Determination of structure of glycans and glycan interactions with proteins by NMR

Into the great wide open

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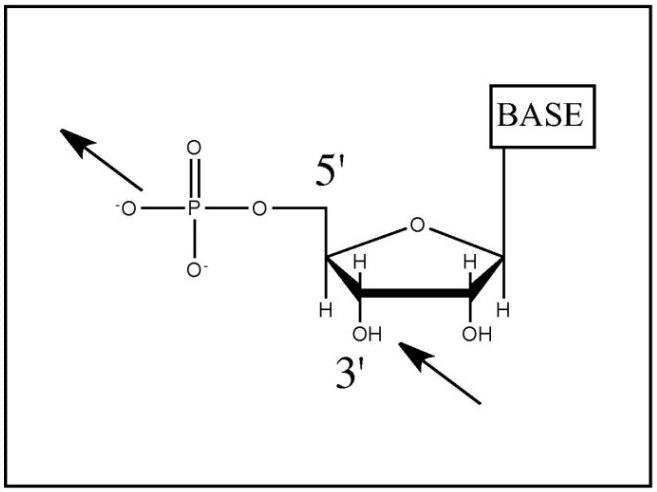
Structure determination of a glycan chain: the steps

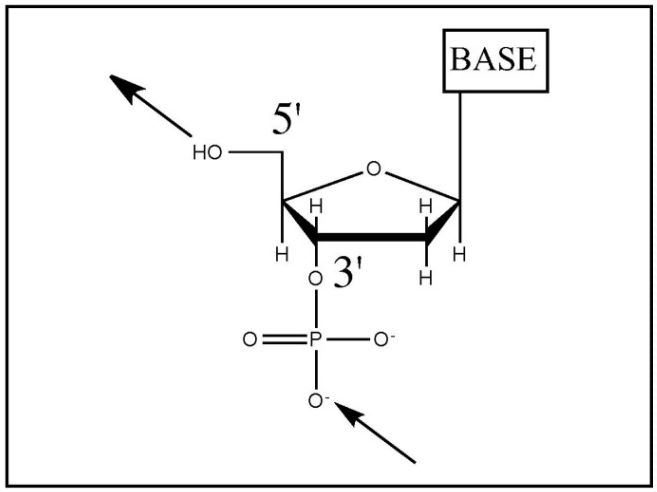
- Quali-quantitative analysis (GC-MS, NMR)
- Absolute configuration (GC-MS, NMR)
- Size of the ring (GC-MS, NMR)
- Anomeric configuration (NMR)
- Linkage analysis (GC-MS, NMR)
- Monosaccharides sequence (MALDI-MS,2D NMR)
- Determination of non-carbohydrate appendages (GC-MS,MALDI-MS, 2D NMR)

Meth. Enzymol., 2010

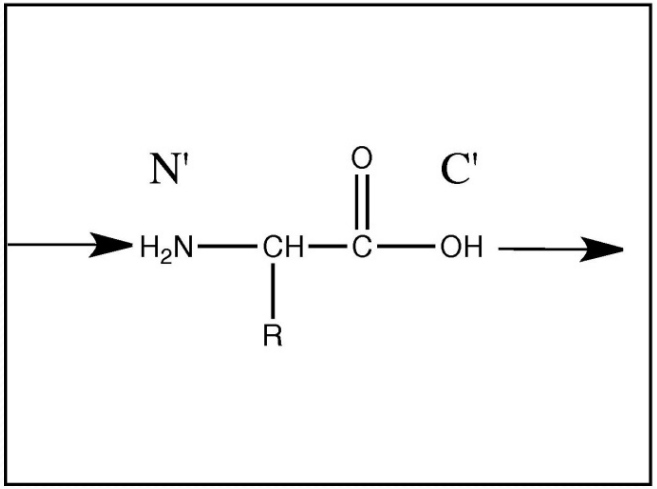
RNA



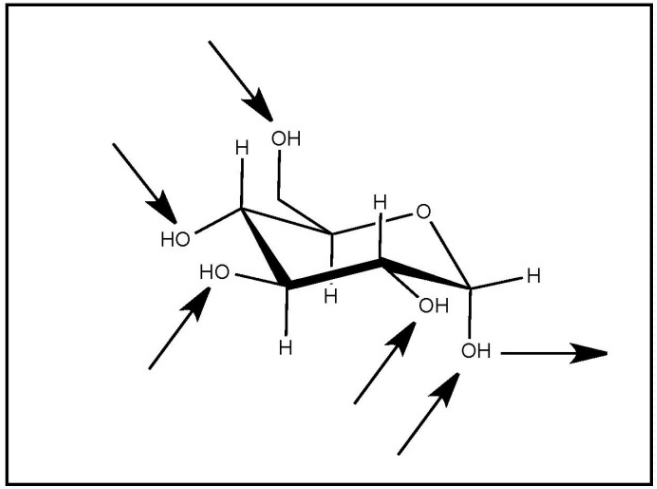
DNA

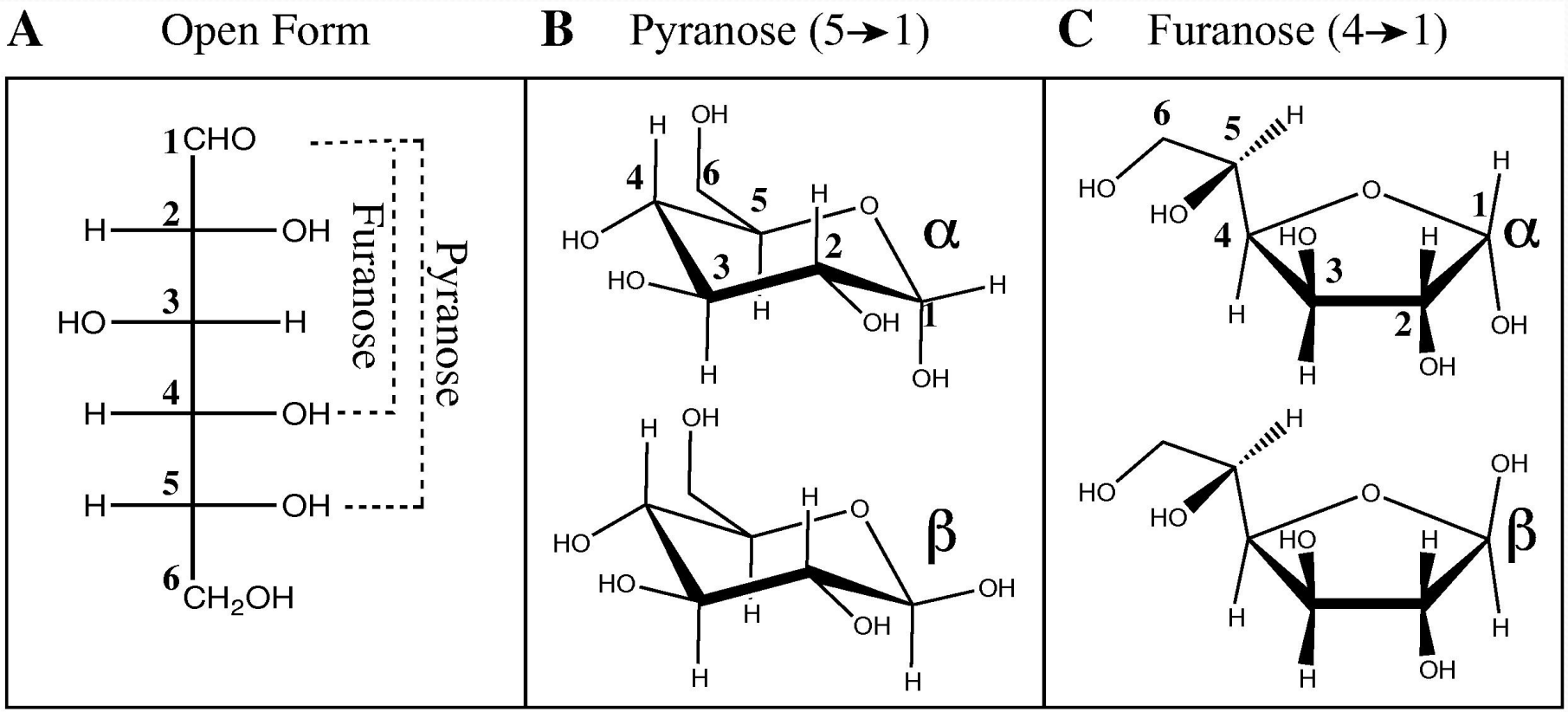


Protein



Glycan





Open, pyranose and furanose forms of an aldose, showing the many equivalent OH groups. The OH groups can be modified by $-PO_4^{-3}$, $-SO_4^{-2}$, $-NH_2$, $-CO-CH_3$, $-NH-CO-CH_3$, -H.

Typical Applications of NMR:

- **Structural (chemical) elucidation**

  Natural product chemistry

  Organic chemistry: Analytical tool of choice for synthetic chemists.

- **Study of dynamic processes**

  Reaction kinetics.

  Study of equilibrium (chemical or structural).

- **Structural (three-dimensional) studies**

  Proteins

  DNA/RNA

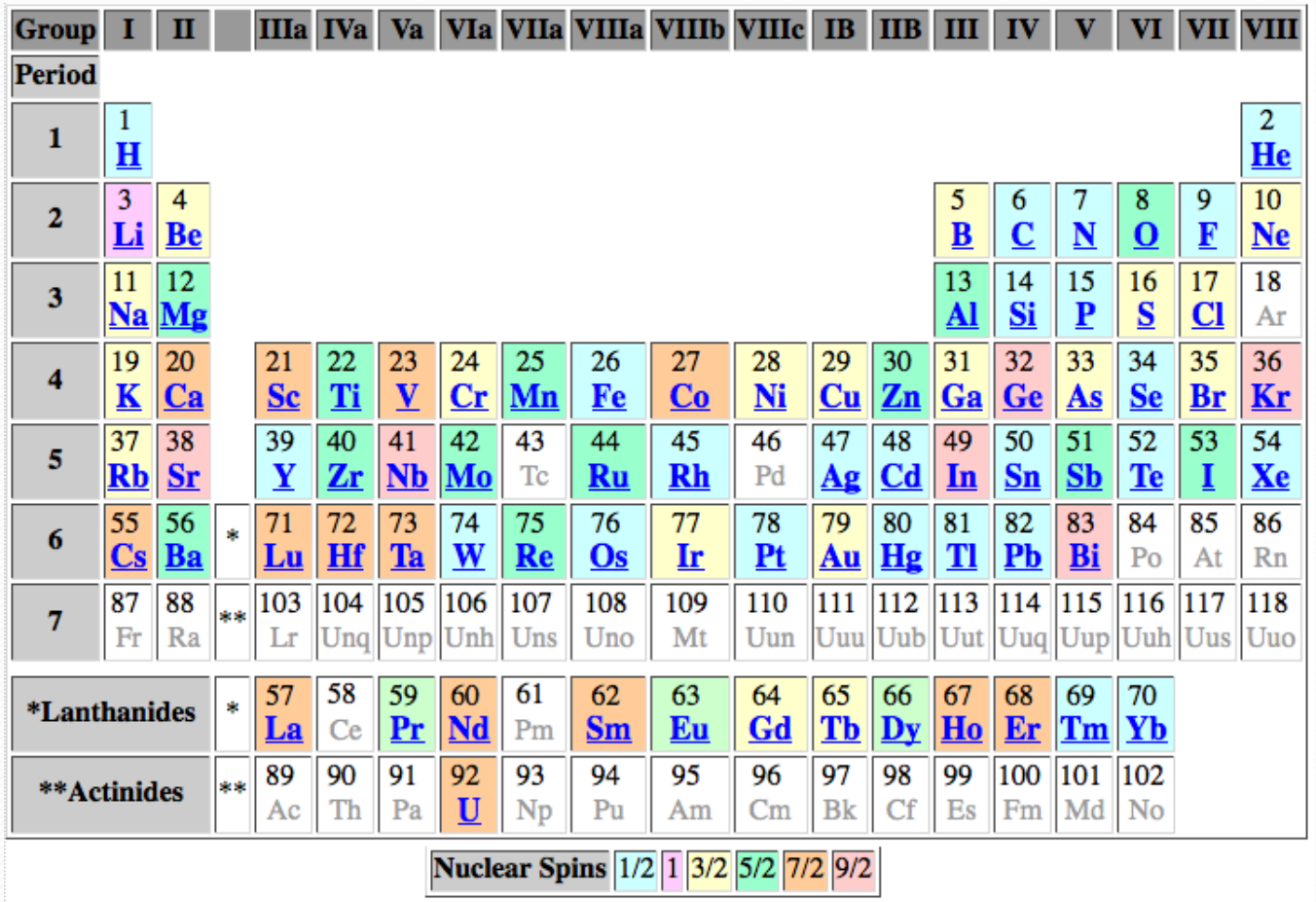
  Polysaccharides

  Drug design

- **Structure Activity Relationships (SAR) by NMR**

- **Medicine - Magnetic Resonance Imaging (MRI)**

NMR active nuclei



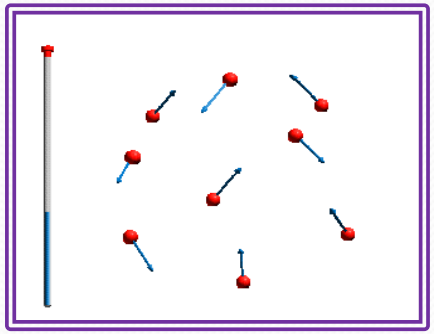
Group	I	II		IIIa	IVa	Va	VIa	VIIa	VIIIa	VIIIb	VIIIc	IB	IIB	III	IV	V	VI	VII	VIII
Period																			
1	1 H																		2 He
2	3 Li	4 Be												5 B	6 C	7 N	8 O	9 F	10 Ne
3	11 Na	12 Mg												13 Al	14 Si	15 P	16 S	17 Cl	18 Ar
4	19 K	20 Ca		21 Sc	22 Ti	23 V	24 Cr	25 Mn	26 Fe	27 Co	28 Ni	29 Cu	30 Zn	31 Ga	32 Ge	33 As	34 Se	35 Br	36 Kr
5	37 Rb	38 Sr		39 Y	40 Zr	41 Nb	42 Mo	43 Tc	44 Ru	45 Rh	46 Pd	47 Ag	48 Cd	49 In	50 Sn	51 Sb	52 Te	53 I	54 Xe
6	55 Cs	56 Ba	*	71 Lu	72 Hf	73 Ta	74 W	75 Re	76 Os	77 Ir	78 Pt	79 Au	80 Hg	81 Tl	82 Pb	83 Bi	84 Po	85 At	86 Rn
7	87 Fr	88 Ra	**	103 Lr	104 Unq	105 Unp	106 Unh	107 Uns	108 Uno	109 Mt	110 Uun	111 Uuu	112 Uub	113 Uut	114 Uuq	115 Uup	116 Uuh	117 Uus	118 Uuo
*Lanthanides			*	57 La	58 Ce	59 Pr	60 Nd	61 Pm	62 Sm	63 Eu	64 Gd	65 Tb	66 Dy	67 Ho	68 Er	69 Tm	70 Yb		
**Actinides			**	89 Ac	90 Th	91 Pa	92 U	93 Np	94 Pu	95 Am	96 Cm	97 Bk	98 Cf	99 Es	100 Fm	101 Md	102 No		

Nuclear Spins: 1/2, 1, 3/2, 5/2, 7/2, 9/2

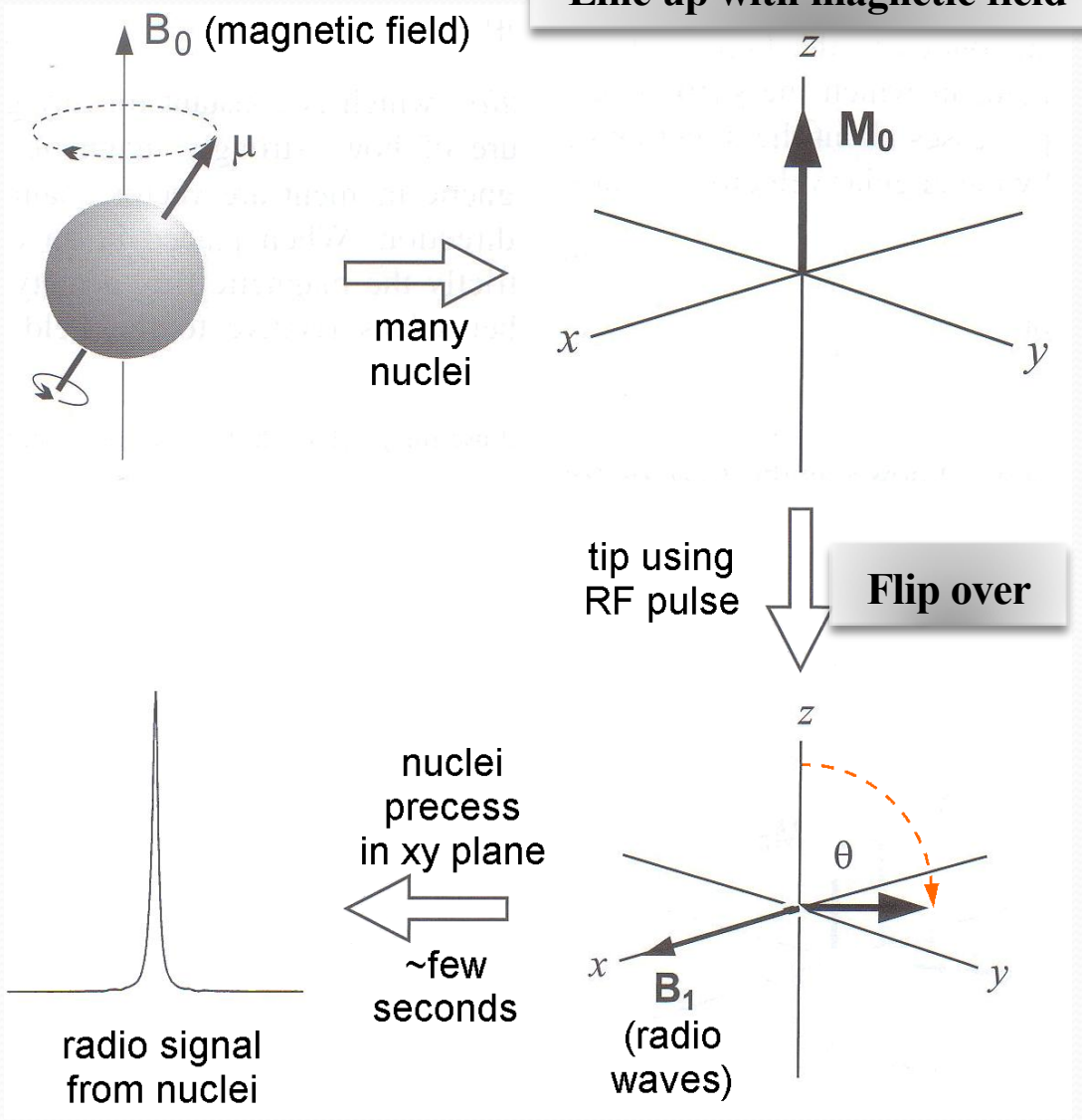
Nucleus (odd atomic number): ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P

How does NMR work?

A spinning charge creates a magnetic moment, so these nuclei can be thought of as tiny magnets.

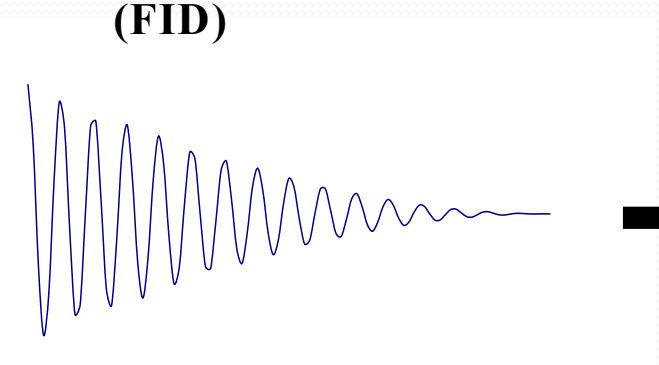


In presence of a magnetic field Magnetic moments precess and orient with or against the field



NMR Signal

Free Induction Decay (FID)



Time

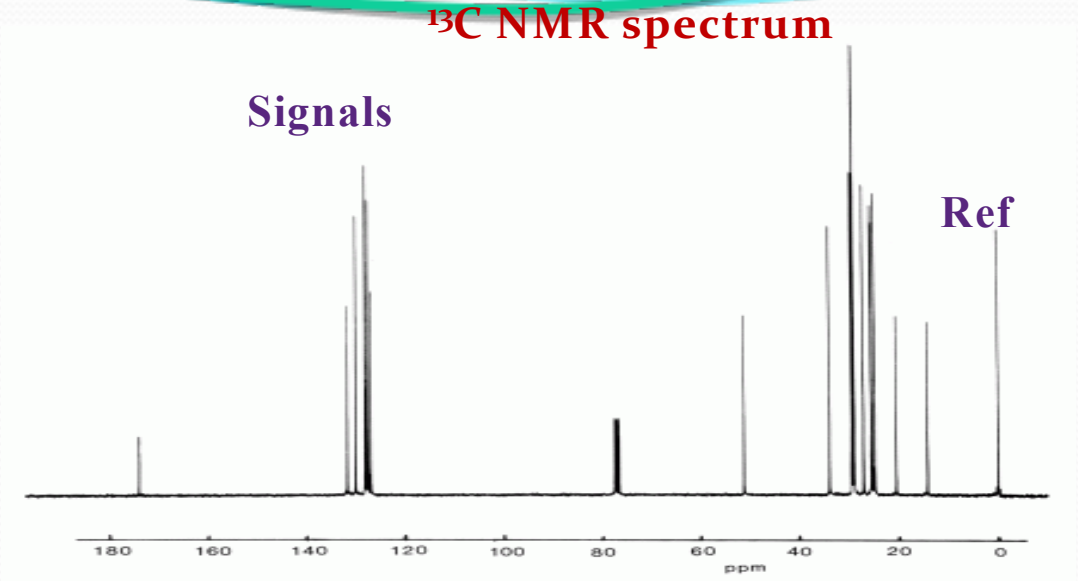
FT

NMR Signal

Frequency (Hz or MHz)

Chemical shift (ppm)

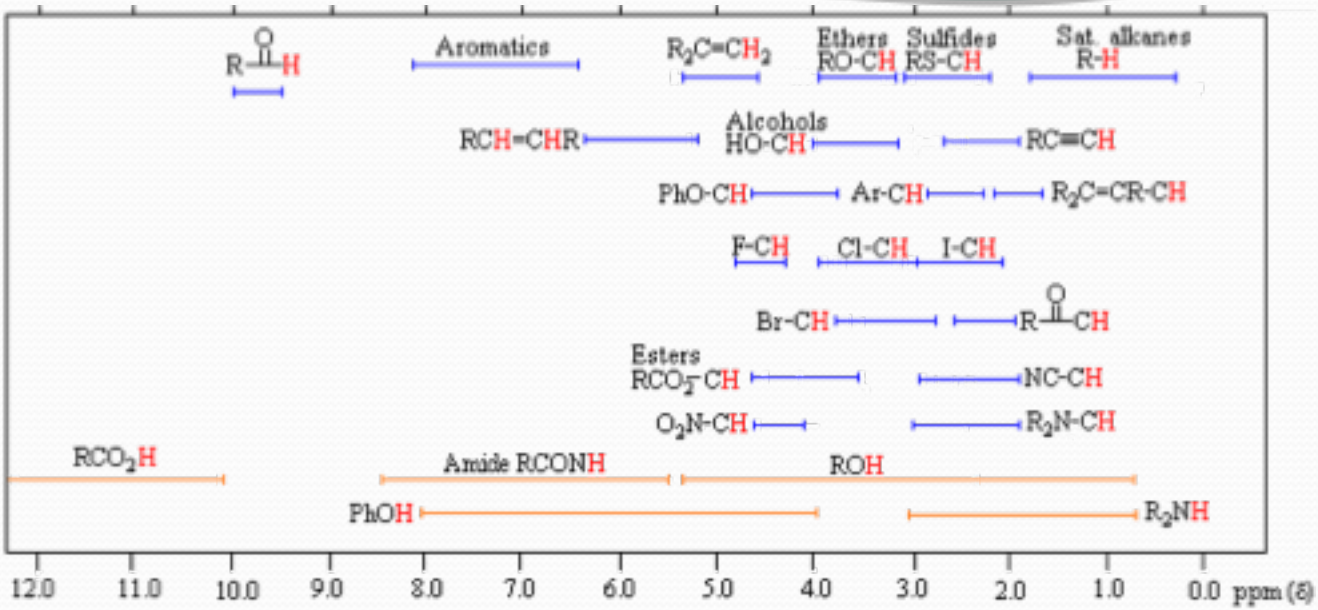
Chemical shift



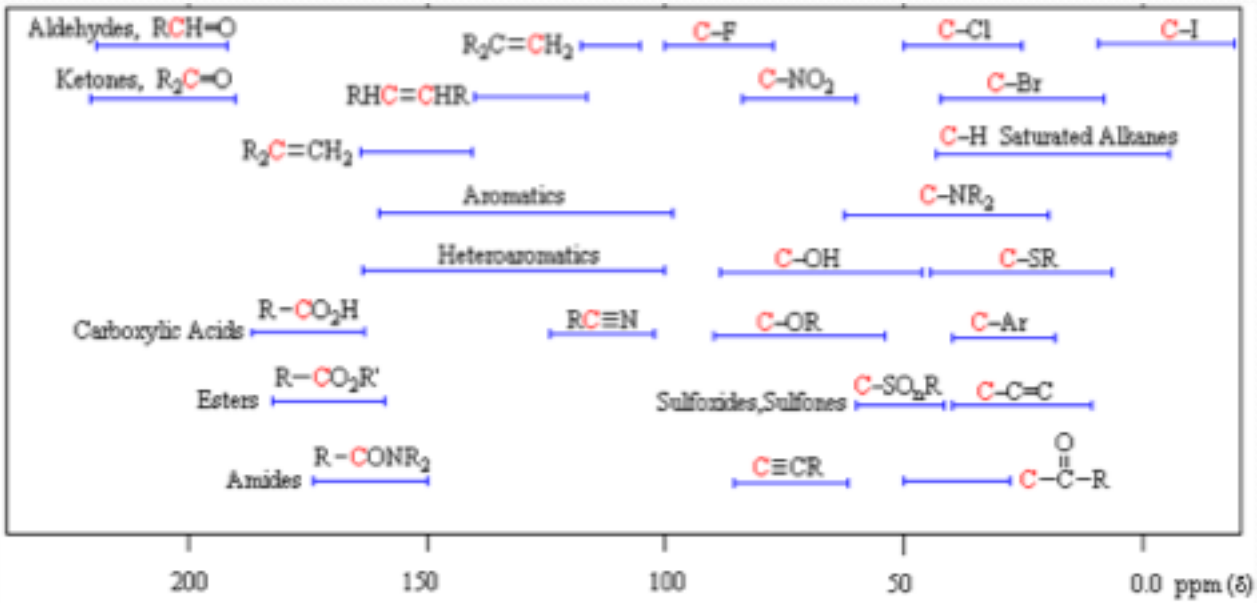
$$\delta\ (\text{ppm}) = \frac{\nu_{PEAK} - \nu_{REF}\ (\text{Hz})}{\text{Freq of the nuclei (MHz)}} = \text{ppm}$$

- Depending on the *chemical environment* we have variations on the magnetic field that the nuclei feel, even for the same type of nuclei. It affects the local magnetic field.

^{1}H chemical shifts

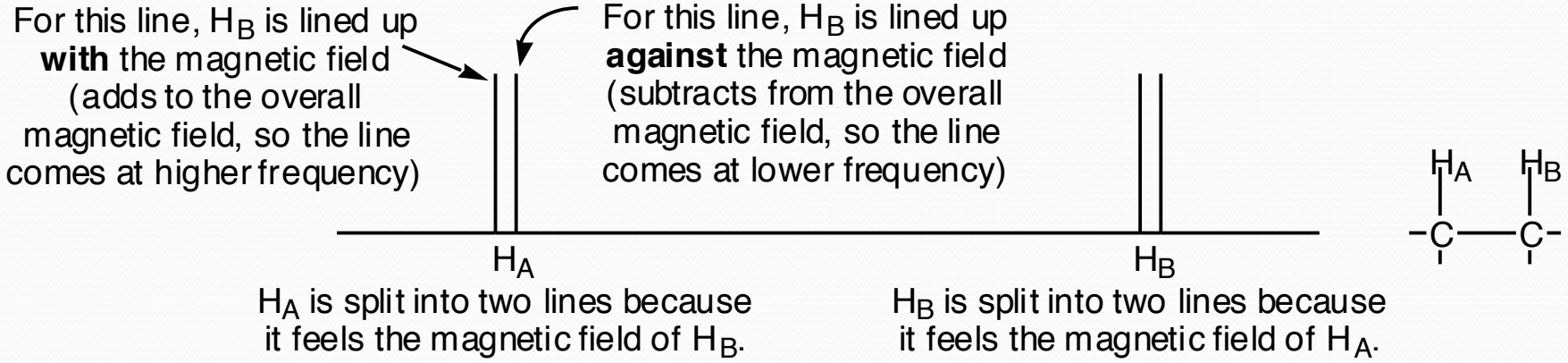


^{13}C chemical shifts



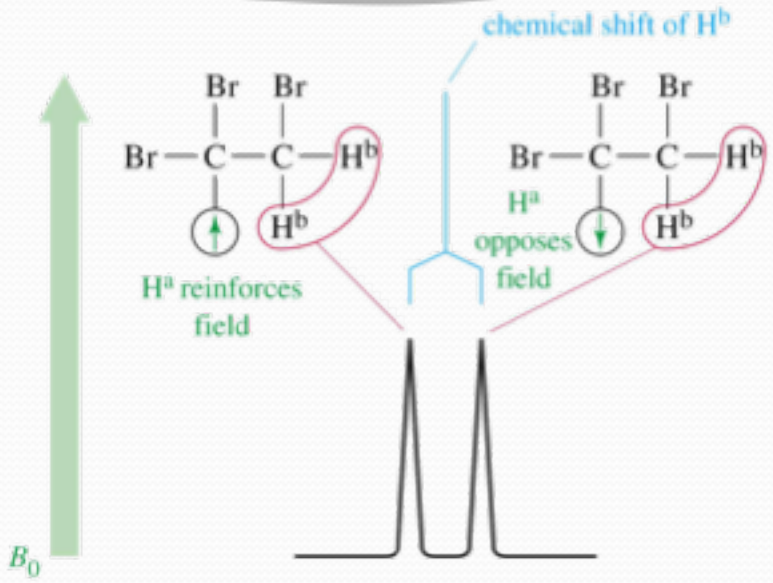
^1H - ^1H Coupling

Signals do not appear as single lines, sometimes they appear as multiple lines. This is due to ^1H - ^1H coupling (also called spin-spin splitting **or J-coupling**).

