Basic NMR Operation for Beginners

Steps for NMR Experiments – larger molecule (biomolecules)

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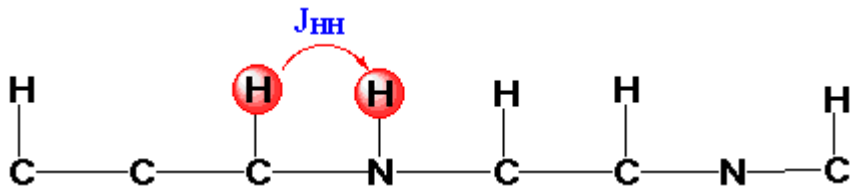
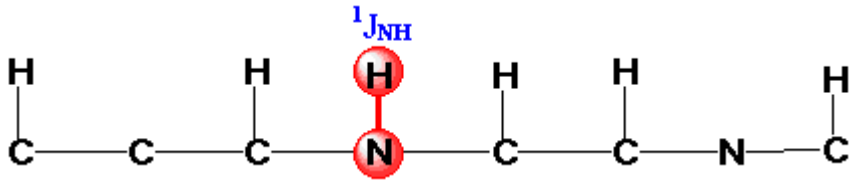
Outline

- introduction of biomolecular NMR
- water suppression techniques
- brief introduction of pulsed field gradients
- 1H,15N-HSQC
- HNCO

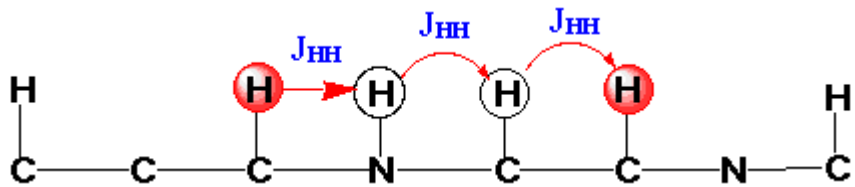
Information Content



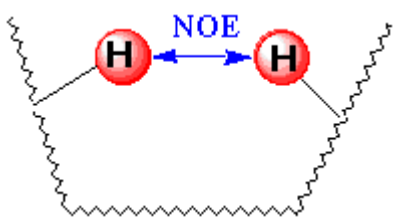
1H-1H COSY (2-, 3-bond)
- **DQF-COSY, E. COSY**
- **Dihedral angle constraints**



Hetero-nuclear COSY (one-bond)
- **1H,15N or 1H,13C-HSQC, HMQC**



TOCSY (2-, 3-bond)
(1-bond for 13C-13C)
Total Correlation



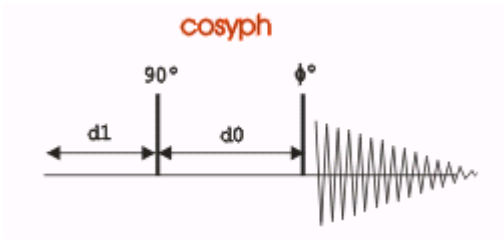
1H-1H NOESY
(through space, <5 Å
- **Intensity & 1/r6**

NOESY or ROESY
- **Distance constraints**

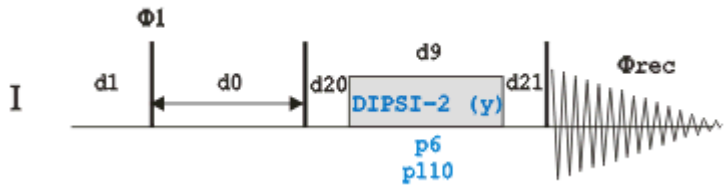
Figures from Bruker's NMR Guides

Some Basic NMR Pulse Sequences

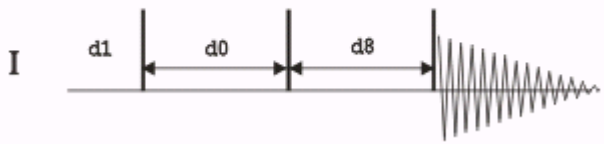
cosyph



dipsi2ph

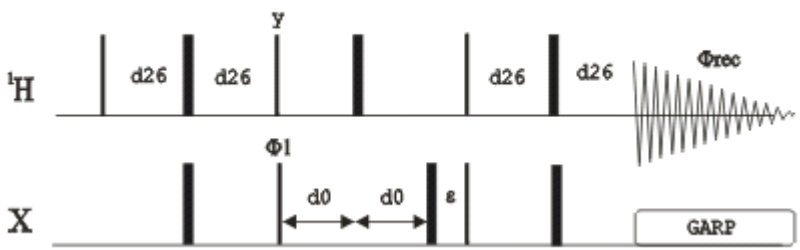


noesyph



roesyph

hsqcf3ph



figures from Bruker's NMR Guides

Peptide/Protein NMR Studies

Peptide/Protein NMR:

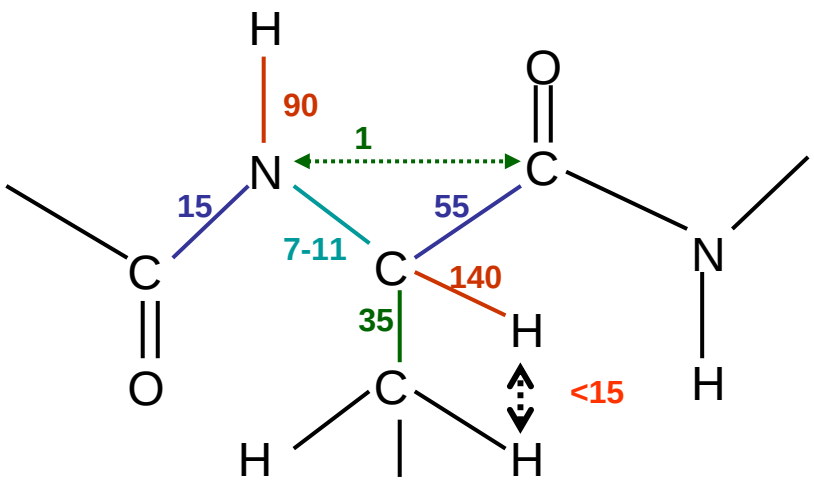
•NMR experiments are usually performed in H_2O (with 5-10% D_2O):

•Signals detected are pH dependent (N-H protons exchange with water).

•Water suppression is required.

$$\text{N-H} + {}^{*}\text{H}_2\text{O} \rightleftharpoons \text{N-}{}^{*}\text{H} + {}^{*}\text{H-O-H}$$

•Higher molecular weight (NOESY V.S. ROESY), shorter T_2, three-bond ^{1}H-^{1}H J-coupling is less efficient with increasing molecular weight (correlation time), isotope labeling (^{15}N/^{13}C/^{2}H) become necessary when #A.A.>50.



•A ^{1}H, ^{15}N-HSQC ($^1J_{NH}$=90 Hz) or a ^{1}H, ^{13}C-HSQC ($^1J_{CH}$=140) is much more sensitive than a ^{1}H-^{1}H COSY ($^3J_{HH}$ <15) experiment.

Typical J-coupling constant values in peptide

Cross Relaxation (NOE) Depends on Tumbling Rates

- ROESY for small molecules (1-2 kDa).
- NOESY for sizeable molecules (>2 kDa)

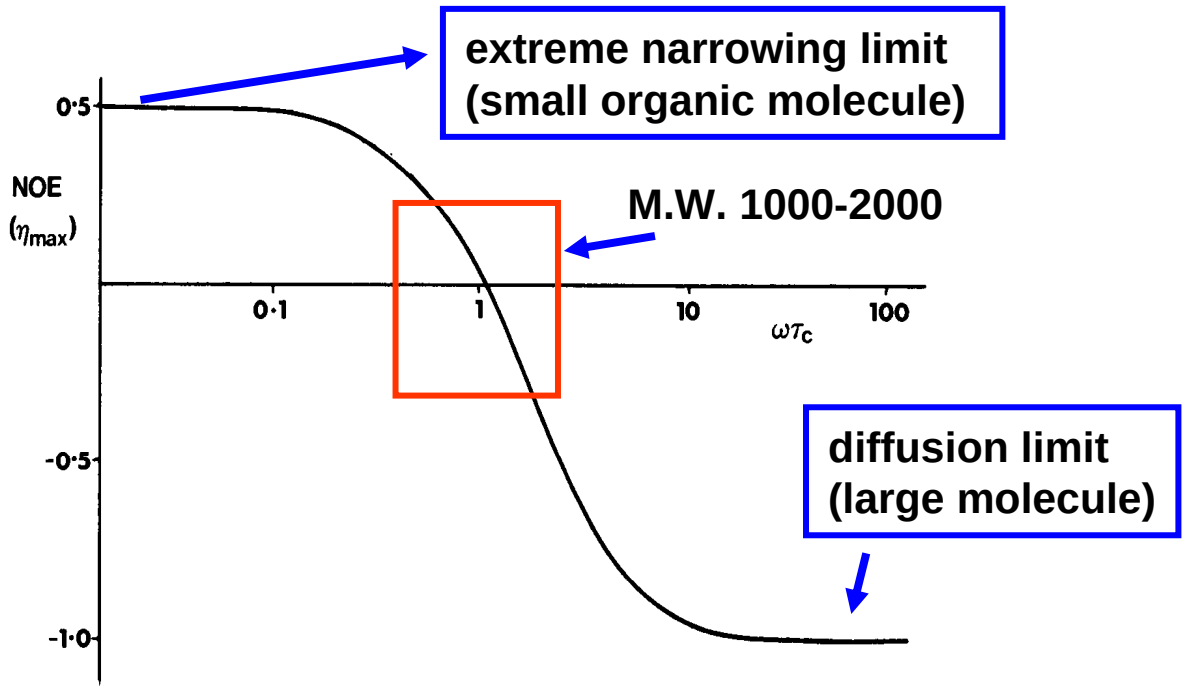


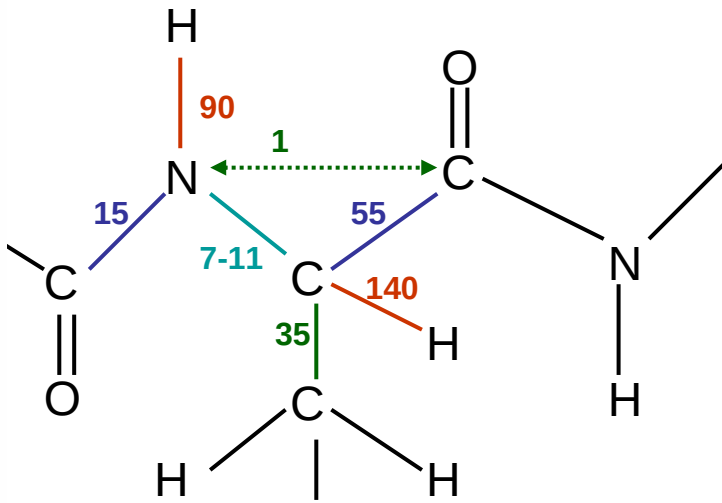
FIGURE 2.6
Dependence of maximum homonuclear NOE enhancement on $\omega\tau_c$. Note the log scale of $\omega\tau_c$.

(figure from p37 of "The Nuclear Overhauser Effect" by D. Neuhaus and M. Williamson

Strategies for Protein NMR Studies

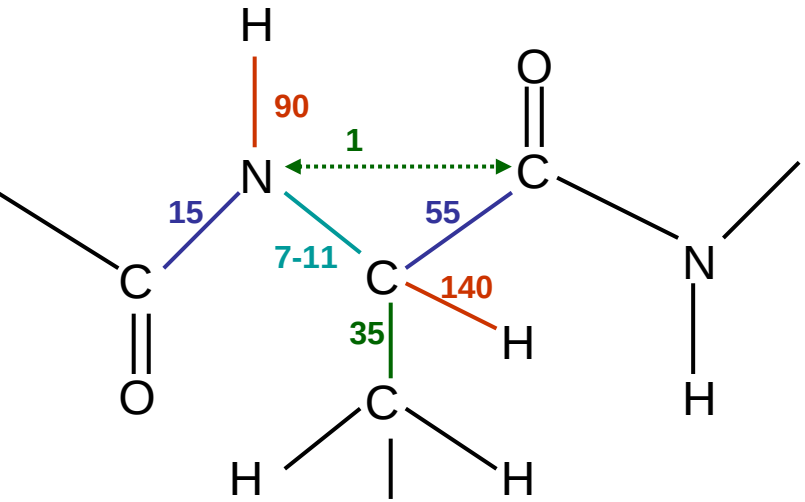
Table 1: Strategies for protein studies

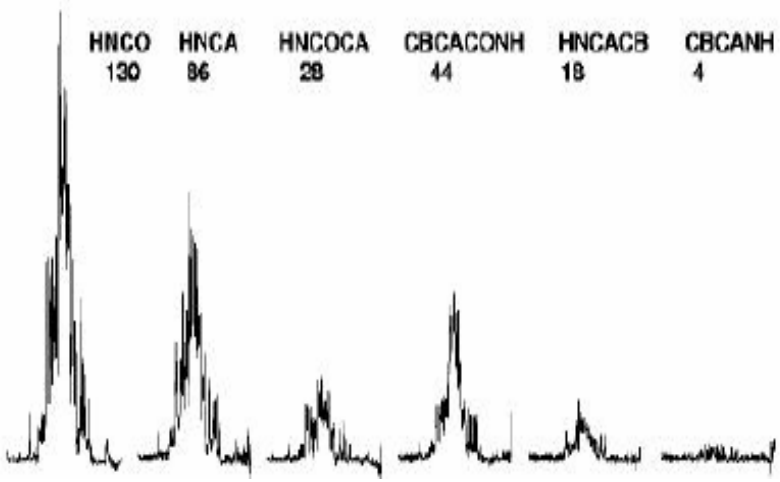
Protein/Size	Experiment	Information obtained	Sensitivity
Unlabeled/ less than 50.a.a.	*2D Homonuclear*		
	COSY, TOCSY	intra-residue assignments	
	NOESY	sequential connectivities NOE distance constraints $^{3}J_{HN\alpha}$ coupling constants	
	E.COSY	$^{3}J_{H\alpha\beta}$ coupling constants	
^{15}N-labeled/ ~ 50-80 .a.a.	*3D Double resonance*		
	^{15}N-TOCSY	intra-residue assignments	
*	^{15}N-NOESY	sequential connectivities NOE constraints	
*	^{15}N-HNHA	$^{3}J_{HN\alpha}$ coupling constants	
	or 2D HMQC-J	$^{3}J_{HN\alpha}$ coupling constants	
	^{15}N-HNHB	$^{3}J_{H\alpha\beta}$ coupling constants	



