Intro to 2D NMR: Homonuclear correlation
COSY, lr-COSY, and DQ-COSY experiments

Use Callisto (Av-500DCH) and Artemis (Av-400) and Calisto for this week's HW.

Many choices exist for samples to be used for this and the following 2D labs:

1. **A research sample is OK (it should be a clean compound of moderate complexity).**

2. **An unknown compound: We can provide these, but they tend to be complex. High quality data will be obtained during this HW, but these compounds are not optimal for first time attempts at using 2D spectra to perform assignments.**

3. **Quinine in C_6D_6. An on-line guide provides all assignments:**
   http://www.chem.wisc.edu/~cic/nmr/NMRdatab/res_cmpd/pdfs/quin_assignment_example-for-pdf.pdf

4. **Quinidine in C_6D_6. This compound is of good complexity, and assignments are similar (but not identical) to those for quinine.**

5. **Sucrose or other samples used in previous homeworks.**

**Read/work through this HW prior to going to your lab session!**

There are a number of areas of discussion interspersed through this HW, labeled by D#, that should be read through prior to working on the spectrometer. Doing so will significantly reduce the time you will need on the spectrometer.

Reading – **Intro to 2D NMR: Claridge sections 5.1-5.5 (esp. 5.1)**
              – **COSY flavors: Claridge sections 5.6, 5.7**

Goals   – **Learn basic terminology of 2D NMR, and the utility of various major flavors of COSY (which includes TOCSY, which will wait until next week)**

D1 2D spectra are acquired in a manner that is analogous to kinetic data sets that have many spectra that are often presented as *stacked plots*. You will learn more about the second dimension in lecture. It is built experimentally by acquiring many one dimensional spectra with a delay in the pulse sequence, usually defined as t_1, being changed incrementally. The number of spectra that are acquired for the 2nd dimension is set by the parameter **TD1** (Bruker) [or **ni** (Varian)].

A COSY pulse sequence has a 2nd 90° pulse added to the standard one pulse 1D sequence:

90°   90°   **Standard COSY sequence**

t_1 (d0) t_2 (aq)
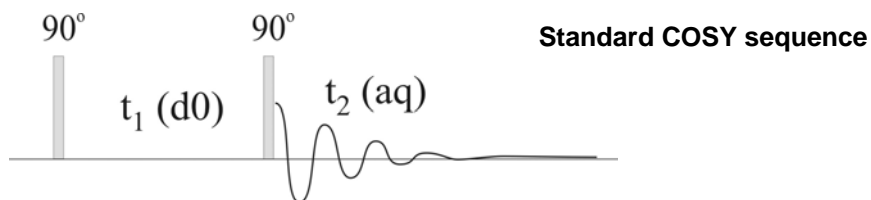
The delay between the two 90° pulses is t_1; on the spectrometer it is the delay **d0** (Bruker) [or **d2** (Varian)]. This delay will be increased by fixed increments (equaling 1/**SW1**) as one 1D spectrum after another is acquired: **d0**=0 for the first spectrum, 2nd spectrum **d0**=1/**SW1**, 3rd spectrum **d0**=2/**SW1**, and the nth spectrum **d0**=(n-1)/**SW1**. t_1 is 1st time period, and is also called the *indirect dimension*; it is also called the *evolution time*. The 2nd time period is t_2, and is the same "normal" acquisition period as used in 1D spectroscopy; it is important to keep in mind that 2D NMR consists of the acquisition of a series of 1D spectra. After two Fourier transform, one along each dimension, t_1 and t_2 are changed to F1 and F2, respectively.

Resolution takes on different requirements in 2D spectra, typically needing to only resolve different spins (or multiplets) from each other. The need to resolve a multiplet to extract J-couplings is removed or reduced. Lower resolution is therefore acceptable in a 2D spectrum. Information content in a 2D experiment arises from **crosspeaks**, which are formed through evolution (during t_1; in COSY, ^1H-^1H J-coupling is the useful component that evolves) and mixing of magnetization (here done by the 2nd 90° pulse).

