Principles and Applications of NMR Spectroscopy
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Module 07
Lecture No 32

In the last class we have seen how the experiment HMBC works, this is Heteronuclear Multiple Bond Correlation experiment compare to what we get in HSQC and HMQC we get longer range correlations in this particular experiment. So the schematic of this spectrum is what we saw last time, so this is shown here. We can see that in this particular spectrum HMBC, you get long range as shown in this case. So you can see here for example in this molecule which is Glutamine.

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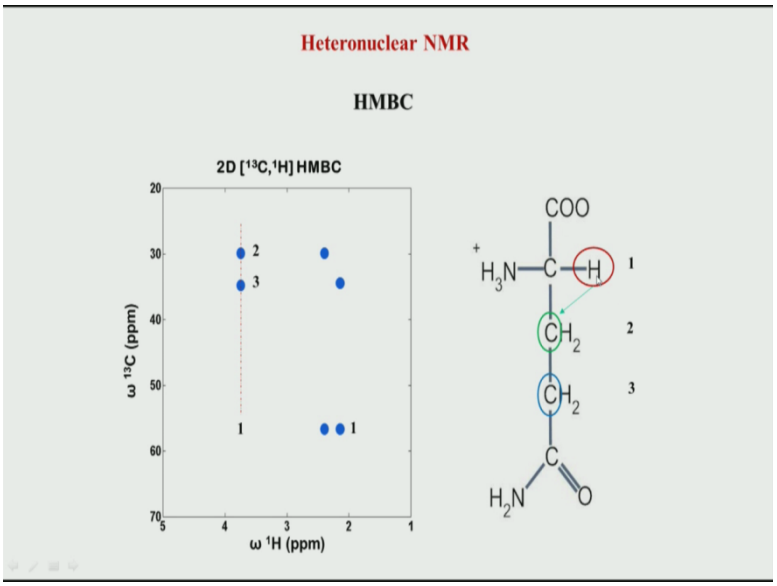

In a Glutamine we saw that you can now get correlations from this hydrogen atom to 2 bonds, 2 bonds carbon and you get a correlation to here carbon. Similarly you can get HH correlation from this proton to the further carbon. So, what is d1 typically is that we suppress the 1 bond correlation in HMBC by tuning the coupling constant or tuning the delays in the coupling INEPT to the frequency or the coupling value for a 2 bond. So, if you recollect in a INEPT experiment in a INEPT block of HSQC, we basically saw that we can tuned that coupling to delay to 1 by 2 J, where J is the coupling constant between the carbon and proton. Similar to that experiment here also we do the same thing, the INEPT here is instead of tuning to 1 by 2 J that is J of 1 bond coupling the 1 by 2 J is taken, but J is tuned to a longer smaller coupling which is typically about 10 to 15 hertz.

In fact, it can be smaller than 10 hertz so about 5 to 15, 5 to 10 let us say for 2 bond and further you can go up to 3 bonds away but that is much weaker coupling. So, therefore that coupling is much smaller than 5 hertz and because of it is weakness in the smaller value we do not normally get correlations further between further than 3 bonds. But 2 bonds coupling from the carbon to proton is very frequent that is most the peaks are in this correlation and that is what we see here.

So, you can see in this particular schematic drawing we can see that when I look at this line here these red line, which corresponds to the line to that means the proton here. We are looking at proton in the X axis and the carbon is on the Y axis, so the proton number 2 this proton has the chemical shift of around the 2.1ppm in the proton axis, but in the carbon axis you do not see the correlation to this carbon 2. Carbon 2 should be here but that is what we do not see very sorry, this is carbon to you do not get that we get correlation to this carbon, which corresponds to 3 on this axis. Okay.

So, on this axis this is a number is given for the carbon and you can see this carbon is number 3 and this carbon is number 1. So, we are getting 1 and 3 correlations for the proton which is number 2. So, that is what is indicated here, that if you a have proton the second proton the second carbon the proton shows correlation to this carbon as well as this carbon. Similarly, we can look at the other proton which is number 3 which had a chemical shift of 2.2 to 2.5 ppm and that shows correlation in the carbon dimension to carbon number 2 now which is here which is this carbon number 2 and if possible that is if there is 3 bond coupling strong enough, then also 2 carbon number 1 okay.

