Solution Phase synthesis

When two amino acids are condensed to form a peptide bond, the two amino groups and two carboxylic acid groups could combine in four different ways (Fig 4.1). In such condensations, several longer peptides would also be synthesized. Hence such strategies are unsuitable for good yields of the desired peptide.

Fig 4.1: Possible products in dipeptide formation.

Chemists have solved this problem by protecting the N-terminal of one amino acid and the C-terminal of the second amino acid by different types of Media:protecting groups (Fig 4.2). This allows only one site for reaction on each amino acid and thereby achieve the desired site-selectivity. Once the new peptide bond is formed in the desired way, the protecting groups could be removed one by one. This is an important facility. Chemists now have the choice of retaining any one of the protections to facilitate further manipulations on this molecule.

Discussion on Protection and Deprotection Strategies in Organic Synthesis, along with important reading references, could be found in Media:Logic of Organic Synthesis, which forms a part of this course series.
As in classical syntheses, protection at the N-terminal could generally be an amide linkage and the acid group could be protected as esters. Simple benzamides or acetamides are similar to peptide bonds in their chemistry. That means removal of the N-protection would hydrolyze the peptide bond as well. Furthermore, such acid or base catalyzed hydrolysis of amide bonds is also reactive at the COOP centers causing deprotection of the ester groups. It is therefore desirable to introduce protecting groups at the C-terminal and the N-terminal that are stable enough to withstand the peptide bond formation, but are more reactive than the peptide bond. Chemists have solved this problem by developing several ingenious reagents for protection at the C-terminal as well as the N-terminal.

**Media:N-terminal protection:**

**Methyloxycarbonate Protection:**

The N-terminal is protected by alkylxycarbonyl groups (N – COOR) (Fig 4.3). Media:Methyloxycarbonate protection at the N-terminal provides an ideal situation.
Fig 4.3 Two different N-protections –acetamide and –N-carbomethoxy groups

Unlike the acetamide carbonyl group, this carbonyl group is an amide at one valency and an ester at the other. On treatment with dilute sodium hydroxide at room temperature, an Media:acyl-oxygen fission occurs at the C – O bond, which is readily cleaved like an ester. The product N-COO⁻, which is very unstable, decomposes to give an amine and CO₂.

Media:Tert-Butyloxycarbonyl- Protection (Boc-):

The most popular protection at the N-terminal is the tert-Butyloxycarbonyl-(Boc-) protection (Fig 4.4). A complimentary C-protection at the COOH group could be a methyl ester. The t-butyloxycarbonyl- group has a hindered C=O group due to the bulky t-butyl group. When reacted with mild alkali such as 1-N NaOH, the Boc- group is stable, but the methyl ester at the C-terminal is readily hydrolyzed to acid. On the other hand, the Boc- group is very unstable to trifluoroacetic acid (in methylene chloride as solvent). The group undergoes an Media:ether-oxygen fission, characteristic of t-butyl ester group. Under such conditions, the methyl ester protection is stable. This deprotection chemistry is depicted below.
Boc protection is introduced at the N-terminal using Boc-chloride (Boc – Cl), which is an acid chloride and triethylamine as a scavenger. A more convenient reagent is the Boc – anhydride ((Boc)₂O) (Fig 4.5).
Fig 4.5 Reagents for –Boc protection

The ester-amine as the hydrochloride of the amino acid is neutralized with base and the free amino ester is quickly added to a solution of Boc – anhydride. The neutral product is extracted in organic solvent (Fig 4.6).

fig4.5a.png
Fig 4.6: Mechanism for Boc protection.

**Media: Benzyloxy carbonyl- protection (Cbz-N):**

Benzyloxy carbonyl- group is introduced by reacting the amino acid with benzyloxy carbonyl chloride. This group is stable to acid conditions. But it is unstable to base hydrolysis. A more interesting point about this group is that it is sensitive to **Media: catalytic hydrogenation**, which is a neutral condition (Fig 4.7).

Fig 4.7 Protection as N-Cbz and Protection / Deprotection steps for C-Cbz group
Media:Fluorenyloxy carbonyl- protection (Fmoc, or FMOC):

This recent reagent is very popular in solution phase and Media:Solid Phase Peptide Synthesis. The reagent for SPPS is discussed later in this chapter.

Protection could be Fluorenyloxycarbonyl chloride. In the example given below, a more recent reagent [Media:N-(9-fluorenylmethoxycarbonyl)oxysuccinimide (FMOC-OSu)] has been used (Fig 4.8). Here the leaving group is N-oxysuccinimide (abbreviated as N-OSu).

