Design and Synthesis of a Glycerol Based Hydrophilic Polymer Support for Solid Phase Organic Reactions.

Thesis submitted in partial fulfilment of the requirements

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By

ROHINI K.C

Research and P.G Department of Chemistry

Christ College, Irinjalakuda, Thrissur

Kerala, India 680125

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Irinjalakkuda

CERTIFICATION OF SUPERVISOR

This is to certify that all the corrections /suggestions from the adjudicators have been incorporated in the thesis entitled ***"Design and synthesis of a Glycerol based Hydrophilic Polymer support for Solid phase Organic Reactions "*** carried out by ***Rohini.K.C***, under my supervision. The contents given in the thesis and CD are the same.

Dr. V.D. John

Research Supervisor

Dr. Tom Cherian

Co- Guide

CERTIFICATE

This is to certify that the thesis entitled "**Design and Synthesis of a Glycerol Based Hydrophilic Polymer Support for Solid Phase Organic Reactions**" submitted to University of Calicut, in partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy in Chemistry is a record of original research work done by **Mrs.Rohini.K.C** ,during the period of her Research in the P.G and Research Department of Christ College, Irinjalakuda, under our guidance and supervision and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship , fellowship or similar other titles and that it is an independent work done by her.

Dr. V.D. John	Dr. K.P. Subhashchandran	Dr. Tom Cherian
(Guide)	(Co-Guide)	(Co-Guide)

Irinjalakuda

DECLARATION

I **Rohini.K.C** hereby declare that the dissertation entitled "**Design and Synthesis of a Glycerol Based Hydrophilic Polymer Support for Solid Phase Organic Reactions**" submitted to the University of Calicut, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Chemistry is my original work and the dissertation has not formed the basis for the award of any degree, diploma, associateship, fellowship or similar other titles. It has not been submitted to any other university or institution for the award of any degree or diploma.

Irinjalakuda. ROHINI.K.C

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PREFACE

Peptides and proteins have a central role in numerous biological and physiological processes in living organisms. They are involved as hormones and neurotransmitters in inter cellular communication, perform as antibodies in the immune system to safeguard organisms against foreign invaders, and are also involved in the passage of various substances through biological membranes. Compared with proteins and antibodies, peptides have the ability to penetrate further in to tissues due to their small size. Therapeutic peptides, even synthetic ones, are generally less immunogenic than recombinant proteins and antibodies. Peptides have other benefits over proteins and antibodies as drug candidates, including lower manufacturing costs (synthetic vs. recombinant production), higher activity per unit mass, lower royalty stack than antibodies for the reason that of a simpler intellectual property landscape during discovery and manufacturing, greater stability (lengthy storage at room temperature acceptable) , less potential for interaction with the immune- system signalling sequence and better organ or tumour penetration. Synthetic peptides find applications in all areas of biomedical research including immunology, neurobiology, pharmacology, enzymology and molecular biology. The first prerequisite for the study of proteins is to evaluate their accessibility in terms of purity and quantity. There are 3 main routes to consider 1) native protein isolation 2) Recombinant techniques for the expression of proteins in micro-organisms and 3) Chemical synthesis.

In 1963 R.B. Merrifield published his historious innovation, the solid phase peptide synthesis, for which he was awarded Nobel Prize. SPPS includes the stepwise

accumulation of protected amino acids to a growing peptide chain which was bound by a covalent bond to a solid resin particle .This offered a procedure whereby reagents and by -products were removed by filtration. The advantages for the new method were speed and simplicity of operation. In detail the method consist of introduction of an N- terminal protected amino acid to the solid matrix. The N- terminal protection is then detached and the successive amino acid is coupled. The deprotection-coupling- washing process is sustained till the required sequence is achieved. Then the peptide is detached from the support and the permanent protecting groups are removed. This is followed by precipitation, characterization and purification.

In this Thesis, the entire work is presented in 7 chapters. First chapter introduces peptides and importance of synthetic peptides in biostudies. Of the many methods used for the synthesis of peptides, Solid Phase Peptide Synthesis is most important. A brief introduction of SPPS and role of solid support is given followed by objectives of study and organization of the thesis. Second chapter is Review of Literature. The purpose of this literature is to provide a summary of information relevant to the subsequent chapters. Development of solid phase peptide synthesis during years is discussed briefly. Each successive developments and related drawbacks are pointed out clearly. The review helps us to identify the areas where information is limited and research is needed

The experimental part mainly describes the polymer preparation, characterisation and the other half of the work explains the detailed procedure for solid phase peptide synthesis. It is included in four chapters. Of which the third chapter describes the development of a new Glycerol dimethacrylate cross linked 4- vinyl pyridine polymer support for solid phase peptide synthesis. Synthesis of the resin support by suspension

polymerisation, characterisation of the resin, swelling and stability studies of the resin in various conditions used in SPPS are described. The hydroxyl functional group of the resin is converted to chloro and amino groups by standard methods and corresponding capacity measurements are described.

In the fourth chapter, the efficiency of the new support in poly peptide synthesis is demonstrated by synthesising a tetra peptide fragment VYGR and a deca peptide sequence KVKRIILARS using solid phase synthetic strategy. The same fragments are prepared using Merrifield resin and Wang resin and a comparison has been done.

Two biologically important peptide hormones Angiotensin II and Angiotensin IV were prepared using improved Fmoc-solid phase peptide synthetic strategy on GDMA-4-VP resin has been discussed in chapter 5. The yield and purity of the peptide products are compared with the existing methods.

The synthesis of biologically active peptides using solid phase methodology is described in chapter 6. Their characterisation was performed with HPLC and ESI-MS. H_2N-PyrLYENKPR-($CONH_2$)- Neurotensin, H_2N- Met-Gln-Met-Lys-Lys-Val-Leu-Asp-Ser-OH- Anti- inflammatory peptide,MRMKHVRAWIPRMR –MR-14,YDGYPTFGEHKQEKDLEY-YY-18,YLFKTNPNYKGNDIK –YK-15,Amyloid beta peptide, Neuromedin N.

Chapter 7 gives a brief summary of the whole work. The chapter concludes by mentioning the scope of the work.

Abbreviations

DIC	-	Diisopropylcarbodiimide
DCC	-	Dicyclohexylcarbodiimide
DIPEA	-	Diisopropyllethylamine
DMAP	-	4-Dimethylaminopyridine
TIS	-	Triisopropylsilane
TFA	-	Trifluoroacetic acid
THF	-	Tetrahydrofuran
EDT	-	Ethanedithiol
HOBt	-	Hydroxybenzotriazole
PyBOP	-	Benzotriazol-1-yi-Oxytripyrrolidinophosphonium hexafluorophosphate
DCM	-	Dicloromethane
DMA	-	N,N-dimethylacetamide
DMF	-	N-Methylpyrolidone
NMP	-	N-methyl-2-pyrolidone
$CHCL_3$	-	Trichloromethane
Fmoc	-	9-Fluorenylmethloxycarbonyl
Boc	-	Butyloxycarbonyl
Trt	-	trityl
TBu	-	tertbutyl
Pbf	-	2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl
AA	-	Amino acid
SPPS	-	Solid phase peptide synthesis
LPPS	-	Liquid phase peptide synthesis
TLC	-	Thin layer chromatography
SEM	-	Scanning electron microscope
FT-IR	-	Fourier transformed infrared
HPLC	-	High performance liquid chromatography
RP-HPLC	-	Reverse phase high performances liquid chromatography
MS	-	Mass spectrometry
MALDI-TOF	-	Matrix –assisted laser desorption –time of flight
ESI	-	Electrospray Ionization

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Chapter 1

Introduction

The primary role of peptides and proteins in living organisms is due to their several biological and physiological functions. They perform various functions of hormones and neurotransmitters in intercellular communication, control the transport of various substances through biological membranes and execute the functions of antibodies in the immune system and thus protect organisms from foreign invaders. Investigating the principles which regulate the structural and functional characteristics of bioactive proteins are the important objective of biological and medical researchers. Compared to proteins and antibodies, peptides have the ability to penetrate further in to tissues due to their small size. Therapeutic peptides, even synthetic ones, are generally less immunogenic than recombinant proteins and antibodies **[1]**. Peptides have other benefits over proteins and antibodies as drug candidates, including lower manufacturing costs (synthetic vs. recombinant production), higher activity per unit mass, lower royalty stack than antibodies for the reason that of a simpler intellectual property landscape during discovery and manufacturing, greater stability (lengthy storage at room temperature acceptable) , less potential for interaction with the immune- system signalling sequence and better organ or tumour penetration. Therapeutic peptides also offer several advantages over small organic molecules that make up traditional medicines **[2]**.

The synthetic peptides and peptide analogues, especially potent bio drugs, have significant therapeutic and commercial applications. The structure and activity correlations of the synthetic peptides and proteins, mainly their high purity, have

prompted biomedical applications **[3]**. Synthetic peptides find applications in all areas of biomedical research including neurobiology, immunology, pharmacology, molecular biology and enzymology [**4]**. The first prerequisite for the study of proteins is their accessibility in high purity and adequate quantity. Three important methods followed for the production of peptides are Native protein isolation, Recombinant techniques for the expression of proteins in micro-organisms and Chemical synthesis. These methods have their own advantages and disadvantages. Among these methods only chemical peptide synthesis allows the integration of unnatural amino acids and also this method helps in the production of large quantities of pure peptides **[5]**. Chemical peptide synthesis is converse of protein bio synthesis, where the synthesis begins at the N- terminal end. Chemical synthesis of peptides can be accomplished by either solution phase or solid phase method. The classical approach to peptide synthesis is liquid phase peptide synthesis. It is beneficial especially in large scale production of peptides for industrial purposes. In classical method, synthesis of peptides containing more than a few amino acids is laborious. Other serious problems include the poor solubility of the intermediate protected peptides in organic solvents and the racemization of the activated C-terminal amino acids. To overcome these problems R.B.Merrifield introduced Solid Phase Peptide Synthesis. The method introduced has made the production of synthetic therapeutic peptides easier **[6]**.

1.1. Solid Phase Peptide Synthesis

In 1963 R.B. Merrifield published his historious innovation, the solid phase peptide synthesis, for which he was awarded Nobel Prize. SPPS includes the stepwise accumulation of protected amino acids to a growing peptide chain which was bound by a

covalent bond to a solid resin particle **[7]**. This offered a procedure whereby reagents and by -products were removed by filtration. Advantages of the method was speed and simplicity of operation **[8]**. In detail the method consists of introduction of a N- terminal protected amino acid to the solid matrix. The N- terminal protection is then detached and the successive amino acid is coupled. The deprotection-coupling- washing process is sustained till the required sequence is achieved. Then the peptide is detached from the support and the permanent protecting groups are removed. This is followed by precipitation, characterization and purification **[9]**.

1.2. Solid support

The basic prerequisite for solid phase peptide synthesis is an apt solid support. Solid supports are small resin beads functionalised with reactive groups that can be used as the binding site for the peptide chain. One of the well-recognised difficulties with the classical synthesis in homogeneous solution is insolubility of some of the intermediates. This problem can be overcome in many cases by the use of solid supports, where the peptide chain and the lightly cross-linked polymer chain become intimately mixed and exert a mutual solvating effect on one another. It becomes thermodynamically less favourable for the peptide to self -aggregate and is therefore remains accessible for the reaction. But still, some problems intrinsic to SPPS have not been eliminated. These difficulties usually hinge on polymer structure, poor solvation property of the resin and the resin bound peptide in different solvents **[10, 11]**. These downsides could lead to imperfect coupling and deprotection reactions in various phases of the synthesis, that result in decreased yield of the target peptide, along with a great amount of various condensed and deletion sequences **[12]**.

Indisputably the polymer matrix has a substantial role in SPPS. It consents simple stepwise synthesis and thus diminishes a large number of discrete steps. The support is made up of diverse materials with upgraded intrinsic properties and so permits more reagent diffusion and swelling in a wider range of solvents. The necessities for a solid support is

1) Uniformity of beads.
2) Mobile, well- solvated and reagent accessible sites.
3) Good swelling in a broad range of solvents.
4) Functionalised beads permitting covalent coupling of the first compound.
5) Accessible loadings.
6) Stability with variation of temperature **[13]**.

As already stated, the success of SPPS depends on the physical and chemical characteristics of the peptide bearing support **[14, 15]**. For operative swelling of the resin and solvation of the peptide, the polymer should have an optimum hydrophobic-hydrophilic balance **[16]**. Various structural parameters of polymeric support such as polarity, nature and extent of crosslinking and the solvation of the support and resin bound species in the solvent medium determines the reactivity of functional groups attached to the polymeric backbone.

Solid support used by Merrifield was 2% DVB –cross linked polystyrene which was less reactive in polar solvents. Peptide synthesis using the classical PS-DVB resin meets some drawbacks because of the rigidity, hydrophobic nature and physico-chemical inconsistency of the polymer with the growing peptide chain **[17,18]**.The strong hydrophobic, macromolecular environment of polymer can persuade the growing peptide

chain to adopt unfavourable conformation that leads to low yield and purity of target peptides **[19]**.

A number of new supports have been established in recent years by presenting various hydrophilic cross-linkers instead of hydrophobic DVB cross-linker in the polystyrene network **[20-23]**.These new cross-linkers have been aimed to increase the swelling characteristics of the polystyrene resin without compromising its mechanical stability and it gives sufficient flexibility to the polymer backbone in order to permit higher diffusion of the reactive intermediates to resin bound reaction centres throughout the resin matrix. These supports were later substituted with different functionalities so as to use them for various organic synthesis **[24-27]**.Peptide chemistry utilises different classes of hydrophilic polymers as support for chemical reactions. The solubility and diffusivity of hydrophilic polymers in water facilitates their biomedical applications.

The rate of incorporation of a particular amino acid residue decreases with increase in chain length. This attributes to steric hindrance at various functional sites at the heterogeneous networks. The incompatibility influences the solvation of the polymer and peptide, rate of coupling and deprotection reactions. Research is going on to overcome all these problems. Development of supports with improved properties and new synthetic strategies for SPPS have become the major areas of interest for researchers. Polyamides, poly ethylene glycol-polystyrene graft resins, PEGA, CLEAR etc. are some of the main supports introduced and tested over the years. Some of the solid supports found to be satisfactory for peptide synthesis are poly methyl methacrylate, polysaccharides, phenolic resins, silica, porous glass and polyacrylamides. But the most widely used one is polyacrylamide resin **[28]**. Polyacrylamide supports were familiarized

as an alternative to the most commonly used DVB cross-linked supports with a view to increase their hydrophilic nature **[29]**. However they undergo only poor swelling in non-polar solvents which are also used in peptide synthesis.

An effective polymer support for repetitive synthesis of biopolymers should facilitate different types of organic reactions occurring in both polar and non-polar medium **[30]**. This is possible only in the case of a macro molecular matrix with optimal hydrophilic- hydrophobic balance. In addition, cross linked polymer matrix should be mechanically stable to withstand the multitude of synthetic operations. The DVB cross linked PS has the necessary mechanical stability whereas the poly acrylamide system lacks this. In order to provide the essential characteristics of an effective polymeric support to a single matrix, we thought of cross linking the hydrophobic 4-VP with hydrophilic cross linking agent GDMA. 4-vinylpyridine is a reactive monomer which when undergoes polymerisation, the nitrogen atom present in the ring becomes more available. The benefit of the addition of a six membered heterocyclic moiety is the increased van der Waals force, which impart better electrostatic forces to the finished polymer and improves adhesion. 4-vinyl pyridine was selected as a monomer suitable for investigation, since in view of its polar character and structural similarity to styrene. Secondary hydroxyl groups, which can be used as growth sites for peptide synthesis, make GDMA an efficient cross-linker. Presence of ester functionalities and hydroxyl groups give hydrophilic character to the polymer. The presence of hydroxyl group limits the necessity of functionalization of the resin with reagents like chloromethyl methylether. The reagent is carcinogenic and also the conversion of the group to other functional groups may not be complete in every case, which will be treated as cost

ineffective. The elimination of all these additional steps makes our resin superior. However some simple functionalizations are made on the resin to make it useful for various reactions.

1.3. Fmoc/tBu- strategy of peptide synthesis

Chemical peptide synthetic strategies are versatile and can be optimized to produce biologically important peptides. In the present study, Fmoc/tBu strategy is used for the synthesis of peptides. The main advantage of Fmoc method is that it offers a mild deprotection scheme. It is cleaved under very mild basic condition but it is stable under acidic conditions. So mild acid labile protecting groups such as Boc and benzyl groups can be used to protect side chains in the target peptide. The reaction starts with anchoring the C- terminal of the first amino acid with the solid support. In case of a support with hydroxyl group as the functional site, this step is performed with esterification reaction using DIC, DMAP in DCM. Fmoc- protection is then removed using piperidine in DMF and the excess reagent can be removed from the system by careful washing with DCM and DMF. The C- terminal of the second amino acid is then coupled to the chain using DIC and HOBt. The coupling and deprotection steps can be monitored using Keiser test. Using the same procedure, all amino acids can be coupled to the chain until the desired sequence is achieved. The final cleavage of the peptide from the resin can be done using TFA and suitable scavengers like TIS and water. From the solution, excess TFA can be removed and crude peptide can be precipitated by adding ice cold ether. Purification and characterization can be done with HPLC, mass spectrum and NMR spectrum and circular dichroism spectrum.

Some changes were made in the selection of solvents, coupling time, amount of amino acids taken and coupling reagents in the preparation of biologically active peptides. The reaction was carried out in NMP since our resin finds maximum swelling in it. Some amount of DCM was added to enhance the solubility. DIC/HOBt was replaced by DIC/ pyBOP in the synthesis of some biologically relevant peptides. These changes brought a significant improvement in the yield and purity of the peptide so it is represented as improved Fmoc SPPS in the current study.

The preparation of the polymer support, functionalization with different anchoring groups, swelling and stability studies of the resin and the illustration of the application of this resin in the peptide synthesis are described in the present study. Some modifications are made on the strategy of Fmoc Solid Phase Peptide synthesis to overcome the experienced difficulties with the method and to increase the quantity and quality of the product so that to make it useful for biological applications. The main objectives of the current work are

1. Preparation of Glycerol dimethacrylate cross linked 4-VinylPyridine resin by free radical suspension polymerisation affording a polymer containing ester and secondary hydroxyl group.
2. Swelling studies of the prepared resin and Stability studies in various synthetic conditions used in solid phase peptide synthesis.
3. Derivatisation of the resin with various functional groups and capacity measurements with standard methods.
4. Synthesis of biologically relevant polypeptides on the resin and comparison with standard resins.

5. Synthesis of biologically active peptides on GDMA-4-VP resin using improved Fmoc solid phase synthetic strategy.

1.4. Organization of the thesis

The Thesis is structured in to 7 chapters.

Chapter 1. Provides the general introduction to the subject matter contained in the thesis. Significance of synthetic peptides, familiarisation of SPPS strategy and related details, observed difficulties and limitations of the method are discussed. The major research aims, objectives, methods and significance of the study have been outlined.

Chapter 2. Presents a general review which will provide details relevant to understand the following chapters.

The experimental part of the thesis is distributed in four chapters.

Chapter 3. Describes the synthesis, Characterization and functional group interconversions of GDMA-4-VP Resin.

Chapter 4. Describes the synthesis of two poly peptides on GDMA-4-VP resin. The results were compared with the results of synthesis of same peptides on Merrifield resin and Wang resin.

Chapter 5. Describes the successful synthesis of Angiotensin II and Angiotensin IV on GDMA-4-VP resin using improved Fmoc strategy.

Chapter 6. Explains the synthesis of seven Biologically active peptides by solid phase methodology.

Chapter 7. Provides Summary and Conclusion of the work.

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Chapter 2

Review of Literature

The purpose of this literature is to provide a summary of information relevant to the subsequent chapters. Development of solid phase peptide synthesis during the years is discussed briefly. Each successive developments and related drawbacks are pointed out clearly. The review helps us to identify the areas where information is limited and research is needed.

2.1. History of peptide synthesis

The preparation of the dipeptide glycylglycine by Emil Fischer in 1901 is regarded as the commencing of peptide chemistry. He described a method based on the use of acyl chlorides **[1, 2]**. However 20 years earlier Theodor Curtius had prepared the first N- protected dipeptide benzoylglycylglycine during his PhD studies. That is known as the azide- coupling method and was the first practical method introduced for peptide synthesis **[3]**. Both these methods confronted the same difficulty of inaccessibility of enantiomerically pure L- amino acids and a simply detachable amino protecting group. The introduction of carbobenzoxy (Cbz) group for the temporary protection of the amino group by M. Bergmann and L. Zervas; students of Fischer, launched a new era in peptide synthesis **[4]**. The use of Cbz- protecting group blocked racemization during the formation of the acyl chloride, which is a general feature of urethane- protecting groups. So the invention became a remarkably lasting contribution. With the discovery of biologically active peptides like oxytocin and insulin, the objective of peptide synthesis

went through a dramatic change **[5]**. L.A. Carpino et al in 1957 introduced tert-butyloxycarbonyl group (Boc), a new acid- labile protecting group. The grouping of Boc- and Cbz-protecting groups was used for the synthesis of several peptides and was found to boost the new research towards the discovery of new coupling methods.

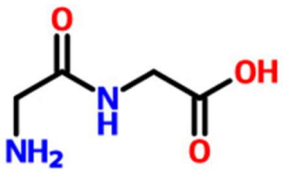


Fig.2.1.The first synthetic peptide

Formation of an amide bond between two amino acids is an energy requiring reaction. Generally this reaction takes place at raised temperatures. Since peptide synthesis is usually executed at or below room temperature, either the carboxyl or amino group of one of the amino acids must be activated. The azide procedure of Curtius and acid chloride approach of Fischer provide simple and efficient ways to peptide bond formation. But the highly reactive reagents used in these methods can affect side chain functions. So investigations continued in order to conduct activation under mild conditions **[6]**. An important invention happened was in 1955 when J.C Sheehan, J.P. Hess and H.G. Khorana presented cabodiimide activation method **[7]**. The racemization associated with the activation of carboxylic group was found to be lessened by the application of additives such as 1-hydroxybenzotriazole (HOBt) because of the formation of less reactive HOBt ester **[8]**.

Carbodiimide based reagents were used in peptide chemistry for the formation of amide bond after the introduction of DCC and racemization suppresser HOBt. Usage of

the liquid reagent DIC is more advantageous since the diisopropylurea by-product formed during the reaction is more soluble in DCM. Urea by product insolubility was the main problem in the case of DCC. Reaction involving DIC is not sensitive to moisture so can be carried out in aqueous medium **[9]**. Thereafter a new group of aliphatic cabodiimides bearing tertiary and quaternary amine substituents were described **[10]**. Louis A Carpino demonstrated the effectiveness of DIC with HOAt than DIC/HOBt in regard to racemization in DCM solvent. The result of this study led to the development of a stepwise procedure for peptide assembly in which collidine is added to enhance activation and subsequently DIEA is added to enhance coupling **[11]**. A number of highly hindered bases which were even more convenient in handling and maintaining the configuration during segment coupling processes were introduced later **[12]**. Ramage has also investigated the triazole based additive HOCt in combination with DIC and reported negligible racemization with all amino acids except histidine **[13]**. Another important invention happened thereafter was the introduction of solid phase peptide synthesis by R.B.Merrifield **[14]**. The generally accepted scheme in those days required the blocking of carboxyl group of one amino acid and the amino group of the second amino acid. Activating the free carboxyl group, the peptide bond could be formed. Finally the removal of the two protecting groups will lead to the free peptide. Merrifield found this method, although very effective, difficult to execute and also time consuming. In order to prepare peptides in larger amounts and for the construction of more complex peptides, he tried to assemble the peptide chain anchored covalently on a solid support, in a step-wise manner. By simple washing after the completion of each step, the intermediate peptides can be washed off instead the tedious crystallisation methods **[15]**. The chloromethylated

divinylbenzene cross linked polystyrene was the polymeric support used in the synthesis which is now known as Merrifield Resin **[16].** Solid phase peptide synthesis offers many advantages over the conventional method. It simplifies and accelerates the multistep synthesis because the whole reaction is carried out in a single reaction vessel. So it avoids the material loss probable to happen during the repeated transfer of materials hence result in high yield of the final product through the use of excess reactant to force the individual reactions to completion. It also increases solvation and decreases aggregation of the intermediate products. An overview of important developments during the history of SPPS is given below.

Table 2.1. History of SPPS

Year	Authors	Development
1963	Merrifield	Development of SPPS, insoluble carrier, cross-linked polystyrene.
1967	Sakakibara	HF- Cleavage **[17]**.
1970	Carpino & Han	Fmoc, a base labile N- α protecting group **[18]**.
1973	Wang	Development of p- alkoxybenzyl alcohol resin **[19]**.
1976	Burgus & Rivier	Application of preparative reversed phase HPLC for the purification of peptides prepared by Boc SPPS **[20]**.
1985	Rapp and others	Poly styrene- poly ethylene glycol grafts like TentaGel.
1987	Rink and others	Introduction of various TFA labile linkers for the Fmoc/tBu SPPS of peptide amides.
1987	Sieber	Xanthenyl linker for the Fmoc/tBu SPPS of fully protected peptide amides **[21]**.

Year	Authors	Development
1987	Mergler et al	Development of 2-methoxy-4-alkoxybenzyl alcohol resin for the Fmoc/tBu SPPS of fully protected peptide fragments **[22]**.
1988	Barlos et al.	2-chlorotrityl chloride resin for the Fmoc/tBu SPPS of fully protected peptide fragments **[23]**.
1993	Hobbs de Witt, Ellman	Combinatorial chemistry **[24]**.
1995	Mutter et al.	Pseudoproline dipeptides **[25]**.
2002	Gogoll and others	Microwave accelerated SPPS **[26]**.
2003	White and others	Fmoc SPPS of long peptides

Recently a combination of Solution-phase synthesis and solid- phase synthesis was reported by Biron et al for the synthesis of class II a bacteriocin pedocin PA-1 and analogs. This method was found to overcome the observed difficulties due to unwanted reactions and instability **[27]**.

2.2. Principles of Merrifield Peptide Synthesis

In 1963 Robert Bruce Merrifield developed solid phase peptide synthesis for which he was awarded Nobel Prize in 1984. The basic technique in this method is the assembly of amino acids in to the desired peptide sequence keeping one end of the chain anchored to an insoluble support. Repeated cycles of coupling-washing-deprotection-washing are followed. Although this method seems to be simple, a large extent of efforts has to be applied to avoid unnecessary products. Merrifield's resin was a gel prepared by suspension co-polymerisation of styrene and DVB as cross linking agent **[28]**. They were spherical beads of 50 μm in diameter when dry. In organic solvents like DCM, it will

swell to 5 to 6 times their original volume. The new method introduced has the following merits over classical solution phase method.

1. During the peptide synthesis its C- terminus is attached covalently to an insoluble polymeric support. This permits the easy separation of the growing peptide from any by products or excess unused amino acid components.
2. The reactions are driven to completion by using excess of reactants and reagents
3. No mechanical loss occurs because the growing peptide is retained on the polymer in a single reaction vessel throughout the synthesis.
4. The final peptide is detached from the polymer support by a single cleavage step at the end of synthesis. The cleavage step does not degrade the assembled peptide.
5. The physical operation involved in the method is simple, rapid and amenable to automation.
6. The spent resin can be recycled.

SPPS made the synthesis of peptides containing 30-40 amino acids and small proteins containing more than 50 amino acids easy by special protocols or chemical ligation of peptide segments. Although Merrifield found that the reaction rates were slower in solid phase than in solution, the final result showed improvements in time and yields since several purification steps could be avoided.

Recently Cherkupally et al. delivered the results of C-terminal and side- chain synthesis and describes both method gives similar results in terms of purity **[29]**.The general protocol of peptide synthesis is given in fig.2.

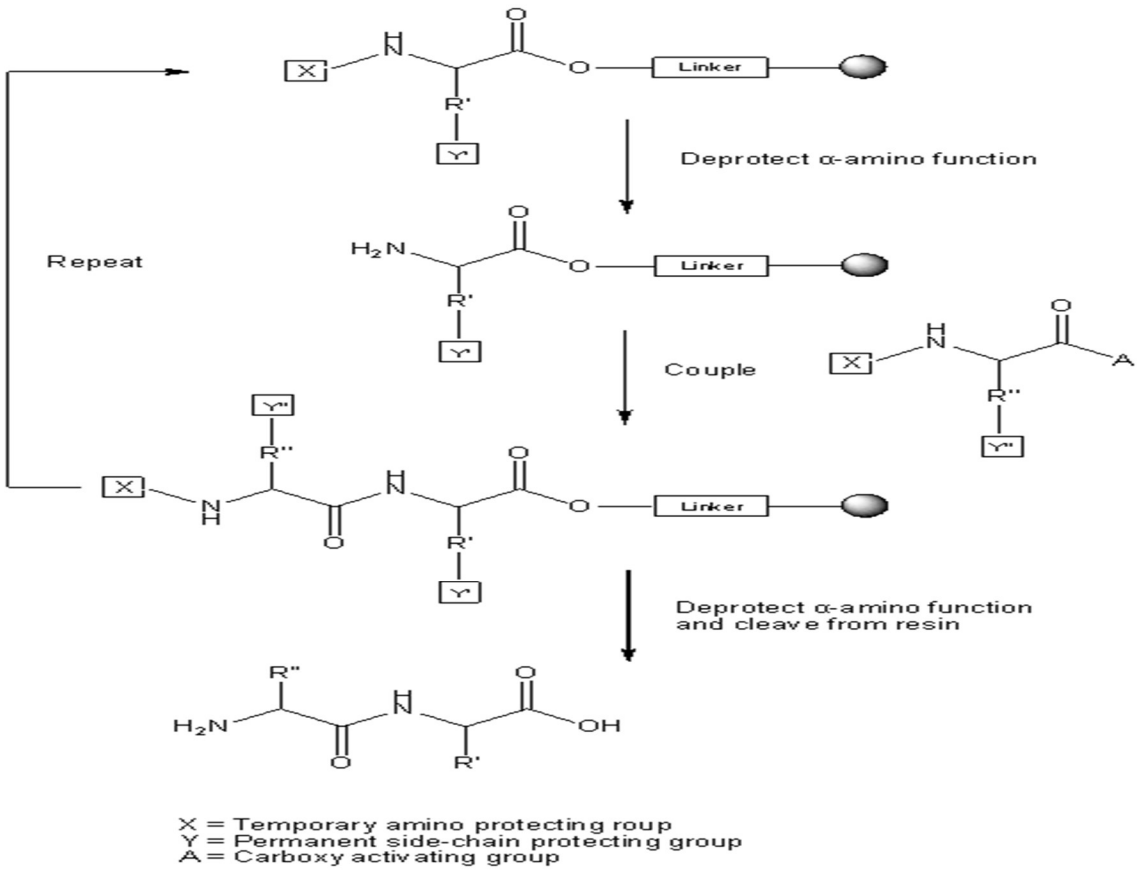


Fig. 2.2. General Protocol of peptide synthesis.