So, typically we look for in HMBC to 2 bond we do not go to at beyond 2 bonds because as I is said beyond 2 bonds correlations can be weak much weaker than 2 bonds. So now let us see where HMBC can be useful, we saw that HSQC in HMQC give correlation between proton and carbon and HMBC is now giving us long range but how can that be used in practical, how can be used in practice and where it will be useful.

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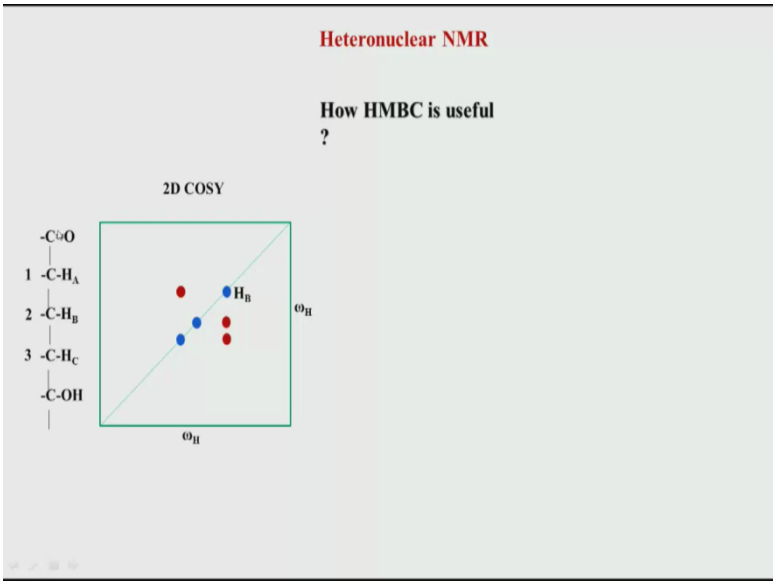

So, let us say we are trying to solve the problem, let us say that we have some molecule we look like this, this is a hypothetical system, but it will be good for illustration purposes. So, let us say that we I have a molecule which is like this which has a structure like this and I record a 2D spectrum 2D COSY spectrum. So, if you recollect a 2D COSY spectrum as hydrogen, protons and protons in both the dimensions, so that is what is shown here. Now if you look at the diagonal, again if you recollect let us say that we have the proton is always this diagonal is always a similar to 1D spectrum.

So, let us say again we do not know exactly the values but let us say that H Beta that H B has a chemical shift here in the proton the axis H the other 2 H B and H C have here, okay. So, now according to the COSY principle the H this H B will show correlation to both H C as well as H A, because H B is 3 bonds separated from H A. So, therefore I would expect a cross peak between H B and H A and also between H B and H C because H B is also suppurated from H C by 3 bonds.

So, based on that we have is shown the schematic here that H B shows correlations to both H B, H B shows to the C and A, so there should be a peak here also so let me draw that. So, there will be a cross peak here also okay. So, this is basically telling us that this is the COSY cross peak between the H Beta proton and the remaining 2 protons. But now the question comes, which have these 2 peaks how do we know which peaks does it this blue color this peak does it belong to this H A or does this belong to H C.

Similarly this one, does it belong to H A or H C, so one of them is H A, one of them is H C but we do not know which is which. So, how do we find out that the one thing is you can say look a chemical shift wise I can probably make a guess that out of these two, one may be more down field shifted other is less down field shifted and so on. But that is not always possible in NMR because we get a broad picture of where the chemical shift will be, but 1 cannot rely exactly on the chemical shift value to decide whether this is H A or H C.

So, what we do in NMR is we have to rely on what is called correlations, chemical shift correlations. A chemical shift correlation is the only way to establish unambiguously means without any doubt, which peak corresponds to which atom. If you do not have chemical shift correlation that is in 1D, for example in 1D, we do not get any chemical shift correlation. So, if we have only 1D spectrum with you, then you have no choice but to rely on the chemical shift values and make a guess.

