Homework for BCH 581 – NMR spectroscopy
Email your answers to klara.briknarova@umontana.edu by 10:30 am on Monday March 6
If you write some parts by hand and scan them, please make sure your writing is legible! Show your work and explain your reasoning. Be concise.

1. (6 points) Which techniques can be used to determine the 3-dimensional structures of biological molecules? List at least one advantage and one limitation for each method.

X-ray crystallography
Advantages: well established, can be used for molecules and molecular assemblies of any size (from small molecules to viral particles) if they crystallize
Limitations: Molecule needs to form crystals, determining phases can be a problem, flexibility can interfere with crystallization and flexible parts are often not observed, conformation may be affected by crystal packing

Nuclear magnetic resonance (NMR) spectroscopy
Advantages: in solution (under close to physiological conditions) – no need for crystals!
Limitations: Not suitable for structure determination of large molecules (larger than ~ 20-30 kDa), low sensitivity (needs relatively high concentrations), isotope labeling very expensive or impossible for proteins expressed in higher eukaryotic cells (insect or mammalian cell lines)

Cryogenic electron microscopy (cryo-EM)
Advantages: in a frozen solution – similar to solution, no need for crystals!, only small amount needed
Limitations: Not suitable for molecules smaller than ~ 100 kDa, often lower resolution (3-4Å)

(discussed at the end of NMR1 lecture, 53:00-1:06:32, and at the beginning of NMR2 lecture, 0-2:10)

2. (3 points) An NMR spectrometer operates at ^{1}H Larmor frequency of 600.13 MHz.
(a) Calculate the Larmor frequency of ^{15}N.

$$\omega_H = 2\pi\, \nu_H = \gamma_H B$$
$$\omega_N = 2\pi\, \nu_N = \gamma_N B$$

$$\frac{2\pi\, \nu_N}{2\pi\, \nu_H} = \frac{\gamma_N B}{\gamma_H B}$$

$$\nu_N = \frac{\gamma_N}{\gamma_H}\, \nu_H = \frac{-2.713 \times 10^7 (Ts)^{-1}}{2.6752 \times 10^8 (Ts)^{-1}}\, 600.13\, MHz = -60.861\, MHz$$

Note that frequencies are typically reported as positive numbers, i.e. 60.861 MHz.

(see NMR2: p.16 for formulas and p.13 for gyromagnetic ratios)

(b) Calculate the magnetic field.

$\omega = 2\pi\nu = \gamma B$

$$B = \frac{2\pi\nu}{\gamma} = \frac{2\pi\ (600.13 \times 10^6 Hz)}{2.6752 \times 10^8 (Ts)^{-1}} = 14.095\ T$$

Note that the frequency provided in MHz units corresponds to ν, not ω ($\omega = 2\pi\nu$). (see NMR2: p.17)

3. (4 points) On the NMR spectrometer described in question 2, the observed splitting of a ^{1}H backbone amide signal due to one-bond J-coupling to the ^{15}N atom is 0.153 ppm.
(a) Calculate the value of the J-coupling in Hz.

$$1\ ppm = \frac{600.13 \times 10^6 Hz}{10^6} = 600.13\ Hz \quad \text{(NMR2: p. 20)}$$

$J = 0.153\ ppm\ \times \frac{600.13\ Hz}{1\ ppm} = 91.82\ Hz$ (NMR2: p.21-22 and NMR3: p.3)

(b) Calculate the value of the splitting, in both Hz and ppm, that is observed for the signal from the ^{15}N atom.

The splitting in Hz is the same for both coupled nuclei: $J = 91.82\ Hz$ (NMR2: p.21-22; see also NMR3: p.3)

$$1\ ppm = \frac{60.861 \times 10^6 Hz}{10^6} = 60.861\ Hz \ \text{(from question 2a above and NMR2: p. 20)}$$

$$J = 91.82\ Hz\ \frac{1\ ppm}{60.861\ Hz} = 1.51\ ppm$$

(Also see NMR5: p.13 coupled HSQC, where this splitting is illustrated for a spectrometer with ^{1}H frequency of 750 MHz.)

4. (2 points) In a 2D ^{1}H-^{15}N HSQC spectrum that was acquired on the NMR spectrometer described in question 2, a signal from backbone amide is observed with a ^{1}H chemical shift of 9.023 ppm and ^{15}N shift of 123.87 ppm.
Calculate the ^{1}H and ^{15}N chemical shifts of this signal in a ^{1}H-^{15}N HSQC spectrum that was acquired on an NMR spectrometer that operates with ^{1}H Larmor frequency of 800.13 MHz.

