Chapter 4

Optimization of Peptide Synthetic Conditions on Tri(propylene glycol) glycerolate diacrylate Cross-linked Polystyrene Supports
4.1 Introduction

Solid support is a synthetic cross-linked polymer possessing reactive functional sites in which the C-terminal amino acid of a peptide is covalently linked to produce an immobilized system. The reactivity of functional group attached to a polymer backbone is governed by various characteristic structural parameters of the polymeric support like its polarity, nature and extent of cross-linking, the solvation of the support and resin bound species in the solvent medium.¹ The most widely used insoluble polymer resin; divinylbenzene cross-linked polystyrene introduced by Merrifield has been one of the choices for the synthesis of oligomeric bio-molecules.² Although this support has been widely used in SPPS with considerable success, the extreme purity and homogeneity of medium to large peptides is still a challenging problem to peptide chemists. The physicochemical incompatibility of this support with the bound peptides in a solvent medium results a negative influence to mass transport of the reagents, effective solvation of the polymer as well as peptide chain and the coupling and deprotection reaction rates. This can lead to the formation of truncated peptides due to incomplete coupling and deprotection reactions, and deletion sequences due to the re-growth of the partially completed sequence. Many attempts have been made to overcome these difficulties encountered in the solid phase peptide synthesis, especially in the modification of polymer supports, which led to the development of a series of new generation supports such as PEGA, POEPOP, POEPS, CLEAR and SPOCC.³⁻⁶ The lack of mechanical stability and the difficulty associated with the handling of the new supports were reported as their major drawbacks. The lack of mechanical stability of polyamide type supports and the physicochemical incompatibility of the hydrophobic macromolecular environment of the polystyrene type supports with the growing peptide chain were overcome to a certain extent by designing a new polymer support, by incorporating tri (propylene glycol) glycerolate diacrylate as cross-linking agent into the polystyrene core by free radical aqueous suspension
polymerization reaction. The PS-TRPGGDA support was found to exhibit optimum hydrophilic/hydrophobic balance, high mechanical stability and excellent swelling properties in various solvents that are used for stepwise synthesis of peptides.

For a polymer support the yield, purity and cost-effectiveness of a particular synthetic peptide essentially depend on the optimization of various reaction conditions. In this chapter the PS-TRPGGDA polymer was subjected to optimization of various reaction conditions in peptide synthesis. A systematic study on the nature of the cross-linker, the effect of cross-linking density on the swelling and the reaction rate, the factors affecting the C-terminal amino acid incorporation, N\textsuperscript{a}-deprotection, coupling and the final cleavage of the peptide from the support was carried out. A kinetic study of peptide bond formation reaction was also carried out to find the correlation between the reactivity of the bound functional groups and the structural parameters of the macromolecular matrix. Quantification of these closely interrelated parameters was necessary for the judicious selection of the optimized reaction conditions such as C-terminal amino acid attachment, coupling and deprotection steps which leads to the successful synthesis of various peptides on the new support with very high purity and yield.

### 4.2 Results and Discussion

**4.2.a C-Terminal amino acid incorporation to PS-TRPGGDA resin**

The initial step in polymer supported polypeptide synthesis is the covalent attachment of C-terminal amino acid to the polymer support. 2% PS-TRPGGDA and PS-TRPGGDA-HMPA supports were used to investigate the optimal reaction conditions of the C-terminal Boc/Fmoc-amino acid incorporation. These results were then compared with the commercially available PS-DVB resin. For the hydroxyl derived polymer supports, the C-terminal attachment can be carried out by preformed symmetric anhydride of Boc/Fmoc-amino acid in presence of p-dimethylamino pyridine as catalyst.
Since the chances of racemisation and dipeptide formation are high in this method, MSNT in presence of MeIm is used for the incorporation of C-terminal amino acid to the resin. The time-dependent attachment of C-terminal Boc/Fmoc Val, Ala, Gly, Leu were carried out by monitoring their percentage incorporation to the resin in presence of MSNT and MeIm in different time intervals. The Boc deprotection reaction was carried out with 30% TFA in DCM followed by neutralization with 5% DIEA in DCM. The percentage incorporation was then calculated by picric acid method.

**Figure 1:** Time dependent C-terminal amino acid incorporation of Boc-Val, Boc-Gly, Boc-Ala and Boc-Leu to the resin.
The percentage incorporation of Fmoc-amino acids to the resin was calculated spectrophotometrically by measuring the OD of piperidenedibenzofulvene adducts released during deprotection by the addition of 20% piperidine in DMF. This was carried out by withdrawing aliquots of the resin at regular time intervals.

