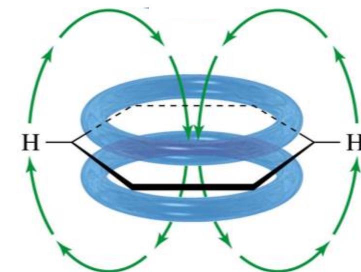
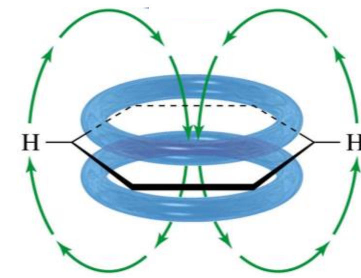


Introduction

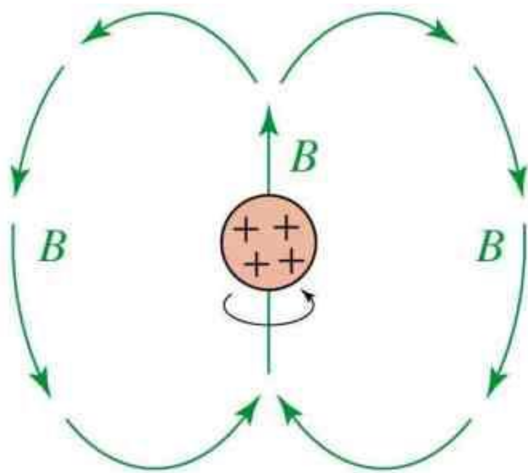
- NMR is the most powerful tool available for organic structure determination.
- It is used to study a wide variety of nuclei:
  - ^{1}H
  - ^{13}C
  - ^{15}N
  - ^{19}F
  - ^{31}P

=>

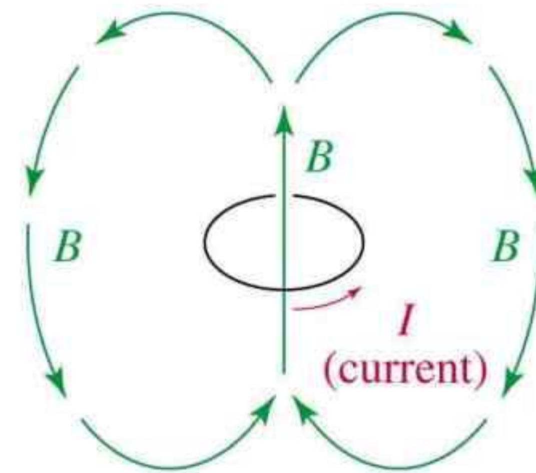
Nuclear Spin



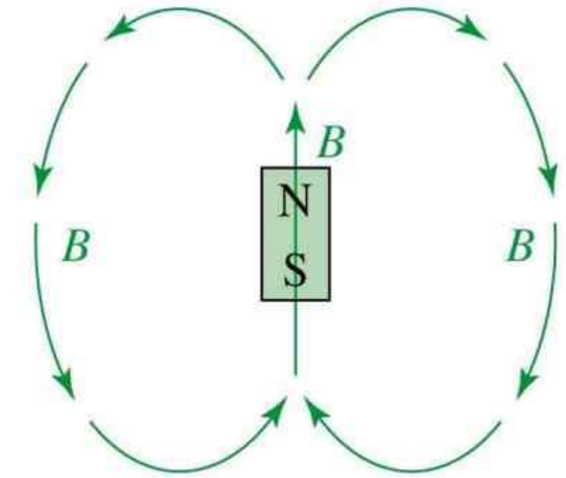
- A nucleus with an odd atomic number or an odd mass number has a nuclear spin.
- The spinning charged nucleus generates a magnetic field.



spinning proton

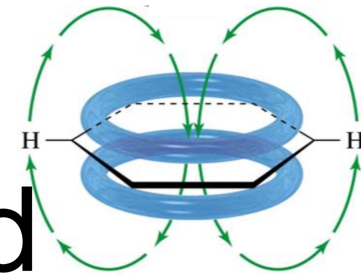


loop of current



bar magnet

=>



External Magnetic Field

When placed in an external field, spinning protons act like bar magnets.

S
twist
B_0
S N
N

S
B_0
N
S
N

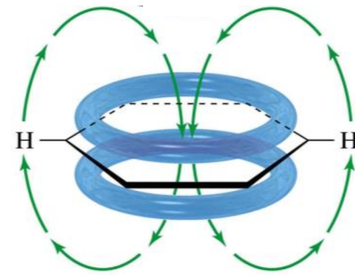
lower energy
more stable

S
B_0
S
N
N

higher energy
less stable

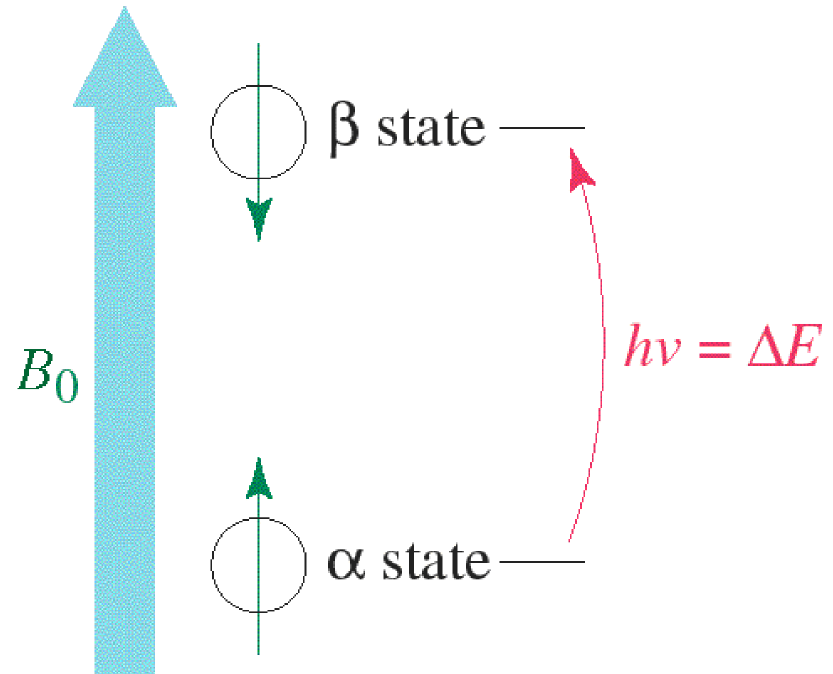
=>

Two Energy States

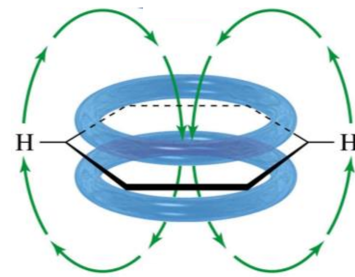


The magnetic fields of the spinning nuclei will align either *with* the external field, or *against* the field.

A photon with the right amount of energy can be absorbed and cause the spinning proton to flip. =>

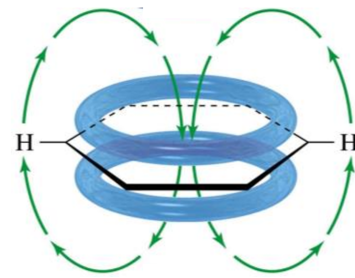


ΔE and Magnet Strength



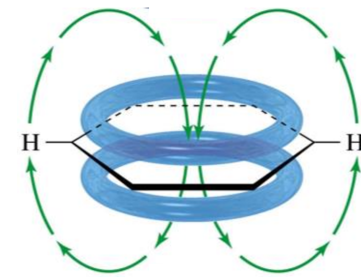
- Energy difference is proportional to the magnetic field strength.
- $\Delta E = h\nu = \gamma \dfrac{h}{2\pi} B_0$
- Gyromagnetic ratio, γ, is a constant for each nucleus (26,753 $s^{-1}gauss^{-1}$ for H).
- In a 14,092 gauss field, a 60 MHz photon is required to flip a proton.
- Low energy, radio frequency. =>

Magnetic Shielding

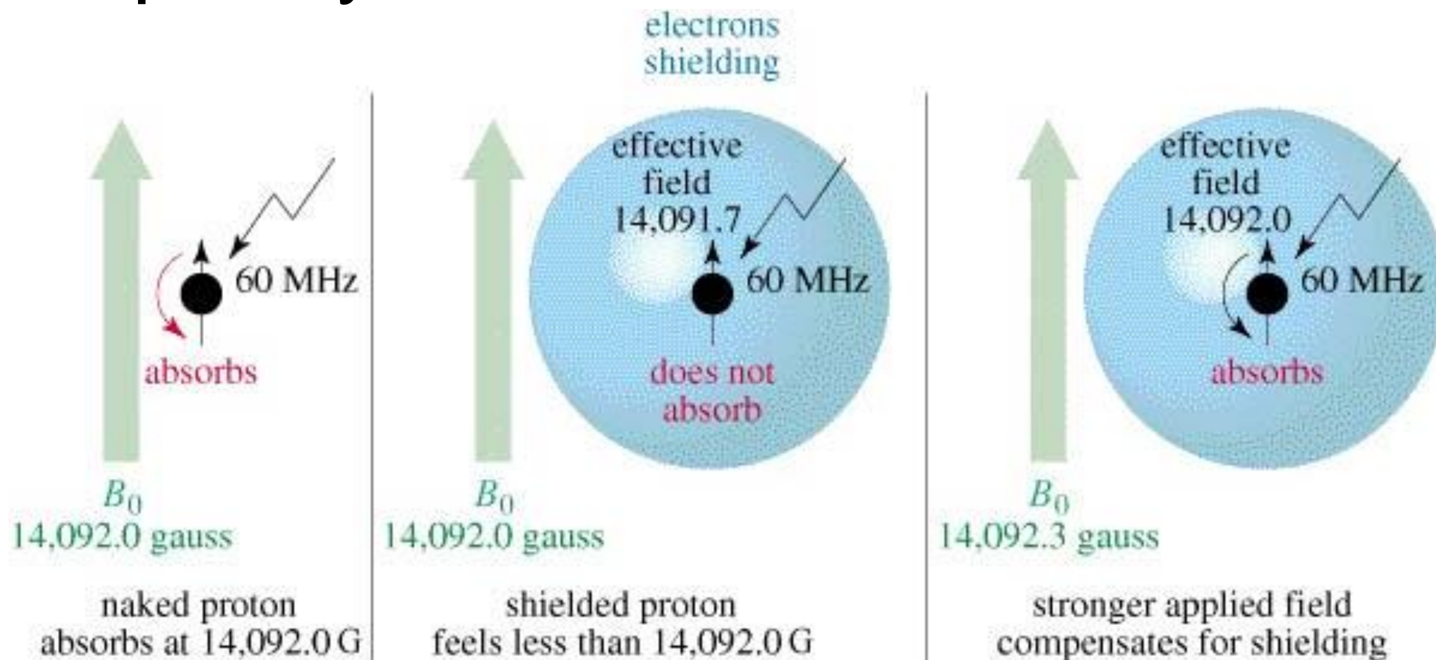


- If all protons absorbed the same amount of energy in a given magnetic field, not much information could be obtained.
- But protons are surrounded by electrons that shield them from the external field.
- Circulating electrons create an induced magnetic field that opposes the external magnetic field. =>

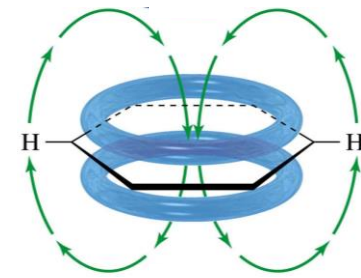
Shielded Protons



Magnetic field strength must be increased for a shielded proton to flip at the same frequency.

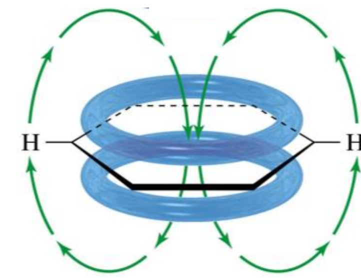


=>



NMR Signals

- The ***number*** of signals shows how many different kinds of protons are present.
- The ***location*** of the signals shows how shielded or deshielded the proton is.
- The ***intensity*** of the signal shows the number of protons of that type.
- Signal ***splitting*** shows the number of protons on adjacent atoms. =>



The NMR Spectrometer

sample tube

magnet

RF
transmitter

detector

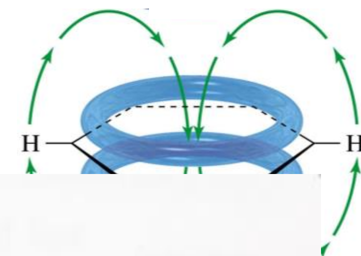
recorder

absorption

B

magnet
controller

magnetic field



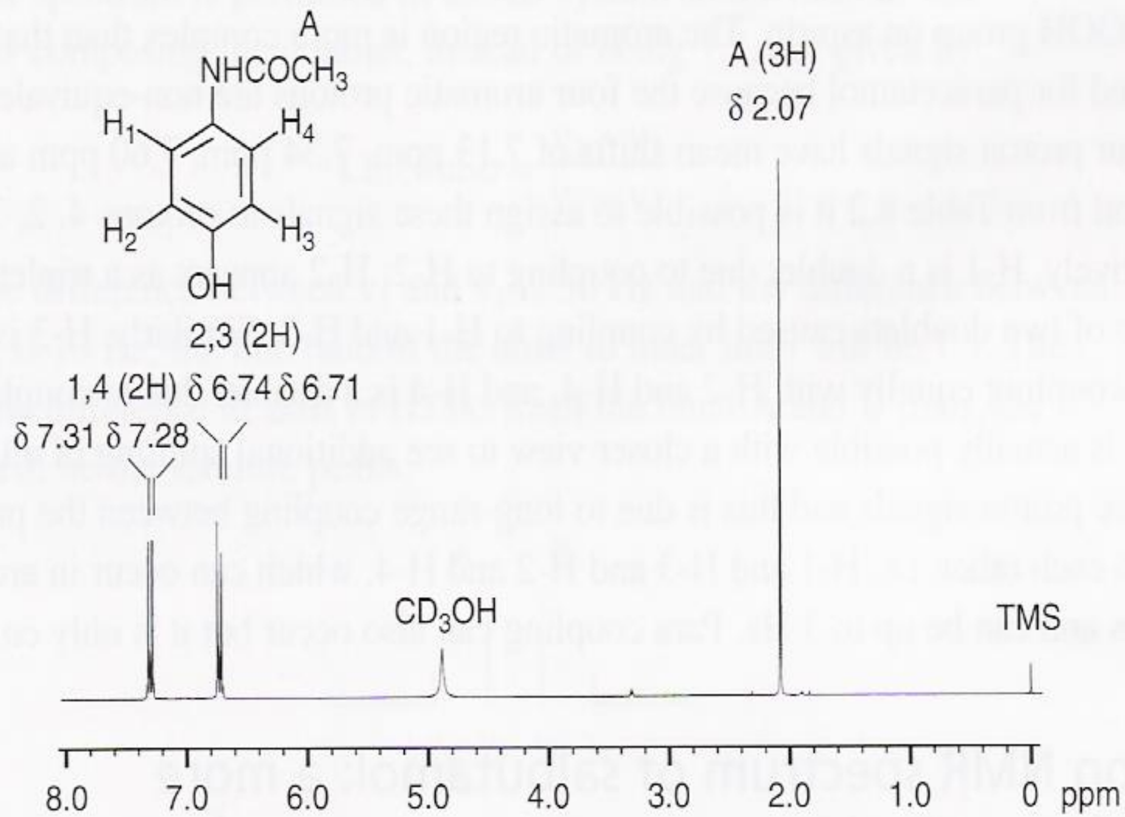


Fig. 8.11
270 mHz ^{1}H NMR spectrum of paracetamol.

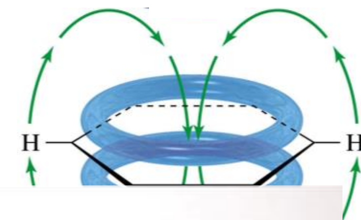


Fig. 8.12
270 mHz ^{1}H NMR spectrum of aspirin.

