Running 13C spectra

^{1}H preliminaries.

As usual, insert a sample, lock on it and shim.

On 2 Nov. the ^{1}H pw90 on Gort at tpwr=62 was 14.25 us. At tpwr of 56, pw90 was 24.75 us (vs. 28.5 =2* 14.25). Thus at a power of 62, the spectrometer is not in the linear regime. At a lower power still, tpwr = 50, pw90 =50.5 (vs. 49.5 = 2* 24.75). This is now linear, as the pw90 is not shorter than predicted.

tof = -431.9 and sw = 3742.5 (you can set these to nearby numbers that are easier to remember, such as -430 and 3740. (The software replaces 3740 with 3742.5).

For a spectrum to save, select 8 scans, acquire and save.

A basic ^{13}C 1D.

Under 'Experiments' in the 'Convert current parameters to do ... section choose 'Standard 1D experiments > Carbon .

The pulse sequence **(Page 2**) consists simply of an excitation pulse [1] followed by data acquisition [2] and recovery [3]. However in contrast to ^{1}H spectra, the ^{13}C excitation pulse is typically < 90 °. Varian's default is a 45 ° pulse [4]. Additionally, ^{1}Hs are continuously irradiated (decoupler = 'dec' channel) [5] (more below).

In Acquire>Acquisition, note the very large spectral width [6]. 25510.2 Hs is more than 250 ppm (253.8 ppm, change the units using the toggle to the right of the number) [7]. Note that although the acquisition time (at) [7] may seem reasonably long (1.285 s) this is actually shorter than is routinely suggested as the default for ^{1}H. This reflects the usually-smaller space between adjacent ^{1}H resonances, and thus a need for higher resolution (lower frequency separations) in ^{1}H spectra. Smaller frequency distinctions requires longer at for ^{1}H spectra. By contrast, there are not only fewer ^{13}C signals, but they are spaced out better over their larger frequency range, so one can compromise more on resolution (shorter at, see lecture notes).

The probe file supplies a pw90 for ^{13}C of 9.30 μs at a tpwr of 56 (on the ^{13}C) [8]. We'll check on that next.

Decoupling parameters are shown in Acquire>Channels (**Page 3**). We noted that this ^{13}C experiment employs continuous ^{1}H is irradiation [1], or ^{1}H decoupling. The pulse sequence is considered to have three stages, denoted by 'A', 'B' and 'C', during each of which aspects of the equipment can have different status. We can have the decoupling turned on in all three stages by setting the decoupler mode (dm) to 'yyy' ('yes' during A, 'yes' during B and 'yes' during C) [2], next to 'Dec On/Off'. If we wished to have the decoupler active only during stage 'C' for signal detection, we

would set dm='nny'.

Continuous decoupling (**dm='yyy'**) has two consequences: decouping and nOe enhancement. It decouples because it rapidly interconverts ^{1}H spin states so that the distinction between them blurs and in fact collapses. Thus, instead of having each ^{13}C resonance be split by J-coupling to each attached ^{1}H, we get a single ^{13}C line for each C atom. Thus signal is more concentrated, the signals are stronger, and because there are fewer signals the spectrum is less congested, facilitating interpretation. The challenge is to have a sufficiently high ^{1}H power to completely average the two spins states of each ^{1}H, without overtaxing the probe. Theoretically, the best irradiation frequency varies form ^{1}H to ^{1}H in accordance with their different resonant frequencies (chemical shifts), so we use a series of pulses rather than continuous irradiation. This enables the decoupling to act over a larger bandwidth. (A popular and robust composite pulse decoupling scheme is Waltz16 [3].) As usual, the bandwidth of the decoupling is proportional to the inverse of the pulse length used for decoupling. For the sake of the probe, a lower power of dpwr = 43 dB is used than for excitation (tpwr = 62 dB), therefore the decoupling pw90 is longer than the one used for ^{1}H detection [4]. The default value is calibrated periodically, but may need customization if you are running an unusual sample which has a ^{1}H pw90 significantly different from the value Varian uses as a default. We can easily calculate the pw90 corresponding to the suggested dpwr. Given that pw90 for ^{1}H was 50.5 at tpwr 50, it would be 101 at tpwr of 44. Given that a 6 dB step down in power produces a doubling (x2) of pw90, each 1 dB step down produces a factor of $\sqrt[6]{2}$ (x $2^{1/6}$ = x 1.12) increase in pw90. Thus at the dpwr suggested, of 43, we calculate a pw90 of 101 x 1.12 = 113 us [4]. Based on the entered pulse width, the software calculates a bandwidth for decoupling [5]. Because we will be applying 90° pulses in the course of decoupling, the decoupling bandwidth will be ≈ 1/pw90. We would like the bandwidth to be ≈ 2x the sw of our ^{1}H spectrum. Thus the parameter set displays 1/113 us = 1000 000/113 s = 8840, which is indeed > 2x my 3700 Hz ^{1}H sw.

The default transmitter frequency for decoupling is 0.00. This is just fine for most purposes. However for gourmet decoupling, or strongly shifted signals, you would set this to the value of tof your had for the ^{1}H spectrum (-430, see above).

The nOe enhancement will be discussed below. However before addressing this, we will collect the suggested ^{13}C 1D. Acquire>Acquisition suggests the use of 256 scans. This will take almost 10 minutes. In order to have the option of stopping early with retention of data, set up a system of 'blocks' (Acquire > Flags **Page 4**) [1]. Thus, every time we complete collection of a 'block' of data, the data set will be saved. We see a default setting of block size (bs) = 64 [2]. That means that if there is a power failure when you have collected 69 scans, the first block of 64 scans will have already been safely saved, and you will only loose 5 scans. If the failure occurs at 130 scans, 128 will have been saved and 2 will be lost. If a failure occurs at 63 scans, these will all be lost. For long runs, be sure you have a block size set. It would be

tragic if a failure at 5 am cost you an entire night's-worth of data collection. I recommend bs= 16, for extra safety. For a first look at my sample, which I know to be strong, I will use 64 scans (2 min). Click 'Show Time' and the 'Acquire'.

