CHAPTER - 2

DESIGN AND SYNTHESIS OF NOVEL HEXAPEPTIDE ANALOGUES RELATED TO IgE Fc FRAGMENT (330-334)
2. INTRODUCTION

Mast cell remain one of the most enigmatic cell in the body and secrete significant amount of numerous proinflammatory mediators which contribute to a number of chronic inflammatory conditions. But there are sufficient reason to implicate mast cell in the pathogenesis of asthma. First this tissue-based effector cells are abundant in the airways of human. Further more, the interaction of specific antigen and surface-bound antigen-specific immunoglobulin(Ig)E, generates mediators that are both redundant and pleiotropic in their capacity to account for many of the symptoms of allergic diseases. The direct correlation between IgE titres and the distribution of mast cells and basophils which bear high affinity receptors for IgE has attracted the attention of many investigators.

Generally four principal strategies are involved in the therapy of immediate hypersensitivity reactions which are as follows:
A) suppression of cell biosynthesis
B) generation of blocking antibodies
C) prevention of cell mediated stimulation of mast cell and
D) neutralization of the effects of the released mediators.

Out of all, the 3rd strategy is of greater significant from therapeutic point of view. Since it blocks IgE-mediated activation of mast cells by interferring with the binding of IgE to the receptor rather than simply treating the aftermath of mediator release. Because of this IgE-receptors have been suggested as possible therapeutic targets for treating allergic disease.

Earlier Noguchi et al\textsuperscript{15} documented the synthesis of various oligopeptides related to immunoglobulin E-fragment crystalline (IgE-Fc) which blocked the allergic response by interferring with the binding of IgE to the receptor of mast cells. One of the Noguchi's hexapeptide having sequence. Ala-Asp-Ser-Asp-Gly-Lys exhibited very
high order of biological response, by suppressing IgE antibody formation and relaxation of the contraction of the blood vessel.

Recently in our laboratory\textsuperscript{216} a series of novel hexapeptide were synthesised related to Noguchi's hexapeptide. Out of which two hexapeptides Ala-Gly-Ser-Asp-Gly-Lys, [CDRI compound, 94/335] and Ala-Gly-Gly-Asp-Gly-Lys, [CDRI compound, 95/220] were found to exhibit high order of activity both by i.p and p.o route in rats and were at least 50 times more potent in terms of dose per dose of disodium cromoglycate (DSCG), a standard antiallergic drug used clinically. The studies led to the findings that Asp\textsuperscript{2} and Ser\textsuperscript{3} are not essential for activity and can be replaced by Gly\textsuperscript{217}.

One of the major drawback associated with Noguchi's hexapeptide, 94/335 and 95/220 is the formation of aspartimide as byproduct during their synthesis due to the presence of troublesome sequences Asp-Ser and Asp-Gly. Aspartimide formation is known to occur in peptides with Asp-Ser and Asp-Gly in their sequences\textsuperscript{218, 219}. This led us to synthesise novel hexapeptides with the view to develop analogues with increased potency and devoid of any aspartimide formation. In the first instance synthesis of novel hexapeptides was taken up in which modifications were introduced at position-1 and -5 of the Noguchi's hexapeptide with the view to carry out structure-activity relationship studies. The peptides were synthesised in order to establish the role of steric, electronic and lipophilic/hydrophobic factors essential for better biological response.

2.1 DESIGN OF NOVEL ANALOGUES

They can be broadly classified into three groups.

2.1.1 Analogues with modification at position-1 of Noguchi's hexapeptide

Structural modification at position-1 have been introduced by replacing Ala with several natural amino acids with the view to establish the role of various side chain
functionality in the expression of biological activity. The following ten analogues have been synthesised for confirming the above points.

I  Pro\textsuperscript{1}-Asp-Ser-Asp-Gly-Lys
II  Glp\textsuperscript{1}-Asp-Ser-Asp-Gly-Lys
III  AcAla\textsuperscript{1}-Asp-Ser-Asp-Gly-Lys
IV  D-Arg\textsuperscript{1}-Asp-Ser-Asp-Gly-Lys
V  Leu\textsuperscript{1}-Asp-Ser-Asp-Gly-Lys
VI  Val\textsuperscript{1}-Asp-Ser-Asp-Gly-Lys
VII  Glu\textsuperscript{1}-Asp-Ser-Asp-Gly-Lys
VIII  Ser\textsuperscript{1}-Asp-Ser-Asp-Gly-Lys
IX  Tyr\textsuperscript{1}-Asp-Ser-Asp-Gly-Lys
X  Arg\textsuperscript{1}-Asp-Ser-Asp-Gly-Lys

