

Triple Resonance NMR Experiments for Proteins

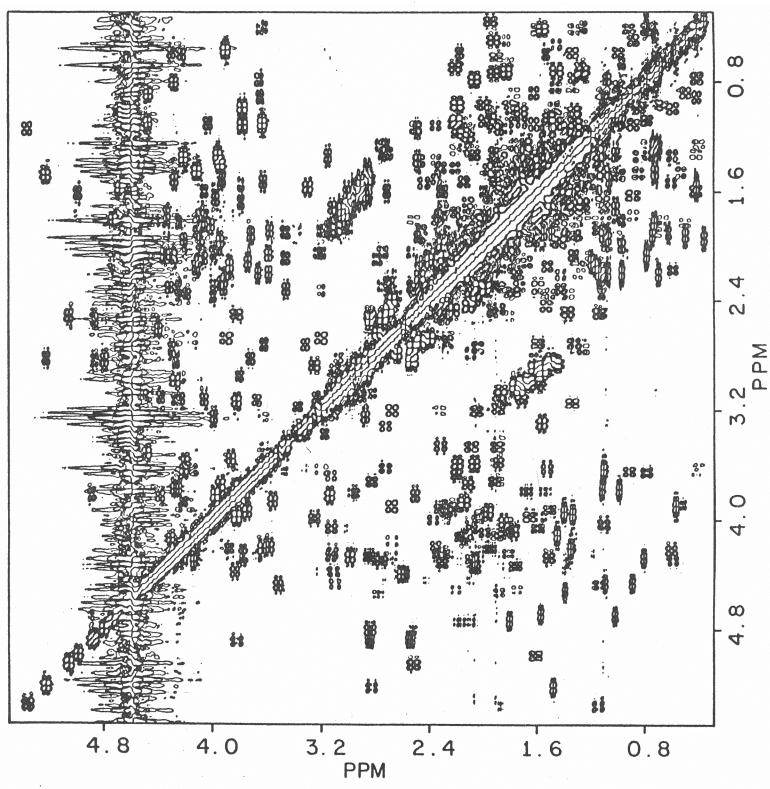
BCMB/CHEM 8190

Limitations of Homonuclear (^1H) Experiments for Proteins

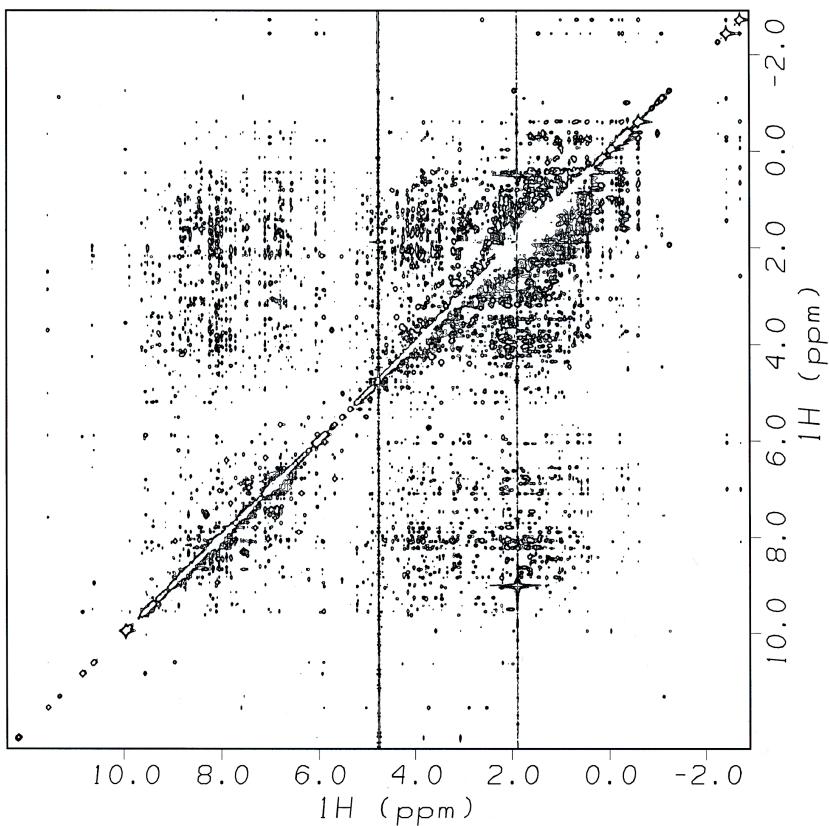
- The utility of homonuclear methods drops quickly with mass (~10 kDa)
 - severe spectral degeneracy
 - decreased magnetization transfer efficiency via the small $^3J(^1\text{H}-^1\text{H})$ couplings

cytochrome c, 12.5 kDa

DQF COSY

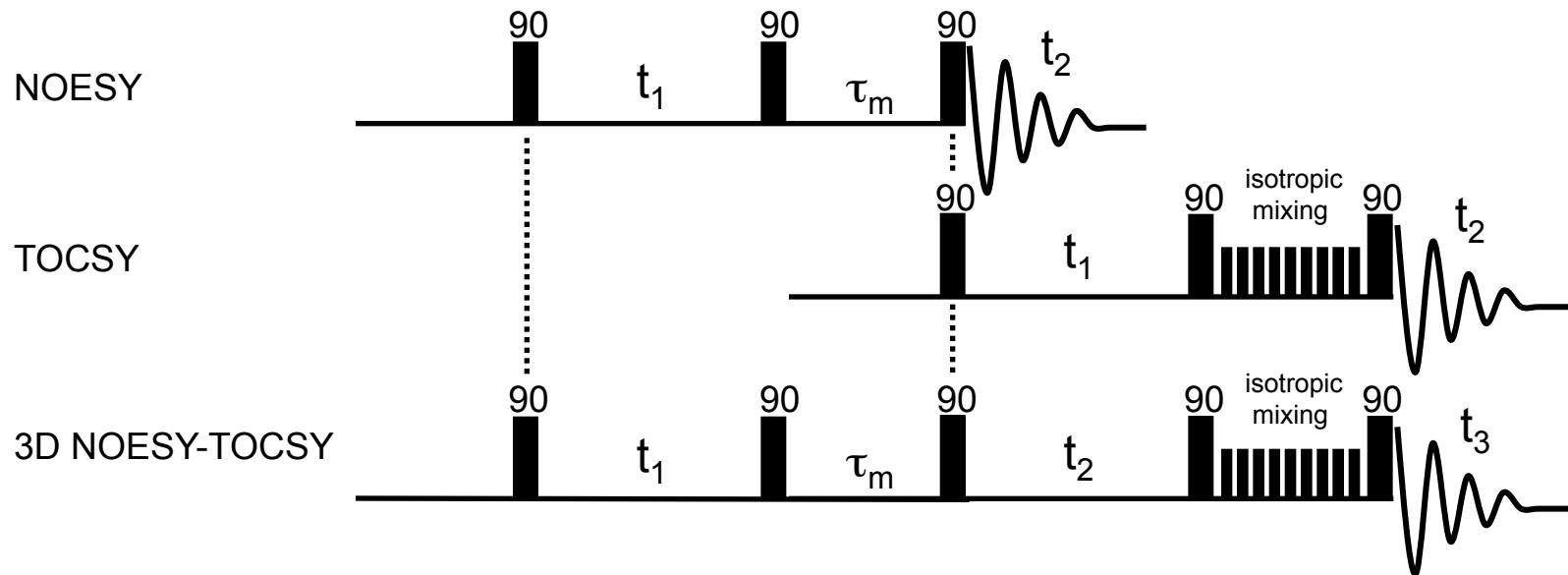


NOESY

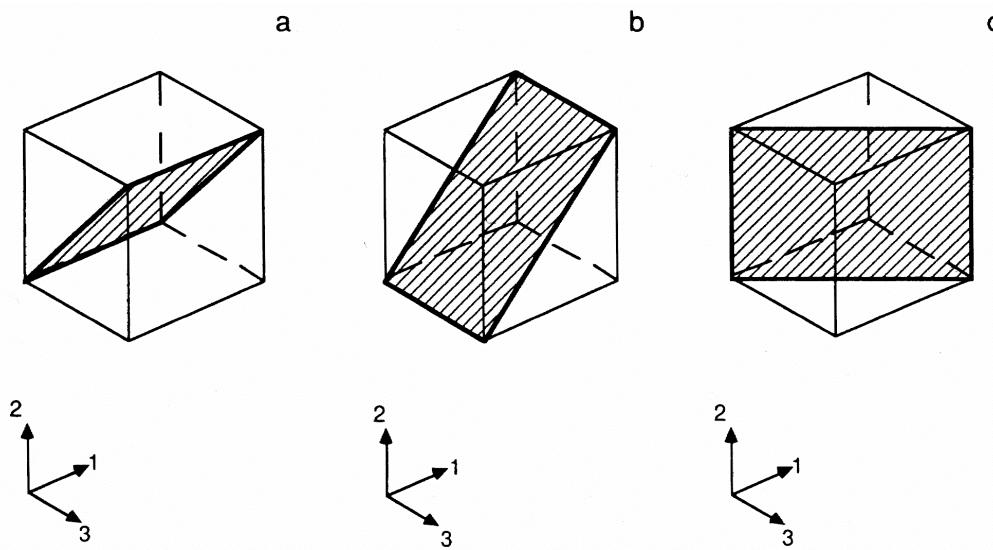


Three-Dimensional Homonuclear Experiments

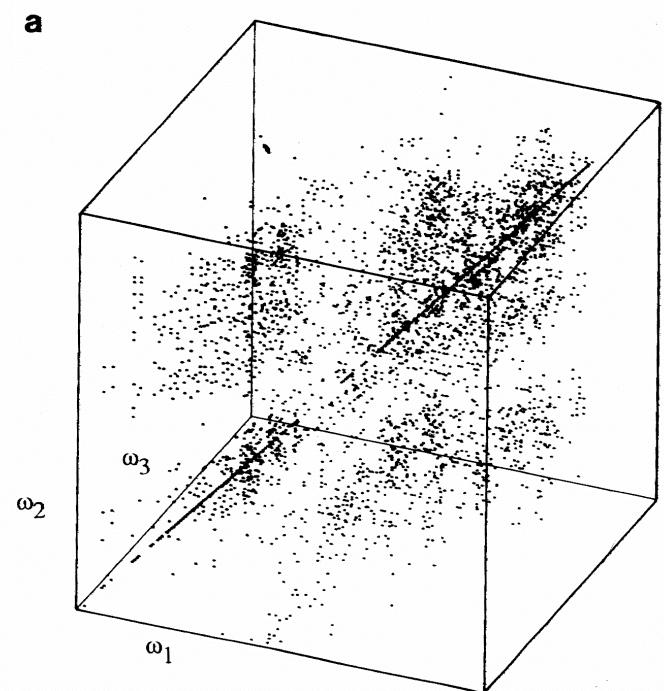
- ^1H 2D experiments can be combined to create 3D experiments
 - one experiment becomes preparation period of a second experiment
 - increased dimensionality can increase resolution and reduce spectral overlap....
 -however, these methods are mostly non-selective and the numbers of signals (peaks) in the resulting spectra are very large
 - the resulting spectra can be very informative but can be difficult to analyze



Three-Dimensional Homonuclear Experiments

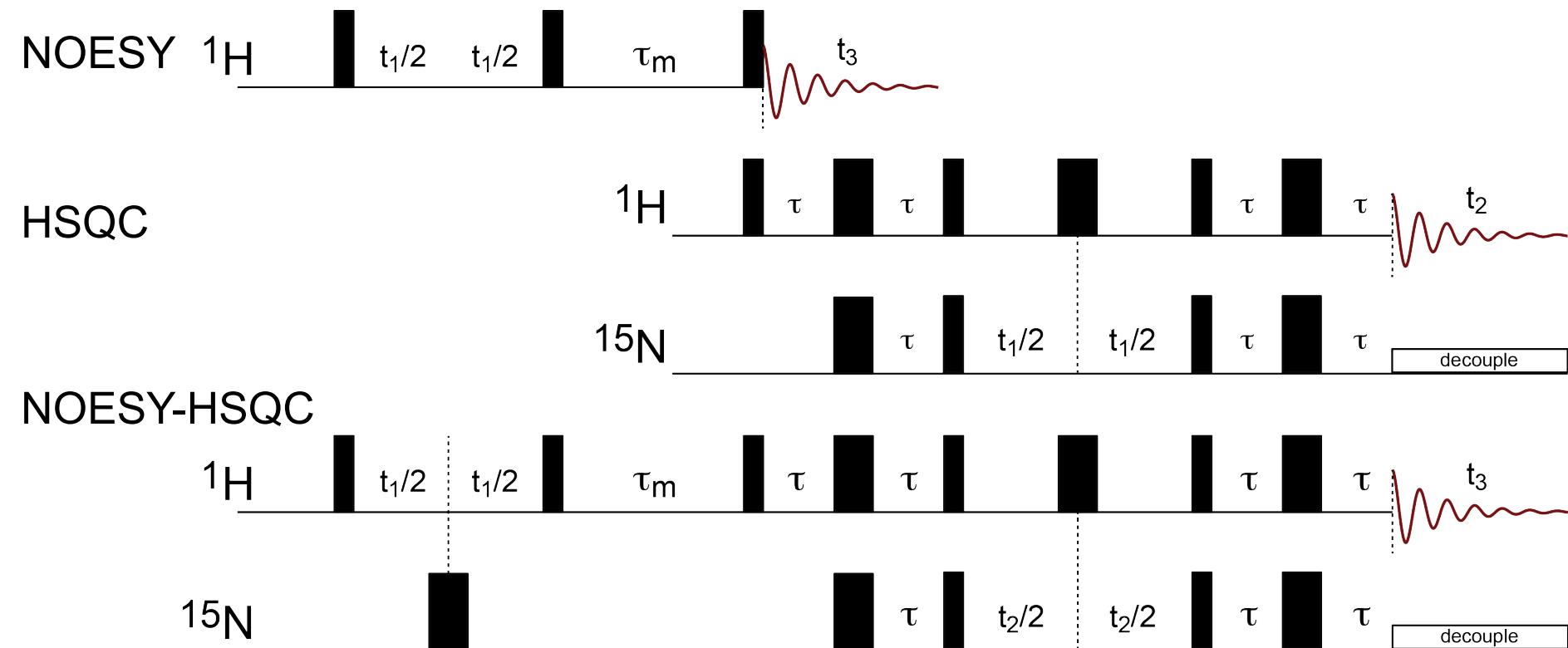


- Example: 3D NOESY-TOCSY of parvalbumin (108 amino acids)
 - 8.7 mM
 - 170 hours (~ 7 days)
 - 50,000 cross peaks !

