Today's Experiments: HOMONUCLEAR CORRELATION

1. **COSY** – ^{1}H-^{1}H COrrelation SpectroscopY (^{2}J-, ^{3}J- & sometimes ^{4}J-coupling)
2. **TOCSY** with NUS (non-uniform sampling) – ^{1}H-^{1}H TO Correlation SpectroscopY (correlations between all protons within a given spin system, up to ^{5}J & ^{6}J-coupling)
3. **1D Selective NOESY** – ^{1}H-^{1}H Nuclear Overhauser Effect SpectroscopY (through-space correlations up to ~ 5Å

For the lab, we'll use **2% 2-ethyl-1-indanone in $CDCl_3$** (white capped NMR tube).

Note: 2D experiments are powerful techniques, but are often a compromise between the amount of information we want and how much spectrometer time we can afford (also, your sample concentration!).

a. Any of the 2D experiments done in class can utilize the Non-Uniform Sampling (NUS) functionality of Bruker's TopSpin. We are using NUS for some 2D applications, but not all. See the NUS handout on Canvas for more info.
b. 1D Selective Experiments: these focus on only one specific region of the spectrum or a specific correlation. In class, we are using 1D selective experiments for some 2D applications, but not all.

1. COSY

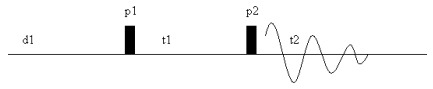


Fig 1: COSY pulse sequence. The standard COSY is two 90° pulses (p1) and (p2), separated by an evolution time (t1). The NMR signal is read out during the detection period (t2).

The 2D COrrelation SpectroscopY experiment (Fig. 1) can yield sequential assignment for interconnected ^{1}H networks, using thru-bond J-coupling correlations. 2D COSY spectra shows thru-bond correlations as cross peaks between two resonances that are separated by 2 or 3 bonds (in rare cases, 4 bonds). The COSY sequence works best for spin-spin coupling constants of 3-15 Hz. There is an alternative COSY experiment for long range correlations (LR-COSY). With LR-COSY you can observe cross peaks between protons which are connected by a small coupling constant (e.g., allylic, homoallylic or W-coupling).

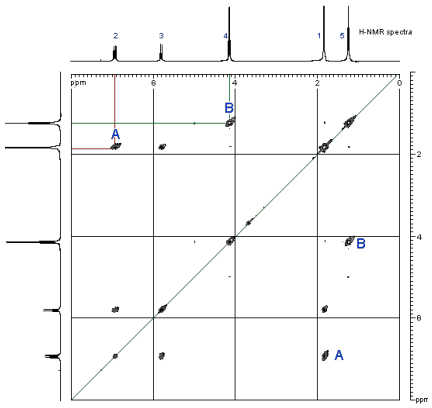
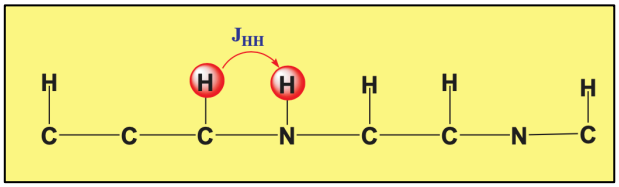


Fig 2: COSY spectrum of ethyl-2-butenoate. The diagonal peaks have the same frequency, and correspond to a projection of the 1D spectrum. The cross peaks have different values for each frequency coordinate and indicate spin-couplings between different protons. Note that there is a second set of equivalent peaks on the other side of the diagonal.



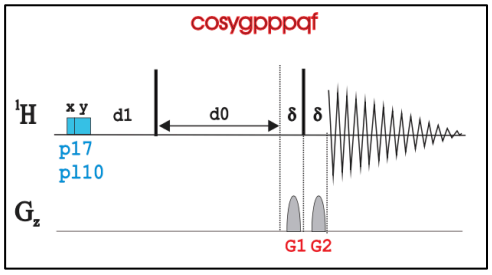
Practically speaking, taking COSY at high magnetic field yields better quality spectra due to increased resolution and sensitivity. This experiment is a proton detection experiment, so the use of an inverse, cryoprobe, or an inverse cryoprobe is recommended.

