Chapter

4

Results and Discussion

PART A: SYNTHESIS OF CROSSLINKED POLYMERS

4.1. Preparation and characterisation of 1,4-butanediol dimethacrylate crosslinked polystyrene (BDDMA-PS)

While new types of resin supports eventually give significant improvements in SPPS, conventionally used polystyrene based supports were most important if certain factors, especially chemical nature and topographical structure of the polymer matrix and the solvents and reagents which are compatible to the polymer, are taken into account. The topography of the polymer matrix is determined by the chemical nature of monomers, degree of crosslinking and polymerization technique. Pillai et al. have recognised the relevance of an optimum hydrophobic-hydrophilic balance in tackling majority of the problems associated with rigid and hydrophobic macromolecular environment created by DVB-crosslinked polystyrene matrix in peptide synthesis. They could tune the macromolecular structure of the styrene based supports by using hydrophilic and flexible crosslinking agents in appropriate mole percentages.

In the present thesis an attempt has been made to prepare a styrene based amphiphilic support by using various percentages of polar and flexible BDDMA as crosslinking agent instead of rigid and hydrophobic DVB,
conventionally used for resin preparation. Destabilised styrene and 1,4-butenediol dimethacrylate were copolymerized by a free radical initiated suspension polymerization to prepare BDDMA-PS (Scheme 4.1). Benzoyl peroxide was used as the free radical initiator and toluene was employed as the diluent. By adjusting the monomer ratio, 1 to 10 mol% BDDMA-crosslinked microporous polystyrene beads were prepared. The polymer was obtained in 80-90% yield in spherical form in varying size ranging from 100-600 mesh size (Table 4.1). Temperature, rate of stirring, atmosphere, molecular weight of suspending agent, nature and amount of diluent, and shape of the polymerization vessel have significant effect on the morphological character of the polymer beads. In the present polymerization, a temperature of 85°C, 600 rpm stirring rate, 1% PVA (M.wt.: 75000) solution as the suspending medium furnished maximum yield in the 200-400 mesh size range. Toluene in 8:1 (diluent to monomer) ratio was found to be optimum for preparing microporous spherical beads of uniform crosslink density. Beads larger than 400 mesh have less total surface area and beads smaller than 200 mesh may clog the sintered disc during the peptide synthesis. Though the polymerization was complete within 3 h, a total reaction time of 8 to 12 h was given to get stable spherical beads. The resulting beads were repeatedly washed with hot water to remove PVA and Soxhleted with toluene and dichloromethane to remove monomers and linear analogues.

![Scheme 4.1. Preparation of BDDMA-PS](image)

Scheme 4.1. Preparation of BDDMA-PS
Table 4.1. Synthetic details of BDDMA-PS

<table>
<thead>
<tr>
<th>BDDMA crosslinking (mol%)</th>
<th>Amount of styrene (ml)</th>
<th>Amount of BDDMA (ml)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.34</td>
<td>0.22</td>
<td>76</td>
</tr>
<tr>
<td>2</td>
<td>11.22</td>
<td>0.44</td>
<td>83</td>
</tr>
<tr>
<td>4</td>
<td>10.99</td>
<td>0.88</td>
<td>84</td>
</tr>
<tr>
<td>5</td>
<td>10.88</td>
<td>1.10</td>
<td>81</td>
</tr>
<tr>
<td>6</td>
<td>10.77</td>
<td>1.32</td>
<td>88</td>
</tr>
<tr>
<td>8</td>
<td>10.54</td>
<td>1.76</td>
<td>89</td>
</tr>
<tr>
<td>10</td>
<td>10.31</td>
<td>2.21</td>
<td>92</td>
</tr>
</tbody>
</table>

The copolymer beads were characterised by IR and $^{13}$C-CP-MAS-NMR spectroscopy. IR spectrum shows a sharp peak at 1720 cm$^{-1}$ corresponding to the ester group of the crosslinking agent, BDDMA (Figure 4.1). NMR gives an intense peak at 130.4 ppm corresponding to the aromatic polystyrene carbons and a small peak at 148.2 ppm due to the C-3 carbon of the styrene. The backbone methylene carbon of the polymer appears as a singlet at 42.7 ppm and the methylene carbon of the crosslinking agent, BDDMA appears at 64 ppm (Figure 4.2). The smooth surface morphology of the polymer beads is evident from SEM photographs (Figure 4.3).
4.2. Swelling studies of BDDMA-PS

The efficiency of a functional polymer is governed by the accessibility of the reactive functional groups anchored on it and which in turn depends upon the extent of swelling and solvation. A good solvent brings the crosslinked polymer to a state of complete solvation and then the polymeric network expands to form a gel. The rate of diffusion of a reagent into the polymer matrix mainly depends on the extent of swelling. So the most effective solvent for a polymer-supported reaction may differ from that commonly used for analogous low molecular weight reactions. Therefore, swelling is an important parameter which controls the success of a solid-phase reaction.

Variously crosslinked (1 to 10 mol%) BDDMA-PS resins swell considerably in solvents of widely varying polarities (Table 4.2). The data show that this resin is compatible with all of the solvents listed, from toluene and ethyl acetate on the one hand to DMSO and water on the other. The resin is truly amphiphilic in nature as evident from the swollen volume of 2 mol% BDDMA-PS in DCM (18.1 ml) and methanol (9.6 ml) which is almost 4.5 and 2.2 times the dry bed volume of 1 g of resin. In water, this styrene based support swells to an extent of 1.3 times its dry volume. It was found that swelling capacity decreases with increase in crosslink density. The reason is
attributed to the reduced tendency of the polymeric backbone to expand and the increased chain entanglement occurred at high crosslink density. As the crosslink density was changed from 1 to 10 mol%, almost 50% decrease was observed in solvent uptake. However, the solvent compatibility extent given in Table 4.2 is not like the liquid inhibition observed for highly porous resins. The solvent uptake of microporous resins reflects the solvation of amphiphilic polymer backbone whereas the macroporous resin represents the storage of the liquid within the pores of the impermeable polymer matrix.9

<table>
<thead>
<tr>
<th>Solvents</th>
<th>BDDMA (mol%)</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
</tr>
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<tr>
<td>DCM</td>
<td>8.92</td>
<td>8.62</td>
<td>5.26</td>
<td>5.71</td>
<td>5.58</td>
<td>4.93</td>
<td></td>
</tr>
<tr>
<td>THF</td>
<td>8.90</td>
<td>9.38</td>
<td>7.71</td>
<td>3.4</td>
<td>2.85</td>
<td>2.62</td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>4.19</td>
<td>4.38</td>
<td>3.86</td>
<td>2.77</td>
<td>1.54</td>
<td>1.82</td>
<td></td>
</tr>
<tr>
<td>Chloroform</td>
<td>7.11</td>
<td>7.24</td>
<td>7.01</td>
<td>7.06</td>
<td>6.52</td>
<td>5.90</td>
<td></td>
</tr>
<tr>
<td>CCl₄</td>
<td>8.71</td>
<td>8.47</td>
<td>7.54</td>
<td>5.93</td>
<td>5.56</td>
<td>4.91</td>
<td></td>
</tr>
<tr>
<td>Benzene</td>
<td>4.87</td>
<td>4.69</td>
<td>3.5</td>
<td>3.11</td>
<td>2.65</td>
<td>2.31</td>
<td></td>
</tr>
<tr>
<td>Toluene</td>
<td>4.21</td>
<td>4.13</td>
<td>3.80</td>
<td>3.53</td>
<td>2.47</td>
<td>2.23</td>
<td></td>
</tr>
<tr>
<td>NMP</td>
<td>6.83</td>
<td>6.69</td>
<td>5.91</td>
<td>4.57</td>
<td>3.96</td>
<td>3.64</td>
<td></td>
</tr>
<tr>
<td>DMF</td>
<td>6.01</td>
<td>5.83</td>
<td>4.82</td>
<td>4.12</td>
<td>3.52</td>
<td>3.33</td>
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<tr>
<td>Methanol</td>
<td>3.68</td>
<td>3.38</td>
<td>3.06</td>
<td>2.89</td>
<td>2.72</td>
<td>2.33</td>
<td></td>
</tr>
<tr>
<td>Pyridine</td>
<td>7.30</td>
<td>6.91</td>
<td>6.81</td>
<td>6.52</td>
<td>5.92</td>
<td>5.80</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2. Swelling capacities of BDDMA-PS resins

The effect of the degree of crosslinking upon swelling and reactivity of the attached functional sites have been well studied.2-14 It was proved that 1% DVB-PS is superior over 2% DVB-PS for the successful synthesis of peptides.13 At high crosslink density, flexibility of the polymer backbone and
hence the solvation and accessibility of the reactive sites by the reagent decreases significantly. In the present BDDMA-PS system, 1 and 2 mol% resins have almost similar swelling behaviour. But, 2 mol% resins were found to have more mechanical strength. A comparison of the swelling capacities of 2 mol% DVB-PS resin with BDDMA-PS resin shows that this new copolymer swells 2 to 3 times more in solvents used for peptide synthesis (Figure 4.4). Such behaviour is self-explainable from the structure of DVB-PS and BDDMA-PS.

![Chemical structures](image)

So relatively high swelling capacities observed for this newly developed resin in solvents of widely varying polarities (amphiphilic nature) is greatly appreciated in peptide synthesis, since the synthetic protocol involves the repeated use of polar and non-polar solvents. Considering the mechanical strength and swelling behaviour, 2 mol% BDDMA-PS seems to be appropriate for SPPS. The increased flexibility offered by the four methylene carbons and the polarity offered by the ester group of BDDMA may furnish sufficient flexibility and room for the entry and interaction by both polar and non-polar solvents. Thus, solvent compatibility can be adjusted by a proper blending of hydrophilic and hydrophobic character, which in turn can tune the fine molecular structure of the polymer network.
4.3. Functionalisation of BDDMA-PS

Functionalised crosslinked polymers have received much attention due to their numerous possible applications in chemistry and biochemistry. Chloromethylation, a key reaction in the synthesis of anion exchange resins, was selected for the functionalisation of the new BDDMA-PS resin. Chloromethylation can be performed either by using \( \beta \)-formaldehyde and hydrogen chloride or by chloromethyl methylether (CMME) in presence of \( \text{AlCl}_3 \), \( \text{ZnCl}_2 \) or \( \text{SnCl}_4 \). Merrifield et al. modified this procedure using CMME and \( \text{ZnCl}_2 \) in THF for the controlled low degree functionalisation. Another procedure developed by Sparrow et al. used varying amounts of boron trifluoride etherate, and this method is useful for preparing resins of chlorine capacity 1 meq\(^1\). Since resins of varying functional group capacities were required for various applications, BDDMA-PS resins were subjected to chloromethylation using \( \text{ZnCl}_2/\text{THF} \) and \( \text{SnCl}_4/\text{DCM} \) as catalysts (Scheme 4.2).

![Scheme 4.2. Chloromethylation of BDDMA-PS](image-url)
As reported for DVB-PS, 1 M ZnCl_2/THF is effective for controlled chloromethylation. The degree of chloromethylation is a function of nature and concentration of catalyst, reaction time and temperature. A comparison of the time required for chloromethylation was performed on 2 mol% DVB-PS and 2 mol% BDDMA-PS using the catalysts 1 M ZnCl_2/THF and 0.1 M SnCl_4/DCM under same temperature (Figure 4.5). As the reaction time increases the extent of functionalisation also increases.

![Graph](image)

**Figure 4.5.** Effect of catalyst and time upon the chloromethylation of BDDMA-PS and DVB-PS

From the graph, it is clear that BDDMA-PS resin is more reactive than DVB-PS. A reaction time of 3.5 h gave a chlorine capacity of 1.97 mmolg^{-1} on 2% BDDMA-PS resin when ZnCl_2/THF was used. But, DVB-PS resin gave only 0.4 mmolg^{-1} after 3.5 h and the reaction was extended to 10 h to get a capacity of 2.04 mmolg^{-1}. However, it is clear from the graph that the reaction is much faster with SnCl_4/DCM. This catalyst is more suitable for high capacity functionalisation of highly crosslinked resins. The 2 mol% crosslinked BDDMA-PS resin gave 3.06 mmolg^{-1} after 24 h reaction, when ZnCl_2/THF was used. SnCl_4/DCM gave 5.8 mmolg^{-1} on the other hand. From Table 4.3, it is clear that as the crosslink density increases, the ease of
functionalisation decreases. The data obtained in the present investigation clearly demonstrated that the reaction is easy and fast in BDDMA-PS resins and the reactivity decreases with increase in crosslink density. All the reactions were carried out in DCM, since it allows the maximum swelling of both DVB-PS and BDDMA-PS resins and hence a faster reaction with CMME takes place, reducing the possibility of undesired intramolecular reactions.

Table 4.3. Effect of crosslink density upon the chloromethylation of BDDMA-PS

<table>
<thead>
<tr>
<th>Crosslink density (mol%)</th>
<th>Chlorine capacity (mmolg⁻¹)</th>
<th>ZnCl₂/THF</th>
<th>SnCl₄/DCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5.74</td>
<td>6.34</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4.98</td>
<td>6.11</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4.52</td>
<td>5.81</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3.64</td>
<td>4.01</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>2.62</td>
<td>3.76</td>
<td></td>
</tr>
</tbody>
</table>

It was noted when SnCl₄ /DCM was used as the catalyst, the resin became yellow in colour. With resins of high crosslink density, especially after prolonged reaction, they turned slightly brownish. Methylene bridging often found to associate with SnCl₄ /DCM catalysed reaction, and/or the binding of catalyst within the resin matrix may be the possible reason for such colouration. Such methylene bridging can be monitored by ¹³C-NMR. However, the colour can be minimised to a large extent by thoroughly washing with 1:1 mixture of HCl and THF or dioxane.

Upon chloromethylation, polarity of the resin increases and hence the uptake of polar solvents also. A comparison of the swelling capacities of chloromethylated 1% DVB-PS and 1% BDDMA-PS is shown in Figure 4.6. Both the resins were chloromethylated to about 1.25 mmolg⁻¹ level. Studies were carried out with solvents used in peptide synthesis. As the support was changed from DVB-PS to BDDMA-PS about 3-5 times increase in the swelling ratio was observed.
CMME was prepared according to the method of Marvel and Porter.\textsuperscript{19} Recently, Lindman has suggested another procedure for the safe preparation of CMME, free from the potent carcinogenic bis (chloromethyl) ether.\textsuperscript{20}

The presence of chlorine in the polymer was detected by sodium fusion extract method. Characterisation of the functionalised support was done by IR spectroscopy. IR (KBr): 670 cm\textsuperscript{-1}; 1425 cm\textsuperscript{-1} (C-Cl stretch) (Figure 4.7). The chloromethyl group was estimated by Volhard's method.\textsuperscript{21} In this method, chlorine bound to the polymer was converted to pyridinium chloride and chloride thus liberated was titrated against ammonium thiocyanate using ferric alum indicator.
Chloromethyl group introduced in BDDMA-PS resin was converted to a variety of other functional groups and thus various resins relevant in peptide synthesis were prepared. Each resin so obtained was characterised by IR and chemical methods. Various resins prepared in the present study are given in Table 4.4.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloromethyl resin</td>
<td><img src="image1" alt="Structure" /></td>
</tr>
<tr>
<td>Aminomethyl resin</td>
<td><img src="image2" alt="Structure" /></td>
</tr>
<tr>
<td>Hydroxymethyl resin</td>
<td><img src="image3" alt="Structure" /></td>
</tr>
<tr>
<td>2-Nitro-1-chloromethyl resin</td>
<td><img src="image4" alt="Structure" /></td>
</tr>
<tr>
<td>4-Hydroxymethyl phenoxy methyl resin (HMP or Wang)</td>
<td><img src="image5" alt="Structure" /></td>
</tr>
<tr>
<td>4-Hydroxymethyl phenoxy acetamidomethyl resin (HEMP or Pam)</td>
<td><img src="image6" alt="Structure" /></td>
</tr>
<tr>
<td>Benzhydryl resin</td>
<td><img src="image7" alt="Structure" /></td>
</tr>
<tr>
<td>PEG grafted resin</td>
<td><img src="image8" alt="Structure" /></td>
</tr>
</tbody>
</table>

### 4.4. Stability of the polymer (BDDMA-PS)

Certain physical and chemical stability are required by the polymer beads, since the resin has to be subjected to vigorous shaking, filtration, drastic acid and base treatments during peptide synthesis and various polymer analogous reactions. Here a temperature of 120°C was applied to 24 h to 2 mol% BDDMA-PS resin in DMF with occasional stirring. Acid stability of the resin was checked by keeping 500 mg of dry resin in neat TFA for 24 h and at 6 N HCl at 110°C for 48 h. Another sample of the resin was
kept in concentrated KOH solution at 70°C for 48 h to check the stability towards the base. IR spectrum of the post treatment resins showed no change in the chemical nature of the support. Therefore, the ester linkages in the BDDMA-PS matrix is sufficiently stable to any synthetic manipulations. The greater stability of the alkyl ester linkage of the crosslinker in BDDMA-PS resin is due to the fact that the chance of formation of alkyl cation (2) is very less, since it is not as stable as the benzylic cation (1) formed during the TFA cleavage of peptide from the support (Scheme 4.3). So, peptides can be conveniently cleaved off from the BDDMA-PS matrix.

Scheme 4.3. Mechanism of acidolysis of benzyl ester linkage and BDDMA-PS during TFA cleaving

So at this instant, the support is seemed to be satisfied all the essential conditions required by a material to be used for SPPS as mentioned by Merrifield. This newly developed resin can be easily prepared in good physical form with suitable physical and chemical stability. The resin swells in solvents of widely varying polarities and it can be easily functionalised to any desired level. Now its utility in peptide synthesis has to be checked.