MoveSW and acquire again (just as for ^{1}H.) (**Page 5**). Note how well dispersed the signals are and how sharp they look, ^{13}C signals are indeed less sensitive to poor shimming, and even at the same number of Hz wide, they are less likely to merge with neighbors, which are spread over a much broader Hz range. The strong set of three lines at 77 ppm is the signal from natural abundance ^{13}C in the chloroform solvent [1]. That C is split into three equal-intensity lines by the one bound ^{2}H, which has a spin of 1 and therefore occurs in three spin states of m_I = -1, 0, 1. The chloroform signal can thus be recognized and used as an internal chemical shift reference. (if TMS is present, it's C is defined as 0 ppm (**Page 6**).

Stepping stones to more advanced experiments: pw90 and T_1 values.

As for ^{1}H, we will want a good value of the pw90 for ^{13}C, for fancy work. Also, as for ^{1}H, the best choice of delays will be based on knowledge of the T_1 values of your signals. Knowledge of the T_1s is essential if you have trying to quantify ^{13}C signals. C atoms that have no Hs attached, such as quaternary C or carboxyl Cs have much longer T_1 values than aliphatic Cs (they also do not benefit from the nOe, below). Therefore typical recycle times result in substantial saturation of these C sites, and therefore smaller signals than they ought to have. It is not uncommon for such a C signal to be overlooked altogether. The best strategy is to know your pw90 and your T_1 values, and to use these to determine an Ernst angle for use in collecting spectra (below). We begin by measuring the pw90, and then the T_1s. The method is exactly the same as that used for ^{1}H, so these notes will be brief.

First measure the pw90, as for ^{1}H. I based my array on the probe file value of 9.3 us for pw90 at tpwr=56. This predicts a pw360 of 37.2, so I had 8 steps beginning at 32 with a step size of 2. Don't forget that in order avoid having the software force your first spectrum upright, you need to go to Acquire > Future Actions and replace 'process' with 'wft dc ds' next to 'when experiment finishes'. I got a pw90 of 13.3 µs at tpwr of 56.

Now that we have a real pw90, we will be able to set up a bona-fide 180 ° pulse too, as required for the inversion-recovery experiment used to measure T_1. If we had used the 9.3 µs value in the measurement of T_1 values, we would have generated a seriously wrong pw180. (Note however that since most ^{13}C 1Ds are collected using a 45 ° excitation pulse, it is not a real problem if the tip angle is 35° or 55°).

Measure T_1 by drawing down 'Experiments' and choosing 'Convert current parameters to do ... > Relax. Measurements > Measure T1 (**Page 7**). In Acquire > Pulse Sequence, next to 'T1 mode' select 'inversion recovery' [1]. Below that enter first guesses for Min T1 [2], Max. T1 [3] and Total Exp Time [4] then click 'Array Relaxation Delay d2' [5]. I made changes to contain the duration of the experiment, such as limiting myself to 6 values of d2, beginning at 0.25 and

extending to 8 sec. by factors of 2.
Once you are satisfied that your array includes at least six times, a time shorter than your shortest-likely value, and at least one time longer that the longest T_1 you expect, click on 'Acquire'.

Once the acquisition is complete, navigate to Process>T1 Analysis (**Page 8**). Click Display Last Spectrum [1], set the threshold and then click Do T1 Analysis [2], check that the number of lines found is acceptable and then click Display all fits [3]. The recovery curves [4] show that different C resonances recover at very different rates. Don't forget that in order to see the results, you will have to go to the right-hand edge of the results panel [1] and drag the *inner* vertical slider down [2]. There are two sliders !! (**Page 9**). The numerical results show that my T_1 values range from ≈ 0.7 s [3] to almost 10 s [4].

If you want to be able to interpret signal integrals in terms of spin concentrations (numbers of identical hydrogens), you must be sure that *all* the hydrogen nuclei are equally relaxed at the beginning of each scan. Therefore you must set (at+d1) > 3*T_1, at least, where T_1 is the longest T_1 in your molecule. (at+d1) > 5*T_1 is slightly better. (You also have to forego the nOe, below.)

Ernst angle

It is common to have long ^{13}C T_1s, and no-one wants to wait for 3x T_1 between scans when T_1 is long, for maximal signal-to-noise per scan. Instead, when you have a weak sample, you are more concerned about amount of signal obtained per hour of spectrometer time. To maximize the latter it is much better to use small excitation tip angles which allow shorter recovery times between scans. For a given value of T_1 and recovery time between scans, the optimal excitation pulse tip angle is called the 'Ernst angle' , α_e , where

$$\cos(\alpha_e) = e^{-del/T1}$$

del is the total relaxation time to be allowed (d1 + at) and T_1 is the longitudinal relaxation time of the spin of interest (Ernst & Sternlicht, 1972, J. Magn. Reson. 6: 167-182). Use the 'ernst' macro to maximize signal per hour of machine time. Type **ernst(T_1, pw90)** where you include your longest T_1 value in units of sec. instead of T_1 , and your pw90 value in μs (example: ernst(7.3, 13.3)). If your experiment already contains a good value for pw90, you can type **ernst(T_1)** (example : ernst(7.3). In both cases, the software will insert the theoretically ideal pulse width, and report the corresponding tip angle. For example, I used a compromise T_1 value of 3 sec. in an experiment containing a calibrated ^{13}C pw90, a d1 of 3 s and an at of 0.6 s, and typed **ernst(3)** . The spectrometer returned with "estimated ersnt angle 72.5 degrees, 10.7 us". When I changed d1 to 1 sec. and typed **ernst(3)** I got "estimated ersnt angle 54.1 degrees, 8.0 us".

Page 10 compares two spectra one collected in 59 sec (no ernst) and one in 61 sec (ernst).

^{1}H nOe

nOe stands for nuclear Overhauser effect. In general, this is a mechanism of magnetization transfer. Here, transfer of magnetization from a large-magnetic-moment nucleus (^{1}H) to a weaker magnetic moment nucleus produces a much stronger spectrum for the weak nucleus than would normally be expected. The mechanism is longitudinal cross-relaxation so the sensitivity enhancement builds up on the time scale of T_1. It also applies only to ^{13}C sites that have nearby ^{1}H. Thus, isolated ^{13}C signals can remain weak when CH_n signals become stronger, and you loose the possibility of interpreting integrals in terms of the concentration or stoichiometry of C sites.

