6. **PEPTIDE SYNTHESIS**

6.1 **Introduction**

Peptide chemistry is currently witnessing tremendous progress in technological and technicological developments. Numerous research possibilities using synthetic peptides in solving biological problems are becoming increasingly recognized. Rapid developments in the biotechnology of new proteins, as well as advances in pharmaceuticals have led to great demands in synthetic peptides. Simultaneous synthesis of completely different peptides, having chain lengths upto 100-150 with 20 amino acids cannot, nowadays, be achieved using solution phase method; though, large scale synthesis of shorter, peptides are in full swing. The solution phase method still is not without problems, the most important difficulty being the low solubility of the growing peptide. The possible racemization at the activated C-Terminal amino acid of the peptide segment in the coupling step and the lack of powerful purification methods are the other problems associated with the synthesis of peptides in solution phase. In spite of these problems, synthesis in solution is one of the major methods of peptide synthesis and continues to find important applications.
One of the most important contemporary methods for the chemical synthesis of peptides is the solid phase peptide synthesis (SPPS) developed by Merrifield.\textsuperscript{106,107} This approach, together with the improvement in chromatographic techniques, particularly the development of HPLC, revolutionised peptide synthesis.\textsuperscript{108,109}

6.2 Solid phase peptide synthesis - An overview

In SPPS, the first amino acid of the required sequence, protected at its \( N_\alpha \) -terminal is attached to a solid polymeric support or resin by a covalent bond. Once the first amino acid is attached, the amino acid sequence of the peptide is built up on the solid support by a series of \( N_\alpha \)-deprotection and amino acid coupling steps. When the desired sequence has been synthesised, the peptide is removed from the resin by acidolysis method affording the crude peptide which is then purified. A schematic representation of the SPPS strategy is given in Scheme 6.1.

The original polymer support used by Merrifield - 2% divinylbenzene crosslinked polystyrene - is still widely used. The polymer functions as a carrier\textsuperscript{110-112} and at the same time acts as a protecting group\textsuperscript{113-115} for the \( N_\alpha \)-protected C-terminal amino acid.
In order to get the final peptide in pure form, modified resins suitable for solid phase synthesis have been developed, nowadays.\textsuperscript{116-121} The important advantages in using the solid phase peptide synthesis are as follows:

i) **Ease of processing:** This is usually the most important consideration since work-up and purification of products is often possible by simple filtrations. The large excess of coupling agent and additive components employed can easily be separated from the
polymer bound peptide by simple filtration. This enables the peptide chemist to avoid the laborious and cumbersome purification of intermediate peptide which are typical of classical solution synthesis.

ii) Ability of the polymer to be recovered, regenerated and reused: In most of the cases the polymeric backbone and the spent material is recovered quantitatively and regenerated without appreciable loss of activity.

iii) Automation: With the success in the standardisation of the steps involved in peptide synthesis the whole process can be automated.\textsuperscript{122,123} This makes the method superior and this is one of the most attractive features of the solid-phase methodology.

The main problem in SPPS, apart from certain sequence-dependent side reactions during couplings\textsuperscript{124} is that of the non-quantitative yields in the amino coupling steps and in the $N_{\alpha}$-deprotection reactions. This leads to small peptides and to terminated peptide sequences all of which are present in the crude mixture after the final cleavage step.\textsuperscript{125,126} Since these undesired peptides may very closely resemble to the target molecule, high resolution separation methods must be used in order to obtain the
required product in a pure form. The inherent problems which limit the yield of peptides are attributed to the polymer effects or by the self-aggregating tendency of the peptides.

To cope with the demand for new strategies and faster syntheses, better coupling reagents and protecting groups have been developed. Methods for the simultaneous preparation and analysis of very large number of peptides in a short time are available, nowadays. Without going into the details of all the above aspects a survey of latest developments in activation/coupling strategy employed in SPPS is presented here.

Polymer-supported mixed carboxylic dithiocarbamic anhydrides were used as acylating reagents for peptide and amide synthesis. The Merryfield’s resin was converted to dithiocarbamate resin and was used for this acylation purpose.

