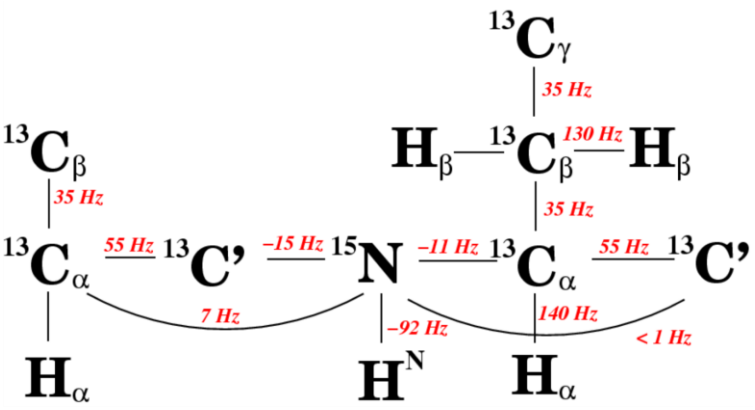
Introduction to 3D Triple Resonance Experiments

Reading: Evans, pp. 79-103
Ikura, Kay & Bax Biochemistry **29**, 4659 (1990)
Cavanagh, pp. 478-528 (reference, for triple resonance methods).
M.J. Sattler *et al.*, Progress in NMR Spectroscopy **34**, p93-158 (1999)

Last time the assignment process using 3D ^{15}N experiments (TOCSY-HSQC and NOESY-HSQC was presented. Although it is an improvement over 2D methods for larger peptides/small proteins, the method also has drawbacks:

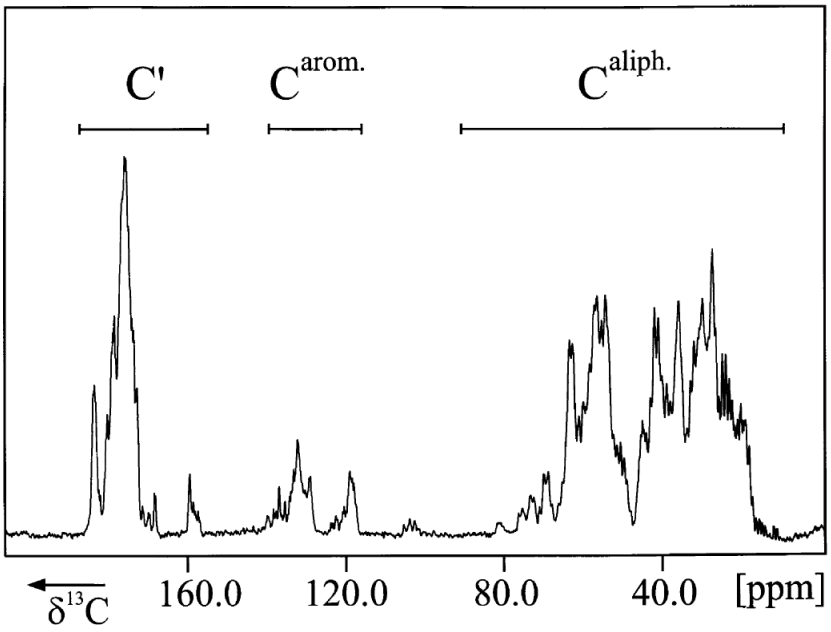
- Not possible to unambiguously assign the side chain proton resonances.
- With increasing protein size the ^{15}N edited TOCSY-HSQC correlations will become weaker and you may not get a complete transfer along the side chain, thus making amino acid typing impossible.
- Assignment relies on HN-X NOEs, which are conformation dependent and degeneracy in the ^{1}H dimension becomes a problem, especially for α-helical proteins and larger proteins.
- For structure determination there is no information about the side chains leading to poor quality structures, especially for α-helical proteins.

In the late 1980s a parallel effort was underway that used heteronuclear (^{15}N and ^{13}C) labeling and 2D NMR to assign the backbone amides. The double-labeling approach made use of large one-bond scalar couplings to correlate the different nuclei along the protein backbone using ^{13}C and ^{15}N: Oh *et al.*, Science 240, 908 (1988); Stockman *et al.*, Biochemistry 28,230 (1989).



The large single bond heteronuclear couplings give efficient transfer through the backbone (*e.g.* ^{13}CO – ^{15}N, ^{1}J=15Hz) when compared to homonuclear 1H couplings, which can be as small as 1Hz. One important feature is that the Cα and C' atoms can be treated as separate nuclei since their chemical shifts are so different (Cα ≈ 40-65 ppm; C' ≈ 165-180ppm). Being able to treat the Cα and CO as different nuclei allows them to be manipulated independently, so magnetization transfer can be selected for N-CO or N-Cα separately.

This new approach in making sequential assignments removed the use of NOEs for assignments and so has the advantage of being less ambiguous and more reliable.



1D ^{13}C spectrum of the protein Rhodniin (103 amino acids) showing the three separate spectral regions containing the C' (carbonyl), aromatic (C^{arom}) and aliphatic (C^{aliph}) ^{13}C resonances.

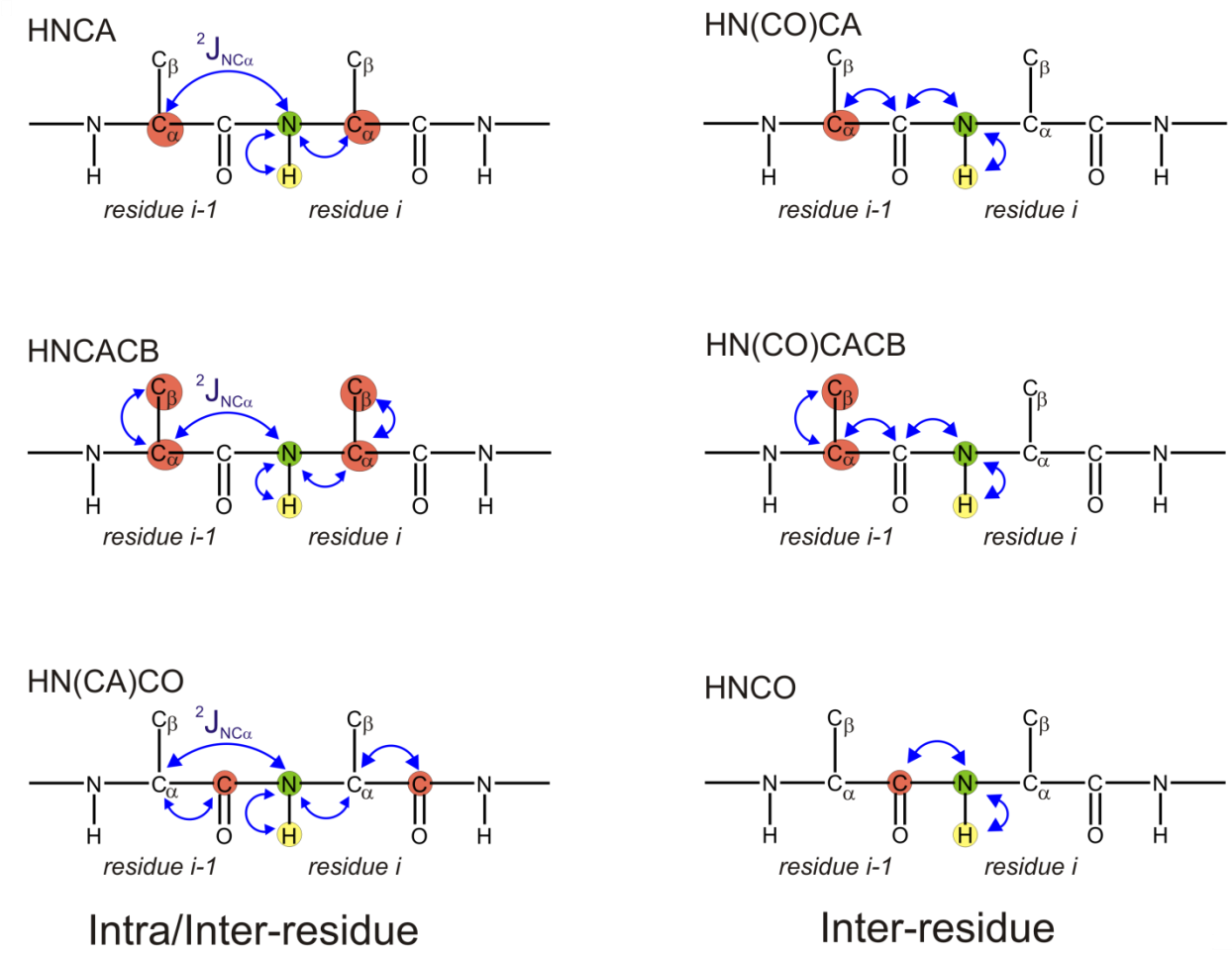
Random Coil ^{13}C chemical shifts for dipeptide X-Ala

