Principles and Applications of NMR Spectroscopy
Professor Hanudatta S. Atreya
NMR Research Centre
Indian Institute of Science Bangalore
Module 06
Lecture No 30

So, in the last class we looked at the basics of 2 D NMR heteronuclear NMR spectroscopy and we started with the HSQC experiment. HSQC stands for Heteronuclear Single Quantum Coherence, so we will continue with that today and see how this experiment works, what kind of the pattern of peaks we can expect to see in this 2 dimensional experiment. So, the shown here is the 2 D pulse sequence which we started with yesterday.

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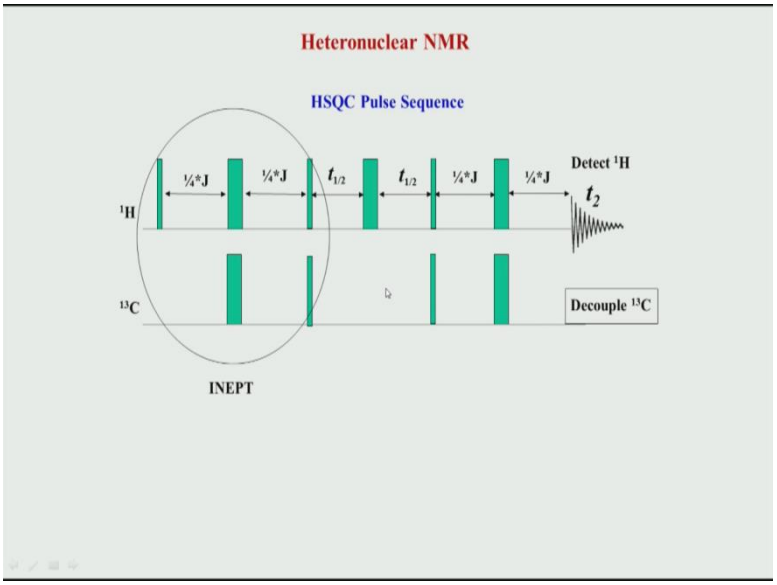

So, here you can see this is a sequence of pulses which are applied on the proton channel okay. So, we saw that yesterday that we called we defined two separate channels one is called proton and other is called carbon and on the proton side we apply series of pulses and delays, so there is a very specific value of the delays which we will see more in detail today and similarly on the carbon channel we apply pulses. So, here this pulse which is narrow which is narrow rectangle is a 90 degree pulse so everywhere here you say it is 90 degree pulse whereas, this fat rectangle is called as a 180 degree pulse so, these 90 and 180 you remember is the flip angle. The angle by which the magnetization rotates from Z axis to Y and this is from Z to - Z or yesterday we defined 2 different, in the last class we looked at 2 different types pulses, one is called Inversion pulse and a Refocusing.

So, inversion is basically taking a magnetization from Z to -Z, whereas refocusing pulse takes some magnetization from X Y; X to -X or Y to -Y, so this is a definition of a refocusing pulse. So, basically a 2 dimensional experiment HSQC consist of a series of or a number of pulses applied like this with a specific delay or difference a delay value a delay period in between, so the first part of the sequence which is this portion we called it has INEPT. So, INEPT is something we saw in the last class is basically method to enhance the polarization of a insensitive nuclei which is carbon here by transferring it from proton. So, this is achieved using this particular series of pulses this particular block and we use in the other word INEPT block.

So, after this pulse is applied after this INEPT is applied, here at this point after the this pulse on carbon is applied this here this point the carbon comes to the X-Y plane. Once carbon comes to the X Y plane then during this whole period from here to here we allow the carbon chemical shift to evolve, evolve meaning we allow the FID of carbon to start. But remember this is a indirect dimension, so we are not going to physically captured the signal this is not a detection, it is indirect detection.

So, similar to all the 2 D is we have seen so far the idea is you allow the chemical shift to go on. And after this period at this point, now what we do is we go back to the hydrogen proton because we have come from proton we come to carbon during this part and from here we go back to proton and then detect the proton magnetization, okay. So this is what the idea is that we saw in the last classes that the idea is to start from hydrogen excite an hydrogen nucleus and then transfer the polarization to carbon and then reverse come back to hydrogen transfer and detect the hydrogen.

