Organic Chemistry In Biology And Drug Development
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Lecture - 53
Mechanistic Enzymology of Isopenicillin N Synthase

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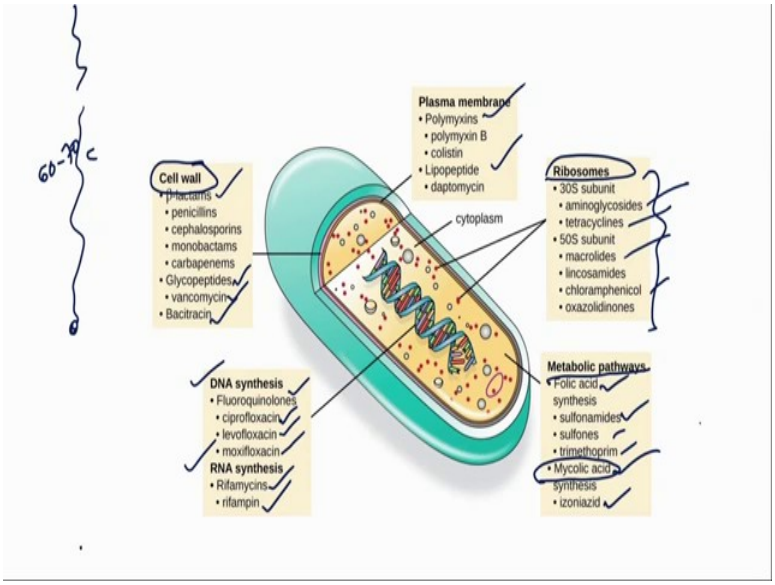

Welcome to this course on Organic Chemistry in Biology and Drug Development. In the last few sessions, we were studying the antimicrobial agents. And we have seen the chemistry of penicillins; how do they work and then, alongside other various kinds of bacteriostatic as well as bacteriocidal antimicrobial compounds; their chemistry has also been told.

Now, I will start with this slide which basically represents the complete spectrum of antibacterial agents that we have and their target of action. Like here it is this bacterial cell and the bacterial cell wall is so important. So, what works against the cell wall? All the β-lactam antibiotics, then the glycopeptides which include vancomycin and bacitracin.

Then you have the some antibacterial agents which work against the DNA replicating enzymes or transcription process; these drugs hamper the transcription process. So, we say that the target is DNA synthesis or RNA synthesis; like the fluoroquinolones which

are DNA gyrase inhibitors; DNA gyrase is basically a kind of topoisomerase which takes care of the super coiling problem when the DNA strand unfolds.

Like ciprofloxacin, levofloxacin, moxifloxacin; RNA synthesis is stopped by rifamycins, or rifampin which are anti-tuberculosis drugs, then plasma membranes are targeted by compounds like polymyxin and lipopeptide. And then there are drugs which target ribosome, like chloramphenicol. This is a big class of compounds which targets the ribosome it could be 30S subunit, it could be 50S subunit aminoglycosides.

Then you have drugs like amikacin, tetracycline, macrolides like erythromycin, chloramphenicol etcetera. And then you have drugs which hamper the metabolism like folic acid metabolism which is inhibited by the sulfonamides, sulfones, trimethoprim. And then in tuberculosis you have mycolic acid biosynthesis; we have not talked about this mycolic acid; that is itself a special topic that how to stop the growth of the tuberculosis causing micro-organisms.

Basically in tuberculosis, the lipid layer is made up of not C20 or C16 or C18 fatty acids like the stearic acid, palmitic acid; it's not like that, which is presenting all other bacteria. But in tuberculosis, the lipid bilayer is made up of extremely long 60 to 70 carbon atom containing fatty acids. And they are extremely difficult to penetrate. Penetration will be very difficult because it is a thick layer of hydrophobic membrane containing about 60 to 70 carbon containing fatty acids; these are called mycolic acids.

So, if you can stop the mycolic biosynthesis which many of the tuberculosis drugs do, you can stop tuberculosis. So, that is done by drugs like isoniazid; it is a very simple pyridine based compound, we have not talked about that; just let you know that mycolic acid is a special class of lipid molecules and they are very difficult to penetrate. So, other antibiotics do not work against the TB causing organisms, so you have to stop the mycolic acid biosynthesis.

