One and Two dimensional NMR Spectroscopy: Concepts and Spectral Analysis

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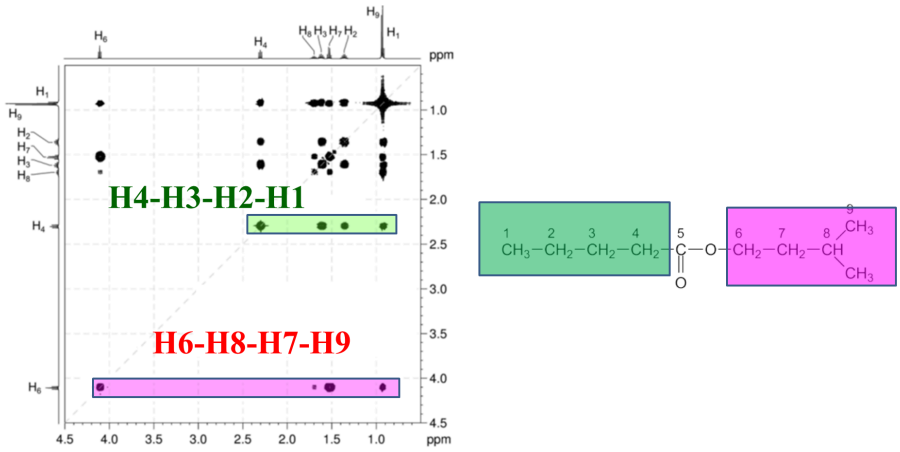
Lecture 43: HSQC-I

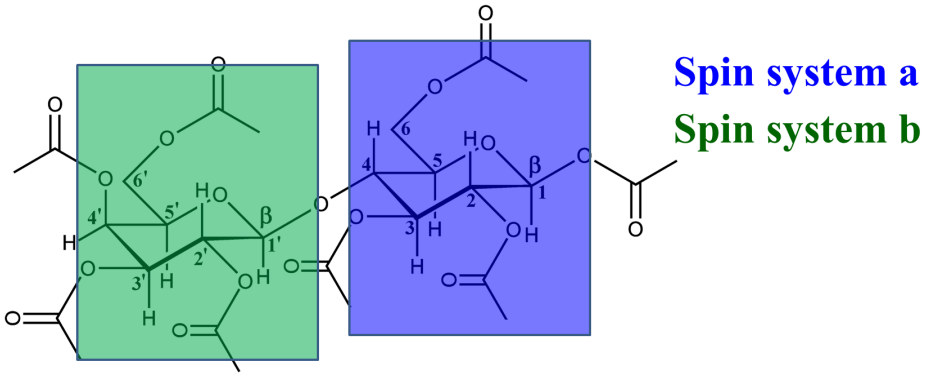
In the last class, we started discussing extensively about total correlation spectroscopy, called TOCSY. Again, it gives you correlation information among the coupled partners of a given spin system. This is an extended version of the COSY, where there is a relay transfer of the magnetization from one spin to another spin to another spin like that among the group of coupled spins. So long as there are couplings among them and forms a coupled spin system. I also told you how it works. It works because during the spin lock pulse which you are going to apply, what is going to happen is the chemical shift difference will becomes 0 and then there exists a J coupling because the spin lock pulse can be treated as if it is like a infinite number of spin echo sequences which are closely spaced. As a consequence, the chemical shift evolution is not there, Whereas J couplings do evolve. So, it creates a strong coupled spin system in which case spins can exchange energy among their coupled partners. And there is a cyclic exchange of energy, it will go from one to other, depending upon the mixing time you are going to give and also depending upon the coupling strengths, that is what I said. It keeps going among all the coupled partners and then if you extend for a long time, it may even come back.

There is always going on as a cyclic exchange. It does not go unidirectionally. It gives to next spin, at the same time it will be coming back. Go to the that spin, again it is coming back. So, the cyclic phenomena is going on. We understood how a TOCSY works. It is much better than a COSY. In one experiment, all the coupled partners can be identified whether you come down vertically or go horizontally from the diagonal peak. All the cross peaks identify the coupled partners. So, that is what we understood and we took one or two examples and compared that with the COSY spectrum, how it is advantageous and whereas in the COSY, we have to go in a stepwise manner. That is what we discussed. We will go further today with one or two more examples and discuss more about TOCSY, advantage of TOCSY. So, this is another example of a 500 MHz 1H NMR spectrum of isoamyl valerate in chloroform, and this is the spectrum. Of course, analysis of the spectrum is not difficult at all. If you carefully see which are the peaks, how do you analyze everything, we know it. But let us not get into the 1D analysis of this. We will straight away see how we can analyze this in a easy way by using TOCSY. We can assign the peaks in one shot. And of course, looking at the molecule, we can identify there are only two groups of coupled spins here. This CH3, CH2, CH2, CH2, 1, 2, 3, 4 form one coupled spin system, and 6 to 9 forms another coupled spin system.

