

# 1D NOE Difference

## Introduction

## 9.1

The Nuclear Overhauser Effect is a net change of the signal intensity from one spin due to the relaxation of a saturated spin that is dipole-dipole coupled to the first spin. An NOE may be positive, meaning that there is a net increase in signal intensity, or it may be negative, meaning that there is a net decrease (this occurs for larger molecules). NOE's develop due to through-space rather than through-bond interactions, and so contain information on the distances between spins.

The buildup of NOE's depends on spin-lattice ( $T_1$ ) relaxation, a process in which energy passes from the nuclear spins to the lattice (i.e., everything else) as heat.  $T_1$  relaxation requires magnetic fields fluctuating at the appropriate frequency. For  $^1\text{H}$ 's and  $^{13}\text{C}$ 's in diamagnetic molecules, the dominant fields are due to the magnetic moments of  $^1\text{H}$ 's in the same molecule as it tumbles in solution (i.e., intramolecular dipole-dipole interactions).

The rate or efficiency of NOE buildup depends on the rate or efficiency of dipole-dipole relaxation. This depends on the strength and frequency of the fluctuating fields, which in turn depend on factors such as the distance between the nuclei involved, the tumbling rate of that portion of the molecule, and the nature of the nuclei themselves. Any other competing  $T_1$  relaxation process will hinder the growth of NOE's. In fact, the presence of paramagnetic molecules (e.g., metal ions, rust, or dissolved oxygen) can be deadly to an NOE experiment, since these may completely dominate  $T_1$  relaxation processes. To get the largest possible useful NOE, it is necessary to maximize the contribution of the intramolecular dipole-dipole relaxation (e.g., eliminate paramagnetic species, use dilute solutions to eliminate problems from intermolecular interactions, also use a solvent without a high  $^1\text{H}$  concentration). It is also necessary to maximize the efficiency of the dipole-dipole relaxation. For example, dipole-dipole relaxation is most efficient when the molecular tumbling rate is intermediate. Small molecules tend to tumble too rapidly, and large molecules in viscous solvents too slowly, both yielding slow dipole-dipole relaxation. So, for a very small molecule with a very slow NOE growth, it is possible to speed up the experiment by using a solvent of higher viscosity (e.g.) to decrease the tumbling rate.

In an NOE difference experiment, a  $^1\text{H}$  resonance is selectively preirradiated until saturation is achieved. During the preirradiation period, NOE buildup occurs at another  $^1\text{H}$  resonance or at other  $^1\text{H}$  resonances. A  $\pi/2$  pulse then creates observable magnetization, which is detected during the acquisition period that follows. The experiment is repeated using different preirradiation frequencies, including one which is off-resonance. The latter is used in obtaining a reference or control spectrum. Each final spectrum is displayed as the difference between a spectrum collected with on-resonance preirradiation and the control spectrum.

Very small phase or frequency shifts between two spectra will give rise to imperfect subtraction of signals that can ruin the NOE difference results. These subtraction artifacts look like dispersive responses (having no net integral) and care should be taken to minimize them. Some artifacts arise from essentially random, short time scale instabilities. To minimize these, it is best to use plenty of signal averaging and

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the maximum line broadening acceptable, and to run the experiment when traffic around the magnet is at a minimum.

Other artifacts arise from longer time scale errors such as temperature or magnetic field drift. These may be minimized by acquiring the preirradiated and the control data in an interleaved manner (i.e., as nearly simultaneously as possible). Thus, each data set samples the same long term changes. Interleaved acquisition may be achieved as follows: Typically, a few dummy scans are acquired (to be sure that any influences of irradiation at the previous frequency in the cycle have completely decayed away) followed by 8 acquisitions with one preirradiation frequency. This is followed by a few dummy scans and 8 acquisitions with a second preirradiation frequency, and so on until the final preirradiation frequency. This entire cycle is repeated as many times as necessary to yield the desired number of signal averages for