The default COSY sequence uses gradients to select the required coherence pathways with only one scan per t1 increment. If your sample is sufficiently concentrated, the experiment takes only ~5 minutes.

There are many versions of the COSY experiment; four are listed in the table below. Most modern versions of the COSY experiment are gradient-COSY (gcosy) experiments. For this lab, we will use the **cosygpppqf** version, but you may find that some of the other versions are more useful in the future.

Parameter Set (rpar input)	Pulse Sequence (pulprog input)	Description
COSY90SW	cosy90qf	Basic magnitude mode COSY, using phase cycles for coherence selection
COSYGPSW	cosygpqf	Magnitude mode COSY using Gradient Pulses for coherence selection
gcosy.av500/600 (UO)	cosygpppqf	Magnitude mode COSY using Gradient Pulses for coherence selection & Purge Pulses
COSYDQFPHSW	cosydfph	PHase-sensitive COSY & Double Quantum Filter, using phase cycles for coherence selection
COSYGPDFPHSW	cosygpmfphpp	PHase-sensitive COSY using Gradient Pulses for selection & Multiple quantum Filter & Purge Pulses

At a glance, what are the differences between the versions? Speaking in general terms: gradient versions are faster and result in cleaner spectra for most samples so they're great for automated operation (but the fine structure of cross peaks is not always apparent); phase-sensitive versions provide better NMR lineshape and resolution (but require high digital resolution and the diagonal peaks may interfere with close cross peaks); the double quantum filter suppresses singlets; purge pulses suppress artifacts from too short of a d1 between scans. See T.W. Claridge "High-Resolution NMR Techniques for Organic Chemistry," chapter 5 for more information. Our standard gcosy experiment is **cosygpppqf**, which provides a good balance of sensitivity and easy analysis.



COSY Data Acquisition

1) Acquire a standard proton spectrum and process the data, making sure that it's well-phased. From this data, you need to determine an optimized spectral window (**sw**), i.e., the region containing your peaks with at least one ppm on either side. Note that all of the peaks outside of this chemical shift range will be eliminated from your 2D spectrum. Write down the following parameters:

a. **sw** [ppm]: the spectral width of your optimized window. Because 2D NMR acquisition is time-consuming, the minimum amount of data collection is desirable. By setting **sw** to an optimized region, you save on acquisition time.
b. **o1p** [ppm]: the center of the optimized window
c. **sr** [Hz]: this parameter gets set automatically, it's usually 0
d. You also need to calibrate your 90° pulse and get the **p1** value [us]

2) Create a new experiment (either with new or iexpno) and read in the gcosy parameter set (e.g., **rpar gcosy.av600**).

3) Type **eda** to view the Acquisition Parameters. There are two columns: on the left is the **F2 dimension** and on the right is the **F1 dimension**. Check the setup for the following parameters:

a. **td(F1)** [points]: 128
b. **aq** [s]: 0.5
c. **o1p(F2)** [ppm]: use the ^{1}H o1p from step 1a
d. **o1p(F1)** [ppm]: use the ^{1}H o1p from step 1a
e. **sw(F2)** [ppm]: use the ^{1}H sw from step 1b
f. **sw(F1)** [ppm]: use the ^{1}H sw from step 1b
g. Set **p1** and **p0** to your calibrated 90^o pulse

4) Click on the "**PulseProg**" tab and check the pulse program text for suggested values for **ns** and **ds**. In 2D experiments, these parameters frequently depend on something called "phase-cycling." Set **ns** = 1, **ds** = 16, and **d1** = 2. Check how long the experiment will take with **expt**. If it's > 10 minutes, reduce your **td(F1)** to 64 or your acquisition time **aq**.

5) Before you start acquiring make sure the probe is tuned to proton (it should be if you acquired a routine 1H in step 1). Set the receiver gain with **rga** and then **zg** to start acquiring.