The first requirement for solid phase peptide synthesis is a suitable support.

2.3. Solid Supports

The basic requisite for solid phase peptide synthesis is the preparation of the polymer support. The solid support allows simple stepwise synthesis thereby avoiding a large number of individual steps. In addition it allows the shortening of the time required and also the synthesis of previously inaccessible peptides. The polymer support should be in regular shape which permits the easy handling of support. Suspension polymerization is the appropriate technique to obtain polymer in beaded form. There are several factors which determine the structure and shape of the polymer beads. Temperature, rate of

stirring, atmosphere, molecular weight of suspending agent, nature of diluent, and even shape of the polymerization vessel have reflective effect on the morphological characteristics of the polymer. Beads of size ranging from 200 to 400 pm are suitable for peptide synthesis. Beads larger than 400 pm have less total surface area, and beads smaller than 200 pm may clog the sintered disc of the peptide synthesizer and cause filtration problems during the synthesis. Other important criteria for the solid support to be used as a support for SPPS are that it should be chemically inert to the reagent used for peptide synthesis and be mechanically stable.

A brief description of the most used resins is given here.

Chloro- methylated polymer of styrene and DVB was used by Merrifield for peptide synthesis. 2% PS-DVB allows the diffusion of reagents when the polymer swells in the presence of suitable solvents. Standard cross linking is about 1-2 per cent DVB therefore it is a highly hydrophobic resin that may distress the peptide assembly. In the case of long peptides, the assimilation of particular amino acids was found to lessening in the case of PS-DVB resin. To solve such inherent problems PEG-PS resin was introduced. CZarnic and collaborators perceived the faster kinetics shown by PEG-PS resin under identical conditions **[30]**. The rate of reaction was found to be more with greater PEG content in the resin. After this finding, many combinations of PEG chains with resin were announced. The formerly introduced Grafted PEG-PS resin was found to have lower loading and chemical stability. Bayer et.al familiarized POE-PS resin which displayed good and uniform swelling in a wide range of solvents **[31]**. However the first generation of this class of resin confronted the problem of low loading and loss of PEG chains during strong acidic treatments, such as TFA applied in the cleavage step. To

overcome these drawbacks, a new generation of PEG-PS resins were developed. These problems were assumed to be because of the direct linkage of the benzyl from the PS to the PEG chain. By using a longer linkage and in some cases by branching it, greater stability and loading can be accomplished. One among such resin is PEG-PS established by Albericio and Barany **[32]**. The moderate loading of the PEG-PS resin (0.15-0.25 mmol/g) was later improved by (0.3-0.5 mmol/g) by introducing a branch point. Another class of PEG-PS resin was developed by Argonaut Technologies. The graft component of Agrogel is a bifunctional PEG chain attached to the PS core at its centre. This class disclosed more inertness to strong acids, nucleophilic reagents and have double loading. Adams et.al developed PEG –PS Champion I and II based on the assimilation of PEG chains on to the PS core by attaching a spacer between them to improve stability and loading.

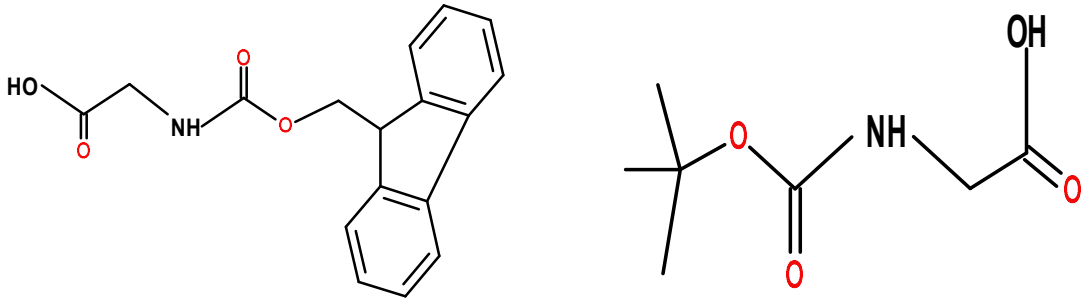
PEGA, TTEGDA-PS, CLEAR are another group of resins in which PEG chains are presented not as a graft but by co polymerisation. As an improvement of Sheppard's polyamide resin, Meldal et al developed PEGA resin in which PEG chains act as cross linkers. PEGA resin has several applications like chemical ligation on the resin, direct screening of phosphor-peptide –binding proteins as a useful technique for proteomics, direct monitoring on the resin by gel phase NMR and biotransformation on solid support **[33]**. In TTEGDA-PS resin, TTEGDA is the cross linker. It resembles a gel more **[34]**. Although the resin has many advantages like mechanical stability, good-swelling and optimum hydrophilic-hydrophobic balance, the internal structure of the resin is found to be damaged during the removal of solvent. This problem is thought to be due to the presence of ester bonds that confer reduced chemical stability. In the case of CLEAR

resin, a trivalent cross linker is used. These supports prepared by radical co-polymerisation of the branched cross-linker trimethylol propane ethoxylate triacrylate with other monomers. The cross-linker contains 4 to 5 oxyethylene units in addition to polymerisable vinyl end group so have PEG like character **[35]**.

2.4. Methods of protection and Deprotection

Amino acids usually contain more than one reactive site which necessitates the application of protection strategy. To avoid polymerisation of activated amino acids, Nα group must be protected. The choice of the Nα protecting group determines the entire synthesis strategy. A good protecting group is the one which can react with the amino or carboxylic group easily and when required, could be easily removed without affecting the peptide bond. Merrifield followed orthogonal protection method which means a set of different classes of protecting groups which can each be selectively removed in the presence of the other classes. In SPPS two major strategies followed are - Fmoc and Boc. The N-terminal of amino acid monomer is protected by these two groups and added in to a deprotected amino acid chain **[36]**. During the first 15 years of peptide synthesis, the Boc group has been used almost exclusively. When R. B. Merrifield invented SPPS, it was according to the tBoc method. To remove Boc from a growing peptide chain, acidic conditions, usually neat TFA are used. Removal of side-chain protecting groups and the peptide from the resin at the end of the synthesis is achieved by incubating in hydrofluoric acid which can be dangerous; for this reason Boc chemistry is generally disfavoured. However for complex synthesis like synthesis of non-natural peptide analogues which are base-sensitive such as depsi-peptides, Boc is necessary. In short, Boc SPPS is recommended mainly for instantaneous cleavage of the peptide affixing

linkage and side chain protecting groups with strong acid, while Fmoc SPPS is designed principally to achieve the same cleavages with moderate strength acids. In both cases, scavengers are used so as to diminish the side reactions **[37].**

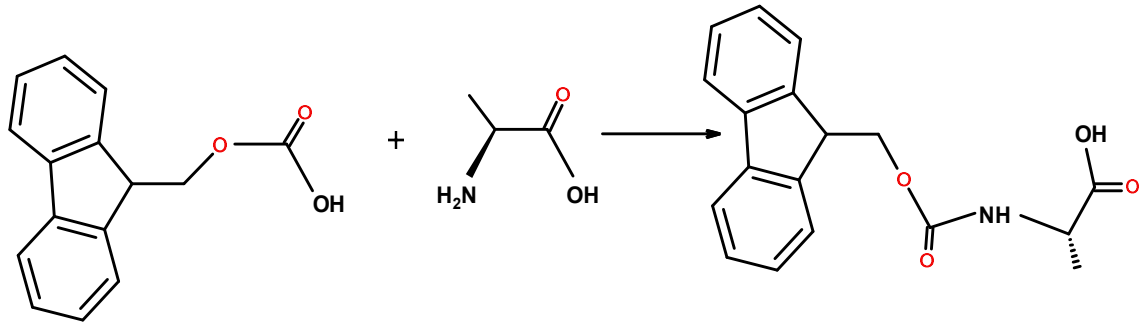


Fmoc Glycine BOC Glycine

Fig.2.3. Amino protected Glycine with Fmoc and BOC groups.

The combination of Fmoc/tBu has met the requirements of peptide synthesis and broadened the scope of SPPS. In the present work Fmoc/tBu strategy is followed. Usually in this method t-Butyl and trityl- based side chain protection groups and alkoxybenzyl based linkers which can be removed with TFA. TFA is an excellent solvent for SPPS **[38]**. Since it is volatile, it can be easily removed by evaporation. C- terminal residue is anchored to a TFA labile linker. Coupling is carried out in DMF or NMP with preformed active esters or using activation reagents that generate benzotriazolyl esters. Convenience of cleavage reaction and the ease with which the method can be adopted to multiple peptide synthesis are the reason for the popularity of Fmoc/tBu approach **[39]**. Side chains containing reactive functional groups like –COOH,-OH, -NH,-SH are present in majority of amino acids commonly present in proteins. These side groups must be masked before using them for coupling **[40]**. Usually the protective groups which can be removed by TFA are employed as this allows the peptide to be totally deprotected at the

same time as it is cleaved from the support. A wide range of groups are present which can be selectively removed thus enabling the selective modification of side chains of individual residues within the peptide chain.



Fmoc-Cl L-Alanine Fmoc-L-Alanine

Fig.2.4. Protection of Alanine with Fmoc

Some of the amino protecting and carboxylic acid protecting groups in use are given below.

Table 2.2. Protecting groups

Protecting groups	**Removal**
Amino protecting groups	
Carbobenzyloxy (Cbz) group	Removed by hydrogenolysis **[41]**
p-Methoxybenzyl carbonyl (Moz or MeOZ) group	Removed by hydrogenolysis
tert-Butyloxycarbonyl (BOC) group	Removed by concentrated strong acid or by heating to >80 °C **[42]**
9-Fluorenylmethyloxycarbonyl (FMOC) group	Removed by base, such as piperidine
Benzyl (Bn) group	Removed by hydrogenolysis **[43]**

Protecting groups	**Removal**
Carbamate group	Removed by acid and mild heating
p-Methoxybenzyl (PMB)	Removed by hydrogenolysis more labile than benzyl **[44]**
3,4-Dimethoxybenzyl (DMPM)	Removed by hydrogenolysis, more labile than *p*-methoxybenzyl
Tosyl (Ts) group	Removed by concentrated acid & strong reducing agents
p-methoxyphenyl (PMP) group	Removed by ammonium cerium(IV) nitrate
Carboxylic acid protecting groups	
Methyl esters	Removed by acid or base **[45]**
Benzyl esters	Removed by hydrogenolysis **[46]**
tert-Butyl esters	Removed by acid, base and some reductants **[47]**
Silyl esters	Removed by acid, base and organometallic reagents **[48]**
Orthoesters	Removed by mild aqueous acid to form ester, which is removed according to ester properties.
Oxazoline	Removed by strong hot acid or alkali

Deprotection reaction is a vital step in Fmoc-SPPS since it ensure good quality product. A good deprotection reagent should satisfy many conditions. Basicity and polarity, toxicity, concentration and availability of reagent are some of the factors which must be considered during the selection of reagent. 20% piperidine in DMF is an excellent reagent usually used for the deprotection of amino group. It is highly reactive and care must be taken to avoid the contact of this reagent with the peptide synthesiser. O.F Luna used 4-methylpiperidine, piperidine, piperazine reagents to remove Fmoc

protection. The study showed the three reagents are interchangeable and suggests the replacement of piperidine will be advantageous considering its toxicity and handling **[49]**.

2.5. Coupling in SPPS- Formation of amide bond

The formation of amide bond is the key step in peptide synthesis. Chemically, the peptide bond is a covalent bond that is formed between a carboxyl group and an amino group by the loss of a water molecule. Although the peptide bond formation can be reversed by the addition of water, amide bonds are very stable in water at neutral pH, and the hydrolysis of peptide bond in cells is also enzymatically controlled **[50]**. The reagent used for coupling should be able to form the peptide bond in mild conditions; the reaction should be fast and should retain the optical integrity of the peptide during the synthesis. Chemical peptide synthesis necessitates activation of the carboxyl group of an amino acid to enable the nucleophilic attack by amino group of the second amino acid.

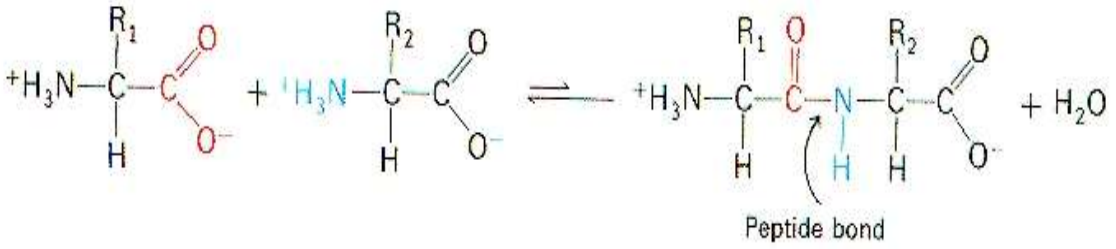
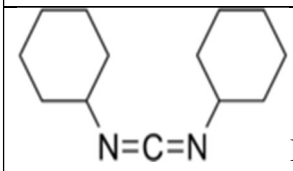
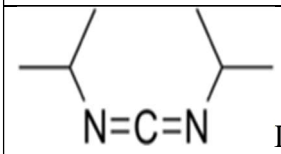
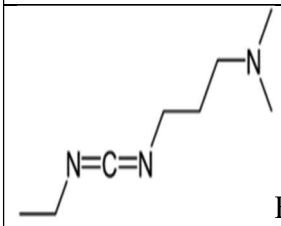
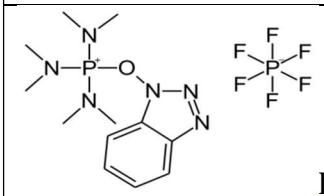
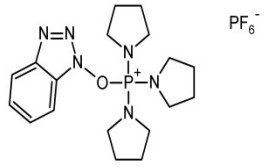


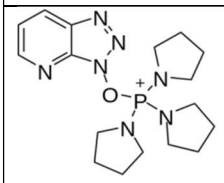
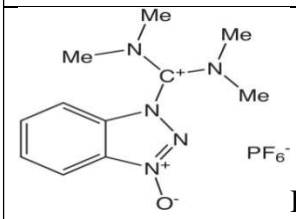
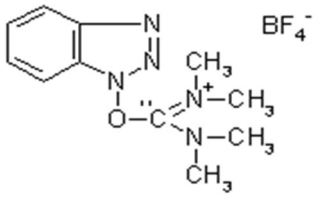
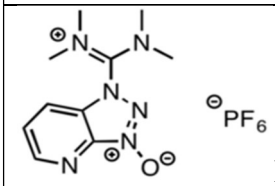
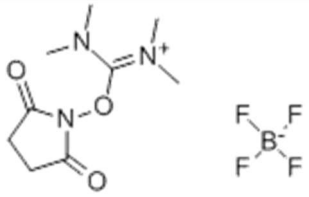
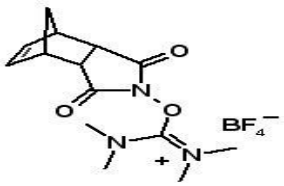
Fig.2.5. Formation of an Amide bond.

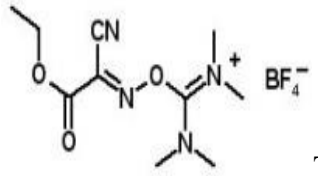
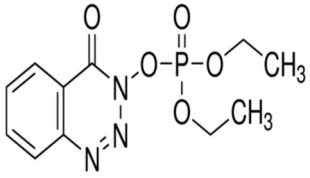
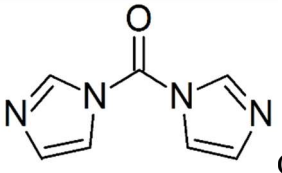
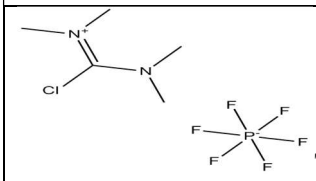
The first coupling reagents synthesised was Carbodiimides. Dicyclohexylcarbodiimide and diisopropylcarbodiimide are common reagents used to

prepare esters, amides and anhydrides from carboxylic acids. DCC is useful in solution phase reactions since the dicyclohexylurea by-product formed from DCC is insoluble in most organic solvents which can be removed by filtration. But for reactions on solid support DCC is not suitable for the same reason. The solubility of urea byproduct formed when DIC is used as coupling reagent is a solution for this problem as mentioned earlier **[51]**. Both the reagent and urea byproduct formed are water soluble in the case of ethyl-(N, N'- dimethylamino) propylcarbodiimide hydrochloride ,EDC. So they can be removed by aqueous extraction. Carpino compared DIC with EDC and some unsymmetrical carbodiimides and found DIC is most efficient coupling reagent when used with HOAt **[52]**. Izdebski proposed two other carbodiimides BMC and BEC, but they found to have no benefit over DIC **[53]**. To avoid the problems related with carbodiimide reagents, many alternative reagents were developed thereafter. The first introduced phosphonium coupling reagent was BOP. Its carcinogenic and respiratory toxic nature leads to the immediate discovery of another coupling reagent PyBOP **[54]**. AOP and PyAOP were prepared by Carpino and a comparative study was made with BOP and PyBOP **[55]**. The study showed the high reactivity of aza- derivatives. Among the aminium coupling reagents, HATU and HBTU shows excellent reactivity. Although a number of immonium coupling reagents like BOMI, BDMP, BPMP, BMMP, and AOMP were introduced by Li, they were not compared with standard reagents. Some of the commonly used coupling reagents are given below.

Table.2.3. Coupling reagents in Peptide synthesis

Coupling reagents	Features
Carbodiimides	
 DCC	• Mainly applicable in solution phase synthesis. • Forms insoluble byproduct. • Low racemization when used with HOBt or HOAt **[56]**.
 DIC	• Mainly applicable in solid phase synthesis. • Urea byproduct is soluble and remains in solution • Efficient at elevated temperatures.
 EDC	• Mainly applicable in applications like modifying proteins. • Reagent and by product are water soluble
Phosphonium	
 Bop	• Mainly used in preparing esters under mild conditions. • Does not generate dehydration byproduct. • Minimum racemization and side reactions **[57]**.
 PyBOP	• Efficient as BOP. • Rapid coupling. • Less hazardous byproduct.

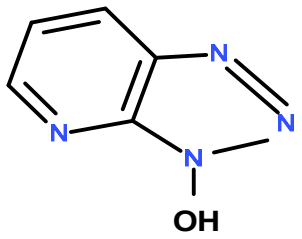
Coupling reagents	Features
 PyAOP	• Mainly useful in difficult coupling,such as coupling N-methylamino acids. • Highly reactive. • Cyclizes linear peptides.
Aminium	
 HBTU	• Very efficient. • Little racemization. • Coupling complete in as little as 6 minutes. • May cause allergic reactions **[58]**.
 TBTU	• Mainly used in macrocyclysation. • Reactivity similar to HBTU.
 HATU	• Reacts faster than HBTU. • Very little racemization **[59]**.
Uronium	
 TSTU	• Coupling in aqueous solutions. • Conjugating oligosaccharides to proteins. • No racemization when used with HOSu and $CuCl_2$.
 TNTU	• Little racemization. • Conjugating biomolecules to linkers. • Couples in aqueous solutions.

Coupling reagents	Features
 TOTU	• Used to prepare PNA monomers from 2-amino ethyl glycine derivatives. • Couples acids to secondary amines.
Other reagents	
 DEPBT	• Mainly used for coupling easily epimerised amino acids like arylglycines. • Good for head-to-tail cyclysation of linear peptides. • Very little epimerisation **[60]**.
 CDI	• Mainly used for coupling peptide fragments to form large peptides and small proteins. • Used in the preparation of urea dipeptides **[61]**.
 TCFH	• Very reactive. • Good for forming esters.

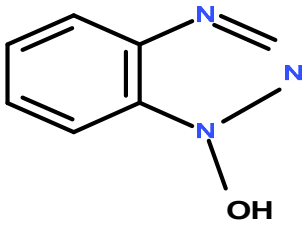
Out of the several alternatives suggested instead of the coupling reagents, native chemical ligation by Bode and ligation of amines to alcohols using a ruthenium complex as catalyst by Milstein are found to be more relevant **[62, 63]**.

2.5.1. Coupling Additives

Carbodiimide activation of amino acids often causes partial racemization of the amino acid. Koenig and Geiger introduced the addition of 1- hydroxyl benzotriazole to minimise this problem. The OBt esters that form as intermediates couple with primary amines with little racemization. L.A carpino demonstrated that the advantage of DIC/HOAt over DIC/HOBt for coupling reactions is due to the effect of the neighbouring nitrogen atom **[64]**. Structures of some common coupling additives are given below.



HOAt



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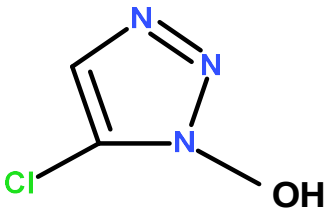

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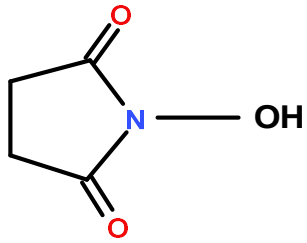

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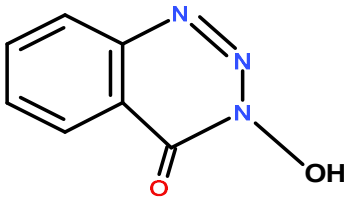

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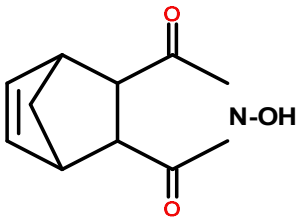

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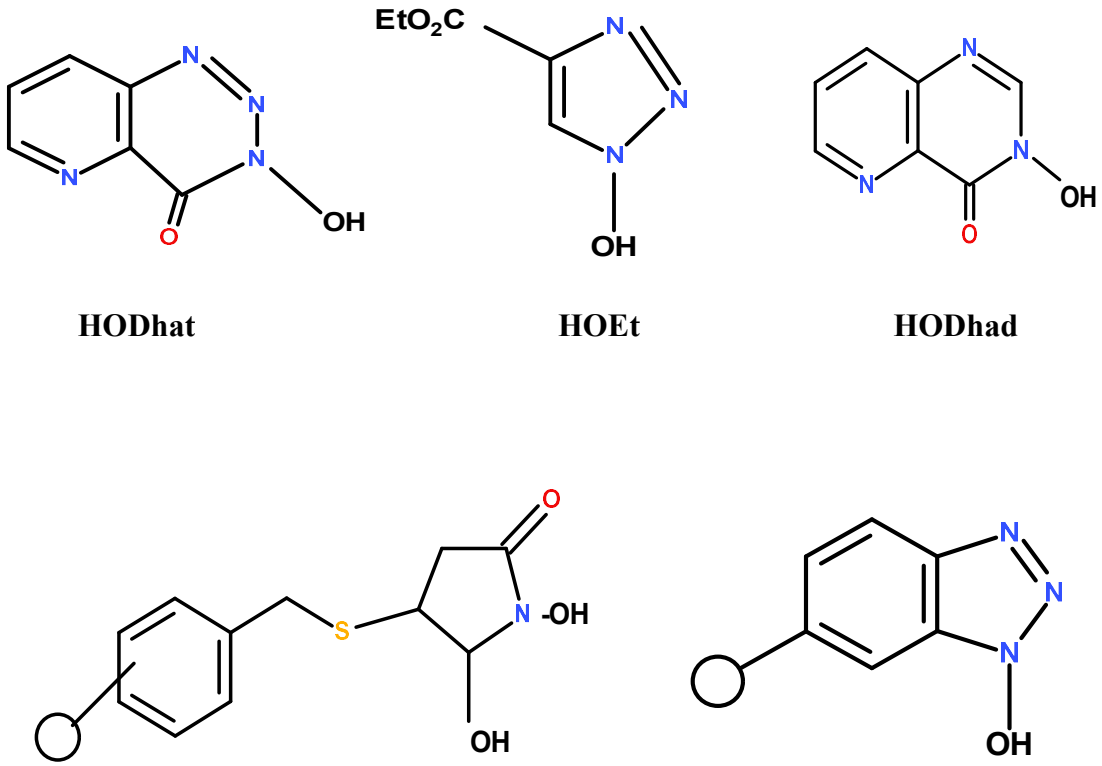

Fig.2.6. Some common coupling additives.

2.6. Monitoring of coupling and deprotection

Monitoring the completion of coupling and deprotection is essential in SPPS. There are a number of colour tests for qualitative monitoring. Performing two independent methods will give more accurate results. In the case of long peptides, double coupling followed by acapping step will be more effective because, with increase in peptide length, detection of free amino group becomes difficult. These tests are performed with small samples of washed resins.

Kaiser test is the most popularly used method to monitor coupling and deprotection reactions. It is based on the reaction of ninhydrin with amines. The presence

of intense blue colour shows primary amines while secondary amines are detected by brownish red colour. To perform the test, three test solutions are needed. Solution A- 5g ninhydrin in 100 ml ethanol, Solution B – 80g phenol in 20 ml ethanol, Solution C – 2ml .001M aq. KCN in 98 ml pyridine. A small sample of washed resin is placed in a small test tube and 2-5 drops of each solutions are added. The tube is placed in an oven and left for 5 minutes at 100° C. Presence of blue colour indicates positive test. Ninhydrin is useful for monitoring the progress of coupling reactions **[65]**.

TNBS test or 2, 4, 6- trinitrobenzenesulfonic acid test can only be used for detecting primary amino groups. To perform the test, Solution A – 10% DIPEA in DMF and Solution B – 1M aq. TNBS are needed. To a small sample of washed resin, added 1-3 drops of the above solution. After mixing, the mixture is left at room temperature for 10 minutes and inspected the beads. Intensely orange or red colour shows positive test **[66]**.

All types of amines can be detected by applying Bromophenol blue test **[67]**. The reagent used is 0.05 % bromophenol blue in DMA. 10-15 drops of reagent is added to washed resin beads and immediately checked. Blue colour shows positive test.

To monitor the progress of the reaction and to examine the purity of the final product TLC can be used. It can be used to detect small and medium sized peptides. A glass plate coated with silica gel containing calcium sulphate binder was heated for 4 h at 100° C to activate. It was cooled and used to perform TLC. The peptide was made in to a solution in methanol and spotted on the plate. It can be developed using appropriate solvent mixture. Butanol: Acetic acid: water mixture can be used in 6:1:5 or 4:1:1 composition. The developed plate can be visualised by spraying a solution of ninhydrin in

acetone and noting the presence or absence of pink colour of free amino group. Iodine can be used as a detector in which brown spots of amino acids can be observed.

2.7. Detachment of the peptide from solid support.

TFA is the most widely used cleaving agent in SPPS **[68]**. By using photolysis, fluoride ion, alkali or hydrogenation, peptide can be separated from the resin in fully protected mode or with unique peptide carboxyl termination. Trimethyl silyl bromide/TFA in the presence of thioanisole and m-cresol and ethanedithiol has been reported as a cleaving reagent by Yajima **[69]**. A mixture of trimethyl silyl triflouromethane sulphonate, TFA and thioanisole was also reported as a cleaving agent by N. Fuji **[70]**. The use of Reagent K for the cleavage of peptide from resin was explained by D.S. King et al. Reagent K is a mixture of 82.5% TFA, 5% phenol, 5% water, 2.5% EDT **[71]**. Time required for the cleavage of peptide from the resin depends on the linker between the peptide chain and the solid support.

2.8. Purification and characterisation of the product

The Final stage of peptide synthesis is careful purification and suitable characterisation of the product obtained. Introduction of HPLC caused a revolution in the field of peptide purification and analysis. The method was very efficient to establish the homogeneity of the peptide because of its high resolving power and operation speed **[72]**. NMR spectroscopy is the unique technique available for determining high-resolution structures of biomolecules in solution **[73]**. It is the only method available for the determination of three dimensional structures of peptides and proteins in solution at atomic resolution **[74]**. Mass spectrum confirms the formation of target peptide **[75]**.

Amino acid analysis is an important method to determine the purity of the peptide. Circular dichroism is an excellent tool for rapid determination of the secondary structure and folding properties of proteins that have been obtained using recombinant techniques. The most widely used applications of protein CD are to determine whether a protein is folded or mutation affects its stability **[76]**.

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Chapter 3

Synthesis, Characterization and functional group interconversion of GDMA-4-VP resin

3.1. Introduction

The field of research in modern peptide chemistry comprise synthesis and analysis, isolation and structure determination, conformational investigations and molecular modelling. The increased demand for synthetic peptides resulted in the development of new solid supports with better qualities like high swelling in broad range of solvents, high mechanical and chemical stability and optimum hydrophilic-hydrophobic balance. Considering all these conditions, we have developed a new solid support by cross linking hydrophilic Glycerol dimethacrylate with hydrophobic 4-vinylpyridine. The secondary hydroxyl functionality of the cross linker is used for the assimilation of the C- terminal amino acid and the successive growth of the peptide. The present chapter describes the synthesis of GDMA-4-VP resin, its swelling and stability studies and functionalization with chloro and amino groups.

