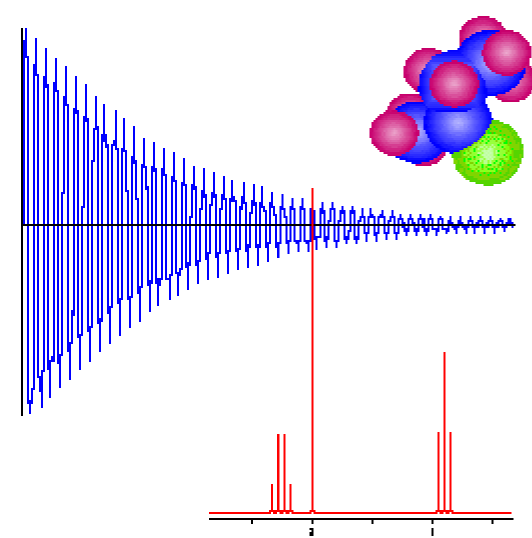
COURSE#1022: Biochemical Applications of NMR Spectroscopy

http://www.bioc.aecom.yu.edu/labs/girvlab/nmr/course/

Lab 4: 2D Proton Homonuclear Spectra and Residue Assignments of a 23 Residue Peptide



LAST UPDATE: 2/28/2007

Reading

Selected Readings for Lab 4:

- refer to lecture on “Resonance assignments using 2D Homonuclear NMR”

Outline

- the primary sequence and NMR sample of a 23-residue peptide

- a strategy for elucidating the structure of peptides/proteins using proton homonuclear 2D NMR methods

- a strategy for assigning the proton signals of peptides/proteins using proton homonuclear 2D NMR methods

- water suppression using WATERGATE

- protocol for collecting NMR spectra of peptide in water/TFE mixture on DRX300

- calibration of ^{1}H 90 degree pulse

- calibration of ^{1}H carrier

- proton NMR experiments used for collecting NMR spectra of peptide in water
    - 1D 1H spectrum with watergate
    - 2D ^{1}H-^{1}H DQF-COSY with watergate
    - 2D ^{1}H-^{1}H TOCSY with watergate
    - 2D ^{1}H-^{1}H NOESY with watergate

A 23-residue peptide

During the next 3 labs (Lab# 4 – Lab# 6), you will assign and calculate the structure of a 23-residue peptide using 2D homonuclear ^1H-^1H NMR methods. The sequence of the peptide is as follows:

Gly_1-Leu-Gly-Lys-Phe_5-Leu-His-Ser-Ala-Lys_{10}-Lys-Phe-Gly-Lys-Ala_{15}-
-Phe-Val-Gly-Glu-Ile_{20}-Met-Asn-Ser-NH_2

We have only <u>one</u> NMR sample of the peptide that will be kept on the rack on top of the DRX300 console – please be very careful handling this sample.

The NMR sample consists of about 4 millimolar peptide dissolved in a mixture of H_2O and trifluoroethanol (TFE)-d_4 and contains 10% D_2O as a lock signal. The pH of the sample is about 4.

The peptide is largely a random coil in water and forms a secondary structure in the presence of TFE.

Solving the structure of this peptide during the next few lab sessions will involve using the procedures described in the last few lectures and outlined on the following page. Briefly, proton 2D NMR spectra, all of which have been collected and made available to you, will be used to assign many of the signals in the proton NMR spectrum of the peptide. Once the signals have been assigned, structural information, mainly in the form of distance restraints obtained from 2D NOESY spectra, will be used to calculate a structure of the peptide. Finally, the resulting structure will be displayed and scrutinized using molecular graphics software.

