Organic Chemistry In Biology And Drug Development
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Lecture - 08
Peptide Synthesis (continued): Protection, Coupling and Deprotection Methods

Well let us continue with the topic that we had been discussing earlier that was on the Synthesis of Peptide molecules ok.

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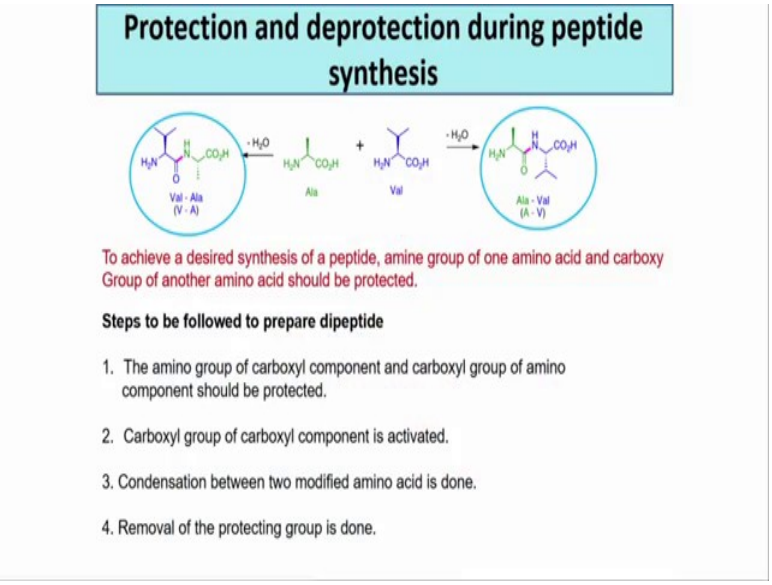

We have done the sequencing, how to know the primary structure, that is how the amino acids are arranged one after another. And, then we started the reversal of sequencing, that is how we can make a designed peptide with particular amino acid at specified position.

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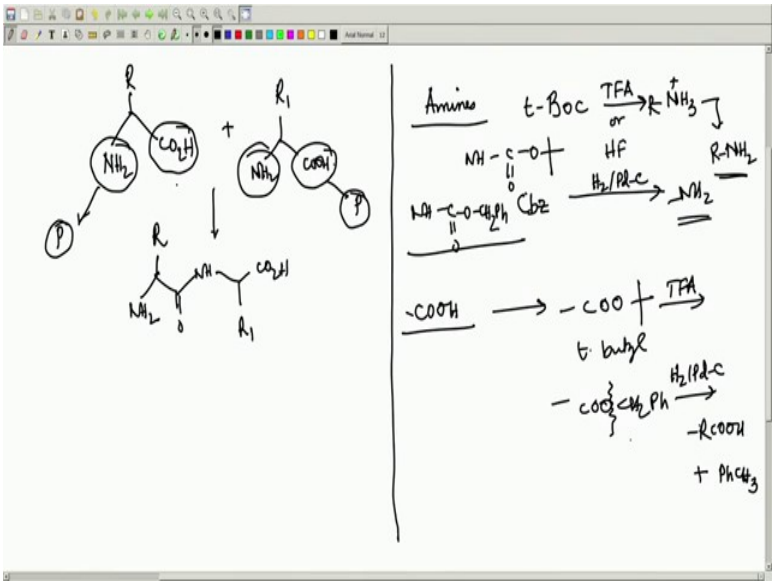

Now, let me just briefly recapitulate what we have said that, in doing a coupling between one amino acid with another one, several things have to be considered. We should be careful that all these functionally active groups, this one and this one as well as this amine and this carboxyl.

What we want is to basically make a peptide bond between this one, this carboxy and this amine this alpha amine. So, our target is this of R and CONH and then R_1 and CO_2H, this is the final target. Now, in order to do that we want to consider following things: first there can be coupling between the same molecules because, in the both the molecules, amine and carboxy functionality are present.