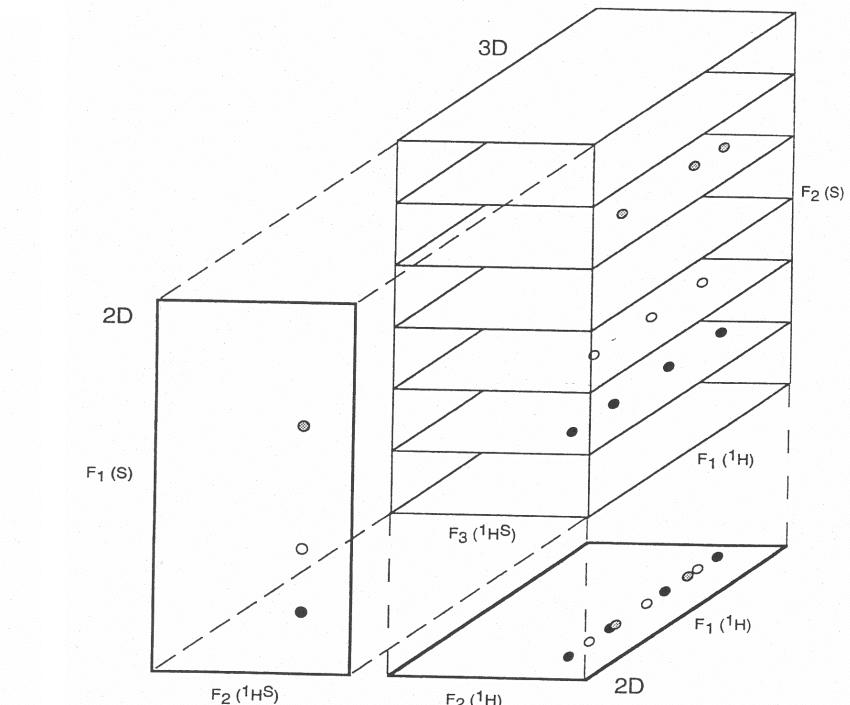
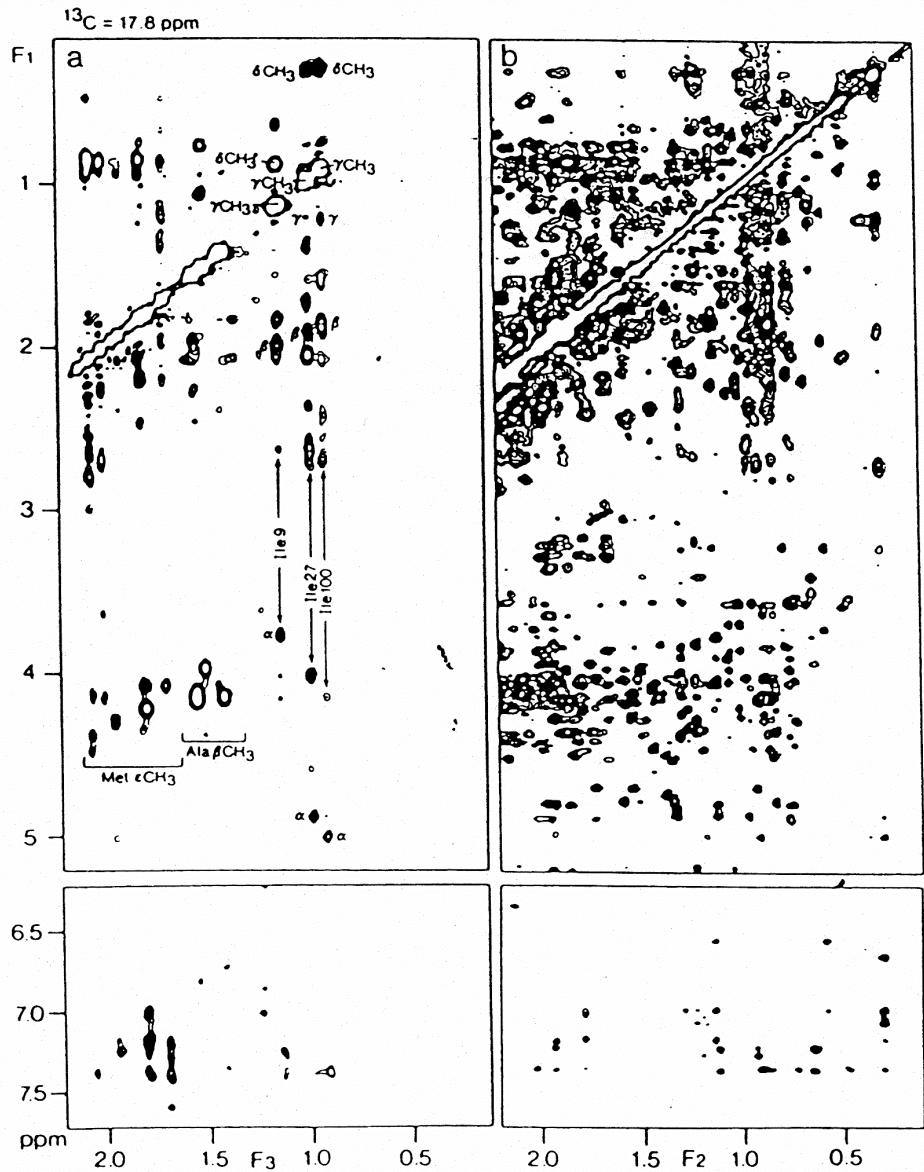


Heteronuclear Resolved Homonuclear Experiments

- pulse sequences: same idea (combine 2D sequences)
- but *selective*: get increased dimensionality and increased resolution *without* an increase in the number of signals (peaks)



Heteronuclear Resolved Homonuclear Experiments



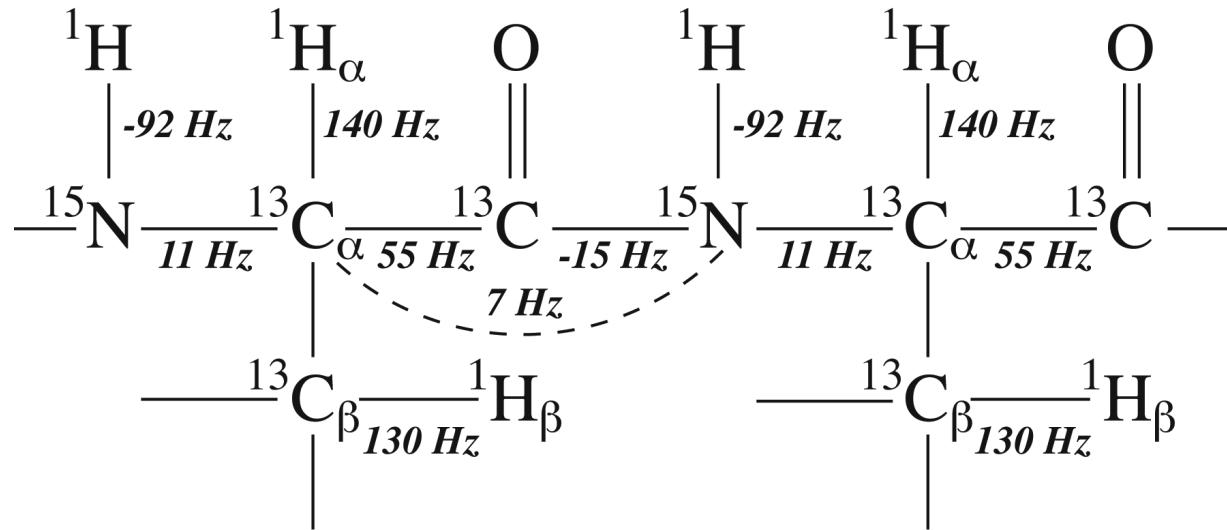
Left: 2D NOESY

Far left: 2D plane of 3D NOESY-HMQC (^1H , ^{13}C)
 ^1H signals resolved by ^{13}C chemical shifts of bound ^{13}C atoms

Triple Resonance Approach

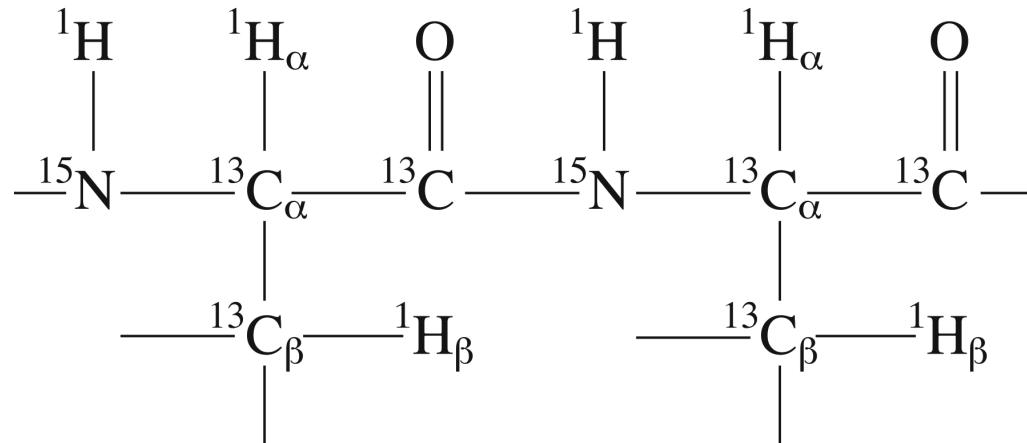
- based on magnetization transfer via (mostly) one bond J couplings
 - most of these couplings are large compared to linewidths for moderate sized proteins (~ 20 kDa)
 - magnetization transfer is efficient
 - indirect (^1H) detection (higher sensitivity)
- applicable to uniformly isotopically enriched proteins
 - uniform ^{13}C and ^{15}N labeling: spin 1/2
- provides *selective* chemical shift correlation
 - spectral degeneracy minimized

1J and 2J Coupling Constants in Polypeptides



- These 1J and 2J couplings are *uniform* throughout polypeptides/proteins
 - these 1J and 2J couplings are virtually *independent of conformation and amino acid type*
 - thus, the signal sensitivity is reliably uniform for all residues in a protein

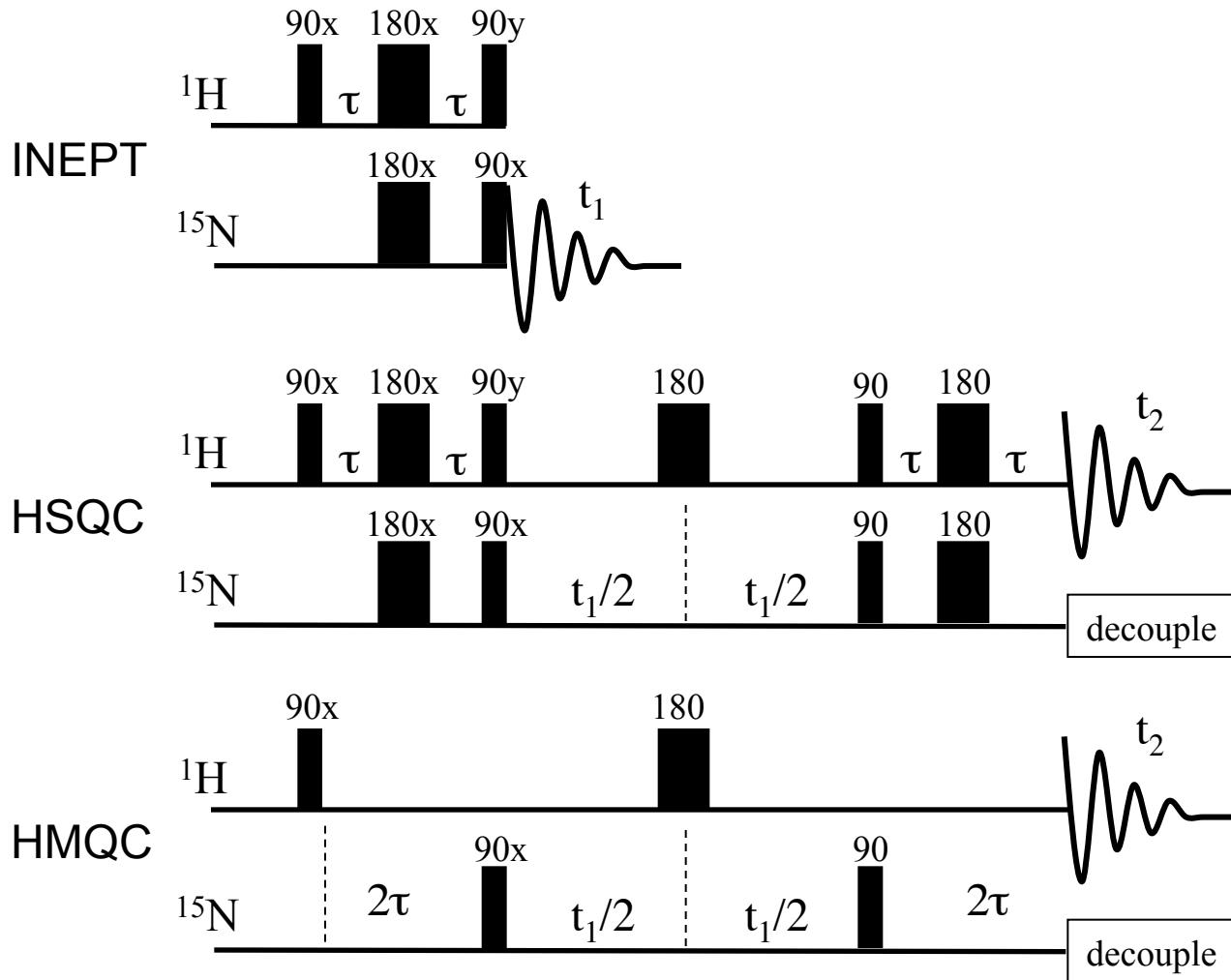
Uniform isotopic labeling of proteins



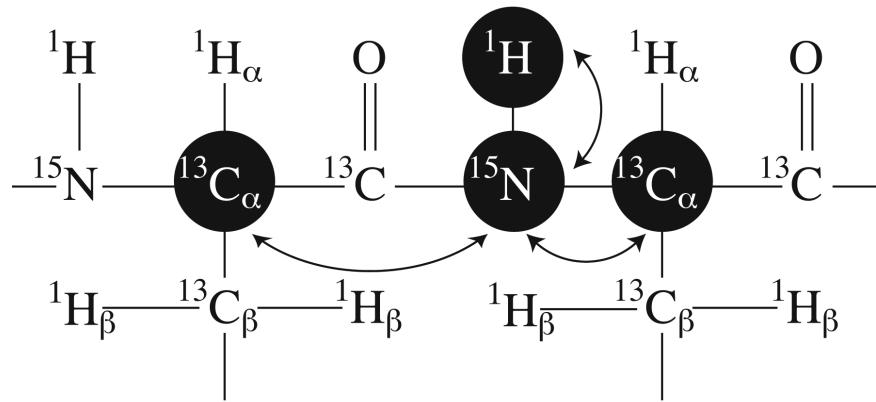
- Proteins can be uniformly isotopically labeled by recombinant expression using defined media
 - bacterial expression most common
 - also yeast, and cell-free systems are being developed
 - minimal media using $^{13}\text{C}_6$ glucose as the sole carbon source and $^{15}\text{NH}_4\text{Cl}$ (or $-\text{SO}_4$) as the sole nitrogen source
 - normally $\geq 98\%$ atom excess
 - also labeled “rich” media (\$\$)
 - for larger proteins, uniform or fractional ^2H labeling also used
 - ^2H , ^{13}C glucose and D_2O

Building Blocks for Triple Resonance Experiments

- Many triple resonance experiments are constructed of common pulse sequence elements such as INEPT and COSY-type magnetization transfers
 - many/most of these are inverse detected (^1H detected in direct dimension)