4.5. Polymer morphology

The influence of polymer morphology upon reactivity was clearly demonstrated. In the present study, polymer samples were sputtered with gold and stained at an accelerated voltage of 15 kV in an electron microscope.
They were micrographed at different magnifications to get the surface details (Figure 4.8). Presence of extensive channels and pores were observed on the surface of the functionalised and non-functionalised resins. They possess an ordered structure with a regular surface feature. This may be due to the uniform crosslinking in the resin matrix. Smooth surface become rough upon polymer analogous reactions, but the spherical shape remains as such even after 20 cycles of operation during the synthesis of a 20-mer peptide. This shows the mechanical integrity of the system. Spherical shape, porous structure and mechanical integrity make the BDDMA-PS resin a very good support for solid-phase reactions, permitting good flow properties even with high molecular weight reagents.

Figure 4.8. SEM of BDDMA-PS: (a) non-functionalised, (b) functionalised and (c) peptidyl resin
PART B: SYNTHESIS OF PEPTIDES

4.6. Synthesis of model tripeptides

To illustrate the utility of newly developed 1,4-butanediol dimethacrylate crosslinked polystyrene (BDDMA-PS) in peptide synthesis, a set of model tripeptides were synthesised in both protected and free form using Boc-chemistry. Though the sequences were randomly selected, their C-terminal end is varied among the sterically hindered hydrophobic amino acids to investigate the efficiency of attachment and detachment of such amino acids with the polymer support under different conditions. Generally, the reactions of such amino acids with the polymer is very slow and incomplete with the conventional supports. The peptides used in the present study are given below.

(i) Val-Ala-Gly
(ii) Ala-Ala-Ala
(iii) Gly-Ala-Val
(iv) Ala-Val-Ile
(v) Gly-Phe-Leu
(vi) Gly-Ala-Pro

The peptides were synthesised on chloromethylated BDDMA-PS resins of varying chlorine capacity ranging from 1.75 to 2.11 mmol g⁻¹. C-terminal amino acid was directly anchored to the support via a benzyl ester linkage. This step was achieved by both cesium salt method and TEA-method. TEA method was found to be comparatively difficult to achieve quantitative conversion; especially with Val and Ile due to steric effect. A certain level of quaternisation leading to the formation of anion exchange resins may take place at this stage which can make serious effects during peptide synthesis. But, cesium salt method was giving nearly quantitative conversion easily. For example, during the attachment of Nα-Boc-Pro to the resin of 1.92 mmol chlorine per g, only 0.98 mmol g⁻¹ amino acid substitution was observed in TEA method after 48 h reaction. The same resin gave a substitution level of 1.83 mmol g⁻¹, in cesium salt method, after 20 h reaction. Amino acid substitution was estimated by picric acid method. The details for the
attachment of various amino acids to BDDMA-PS support are given in Table 4.5. It was clear that all amino acids gave more than 95% substitution on reaction with cesium salt of N\textsuperscript{\text{a}} Boc amino acids with chloromethylated resin in NMP at 50°C for 20 h. With Val and Ile, reaction was extended to 30 h.

Table 4.5. Details of the C-terminal attachment to the chloromethylated BDDMA-PS

<table>
<thead>
<tr>
<th>Chlorine capacity of resin (mmol g(^{-1}))</th>
<th>Amino acid</th>
<th>Reaction time (h)</th>
<th>Amino acid substitution level (mmol g(^{-1}))(^{\text{a}})</th>
<th>Cesium salt method</th>
<th>TEA method</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.11</td>
<td>Gly</td>
<td>20 (48)</td>
<td>1.97 (98%)</td>
<td>1.87 (92%)</td>
<td></td>
</tr>
<tr>
<td>1.92</td>
<td>Ala</td>
<td>20 (48)</td>
<td>1.80 (97%)</td>
<td>1.60 (83%)</td>
<td></td>
</tr>
<tr>
<td>2.23</td>
<td>Leu</td>
<td>20 (48)</td>
<td>2.04 (97%)</td>
<td>1.43 (67%)</td>
<td></td>
</tr>
<tr>
<td>1.75</td>
<td>Val</td>
<td>30 (48)</td>
<td>1.53 (91%)</td>
<td>1.20 (71%)</td>
<td></td>
</tr>
<tr>
<td>1.75</td>
<td>Ile</td>
<td>30 (48)</td>
<td>1.58 (93%)</td>
<td>0.81 (47%)</td>
<td></td>
</tr>
<tr>
<td>1.92</td>
<td>Pro</td>
<td>24 (48)</td>
<td>1.74 (94%)</td>
<td>0.98 (51%)</td>
<td></td>
</tr>
</tbody>
</table>

\(^{\text{a}}\) The reaction time required for TEA method was given in brackets.

Peptides were synthesised manually on a silanised reaction vessel. 100 mg of C-terminal amino acid attached resin was used for each synthesis. Each amino acid was then incorporated using preformed DCC/HOBt active ester coupling procedure described in Section 4.7. 2.5 mmol excess of each amino acid was dissolved in NMP and mixed with HOBt and DCC (1:1:1 molar ratio). Precipitated DCU was filtered off and solution was added to the resin and was shaken for 45 min. Then Kaiser test was conducted and coupling was repeated till a negative result was obtained. All the coupling steps were complete in the first time itself; except Ala to Val in (ii) and Val to Ile in (iii). Generally, sterically hindered amino acids are difficult to couple quantitatively.\(^{\text{a}}\). In general, the assembly of these peptides were easy as evident from the total reaction time required for each peptide (Table 4.6).
The finished peptides were cleaved from the support by TFA/thioanisole and transesterification (TEA/methanol) methods. The weight increment observed with each peptide was in good agreement with a nearly quantitative coupling in all stages. Upon TFA cleavage, all the peptides were obtained in more than 90% yield. Transesterification also gave 75-85% cleavage yield in the first attempt itself (Table 4.6). Repetition of the cleavage did not make any significant change in the overall yield. It was noted that the cleavage of peptides from the conventional DVB-PS support is very difficult and often ended up on very poor yield, especially when the sterically hindered amino acids were directly incorporated to benzyl ester linkage. Yields reported here were based on the first amino acid substitution level. A probable explanation for the easy synthesis and high yield of protected peptides may be the change in the macromolecular character of the polymer matrix, offered by the polar and flexible BDDMA units. This resin can swell extensively not only in NMP but also in methanol unlike DVB-PS.
and hence the reagents can easily enter into the reactive sites for a quantitative reaction.

The peptides obtained were repeatedly washed with ether, and then dissolved in 1% acetic acid/water and then lyophilised. Purity of these peptides was ascertained by thin layer chromatography (Rf values are given in Table 4.6) and characterised by elemental analysis (Table 4.7). Though these methods are not adequate for medium to large size peptides, for small peptides the method has been widely used.

<table>
<thead>
<tr>
<th>Table 4.7. Elemental analysis of the synthetic peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide*</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>VAG</td>
</tr>
<tr>
<td>AAA</td>
</tr>
<tr>
<td>GAV</td>
</tr>
<tr>
<td>AVI</td>
</tr>
<tr>
<td>GAP</td>
</tr>
<tr>
<td>GFL</td>
</tr>
</tbody>
</table>

* One letter symbols are used to express amino acids.

b Experimental values are given in brackets.

4.7. Synthesis of model difficult sequence peptides on BDDMA-PS and DVB-PS: A correlation between coupling difficulty and nature of the support

The occurrence of so-called 'difficult sequences' which undergo strong internal association within the peptide resin network due to the formation of β-and/or related structures of the growing peptide chain is a serious problem encountered in SPPS. Such sequences exhibit chronic repetitive stretches of incomplete aminoacylations and deprotections irrespective of the resin or strategy. This phenomenon gives rise to a variety of peptide side products lacking one or more internal amino acid residues which hamper purification.
and reduce yields. Many attempts have been made in the last decade to identify such sequences prior to the synthesis. Some efforts in this direction focused on computational approaches related to the prediction of protein conformations were significant, though not completely successful. Narita et al. assigned an arbitrary value, $SP_\beta$, the $\beta$-sheet stabilising potential, for each amino acid based on the solubility of several hydrophobic peptides in different solvents. As the $SP_\beta$ value increases, $\beta$-sheet structure propensity also increases.

It has been shown that peptides with $<SP_\beta>$ value greater than 5.0, will be highly stabilised in the $\beta$-sheet structure and hence difficult to synthesise either by solution phase (due to insolubility of peptides) or solid-phase (due to incomplete reactions) technique. Using this parameter, Narita has predicted the solubility of several protected fragments and then designed synthetic routes for the selection of peptides for solution phase fragment condensation strategy of synthesis. The studies on 77 kinds of tri to hepta protected hydrophobic peptide fragments of E-coli ribosomal protein, have demonstrated that $SP_\beta$ values are independent of the protection strategy used.

Milton et al. suggested a predictive method based on Chou and Fasman type coil conformational parameters and tested on 986 aminoacylation reactions. Considering that the addition of each amino acid in SPPS, creates a new segment, they derived a statistically refined parameter $<SP_\beta>$, referred to as arithmetic average random coil induction parameter, for each sequence. In this manner, a series of cumulative $Pc^*$ values were generated for each residue in the C→N synthesis of target sequence to predict the ease of aminoacylation for the next residue in the synthesis. Generally, $<Pc^*>$ values greater than 1.0, are associated with easy quantitative coupling while values between 0.9-1.0, required a longer coupling time or recoupling and values less than 0.9 were normally associated with persistent aminoacylation difficulties.
Results and Discussion

In the present work, a set of hydrophobic peptides were selected and built on both 2 mol% DVB-PS and 2 mol% BDDMA-PS resins under identical conditions using Boc-chemistry. The peptides were characterised to be typical difficult sequences, from their high $<\text{SP}_p>$ values (> 5.0) low stepwise $<\text{Pe}>$ values (<0.9) and optimum $<\text{P}_\text{e}>$ values (Table 4.8). A comparison of the synthetic results, namely the stepwise coupling time, total reaction time required for the chain assembly, yield of the peptide obtained on both TFA and transesterification cleavage, and purity of the synthetic products were made to correlate between nature of the support and success of solid-phase peptide synthesis.

Table 4.8. Average conformational parameters of the peptides (I) to (vii)

<table>
<thead>
<tr>
<th>No</th>
<th>Sequence</th>
<th>$&lt;\text{P}_\text{a}&gt;$</th>
<th>$&lt;\text{P}_\text{e}&gt;$</th>
<th>$&lt;\text{P}_\text{e}&gt;$</th>
<th>$&lt;\text{SP}_p&gt;$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i)</td>
<td>VAVV</td>
<td>1.18</td>
<td>1.46</td>
<td>0.69</td>
<td>5.5</td>
</tr>
<tr>
<td>(ii)</td>
<td>KVAVV</td>
<td>1.16</td>
<td>1.32</td>
<td>0.76</td>
<td>5.0</td>
</tr>
<tr>
<td>(iii)</td>
<td>NVKAVV</td>
<td>1.08</td>
<td>1.21</td>
<td>0.86</td>
<td>5.3</td>
</tr>
<tr>
<td>(iv)</td>
<td>ANKVAVV</td>
<td>1.14</td>
<td>1.17</td>
<td>0.83</td>
<td>5.1</td>
</tr>
<tr>
<td>(v)</td>
<td>VQELG</td>
<td>1.14</td>
<td>1.03</td>
<td>0.88</td>
<td>4.4</td>
</tr>
<tr>
<td>(vi)</td>
<td>QNGQELG</td>
<td>1.06</td>
<td>1.10</td>
<td>0.91</td>
<td>4.7</td>
</tr>
<tr>
<td>(vii)</td>
<td>VAVAAG</td>
<td>1.19</td>
<td>1.17</td>
<td>0.78</td>
<td>5.3</td>
</tr>
</tbody>
</table>

High capacity resins were purposefully selected to investigate the effect of functional group capacity, since the intrasite interactions are known to be dependant on the local density of peptide chains which may contribute to coupling difficulty. C-terminal amino acid was attached to the chloromethylated support by the cesium salt method. The reaction time required by the cesium salt of N\textsuperscript{N}-Boc-Gly and N\textsuperscript{N}-Boc-Ile to get attached to the BDDMA-PS resin was considerably less than the time required for DVB-PS. But, the reaction of N\textsuperscript{N}-Boc-Ile was somewhat difficult with both the
supports. Reaction was continued to 56 h to get about 82% conversion with N\textsuperscript{\text{\textregistered}}-Boc-Ile on DVB-PS resin while 96% attachment was taken place with BDDMA-PS resin during 30 h reaction. NMP was used as the solvent for the reaction.

Peptide synthesis was performed on a silanised reaction vessel manually. 100 mg of C-terminal amino acid attached resin was used for each synthesis. Considering the early attempts of difficult sequence synthesis and the swelling behaviour of BDDMA-PS support, a special protocol was suggested for the chain assembly (Table 4.9). 2.5 meq. excess of preformed HOBt active esters of amino acids were used for each coupling. HOBt is a very useful coupling reagent which was proved to be immune to racemisation.\textsuperscript{41} DCC/HOBt coupling was shown to be equally effective as HBTU and TBTU.\textsuperscript{42} NMP containing 10% v/v of DMSO was used as coupling medium. DMSO is a powerful desegregating solvent.\textsuperscript{43} Then after 45 min, 0.25 ml (8% v/v) of DIEA was also added. Addition of small amounts of DIEA was shown to be catalysing the DCC/HOBt coupling.\textsuperscript{44} But, DIEA was avoided during the coupling of second and third residues to avoid the danger of racemisation.\textsuperscript{45} After the third amino acid attachment, a small portion of the resin (5 mg) was removed for determining the chain loss by quantitative ninhydrin test. 30% TFA/DCM was employed for Boc group removal. Neutralisation was performed in two step, first by using 5% DIEA in DCM and then using 10% DIEA in NMP for 2 min. A standard reaction time was given for each coupling. No capping reaction for unreacted amino group was performed. Synthesis of all the peptides progressed smoothly on BDDMA-PS resin and the reaction was complete in the first time itself. But many instants especially the attachment of Val, Ile, Lys and Gln were rather complicating in DVB-PS. This was evident from the total reaction time required for the synthesis of each peptide on both supports (Table 4.9).
Results and Discussion

Table 4.9. Synthetic results of the peptides (i) to (vii) on both DVB-PS and BDDMA-PS resins

<table>
<thead>
<tr>
<th>No</th>
<th>Sequence</th>
<th>Acylation Time (h)</th>
<th>Yield of the Crude Product (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DVB-PS</td>
<td>BDDMA-PS</td>
</tr>
<tr>
<td>(i)</td>
<td>VAVI</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>(ii)</td>
<td>KAVI</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>(iii)</td>
<td>NKAVI</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>(iv)</td>
<td>ANKAVI</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>(v)</td>
<td>VQELG</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>(vi)</td>
<td>QVQELG</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>(vii)</td>
<td>VAVAAG</td>
<td>8</td>
<td>5</td>
</tr>
</tbody>
</table>

The completed peptides were cleaved from the support in both free and protected form using TFA and transesterification methods respectively. All the sequences were cleaved by both ways nearly quantitatively form BDDMA-PS support. But the yield of the peptides obtained from DVB-PS resin was very poor, especially with nucleophilic cleavage. It was observed that the nucleophilic cleavage of the direct benzyl ester linkage between the polymer and peptide chain is very difficult, especially with sterically hindered amino acids. Moreover, the present synthesis used high functional density support, which is again a real bottleneck with conventional supports. Despite these complications, BDDMA-PS support furnished 80-90% yield against 45-60% yield from DVB-PS resins (Table 4.9).

The cleaved peptides were repeatedly washed with ether and dissolved in acetic acid-water mixture, or acetic acid-methanol mixture, depending on the solubility, and lyophilised. As evident from the analytical HPLC profile, recorded on reverse phase C18 column under a linear gradient of 10 to 70% B over 25 min using 0.1% TFA containing H2O (A) and 0.1% TFA containing acetonitrile (B) was used as the solvent system, the products obtained from BDDMA-PS resin were highly pure compared to the products from DVB-PS resin (Figure 4.9).
Results and Discussion

i(a)

i(b)

ii(a)

ii(b)

iii(a)

iii(b)

iv(a)

iv(b)
Figure 4.9. HPLC profiles of the peptides (i) to (vii) synthesised on: (a) BDDMA-PS and (b) DVB-PS

Finally the peptides were characterised by amino acid analysis (Table 4.10). Amino acid analysis was performed by hydrolysing the sample with 6N HCl:TFA (1:1) mixture at 120°C for 24 h to achieve the quantitative breakage of the amide linkage of all hydrophobic amino acids.
An investigation into the solubility of these peptides was quite interesting as it can provide information regarding the internal aggregation. As the peptide chain length increases, β-structure predominates and the peptide becomes insoluble in protic solvents. Solubility characteristics were in good agreement with Narita's observation of β-structured peptides (Table 4.11). All the sequences used in the present study contain Val and Ile which have a strong β-sheet propensity due to the β-branched hydrophobic side chains and hence such peptides can stabilise the β-structure in the solid state. Moreover, the presence of Ala-Val-Ala moiety, a strong β-sheet inducer, also favours this conformation.
The results of the present investigation point towards the superiority of BDDMA-PS support over conventional DVB-PS resin. The increased flexibility offered by the long four methylene chain has significant influence upon the mobility of polymer backbone; which in turn is effective in reducing the steric factors. This is evident from the easy chloromethylation and attachment of even sterically hindered N\textsuperscript{\textsteric}-Boc-Ile to the support. The presence of DMSO, can well solvate the peptide chain reducing the polymer-peptide interactions, a well pronounced effect for the coupling difficulty. The heat generated by the addition of DCM to NMP also favours fast coupling. The temperature effect of coupling has already been studied. These factors might have helped the flexible BDDMA-PS bound peptide chains to desegregate unlike in the rigid DVB-PS resin. A kind of desegregation was also evident from the high cleavage yield of these aggregating sequences from this new support. Therefore, BDDMA-PS resin when present in polar aprotic solvents like NMP, DMSO etc. may reduce the various inter and

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**Table 4.11. Solubility studies of peptides (i) to (vii)**

<table>
<thead>
<tr>
<th>Amino acid sequence*</th>
<th>Hexane</th>
<th>CCl(_4)</th>
<th>Acetonitrile</th>
<th>MeOH</th>
<th>DCM</th>
<th>DMSO</th>
<th>HOAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAVI</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>b</td>
<td>c</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td>KVAVI</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td>NKVAVI</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td>ANKVAVI</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td>VQELG</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>b</td>
</tr>
<tr>
<td>QVGVQELG</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>c</td>
<td>b</td>
</tr>
<tr>
<td>VAVAAG</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>c</td>
<td>c</td>
</tr>
</tbody>
</table>

\*One letter symbol was used to express the amino acids
a - insoluble, b - partially soluble, c - soluble.
intramolecular interactions responsible for the difficulty in the synthetic transformations often associated with rigid and hydrophobic styrene based supports. Therefore, the nature of the solid support has a significant dynamic influence upon the coupling difficulty and hence success of SPPS, though some sequence specific coupling problems are of course independent of the resin support.