Typical J-coupling constant values

Table taken from Bruker's Avance 3D/ Triple Resonance manual





1st increment of 3D triple resonance experiments

From Bruker's Avance 3D/ Triple Resonance manual

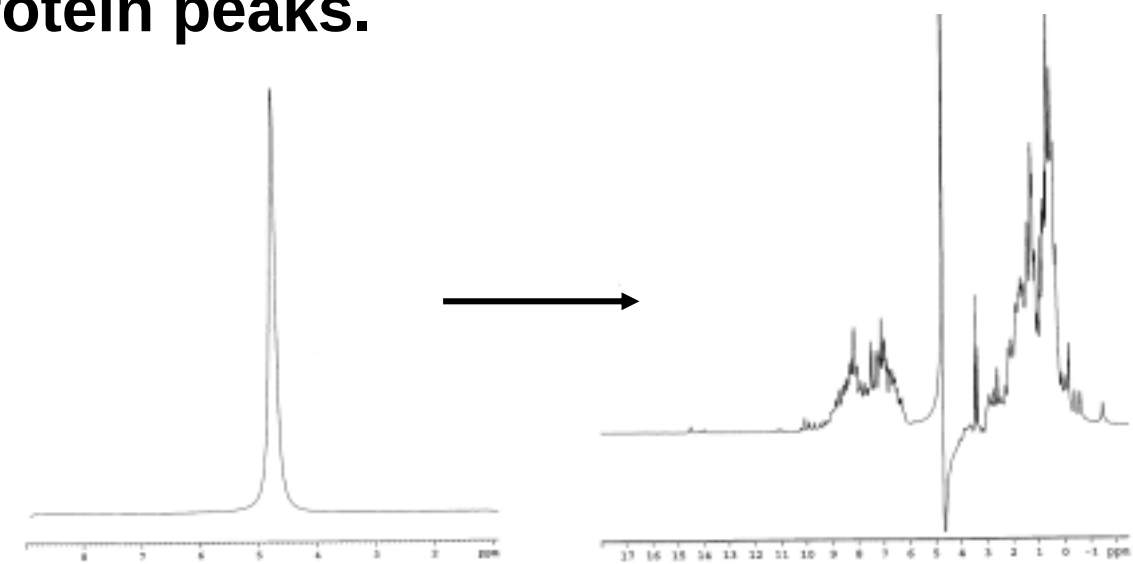
Protein/Size	Experiment	Information obtained	Sensitivity
^{13}C, ^{15}N-labeled/ – 80-150 .a.a.	*3D Double resonance*	*NB. Possibly fractionally ^{2}H-labeled*	
*	^{15}N-NOESY	NOE constraints	
*	^{15}N-HNHA	$^{3}J_{HN\alpha}$ coupling constants	
	^{15}N-HNHB	$^{3}J_{H\alpha\beta}$ coupling constants	
	^{13}C HCCH-COSY	intra-residue assignments	
*	^{13}C HCCH-TOCSY	intra-residue assignments	
	^{13}C NOESY	sidechain NOE constraints	
	3D Triple resonance		
	HNCO	sequential connectivity	100 inter
	HN(CA)CO	sequential connectivity (combine with HNCO)	13/4 intra/inter
*	HNCA	sequential connectivity $^{13}C^{\alpha}$ chemical shift constraints	50/15 intra/inter
*	HN(CO)CA	(combine with HNCA)	71 inter
*	CBCA(CO)NH	sequential connectivity $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ chemical shifts	13/9 $^{13}C^{\alpha}$ / $^{13}C^{\beta}$ inter
	CBCANH	for smaller proteins (combine with CBCA(CO)NH)	4/1.7 $^{13}C^{\alpha}$ / $^{13}C^{\beta}$ intra 1.3/0.5 $^{13}C^{\alpha}$ / $^{13}C^{\beta}$ intra
	HNCACB	for bigger proteins (combine with CBCA(CO)NH)	
*	HBHA(CO)NH	$^{1}H^{\alpha}$ and $^{1}H^{\beta}$ assignments	13/9 $^{1}H^{\alpha}$ / $^{1}H^{\beta}$ inter
*	H(CCCO)NH	sidechain ^{1}H assignments	
	(H)CC(CO)NH	sidechain ^{13}C assignments	
^{13}C, ^{15}N,^{2}H-label. >160 .a.a.	*3D Triple resonance with ^{2}H-decoupling*		
	CT-HNCA	sequential connectivity	
	HN(CO)CA	(combine with HNCA)	
	CBCA(CO)NH	sequential connectivity $^{13}C^{\alpha}$/$^{13}C^{\beta}$ chemical shifts	
	CT-HNCACB	(combine with CBCA(CO)NH)	
	C(CO)NH	sidechain ^{13}C assignments	
	^{15}N-HSQC-NOESY-HSQC	sequential and long-range NH-NH NOE constraints	

The experiments presented in this manual are denoted by an asterisk.

Water Suppression

Why water suppression?

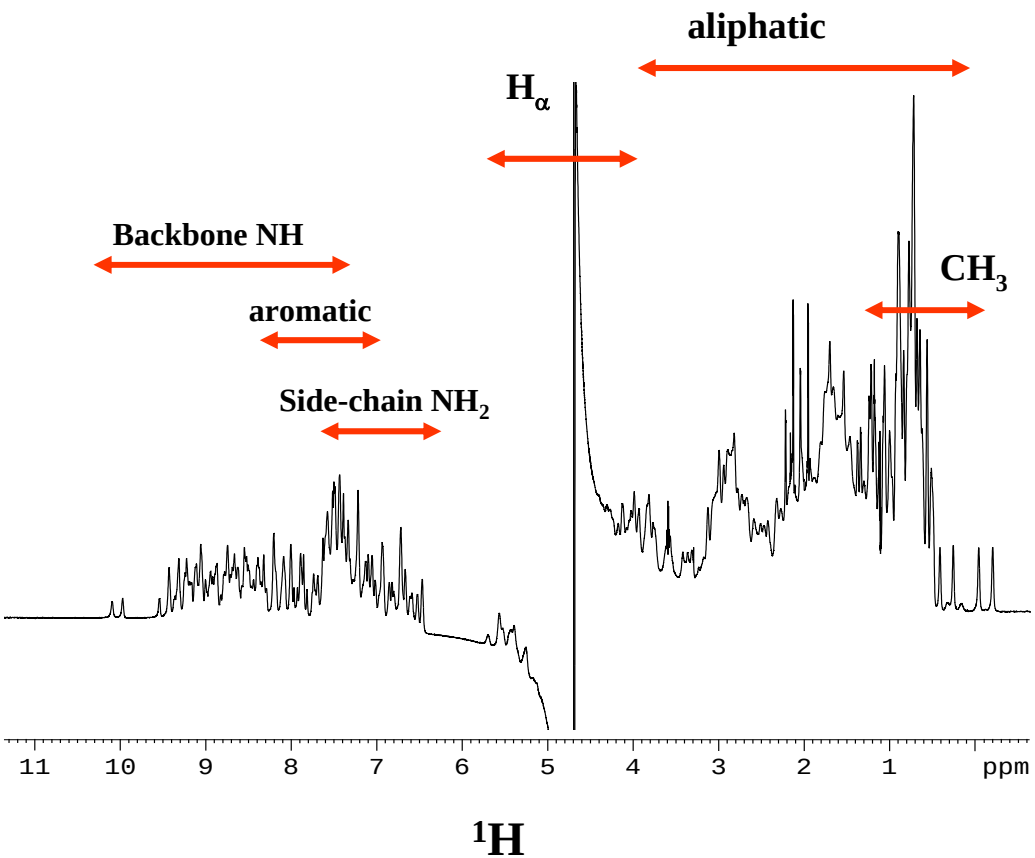
- **[H_2O]~110 M, [Protein]~1 mM**
- **110 M/1 mM=110,000**
- **Suppression of the strong solvent signal is necessary in order to obtain high signal to noise for the protein peaks.**



Water Suppression: Some Consideration

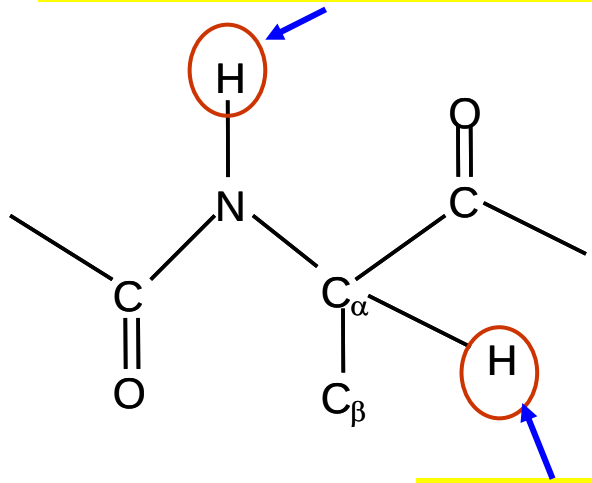
$$N\text{-}H + {}^{*}H_2O \rightleftharpoons N\text{-}{}^{*}H + {}^{*}H\text{-}O\text{-}H$$

Saturation transfer: saturation of water also reduce the intensity of the resonances that are in exchange with water.



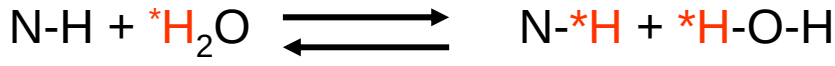
^1H

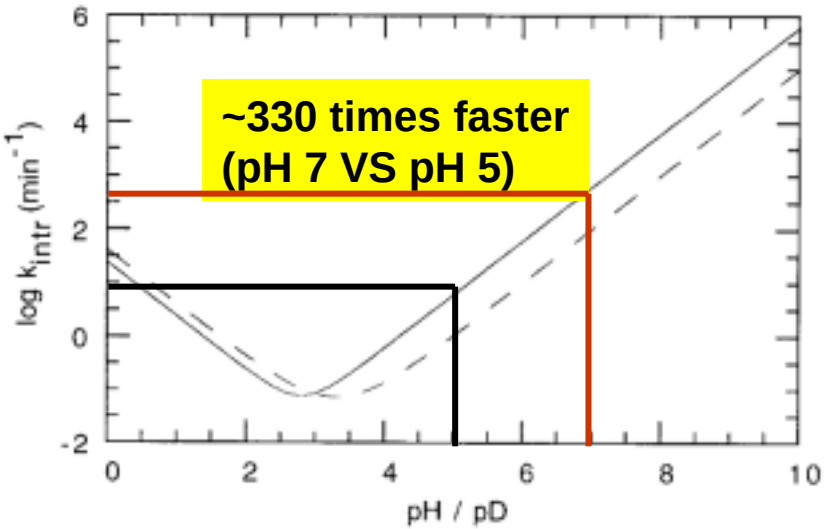
Labile, exchange with water (pH-, structure-, temperature-dependent)



near water

Amide Proton Exchange Rate





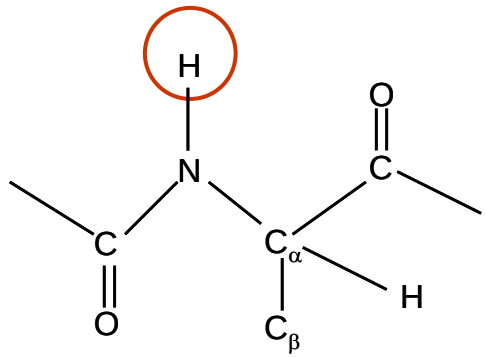


FIGURE 3.26 Intrinsic backbone amide proton exchange rates calculated according to Connelly *et al.* (*63*). The intrinsic exchange rate, k_{intr}, is shown for exchange of a backbone amide proton with (—) H_2O or (- - -) D_2O as a function of pH or pD. The pD values are corrected for isotope effects; uncorrected pH meter readings would be 0.4 units smaller.