![Sodium Dithiocarbamate resin](attachment://sodium_dithiocarbamate_resin.png)
Polymeric active esters have been used as acylating reagents in peptide synthesis and in the preparation of semisynthetic penicillins.$^{135-137}$ Shambu et al. used polymeric anhydrides as acylating agents for the synthesis of amides and esters extensively.$^{138}$

Matsueda et al. described the synthesis of LHRH, Luteinizing hormone-releasing hormone (Luliberin), by coupling reaction using oxidation reduction method.$^{139,140}$ The segment condensation approach was carried out on a polymeric support using both C to N and N to C elongation methods. In C to N strategy 1.5 fold excess of the protected peptide segment was coupled onto a tetrapeptide bound to a hydroxymethyl resin using a 3 fold excess of triphenyl phosphine and of DPD (2,2'-dipyridyldisulphide) as coupling agent. The same research group synthesized ACTH (Adreno cortico tropin) by N to C strategy using a thirty-fold excess of triphenylphosphine, DPD and of 2-mercaptopyridine as coupling agent.$^{141}$

The early coupling procedures such as azide and oxidation reduction methods were superseded by the introduction of DCC. The DCC/HOBt coupling method has been used extensively for the solid phase coupling of protected
peptides and repetitive sequences like, valinomycin, glutelin-2, apamin etc.\textsuperscript{142-144}

DCC/HOSu (N-hydroxy succinimide) was used in many cases for the synthesis of different polypeptides. Here, 1-5 fold excess of the coupling reagent is used in the synthesis of asymmetrical insulin intermediate.\textsuperscript{145}

The total synthesis of glucagon was carried out using a two to three fold excess of protected amino acid and using either DCC or EEDQ as the coupling agent, in the presence of HOBr.\textsuperscript{146}

Birr used the DCC/HOBt combination for coupling in the synthesis of A and B chains of human insulin, and also investigated the use of CDI (carbonyl diimidazole) in the presence of HOBt for similar coupling reactions.\textsuperscript{147} In general couplings carried out using the CDI/HOBt were complete in shorter reaction times than with DCC/HOBt.\textsuperscript{148}

Coupling reagents based on phosphonium and uronium salts are now increasingly used in SPPS. BOP reagent was used extensively as coupling reagents for peptide synthesis and give results better than DCC.\textsuperscript{60-63}
In the synthesis of calcitonin the combination of BOP, HOBt and DIEA (N,N'-diisopropyl ethylamine) has been used by Chang. The same combination has also been used by Nokihara for solid phase peptide synthesis.

\[
\text{CH}_3-\text{CH}_2-N\left(\text{CH}_{\text{CH}_3}\right)\text{CH}_{\text{CH}_3}
\]

DIEA

Jung has used a combination of TBTU [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate], HOBt and DIEA in order to couple the various protected peptide segments in the synthesis of nucleocapsid protein of the HIV virus.

\[
\text{CH}_3-\text{N}^+\left(\text{CH}_{\text{CH}_3}\right)\text{BF}_4^-
\]

TBTU

Recently, DIC, [N,N'-diisopropylcarbodiimide], an analogue of DCC is used for the effective synthesis of
peptides. Vagner et al. used DIC along with HOBt for the activation purpose on a polymeric support. The same coupling procedure was used by Eichler et al. for the synthesis of corticotropin on a cellular support.

\[
\text{DIC}
\]

A combination of DIC/HOBt or TBTU gave excellent results either in PS-DVB support or cellulose support. PyBOP \([\text{benzotriazo-1-yl-} \text{N-oxytripyrrolidino-phosphonium hexafluorophosphate}]\) along with HOBt is very much used at present for the stepwise synthesis of peptides.

\[
\text{PyBOP}
\]

Geyseren et al. used Pfp (Pentafluorophenyl) ester for the activation and prepared hundreds of peptides using this. Amino functionalised polyethylene 'rods' were employed as support. Another active ester DHBt (3-hydroxy-
4-oxo-3,4-dihydro-1,2,3-benzotriazine was effectively used by Meldal et al. They successfully prepared a number of tetrapeptide using a semi-automatic synthesizer.

A new coupling reagent, tetrabutyl ammonium hydroxide/p-toluene sulphonyl chloride, was used for the recemization free coupling of peptides. This method gave good result comparable with that of DCC/HOBt method.