3.2. Experimental Details

3.2.1. Materials and Methods

Glycerol dimethacrylate, Polyvinyl alcohol, Benzoyl peroxide, 4-Vinylpyridine were purchased from Sigma-Aldrich. Toluene, sodium hydroxide, dimethylformamide, dichloromethane, Trifluoroacetic acid, Pyridine, Acetic anhydride, Ammonia, Piperidine,

Acetone, Dioxane, Ether, Thionyl Chloride, Tetrahydrofuran, Nitric acid, Silver nitrate, Ammonium thiocyanate, N-Methylpyrrolidone, Potassium phthalimide, hydrazine hydrate, Cyclohexane, Calcium chloride, Sodium carbonate, Ethyl acetate, methanol and chloroform were purchased from Merck group.

IR spectrum was recorded in a Thermo Nicolet, Avatar 370 model FT-IR spectrometer working in a spectral range of 4000-400 cm $^{-1}$ with resolution 4 cm^{-1}. SEM image was taken in a JEOL model-JSM-6390LV model scanning electron microscope with tungsten filament at a specimen stage 4 nm. NMR spectra was taken on a Bruker Avance III, 400 MHZ model FT-NMR spectrometer with a 9.4 Tesla super- conducting magnet at an operational temperature 360°C.

3.2.2. Purification of Solvents

Dichloromethane was purified by keeping over anhydrous sodium carbonate and then distilled and kept in dark coloured bottle. N-Methyl-2-pyrrolidone was distilled under reduced pressure and kept over molecular sieve in dark coloured bottle. Trifluoroacetic acid was distilled over 10% concentrated (v/v) sulphuric acid to eliminate water and extraneous substances. Repeated distillation was avoided considering the chance of formation of anhydride and kept in amber coloured bottle. Diethyl ether was dried over fused calcium chloride overnight. All other solvents were purified with standard procedures.

3.2.3. Destabilisation of GDMA and 4-VP

NaOH (1 g) was dissolved in 100 ml distilled water to prepare a 1% aqueous solution. 25 ml of 4-VP was added to 15 ml of NaOH taken in a separating funnel and

shaken well and allowed to settle. Then the destabilised 4-Vinyl pyridine is collected in well stoppered bottle. Repeated for three times and it was treated with anhydrous calcium chloride. Similarly 10 ml GDMA was destabilised by using 1% NaOH and treated with anhydrous $CaCl_2$. Inhibitors were removed by washing with distilled water.

3.2.4. Synthesis of GDMA-4-VP resin

3.2.4.1 Suspension polymerisation

The polymerisation was carried out in a conventional suspension polymerisation reactor. Glycerol dimethacrylate and 4-Vinylpyridine were destabilised using 1% NaOH. 1 g PVA was dissolved in 100 ml distilled water at 80^{o}C to prepare a 1% aqueous solution and added to the reaction vessel. The solution was deoxygenated by bubbling nitrogen gas. The amount of monomer and cross-linker required for the synthesis of a particular mol percentage can be calculated using the same equation given below.

Amount of Monomer = Mol.wt of monomer × mol% ÷ 1000 × Density.

To synthesise 1% GDMA-4-VP resin, GDMA (0.20 ml), 4-VP (10.67 ml) along with toluene (10 ml) were added to the reaction vessel. Benzoyl peroxide (600 mg) was added to PVA solution keeping it stirred mechanically at 2000 rpm. The temperature of the system was maintained at 80^{o}C using a thermostated oil bath and the reaction was continued to 6 h. Resin beads began to appear on the wall of the vessel. The system was kept overnight as such. The beaded resin was then filtered and washed with hot water to remove PVA. The unreacted monomers were washed off using acetone, toluene and methanol. The polymer was soxheletted with acetone followed by methanol to remove all linear polymers and then dried. The beads were meshed to 100-200 range. The beads

were sieved and the major fraction was suspended in MeOH and repeatedly sedimented and decanted to remove remaining smaller beads. Washed with water and MeOH

3.2.5. Hydroxyl capacity of the resin.

Taken 1% GDMA-4-VP resin (100 mg) and allowed to swell DCM (10 ml) overnight in a stoppered bottle. It was then acetylated with acetic anhydride-piperidine mixture (1:10, 2 ml) for 6 h. A blank is also kept for refluxing. Added distilled water (10 ml) to each reaction mixture and it was refluxed for 3 h in a water bath. The mixture was cooled, filtered and acetic acid formed was back titrated with standard NaOH solution (0. I N). The blank titration was also carried out. From the titre values, hydroxyl capacity of the resin was calculated. If the volume of NaOH required for blank solution is taken as 'X' and the volume of NaOH required for resin is 'Y', then the capacity of the resin can be calculated using the equation

Capacity of resin = (X-Y) ×Normality of resin ÷ Weight of resin in gram

3.2.6. Swelling studies

1% GDMA-4-VP resin (100 mg) was taken in a syringe fitted with a sintered Teflon filter. The solvents were discharged from the top of the syringe and solvent flow was created by applying a continuous suction at the syringe outlet. The suction was structured to maintain a flow rate of 1 mL/min. The solvent was permitted to flow through the resin for 30 min. The syringe outlet was blocked and the resin was immersed in the solvent for 1 h. The swollen resin was pressed with the piston of the syringe and the pressure was gradually released. Current volume of the resin was noted and correlated to the sample weight to get the swelling ability of the resin. The experiment was repeated

to confirm reproducible values. The increase in weight of the solvent swollen resin beads were also noted and compared with the dry resin.

3.2.7. Chemical stability studies

The stability studies of the resin in various solvents such as 100% TFA, 20% piperidine in DMF, pyridine, 2 M aqueous NaOH , 2 M NH_2OH in aqueous methanol and ammonia were conducted.100 mg of the resin samples were stirred with 10 ml each of the above stated solvents. The resin samples were kept as such for 48 hrs. Then the respective resin samples were filtered and washed with ethanol (3 x 30 mL), water (3 x 30 mL), acetone (3 x 30 mL), DCM (3 x 30 mL), dioxane (3 x 30 mL), and ether (3 x 30 mL) and dried under vacuum. FT-IR (KBr) spectra of these resins were measured and compared with the spectrum of the untreated resin.

3.2.8. Functional group interconversions

3.2.8.1. Chlorination

1% GDMA-4-VP resin (100 mg) was immersed in DCM (25 ml). Excess solvent was filtered off after 1 h. Thionyl chloride was added drop wise to the swollen resin. The reaction mixture was heated at 55ºC for 6 h with occasional stirring. The reaction mixture was cooled, washed with THF (5 x 10 mL), THF/H_2O (1: 1, 5 x 10 mL), THF (5 x 10 mL), DCM (5 x 10 mL), Methanol (5 x 10 mL) and ether (5 x 10 mL) and dried under vacuum. The above procedure was followed for chlorination of resins with higher cross-linking densities; the reactions were carried out using 10 m molar excess of the reagent with respect to hydroxyl capacity of the resin.

3.2.8.2. Estimation of chlorine capacity - Volhardt's method

1% Chlorinated GDMA-4-VP resin (100 mg) was digested with 5ml pyridine in a Kjeldhal's flask for 5 h at 100ºC. Pyridinium chloride thus formed was quantitatively transferred to a conical flask using 50% acetic acid (30 mL). Conc. HNO_3 (5 ml) was added to this followed by slow addition of standard 0.1 N $AgNO_3$ solution (10 ml) with stirring.50 ml water was added to the reaction mixture followed by the addition of sufficient amount of toluene to form a layer over water surface. The suspension was mixed well. The excess $AgNO_3$ was determined by back titration with 0.1N standard ammonium thiocyanate solution. Ferric alum was used as indicator and the end point is the appearance of a dark brown colour. A blank reaction was also conducted. From the titre values, the chlorine capacity of the resin was calculated **[1].**

3.2.8.3. Amination

1% Chloro GDMA-4-VP resin (100 mg) was swelled in excess NMP for 1 h and the excess solvent was filtered off. Potassium phthalimide in 20 ml NMP was added and the reaction mixture was stirred at 120ºC for 12 h. The resin was filtered and washed with NMP (5 x 10 mL), dioxane (5 x 10 mL), ethanol (5 x 10 mL), methanol (5 x 10 mL) and ether (5 x 10 mL) and dried in vacuum. The resin was immersed in distilled ethanol (20 mL) and hydrazine hydrate was added and refluxed for 8 h. The resin was filtered and washed with hot ethanol (5 x 10 mL), methanol (5 x 10 mL) and ether (5 x 10 mL) and dried under vacuum. Amination was also carried out for 2% GDMA-4-VP resin also. Amino capacity was calculated using standard picric acid method.

3.3. RESULTS AND DISCUSSION

3. 3.1. Synthesis of GDMA-4VP resin

GDMA-4VP RESIN support was prepared by suspension polymerisation. Benzoyl peroxide was used as the radical initiator and toluene was used as the diluent. The product obtained by bulk polymerisation has to be crushed and sieved to required particle size. But by suspension polymerisation, the polymer obtained was beaded which lends itself to further conversions. Principally suspension polymerisation is the most useful technique for preparing cross linked polymer support. Polyvinyl alcohol is the suspension stabiliser used here to prevent coagulation. A schematic diagram of GDMA-4-VP resin is given in Fig.**3.1.**

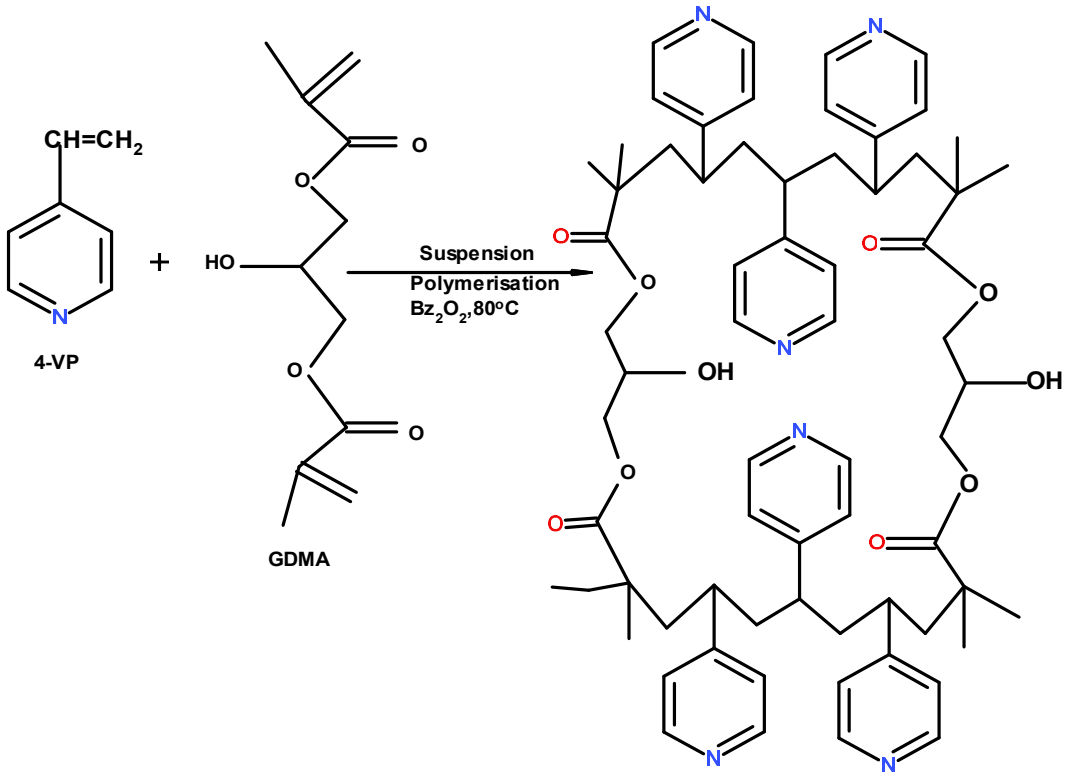


Fig. 3. 1. Suspension polymerisation of GDMA and 4-VP

GDMA-4VP supports with different cross linking densities of hydrophilic cross-linker (1%, 2%, 3%, 5%, and 10%) were prepared using the same procedure. The amount of monomer, cross linker and yield of the product obtained in each case is given in Table **3.1**. A random selection was made in the percentage of cross- linker. The yield of the peptide was about 95% after appropriate washing and soxhletting with various solvents to eliminate dissolved linear polymers and associated reagents. The yield and topography of the polymer support vary with the chemical nature of monomer, mole percentage of the cross linker, type of diluents, geometry of the reaction vessel and organic phase to aqueous phase ratio.

Table 3.1. Details of Preparation of GDMA-4VP resin

Mole percentage of GDMA	Volume of 4-VP in ml	Volume of GDMA in ml	Yield of the polymer in gram
1	10.67	0.20	8.05
2	10.56	0.40	8.73
3	10.46	0.61	9.23
5	10.24	1.01	9.72
10	9.70	2.03	11.27
15	9.16	3.05	14.94

From the table we can see the volume of monomer and cross linker corresponding to the percentage of crosslinking. The yield of the product was found to be increasing with the degree of cross linking. The polymerization reaction was carried out using various solvents such as carbon tetrachloride, toluene, cyclohexane and ethyl acetate. The yield of the product obtained was found to be almost same in all cases. Different solvents gave polymer with different mesh size. Suitable size for peptide synthesis, 100-200 mesh

size was found to be obtained when the diluent used was toluene as given in Table **3.2.** The cross linker used in each case was 2% GDMA. Beads in the range of 100-200 mesh in diameter offer a good balance of reaction kinetics versus reliability. Bead size is commonly reported in Tyler mesh size, which is inversely proportional to nominal diameter.100-200 mesh means 75 to 150 microns 200-400 mesh means35 to 75 microns.

Table 3.2. The bead size and yield of the resin obtained using different diluents.

Diluent	**Yield(g)**	**Bead size (Mesh Size)**
Cyclohexane	7.57	20-50
Chloroform	8.32	50-100
Ethyl acetate	8.79	50-100
Toluene	8.73	100-200

The size, shape and morphology of the peptide were studied using SEM. The insoluble polymer support was obtained as spherical uniform beads. The scanning electron micrograph of the resin is given in Fig **3.2.**

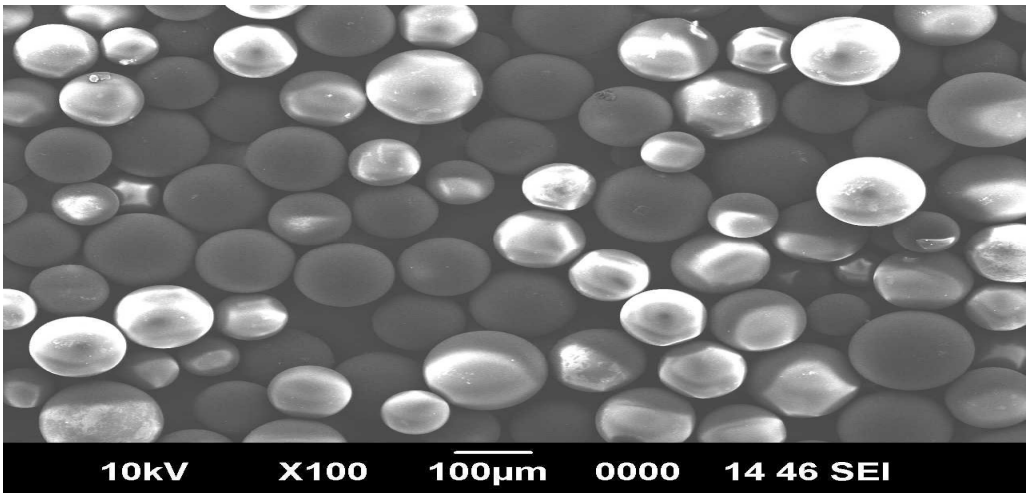


Fig.3.2. SEM image of Resin

The incorporation of cross-linking agent was confirmed by IR spectroscopy. It is the apt technique for studying the polymer identification. The IR spectrum of GDMA-4VP resin gave a sharp and intense peak at 1725 cm^{-1}corresponding to the ester carbonyl group of the cross linker. The broad peak at 3419 cm^{-1} corresponding to hydroxyl group of the cross linker. Peak at 1600 cm^{-1} show medium c=c stretching and that at 1418 cm^{-1} corresponds to medium –OH bending. A peak at 3030 cm^{-1}shows alkenyl C-H stretching. Absorption peaks above 3000 cm^{-1} are frequently diagnostic of unsaturation. So the obtained spectrum contains peaks corresponding to the monomer 4-VP and cross-linker GDMA.

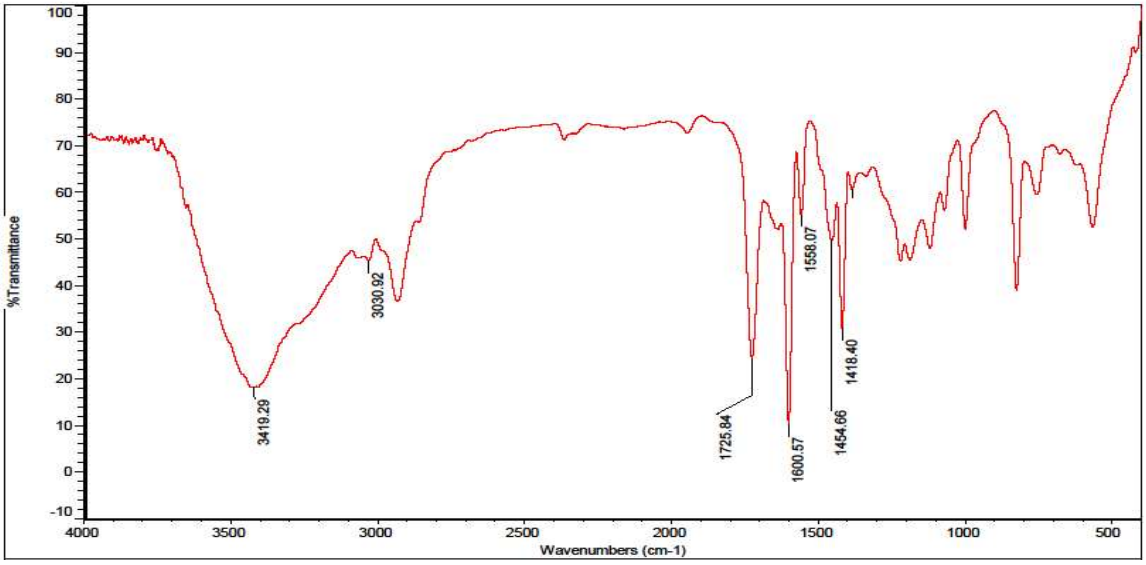


Fig.3.3. FT-IR spectral analysis of GDMA-4VP

The hydroxyl capacity of the resin was determined with standard procedures. It was found to be 0.78 mmol /g for 1% GDMA-4VP resin. The ^{1}HNMR spectrum of GDMA-4-VP resin is given in Fig.**3.4.** Only few peaks are seen in the spectrum.

Conventional NMR techniques are not suitable for the characterization of the cross-linked polymer support because of its insolubility in all types of solvents.

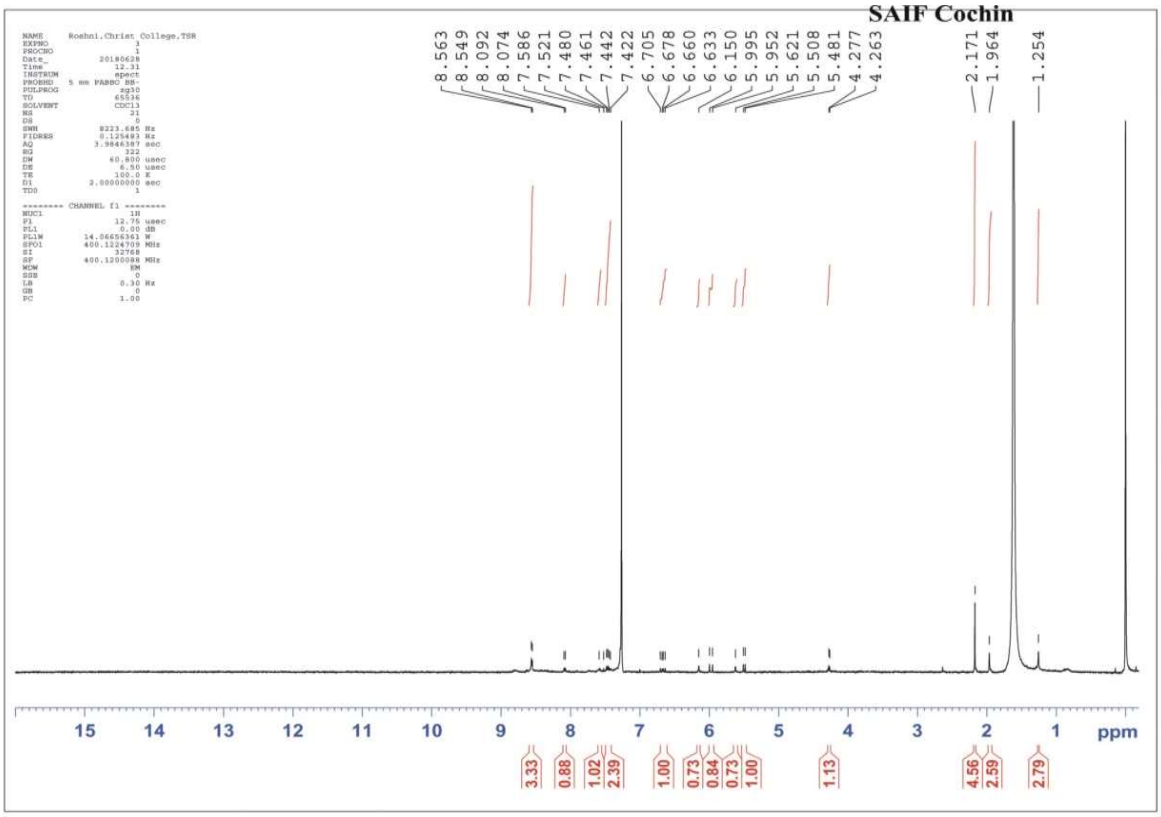


Fig. 3.4. ^{1}H NMR spectrum of GDMA-4-VP resin

3.3.2. Swelling studies

Swelling of a polymer in a specific solvent indicates the amount of solvation. Solvation is a necessary condition that favours the reaction. During swelling of the resin, the solvent diffuses in to the polymer bound functional groups. To enhance the chemical reaction, the reactive functional groups in the resin should have maximum accessibility towards the reagents. This will happen in the case of highly swelling resin. The GDMA-4VP resin showed good swelling properties in a wide range of solvents usually used for

SPPS. Swelling of the resin in four different solvents NMP, DCM, DMF and TFA was measured and represented graphically in Fig. **3.5**.

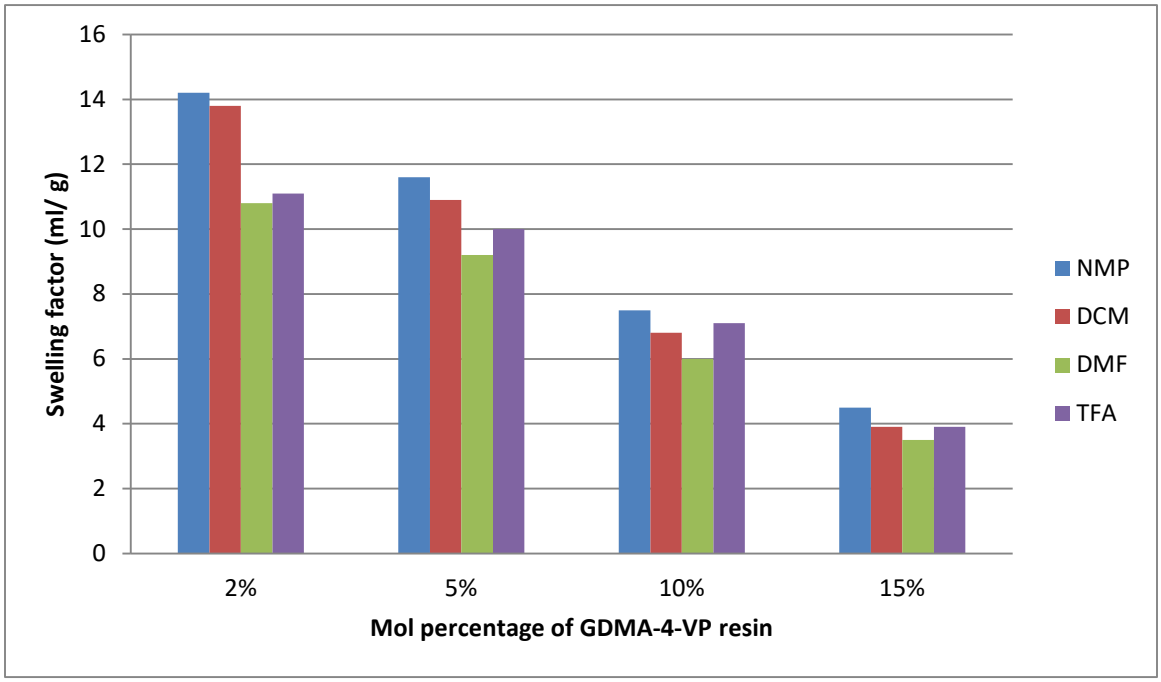


Fig. 3.5. Swelling comparison of GDMA-4-VP resin with different crosslinking.

From the graph it is evident that our resin shows maximum swelling in NMP. As the degree of crosslinking increases, swelling capacity is found to be decreasing. The study utilises a random selection of crosslinking percentage and it was found that 2% resin shows superior swelling properties.

The swelling of GDMA-4-VP resin was compared with that of Merrifield resin which is represented in Fig. **3.6.** The swelling volume of GDMA-4-VP resin obtained from the current study was compared with the values of Merrifield resin from literature. From the graph we can see GDMA-4-VP resin is far more superior to Merrifield resin in swelling.