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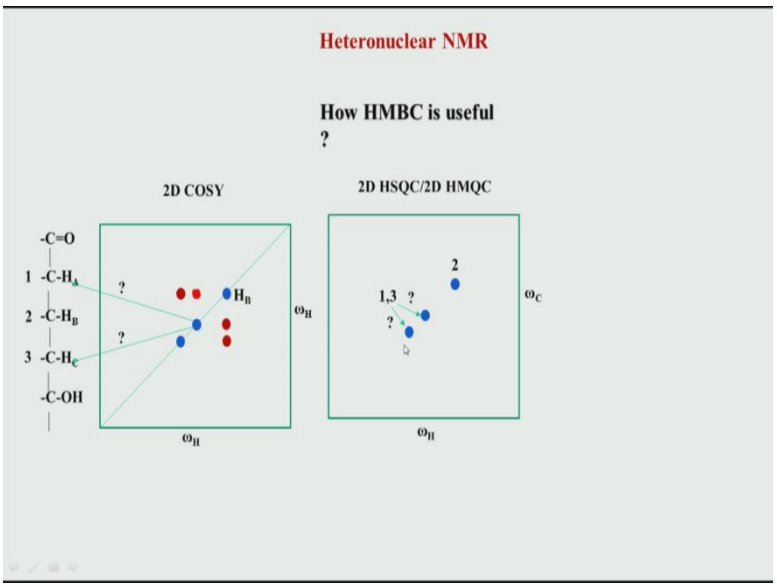

But that guess can go wrong many times, so therefore the whole idea of 2D NMR was invented because that helps us to give chemical shift correlation connection between 2 protons or 2 carbons and that information really helps to find out which peak will corresponds to which atom. So, in this example we have 2D correlations spectroscopy that is COSY, but you can see this is also not sufficient. This is not giving me a clear picture of which atom belongs to which peak and vice a versa okay.

So how do we solve this problem? We will see that HMBC helps us here. Now let us say that I record 2D HSQC of this molecule, which is shown here and the 2D HSQC and HMQC

remember will give us the same information. So, as for as that chemical shift correlations are concerned absolutely there is no difference between these 2 spectrums, minor differences are there in terms of sensitivity, resolution etc, but the chemical shift correlation information is same in both.

So, let us say I record such a spectrum of for this hypothetical molecule shown here, I may get something like this. So, here this is on carbon number 2, this is shown proton number 2. How did I get this information? I got the information because I looked here and I have somehow assigned this as H beta as H B because H B is show into both, so I know that H B has to be in the center of the 2.

So, again we know in this kind of system what we are trying to use is a simple logical based argument. We are not relying on the chemical shift value our all arguments here are logical or based on simple intuitively based on the connectivity and this is how NMR typically works that we normally go by first the chemical shift correlations, then by the connectivity information and then third is by using chemical shift values also. But chemical shift values as I mentioned is not always reliable because they could be many factors which affect the chemical shift values.

So, one should be cautious in directly using chemical shift value information, therefore we go for this correlation. So now the logic which I am using here is as fallows, so I am saying that is H beta this peak gives me correlation to 2 protons, so using the argument that in a COSY you get correlations only 2 and 3 bond neighboring proton and not further away, I can say that this H beta or H B has to be somewhere, where it is coupled to both protons on either side and that only has this here that is a structure is telling me that only this proton can be such that it will be coupled to 1 proton this side and another proton this side. So, based on this kind of an argument I have assigned or I have chosen this H beta to be this peak okay.

Now coming knowing the chemical shift of the hydrogen which is here in this line vertical line which I am drawing that this arrow, I can use the same value here, which is on this line vertical and therefore I can say that this proton this correlation in HSQC belongs to carbon number 2 because the proton number 2 that is this proton H B is having this chemical shift value which is what I have also seen in this spectrum okay. So, you can see we are trying to now use a simple connection based connectivity based arguments. Now let us come to this again, so these 2 still are not clear is a question mark says that we do not know whether

which is 1 proton or carbon pair number 1 or which could be proton or carbon pair number 3, so this either it is this pair or this pair here.

