be conveniently employed for peptides containing sterically hindered C-terminal amino acids like Val and Ile.

V.3. Synthesis of Boc-(Ala-Leu-Ala)_{4}OMe on a High Capacity Chloromethyl TTEGDA-Crosslinked Polystyrene Resin (4)

The synthesis of a hydrophobic 12-residue
peptide was carried out on the new high capacity chloromethyl TTEGDA-crosslinked polystyrene solid support employing established protocols for solid phase peptide synthesis as shown in Scheme V.3. Boc-Ala resin (1.8 m mol Ala/g) (5) was prepared by the cesium salt procedure in high capacity from chloromethyl resin (4). This resin (2) was taken in a solid phase reaction vessel and the remaining amino acid residues were incorporated in the manual mode according to the schedule indicated in Table V.2. N-Boc-protection was used throughout the synthesis. Boc-amino acids were coupled by the dicyclohexylcarbodiimide method. After each coupling reaction dicyclohexylurea formed was removed by washing with 30% EtOH-CH₂Cl₂ mixture. Completion of the coupling was confirmed by the semiquantitative ninhydrin method. Removal of Boc group was effected by anhydrous formic acid. The solvation, swelling and the overall size of the polymer beads increased with the elongation of peptide chain. There was a 2.5-fold weight increase in the case of the peptide resin (6) after the synthesis. This agrees approximately with the molecular weight of the target peptide. The scanning electron micrographs (Fig. V.1) show an increase in bead size after the synthesis. This
Scheme V.3 Synthesis of Fully Protected Hydrophobic 12-Residue Peptide on the TTEGDA-Crosslinked Polystyrene Support. (R = TTEGDA-Crosslinked Polystyrene Resin)
Table V.2 Schedule of Operations for the Solid Phase Assembly of 12-Residue Peptide on the Chloromethyl TTEGDA-Crosslinked Polystyrene Support* (1)

<table>
<thead>
<tr>
<th>Operation</th>
<th>Reagent or Solvent</th>
<th>Volume mL</th>
<th>Mixing time (min)</th>
<th>Application No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wash CH\textsubscript{2}Cl\textsubscript{2}</td>
<td>10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Deprotection</td>
<td>20</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Anhydrous Formic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Wash DMF</td>
<td>10</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Wash CH\textsubscript{2}Cl\textsubscript{2}</td>
<td>10</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>Neutralize</td>
<td>20</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10% DIEA in DMF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Wash DMF</td>
<td>10</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>Wash CH\textsubscript{2}Cl\textsubscript{2}</td>
<td>10</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>Boc-Amino acid, DCC, HOBt</td>
<td>10</td>
<td>45</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>33% EtOH/CH\textsubscript{2}Cl\textsubscript{2}</td>
<td>10</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>Repeat steps 5-8 for second coupling</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*460 mg resin was used in synthesis.
Fig. V.1.a) Scanning Electron Micrographs of TTEGDA-Crosslinked Polymer Beads before (i) and after (ii) the Synthesis of 12-Residue Peptide
Fig. V.1.b) A Single Bead Enlarged from Fig. V.1.a
increase in size of the beads is due to the accommodation of the peptide chain in the high capacity TTEGDA-crosslinked polystyrene resin. The high capacity resin has the advantage of obtaining peptides in relatively large amount as in the solution phase method\textsuperscript{204,205}. The monitoring of the reaction by ninhydrin test is much easy in the case of these high capacity and highly swelling resins. In the \textsuperscript{13}C-CP-MAS-NMR spectra (Fig.V.2) of the peptide resin (6), the intensity of the peaks in the methylene region of peptide chain is enhanced and the styrene carbon peaks are diminished showing that the peptide is the major component in the polymer matrix.

The cleavage of the peptide from the solid support was accomplished in high yield (95\%) by trans-esterification with anhydrous methanol-TEA at 50°C.\textsuperscript{117} Although cleavage by anhydrous HF had been extensively used in solid phase peptide synthesis, considerable difficulties were experienced in the purification of hydrophobic peptides in these cases. Apart from generating protected peptides suitable for post-synthetic modification for physicochemical studies, the trans-esterification method of cleavage also aids in purification, as the protected peptide
Fig. V.2 75.47 MHz $^{13}$C-CP-MAS (Solid state) NMR spectrum of Boc(Ala-Leu-Ala)$_4$-resin (6)

LB, 6 Hz; NS, 1035; $\delta$(ppm)-assigned carbon: 172.59 C-5; 156.2 C-3; 126.01 C-styrene ring; 79.18 C-2; 71.38 C-ether linkages of polymer; 55.21-48.7 C-4/6; 40.20 C-polymer backbone methylene; 28.38 C-1; 15.6-25.39 C-7.
Fig. V.3 270 MHz $^1$H NMR Spectrum of Boc(Ala-Leu-Ala)$_4$-OMe at 60°C.
Fig. V.4. CD Spectrum of Boc(Ala-Leu-Ala)$_4$OMe in TFE; 
$\text{c} = 1 \text{ mg Peptide/mL}$
esters are relatively more soluble in organic solvents. But in the present case the peptide was highly insoluble due to the extreme hydrophobic nature of the peptide. Hence purification cannot be carried out by the usual chromatographic techniques. The crude product was partially soluble in warm DMSO. Hence purification was carried out by reprecipitation in DMSO/ether mixture and washing off the soluble impurities. The final product shows a single spot on tlc and was identified as the target peptide by amino acid analysis and by $^1$H-NMR spectroscopy as shown in Fig. V.3.

The circular dichroic spectra of the fully protected Boc(Ala-Leu-Ala)$_4$-OMe peptide in the helicogenic solvent trifluoroethanol (TFE) is shown in Fig. V.4. The CD spectrum has a single sharp negative band in the 203 nm region.

V.4. Synthesis of Protected Peptide Segments on Photosensitive 4-Bromomethyl-3-nitrobenzyl TTEGDA-Crosslinked Polystyrene Resin (7)

The observation by Rich and Gurwara that ortho-nitrobenzyl ester anchoring linkages can be cleaved to the parent carboxylic acid by photolysis
has spawned considerable work with large implication for practical solid phase peptide synthesis. Photolysis is recognized as a useful technique to achieve the dimensions of orthogonality needed in general routes to protected peptide segments. The unique advantage of this method is that it avoids the drastic conditions of the acid- and the base-catalysed cleavage of the finished peptide from the support involved in the final cleavage step in the solid phase method of peptide synthesis. The photolytic cleavage method under neutral conditions at room temperature offers the possibility of obtaining fully protected peptide segments that can be subsequently used for segment condensation in solution or in the solid phase. A number of polymeric supports with different types of photo-detachable anchoring linkages for the synthesis of peptides on insoluble and soluble polymeric supports have been described. They facilitate the preparation of protected C-terminal peptides, peptide amides and substituted peptide amides under neutral conditions.