So, isoniazid is one of the very well-known anti-tuberculosis drugs. Now, last time we took up a topic which is called the engineered antibiotics or engineered antibacterial agents; that means, where you manipulate the protein that makes the antibacterial compounds, specifically the antibiotics.

Because now we are talking about proteins; that means, they are made by the living organisms. So, now, we started with this engineered antibiotics; that means, change the structure of the antibiotic. We started with penicillin; and to make that engineered antibiotics, we need to know that how the bacteria first makes that antibiotic.

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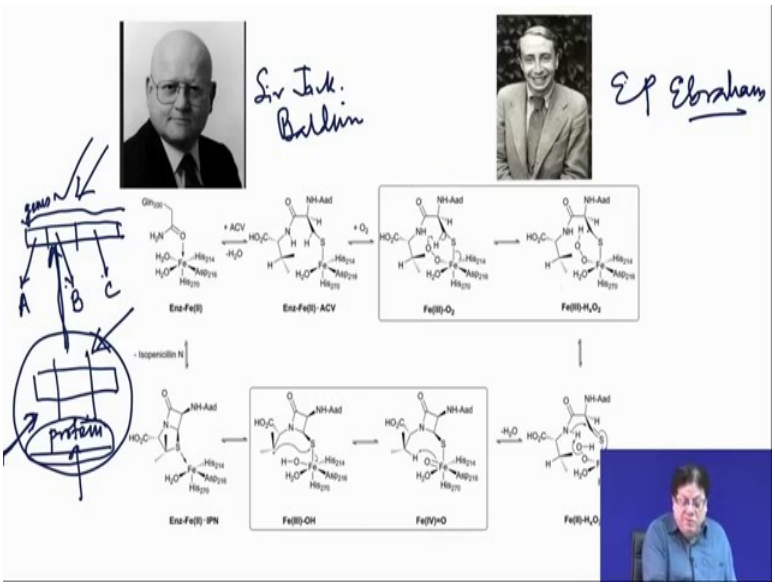

And then you modulate the different enzyme systems that are involved in the biosynthesis of that antibiotic. Now, remember there are basically enzymes which ultimately makes antibiotic. So, you can make new antibiotics by changing or by forcing the enzyme to accept modified substrates; that is one strategy that you take at the protein level, you can dictate the protein to accept modified substrates.

Because, we know in penicillin there are three amino acids which combine to make a tripeptide and that tripeptide undergoes cyclization by a single enzyme mediated double cyclization. Say the tripeptide is actually LLD-ACV; A stands for α-aminoadipic acid, and then C for cysteine, and V for valine.

Now, if we change valine and put some alanine there, so ACA, the question is whether that will be accepted as a substrate by the enzyme; if it is accepted then you will get a penicillin where two methyls are not present only one methyl will be there. So, that is one way that of giving modified substrates to the proteins that are involved in the biosynthesis; that was the old strategy; that is not the new strategy. The protein at some point of time especially in the 80's or 70's, it was a very precious item.

Because ultimately if you try to isolate the protein it will be very few amount; it is a very small quantity of micrograms, to get milligrams of protein, it was a challenge at that time. This approach is restricted by the ability of the enzyme to accept modified substrate. Because, if alanine is not accepted as a substrate, then you are not getting the penicillin which you wanted to get.

So, it all depends on how specific is the protein, if it is too specific then it has very narrow substrate dependence, so you will not be able to get newer modified penicillins. On the other end, if it really has a broad specificity then you can change the tripeptide, put different amino acids and then you can get new molecules. But, again I repeat, this was the earlier strategy, today people do the reverse engineering.

So, they see this protein is involved in biosynthesis of antibiotics, they go for the gene that are involved; what are genes? Genes are the functional part of the genome. So, there may be several genes which may be involved. Suppose this gene expresses one protein say A, this gene another protein like B, and this third one again produces C.

You can change at the genetic level; which these days is quite easy after the advent of polymerase chain reaction, and then now the latest technology of gene editing is CRISPR-Cas9 where you can edit the gene. So, if you edit the gene at the genetic level, then you put the gene in a live microorganism then you always get the modified systems.

So, there are two approaches; you can approach from the protein side, or you can approach from the DNA side, the gene editing. I will show you both the approaches. First, it is the proteins that are we are just discussing. In case of penicillin, what happens last time I said that penicillin requires basically this assembly of the three amino acids LLD-ACV and that LLD-ACV then cyclizes in a single step.