4.8. Synthesis of hydrophobic peptides

Hydrophobic peptides are derived from amino acids with apolar side chains. Such peptides have a very crucial role in the biological function of proteins and peptides. Signal sequences, membrane proteins, toxins are all essentially hydrophobic. Antimicrobial peptides have hydrophobic regions which take part in the interaction between the lipids. Synthetic hydrophobic peptides are also very important in elucidating the importance of hydrophobicity to the folding of proteins. But, the synthesis of these peptides is always difficult. The classical solution phase synthesis is severely hampered by the insolubility of intermediate peptide segments. Insolubility is mainly caused by the aggregation of the sequences through various side chain interactions of the amino acids constituting the peptide. Solid-phase techniques also give poor results due to the incomplete coupling at various stages. Internal aggregation of the pendant peptide chains is the reason here also. But, such problems in SPPS can be well tackled, if it is possible to increase the segmental mobility of the polymer chains to which the peptides are to be attached. So from the swelling behaviour of the BDDMA-PS resins it is clear that the polymer chains of this support are well separated than the rigid DVB-PS. So, lightly crosslinked BDDMA-PS resins were selected in the present study to check its efficiency during the synthesis of highly hydrophobic peptides.
4.8.1 A decapeptide corresponding to the chemotactic domain of elastin

Val-Pro-Gly-Val-Gly-Val-Pro-Gly-Val-Gly

Biological elastic fibres are composed of a core of fibrous elastin and a fine surface layer of microfibrillar protein.\textsuperscript{53} It has been demonstrated that the most striking structural feature of elastin is a repeating pentapeptide sequence, Val-Pro-Gly-Val-Gly, which repeats some 11 times in pig and 13 times in chick.\textsuperscript{54} Additional repeating sequences have also been observed which generally occur for less extensive stretches, with less fidelity. Structural investigations of this polypentapeptide have been carried out and it comprised of regularly recurring type-II Pro-Gly $\beta$-turns which repeat on a helical axis.\textsuperscript{55} To investigate the effect of intramolecular hydrogen bonding in SPPS, this peptide with a $\beta$-turn structure has been selected. Intramolecular hydrogen bonding mostly occurs at reverse turns.\textsuperscript{56} It has been reported that inter and intramolecular hydrogen bonding severely affects the success of SPPS.\textsuperscript{52}

To the chloromethylated BDDMA-PS resin, the C-terminal amino acid, Gly, was attached by Gisin's cesium salt method. Substitution level was found to be 1.34 mmol g$^{-1}$ of NH$_2$/g by picric acid method. This corresponds to approximately 97% conversion. 100 mg of N$^2$-Boc-Gly attached resin was used for synthesis. Synthesis was performed on silanised filter-frit reaction vessel. 30\% TFA/DCM was employed for Boc group removal and the completion of reaction was checked by Kaiser test. Deprotection of 9th Pro was found to be very difficult. So, a second treatment using 50\% TFA/DCM for 20 min was also given. Each amino acid was coupled by preformed DCC/HOBt active ester method. 2.5 meq, excess of the initial amino capacity, was taken for each coupling and the coupling was performed in NMP. Up to the 7th Val, all the couplings were complete within 45 min. Attachment of 8th Gly was performed in NMP-DMSO (20\% v/v) mixture for 1 h. Then a second coupling was also given in DMF-DMSO (10\% v/v) mixture containing
5% v/v of DIEA for 2 h. The reaction was found to be 99.7% complete at this stage. A similar difficulty was observed for 10th Val also. Attachment of Val was very difficult and even after 3 cycles of coupling, the reaction was found to be only 98.7% complete. In performing these difficult couplings, DMF-DMSO mixture was found to be more effective. DMSO has already proven to be a good solvent for the desegregation of resin bound peptides. Attempts to improve the degree of incorporation of amino acids by repeating the couplings, using large excess of carboxyl components, changing the solvent etc. have only a very little impact. In fact, this sequence shows all the characteristic features of a difficult sequence peptide. An examination of the extent of coupling at each stage by the quantitative ninhydrin test was made (Figure 4.10).

![Graph](image)

**Figure 4.10.** Diagrammatic representation of the efficiency of coupling during the synthesis of (VPGVG)$_2$.

An attempt to prepare the 15 residue analogue, (Val-Pro-Gly-Val-Gly)$_3$, failed after the attachment of thirteen residues. The Boc-group removal at this stage was found to be extremely difficult even after repeated treatment with 50% TFA/DCM. So a sequential polypeptide as observed in elastin cannot be prepared by stepwise elongation strategy.
Results and Discussion

It was reported that the difficulties of this sought observed during the chain assembly is a characteristic feature of sequences which undergo strong internal association via. a definite secondary structure formation, especially β-sheet structure (difficult sequences). Moreover, this peptide consists of sterically hindered Val and Pro at regular intervals. After the assembly of 4 residues, Val-Gly-Val moiety flanked by Gly-Pro was repeated in this sequence. These short sequences can be observed in the globular and fibrous proteins. Intramolecular hydrogen bonding in addition to the intermolecular hydrogen bonding predominates at the reverse turns. In addition, the peptide was synthesised on a high capacity resin. With a few exceptions, synthesis on high capacity resins are generally ended up with low yield and purity. The present sequence can well associate by various hydrophobic interactions of the side chains of constituent amino acids and by inter and intramolecular hydrogen bonding facilitated by the sequence propensity at high loading levels in the resin matrix. Local clusters thus formed and hence the microphase transformations occurred in the peptidyl resin network due to the site-site interactions may be responsible for the failure of synthesis.

The completed peptide was cleaved from the support by neat TFA in presence of thioanisole as a catalyst and scavenger. The reaction was continued for 24 h at 35°C. Peptide was precipitated using dry diethylether and washed several times. The yield of the crude peptide obtained was 68%, based on the first amino acid substitution level.

The crude peptide was dissolved in TFA and diluted with acetic acid-water. It was passed through a Sephadex-G10 column and the fractions were eluted using 20% acetic acid-water mixture. Fractions were collected and lyophilised.

On HPLC analysis using the solvents, water and acetonitrile containing 0.1% TFA, two major peaks were obtained (Figure 4.11). They were separately collected and subjected to amino acid analysis. The peak 2 corresponds to the target peptide, whose amino acid composition is given in Table 4.12.
Amino acid analysis was performed by hydrolysing the sample with 6N.HCl:TFA (1:1) mixture at 130°C for 30 h to achieve the quantitative breakage of the amide linkage of all hydrophobic amino acids.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Theoretical</th>
<th>Peak 1</th>
<th>Peak 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Val</td>
<td>4</td>
<td>2.96</td>
<td>3.61</td>
</tr>
<tr>
<td>Pro</td>
<td>2</td>
<td>1.14</td>
<td>1.89</td>
</tr>
</tbody>
</table>

### 4.8.2 Synthesis of a decapeptide corresponding to the Bombyx-A-fragment (19-28)

Ile-Val-Lys-Ala-Gly-Pro-Ala-Ile-Glu-Val

The decapeptide is a (19-28) fragment of the antimicrobial peptide, Bombyx-A, a 35 residue peptide isolated from Cecropins. The sequence is entirely straight forward and assembled well using Fmoc-amino acids using DCC/HOBt active ester method, on a 4-hydroxymethyl phenoxy methyl resin (Wang), built on chloromethylated 2 mol% BDDMA-PS. The wang resin was synthesised by attaching 4-hydroxymethyl phenol to the
chloromethylated resin using the base NaOCH₃ in DMA at 50°C (Scheme 4.4). The resin was characterised by IR spectroscopy (Figure 4.12).

Scheme 4.4. Preparation of HMP resin on BDDMA-PS

Figure 4.12. IR spectrum of HMP resin

HMP resin was synthesised using optimal conditions to avoid the side reactions leading to the formation of polymerisation products of 4-HMP. Ability of 4-HMP to undergo 1,6-elimination to 4-quinone methide in base or at elevated temperature was also documented. Since this reaction is highly sensitive to temperature, the conditions for the attachment of 4-HMP to chloromethyl BDDMA-PS was optimised by measuring the loss of chlorine from the resin (Figure 4.13).
At 40°C about 75% of the reaction takes place within 4 h ($T_v - 2.6$ h), but, the competing side reactions stopped the reaction at 75-80% stage. At 80°C the reactions was fast ($T_v - 18$ min), but again not complete. At this temperature, the resin obtained was reddish brown in colour and heterogeneous, probably due to the polymerisation of 4-HMP and the subsequent displacement of chlorine by polymeric phenolate anions. At 50 to 60°C, the reaction takes place reasonably well and >95% conversion results within 4 to 6 h ($T_v - 9$ min).

C-terminal valine was attached to the support using symmetric anhydride procedure using DMAP as a catalyst. The attachment of Val was very easy and gave 91% substitution within 1 h reaction. Amino acid incorporation level was determined by measuring the absorbance of the fluorene derivative at 30 nm. Benzylation was conducted using benzoyl chloride in pyridine to block the free hydroxyl groups. Opportunity was also taken to determine the dipeptide, Val-Val, content of the resin, after base catalysed reaction. A small sample of the deprotected Val attached resin was cleaved using TFA and the product was examined by tlc. No spot corresponding to Val-Val was observed, indicating the absence of side reaction.
Results and Discussion

Chain assembly was carried out manually on a filter-frit reaction vessel using 2.5 meq. of preformed DCC/HOBt active esters in DMF containing 5% v/v of DIEA. The attachment of second and third amino acid residues were conducted in NMP by adding no DIEA to suppress racemisation. All couplings were complete at the end of 30 min as indicated by the negative Kaiser test. However, the first coupling was extended to 45 min and a second coupling using 1 meq of amino acids was also given to ensure quantitative reaction. The presence of Pro at the middle of this sequence might be contributed significantly to the straightforward synthesis. The effect of tertiary peptide bonds in the desegregation of the pendant peptide chains was already reported. After the synthesis, 554.2 mg of weight increment of the peptidyl resin was observed.

The crude peptide was detached from the resin using 50% TFA/DCM containing 5% water, 1% thioanisole and 1% EDT for 1.5 h. The crude peptide was obtained in 82% yield, as calculated from first amino acid substitution level. The crude peptide was repeatedly washed with ether and dissolved in 10% acetic acid water mixture and applied to HPLC. The crude peptide itself was highly pure (> 90%) as seen from single peak HPLC (Figure 4.14). Chromatogram was run using the binary gradient 0.1% TFA/water (A) and 0.1% TFA/80% aqueous acetonitrile (B) under the gradient 10 to 80% B over 30 min.

Figure 4.14. Analytical HPLC profile of Bombyx-A fragment (19-28)
10 mg of peptidyl resin was subjected to amino acid analysis to check the progress of the reaction at the end of synthesis. Amino acid compositions obtained were in good agreement with the theoretical value and it corresponds to 92% peptide content. No indication of the deletion or insertions was found. The results are summarised below.

Val - 1.91 (2.0); Glu - 0.94 (1.0); Ile - 1.84 (2.0); Ala - 2.11 (2.0);
Pro - 1.01 (1.0); Gly - 0.98 (1.0); Lys - 0.96 (1.0).

4.8.3 Synthesis of [Val] [Pro] Bombyx-A fragment (19-28)

Ile-Val-Lys, Ala-Gly-Val-Ala-Val-Glu-Pro

This decapeptide is an analogue of fragment (19-28) of Bombyx-A, in which 6th Pro and 10th Val are interchanged to study the effect of the proline residue at the middle of the sequence upon synthesis. It was reported that the presence of proline at regular intervals in a given sequence facilitate the quantitative coupling\(^{33}\) by disrupting the formation of regular structures, especially \(\beta\)-sheets. Moreover sequences containing Gly or Pro as the C-terminal residues are preferred for fragment condensation methods.\(^ {34}\)

The peptide was assembled on the 4-hydroxymethyl phenoxy methyl resin (Wang) built on chloromethylated 2 mol% BDDMA-PS resin using Fmoc-chemistry. C-terminal proline was attached to the resin as its symmetric anhydride in DMF. In the presence of DMAP as a catalyst, attachment was 73% complete after 1 h and 89% complete after 3.5 h. Incorporation of first amino acid was estimated by monitoring the absorbance of the fluorene chromophore at 301 nm. The dipeptide, Pro-Pro, formation was checked after 3.5 h base catalysed reaction by cleaving a small portion of the resin and checked using tlc. No significant effect was observed.
Chain assembly was carried out exactly as in the case of Section 3.8.2; under identical conditions. Second and third amino acids were coupled in NMP without DIEA and the other amino acids were coupled using DMF containing 5\% v/v DIEA. All the reactions were complete within 30 min except the attachment of Lys to Ala and Ile to Val. Lys to Ala gave a clear negative Kaiser test at the end of 1 h. But Ile to Val was extended to 1 h more to get the negative test. All the coupling were repeated after getting the negative result to once more using 1 meq. of amino acids to ensure 100\% attachment. Therefore, it can be seen that the substitution of \(\beta\)-branched Val in the midway of a sequence instead of Pro has created some delay in the chain assembly, although it was not significant. After the synthesis, the resin showed an increment of 541.3 mg.

The crude peptide was cleaved from the support using 50\% TFA/DCM containing 5\% water, 1\% thioanisole and 1\% EDT for 1.5 h. The crude peptide was obtained in 86\% yield as calculated from the first amino acid substitution level. The peptide was dissolved in 10\% acetic acid-water mixture and applied to HPLC fitted with a C\textsubscript{18} column. Solvent systems used were 0.1\% TFA/water (A) and 0.1\% TFA/80\% acetonitrile (B) under the gradient 10 to 80\% B over 30 min. The peptide was more than 90\% pure as can be seen from the single peak chromatogram (Figure 4.15).

![Figure 4.15. Analytical HPLC profile of [Val\textsuperscript{36}] [Pro\textsuperscript{38}] Bombyx-A fragment (19-28)](image-url)
10 mg of peptidyl resin was subjected to amino acid analysis, after hydrolysing with 6 N HCl-propionic acid mixture (1:1) at 110°C for 20 h. Amino acid compositions obtained were in good agreement with the theoretical values. The total peptide content was calculated as 89.6%. No major deletions or insertions were also observed.

Pro - 1.0 (1.0); Glu 1.03 (1.0); Val - 1.86 (2.0); Ala - 2.04 (2.0);
Gly - 1.01 (1.0); Lys - 0.88 (1.0); Ile - 0.84 (1.0)

The above syntheses (2 and 3) clearly illustrates the utility of BDDMA-PS supports for efficient solid-phase synthesis of hydrophobic peptides using Fmoc-chemistry.

4.8.4 Synthesis of CKS-17 fragment (6-17)

Gly-Leu-Asp-Leu-Leu-Phe-Leu-Lys-Glu-Gly-Gly-Leu

CKS-17 is a synthetic peptide which inhibits the lymphocyte proliferation. This peptide was assembled on 4-hydroxymethyl phenoxy acetamidomethyl resin (Pam) built on 2 mol% BDDMA-PS using Fmoc-chemistry (Scheme 4.5). The resin was characterised by IR spectroscopy (Figure 4.16). The carboxyl terminal leucine residue was esterified to the support as its Boc-derivative. Symmetric anhydride coupling was employed at this stage. This method is useful for Pam linkage and for amino acids which do not bear acid labile side chain protecting groups. It avoids any risk of partial removal of Fmoc groups by DMAP, resulting in dipeptide formation. After cleavage of the Boc group with 30% TFA/DCM for 30 min, the resin was neutralised with 10% DIEA/DCM. Amino acid substitution level was obtained by picric acid method. The remaining amino acids were all added using the Fmoc procedure. All couplings were complete within 30 min except Asp to Leu. But a clear negative Kaiser test was obtained after 1 h. The weight of the peptidyl resin was increased to 754 mg, after the complete assembly.
Scheme 4.5. Preparation of HEMP (Pam) resin on BDDMA-PS

The completed peptide was cleaved from the support using TFA containing 0.1 ml thioanisol, 0.1 ml water and 0.1 ml EDT. N-terminal Fmoc group was removed using 20% piperidine/DMF, prior to the final cleavage. Peptide was precipitated by cold dry ether and washed by centrifugation. The crude peptide corresponding to 87% cleavage yield was obtained.