So, this is the most optimal way to achieve the best sensitivity, so you start from a hydrogen come back to hydrogen, so this is how all NMR experiments many of them are typically designed, so that you get maximum sensitivity and therefore you do not even if the natural abundance of carbon is low at least you have taken advantage of the high Gamma value of proton. And now let us look at the each of these details or let us see how the magnetization mathematically goes through this pulse sequence, so this is how is shown line here.

So, you start so we are looking at a carbon proton pair okay, so we are looking at a system like this where one hydrogen is attached to directly through one bond, so this is one bond through to carbon. So, our aim in this whole exercise in this whole experiment is to correlate

the chemical shift of this proton with the chemical shift of the directly attached carbon. So, that is our aim in all these experiments, so we are correlating hydrogen and carbon.

So, we start from a proton pulse that is this pulse here and we are applied a 90 degree pulse the first pulse on proton, then the proton comes to X-Y plane, but we do not we do some INEPT experiment here, INEPT block here because of which the carbon polarization has now gone to carbon. So, we are not going to go in details of a mathematical analysis of how this INEPT works that will be a not covered here but will have to take it for granted that a proton polarization is transferred to carbon at the end of this period.

Now during this period which is now labeled as T 1, so you see here we have a T 1 by 2 that is half of T 1 and another half here and between them is one 180 degree port pulse on the hydrogen. And why is this here, the reason for this is at during this period, when the carbon is free to evolve because of its chemical shift during this period it will be coupled to protons because by J coupling because that is what is here, so we are exploiting the J coupling to in the first place to get that transfer by INEPT.

So remember, INEPT works by using J coupling between two atoms. So, here during this period, when it is evolving it will be coupled to the hydrogen, so to avoid that, that is to decouple protons from carbon, when carbon is evolving we apply a 180 degree pulse on the proton in the center of the evolution time. So, you see here this is symmetrical that is half T 1 this side, half T 1 this side, so right in the center is applied this pulse, so during this period the carbon chemical shift has a evolution okay.

This is again similar to many 2 D experiments we have seen earlier where in the indirect dimension that is during the T 1 dimension we have a chemical shift, then you transfer back to proton during this period that is reverse INEPT. So, it is exactly a reverse or what you get here, so that is why if you look at the picture they are very symmetrical, so, if I for example draw a line here in the center if I draw a line it is exactly symmetrical.

So, it is a reverse of the INEPT here. So, when I up to the end of these reverse process, now the proton magnetization is on the X Y plane and during that period it starts evolving. So, as we can see here, there is a chemical shift of hydrogen which is captured during the T 2 period, T2 evolution. So, now if you look at the whole picture in total what we have achieved is that we have achieved a transfer of magnetization from hydrogen to carbon got the chemical shift of carbon went back to proton and got the chemical shift of proton.

So, what we have done we have correlated or connected two atoms here one is hydrogen another is carbon. So, therefore when I do a Fourier transform of this combined signal which is now T 1 and T 2, I would expect a 2 D spectrum, where the carbon and proton are correlated. So, now this is just a little bit of detail a technical detail which you can see is that what is the value of this delay period, so this is delay period as it is shown here it is 1 over 4 times into J, so that means this is 1 over 4 times J and this is also 1 over 4 times J. So, the total period from this pulse to this pulse is going to be one over 2 times J, double of each period here. So, typically what is done is this period one over 4 times J is chosen J depends. Now J is shown here this is an approximate value this is for a hydrogen proton pair direct connection from S P 3 carbon going all the way to S P carbon.

So, we saw that in the previous classes that the coupling constant is weak for CH 1 bond that is CH that is SP3 and as we go to SP2 and that is in aromatic and SP in Acetylene for example, the the coupling constant is very high. So, this is a large variation you can see here, so what one does this based on what type of you came functional group you are trying to look at then you decide the delay value. So, for example you are only interested in aliphatic within only SP3 carbons, then your value can be somewhere around 140. So that value you choose a delay accordingly so 1 over 4 into 140 into 2. So, if you want to look at a SP aromatic systems in aromatic you then tuned your J we it as you have to coupling will not be 140, it will be around 160 to 180, so if you choose that value accordingly J value this delay value will change.

