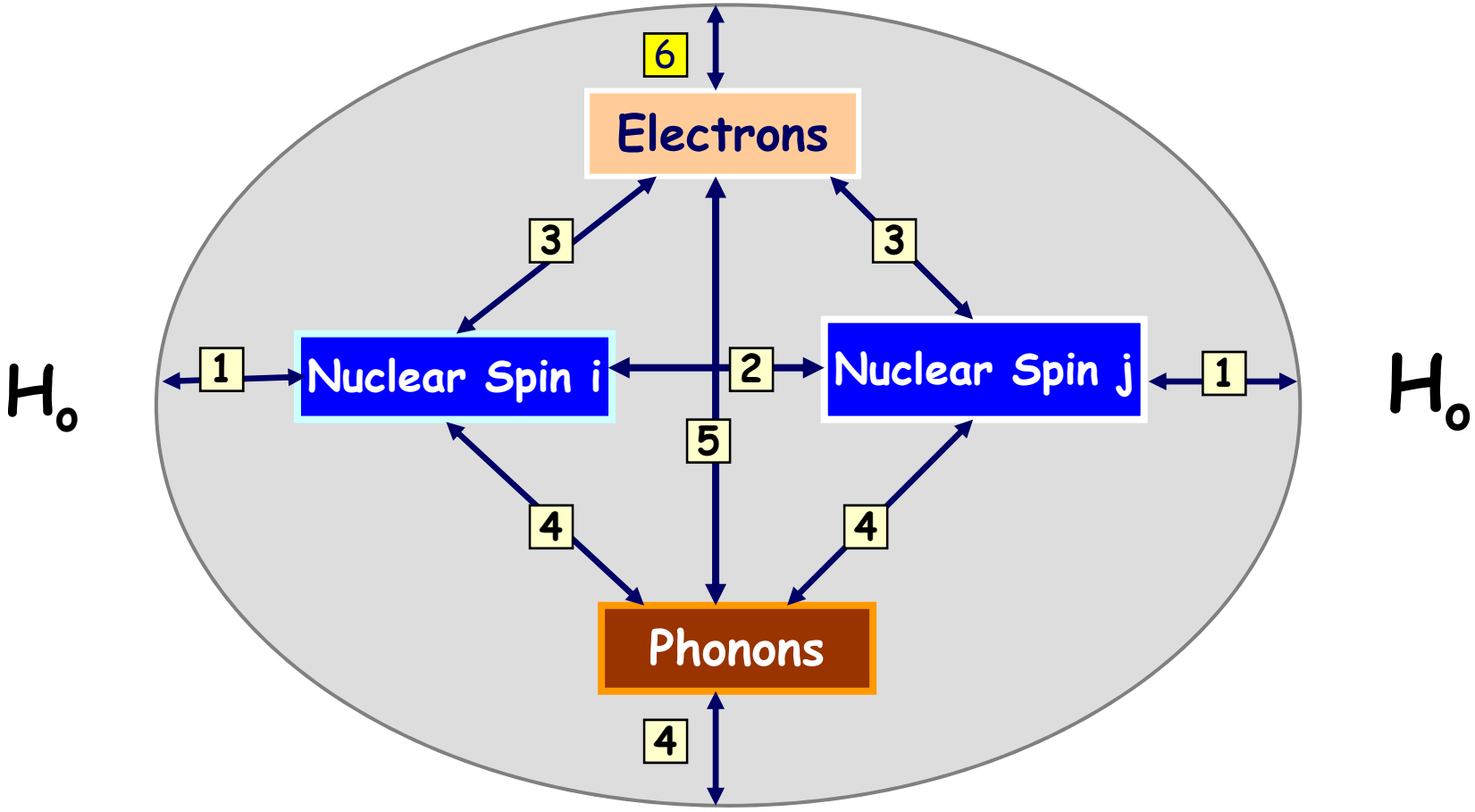
Lecture 13 – Review

1. 3-hour Open book exam. No discussion among yourselves.
2. Simple calculations.
3. Terminologies.
4. Decriptive questions.
5. Analyze a pulse program using density matrix approach (Homonuclear 2D).
6. Analyze a pulse program using product operator approach (Heteronuclear 2D).

Lecture 13 – Protein Structure determination by NMR

Nuclear Spin Interactions (核子自旋交互作用)



Dominant interactions: $H = H_Z + H_D + H_S + H_Q$

H_Z = Zeeman Interaction

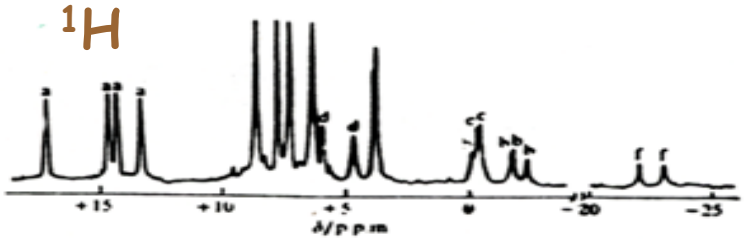
H_D = Dipolar Interactions

H_S = Chemical Shielding Interaction.

H_Q = Quadrupolar Interaction

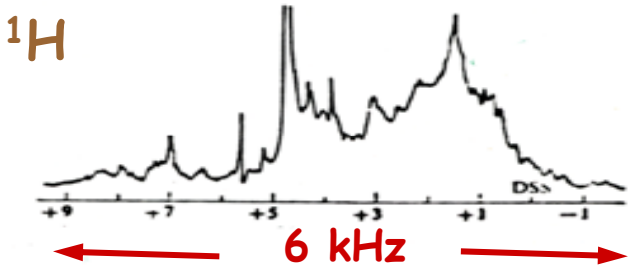
NMR spectra of samples in different physical states

Small molecule in solution



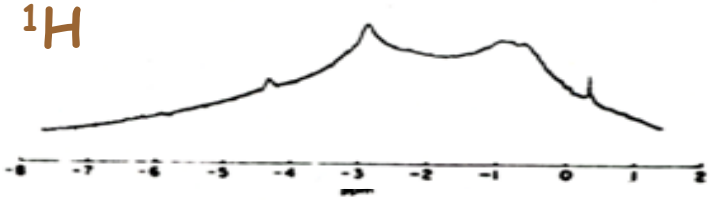
Porphorin

Macromolecule in solution



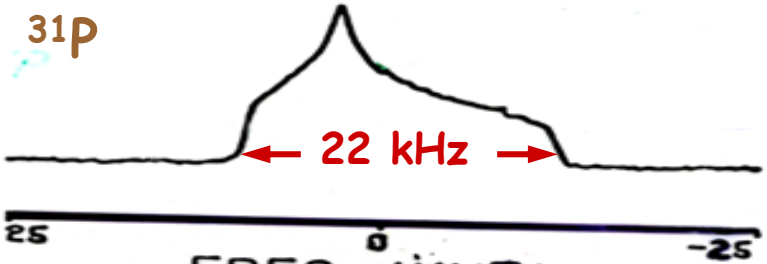
Myoglobin

Gel



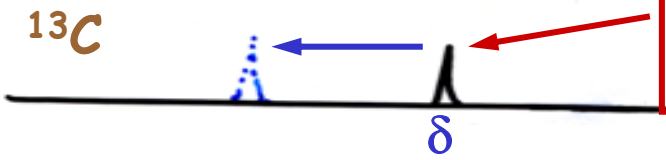
Fat

Solid sample



3'-CMP

Single crystal



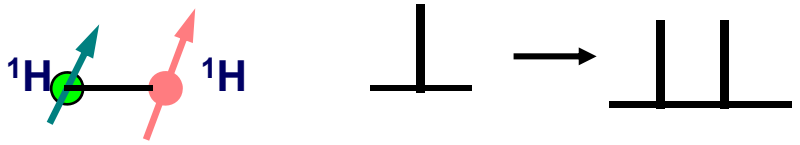
Chemical shift is orientation dependent

NMR Parameters (參數) (Measurable quantities)

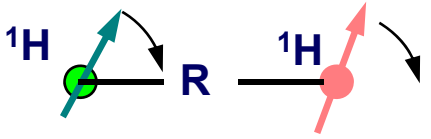
1. Chemical Shift : Difference in resonance frequency due to chemical structure difference (in ppm).

2. Resonance Intensity: Determine number of spins..

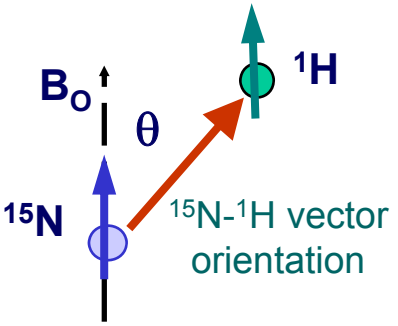
3. J-coupling: Resonance splitting due to through-bond spin coupling.



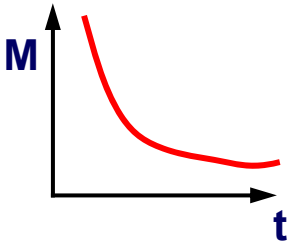
4. Nuclear Overhauser Effect (NOE):
Energy transfer through dipolar coupling.



5. Residual dipolar coupling:
Non-vanishing dipolar coupling in oriented media.



6. Relaxation rates (T_1, T_2 etc):
Lost of magnetization due to dephasing (T_2) or energy dissipation (T_1)



NMR Parameters

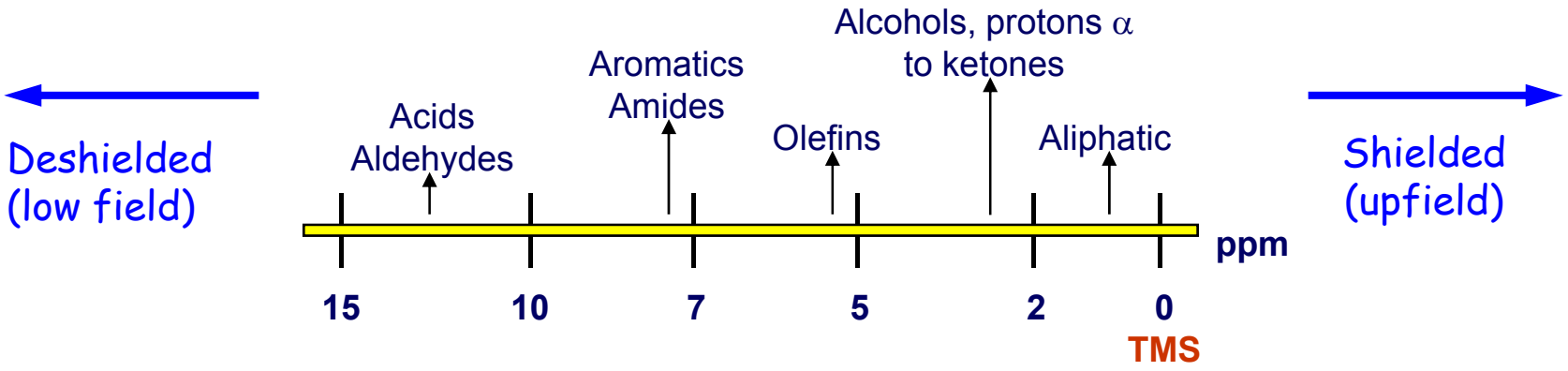
1. Chemical Shift

- The chemical shift of a nucleus is the difference between the resonance frequency of the nucleus and a standard, relative to the standard. This quantity is reported in ppm and is given by the symbol δ ,

$$\delta \equiv (\omega - \omega_{REF}) \times 10^6 / \omega_{REF}$$

- Where ω_{REF} is the reference frequency of the standard compound, i.e. the methyl resonance of tetramethylsilane (TMS) or 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

- In this relative scale, the δ value is independent of magnet field used. (i.e same in 100 MHz magnet (2.35 T) or in a 600 MHz magnet (14.1 T).



Chemical Shift Referencing: The ^{1}H chemical shift was referenced to 2,2-dimethyl-2-Silapentane-5-sulfonate (DSS) at 0 ppm. The ^{15}N and ^{13}C chemical shift values were referenced using the consensus ratio of Ξ of 0.101329118 and 0.251449530 for $^{15}N/^{1}H$ and $^{13}C/^{1}H$, respectively

(Wishart and Case, Method. Enzymol. 338, 3-34 (2001))

TABLE I

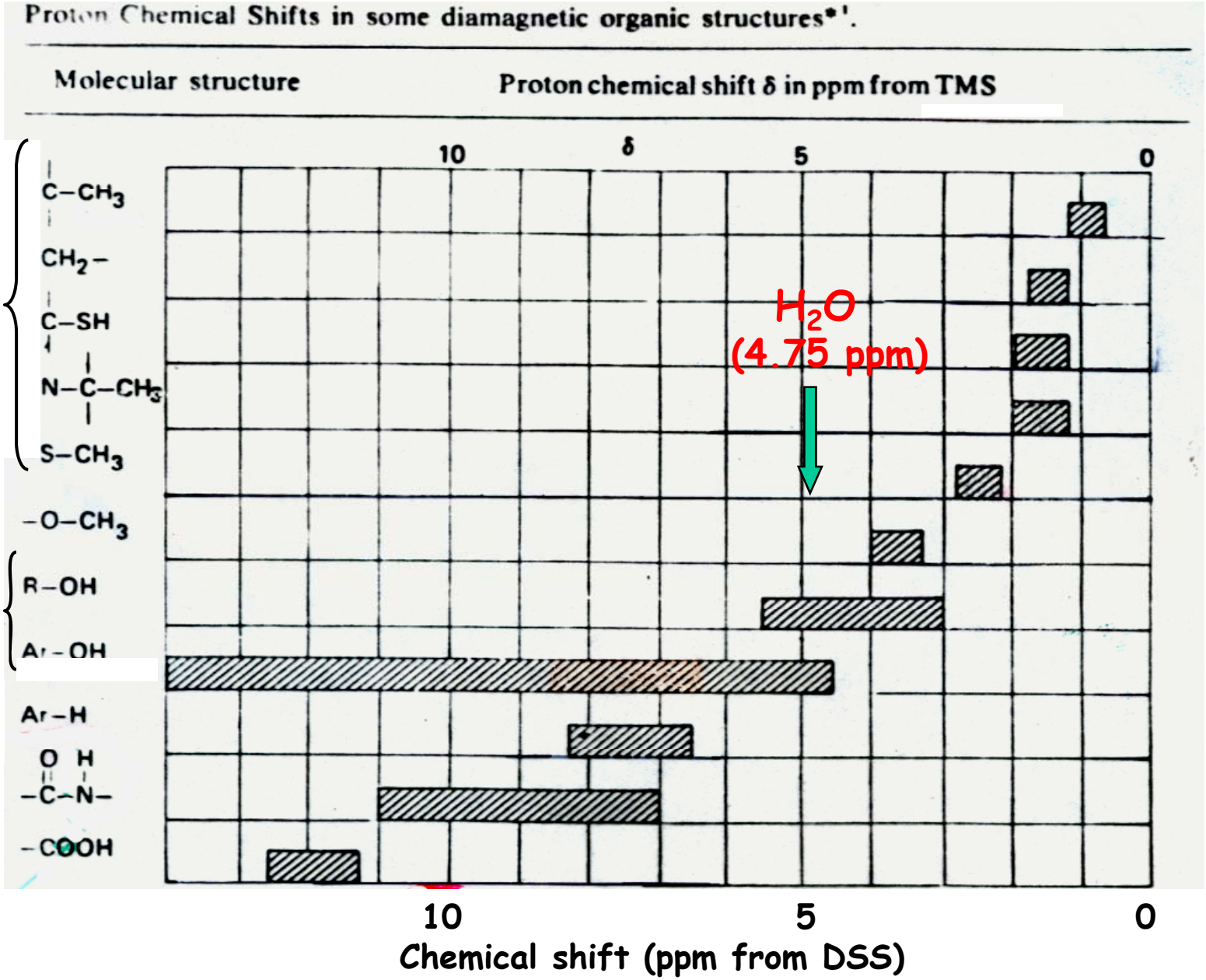
IUPAC/IUBMB RECOMMENDED Ξ (XI) RATIOS FOR INDIRECT CHEMICAL SHIFT REFERENCING IN BIOMOLECULAR NMR[a]

Nucleus	Compound	Ξ Ratio
^{1}H	DSS	1.000 000 000
^{13}C	DSS	0.251 449 530
^{15}N	Liquid NH_3	0.101 329 118
^{19}F	CF_3COOH	0.940 867 196
^{31}P	$(CH_3)_3PO_4$	0.404 808 636

[a] Relative to DSS.

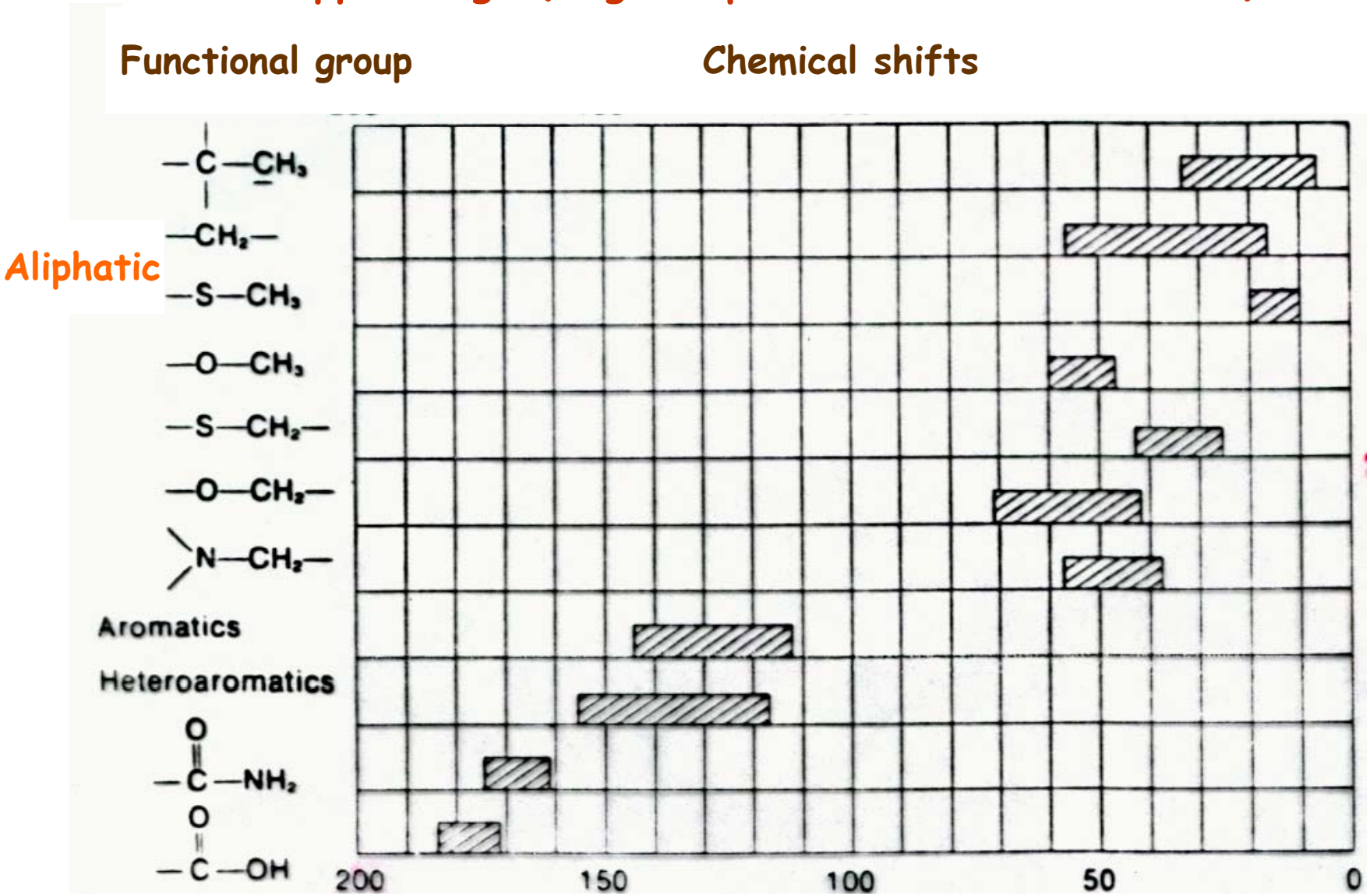
Ξ ratio (Nucleus-specific frequency ratio: Determine the precise ^{1}H resonance frequency of DSS then multiply this frequency by Ξ of a particular nucleus one obtains the exact resonance frequency reference at 0 ppm of that nucleus.

