

Recent Advances in Trace Quantity Sample Identification and Determination of Absolute Stereochemistry by NMR

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Wyeth
Research

Overview

- **Tools available in NMR for trace quantities**
 - **Hardware**
 - **Sequences**
 - **Structures**
 - **Absolute Stereochemistry by NMR**
 - **Tools employed**
 - **NMR hardware needed**
 - **Perspectives**
-

Small Sample Quantities

- **NMR - Sensitivity, Sensitivity, Sensitivity**
- Structure elucidation of metabolites, impurities
 - Limited quantity - ug scale, not only single chemical species
 - Low sensitivity and only very limited information we can obtain

Tools

- Small Volume Probes - Nanoprobe, Capillary Probes, Micro-flow Probes
 - High sensitivity Probes - Cryoprobes, Cold Probes
-

Tools for Small Quantities Small Volume Probes

- **Nanoprobe , HR MAS probe** 10 – 40 ul volume

Rapid spinning 2000 –6000 Hz they have gradient capability for modern 2D experiments

- **Capillary Probes** – 8 ul volume. Sample changer is a liquid handler or syringe.

- **Classic 1.7 and 1 mm probes** with sample changers can be used in routine mainly ^1H analysis, but they allow also 2D experiments.

- **Require small volume of solvent. Careful sample handling and high solubility is required**
-

Tools for Small Quantities

Small Volume Probes

- **Nanoprobe 10 – 40 ul volume**
 - Strong gradient field 150 – 180 Gauss/cm
 - Gradient field is along Magic Angle
 - Tuning range as in regular indirect gradient probes ^1H / $\{^{31}\text{P}-^{15}\text{N}\}$
 - Gradient sequences with spinning rate taken into account
 - High resolution spectra in heterogeneous samples
 - Nanomole quantities of sample dissolved in 10 – 40 ul of solvent
 - Comfortable to work with
-

Tools for Small Quantities

Small Volume Probes

Classic 1 and 1.7 mm probes

- 5 -15 ul active volume, 25 ul push volume
 - ^1H detection ^{13}C decoupling gradient
 - Use of syringe with fused silica
 - Requires high solubility and good preparation of
 - sample without particles
 - Quantitative sample pickup after purification and drying is challenging
-

Tools for Small Quantities

Small Volume Probes

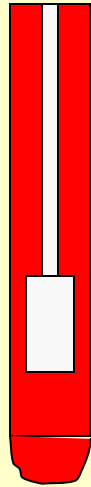
Capillary probe

- 8 ul active volume, 25 ul push volume
 - ^1H detection ^{13}C decoupling gradient
 - Use of syringe or high pressure microflow pump
 - Possibility of automation and open access using proper software
 - Requires high solubility and good preparation of sample without particles
-

Tools for Small Quantities

Nanoprobe tubes

Low viscosity liquids,
swollen beads from



Narrow mouth

Tissues, seeds,
leaves etc



Wide mouth

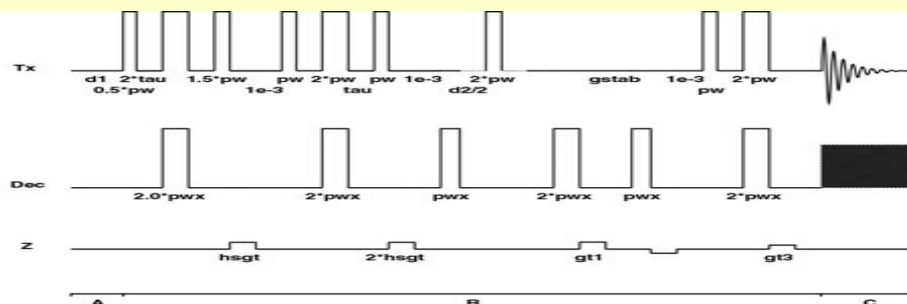
Tools for Small Quantities Nanoprobe tubes



Source of Increased Sensitivity

- **Filling factor** is the key to increase sensitivity in small volume probes
 - Compare to 5 and 3 mm probes the same number of nuclei are packed into 20 to 100 times smaller volume and they are getting closer to receiver coil of the probe
 - This increase dramatically Signal/Noise
 - Having smaller volume of solvent prevent contamination of sample with solvent impurities
-

15N gHSQC of 1H 15N Labeled Ubiquitin in gHX Nanoprobe approx 1 Nanomole



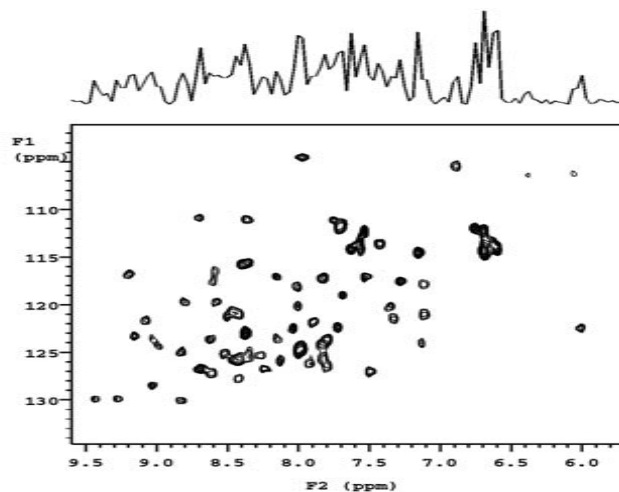
Pulse sequence gHSQCs

Doubly labeled Ubiquitin 40 nanomoles
in gHX nanoprobe 21 min
spinning speed 2600Hz

Pulse Sequence: gHSQCs

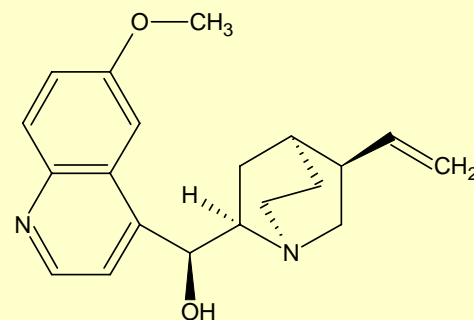
Solvent: D2O
Temp. 25.0 C / 298.1 K
File: UBIQUITIN_gHSQC
INNOVA-500 "fpi500"

PULSE SEQUENCE: gHSQCs
Relax. delay 1.000 sec
Acq. time 0.128 sec
Width 8000.0 Hz
2D Width 2532.9 Hz
16 repetitions
2 x 32 increments
OBSERVE H1, 499.9452761 MHz
DECOUPLE N15, 50.6647299 MHz
Power 40 dB
on during acquisition
off during delay
GARP-1 modulated
DATA PROCESSING
Gauss apodization 0.030 sec
F1 DATA PROCESSING
Gauss apodization 0.017 sec
FT size 1024 x 1024
Total time 21 min, 5 sec

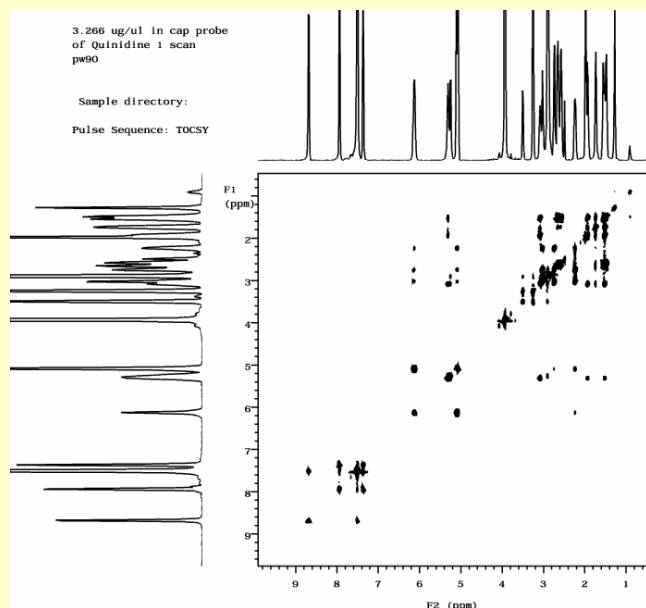


Example Quinidine in Capillary and Nano probes

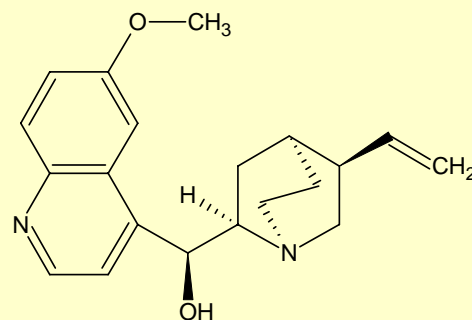
Quinidine 3.26ug/ul in Cap probe
Total amount 26 ug in 8 ul volume



TOCSY 1 scan per increment 256
complex increments



Quinidine in Nanoprobe



In gHX nanoprobe the same amount 26 ug gives about 1/2 signal/noise ratio this translates to 4 times increase of measurement time.

Larger volumes 120 ul 3 mm probe

Thyroxine glucuronide metabolite structure
-where the glucuronidation occurs?

NMR experiments ^1H , TOCSY
gHSQC
gHMBC

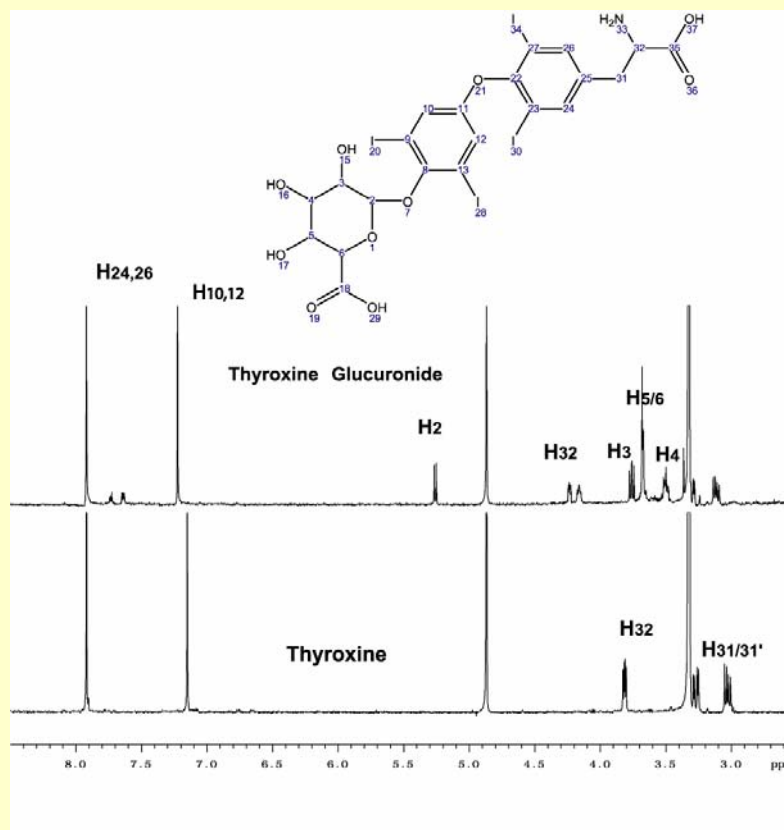


Figure 1. Structure of O-phenoxy-glucuronidated thyroxine.

Thyroxine 2D spectra

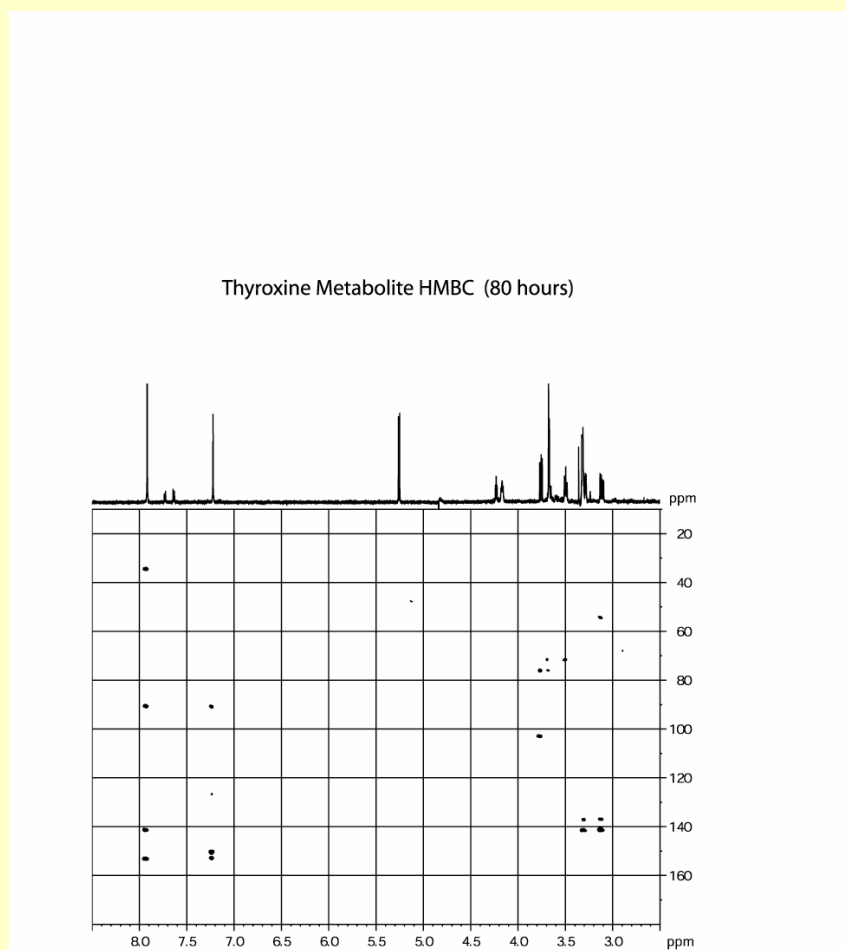


Figure 2. Gradient selected heteronuclear multiple bond correlation spectrum of O-phenoxy-glucuronidated thyroxine taken on the Bruker Avance 600 using a 3 mm indirect detection probe. Spectrum took more than 80 hours to acquire. Key correlations are found in the aromatic region.