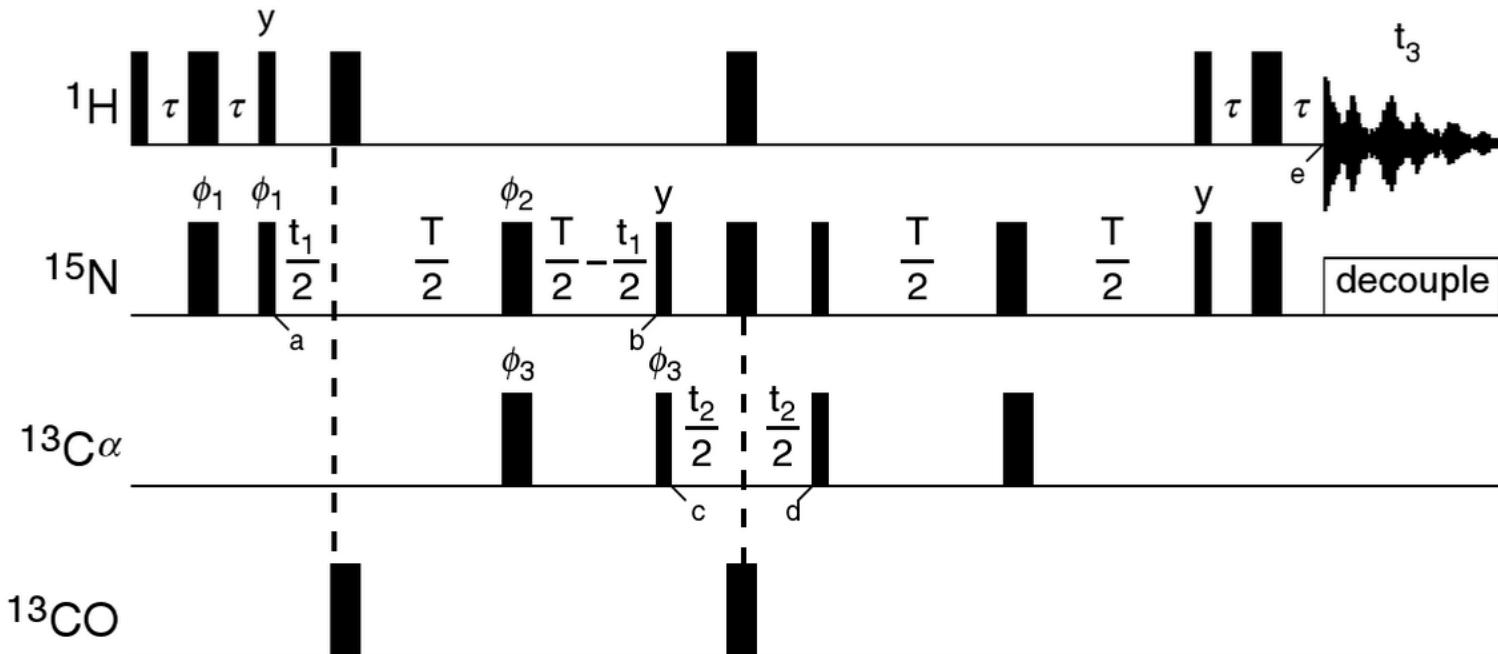


Prototypical Triple Resonance Experiment: HNCA



- correlates the chemical shifts of $^1\text{H}^N_i$, $^{15}\text{N}_i$, $^{13}\text{C}_\alpha^i$ and $^{13}\text{C}_{\alpha-1}^i$

$$^1\text{H}^N_i \xrightarrow{^1J_{\text{HN}}} {^{15}\text{N}_i(t_1)} \xrightarrow{^1J_{\text{NC}\alpha i}, {^2J_{\text{NC}\alpha i-1}}} {^{13}\text{C}_{\alpha i, i-1}(t_2)} \xrightarrow{^{1,2}J_{\text{NC}\alpha}} {^{15}\text{N}_i} \xrightarrow{^1J_{\text{HN}}} {^1\text{H}^N_i(t_3)}$$



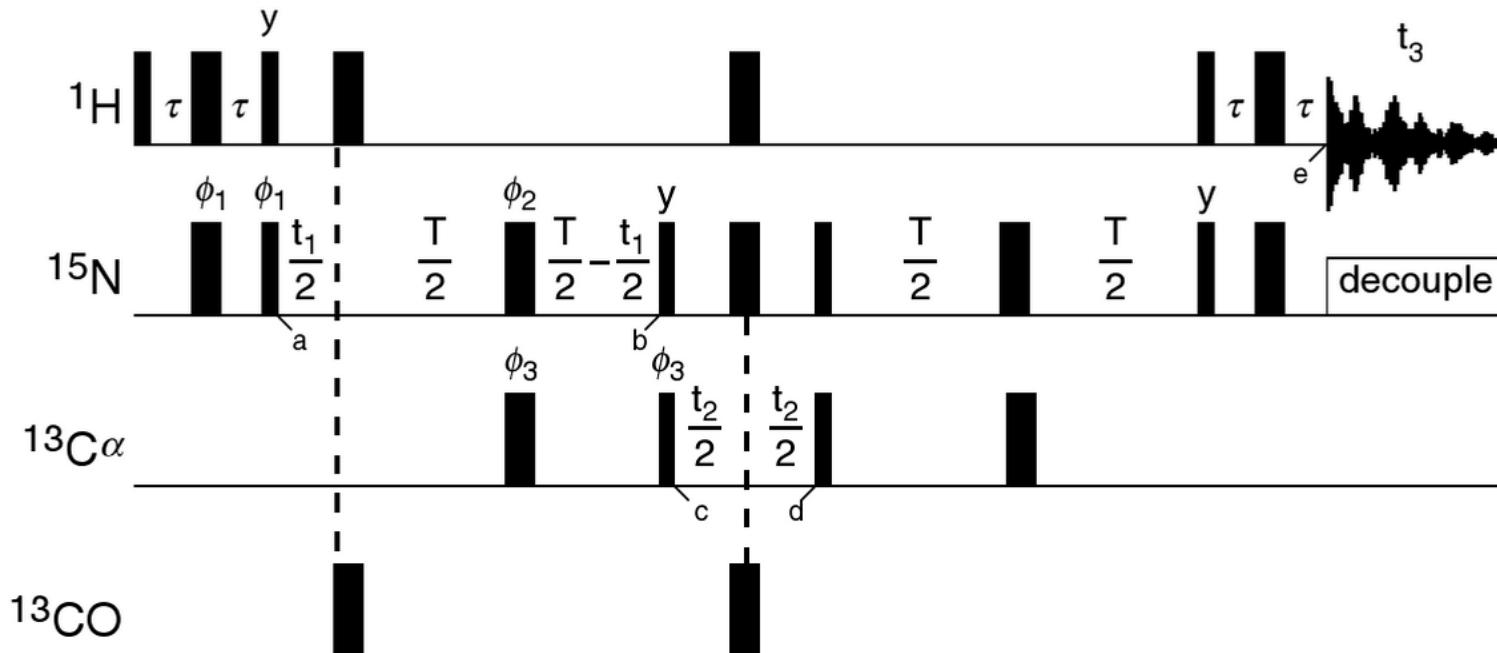
Prototypical Triple Resonance Experiment: HNCA

$$I=H^N, N=N, A=C_{\alpha i}, E=C_{\alpha i-1}$$

- “a” $IzNy$ (antiphase ^{15}N magnetization)
- “b” $NxIzAz \cos(\Omega_N t_1) + NxIzEz \cos(\Omega_N t_1)$ (trigonometric terms omitted)
- “c” $AyNzIz + EyNzIz$ (trigonometric terms omitted)
- “d” $AyNzIz \cos(\Omega_A t_2) + EyNzIz \cos(\Omega_E t_2)$ (important terms only)

At the beginning of t_3 :

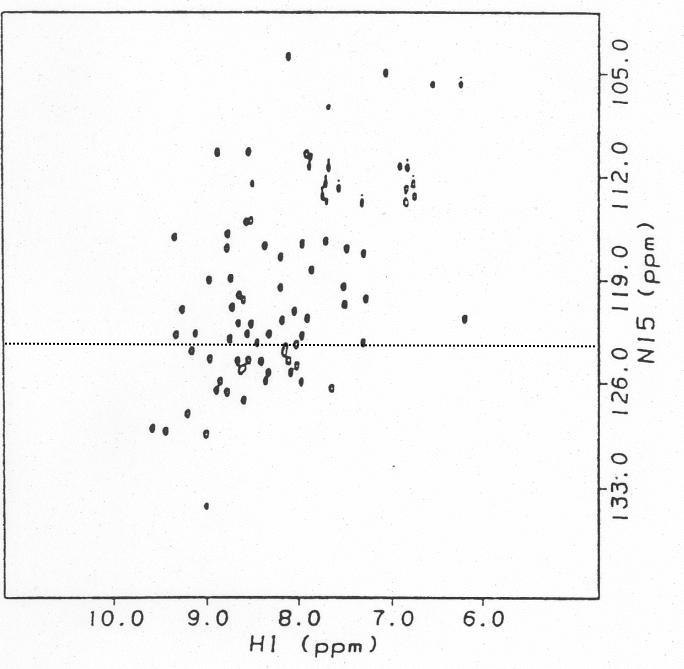
$$Ix \cos(\Omega_N t_1) [\cos(\Omega_E t_2) + \cos(\Omega_A t_2)]$$



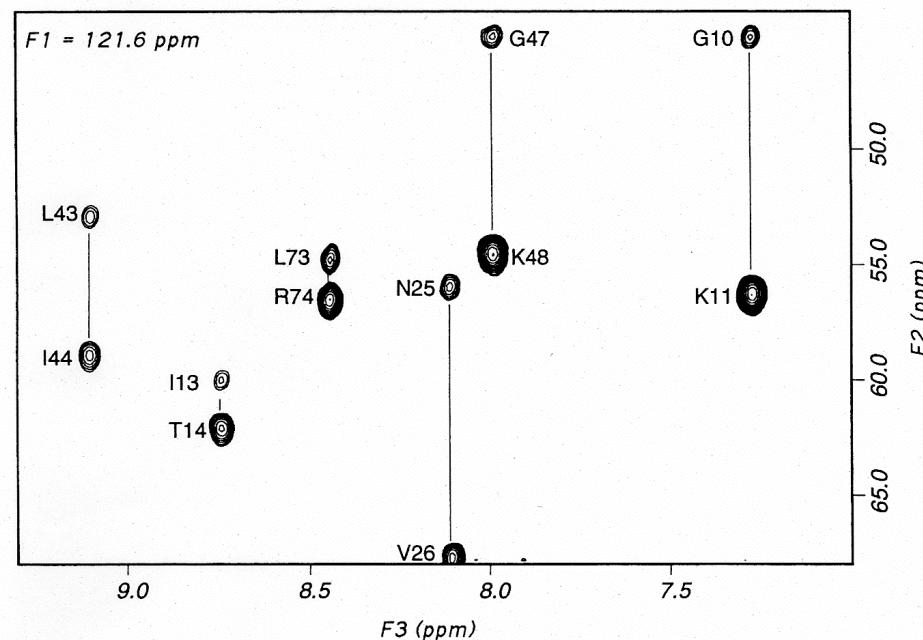
Prototypical Triple Resonance Experiment: HNCA

- Both $^{13}\text{C}_\alpha^i$ and $^{13}\text{C}_{\alpha_{i-1}}$ chemical shifts are correlated
 - the peak for the intra-residue correlation is usually more intense (11 Hz $^1J_{\text{NC}\alpha}$ coupling vs 7 Hz $^2J_{\text{NC}\alpha}$ coupling)

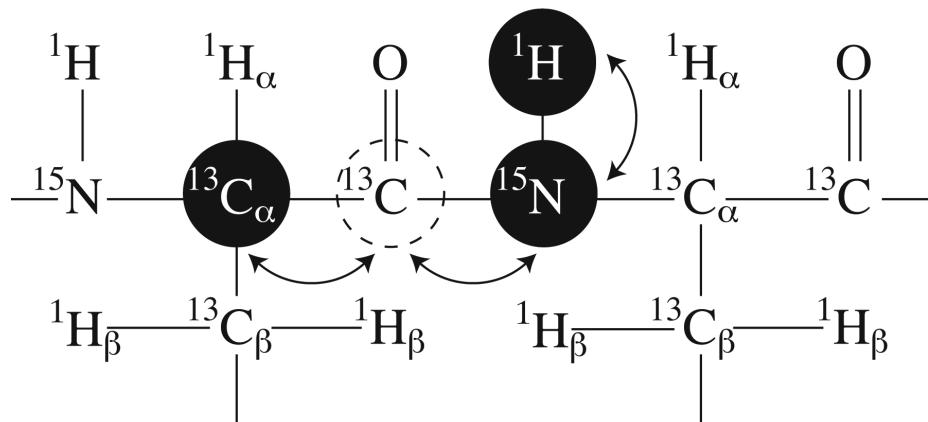
$^1\text{H}, ^{15}\text{N}$ -HSQC



HNCA

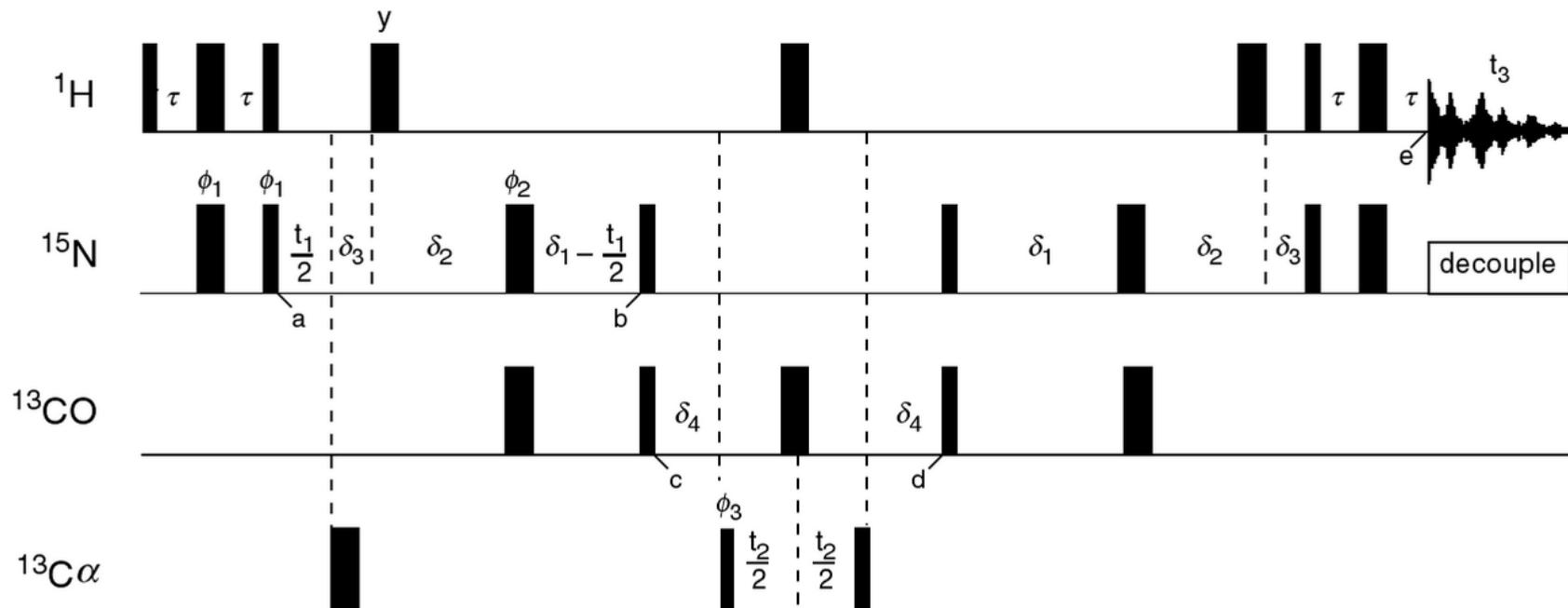


HN(CO)CA



- correlates the chemical shifts of $^1\text{H}^N_i$, $^{15}\text{N}_i$, and $^{13}\text{C}^\alpha_{i-1}$
- magnetization is transferred through the carbonyl ^{13}C , but no evolution of $^{13}\text{C}=\text{O}$ magnetization (hence the parentheses)

