H-C multiple-bond correlations: HMBC

The HSQC is a high-sensitivity way to observe Cs that have an attached H, get the chemical shifts of each, and know which Hs are attached to which C. This wealth of information in addition to the fact that the gradient HSQC color-codes CH_2 groups make this an extremely useful experiment. However it is blind to Cs that lack an attached H. For this, we use one of the several Heteronuclear Multiple Bond Coherence HMBC experiments. This experiment produces cross-peaks combining the chemical shift of an ^{1}H with that of a ^{13}C two or three bonds away (*multiple* bonds). Thus, instead of one-bond J-couplings of ≈140 Hz (or 170 Hz for aromatics), the HMBCs use J_{HC} coupling constants on the order of 5-8 Hz. They also tend to incorporate features designed to suppress peaks from one-bond correlations. The CIGAR variant is attractive because the duration of the delay used to accrue J-coupling is varied, so that a single experiment produces a compromise over a range of J values. The virtue of this is that any single choice of delay will allow certain values of J to produce no cross peak at all. Thus a conventional HMBC can lack a peak or two and one is advised that if using the gHMBC, you should collect two: one assuming J_{HC} = 5 Hz and a second assuming J_{HC} = 8 Hz. (These can even co-added, using Varian software.) The following describes collection of a CIGAR spectrum, however a single click of a button can produce a gHMBC instead, via the Varian set-up panels.

Preliminaries.

As for the HSQC, you need a good power-pw90 pair for both ^{1}H and ^{13}C and you should have good values for both sw and tof for both nuclei. In short, you should have a beautiful calibrated ^{1}H spectrum in one workspace (experiment) and beautiful calibrated ^{13}C 1D conveniently loaded in another workspace. Having both these spectra loaded before you begin makes it easy to check on parameters you will need. It will prove convenient to have a ^{13}C pw90 determined using a tpwr of 55.

Once again, a good way to keep track of these is to fill out the table below:

Nucleus	power	pw90	spectral width	offset
^{1}H	(→tpwr)	(→pw)	(→sw)	(→tof)
^{13}C	(→pwxlvl)	(→pwx)	(→sw1)	(→dof)

Begin with your beautiful calibrated 1H 1D.

The CIGAR set-up is based on the parameters of a beautiful ^{1}H 1D, so work in an experiment that has this spectrum in it. If you are using a spectrum collected on another day, it is a good precaution to reacquire it immediately before beginning the CIGAR (takes 30 sec.). Type **setlp0** then recollect and adjust the phase. Save that spectrum. Also load a ^{13}C spectrum of your sample in another workspace, for reference.

Move the parameters of your beautiful ^{1}H1D to an additional workspace from whence you will run the 2D. For the example of your ^{1}H1D spectrum in workspace 1 and workspace 3 as the workspace to be used for the 2D, type mp(1,3). Go to the 2D workspace by typing jexp3 (in the above example).

Setting up CIGAR

Under 'Experiments' choose 'Convert current parameters to do... > Indirect Het. Corr. (Basic) > CIGAR (**Page 2**). This pulse sequence has more going on at once than did the HSQC sequence, so it is not easy for us identify motifs. However it begins with gradient-90-gradient pre-sequence [1] to prevent any new magnetization from carrying over from the preceding scan. You see that there are not multiple spin echos, indeed there is only one ^{1}H 180° pulse [2]. This tells you that any INEPT transfer to ^{13}C is not refocussed. Therefore we cannot decouple during acquisition [3]. On order to not have to look at the complicated splittings that will be present, we will simply use absolute value mode for the F2 axis (later).

^{1}H1D parameters should transfer over to your 2D parameter set. In Acquire>Parameters (**Page 3**) you should see sw [1], tof [2], pw90 [3] and tpwr [4]. (Beware, [3] brings the 'observe pulse' pw from your ^{1}H 1D and this is not necessarily pw90, make sure you have the pw90 value here.) As for the HSQC, we will want to manually optimize the corresponding values for ^{13}C from our beautiful calibrated ^{13}C 1D. The ^{13}C1D pw90 becomes pwx in CIGAR [5], the ^{13}C tpwr becomes pwxlvl [6]. The dof for ^{13}C is based on the tof of your *full*-width ^{13}C 1D (not just the Cs with attached Hs) [7]. To install your desired sw1 (f*ull* ^{13}C spectral width, derived from the sw in the ^{13}C 1D), go to Acquire>Acquisition * (**Page 4**) [1].

You now have the basics in place. However in order for the experiment to work well you will either need to disable fancy options, or make sure they are properly set-up. The Acquire>Pulse Sequence panel (**Page 5**) is where you can decide either to run an experiment optimized around a choice of J_{HC} or the CIGAR, which will produce a compromise. For CIGAR, click on the CIGAR button [1]. The suggested maximum and minimum expected J_{HC} values are good [2], as is the range of one-bond J_{HC} to be suppressed [3]. The one item that may need adjustment is the S6 refocussing pulse length [4]. This will have been drawn from a list of calibrations performed when the probe was first installed. In an ideal world, these calibrations would always be up to date. However each sample is a little different, and our probe's X-channel is most sensitive to sample variations. If your calibrated ^{13}C pulse agrees with the stored value that appeared when we first invoked CIGAR, then you know you can use the other stored X-values too. However my ^{13}C pw90 was 14.5 µs using a power of 55 whereas the stored value was 10.7 µs using a power of 55. This produces a correction factor of 14.5/10.7 = 1.36). With that in mind, I multiplied the stored value of 28.9 µs by 1.36 to get 39.3 and used that for the S6 pulse width [4]. (Instead of using my pulse width, you should use your own most recent value.) For the simplest math, use a ^{13}C pw90 determined at a power of 55, which will be directly comparable to the stored value. If you calibrated ^{13}C at 56 dB, then multiply your pw90 by 1.12 to estimate the value you would have obtained at 55 dB (-1 dB

corresponds to x1.12 in pw) Back in Acquire>Acquisition (**Page 4**), Find Gain [2]. My experimental parameters came up with a suggested value of 10. Hopefully you recognize this as a bad choice. The Find Gain operation chose 60. I used 46 (even numbers only are allowed). I also set the acquisition time to 0.3 s [3], as this pulse sequence does not decouple during acquisition so there is no danger to the probe. 'Show Time' said that this experiment would take 40 minutes [4]. I have 4 scans per FID [5], but I really need that many. Count on roughly as many scans as it takes to see signals from your molecule (not solvent) in a ^{13}C 1D.

I also have 200 increments [6]. That too should not be decreased as the CIGAR will have many more peaks than the HSQC. The peaks will be also be closer together than they were in the HSQC because there may be several Cs within two or three bonds of a given H. Therefore we will have a greater need for ^{13}C resolution than we did for a comparable HSQC. Finally, because the CIGAR ^{13}C spectral width (sw1) has to retain the *full* ^{13}C width, in contrast to the HSQC which could cut out the region of quaternary Cs), the CIGAR will have a shorter dwell time (DW = 1/sw1, see lecture notes). This is used as the increment by which the d2 delay is increased with each 1D spectrum that contributes to the 2D. Thus, the maximum value of d2 = at1 = (ni-1)/sw1 [7] will be smaller in an HMBC of the same ni than it was in an HSQC. Because the resolution in F1 is 1/at1 [8], the same number of increments results in inferior resolution in an HMBC than in an HSQC (resolution ≈ sw1/(ni-1), recall that large is *bad* here.) Varian's fix is aggressive linear prediction (later).

After clicking on 'Acquire' to launch the experiment, we can look at the 1Ds as they accumulate. For **Page 6** I typed **wft** then **dssa(1,100,10)**. This performs the Fourier transformation of all the 1Ds acquired so far (which was 174 at the time [1]), and then displays every 10th spectrum of spectra 1 to 160, stacked vertically (display stacked spectra automatically). This page also shows the set of main parameters, along with the parameter names. Try to locate a few familiar ones.

Page 7 shows that this experiment is collecting complex data (real and imaginary) for the ^{13}C indirect (F1) dimension. Although ni is 200, indicating apparently that 200 1Ds will be collected, there will actually be two of each, one collecting X-magnetization and the other collecting Y-magnetization for ^{13}C (phases 1 and 2) [1]. Thus you see that a total of 400 1Ds will be collected [2].