A Strategy for Elucidating the Structure of Peptides/Proteins Using 2D Proton Homonuclear NMR Experiments

Obtain primary sequence of peptide/protein

Collect 2D NMR spectra

^{1}H-^{1}H DQF-COSY
^{1}H-^{1}H TOCSY, short and long mixing times
^{1}H-^{1}H NOESY, short and long mixing times

Assign spin systems

use TOCSY and DQF-COSY spectra and, if necessary, NOESY spectra

Assign sequential amino acids

use NOESY HN-HN and HN-Hα regions

Obtain structural information from NMR spectra

NOESY	cross peak intensities	r_{HH}
DQF-COSY	$^3J_{HN\text{-}H\alpha}$	ϕ
PE COSY	$^3J_{H\alpha\text{-}H\beta}$	χ_1
H2O/D2O exch.	HN exchange rates	h-bonds

Generate structure

Use software that “folds” primary sequence on basis of structural info.

Visualize structures using molecular graphics

Refine structure, if needed, with additional data

A Strategy for Assigning the Spin Systems of Peptides/Proteins Using 2D Proton Homonuclear NMR Experiments

• Try to determine as many spin systems as possible by identifying through bond connections obtained from COSY and TOCSY spectra. Start with "fingerprint region" (HN-Hα) of DQFCOSY to "anchor" each spin system (note that glycines will often show two HN-Hα crosspeaks).

• Use TOCSY crosspeak pattern in "amide towers" (vertical slice through each HN position) to "type" each spin system:

  • Glycines will have no more than 1 or 2 crosspeaks in the HN tower, even in long-mix TOCSY. Also, the αCH2 group usually gives an "AX" spectrum with a large doublet splitting from the geminal proton coupling constant of 15 hz.

  • Identify αCH-βCH_2 "AMX" spin systems of those spin systems that have β proton chemical shifts in the 2.5-4.0 ppm range and *no* additional crosspeaks even in long-mix TOCSY:

Ser, Asp, Asn, Cys, Trp, Phe, Tyr, His

Note: Ser β is often found around 4.0 ppm close to a shift.

- For aromatic residues, use NOESY to make connection between proton signals of aromatic ring and $H\alpha$ and $H\beta$ proton signals (these correlations do not appear in either TOCSY or DQFCOSY).

- Identify Alanines by TOCSY connection from HN–$H\alpha$–βCH_3 where methyl signal is around 1.5 ppm.

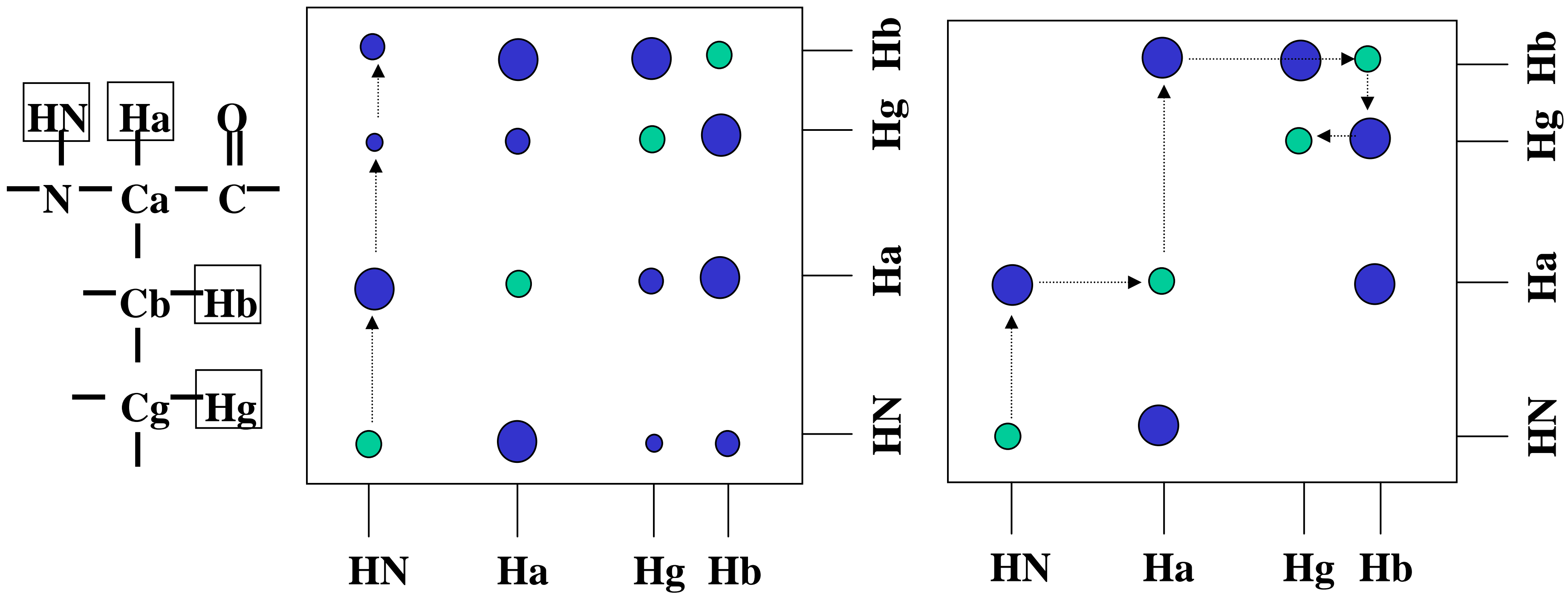
- Identify other methyl containing sidechains Ile, Val and Leu by long mix TOCSY connections between HN and signals in methyl region around 1 ppm (note: the methyl group of Met does not give correlation to HN because of intervening sulfur). The methyl signals of Val are generally more intense because of the shorter chain. Also, the signal from one methyl group of Ile will be more intense than the other because it is closer in bonds to the NH. The methyl signals of Leu are often very weak.