6) While this is running, you can save time by setting up the TOCSY (see below). Type **iexpno** or **new** to create a new experiment and follow the instructions in Part 2. You can do everything except type "rga" and "zg" ☺

COSY Data Processing in TopSpin:

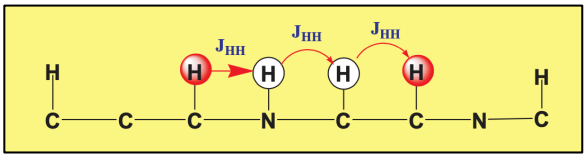
1) Type **edp**. Change the following parameters in both **F2** and **F1** dimensions:
   a. **sr**: set to the value determined above.
   b. **window functions**: use **Sine**
   c. **si**: set SI ≥ TD. Note that **SI**(F1) >512 will slow down data processing.
2) Fourier transform in both dimensions with **xfb**. The default COSY experiment is in magnitude mode, so you will not need to phase anything.
3) Run **abs2;abs1** to do a baseline correction in both dimensions.
4) On the 2D spectrum, you can adjust the peak intensities using the "**x2..etc**" buttons, or by scrolling up/down with the center button.
5) Sometimes manual peak picking (**.pp**) is best.
6) To change the **External Projections** on both axes, make sure you have the appropriate 1D spectra processed. Right click on the projection and select "External Projection," and pick the dataset to use. External projections usually look better than internal ones.
   a. Right-click on the □ to the right of the projection on the horizontal axis, then **select External Projection**. Enter the full data set specifier for the 1H spectrum to be used as the “projection” plot for the F2 dimension.
   b. Right-click on the □ to the right of the projection on the vertical axis, then **select External Projection**. Enter the full data set specifier for the 1H spectrum to be used as the “projection” plot for the F1 dimension.

COSY Data Processing in MestReNova

Mnova will do the data processing for you, although you may want to change the *window function (apodization)* and *zero-filling* parameters. One additional thing that Mnova can

do (but isn't enabled by default) is to *Symmetrize* your data. This is a processing function which is used in homonuclear correlation spectroscopy (e.g., COSY, NOESY), which replaces all signals symmetrically positioned about the diagonal by the smaller of the two signals. Why is that useful? Because this process removes asymmetrically positioned noise and artifacts. Use this with caution as any symmetrical noise/artifact is preserved and might be mistaken for as a real signal. See the Mnova Manual for more information.

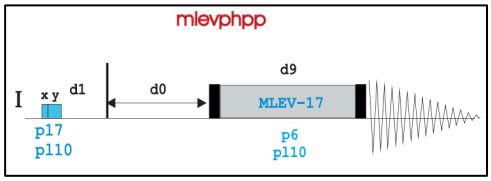
2. TOCSY (using NUS)



TOCSY is related to COSY in that we look at through-bond (J-coupling) spin-system correlations between different ^{1}H nuclei. The difference is that TOCSY maps out all the neighboring proton spins that are coupled to each other, even if there are heteronuclear atoms or quaternary carbons in between. On the other hand, COSY's correlations get "broken up" by heteronuclei that don't have a directly bonded proton like a carbonyl.

How does this happen? Coherence transfer occurs during a "spin-lock" field, which is a B_1 field much weaker than the main B_0 field. In the spin-lock field, magnetization is transferred throughout the entire coupling network, creating correlations between all protons in a given spin system. Even if each spin is not directly coupled, each spin (up to 5 or 6 bonds) is correlated (shows a cross peak), as long as there are couplings between each intervening proton.

Just like the COSY experiment, there are multiple versions of TOCSY. In class we will use the PHase-sensitive **mlevphpp** sequence that uses the MLEV-17 spin-lock and Purge Pulses. The full TOCSY experiment is quite long; to reduce scan time we will make use of non-uniform sampling (NUS). NUS partially samples the full NMR data set and then utilizes reconstruction algorithms in order to convert the NUS data into a spectrum. This easily reduces measurement time by a factor of 2 in routine 2D small molecule NMR.



TOCSY Data Acquisition

1) For TOCSY, we can use the same **sw**, **o1p**, **sr**, and **p1** as in the COSY experiment.

2) Create a new experiment (either with new or iexpno) and read in the TOCSY parameter set (e.g., **rpar gtocsy.av500**).