Amino Acid	CO	Cα	Cβ	Cγ	Cδ	Cε	Cζ	Cη
A	177.8	52.5	19.1					
C (S-S)	174.6	55.4	41.1					
C (S-H)	174.6	58.2	28.0					
D	176.3	54.2	41.1					
E	176.6	56.6	29.9	35.6				
F	175.8	57.7	39.6		131.9	131.5	129.9	
G	174.9	45.1						
H	174.1	55.0	29.0		120.1	131.1		
I	176.4	61.1	38.8	27.2 γ1	12.9			
				17.4 γ2				
K	176.6	56.2	33.1	24.7	29.0	41.9		
L	177.6	55.1	42.4	26.9	24.1			
M	176.3	55.4	32.9	32.0		16.9		
N	175.2	53.1	38.9					
P	177 3	63.3	32.1	27.2	49.8			
Q	176.0	55.7	29.4	33.7				
R	176.3	56.0	30.9	27.1	43.3			
S	174.6	58.3	63.8					
T	174.7	61.8	69.8	21.5				
V	176.3	62.2	32.9	20.7				
W	175.9	57.5	29.6		127.4δ1	122.2ε3	114.7 ζ2	121.0 η2
							124.8 ζ3	
Y	175.9	57.9	38.8		133.3	118.2		

Wishart *et al.* (1995) J. Biomolecular NMR 5(1), 67-81

Bax's lab at the NIH brought these two themes (resolution by 3D experiments and efficiency of direct scalar coupling transfers via $^{13}C/^{15}N$) together in 1990: Ikura et al, Biochemistry 29, 4659. These "triple resonance" ($^{1}H^{13}C^{15}N$) methods have become *the way* that proteins larger than 10-15 kD are studied by NMR. We won't go through their assignment strategy in detail, because the preferred set of triple resonance experiments has changed a bit as new experiments have been developed.

The current set of triple resonance experiments that are used for sequence specific backbone assignments are the HNCA, HN(CO)CA, HNCACB, HN(CO)CACB, HNCO and HN(CA)CO.



The naming of the experiments describes the magnetization transfer, with the chemical shifts of the nuclei in paranthesis not being mapped.

The experiments are run in pairs with one experiment giving rise to correlations to both the residue itself (residue i) and the previous residue (residue i-1) and its partner experiment giving only the inter-residue (i-1) correlation.

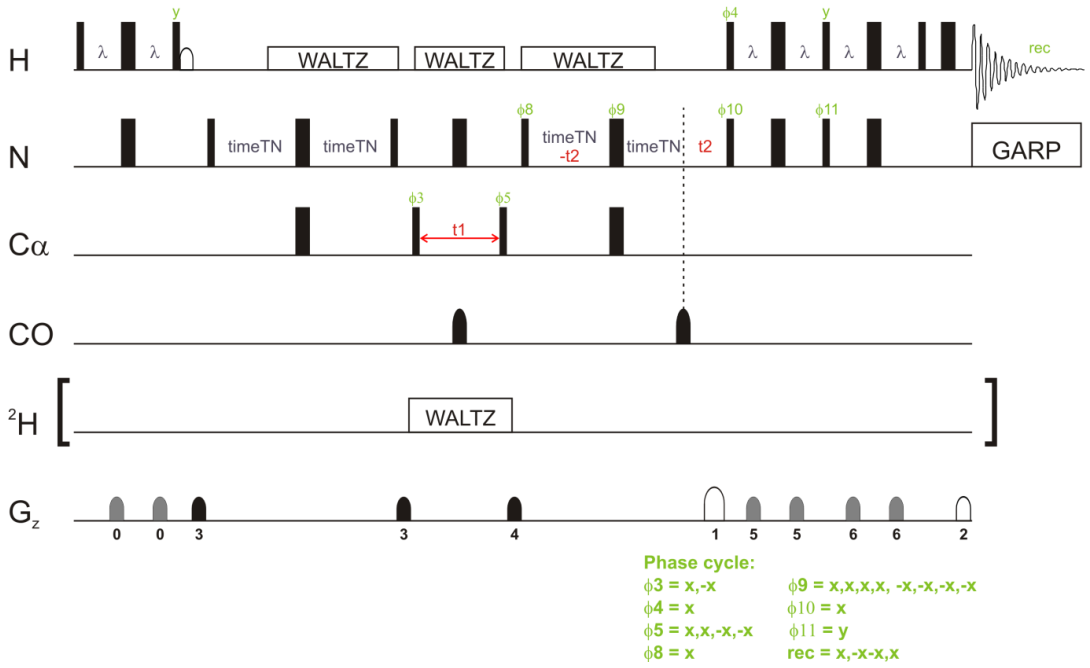
Any experiment that contains the magnetization transfer step N → Ca gives rise to the two sets of correlations because of the similar size of the intra-residue coupling, $^{1}J_{NCA}$=11Hz, and the inter-residue coupling, $^{2}J_{NCA}$=7Hz.

The correlations that you see for a given NH (*residue i*) in each of the spectra are:

HNCA	$C\alpha_i$	$C\alpha_{i-1}$		
HN(CO)CA		$C\alpha_{i-1}$		
HNCACB	$C\alpha_i$	$C\alpha_{i-1}$	$C\beta_i$	$C\beta_{i-1}$
HN(CO)CACB		$C\alpha_{i-1}$		$C\beta_{i-1}$
HN(CA)CO	CO_i	CO_{i-1}		
HNCO		CO_{i-1}		

HNCA

Let us consider one of the triple resonance experiments, the HNCA, in more detail. This is an out-and-back triple resonance experiment, where the magnetization starts on the H^N is transferred to N and then Cα and then back to N and then to H^N for acquisition. The chemical shifts for the N and Cα nuclei are both mapped to make this a 3D experiment with one dimension being the H^N, one the N and the other Cα.