The nOe is produced by irradiation of ^{1}H, as for decoupling, In addition to setting up decoupling in the Acquire > Channels page (above), you can go to Acquire >Default C13 where you see H1 dec. mode (**Page 11**). The 'yyy' setting for decoupler mode (dm) is equivalent to 'Decoupled + NOE' here. The NOE benefit accrues during d1, which is segment A of the sequence [1]. Other options are 'Decoupled - NOE', 'Coupled + NOE' and 'Coupled - NOE'. These correspond to 'nny', 'ynn' and 'nnn'.

For demonstration purposes, I set a long d1 and a short at, so that any nOe that accrues during at can dissipate again, if nOe is not desired. To compare all possible combinations of decoupling and nOe, I would like ^{1}H irradiation on in A and on in C, off in A and on in C, on in A and off in C, and off in both A and C. I set this up by typing **dm='nnn', 'ynn','nny', 'yyy'**. (The value given for interval B in principle does not matter since the duration of B is zero.) As shown in **page 12**, you can check on these non-standard arrays by selecting Acquire>Overview [1] and choosing 'Array' [2]. You can see that turning on the ^{1}H irradiation during interval A (second and 4th scans) strengthens the signal compared to the analogous scans with the ^{1}H irradiation off (1 and 3) [3] . This is purely an nOe effect. The simplification provided by decoupling during interval C is seen by comparing spectra 1 and 3 (**Page 13**) . (Note that it is very difficult to decouple without also producing some nOe enhancement.) To see just spectra 1 and 3, type dssa(1,3,2) (display spectra 1 to 3 in steps of 2).

Page 14, lower pair, compares the effect of decoupling during A (designed to produce an nOe) (**dssa(3,4)**). The upper pair demonstrates the effect of turning the decoupler on during interval C (designed to only decouple) (**dssa(2,4)**) .

DEPT: distortionless enhancement by polarization transfer

Begin with your beautiful calibrated ^{13}C 1D.

Under 'Experiments' go to 'Convert current parameters to do ... >X-H Multiplicity Determination'.

The DEPT pulse sequence employs a combination of delays and pulses that allow the spin states of attached ^{1}H to affect the sign of ^{13}C signals (**Page 15**). You must use a correct pw90 for ^{13}C [1] at the power to be used [2]

The ^{1}H pulses used here must also be a ^{1}H pw90 calibrated for the power used. The current value for the ^{1}H pw90 can be seen in the pulse sequence display however I was not able to find a place to enter this information in any of the panels. The ^{1}H pulse width is associated with the parameter name pp in this sequence ('pp' for proton pulse) and the corresponding ^{1}H power is associated with the parameter name pplvl (proton pulse level) (see below). Therefore, to enter correct values, type in **pp=13.6 pplvl=62** (substitute your values for mine). You can confirm that the spectrometer will use them by displaying the sequence again and viewing the ^{1}H channel (second row) 90° pulse length [1] or looking at the values in Acquire>Overview [2] for pp and [3] for pplvl (**Page 16**).

Spectra in which ^{13}C sites with different numbers of H attached acquire different signs are added or subtracted one from another to give sum and different spectra in which the only signals that survive have either one H, two H or three H attached to the C. The collected subspectra are shown on **Page 17**. Notice that different signals change signs between different pairs of spectra [1], or do not change signs [2]. Thus addition of some pairs will cancel some signals and reinforce others.

The sums and differences are shown on **Page 18**. The resulting spectrum at the bottom of the stack is a subspectrum containing only signals from C with no H attached, the second from the bottom includes only methyne Cs, the second spectrum from the top includes only methylene Cs and the top subspectrum would contain the methyl Cs if strychnine had any. This is the set of results that will automatically appear upon completion of the experiment, provided that you leave 'Acquire>Future Actions' with 'when experiment finishes' set to 'process'. If you deviate from the default expectations and do other processing, you can still go back and have Varian repeat the automatic processing by typing **process**.

Viewing multiple spectra from multiple experiments, at once.

Make sure that all the data sets you want to compare are loaded into different experiments ('workspaces'). Go to one of those experiments. Under 'Edit' choose Viewports and choose as many viewports as you will need, then close. The current example calls for 5 viewports.

Select the 'Viewports' tab [1]. (**Page 19**)

Select the viewports [2] that contain the experiments of interest [3] by activating their checkboxes [2]. Select either one spectrum over the other or one beside the

other [4]. Activate the one containing the DEPT analysis [5] and type **dssa** to get all four subspectra. (You can activate a workspace either by using its radio button [5] or by clicking in its header bar [6]. The example shows the ordinary ^{13}C spectrum as the active one.) Adjust their vertical scale, for example by typing **vs=vs/2** (cut vertical scale (vs) in half) and then **dssa** again (dssa = display stacked spectra auotmatic). The example compares the DEPT results in the upper viewport with a standard ^{13}C spectrum in the lower viewport. I have activated color-coding to present the different data sets in different colors [7]. On **page 19**, the spectra do not have the same frequency axis. The software can fix this. Click on 'Overlay Viewports' [8] then a new option appears, 'Stack Spectra' [9]. Select this to cause the software to reconcile the two frequency axes (very cool).

Under Viewport Layout again click on the icon for horizontal panels one above the other to get our display [1] (**Page 20**). The upper panel now contains the first DEPT subspectrum Activate the upper panel either by clicking in the header bar that contains its name and address [2], or by selecting its button in the left-hand side panel [3].