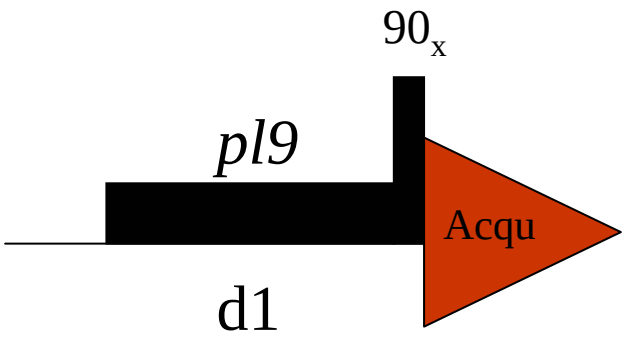
Figure modified from p151 by Cavanagh et. al "Protein NMR Spectroscopy"

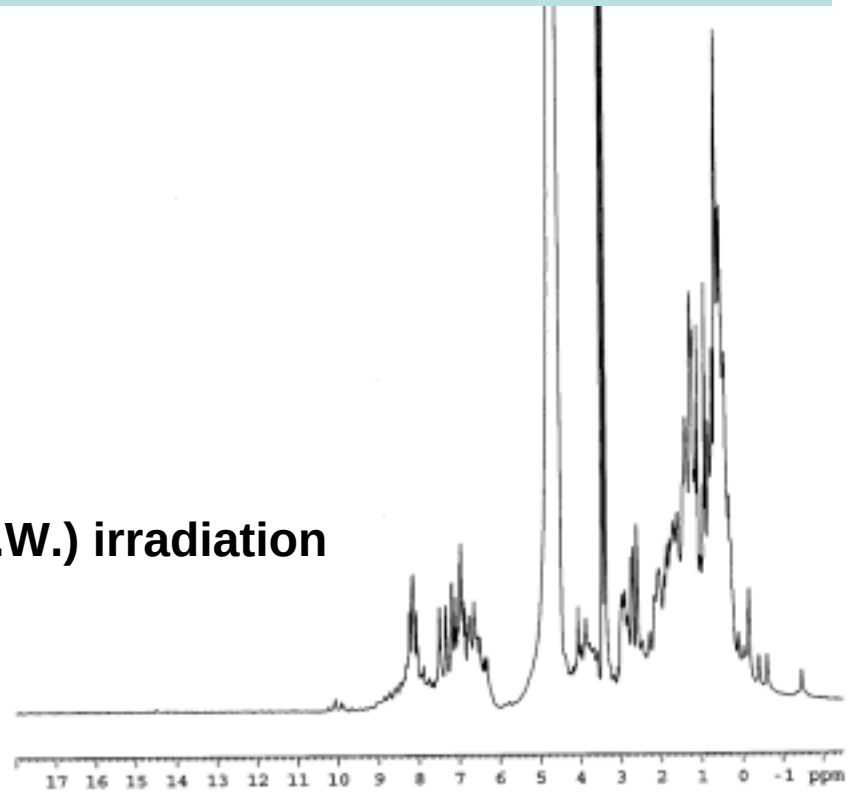
In practice: the pH value for a protein sample for NMR studies is kept below 7.5

Water Suppression

- Presaturation (‘zgpr’)
- Watergate (‘zggpwg’)
- Water flipback (‘*fp*’)
- Jump and return, 1-1, 1331
- Coherence pathway rejection (Echo/anti echo)

Presaturation





Usually during the relaxation delay ,apply a low power continued wave (C.W.) irradiation before the first 90 degree pulse.

Pulprog: zgpr

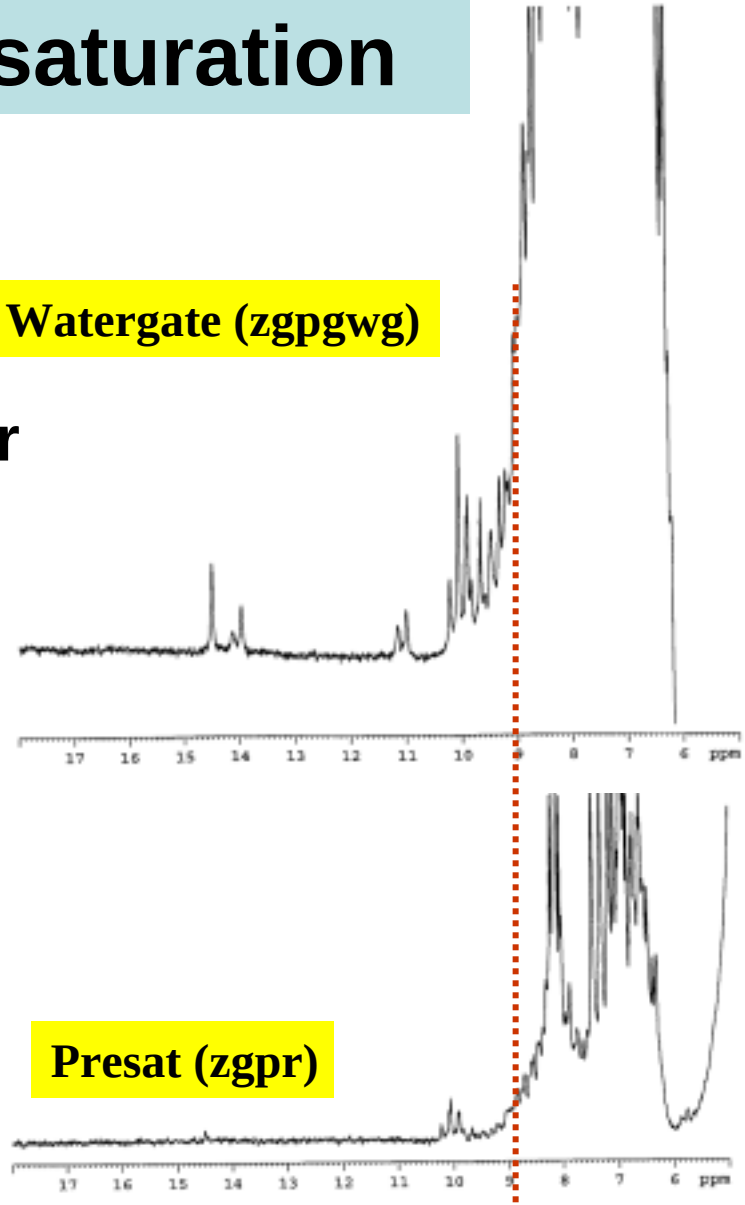
Power level:

pl9: for weak saturation on channel 1

pl1: high power level for channel 1

Drawback of Presaturation

- **Saturation transfer to the exchangeable NH protons**
- **Bleaching of signals near water**
- **Large dispersive tail of water signal: tilted baseline**



Pulsed Field Gradient

- **A field-gradient pulse is a pulse or a period during which the magnetic field is made deliberately inhomogeneous.**

$$B=B_0+B_g(z)$$

- **The magnetic field, generated by a gradient pulse, Bg(z) varies linearly along the Z-axis**

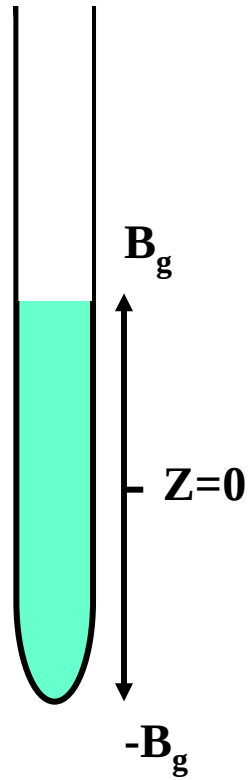
$B_g(z)=zG_z$, where

G_z: gradient strength (G/cm), Z: z-axis position

- ***Viewing on the rotating frame, spins at different z-position acquire different phase (Larmor frequencies):***

$$\phi(z)=\gamma z G_z t,$$

where γ: gyromagnetic ratio, τ: gradient duration

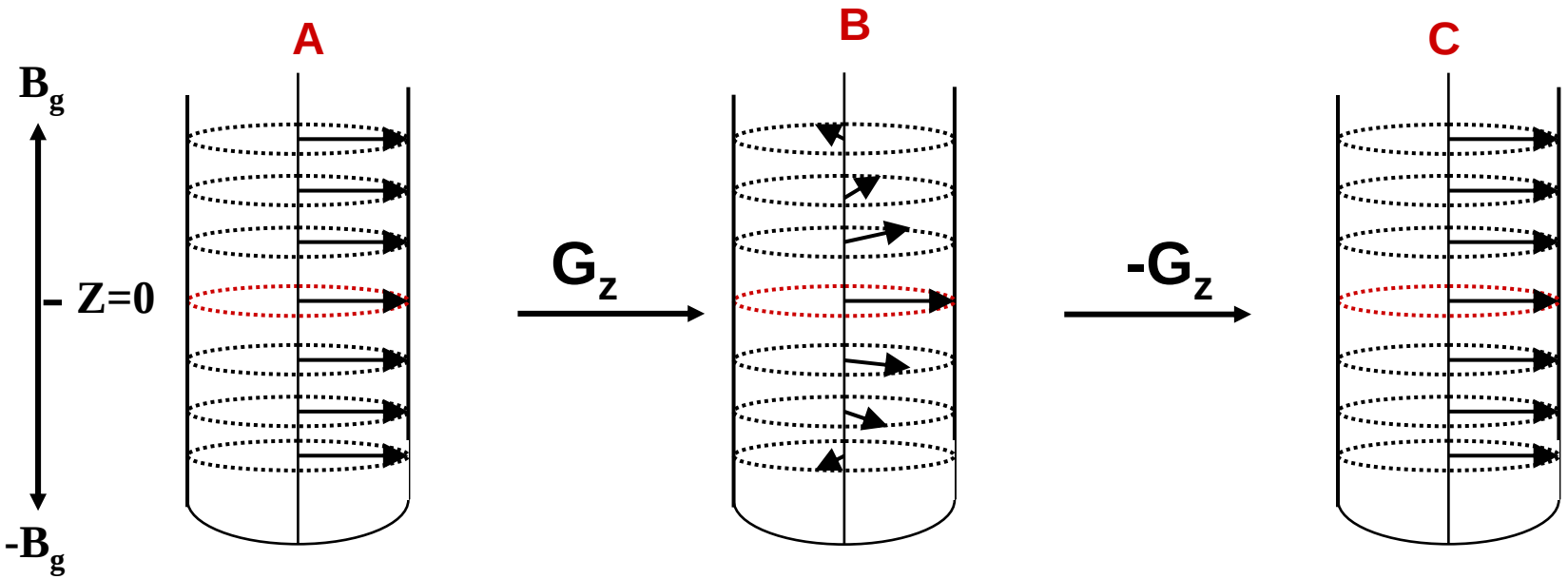


- **A coherence can be dephased by a strong pulse field gradient.**
- **A dephased coherence can be refocused by a refocus-gradient providing the "overall phase change " is zero.**

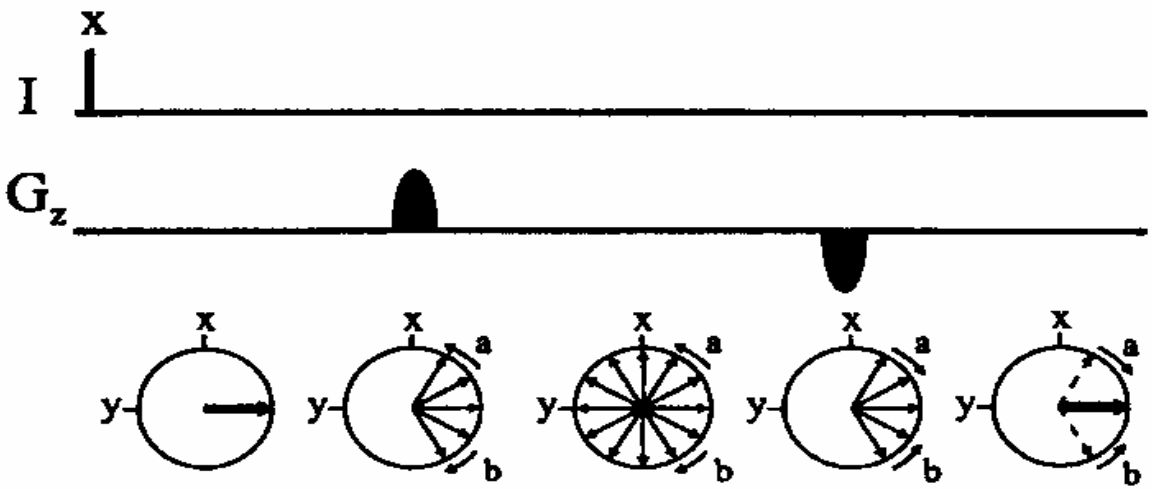
$\phi_i+\phi_f=0$;Coherence refocused.

$\phi_i+\phi_f\neq 0$;Coherence dephased.