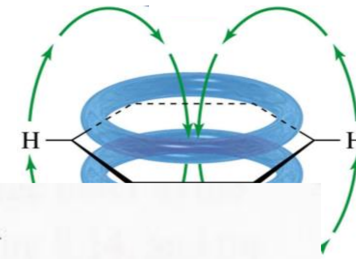
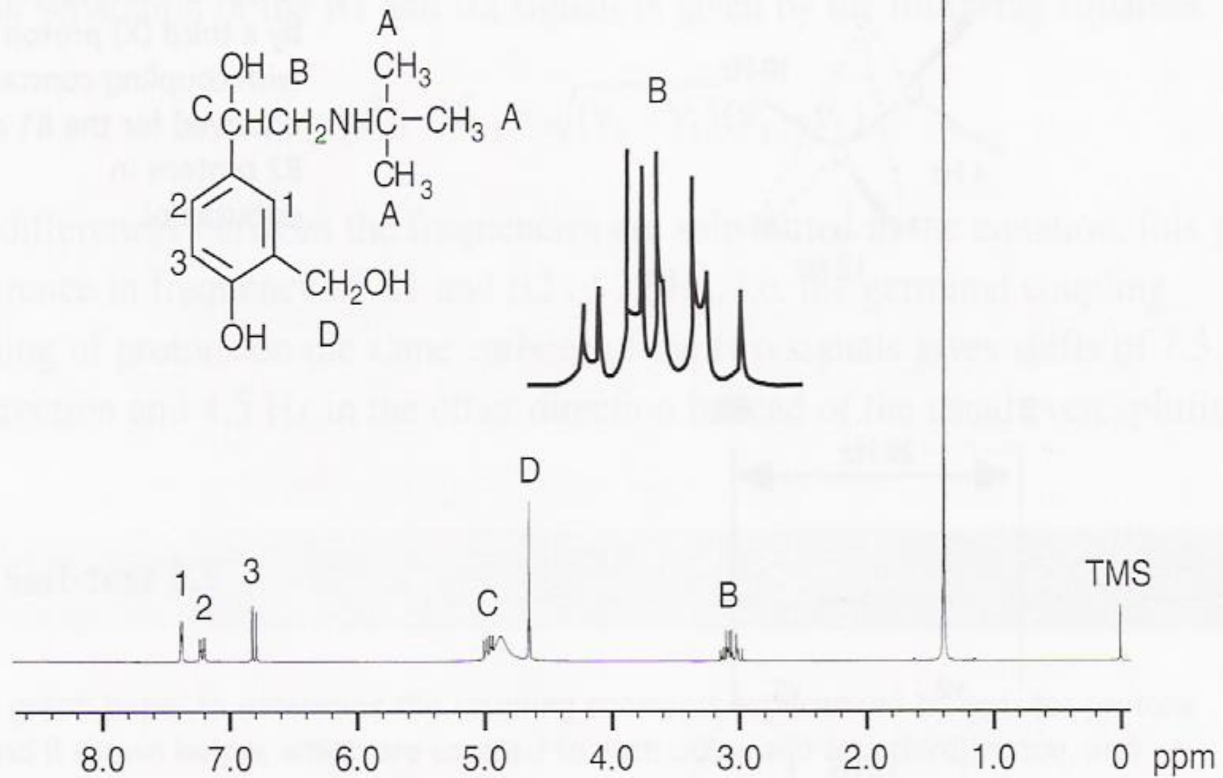
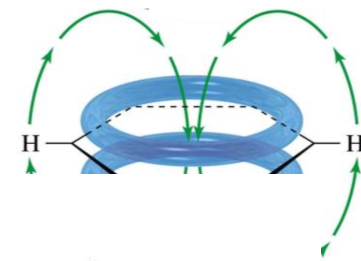


Fig. 8.13
270 mHz ¹H NMR
spectrum of salbutamol.



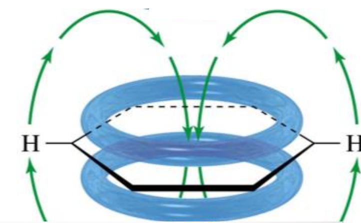


Application of NMR to quantitative analysis

NMR can be used as a rapid and specific quantitative technique. For example, a drug can be rapidly quantified by measuring suitable protons (often isolated methyl protons) against the intense singlet for the methyl groups in t-butanol. The amount of drug present can be calculated using the following formula for the methyl groups in t-butanol used as an internal standard (int. std.):

$$\text{Amount of drug} = \frac{\text{Area signal for drug protons}}{\text{Area signal for int. std. protons}} \times \text{mass of int. std. added} \times \frac{\text{MW drug}}{\text{MW int.std.}} \times \frac{\text{No. protons from int. std.}}{\text{No. protons from drug}}$$

An advantage of this method of quantitation is that a pure external standard for the drug is not required since the response is purely proportional to the number of protons present and this can be measured against a pure internal standard. Thus the purity of a substance can be determined without a pure standard for it being available. Figure 8.19 shows the spectrum of an extract from tablet powder containing aspirin, paracetamol and codeine with 8 mg of t-butanol added as an internal standard. In the analysis, deuterated methanol containing 8 mg/ml of t-butanol was added to the sample of tablet



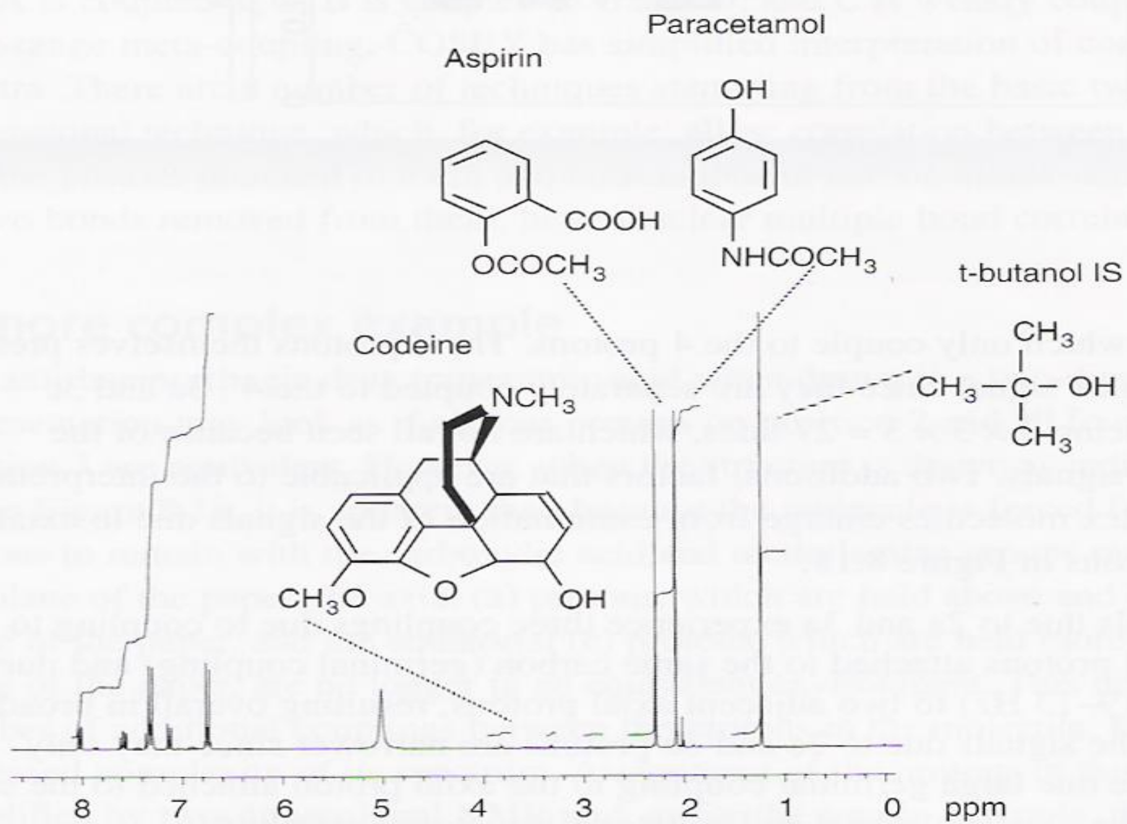
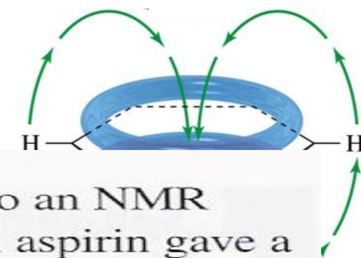


Fig. 8.19
NMR spectrum obtained from a tablet containing aspirin, paracetamol and codeine with 8 mg of t-butanol added as an internal standard.

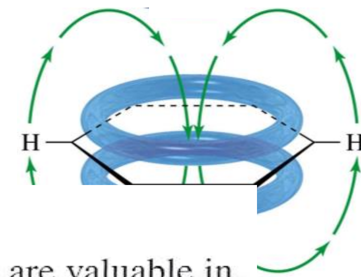


powder, and the sample was shaken for 5 min, filtered and transferred to an NMR tube.The t-butanol protons gave a signal at ∂ 1.31; the CH_3CO group in aspirin gave a signal at ∂ 2.35; the CH_3CON group in paracetamol gave a signal at ∂ 2.09; and the CH_3O group in codeine gave a signal at ∂ 3.92. The low amount of codeine present would be likely to make its quantitation inaccurate in the example shown, which was only scanned for a few minutes. Since its signal is close to the baseline, a longer scan would improve the signal:noise ratio, giving better quantitative accuracy.

The data obtained from the analysis is as follows:

- Stated content/tablet = aspirin 250 mg, paracetamol 250 mg, codeine phosphate 6.8 mg
- Weight of 1 tablet = 0.6425 g
- Weight of tablet powder taken for analysis = 0.1228 g
- Weight of t-butanol internal standard added = 8.0 mg
- Area of internal standard peak = 7.2
- Area of aspirin CH_3 peak = 5.65
- Area of paracetamol CH_3 peak = 6.73
- Area of codeine phosphate CH_3 peak = 0.115
- MW t-butanol = 74.1
- MW aspirin = 180.2
- MW paracetamol = 151.2
- MW codeine phosphate = 397.4
- Number of protons in t-butyl group = 9
- Number of protons in methyl groups of aspirin, paracetamol and codeine = 3.

Calculation of the paracetamol in the tablets is shown in Example 8.2.



Other specialised applications of NMR

There are a number of other specialised applications of NMR which are valuable in pharmaceutical development. Chiral NMR employs chiral shift reagents, e.g.

Calculation example 8.2

Weight of aspirin and paracetamol expected in the tablet powder $= 250 \times \dfrac{0.1228}{0.6425} = 47.97$ mg

Weight of codeine expected in the tablet powder $= 6.8 \times \dfrac{0.1228}{0.6425} = 1.300$ mg

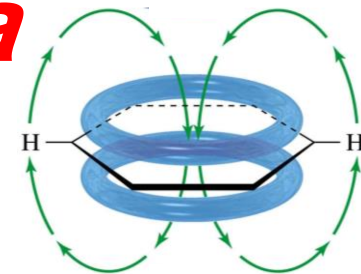
Calculation for aspirin

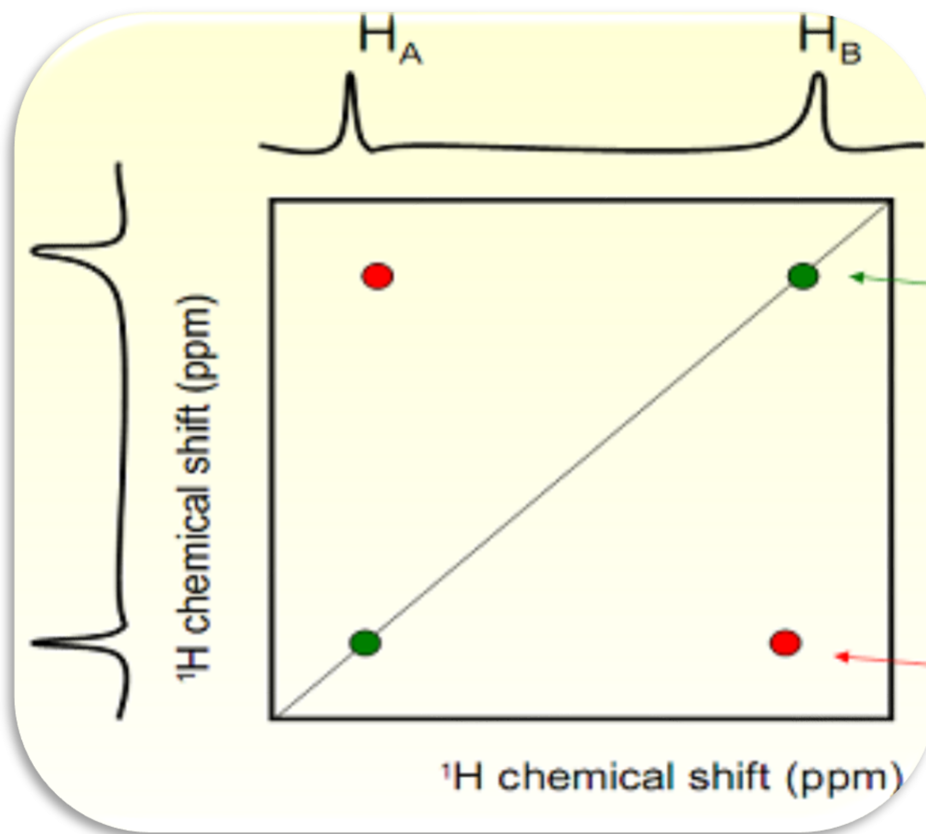
Substituting into the formula given above:

mg of aspirin present in extract $= \dfrac{5.65}{7.2} \times 8 \times \dfrac{180.2}{74.1} \times \dfrac{9}{3} = 45.80$ mg

Percentage of stated content $= \dfrac{45.8}{47.97} \times 100 = 95.48\%$

Basic features of 2D spectra

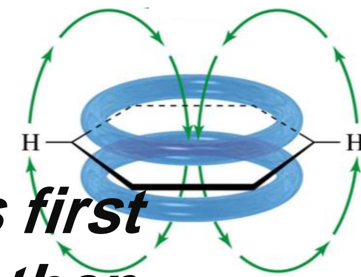




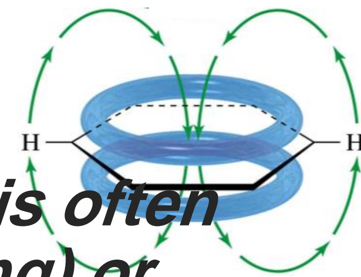
Diagonal peak($F_1=F_2$)

Crosspeak:correlation of two different resonances by short interatomic distance or through-bond connection