Fundamental questions in 2D NMR then become how do crosspeaks arise, how intense are they (what needs to be optimized to insure that they are sufficiently intense), and what else needs to be optimized (including resolution) to optimize experiment times.

> ➜ *Use Callisto 1st in TopSpin mode to acquire spectra 1, 2, and 4 (in sections I, II, III).*
> *Then move to the Av400 and use it in standard IconNMR mode to acquire spectra 3 and 5.*

I. _Resolution in absolute-value COSY:_   (this is the most common type of COSY run in our lab)

D2 In this HW, we'll explore the issues of resolution and crosspeak intensity in COSY 2D NMR. Resolution is (relatively) easy to understand: it is controlled by the acquisition time in the 2nd dimension, just as it is in 1D NMR. In the detected dimension ($t_2 \rightarrow F2$):

$$\mathbf{AQ} = \text{(number of points acquired)} \times \text{(time per point)} = \mathbf{TD \times DW} \qquad [1]$$

The tricky part is Nyquist's theorem:

$\mathbf{SW = 1/(2DW)}$, so $\mathbf{AQ = TD / (2 \times SW)}$.

In the indirect dimension ($t_1 \rightarrow F1$), we just label everything with a one:

$\mathbf{AQ1 = TD1 / (2 \times SW1)} \qquad [2]$

Annoyingly, you can't type the parameters this way into TopSpin, but they show up in columns (1 *after* 2, of course!) in the ACQUPARS (**eda**) panel.

For a 2D COSY spectrum:

digital resolution ~ 1/AQ1 $\qquad$ [3]

Note that in normal COSY data, **SW = SW1** and **TD1 < TD**.

	F2	F1	Frequency axis
⌃ Experiment			
PULPROG	cosygpppqf ... E		Current pulse pro
AQ_mod	DQD		Acquisition mode
FnMODE		QF	Acquisition mode
FnTYPE	traditional(planes)		nD acquisition mo
TD	2048	400 **TD1**	Size of fid
DS	8		Number of dumm
NS	1		Number of scans
TD0	1		Loop count for 'to
⌃ Width			
SW [ppm]	12.9864	12.9864 **SW1**	Spectral width
SWH [Hz]	6493.506	6493.506	Spectral width
IN_F [µsec]		154.00	Increment for del
AQ [sec]	0.1576960	0.0308000 **AQ1**	Acquisition time
FIDRES [Hz]	6.341315	32.467533	Fid resolution

Probe: not defined

> *The digital resolution in COSY spectra is dominated by the indirect dimension parameter **TD1**.*
I.e., the number of ^{1}H spectra acquired to form the 2D stack is a crucial parameter during COSY experiment setups. [The other three crucial parameters are the *COSY-type selected*, **D1**, and **NS**.]

We're now ready to play around with resolution. In this section, we'll use the simplest, most common form of COSY utilizing the experiment (parameter set) **COSYGPSW**.

One might wonder how small **TD1** can be and still obtain usable data, since experiments get shorter in total time linearly with **TD1**.

a) **Spectrum 1**: <u>Setup a very low resolution cosy</u>, using experiment/parameter set **COSYGPSW**.

    i) Optimize the spectral window using [SetLimits] (as done with the HSQC in HW#5) with the ^{1}H 1D spectrum (that you should have already taken to check shimming and as a chem. shift reference). Since COSYs are homonuclear, the SETLIMITS will setup **SW/SW1** and **O1p** for both F1 and F2.

    ii) Write down the **SW** and **O1p** values from the SETLIMITS section. These will be needed in section III.

    iii) Now setup the experiment to have 0.5 to 1 ppm resolution (compute **TD1** to match this resolution from eqns 2 and 3).