The experiment is built on INEPT transfers with the delays set to:

$\lambda = 1/(4x{}^1J_{HN}) = 2.5$ms for the INEPT and reverse INEPT transfers between ^{1}H and ^{15}N

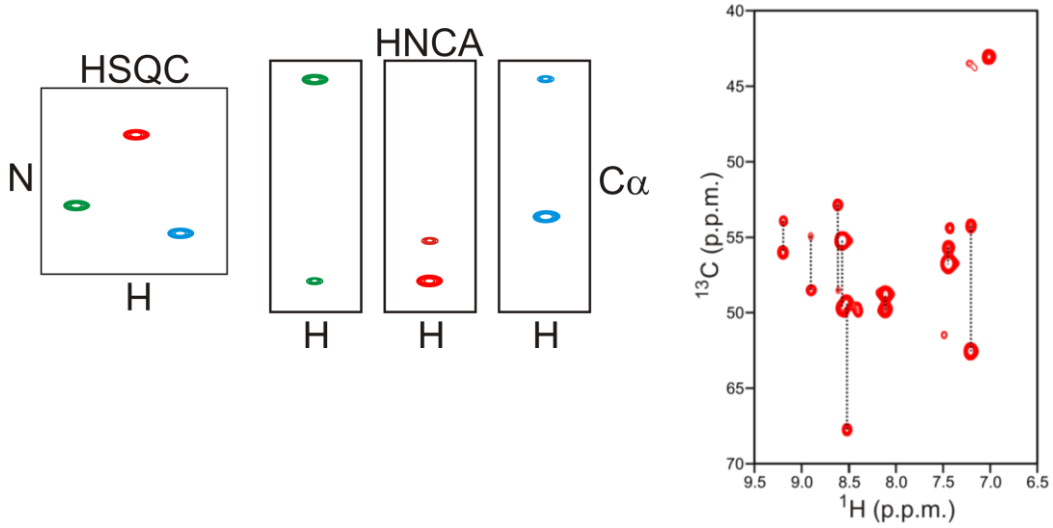
timeTN = $1/(4x{}^1J_{NC\alpha}) = 12$ms to develop antiphase ^{15}N,^{13}C magnetization ($N_y \rightarrow N_xC_z$)

1. INEPT transfer $^1H \rightarrow {}^{15}N$, giving $2H_zN_y$
2. INEPT transfer $^{15}N \rightarrow {}^{13}C\alpha$ that also incorporates refocusing of the ^{15}N, ^{1}H coupling. So at the end of the two timeTN delays we have N_yC_z antiphase magnetization.
3. $\underline{90N_x, 90C_x} \rightarrow N_zC_y$
4. **t_1** period to evolve Cα chemical shift, decoupling ^{1}Hα (WALTZ decoupling sequence), ^{15}N and ^{13}CO with 180° pulses, giving $N_zC_y cos(\omega_C t_1)$

Now, back to H^N:

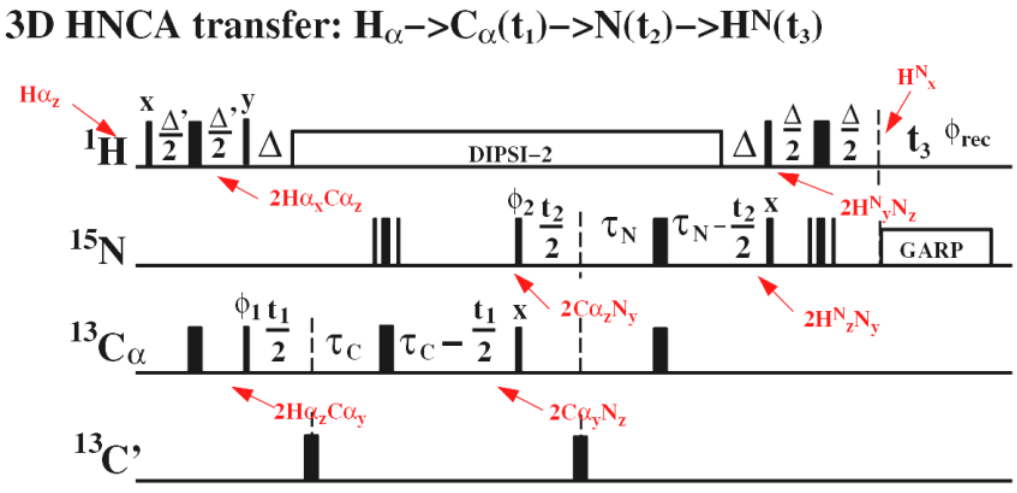
5. $\underline{90N_x, 90C_x} \rightarrow N_xC_z cos(\omega_C t_1)$
6. Reverse INEPT transfer for N → CA, giving $N_y cos(\omega_C t_1)$
7. t_2 evolution period on ^{15}N including gradient encoding, Gz1(N), $N_y cos(\omega_C t_1) cos(\omega_N t_2)$
8. Evolution of ^1H,^{15}N coupling, $H_zN_y cos(\omega_C t_1) cos(\omega_N t_2)$
9. Sensitivity enhanced reverse INEPT transfer $^{15}N \rightarrow {}^1H$, $H_y cos(\omega_C t_1) cos(\omega_N t_2)$ including the decoding gradient, Gz2(H)
10. Detection, t_3, modulated by $cos(\omega_C t_1)$ and $cos(\omega_N t_2)$

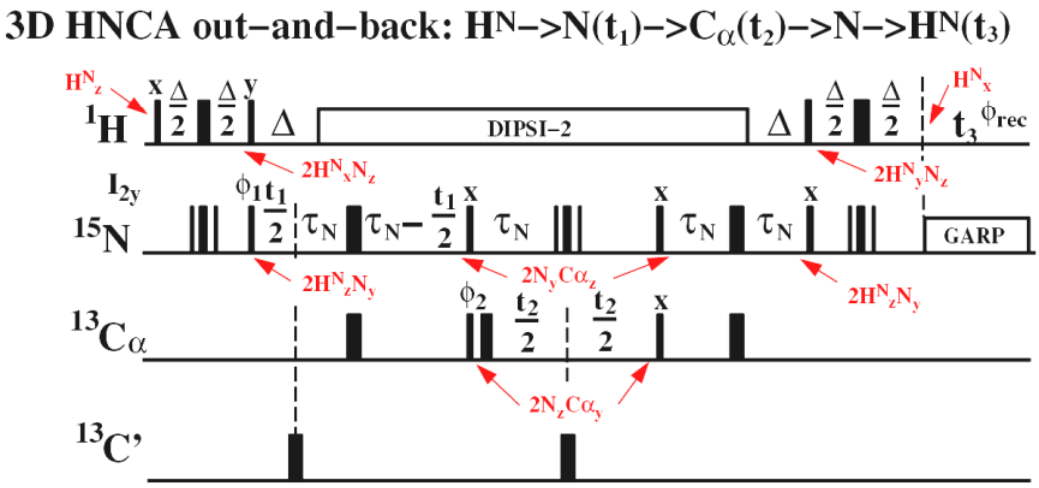
This experiment correlates the $^1H^N$, ^{15}N, and $^{13}C\alpha$ resonances within each residue, giving one intra-residue cross-peak for each non-proline and a weaker correlation for the inter-residue, $^{13}C\alpha_{i-1}$.



