Molecular Spectroscopy

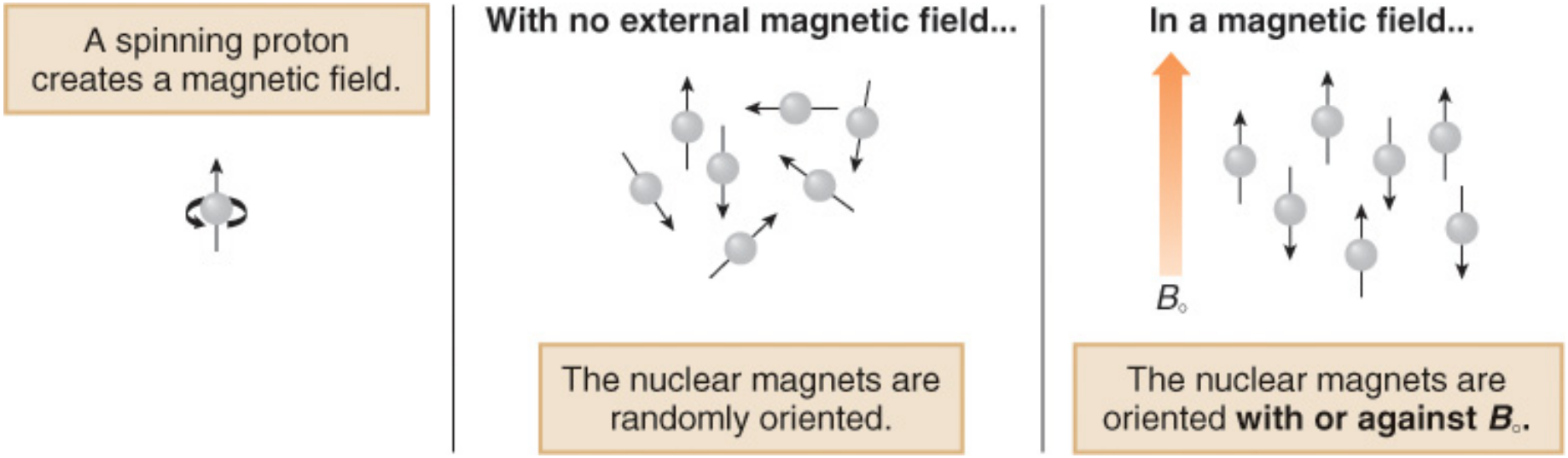
- We study three types of molecular spectroscopy

Region of the Electromagnetic Spectrum	Absorption of Electromagnetic Radiation Results in Transition Between
radio frequency	nuclear spin energy levels
infrared	vibrational energy levels
ultraviolet-visible	electronic energy levels

Nuclear Magnetic Resonance Spectroscopy

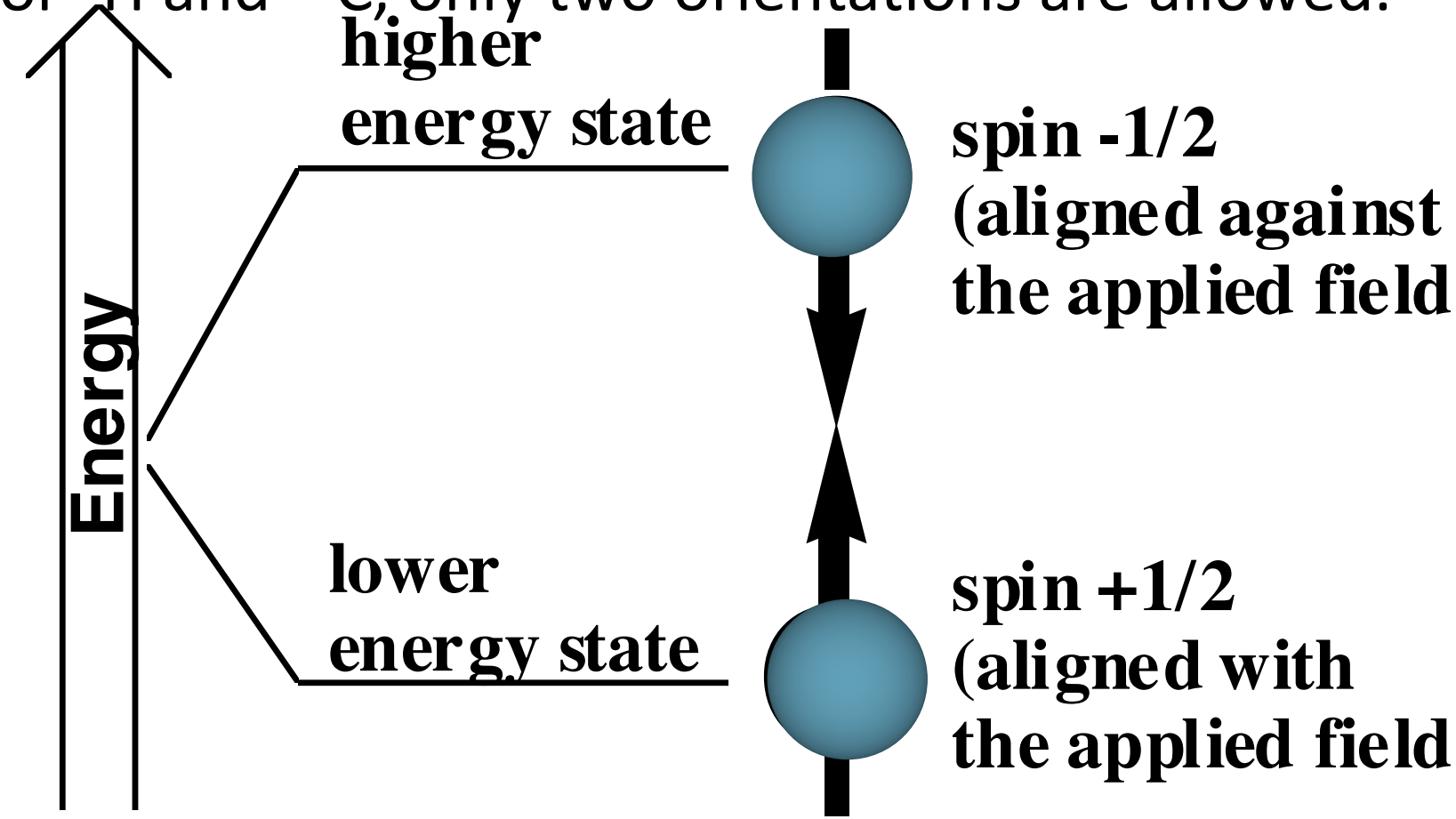
Introduction to NMR

- **When a charged particle such as a proton spins on its axis, it creates a magnetic field. Thus, the nucleus can be considered to be a tiny bar magnet.**
- **Normally, these tiny bar magnets are randomly oriented in space. However, in the presence of a magnetic field B_0, they are oriented with or against this applied field.**
- **The energy difference between these two states is very small (<0.1 cal).**



Nuclear Spins in B_0

- For ^{1}H and ^{13}C, only two orientations are allowed.



Nuclear Spins in B_0

- In an applied field strength of 7.05T, which is readily available with present-day superconducting electromagnets, the difference in energy between nuclear spin states for
  - ^{1}H is approximately 0.0286 cal/mol, which corresponds to electromagnetic radiation of 300 MHz (300,000,000 Hz)(300MHz)
  - ^{13}C is approximately 0.00715 cal/mol, which corresponds to electromagnetic radiation of 75MHz (75,000,000 Hz)(75 MHz)

Population in high vs low

- ΔE= 0.0286 cal/mol RT=582cal/mol
- If pop in high E state is 1,000,000 then pop in low energy state is 1,000,049

$$\frac{nuclei\ in\ high\ E\ state}{nuclei\ in\ low\ E\ state} = e^{-\Delta E / RT}$$

NMR Spectroscopy

- NMR uses radiowaves, measured in MHz
- The energy transitions depend on the strength of the magnetic field which is different from machine to machine
- We define the machine independent ppm as

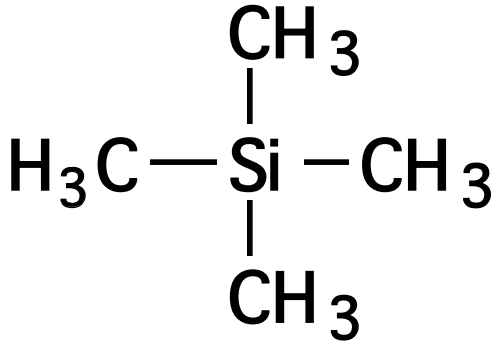
$$\delta = \frac{\Delta \nu}{Oscillatorfrequency} \times 10^6$$

Nuclear Magnetic Resonance

- If we were dealing with ^{1}H nuclei isolated from all other atoms and electrons, any combination of applied field and radiation that produces a signal for one ^{1}H would produce a signal for all ^{1}H. The same is true of ^{13}C nuclei
- But hydrogens in organic molecules are not isolated from all other atoms; they are surrounded by electrons, which are caused to circulate by the presence of the applied field

Nuclear Magnetic Resonance

- It is customary to measure the resonance frequency (signal) of individual nuclei relative to the resonance frequency (signal) of a reference compound
- The reference compound now universally accepted is tetramethylsilane (TMS)

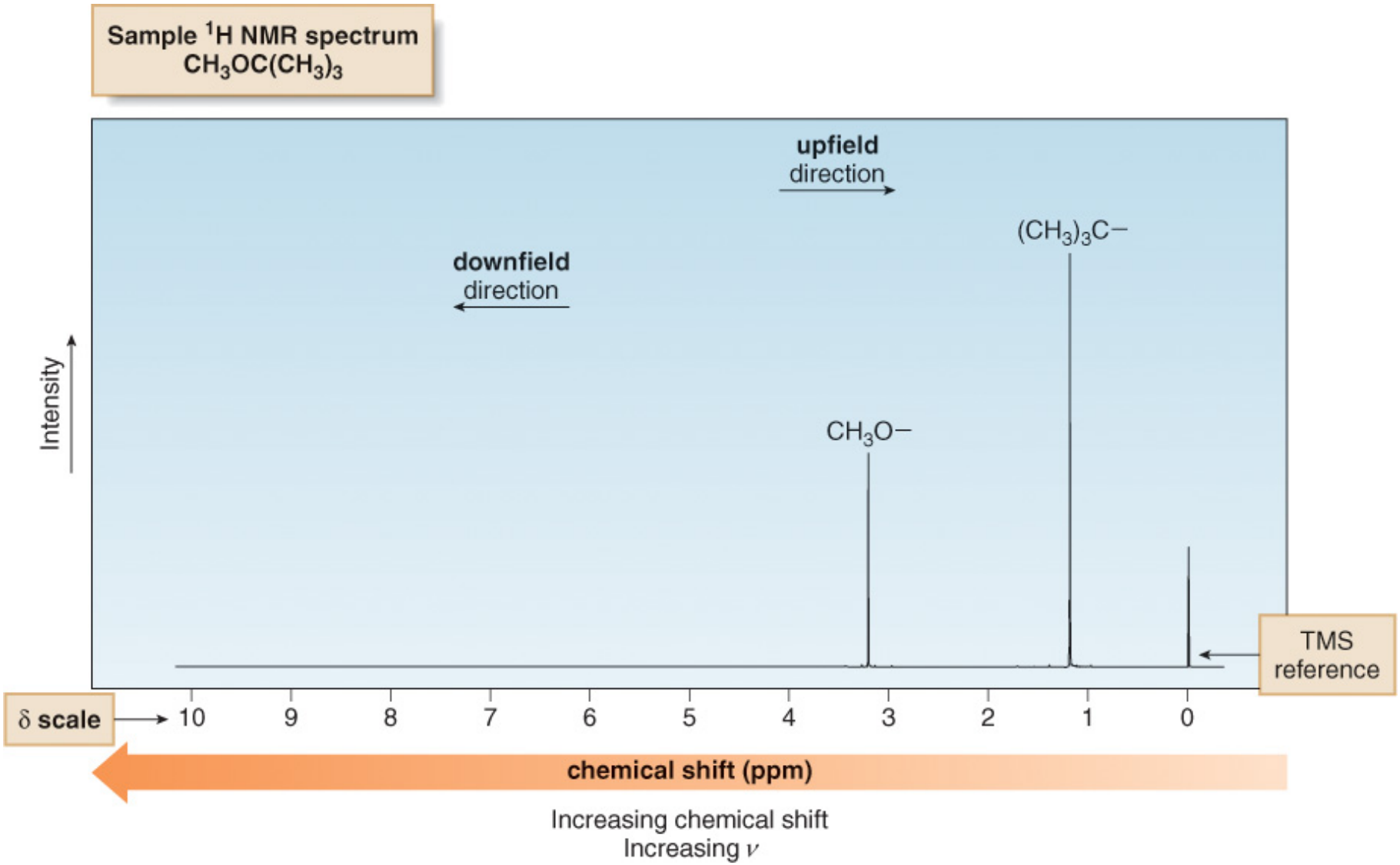


Tetramethylsilane (TMS)

Nuclear Magnetic Resonance Spectroscopy

^{1}H NMR—The Spectrum

- **An NMR spectrum is a plot of the intensity of a peak against its chemical shift, measured in parts per million (ppm).**



Nuclear Magnetic Resonance

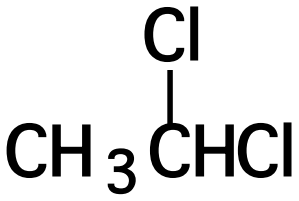
- For a ^{1}H-NMR spectrum, signals are reported by their shift from the 12 H signal in TMS
- For a ^{13}C-NMR spectrum, signals are reported by their shift from the 4 C signal in TMS

- Chemical shift (δ): the shift in ppm of an NMR signal from the signal of TMS

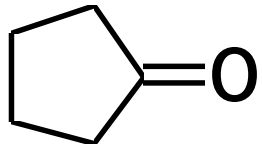
$$\delta = \frac{\text{Shift in frequency from TMS (Hz)}}{\text{Frequency of spectrometer (Hz)}}$$

Equivalent Hydrogens

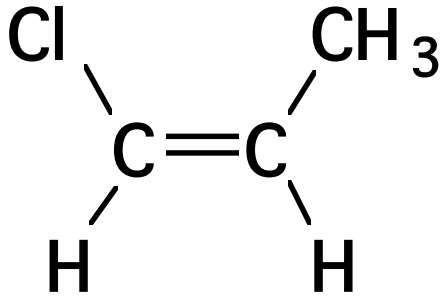
- Equivalent hydrogens: have the same chemical environment (Section 2.3C)
- Molecules with
  - 1 set of equivalent hydrogens give 1 NMR signal
  - 2 or more sets of equivalent hydrogens give a different NMR signal for each set



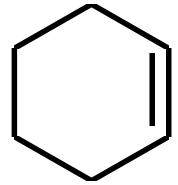
1,1-Dichloro-ethane (2 signals)



Cyclopent-anone (2 signals)



(Z)-1-Chloro-propene (3 signals)

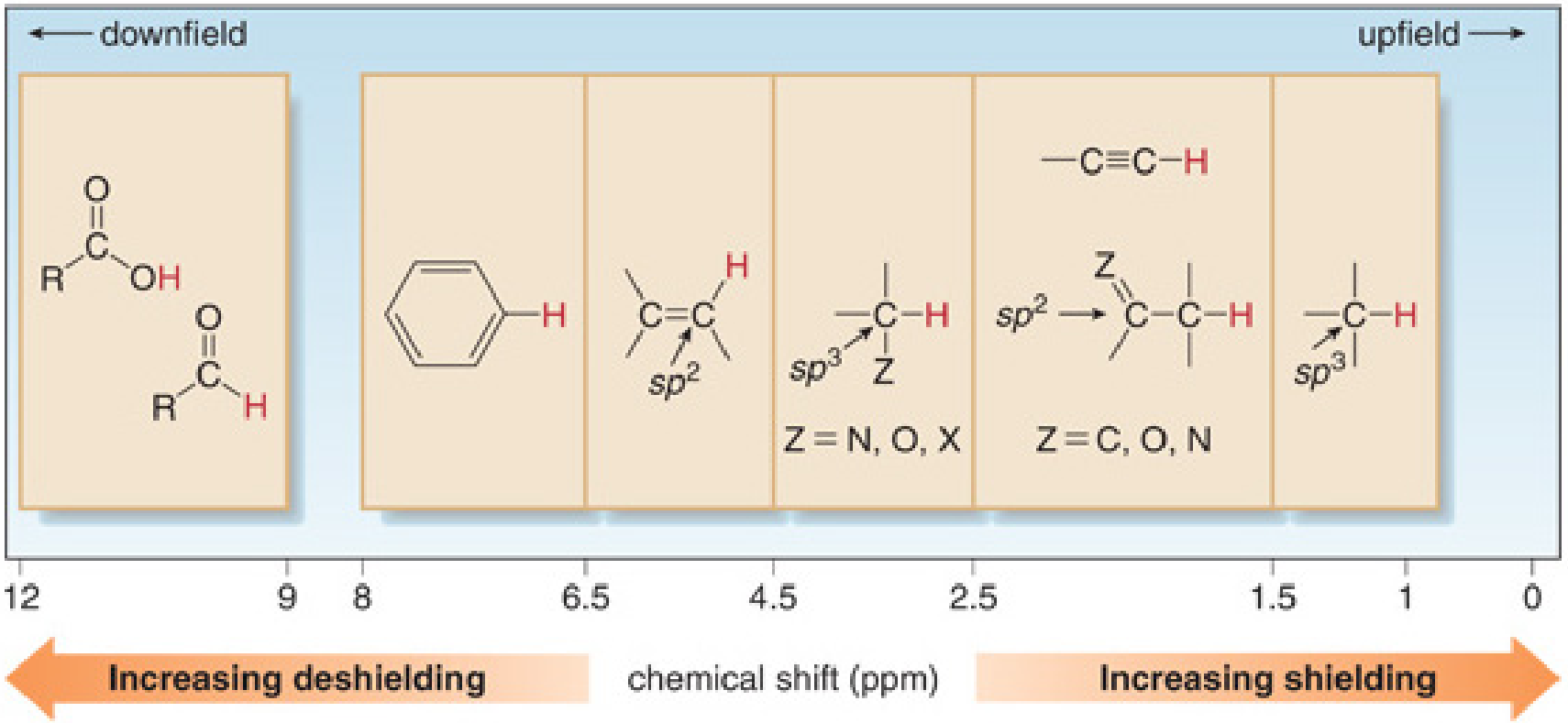


Cyclohexene (3 signals)

Nuclear Magnetic Resonance Spectroscopy

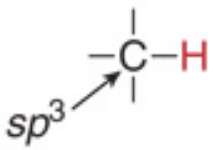
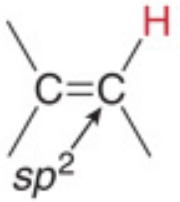
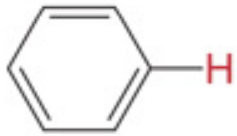
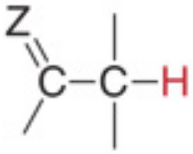
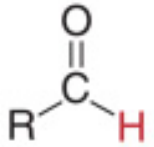
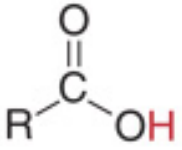
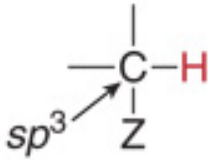
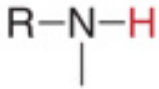
^{1}H NMR—Chemical Shift Values

Figure 15.5 Regions in the ^{1}H NMR spectrum



- Shielded protons absorb at lower chemical shift (to the right).
- Deshielded protons absorb at higher chemical shift (to the left).

TABLE 15.1 Characteristic Chemical Shifts of Common Types of Protons

Type of proton	Chemical shift (ppm)	Type of proton	Chemical shift (ppm)
	0.9–2		4.5–6
• RCH_3 • R_2CH_2 • R_3CH	~0.9 ~1.3 ~1.7		6.5–8
 Z = C, O, N	1.5–2.5		9–10
—C≡C–H	~2.5		10–12
 Z = N, O, X	2.5–4	RO–H or 	1–5

Chemical Shift

- Depends on (1) electronegativity of nearby atoms, (2) the hybridization of adjacent atoms, and (3) magnetic induction within an adjacent pi bond
- Electronegativity

CH_3-X	Electroneg-ativity of X	δ of H
CH_3F	4.0	4.26
CH_3OH	3.5	3.47
CH_3Cl	3.1	3.05
CH_3Br	2.8	2.68
CH_3I	2.5	2.16
$(CH_3)_4C$	2.1	0.86
$(CH_3)_4Si$	1.8	0.00 (by definition

Signal Splitting (n + 1)

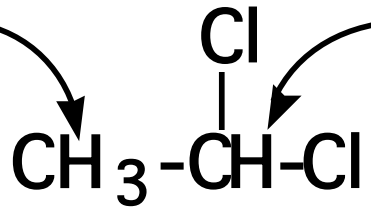
- Peak: the units into which an NMR signal is split; doublet, triplet, quartet, etc.