For synthesising model peptides using the newly developed TTEGDA-crosslinked polystyrene
Table V.3. General Protocol for Peptide Synthesis on Photolabile TEGDA-Crosslinked Polystyrene Resin using Symmetric Anhydride Method

<table>
<thead>
<tr>
<th>Operation</th>
<th>Reagents or Solvents</th>
<th>Vol mL</th>
<th>time min</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Suspend - CH₂Cl₂</td>
<td>10</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Deprotect - 4N HCl-dioxane</td>
<td>10</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Wash - dioxane</td>
<td>10</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Wash - CH₂Cl₂</td>
<td>10</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>Neutralize* - 7% TEA in CH₂Cl₂</td>
<td>10</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Wash - CH₂Cl₂</td>
<td>10</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>Preformed Boc-amino acid Symmetric anhydride</td>
<td>-</td>
<td>60</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>Wash - MeOH</td>
<td>10</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>Wash - CH₂Cl₂</td>
<td>10</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>Repeat 6-9 for second coupling</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*In-situ neutralization procedure was followed for first 2 cycles.
supports, N-protected amino acids were attached to the bromo resin (7) by refluxing in the presence of triethylamine. The degree of incorporation of Boc-amino acids was determined by picric acid titration method. The N-Boc group was deprotected using 4N HCl-dioxane. The amino acid residues were incorporated in the manual mode according to the schedule shown in Table V.3. The protected peptides were removed from the resin by photolysis in a water-cooled immersion-type photochemical reactor. The peptides were finally purified by column chromatography.

i) Synthesis of Boc-Leu-Leu-Leu-Leu-Leu

The synthetic sequence is depicted in Scheme V.4. The C-terminal Leu residue was attached to the resin by esterification in the presence of triethylamine (TEA). Picric acid method of estimation indicated a capacity of 0.9 m mol of Boc-Leu/g of the resin. The Boc group was deprotected with 4N HCl-dioxane and neutralized with 10% solution of triethylamine in CH₂Cl₂. Symmetric anhydride coupling procedure was used in the entire synthesis. During the addition of third amino acid, in-situ neutralization procedure was employed in
Scheme V.4  Solid Phase Synthesis of Boc-Leu-Leu-Leu-Leu-Leu
Scheme V.4  
Resin (8)

1. 4 NHCl - DIOXANE
2. TEA-CH₂Cl₂
3. Boc-Ala-DCC-CH₂Cl₂

Boc-Ala-Leu-O-CH₂-CONHCH₂-

O₂N

Boc-Val-DCC-TEA
CH₂Cl₂

Boc-Val-Ala-Leu-O-CH₂-CONH-CH₂-

O₂N

Boc-Gly-Val-Ala-Leu-OCH₂-CONH-CH₂-

(10)

Boc-Gly-Val-Ala-Leu-OH

Scheme V.5 Solid Phase Synthesis of Boc-Gly-Val-Ala-Leu-OH
order to avoid diketopiperazine formation. Finally the tetrapeptide Boc-Leu-Leu-Leu-Leu-OH was obtained by photolysis of the peptide resin (9).

ii) Synthesis of Boc-Gly-Val-Ala-Leu

Boc-Leu resin (8) was taken in a solid phase reaction vessel and the coupling of the next three amino acids were carried out by the symmetrical anhydride method. In-situ neutralization was done for third amino acid incorporation to avoid diketopiperazine formation. Photolysis of the resin (10) in methanol released the tetrapeptide. The steps involved are depicted in Scheme V.5.

V.5. Synthesis of Peptide Amides of 4-Aminomethyl-3-nitrobenzamidomethyl TTEGDA-Crosslinked Polystyrene Resin (11)

The synthesis of C-terminal peptide amides is important for the structure-activity correlation and conformational studies. In the conventional method, peptide amides are generally prepared by converting the N-protected C-terminal amino acid to amide by treating with ammonia. In the solid phase polymer-supported
method of Merrifield, these C-terminal peptide amides are usually synthesised by ammonolysis or trans-esterification of peptidyl polymer which usually involves a number of side reactions\textsuperscript{212}. 2-Nitrobenzamide-type photolabile anchoring groups have been reported for the synthesis of peptide amides by making use of insoluble and soluble polymer supports\textsuperscript{169-174,177,178}.

2-Nitrobenzamido linkage was used here for the synthesis of segment 12-17 of Bovine Insulin chain B on the TTEGDA-crosslinked polystyrene resin. Boc-Leu was coupled to the amino resin (11) by dicyclohexylcarbodiimide-mediated coupling. N-\textsuperscript{N}-Boc protection was used throughout the synthesis with suitable selection of side chain protecting groups. Boc-amino acids were coupled by the symmetric anhydride method. Completion of coupling was monitored by semiquantitative ninhydrin method. Cleavage of the protected hexapeptide amide was accomplished by irradiation of a suspension of the peptide polymer (12) in 30\% solution of trifluoroethanol (TFE) in CH\textsubscript{2}Cl\textsubscript{2} for 9-14 hours. The crude peptide was purified by chromatography on a silica gel column. The purity of the peptide was checked by tlc and amino acid analysis.
Scheme V.6. Synthesis of Segment 12-17 of Bovine Insulin Chain B on 4-aminomethyl-3-nitrobenzamidomethyl TTEGDA-Crosslinked Polystyrene Resin (11)

\(\alpha\)-Methylphenacyl linkage, originally developed by Wang in 1976, is now being extensively used in polymer-supported peptide synthesis. The peptide can be conveniently cleaved in the protected form from the support by mild photolytic method. This approach was extended to the new resin in the present case. A model tripeptide was synthesised on this support using solid phase methodology (Scheme V.7). Boc-Leu was esterified to the resin (13) in the presence of triethylamine (TEA). The amino acid analysis gave 0.82 m mol Boc-Leu/g resin. The Boc-group was deprotected using 4N HCl-dioxane.