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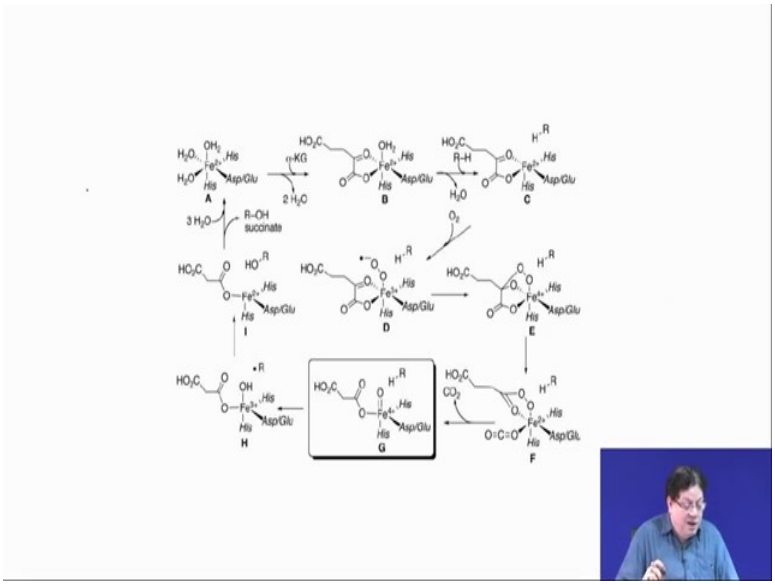

Also we told you that what is the rate-determining stepp.

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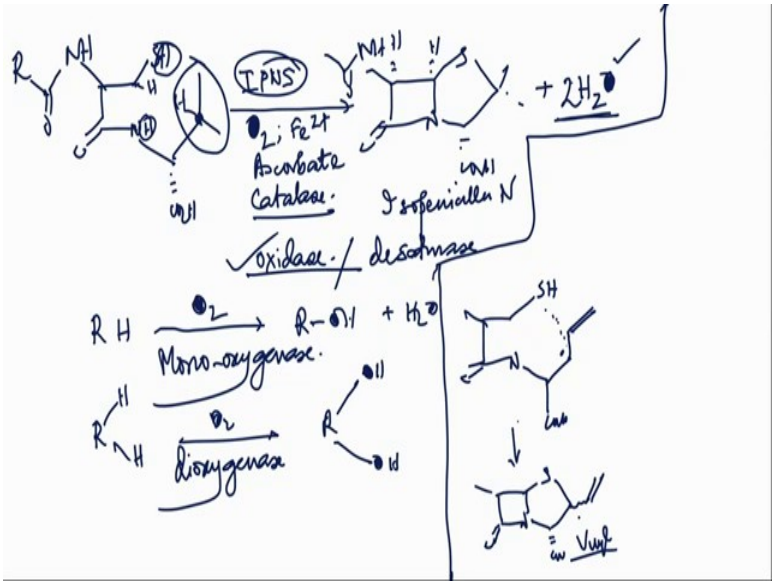

In the tripeptide ACV, that is SH and we are not writing the rest here; those are the α-hydrogens and then CO and then R. Now, you know the enzyme that cyclizes this is called IPNS, Isopenicillin N Synthase; it does not require any ATP. There is a co-substrate requirement which is oxygen and there is a cofactor requirement that is ferrous and to keep the iron in the ferrous state you need ascorbate. However, another enzyme which also gives better yield is called catalase.

I will tell you why catalase is added; why does it improve the yield, first of all let us look at the reaction; the reaction gives you straight away the penicillin. But this penicillin is what is called the isopenicillin N because it has L-α-amino adipoyl side chain.

So this is called isopenicillin N, these are α-hydrogens; what is the other product? What is the status of oxygen? This comes as two molecules of water. You are removing four hydrogens; one is here, one is here, another is this, and another is the sulfur hydrogen. I told you about a kinetic isotope effect experiment that demonstrated that it is the carbon-nitrogen bond that is formed first followed by the carbon-sulfur bond.