The irradiation power level should be minimized to get the appropriate frequency selectivity, but should not be so low that the saturation is incomplete and the resulting NOE's are very low, or that the frequency spread is so narrow and asymmetric that different lines in an irradiated multiplet are saturated to different extents. In this case it is possible to get selective population transfer (SPT) in spins J-coupled to the multiplet. The pulse sequence used in this chapter allows the user to loop through several frequencies of a multiplet during the preirradiation period of a given acquisition. Such cycling causes the NOE's to co-add but the SPT effects to cancel, and enables one to use lower power, more selective preirradiation. When cycling through the frequencies of a multiplet, the irradiation time per line should be short so that there is little relaxation of previously irradiated lines during the final irradiation prior to the  $\pi/2$  pulse, otherwise SPT effects reappear. On the other hand, the irradiation time per line should be long enough to avoid significant artifacts due to frequency modulation of the irradiation.

**Reference:** D. Neuhaus and M. P. Williamson, "The Nuclear Overhauser Effect in Structural and Conformational Analysis," New York: VCH Publishers, Inc., 1989.

### Sample

The sample used to demonstrate a 1D NOE difference experiment in this chapter is 100mM Pamoic Acid in DMSO-d<sub>6</sub>. (Almost any sample can be used, as long as it has an observable nuclear Overhauser enhancement.)

## Pulse Sequence Diagram

## 9.2

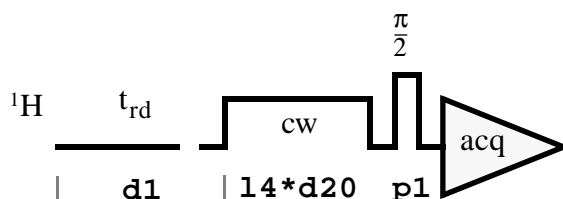
The NOE difference pulse sequence is shown in Figure 29. The pulse sequence begins with the recycle delay time **d1**. This is followed by the cw irradiation period of total time **14\*d20**. Here **d20** is the irradiation time for one particular frequency. The pulse program makes use of a frequency list (**fq2list**) to determine the frequency or frequencies for cw irradiation. Several frequencies are required only if the resonance to be irradiated is a multiplet. (Here it is necessary to define more than one frequency only if there is concern that a single irradiation point will not excite the full multiplet adequately.) For each of the **14** time intervals **d20**, the next frequency of the **fq2list** is used. In this way, lines of a multiplet are irradiated in an interleaved manner so that those irradiated first have not relaxed by the time the last peaks are irradiated.

The final 90° pulse **p1** creates the observable magnetization and is followed immediately by the acquisition period.

Several spectra are acquired during an NOE difference experiment, and for each spectrum a different **fq2list** is used. In one of the spectra, the control or reference spectrum, the cw pulse is applied off-resonance, while for each of the other spectra, the cw pulse is applied on-resonances for one of the peaks, in order to saturate that peak. An NOE difference spectrum is then the difference between a spectrum with a saturated resonance, and the control spectrum.

The au program **noemult** is used to acquire the spectra in an interleaved manner.

Figure 29: 1D NOE Difference Pulse Sequence



## Acquisition and Processing

## 9.3

Make sure the following preliminary steps have been completed: Insert the sample in the magnet. Lock the spectrometer. Readjust the Z and Z<sup>2</sup> shims until the lock level is optimized. Tune and match the probehead for <sup>1</sup>H observation.

For best results it is recommended to optimize the lock parameters as described on page 16. Also note that NOE difference experiments should be run without sample spinning.

The parameters and spectra shown below are from a 300MHz spectrometer. The signal enhancements for Pamoic Acid in DMSO-d<sub>6</sub> at other field strengths will be different than those shown here. If an NOE response is difficult to obtain, it may be necessary to change the sample temperature or solvent. In particular for this sample at 400MHz, it is recommended to use a temperature of 40°C.

