

**H**eteronuclear **M**ultiple **B**ond **C**orrelation spectroscopy is a modified version of HMQC suitable for determining long-range  $^1\text{H}$ - $^{13}\text{C}$  connectivity. This is useful in determining the structure and  $^1\text{H}$  and  $^{13}\text{C}$  assignments of molecules. Since it is a long-range chemical shift correlation experiment, HMBC provides basically the same information as COLOC; however, since it is also an inverse experiment, HMBC has a higher sensitivity than COLOC.

The HMBC pulse sequence may be described simply as follows: The first  $^{13}\text{C}$   $90^\circ$  pulse, which occurs  $1/(2^1J_{\text{XH}})$  after the first  $^1\text{H}$   $90^\circ$  pulse, serves as a low-pass J-filter to suppress one-bond correlations in the 2D spectrum. It does this by creating heteronuclear multiple quantum coherence for  $^1\text{H}$ 's directly coupled to a  $^{13}\text{C}$  nucleus. This unwanted coherence is removed from the 2D spectrum by phase cycling the first  $^{13}\text{C}$   $90^\circ$  pulse with respect to the receiver. After the interval  $\Delta_2$  (which is about 60 msec), the second  $^{13}\text{C}$   $90^\circ$  pulse creates the desired heteronuclear multiple quantum coherence for  $^1\text{H}$ 's J-coupled to a  $^{13}\text{C}$  nucleus 2 or 3 bonds away. This is followed by the evolution time  $t_1$ . A  $^1\text{H}$   $180^\circ$  pulse placed halfway through  $t_1$  removes the effect of  $^1\text{H}$  chemical shift from the  $t_1$  modulation frequency. The final  $^{13}\text{C}$   $90^\circ$  pulse occurs directly after the evolution period, and is followed immediately by the detection period  $t_2$ . After the final  $^{13}\text{C}$   $90^\circ$  pulse, the  $^1\text{H}$  signals originating from  $^1\text{H}$ - $^{13}\text{C}$  multiple quantum coherence are modulated by  $^{13}\text{C}$  chemical shifts and homonuclear  $^1\text{H}$  J-couplings. Phase cycling of the second  $^{13}\text{C}$   $90^\circ$  pulse removes signal from  $^1\text{H}$ 's that do not have a long-range coupling to  $^{13}\text{C}$ . The signal detected during  $t_2$  is phase modulated by the homonuclear  $^1\text{H}$  J-couplings. The 2D spectrum is generated by a Fourier transform with respect to  $t_1$  and  $t_2$ .

Because of phase modulation, the final spectrum has peaks which are a combination of absorption and dispersion lineshapes. It is not possible to phase correct the spectrum so that the peaks are purely absorptive, and so the spectrum must be presented in magnitude mode.

If more than one long-range  $^1\text{H}$ - $^{13}\text{C}$  connectivity is detected for one particular proton, the relative intensities of the corresponding resonances are directly related to the magnitude of the coupling constant.

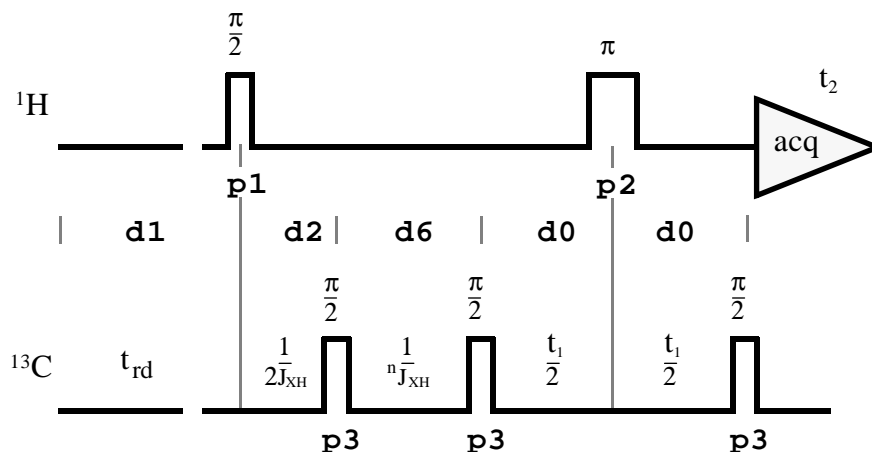
**Reference:** A. Bax and M. F. Summers, *J. Am. Chem. Soc.*, **108**, 2093 (1986).

### Sample

The sample used to demonstrate HMBC in this chapter is 50mM Gramicidin in DMSO-d<sub>6</sub>. This is the same sample that was used to demonstrate COSY, NOESY, ROESY, and TOCSY, and HMQC.

The HMBC pulse sequence is shown in Figure 49. Notice that the pulses **p1** and **p3** must be set to the appropriate  $90^\circ$  times found in Chapter 5 ‘Pulse Calibration’. The  $180^\circ$  pulse length **p2** is determined by the pulse program itself.