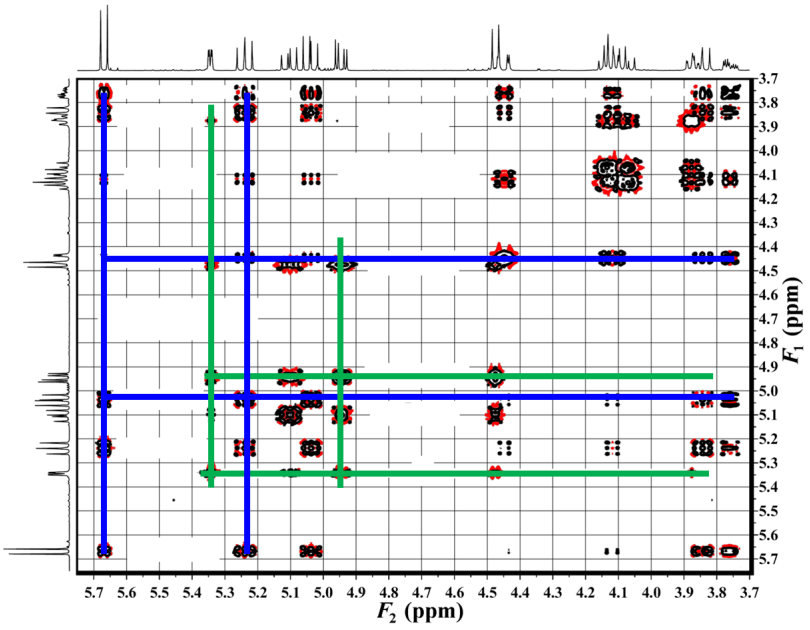
If you do a COSY, there is a breakage here because you can go from 1 to 2, 3 to 3 to 4 like that. And again, you have to identify another peak from the diagonal, start with 6, 7, 8, 10, you go systematically. But a simple TOCSY spectrum of this molecule is like this. You start with one of the peaks, any peak, horizontally or vertically, you can see from this diagonal, you come horizontally, you get four peaks. And I will say this peak, this peak and this peak, if you go vertically, this one, this one and this one, they are all coupled partners, that forms a spin system.



And you can get this, this is H1, 2, 3, 4. I started with one of them, this was H4 identified because it is attached to C=O. So, there is a confidence in the assignment. Using that peak on the diagonal, we went horizontally and identified all the peaks. Of course, you can go vertically also. As I told you, you can identify all the same four peaks. So, if you go vertically, you can go back here and identify the coupled partners very easily. So, this is one spin system. Go further, you can see there are four peaks here, start with this diagonal, come here 1, 2, 3, 4. So, you can identify all these four coupled partners. See, started with this proton, which is 6, which is attached to oxygen and then it comes here, here and here, everything can be easily identified. One of them of course, has a very weak intensity. As I told you, it depends upon the coupling strength and the mixing time. Another spin system is identified. So, easily TOCSY identified the two spin systems.



Now, we will start with analysis of the 2D TOCSY spectrum of β-D-Lactose Peracetate in CDCl3. This is a disaccharide. Let us see how we analyze this disaccharide spectrum. It has a glycosidic linkage also. But there are eight acetylesters here. Each of them consisting of 1, 2, 3, 4, 5, 6, etc, in the beta glycosidic linkage. You can see which are those acetyl esters, like these 4 and these 4. These are the eight acetyl esters, which are present. And each saccharide has seven chemically inequivalent protons here, here. I have marked 6, but remember H6 has 6 and 6 prime both. All others have single protons, but on the other hand 6 has two protons. So, there are seven chemically inequivalent protons here in each of the ring, each of the saccharide group. So, we have two coupled spins. Definitely, we should identify because we analyzed the glucose spectrum of the COSY and earlier few examples. We always start with anomeric proton and then we could identify all the coupled partners. Here also, we should do that. But we can also, instead of doing through COSY, we can also do through the TOCSY. This is one coupled spin system, this is another coupled spin system. We will do the TOCSY now.