So, that requires what is called protection. So, since we want to have coupling between this carboxy and this amine so, what we need is to protect this amine and this carboxyl. We have introduced last time the different types of protection. I introduced two groups for amines: one is called t-butyloxycarbonyl 'Boc which is cleavable under acid condition like TFA (trifluoroacetic acid) or HF. And, the other group I introduced is called Cbz. Cbz protected amino to be precise is $NHCOOCH_2Ph$ and the Boc protected amine is NHCOO and then a tertiary butyl group.

So, now this Cbz group comes off with hydrogen in presence of palladium and charcoal; then you get the free amine; and here in case of Boc protected amines, to get the free amine, you deprotect with acid. With TFA, first we will get the TFA salt, and then you

have to basify and then remove the TFA and you get the RNH_2 as free amine. So, these are the two protecting groups, they are frequently used because one does not affect the other (mutually orthogonal). And, regarding the carboxy group, what we have is very similar type of protecting groups; one protecting group which comes off in acid that is the t-butyl ester and this also comes off with TFA.

And, the other protecting group is benzyl, a very similar one like the earlier one (Cbz); this also like Cbz comes off with palladium hydrogen, palladium, which is basically carbon supported or hydrogen, resulting in hydrogenolysis. Remember this is hydrogenolysis because, the product that comes out is RCOOH (that is the free acid) and you get toluene as byproduct. So, we are breaking this bond which is called a lysis. So, this is not hydrogenation, this is the hydrogenolysis. So, these are the things we covered last time; next is first of all these are the deprotection methods, but I did not say the protection that how to introduce these groups ok.

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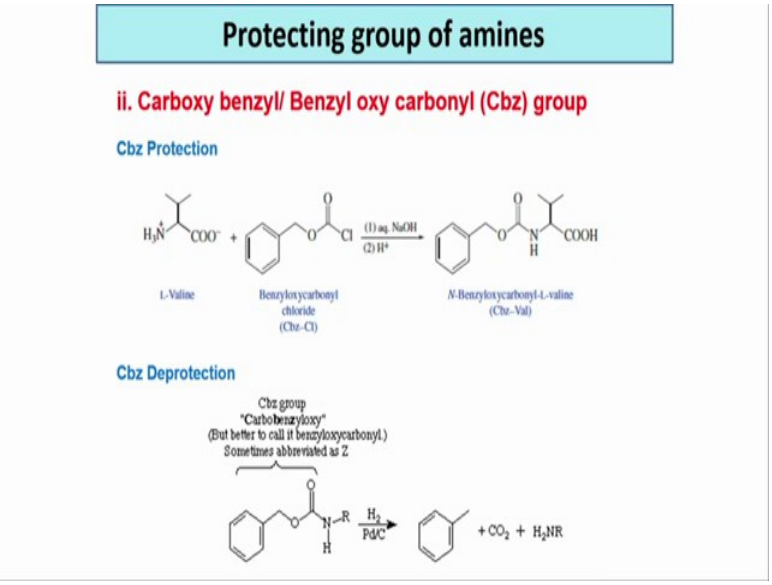

So, there are some slides I can show you, here it is shown that to put the Cbz group, you take the amino acid. This is your amino acid which is shown as valine and you take this benzyloxycarbonyl chloride. This is the ester of chloroformic acid. Formic acid is H C O O Hand if you just replace the hydrogen with chlorine, you get chloroformic acidThis is sometimes called benzyl oxy carbonyl chloride and it is also known as benzyl chloroformate; as it is an ester of chloroformic acid.

So, in presence of alkali, because there is HCl that will be liberated so, you need one equivalent of alkali and then you get the free acid, ok. This H plus is to extract the system because, you have a carboxylic acid. So, in presence of alkaline that will be CO_2 minus. So, to get the free acid you have to add the acid and that get the free acid ok.