Very recently, Carpino has been described 1-hydroxy-7-azabenzotriazole (HOAt) as a superior peptide coupling additive which increases the yield in solution about 6-32 times than HOBt. After this tremendous progress a number of coupling additives [eg. AOP - 7-azabenzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluoro phosphate] were prepared starting from HOAt counter to HOBt. A number of peptides have been successfully prepared using this new reagents. The results clearly indicate that these activating reagents are highly superior to their HOBt counter-parts. The enhanced reactivity of the HOAt esters
is proposed to be due to the neighbouring group participation.

\[
\text{OAt} \quad \text{PF}_6^-
\]

\[
\text{Me}_2\text{N} \quad \text{P} \quad \text{N(CH}_3\text{)}_2 \quad \text{Me}_2\text{N}
\]

6.3 Synthesis of peptides

Application of a number of activating and coupling reagents – the key steps in peptide synthesis – has been the subject matter of the above review. Nowadays, the need for better and mild carboxyl activating group has become a challenge towards peptide chemistry. Heterocyclic thiol compounds have been found to be an attractive alternative and the use of 2-mercaptopbenzothiazole (1) and 2-mercaptopbenzoxazole (2) as mild carboxyl activating group in general organic synthesis is discussed in chapter 3 and 4. The ease in which amides and esters are formed from 3-acyl benzo-(thiazoline/oxazoline)-2-thione by the reaction with amines and alcohols respectively, prompted the attention towards the synthesis of peptides.
Thus, the following part in this chapter deals with

i) the derivatization of different N-protected amino acids with 2-mercaptobenzothiazole and also with 2-mercaptobenzoxazol.

ii) characterisation of these N-protected amino acyl benzo(thiazoline/oxazoline)-2-thiones using analytical and spectral methods.

iii) synthesis of small peptides by making use of carboxyl activation strategy.

iv) synthesis of small chain peptide by SPPS method

v) characterisation of synthesised peptides by different analytical and spectral techniques.

6.3.1 Synthesis of 3-[(N-benzoyl)glyclyl] benzothiazoline-2-thione (4f)

Equal molar proportions of 2-mercaptobenzothiazole and hippuric acid (3f) were dissolved in a mixture of THF-methylene chloride (1:4). DCC was added slowly with stirring. The precipitated DCU was filtered off and the
product was separated from the reaction mixture by column chromatography. Recrystalisation from alcohol afforded orange crystals of 3-[(N-benzoyl)glycyl] benzothiazoline-2-thione (4f). Yield: 80%, m.p.: 133 °C; UV $\lambda_{\text{max}}$ (CHCl₃): 295 nm, IR (KBr) spectrum (Fig. 6.1) showed characteristic NH band at 3330 ester carbonyl at 1740, NHCO at 1630 and thiocarbonyl at 1130 cm⁻¹.

Fig. 6.1 IR (KBr) spectrum of 3-[(N-benzoyl)glycyl] benzothiazoline-2-thione
6.3.2 Synthesis of protected model peptides (21a-d)

\[
\begin{align*}
C_6H_5-CO-NH-CH_2-CO-NH-CH_2-COOMe & \quad 21a \\
C_6H_5-CO-NH-CH_2-CO-NH-CH-COOMe & \quad 21b \\
C_6H_5-CO-NH-CH_2-CO-NCH-COOMe & \quad 21c \\
C_6H_5-CO-NH-CH_2-CO-NH-CH_2-COOH & \quad 21d
\end{align*}
\]

A solution containing 3-[(N-benzoyl)glycyl] benzo-thiazoline-2-thione and freshly prepared glycyl methyl ester in chloroform were stirred for half an hour. The disappearance of yellow colour was an indication of the reaction. The course of the reaction was also monitored by tlc. After concentration, the mixture was separated using neutral alumina column and was purified. Z-Gly-Gly-OMe (21a) was formed in 90% yield. m.p. : 181°C. Mixed m.p. of 21a prepared by an independent route did not show any depression. Also the IR spectrum (Fig. 6.2) shows -NH bands at 3446, ester carbonyl at 1747 and amide carbonyl at 1648 and 1635 cm\(^{-1}\). Rf: 0.52.
Repeating the reaction with other amino acids such as alanine methyl ester, proline methyl ester and glycine gave Z-Gly-Ala-OMe (21b), Z-Gly-Pro-OMe (21c) and Z-Gly-Gly (21d) in 80-90% yields. The products formed were characterised by analytical and spectral data and also by comparison with authentic samples. The details of the model dipeptides prepared and their characterisation data are presented in table 6.1.