So typically the delay which we used is about this much time 2.5 to 3.5 millisecond coming from this pulse this pulse what it is here to this side here. So, we are looking at the entire delay period between these two pulses. So, this is why is it important to know this, this is from a practical aspect because you want to the maximum sensitivity is obtained, when you achieve a 100% transfer. So, now coming back to the concept of transfer efficiency of transfer if you recollect we saw in the hydrogen proton spectrum you will never get 100% transfer from one proton to another proton in any experiment typically when if you use COSY, NOSEY, TOCSY and so on. Efficiency is not 100% typically it is in fact, in NOSEY it is just about few% 5 to 10% not even 10.

So, you see the + efficiency of transfer is low even if comes to proton-proton transfer. But when it comes to proton carbon transfer, the efficiency is high the reason being that we use this experiment a block known as INEPT and that achieve that has heteronuclear transfer is

100% can be 100% of course again not ideally, ideally 100 % but not practical but you can reach a level of 90% efficiency provided you take the correct values of these delays. If you choose incorrect values, then efficiency goes down tremendously. So that is why 1 has to know what type of systems we are looking at therefore one should know a have an rough idea of what are the coupling values you expect and accordingly you have to decide the time delay between the pulses. So this idea and HSQC whatever we discuss right now is applicable to many 2D experiments in hetero nuclear NMR in general.

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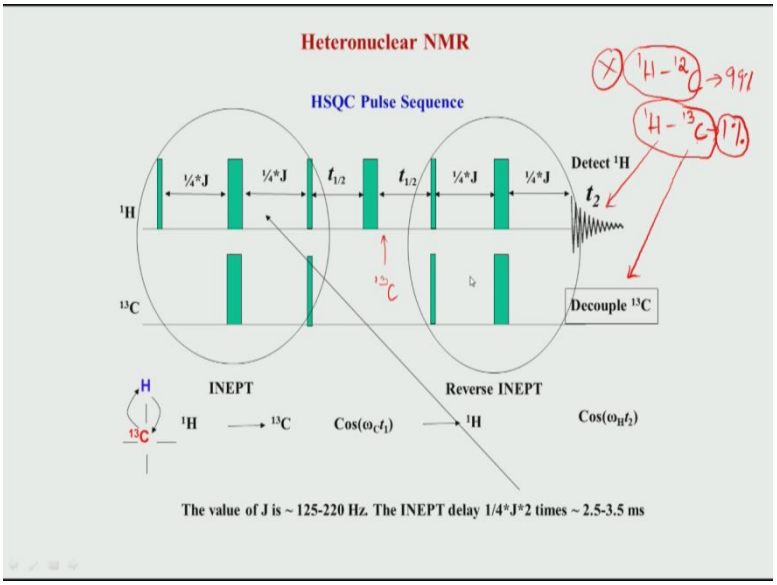

So, that is why you spend more time on this experiment to understand what the integrities of these experiments are. So, one thing which we discuss yesterday again was decoupling is that, when you are acquiring or detecting protons during this period you have to decouple it from the carbon because carbon is what is couple to proton by J coupling. Now one as to keep it mind the following so let me show you one thing which is very important to know here is that we are now get. So, we have in a molecule we have this combination and this combination okay. So, basically this is rough above 99% because a natural abundance of C12 is 99% and this is 1% okay. So, this is so our whole experiment is based on detecting this much this moiety because remember carbon 12 is NMR inactive.

So, we are only this is not possible to detect because there is no way to get C12, it is not active NMR, NMR active nucleus. So, the only thing we are looking at this carbon to 13 C, but in this experiment therefore what we are looking at is only signals from 1% of the sample. So, during this period during this period you see we have to decouple C13 because 1H is getting detected, so whenever you have detecting 1H you have to decouple carbon 13 because

this anyway is gone we are not looking at this. Similarly, when you are evolving carbon here in this period, we should decouple from proton to that is what this pulse is. So therefore in both T 1 and T 2 we apply decoupling okay. But again going to little deeper, this decoupling is slightly different from this type of decoupling, this is just one pulse okay.