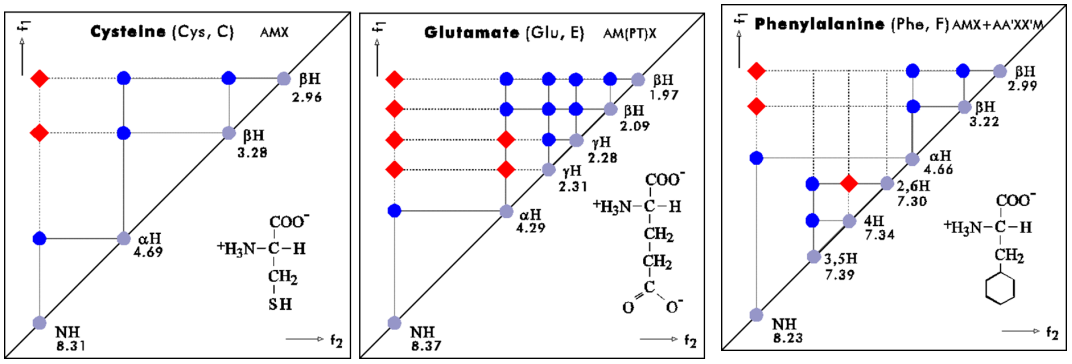


The TOCSY should exhibit correlation among all the coupled protons, of course, depending upon the mixing time because it is a big system. From one proton, for all the six protons if the magnetization has to go, we have to have mixing time quite large, because remember, mixing time will be of the order of milliseconds here, 30 milliseconds, 50 milliseconds up to sometimes 80 milliseconds we can go, 100 milliseconds like that. Mixing time becomes larger. But you cannot give enormously. If you give more than whatever is required, more 200, 300 like that, whatever the gain you have got, you will be losing because the magnetization again comes back to the starting

spin. This is what happens. This is the TOCSY spectrum of β-D-Lactose Peracetate with a mixing time of 70 milliseconds. Mixing time is of the order several milliseconds I told you. Now, can you identify two coupled spin systems. Very easily we can identify. Start with any one of the row, the horizontal row, this correspondence to spin system A. We can identify seven chemical inequivalent groups I told you, 1, 2, 3, 4, 5, 6, 7. So, this and there is an overlap here and there is an overlap here, this one and this one and this one. See, in a coupled spin system, there is a severe overlap. Just going horizontally, in one row, you will identify all the coupled spins. Of course, everything remains identical. You can go to any row because it is symmetric. You can go horizontally, vertically, column wise or row wise, you can still identify all the spin systems. Go to the other one, it identifies another spin system. But you may see all the seven peaks may not be seen there in the other one. You see, there are some issues. That is because beyond a certain level, magnetization transfer may be not become efficient because the coupling between one of them become very weak, and there is a stop there, it reaches a dead end. But then, do not stop at that. You can look for other rows. You can combine one or two rows if there is enough transfer of magnetization is not there among all the coupled spins, because of weak coupling or so in between. But you can use two or three rows and then get the required information. Like this you can do. This one column, other column. If that way identified all. See here in this row, only four are identified. Therefore, the remaining three, you can go like this. It is possible. So, we can go like that and identify everything. And some of the TOCSY transfer peaks are barely visible. A simple doublet, you start with this thing that correspond to anomeric proton and of course, with that, we can draw the line and analyze everything. Only second spin system has a bottleneck, I told you. We are seeing only four because it does not go to the entire spin system. It is because bottleneck is at the proton C4, in that there is less coupling. There is equatorial axial coupling which is smaller. As a consequence, beyond that, TOCSY does not transfer the magnetization to spin system. Very weak coupling. As a consequence, it may not happen. But you can find out from the other one also, other peaks. There are several rows. Some rows, you can use. We can identify all the spin system and then assign all the peaks. Of course, you do the same COSY. You look at the advantage of TOCSY. COSY, if you start like this and goes, you identify one spin system. Start with this one and I will go like this. From one, it will come to two. I can identify two. Two to three, identify three. Three to four and then four to five there. Four to five, identify and five to six here. Six and six prime, six for A, both are there. Six A and B. Whole spin system you can identify. Here there is no bottleneck. Even for the other one also, other spin system also, you can keep going like this. All six you can identify. The advantage here, you can do it in a better way, but only step by step. In the TOCSY, there are some issues where there is no complete transfer of magnetization. But of course, you can combine both of them and analyze this one. Where do you use this TOCSY? Very efficiently, if you want to use the TOCSY, think of a situation. Let us say, you are working on a biomolecule like a peptide or a

protein. What does this contain? What is the primary structure? They are consisting of amino acids in the primary structure, several sequence of amino acids and each amino acid forms spin system. For example, take glycine here. Starting from NH3, CH2, COOH, this forms one spin system. Go to alanine, starting from CH3, CH, NH3 forms one spin system and go for the leucine, (CH3)2CHCH2CH, and NH3. All of them form a coupled spin system. So, each amino acid forms a coupled spin system. So, there is a TOCSY transfer of magnetization among all the protons right from CH3 to NH group. So, what you will do? If there is a protein or a peptide, you are analyzing, sequence of amino acids are there. Simply draw number of vertical lines. Each of them will identify how many amino acids are there. And of course, further you can interpret the spectrum very easily. So, each amino acid forms a spin system and they are identifiable by one vertical row or horizontal row. That is what it is. Vertical line or horizontal line you draw and identify everything.