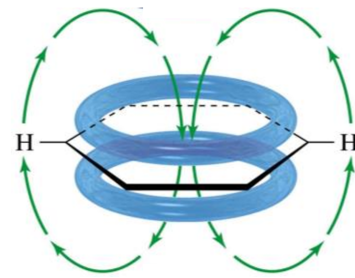
Introduction



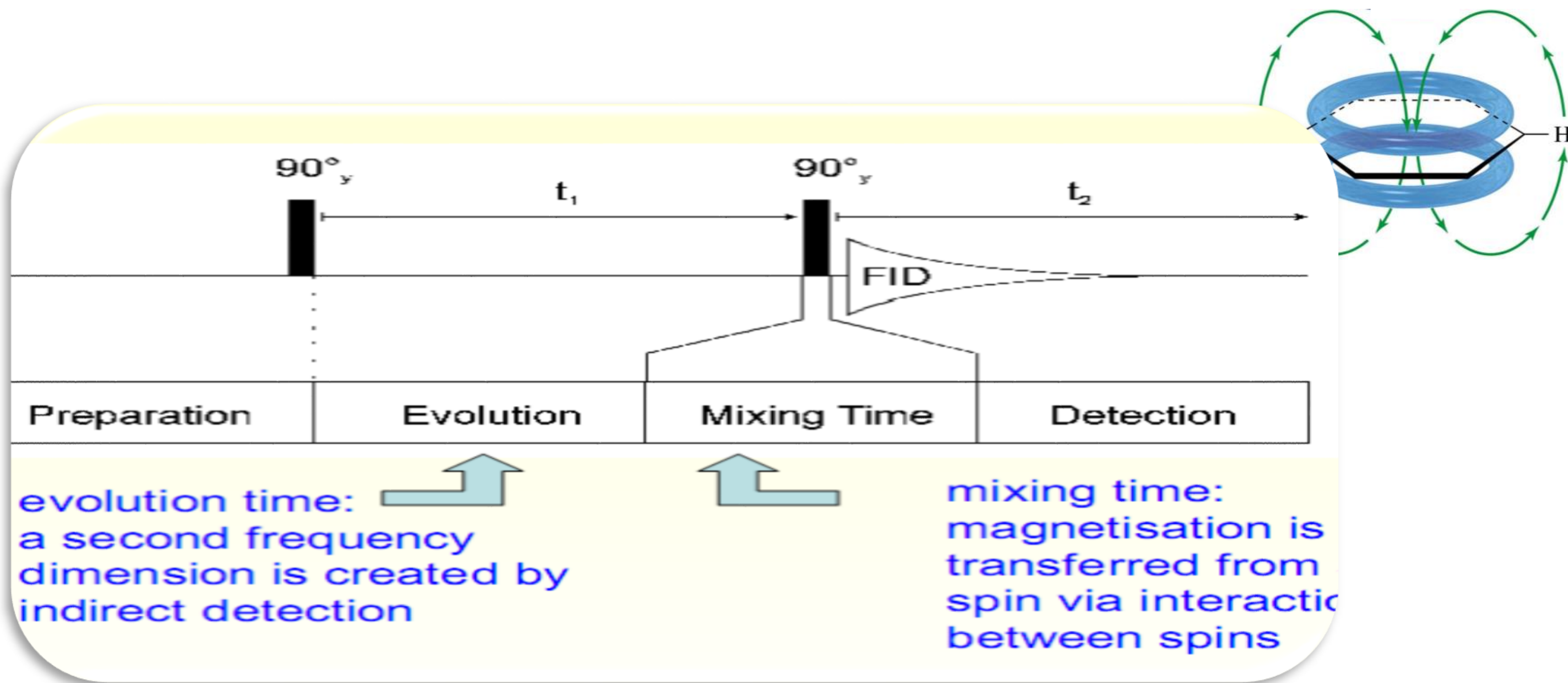
A two dimensional variation of NMR was first proposed by Jean Jeener in 1971; since then, scientists such as Richard Ernst have applied the concept to develop the many techniques of 2D NMR. Although traditional, one-dimensional NMR is sufficient to observe distinct peaks for the various funtional groups of small molecules, for larger, more complex molecules, many overlapping resonances can make interpretation of an NMR spectrum difficult. Two-dimensional NMR, however, allows one to circumvent this challenge by adding additional experimental variables and thus introducing a second dimension to the resulting spectrum, providing data that is easier to interpret and often more informative.



- ***Two-dimensional (2D) NMR spectroscopy is often used to provide through-bond (J-coupling) or through-space (NOE) correlation. These correlations, shown as "crosspeaks" in the spectrum, can be analyzed to examine detailed molecular structure. In a 2D experiment, the direct detection dimension is usually 1H while the indirect (transient) dimension could be 1H, 13C, 15N, 31P, or other nuclei. The low sensitivity of the broadband nucleus is overcome by coherence transfer with 1H and encoding their chemical shift in the indirect dimension. The advantage of 2D NMR also lies in its ability to resolve ambiguous or overlapping resonances resulting from one nucleus (i.e 1H) along the indirect dimension of another nucleus (i.e. 13C).***



- ***Anatomy of a 2D experiment:***
- ***The appearance of 1D-spectra is determined by the three blocks of preparation, mixing and detection (=acquisition).***
  - ***The pulse sequence of a 2D experiment adds a 4th block, the so called evolution time t_1.***



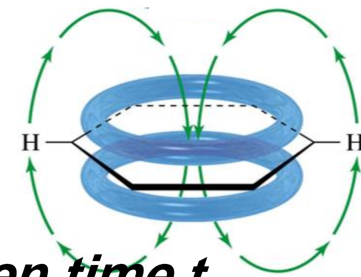
This scheme can be viewed as:

Do something with the nulcei

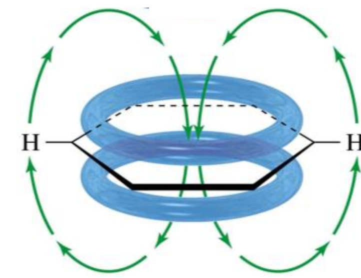
(preparation),

let them precess freely (evolution),

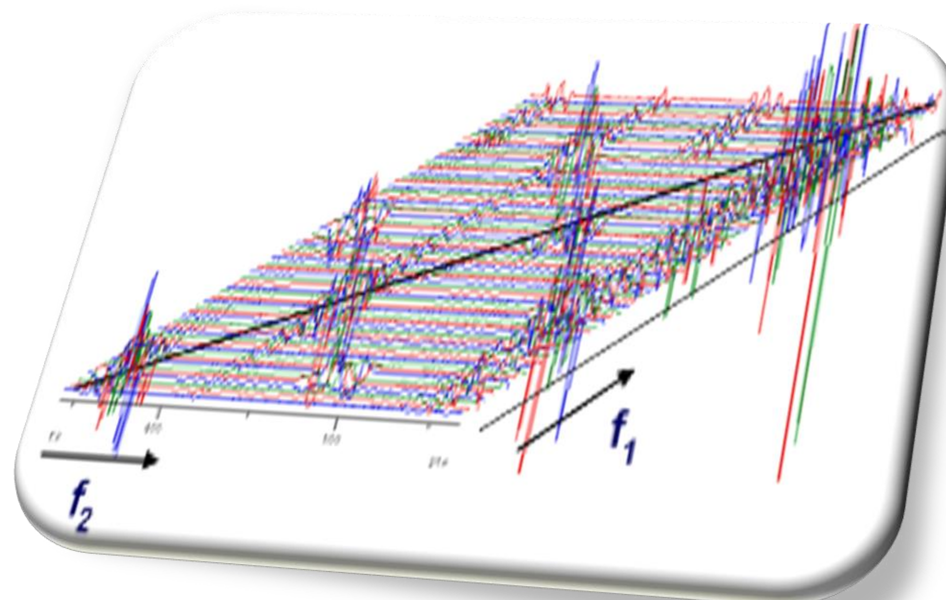
do something else (mixing),



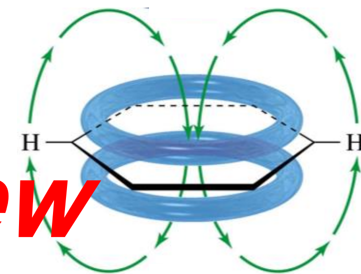
****After preparation the spins can precess freely for a given time t_1. During this time the magnetization is labelled with the chemical shift of the first nucleus. During the mixing time magnetization is then transferred from the first nucleus to a second one. Mixing sequences utilize two mechanisms for magnetization transfer: scalar coupling or dipolar interaction (NOE). Data are acquired at the end of the experiment (detection, often called direct evolution time); during this time the magnetization is labelled with the chemical shift of the second nucleus. Two dimensional FT yields the 2D spectrum with two frequency axes.***

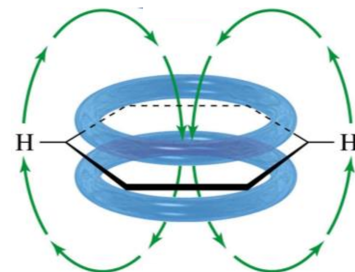


a. ***Stack of several 1D spectra***
b. ***Each 2D is different from the next by a small change in the evolution time t1***
c. ***Parameters for each successive expriment in the series are constant except the phase of the pulses***
d. ***FT of the two time domains provides a map of spin-spin correlations***

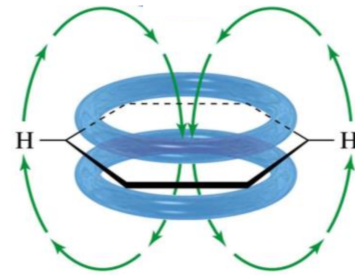


2D Spectrum –actual view



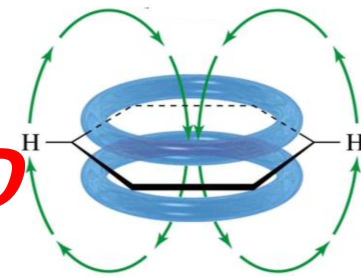


-In two dimensional experiments, both the x and the y axes have chemical shift scales and the 2D spectra are plotted as a grid like a map.
Information is obtained from the spectra by looking at the peaks in the grid and matching them to the x and y axes.



2D-NMR

a.HOMCOR(homonuclear [H-H]correlation b. HETCOR(heteronuclear correlation spectroscopy) c.INADEQUATE (Incredible Natural Abundance DoublE QUAntum Transition Experiment)

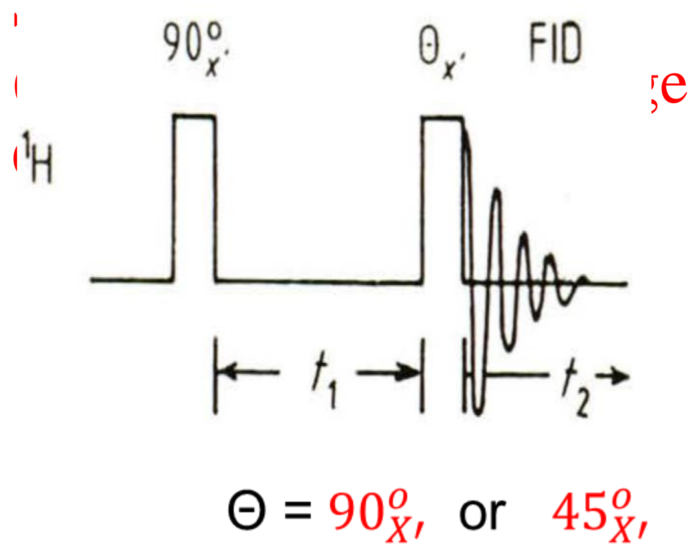


Two main type of homonuclear 2D techniques

- Scalar coupling(J)based
- a.COSY90&45 pulse sequence (Correlation spectroscopy)
- b.TOCSY(Total correlation)
- c.DQF(Double quantum filter spectroscopy)
- d.ECOSY(Exclusive correlation spectroscopy
- e.LR COSY(Long-rang cosy)
- f.RELAY-COSY
- Homonuclear 2D J-resolved.g

- NOE based
- a.NOESY(Nuclear overhauser)effect spectroscopy
- b.ROESY(Rotating frame spectroscopy)