Processing

Once again, we have to think about weighting, linear prediction, phase correction and chemical shift referencing for each of F2 and F1. However linear prediction is rarely needed for F2, and the phase correction is not applicable here because we will look at this spectrum in absolute value (av) mode. Thus the processing in simpler than HSQC.

-1- Weighting F2. As usual, in Process>Weighting, click on 'FT 1D- 1st increment' [1] (**Page 8**) and confirm that the weighting function is a good fit for the intensity

(signal) in the FID. You see that as for the COSY, this weighting is designed to emphasize magnetization that is growing in during at. This is because our pulse sequence is in-essence an INEPT that is not refocussed. Antiphase magnetization comes back in phase during at. If the FID is dominated by ^{1}H coupled to ^{13}C with J_{HC} ≈5-8 Hz, then it will rephase after 1/2J = 100 ms - 62 ms, and then dephase again. Thus the ideal FID would be 240-400 ms long and its weighting function would peak in its middle. Our FID is 300 ms long because we set at=.300 (good choice). If we were to use a Gaussian, we would shift it by .15 and have it decay to half in .075. (Review your earlier problem set.) Varian does something similar using a squared sine function (called 'sinebell') [2]. Because the sine naturally has a value of zero when its argument is zero, no shifting is needed. The sine is set to reach half-amplitude in 0.075s in order to just fit the FID [3]. (Note that we are working in the 'F2' column [4].) As usual, you need to be sure that your window drops to zero by the end of the FID. (If the signal itself is still evident at the end of the FID, then you have the option to linear predict to extend it in Process>More 2D. If you double the F2 data then also double the F2 weighting function that was a good fit to the original data.) Confirm that the size of the F2 transform is approximately 2x the number of points collected (or collected plus predicted) [5]. Then click on 'Transform F2' [6].

-2,3- Weighting and Linear Predicting F1.
Use the middle mouse to increase the vertical scale of the resulting interferrogram (**Page 9**), place your cursor on a streak [1] and click on 'Interactive Weighting' [2] in Process>Weighting. In the resulting window (**Page 10**), we see an unshifted Gaussian ([1] magnitude begins at 1 and drops to zero). First click with the middle mouse in the lower panel to see the indirect 'FID' (interferrogram) [2]. Assess the strength of the signal. If you can see good signal oscillating to the end of the detection interval, then it is worth extending by linear prediction (the example does have good oscillating signal). If you have the bad fortune that your signal has already vanished by the end of the acquired points, there is no point in extending what would at later times only be noise. Assuming you have signal that has not vanished by the end of the interferrogram, decide how strong it looks. The stronger it is, the more you can predict from it. Varian quadruples ! The weighting function you see is designed to accommodate this.
In Process>More 2D (**Page 11**), you see that ni x 3 calculated points are added to the data [1] (ni = 200 [2]), so whatever weighting you apply in Process>Weighting should assume a four-fold longer interferrogram.
If you would rather use numbers that your eye, fit your weighting function in Process>Weighting to the data you see [1] (no linear prediction visible, **Page 12**), and then multiply the gf1 value (length of the Gaussian applied to F1 [2]) by the factor by which linear prediction increases your data set size (**gf1=gf1*4**, in our case). (Note that we are now working in the F1 column [3].)
Page 13 displays the 4-fold extended Gaussian. Check that the size of the F1 transform is ≈ 2 times the size of your linear predicted data set size [1]. Click 'Full 2D transform' [2].

-4- Phasing. Because we are displaying the F2 dimension in 'absolute value' (av) mode, all peaks are positive in sign, and there is no phase correction to be made (**Page 14**).

-5- Chemical shift referencing
As for the HSQC, locate signals in the HMBC that obviously correspond to signals in the ^{13}C 1D. Based on the calibrated chemical shift of that line in the ^{13}C 1D, lace the cursor on the corresponding peak in the HMBC and type **rl1(123.4 d)** where 123.4 is the chemical shift, the '1' in rl1 tells the software that this correction applied to the F1 axis and the 'd' tells the spectrometer to use the carrier frequency of the X-channel. Similarly for the ^{1}H axis , place your cursor on a resonance of known ^{1}H chemical shift and type **rl(5.67p)** where 5.67 is the chemical shift and the 'p; tells the spectrometer to use the H-channel frequency to calculate ppm. The referenced spectrum is on **page 15** (changes are slight only, but they matter.)

Page 16 shows the CIGAR of pulegone with the two 1D spectra collected as references. Note that in the CIGAR has a few peaks apparent at ^{13}C chemical shifts where there is no ^{13}C signal in the 1D [1]. These are obviously artefacts. However with your ^{13}C 1D in front of you, these can be identified (do not use a projection of the 2D as your reference 1D, as a projection will carry the artefacts). These artefacts are mirror-images of actual CH resonances, and indicate an imperfection in a ^{13}C pulse. Normally they are weaker than their bona-fide counterparts.

Also note however that we have lots of signals at the chemical shifts of quaternary Cs !! (green dashed lines [2]). These are at the ^{1}H chemical shifts of neighbouring positions, permitting tentative assignments, in conjunction with our chemical shift expectations.

The CIGAR of strychnine (**Page 17**) does not show such artefacts. All signals in the 2D have corresponding signals in the ^{1}H and ^{13}C 1Ds. At this point I remain unsure as to the cause of the artefacts in the pulegone spectrum, however they illustrate the lesson that none of these experiments should be interpreted in isolation, as all can contain deceptive and misleading features, and these become more common when substandard samples, incorrect pulse widths or inappropriate delays are used.

You also see that the CIGAR does a good job of suppressing one-bond CH couplings, as the crosspeaks characteristic of the HSQC should be absent from the CIGAR (**page 18**) [1].

Overlays and synchronized cursor in the viewport overlay tool

The CIGAR is most useful in combination with an HSQC. To exploit Varian's extremely convenient tool for directly comparing two spectra, you first need to make sure that both are correctly referenced with respect to chemical shift (see the HSQC instruction handout), and loaded in separate workspaces. In the example my CIGAR is in workspace 8 and my HSQC is in workspace 6.

Beginning in the CIGAR experiment (**Page 19**) and under 'Edit' select 'Viewports'; (4th from bottom). Activate number of viewports = 2. The current experiment (CIGAR) comes up as the active viewport [1].
Next, make the other viewport active by clicking in the 'eyeball' [2]. Enter the number of the workspace in which the comparison HSQC is loaded [3]. Click 'Display Spectrum' [4] Click on stacked panels [1] under 'Viewport layout' to see your two spectra together (**Page 20**). Activate the top spectrum by clicking in its frame [2] or in the 'Active' button [3]. Click in the blue bulls-eye [4] to get the display tools (you may have to chose this option twice) then decrease the vertical scale [5] as needed. This will affect only the 'active experiment. You can go back and forth between them activating one and adjusting it to achieve a better comparison with the other and then switching back. In order to have more space for the spectra, I have inactivated 'show fields'. If the axes are not perfectly aligned click on 'Overlay Viewports' [6] (for example, you collected the spectra using different spectral widths, which is fine , the software can handle it !!).