This is the glycine you see. Start with Calpha, come here and then NH. Similarly, for alanine, CH3 to Calpha and then NH. This is COSY, but TOCSY identify in one row. Same way here, in the case of leucine, start with CH3, then it comes here and then here you have to come like this for COSY, step by step manner. Instead of that, what we do here is, in one shot, CH3 to NH proton, all of them assigned in one shot. Otherwise, you go here, NH to here, here, here, like that. Several steps you have to do in COSY, but TOCSY you see, from CH3 to NH, all the coupled partners give rise to cross peaks. Any row you take, does not matter.



Similarly, an example of TOCSY of cysteine, glutamate and some cases, you can have two or three spin systems present. For example, in this molecule, if you consider tryptophan, this forms one spin system, this forms another spin system. So, there will be two sets of peaks like we saw in the previous example. So, you can have two different spin systems to identify, you can identify everything. So, you can see the advantage of the TOCSY. It is in biomolecules, especially when you want to analyze bigger molecules like

peptides, proteins, TOCSY is very, very useful to make the assignment. COSY is going to be very cumbersome there. You can use TOCSY like this. Look at this leucine, starting from NH3, completely everything has been taken and methionine starting from CH3 here up to NH, all the peaks can be identified.

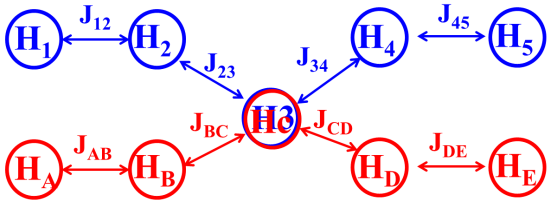
Now, I will tell you one advantage of TOCSY over COSY. What is an advantage? That is very interesting. Let us understand that. Where do you find it? Of course, already I showed the advantage. If you want to analyze a biomolecule like proteins or nucleic acids, nucleotides, there are several of them, you know. If you consider a big protein, there is a limit of course to get the size of the protein. Then doing a TOCSY helps you in identifying each amino acid by a single row or a single column, fine. Apart from that, there are certain advantages. One of the advantage I am going to tell you now.

Spin System 1

Spin System 2

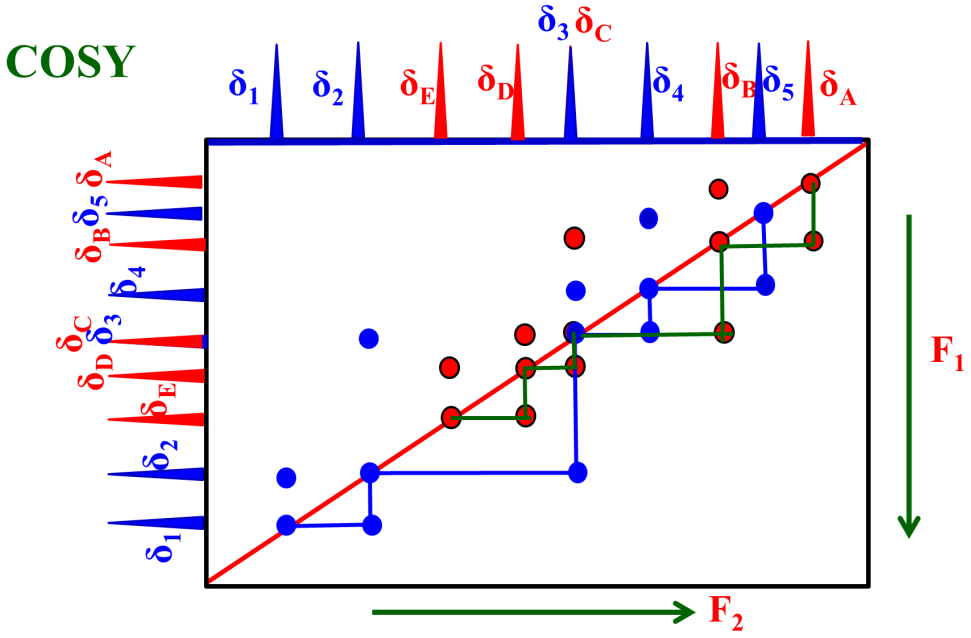
Consider a situation, we have one spin system like this. There are five protons coupled. This is one coupling 1, 2, 2, 3, 3, 4 and 4, 5. There are five different couplings for one spin system. Go to another spin system, same five protons. This I am going to call it as A B C D E. We have JAB, JBC, JCD, JDE. Two different spin systems and it is mixed. You are going to run a TOCSY. It so happens when you look at the spectrum, this chemical shift of 3 and chemical shift of 3 are overlapped, indistinguishable.