- Signal splitting: splitting of an NMR signal into a set of peaks by the influence of neighboring nonequivalent hydrogens

- (n + 1) rule: the ^{1}H-NMR signal of a hydrogen or set of equivalent hydrogens is split into (n + 1) peaks by a nonequivalent set of n equivalent neighboring hydrogens

Signal Splitting (n + 1)

n = 1. Their signal is split into (1 + 1) or 2 peaks ; a doublet



n = 3. Its signal is split into (3 + 1) or 4 peaks; a quartet

- Problem: predict the number of ^{1}H-NMR signals and the splitting pattern of each

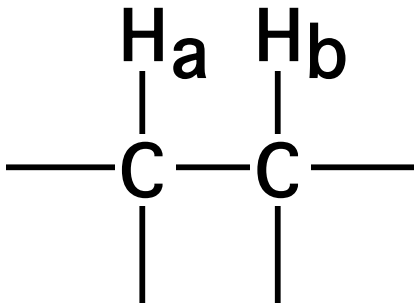
(a) $CH_3CCH_2CH_3$ (O above C)

(b) $CH_3CH_2CCH_2CH_3$ (O above C)

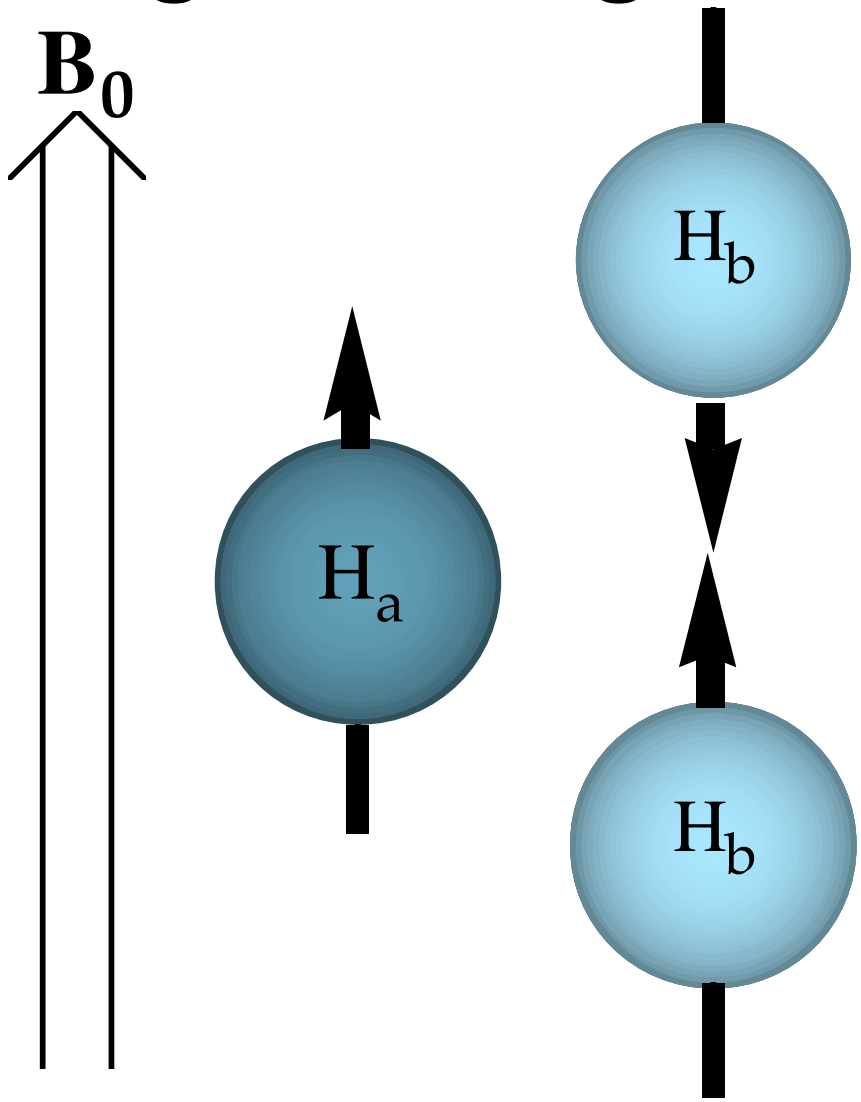
(c) $CH_3CCH(CH_3)_2$ (O above C)

Origins of Signal Splitting

- When the chemical shift of one nucleus is influenced by the spin of another, the two are said to be coupled
- Consider nonequivalent hydrogens H_a and H_b on adjacent carbons
  - the chemical shift of H_a is influenced by whether the spin of H_b is aligned with or against the applied field



Origins of Signal Splitting

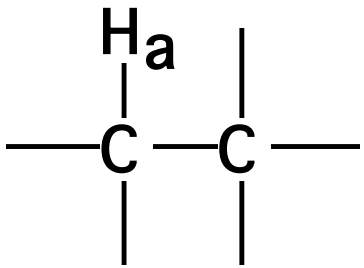
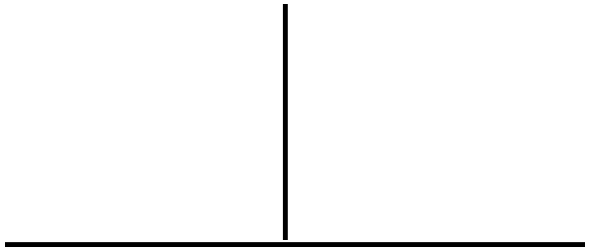
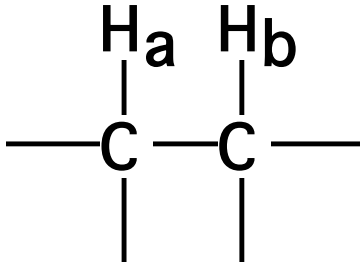
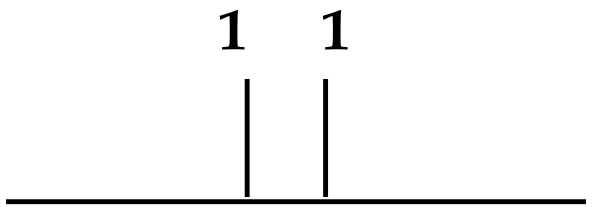


Magnetic field of H_b subtracts from the applied field; H_b signal appears at a higher applied field

Magnetic field of H_b adds to the applied field; H_a signal appears at a lower applied field

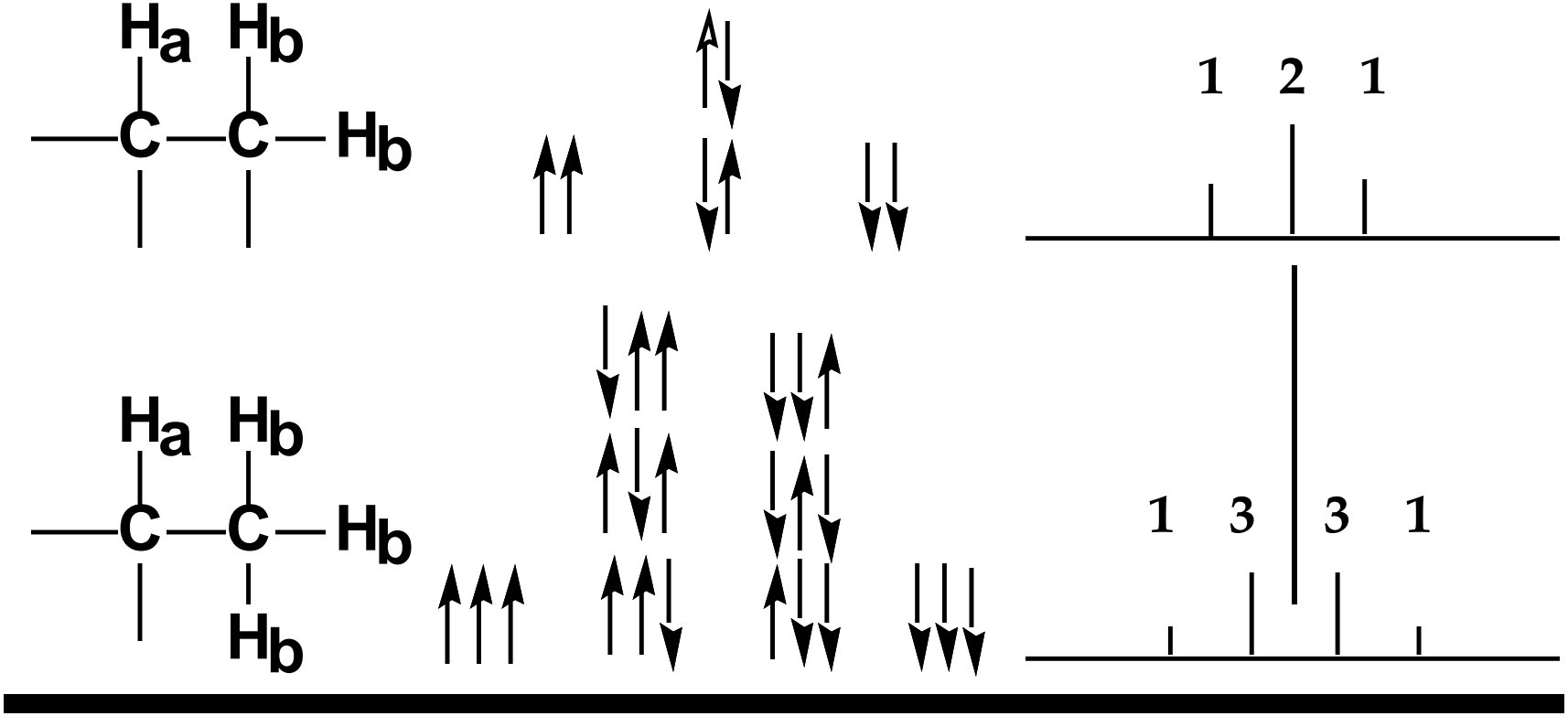
Origins of Signal Splitting

- Table 13.8 Observed signal splitting patterns for an H with 0, 1, 2, and 3 equivalent neighboring hydrogens

Structure	Spin States of H_b	Signal of H_a
		
		

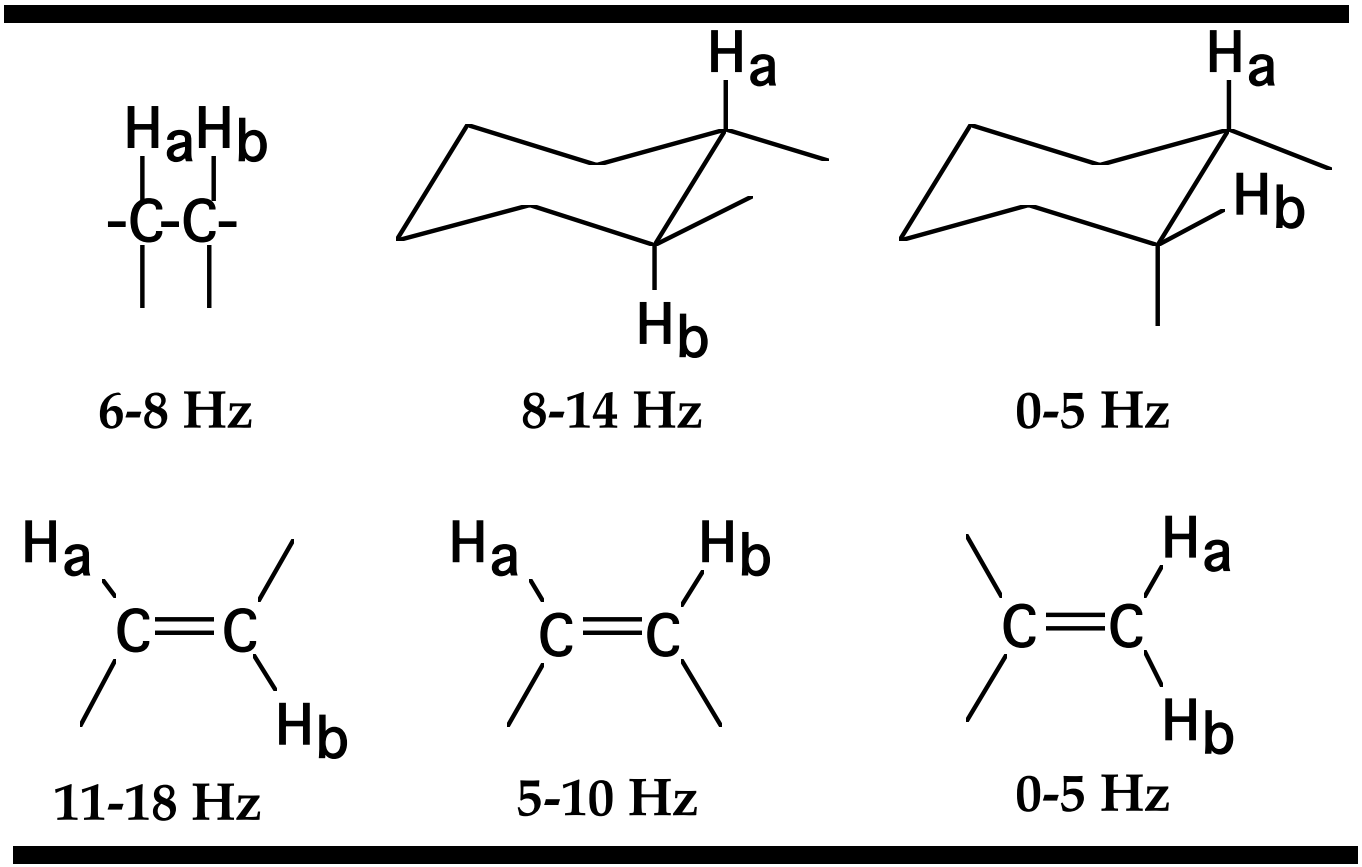
Origins of Signal Splitting

- Table 13.8 (contd.)

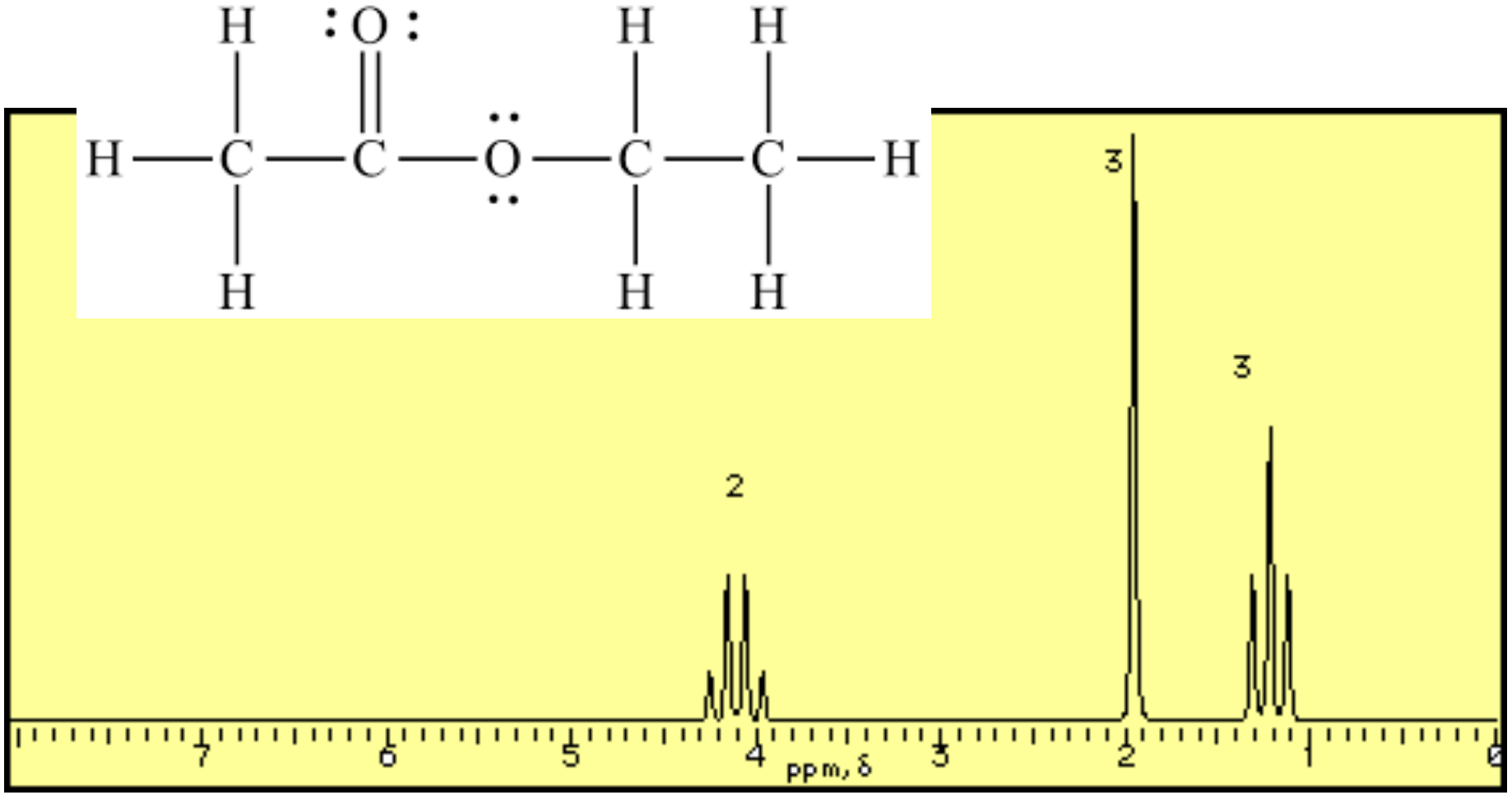


Coupling Constants

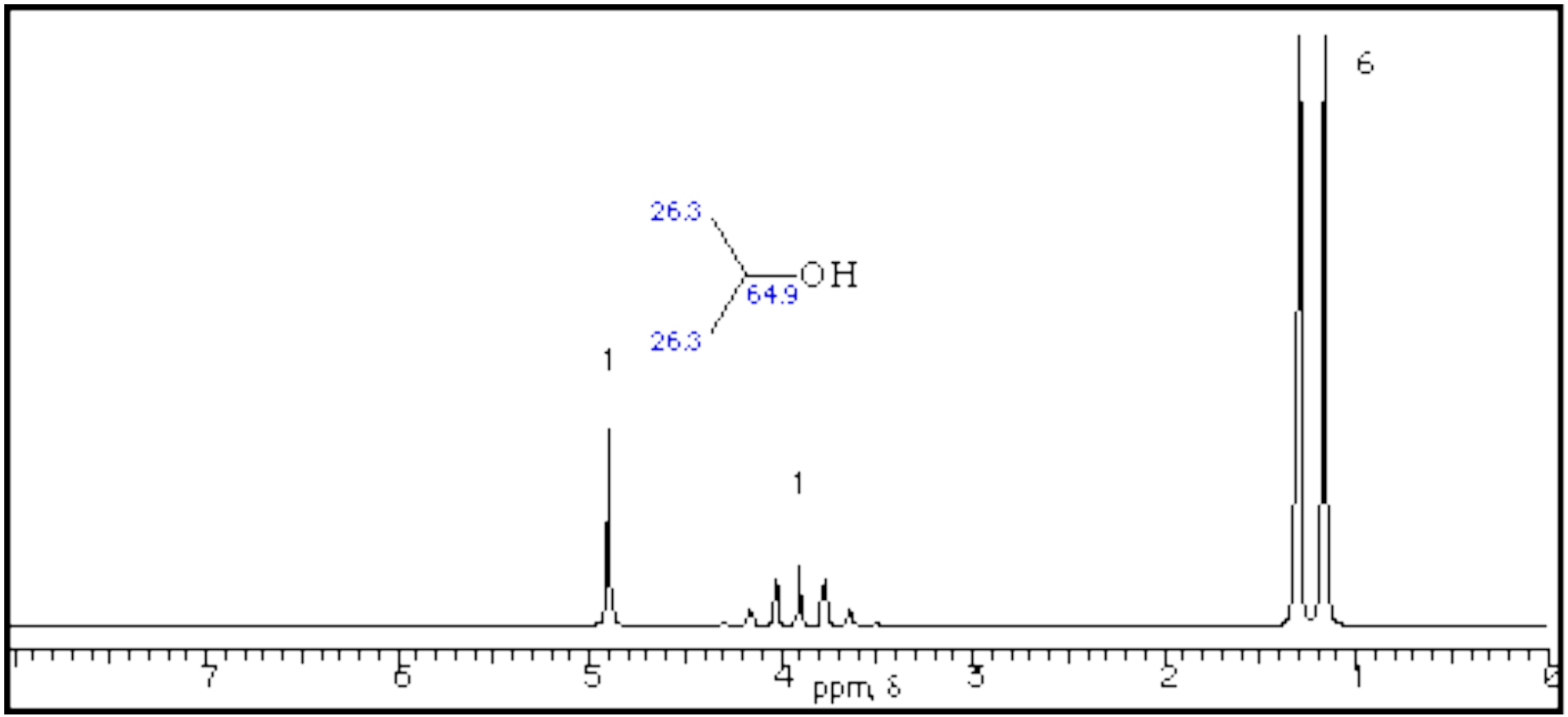
- Coupling constant (J): the distance between peaks in an NMR multiplet, expressed in hertz
  - J is a quantitative measure of the magnetic interaction of nuclei whose spins are coupled