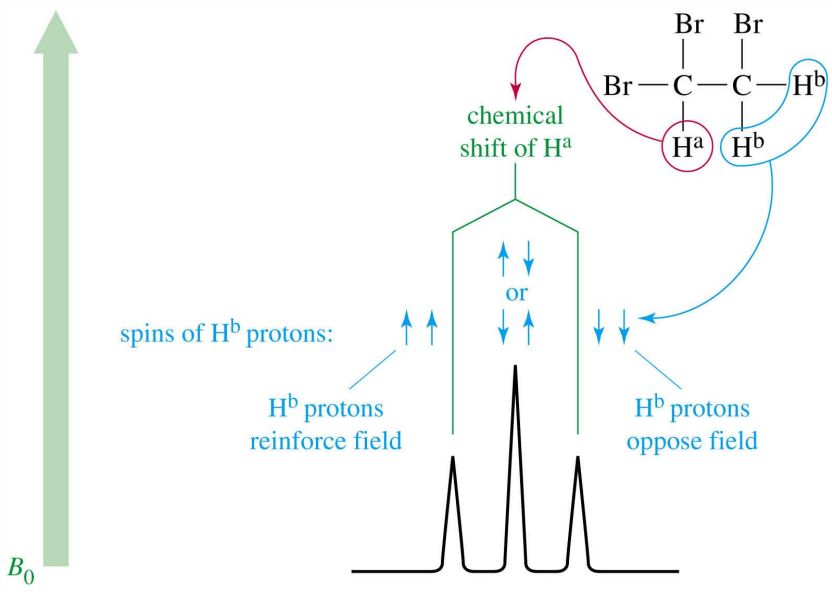


A and B are scalarly coupled nuclei

Doublet: 1 Adjacent Proton



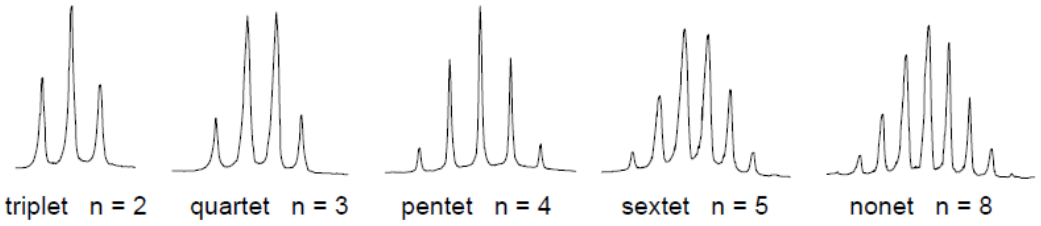
Triplet: 2 Adjacent Protons



The *N* + 1 Rule

If a signal is split by *N* equivalent protons, it is split into *N* + 1 peaks.

	Intensities
n = 0	1
n = 1	1 1
n = 2	1 2 1
n = 3	1 3 3 1
n = 4	1 4 6 4 1
n = 5	1 5 10 10 5 1
n = 6	1 6 15 20 15 6 1



n	2^n	Multiplet Intensities
0	1	1
1	2	1 1
2	4	1 2 1
3	8	1 3 3 1
4	16	Pentet 1 4 6 4 1
5	32	Sextet 1 5 10 10 5 1
6	64	Septet 1 6 15 20 15 6 1
7	128	Octet 1 7 21 35 35 21 7 1
8	256	Nonet 1 8 28 56 70 56 28 8 1

^{1}H NMR

- ☞ Number of signals (number of non-equivalent H)
- ☞ Chemical shift (differences in chemical environment)
- ☞ Splitting or Coupling (number of neighboring H)
- ☞ Integration (relative number of H at each signal)

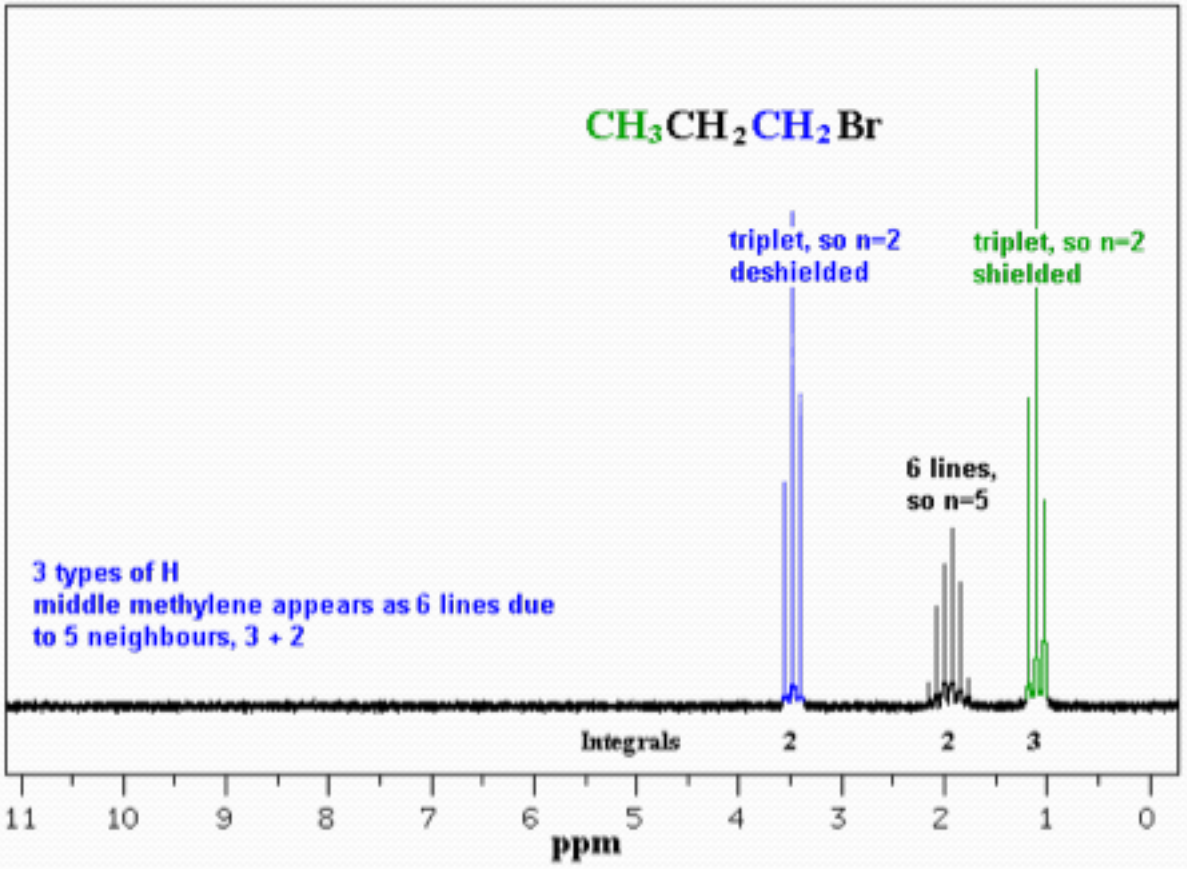
- Chemical shift data - tells us what kinds of protons we have.
- Integrals - tells us the ratio of each kind of proton in our sample.
- ^1H - ^1H coupling - tells us about protons that are near other protons.

- Number of signals

- Chemical shift

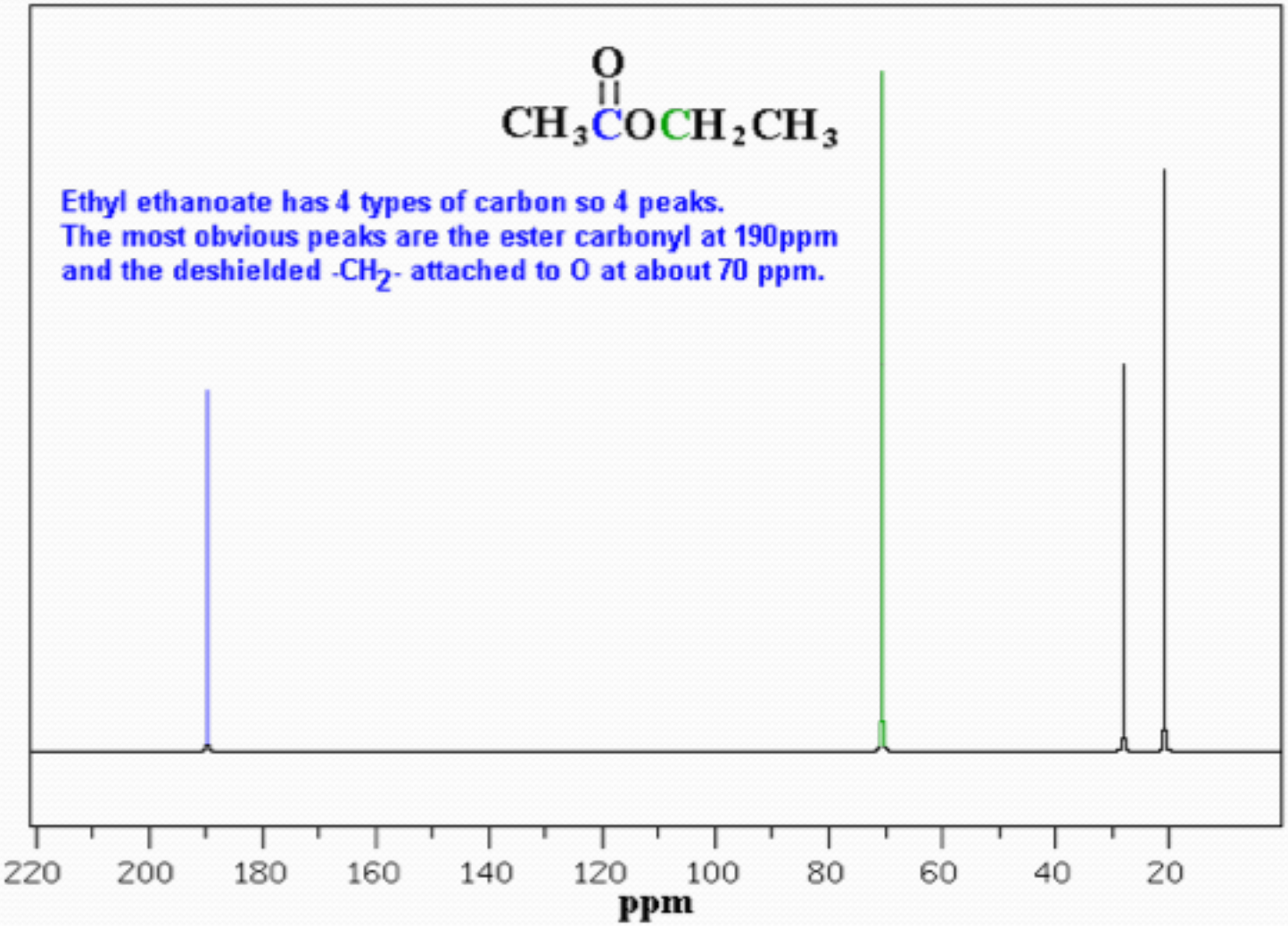
- Splitting or coupling

- Integration



^{13}C NMR

- Chemical shift is normally 0 to 220 ppm
- Similar factors affect the chemical shifts in ^{13}C as seen for H NMR
- Long relaxation times (exited state to ground state) mean no integration
- Number of peaks indicates the number of types of C


O
CH3COCH2CH3
Ethyl ethanoate has 4 types of carbon so 4 peaks.
The most obvious peaks are the ester carbonyl at 190ppm
and the deshielded -CH2- attached to O at about 70 ppm.
220
200
180
160
140
120
100
80
60
40
20
ppm

Types of NMR experiments

- **1D Experiments:**

  - ^{1}H
  - ^{13}C
  - ^{31}P

- **2D Experiments:**

  - COSY (Correlation Spectroscopy)
  - TOCSY (Total Correlation Spectroscopy)
  - NOESY (Nuclear Overhauser effect spectroscopy)

  $^{1}H - ^{1}H$

  - HSQC (Heteronuclear Single Quantum Coherence)
  - HMBC (Heteronuclear Multiple Bond Coherence)

  $^{1}H - ^{13}C$

Structure Determination

- **Each observable NMR resonance needs to be assigned or associated with the atom .**

- Molecular Formula
- Functional groups
- Carbon Connectivity
- Position of substitution on the carbon framework
- Stereochemical properties.

NMR of carbohydrates:

- Sugar assignment (COSY, TOCSY, HSQC, HMBC)
- Anomeric configuration ($^{3}J_{H,H}$ coupling constants, NOE)
- Ring size (^{13}C chemical shift, HMBC)
- Non carbohydrate substituents (Homo- and Heteronuclear NMR)
- Sugar sequence: Linkage analysis and glycosylation pattern (HMBC, NOE)

^{1}H and ^{13}C typical regions of carbohydrates:

The ^{1}H NMR Spectra can be roughly divided into the following regions:

Anomeric and Acylated Protons : 5.5-4.5 ppm.

Ring Protons : 4.5-3 ppm

Acetyl Groups, Methylene Protons: 3-2 ppm

Methyl Groups: 0.8-2.0 ppm

The ^{13}C NMR Spectra can be roughly divided into the following regions :

Anomeric Carbons Resonate Between 90-105 ppm

Ring Carbons Between 60-85 ppm

Nitrogen Bearing Carbons (In Amino Sugar) 50-60 ppm

Acetyl Groups 20-25ppm

Methylene Protons: 25-35 ppm

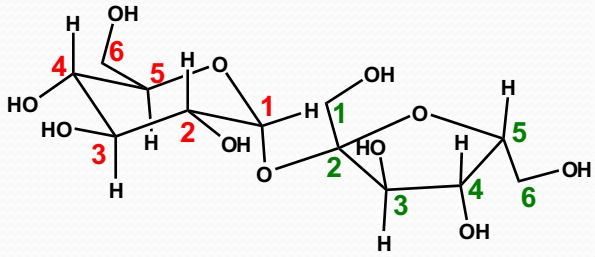
Methyl Groups: 15-20ppm

Sucrose

^{1}H

Ring proton region

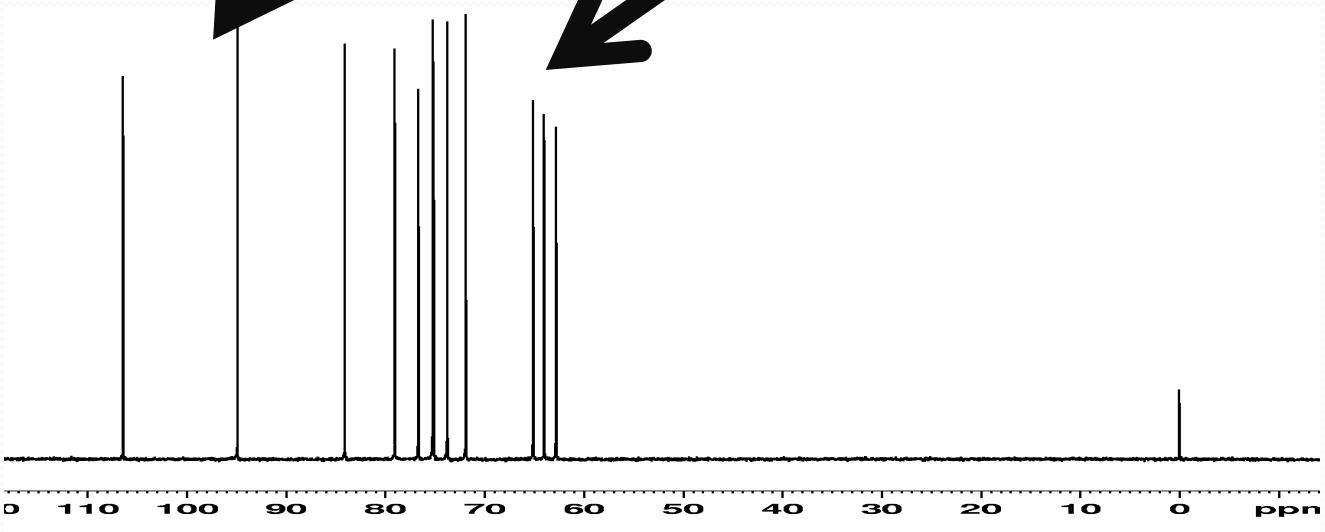
Anomeric region



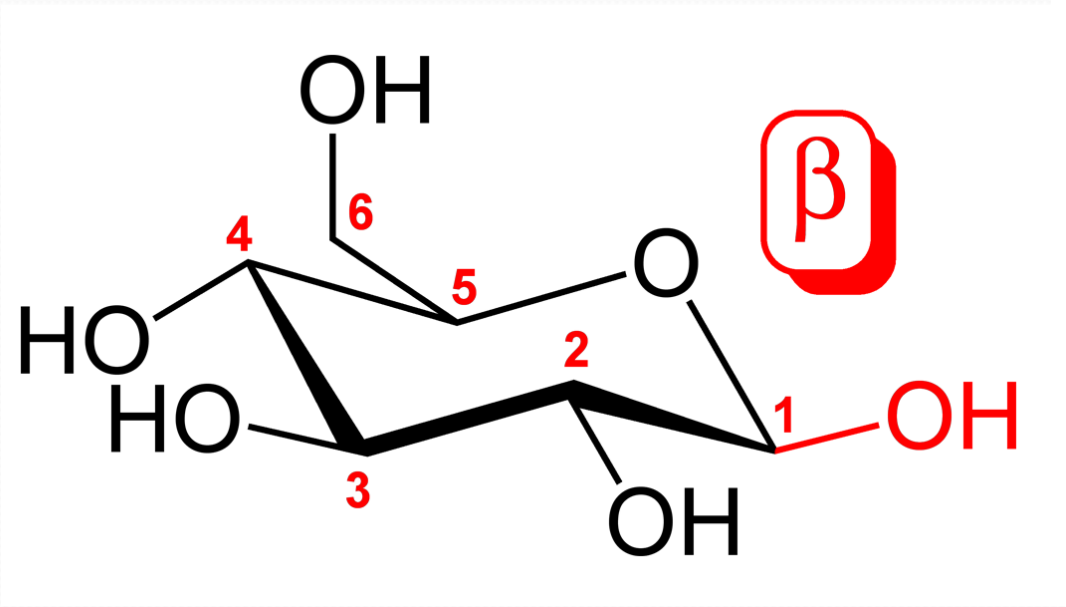
^{13}C

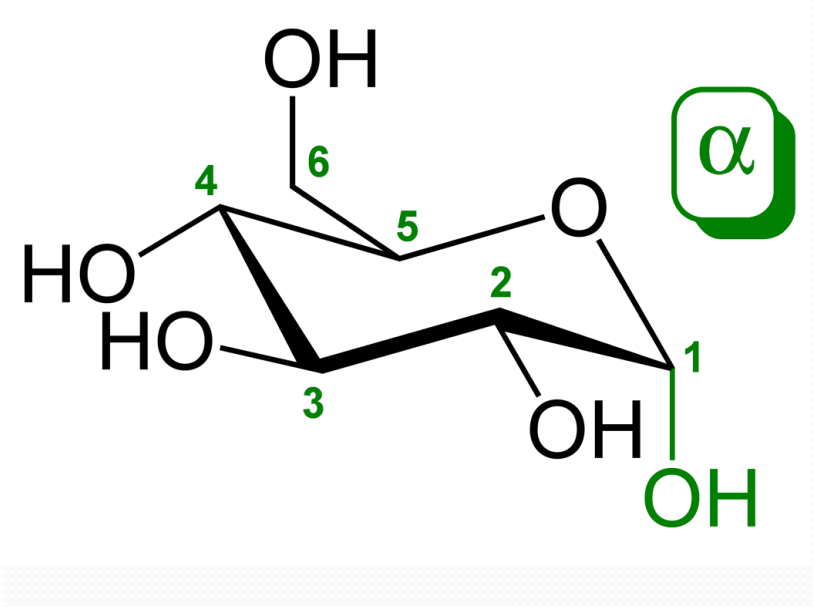
Anomeric region

Ring proton region

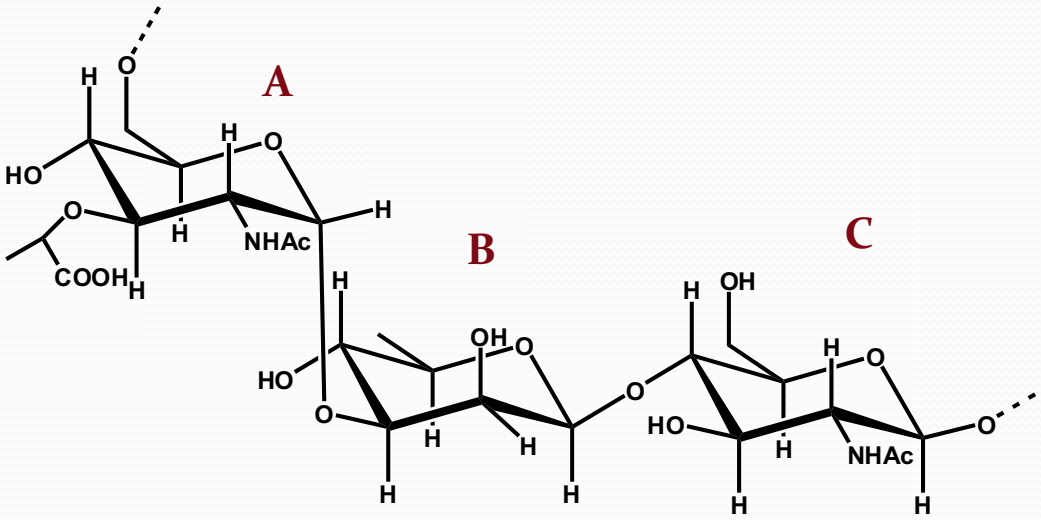


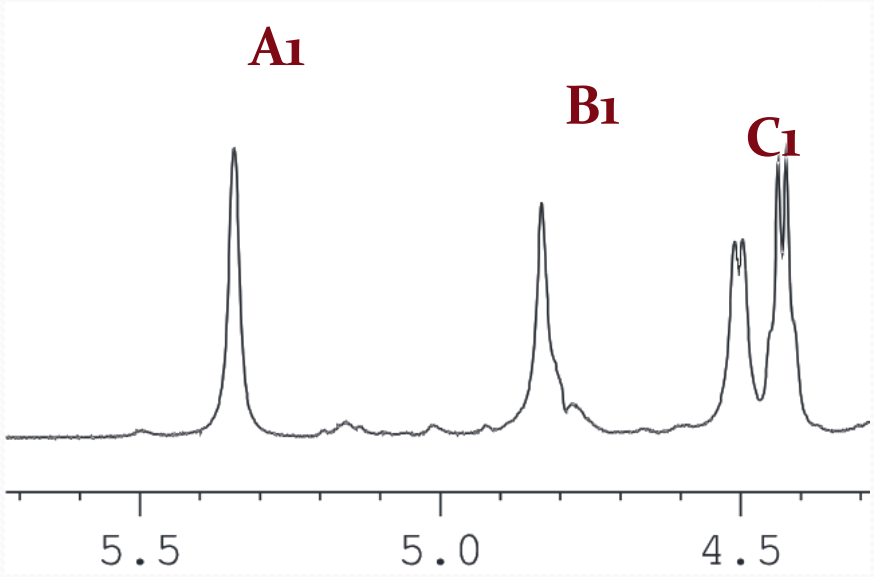
❖Anomeric configuration

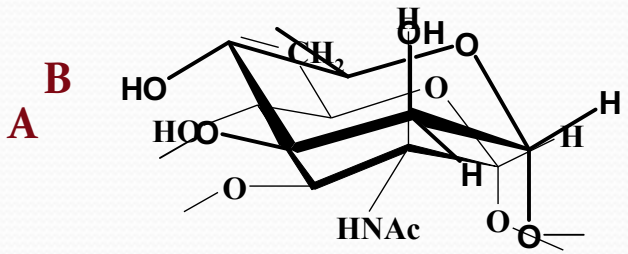




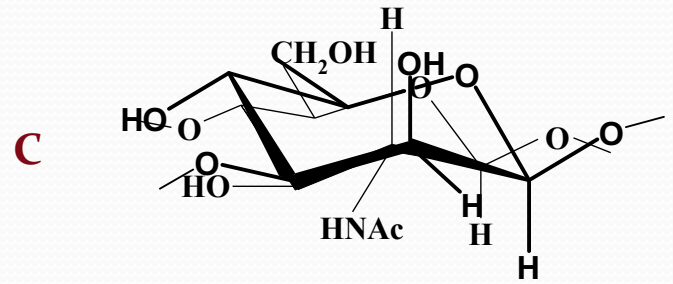
^{1}H NMR spectrum contains information on the configuration of glycosidic linkages