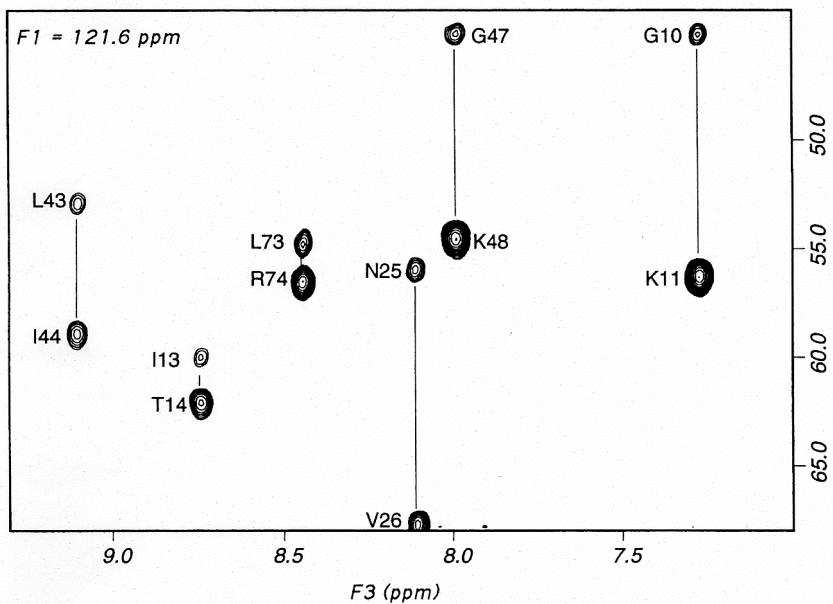
$$^1\text{H}^N_i \xrightarrow{^1J_{\text{HN}}} {}^{15}\text{N}_i(t_1) \xrightarrow{^1J_{\text{NC}'}} {}^{13}\text{C}'_{i-1} \Rightarrow {}^{13}\text{C}^\alpha_{i-1}(t_2) \xrightarrow{^1J_{\text{C}'\text{C}\alpha}} {}^{13}\text{C}'_{i-1} \xrightarrow{^1J_{\text{NC}'}} {}^{15}\text{N}_i \xrightarrow{^1J_{\text{HN}}} {}^1\text{H}^N_i(t_3)$$



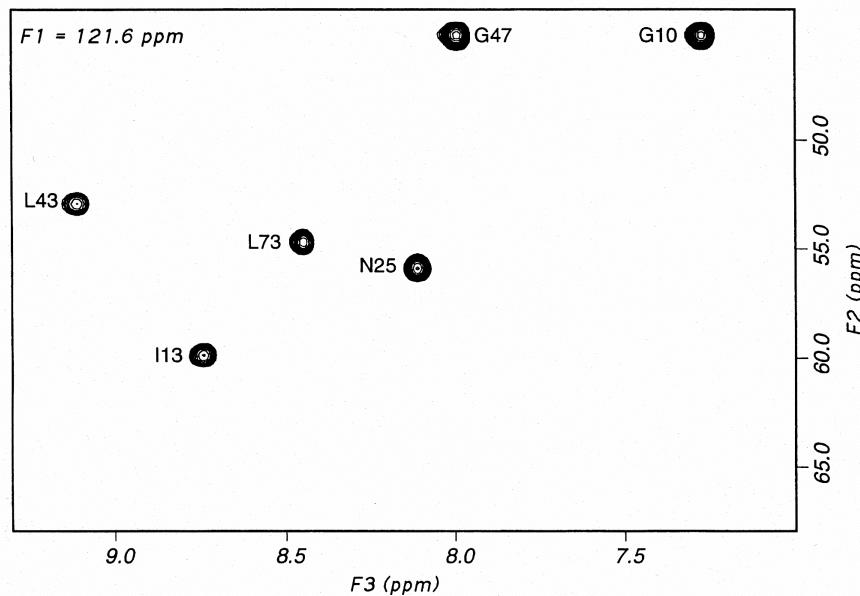
HN(CO)CA

- only the $^{13}\text{C}_{\alpha_{i-1}}$ chemical shifts is correlated
 - this provides confirmation of the inter-residue correlation in the HNCA experiment

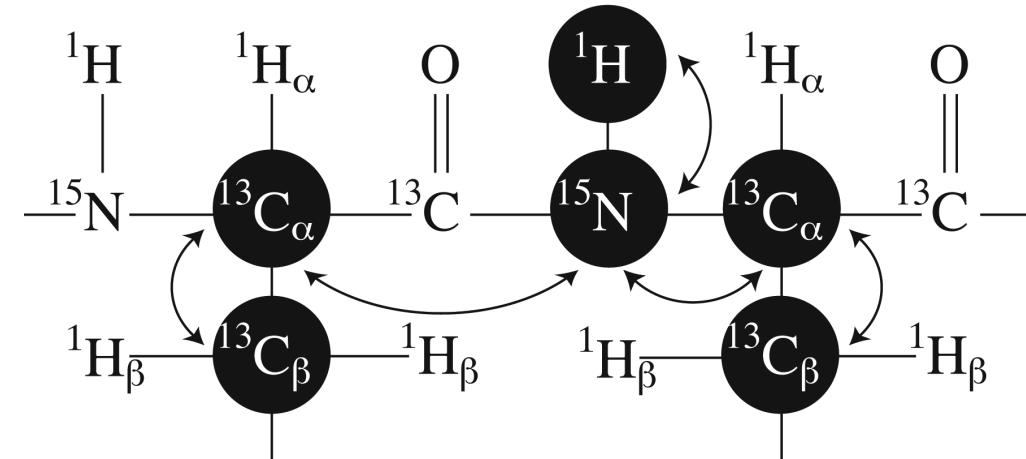
HNCA



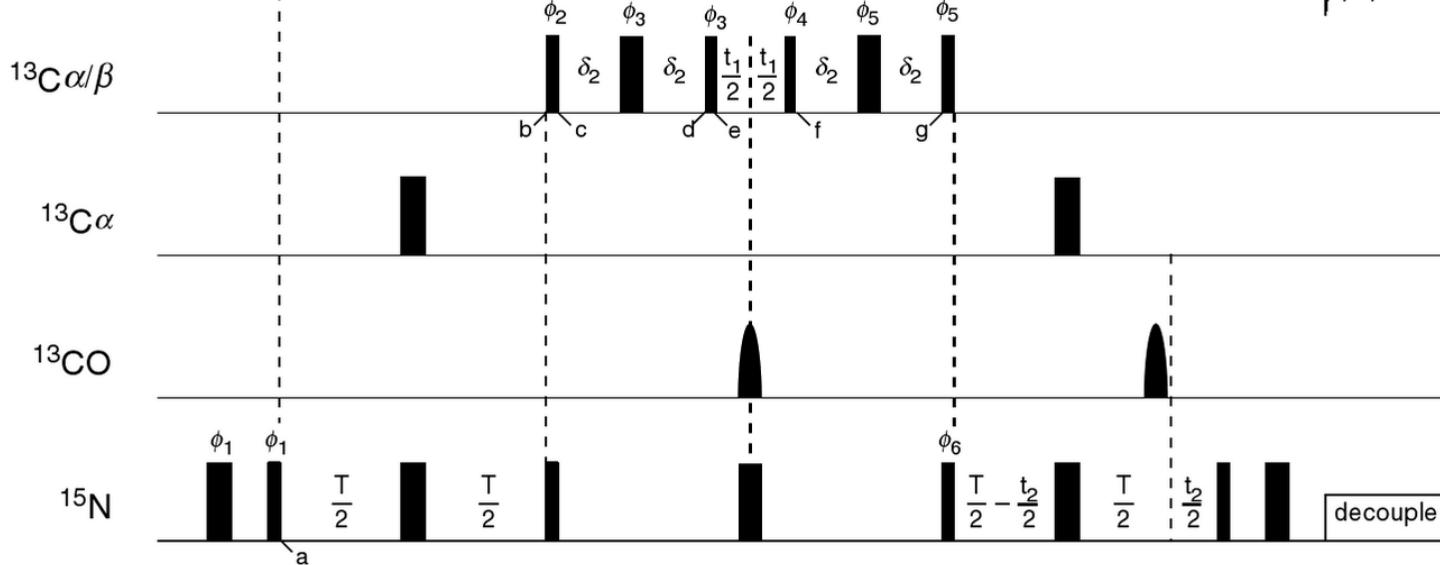
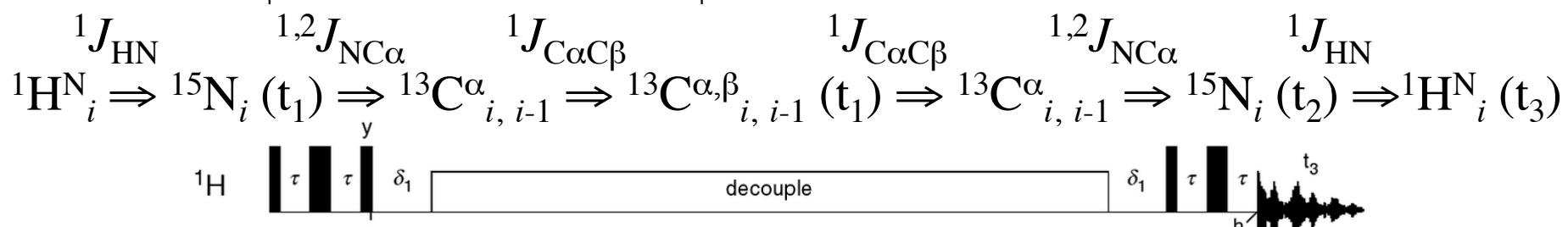
HN(CO)CA



HNCACB

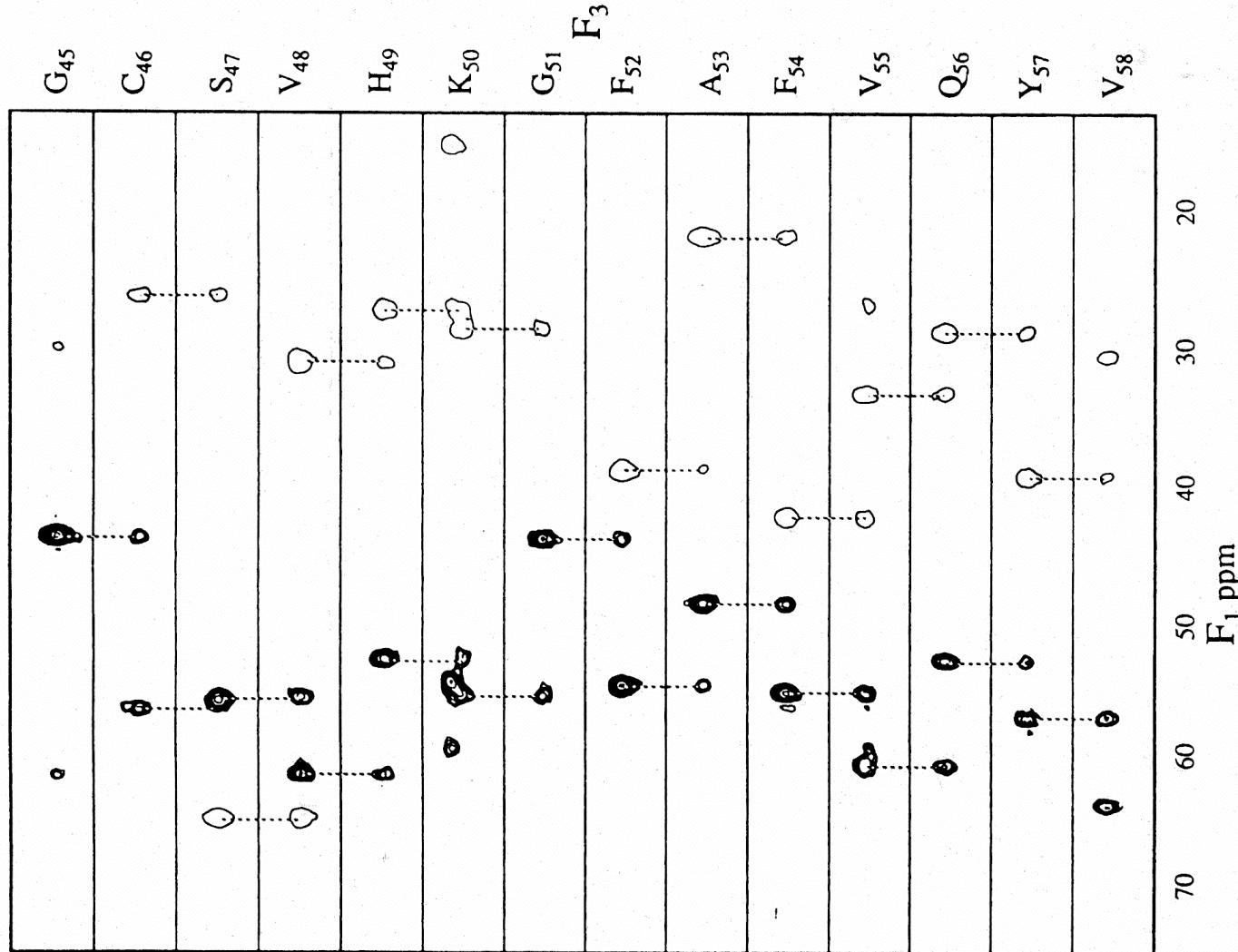


- correlates the chemical shifts of $^1\text{H}^N_i$, $^{15}\text{N}_i$, $^{13}\text{C}^\alpha_i$, $^{13}\text{C}^\alpha_{i-1}$, $^{13}\text{C}^\beta_i$, $^{13}\text{C}^\beta_{i-1}$
- also CBCANH