Pulsed Field Gradient (PFG)

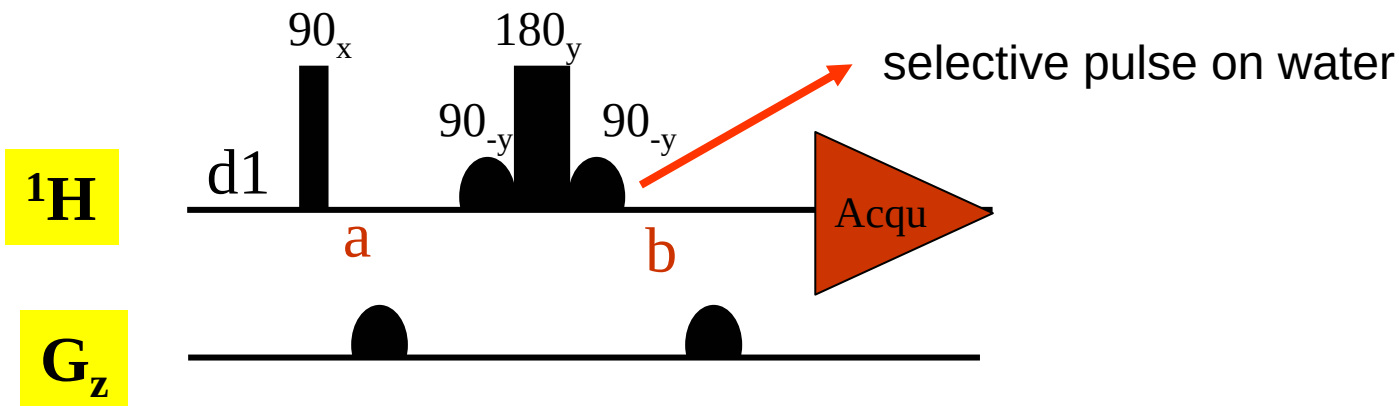


Viewing from the Z-axis:



(figure from p106 of Sattler et al. Prog. In Nucl. Mag. Reson. Spect. 34 (1999)

Watergate



•**Z-gradient destroy transverse magnetization.**

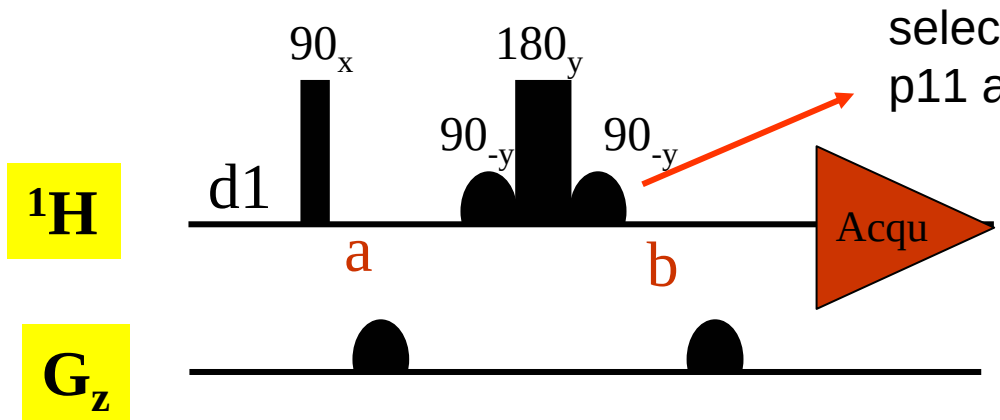
•**The destroyed magnetization can be refocused by another z-gradient pulse of the same amplitude but of opposite phase. (or use a 180 pulse in between two identical z-gradient pulse).**

•**H_2O: the two extra selective 90 pulse on water makes the 2nd z-gradient pulse act as another defocus gradient pulse. Water is suppressed.**

•**Protein signals: the 180 pulse makes the 2nd Z-gradient act as a refocus gradient. Protein signals are observed.**

Ref: M. Piotto, V. Saudek & V. Sklenar, ***J. Biomol. NMR*** *2, 661 - 666 (1992)*

Watergate

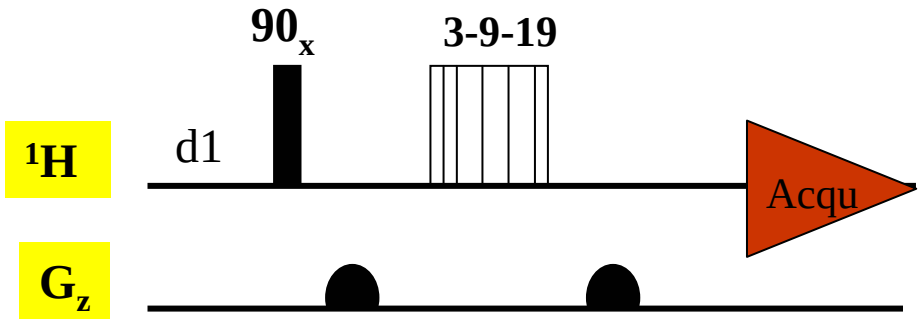


- **Parameter adjustment, Pulprog=zggpwg**
- **p11: pulse length for 90 degree shaped pulse**
- **sp1: power level for 90 degree shaped pulse**
- **spnam1: type of shaped pulse**

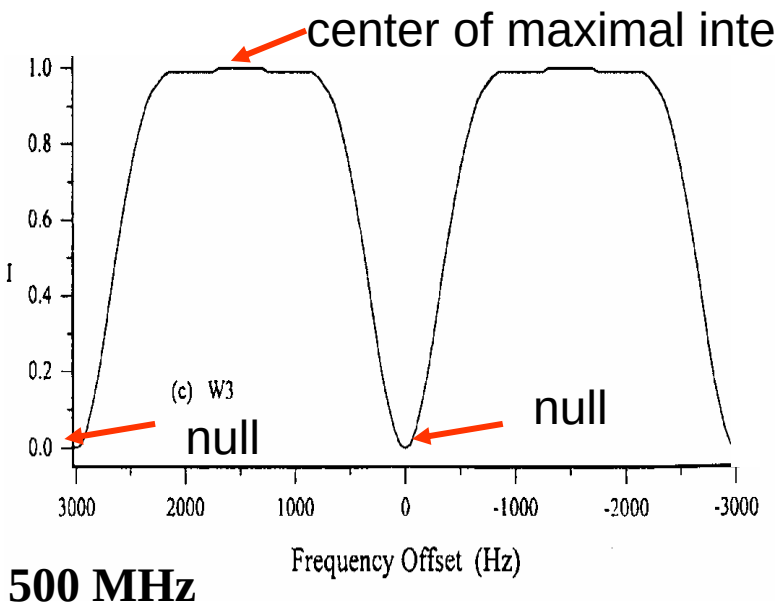
For example: set spnam1=Sinc1.1000, p11=1.5-2 msec,

Minimize the fid (less water signal) by adjusting the power level "sp1" under the "gs" utility.

3-9-19 Watergate



- **Off resonance DANTE excitation technique.**
- **3-9-19: 3α-τ-9α-τ-19α–τ-19α-τ-3α, where α=180/26-degree hard pulse, τ=delay.**



500 MHz

Delay $\tau = 1/(4\,\Delta\upsilon_{max})$,
where $2\Delta\upsilon_{max}$=distance of next null (Hz).
(The delay τ is field-dependent !!)

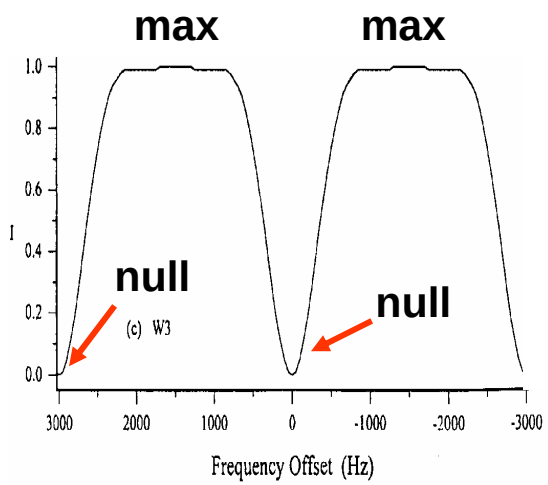
*Sklenar et al., **J. Magn. Reson.**, A102, 241-245 (1993)*

3-9-19 Watergate

Delay τ =1/(4 $\Delta\upsilon_{max}$), where $2\Delta\upsilon_{max}$=distance of next null (Hz).
(The delay τ is field-dependent !!)

Parameter adjustment: Pulprog=p3919
Set pl18=pl1, p27=p1, p0=p1
;d19: delay for binomial water suppression
;d19 =1/2*d, d = distance of next null (in Hz)

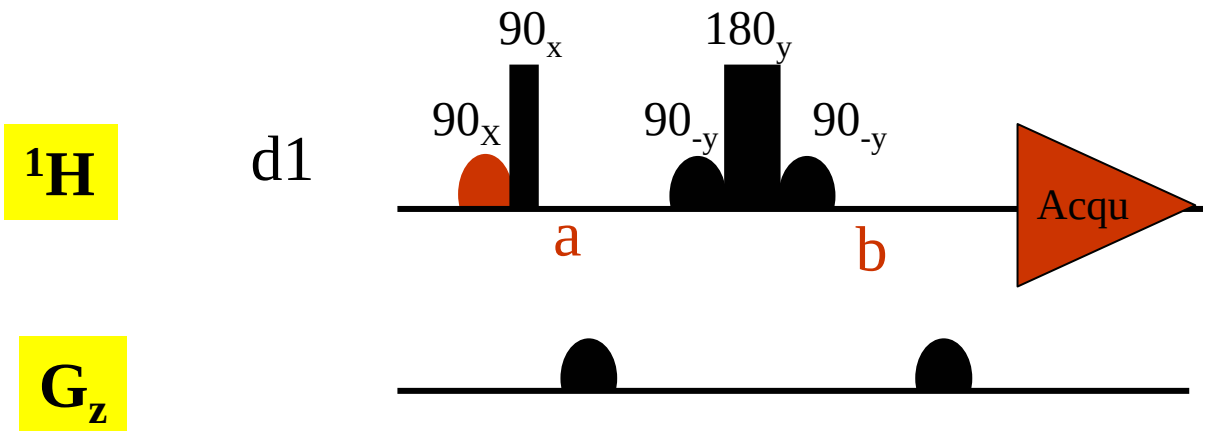
Adjust d19 according to the magnetic field strength and where you want the center of maxima excitation to be.



For example: Have the center of NH region (i.e. 8.2 ppm) to be the center of maximal excitation region:

t=1/[4*(8.2-4.75)*600.13]=121 usec @600 MHz machine
t=1/[4*(8.2-4.75)*500.13]=145 usec @500 MHz machine
(Carrier frequency on H_2O at 4.75 ppm)

Water Flip-back Watergate

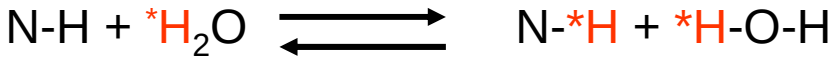


S. Grzesiek and A. Bax, ***J. Am. Chem. Soc.****, 115, 12593-12594 (1993)*

- **Water is aligned along the z axis before any Z-gradient pulse point "a".**
- **Water is not destroyed by the z-gradient pulse; this reduces the signal loss of exchangeable protons due to attenuation of water Signal.**

Parameter adjustment:
Pulprog=*fp*, i.e "hsqcetfpf3gp" calibrate the shape pulse as describe in WATERGATE.

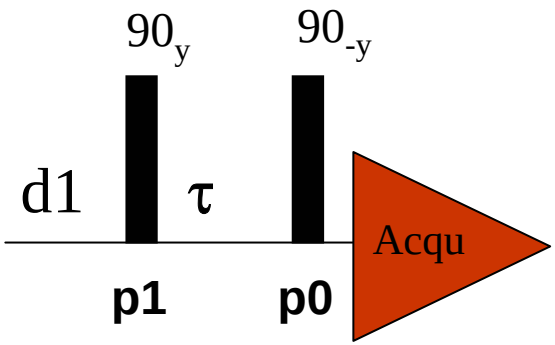
Pulse Sequence for Observing Fast-Exchanging Protons



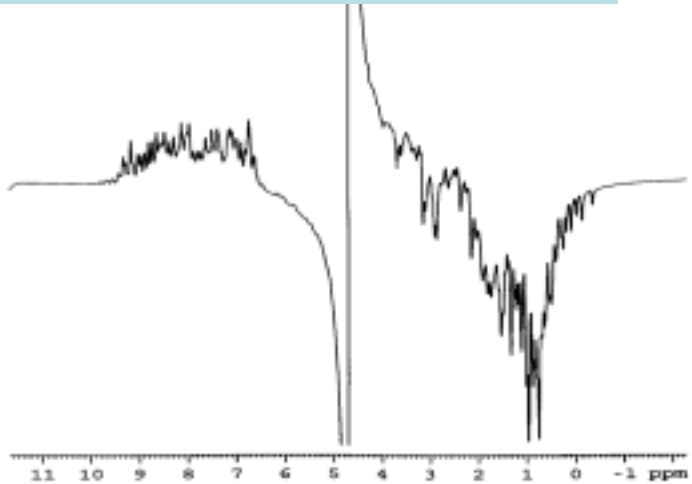
Imino protons in DNA, hydroxyl protons (-OH), Histidine side chain protons in proteins are usually in a fast exchange process with water.

- **Flip-back WATERGATE (marginal performance)**
- **Jump and return 1-1**
- **1-3-3-1**

Jump and Return: 1-1



*P. Plateau et and M. Gueron, al., **J. Am. Chem. Soc**. 1982, 104, 7310-7311*



- **Water signal: "on resonance", aligned to the "z" axis, null signal.**
- **Protein signals: free to precess on the transverse plan**
- **Peak Intensity: I_x Sin($\Omega\tau$)**
- **Delay $\tau = 1/(4\Delta\upsilon_{max})$, $\Delta\upsilon_{max}$=distance of maxima intensity**

Parameter adjustment: Pulprog=p11

p1: 90 pulse, p0: 90 degree "return" pulse, adjust p0 to be 0.1-0.2 usec less than p1.

d19: d19= 1/2*d, d = distance of next null (in Hz)=2*distance of maxima intensity

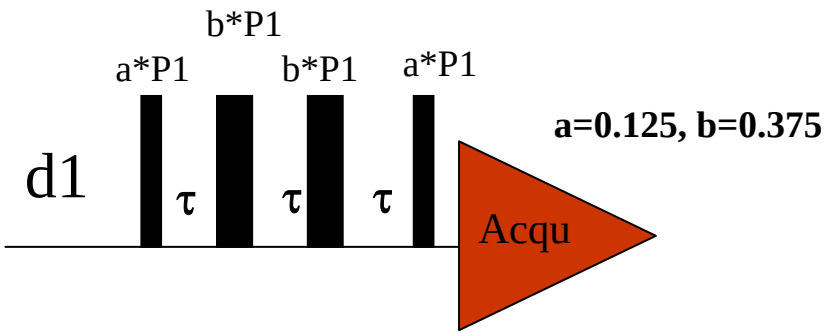
The delay time is magnetic field-strength dependent !