So, this is the protection how you protect the amine with the reagent; the reagent is this benzyl chloroformate or benzyloxycarbonyl chloride. For the deprotection, I already have said that this is hydrogen and palladium; it goes to toluene; the breakages is at the benzylic position. So, that goes to OH and that is a carbamic acid derivative and that loses carbon dioxide immediately and it goes to RNH_2 ok.

Very clean reaction; carbon dioxide is just liberated, that is a gas and toluene is a volatile solvent so, that you can remove. So, ultimately what is remaining is the free amine.

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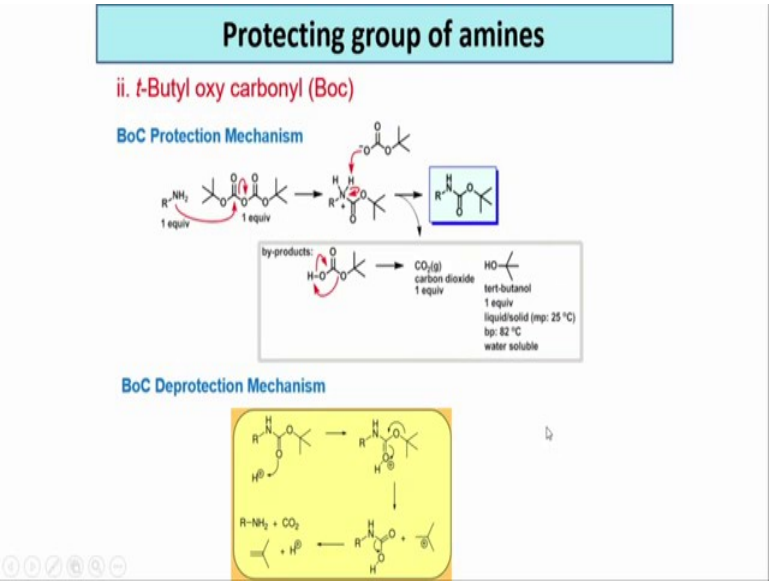

For protection by the amine, there are many reagents, I am just showing you few of those. The most commonly used is what is called 'Boc anhydride; this is the t-Butyloxycarbonyl anhydride because you had a CO O CO so, that is the anhydride portion. So, it is commonly called Boc anhydride. So, you take Boc anhydride, 1 equivalent, add the amine; you do not have to add any other thing, that is in a solvent. And, what will happen? This NH_2 will attack one of the anhydride carbonyls.

The other oxygen takes up the electrons and then in a similar way, that loses carbon dioxide and finally, what you get is the protected t-butyl oxy group ok. So, this is not shown here, this will be O minus, then O minus comes back and carbon dioxide is liberated.

Thus now you are getting a carbonate and when that is lost ultimately, you get t-butyl alcohol, carbon dioxide and this ′Boc protected amine. And the deprotection is by acid so, in the acid what you do? If you treat that with organic acids like TFA or anhydrous inorganic acids like a HF,

what happens? here is that H plus, this carbonyl oxygen is the centre for negative charge because, of the donation from the adjacent and nitrogen. So, that will be protonated and after protonation the t-butyl group is lost, we know that t-butyl cation is a very stable cation. So, that is lost and ultimately that will lose 1 proton and what you get it isobutylene as a gas.

And, then you get H plus and this carbamic acid derivative (intermediate) from this part will decarboxylate; carbamic acids are very unstable. So, that will decarboxylate and you get the free amine. So, these are the methods of protection and deprotection and the corresponding mechanistic aspects.