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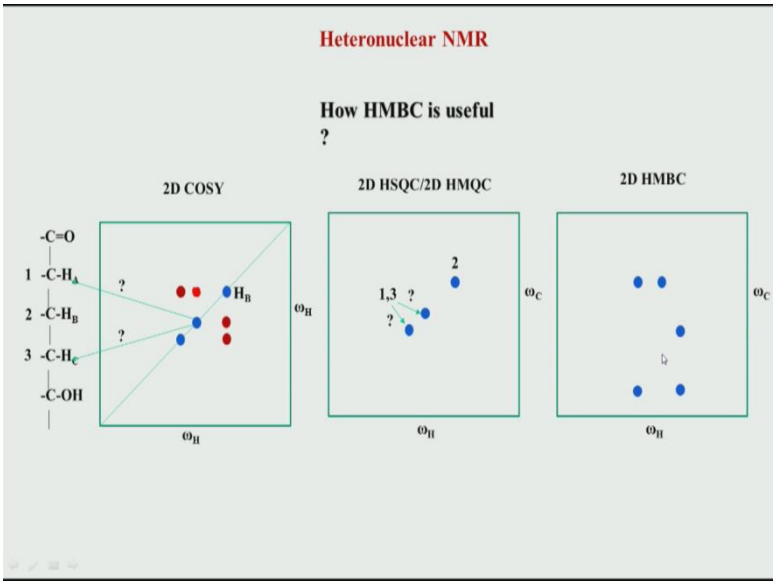

So, you can see that this ambiguity remains it is not HSQC is not helping us any way in resolving this problem, okay. So, then what should be do next, we can go to the HMBC. Now let us look at HMBC what information it comes and now it helps. So let say I record a 2D HMBC on this molecule and as we saw in the previous slide, this verticals scale axis is for carbon and the horizontal axis is proton. Now let us say that I am seeing a peak like this at 170ppm in the carbon along the carbon axis and that remember according to our carbon chemical shifts scales carbonyl comes around 170ppm and we have a carbonyl in our molecule. So what is happening is that here for these particular region here I am seeing a peak here correlation corresponding to a carbonyl.

So remember this proton where am I arrows a pointing now, they correspond the proton and carbon number 2 this proton okay. Now that is showing a correlation if you draw the line this is this line is number 2 it is showing correlation to carbonyl, which means this beta proton is coupled to a carbonyl and that is not very surprising because we aspect a long range correlation in a HMBC. In this case it is 3 bonds away from this hydrogen oh fine so that is not very surprising and H B now should show me a correlation to the next proton which is either H A or H C okay. So, that is what is you will come in this line but if you go to this particular case, now if you look at this line here what is happening in this line you see this particular proton which is this 1 of the 2, so these 2 are this 2 if you draw a vertical line here.

So, remember we are all hydrogen axis in all the 3 spectra, so my horizontal vertical line if I draw like this it will give me the proton value. So, this proton value here and this proton value here these 2 proton values corresponds to these 2 here, okay. So, if you look at the proton value here it is giving me correlation now to a peak here which is carbonyl. Now if you look at the structure here, only H A and H B are closed to this carbonyl in fact H A is very closed 2 bonds away. So, H alpha H A is expected to give a strong HMBC correlation to a carbon to the carbonyl carbon and I would not aspect H C to give correlation to this carbonyl carbon. Therefore, out of these 2 the 1 which gives me correlation to the carbonyl peak will definitely be the HA that is will definitely be that 1 okay. So, that is what is shown here that based on this information that this peak at this line gives me correlation to carbonyl and 2, so this is 2

So let me write that down, so this is number 2 carbons and this is CO, so therefore, it becomes this is H A is basically that number 1, so now I can say that now this proton belongs to number 1. So, based on that now I can say that this line which was what was a problem remembers we had a problem saying which of these 2 corresponds to H or C. Now this having seeing that this line here this proton gives me correlation in HMBC to carbonyl, then I conclude that this line has to belong to H A and that is this one here

So, in the second 1 here does not give me any correlation to carbonyl, but it gives me correlation to 2 and which is expected for C. If you look at C, C is expected to give a correlation to B that is use this second carbon, but it is not expected to give a correlation to carbonyl which is very far away it is 4 bonds way. So, therefore this kind of a spectrum hence now what is this line coming back to the last one, this is nothing but a B and B is giving me cross is a being me a correlation to C carbonyl and it should give me correlation to both A and C. But remember both A and C are very close, so let me draw the correct this 1 I would expect let us say this is number 3 that is H C. So, therefore this will be there will be a peak here which will be carbon of H A this is C A which is number 1 okay.