In the case of the phenacyl resin there are chances of significant loss of \(\alpha\)-amino group during the first two cycles of the synthesis. These losses were sometimes as high as 50%. They were attributed to (i) loss of the first amino group with the ketone of the phenacyl group to form a dihydrooxazinone A and (ii) loss of the first two residues due to the formation of dioxopiperazine B which is prompted by the phenacyl leaving group.
Scheme V.7. Synthesis of Boc-Met-Phe-Leu using α-bromopropionyl TTEGDA-Crosslinked Polystyrene Resin (13)
A modified coupling using the Suzuki procedure\textsuperscript{119} was adopted for the first two coupling steps in the present synthetic procedure. This avoids the side reactions. After the synthesis the tripeptide Boc-Met-Phe-Leu-OH was cleaved from the resin (14) by photolysis in a mixture of dichloromethane and ethanol.

V.7. Synthesis of Peptide amides Using $\alpha$-Amino-propionyl TTEGDA-Crosslinked Polystyrene Resin (15)

The synthesis of C-terminal peptide amides using $\alpha$-methylphenacylamido linkages has been reported. The application of the new TTEGDA-crosslinked polystyrene resin in solid phase peptide synthesis is illustrated here by the synthesis of model peptide amides and substance P segment using $\alpha$-methylphenacylamido anchoring group. Amino propionyl resins with a capacity of 2.1 and 2.61 mmol/g amino group was used for the peptide synthesis. The peptides were assembled on the resin by the stepwise incorporation of Boc-amino acids following the symmetric anhydride procedure. In-situ neutralization procedure was employed during the first cycle in order to avoid the formation of dihydrooxazinone. In all the cases a second coupling
Scheme V.8. Solid Phase Synthesis of Boc-Leu-Gly-Val-NH₂ on α-aminopropionyl TTEGDA-Crosslinked Polystyrene Resin
was performed to ensure maximum incorporation of the Boc-amino acid. The Boc-group was removed by 50% TFA-CH₂Cl₂ or 4N HCl-dioxane for 30 min. The progress of each coupling was monitored by the ninhydrin test. The completed peptide was removed in the C-terminal amide form from the resin (16) by the photolysis of the peptide resin in CH₂Cl₂:EtOH mixture at 350 nm for 25 h. The crude peptide was purified by chromatography and crystallization. The purity was checked by tlc. The synthesis of the model peptide Boc-Leu-Ala-Gly-Ala-NH₂ is depicted in Scheme V.8.

V.8. Synthesis of Substance P (7-11) Segment on α-Aminopropionyl Tetraethyleneglycol Diacrylate (TTEGDA)-Crosslinked Polystyrene Resin (15)

In order to demonstrate the application of the α-aminopropionyl resin further, Substance P (7-11) segment was synthesised on the new support. Substance P (SP) was discovered as far back as 1931, but was not isolated in pure form until 1970. In 1971 the amino acid sequence of SP from bovine hypothalami was elucidated. This sequence was found identical with that of SP from horse intestine:
Scheme V.9. Synthesis of Substance P (7-11) Segment on α-Aminopropionyl TTEGDA-Crosslinked Polystyrene Resin (15)
(H-Arg-Pro-Cys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂).

1 2 3 4 5 6 7 8 9 10 11

Since then several synthesis have been described using the conventional and solid phase methodologies. Recently solid phase synthesis of substance P and its analogues employing Fmoc and Boc strategy on photolabile resins have been reported.

The synthetic strategy for SP(7-11) Boc-Phe-Phe-Gly-Leu-Met-NH₂ is depicted in Scheme V.9. After the stepwise synthesis the peptide amide was obtained by photolysis in 55% yield and purified on a silica gel column. The pure peptide was characterised by amino acid analysis.

V.9. Synthesis of Opioid Agonist Deltorphin on High Capacity Chloromethyl Tetraethyleneglycol diacrylate -Crosslinked Polystyrene Resin by Fmoc N-Protection Strategy and Ammonolytic Cleavage

The synthesis of the 7-residue peptide amide was carried out on chloromethyl TTEGDA-crosslinked polystyrene support using Fmoc N-protection and final cleavage of the peptide from the resin by ammonolysis. Recently, two opioid heptapeptides were
isolated from the skin extracts of South American frog, *Phyllomedusa bicolor*. They were named deltorphins, the amino acid sequence being Tyr-D-Ala-Phe-Asp/Glu-Val-Val-Gly-NH₂. They possess a high affinity and selectivity towards opioid binding sites, they are several times more specific than the synthetic peptide, DPDPE ((D-Pen², D-Pen⁵) enkephalin) which is routinely used at present as a prototypical 6-probe.

The versatile nature of the TTEGDA-crosslinked polystyrene resin is established by the synthesis of another 7-residue peptide, deltorphin. The first amino acid, Boc-Gly was anchored to chloromethyl resin (4) by the triethylamine method in high capacity (1.65 m mol/g). The Boc-group was removed by treatment with 30% TFA/CH₂Cl₂ and the resulting amine salt was neutralized with triethylamine. The peptide chain was built by sequentially extending it towards the amino terminus by stepwise addition of Fmoc amino acid (3 equiv), DCC (3 equiv) and HOBT (1 equiv). The coupling reactions were monitored by the ninhydrin test. The N-terminal amino acid Tyr was incorporated as benzyloxycarbonyl (Z)-Tyr-(Bz₁)-OTCP (trichlorophenylester) (3 equiv) in the presence of HOBT (1 equiv). The protected heptapeptide amide was
Boc-Gly-OH + Cl-CH₂ = R (4)

   Cesium carbonate;
   DMF 60°C 24 h.

Boc-Gly-O-CH₂ = R

   1) 30% TFA-CH₂Cl₂
   2) 10% Et₂N in DMF
   3) Fmoc-Val (3 equiv)
      DCC (3 equiv) HOBT
      (1 equiv) in CH₂Cl₂/DMF

Fmoc-Val-Gly-O-CH₂ = R

   4) 50% Piperidine in DMF
   5) Appropriate
      Fmoc-amino acid
      (3 equiv) DCC (3 equiv)
      HOBT (1 equiv) in DMF/CH₂Cl₂

Fmoc-D-Ala-Phe-Asp(OBzl)-Val-Val-Gly-O-CH₂ = R

   6) Z-Tyr(Bzl)-OTCP
      (3 equiv) HOBT (1 equiv)
      in DMF:CH₂Cl₂

Z-Tyr(Bzl)-D-Ala-Phe-Asp(OBzl)-Val-Val-Gly-O-CH₂ = R (18)

   7) Ammonolysis(NH₃-MeOH)
   8) Catalytic transfer-
      hydrogenation
      (Pd-black/85%HCOOH)
   9) Purification

Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH₂

Scheme V.10. Synthesis of Deltorphin using High Capacity TTEGDA-Crosslinked Polystyrene Resin (4); (R) = TTEGDA-Crosslinked Polystyrene Resin)
cleaved from the support by ammonolysis in 85% yield. The benzyl group from Tyr(Bz1) and Asp (OBzl) residues and terminal Z group were removed by catalytic hydrogenation (Pd black/85% HCOOH at room temperature). The resulting free peptide amide was purified by gel filtration on sephadex G-15. The pure peptide was characterised by amino acid analysis and tlc. (Scheme V.10).