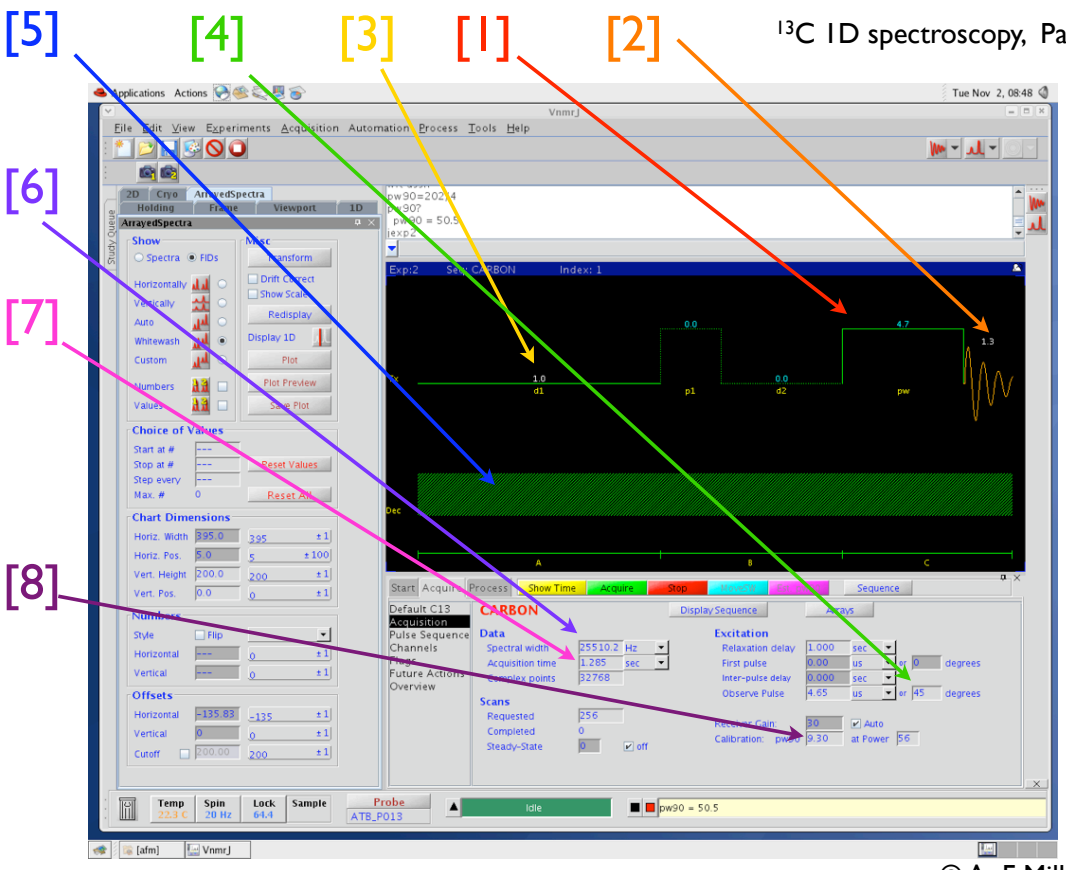
Then type **dssa** (display stacked spectra automatic).

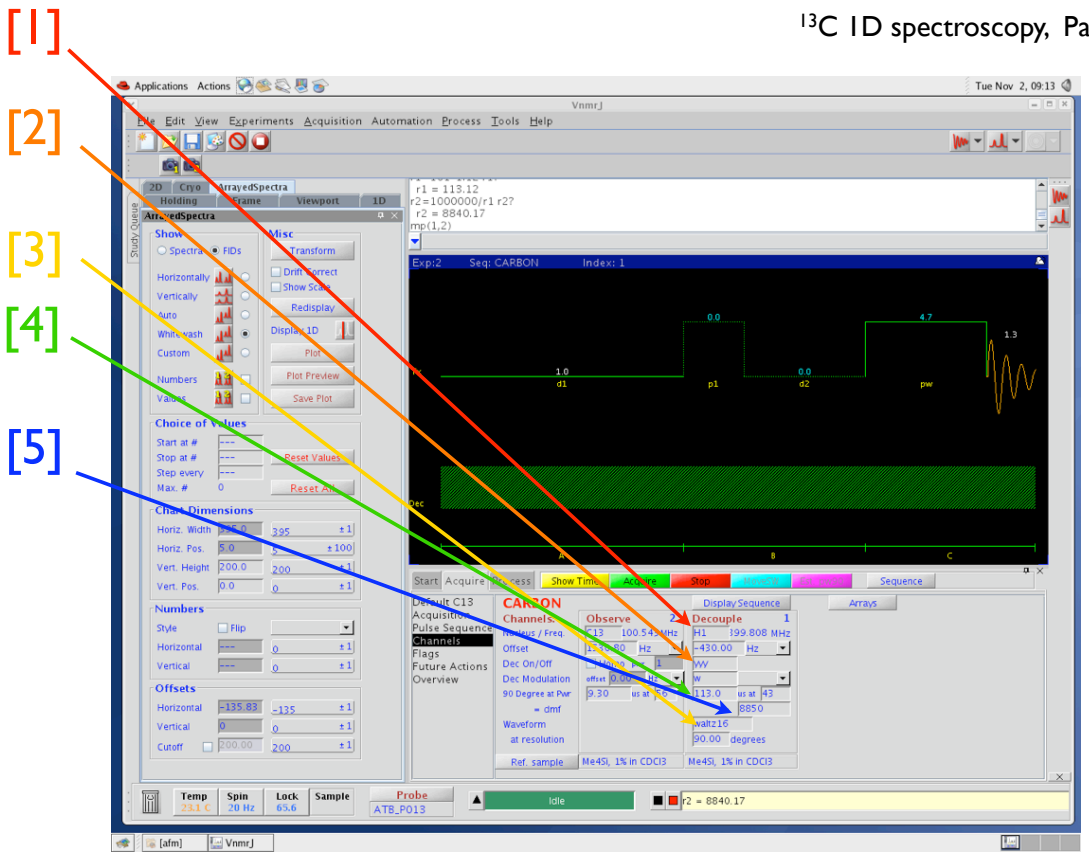
This produces the view on **page 21**. Notice how each of the signals in the complete ^{13}C spectrum in the bottom viewport is present in one of the rows of the upper panel. Note that the bottom row of the DEPT experiment is very weak though. These are the quaternary Cs and they are not strong in any of the individual DEPT spectra (see page 17). The fact that the second row has intensity at the position of the chloroform signal suggests that strychnine has a ^{13}C at that chemical shift as well. The first row also displays the four aromatic Cs that each have one H, near 12 ppm We will test that possibility with future experiments. The emptiness of the top row indicates that strychnine has no methyl groups, which is correct.