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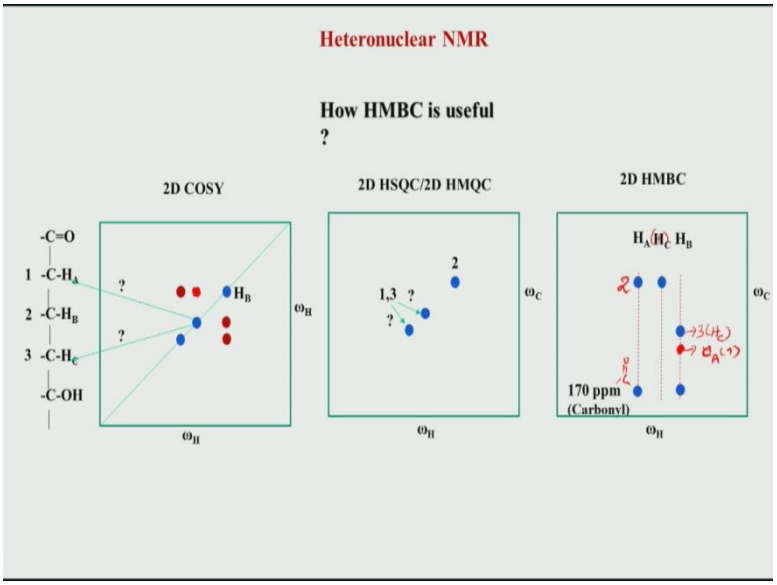

So, this particular line if you see the last line here corresponds to H B, H B, H B which is here and it is showing correlation to 3 peaks 1 is the 3, 2 is shown into 3 this H beta is show into 3 which is this carbon here and it is showing to 1 which is this carbon here and both of these are only 2 bonds away and it is seeing that there is a correlation to carbonyl which is far away, but this peak may not come or may come with does not matter to us because a remember we only focusing on the assignment of 1 and 3.

The problem was here 1 and 3, and that may is now resolved or solved using this HMBC spectrum, which helps me to give this information. So therefore, HMBC it is way this helps a lot to unambiguously figure out which carbon is coupled to which proton and the long range information helps us to resolve any doubts like we saw in this particular example. One thing you may asked and you make have in mind is that why we are not discussing about this carbon, but remember it is fine if there is a this carbon will come around 60ppm, so there may be a correlation between H C and this carbon which is not I am not showing in this spectrum at all because for me just getting a correlation to carbonyl is sufficient to resolve between the doubts of which is H A which is H C, okay.

If I get a information for this carbon that is this COH carbon, good that is also will helps me to resolve a doubts between which is H C and which is H A, for example, H C will give me correlation to this carbon but H A will not give me any correlation to this carbon okay. So therefore, yes we having this information also helps, but already I am able to find out clarify my doubts by simply looking at the carbonyl correlation, okay. So therefore chemical shift correlations which are given by 2D NMR are very useful for assignment for of peaks and

remember assignment of peaks in NMR is the very important part is not just recording 1D and we cannot figure out which peak correspond to which atom, we needs this kind of information to complete the assignment.

So, let us move on to the 1 experiment which is the HSQC TOCSY now, so this is going to be the last experiment in the list of heteronuclear correlation 2D NMR. So, this is a different type so if you recollect we discuss fact that you can combined many experiments together. So, in NMR this is how 3Dimensional NMR is built a 3D NMR typically many of the 3D NMR experiments are nothing but combinations of to 2 2 Dimensional experiments. So, this is 1 such combination, but this is not a 3D experiment it is still 2D and these experiments helps many times, when there is a doubts or ambiguity on what if the TOCSY is not sufficient to resolve the peaks, it helps to record this experiments. So, let us see how this experiment works and this is a very standard experiment used for small molecules and sometimes you want for peptides and proteins.