So this is just one 180 degree pulse, which is achieving the decoupling, but here it is a series of 180 a not simple 180 was as it is not just one pulse. So, this is that is why we show it in a box like this okay. So, this is called Composite Pulse Decoupling CPD is used for the word, when you apply decoupling like this and this is simple 180 degree decoupling pulse. So, this experiment captures many of the details of a typical heteronuclear NMR in fact, although we are not going to do this in this course, you can also use this ideas to designed a 3 dimensional experiment and N dimensional experiment because this blocks what we have seen are called building blocks.

So we can actually build when a n number of blocks you can put and choose at depending on what experiment you have doing you can design new experiments. We will see one such 2 D experiment we are not going to go into 3 D in this entire course but we will look at in the 3 D experiment, where we will combine the idea of this with a 2 D homonuclear experiment.

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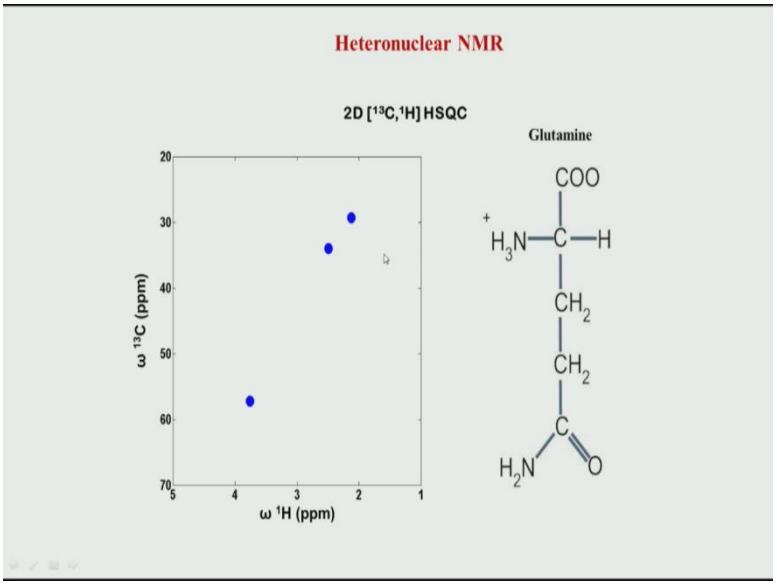

So, let us continue now to see this spectrum will look like now that we have understood how the experiment works. So, this is the HSQC spectrum of this molecule Glutamine, it is an Amino acid and it has a structure which looks like this. So, there are how many pairs of hydrogen carbon, so you have to keep in mind that we are only detecting pairs, we are not

detecting any other come system we are only looking at CH pairs. So, there is one CH here, there is one CH here and there is one CH here, so we will assume that these two are equivalent and we will assume these two are equivalent, but you have to keep in mind that it is not so the reason is this is a chiral carbon. This carbon which is shown here is a chiral carbon, so whenever you have a chiral carbon in your molecule it is asymmetric means you have a R combination or you can have a S combination S isomer and R enantiomers.

So, we are looking at L Amino acid in general, so this is typically an L Amino acid, which is there in our chemistry in biology we use. So, therefore this is only this is only one enantiomers it is asymmetric, so moment you have an asymmetry in your molecule, it need not be only it is geometrical symmetry, but even this symmetry mix all the hydrogens equivalent. Now remember we saw the chemical equivalence concept, the idea was that there is a asymmetry in the molecule, then there is chance of equivalence chemical equivalence. But there is no symmetry here, it is a very asymmetric system because of this chiral center here therefore, these two hydrogens are actually not equivalent chemically and these are also chemically not equivalent.

What it means is that these two hydrogens will have two different chemical shifts values, but they may be very close to each other in fact, they may be even overlapping means they may have the same value, but having chemical shift same is not that they are chemically equivalent okay. They are same because they are accidently closed in environment chemical environment and therefore they show up to same. So, therefore in this whole analysis and HSQC we will assume that that two chemical shifts are same but they are not necessarily chemical equivalent. So, therefore you see we have only see 1 peak you do not see 2 peaks for each hydrogen. So, now let us understand how this spectrum can be interpreted, so on this axis Y axis is your carbon chemical shift as you can see the values and on the axis is the chemical shift of hydrogen.

