Organic Chemistry In Biology And Drug Development
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Lecture - 09
Recent Development of Coupling Agents: Merrifield's Method of Solid Phase Peptide Synthesis

We will start from where we have ended. We just told you that how to do the coupling reaction and what is the coupling reagent? The coupling reagent is DCC; however, DCC has some limitations and one of them is racemization.

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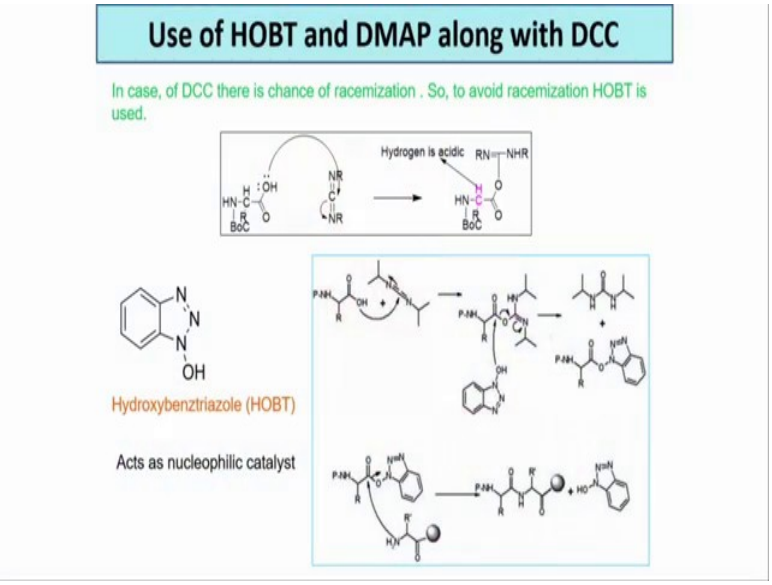

In the slide that is projected, you can see the mechanism that first it is the carboxylic acid of the amine protected amino acid, that attacks the electrophilic carbon DCC and this imine N becomes NH.

If you do not have any other reagent other than only DCC then the amine comes and attacks here and this DCU is released. The carbodiimide shown in the slide is diisopropylcarbodiimide. If you have the hydroxy benzotriazole, after the formation of the intermediate, the N-hydroxy moiety bonded to benzotriazole comes and kicks the DCC part out which comes out as the urea derivative and in the process, it forms another new intermediate. But this is also very labile; immediately the amine comes and attacks

this carbon and in the form you get again the hydroxybenzotriazole. So, that was what I wrote on the board that time ok.

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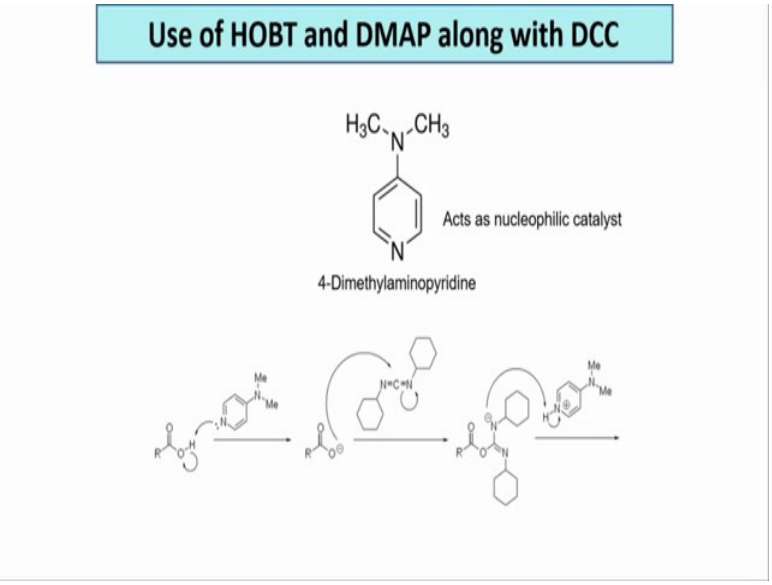

DCC also fails as a coupling reagent; hence there are some new protecting groups which are there. Sometimes a little amount of DMAP also helps; because the first reaction is between the carboxylic acid and the carbodiimide. But if you can make a carboxylate, that will be a better nucleophile than a carboxylic acid; so a catalytic amount of DMAP, if you add, that speeds up the reaction by forming the carboxylate.