V.10. Synthesis of Cytoadhesive Inhibitory Peptide Gly-Arg-Gly-Asp/Glu-Ser-Pro on High Capacity Chloromethyl TTEGDA-Crosslinked Polystyrene Resin

High capacity chloromethyl resins were found to be efficient in the synthesis of medium peptides in the case of this new support. This appears to be due to the additional space provided by the flexible and hydrophilic crosslinking agent TTEGDA. For establishing the utility of new resin in high capacity solid phase peptide synthesis a few biologically important sequences were selected.

It has been well-established that Arg-Gly-Asp (R.G.D.) motif is very important for the cytoadhesive property of several proteins to the
Most of these proteins are found in the extra cellular matrix and are attached to the cell surface by means of specific receptors called integrins. The main examples of such cytoadhesive proteins which are involved in cell adhesion through the Arg-Gly-Asp (R.G.D.) orif are fibronectin, thrombospondin, vitronectin, collagen type III and IV. In all these cases it has been demonstrated that interaction between the proteins and their corresponding integrin can be abolished by synthetic peptides that contain R.G.D. sequence as in Gly-Arg-Gly-Asp-Ser-Pro. Even a single amino acid change in this sequence abolishes the inhibitory effect of these peptides and therefore the peptide Gly-Arg-Gly-Glu-Ser-Pro does not show this inhibitory effect. As a further demonstration of the application of the high capacity TTEGDA-crosslinked polystyrene resin in solid phase peptide synthesis two hexapeptides Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) and Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) were synthesised.

Boc-Pro was quantitatively attached to the chloromethyl resin by the cesium salt method (1.7 m mol/g). The incorporation of Boc-Pro was estimated by picric acid method and solid phase manual
Boc-Pro + Cl-CH$_2$ (R) (4)

Cesium Carbonate/DMF 60\%  24 h.

Boc-Pro-O-CH$_2$ (R)

1) 30\% TFA-CH$_2$Cl$_2$
2) 5\% DIPA/CH$_2$Cl$_2$
3) Boc-Ser/ DCC/CH$_2$Cl$_2$

Boc-Ser(Bzl)-Pro-O-CH$_2$ (R)

(1) & (2)
4) Boc-Asp(Obzl)/DCC
   Boc-Glu(Obzl)/CH$_2$Cl$_2$

Boc-X(Obzl)-Ser(Bzl)-Pro-O-CH$_2$ (R)

(1) & (2)
5) Boc-Gly/DCC/CH$_2$Cl$_2$

Boc-Gly-X(Obzl)-Ser(Bzl)-Pro-O-CH$_2$ (R)

(1) & (2)
6) Boc-Arg(Mts)/DCC
   HOBt/CH$_2$Cl$_2$/DMF

Boc-Arg(Mts)-Gly-X(Obzl)-Ser(Bzl)-Pro-O-CH$_2$ (R)

(1) & (2)
7) Boc-Gly/DCC/CH$_2$Cl$_2$

Boc-Gly-Arg(Mts)-Gly-X(Obzl)-Ser(Bzl)-Pro-O-CH$_2$ (R)
(19/20)

TFA: Thioanisole&m-cresol
40\°C, 24 h.

H-Gly-Arg-Gly-X-Ser-Pro-OH

Scheme V.11. Synthesis of Cytoadhesive inhibitory peptide Gly-Arg-Gly-Asp-Ser-Pro and Gly-Arg-Gly-Glu-Ser-Pro on High Capacity TTEGDA-Crosslinked Polystyrene Resin (4); (X = Glu or Asp; R = TTEGDA-Crosslinked Polystyrene Resin)
synthesis was carried out on this resin (Scheme V.9). N-Boc-protection was used throughout the synthesis with mesitylene sulfonic acid side chain protection for Arg and benzylester side chain protection for Glu/Asp. Dicyclohexylcarbodiimide double coupling procedure was followed throughout the synthesis with HOBt coupling for Boc-Arg (Mts). Coupling reactions were followed by the ninhydrin test. After the synthesis, the peptide was cleaved from the resin by treatment with trifluoroacetic acid in the presence of thioanisole and m-cresol. In order to ensure complete removal of the peptide from the resin, a second cleavage was conducted. The deprotected peptide was obtained in 95% yield as evidenced by amino acid analysis of the residual resin after cleavage of the peptide. The peptide was purified by reprecipitation from methanol/ether mixture and characterised by amino acid analysis. FPLC profile of the purified peptide is shown in Fig. V.5. The same procedure was adopted for the synthesis of control peptide Gly-Arg-Gly-Glu-Ser-Pro. The peptide was cleaved in 95% yield and purified by recrystallization from methanol/ether. The pure peptide was characterised by tlc and amino acid analysis.
Fig. V.5. FPLC Purified Gly-Arg-Gly-Asp-Ser-Pro on a Pharmacia C-18 column Using 0.1% TFA in Water (A), 0.1% TFA in Acetonitrile (B) Solvent System. Flow rate 0.5 mL/min.
Biological studies were in perfect agreement with the previously reported results\textsuperscript{227}. Cell adhesion experiments were conducted according to reported procedures using AK-5 tumor cells\textsuperscript{228} and it was found that Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) synthetic peptides inhibit the cell adhesion while the control peptide Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) does not. It was also found that the synthetic peptides were not toxic on AK-5 cells and they can be directly used for biological studies.

V.11. Synthesis of Nuclear Signal Sequence on Chloromethyl Tetraethyleneglycol diacrylate (TTEGDA)-Crosslinked Polystyrene Support (4)

It has been well-established that certain medium-to short-range polypeptides are essential for the transport of proteins from the extracellular matrix to the nucleus through the nuclear envelope\textsuperscript{229,230}. Specific signal peptides are recognised by the nuclear envelope at certain positions and they allow the transport of specific proteins through these pores. As soon as their function is over signal peptides are degraded and hence they cannot be isolated from natural sources. Synthesis of nuclear signal sequences is essential
for the study of the mechanism of transport of proteins through the nuclear envelope. In the present study a 11-residue nuclear signal sequence was synthesised on the chloromethyl TTEGDA-crosslinked polystyrene support.