Compare chemical shifts to parent compound thyroxine

SUMMARY

Based on 1- and 2-D NMR analysis of your Phase II metabolite of L-Thyroxine, the glucuronide appears to be located on the distal di-iodophenoxy group rather than on the α -amino acid group as a glucuronide ester. This tentative conclusion is based on the clear changes in proton and carbon chemical shifts in this group relative to the rest of the molecule. The NMR data are summarized in Table I and Figures 2 and 3. In particular, there is a significant downfield shift of more than 6 ppm at the iodinated carbons, C-9 and C-13, going from thyroxine to thyroxine glucuronide, which is consistent with the presence of an ether linkage at C-8 in the metabolite. The protons observed at H-10 and H-12 are also observed downfield, going from thyroxine to thyroxine glucuronide, whereas all of the carbons and protons in the di-iodophenoxy group closest to the amino acid group do not express significant changes. Further, the anomeric proton of the β -D-glucuronide in aromatic ethers is found at higher field than in acylglucuronides (see example structures and corresponding references below.), although the proton chemical shifts are too variable to be diagnostic. In the thyroxine metabolite, the anomeric proton is observed at δ 5.27, which is more consistent with the presence of an aromatic ether.

Number	Thyroxine		Thyroxine Glucuronide	
	¹ H	¹³ C	¹ H	¹³ C
2			5.27	103.14
3			3.75	
4			3.5	76.14
5			3.69	71.9
6			3.69	76.06
8		151.1		150.1
9		84.4		90.65
10	7.15	125.9	7.23	126.7
11		150.4		152.7
12	7.15	125.9	7.23	126.7
13		84.4		90.65
18				
22		153.14		153.01
23		90.4		90.4
24	7.93	141.5	7.93	141.2
25		141.5		141.1
26	7.93	141.5	7.93	141.2
27		90.51		90.4
31	3.04/3.27	35.32	3.11/3.31	35.0
32	3.81	55.71	4.16	55.0

Cryoprobes and Cold Probes

- Increase of signal/noise by eliminating thermal noise in the probe by cooling the receiver coil and cooling the preamplifier to 15 K
 - Increase of S/N by factor 3 –5 times – translates into 9 to 25 time increase of productivity
 - Extremely sophisticated and complex technology
 - Increased maintenance costs
 - Comfortable sample preparation and manipulation
 - Sample solubility is not a problem
 - Suitable for implementation of modern fast acquisition methods as Hadamard, FDM, GFT etc.
-

Choosing Probes and Sequences

Conclusions

- **Nature of the sample**

- **quantity and solubility are two major factors**

- **Highly soluble and easy transferable sample – low volume probes**

- **Tissues and beads use HR MAS probes – very universal**

- **Limited solubility and difficult to concentrate sample - cryoprobes**

- **Sequences**

- **choose sequences with maximum final s/n**

- **example – TOCSY is much more sensitive than gCOSY**

- **NOESY sensitivity is limited be careful with correlation**

- **times ROESY is good alternative**

- **HSQC instead of gHSQC if possible use sensitivity**

- **enhanced sequences.**

- **comparative methods**

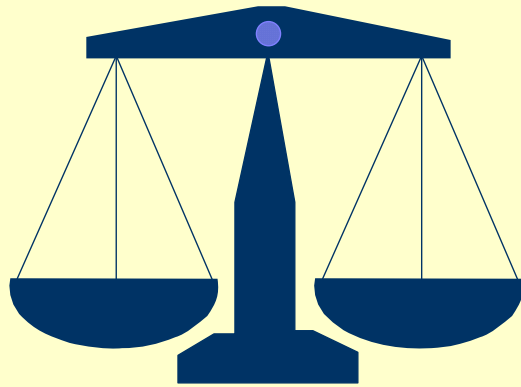
Fast NMR Methods

- Substantial Increase of Sensitivity (cold probes, cryoprobes)
 - In ND spectroscopy the main obstacle is large number of FID's required for desired resolution
 - Forward Linear prediction
 - Filter Diagonalization Method
 - Frydman's methods
 - Hadamard
 - Projection Reconstruction
 - GFT
-

Hadamard Spectroscopy

The HADAMARD Principle

(Hadamard, J, Bull.Sci.Math, 17, 240-248, 1893)



+ + + +
 + + - -
 + - + -
 + - - +



d
e
c
o
d
i
n
g

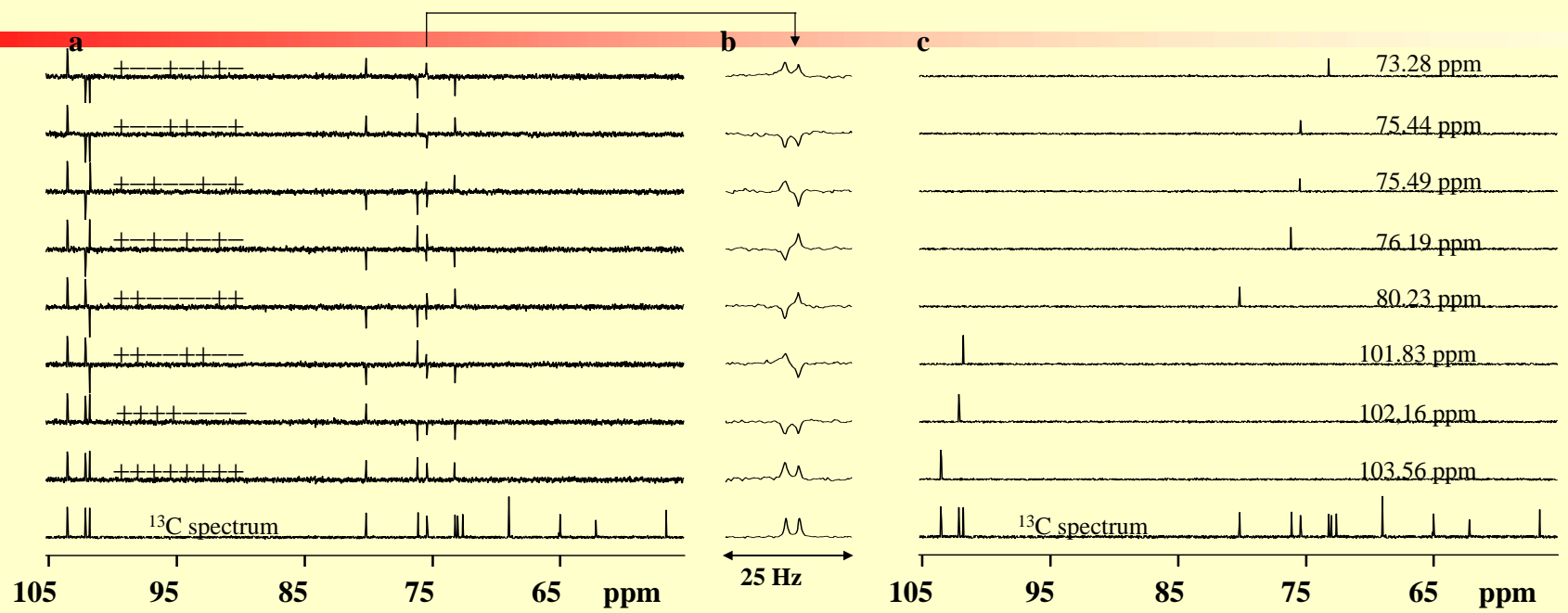
A B C D	=	+A +B +C +D	+
A B	C D	=	+A +B -C -D
A C	B D	=	+A -B +C -D
A D	B C	=	+A -B -C +D

encoding

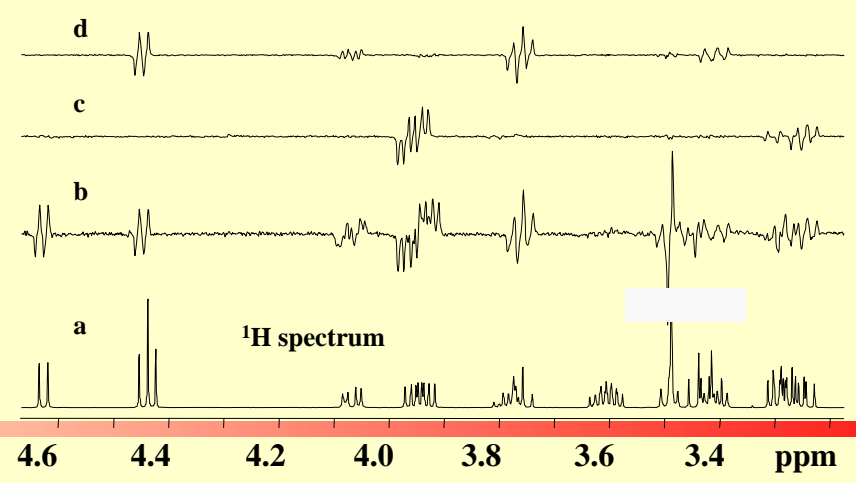
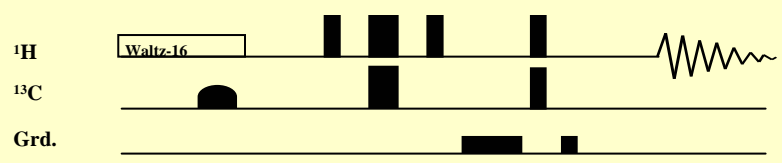
Hadamard matrices: 4^n ($n=1-267$) (4,8,12,16

2^n ($n=integer$) (2,4,8,16,....

The Hadamard Principle in Selective 1D NMR Experiments

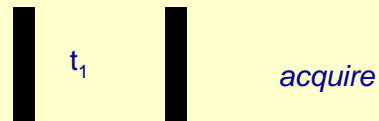


+	+	+	+	+	+	+	+
+	+	+	+	-	-	-	-
+	+	-	+	+	-	-	+
+	-	+	-	+	-	+	+
+	-	+	-	+	-	+	+
+	-	+	+	+	-	+	+
+	-	-	+	-	+	+	-



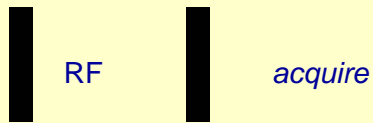
Principle of the nD Hadamard Spectroscopy

Conventional:

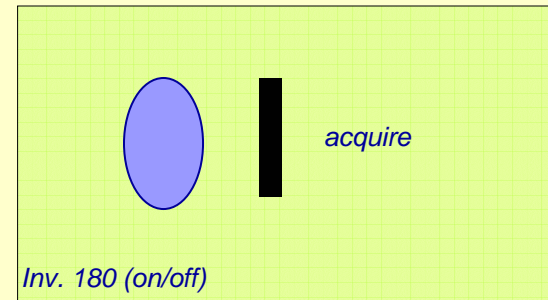
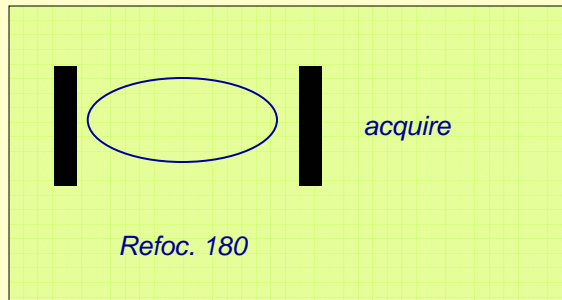
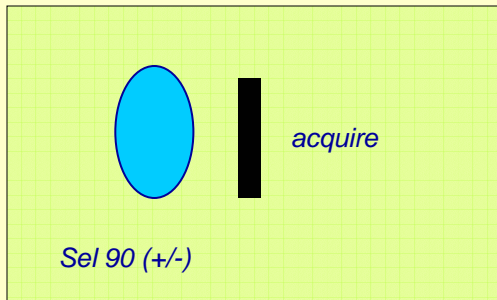


Free precession

RF driven:



RF driven evolution



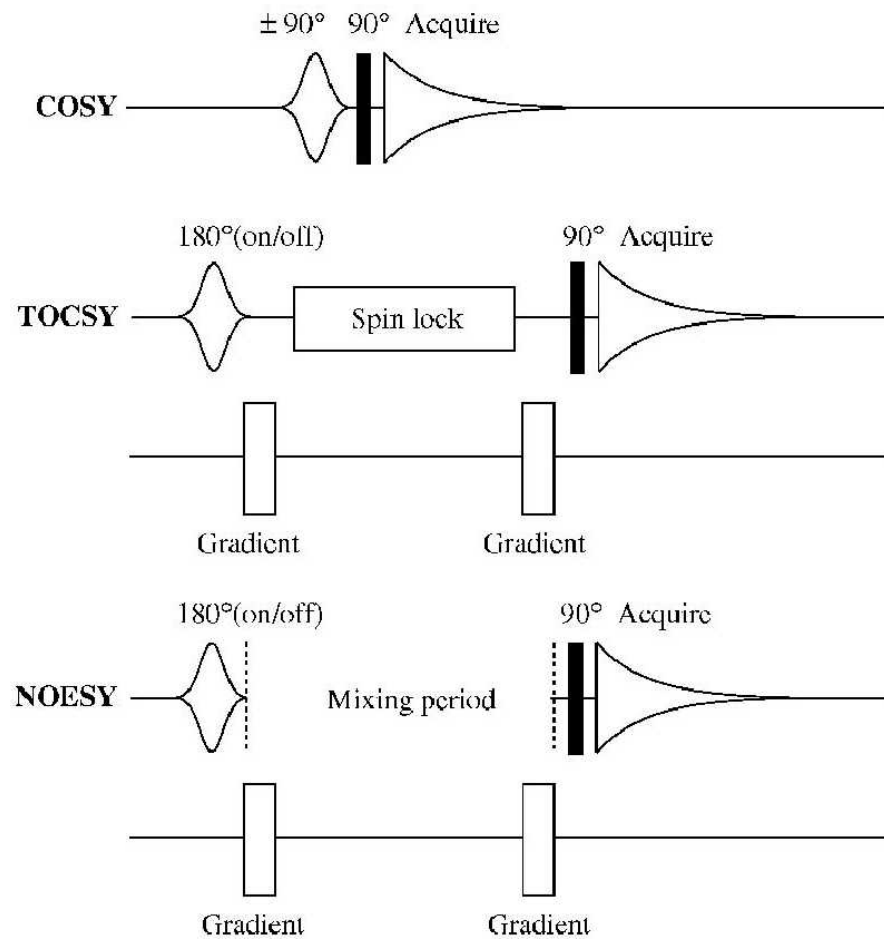
Phase Encoding (FT)

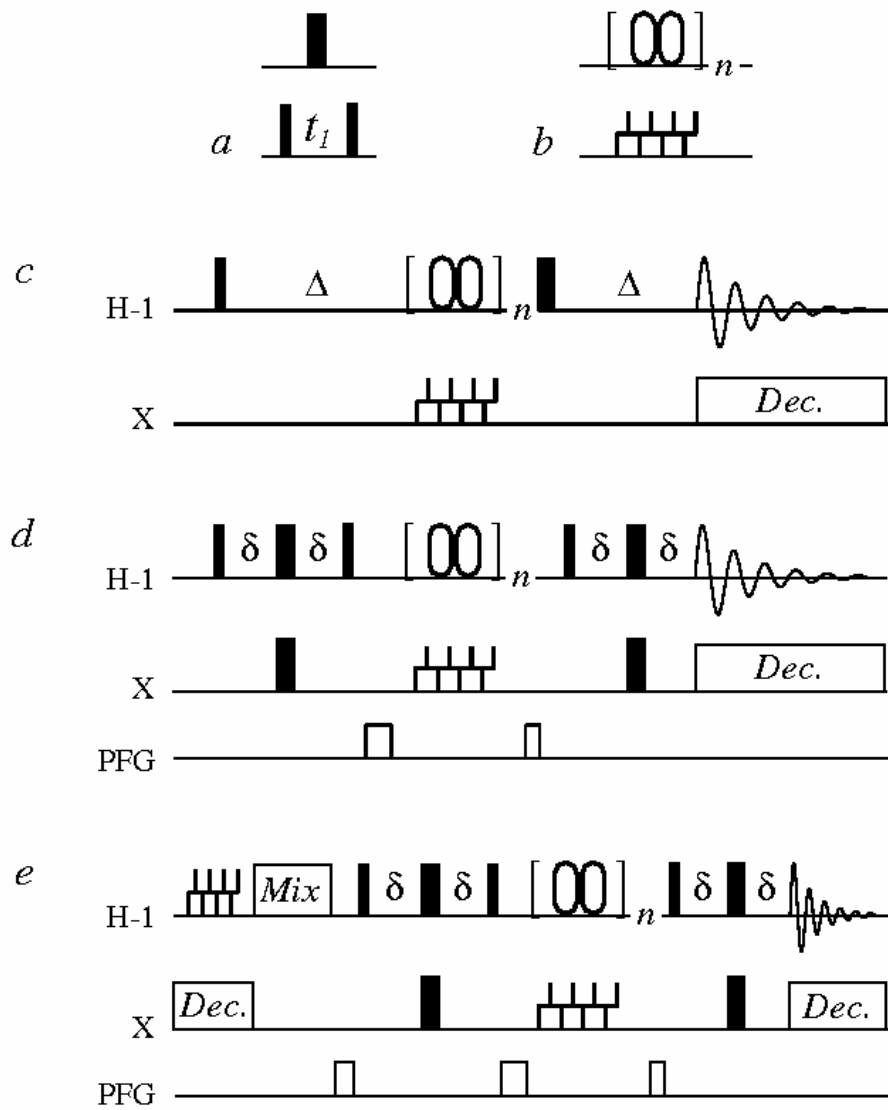
Hadamard Encoding (HT)

⊗ E. Kupce and R. Freeman *J. Magn. Reson.*, v.162, p.158, v.162, p. 300; v.163, p.56 (2003).

⊗ E. Kupce and R. Freeman *J. Biomol. NMR.*, v.25, p349 (2003).

Homo-Nuclear 2D Pulse Sequences with Hadamard Encoding





*Hadamard Encoded Hetero-Nuclear
2D and 3D Experiments*

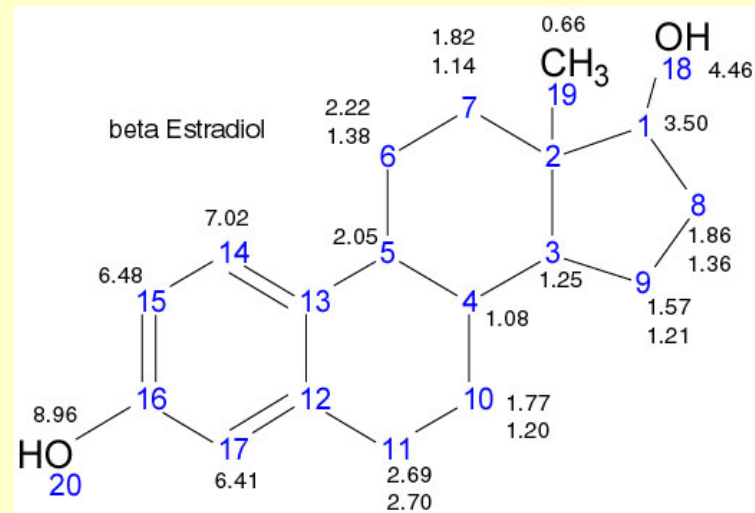
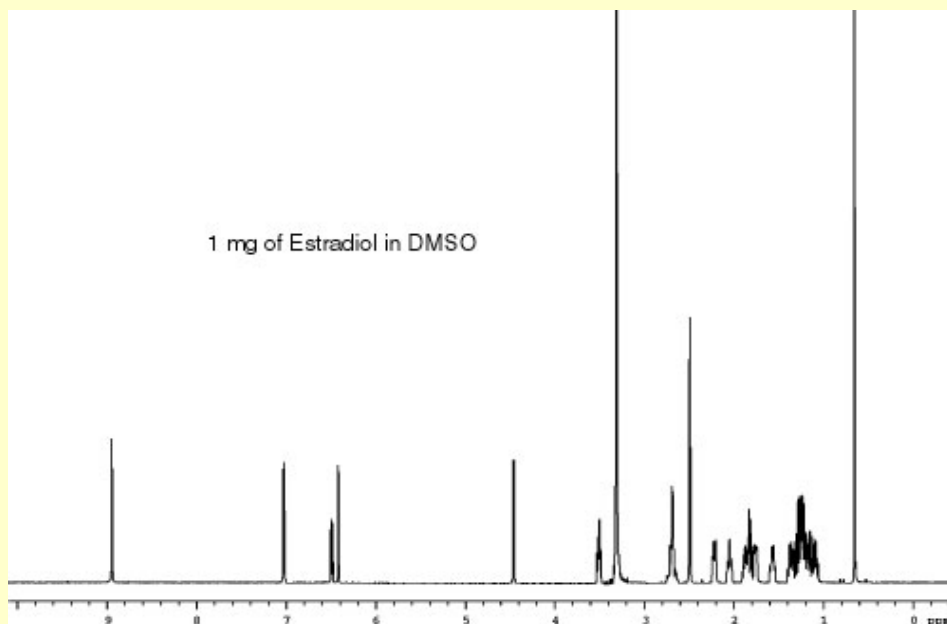
2D HMQC (c)

2D HSQC (d)

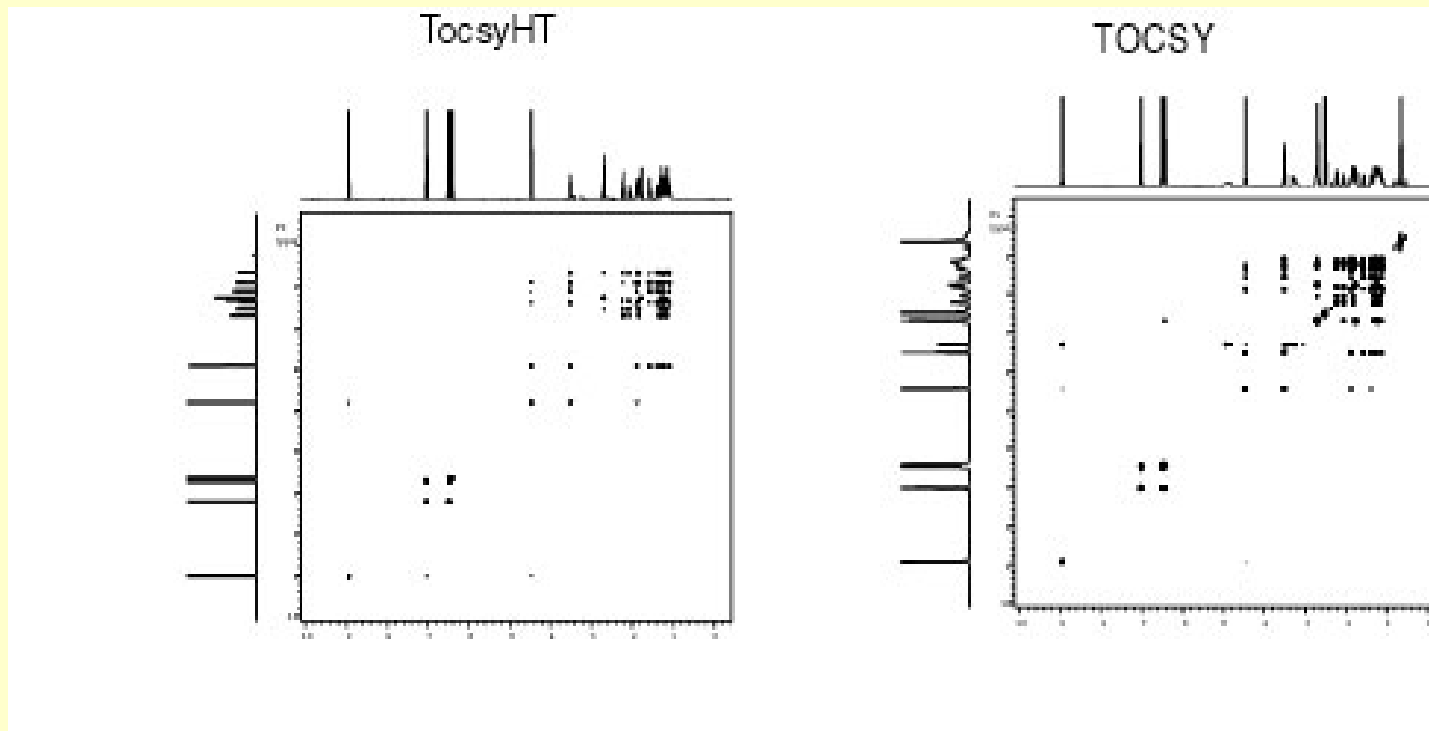
3D TOCSY-HSQC

Hadamard Transformation

Beta Estradiol



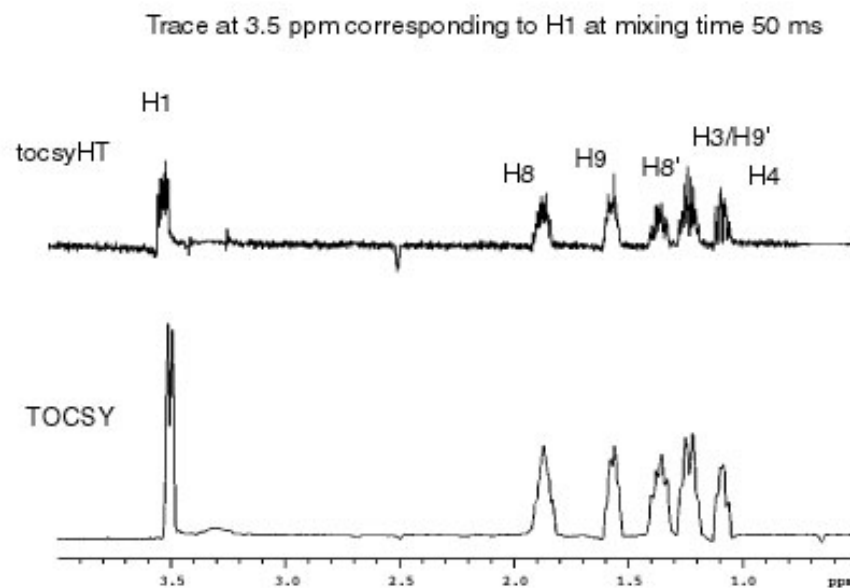
Hadamard Transformation



On the left is Hadamard TOCSY of Estradiol acquired in 42 seconds Mixing time is 50 ms. On the right is a classical phase sensitive TOCSY spectrum acquired in 45 minutes with 200 complex increments and 4 scans per increment in indirect f1 dimension.