Fig 4.8 Fmoc protection of an aminoester using Fmoc-OSu.

The special feature of the Fmoc protection is the ease with which it is removed with piperidene. The base abstracts a proton to initiate the cleavage reaction (Fig 4.9).
Fig 4.9 Deprotection of Fmoc group

**Media:** C-terminal protection:

**COOR protection:**

The most popular protection is the alkyloxycarbonyl protection, where R = Ethyl or Methy residues. Dilute NaOH easily cleaves these groups. When R = benzyl group we have a group that is removed by hydrogenolysis under neutral conditions *(Fig 4.10).*

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Fig 4.10 Deprotections at the C-terminal.

**Media:** Coupling Reaction:
Acid halides: The acid chloride / triethylamine procedure for peptide bond formation is no doubt efficient, economical and ecologically acceptable. However, the procedure suffers from the shortcoming that racemisation is mostly observed to unacceptable levels. However, in recent years, the process has been successfully applied in solid phase synthesis protocols.


A better procedure is the Acid fluoride coupling. Acid fluorides are more stable to aqueous conditions and the byproducts are nontoxic. Hence, this procedure is more suited for Green Chemistry protocols. The old problem of preparation of acid fluoride has also been solved with modern reagents. Two such reagent are Tetramethylfluoroformamidinium hexafluorophosphate (TFFH) and bis(tetramethylene)fluoroformamidinium hexafluorophosphate (BTFFH).

TFFH is a nonhygroscopic, air-stable salt that can be handled under routine conditions. The main advantage in using BTFFH over TFFH is that BTFFH does not form volatile or toxic byproducts (Fig 4.11). The acid fluoride coupling in solution phase is carried out in a two phase(CH₂Cl₂–H₂O) system. The acid fluoride of the first acid is taken in the organic phase and the second amino acid as ester hydrochloride is taken in aqueous phase with sodium bicarbonate as the base to neutralize the acids generated. Coupling is complete within 10 minutes and could be followed by observing the disappearance of the IR band at 1845 cm⁻¹. These coupling reactions are milder compared to acid chloride coupling. The condensation is achieved with less than 1% racemisation, an important parameter in peptide synthesis.
**Fig 4.11 Some useful reagents for acyl fluoride formation**

**Media: Dicyclohexylcarbodiimide (DCC):**

The most popular coupling protocol is the Dicyclohexylcarbodiimide (DCC) procedure. The N-protected acid (1 eq) and the C-protected amine (1.1 eq) are stirred in an organic solvent with DCC (1 eq). The by-product is dicyclohexylurea, which is generally insoluble in solvents like methylenedichloride. The simplicity of this procedure is impressive. Note that an active ester is generated in-situ in this procedure as well. Hence, activation and condensation are achieved in one simple step (Fig 4.12).
Racemisation is sometimes observed in DCC reactions. In such cases, [[Media:Hydroxybenzotriazole (HOBt)]] (Fig 4.13) is added to the reaction mixture.

This reagent is believed to react with the activated product of DCC (with acid group) to provide a new active ester, which reacts with the amine group to form the peptide bond.

“Recent development of peptide coupling reagents in organic synthesis”, So-Yeop Han and Young-Ah Kim, Tetrahedron, 60, 2447–2467 (2004); Tetrahedron report number 672;
The main advantage in solution chemistry (viz., the insolubility of DCU) becomes a problem when used in solid phase peptide synthesizers. The columns are choked by the precipitation of urea. Using modified DCC reagents that provide soluble urea derivatives this problem has been solved. An example of this class of reagents is $N,N'$-diisopropylcarbodiimide (DIC) (Fig 4.14).

**Fig 4.14** A reagent that yields soluble urea derivative in peptide bond condensation reaction

**Active Esters:** The leaving group on the ester carbonyl group could as well be an activating group. Such esters are called Active Esters. The methyl ester itself is sufficiently active if the reaction is at the terminal step and a terminal amide is desired. Ammonia, methylamine, ethylamine. Diethylamine etc., are used in such cases. However, when a peptide bond is desired, such displacements are slow, leading to racemisations. In such cases, activated leaving groups are preferred. Some of the commonly encountered active esters in peptide synthesis are the following (Fig 4.15).

**Fig 4.15** Some commonly used active esters.
These types of C-terminal activations are frequently used in SPPS protocols. A simple tripeptide Gly – Ala – Gly, which has both the terminals free, could be synthesized as follows (Fig 4.16). This protocol is called Media:solution phase synthesis.