2D ^1H,^{13}C plane from the 3D HNCA experiment for Ubiquitin

The HNCA experiment described above is an example of an out-and-back experiment. The magnetization starts on the HN and goes out to the Ca and then returns back to the HN for acquisition. It is also possible to run an equivalent experiment that starts on the Ha spins and transfers straight-through to the HN, HACANNH. This experiment can be run to map the CA(t1), N(t2) and HN(t3) chemical shifts.



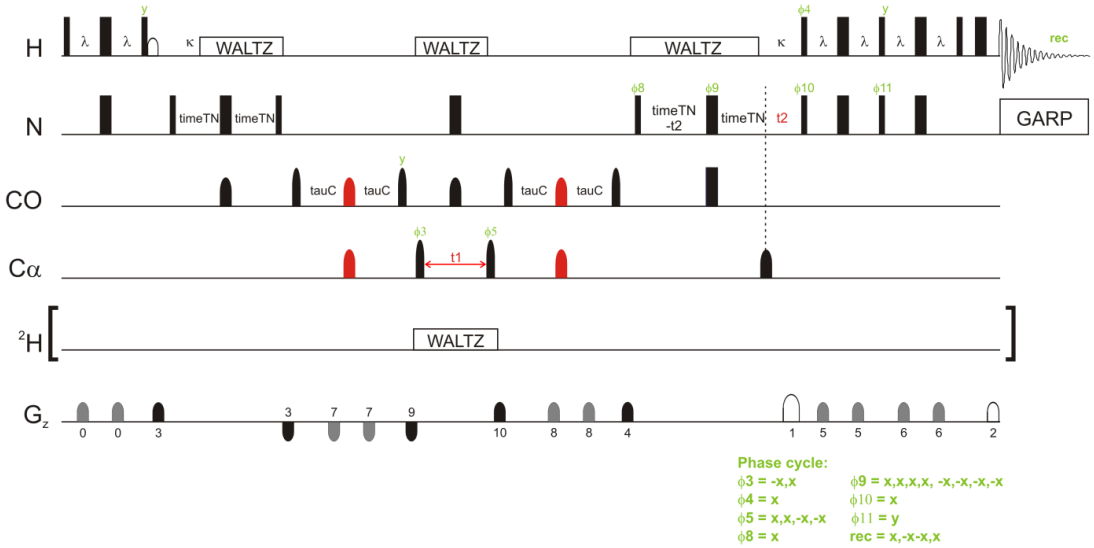


Comparison of the two experiment shows that the straight-through experiment contains fewer pulses and the overall length of the experiments is approximately the same:
HACANNH experiment is $\Delta' + 2\tau C + 2\tau N + \Delta$
HNCA experiment is $\Delta + 2\tau N + 2\tau N + \Delta$
and the transfer efficiency is identical to the out-and-back experiment. So why does no-one ever use this experiment?

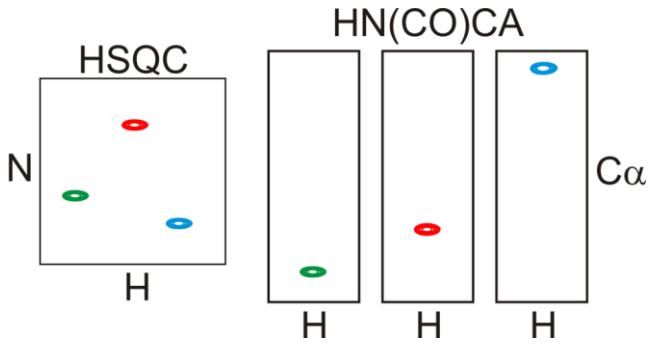
Comparing what nuclei are active during the sequences gives the answer. During the first part of the HACANNH experiment the magnetization is along HA(Δ') and CA($2\tau C$), whilst in the HNCA experiment the magnetization is along HN(Δ) and N($2\tau N$). The HA and CA nuclei have significantly shorter T2 relaxation times compared to the HN and N nuclei. This means that once relaxation effects are taken into account the HNCA version of the experiment is almost 4 times more sensitive than the HACANNH version.

HN(CO)CA

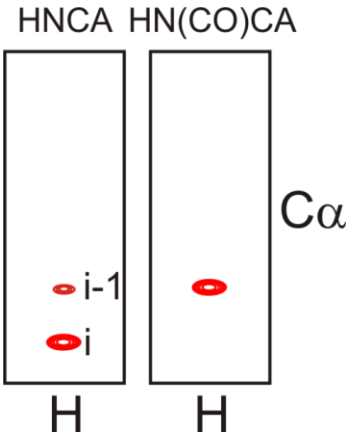
The partner experiment to the HNCA is the HN(CO)CA:



The magnetization transfer is H → N → CO → Ca (t_1) → CO → N (t_2) → H (t_3) all using INEPT/Reverse INEPT steps. This correlates $^{1}H_i$, $^{15}N_i$ and $^{13}C\alpha_{i-1}$

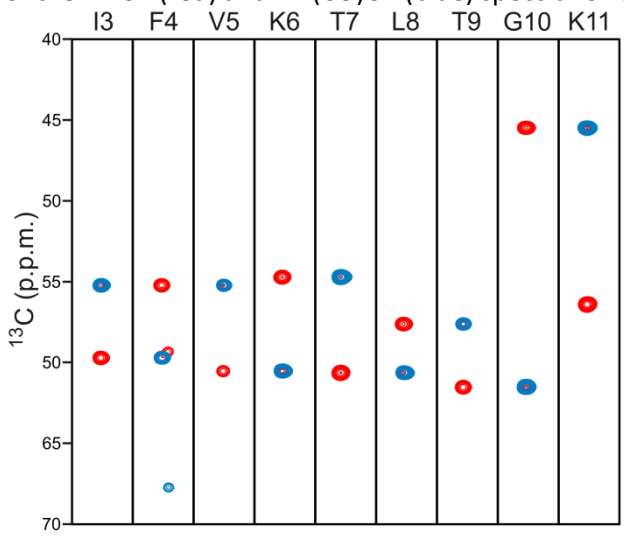


Comparison of the HNCA and HN(CO)CA spectra allows easy identification of the $C\alpha_i$ and $C\alpha_{i-1}$ correlations:



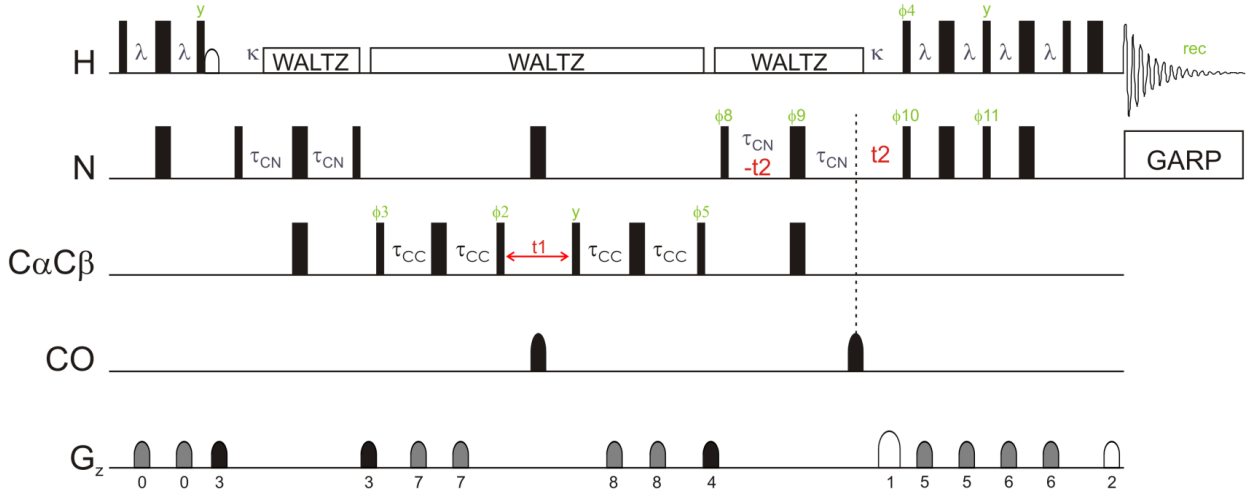
The preceding residue can be found by looking for a residue that has a $C\alpha_i$ correlation at the chemical shift of $C\alpha_{i-1}$ correlation of this residue