β-linkage: $J_{1,2} \leq 4$ Hz



β-linkage: $J_{1,2} > 6$ Hz

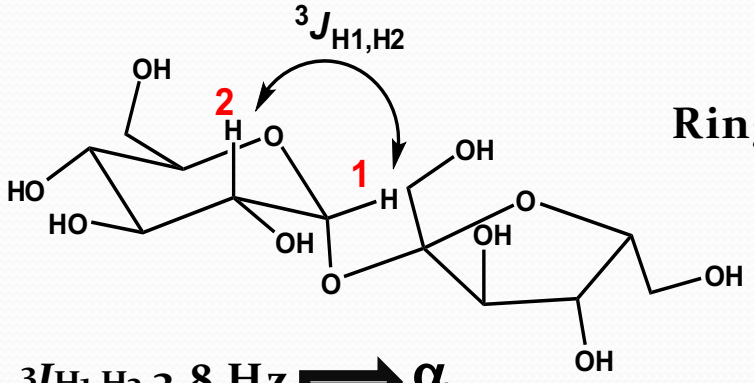
α-linkage: $J_{1,2} < 2$ Hz

α-linkage: $^1J_{C1,H1}$ 170-175 Hz

β-linkage: $^1J_{C1,H1}$ 160-165 Hz

Sucrose

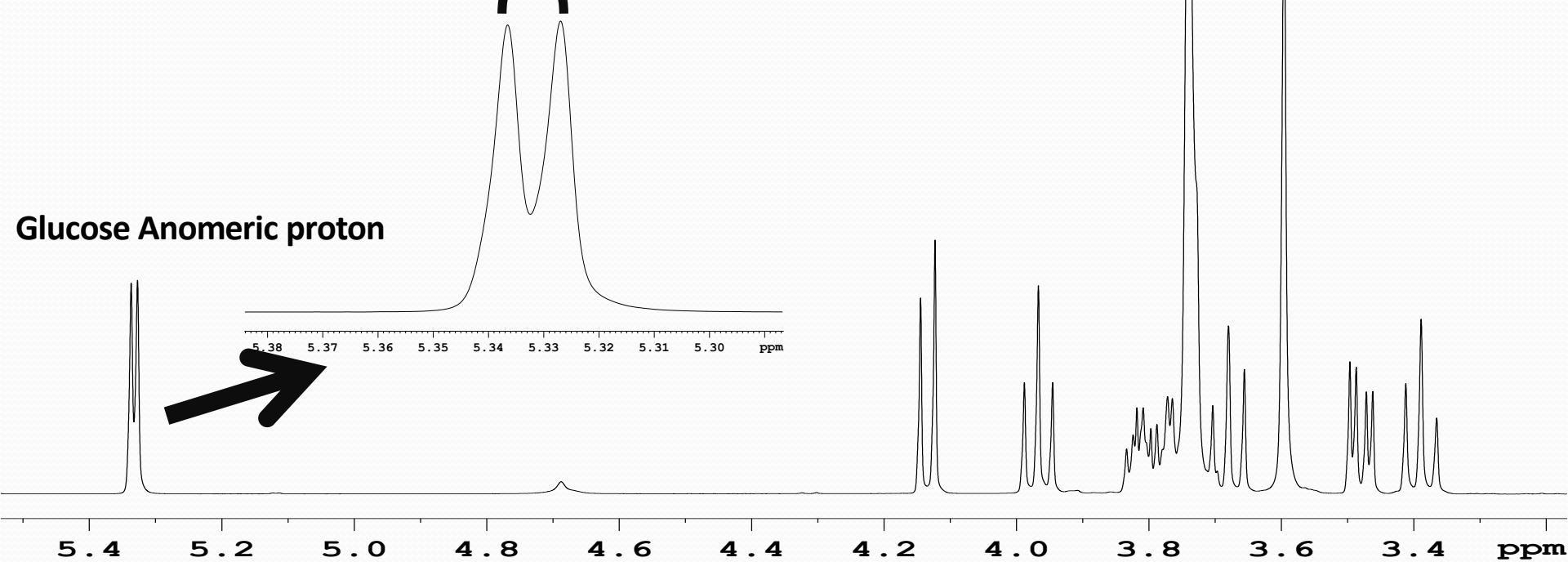
^1H



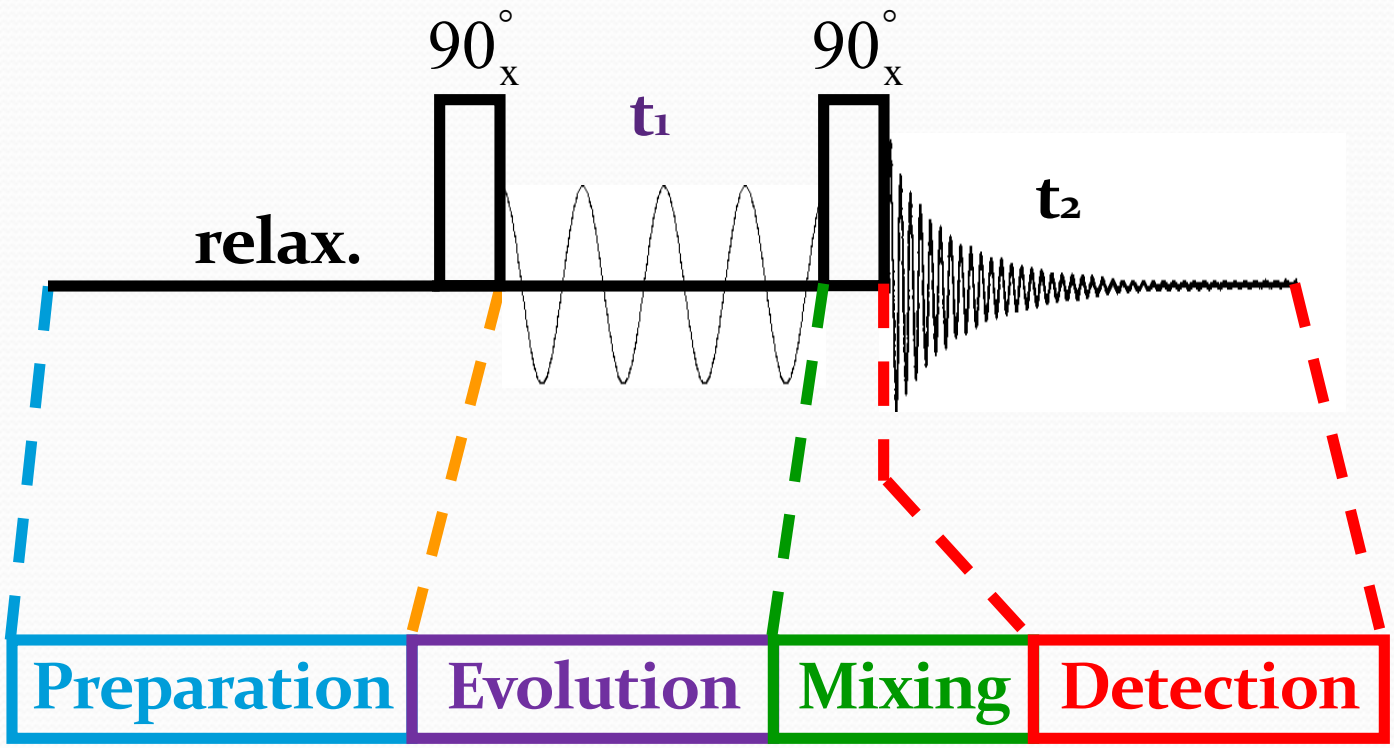
Ring proton region

$^3J_{H1,H2}$ 3.8 Hz ⟹ α

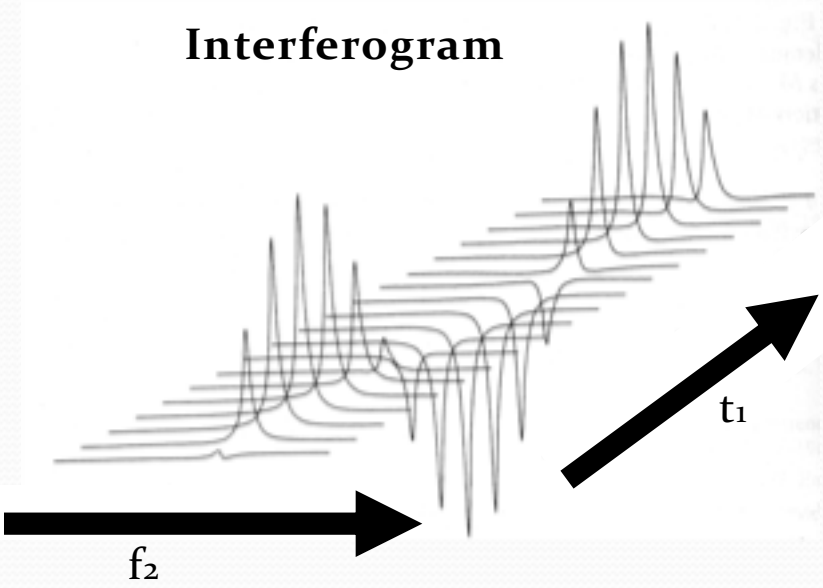
Glucose Anomeric proton



Anatomy of a 2D NMR Experiment



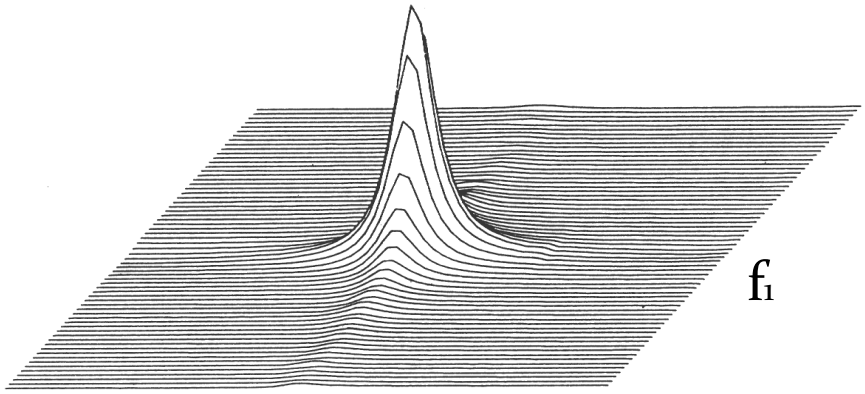
2D NMR - The Interferogram



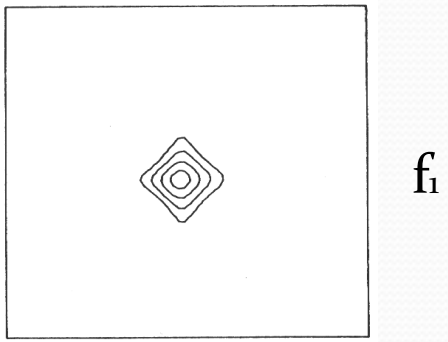
A 2D data set can be thought of as a series of 1D . Each 1D file is different from the next by a change in t_1.

Fourier transformation of each 1D in the t2 domain creates an interferogram.

2D plot of data



Contour plot.

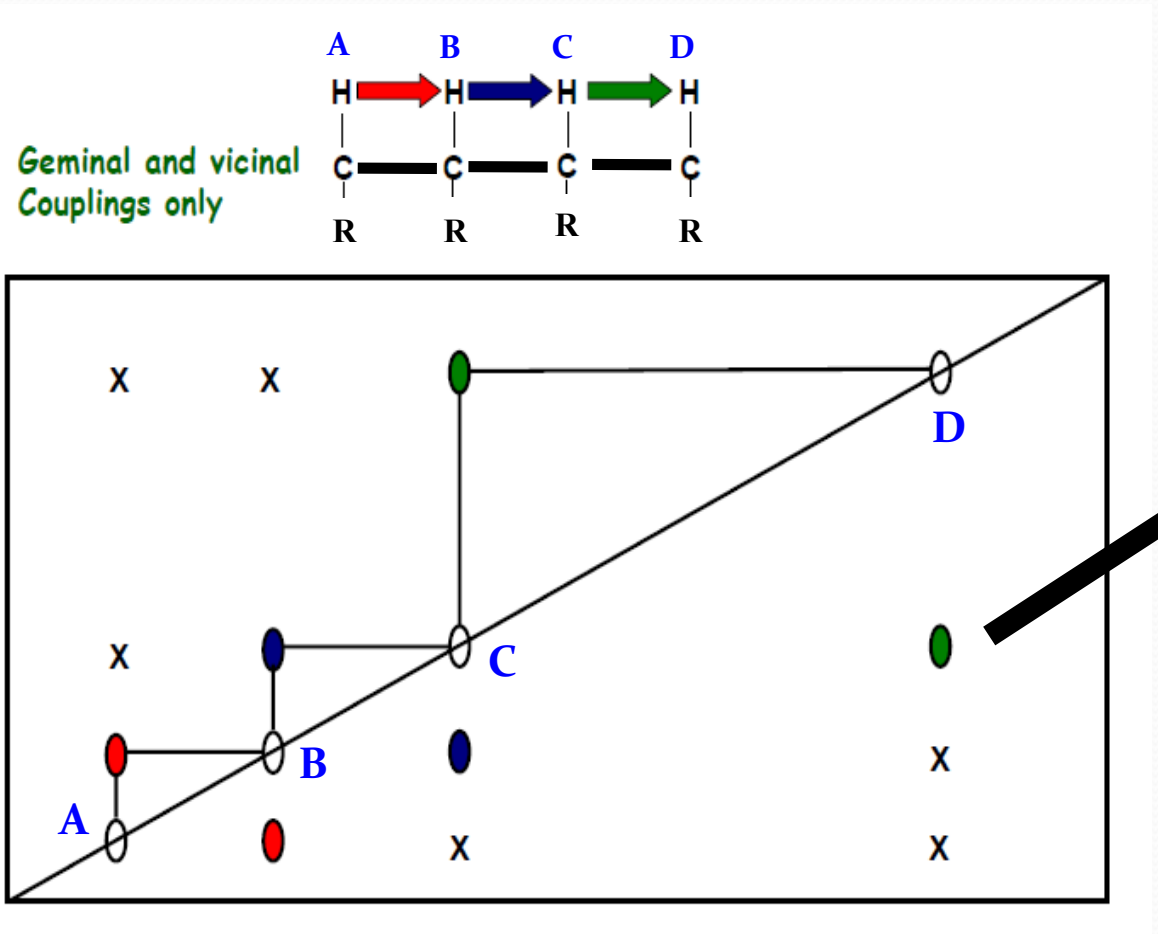


Two Dimensional NMR

- ✓A 2D data set can be thought of as a series of 1D experiments collected with different timing.
- ✓Fourier transformation of each 1D in the t2 domain creates an interferogram.
- ✓The t1 domain is then Fourier transformed resulting in a 2D file with the frequency in each dimension.
- ✓This 2D file will provide a map of all spin-to-spin correlations
- ✓Each 2D experiment can provide either through bond (COSY type) or through space (NOESY type) correlation

COrrelation SpectroscopY (COSY)

In a 2D COSY spectrum, cross-peaks will exist where there is spin-spin coupling between nuclei.

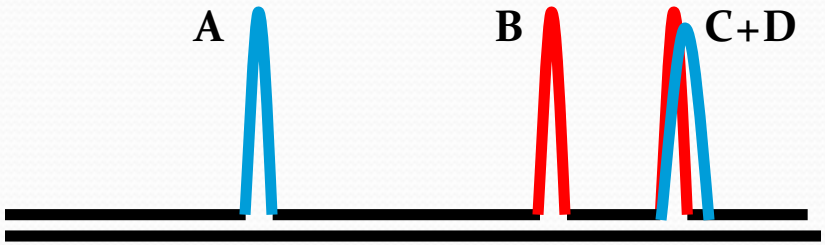


Used to identify spins which are coupled to each other.

2D Experiments – COSY

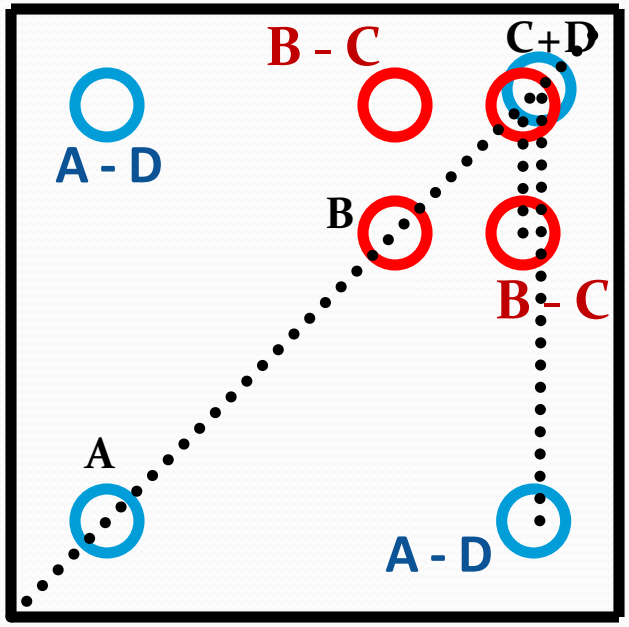
The Power of 2D NMR:
Resolving Overlapping Signals

1D



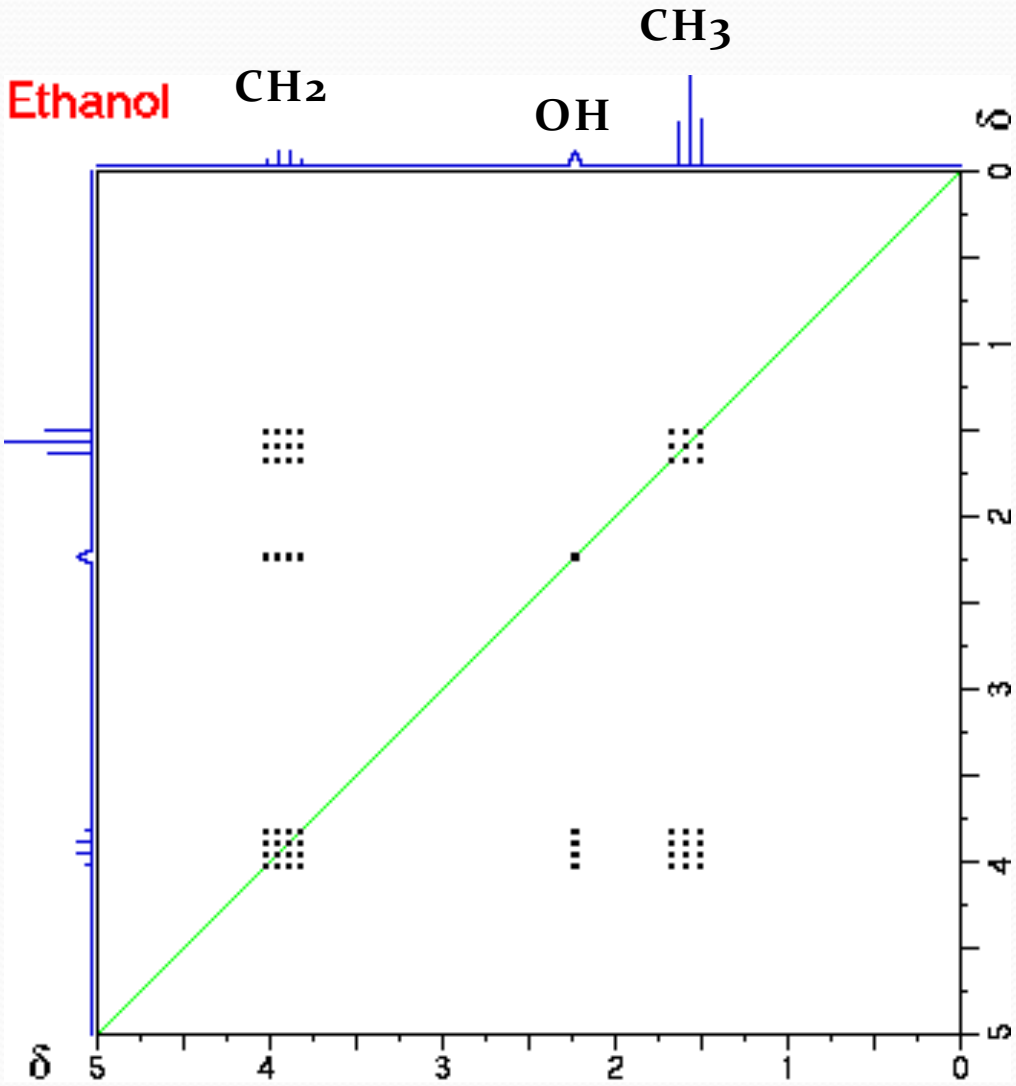
2 signals overlapped

2D



2 cross peaks resolved

2D Experiments – COSY

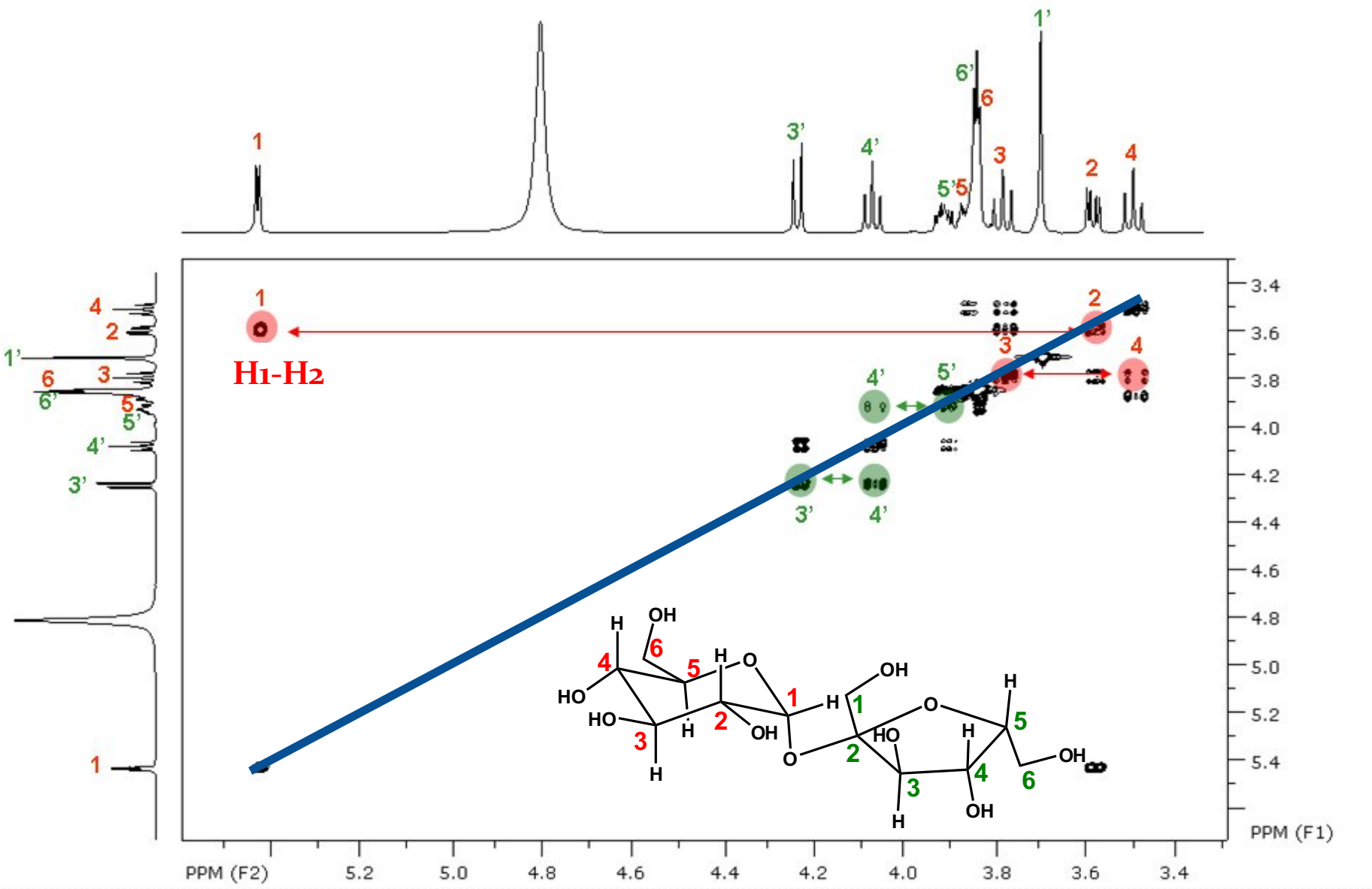


Cross peaks due to geminal and vicinal coupling

CH_3-CH_2-OH

Sugar assignment

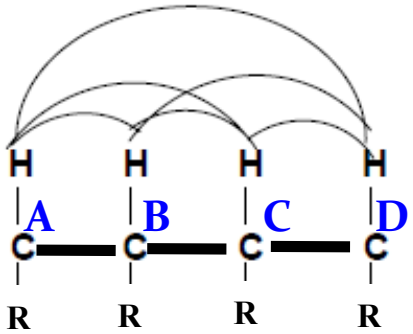
COSY of sucrose



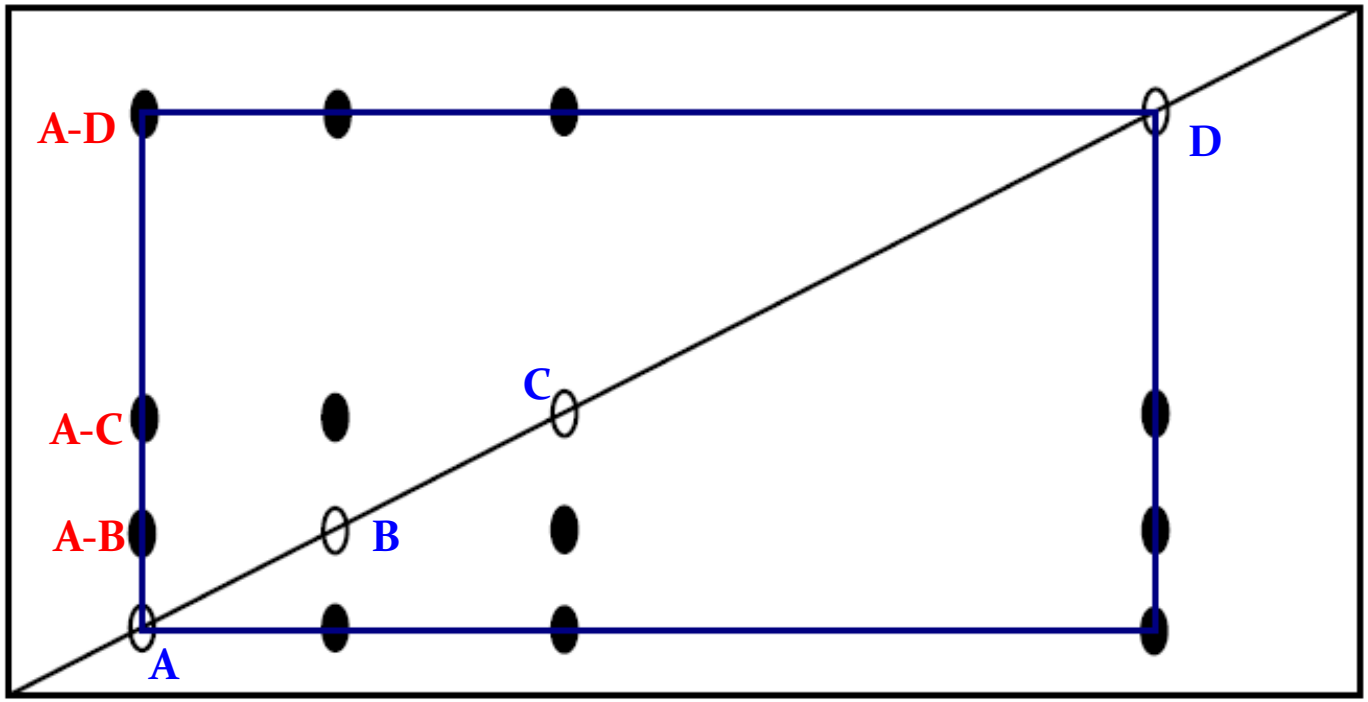
TOtal Correlation SpectroscopY (TOCSY) experiment

TOCSY Experiment

In general, the TOCSY mixing time determines the number of bonds over which signal can be Transferred, assuming that none of the coupling Constants = 0

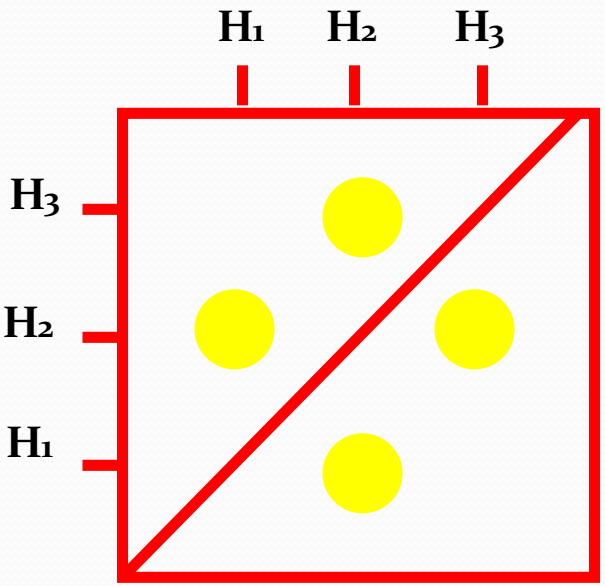


•Cross peaks generated between all members of a coupled spin network

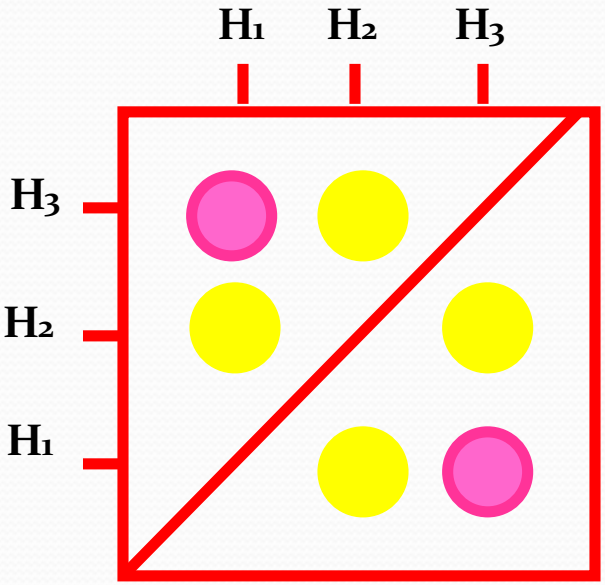


TOCSY

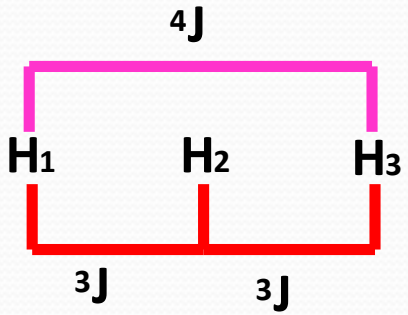
- Cross peaks are generated between all members of a coupled spin network
- Coherence transfer period occurs during a multi-pulse spin-lock period;
- Length of spin-lock and J-coupling constants determine how far the spin coupling network will be probed