![Graphs showing time-dependent incorporation of Fmoc-amino acids](image)

**Figure 2:** Time dependent C-terminal amino acid incorporation of Fmoc-Val, Fmoc-Gly, Fmcc-Ala and Fmoc-Leu to the resin.

The results showed that for PS-TRPGGDA-HMPA and PS-TRPGGDA-OH resins the quantitative incorporation of C-terminal amino acids takes place...
within 25 min after the addition of the reaction mixture (Fig. 1-2). But under the same reaction conditions the commercially available PS-DVB-HMPA and PS-DVB resins were found to require more than 35 min for the quantitative incorporation of the C-terminal amino acid. The higher rate of incorporation of C-terminal amino acid in PS-TRPGGDA resin may be due to its higher swelling in the reaction medium that can lead to the free interaction between the resin bound hydroxy functional groups and the activated C-terminal amino acid in the medium. Since the secondary hydroxyl group present in the cross-linker is selected for the C-terminal amino acid incorporation the incompatibility arising due to the hydrophobic polystyrene backbone to the activated amino acid in the medium is very much minimised in the swollen state of the resin. The free resin as well as HMPA attached resins does not show any marked deviation in the rate of incorporation, which substantiates the above hypothesis.

4.2.b Optimization of Nα-deprotection studies

1. Time-dependent cleavage of Nα-Boc amino protection

Incomplete deprotection of the temporary amino protecting group (Boc/Fmoc) during various stages of the synthesis can lead to the formation of truncated peptide sequences and deletion sequences due to the regrowth of partially completed sequences. The higher swelling of the resin in a solvent create a solvent porosity with in the resin and allows the ready access of the small reactant molecules to the resin bound functional groups within the polymer network. The swelling process occurs largely from the out side to the interior. The temporary Boc-protection was removed by exposing the resin to 30% TFA in DCM. A sudden change of solvent from DCM to 30% TFA in DCM results a change in the swelling of the resin bead that can result a sudden attenuation of reaction and results incomplete deprotection. This can also results in the trapping of the reagents and by-products, which can create various problems in the successive steps. The swelling studies showed that the change in solvents affects the swelling character of both resins (Fig. 3).
But in the case of PS-TRPGGDA may be because of the very high swelling compared to commercially available PS-DVB resin in DCM and 30\%TFA in DCM mixture the complete Boc deprotection takes place in a short time.

![Swelling studies](image)

**Figure 3:** Swelling studies of the resin, N-terminal amino acid incorporated resin and peptidyl resin in DCM and Boc-deprotecting reagent.

Another reason behind incomplete deprotection is the internal aggregation of resin bound peptide and steric hindrance due to side chain/side chain protecting group present in the N-terminus of the resin bound peptide. The deprotection studies in solid phase peptide synthesis could be used as a useful tool to follow the aggregation phenomenon and steric hindrance exhibited by various amino acids.

The rate of Boc-deprotection of various Boc-amino acid incorporated PS-TRPGGDA resins were studied and compared with PS-DVB resin under identical conditions by measuring the percentage of resin bound free amino group at different time intervals using picric acid method (**Fig. 4**). The quantitative Boc removal from the C-terminal amino acid is completed within 15 min in PS-TRPGGDA resin where as PS-DVB resin required 20 min. The free and fair permeation of cleavage reagents to PS-TRPGGDA resin matrix can be attributed to its high swelling in the cleavage reagent mixture and also the balanced polar character of the resin matrix.
2. **Time-dependent cleavage of Fmoc-amino protection**

The temporary Fmoc-removal is carried out by a 20% piperidine in DMF solution. The rate of Fmoc-deprotection from the C-terminal amino acid incorporated PS-TRPGGDA resin was studied and compared with Merrifield resin. A comparative study of Fmoc-removal was also carried out using various concentrations like 5%, 10%, 15%, 20% and 25% piperidine in DMF (Fig. 5a). The time dependent Fmoc-cleavage studies on PS-TRPGGDA resin showed that a 5% piperidine in DMF solution required 22 min, where as a 25% piperidine in DMF solution required only less than 8 min. for the complete Fmoc-removal. It is also found that the base concentration does not affect the swelling characteristics of the resin. The rate of cleavage is found to increase with increase in base concentration. To avoid the chance of racemisation and any unwanted side reactions the usual 20% v/v solution of piperidine in DMF was employed throughout the synthesis. With this solution the 100% Fmoc-removal was found to takes place within 10 min. of base addition (Fig. 5b). The rate of Fmoc-deprotection was monitored spectrophotometrically.
by measuring the optical density of the piperidine-dibenzofulvene adducts. The PS-TRPGGDA-HMPA and PS-TRPGGDA does not show any marked deviation in their reactivity.