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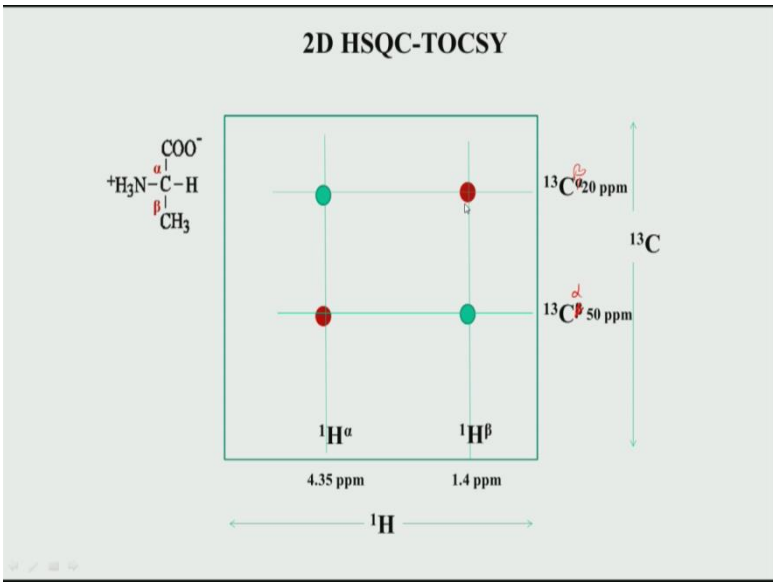

So, the HSQC TOCSY works like this, so on the left side you see we have there is a structure of molecule this is alanine amino acid called as alanine. Now in alanine, there are 2 protons okay; we will ignore this NH3 for time being we are only looking at carbon proton pairs. So this proton, we called it as alpha proton and this proton we will call it as beta proton. Now in a regular COSY or a TOCSY, you will get correlation between these 2 okay. Now in a regular HSQC you get correlation between these 2, okay.

So, what is done in HSQC is this 2 information that is information of direct proton carbon correlation and information of proton-proton correlation is combined together and both the information what just now I mentioned are available in one spectrum. So, how is that let look at look at it, so this is let us say this is alanine and alanine beta. So, there is mistype here, we will correct it this is, this is beta and this is alpha, so there is a so so, now it is known that, this is expected that the carbon beta should come around 20 ppm in alanine and carbon alpha comes around 50ppm. So, now what happens in HSQC TOCSY let us understand the peak pattern, so first let us see HSQC peak, so in H for in alanine where do you expects the HSQC peaks to come, you aspect HSQC peak to come at this position this cross here because a carbon beta is correlated to proton beta.

So that will come here and similarly carbon alpha is correlated to carbon proton alpha, so this is the standard HSQC peak, this is what we expect. Please note that this is not a diagonal peak all though in this picture it is looks like a diagonal, it is not diagonal because 1 axis is proton, other axis is carbon, so you cannot expect a diagonal peak in this kind of a spectrum. Now next is the TOCSY peak, so HSQC peaks have come; now the word TOCSY has to come now I mean the spectrum correlation corresponding TOCSY has to come. So, what is the TOCSY correlation what do you except? You expect this proton and this proton to be coupled in a regular TOCSY.

So, that is what we will see now that you these 2 protons are the cross peaks we these 2 peaks are the cross peaks corresponding to the TOCSY coupling. Now if you see along this line straight along this line for a given carbon that is beta I am seeing the full proton connectivity means all the protons in a spin system remember the word spin system which is very important to understand in TOCSY.

So, the complete spin system will appear in the line of a given carbon peak. Similarly, if you go to any other carbon in the same molecule, you will get a full line of the full spin system which will show off, okay. So, this is how the HSQC TOCSY works that given a molecule or a spin system to take any carbon in the spin system and take the line along that horizontal and you will see all the proton peaks in that spin system will show off here, we will see examples just in other few slides. Similarly, you take to any other carbon you will again get the same full spin system along that, so it means all the carbons we will show to all the protons okay. So, you see this is combination of proton proton TOCSY to carbon HSQC.

