CHAPTER 2

MATERIALS

and

EXPERIMENTAL METHODS
2.1 Source of chemicals:

**Sigma Chemical Company, USA**: All L-amino acids, Boc-ON, t-butyl carbazate, dicyclohexylcarbodiimide, diisopropylethyl amine, and hydroxybenzotriazole.

**Fluka AG, Switzerland**: benzyloxy carbonyl chloride, cesium carbonate, thioanisole.

**Merck, India**: acetonitrile, dichloromethane, N-N-dimethylformamide, dimethyl sulfoxide, glacial acetic acid, methanol, silica gel 60 (230-400 mesh).

**Peninsula Labs, Inc. USA**: Boc-Lys-Z-CI, Ser-OBzI.

Glaxo, Loba Chemie, Qualigens fine chemicals, SD fine chemicals, Sisco Research Labs and Spectrochem Pvt Ltd provided the following:

Acetone, acetonitrile (UV spectroscopy grade), CHCl₃, diethyl ether, hexane, m-cresol, methanol, methanol (UV spectroscopy grade) ninhydrin, petroleum ether AR (40-60°C), phosphorous pentoxide, silica gel G for TLC (incorporating 13% calcium sulfate as binder), tetrahydrofuran and trifluoroacetic acid.

2.2 Purification of solvents:

1. Dry benzene was obtained by storing benzene over metallic sodium.
2. DIEA and TEA were each distilled over ninhydrin.
3. Ethanol was twice distilled.
4. Absolute MeOH was prepared by refluxing MeOH over acid washed-magnesium wire and iodine crystals for an hour and then distilling.
5. THF was refluxed over metallic sodium for one hour and distilled.
6. TFA was distilled.
7. Pyridine was first distilled over phosphorous pentoxide and then over ninhydrin before use.

All other solvents were used as supplied without further purification.
2.3 Preparation of amino acid derivatives:

**Boc-azide** (Carpino et al, 1959):
30 gm t-butyl carbazate was dissolved in 27 ml glacial AcOH and 27.5 ml water. It was cooled to 0°C with constant stirring and 17.4 gm sodium nitrite added slowly, over 15 min. This was stirred for 90 min and the oily layer formed separated, using a separating funnel. The aqueous layer was washed with ether and the ether wash added to the previous oily layer. This layer was now washed with water, sodium bicarbonate (saturated solution) and water again, about 5-7 times each, till the aqueous layer was clear. The oily layer was dried over sodium sulphate and the residual ether evaporated under reduced pressure. The golden yellow liquid so obtained was stored at 4°C.

yield : 90%

**Boc amino acids**:
Side-chain protected amino acids (OBzl/Ser and OBzl/Glu) were derivatized with the t-butyloxy carbonyl group by the DMSO method.

**DMSO method** (Stewart and Young, 1984):
10 mmol of the given amino acid was dissolved in 20 ml DMSO, 3ml TEA and 2 ml Boc-N3. The solution was stirred for 24 hours. 25 ml of water was then added to effect phase separation. This was twice washed with ether. The ether wash was reextracted with 1% sodium carbonate, and the sodium carbonate extract added to the previous aqueous phase. The pH of the mixed aqueous phases was lowered to 2.0 rapidly with 6N HCl, and sodium chloride added to supersaturation. Ether was used for multiple extractions. This ether extract was dried over sodium sulphate and then evaporated under reduced pressure. The residual oil was weighed and the TLC checked to ensure Boc amino acid purity (any residual unprotected amino acid was washed off by resuspending the oil in ether and washing with very dilute HCl).

All other amino acids were Boc-protected according to Schnabel's procedure:

**Schnabel's method** (Schnabel, 1967):
10 mmol of amino acid was suspended in 1:1 dioxane-water (10 ml). 1.55 ml Boc-N3 was added and enough of 4 N NaOH to raise the pH to 9.0. The solution was stirred for 24 hrs, maintaining pH 9.0. 25 ml water was added to the solution and a couple of ether washes done. The aqueous layer was acidified to pH 2.0 with 6N HCl and then extracted with
EtOAc several times (in case of Boc-Leu and Boc-Lys/Z, ether was used for the extraction) The EtOAc was dried over sodium sulphate and then rotavaporated, and the residual oil triturated with Pet ether in the cases of Boc-Gln, -Gly, -Leu, -Ala, and -Pro. The resulting solids were stored at room temperature. The following protected derivatives were however stored as dicholoromethane solutions at 0°C: Boc-Ile, -Val, -Thr, -Phe and -Lys/Z.