![Graph](image-url)

**Figure 5:** (a) Time dependent Fmoc-removal from C-terminal Fmoc-Val attached PS-TRPGGDA-HMPA resins with various concentrations of piperidine in DMF (b) Time-dependent Fmoc-removal of the C-terminal Fmoc-Val from various supports using 20% piperidine in DMF.

A comparative study of Fmoc-deprotection was also carried out using PS-DVB resin. The deprotection studies showed that the presence of HMPA linker in PS-DVB resin influences the deprotection rate whereas it is not influencing the performance of Fmoc-deprotection in PS-TRPGGDA resin. The commercially available PS-DVB resin took about 18 min. for complete Fmoc-removal whereas in the case of PS-DVB-HMPA resin the deprotection time was reduced by four min. The high rate of deprotection in PS-DVB-HMPA resin compared to PS-DVB resin may be due to the spacer effect resulted by the incorporation of the HMPA linker which reduces the effect of the hydrophobicity of the PS-DVB resin network. In the case of PS-TRPGGDA
resin 100% Fmoc-removal took place within 10min. after the addition of cleavage mixture.

Fmoc-cleavage study of both HMPA attached PS-TRPGGDA and PS-DVB peptidyl resins containing six amino acid residues was also carried out to study the effect of secondary structure formation of the resin bound peptide.

Figure 6: Swelling studies of the resin, N-terminal amino acid incorporated resin and peptidyl resin in DMF and Fmoc-deprotecting reagent.

Peptide containing several Ala residues tends to form β-forming oligomers, which may result in the aggregation of the peptide. The studies showed that the tetra Ala incorporated PS-TRPGGDA resin had higher swelling characteristics in the deprotection medium and the rate of cleavage is not affected by the presence of the peptide chain (Fig. 6). Results revealed the positive role of the TRPGGDA cross-linker in assisting the free interaction between the cleavage mixture and the Nα-protected peptidyl resin.

4.2.c Comparative study of solid phase amide bond formation using Leu-Ala-Gly-Val as model peptide

To optimize the PS-TRPGGDA support in peptide synthetic conditions a time and temperature-dependent coupling reaction was carried out using the
Merrifield peptide Leu-Ala-Cly-Val as the model peptide. The results were compared with Merrifield resin under identical conditions (Fig. 7(1-4)). Each coupling step was compared by adding the respective activated Fmoc-amino acids to the resin bound amino acid/peptide and then monitored by the degree of coupling at various time intervals. The time/temperature dependent studies reveal that the amino acid coupling reaction takes place at a higher rate when PS-TRPGGDA support was used for the test peptide synthesis. The amide formation was completed within 25 min., 20 min., and 15 min. in all the different coupling steps when the new support was used at 30°, 40° and 50° C respectively. In PS-DVB-HMPA resin the coupling was completed within 45 min. and 30 min. and for PS-DVB support within 75 min., 60 min., and 45 min. at 30°, 40° and 50° C respectively.

The major reason for low coupling reaction in various stages of peptide synthesis is the steric occlusion of the peptide chain within the polymer network. This in turn depends upon the type of the resin and its swelling characteristics in the coupling medium. In solvents like DCM, NMP and DMF the PS-TRPGGDA swells to its elastic limit and creates a space or solvent porosity within the resin and allow ready access of the activated amino acid molecule to the polymer network and which in turn becomes saturated with free flowing activated amino acid. This can lead to the free interaction between the activated amino acid and the amino terminus of the resin bound amino acid/peptide. The steric effect, which caused by the peptide chain or the sterically hindered amino acid, is extremely low in PS-TRPGGDA resin compared to PD-DVB resin.

