CHAPTER 3

PEPTIDE SYNTHESIS
Peptides were synthesized by both solution-phase and solid-phase methods.

3.1. Solution phase synthesis:

The following short peptides were obtained by solution phase methods:

1. Boc-Gly-Phe-Phe-Ala-OMe
2. Boc-Leu-Ile-Pro-Lys(Z)-OMe
3. Boc-Ser(Bzl)-Ser(Bzl)-Pro-Leu-Phe-OMe
4. Boc-Lys(Z)-Thr-Leu-OMe
5. Boc-Leu-Ser(Bzl)-Ala-Val-OMe
6. Boc-Gly-Ser(Bzl)-Ala-Leu-OMe

(Note: The side chain protecting groups were Bzl for Ser, and Z for Lys.)

The cycle of operations followed was along the following lines (Lys-Thr-Leu is used as a sample synthesis):

1. Leu was converted to Leu-OMe, as described in chapter 2 (pg. 38). Yields of this reaction were 70-90%. The Leu-OMe was coupled to Boc-Thr immediately, as described below.

2. 1.0-1.2 equivalents of Boc-Thr were dissolved in DCM and cooled on ice, and Leu-OMe dissolved in minimal volumes of DCM, was added. DCC was added in amounts equivalent to Boc-Thr. The reaction mixture was kept on ice for half an hour, after which it was allowed to stir overnight, at room temperature. The white residue of DCU was visible within the first half hour.

3. Most of the DCU was filtered off using a sintered funnel, and the DCM eluate washed with 1N HCl, water and a saturated solution of sodium bicarbonate. The DCM layer was then dried over sodium sulphate and the solvent removed under reduced pressure. The oil so obtained was contaminated with DCU. The mixture was therefore suspended in minimum volume of acetonitrile, which was then filtered through a
cotton plug. The clear acetonitrile solution was evaporated to dryness and the oil triturated with Pet ether. If pure, the resultant solid was stored at room temperature. If not, the peptide was subjected to silica gel chromatography (described below).

4. After purification, the dipeptide was subjected to 85% formic acid treatment overnight, to remove the Boc protecting group. This reaction was invariably complete as judged by TLC. The formic acid was removed by rotavaporating at 70°C. Water was added, and the pH made alkaline with sodium carbonate. Multiple CHCl₃ extractions were performed immediately, the CHCl₃ extract dried over sodium sulphate, and then evaporated to dryness. The dipeptide methyl ester free bases were used immediately for generation of tri- or longer peptides.

5. 1.0-1.2 equivalents of Boc-Lys/Z were dissolved in a small volume of DMF and cooled over ice. Equivalent amounts of HOBt and DCC were added, and then the Thr-Leu-OMe. The reaction was kept on ice for half an hour and then allowed to stir at room temperature overnight. It was ensured that the reaction flask was sealed well.

6. The tripeptide was filtered of its DCU and diluted with water and EtOAc. The organic layer was washed with HCl, water and sodium bicarbonate, as above. The EtOAc layer was dried over sodium sulphate and evaporated to dryness. This was then purified as described below.

7. Tripeptides and longer peptides were always coupled in DMF using HOBt and DCC.

Details regarding individual syntheses are listed in Table 3.1.
(Note: all amino acid analyses are listed in Table 3.4)

Text continued after Table 3.1
Table 3.1: Details regarding individual syntheses
Table 3.1:

**Gly-Phe-Phe-Ala** :

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<tr>
<th>Reaction</th>
<th>Product</th>
<th>Yield</th>
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<tr>
<td>Ala $\rightarrow$ Ala-OMe</td>
<td>(0.85 gm, 8.1 mmol, 54% yield)</td>
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<tr>
<td>Ala-OMe $\rightarrow$ Boc-Phe-Ala-OMe</td>
<td>(solid, 2.2 gm, 6.28 mmol, 77% yield)</td>
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<tr>
<td>Boc-Phe-Ala-OMe $\rightarrow$ Boc-Phe-Phe-Ala-OMe</td>
<td>(solid, 1.28 gm, 3.6 mmol, 60% yield)</td>
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<tr>
<td>Boc-Phe-Phe-Ala-OMe $\rightarrow$ Boc-Gly-Phe-Phe-Ala-OMe</td>
<td>(solid, 0.8 gm, 1.6 mmol, 80% yield)</td>
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**Leu-Ile-Pro-Lys** :