3) Type **eda** to view the Acquisition Parameters. There are two columns: on the left is the **F2 dimension** and on the right is the **F1 dimension**. Change the following parameters to your optimized values from earlier:

a. **td(F1)** [points]: 128
b. **o1p(F2)** [ppm]: use the ^{1}H o1p from step 1a
c. **o1p(F1)** [ppm]: use the ^{1}H o1p from step 1a
d. **sw(F2)** [ppm]: use the ^{1}H sw from step 1b
e. **sw(F1)** [ppm]: use the ^{1}H sw from step 1b
f. Set **p1** to your calibrated 90^o pulse

4) Click on the "**PulseProg**" tab and check the pulse program text for suggested values for **ns** and **ds**. Set **ns** = 8, **ds** = 16, and **d1** = 2. Check how long the experiment will take with **expt**.

5) We will enable non-uniform sampling (NUS) for this experiment. Set the **FnTYPE** to non-uniform sampling and set **NusAMOUNT** to 25%. (Reduce scan time by a factor of 4, so that expt ≈ 11 minutes). If you have time, you could try NusAMOUNT = 50 % (reduces scan time by a factor of 2), but this will take ~ 22 minutes.

6) The default **d9** spin-lock mixing time is 80 ms. You could set **d9 to** anything between 60-250 ms. The longer the time, the more correlations you will see. do **¡DO NOT EXCEED 250 MILLISECONDS BECAUSE YOU COULD DAMAGE THE PROBE BY COOKING IT RF POWER!**

7) Before you start acquiring make sure the probe is tuned to proton (it should be if you acquired a routine ^{1}H earlier). Set the receiver gain with **rga** and then **zg** to start acquiring.

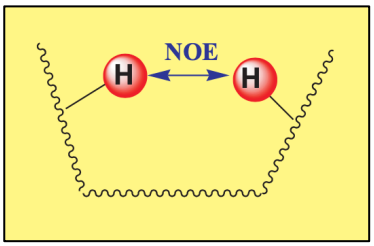
8) While this is running, you can save time by setting up the selNOE (see below). Type **iexpno** or **new** to create a new experiment and follow the instructions in Part 3. You can do everything except type "rga" and "zg" ☺

TOCSY Data Processing in TopSpin:

1) Type **edp**. Change the following parameters in both **F2** and **F1** dimensions:
   a. **sr**: set to the value determined above.
   b. **window functions**: use **Sine**
   c. **si**: set SI ≥ TD. Note that **SI**(F1) >512 will slow down data processing.
2) For NUS data there are two extra steps before the 2D Fourier transform. TopSpin will need to perform 2D Hilbert transform (**xht1**; **xht2**). This can take a *long* time in TopSpin (but it can be done!), so we recommend just opening it up in Mnova. ☺

TOCSY Data Processing in MestReNova

Mnova will do the data processing for you, although you may want to change the *window function (apodization)* and *zero-filling* parameters. One additional thing that Mnova can do (but isn't enabled by default) is to *Symmetrize* your data.

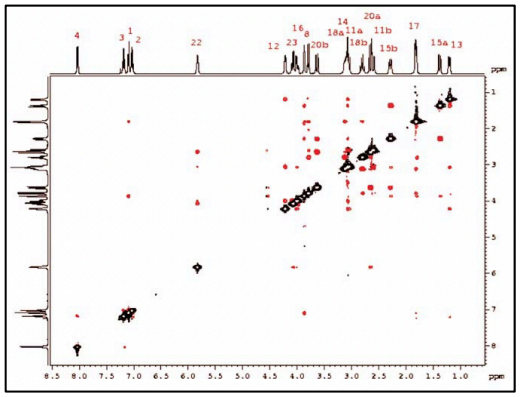


3. NOESY (1D Selective NOE)

The Nuclear Overhauser Effect SpectroscopY (NOESY) experiment is used in determining thru-space correlations between ^{1}H nuclei. This technique allows solving 3D structure of small and large molecules and is especially useful for determining the characteristics of stereocenters beyond the topological connectivity of the molecule. Meaningful NOESY experiments require that ^{1}H spectra have been assigned previously.