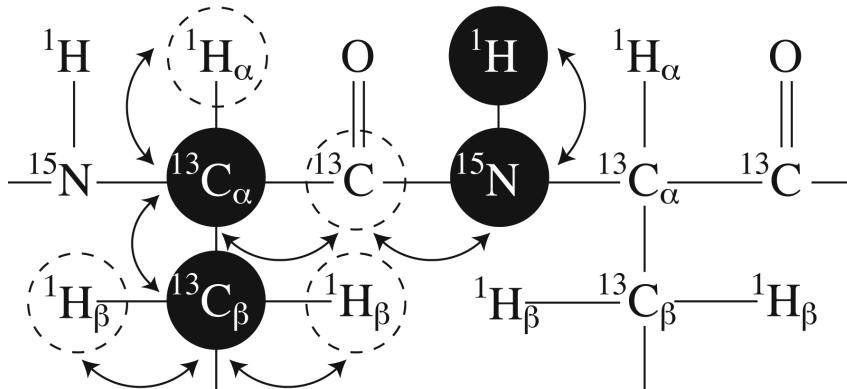


HNCACB

- $^{13}\text{C}^\alpha_i$ and $^{13}\text{C}^\alpha_{i-1}$ as well as $^{13}\text{C}^\beta_i$ and $^{13}\text{C}^\beta_{i-1}$ chemical shifts are correlated
 - the phase of the $^{13}\text{C}^\beta$ peaks is opposite to that for the $^{13}\text{C}^\alpha$ peaks

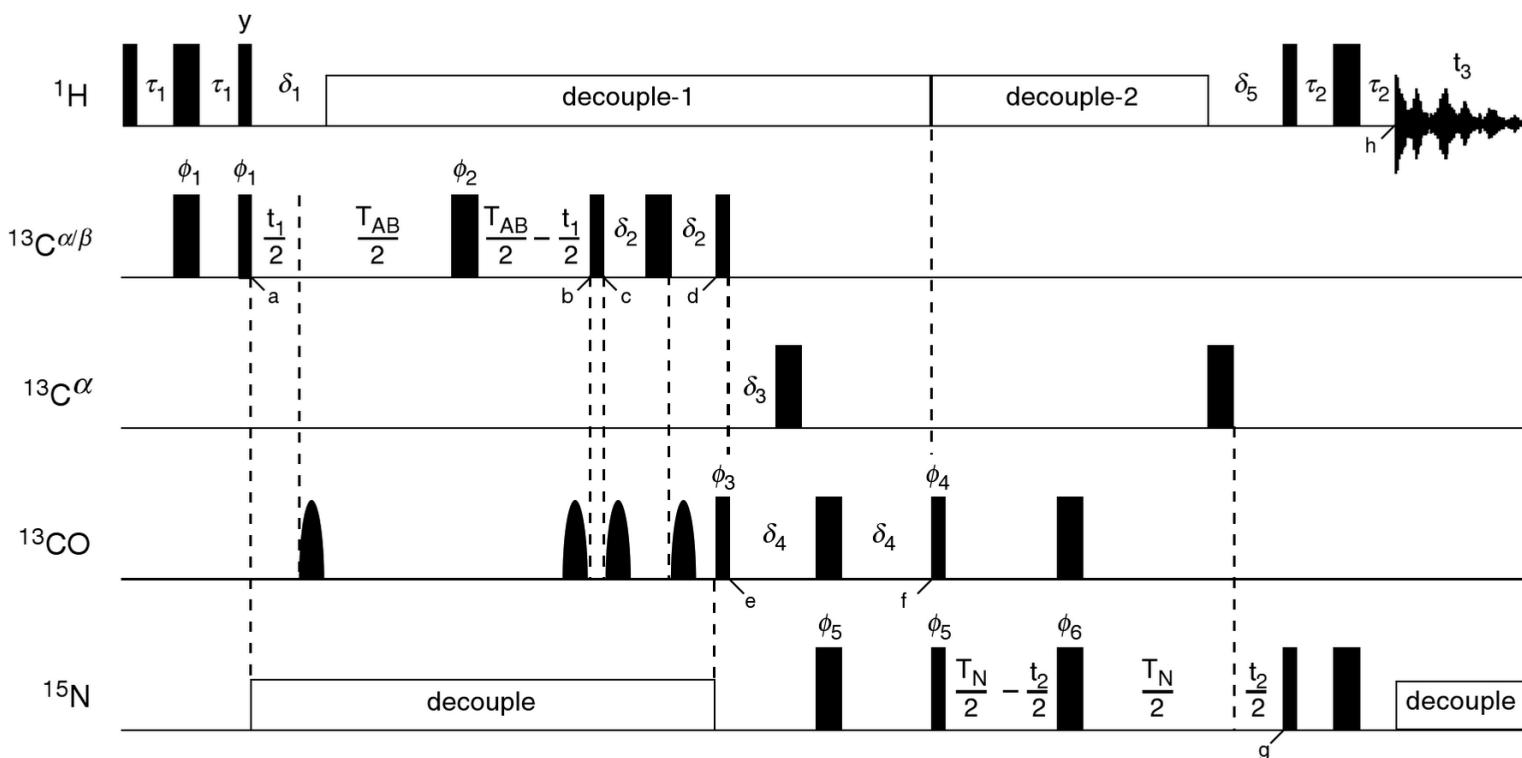


CBCA(CO)NH or (HBHA)CBCA(CO)NH

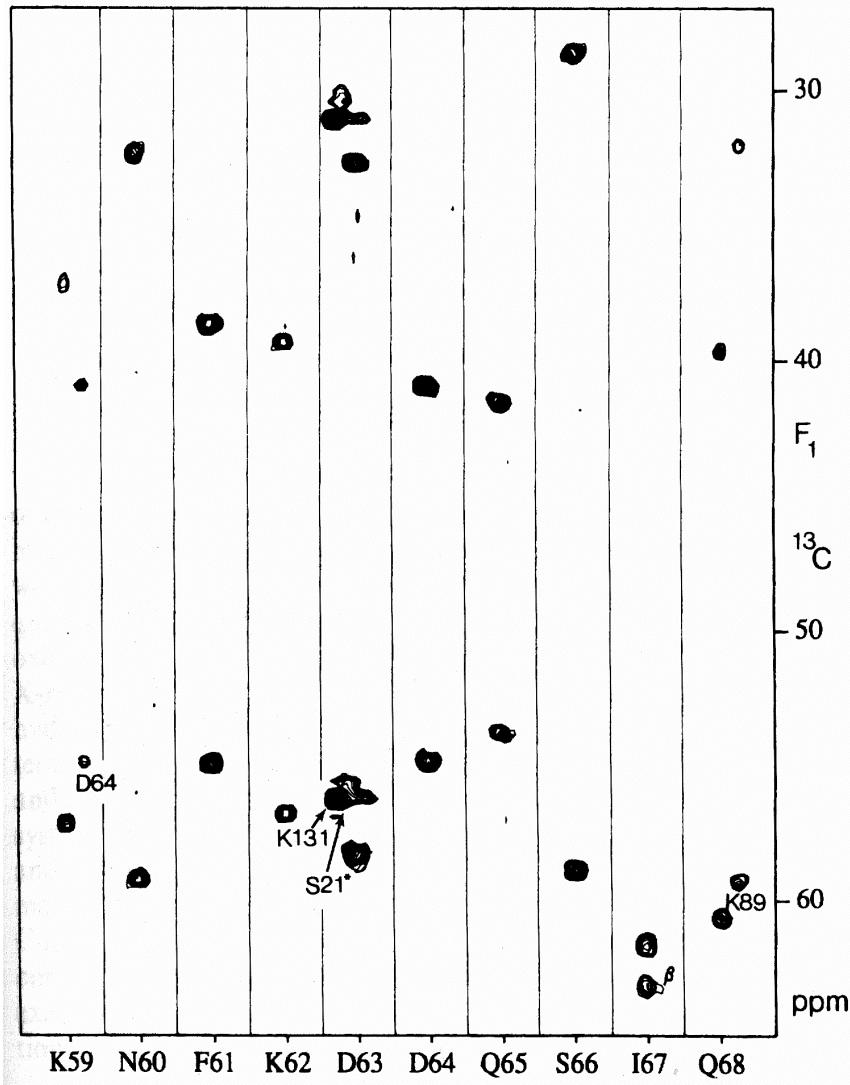


- correlates the chemical shifts of $^1\text{H}^{\text{N}}$, ^{15}N , $^{13}\text{C}^{\alpha}_{i-1}$, and $^{13}\text{C}^{\beta}_{i-1}$
- not an “out and back” experiment

$$^1\text{H}^{\alpha,\beta}_{i-1} \Rightarrow ^{13}\text{C}^{\alpha,\beta}_{i-1} (t_1) \Rightarrow ^{13}\text{C}^{\alpha}_{i-1} \Rightarrow ^{13}\text{C}'_{i-1} \Rightarrow ^{15}\text{N}_i (t_2) \Rightarrow ^1\text{H}^{\text{N}}_i (t_3)$$

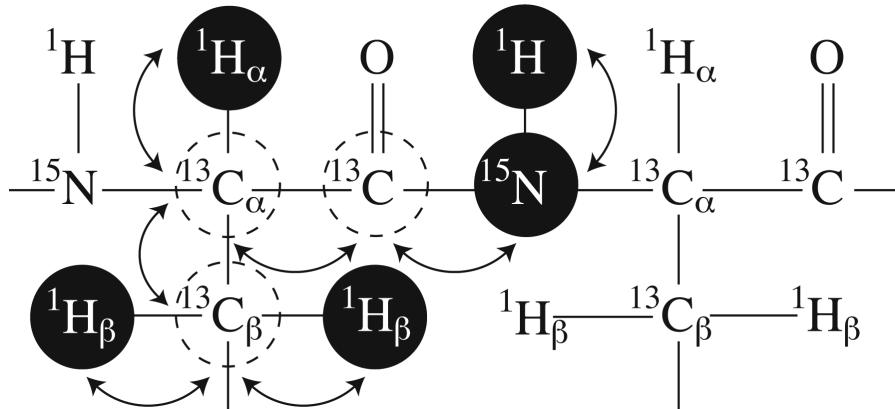


CBCA(CO)NH



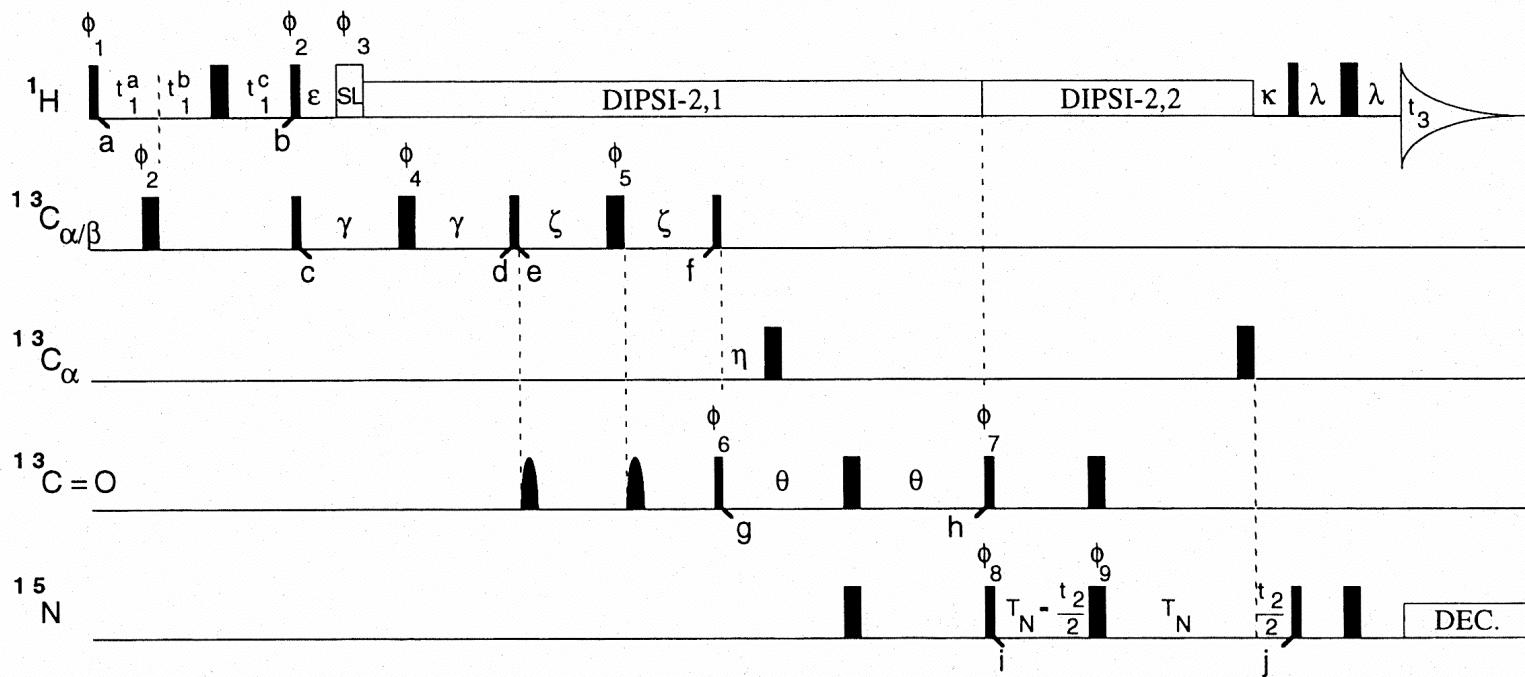
- only the $^{13}\text{C}^{\alpha}_{i-1}$ and $^{13}\text{C}^{\beta}_{i-1}$ chemical shifts are correlated
 - this provides confirmation of the inter-residue correlations in the HNCACB experiment

HBHA(CBCACO)NH



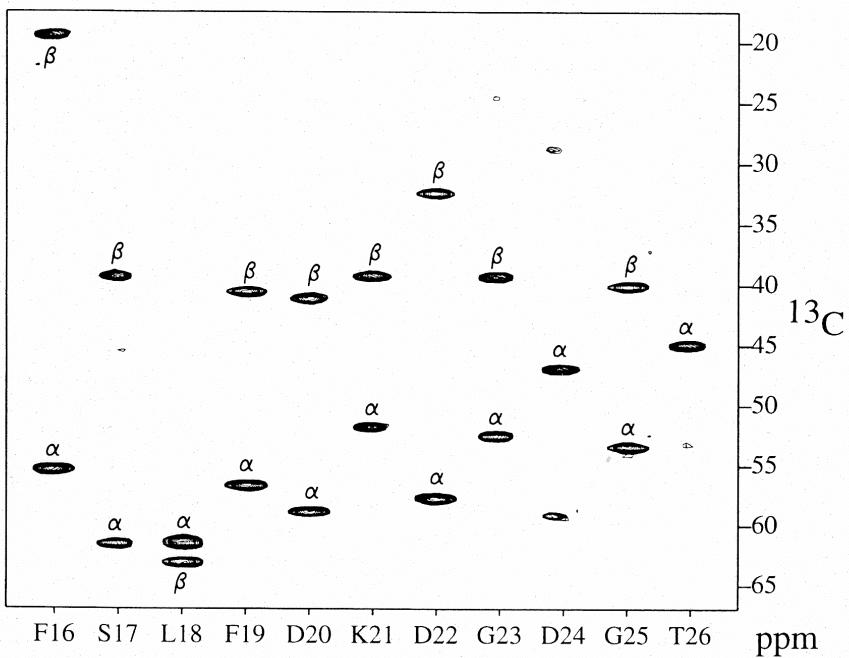
- correlates the chemical shifts of $^1\text{H}^N$, ^{15}N , $^1\text{H}_\alpha$, and $^1\text{H}_\beta$
- very similar to CBCA(CO)NH