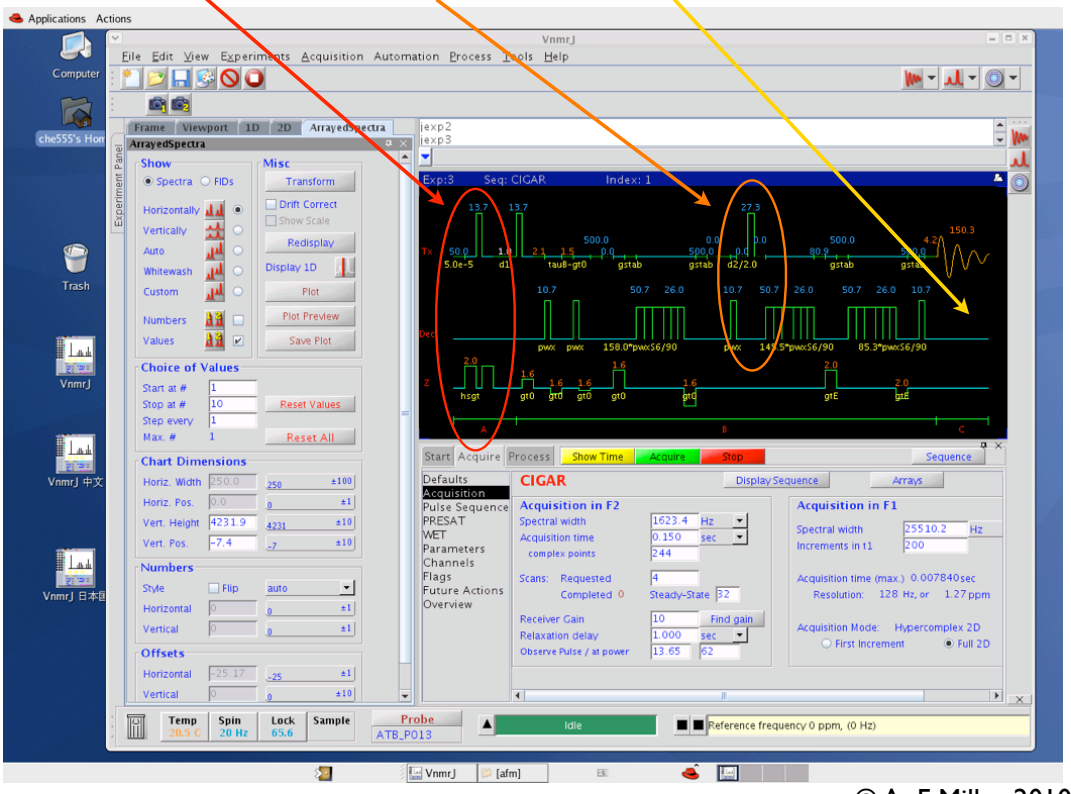
An option should then appear under Overlay Viewports. [7] 'Stack Spectra' should reconcile the chemical shifts (make sure that both spectra are referenced IN ADVANCE). Go back to panels one above the other, and make sure that the cursor is synched (as well as the axis) [8].

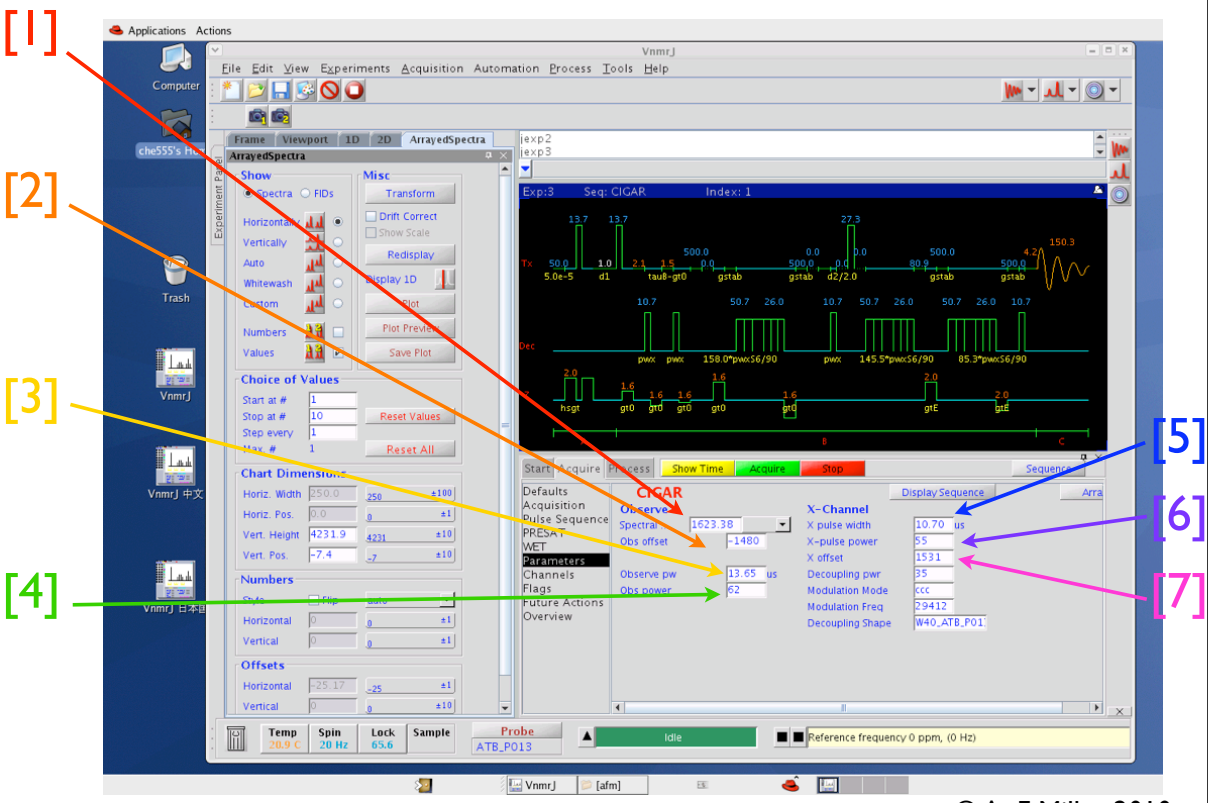
I find that the stacked display is the more useful one, however for comparison, **Page 21** shows the result of the 'Overlay Spectra' option. The CIGAR peaks are in aqua, the CH_3 and CH from the HSQC are in green and the CH_2 of the HSQC are in red.

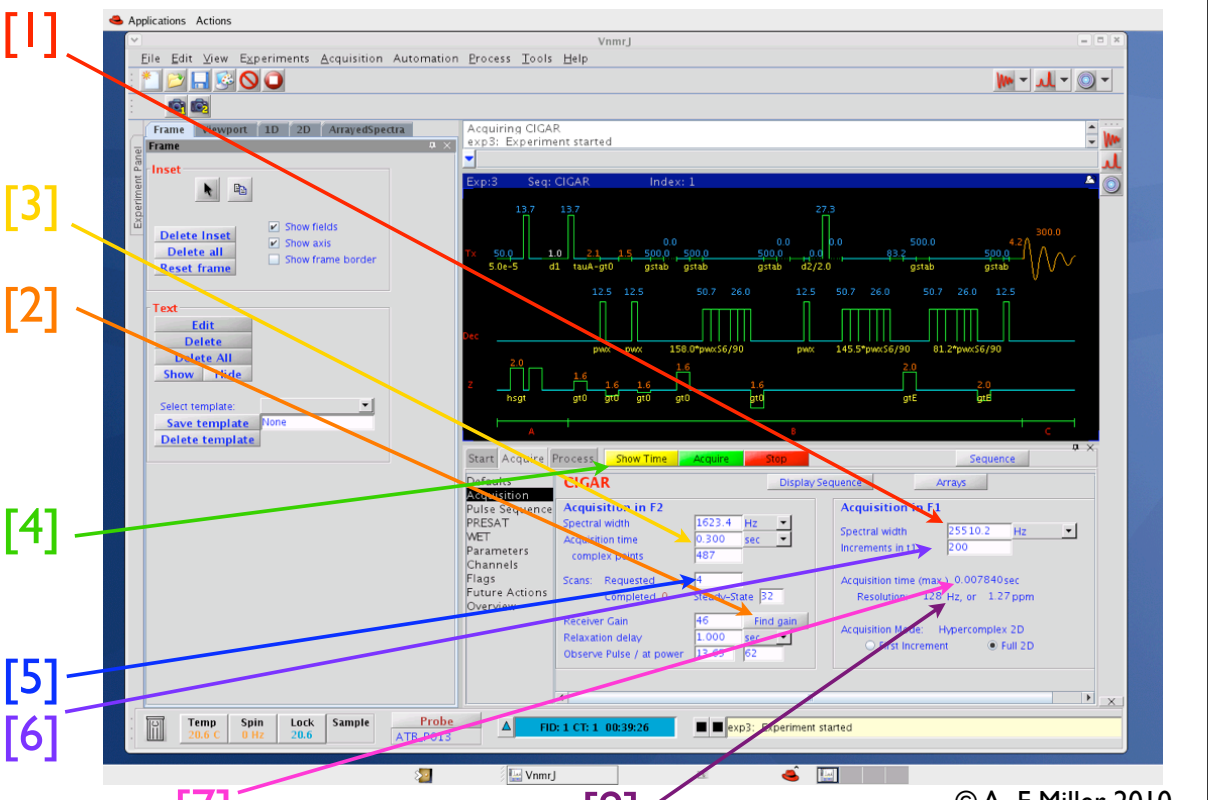
Back in the stacked display (**Page 22**), you can see how each 1-bond cross peak is related to 2- and 3-bond cross peaks. . The methyl ^{1}H at 1.01 ppm is 2 or three bonds from the CH_2 Cs at 51 ppm and 33 ppm [1] but not the CH_2 C at 29 ppm. This suggests that the methyl at 1.01 ppm is between the aliphatic CH_2s, not attached to the double bond. The olephenic Cs see a CH_2 C at 29 ppm [2] but not the CH_2 Cs at 33 ppm or 51 ppm. This identifies the CH_2 C at 29 ppm as the one adjacent to the double bond. The two methyls remaining methyls have CIGAR cross-peaks to one-another, indicating that they are the two adjacent to the double bond [3]. The carbonyl C also 'sees' the two methyls adjacent to the double bond [4].