All Boc- amino acids were prepared with over 80% yield.

A more rapid procedure used to obtain Boc-amino acids was the following:

**Boc ON method** (Itoh et al, 1975):

10 mmol of amino acid was added to 11 mmol Boc ON, 11 mmol TEA, and 12 ml dioxane-water (1:1) and stirred for 12 hours at room temperature. The reaction mixture was diluted with 25 ml water, and EtOAc washes done twice. The aqueous layer was acidified with 6N HCl and extracted with EtOAc rapidly a few times. As above, the EtOAc extract was dried over sodium sulphate and rotavaporated.

**Z/Lysine** (Greenstein and Winitz, 1961):

2.6 gm (20 mmol) of lysine was dissolved in minimum volume of water. 3.6 gm (30 mmol) of copper carbonate was suspended in 100 ml of water and heated. The two solutions were mixed and the resultant copper complex of lysine boiled for half an hour and filtered. Over 30 min, 7.8 ml (23 mmol) of benzyl oxycarbonyl chloride (50% in toluene) was added, using 4N NaOH to keep the pH 8-9. The suspension was stirred overnight and then filtered. The residue was washed thoroughly with water, MeOH and ether. The resultant dry solid was dissolved in warm 2N HCl and a saturated solution of EDTA (8.9 gm, 24 mmol) added to it. NaOH was added to neutralize the Z/Lys solution. After completing the precipitation by storing at 0°C awhile, the precipitate was washed with water, MeOH and then ether. If any trace of blue remained in the solid thus obtained, the EDTA treatment was repeated.

Yield: 16.4 mmol, 82% .

**Amino acid methyl esters**:

The C-terminally protected amino acid methyl esters were prepared by the thionyl chloride-methanol procedure (Brenner and Huber, 1953).
15 ml of absolute MeOH was taken in a round bottom flask and cooled in a -20°C mixture of NaCl-ice. Over 5 min, 2ml of thionyl chloride was added. 10 mmol of preweighed amino acid was added to this flask. After half an hour the mixture was allowed to come to room temperature and thereafter kept overnight as such.

The methanol and acid fumes were evaporated under reduced pressure and about 25 ml water added. Immediately, sodium carbonate was added to raise the pH, and the C-terminally protected amino acid extracted with several CHCl₃ washings. The combined CHCl₃ extract was dried over sodium sulphate and rotavaporated to remove all solvent. The amino acid methyl ester oil was coupled to a Boc-amino acid immediately.

**Palladium black** (Greenstein and Winitz, 1961):
100 mg of palladium chloride was suspended in 2 ml concentrated HCl and 18 ml water. This was heated in a boiling water bath and 5 drops of 98% formic acid added. 5N KOH was then added to ensure pH 10.0. A few drops of formic acid were then added rapidly to neutralise the solution. The resulting suspension of black particles was then washed with distilled water several times and kept under water till use. Since Palladium Black is pyrophoric it was never allowed to dry. It was used almost immediately.

**2.4 General procedures used in solid phase peptide synthesis:**

**Purity of Boc amino acids** (Stewart and Young, 1984):
This was checked by TLC using either the solvent system CHCl₃ : AcOH (95:5) in most cases, or CHCl₃ : MeOH : AcOH (85:10:5) in the case of Boc-Gln.

**Incorporation of the first Boc amino acid into the resin**:
This was accomplished by the cesium salt method of Gisin (Gisin, 1973).

Two mmol of the Boc amino acid was dissolved in 10 ml EtOH and 2 ml water in a round bottom flask. A cesium carbonate solution (800 mg in 1 ml) was added till pH 8.0 was achieved. The EtOH was evaporated and benzene added to form an azeotrope with water. This was evaporated and the benzene treatment repeated several times till a dry solid obtained.