$\delta(1H) = 9.023\ ppm$ and $\delta(15N) = 123.87\ ppm.$

$$\delta = \frac{\nu - \nu_0}{\nu_0} \times 10^6 \ \text{(NMR2: p. 20)}$$

$\nu \propto B_0$ and $\nu_0 \propto B_0 \Rightarrow$ dependence on B cancels out (NMR2: p. 16, 20)
Chemical shifts in ppm units are independent of magnetic field! (see also NMR 3: p.2)

Note that the tables in NMR3: p.5-6 with random coil ^{1}H and ^{13}C chemical shifts do not contain any information about magnetic field.

5. (2 points) Explain what a 90º pulse is and what it does.

A 90º pulse is a pulse of electromagnetic radiation, with the magnetic field vector perpendicular to the static magnetic field B_o and with frequency equal (or close) to the Larmor frequency of the nuclei of interest, applied for a duration t such that the magnetization in the sample is rotated 90º around this magnetic field vector. A 90º pulse is typically used to rotate magnetization from vertical orientation (aligned with external magnetic field) into the transverse plane (the plane perpendicular to the external magnetic field), or vice versa. (NMR3: p. 10-11, also NMR4: p.2)

Note: The pulse rotates the magnetization in the sample. The sample itself and the molecules in it are not rotated in the process.

6. (2 points) Provide two reasons for why the absorption line shape is preferred over the dispersion line shape.

Absorption line shape decreases away from the center faster ($\propto \omega^{-2}$) than dispersion line shape ($\propto \omega^{-1}$) $\Longrightarrow$ narrower signals, less overlap
Dispersion line shape has a positive and negative lobe $\Longrightarrow$ in a crowded spectrum, dispersion signals will tend to cancel
(NMR3: p.26)

Note: Lorentzian refers to both the real (absorption) and imaginary (dispersion) components.

7. (2 points) Explain what effect fast transverse relaxation will have on NMR spectra.

Faster transverse relaxation rate (R_2) leads to:
Broader signals (full width at half height $\propto R_2$) $\Longrightarrow$ more overlap
Lower peak amplitude (peak intensity at the center $\propto \frac{1}{R_2}$) $\Longrightarrow$ lower signal-to-noise ratio
(NMR3: p.26)

More signal lost due to relaxation during experiments with multiple pulses and delays, i.e. all 2D and 3D experiments: every time the magnetization is transverse during the experiment, it relaxes ($\propto e^{-R_2 t}$) $\Longrightarrow$ lower signal-to-noise ratio

8. Analysis of magnetization during 2D NOESY experiment:
(a) (3 points) When analyzing magnetization during 2D NOESY, I suggested (NMR4: p.11) that we ignore the M_y term that contains sin (Ωt_1) after point D. Show what will become of this term after the mixing time (t_{mix}) and the last 90º pulse if you do not ignore it, i.e. derive equations for M_x, M_y and M_z at point F that originate from this term. Use the right handed rotation convention and ignore transfer of magnetization to other spins during the mixing time (t_{mix}).

To answer question 8, you need to be familiar with several rules for analysis of NMR signal in the rotating coordinate frame:

1. At the beginning of an NMR experiment, the magnetization is at equilibrium, i.e. aligned with the direction of the external magnetic field: $M_z = M_0$ (see e.g. NMR3: p.1, NMR4: p.1 & 9).
2. A 90º pulse applied along a certain axis will rotate magnetization 90º around the axis (NMR 3: p.11 & 13, NMR 4: p.2). If the magnetization is aligned with the axis of the rotation, the rotation has no effect (i.e. a pulse along y or –y will have no effect on the M_y component of magnetization).
3. In the absence of pulses, magnetization rotates (precesses) around the static magnetic field along the z axis (and undergoes relaxation, which we will ignore here). In the rotating frame, the angular velocity of the rotation (Ω) is the difference between the angular velocity of the spin precession (ω) and the angular velocity of the rotating frame, which is set equal to the frequency of the radiofrequency pulses (ω_{rf}): $\Omega = \omega - \omega_{rf}$. When the magnetization is aligned along z, there is no observable precession. (see e.g. NMR3: p.9, 17 & 19-20)
Note: This rule applies during ANY time delay including t_{mix} !
4. The effect of pulses and precession on the $M_x, M_y,$ and M_z components of magnetization can be considered separately (NMR 4: p.11).