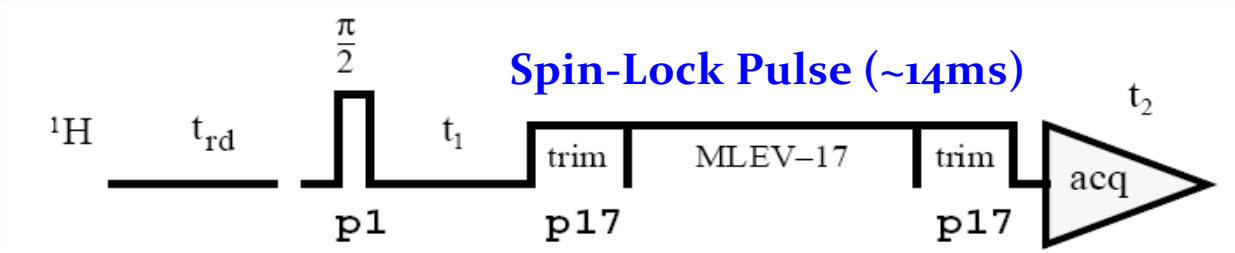


COSY

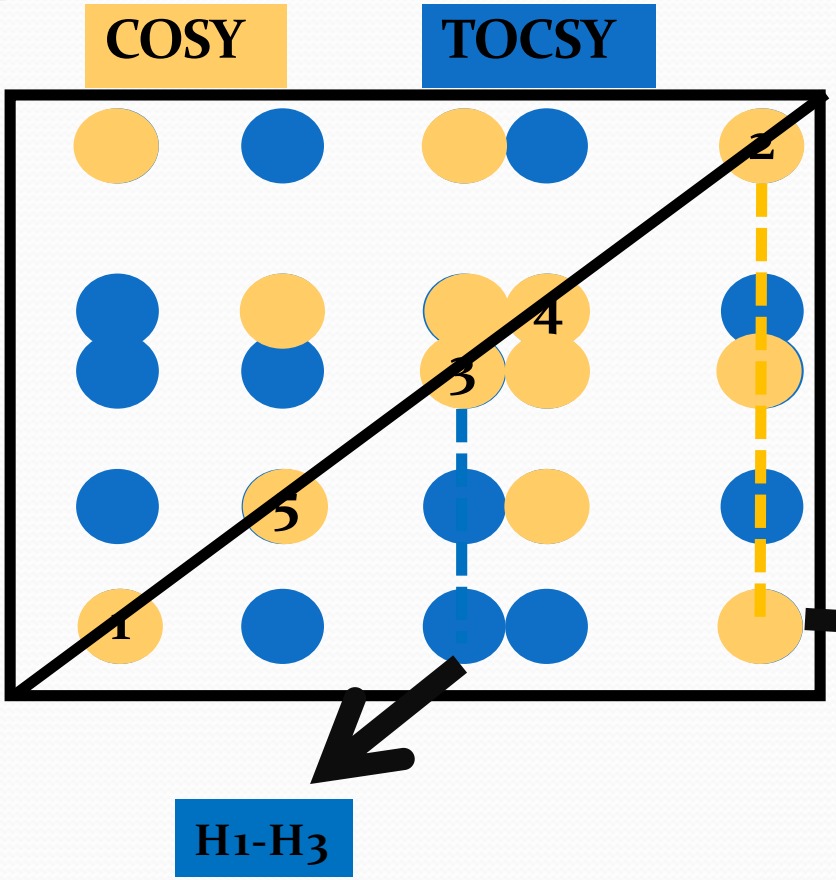


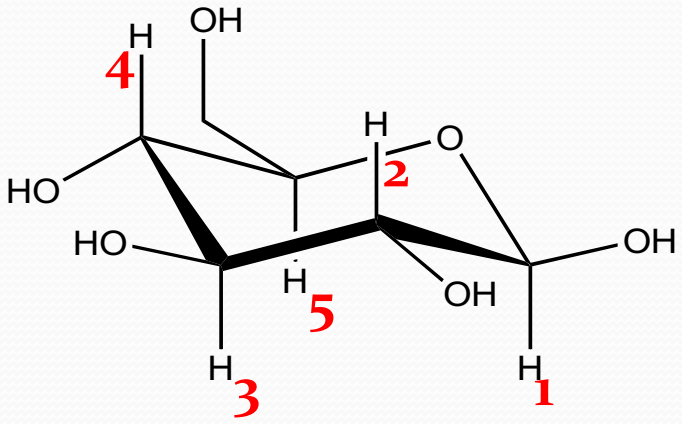
TOCSY





COSY and TOCSY – Sugar assignment





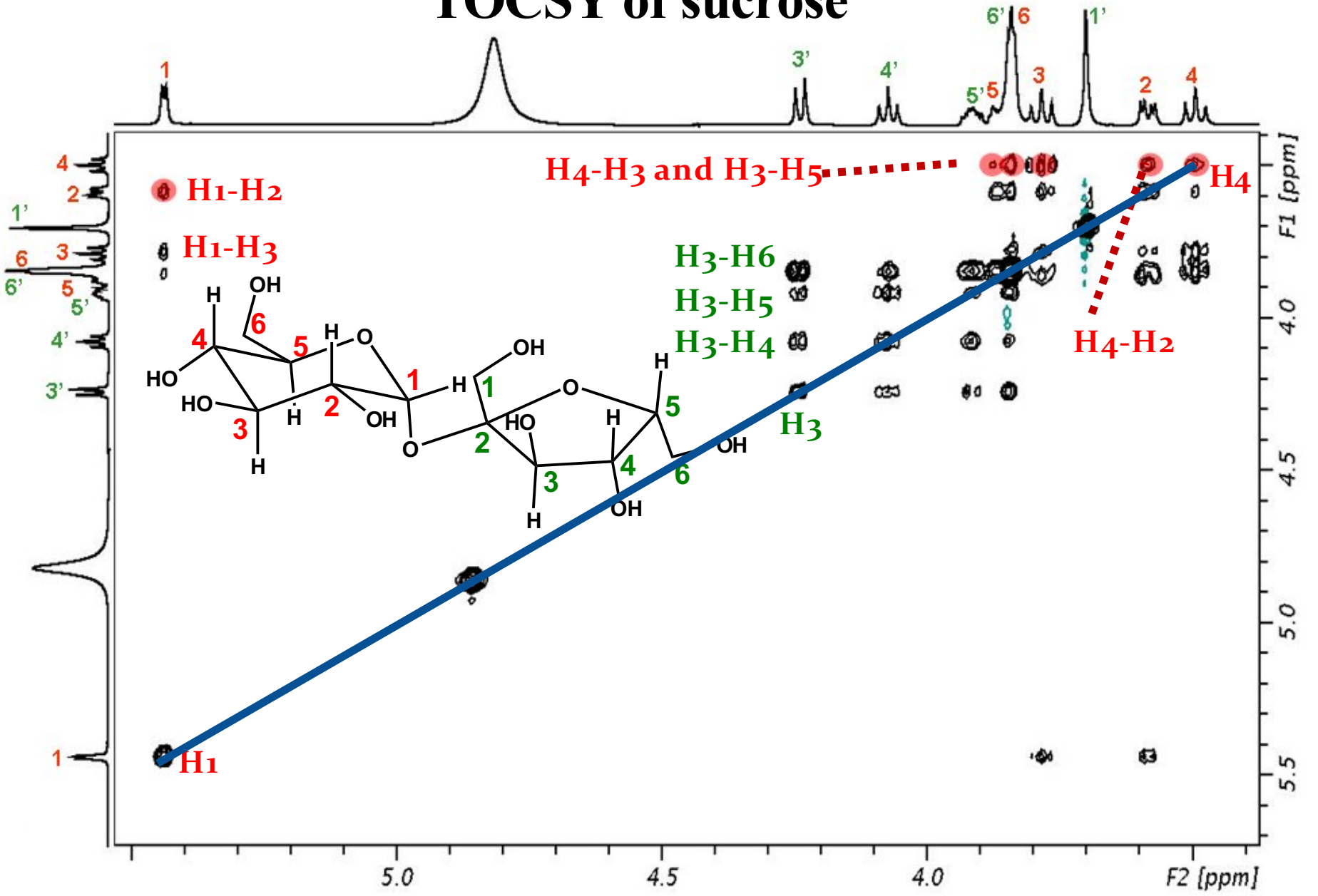
In Glucose, H1 and H2 protons are scalarly coupled, H1 and H3 are not.

In COSY spectra ⟶ H1 andH2 correlation observed ;

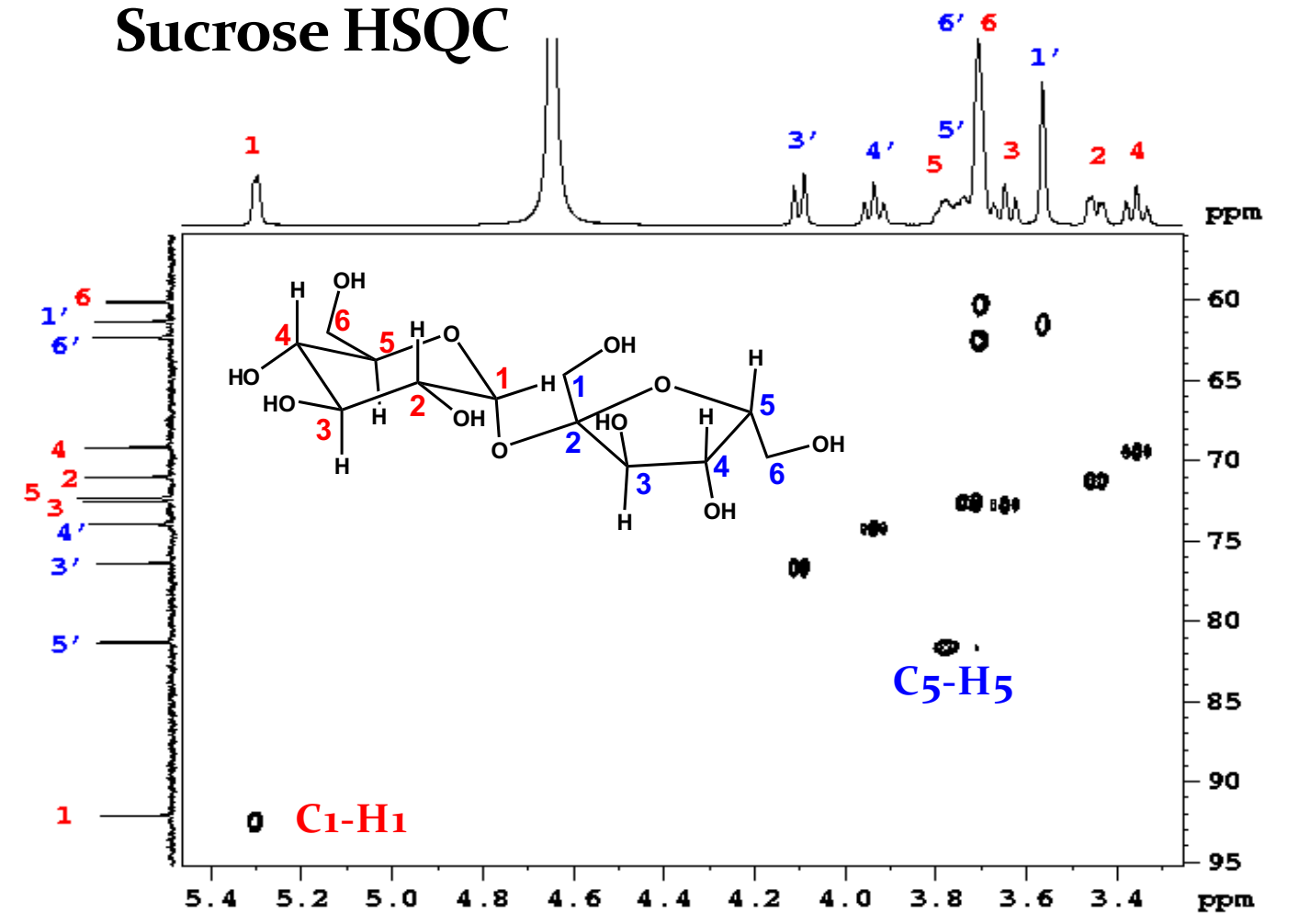
In TOCSY spectra ⟶ H1 and H3 observed

COSY and TOCSY – Sugar assignment

TOCSY of sucrose



HSQC: Heteronuclear Single-Quantum Correlation

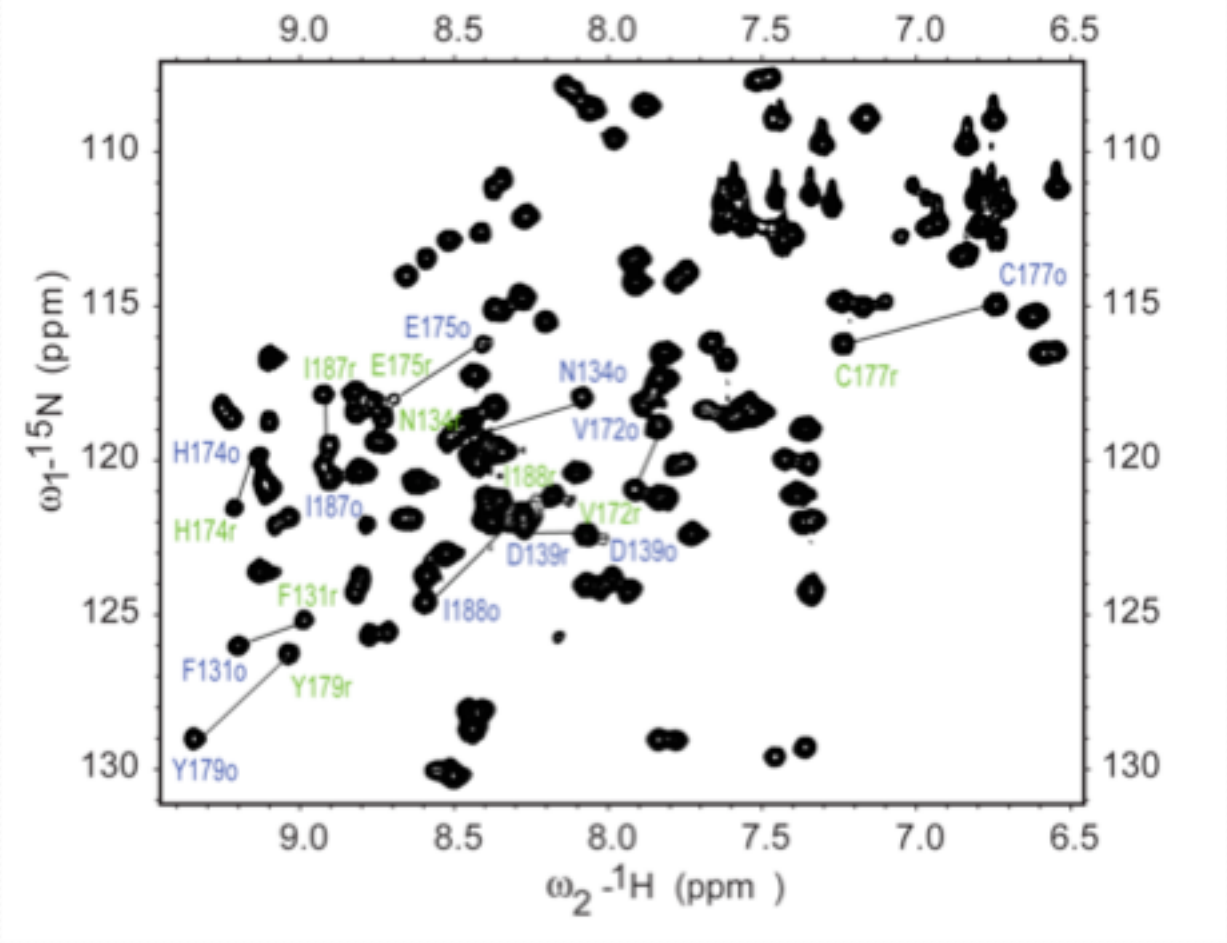


The spectrum contains a peak for each unique proton attached to the heteronucleus being considered.

The **2D HSQC experiment** permits to obtain a 2D heteronuclear chemical shift correlation map between directly-bonded ^{1}H and X-heteronuclei (an atomic nucleus other than a proton), often ^{13}C or ^{15}N.

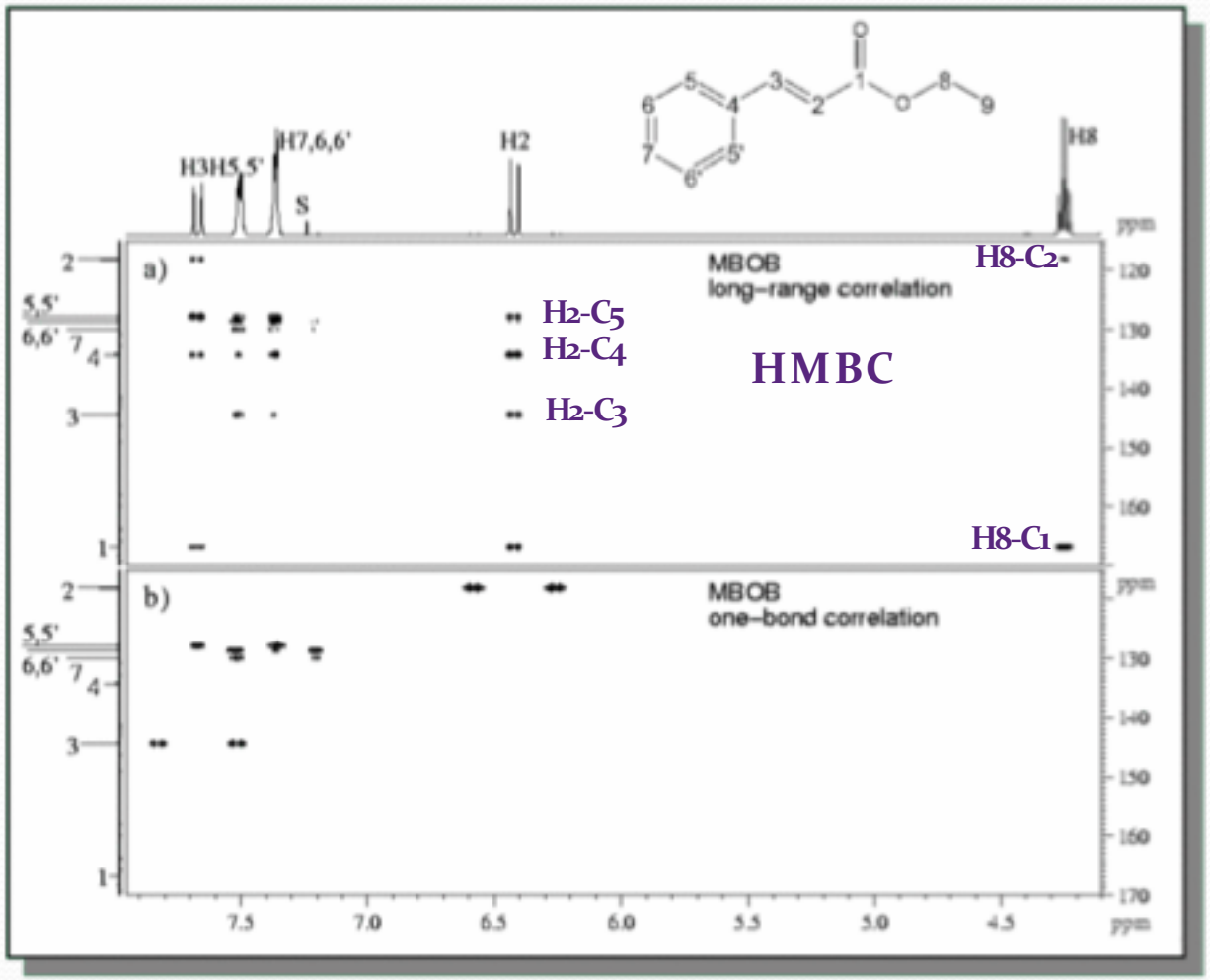
HSQC: Heteronuclear Single-Quantum Correlation

$^{1}H-^{15}N$ HSQC spectrum of a fragment of the protein NleG3-2.
Each peak in the spectrum represents a bonded N-H pair, with its two coordinates corresponding to the chemical shifts of each of the H and N atoms. Some of the peaks are labeled with the amino acid residue that gives that signal

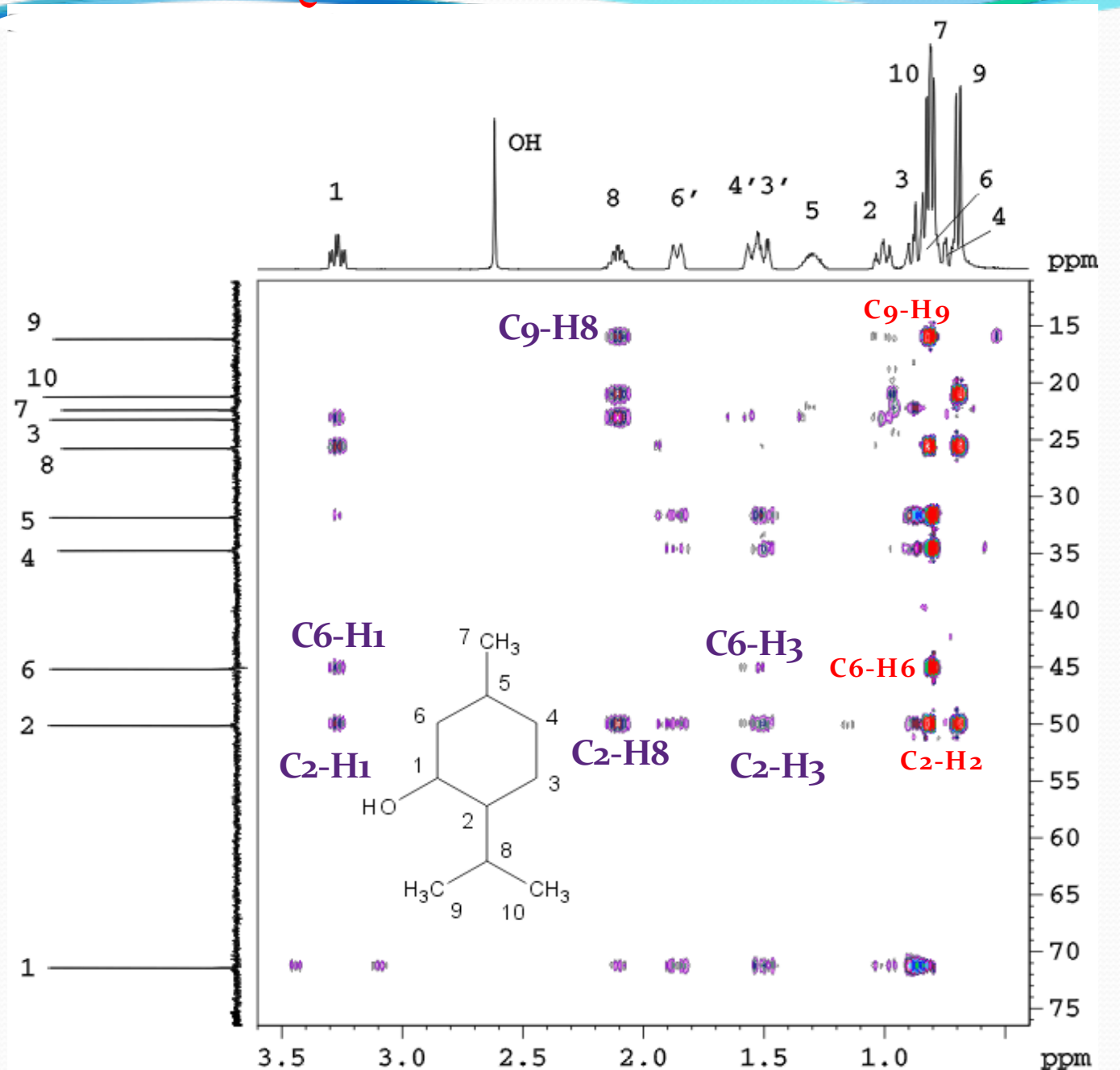


HMBC (Heteronuclear Multiple Bond Correlation)

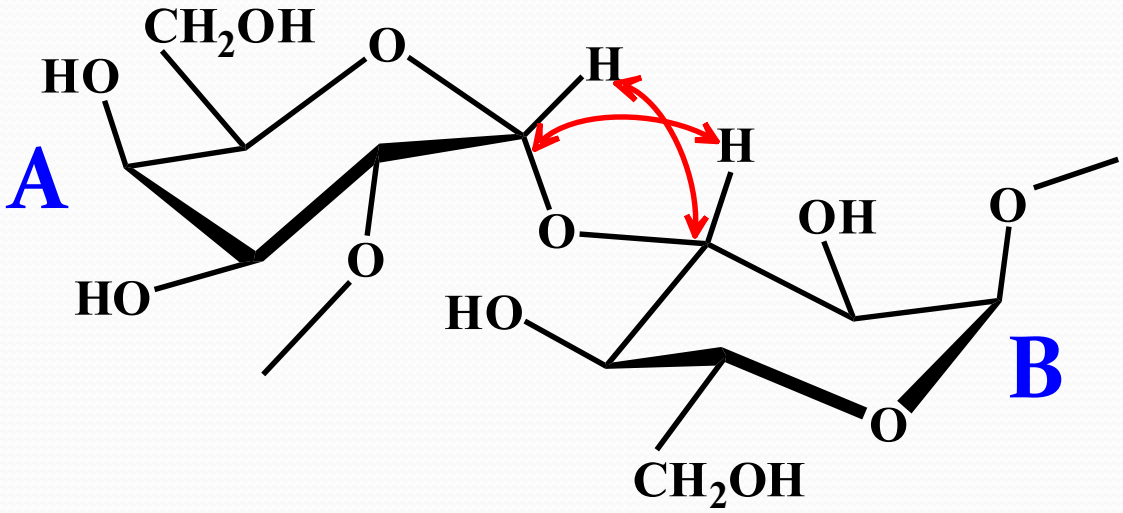
2D HMBC experiment correlates chemical shifts of two types of nuclei **separated from each other with two or more chemical bonds.**



HSQC and HMBC of Menthol



Long range *inter*-residual correlations in the HMBC spectrum

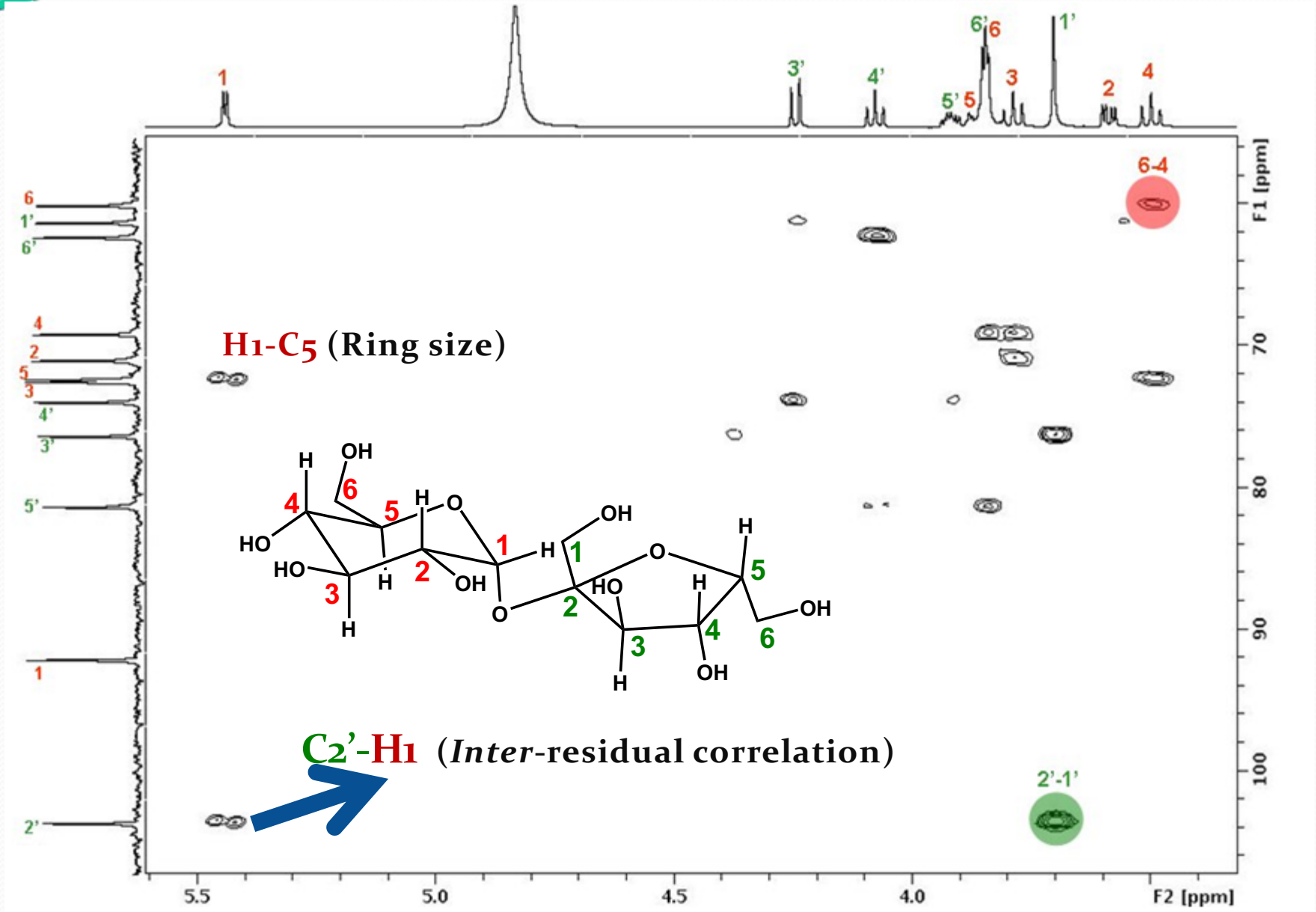


A1 – B3 long range correlations
(H1 A – C3 B and C1 A – H3 B)

A1- B3 linkage / A and B are 1-3 linked

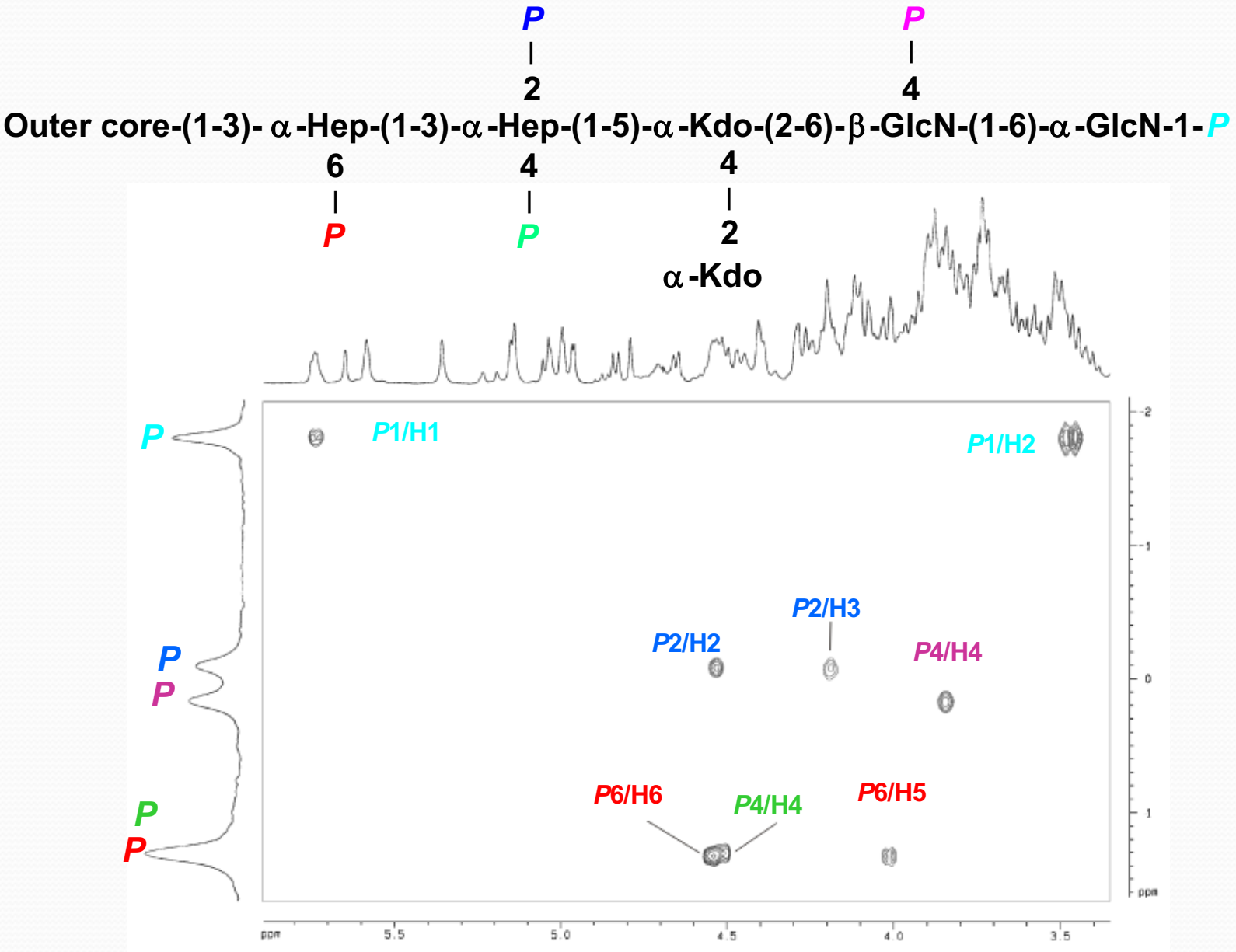
Sugar Assignment, Ring size, Linkage analysis

HMBC of Sucrose



Sugar Assignment

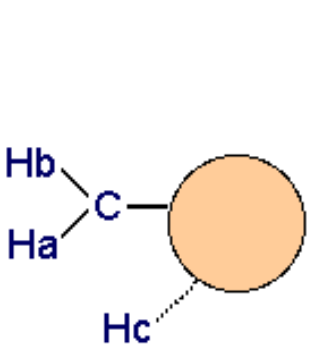
$^{1}H,^{31}P$ HMBC spectrum of the deacylated core-lipid A backbone of *P. aeruginosa*

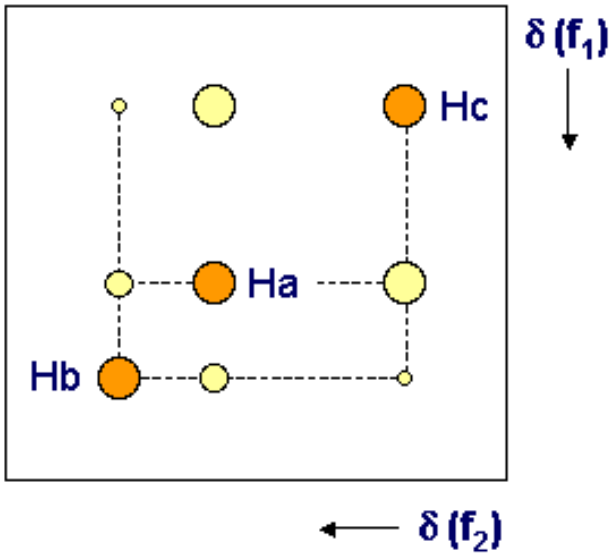


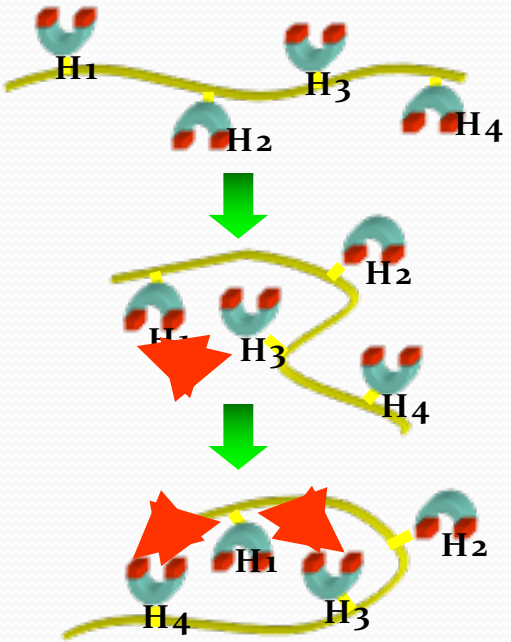
Nuclear Overhauser Effect (NOE) Spectroscopy

The 2D spectrum will have chemical shifts in f1 and f2.