Out of these two bonds, it is more difficult to form the carbon-sulfur bond, because this carbon is not functionalized; this is a hydrocarbon side chain. So, to chemically do a reaction is very difficult, but enzyme does it because enzyme has a different approach to do reactions which are chemically very difficult to do. Now, it has been shown that this oxygen of water comes from the molecular oxygen that is used in the in the reaction, it does not come from any water.

So, source of oxygen is molecular oxygen, now these type of enzyme is called oxidase means where the oxygen is not incorporated in the substrate, there is no incorporation of oxygen in the substrate. If you see the incorporation of one oxygen in the substrate like suppose if you have R H and you have an enzyme which utilizes oxygen and you get ROH. Then that enzyme will be called mono-oxygenase, mono-oxygenase because out of the 2 oxygen atoms here, one ends up here and the other ends up as water.

Two hydrogens are there, if both the oxygens are incorporated in the substrate OH; R is just a general group. So, if both the oxygens are incorporated in the substrate that is called dioxygenase. So, penicillin is not a mono-oxygenase, it is not a dioxygenases, if nothing happens then it is called oxidase.

It can be called desaturase also; desaturase means, you are actually desaturating it, saturation means addition of hydrogen and desaturation means taking out the hydrogen. So, you are taking out four hydrogens, so that is desaturation; now what is the mechanism of this reaction now. Now, we know that which is the first bond that is formed maybe I can write here.

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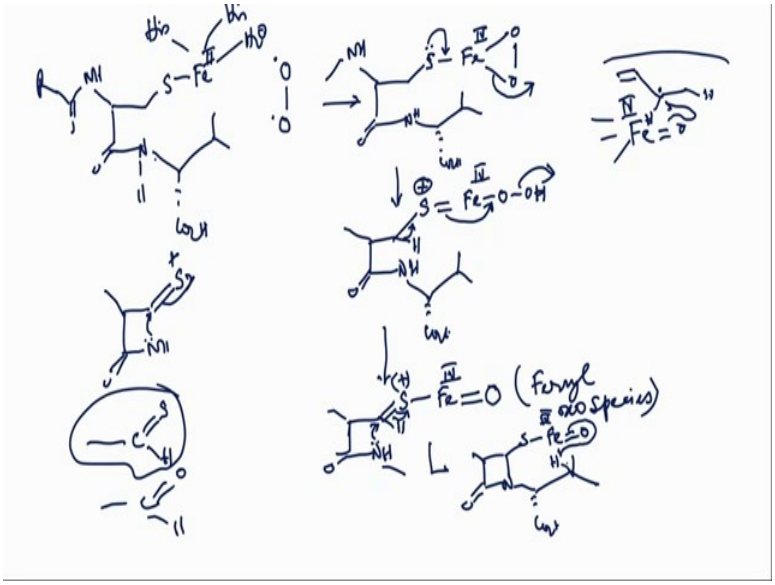

Now, the mechanism that has been now quite well established is that that you have the side chain R, and this is NH, this is S, and then you have this CO_2H and you have methyl So, the first step that happens is the iron which is bound to the sulfur, and then iron is having 6 coordination.

So, there may be some water or some maybe histidine, but we are not writing all those; Fe is in the plus two oxidation state. Now, you have the oxygen, you know oxygen is in the triplet state, so two unpaired electrons, it's triplet oxygen. This is S Fe; we are not writing the other arms, this reacts with the oxygen forming this cyclic dioxygen system.

That means iron is giving one electron to this oxygen, and oxygen is giving one electron. That means, in this bond, one of the electrons has come from iron, one from the oxygen and this bond also, one of the electron has come from oxygen and one of the electrons from that iron. So, iron has now donated two electron, so now this will be in the plus four oxidation state.

The iron has already involved its electrons to form these bonds, so NH and then CO_2H this NH and that. Now, what will happen? The sulfur lone pair comes here because this is a strained system, so that now breaks. So, N, so you have to form the β-lactam and what people thought that it must be a kind of double bond here.

So, if you can generate this type of system then you can get a cyclization like this 4-*exo*-trig. So, that you have to generate, so this is now S double bond Fe and then O OH; this will take a proton. Now, this you have to also think of what is the oxidation state of the iron, see iron is not gaining any electron nor losing any electron.