Example of the HNCA (red) and HN(CO)CA (blue) spectra for Ubiquitin



HNCACB/CBCANH

The HNCAB, or the one-way version, CBCANH (both are equally efficient unlike in the HNCA case), correlate the amide proton and nitrogen to the CA and CB for both the i and i-1 residues.
The HNCACB pulse sequences is shown below and is again a series of INEPT and Reverse-INEPT transfers starting on the amide proton and transferring magnetization in a stepwise fashion to N then CA then CB.
H → N → CA → CA/CB (**t_1**) → CA → N (**t_2**) → H (**t_3**)

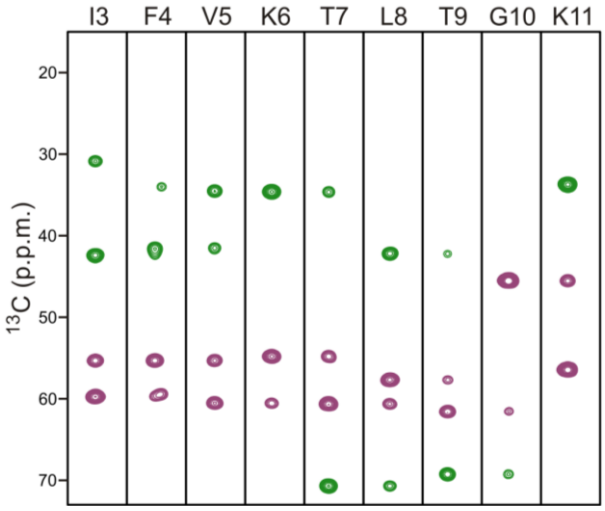


For the CBCANH experiment the magnetization transfer is:
HA/HB → CB/CA (**t_1**) → CA → N (**t_2**) → H (**t_3**)

The HNCACB (not CBCANH) experiment can be run in two different ways depending upon the length of the delay τCC. For τCC=3.4ms both Cα and Cβ correlations are observed, experiment is HNCACB. For τCC=6.8ms, all the magnetization is transferred to the Cβ nuclei so only correlations to Cβ are observed, experiment is HN(CA)CB. If neglecting relaxation losses, the HN(CA)CB experiment would be more

sensitive for the Cβ correlations, however, because of the increased length of the sequence and because the magnetization at that point is on the Cα nuclei, there is significant relaxation losses and the signal-to-noise is decreased. The HN(CA)CB version of experiment is often used only for either very small proteins (but then not often as the signal-to-noise is generally high even for the HNCACB version) or for larger proteins that are deuterated, where the relaxation losses of the Cα nuclei are reduced due to the attached deuteron versus proton (see class on larger proteins).

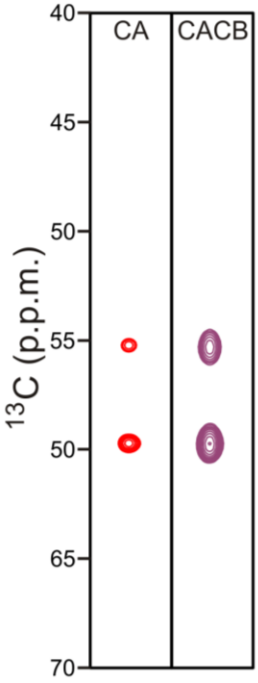
One useful feature of the HNCACB experiment is that the correlations for the CA and CB nuclei are of opposite phase. In the panel below is shown the amide strips from the 3D HNCACB experiment on Ubiquitin. The CA resonances are in purple whilst the CB resonances are in green. The opposite phase can be very helpful in quickly determining the Ser and Thr residues as they (usually) have CB resonance downfield of the CA residues.



So if the HNCACB contains both the CA and CB correlations why do we run the HNCA experiment?

The reason is two-fold:

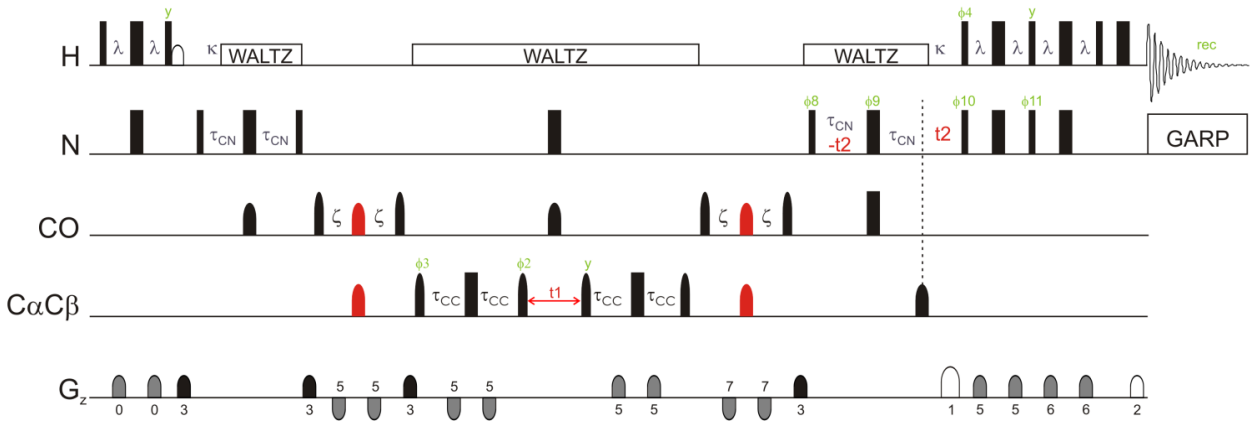
1) First the CA correlations in the HNCA experiment are about 10 times the signal-to-noise compared to those in the HNCACB experiment.
2) The resolution in the HNCA experiment is higher. In the HNCA experiment the carbon region that is acquired is from 65-40ppm, whilst in the HNCAB experiment the region is 75-10ppm. This means that for the same number of increments in the indirect dimension the HNCACB has a much lower resolution for the CA correlations. To obtain a similar resolution for the HNCACB as HNCA would increase the experimental time by up to 3 times, which in the same amount of time you can run the HNCA, HN(CO)CA and 'normal resolution' HBCACB.



HN(CO)CACB/CBCA(CO)NH

The HN(CO)CACB or CBCA(CO)NH experiments are the companion experiments to the HNCACB/CBCANH experiments and are used to identify the Cαi-1 and Cβi-1 correlations.

Like all the triple resonance experiments the magnetization is transferred via INEPT/Reverse INEPT steps and the pulse sequence for the HN(CO)CACB experiment is shown.