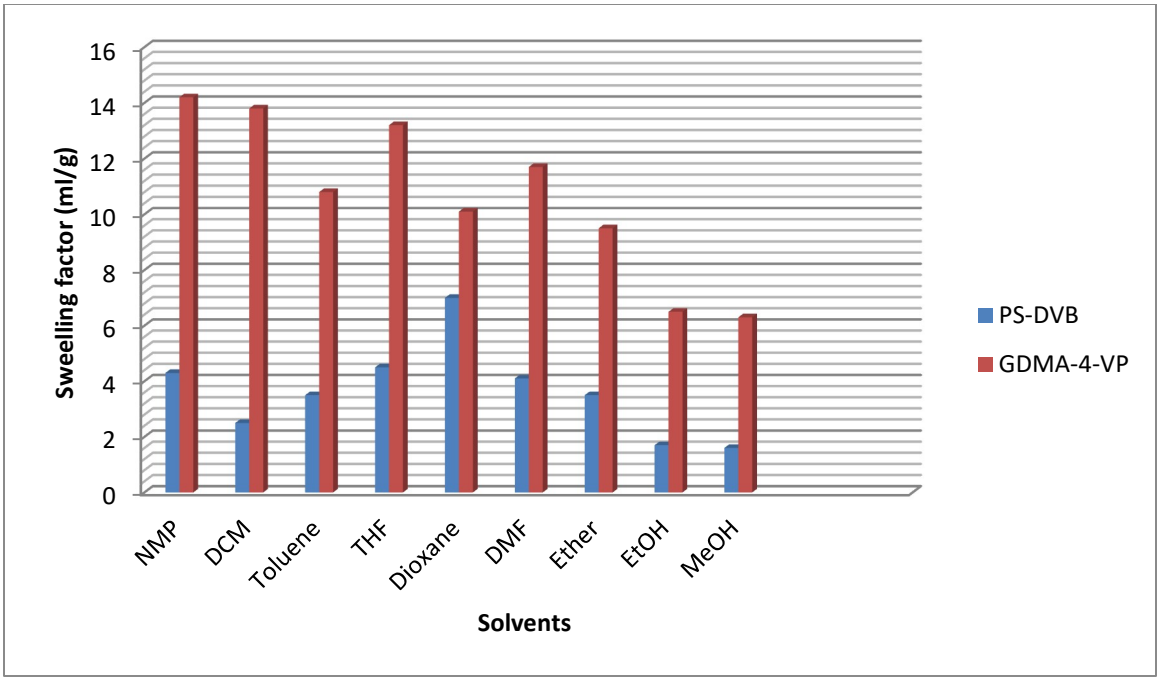


Fig.3.6. Swelling comparison of GDMA-4-VP with PS-DVB resin

3.3.3. Chemical stability studies

The polymer should be chemically stable throughout the synthesis until the peptide is cleaved from it. If any cleavage of cross-linker occurs in between, that will affect the purity and quantity of the product. There is a chance for the formation of some linear peptides or the integrity of polymer support may lose. So the stability of polymer support towards TFA is an important criterion for the peptide to be used in SPPS. The GDMA-4VP resin showed good stability in 100% TFA, 20% piperidine in DMF, pyridine, 2 M aqueous NaOH, 2 M NH_2OH in aqueous methanol and ammonia. The IR spectrum taken after 48 hours treatment with these resins showed similar peaks as there are in original resin. The IR spectra of GDMA-4VP resin treated with TFA and Piperidine in DMF is shown below in Fig.**3.7** and Fig.**3.8.**

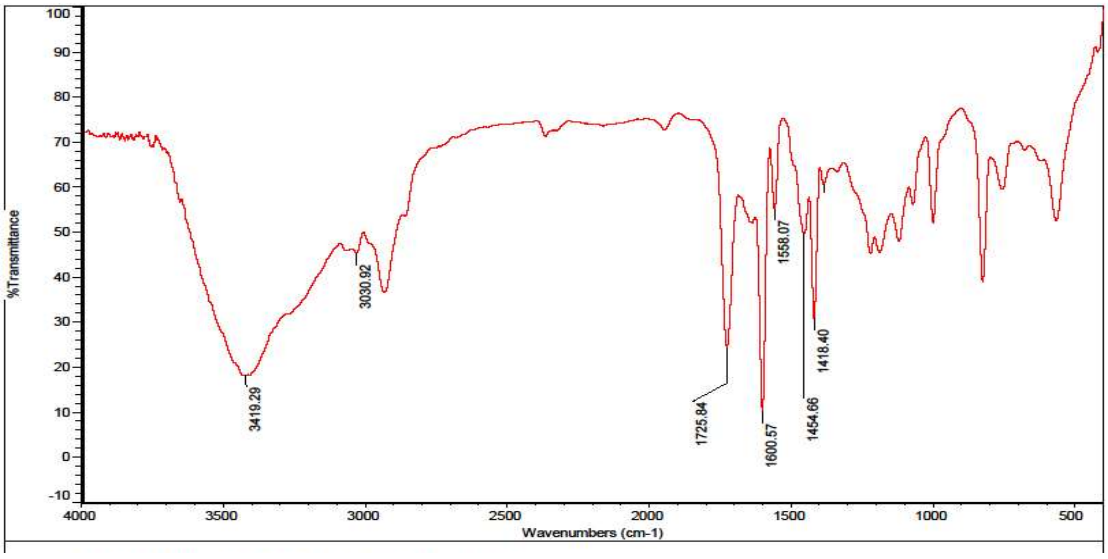


Fig.3.7. IR spectrum of GDMA-4-VP in 20% piperidine in DMF

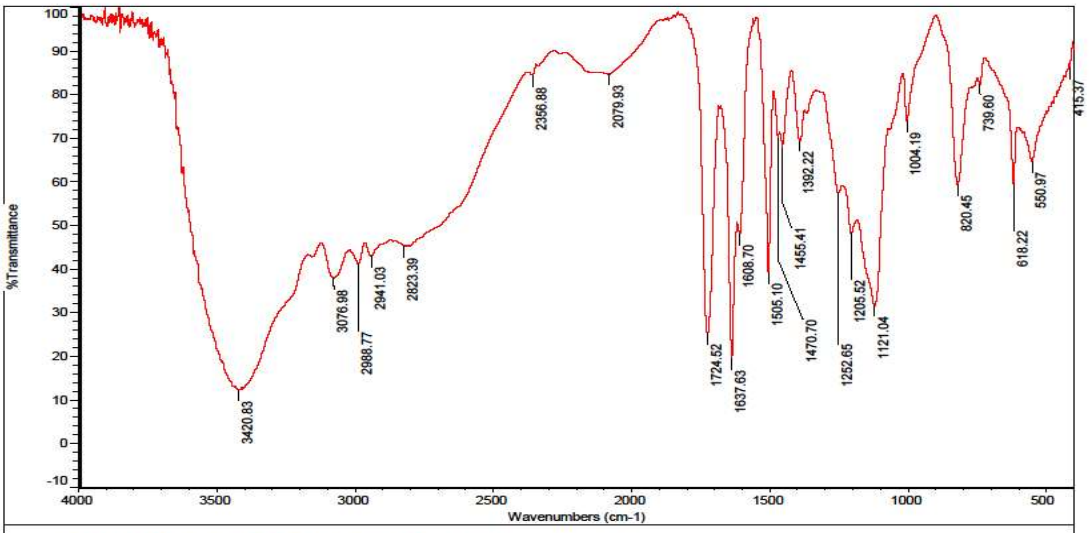


Fig.3.8. IR spectrum of GDMA-4-VP resin in TFA

3.3.4. Functional group interconversions

The –OH functional group present in GDMA-4-VP resin was converted to chloro and amino group by standard procedures. The conversion was found to be nearly quantitative. Chlorination and amination reactions were performed using thionyl chloride and Gabriel

phthalimide synthesis respectively. The calculated capacity of chloro functionalised resin was 0.77 mmol/g and amino functionalised resin was 0.75 mmol /g. A schematic representation of functional group conversion is given in Fig. **3.9.**

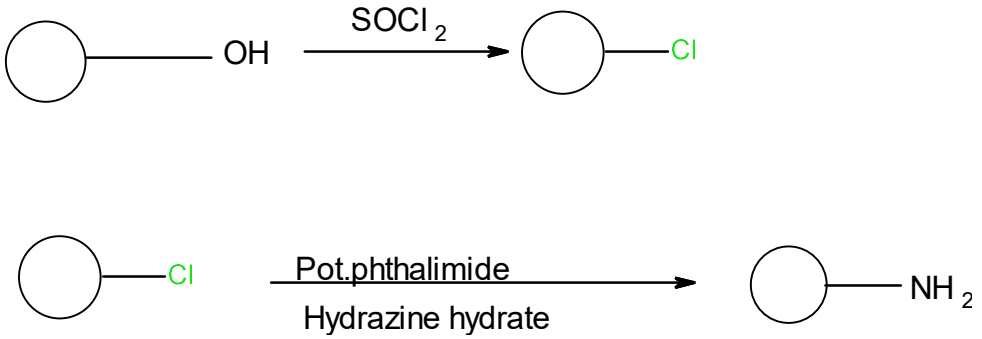


Fig.3.9. Schematic representation of functional group conversion

Scheme for conversion of chlorinated resin in to aminated resin is given in Fig.**3.10.** In Gabriel synthesis, Potassiun phtalimide when reacted with an alkyl halide is converted in to N-alkyl pthalimide which on hydrolysis yield primary amine.

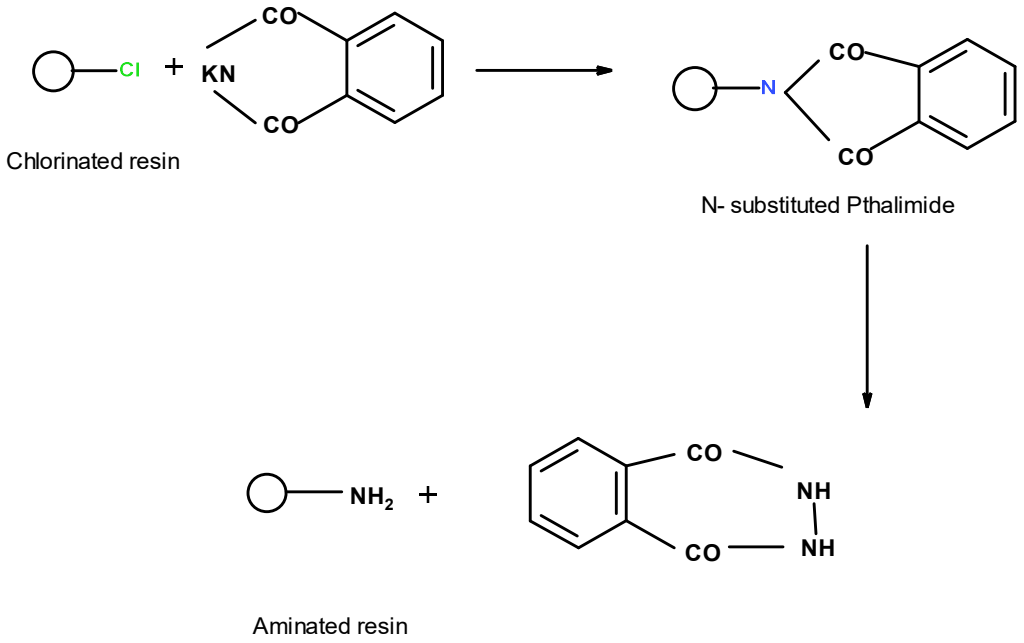


Fig.3.10. Conversion of chlorinated resin to aminated resin

The IR spectrum of aminated resin is given in Fig.**3.11**. Two NH stretch absorption produced in the range of 3200- 3400 shows the formation of aminated resin.

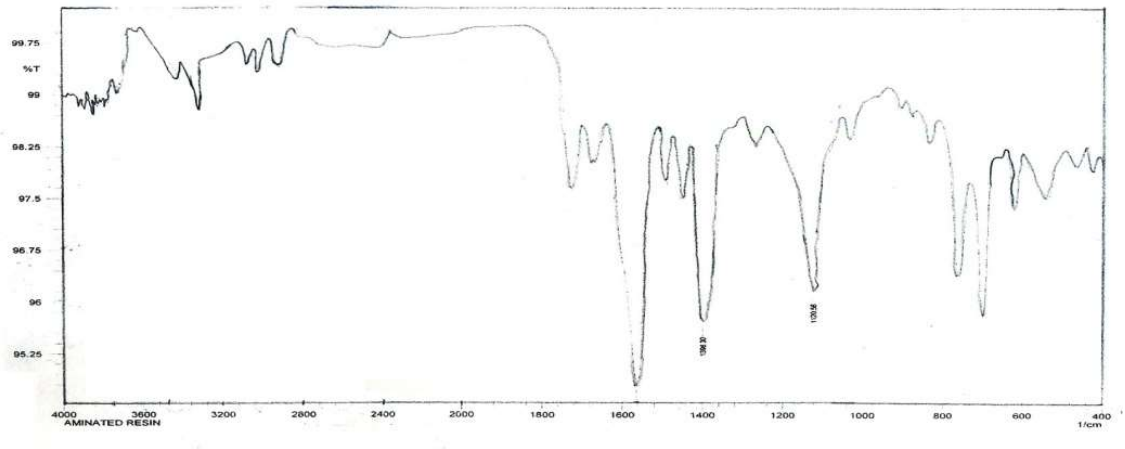


Fig.3.11. IR spectrum of aminated resin

References

1. Sasikumar, P.G, Arunan, C, Pillai,V.N.R. (2002). Synthesis and optimization of tri (propylene glycol) glycerolate diacrylate cross-linked polystyrene resin in polypeptide synthesis: role of the macromolecular support in solid phase peptide synthesis., Journal of the chemical Society,24.

Chapter 4

Study on the Efficiency of GDMA-4-VP resin as solid support in SPPS – A comparison with Merrifield resin and Wang resin

4.1. Introduction

Synthesis of products with low cost, less pollution, least time and good yield is always the aim of chemical industry. Researches are going on with this aim in the synthesis of peptides also. Peptides can be hydrophobic or hydrophilic depending on the nature of amino acids present in the sequence. Compared to hydrophilic peptides, the synthesis of neutral peptides are difficult because of its solvation and intermolecular aggregation property since SPPS is carried out in homogeneous medium. In this chapter, the efficiency of Glycerol dimethacrylate cross-linked 4-Vinyl Pyridine support for peptide synthesis using solid phase methodology was studied by synthesising two biologically active peptide fragments VYGR and KVKRIILARS using F-moc strategy. VYGR and KVKRIILARS sequences contain equal number of hydrophobic and hydrophilic amino acids which will affect the coupling efficiency and will reduce the yield of the final product. Therefore they are considered as 'difficult peptide sequences' **[1]**. So some special resin is needed for the synthesis of these two peptides. GDMA-4VP resin was proved to be suitable for the synthesis of these difficult sequences because of its high swelling and solvation power. The same peptide fragments were prepared on Merrifield resin and Wang resin respectively and a comparison was made.

VYGR is a peptide fragment which is capable of binding to insulin or insulin type growth factor receptors with either agonist or antagonist activity. The structure of VYGR tetra peptide is given below in Fig. **4.1.**

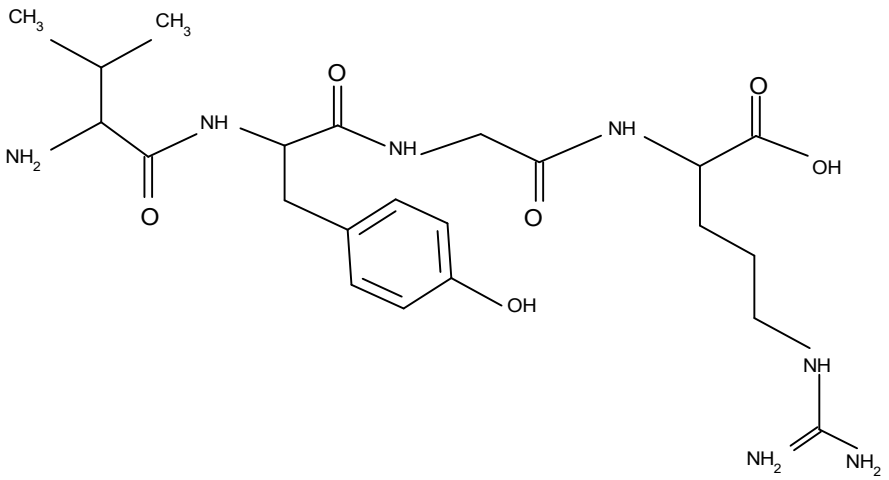
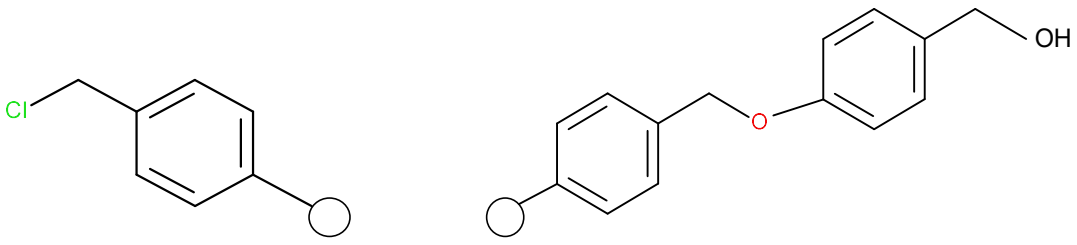


Fig. 4.1. Structure of VYGR

The structures of Merrifield resin and Wang resin are shown in Fig. **4.2.**



(A) (B)

Fig. 4.2. Structure of Merrifield resin (A) and Wang resin (B)

4.2. Experimental Details

4.2.1 Materials and methods

All amino acids, HOBt, PyBOP, TIS, DIC, DIPEA, and DMAP were purchased from Sigma Aldrich, Germany. TFA, Chloranil, Ninhydrin, Petroleum ether, Piperidine were purchased from Merck.

The HPLC system used was a Shimadzu LCMS-2010EV model with RP-C 18 column diameter 150 mm × 2.6 mm, 25 cm length, 5 μm particle size, run time 30 min, linear gradient 5% acetonitrile : 95% water at 0 min, 100% acetonitrile : 0% water at 30 min., flow rate 1 ml per min.

ESI-MS spectra were recorded on Finnigan MAT SSQ 710 spectrometer equipped with an IBM PS1295 x p486 in positive ionization mode with $CH_3CN/H_2O/CH_3COOH$--50:50:1 as solvent.

4.2.2. Synthesis of VYGR on GDMA-4-VP resin

4.2.2.1. Esterification of Fmoc-Arg (pbf) to polymer support.

GDMA- 4 -VP resin (120 mg, 0.78 mmol, 100-200 mesh size) was taken in a peptide synthesizer and swelled in minimum dry DCM for 1 hour. Fmoc-Arg (pbf) (3 eq) was dissolved in minimum dry DCM in a tiny R.B flask and Diisopropylcarbodiimide (3 eq) and Dimethylaminopyridine (0.1 eq) were added to the resin and shaken well. After 1 hour, the excess reactants were filtered off from the synthesiser and coupling process is repeated on the resin using the same procedure. After 30 min the reactants were washed off with DMF and NMP (each 5 x 3 ml). DMF used was degassed with nitrogen taking 2

litre DMF in a bottle and passing a continuous flow of nitrogen about 2 hours. NMP bottle was closed immediately after taking in order to avoid contact with air. The Fmoc protection was removed by 20% Piperidine in DMF (2 x 3 ml). After 30 min, the reagents were washed off with sufficient volume of DMF followed by NMP. Keiser test was conducted to ensure the completion of reaction. A pinch of resin beads were taken in a small glass tube and washed with ethanol several times. Ninhydrin solution was added and placed the sample for five min. Presence of blue colour shows completion of the reaction. Freshly prepared ninhydrin was used for each synthesis.

4.2.2.2. Coupling of GLY-TYR-VAL

Fmoc-Arg (pbf)-resin in the peptide synthesizer was wetted with minimum DCM. Fmoc-Gly (2.5 eq) was attached to the resin in the presence of minimum DCM, HOBT (2.5 eq), DIC (2.5 eq) and DIEA (0.3 eq). After 45 min, the resin was filtered and washed thoroughly with DMF (5 x 3 ml) and NMP (3 x 3 ml). The completion of the reaction can be tested with Keiser test. The absence of blue colour shows completion of the reaction.

The remaining amino acids in the sequence Fmoc-Tyr, Fmoc-Val were successively incorporated using the above procedure. Resin was washed with DMF and NMP. Acetylation reactions were performed twice for confirming the quantitative conversion. Each coupling and deprotection step was monitored by Kaiser Test. After the synthesis, Fmoc protection was removed and the resin was washed with DMF and NMP. The details of synthesis are given in Table **4.1.**

4.2.2.3. Detachment of peptide from the resin

The peptidyl-resin was suspended in TFA (9.75 ml) and TIS (0.25 ml) at room temperature for 2 Hrs. The resin was filtered and the filtrate was collected in an R. B. flask. The resin washed several times with TFA and all the washings were collected. The collected filtrate was evaporated using Rota vacuum evaporator to a small volume by removing excess TFA. The peptide was precipitated by adding ice cold ether, washed thoroughly with ether and was collected by centrifugation in white colour.

Table 4.1. Details of Synthesis of VYGR

Amino Acid Residues	**Quantity (g/Equivalent)**	**Coupling Time (min.)**	**Test results Decision for next step**
Fmoc-Arg(Pbf)-OH	0.151/3 eq.	30	Ninhydrin (-ve)
Fmoc-Gly-OH	0.0579/2.5 eq.	30	Ninhydrin (-ve)
Fmoc-Tyr(TBu)-OH	0.0896/2.5 eq.	35	Ninhydrin(-ve)
Fmoc-Val-OH	0.066/2.5 eq.	60	Chloranil (-ve)

The peptide fragment VYGR was prepared on Merrifield resin using standard procedures and the result obtained was compared with that of GDMA-4-VP resin.

4.2.3. Synthesis of KVKRIILARS

Sequence: Lys-Val-Lys-Arg-Ile-Ile-Leu-Ala-Arg-Ser

Synthesis of the decapeptide was carried out on 1%GDMA-4-VP (250 mg resin, capacity 0.65 mmol/g, 100-200 mesh size) using Fmoc/TBu method.

4.2.3.1. Esterification of Fmoc-Ser-OH to polymer support.

GDMA- 4- VP resin (250 mg, 0.65 mmol/g) was taken in a silylated peptide synthesizer and swelled in Dichloromethane for 1 hour. The synthesiser was washed with dichloro dimethylsilane since it can form a monolayer coating inside the vessel which will prevent the sticking of micro particles on the walls **[2]**. Fmoc-Ser (TBu)-OH (3 eq), Diisopropylcarbodiimide (3 eq) and Dimethylaminopyridine (0.1 eq) were added to the resin and shaken well. Dimethylaminopyridine is a colourless solid and useful nucleophilic catalyst for a variety of reactions such as esterification. Coupling of an amino acid to a hydroxyl functionalised resin requires a catalytic amount of Dimethylaminopyridine. It is capable to produce undesirable levels of racemization so its amount should be less than 0.15 equivalents. After 1 hour, the excess reactants were filtered off and coupling process is repeated using the same procedure described above. After 30 min the excess reactants were washed off with DMF and NMP (each 5 x 3 ml). The Fmoc protection was removed by 20% Piperidine in DMF (2 x 3 ml). It is a commonly used base for the deprotection of Fmoc group. After 20 min, the excess reagents were washed off with sufficient volume of DMF followed by NMP. Keiser test was conducted to ensure the completion of reaction as explained before. Freshly prepared ninhydrin was used for each synthesis.

4.2.3.2. Coupling of Arg-Ala-Leu-Ile-Ile-Arg-Lys-Val-Lys

Fmoc-Ser (TBu)-resin in the peptide synthesizer was wetted with minimum DCM. Fmoc-Arg (Pbf) - OH (2.5 eq) was attached to the resin in the presence of minimum DCM, HOBT (2.5 eq), DIC (2.5 eq) and DIEA (0.3 eq). DIEA is a good base but a poor

nucleophile, which makes it a useful organic reagent. After 45 min, the resin was filtered and washed thoroughly with DMF (5 x 3 ml) and NMP (3 x 3 ml).

The remaining amino acids in the sequence, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Ile-OH, Fmoc-Arg (Pbf)- OH, Fmoc- Lys (Boc)-OH, Fmoc-Val-OH and Fmoc-Lys (Boc)-OH were successively incorporated. Resin was washed with DMF and NMP. Acetylation reactions were performed twice for confirming the quantitative conversion. Each coupling and deprotection step was monitored by Kaiser Test. After the synthesis, Fmoc protection was removed and the resin was washed with DMF and NMP. The details are given in Table **4.2.**

Table 4.2. Fmoc-SPPS for KVKRIILARS

Amino acid residue	**Quantity(mg)/ Equivalent**	**Coupling time (min)**	**Test results**
Fmoc-Ser(TBu)-OH	153/2.5 eq.	45	Esterification repeated
Fmoc-Arg(Pbf)-OH	207/2 eq.	45	Ninhydrin (-ve)
Fmoc-Ala-OH	99/2 eq	45	Ninhydrin (-ve)
Fmoc-Leu-OH	113/2 eq	50	Chloranil (-ve)
Fmoc-Ile-OH	113/2 eq	100	Ninhydrin (+ve).Coupling repeated. Chloranil (-ve)
Fmoc-Ile-OH	113/2 eq	120	Chloranil (+ve)Coupling repeated.Chloranil (-ve)
Fmoc-Arg(Pbf)-OH	207/2 eq	30	Ninhydrin (-ve)
Fmoc-Lys(Boc)-OH	149/2 eq	50	Ninhydrin (-ve)
Fmoc-Val-OH	108/2 eq	120	Chloranil (-ve)
Fmoc-Lys(Boc)-OH	132/2 eq	45	Ninhydrin (-ve)

4.2.3.3. Detachment of peptide from the resin

The peptidyl resin was suspended in TFA (9.75 ml) and TIS (0.25 ml) at room temperature for 2 Hrs. The resin was filtered, washed two times with TFA. Collected filtrate was evaporated using rotary evaporator to a small volume. The peptide was precipitated by adding ice cold ether, washed thoroughly with ether and was collected by centrifugation. The crude peptide was isolated and purified with suitable techniques. After purification by preparative HPLC, peptide was obtained as white powder.

The same peptide fragment was prepared using commercially available Wang resin and the yield and purity of the product obtained was compared with the product obtained using GDMA-4-VP resin. Wang resin is a popular resin used for Fmoc Solid phase synthesis. Using this resin, acids and phenols can be immobilized on it. To couple an amino acid to the resin, esterification reaction is needed. The first amino acid is attached to the resin through ester linkage and rest of the amino acids can be coupled using the above said procedure. Cleavage of the peptide fragment is carried out as in the above case.

4.3. Results and Discussion

4.3.1. Result and Discussion of tetra peptide VYGR

The biologically active neutral peptide sequence VYGR was prepared successfully with high yield and purity on GDMA-4VP resin. The first amino acid arginine was attached to the resin by esterification using DIC/DMAP. Elimination reaction took place between the –OH group of resin and –COOH group of arginine resulting in the formation of a strong ester bond. To this sequence amino protected

glycine was added through coupling using DIC/HOBt after removing the protection of amino group of arginine. Formation of the first amide bond takes place here. To this chain, tyrosine and valine are added by reverse amidation. The coupling and deprotection reactions were monitored by TLC. After the coupling of complete sequence, it was detached from the resin using TFA and suitable scavengers. Using ice cold ether, peptide was precipitated. The crude peptide obtained was purified and checked the purity by HPLC. The sharp single line obtained shows our peptide is more than 90% pure as shown in Fig. **4.3**

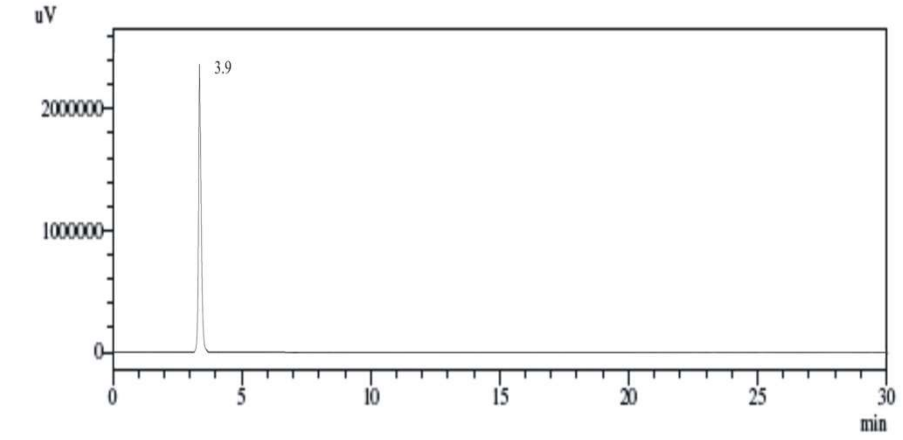


Fig.4.3. HPLC of VYGR synthesised on GDMA-4-VP

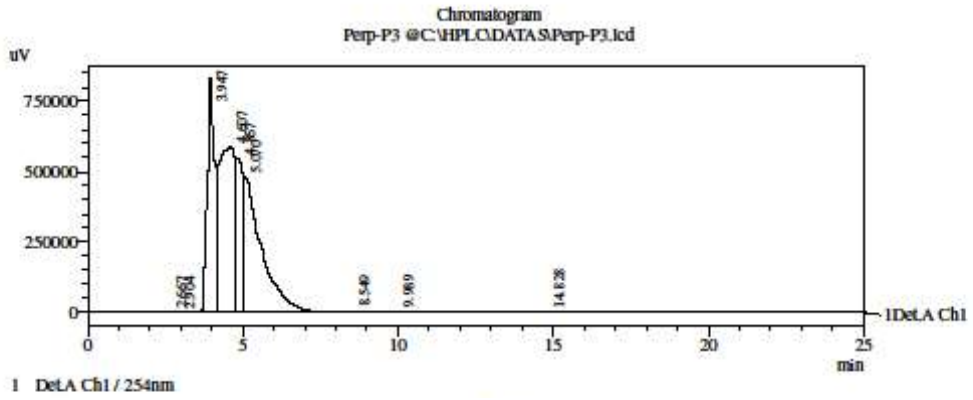


Fig. 4.4. HPLC of VYGR synthesised on Merrifield Resin.

4.3.2. Comparison of GDMA-4-VP and Merrifield resin

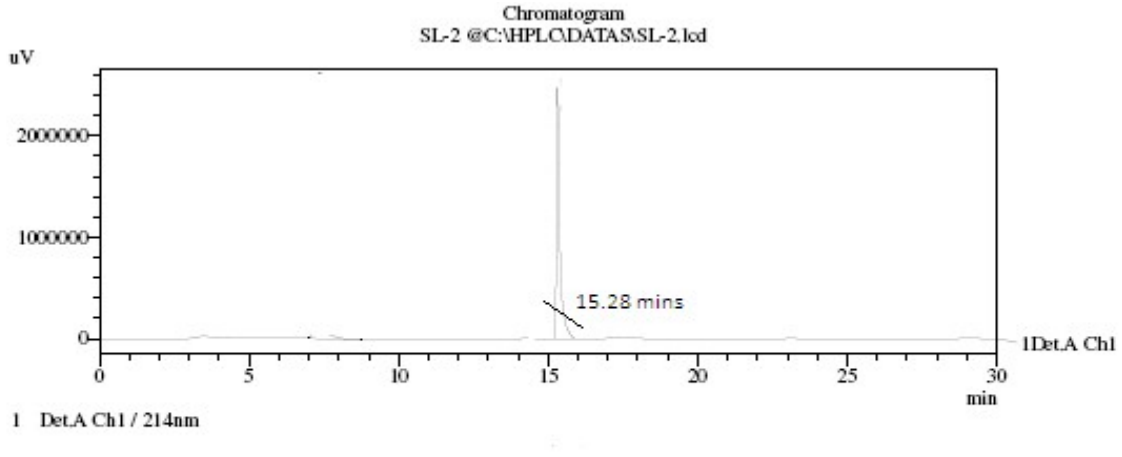
The sharp single line obtained in HPLC shows the high purity of the target peptide when GDMA-4-VP resin is used as polymer support. The chromatogram obtained when Merrifield resin was used as the support contains additional peaks at 4.607, 4.867 and 5.070 along with peak at 3.947 ret.time. This shows GDMA-4-VP resin is more efficient than Merrifield resin. Swelling and solvation of peptide attached resin in the reaction medium is important in SPPS. The insufficient accessibility of reaction sites along with its hydrophobicity is the main shortcomings of Merrifield resin. The additional peaks obtained are thought to be due to the formation of some additional sequences and impurity associated with the product.

4.3.3. Result and discussion of KVKRIILARS

In the first step of the reaction a strong ester bond is formed between the –OH group of the resin and –COOH group of the first amino acid Fmoc-Ser. This reaction is very important because as the length of the amino acid chain increases, the degree of coupling decreases. So 100% esterification ensures good yield of the final product. After esterification, the Fmoc protection of Serine is removed using Piperidine in DMF solution. Once the $–NH_2$ group of Serine is made free, it is coupled to –COOH group of second amino acid Fmoc-Arg using DIC/HOBT. Reverse Amidation coupling is used for the remaining amino acids also. The fourth amino acid in this sequence is Leucine, a hydrophobic amino acid. The problem of aggregation starts from the coupling between Leucine and Isoleucine because of their orientation directions. The optimum hydrophilic-hydrophobic balance of the GDMA-4-VP resin results in high solvation of the growing

sequence and avoids the problem. After the coupling of all the ten amino acids, the peptide is separated from the resin using TFA and suitable scavengers. Cold diethyl ether precipitates the crude peptide from TFA solution which can be further purified and characterized by HPLC and mass spectra. The prepared GDMA-4-VP resin satisfied all properties required for a solid support and reduces the propensity of hydrophobic amino acids and break up the self-aggregation of the amino acids during the synthesis.