So, iron is still in the plus four oxidation state and this is NH and that is CO_2H, then methyl and that part; so now, sulfur is plus. Now, what will happen? One of the hydrogen will come here, that will go there, these goes out as water. And that forms double bond S; see that is how the thio aldehyde is formed. There is a hydrogen here, C double bond SH. There are so many aldehydes you can see, but it is very difficult to isolate a thio aldehyde, thioketones you can isolate, but thio aldehydes are extremely unstable.

So, what nature has done; it has not generated any free thio aldehydes, remember I told you at that time that when the there is a debate which bond is first formed; they tried to prepare an intermediate containing an SH in a four membered ring. But that was not stable; so nature bypass that by putting the sulfur attached to the iron ok, so that it stabilizes.

Now, the sulfur has a positive charge and this, iron double bond O and iron is still in the plus four oxygen state; this is called a super oxidant; this is what is called a ferryl-oxo species. So, now, this nitrogen will attack here in a 4-*exo*-trig fashion. So, the first ring formation has taken place. S Fe double bond O that is the plus four oxidation level and this was CO_2H and then two methyls.

This is a super oxidant, if it sees a nearby hydrogen which it can abstract, it will abstract that hydrogen, provided that after the abstraction of the hydrogen, the resulting species, whether it is a radical or cation or anion that has to be stabilized. I will give you some examples, suppose if you have allyl and if you generate a ferryl oxo.

We can actually generate ferryl oxo species; there are techniques for generating; hydrogen peroxide and Fe (II) constitutes the Fenton's reagent. Then Fe(II), EDTA, O_2 and L-ascorbic acid constitutes to form the Udenfriend's reagent; these are some reagent systems which make the ferryl oxo species. So, the oxygen will abstract the hydrogen usually in a radical fashion, the hydrogen that will be abstracted will produce a radical that is stabilized.

It will not abstract hydrogen from the homoallylic hydrogen, it will abstract the hydrogen from the allylic carbon because, the allylic radical or the cation or radical. Because, there was again this debate whether it goes *via* radical or it goes *via* cation, but to cut the story short, we are not going into big details. But, to cut the story short that this is the mechanism; the oxygen abstracts the hydrogen and the hydrogen forms a radical here. So now what is going to happen let us see.

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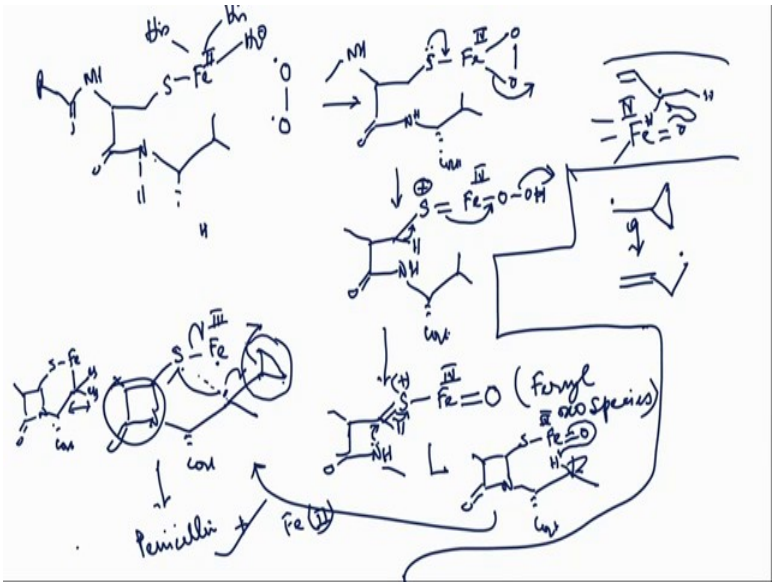

So, after this, you have the β-lactam already formed, you have sulfur, you have iron, and you have the CO_2H and two methyls, a carbon radical; and remember this is one electron shift. So, iron must be having one electron back to it, so iron is in now in plus three oxidation level; however, this iron with one electron and the carbon has one electron, so they can actually form a bond also.

So, it says that they exist as a resonance hybrid of an organometallic compound where there is a carbon-iron bond, so there are two methyls; and also it has a radical character. So, the intermediate is an organometallic compound, but because iron-carbon bonds are not very stable (at least in this case), so you can think of that they equilibrate between these two structures.