Boc-Cys(Acm) was quantitatively attached to the chloromethyl resin by cesium salt procedure. N-Boc protection was used for the synthesis of the 11-residue signal sequence. A resin of 0.33 m mol Boc-Cys(Acm)/g capacity was employed in the stepwise synthesis by manual mode according to the schedule shown in Table V.4. Upto two amino acid residues could be attached per day by the dicyclohexylcarbodiimide-mediated double coupling procedure. The duration of HOBt coupling in the case of Boc-Arg(Mts) was increased from 45 to 120 min (Scheme V.12). The finished peptide was cleaved from the support using trifluoroacetic acid in the presence of thioanisole and m-cresol as scavengers in 90% yield. The purification was achieved using a reverse phase C-18 column on a fast protein liquid chromatograph. A linear gradient of 0.1% TFA-water (A)-0.1% TFA-acetonitrile (B) solvent mixture was employed here (Fig. V.6). The purified peptide gave a single peak on FPLC and this was found to be the
Table V.4. Schedule for the Coupling of Boc Amino Acids by DCC Method

<table>
<thead>
<tr>
<th>Operation No.</th>
<th>Reagent/Solvent*</th>
<th>Time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CH₂Cl₂ Wash</td>
<td>x 3</td>
</tr>
<tr>
<td>2</td>
<td>30% TFA-CH₂Cl₂</td>
<td>x 1</td>
</tr>
<tr>
<td>3</td>
<td>CH₂Cl₂ Wash</td>
<td>x 4</td>
</tr>
<tr>
<td>4</td>
<td>Pre wash 5% TEA-CH₂Cl₂</td>
<td>x 1</td>
</tr>
<tr>
<td>5</td>
<td>Neutralize 5% DIEA-CH₂Cl₂</td>
<td>x 1</td>
</tr>
<tr>
<td>6</td>
<td>CH₂Cl₂</td>
<td>x 4</td>
</tr>
<tr>
<td>7</td>
<td>Boc Amino acid (2.5 equiv)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>DCC (2.5 equiv) in CH₂Cl₂</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>33% EtOH-CH₂Cl₂</td>
<td>x 4</td>
</tr>
</tbody>
</table>

Steps 5 to 8 were repeated for second coupling

*20 mL per gm resin.
Boc-Cys(Acm)-OH + Cl-CH₂-R (4)

Cesium Carbonate/
DMF 60°C,
24 h.

Boc-Cys(Acm)-OH-O-CH₂-R

1) 30% TFA-CH₂Cl₂
2) 5% DIEA-CH₂Cl₂
3) Boc amino acid/DCC/
   HOBt in CH₂Cl₂/DMF

Boc-Ser(Bzl)-Thr(Bzl)-Pro-Pro-Lys(ClZ)-Lys(ClZ)-Lys
(ClZ)-Arg(Mts)-Lys(ClZ)-Val-Cys(Acm)-O-CH₂-R (21)

TFA: Thioanisole:m-cresol
40°C

H-Ser-Thr-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-Cys(Acm)-OH

Scheme V.12. Synthesis of 11-residue Nuclear Signal Sequence on Chloromethyl TTEGDA-Crosslinked Polystyrene Resin (4); R = TTEGDA-Crosslinked Polystyrene Resin
Fig. V.6. FPLC of H-Ser-Thr-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-Cys(Acm)-OH on a Pharmacia Prep. C-18 Column Using 0.1% TFA in Water (A), 0.1% TFA in Acetonitrile (B) Solvent System. Flow rate 2 mL/min.
target peptide by 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).

These syntheses clearly demonstrate that the new TTEGDA-crosslinked polystyrene resin is an efficient support for the stepwise synthesis of peptides. The crosslinked copolymer has the desired physicochemical properties such as optimum polarity and greater chain mobility. This makes the polymer matrix more compatible with the growing peptide chain and thereby increases the stepwise coupling yield and final product yield.

V.12. Synthesis of Pardaxin, a 33-residue Peptide Toxin, on High Capacity Tetraethyleneglycol (TTEGDA)-Crosslinked Polystyrene Support

Investigation on the synthesis of 33-residue peptide toxin Pardaxin Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val-Gly-Ser-Ala-Leu-Ser-Ser-Ser-Gly-Glu-Gln-Glu (GFFAL'IPIKIISSPLLFFKTLLSAGSALSSEGSQE) was carried out using the newly developed tetraethyleneglycol diacrylate-crosslinked polystyrene (2% crosslinked). These
sequences were required in other studies related to the effect of chain length and secondary structure on the activity of the peptide\textsuperscript{231}. Although discovered two decades ago, relatively little work has been done with pardaxin and the experimental findings are complicated by the discrepancies over its composition and sequence. The pardachirus toxins or pardaxins, were first isolated and described in 1972\textsuperscript{232}. The Mose Sole of the Red sea \textit{Pardachirus marmoratus}, is an ichthocrinotoxic fish, and thus secretes toxins from special glands in its skin. \textit{P. marmoratus} has 200 glands along its dorsal and anal fins and crude pardaxin (Px) is isolated by applying pressure on these glands. Preliminary fraction of the 'crude' exudate yielded a monomeric polypeptide of Mr=13 Kd with 160 amino acid and 4 disulfide bridges. This peptide was found to be lethal to teleost fish and to repel sharks, suggesting a function in defense against predators\textsuperscript{233,234}. Further attempts to characterize Px yielded Px\textsubscript{i} and Px\textsubscript{ii} with the first 10 residues as Gly-Phe-Phe-Ala-Leu-Ile-Pro-Gly-Ile-Glu (G F F A L I P G I E). In 1986 the isolation of three closely related pardaxins (P-1, P-2, P-3) from another species of pardachirus (\textit{P. pavoninus}, the peacock sole of the Western Pacific) was reported\textsuperscript{235}. This
was the first report of entire sequence of any pardaxin isolated, and the first 10 residues Gly-Phe-Phe-Ala-Leu-Pro-Lys-Ile-Ile (GFPA~LIPKII) were different from that obtained for the $P_{xi}$ and $P_{xi_{ii}}$. Moreover no Asp/Asn, His or Cys was present in any of the three sequences which are as follows:

P-1 GFFALIPDIISSPLFDTLLSAVGSALSSEGQE

P-2 I G

P-3 F L (-)E

The peptides were found to be erythroleptic and ichthyotoxic ($P\text{-}1$ and $P\text{-}2$ killed killer fish on 30 min at 25 g/mL), possess strong surface activity and aggregate unto tetramers at high concentration.
In an effort to confirm the sequence, the peptide was synthesised by solid phase peptide synthetic method on PAM resins. Synthesis of Pardaxin partial sequences have also been reported by solution phase and segment condensation method. Although the 'native' pardaxin reported as two peaks in an HPLC procedure using C-4 column, the synthetic peptides corresponded to only one of these peaks. The channel forming ability of the synthetic peptide was found to be 10-fold greater. A synthetic N-terminal 26-residue peptide was observed to have 1000-fold decreased ability to form channels, and the N-terminal decamer was totally inactive.