HPLC of the decapeptide shows a sharp single peak at ret. time 15.28 min as shown in Fig.**4.5** which shows our target peptide is 99.99% pure. The peptide obtained by using the new GDMA-4-VP support was compared to the previous conventional methods and found to have higher purity equal to the biological peptide.



Detector A Ch1 214nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	15.287	22011802	2510444	100.000	100.000

Fig. 4.5. HPLC of KVKRIILARS

The ESI- MS spectrum of the deca peptide is shown in Fig.**4.6**. The charge state of the peptide detected at m/z 1184.4[M+H] $^{+}$ verifies a molecular mass of 1183.54 Da which is the mass of our target peptide.

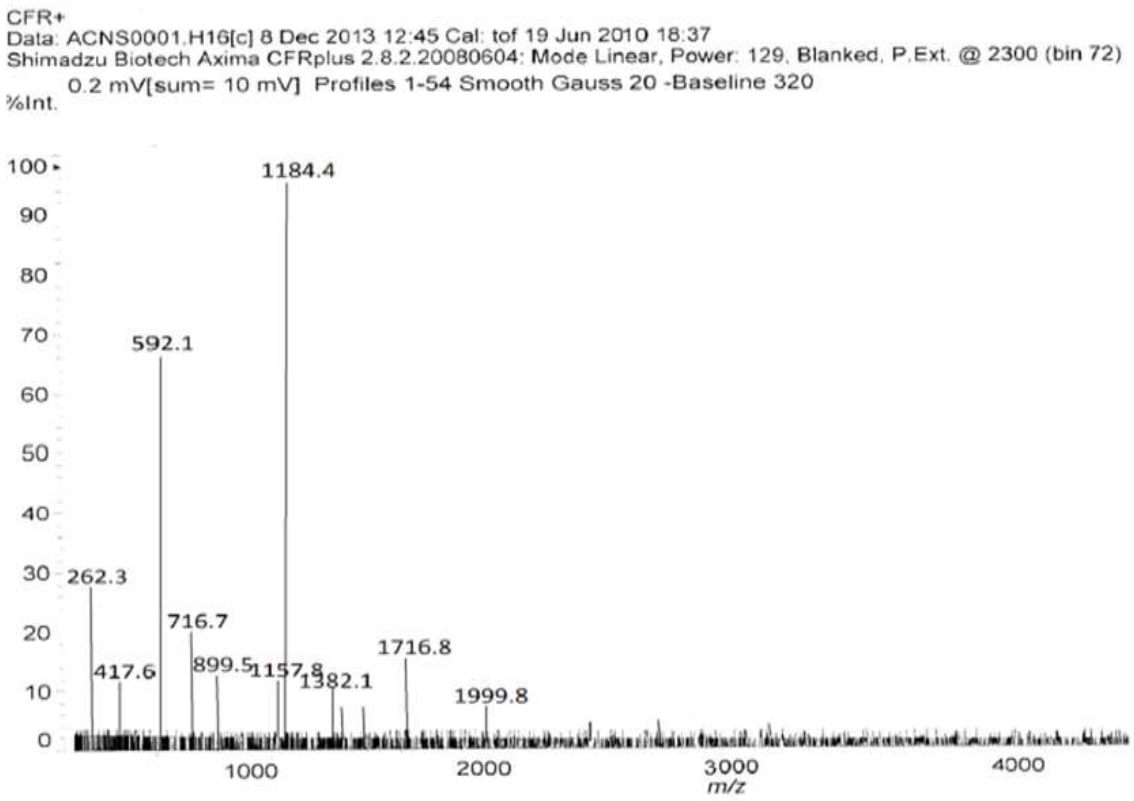


Fig. 4.6. ESI- MS of KVKRIILARS

The deca peptide KVKRIILARS was successfully synthesized on the prepared GDMA-4-VP resin using improved Fmoc SPPS. The observed properties of the deca peptide are given in the following Table.**4.3**

Table 4.3. Observed Properties of KVKRIILARS

Properties	**Values/Result**
Appearance	White coloured fine powder
Molecular Weight	1183.54g/mol
Iso- electronic point	12.53
Solubility	Soluble in water and acidic solvents
Hydrophobicity/ Hydrophilicity	50/43

4.3.4. Comparison between GDMA-4-VP and Wang resin

Both GDMA-4-VP resin and Wang resin contains hydroxyl group as reactive site. Number of coupling and time for coupling were similar in both cases. In some cases, incomplete coupling after a second coupling was seen from Keiser test. The amount of amino acids required for the synthesis was found to be less for GDMA-4-VP resin. The coupling was based on active ester method in which DIC/HOBt in NMP was used. Minimum amount of DCM was added to enhance solubility. For almost all cases, reaction was found to be complete in first coupling itself. The yield of peptide from 100 mg of our resin was 38 mg and that from Wang resin was 35 mg. The single peak in HPLC was obtained from both resins. However the product obtained from the GDMA-4-VP support was hygiene pure. So GDMA-4-VP resin is cost- effective and less time consuming as compared to other resins since the use of excess reagents or amino acids is not required. It is superior to other resins in respect of yield and purity. The result shows our resin is more efficient than the commercially established Wang resin.

Reference

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Chapter 5

Synthesis of Biologically active Angiotensin II and Angiotensin IV on GDMA-4-VP resin using improved Fmoc Strategy.

5.1. Introduction

Solid phase peptide synthesis is the most popular way followed now-a-days to synthesise peptides on small scale and large scale. Commercially available anti-HIV peptide, Fuzeon, is a most obvious example which is manufactured in multi kilograms using solid phase synthetic strategy **[1]**. The success of solid phase technique is determined by the properties of solid support. The strong hydrophobic, macromolecular environment of polymer can persuade the developing peptide chain to adopt unfavourable conformation that lead to low yield and purity of target peptides **[2]**. Peptide chemistry utilises different classes of hydrophilic polymers as support for chemical reactions. The diffusivity and solubility of hydrophilic polymers in water facilitates their biomedical applications. In this work, the Influence of Glyceroldimethacrylate cross-linker in the 4-Vinyl Pyridine support for peptide synthesis was studied by synthesising a biologically active Angiotensin II peptide fragment by improved F-moc strategy.

It is fragment of the renin-angiotensin system, which is a major aim for drugs that lessen blood pressure **[3]**. Angiotensin also arouses the discharge of aldosterone, another hormone, from the adrenal cortex. Aldosterone stimulates sodium retention in the distal nephron, in the kidney, which also pushes blood pressure up. Angiotensin II

performances on the adrenal cortex, producing it to discharge aldosterone, a hormone that causes the kidneys to recollect sodium and expel potassium. Raised plasma angiotensin II levels are liable for the high aldosterone levels present during the luteal phase of the menstrual cycle **[4, 5].**

Angiotensin II is an octa peptide which is produced from angiotensin I by removing two amino acids. Angiotensin II is a hormone that acts on the central nervous system to control renal sympathetic nerve activity, renal function and consequently blood pressure. It is produced inside the kidney and mediates tissue injury over a series of nonhemodynamic effects **[6].** Besides regulation of blood pressure Angiotensin II leads to excessive production of reactive oxygen species. Angiotensin II is one of the main factors involved in hypertension- induced tissue damage. This peptide regulates the inflammatory processes. AngiotensinII also control the regulation of hormone formation and release, the organizing of the central and peripheral sympatho adrenal systems and the management of water and sodium intake.

The chemical arrangement of Angiotensin II is presented in Fig **5.1.**

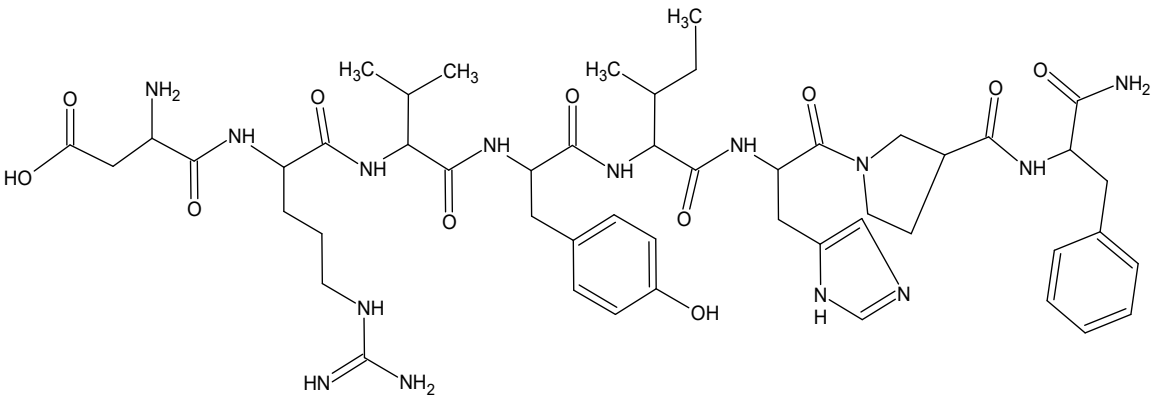


Fig.5.1. Structure of Angiotensin II

Angiotensin IV is one of the degradation products of angiotensin II. It umpires important functions in the central nervous system as blood flow regulation, processes essential to learning and memory **[7].** Angiotensin IV specific binding sites are found to be present in various mammalian tissues like blood vessels, heart, kidney and brain. The structure of Angiotensin IV is given in Fig. **5.2.**

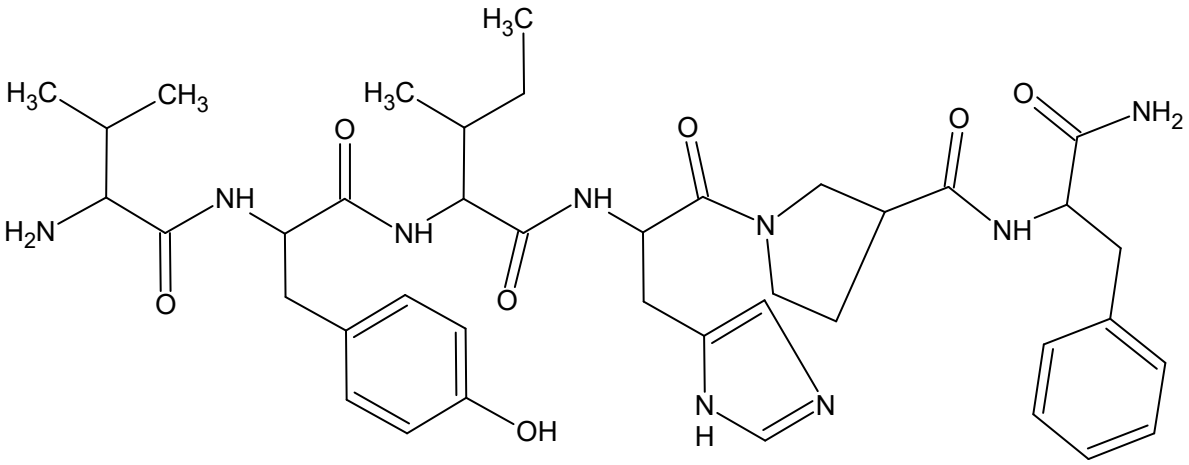


Fig.5.2. Structure of Angiotensin IV

5.2. Experimental Details

5.2.1. Synthesis of Angiotensin II

Sequence: Angiotensin II - NH_2 -Asp-Arg-Val-Tyr-Ile-His-Pro-Phe –COOH

The peptide was synthesised on GDMA-4-VP resin (250 mg resin, 0.65 mmol/g capacity and 100-200 mesh size) using improved F-moc chemistry. Each coupling was performed with 2.5 equivalents of HOBt with respect to resin capacity and 2.5 equivalence of amino acid.

The first amino acid Fmoc- Phe - OH was anchored by esterification to the resin using the following procedure.

The resin was transferred to a clean, dry, sililated peptide synthesiser, added sufficient amount of NMP and kept for an hour for swelling and the excess NMP was removed. Fmoc-Phe-OH (3 eq), DMAP (0.1 eq) and DIC (3 eq) were added to the swollen resin and shaken for 60 minutes. Washing of the resin was performed with NMP (2 times), DMF (2 times), MeOH (1 time). The above esterification reaction was repeated to ensure the 100% loading of the first amino acid which was checked using ninhydrin solution.

The second amino acid Fmoc-Pro-OH was attached to the resin through amide bond formation using the following method.

Fmoc protection was removed from the resin bound amino acid. Fmoc- Pro-OH (2 eq) dissolved in minimum quantity of NMP in a 25 ml RB flask. To that HOBt (2.5 eq) and DIC (2 eq) were added and shaken well for 3 min and immediately the content was transferred in to the resin with moisture free atmosphere and shaken well for 5 min, to that DIPEA was added and shaken well for 45mins. Reaction progress was monitored by TLC. Small pinch of the resin was taken and washed and tested with ninhydrin. In the case of negative result, washing and deprotection was performed. The remaining amino acids were coupled following the above method. The detailed synthetic strategy, time duration of reaction process and conditions are given in Table **5.1.**

Table 5.1. Synthetic Strategy of Angiotensin II

Amino acid Residue	Coupling (min)			Ninhydrin	Washing	Deprotection 15min (×2)	Washing
	1st	2nd	3rd				
Fmoc-Phe-OH	45	40	-	-ve	Done	Done	Done
Fmoc-Pro-OH	30	35	-	-ve	Done	Done	Done
Fmoc-His(trt)-OH	30	30	-	-ve	Done	Done	Done
Fmoc-Ile-OH	30	30	45	-ve	Done	Done	Done
Fmoc-Tyr(tbu)-OH	30	40	-	-ve	Done	Done	Done
Fmoc-Val-OH	30	35	40	+ve	Done	Done	Done
Fmoc-Arg(pbf)-OH	30	35	-	-ve	Done	Done	Done
Boc-Asp(tbu)-OH	30	40	-	-ve	Done	-	-

5.2.2. Synthesis of Angiotensin IV

Sequence: Angiotensin IV- NH_2- Val-Tyr-Ile-His-Pro-Phe-COOH

The peptide was synthesised on GDMA-4-VP resin (250 mg resin, 0.65 mmol/g capacity, 100-200 mesh size) using improved F-moc chemistry. The first amino acid Fmoc- Phe - OH was anchored by esterification to the resin using the following procedure. The resin was transferred to a clean, dry, sililated peptide synthesiser, added sufficient amount of NMP and kept for an hour for swelling and the excess NMP was removed. Fmoc-Phe-OH (3 eq.), DMAP (0.1 eq.) and DIC (3 eq.) were added to the swollen resin and shaken for 60 minutes. Washing of the resin was performed with NMP (2 times), DMF (2 times), MeOH (1 time). The above esterification reaction was repeated to ensure the 100% loading of the first amino acid which was checked using Keiser test.

The second amino acid Fmoc-Pro-OH was attached to the resin through amide bond formation using the following method.

Fmoc protection was removed from the resin bound amino acid. Fmoc- Pro-OH dissolved in minimum quantity of NMP in a 25 ml RB flask. To that HOBt (2.5 eq) and DIC (2.5 eq) were added and shaken well for 3 min and immediately the content was transferred in to the resin with moisture free atmosphere and shaken well for 5 min, to that DIPEA (2.5 eq) was added and shaken well for 45 min. Reaction progress was monitored by TLC. Small pinch of the resin was taken and washed and tested with ninhydrin. In the case of negative result, washing and deprotection was performed. The remaining amino acids were coupled following the above procedure.

The detailed synthetic strategy, time duration of reaction process and conditions are given in Table **5.2.**

Table 5.2. Synthetic Strategy of Angiotensin-IV

Amino acid Residue	**Coupling (min)**			**Ninhydrin**	**Washing**	**Deprotection 15min (×2)**	**Washing**
	1st	**2nd**	**3rd**				
Fmoc-Phe-OH	45	40	-	-ve	Done	Done	Done
Fmoc-Pro-OH	30	30	-	-ve	Done	Done	Done
Fmoc-His(trt)-OH	30	30	-	-ve	Done	Done	Done
Fmoc-Ile-OH	30	40	45	-ve	Done	Done	Done
Fmoc-Tyr(tbu)-OH	30	35	-	-ve	Done	Done	Done
Boc-Val-OH	30	35	40	+ve	Done	-	-

5.2.3. Cleavage of crude peptide from resin

After synthesis the resin was washed with hexane, DCM, $CHCl_3$ and MeOH and dried. The cleavage was performed with TFA-TIS-Water- m-Cresol in a ratio 95%: 2.5%: 2%: 0.5%. The resin was treated with these solvents for 3 hours under nitrogen atmosphere and the resin was washed 4 times with TFA. The filtrate was collected in 50 ml R. B-Flash and all the traces of TFA was evaporated by using Rota vacuum evaporator .The peptide was isolated with excess of peroxide free pure cold diethylether and the peptide was washed 3 times with diethyl ether and centrifuged. The white powder form of peptide was taken in small tubes and sealed .

5.3. Results and Discussion

Biologically active angiotensin II is an octa peptide and angiotensin IV is a hexa peptide and were synthesised earlier in many studies which was not as pure as needed for therapeutic studies. One of the major problems in chemical peptide synthesis is the purification of the end product since it will lead to loss of the crude peptide during the process. Here the hydrophilic polymer support GDMA-4-VP was used to prepare angiotensin II and IV. The –OH functional group present in the resin was made to react with –COOH group of the first amino acid Fmoc-Phe-OH. Freshly prepared 20% Piperidine in DMF was used to remove the amino protecting group. All other amino groups were kept protected using groups like OtBu, Pbf, Trt wherever suited. The second amino acid Fmoc-Pro was added to the resin by reverse amidation coupling. TFA was used to detach the completed fragments from the resin and purified through HPLC analysis.

The accuracy of HPLC assay method was assessed by standard method (the known peptide sample purity were recorded and compared with standard reference). The HPLC analysis of angiotensin II is given below in Fig. **5.3**. From the sharp single peak at ret. Time 7.24 purity of the peptide is clearly visible. Crude peptide yield was found to be 83.67%.

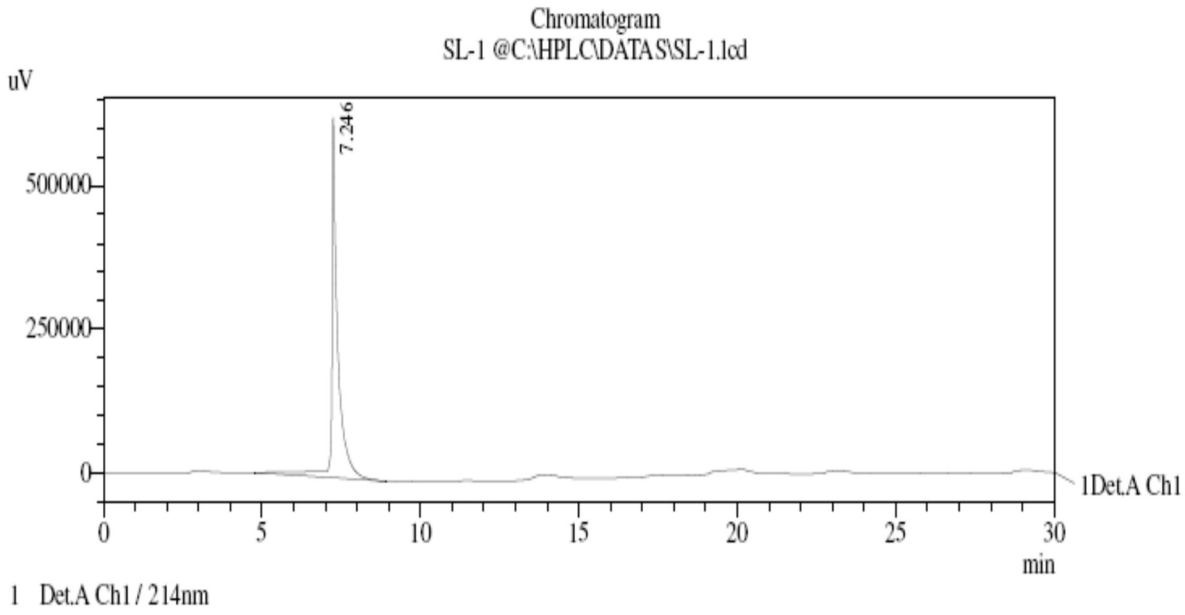


Fig.5.3. HPLC of Angiotensin II

The ESI MS spectrum of angiotensin II is shown in Fig.**5.4**. The parent peak obtained at m/z 1046.35$[M+H]^{+}$ verifying a molecular mass of 1046 Da which is evidence of the target peptide. The obtained spectrum $[M+H]^{+}$ and $[M+2H]^{2+}$ confirmed the expected target peptide.

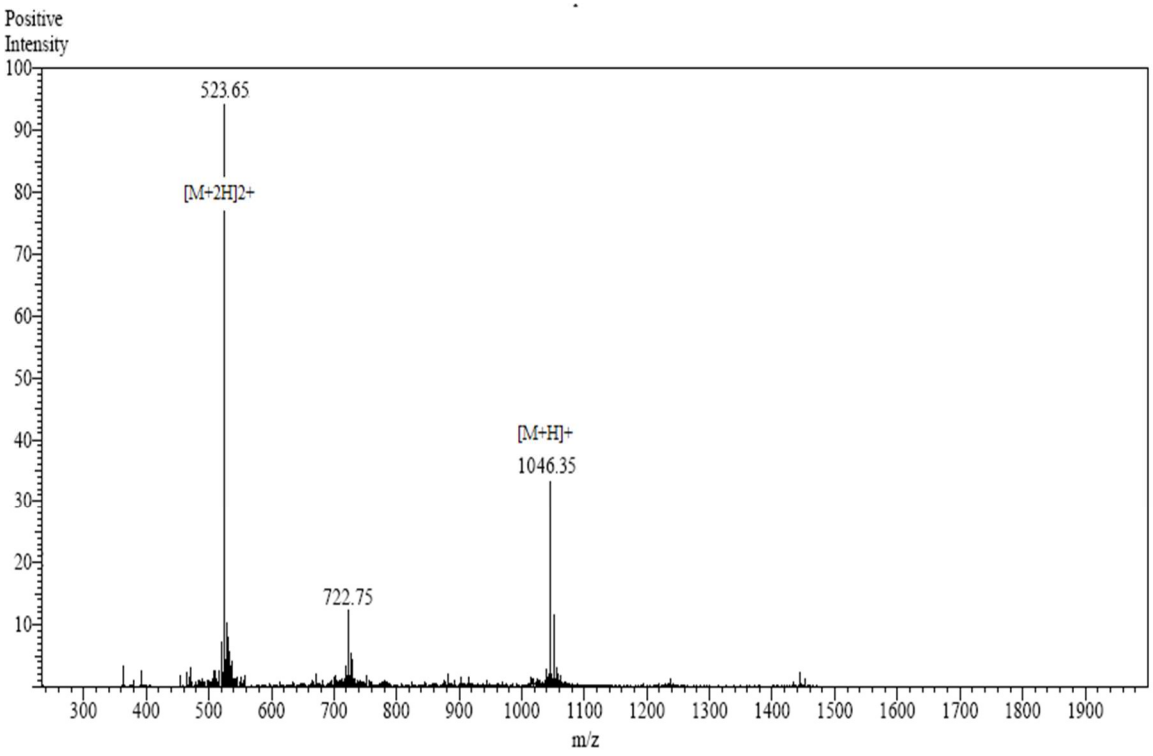


Fig. 5.4. ESI MS of angiotensin II

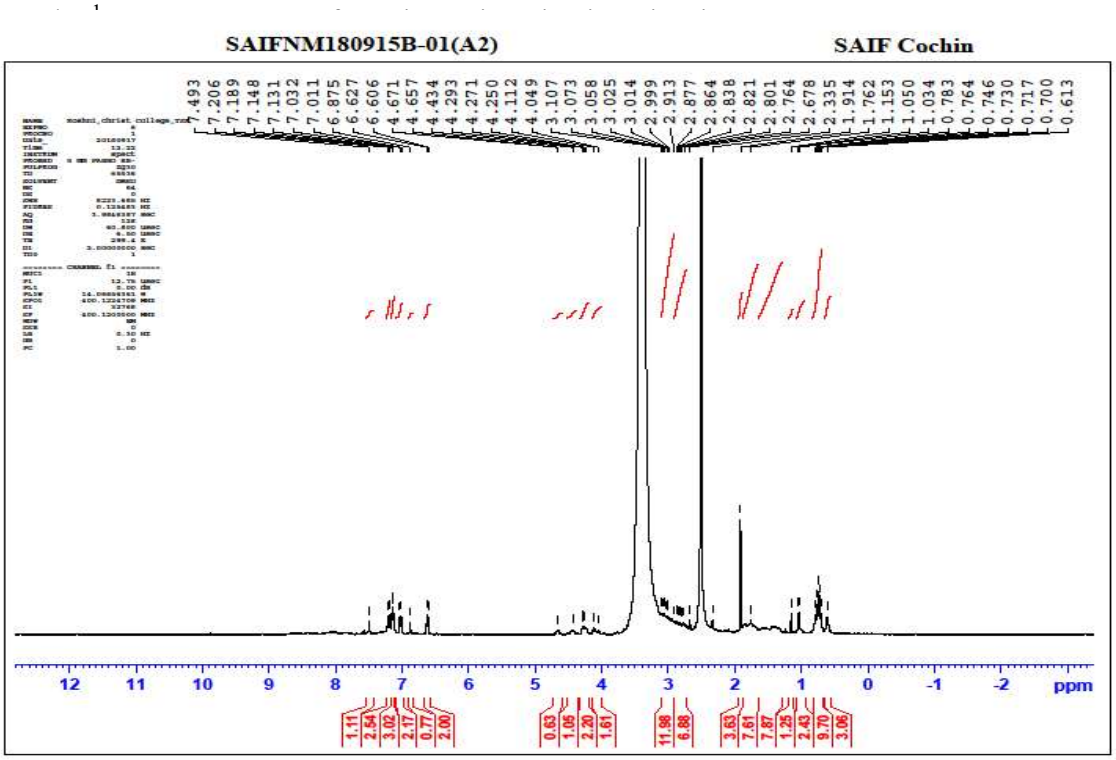
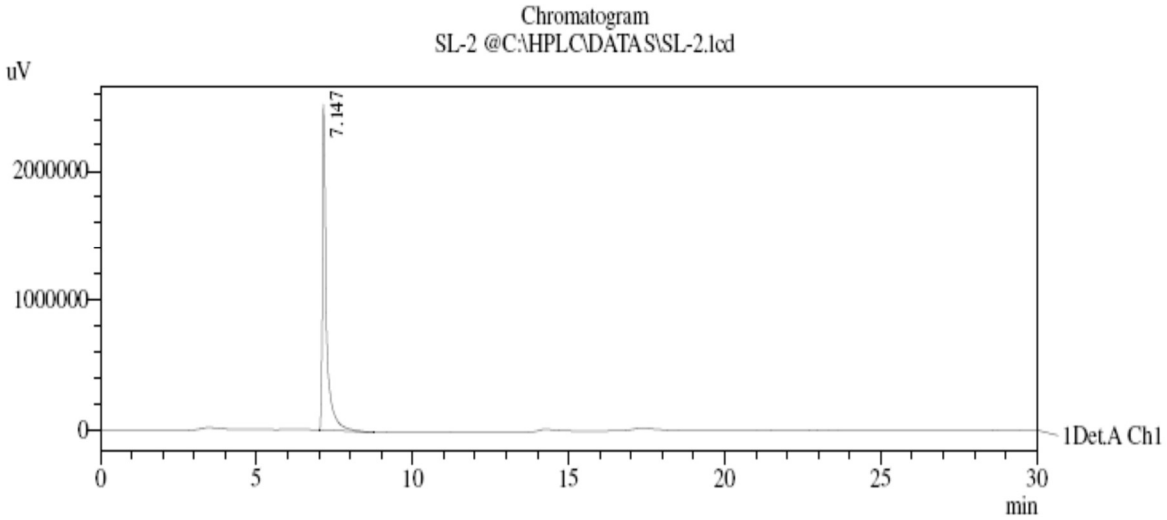


Fig. 5.5. ^{1}H NMR spectra of Angiotensin II

The purity of biologically active angiotensin IV was determined by HPLC as shown in Fig.**5.6**.below. The single sharp peak at ret. time 7.14 min shows our target peptide purity. Crude peptide yield: 85.78%



PeakTable

Detector A Ch1 214nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	7.147	22011802	2510444	100.000	100.000
Total		22011802	2510444	100.000	100.000

Fig. 5.6. HPLC of Angiotensin IV

The ESI-MS spectrum of the biologically active angiotensin IV is given below in Fig.**5.7**. Molecular peak at m/z 775.35 $[M+H]^+$ verifies the molecular mass of 775 Da. The obtained spectrum confirmed the expected target peptide.