Spin System 1

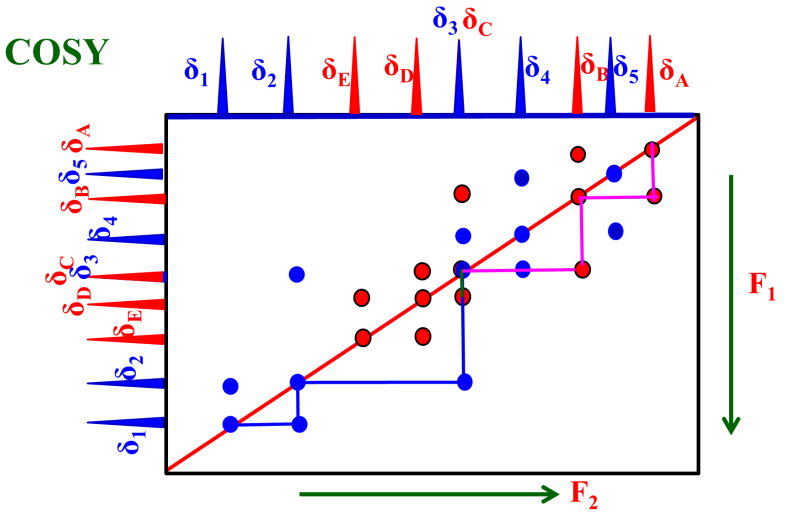


Spin System 2

Both of them come at the same chemical shift. What you do now? Is there a problem in this analysis? Of course, there is a problem in the analysis if you are not careful. Look at this one spin system. I can come here and then go. We will look at the COSY spectrum of this where you get into problems and this is the one spin system 1, 2, 3, 4, 5, this is the other one. Now, how do you make the assignment?



Start on the diagonal of one of them, 1 to 2 you go, then 2 to 3 you go to 3 to 4, 4 to 5, perfect. You can analyze. Other one, start with E to B, D, D to C, B, A. Both the spin systems are clearly analyzed, identified. There is no problem at all if my assignment is correct. In spite of the fact both are overlapped, both 3 and C are overlapped there. Same chemical shifts. Accidentally it was right, but I can also make a mistake like this.



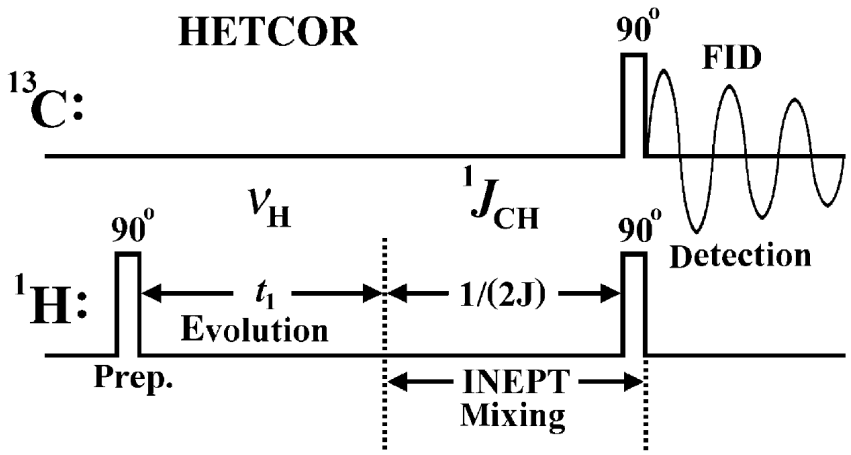
Look at this. I can start with 1, 1 to 2, 2 to 3, I will go. Here there is an overlap of 3 and C. From 2 different spins, at the same chemical shifts, both the peaks are giving peaks. Now, where do you go? This is also possible. Instead of going here to complete this, if I complete the analysis like this, you are right. Accidentally I showed you it is possible in the previous slide, but there is also possibility you can go like this. You have crossed over from one spin system to another spin system, 2 different molecules. You can make a mistake. Or you can also do like this. It is quite likely you can start with this. Now, instead of going to red, I can go through blue. What happened? Here again I crossed over. When there is an overlap, when I am analyzing the COSY spectrum, there is every possibility that I can make a mistake if there is overlap. If they are completely overlapped and it is difficult for you to distinguish, we can make a mistake like this. We can cross over to the different spin system while analyzing. Alternately, what we can do is, I run a TOCSY.

What does TOCSY give? TOCSY gives cross peaks among all the, coupled partners. For example, you take the blue spin. Start from here, go like this. All the peaks you can identify, come like this. All the peaks you can identify. Similarly, for the C, this one you can identify all the 5 peaks. Even though there is an overlap here, you can distinguish them, which is not possible in the COSY. There is possibility one can make a mistake and cross over to a different spin system. You see the advantage of it. So, TOCSY has a better advantage at times when there is a severe overlap of the peaks. Simply TOCSY, you can do and then identify all the spin systems like this. So, this is what I just wanted to tell you about TOCSY. I showed you what is a TOCSY, where do we use and advantages of TOCSY over COSY, \etc. These are homonuclear correlation experiments. With this, we

will now switch over to a different topic, that is, heteronuclear correlation experiment. I will start it right now.