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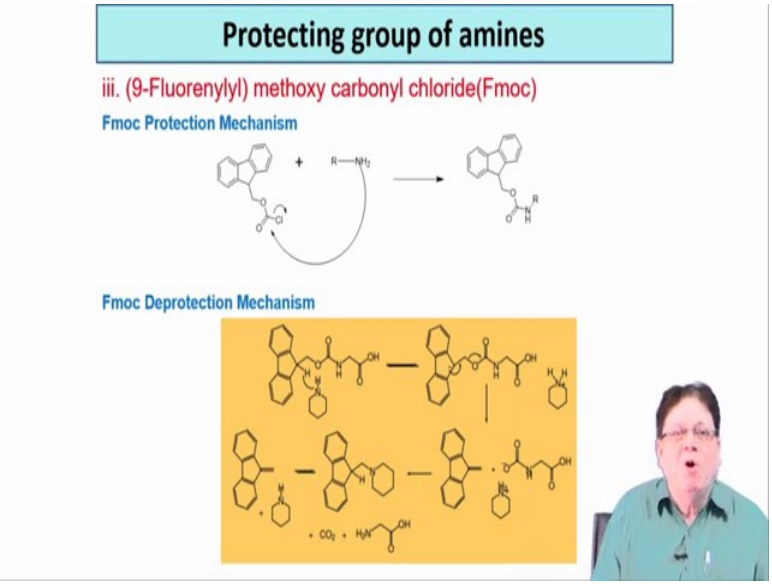

Next comes another protecting group, that is now routinely used in peptide synthesis, it is called the Fmoc group. Fmoc stands for (9-Fluorenylyl) methoxy carbonyl chloride; it is very similar to rest of the protecting groups. Actually this is commercially available; this is nothing but 9-fluorenylmethyloxy chloroformate; 9-fluorenylmethyl chloroformate like earlier benzyl chloroformate.

This fluorenylmethyl group has nothing to do with your elemental fluorine (F). Their spellings are different; elemental fluorine has 'i' here and in case of this aromatic hydrocarbon (Fmoc), that is called fluorenylmethyloxycarbonyl. Because, this is fluorene and then there is a carbon and then you have fluorenylmethyl oxo. So, it is a chloformate again, the same thing like a benzyl chloroformate and RNH_2 attacks at the carbonyl carbon. So, it requires definitely 1 equivalent of base, because 1 equivalent of HCl is formed. So, the group that is generated is this OCONHR and on this part it is a fluorene derivative.

This is abbreviated as Fmoc like you have ^{t}Boc and this is fluorenylmethyloxy carbonyl group. And, this group is little different from the earlier ones like the ^{t}Boc. In case of Fmoc deprotection, the hydrogen of this group comes off with aqueous piperidine. Piperidine is the mild organic base. As I told you that inorganic bases cannot be used because that will cause racemisation of the α centre. So, you can use mild basic conditions like this aqueous piperidine, when you use piperidine, what happens is this hydrogen (which is double benzylic because it is a benzylic carbon where there is one aromatic ring here and another aromatic ring there), will be lost very in a facile manner and the result is that this electron goes there and then this electron comes here and then this goes out. So that means, you are getting liberation of carbon dioxide and you get liberation of this compound. This comes out from the fluorine. This is called methylene fluorene because you have a methylene group in the fluorene. Now, by the way this is this fluorenylmethylene group is fluorescent. So, it is very easy to detect its detection limit, that if a amino acid is deprotected or protected that can be performed by doing the by detecting with fluorescence.

So, when you do HPLC, if you use a fluorescence detector then that can immediately tell you how much fluorine has been converted into methylene fluorene, if you are doing a deprotection. And, you can calculate that this is the number of moles that one should get and if that is the case; that means, the reaction is over. So, these are the three groups so,

one group comes off with acid, another group comes off with hydrogen and there is a third group which is this Fmoc group that comes off under basic condition (organic base like piperidine).