D3   Does the above experiment make any sense? One might well think not — are you kidding me, 1 ppm resolution?? — and in general that negativity would be correct. You'll of course work the data up and see what you think based on actual spectra. But note how fast the experiment can be done: you're asking for just a few ^{1}H spectra to be acquired. **NS**=2 is best to do, but **NS**=1 works ok and is the correct choice when rapid acquisitions are required. Perhaps monitoring of a moderately fast reaction can be done with this type of COSY spectrum, and provide useful information (e.g., the structure of an intermediate compound)? Maybe…. There are better methods for taking fast data, and we'll discuss some of those briefly in lecture. Published data using those other methods have included fast TOCSY (a similar experiment to COSY) and HSQC/HMQC.

b) **Spectrum 2:** <u>Acquire a "standard" cosy</u> (use new↵ and read in a new set of parameters using **COSYGPSW**). Calculate the digital resolution for this spectrum.

D4   If you find it amazing that even this "improved" resolution works, you're not alone in your first impressions. For years after COSY was first invented, prominent NMR spectroscopists believed that **TD1** had to be larger than the value used here (128). It took much empirical evidence, and a more precise understanding of COSY mixing and evolution to convince everyone otherwise. We'll investigate these issues further in the next section. The key requirement for digital resolution in 2D NMR is that it resolves one multiplet from another. That is a far simpler requirement than that of 1D NMR, where the multiplet itself must be resolved to enable measurement of small ^{1}H-^{1}H J-couplings (or ferret out overlapping peaks). The ability to resolve coupling within the multiplet is removed by the experiment itself: *crosspeaks* tell us about the coupling, not measurement of the coupling itself. Even so, it is important to keep in mind that **TD1**=128 is not sufficient for many research situations, so **TD1** is a critical parameter to optimize.

   c) **Spectrum 3:** **Move to the next section and acquire Spectrum 4 in TopSpin mode first. Then use IconNMR on the Av400 to queue this and spectrum 5 to the nightqueue there.**

Last in this section is to acquire a "high-resolution" spectrum, by improving the digital resolution by a factor of four over that for the default setup (i.e., still using **COSYGPSW**, but now in IconNMR). Use the default settings applied in IconNMR, but with:

i) **NS** = 1 (for a research compound, you should leave **NS** $\geq$ 2; it gives much better data quality).

ii) **TD1**=512, to provide higher resolution.

In research involving complex compounds, **TD1**=512 is fairly common. **TD1**=1024 would not be unusual, and **TD1**=1600 occasionally going to 2048 are also used when multiplets are strongly overlapped. [NOTE: Going to such large values with HSQC is *not* recommended, as will be discussed in the next lab.]

123☞ Work up the 3 COSY spectra acquired above. Spend some time with the processing, using the following steps as a guide:

   a) In properties (double-left-click the spectrum background), I (cgf) prefer 40 contours (positive and negative) and Scaling=1.2 as defaults for 2D spectra. This will slow down some operations, so you may need to back off to fewer contours on occasion if things get too slow.

   b) The Fourier transform sizes in both dimensions need to be identical in order to "symmetrize" the data (see step f below). This can be checked using **Zero Filling and LP…** within the button.

   c) The apodization (**W**) should be set to Sine Square at 0° in both dimensions; click f1 f2 to switch between the two dimensions. For F1, the **First Point = 0.5** is normal.
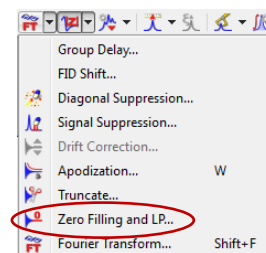
   d) Set traces (as done in an earlier lab) by loading the proton spectrum with the COSY into the same document. Then drag the proton spectrum onto the COSY.

   e) The COSY should look fine at this point. But occasionally MNova does not put the data into magnitude mode. Expansion of a section of the spectrum will then show many positive-to-negative transitions, shown below as Phased. If so, click and choose "Magnitude along F2". Your display will then look as shown in the middle (as long as you're displaying a Contour Plot (in).

   f) Usually the data is improved by symmetrizing it, shown on the far right below. Be wary of symmetrization artifacts; compare spectra prior to and after processing to decide if correlations are real or potential artifacts. Perform symmetrization thru the menu selections: **Processing → Symmetrize → Cosy-Like** .