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<tr>
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</thead>
<tbody>
<tr>
<td>Lys/Z $\rightarrow$ Boc-Pro-Lys/Z-OMe</td>
<td>(solid, 4.65 gm, 9.45 mmol, 63% yield)</td>
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<tr>
<td>Boc-Pro-Lys/Z-OMe $\rightarrow$ Boc-Ile-Pro-Lys/Z-OMe</td>
<td>(solid, 4.7 gm, 7.83 mmol, 83% yield)</td>
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<tr>
<td>Boc-Ile-Pro-Lys/Z-OMe $\rightarrow$ Boc-Leu-Ile-Pro-Lys/Z-OMe</td>
<td>(oil, 1.7 gm, 2.37 mmol, 30% yield)</td>
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</table>

... contd.
Ser-Ser-Pro-Leu-Phe :

Phe \[\text{--------->} \] Z-Leu-Phe-OMe  
(solid, 2.17 gm, 5.1 mmol, 51% yield)

Z-Leu-Phe-OMe \[\text{--------->} \] Boc-Pro-Leu-Phe-OMe  
(solid, 0.68 gm, 1.41 mmol, 30% yield)

Boc-Pro-Leu-Phe-OMe \[\text{--------->} \] Boc-Ser/Bzl-Pro-Leu-Phe-OMe  
(solid, 0.6 gm, 1.16 mmol, 83% yield)

Boc-Ser/Bzl-Pro-Leu-Phe-OMe \[\text{--------->} \] Boc-Ser/Bzl-Ser/Bzl-Pro-Leu-Phe-OMe  
(solid, 0.27 gm, 0.32 mmol, 30% yield)

Lys-Thr-Leu :

Leu \[\text{--------->} \] Leu-OMe  
(1.73 gm, 12 mmol, 80% yield)

Leu-OMe \[\text{--------->} \] Boc-Thr-Leu-OMe  
(solid, 2.4 gm, 7.0 mmol, 90%)

Boc-Thr-Leu-OMe \[\text{--------->} \] Boc-Lys/Z-Thr-Leu-OMe  
(solid, 2.15 gm, 3.58 mmol, 40% yield)

... contd.
**Leu-Ser-Ala-Val :**

Val

\[
\text{-----------------------------} \rightarrow \text{Boc-Ala-Val-OMe}
\]

(solid, 1.11 gm, 3.7 mmol, 37% yield)

Boc-Ala-Val-OMe

\[
\text{----------------} \rightarrow \text{Boc-Ser/Bzl-Ala-Val-OMe}
\]

(solid, 1.1 gm, 2.3 mmol, 62% yield)

Boc-Ser/Bzl-Ala-Val-OMe

\[
\text{------} \rightarrow \text{Boc-Leu-Ser/Bzl-Ala-Val-OMe}
\]

(solid, 0.7 gm, 1.16 mmol, 74% yield)

**Gly-Ser-Ala-Leu :**

Leu

\[
\text{-----------------------------} \rightarrow \text{Boc-Ala-Leu-OMe}
\]

(solid, 2.6 gm, 7.3 mmol, 60% yield)

Boc-Ala-Leu-OMe

\[
\text{-----------} \rightarrow \text{Boc-Ser/Bzl-Ala-Leu-OMe}
\]

(solid, 1.7 gm, 3.44 mmol, 47% yield)

Boc-Ser/Bzl-Ala-Leu-OMe

\[
\text{-----} \rightarrow \text{Boc-Gly-Ser/Bzl-Ala-Leu-OMe}
\]

(solid, 0.6 gm, 1.1 mmol, 54% yield)
Segment condensation:
The following peptides were obtained by segment condensation:

1. Boc-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys(OMe) (8P)
2. Boc-Lys(Z)-Thr-Leu-Leu-Ser(Bzl)-Ala-Val-OMe (KTLLSAV)
3. Boc-Leu-Ser(Bzl)-Ala-Val-Gly-Ser(Bzl)-Ala-Leu-OMe (LSAVGSAL)
4. Boc-Lys(Z)-Thr-Leu-Leu-Ser(Bzl)-Ala-Val-Gly-Ser-Ala-Leu-OMe (11P)
5. Boc-Gly-Phe-Ala-Leu-Ile-Pro-Lys(Z)-Ile-Ile-Ser(Bzl)-Ser(Bzl)-Pro-Leu-Phe-OMe (15P)
6. Boc-Ile-Ile-Ser(Bzl)-Ser(Bzl)-Pro-Leu-Phe-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys(Z)-OMe (Rev 15P)
7. Boc-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys(Z)-Ile-Ile-Ser(Bzl)-Ser(Bzl)-Pro-Leu-Phe-Lys(Z)-OMe (16P)
8. Boc-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys(Z)-Ser(Bzl)-Ser(Bzl)-Ser(Bzl)-Gly-Glu-OMe-Gln-Glu-OMe (15Q)

(Note: the underlined fragments were used for the segment condensation)

The steps involved were as follows:
1. The C-terminal peptide was subjected to HCl/THF (fresh): THF (2:1) for a few hours, with continual monitoring by TLC to remove the Boc protecting group. After the reaction was complete, HCl/THF was evaporated under reduced pressure, the residual oil diluted with water, made alkaline with sodium carbonate, and extracted into CHCl₃ immediately. Multiple CHCl₃ extractions were done. The CHCl₃ was dried over sodium sulphate and evaporated once the N-terminal peptide was ready.

2. The N-terminal peptide was saponified to remove the OMe protecting group. The peptide was dissolved in MeOH, and NaOH added to make it pH 10.0. It was then kept at 50°C for a few hours with continual monitoring by TLC. Once the reaction was complete, the reaction mixture was diluted with water and made acidic with 1N HCl. This was rapidly extracted into EtOAc and dried over sodium sulphate.

3. After the two fragments were dried to oils, the N-terminal segment was dissolved in minimal DMF and cooled on ice. Equivalent amounts of HOBt and DCC were added, and after a few minutes the C-terminal fragment was also dissolved in minimal DMF.
4. The reaction flask was tightly sealed and the coupling allowed to take place for a few days. About half way through, another aliquot of DCC was added to try to maximise coupling.

Details of the synthesis of peptides by segment condensation are listed in Table 3.2.

3.2 Solid Phase Synthesis:

The following peptides were synthesised by solid phase methods:

1. Boc-Ile-Ile-Ser(Bzl)-Ser(Bzl)-Pro-Leu-Phe-OMe, (IISSLF)
2. Boc-Ser(Bzl)-Ser(Bzl)-Ser(Bzl)-Gly-Glu(OMe)-Gln-Glu(OMe, OMe), (SSSGEQE)
3. Boc-Lys(Z)-Thr-Leu-Leu-Ser(Bzl)-Ala-Val-Gly-Ser(Bzl)-Ala-Leu-OMe, (11P)
4. Boc-Lys(Z)-Ile-Ile-Ser(Bzl)-Ser(Bzl)-Pro-Leu-Phe-OMe, (K7P)
5. Boc-Ile-Ile-Ser(Bzl)-Ser(Bzl)-Pro-Leu-Phe-Lys(Z)-OMe, (7PK)
6. Gly-Phe-Phe-Ala-Lys-Thr-Leu-Leu-Ser-Ala-Val-Gly-Ser-Ala-Leu, (15T)
7. Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val, (14P)
8. Ac-Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val, (Ac-14P)
9. Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val-Am, (14P-Am)
10. Ac-Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys-The-Leu-Leu-Ser-Ala-Val (Ac-K15P)

The general protocol followed for the solid phase synthesis was as follows:

1. 1.1-2 gm of Merrifield resin was suspended in DCM. The broken, very fine resin particles which failed to float in DCM were discarded.