The magnetization transfer is:

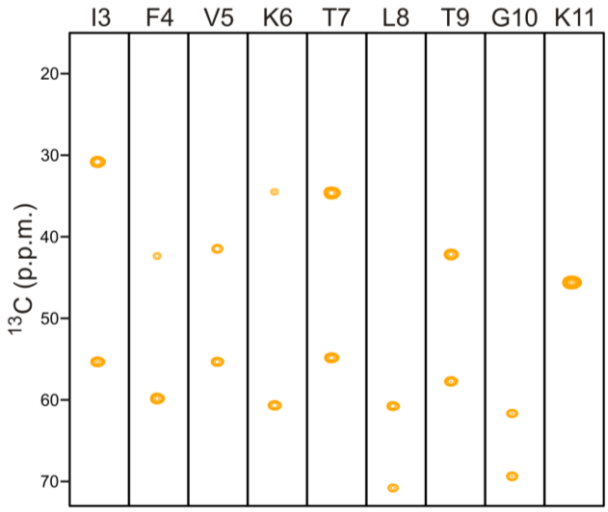
HN(CO)CACB: H → N → CO → CA → CA/CB (t_1) → CA → CO → N (t_2) → H (t_3)

CBCA(CO)NH: HA/HB → CB/CA (t_1) → CA → CO → N (t_2) → H (t_3)

Like the HNCACB experiment, the HN(CO)CACB experiment can be run to select either both the CA/CB or only the CB correlations, by adjustment of the τCC delay.

In general, the CBCA(CO)NH experiment is the preferred version for smaller protonated proteins, whilst for larger, deuterated proteins the HN(COCA)CB experiment is preferred.

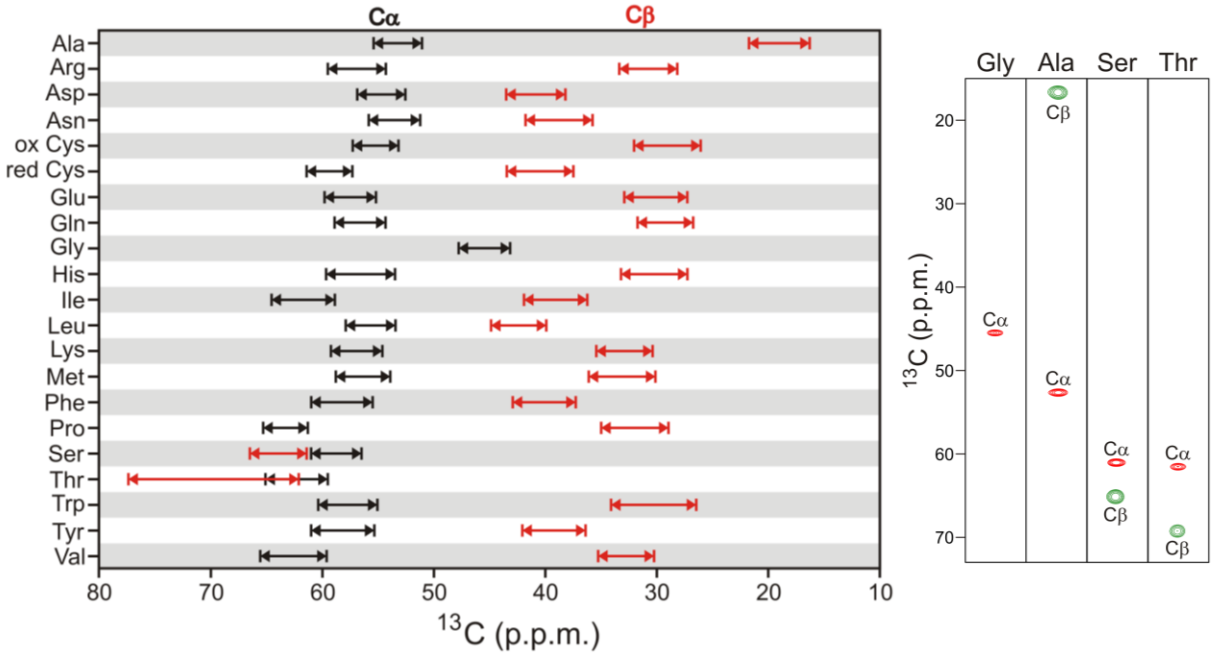
Note that the CBCA(CO)NH experiment cannot be used for deterated proteins because the magnetization starts on the HA/HB nuclei, but these are not present in deuterated proteins.



Strip plot from the 3D CBCA(CO)NH experiment for Ubiquitin

Starting points in the sequential assignment

The range of chemical shifts for Cα and Cβ resonances for each amino acid is shown below. Whilst the Cα resonances alone cannot be used determine what the amino acid is, the combination of both the Cα and Cβ resonances can limit the possibilities, or in some cases be type-specific.



The resonances cluster into a number of classes:

Cβ >60ppm – Ser/Thr

> 35ppm – Asp/Asn/Cys(reduced)/Ile/Leu/Phe/Tyr

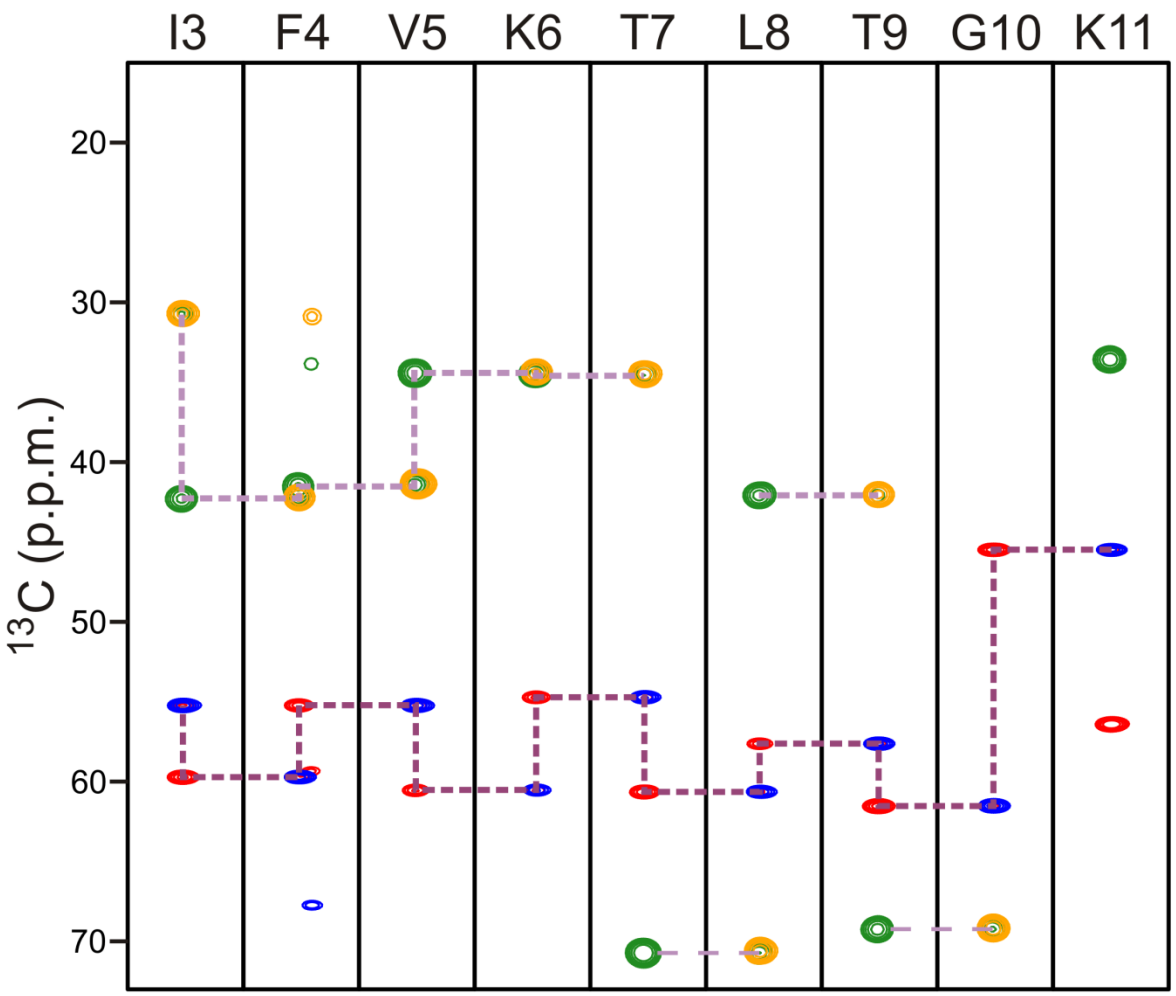
< 35ppm, Cα < 60ppm – Arg/Cys(oxidized)/Glu/Gln/His/Lys/Met/Trp