So, we are only looking at CH system. So, we are only have it 3 such 3H system and therefore there are 3 peaks here. Now let us see how to get look at the positions, so now if you look at this pair this is attached to 2 electro negatives functional group one is carboxylate and other is NH3. So, therefore this comes down field shifted for both croton and carbon and therefore the carbon value is closed to 60 ppm and proton value is down field shifted to 3 to 4 ppm.

So, if you look at the individual 1D and carbon if you recollect our 1D proton and carbon NMR part. In this course, you saw that if there is a carbon attached to electro negative group or hydrogen attached to carbon, which is attached to electro negative they come down field shifted because of the shielding deshielding effect. Now let us move to the second one this is CH2 and this is now coming in the center the reason being we will look at it this is Aliphatic spin system, so there is shielded like we saw the inductive effect, so it is up field shielded.

But if you look at, compared to this hydrogen carbon pair here it is now Amide, so it is again an electro negative we electron withdrawing functional group. Because of this, these pair comes now down field shifted compare to this, down field means it is shifted down both in carbon and both in proton. So you can see that this is why this whole this CH pair comes down field shifted and it is around 2.5.

So, this is how the spectrum of a simple molecule like this we looked like in HSQC. So, here the interpretation is not based on the J coupling and so on like we did in 1 D, it is purely based on the knowledge of where the chemical shifts are expected to come and thus how we can look at it. So this HSQC is only telling you the peak positions okay, so it is not giving you the information of proton-proton connectivity which is can be obtained only from COSY okay.

So this for, you see this experiment it gives you some information, but to get the connection that this is this peak this pair corresponds to this peak and this pair corresponds to this peak that kind of information we have to obtain some more experiments of course, from experience just now as we analyze we can do that also, but more detail molecule a structure one has to not only delay on HSQC one has to also combine this with other experiments and we will see that, when we come to the assignment part or the structure determination part of this course.

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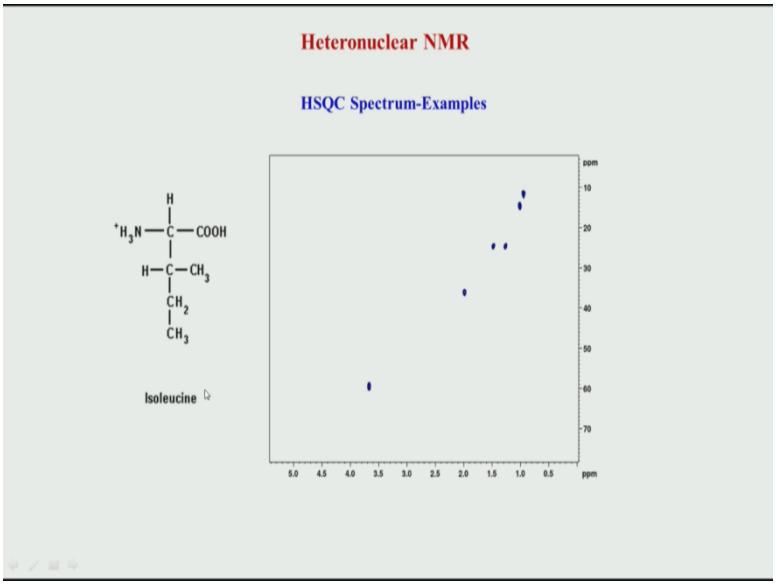

So let us move on to another example, so that we can go through all different combinations and you can see here that in this case. Now this is again an Amino acid this is Isoleucine, so it has branch chain Amino acid okay, so here it has two chiral centers, this is also chiral this carbon and this carbon is also chiral. So, because of this it has this is a chiral and both are L configuration. So, now let us start from looking at which peak can correspond to which of this CH. So how many CH pairs do we have? We have one here that is CH3 is H3 remember H3 always are equivalent, so we have CH here, these 2 are not equivalent, but for the time being will considered them as same chemical shift. So we have one CH here that is one here, one here is the second, this is a third system third CH pair, another fourth and a fifth CH pair, okay.