The use of these rules was illustrated during analysis of the single pulse experiment (NMR3: p.19-20) and the 2D NOESY experiment (NMR 4: p.9-12).

Starting point for part a: the M_y term at point D: $M_y = M_0 sin\Omega t_1$

Point D to E (t_{mix}): precession from y into negative x with angular frequency Ω
Point E: $M_y = M_0 sin\Omega t_1\, cos\Omega t_{mix}\,, M_x = -M_0 sin\Omega t_1\, sin\Omega t_{mix}$

Point E to F (90º pulse along –y): the negative M_x component is rotated into negative M_z
Point F: $M_y = M_0 sin\Omega t_1\, cos\Omega t_{mix}\,, M_z = -M_0 sin\Omega t_1\, sin\Omega t_{mix}$

Note: These terms will be present at point F in addition to $M_x = M_0\, a\, cos\Omega t_1$ (NMR 4: p.12), and the M_y term will give rise to undesirable observable signal during the FID.

(b) (7 points) Consider the same 2D NOESY pulse sequence as shown on p.9 of the NMR4 notes, but with the phase of all three 90º pulses set to **–y** (i.e. **negative y**). Show the magnetization that you will have at the beginning of the FID, i.e. derive equations for M_x, M_y and M_z at point F. Ignore transfer of magnetization to other spins during the mixing time (t_{mix}) but do not ignore any other terms along the way. Can you imagine any use for the data acquired in this way?

Point A: equilibrium magnetization: $M_z = M_0$

Point A to B (90º pulse along –y): M_z is rotated into negative M_x
Point B: $M_x = -M_0$

Point B to C (t_1): precession from negative x into negative y with angular frequency Ω
Point C: $M_x = -M_0 cos\Omega t_1\,, M_y = -M_0 sin\Omega t_1$

Point C to D (90º pulse along –y): the negative M_x component is rotated into negative M_z
Point D: $M_z = -M_0 cos\Omega t_1$, $M_y = -M_0 sin\Omega t_1$

Point D to E (t_{mix}): precession from negative y into x with angular frequency Ω, no change for M_z term (except perhaps for a factor a to account for relaxation, for consistency with NMR4: p.11-12)
Point E: $M_z = -M_0\ a\ cos\Omega t_1$, $M_y = -M_0 sin\Omega t_1\ cos\Omega t_{mix}$, $M_x = M_0 sin\Omega t_1\ sin\Omega t_{mix}$

Point E to F (90º pulse along –y): the negative M_z component is rotated into M_x, and M_x is rotated into M_z
Point F: $M_x = M_0\ a\ cos\Omega t_1$, $M_y = -M_0 sin\Omega t_1\ cos\Omega t_{mix}$, $M_z = M_0 sin\Omega t_1\ sin\Omega t_{mix}$

Now compare with the result for the original experiment with phases of the pulses set to y, y, and –y (see NMR4: p.12 and question 8a above):
Point F: $M_x = M_0\ a\ cos\Omega t_1, M_y = M_0 sin\Omega t_1\ cos\Omega t_{mix}$, $M_z = -M_0 sin\Omega t_1\ sin\Omega t_{mix}$

The term that we want, $M_x = M_0\ a\ cos\Omega t_1$, is the same in both experiments, but the M_y and M_z terms, which we do not want, have opposite signs! If you acquire data with both the original experiment and the experiment in 8b and add the data, the desirable signals ($M_x = M_0\ a\ cos\Omega t_1$) will add up while the undesirable signals will cancel. This method to cancel unwanted signals is called phase cycling (mentioned in NMR4: p.11).

(c) (7 points) Consider the same 2D NOESY pulse sequence as shown on p.9 of the NMR4 notes, but with the phase of the first 90º pulse set to **x**, the phase of the second 90º pulse set to **y**, and the phase of the third 90º pulse set to **-y** (i.e. **negative y**). Show the magnetization that you will have at the beginning of the FID, i.e. derive equations for M_x, M_y and M_z at point F. Ignore transfer of magnetization to other spins during the mixing time (t_{mix}) but do not ignore any other terms along the way. Can you imagine any use for the data acquired in this way?