Two-Dimensional Homonuclear (H,H)-Correlated NMR

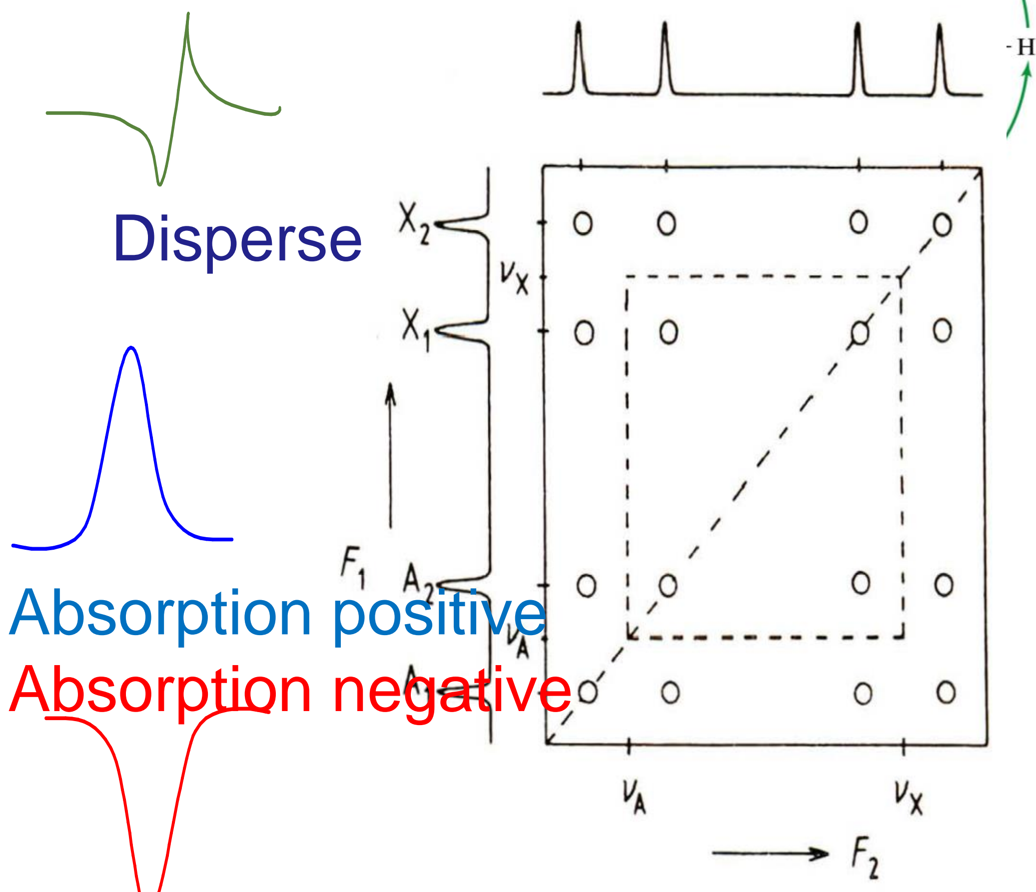


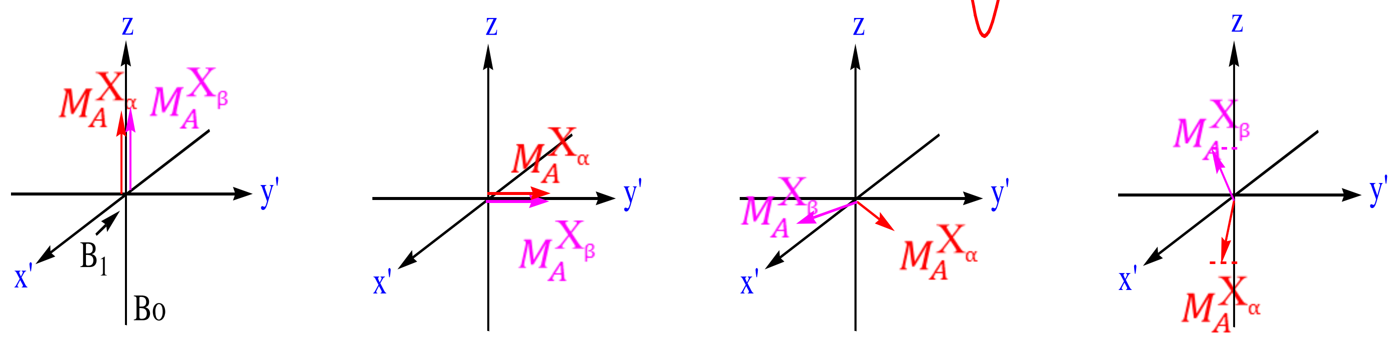
$\Theta = 90^o_{X'}$, or $45^o_{X'}$

Disperse

Absorption positive

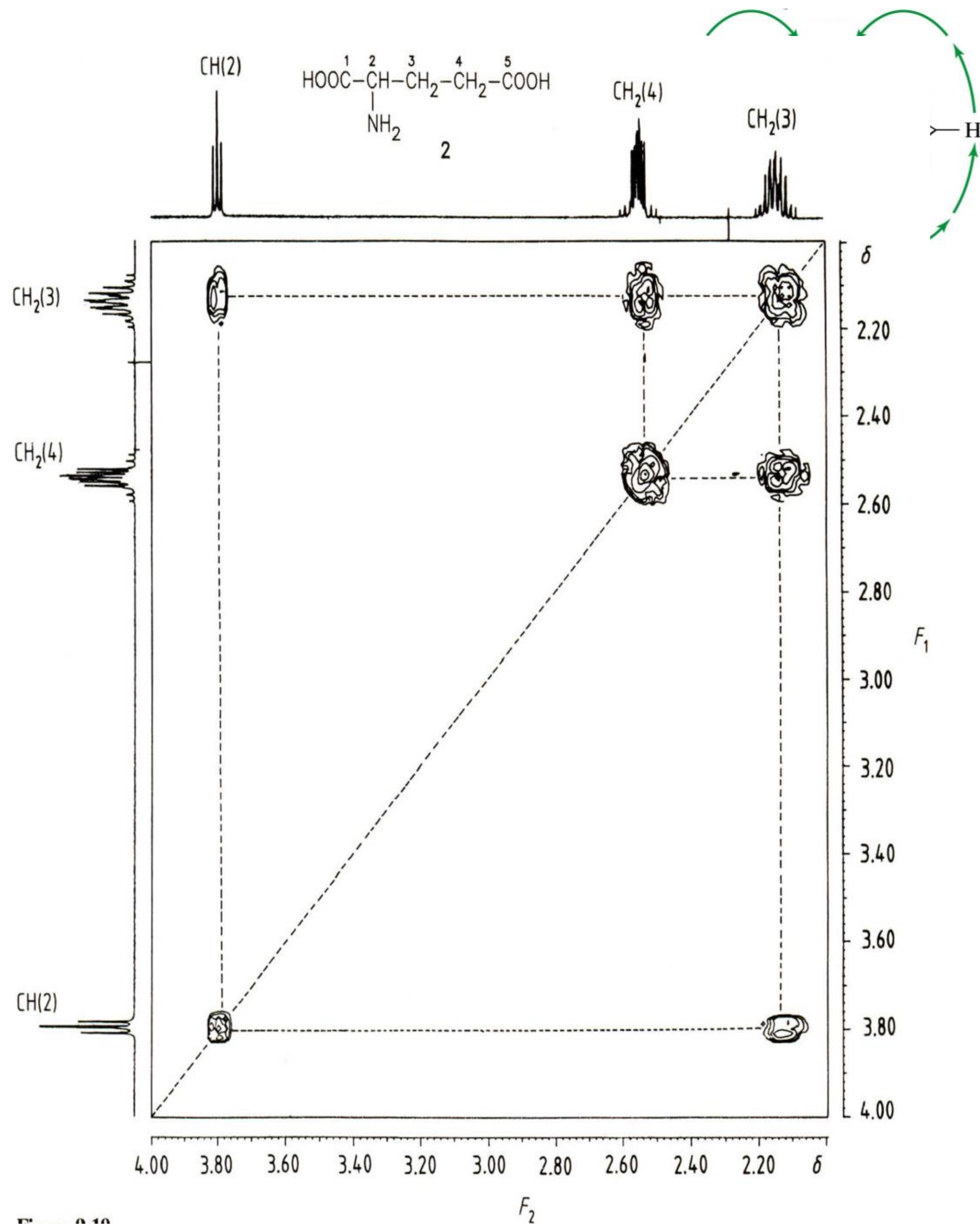
Absorption negative

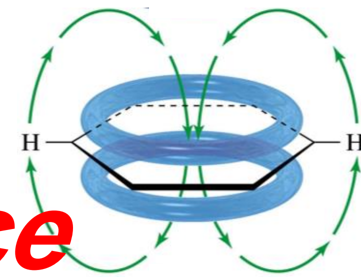




500 MHz COSY-90 spectrum of glutamic acid

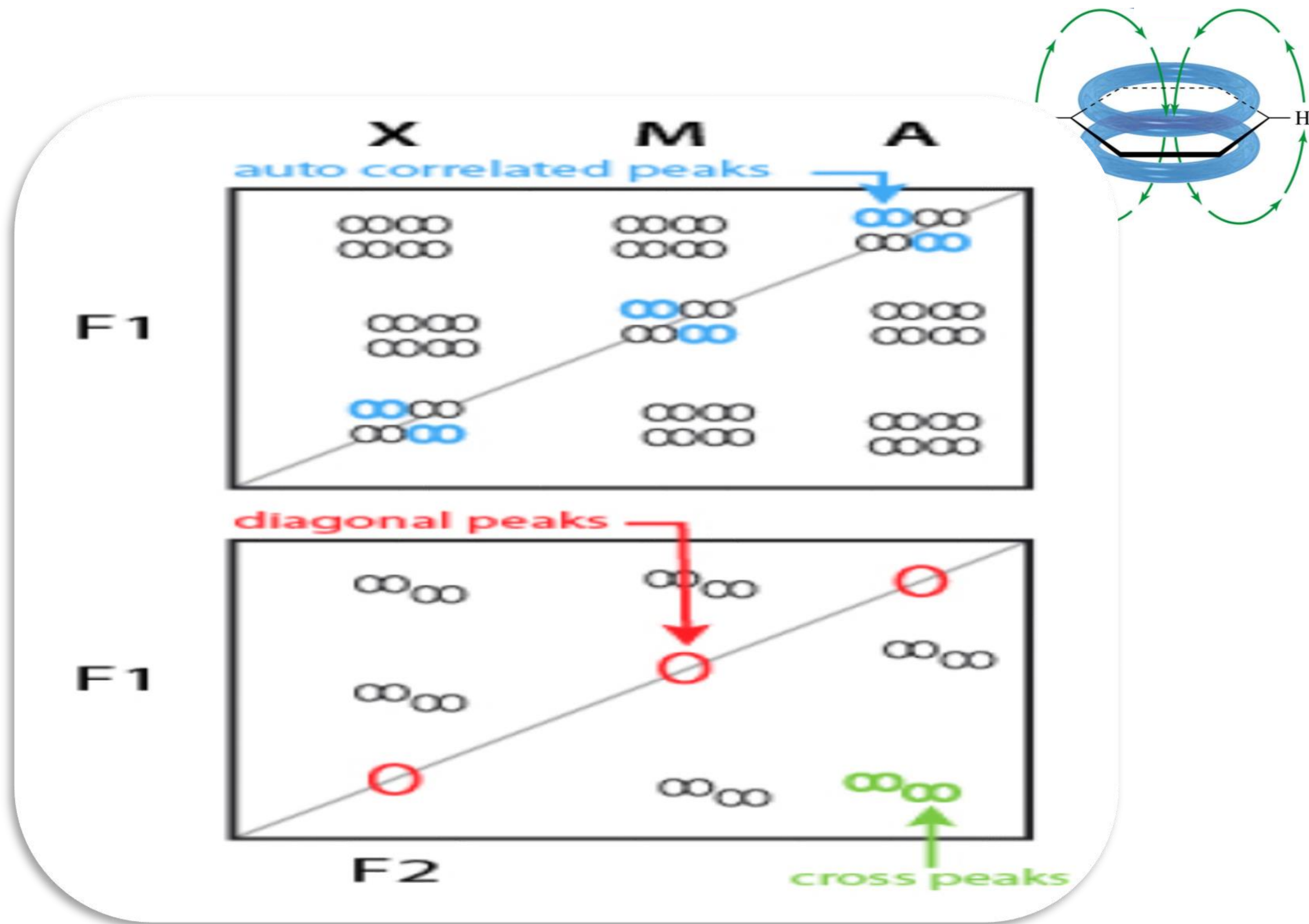
256 measurements with different values of t_1, each measurement with 16 FIDs





COSY 45 pulse sequence

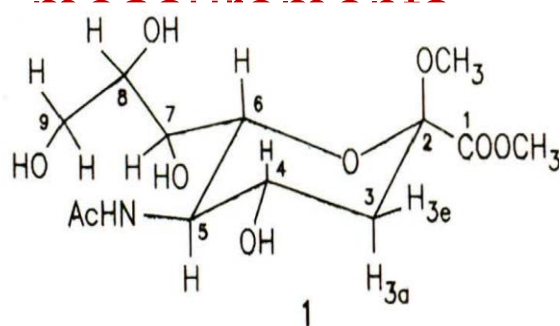
- **COSY-90 is the most common COSY experiment. In COSY-90, the p1 pulse tilts the nuclear spin by 90°. Another member of the COSY family is COSY-45. In COSY-45 a 45° pulse is used instead of a 90° pulse for the first pulse, p1. The advantage of a COSY-45 is that the diagonal-peaks are less pronounced, making it simpler to match cross-peaks near the diagonal in a large molecule. Additionally, the relative signs of the coupling constants can be elucidated from a COSY-45 spectrum. This is not possible using COSY-90.-Overall, the COSY-45 offers a cleaner spectrum while the COSY-90 is more sensitive.*
-



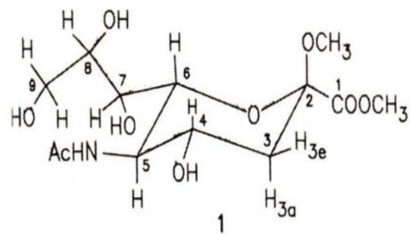
Comparison of the COSY spectrum (upper part) and COSY 45 of an AMX type system.

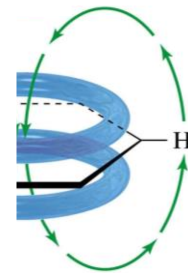
400 MHz COSY-45 spectrum of the neuraminic acid derivative **1**

512



measurement with 32 FIDs





COOCH3

H-4 H-9′ H-7

δ

H-7

H-9′

H-9

H-8

H-5

H-4

H-9

H-8

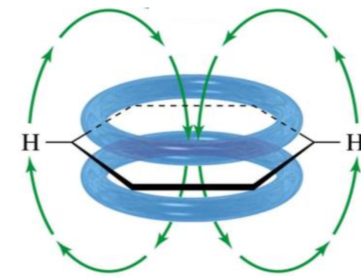
H-5

3.4
3.5
3.6
3.7
3.8
3.9
4.0
4.1
4.2

F_1

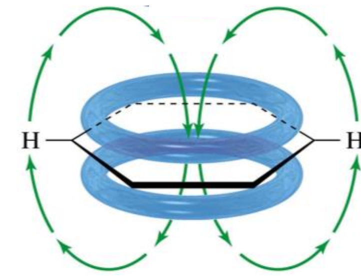
4.1 4.0 3.9 3.8 3.7 3.6 3.5 δ

F_2

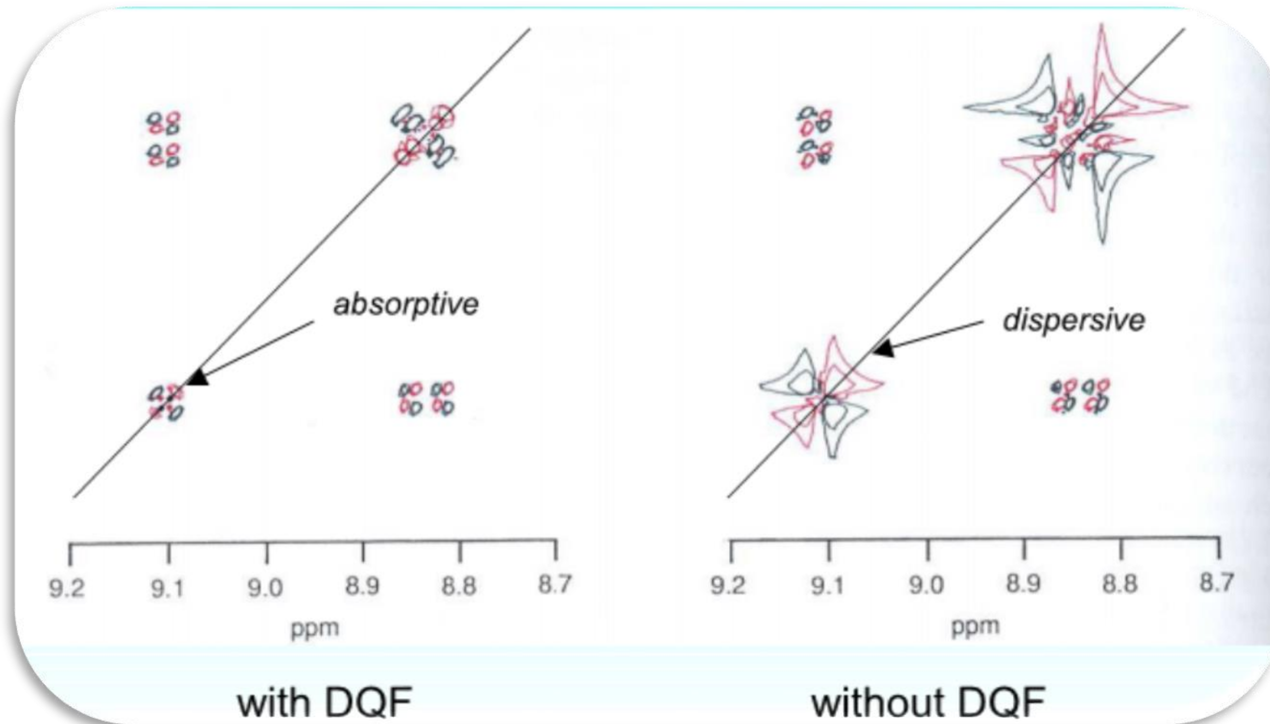


DQF COSY

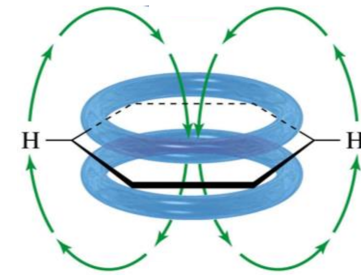
- *a.Phase-sensitive,pure absorption lineshapes with +ve & +ve components*
- *b.Higher resolution*
- *c.J values can be determined*
- *d.Less pronounced diagonal ridge, so easy to assign crosspeak close to diagonal*
- *e.Elimination of strong(solvent) signals not involved in homonuclear J-coupling*

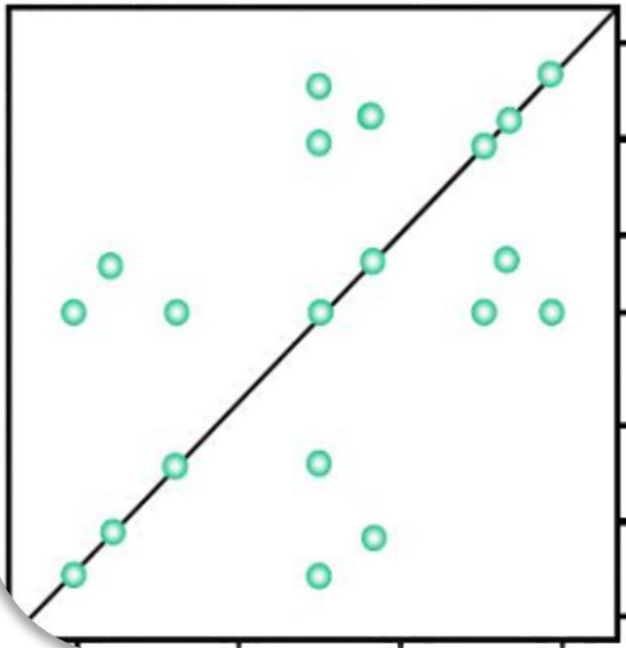


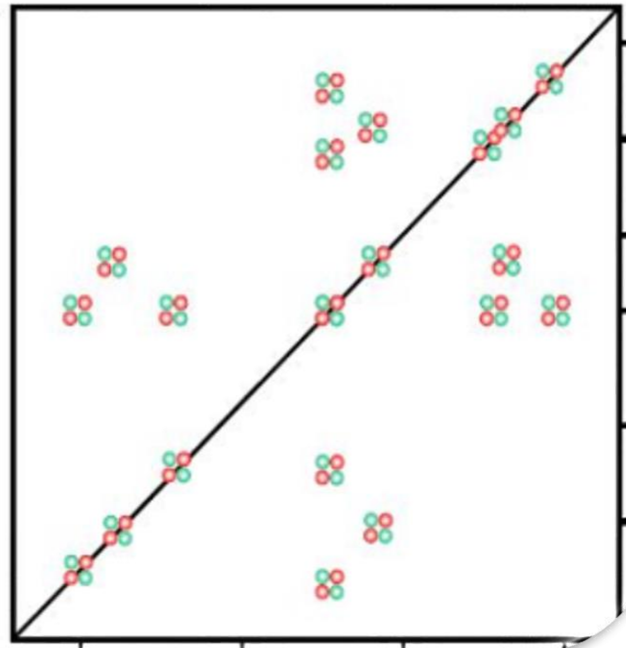
a. ***Improved lineshapes and resolution.***
b. ***Singlet(uncoupled) peaks suppressed.***