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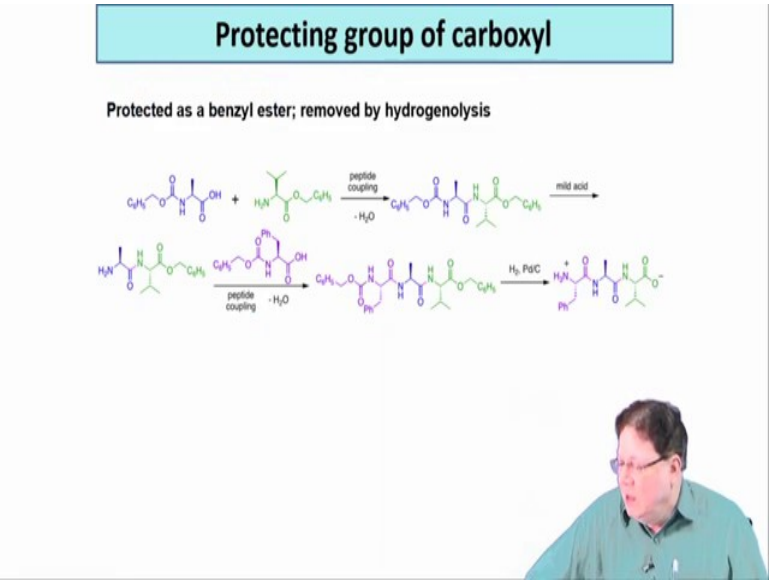

So, these are the protecting groups. Now let us consider, now what to do?

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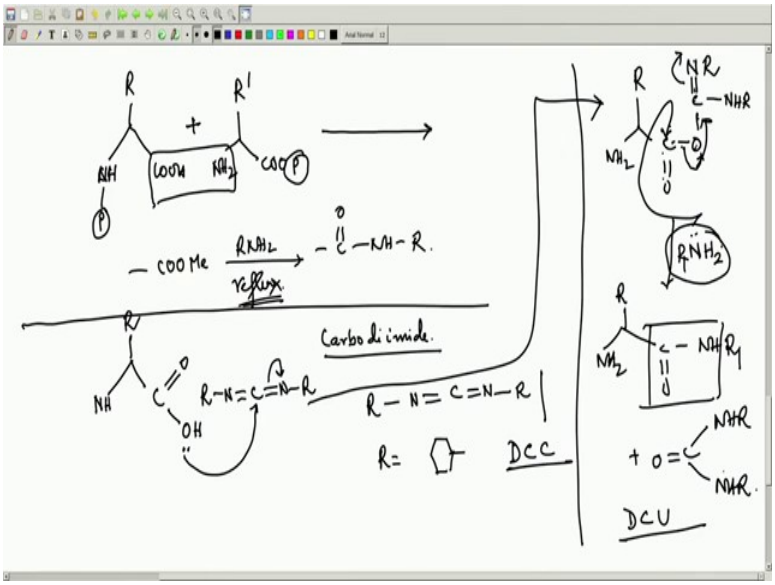

After the protection, this amine is protected and this carboxy is free. And, for the other amino acid, this amine is free and the carboxy is protected. Now, what type of protecting group we want to use, that we will discuss later.

Let us talk about that what are the conditions that should be used to have this coupling, which involves removal of water. Amides are usually made from acid chloride or they can be made from esters which is also possible; some esters like methyl ester is refluxed with a free amine.

So, that will definitely form an amide, but you have to reflux it; the problem arises as when you reflux, the chances of racemization will increase. So, you cannot raise the temperature very much that is one restriction, you cannot use acid chloride where although you do not have to reflux as it is a very spontaneous reaction, but that forms an azlactone intermediate where the α-hydrogen becomes very acidic and that leads to racemization. So, acid chloride with amine, that reaction to give the amide is ruled out in case of peptide chemistry. So, we have to use a very mild reagent, but it should be so mild that the carboxy is not even activated.

We have to activate the carboxy but not too much, rather to an optimum level. Now, what are those activation processes? Acid chloride is ruled out, esterification is ruled out.