Ethyl acetate



Isopropyl alcohol



^{13}C-NMR Spectroscopy

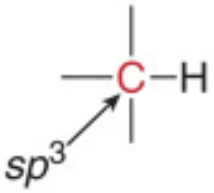
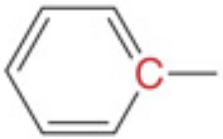
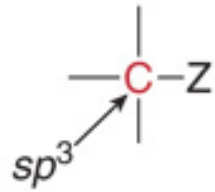
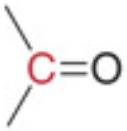
- Each nonequivalent ^{13}C gives a different signal
- A ^{13}C is split by the ^{1}H bonded to it according to the (n + 1) rule
- Coupling constants of 100-250 Hz are common, which means that there is often significant overlap between signals, and splitting patterns can be very difficult to determine
- The most common mode of operation of a ^{13}C-NMR spectrometer is a hydrogen-decoupled mode

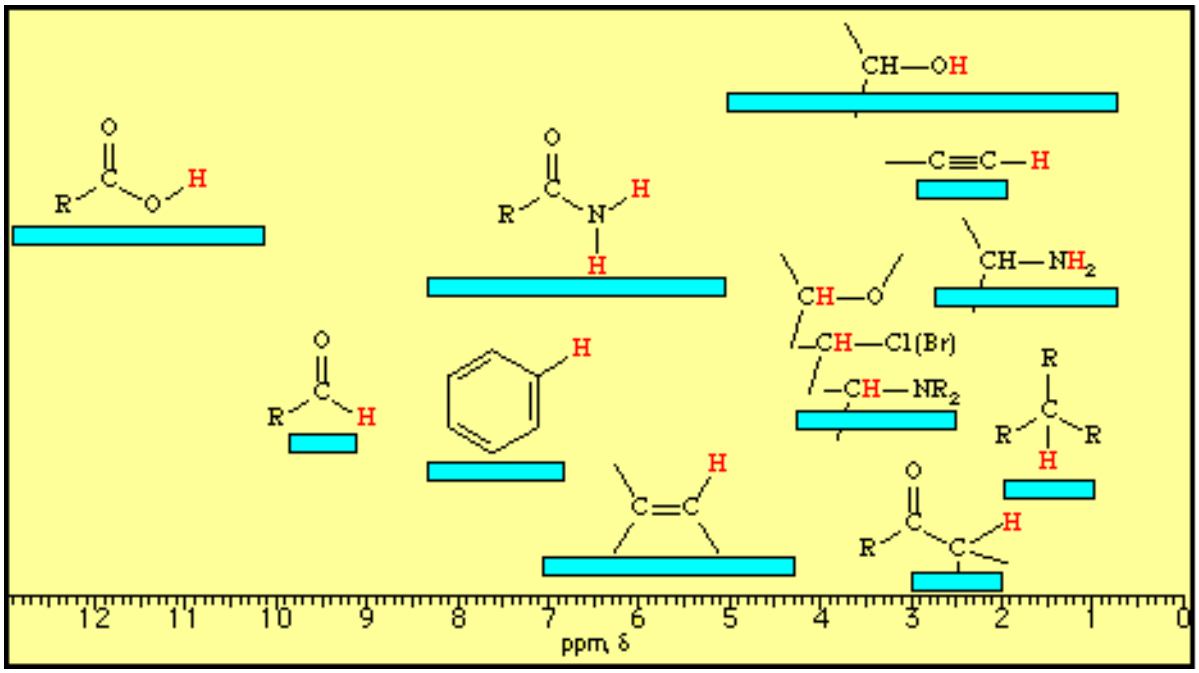
^{13}C-NMR Spectroscopy

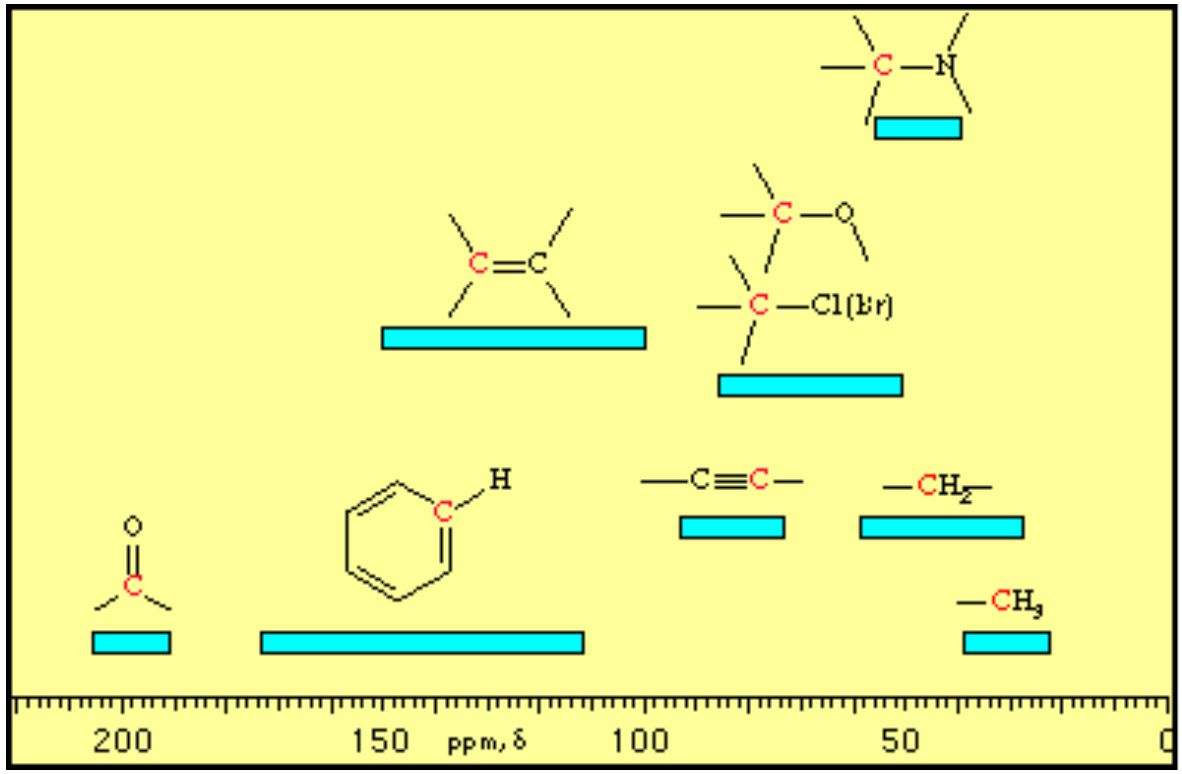
- In a hydrogen-decoupled mode, a sample is irradiated with two different radio frequencies
  - one to excite all ^{13}C nuclei
  - a second is a broad spectrum of frequencies that causes all hydrogens in the molecule to undergo rapid transitions between their nuclear spin states
- On the time scale of a ^{13}C-NMR spectrum, each hydrogen is in an average or effectively constant nuclear spin state, with the result that ^{1}H-^{13}C spin-spin interactions are not observed; they are decoupled

Carbon – 13 shifts

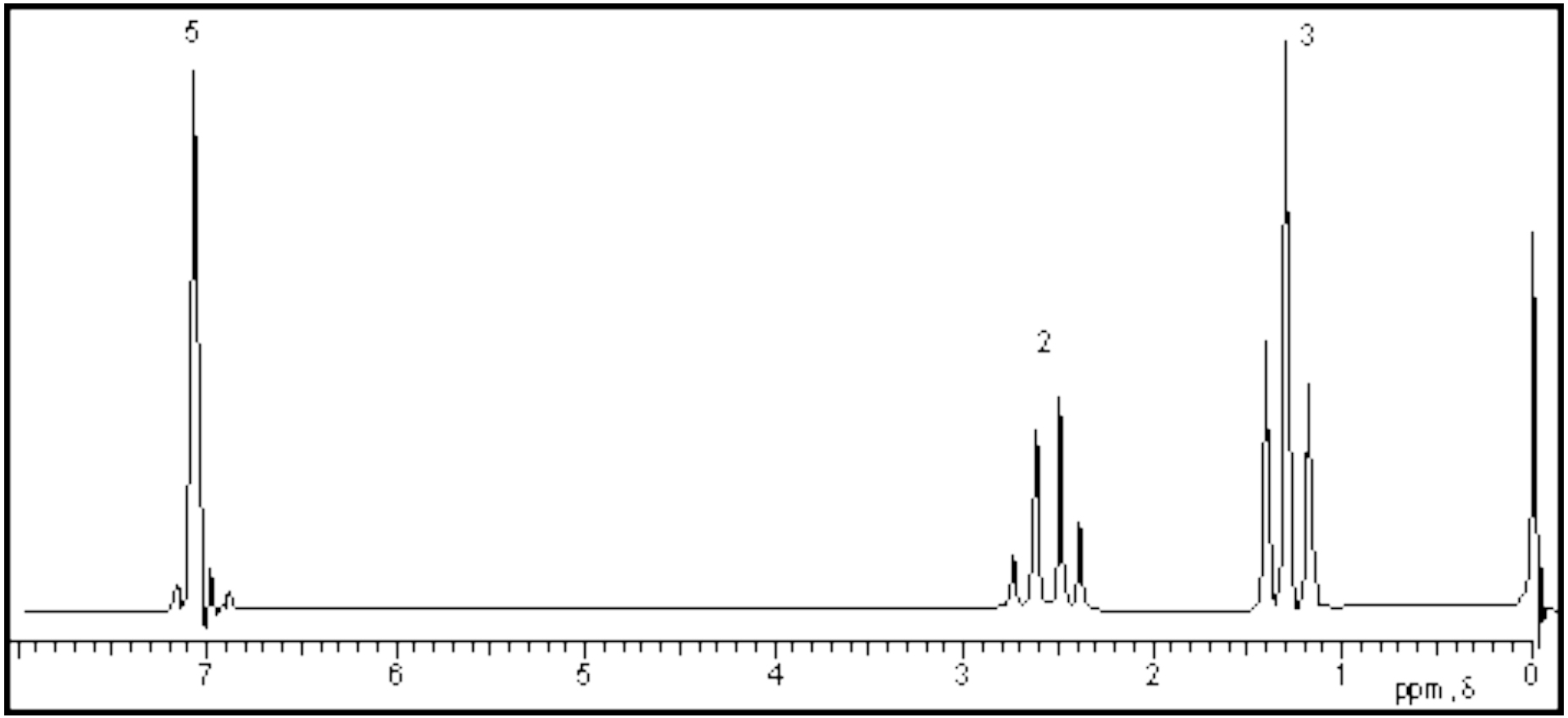
TABLE 15.5 Common ^{13}C Chemical Shift Values

Type of carbon	Chemical shift (ppm)	Type of carbon	Chemical shift (ppm)
	5–45		100–140
	65–100		120–150
 Z = N, O, X	30–80		160–210