Proton chemical shift in some diamagnetic structures (12 ppm)

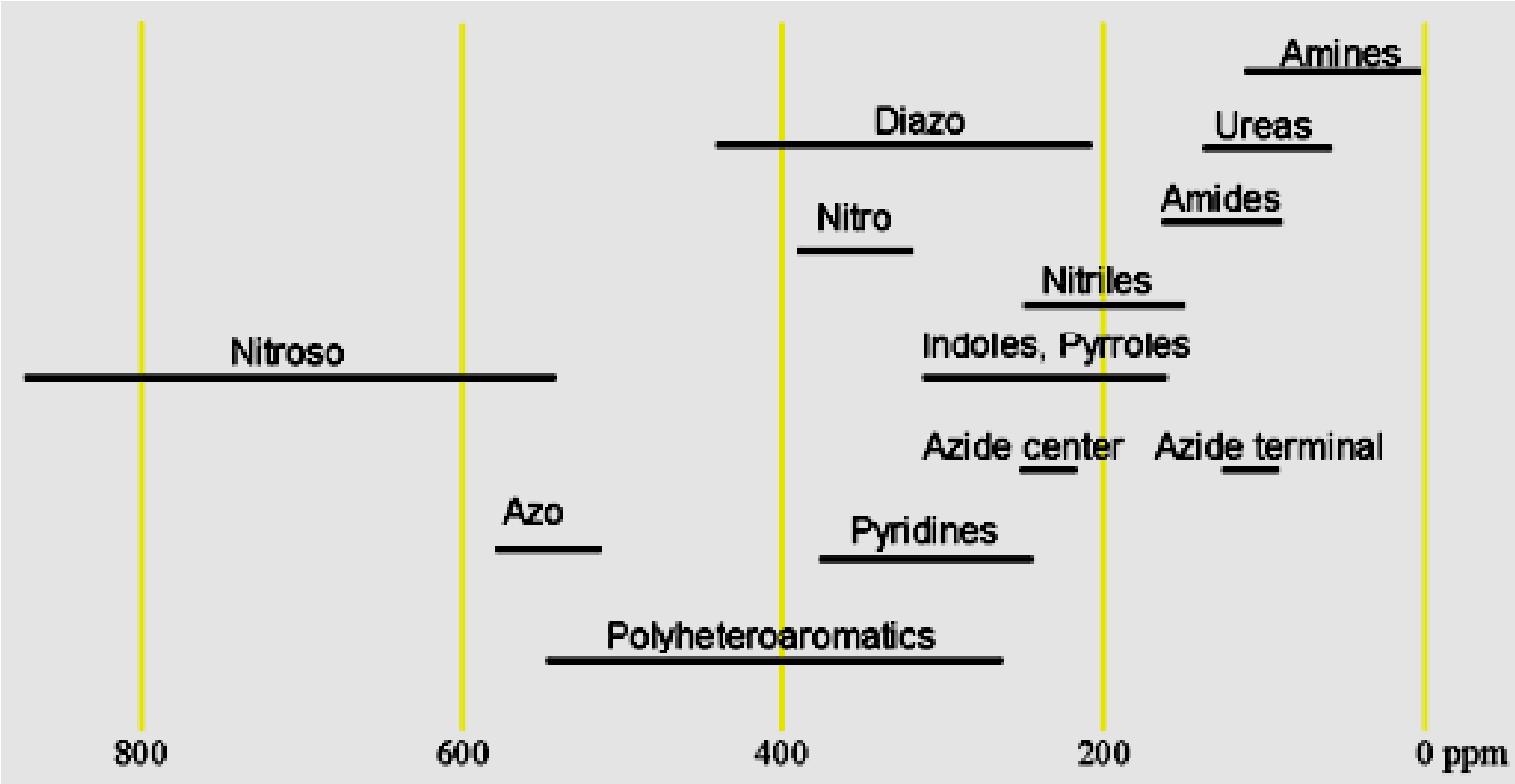


^{13}C chemical shift in some diamagnetic structures

200 ppm range (large dispersion, better resolution)



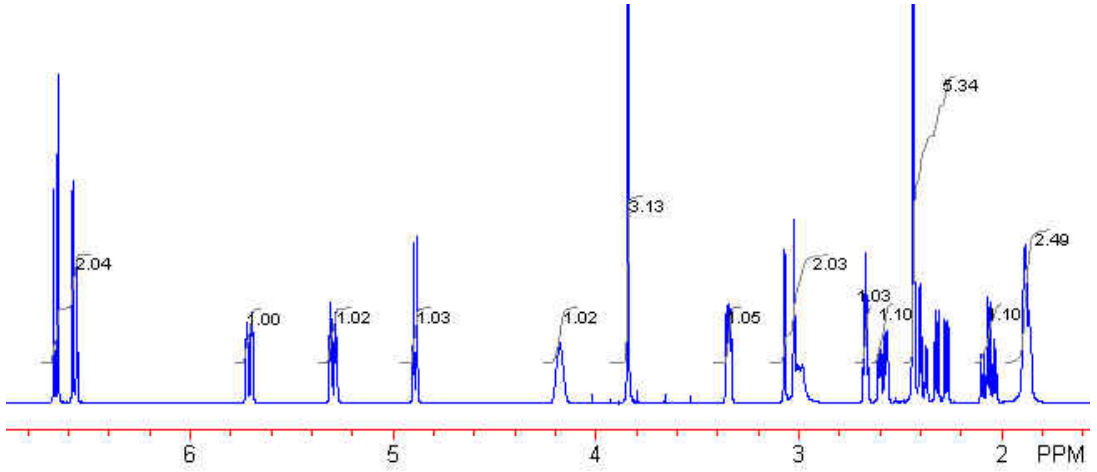
Chemical shift ranges of ^{15}N (800 ppm)

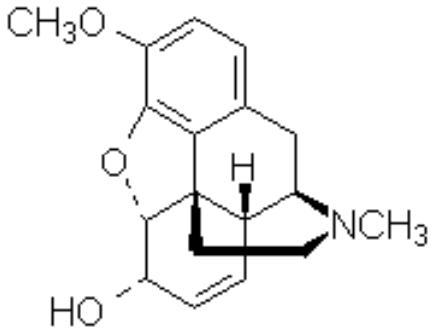


In biomacromolecular NMR one observe mostly amide nitrogen (-^{15}NH-) and side chain amino nitrogens (Arg and Lys) (-$^{15}NH_3$ or -$^{15}NH_2$). Amide nitrogen resonates at ~100 -140 ppm range and 80 ppm for NH_2. **Notice, amide nitrogen shift spans ~ 40 ppm.**

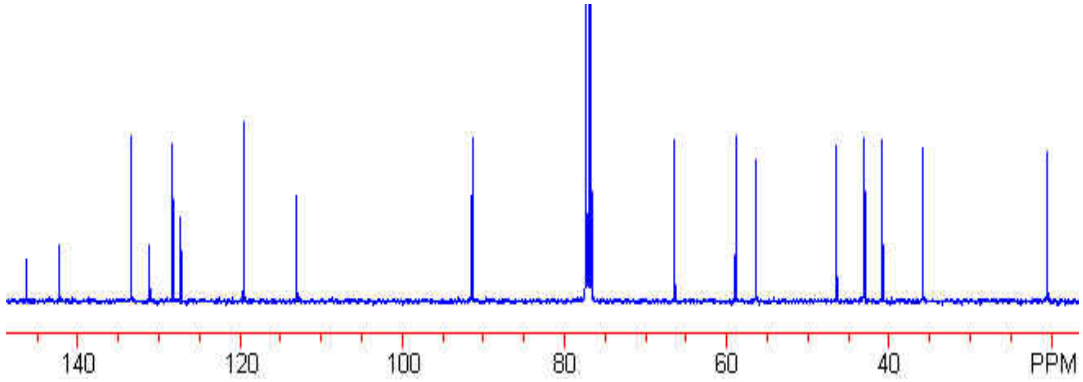
➢**Example of 1D : 1H spectra, 13C spectra of Codeine $C_{18}H_{21}NO_3$, MW= 299.4**

1H



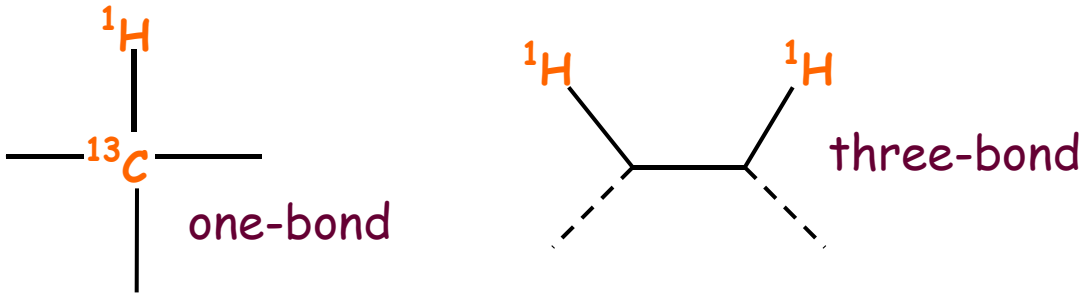


13C

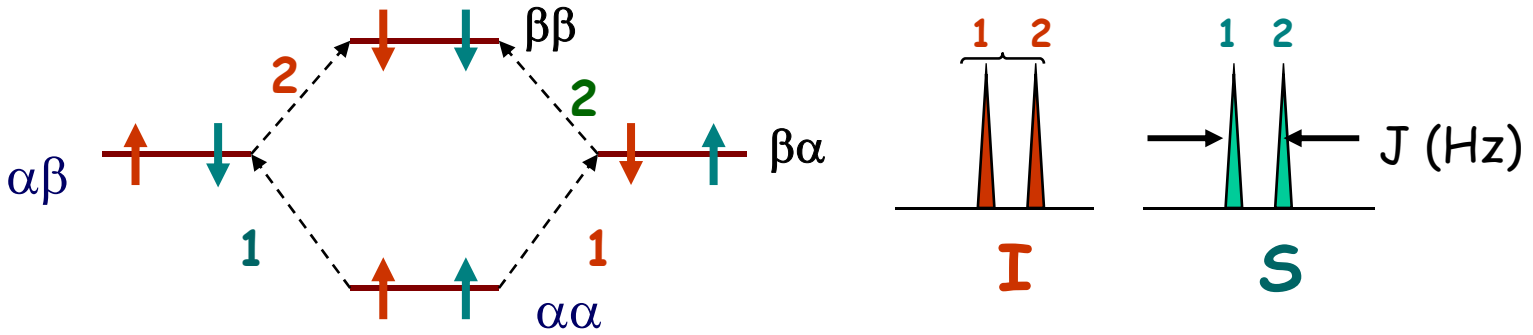


2. J-coupling (More than one spins)

- Nuclei which are connected by chemical bonds form a coupled system and cause splitting on the energy level, thus cause resonance splitting This is called **spin-spin coupling** or **J coupling**.



- Energy diagram of two spin system: Each spin now seems to has two energy 'sub-levels' depending on the state of the spin it is coupled to:

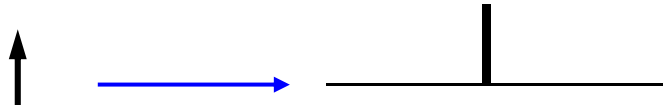


- The magnitude of the separation is called *coupling constant* (J) and has units of Hz.

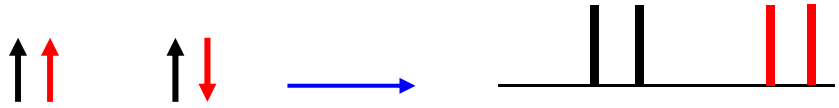
Number of lines

N neighboring spins: split into N + 1 lines

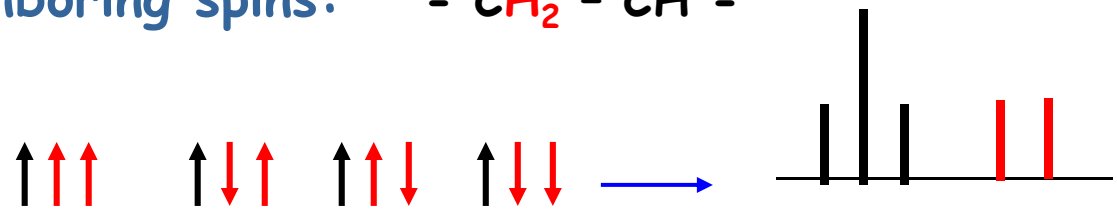
1. Single spin:



2. One neighboring spins: - CH - CH -

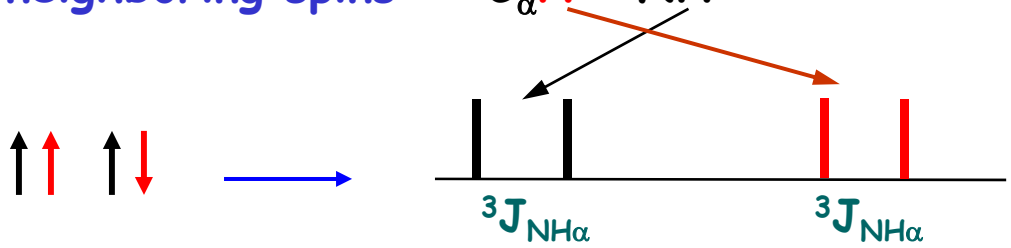


3. Two neighboring spins: - CH_2 - CH -



Use of J-coupling for structure determination (Dihedral angle)

- One neighboring spins: - $C_\alpha H$ - NH -

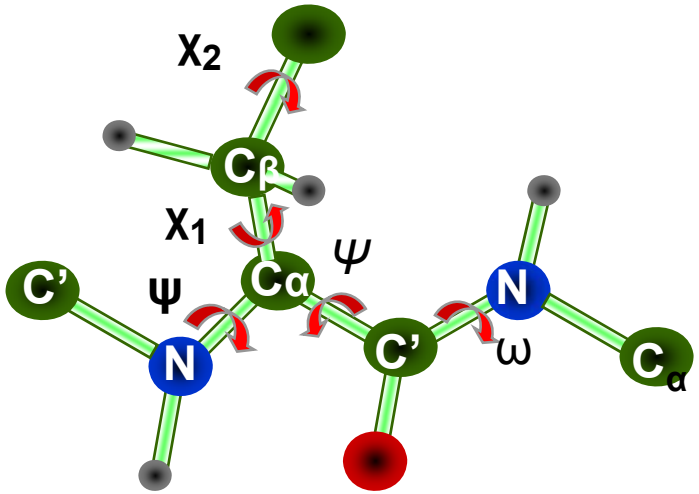


- From *coupling constant* (J) one can determine the dihedral angles from the following Karplus equations, where $^3J_{NH\alpha}$ is the coupling constant between $C_\alpha H$ - NH.

$$^3J_{NH\alpha} = 6.4\cos^2(\phi - 60) - 1.4\cos(\phi - 60) + 1.9$$

$$^3J_{\alpha\beta 1} = 9.5\cos^2(\chi_1 - 120) - 1.6\cos(\chi_1 - 120) + 1.8$$

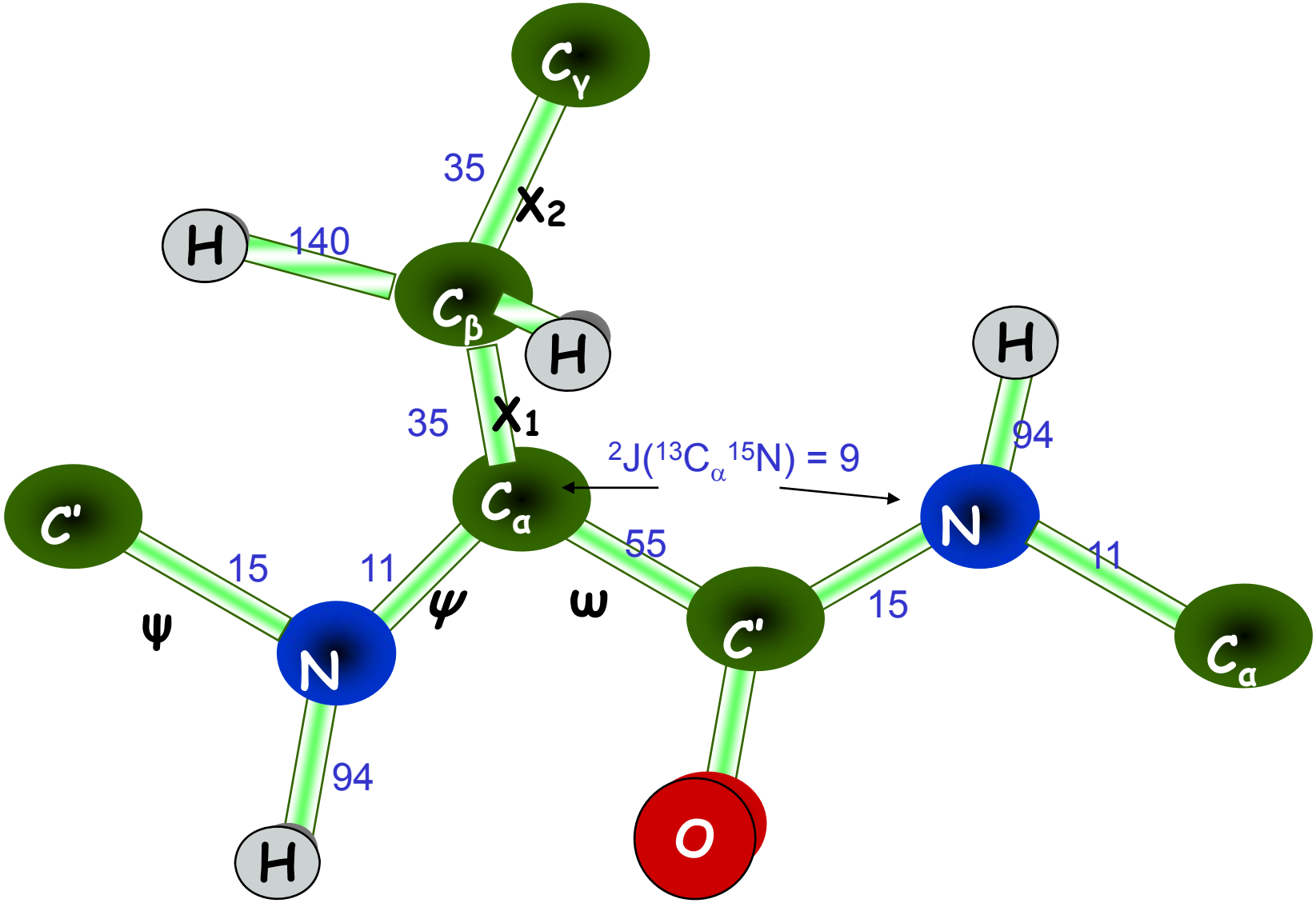
$$^3J_{\alpha\beta 2} = 9.5\cos^2\chi_1 - 1.6\cos\chi_1 + 1.8$$



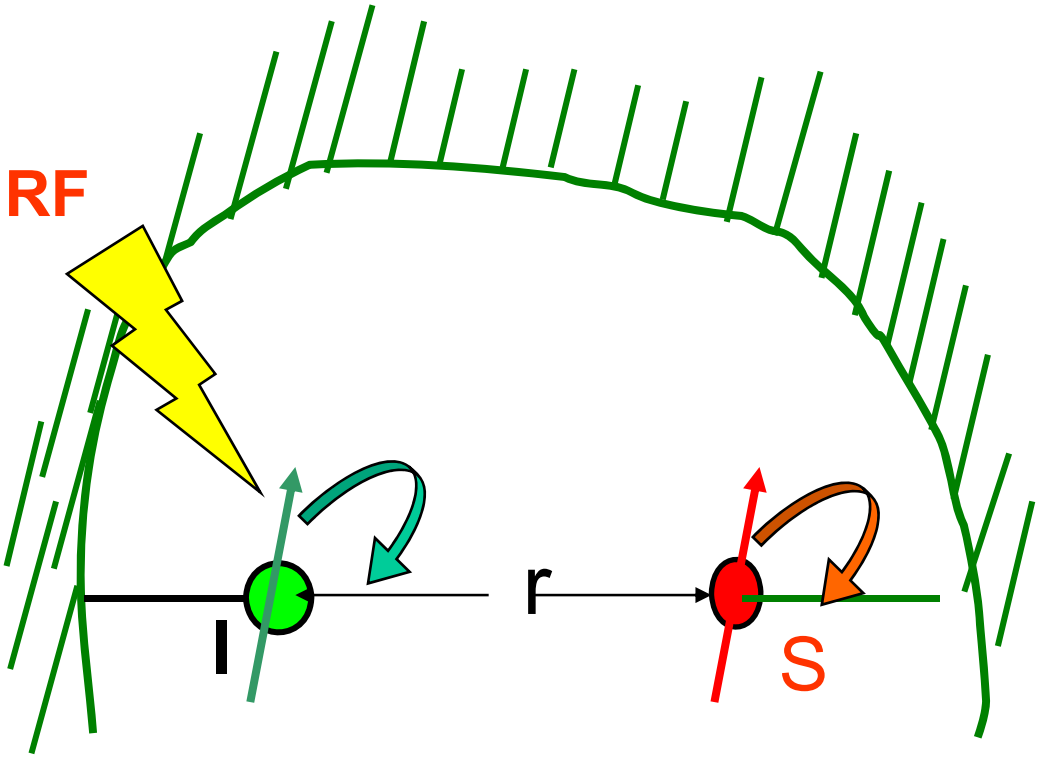
- $^3J_{NH\alpha}$ = 4 - 11 Hz depends on secondary structure.
  $^3J_{NH\alpha}$ < 6 Hz → α-helix; $^3J_{NH\alpha}$ > 8 Hz → β-stand

For through-bond 3D NMR (Magnetization transfer)

- J-coupling of backbone nuclei (Hz)