2. The first Boc-amino acid was incorporated into the resin as described in Chapter 2.
Table 3.2: Details of fragment condensation:
(* indicates that the synthesis was done more than once: representative data is listed)

1. Gly-Phe-Phe-Ala (0.83 gm, 1.5 mmol) + Leu-Ile-Pro-Lys (0.9 gm, 1.25 mmol) -> Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys (solid, 1.0 gm, 0.87 mmol, 70% yield)

2. Lys-Thr-Leu (0.6 gm, 1.0 mmol) + Leu-Ser-Ala-Val (0.47 gm, 0.8 mmol) -> Lys-Thr-Leu-Leu-Ser-Ala-Val (solid, 0.53 gm, 0.53 mmol, 66% yield)

3. Leu-Ser-Ala-Val (0.1 gm, 0.16 mmol) + Gly-Ser-Ala-Leu (0.113 gm, 0.2 mmol) -> Leu-Ser-Ala-Val-Gly-Ser-Ala-Leu (solid, 0.08 gm, 0.08 mmol, 47% yield)

4. Lys (0.045 gm, 0.12 mmol) + Ile-Ile-Ser-Ser-Pro-Leu-Phe (0.097 gm, 0.1 mmol) -> Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe (solid, 0.08 gm, 0.06 mmol, 60% yield)

5. Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys (0.09 gm, 0.09 mmol) + Ile-Ile-Ser-Ser-Pro-Leu-Phe (0.09 gm, 0.09 mmol) -> Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe, 15P (solid, 0.06 gm, 0.03 mmol, 30% yield)

6. Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys (205 mg, 180µmol) + Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys (200 mg, 150 µmol) -> Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys, 16P (solid, 110 mg, 47 µmol, 30% yield)

7. Ile-Ile-Ser-Ser-Pro-Leu-Phe (100 mg, 100 µmol) + Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys (150 mg, 130 µmol) -> Ile-Ile-Ser-Ser-Pro-Leu-Phe-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys, Rev 15P (solid, 30 mg, 15 µmol, 15% yield after FPLC purification)
3. The derivatized resin was swollen in DCM in a reaction vessel. After a couple of washes with DCM it was subjected to 5% TFA/DCM for 5 min. (all steps were performed on the basis of 15 ml solvent per gm resin). This prewash was followed by 30 min. of 30% TFA/DCM followed by six 2 min. washes with DCM.

4. The free amino group of the peptide was then neutralised using a prewash of 5% TEA/DCM for 2 min. followed by a 10 min. wash with 5% DIEA/DCM. This was then washed off with six 2 min. washes of DCM.

5. A three-fold excess of Boc-amino acid was added (in 10 ml DCM per gm resin) and allowed to mix thoroughly with the resin for about 5 min. An equivalent amount of DCC, dissolved in 5 ml DCM, was then added and the reaction allowed to proceed for half an hour. For peptides longer than ten residues, coupling time was extended by 5 min. per residue.

6. The reaction byproduct DCU was washed away with three 2 min. washes of 33% EtOH/DCM followed by three DCM washes. Steps 5 and 6 were repeated.

7. If Boc-Ile was being added, steps 5 and 6 were performed a total of three times before checking for the extent of noncoupling by the Picric acid test (see Chapter 2). If there was a significant percentage of unattached amino groups, these steps were executed up to five times and the resin capped as described below.

8. Capping involved acetic anhydride : benzene : pyridine 1:3:3 for two 10 min. intervals. Six 2 min. washes of DCM were used to wash off the capping reagents.

9. An aliquot of the \textsuperscript{14}P resin and the entire \textsuperscript{K15}P resin was subjected to the capping reaction after acidolytic removal of the Boc group and subsequent neutralisation (see above). This generated acetyl-\textsuperscript{14}P (Ac-\textsuperscript{14}P) and acetyl-\textsuperscript{K15}P (Ac-\textsuperscript{K15}P).

10. Most shorter peptides were cleaved from the resin in the completely protected form, by trans-esterification. \textsuperscript{14}P, Ac-\textsuperscript{14}P, Ac-\textsuperscript{K15}P and \textsuperscript{L5T} were obtained in a completely deprotected condition after cleavage from the resin by the TFA/thioanisole method.

Details of individual solid phase syntheses are listed in Table 3.3.
Table 3.3: Details of syntheses done by solid phase.
(* indicates that the synthesis was done more than once: representative data is listed)

1. Ile-Ile-Ser-Ser-Pro-Leu-Phe (7P) : 1.5 gm resin, 0.42 mmol/gm resin substitution of first residue, 0.444 gm (0.415 mmol) pure peptide (protected) obtained after purification by silica gel chromatography, 66% yield.