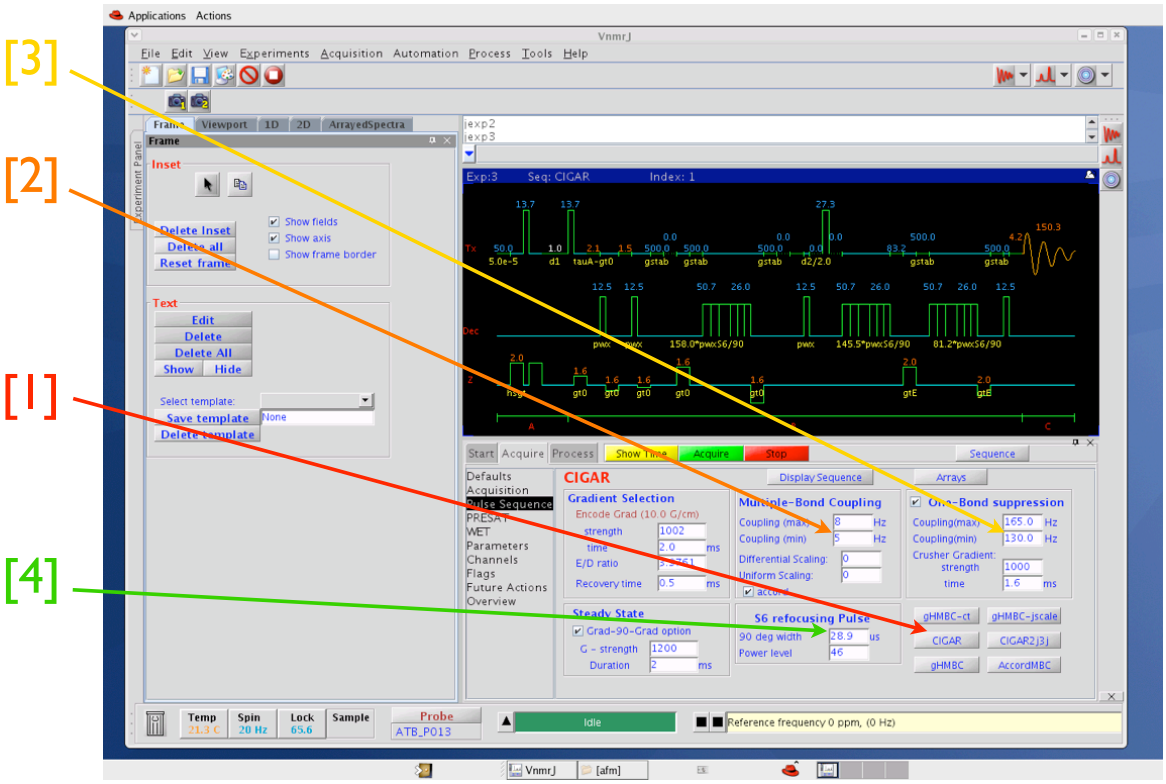
The comparison of the Strychnine CIGAR and HSQC is even more striking (**Page 23**). The strychnine data set was processed with a 2k Fourier transform size in F2 vs. the 1k used for pulegone in Page 13. Compare resolution of the strychine data set (**Page 24**) with that of the pulegone spectrum on page 13.

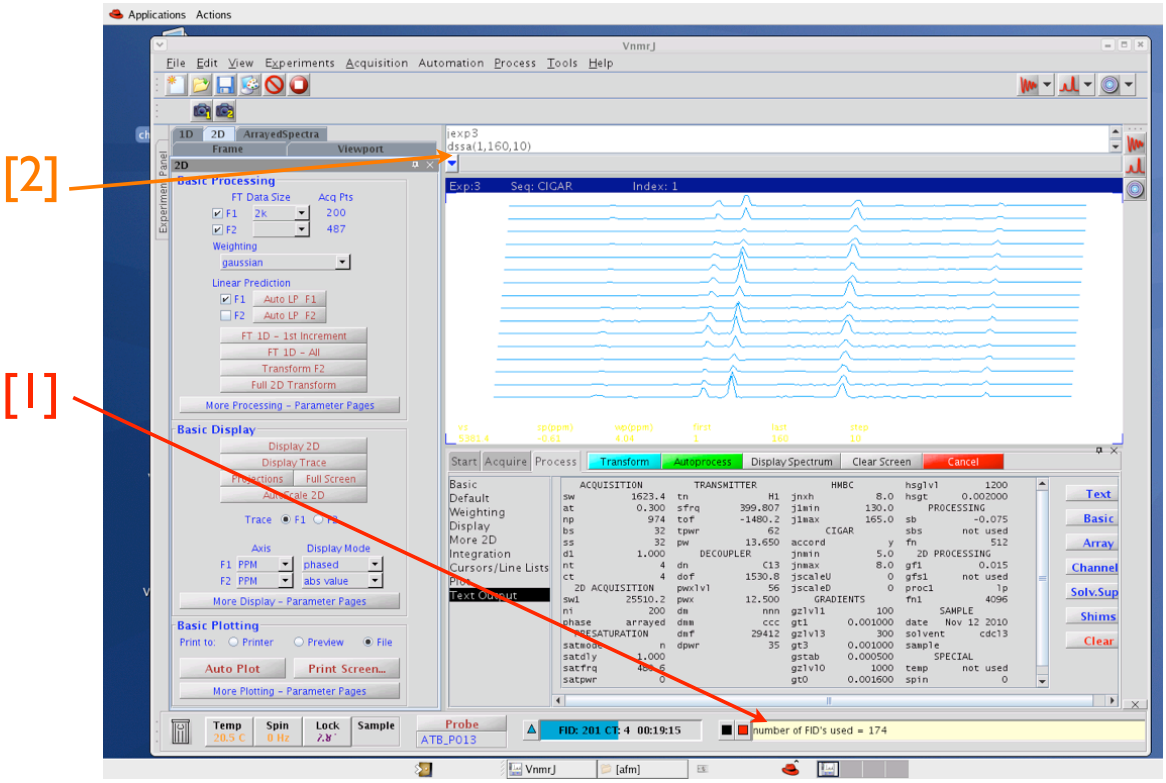
^{13}C Heteronuclear Multiple Bond Coherence spectrum: HMBC (CIGAR)