The hydrogen bonding interaction between the resin bound peptide and the resin backbone is another major factor contributing to the poor coupling reactions especially in polyamide resin and PEG resins. This negative factor can be completely avoided in the new support because of the resin molecular nature. The interchain hydrogen bonding interactions between the various growing resin bound peptide chain can be avoided to an extent since the peptide growth point on the resin is selected from the cross-linker. In the swollen state the fluctuating
cross-linker chain in the solvent will not favour an inter chain hydrogen bonding between the resin bound peptide chains. These inter chain hydrogen bonding is also depending upon the degree of loading on the resin.

Figure 7-1: Time/temperature dependent coupling reaction
(a) PS-TRPGGDA-HMPA-Val-Gly (b) PS-TRPGGDA-HMPA-Val-Gly-Ala (c) PS-TRPGGDA-HMPA-Val-Gly-Ala-Leu

Figure 7-2: Time/temperature dependent coupling reaction (a) PS-TRPGGDA-Val-Gly (b) PS-TRPGGDA-Val-Gly-Ala (c) PS-TRPGGDA-Val-Gly-Ala-Leu
Figure 7-3: Time/temperature dependent coupling reaction (a) PS-DVB-HMPA-Val-Gly (b) PS-DVB-HMPA-Val-Gly-Ala (c) PS-DVB-HMPA-Val-Gly-Ala-Leu

Figure 7-4: Time/temperature dependent coupling reaction (a) PS-DVB-Val-Gly (b) PS-DVB-Val-Gly-Ala (c) PS-DVB-Val-Gly-Ala-Leu
The capability of the new polymer support in solid phase peptide synthesis depends upon the efficiency in chain assembly. One hundred percentage yields in each amide bond formation to the growing resin bound peptide chain is an important factor to the successful synthesis of long chain polypeptide.

4.2.d Peptide cleavage studies of PS-TRPGGDA support

The time and temperature-dependent cleavage studies of the PS-TRPGGDA supports were followed by the cleavage study of Leu-Ala-Gly-Val peptide and compared with Merrifield resin (Fig. 8). The studies revealed that PS-TRPGGDA-HMPA support required 3h reaction time for 98% cleavage of the peptide at 30°C whereas same cleavage yield was found to occur at 40°C within 2h of the reaction time. Un-derivatized PS-TRPGGDA supports required 12 h for 85% cleavage of the bound peptide at 30°C whereas it required 10 h for 87% cleavage at 40°C. The studies carried out with Merrifield supports showed that the linker attached support required 2 h for 90% cleavage at 30°C and it took 3 h for 91% cleavage at 40°C. Un-derivatized Merrifield supports required 12 h for 77% cleavage of the bound peptide at 30°C whereas it required 10 h for the 80% cleavage at 40°C. The enhanced rate of cleavage of linker attached PS-TRPGGDA resin compared to its un-derivatized resin may be due to the formation of more stable carbocation intermediate during the cleavage conditions.
Figure 8: Percentage cleavage of tetra peptide (Leu-Ala-Gly-Val) from different supports (a) cleavage at 30°C (b) cleavage at 40°C

4.2.e Kinetic comparison of PS-TRPGGDA supports with Merrifield resin

Chemical reactions on cross-linked polymer matrices are diffusion-controlled and therefore the topographical features of the matrix play a decisive role in driving the reaction to completion. To gain the full advantage of solid supported organic synthesis; the reaction performed on the resin should go to completion preferably at high rate. This ensures high purity of the end product. A better understanding of the reaction rate in the solid support enables its use in solid-phase organic synthesis, especially in peptide synthesis, where multiple operations are employed repeatedly. Even though the reactivity’s of groups anchored on polymer supports do not vary from their low molecular weight counterpart, the rate of the reaction in cross-linked resin is very much retarded. This is primarily because of the steric effect and subsequent diffusion problems encountered by the soluble reagent. The functional groups in resin beads are uniformly distributed. On a gelatinous resin up to 99% of the reactive site is located inside the bead and only 0.1-1% on the outer surface. The total polymeric reaction spaces represented by a
certain amount of beads are divided into small individual reaction compartments represented by each bead.