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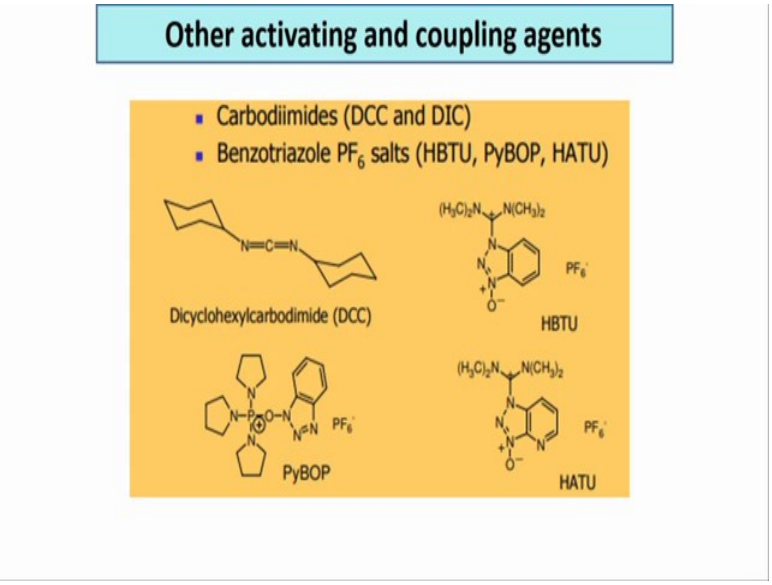

And then this is DCC; I showed the mechanism of this, but there are other modern reagents which are which are not based on carbodiimides; these are based on only the hydroxybenzotriazole framework. As you see, in all this reagents, similar sort of hydroxybenzotriazole system is present.

This is a reagent which is called PyBOP because there are these three pyrrolidine rings which are present and phosphorus. These are phosphorus based coupling agents and, these are also called uronium salt based coupling agent; this is called HBTU and this is called HATU.

In some cases, they are actually much more efficient than DCC; sometimes coupling fails with DCC as DCC is quite sterically bulky; in those cases, this type of HATU, HBTU or PyBOP may be used. There are many uses for these coupling agents; it is not necessarily always amide coupling between amino acids; sometimes an aromatic amine has to be coupled with an acid, then you might have to use this HBTU or HATU.

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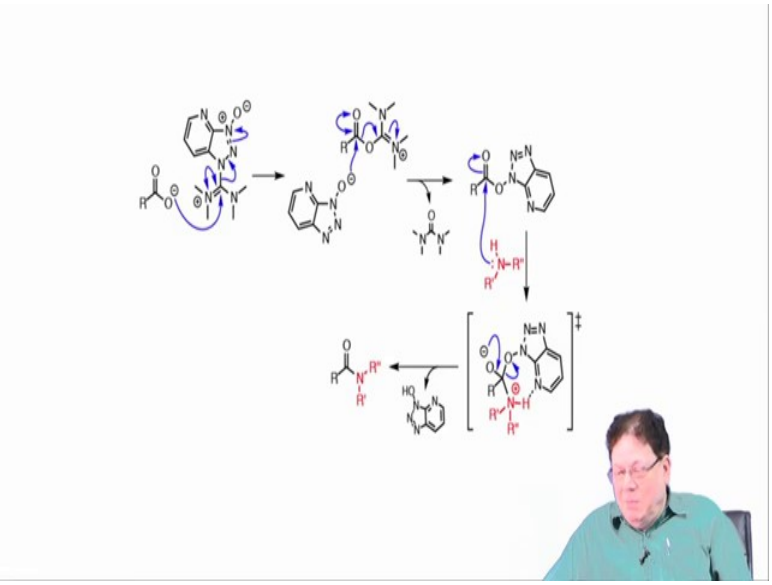

The mechanism of one of these is shown here; this is HATU. There is a pyridine ring also. So, instead of benzene you have a pyridine in HATU; if it is benzene then it is HBTU. Here the mechanism is shows that this is a guanidinium type of system and this nitrogen is a good is good leaving group because of the pull from the other N double bond N.