V.13. Synthesis of Pardaxin Sequence (16-33), (5-33) and (1-33)

Chloromethyl resin of high capacity (1.5 m mol Cl/g) was used for the synthesis. Boc-Glu (OBzI) was first attached to the chloromethyl tetraethyleneglycol diacrylate-crosslinked polystyrene resin by the cesium salt method. Boc-Glu(OBzI) was quantitatively attached to the solid support. The extent of incorporation of amino acid residue was estimated by amino acid analysis.
and by picric acid method. The completion of reaction was also confirmed by absence of any detectable amount of residual chlorine by Volhard's method. Standard procedures for the solid phase method with certain modifications were followed for the synthesis (Scheme V.13). The reaction flask with the Boc-Glu (OBzl) polymer was placed in a shaker and the remaining amino acid residues were incorporated in the manual mode according to the schedule shown in Table V.4. Upto 3 residues could be incorporated per day using this manual procedure. N-Boc protection was used throughout the synthesis with suitable selection of side chain protecting group as detailed in the experimental section. After Boc deprotection using 30% TFA in CH₂Cl₂ and neutralization with 5% DIEA in CH₂Cl₂, Boc-amino acid (2.5 equiv) was added to the resin followed by dicyclohexylcarbodiimide (2.5 equiv) in CH₂Cl₂. HOBt procedure was used for Boc-Gln coupling, and DCC double coupling was followed throughout the synthesis. Completion of the coupling was verified by the ninhydrin test. A third coupling was performed wherever necessary to bring the coupling to completion. The synthesis was stopped at the 16th amino acid residue from the N-terminal and 1/3 of the resin-bound peptide was removed. The
Boc-Glu(OBzl) + Cl-CH$_2$-C$_6$H$_4$- (4)

Cesium Carbonate/DMF 60°, 24 h.

Boc-Glu(OBzl)-O-CH$_2$-C$_6$H$_4$- (R)

1) 30% TFA/CH$_2$Cl$_2$
2) 5% DIEA/CH$_2$Cl$_2$
3) Boc Amino acids/DCC/HOBt Stepwise Synthesis

Boc-Lys(ClZ)-Thr(Bzl)-Leu-Leu-Ser(Bzl)-Ala-Val-Gly-Ser(Bzl)-Ala-Leu-Ser(Bzl)-Ser(Bzl)-Ser(Bzl)-Ser(Bzl)-Ser(Bzl)-Ser(Bzl)-Gly-Glu(OBzl)-Gln-Glu(OBzl)-O-CH$_2$-C$_6$H$_4$- (R) (22)

TFA : Thioanisole: m-Cresol (10:1:1) 37°C 24 h.

H-Lys-Thr-Leu-Leu-Ser-Ala-Val-Gly-Ser-Ala-Leu-Ser-Ser-Ser-Gly-Glu-Gln-Glu-OR

Scheme V.13. Outline of the Synthesis of 16-33 Pardaxin Peptide using Tetraethyleneglycol diacrylate-Crosslinked Polystyrene as Solid Support; (R) = TTEGDA-Crosslinked Polystyrene Resin
(16-33) pardaxin peptide was cleaved from the resin by treating with trifluoroacetic acid in the presence of thioanisole and m-cresol at 37°C under anhydrous condition. A second cleavage was carried out to make sure that complete cleavage of the peptide from the support has occurred. The cleavage yield was 98% as indicated by the estimation of remaining peptide bound to the resin. The cleaved peptide was separated by filtration and subsequent removal of trifluoroacetic acid under vacuum. The peptide was precipitated using cold ether and washed thoroughly with ether to remove the scavengers. The completely deprotected peptide was purified on a Fast protein Liquid Chromatograph (FPLC) using a C-18 reverse phase semi prep Pharmacia FPLC column. The solvent system used was 0.25% TFA in water (A) and 0.25% TFA in acetonitrile (B) at a flow rate of 0.5mL/min. The gradient used is shown in Fig.V.7. A single major peak which occurred at 38% acetonitrile was collected and the solvent evaporated to get 67.5% overall yield of the pure 18-residue peptide. Amino acid analysis of the fraction agrees with the target sequence. From the analytical HPLC profile of the purified peptide (Fig. V.7) the homogeneity of the 18-residue peptide was confirmed. The free peptide in methanol showed
Fig. V.7. FPLC of unpurified 18-residue pardaxin sequence after TFA cleavage: Gradient used 0 time 0% B; 20% B in 15 min; 39% B in 30 min; 100% B in 5 min; a flow rate of 0.5 mL/min. (Inset) HPLC profile of the purified peptide: Gradient used: 0 time 5% B; 40% B in 35 min; 40% for 5 min; 95% B in 25 min; flow rate 0.8 mL/min.
Fig. V.8. C.D. Spectrum of 16-33 Pardaxin Sequence in Methanol.
a circular dichroism curve with a weak positive peak at 215 nm and strong negative band near 197 nm confirming a random coil structure (Fig. V.8).