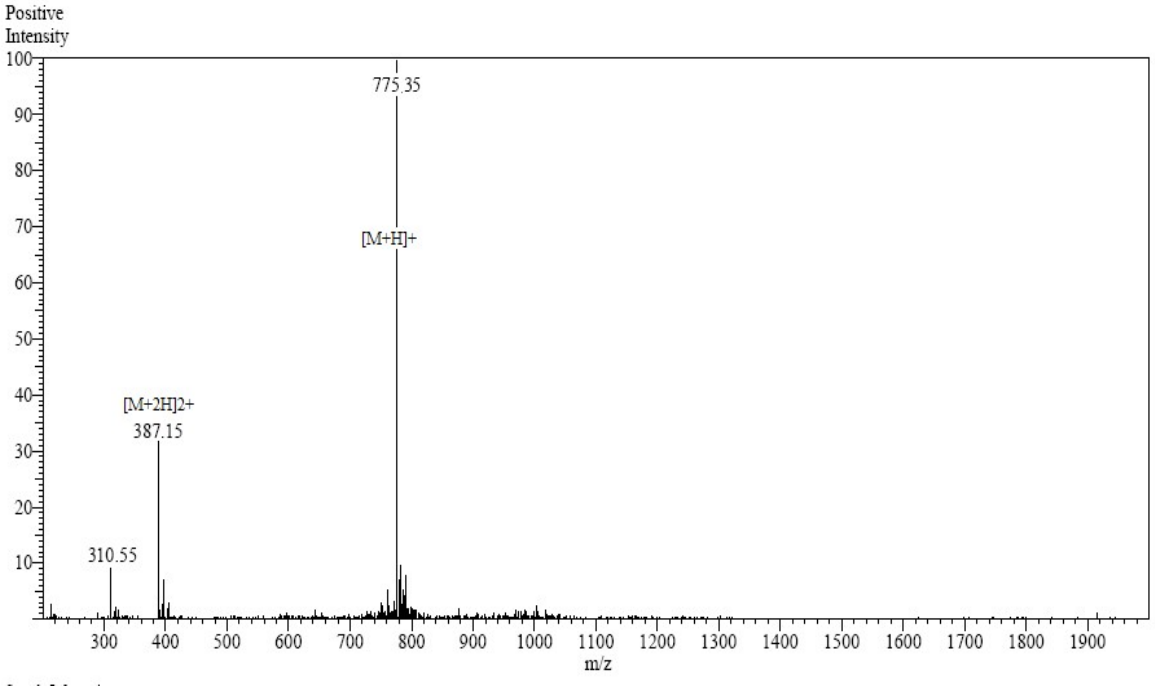


Fig.**5.7.** ESI MS of Angiotensin IV

Biologically important angiotensin II and IV were synthesised successfully on GDMA-4-VP resin using improved Fmoc synthetic strategy. The observed common properties of angiotensin II and angiotensin IV are given below in Table **5.3** and Table **5.4.**

Table 5.3. Observed properties of Angiotensin II

Property	**Result**
Appearance	Clear white powder
Molecular weight	1046 g/mol
Isoelectric point	9.9
Purity (HPLC)	99%

Table 5.4. Observed properties of Angiotensin IV

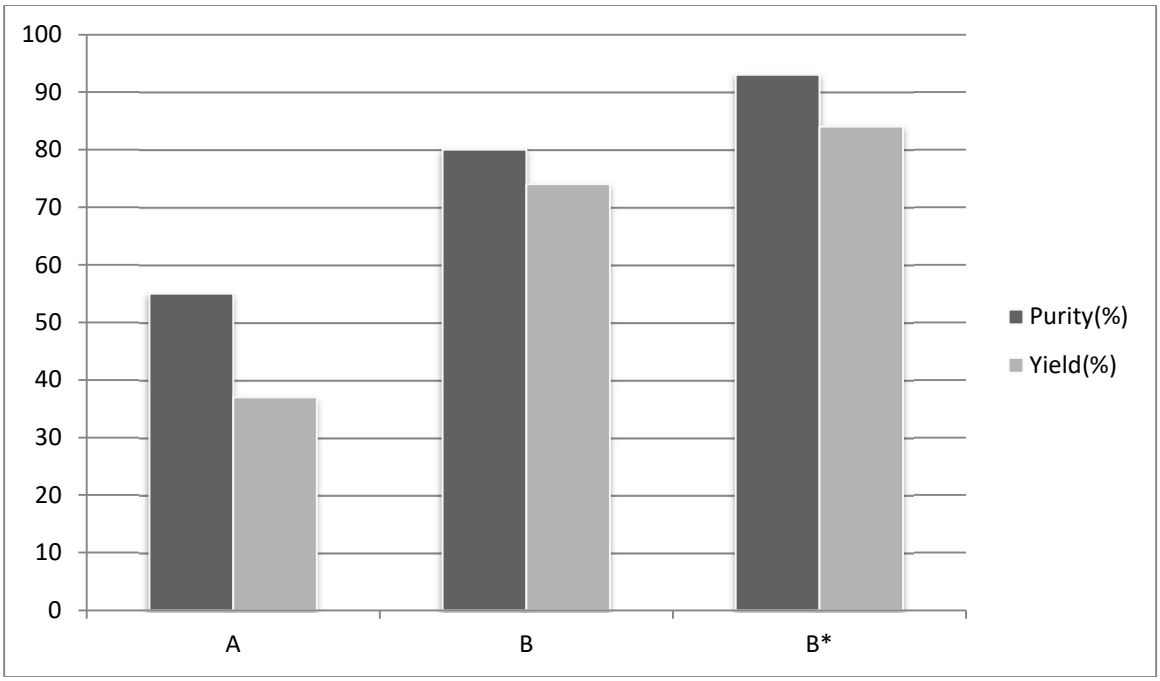
Property	Result
Appearance	White powder.
Molecular weight	775 g/mol
Isoelectric point	9.81
Purity(HPLC)	99%

4. Comparison between SPPS and other methods

The success of this modified synthetic approach was compared with other known methods. Here maximum crude peptide purity and yield was obtained in new modified Fmoc synthetic approach. When compared to other approach, the result and observations of this work is found to be superior. The purity and yield of Crude sample and purified sample of angiotensin II obtained from various methods is given in Table **5.5**. The following graphical evidence clearly reveals the success of this method.

Table.5.5. Comparison of purity and yield.

Methods of peptide synthesis	**Crude peptide**		**Purified Peptide**	
	Yield	Purity	Yield	Purity
LPPS	>70%	>80%	>63%	>85%
SPPS	>71%	>82%	>65%	>80%
Improved Fmoc SPPS	>82.98%	>90%	70.53%	>90%



A. Solution phase peptide synthesis

B . Fmoc peptide chemistry

B*. Modified Fmoc peptide chemistry

Fig.5.8. Comparison of Modified Fmoc Method with conventional methods.

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Chapter 6

Synthesis of biologically active peptides by Solid phase methodology

6.1. Introduction

The concept of total chemical synthesis of peptides and proteins has always been a powerful tool in Bio-Organic Chemistry. Research on resins shows that the success of SPPS for longer fragments in special, depends on the property of resin. It must be applicable in different solvents of different polarity that means a kind of hydrophilic-hydrophobic balance is essential requirement for a successful solid support. This chapter describes the application of GDMA-4-V-P resin for the synthesis of a number of biologically active peptides with high purity and good yield. Anti-inflammatory peptide, Neurotensin 1-8, Neuromedin-N, MR-14, YY-18, YK-15, Amyloid β peptide were prepared on GDMA-4-VP resin.

Anti-inflammatory peptides are useful to inhibit inflammation of a mammal's skin, mucous membranes, or lacerations of the musculature or injury to the brain or leakage of fluids into the air spaces of the lungs. Camussi G in 1986 described platelet-activating factor as a phospholipid mediator of inflammation and endotoxic shock. Polymorphonuclear neutrophils, peritoneal macrophages, vascular endothelial cells, basophils, and platelets synthesize PAF rapidly after appropriate stimuli **[1]**. Anti-inflammatory peptide 1 has its place among the group of synthetic oligo peptides corresponding to a zone of high amino acid sequence similar to uteroglobin and

lipocortin1. Lipocortins belong to a family of related proteins that mediate the anti-inflamatory activity of Corticosteroids. The name antiflammins is anticipated for those peptides, which are important models for the synthesis of novel anti-inflamatory agents of therapeutic prominence. A1P1 possesses effective anti- inflammatory activity in vivo and is a strong inhibitor of phospholipase A2 (PLA2) whose amplified occurrence and activity leads to inflammation and pain at certain bodily sites **[2].** The structure of anti-inflamatory peptide is shown in the Fig.**6.1.**

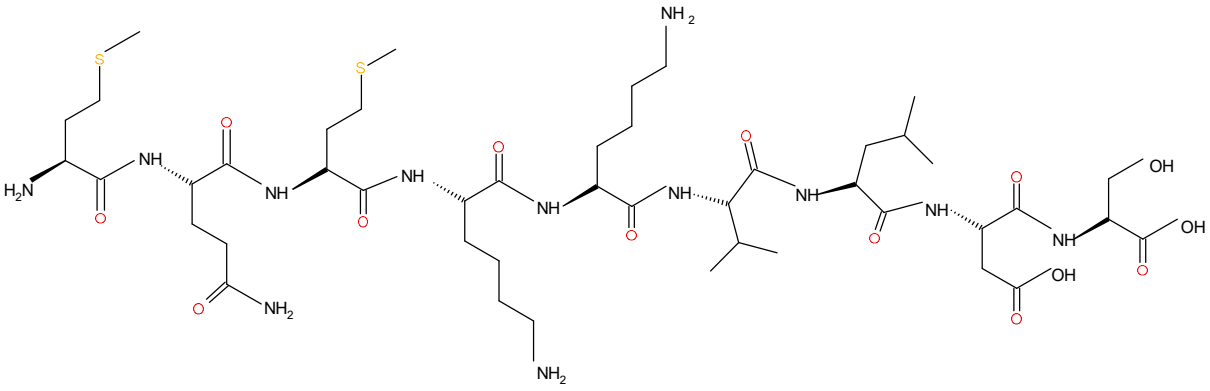

Fig. 6.1. Structure of anti- inflammatory peptide.

Neurotensin and Neuromedin N are two biologically active gastro- intestinal hormones also found abundantly in brain and adrenal gland. They are synthesised from a common precursor by prohormone convertases. Both of them are located near the carboxy- terminal region of the propeptide. Neuromedin N is structurally similar to Neurotensin. It is a hexapeptide found in mammals.Neurotensin is an endogenous antipsychotic. It has major role in the control of appetite, modulation of stress responses, pain perception and psychostimulant responses. The development of Neurotensin

agonists is thought to be able to deliver new drugs for curing stress related neuropathic pain syndromes and drug addiction**[3].**

The structure of Neurotensin peptide is given in Fig.**6.2**.

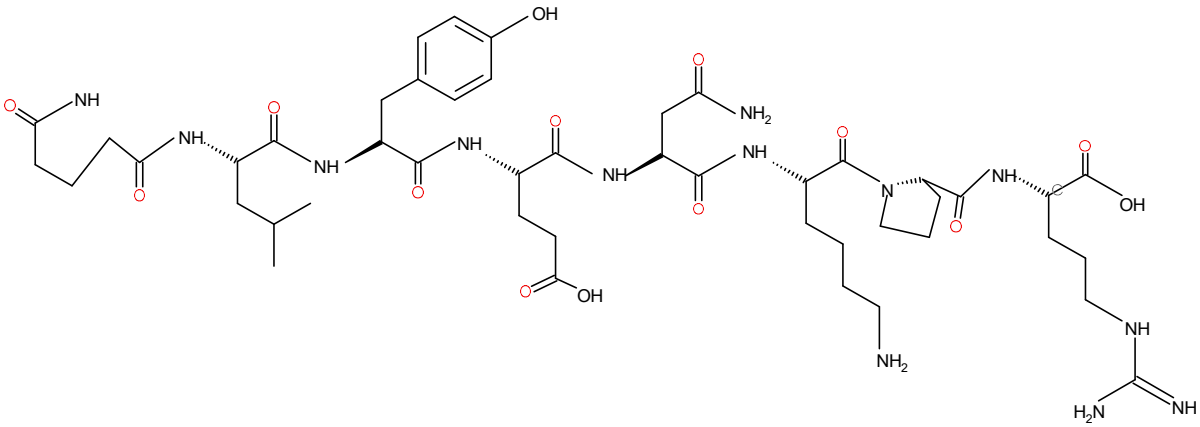

Fig.6.2. Structure of Neurotensin 1-8

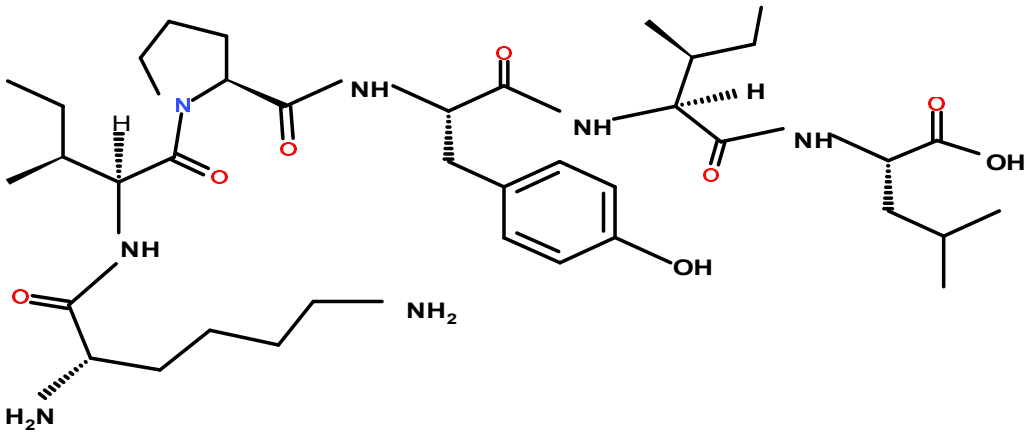

Fig. 6.3 Structure of Neuromedin N

Beta- Amyloid is an amyloid that is derived from a larger precursor protein and is the primary component of plaques characteristic of Alzheimer's disease. Amyloid β protein 1-28 is also known as Alzheimer's disease β- protein.

6.2. Experimental Details

6.2.1. Synthesis of Anti- inflammatory peptide.

Sequence: H_2N- Met-Gln-Met-Lys-Lys-Val-Leu-Asp-Ser-OH

Synthesis was performed on 2% amino GDMA-4VP resin (400 mg, loading 0.75 mmol/ g and 100-200 mesh size) using improved Fmoc spps method. Each coupling was performed with 2.5 eq of PyBOP with respect to resin capacity. Washings were performed with DMF and DCM. 20% Piperidene in DMF was used to remove Fmoc group.

The first amino acid Fmoc-Ser–OH was attached to the resin by amidation reaction using the following procedure.

The cross linked resin was taken in a peptide synthesizer and swelled in DCM for 1 hour and filtered under vacuum. Fmoc-Ser-OH (2.5 eq) dissolved in minimum dry DCM, DIC (3 eq) and DMAP (0.1 eq) were added to the resin and shaken well. After 1 hour, the reactants were filtered off and coupling process is repeated using the same procedure. After 30 min the reactants were washed off with DMF and DCM (each 5 x 3 ml). The amidation reaction was performed once more to ensure complete loading of the first amino acid on the resin.

The second amino acid Fmoc-Asp-OH was attached to the resin through peptide coupling method.

Fmoc protection was removed from the first amino acid Fmoc-Asp-OH (2 eq), PyBOP (2 eq) was dissolved in minimum dry DCM. Added some drops of DMF to enhance solubility. To that mixture, added DIC (2 eq) and shaken for 30 minutes. The mixture is then transferred to the resin in dry condition and shaken vigorously for 3 minutes. To the reaction mixture added DIPEA (3 eq) and the reaction was continued for 30 minutes. The completion of the reaction was checked with ninhydrin solution taking a small pinch of resin which is washed enough. The reaction was found to be complete from the absence of blue colour. Then the resin is washed with DCM, MeOH, DMF and DCM (2 x 3 ml). Then Fmoc protection of the second amino acis, ie Pro-OH is removed using piperidine in DMF. The reagents were removed by thorough washing using the above solvents. The completeness of deprotection was checked with ninhydrin solution again. Now the presence of blue colour indicates free amino group. The above process was repeated until the remaining amino acid residues in the peptide sequence are successfully attached to the resin. They are Leu, Val, Lys, Lys, Met, Gln, Met. The reaction can be monitored by thin layer chromatography wherever required.

The cleavage of the synthesised peptide fragment from GDMA-4-VP resin was done by the following procedure.

When the whole sequence was achieved, the resin was washed well with $CHCl_3$, DCM and methanol and dried under vacuum. The cleavage was executed with TIS as scavenger (2.5%) and TFA/ water /m-cresol, (95%:2%:0.5%). This solvent mixture was

added to resin and kept for 3 hours under nitrogen atmosphere. After the time limit, the whole liquid part was collected. The resin was washed 2 times with TFA and the liquid part is collected to the vessel. The crude peptide can be isolated and purified with analytical techniques. The peptide was purified by preparative HPLC to yield a clear white powder. The complete synthesis is given in Table **6.1.**

Table 6.1. Details of synthesis of Anti-inflammatory peptide

Amino acid residue	Quantity (g) /eq.	Coupling time (min.)	Test result
Fmoc-Ser(TBu)-OH	0.2875/ 2.5 eq.	45	Coupling repeated
Fmoc- Asp(OtBu)	0.246/2 eq.	40	Ninhydrin –ve
Fmoc-Leu-OH	0.212/2eq.	50	Chloranil +ve, Coupling repeated,Chloranil –ve.
Fmoc-Val-OH	0.2036/2eq.	30	Chloranil –ve
Fmoc-Lys(Boc)-OH	0.281/2eq.	100	Ninhydrin (-ve)
Fmoc-Lys(Boc)-OH	0.281/2eq.	100	Ninhydrin (-ve)
Fmoc-Met-OH	0.222/2eq.	45	Ninhydrin (-ve)
Fmoc Gln(Trt)-OH	0.366/2eq.	45	Chloranil(-ve)
Fmoc-Met-OH	0.222/2eq.	45	Ninhydrin(-ve)

6.2.2. Synthesis of Neurotensin peptide.

Sequence: H_2N-pELYENKPR-($CONH_2$)

Synthesis was performed on 2% amino GDMA-4VP resin (350 mg, loading 0.75 mmol / g and 100-200 mesh size) using improved Fmoc spps method. Each coupling was performed with 2.5 eq of PyBOP with respect to resin capacity. Washings were performed with DMF and DCM. 20% Piperidene in DMF was used to remove Fmoc group.

The first amino acid Fmoc-Arg (pbf) –OH was attached to the resin by amidation reaction using the following procedure.

The cross linked resin was taken in a peptide synthesizer and swelled in DCM for 1 hour and filtered under vacuum. Fmoc-Arg (pbf) (2.5equiv) dissolved in minimum dry DCM, DIC (3equiv) and DMAP (0.1equiv) were added to the resin and shaken well. After 1 hour, the excess reactants were filtered off and coupling process was repeated using the same procedure. After 30 min the reactants were washed off with DMF and DCM (each 5 x 3 ml). The amidation reaction is performed once more to ensure complete loading of the first amino acid on the resin.

The second amino acid Fmoc-Pro-OH was attached to the resin through peptide coupling method.

Fmoc protection was removed from the first amino acid .Fmoc-Pro-OH (2 eq), PyBOP (2 eq) were dissolved in minimum dry DCM. Added some drops of DMF to enhance solubility. To that mixture, added DIC (2 eq) and shaken for 30 minutes. The mixture is then transferred to the resin in dry condition and shaken vigorously for 3 minutes. To the reaction mixture added DIPEA (3 eq) and the reaction was continued for 30 minutes. The completion of the reaction was checked with ninhydrin solution taking a small pinch of resin which is washed enough. The reaction was found to be complete from the absence of blue colour. Then the resin is washed with DCM, MeOH, DMF and DCM (2 x 3 ml). Then Fmoc protection of the second amino acis, ie Fmoc-Pro-OH was removed using Piperidine in DMF. The reagents were removed by washing using the above solvents. The apparatus used to take piperidine must be washed well as soon as the reagents are added and their contact with the reaction vessel must be strictly avoided. The completeness of deprotection can be checked with ninhydrin solution again. Now the presence of blue colour indicates free amino group.The above process is repeated until

the remaining amino acid residues in the peptide sequence are successfully attached to the resin. They are Lys, Asn, Glu, Tyr, Leu and Glu. The reaction can be monitored by thin layer chromatography wherever required.

The Neurotensin peptide can be cleaved and isolated from the resin when the whole sequence is achieved. The resin was washed well with $CHCl_3$, DCM and methanol and dried under vacuum. The cleavage was executed with TFA/ TIS/ water / m-cresol (95%:2.5%:2%:0.5%). This solvent mixture is added to resin and kept for 3 hours under nitrogen atmosphere. After the lime limit, the whole liquid part is collected. The resin was washed 2 times with TFA and the liquid part is collected to the vessel. The crude peptide can be isolated and purified with analytical techniques. The peptide was purified by preparative HPLC to yield a clear white powder. The complete synthesis is given in Table **6.2.**

Table 6.2. Details of Synthesis of Neurotensin peptide.

Amino acid Residue	**Quantity (mg) / Eq**	**Coupling Time (Min)**	**Test results**
Fmoc-Arg(pbf)- OH	0.433/2.5 eq.	35	Ninhydrin (-ve)
Fmoc-Pro-OH	0.180/2eq.	40	Chloranil (-ve)
Fmoc-Lys (pbf)-OH	0.250/2eq.	45	Ninhydrin(-ve)
Fmoc-Asn (Trt)-OH	0.318/2eq.	35	Ninhydrin(-ve)
Fmoc-Glu(TBu)-OH	0.227/2eq.	50	Chloranil (+ve) Coupling repeated.
Fmoc-Tyr(TBu)-OH	0.245/2eq.	50	Ninhydrin(-ve)
Fmoc-Leu-OH	0.188/2eq.	120	Chloranil(-ve)
Fmoc-Glu(TBu)-OH	0.227//2eq.	50	Chloranil (-ve)

6.2.3. Synthesis of Neuromedin N

Sequence: H-Lys-Ile-Pro-Tyr-Ile-Leu-OH

Synthesis was performed on 2% amino GDMA-4VP resin (300 mg, loading 0.75 mmol/g and 100-200 mesh size) using improved Fmoc SPPS method. Each coupling was performed with 2.5 eq of PyBOP with respect to resin capacity. Washings were performed with DMF and DCM. 20% Piperidene in DMF was used to remove Fmoc group.

The first amino acid Fmoc- (Leu) –OH was attached to the resin by amidation reaction using the following procedure.

The cross linked resin was taken in a peptide synthesizer and swelled in DCM for 1 hour and filtered under vacuum. Fmoc- Leu-OH (2.5equiv) dissolved in minimum dry DCM, DIC (3equiv) and DMAP (0.1equiv) were added to the resin and shaken well. After 1 hour, the reactants were filtered off and coupling process was repeated using the same procedure. After 30 min excess reactants were washed off with DMF and DCM (each 5 x 3 ml). The reaction was performed once more to ensure complete loading of the first amino acid on the resin.

The second amino acid Fmoc-Ile-OH was attached to the resin through amino acid coupling method.

Fmoc protection was removed from the first amino acid. Fmoc-Ile-OH (2 eq), PyBOP (2 eq) was dissolved in dry DCM. Added some drops of DMF to enhance solubility. To that mixture, added DIC (2 eq) and shaken for 30 minutes. The mixture is

then transferred to the resin in dry condition and shaken vigorously for 3 minutes. To the reaction mixture added DIPEA (3 eq) and the reaction was continued for 30 minutes. The completion of the reaction was checked with ninhydrin taking a small pinch of resin which is washed enough. The reaction was found to be complete from the absence of blue colour. Then the resin is washed with DCM, MeOH, DMF and DCM (2 x 3 ml). Then Fmoc protection of the second amino acis, ie Fmoc-Ile-OH is removed using Piperidine in DMF. The reagents are removed by thorough washing using the above solvents. The completeness of deprotection can be checked with ninhydrin solution again. Now the presence of blue colour indicates free amino group.

The above process is repeated until the remaining amino acid residues in the peptide sequence are successfully attached to the resin. They are Tyr, Pro Ile , Lys. The reaction can be monitored by thin layer chromatography wherever required.

Cleavage and isolation of the peptide was done as follows.

When the whole sequence is achieved, the resin was washed well with $CHCl_3$, DCM and methanol and dried under vacuum. The cleavage was executed with TFA, TIS, water, m-cresol (95%:2.5 %:2%:0.5%). This solvent mixture is added to resin and kept for 3 hours under nitrogen atmosphere. After the lime limit, the whole liquid part is collected. The resin was washed 2 times with TFA and the liquid part is collected to the vessel. The crude peptide can be isolated and purified with analytical techniques. The peptide was purified by preparative HPLC to yield a clear white powder.The complete synthesis is given in Table **6.3.**

Table 6.3. Details of Synthesis of Neuromedin N

Amino acid Residue	Quantity (mg) / Eq.	Coupling Time (Min)	Test results
Fmoc-Leu- OH	0.433/2.5 eq.	35	Ninhydrin (-ve)
Fmoc-Ile-OH	0.180/2eq.	40	Chloranil (-ve)
Fmoc-Tyr(TBu)-OH	0.250/2eq.	45	Ninhydrin(-ve)
Fmoc-Pro-OH	0.318/2eq.	35	Ninhydrin(-ve)
Fmoc-Glu(TBu)-OH	0.227/2eq.	50	Chloranil (+ve) Coupling repeated.
Fmoc-Ile-OH	0.245/2eq.	50	Ninhydrin(-ve)
Fmoc-Lys(Boc)-OH	0.188/2eq.	120	Chloranil(-ve)

6.2.4. Synthesis of MR- 14

Sequence: MRMKHVRAWIPRMR

Esterification of Fmoc-Arg (pbf) to polymer support was carried out by the following procedure.

The cross linked resin (120 mg, 0.78 mmol) was taken in a peptide synthesizer and swelled in NMP for 1 hour. Fmoc-Arg (pbf) (2.5equiv), DIC (2.5 eq) and DMAP (0.1 eq) were added to the resin and shaken well. After 1 hour, the unreacted reactants were filtered off and coupling process is repeated using the same procedure. After 30 min the excess reactants were washed off with DMF and NMP (each 5 x 3 ml). DMF used was degassed with nitrogen taking 2 litre DMF in a bottle and passing a continuous flow of nitrogen about 2 hours. NMP bottle was closed immediately after taking in order to avoid contact with air. The Fmoc protection was removed by 20% piperidine in DMF (2x3ml). After 20 min, the reagents were washed off with sufficient volume of DMF

followed by NMP .Keiser test was conducted to ensure the completion of reaction. Freshly prepared ninhydrin was used for each synthesis.

Coupling of Met-Arg-Pro-Ile-Trp-Ala-Arg-Val-His-Lys-Met-Arg-Met was carried out by the following procedure.

Fmoc-Arg (pbf) –resin in the peptide synthesizer was wetted with minimum NMP. Fmoc-Met-OH (2.5 eq) was attached to the resin in the presence of NMP, HOBT (2.5 eq), DIC (2.5 eq) and DIEA (0.3equiv). After 45 min, the resin was filtered and washed thoroughly with DMF (5 x 3 ml) and NMP (3 x 3 ml).

The remaining amino acids in the sequence Fmoc-Arg(Pbf)- OH, Fmoc-Pro-OH, Fmoc-Ile-OH, Fmoc-Trp(Boc)-OH, Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Val-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Met-OH were successively incorporated. Resin was washed with DMF and NMP. Acetylation reactions were performed twice for confirming the quantitative conversion. Each coupling and deprotection step was monitored by Kaiser Test. After the synthesis, Fmoc protection was removed and the resin was washed with DMF and NMP. The details are given in Table **6.4.**

Detachment of peptide from the resin was performed after completing the sequence as explained below.

The peptidyl resin was suspended in TFA (9.75 ml) and TIS (0.25 ml) at room temperature for 2 Hrs. The resin was filtered, washed with TFA. Collected filtrate was evaporated using Rota vacuum evaporator to a small volume. The peptide was precipitated by adding ice cold ether, washed thoroughly with ether and was collected by

centrifugation. The crude peptide obtained was purified with HPLC and characterized using mass spectrum.

Table 6.4. Details of synthesis of MR-14

Amino Acid Residues	Quantity (g / Equivalent)	Coupling Time (min.)	Test results Decision for next step
Fmoc- Arg(Pbf)-OH	0.182/3 eq.	45	Coupling repeated
Fmoc-Met-OH	0.0871/2.5 eq.	45	Ninhydrin(-ve)
Fmoc-Arg(Pbf)- OH	0.1523/2.5 eq.	45	Ninhydrin(-ve)
Fmoc-Pro-OH	0.0791/2.5 eq.	45	Ninhydrin(-ve)
Fmoc-Ile-OH	0.083/2.5 eq.	60	Chloranil(-ve)
Fmoc-Trp(Boc)-OH	0.123/2.5eq.	45	Chloranil(-ve)
Fmoc-Ala-OH	0.0730/2.5 eq.	45	Ninhydrin(-ve)
Fmoc-Arg(Pbf)-OH	0.1523/2.5 eq.	30	Ninhydrin(-ve)
Fmoc-Val-OH	0.0796/2.5 eq.	60	Chloranil(-ve)
Fmoc-His(Trt)-OH	0.145/2.5 eq.	35	Ninhydrin(-ve)
Fmoc-Lys(Boc)-OH	0.1099/2.5 eq.	50	Ninhydrin(-ve)
Fmoc-Met-OH	0.0871/2.5 eq.	45	Ninhydrin(-ve)
Fmoc-Arg(Pbf)-OH	0.1523/2.5 eq.	30	Ninhydrin(-ve)
Fmoc-Met-OH	0.0871/2.5 eq.	40	Ninhydrin(-ve)

6.2.5. Synthesis of YY-18

Sequence: YDGYPTFGEHKQEKDLEY

To perform esterification of Fmoc-Tyr (TBu)-OH on the cross linked resin; initially GDMA-4-VP resin (120 mg, 0.78 mmol) was taken in a peptide synthesizer and swelled in N-Methylpyrrolidone for 1 hour. Fmoc-Tyr (TBu)-OH (2.5 eq), diisopropyl carbodiimide (2.5 eq) and Dimethylaminopyridine (0.1 eq) were added to the resin and shaken well. After 1 hour, the reactants were filtered off and coupling process is repeated using the same procedure. After 30 min the reactants were washed off with DMF and

NMP (each 5 x 3 ml). DMF used was degassed with nitrogen taking 2 litre DMF in a bottle and passing a continuous flow of nitrogen about 2 hours. NMP bottle was closed immediately after taking in order to avoid contact with air. The Fmoc protection was removed by 20% piperidine in DMF (2x3ml). After 20 min, the reagents were washed off with sufficient volume of DMF followed by NMP .Keiser test was conducted to ensure the completion of reaction. Freshly prepared ninhydrin was used for each synthesis.