Figure 49: HMBC Pulse Sequence



Make sure the following preliminary steps have been completed: Insert the sample in the magnet. Lock the spectrometer. Readjust the Z and  $Z^2$  shims until the lock level is optimized. Tune and match the probehead for  $^1\text{H}$  observation  $^{13}\text{C}$  decoupling.

It is generally recommended that HMBC, like all 2D experiments, be run without sample spinning.

#### $^1\text{H}$ reference spectrum

Since HMBC is a  $^1\text{H}$ -observe experiment, the first step is to obtain a reference  $^1\text{H}$  spectrum of the sample. This reference spectrum will be used to determine the correct **o1** for  $^1\text{H}$ , the correct **sw** for the F2 dimension, and can also be used as the F2 projection of the HMBC spectrum.

A  $^1\text{H}$  reference spectrum of this sample was already created for the magnitude COSY experiment. This spectrum is found in the data set proton/5/1.

#### $^{13}\text{C}$ reference spectrum

It can be assumed that the sample used for an inverse experiment such as HMBC has too small a  $^{13}\text{C}$  signal to make it practical to obtain a  $^{13}\text{C}$  reference spectrum. Thus, the user will need to make an educated guess as to the appropriate values of **o2** and

**sw** for the F1 dimension. Actually, it is easier to use **o2p** (in ppm) rather than **o2** (in Hz). This is because the UXNMR **lock** routine was used to lock the magnetic field, and so 0ppm (for a given nucleus) is at the same absolute frequency regardless of the lock solvent.

Note that because HMBC is a multiple bond correlation experiment, we can expect to detect signals from  $^1\text{H}$ 's coupled to quaternary  $^{13}\text{C}$ 's, in addition to primary, secondary and tertiary  $^{13}\text{C}$ 's. Thus, the  $^{13}\text{C}$  spectral width should be larger than that used for HMQC. An appropriate spectral width would cover the range from  $-10\text{ppm}$  to  $250\text{ppm}$ . This corresponds to an **o2p** value of  $120\text{ppm}$  and an **sw** value of  $260\text{ppm}$ .

### Create a new file directory for the 2D data set

Enter **re proton 5 1** to return to the optimized  $^1\text{H}$  spectrum. From this data set, enter **edc** and change the following parameters:

NAME	hmbc
EXPNO	1
PROCNO	1 .

Click **SAVE** to create the data set hmbc/1/1. By creating the HMBC data set from data set of the  $^1\text{H}$  reference spectrum, most of the F2 parameters for HMBC are already set.

Enter **edsp** and set NUC2 to 13C and OFSH1 to **o1** of the  $^1\text{H}$  reference spectrum proton/5/1. The parameter OFSX1 should have the value of **o2** corresponding to **o2p** =  $120\text{ppm}$ , but the best way to set this is simply to set **o2p** correctly in the main UXNMR window.

### Change to 2D parameter mode

Enter **eda** and set PARMODE = 2D. Click on **SAVE** and ok the message "Delete 'meta.ext' files?". The window now switches to a 2D display and the message "NEW 2D DATA SET" appears.

### Set up the acquisition parameters

Enter **eda** and set the acquisition parameters as shown in Table 51. Use the values determined in Chapter 5 'Pulse Calibration' for the parameters **p11** and **p1** ( $^1\text{H}$  observe high power level and  $90^\circ$  pulse time), and **p12** and **p3** ( $^{13}\text{C}$  decouple high power level and  $90^\circ$  pulse time). Note that the pulse program inv4lplrnd calls an include file in which **cnst2** is used to calculate **d2** ( $\text{d2} = 1/(2 * \text{cnst2})$ ). Thus, it is only necessary for the user to set the value of **cnst2**. Similarly, the  $180^\circ$  pulse length **p2** is calculated from the corresponding  $90^\circ$  pulse length **p1**, so the user need only set the value of **p1**. On the other hand, **d6** is not defined in the include file, and so must be set explicitly in **eda**.

The F2 parameters **o1** and **sw** (not shown in the table) should be identical to the values used in the optimized  $^1\text{H}$  reference spectrum (proton/5/1). Make sure to set **o2p** to  $120\text{ppm}$  as discussed above. The F1 parameter **sw** should also be set to  $260\text{ppm}$  as discussed above.

Finally, notice that **in0** and **sw(F1)** are not independent. A convenient way to set **in0** is to set the F1 parameters **nuc1** by clicking **NUCLEI** for F1 parameters, **nd0**, and **sw** correctly. This automatically sets **in0** to the correct value.