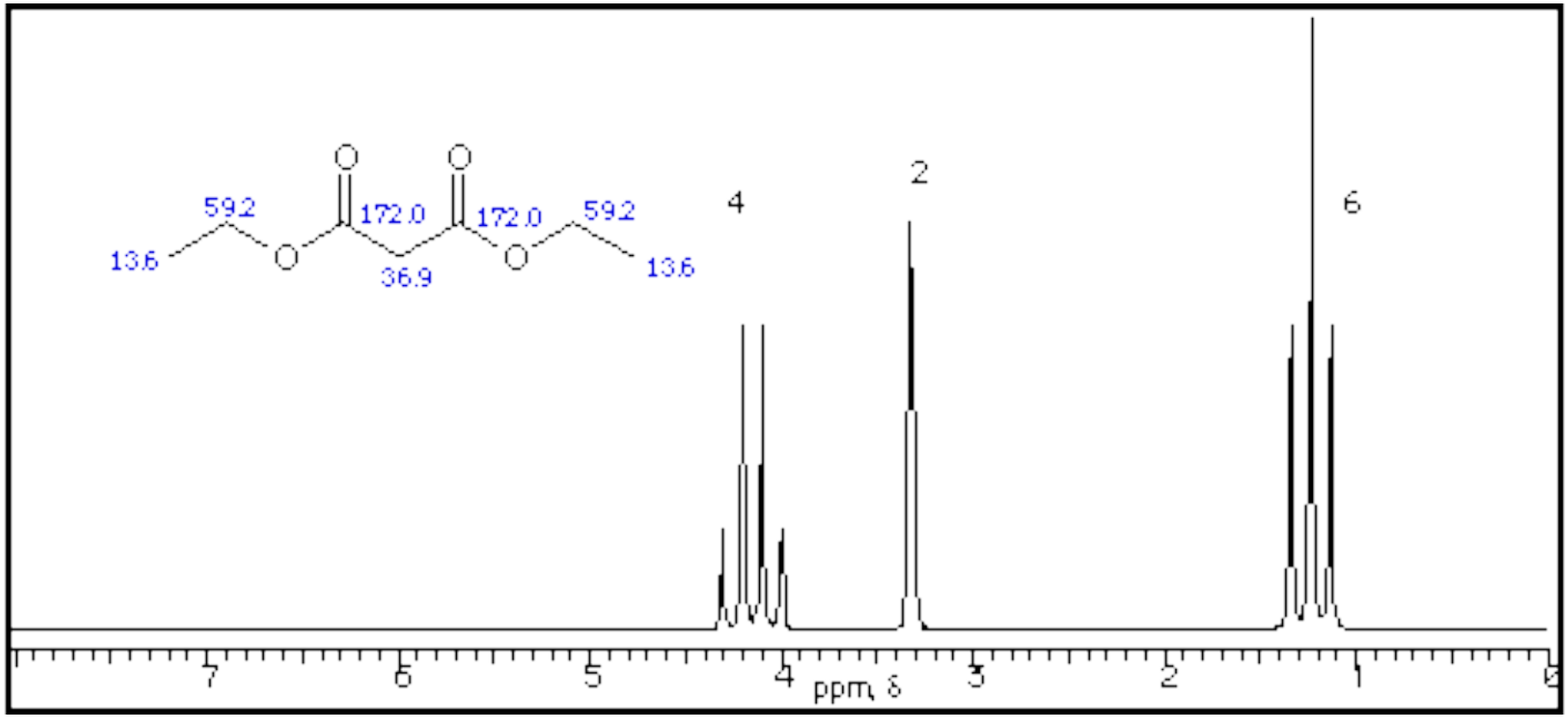

CH—OH
—C≡C—H
CH—NH2
CH—O
CH—Cl(Br)
CH—NR2
12 11 10 9 8 7 6 5 4 3 2 1 0
ppm, δ


C—N
C—O
C—Cl(Br)
—C≡C—
—CH2—
—CH3
200 150 ppm, δ 100 50 0

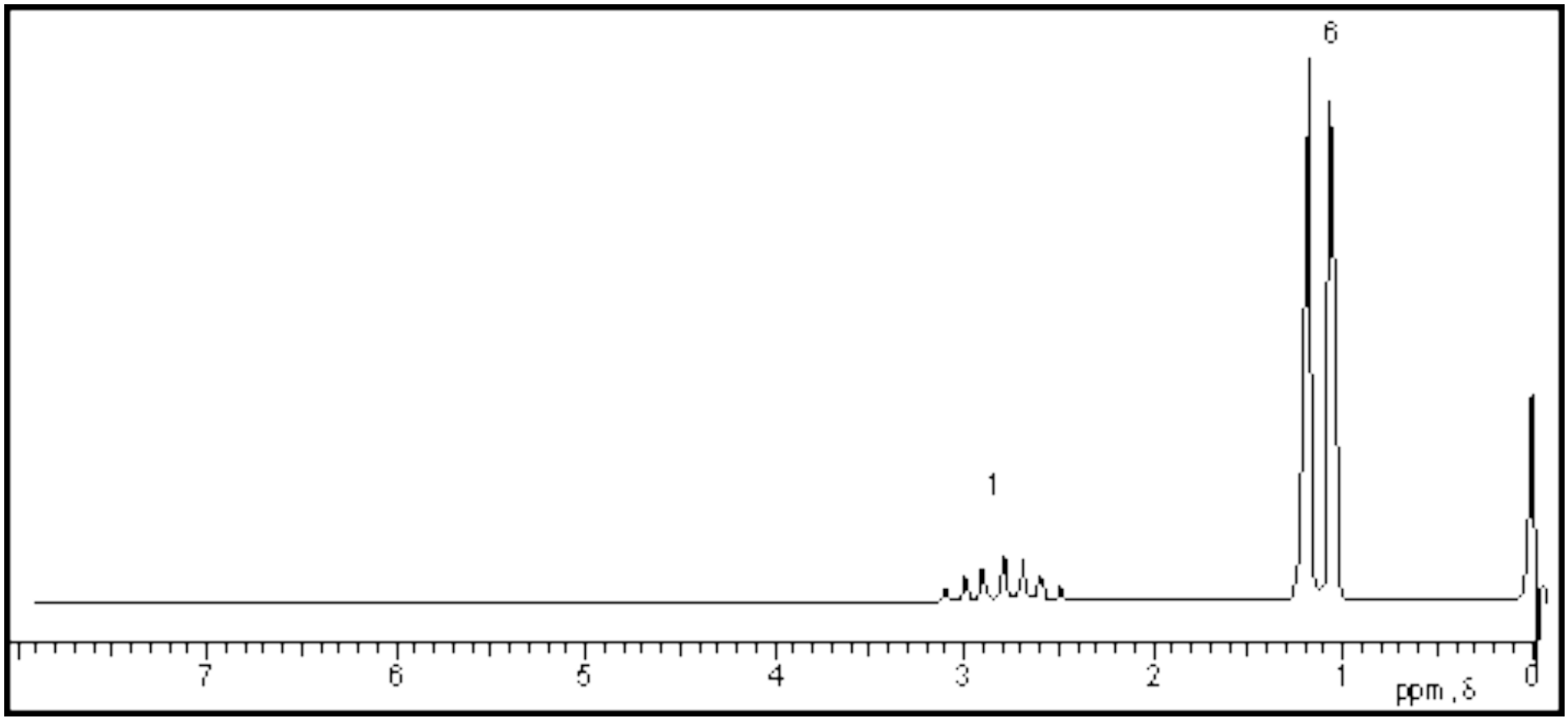
C8H10



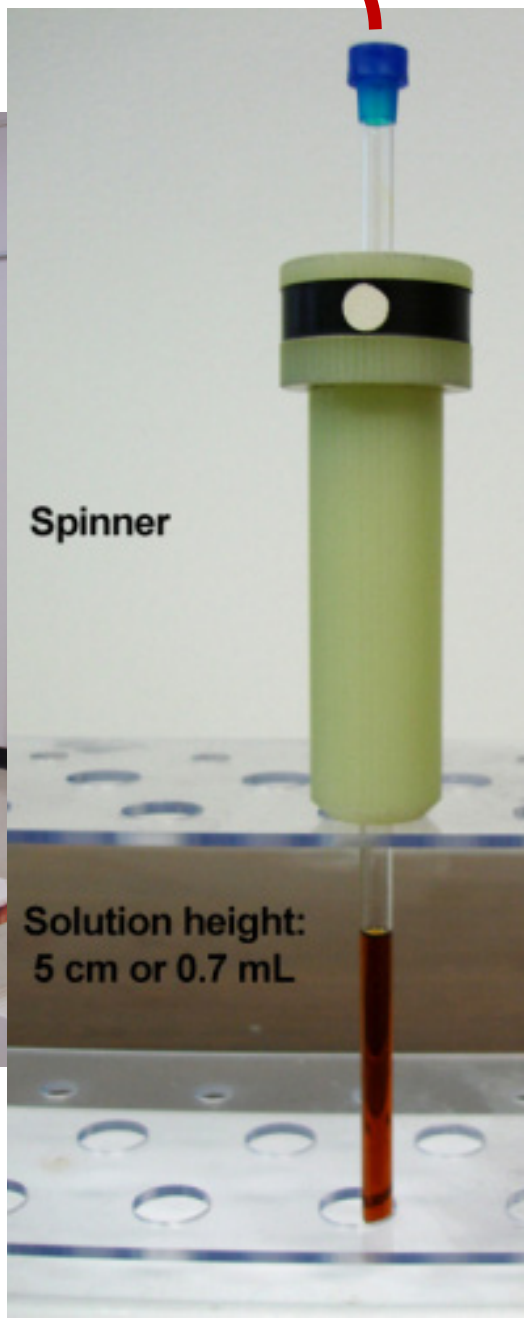
C7H12O4



C7H14O



Typical NMR spectrometer setup



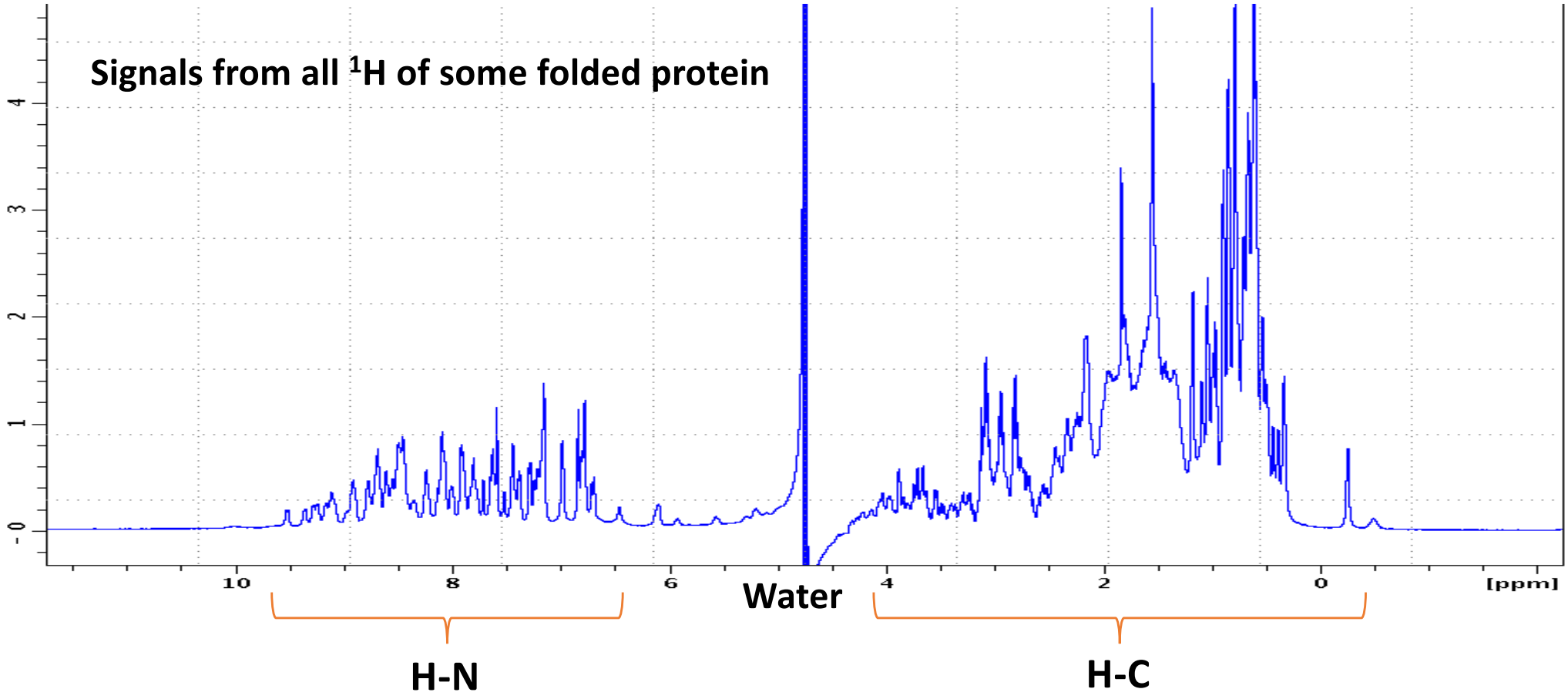
“The Magnet is always ON”

Magnetic field strength 11.74 Tesla (500 MHz for proton)
other common field strengths 600 or 800 MHz

1D NMR spectra

Application of RF pulses of specified lengths and frequencies can make certain nuclei detectable

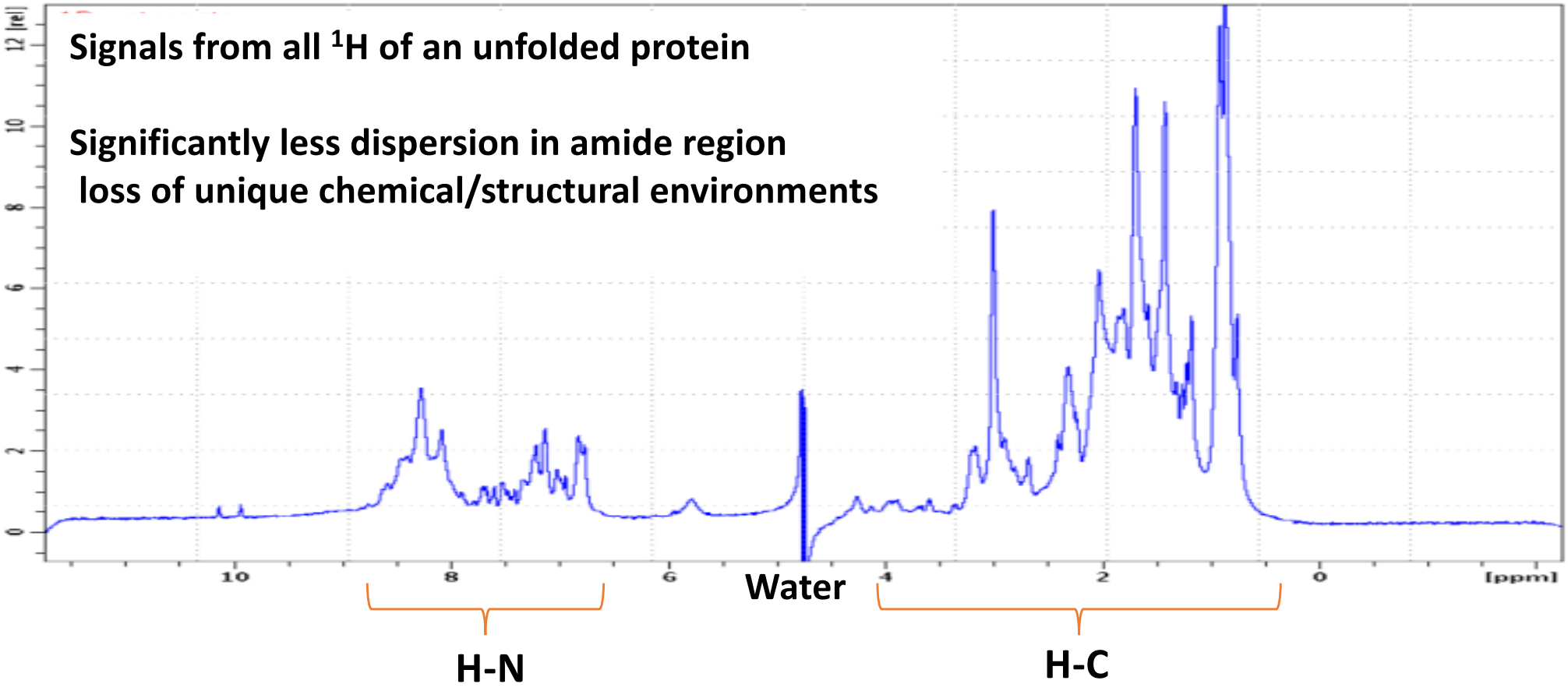
We can selectively excite nuclei of interest.



1D NMR spectra

Application of RF pulses of specified lengths and frequencies can make certain nuclei detectable

We can selectively excite nuclei of interest.

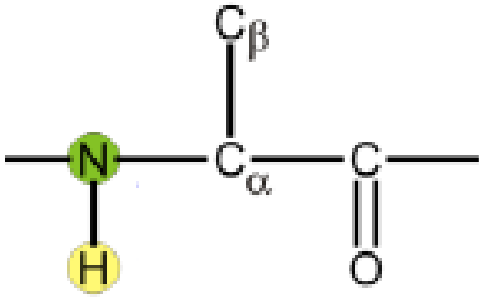


Multidimensional NMR

1D NMR gives signals of just one nuclei (e.g. ^{1}H, ^{13}C, or ^{15}N)

Much more information when we add dimensions.

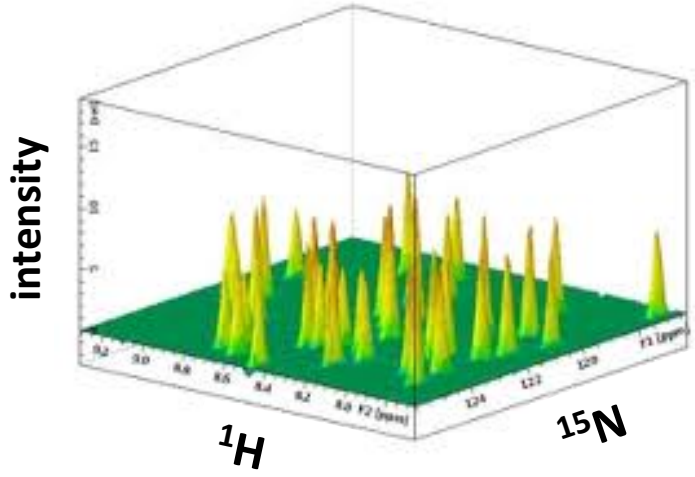
We use the through-bond J couplings to pass around the magnetization



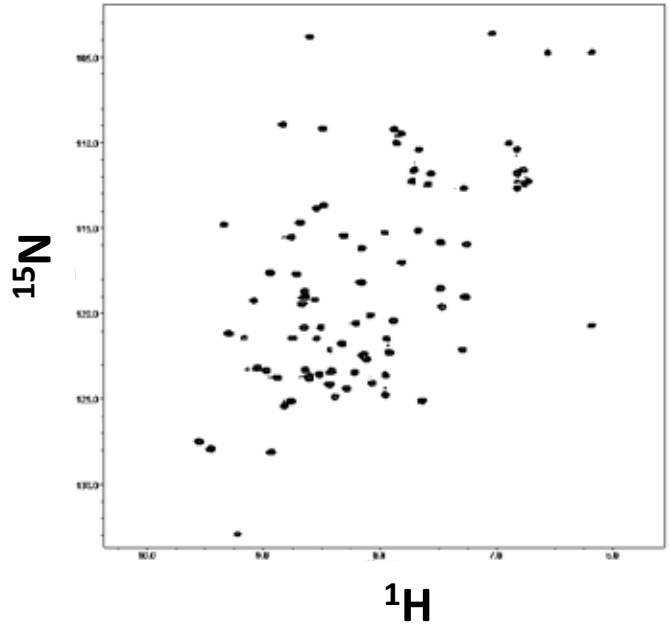
Most frequently used 2D NMR spectra is the HSQC
(heteronuclear single quantum coherence)
Magnetization is transferred from the H to the attached ^{15}N nuclei via the J-coupling

2D Spectra

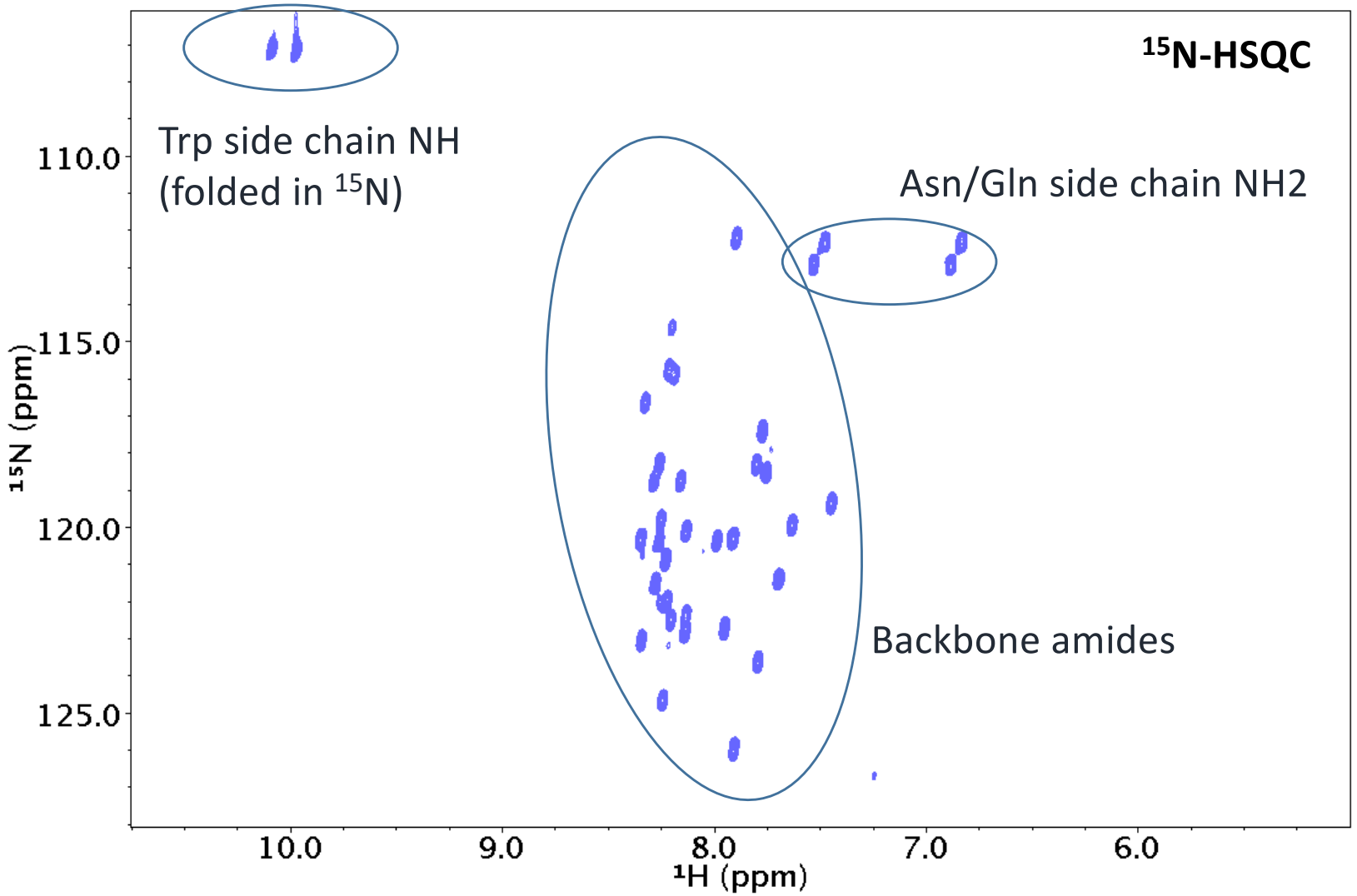
Stacked Plot



Contour Plot



NMR Assignments – A simple example assigning a small Intrinsically Disordered Peptide



110 120 130

34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD

1) Protein sample preparation

- Overwhelming majority of the proteins studied by NMR are over-expressed in and purified from *E. Coli*

- M9 (minimal media) with ^{13}C-enriched glucose and ^{15}N-enriched ammonium chloride as sole carbon and nitrogen sources is used for $^{13}C/^{15}N$ labeling

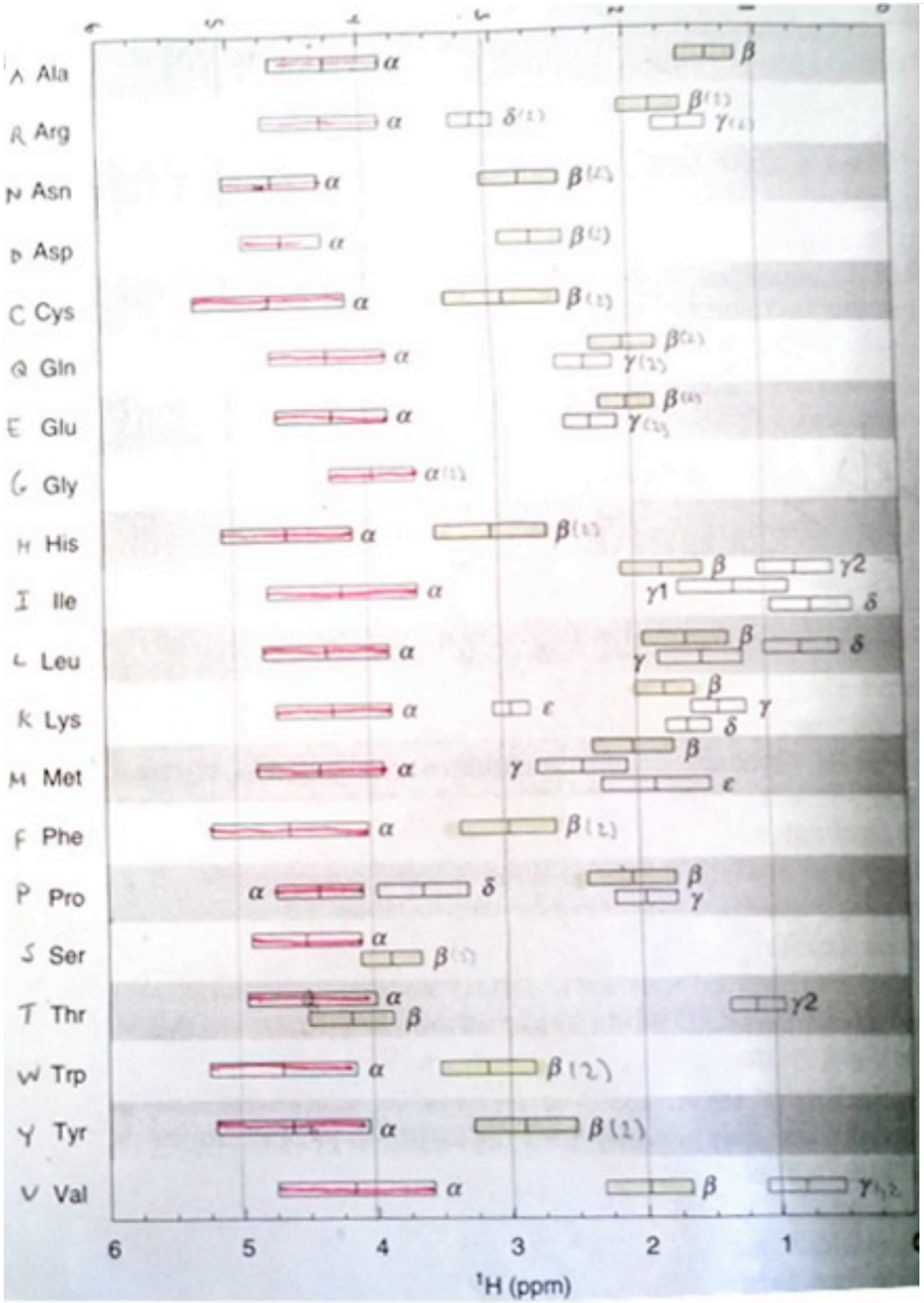
- *E. Coli* growth in D_2O is used to introduce deuterium into <u>non-exchangeable</u> protein sites. Partial deuteration is useful for NMR studies of proteins > 25 kDa