Now, this radical attacks the sulfur and the iron gets back its electron; so you have this five membered ring formation; now that is the penicillin. So, that gives you penicillin and you see the iron is back to the ferrous state, because it has got back both the

electrons with which it started. One electron it got back from this when the oxygen attacked the hydrogen, in a radical fashion. It is a homolytic cleavage so one electron remains with iron and the other electron again comes from the breakage of the iron-sulfur bond, so this is the mechanism.

Now, you can say that how do we know that there is a radical that is formed here; it could be an ion as well. Now, the radical was proved by EPR spectroscopy, or ESR; Electrons Spin Resonance spectroscopy. That usually is that is the gold standard for making these type of statements that a reaction going through radicals.

However, if the radical is very short lived and if it is inside an enzyme system; you may not be able to get any EPR signal, and people tried they did not get any EPR signal. But there are indirect ways of proving that a mechanism is radical based or cation based, or anion based; what was that? If you have a radical which is α to a cyclopropyl ring then what happens? It immediately breaks down into double bond and then this happens.

This rate of reaction is so fast that when you generated radical here in this valine moiety, suppose I have a cyclopropyl here in the amino acid. If it is radical then I am sure that before this attack can take place, it will open up, because it is so fast. This is called a radical clock and this is a test whether a reaction is going through radical or not; this is called a cyclopropyl radical clock.

So, scientists have made this cyclopropyl and they have shown, if there is a cyclopropyl, so the radical will now be formed here and you will get a bigger ring. You will get 4-membered ring β-lactam fused to a much larger ring and you will get also this. So, some radical will be here, it will be equilibrating between this radical α to the cyclopropyl and also the ring open form.

So, that means, you will get a 4-membered ring fused with the 5-membered, but with the cyclopropyl at the arm or you can get a 4-membered ring fused to an 8-membered ring . This is one way of getting new penicillins. I will give some examples because now we know that it is a radical mechanism.

So, what you can do? You take different peptides; first of all you can take an allyl because, the first thing that will come to your mind that better have an allyl and this

radical will be very stable in that case. So, if you take an allyl, you will get penicillin which is having allyl moiety this is actually vinyl penicillin.

Remember I told you that in penicillin skeleton, it is very difficult to change the pharmacophore. So, this is the first example of changing the skeleton of the penicillin with different substituents, because synthetically it is not possible. Even if you make the tripeptide, this bond is very difficult to form.

So, that, so now synthetic methodology is available; the enzyme is really very generous, it accepts lot of different side chains in the amino acid. The natural substrate is valine, but you can take allyl; this is called allyl glycine derived peptide or you can take even the higher homoallyl also; homoallyl glycine. But now if you take homoallyl glycine; that means, your double bond is here now, so the radical will be formed here and not there.

So, you will not get penicillin you will get cephalosporin. And then you can actually have different substituents were substituted amino acids were taken in place of valine and these was all were basically the works of Sir Jack Baldwin and E. P. Ebraham. They combined together and then collaborated.

And then finally, at least a 100 new penicillins were made and many of these pencillins are very very effective against gram negative organisms. So, that is all about penicillin. I think this mechanism which I told you is shown in this slide.

So, later on you can take this to see this slide and what I explained the oxidation level of the iron in a very critical way, because that the important aspect of this mechanism. If you study bioinorganic chemistry, this will be very important that how to calculate the oxidation level of metal ions and one example is this iron oxo complex. Till today many papers are being published in very well-known journals like JACS or Angewandte Chemie where the chemistry of ferryl oxo has been studied, because this is called a super oxidant.

C-H activation is a very important aspect in today's research, and ferryl oxo is one way to do the C-H activation. So, now, let us very briefly talk about cephalosporin; cephalosporin comes from penicillin and there was one reaction; The problem with cephalosporin initially although it was discovered in the early in the late 50's, but the cephalosporin came into the market much later because, you know that the 6-APA (6-

aminopenicillanic acid) was made available by Beechams and from that amine you can derivatize and get different penicillins. However, they could not get the corresponding 7-amino cephalosporonic acid, if you do not get the amine then you cannot make different cephalosporins.

So, that was a stumbling block in getting cephalosporin because, it will be much more costly if you do want to do it synthetically. So, cephalosporin took a long time finally, there were systems by which you can get 7-aminopenicillinic cephalosporin acid. Penicillin after a few more steps goes to the cephalosporin, what are those two steps? First the L-alpha amino adipoyl goes to the D isomer.