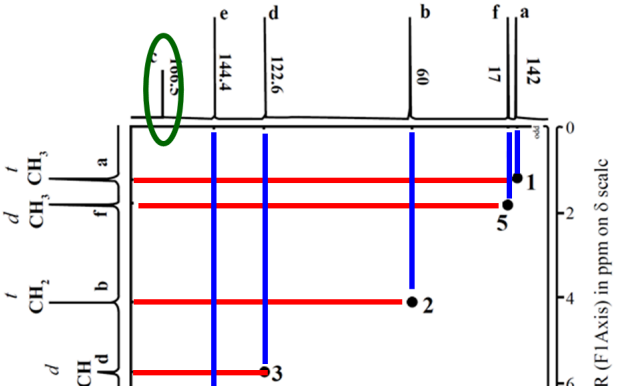
We will start with the heteronuclear correlation experiment. So far, we discussed about homonuclear. Now, we will switch out entirely a different experiment, where we are not correlating the same spins, we are correlating unlike spins, different spins, not like spins. We will start with this one. What it does? Earlier case, it was correlation from proton to proton, whether it is COSY or TOCSY, does not matter. But here, correlation is from proton to carbon or nitrogen or any other nuclei apart from the same spin. It will always have different heteronuclear correlation. What is the requirement for this? Again, there must be a J coupling. Using J coupling only, we can do the correlation. For all these correlation experiments, whether it is homonuclear or heteronuclear, J coupling is essential. In the homonuclear case, COSY and TOCSY, coupling among protons, among homonuclear nuclear spins are needed. But here, coupling among heteronuclear spins are needed. That is what it is. And you can do the chemical shift connectivity between carbon 13 and proton, nitrogen 15 and proton, fluorine and proton, etc. Any heteronuclear can be correlated. Usually, one of them is abundant spin, other is a rare spin. You may ask me what about the correlation among dilute spins. That is a different thing. I will come to that later. So, heteronuclear correlation experiment if I do, few important points you must remember. They are not symmetrical unlike in COSY and TOCSY. Why? Because in COSY and TOCSY both the dimensions, the chemical shift range is identical. If I am doing protons, homonuclear COSY of protons, it is 10 ppm chemical shift range along F1 and 10 and in the F2, identical. It is symmetrical and there will be a diagonal. So, with respect to diagonal, they are all symmetric. No doubt about it. That is true. But here, they are not symmetrical because heteronuclear chemical shift ranges are different. Proton may go from 0 to 10 ppm, carbon 13 may go to 0 to 300 ppm. It is not square at all. So, question of symmetry does not come at all. Chemical shift ranges are entirely different. So, heteronuclear correlation spectrum, first thing is not symmetrical and there are no diagonal peaks here. Because in the homonuclear case, in COSY, both the dimension you are detecting the same spin. For example, the proton is having a frequency, some frequency in t1 period. You are detecting the same thing. Suppose, if it remains unchanged, same frequency is retained in the t2 dimension also, we are going to get a diagonal peak because we are detecting both protons in both the dimensions. But in the second dimension here, I am not detecting proton. So, the question of diagonal does not come at all. There are no diagonal peaks in the heteronuclear correlation. And normally, what we do is, the indirect dimension is less abundant spin. What do you mean by that? In the case of a 2D, the direct dimension is F2, this is F1, indirect dimension. In yhr Indirect dimension, we always take less abundant spin like carbon 13, nitrogen 15, etc. Abundant spin is here, proton, fluorine, etc. That is a convention. Of course, you can do

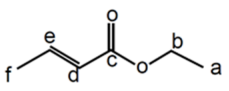
the reverse also. That is also possible. Nowadays, so many experiments are possible. We can even detect in indirect dimension abundant spin and direct dimension less abundant spin. Both are possible, inverse experiments are also possible. But this was the convention for HETCOR, the indirect dimension less abundant, direct dimension, abundant spin. What is the simplest HETCOR pulse sequence? It is like this.



Heteronuclear pulse sequence is very simple. On the carbon 13 channel, you have a detecting pulse, 90 pulse. The proton channel, you apply 90-degree pulse and allow it to evolve for a time t1, that is a t1 period, evolution period. Then after that, you give some delay where you apply INEPT mixing to do the polarization transfer. And then two 90-degree pulses there. What do 90-degree pulses will do? I already explained to you. It will ensure that the anti-phase magnetization gets transferred from one spin to other spin, from proton to carbon, it can be transferred. So, this is the very simple pulse sequence, two 90-degree pulses on the proton channel, t1 period evolution. But after the evolution, there is INEPT mixing for polarization transfer. How this pulse sequence works without going into the detail, I will explain to you.