The cesium salt of the Boc amino acid was dissolved in 10 ml of DMF (stored for several days over 4A molecular sieves) and added to 1 gm. Merrifield resin (chloromethylated polystyrene co-1% divinyl benzene, 200-400 mesh, 1.03 meq/gm, Sigma, USA). The
suspension was gently stirred for 48 hrs at 50°C in an oil bath. Thereafter, it was filtered using a G-2 funnel and washed with DMF, DMF-water, MeOH, and DCM very thoroughly. After drying under mild vacuum, the derivatised resin was weighed and the increase in weight noted. This value was, however, not found to be as accurate an estimation of the degree of incorporation of the first amino acid as the picric acid method given below.

Picric acid estimation (Gisin, 1972):
An aliquot of ~4 mg deprotected or protected resin was taken in a 5 ml sintered funnel. If the resin was protected, it was subjected to 30% TFA/DCM for half an hour, washed thoroughly with DCM, neutralised with 5% DIEA/DCM, and again washed thoroughly with DCM. (Thereafter, the two resins were treated identically). The resin was treated with 0.1 M picric acid/DCM for 5 min and all unbound picrate was washed off with multiple DCM washings. The stoichiometrically bound picrate was then eluted with 5% DIEA/DCM, till the eluate was clear. 0.2 ml of the eluate was diluted to 2 ml with 95% EtOH/DCM and the OD358 determined. $\varepsilon_{358}$ for picrate is 14,500, and the degree of substitution was calculated. This was found to be within 0.1 mmol of the substitution, as calculated from peptide (crude) recovery after the synthesis.

Cleavage of peptide from the resin:
The transesterification method was used to obtain peptides (~8 residues in length) in a completely protected form. The TFA/thioanisole method was, however, used where the transesterification method gave unsatisfactory yields, as in the case of the longer (~14 residue) peptides.

Transesterification method (Stewart and Young, 1984):
0.5 gm resin was suspended in 30 ml MeOH and 3 ml freshly distilled TEA and gently stirred overnight in an oil bath set at 40°C. The resin was allowed to settle and the MeOH supernatant decanted and rotavaporated. The white peptide residue was washed with ether to yield a solid. After the ether was filtered off, the dry powder was stored at room temperature. (The resin was subjected to this entire procedure two to three times to maximize peptide recovery.)

If MeOH failed to effect good recovery of the peptide, the peptidyl resin was suspended in 2 : 2 : 1 DMF : MeOH : TEA and the rest of the procedure followed as above. DMF was removed using the Savant speed vac.
If the DMF based transesterification procedure also failed to cause adequate recovery of the peptide, then the TFA/thioanisole method was resorted to.

**TFA/thioanisole method** (Bodanszky and Bodanszky, 1984):
0.5 gm peptidyl resin was suspended in 10 ml TFA : 1.1 ml thioanisole : 1.1 ml m-cresol and gently stirred in an oil bath at 40°C overnight. The TFA was filtered using a 5 ml sintered funnel and then rotavaporated. The peptide remained dissolved in the thioanisole-m-cresol mixture and was cooled on ice. Cold ether was added to make up to 15 ml. This was centrifuged at 2,000 rpm and the ether decanted. Fresh ether was added to resuspend the peptide. Washing was done 4-6 times to ensure a white powder devoid of any contaminating deprotecting reagents. The peptide was then stored at room temperature. The same aliquot of resin was subjected to another cycle of deprotection, to optimise recovery of the peptide.

The peptide so obtained lacks the Boc-, Z- and OBzI- protecting groups. If, however, a peptide has been obtained by the transesterification method, in a completely protected form, and subsequently subjected to this procedure of deprotection, then the C terminal OMe protecting group is still intact.

**Visualization of peptide by TLC:**

**Ninhydrin spray:**
A 1% solution of ninhydrin in acetone was sprayed onto the TLC plate (in case of protected peptides the plate was first exposed to HCl gas for a few minutes and then heated at 100°C to evaporate all traces of the acid fumes). The plate was then heated at 100°C for a few minutes till purple spots (yellow for N terminal proline) appeared.

**Starch KI test** (Rydon and Smith, 1952):
For peptides longer than tripeptides, this method was preferred. After running the TLC plate (in varying percentages of MeOH/CHCl3, see Chapter 3) and drying it at 100°C, the plate was exposed to chlorine gas (the chlorine chamber was prepared by adding 2N HCl to potassium permanganate just before use). After 3 min., the plate was heated at 100°C for 5 min. precisely. It was then sprayed with a freshly prepared 1% starch-1% KI solution (made in distilled water). Discreet purple spots appeared which, however, faded over a few hours.
Amino acid analysis:

Amino acid analysis was used for characterization of the peptides, as also for quantitation of peptide stock solutions.