Plot each spectrum as .pdf and .mnova and upload.

| **Phased** | **Magnitude Mode** | **Svmmetrized** |
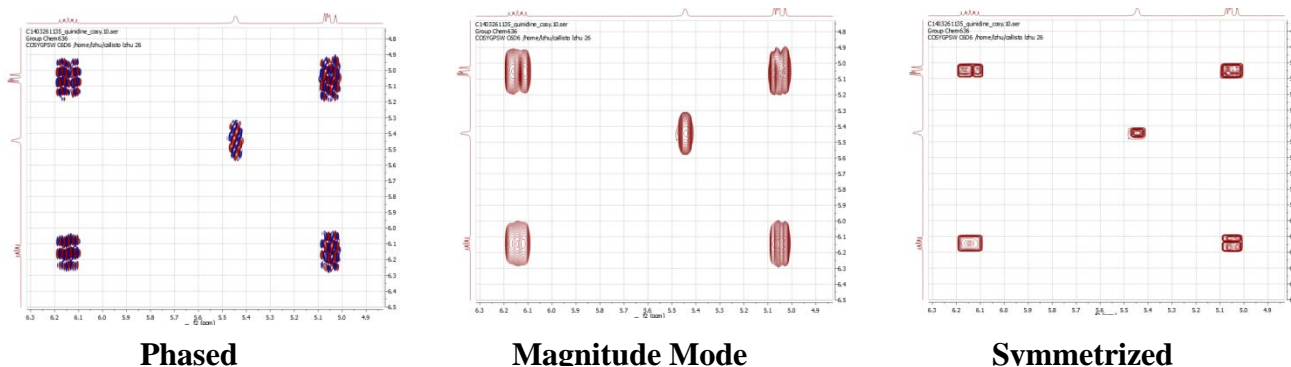
II. *Crosspeak intensities — Long-Range COSY:*

➔ *Use Callisto in TopSpin mode for this section of the HW.*

D5 So what's the deal with the crosspeaks? You should have observed that most crosspeaks are least intense in the poorly resolved (first) spectrum you acquired above. But they won't consistently get larger when going from **TD1**=128 to **TD1**=512; some will, but some will not. Crosspeaks arise from transfer of magnetization from one spin in the first time period to a coupled spin in the 2nd time period, e.g.: $I_Y^A(t_1) \rightarrow I_Y^B(t_2)$. The transfer is sinusoidal in character: during t_1, the magnetization evolves as:

$$I_Y^A \xrightarrow{\ t_1\ } I_Y^A \cos(\pi J t_1) + I_Y^A I_Z^B \sin(\pi J t_1) \qquad [4]$$

During the acquisition (and ignoring chemical shift), this magnetization is detected as:

$$\xrightarrow{\ t_2\ } I_Y^A \cos(\pi J t_1)\cos(\pi J t_2) + I_Y^B \sin(\pi J t_1)\sin(\pi J t_2) \qquad [5]$$

The cos×cos term (starting as I_Y^A) forms the diagonal in the COSY spectrum. The sin×sin term (starting as $I_Y^A I_Z^B$) forms the crosspeaks. The larger the sin×sin term gets, the larger the crosspeak intensity at $\delta_A(t_1,\text{F1}),\delta_B(t_2,\text{F2})$ will be. From eq 5, when $t_1 \rightarrow 1/2J$, crosspeaks intensity maximizes since $\sin(\pi J t_1) \rightarrow 1$. Once past 1/2J, the crosspeaks will get smaller. At $t_1 = 1/J$, the crosspeak will vanish: $\sin(\pi J t_1) = 0$.