2. Ser-Ser-Ser-Gly-Glu-Gln-Glu : 1.5 gm resin, 0.35 mmol/gm resin substitution of first residue, 0.4 gm (0.36 mmol) pure peptide (protected) obtained after purification, 68% yield.

3. Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe (K 7P) : 1.5 gm resin, 0.46 mmol/gm resin substitution, 0.66 gm (0.49 mmol) pure peptide (protected) obtained after purification, 72% yield.

4. Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys (7 PK) : 1.5 gm resin, 0.44 mmol/gm resin substitution, 0.325 gm (protected peptide), 0.244 mmol (from half the resin), 74% yield.

5. Lys-Thr-Leu-Leu-Ser-Ala-Val-Gly-Ser-Ala-Leu (11P) : 0.8 gm resin, 0.55 mmol/gm resin.

6. Ile-Ile-Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val (14P) : 1.5 gm, 0.3 mmol/gm resin substitution. (The 14P resin was the basis for obtaining Ac-14P and Ac-K15P). Yield (for all three deprotected peptides, after FPLC purification) < 10%.

7. Gly-Phe-Phe-Ala-Lys-Thr-Leu-Leu-Ser-Ala-Val-Gly-Ser-Ala-Leu (15T) : 0.8 gm resin, 0.55 mmol resin, yield of deprotected peptide (after FPLC purification) 8.8%.
Table 3.4: Amino acid analysis of peptides synthesized
**Table 3.4**: Amino acid analysis of peptides synthesized

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*: Structure confirmed by $^1$H NMR at 300 MHz

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<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.63</td>
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<td></td>
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<tr>
<td>Lys-Thr-Leu-Leu-Ser-Ala-Val-Gly-Ser-Ala-Leu (11P)</td>
<td>2.18</td>
<td>0.94</td>
<td>3.24</td>
<td>0.99</td>
<td>1.94</td>
<td>0.55</td>
<td>1.00</td>
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<tr>
<th>Peptide</th>
<th>Ala</th>
<th>Gly</th>
<th>Ile</th>
<th>Leu</th>
<th>Lys</th>
<th>Phe</th>
<th>Pro</th>
<th>Ser</th>
<th>Thr</th>
<th>Val</th>
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<tbody>
<tr>
<td>Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe (15P)</td>
<td>0.95</td>
<td>1.18</td>
<td>2.75</td>
<td>2.02</td>
<td>0.91</td>
<td>2.79</td>
<td>2.0</td>
<td>1.34</td>
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<tr>
<td>Ile-Ile-Ser-Ser-Pro-Leu-Phe-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys (Rev 15P)</td>
<td>1.06</td>
<td>1.02</td>
<td>2.46</td>
<td>2.06</td>
<td>1.07</td>
<td>2.90</td>
<td>1.89</td>
<td>0.90</td>
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<tr>
<td>Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys (16P)</td>
<td>1.13</td>
<td>1.09</td>
<td>2.47</td>
<td>2.13</td>
<td>1.88</td>
<td>2.75</td>
<td>1.87</td>
<td>1.18</td>
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<td></td>
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<tr>
<td>Gly-Phe-Phe-Ala-Lys-Thr-Leu-Leu-Ser-Ala-Val-Gly-Ser-Ala-Leu (15T)</td>
<td>2.85</td>
<td>2.16</td>
<td>2.78</td>
<td>1.00</td>
<td>1.78</td>
<td>1.46</td>
<td>0.76</td>
<td>1.0</td>
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<tr>
<td>Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val (14P)</td>
<td>1.02</td>
<td>1.29</td>
<td>2.97</td>
<td>0.90</td>
<td>0.95</td>
<td>0.91</td>
<td>1.57</td>
<td>0.63</td>
<td>1.11</td>
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<tr>
<td>Acetyl-Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val (Ac-14P)</td>
<td>1.03</td>
<td>1.59</td>
<td>2.90</td>
<td>0.93</td>
<td>0.97</td>
<td>0.89</td>
<td>1.20</td>
<td>0.55</td>
<td>1.05</td>
<td></td>
</tr>
<tr>
<td>Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val-Am (14P-Am)**</td>
<td>1.06</td>
<td>0.30</td>
<td>3.03</td>
<td>1.03</td>
<td>0.97</td>
<td>1.15</td>
<td>2.60</td>
<td>0.90</td>
<td>1.06</td>
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... contd.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Ala</th>
<th>Gly</th>
<th>Ile</th>
<th>Leu</th>
<th>Lys</th>
<th>Phe</th>
<th>Pro</th>
<th>Ser</th>
<th>Thr</th>
<th>Val</th>
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<tbody>
<tr>
<td>Acetyl-Lys-Ile-Ile-Ser-</td>
<td>0.96</td>
<td>1.38</td>
<td>3.13</td>
<td>2.0</td>
<td>1.11</td>
<td>0.99</td>
<td>1.94</td>
<td>0.90</td>
<td>1.05</td>
<td></td>
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<tr>
<td>Ser-Pro-Leu-Phe-Lys-Thr-</td>
<td>(1.0)</td>
<td>(2.0)</td>
<td>(3.0)</td>
<td>(2.0)</td>
<td>(1.0)</td>
<td>(1.0)</td>
<td>(3.0)</td>
<td>(1.0)</td>
<td>(1.0)</td>
<td></td>
</tr>
<tr>
<td>Leu-Leu-Ser-Ala-Val (Ac-K15P)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** : Stronger hydrolysis conditions resulted in 1.57 : 1 (Ile : Phe) ratios, confirming a correct Ile content.
3.3 Purification of peptides