In analogue I, Ala has been replaced by Pro as it is known to play a central role in directing peptide folding and due to the presence of five-membered pyrrolidine ring it contributes to conformational rigidifying effect in the molecule. In analogues V and VI Ala has been replaced by neutral and hydrophobic amino acids Leu and Val in order to get an insight into the nature of branching required in the side chain for the expression of biological activity. In analogue VII Ala has been replaced by Glu so as to introduce acidic character in the side chain functionality by providing additional site for ionic interaction. In analogues VIII and IX Ala has been replaced with Ser and Tyr containing hydroxylic side chain functionality, to enhance its binding with the receptors of the mast cell surface through the -OH group present in their side chain. In analogue X Ala has been replaced with Arg in order to study the effect of basic amino acid in the expression of biological activity. Beside this Ala at position-I has been also replaced with Glp (II), AcAla (III) and D-Arg (IV) to prevent the degradation of peptides by various aminopeptidase.
2.1.2 Analogues with modification at position-5 of Noguchi’s hexa peptide

Structural modifications at position-5 has been carried out with the view to ascertain the role of Gly\(^5\) in the expression of biological activity. Beside this replacement of Gly with other amino acids may also abolish aspartimide formation. Accordingly following two analogues have been synthesised.

**XI** Ala-Asp-Ser-Asp-\(\beta\)Ala-Lys

**XII** Ala-Asp-Ser-Asp-Sar-Lys

In analogues XI and XII Gly has been replaced with \(\beta\)-Ala and Sar respectively as such changes in stereochemistry at position-5 would disturb the process of enzyme substrate recognition and make the adjacent peptide bond resistant to enzymatic degradation. Beside, N-methyl amino acids are known to restrict the conformational flexibility and may enhance the potency of the molecule.

2.2. Synthesis of Peptides (I-XII)

All the peptides from I to XII described in this chapter were synthesised manually using solid phase method of peptide synthesis\(^{220}\). Fmoc-chemistry\(^{221-223}\) was used to synthesise all the peptides using p-benzyloxy benzyl alcohol (Wang) resin\(^{224}\),\(^{225}\) as solid support. The side chains of Asp, Ser, Tyr and Glu were protected by acid labile t-butyl (Bu') group. The side chains of Lys and Arg were protected by acid labile t-butoxy carbonyl (Boc)\(^{226}\) and 2,2,5,7,8-pentamethyl chroman-6-sulphonyl (Pmc)\(^{227}\) group respectively. All the protecting groups used for side chain functionality have been chosen in a manner that they can be readily removed during the final cleavage of the peptide from the resin. The synthesis involved following steps.

2.2.1 Loading of c-terminal amino acid to the resin

Loading of N\(^\alpha\)-Fmoc protected c-terminal amino acids on Wang’s resin was carried out using (Boc)\(_2\)O/pyridine/DMAP (scheme 1) procedure developed in our laboratory\(^{228}\). In this procedure Wang’s resin was esterified with N-terminal protected
Fmoc-amino acids in the presence of (Boc)_2O-pyridine-DMAP system using a low polarity solvent (such as CH_2Cl_2) and low temperature (0°C).

![Chemical Reaction Diagram](image)

**Scheme 1**

2.2.2 Estimation of the Loaded Resin

The extent to which the Fmoc-protected C-terminal amino acid was loaded to the resin was estimated via optical density calculation\(^{229}\) described as follows:

A sample of derivatised resin (5-15 mg) was removed, washed with 40% MeOH in CH_2Cl_2 on a sintered funnel and dried under suction for 5-10 min. The sample was weighed, then the Fmoc-group was cleaved using a solution of 20% piperidine in DMF (x ml) for 15-20 min. After allowing the resin to settle, a sample (y ml) of the supernatant was diluted to Z ml with MeOH and UV absorbance recorded at 301 nm. Substitution (in mmol g\(^{-1}\)) was determined by following equations.

\[
\text{Substitution} = \frac{\text{absorbance} \times Z \times X}{7200 \times \text{Weight} \times Y} \times 1000
\]
The absorbance for the dibenzo fulvene piperidine adduct ($\lambda=301$ nm, $\Sigma = 7200$ mol$^{-1}$ dm$^3$ cm$^{-1}$) was determined experimentally from Fmoc-protected amino acids. In a typical experiment $X = 2$ ml, $Y = 0.1$ ml, and $Z = 2.5$ ml.

2.2.3 Deprotection

20% piperidine-DMF solution was used for the removal of Fmoc group from the peptidyl resin.