Hadamard Transformation

Comparison of traces from tocsyHT and TOCSY at 3.5ppm. Note the considerably higher resolution in the Hadamard experiment



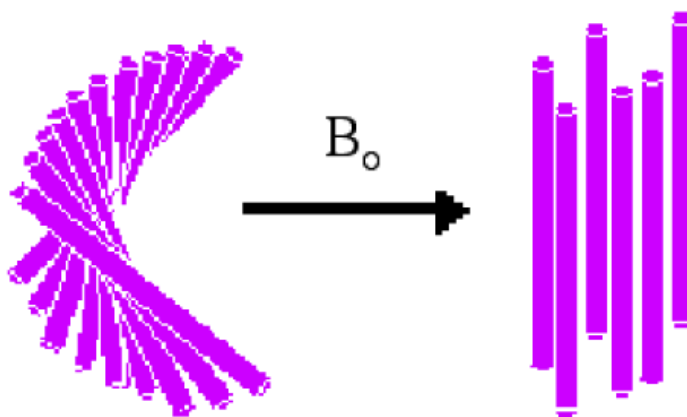
NMR and Chiral Compounds

- **Strategy**
 - **Get additional parameters as dipolar, quadrupolar interaction and chemical shift anisotropy into NMR spectra using orientation media in magnetic field**
 - **This information is R / S dependent**
 - **Spectra become more complex and they are stereospecific**
 - **Extract this information using various methods as ^1H selective decoupling, FCHSQC, INADEQUATE etc.**
 - **Compare extracted parameters with calculated using molecular modeling and assign stereochemistry**
-

Chiral Liquid Crystals

Magnetic Ordering of PBLG Liquid Crystals

PBLG forms twisted liquid crystalline phase in organic solvents such as CDCl_3 , CD_2Cl_2 , THF and DMF

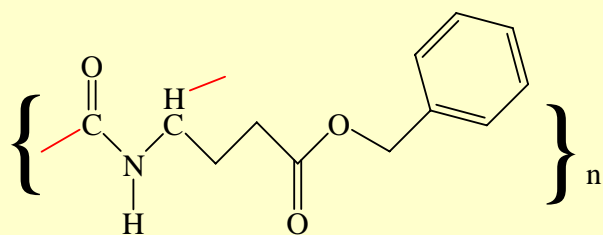


Chiral nematic liquid crystalline phase
(partial alignment
In magnetic field)

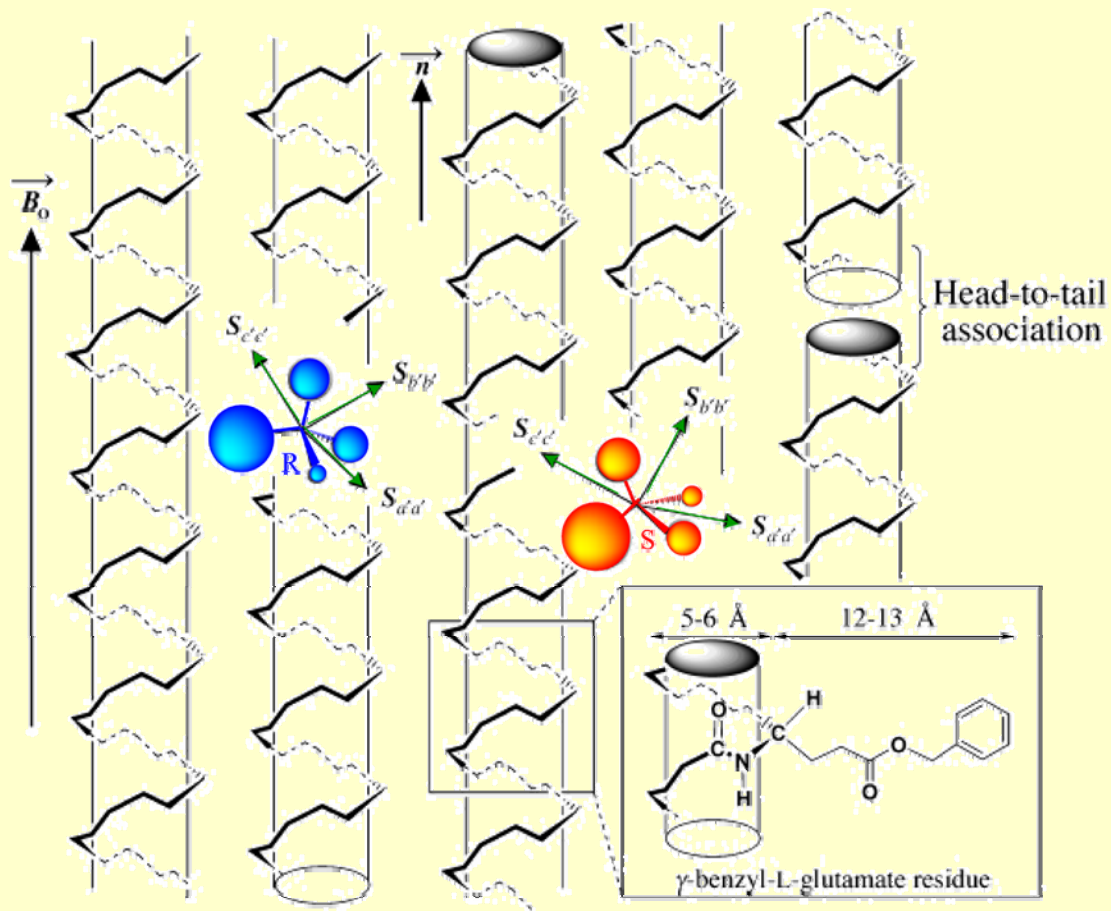
Magnetic torque induces an uncoiling of the twisted state to form the nematic phase

M. Panar & w. E. Phillips; JACS 1968, 90, 3880

NMR and Chiral Compounds



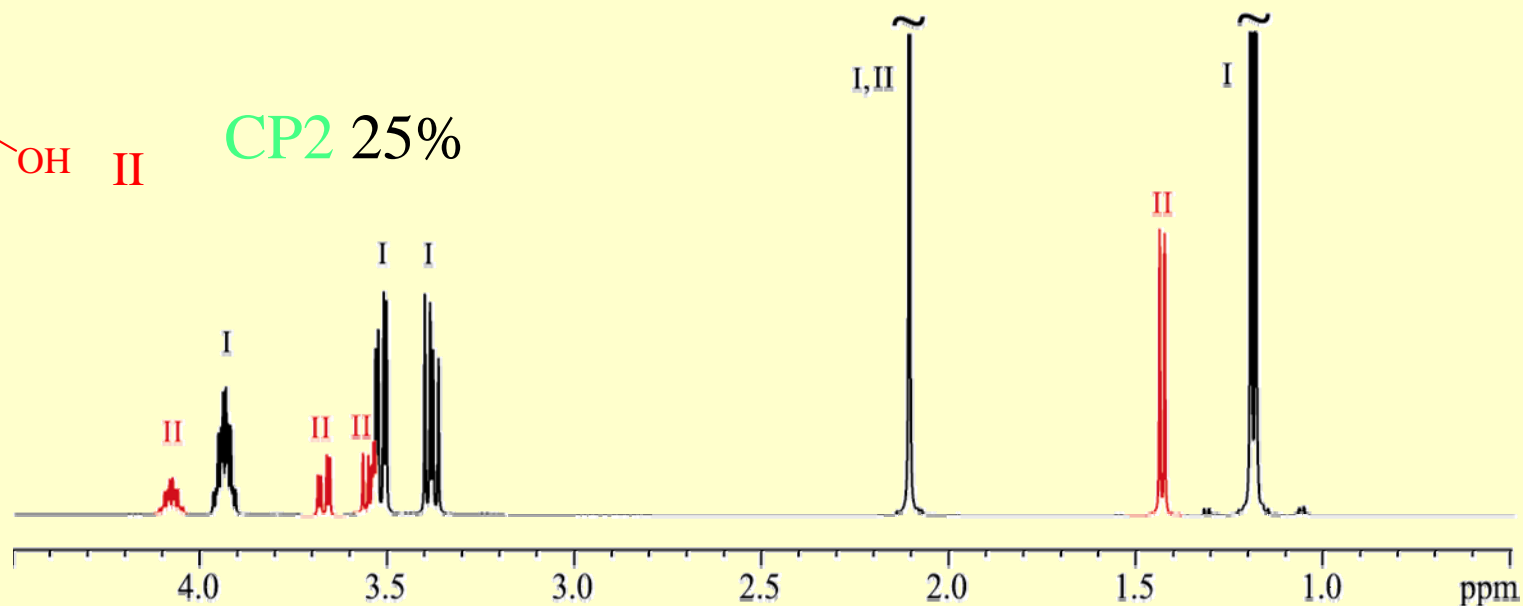
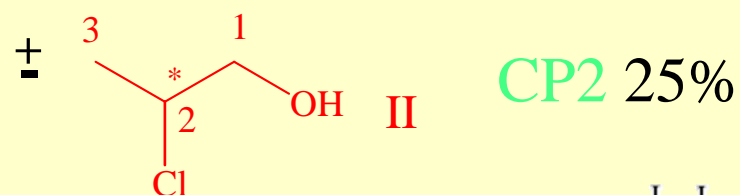
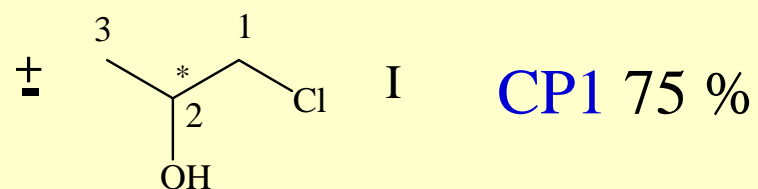
- Liquid crystalline phase is composed of a polypeptide (poly- γ -benzyl-L-glutamate, PBLG) dissolved in an organic solvent.