So, the carboxylate anion or the carboxylic acid, it attacks this carbon first, as a result that goes here and then it comes back. Then basically what is happening is that this comes and attacks at this electrophilic carbon and resuls in this carbon nitrogen bond breakage. And why this will be facile? Because there is a N double bond N here which is having a plus charge (because it's an N oxide to start with). I can go back to the earlier shown structure and see it was n N oxide, which is slightly different than the N hydroxybenzotriazole.

So, now this nitrogen is a a much better leaving room. So, once that happens, you get a benzhydroxytriazole where the hydroxy is actually in the oxide form; it's not in the OH form is in the oxide form, because the nitrogen is now neutral and this amidine group is attached to the carboxy now. So, it is very similar to the carbodimide chemistry; now this type of intermediate was there in the carbodimide chemistry. So, now, this pyridine triazole (or azabenzotriazole), that O minus now comes and attacks the carbonyl and this goes out, this is very similar to the later part of the earlier one and then the hydroxytriazole becomes a leaving group and then the amine comes and attacks this carbonyl and this hydroxy pyridinetriazole (or hydroxy azabenzotriazole) goes out.

So, you see, there are a lot of things here to notice; one is the ultimate mechanism; that means, the hydroxybenzotriazole that we were using to suppress racemization, is again formed here, but via another new type of reagent which is this N-oxide and coupled with the uronium salt and the mechanism is shown here.

So, you can write it down or these slides will be made available. So, you can see it if you have any problem. The byproduct is urea with substitutions. So, these are very good peptide coupling reagents; only problem is that they (HBTU or HATU) are extremely expensive .

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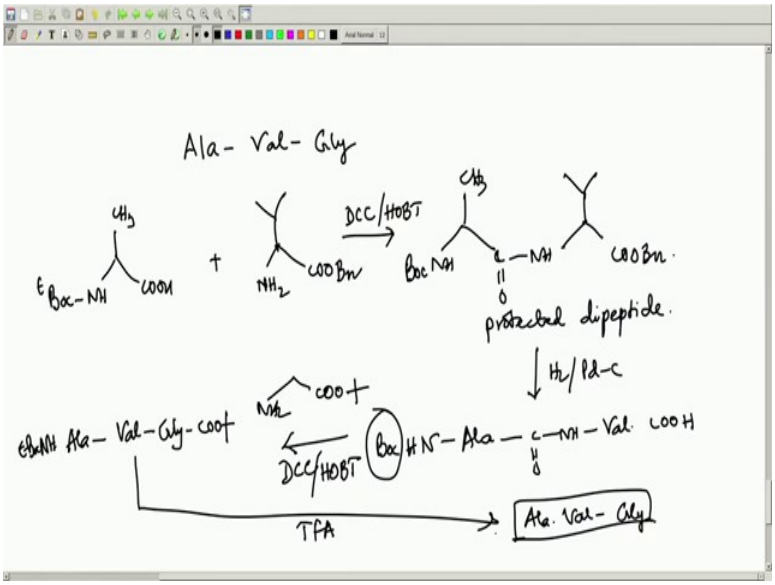

Now, let us do one problem; see we go beyond dipeptide suppose I want to make alanine, valine and suppose glycine. So, in this case, what we will do? We will first make protect the alanine NH_2 with ^{t}Boc and this carboxyl is free; for valine, we keep free amine, because we have to couple this side; and the carboxyl has to be protected.

Here you have to pause a little and then think what type of protecting group I should use. Remember, here I have to do two peptide couplings; one is between alanine and valine another is between this valine and glycine.

So, at the first stage, when I make dipeptide, I should not use protecting groups where both the protecting groups will come off together by the treatment of a single reagent system. We should use a protecting group which can be deprotected without disturbing the Boc group of the alanine (mutually orthogonal protecting groups); so best is to use a benzyl protecting group. Thus by using the benzyl protecting group, you have the protected dipeptide (Boc-Ala-Val-CO_2Bn).

Now, these two groups are basically mutually orthogonal to each other, in this case, what you need is to deprotect benzyl protection because in the next coupling is between this carboxyl of the dipeptide with the free amine of glycine. So, we should deprotect it by using hydrogen, palladium, charcoal; I am just writing alanine to save time, so that will be alanine Boc.