So, now other people searched in the 1950s that what type of reagent can activate the acid to form an amide without racemizing the α carbon. And, one scientist John Sheehan from MIT (Massachusetts Institute of Technology) developed a reagent which was called carbodiimide. Now, what is carbodiimide? Carbodiimide is basically where you have a C double bond N it is a di imide.

C double bond N on the other side, C double bond N and one side R, another side is another R; that is called carbodiimide. Now, these are very good reagents to do the activation of the carboxy and subsequent coupling with the amine. The reagent that John Sheehan used was where R was equal to cyclohexyl; cyclohexyl means, the fully reduced benzene.

So, that reagent will be called DCC: Dicyclohexylcarbo diimide. What DCC does? It basically activates the carboxy and makes it a very good leaving group. OH is a bad leaving group unless you protonate it or you convert it into a good leaving group; DCC or any carbodiimide can convert OH into a better leaving group.

So, what happens first this OH reacts with DCC. So, this oxygen reacts with the carbon, the carbon is highly electrophilic because it is flanked by this two imine nitrogens. And,

one of the nitrogen takes up the electrons and then subsequent that will take up the proton that is available because it will release the proton, the proton will go to the nitrogen. So, you have R and then NH_2 and you have CO and then O and then you have C on one side, it is now NHR and on the other side is NR the double bond is still there.

So, this is the intermediate, now what you do? Once the reaction of the acid and the DCC is over, then you add your amine. If you add the amine now look at the structure, this is now no longer the carboxy; this is the carbonyl carbon of the earlier carboxylic acid. And, this OH is now converted into a system which resembles like urea but not urea, but it is a urea derivative. But, the thing to notices is that if this goes off, this O minus is highly delocalized. Because, this O minus can go here and this can take the N minus and then this minus come back and forth. So, this is a very good leaving group both are highly electronegative.

So, this is pooling electrons from the carbon and so, the carbon wants the electrons from the oxygen and the oxygen now wants the electron from this carbon oxygen bond. So, the bottom line is that, this has become very good leaving group. So, RNH_2 now goes and attacks the carbon and these goes out. This is exactly the reaction that takes place.

So, what you are getting? We are getting peptide CONH

So, you get your desired peptide bond if this happens to be an amino acid, and what comes out as a byproduct here is also interesting. So, you get 1 equivalent of this C double bond ONHR and NHR. This is nothing, but an urea derivative, which is called DCU: Dicyclohexyl Urea. Urea is only NH_2CONH_2. So, let us go to the next page.

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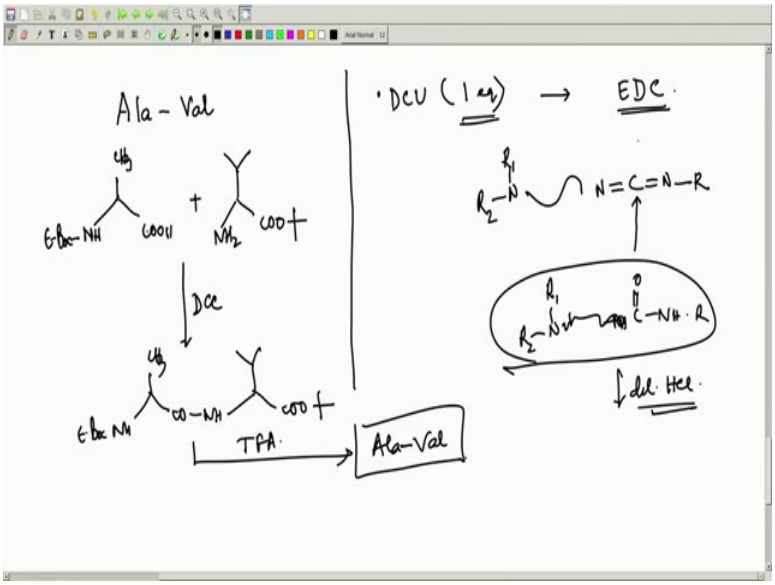

Now, once we have learnt the mechanism of DCC coupling;I will just write all the steps; suppose I want to make alanine and valine (this di peptide). So, what I will do? I will first take alanine I will protect its amine as suppose ^{t}Boc derivative and this acid is free. And, then I take the valine where the valine carboxy is protected; which protection I should use? I should use the same protecting group which comes under the same condition as the protecting group on the nitrogen or the other amino acid.