2.2.4 Couplings

In order to elongate the peptide chain, the subsequent Fmoc-amino acid was coupled with the free amino terminal of the peptidyl resin by diisopropyl carbodiimide (DIC/HOBt) method.$^{230,231}$

Deprotection and coupling steps were repeated for the attachment of the remaining Fmoc-amino acids in successive steps until the desired peptide sequence was built up on the solid support. This was followed by coupling of N-terminal BOC (tert-butyloxy carbonyl) protected amino acids to peptidyl resin.

2.2.5 Final Cleavage of the Peptide

The cleavage of all the peptides (I-XII) from the solid support was achieved by treatment of the cocktail of TFA-water-anisole (40 : 1 : 1) at room temperature to yield crude peptide, which was purified on RP HPLC to get the desired peptide.

2.2.6 Synthetic protocol Adopted for Solid Phase Peptide Synthesis

For the synthesis of compounds I to XII the protocol adopted is given in the following table. The volume of the reagents are given for 250 mg of the resin loaded with the C-terminal amino acid.

All the steps described in table 1 were carried out under nitrogen current, in a reaction vessel, especially designed for solid phase peptide synthesis. A gentle nitrogen current was used to agitate the resin in the presence of the reagents described in the table as well as to provide totally anhydrous atmosphere during the synthesis.
Table 1: Standard Synthetic Protocol for Solid Phase Peptide Synthesis

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Volume (ml)</th>
<th>No. of Time</th>
<th>Time of each min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMF</td>
<td>8</td>
<td>Thrice</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>20% Piperidine-DMF</td>
<td>8</td>
<td>Once</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>DMF</td>
<td>8</td>
<td>Thrice</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>iPrOH</td>
<td>8</td>
<td>Thrice</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>DMF</td>
<td>8</td>
<td>Thrice</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>Fmoc-amino acid (3 molar excess)</td>
<td>8</td>
<td>Once</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>HOBt (3 molar excess)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DIC (3 molar excess)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>DMF</td>
<td>8</td>
<td>Thrice</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>MeOH</td>
<td>8</td>
<td>Thrice</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>DCM</td>
<td>8</td>
<td>Thrice</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>Recoupled if positive Kaiser test by repeating steps 5-9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.2.7 Monitoring of Coupling Reaction

Usually Kaiser’s test\(^{232}\) has been used for monitoring all the coupling reactions involved in the solid phase peptide synthesis. For this test a small aliquot of the peptidyl resin was taken in a sample tube and three drops of each solution i.e. \(2 \times 10^{-4}\) mole KCN/Pyridine, 76% phenol/ethanol (w/w) and 0.28 mole ninhydrin/ethanol added to it. The sample was heated for 5-6 min at 100°C and the colour of the beads examined. Absence of blue colouration of the resin beads indicated a negative Kaiser’s test thereby confirming the completion of the coupling reaction.
2.3. EXPERIMENTAL

Except for D-Arg and D-Ala, all the amino acid used were of L-configuration. All the reactions were carried out exclusively by using dry reactants and solvents. Homogeneity and purity of all the peptides were determined by TLC on silica gel-G plates using the following solvent systems.

A) n-BuOH-pyridine-AcOH-H₂O (9:3:3:5)
B) n-BuOH-AcOH-H₂O (3:3:1)
C) n-BuOH-AcOH-pyridine-H₂O (15:3:10:3)

The final purification and confirmation of the homogeneity of peptides was done with the help of reverse phase HPLC. The solvent system used for analytical HPLC was a binary gradient system of 20-30% acetonitrile-water-0.1% TFA over 15 min. at a flow rate of 1.5 ml/min. A C₁₈ µ bond pack column (7.8 x 300 mm) was used and detection was done at 220 nm.

Amino acid composition of final peptides was determined by hydrolysis of the sample in vacuo in constant boiling 6N HCl for 22 hrs at 110°C. Samples were then analysed using the pico Tag (Millipore) amino acid analyser. Toshniwal melting point apparatus was used for determination of the corrected capillary melting points. Optical rotations were recorded on Autopol polarimeter III. The mass spectra of final purified peptides were characterised by their FABMS on Jeol, JMS sx-102 FAB mass spectrometer.