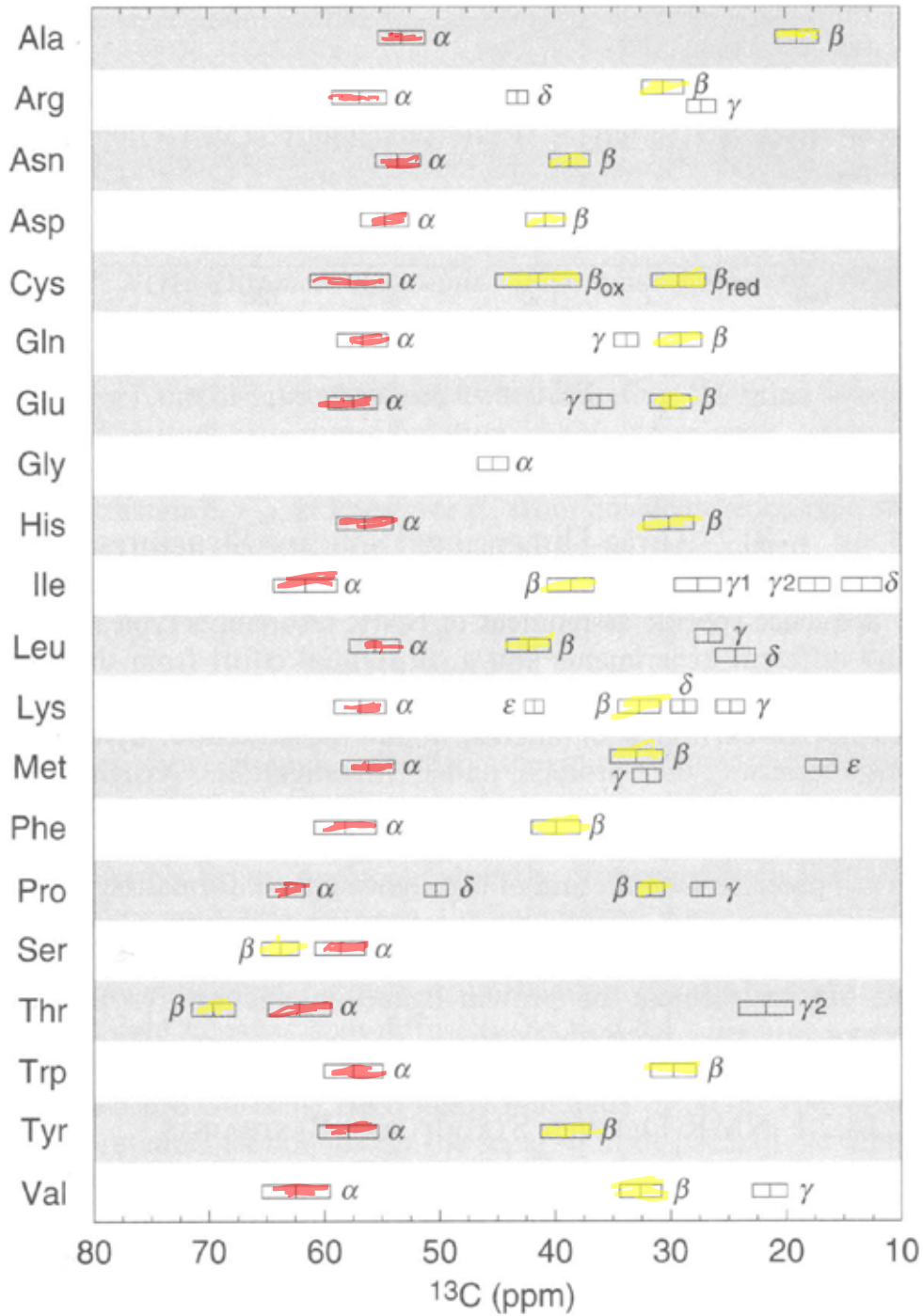
- Insect cell medium and in-vitro translation systems enriched with stable isotopes are available; but still prohibitively expensive

2) Optimization of sample conditions

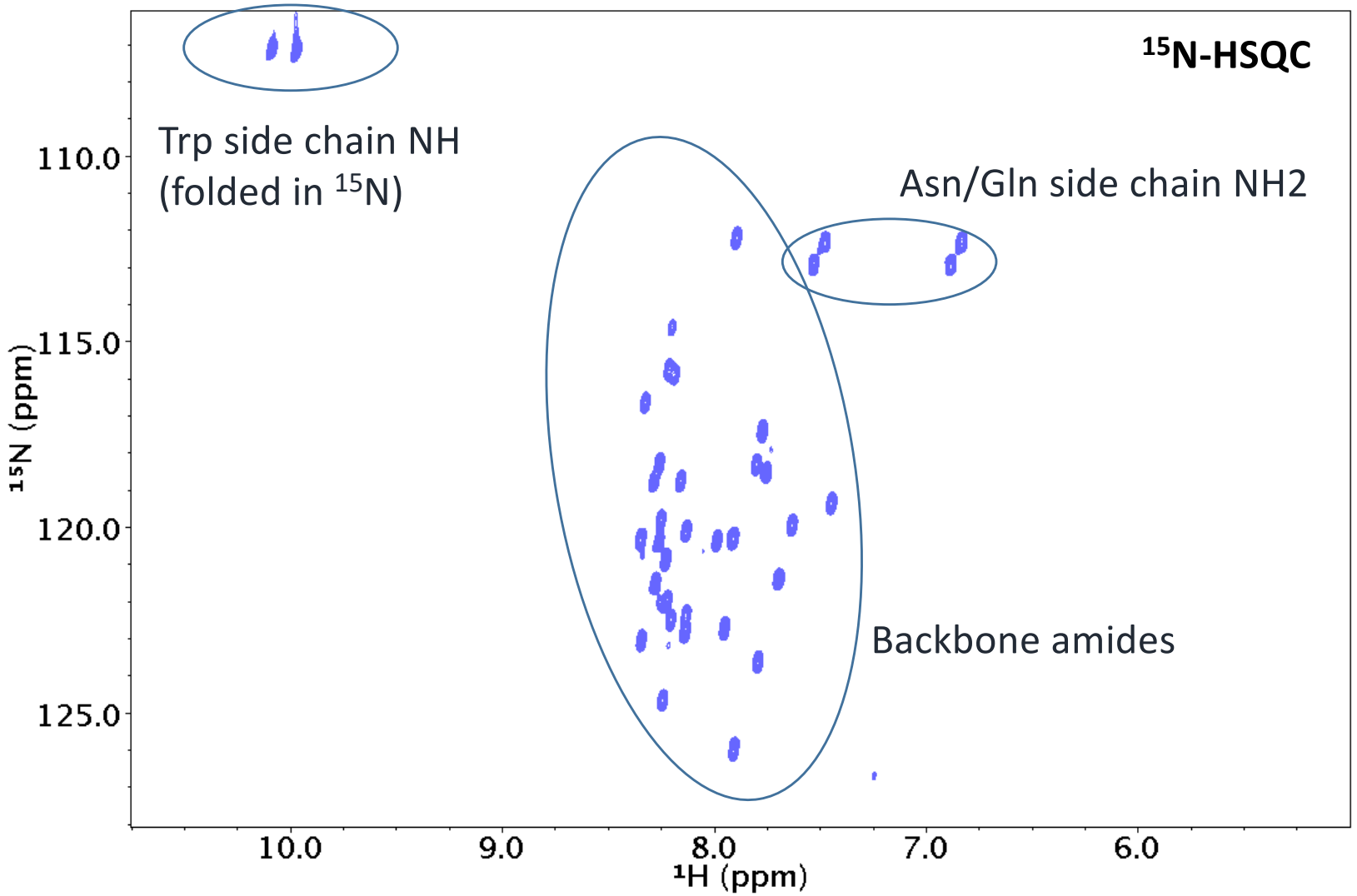
- Buffers with non-negligible temperature dependence of pH (e.g. Tris) should be avoided.
- pH < 7 is preferred, as it minimizes the loss of ^{1}H sensitivity due to exchange with water protons.
- The protein must be in a well-defined oligomeric state
- 0.5 - 1 mM is the optimum protein concentration for structural and dynamical studies
- The NMR sample should be stable over periods of time required to collect the NMR data
  - days > binding studies
  - weeks > assignments or dynamics
  - months > all atom assignments / full dynamics characterization

Characteristic amino acid proton and carbon chemical shifts





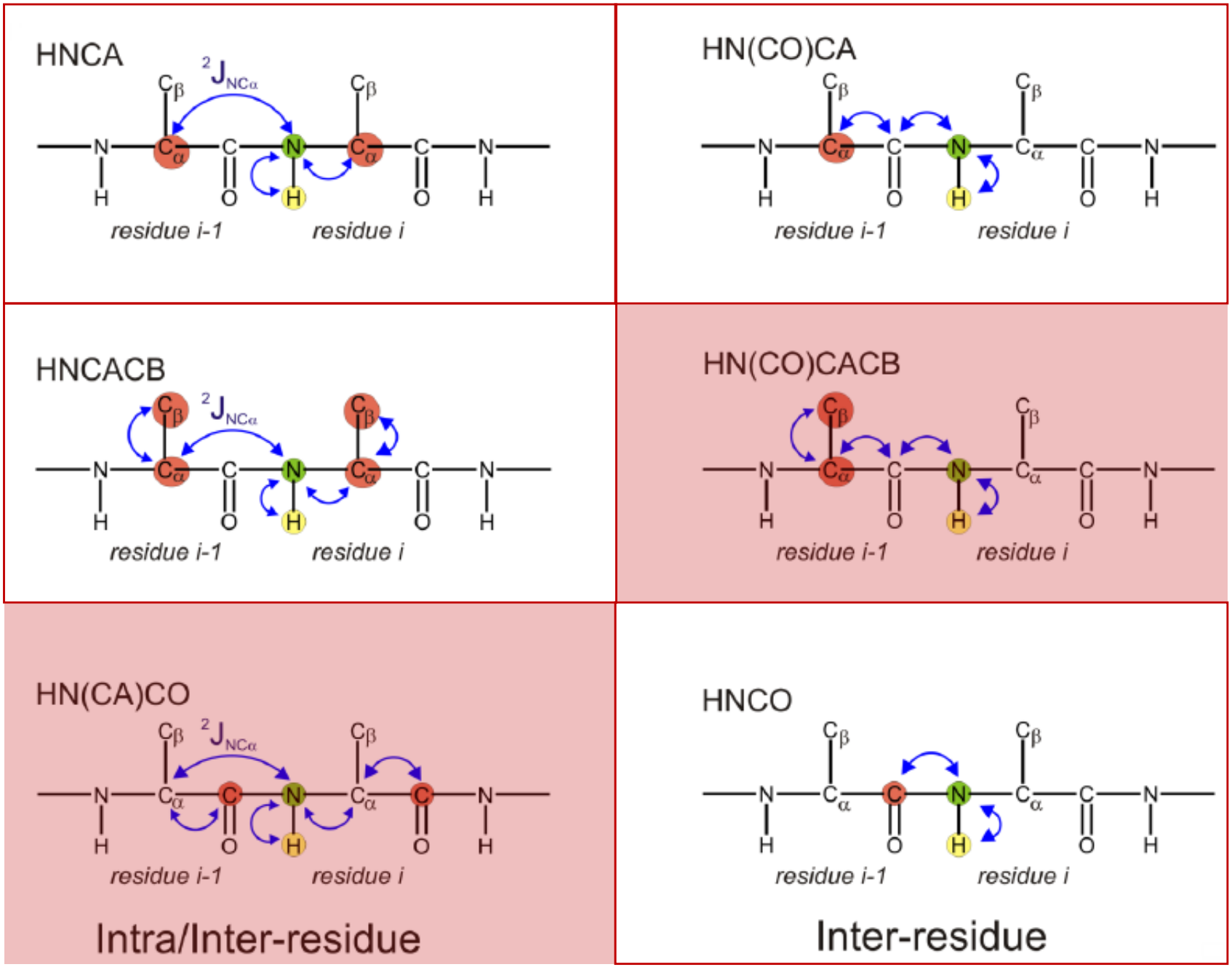
NMR Assignments – A simple example assigning a small Intrinsically Disordered Peptide



110 120 130

34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD

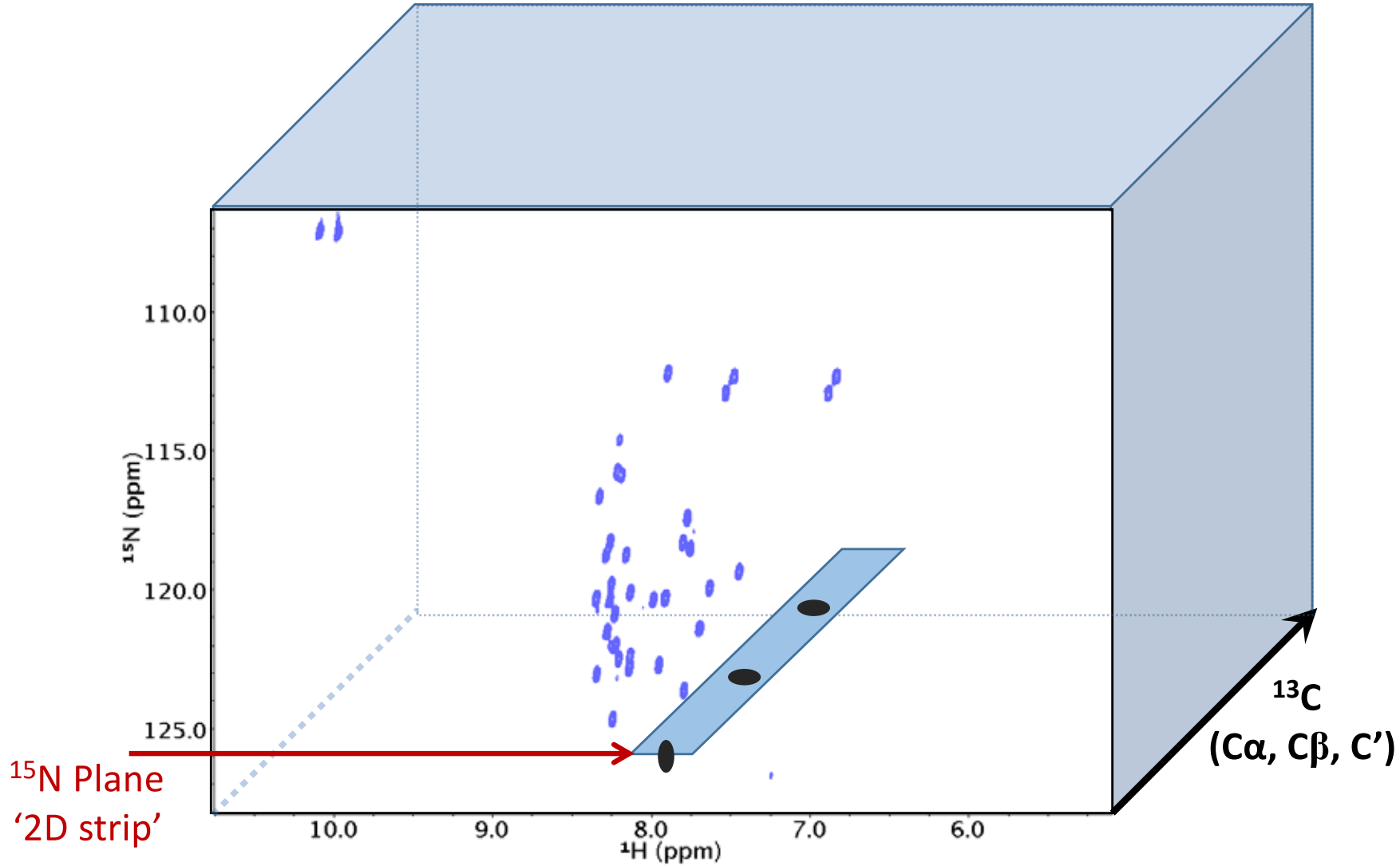
Backbone triple resonance experiments (need ^{1}H, ^{13}C, ^{15}N sample)



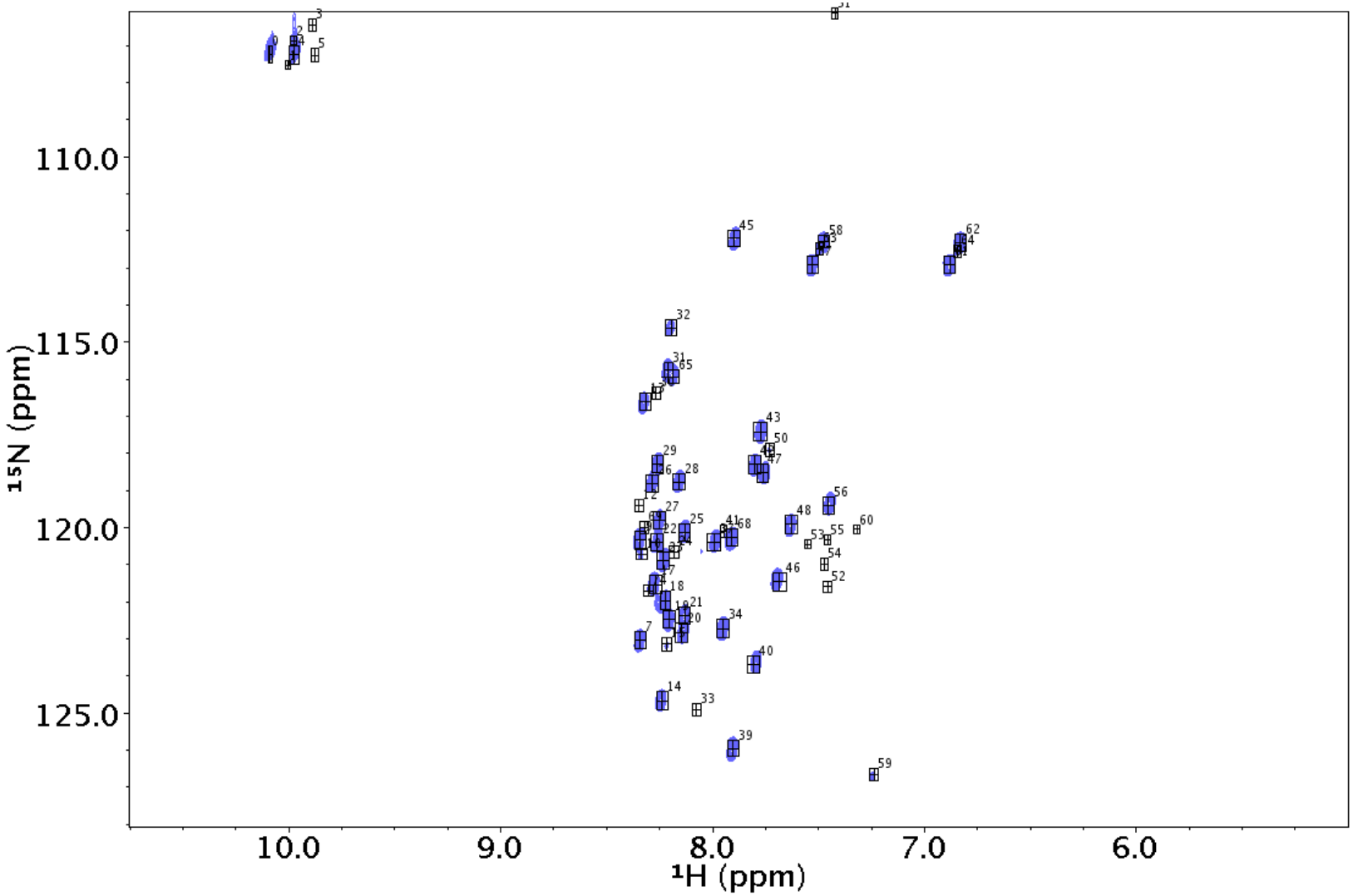
***i* and *i-1* peaks**

***i-1* peaks**

3D spectra for backbone assignments



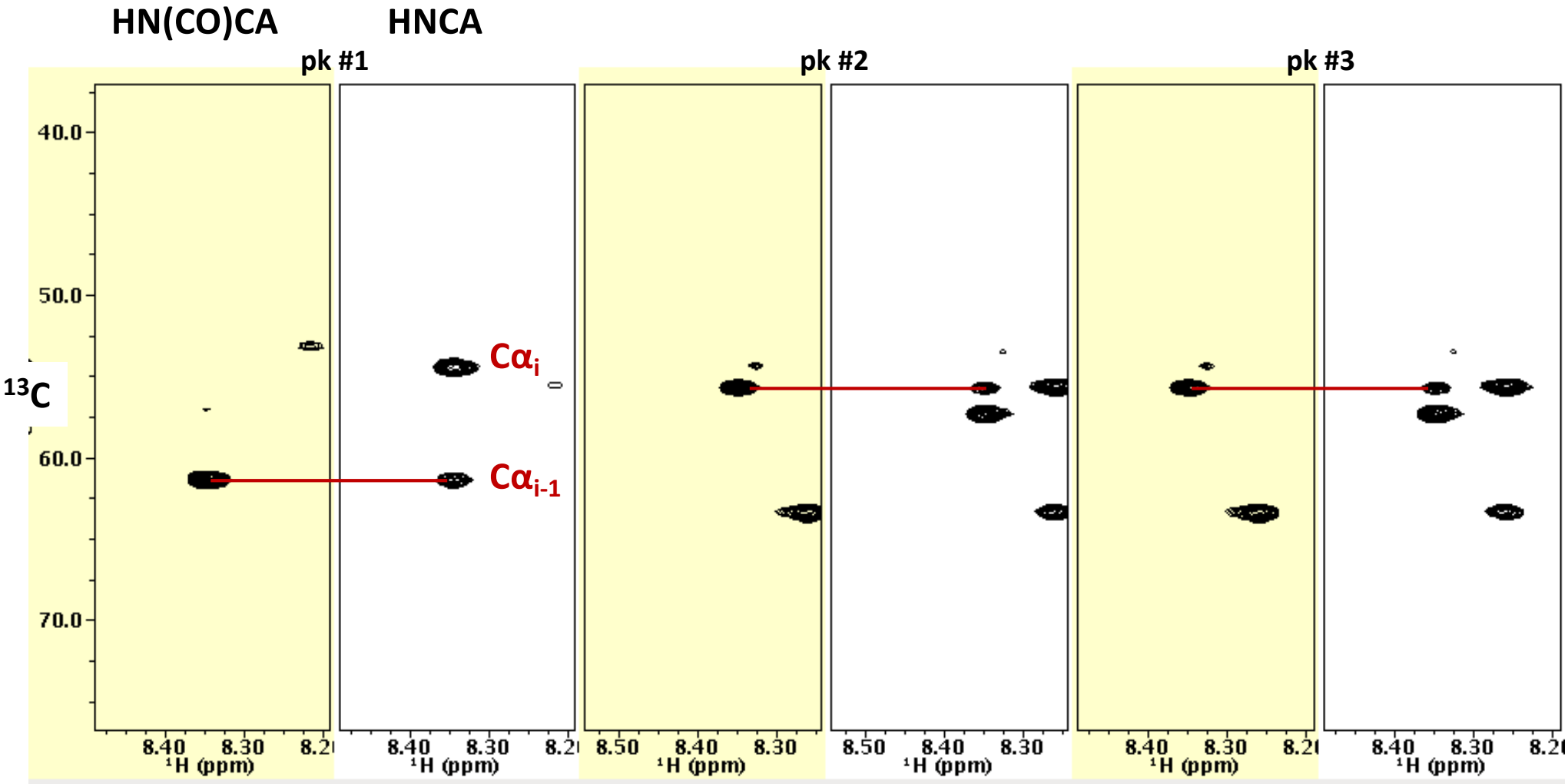
Backbone Assignments – Step 1: Pick the peaks