3. Nuclear Overhauser Effect (NOE)



$$XNOE = 1 + (d^2/4)(\gamma_H/\gamma_N)[6J(\omega_H + \omega_N) - J(\omega_H - \omega_N)]\, T_1$$

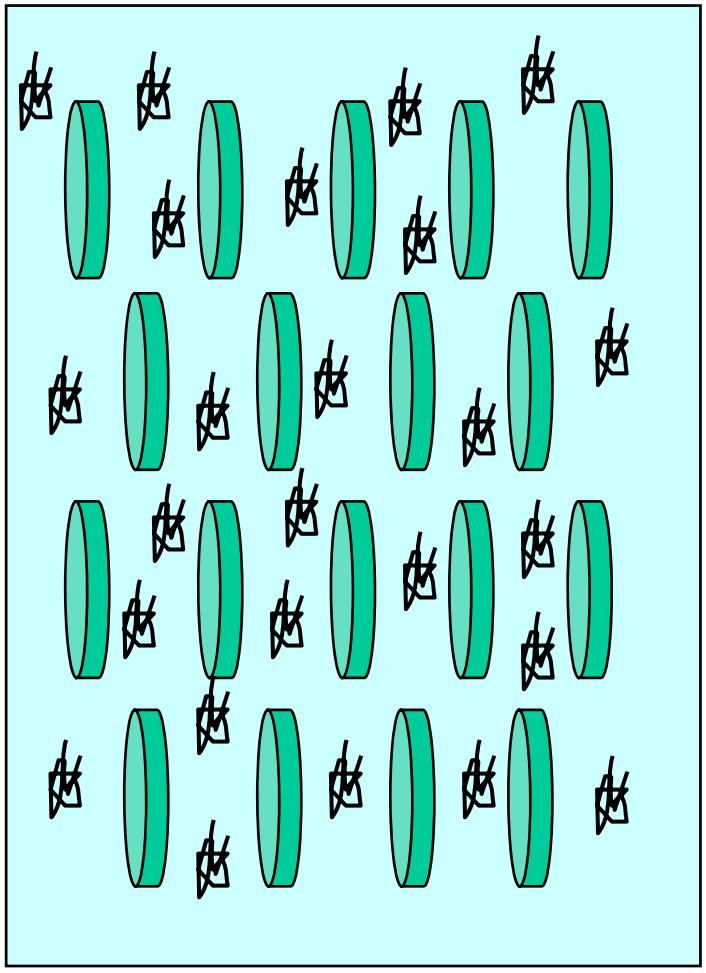
where $d = (\mu_o h \gamma_N \gamma_H / 8\pi^2)(r_{NH}^{-3})$, $J(\omega)$ is the spectral density function

➔ **1. Distance info: XNOE $\propto$ r^{-6} ;**

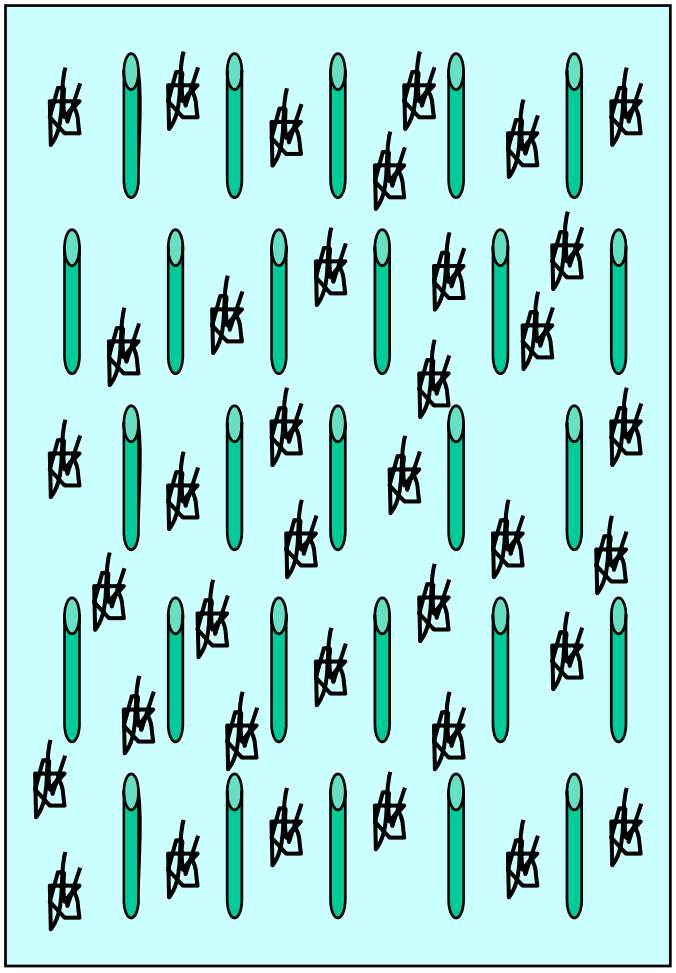
2. Dynamics: XNOE $\propto$ J (ω)

4. Residual dipolar coupling in partially oriented media

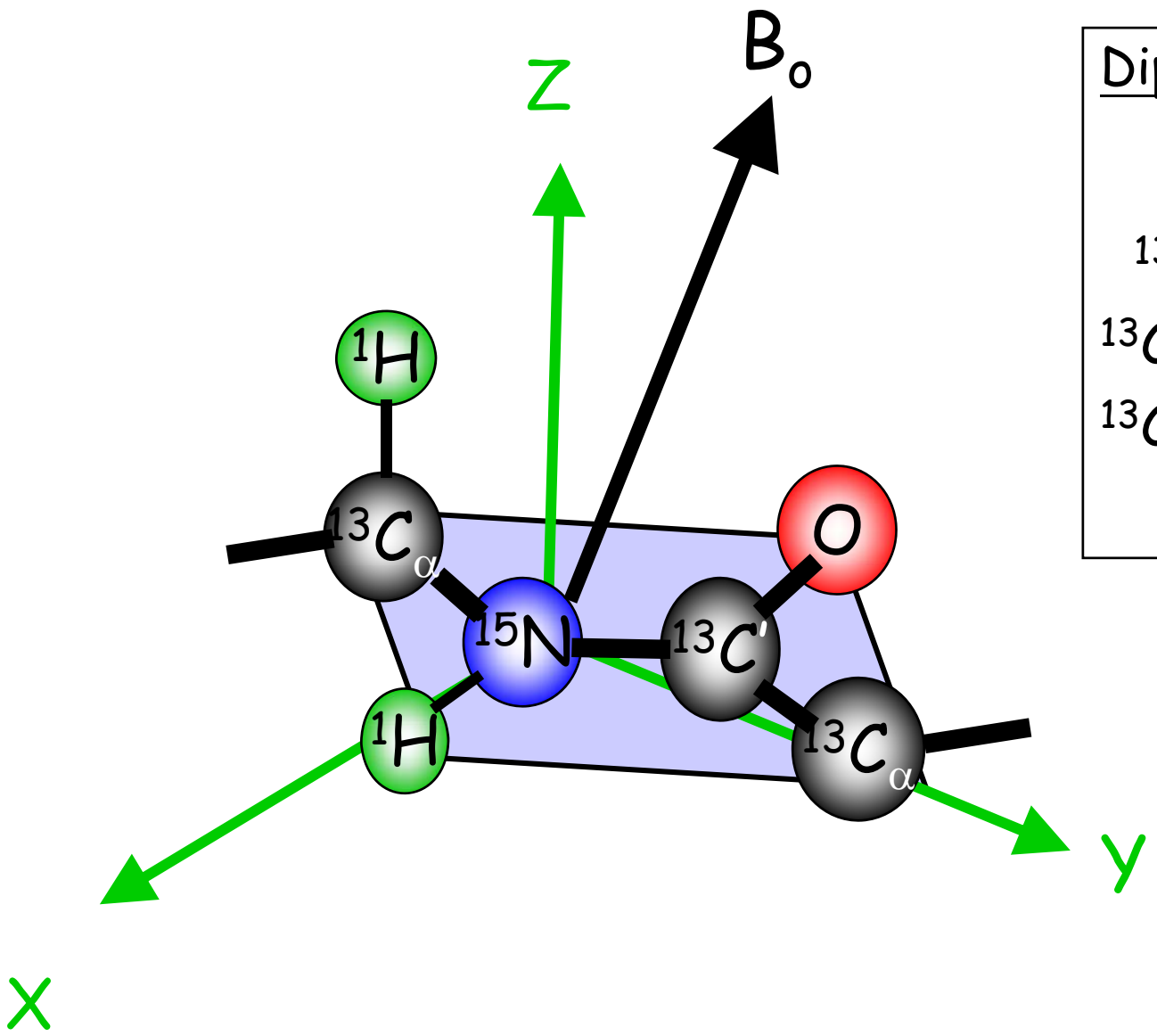
Bicells



Phage

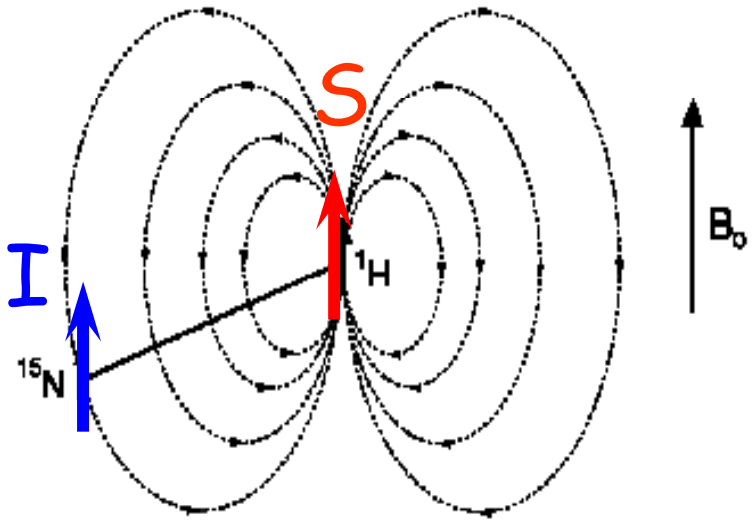


Dipolar interactions of peptide plane nuclei



Dipolar pairs
^{1}H - ^{15}N
$^{13}C_{\alpha}$ - ^{15}N
^{13}CO - ^{15}N
^{13}CO - $^{13}C_{\alpha}$
^{1}H - $^{13}C_{\alpha}$

Dipolar Field



Residual Dipolar Coupling:

S: Scaling factor = 0 in non-viscous liquid media

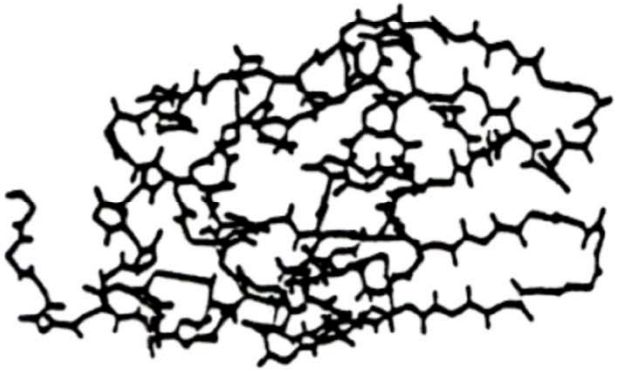
γ: Nuclear gyric ratios

$$D_{IS}(\theta,\varphi) = -\left(\frac{\mu_o h S \gamma_I \gamma_S}{16\pi R^3}\right)\left\{(3\cos^2\theta - 1) + \frac{3}{2}A\sin^2\theta\cos 2\varphi\right\}$$

R: Distance between spins I and S

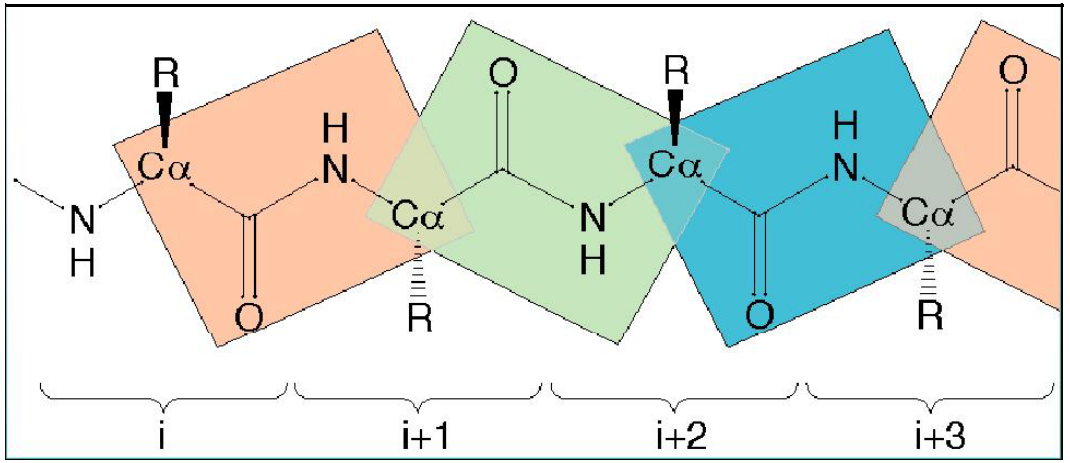
Protein structure

Stick & ball representatior
(Cartesian space)

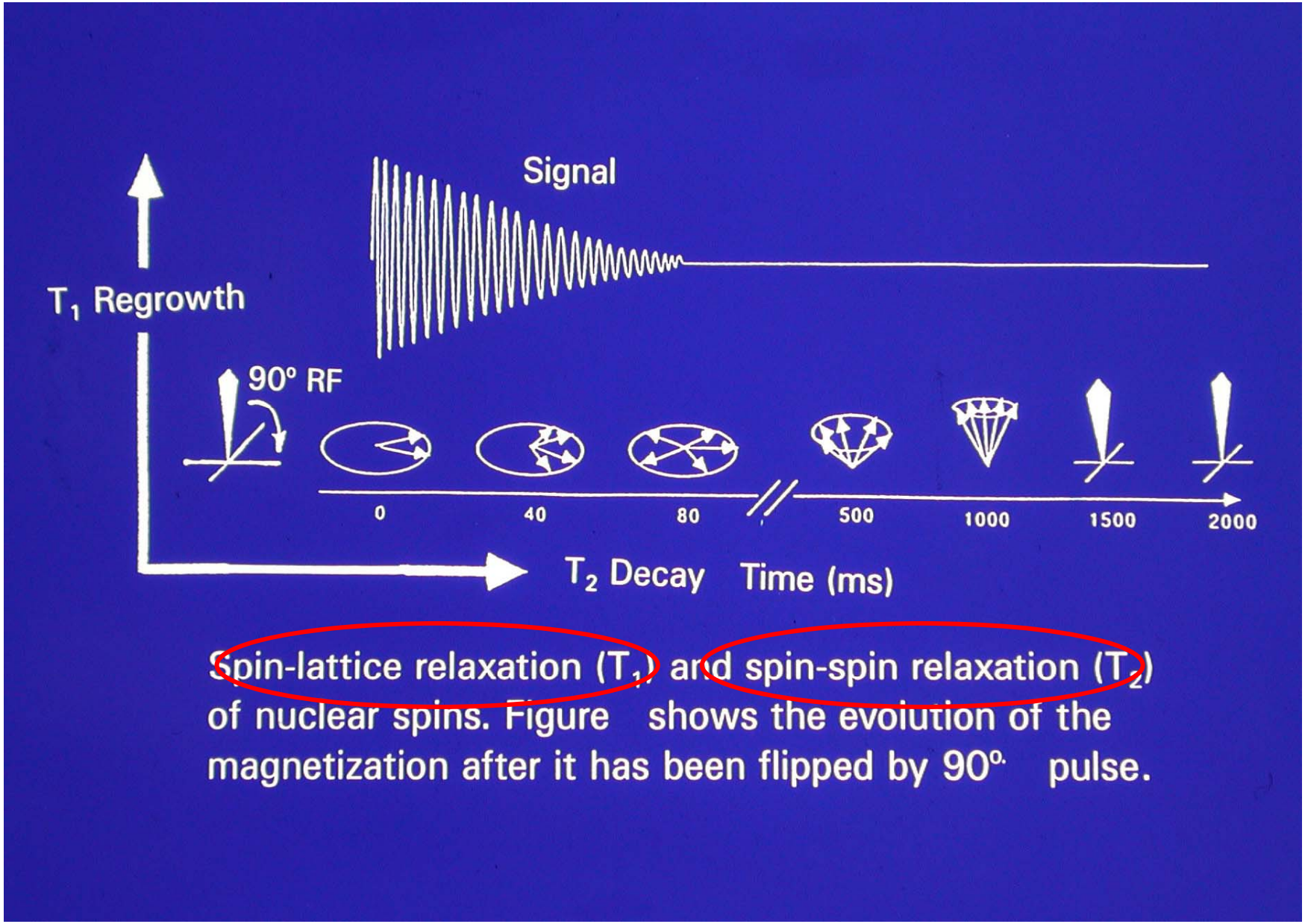


Peptide plane representation
(Angular space)

Strings of Peptide planes



4. NMR Relaxation



Spin-lattice relaxation (T_1) and spin-spin relaxation (T_2) of nuclear spins. Figure shows the evolution of the magnetization after it has been flipped by 90° pulse.

Applications

I. Structure:

- Protein structure up to 60 kDa has been reported (easier for < 20 kDa)
- Can observe good protein signal up to 800 kDa.

II. Dynamics (Motion):

- Characterize molecular motion (4th dimension)

III. Drug screening:

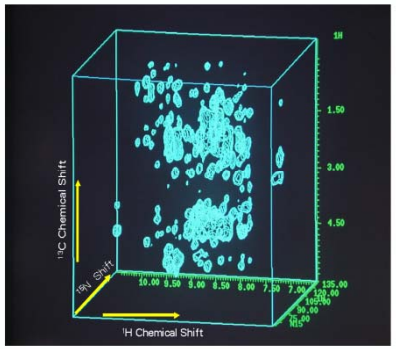
- High throughput (1000 samples per day)
- Atomic details
- Lead discovery.

IV. Magnetic Resonance Imaging (MRI):

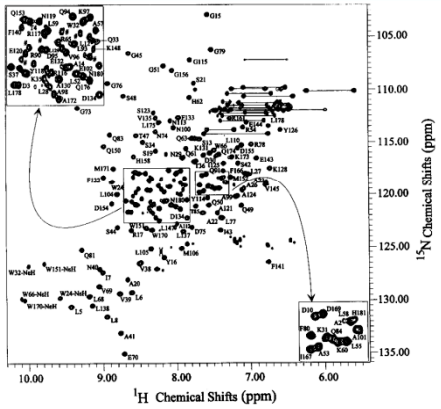
V. Metabolomics (Small molecule identification):

Determine Protein Structure by NMR

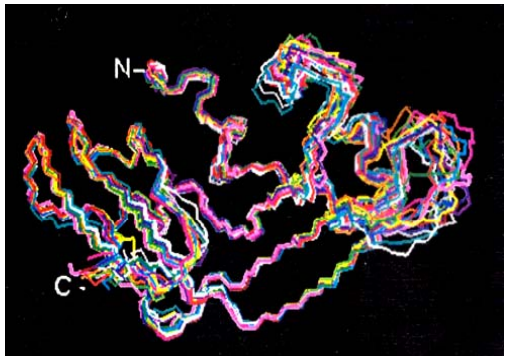
NMR Sample
(1 mM, 0.4 ml)
^{2}H, 13C, ^{15}N-label



Obtain NMR spectra
(3 weeks)

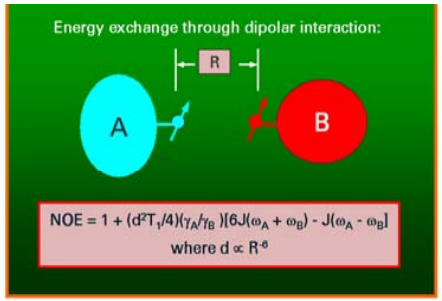


Assign resonances
Automation ?