Point A: equilibrium magnetization: $M_z = M_0$

Point A to B (90º pulse along x): M_z is rotated into negative M_y
Point B: $M_y = -M_0$

Point B to C (t_1): precession from negative y into x with angular frequency Ω
Point C: $M_y = -M_0 cos\Omega t_1$, $M_x = M_0 sin\Omega t_1$

Point C to D (90º pulse along y): M_x is rotated into negative M_z
Point D: $M_y = -M_0 cos\Omega t_1$, $M_z = -M_0 sin\Omega t_1$

Point D to E (t_{mix}): precession from negative y into x with angular frequency Ω, no change for M_z term (except perhaps for a factor a to account for relaxation, for consistency with NMR4: p.11-12)
Point E: $M_y = -M_0 cos\Omega t_1\ cos\Omega t_{mix}$, $M_x = M_0 cos\Omega t_1\ sin\Omega t_{mix}, M_z = -M_0\ a\ sin\Omega t_1$

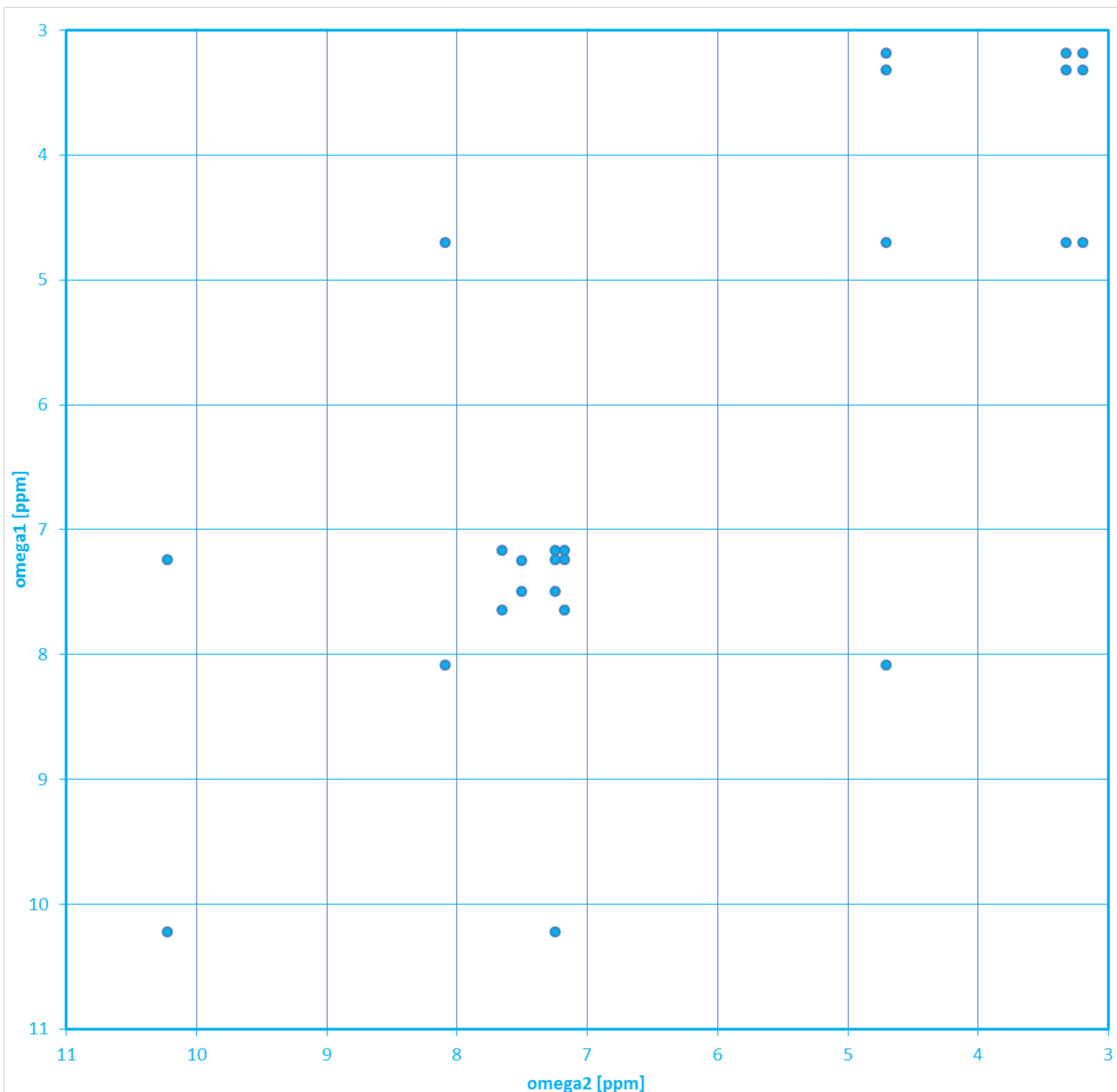
Point E to F (90º pulse along –y): the negative M_z component is rotated into M_x, and M_x is rotated into M_z
Point F: $M_y = -M_0 cos\Omega t_1 \, cos\Omega t_{mix}$, $M_z = M_0 cos\Omega t_1 \, sin\Omega t_{mix}$, $M_x = M_0 \, a \, sin\Omega t_1$