For example: To observe a peak at 14 ppm (o1p on water at 4.75 ppm)

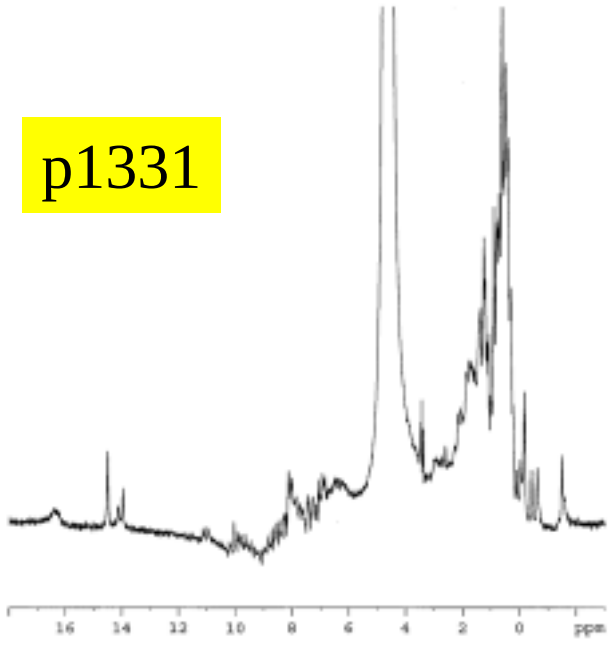
d19=1/[4*(14-4.75)*600.13]=45 usec (at 600 MHz)

d19=1/[4*(14-4.75)*500.13]=54 usec (at 500 MHz)

Binominal: 1-3-3-1



p1331



•**Delay $\tau = 1/(2\Delta\upsilon_{max})=1/d$,**
$\Delta\upsilon_{max}$=distance of maximal intensity
d=distance of next null

Parameter adjustment:

- **Pulpro=p1331**
- **d19: delay for binomial water suppression**
- **d19 = (1/d), d = distance of next null (in Hz)=2*distance of maximal intensity**
- **d19=τ as defined above**

For example: To observe a peak at 14 ppm (o1p on water at 4.75 ppm)

d19=1/[2*(14-4.75)*600.13]= 90 usec at 600 MHz

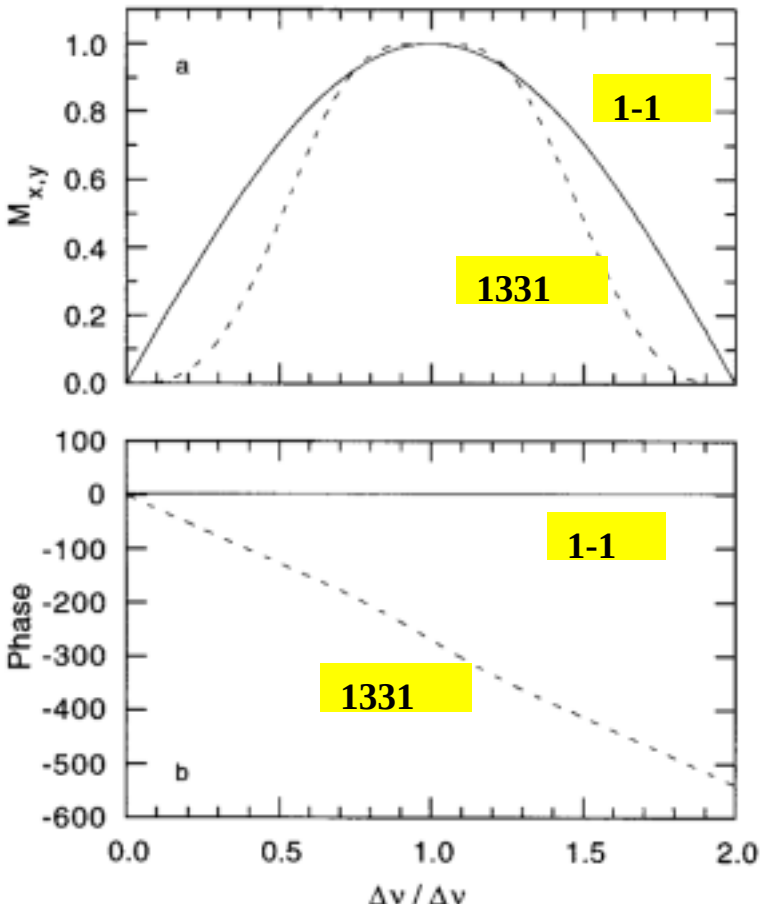
d19=1/[2*(14-4.75)*500.13]=108 usec at 500 MHz

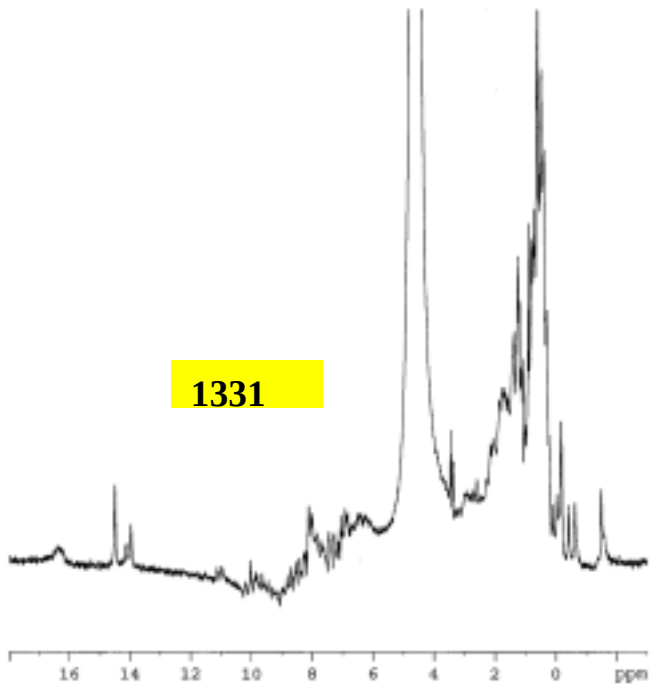
P.J. Hore, **J. Magn. Reson.**, 55, 283-300 (1983)

Jump-Return 1-1 and Binominal 1-3-3-1

Intensity

Phase





Binomial excitation profiles of 1-1 and 1-3-3-1.

John Cavanagh et al., page 154, "Protein NMR Spectroscopy: Principles and Practice", Academic Press (1995)

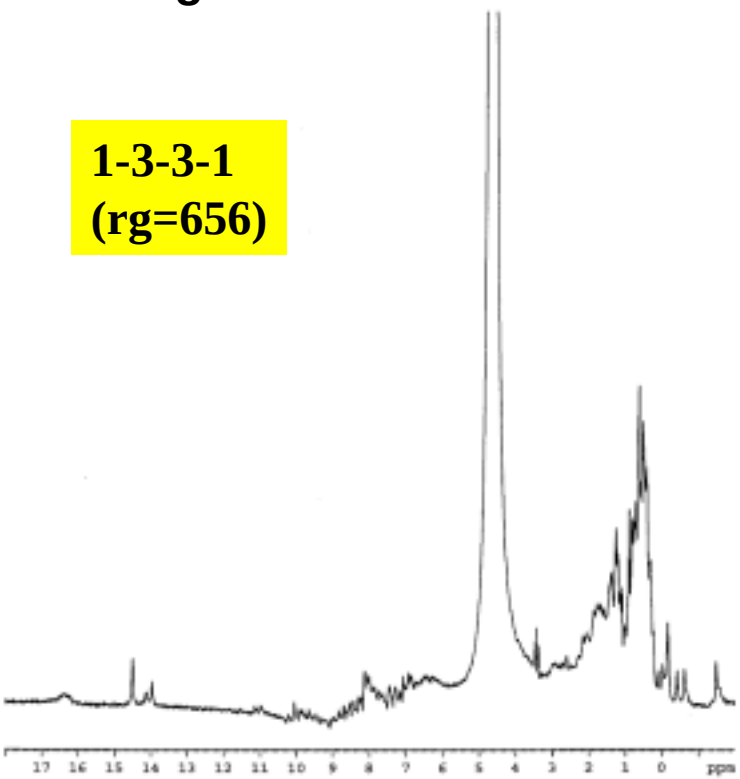
Jump-Return 1-1 and Binominal 1-3-3-1

Both are for observing fast exchanging protons.

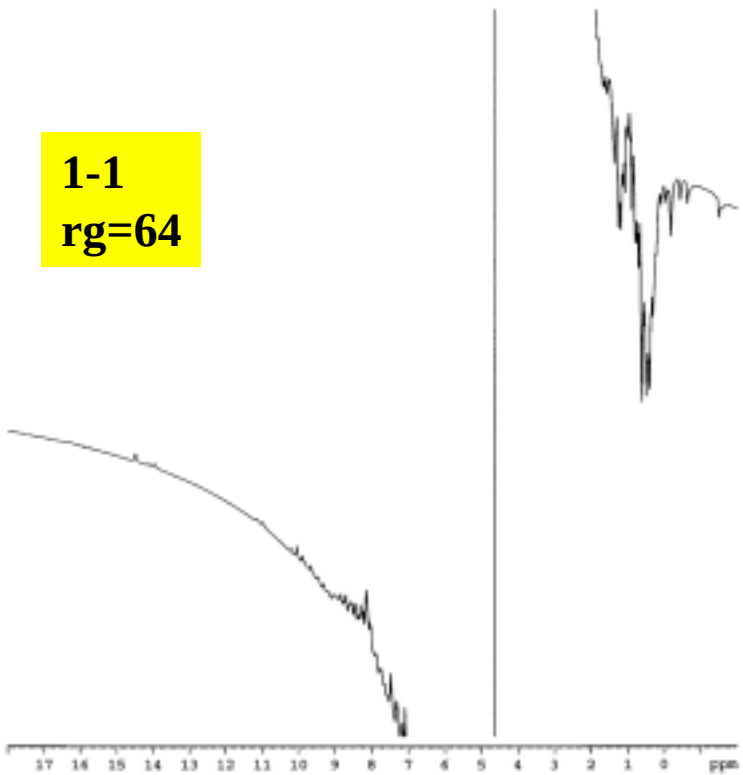
•1-3-3-1: Better water suppression (higher receiver gain), but with offset-dependent phase distortion (unsuitable for 2D)

•1-1: low receiver gain, the dispersive tail of water interferes with the signals of interest.

1-3-3-1 (rg=656)

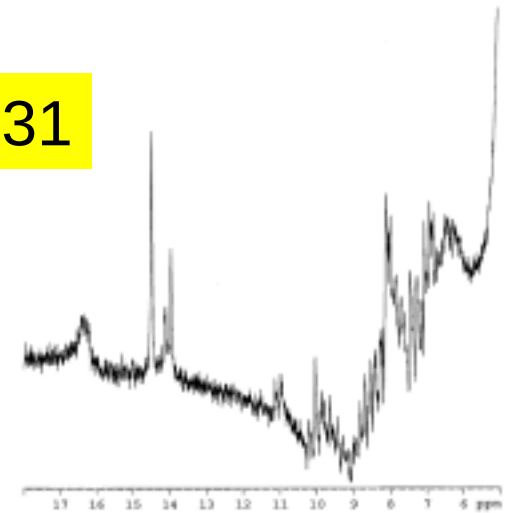


1-1 rg=64

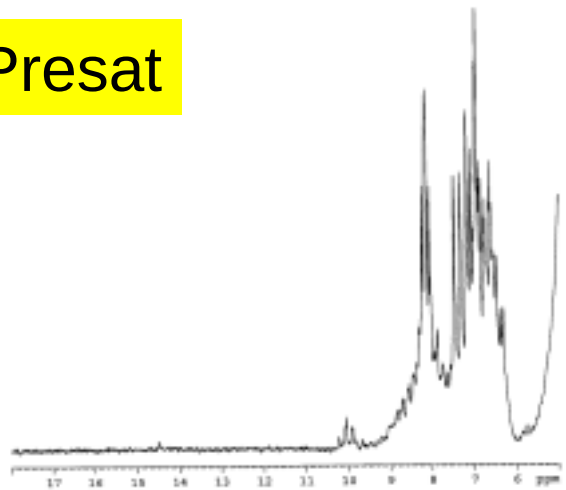


What are you trying to detect ?

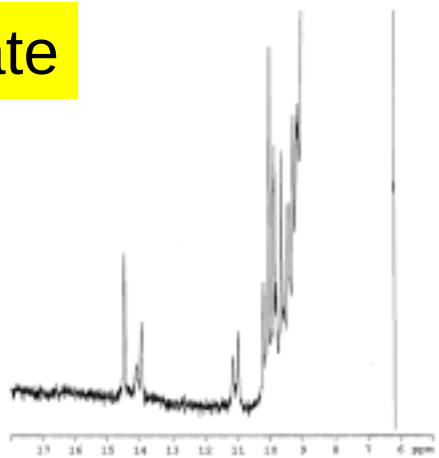
p1331



Presat



Watergate



Water Suppression via Coherence Pathway Rejection

Coherence pathway selected by gradients:

In a gradient selection experiment (echo/antiecho), the water coherence is not "refocused" by the refocus gradient (therefore, is not selected), this naturally suppresses the water signal.