NOESY experiments determine through space correlations via the Nuclear Overhauser Effect (NOE). NOE correlations will be strong for nuclei separated by < 2 Å and can be observed, albeit weakly, for nuclei separated up to ~6 Å. NOE interactions depend on the rigidity, T_1 relaxation time, and the correlation time of the molecule (the time it takes for the molecule to tumble 2π radians). Factors that can affect the tumbling motion of the molecules can have a significant effect on the NOE transfer rate, e.g. size of the molecule, the viscosity of the solvent, or the temperature. For most small molecules, NOE

correlation peaks will have opposing polarity as the diagonal peaks on the 2D spectrum or the selectively excited resonance in the 1D spectrum. However, with increasing molecular weight or viscosity of the solvent, NOE enhancements will change sign, and show positive correlation peaks. This sign change can be observed at ~700 molecular weight. Note that the NOE correlations will be very weak for this regime due to low enhancement rate, and it will be more advantageous to run a ROESY experiment.



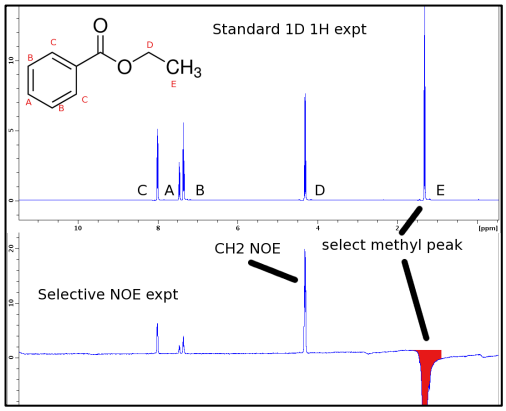
A 2D NOESY experiment will present *all* thru-space correlations for *all* resonances in a sample. NOE correlations are weak, and the pulse sequences require phase cycling in multiples of 8. Consequently, NOE experiments take much longer than COSY or TOCSY. For moderately concentrated samples (>0.5 mM), a 2D NOESY can 45 minutes-12 hours, even with a cryoprobe. There are two alternatives to the long 2D acquisition: non-uniform sampling (NUS) or the 1D selective NOE (selNOE) experiment. For moderately concentrated samples, NUS can reduce the scan time by a factor of 4. For lower concentration samples, the 1D selNOE experiment is suggested.

The version we will perform in class is the 1D selNOE experiment. Instead of determining thru-space correlations for all ^{1}H resonances in a sample, one can selectively excite a specific resonance and observe a limited set of correlations. This approach can reduce the experimental time dramatically compared to 2D NOESY. However, it is *crucial* to have a complete assignment for ^{1}H resonances before running the selNOE experiment. Another advantage of using a selective 1D NOE experiment is that it is not limited by resolution; a large number of increments might be needed for a 2D NOESY experiment in order to clearly see thru-space correlations for peaks with similar chemical shifts. Conversely, these peaks need to be separated by only ~10 Hz for selNOE experiments. It would require a long 2D NOESY (on the order of hours) to achieve that resolution limit, whereas a selNOE experiment could finish in a matter of minutes. (Aside: there is yet *another* version 1D NOE method called the NOE difference experiment.)

In class, we will perform the 1D selNOE. Even with a moderately concentrated (>0.5 mM) sample, it is a good idea to perform a 1D selNOE experiment before your 2D measurement, in order to make sure your parameters are set properly.

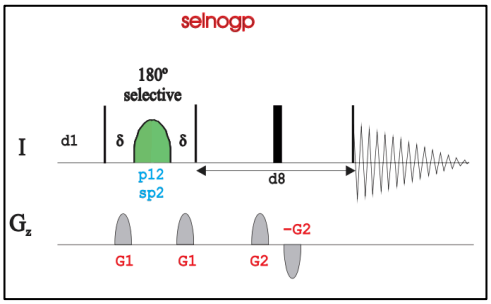
NOESY Experimental Notes:

- It is often a good practice to run 1D selNOE experiments with a range of "mixing times" (e.g., 0.3 s, 1 s, 3 s, depending on T_1) for a better understanding of relative distances in the spin network. As the mixing time gets longer, peaks correlated through stronger NOE's will build up intensity faster than peaks that are further away.
- A proper "mixing time" (**d8**) is crucial in order to get optimal spectra. Setting **d8** ≈ 1xT_1 is generally a good compromise between SNR and



a decent NOE build-up. Setting **d8** much longer can cause significant signal loss due to T_1 relaxation. Furthermore, long mixing times can lead to spin diffusion and higher-order terns in NOE correlations, resulting in false conclusions.