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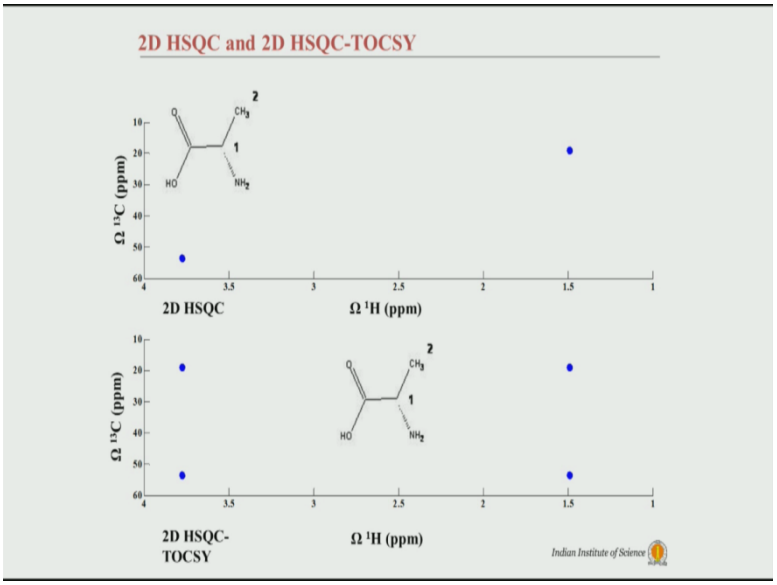

Let us see some more examples let us say that you have this molecule this is same alanine. So, this is what is shown here this is peak number 1 and this is a beta peak now I come this is similarly, a peak this is a TOCSY because proton to proton TOCSY and I will get this 4 peak pattern. So, where does HSQC TOCSY helps? the HSQC TOCSY helps in when suppose you have a mixture of molecules, okay. So, remember in NMR typically when you do organic compounds we have only a pure compound in hand and all our spectra that we record is typically a pure compound. But let us say you are working on some project or some work, where you have mixture of compounds then how do I figure which peaks corresponds to which compound, okay. So, this is typically a situation in some a areas in NMR known as metabolomics, where you are looking at small molecules in a system and what happens is many molecules will be mixed together in the same sample.

So, therefore we need to find out which peak belong to which molecule and this experiment HSQC TOCSY comes into helps for that kind of a system, when you want to find out what are the molecules mixture present in a mixture.

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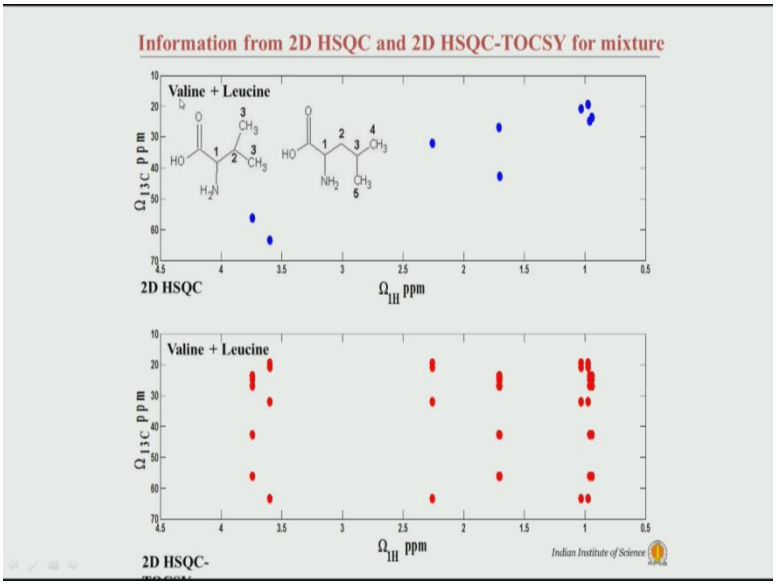

So, let us say that we take an example here; we take a mixture of Valine molecule amino acid called Valine with another amino acid Leucine. So, let us say I have a mixture means I have combined the 2 and in my sample and I have mix 3. Remember this is different from a dipeptide right, because in a dipeptide you will have a connection between the 2 by a bond known peptide bond and that we will see later down the line, but in this example I have simply mixed the 2 and there is no direct physical connection in terms of between the 2 molecules.

So, therefore each you can say is a spin system okay, so 1 now each Valine is denotes 1 spin system and Leucine denotes another spin system. So, let us see how this spectrum will come, so now this on the top portion what you have seeing here is an HSQC a 2D HSQC of the mixture. So, what do you seen in a mixture, you will see all the peaks corresponding to each proton carbon pair okay this is the standard HSQC. Now how do I figure out which peaks here corresponds to which molecule, one way to do that is run this TOCSY HSQC TOCSY. What will happen is shown in the bottom picture, here you see all the peaks which will belong to 1 amino acid will be denoted as a spin system and they will show chemical shift correlation with each other, but they will not show any chemical shift correlation with another amino acid because this amino acid is completely a different spin system compare to this, there is no connection between them.