< 35ppm, Cα > 60ppm – Pro/Val

< 22ppm - Ala

So the amino acids Ala, Gly, Ser and Thr have unique Cα/Cβ chemical shifts and can be used as starting points in sequential assignment strategy.

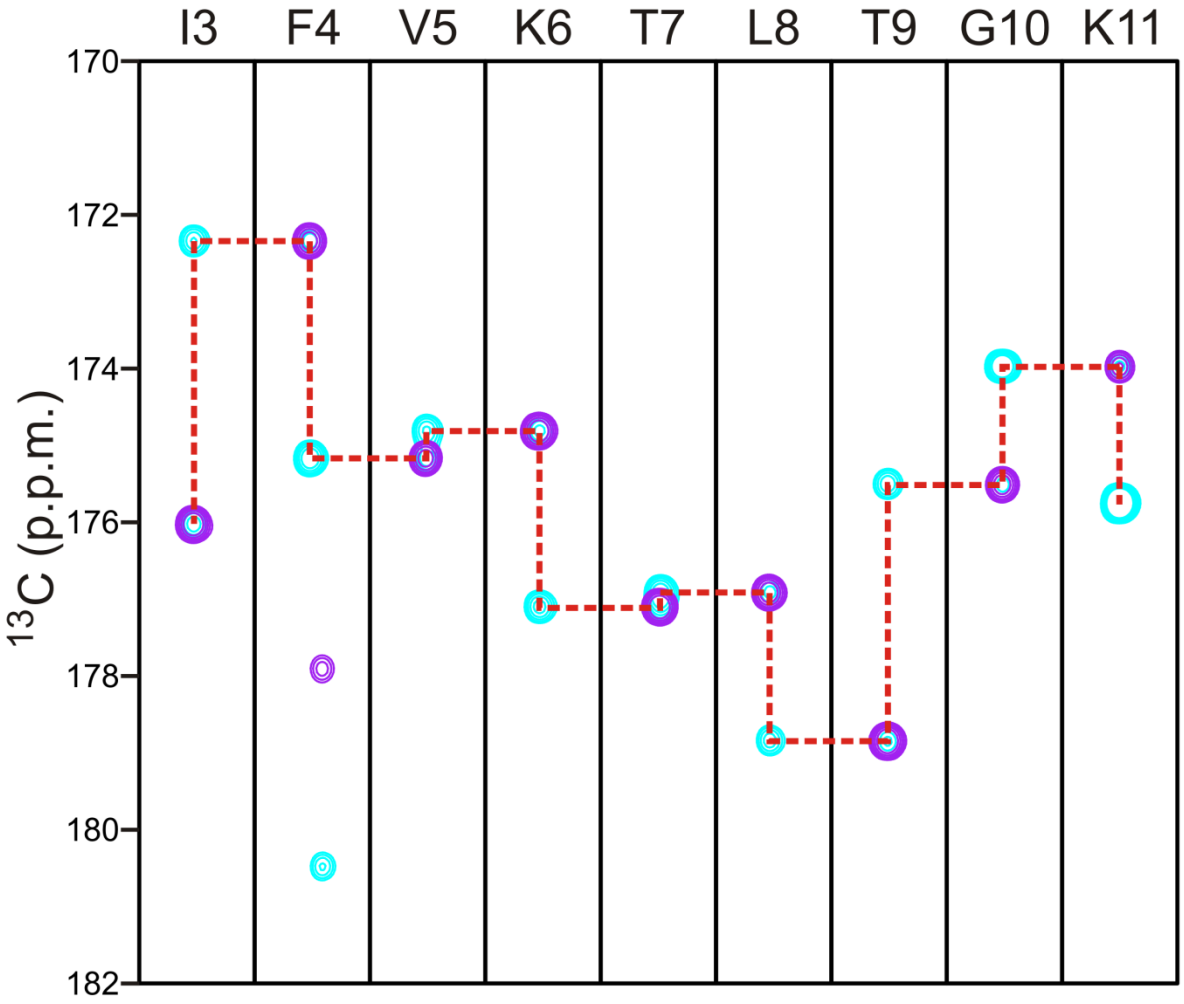
Example of the backbone walk for residues Ile-3 to Lys-11 for Ubiquitin. The correlations are HNCA(red), HN(CO)CA (blue), HN(CA)CB (green) and HN(COCA)CB (orange). Lines marking the sequential assignments for the Cα (light green) and Cβ (purple)



HNCO/HN(CA)CO

The final pair of triple resonance experiments are the HNCO and HN(CA)CO, which correlate the amide proton and nitrogen to the carbonyl carbon of residues i and i-1.
The pulse sequences are identical to the HNCA (HNCO) and HN(CO)CA (HN(CA)CO), except that the pulses on the CA and CO nuclei are reversed.