To investigate the influence of the nature of the polymer backbone chain on the side chain reactivity in peptide synthesis a kinetic study of amide bond formation was carried out. In SPPS the time consuming reaction is amide bond formation as compared to deprotection. Hence monitoring the peptide bond formation assumes more importance than monitoring any other reactions in order to study the influence of various factors of the resin on reaction rate. PS-TRPGGDA-Ala resins of same functional group capacity was used for the present investigation and a comparative study of the same reaction was carried out with PS-DVB in order to establish the superiority of the new resin as an effective polymer support for the solid phase organic synthesis. The effect of various cross-linking densities of the resin on reaction rate of PS-TRPGGDA resin supported synthesis were also investigated by selecting the resins with 2%, 3%, 4% and 8% cross-linking densities.

\[ \text{Scheme 1: Aminolysis of N-benzoylglycine p-nitro phenyl ester} \]

The reaction is carried out by following the course of aminolysis of N-benzoylglycine 4-nitrophenyl ester in DMF with amino acid incorporated polymer support (Scheme 1). During aminolysis reaction, p-nitrophenol was liberated as by-product. The \( \lambda_{\text{max}} \) of p-nitrophenol was found at 432 and 318 nm in DMF which can be utilised for the analysis of the rate of the reaction.
However, p-nitrophenyl ester of Benzoylglycine also showed a similar peak at 431 nm obviously due to NO\textsubscript{2} chromophore, in addition to a peak at 275 nm. When amine was added to the ester, the intensity of the peak at 275 nm was drastically reduced indicating participation of ester in the amide bond formation. Hence this peak was chosen for monitoring the reaction rate. The variation in the peak intensity gives an idea about the amount of ester consumed during the reaction, and hence the reactivity of resin bound amine (Fig.9). In 2%, 3% and 4% PS-TRPGGDA support, the reaction started instantly when the ester was added (between 3-5 min) and quantitative conversion of the amino groups was achieved in 10 min. In PS-DVB only 69% of reaction was completed even after 16 min of the aminolysis. The slow reaction rate of PS-DVB at the initial stage may be attributed to its slow swelling rate. Swelling of the cross-linker polymer in the appropriate solvent is one of the important factor that determines its efficiency as a polymer support for organic synthesis. The swelling of the resin creates enough space within the polymer and results in the ready access of small molecules present in the solvent to the resin bound
reactive centres. In solvents like DCM, DMF and NMP the PS-TRPGGDA resin network swells to its elastic limits. The amine groups residing inside the matrix were readily available for reaction under this condition. In the case of the new resin the solvent uptake was very fast and swelling characteristics is higher than DVB-PS. The difference in the rate of aminolysis among 2%, 3% and 4% PS-TRPGGDA resin was negligible. Even 8% cross-linked PS-TRPGGDA showed a higher rate of aminolysis compared to PS-DVB resin. These studies revealed that there is a significant influence of the nature and extent of cross-linking on the polymer supported aminolysis reaction. The enhanced reactivity in PS-TRPGGDA support is due to the increased availability of amino groups, which are proximal to the hydrophilic core than to the rigid polystyrene core.

4.2.f Optimization of the PS-TRPGGDA support

All the above studies showed that the swelling and solvation characteristics of the support in a particular solvent govern the reactivity of the resin bound functionality. The swelling studies were carried out on different cross-linking densities of PS-TRPGGDA resin and compared with PS-DVB. PS-TRPGGDA supports with 2%, 3%, 4% and 8% cross-linking densities were used for the swelling studies. The 2% PS-TRPGGDA support showed maximum solvation in wide range of solvents employed in the synthesis. The deviation in the swelling character was found to be marginal for 2%, 3%, and 4% cross-linked resins. Degree of cross-linking controls the swelling and solvation characteristics, diffusion of solvents and reagents in the matrix and the extent of functionalisation since the secondary hydroxyl functionality of the cross-linker is utilised for the C-terminal amino acid incorporation. In the PS-TRPGGDA system negligible variation in the swelling factors of 2%, 3% and 4% system could be attributed to the effect of cross-linker in the system. The effect due to the increased cross-linking density is suppressed by the hydrophilic nature of the cross-linker. In Merrifield system the functional groups are situated close to the hydrophobic core of the cross-linker which
may not be accessible to reagents in continuous phase due to diffusional restrictions (Fig. 10). The kinetic studies also revealed that the difference in the rate of aminolysis among 2%, 3% and 4% PS-TRPGGDA resin was negligible and even 8% cross-linked PS-TRPGGDA showed a higher rate of aminolysis compared to PS-DVB resin. The modelled lipophilic structures of the two different resins also validate the findings of enhanced rate of reaction in PS-TRPGGDA support. The functional group present in the cross-linker in this support moves away from the hydrophobic phenyl rings of polystyrene thus creating a hydrophilic cavity, which results in the enhanced efficiency in peptide synthetic conditions.