So this will exist independently of this spin system okay. So, now if you look at this picture I have join the lines corresponding to a given spin system means all these peaks red colored, they all show correlations to each other. If you look at this lines this whole spin system this

vertical line belongs to 1 atom molecule similarly, this line belongs to 1 molecule, so this is like a TOCSY but now the horizontal lines are carbons this lines vertical is proton.

So, this entire 1 molecule will have 1 connection and similarly the other molecule. Now if you look this corresponds to this HSQC peaks okay, so this line vertical line corresponds to this peaks in the horizontal X axis and Y axis and similarly so each line corresponds to 1 peak in the HSQC and this whole thing corresponds to 1 spin system, okay. Now the second spin system is basically the other peaks whereas draw that also and this is basically this, this connection.

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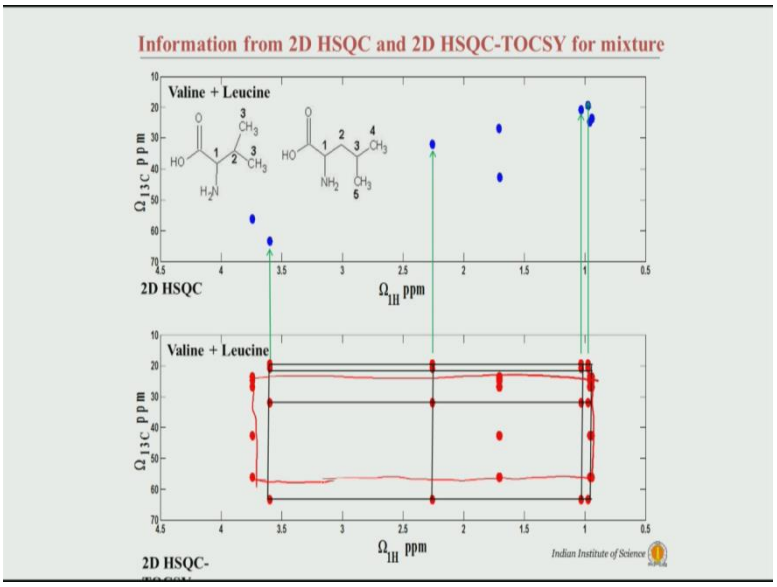

This connection corresponds to another spin system and it is completely different from the previous connections shown by the black line okay. So, what we have done is that we have identify a complete spin system within which are coupled to each other and this spin system corresponds to this atom the remaining one. So what is done next, you just simply look at the expected peak pattern, which is what you are seen here and then match it with a data base or with a literature or with a reference data base values and you can figure out that these arrows which are pointing to these peaks corresponds to 1 spin system and this spin system could be which amino acid.

Similarly, the remaining peaks can now we club together because they all belong to 1 spin system and those together based on the data base or any reference values, you can then figure out which molecule that could be because in a data base 1 would have stored all the chemical shift values of a given amino acid or a given molecule and that molecule will have a known

expected chemical shift, but now we have connected them by the HSQC TOCSY and that connection reveals that that has that is 1 spin system and now we can identify then that molecule could be either this Valine or Leucine okay.

So, we do not need a more than TOCSY and HSQC to identify of course the thing here you can think of it, suppose I have a completely new molecule then I will not be able to figure out just from this spectra where I would need additional data. But if I have a known molecule means some molecule exactly I do not know which molecule, but I know that it has to be one of the X and n number of molecules in my list, then which molecule is that can be figured out by simply identifying the spin system based on TOCSY.

So, this brings us to the end of the heteronuclear NMR part of the course we have seen at least 3-4 different experiments that is HSQC, HMBC, HMQC and HSQC TOCSY. These are the experiment which covers almost 90% of the NMR experiments, 90% of the problems which are looked down by or by chemist. Now we will moved to the next part of the course in the next class, where we start looking at how we can apply these in the experiment for structure determination of a molecule and we will look at specifically a peptide molecule.