NMR structures
(Ensemble of 20 structures)

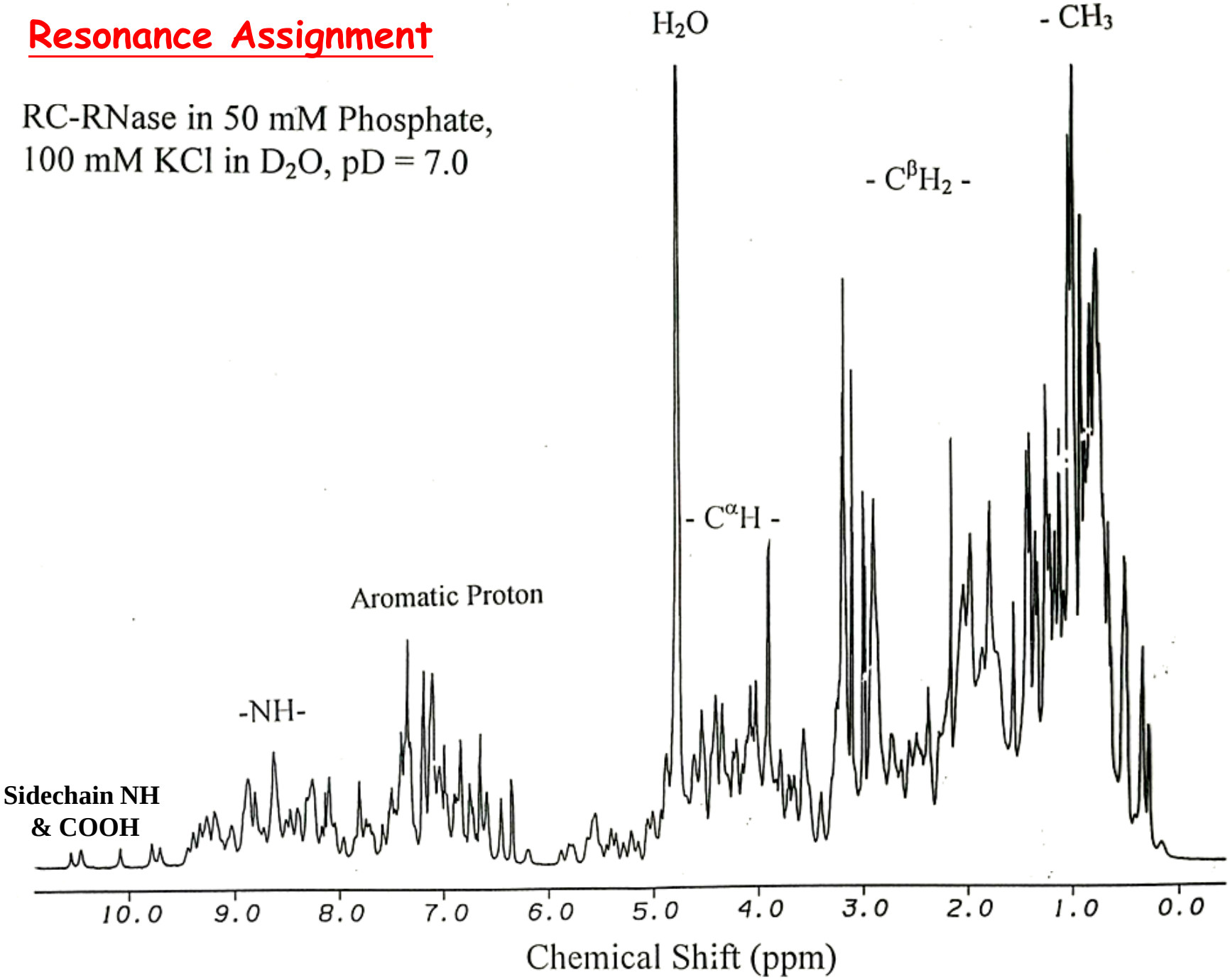
Calculate structures

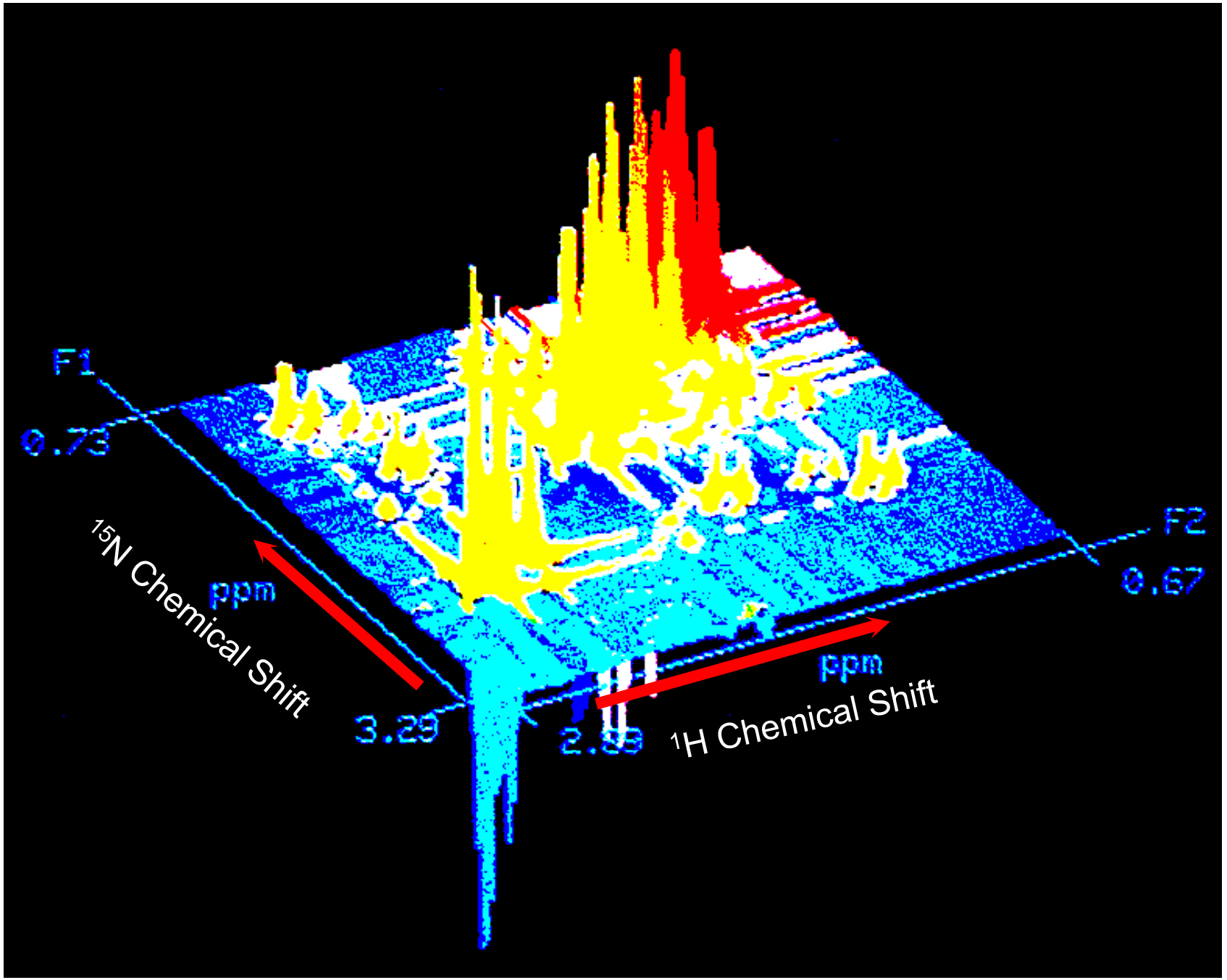


Obtain restrains
(Distances, angles,
Orientations etc)

Resonance Assignment

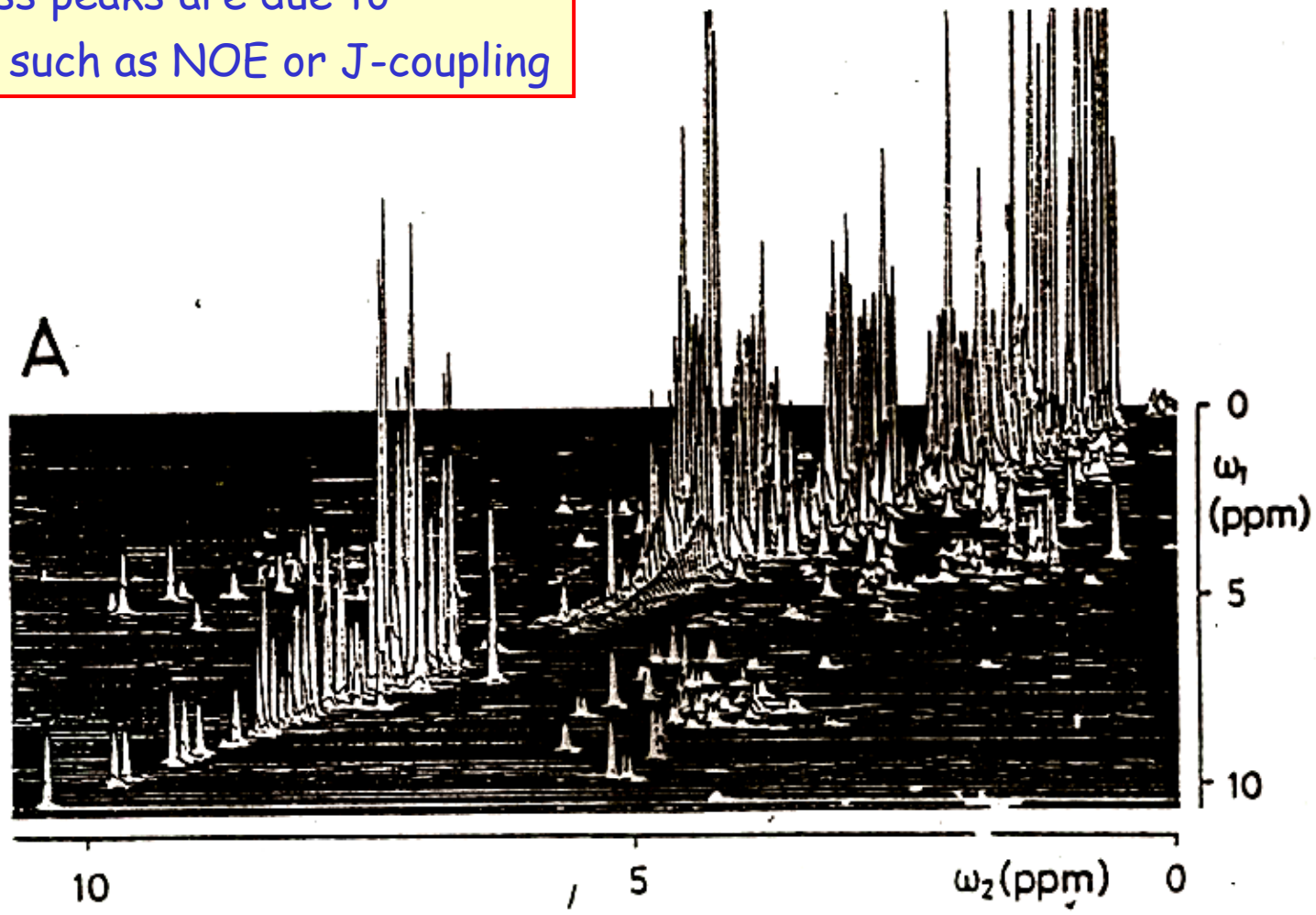
RC-RNase in 50 mM Phosphate,
100 mM KCl in D_2O, pD = 7.0



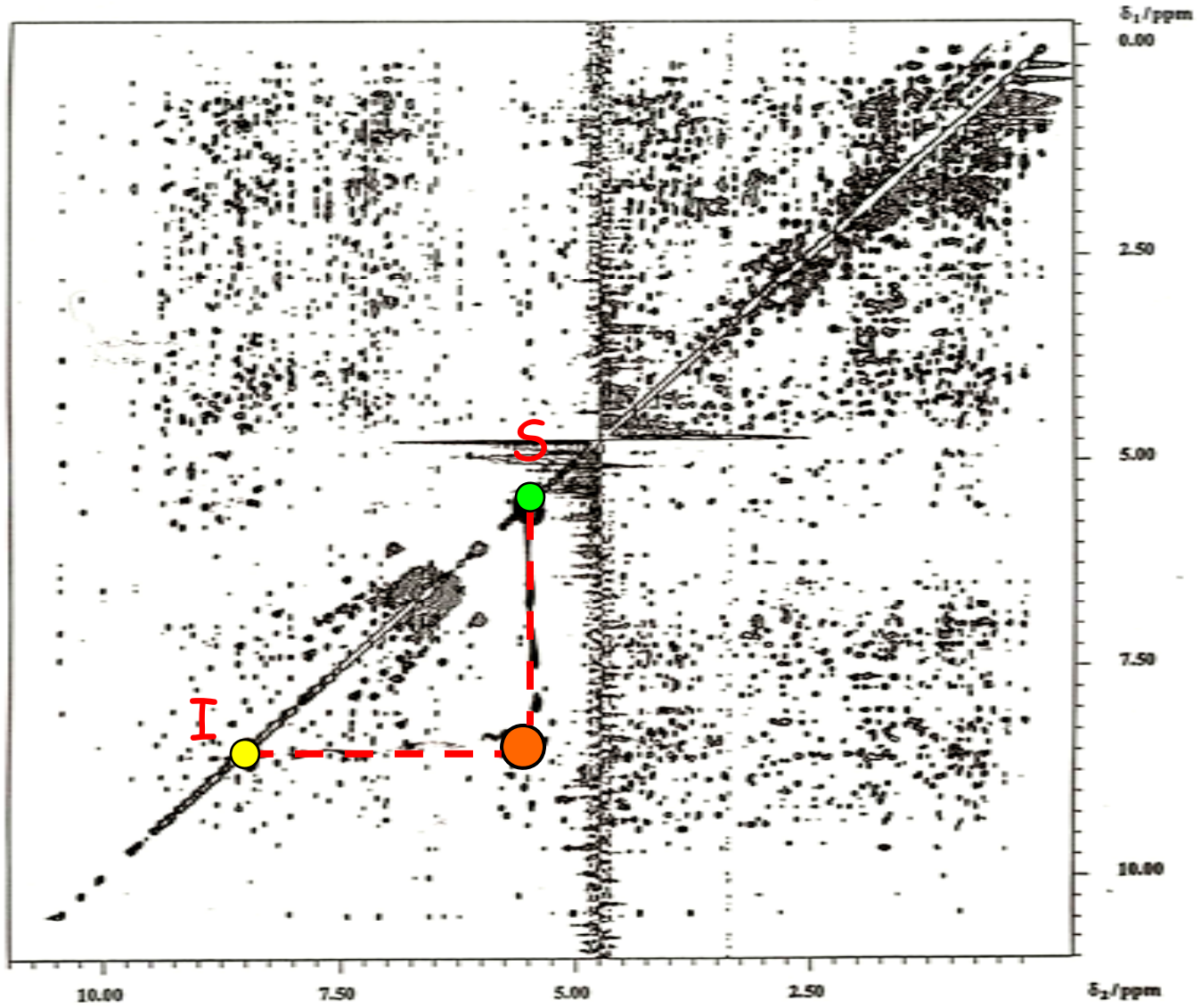

F1
0.73
^{15}N Chemical Shift
ppm
3.29
F2
0.67
ppm
^{1}H Chemical Shift

2D-NMR Spectrum

- Diagonal resonances same as in 1D spectrum
- off-diagonal cross peaks are due to **interactions** such as NOE or J-coupling



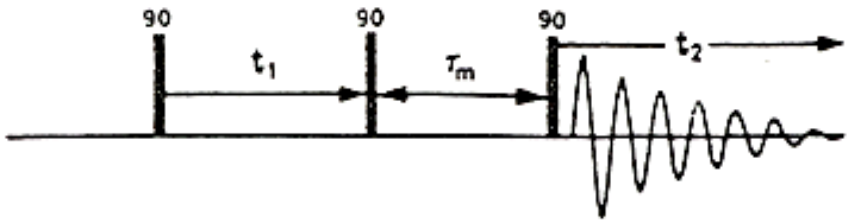
^1H - ^1H NOESY of RC-RNase



Homonuclear 2D NMR experiments

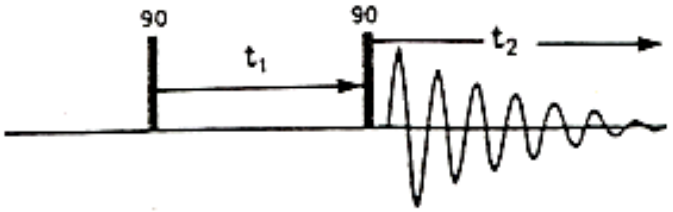
(**N**uclear **O**verhauser **E**ffect **S**pectroscop**Y**)
→ Through space dipolar effect
→ Determine NOE
→ Measuring distance
→ Assign resonances

NOESY



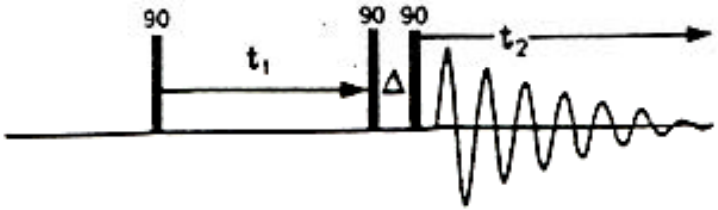
(**CO**rrelated **S**pectroscop**Y**)
→Through bond J-coupling
→ Assign adjacent resonances

COSY



(**M**ultiple **Q**uantum **F**iltered **CO**rrelated **S**pectroscop**Y**)
→Through bond J-coupling similar to COSY
→ Assign adjacent resonances
→ More sensitive

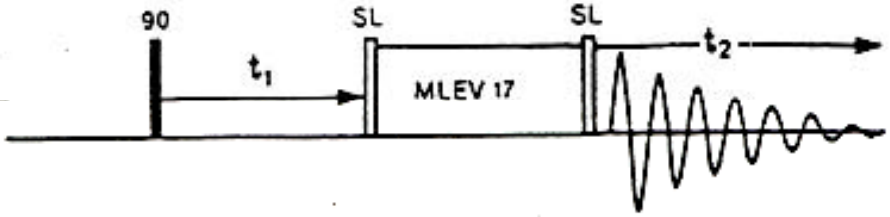
MQF-COSY



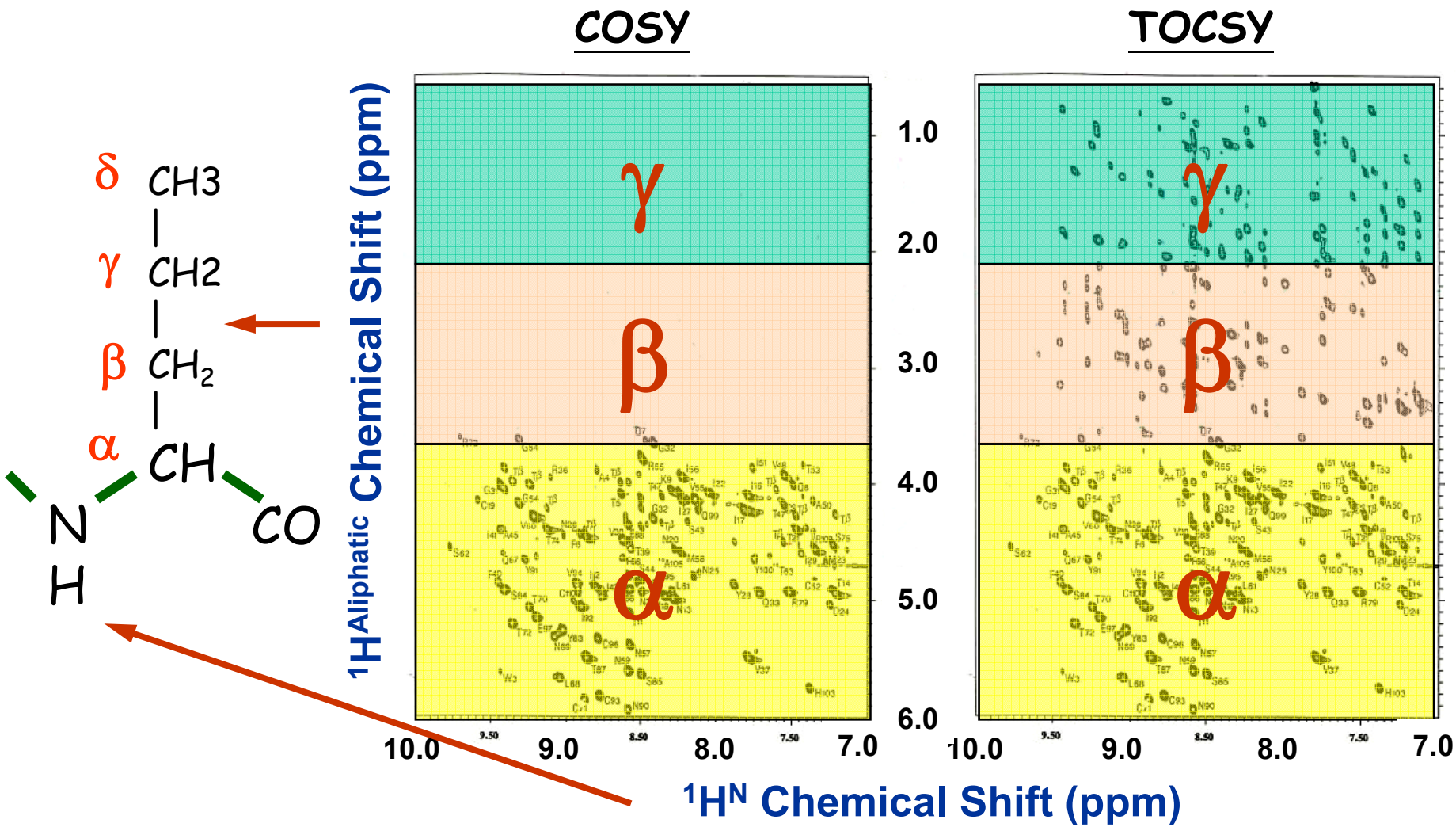
(**TO**tal **C**orrelated **S**pectroscop**Y**) (TOC SY)

→Through bond relayed J-coupling
→ Assign full spin system (residues type)