The cross peaks are for nuclei that are dipolar coupled.

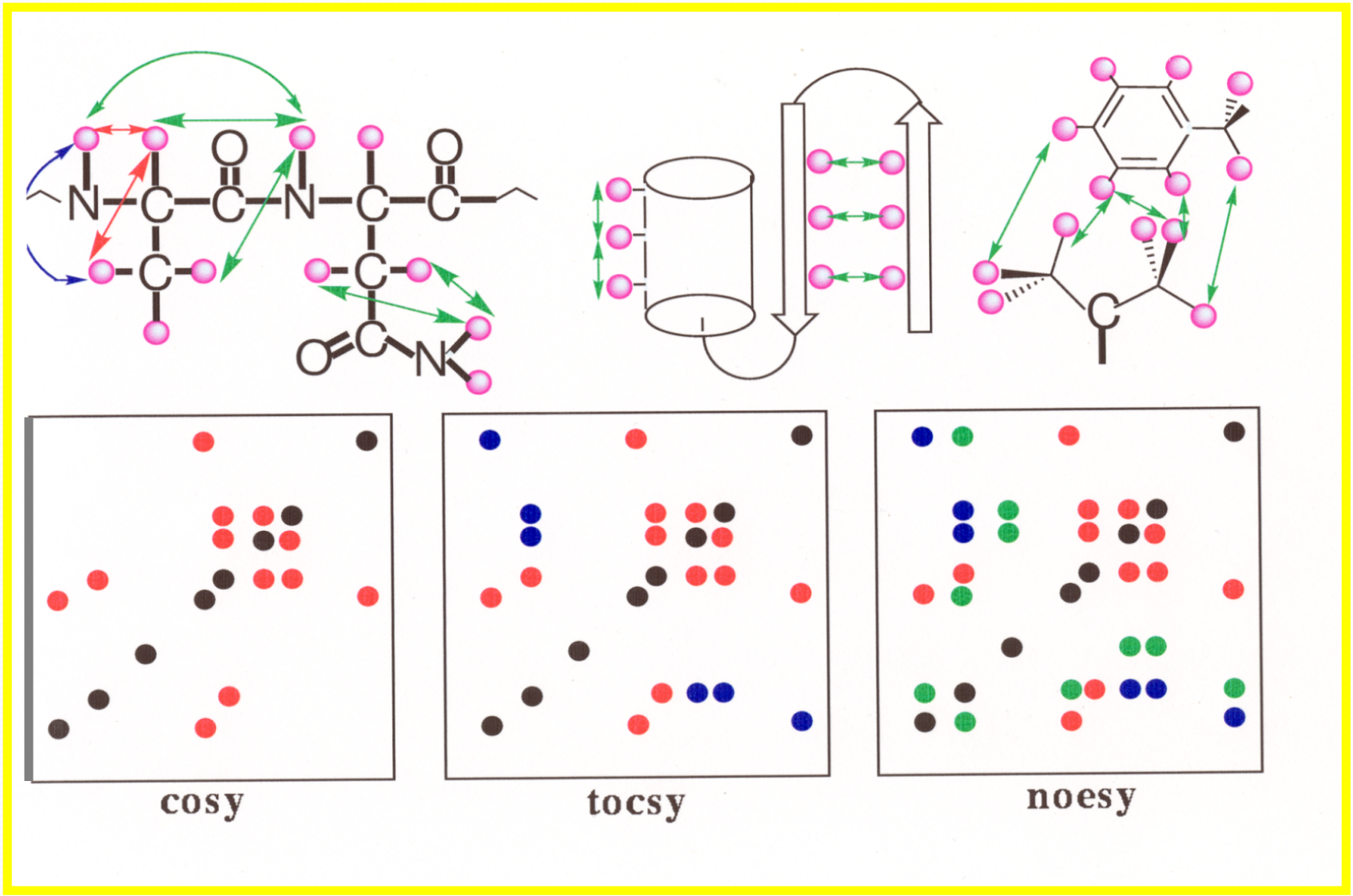




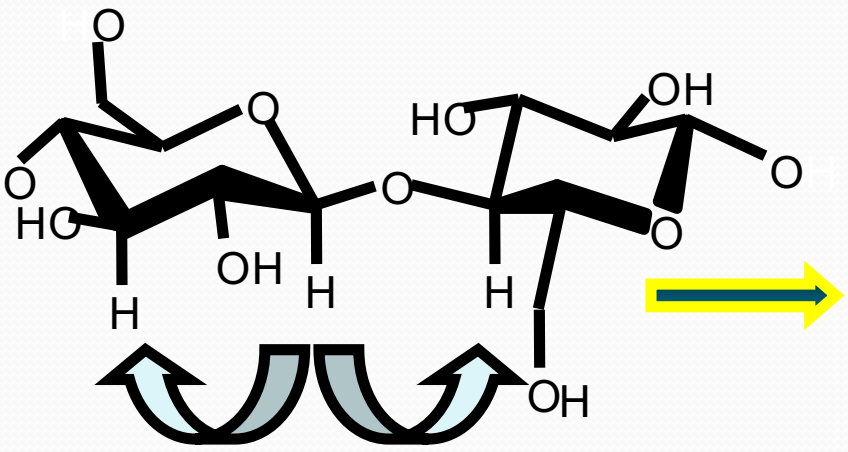


NOE contact: C is close in space to spin A

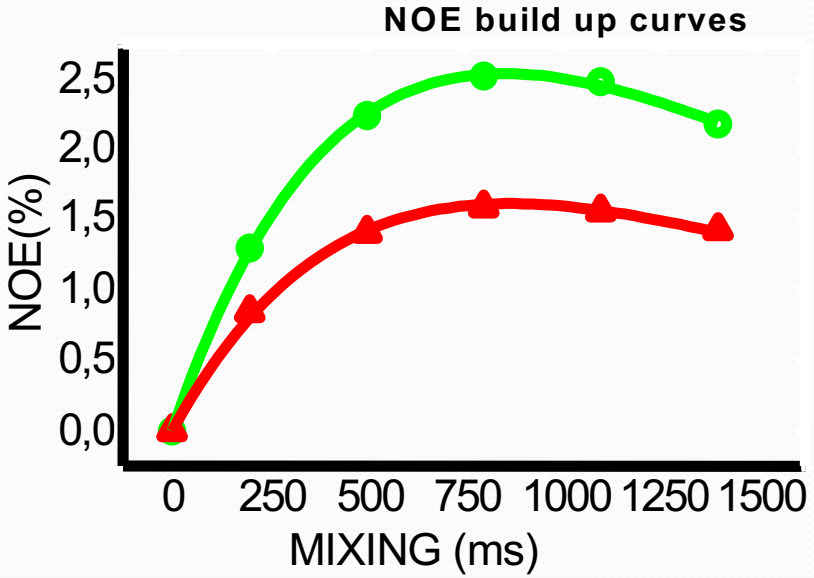
The NOE effect is the method for the elucidation of 3D structural features and stereochemistry



NOE and Distances
Isolated spin pair aproximation (ISPA)



NOE H1'-H3' **NOE H1'-H4**

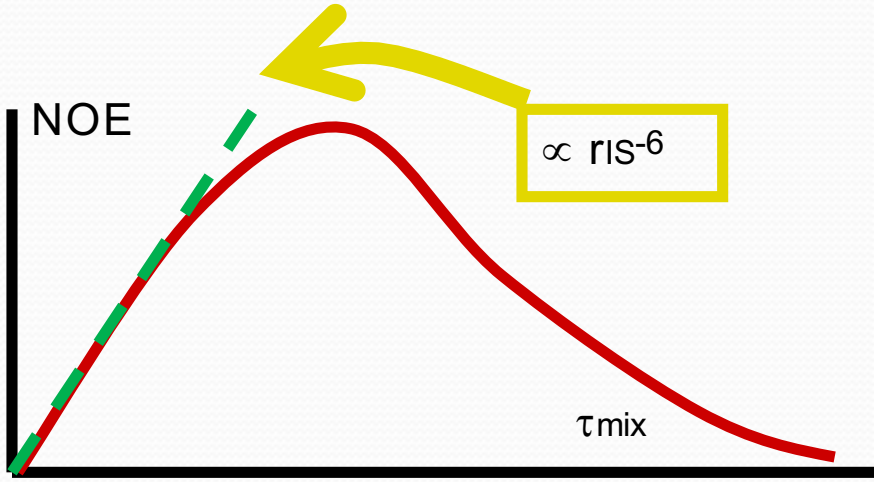


$$\frac{\sigma_{H1'-H4}}{\sigma_{H1'-H3'}} = \frac{r^{-6}_{H1'-H4}}{r^{-6}_{H1'-H3'}}$$

$\eta_{ab} \propto r_{ab}^{-6}$

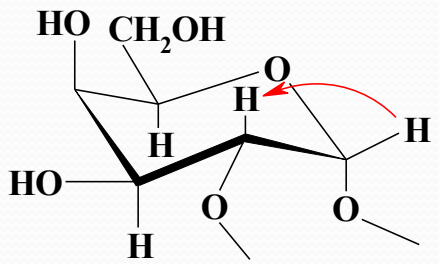
$\eta_{ac} \propto r_{ac}^{-6}$

$$r_{ac} = r_{ab} * (\eta_{ab} / \eta_{ac})^{-1/6}$$

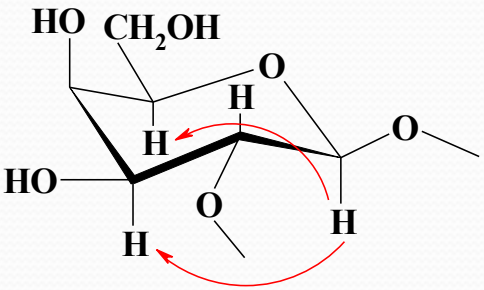


Intra-residue NOE contacts in monosaccharides: relative configuration of sugar residues

gluco, galacto configuration

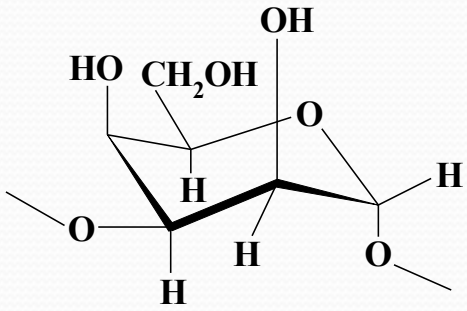


α-linkage: H1/H2

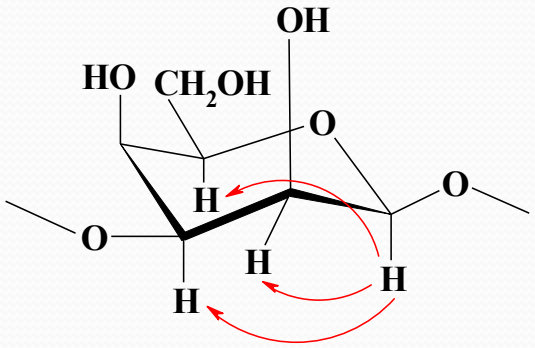


β-linkage: H1/H3, H1/H5

manno configuration



α-linkage: no contact

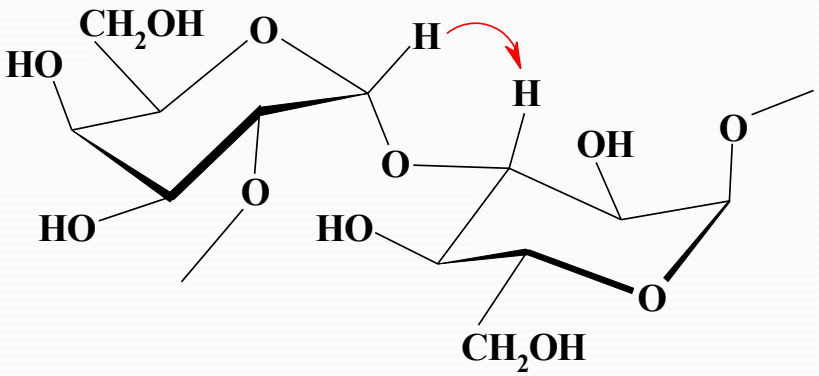


β-linkage: H1/H2, H1/H3, H1/H5

Monosaccharide Sequence

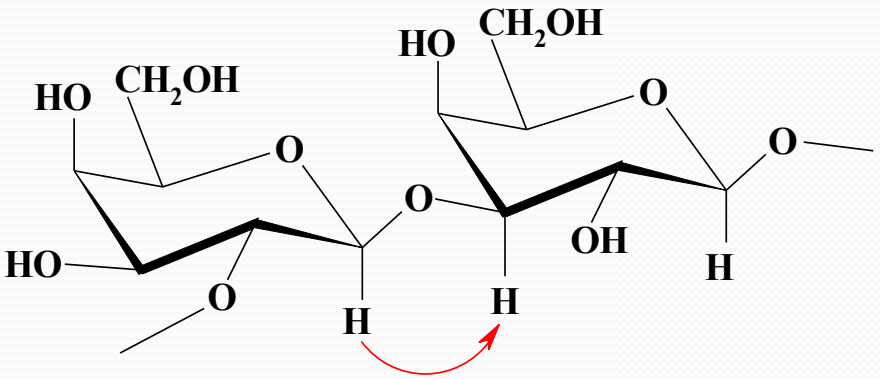
- ***Inter*-residue NOE**
- Glycosylation shift (HSQC spectrum)
- Inter-residual long range correlation (HMBC spectrum)

Inter-residue NOE contacts in saccharides (Linkage analysis)



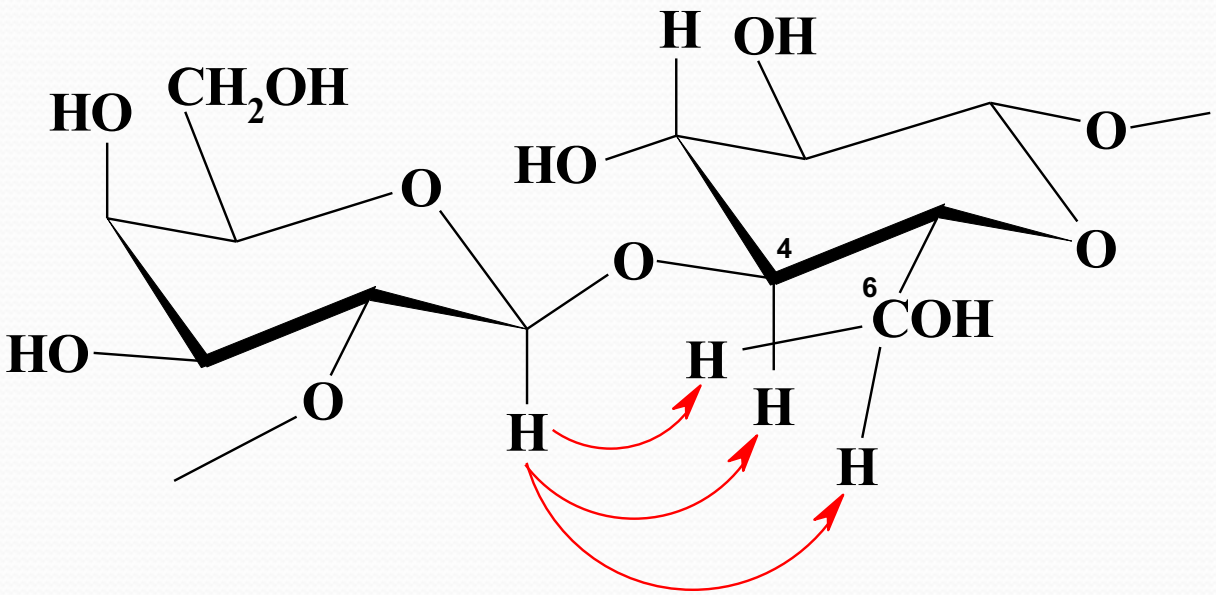
α-(1-3)linkage

β-(1-3) linkage



Sugar sequence - Linkage analysis

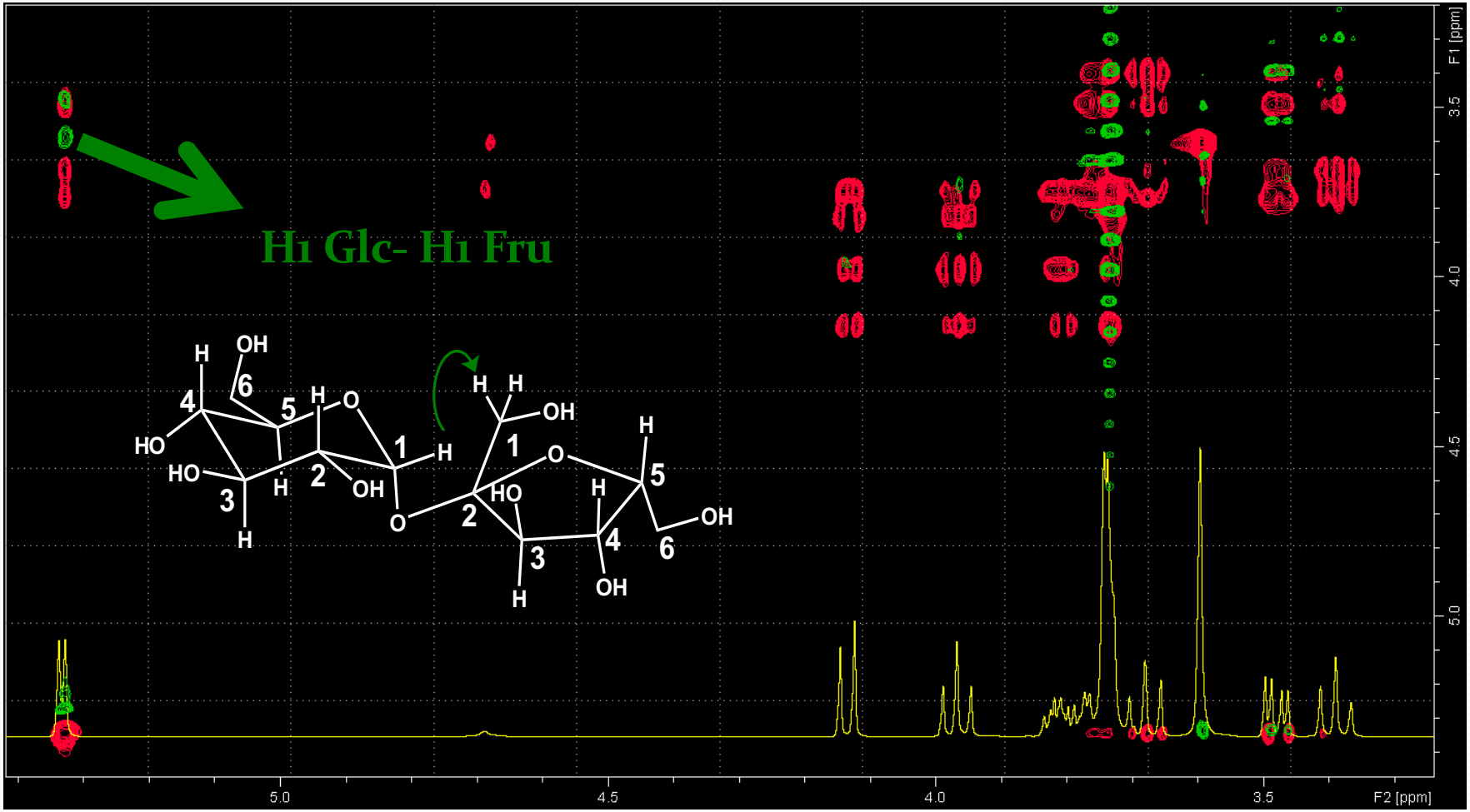
NOE in disaccharides may occur not only at the linkage protons but also at the neighbouring protons



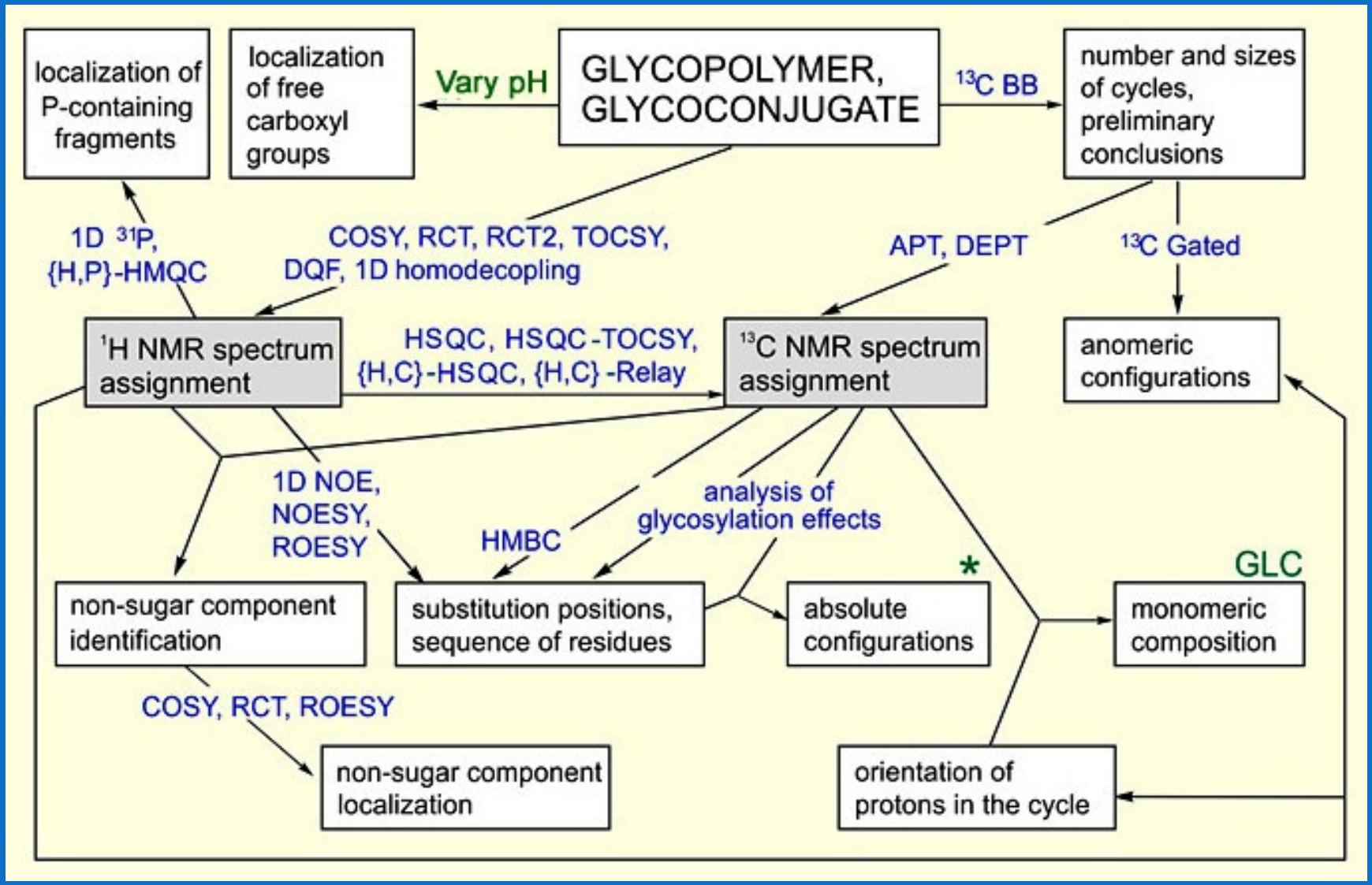
....Saccharide conformation...

Sugar sequence - Linkage analysis

Sucrose NOESY and TOCSY

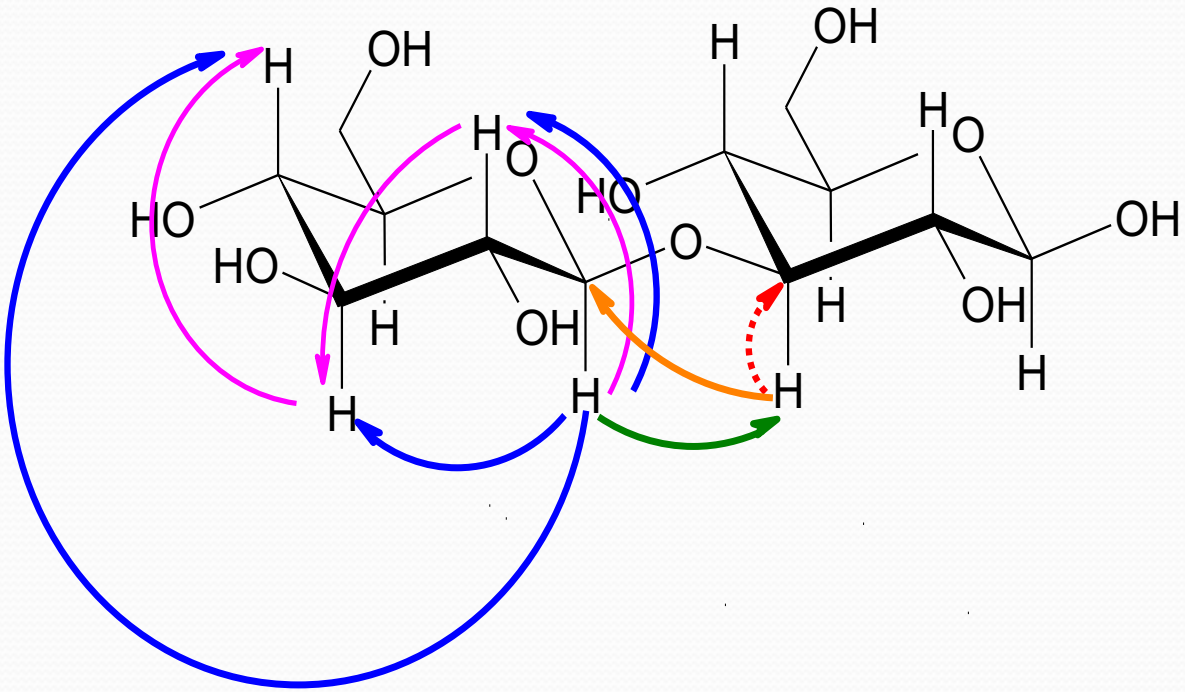


A labirinth?