3.3.1 Protected peptides:
Peptides obtained in the completely protected form (either by solution phase or by the transesterification method from the resin of a solid phase synthesis) were always purified by flash chromatography. Silica gel of 70-325 or 230-400 mesh was packed as a 30 cm by 2 cm column in a suitable organic solvent or combination of solvents i.e. hexane/chloroform (H/C), chloroform or methanol/chloroform (M/C).

After loading the crude peptide, polarity of the solvent was increased in the following order:

70% H/C, 50% H/C, 30% H/C, 10% H/C, CHCl₃, 1% M/C, 2% M/C 3% M/C etc. i.e. 10-20% jumps in solvent composition were made for hexane/CHCl₃ mixtures, while as low as 0.5-1.0% jumps were necessary for MeOH/CHCl₃ ones.

In each case 120-150 ml of one particular eluting solvent was used, unless a component was found to be eluting, in which case that particular solvent mixture was continued.

For each peptide, optimal TLC running solvent (MeOH/CHCl₃ mixtures) was determined to within 1-2%.

Representative data on three peptides' purification is shown in Table 3.5. These were:

i. all the peptides of one of the short solution phase syntheses;

ii. the eight residue product of a solid phase synthesis;

iii. a fifteen residue peptide obtained by fragment condensation.

No problems of any sort were encountered in any of the peptides so purified, except Protected 11P. This peptide presented solubility problems, when obtained by either solid phase or solution phase methods. Loading a highly diluted solution on a silica gel column resulted in very severe losses, with recovery of the order of 40%. This contrasts with other peptides, where for e.g., 110 mg of pure Protected 7P was recovered from 120 mg slightly impure peptide loaded on the column, or 61mg Protected 15P of the 64 mg slightly impure peptide loaded.
Table 3.5: Representative data on three peptides' purification.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Mesh type</th>
<th>Starting solvent</th>
<th>Peptide elution %</th>
<th>TLC M/C %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boc-Phe-Ala-OMe</td>
<td>70-325</td>
<td>70% H/C</td>
<td>60% H/C</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td>230-400</td>
<td>60% H/C</td>
<td>40% H/C</td>
<td></td>
</tr>
<tr>
<td>Boc-Phe-Phe-Ala-OMe</td>
<td>230-400</td>
<td>50% H/C</td>
<td>30% H/C</td>
<td>5%</td>
</tr>
<tr>
<td>Boc-Gly-Phe-Phe-Ala-OMe</td>
<td>230-400</td>
<td>20% H/C</td>
<td>CHCl₃</td>
<td>5%</td>
</tr>
<tr>
<td>Boc-Lys/Z-Ile-Ile-Ser/Bzl-Ser/Bzl-Pro-Leu-Phe-OMe</td>
<td>230-400</td>
<td>10% H/C</td>
<td>CHCl₃</td>
<td>4%</td>
</tr>
<tr>
<td>Protected _15P</td>
<td>230-400</td>
<td>CHCl₃</td>
<td>2% M/C</td>
<td>7%</td>
</tr>
</tbody>
</table>
Protected Rev 15P was not completely pure after flash chromatography, and was further purified by FPLC in a protected or deprotected condition.