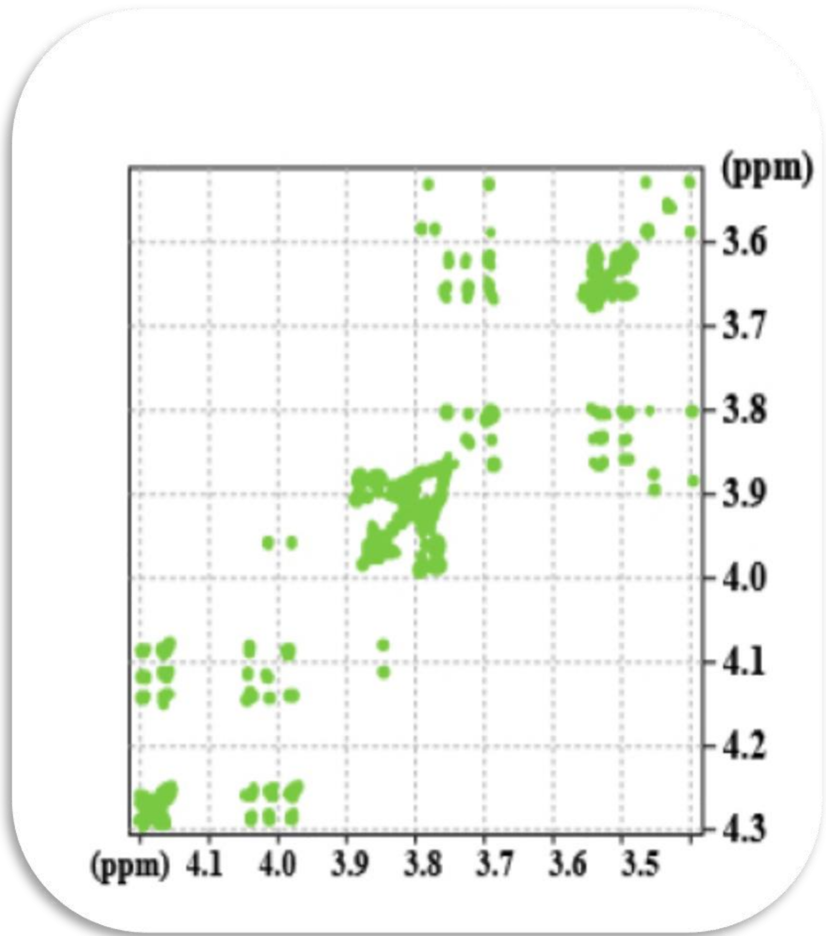


COSY Vs DQFCOSY

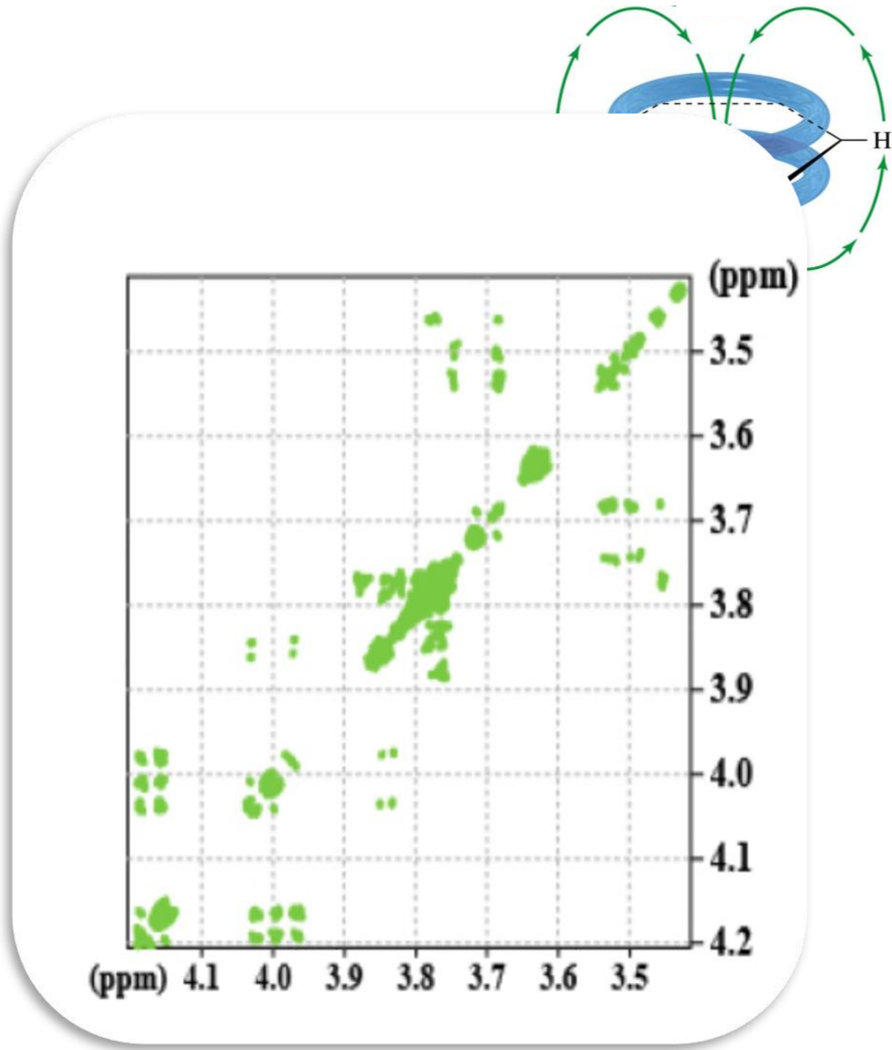




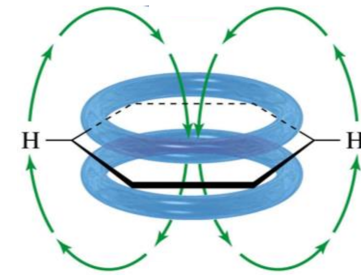




DQF COSY



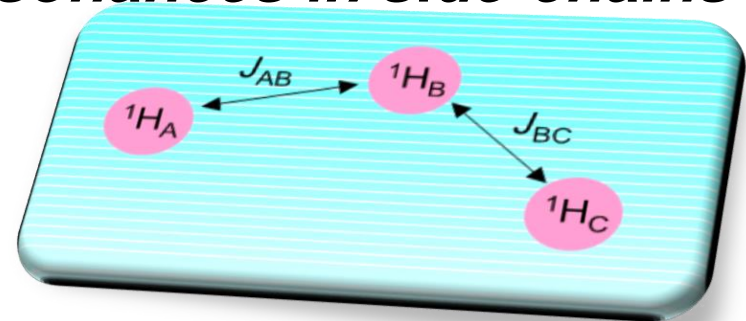
COSY

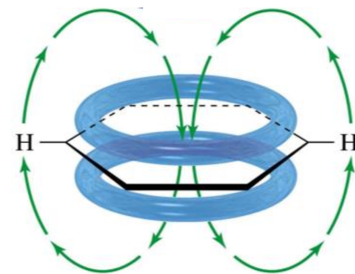


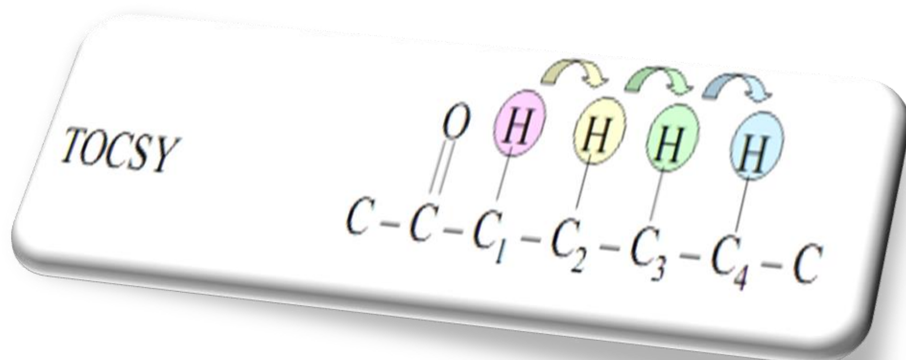
TOCSY

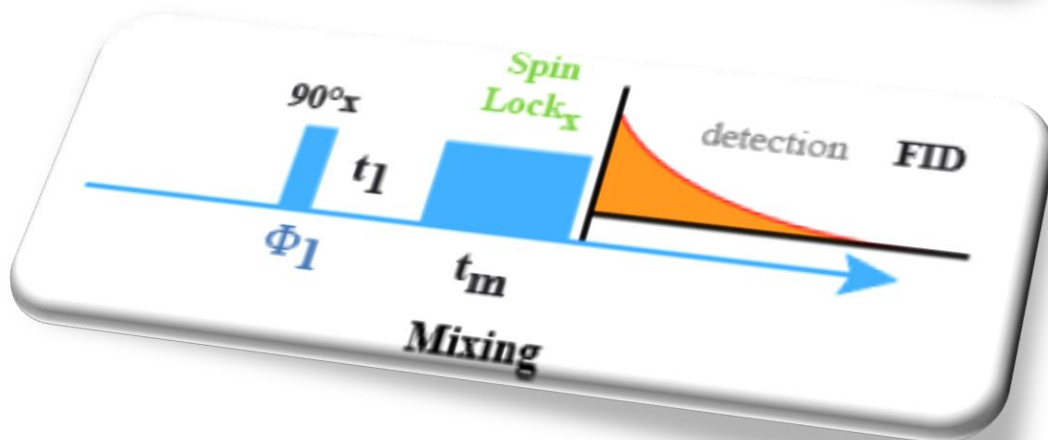
- a. Correlation between all protons within spine system (not just those directly coupled.
- b. Useful when key COSY cross-peaks are obscured.
- c. Useful for assigning resonances in side-chains of proteins.

COSY crosse peaks:A-B,B-C
TOCSY crosse peaks:A-B,B-C and A-C




H
H


TOCSY
O
H
H
H
H
C – C – C1 – C2 – C3 – C4 – C


90°x
Spin
Lockx
t1
detection
FID
Φ1
tm
Mixing

Analysis of mixture: COSY

$CH_3-CH_2-CH_2-CH_2-I$

$CH_3-CH_2-CH_2-CH_2-OH$

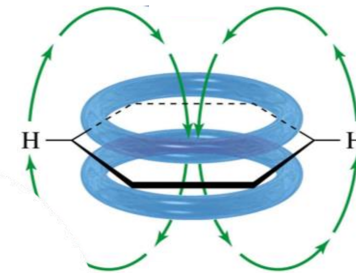
^1H - ^1H COSY Specrtum
(500 MHz, C_6D_6 solution)

2 2 3 3 4 4

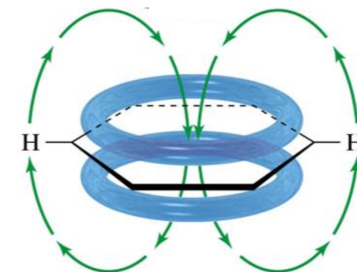
1 ? 1 ?

1 CH_2 OH 2

1.0 2.0 3.0 ppm



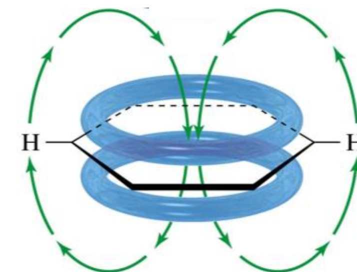
Analysis of mixture: TOCSY



SUMMARY

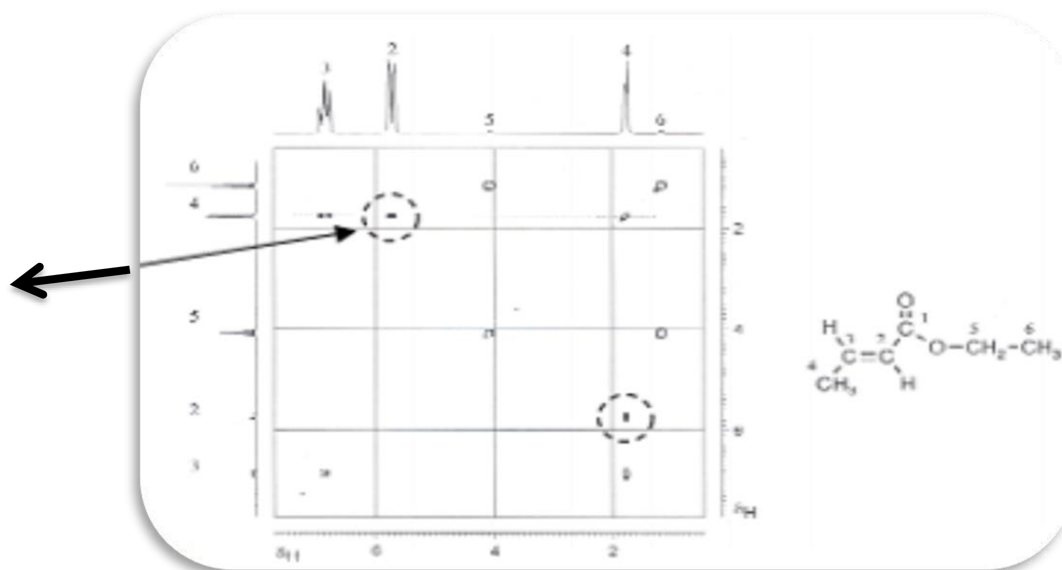
COSY	DQF COSY	TOCSY
H^1-H^1 through-bond correlations	The best standard cosy expriment	Shows correlations between all protons in same spin system
Mostly 2-bond/3-bond correlation	High resolution and narrow lineshapes	Narrow lineshapes
	Singlet peaks suppressed	

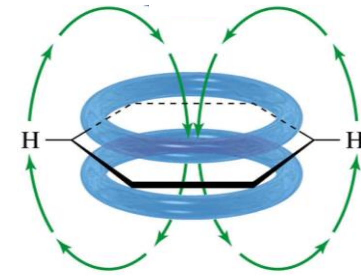
Long-range cosy



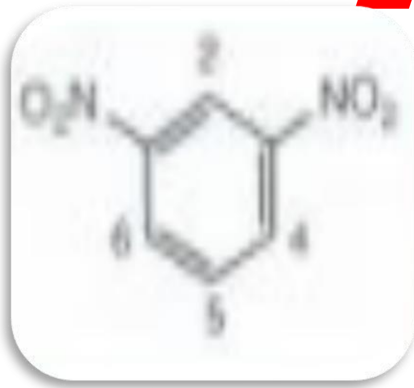
- a.Correlations via small JH-H enhanced e.g. allylic coupling, W and para coupling in aromatic rings
- b.Short-range correlations may be weakened

H_2-H_4 allylic 4-bond correlation ^4JH-H~2HZ

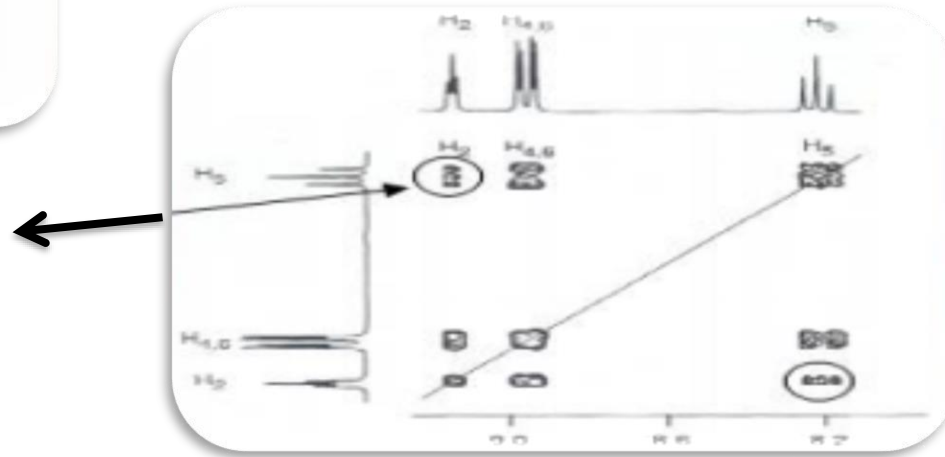


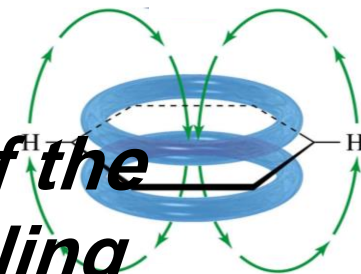


Long-range cosy

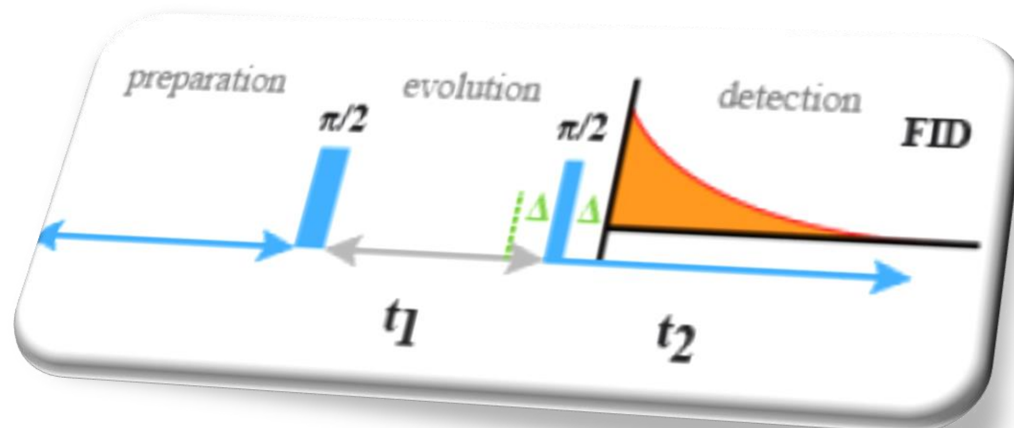


H_2-H_5 para 5-bond correlation $^5J_{H-H} < 0.5$ HZ





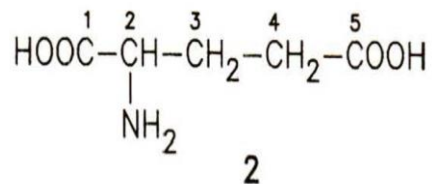
This experiment allows the detection of the correlation peaks caused by weak coupling constant (less than one Hz) and which are not detectable with .the COSY 9O° pulse sequence