GLYCOPOLYMER, GLYCOCONJUGATE
Vary pH
localization of free carboxyl groups
localization of P-containing fragments
^{13}C BB
number and sizes of cycles, preliminary conclusions
1D ^{31}P, {H,P}-HMQC
COSY, RCT, RCT2, TOCSY, DQF, 1D homodecopling
APT, DEPT
^{13}C Gated
^{1}H NMR spectrum assignment
HSQC, HSQC-TOCSY, {H,C}-HSQC, {H,C}-Relay
^{13}C NMR spectrum assignment
anomeric configurations
1D NOE, NOESY, ROESY
HMBC
analysis of glycosylation effects
*
GLC
non-sugar component identification
substitution positions, sequence of residues
absolute configurations
monomeric composition
COSY, RCT, ROESY
non-sugar component localization
orientation of protons in the cycle

Application of various NMR techniques to carbohydrates

- **HOMONUCLEAR (^{1}H-^{1}H)**
- **HETERONUCLEAR (^{1}H-^{13}C)**



HOMONUCLEAR ^{1}H-^{1}H

COSY

TOCSY

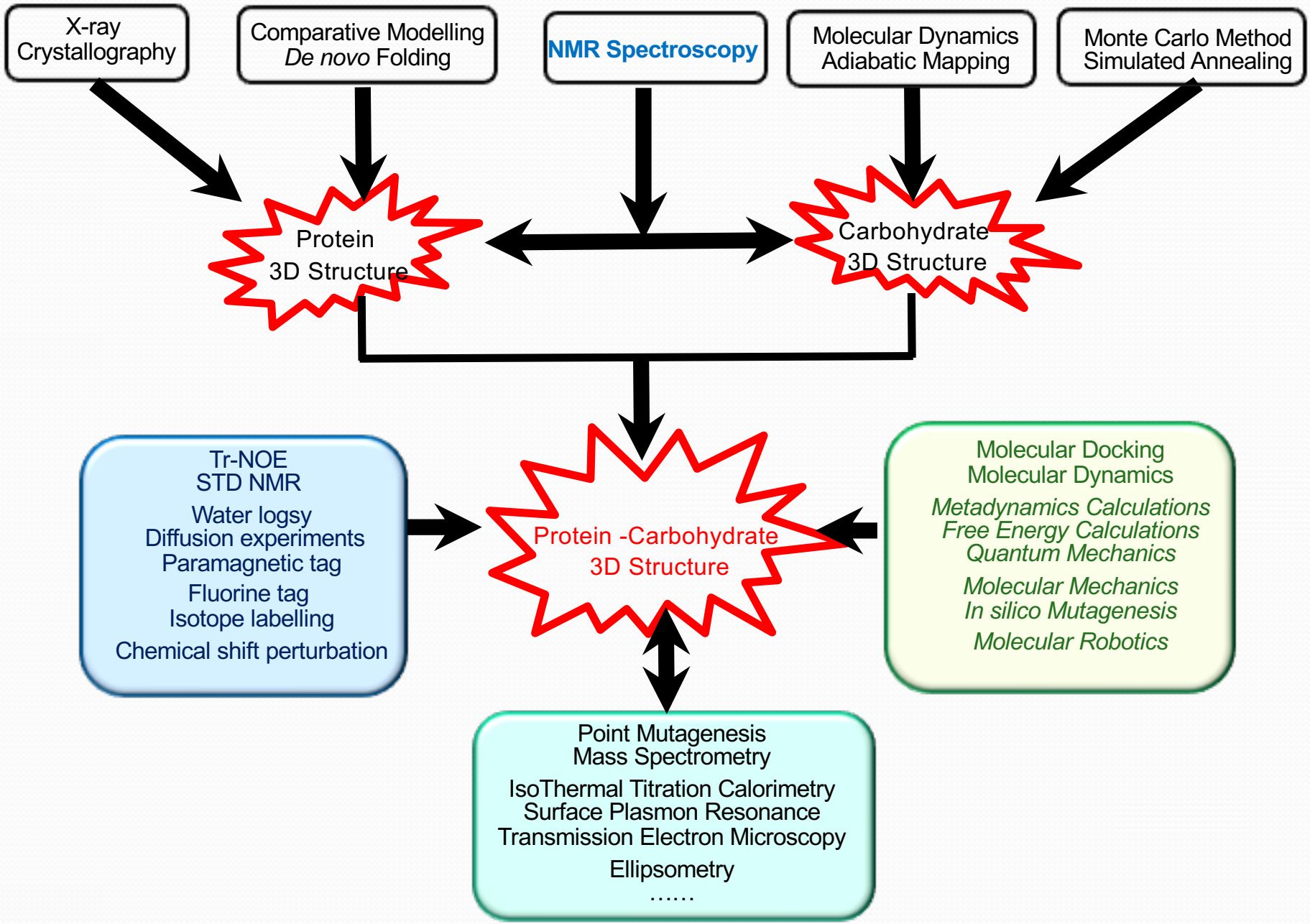
NOESY/ROESY

HETERONUCLEAR ^{1}H-^{13}C

HMQC/HSQC

HMBC

Interplay of NMR with other biophysical methods in the 3D structure determination of carbohydrates, proteins and proteins-glycoconjugates



MOLECULAR INTERACTION by NMR

The ligand-based approach

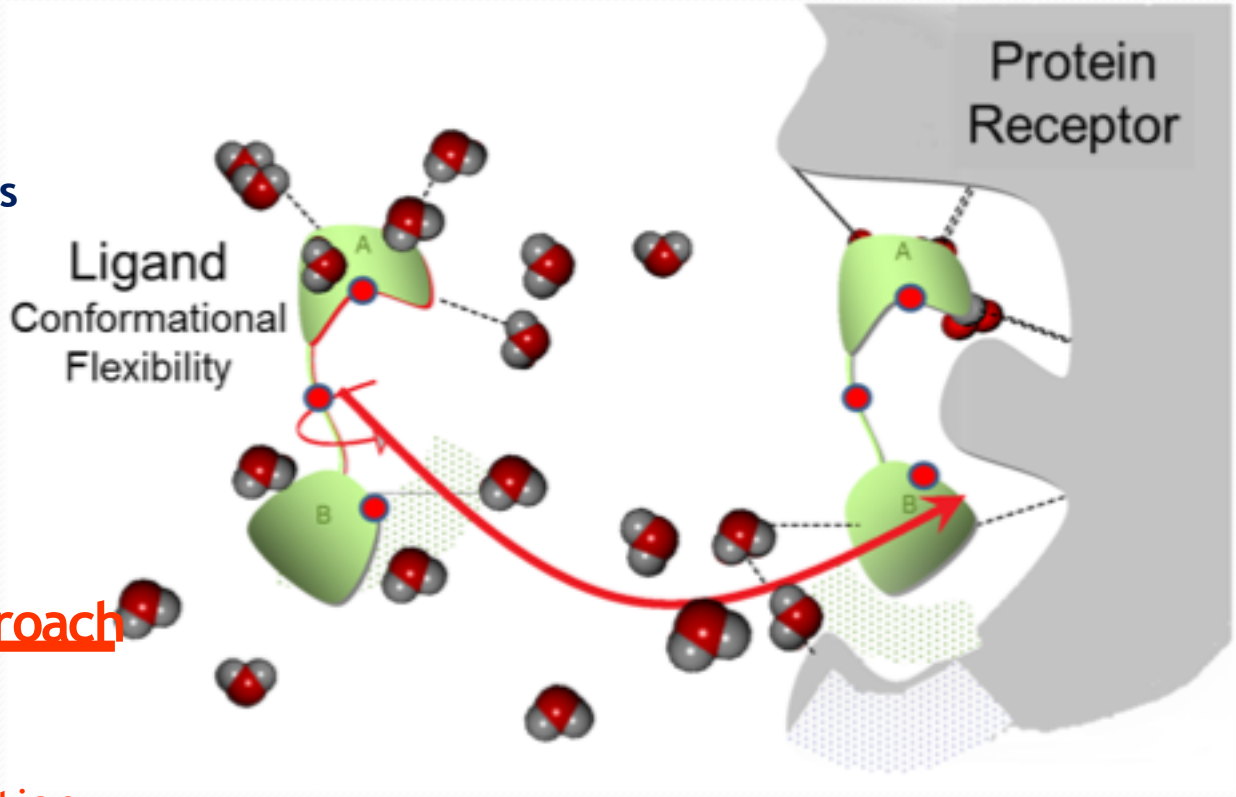
- STD NMR
- TrNOESY
- WaterLOGSY
- Relaxation experiments
- Diffusion experiments

- Other nuclei: ^{19}F
- Paramagnetic tagging
- Other methods

The receptor based approach

- Isotope labelling
- Chemical shift perturbation mapping
- Paramagnetic tagging
- Other variations

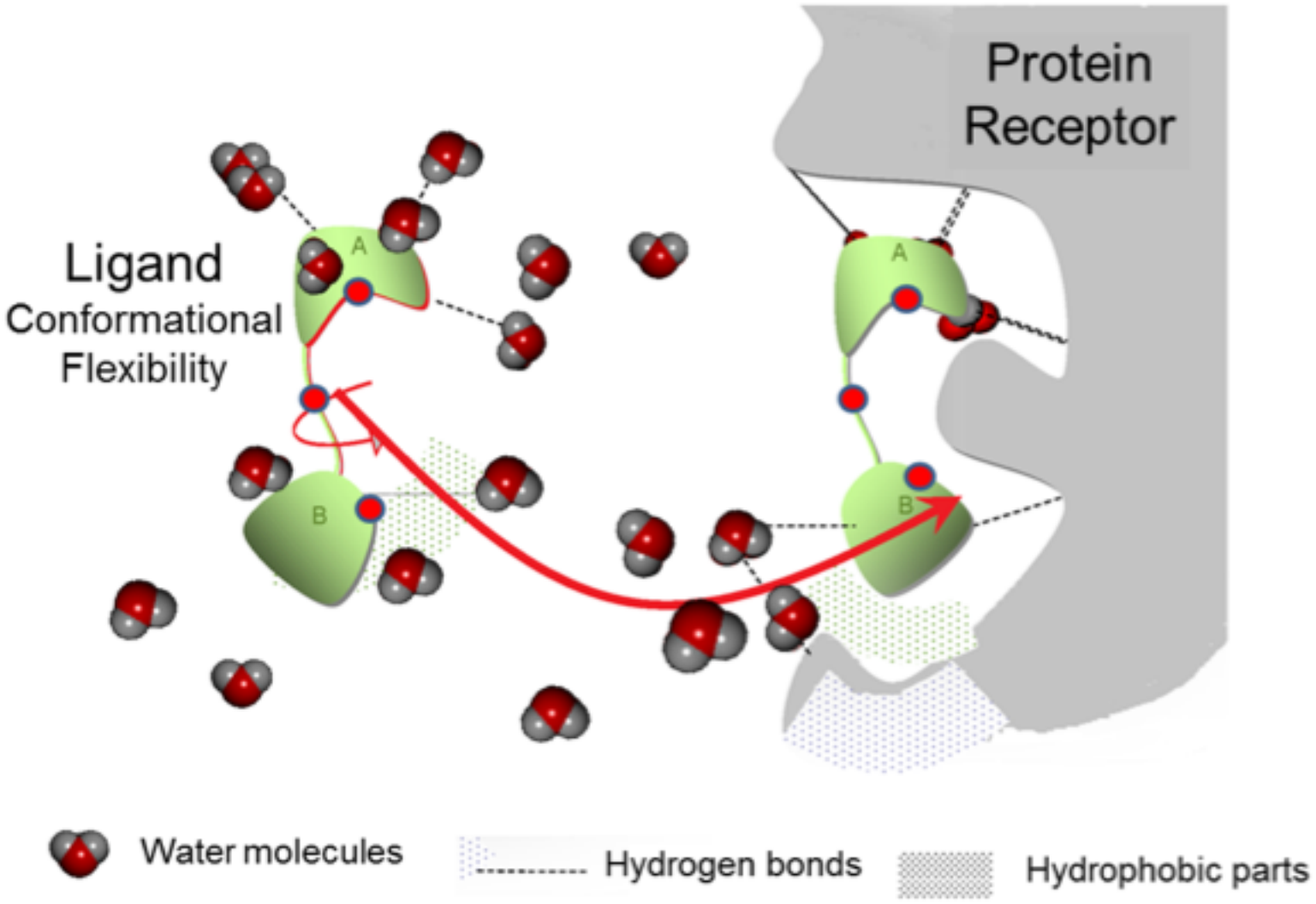
Representation of protein-ligand interactions



Chemistry Open, 2016

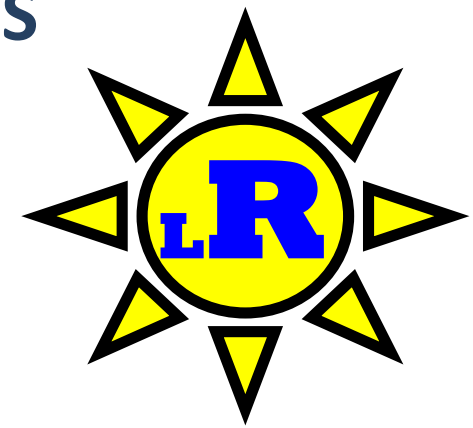
Representation of protein-ligand interactions.

Molecular interaction by NMR. Ligand- based and receptor based approach



Theoretical and computational methods are used to predict ligand orientation in the binding pocket.

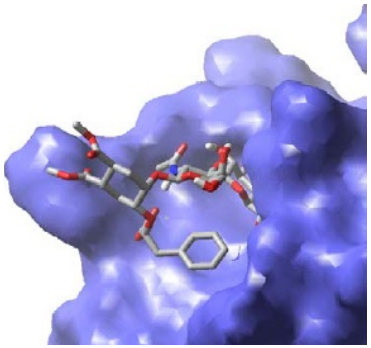
LIGAND-RECEPTOR INTERACTIONS



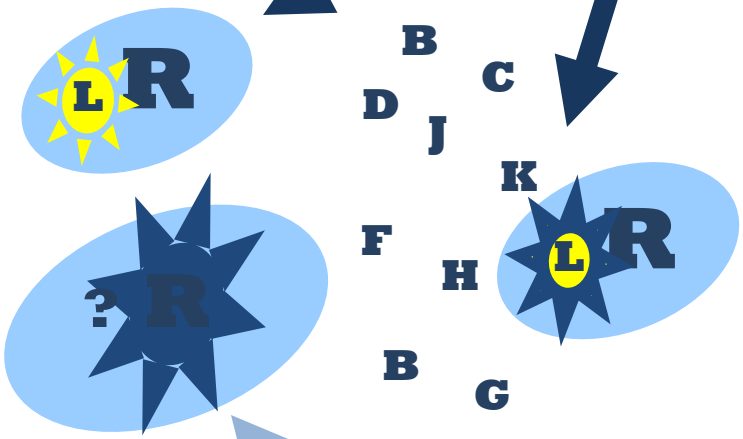
Ligand

L + R ▶ LR

$$K_d = \frac{[L]*[R]}{[LR]}$$



STRUCTURE



IDENTIFICATION

BINDING

DETECTION

Receptor

Ligand observation

Two states equilibrium

L_{free} L_{bound} Molar fractions

FAST EXCHANGE

$$K_d = \frac{k_{off}}{k_{on\ diffusion\ controlled}}$$

k_{off} = $>10^2$ (s^{-1})

k_{on} = $>10^7$ ($s^{-1}M^{-1}$)

$$R_{Lobs} = L_f * R_{Lf} + L_b * R_{Lb}$$

$$\Delta R = L_b * (R_{Lb} - R_{Lf})$$

Experimental procedure: $L_0 >> R_0$;

$L_0/R_0 > 10 - 100....$ $L_f >>> L_b$

Necessary condition: $|(R_{Lb} - R_{Lf})| >> 0$

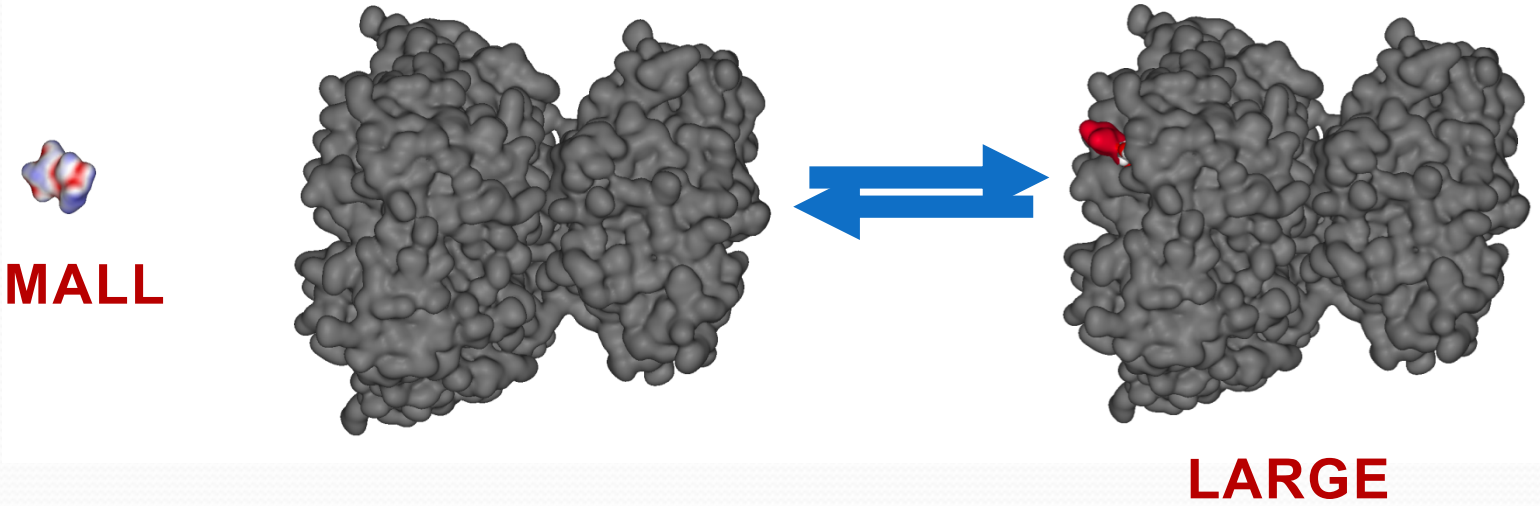
R_{Lb} Strong dependency on molecular size

NMR observable parameter R : **NOE; Diffusion; Line Shape**

Recapitulation

- K_{off} fast in the relaxation time scale, dissociation must occur before relaxation.
- Kon related to the efficacy of the interaction.
- Consider the molar fraction of ligand free and bound to the protein.
- Fast exchange in the chemical shift time scale
- **$L_0 >> R_0$ means excess of free ligand but since we are in conditions in which the exchange is high (rate) the system is dominated by the bound state**
- K_{off} Dissociation before relaxation takes place; dissociation rate high since the molecule relax in the binding site

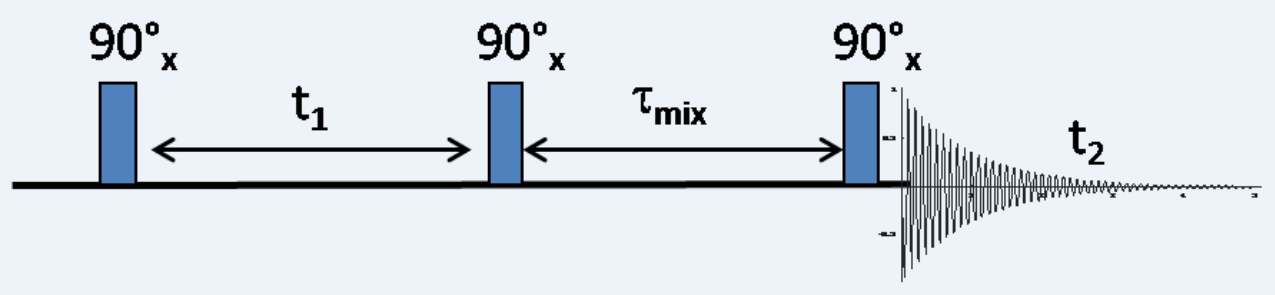
When monitoring the ligand binding you realize that relaxation is perturbed...



Upon binging of a small molecule (L) to a macromolecular receptor (R), L will take on the motional properties of R, and consequently, the NMR properties of L will be altered.

TRANSFERRED NOE

Information on the ligand bioactive conformation



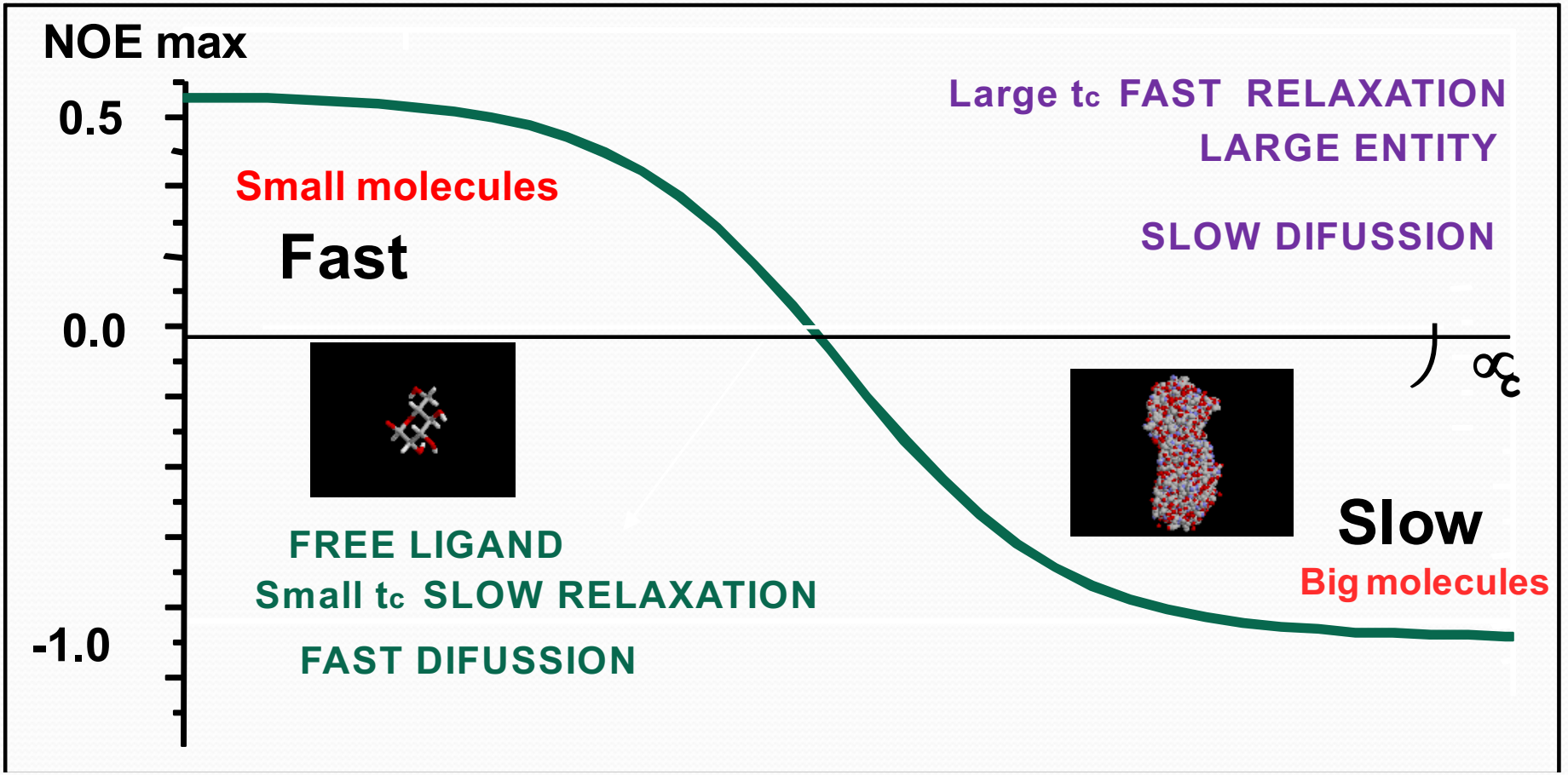
Ligand-protein 1:5

- **During the mixing time *inter* and *intra*-molecular NOE effects build up**
- ***Inter*-molecular tr-NOE effects are visible, intermolecular trNOEs are usually much larger than intramolecular effects**

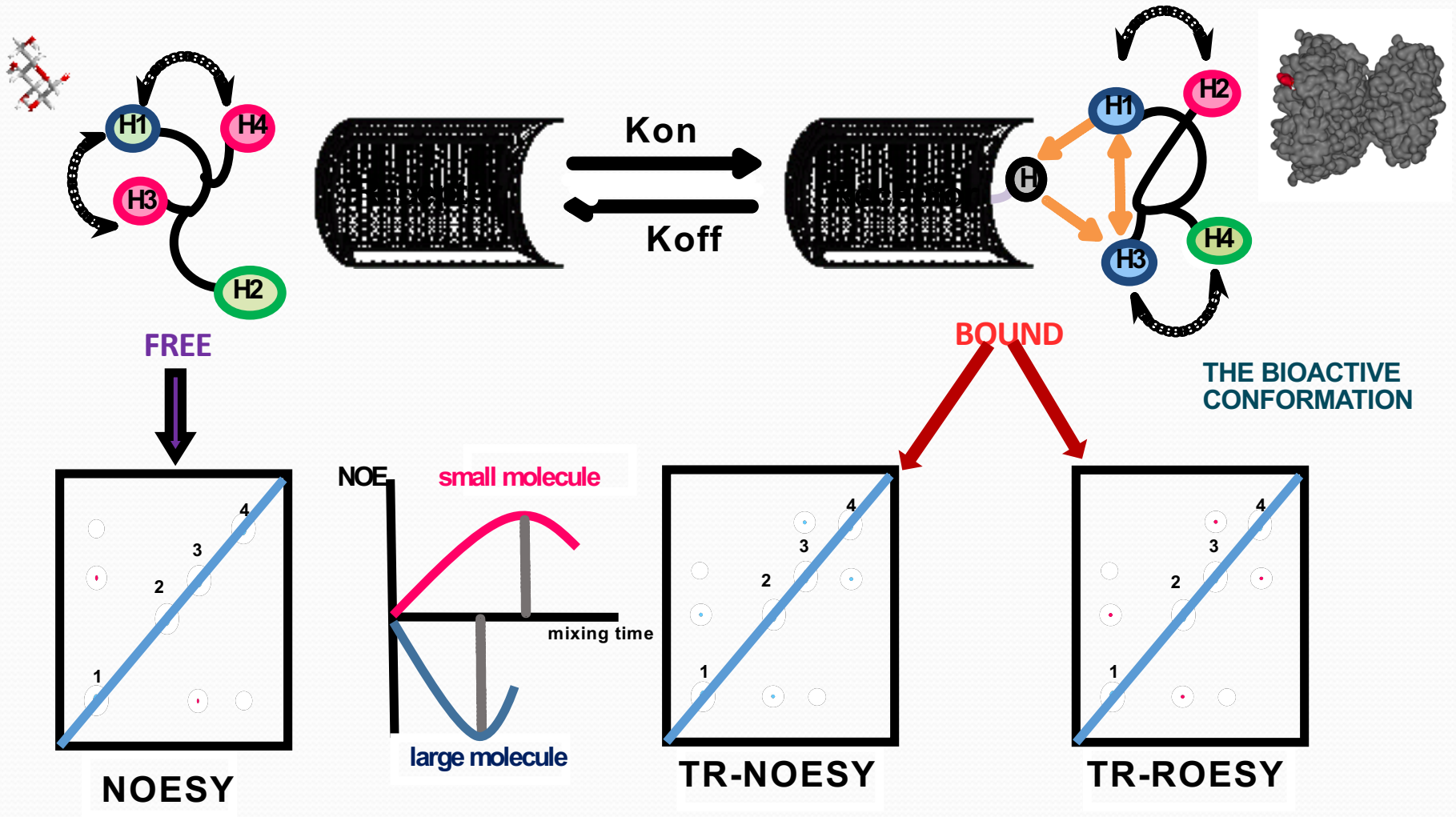
Important notes

- The mixing time must be short enough so that the contribution of the free ligand is negligible and long enough to allow visualization of the signal in the spectrum
- One molar ratio of ligand to receptor. It should be emphasized that the trNOESY experiment works well for ligands that have K_D in the range 10^{-3} – 10^{-6} M / mM range
- Small amount of purified receptor
- Routinely used to probe ligand-receptor interaction

TRANSFERRED NOE and MOLECULAR MOTION



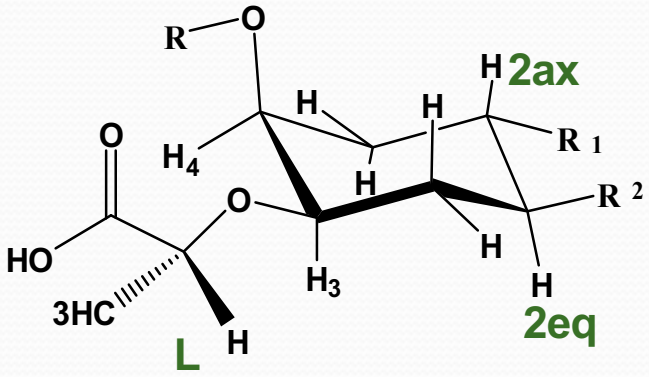
The bioactive conformation: Transfer NOESY

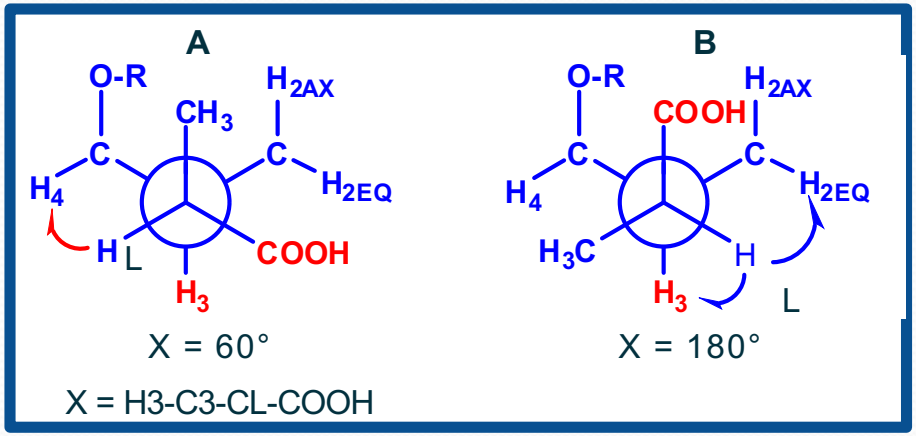


- Is There Any Binding?
- Which Is The Ligand Bioactive Conformation?