Chiral Liquid Crystals

^1H - NMR
 CDCl_3 at 25 °C

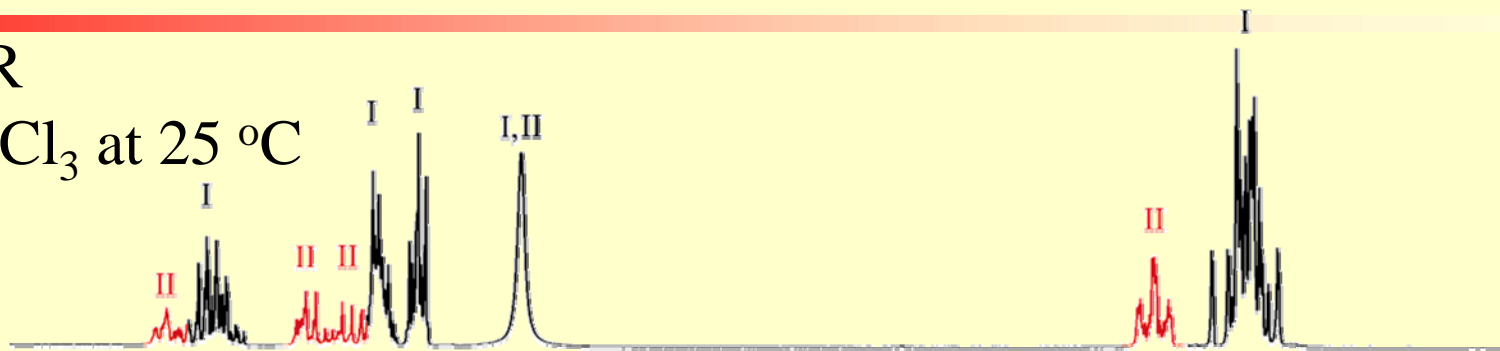
Experimental Results



Chiral Liquid Crystals

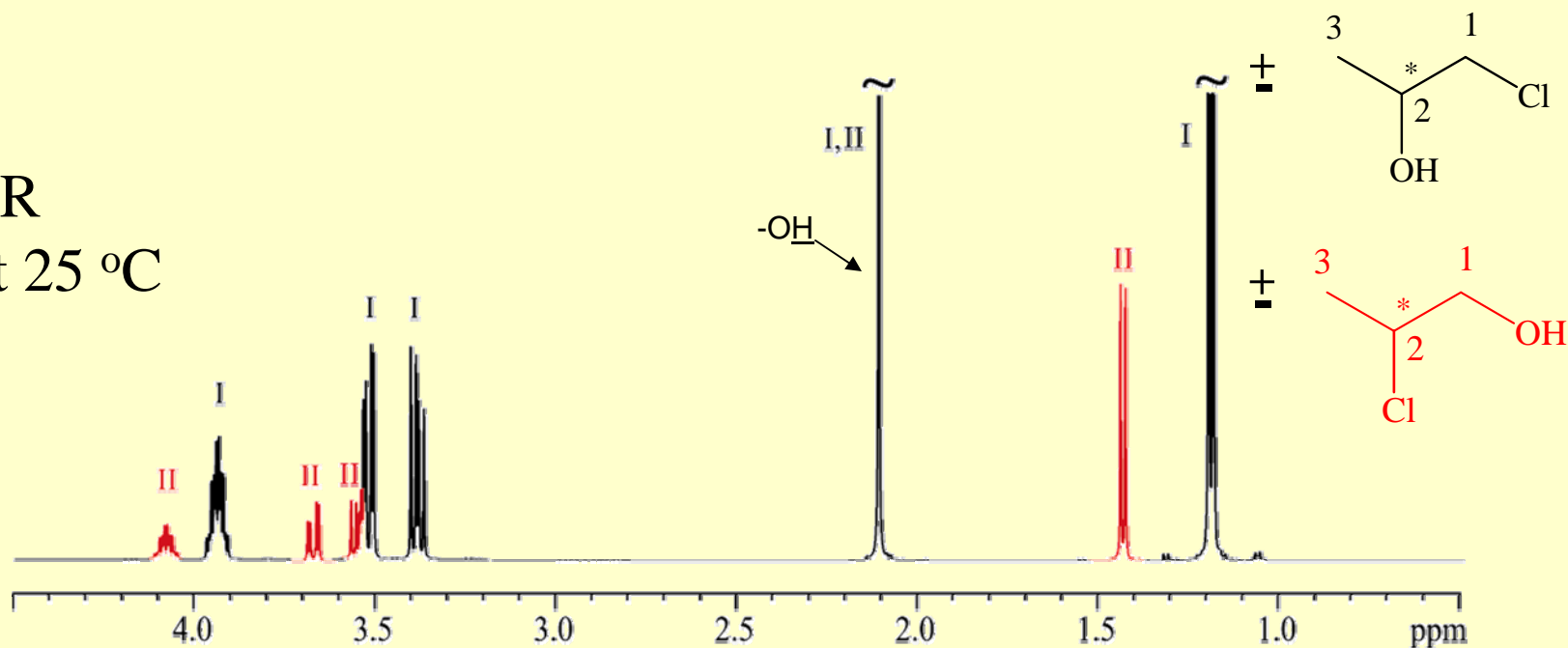
^1H - NMR

LQ + CDCl_3 at 25 °C



^1H - NMR

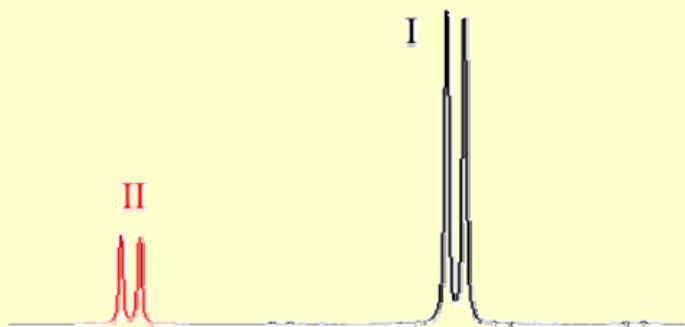
CDCl_3 at 25 °C



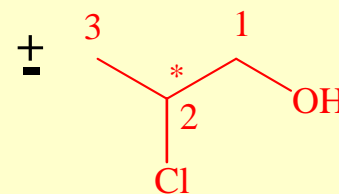
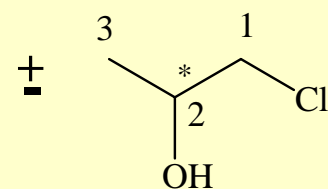
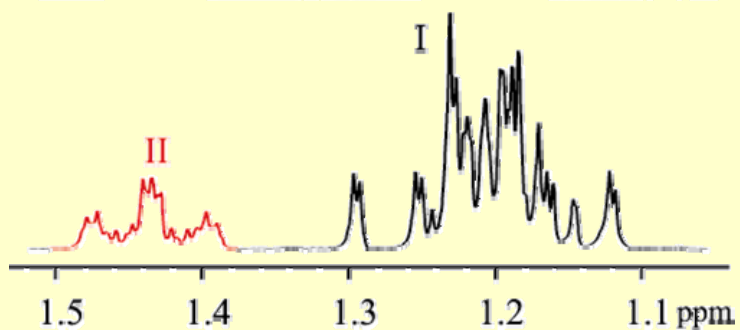
Chiral Liquid Crystals

Methyl Groups

^1H - NMR
 CDCl_3 at 25 °C

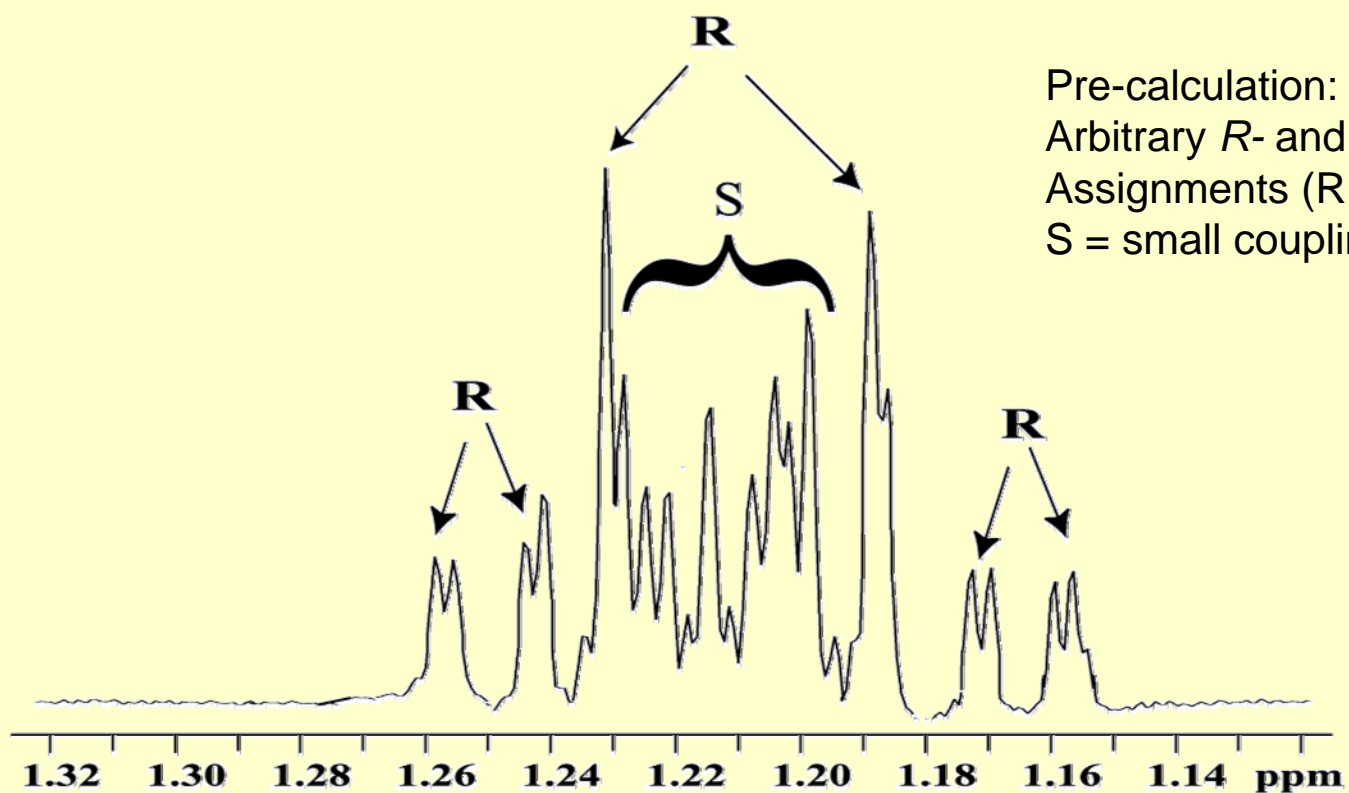


^1H - NMR
LQ + CDCl_3 at 25 °C



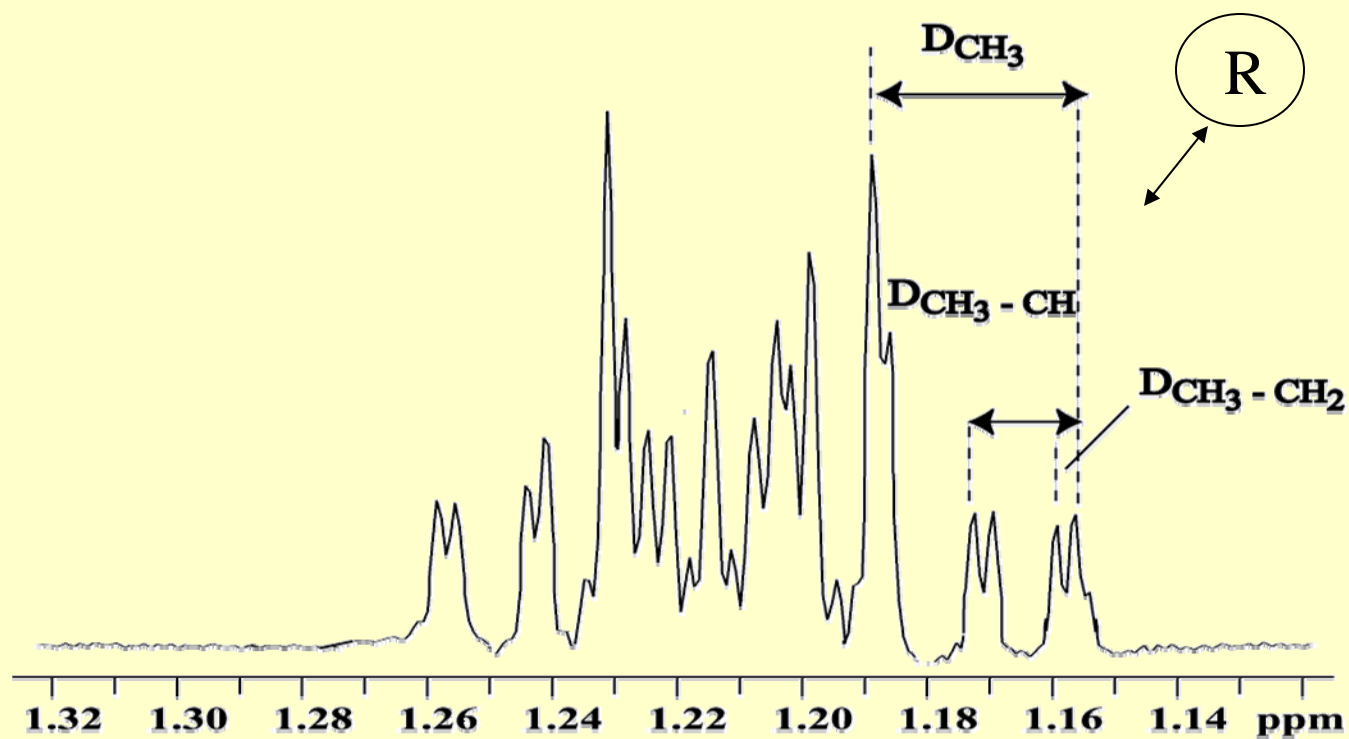
Chiral Liquid Crystals

Experimental Results



Chiral Liquid Crystals

Experimental Results



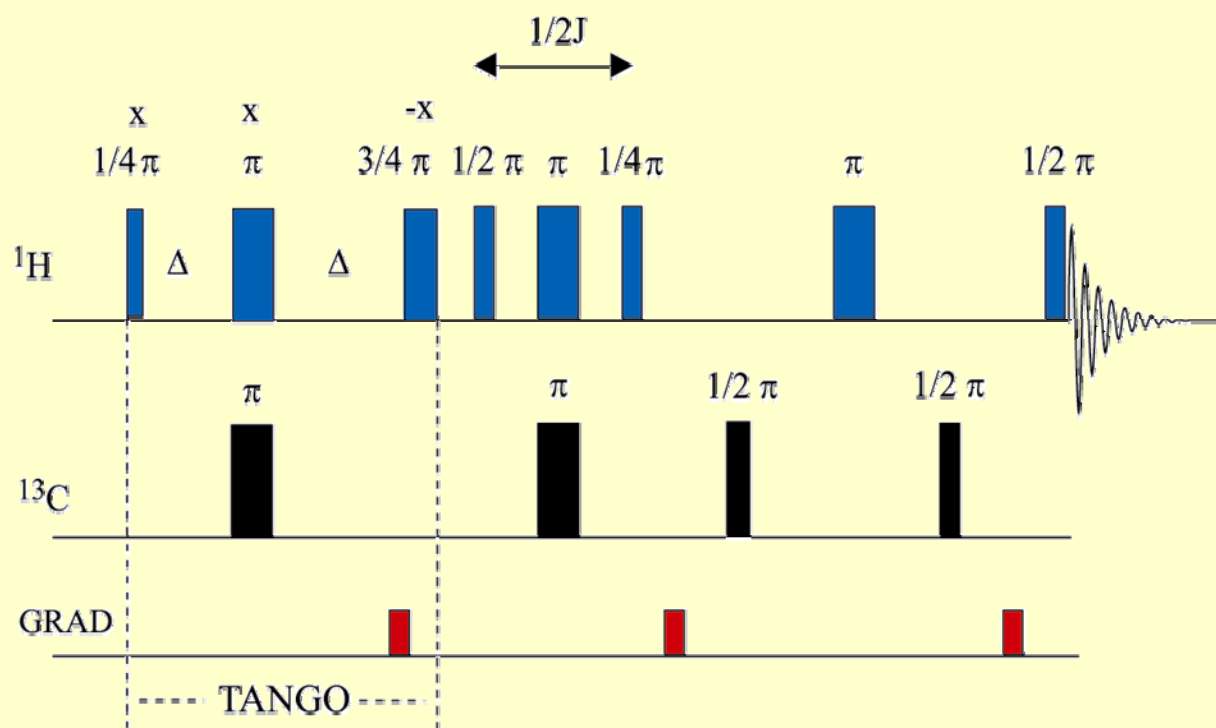
Chiral Liquid Crystals

Current Experimental Requirements

- 10 mg sample total (mixture of 7.5 mg CP1 and 2.5 mg CP2)
- Natural abundance indirect ^1H – detect experiments completed in 1.5 hours
- No Chemical modification required