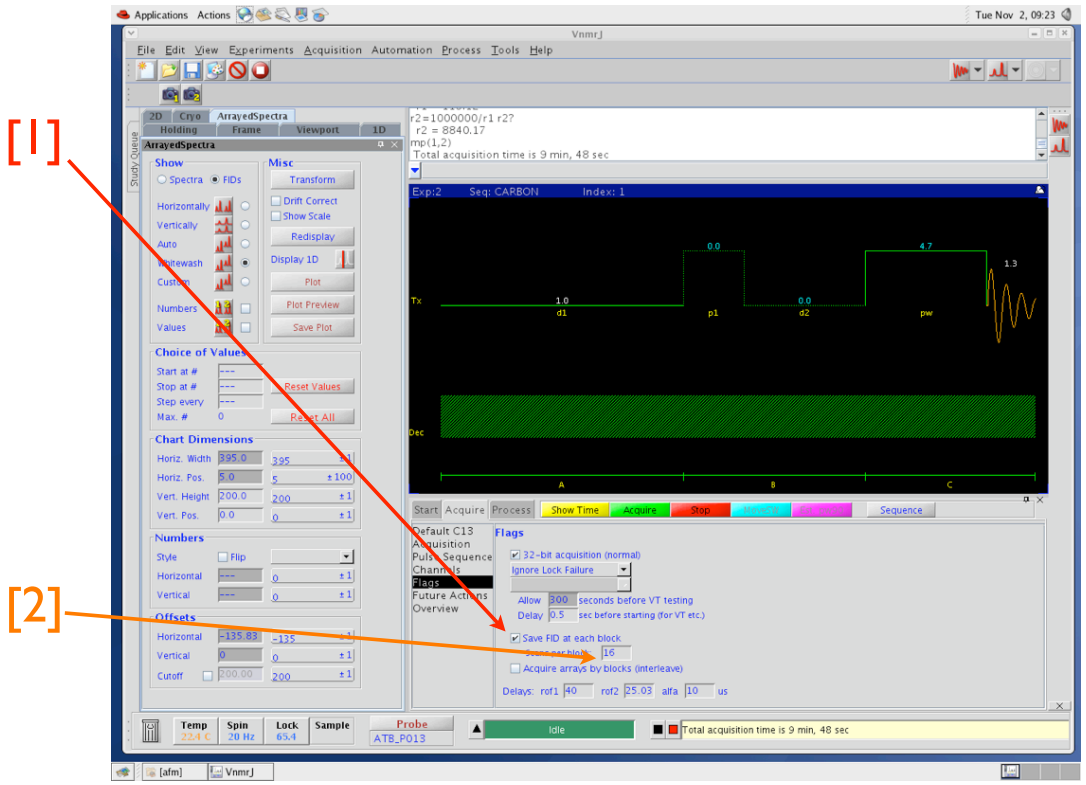
As you can see, a clean DEPT experiment provides very valuable categorization of the ^{13}C resonances that is extremely useful when assigning the signals of a spectrum to the structure of a molecule. Despite the fact that 1D spectra are given less prestige than 2Ds, this one is really useful.

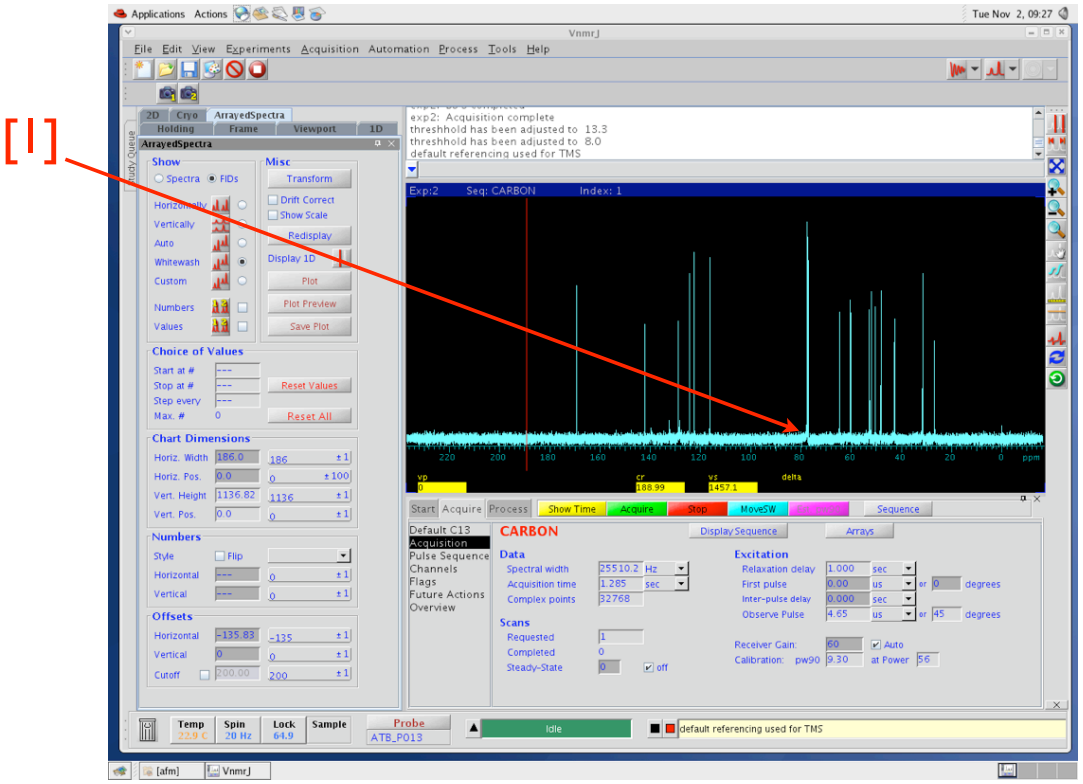
^{13}C spectra

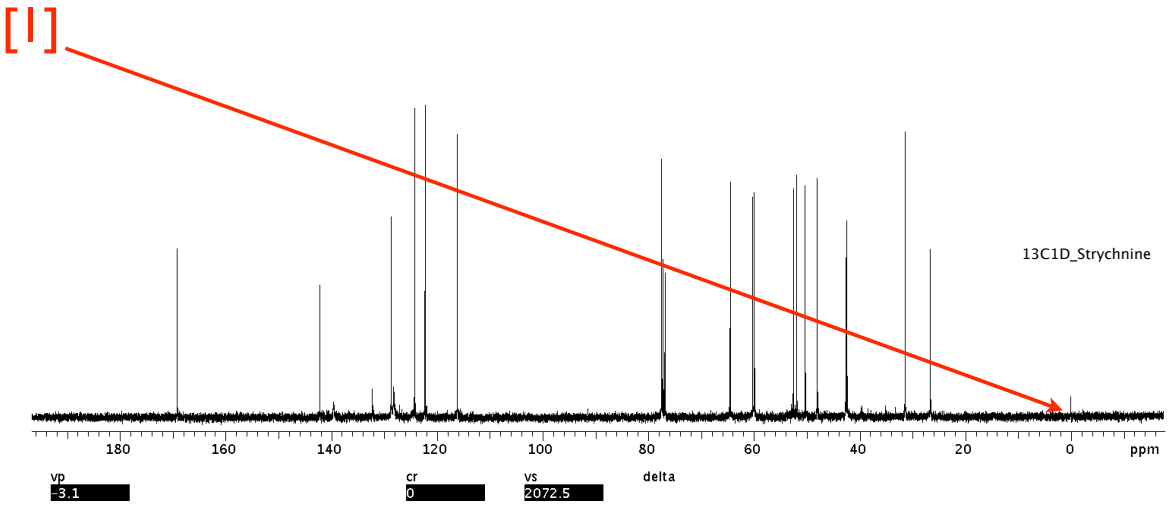
A simple 1D,
Measurement of ^{13}C T_1 and
DEPT to distinguish Cs bearing different numbers of ^{1}H.