$^1\text{H}^{\alpha,\beta}_{i-1} (t_1) \Rightarrow ^{13}\text{C}_{\alpha,\beta}_{i-1} \Rightarrow ^{13}\text{C}^\alpha_{i-1} \Rightarrow ^{13}\text{C}'_{i-1} \Rightarrow ^{15}\text{N}_i (t_2) \Rightarrow ^1\text{H}^N_i (t_3)$

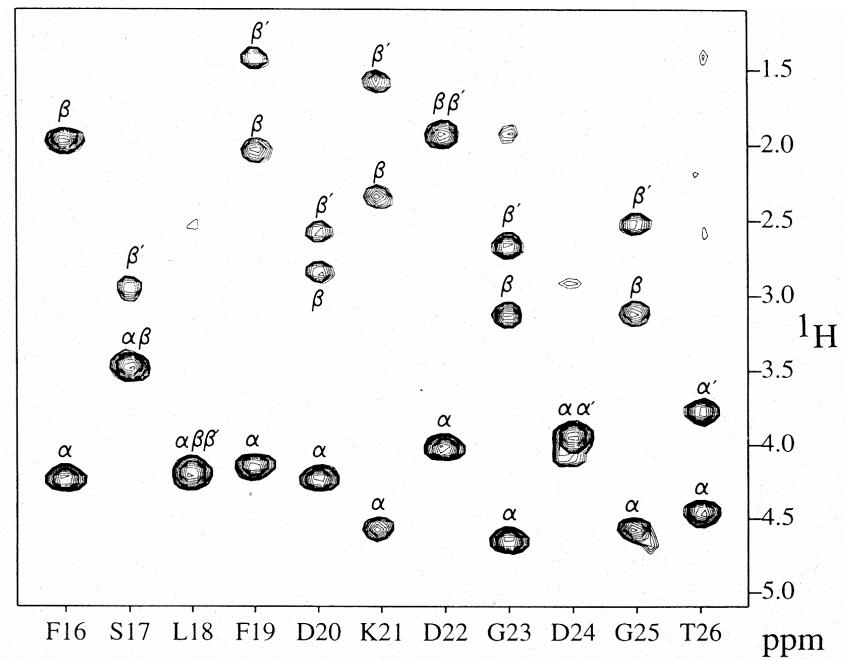


HBHA(CBCACO)NH

(HBHA)CBCA(CO)NH

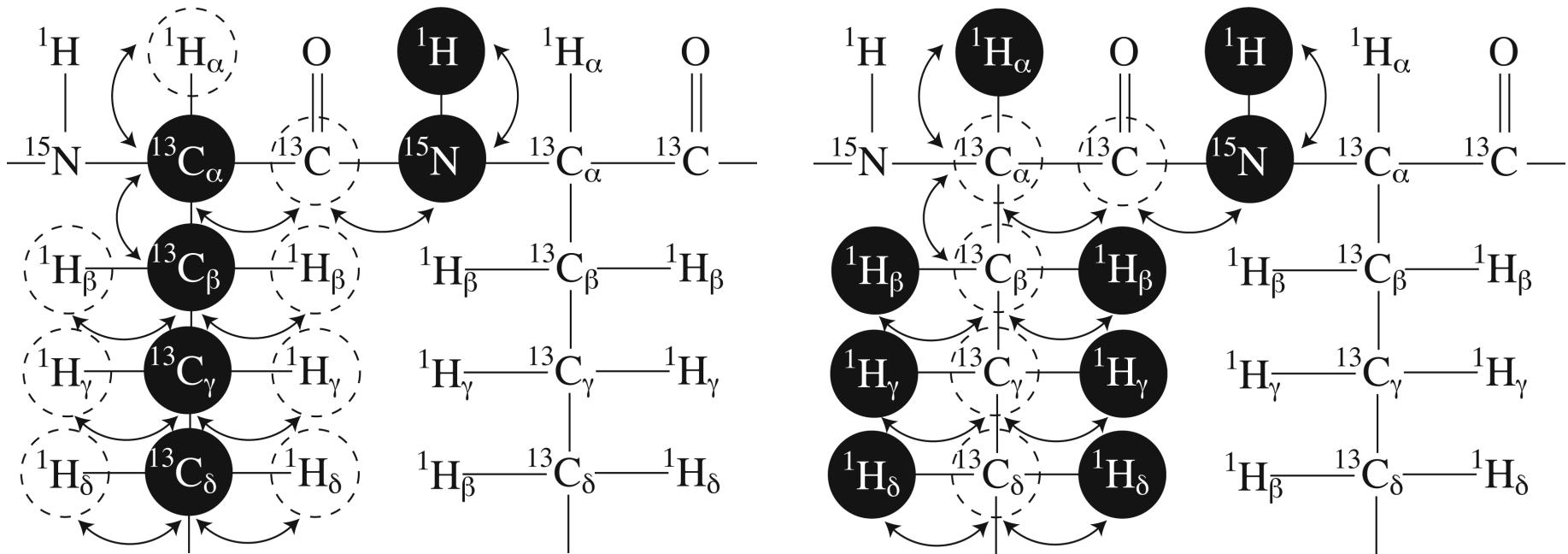


HBHA(CBCACO)NH



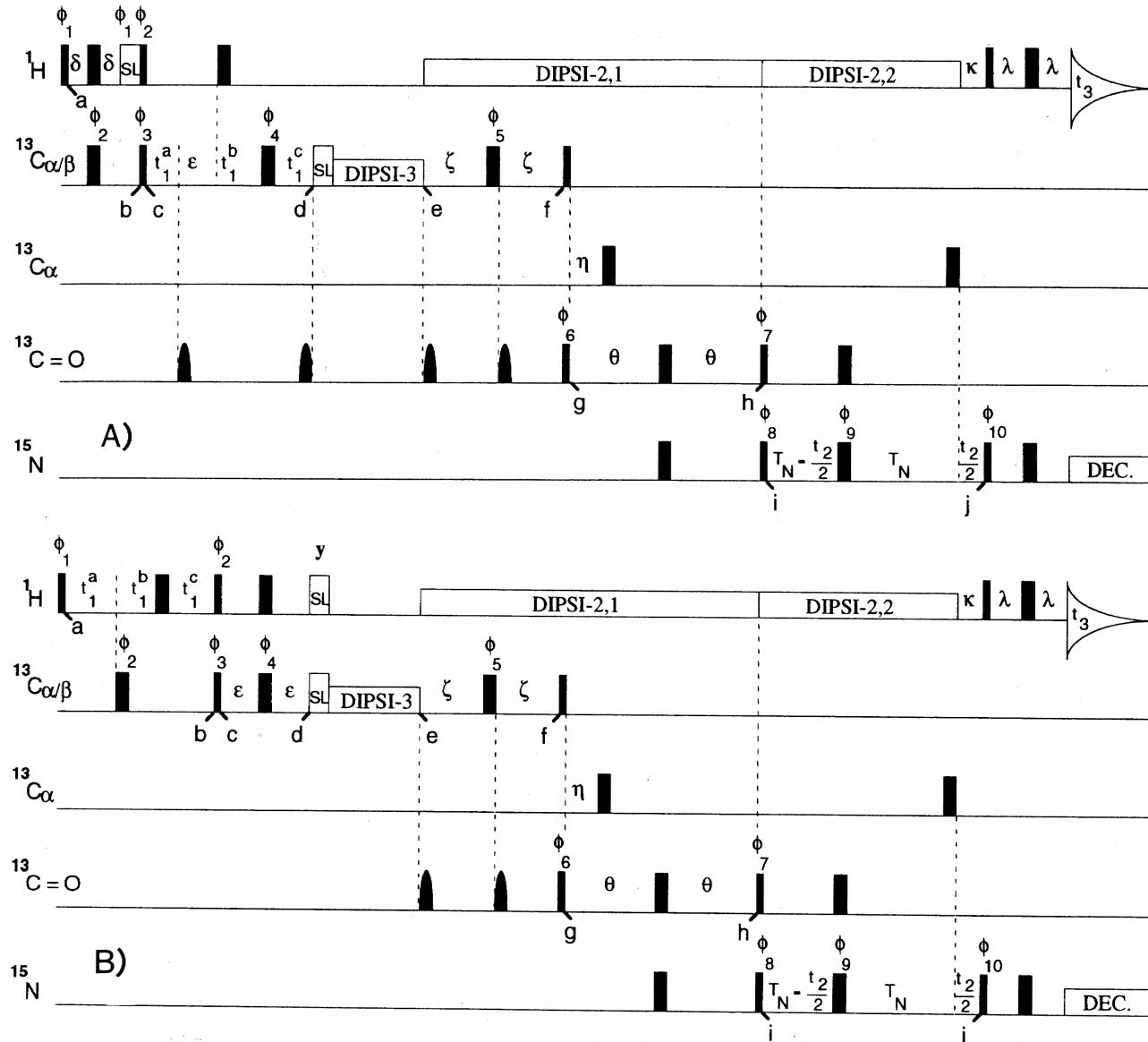
C(CO)NH and H(CCO)NH

- perhaps more appropriately called (H)C(CCTOCSY)(CO)NH and H(CCCTOCSY)(CO)NH, or something like that
- correlate $^1\text{H}^N$ and ^{15}N with $^{13}\text{C}_\alpha{}_{i-1}$, $^{13}\text{C}_\beta{}_{i-1}$, $^{13}\text{C}_\gamma{}_{i-1}$, $^{13}\text{C}_\delta{}_{i-1}$, etc. or with $^1\text{H}_\alpha{}_{i-1}$, $^1\text{H}_\beta{}_{i-1}$, $^1\text{H}_\gamma{}_{i-1}$, $^1\text{H}_\delta{}_{i-1}$, etc.

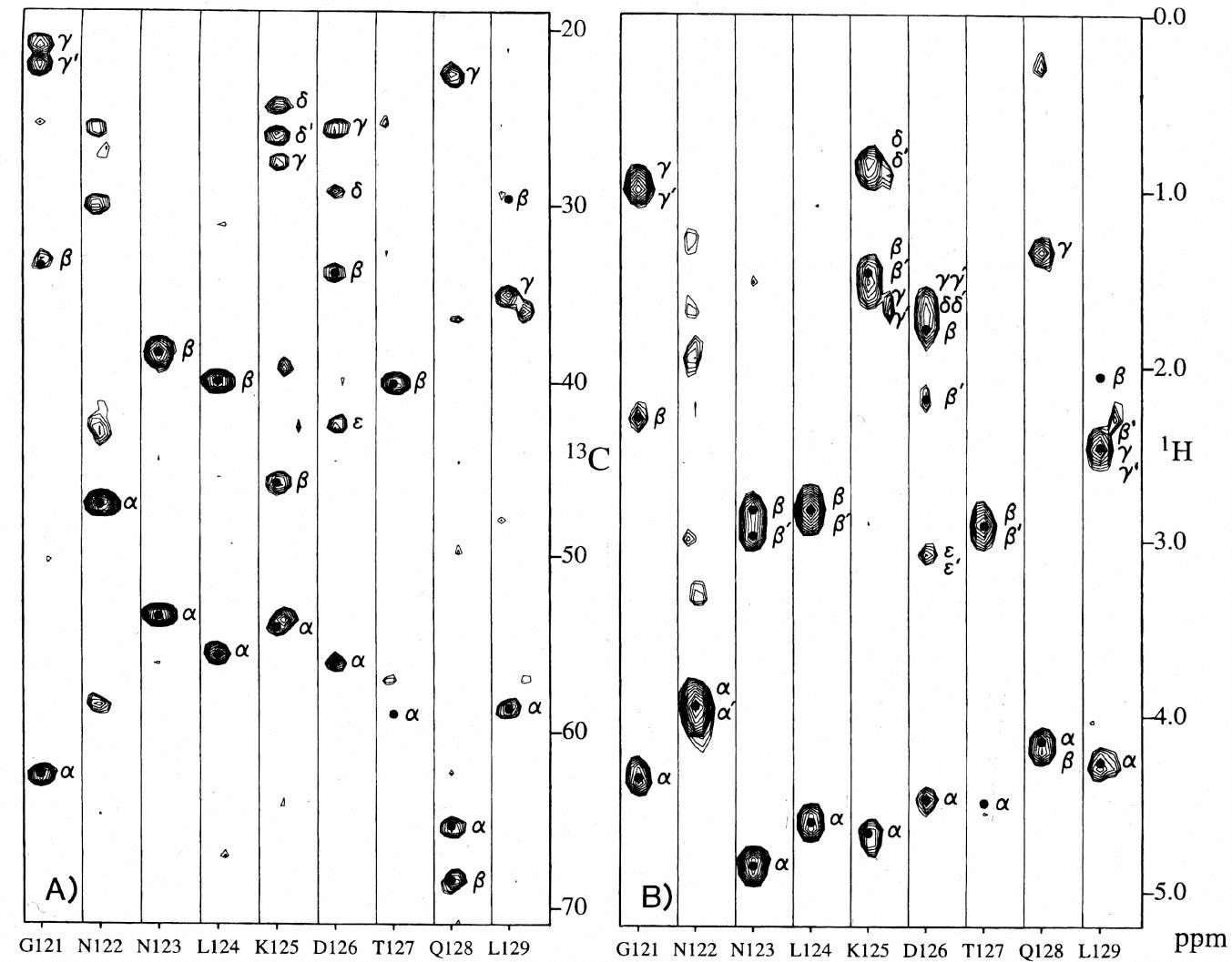


C(CO)NH and H(CCO)NH

- very similar to CBCA(CO)NH and HBHA(CBCACO)NH
 - use isotropic ^{13}C mixing to transfer magnetization to $^{13}\text{C}^\alpha$

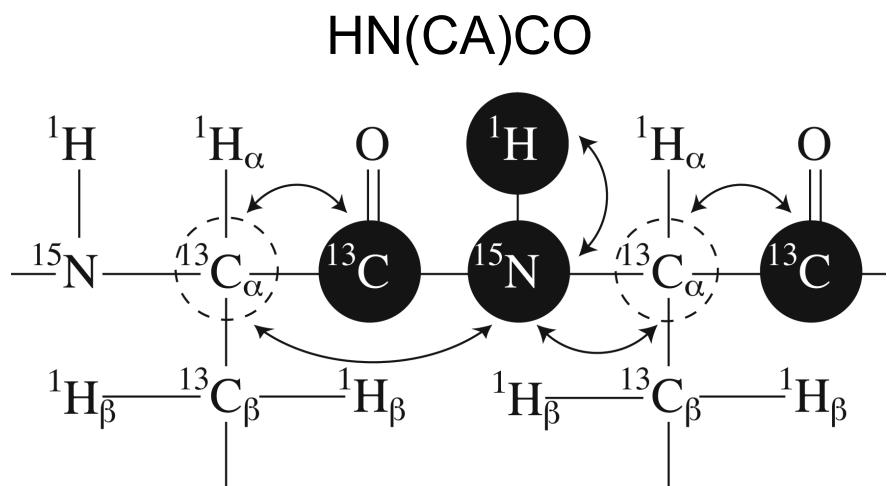
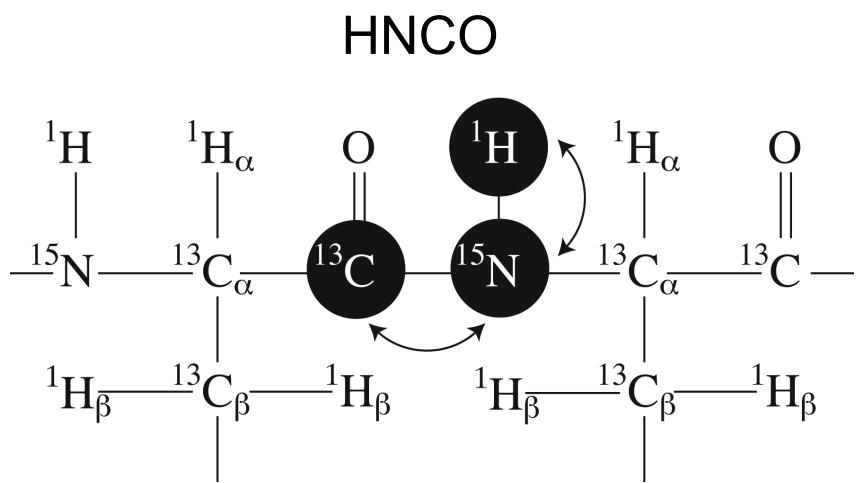