110 120 130

34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD

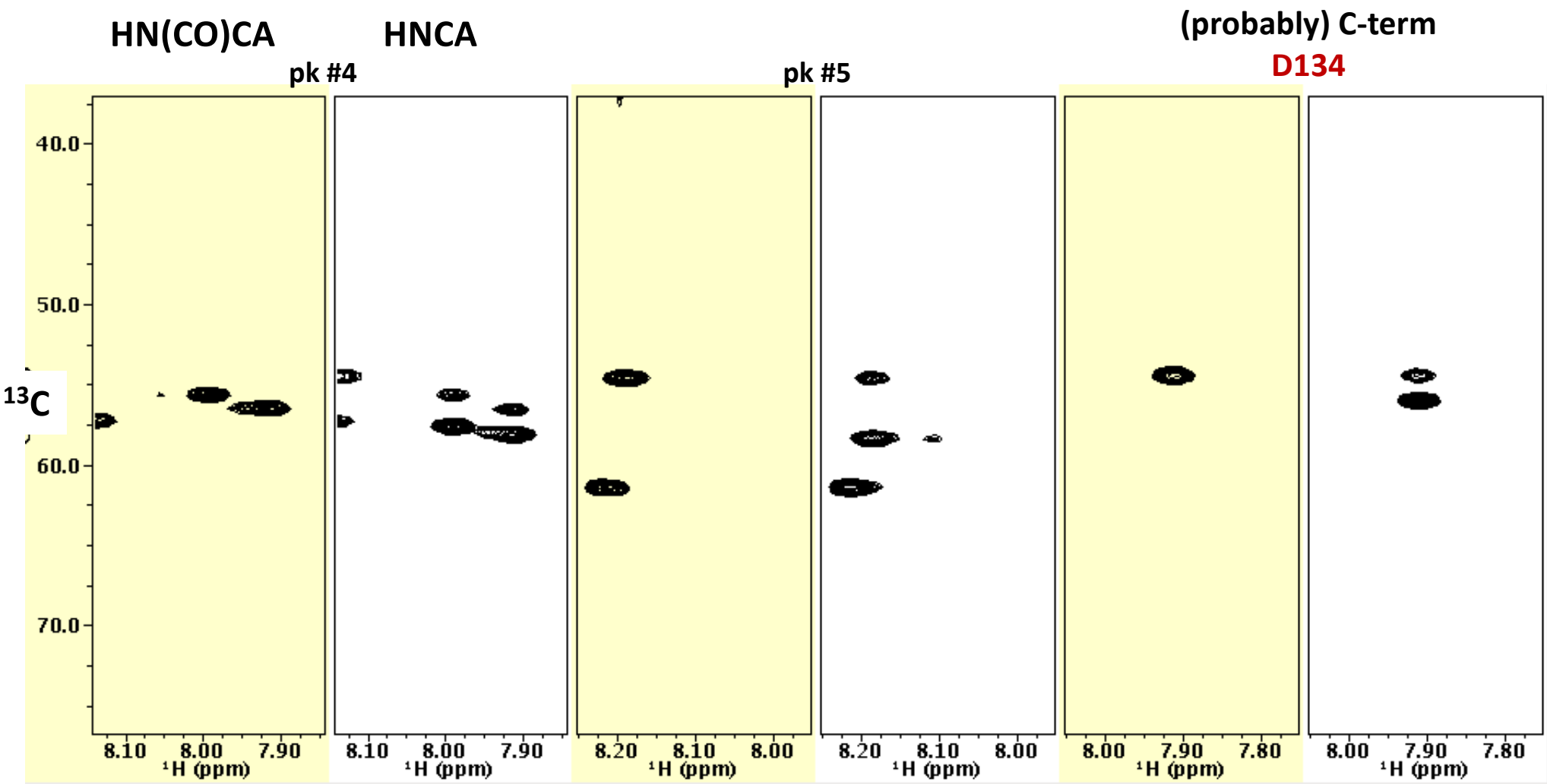
Backbone Assignments – Usually look at 2D strips taken from 3D experiment



110 120 130

34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD

Backbone Assignments

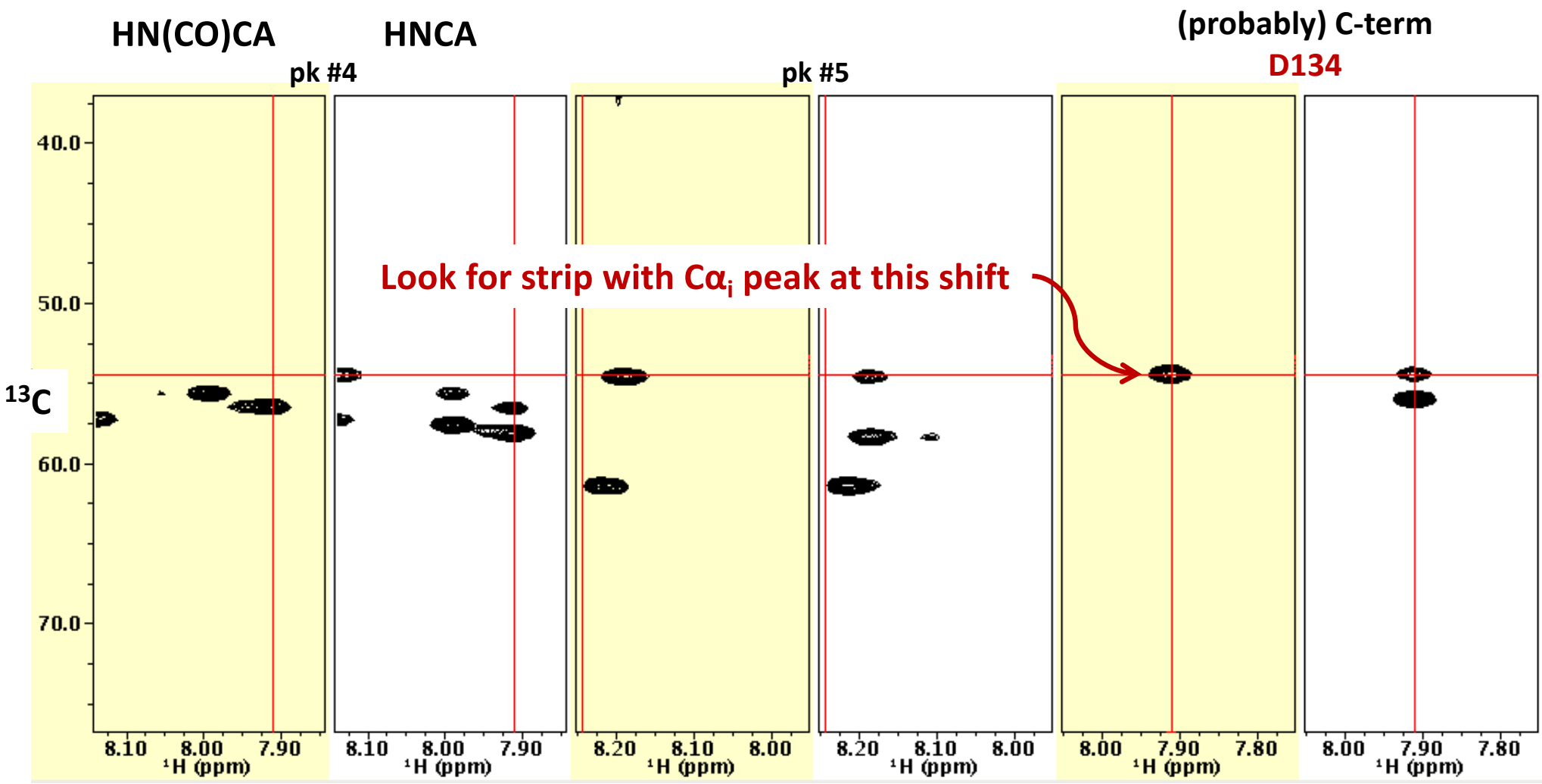


110 120 130

34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD

Backbone Assignments

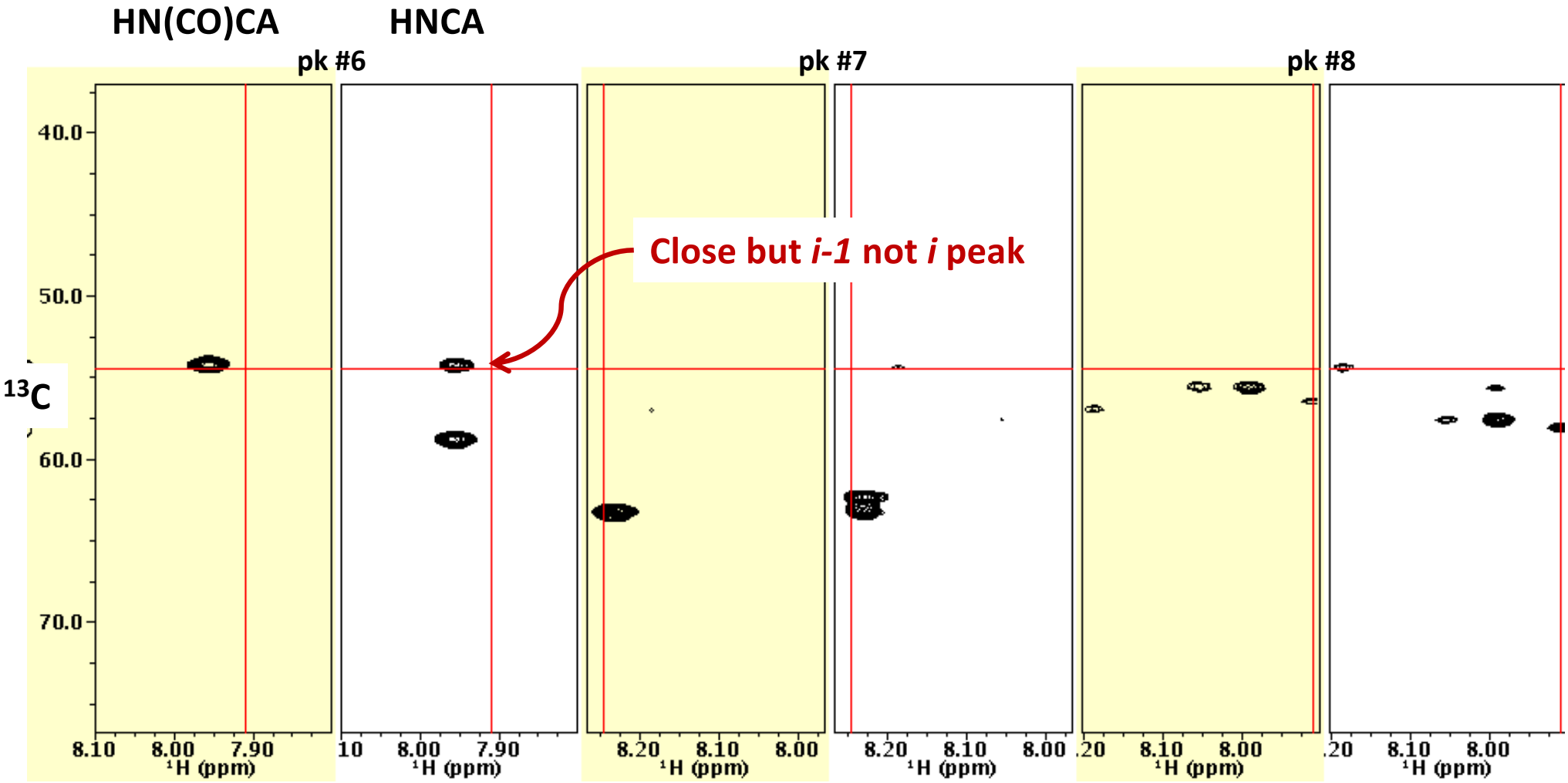
Have to start somewhere ...