![Figure 10: Lipophilic structure of PS-DVB and PS-TRPGGDA resin](image)

Resins with various cross-linking densities ranging from 2-8% PS-TRPGGDA supports were utilised for polypeptide synthesis based on the nature of the sequence to be synthesised and on the requirement of the peptides.

### 4.3 Experimental

#### 4.3.a Materials

Boc-amino acids, Fmoc-amino acids, DCC, 4-(hydroxymethyl)phenoxyacetic acid (HMPA), 2-(1 H-benzotriazol-1-yl) 1, 1, 3, 3- tetramethyl uronium hexafluoro phosphate (HBTU), 1- hydroxy benzoatriazole (HOBr),
1-(2-mesitylenesulfonyl)-3-nitro-1H-1, 2, 4-triazole (MSNT) and Merrifield resin were purchased from Nova Biochem Ltd. UK. 4-Nitrophenyl ester, Benzoylglycine, DIEA, N-methylimidazole, trifluoroacetic acid, ethanedithiol, phenol, thioanisole and piperidine were purchased from Sigma-Aldrich Corp., USA. Solvents (HPLC grade) were purchased from E. Merck (India) and BDH (India).

4.3.b Optimization of C-terminal amino acid incorporation studies

1. Time-dependent C-terminal Boc-amino acid incorporation

PS-TRPGGDA resin (200 mg, 0.02 mmol) was used to study the optimization of C-terminal N"-Boc esterification reaction. A comparative study of the same reaction was also carried out using PS-DVB resin (182 mg, 0.02 mmol). Boc-Val, Boc-Gly, Boc-Ala and Boc-Leu were the amino acids used for the present study. These amino acids were attached to the support as Boc-active ester using MSNT (2 equiv., 0.04 mmol) in presence of MeIm (1.5 equiv., 0.03 mmol). The reaction mixture was kept for coupling in a septum-stoppered flask under nitrogen atmosphere. About 5 mg of the resin was withdrawn from the reaction mixture at every 5 min. interval up to 60 min. The aliquot withdrawn was washed thoroughly with DCM (5 x 6ml), MeOH (5 x 6ml) and ether (5 x 6ml). The Boc-protection of the C-terminal N"-Boc attached resin was removed by treating with 30% TFA in DCM for 30 min. The resin was then filtered and washed with DCM (5 x 6ml) and neutralized with 5% DIEA in DCM (3 x 3ml). The neutralized resin was washed thoroughly with DCM (5 x 6ml), MeOH (5 x 6ml) and ether (5 x 6ml). A definite quantity of absolutely dried resin was swelled in DCM and 0.1 M picric acid was added (2 x 5ml) and kept for 10 min. The unbound picric acid was removed by washing with DCM (5 x 6ml). The bound picrate was then eluted using 5% DIEA in DCM (2 x 3ml) and from the optical density of the elute, at 358 nm the amino capacity and the extent of amino acid incorporation was calculated.
2. **Time-dependent C-terminal Fmoc-amino acid incorporation**

Optimization of time-dependent C-terminal N\textsuperscript{\textalpha}—Fmoc esterification reaction was carried out with PS-TRPGGDA, PS-TRPGGDA-HMPA, PS-DVB-HMPA and PS-DVB resins. Resin (~ 500 mg, weighed to 0.05 mmol) was swelled in dry DCM (distilled over anhydrous P\textsubscript{2}O\textsubscript{5}) in a RB, after 1h the excess DCM was removed. Fmoc-amino acid (2 equiv, 0.1 mmol), MSNT (2 equiv., 0.1 mmol) and N-methyl-imidazole (1.5 equiv, 0.075 mmol) dissolved in dry DCM was added to the preswollen resin. The reaction mixture was kept for coupling in a septum-stoppered flask under nitrogen atmosphere. About 5 mg of the resin was withdrawn from the reaction mixture at every 5 min. interval up to 60 min. The resin was washed with dry DCM (5 x 50 mL) and dried under vacuum. An accurately weighed amount of dried resin (10 mg) was mixed with 3 mL 20% piperidine in DMF for 30 min. The percentage incorporation of amino acid was estimated by measuring the OD of the above solution containing dibenzofulvene-piperidine adducts at 290 nm.