3.3.2 FPLC purification of deprotected peptides:
The above-mentioned four long sequences, 14P, Ac-14P, Ac-K15P, and 15T were cleaved from the resin in a completely deprotected condition, and then purified by FPLC.

Purification conditions included i) Analytical or semi-preparative reverse phase C-18 column (Silica Reverse Phase C18, Pep RPC 5/5 or HR 10/10 from Pharmacia Fine Chemicals AB, Sweden); ii) Solvent A : 0.1% TFA in water; iii) Solvent B : 0.1% TFA in acetonitrile. Detection was at 214nm.

(Note: Certain batches of acetonitrile were suspected to contain impurities, as the baseline rose gradually (or in steps, if a step gradient was used) as %Solvent B was increased. These batches of solvent were not used.)

All the peptides were found to give several peaks (often of similar intensity), when the crude was loaded onto the column. The use of very low percentage Solvent B, and very shallow gradients was found, however, to resolve the different peaks extremely well. These 'baseline separated' peaks were found to give close to integer value amino acid analysis values for each residue, unlike the non-'baseline separated' peaks. Given in Table 3.2 are some details of each of the syntheses. FPLC traces of some of the purifications are shown in Figs. 3.1 - 3.7.

Dotted line on each trace indicates gradient.

Text continued after Fig. 3.7
Fig. 3.1: FPLC of a 'crude' peptide. A very large unresolved area at the base of the sharp peaks is seen.
Fig. 3.2: The improved profile of a slightly lower percentage Sovent B. The peaks are better resolved, and the unresolved area beneath each peak, reduced.
Fig 3.3 : Preliminary baseline separation, to identify the peak of interest.
Fig 3.4: An optimized flattened gradient to collect the peak of interest. Note the bunched up peaks (not of interest) in the early part of the run, as also those at the end of the run. In other runs, the time of 100% solvent B was extended to ensure complete elution of the later peaks.
Fig 3.5: 15T, collected by baseline separation, reinjected to check purity.
Fig. 3.6: FPLC profile of reinjected 14P-Am after purification.
Fig. 3.7: 16P-OMe, obtained after silica gel chromatography of Pr16P, had this profile.

A 60 ml (230-400 mesh) column was loaded with ~200 mg of partially purified Pr16P. TLC indicated that the spot of interest had two closely moving impurities. 8.0 litres of solvent were utilized to purify the peptide. 90 mg peptide was obtained, which, on TFA-deprotection, has the profile shown above. This peptide was used without further purification.
3.3.3 **Problems encountered in the purification of 15P-OMe:**

15P was synthesized by solution phase methods and purified by silica gel chromatography (details in Table 3.5). TLC in different percentages of methanol yielded a single discreet spot, with no other closely moving spot or haze. Several batches of synthesis yielded this single spot with a similar Rf, and without any detectable impurity. Yet, different batches of protected 15P (Pr 15P) gave two major peaks on FPLC, as shown in Fig. 3.8. The amino acid analysis of both the peaks was similar:

- **Peak 1**: Ala 1.17 (1.0), Gly 1.07 (1.0), Ile 2.53 (3.0), Leu 2.24 (2.0), Lys 0.86 (1.0), Phe 3.0 (3.0), Ser 1.76 (2.0). (The value of Pro was not calculated)
- **Peak 2**: Ala 1.13 (1.0), Gly 1.13 (1.0), Ile 2.37 (3.0), Leu 2.05 (2.0), Lys 1.13 (1.0), Phe 3.0 (3.0), Ser 1.7 (2.0). (The value of Pro was not calculated)

The two peaks were collected separately and deprotected using the TFA-procedure (see below). The profile of the two peptides after deprotection was very different, with peak 1 eluting much earlier than peak 2. Peak 1 was resolved into three major peaks in the deprotected condition, with the amino acid analysis of one peak corresponding to 8P, and the other to 7P. Thus, the almost equivalent amounts of unreacted Boc-GFFALIPK(Z)OH- and Bzl(Bzl)S(Bzl)PLF-OMe, used in the fragment condensation to generate 15P, coeluted from the C8 or C-18 FPLC columns. Peak 2 gave one major peak. Amino acid analysis and sequencing confirmed this as 15P (See Fig. 3.9 for FPLC profile).