2.3.1 Synthesis of Analogues I to XII via Solid Phase Peptide Synthesis on Wang Resin using Fmoc-Strategy

![Fmoc-Lys(Boc)-OCH₂-OCH₂-P](image)

To a p-benzyloxy benzyl alcohol resin i.e. Wang's resin (1g, 0.72 mmolg⁻¹, prewashed with dry DCM) in DCM (10 mol), the Fmoc-Lys(Boc)-OH (1.01 g, 2.16 mmol) (Boc)₂ (0.47g, 2.16 mmol), pyridine (0.17 ml, 2.16 mmol) and DMAP (26.40
mg, 0.22 mmol) were added at 0°C. The reaction mixture was gently stirred at the same temperature under anhydrous conditions for 16-20 hrs after which the resin was washed with DMF (3 × 20 ml), MeOH (3 × 20 ml) and DCM (3 × 20 ml). The substitution of Fmoc-Lys(Boc)-OH on the resin was estimated to be 0.43 mmol/gm (64%) of resin.

**Pro-Asp-Ser-Asp-Gly-Lys (I)**

Fmoc-Lys(Boc)-resin (0.25 g, 0.11 mmol) was placed in the reaction vessel and the required Fmoc- amino acids/terminal Boc amino acid (0.33 mmol) viz. Fmoc-Gly, Fmoc-Asp(Bu'), Fmoc-Ser(Bu'), and Boc-Pro successively added to it for condensation in a stepwise manner for elogation of the complete peptide chain using the synthetic protocol earlier.

The protected peptide resin obtained above, was treated with TFA-Water-Anisole (9.50 + 0.25 + 0.25) mixture for 2 hrs at room temperature under N₂ current agitation. Resin was then filtered and the filtrate collected. The resin was further washed with TFA (3 × 3 ml) and the combined filtrate evaporated *in vacuo*. The crude product was precipitated from MeOH-ether, filtered, washed with ether and dried over P₂O₅ and NaOH pellets in a vacuum dessicator. The product was thus purified by HPLC on a semi preparative C₁₈ column (78 x 300 mm) with 10% ACN-water as eluant. The main peak was collected and lyophilized.

Yield (86%); m.p. 169°C; Rf: 0.56(A), 0.74(B), 0.37(C); [α]D²⁵−20.00 (c, 0.10 MeOH) FABMS: 617[M +H]+, Amino Acid Analysis; Asp 1.92(2), Ser 0.96(1), Gly 1.09(1), Lys 0.97(1), Pro 0.94(1).

**Glp-Asp-Ser-Asp-Gly-Lys (II)**

Fmoc-Lys(Boc)-resin (0.25 g, 0.11 mmol) was added to the reaction vessel and Fmoc-protected amino acids (0.33 mmol) viz. Fmoc-Gly, Fmoc-Asp(Bu'), Fmoc-Ser(Bu') and Glp were successively added to it for assembling the complete sequence using the same protocol.
Glp-Asp-Ser-Asp-Gly-Lys was obtained after treating the peptide resin with TFA:Water:Anisole (9.50 + 0.25 + 0.25) mixture for 2 hrs at room temperature under N₂ agitation. The product was precipitated from MeOH-ether, filtered and purified by HPLC as described above.

Yield (88%); m.p. 162°C; Rf : 0.55(A), 0.73(B), 0.72(C); [α]₀^25 -25.00 (C,0.20 MeOH); FABMS: 631 [M+H]^+, Amino Acid Analysis: Asp 2.06(2), Ser 1.04(1), Gly 0.96(1), Lys 0.95(1), Glp 0.94(1).

AcAla-Asp-Ser-Asp-Gly-Lys (III)

Compound III was synthesised in the same manner as that employed for compound II except for the acylation of the protected peptide which was carried out with Ac₂O : DIEA : DMF (0.3:0.6:6.0) mixture for 2 hrs.

Yield: (90%); m.p. 183°C; Rf: 0.61(A), 0.74(B), 0.49(C); [α]₀^25 -45.00 (C 0.20 MeOH); FABMS: 633 [M+H]^+; Amino Acid Analysis: Asp 1.97(2), Ser 0.98(1), Gly 0.97(1), Lys 0.95(1), Ala 0.98(1).

D-Arg-Asp-Ser-Asp-Gly-Lys (IV)

Compound IV was synthesised in the similar manner as that employed for compound II. However Fmoc-D-Arg (Pmc) was used at the last coupling step.

Yield: (87%); m.p. 242°C; Rf: 0.59(A), 0.46(B), 0.65(C); [α]₀^25 + 10.00 (C, 0.10 MeOH); FABMS: 676 [M+H]^+; Amino Acid Analysis: Asp 1.97(2), Ser 0.98(1), Gly 1.02(1), Lys 1.03(1), Arg 0.97(1).

Leu-Asp-Ser-Asp-Gly-Lys (V)

Compound V was synthesised in the usual manner as that applied for compound II. However Boc-Leu was used at the final coupling step.