- Some other amino acids also have unique cross peak patterns (eg. Lys) – see Wuthrich handouts from lecture for details.

• Once the spin systems are "typed" or at least classified to a group of amino acids, analysis of NOESY spectra will help "map" the spin system to the primary sequence through sequential assignments.

Strategy for Assigning Spin Systems of Peptides/Proteins using 2D TOCSY and 2D DQF-COSY Methods

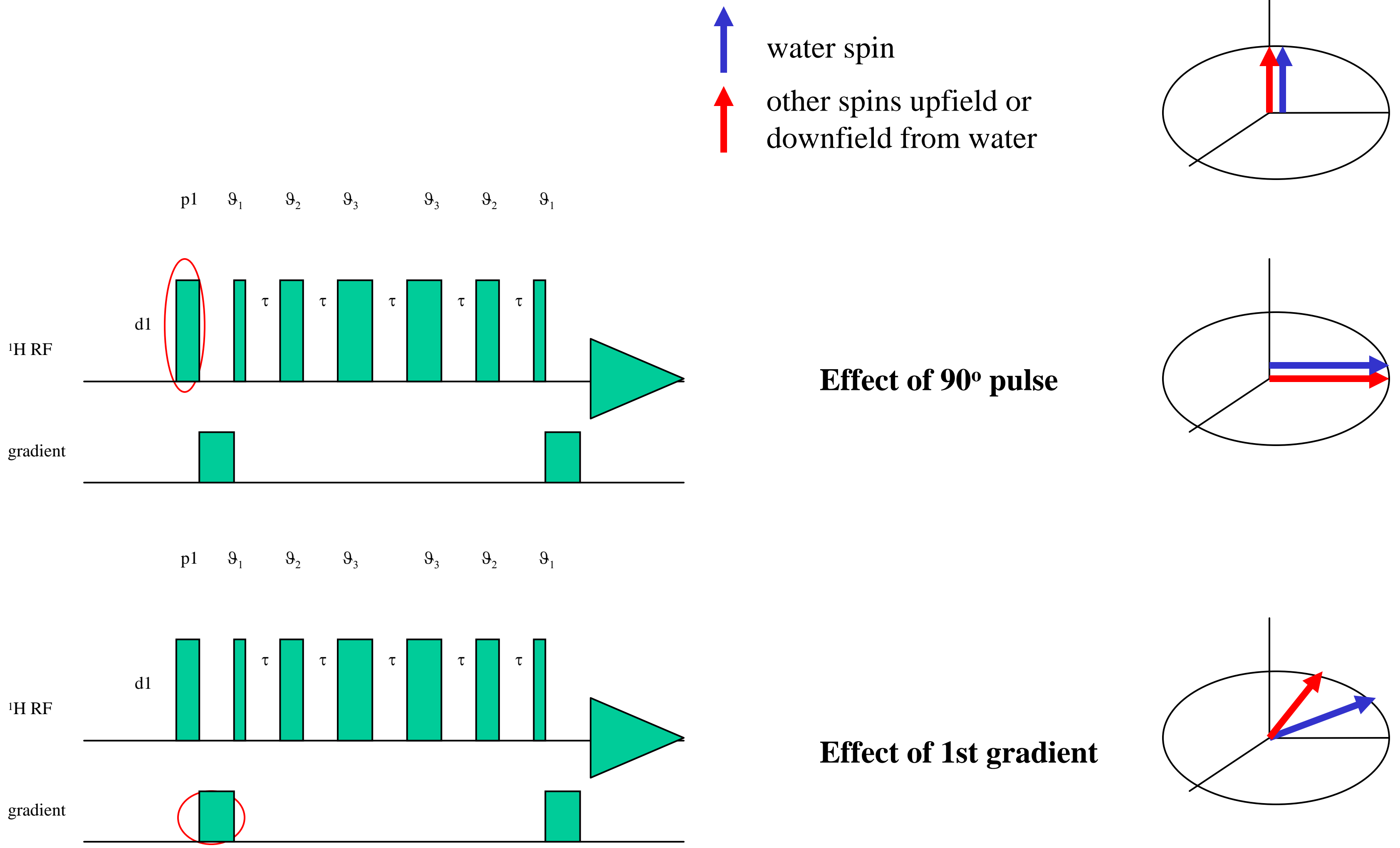
use TOCSY to group all protons within a spin system then use COSY to "order" the protons within the group:



2D long-mix TOCSY

2D DQF-COSY

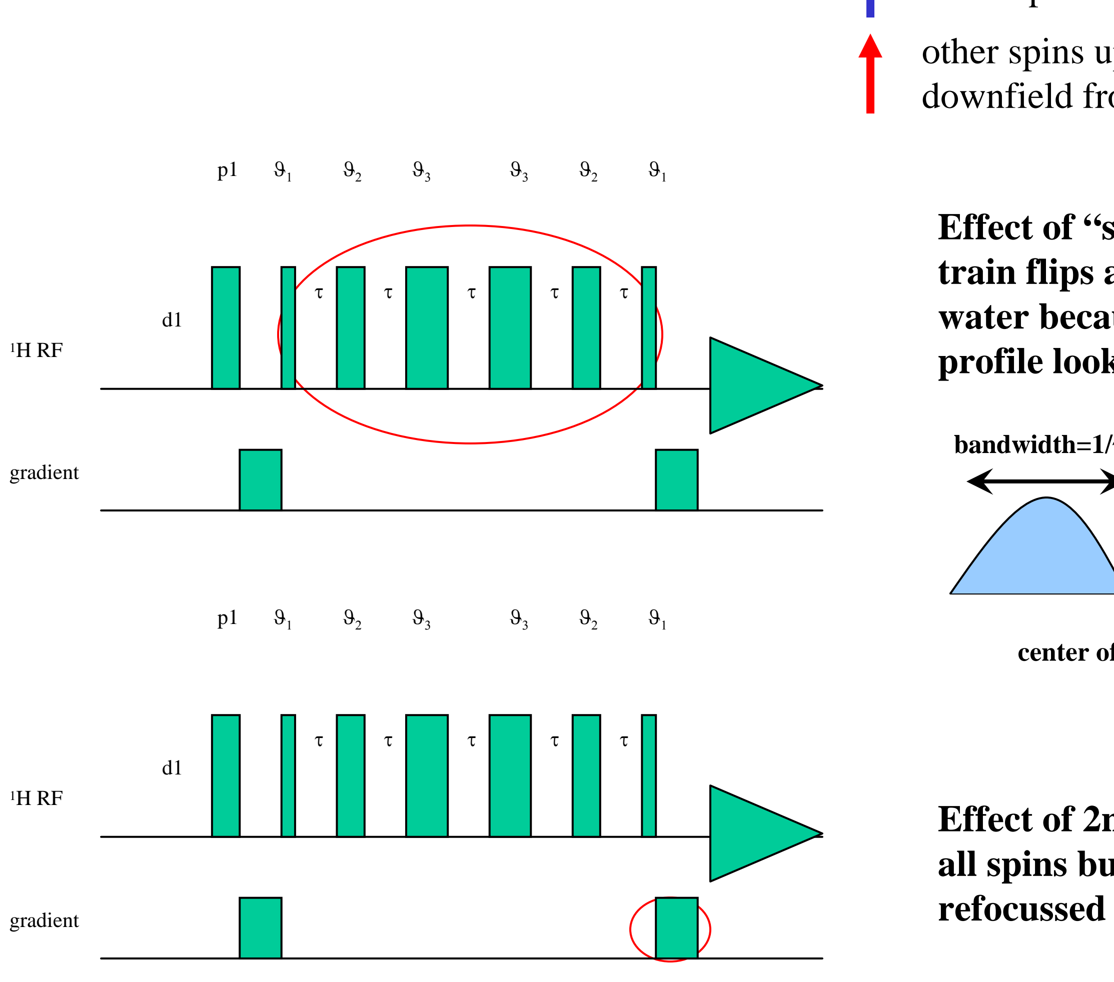
Water Suppression Using WATERGATE



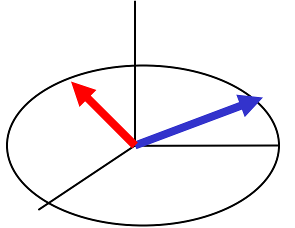
Water Suppression Using WATERGATE, cont.

water spin

other spins upfield or downfield from water



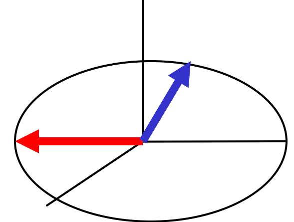
Effect of "selective" pulse train flips all spins but water because excitation profile looks like:



bandwidth=1/τ

center of spectrum

Effect of 2nd gradient: all spins but water are refocussed



Protocol for Collecting NMR Spectra of Peptide in Water/TFE mixture on DRX300

- set temperature to 25 ^{o}C using the command "**edte**"
- load NMR sample
- check tuning of probe using command "**h1tune**"
- bring up lock display by typing "**lockdisp**"
- read default shimfile by typing "**rsh LINESHAPE**"
- lock onto sample using command "**lock D2O**"

Note: since our peptide sample contains two deuterated components (D_2O and TFE), it is important to check that the instrument has properly locked onto the D_2O signal and not the TFE signal. Check by pressing the "LOCK ON/OFF" button on the BSMS keypad so that the lock display shows the signals (should see two). Lock onto the D_2O signal (the "downfield" signal) by moving the "FIELD" button until the D_2O signal is located on the center vertical grid line of the lock display and then press "LOCK ON/OFF" button to relock. If the lock signal is not visible (likely because it is maximized at top of lock display), lower the lock gain by pressing the "LOCK GAIN" button on the BSMS keypad and use knob to lower gain until lock signal becomes visible within lock display.

Protocol for Collecting NMR Spectra of Peptide in Water/TFE mixture on DRX300, cont.

• shim the sample using gradient shimming by typing "**gradshim**" then click on the "Start Gradient Shimming" button of the "Gradient Shimming" window. Wait for new window, "Shim Results", to appear that will ultimately contain several profiles. Make sure that the profile from the last iteration is relatively flat indicating the sample is properly shimmed. Click on "OK" in the "Shim Results" window. Click on "Exit" in the "Gradient Shimming" window to exit from gradshim.