So, this is actually the experimental spectrum recorded for this molecule. So, one is one can say that the most up field shift which is closed to 10 ppm and around 1 ppm has to be Methyl's according to our earlier analysis where we saw that Methyl's are always up field shifted. There are 2 Methyls in this molecule, so the second one is this Methyl. Now which is which methyl how did we get that answer; again this is more or less based on kind of experience and data base means we will have a known values for these Amino acids and one can based on that one can say that the most up field is usually this methyl and the next up field is this. One can always argue based on shielding de-shielding effects, but this is not how it is done, typically we have to assign this each and every peak to the respective pair based on more experiment. So, right now I am only showing you the results means given in

spectrum which how the peaks look, but how did we arrive at which peak is which that is going to come a later where we look at more experiments.

Now this is a CH2 this comes here you can see there are two peaks here one peak is from one hydrogen and another peak is from another hydrogen. So, again we do not know which is which these are called pro-chiral protons. So, this is like stereotypic protons and you can see there are 2 protons here, so 2 peaks which one is which we do not know. So, we usually have to arbitrarily says that one of the peak is the one proton other peak is another proton, but the fact the important point to note is that they are not equivalent. Many times you may thing that because there is a CH2 here it becomes equivalent or that is not the case that is non-equivalent that is why there are 2 peaks, now coming to this peak here. Now this peak we can go to this peak, so that 2 pulse 2 peaks left, one is this CH here and another is this CH.

Now, if you look at this CH similar to what we saw in the previous example in Glutamine this comes down field shifted because it is attached to two electro negative groups. So, therefore this down field this one down here will be most likely from and going to be form this, so therefore this one it has to be this peak okay, that is the beta carbon of isoleucine and the alpha carbon this is an alpha Amino acid, so, this carbon is denoted as alpha, it has a peak which is coming here amd this position is a down field shift. So, this is how we can interpret or analyze the HSQC spectrum of molecules. Let us look at some one more examples before we move on to HMQC.

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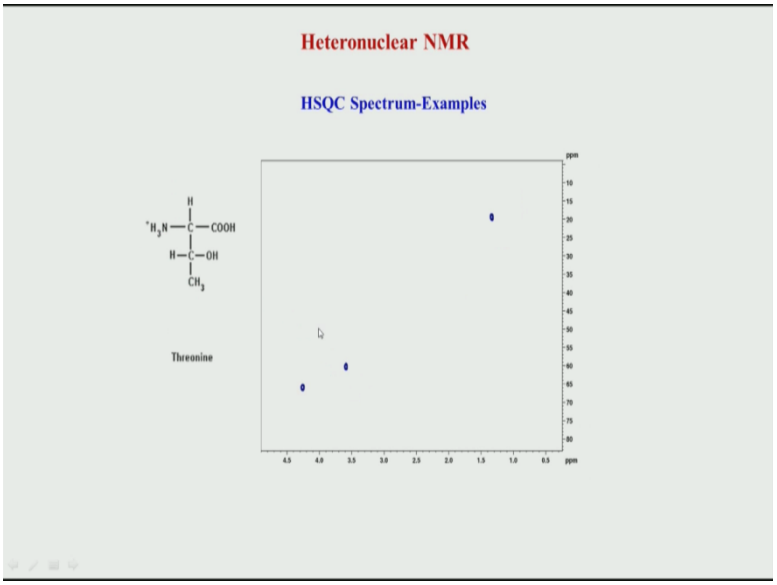

So, this is a threonine atom amino acid called threonine and here or you can see that there are only 3 types of carbon proton pairs; one is this pair, one is this pair and one is this pair, so again this is a alpha amino acid. So, I like we saw in the previous two examples this will comes somewhere around 60ppm which we saw earlier, so here we will also I can see a peak at 60 and that could be this peak.

Now methyl's again remember come up field shifted and therefore it here it will be somewhere around 1.5 to the 1.8 or we save on 1.5 ppm okay. And this carbon this is here the Beta, so this is Alpha, Beta, Gamma, so the Beta carbon is directly attached to an Oxygen. So, therefore we expect a down field shift because of that and therefore this Beta carbon comes way down here, where I am pointing the arrow that around 70ppm, 65 to 70 and also the alpha is down field shifted sorry the proton is down field shifted and alpha is here and beta is here. So, let us assign now which is which, so methyl is this peak up field shifted this CH OH is the down field shifted and this the middle one is the CH alpha amino acid peak.