Chiral Liquid Crystals

J-D Resolved HSQC (JD-HSQC) NMR Experiment



Removed refocusing pulses and delays and ^{13}C decoupling

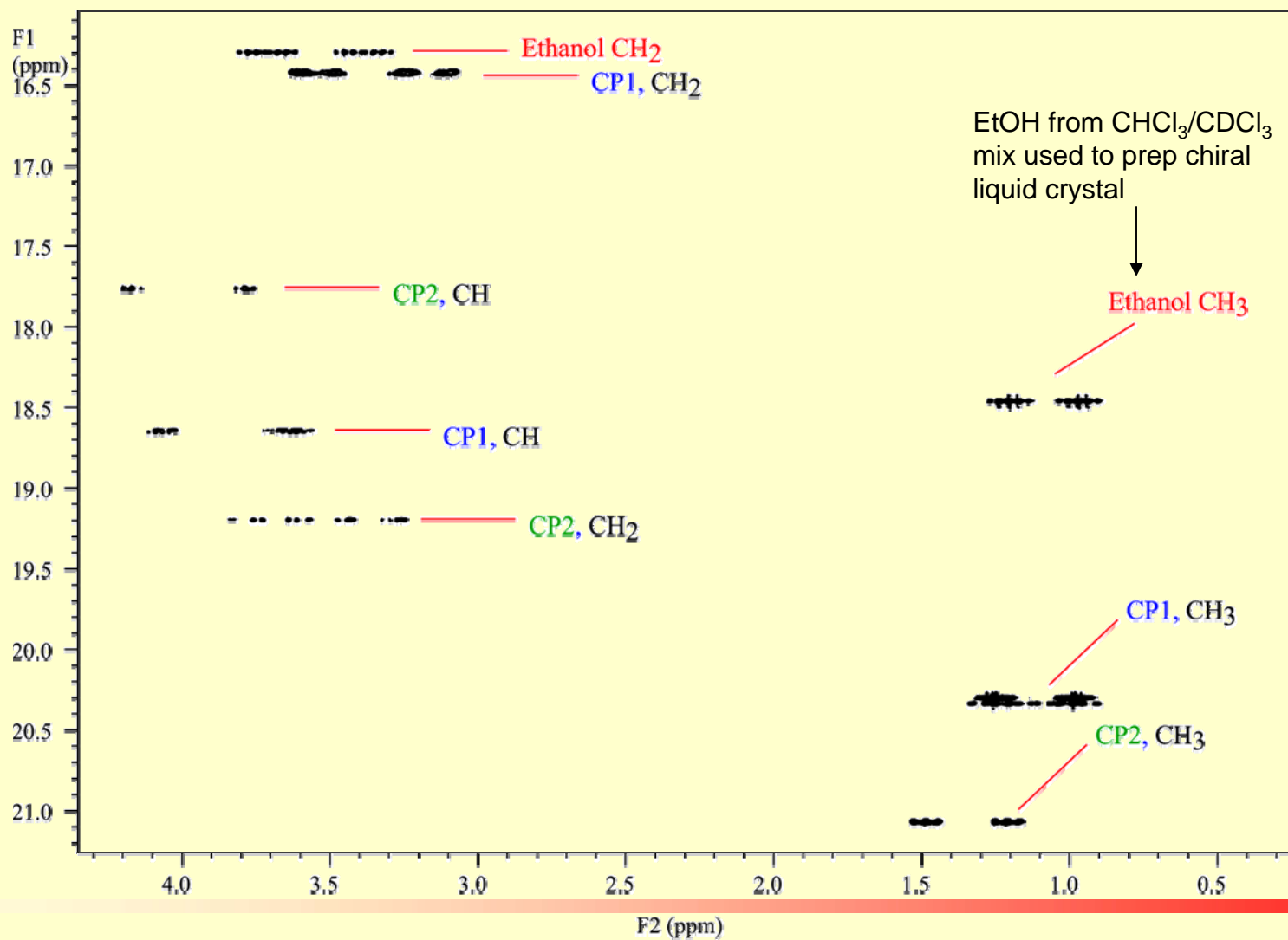


Leads to in-phase and anti-phase peaks

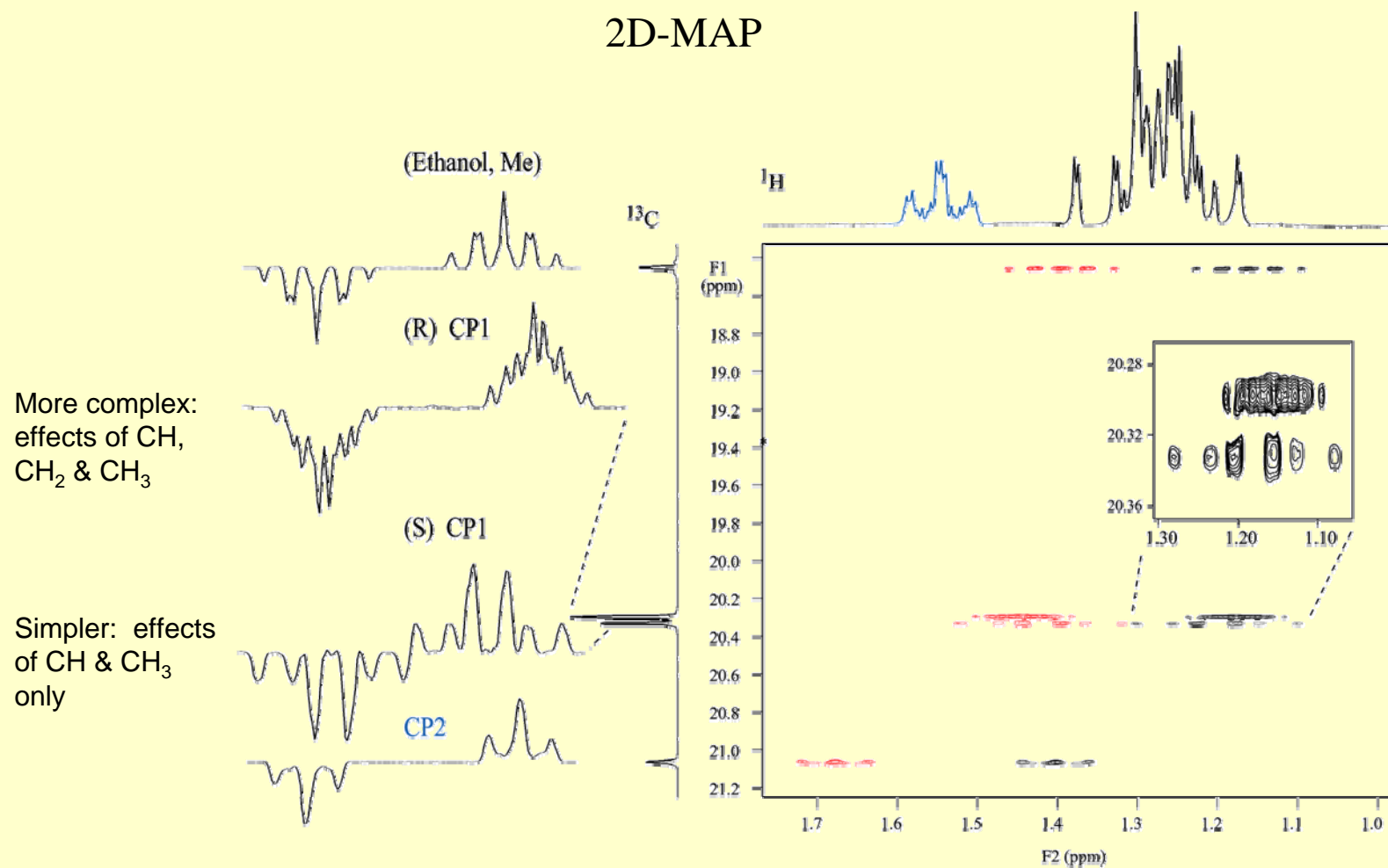
J-D Resolved HSQC (JD-HSQC) NMR Experiment

^1H Full 2D HSQC MAP

^{13}C

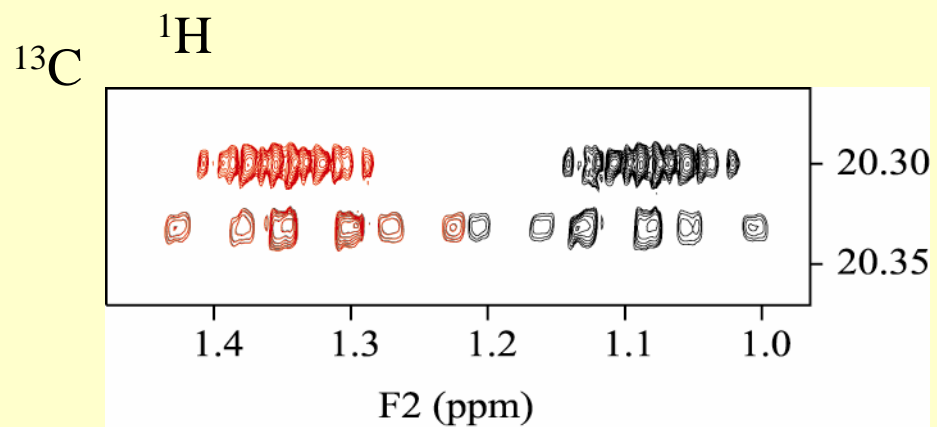


J-D Resolved HSQC (JD-HSQC) NMR Experiment



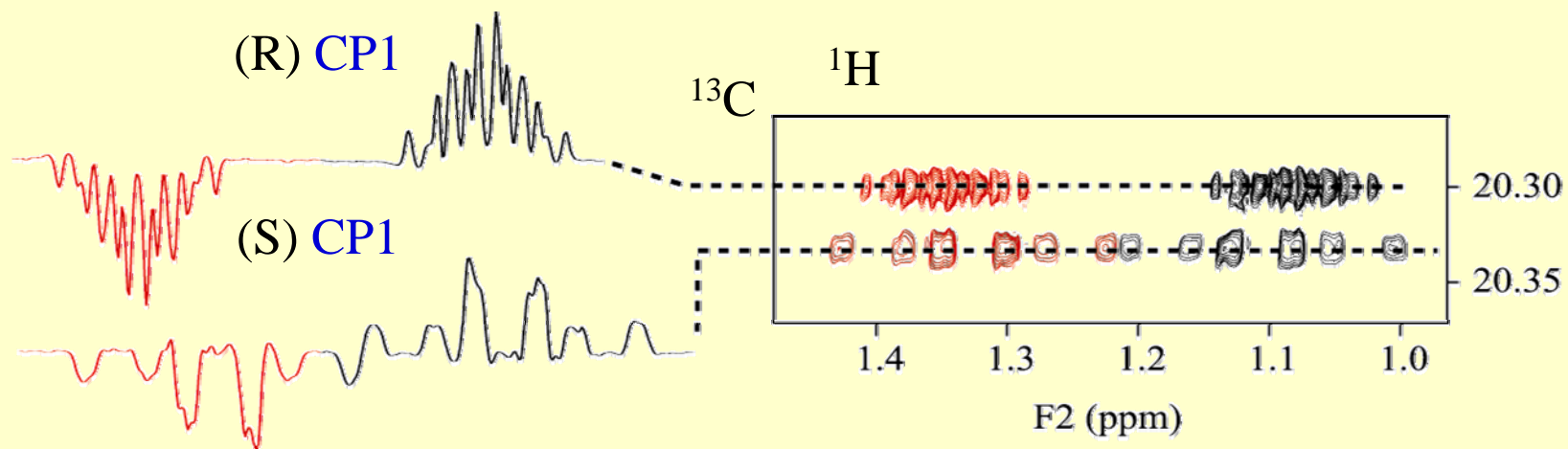
J-D Resolved HSQC (JD-HSQC) NMR Experiment