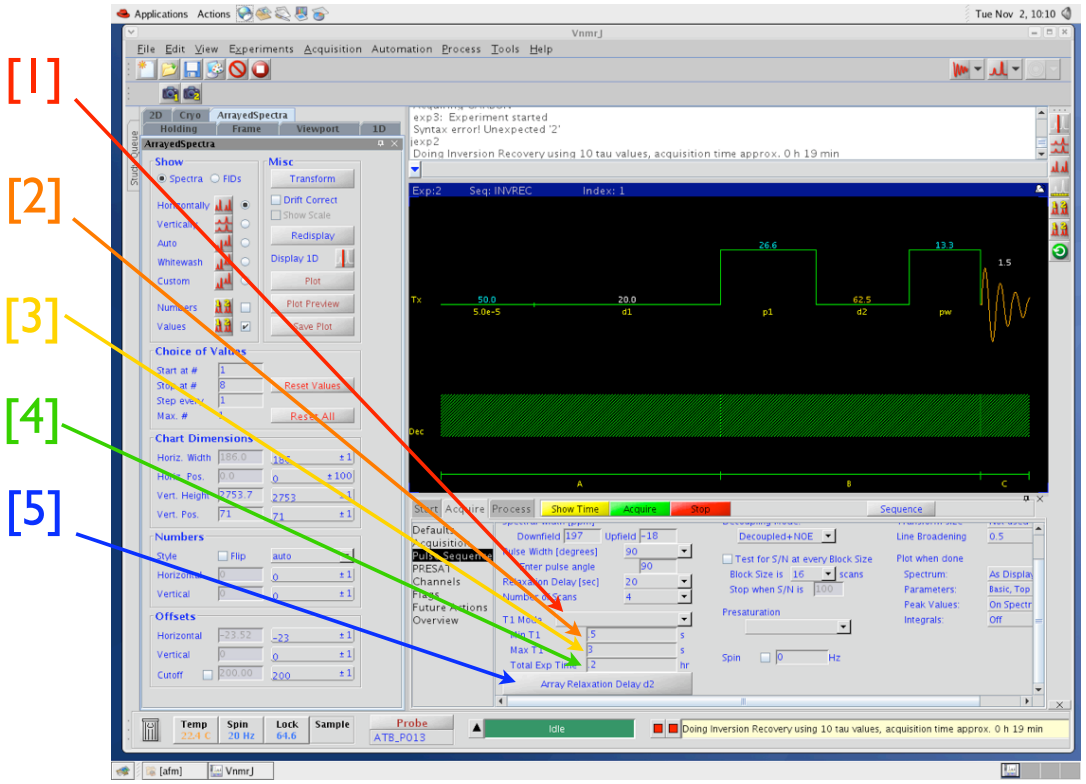


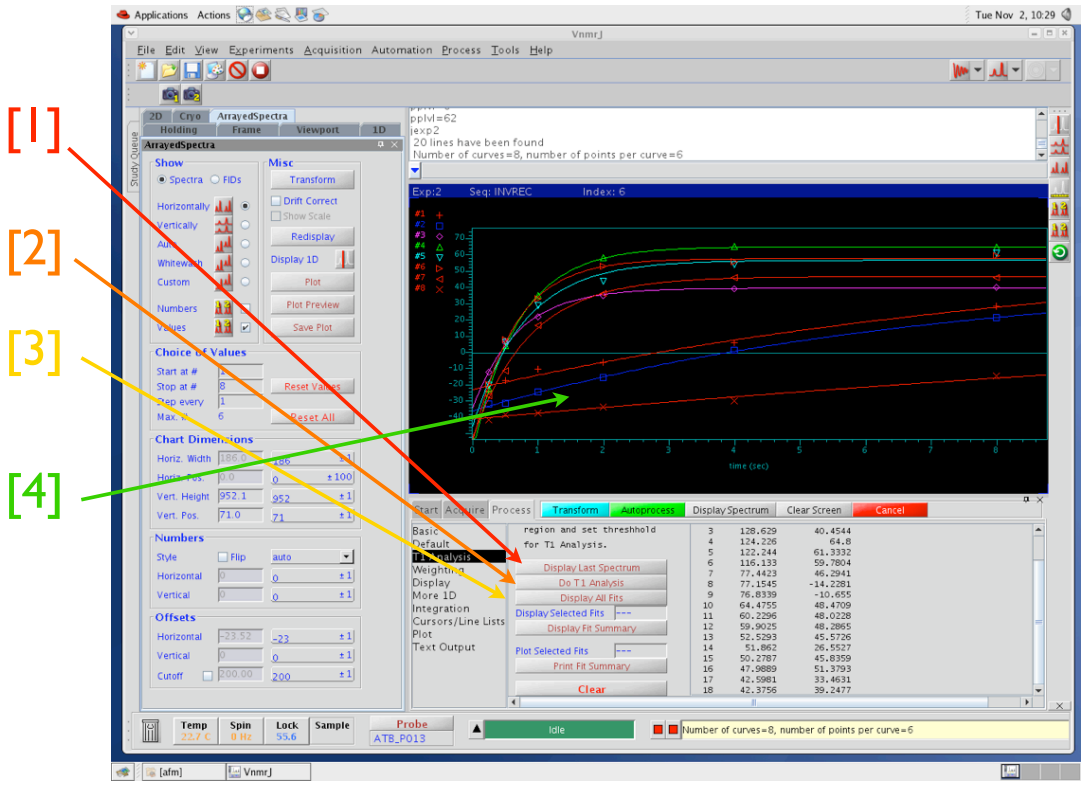


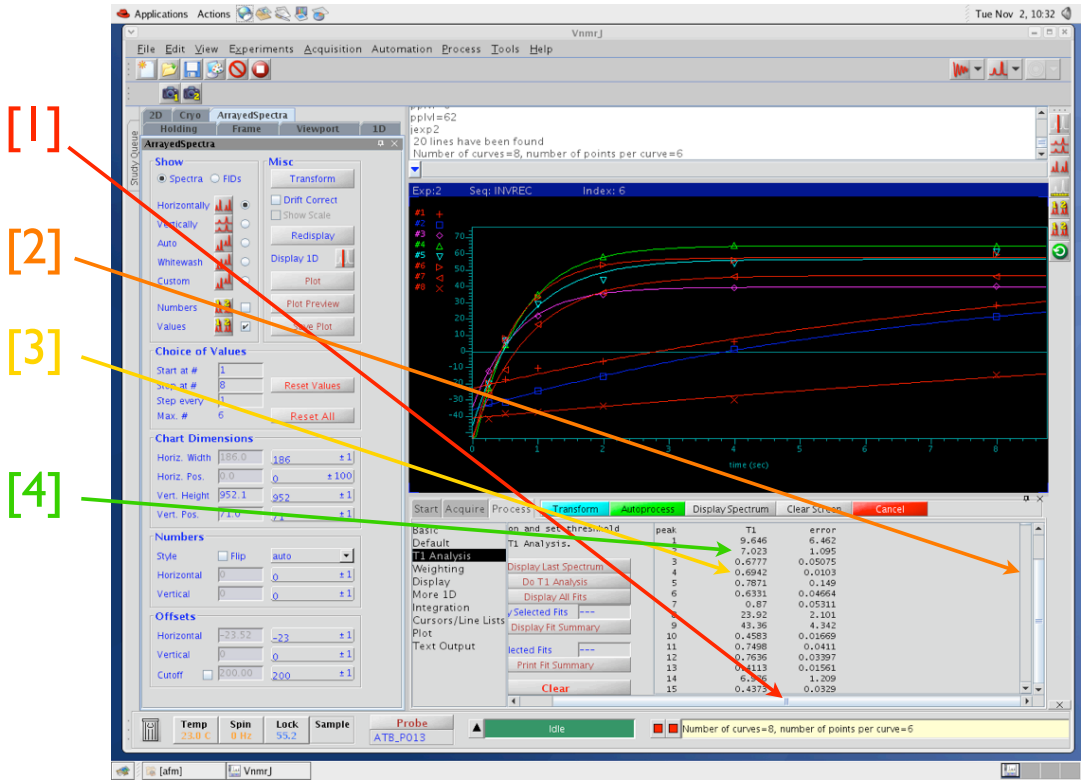




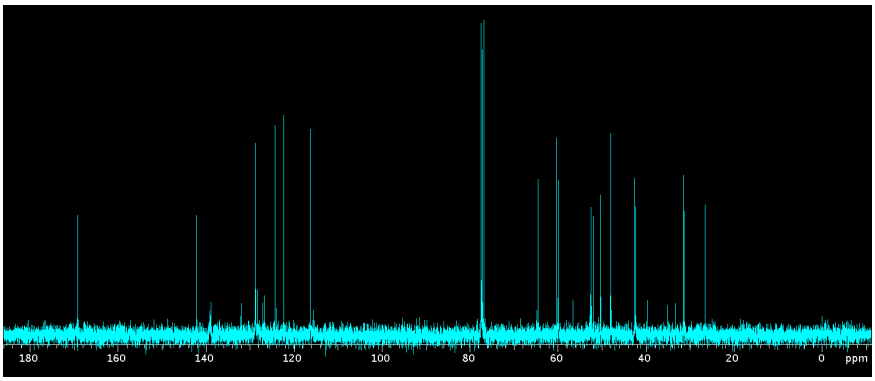




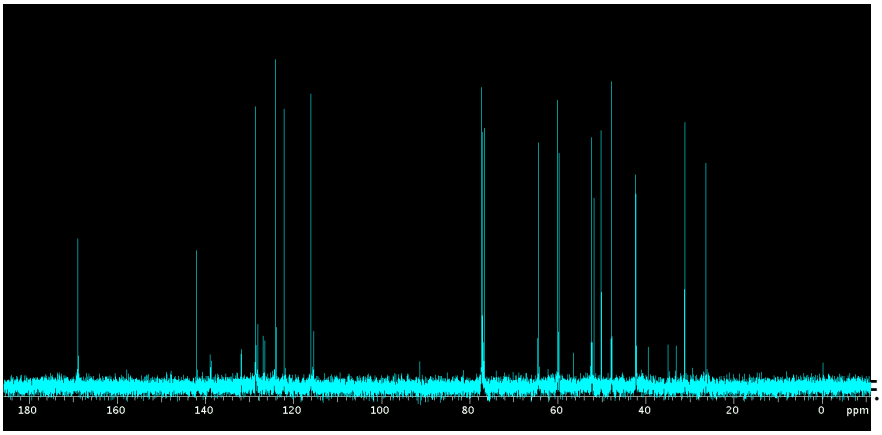