- For NOE experiments, sample preparation is very important. For optimal experimental conditions, the sample should be degassed in order to remove dissolved O_2 gas. Oxygen is paramagnetic and can inhibit observation of NOE's.
- As with any proton-detected experiment, using an inverse probe, cryoprobe, or inverse cryoprobe gives better sensitivity.
- Temperature stability is very important for NOE experiments.
- NOE correlation spectra may demonstrate out-of-phase features due to through-bond (COSY) artifacts. Chemical exchange can also give false positives; these can be distinguished from NOE correlations because they will be of opposite polarity compared to true NOE correlation peaks.



1D selNOE Data Acquisition

1) You will need to have a standard 1D proton spectrum of your sample. In this dataset, set **sr** = 0. We want this to be zero so that we have the unshifted ppm value for our peak to be selectively excited. Zoom in on the target peak (i.e., the peak you want to selectively excited and observe NOEs with). Record the value of its chemical shift in ppm by reading it in with the cursor. If your target peak is a multiplet, record the central value. For this lab, please use the methyl (CH_3) peak of our ethyl-indanone.

2) You will also need the 90° pulse value **p1** from pulsecal and the approximate T_1 that you determined in Lab 2.

3) Create a new experiment (either with new or iexpno) and read in the selNOE parameter set (e.g., **rpar selnoe.av500**).

4) Set **o1p** = the target peak from step 1. Set **p1** = your calibrated 90° pulse.

5) SelNOE is one of the 1D experiments that depend on something called "phase-cycling." Click on the "**PulseProg**" tab and check the pulse program text for suggested values for **ns** and **ds**. Adjust any other spectral parameters as necessary (**sw** = 20, **ds** = 4). You want **sw** to be large enough to cover the entire spectrum around **o1p**.

6) Set **d8** (the mixing time) ≈ 1xT_1.

7) Although the sensitivity of the selNOE experiment is better than that of the full 2D NOE experiment, selNOE is still much less sensitive than routine proton NMR. Set **ns** = 8 and **td0** = 5. This way after every 8 scans, the data will automatically save and you can type **ef** to fourier transform the NMR spectrum. Manually phase the spectrum to show the peak that was selectively excited as upside down. Evaluate the quality of the resulting spectrum and **halt** the experiment if you think it's good enough SNR.

8) Check how long the experiment will run with **expt**. Then type **rga;zg;ef**. Phase the spectrum with **.ph** such that the peak that was selectively excited is upside down. Evaluate the quality of the resulting spectrum.

9) Use **iexpno** to create another experiment with the same parameters. Try another **d8** mixing time. Repeat this for: 0.5xT_1 and 2xT_1.

When you are done in the lab, make sure to log out. Type **CTRL + ALT + BACKSPACE** to log out of the computer.

What to turn in:

- The full gCOSY spectrum, with any expanded regions that you think are important. Assign all of the cross peaks on the COSY spectrum.
- The full gTOCSY spectrum, with any expanded regions that you think are important. Assign all of the cross peaks on the TOCSY spectrum.
- Stacked 1D selNOE spectra for each **d8** mixing time you chose, with expanded regions that you think are important. Make note of any through-space connectivity as revealed by the experiment.
- Answers to the following questions:

Questions

1. Discuss the differences between COSY, TOCSY, and NOESY.
2. How can you be sure that your NOE correlations are real and not artifacts?
3. How might incomplete relaxation between scans affect the results? Why are your T_1 relaxation times different from one proton to another?
4. How does the choice of spin-lock time in the TOCSY affect your results? What about mixing time in the NOE?
5. When should you use ROESY instead of a NOESY?