Peptide (0.1-1.0 mg) was weighed accurately and transferred to a hydrolysis tube. 200 μl of TFA : 12N HCl (1:1) was added and the tube sealed under vacuum. It was then kept at ~110°C overnight. The tube was broken open, and the acid dried over NaOH and phosphorous pentoxide in a dessicator under strong vacuum. The amino acid mixture was dissolved in amino acid - analyser loading buffer and an aliquot loaded in the analyser capsule such that every amino acid was expected in the 3-10 nmol range.

For quantitative analysis, an aliquot of peptide stock solution in MeOH was transferred to a hydrolysis tube. A known amount of nor-Leu was added and hydrolysis carried out as above. A known amount of nor-Leu was included in the standards being run on the analyser that day, and from the recovery of the nor-Leu in the sample, suitable calculation of peptide concentration was done. Nor-Leu recovery was always more than 80%.

The above hydrolysis conditions lead to losses of serine and threonine, and for better estimation of these two residues, 6N HCl for 24 hrs at 105°C was preferred. It has also been found that 1:1 3N HCl : TFA, 12 hours at 110°C, while giving complete recovery of serine and threonine, gave as good recovery for the hydrophobic residues (e.g., isoleucine and leucine).

Differences were observed in the amino acid analysis of samples taken from MeOH stocks and those taken from DMSO stocks (the ~10 μl stock was not evaporated before hydrolysis in the latter solvent). The 15T peptide gave solubility problems in MeOH, and the stock solution was therefore made up in DMSO. The amino acid analysis obtained was as follows (values in brackets indicate the expected values):

Thr 0.83 (1.0) Ser 0.61 (2.0) Gly 2.08 (2.0) Ala 2.4 (3.0) Val 1.01 (1.0) Leu 2.2 (3.0) Phe 0.48 (2.0) Lys 0.9 (1.0)

The same sample, after evaporating the DMSO (Savant Speed Vac) and dissolving in MeOH, gave the following analysis:

Thr 0.76 (1.0) Ser 1.46 (2.0) Gly 2.16 (2.0) Ala 2.85 (3.0) Val 1.0 (1.0) Leu 2.78 (3.0) Phe 1.78 (2.0) Lys 1.0 (1.0)
This very low recovery of several residues was observed for DMSO stocks of L5T more than once, although the peptide's FPLC profile was identical to that of the MeOH stock's.

Amino acid analysis standards were compared for reproducibility. On days on which several runs had been made, the values of a given amino acid for a later standard were divided by the values for the first standard. The results obtained are shown in Table 2.1. These standards were obtained for good-to-excellent baselines. As can be seen, most amino acids deviate from each other by up to ~6%. However, particular amino acids can, at random, deviate much more. Since the standards are run by loading a commercially available premixture of amino acids, the observed difference is unlikely to be due to loading errors. Instead, slight differences in elution from the analyser column, and/or integration errors may be the cause for the deviation.

Although it is advisable to check the day's standard with another day's, before using it as a reference, no correction for this deviation has been applied in the amino acid analyses reported (see chapter 3).

It is observed that the amino acid analysis of residues of 'pure' peptides (single peak on FPLC, baseline separated from the others, see chapter 3), excluding isoleucine, serine and threonine, are +/- 0.20 an integral value. This is in spite of the fact that other peaks are clearly missing one or more residues in the case of a peptide obtained by solid phase synthesis. Likewise, for a solution phase synthesis, if the peptide is examined from the view point of the fragments that it was synthesized from, it is clear that the deviation from integer values of a given amino acid are not correlated with the synthetic strategy. This variation is higher than that obtained by some other workers (Houghton et al, 1984). The discrepancies between standards, described above, would contribute to this variation.

It is also possible, though this has not been demonstrated, that the C-18 column matrix retains small fractions of different peptides which co-elute with any given peak. Thus, the difference in amino acid analysis is not that of one or more residues missing, but very small fractions of different residues varying from an integer value. The peptides may be even purer than the already satisfactory analyses would indicate.