Example: cosydf*etgp*.1, hsqc*et*f3*gp*

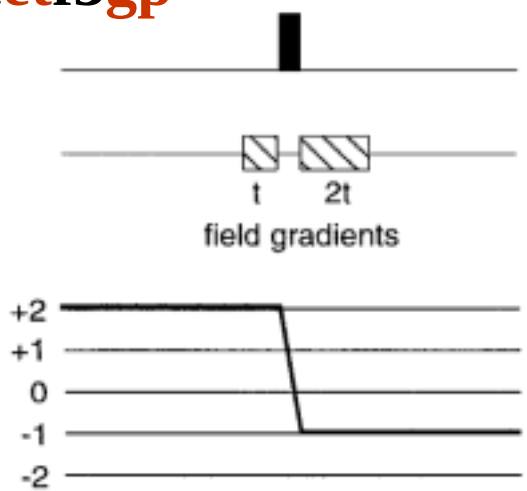


Figure from John Cavanagh et al.,"Protein NMR Spectroscopy: Principles and Practice", Academic Press (1995)

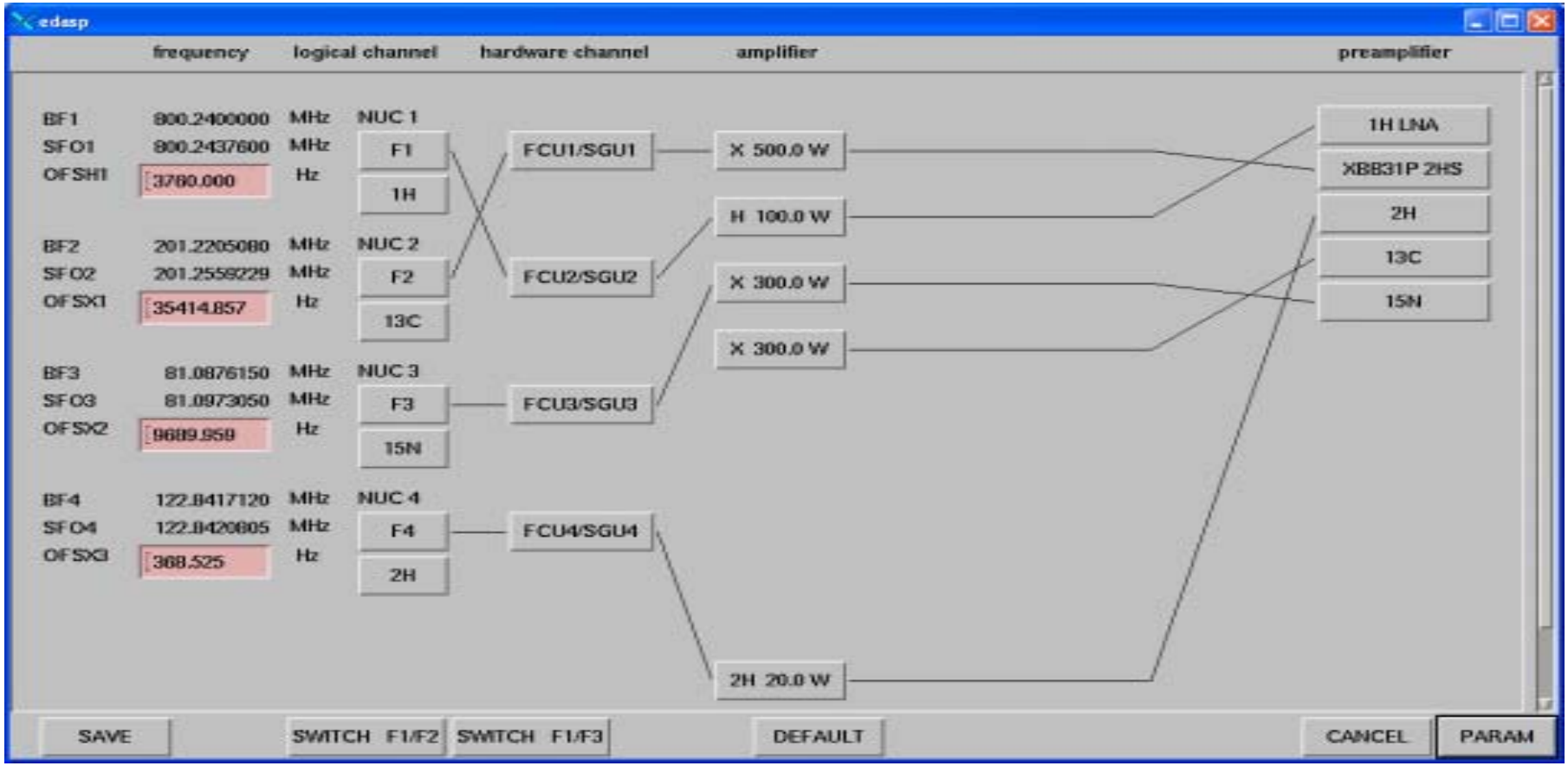
Practical Implementation: 1D, 2D and 3D

- **Exchangeable NH: Water-flip-back HSQC, Fast-HSQC, echo/anti echo WATERGATE HSQC (compare sensitivity with real samples).**

- **Signals near water (i.e. H_α) : (i.e. TOCSY, COSY, NOESY) WATERGATE with selective pulse, echo/anti echo PFG.**

- **Fast exchangeable proton (His sidechain, -OH): 1-1 (good for 2D), 1331 (not suitable for 2D, 3D).**

Standard Configuration of NMR Channels in IBMS



1H: F1 (channel 1)
13C: F2 (channel 2)
15N: F3 (channel 3)
2H: F4 (channel 4)

The pulse sequence “hsqcetf3gpsi” is by default 15N-HSQC

The pulse sequence “hsqcetgpsi” is by default: 13C-HSQC

Power level and limitation

•Bruker uses "attenuation level" (dB) to represent power level.
So, the larger dB number, the more attenuation and therefore, weaker power.

For example: on 1H (F1 channel), a power level of 55 dB is stronger than 61 dB. -2 dB stronger than 0 dB. -3 dB stronger than -2 dB,

•Each channel (1H, 13C, 15N, 2H) at each probe at each spectrometer (500-800 MHz) head has its limitation on maximal power, so please DO NOT exceed the maximal power level limitation (see "pulse width calibration table" for each probe.) If the P90 of 13C is 10.2 us at -3 dB, don't use -4 dB etc.

•After you read in our standard parameter file, you only have to change the 90-degree pulse on 1H (salt-dependent), the pulse widths for 13C and 15N are quite insensitive; use our default values, and Do Not change them.

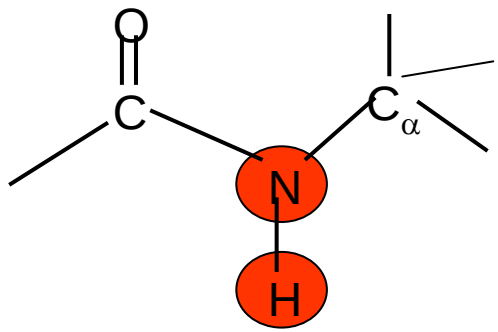
Coherence Order

- Zero quantum coherence: I_zS_z…etc
- Single quantum coherence: I_xS_z, I_yS_z, I_zS_y …etc
- Double quantum coherence: I_xS_x, I_xS_y…etc
- Triple quantum coherence: $I_xK_xS_x$, $I_xK_xS_y$ …etc

- **HSQC:** Hetero-nuclear single quantum coherence. (I_xS_z)
- **HMQC:**Hetero-nuclear multiple quantum coherence.

HSQC

HSQC: Hetero-nuclear single quantum coherence



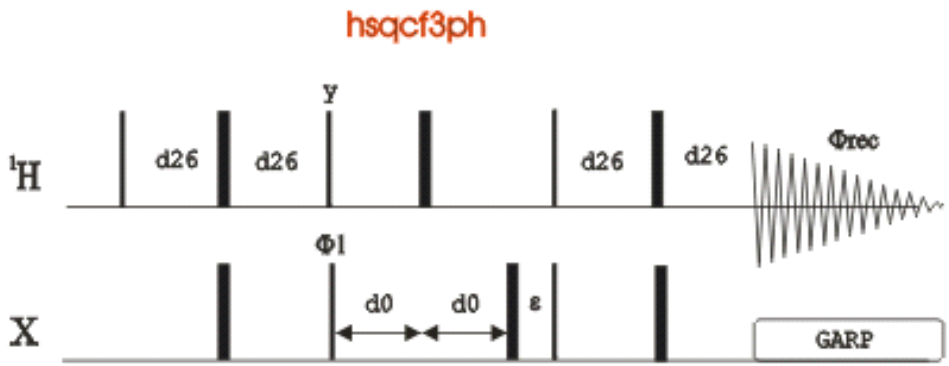
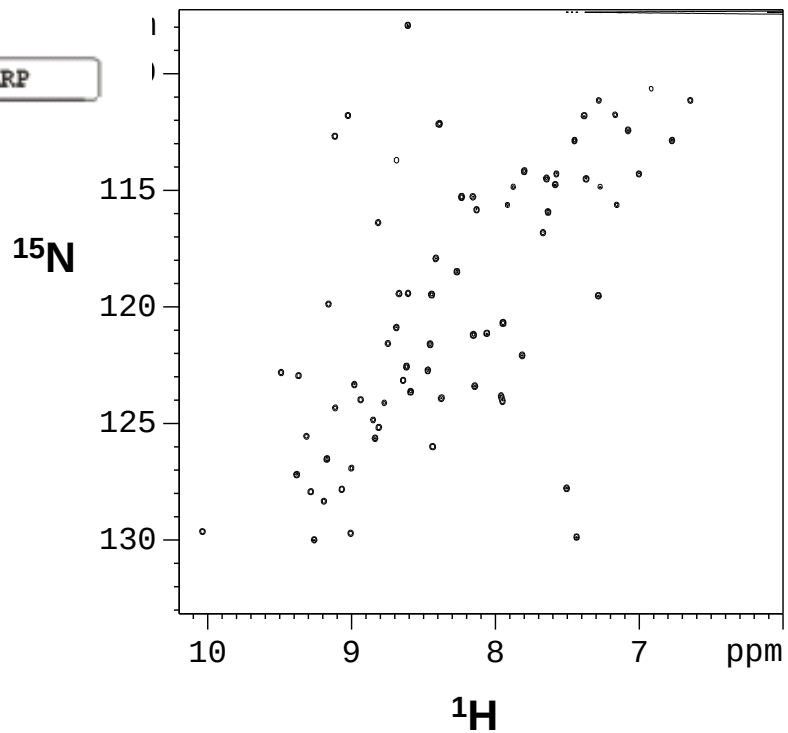


figure from Bruker's NMR Guide



$d26=(1/4J_{XH})$

J_{CH}=140 Hz (aliphatic)

J_{NH}=90 Hz

Setting up an ^{15}N-HSQC Experiment

(1) read in the standard parameter file for **2D 1H-15N HSQC**
(pulprog=hsqcetf3gpsi2):
rpar 2d_15n_hsqc_etsi2 *(on the 800 MHz spectrometer)*
rpar CRP_2D_15N_HSQC_ETSI *(on the AV600 with a CryoProbe)*
(check the specific spectrometer manual for the exact name)

(2) change p1 (1H), o1, sw (1H) that you've determined from 1D, take a quick spectrum with a large spectral width (40 ppm for 15N for o3p at 116-120 ppm)
Use minimal number of scans required, use small ni (*i.e.* ni=32).