The evolution time t_1 is therefore critical to crosspeak intensity. Remember that t_1 is the incrementally increasing separation between the two 90° pulses in a COSY. It takes another spectrum to increase t_1 by another increment of 1/**SW1**. Getting to large t_1 values costs in increased experiment time. <u>What we want is just enough $\sin(\pi J t_1)$ to get the crosspeak well out of the baseline noise of the 2D spectrum.</u>

After a lot of experimentation and more detailed analysis, for standard COSY we end up with a relationship as follows (as discussed in lecture):

$$J_{\text{observed}} \ \geq \ \frac{1}{6 \times \mathbf{AQ1}} = \frac{\mathbf{SW1}}{3 \times \mathbf{TD1}} \qquad [6]$$

where $J_{observed}$ is the smallest J-coupling that we can be certain will give a crosspeak with sufficient intensity to observe. For a range of normal proton-proton couplings, 3-15 Hz, **TD1** = 128 to 512 works nicely. For smaller **TD1** values (and therefore faster experiments), crosspeaks involving smaller J-couplings may be difficult to identify. In such cases, increasing **NS** would help, but increasing **TD1** is a better option for small molecules. Larger **NS** gives somewhat better overall signal-to-noise; larger **TD1** definitely increases crosspeak intensity where J_{HH} is small.

What about couplings smaller than 3 Hz? Suppose you're interested in verifying a small coupling of 0.8 Hz (rotenone has many small couplings, as shown on pg 3 of the <u>document</u> at:

> http://www.chem.wisc.edu/~cic/nmr/NMRdatab/res_cmpd/pdfs/rotanone_assignm-COSY-new-driver.pdf.

Q1   From the analysis above, what would t_1 need to equal to insure observing a 0.8 Hz J-coupling?

Q2   If **SW=SW1**=8ppm, what would **TD1** have to equal?[1]

Q3   For a 2 scan COSY, how long would the experiment take? (You could use the spectrometer to estimate this, or realize that **D1**×**NS**×**TD1** is a good estimate.)

The key to all this is the rather extreme value you should have arrived at for **TD1** (see eqn 2 above). [**TD1**=12,000 on a 600 when the sinebell apodization correction is included!]

One way around this issue is simple: acquire spectra only when t_1 is closer to 1/2J. This is the essence of the long-range COSY experiment. If we put in a delay **D4**, typically ranging 50 to 200ms (for small molecules, up to 500ms works fine), crosspeak intensities arising from small J-couplings will be much larger.
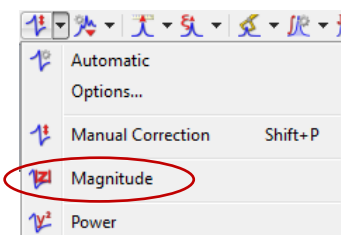
Long-range COSY sequence
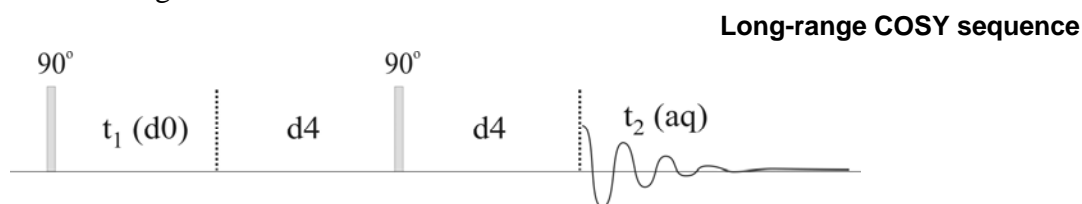
4☞   **Spectrum 4:** <u>Acquire a long-range COSY</u> (use that parameter set **cosylr.UW**) with **D4**=0.2s, **TD1**=128, and **NS** ≥ 2. Process, symmetrize, and plot. Identify a couple crosspeaks that increased in size from the standard COSY.