CH₃ region

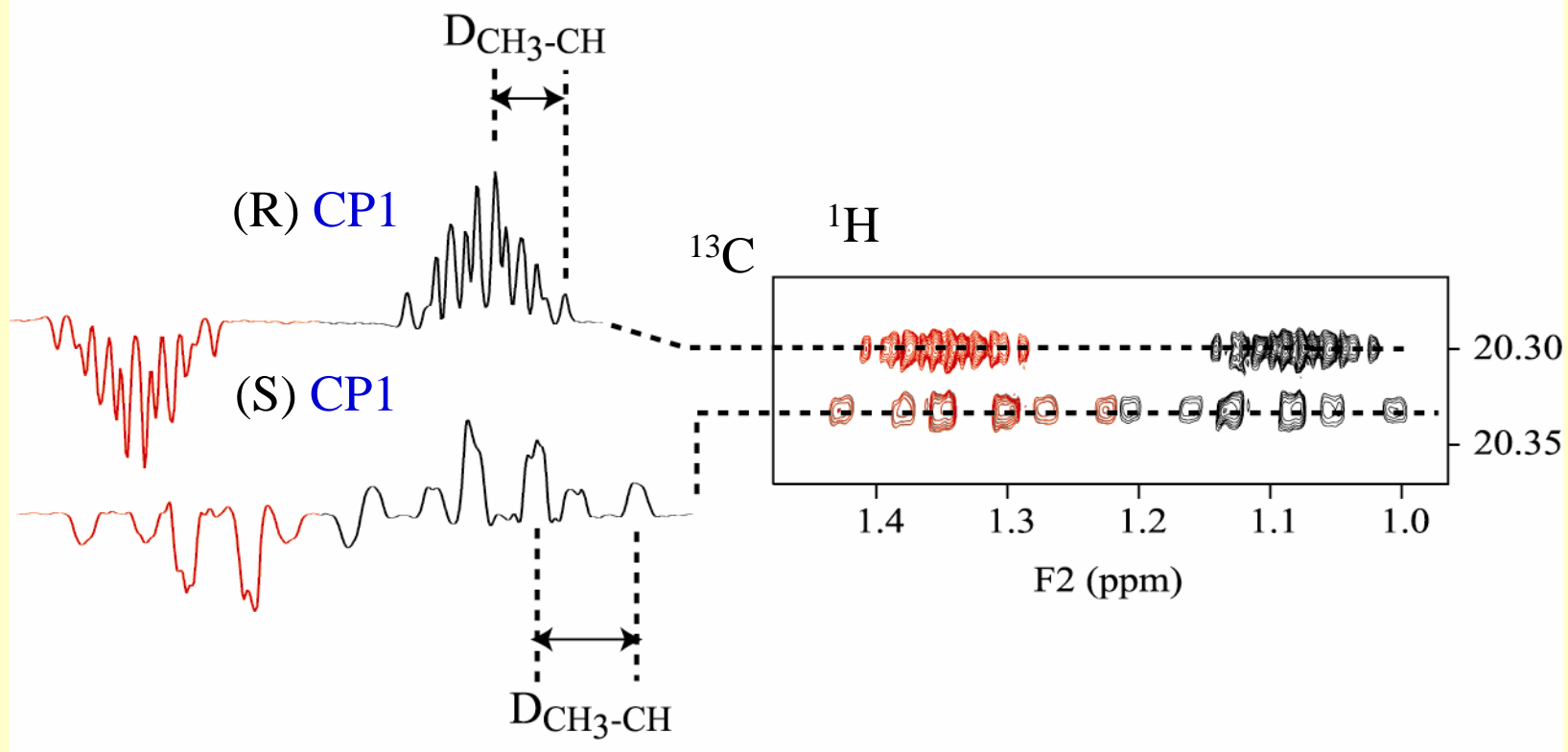


J-D Resolved HSQC (JD-HSQC) NMR Experiment

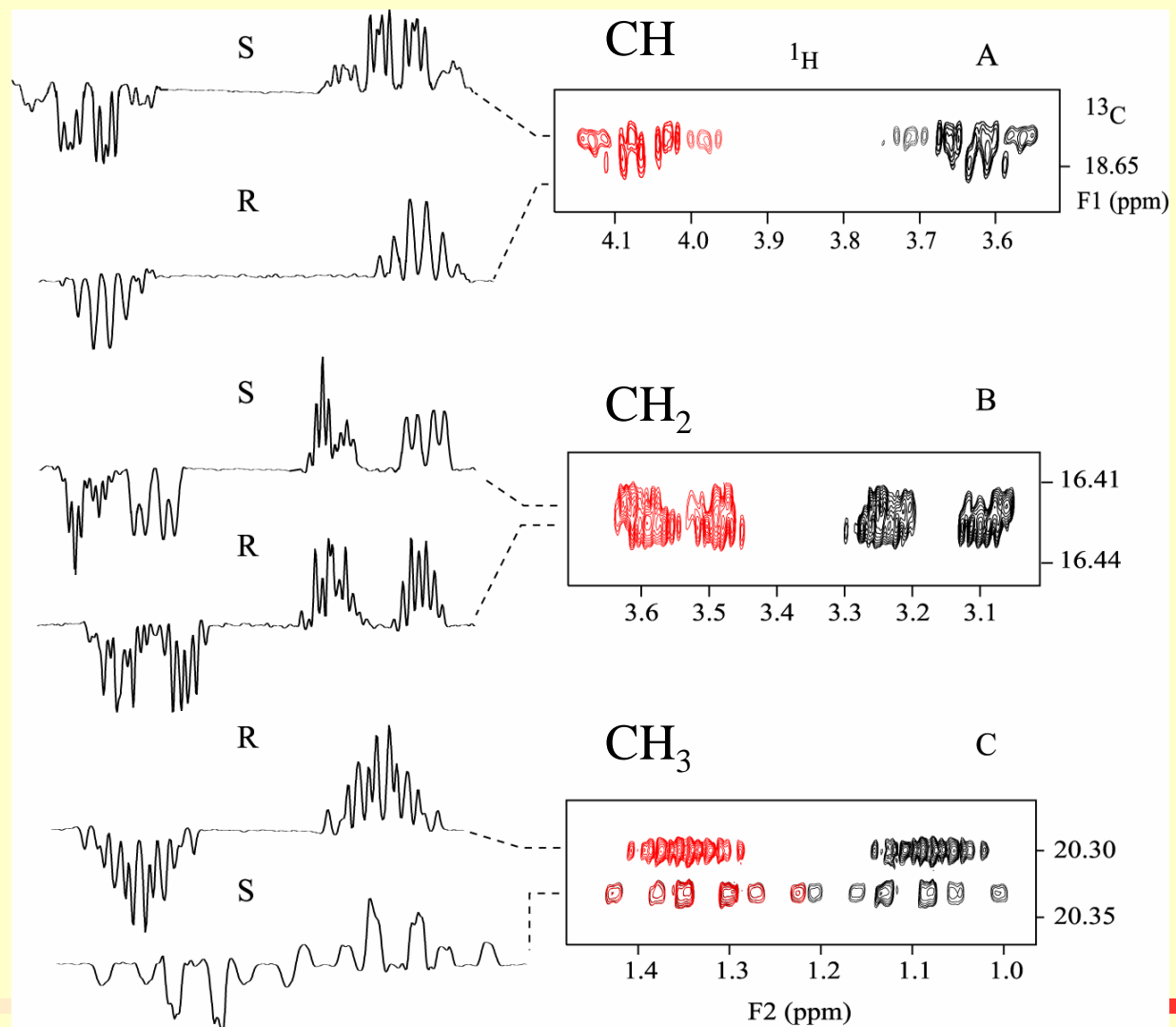
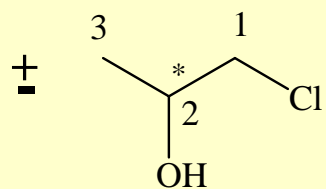
CH₃ region



J-D Resolved HSQC (JD-HSQC) NMR Experiment

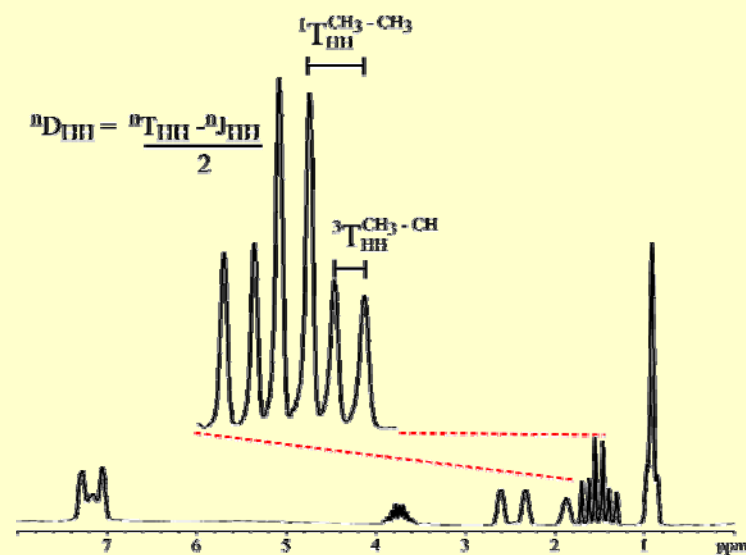
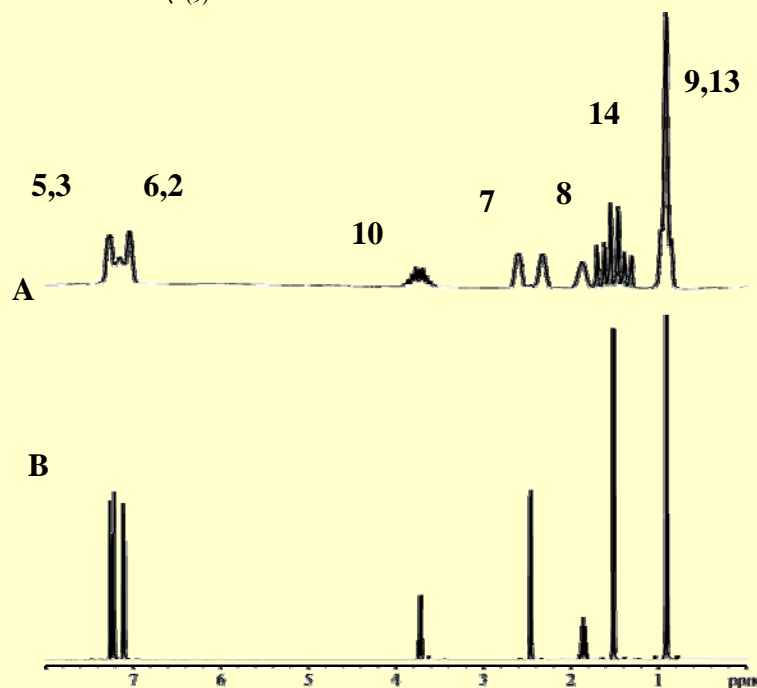
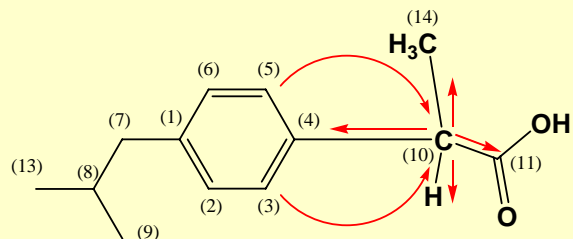


J-D Resolved HSQC (JD-HSQC) NMR Experiment



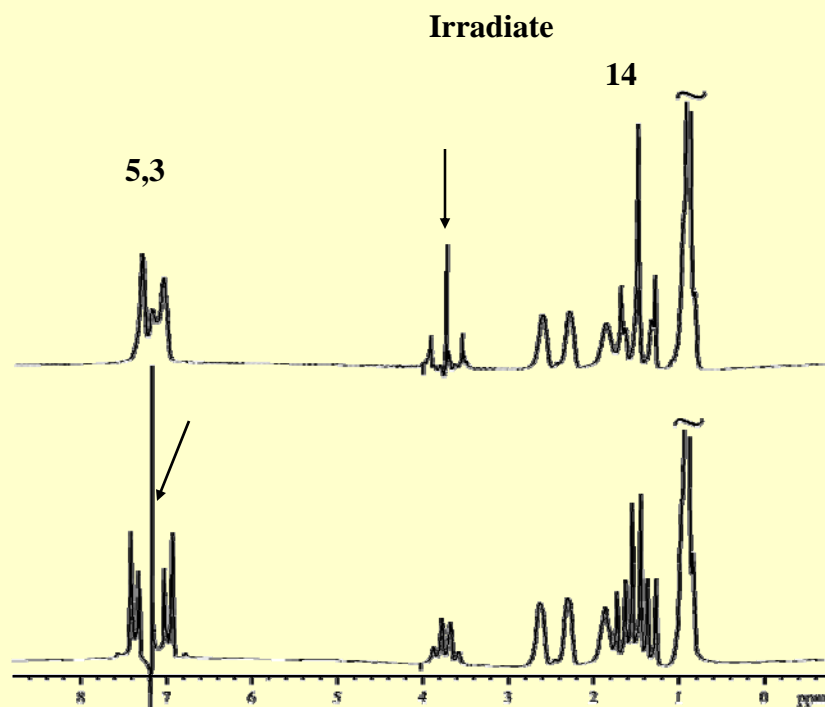
Spectra in oriented media

In oriented media we rely on dipole-dipolar interactions ^1H - ^1H , ^1H - ^{13}C , ^{13}C - ^{13}C etc