C(CO)NH and H(CCO)NH

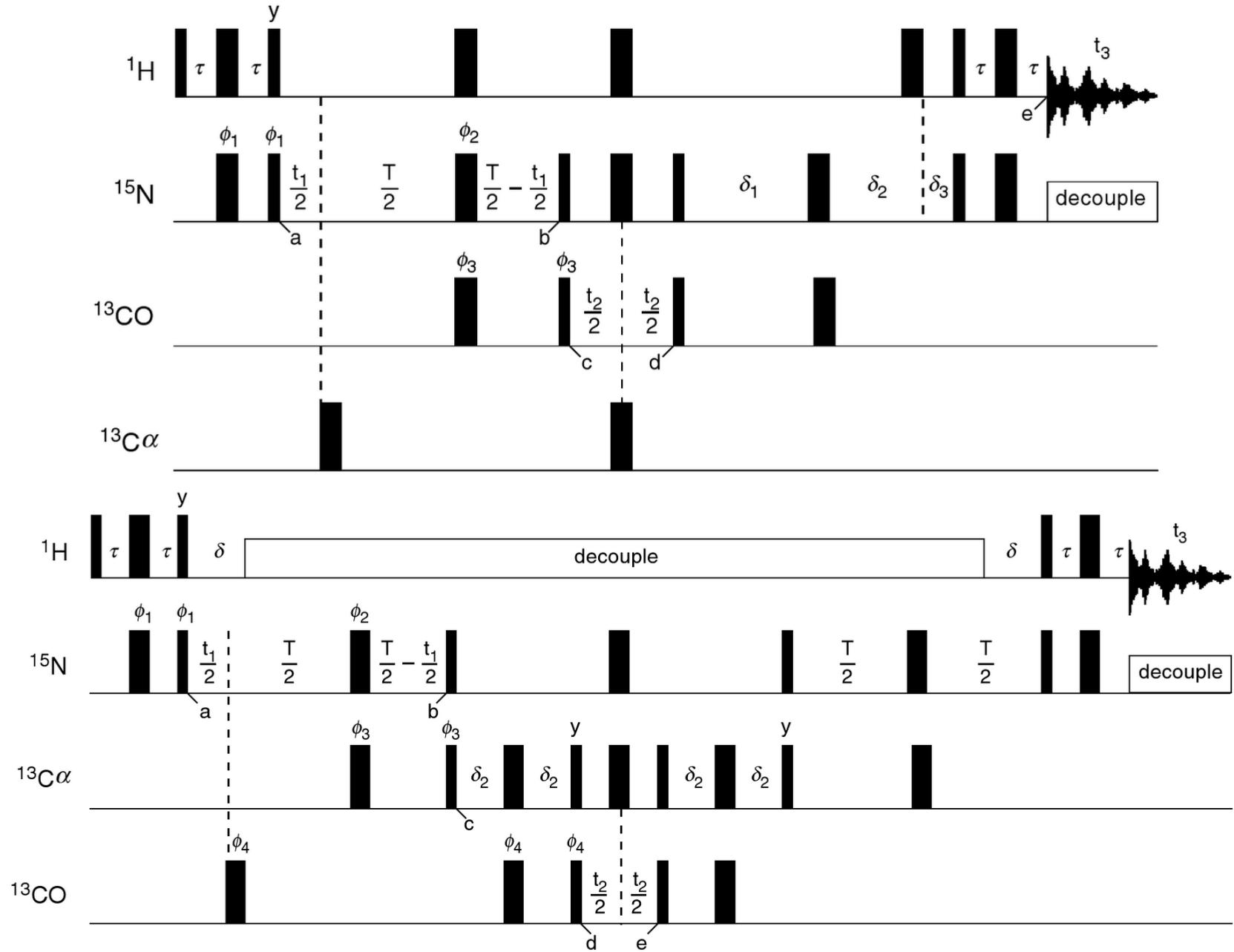


HNCO and HN(CA)CO

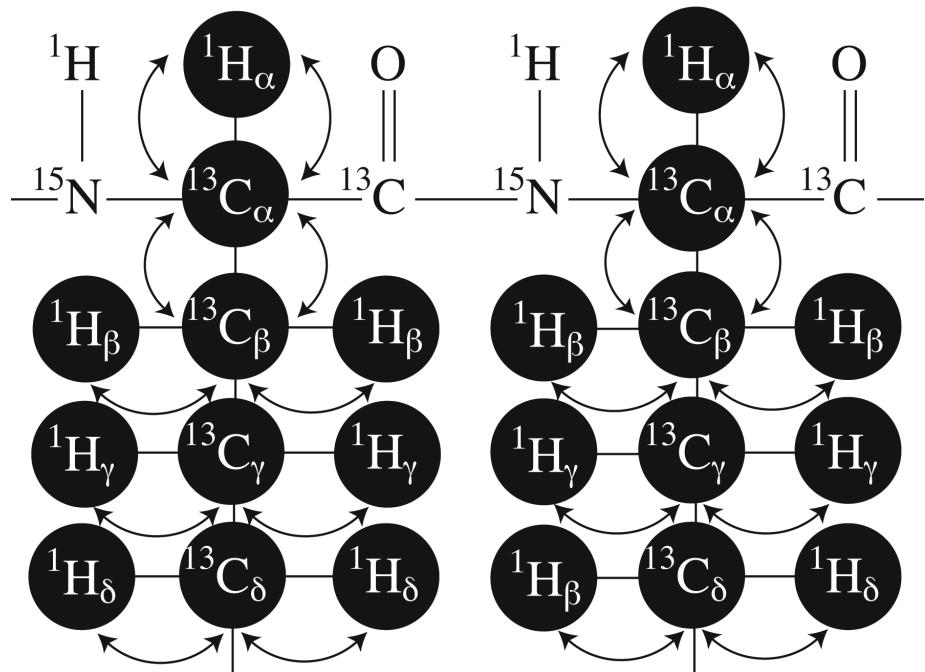
- HNCO is the most sensitive triple resonance experiment
- correlate $^1\text{H}^{\text{N}}$ and ^{15}N with $^{13}\text{C}'_i$ (HNCO) and with $^{13}\text{C}'_i$ and $^{13}\text{C}'_{i-1}$ (HN(CA)CO)



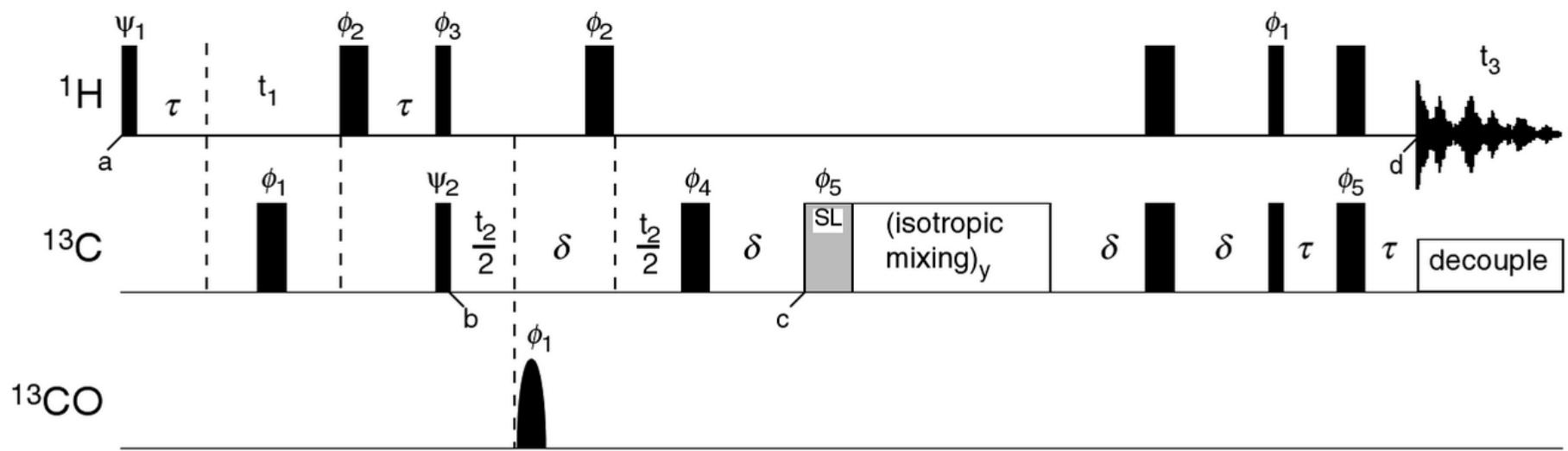
HNCO and HN(CA)CO



HCCH-TOCSY

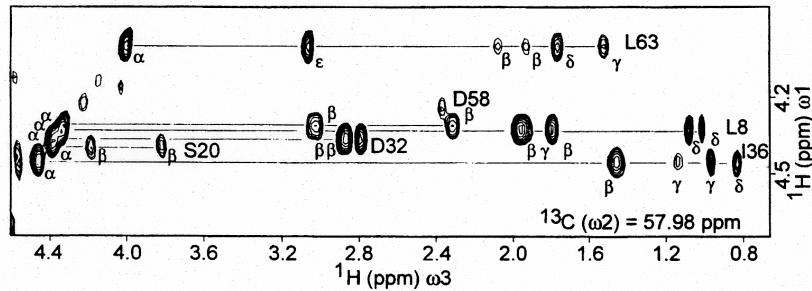
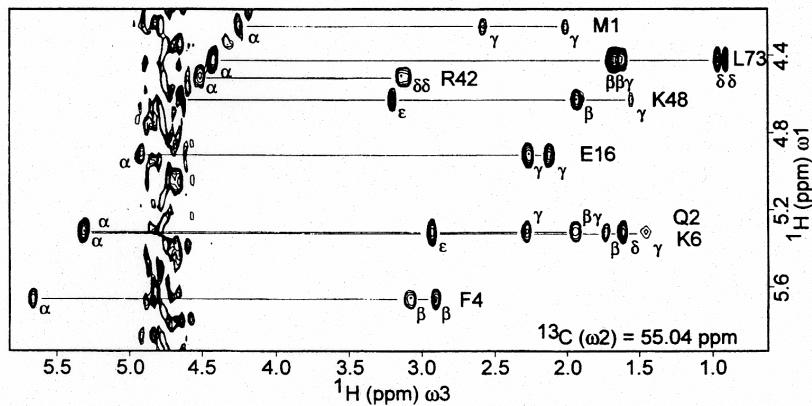
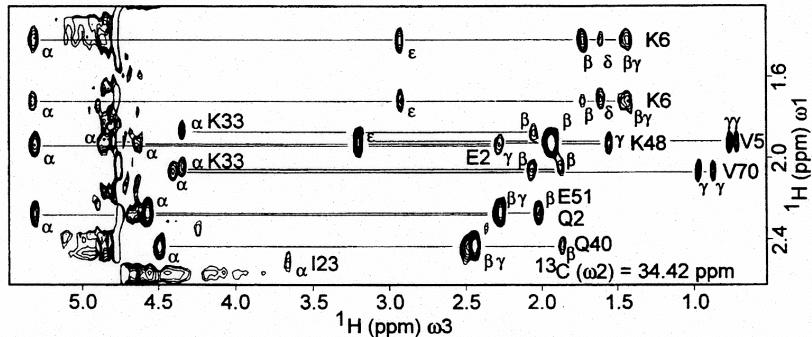


- correlates all ^1H and ^{13}C in aliphatic side chains
- very useful for assigning side chain resonances
 - somewhat more difficult to analyze than NH resolved TOCSY data

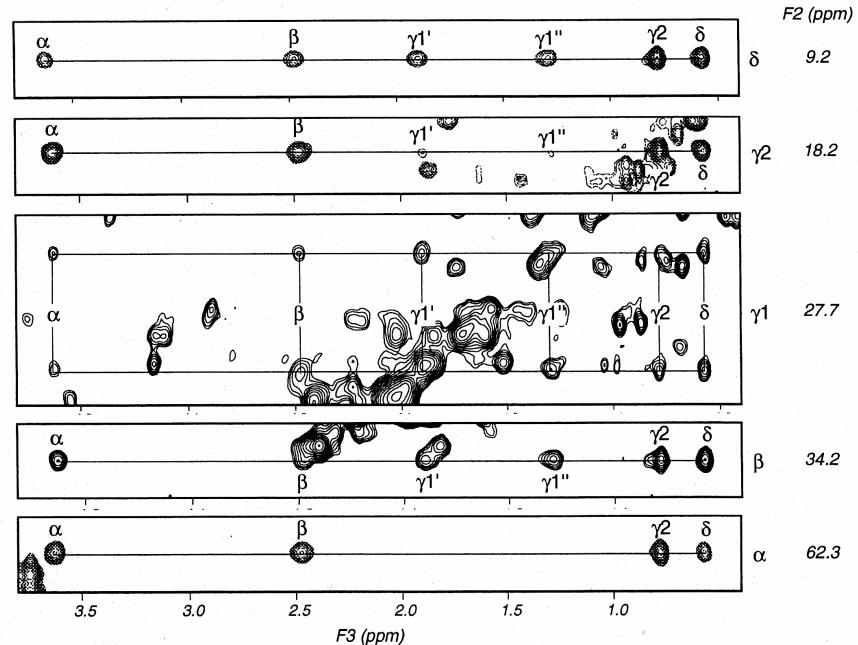


HCCH-TOCSY

- the HCCH-TOCSY is a very sensitive experiment, and the quality of the data are very good



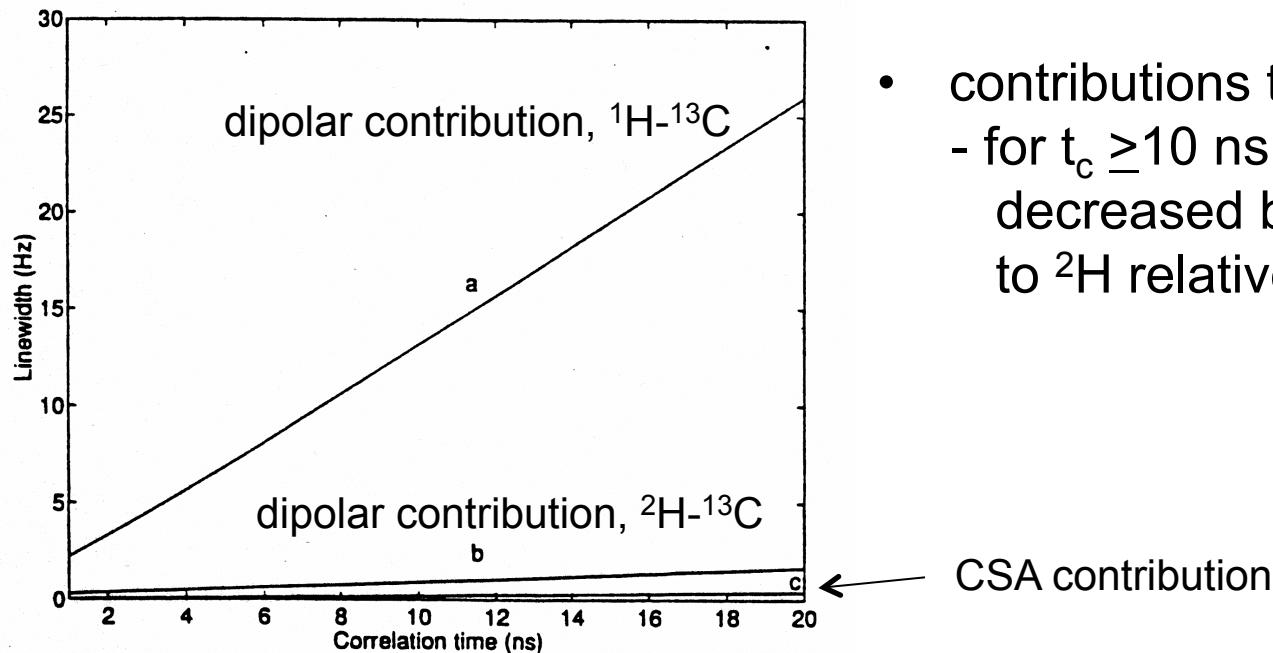
- data are also redundant !