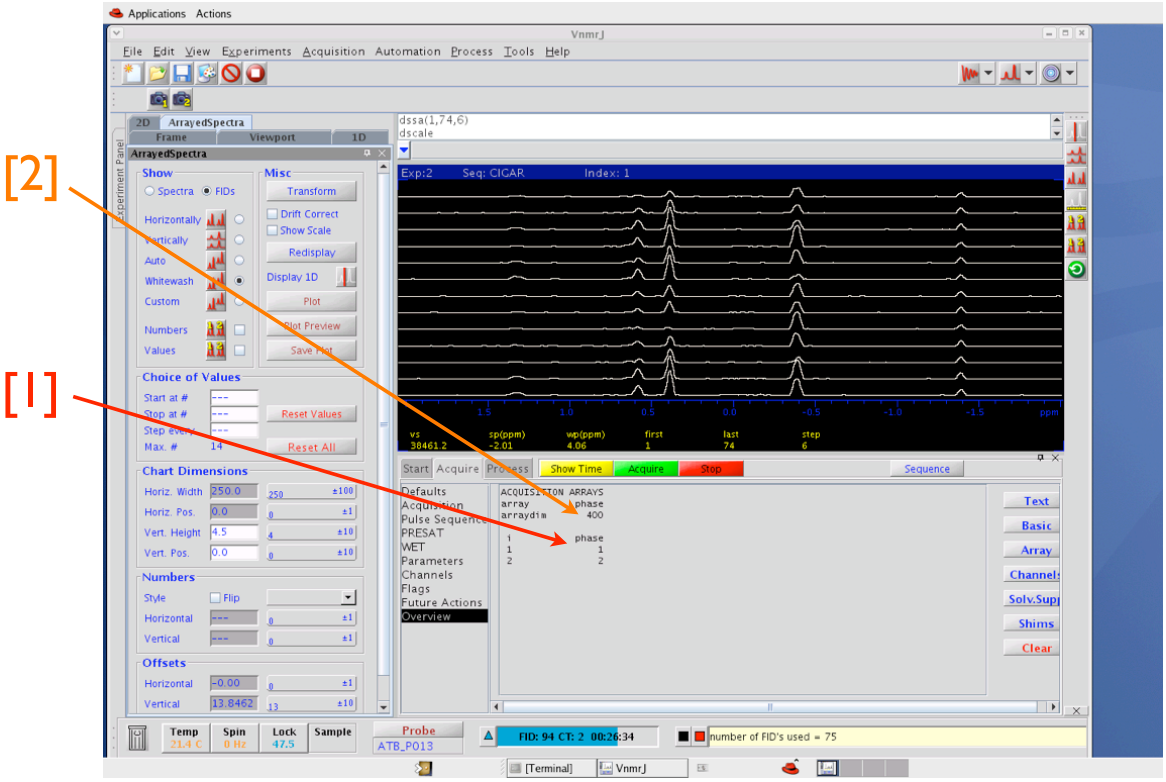


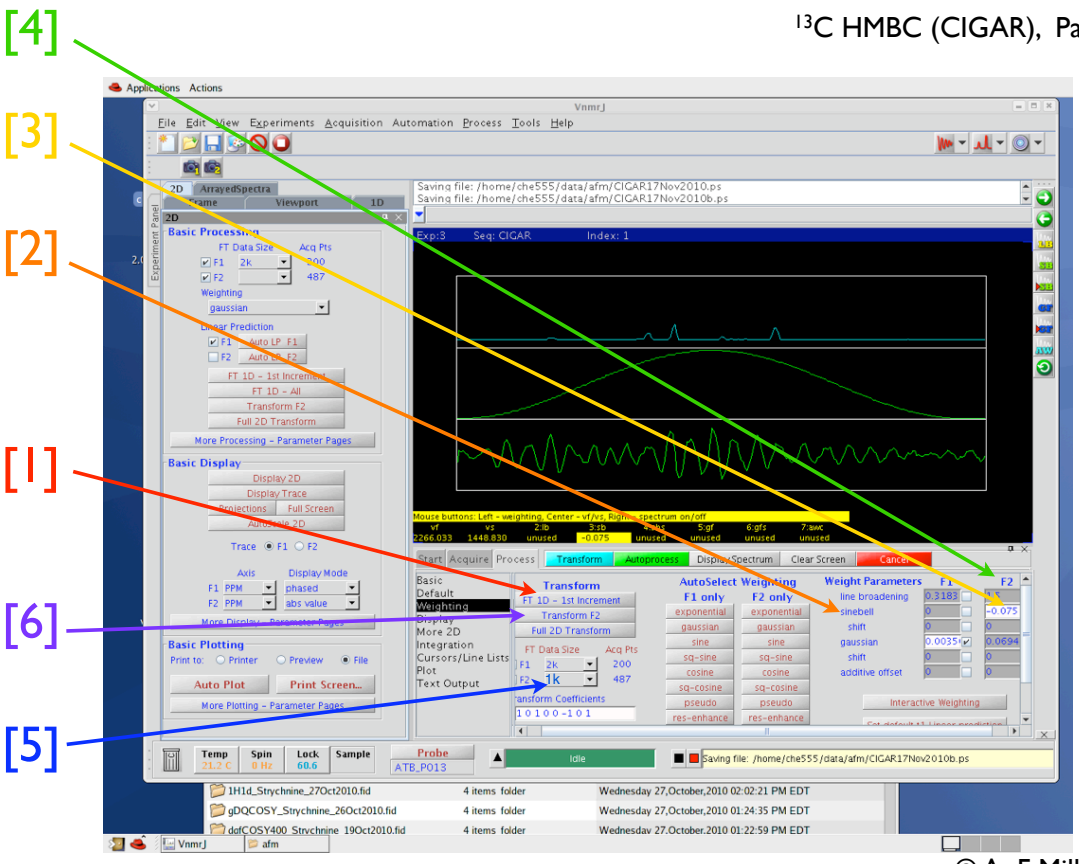


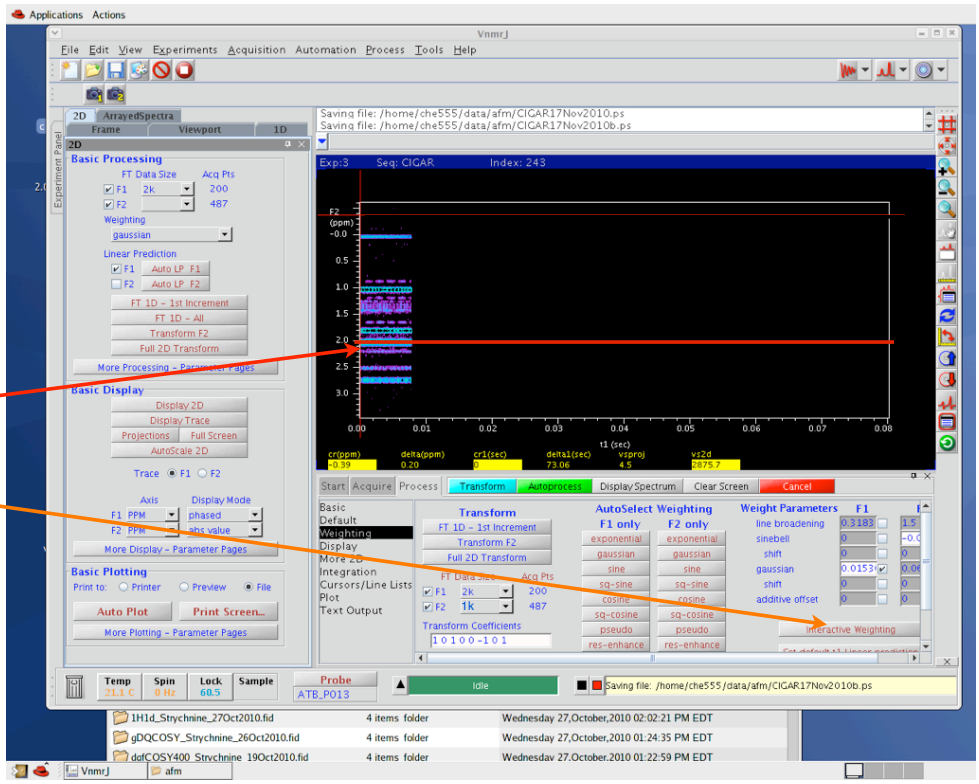


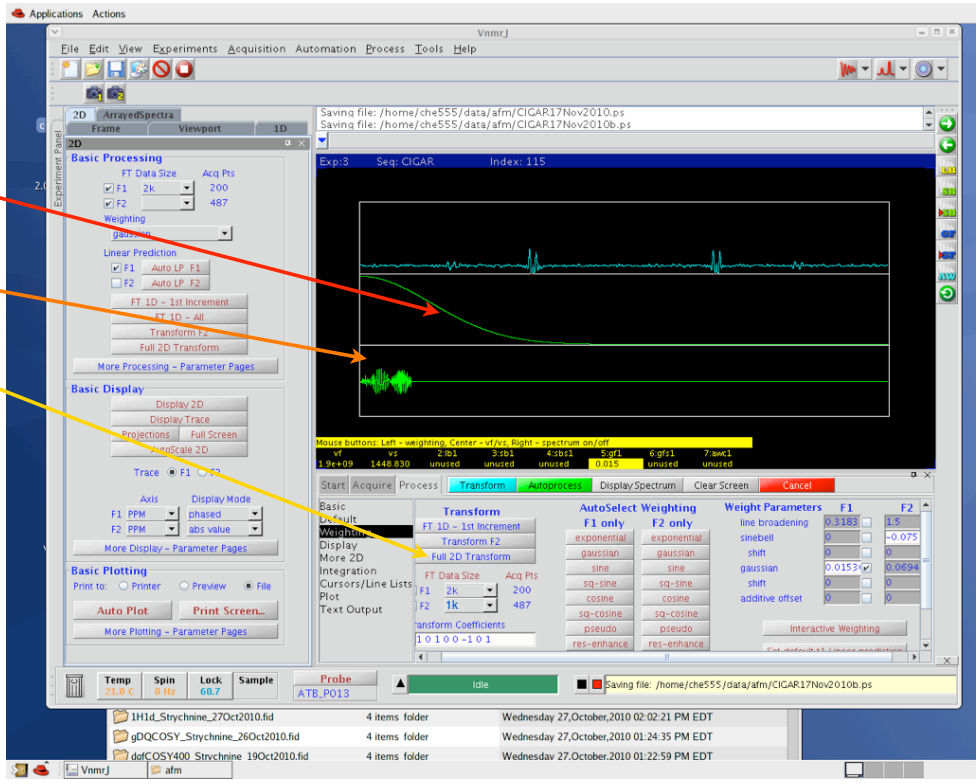