And this will be CONH and then you have valine and the valine will come off as the carboxyl (Boc-Ala-Val-COOH). So, then what I do, here I use DCC/HOBT and now I should use glycine; the glycine carboxy now should be protected and the amine should be free, the carboxyl should be protected in such a way that the protecting group here should be similar (similar means they should come off under the same condition) to the protecting group of the amine of alanine part.. So, glycine should be protected as t-butyl ester. So, I take glycine as the t-butyl ester and its NH_2 should be free, and then I use DCC/HOBT and then what I will get is a protected tripeptide having alanine with NH ^{t} Boc, then I get valine and I get glycine with COO tbutyl ester.

And now with one shot using TFA, I can get alanine valine and glycine tripeptide. Although the tripeptide remains more or less pure if done very carefully, but final purification of these peptides are usually done by high performance liquid chromatography or ion exchange column; I will actually discuss the purification of the peptides and proteins in our next session.

So, right now what we have learnt that, if I want to make a tripeptide, then the protecting groups need to be selected in such a way that the first two should be orthogonal protecting groups so that I can take one of keeping the other intact. And the second one (in this case protecting glycine) should be a protecting group, which will undergo removal under similar kind of deprotection conditions as the amine group at the N terminus. If you have tetrapeptide, so, you can slowly build you are peptides using a similar strategy..

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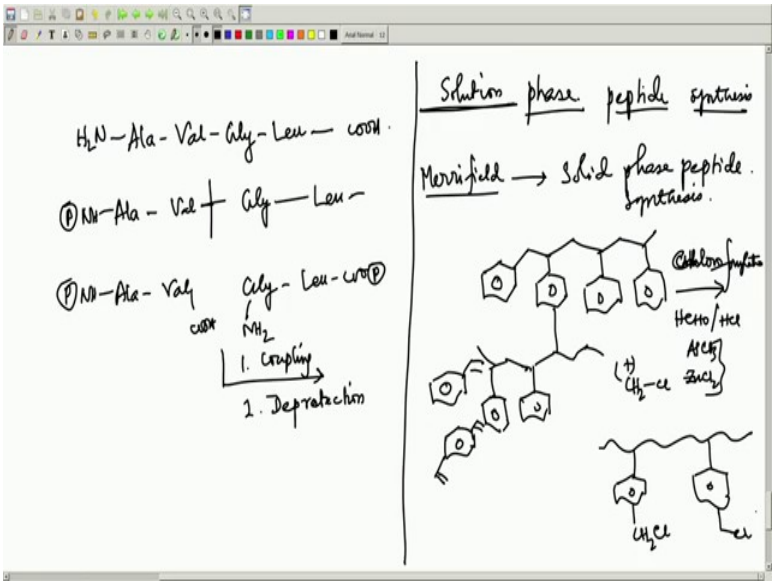

If you have a tetrapeptide, you have see more choices because it can be built up by adding amino acids one after another; or it can be made by making blocks and then attaching the blocks together. Suppose, I have to make this tetra peptide (Ala-Val-Gly-Leu). Always remember, that on left side if nothing is written, this should be the amine and the right side should be the carboxyl.

Now, in this case, you can start form alanine, valine-dipeptide; alanine should be protected and then you should couple that with glycine; that is one possibility. And then couple glycine with leucine and leucine should have a protection which is similar to the amine protection here; that is one possibility or what you can do? you can do it in blocs?

Alanine, valine (where the N-terminal of alanine is protected and the C-terminal of valine is free) and you have glycine, leucine (where the C-terminal of leucine is protected and the N-terminal of glycine is free); . So, now, these two blocks are coupled and then you deprotect, so first coupling and then deprotection. So, rather than adding one after another, you can actually do it in blocks; if you have hexapeptide you can make two tripeptides and then join the two tripeptides together.

Now, all these reactions that we have discussed so far are done in solution. Remember I said that amino acids are not soluble in organic solvents, but these reagents that we are discussing are all soluble in organic solvents; actually water should be avoided in these coupling reactions..