• determine the 90° proton pulse

• determine the proton carrier frequency (o1) for water

• create a new dataset and use the appropriate macro to set-up the desired experiment.

Calibrating the 90 Degree ^{1}H Pulse

Method 1 for determining the 90° ^{1}H pulse:

1. make sure your sample is in, the probe is tuned and your sample is locked and shimmed. **NOTE:** *This procedure will overwrite data so make sure you create a new dataset if needed.*
2. run the macro "**h190**" which will automatically acquire and process a single scan spectrum
3. run the macro "**p1see**" and input a pulse value expected for 360° pulse (should be about 4*(default p1 value)).
4. if signal appears negative, pulse is too short and repeat step 3 with a longer pulse length; if signal appears positive, pulse is too long and repeat step 3 with a shorter pulse length
5. repeat until null signal is obtained; 90° pulse = (360° pulse/4)

Calibrating the 90 Degree ^{1}H Pulse

Method 2 for determining the 90° ^{1}H pulse:

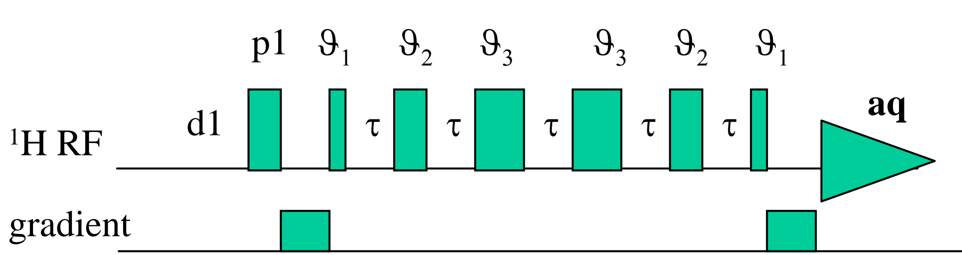
1. make sure your sample is in, the probe is tuned and your sample is locked and shimmed. **NOTE:** *This procedure will overwrite data so make sure you create a new dataset if needed.*
2. run the macro "**h190**" which will automatically acquire and process a single scan spectrum
3. zoom ~0.5 ppm about a signal in spectrum using the **dp1** button
4. run the macro "**paropt**" – answer that p1 is to be varied and input a starting value, increment and # of p1 values to be tried (try to cover a series of p1 values that are close to 360° pulse (should be about 4*(default p1 value)).
5. examine resulting plot to determine where null is achieved and calculate 90° pulse = (360° pulse/4)
6. This procedure will process and display data using proc# 999; use the command "**edc**" to return to proc# 1 when calibration is complete.

Calibrating the ^{1}H Carrier Frequency

1. make sure your sample is in, the probe is tuned and your sample is locked and shimmed. **NOTE:** *This procedure will overwrite data so make sure you create a new dataset if needed.*
2. run the macro "**h190**" which will automatically acquire and process a single scan spectrum
3. go into the UTILITIES menu by clicking on the **utilities** button
4. click on **O1** and use the mouse button to position the cursor at the top of the water signal then press the middle mouse button.
5. type "**o1**" and record the o1 value – this is the carrier position of water.
6. click on the **return** button to exit out of the utilities menu