Yield: (91%); m.p.182-189°C; Rf: 0.76(A), 0.72(B), 0.67(C); [α]₀^25 0.00(C, 0.11 MeOH); FABMS: 633 [M+H]^+; Amino Acid Analysis: Asp 1.93(2), Ser 1.02(1), Gly 0.98(1), Lys 1.06(1), Leu 0.98(1).
Val-Asp-Ser-Asp-Gly-Lys (VI)

Compound VI was synthesised in a similar manner as that employed for compound II except that Boc-Val was used in the last coupling step.
Yield: (90%); m.p. 169-173°C; Rf: 0.54(A), 0.76(B), 0.81(C); $[\alpha]_D^{25}$ 0.00 (C, 0.10 MeOH); FABMS: 619 [M+H]$^+$; Amino Acid Analysis; Asp 1.97(2), Ser 0.97(1), Gly 0.96(1), Lys 1.04(1), Val 1.06(1).

Glu-Asp-Ser-Asp-Gly-Lys (VII)

Compound VII was synthesised in a usual manner as that employed for compound II. However Fmoc-Glu(Bu') was used for the last coupling step.
Yield: (87%); m.p. 187°C; Rf: 0.64(A), 0.76(B), 0.81(C); $[\alpha]_D^{25}$; FABMS: 649 [M+H]$^+$; Amino Acid Analysis; Asp 1.96(2), Ser 1.02(1), Gly 1.04(1), Glu 0.97(1), Lys 0.96(1).

Ser-Asp-Ser-Asp-Gly-Lys (VIII)

Compound VIII was synthesised in a similar manner as that employed for compound II. However, Fmoc-Ser(Bu') was used for the last coupling step.
Yield: (88%); m.p. 183-185°C; Rf: 0.47(A), 0.63(B), 0.67(C); $[\alpha]_D^{25}$-20.00 (C 0.20 MeOH); FABMS: 607 [M+H]$^+$; Amino Acid Analysis; Asp 1.94(2), Ser 1.98(2), Gly 1.02(1), Lys 1.06(1)

Tyr-Asp-Ser-Asp-Gly-Lys (IX)

Compound IX was synthesised in a usual manner as that employed for compound II except that Fmoc-Tyr (Bu') was used in the last coupling step.
Yield: (90%); m.p. 186-187°C; Rf: 0.82(A), 0.71(B), 0.36(C); $[\alpha]_D^{25}$ -15.00 (C 0.20 MeOH); FABMS: 683 [M+H]$^+$; Amino Acid Analysis: Asp 1.93(2); Ser 1.03(1); Gly 0.96(1); Lys 1.04(1) Tyr 1.03(1)
**Arg-Asp-Ser-Asp-Gly-Lys (X)**

Compound X was synthesised in a similar manner as that employed for compound II. However, Fmoc-Arg (Pmc) was used in the last coupling step.

Yield: (89%); m.p. 191-192°C; Rf: 0.76 (A), 0.75(B), 0.62(C); \([\alpha]_D^{25} + 38.09\) (C, 0.11 MeOH); FABMS: 676 \([M+H]^+\); Amino Acid Analysis; Asp 2.09(2), Ser 0.94(1), Gly 0.98(1), Lys 0.97(1), Arg 0.93(1).

**Ala-Asp-Ser-Asp-β-Ala-Lys (XI)**

Compound X was synthesised in a similar manner as that employed for compound II. However, in place of Fmoc-Gly, Fmoc-β-Ala is used at position-5.

Yield: (90%); m.p. 176°C; Rf: 0.85 (A), 0.52(B), 0.44(C); \([\alpha]_D^{25} -20.00\) (C 0.20 MeOH); FABMS: 598 \([M+H]^+\); Amino Acid Analysis; Ala 1.03(1), Asp 1.93 (2), Ser 0.97(1), β-Ala 1.04 (1), Lys 0.93(1).

**Ala-Asp-Ser-Asp-Sar-Lys (XII)**

Compound XII was synthesised in a usual manner as that employed for compound II. However, Fmoc-Sar is used at position-5 in place of Fmoc-Gly.

Yield: (91%); m.p. 168-170°C; Rf: 0.63 (A), 0.51(B), 0.39(C); \([\alpha]_D^{25} -35.00\) (C0.22, MeOH); FABMS: 606 \([M+H]^+\); Amino Acid Analysis; Ala 1.03(1), Asp 1.90(2), Ser 0.95(1), Sar 1.01(1), Lys 0.98(1).