So, this is what is called a solution phase peptide synthesis, where these organic solvents are used to have a homogenous mixture; you cannot use water; you have to avoid water in all these reactions because water is a competing nucleophile in all in these reactions.

Later on, a scientist named Robert Bruce Merrifield actually developed a method which is called solid phase peptide synthesis (Refer Time: 15:31).

Today, if you want to make a larger peptide, then solution phase synthesis does not work; then only Merrifield's method of solid phase peptide synthesis is used. Now what is the solid phase method? What Merrifield did? Say he took a polymer bead which is insoluble in water.

And on this bead, he developed a functionality and utilized this functionality to attach amino acids. But even if you attach amino acid, the whole thing is still remaining soluble in organic solvent or in water, whatever you use.

But as I said water is a competing nucleophile, so one should avoid water. So, you do the reaction on a resin bead; add your reagent; suppose I want to protect the amine and this amine belongs to an amino acid where it is already hooked up to a resin bead. Now you add the reagent in a solvent, the bead will not be soluble in the solvent, but the reagent will ultimately react because this bead has a functionalized system.

So, the reagent will react with whatever functionality is present on the bead. And after the reaction, how to purify? If you filter, you isolate the bead and the bead has your required product(s) since the reaction was carried out on the bead; the other reagents will be washed out of the bead.

So, basically purification is extremely easy, virtually there is no purification because it is already tied to a bead like a marble; small marble balls; and their functionality is present there. So, you add one amino acid that goes in then, you add the next amino acid that goes in, but everything is attached to the marble. So, after every reaction you just wash out the marbles, wash out the beads and your desired peptide will be on the bead.

So, in the final stage, you have to take out the peptide from the bead and then you remove the bead; what you are left out is only the peptide; that is the method. So, Merrifield's method is like this: There is a resin which is called polystyrene. Polystyrene

is derived from styrene you know styrene is actually vinyl benzene. And polystyrene has various pendant phenyl groups and the backbone is a saturated carbon network. So, all these are derived from vinyl benzene; that means, styrene; but while making polystyrene, you add divinyl styrene; that means, you take vinyl styrene and a little bit of this divinyl benzene.

Now, what it does? Styrene has got only one functional group and divinyl benzene has got two points where polymerization can take place. So, what will happen? Suppose this benzene ring comes from the divinyl system. So, it will also again have another chain of these aromatic rings. Divinyl is actually a cross linking agent; that means, there are some cross linkings that happens. So, basically what you have are pendant aromatic rings, but they are also cross linked and he used these polystyrene resins which are available in beads. And then, he did a reaction which is called chloro formylation ; this is done by formaldehyde and HCl. And in presence of Lewis acid like aluminum chloride or anhydrous zinc chloride. And what is the ultimate effect? You have all these phenyl groups; some of these phenyl rings will undergo chloromethylation because HCHO HCl means actually you are generating CH_2Cl plus which is basically an electrophile. So this chloroformylation reaction is just a type of Friedel craft reaction, . So, many of these aromatic rings will be chloro formylated. So, they are nothing, but benzyl chlorides ok, so a polymer bead with lot of CH_2Cl groups.

So, this is called Merrifield resin; these Chloro formylated polystyrene beads are called Merrifield resin. The fact that how much chloro formylation has taken place, that will ensure that how many molecules of peptide you can attach to the this chloro formylation hands.

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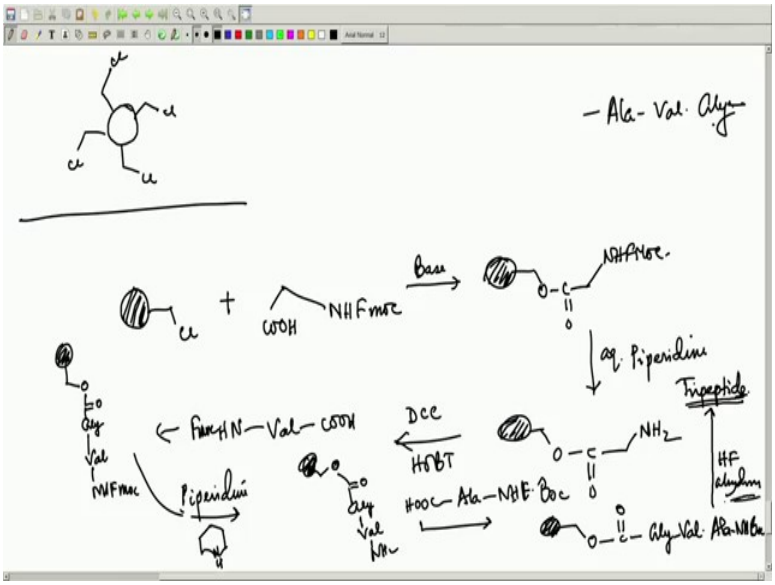