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### Create a new file directory

Enter **re proton 2 1** to call up the data set proton/2/1. Enter **edc** and change the following parameters:

NAME	noe
EXPNO	1
PROCNO	1 .

Click **SAVE** to create the data set noe/1/1.

Enter **edasp** and set both NUC1 and NUC2 to 1H. It is necessary to set NUC2 to 1H because the f2 channel will be used to provide the cw irradiation during the NOE experiment. By using **edasp** rather than **edsp**, the value of **o1** is preserved rather than overwritten by the default value (i.e., OFSH1 remains equal to the **o1** value of proton/2/1). For now **o2** is unimportant, so let OFSH2 = 0.


### <sup>1</sup>H reference spectrum

Enter **rga** to perform an automatic receiver gain adjustment. Acquire and process a standard <sup>1</sup>H spectrum, as described in Chapter 3 ‘Basic 1H Acquisition and Processing’ (notice that since the data set noe/1/1 was created from proton/2/1, most acquisition parameters are already set). Calibrate the spectrum and optimize **sw** and **o1**. Keep in mind that the control spectrum should be irradiated well off-resonance (here -2 ppm is suggested), so do not choose **sw** to be too small. Acquire and process an optimized spectrum. This reference spectrum can be compared with the off-resonance control spectrum acquired during the NOE difference experiment.

### Select the resonances for irradiation

The next step is to define the frequencies that will be used by the f2 channel during the preirradiation periods of the NOE experiment. These frequencies will be written to fq2lists. A separate list should be created for each resonance to be irradiated (be it a multiplet or singlet). A given list may have several frequencies if it is desired to irradiate a given resonance at several points. One of the lists must define a frequency well off-resonance to be used in generating the control spectrum.

For this example we will create lists with frequencies for the resonances at 4.8 ppm and 8.5 ppm, and one with the off-resonance frequency -2 ppm. The frequency lists are defined in the **frqlist** routine, which is found in the **utilities** submenu. Note that if it is necessary to expand the spectrum in order to define the irradiation points accurately enough, this must be done before entering the **frqlist** routine. Also, within the calibration submenu it is not possible to expand the spectrum using the mouse.

Click **utilities** to enter the corresponding submenu. If desired, expand the spectrum and display the peak at 4.8 ppm using the  buttons. Within the **utilities** submenu, click **frqlist**. Answer the questions as shown below:

```
Please enter type of list (f1, f2, f3):      f1
Please enter name of f1 list:                 noedif.1
Write name of f1 list to acqu parameters? n .
```

If an f1 frequency list of the same name already exists, the following option also appears:

```
Frequency list exists, append (a), overwrite (o) or quit (q):.
```

Answer **a** if you wish to add new frequencies to the old list, **o** if you wish to overwrite the old list with the new list, or **q** if you wish to quit the **frqlist** routine and keep the old list.

**(Point of clarification:** Here, the “type of list (f1, f2, f3)” actually refers to the *directory* where the frequency list will be stored (not the spectrometer channel for which the list will be used). This must be set to f1. In any given acquisition parameter set, it is possible to define eight separate frequency lists (**fq1list**, **fq2list**, etc.). The pulse program **noemul** uses only one frequency list: **fq2list**. Therefore, within the **eda** menu, it is necessary to set the parameter **fq2list** to the appropriate list name (here, noedif.1). The automation program **noemult** redefines **fq2list** each time **noemul** is to be run with a different frequency list.)

Once the questions have been answered, move the mouse until the cursor is in the spectral window. The cursor is now tied to the spectrum. Click on the peak at 4.8ppm with the middle mouse button. Finish the list by clicking the left mouse button. Remember that for a given list, multiple irradiation points should all be part of the same multiplet. A separate frequency list should be generated for each multiplet irradiated.