34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD

110 120 130

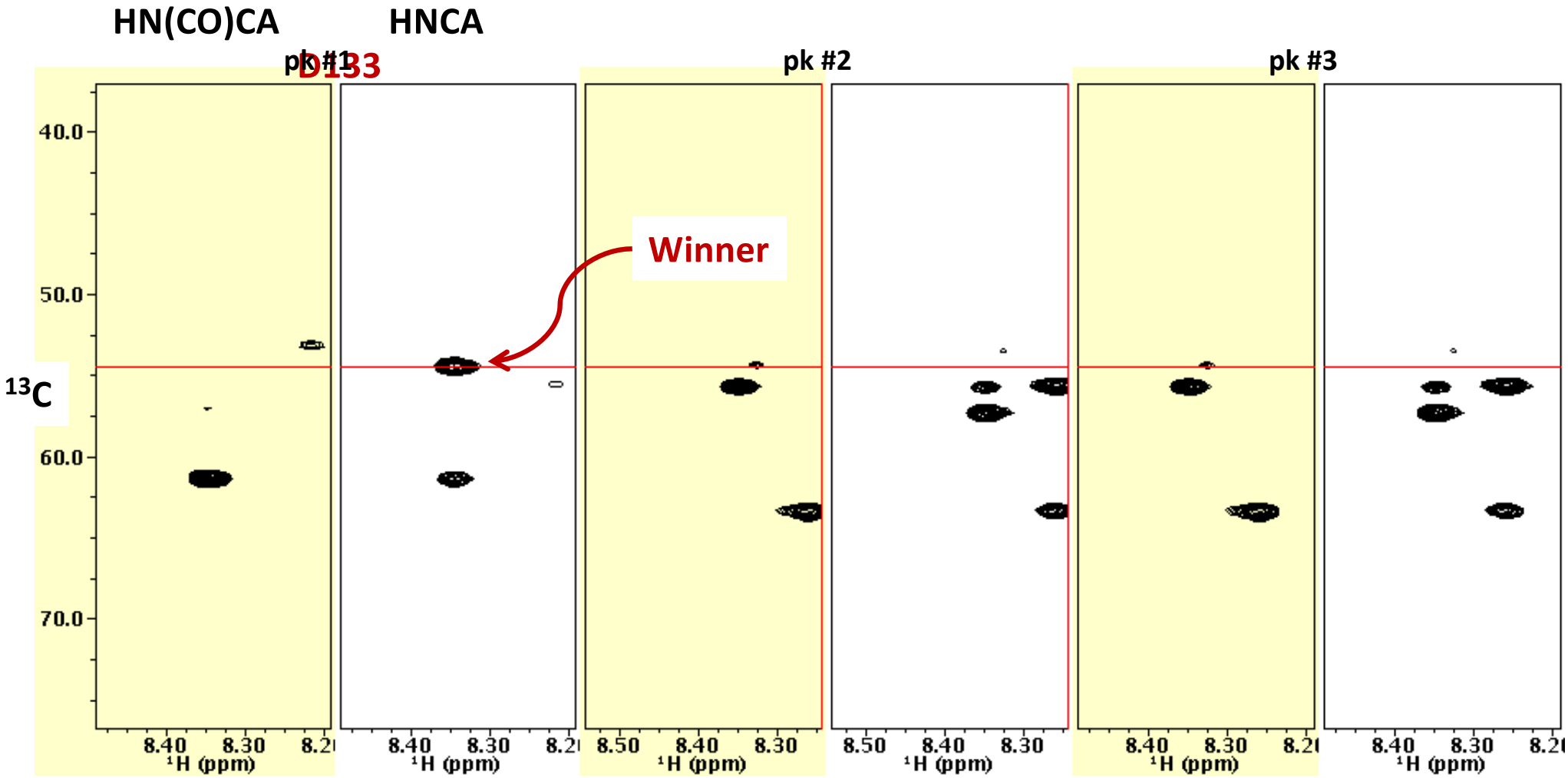
Backbone Assignments



34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD

110 120 130

Backbone Assignments

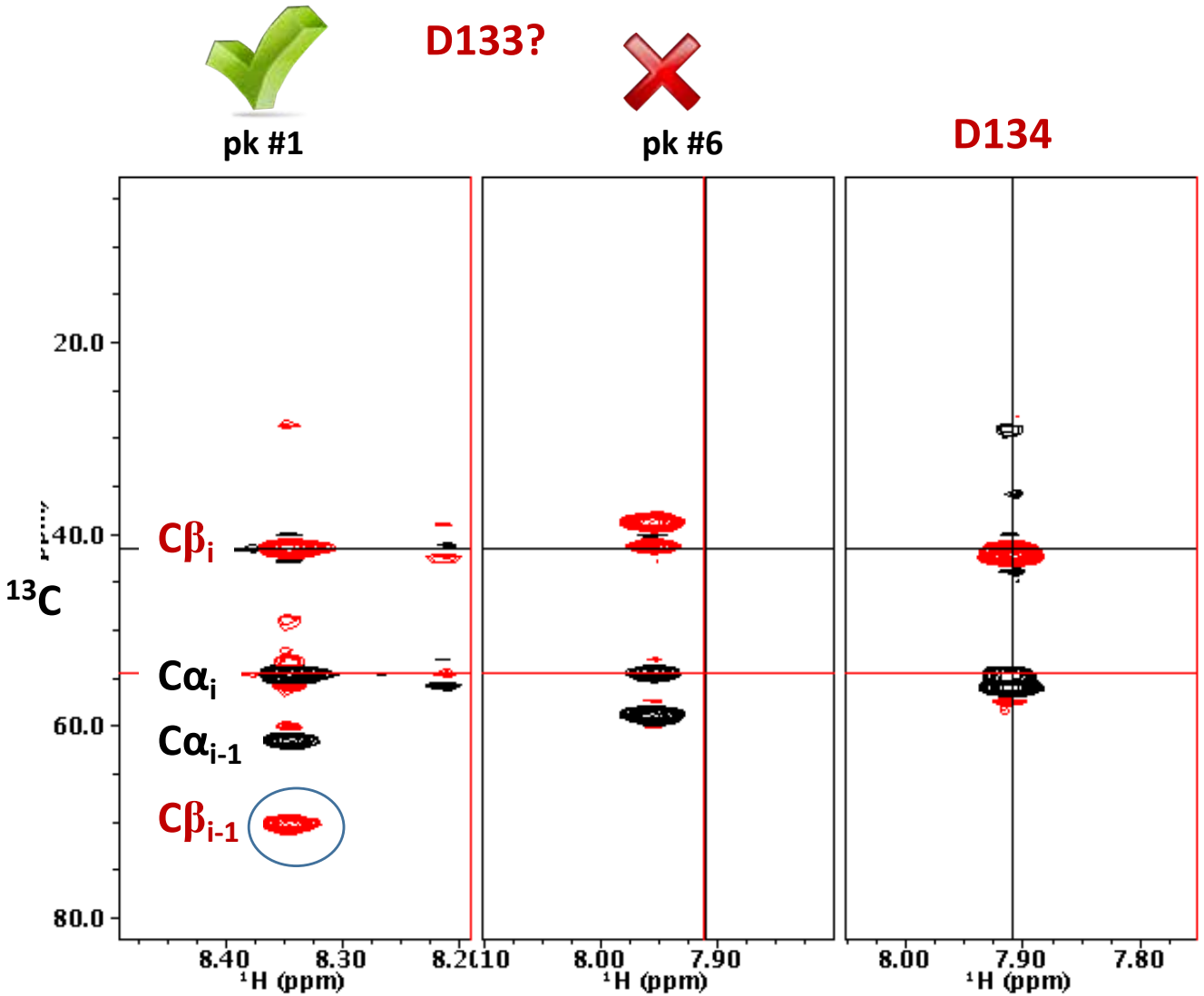


34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD

110 120 130

Backbone Assignments

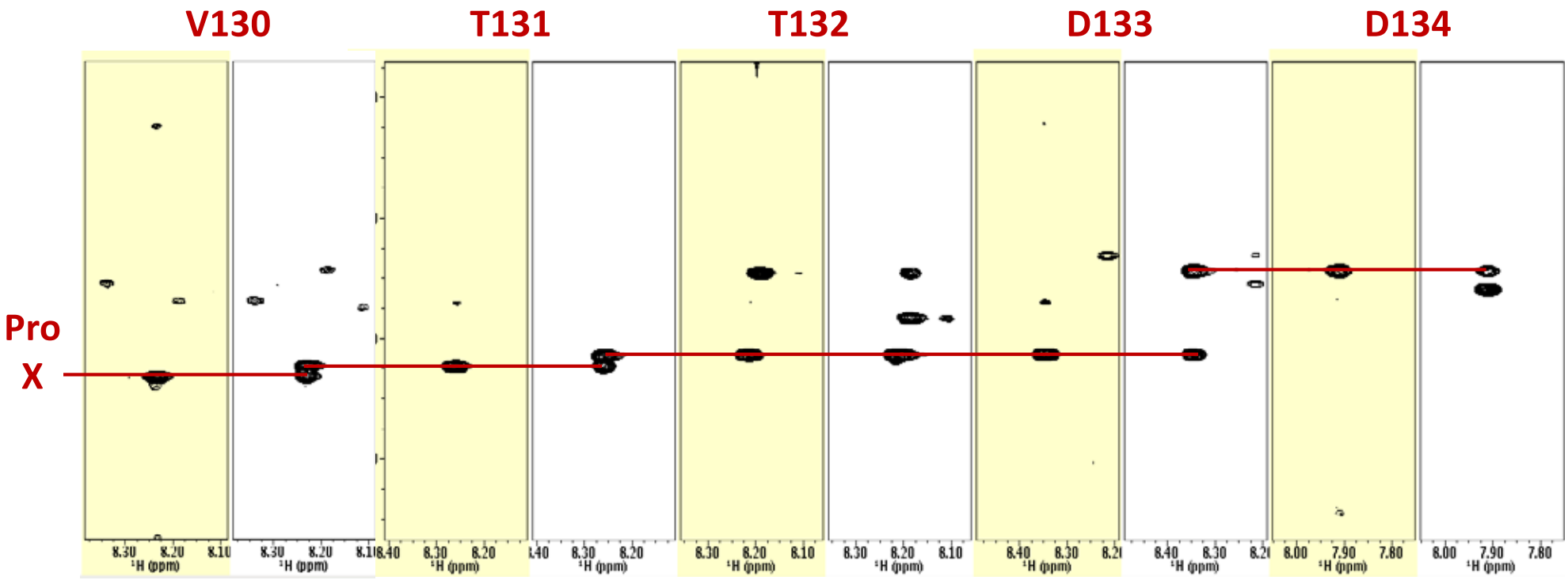
Can confirm with HNCACB



110 120 130

34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD

Backbone Assignments



Chain stops here

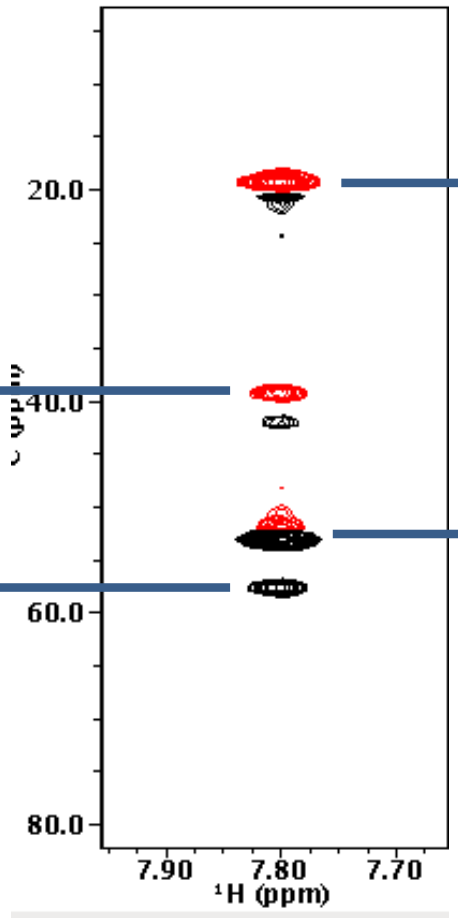
110 120 130

34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD

Backbone Assignments

Alanine
118 or 125?

Alanines have distinctive Cβ shifts



Look for *i-1* peaks

Look for *i* peaks

Peak is **A118** if the previous strip looks like a **Ser**

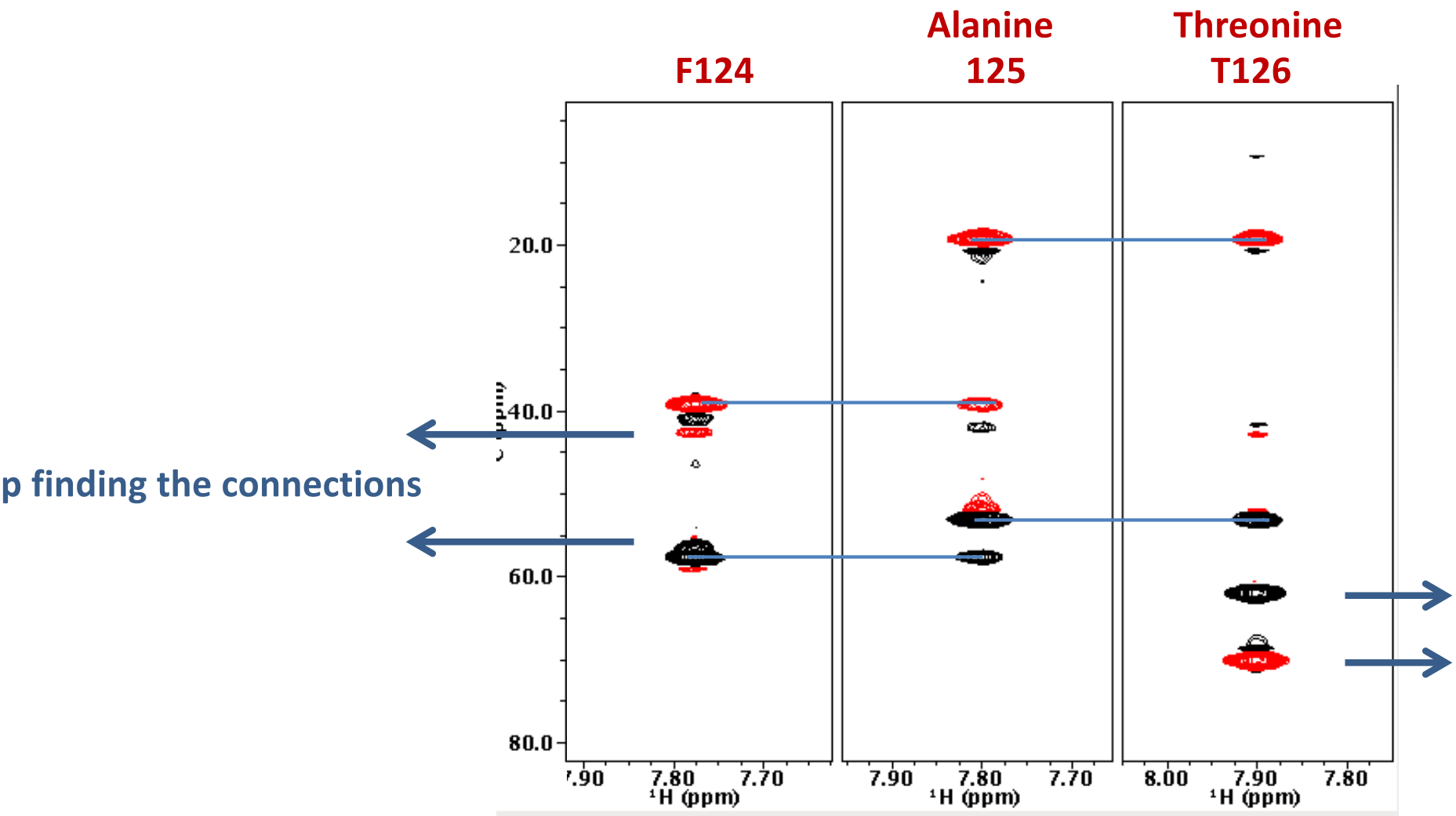
Peak is **A125** if the next strip looks like a **Thr**

So do Thr & Ser

34 Residue peptide: STDST PMFEY ENLED NSAFW MWLFA TDIPV TTDD

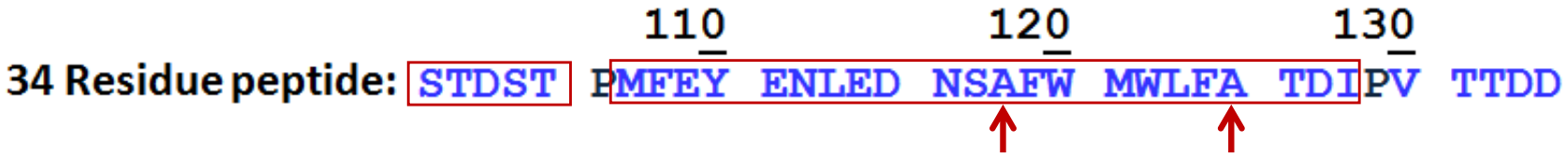
110 120 130

Backbone Assignments

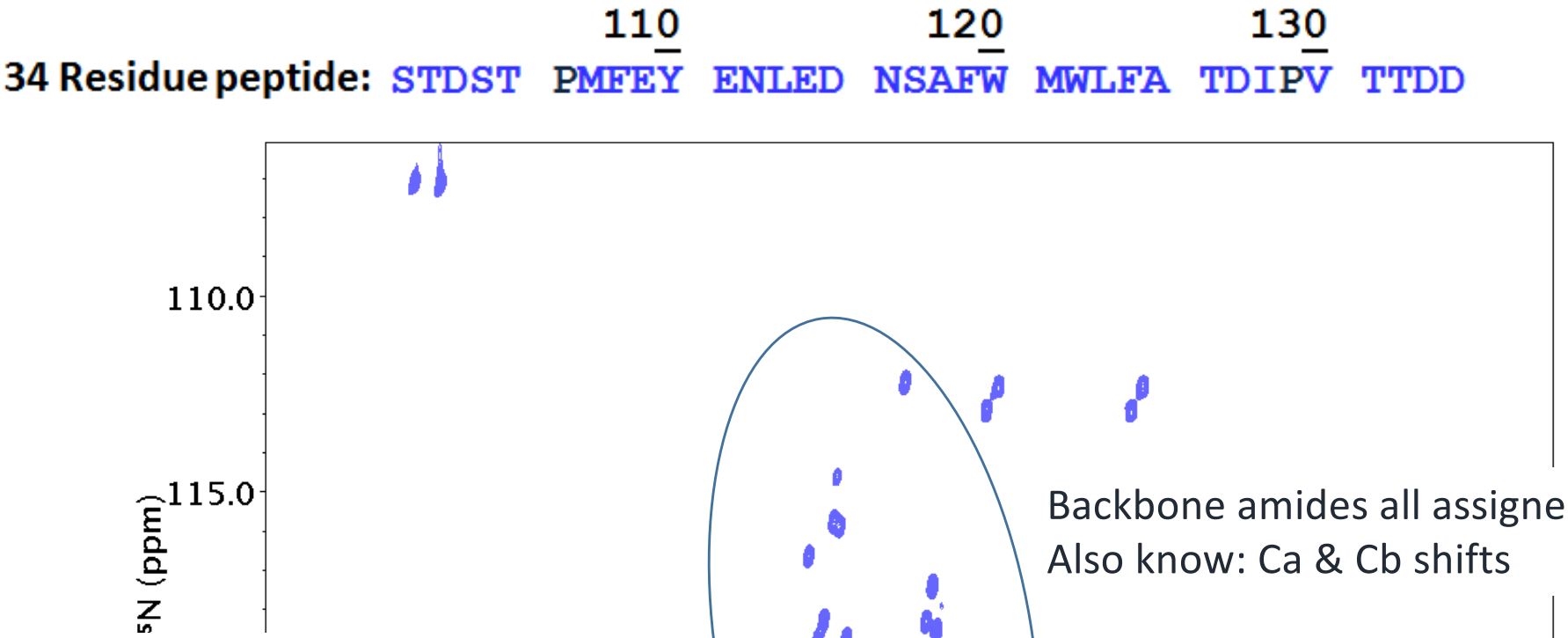


Keep finding the connections

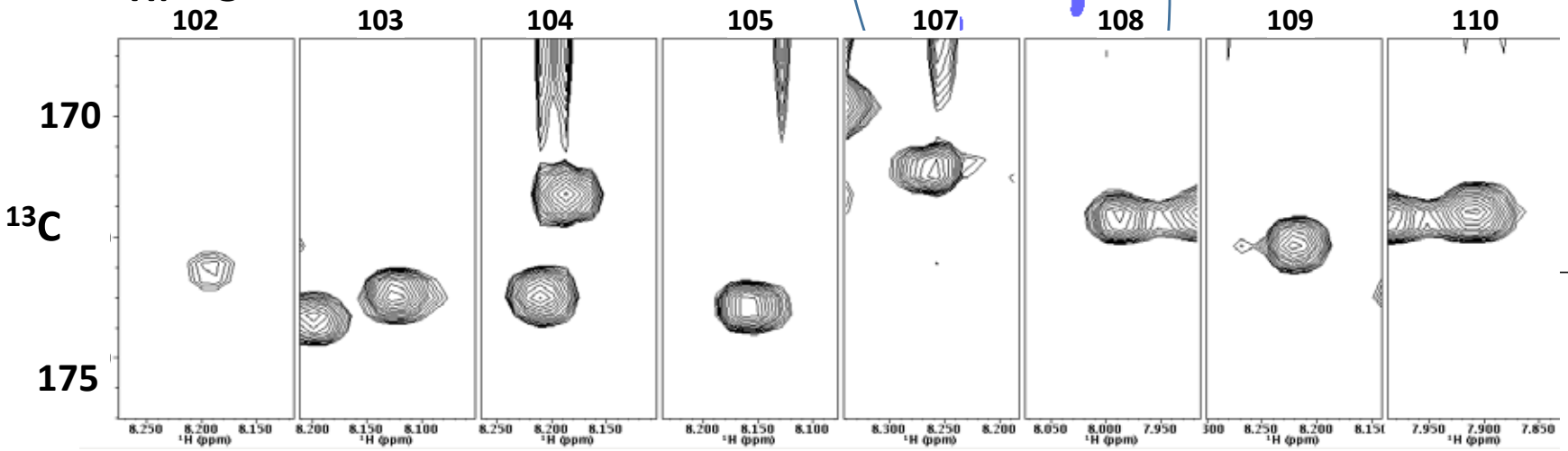
Repeat for remaining sections ...



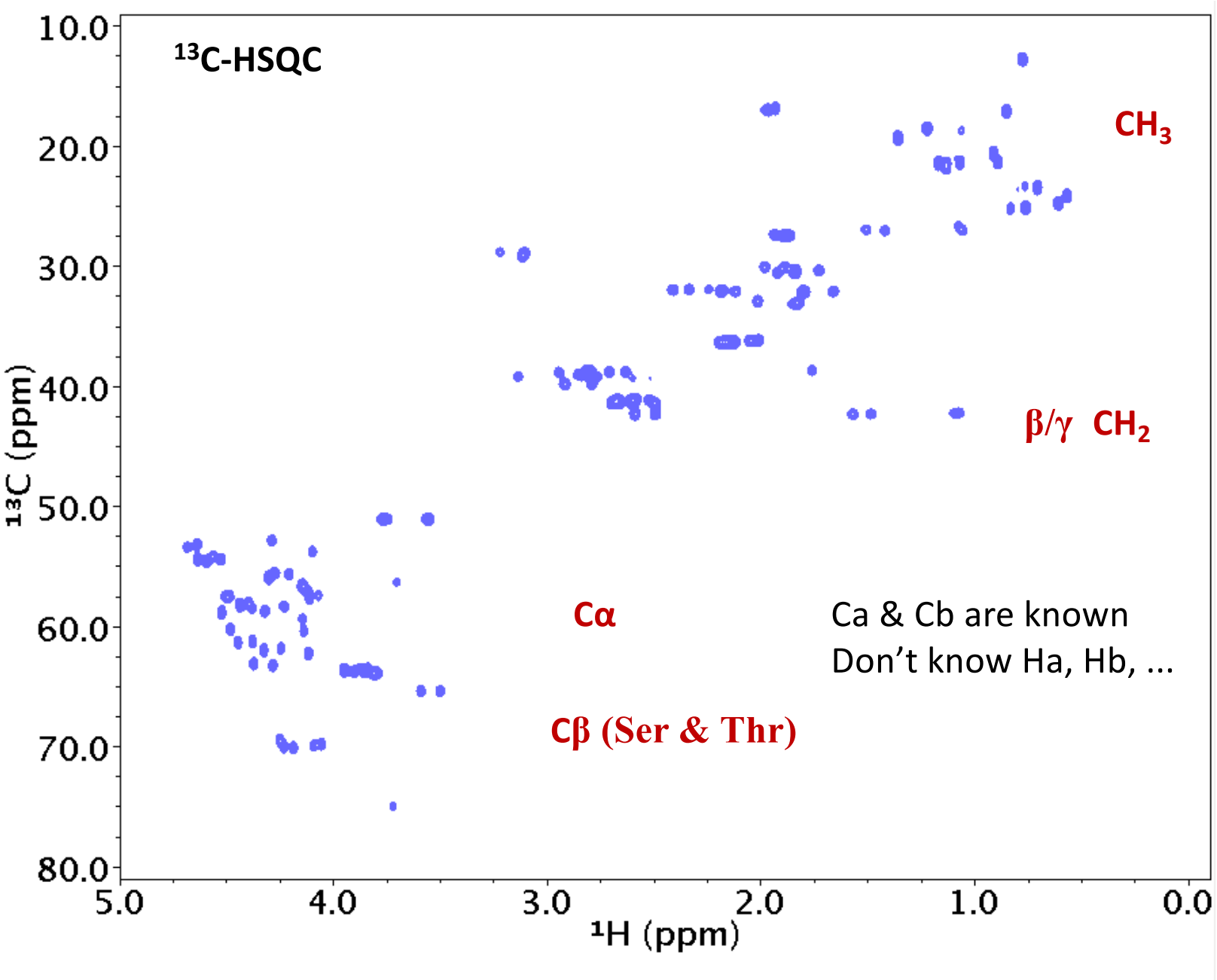
Backbone Assignments: HN, N, Ca, Cb, C’



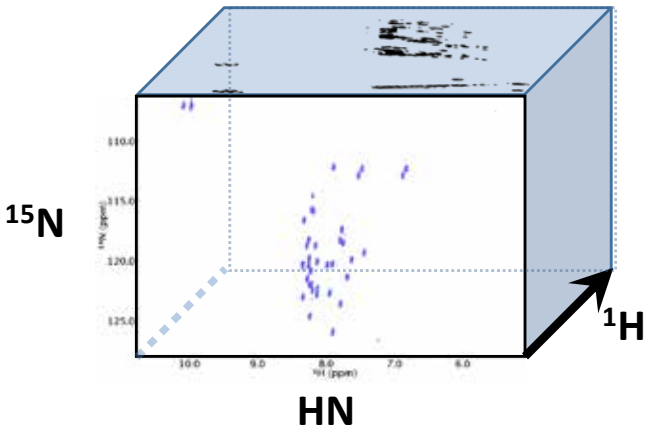
Trivial to add the C’ shifts:



Side chain assignments



Side chain assignments



15N-TOCSY (flattened)

Methyls

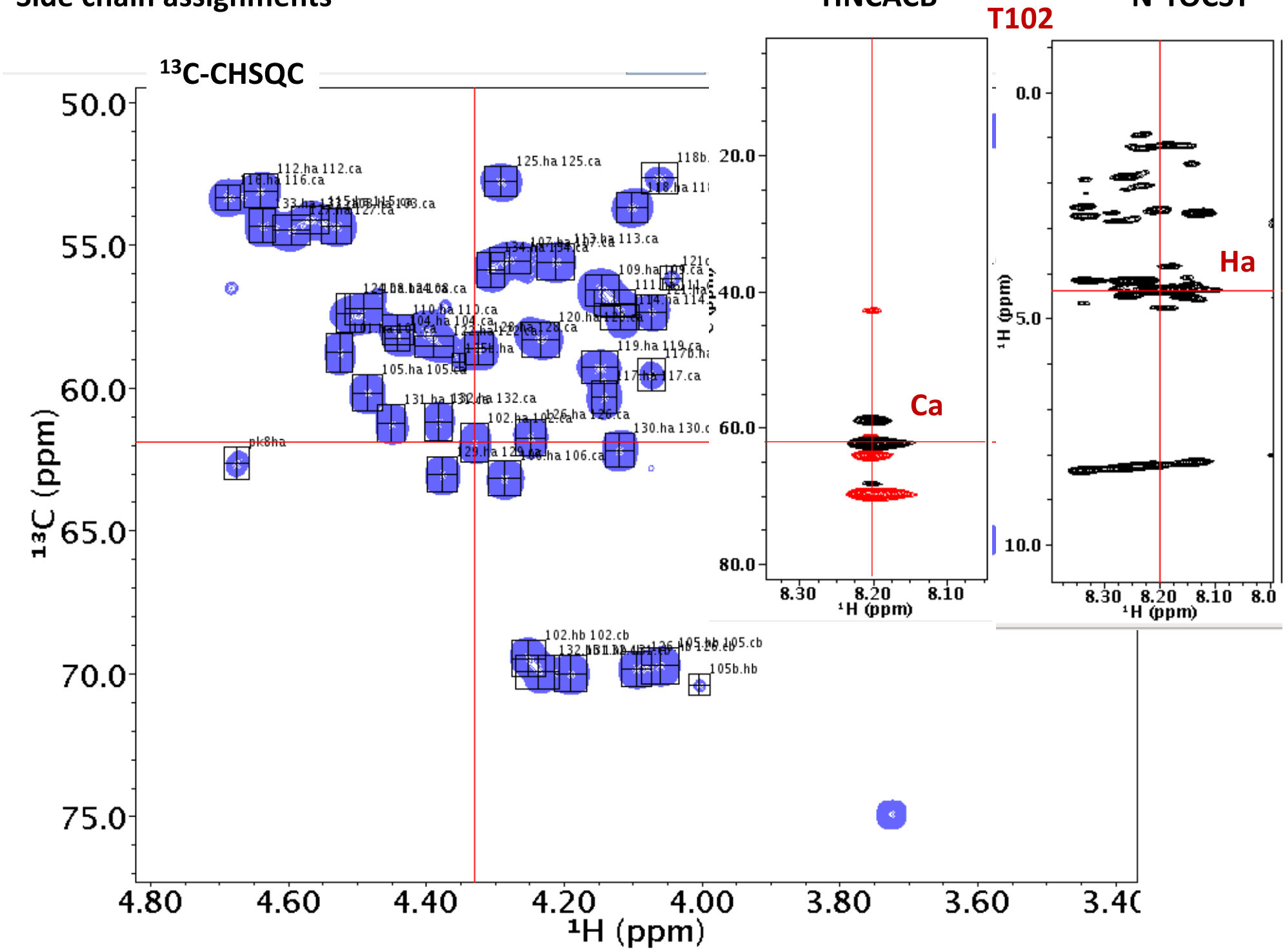
Hβ/γ

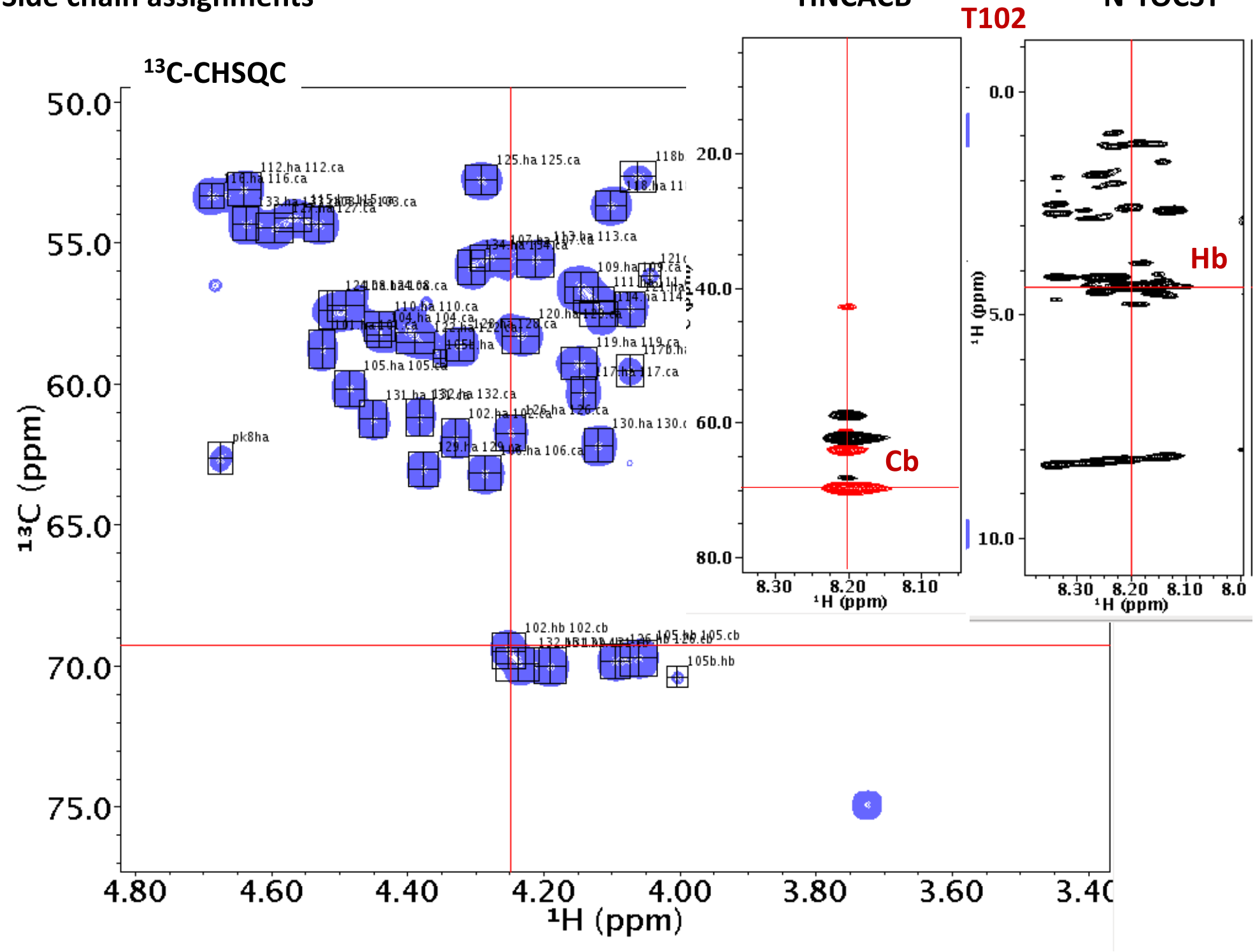
Hα

Side chain protons

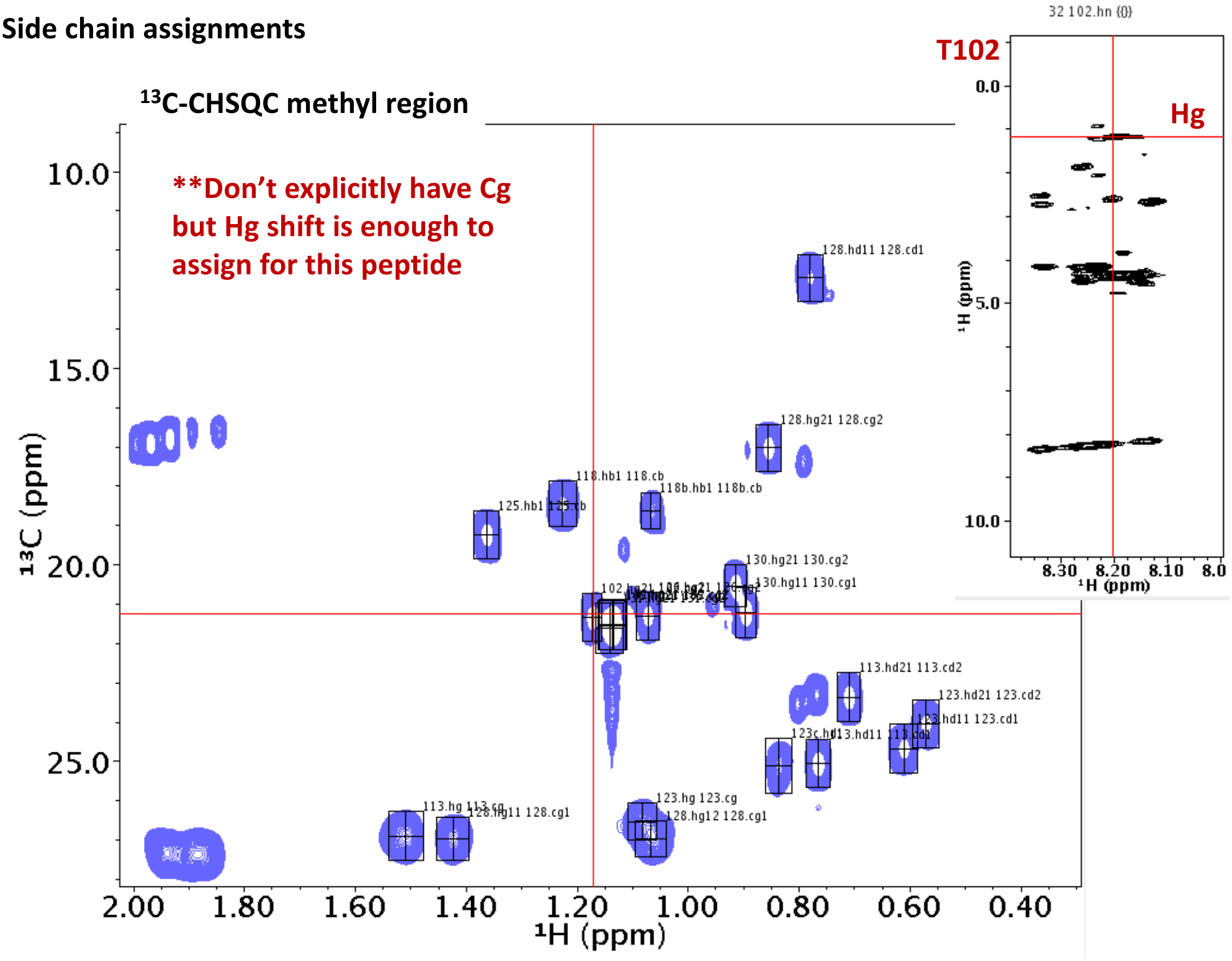
Amides on diagonal

0.0
2.0
4.0
6.0
8.0
10.0
1H (ppm)
10.0
9.0
8.0
7.0
1H (ppm)

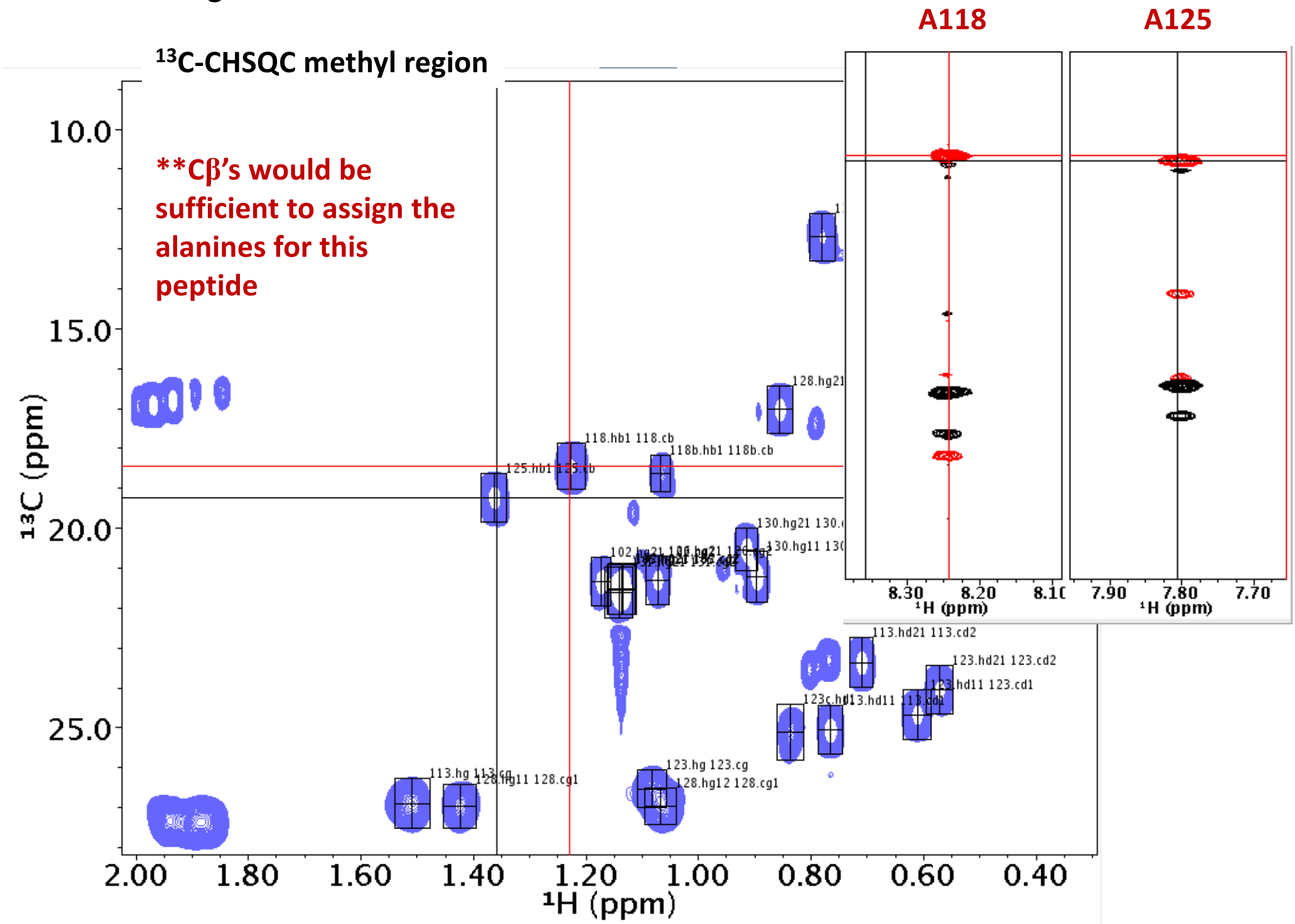

Side chain assignments
HNCACB
15N-TOCSY
T102
13C-CHSQC
Ca
Ha
13C (ppm)
1H (ppm)
1H (ppm)
1H (ppm)


Side chain assignments
HNCACB
15N-TOCSY
T102
13C-CHSQC
Cb
Hb
13C (ppm)
1H (ppm)
1H (ppm)
1H (ppm)

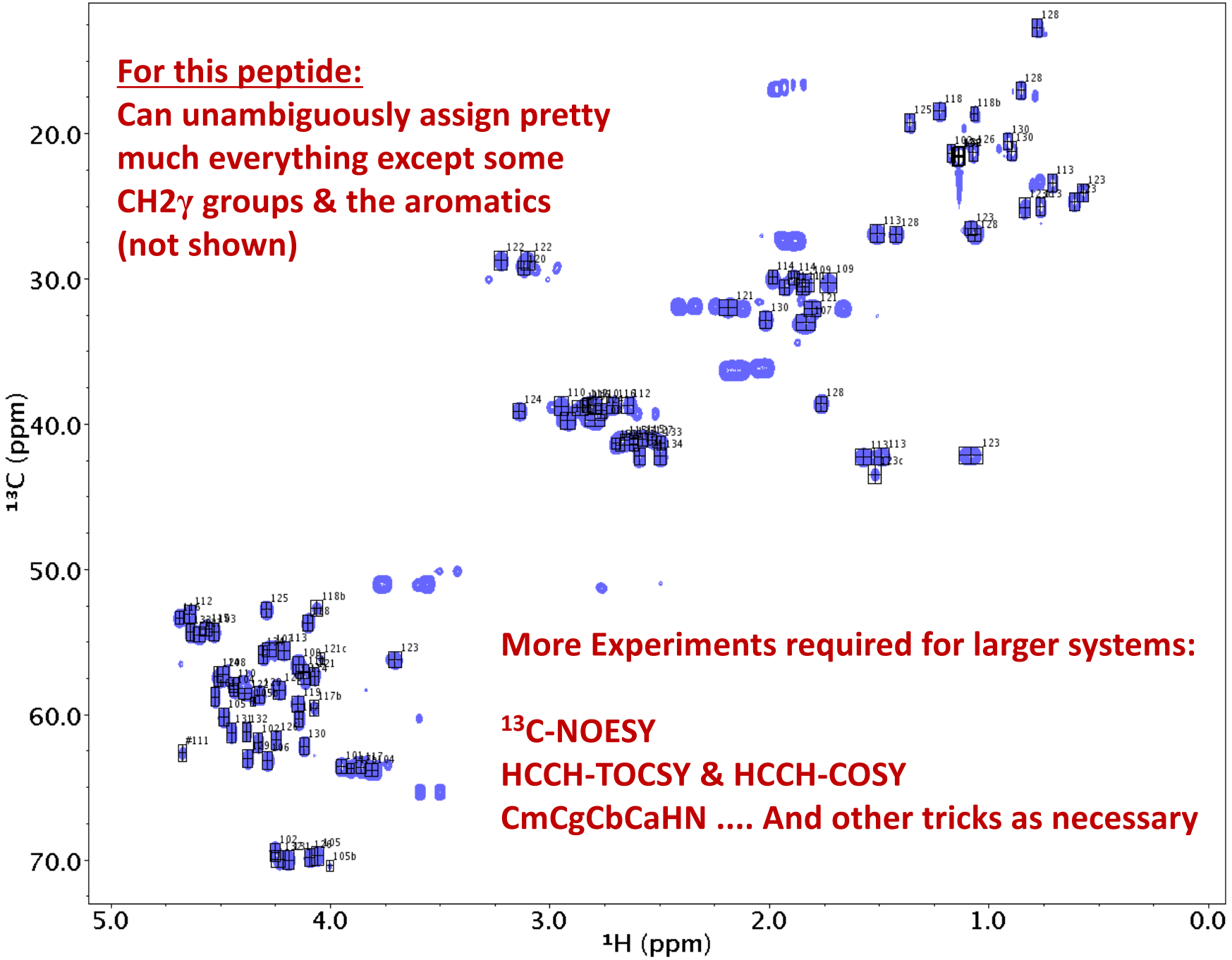
Side chain assignments



Side chain assignments



Side chain assignments: Ha, Ca, Hb, Cb, Hg, Hd ... Cg, Cd inferred



References

Good old school, short intro video on nuclear spin (other episodes are good, too)
https://www.youtube.com/watch?v=jUKdVBpCLHM

UC Davis NMR wiki (source of spin graphics)
http://chemwiki.ucdavis.edu/Physical_Chemistry/Spectroscopy/Magnetic_Resonance_Spectroscopies/Nuclear_Magnetic_Resonance/Nuclear_Magnetic_Resonance_II

Duke intro to NMR
http://www.cs.duke.edu/brd/Teaching/Bio/asmb/current/2papers/Intro-reviews/flemming.pdf

Excellent practical guide for NMR experiments (pulse programs & how they work)
http://www.protein-nmr.org.uk/

MOOC course on NMR, might be good (starts Nov 16; free registration)
https://www.france-universite-numerique-mooc.fr/courses/lille1/54002/session01/about