So, basically what I am saying that if a bead has lot of chloroformylation hands, then from one bead you can make three molecules of the peptide. If there are more number of hands, then from each bead, you get more number of peptide bonds. So, the extent of chloromethylation will tell you that how much is the efficiency of your process and you can get lot of Merrifield resins having different extents of chloromethylation content.

Now let us consider a bead with just one chloromethylation. It is first reacted with an amino acid in which the NH_2 is protected as Fmoc; here we use Fmoc. Since this is a very reactive benzyl chloride moiety attached to the bead; we know benzyl chloride are susceptible to nucleophilic displacement; carboxylic acid in presence of a base will react with the benzyl chloride. So, what get the formation of new C-O bond between the carboxylate part of the amino acid whose N-terminal is protected as Fmoc.

Now the question is why Fmoc here? Why not 'Boc? Because now if you deprotect Fmoc, it generates methylene fluorene which is a a very fluorescent material. So, seeing the fluorescence and the extent of fluorescence, and you can calculate how much Fmoc group has been deprotected; you can be sure that yes the bead is now free from Fmoc group. So, by using aqueous piperidine, you remove the Fmoc and free the NH_2 group. As I said, the reaction is very easy to work because you take this resin which is insoluble in water, add the Fmoc amino acid, a base like triethylamine and then do this reaction, stir for some time. And then take the bead out; that means, you remove; its the other way

around with respect to solution phase; remove the solution phase and take the solid phase; you give a couple of washings which will take care of any of the adherent solvent molecules or reagents.

Suppose I want to make the tripeptide, alanine, valine, glycine. Only difference and that in this is very important, is that in case of Merrifield system, if you want to use solid phase, you start from the C terminal side; you do not start from the N-terminal side because, it is the C-terminus that is first attached. So, you have to first attach glycine first.

So, I attached glycine; now what I will do? Here DCC usually does not pose any problem because it's a solid phase method. So, DCC and HOBT have to be added. . So, now, with priorly obtained bead attached to CH_2, then O then CO then your glycine, we form amide bond with valine having free carboxylic acid and its NH_2 should be Fmoc protected.

So, now F moc will be deprotected again by piperidine. And you get the bead coupled with CH_2O and then CO. And then you have glycine that is attached to valine.; Now F moc will be gone which you can monitor by fluorescence and this valine has free NH_2. Now what you give you can write your alanine, amine should be there. Remember, again I repeat the same thing that it is just the opposite we started from the right side from the carboxyl.

So, now, we should have alanine and whose carboxylic acid should be free and the NH should be protected now ok. So, we will get the tripeptide. At this point I take a pause; I did not put any protecting group here. So, what should the protecting group that I should use here? If I use Fmoc again? But after that, there is no further peptide extension. So, then for the Fmoc deprotection I have to use piperidine, and for the deprotection from the bead, I have to use another method.

So, the last amino acid that has to be incorporated which isin fact, the first N terminus amino acid, should be protected with a group which is similar to the deprotecting agent for the resin deprotection. So, you take the ^{t}Boc. So if you take ^{t}Boc then, this is the resin then you have CH_2 then O then CO and then first you have glycine, then you have valine, then you have alanine and then you have NHBoc.

Now, to the whole thing, you add HF and you get the tripeptide. So, this is the Nobel Prize winning work of Robert Bruce Merrifield; he incidentally received the Nobel Prize in 1984; just single recipient of the Nobel Prize for that year. So, remember that it starts from the carboxyl end and then slowly use FMoc till you come to the first amino acid which has to be protected with Boc. And then, the last deprotection is with anhydrous HF.

Thank you.