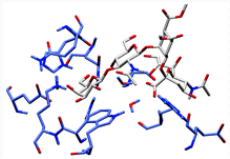
Chem Soc Rev 27 (1998) 133; Methods Enzymol (2003) 417; Curr Opin Struct Biol (1999) 549 , ibid (2003) 646

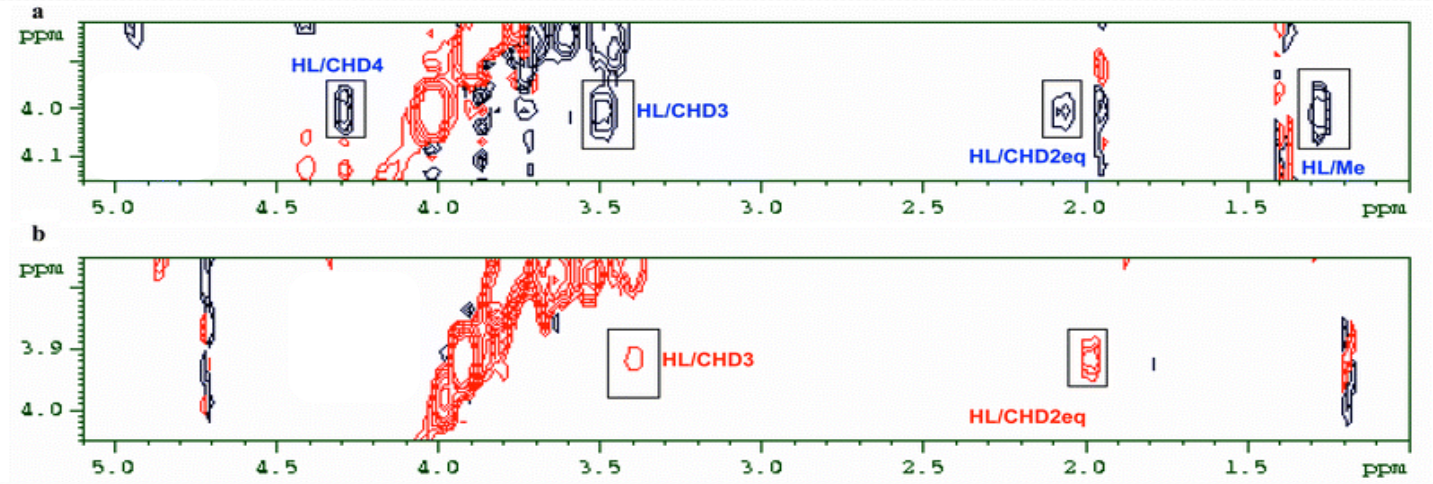
Ligand bioactive conformation tr-NOESY





Cholera toxin ligand





NOESY free state

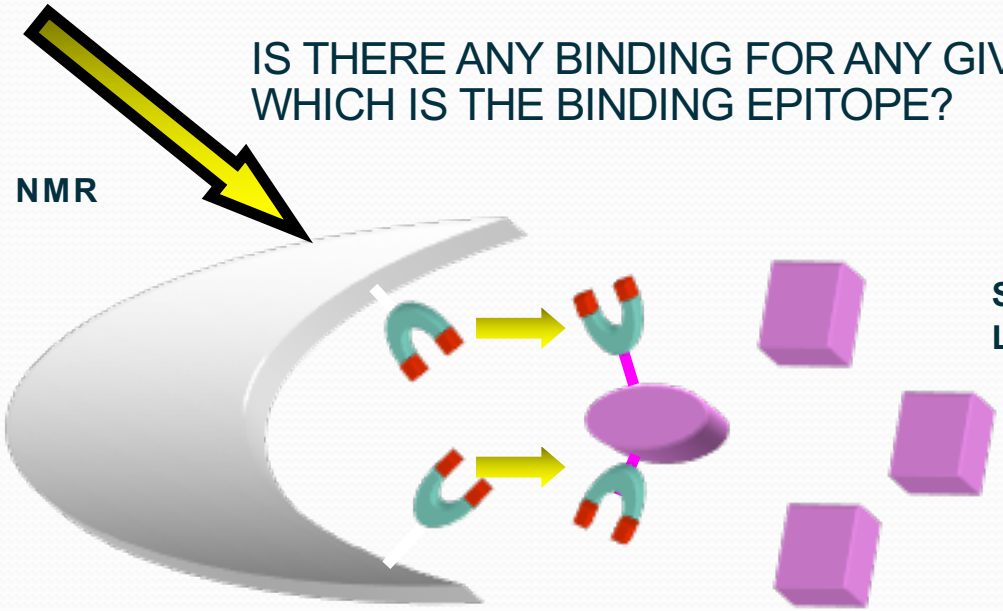
tr-NOESY bound state

Bernardi, A.; Arosio, D.; Manzoni, L.; Monti, D.; Posteri, H.; Potenza, D.; Mari, S.; Jimenez-Barbero, J. *Org. Biomol. Chem.*, **2003**, *1*, 785-792.

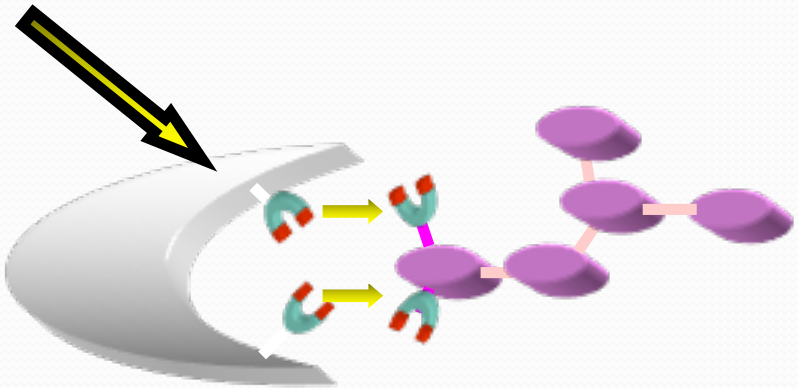
Saturation Transfer Difference NMR Spectroscopy – STD NMR

IS THERE ANY BINDING FOR ANY GIVEN COMPOUND?
WHICH IS THE BINDING EPITOPE?

IRRADIATION at the aromatic or aliphatic NMR regions



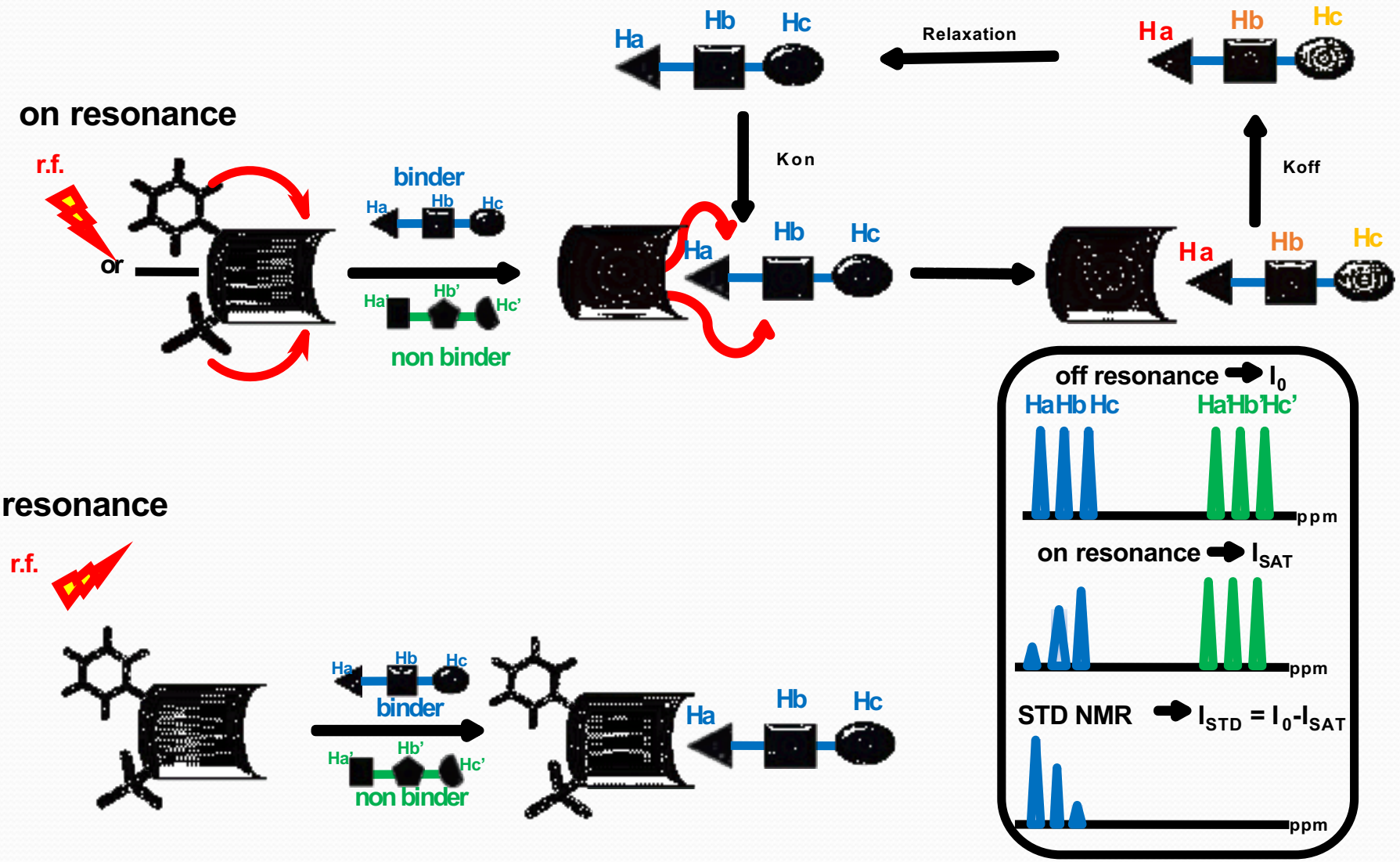
Single Compound or Library



At long irradiation times, the saturation is transferred to the bound ligand, first to the protons belonging to the ligand epitope, then to the rest of the ligand

(Meyer and Mayer, Peters, 1999, 2000)

Schematic representation of STD NMR method.



Key elements of protein-substrate binding

H close to receptor

H far from receptor

H furthest away from receptor

STD (Meyer and Mayer, Peters, 1999, 2000)

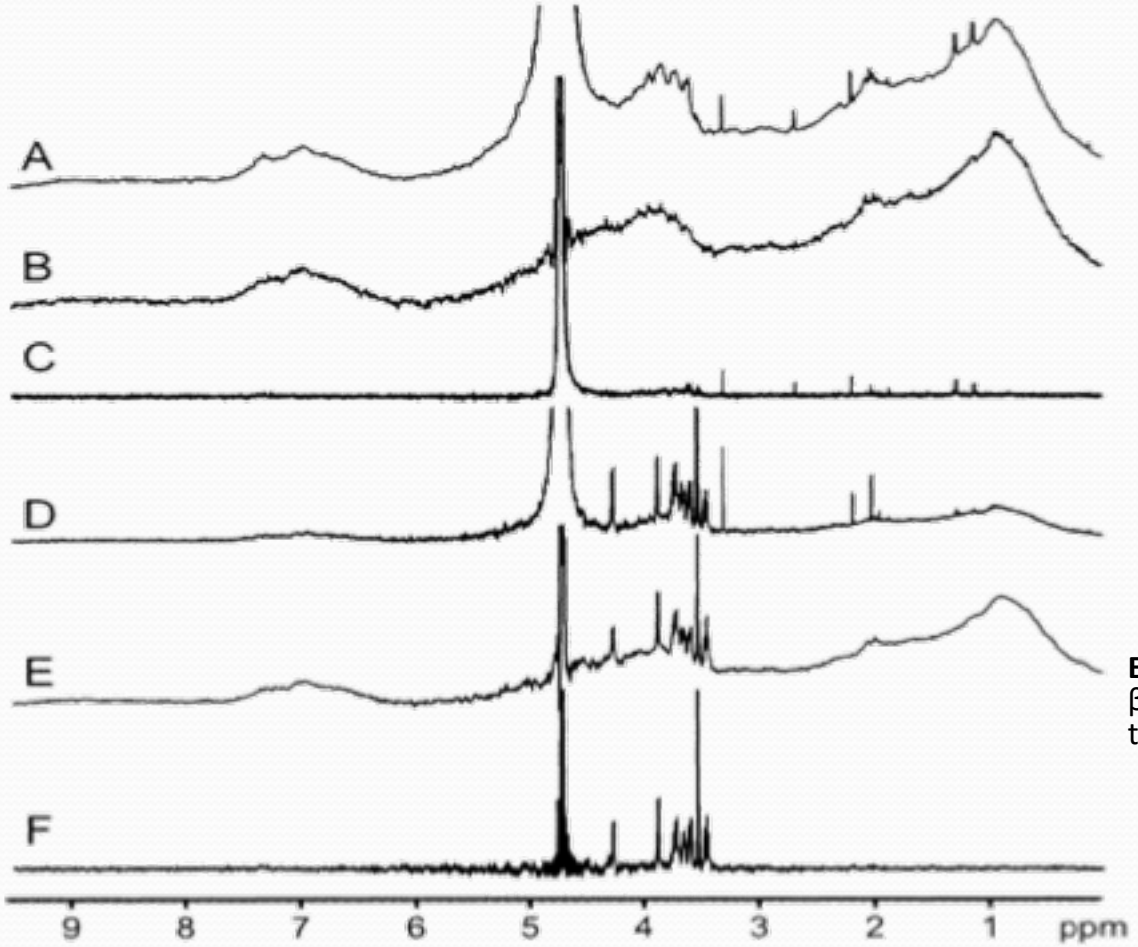
RECAPITULATION...

- **The saturation is transferred first to the protons belonging to the ligand epitope, then to the rest of the ligand**

- STD involves selective perturbation of protein-specific methyl proton resonances (0.5 p.p.m.–1 p.p.m.). This perturbation rapidly diffuses throughout the protein.
- This is usually done via a selective 180-degree pulse and results in a transfer of magnetization from the protein to any transiently bound ligands via the nuclear Overhauser effect (NOE).
- If the fragment binds to the target protein, the buildup of NOE that is transferred to the ligand results in enhanced signal corresponding to the resonances of that ligand in the STD spectrum.
- A number of factors affect the signal strength in an STD experiment, including protein size, duration of on-resonance irradiation, the frequency of irradiation, the dissociation constant of the ligand, the ligand/protein ratio and the field of the spectrometer.
- As such, it is useful to vary parameters such as ligand and protein concentration, the frequency of irradiation and the duration of irradiation

NMR Spectroscopy Techniques for Screening and Identifying Ligand Binding to Protein Receptors

β-Gal-Ome and RCA120 (Ricinus communis agglutinin), 50 μM and 1.2 mM, 1:40



A) Reference 1D NMR spectrum of the 120 kDa protein RCA120

B) Corresponding STD NMR spectrums, ON at -2 ppm

C) 1D NMR spectrum recorded with a T11 filter (ms spin-lock pulse)

D) Reference 1D NMR spectrum of RCA120 in presence of 1.2 mm b-GalOMe

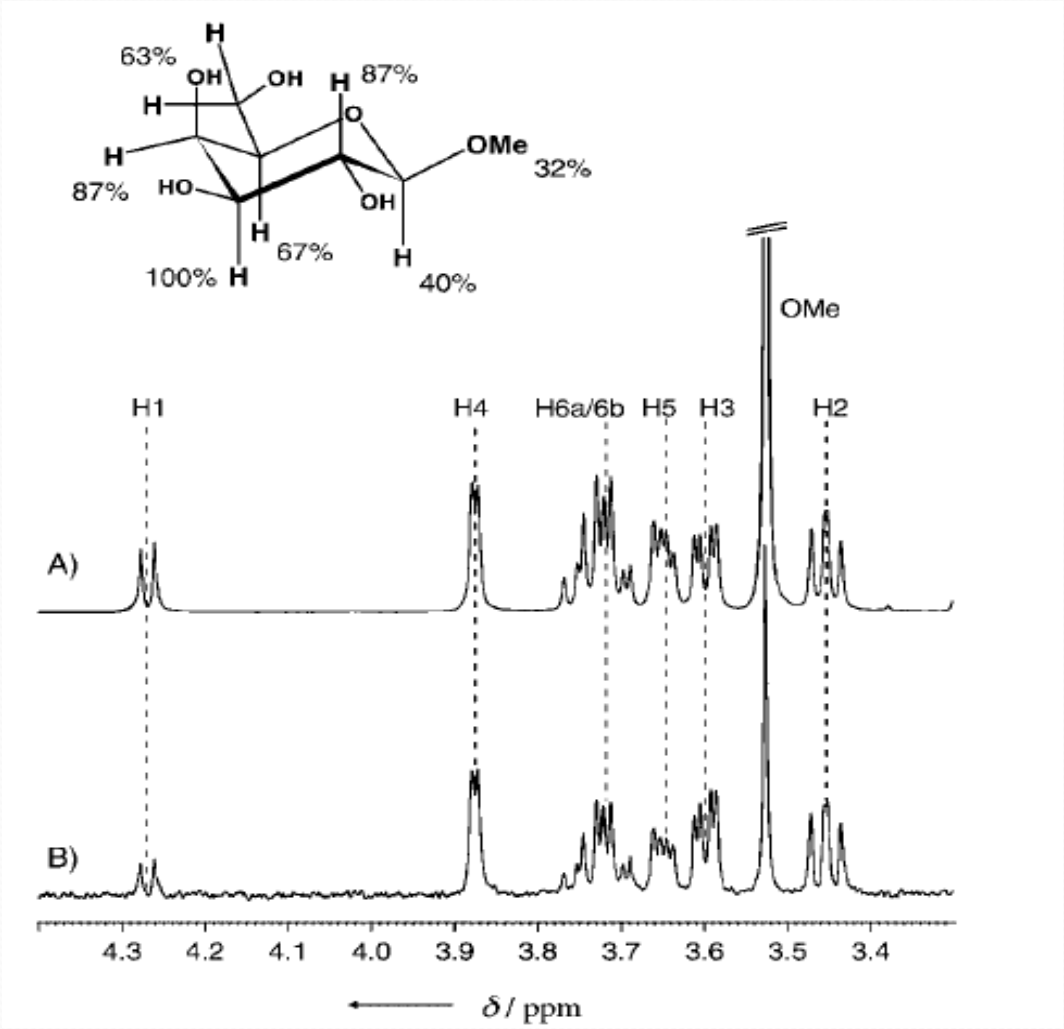
E) Corresponding STD NMR spectrum, which shows the β-GalOMe resonance signals and therefore its binding to the receptor

F) STD NMR spectrum as in (E) but with the $T_{1\rho}$ filter eliminating all protein background signals.

STD effect: $(I_0 - I_{sat})/I_0$

M. Mayer, B. Meyer, J. Am. Chem. Soc. 2001;123(25):6108-17; Bernd Meyer, Thomas Peters, *Angewandte Chemie International Edition*, *42*, 8, 864-890

β-Gal-Ome and RCA120 (Ricinus communis agglutinin), 50 µM and 1.2 mM, 1:40



^{1}H NMR

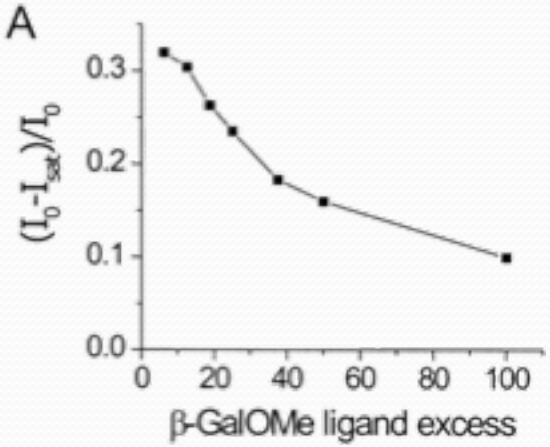
STD

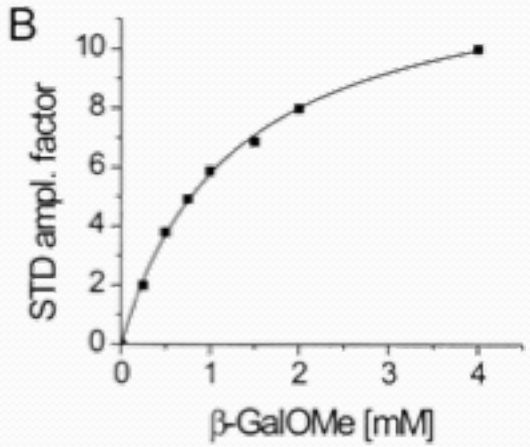
In order to determine the magnitude of the STD effects, the intensity of the signal in the STD NMR spectrum are compared with the signal intensities of a reference spectrum (off-resonance).
The STD signal with the highest intensity is set to 100% and the others are normalized to this signal.

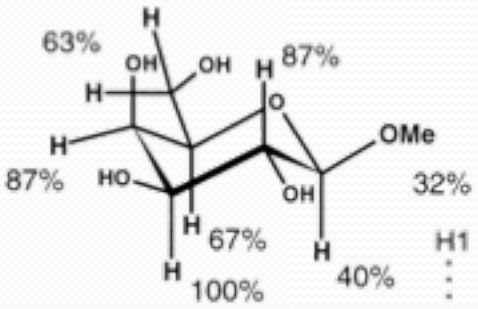
M. Mayer, B. Meyer, J. Am. Chem. Soc. 2001;123(25):6108-17; Bernd Meyer, Thomas Peters, *Angewandte Chemie International Edition*, *42*, 8, 864-890

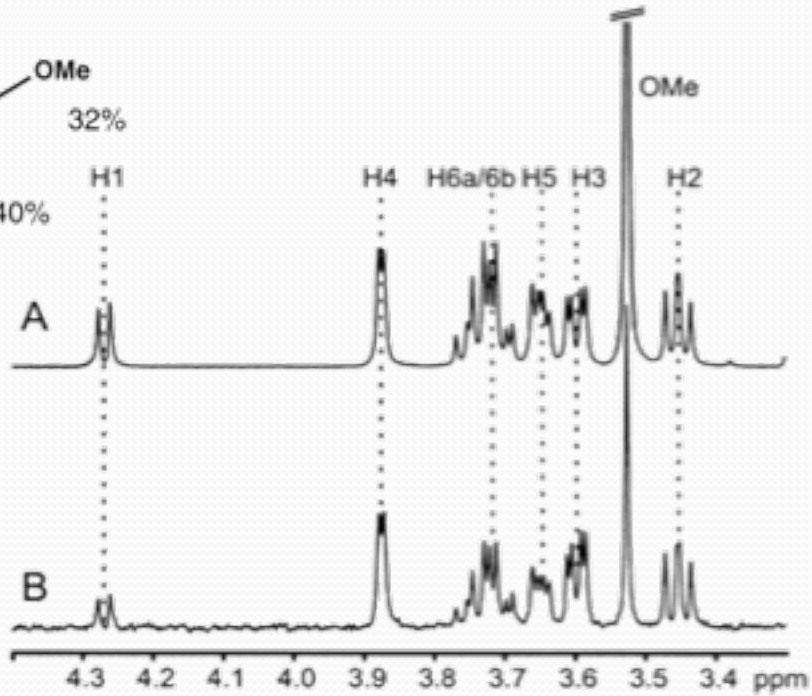
Binding Constants from STD NMR Experiments

STD intensity = (STDmax + [L])/ kd + [L]







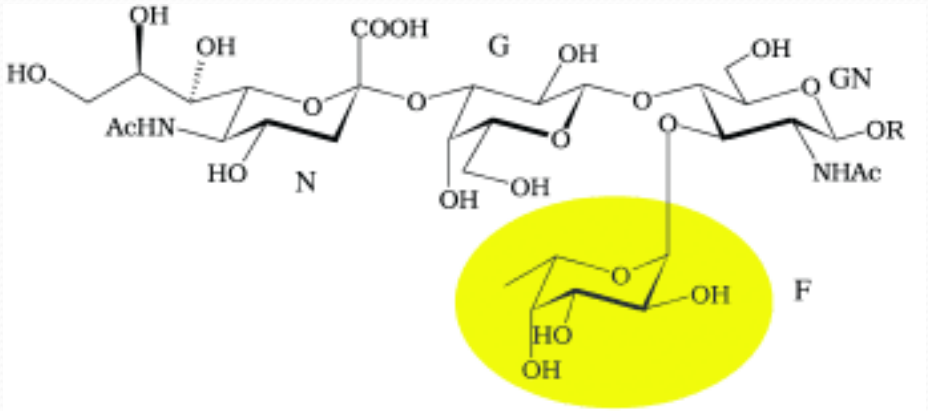


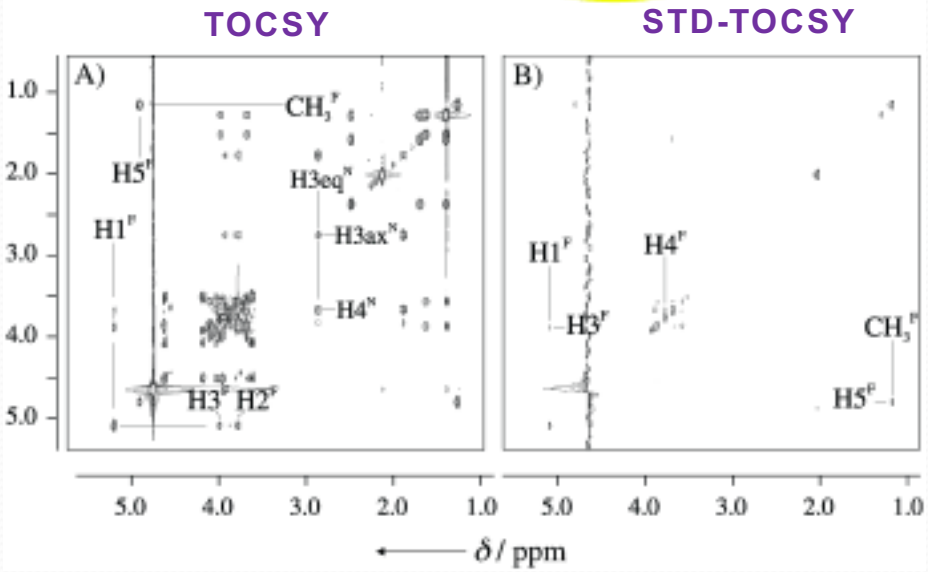
RCA_{120} and *β*-GalOMe (4 mM) in a ratio of 1:100

Diagram showing the STD amplification determined from STD spectra on titration of β-GalOMe to a sample of RCA_{120} (binding site concentration 50 μM) and NA_2 (0.55 mM). The STD amplification factor of the signal corresponding to NA_2 decreases from 1 to 0.66 with increasing concentration of β-GalOMe. This competition experiment gives evidence to the specificity of the RCA_{120} towards galactose containing saccharides. The K_D of NA_2 can be calculated to 27 μM.