Proton NMR Experiments Used for Collecting NMR Spectra of Peptide in Water

• 1D ^{1}H spectrum with watergate



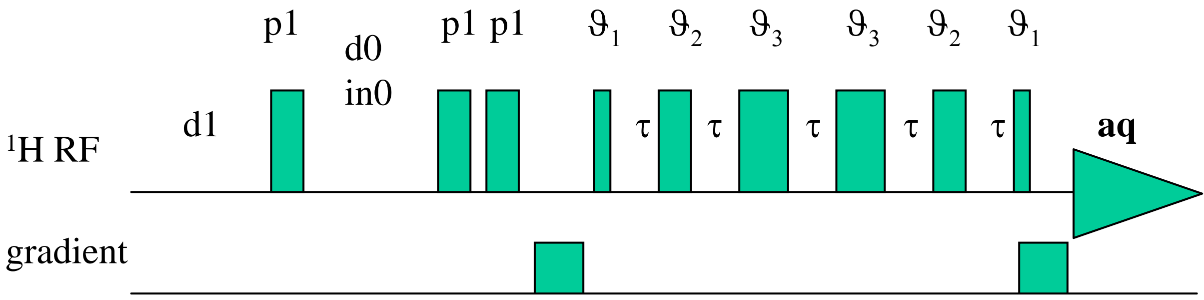
$\vartheta_1 = 0.231 * p1$
$\vartheta_2 = 0.692 * p1$
$\vartheta_3 = 1.462 * p1$
$\tau = d19*2$
$BW = 1/\tau$

Set-up:

1. make sure that instrument is set-up properly (probe is tuned for ^{1}H and sample is locked and shimmed).
2. determine the ^{1}H 90 degree pulse and the carrier position for water.
3. create a new dataset.
4. run macro by typing “1D1Hwgate_peptide” – this macro will set up your dataset with the appropriate parameters for running a 1D ^{1}H spectrum with watergate and will ask for the ^{1}H 90 degree pulse and the carrier frequency (o1) for water.
5. change any acquisition parameters to suit your experimental needs
6. type “rga” to automatically adjust receiver gain. Default receiver gain is 64 – if value of “rg” is larger than 64, set back to 64 using command “rg”; if value of “rg” is smaller than 64, don’t change value but it may indicate problem with water suppression.
7. run the experiment using the command “zg” (starts the experiment) or “azg” (starts the experiment and puts you into acqu window to monitor the data collection).
8. after the experiment ends, the data can be processed using the command “trf”. The spectrum may need to be phased.

Proton NMR Experiments Used for Collecting NMR Spectra of Peptide in Water

• **2D ^{1}H-^{1}H DQF-COSY spectrum with watergate**

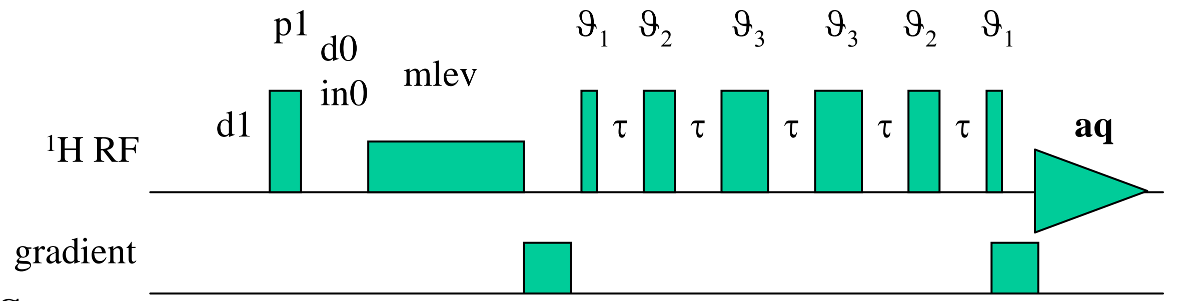


Set-up:

1. **make sure that instrument is set-up properly (probe is tuned for ^{1}H and sample is locked and shimmed).**
2. **determine the ^{1}H 90 degree pulse and the carrier position for water.**
3. **create a new dataset.**
4. **run macro by typing "2DhhDQFCOSYwgate_peptide" – this macro will set up your dataset with the appropriate parameters for running a 2D ^{1}H-^{1}H DQF-COSY spectrum with watergate and will ask for the ^{1}H 90 degree pulse and the carrier frequency (o1) for water.**
5. **change any acquisition parameters to suit your experimental needs**
6. **type "rga" to automatically adjust receiver gain. Default receiver gain is 64 – if value of "rg" is larger than 64, set back to 64 using command "rg"; if value of "rg" is smaller than 64, don't change value but it may indicate problem with water suppression.**
7. **run the experiment using the command "zg" (starts the experiment) or "azg" (starts the experiment and puts you into acqu window to monitor the data collection).**
8. **after the experiment ends, the data can be processed using the command "xfb". The spectrum may need to be phased.**