The purity of the peptide was checked on an analytical HPLC fitted with C18 column. 0.1% TFA/water (A) and 0.1% TFA/acetonitrile (B) under the gradient 10 to 80% B for 30 min was used for running the chromatogram. The peptide was highly pure, as can be seen from the single peak HPLC (Figure 4.17).
A small portion of the peptidyl resin was subjected to amino acid analysis, after hydrolysing with 6 N HCl-propionic acid mixture at 110°C for 24 h. The results were in agreement with the target sequence and which corresponds to 94% peptide content.

Leu - 4.72 (5.0); Gly - 2.84 (3.0); Glu - 1.04 (1.0);
Lys - 0.91 (1.0); Phe - 1.09 (1.0); Asp - 0.86 (1.0)

4.8.5 Synthesis of β-amyloid protein (33-42) fragment on PEG<sub>400</sub> grafted copoly(styrene-2% BDDMA) support

Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala

An insoluble graft copolymer, composed of covalently linked polyethylene glycol of molecular weight 400 to copoly (styrene-2% BDDMA), was employed for the synthesis. Divinylbenzene crosslinked polystyrene-polyethylene graft copolymer, so called tentagel support was originally developed by Bayer et al. in an effort to combine the advantages of liquid phase synthesis with solid-phase methods. Liquid-phase synthesis on PEG is a well known technique for peptide synthesis. In such systems, PEG
chains not only increase the polarity of the system but provide a well pronounced spacer effect. Although short spacer molecules have no significant effect, long aliphatic spacers have shown to have a significant impact upon the overall yield of a 19-residue peptide. The graft copolymer thus obtained swells well in all solvents which dissolves PEG. $^{13}$C-NMR relaxation time measurement studies of the peptides bound to such graft copolymer show a high mobility than the conventional DVB-PS resins. A variety of anchoring linkages can be suitably attached to the end of PEG chains to prepare different peptide derivatives. These supports exhibit sufficient mechanical strength and compatibility to use even in continuous flow synthesisers. In fact, synthesis of proteins such as HIV-1 protease, human insulin, metallothionine, and cardiodilatin are the milestones in the history of tantagel supports.

The graft copolymer can be prepared by coupling one of the hydroxyl group of PEG to chloromethylated crosslinked polystyrene. But, when long PEG chains (> 800 Da) were used, both the terminal hydroxyl groups may be attached to the support making additional crosslinking. This difficulty was overcome by means of anionic polymerization of ethylene oxide. Graft copolymers with PEG chains of about 3000 Da were reported to be optimal.

In the present study, an attempt was made to graft PEG of molecular weight 400 Da to chloromethylated BDDMA-PS resins, with a view to enhance the swelling and solvation of the polymer networks. Chloromethylated BDDMA-PS resin (1) was first of all reacted with 4-fold excess of PEG$_{400}$ in presence of sodium hydride or metallic sodium in dry THF. PEG and NaH were taken in 1:1 molar ratio to minimise the pseudo crown ether formation. Hydroxyl group in the resin was then converted to amino group by a three step reaction. The resin (3) was characterised by IR spectroscopy (Figure 4.18). BDDMA-PS-PEG-OH (3) resin was treated with SOCl$_2$ to produce BDDMA-PS-PEG-CI (4) which on treatment with potassium phthalimide gave phthalimido resin. The resin was then subjected to
hydrazinolysis using 5% hydrazine hydrate in absolute alcohol to get BDDMA-PS-PEG-NH$_2$ (5). Methyl phenoxy acetic acid was the coupled to the amino group to get a PAM resin on PEG grafted BDDMA-PS (6). The polymer-analogous reactions for the preparation of the resin are given below (Scheme 4.6).

**Figure 4.18.** IR spectrum of PEG grafted BDDMA-PS

**Scheme 4.6.** Preparation of PAM resin on PEG grafted copoly(styrene-BDDMA)
The swelling studies of the resin (2) was carried out in a variety of solvents (Table 4.13). In polar solvents like methanol, PEG grafted resins swell more. Though it swell well in non polar solvents like toluene, the variation from BDDMA-PS resin is not significant. Good solvation can lead to a high degree of mobility not only to the PEG chains but also to the peptides bound to them. It has been proved that the extent of swelling, microscopic uniformity and degree of substitution have tremendous impact upon the efficiency of synthesis. When the peptide to be synthesised is branched or with a definite secondary structure, swelling capacity becomes very critical. Importance of swelling is evident from the efficiency of HCl-dioxane for Boc group removal in SPPS whereas HCl-acetic acid is not effective, due to the poor swelling of polystyrene in acetic acid.

Table 4.13. Swelling capacity of BDDMA-PS-PEG₄₀₀ in comparison with DVB-PS and BDDMA-PS

<table>
<thead>
<tr>
<th>Solvent</th>
<th>2% DVB-PS</th>
<th>2% BDDMA-PS</th>
<th>2% BDDMA-PS-PEG₄₀₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>2.10</td>
<td>4.33</td>
<td>7.96</td>
</tr>
<tr>
<td>DCM</td>
<td>5.61</td>
<td>11.22</td>
<td>13.78</td>
</tr>
<tr>
<td>Toluene</td>
<td>5.11</td>
<td>9.86</td>
<td>10.25</td>
</tr>
<tr>
<td>DMF</td>
<td>3.62</td>
<td>8.44</td>
<td>10.42</td>
</tr>
<tr>
<td>NMP</td>
<td>4.96</td>
<td>9.87</td>
<td>12.03</td>
</tr>
<tr>
<td>THF</td>
<td>5.31</td>
<td>9.52</td>
<td>14.21</td>
</tr>
<tr>
<td>Dioxane</td>
<td>3.43</td>
<td>8.91</td>
<td>10.59</td>
</tr>
</tbody>
</table>

To check the efficiency of this support, a typical aggregating sequence corresponding to the C-terminal region, (33-42) fragment, of β-amyloid protein was selected. The peptide was assembled using Boc-chemistry. Coupling was performed in DCM-DMF mixture (1:1) due to the extensive swelling of resin in these solvents. DMSO (20% v/v) was added after 30 min, considering the β-sheet dissociating property of DMSO. Each coupling was
given 1 h time. Coupling problems started from Val19. The attachment of Val19 to Val20 and Gly27 to Gly38 were repeated 4 times to get a negative Kaiser test. No DMSO was added during methionine coupling to prevent oxidation.

The crude peptide was obtained in 88% cleavage yield based on the first amino acid substitution. Thioanisole was added during cleavage since it is an effective scavenger for trifluoroacetate and it prevents methionine sulphoxide formation. The peptide is insoluble in methanol, acetic acid, water, DCM and DMF. But a slight solubility was observed in DMSO. Then 0.4 M KSCN in DMF was used to dissolve the peptide. Chaotropic salts like KSCN, LiBr, LiClO4, etc. were reported to be excellent for solubilisation of hydrophobic peptides. The peptide was then lyophilised. It was also reported that lyophilisation can reverse tert-butyl modifications of methionine containing peptides.

Purity was checked on a RP-HPLC fitted with a C18 column (Figure 4.19). Gradient elution using the solvent systems 0.1% TFA containing water (A) and 0.1% TFA containing acetonitrile (B) were used for elution. A major peak at 21.2% was eluted out. 5-80% B over 40 min was used as the gradient for elution. Sample was prepared by dissolving 1 mg ml\(^{-1}\) of TFA and diluted to 2 ml with water. Then 20 ml was injected. Flow rate of 1 ml min\(^{-1}\) was maintained. The fraction corresponding to this peak was collected and lyophilised. Amino acid analysis of this sequence was performed by hydrolysing the peptide at 130°C for 48 h using 6 N HCl : TFA (1:1) mixture. Such a drastic hydrolytic condition was necessary to break the bonds like Val-Val and Val-Ile. But methionine was completely lost during hydrolysis. The observed values are in agreement with the target sequence and which correspond to 94.3% purified yield.

Ala - 1.0 (1.0); Ile - 0.91 (1.0); Val - 2.83 (3.0); Leu - 1.04 (1.0); Gly - 2.92 (3.0)
The purified peptide thus obtained was subjected to solid state FTIR spectroscopic analysis. The spectrum shows that the sequence is a typical β-sheet structured peptide (Figure 4.20) as evident from the characteristic peaks at 3274 cm⁻¹ (Amide-I); 1639 cm⁻¹ (Amide I) and 1523 cm⁻¹ (Amide II). We have made no attempt to check the conformational behaviour of resin bound peptides during synthesis, since many peaks of the resin were also present in the conformationally sensitive region.
The identity of the compound was established by recording ESI-MS. The peptide was dissolved in TFA and applied to the nitrocellulose matrix. The spectrum showed a molecular ion peak at 915.2 while calculated was 914.1 (Figure 4.21). Thus, the present results pave the way for a reliable strategy for the synthesis of aggregating and hydrophobic sequences on PEG grafted flexible crosslinked supports.

![Figure 4.21. ESI-MS of β-amyloid fragment (33-42)](image)

For DVB-PS resins PEG\textsubscript{600} is found to be optimal. But in BDDMA-PS, even PEG\textsubscript{600} has made significant improvement in the overall yield and purity of the synthetic peptide. Flexibility of the core matrix may be the reason for this. The enhanced swelling and solvation of the polymer-peptide system and low substitution level may be the two major factors responsible for the success of the present synthesis. PEG chain also furnishes a spacer effect which minimises the steric effect. A well solvated BDDMA-PS-PEG resin carrying the peptide chains can be typically represented in Figure 4.22.
4.9. Synthesis of difficult sequence peptides

4.9.1 Synthesis of acyl carrier protein fragment (65-74) on both BDDMA-PS and DVB-PS: The role of macromolecular character of the matrix upon solid-phase peptide synthesis

Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly

ACP (65-74) fragment, a small decapeptide, is one of the well studied examples for sequence dependant problems in SPPS.\textsuperscript{81} Although its synthesis may seem to be straight forward, the earliest attempts were quite discouraging.\textsuperscript{81-84} Hancock et al. studied the sequence in detail and found that the addition of Asn\textsuperscript{74}, Ile\textsuperscript{72} and Tyr\textsuperscript{71} were very difficult even after repeated couplings.\textsuperscript{81} The peptide was also shown to undergo strong internal association after the deprotection of Gln\textsuperscript{63}. So the attachment of Val\textsuperscript{64} was found to be very difficult.\textsuperscript{81} NMR studies of the 1% DVB-PS resin carrying this sequence revealed a marked decrease in the segmental mobility of the polystyrene backbone.\textsuperscript{85} Swelling studies of the peptidyl resin showed a considerable decrease in the swelling capacity.\textsuperscript{81} These observations were in good agreement with the β-sheet formation of pendant peptide chains.

Earlier attempt of Hancock et al., using 3 meq. excess Boc-amino acids and DCC coupling gave only 20% yield.\textsuperscript{81} Later Sheppard et al. synthesised
this sequence in polyacrylamide support using six-fold excess of Boc amino acid anhydrides in DMF and obtained 48% yield. Using Fmoc-anhydrides, they got 75% yield. Then Fmoc-amino acid pentafluorophenyl esters and trichlorophenyl esters were used in 5 to 10 fold excess to get >90% yield. However, this sequence has been used by many group of peptide chemists to check the utility of their support, reagents or strategy in peptide synthesis.

So in the present study, this sequence was built on both conventional DVB-PS and newly developed BDDMA-PS resins under identical conditions to compare the efficiency of supports. Various synthetic parameters like acylation time, yield and purity were monitored. Conformational behaviour of the growing peptide chain was also analysed using FTIR spectroscopy.

In the present study, the synthesis of this peptide on high capacity DVB-PS and BDDMA-PS resins under the same conditions was described. Only 2.5 mmol excess of the N-Boc-protected amino acids were used for coupling as their HOBT active esters. C-terminal amino acid, Gly was attached to the resin by cesium salt method. A reaction time of 24 h was given to BDDMA-PS resin and 38 h to DVB-PS resin to get more than 90% amino acid substitution. NMP containing 10% v/v of DMSO was selected as the coupling medium. DMSO was reported to be an effective solvent for the desegregation of the peptide chains. Each coupling was given a reaction time of 1 h and couplings were repeated till the Kaiser test is negative. No capping was performed to block the unreacted amino groups. The same protocol was used to assemble the peptide chain on DVB-PS also. But the attachment of most of the amino acids on DVB-PS resin was very difficult. Coupling of Tyr to Ile was performed 5 times, Ile to Asp, Ala to Ile and Gln to Ala were carried out 3 times to get a negative Kaiser test. Finally, the attachment of Val to Gln was repeated 4 times.

Compared to DVB-PS resins, the synthesis of ACP fragment (65-74) on BDDMA-PS resin was easy. All the couplings except Tyr to Ile and Val to Gln were complete in the first time itself. But, in the second coupling these amino acids were quantitatively attached. In each step, even a very slight blue colour in the Kaiser test to either the solution or the resin was taken as
positive. It was observed that a total acylation time of 20 h was required to assemble the peptide on DVB-PS resin, but, only 11 h was sufficient to build on BDDMA-PS resins. This further confirmed the high reactivity of BDDMA-PS resins as compared to DVB-PS resins.

The completed peptide was cleaved from the support using TFA/thioanisole method. The crude peptide was dissolved in methanol and the purity was checked by tlc and HPLC techniques. On tlc analysis, using (a) pyridine : water : acetic acid (85:10:5) and (b) n-butanol : acetic acid : water (66:12:22), the peptide obtained from BDDMA-PS resin gave a single spot $R_{f(a)} = 0.52$ and $R_{f(b)} = 0.14$. However, the product from DVB-PS gave a major spot at the same $R_f$-value with a significant tailing. The homogeneity was further ascertained by HPLC analysis on a reverse phase C18 column using the solvent $A = 0.1\%$ TFA containing water and $B = 0.1\%$ TFA containing acetonitrile. The crude peptide itself obtained after TFA cleavage from BDDMA-PS resin was highly pure (> 95%) as evident from Figure 4.23a, but, the product from DVB-PS resin was contaminated with deletion sequences (Figure 4.23b).

![Figure 4.23.](image)
Efficiency of chain assembly on the different supports was further checked by subjecting the peptidyl resin to the amino acid analysis. 10 mg of the peptide containing resin was hydrolysed using 6 N HCl in propionic acid (1:1) at 110°C for 22 h. The resin was removed and the solution was checked by o-phthalaldehyde procedure. The result clearly demonstrated that the peptide obtained from BDDMA-PS resin was highly homogeneous. The product from DVB-PS was contaminated with both insertion and deletion sequences. The very low value of Tyr, Gln, Val and the exceptionally high value of Ala clearly indicated the above fact (Table 4.14).

Table 4.14. Amino acid analysis of ACP (65-74) fragment obtained from DVB-PS and BDDMA-PS supports

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Theoretical</th>
<th>DVB-PS</th>
<th>BDDMA-PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>2</td>
<td>3.37</td>
<td>2.07</td>
</tr>
<tr>
<td>Asp</td>
<td>2</td>
<td>1.47</td>
<td>2.04</td>
</tr>
<tr>
<td>Glu</td>
<td>1</td>
<td>0.48</td>
<td>0.89</td>
</tr>
<tr>
<td>Gly</td>
<td>1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ile</td>
<td>2</td>
<td>1.61</td>
<td>1.97</td>
</tr>
<tr>
<td>Tyr</td>
<td>1</td>
<td>0.85</td>
<td>0.98</td>
</tr>
<tr>
<td>Val</td>
<td>1</td>
<td>0.41</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Despite the fact that the synthetic product from BDDMA-PS gives a single peak on reverse phase HPLC analysis it is unfortunate that this most extensively used technique for assessing the homogeneity of the peptide product is not satisfactory. There are some cases where the single peak compound on RP-HPLC was shown to be non-homogeneous on mass spectroscopic analysis. So the mass spectrum of the product obtained from BDDMA-PS resin was recorded on a fast atom bombardment mass spectrometer. The spectrum showed that the product is highly homogeneous with molecular weight 1063.9 while calculated molecular weight is 1063.1 (Figure 4.24).
Thus, the high yield synthesis of pure peptide was achieved rapidly on the newly developed BDDMA-PS resins. In the present investigation, a high capacity resin (about 2 mmolg$^{-1}$ chlorine capacity) was used for synthesis. The high capacity and 2% crosslinking was affected the efficiency of DVB-PS resins to a great extent. This was clear from the yield, purity and coupling difficulty observed during the synthesis of ACP (65-74) on this resin. Hancock et al. already studied the synthesis of this peptide in detail and reported that the difficulty is mainly due to the internal aggregation of the sequence. But when the support was changed to BDDMA-PS, the synthesis became more easy, probably due to the desegregation of resin bound peptides. For a detailed understanding on this aspect, conformational developments of the resin bound peptides during its synthesis on both resins, especially after 8th, 9th and 10th residues, were carried out using solid state FTIR spectroscopy. Narita et al. extensively used this technique for conformational analysis of peptides bound to linear and crosslinked resins. Though there can be nine bands of stretching frequencies characteristics of an isolated planar amide bond, the most significant amide-I (1600-1700 cm$^{-1}$)
region was selected for conformational assignments. DVB-PS sample collected after the attachment of 8th residue gave a sharp peak at 1639 cm⁻¹ and a small peak at 1658 cm⁻¹. Similarly peaks were obtained at 1646 cm⁻¹ and 1638 cm⁻¹ in the amide I region for samples collected after the attachment of 9th and 10th residues respectively. These peaks are the characteristic of the β-sheet structure, in accordance with the early reports. The same sequence, when built on BDDMA-PS resin, gave peaks at 1660, 1662 and 1665 cm⁻¹ respectively after the attachment of 8th, 9th and 10th residues on FTIR analysis. These are characteristic of the random coil conformation. The shoulder observed at 1648 cm⁻¹ for the sample collected after the attachment of 9th residue showed the presence of a fraction of β-sheet aggregation in BDDMA-PS bound sample also (Figure 4.25). Hence it was seen that as the support was changed from DVB-PS to BDDMA-PS, a major conformational transition from β-sheet structure to random coil of the resin bound peptides was observed. Such a desegregation caused by the disruption of the resin bound β-sheet structure on BDDMA-PS resin may be the reason for the improved synthetic results on this new support.