![Graph: IR (KBr) spectrum of Z-Gly-Gly-OMe](image-url)
Table 6.1. Synthesis of protected model peptides (21b-d)

<table>
<thead>
<tr>
<th>Amino acid used</th>
<th>Solvent</th>
<th>Time of reaction (min)</th>
<th>Peptide</th>
<th>m.p. (°C)</th>
<th>Rf</th>
<th>Yield (%)</th>
<th>IR (KBr cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine methyl ester</td>
<td>CHCl₃</td>
<td>30</td>
<td>Z-Gly-Ala-OMe (21b)</td>
<td>110</td>
<td>0.44</td>
<td>85</td>
<td>3350, 1750, 1650</td>
</tr>
<tr>
<td>Proline methyl ester</td>
<td>CHCl₃</td>
<td>30</td>
<td>Z-Gly-Pro-OMe (21c)</td>
<td>133</td>
<td>0.50</td>
<td>82</td>
<td>3400, 1740, 1670, 1640</td>
</tr>
<tr>
<td>Glycine</td>
<td>Dioxan + water</td>
<td>30</td>
<td>Z-Gly-Gly (21d)</td>
<td>172</td>
<td>0.46</td>
<td>80</td>
<td>3400, 1690, 1640</td>
</tr>
</tbody>
</table>

6.3.3 Synthesis of 3-[(Boc)glycyl] benzothiazoline-2-thione (4g)

\[
\text{Equimolar mixture of 2-mercaptobenzothiazole and Boc-Glycine were dissolved in a mixture of THF-methylene chloride (1:4). DCC in methylene chloride was added in equal quantity with stirring. The DCU was filtered off and}
\]
the product was separated by column chromatography. Orange vaxy material obtained was identified as 3-[(Boc)glycyl] benzothiazoline-2-thione from analytical and spectral techniques. IR (KBr) spectrum (Fig. 6.3) shows -NH bands at 3420, ester carbonyl at 1710, amide carbonyl 1690 and the thiocarbonyl at 1150 cm⁻¹. ¹H NMR (DMSO) spectrum (Fig. 6.4) shows peaks at 6 7.4 (4H,m) -phenyl protons, 5.5 (1H,m) -NH proton, 3.2 (9H,m) -methyl protons and 1.6 (2H,d) -CH₂ protons.

![IR (KBr) spectrum of 3-[(Boc)-glycyl]-benzothiazoline-2-thione](image-url)
Fig. 6.4  $^1$H NMR (90 MHz) spectrum of 3-[((Boc)glycyl]
6.3.4 Synthesis of dipeptide Boc-Gly-Phe-OMe (21e)

\[ \text{(CH}_3\text{)}_3\text{C} \rightarrow \text{O} \rightarrow \text{CO} \rightarrow \text{NH} \rightarrow \text{CH}_2 \rightarrow \text{CO} \rightarrow \text{NH} \rightarrow \text{CHCOO} \rightarrow \text{CH}_3 \]

0.1 Molar 3-[(Boc)-glycyl] benzothiazoline-2-thione was dissolved in chloroform and an equivalent amount of freshly prepared phenyl alanine methyl ester was added to it. Yellow colour of the solution started disappearing. After stirring for half an hour, the mixture was concentrated and separated by column chromatography. The product obtained in 82% yield was identified as Boc-Gly-Phe-OMe. m.p.: 112°C; IR (KBr) spectrum (Fig. 6.5) shows bands at 3400 cm\(^{-1}\) (NH), 1730 cm\(^{-1}\) (ester) and 1660 cm\(^{-1}\) (NH-CO); Rf: 0.36.