Long range COSY

$90^\circ_{x'} - t_1 - \Delta - 90^\circ_{x'} - \Delta - \mathrm{FID}$

Δ = 0.1 sec to 0.4 sec



128 measurements with t_1 altered in 600 μs increments; each measurement with 8 FIDs

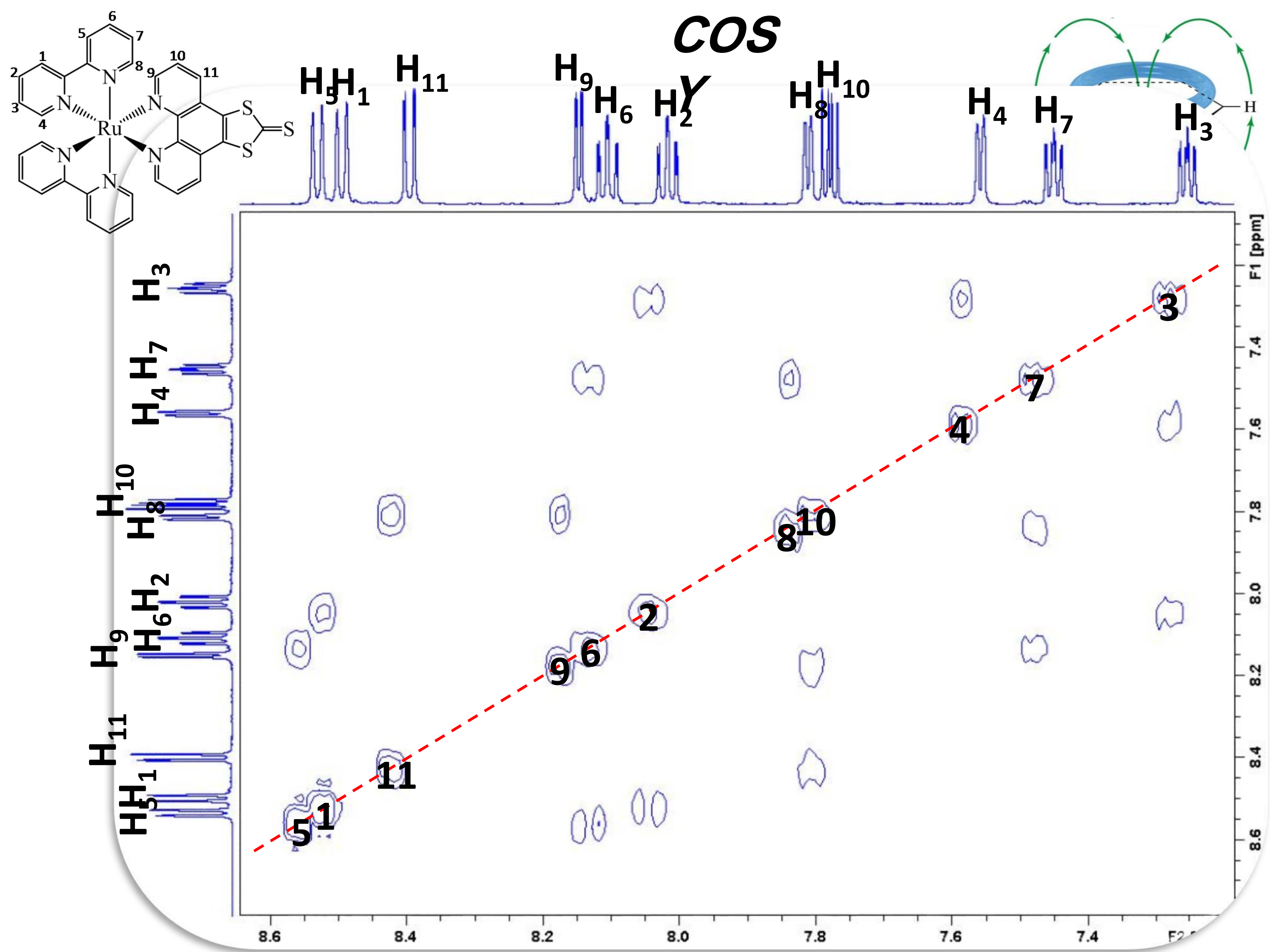
H-2 H-4 H-3

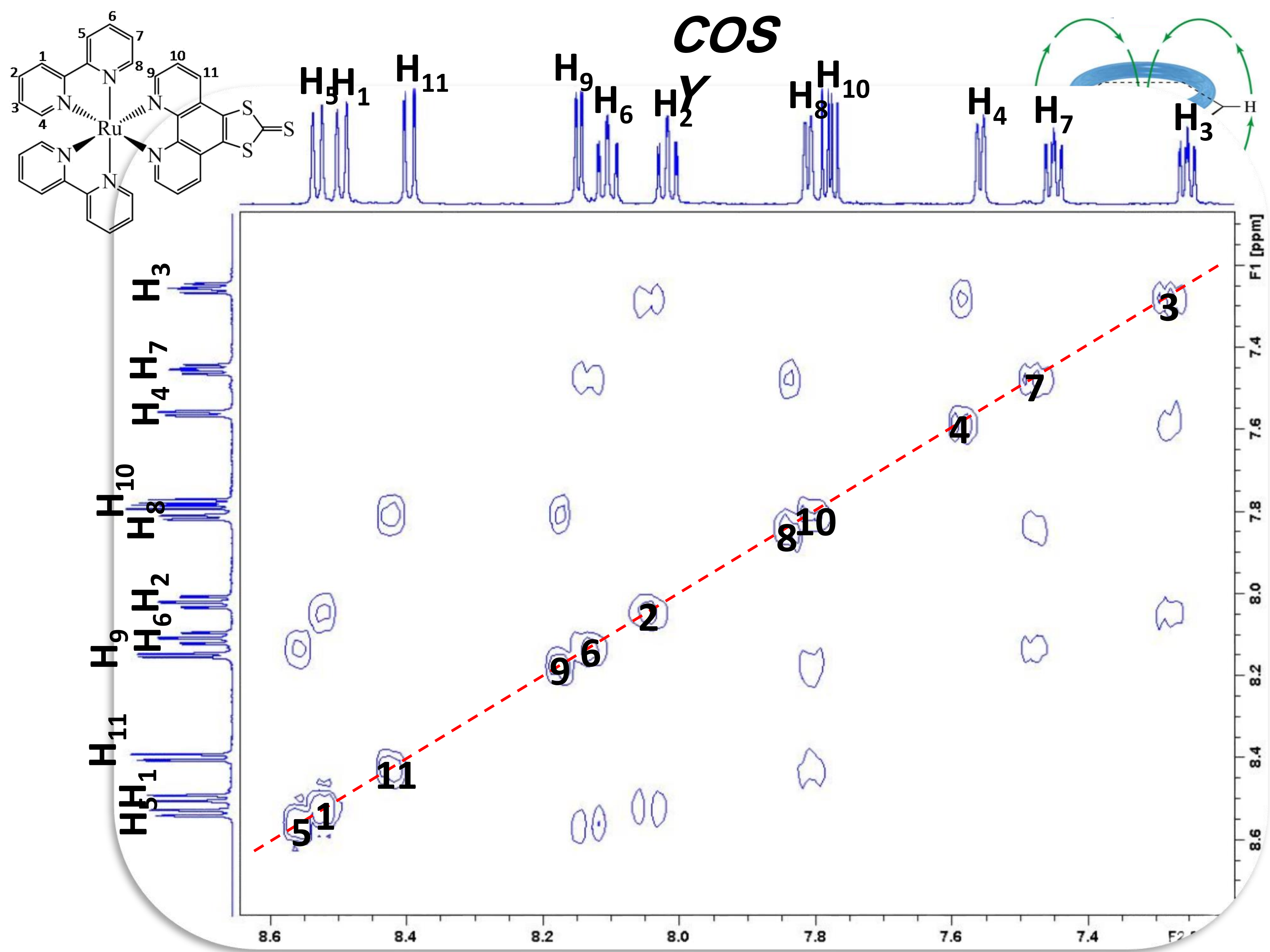
H-3
H-4
H-2

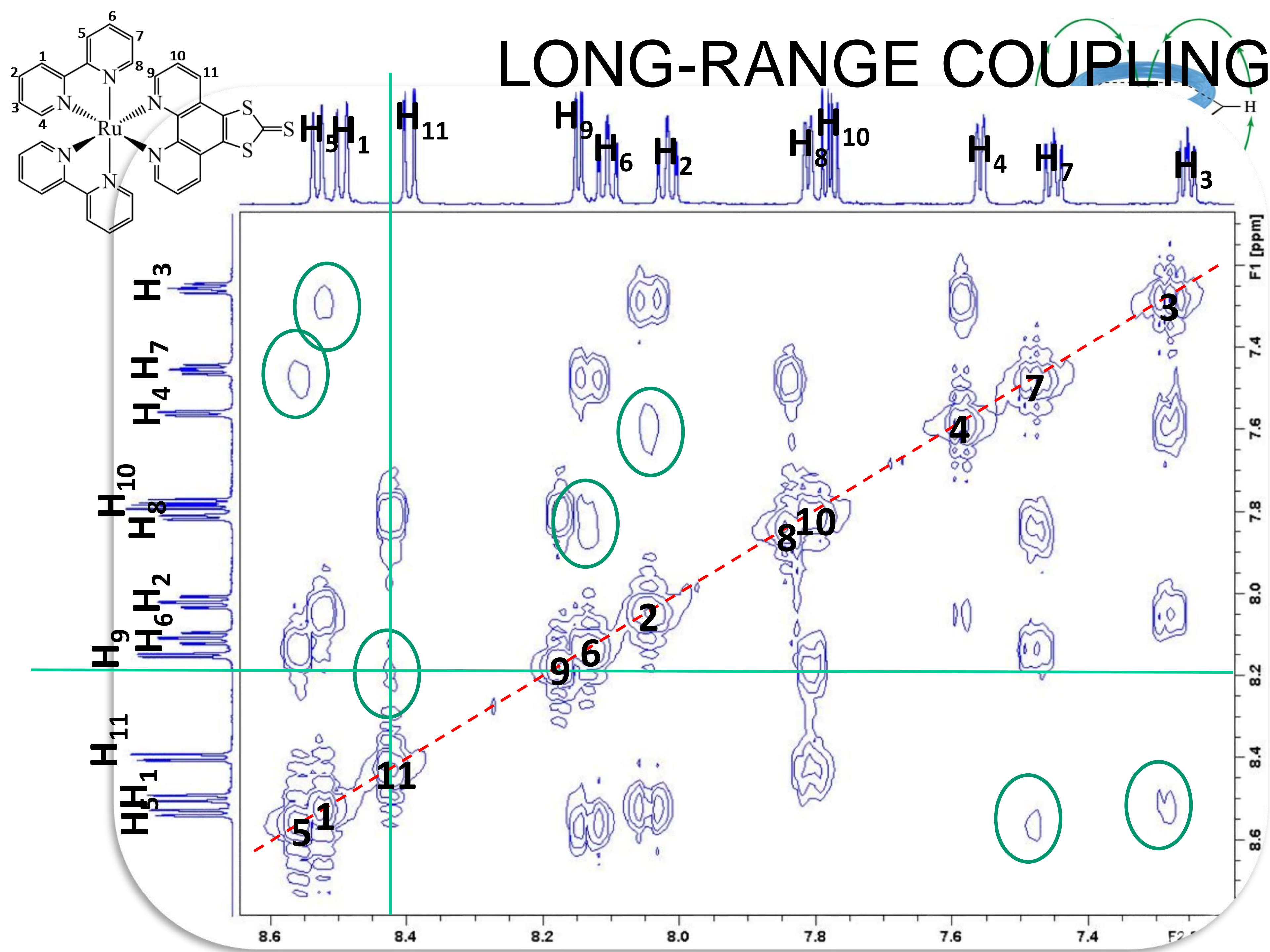
2.5
3.0
3.5
δ

3.5 3.0 2.5 δ

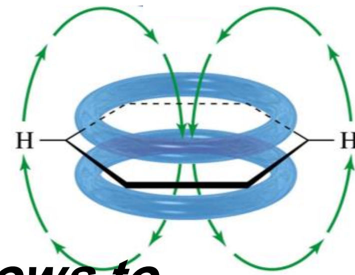
H


COSY
H5 H1 H11 H9 H6 H2 H8 H10 H4 H7 H3
F1 [ppm]
F2
8.6 8.4 8.2 8.0 7.8 7.6 7.4
Ru
S
N
5 1 11 9 6 2 8 10 4 7 3

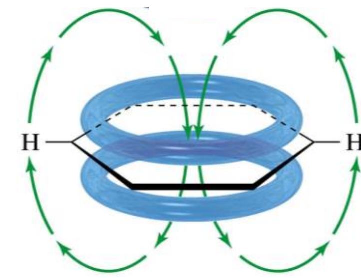

COSY
H5
H1
H11
H9
H6
H2
H8
H10
H4
H7
H3
F1 [ppm]
F2
5
1
11
9
6
2
8
10
4
7
3
Ru
N
S
8.6
8.4
8.2
8.0
7.8
7.6
7.4


LONG-RANGE COUPLING

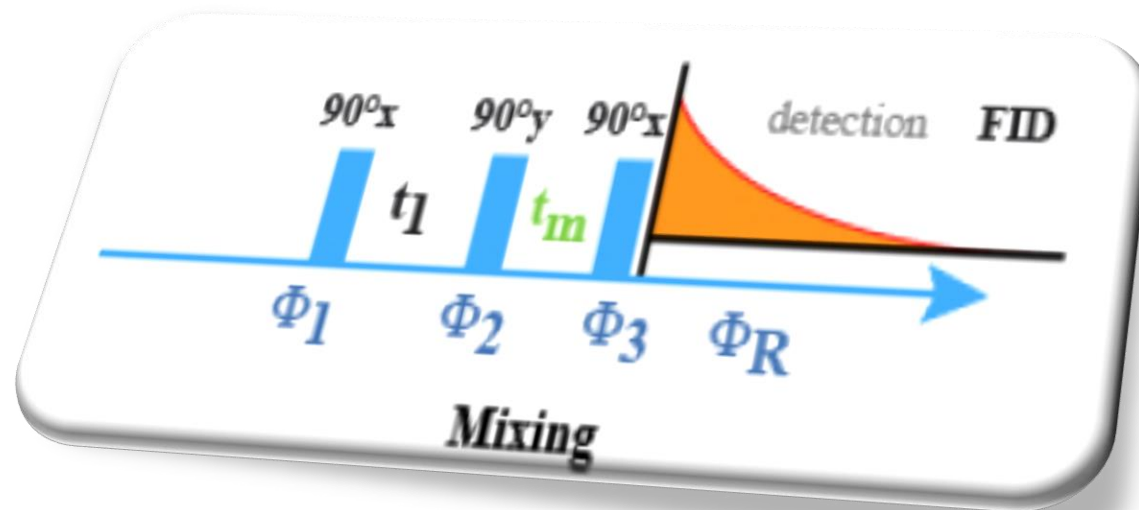
NOESY)(NOE-based expriments



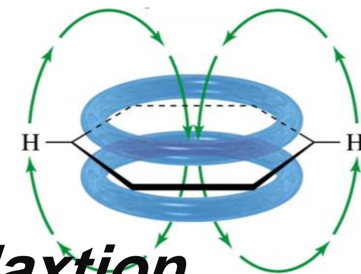
- **NOESY is one of the most useful techniques as it allows to correlate nuclei through space (distance smaller than 5Å). By measuring cross peak intensity, distance information can be extracted.*
- *The pulse sequence starts as usual with a 90` pulse followed by an evolution time t1. This delay is varied systematically as usual to provide chemical shift information in the F1 domain. Then a 90` pulse transmit some of the magnetization to the Z axis and during the following mixing period, the non-equilibria Z component will exchange magnetization through relaxation (dipole-dipole mechanism). This exchange of magnetization is known as NOE (Nuclear Overhauser Effect). After some time (shorter than the relaxation time T1), the transverse magnetization is restored and detected. If relaxation exchange (or chemical exchange) have taken place during the mixing time, cross peaks will be observed in the spectra.*



This experiment allows the determination of large molecules such as the proteins.