Figure 4.25. Amide A and Amide I regions of the FTIR spectra of resin bound ACP fragments: (I-III) amide A region after 8th, 9th and 10th residues; (IV-VI) amide I region after 8th, 9th and 10th residues
The present investigation paves the way to a new class of versatile supports in which the physicochemical characteristics of the support resemble those of the growing peptide chain. The high reactivity, rapid synthesis, high yield and purity of the product, etc. even with resins of high loading capacity may be due to the optimum hydrophobic-hydrophilic balance of this support. High swelling capacities of BDDMA-PS support in both polar and nonpolar solvents is also responsible for the superiority of the resin. As the swelling increases the segmental mobility of the polymer backbone may increase which in turn facilitates the rapid diffusion of the reagents into the peptidyl resin network. So all the reactive sites are well exposed and quantitatively available for reaction at each step. It has been reported that the microenvironment surrounding the active site has a direct influence upon the kinetics and yield of the organic reactions on polymers. In BDDMA-PS resin, the polarity and flexibility offered by 1,4-butanediol dimethacrylate provides a slightly polar microenvironment which facilitates the rapid reaction even at high substitution levels. At this point, it is interesting to note that as the support was changed from DVB-PS to BDDMA-PS, many of the synthetic problems could be solved to some extent, though it was reported that the coupling difficulty was independent of the nature of the support. So it may be possible to tune the molecular character of the polymer support by changing the nature and degree of crosslinking to effect the successful synthesis of peptides. In this report, both DVB-PS and BDDMA-PS resins were prepared under identical conditions to cancel the effect of the local clustering of functionalities and hence microenvironmental phase transformation effects.
4.9.2 Synthesis of DAla\textsuperscript{17} Analogue of Human Gonaodotropin Releasing Hormone precursor protein fragment (14-36)

Asp-Ala-Glu-DAla-Leu-Ile-Asp-Ser-Phe-Gln-Glu-Ile-Val-Lys-Glu-Val-Gly-Gln-Leu-Asp-Glu-Thr-Gln

The human gonadotropin releasing hormone (hGnRH) precursor consists of the gonadotropin releasing hormone (GnRH) sequence followed by a 59-amino acid carboxyl-terminal extension.\textsuperscript{98} A 56-amino acid peptide within this extension has been shown to stimulate gonadotropin release.\textsuperscript{99} A decapeptide sequence, \textit{Asn-Leu-Ile-Asp-Ser-Phe-Gln-Glu-Ile-Val}, corresponding to (17-26) fragment of the carboxyl extension of the GnRH precursor is regarded as the minimal structural requirement delineated for gonadotropin releasing activity.\textsuperscript{100} A further flanking sequence extending this active region from its carboxyl terminal is found to enhance the releasing activity, although the flanking sequence itself is inactive.\textsuperscript{100}

GnRH is a decapeptide \textit{pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH\textsubscript{2}} which is secreted by hypothalamic neurones in a pulsatile manner to effect the release of the gonadotropins, luteinizing hormone and follicle stimulating hormone from pituitary gonadotrophs.\textsuperscript{96,99} The determination of the nucleotide sequences of human placental GnRH cDNA, human hypothalamic GnRH cDNA, and rat hypothalamic cDNA, revealed the following structure to human prepro GnRH (Figure 4.26).\textsuperscript{100,101}

![Figure 4.26. Diagrammatic representation of human GnRH](image-url)

\begin{center}
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|}
\hline
1 & 10 & 11 & 14 & 27 & 37 & 46 & 50 & 53 & 66 & 69 \\
\hline
QHWSYGLRPG & G & KR & K & R & R & R & L & KK & I \\
\hline
Pre & GnRH & cs & Carboxyl extension & pcs \\
\hline
\end{tabular}
\end{center}

where the signal sequence (Pre) was followed by GnRH sequence and Lys-Arg cleavage site (cs). The second potential cleavage site (pcs) precedes the carboxyl terminal Ile. Gln undergo cyclisation to pGlu at the amino terminus of GnRH.

\textbf{Figure 4.26.} Diagrammatic representation of human GnRH
Results and Discussion

In an attempt to check whether the further processing of the carboxyl terminal extension occurs in addition to the cleavage of GnRH sequence from the precursor, a series of peptides containing the active (17-26) fragment were chemically synthesised. Then it was found that the (14-30) fragment has all the characteristic features of a typical difficult sequence peptide. An exceptionally low yield was noticed in the incorporation of Val to Lys. Also during the synthesis of this peptide, a marked decrease in DCM and DMF swelling of the peptidyl resin was also noticed. This observation was consistent with a model which has undergone a sudden increase in the effective crosslinking of the polymer matrix. FTIR spectroscopic investigation of the 1% DVB-PS resin carrying the (14-36) hGnRH sequence clearly demonstrated the aggregation of the resin bound peptide chains by β-sheet formation. So this sequence was selected to establish the potentiality of the newly developed BDDMA-PS resin for the synthesis of difficult sequences.

Peptide synthesis was carried out on a o-nitrochloromethyl copoly(styrene-2 mol% BDDMA) resin C-terminal Gln was attached to 1.05 mmolg⁻¹ level. Amino acids were stepwisely assembled using Boc/Bzl strategy. All the couplings were performed in NMP-DMSO (20% v/v) mixture. All the couplings were repeated till a negative Kaiser test was obtained. Then quantitative ninhydrin test was performed to check the coupling level. 3 meq. excess of preformed HOBT esters were used for each coupling. The difficulty in coupling was started with Val. It was found that the attachment of Val to Lys was very difficult and in a prior synthesis using symmetric anhydrides only 50% coupling was reported. But in the present attempt, after 1 h, 86% of coupling was found to occur. On repetition of the coupling using 5% v/v of DIEA towards the end improved the efficiency to 96.3%. The unreacted amino groups were capped using acetic anhydride. Similarly, the problem was noticed in the coupling of Ser to Phe also. But after three couplings Kaiser test was obtained negative. No more complications were observed in the remaining chain assembly. All
couplings to the Glu (OBzl) residues were mediated by symmetric anhydrides in NMP to minimise pyroglutamyl formation.\textsuperscript{103} Total amino acylation time required for each amino acid coupling is given in Table 4.15.

Table 4.15. Coupling details of amino acids during the synthesis of hGnRH fragment (14-36)

<table>
<thead>
<tr>
<th>Coupling position</th>
<th>Time\textsuperscript{a} (min)</th>
<th>% coupled\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr\textsuperscript{16}-Gln\textsubscript{36}</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Glu\textsuperscript{17}-Thr\textsubscript{15}</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Asp\textsuperscript{18}-Glu\textsubscript{34}</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Leu\textsuperscript{12}-Asp\textsubscript{13}</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Gln\textsuperscript{10}-Leu\textsubscript{12}</td>
<td>45</td>
<td>99.86</td>
</tr>
<tr>
<td>Gly\textsuperscript{11}-Gln\textsubscript{31}</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Val\textsuperscript{19}-Gly\textsuperscript{40}</td>
<td>90</td>
<td>-</td>
</tr>
<tr>
<td>Glu\textsuperscript{28}-Val\textsuperscript{29}</td>
<td>75</td>
<td>-</td>
</tr>
<tr>
<td>Lys\textsuperscript{26}-Glu\textsubscript{28}</td>
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<td>99.82</td>
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<td>Val\textsuperscript{20}-Lys\textsubscript{27}</td>
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<td>96.30</td>
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<td>Ile\textsuperscript{23}-Val\textsubscript{26}</td>
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<td>99.91</td>
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<td>Glu\textsuperscript{21}-Ile\textsuperscript{25}</td>
<td>60</td>
<td>98.89</td>
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<tr>
<td>Gln\textsuperscript{22}-Glu\textsubscript{24}</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Phe\textsuperscript{23}-Gln\textsubscript{29}</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Ser\textsuperscript{27}-Phe\textsubscript{22}</td>
<td>75</td>
<td>99.51</td>
</tr>
<tr>
<td>Asp\textsuperscript{28}-Ser\textsuperscript{21}</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>Ile\textsuperscript{26}-Asp\textsubscript{29}</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Leu\textsuperscript{16}-Ile\textsubscript{19}</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>D-Ala\textsuperscript{17}-Leu\textsubscript{18}</td>
<td>60</td>
<td>99.93</td>
</tr>
<tr>
<td>Glu\textsubscript{16}-Ala\textsubscript{17}</td>
<td>60</td>
<td>99.74</td>
</tr>
<tr>
<td>Ala\textsubscript{15}-Asp\textsubscript{14}</td>
<td>45</td>
<td>99.90</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Time of complete coupling was calculated on the basis of Kaiser test. First test was performed after 45 min. In case of incomplete reaction, tests were performed at intervals of 15 min. A maximum time of 1.5 h was given to each coupling.

\textsuperscript{b} As determined from quantitative ninhydrin test and picric acid method. Percentage was calculated on the basis of first amino acid substitution level.
After the attachment of Asn and Gln, IR spectrum was recorded to check the nitrile formation. No problem was found from the spectrum, during HOBt mediated couplings. In the earlier attempts to synthesise this peptide on DVB-PS resins, a decrease in the swelling behaviour was noticed after the attachment of a few protected amino acids.\textsuperscript{102} But, BDDMA-PS resin showed remarkable swelling throughout the chain assembly. Decrease in swelling was attributed to the formation of additional crosslinking in the resin matrix due to $\beta$-sheet aggregation.\textsuperscript{34} The relatively easy and quantitative coupling of various amino acids and increased swelling clearly illustrates the desegregation of the pendant peptide chains on BDDMA-PS networks. Moreover, the repeated couplings made considerable enhancement in the coupling yield unlike in DVB-PS resins.\textsuperscript{31} So, the observed improvements in the difficult coupling stages is primarily the new polymer matrix effect. In addition, the presence of polar nitro group in the ortho position of the peptide-resin linkage might have played crucial role in the reactivity by increasing the polarity of the microenvironment in the resin networks. The significance of local polarity upon the yield and efficiency of polymer-supported synthesis had been proved by Alexandratos et al.\textsuperscript{90} Highly hydrophobic clusters in DVB-PS resin can well promote the $\beta$-sheet aggregation of sequences containing amino acids with preferable $\beta$-sheet propensity. But, the polar crosslinking agent, BDDMA and polar functional moieties present in the relatively hydrophobic styrene unit will be sufficient to swell and solvate the polymer and peptide chains, especially in solvents of $\beta$-sheet disrupting property like NMP, DMSO etc. and to establish peptide polymer interactions to disrupt the permanent aggregation.

Finally, the peptide was cleaved from the support using TFA at 35°C for 20 h. In view of the presence of four glutamic acid residues in the sequence, only a little thioanisole was added just to catalyse the cleavage
reaction. Glutamic acid side chain can undergo reaction with anisole as in Scheme 4.7.³⁰ So the scavenger mixture p-cresol : EDT : thioanisole (2:2:0.5) was used to cleave the peptide. Addition of p-cresol was found to be very effective in such sequences to suppress the side reactions and to increase the yield.³⁰ However, synthesis of such peptides containing several acid sensitive groups or linkages can be conveniently carried out on a benzhydrylamine support using Boc/Bzl strategy and low/high HF cleavage procedure using excess dimethyl sulphide. Thus, the usual \( S_N1 \) mechanism of the acidolysis can be changed to \( S_N2 \) and hence can minimise the side reactions due to carbocations.³⁰

\[
\text{Scheme 4.7 Anisylaton of glutamic acid}
\]

The crude peptide was obtained in 93% cleavage yield based on the first amino acid substitution level. The peptide was injected into RP-HPLC fitted with a C₁₈ column (Figure 4.27). A prior run was made for 20 min at isocratic condition using 10% acetonitrile to remove the side products and scavenger impurities. Amino acids containing products will not appear in this region. Then the chromatogram was run under water (A) and acetonitrile (B) \( \text{pH} = 6.5 \) adjusted using triethylammonium phosphate (B). Then the major peak was obtained at 10.98 min which corresponds to the target peptide as seen from amino acid analysis (Peptide content 95.6%) (Table 4.16).
Figure 4.27. Analytical HPLC profile of crude hGnRH fragment (14-36). HPLC profile of the purified fragment is given in inset.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Expected</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>7.0</td>
<td>6.84</td>
</tr>
<tr>
<td>Thr</td>
<td>1.0</td>
<td>0.63</td>
</tr>
<tr>
<td>Asp</td>
<td>3.0</td>
<td>2.91</td>
</tr>
<tr>
<td>Lys</td>
<td>2.0</td>
<td>2.04</td>
</tr>
<tr>
<td>Gly</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Val</td>
<td>1.0</td>
<td>1.82</td>
</tr>
<tr>
<td>Lys</td>
<td>1.0</td>
<td>1.03</td>
</tr>
<tr>
<td>Ile</td>
<td>2.0</td>
<td>1.90</td>
</tr>
<tr>
<td>Phe</td>
<td>1.0</td>
<td>1.08</td>
</tr>
<tr>
<td>Ser</td>
<td>1.0</td>
<td>1.71</td>
</tr>
<tr>
<td>Ala</td>
<td>1.0</td>
<td>1.10</td>
</tr>
<tr>
<td>D Ala</td>
<td>1.0</td>
<td>1.03</td>
</tr>
</tbody>
</table>

"Asn and Gln were converted to Asp and Glu during hydrolysis.
"Low value of Thr and Ser were due to partial destruction during hydrolysis.
"Ile-Val has also a low value, probably due to the incomplete hydrolysis of Ile-Val bond."
Comparison of this synthesis with the early attempts illustrates the utility of present resin, BDDMA-PS. In the present synthesis, repetition of the coupling cycle could improve the difficult couplings considerably. Near quantitative coupling (99.9%) was achieved within 15 to 30 min. In an early attempt using DVB-PS, this sequence made no improvement in coupling yield even after repeated couplings and capping times of about 4 h to achieve >99.5% acylation. Addition of HFIP, a strong β-sheet destabilising solvent, improved the coupling efficiency to an extent of 0.5 to 9.6%. HFIP synthesis revealed a reduction in total amino acylation time. That is, 20 h vs. 68 h with an average post-cycle acylation efficiency of 99.91%. The present synthesis required 22 h, but many of the couplings were 100% complete within 30 min. However, a minimum of 45 min was given for each coupling to get an average coupling efficiency of 99.86%. Then an improved yield of 26.2% (mmol peptide/initial substitution of resin) was obtained. Earlier it was 12.8% and 17.3% without and with HFIP respectively. But, one important thing here is that all the earlier attempts were made on 1% DVB-PS resins of low chlorine capacity (0.4 to 0.7 mmol g⁻¹). The present synthesis was achieved on a relatively high capacity resin (1.05 mmol g⁻¹). Even then a satisfactory synthesis could be achieved. So, though the present system is not the ultimate for difficult sequence problems, it would seem to be a considerable aid for the prevention of deletion sequences by allowing quantitative reactions.
4.9.3 Synthesis of Human Immuno Deficiency Virus Protease (HIV-1) fragment (80-99)

Thr-Pro-Val-Asn-Ile-Ile-Gly-Arg-Asn-Leu-Leu-Thr-Glu-Ile-Gly-Cys-Thr-Leu-Asn-Phe

A key step in the maturation of retroviruses is the post transitional cleavage of polyprotein fusion into their constituent functional proteins. For HIV virus, the proteases responsible for this process was encoded by the pol gene as a 99-residue polypeptide. The importance of such proteases in the infectivity of their viruses is well known. It was also reported that the replacement of Asp25 with Asn eliminated the infectivity of the virus. So these findings stimulated the development of HIV-protease inhibitors as potential therapeutic agents in the treatment of acquired immuno deficiency syndrome (AIDS). These inhibition studies were mainly carried out by specifically labelling the active centre amino acids with $^{13}$C and then using NMR. Thus, the chemical synthesis of this enzyme is envisioned as the reliable approach to rapidly obtain this protease in useful quantities.