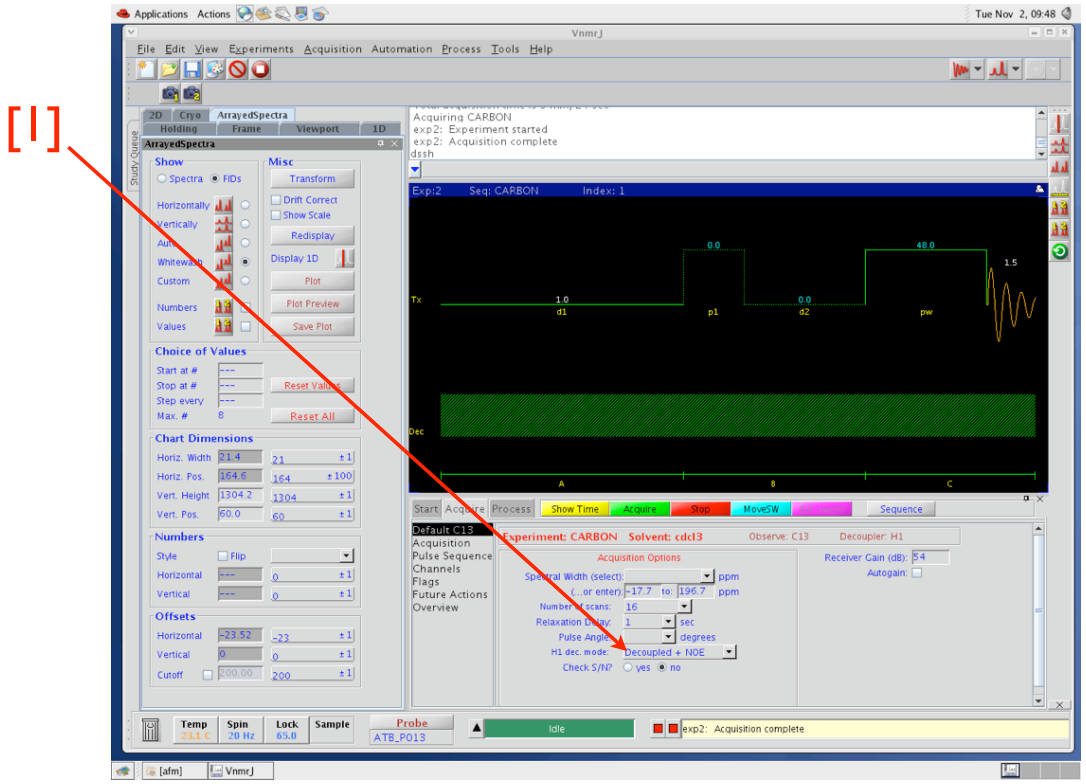


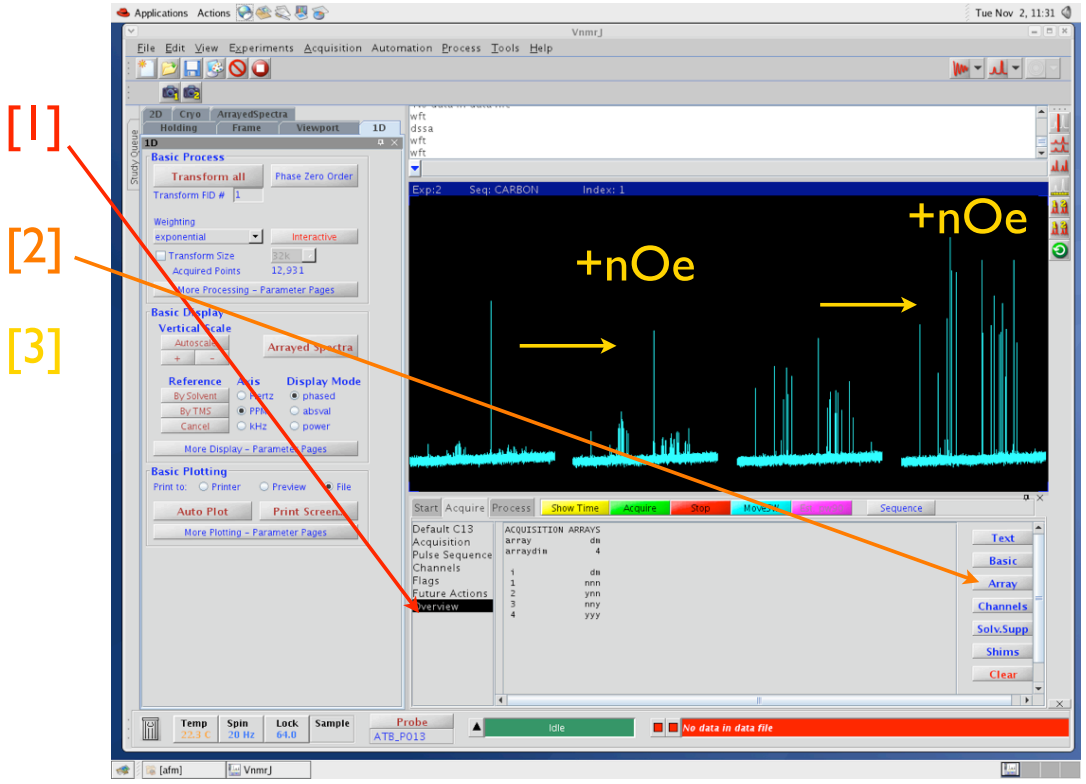
Decoupled
+ nOE
3 sec. d1,
0.6 sec at
90° excite
16 scans



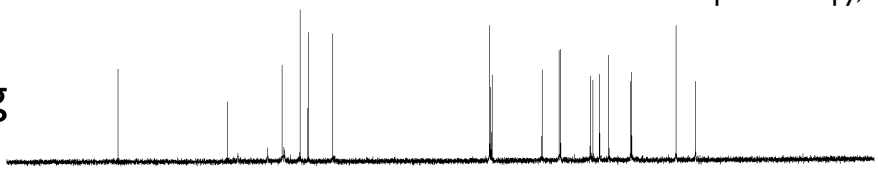
Decoupled
+ nOE
1 sec. d1,
0.6 sec at
54° excite
30 scans