Coupling of the remaining amino acids were performed using the standard procedure.Fmoc-Tyr (TBu)-OH –resin in the peptide synthesizer was wetted with minimum NMP. Fmoc-Glu (TBu)-OH (2.5 eq) was attached to the resin in the presence of minimum NMP, HOBT (2.5 eq), DIC (2.5 eq) and DIEA (0.3 eq). After 45 min, the resin was filtered and washed thoroughly with DMF (5 x 3 ml) and NMP (3 x 3 ml).

The remaining amino acids in the sequence Fmoc-Leu, Fmoc-Asp, Fmoc-Lys, Fmoc-Glu, Fmoc-Gln, Fmoc- Lys, Fmoc-His, Fmoc-Glu, Fmoc-Gly, Fmoc- Phe, Fmoc-Thr, Fmoc-Pro, Fmoc-Tyr, Fmoc-Gly, Fmoc-Asp, Fmoc- Tyr were successively incorporated. Resin was washed with DMF and NMP. Acetylation reactions were performed twice for confirming the quantitative conversion. Each coupling and deprotection step was monitored by Kaiser Test. After the synthesis, Fmoc protection was removed and the resin was washed with DMF and NMP. The details are given in Table **6.5**.

To detach the peptide from the resin, the peptidyl resin was suspended in TFA (9.75 ml) and TIS (0.25 ml) at room temperature for 2 Hrs. The resin was filtered, washed with TFA. Collected filtrate was evaporated using Rota vacuum evaporator to a

small volume. The peptide was precipitated by adding ice cold ether, washed thoroughly with ether and was collected by centrifugation. The product is then purified and characterized by ESI-MS.

Table 6.5. Details of Synthesis of YY 18 peptide fragment

Amino Acid Residues	Quantity (g/Equivalent)	Coupling Time (min)	Test results Decision for next step
Fmoc-Tyr (TBu)-OH	0.129/3 eq.	60	Ninhydrin(-ve)
Fmoc-Glu (OtBu)-OH	0.0998/ 2.5 eq.	45	Ninhydrin(-ve)
Fmoc-Leu-OH	0.08296/ 2.5 eq.	60	Chloranil(-ve)
Fmoc-Asp(OtBu)-OH	0.0965/ 2.5 eq.	50	Ninhydrin(-ve)
Fmoc-Lys(Boc)-OH	0.1099/2.5 eq.	40	Ninhydrin(-ve)
Fmoc-Glu (OtBu)-OH	0.0998/2.5 eq.	45	Ninhydrin(-ve)
Fmoc-Gln(Trt)-OH	0.1433/2.5 eq.	45	Ninhydrin(-ve)
Fmoc-Lys(Boc)-OH	0.1099/2.5 eq.	40	Ninhydrin(-ve)
Fmoc-His(Trt)-OH	0.145/2.5 eq.	35	Ninhydrin(-ve)
Fmoc-Glu (OtBu)-OH	0.0998/2.5 eq.	60	Chloranil(-ve)
Fmoc-Gly-OH	0.0579/ 2.5 eq.	30	Ninhydrin(-ve)
Fmoc-Phe-OH	0.0909/2.5 eq.	60	Ninhydrin(-ve)
Fmoc-Thr(TBu)-OH	0.0933/2.5 eq.	40	Ninhydrin(-ve)
Fmoc-Pro-OH	0.07919/2.5 eq.	40	Ninhydrin(-ve)
Fmoc-Tyr (TBu)-OH	0.0896/2.5 eq.	35	Ninhydrin(-ve)
Fmoc-Gly-OH	0.0579/2.5 eq.	30	Ninhydrin(-ve)
Fmoc-Asp(OtBu)-OH	0.0965/2.5 eq.	60	Ninhydrin(-ve)
Fmoc-Tyr (TBu)-OH	0.0896/2.5 eq.	60	Ninhydrin(-ve)

2.6. Synthesis of YK- 15

Sequence: YLFKTNPNYKGNDIK

Esterification of Fmoc-Lys (Boc)-OH to polymer support was done as follows.

GDMA-4-VP resin (120 mg, 0.78 mmol) was taken in a peptide synthesizer and swelled in N-Methylpyrrolidone for 1 hour. Fmoc-Lys (Boc) - OH (2.5 eq), diisopropylcarbodiimide (2.5 eq) and Dimethylaminopyridine (0.1 eq) DIC (3 eq) and DMAP (0.1eq) were added to the resin and shaken well. After 1 hour, the reactants were filtered off and coupling process is repeated using the same procedure. After 30 min the ghbreactants were washed off with DMF and NMP (each 5x3ml). DMF used was degassed with nitrogen taking 2 litre DMF in a bottle and passing a continuous flow of nitrogen about 2 hours. NMP bottle was closed immediately after taking in order to avoid contact with air. The Fmoc protection was removed by 20% Piperidine in DMF (2 x 3 ml). After 20 min, the reagents were washed off with sufficient volume of DMF followed by NMP .Keiser test was conducted to ensure the completion of reaction. Freshly prepared ninhydrin was used for each synthesis.

Coupling of the second amino acid was done as follows.

Fmoc-Lys (Boc) – OH -resin in the peptide synthesizer was wetted with minimum NMP. Fmoc-Ile-OH (2.5 eq) was attached to the resin in the presence of NMP, HOBT (2.5 eq), DIC and DIEA (0.3equiv). After 45 min, the resin was filtered and washed thoroughly with DMF (5 x 3 ml) and NMP (3 x 3 ml).

The remaining amino acids in the sequence were successively incorporated. Resin was washed with DMF and NMP. Acetylation reactions were performed twice for confirming the quantitative conversion. Each coupling and deprotection step was monitored by Kaiser Test. After the synthesis, Fmoc protection was removed and the resin was washed with DMF and NMP. The details are given in Table **6.6.**

Detachment of peptide from the resin is a crucial step in peptide synthesis which can be done as follows.

The peptidyl resin was suspended in TFA (9.75 ml) and TIS (0.25 ml) at room temperature for 2 Hrs. The resin was filtered, washed with TFA. Collected filtrate was evaporated using Rota vacuum evaporator to a small volume. The peptide was precipitated by adding ice cold ether, washed thoroughly with ether and was collected by centrifugation. The crude peptide obtained was purified by HPLC and characterized with ESI MS.

Table 6.6. Details of Synthesis of YK 15

Amino Acid residue	Quantity (g/eq.)	Coupling Time (Min)	Test result (Decision to the next step)
Fmoc –Lys(Boc)-OH	0.1319/ 3 eq.	60	Coupling repeated
Fmoc-Ile-OH	0.08296/ 2.5 eq.	60	Chloranil(-ve)
Fmoc-Asp(OtBu)-OH	0.0965/ 2.5 eq.	45	Ninhydrin(-ve)
Fmoc-Asn(Trt)-OH	0.14006/ 2.5 eq.	35	Ninhydrin (-ve)
Fmoc-Gly-OH	0.0579/ 2.5 eq.	60	Ninhydrin (-ve)
Fmoc-Lys(Boc)-OH	0.1099/ 2.5 eq.	50	Ninhydrin (-ve)
Fmoc-Tyr(TBu)-OH	0.0896/ 2.5 eq.	35	Ninhydrin (-ve)
Fmoc-Asn(Trt)-OH	0.14006/ 2.5 eq.	35	Ninhydrin (-ve)
Fmoc-Pro-OH	0.07919/ 2.5 eq.	45	Ninhydrin (-ve)
Fmoc-Asn(Trt)-OH	0.14006/ 2.5 eq.	35	Ninhydrin (-ve)
Fmoc-Thr(TBu)-OH	0.0933/ 2.5 eq.	40	Ninhydrin (-ve)
Fmoc-Lys(Boc)-OH	0.1099/ 2.5 eq.	50	Ninhydrin (-ve)
Fmoc-Phe-OH	0.0909/ 2.5 eq.	60	Ninhydrin (-ve)
Fmoc-Leu-OH	0.0829/ 2.5 eq.	60	Chloranil (-ve)
Fmoc-Tyr(TBu)-OH	0.0896/ 2.5 eq.	35	Ninhydrin (-ve)

2.7. Synthesis of Amyloid β peptide

Sequence: DAEFRHDSGYEVHHQKLVFFAEDVGSNK

The first amino acid Fmoc-Lys (Boc)-OH was attached to GDMA-4-VP resin through an ester linkage as described below.

The cross linked resin (500 mg, 0.78 mmol) was taken in a peptide synthesizer and swelled in minimum NMP for 1 hour. Fmoc-Lys (Boc)-OH (5 eq), DIC (5 eq.) and DMAP (0.1 eq) DIC (3 eq) and DMAP (0.1eq) were added to the resin and shaken well. After 1 hour, the reactants were filtered off and coupling process was repeated using the same procedure. After 30 min the excess reactants were washed off with DMF and NMP (each 5 x 3 ml). DMF used was degassed with nitrogen taking 2 litre DMF in a bottle and passing a continuous flow of nitrogen about 2 hours. NMP bottle was closed immediately after taking in order to avoid contact with air. The Fmoc protection was removed by 20% piperidine in DMF (2 x 3 ml). After 20 min, the reagents were washed off with sufficient volume of DMF followed by NMP .Keiser test was conducted to ensure the completion of reaction.

Coupling of Fmoc-Asn-OH to the amino acid attached resin can be carried out using the following procedure.

Fmoc-Lys (Boc) – OH -resin in the peptide synthesizer was wetted with minimum NMP. Fmoc-Asn-OH (4 eq) was attached to the resin in the presence of NMP, HOBT (4 eq), DIC (4 eq) and DIEA (0.3equiv). After 45 min, the resin was filtered and washed thoroughly with DMF (5 x 3 ml) and NMP (3 x 3 ml).

The remaining amino acids in the sequence were successively incorporated. Resin was washed with DMF and NMP. Acetylation reactions were performed twice for confirming the quantitative conversion. Each coupling and deprotection step was monitored by Kaiser Test. After the synthesis, Fmoc protection was removed and the resin was washed with DMF and NMP.

Peptide was detached from the resin using the following procedure.

The peptidyl resin was suspended in TFA (9.75 ml) and TIS (0.25 ml) at room temperature for 2 Hrs. The resin was filtered, washed with TFA. Collected filtrate was evaporated using Rotary evaporator to a small volume. The peptide was precipitated by adding ice cold ether, washed thoroughly with ether and was collected by centrifugation. The crude peptide obtained was purified by HPLC and characterized with ESI MS. The detailed synthetic strategy is given in the Table **6.7.**

Table 6.7. Details of Synthesis of Amyloid β peptide

Amino Acid residue	**Quantity (g / eq.)**	**Coupling Time (Min)**	**Test result (Decision to the next step)**
Fmoc –Lys(Boc)-OH	0.914/ 5 eq	60	Coupling repeated
Fmoc-Asn(Trt)-OH	0.930/ 4 eq	35	Ninhydrin (-ve)
Fmoc- Ser(TBu)-OH	0.598/ 4 eq	45	Ninhydrin (-ve)
Fmoc-Gly-OH	0.463/ 4 eq	45	Coupling repeated. Chloranil (-ve)
Fmoc-Val-OH	0.529/ 4 eq	60	Ninhydrin (-ve)
Fmoc-Asp(OtBu)-OH	0.641/ 4 eq	45	Ninhydrin(-ve)
Fmoc-Glu(TBu)-OH	0.663/ 4 eq	60	Ninhydrin (-ve)
Fmoc-Ala-OH	0.513/ 4 eq	45	Ninhydrin (-ve)
Fmoc-Phe-OH	0.604/ 4 eq	60	Coupling reapeated. Acetylated.

Amino Acid residue	Quantity (g / eq.)	Coupling Time (Min)	Test result (Decision to the next step)
Fmoc-Phe-OH	0.604/ 4 eq	60	Coupling reapeated. Acetylated.
Fmoc-Val-OH	0.529/ 4 eq	60	Coupling reapeated. Acetylated.
Fmoc-Leu-OH	0.551/ 4 eq	60	Coupling repeated. Chloranil (-ve)
Fmoc-Lys(Boc)-OH	0.731/ 4 eq	50	Ninhydrin (-ve)
Fmoc-Gln(Trt)-OH	0.952/ 4 eq	90	
Fmoc-His(Trt)-OH	0.967/ 4 eq	45	Ninhydrin (-ve)
Fmoc-His(Trt)-OH	0.967/ 4 eq	60	Ninhydrin (-ve)
Fmoc-Val-OH	0.529/ 4 eq	60	Coupling repeated. Chloranil (-ve)
Fmoc-Glu(TBu)-OH	0.663/ 4 eq	60	Ninhydrin (-ve)
Fmoc-Tyr(TBu)-OH	0.717/ 4 eq	60	Ninhydrin (-ve)
Fmoc-Gly-OH	0.463/ 4 eq	90	Ninhydrin (-ve)
Fmoc- Ser(TBu)-OH	0.598/ 4 eq	90	Ninhydrin (-ve)
Fmoc-Asp(OtBu)-OH	0.641/ 4 eq	120	Ninhydrin(-ve)
Fmoc-His(Trt)-OH	0.967/ 4 eq	90	Coupling repeated. Chloranil (-ve)
Fmoc-Arg(Pbf)-OH	1.012/ 4 eq	80	Ninhydrin (-ve)
Fmoc-Phe-OH	0.604/ 4 eq	60	Coupling repeated. Ninhydrin (-ve)
Fmoc-Glu(TBu)-OH	0.663/ 4 eq	80	Ninhydrin (-ve)
Fmoc-Ala-OH	0.513/ 4 eq	90	Coupling repeated. Chloranil (-ve)
Fmoc-Asp(OtBu)-OH	0.641/ 4 eq	45	Ninhydrin(-ve)

6.3. Results and Discussion

6.3.1. Results and discussion of Anti-inflammatory peptide

The reactive site present in GDMA-4-VP resin is –OH group so usually reaction takes place between –OH groups of the resin with Fmoc- amino acid. Here amino

functionalised GDMA-4-VP resin is used as solid support. So $–NH_2$ group of the resin reacts with Fmoc amino acid. The side chain protecting groups OtBu, TBu, Trt are used in selective Fmoc amino acids. The deprotection and coupling reactions are followed by ninhydrin test and chloranil test. The reverse amidation coupling of the remaining amino acids was performed with DIC/HOBt in DIEA. The final cleavage of the peptide was done with TFA.

The ESI-MS spectrum of the peptide is shown below in Fig.**6.4.** The molecular mass 1079.33 Da of the target peptide was evident from the mass spectrum.

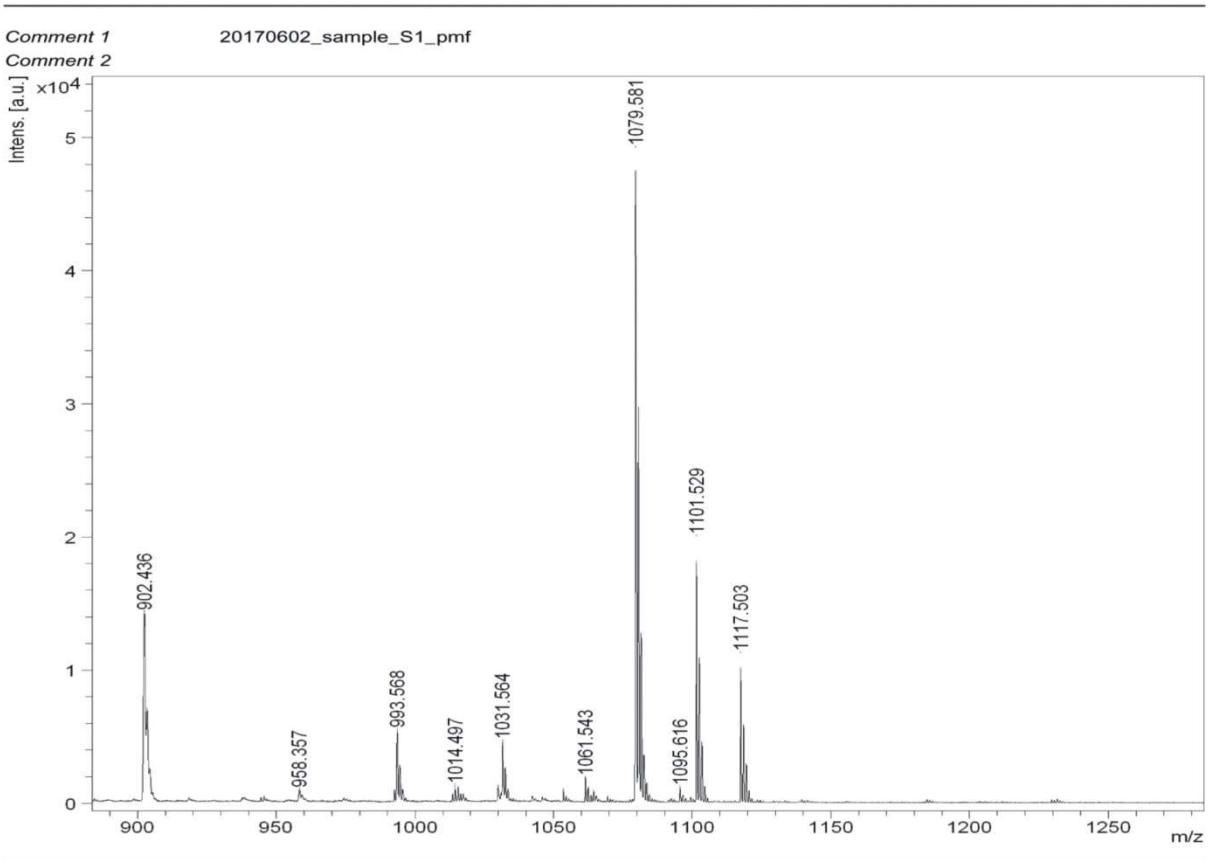

Fig. 6.4. ESI-MS of Anti –inflammatory peptide.

The ^{1}H NMR spectrum of Anti- inflammatory peptide is given in Fig.**6.5.** The signals obtained in the range 6-8 ppm shows the presence of protons in the aromatic ring. Because of the large size of the molecule, the coupling of individual protons undergoes overlapping so is not visible clearly.

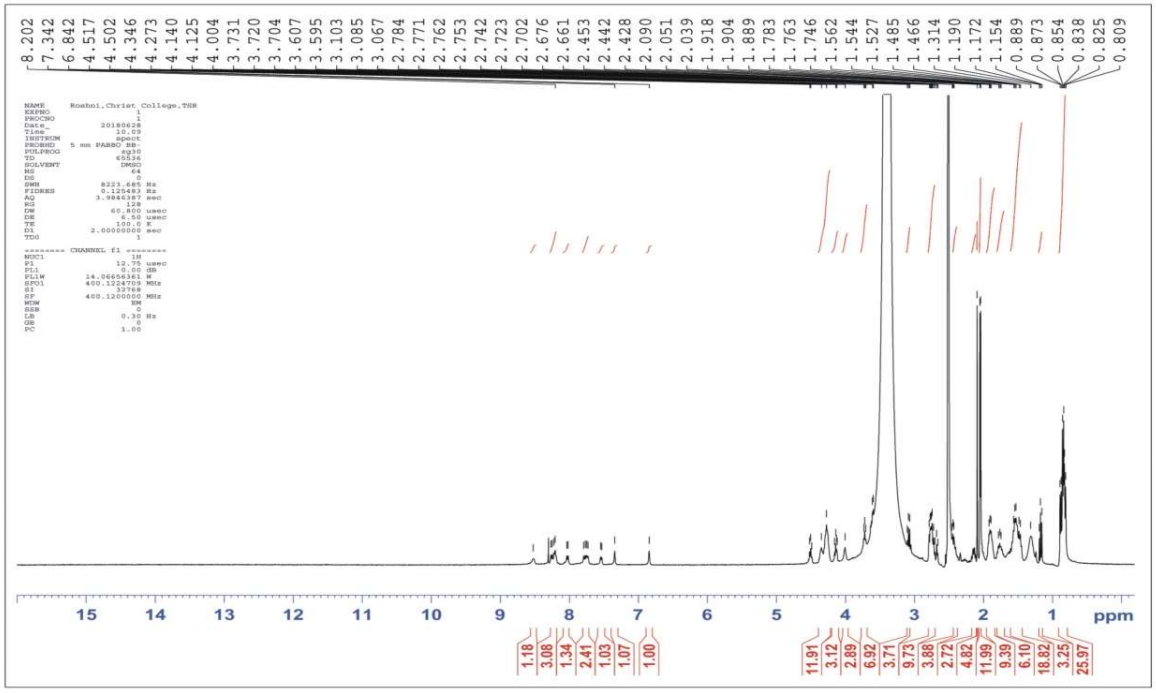

Fig. 6.5. ^{1}H NMR spectrum of Anti-inflammatory peptide.

6.3.2. Results and Discussion of Neurotensin peptide

The first amino acid Fmoc-Arg was attached to amino functionalised GDMA-4-VP resin through the $–NH_2$ group present in the resin. The other amino acids present in the sequence were attached through reverse amidation coupling using DIC/ HOBt in DIEA. After the coupling of all amino acids in the sequence, the product can be cleaved using TFA. The completion of deprotection and coupling reactions were ensured using Ninhydrin test. Depending on the test results, coupling was repeated wherever required.

The ESI-MS spectrum of neurotensin peptide is given in Fig.**6.6**. The molecular mass of 1030.13 Da is evident from the mass spectrum obtained.

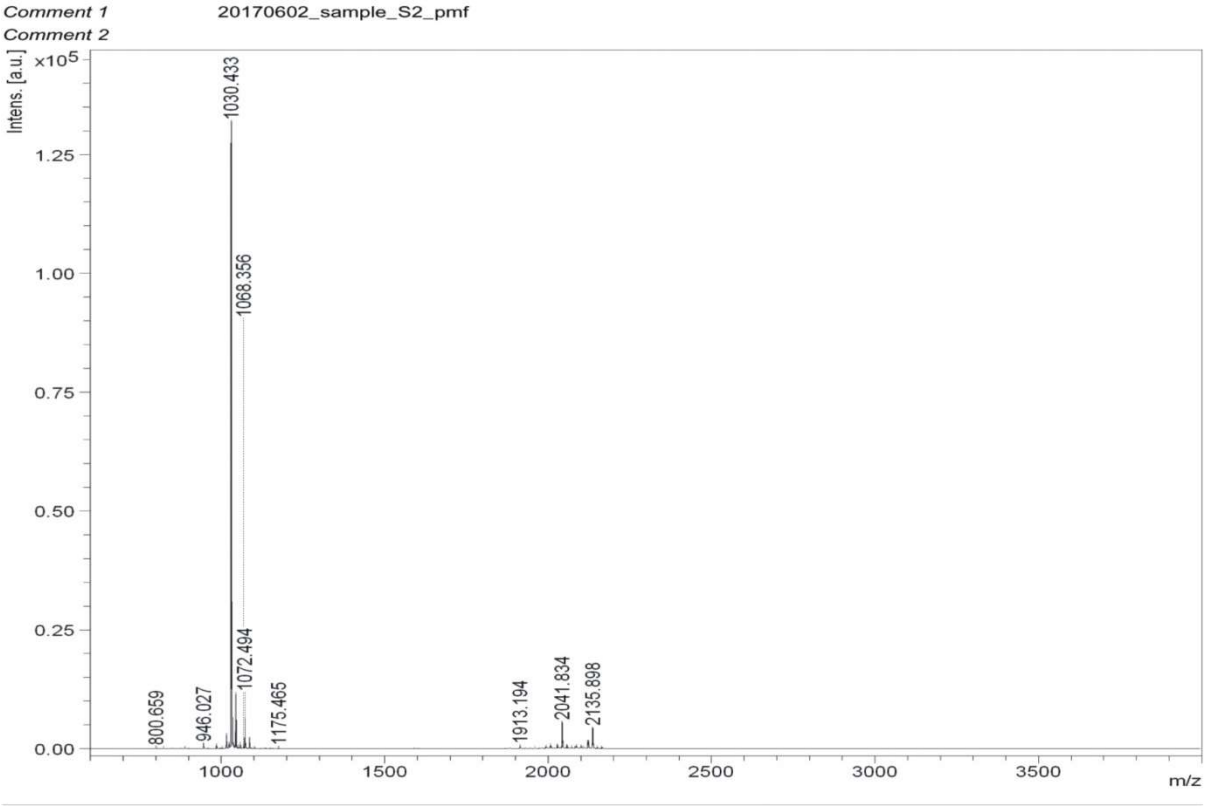

Fig. 6.6. ESI-MS spectrum of Neurotensin peptide.

6.3.3 Result and discussion of Neuromedin N

Here the solid support amino functionalised GDMA-4-VP resin was used for the synthesis of Neuromedin N peptide. For excellent swelling of the resin, NMP was added. For effective coupling of amino acids, DIC/HOBt in DIEA was used.

The ^{1}H NMR spectrum of Neuromedin N is shown in Fig.**6.7**.Aromatic protons are visible in the range 6-8 ppm. Coupling of individual protons are not clear because of the overlapping due to large size of molecule.

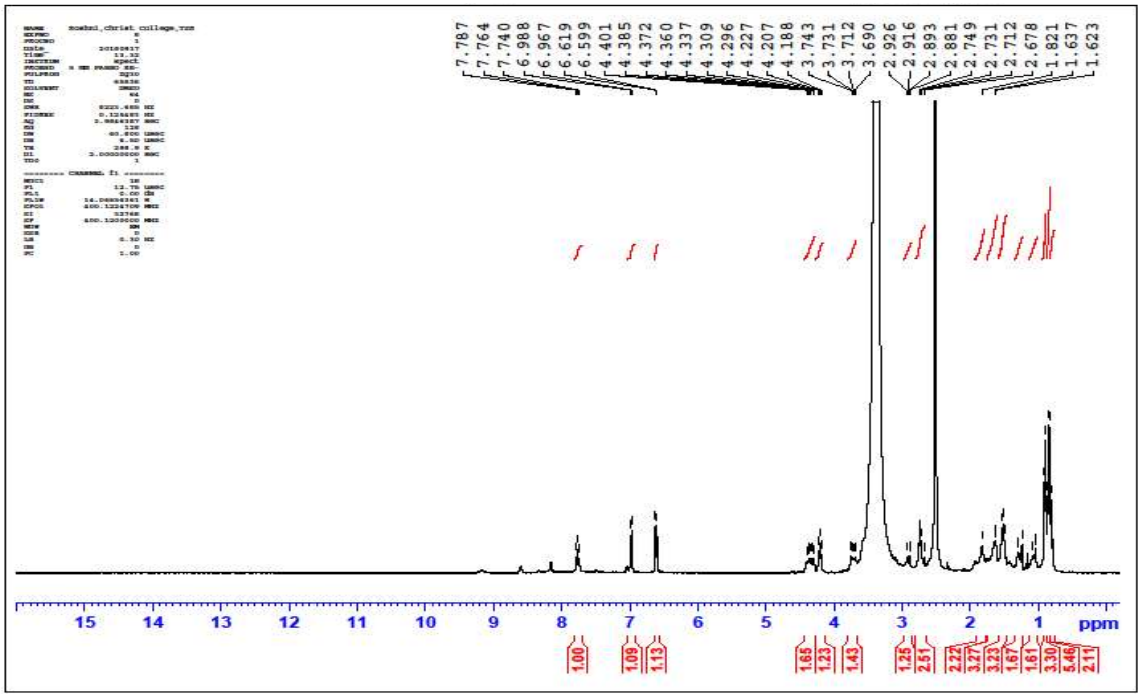

Fig.6.7. ^{1}H NMR spectrum of Neuromedin N.

3.4. Result and Discussion of MR-14 peptide

The first amino acid Fmoc- Arg was attached to resin by esterification reaction and the second amino acid Fmoc- Met was coupled to arginine by amidation. Coupling time needed for most amino acids were found to be less except hydrophobic ones which is evident from the above Table.**6.4.** When the 14 amino acids are coupled to the resin, HF is added to remove all protection groups. When the excess solvent is removed from the mixture, the peptide is precipitated as white powder by adding ice cold ether. It was then purified and characterised using HPLC as given in Fig.**6.8.**

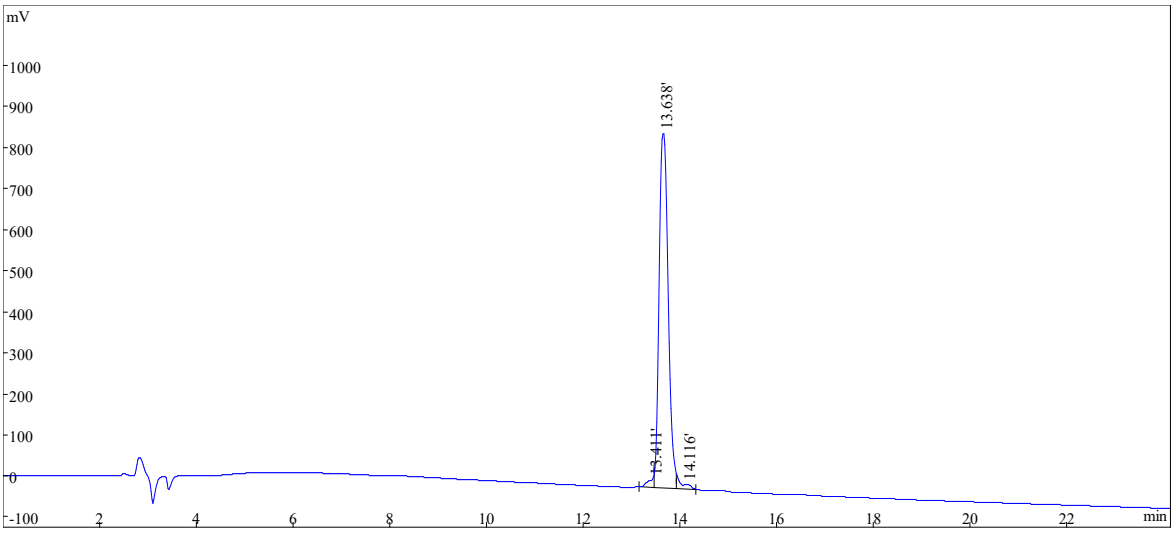

Fig. 6.8. HPLC of MR14 peptide.