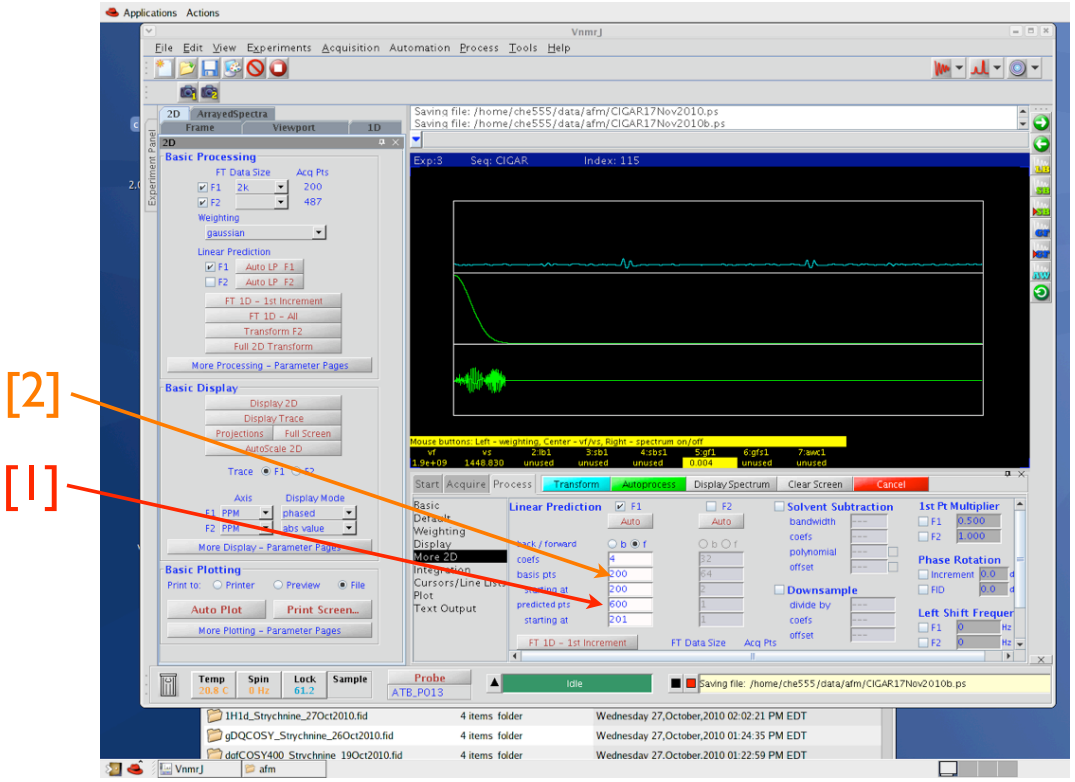


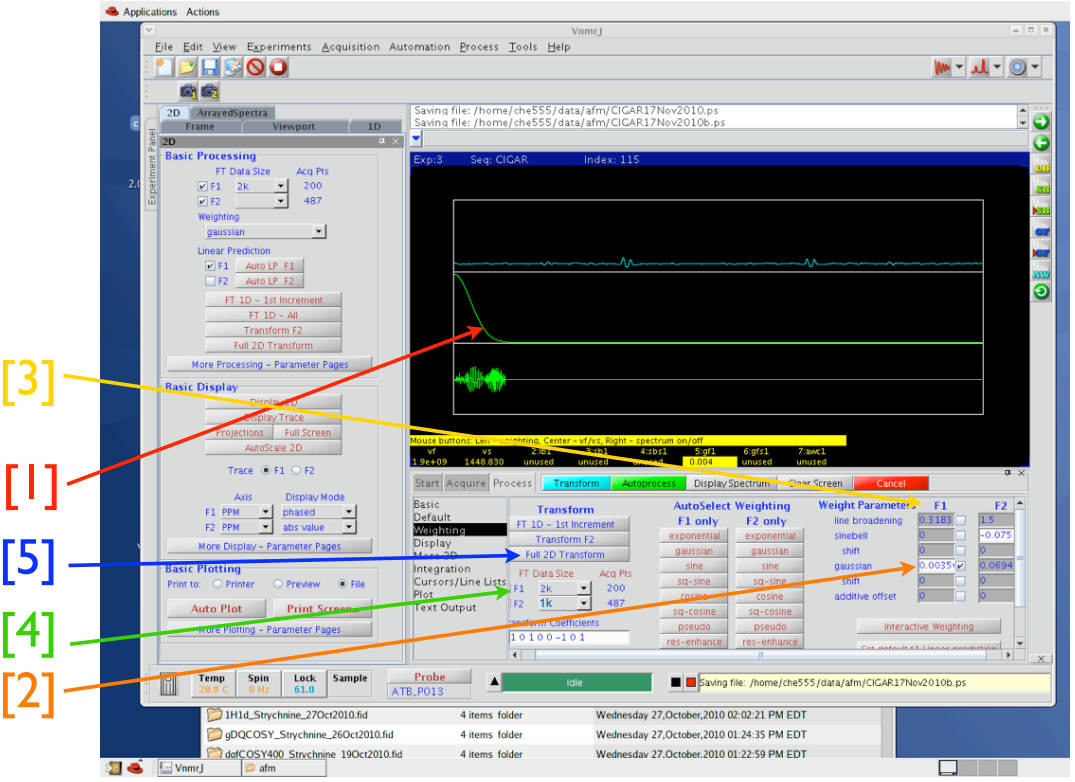


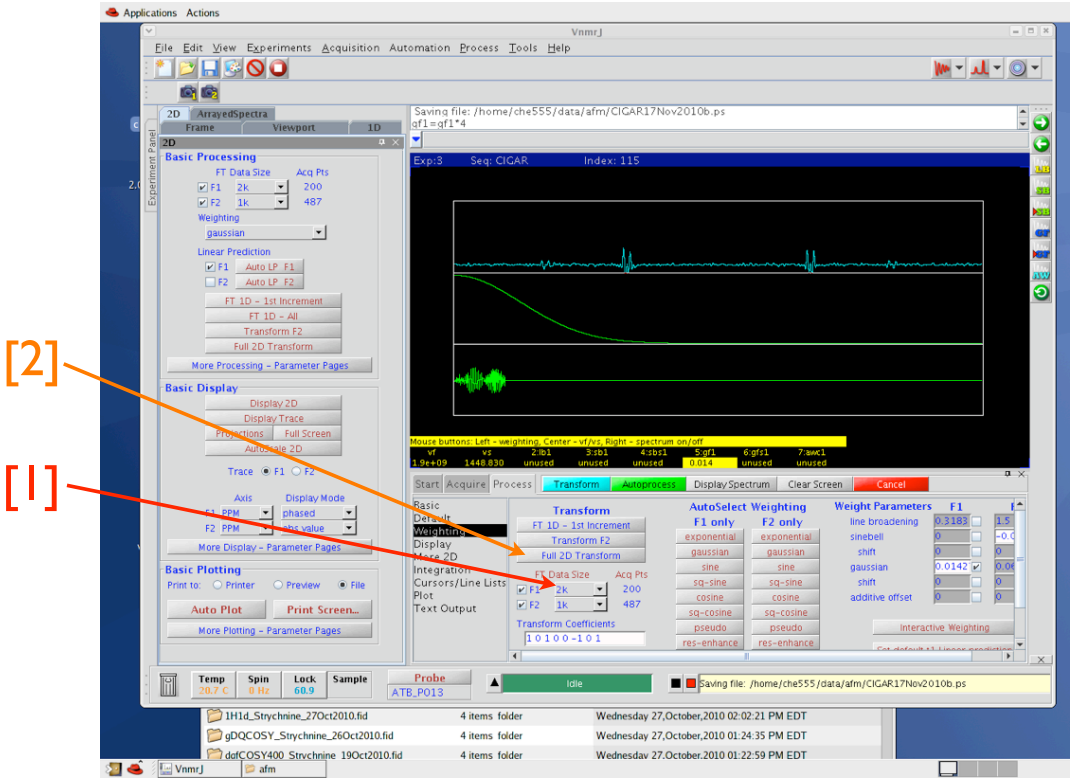