The stepwise synthesis of the Pardaxin sequence, a 33-residue peptide, was continued with the remaining resin adopting the same DCC coupling procedure (Scheme V.14). The synthesis was stopped at fifth amino acid residue from the N-terminal to get 5-33 Pardaxin peptide-resin, a portion of which was kept aside. The weight increase of the resin was not as expected indicating some problems in the incorporation of the amino acid from 18-residue peptide (16-33 Pardaxin) onwards. Further four more amino acids were added to the remaining portion of the resin to yield the 33-residue Pardaxin peptide resin (Scheme V.15). During the final stage of the synthesis 5% DIEA in DMF was found to be more effective than CH₂Cl₂ as the solvent for neutralization step as indicated by ninhydrin test. The peptides (33- and 29-residues) were cleaved from the support by TFA cleavage procedure as described in the case of the 16-33 Pardaxin (18-residue) peptide. The purification of these peptides were attempted on a FPLC and HPLC using C-18 reverse phase column (RPC). The solvents used were 0.1% TFA in water (A) and
Boc-Glu(OBzl) - O-CH$_2$-C$_6$H$_4$ - R

1) 30% TFA-CH$_2$Cl$_2$
2) 5% DIEA-CH$_2$Cl$_2$
3) Boc Amino Acids/DCC/HOBt
   Stepwise Synthesis

Boc-Leu-Ile-Pro-Lys(ClZ)-Ile-Ile-Ser(Bzl)-Ser(Bzl)-
Pro-Leu-Phe-Lys(ClZ)-Thr(Bzl)-Leu-Leu-Ser(Bzl)-Ala-
Val-Gly-Ser(Bzl)-Ala-Leu-Ser(Bzl)-Ser(Bzl)-Ser(Bzl)-
Gly-Glu(OBzl)-Gln-Glu(OBzl) - O-CH$_2$-C$_6$H$_4$ - R (23)

TFA : Thioanisole: m-Cresol
      (10:1:1)/37°C
      24 h.

H-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys-
Thr-Leu-Leu-Ser-Ala-Val-Gly-Ser-Ala-Leu-Ser-Ser-Ser-
Gly-Glu-Gln-Glu-OH

Scheme V.14 Synthetic Strategy for 5-33 Pardaxin
Sequence using TTEGDA-Crosslinked
Polystyrene (2%) Support; (R) =
TTEGDA-Crosslinked Polystyrene Resin)
Boc-Glu(OBz1)-O-CH$_2$-C$_6$H$_4$-\(\text{R}\)

1) 30\% TFA/CH$_2$Cl$_2$
2) 5\% DIEA/CH$_2$Cl$_2$
3) Boc Amino Acid/DCC/HOBt Stepwise Synthesis

Boc-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys(ClZ)-Ile-Ile-Ser(Bz1)-Ser(Bz1)-Pro-Leu-Phe-Lys(ClZ)-Thr(Bz1)-Leu-Leu-Ser(Bz1)-Ala-Val-Gly-Ser(Bz1)-Ala-Leu-Ser(Bz1)-Ser(Bz1)-Ser(Bz1)-Gly-Glu(OBz1)-Gln-Glu(OBz1)-O-CH$_2$-C$_6$H$_4$-\(\text{R}\) (24)

TFA : Thioanisole: m-Cresol
(10:1:1)/37°C
24 h.

H-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val-Gly-Ser-Ala-Leu-Ser-Ser-Ser-Gly-Glu-Gln-Glu-OH

Scheme V.15, Synthetic Strategy for the 1-33 Pardaxin, Peptide Toxin Sequence using High Capacity TTEGDA-Crosslinked Polystyrene (2\%) Resin; \(\text{R}\) = TTEGDA-Crosslinked Polystyrene Resin
0.1% TFA in acetonitrile (B) at a flow rate of 0.5 mL/min. Purification of these peptides was very difficult. The biological studies were carried out using the crude peptide. These studies show that 18-residue peptide (18-P) is inactive, 29-residue peptide (29-P) is less active compared to 33-residue synthetic Pardaxin (33-P) in the case of membrane lytic activity.

Since the purification of the 33-residue and 29-residue peptides was difficult using C-18 RPC the crude peptides were sequenced by manual procedure to probe the sequence of the synthesised peptide. Tarr's manual sequencing procedure was employed in sequencing of 29-P and 33-P simultaneously. Using Tarr's batchwise procedure, HPLC peptide map fraction could be quickly screened for amino terminal purity and identification of peptides. Four samples: 29-P and 33-P, one peptide with known sequence (RNA polymerase) and a blank were handled simultaneously. Using this procedure it was possible to go up to the eighth residue; after that the peptide got washed off from the tube. The results (Table V.5) indicate that the target peptide is present in the crude product along with deletion sequences.

In the attempted synthesis of 33-residue
Table V.6. Manual sequencing of unpurified pardaxin sequences after cleavage from the support using Tarr's method 123

<table>
<thead>
<tr>
<th>Residue</th>
<th>5-33 (29-P)</th>
<th>1-33 (33-P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L &gt; E/S&gt;G&gt;A</td>
<td>G &gt;L&gt;K&gt;A&gt;P</td>
</tr>
<tr>
<td>2</td>
<td>I &gt;A&gt;L&gt;P</td>
<td>F &gt;G&gt;A&gt;T</td>
</tr>
<tr>
<td>3</td>
<td>P &gt;L/I&gt;A&gt;V</td>
<td>F &gt;G&gt;A&gt;P</td>
</tr>
<tr>
<td>4</td>
<td>K &gt;L&gt;A&gt;G</td>
<td>A &gt;L&gt;K&gt;V</td>
</tr>
<tr>
<td>5</td>
<td>I &gt;L&gt;G&gt;E/S</td>
<td>P &gt;L&gt;A&gt;V</td>
</tr>
<tr>
<td>6</td>
<td>I &gt;L&gt;A&gt;G&gt;R</td>
<td>I &gt;F&gt;K&gt;G</td>
</tr>
<tr>
<td>7</td>
<td>S &gt;G&gt;E&gt;A&gt;L</td>
<td>P &gt;A&gt;G&gt;L</td>
</tr>
<tr>
<td>8</td>
<td>S &gt;Q</td>
<td>K &gt;F&gt;G</td>
</tr>
</tbody>
</table>

Pardaxin peptide using tetraethyleneglycol diacrylate crosslinked polystyrene resin the synthesis proceeded very well upto the 18-residue peptide. Further chain elongation was difficult as indicated by product analysis and weight increment of the peptide resin. This might be due to some conformational changes occurring during the stepwise elongation. It has been shown that conformational transition of the growing peptide chain may cause a dramatic decrease in the reactivity. Thus
conformationally induced changes in the physicochemical properties of a growing peptide chain were revealed to be a general source of trouble\textsuperscript{239-241}. Such studies were not undertaken in the present context since they are beyond the scope of this work.

V.14. Synthesis of 26-residue Peptide Corresponding to the N-terminal Hydrophobic Region of Pardaxin on High Capacity Tetraethyleneglycol Diacrylate -Crosslinked Polystyrene (4%) Supports

Tetraethyleneglycol diacrylate (TTEGDA)-crosslinked polystyrene supports were found suitable for the synthesis of many medium-sized peptides by standard solid phase methodology. The use of this resin was further illustrated by the synthesis of bioactive peptides. The 26-residue corresponding to the N-terminal hydrophobic region of Pardaxin from Pardachirus Pavoninus was synthesised on the high capacity chloromethyl TTEGDA-crosslinked polystyrene (4%) resin. The study of interaction of synthetic peptides with small unilamellar vesicles of phosphatidyl choline and phosphatidyl serin is of current interest for the study of mechanism by which peptide toxins act on biological membranes\textsuperscript{231}. The
26-residue peptide forms a useful tool for the study of biophysical properties of pardaxin peptide sequences.