Fig 4.16 Tripeptide synthesis using a solution protocol.

There are a few important points that need attention. Note that these solution phase syntheses start from the N-terminal. Another important point to remember is that peptides with free NH₂ groups could internally cyclise to diketopiperizine(s) and cause Deletion of the chain (Fig 4.17). This is particularly so with the side chain of Lysine.

Fig 4.17 Formation of dikitopiperizine by internal cyclisation reaction.
Such linear syntheses are reasonable only for small peptides. Beyond pentapeptide it is desirable to resort to fragment condensation procedure. In this technique, the given peptide is dissected into fragments of di-, tri- and / or tetrapeptides and the fragments are assembled. This is called Media:'Convergent synthesis'. Such protocols provide more efficient routes than Media:'Linear synthesis'.

Have a close look at the following octapeptide which was required for conformational studies. Since there are two repeating sequences

Boc-Leu-Val-Val-DPro-Ac6c-Leu-Val-Val-OMe

Leu-Val-Val, the authors preferred to synthesize three sets of fragments and condense the fragments to synthesise the octopeptide as shown below (Fig 4.18).

Fig 4.18 Solution phase fragment condensation procedure.

Rajkishore Rai, Ph.D. Theses, Banaras Hindu University, Varanasi, India (2002).
Several peptides have been synthesized using similar protocols. Since all reactions take place in conventional solution phase, these synthetic procedures are called Solution Phase Peptide Synthesis.

Solid Phase Peptide Synthesis (SPPS)

Generally all organic reactions take place in solution phase. Even when two liquids reagents are mixed without a solvent to perform reactions like Diels-Alder reaction, the reagents generally form a uniform solution. An exception to this concept is the catalytic hydrogenation reactions with Pt / C or Pd /C. In such reactions, these catalysts are insoluble solids. The noble metal occludes hydrogen and activates the bonds concerned. The olefinic compound is either in solution or is a neat liquid. After the reaction is complete, the catalyst is simply filtered off and the product purified by distillation, crystallization or chromatography. In 1969 R.B. Merrifield described a Solid Phase synthesis methodology that revolutionized peptide synthesis. The process was found to be suitable for Automated Synthesis of Peptides. For this achievement Merrifield was awarded the 1984 Nobel Prize In Chemistry. Later the procedure was applied to other organic syntheses as well. Let us have a brief look at solid phase synthesis.

Merrifield first demonstrated this methodology using polystyrene beads as solid support. The styrene polymer is a long chain, which is randomly coiled. This polymer is commercially available as insoluble beads. When suspended in a solvent, the solvent molecules enter the holes in the bead, causing the beads to swell. While most of the benzene rings are buried into the folds of the coil, less than 10% of the benzene rings of the polymer chain are available for reaction with the reagents in solution phase. A cartoonic representation of one such bead is shown below (Fig 4.19).

Fig 4.19 Representations of a polymer bead while writing chemical equations.

In practice, the bead is shown either as a rectangle, a spear or a triangle or any such shapes to simplify the drawing.
The first solid phase synthesis of peptides on beads reported by Merrifield is discussed here to illustrate the concept (Fig 4.20). The swollen beads were chloromethylated using [(CH₂O)n / HCl ] reagent. Note that these Media:Linker groups could be attached only to the phenyl groups that are readily available to the reagents which are dissolved in the solvent. The excess reagents and the soluble byproducts were removed by filtration of the beads and repeatedly washed with suitable solvent(s). These phenyl rings were then nitrated with conventional reagents and repeatedly washed. The nitration step facilitated easy hydrolytic cleavage of the bead at the last step. The last amino acid (the C-terminal) in the peptide sequence was first attached to the bead. This was done using the N-Cbz-protected amino acid as the sodium salt of carboxylic acid. An ester was formed by displacement of the C – Cl bond. The wash cycle was repeated. The Cbz-protection was removed using HBr / HOAc, followed by a wash cycle. To this free amine attached to the bead, the next N-Cbz- protected amino acid was coupled using DCC procedure. The deprotect / couple protocol was repeated until the desired length was achieved. Each step was followed by a wash cycle. The final peptide was cleaved from the resin by base hydrolysis. This step also cleaved the Cbz- protection. Thus, the workers synthesized several tetrapeptides within a remarkably short time. Note that the synthesis on beads starts at the C-terminal.
Fig 4.20 Merrifield’s solid phase synthesis of a dipeptide.

In 1984, Merrifield reported the synthesis of a nonapeptide - Media:Bradykinin - using a modified solid phase procedure. In this synthesis several improvements were reported.