Repeat this procedure for the peak at 8.5 ppm and for the off-resonance frequency of -2 ppm. Write the lists to the files noedif.2 and noedif.3, respectively. Note that the automation program **noemult** used to run the NOE experiment requires that all frequency lists have the same base name and increasing extension numbers.

Click on **return** to leave the utilities submenu and return to the main menu.

### Set up the acquisition parameters

Enter **edc** and change EXPNO to 2. Click **SAVE** to create the data set noe/2/1.

Enter **eda** and set the acquisition parameters as shown in Table 33. Use the values determined in Chapter 5 ‘Pulse Calibration’ for the parameters **p11** and **p1** (<sup>1</sup>H observe high power level and 90° pulse time).

Be sure that NUC2 has been set to 1H in **edasp**.

**Table 33. 1D NOE Difference Acquisition Parameters**

Parameter	Value	Comments
PULPROG	noemul	see Figure 29 for pulse sequence diagram.
TD	16k	
NS	8	the number of scans must be 8*n in order for the phase cycling to work properly.
DS	4	number of dummy scans.
PL1		high power level on f1 channel (see “An Important Note on Power Levels” on page 7).
PL14	70dB	power level for NOE buildup.
P1		90° <sup>1</sup> H high power pulse on f1 channel.

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D1	1sec	relaxation delay
D12	20 $\mu$ sec	delay for power switching; predefined.
D13	3 $\mu$ sec	short delay; predefined.
D20	50msec	irradiation time.
L4	50	loop counter to determine overall irradiation time (L4 * d20).
FQ2LIST	noedif.1	f2 frequency list for frequency of selective irradiation; this gets set by the automation program.

The pulse program **noemul** operates such that **o2** is set to the first frequency of the fq2list and the selected multiplet is irradiated with this frequency for a time **d20**. Then **o2** is set to the next frequency (if there is one) of the fq2list and the selected multiplet is irradiated with this new frequency (or the first frequency if the fq2list has only one entry) for a time **d20**. This process continues until the multiplet is irradiated a total of **l4** times, using as many different frequencies as are in the fq2list.

### Optimize the irradiation power and duration

The next step is to optimize the irradiation power and duration. An easy way to do this is, using the parameters shown above, to start the pulse program **noemul** with the command **zg**. For the full NOE difference experiment, the au program **noemult** will run the pulse program **noemul** with successive fq2lists. For optimizing the irradiation power and duration, however, it is only necessary to irradiate one resonance, and so the au program is not necessary.

Make sure that PULPROG is set to **noemul** and that FQ2LIST is set to **noedif.1** (or **noedif.2**, i.e., make sure that the cw irradiation will be applied on resonance for one of the multiplets). Start the acquisition with **zg**. Process the spectrum with **ef** (see the processing parameters listed below in Table 34). Manually phase correct the spectrum.

Compare this spectrum with the reference spectrum **noe/1/1**. It may be useful to use the dual routine. From the current data set (**noe/2/1**), enter **edc2** to define the second data set to be shown in the dual display. Set **EXPNO2** to 1 and **PROCNO2** to 1 and click **SAVE**. Click **dual** to enter the dual display. Both **noe/1/1** and **noe/2/1** should appear in the window. When finished comparing the spectra, click **return** to return to the main 1D processing window.

Ideally, the target resonance is completely saturated by the selective irradiation, while all other signals are completely unaffected by the irradiating field. In practice, the chemical shift difference between signals is often too small for this to be possible. For example, if a high enough power is used to saturate all lines of a multiplet, neighboring resonances may also be saturated.

It is almost always preferable to use low-power (and hence selective) irradiation rather than risk unwanted saturation of nearby resonances. However, uneven partial saturation of a multiplet leads to selective population transfer which may obscure NOE effects. To avoid this, as mentioned above, the lines (or several frequencies) of

a target multiplet are irradiated in an interleaved manner during the preirradiation period before each scan.

If needed, adjust **p114** to change the power level of the cw irradiation.