4.3.c **Synthesis of PS-TRPGGDA-Val-(Ala)\textsubscript{4}-Val-Boc and PS-DVB-Val-(Ala)\textsubscript{4}-Val-Boc resins**

PS-TRPGGDA-Val-Boc (250 mg, 0.025 mmol) and PS-DVB-Val-Boc (228 mg, 0.025 mmol) resins were used for the present study. Synthesis was carried out manually in a silanised glass reaction vessel of 25 ml volume. The resin bound C-terminal amino acid was made to swell in DCM (10 ml). HOBr active esters of Boc-amino acids were prepared by the reaction of DCC (18 mg, 0.086 mmol), HOBr (11.6 mg, 0.086 mmol) and respective Boc-amino acid (3.5 equiv., 0.086 mmol) for 1 h at room temperature. The precipitated DCU was filtered off and the active ester solution was added to the pre-swollen resin in DCM. The contents were shaken for 50 min., the resin was filtered and washed with DCM (6 x 10 ml). Each coupling step was monitored by ninhydrin test to ensure the completion of coupling. After incorporating each Boc-amino acid, the resin was treated with 30% TFA in DCM for 30 min to remove the Boc-protection. The resin was filtered and washed with DCM (6 x 10 ml). The deprotected resin was
neutralised by adding 5% DIEA in DCM (3 x 3 ml) for 10 min and then washed with DCM (6 x 10 ml). The coupling, deprotection and washings were repeated until the desired sequence was achieved. Boc-protection of the N-terminal amino acid was not removed.

4.3.d Synthesis of PS-TRPGGDA-HMPA-Val-(Ala)$_4$-Val-Fmoc and PS-DVB-HMPA-Val-(Ala)$_4$-Val-Fmoc resins

PS-TRPGGDA-HMPA-Val-Fmoc (250 mg, 0.025 mmol) and PS-DVB-HMPA-Val-Fmoc (228 mg, 0.025 mmol) resins were used for the present study. C-terminal amino acid attached resins were swelled in DMF in a manual peptide synthesiser. Fmoc-protection was removed using 20% piperidine solution in DMF (25 ml x 20 min), followed by the washing of the resin with DMF (6 x 10 ml). Coupling reactions were carried out using the respective amino acids (3.5 m eq. excess with respect to amino capacity of C-terminal amino acid attached resin) with a mixture of HBTU (3.5 eq), HOBt (3.5 eq) and DIEA (3 eq) in DMF for 40 min. The resin was washed with DMF (6 x 10 ml). The following sequence of operations was carried out for the introduction of each amino acid residue. (a) Washing with DMF (6 x 10 ml), (b) washing with 20% piperidine in DMF (1 x 25 ml), (c) Deprotection with 20% piperidine in DMF (1 x 25 ml x 20 min). (c) Washing with DMF (6 x 10 ml) (d) Acylation was carried out with 3.5 mmol excess of Fmoc-amino acid, HBTU, DIEA and HOBt relative to amino capacity of C-terminal amino acid present in the HMPA resin. After the incorporation of all amino acids, Fmoc-protection of N-terminal amino acid in peptide resin was not removed.

4.3.e Optimization of N\(^\text{a}\)-deprotection studies

1. Time-dependent Boc-deprotection

The time-dependent Boc deprotection was carried out using Boc-Val-PS-TRPGGDA resin and Boc-Val-(Ala)$_4$-Val-PS-TRPGGDA resin. Boc-Val-PS-DVB and Boc-Val-(Ala)$_4$-Val-PS-DVB resin were also used for a Boc-deprotection comparative study. A definite amount of (200 mg) these resins
were treated with 30% TFA in DCM. About 5 mg of the resin was withdrawn from the reaction mixture in every 1 min. interval for 30 min. The Boc deprotected resin was washed with DCM (5 × 10 ml) and neutralized with 5% DIEA in DCM. Neutralized resin was washed with DCM (5 × 10 ml), MeOH (5 × 10 ml) and ether (5 × 10 ml) and dried. An accurately weighed amino acid attached resin was swelled in DCM. To the swelled resin 0.1 M picric acid was added (2 × 5ml) and kept for 10 min. The bound picrate was eluted with 5% DIEA in DCM (2 × 3ml). From the optical density, the amino capacity and the percentage of deprotection was calculated.