That means, I take a protecting group here which comes off under a condition that also removes the protecting group of the other amino acid. So, that I do not have to repeat steps and thus this strategy will cut down my steps. So, I should use this is ^{t}Boc which comes off in presence of TFA. So, here I take a t-butyl and then this is NH_2. So, then I add this DCC and I do the coupling; C H_3NH ^{t}Boc and then CO now, that will form the peptide and then this will be the valine part. And what is the last step? The last step is deprotection with TFA so, you get alanine and valine ok.

Is it the end of the story the peptide synthesis? Certainly not, there are other important points that we have to now consider. What are the problems with DCC coupling? DCU that is generated as 1 mole equivalent as a byproduct. That means, you have a separation process now.

You separate your protected dipeptide from DCU, now luckily DCU is has less solubility in dichloromethane. So, you can separate most of the DCU by just crystallization form

dichloromethane or methylene chloride. Still there are some DCUs left in the procedure and sometimes it is really difficult to separate the protected dipeptide or protected peptide from the DCU. So, you have to do a series of columns or series of crystallizations in order to get the DCU out. Now, that problem has been taken care of by a reagent which is called EDC, that is also a carbodimide.

The theory is like this that if you have C double bond N and C double bond N, in one arm you have one type of alkyl group and the other arm you put an amine functionality, but that is a your tertiary amine. So, if that happens, then the reaction will take place like what happened for the case of DCC; but the end product here will be C double bond O. And, urea which is having C O then another NH which is having an amine group at the end; due to this amine group now, this urea derivative will be soluble in dilute HCl.

So, if you just give a wash with dilute HCl, your urea derivative coming from EDC will go out. So, that is a good; when you have problems with DCU you can use EDC. However, this is a much costlier agent than DCC, DCC is much cheaper ok. So, that is one problem which can be taken care of by other coupling agents, another problem is there which is more serious.

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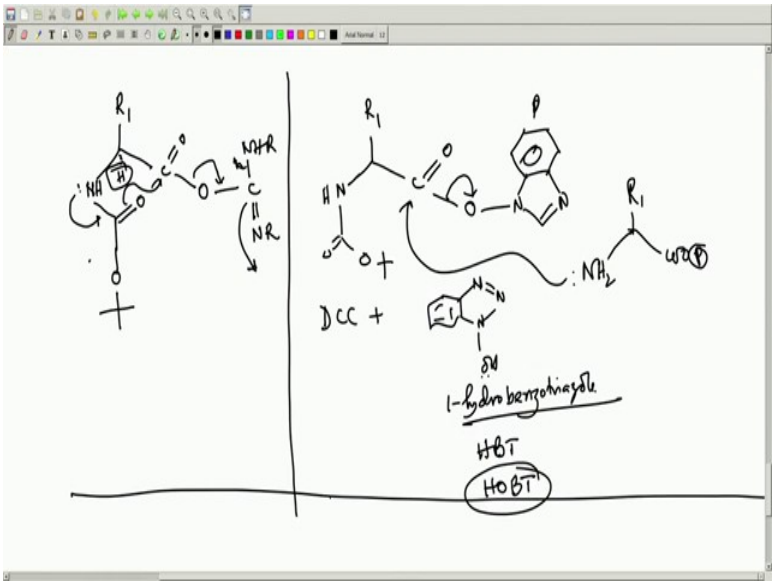