Arg - Pro - Pro - Gly - Phe - Ser - Pro - Phe - Arg (Bradykinin)

t-Butyloxycarbonylamino acids were used throughout the synthesis in order to permit mild conditions for the deacylation. The condensation reactions were carried out with excess reagents in DMF solvent to drive the reaction near to 100% efficiency. The final peptide was cleaved with HBr-TFAA. Final purification was achieved by chromatography. Overall yield reported was 68% and time taken was 8 days.
This protocol developed by Merrifield has now undergone several modifications. Protocols are now available for fully automated peptide synthesizers, which are controlled by computers. A wide range of choice (of materials) is now commercially available. The beads could be modified polystyrenes, amide polymers, polyethers etc. Commercially available beads come with different Spacers that remove the reaction center away from the crowded bead surface and also help to keep the reaction sites far apart to prevent the sites from ‘talking’ with each other. These spacers are then attached to different type of linkers. Linkers permit easy cleavage of the peptides from the resin, under a variety of reaction conditions. While most of the linkers leave their ‘residues’ like amino group, carboxylic acid group or olefin, some linkers are said to be ‘tracerless’. Beads are also commercially available with different first amino acids. Fmoc protection is now most popular for N-protection. Several natural and synthetic amino acids are commercially available with C- as well as N-Protections of choice. Several new condensing agents, activating groups etc., are now commercially available for lab scale or large-scale batches. With these improvements in chemistry and technology, a synthesis that takes months by solution chemistry protocol, is now completed within a few hours, using SPPS and Medium Pressure Liquid Chromatography (MPLC) purification protocols. Since the procedures are repetitious in nature, a cartoon drawing would be easier to vitalize for such schemes. An SPPS protocol using Fmoc- protection is shown below (Fig. 4.21).
Fig. 4.21 Fmoc protocol for SPPS.

A few of the commercially available beads are briefly discussed below.

**Merrifield Resin:**

![fig4.21a.png](fig4.21a.png)

Merrifield Resin is a polystyrene resin based on a copolymer of styrene and chloromethylstyrene. In addition, this polymer is also cross-linked with divinylbenzene present in the monomer composition up to 5%. Merrifield resin is
named after its inventor, Robert Bruce Merrifield (1984 winner of the Nobel Prize in Chemistry).

**Wang Resin:**

![fig4.21b.png](fig4.21b.png)

Wang resin is the most popular support for solid phase organic synthesis (SPOS) using Fmoc chemistry. As a standard support it can be used for the solid phase immobilization of acids and phenols. The ester linkage formed has good stability to a variety of reaction conditions, but can be readily removed with the moderate acid treatment, generally with trifluoroacetic acid.

**Polyamide resin:**

These resins are normally polyacrylamide resins made up of the following monomers and functionalised backbone monomers. Polyamide resin is also a useful and versatile resin. It seems to swell much more than polystyrene.

![fig4.21c.png](fig4.21c.png)

Precursors are used in the preparation of polyamine resins

**PEG hybrid polystyrene resin**
An example of this type of resin is the Tentagel resin. The base resin is polystyrene onto which is attached long chains (Mw ca. 3000 Da) of polyoxyethylene (PEG). Synthesis is carried out on the distant end of the PEG spacer making it suited for long and difficult peptides. In addition it is also attractive for the synthesis of combinatorial peptide libraries and on resin screening experiments. It does not expand much during synthesis making it a preferred resin for robotic peptide synthesis.

For convenience of remembering, a simplified scheme for SPPS is given below (Fig 4.22)
The SPPS methodology is remarkably simple compared to solution phase procedures that demand considerable skill even to synthesize small peptides. For this reason, SPPS is very popular even in the hands of non-chemists. Peptide synthesizers are now available to routinely plan and execute the steps in Media:Automated Peptide synthesizers. The reactions take place on columns. The swollen beads, bearing the spacer and linker (and often bearing the first amino acid), are loaded on the column and the reagents, protected amino acids and wash solvent(s) are placed in labeled compartments. A solution of the amino acid reagents with coupling agent is repeatedly recycled for fixed time as determined in the program to ensure completion of the coupling process. Since the protecting groups (e.g. Fmoc) are UV active, the progress of each cycle could be monitored and plotted. One such plot (for one cycle) is shown below (Fig 4.23). This is then repeated with different monomers units in the desired sequence.

![Monitoring a coupling on SPPS using a UV monitor](Image)

Once the synthesis is completed, the resin is removed from the column and delinked in a separate flask. Final purification is accomplished using HPLC / MPLC procedures.