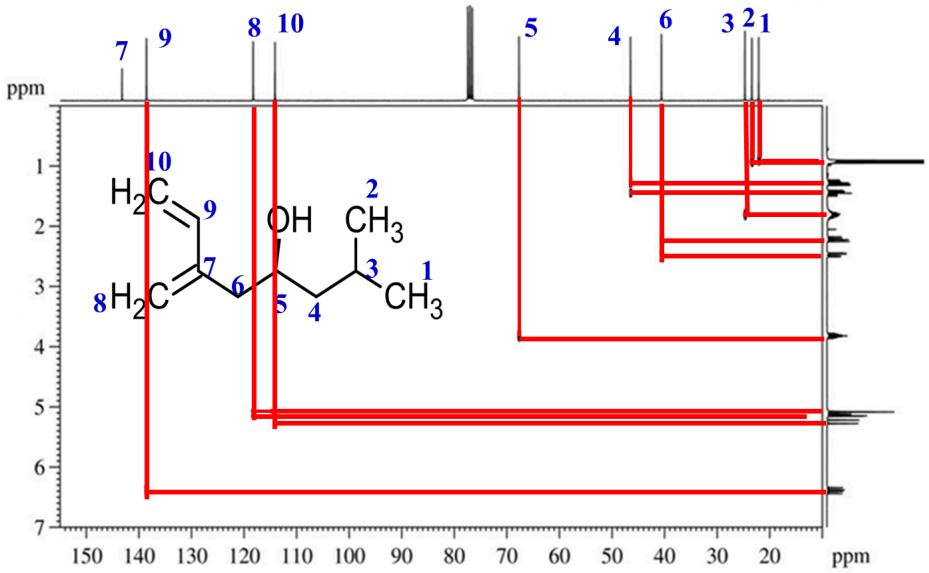
What does first 90-degree pulse do? It will bring the magnetization to xy plane. That is what it does. And here, magnetization starts evolving because I have applied 90-degree pulse. What does evolve? Carbon 13 chemical shift will not come here because I am applying pulse only on protons. So, proton





chemical shifts and proton couplings start evolving here. All right. And after some time, what I am going to do is, after the t1 period, I am going to do the INEPT here. What does INEPT does? It converts proton magnetization into anti-phase with respect to carbon 13 for polarization transfer. We have understood what is INEPT. We discussed extensively for two or three classes. So, INEPT creates anti-phase magnetization of the proton with respect to carbon 13. Now, simultaneous application of two 90-degree pulses, what will it do? It will transfer the proton magnetization to carbon 13. So, that means anti-phase magnetization gets jumped. It jumps from proton to carbon and then start detecting the carbon 13 signal. That is what basically you do. Carbon 13 signal is detected, bring the magnetization to x-y plane, allow it evolve, do an INEPT for magnetization transfer, create anti-phase magnetization of proton, apply two simultaneous 90-degree pulses, transfer to carbon 13 and start detecting the signal. This is the basic simple experiment. So, HETCOR if you want to understand in brief, it gives information about which carbon is attached to which proton. It is done by inverting the proton population and varying the transfer of proton magnetization during t1. t1, when I vary this one, I will first bring down the magnetization of the proton, and keep on varying the t1 period. That is how the two-dimensional experiment is going to be done and vary the transfer of polarization to carbon to 13 as a function of t1 and this depends upon JCH we use. You can have HETCOR experiment both decoupled version and coupled version. What is mean by decoupled version? I can see the carbon 13 with proton decoupling. I use decoupling power on the proton channel, I can decouple proton. Or I can see proton with carbon 13 decoupling, that is also possible, both are possible or I do not do decoupling at all, in both the dimensions and in both the dimensions, I am going to get the coupled spectrum. So, this what happens and this is a HETCOR spectrum decoupled in both the dimensions. When you decouple in both the dimensions, interpretation of the HETCOR spectrum is fairly simple. You do not need to break your head at all, very simple we have to analyze. First of all, these are the proton peaks here. This dimension is proton and in this dimension it is carbon 13. In the proton somehow we have assigned this to be CH3, proton assignment has been done either by one dimensional way or by COSY, whatever the experiment you have done. This is another CH3 and then we have a CH2, CH2, all the three are there, everything has been easily identified, this is CH3, CH3, CH2, there are two CHs, one CH3 and two CH3 and one CH2, this is what we have.