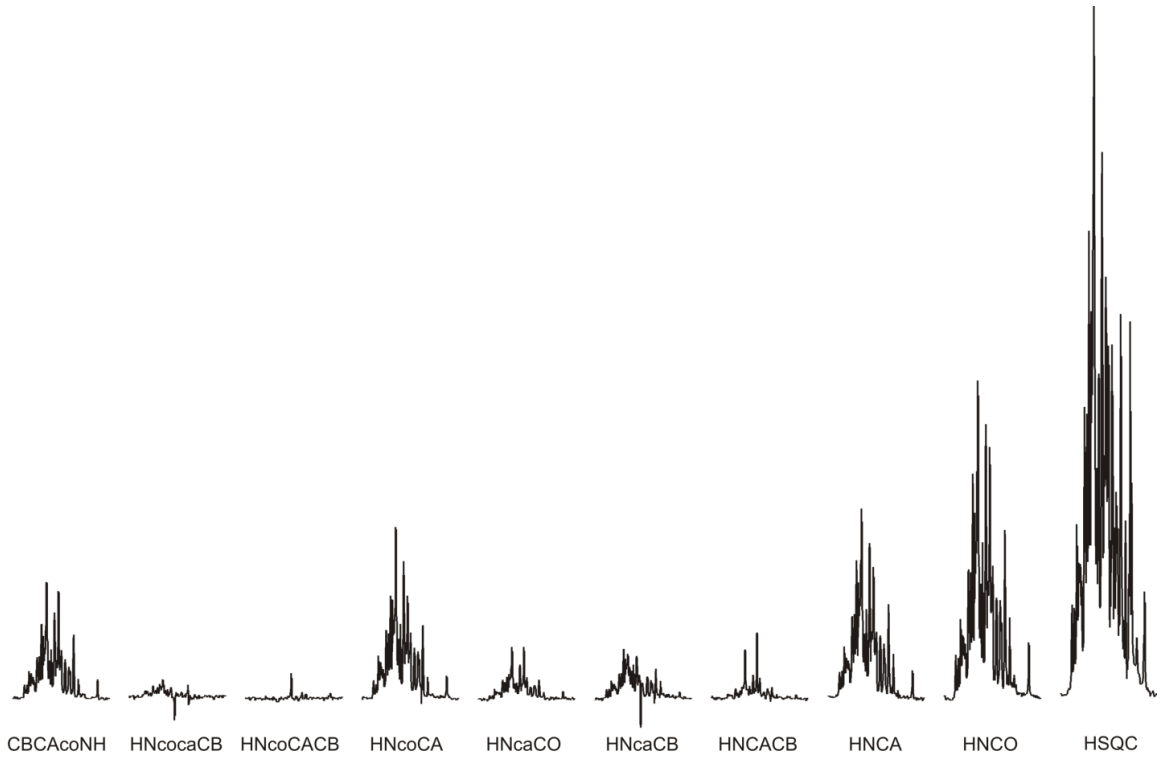
Example of the backbone walk for the CO correlations; HN(CA)CO (cyan) and HNCO (purple) with the sequential correlation marked by the red line.



So what is the sensitivity of the experiments?

Experiment	Assignment	Comment	Relative S/N (%)
HNCO	H_i N_i CO_{i-1}	<20kD, above use ^2H labeling	100
HNCA	H_i N_i $C\alpha_i$ $C\alpha_{i-1}$	<20kD, above use ^2H labeling	50/15
HNcoCA	H_i N_i $C\alpha_{i-1}$	<20kD, above use ^2H labeling	71
HNcaCO	H_i N_i CO_i CO_{i-1}	<20kD, above use ^2H labeling	13/4
CBCAcoNH/HNcoCACB	H_i N_i $C\alpha_{i-1}$ $C\beta_{i-1}$	<20kD, above use ^2H labeling	13/9 α_{i-1}/β_{i-1}
CBCANH/HNCACB	H_i N_i $C\alpha_i$ $C\beta_i$ $C\alpha_{i-1}$ $C\beta_{i-1}$	<15kD, above use ^2H labeling	4/1.7 α_i/β_i 1.3/0.5 α_{i-1}/β_{i-1}
hCCcoNH-TOCSY	H_i N_i C^{aliph}_{i-1}	<15-20kD, above use ^2H labeling	
HcccoNH-TOCsY	H_i N_i H^{aliph}_{i-1}	<15-20kD, above use fractional ^2H labeling	
HCCH-TOCSY	H^{aliph} C^{aliph}	<25kD, sensitive but tedious to analyse, combine with HCCONH type experiments	

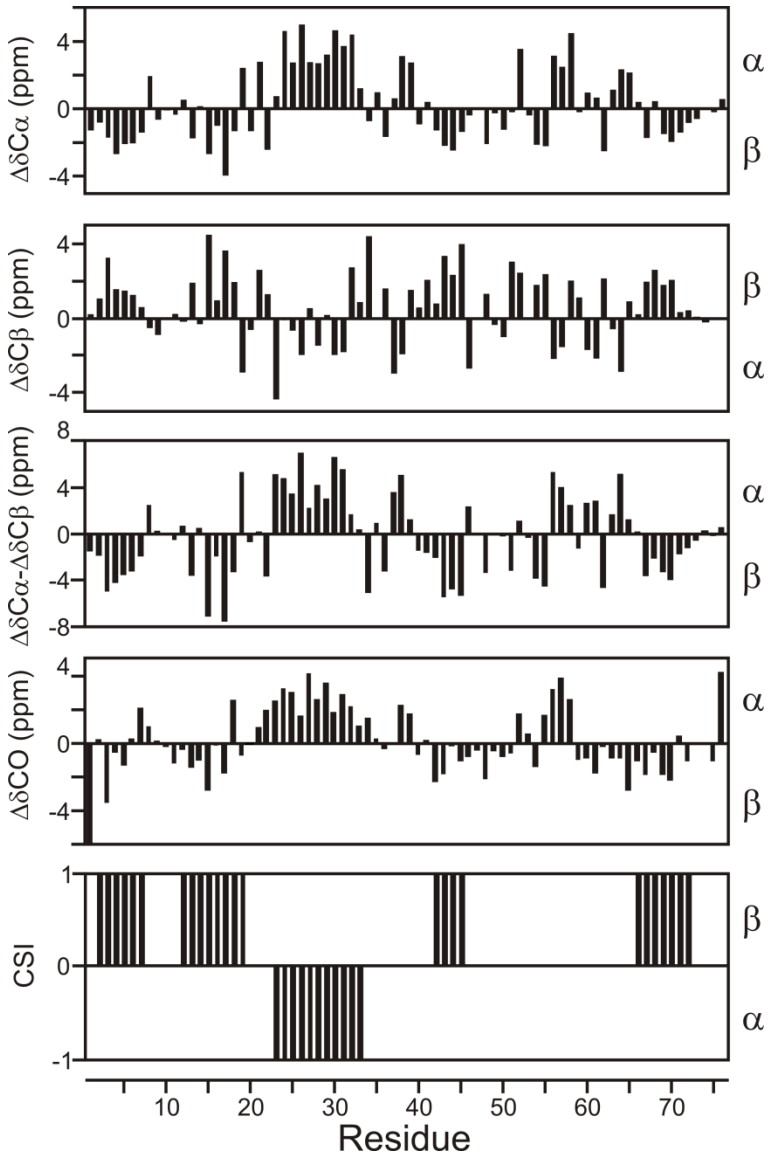
1D versions of common triple resonance experiments showing the relative sensitivity of the experiments:



Note that in the HNCACB and HN(CO)CACB the Cα and Cβ correlations are of opposite sign, so that in the 1D versions of these experiments the signal cancel resulting in little signal.

Using ^{13}C chemical shifts to predict secondary structure

The Cα, Cβ and CO chemical shifts relative to random coil shifts have a clear correlation with the polypeptide torsion backbone angles ϕ and ψ (Spera and Bax, *J. Am. Chem. Soc.* **1991**, *113*, 5490-5492; Wishart *et al.*, *J. Mol. Biol.* **1991**, *222*, 311-333). For example, for the Cβ resonance a downfield shift from the random coil position is observed for extended (β-) structure, with ψ ~ 130°, whereas for helical structures a small upfield shift is observed (ψ ~ -50°). On this basis the local secondary structure can be predicted using the Cα, Cβ and CO chemical shifts by either plotting the secondary shifts (Δδ = observed shift – random coil shift) or by using the chemical shift index (CSI) program (Wishart and Sykes, *J. Bio. NMR* **1994**, *4*, 171-180), which gives a consensus value using all of the secondary shifts from the Cα, Cβ and CO nuclei and has an output of +1 for beta-strand, 0 for random coil and -1 for α-helix.



Secondary Structure prediction for Ubiquitin