![IR spectrum](image)

**Fig. 6.5** IR (KBr) spectrum of Boc-Gly-Phe-OMe
6.3.5 Synthesis of 3-[(N-benzoyl)glycyl] benzoxazoline-2-thione (5f)

Equimolar solutions of 2-mercaptobenzoxazole and hippuric acid (3f) in THF and methylene chloride mixture (1:4) were stirred along with an equivalent amount of DCC in methylene chloride. After stirring for 1 h in an ice bath, the precipitated DCU was filtered off and the mixture was separated using a silica gel column. The formation of the new product was evidenced by tlc. Recrystallisation from alcohol afforded pale brown crystals with m.p. 145 °C in 75% yield. The product was characterised as 3-[(N-benzoyl)glycyl] benzoxazoline-2-thione (5f). IR (KBr) spectrum (Fig. 6.6) shows bands at 3330 (-NH), 1702 (ester C=O), 1647 (NH-C=O) and 1140 cm⁻¹ (thiocarbonyl).

6.3.6 Synthesis of protected peptides

The synthesis of some model protected peptides (21a-d) from N-benzoyl glycine activated by benzothiazoline-2-thione
prompted to carry out the synthesis of some peptides also from 3-[(N-benzoyl)glycyl] benzoxazoline-2-thione (5f). Thus, a solution of 3-[(N-benzoyl)glycyl] benzoxazoline-2-thione (5f) and freshly prepared glycyl methyl ester was stirred for half an hour. The reaction was followed by tlc and the mixture was separated using column chromatography (neutral alumina column) and was purified. White crystals obtained in 85% yield was identified as Z-gly-gly-OMe. m.p.: 180 °C. Mixed m.p. using the compound prepared as...
described in 6.3.2 did not show any depression. IR (KBr) spectrum shows bands at 3440 cm$^{-1}$ (NH), 1740 cm$^{-1}$ (ester) at 1650 cm$^{-1}$ and at 1635 cm$^{-1}$ (NH-CO); Rf = 0.50.

Similar synthesis was also done with different protected amino acids such as alanine methyl ester, proline methyl ester and glycine. The peptides Z-Gly-Ala-OMe, Z-Gly-Pro-OMe and Z-Gly-Gly were formed in 80-90% yield. The products formed were characterised by analytical and spectral data and also by comparison with samples synthesised by the method described in Chapter 6.3.2, the details of which are presented in Table 6.2.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Solvent used</th>
<th>Time of reaction (min)</th>
<th>Peptide</th>
<th>m.p (°C)</th>
<th>Rf</th>
<th>Yield (%)</th>
<th>IR (KBr) cm$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine methyl ester</td>
<td>CHCl$_3$</td>
<td>45</td>
<td>Z-Gly-Ala-OMe (21b)</td>
<td>110</td>
<td>0.45</td>
<td>80</td>
<td>3300, 1780, 1660, 1640</td>
</tr>
<tr>
<td>Proline methyl ester</td>
<td>CHCl$_3$</td>
<td>40</td>
<td>Z-Gly-Pro-OMe (21c)</td>
<td>132</td>
<td>0.50</td>
<td>82</td>
<td>3400, 1750, 1650</td>
</tr>
<tr>
<td>Glycine</td>
<td>Dioxan + water</td>
<td>45</td>
<td>Z-Gly-Gly (21d)</td>
<td>172</td>
<td>0.46</td>
<td>80</td>
<td>3450, 1670, 1640</td>
</tr>
</tbody>
</table>
6.4 Peptide Synthesis using solid phase strategy

Synthesis of different peptides using the solution phase method as described above in 6.3.2 and 6.3.6 affords often, contaminated peptides. To separate peptides in a pure form is very difficult and it involves time consuming column chromatographic techniques. Even then, the product may contain minor quantities of heterocyclic thiol compound since this is a by-product in this reaction. Also to get small chain peptides in the solution phase method according to the previous procedures, as the number of steps increases, the impurities are carried throughout.