In contrast to Pr 15P, the closely related Pr 16P gave a single peak on FPLC. TFA-deprotected 16P-OMe also gave a single peak on the FPLC. In view of the behaviour of Pr 15P, it is advisable to check the FPLC profile of the TFA-deprotected peptide to confirm purity.

Text continued after Fig. 3.9
Fig. 3.8: FPLC of Pr15P. Peaks 1 and 2 had very similar amino acid analysis.
Fig. 3.2: 15P-OMe, collected by FPLC, reinjected to give this profile.
3.4. Generation of charge variants of peptides:

In order to study the effect of charges, positive and negative, at different positions of the 14-16 residue peptides, 16P, obtained in a completely protected form, was selectively deprotected in different ways, as described below.

1. The de-Boc reaction:
The Boc group of a completely protected peptide was removed by treatment with 85% formic acid overnight. The formic acid was then evaporated at 70°C and pumped under very strong vacuum to remove all traces of acid.

It was confirmed that deBoc 16P has a lower retention time on FPLC than Pr 16P. This procedure is used routinely in solution phase synthesis to prepare the C-terminal fragment.

2. The Saponification (or OH-) reaction:
2-3 mg of a completely protected peptide was dissolved in MeOH (~4ml) at 50°C and ~60µl 0.4N sodium hydroxide was added. The reaction was incubated at this temperature for 2 hours. K7P was used as a test peptide, and completion of the reaction was ensured by FPLC, with a lesser retention of K7P OH- than Pr K7P. The medium was made acidic with 2N HCl and quickly extracted into EtOAc several times. The EtOAc was dried over sodium sulphate and evaporated.

This procedure is also used during segment condensation reactions of solution phase synthesis.

3. The Transhydrogenation (or de Z) reaction:
2-3 mg of completely protected peptide was dissolved in 5% of 98% formic acid/MeOH, and Palladium Black added. FPLC was used to monitor the reaction. Disappearance of the Pr K7P peak (and appearance of the dZ-K7P peak) was taken as end-point. The suspension was stirred for about 4 hours. The catalyst was removed by centrifugation, and the solvent dried using the Savant speed vac, since the high temperatures necessary to evaporate the formic acid using rotavaporation would cause the Boc group to be removed. The ongoing de Z reaction is depicted in Fig. 3.10.

4. The TFA - deprotection reaction:
The peptide was dissolved in TFA : thioanisole : m-cresol (5 : 0.44 : 0.44) and kept at 40°C for 12 hours. The acid was evaporated, the tube cooled on ice, and cold ether added to
Fig. 3.10: Ongoing hydrogenation of Pr K7P. Peak 1 is non-peptidyl, as determined by amino acid analysis. It is probably related to the cleaved side chain protecting group. Peak 2 is hydrogenated K7P, and peak 3 is unreacted Pr K7P.
precipitate the peptide. Several ether washes were done to rid the peptide of the deprotecting reagents. Although the methyl ester group is still intact, all the other protecting groups are removed by this method. It was confirmed that the FPLC profile of deprotected peptide so obtained did not change with another cycle of deprotection.

Taking accurately weighed samples of the protected peptide for deprotection and/or quantitating a methanolic solution of the deprotected peptide (using the amino acid analyser, see Chapter 2), indicates that yields of this reaction may be only 50-60%. The losses are probably due to incomplete precipitation by ether.

Pr 16P was subjected to these reactions in 1-3 mg. amounts, and the following peptides obtained:

Pr 16P -------> +16P(++)-
16P-OMe
deZ 16P
de Boc 16P
16P OH^-