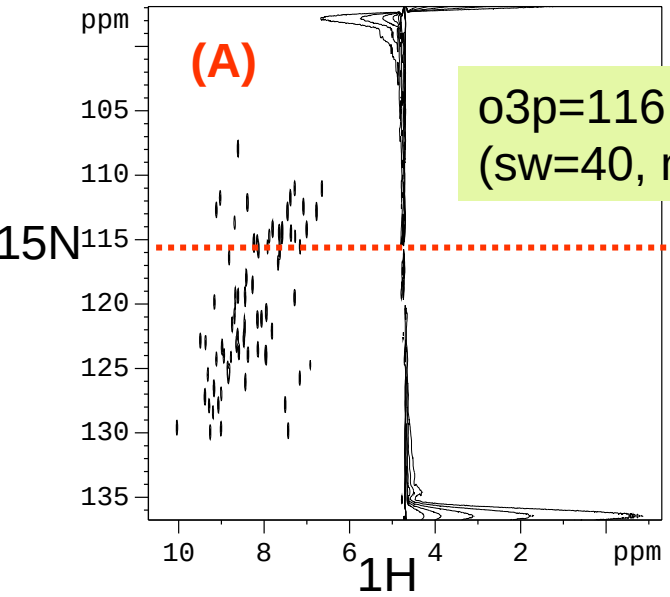
(3) process and inspect the spectrum

(5) change o3p to the center of 15N region
under the **eda** window (not just under the command line)

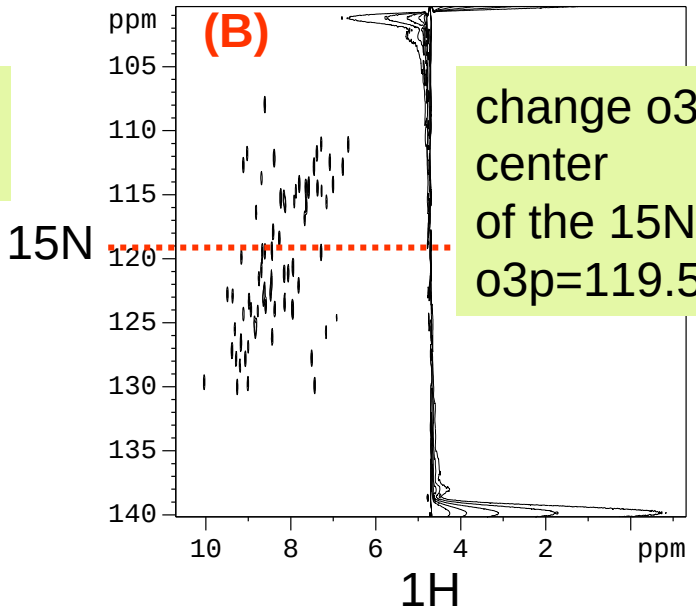
(6) acquire another quick spectrum

(7) acquired spectrum with minimal spectra width, and sufficient t1 experiments in the 15N dimension (i.e. ni=128)

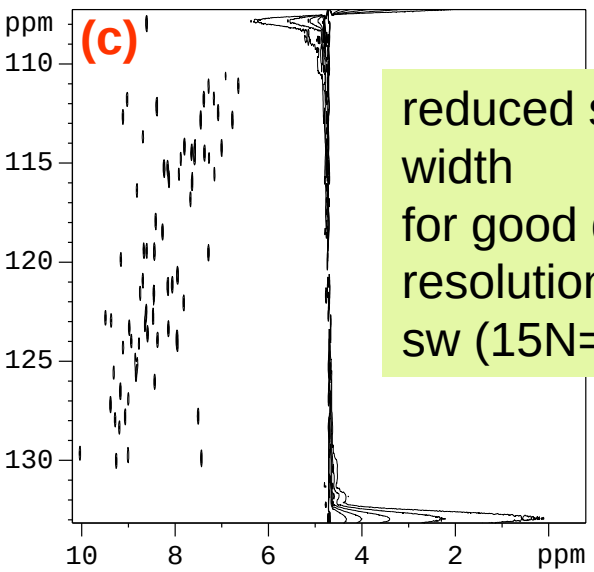
Setting up an ^{15}N-HSQC Experiment



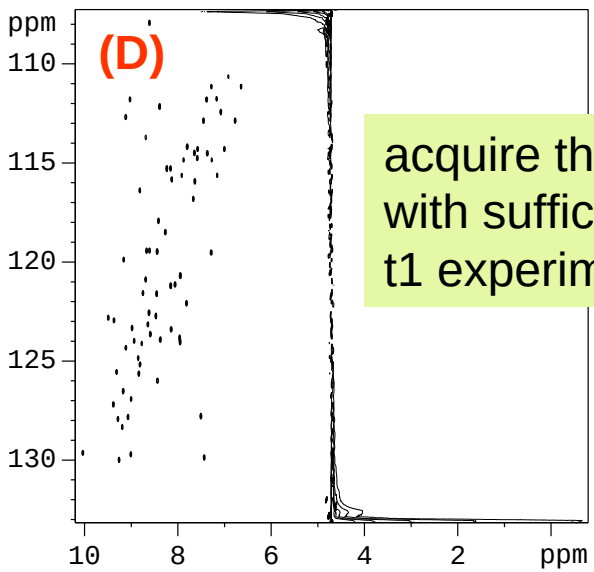
o3p=116 ppm
(sw=40, ni=32)



change o3p to the
center
of the 15N region
o3p=119.5 ppm

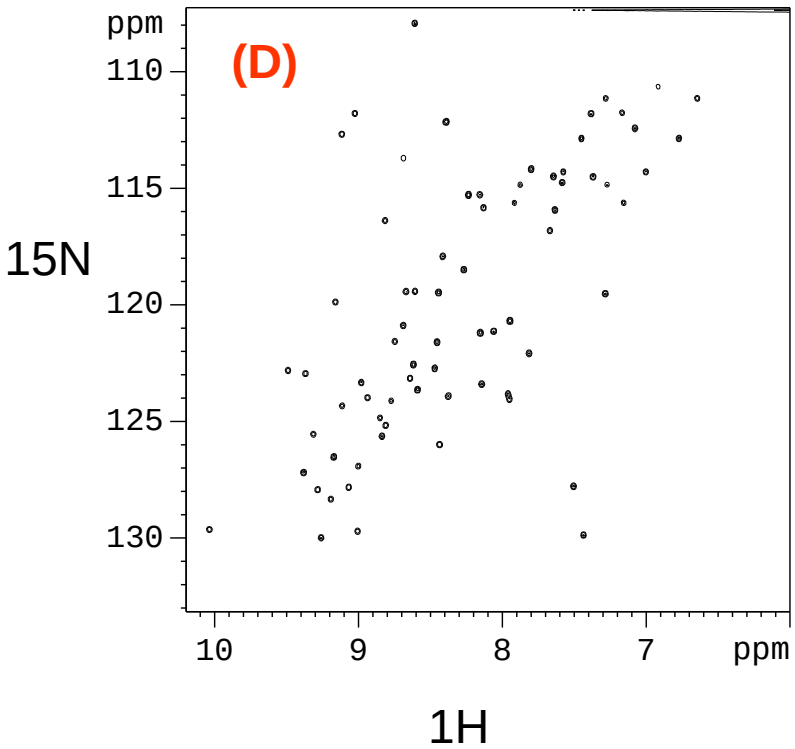


reduced spectral
width
for good digital
resolution
sw (15N=32 ppm)

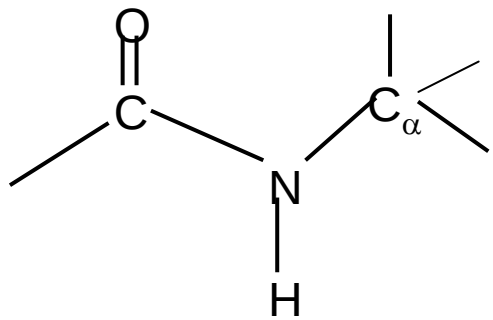


acquire the final spectrum
with sufficient NS and
t1 experiment (ni=128)

Setting up an ^{15}N-HSQC Experiment



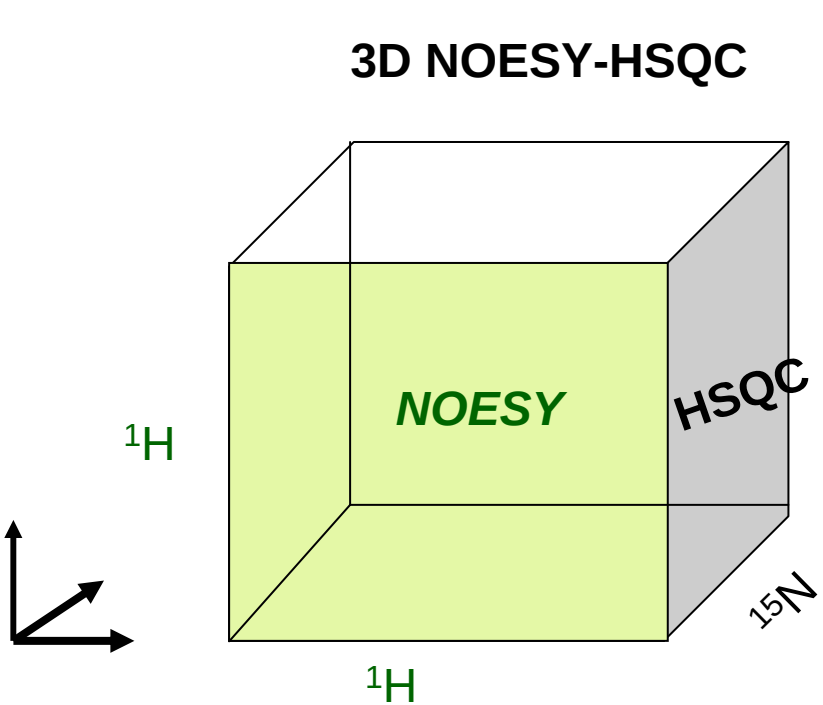
Final 1H, 15N spectrum

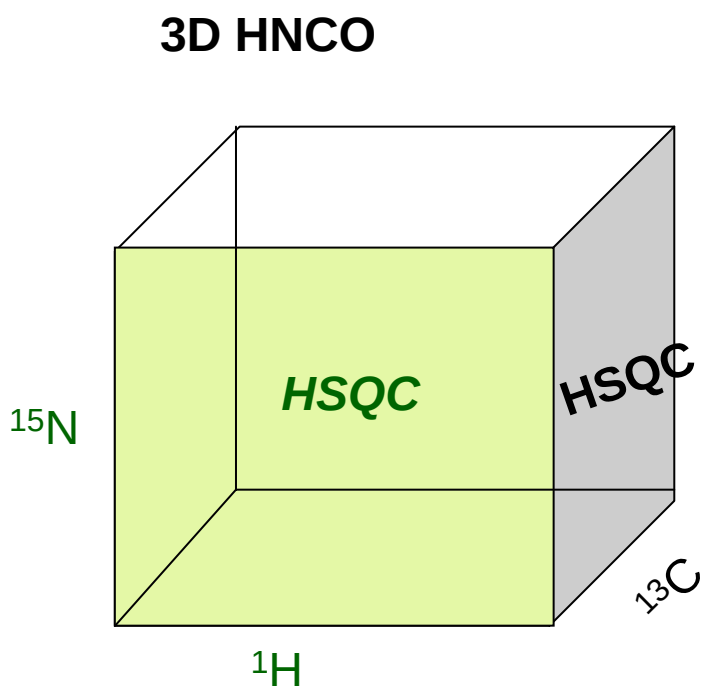


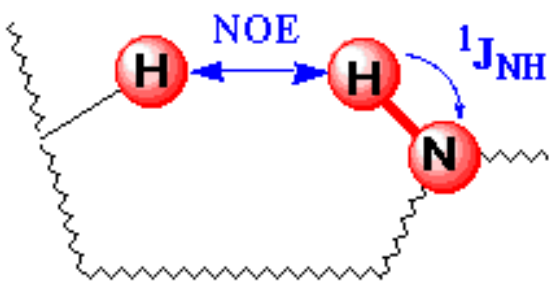
Note: If the protein is double labeled with both 13C&15N, apply adiabatic 13C inversion pulse during 15N evolution to remove the 13C-15N J-coupling.

enter “-DLABEL_CN” in the ZGOPTNS field under the EDA window.

Spread the Resonances into the Third Dimension







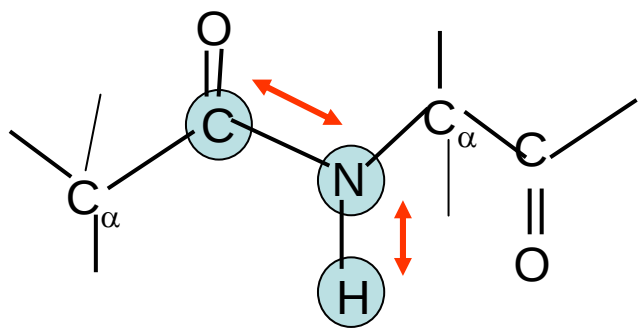
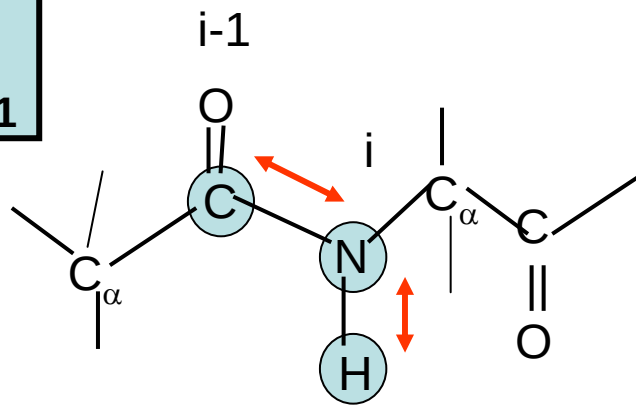
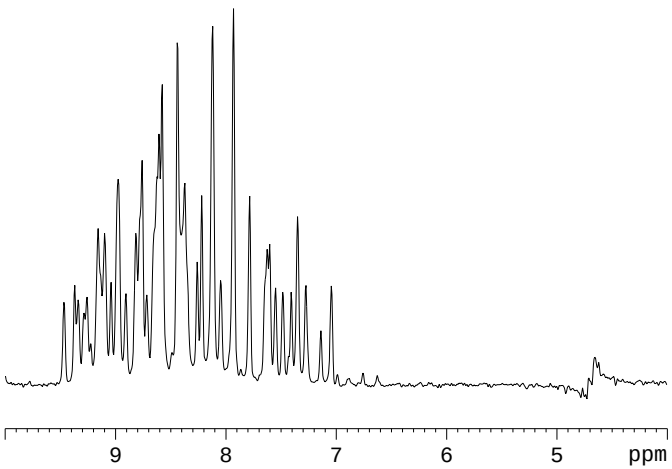