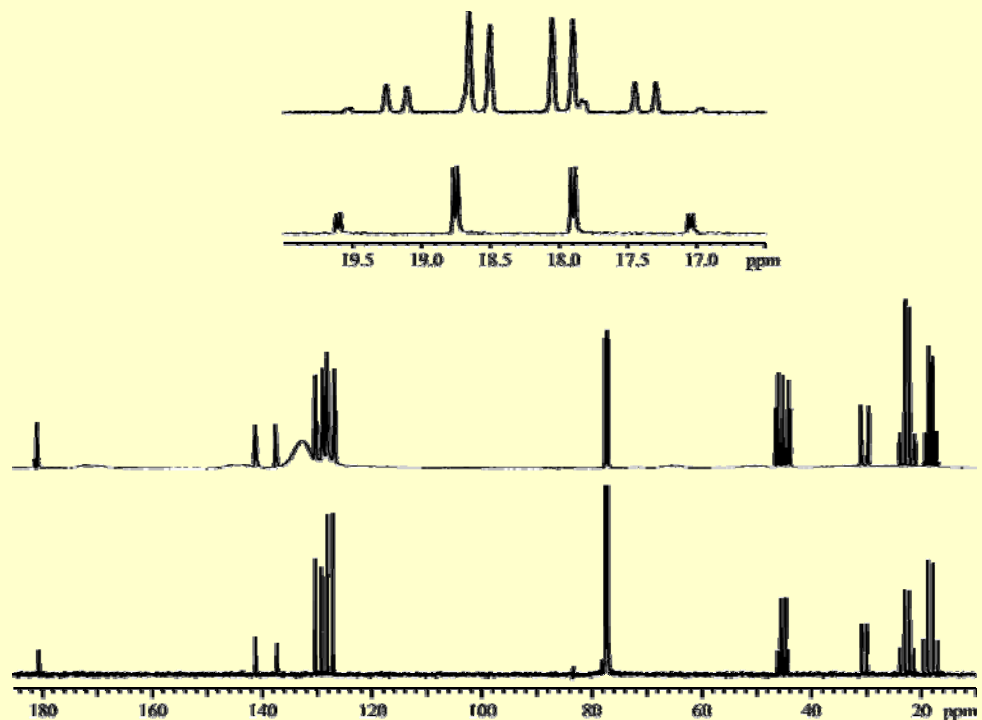
$$^nD_{\text{HH}} = \frac{nT_{\text{HH}} - nJ_{\text{HH}}}{2}$$

Spectra in oriented media



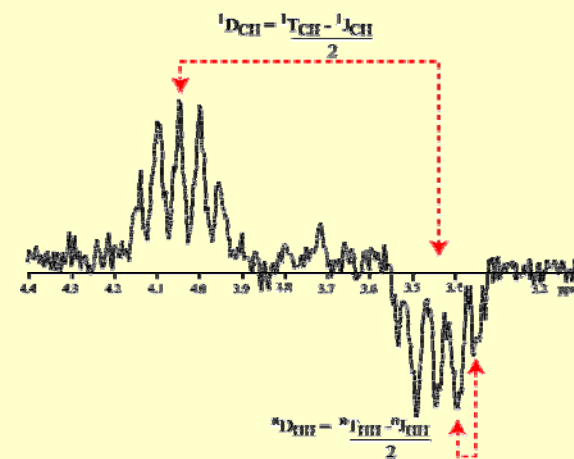
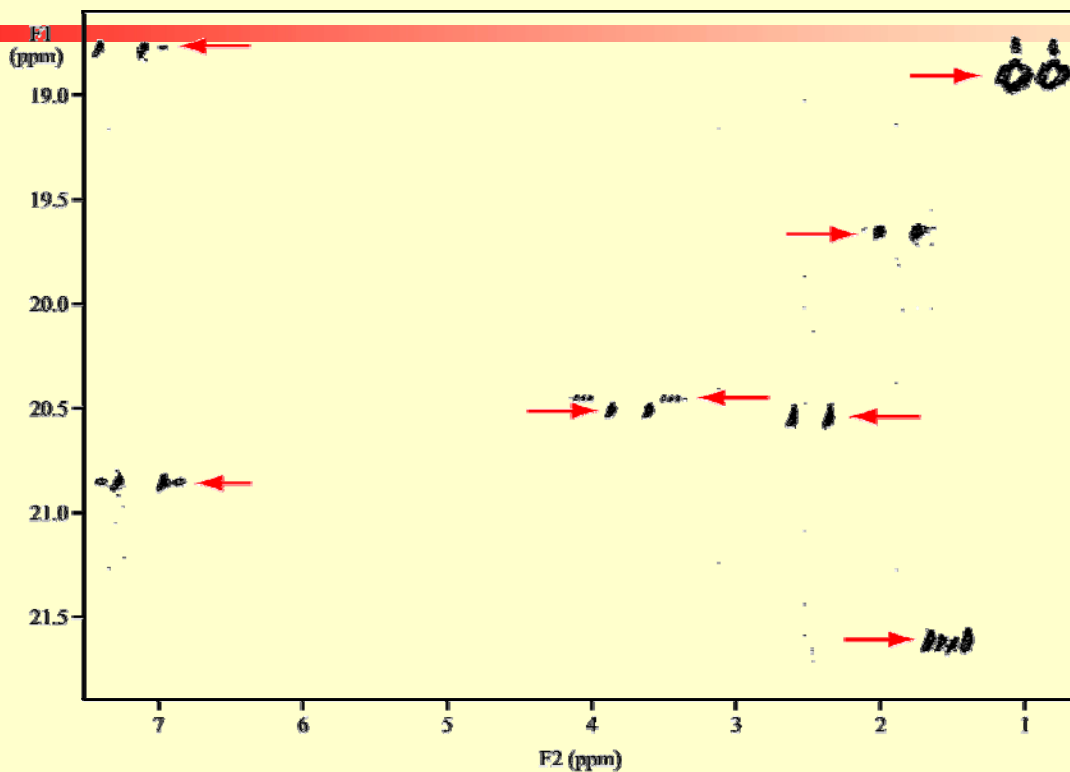
$^1\text{H-NMR}$ Spectra with selective proton decoupling of (S)Ibuprofen in PBLG/ CDCl_3 alignment media. When the methyne resonance (10) is irradiated, both the aromatic protons (5,3) and the methyl (14) simplify (A). When the aromatic protons are irradiated, only the methyne (10) simplifies (B). This indicates that proton (10) is coupled to both (5,3) and (14).

Spectra in oriented media



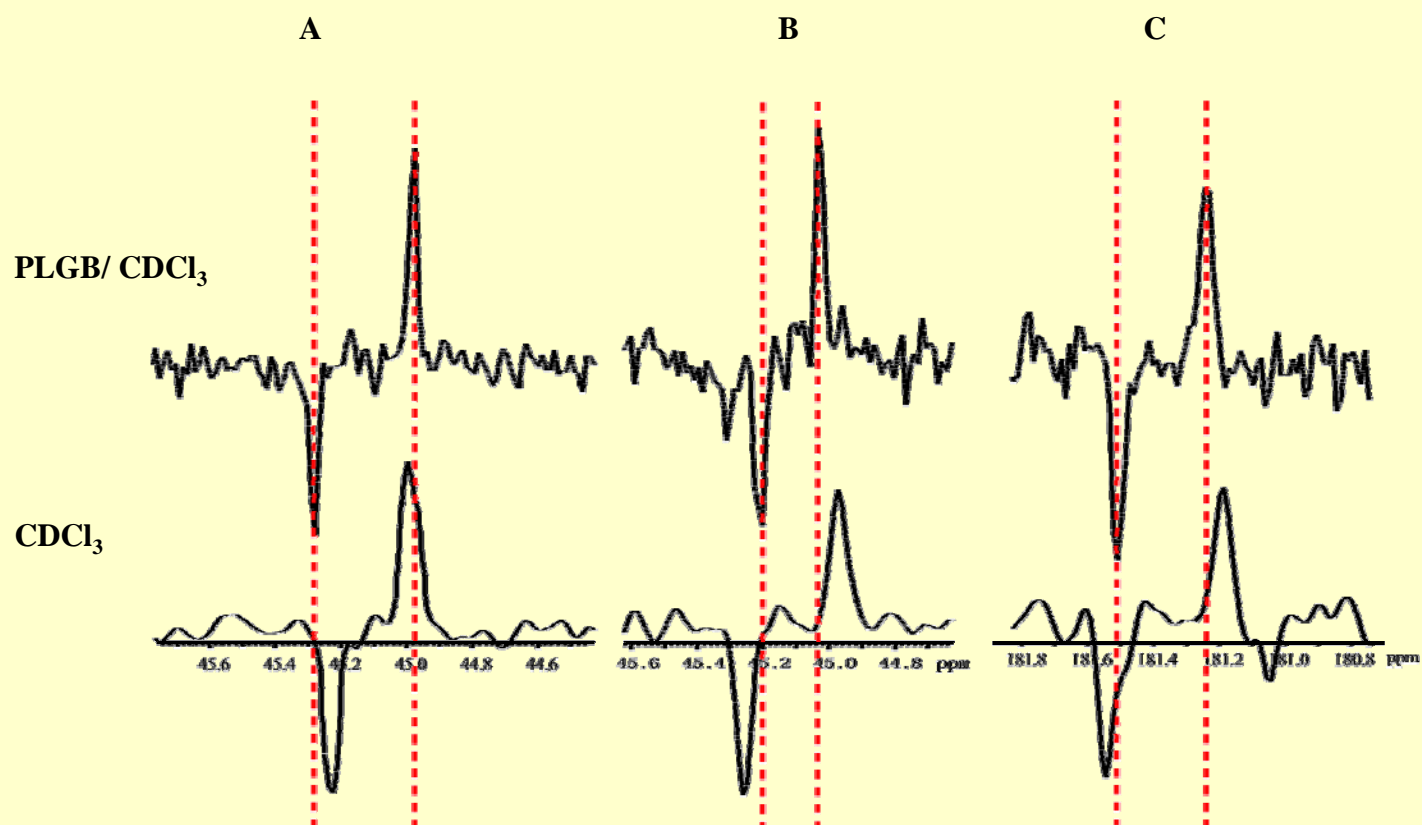
^{13}C -NMR (^1H) coupled Spectra. DCH coupling can be obtained from this experiment. (S) ibuprofen present in PBLG/ CDCl_3 (A) and **ibuprofen, in standard CDCl_3 , (B)** have very distinctive couplings. This is more apparent in the expansion of the methyl (14) resonances (C). Selective decoupling was performed for these experiments as well.

Spectra in oriented media



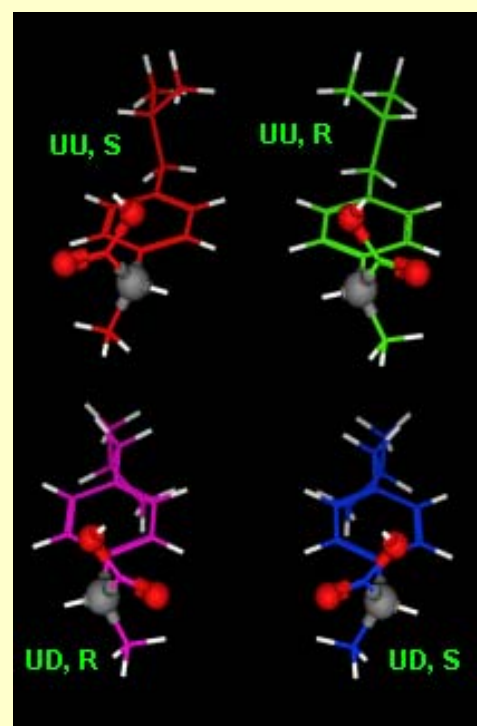
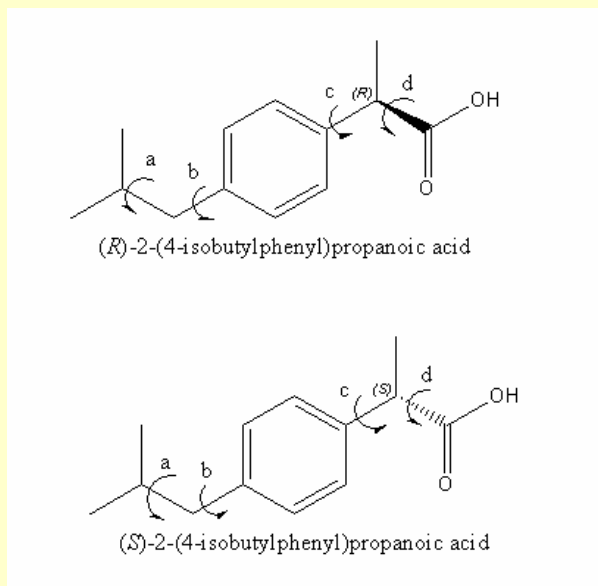
1H - ${}^{13}C$ -NMR F2HSQC 2D experiment of (S) Ibuprofen. Both DHH and DCH coupling can be simultaneously obtained from this experiment. By going to a two dimensional experiment the resolution can be substantially increased when compared to a standard ${}^{13}C$ -NMR (1H) coupled experiment. A small amount of (R) ibuprofen can also be observed in the 2D map.

Spectra in oriented media



Traces from the 2D ¹³C Inadequate experiment of (S) Ibuprofen. The figure shows the differences in the TCC couplings between CH(10) –CH₃(14) (A), CH(10) –CH(4) (B), and CH(10) –CO(11) (C).

Spectra in oriented media



Analysis of the two lowest energy lead structures for Ibuprofen (R) and (S) reveal two geometry types – those with carbonyl oxygen and isopropyl above the plane of the central aromatic (UU) and those with carbonyl oxygen and isopropyl above and below the plane of the central aromatic (UD) respectively. Since both R and S enantiomers were considered, there are 4 structures in all:

Spectra in oriented media

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Acknowledgements

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