➔ *Use Callisto in TopSpin mode to acquire Spectrum 4.*

III.   *Phase-sensitive, double-quantum filtered COSY:*

D6 We'll discuss the conceptual part of dqf-COSY in two bites, starting with *phase sensitive*. To prove that phase sensitive is better, go to your ^{1}H spectrum in MNova, and put the spectrum into magnitude mode: each complex point $a + ib$ in the spectrum is replaced by $\sqrt{a^2+b^2}$. You'll see that this is not good for resolution. So why do magnitude processing at all? Well, we might be lazy and not want to

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[1] Since we're applying sinebell apodization, the 1/2J point needs to be in the *middle* of the acquisition, not at the end. That means that one needs **AQ**=1/J (not 1/2J) for maximum crosspeak intensity.

have to phase the spectra. But the result seems to be awful. We can improve it by applying a sinebell apodization. Try it on the 1D ^{1}H spectrum: using **W**, switch the apodization to **Sine Bell** at 0°. You'll see that this greatly improves the spectrum: the sinebell narrows the long peak tails arising from the magnitude processing. Unhappily, the ability to quantify the data is seriously degraded by sinebell apodization. Sinebell apodization is therefore not usually applied to 1D spectra. Exponential or gaussian apodization followed by the (relatively simple) phasing of 1D data is used instead to achieve both good resolution and quantitative spectra.

D7 Why then do COSY as absolute value (magnitude mode), as we've done in the previous two sections? Turns out it's not so simple to get a properly phased 2D COSY spectrum.[2] Claridge provides more detail on this topic if you are interested. But eqs 4 and 5 above tell the tale: the diagonal (arising from the I_Y^A term) has cosine phase, whereas the crosspeaks (arising the $I_Y^A I_Z^B$ term) are sin. The two sets of peaks in a COSY spectrum will always be 90° out-of-phase (in both dimensions): there is no way to phase this simple form of COSY spectra. Sinebell apodization recovers much of the lost resolution, and magnitude mode processing removes the issue of having to phase. Simple!

There is a more complex version of COSY that gets diagonals and crosspeaks in-phase by implementing a double-quantum filter (cool spin-physics! ☺). This improves resolution, and adds two features that can be valuable:

DQ-COSY: 1) The coupling *actively* involved in forming a crosspeak is always *anti-phase* (e.g., a doublet going plus-minus). Other couplings are inactive, and are in-phase (e.g, ++ or − −). See discussion and examples in Claridge, Figs 5.45 thru 5.51.

2) The double-quantum filter removes large singlets. To create double-quantum magnetization, two coupled spins are *required*. tert-butyl groups, a large HOD peak, etc., will be removed in this experiment.

Spectrum 5: **Queue this and spectrum 3 to the nightqueue on the Av400 running IconNMR in the standard mode (i.e., for all to use; do not stop or change the default setup!).**

Acquire a dqf-COSY[3] spectrum (using the parameter set **COSYGPDFPHPP**) using IconNMR. If the sample you've selected won't go on the 400, you can use Callisto. But otherwise (e.g., for quinine or quinidine, where we've provided samples), use the 400 for this experiment. On either spectrometer, put the experiment into the nightqueue.

Automation is often the simplest way to acquire such data, but it can present issues that are often more easily solved in TopSpin. A major issue with IconNMR in getting 2D spectra on many research samples is that by default the software attempts to automatically determine the optimum ^{1}H spectral window, and fails. This is particularly a common when the solute peaks of interest are small compared to other peaks in the spectrum. For the dqf-COSY, we will have you avoid the automatic **SW** determination.

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[2] TOCSY 2D data provide similar information as COSY, and TOCSY spectra are phased, providing better overall resolution and sensitivity (the latter is a big issue for large molecules, due to relaxation). Biochemists therefore prefer TOCSY. COSY is better for observing small J-couplings, and is therefore preferred for small molecule work.