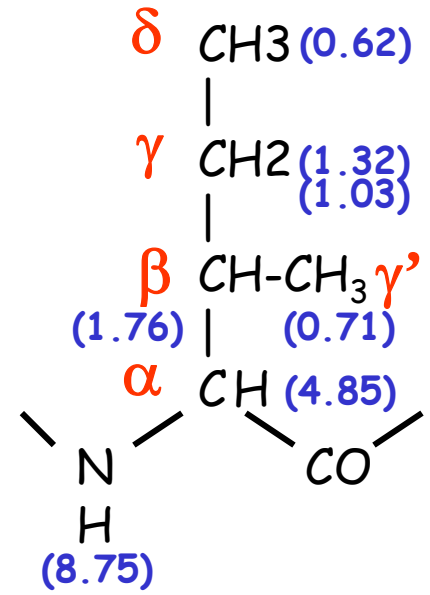
TOSY

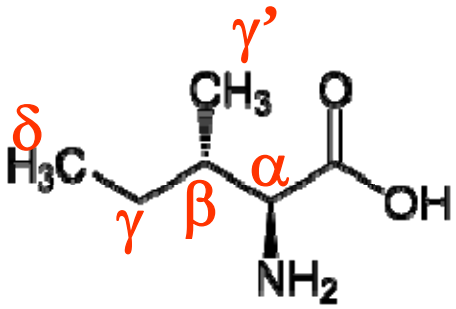


COSY v.s. TOCSY spectra (Fingerprint region)

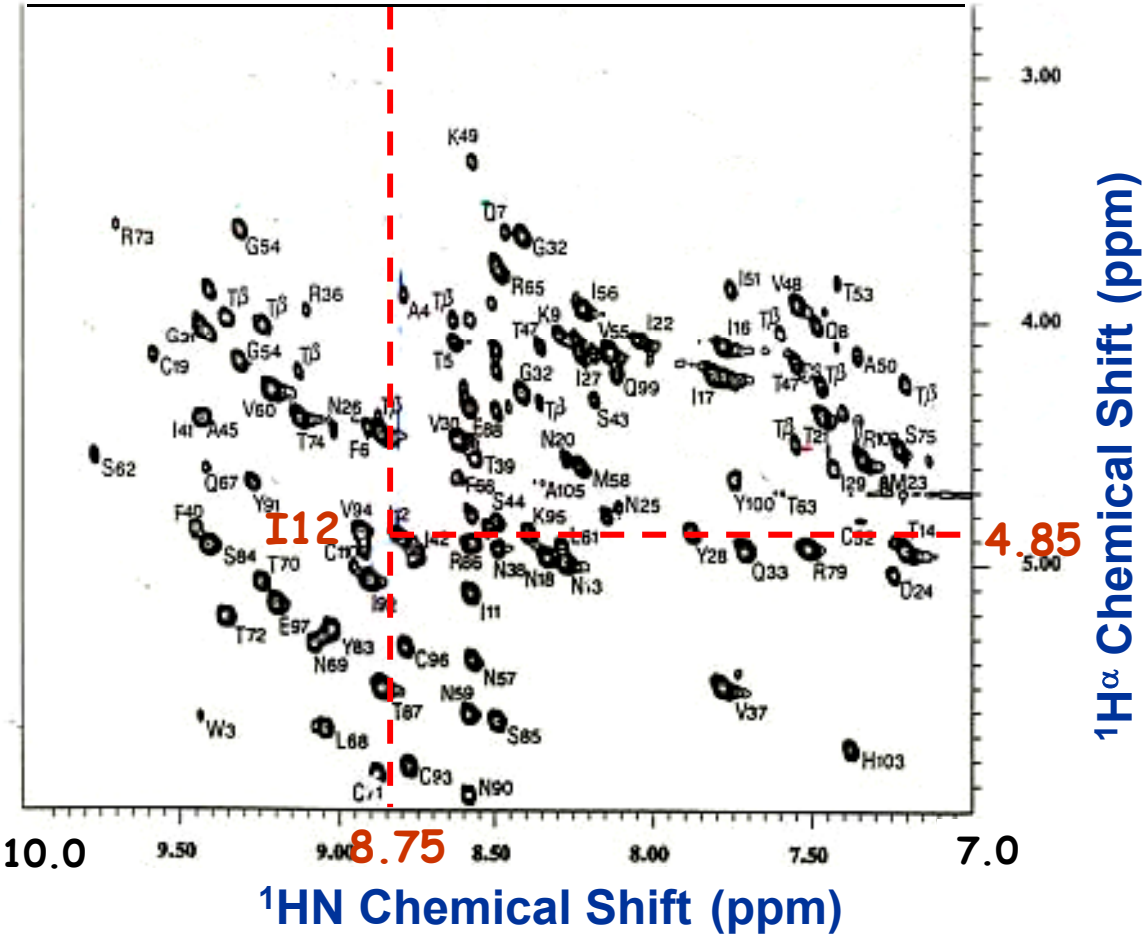


COSY (Fingerprint region)





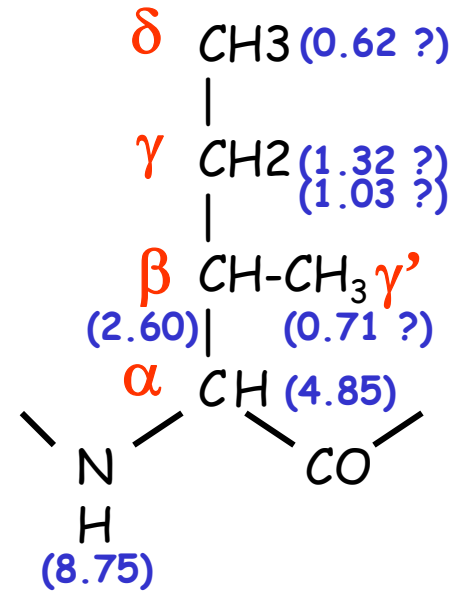
Isoleucine

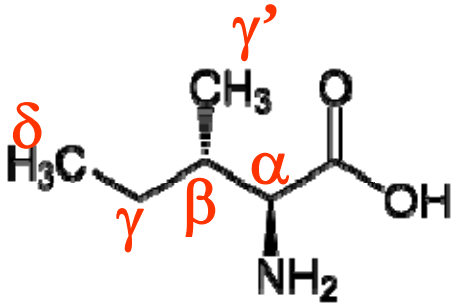


See only H^N and H^α correlation

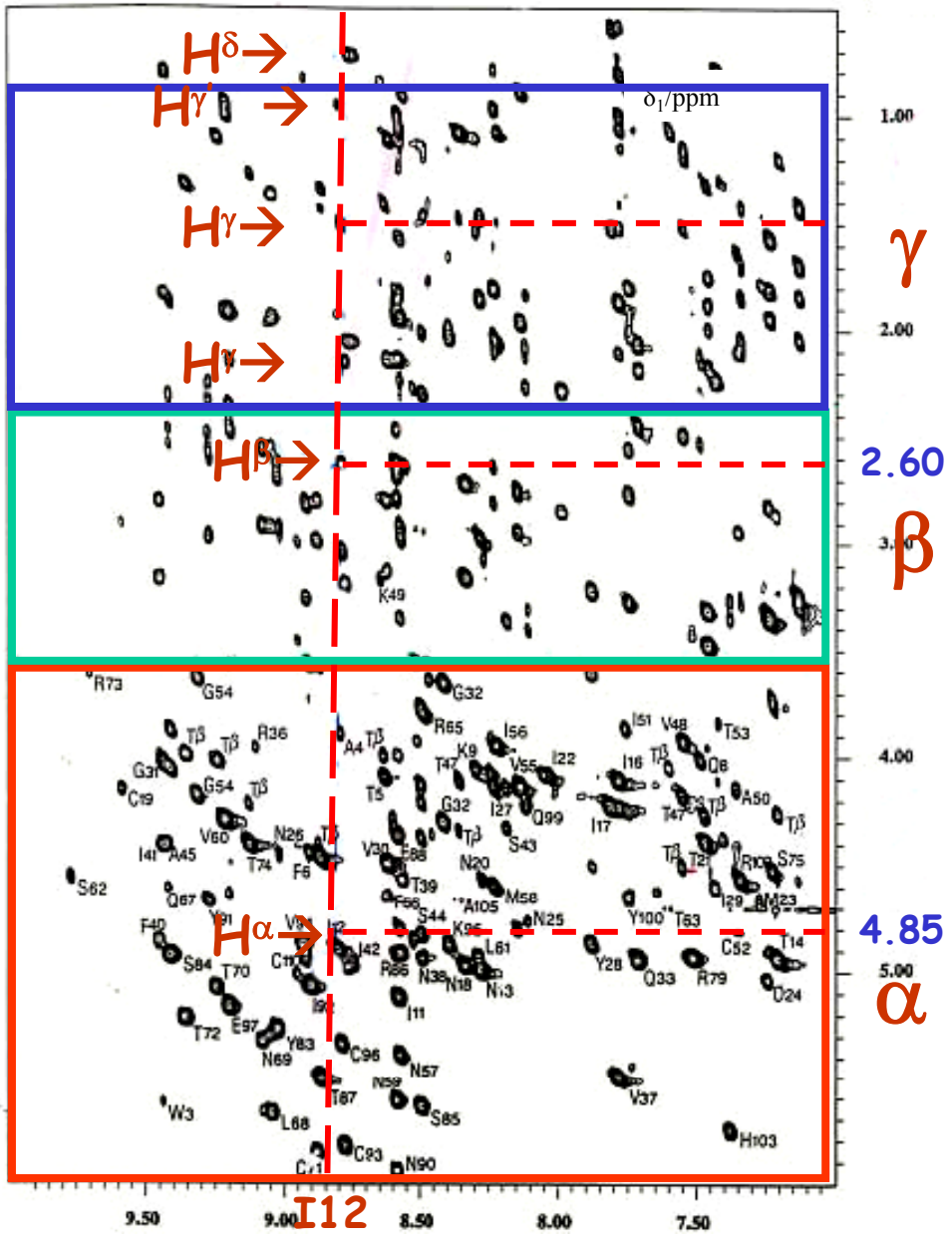
TOCSY (Spin System Identification) RC-RNase

1. J-Coupling: HN→H_α→H_β.....2. Identify Spin System(a.a. type)

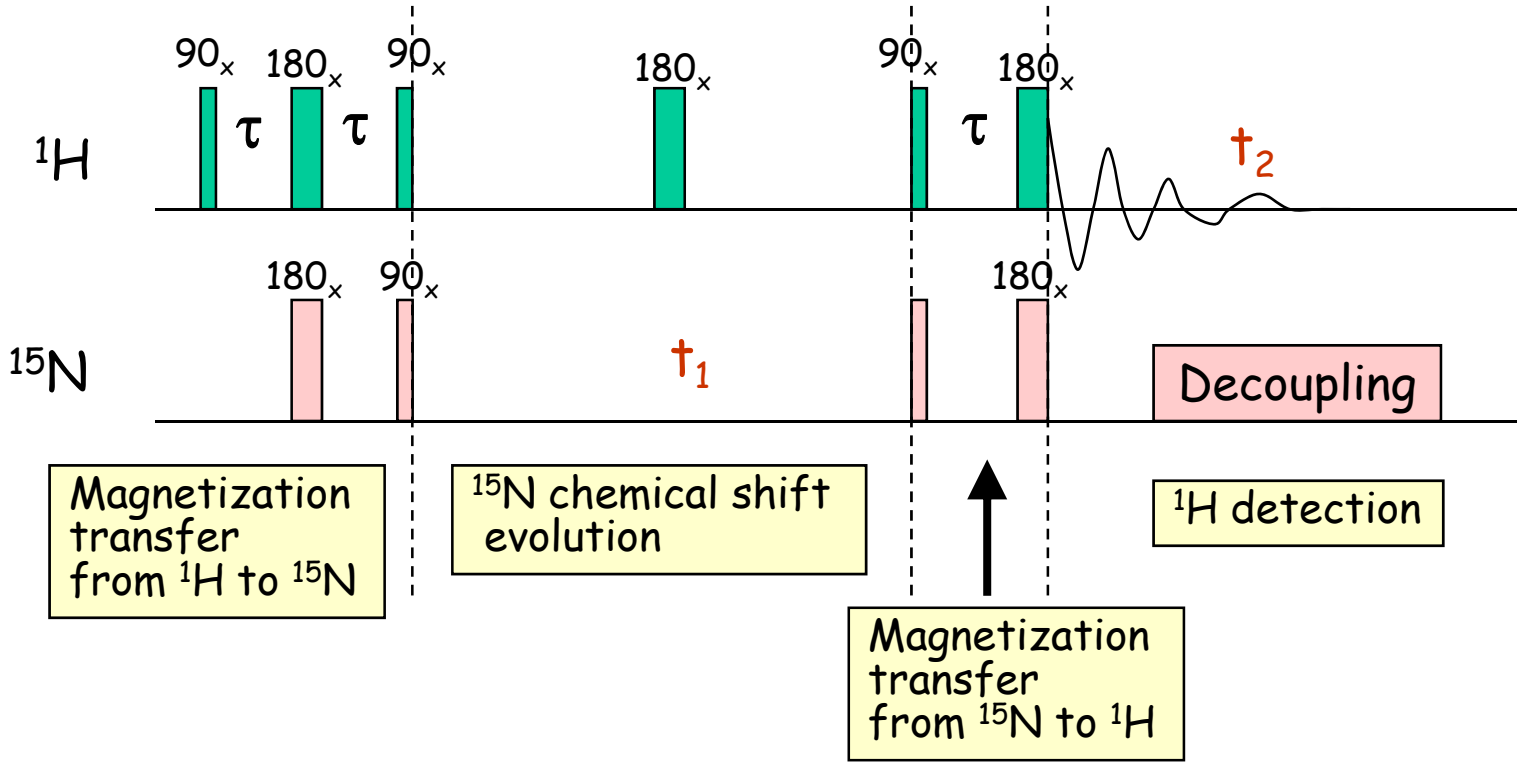




Isoleucine

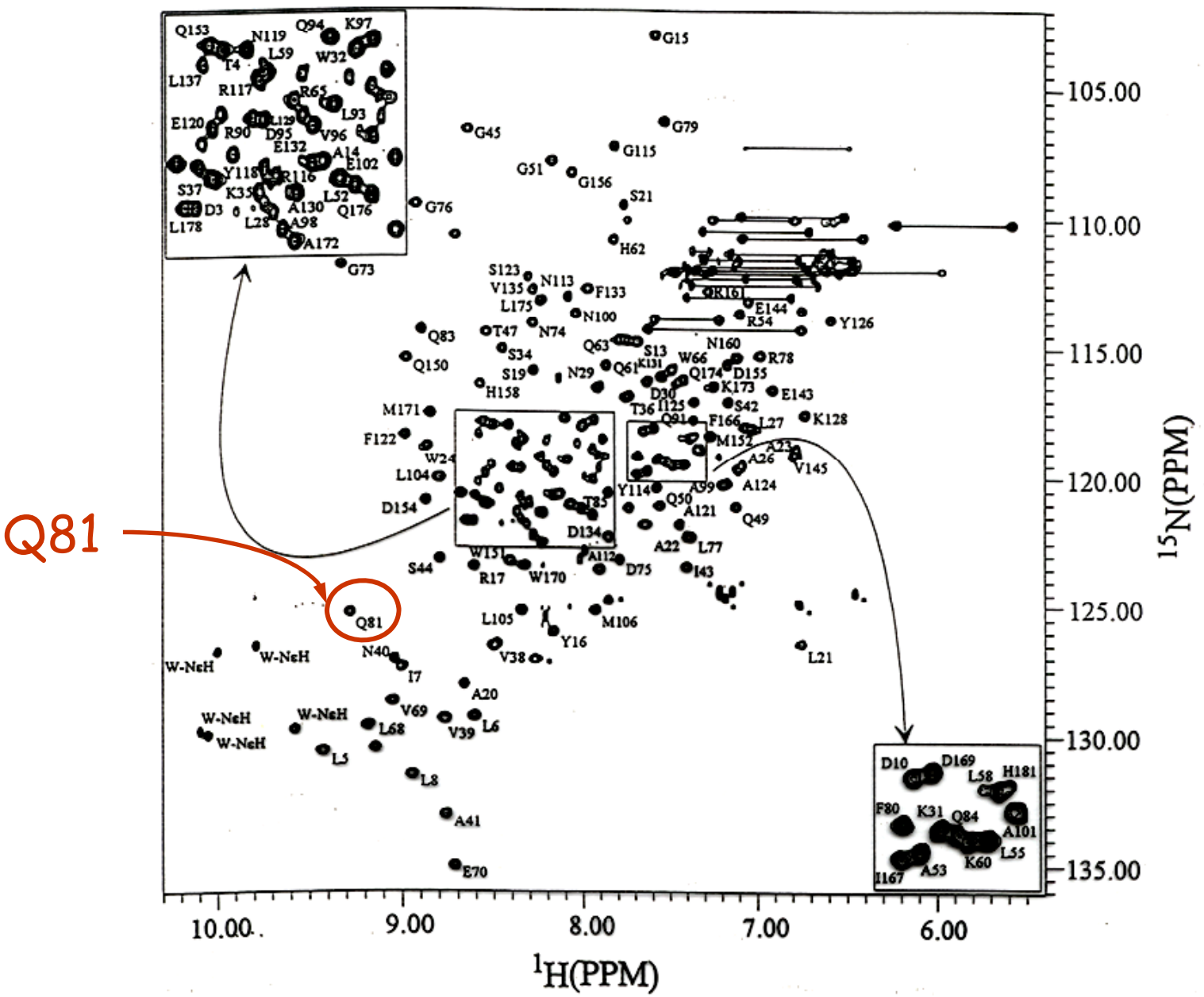


2D ^{15}N-^{1}H Heteronuclear Single Quantum Correlation Spectroscopy (^{15}N-HSQC)



- Observe ^{15}N spectrum in t_1 and ^{1}H spectrum in t_2 dimension
  → Excellent resolution
- Each peak codes for one amide group (^{15}N-^{1}H), i.e. one amino acid.
- Detect ^{15}N at ^{1}H sensitivity.

^{15}N-Heteronculear Single Quantum Spectroscopy (^{15}N HSQC)

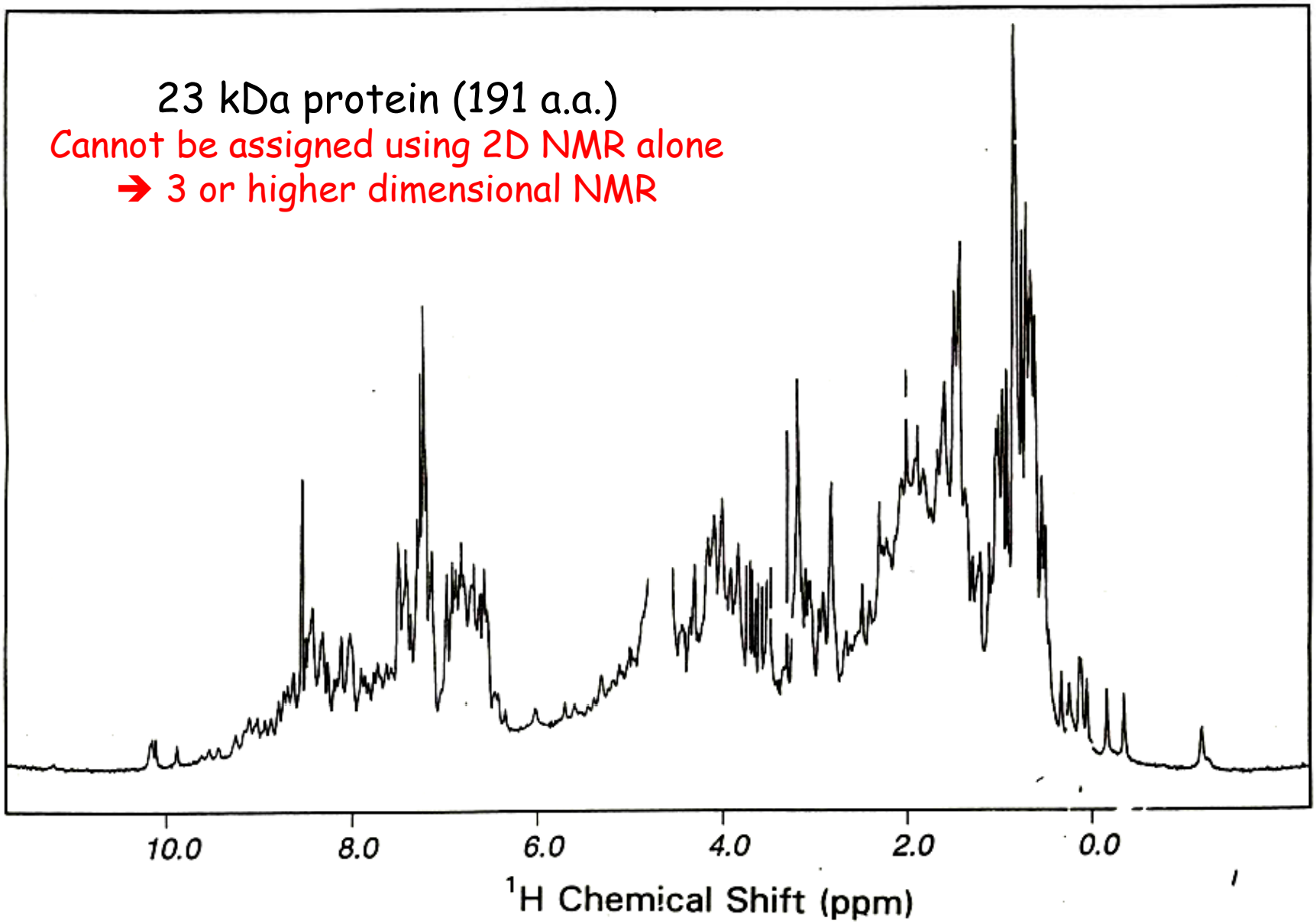


^{1}H NMR Spectrum of Thioesterase (pH 3.6, 303K)

23 kDa protein (191 a.a.)