The first enzyme synthesised were ribonuclease A and S. Total synthesis of HIV-protease has been accomplished by Nutt et al. The early attempts of the synthesis of this protein have shown that repetitive incomplete amino acylations occur in the region Thr42 to Val91. From a preview synthesis of 59 to 99 fragment on a tentagel support by Bayer et al., and the subsequent separation, purification and characterisation of the intermediates by ESI-MS and FAB-MS, they concluded that the formation of rigid secondary structure ($\beta$-sheet) as the reason for the synthetic difficulty. So, the fragment containing the difficult region, (80-99) was selected to illustrate the usefulness of BDDMA-PS support. The resin has already showed a tendency to disrupt $\beta$-sheet aggregation.
α-Nitrochloromethyl copoly (styrene-2% BDDMA) was used for synthesis. Chlorine capacity of the resin was estimated by Volhard's method (1.38 mmol g⁻¹). N-Boc-Phe was attached to the resin by cesium salt method (1.26 mmol g⁻¹ amino capacity). All the couplings were repeated until a clear negative Kaiser test was obtained. Even a slight blue or green colour either to the solution or the resin beads was taken as positive. Couplings were very smooth up to Asn₈⁵. N-Boc-Arg (mtry) was coupled to Asn₉⁷ using 5 mmol excess of HOBt active esters. The first two couplings were performed in NMP (1 h each) and then two more couplings were given in DMF-DMSO mixture (20% v/v) 5% DIEA was also added towards the end of reaction. Then 97.9% attachment was obtained. The other difficult stages were Ile₈⁴ to Ile₉⁵, Asn₉⁵ to Ile₉⁴ and Thr₉⁶ to Pro₉⁷. But quantitative ninhydrin analysis performed after each difficult stages showed no such major problems in the present synthesis. Capping of the three amino groups using acetic anhydride was also performed to avoid deletion sequences. The progress of the reaction is evident from the following Table 4.17. Kent et al. have shown that the percentage coupling of Val, Ile, Thr and Leu in incomplete reactions of the order of 13, 19, 19 and 21% respectively. Van Woekom et al. has confirmed this for Bmoc-amino acids also. This is mainly due to the steric effect of these amino acids. Thus a total acylation time of 20.5 h was required for synthesis. From the ninhydrin analysis an average coupling yield of 99.77% was evident. This is an indication of the success of chain assembly. In the earlier synthesis progressively decreasing coupling has been observed from Asn₈⁵ to Pro₉⁷. But here, though some problems were found, nearly quantitative coupling was achieved by repeated coupling or extending the time of coupling.
Table 4.17. Coupling details of amino acids during the chain assembly of HIV-1 aspartyl protease fragment (80-99)

<table>
<thead>
<tr>
<th>Coupling position</th>
<th>Time (min)</th>
<th>Coupling yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asn98 - Phe99</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Leu97 - Asn98</td>
<td>45</td>
<td>99.96</td>
</tr>
<tr>
<td>Thr96 - Leu97</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Cys95 - Thr96</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Gly94 - Cys95</td>
<td>45</td>
<td>99.99</td>
</tr>
<tr>
<td>Ile93 - Gly94</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Gln92 - Ile93</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>Thr91 - Gln92</td>
<td>45</td>
<td>99.01</td>
</tr>
<tr>
<td>Leu90 - Thr91</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Leu89 - Leu90</td>
<td>45</td>
<td>98.90</td>
</tr>
<tr>
<td>Asn89 - Leu89</td>
<td>90</td>
<td>99.24</td>
</tr>
<tr>
<td>Arg56 - Asn88</td>
<td>240</td>
<td>97.61</td>
</tr>
<tr>
<td>Gly86 - Arg57</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Ile85 - Gly86</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Ile84 - Ile85</td>
<td>90</td>
<td>99.11</td>
</tr>
<tr>
<td>Asn84 - Ile84</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Val82 - Asn83</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Pro81 - Val82</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>Thr80 - Pro81</td>
<td>90</td>
<td>99.89</td>
</tr>
</tbody>
</table>

The completed peptides were cleaved from the support using neat TFA containing thioanisole, EDT and m-cresol as scavengers. The crude peptide was obtained in 91% yield, based on the weight increment and first amino acid substitution level. The crude peptide was dissolved in 50% acetic acid and injected into RP-HPLC fitted with a C18 column. The peptide was more than 90% pure as seen from the chromatogram (Figure 4.28). The fraction corresponding to the major peak at 27.8 min was subjected to amino acid analysis (Table 4.18). The values were in good agreement with the theoretical value and which correspond to a peptide content of 96.4%.
Figure 4.28. HPLC profile of crude HIV fragment (80-99). HPLC profile of the purified sample is given in inset.

Table 4.18. Amino acid analysis of the synthetic HIV-1 fragment (80-99)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Expected</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Asp&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0</td>
<td>2.92</td>
</tr>
<tr>
<td>Leu</td>
<td>3.0</td>
<td>3.01</td>
</tr>
<tr>
<td>Thr&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.0</td>
<td>1.31</td>
</tr>
<tr>
<td>Cys&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0</td>
<td>0.82</td>
</tr>
<tr>
<td>Glv</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Ile&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.0</td>
<td>2.63</td>
</tr>
<tr>
<td>Glu</td>
<td>1.0</td>
<td>1.02</td>
</tr>
<tr>
<td>Arg</td>
<td>1.0</td>
<td>0.81</td>
</tr>
<tr>
<td>Val</td>
<td>1.0</td>
<td>1.03</td>
</tr>
<tr>
<td>Pro</td>
<td>1.0</td>
<td>0.98</td>
</tr>
</tbody>
</table>

<sup>a</sup> Asn and Gln were hydrolysed to Asp and Gln respectively.

<sup>b</sup> Low value was due to the incomplete hydrolysis of lle-Ile bond

<sup>c</sup> Thr and Cys was lost during hydrolysis.
Finally the peptide was subjected to ESI-MS (Figure 4.29). The molecular ion peak observed at 2187.4 was also in agreement with the calculated value 2186.1.

![Graph](image)

**Figure 4.29.** ESI-MS of HIV fragment (80-99)

### 4.10. Synthesis of biologically active peptides

#### 4.10.1 Synthesis of Leu$^{51}$-enkephalin

**Tyr-Gly-Gly-Phe-Leu**

Enkephalins belong to opiate family of peptides consisting of endorphins, dermorphins, casmorphins, dynorphins and deltorphins. They display morphin like properties in several *in vitro* and *in vivo* test systems. The most important derivatives are Leu$^{51}$-enkephalin and Met$^{51}$-enkephalin. These are under clinical trial as a novel analgesics instead of morphins. Leu$^{51}$-enkephalin was synthesised on chloromethylated BDDMA-PS resin of chlorine capacity 2.23 mmolg$^{-1}$. C-terminal amino acid leucine was attached by a Gisin's cesium salt method to a level of 2.11 mmolg$^{-1}$; which corresponds to 97% conversion. Boc-chemistry was employed for synthesis. The following methods of coupling were employed for the chain assembly to optimise the conditions highly compatible with the BDDMA-PS support: (a) direct DCC
mediated coupling in DMF, (b) direct DCC mediated coupling in NMP, (c) preformed DCC/HOBt active ester coupling in DMF and (d) preformed DCC/HOBt active ester coupling in NMP.

Each amino acid was then attached stepwisely and the completion was checked by Kaiser test. At the tripeptide stage, a portion of the peptidyl resin was subjected to quantitative ninhydrin analysis to estimate the chain loss via DKP formation. DKP formation has been widely investigated and recognised as a sequence dependent problem. But, this side reaction is known to be both acid and base catalysed and dependent on solvents, additives added during coupling, resin-peptide linkage, etc. In the present study, about 14.6% loss was observed in (a) and 8.6% in (b). In methods (c) and (d) no significant chain loss was observed. This is consistent with the observation that DCC/HOBt coupling is relatively immune to racemisation.

A comparison of the above coupling methods shows that active ester coupling was more effective than DCC mediated direct coupling. Unlike other active esters like nitrophenyl esters, pentachlorophenyl esters and pentafluorophenyl esters, HOBt ester coupling and symmetrical anhydride coupling were fast and devoid of side reactions. Among the solvents DMF and NMP, the former is the best for DCC coupling, but both were found to be equally effective for active ester coupling. Superiority of DMF over NMP in Boc-amino acid N-carboxyanhydride mediated couplings was already established. The above inference was made out of the total and stepwise acylation time required for the synthesis of this peptide in each method. In (a) attachment of Tyr to Gly was extended to 60 min. In (b) the coupling of Gly to Gly and Tyr to Gly was repeated twice. In (c) and (d) all the couplings were equally effective, and completed within 30 min. The total time required for these methods were 3, 3.5, 2.5 and 2.5 h respectively. The superiority of DMF is due to its comparable polarity with the peptide chain and BDDMA-PS support. So DMF lead to optimal peptide-resin solvation and thus enhanced the accessibility of the resin bound amine to acylations species.
The finished peptide was cleaved from the support using TFA/thioanisole method. When allowance was given for the chain loss at the peptide stage, all the couplings have gone to 99.9% completion. The peptide was cleaved from the support in >96% yield.

The purity of the products obtained from each method was ascertained by tlc. Two solvent systems (A) pyridine : water : acetic acid (85:10:5) and (B) n-butanol : water : acetic acid (4:1:1 v/v) were used for analysis. The product from (a) gave single spot at $R_f(A)$ and $R_f(B)$. $R_f$ values of (b) were also identical. Similarly, the products from (c) and (d) were also single spot at same $R_f$ values. But a slight tailing was observed for the product from (d) in the solvent system (B). So the observations in tlc do not furnish a definite inference. However, HPLC analysis on a C18 column gave a single peak showing >95% purity (Figure 4.30).

![Figure 4.30](image)

**Figure 4.30.** Analytical HPLC of Leu-enkephalin

Homogeneity of the compound was further confirmed by amino acid analysis: Leu 1.01 (1.0); Phe 0.94 (1.0); Gly 1.0 (1.0) and Tyr 0.76 (1.0). Low value of Tyr was due to its partial destruction during hydrolysis.

Finally the identity of the compound was established by mass spectrometry. The spectrum shows a molecular ion peak at 555.9 while
calculated is at 554.6 (Figure 4.31). Thus a satisfactory synthesis of Leu\textsuperscript{5}-enkephalin was achieved on BDDMA-PS support.

![FAB-MS of Leu\textsuperscript{5}-enkephalin](image)

**Figure 4.31.** FAB-MS of Leu\textsuperscript{5}-enkephalin

### 4.10.2 Synthesis of delta sleep inducing peptide (DSIP)

Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu

DSIP is a nonapeptide isolated from the hemodialyzate of rabbits by Monniev et al.\textsuperscript{121} This peptide increases the δ-wave on electroencephalograms after ventricular infusion. Both chemical and enzymatic syntheses of this peptide have been reported.\textsuperscript{122,123} Although it is a simple peptide, it contains highly acid sensitive Gly-Asp and Ser-Gly linkages\textsuperscript{124} and tryptophan at the N-terminal. So this sequence was selected as a test peptide to optimise the conditions for the successful synthesis of large peptides containing more acid labile linkages and side chain protected amino acids.

The peptide was assembled on a benzhydryl resin, built on copoly (styrene-2 mol% BDDMA) support using Fmoc-chemistry. Benzhydryl type resins, originally developed by Southard et al.,\textsuperscript{125} is less stable towards acidolysis and hence cannot be used with Boc-amino acids. An important
modification of this type of resins is the development of benzhydrylamine resin,\textsuperscript{126} which facilitates the attachment of the growing peptide chain by a C-terminal amide bond to the support. Various kinds of substituted benzhydrylamine supports are also widely used in peptide synthesis.\textsuperscript{127} This resin can be conveniently used for the production of peptide amides by treatment with anhydrous HF.\textsuperscript{126}

(a) \textit{Preparation of benzhydryl resin (BH-resin)}

The BH-resin was prepared from 2 mol \% crosslinked BDDMA-PS resin by a two step reaction. In the first step, Friedel-Crafts reaction of BDDMA-PS (1) with benzoyl chloride (2) was carried out in presence of anhydrous aluminium chloride to give a keto resin (3) characterised by the IR band at 1660 cm\textsuperscript{-1} of the keto group. The keto resin thus obtained was reduced in the second stage to the hydroxy resin (4) with NaBH\textsubscript{4} in diglyme (Scheme 4.8).

\begin{center}
\includegraphics[width=\textwidth]{scheme4.8.pdf}
\end{center}

\textbf{Scheme 4.8. Preparation of benzhydryl resin}
The reduction was followed by the disappearance of the carbonyl peak at 1660 cm\(^{-1}\) and the appearance of a peak at 3500 cm\(^{-1}\), characteristic of the OH group. IR spectrum is given in Figure 4.32.

![IR spectrum of benzhydryl resin](image)

**Figure 4.32.** IR spectrum of benzhydryl resin

(b) Attachment of C-terminal amino acid

The first step of the SPPS is the attachment of C-terminal amino acid to the support. So the polymer carrier plays not only the role of an inert solid medium but also as a carboxyl protecting group. Thus, the support should undergo facile reaction with the carboxyl group of N\(\text{\textsuperscript{\textalpha}}\)-protected amino acids and the linkage formed must be stable under all conditions of stepwise elongation. Finally, the bond must be easily cleavable to detach the peptide from the support without side reactions. To check these conditions, N\(\text{\textsuperscript{\textalpha}}\)-Boc-Ala was attached to the newly developed BDDMA-PS-BH resin using the DMAP catalysed symmetric anhydride procedure. Boc-Ala substitution level was judged to 86% from elemental analysis and picric acid method. In order to check the peptide-resin linkage stability, Boc-Ala attached resin was treated with 25% TFA/DCM, 50% TFA/DCM and neat TFA separately. A small portion of the sample was withdrawn at regular intervals and the residual amino acid on the support was estimated by picric acid method.
(Figure 4.33). It can be inferred that about 26.5% loss was obtained with 50% TFA/DCM after 10 h reaction and 21% loss was obtained for 25% TFA/DCM. When neat TFA was used, 40% loss was noticed within 30 min and at the end of 1.5 h, 89% of cleavage was taken place. So it is better to use Fmoc-chemistry for temporary deprotection and TFA for the final cleavage of the peptide from the resin.

![Figure 4.33. Relative acid stability of amino acyl BH resin](image)

(c) Peptide synthesis

Fmoc-chemistry has been proved to be valuable for the synthesis of sequences containing highly acid labile linkages and acid sensitive amino acid residues.\(^{28}\) C-terminal N\(^{\alpha}\)-Fmoc-Glu (OEt-Bu) was attached to the benzhydryl resin by symmetric anhydride procedure. The degree of substitution after 3 h reaction was found to be 81% by UV-method. The remaining hydroxyl group on the resin was benzoylated. 20% piperidine in DMF was used for Fmoc removal. The remaining amino acids were coupled
to the support using 2.5 meq. excess of preformed DCC/HOBt active esters. All coupling reactions were performed in DMF-DMSO (20% v/v) mixture containing 5% v/v of DIEA. Second and third amino acids were coupled in NMP-DMSO mixture and no DIEA was added to minimise the DKP formation. Solvent DMF and DIEA was found to be catalysing the DKP formation. DKP formation rate in various linkages like Wang, Pam and Nbb polystyrene resins have been studied by Giralt et al. All the couplings were complete within 45 min as seen from Kaiser test. N-terminal tryptophan was incorporated as the formyl derivative, i.e., N^Fmoc-Trp (CHO). The typical protocol adopted for the synthesis is given in Table 4.19.

<table>
<thead>
<tr>
<th>Table 4.19. Protocol adopted for the chain assembly of DSIP on BH support built on BDDMA-PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DCM wash</td>
</tr>
<tr>
<td>2. DMF wash</td>
</tr>
<tr>
<td>3. 20% Piperidine/DMF</td>
</tr>
<tr>
<td>4. DMF wash</td>
</tr>
<tr>
<td>5. 2.5 meq. DCC/HOBt active ester coupling</td>
</tr>
<tr>
<td>6. 30% DCM/methanol wash</td>
</tr>
<tr>
<td>7. DCM wash</td>
</tr>
<tr>
<td>8. Kaiser test (If positive, repeat steps 2 to 8; if negative couple the next amino acid)</td>
</tr>
</tbody>
</table>

After the chain assembly, the resin was washed with DCM and dried in vacuo. The peptidyl resin was then treated with 1 ml piperidine in 10 ml of DMF (for 200 mg peptidyl resin) for 2 h at 0°C with occasional stirring to deformylate the peptide. Then the resin was filtered and washed with DMF and treated with 20% piperidine in DMF for 20 min, to remove the residual N^Fmoc group. Washed and dried resin was subjected to the final cleavage.
The peptidyl resin was treated with neat TFA containing 5% phenol, 5% water, 5% thioanisole and 2.5% EDT. This cleavage mixture was developed by King et al. for the successful cleavage of the completed peptides containing residues like Trp, Arg, Met, Glu, Asp, Tyr, etc. The scavengers EDT and water have been shown to be highly efficient for quenching trifluoroacetate and tert-Bu cations respectively. After 1.5 h, the resin was filtered and the filtrate was rotary evaporated. Peptide was then precipitated by the addition of ether. Crude peptide thus obtained was about 80%, based on the first amino acid substitution level.

The peptide was then dissolved in 50% acetic acid and passed through Sephadex G-10 column to remove the low molecular weight impurities mainly resulting from the scavenger mixture. Purity was then checked by RP-HPLC on a C18 column using the gradient 0 to 60% B over 30 min where A is 0.15 TFA containing water and B is 0.08% TFA containing acetonitrile (Figure 4.34). Detection was done at 214 nm and 280 nm to check the presence of Tryptophan and finally the presence of Tryptophan was assessed by UV-spectroscopy (Figure 4.35). The absorbance at 280 nm is a clear indication of Trp presence. Both the chromatograms indicated a purity of > 90%, as judged from the major single peak, for the synthesised product.