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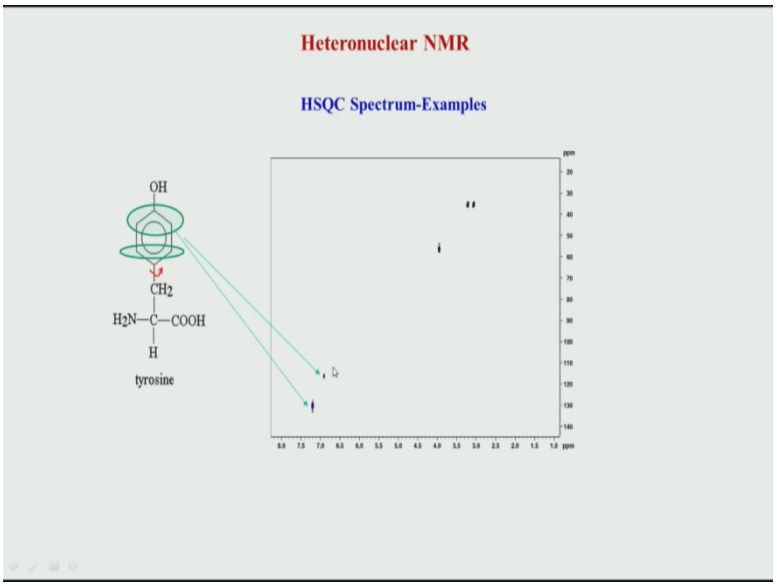

So, let us look at some more example this is on aromatic Amino acid, so in the aromatic Amino acid now we have so we are looking at different types of amino acids and because there different functional groups are there, it will help us to look at where the peaks come in HSQC. So, if you look at this tyrosine molecule then you have a CH pair here, there is a CH pair here CH2 and there is many CH here. So, one is there is a CH here this side and this side that is CH here and here, and there is a there is no CH here. So, now if you see you may again think like we saw in the case of simple aromatic compound whether they are a chemically

equivalent but these two are not chemically equivalent because there is asymmetry here this carbon which is chiral.

But what happens is that this Amino acid in a in a molecule rotates rapidly around this axis. So let me show you that axis, there is a typically a very fast rotation around this axis. Therefore because of this fast rotation these 2 hydrogens that is the these the 2 hydrogens in the ortho protons and the meta protons are equivalent because of this rotation similar to what we see in the case of methyl's, but there are been research in this area where people have gone down to very low temperature and found that these ring freezes means it does not rotate around this because of low temperature and at that temperature people have seen that these 2 hydrogens they become nonequivalent and they show different chemical shifts. But in our case in a simple molecule like this or in even in proteins and peptide at room temperature these rings rotate very fast is called ring flipping.

So, the ring flipping occurs so fast that these 2 hydrogens are more or less averaged out like in the CH3 system and they have chemical equivalent chemical shifts I mean equivalent interchange. So, now let us look at the different hydrogen carbon pairs. So, again as you can see in this spectrum there are tw2o peaks coming here which is around 30ppm there is one peak coming at 60, so this is an alpha Amino acid, so again like in the previous example this hydrogen carbon pair which is alpha carbon is expected for this peak here and the Beta that is this CH2 again they are not equivalent, so they show two different peaks at 30ppm, 35ppm and then aromatic portion remember we saw in the hydrogen proton NMR it comes around 7 to 7.5 and the carbon comes around 120 and that is what we are seen here in this portion of these spectrum.

So, it is very now easy to assign which is which the CH is this peak because of this down field shifted, the CH2 are these 2 hydrogens protons and then the aromatic one is up field shifted and the second one which is down field shifted is this because of close is close to this hydroxyl group, so we expect two equivalence one pair here and another pair here and that is what is shown here okay. So, we can see you like this or we can so we have covered a different types of Amino acids and different type of structures and look at how the peak pattern looks. So, in the next class we will move on to HMQC which is another experiment very similar to HSQC and then another experiment further in HMDC and we will that will tell us how the different experiments works as we saw in HSQC is not sufficient just for

assignment HMBC helps sometimes then there is an ambiguity that we will see in the next class.