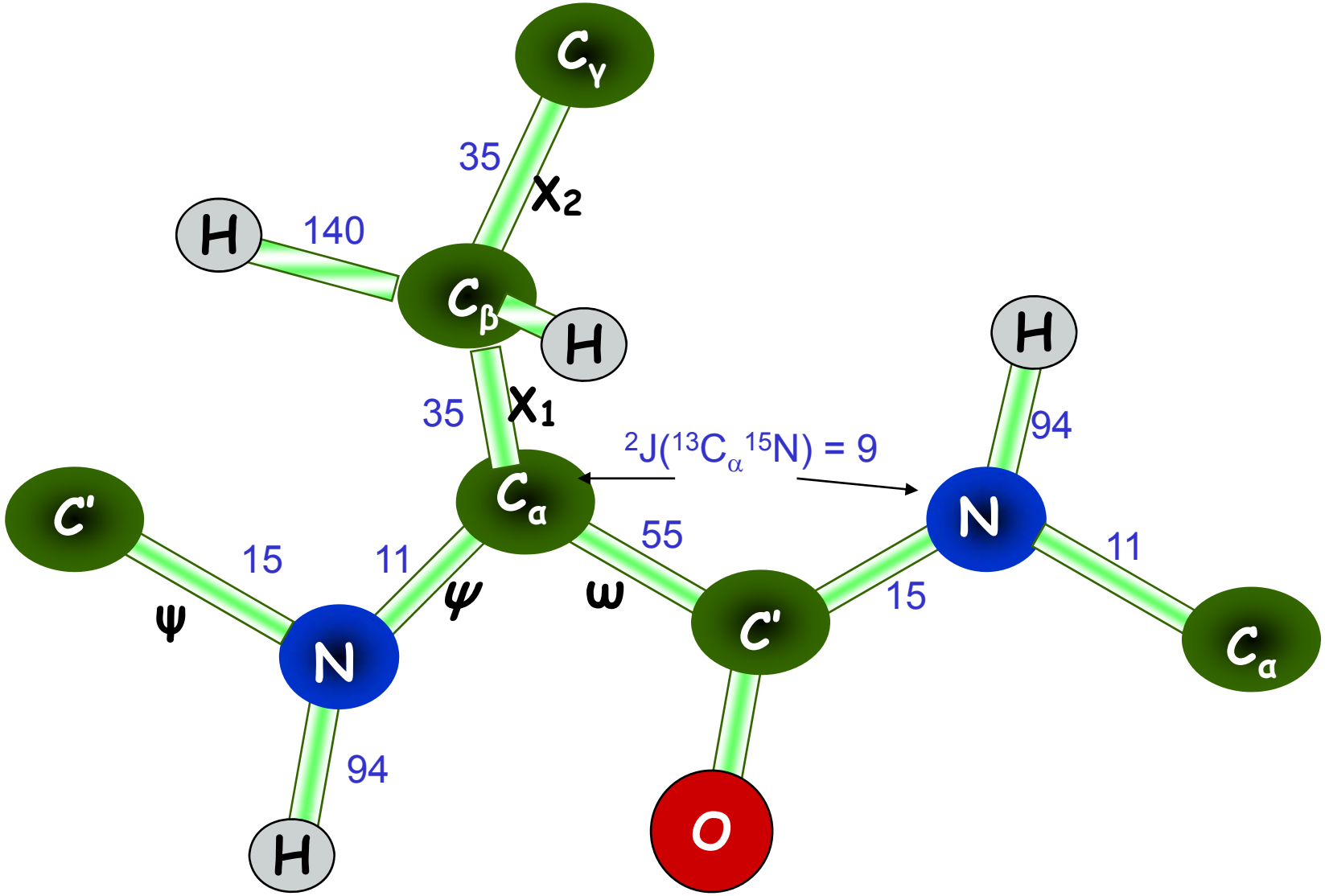
Cannot be assigned using 2D NMR alone

➔ 3 or higher dimensional NMR



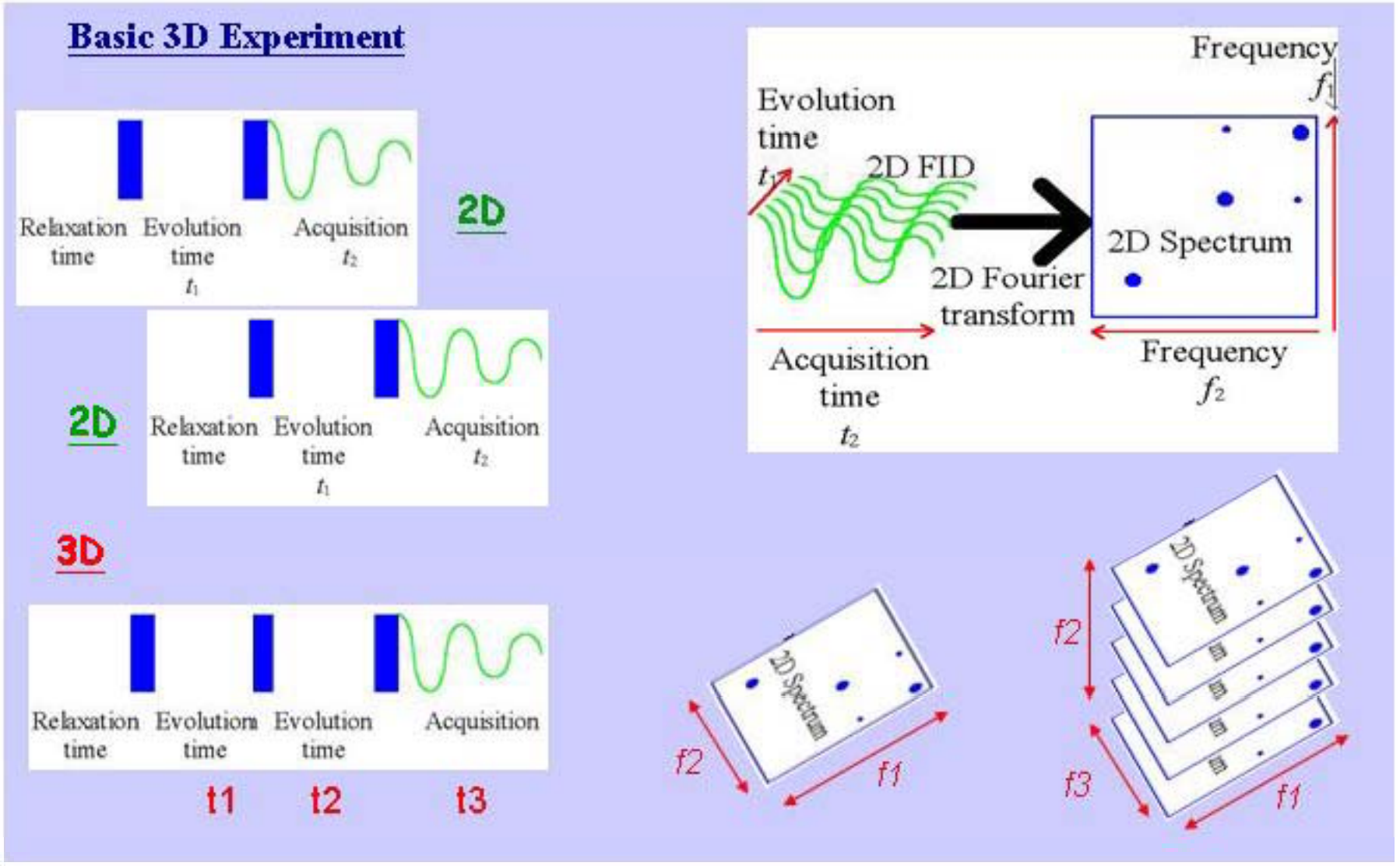
Magnetization transfer thru bonds

J-coupling of backbone nuclei (Hz)

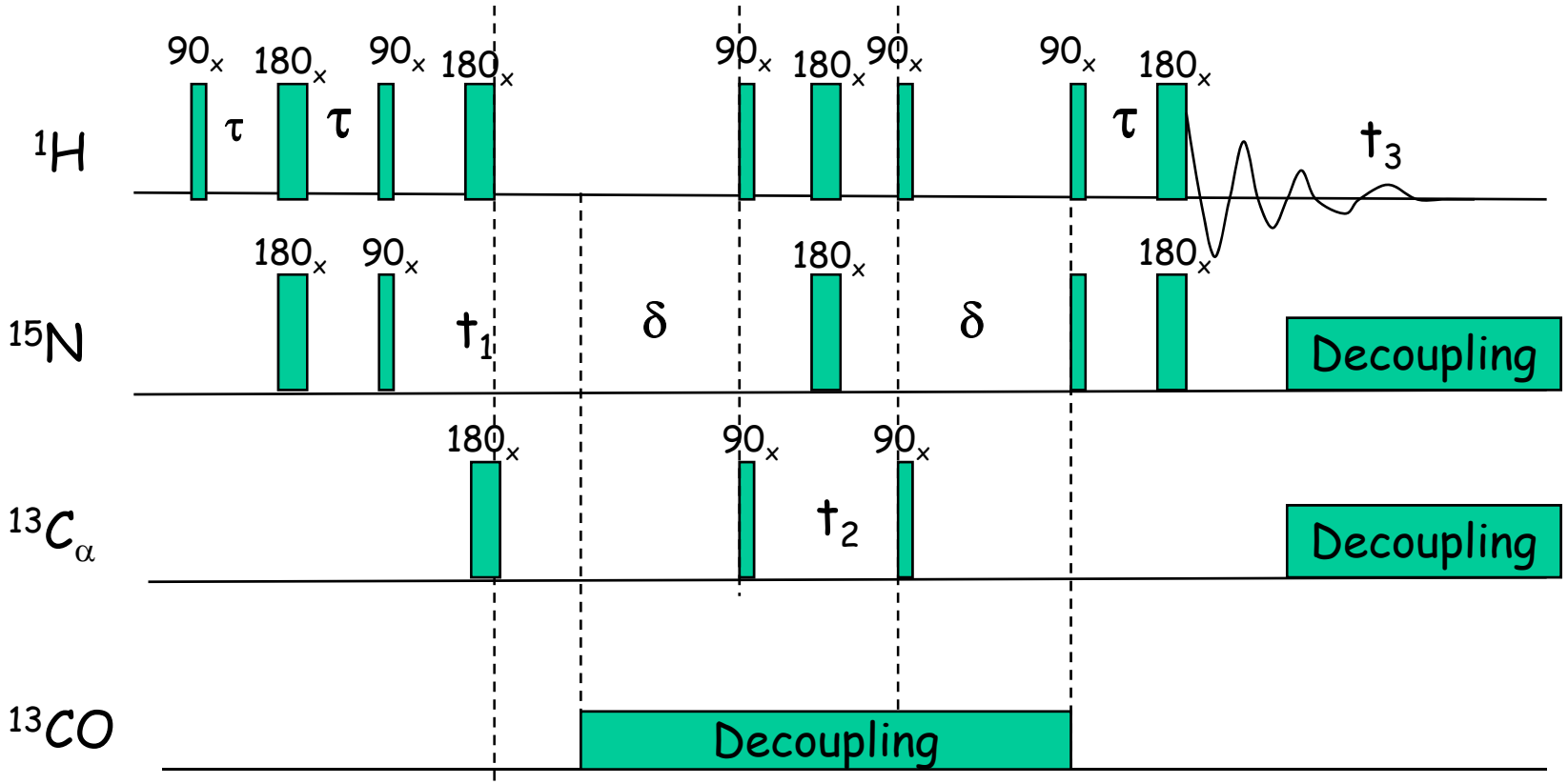


Multi-Dimensional NMR

Magnetization transfer thru bonds



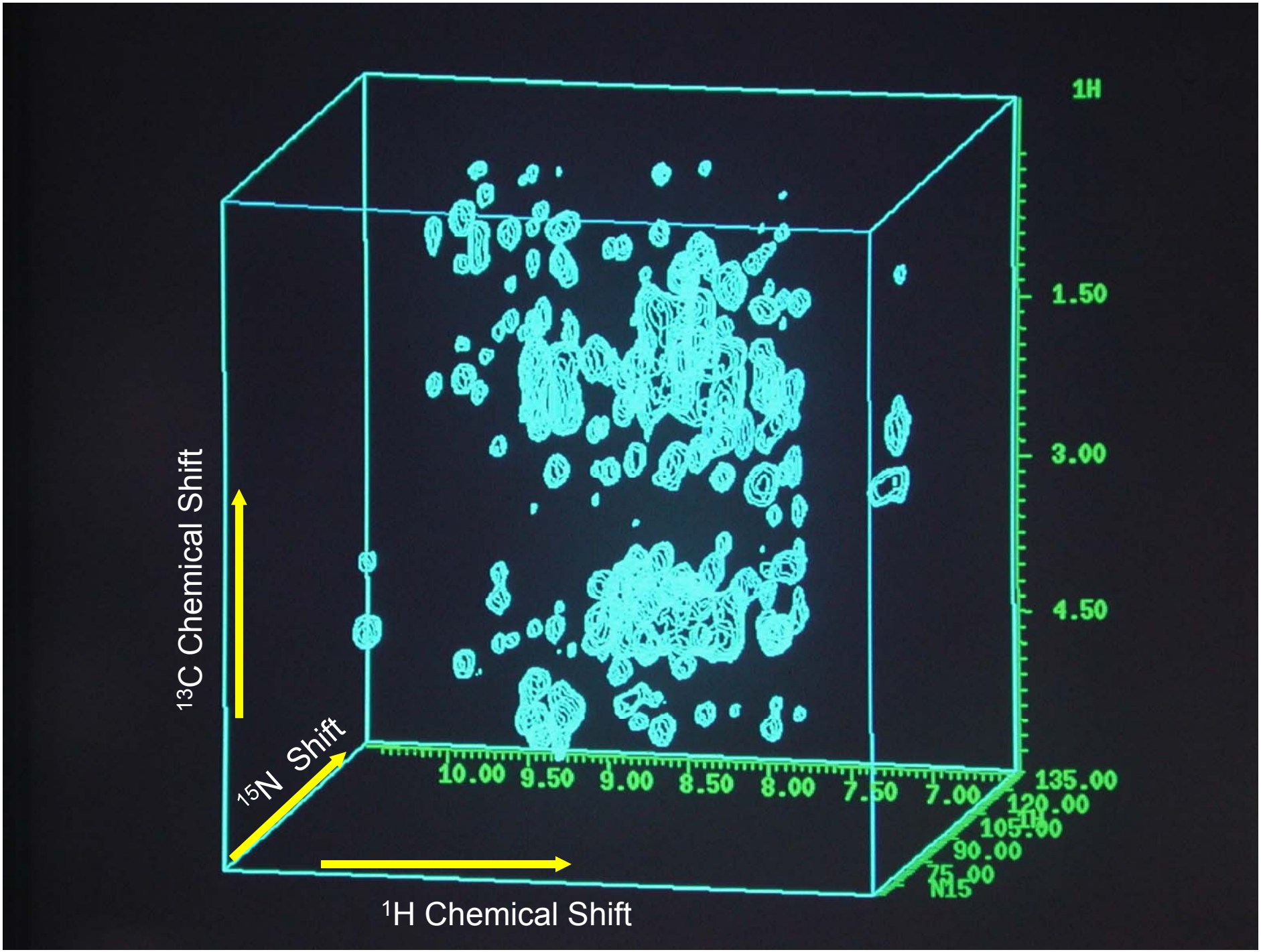
3D HNCA



Detect: $^{1}H^{N}$, ^{15}N and $^{13}C_{\alpha}$

$\delta = 1/4J_{N\text{-}CA} = 1/4x10 = 25$ ms for optimal detection

$\tau = 1/4J_{H\text{-}N} = 1/4x94 = 2.5$ ms


1H
1.50
3.00
4.50
13C Chemical Shift
15N Shift
10.00 9.50 9.00 8.50 8.00 7.50 7.00
135.00
120.00
105.00
90.00
75.00
N15
1H Chemical Shift

Heteronuclear multidimensional NMR experiments for resonance assignments

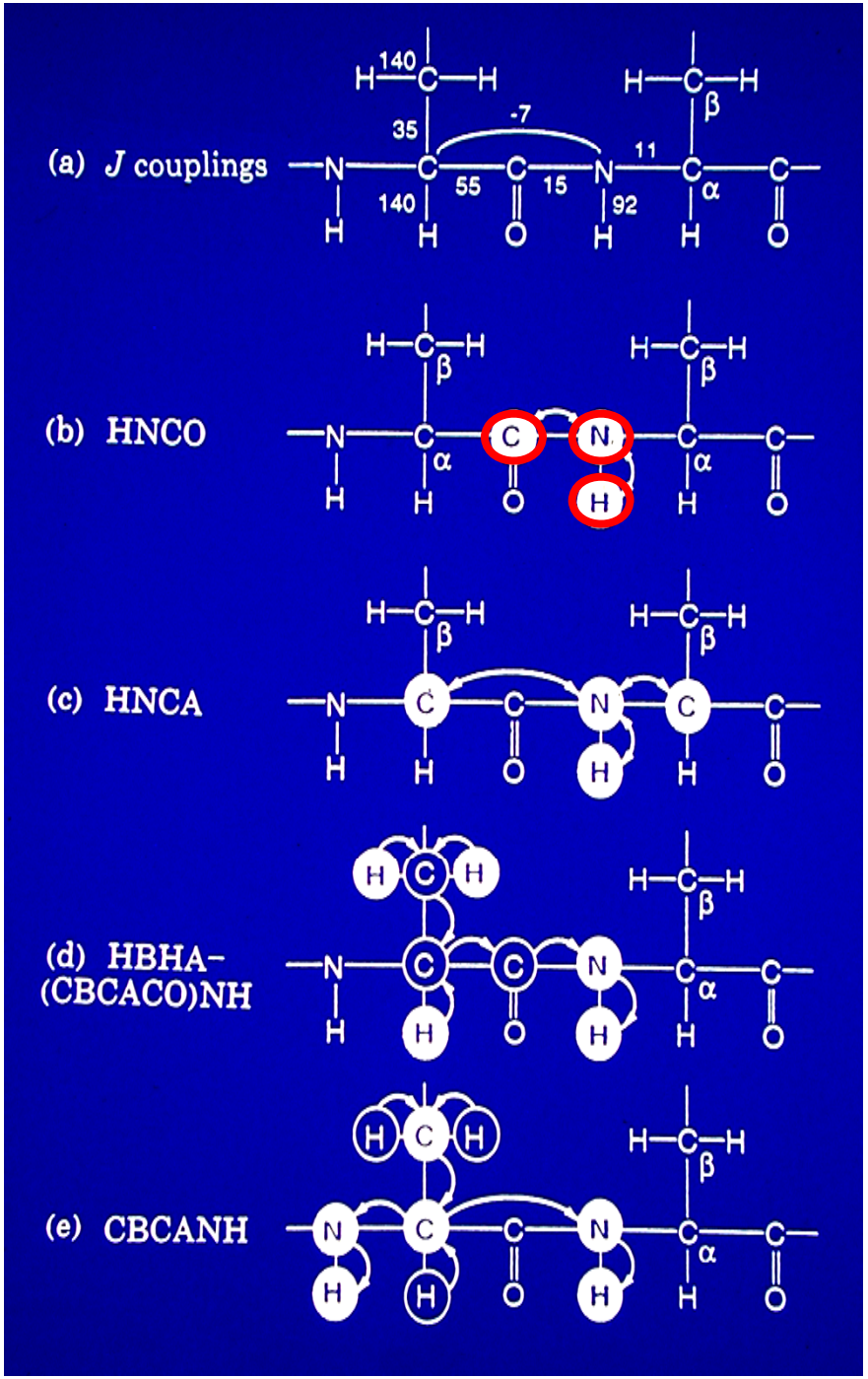
Magnetization transfer pathway:

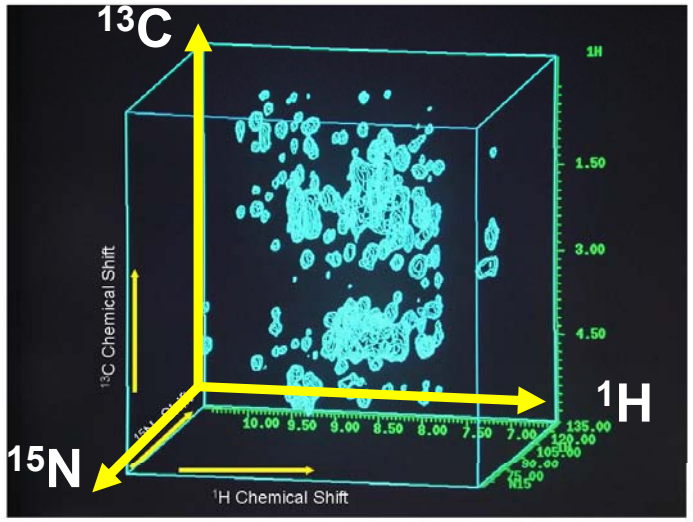
$^{1}H \rightarrow {}^{15}N \rightarrow {}^{13}C \rightarrow {}^{15}N$

$\rightarrow {}^{1}H \rightarrow {}^{1}H$ Detection

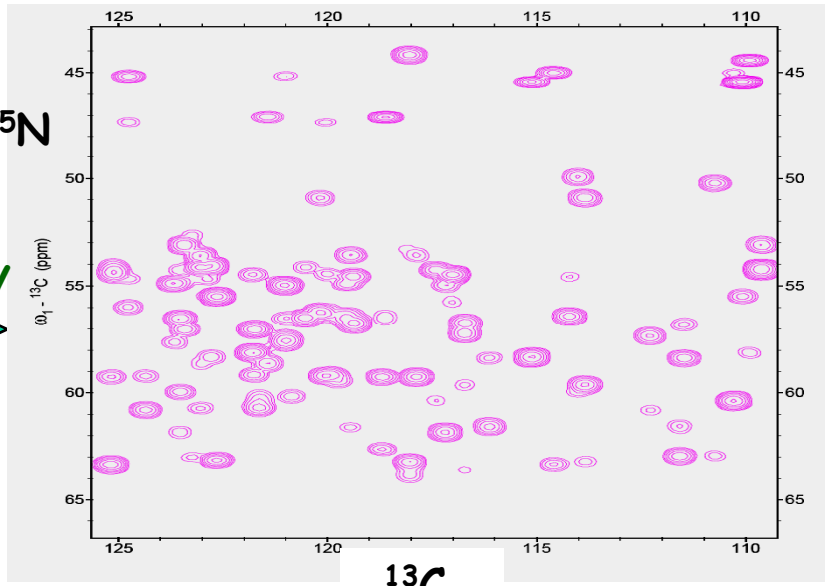
➔ Detect ^{1}H, ^{13}C, ^{15}N resonances

➔Permit sequential correlation of backbone ^{1}H-^{13}C-^{15}N resonances !!!