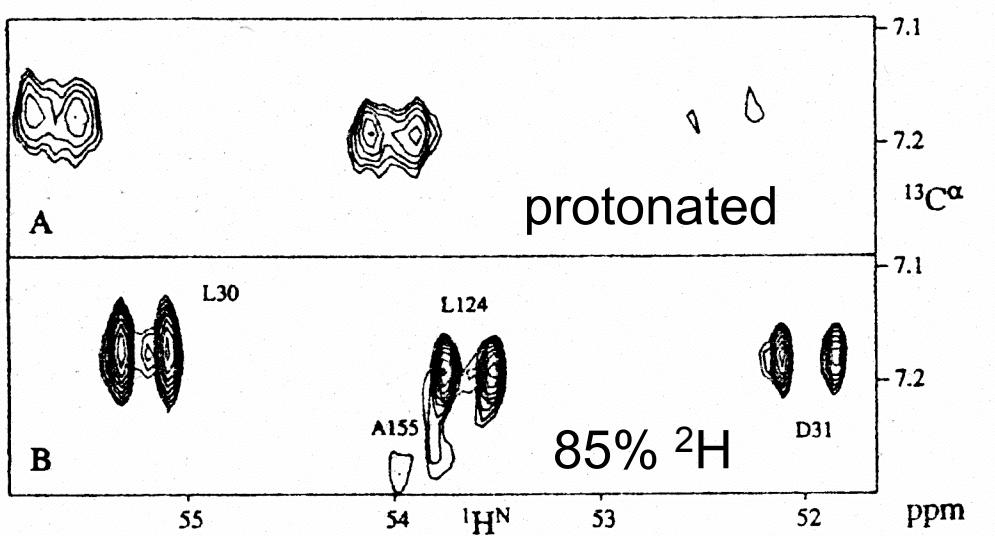
Fractional Deuteration

- for larger proteins, fractional deuteration is useful to improve S/N
 - transverse ^{13}C relaxation times (T_2) in proteins are short and increase quickly with MW
 - in heteronuclear and triple resonance experiments, magnetization must reside on ^{13}C nuclei for finite time
 - so, as size increases, T_2 decreases, linewidths increase, S/N decreases
- the dipolar ^1H - ^{13}C interaction dominates T_2 for ^{13}C nuclei with bound ^1H
 - the efficiency of this dipolar relaxation mechanism is greatly reduced by replacing ^1H with ^2H
 - so, deuteration can potentially offset sensitivity decreases introduced by T_2
- the ^2H - ^{13}C coupling (~20 Hz) results in a broad ^{13}C line width
 - ^2H decoupling reduces ^{13}C line width

Fractional Deuteration

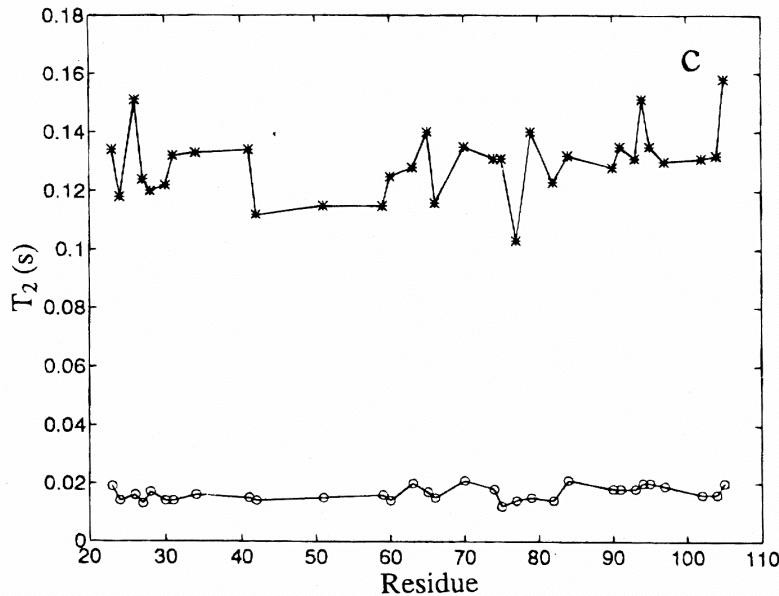


- contributions to $^{13}\text{C}^\alpha$ line width
 - for $t_c \geq 10$ ns, ^{13}C line width decreased by ~ 15 for $^{13}\text{C}^\alpha$ coupled to ^2H relative to ^1H

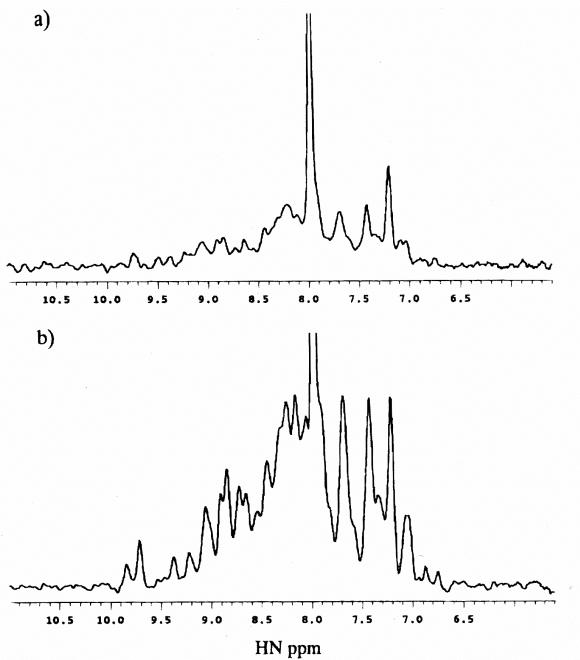


- example: 2D HNCA spectrum, protonated protein vs 85% deuterated protein (with ^2H decoupling)

Fractional Deuteration



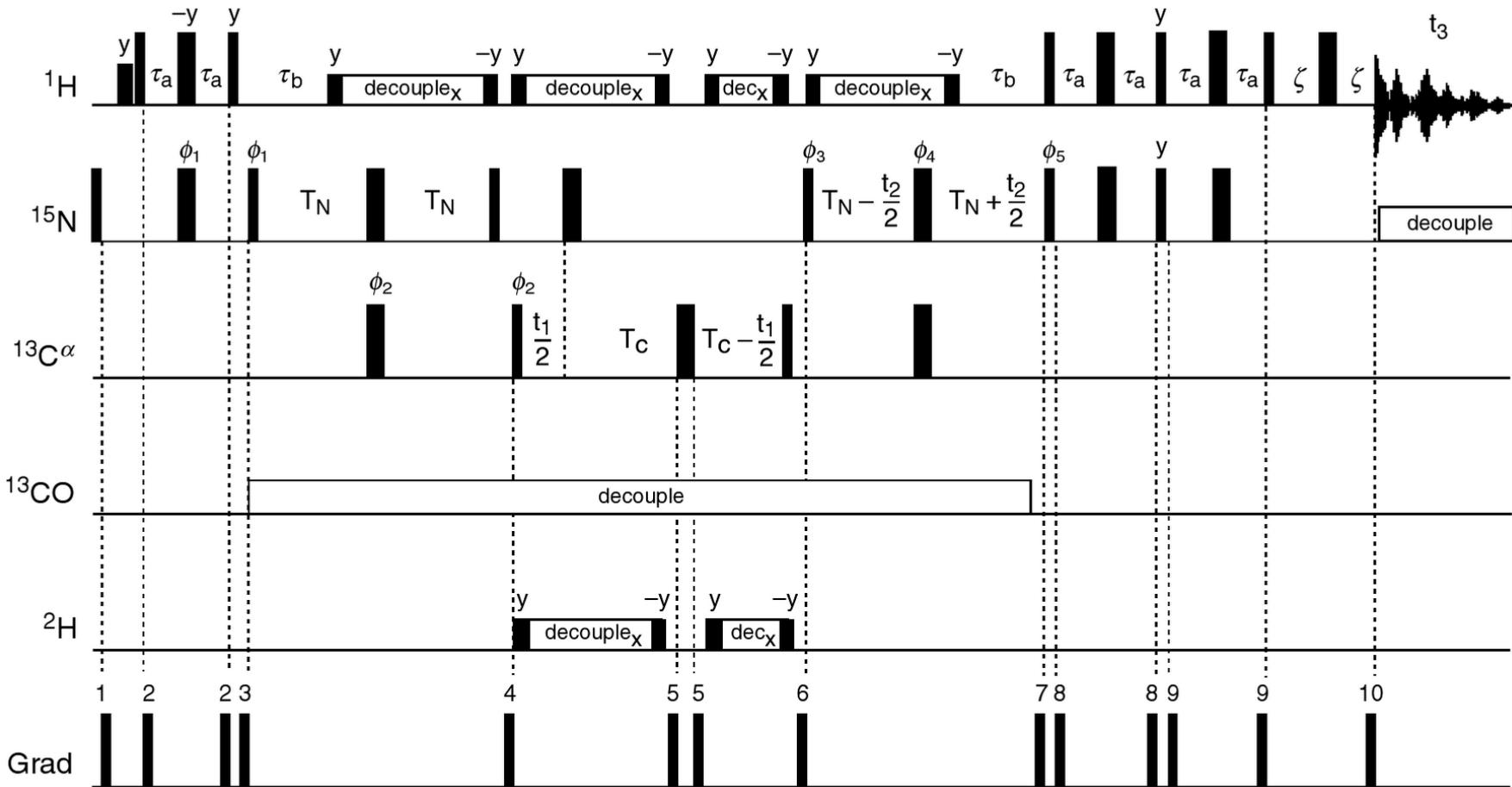
- example: $^{13}\text{C}^\alpha T_2$ magnitudes for protonated protein vs 70% deuterated protein



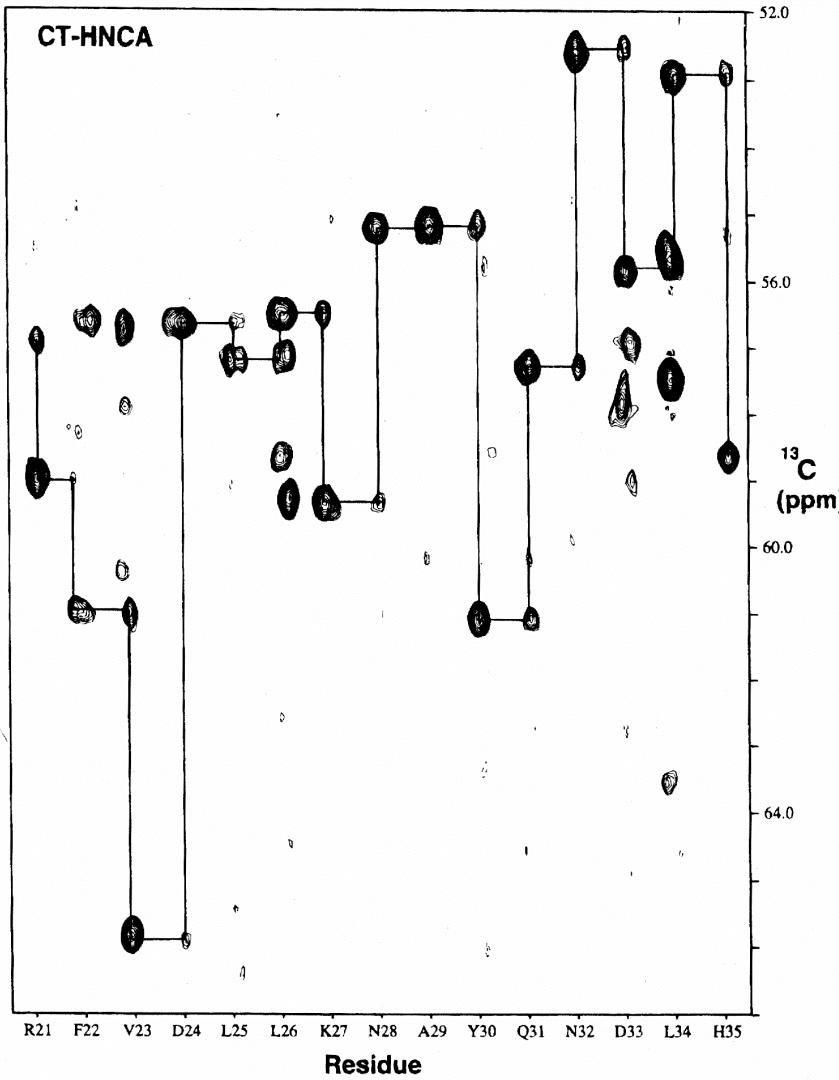
- example: 1D HNCACB spectrum, S/N for protonated protein vs 70% deuterated protein

Modernization and Improvements

- Gradient sensitivity-enhanced HNCA with ^2H decoupling
 - field gradient pulses for artifact removal and coherence selection
 - (gradient) sensitivity enhancement ($\sqrt{2}$ improvement in S/N)
 - water-selective pulse for H_2O suppression
 - ^2H decoupling for deuterated proteins



Gradient Sensitivity-Enhanced HNCA with ^2H Decoupling



- protein: ^{13}C , ^{15}N , **70% ^2H labeled**
- sample, **37 kDa** protein/DNA complex