Figure from Bruker NMR Guide

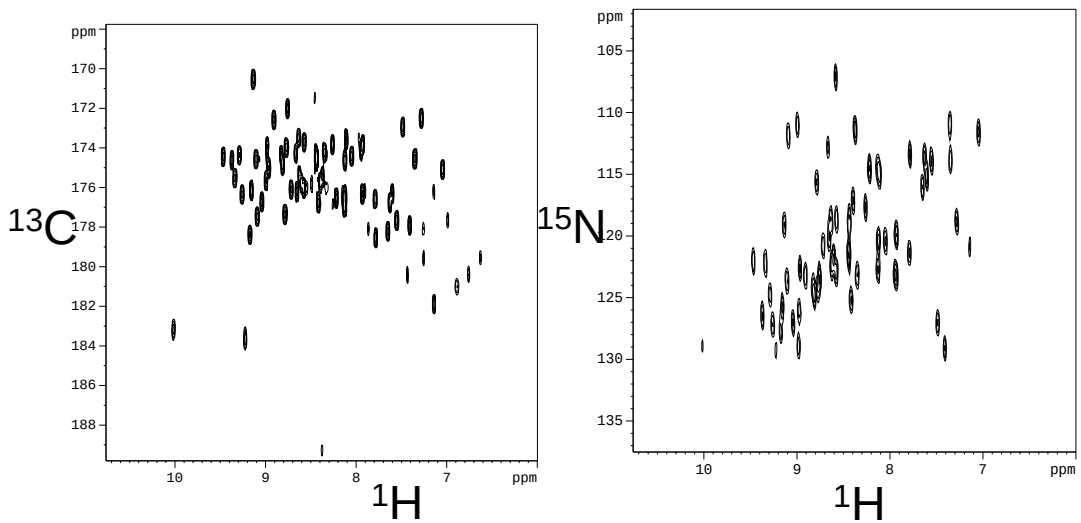
3D HNCO:
Correlation between $^{1}H^{N}_{i}$ – $^{15}N_{i}$-$^{13}C'_{i-1}$

o3p (^{15}N) at ~ 119 ppm, sw~32 ppm
o2p (^{13}C) at ~176 ppm, sw~22 ppm





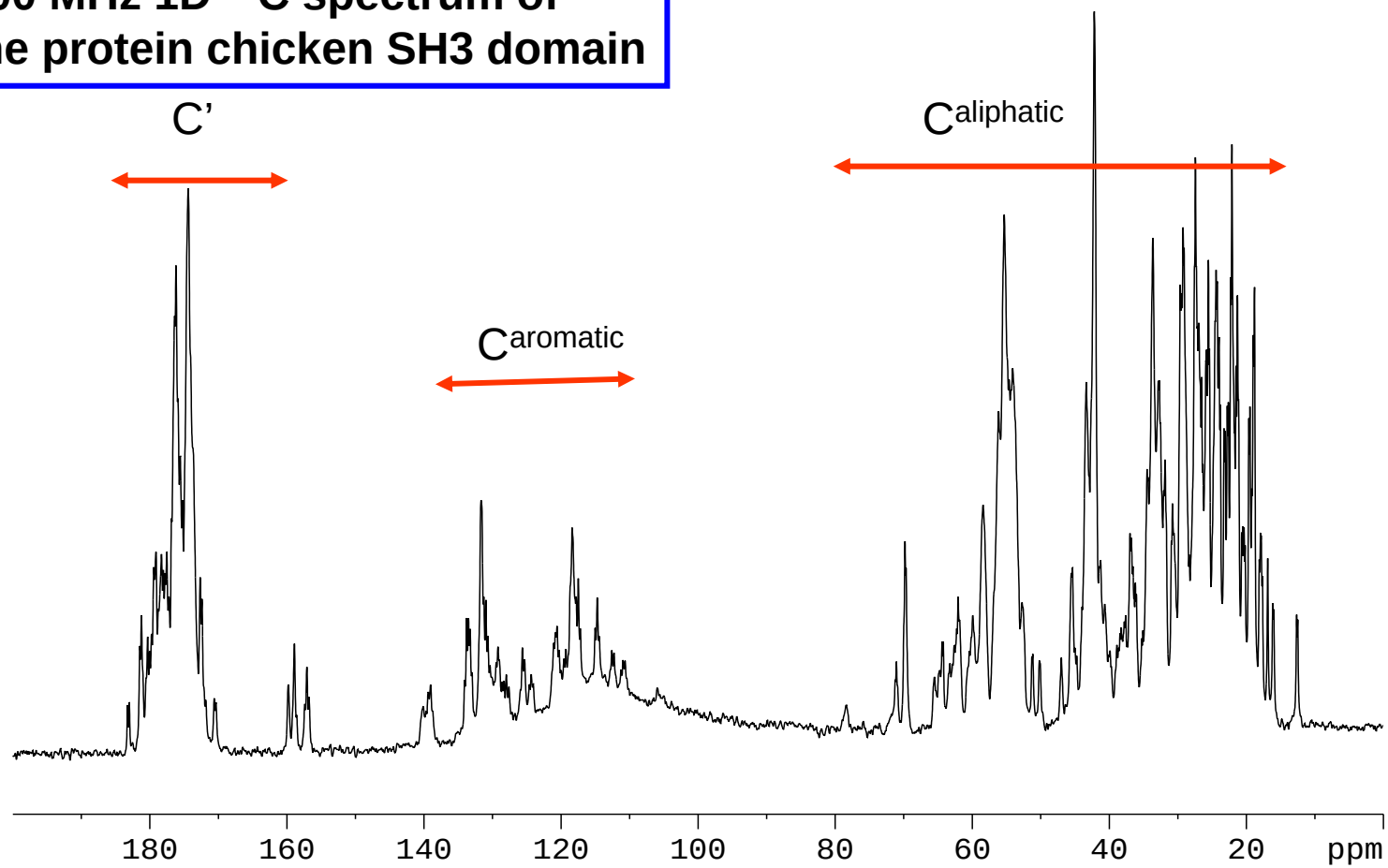
The first increment of a 3D HNCO



The ^{1}H-^{15}N (left) and ^{1}H-^{13}C (right) planes of the HNCO spectrum

Look at the 1st fid to optimize parameters, and acquire a short 2D version of a 3D to optimize SW, ni, o2p, o3p, and make sure the first 2D plane look normal before a long 3D experiment.

200 MHz 1D ^{13}C spectrum of the protein chicken SH3 domain

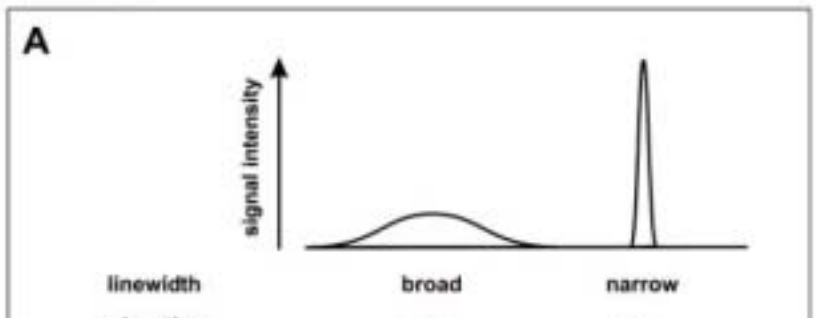


Carbonyl (C') center ~176 ppm (15-25 ppm wide)
The aromatic resonances center at ~ 120 ppm (~ 54 ppm wide),
The aliphatic resonance ~ 10-75 ppm
C_α centers at ~ 56 ppm (sw ~35 ppm)
C_α-C_β center at ~ 39 ppm (sw~75 ppm)

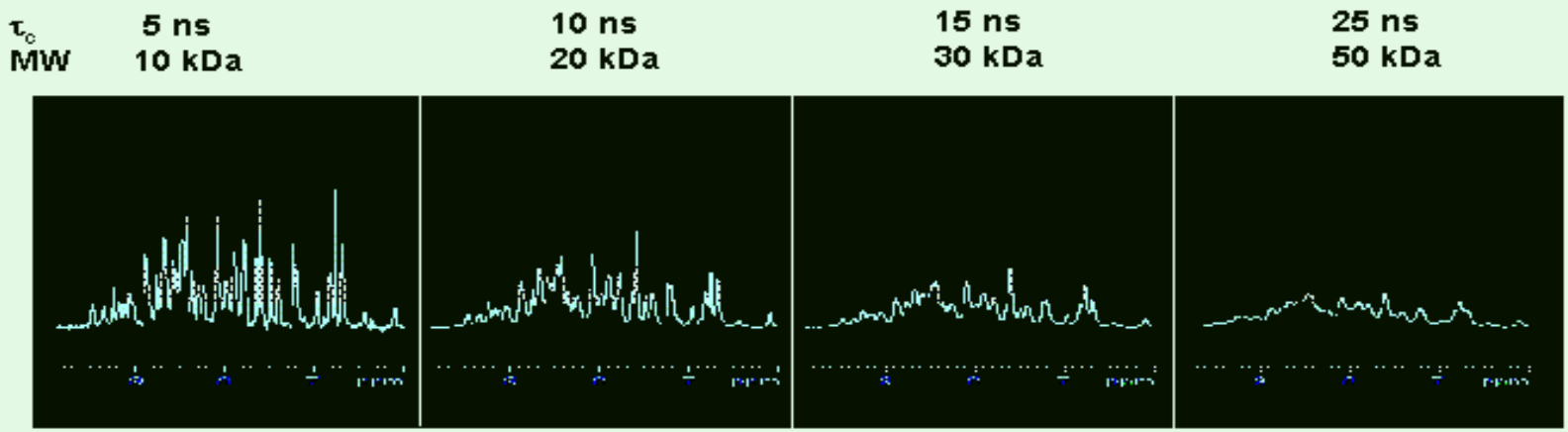
Intrinsic Problems of Solution NMR of Large Molecules

- Many more signals: spectral overlap
- Slow tumbling: fast transverse relaxation rate (short T_2)
- faster decay of signal: poor signal to noise (s/n)

  Linewidth $\Delta\upsilon_{1/2} = 1/(\pi T_2)$

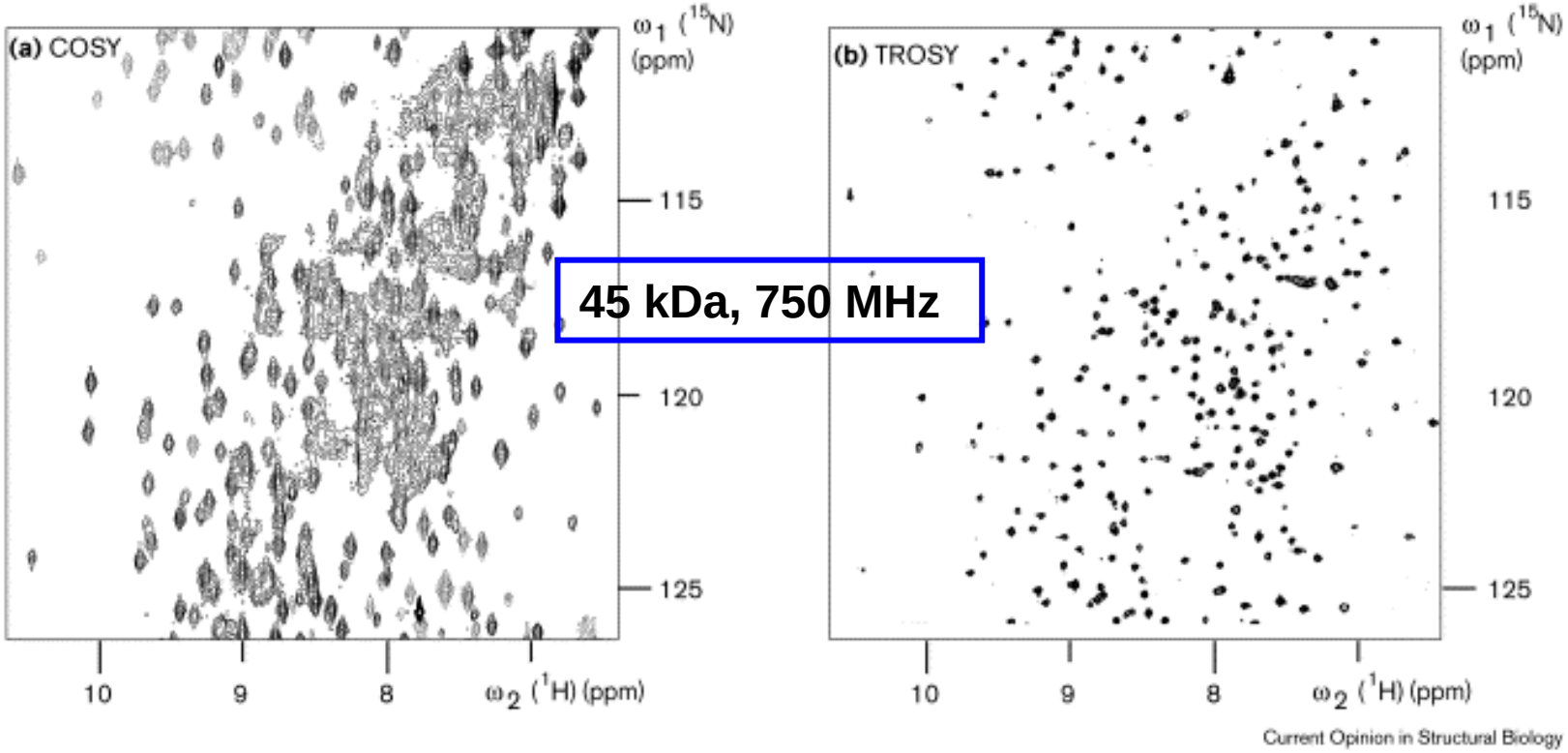


Figures from
M. Sattler's website



When Molecular Weight >20 KDa: TROSY at High Magnetic Field

TROSY: Transverse Relaxation-Optimized Spectroscopy



Wider and Wuthrich, ***Current Opinion in Structural Biology****, 1999, 9:594-601*