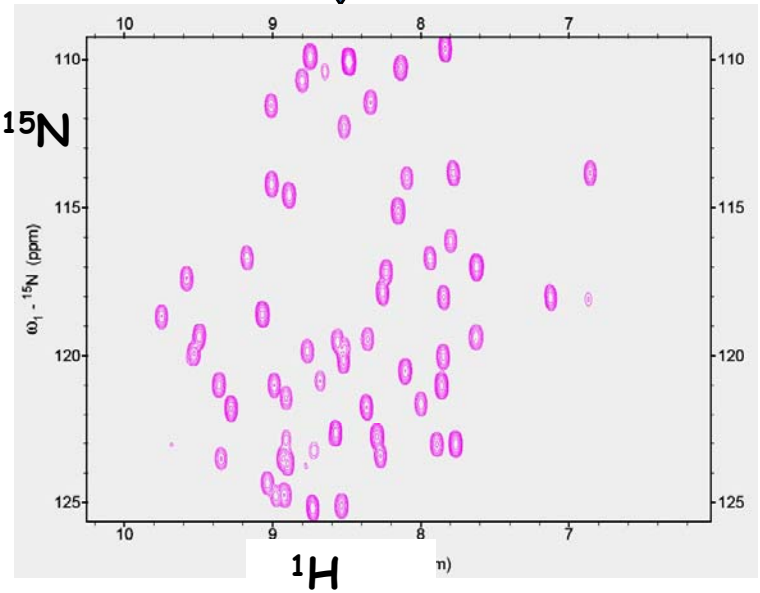


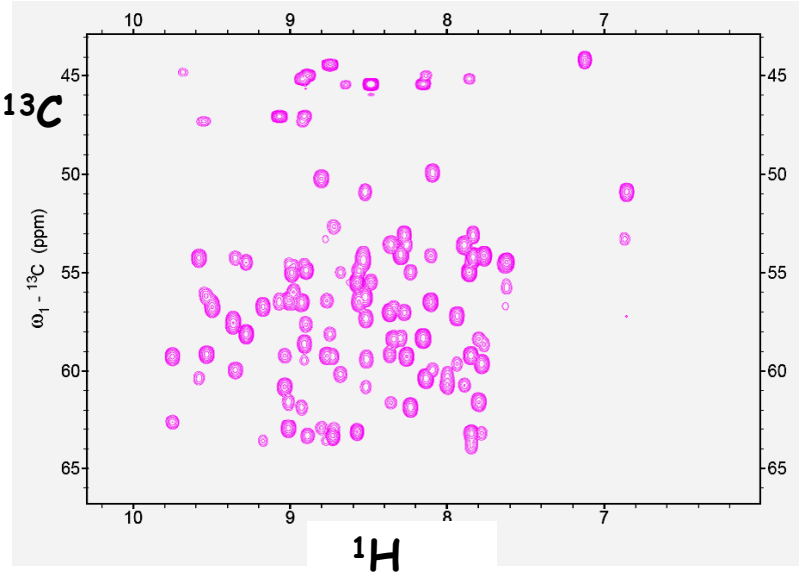
Select a ^{1}H frequency

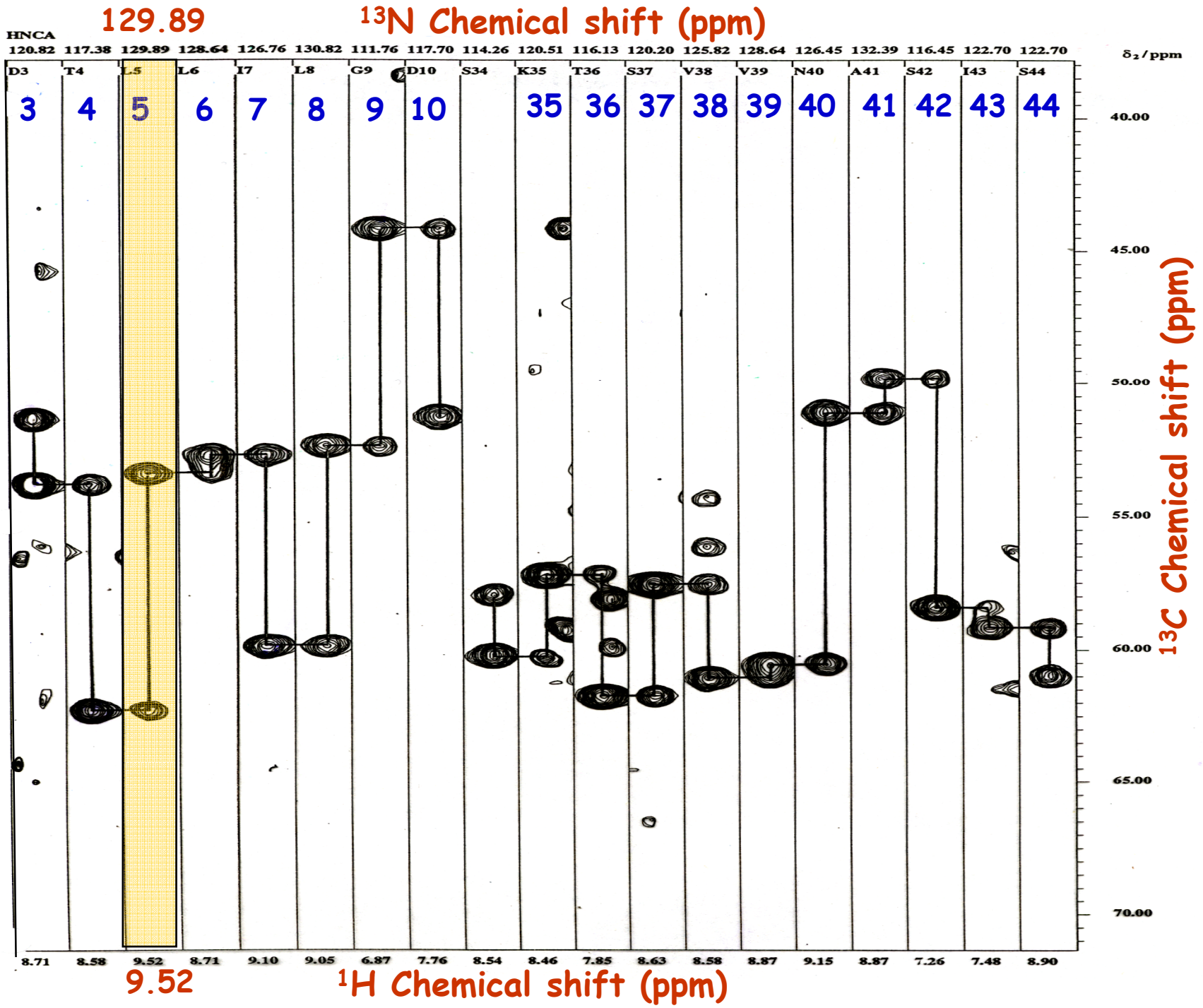


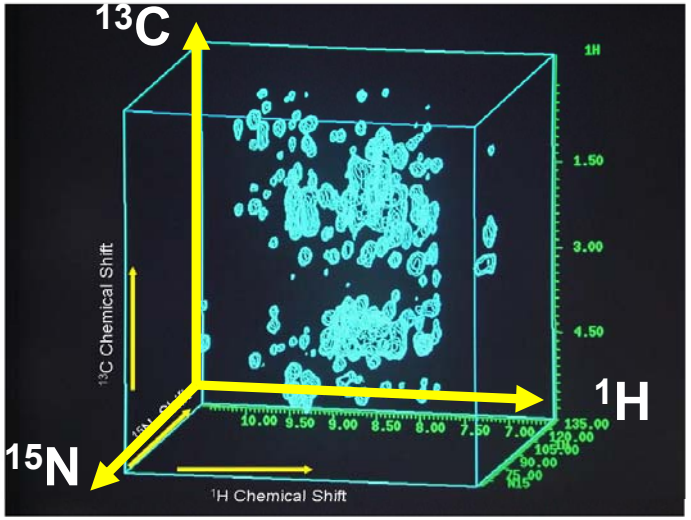
Select a particular ^{13}C frequency

Select a ^{15}N frequency

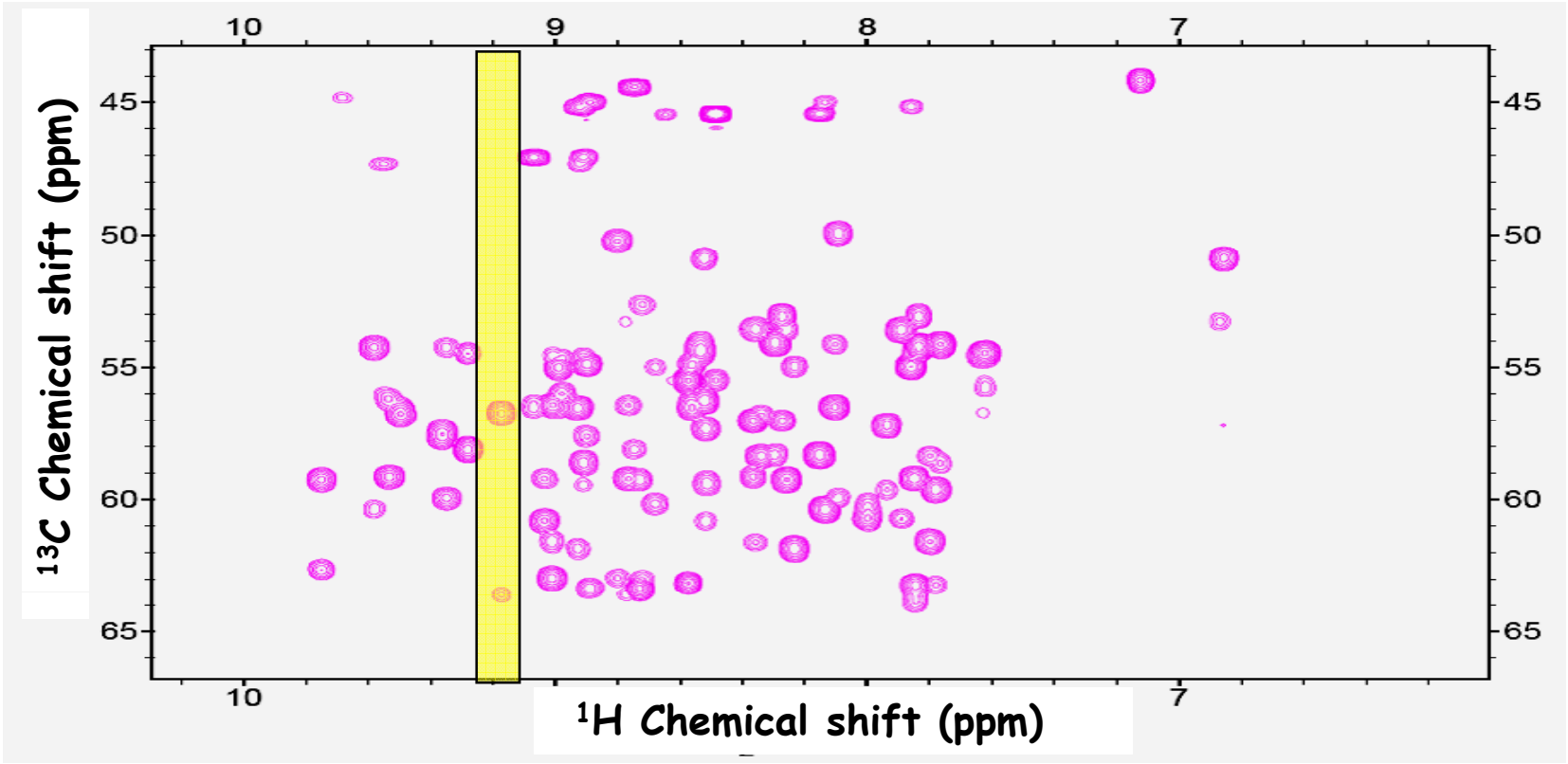


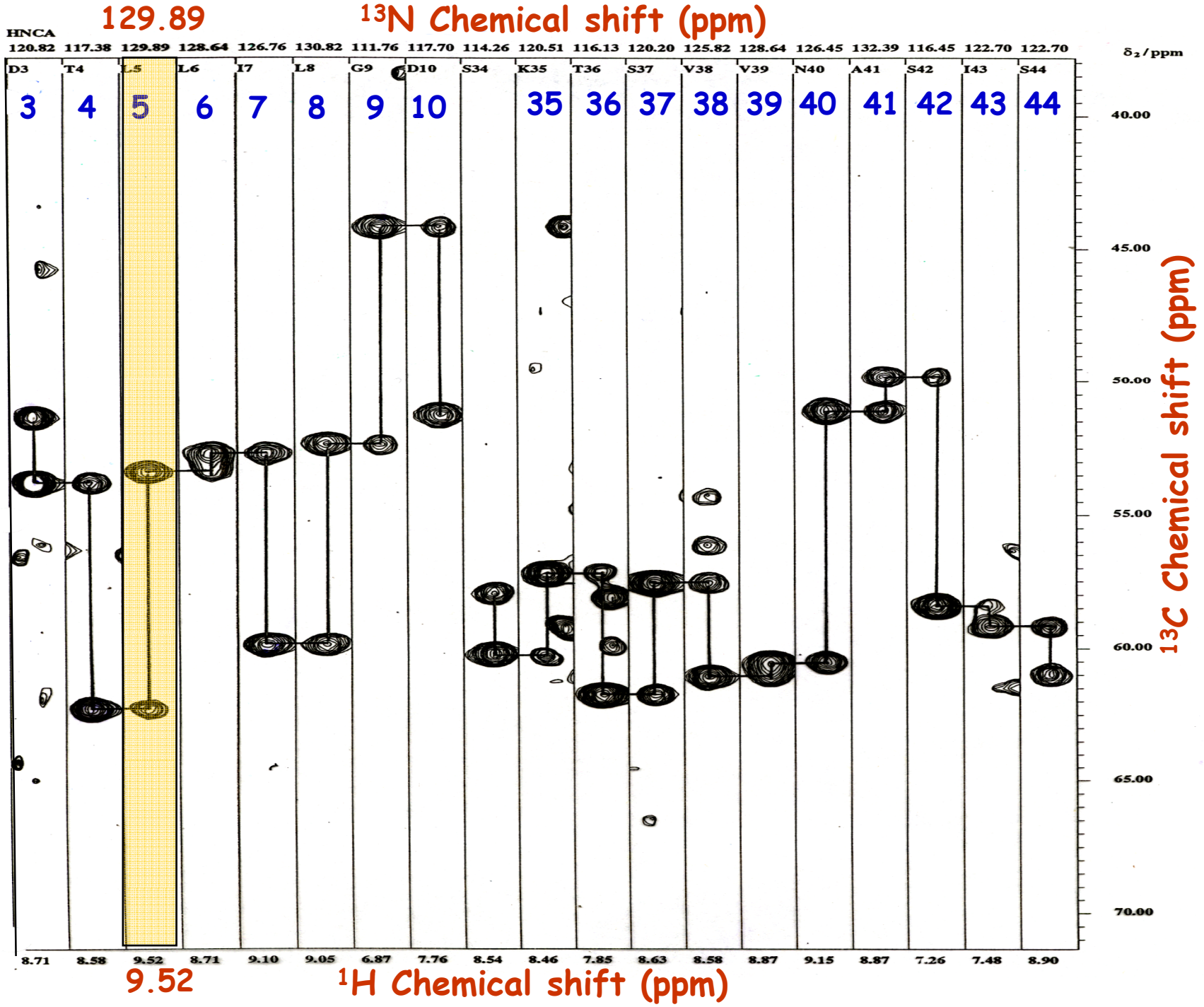


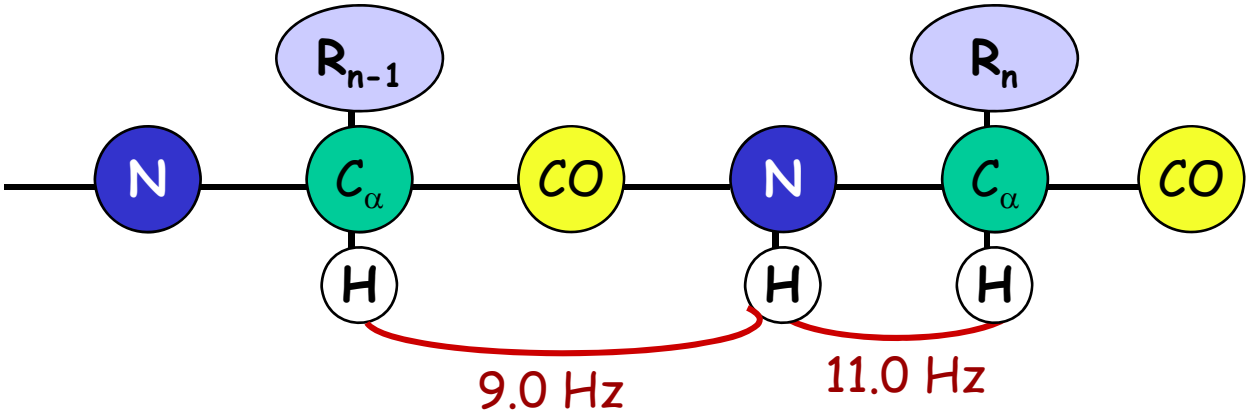

129.89
^{13}N Chemical shift (ppm)
HNCA
120.82 117.38 129.89 128.64 126.76 130.82 111.76 117.70 114.26 120.51 116.13 120.20 125.82 128.64 126.45 132.39 116.45 122.70 122.70
D3 T4 L5 L6 I7 L8 G9 D10 S34 K35 T36 S37 V38 V39 N40 A41 S42 I43 S44
3 4 5 6 7 8 9 10 35 36 37 38 39 40 41 42 43 44
δ_2/ppm
40.00
45.00
50.00
55.00
60.00
65.00
70.00
^{13}C Chemical shift (ppm)
8.71 8.58 9.52 8.71 9.10 9.05 6.87 7.76 8.54 8.46 7.85 8.63 8.58 8.87 9.15 8.87 7.26 7.48 8.90
9.52
^{1}H Chemical shift (ppm)



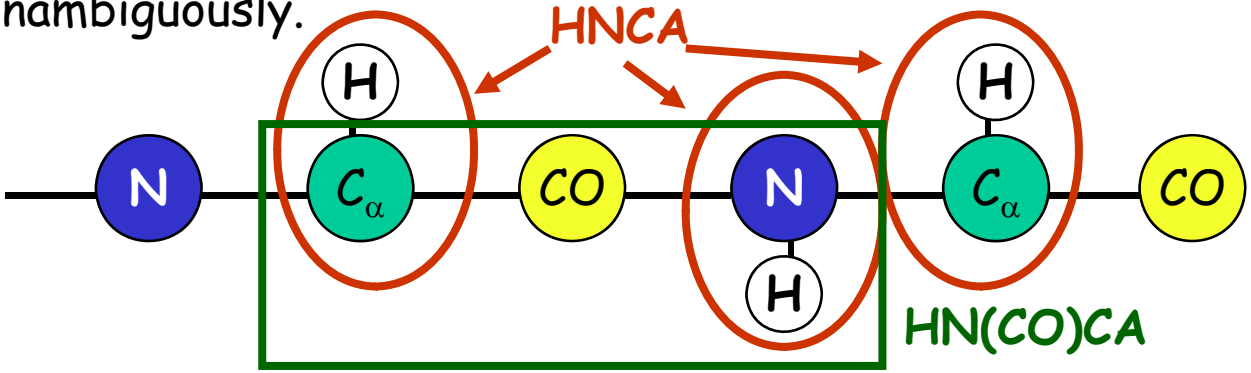
Select a ^{15}N frequency




129.89
^{13}N Chemical shift (ppm)
HNCA
120.82 117.38 129.89 128.64 126.76 130.82 111.76 117.70 114.26 120.51 116.13 120.20 125.82 128.64 126.45 132.39 116.45 122.70 122.70
D3 T4 L5 L6 I7 L8 G9 D10 S34 K35 T36 S37 V38 V39 N40 A41 S42 I43 S44
3 4 5 6 7 8 9 10 35 36 37 38 39 40 41 42 43 44
δ_2/ppm
40.00
45.00
50.00
55.00
60.00
65.00
70.00
^{13}C Chemical shift (ppm)
8.71 8.58 9.52 8.71 9.10 9.05 6.87 7.76 8.54 8.46 7.85 8.63 8.58 8.87 9.15 8.87 7.26 7.48 8.90
9.52
^{1}H Chemical shift (ppm)



1. In HNCA experiment the stronger cross peak belongs to its own CA and the weaker one belongs to precedent amino acid.
2. Combine HNCA with HN(CO)CA one can assign the CA resonances unambiguously.