So, there is an isomerase, or racemase there which racemizes that; that is called penicillin N, penicillin N is the side chain with D configuration. And then that cyclizes by another enzyme system and gives the cephalosporin, but we are not talking about that. If you want to know, you can study these slides and then check how the cephalosporins are formed. I told you about the difficulty in getting the cephalosporins; just maybe a quick brush up before I go into another type of antibiotics where the engineering has been done at the genetic level.

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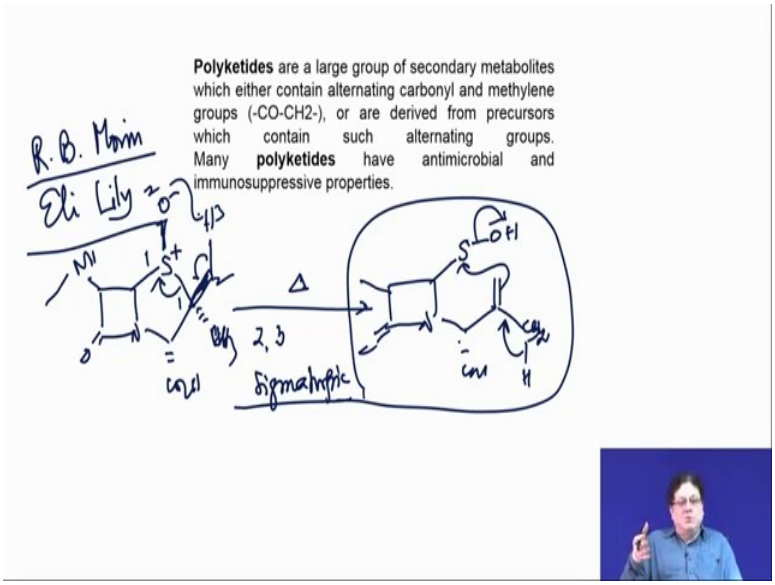

This was done at the protein level, you are giving different substrates and then getting different penicillins. Only one reaction from organic chemistry point of view; when it was found that cephalosporins were hard to get, R B Morin (one chemist) who was

working at the company Eli Lilly, a very famous company which makes many of these antibiotics, discovered one reaction and that starts from penicillin being oxidized to sulfoxide.

See you can always oxidize the sulfur and make the sulfoxide and then heat it by a pericyclic reaction like this, this is a [2,3]-sigmatropic shift and then followed by cyclization. So, if you do this pericyclic reaction, what you get is sulfur, you have broken the bond here, you have broken the bond a double bond here, this is methyl and a methyl here. And then you have CO_2H and you have S OH and then very interesting CH_2 this H leaves these attracts the sulfur and the OH leaves as water.

So, you get the cephalosporin; in fact, this was the method in earlier days when 7-amino cephalosporonic acid was not available. So, they were converting the penicillin *via* this Morin reaction which is nothing but a [2,3]-sigma tropic shift. And because these are 1 1 that is the bond which is a 2 3 and these 2, so this is a [2,3]-sigma tropic shift, also called the Morin reaction.

So, that is the way you can convert penicillin into cephalosporin that also gave some clue that how cephalosporin can be obtained in the microorganisms from penicillin. Because, if penicillins can be converted by this way, people thought that maybe this is the mechanism by which penicillin is converted to cephalosporin by the enzymes.

However that is not true; it was not by the sulfoxide; people made this sulfoxide and shown it to the microorganism, but the microorganism refused to accept these as the substrate to make the cephalosporin. But, as I told you, if you are interested, I have the slide, you can go back and then see how cephalosporin are formed.

So, that is for the biosynthesis of penicillins; the engineered different structural variation of penicillin can be made by changing the substrate; instead of ACV you take other tripeptides where valine can be changed. Now, you can say why not change the cystine? If the cystine is changed by a serine then you should get an oxa penicillin.

But unfortunately that is not accepted as a substrate; you need the sulfur; so not everything can be accepted; that is not accepted. So, you have to stick to the cystine and then variation can be done with valine; there are lot of perturbations you can do. So, different substituted penicillins can be made and have been made.

Thank you, so just wait for the next session.