[3] **D**ouble-**q**uantum **f**iltered COSY: double-quantum arises from two coupled spins, producing magnetization having product operator terms such as $I_X S_X$, $I_Y S_X$, etc. Singlets cannot produce such terms (having no coupling), and therefore are filtered out of dqf-COSY spectra.

a) Using the **Parameters → Edit all Acquisition Parameters** , set **SW, SW1** (for cosy-types, always **SW1=SW**) and **O1p** as found in section I. a) ii). You can find these parameters in either the pulse-sequence-optimized listing, **ased↵** or ⊓ , or the all-parameters listing, **eda↵** or **A** .

b) IconNMR will still attempt the automatic spectral window optimization unless you "turn off" the automation routine that performs that chore. You can most easily find this automation routine in the **eda↵** or **A** listing, under the AUTOMATION link:
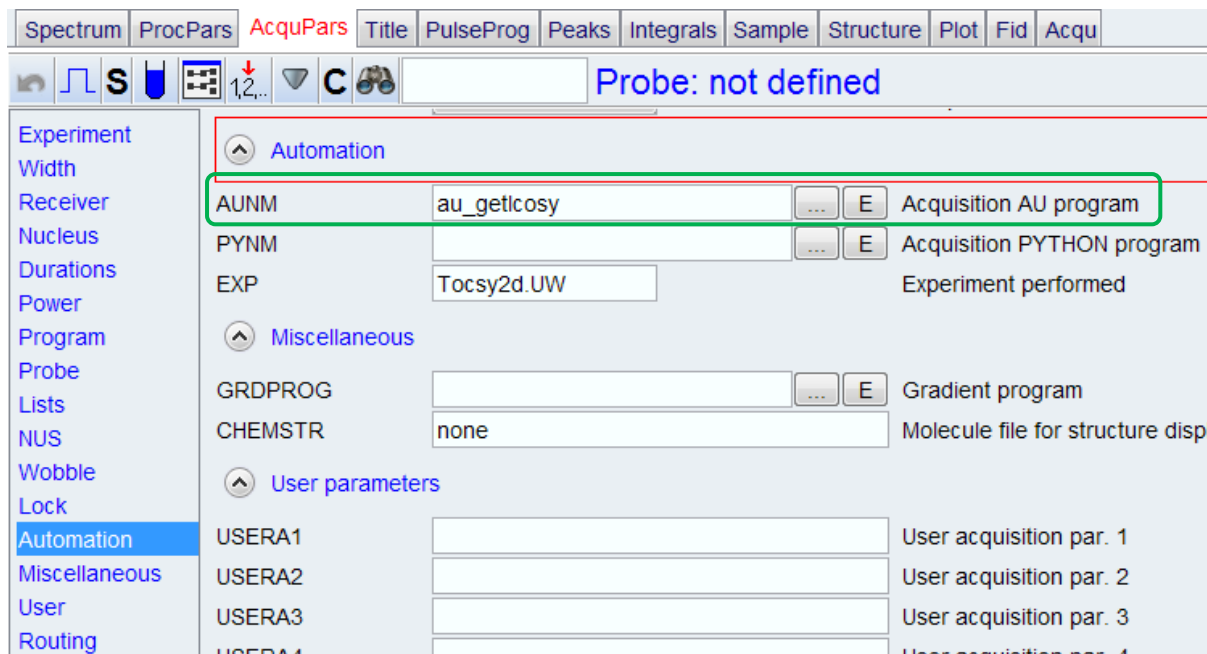
AUNM = **au_getlcosy** will "get limits" from the proton spectrum pointed to in the Icon setup.

Change this to: **AUNM = au_zgcosy** to turn off the spectral window optimization.

The automation routine above also does not perform an RGA on the COSY spectrum itself, but rather on the proton spectrum.

Use **TD1**=512, and setup a minimum phase cycle (remember to consult the end of the pulse sequence listing). Queue the experiment to the nightqueue.

5☞ Process, phase (should be close to correct initially), and plot the full dqf-COSY.

6☞ Zoom in and identify active versus inactive couplings for at least one coupled pair. Plot this expanded spectrum with annotation pointing out the identified correlations.

Upload 6 plots as .mnova and .pdf files, and answer 3 questions.