Note that the total cw irradiation time (**14\*d20**) should be approximately equal to  $T_1$  of the irradiated peak, but with the au program **noemult**, it is necessary to use the same total irradiation time for each peak irradiated. Thus, the irradiation time should be chosen based on the longest  $T_1$ . Here a total irradiation time of 2.5 sec is used, which is longer than the  $T_1$  of the peak at 8.5 ppm.

### Perform the multiple NOE experiment

The automation sequence **noemult** is used to run the multiple NOE experiment. Simply type **xau noemult** and answer the questions as follows:

base name of all frequency lists:	noedif
# of frequency lists:	3
# of cycles through each list:	50
# of average cycles:	10 .

Here, the number of frequency lists is the number of fq2lists written above and will be the number of spectra acquired. The number of cycles through each list is the loop counter **14**. The number of average cycles controls the total number of scans for each frequency list. For each frequency list (and hence, for each spectrum) the total number of scans is **ns** × (number of average cycles). So that each spectrum may reflect any long term drift equivalently (which is important in minimizing subtraction artifacts in the difference spectra), it is best to keep **ns** small (e.g., 8) and then improve the signal-to-noise ratio by increasing the number of average cycles (to, e.g., 10).

Note that since this is a difference experiment, the observation of small NOE responses requires a high signal-to-noise ratio in the individual spectra, as well as careful temperature control. Be sure to run this experiment non-spinning, and it is also recommended to run it at night (or at some time when activity around the spectrometer is at a minimum).

The au program **noemult** automatically calls the pulse program **noemul**. Using the acquisition parameters defined in the current data set (here noe/2/1), and the **o2** frequency or frequencies defined in the first fq2list (here noedif.1) for the preirradiation, **ds** dummy scans are run and **ns** signal averages are acquired and written to the current data set. Next, **ds** dummy scans and **ns** signal averages are performed using the **o2** frequency or frequencies defined in the second fq2list (here noedif.2). The results are written to the next data set (here noe/3/1). This process is continued until the results obtained using the last frequency list (here noedif.3) are written to the last data set (here noe/4/1). Notice that new data sets created by **noemult** have the same name as the original data set, but increasing EXPNO's. Here the spectrum irradiated at 4.5 ppm is noe/2/1, that at 8.5 ppm is noe/3/1, and that at -2 ppm is noe/4/1.

The entire cycle is repeated until the experiment is finished. The number of times this cycle is performed is determined by the value entered for the number of average cycles.

The total experiment time with the parameters shown here is approximately 15 mins.

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### Set up the processing parameters

Enter **edp** and set the processing parameters as shown in Table 34.

**Table 34. NOE Difference Processing Parameters**

Parameter	Value	Comments
SI	8k	
WDW	EM	
LB	0.30Hz	
PKNL	TRUE	necessary when using the digital filter.

### Process the spectra

To process the spectra acquired by **noemult** it is first necessary to define the phase correction parameters.

Read in the first file (**re 2 1**). Apply the window function and Fourier transform with the command **ef**. Manually phase correct the spectrum and store the correction.

The remaining spectra must be processed identically. An easy way to accomplish this is by using the automation program **multiefp**. Simply enter **xau multiefp** and answer the questions as follows:

```
Enter first expno to process:      2
Enter number of expnos:          3 .
```

Here, the “first expno to process” indicates the spectrum that is already Fourier transformed and phased correctly. The program **multiefp** reads all processing parameters (including 0<sup>th</sup>- and 1<sup>st</sup>-order phase corrections) from this data set and uses them to process the remaining spectra. Notice that this automation program assumes all spectra to be processed have the same name and increasing EXPNO's.

At this point the data consists of a series of spectra with various saturated resonances (whether singlets or multiplets) and one reference spectrum. The procedure for creating the difference spectra is outlined below.