2. **Time-dependent Fmoc-deprotection**

Optimization of the time-dependent Fmoc-deprotection was carried out using HMPA attached and underivatised Fmoc-Val-PS-TRPGGDA resin and Fmoc-Val-(Ala)_4-Val-HMPA-PS-TRPGGDA resin. A comparative study with both HMPA attached and underivatised Fmoc-Val-PS-DVB and Fmoc-Val-(Ala)_4-Val-HMPA-PS-DVB resin were also carried out. Fmoc-amino acid attached resin (500 mg) was treated with 20% piperidine in DMF. About 5 mg of resin was withdrawn from the reaction mixture at 1 min. interval for 30 min. The Fmoc-cleaved resin was washed with DMF (5 × 6ml), MeOH (5 × 6ml), ether (5 × 6ml) and dried. Accurately weighed resin was treated with 0.1 M picric acid. The bound picrate was eluted with 5% DIEA in DCM (2 × 3ml). From the optical density of the eluate at 358 nm the amino acid capacity and the percentage of cleavage was calculated. This was further confirmed by measuring the Fmoc-released at regular intervals of accurately weighed resin spectrophotometrically. From the optical density of dibenzofulvene adduct at 290 nm, the amino capacity and the percentage of Fmoc-cleavage was calculated.

4.3.f **Optimization of time and temperature dependent couplings**

The PS-TRPGGDA-HMPA, PS-TRPGGDA, PS-DVB-HMPA, and PS-DVB (~500 mg, 0.05 mmol) resins were used for the Optimization of time and temperature-dependent coupling studies. The coupling study of a model
peptide Leu-Ala-Gly-Val was used for the present study. The Fmoc-Val attached resins were deprotected with 20% piperidine in DMF. The resins were washed with DMF (6 × 20 ml). Each coupling step was carried out using HOBt and HBTU as coupling reagents in presence of DIEA. About 5 mg of the resin was withdrawn from the reaction mixture in every 5 min up to 90 min. Resin was washed with DMF (6 × 20 ml), MeOH (6 × 20 ml), ether (6 × 20 ml) and dried. The Fmoc-content in the resin after regular intervals were measured by monitoring the optical density of the dibenzofulvene adducts formed at 290 nm. From the optical density the percentage of coupling was calculated. Same protocol was adopted for carrying out the reaction at 40 and 50 °C. At elevated temperature, peptides were synthesised manually in a water-jacketed vessel connected to a heated water-bath circulator.

4.3.g Time and temperature dependent cleavage of the peptide

Accurately weighed amounts of Leu-Ala-Gly-Val attached PS-TRPGGDA, PS-DVB and their HMPA attached derivatives were treated separately with TFA (2.85 mL), and water (150 mL) for a time period of 1 h to 24 h at two different temperatures, 30 °C and 40 °C respectively. The percentage yield of the peptide was calculated by noting the weight of peptidyl resin and the amount of peptide obtained.

4.3.h Preparation of N-Benzoylglycine-4-nitrophenyl ester

To a solution of benzoylglycine (1.79 g, 10 mmol) in THF (20 mL), dicyclohexylcarbodiimide (2.06 g, 10 mmol) and 4-nitrophenol (1.39 g, 10 mmol) were added and the reaction mixture was stirred at 0 °C for 60 min. The precipitated dicyclohexylurea was removed by filtration and the solvent evaporated slowly from the filtrate. The residue was washed repeatedly with ethanol to remove free 4-nitrophenol, dissolved in dichloromethane, and precipitated by the addition of petroleum ether.
4.3.1 Aminolysis of N-benzoylglycine-4-nitrophenyl ester by C-terminal amino acid attached polymeric supports

2% PS-TRPGGDA-Ala-NH₂ (21 mg, 0.002 mmol), 3% PS-TRPGGDA-Ala-NH₂ (16.6 mg, 0.002 mmol), 4% PS-TRPGGDA-Ala-NH₂ (11.8 mg, 0.002 mmol), 8% PS-TRPGGDA-Ala-NH₂ (4.8 mg, 0.002 mmol) and 1% PS-DVB Ala-NH₂ (18 mg, 0.002 mmol) resin beads were used for kinetic studies. A 10 mmol excess of N-benzoylglycine-4-nitrophenyl ester (6 mg, 0.02 mmol) in DMF was added to the cuvette containing each of the resin beads. The reaction mixture was gently stirred with a small magnetic bead. The spectrometer was programmed to read optical density at regular intervals (1 min) for 20 min. From the optical density values of N-benzoylglycine-4-nitrophenyl ester measured at 275 nm the percentage of peptide bond formation was calculated.
References