NOESY

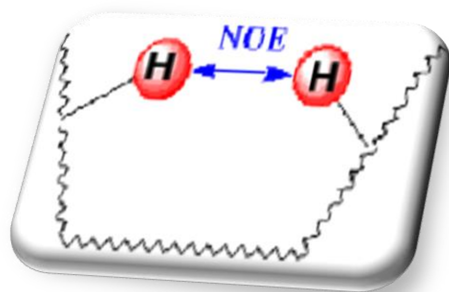


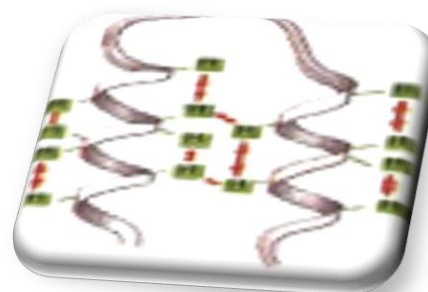
a. Useful to identify spins undergoing cross-relaxtion

b. Direct dipolar couplings provides primary means of cross-relaxtion,& so spins undergoing this are close to one another in space(<5Å)

c. Intensity of peaks depends on parameter,τ_m the mixing time

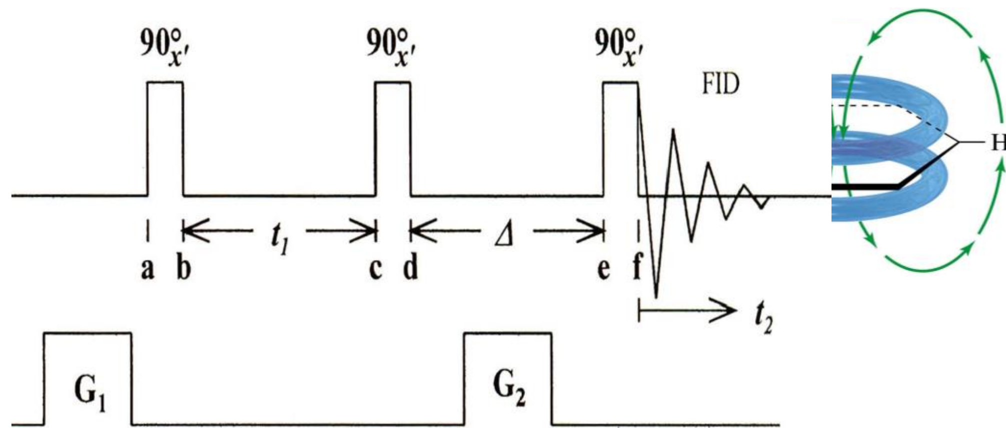
d. Indicated in the form of cross peaks in the NOESY spectrum

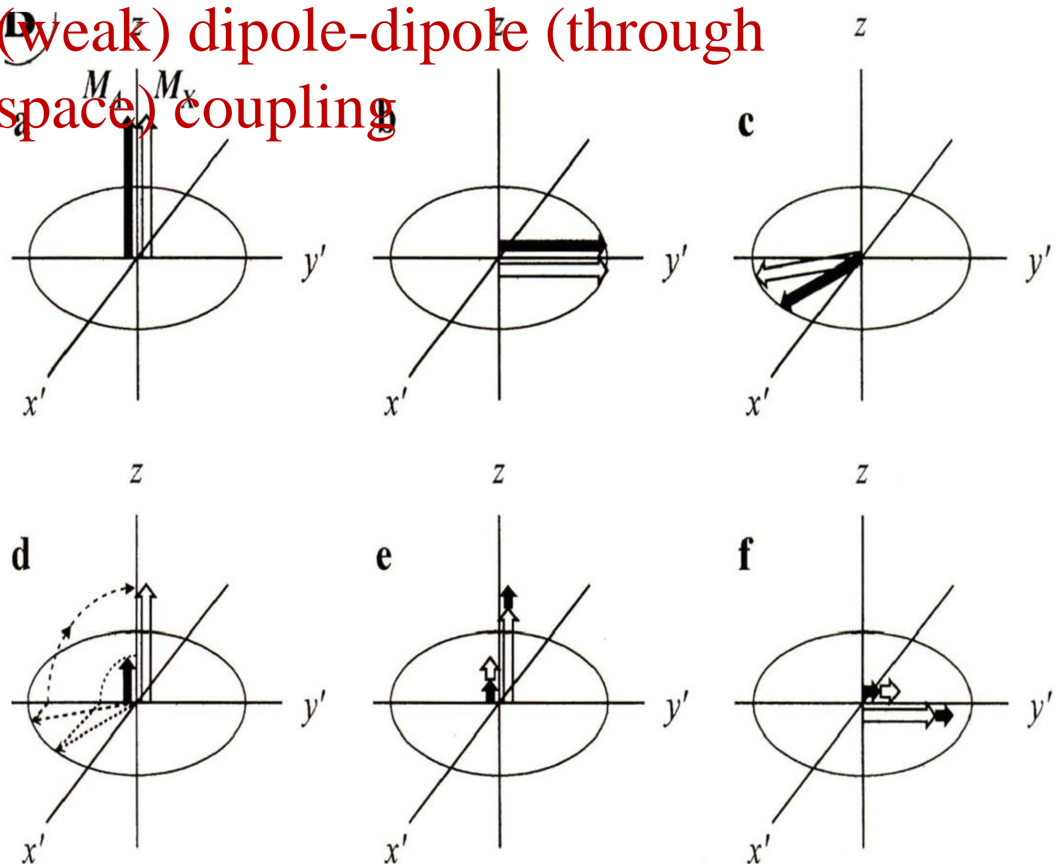




NOESY [Nuclear Overhauser Enhancement (or Effect) SpectroscopY]

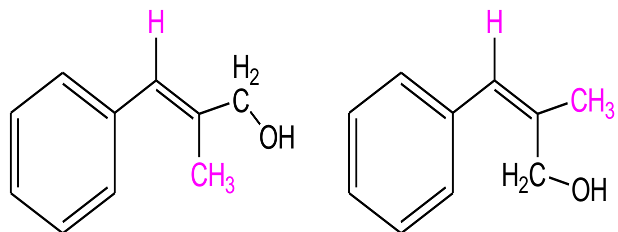
Transfer of magnetization between the nuclei that have (weak) dipole-dipole (through space) coupling



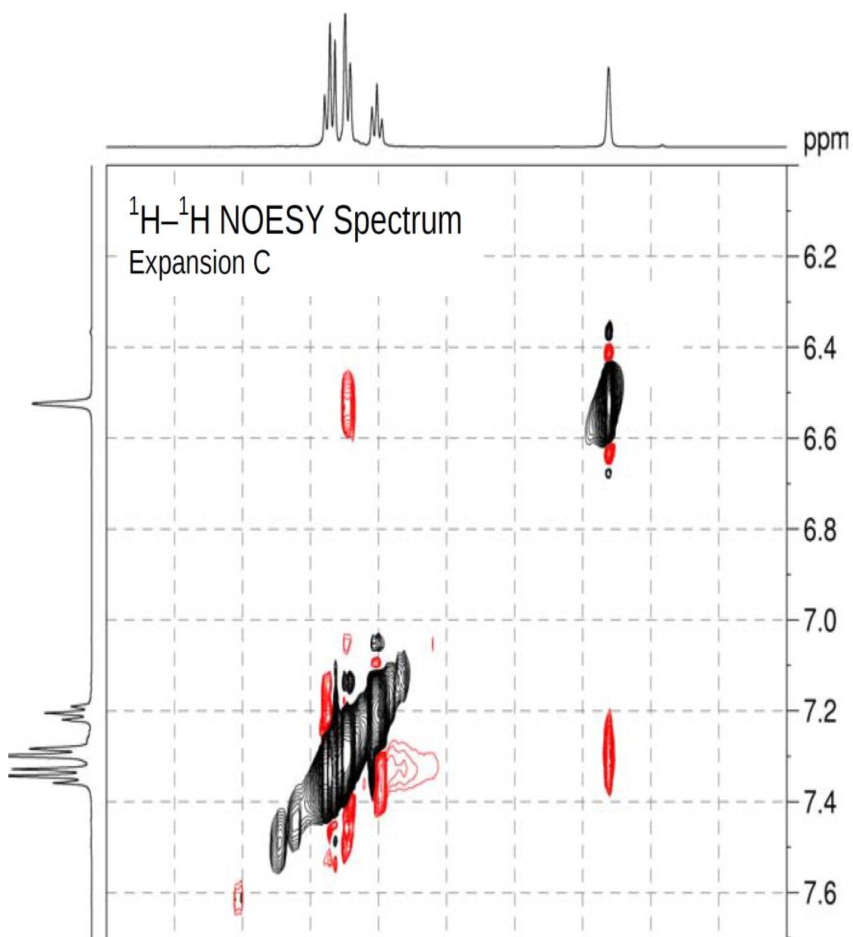


Δ = (the mixing time)

The same order as T_1



2-Methyl-3-



^1H–^1H NOESY Spectrum

(DMSO-d_6, 500 MHz)

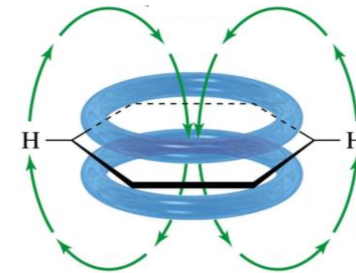
Expansion C

ppm

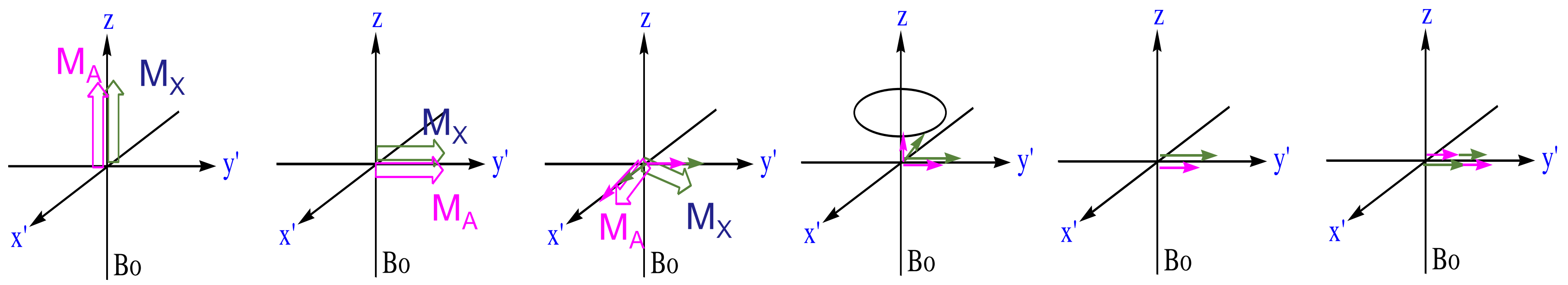
2
3
4
5
6
7
8

8 7 6 5 4 3 2 ppm

R*O*ESY [Rotating frame *O*verhauser Enhancement (or Effect)

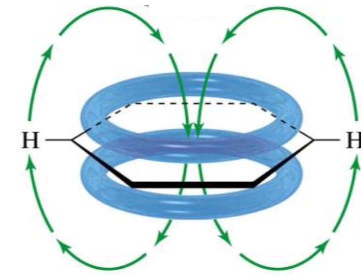


$90^0{}_{\phi 1} - t_1 - \text{spin lock (phase } \phi_2) - \text{FID}$



NOESY and ROESY are similar except that for ROESY
The amplitude of diagonal and cross peaks are always opposite

The spin-lock pulse used in ROESY is about 1/5 that used in TO
The cross peaks of ROESY and TOCSY are opposite



2D INADEQUATE

$$1D: 90^\circ_{x'} - \tau - 180^\circ_{y'} - \tau - 90^\circ_{x'} - \Delta - 90^\circ_{\Phi'} - \text{FID}\ (t_2)$$

$$2D: 90^\circ_{x'} - \tau - 180^\circ_{y'} - \tau - 90^\circ_{x'} - t_1 - 90^\circ_{\Phi'} - \text{FID}\ (t_2)$$

Double quantum coherence

Evolution

Single quantum transition

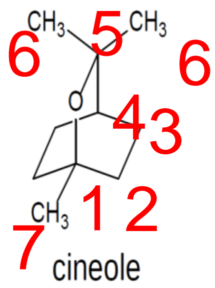
Observation of ^{13}C sattelites

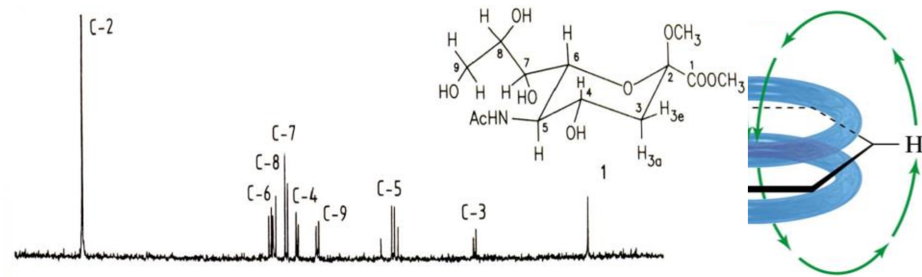
Each carbon nucleus gives a doublet consisting of the two satellite signals. The chemical shift would be at the centers of these doublets

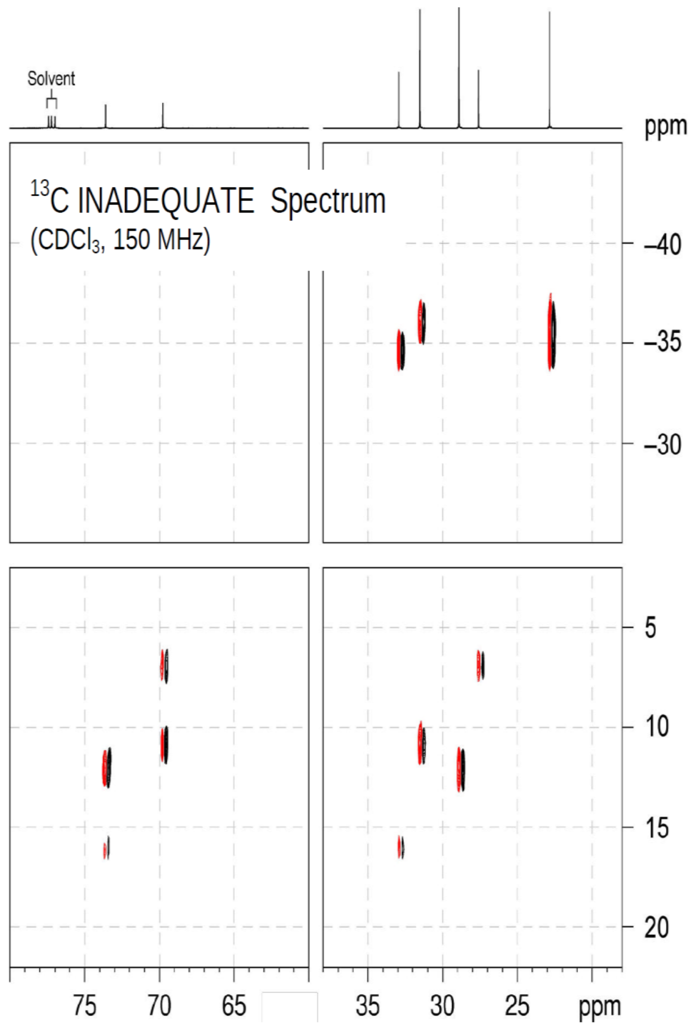
$$\tau = \frac{2n+1}{4J(C,C)} \qquad n = 0, 1, 2, 3$$