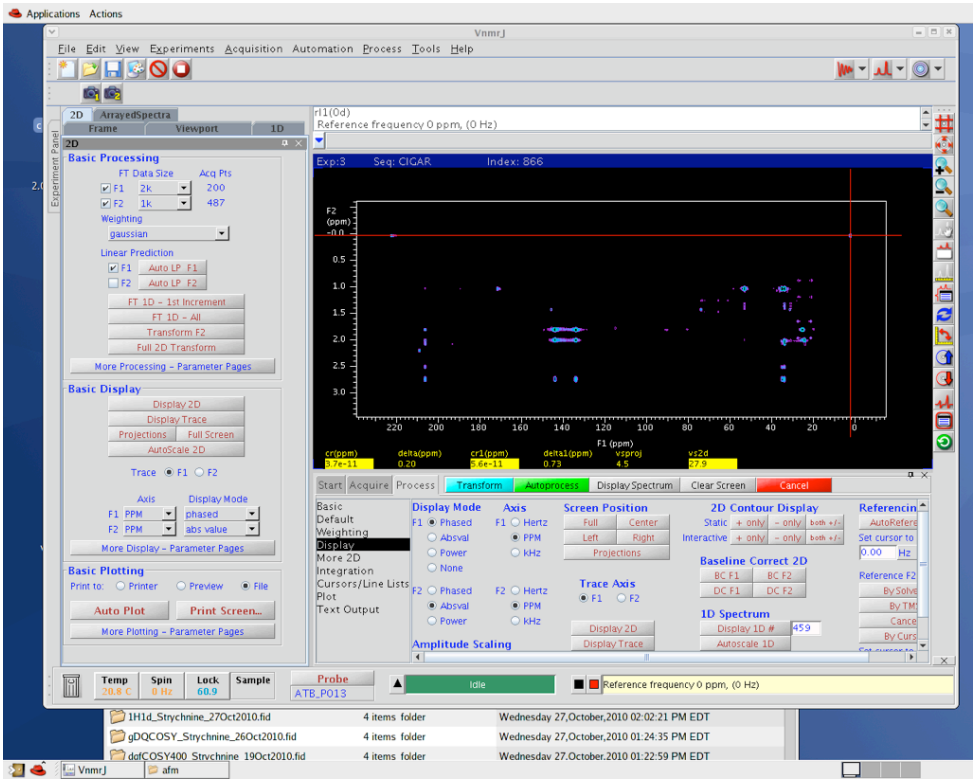


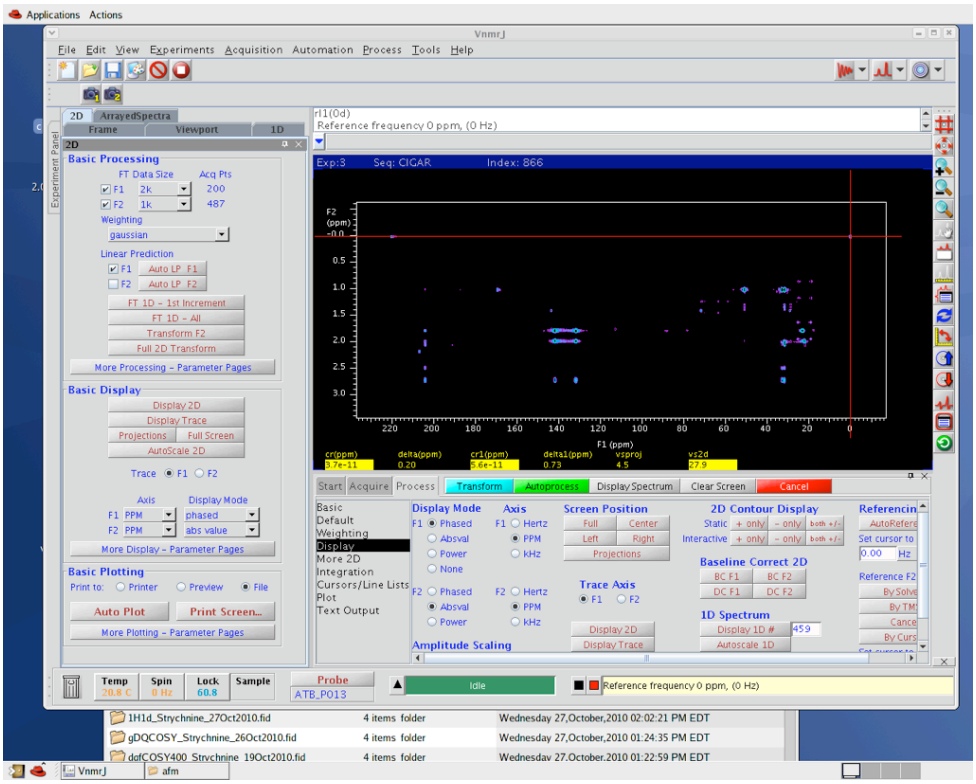


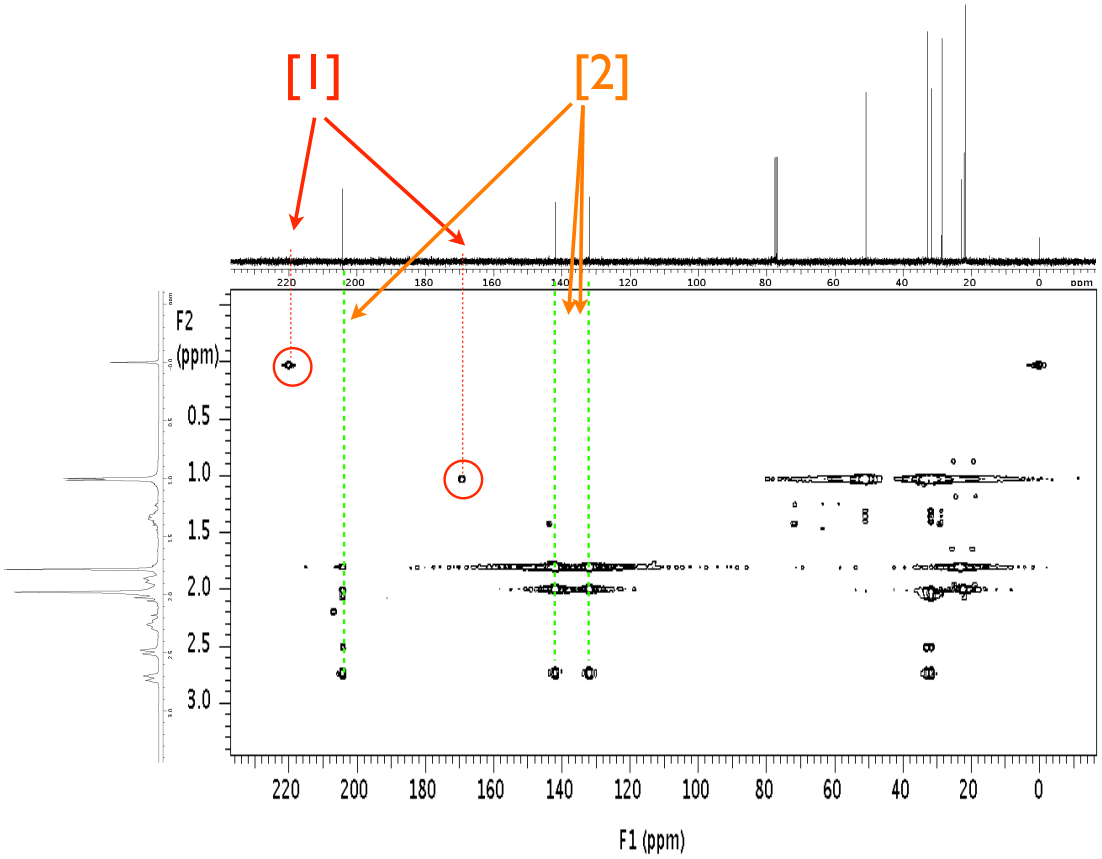




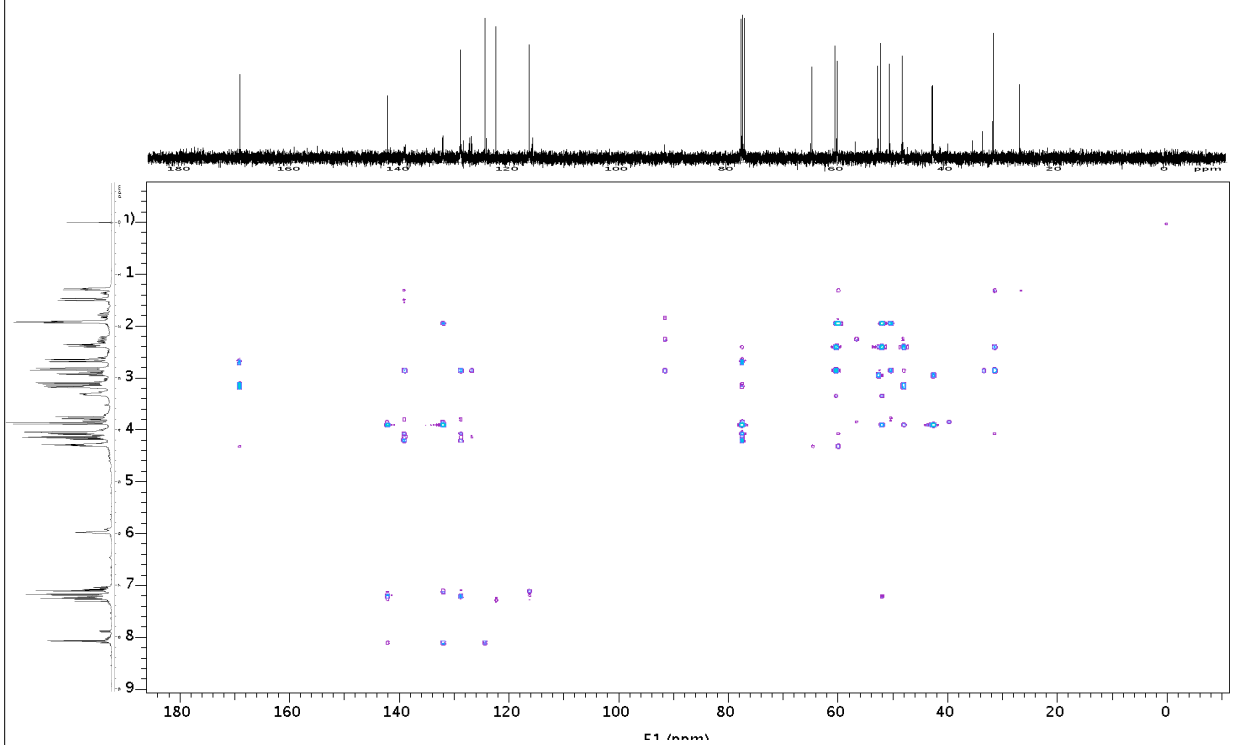