### Create the NOE difference spectra

The NOE difference spectra are created by subtracting the control spectrum from each of the spectra preirradiated on-resonance. Within the data set of each preirradiated spectrum, then, it is necessary to use **edc2** to define the second and third data sets. The second data set refers to the control spectrum and the third data set is where the difference spectrum will be stored.

To create the first difference spectrum, first make sure the first preirradiated spectrum is displayed (e.g., enter **re 2 1**). Enter **edc2** to set up the second and third data sets. Within the **edc2** menu set EXPNO2 and PROCNO2 to the values for the control spectrum, here EXPNO2 = 4 and PROCNO2 = 1.

Next, set EXPNO3 to the value of the current experiment number, and PROCNO3 to one greater than the current PROCNO, here EXPNO3 = 2 and PROCNO3 = 2. Click on **SAVE** to exit **edc2** and return to the main menu.

Enter the dual submenu by clicking on **dual**. Both the current spectrum and the reference spectrum should now appear on the screen.

Click on **Return** and select **Save & return** to subtract the control spectrum from the current preirradiated spectrum. The difference spectrum appears automatically in the window.

Click **store** to save the results and return to the main menu. The message “result will be put into: DU = u, USER = <username>, NAME = noe, EXPNO = 2, PROCNO = 2, click OK if ok” appears. Click **OK** and notice that the current data set is now noe/2/2.

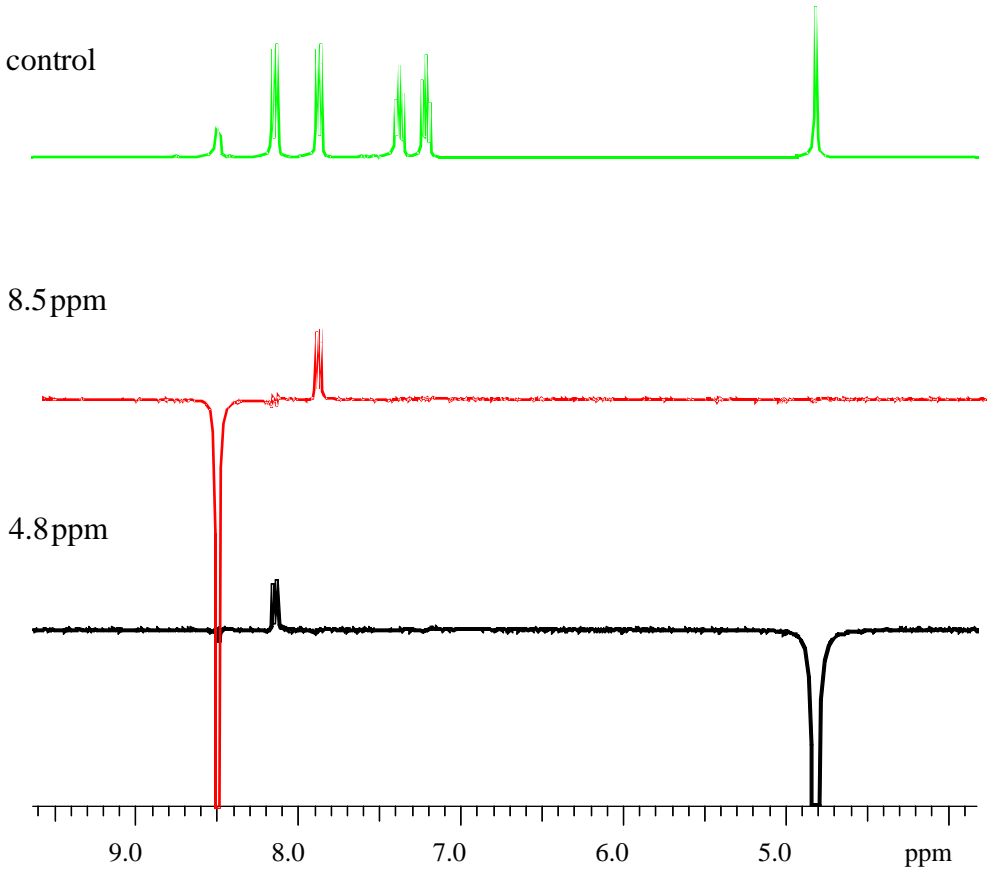
Move to the next preirradiated spectrum (e.g., enter **re 3 1**) and repeat the above procedure. Here EXPNO2 = 4, PROCNO2 = 1, EXPNO3 = 3 and PROCNO2 = 2, and the difference spectrum is stored in noe/3/2.