The amino acid analysis was done on an LKB 4151 ALPHA PLUS amino acid analyser.
## Table 2.1: Comparison of amino acid analysis standards:

<table>
<thead>
<tr>
<th></th>
<th>Run 16 / Run 2</th>
<th>Run 9 / Run 2</th>
<th>Run 8 / Run 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr</td>
<td>0.94</td>
<td>0.83</td>
<td>0.81 (17% l)</td>
</tr>
<tr>
<td>Ser</td>
<td>0.81 (16% l)</td>
<td>0.87</td>
<td>0.99</td>
</tr>
<tr>
<td>Gly</td>
<td>0.95</td>
<td>0.65 (22% l)</td>
<td>0.86 (12% l)</td>
</tr>
<tr>
<td>Ala</td>
<td>1.04</td>
<td>0.76 (9% l)</td>
<td>1.01</td>
</tr>
<tr>
<td>Val</td>
<td>1.01</td>
<td>0.80</td>
<td>0.99</td>
</tr>
<tr>
<td>Ile</td>
<td>0.91</td>
<td>0.80</td>
<td>0.95</td>
</tr>
<tr>
<td>Leu</td>
<td>1.01</td>
<td>0.87</td>
<td>1.0</td>
</tr>
<tr>
<td>Phe</td>
<td>1.01</td>
<td>0.96 (15% h)</td>
<td>0.98</td>
</tr>
<tr>
<td>Lys</td>
<td>0.96</td>
<td>0.82</td>
<td>1.16 (18% h)</td>
</tr>
</tbody>
</table>

+/- 6.5%  
+/- 4%  
+/- 3%

(l = lower, h = higher)
2.5 Purification of lipids:

Purification of phosphatidyl choline from egg (modification of Singleton et al., 1965):
PC was purified by column chromatography using neutral alumina, as follows:
A 60 ml bed volume column was packed with neutral alumina in 4% MeOH/CHCl₃. Crude Sigma egg PC was loaded and a 100 ml wash of 4% M/C done. 200 ml 30% M/C was then used to elute pure PC. Purity of the fractions collected during purification, as also of the final lipid solution, was checked by running the TLC in CHCl₃ : MeOH : H₂O, 35:25:8. The lipid was stored in dilute CHCl₃ at -20°C, and was found to be stable for several weeks.

Purification of phosphatidyl ethanolamine and phosphatidyl serine from bovine brain:
Freshly obtained bovine brain was largely denuded of meninges and white matter, and then pulverized in CHCl₃ : MeOH 2:1. The lower, chloroform-rich phase was separated and washed extensively with 1% NaCl. The organic layer was then dried over sodium sulphate and evaporated. It was stored at -70°C as a CH₃Cl solution till further purification.

Pure PE and PS were obtained as follows:

230-400 mesh silica gel was prewashed with 1 volume MeOH, 3 vol. CHCl₃, 3 vol. CHCl₃ : MeOH 1:1 and 1 vol. MeOH, before being activated at 100°C overnight.

The silica gel was then packed as a 60 ml bed volume column and prewashed with three bed volumes of 5% MeOH/CHCl₃. After loading the lipid, three bed volumes each of 20% and 30% MeOH/CHCl₃ were used to elute the lipid. Phosphatidyl ethanolamine is obtained in these washes of the column, with 30% MeOH/CHCl₃ washing continuing till all PE is eluted. Further washing is continued with 40% MeOH/CHCl₃, and pure PS is obtained before PS contaminated with lyso-PS elutes from the column. PE and PS were stored as separate CHCl₃ solutions at -20°C. Whereas PE could be stored for several weeks, PS was stable for only a week, when stored as stated above. TLC using the solvent system CHCl₃ : MeOH : AcOH : water (65 : 15 : 10 : 4), was used to check the purity of the eluting lipid, as also of the lipid stock prior to use.
Quantitation of phospholipids (Stewart, 1980):

A 0.1N ferric thiocyanate solution was prepared by mixing 27 g/L ferric chloride hexahydrate and 30 g/L ammonium thiocyanate. 2 ml of the ferric thiocyanate solution was mixed with 2 ml of lipid-in-chloroform (in triplicate). After 1 min. vortexing, the lower chloroform layer was transferred to another test tube, ensuring that no ferric thiocyanate was transferred. The OD488, OD470 and OD452 were checked for PC, PE and PS respectively.

Concentration of the lipid was determined from previously obtained standard plots of the different lipids.