DCU can also you give rise to racemization; exactly in the same way like your azlactone formation. Suppose you have this tbutyloxy carbonyl (^{t}Boc) and when you activate the carboxy with DCC, the intermediate that you get is this NHR double bond N and this is

R. This is the intermediate, this is the α hydrogen; you want to retain that so that it never gets lost otherwise there will be racemization, but this is also quite reactive. You want less than 0.0001 percent of racemization because, ultimately this peptides are being made for biological study. So, you have to ensure that my molecule is optically pure. So however, what happens in DCC? There is this azlactone formation when this azlactone formation takes place, you know this hydrogen becomes more acidic. So, there is some racemization in DCC coupling. So, what to do? Then people thought about it and they could find other reagents along with DCC; if you use these reagents, then this racemization can be suppressed.

And, what is that reagent? Along with DCC, you use 1-hydroxy benztriazole; We generally abbreviate as HBT or HOBT. So, what happens here? As soon as this intermediate is formed, 1-hydroxybenzotriazole now comes instead of the amine, which was supposed to attack the carbonyl and make the peptide bond, this 1-hydroxybenzotriazole comes and attacks the carbonyl and kicks out the DCC part, as urea derivative. So, what you get is this R, what you get it is this is t-butyl and you will get C double bond O then O then, the traiazole part

So, you will get this as the intermediate, now this is reactive; this does not form azlactone. So, before the azlactone is formed, you form this compound. So, this acts as a nucleophile and then what happens? Your other amino acid that you add as the protected acid, its amine will now come and attack the carbonyl and this will go out.

So, you see N-hydroxybenzotriazole is again liberated and you can use it in catalytic amount. So, 1-hydroxybenzotriazole can be used in catalytic amount with DCC. Because you are releasing the 1 hydroxybenzotriazole, but in practice sometimes we say that to suppress this racemization, it is better that you use 1 equivalent of 1-hydroxybenzotriazole.

Because, if it is catalytic by the time the catalytic reaction takes place, there may be some azlactone formation. So, it wise to use 1 equivalent of this. So, that is the coupling agent DCC-HOBT. But, it is worthy to remember that DCU creates a few problems; alternatively you may use EDC, which has an amine group at one of the arm as a tertiary amine group.

So, that is called water soluble carbodiimide reagents, you just wash out with actually not water, but with slightly acidic water. And, to suppress the racemization, you use DCC along with HOBT, but there are various other type of reagents; many coupling reagents are available now and we will do that in the next session.

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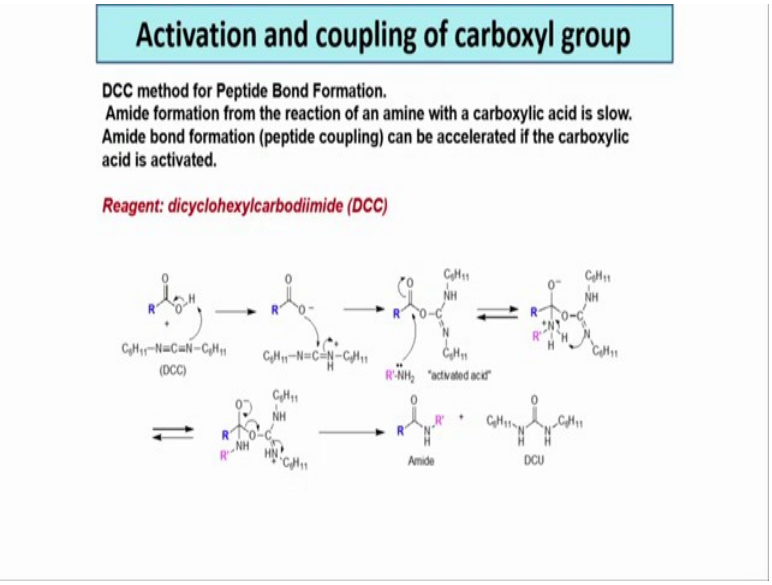

Thank you.