Proton NMR Experiments Used for Collecting NMR Spectra of Peptide in Water

• 2D ^{1}H-^{1}H TOCSY spectrum with watergate



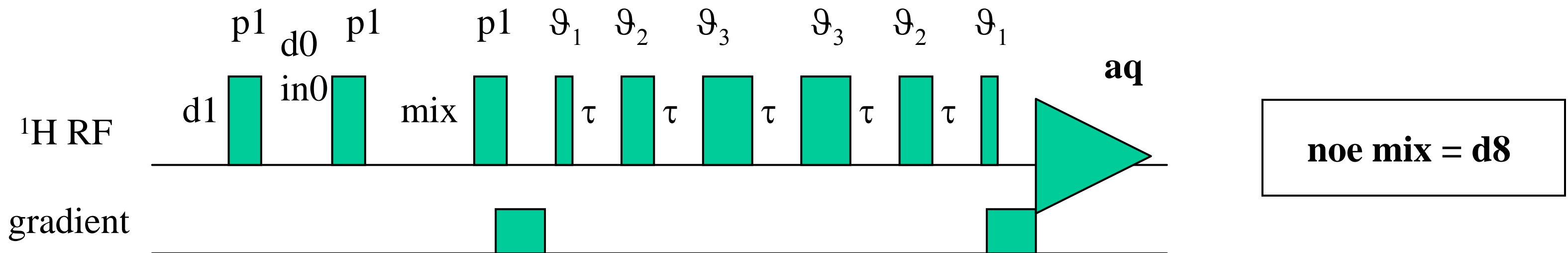
mlev spin lock: mix = p6 * 64 *l1 @ pl10
= 2.9ms * l1

Set-up:

1. make sure that instrument is set-up properly (probe is tuned for ^{1}H and sample is locked and shimmed).
2. determine the ^{1}H 90 degree pulse and the carrier position for water.
3. create a new dataset.
4. run macro by typing “2DhhTOCSYwgate_peptide” – this macro will set up your dataset with the appropriate parameters for running a 2D ^{1}H-^{1}H TOCSY spectrum with watergate and will ask for the ^{1}H 90 degree pulse and the carrier frequency (o1) for water.
5. change any acquisition parameters to suit your experimental needs
6. type “rga” to automatically adjust receiver gain. Default receiver gain is 512 – if value of “rg” is larger than 512, set back to 512 using command “rg”; if value of “rg” is smaller than 512, don’t change value but it may indicate problem with water suppression.
7. run the experiment using the command “zg” (starts the experiment) or “azg” (starts the experiment and puts you into acqu window to monitor the data collection).
8. after the experiment ends, the data can be processed using the command “xfb”. The spectrum may need to be phased.

Proton NMR Experiments Used for Collecting NMR Spectra of Peptide in Water

• 2D ^{1}H-^{1}H NOESY spectrum with watergate



Set-up:

1. make sure that instrument is set-up properly (probe is tuned for ^{1}H and sample is locked and shimmed).
2. determine the ^{1}H 90 degree pulse and the carrier position for water.
3. create a new dataset.
4. run macro by typing "2DhhNOESYwgate_peptide" – this macro will set up your dataset with the appropriate parameters for running a 2D ^{1}H-^{1}H NOESY spectrum with watergate and will ask for the ^{1}H 90 degree pulse and the carrier frequency (o1) for water.
5. change any acquisition parameters to suit your experimental needs
6. type "rga" to automatically adjust receiver gain. Default receiver gain is 512 – if value of "rg" is larger than 512, set back to 512 using command "rg"; if value of "rg" is much smaller than 512, don't change value but it may indicate problem with water suppression.
7. run the experiment using the command "zg" (starts the experiment) or "azg" (starts the experiment and puts you into acqu window to monitor the data collection).
8. after the experiment ends, the data can be processed using the command "xfb". The spectrum may need to be phased.