![Figure 4.34. HPLC profile of DSIP](image1)

![Figure 4.35. UV spectrum of DSIP](image2)
The tlc analysis of the purified product gave a single spot corresponding to the $R_f$ value 0.15 in $n$-Butanol : water : acetic acid (4:1:1 v/v). Presence of Trp was clear from the tlc development using Erlich reagent specific for Tryptophan. Finally the peptide was characterised by amino acid analysis. The values obtained were: Glu 1.03 (1.0); Gly 3.01 (3.0); Ser 0.70 (1.0); Ala 1.96 (2.0); Asp 0.94 (1.0) Trp 0.49 (1.0) Low value of Ser and Trp were due to the partial destruction during hydrolysis. The identity of the compound was established by recording ESI-MS (Figure 4.36).

![Figure 4.36. ESI-MS of delta sleep inducing peptide](image)

### 4.10.3 Synthesis of C3-peptide involved in the cell proliferation regulation

**Lys-Phe-Lys-Trp-Asp-Asp-Pro-Gly-Lys-Asn-Leu-Tyr-Glu-Val-Glu-Ala**

This peptide contains some problematic amino acid residues like Arg, Tyr, Trp, Gln, Asn, Asp, etc. Synthesis was carried out on an HMP resin built on 2 mol% BDDMA-PS using $N^\alpha$-Fmoc-amino acids. Fmoc-Arg (mtr) Fmoc-Asp (OtBu); Fmoc-Glu (OtBu) Fmoc Lys (Boc) Fmoc-Trp (CHO) and Fmoc-Tyr (tBu) derivatives were used for side chain protection. Asn and Gln
were incorporated with the carboxamide side chain protection. The details of synthetic steps have given in experimental chapter.

After the attachment of Asn and Gln, a small portion was withdrawn for recording the IR spectrum using KBr discs. No nitrile formation was detected. Attachment of Arg to Trp was conducted 4 times using 4 meq of active esters for each coupling. Then two more couplings were given using symmetric anhydrides in DMF. The remaining amino groups were capped using acetic anhydride. Similarly attachment of Lys to Tyr, Asp to Pro, and Gln to Arg were also repeated 3 times each, to get a negative Kaiser test.

The peptide was cleaved from the support using 50% TFA/DCM containing scavengers phenol, water, thioanisole and EDT in the ratio (1:2:2:2 v/v). The reaction was continued for 1.5 h at room temperature. The resin was filtered and the peptide was precipitated using ether. Crude peptide was obtained in 87% yield. It was then purified by passing through a Sephadex-G25 column.

Formyl group was cleaved prior to the cleavage from resin using piperidine in DMF at 0°C for 2 h. Analytical RP-HPLC analysis shows the presence of 2 peaks (Figure 4.37). The major peak observed at retention time 27.1 min was separately collected and amino acid analysis was performed using e-phthalaldehyde procedure. The results were in good agreement with the expected values.

![Figure 4.37. HPLC profile of the synthetic C3 peptide](image)
Amino acid analysis:

 Ala - 1.0 (1.0) ;  Glu - 3.88 (4.0) ;  Asp - 1.96 (2.0) ;  Val - 1.02 (1.0) ;
 Tyr - 0.51 (1.0) ;  Lys - 3.04 (3.0) ;  Gly - 1.0 (1.0) ;  Pro - 0.99 (0.1) ;
 Arg - 0.82 (1.0) .

where Asn and Gln were converted to Asp and Glu during hydrolysis; Tyr
was also partially lost; Trp was completely lost. The identity of the
compound was established by ESI-MS (Figure 4.38).

![Figure 4.38. ESI-MS of C3-peptide](image)

4.10.4 Synthesis of CKS-17


The retroviral transmembrane envelope protein is a hydrophobic
protein that inhibits immune responses of lymphocytes, monocytes, and
macrophages. A region of this protein is found to be conserved among the
transmembrane envelope proteins of the human retroviruses HTLV-I and
HTLV-II and in retroviral DNA. CKS peptide is a synthetic peptide
corresponds to this region of homology which inhibited the proliferation of
an interleukin-2 dependent lymphocytes. This 17-peptide was assembled on 2 mol% BDDMA-PS resin of chlorine capacity 1.36 mmol g\(^{-1}\). The C-terminal Leu was attached to the resin using Gisin's cesium salt procedure. Amino acid substitution level was ascertained by picric acid method (1.22 mmol g\(^{-1}\)) and confirmed by elemental analysis for nitrogen. It was reported that polar aprotic solvents like DMF is highly efficient for peptide bond formation. It was also reported that addition of tertiary amines like DIEA has a catalytic effect upon coupling reactions. But, Bodanszky et al. and Naider et al. found that addition of tertiary amines has no profound effect upon the acylation of HOBt salts of amino acids or N-carboxyanhydride mediated coupling reactions. So the present synthesis was carried out in DMF using 2.5 meq. of symmetric anhydrides without the addition of DIEA. Symmetric anhydrides were reported to be equally effective and devoid of side reactions as in the case of DCC/HOBt method. Difficult couplings were performed in DMF-DMSO (10% v/v) mixture. The third amino acid was coupled as its DCC/HOBt active ester in NMP to minimise the DKP formation. Quantitative ninhydrin tests were performed after 2, 4, 6, 8, 10, 12, 13, 14 and 17 amino acid attachment, from the C-terminal end (Figure 4.39).

![Figure 4.39: Diagrammatic representation of the coupling efficiency](image-url)
Couplings of Arg to Arg and Asn to Arg were very difficult due to the competitive lactum formation reaction with the peptide bond formation. All the other couplings were complete within 45 min. So it is found that the absence of tertiary amines has not made any significant loss of reactivity during peptide bond formation. However, in the earlier syntheses, DIEA was used for efficient coupling. In the present synthesis, Asn and Gln were incorporated as DCC/HOBt active esters to avoid the nitrile formation. The synthetic steps involved in one cycle of operation is given below (Table 4.20).

Completed peptide was cleaved from the support using neat TFA containing thioanisole and EDT at 35°C for 24h. The peptide was precipitated with diethylether and washed successively to remove the scavengers. Cleavage yield was 89.2% based on the first amino acid substitution.

<table>
<thead>
<tr>
<th>Step</th>
<th>Protocol Adopted for the Chain Assembly of CKS-17</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>DCM wash 5 x 3 ml</td>
</tr>
<tr>
<td>2.</td>
<td>30% TFA/DCM 1 x 5 ml (30 min)</td>
</tr>
<tr>
<td>3.</td>
<td>DCM wash 3 x 3 ml (2 min)</td>
</tr>
<tr>
<td>4.</td>
<td>5% DIEA/DCM 1 x 5 ml (2 min)</td>
</tr>
<tr>
<td>5.</td>
<td>10% DIEA/NMP 1 x 5 ml (2 min)</td>
</tr>
<tr>
<td>6.</td>
<td>DCM wash 5 x 5 ml (2 min)</td>
</tr>
<tr>
<td>7.</td>
<td>2.5 meq amino acid anhydride/DMF (AA+ DCC; 2:1)</td>
</tr>
<tr>
<td>8.</td>
<td>30% Methanol/DCM 3 x 5 ml (2 min)</td>
</tr>
<tr>
<td>9.</td>
<td>DCM wash 3 x 5 ml (2 min)</td>
</tr>
<tr>
<td>10.</td>
<td>Kaiser test; repeat steps 6 to 10, if positive</td>
</tr>
<tr>
<td></td>
<td>repeat 1 to 10 for next amino acid if negative</td>
</tr>
</tbody>
</table>

The peptide was subjected to a prior HPLC purification, by running in 10% acetonitrile for 20 min. Then a gradient of 5 to 60% B over 40 min was applied where solvent A is 0.1% TFA/H2O and B is 0.1% TFA/acetonitrile. The chromatogram gave a major peak at 22.5 min corresponding to the target peptide (Figure 4.40).
The peptide corresponding to the major fraction was subjected to amino acid analysis: Leu - 5.94 (6.0); Gly - 3.01 (3.0); Glu - 1.92 (2.0); Asp - 1.89 (2.0); Phe - 1.08 (1.0); Arg - 1.81 (2.0). Asn and Gln were hydrolysed to Asp and Gln. ESI-MS of the purified peptide is given in Figure 4.41.
4.10.5 Synthesis of [Thr$^{14}$] [Thr$^{19}$] galanine fragment (1-19)

Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Thr-Ala-Val-Gly-Asn-Thr

Galanine, a 29-amino acid residues containing C-terminally amidated neuropeptide, widely distributed throughout the central and peripheral nervous system.$^{135}$ It was first isolated from porcine by Tatemoto et al. and its primary structure was deduced from cDNA precursor.$^{136}$ To define the physiological role of this regulatory peptide in humans, Schimidt et al. studied the sequence and biological activity of human pituitary galanine which is a 30-residue non-amidated peptide.$^{135}$ The primary structure of the peptide was shown to be Gly-Trp-Phe-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Phe-His-Ala-Val-Gly-Asn-His-Asp-Ser-Phe-Ser-Asp-Lys-Asn Gly-Leu-Thr-Ser.

Biological actions of galanine include relaxation of gut smooth muscles, inhibition of somatostatin release, and modulation of hormone secretion from pituitary and adrenal glands.$^{137}$ Galanine also plays an important role as neuro modulator of endocrine and synaptic transmission.$^{138}$

In an attempt to gather information regarding the charge, structural requirement and hydrophobicity of the peptide to exhibit biological activity and hence to derive peptide with varying degrees of activity, this 19-residue fragment corresponding to (1-19) of Galanine in which 14th and 19th histidines were replaced with threonine, was synthesised on 2 mol% crosslinked BDDMA-PS resins using the standard solid-phase synthesis using N$^\text{Boc}$-protected amino acids. Derivation of short peptides from the entire sequences of large peptides and proteins as potent therapeutic agents is of modern research interest since they can be synthesised in high yield and purity by chemical methods. Some short peptides thus desired were shown to be more active than the naturally occurring one.$^{139}$ Moreover, the substitution of Thr increased the hydrophobicity of the sequence and hence this sequence
is adequate to check the efficiency of BDDMA-PS support for the synthesis of hydrophobic peptides.

(a) Peptide chain assembly

Peptide was synthesised by stepwisely incorporating the amino acids from the carboxyl terminal on 2 mol% BDDMA-PS resin of chlorine capacity 2.36 mmolg⁻¹. Chloromethylated resin was subjected to nitration using fuming nitric acid to introduce nitro group ortho to the chloromethyl group. Nitration was performed to increase the acid stability of the benzyl ester linkage of the C-terminal threonine to the support, because 19 cycles of repetitive acid treatment has to be performed during synthesis. Nitro group was reported to be effective for increasing the acid stability of the benzyl ester linkage and hence to prevent the peptide chain loss. N"-Boc-Thr (Bzl) was attached to the support using cesium salt method and the substitution level was found to be 1.97 mmolg⁻¹ by picric acid method. Thus 94% conversion was obtained within 24 h reaction. Boc-group was removed using 30% TFA/DCM for 30 min and the free amino group was liberated by treatment with 5% DIEA/DCM followed by 10% DIEA/NMP for 2 to 3 min. Threonine, serine and tyrosine were incorporated as their OBzl ethers, and tryptophan as Nω-formyl derivative. Asparagin was attached by HOBT active ester method in NMP to avoid nitrile formation. In all the coupling steps, care was taken to avoid prolonged treatments with nucleophilic additives such as HOBT, pyridine etc., since the phenyl ester linkage is susceptible to such reagents. Each couplings was given 1 h and repetitive couplings were performed for completion.
All couplings were monitored by the Kaiser test and quantitative ninhydrin method and found no apparent difficulties during synthesis. Quantitative ninhydrin test after synthetic cycles 2, 4, 8, 10, 14, 16 and 18 indicated 99.6 to 99.9% completion (Figure 4.42). But the attachment of Tyr to Leu and Ser to Ala were only 97% complete even after the repetition of coupling in NMP and DMF. There was only a little loss of peptide chains from the resin support, as 98% of the starting material was bound to the support even after 18 cycles of acid and base treatment. IR spectrum of the peptidyl resin was recorded after 3rd and 15th Asn residue attachment to check the nitrile formation, but showed no serious effect of nitrile formation as the peak at 1630 cm⁻¹ was absent.

(b) Cleavage of the peptide

Protected galanine fragment carrying peptidyl resin was treated with 50% TFA/DCM for 20 min to remove the N-terminal Boc group. Dry resin, was then treated with 1 ml piperidine in 10 ml DMF (500 mg resin) at 0°C for 2 h to deformylate Trp (CHO). The resin was filtered and wash with DMF, DCM and Methanol, then dried in vacuo 100 mg of peptidyl resin was first treated with 50% TFA/DCM for 5 min to remove extraneous materials absorbed or precipitated on the resin. After washing with DCM, the resin was dried and treated with neat TFA in presence of thioanisole, m-cresol, ethanedithiol, p-thiocresol and H₂O. Thioanisole, in addition to promoting acidolysis, plays the role of an acceptor of benzylic group of polymer during cleavage. Thioanisole is also preferable to anisole for preventing
C-alkylation of tyrosine. But, thioanisole cation adducts are not stable and shown to undergo reversible reactions to alkylate other amino acids like Met and Trp during HF cleavage. So p-thiocresol was added to prevent alkylation of Trp. EDT is the most efficient scavenger for t-butyl trifluoroacetate. It can prevent the alkylation of Trp when used with water. m-Cresol is also used as a scavenger for t-butyl trifluoroacetate and t-butyl carbonium ion. The reaction was continued for 20 h at 35°C. The peptide was precipitated with cold diethylether and washed and dried. The crude peptide was obtained in 86% yield, based on the first amino acid substitution level.

(c) Purification

Gel-filtration on Sephadex G25 using 2% acetic acid/water as the eluent was primarily used for purification. The peptide obtained was dissolved in methanol and loaded in a C18-reverse phase column and the chromatogram was run for the first 20 min under isocratic condition of 10% acetonitrile to remove the impurity peaks due to p-thiocresol, EDT, thioanisole, m-cresol, oxidised and reduced species of EDT and other p-thiocresol adducts Amino acid containing products were not found in this region. Then the chromatogram was run under the binary gradient of 5 min 10% B; 45 min 80% B and 50 min 100% B, where A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile. The analytical HPLC profile of the crude product showed only one major peak (Figure 4.43) and which was collected and lyophilised to get pure peptide. The high purity of the peptide thus obtained is a clear evidence of the utility of BDDMA-PS support and the effectiveness of the tactics employed for the synthesis.
(d) Characterisation

Efficiency of the chain assembly and the homogeneity of the peptide thus obtained was compared by amino acid analysis of the peptidyl resin and the purified peptide. The amino acid compositions obtained are given in Table 4.21. It can be seen that the values of crude peptides clearly indicate the presence of one or two minor deletion sequences but there was no insertion sequences. This shows the effectiveness of the protocol used and the purity of the amino acids employed for synthesis. The values of the purified peptides are in good agreement with the theoretical value and which corresponds to 92% of the peptide content.

The presence of Trp and Tyr residues was further confirmed by HPLC profiles monitored at 214 nm and 280 nm respectively. No product was detected by monitoring at 310 nm [$\lambda_{max}$ for Trp (CHO)] showing the complete removal of formyl group. The presence of Trp residue was established by recording the UV-spectrum which also suggests the complete deformylation of Trp residue (Figure 4.44).
Table 4.21. Amino acid composition of galanine fragment (1-19)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Theoretical</th>
<th>Peak 1</th>
<th>Peak 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr</td>
<td>3</td>
<td>2.76</td>
<td>2.89</td>
</tr>
<tr>
<td>Asp</td>
<td>2</td>
<td>1.42</td>
<td>2.04</td>
</tr>
<tr>
<td>Gly</td>
<td>4</td>
<td>3.84</td>
<td>3.98</td>
</tr>
<tr>
<td>Val</td>
<td>1</td>
<td>-</td>
<td>0.88</td>
</tr>
<tr>
<td>Ala</td>
<td>2</td>
<td>1.01</td>
<td>2.08</td>
</tr>
<tr>
<td>Pro</td>
<td>1</td>
<td>1.34</td>
<td>0.92</td>
</tr>
<tr>
<td>Leu</td>
<td>3</td>
<td>2.11</td>
<td>3.11</td>
</tr>
<tr>
<td>Tyr</td>
<td>1</td>
<td>-</td>
<td>0.76</td>
</tr>
<tr>
<td>Ser</td>
<td>1</td>
<td>0.41</td>
<td>0.71</td>
</tr>
<tr>
<td>Trp</td>
<td>1</td>
<td>0.32</td>
<td>0.81</td>
</tr>
</tbody>
</table>

Low values of Thr, Ser, Tyr and Trp were due to its partial destruction during hydrolysis.

![UV spectrum of deformylated galanine fragment (1-19)](image)

Figure 4.44. UV spectrum of deformylated galanine fragment (1-19)

Finally the identity of the synthesised galanine fragment was established by recording ESI-MS. The spectrum showed the peaks at 1815.1 and 1816.4 corresponding to M + H and M + 2H respectively. The calculated molecular weight is 1814.8 (Figure 4.45).
So, it is clear that this relatively hydrophobic galanine (1-19) fragment was synthesised in excellent purity and yield on high capacity BDDMA-PS resins. Except a very few reports, synthesis on high capacity resins are generally a failure due to the possibility of site-site interactions and hence the aggregation of peptide chains to local clusters within the resin matrix. But the flexibility and hence the increased mobility of polymer backbone of the BDDMA-PS minimises this effect and makes the reactive sites quantitatively available for reaction. One such support TTEGDA-PS was also reported to be good for high capacity synthesis.\textsuperscript{5} In addition, the support effect, a proper choice of the side-chain protected amino acids, coupling procedures and additives, cleavage procedures and scavengers and the skill of the person conducting the synthesis are all crucial in the success of a peptide synthesis.
4.10.6 Synthesis of chromostatin

Ser-Asp-Glu-Asp-Ser-Asp-Gly-Asp-Arg-Pro-Glu-Ala-Ser-Pro-Gly-Leu-Gly-Pro-Gly-Pro

Chromogranine A, is a ubiquitous 48 kD secretary protein present in adrenal medulla, anterior pituitary, central and peripheral nervous system, thyroid, parathyroid and endocrine pancreas. Chromostatin, a 20-residue peptide has been derived from this protein, which modulate the catecholamine secretion from cultured adrenal medullary chromaffin cells. It was shown that chromostatin is extra cellurally generated from chromogranine A by a calcium dependant proteolytic mechanism. This peptide may be an endocrine modulator of catecholamine associated responses.