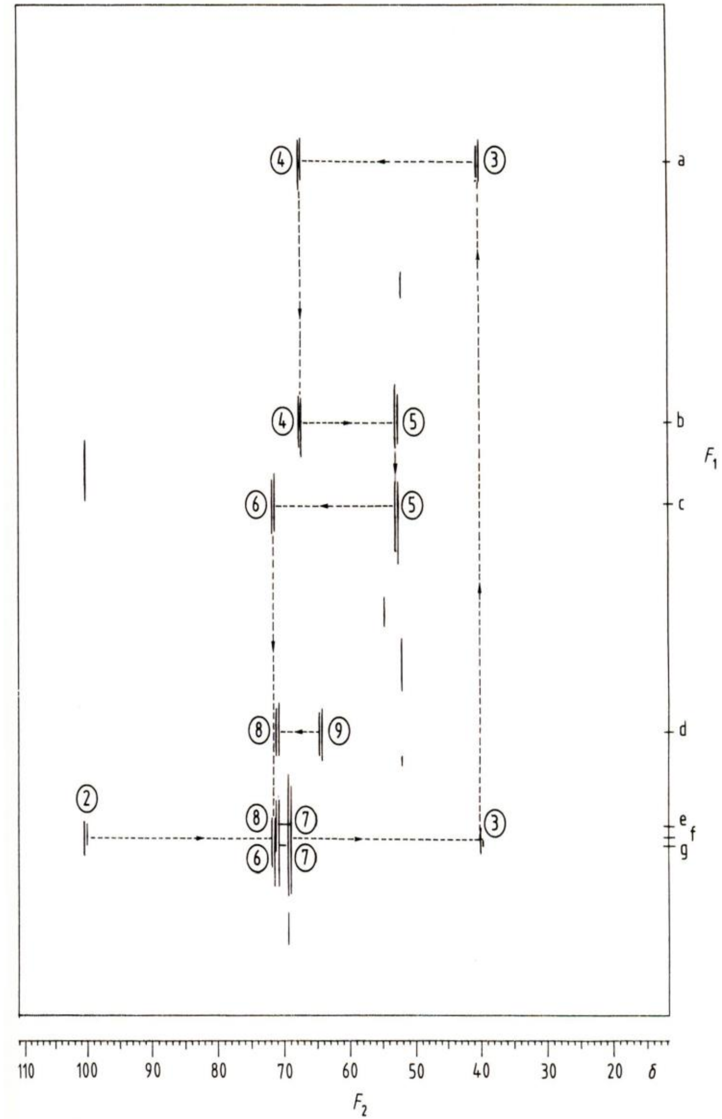
There is no enhancement of signal intensities by polarization Transfer

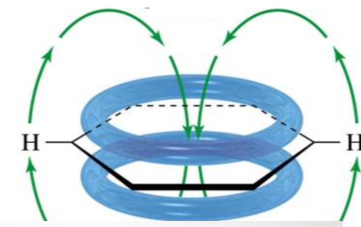
It is time consuming (over night)

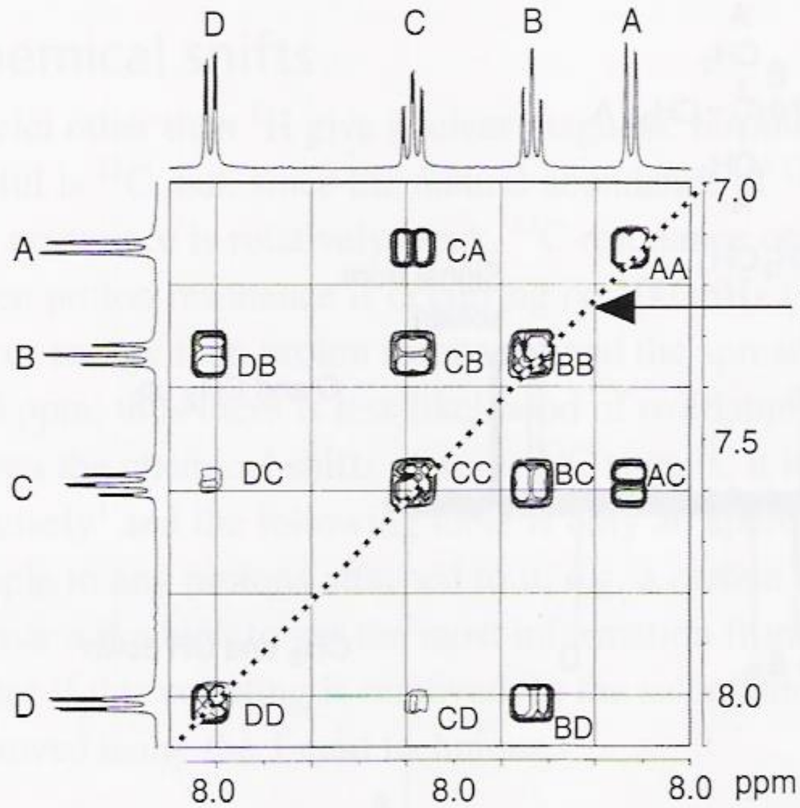

CH3
CH3
5
6
6
O
4
3
CH3
1
2
7
cineole


C-2
C-7
C-8
C-6
C-4
C-9
C-5
C-3
OCH3
COOCH3
AcHN
OH
HO
H


Solvent
ppm
^{13}C INADEQUATE Spectrum
(CDCl3, 150 MHz)
–40
–35
–30
5
10
15
20
75
70
65
35
30
25
ppm


a
b
c
d
e
f
g
F1
F2
110
100
90
80
70
60
50
40
30
20
δ





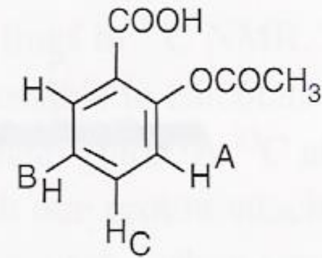
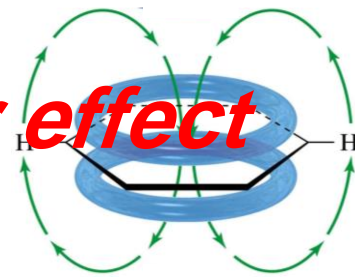
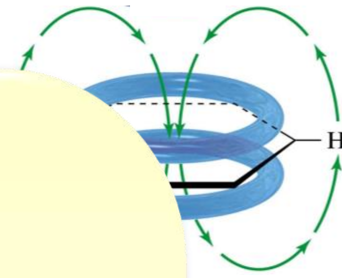


Fig. 8.17
The proton–proton correlation spectrum of the aromatic region of aspirin.

)HOESY(heteronuclear overhauser effect spectroscopy

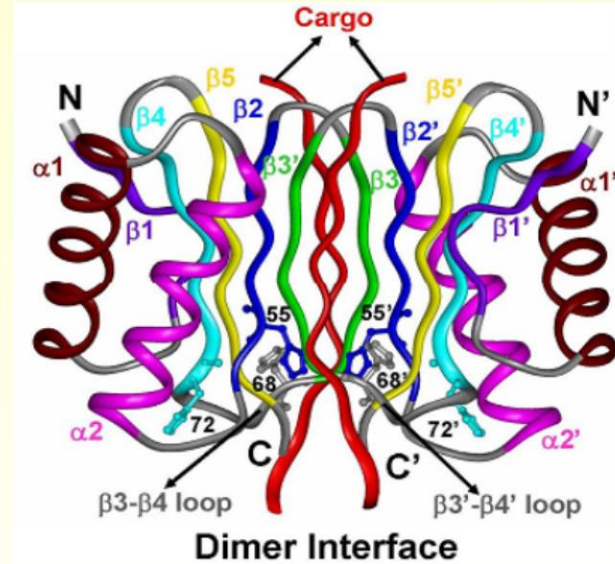


Correlation between protons and heteronuclei that are close in space.Requires that the heteronucleus be relaxed significantly by dipole-dipole interactions with nearby protons or other magnetic nuclei,and so fails with nuclei relaxed exclusively by chemical shift anisotropy or quadrupolar mechanisms used extensively in the spectroscopy oforganolithium reagents.

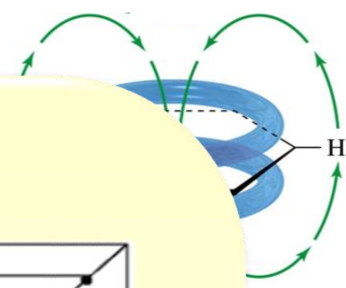


NMR of Biomolecules

- NMR is the only method for residue level structure determination of biomacromolecules in aqueous solutions at near physiological conditions
- Depending on the size of the molecule, one can go for homunuclear/heteronuclear 2D corelation or even 3D or 4D!

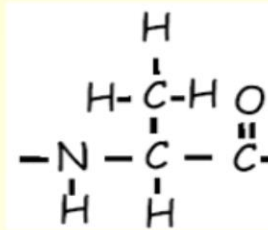


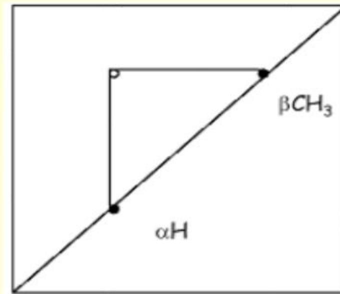
Dynein light chain protein (DLC8)
89 residues, MW~10.3kD



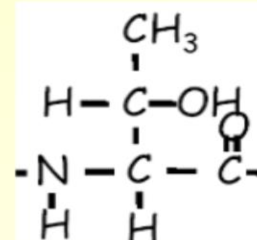
COSY peak patterns of amino acids

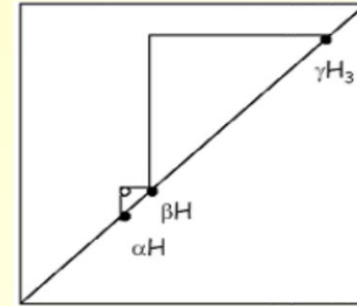
Ala



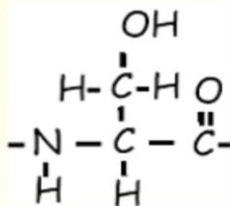


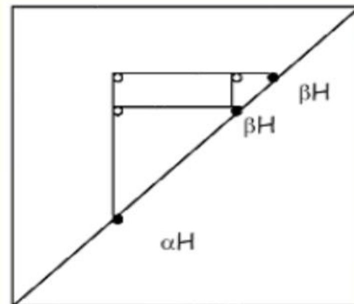
Thr



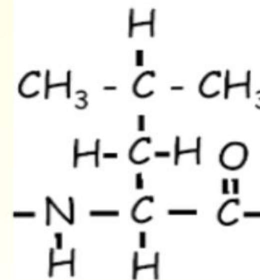


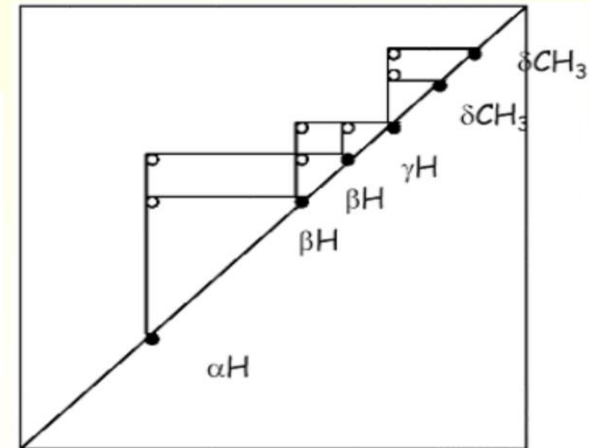
Ser, Cys, Asp, Asn

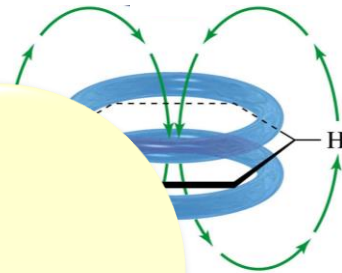




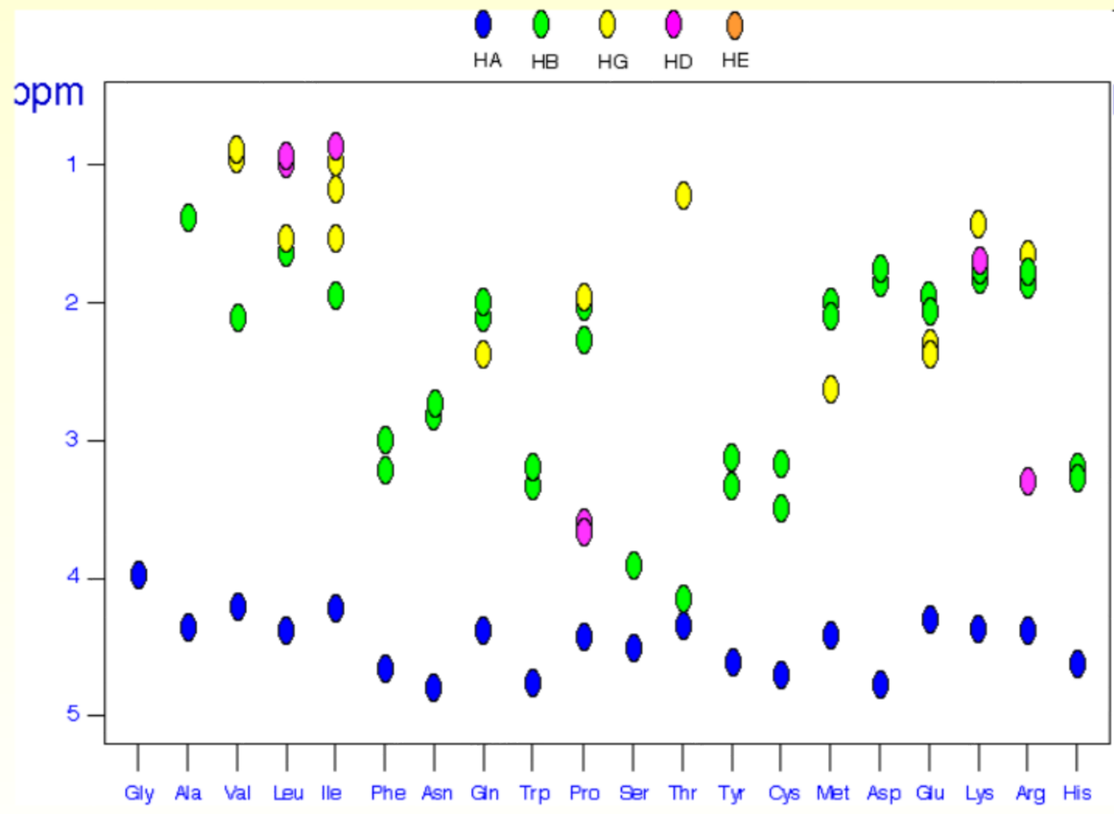
Leu

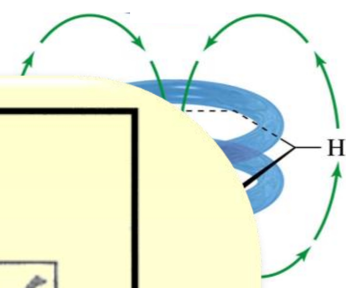






TOCSY patterns of amino acids





NOESY-DNA

H2’/2” -> Base

H1’ -> Base