The original experiment with phases of the pulses set to y, y, and –y (NMR4: p.12) had $M_x = M_0 \, a \, cos\Omega t_1$ at point F, but now we have $M_x = M_0 \, a \, sin\Omega t_1$ instead! If we manage to get rid of the M_y and M_z terms (for example by phase cycling, similar to the process outlined in 8a & 8b above), the experiment in 8c will provide signal modulated by $sin\Omega t_1$. This signal is complementary to the signal modulated by $cos\Omega t_1$, and the two signals can be used together in a similar manner as the M_x and M_y components of magnetization during FID to distinguish the direction (sign) of rotation during t_1 (see NMR 3: p.20-22).

9. (3 points) What is the main difference between COSY and NOESY spectrum? What are these experiments used for?

COSY spectrum contains crosspeaks between nuclei that are J-coupled, i.e. 2 or 3 bonds apart. NOESY spectrum contains crosspeaks between nuclei that are close in space, i.e. less than ~ 5 Å apart. (NMR 5: p.4)
Both experiments are used for assignment, i.e. to determine which signal originates from which nucleus in the molecule. In proteins, COSY is used to identify spin systems, i.e. to determine which signals belong to the same amino acid residue and which amino acid type (e.g. leucine, proline, etc.) this might be. NOESY is used for sequential assignment, i.e. to identify neighboring residues, and this, together with COSY, facilitates assignment to a particular residue and atom in the protein. NOESY also contains additional information about short distances in the molecule, and this information can be used to determine the 3-dimensional structure of the molecule. (NMR 5: p.7-9 & 24)

10. (4 points) Prepare a sketch of a COSY spectrum for a tryptophan residue that is part of a larger polypeptide. Assume that the chemical shifts of the atoms are close to their random coil values.
Use the data from the attached table, which contains chemical shift for one more atom than the table provided in class.



11. (5 points) For a particular backbone amide signal in a 2D ^{1}H-^{15}N HSQC spectrum, three peaks were observed in an HNCACB spectrum with ^{13}C chemical shifts of 64.0 ppm, 58.5 ppm, and 45.0 ppm, and one peak was observed in a CBCACONH spectrum with ^{13}C chemical shift of 45.0 ppm. To which residue type (i.e. amino acid) does this amide signal most likely belong? What is the most likely identity of the preceding residue, i.e. of the amino acid that is N-terminal of this residue? What is the most likely identity of the following residue, i.e. of the amino acid that is C-terminal of this residue? Can you say anything about the secondary structure that this residue is part of?
A note: CBCACONH and CBCA(CO)NH are two slightly different names for the same experiment. In addition, HNCOCACB (shown in some of the slides in nmr5 notes) will yield signals in the same locations and so can be considered identical for our purposes.