## HMBC

Table 51. HMBC Acquisition Parameters

F2 Parameters		
Parameter	Value	Comments
PULPROG	inv4lplrnd	see Figure 49 for pulse sequence diagram.
TD	4k	
NS	64	the number of scans should be $16 * n$ in order for the phase cycling to work properly.
DS	32	number of dummy scans.
PL1		high power level on f1 channel (see “An Important Note on Power Levels” on page 7).
PL2		high power level on f2 channel (see “An Important Note on Power Levels” on page 7).
P1		$90^\circ$ $^1\text{H}$ high power pulse on f1 channel.
P2		$180^\circ$ $^1\text{H}$ high power pulse on f1 channel; calculated internally.
P3		$90^\circ$ $^{13}\text{C}$ high power pulse on f2 channel.
D0	3 $\mu\text{sec}$	incremented delay ( $t_1/2$ ); predefined.
D1	1.5 sec	relaxation delay; should be about $1.25 * T_1(^1\text{H})$ .
D2	3.45 msec	delay for creation of anti-phase magnetization ( $1/(2J_{\text{XH}})$ ); calculated internally.
D6	$\sim 50$ msec	delay for evolution of long range couplings ( $1/(^n J_{\text{XH}})$ ).
CNST2	145 Hz	one-bond heteronuclear J-coupling ( $J_{\text{XH}}$ ).
F1 Parameters		
Parameter	Value	Comments
TD	256	number of experiments.
ND0	2	there are two d0 periods per cycle and $\text{MC2} = \text{QF}$ .
IN0	$1/(2 * \text{SW}_X) = \text{DW}_X$	$t_1$ increment.
SW		sw of the $^{13}\text{C}$ spectrum (here typically 260 ppm).
NUC1		select $^{13}\text{C}$ frequency for F1

**Acquire the 2D data set**

Enter **zg** to start the HMBC experiment. With the acquisition parameters shown above, the approximate experiment time is 13.5 hours.

### Set up the processing parameters

Enter **edp** and set the processing parameters as shown in Table 52.

**Table 52. HMBC Processing Parameters**


F2 Parameters		
Parameter	Value	Comments
SI	2k	
SF		spectrum reference frequency ( $^1\text{H}$ ).
WDW	QSINE	multiply data by phase-shifted sine-squared function.
SSB	0 (4)	choose pure sine wave (or optimize the phase shift of the sine-squared function).
PH_mod	no	this is a magnitude spectrum.
PKNL	TRUE	necessary when using the digital filter.
BC_mod	quad	
F1 Parameters		
Parameter	Value	Comments
SI	256	
SF		spectrum reference frequency ( $^{13}\text{C}$ ).
WDW	SINE	multiply data by phase-shifted sine function.
SSB	2	choose pure cosine wave.
PH_mod	mc	this is a magnitude spectrum.
BC_mod		
MC2	QF	determines type of FT in F1; QF results in a forward quadrature complex FT.

### Process the 2D data set

It is especially useful to do an automatic baseline correction in the F1 dimension of this 2D spectrum, in part because HMBC spectra usually have quite a bit of  $t_1$  noise and also because they are magnitude mode.

Enter **xfb** to multiply the time domain data by the window functions and also perform the 2D Fourier transform.

### Adjust the contour levels

The threshold level can be adjusted by placing the cursor on the  button, holding down the left mouse button, and moving the mouse up and down.

Since this is a magnitude spectrum, click on **+/-** with the left mouse button until only the positive peaks are displayed.

The optimum display (both the threshold and which peaks are displayed) may be saved by clicking on **DefPlot**.

### Phase correct the spectrum

Since this is a magnitude spectrum, no phase adjustment can be made.

### Plot the spectrum

Read in the plot parameter file standard2D, e.g., enter **rpar standard2D plot**. This sets most of the plotting parameters to values which are appropriate for this 2D spectrum, assuming that the paper size to be used here is the same as the default paper size defined when the spectrometer was configured.

More information about plotting parameters and the file standard2D can be found in Appendix C '1D and 2D Plotting Parameters'.

To set the region (full or expanded), threshold, and peak type (positive and/or negative), to be used in plotting the spectrum, first make sure the spectrum appears as desired on the screen, and then click **DefPlot** and answer the following questions.

```
Change levels?          y
Please enter number of positive levels?      6
Display contours?      n .
```

Enter **edg** to edit the plotting parameters.

Click the **ed** next to the parameter EDAXIS to enter the axis parameters submenu. Change the value of the parameter X2TICD from 0.1 to 2.5. Click **SAVE** to save this change and return to the **edg** menu.

Since there is no  $^{13}\text{C}$  reference spectrum of this sample, the user may choose not to plot an F1 projection for the HMBC spectrum. To do this, simply click the YES adjacent to PROJ1 in the **edg** menu to toggle it to NO.

Click the **ed** next to the parameter EDPROJ2 to enter the F2 projection parameters submenu. Edit the parameters from PF2DU to PF2PROC as follows:

```
PF2DU          u
PF2USER        (name of user for file proton/5/1)
PF2NAME        proton
PF2EXP         5
PF2PROC        1 .
```

Click **SAVE** to save these changes and return to the **edg** menu.

Click **SAVE** to save all the above changes and exit the **edg** menu.

Next create a title for the spectrum. Enter **setti** to use the editor to open the title file. Write a title and save the file.

To plot the spectrum, simply enter **plot** (provided the correct plotter is selected in **edo**).

An HMBC spectrum of 50mM Gramicidin in DMSO-d6 is shown in Figure 50.

Acquisition and Processing

Figure 50: HMBC Spectrum of 50 mM Gramicidin in DMSO-d6