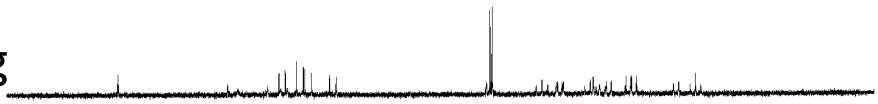




\+ decouping

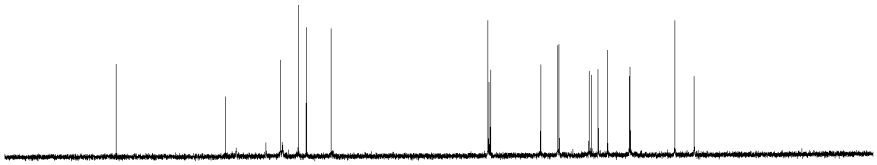


no decouping

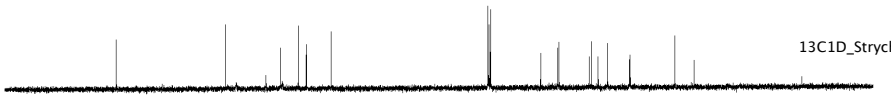


13C1D_Strychnine_NOEPMdcpl

\+ NOE



no NOE



13C1D_Strychnine_dcplPMNOE