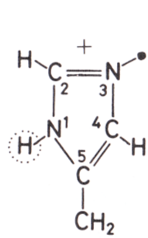
The CBCACONH spectrum correlates the backbone amide ^{1}H chemical shift (in the ^{1}H dimension) and the backbone amide ^{15}N chemical shift (in the ^{15}N dimension) of one residue with ^{13}C chemical shifts of Cα and Cβ atoms of the **previous residue** (in the ^{13}C dimension). The single observed signal with a chemical shift of 45.0 ppm is in excellent agreement with random coil Cα chemical shift of glycine (45.1 ppm), and assignment to a glycine is also supported by the absence of a second signal, consistent with absence of Cβ.
The HNCACB spectrum correlates the backbone amide ^{1}H chemical shift (in the ^{1}H dimension) and the backbone amide ^{15}N chemical shift (in the ^{15}N dimension) of one residue with ^{13}C chemical shifts of Cα and Cβ atoms of **the same residue** and Cα and Cβ atoms of the **previous residue** (in the ^{13}C dimension). The signal at 45.0 ppm is also observed in CBCACONH and is therefore from the previous residue. The remaining two chemical shifts, 58.5 ppm and 64.0 ppm, are in excellent agreement with random coil Cα and Cβ chemical shifts of serine (58.3 ppm and 63.8 ppm). The data therefore suggest that the amide signal belongs to a serine preceded by a glycine. (NMR5: p.14, 17, 20-21)

^{13}C chemical shifts of one residue cannot be used to predict the type of neighboring residues. The experiments therefore do not contain any information about the residue that is C-terminal of the serine. (NMR5 lecture 1:02:35-1:02:55)

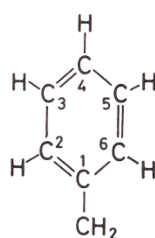
The difference from random coil chemical shift (called secondary chemical shift) is -0.1 ppm for Gly Cα, +0.2 ppm for Ser Cα, and +0.2 ppm for Ser Cβ. In an α-helix, we would expect a positive Cα and negative Cβ secondary chemical shifts, and in a β-sheet, negative Cα and positive Cβ secondary chemical shifts (NMR5: p.18). However, the signs of the observed secondary shifts are not consistent with a single secondary structure, and their magnitudes are much smaller than what would be expected for fully formed secondary structure (NMR5: p.18). Hence, the serine and the preceding glycine are most likely not part of any regular secondary structure.

TABLE 2.3. Random Coil ^{1}H Chemical Shifts for the 20 Common Amino Acid Residues[a]

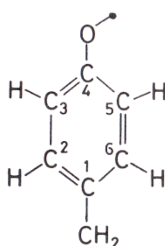
Residue	NH	αH	βH	Others
Gly	8.39	3.97		
Ala	8.25	4.35	1.39	
Val	8.44	4.18	2.13	γCH_3 0.97, 0.94
Ile	8.19	4.23	1.90	γCH_2 1.48, 1.19
				γCH_3 0.95
				δCH_3 0.89
Leu	8.42	4.38	1.65,1.65	γH 1.64
				δCH_3 0.94, 0.90
Pro [b]		4.44	2.28,2.02	γCH_2 2.03, 2.03
				δCH_2 3.68, 3.65
Ser	8.38	4.50	3.88,3.88	
Thr	8.24	4.35	4.22	γCH_3 1.23
Asp	8.41	4.76	2.84,2.75	
Glu	8.37	4.29	2.09,1.97	γCH_2 2.31, 2.28
Lys	8.41	4.36	1.85,1.76	γCH_2 1.45, 1.45
				δCH_2 1.70, 1.70
				ϵCH_2 3.02, 3.02
				ϵNH_3^+ 7.52
Arg	8.27	4.38	1.89,1.79	γCH_2 1.70, 1.70
				δCH_2 3.32, 3.32
				NH 7.17, 6.62
Asn	8.75	4.75	2.83,2.75	γNH_2 7.59, 6.91
Gln	8.41	4.37	2.13,2.01	γCH_2 2.38, 2.38
				δNH_2 6.87, 7.59
Met	8.42	4.52	2.15,2.01	γCH_2 2.64, 2.64
				ϵCH_3 2.13
Cys	8.31	4.69	3.28,2.96	
Trp	8.09	4.70	3.32,3.19	2H 7.24
				4H 7.65
				5H 7.17
				6H 7.24
				7H 7.50
				NH 10.22
Phe	8.23	4.66	3.22,2.99	2,6H 7.30
				3,5H 7.39
				4H 7.34
Tyr	8.18	4.60	3.13,2.92	2,6H 7.15
				3,5H 6.86
His	8.41	4.63	3.26,3.20	2H 8.12
				4H 7.14



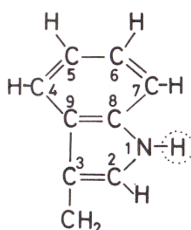
His, H



Phe, F



Tyr, Y



Trp, W