Two NOE difference spectra (with cw irradiation on-resonance at 8.5 ppm and 4.8 ppm) and the control spectrum (with cw irradiation off-resonance at -2 ppm) of the sample 100 mM Pamoic Acid in DMSO-d6 are shown in Figure 30. Notice that only the relevant portion of the spectral width is displayed (**sw** was actually much greater than what is shown).

For both the difference spectrum with cw irradiation at 8.5 ppm and that with cw irradiation at 4.8 ppm, the large negative peak is the irradiated resonance and the small positive doublet is the NOE. Note that these spectra were recorded on a DPX300 at 298 K. Similar spectra of this sample recorded at 500 MHz and 298 K will have negative NOE peaks, while those recorded at 400 MHz and 298 K may show no NOE peaks at all.

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Figure 30: NOE Difference Spectra of 100mg Pamoic Acid in DMSO-d6



### Quantitate the NOE's

To quantitate (roughly) an observed NOE, the integrated intensity of the NOE peak in the difference spectrum is compared with the integrated intensity of the peak that was irradiated to cause the NOE. However, this latter intensity should be measured in the control spectrum. Thus, it is necessary to integrate peaks in both the control and the difference spectrum, and to use the same normalization constant for the integrals in both spectra.

First integrate the appropriate peak in the control spectrum and set the normalization constant. Call up the reference spectrum (**re 4 1**). Click **integrate** to enter the integration submenu. Integrate the peak of interest (e.g., the peak at 8.5 ppm). To integrate the peak, first move the cursor into the spectral window and click the left mouse button to tie the cursor to the spectrum. Next, click the middle mouse button once at each end of the range of interest; the integral appears automatically. Click the left mouse button again to release the cursor from the spectrum. An asterisk should appear next to the integral (if not, select the integral with the left mouse button). Then, correct the baseline of the integral: with the cursor in the spectral window but not directly on the spectrum, move the mouse while holding down the middle mouse button to adjust the bias, and while holding down the right mouse button to adjust the slope. Again make sure the integral is selected (an asterisk appears next to it). Click on **calib** and enter 100 to calibrate this integral to 100%. Click on **return** and select **Save & store 'intrng'** to save the integral and normalization constant and return to the main 1D processing window.

Next call up the difference spectrum with the NOE you wish to quantitate (here the difference spectrum with cw irradiation frequency of 8.5 ppm, so enter **re 2 2**). Click on **integrate** to enter the integration subroutine. Integrate the NOE peak and adjust the slope and bias. Now click **lastscal** so that this integral is scaled using the same normalization constant that was defined for the previous data set. The number appearing below the NOE integral now indicates the percent integrated intensity (e.g., a value of 2.5 would mean that the NOE peak is 2.5% as intense as the irradiated peak). Note this value and then click on **return** to return to the main 1D processing window.

Repeat this procedure for other NOE peaks. For example, here read in the control spectrum (**re 4 1**), integrate the peak at 4.8 ppm, adjust the slope and bias, calibrate the integral to 100%, and click on **return** and select **Save & store 'intrng'** to save the integral and normalization constant and return to the main 1D processing window. Next read in the difference spectrum (**re 3 2**), integrate the NOE peak, adjust the slope and bias, and click **lastscal** to scale the integral.

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