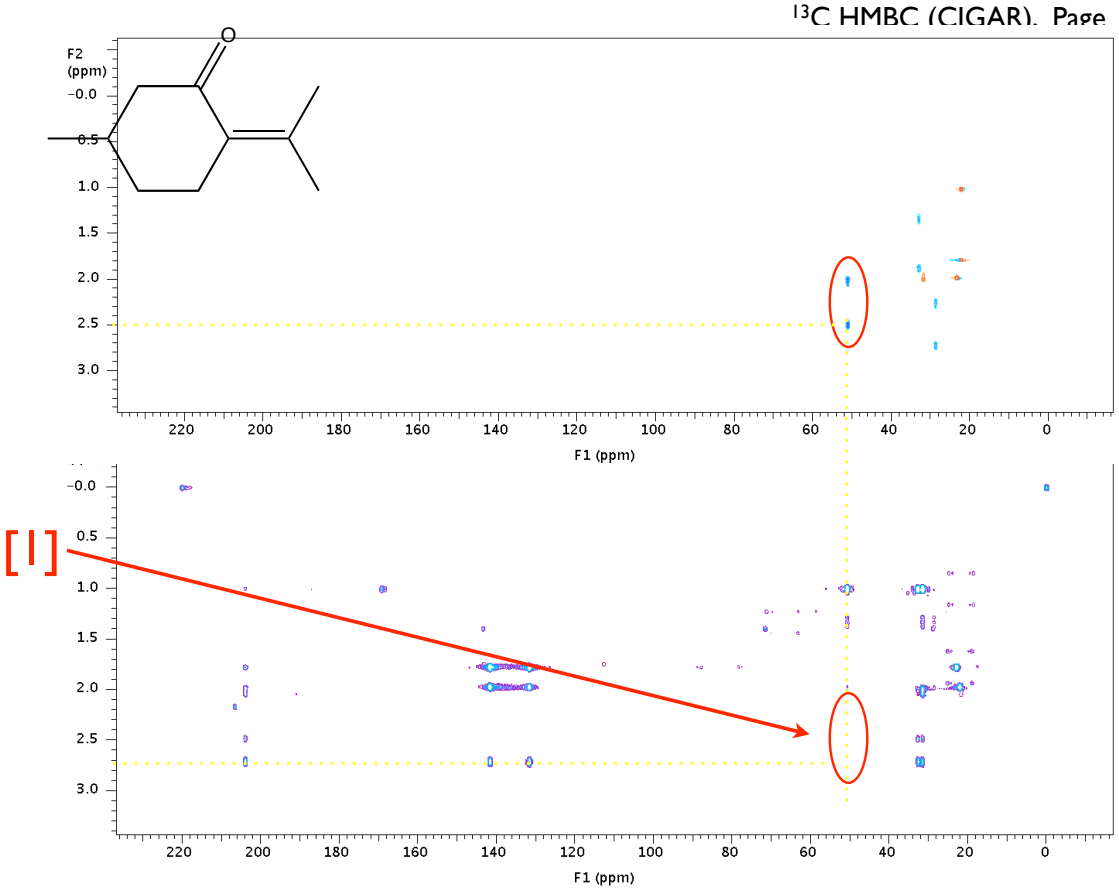




^{13}C HMBC (CIGAR), Page 17



^{13}C HMBC (CIGAR), Page 18



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