3. Use several sets of thru-bond 3D experiment one can assign all Backbone resonances.
4. Side chain resonances: HCCH-TOCSY, TOCSY-HSQC or NOESY-HSQC.

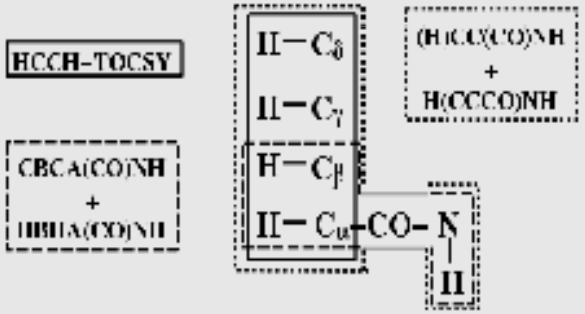
Assignment based on J-correlations

a)

b)

c)

d) side chain assignments

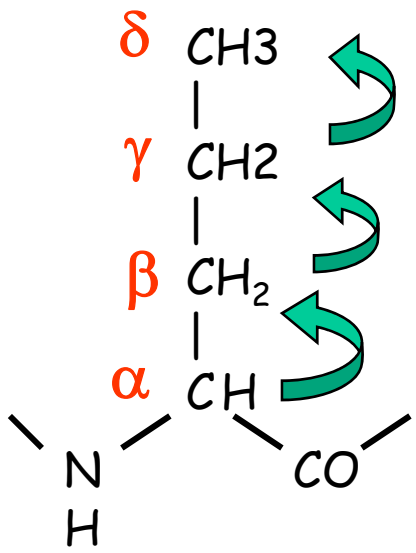


Side-Chain assignments

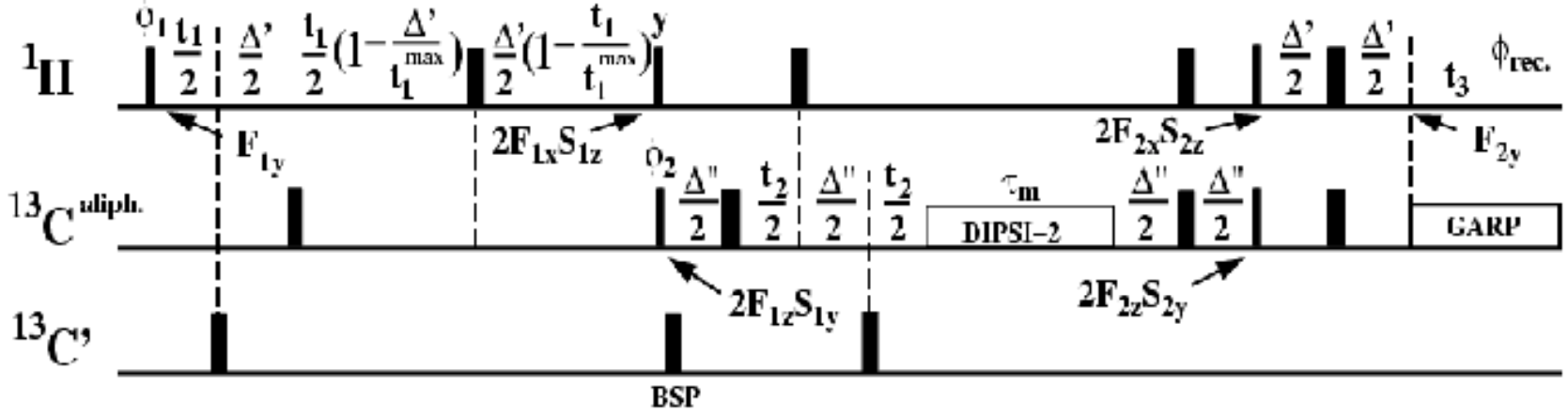
3D HC(C)H-TOCSY (^1H-^{13}C-^1H)

➔ Use ^{13}C to resolve the proton overlaps.

➔ Use ^1H-^1H coupling as in 2D TOCSY to detect side chain connectivity.



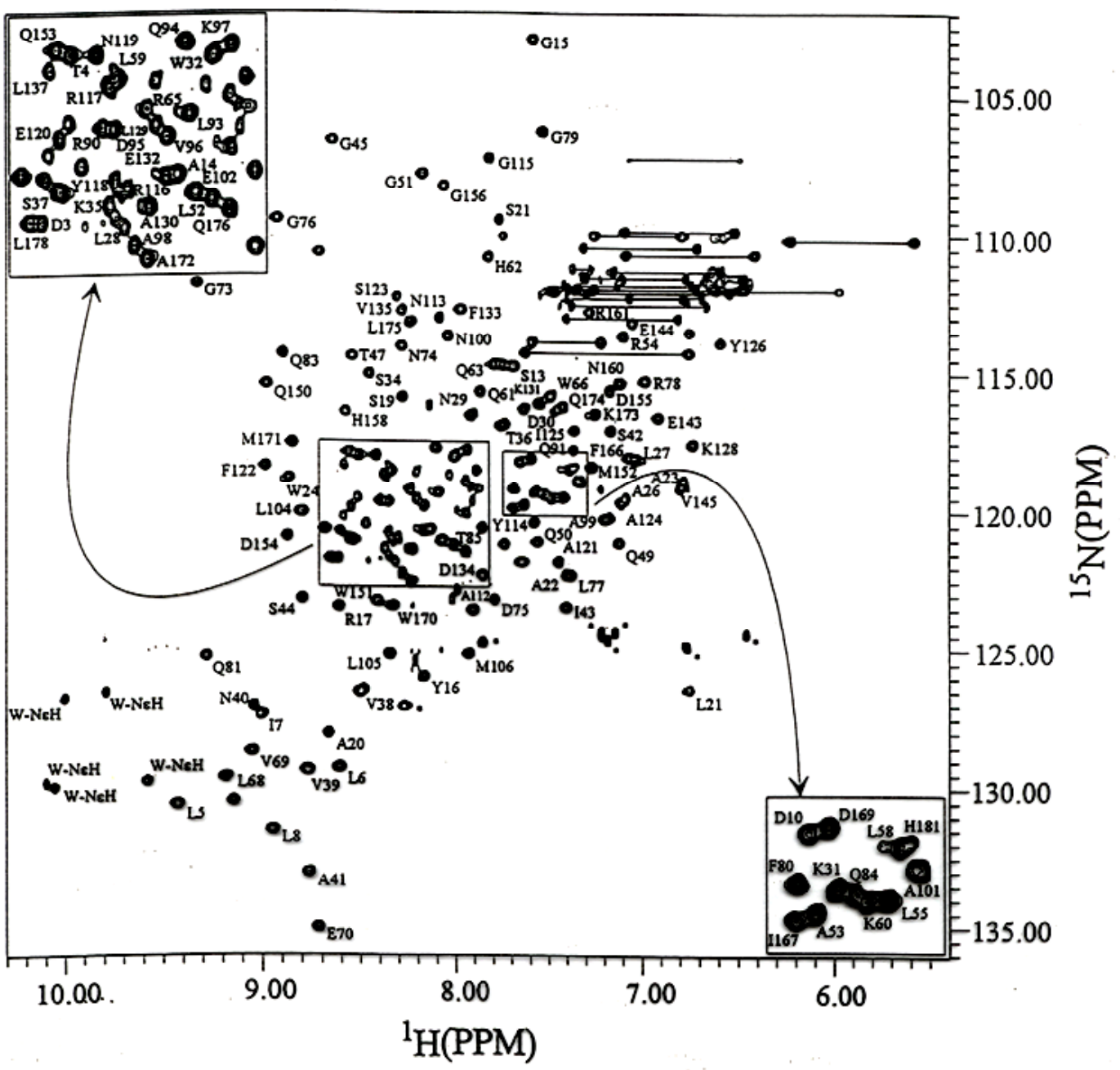
a) 3D HC(C)H–TOCSY



ϕ_1= x, –x + TPPI(t_1); ϕ_2 = 2(x), 2(–x)+ TPPI(t_2); ϕ_{rec}= x, 2(–x), x, –x, 2(x), –x.

Δ'=3.6 ms, Δ''=2.4 ms.

^{15}N-Heteronculear Single Quantum Spectroscopy ($^{15\text{-}N}$ HSQC)



Assignment Table

Table 1. ^{1}H chemical shifts for RC-RNase in 90%/10% H_2O/D_2O at 310 K, pH 3.5, taking TSP resonance (0.00 ppm) as a reference.

Residues	Chemical Shift (ppm)				
	NH	CαH	CβH	CγH	Others
PE1		3.89			
Asn2	8.04	4.65	3.31, 3.21		$N\delta H_2$ 7.82
Trp3	9.36	5.54	3.17, 3.04		$N\varepsilon H$ 10.41; 2H 7.14; 4H 8.42; 5H 6.87; 6H 7.37; 7H 7.46
Ala4	8.72	3.80	1.40		
Thr5	8.55	4.00	3.90	1.31	
Phe6	8.83	4.34	3.62, 3.20		$C_{2,6}H$ 7.20; $C_{3,5}H$ 7.01; C_4H 6.83
Gln7	8.39	3.55	1.30, 1.67	1.93, 1.85	
Gln8	7.41	3.91	2.20, 2.03	2.43, 2.43	
Lys9	8.22	3.96	1.44		
His10	7.90	4.65	2.75, 2.19		$C\varepsilon_1H$ 8.52; $C\delta_2H$ 6.59
Ile11	8.50	5.02	2.03, 1.72	1.13	0.95, 0.95
Ile12	8.72	4.81	1.83	1.35, 1.35	0.86
Asn13	8.19	4.90	2.88, 2.88		
Thr14	7.14	4.84	4.17	1.11	
Pro15		4.35	2.23, 2.23	1.96, 1.96	$C\delta H_2$ 3.84, 3.67
Ile16	7.70	4.01	1.76	1.32, 1.03	0.71, 0.62
Ile17	7.72	4.13	1.42		0.71, 0.49
Asn18	8.26	4.88	3.07, 2.62		$N\delta H_2$ 7.54, 7.04
Cys19	9.51	4.06	2.81, 2.43		
Asn20	8.19	4.48	2.98, 2.98		
Thr21	7.39	4.30	4.18	1.23	
Ile22	7.96	3.99	1.53		0.64
Met23	7.19	4.56	1.39, 0.51	2.17, 1.70	
Asp24	7.16	4.94	3.12, 2.63		
Asn25	8.03	4.69	2.85, 2.66		
Asn26	8.94	4.36	2.87, 2.82		$N\delta H_2$ 7.45, 6.81
Ile27	8.15	4.04	1.53		0.55, 0.28
Tyr28	7.80	4.76	3.51, 3.13		$C_{2,6}H$ 6.96, $C_{3,5}H$ 6.54
Ile29	7.35	4.51	2.14		0.69
Val30	8.53	4.40	2.04	1.01, 1.01	
Gly31	9.36	3.93, 3.93			
Gly32	8.34	4.20, 3.56			
Gln33	7.64	4.85	2.09, 1.97	2.35, 2.35	
Cys34	8.43	3.83	2.40, 1.08		
Lys35	8.38	4.25			
Arg36	9.02	3.87	2.08, 1.90	1.79, 1.65	$C\delta H_2$ 3.38, 3.23; $N\varepsilon H$ 7.37
Val37	7.71	5.41	2.01	0.96, 0.91	
Asn38	8.41	4.84	2.21, 1.92		
Thr39	8.80	4.46			
Phe40	9.38	4.77	3.07, 2.71		$C_{2,6}H$ 7.08; $C_{3,5}H$ 6.93; C_4H 7.03
Ile41	9.36	4.32	1.74	0.78	0.70, 0.58
Ile42	8.68	4.89	1.96	1.27, 1.27	0.74, 0.62
Ser43	8.10	4.65	4.23, 3.26		
Ser44	8.41	4.73	4.27, 4.10		
Ala45	9.34	4.32	1.77		
Thr46	8.28	4.01	4.24	0.98	
Thr47	7.47	4.08	4.41	1.42	
Val48	7.47	3.83	2.40	1.09, 1.06	
Lys49	8.56	3.07	1.80, 1.68	1.03, 0.60	$C\delta H_2$ 1.32, 1.32; $C\varepsilon H_2$ 2.59, 2.28
Ala50	7.28	4.05	1.54		
Ile51	7.68	3.77	1.70	1.00	0.78
Cys52	7.27	4.72	2.85, 2.01		
Thr53	7.34	3.75	4.01	1.20	
Gly54	9.24	4.08, 3.54			
Val55	8.05	4.04	1.87	0.79, 0.79	

pH-dependent of proton exchange rates

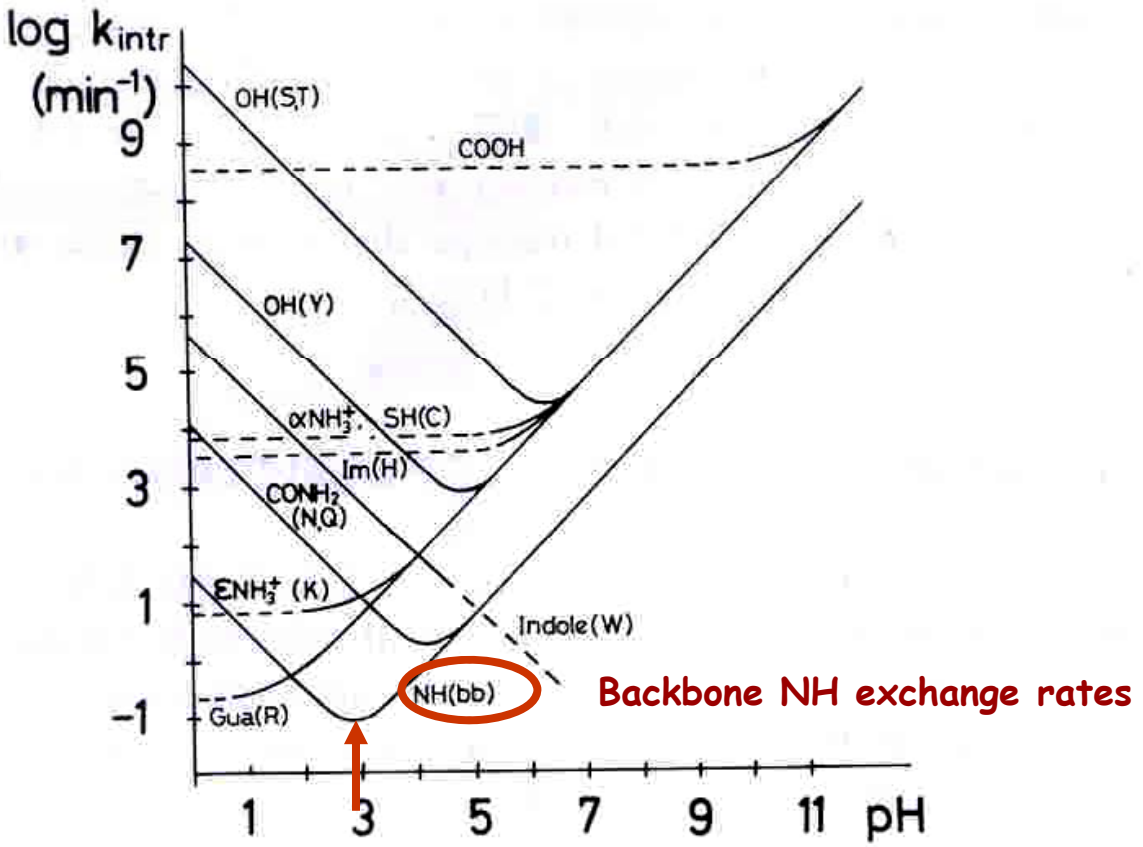


Figure 2.3. Logarithmic plots versus pH of approximate exchange rate constants k_{intr} computed with Eq. (2.2) for solvent accessible, labile protons of polypeptides in H_2O solution at 25°C. Broken lines represent lower limits for k_{intr} in situations where pK_a data were available either only for the base-catalyzed regime, or only for the acid catalysis. The individual curves are identified with the proton types and, where applicable, the residues types (Im stands for imidazole ring NH, Gua for guanidinium NH, bb for backbone) (adapted from Wüthrich and Wagner, 1979).

Nucleotide NH exchange rates

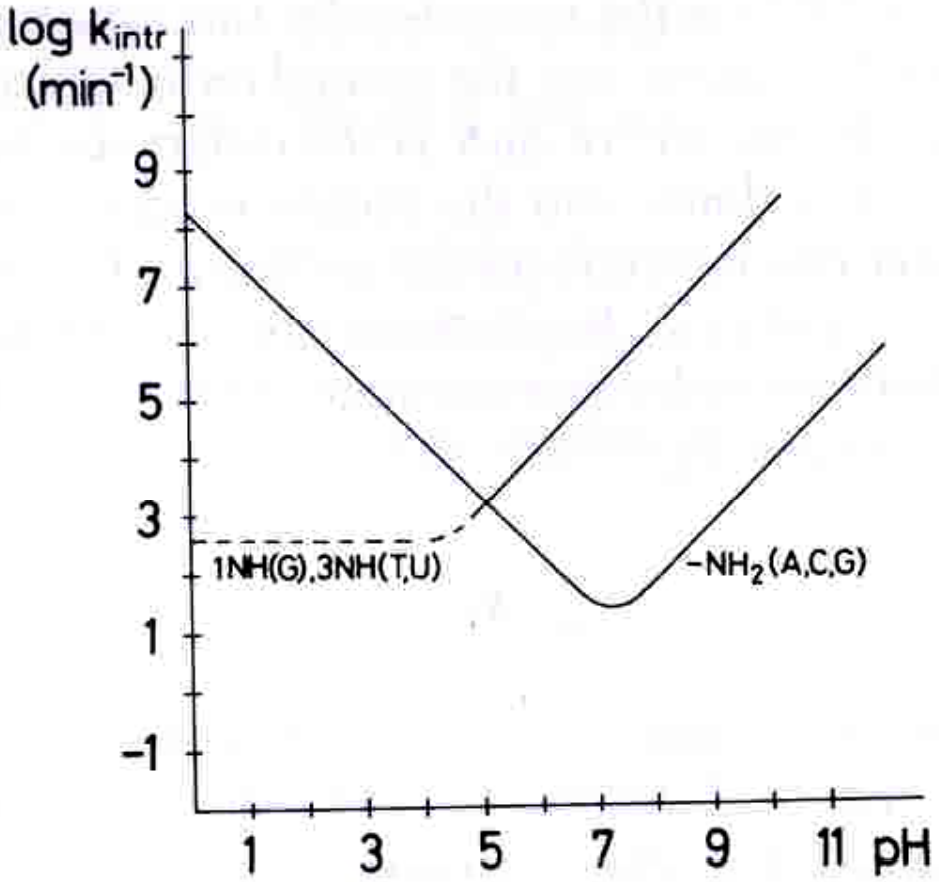


Figure 2.4. Logarithmic plots versus pH of approximate exchange rate constants k_{intr} for solvent accessible, labile base protons of polynucleotides in H_2O solution at 25°C. For all additional labile protons in polynucleotides (Fig. 2.2, Table 2.5), $k_{intr} \gtrsim 10^6$ min^{-1} over the entire pH range.