In order to avoid these experimental problems, solid phase peptide strategy has been used. Thus, the usual Merrifield resin (1 mmol Cl capacity/g) is used and the amino acid residues are stepwisely attached on to the polymer by the new activation strategy using 2-mercaptobenzothiazole. The finally derived peptide is then cleaved from the resin and purified. A usual protocol for the stepwise attachment is given in Scheme 6.2.
Scheme 6.2. Schematic representation of SPPS. Amino acids are represented by squares and the protected \( \alpha \)-amino groups by diamond. The C-terminal of the peptide is bound to an insoluble solid support designated \( R \) for resin. The carboxyl group of amino acid is activated by thiol designated \( T \).
6.4.1 Synthesis of dipeptide Ala-Gly (22a)

6.4.1(i) Attachment of first amino acid - (Boc-Ala) on the resin

2.5 Mmol of Boc-alanine was dissolved in ethanol. Then it was neutralised with cesium carbonate solution dropwise. Ethanol was evaporated and the residue was dried by co-evaporating with benzene as azeotrope under vacuum for 1 h. White powdery cesium salt of Boc-alanine was formed and dried.

The resin (1 mmol Cl capacity/g) was swelled in DMF. The above powdery cesium salt of Boc-alanine was added and stirred at 50-60 °C for 48 h. Washed with DMF, H₂O, MeOH & DCM and dried under vacuum and polymeric Ala-Boc was formed.

6.4.1(ii) Preparation of activated Boc-Gly (4g)

In a separate vessel 3-[(Boc)glycyl] benzothiazoline-2-thione (4g) was prepared. For this equal molar quantities of Boc-Gly and 3-mercaptobenzothiazole were mixed using DCC. The product was separated and purified.
6.4.1(iii) Synthesis of dipeptide Ala-Gly (22a)

The polymeric Ala-Boc was taken in a peptide synthesizer and was deprotected using 30% TFA. The reaction was done in 30 minutes with vigorous shaking. The Boc-deprotected resin was washed with DCM, then neutralised with 5% TEA and again washings were repeated.

The carboxyl activated glycine was added to the resin in nearly twice the equivalent. The stirring was continued for half an hour. The reaction was monitored by the slow disappearance of the yellow colour of the carboxyl activated glycine 4g. The resin was thoroughly washed with DCM. A second coupling was also carried out to complete the reaction and repeated the washings. A negative ninhydrin test (Kaiser test) was observed which indicated complete conversion. After the incorporation of the amino acid to the peptide resin, it was subjected to cleaving. For this the peptide resin was dipped in 100% TFA in presence of thioanisol for 24 h. The product was analysed using a tlc which gave a single spot and having an Rf value 0.46. The yield of crude peptide was 100% as calculated. m.p. 252 °C. IR (KBr) spectrum shows bands at 3440 cm⁻¹ (NH), 1670 cm⁻¹ (C=O) (Fig. 6.7). The analytical and spectral data were compared with samples prepared by another method.
6.4.2 Synthesis of dipeptide Ala-Ala (22b)

The first amino acid alanine was attached to the polymeric resin by the procedure described already in 6.4.1(i). Activated Boc-Ala (4h) was prepared analogous to the preparation of activated Boc-Gly (6.4.1(ii)).
Then the synthesis of dipeptide was done using the protocol given below. The synthesis consists of the following general operations:

1. Washed the peptide resin with DCM
2. Shaken the resin with 30% TFA for 30 min (Deprotection)
3. Washed with DCM
4. Neutralised with 5% TEA in DCM
5. Washed with DCM
6. Two equivalents of activated Boc-Ala was added and shaken for 30 min (1st coupling)
7. Washed with DCM
8. Activated Boc-Ala was added and shaken for another 30 min (2nd coupling for complete conversion)
9. Washed with DCM
10. Performed Kaiser test - A negative result obtained
11. Dipped in 100% TFA and thioanisol (cleavage)
12. Extracted the crude peptide with ether

The crude peptide Ala-Ala was analysed using tlc which gave a single spot and having an Rf value = 0.52. The yield was calculated as 100%, m.p.: 265 °C, IR (KBr) spectrum (Fig. 6.8) shows bands at 3430 cm⁻¹ (NH) and 1660 cm⁻¹ (C=O). The analytical and spectral data were compared with samples prepared by another method.¹²¹
6.4.3 Synthesis of tripeptide Ala-Ala-Ala (22c)

The attachment of the first resin to the polymer and the preparation of activated Boc-Ala (4h) was done according to the procedures given in 6.4.1(i) and 6.4.1(ii) respectively. Then the synthesis of Ala-Ala-Ala was done using following operations:

1. Washed with peptide resin with DCM
2. Shaken the resin with 30% TFA for 30 min (Deprotection)
3. Washed with DCM
4. Neutralised with 5% TEA in DCM
5. Washed with DCM
6. Two equivalents of activated Boc-Ala was added and shaken for 30 min (1st coupling)
7. Washed with DCM
8. Activated Boc-Ala was added and shaken for another 30 min (IIInd coupling for complete conversion)
9. Washed with DCM
10. Performed Kaiser test - A negative result
11. Repeated steps 2-9 (For the coupling of third amino acid to the resin)
12. Performed Kaiser test - A negative result obtained
13. Dipped in 100% TFA and thioanisol (cleavage)
14. Extracted the crude peptide with ether

Thin layer chromatography of the crude peptide Ala-Ala-Ala gave a single spot, having an Rf value 0.50. m.p.: 270°C. The conversion was found to 100%. IR (KBr) spectrum (Fig. 6.9) shows characteristic bands at 3450 cm⁻¹ (NH), 1680, 1650 cm⁻¹ (C=O); ¹H NMR (DMSO) (Fig. 6.10) gave characteristic signals for -CH protons at δ 4.42 and methyl protons at δ 1.3. The analytical and spectral data of the sample were compared with samples prepared by another method.
Fig. 6.9 IR (KBr) spectrum of Ala-Ala-Ala

6.4.4 Synthesis of Tetrapeptide Ala-Gly-Gly-Gly (22d)

First amino acid attachment to the resin and the preparation of activated Boc-Gly (4g) was done according to the procedures given in 6.4.1(i) and 6.4.1(ii) respectively. Then the further attachment of the amino acid was done using the following protocol.

1. Washed the peptide resin with DCM
2. Shaken the resin with 30% TFA for 30 min (Deprotection)
3. Washed with DCM
4. Neutralised with 5% TEA in DCM
5. Washed with DCM
6. Two equivalents of activated Boc-Gly was added and shaken for 30 min (1st coupling)
7. Washed with DCM
8. Activated Boc-Ala was added and shaken for another 30 min. (IIInd coupling for complete conversion)
9. Washed with DCM
10. Performed Kaiser test - A negative result
11. Repeated steps 2-9 (For the coupling of third amino acid to the resin)
12. Performed Kaiser test - A negative result
13. Repeated steps 2-9 (For the coupling of fourth amino acid)
14. Performed Kaiser test - A negative result obtained
15. Extracted the tetrapeptide with ether

The crude peptide Ala-Gly-Gly-Gly (22d) was analysed using tlc, gave a single spot and having an Rf value = 0.48. m.p. > 270 °C. IR (KBr) spectrum (Fig. 6.11) shows bands at 3450 cm⁻¹ (NH) and carbonyl peaks at 1700, 1660 & 1620 cm⁻¹. The analytical and spectral details were compared with samples prepared by another method.\(^{120}\)
6.5 Materials and methods

The solvents used were of reagent grade and further purified according to literature procedure. Merrifields chloromethylated polystyrene resin (2% DVB cross linked and 1 mmol Cl/g) was purchased from Fluka, Switzerland. Boc-amino acids were purchased from Sigma Chemical Company, U.S.A. TFA, TEA and thioanisol were purchased from E. Merck.

Solid phase peptide syntheses were carried out in a peptide synthesizer. The resin (200 mg) was taken in the
vessel and swelled in the specific solvent (DCM). After deprotection, activated amino acid was added, stirred well manually and after coupling the product was washed repeatedly with DCM. For cleavage, the resin was kept in TFA for 24 h in the vessel itself. After the cleavage step, the peptide which is in the solution was extracted with ether. The resin which left in the synthesizer was recycled.

Capacity of chlorine in chloromethylated support was determined by Volhard's method. Here, accurately weighed resin (100 mg) was fused with pyridine at 100 °C for 4 h. To the pyridinium hydrochloride thus formed, known volume of standard silver nitrate solution was added. The volume of unreacted silver nitrate solution was found out by back-titrating with ammonium thiocyanate solution. From the volume of ammonium thiocyanate, the chlorine capacity was determined.

For analytical tlc, the solvent mixture used was pyridine:water: acetic acid (50:15:30) and the chromatographic paper was sprayed by ninhydrin.
SUMMARY AND OUTLOOK