The single peak in HPLC at ret.time 13.6 shows the purity of our peptide sequence. The ESI-MS spectrum of MR14 is given below in Fig. **6.9**. It consists of three peaks at M+2H, M+3H, and M+ 4H which verifies the formation of MR 14.

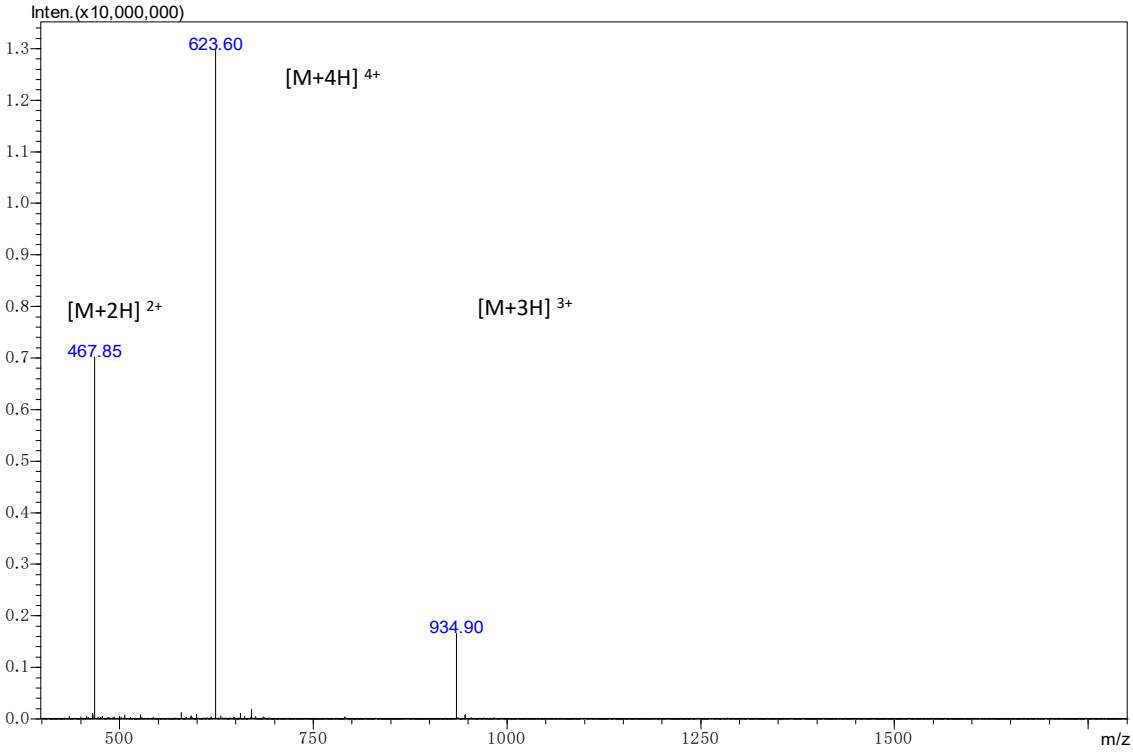

Fig.6.9. ESI- MS of MR 14

The hydrophilic peptide fragment MR 14 was successfully synthesised on GDMA-4VP resin using Fmoc strategy.The observed common properties of MR 14 are given below in Table **6.8.**

Table 6.8. Observed Properties of MR 14

Test	**Result**
Appearance	White to off white powder
Purity(HPLC)	95.85%
Mass	Consistent
Molecular weight	1868.39

6.3.5. Results and Discussion of YY-18 peptide

The first amino acid Fmoc- Tyr (TBu)-OH was esterified to resin and the second amino acid Fmoc- Glu(OtBu)-OH was coupled to arginine by amidation. Coupling time needed for most amino acids were found to be less except hydrophobic ones which is evident from the above Table **6.5**. When the 18 amino acids are coupled to the resin, HF is added to remove all protection groups. After removing the excess solvent from the mixture, the peptide is precipitated as white powder by adding ice cold ether. It was then purified and characterised using HPLC and ESI-MS.

HPLC of YY 18contains a sharp single peak at ret. Time 10.50 as shown in Fig.**6.10.** This shows the purity of the obtained peptide.

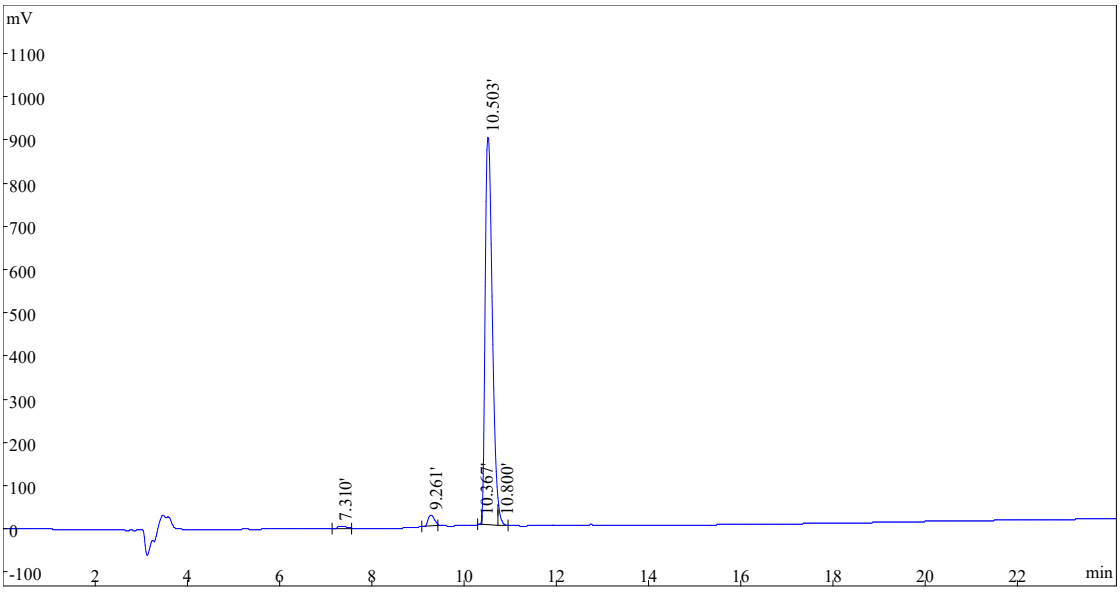

Fig.6.10. HPLC of YY 18 peptide

The ESI-MS of YY 18 shows three peaks as shown in Fig.**6.11**. This confirms the molecular mass of the peptide synthesised.

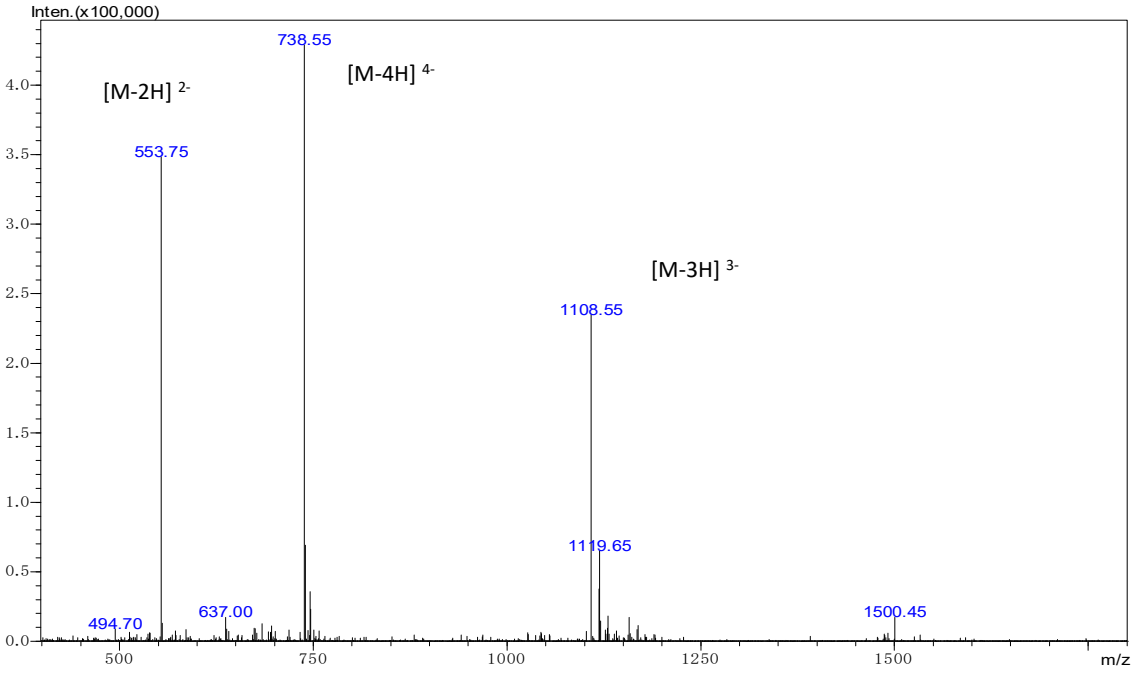

Fig.6.11. ESI-MS spectra of YY 18 peptide

The hydrophilic peptide YY 18 was successfully synthesised on GDMA-4VP resin. The observed common properties of the peptide fragment are given in the table below.

Table 6.9. Observed Properties of YY 18

Test	Result
Appearance	White to off white powder
Molecular weight	2219.37
Purity(HPLC)	95.75%
MS	Consistent

6.3.6. Result and Discussion of YK 15

The novel polymer support GDMA-4-VP resin was used for the synthesis of hydrophilic peptide YK 15. The resin shows excellent swelling properties in NMP which will help to avoid the folding of the sequence which will result in aggregation. The side chains of amino acids are protected until all the 15 amino acids are being coupled and Keiser test was used to detect free amino group. Using esterification the first amino acid was attached to the resin and the rest fourteen were coupled through amide bond. Finally the resin was detached from the peptide using TFA and suitable scavengers. The HPLC of the peptide YK15 is given below in Fig.**6.12**. The sharp single peak at ret. time 10.5 shows our target peptide is highly pure. The ESI-MS spectrum of YK 15 is given below in Fig.**6.13.** Two charge states of peptide were readily detected: m/z 605.85[M+3H] $^{3+}$ and 908.30 m/z [M+2H] $^{2+}$ verifying a molecular mass of 1815.08 Da as proof of target peptide.

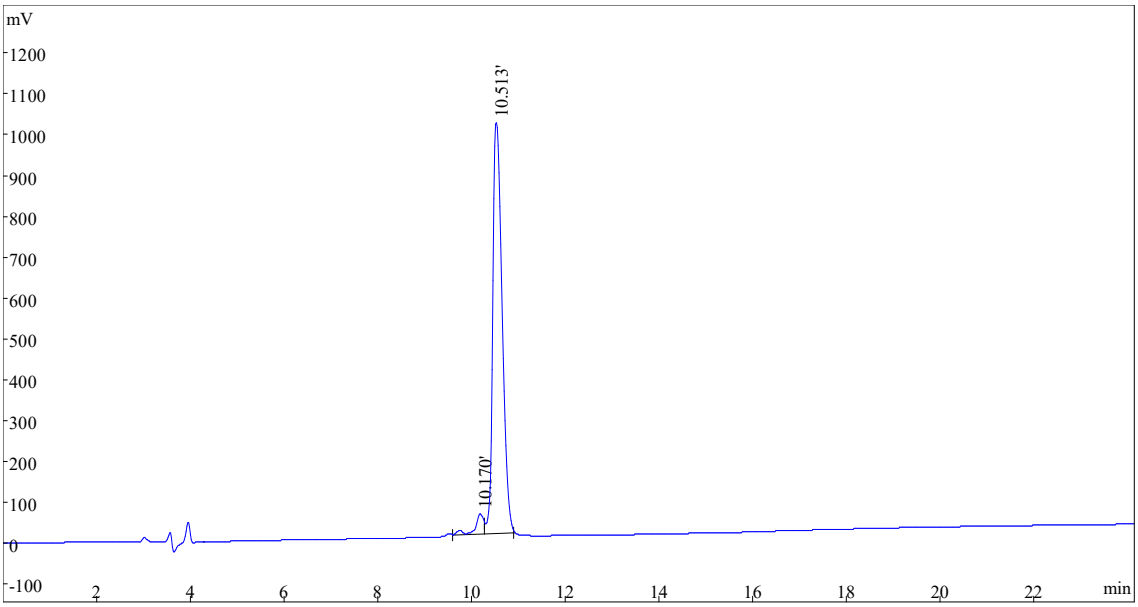

Fig.6.12. HPLC of YK- 15 Peptide.

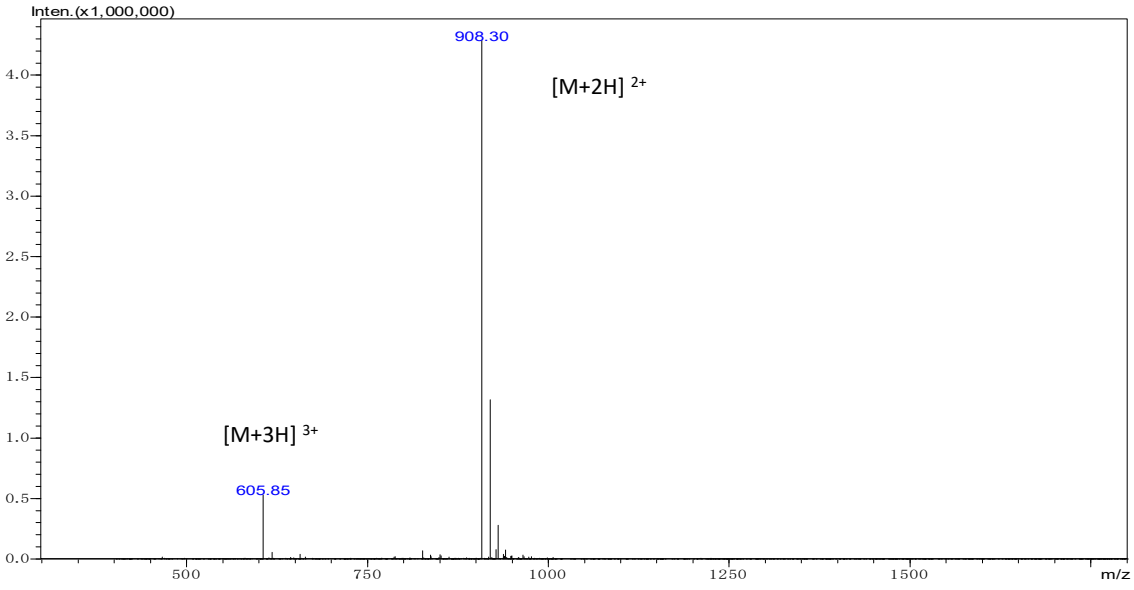

Fig. 6.13. ESI- MS spectra of YK- 15 peptide.

Here we successfully synthesized hydrophilic YK-15 peptide on Hydrophilic polymer support GDMA-4VP resin. The product was obtained in good yield and high purity. The observed common properties of MK 15 are given below in Table **6.10**.

Table 6.10. Observed common properties of YK- 15

Test	**Result**
Appearance	White to off white powder
Molecular weight	1815.08
Purity(HPLC)	95.8%
MS	Consistent

6.3.7. Result and discussion of Amyloid β peptide

Biologically active Amyloid β peptide was prepared on GDMA-4-VP resin. In order to ensure the yield of the product, esterification reaction was carried out with more resin and more reagents. The loading of first amino acid determines the yield of the end product since, amount of coupling decreases with number of amino acids in the sequence. Coupling also was conducted two times to ensure the reaction. Coupling after the eighth amino acid was found to be difficult due to the presence of Ala-Phe-Phe-Val sequence. Although we have prepared the product, the yield of the product was found to be less compared to other products prepared. The ESI-MS spectrum of amyloid β peptide is shown in Fig.**6.14**. The mass of the product is confirmed from the spectrum.

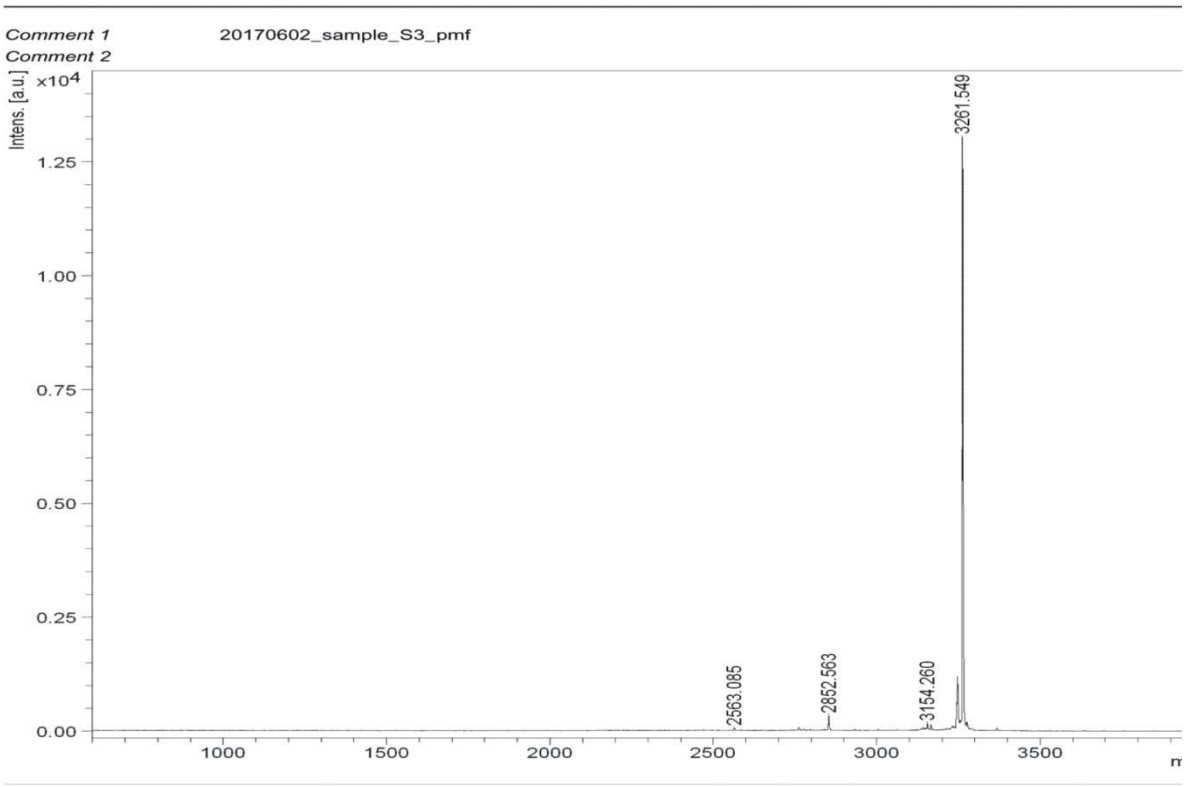

Fig.6.14.ESI- MS of Amyloid β peptide

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Chapter 7
Conclusion

The demand for therapeutic peptides is increasing day by day than biological peptides can alone be fulfilled. Synthetic peptides optimized by equivalent of biological peptides with high purity could be used commercially. Solid phase peptide synthesis introduced by R.B. Merrifield is a powerful method in the synthesis of peptides and it is continuously undergoing improvements thereafter. One of the major drawbacks of Merrifield's method was yield and purity of the final product. It is reduced to a large extent by the careful selection of the polymer support in the present study. An efficient polymer support is the first requirement for the preparation of peptides by solid phase synthesis.

Synthesis of Glycerol Di Methacrylate cross-linked 4-Vinyl pyridine resin and its application in the synthesis of peptides is the main objective of this work. The resin was prepared successfully by suspension polymerisation where toluene was used as the organic medium and PVA was used as the suspension stabiliser. The shape, size and morphological topographies of the cross-linked polymer beads were evaluated by scanning electron microscopy. SEM analysis of the polymer indicated that they are uniform spherical beads. The new resin is hydrophilic and flexible and by using this resin some biologically relevant peptides were synthesised. It can be used in diverse applications without losing stability because of its extra ordinary swelling and stability in solvents used for SPPS. Moreover the new support is easy to handle besides the ease of preparation. The resin contains a secondary hydroxyl group as the reactive site. So vigorous functionalization steps are not required. The optimum hydrophilic- hydro phobic balance of the resin is exploited for the preparation of these peptides. The effective N- terminal protective Fmoc group, highly penetrating reagents and solvents along with effective side chain protecting groups were used to avoid major drawbacks of previous studies.

The functional groups accessible for C-terminal amino acid incorporation can be easily determined by selecting the degree of cross-linking in the resin. The performance of GDMA-4-VP resin in polypeptide synthesis, especially in the case of peptide sequences which form secondary structures during the synthesis, makes it better choice when compared to various styrene based supports.

GDMA-4-VP resin in various cross-linking ratio were prepared, out of which 1%, 2% and amino functionalised GDMA cross- linked 4-VP resin were used for peptide synthesis. During the synthesis, 2% cross-linked resin was found to be more efficient than 1%. GDMA-4-VP resin with lower crosslinking ratio contain relatively low hydroxyl functional groups and found to be less favourable for using as a solid support. Resin with low cross-linking also decreases mechanical stability and causes difficulties during filtration of reagents and solvents. Mechanical stability increases with increase in degree of crosslinking. Capacity of hydroxyl functional group and rigidity of the support was also found to be increasing. So 2% GDMA cross-linked 4-VP resin was found to be satisfactory for peptide synthesis compared to 1% GDMA-4-VP resin.

In order to check the efficiency of GDMA-4-VP resin as a solid support in SPPS, a tetra peptide VYGR was synthesised on the resin using Fmoc/tBu strategy. The peptide was obtained in good yield and purity. The same sequence was synthesised on Merrifield resin. The HPLC of VYGR obtained from GDMA-4-VP resin showed a sharp single peak at retention time 3.9 min. and that obtained from Merrifield resin showed some additional peaks. Since this was the initial study, no other characterisation was done. As a next stage a decapeptide KVKRIILARS was synthesised on GDMA-4-VP resin. The product was obtained in good yield. HPLC and ESI-MS of the peptide confirmed its purity and formation. So it was concluded that our resin gave superior results than Merrifield resin and Wang resin in terms of purity and yield.

We have successfully synthesised biologically relevant Angiotensin II and AngiotensinIV on GDMA-4VP resin and compared with the previously used methods and anticipating profitable to the biodrug community. Angiotensin II and Angiotensin IV showed a crude yield of >77% and >64% purity which became >89% in yield and >99%in purity.

We have successfully synthesised 7 biologically active peptides on GDMA-4-VP resin. Anti-inflammatory peptide, Neurotensin, Neuromedin, MR-14, YY-18, YK-15, were synthesised successfully with good yield and purity. Amyloid β peptide which is a difficult sequence was obtained in high purity. But the yield of the product was very poor because of aggregation. Introducing suitable Pseudoproline dipeptides to the peptide sequence, self- aggregation during reaction can be eliminated.

Nowadays synthetic peptides attracted much attention in the bio drug community. Naturally occurring peptides have a short biological half-life due to rapid degradation by various peptidases and doesn't fulfil our requirements continuously. Synthetic peptides are attributed to promoting biological natured peptides by using chemical methods. Definitely all therapeutic peptides associated with lengthy and difficult sequences so it couldn't guarantee the expected purity and yield, enough to fulfill all requirements in the biological community. The study delivers a new solid support which can be used for the synthesis of biologically active peptides in large scale. The use of this resin with other new methods thought to give better results. Improved Fmoc strategy improves the quality of difficult sequences and hope the present work help to biological community like an extension of the hands on research work.

List of Publication

1. **K.C.Rohini**, V.D.John, K.P.SubhahChandran. Synthesis and characterisation of a Hydrophilic support for solid phase organic reactions. *International Journal of Scientific Research in Chemical Sciences*.Vol.4, issue 3, 2017.

2. **K.C.Rohini**, V.D.John, K.P.SubhahChandran. Cost effective synthesis of Angiotensin II and Angiotensin IV on a hydrophilic polymer support. *International journal of Current research.* Vol.9, issue.5, 2017.

3. R. Selvam, **K.C. Rohini**, C. Arunan and K.P. Subashchandran, Synthesis of biologically important Angiotensin II and Angiotensin IV peptides by using 4-(2', 4'-Dimethoxypenyl-F- moc-Aminomethyl) phenoxy resin. *, Asian journal of chemistry,* Vol.25, No.15, 8673-8676, 2013.

Conference Papers

1. **Rohini.k.c**, Subhashchandran.K.P and John.V.D "Cost- effective synthesis of a biologically active decapeptide on hydrophilic polymer support by Solid phase peptide synthesis", *Book of Abstracts, Internationa Conference on materials for the Millenium,January 14-16,2016.*Organized by CUSAT on January 2016.

2. **Rohini.K.c,** Dr.V.D.John and Dr.K.P.Subhashchandran, "Modified Fmoc Chemistry for the Solid phase synthesis of Angiotensin II on 4-(2', 4'-dimethoxyphenyl-fmoc-amino methyl)-Phenoxy resin- A relatively clean method". National seminar at St.Mary's College,Thrissur on july 2014.

3. **Rohini.k.c**, Subhashchandran.K.P and John.V.D, "Synthesis of a tetrapeptide on GDMA-4-VP polymer support using improved Fmoc strategy." National Conference at SVNSS College, December 2015.

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Research Paper

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Synthesis and Characterisation of a Hydrophilic Support for Solid Phase Organic Reactions.

K.C. Rohini[1*], V.D. John[2], K.P. Subhashchandran[3]

[1*] Department of Chemistry, Christ College, University of Calicut, Calicut, India
[2] Depatment of Chemistry, Christ College, University of Calicut, Calicut, India
[3] Department of Chemistry, Sri Vyasa NSS College, University of Calicut, Calicut, India

Corresponding Author: rohinichndrn@gmail.com, Tel.: +00-96056-13695

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Abstract—Glycerol dimethacrylate cross-linked 4-vinyl pyridine (GDMA-4-VP) support with optimum hydrophobic-hydrophilic balance has been introduced as a new class of polymer support in manual solid phase organic synthesis. The synthesis of GDMA-4-VP resin is based on the crosslinking of GDMA with 4-VP (in various cross linking densities) using free radical polymerization, affording a polymer containing secondary hydroxyl groups in great yield and purity. Benzoyl peroxide was used as initiator for the synthesis of polymer either via bulk polymerization or via suspension polymerization in polyvinyl alcohol, the latter yielding a beaded resin. Characterization was done by SEM and FT-IR spectroscopic techniques. The resin was found to undergo good swelling in various solvents, polar and non- polar. The synthetic utility of GDMA-4-VP resin was demonstrated by preparing biologically active Angiotensin II. The purity of the peptide was checked by HPLC.

Keywords— Solid phase synthesis, GDMA-4VP resin, suspension polymerization

I. Introduction

Solid phase peptide synthesis is the most popular way followed now-a-days to synthesise peptides on small scale and large scale. Commercial anti-HIV peptide, Fuzeon, is a most obvious example which is manufactured in multi kilograms using solid phase synthetic strategy [1]. The success of solid phase technique depends on the properties of solid support [2, 3]. Peptide synthesis using the classical PS-DVB resin meets some drawbacks because of the rigidity, hydrophobicity and physico-chemical incompatibility of the polymer with the growing peptide chain. [4,5].The strong hydrophobic, macromolecular environment of polymer can persuade the growing peptide chain to adopt unfavourable conformation that lead to low yield of purity of target peptides[6]. Peptide chemistry utilises different classes of hydrophilic polymers as support for chemical reactions. The solubility and diffusivity of hydrophilic polymers in water facilitates their biomedical applications. In this work, the Influence of Glyceroldimethacrylate cross-linker in the 4-Vinyl Pyridine support for peptide synthesis was studied by synthesising a biologically active Angiotensin II peptide fragment by improved F-moc strategy.

Angiotensin was individually isolated in Indian apolis and Argentina in the late 1930s (as 'angiotonin' and 'hypertensin', respectively). Angiotensin is a peptide hormone that reasons vasoconstriction and a succeeding rise in blood pressure. It is fragment of the renin-angiotensin system, which is a major aim for drugs that lessen blood pressure. Angiotensin also arouses the discharge of aldosterone, another hormone, from the adrenal cortex. Aldosterone stimulates sodium retention in the distal nephron, in the kidney, which also pushes blood pressure up. Angiotensin II performances on the adrenal cortex, producing it to discharge aldosterone, a hormone that causes the kidneys to recollect sodium and expel potassium. Raised plasma angiotensin II levels are liable for the high aldosterone levels present during the luteal phase of the menstrual cycle [7, 8]. The chemical arrangement of Angiotensin II is presented in figure 1.

(NH_2)Asp-Arg-Val-Tyr-Ile-His-Pro-Phe ($CONH_2$)

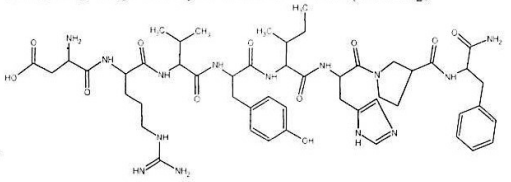

Fig. 1. Chemical composition and structure of Angiotensin II contains 8 amino acid residues (m.wt:1046 g/mol)

After a brief introduction, the synthesis of GDMA-4VP resin by suspension polymerisation is discussed in detail. The experimental part also contains the synthesis of Angiotensin II using Fmoc strategy, on the resin synthesised followed by the result and discussion. In this work we concerned purity which should applicable for biological research and yield for