When I make the assignment, I know the proton chemical shift. If I get a peak in the HETCOR, heteronuclear correlation, sit on that peak, go horizontally, you hit the proton, in the proton dimension I get proton chemical shift. Go vertically up, you get carbon 13 chemical shift, very easy. So, it correlates proton and carbon in this experiment, it is a proton carbon HHQC, heteronuclear correlation, I can correlate the chemical shift of two coupled nuclei, one bond, directly attached, which carbon is attached to which proton I can find out. So, this is what I told you. Remember, I was telling you when I was



discussing the introduction to 2D, I said the simultaneous detection of two nuclei is not possible in 1D, that was a limitation, but now it is possible, look at it, I get a peak, simultaneously I can get proton and carbon chemical shifts. If I do the coupled experiment, I can get heteronuclear coupling and also homonuclear coupling both, depending upon the type of experiment I do. So, in one experiment, both the nuclei can be detected, that was the limitation of 1D. So, I showed you the advantage of a 2D, especially in HETCOR, I can correlate. For example, come to this peak, now go horizontally, I will identify this proton CH3 peak, go vertically, I get the carbon 13 peak. Similarly, for this, this, very easily you have to draw horizontal line and vertical lines, horizontally you come to proton axis, you get proton chemical shift and this peak tells me CH chemical shift of proton and also carbon 13 chemical shift of the same CH group.

This CH has carbon and proton, its carbon chemical shift is this, and its proton chemical shift is this. For this proton, this is the proton chemical shift and this is the carbon chemical shift of the CH3 group, same way this is for CH2 group. So, very easily you can identify, do not get confused, take the CH2 group, in the CH2 group, I go vertically, I get carbon chemical shift, come horizontal, I get proton chemical shift. Here this is chemical carbon 13 axis, this is proton axis. It can be reversed depending upon the type of experiment you have done, you can identify chemical shifts in either dimension, go vertically and horizontally, you can get the correlation information, heteronuclear correlation.

Advantage also I can tell you, take an example of molecule IPSENOL, you can identify proton 1, CH3, this is the proton, the carbon dimension, this is proton chemical shift, that is the proton chemical shift and vertically if you go, you get carbon chemical shift. Similarly, for 2, they are overlapped, you can find out, you see this one, for the proton 3, we get carbon chemical shift and proton chemical shift. Look at this one, the proton 6, this is an advantage, this has two different chemically inequivalent protons, like we saw in the glucose, 6 and 6 prime, they have different chemical shifts, but attached to the same carbon. Similarly, here these are two chemically inequivalent protons attached to same carbon. Now, if I go vertically up here, I get the carbon chemical shifts and then go horizontally, I get two different chemical shifts, one chemical shift of the one proton here, other chemical shift here for other proton. So, this is an advantage. Here one cross peak of the carbon correlates to two proton chemical shifts like this. So, you can find out this thing, you look at this one also, this carbon 10 again has two protons, both of them very easily you can identify. So, all you have to do, HETCOR interpretation is very simple, go horizontally or vertically, you get proton chemical shifts and carbon chemical shifts. If two different chemically equivalent protons are attached to the same carbon, you will get one chemical shift in the carbon 13 dimension, and for the same carbon, you have two different proton chemical shifts. So, this is an advantage. So, very easily you can do the experiment and do the interpretation like this.

So, now since the time is getting up. What I am going to do is to extend this for the inverse spectroscopy. The heteronuclear correlation can be done in a different way. I am going to discuss that one in the next class, but what we discussed today is we discussed more about the TOCSY, few more examples. I showed you what is the advantage of TOCSY over COSY. TOCSY easily identifies all the coupled spins in a spin system in

one single experiment, either you go vertically, draw a vertical and a horizontal line from the diagonal, all the cross peaks identify its coupled partners. Similarly, if you go horizontally also, it identifies all its coupled partners. And I showed the advantage when there is an overlap, how COSY can be confusing and it can mislead the interpretation, whereas TOCSY can easily overcome, circumvent that problem. Then we switched over to heteronuclear correlation. I showed you how heteronuclear correlation works. It is simple correlation let us say proton to carbon, nitrogen, etcetera to any other heteronucleus. So, this is possible. And this is the pulse sequence. Also the heteronuclear correlation is very simple. In the case of proton, we allow it to evolve and do the INEPT polarization transfer, create an anti-phase coherence for proton, then simultaneously apply two 90 degree pulses on proton and carbon, then the coherence jumps from proton to carbon and then detect the carbon. It is a very simple experiment. That is what we discussed. And then I showed the example of how we can interpret the carbon-13 HETCOR experiment. First of all, heteronuclear is not symmetrical, and there are no diagonal peaks. And usually in one dimension, indirect dimension will have a less abundant spin, direct dimension is the abundant spin. Other way is also possible. And we took the example of one or two molecules and very easily we knew how to interpret the spectrum. So, with this, I will stop here and we will continue with the inverse experiments in the next class. Thank you.