M. Mayer, B. Meyer, *J. Am. Chem. Soc.* 2001, 123, 6108–6117.

Epitope mapping for sialyl LewisX bound to the the lectin *Aleuria aurantia* agglutinin (AAA)



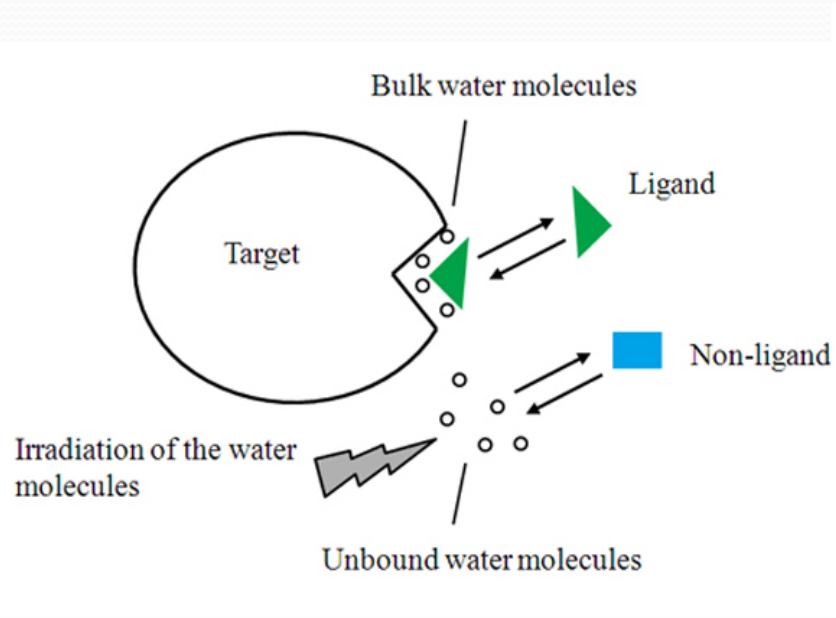


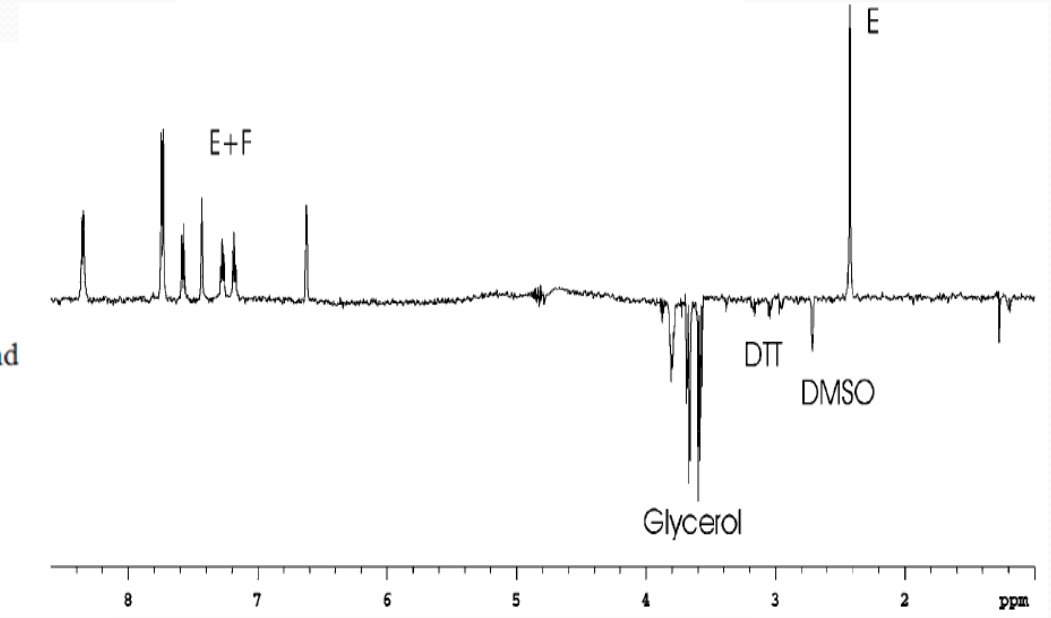
STD TOCSY spectrum of sialyl LewisX in the presence of AAA (molar ratio 100:1): only the spin system of the l-fucose residue (F, yellow circle) is visible

1D and 2D STD spectra shows unambiguously that only the fucose interact with the protein

M. Mayer, B. Meyer, Angew.Chem. 1999, 111, 1902 – 1906 ;Angew.Chem.Int.Ed. 1999, 38, 1784 – 1788.

Water-LOGSY NMR



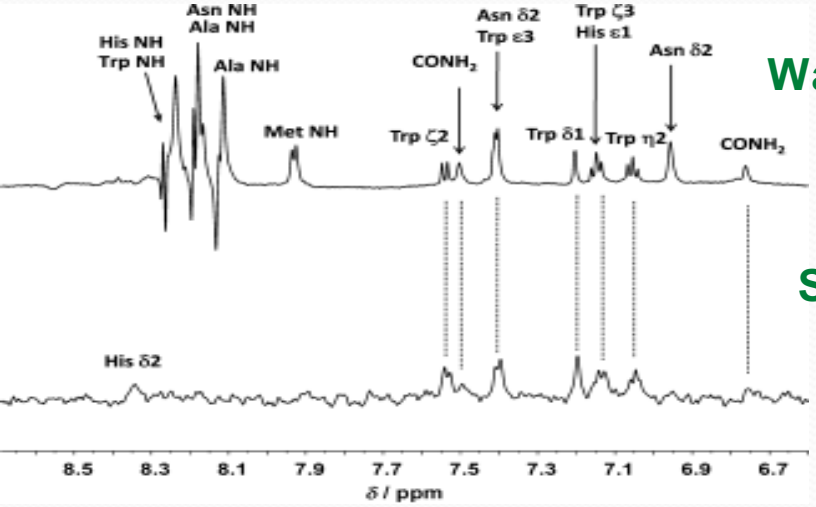


The resonances of non-binding compounds appear with opposite sign and tend to be weaker than those of the interacting ligans.
Experimentally, the first step is the selective water excitation; during the mixing time (to be optimized based on the size of the complex) the water magnetization that has migrated to the protein is transferred to the ligand via direct of relay processes.

Dalvit C., Fogliatto G., Stewart A., Veronesi M, Stockman B., J Biomol NMR. 2001, 21(4):349-59.

A carbohydrate mimic MDWNMHAA bound to an anti-*Shigella flexneri* Y mAb SYA/J6

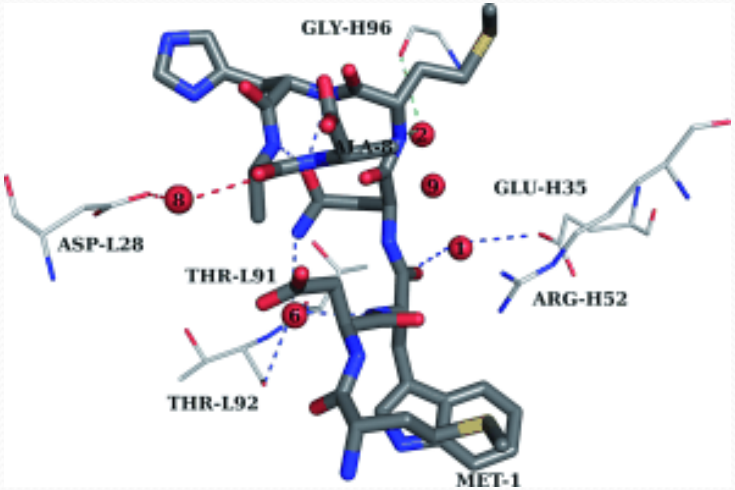
WaterLOGSY was used in conjunction with STD-NMR spectroscopy to probe the existence of immobilized water molecules in the complex of MDWNMHAA **1** bound to mAb SYA/J6.

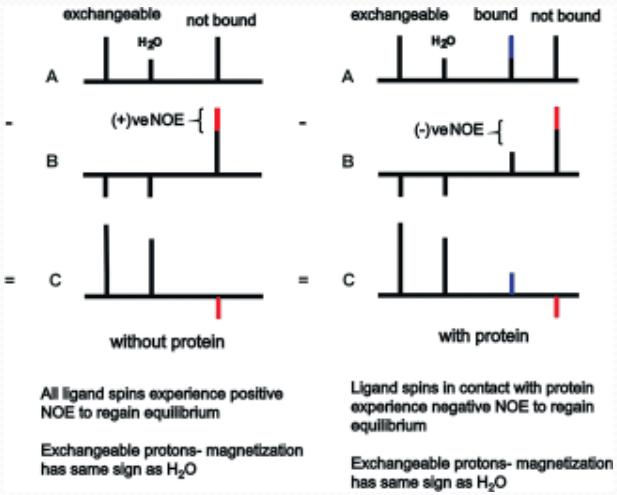


Water-LOGSY

STD-NMR

Mapping of immobilized water molecules identified from combined WaterLOGSY and STD-NMR experiments and molecular dynamics simulations, onto the X-Ray structure of peptide **1** and to mAb SYA/J6.





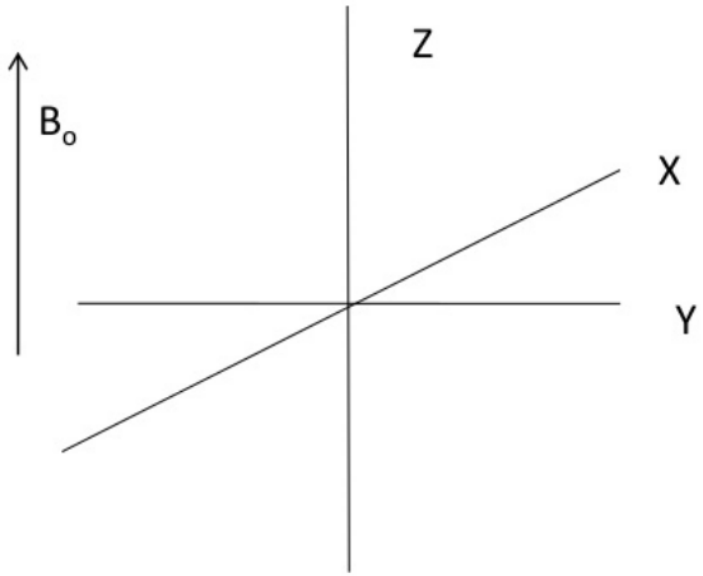
Schematic representations of WaterLOGSY spectra of a hypothetical ligand in the absence (left) and presence (right) of a protein receptor. **State A** refers to the equilibrium state, or immediately after off-resonance irradiation. **State B** refers to irradiation at the water frequency. **State C** is the WaterLOGSY spectrum and is the difference between states A and B.

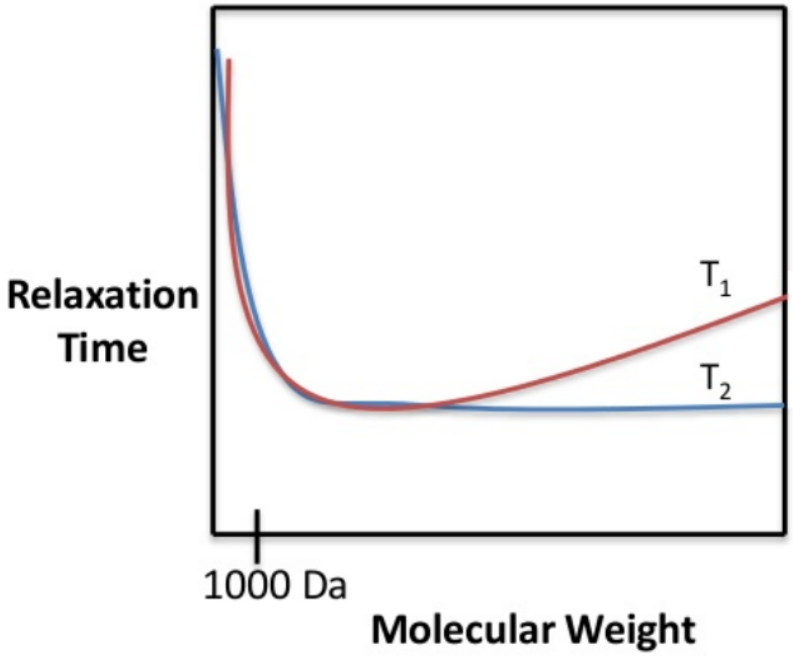
Szczepina MG, Bleile DW, Müllegger J, Lewis AR, Pinto BM., Chemistry. 2011 Oct 4;17(41):11438-45.

Use of Relaxation Times to Identify Ligands

After resonance, where $v_1 = v_o$, magnetization relaxes back to equilibrium

- T_1 = relaxation of nuclear spin magnetic vector parallel to the magnetic field, B_o
- T_2 = relaxation of nuclear spin magnetic vector perpendicular to the magnetic field, B_o





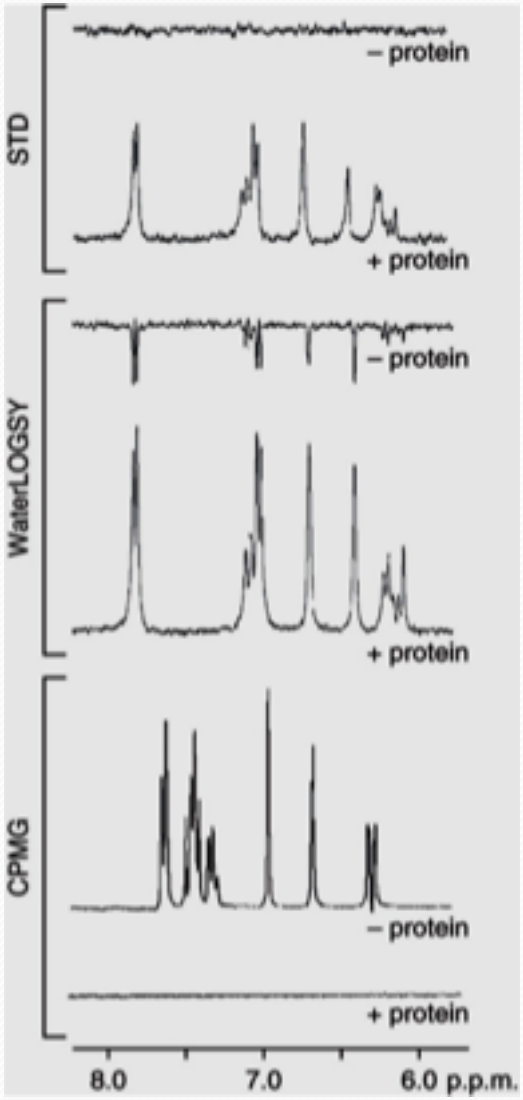
- **Small, rapidly tumbling molecules:** high (longer) relaxation times
- **Macromolecules that move slowly through solution:** low (shorter) relaxation times

Shortening T_2 relaxation time leads to peak broadening

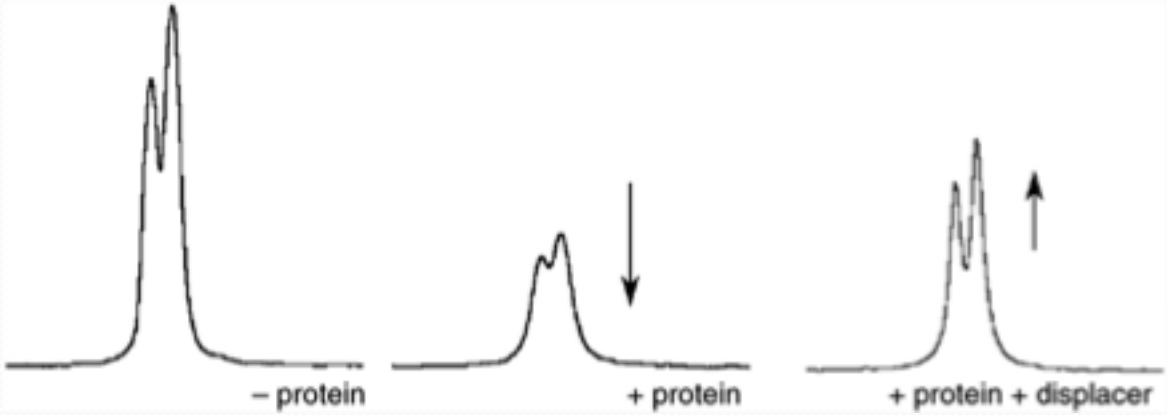
Pellecchia, M.; Sem, D.S.; Wuthrich, K. *Nature* **2002,** *1*, 211.
Meyers, B.; Peters, B. *Angew. Chem. Int. Ed.* **2003**, *42*, 864.

Carr Purcell Meiboom Gill (CPMG) experiments

CPMG, is a relaxation-time-edited NMR experiment that exploits differences in transverse relaxation time (*T2). Proteins (and bound ligands)* have a small *T2 while free ligands have a large T2. Thus monitoring T2, binding* can be detected when the signal of the ligand decreases.



In the CPMG experiment, we should see the signal decrease (or disappear) in the presence of protein and a binding fragment



In the presence of protein, the signal decreases, which is indicative of fragment binding. In the presence of both protein and a molecule known to bind the target (displacer), the signal is restored by ~30%. The protein target is *M. tuberculosis* CoaBC (10 μM). Fragment and displacer are present at 0.5 and 0.25 mM, respectively

Carr, Y.H. & Purcell, M.E. Phys. Rev. 94, 630–638 (1954).; Hajduk, P.J., Olejniczak, E.T. & Fesik, S.W. J. Am. Chem. Soc. 119, 12257–12261 (1997).; E.H Mashalidis, P. Śledź, S. Lang, C. Abell, Nature Protocols, 8, 2309–2324 (2013)

TLR4/MD-2 activation by a synthetic agonist with no similarity to LPS

Direct interaction between Neoseptin-3 and highly purified TLR4/MD-2 complexes demonstrated in vitro by Carr PurcellMeiboom Gill (CPMG) experiments

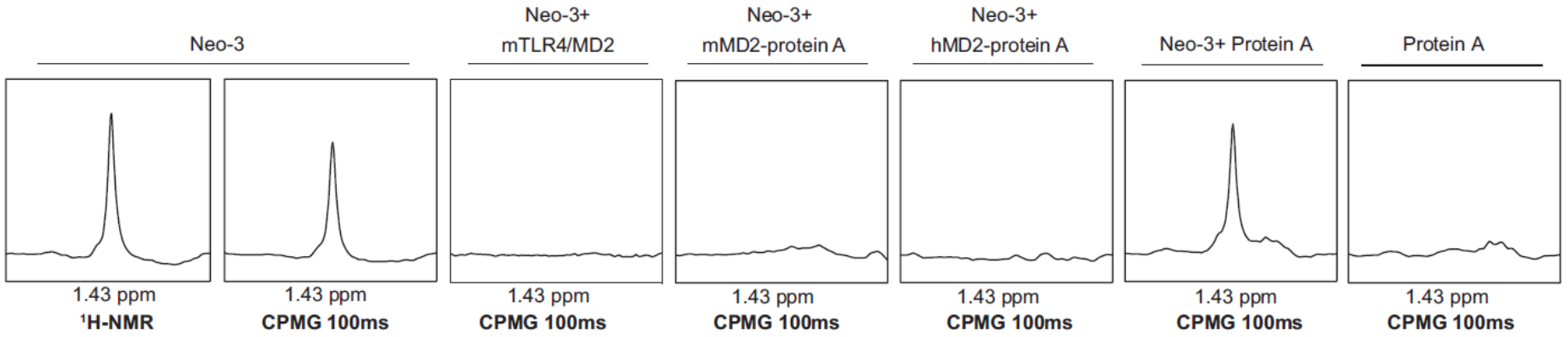
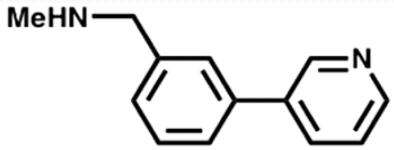


Fig. 4. NMR spectroscopy of Neoseptin-3 with mTLR4/MD-2. One-dimensional ^{1}H-NMR spectra of the methyl regions of Neoseptin-3 alone, with mTLR4/MD-2, with mouse MD-2/protein A, or with human MD-2/protein A. Controls were Neoseptin-3 plus protein A and protein A alone. A CPMG sequence was applied for 100 ms (CPMG 100ms) as indicated.

Neoseptin-3 alone showed a relaxation time greater than 100 ms, which was reduced upon addition of mMD-2, hMD-2, or mTLR4/mMD-2, consistent with binding of Neoseptin-3 to h- or mMD-2 or the mTLR4/ MD-2 complex (Fig. 4). We concluded that the biologically relevant molecular target for Neoseptin-3 is the TLR4/MD-2 complex.

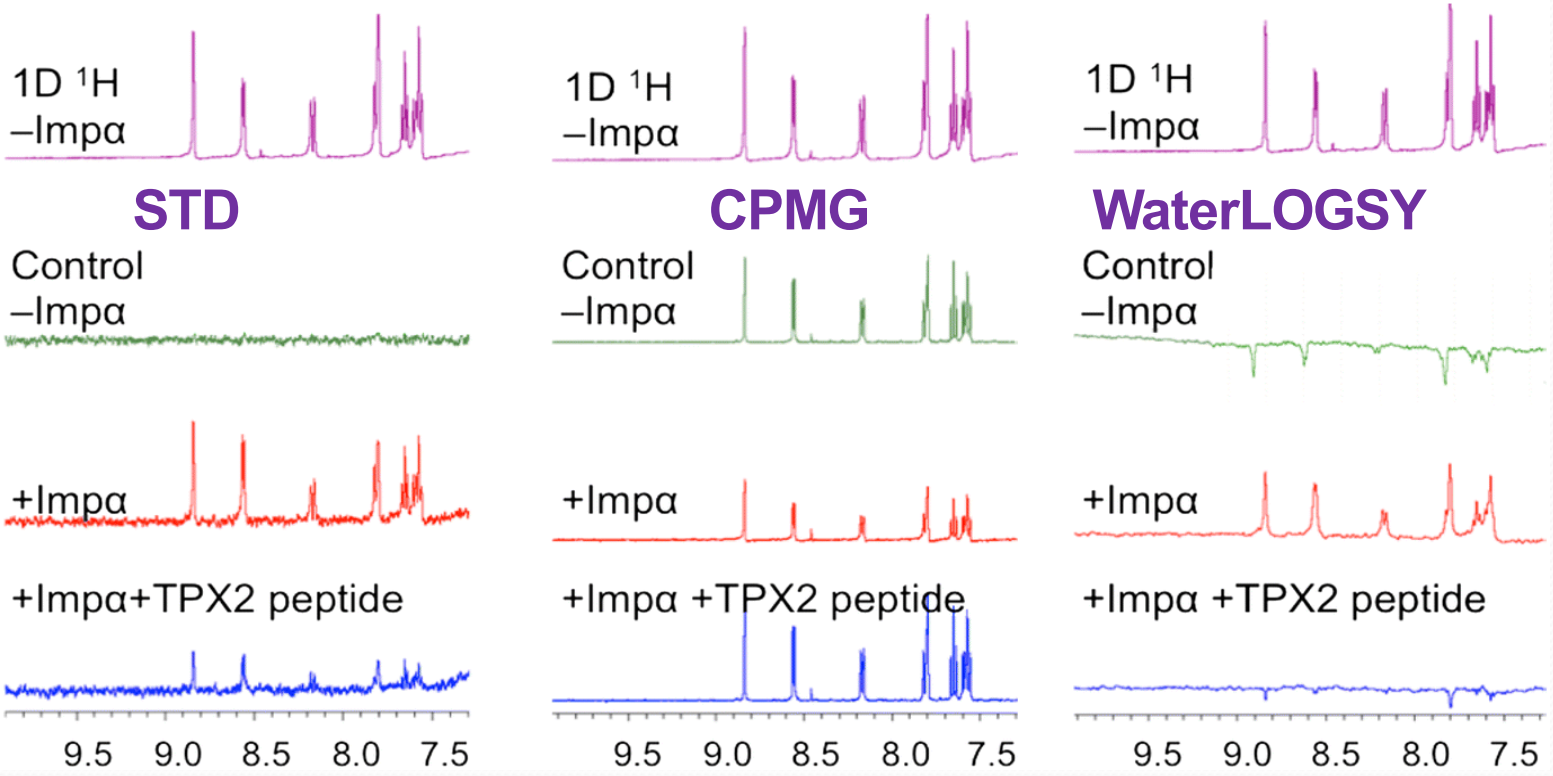
Wang Y, Su L, Morin MD, Jones BT, Whitby LR, Surakattula MM, Huang H, Shi H, Choi JH, Wang KW, Moresco EM, Berger M, Zhan X, Zhang H, Boger DL, Beutler B, Proc Natl Acad Sci U S A. 2016;113(7):E884-93.

Ligand-observed NMR techniques STD, CPMG and WaterLOGSY



1

Binding of **fragment 1 to importin-α (Impα).**



^{1}H NMR Fragment 1

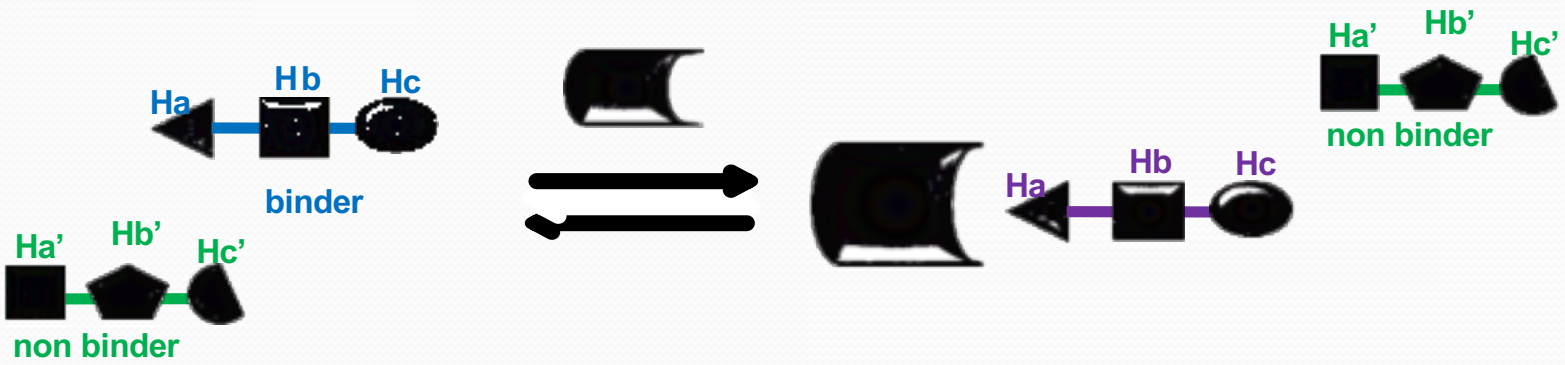
Fragment 1 and importin-α (Impα).

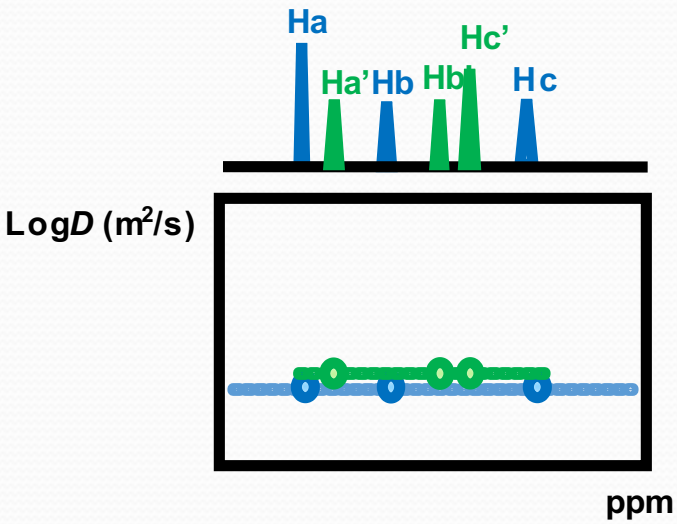
Control −Impα (green): fragment in the absence of the protein. +Impα (red): fragment in the presence of the protein, changes in signal indicating binding. +Impα +TPX2 peptide (blue): displacement study by addition of the TPX2 peptide.

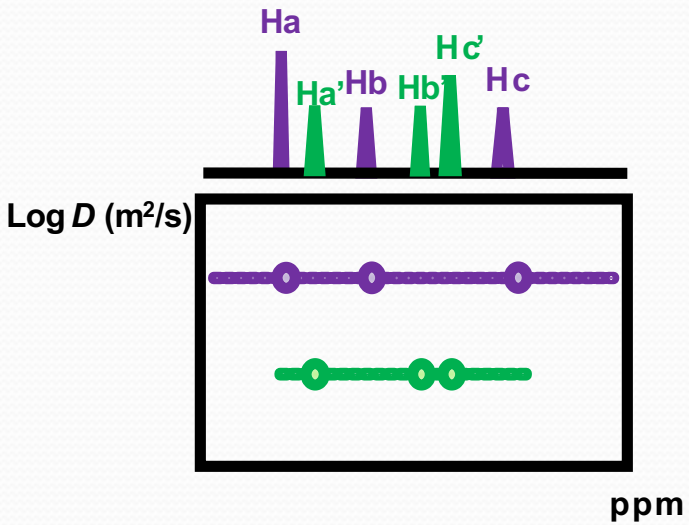
C. Abell, C. Dagostin *Fragment-Based Drug Discovery*, 2015, pp. 1-18

DOSY NMR

Schematic representation of a pseudo 2D DOSY experiment. Upon the addition of the receptor in solution, a change in the diffusion coefficient is observed only for binders molecules.







Typical range of applicability and delivered information from the main NMR methods for the study of protein –ligand interactions.

NMR Methods	Range of applicability				Delivered information		
	Kd (M)	Target MW	Typical protein:ligand ratio	Labeled target required	Target binding site	Ligand epitope mapping	Ligand selectivity in a mixture
TR-NOE	10^{-6} - 10^{-3}	No limit	1:5/ 1:50	no			✓
STD NMR	10^{-6} - 10^{-3}	> 15 kDa	1:50/ 1:200	no		✓	✓
Water Logsy	10^{-6} - 10^{-3}	No limit	1:5/ 1:50	no		✓	✓
Diffusion Experiments	10^{-6} - 10^{-3}	No limit	1:1/ 1:20	no		✓	✓
CSP	10^{-9} - 10^{-3}	< 100 kDa	1:1/ 1:10	yes	✓		

Napoli

A. Silipo, C. de Castro,
M. Parrilli, R. Lanzetta
R. Marchetti, F. Di Lorenzo,

Catania

D. Garozzo, L. Sturiale, A. Palmigiano
ICTMP CNR

TONY

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COST – EU 2010-2014
EU- FP7; H2020
FFC2006-2012
PRIN 2008, 2009,2010
Mizutani Foundation
PNRA, 2017
Japan JSPS,
KOREAN KAIST


Questions?