The chemical synthesis of this sequence is a challenging problem due to the presence of four alternate aspartic acid residues at the N-terminus. It becomes more complicated due to the two Asp-Ser linkages and Asp-Gly linkages which are highly sensitive to acids. So the major side reaction during the synthesis of such sequences will be aspartimide formation and subsequent mild opening to give mainly the β-isomer. Since many peptide hormone, biologically active factors, enzymes and toxins contain these sequences, this sequence was selected to optimise the conditions for best results. In addition, this sequence contains an Arg-Pro linkage, which is very difficult to establish. C-terminal end is Gly-Pro-Gly-Pro a very sensitive sequence for diketopiperazine formation. Similarly the N-terminal ten residues of this peptide is hydrophilic, and the remaining ten residues of the C-terminal is hydrophobic. Since these specialities make this sequence a real challenge to peptide chemists, its synthesis was attempted.

(a) Chain assembly using Boc-chemistry

Synthesis was started by the anchoring of the C-terminal proline as its cesium salt (N'-Boc-Pro-Cs) to chloromethylated BDDMA-PS resin of
1.65 mmolg\(^{-1}\) chlorine capacity. Amino acid substitution level was found to be 1.49 mmolg\(^{-1}\) which corresponds to 93\% of conversion. The second and third amino acid was coupled to the support using 4 mmol excess of HOBT-active esters in NMP. No tertiary amine was added as a catalyst during coupling. The coupling was extended to 45 min and then recoupling was given at the earliest to avoid the DKP formation and acyldioxopiperazines, particularly of Gly-Pro sequences. The present sequence with Gly-Pro-Gly-Pro at the C-terminal is quite liable to chain loss via the following reaction (Scheme 4.9).\(^{146,147}\)

![Scheme 4.9. DKP formation in resin bound Gly-Pro](image)

The unusual geometry of proline residues contributes to side reactions such as acyliso urea formation, because the bulky ring can interfere with the desired attack on the O-acyl isourea.\(^{148}\) Such O→N shift is very pronounced in DMF and hence this solvent was avoided. After the attachment of 5 residues, the amino capacity was determined by picric acid method. 8.2\% chain loss was observed. N\(^\alpha\)-Boc-glutamine was incorporated using 3 mmol excess of HOBT active ester. The reaction was complete within 30 minuets as evident from the Kaiser test. Boc-group of the Gln was removed using 30\% TFA/DCM and the next amino acid proline was attached using 5 mmol excess of the active ester. This is to prevent the pyroglutamyl formation of the N-terminal Gln in acid media.\(^{149}\) This side reaction was shown to be very serious if acetic acid is used for deprotection.\(^{150}\) The most serious damage is caused by the dehydration of the carboxamide side chain to γ-cyanobutyrine (Scheme 4.10). But HOBT active ester coupling was shown to be immune to this side reaction.\(^{150}\) After the attachment of proline, a small portion of the
Resin was subjected to IR analysis, but no peak corresponding to the CN group was observed.

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{CH}_2-\text{CH}_2-C=\text{NH}_2 & \quad \text{CH}_2-\text{CH}_2-C=\text{N} \\
\text{NH}-\text{CH}-\text{C}-\text{X} & \quad + \quad \text{H}_2\text{N}-\text{R} \quad \xrightarrow{\text{HX} \cdot \text{H}_2\text{O}} \quad \text{NH}-\text{CH}-\text{C}-\text{NH}-\text{R}
\end{align*}
\]

Scheme 4.10. Nitrile formation in glutamine

\(N^\alpha\)-Boc-Arg (mts) was attached to proline by both HOBT active ester method of coupling and symmetric anhydride coupling. Active ester couplings were repeated thrice in NMP-DMSO (20% v/v) using 3 mmol excess of amino acids. Still the Kaiser test was found to be positive. Then two more couplings were given using 5 meq. of symmetric anhydrides in DMF-DMSO (10% v/v) mixture containing 5% v/v of DIEA, for 2 h. After the attachment, 96.3% Arg was found to be incorporated from quantitative ninhydrin test and the remaining amino groups were capped using acetic anhydride. At this stage 10 mg of the peptidyl resin was withdrawn and subjected to amino acid analysis. From the results, it is clear that no serious problem has occurred during synthesis, except the low incorporation of arginine residues.

- Pro - 3.86 (4.0); Gly - 3.04 (3.0); Leu 0.94 (1.0); Ser - 0.26 (1.0);
- Ala - 0.99 (1.0); Glu - 0.89 (1.0); Arg - 0.78 (1.0).

Next amino acid, aspartic acid was incorporated using \(N^\alpha\)-Boc-Asp-(OBzl). The most serious side reaction of this residue is the \(\beta\)-aspartyl peptide formation as depicted below (Scheme 4.11).
This reaction is fast especially when this residue was followed by Glycine or serine. Cyclohexyl ester protection for the side chain of aspartic acid was found to be better for eliminating this problem, but, it is cleavable only in HF. For benzyl ester protection 0.34% β-peptide formation was calculated in each step. However, benzyl ester side chain protection was used for Asp in the present synthesis.

After the chain assembly, the weight increment observed agrees with only 80-85% of average coupling yield. Boc-protection of the N-terminal group was removed using 30% TFA/DCM and neutralised with 5% DIEA in DCM or NMP. The peptidyl resin was washed and dried. Then the resin was given a prior treatment with 50% TFA/DCM for 5 min and washed with DCM to remove the extraneous contaminants. The completed peptide was then cleaved using TFA containing thioanisole, m-cresol, EDT and 2,4-dinitrophenol, which is able to reduce the succinimide formation to a minimum. The peptide was precipitated with ether and washed.

The peptide was then dissolved in 10% acetic acid-water mixture and passed through a column of Sephadex G25. The fraction on evaporation and lyophilisation gave white powder. The yield of the peptide was 69% based on the first amino acid substitution.
Purity of the synthetic product was checked on analytical HPLC fitted with RP C_{18} column. Using the gradient 0 min-5% B to 45 min 70% B and 50 min 100% B, where solvent A = 0.1% TFA/water and B = 0.1% TFA/80% acetonitrile-water. The chromatogram showed 3 peaks in which the major peak corresponds to the target peptide (Figure 4.46). The amino acid analysis of the peaks are given in Table 4.22. The peak 3 lacks some Asp and Pro residues. The peak may be probably the truncated sequence results after arginine incorporation. Purified peptide was obtained in an overall yield of 32% of the total crude product.

![HPLC profile](image)

**Figure 4.46.** HPLC profile of crude chromostatin synthesised using Boc chemistry

**Table 4.22.** Amino acid composition of synthetic chromostatin synthesised using Boc chemistry

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Theoretical</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro</td>
<td>4.0</td>
<td>3.01</td>
<td>3.92</td>
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<tr>
<td>Gly</td>
<td>4.0</td>
<td>3.68</td>
<td>4.11</td>
<td>4.51</td>
</tr>
<tr>
<td>Leu</td>
<td>1.0</td>
<td>1.45</td>
<td>1.04</td>
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</tr>
<tr>
<td>Ser</td>
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<td>Ala</td>
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</tr>
<tr>
<td>Glu</td>
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<td>1.10</td>
<td>1.81</td>
<td>3.11</td>
</tr>
<tr>
<td>Arg</td>
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<td>-</td>
<td>0.80</td>
<td>0.16</td>
</tr>
<tr>
<td>Asp</td>
<td>4.0</td>
<td>2.84</td>
<td>3.72</td>
<td>3.13</td>
</tr>
</tbody>
</table>
(b) **Synthesis of chromostatin using Fmoc-chemistry**

The results of the synthesis of this sequence using Boc-chemistry reveal that the difficulties encountered in the synthesis are not mainly due to the incomplete coupling, but, due to the residue and sequence specific side reactions, especially the acid catalysed reactions. A similar problem was encountered during the synthesis of human Gastrin-1 using Boc/Bzl strategy and it was then tackled using Fmoc/t-Bu strategy. So, the synthesis of this peptide was attempted using Fmoc-chemistry also.

Synthesis was carried manually on a PAM resin built on 2 mol% BDDMA-PS. The carboxyl terminal proline was attached to the resin as its Boc derivative using symmetric anhydride method, catalysed by DMAP. Boc group was removed using 30% TFA/DCM for 30 min and free amino group was liberated using 5% DIEA/DCM. The amino acid substitution level was estimated to be 0.86 mmol/g by picric acid method. The unreacted hydroxyl groups on the support were capped by benzoylation using benzylochloride and pyridine.

Next three residues were coupled using the Boc-procedure as explained above. Quantitative ninhydrin reaction has showed about 4.6% chain loss at this stage of the synthesis. The remaining amino acids were incorporated as their Fmoc derivatives. Fmoc-amino acids were avoided at the initial stage to minimise the base catalysed DKP formation of the highly susceptible sequence, Gly-Pro-Gly-Pro. All the couplings up to Arg were complete within 30 min. (2.5 fold excess of amino acids). Quantitative monitoring of the deprotection of Fmoc group using 20% piperidine in DMF showed 99.8% completion. Attachment of N\(^\circ\)-Fmoc-Arg (mtr) to Pro was 99.7% complete when performed for 4 times in DMF as its HOBt active ester. 3rd and 4th couplings were given 2 h. 5% DIEA was also added during coupling to catalyse the reaction. The unreacted amino groups were then capped using acetic anhydride. The reason for the slow acylation of Arg
residues was attributed to the competing intramolecular aminolysis to δ-lactum formation with the intermolecular peptide bond formation.152 Once formed, δ-lactum can transfer the protected guanidino group to a free amino group, terminating the peptide chain. Transfer of NO2 protected guanidino group to the imino terminus of Pro was documented.154 This side reaction can be effectively minimised using HOBT.

The remaining aspartic acid rich sequence containing Asp-Gly and Asp-Ser linkages were assembled using Nf-Fmoc-Asp (Ot-Bu) derivative. Amongst the bases tested, piperidine catalyses the aspartimide formation far more rapidly than DIEA or TEA.155 Treatment with 20% piperidine/DMF, for 10 min has made 100% side reaction for Asp(OBzI)-Gly sequence. Use of cyclohexyl ester has reduced it to 67% after 4h, while Asp (t-Bu) is only 11% cyclised after 4 h. To assemble Asp-Ser, side chain hydroxyl group was protected by t-Bu ethers. Each couplings has given 1 h without the addition of DIEA. Repeated couplings were given to achieve 100% coupling. To minimise the pyroglutamyl formation, Nf-Fmoc-Gln (tBu) was deprotected using 50% piperidine in DMF for 2 min.156 Finally the N-terminal Fmoc group was also removed using 50% piperidine/DMF for 2 min.

The peptidyl resin was washed with 30% TFA/DCM for 5 min and dried. Then the resin was suspended in the cleaving mixture, 83% TFA; 5% phenol; water; 5% thioanisole and 2.5% EDT for 1 h (Reagent K). Filter the resin and TFA was evaporated. The peptide was then precipitated using cold dry diethyl ether and washed with ether. The crude yield of the peptide was about 83% based on the first amino acid attached level. This corresponds to about 93% of the observed weight increment after synthesis.

The crude peptide was dissolved in acetic acid and purity was checked using RP-HPLC fitted with a C18 column. The same gradient as applied to Boc-chemistry product was used here also. The peptide showed only one major peak and a small peak (Figure 4.47). A prior run of the HPLC using
10% acetonitrile was used for 20 min to remove all side products resulting from the scavenger mixture.

Figure 4.47. HPLC profile of the crude chromostatin synthesised using Fmoc chemistry

The two peaks were separately collected and amino acid analysis was performed using 1:1 mixture of 6 N HCl-TFA at 110°C for 20 h. The small peak contains no Asp; so it is probably the capped sequence resulted from the incomplete acylation of Arg. The values of the major peaks are in good agreement with the theoretical value, which corresponds to 89% of the peptide content. The purified peptide was characterised by ESI-MS (Figure 4.48).

Pro: 4.09 (4.0); Gly: 3.98 (4.0); Leu: 0.71 (1.0); Ser: 2.72 (3.0);
Ala: 1.09 (1.0); Glu: 2.12 (2.0); Arg: 0.86 (1.0); Asp: 3.83 (4.0)

From the synthesis of highly acid-sensitive chromostatin, using both Boc and Fmoc chemistries, the procedure avoiding the repetitive acidolysis was found to be reliable for the synthesis of Asp and Glu rich sequences, especially for those containing Asp-Gly, Asp-Ser, Asp-Thr and Asp-His linkages. On changing the tactics from Boc/Bzl to Fmoc/t-Bu, almost 2-fold increase in the overall yield of the purified peptide was obtained. This work further confirmed the utility of the cleavage mixture, reagent K. Since all the
coupling steps and deprotection steps were fairly rapid and complete, on both the occasions, except with arginine, the solid support has no influence upon the success of synthesis of such sequence.

![Figure 4.48. ESI-MS of chromostatin](image)

4.11. Conclusion

Thus, the newly developed flexible crosslinked polymeric support is excellent for the synthesis of medium to large sized peptides of unordered structure. The support, BDDMA-PS also provides a very much reliable solution for the potent problem of difficult sequence peptides; especially when used in conjugation with NMP-DMSO or DMF-DMSO mixture. Hydrophobic peptides are also prepared satisfactorily on this support. A comparative investigation of the synthetic parameters on this support and conventional DVB-PS resin for the synthesis of some model β-sheet structured peptides and typical difficult sequence peptide viz. ACP (65-74) fragment has clearly demonstrated the superiority of BDDMA-PS support.
The various factors responsible for the success of BDDMA-PS resin can be explained as follows. Primarily the resin is polystyrene based and hence it possess all the characteristic features of DVB-PS systems like mechanical strength, physical form, easy synthetic manipulations, easy functionalisation etc. The presence of four methylene chain and two ester linkages in the crosslinker, BDDMA, makes the system polar and flexible. So BDDMA-PS resin matrix extensively swells in both polar and non polar solvents, unlike DVB-PS. Hence the reagents can well penetrate into the polymer network and reactions employed can be comparable to solution phase, at molecular level.

The flexible systems can adjust the relative spacing in the polymer network in various solvents depending on the polarity. Pillai et al. has delineated the dependence of the chemical nature of monomers, nature and extent of crosslinking, hydrophilic-hydrophobic balance of the matrix, rigidity-flexibility balance of the network, swelling-solvation behaviour, porosity and pore dimensions of the particles, and chemical reactivity of the attached functional sites with a view to design tailor-made macromolecular architecture for specific applications. Decrease in reactivity with increase in rigidity has also been proved by Pillai et al. and explained as due to the decrease in the molecular mobility of the chain segments in a tightly packed system.²

The hydrophobic-hydrophilic balance of the support can be adjusted by varying the nature and extent of crosslinking. This is clear from the kinetics measurements of the peptide bond formation reaction on a series of variously crosslinked polyacrylamides in which the crosslinking agents are varied among hydrophobic to hydrophilic.³ The enhanced reactivity and high yield of the pure synthetic peptides point out the attainment of optimum hydrophobic-hydrophilic balance in 2 mol% BDDMA-PS. However, a detailed investigation on this aspect is required to improve the support.

Another factor behind the success of this support is the polarity of the system. The presence of polar ester linkages not only increases the solvent
compatibility, but create a polar microenvironment for the easy progress of polar reactions like peptide bond formation. The importance of a suitable microenvironment surrounding the active site in a polymer-supported reaction has been tailored by Alexandratos and Miller for maximised kinetics and yield. Morawetz has observed a dependence of solvent polarity on the kinetics of the aminolysis of the resin-bound active esters. Later, many reports have clearly demonstrated the relevance of the local effective medium in the vicinity of the polymer. BDDMA-PS, when used in conjugation with NMP, DMF and DMSO, effectively solvate the functional sites and create a dry polar environment within the styrene units.

The most important property of BDDMA-PS support is its tendency to disrupt the β-sheet aggregates during SPPS. When the macromolecular structural parameters are properly adjusted the ester linkage in BDDMA interacts with the growing peptide chains and disrupt the aggregates more strongly in solvents like DMSO. Though intra-resin site-site interaction and hence the formation of local clusters and microphase separations have been noted even with less substituted DVB-PS resins, the desegregating capacity of BDDMA-PS supports has made it possible to use resins of ~ 2 mmolg⁻¹ capacity for the synthesis of even difficult sequences. Thus, BDDMA-PS supports offer a role rather than an aid for the synthesis of all kinds of medium to large size peptides, especially hydrophobic and β-sheet structured. Though BDDMA-PS support can definitely improve the role of solid-phase in SPPS, a proper selection of strategy and tactics recognising all the possible side reactions of a particular sequence and the hands of a skilful person are indispensable for the successful synthesis of any peptide.
References

Results and Discussion

Results and Discussion