Chloromethyl TTEGDA-crosslinked polystyrene resin of uniform bead size (100-200 mesh) was used in solid phase synthesis of the 26-residue peptide. Boc-Leu was attached to the high capacity (2.1 m mol Cl/g) chloromethyl resin(4) using the cesium salt procedure. Stirring of the resin during this step caused problem of clogging during the synthesis. Hence stirring was avoided. Amino acid analysis of this resin showed quantitative incorporation of Boc-Leu (1.8 m mol Boc-Leu/g). There was no residual chlorine after the reaction as indicated by Volhard's method. A portion of the Boc-Leu-resin was used for the synthesis of the 26-residue peptide. Boc-group was deprotected using 30% TFA-CH₂Cl₂ and stepwise synthesis was carried out as shown in Scheme V.16. The peptide was assembled by the stepwise incorporation of the Boc-amino acids using the DCC coupling procedure manually. Double coupling procedure using 2.5-fold molar excess of Boc-amino acid was followed throughout the synthesis. HOBt coupling in DMF was found effective for Boc-Ile. The stepwise coupling and deprotection
Boc-Leu-OH + Cl-CH$_2$-C$_6$H$_4$-\(\text{R}\) \[\downarrow\]
Cesium Carbonate/
DMF 60°C,
24 h.
\[\downarrow\]
Boc-Leu-OCH$_2$-C$_6$H$_4$-\(\text{R}\) \[\downarrow\]
1) 30% TFA/CH$_2$Cl$_2$
2) 5% DIEA/CH$_2$Cl$_2$
3) Boc Amino Acid/DCC/HOBt
Stepwise Synthesis

Boc-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys(Cl$_2$)-Ile-Ile-
Ser(Bzl))-Ser(Bzl)-Pro-Leu-Phe-Lys(Cl$_2$)-Thr(Bzl)-Leu-
Leu-Ser(Bzl)-Ala-Val-Gly-Ser(Bzl)-Ala-Leu-
O-CH$_2$-C$_6$H$_4$-\(\text{R}\) (25) \[\downarrow\]
TFA:Thioanisol:anisole:cresol
(10:1:1), 40°C

H-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser
Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val-Gly-
Ser-Ala-Leu-OH

Scheme V.16. Synthesis of Pardaxin 1-26 Segment on
High Capacity TTEGDA-Crosslinked
Polystyrene (4%) Support; \(\text{R}\) =
TTEGDA-Crosslinked Polystyrene Resin)
steps in this synthetic strategy were observed to proceed in near-quantitative yield supporting the positive role of the hydrophilic and flexible polyoxyethylene crosslinks in facilitating the synthetic reactions. The diacrylate crosslinks withstand all the conditions which are commonly employed in solid phase peptide synthesis like TFA treatment. It is possible that the ester linkages in the interior of the crosslinked matrix resist the hydrolysis due to the relative inaccessibility for hydrolytic reagent. The progress of the coupling reaction in the synthesis was monitored by semiquantitative ninhydrin method. Coupling yields averaging > 99.8% were observed in each coupling. After the synthesis, the resin showed a five-fold weight increase which is in agreement with the molecular weight of the 26-residue peptide.

Cleavage of the completely deprotected 26-residue peptide was accomplished by treatment with trifluoroacetic acid at 37°C in presence of thioanisole and m-cresol for 24 h under completely anhydrous conditions. The yield of acidolytic cleavage was about 95% which was determined by the measurement of the remaining peptide still bound to the resin. The cleaved peptide was isolated by
filtration and evaporation of the filtrate under vacuum. The peptide was precipitated as a white powder using cold ether. The white residue was thoroughly washed with cold ether and the solid product was reprecipitated from methanol-ether mixture. The crude product was further purified on a fast protein liquid chromatograph using reverse phase C-18 FPLC semi prep column to obtain 26-residue pure peptide with an overall 20% yield based on the starting Leu-polymer (Fig. V.9). The solvent system used was 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 pure peptide.

Amino acid analysis; Leu, 4.91 (5); Ala, 2.87 (3); Ser, 3.51 (4); Gly, 2.07 (2); Val, 0.97 (1); Thr, 0.904 (1); Lys, 2.00 (2); Phe, 3.02 (3); Pro, 2.10 (2); Ile, 2.35 (3); The values 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. The free peptide in methanol showed a circular dichroism curve with a strong positive peak at 195 nm and two strong negative bands at 209 nm and 222 nm confirming a helical structure (Fig. V.10).
The peptide was characterised by amino acid analysis and Edman degradation on an Applied Biosystem gas phase protein sequencer. With the pure 26-residue peptide the analysis was possible only up to thirteenth residue from the N-terminal. This is due to the hydrophobic nature of the peptide which got washed off from the solid support. Therefore the resin-bound peptide was sequenced using a different programme on the protein sequencer (03R. REZ Program)\textsuperscript{124}. Sequence analysis confirmed the purity of the target peptide supporting the FPLC profiles of the 26-residue peptide.

The above results indicate that tetraethyleneglycol diacrylate crosslinking agents can be successfully employed in the design of efficient polymeric supports for solid phase peptide synthesis. These supports are extremely stable under all conditions of peptide synthesis and they have the added advantage of high swelling and increased reactivity in aminoacylation and deprotection steps driving the gel phase reaction nearly to completion. Compared to the classical solid phase synthesis at conventional loading, these high capacity resins have a number of advantages, which stem from the much more efficient use of
Fig. V.9. FPLC of Total Crude (A) and Purified (B) 1-26 Pardaxin Peptide Sequence. A Pharmacia Semi prep C-18 Column was used. Elution was carried out with a gradient of 0.1% TFA in Water and 0.1% TFA in Acetonitrile (B); Flow rate: 0.5 mL/min
Fig. V.10. CD Spectrum of 1-26 Pardaxin Peptide Toxin in Methanol
available volume within each gel particle. These advantages include enhanced coupling rates during peptide bond formation, major saving in cost due to the more effective use of reagents and of reaction and washing solvents, greatly improved sensitivity in the monitoring of the coupling reaction to effect peptide bond formation and the synthesis of peptides in quantities approaching those obtainable by solution phase method.